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Emerging Technologies and New Directions in Drug Abuse Research

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Foreword

In October 1989 the National Institute on Drug Abuse (NIDA) organized a conference titled “Emerging Technologies for Drug Abuse Research” to discuss the impact of new technological advances on drug abuse research. The conference set as its principal goal the discussion of novel methodologies with an emphasis on the important current and future directions in research related to drugs of abuse.

In the past two decades we have witnessed not only the emergence of many scientific discoveries but also an explosive growth in the development of new methods that greatly enhance the quality and productivity of research. These include the application of solid-phase peptide synthesis methods in the design of novel peptide analogs, cDNA and cloning techniques in biological research, spectroscopic methods in biochemical and biophysical research, novel imaging techniques in biological research, high-performance, liquid chromatographic methods in analysis and purification of biomolecules, and, importantly, the application of computer technology across all scientific fields of research, including the area of computer-aided drug design (CADD).

Hence, NIDA believed that it was timely to call together experts in these areas and seek new directions and applications for NIDA-related research. Discussions at this conference were centered on CADD of benzodiazepine drugs, 3D-searching methodologies for drug design, design of selective agonists for 5-HT receptors, application of spectroscopic techniques such as 2D and 3D nuclear magnetic resonance (NMR), solid-state NMR, small-angle x-ray diffraction, differential scanning calorimetry, positron emission tomography (PET) and its application for studies on cocaine, autoradiographic and PET studies of the cannabinoid receptor, cocaine antagonists, and modified solid-phase peptide synthesis methods. It was clear from the discussions that these techniques hold great promise in such areas as understanding the mechanisms

of action of drugs, understanding the molecular determinants for interaction at receptor types, understanding addictive behavior, and, finally, design of new pharmacotherapies.

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Contents

Emerging Technologies and New Directions in Drug Abuse Research: An Overview

Rao S. Rapaka, Alexandros Makriyannis, and Michael J. Kuhar

During the past decade, many important advances in physicochemical methodologies have been adopted successfully in drug research. The concerted use of these methods coupled with computer-aided approaches for drug design have produced a new era in drug discovery and in the understanding of the molecular mechanisms of drug action. In October 1989, the National Institute on Drug Abuse organized a conference to discuss these advances as they apply to research on drugs of abuse. This volume draws to a large extent from this event, and a summary of these contributions follows.

Of the 17 chapters included in this volume, 4 are dedicated to the use of computer technology in drug research. Dr. Crippen discusses methods for gaining information on receptor structure and the energetics involved in drug-receptor interactions. Calculations are based on observing the binding constants of small ligands with several receptor preparations. The approach consists of developing simple binding site models and, through computational methods, exploring their interactions with drug molecules. In the second chapter, Drs. Osman and Weinstein add an extra level of complexity in the theoretical model of ligand-receptor interactions. Again, using biochemical and pharmacological data as a starting point, the authors undertake computational slmulations to develop a mechanism by which ligands can activate the 5-HT_{1A} receptor. This molecular model for receptor activation can differentiate between agonist and antagonist response. Dr. Loew and coworkers discuss a further expansion of this approach in describing a computer-aided approach for drug design aimed at the benzodiazepine receptor site. As with other computational approaches, receptor-binding and pharmacological data for a series of analogs are used to identify and characterize the stereoelectronic properties associated with agonist and antagonist activities, These stereoelectronic criteria are used in turn in a three-dimensional search of databases of known compounds. Finally, using stereoelectronic criteria complementary to those developed for the ligands, the entire set of data is used to characterize the receptor-binding site.

The information developed in this manner can be used to characterize regions in the GABA receptor subunits that could be good active site candidates. Alternatively, the results can be used to develop receptor site models in different, functionally unrelated protein structures already known.

The increasing importance of computer-aided searches for the discovery of new lead compounds in drug therapy is outlined by Dr. Pearlman. The approach described here is especially useful for designing novel drug molecules in cases where only a limited knowledge is available on the structure of the site of action. It Involves searching large databases of molecular structures for molecules that satisfy the three-dimensional geometric criteria describing the pharmacophore. The method was shown to be successful in the comprehensive computer-aided design of ligands for the benzodiazepine receptor site described in the previous chapter.

The next two chapters deal with novel developments in methodologies used to probe macromolecular structures and ligand-biopolymer interactions. Drs. Gronenborn and Clore describe the uses of multidimensional nuclear magnetic resonance (NMR) methods to study the structure of proteins and their interactions with ligands of biological significance. Indeed, two- and three-dimensional NMR experiments now can be used effectively to determine the structure and conformation of soluble proteins up to 25 kilodaltons in size. In the other chapter, Dr. Makrlyannis and coworkers describe the combined use of small-angle x-ray diffraction and solid-state NMR to study membrane structure and drug membrane interactions. Because of their complexity and inherent instability, membranes do not lend themselves to a detailed analysis of their structure and dynamics by means of a single physicochemical method. However, the combined techniques can provide detailed information on the topography, orientation, and conformation of the drug molecule in the membrane. The work described here deals with cannabinoids and model membranes; however, the methods used should have more general applicability for studying drug interactions with membrane-bound receptors.

Dr. Herkenham also discusses cannabinoids; he describes his experiments with slide-mounted tissue sections to characterize and localize the newly discovered “cannabinoid receptor.” This membrane-associated protein, which stereoselectively binds cannabinomimetic molecules, recently has been cloned. These new findings open a new era in cannabinoid research and enhance the opportunities for designing novel analogs of therapeutic value.

Positron emission tomography (PET) is a radioimaging method that can be used to measure receptor concentration and neurotransmitter activity, brain

glucose metabolism, cerebral blood flow, and pharmacokinetics in the living brain. Therefore, the technique is uniquely suited for investigating the neurochemical and physiological processes underlying drug addiction. The group from the Brookhaven National Laboratory headed by Dr. Wolf has contributed two chapters outlining the principles and applications of this method, which requires muitidisciplinary skills. Drs. Fowler and Wolf briefly describe the basis for and new developments in PET methodology, including the different accelerators for isotope production and the synthesis of labeled tracers. They then review the use of radiotracers for PET studies of the brain and for studying the binding of therapeutic drugs and substances of abuse. In the other chapter, Dr. Volkow and colleagues address the pharmacological and medical aspects of PET. They focus on the action of cocaine in the human brain and review the different experimental strategies currently used to study the drug's effects on the dopaminergic system.

The most extensive contribution in this volume deals with the structure-function correlations of opioids. Dr. Schiller first reviews the current status of selective opioid receptor ligands. There are three approaches used for the design of opioid peptide analogs, The most direct one deals with substitution, deletion, or addition of amino acids in the native ligands, which has resulted in the synthesis of a variety of linear peptides. The second approach introduces conformational restriction through cyclization as an additional element in drug design, and the third uses bivalent compounds containing two opioid-receptor ligands. The author concludes that substantial progress has been made toward the development of analogs with high selectivity for mu- and delta-opioid receptors, while promising efforts are being made for the development of ligands with specificity for subtypes of kappa-, delta-, and mu-receptor classes.

Early clinical trials with enkephalin analogs are disappointing because of serious side effects. However, it is hoped that these side effects will be minimized when analogs with higher selectivity for the different opioid receptor classes and subtypes are introduced. Also, encouraging from the therapeutic point of view are the recent data indicating that opioids are able to produce peripherally mediated analgesic effects, thus offering potential therapeutic usefulness in hyperalgesia or painful inflammatory conditions.

The two additional chapters dealing with novel opioids introduce the combined use of high-resolution NMR and computational methods as aids in drug design. Small linear peptides are known to be flexible and adopt multiple conformations that may allow them to interact with more than one receptor. It is hypothesized that, through conformational constraints, the peptides will be forced to adopt a single conformation and, consequently, show increased selectivity toward a

single receptor. Dr. Hruby and colleagues reflect on how conformational constraints and topographical considerations can be used for the design of receptor-specific opioid peptide ligands. The highly delta-opioid receptor-selective [D-Pen^2,D-Pen^5]-enkephalin is used as a model starting point for such a design. In a different approach, cyclic somatostatin peptides are modified strategically to give a cyclic octapeptide with high selectivity for opioid receptor end only marginal somatostatin activity.

Dr. Goodman and colleagues, in dealing with the design of stereochemically defined opioids, describe their "peptidomimetic retroinverso" modified enkephalins and modified cyclic demorphins, whose conformations are stabilized through intramolecular hydrogen bonding.

Three chapters discuss the use of organic synthesis for drug abuse research. Dr. Houghten and coworkers deal with modified solid-phase methods for the rapid synthesis of opioid peptides. The technique of multiple peptide synthesis in small- and large-scale preparations using solid-phase methodology is described. These relatively straightforward approaches are efficient and can be used to obtain large numbers of synthetic peptides in milligram as well as multigram quantities. They should be especially useful for applications in small- or medium-size research laboratories.

Dr. Newman reviews the use of irreversible ligands as probes for drug receptors. Electrophilic and photoactivatable groups can be introduced on high-affinity receptor ligands to obtain useful receptor-selective irreversible probes that attach covaiently to the receptor protein. These receptor probes can be used for the neuroanatomical localization of receptor sites, the isolation and purification of drug receptors, and in understanding the physiological and pharmacological properties of these receptors. Such affinity labels are described for the opioid, N-methyl-D-spartate, sigma, benzodiazepine, GABA, dopamine, and serotonin receptors, all of which are of interest in drug abuse research.

Because of its dominant role as a drug of abuse, cocaine has been the focus of extensive work in search of a cocaine "antagonist." Drs. Carroll and Lewin provide an overview of the different approaches for the synthesis of cocaine analogs.

The AIDS epidemic has intensified interest in the immunomodulatory effects of drugs of abuse. Receptors for opiates, opioid peptides, phencyclidine, benzodiazepine, and dopamine have been found on the cells of the immune system. It also has been shown that several groups of drugs of abuse have

immunomodulatory effects. For example, morphine has immunosuppressive effects, whereas many opioid peptides enhance the immune function. Marijuana and THC have been found to suppress the immune system in animals, but there is no conclusive evidence for such effects in humans. Drs. Rapaka and Hoiberg make a case that immunomodulatory activity is subject to specific structural requirements, and they stress the need for evaluation of these effects for ail potential new drugs. Structure-activity relationship studies on these drugs would be useful while evaluating the overall effects of drugs of abuse.

Drs. Hoiberg and Rapaka describe the recent status of Food and Drug Administration regulatory requirements for investigational new drugs and new drug approval. They also describe the recent changes regarding requirements for the submission of applications for investigational new drugs and new drugs. Other topics of interest include orphan drug products and the innovative procedures intended to facilitate and improve the review process.

This National Institute on Drug Abuse monograph mirrors the multidisciplinary nature of current chemical research related to drugs of abuse and serves to highlight some of the most important developments in methodology currently used or of potential applicability in research related to drugs of abuse.

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Voronoi Binding Site Models

Gordon M. Crippen

INTRODUCTION

The goal to develop improved methods for deducing the receptor structure and energetics, given the observed binding constants for a series of small ligands, is a commonly occurring problem in the search for better drugs, and many research groups are turning to computer-aided drug design techniques for help. For example, what can we say about the shape and intermolecular forces governing the binding of competitive inhibitors of an enzyme, given their binding constants but without knowing the enzyme's x-ray crystal structure? The algorithm for deducing this should be as little influenced as possible by the preconceptions of the investigator, and the amount of detail in the result should correspond to the quality of the data. The technique is to construct a simplified picture of the site that still allows the ligands to explore their full range of energetically allowed conformations and alternate orientations within the site. Binding energies are modeled as a sum of interactions between ligand atoms and the regions of the site they occupy.

A review of the *Journal of Medicinal Chemistry* indicates at least 10 articles per issue are published in which the authors have measured some biological activity or the binding affinity to some receptor for a series of compounds, and they attempt to comprehend the resulting data. Nearly all these discussions are cast in the language of the organic/medicinal chemist: What is the effect of different substituents at different positions on the parent compound? There are two pervasive assumptions in these qualitative discussions and in most quantitative structure-activity relationship (QSAR) methods—namely, that the compounds are clearly so closely related structurally that one may unambiguously superimpose them; indeed, they all bind in analogous orientations at the receptor site. The first assumption is often a consequence of everyone's natural tendency to modify promising compounds in hopes of improving them but sometimes the set of compounds is extremely varied, as in the case of the benzodiazepine receptor, which also binds ß-carbolines (Haefely 1988). The second assumption is often valid (Cheung et al. 1986), even though we know that the ligand-receptor complex forms by a random approach of the ligand tumbling in the solvent and tending to remain for longer periods in

whatever states correspond to exceptionally low free energy. There are, however, dramatic exceptions to the assumption, where small changes in the chemical structure lead to complete reversals in the mode of binding (Roberts et al. 1981).

Many methods have been devised to correlate the structure of compounds with their quantitatively measured binding to various receptors, starting with the pioneering work of Free and Wilson (1964) Hansch and Fujita (1964) and Kier (1971). Hansch's approach is probably the most frequently used method today, and it virtually defines the QSAR field. The investigator must assume that chemically similar compounds bind to the receptor such that atoms common to several compounds lie always at the same positions. Then the binding is fit in a least squares sense to a linear combination of selected physicochemical properties of the entire molecules and the various substituents. For example, a favorite property is the octanol/water partition coefficient. Some methods are intended to correlate chemical structural and topological (Ray et al. 1981) features with activity even more empirically by cluster analysis and related approaches (Rubin and Willett 1983; Wiliett 1982; Peredunova and Kruglyak 1983). If the chemical similarity between active compounds is minimal, then searches for common atoms responsible for activity (Danziger and Dean 1985), called the pharmacophore, sometimes take into account conformational flexibility, but generally rely on some superposition hypothesis from the investigator (Andrews and Lloyd 1983; Brint and Willett 1987). Marshall's group has devoted considerable effort to examining different conformations of active compounds and comparing the geometric and steric differences between active and inactive compounds, given a hypothesis for superimposing the molecules (Humblet and Marshall 1981; Motoc 1984; Motoc et al. 1985; Cramer and Bunce 1987). See the following references (Karfunkel 1986; Kuz'min and Krutius 1986; Motoc 1979) for other methods of overlapping molecules to develop an image of the receptor.

Although the usual methods are varied in detail and in the degree of conformational, geometric, and steric realism, the site tends to be viewed as the complement of the commonality of the active molecules in a chosen superposition. There may be a geometric component to the site model, at least implicitly, embodied in the hypothesized molecular superposition. The model also may be quantitative, assigning contributions to the total binding due to the various substituents at different positions on the parent compound. We always have advocated making an explicit model of the site so as to make the geometric and energetic features clear and to facilitate comparisons with other experiments such as x-ray crystallography. But given explicit site geometry and interactions with the ligands, would the ligands choose to bind to the site in the hypothesized modes? The answer generally is "no": They would change their

conformation and positioning relative to the site in a manner unanticipated by the investigator so as to achieve a more favorable energy of interaction. Revising the site model to incorporate this additional aspect of realism requires a basic rethinking of how QSAR is done. The ideas of pharmacophore, steric tolerance, conformational analysis on the ligands, and substituent effects all become intertwined It is fair to say that our QSAR studies are unique in the field for having directly addressed the issue. The costs are much lengthier computer calculations by much more complicated algorithms, but we believe the benefit is a shift away from purely empirical correlations toward models that can be verified by other physical experiments and that will have enhanced predictive ability.

METHODS

An enormous amount of work over the past 2 years has been summarized in a few papers. It is difficult to describe because of the number of new concepts we have introduced (rather than their difficulty): (1) the "linearized" representation of the rigid valence geometry of organic compounds, (2) a *global* summary of the conformation space of a molecule so represented, (3) modeling of binding sites in terms of Voronoi polyhedra, and (4) the relationship between the accuracy of the experimental binding data and the geometric resolution of the deduced binding site. An overview is presented here, and the reader may refer to other references as well (Crippen and Havel 1988; Crippen 1987).

1. We need a compact description of organic molecules that lends itself to receptor site modeling, and it is particularly important to be able to treat the conformational flexibility that arises from rotations about single bonds. Our linearized representation of a molecule involves assuming fixed bond lengths and vicinal bond angles, specifying the overall translation of the molecule with respect to an external reference frame by a vector w pointing to a centrally located atom, and setting up local coordinate systems in terms of unit vectors u_1, u_2, ... for mutually rigid groups of atoms, as illustrated in figure 1. Then the position of every atom can be given as a linear combination of the translation vector and the unit vectors. This turns out not only to facilitate binding site modeling, but also has led to a new algorithm for calculating atomic coordinates subject to distance constraints, as in the determination of solution conformation by two-dimensional nuclear magnetic resonance (Crippen 1989).

2. Most methods for developing QSARs make assumptions about the orientation of the ligand molecule in the binding site and its conformation upon binding. To avoid making such assumptions, we must have a convenient summary of the global range of conformations that are

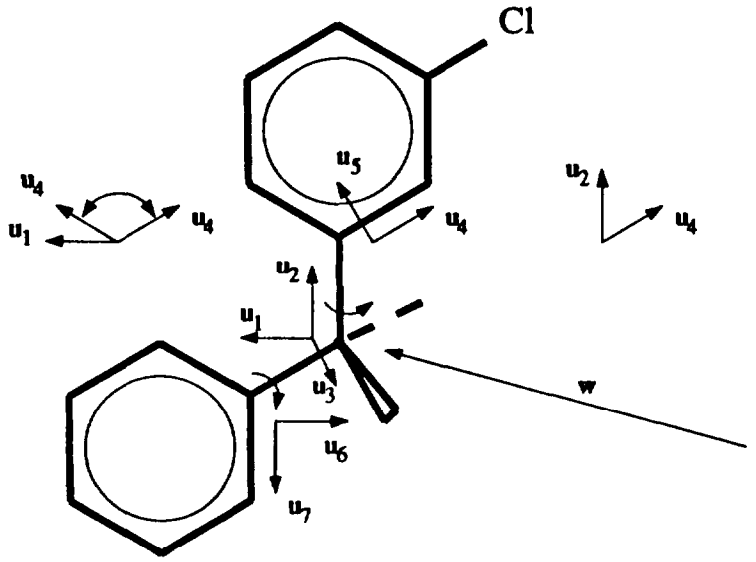

FIGURE 1. *Linearized representation of the rigid valence geometry of a molecule. Note that planar groups require only two-dimensional local coordinate systems.*

energetically accessible. The linearized representation of a molecule automatically incorporates correct bond lengths and angles, and it holds such items as benzene rings rigid. As can be seen in figure 1, some unit vectors, such as 2 and 4, have a fixed dot product by construction; but others, such as 1 and 4, have a range of dot product values as the molecule rotates about single bonds. Thus, we see in figure 2 that the rigid valence geometry is represented as points in the unit vector space, while the entire (energetically or sterically) allowed conformation space can be summarized as a rectangular region in the space of unit vector dot products.

3. Instead of concentrating on the common features of several tightly binding molecules, we construct an explicit model of the binding site. To be able to express precise and indeterminate structural features of the site, we have chosen to represent it as a set of Voronoi polyhedra. These constitute a mathematically convenient way to divide up all three-dimensional space

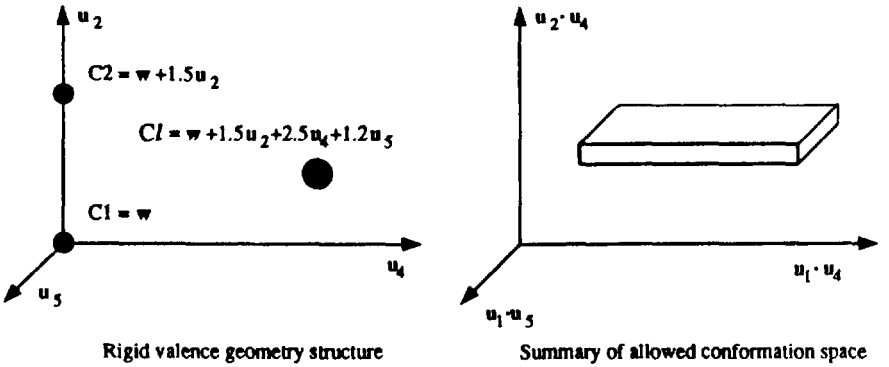

FIGURE 2. *How to describe the molecular structure and conformational flexibility in the linearized representation. Each program should have more than three axes, but these illustrate features seen in figure 1.*

into convex, nonoverlapping regions bounded by planar surfaces. To specify a region, one only needs to choose the Cartesian coordinates of the corresponding "generating point," and then the region is defined as the set of points closer to that generating point than to any other generating point. For example, figure 3 shows a model of a binding site built out of one finite tetrahedral region surrounded by four infinite regions. The central region can be thought of as the empty space inside the real receptor site, the left infinite region may represent the solvent, and the other three might be sterically forbidden regions, representing the walls of the real site. Adding more (finite) regions would add detail to the picture, if justifiable. At the opposite extreme, the trivial site, consisting of a single infinite region, corresponds to interaction with only solvent and puts no geometric demands on the molecule. A two-region site would have a single plane slicing all space in half and might be used to represent an interface between aqueous solvent and a lipid layer.

4. Generally, one fits a model to experiment in a least squares sense to find a single "best" approximation to the given data. There is a dangerous tendency to overinterprat imprecise data and not to realize the range of models that adequately explain the data, especially when there are so few observations that the usual statistical tests cannot be applied. Voronoi binding site models avoid these pitfalls by insisting that the calculated free energy of binding must lie within the experimentally determined range (i.e., anywhere within the error bars) for every molecule in the study. In a study

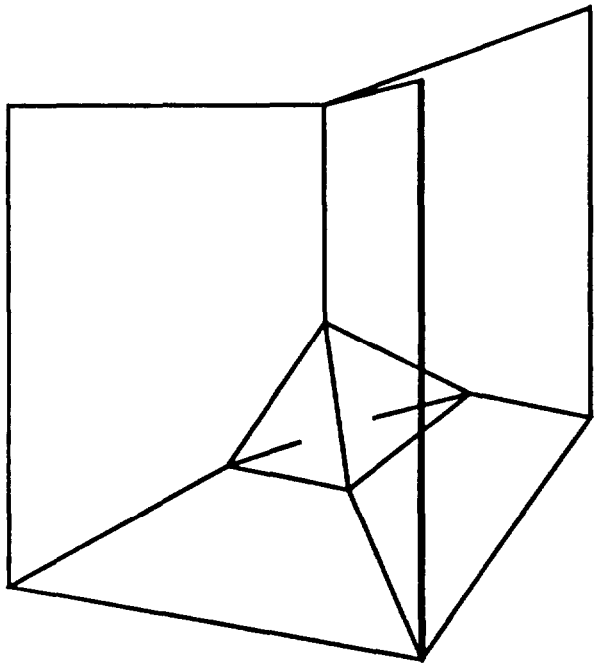

FIGURE 3. *An example of a five-region Voronoi site model consisting of a finite central tetrahedral region surrounded by four infinite regions, indicated by portions of their boundary planes*

on 12 polychlorinated biphenyls binding to prealbumin (Boulu and Crippen 1989b), it was shown that a three-region site model is necessary and sufficient to account for relatively high-accuracy data; but if the assumed experimental error expands beyond a certain point, only a single featureless region is required. There is apparently no other method in the literature that gives this sensible linkage between accuracy of the input data and spatial resolution of the resulting model.

Although it is not immediately obvious, these four ideas tightly interlock mathematically to permit efficient computer programs for generating the linearized representation of a series of ligand molecules, searching over the entire allowed conformation space of each, and examining all geometrically allowed ways each molecule has of fitting into a given Voronoi site model. From this, another program can be used to adjust the energies of interaction between the various regions and the atoms lying within them to achieve the "absolute" fit mentioned above. Not surprisingly, bringing all these computer

algorithms up to a practical level has been extremely time consuming. Since our first version of these programs (Crippen 1987), we have been able to treat molecules with 36 atoms, including all hydrogens so that editing out presumably unimportant atoms is no longer necessary. In addition, many subtle errors have been eliminated from the determination of all allowed binding modes; and this, the most time-consuming part of the calculation, has been speeded up by orders of magnitude.

To explain what we intend to do next with this approach to modeling ligand-receptor interactions, it will help to illustrate the current state of the art on a simple example. Suppose we have a single, rigid, linear molecule having atoms A, B, and C, as shown in figure 4. If the investigator proposes a two-region site, both regions will be infinite and separated by a plane, as shown. The next step is to find all geometrically allowed binding modes, where a mode states the region in which each atom lies. Thus, having all three atoms in region 1 is one possible mode, but with such a low resolution site, there is no attempt to specify where in that region the molecule is located or what its orientation might be.

Even in such a simple example, not all 2^3=8 modes are possible because the linear molecule cannot bend enough to put atom B in one region while keeping A and C in the other. It is convenient to think of enumerating all the allowed modes by traversing a tree of possibilities, as shown in figure 5. Every "leaf" of the tree along the bottom row of the figure corresponds to a placement of the entire molecule in the site in a particular mode, and our programs for doing this come up with a set of Cartesian coordinates for the atoms. For a site with many regions, the tree becomes very broad, and for a molecule with many atoms, the tree becomes very deep. So, it is important to prune the tree at as high a level as possible, with as little computational expense as possible. In this example, the two disallowed modes can be excluded immediately on noting that B is in the convex hull defined by A and C, and Voronoi regions are always convex; we have found similar rules extremely helpful with realistic ligand molecules (Boulu and Crippen 1989a).

Suppose the molecule in this example is nitrous oxide, so that atoms A and B are of type N, and atom C is of type O. If we denote the interaction between region 1 and nitrogen by $\varepsilon_{1,N}$ and so on, then the calculated free energy of binding for a particular mode is assumed to be

$$\Delta G_{mode} = \sum_{\substack{\text{region} \\ r}} \sum_{\substack{\text{atoms } a \\ \text{in } r}} \varepsilon_{r,\,type(a)} \qquad (1)$$

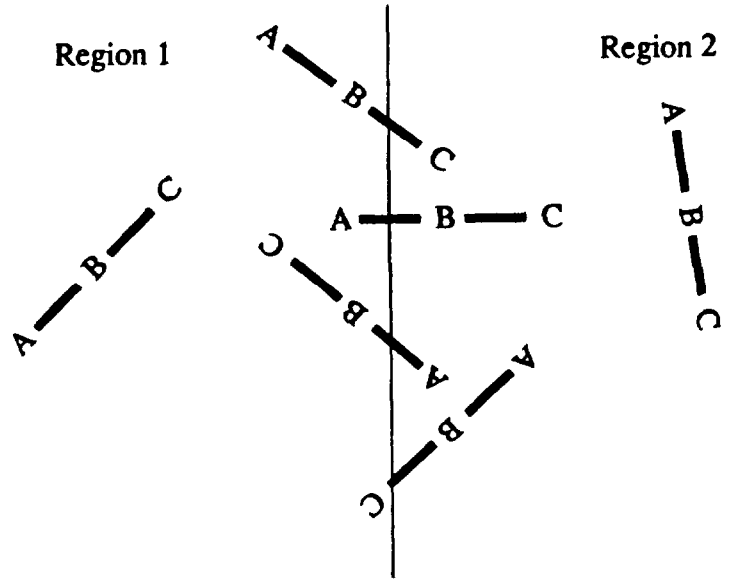

FIGURE 4. *All geometrically allowed binding modes for a linear molecule having three atoms in a two-region site*

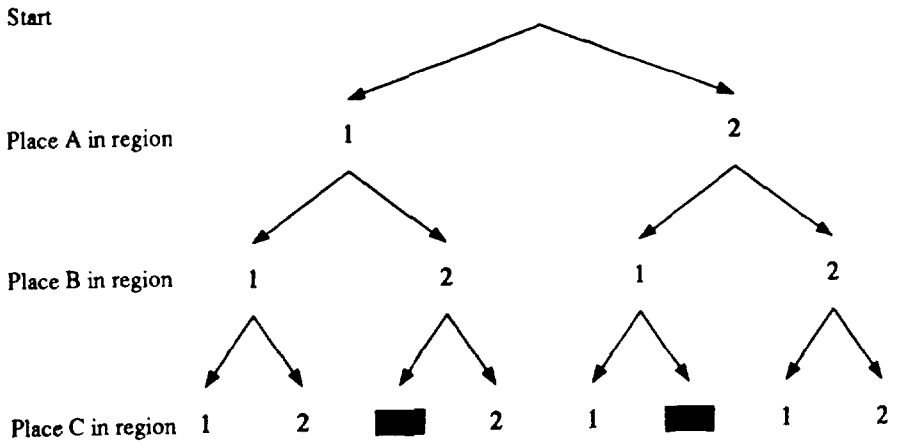

FIGURE 5. *The tree of possible binding modes corresponding to figure 4*

and the molecule will choose the energetically best mode

$$\Delta G_{calc} = \max_{modes} \Delta G_{mode} \qquad (2)$$

(using the sign convention that greater values denote stronger binding). If there is an experimentally determined range of binding, ΔG_- to ΔG_+, then we insist that the model must agree with experiment completely, rather than approximately

$$\Delta G_- \leq \Delta G_{calc} \leq \Delta G_+. \qquad (3)$$

Equation 3 is linear in the εs, so it is relatively easy to adjust the four parameters, $\varepsilon_{1,N}$, $\varepsilon_{1,O}$, $\varepsilon_{2,N}$, and $\varepsilon_{2,O}$, so that equation 3 is satisfied. This can be done by defining a penalty function $F(\varepsilon_{1,N}, \ldots)$ that is zero only when the best mode for each molecule in the study has a calculated binding energy in the correct range. Because the best mode may change when the interaction parameters are varied, the penalty function can be constructed to be continuous, but only piecewise linear (for example, as in figure 6). Note also that even in simple situations, the penalty function can have multiple minima of zero depth, corresponding to different sets of optimal binding modes being equally able to explain the data, or a minimum may not reach to zero, corresponding to almost but not quite satisfying equation 3 for some of the molecules. We have been successful in locating completely correct solutions by the subgradient optimization technique.

To show how this works with real data (Boulu and Crippen 1990), consider the noncovalent, reversible, competitive binding of 10 polycyclic aromatic hydrocarbons to a carcinogen binding protein isolated from mouse liver. The experimental binding data determined by Marietta and coworkers (Collins and Marletta 1984, 1986; Barton and Marletta 1988) includes an estimate of the experimental errors, as shown in table 1.

The given binding data show that the affinity of a ligand increases roughly with the number of carbons and hydrogens until a critical molecular size is reached and then remains constant for molecules bigger than pyrene. Hence, a simple correlation between logP and binding, for example, is impossible. In fact, most methods of QSAR would have difficulty in the initial step of superimposing these compounds to find a pharmacophore, because they do not form a congeneric series. Apparently, there must be a hydrophobic-preferring binding pocket of some limited size, large enough to accept all of pyrene, but not much bigger. Note also that because compound 2 binds better than 5 while having similar numbers of atoms, the central hydrophobic pocket may be narrow. The simplest way to achieve such a Voronoi model is to use five regions, as shown

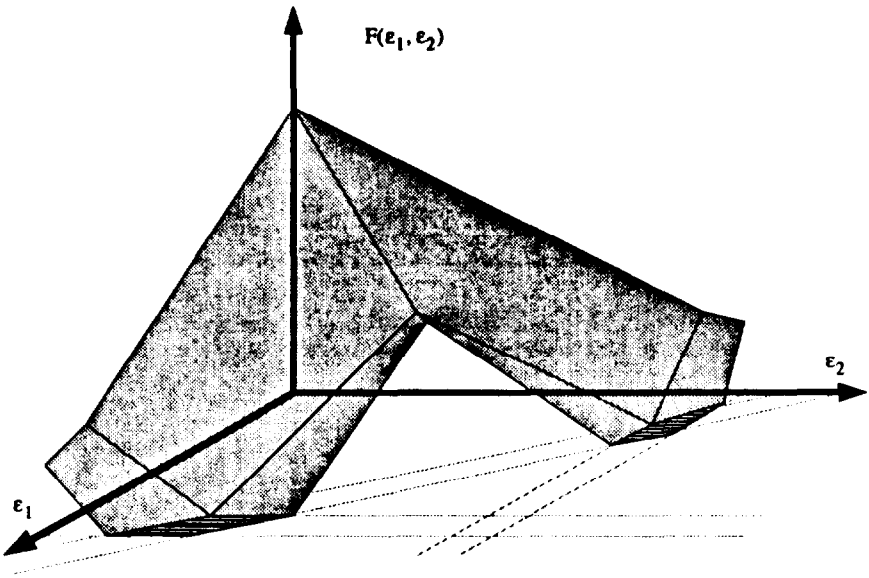

FIGURE 6. *A portion of one example of the continuous but piecewise linear function F that must be minimized to determine the interaction energy parameters—hatched areas correspond to solution sets.*

in figure 3. The central tetrahedron, r_1, is supposed to be the hydrophobic binding pocket; the infinite region opening up on the left, r_2, represents access to the solvent; and the other three regions are a *priori* chosen to be infinitely repulsive, so as to represent the sterically forbidden walls of the binding pocket. After some experimentation, we adjusted the geometry so that the leftward facing side of the inner tetrahedron (which opens out onto the solvent region) is an equilateral triangle having side length 9 Å, and the remaining three edges have length 13 Å. Then the program for determining interaction parameters automatically was able to find the values shown in table 2. An amino group is mildly repelled by the solvent region, but more so by the interior pocket, and C and H atoms experience virtually no interaction with the solvent region. Because we are fitting binding affinities that are proportional to the free energy of transfer from solvent to the bound state, these calculated weak interaction parameters with r_2 are indeed gratifying bonuses.

TABLE 1. *Observed and calculated binding of the compounds of figure 7 for a five-region site shown in figure 3*

Compound	ΔG°_{-}	ΔG°_{+}	ΔG°_{calc}	Optimal Mode[b]
1 benzo[a]pyrene	17.7	19.0	18.9	15C&8H
2 dibenzo[a,c]anthracene	17.3	18.6	18.2	14C&8H
3 chrysene	16.7	18.0	18.0	14C&8H
4 pyrene	16.3	17.6	16.4	13C&7H
5 cyclopenta[c,d]pyrene	15.6	17.6	15.6	13C&6H
6 fluoranthrene	16.7	18.0	18.0	14C&8H
7 1-aminonaphthalene	10.6	11.9	11.6	10C&6H (NH_2 in r_1)
8 2-aminofluorene	13.8	15.1	14.1	11C&7H (NH_2 in r_2)
9 1-aminoanthracene	14.9	16.2	14.9	12C&7H (NH_2 in r_2)
10 9-aminophenanthrene	14.9	16.2	14.9	13C&7H (NH_2 in r_1)

[a] The ΔG are given as in K_i, where K_i is the association constant of the competitor with the receptor.

[b] The optimal modes are given as the number of C and H atoms lying in r_1, the rest being in r_2.

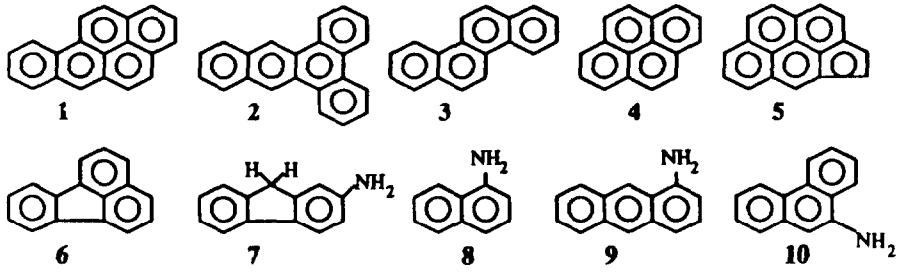

FIGURE 7. *Competitive inhibitors of [^{3}H]benzo[a]pyrene binding. Numbering corresponds to table 1.*

TABLE 2. *Interaction parameters in in K_l units for the site model corresponding to table 1*

Atom	Site Regions r_1	r_2
H	0.81	-0.01
C	0.83	0.03
NH_2	-1.43	-0.65

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A Mechanistic Basis for Design of Selective Agonists for 5-HT Receptors

Roman Osman and Harel Weinstein

INTRODUCTION

Insight into the processes that define receptor function has come from experimental efforts at the cellular, tissue, and whole-organ levels (Black et al. 1987). However, an understanding of the molecular pharmacology of receptors still is hampered by the lack of structural information, at the detailed atomic level, about the receptor and effector molecules responsible for most measurable biological activities. With the development of theoretical approaches and efficient computational algorithms (Dean 1987; Brooks et al. 1988; Beveridge and Jorgensen 1986) the experimental approaches are complemented by molecular models of receptor systems constructed with the aid of theoretical methods. Such models are based on inferences from studies carried out in a variety of disciplines and experimental approaches, including chemistry, molecular pharmacology, enzymatics, and molecular biophysics of proteins (Richards 1983; Fersht 1985; Weinstein 1986, 1987; Dean 1987; Austin et al. 1987; Oxender 1987). It is now evident that theoretical chemistry, in a variety of forms (e.g., quantum chemistry, molecular modeling, molecular mechanics), has provided the formal means to unify the inferences obtained in a heuristic process of learning from the various models about the molecular mechanisms that describe the interaction of ligands with protein targets and the consequences of these interactions (Weinstein 1986; Dean 1987; Brooks et at. 1988). Key components of activity on receptors (e.g., selective recognition and triggering of response) can be understood from such models at the molecular level, based on the properties of the interacting molecules and the results of their interactions.

As reviewed and described below, a combination of theoretical and experimental studies led to the proposed mechanistic models for the selectivity of different 5-HT receptors and the activation of the 5-HT_{1A} receptor. These studies also provided the first indications of the possible mechanisms for recognition and triggering of a response at the 5-HT_2 receptor. The computational exploration of such processes inside proteins that serve as

receptor models, as described briefly in this review and in more detail in previous publications (Mercier et al. 1988a, 1988b; Weinstein et al. 1988; Weinstein and Osman 1989a), demonstrates the significant progress achievable from heuristic models based on solid physicochemical principles and cognate experimental data. Notably, these types of models have made it possible to probe the molecular basis of the observed efficacy of ligands in eliciting the pharmacological responses, thus yielding insight into the molecular details of the difference between agonists and antagonists. Such explorations of activation models provide a new level of understanding of the mechanisms underlying the classical pharmacological properties of agonists, partial agonists, or antagonists and lead to the definition of structural correlates of pharmacological efficacy. They also provide a basis for a rational approach to the design of compounds with predetermined efficacy (i.e., full and partial agonists and antagonists).

HEURISTIC SCHEMES FOR THE STUDY OF RECEPTOR MECHANISMS AND LIGAND DESIGN

Because the mechanism of receptor occlusion by binding appeared to be understood, antagonists have been the subject of rational drug design for some time. In contrast, compounds with agonist activity still are obtained mostly by serendipity or from extensions of known templates through structural analogy. As shown in the heuristic approach to design (figure 1), which was defined recently (Weinstein and Osman 1989b, 1990) the rational design of antagonists is guided by structural correlates of affinity (e.g., the “interaction pharmacophore”) (Weinstein 1975; Weinstein et al. 1978) which is based on the mechanistic hypothesis that certain structural elements of the ligand (components of the pharmacophore) confer selectivity and affinity at receptors through specific types of interactions. The assumption is that these interactions are quantified by the results of receptor-binding assays. In spite of caveats and limitations of this assumption relating structure to measured affinity (Weinstein 1987), this approach to structure-activity relations often has been successful when applied to the design of antagonists. But the situation is more complicated for agonists. Although efficacy, like binding affinity, is readily measurable in pharmacological assays (Clancy and Maayani 1985), there has been no way to identify an “activation pharmacophore” that includes structural elements of the ligand that are involved in eliciting the response. Thus, guidelines for the design of agonists based on connecting molecular structure to efficacy (as opposed to affinity) did not exist. This shortcoming is attributable, at least in part, to the lack of mechanistic models for the way in which ligands trigger a response at the receptor. Simulations of recognition mechanisms as part of a heuristic approach (figure 1) help identify possible molecular mechanisms for receptor activation based on the changes induced in the

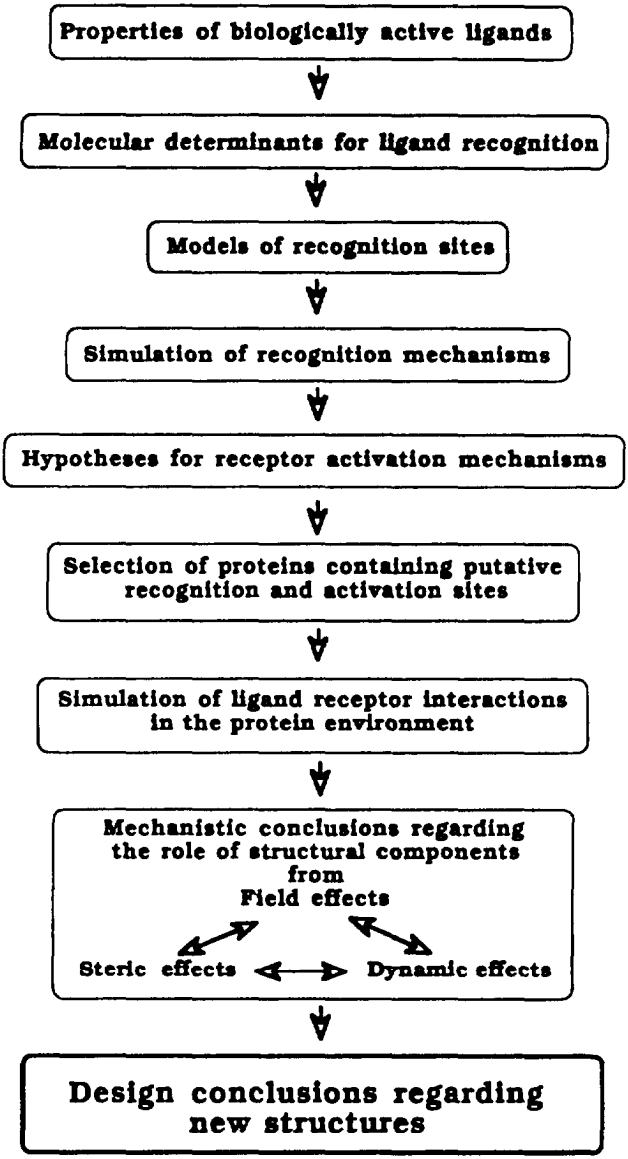

FIGURE 1. *Outline of a hierarchical approach to ligand design based on a procedure of heuristic modeling of ligand-receptor interaction that leads to the identification of structures with predetermined pharmacological properties*

models of recognition sites and thus provide a link to the pharmacological concepts of affinity and efficacy.

Our approach in relating the molecular mechanisms to the classical pharmacological concepts is anchored in the relatively recent formulation of the operational model of agonist action by Black and Leff (1983). In this formalism, the classical pharmacological concept of efficacy has been identifled with a parameter, K_E, that relates to a specific mechanism: the transduction of the agonist-receptor complex into a pharmacological effect. This approach helps overcome a major shortcoming of earlier versions of receptor theory (Clark 1926; Ariens 1954; Stephenson 1956; Furchgott 1964; Furchgott and Bursztyn 1967), which had difficulties in proposing practicable ways to measure efficacy or, better yet, intrinsic efficacy of agonists (DeLean et al. 1978; Kenakin 1982). The operational model of Black and Leff (1983) demonstrated that a hyperbolic nature of the stimulus-effect relationship is a necessary outcome from the hyperbolic nature of the relationship between the pharmacological effect and the concentration of the agonist. This relationship also is consistent with many experimental findings. More importantly, efficacy, named in this model the "transducer ratio," assumed the form $\tau=[R_o]/K_E$, where $[R_o]$ is the total number of receptors and K_E is defined operationally as the concentration of the agonist-receptor complex that yields half-maximal response. Black's K_E differs from Furchgott's ε, which remains unexplained in chemical terms, because it has a clear meaning in well-understood responsive systems such as the activation of adenylate cyclase by ß-adrenergic agonists (DeLean et al. 1978). In such systems K_E becomes the affinity of the agonist-receptor complex to the effector and thus acquires a well-defined chemical identity. As stated explicitly, because "intrinsic efficacy is attributed to chemical binding it makes this property of agonists accessible in the search for new drugs so that efficacy as well as affinity can be associated with the chemical properties of substances and thus incorporated into structure-activity calculations" (Black and Leff 1983). These concepts then can be used to relate the results from pharmacological characterizations of receptor systems to those from the simulations of molecular mechanisms in protein models of receptors. Thus, the expression of drug efficacy through the effects on the energetics of the activation mechanisms should correlate with the efficacy obtained from pharmacological measurements. As described below, this expression of efficacy in terms of the energetics of the underlying mechanism is obtained from the application of methods from theoretical chemistry in computational simulations of the molecular processes occurring at the receptor.

MOLECULAR DETERMINANTS FOR RECOGNITION AT THE 5-HT_{1A} AND 5-HT_2 RECEPTORS

Recognition Elements at the 5-HT_{1A} Sites

The major determinants for ligand selectivity at these receptors were determined in early studies to reside in the electrostatic properties of 5-HT ligands, such as those expressed in the molecular electrostatic potential (MEP) they generate (Weinstein et al. 1979, 1981a). The directionality and the shape of the MEP, in its relation to the positively charged end of the side chain of 5-HT congeners, were shown to be predictive of the relative affinities of these compounds as well as of other classes of compounds, including fused ring structures derived from tryptamine and ergoline derivatives (Weinstein et al. 1987). Experimental testing of compounds designed to probe the spatial relationship among the various molecular determinants for recognition in each molecule showed (Mazurek et al. 1984) that the molecular determinants for 5-HT-like recognition have to be assembled in a specific geometric template. This template was defined from the properties of the more rigid ligands to make it predictive of the activity ranking of new compounds (Weinstein et al. 1979, 1981a).

Topological considerations of the relations between the molecular determinants for ligand recognition at the receptor (i.e., the recognition template) have gained in importance with the recent discoveries of sequence similarities between the genes encoding various subtypes of 5-HT receptors as well as ß-adrenergic receptors. These new findings (Fargin et al. 1988; Julius et al. 1988; Strader et al. 1989) show that the receptor proteins are likely to include anionic binding sites for the cationic side chains of the natural ligands that are similar or even identical (e.g., the conserved Asp observed in 5-HT and $ß_2$-adrenergic receptor sequences). However, the recognition sites in the receptors most likely will discriminate between the ligands on the basis of the topology of their other recognition elements. Studies of this type of topology (Weinstein et al. 1981a; Mazurek et al. 1984) must be continued in the context of stearic probing of the ligand orientation in the receptor recognition site of structural models of the authentic receptors (see below).

To be believable and useful, the proposed molecular determinants for recognition and the specific requirements for the orientation of ligands at the receptor (Weinstein et al. 1981a, 1987) must be consistent with chemical information and the principles of chemical reactivity. Computational simulations based on quantum mechanical methods provide excellent means to test the validity of such a reactivity characteristic. For example, an electrostatic orientation vector has been defined that describes an optimal 5-HT-like

orientation of the electrostatic potential of any ligand designed to mimic 5-HT at the receptor (Weinstein et al. 1978, 1979) and has been tested as to whether it determines the mode of interaction of the molecules with compatible reactants. Because the nature of the recognition elements proposed for the 5-HT congeners and the related compounds was primarily electrostatic (including polarization), chemical intuition suggested the choice of stacking as a likely form of interaction. Experimental data pointed to stacking complexes with imidazolium cation as a specific model (Shinitzky and Katchalski 1968). Using a stacking model of interaction between imidazolium and a large number of 5-HT congeners, the computational simulation of complexation revealed the clear energetic preference for specific mutual orientations in the stacking complexes, thus explaining the success of criteria based on the shape of the MEP and the orientation vector defined from it (Weinstein et al. 1981b). However, the quantum mechanical simulations of the interactions between the various 5-HT congeners and the model recognition site provided more than a validation of the reactivity criteria that were used as molecular determinants (Weinstein et al. 1978, 1981b). They also yielded valuable insight into the consequences of ligand-receptor complexation. Rigorous evaluation of these consequences in terms of energy and structure lead (see below) to the identification of molecular determinants for receptor activation and to criteria for design of agonists.

Recognition Elements at 5-HT_2 Sites

Current evidence shows that the 5-HT_{1A} and the 5-HT_2 receptors are different protein molecules. The effector mechanisms associated with each receptor are also different. The 5-HT_{1A} receptor modulates adenylate cyclase activity in the brain (Shenker et al. 1987; DeVivo and Maayani 1986), whereas the 5-HT_2 receptor stimulates phosphoinositide turnover (Sanders-Bush 1988). The amino acid sequences of the 5-HT_{1A} and 5-HT_2 receptors point to significant differences in specific regions (Hartig 1989) suggesting that different mechanisms might be involved with the recognition process at the two receptors. Binding studies support this idea: 8-OH-DPAT has a high affinity (K_D=1.7nM) for 5-HT_{1A} sites but a low affinity (K_D=7,100 nM) for 5-HT_2 sites. In contrast, ketanserin has a high affinity (K_D=1.4 nM) for 5-HT_2 sites but a low affinity (K_D=1,900 nM) for 5-HT_{1A} sites. Serotonin has a high affinity (K_D=2.9 nM for the 5-HT_{1A} receptor sites and a low affinity (K_D=2,950 nM) for 5-HT_2. We have begun to explore the differences between the recognition processes at the two receptors by comparing the determinants for recognition at the 5-HT_2 receptor to those at the 5-HT_{1A} receptor modeled by the imidazolium cation in our previous work (see section above and references therein). To this end, we explored the interaction of 5-HT and 6-HT with an indole molecule, which represents a possible tryptophan residue, as the ligand-recognition site in the 5-HT_2 receptor.

The energy of interaction (E_{SCAN}) between the indole molecule, modeling the 5-HT_2 recognition site, and 5-HT or 6-HT was scanned over an entire surface by means of an interaction potential summing the electrostatic and dispersion energies of interaction for a given configuration of ligand-indole dimer $E_{SCAN}=E_{QQ}+E_{DISP}$, where E_{QQ} designates the electrostatic component of the energy and E_{DISP} the dispersion component energy. The electrostatic interaction energy was calculated as described before (Weinstein et al. 1981b) with a monopole-monopole approximation using atomic Mulliken charges obtained from a self-consistent field calculation with an LP-3G basis set (Topiol and Osman 1980). The dispersion energy was calculated from the interaction of bond polarizabilities according to the method of Claverie (1978).

The scan of the interaction surface of indole with 5-HT is different from that with 6-HT (Rubenstein and Osman, in press). Each map of the interaction energy as a function of the mutual positions of the ligand (5-HT or 6-HT) and the recognition site (indole) is characterized by a region of weak interaction that spans the indole ring and the hydroxyl group. The difference in the positions of the OH substitution in the two ligands leads to a large difference in the character of the contour maps. When the scanning molecule is close to the oxygen atom, the magnitude of E_{SCAN} is small. As a result of the pronounced effect of the oxygen atom on the shape of the contour map, the position of a local minimum in the 5-HT scan corresponds to a position of weak interaction in 6-HT. For this minimum In the 5-HT scan, the value of E_{SCAN} is -7.39 kcal/mol at an orientation angle of 260°, whereas for the same position in the 6-HT scan, the value of E_{SCAN} is -1.61 kcal/mol at a similar orientation angle of 290°. This difference of 5.78 kcal/mol in the interaction energy comes primarily from the electrostatic interaction. For 5-HT at this position, E_{QQ} is -3.72 kcal/mol and E_{DISP} is -3.67 kcal/mol. The corresponding values for the 6-HT are +2.45 and -4.06, respectively. Thus, the difference in E_{QQ} is responsible for the selectivity demonstrated by the model for a neutral recognition site in the receptor. If one assumes that the interaction geometry of 5-HT relative to the recognition site is optimal at the local minimum identified by the scan, then it corresponds In this recognition model to the high-affinity binding configuration of 5-HT at the 5-HT_2 receptor. Based on the much lower interaction energy of 6-HT at this position, the latter ligand would be expected to have a much lower affinity for the 5-HT_2 receptor because it will have an unfavorable electrostatic interaction with the receptor site. Experimental measurements show a large difference in the affinities of 5-HT and 6-HT for the 5-HT_2 receptor (250 nM vs. >10,000 nM in the isolated rabbit aorta (Clancy and Maayani 1985).

The scanning position that most clearly distinguishes between 5-HT and 6-HT was selected for more detailed investigations of the variation in scan energies with the orientation angle (Rubenstein and Osman, in press). These showed

that the magnitude of E_{DISP} is generally greater than that of E_{QQ} and that the variations of these components of the interaction energy are almost exactly out-of-phase. E_{QQ} varies over a range of approximately 4.8 kcal/mol, whereas E_{DISP} varies only over a range of approximately 1.5 kcal/mol. Consequently, the electrostatic interaction determines the preferred orientation and establishes a molecular correlate of selectivity, whereas the dispersion energy provides the major contribution to the stabilization of the complex (i.e., a major component of ligand affinity; E_{SCF} is -2.70 kcal/mol, whereas the E_{DISP} is -3.67 kcal/mol). This is in clear contrast to the results obtained from the model recognition site of the 5-HT_{1A} receptor—charged imidazolium/the proton-transfer model (Osman et al. 1987)—for which the preferred orientation and the major component of stabilization were electrostatic.

The charge redistribution upon formation of the recognition complex, calculated by integration In planes parallel to the molecules in the complex in a manner described earlier in our publications (Weinstein et al. 1978), shows a depletion of electron density midway between the indole planes. This is attributable to electron repulsion between the molecules. Simultaneously, the electrons in each molecule are polarized in a direction perpendicular to the indole plane, so that the negative end of each induced dipole points toward the region between the planes. The electron density redistribution in the 5-HT/indole complex contrasts sharply with that in the 5-HT/imidazolium cation complex (i.e., the 5-HT_{1A} recognition model), which we reported previously (Weinstein et al. 1978). In the latter case, 5-HT was more extensively polarized (toward the imidazolium), and the imidazolium was slightly polarized away from the 5-HT. In the imidazolium complex, the 5-HT was polarized nearly eight times more than in the complex with indole. The polarization pattern in the 5-HT/ imidazolium complex was shown to be characteristic of a strong electrostatic interaction; in the complex with indole, the electrostatic contribution to the stabilization is much less important.

Assessment of the roles that receptor-recognition models (indole for 5-HT_2 and imidazolium for 5-HT_{1A}) play in the interactions of ligands at 5-HT receptors requires additional experimental information about the structures of the receptor proteins. At the present stage, models of the kind used in the work on 5-HT_{1A} (see below) can help explore whether the demonstrated selectivity of different drugs toward the known 5-HT receptors can be correlated with the discriminating properties of the models. The different mechanisms that are responsible for affinity in the two models—dispersion in the neutral model and electrostatics In the charged model—illustrate the possible basis for specificity of ligands toward the various 5-HT receptors. Such differences can be used in probing the validity of the models (e.g., by simulating interactions with ligands designed to test the ability of the models to discriminate specificities for the

different receptor models and by testing the ability to predict ligand selectivity toward specific receptor sites). Once proven to have predictive value, the models can serve as direct guides in the design of new selective ligands.

AN ACTIVATION MECHANISM OF A 5-HT$_{1A}$ RECEPTOR

The computational simulation of the recognition complex between imidazolium and 5-HT congeners led to the suggestion that interaction with the ligand could induce the imidazolium to donate a proton to an acceptor (Osman et al. 1985). The mechanism was simulated computationally with quantum mechanical methods and was found to constitute a plausible primary event in the activation of a 5-HT receptor (Osman et al. 1987). The simulations revealed the mechanism by which the binding of the ligand to the receptor model lowered the barrier for the transfer of the proton from imidazolium to an acceptor and increased the energetic driving force for the process (i.e., the energy difference between the reactants and the products). More importantly, these quantum mechanical simulations pointed to the properties that made the model capable of discriminating between agonists (receptor activators) and antagonists (receptor blockers). However, because the electrostatic field generated by the ligand was shown to be a crucial factor in the activation and because of specific steric requirements for the mutual orientation of the recognition site and the ligand, the activation model was expected to be affected by its environment in the receptor. The exploration of this effect required the simulation of the activation process inside the protein environment.

The Receptor Activation Mechanism in a Protein Environment

Following the heuristic strategy (figure 1) (Weinstein and Osman 1989b), the lack of three-dimensional structure for a receptor makes it necessary to identify a suitable protein to explore the effect of an environment on the activation mechanism. Actinidin, a thiol proteinase for which the structure is known from x-ray crystallography (Baker 1980; Baker and Drenth 1987) and is available in the Protein Data Bank (Bernstein et al. 1977), was selected for this heuristic study. This protein was chosen because it contains an appropriate juxtaposition of the groups that form the proton transfer complex proposed for the activation mechanism (Osman et al. 1985, 1987) and because it also contains an intrinsic model for an indole-derivative ligand that can be mutated to simulate known and putative ligands (figure 2). One possible proton transfer system in actinidin consists of His-162 hydrogen bonded to Cys-25 through ND1; another consists of Asn-182 hydrogen bonded to NE2 of His-162. The indole side chain of Trp-184, which can mimic ligands that are 5-HT derivatives, is in close proximity to this system and is aligned in a nearly stacking geometry with the imidazole ring of His-162 (figure 2). The structure of the protein is well

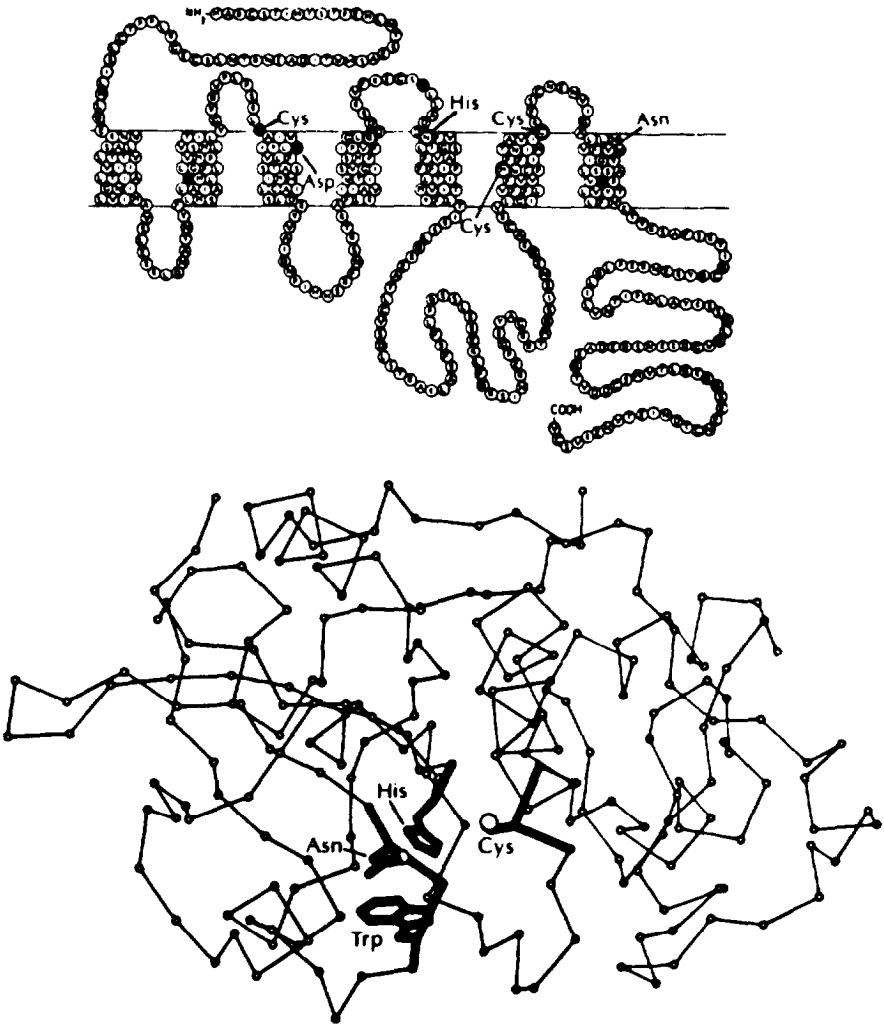

FIGURE 2. *Proposed folding model for 5-HT receptor sequences (fop part of the figure), taken from Hartig (1989), and the α-carbon chain of actinidin, our receptor model protein (in the lower part of the figure). The proton transfer system in the receptor recognition model in actinidin consists of His-162 hydrogen bonded to Cys-25 through ND1 and of Asn-182 hydrogen bonded to NE2 of His-162. The indole side chain of Trp-184, which can mimic ligands that are 5-HT derivatives, is in close proximity to this system and is*

aligned in a nearly stacking geometry with the imidazole ring of His- 162. The side chains of these residues are outlined in the figure over the α-carbon structure of actinidin. As described in the text, the α-helices can modulate the activation mechanism simulated inside the protein. Therefore, it is interesting to note that the tentative model of the folding of the 5-HT_2 receptor—to which we added the indication of the residues specific to the 5-HT_{1A} receptor and their location in the otherwise highly homologous sequence of the 5-HT_2 receptor—places the putative binding sites so that they are surrounded by the transmembranal helices. The same residues considered in the heuristic model to be involved in the proton transfer-based activation model explored inside actinidin are present in a favorable juxtaposition in the folding model shown in the top part of the figure. The His and Asn residues identified in the figure are specific to the 5-HT_{1A} sequence and are not present in the other 5-HT receptor sequences; they flank a Cys residue common to the other sequences and are close to the Asp considered to be involved with the recognition of the cationic side chain of neurotransmitters (Strader et al. 1989 and text).

characterized and contains the same elements of secondary and tertiary structure that have been proposed for a variety of membrane proteins, including the 5-HT receptors (Hartig 1989) (figure 2). The general basis for the heuristic approach to simulate the receptor activation mechanism inside this protein model is the premise that the structural elements (e.g., helices, ß-sheets), which are conserved across different proteins and have similar properties (e.g., electrostatic), also conserve the role they would play in intramolecular processes. It must be emphasized, however, that the proton transfer that simulates the activation mechanism is in a direction opposite to that considered to occur in the enzymatic process of thiol proteases.

Given the nature of the proposed activation mechanism, the strong effect of the protein on the simulated activation process would be expected to come from the electrostatic field it generates in the vicinity of the proton transfer complex. Such effects can be calculated in several variants of the semiclassical approaches combining quantum mechanical methods and classical electrostatics (Dijkman et al. 1989; Mercier et al. 1988b). In this approach, the entire system is divided into a domain described by quantum mechanics (termed “the quantum motif”) and a domain described with classical electrostatics. An iterative perturbation scheme developed recently (Mercier et al. 1989) also includes terms representing the polarization of the protein

induced by the quantum motif and the polarization of the quantum motif induced by the polarized protein (Mercier et al. 1988b). The energy and charge distribution of the quantum motif in the classical electrostatic field can be calculated following either the direct reaction field approach (Dijkman et al. 1989 and references therein) or within the perturbation scheme (Mercier et al. 1989), which is based on the electron group formalism of McWeeny and Sutcliffe (1969).

Results from such calculations (Mercier et al. 1988b, 1989; Weinstein et al. 1988; Weinstein and Osman 1989a) showed that the six α-helices in actinidin (figure 2 in Mercier et al. 1988b) contribute differently to the energy of proton transfer between His-162 and the thiol side chain of Cys-25. For example, the interaction with the largest helix, A1, acts in opposition to the proton transfer through the effect of a large helix dipole, whereas helix A3 affects the proton transfer in nearly equal proportions through the field of its charged residues (which favors the process) and its helix dipole (which opposes it). The effect of the other helices, A2, A4, and A5, reflects mainly the contribution from their charged residues; but when these residues are eliminated in computer-simulated mutations, the effects of their helix dipoles are revealed (Mercier et al. 1988b). These significant effects of α-helical structure indicate the essential role that the helical motifs predicted for the structures of the membrane-bound receptors (figure 2) could play in receptor mechanisms.

The other possible proton transfer model in actinidin also involves His-162 from which the proton can move to Asn-182. This proton transfer process shows greater sensitivity to the ß-sheet structures in the protein (Weinstein et al. 1988; Weinstein and Osman 1989a), but neither the His-162/Cys-25 nor the His-162/ Asn-182 complex is strongly affected by the near neighbors surrounding them. When the effect of the entire protein structure is calculated, the protein environment is found to oppose the transfer of a proton in either the His-162/ Cys-25 or the His-162/Asn-182 systems. Yet it became clear that specific contributions from secondary elements such as the helices, or from specific charged residues, can modulate the magnitude of this effect. It is therefore interesting to note that a tentative model of the folding of the 5-HT_2 receptor (Hartig 1989) places the putative binding sites so that it is surrounded by the transmembranal helices. In particular, a speculative model (figure 2) would indicate that the same residues considered to be involved in the proton transfer-based activation model explored inside actinidin are present in a favorable juxtaposition in the folding model shown in figure 2. Quite intriguing is the fact that the His and Asn residues highlighted in the figure are specific to the 5-HT_{1A} sequence and are not present in the other 5-HT receptor sequences discussed recently (Hartig 1989). They flank a Cys residue common to the other sequences and are close to the Asp considered to be involved with the

recognition of the anionic side chain (Strader et al. 1989). This situation could be fortuitous, but it deserves and will receive additional attention in relation to our models of recognition and activation of the 5-HT receptors, especially in collaborative studies with molecular biologists interested in using selected mutations to probe the residues essential for receptor function.

The finding that the protein structure acts to reduce the probability of the proton transfer supports the use of this protein as a heuristic model for receptor activation, for it shows how the protein can prevent a spontaneous triggering of the receptor activation mechanism. This modulation of the barrier, and the concomitant increase in the energy drive for the reaction (i.e., the difference in energy between the system before and after the proton transfer), is achieved through the difference in the interaction of the proton acceptor, and of the proton donor, with the protein. The difference is attributable to the change in the electronic structure of the quantum motif as a result of the movement of the proton.

More importantly, the calculations described below showed how the barrier to proton transfer may be overcome if the charge distribution of the proton transfer complex is altered by interaction with a ligand. Because not all the ligands will affect equally the electronic structure of the proton transfer complex, they will have different effects on the height of the barrier and the driving energy. On this basis, they will be expected to exhibit different efficacies in activating the receptor (see below).

Role of the Ligand In the Activation Inside a Protein: An Expression of Pharmacological Efficacy

To probe the effect of the ligand on the proton transfer model of receptor activation inside the protein environment, we turned to the His-162/Asn-182 complex because it interacts more closely with Trp-184, which represents the ligand. Like the His-162/Cys-25, the His-162/Asn-182 complex also is not much affected by the near neighbors in the active site. When the entire protein structure is considered, the effect of the protein environment again is found to oppose the transfer of a proton in the His-162/Asn-182 system, as in the His-162/Cys-25 complex. Yet it became clear from calculations (Weinstein et al. 1988; Weinstein and Osman 1989a) that the ligands—simulated by the side chain of Trp-184 or by other structures replacing that residue with side chains representing compounds with known activities at the 5-HT_{1A} receptor—will lower the energy barrier of the proton transfer process. The ligands shown in table 1 lower the energy barrier **(ΔE** Barrier) for proton transfer and increase the driving force **(ΔE** Minima) for this process. (For descriptions of the electronic mechanism involved and the method of calculation, see Mercier and colleagues

1988b, 1989; Weinstein and coworkers 1988.) As shown in table 1, the effect of the listed ligands follows, within each structural group, a rank order that is compatible with the pharmacological data on the intrinsic activity of these compounds. (Note that these results of the simulation of the receptor activation process do not reflect the affinities of the compounds.) The ligand-protein interactions were calculated here without the required optimization of the orientation between the ligand and the recognition (activation) site (Osman et al. 1987). Even in the common orientation, the calculations show that the ligands do not affect equally the electronic structure of the proton transfer complex, so that they produce different effects on the height of the barrier and on the driving force.

TABLE 1. *The effect of 5-HT agonists on the barrier and driving force for the proton transfer from His-162 to Asn-182 inside actinidin*

Ligand[a]	ΔE Barrier[b]	Δ|E Minima[b]
5-OH TRYP	-2.5	-4.4
TRYP	-2.4	-4.2
4-OH TRYP	-2.1	-3.6
6-OH TRYP	-1.4	-2.5
7-OH TRYP	-1.0	-1.8
4,5-MDOX TRYP	-1.7	-3.4
5,6-MDOX TRYP	-1.6	-2.8
5-$CONH_2$ TRYP	-1.5	-2.5

[a]The indole portion of the ligands is superimposed on the indole of Trp-184.
[b]In kcal/mol

As with the effect of the total protein environment on the His-162/Cys-25 complex (Mercier et al. 1988a) and on the His-162/Asn-182 pair (Weinstein and Osman 1989a), the lowering of the barrier for the His-162/Asn-182 complex by the ligands is determined by their effect on the proton donor/acceptor pair and not by an effect on the moving proton (Weinstein et al. 1988; Weinstein and Osman 1989a). This interaction changes with the rearrangement of the electronic structures of the donor and acceptor moieties. It follows that the evaluation of the effect of both the protein and the ligands on the barrier is dependent on the adequate representation of the changes that occur in the electronic structure of the complex during the process of proton transfer.

Taken together, the results show that an exploration of the determinants for the formation of a ligand-receptor complex inside the protein produces information concerning the elements of recognition within the constraints of a protein structure, as well as the conditions necessary to overcome the barrier and to trigger the proton transfer as a result of the interaction with the recognition site. A mechanistic picture emerges in which receptor occupancy by a partial agonist does not result in the proton transfer (the trigger is not activated because the barrier is still too high or because the driving energy is not negative enough; see table 1), so that the conformational change required for interaction with the effector (e.g., G-protein) does not occur. Thus, although occupied, the receptor is not activated and does not produce a response. It is possible to envision a mechanism in which the kinetics of the proton transfer is the rate-limiting element, so that the height of the barrier (and the ability of various ligands to reduce it) determines efficacy. However, at this point we have not distinguished this criterion from the one that would be established if the conformational change induced in the receptor structure as a result of the proton transfer (a change required to increase the affinity of the receptor-effector complex) is the rate-determining step. in the latter case, the energy drive (i.e., ΔE Minima in table 1) would be the discriminant criterion for efficacy. Results in table 1 indicate that at this stage the rank order predicted by both criteria would be the same for the ligands we have considered at this first level of computational simulation.

The receptor activation model we have simulated inside the protein clearly is predictive of a relative pharmacological efficacy. As with the mechanistic picture of partial agonism on ß-adrenergic receptors that emerges from experimental results (Benovic et al. 1988), the present model still must consider the possibility that partial agonism also may result from a partial rearrangement of the structure that would affect the rate of the formation of receptor-effector complexes due to an enhanced rate of reversal of the activated state. This situation also is reflected in the effect of ligand-binding on the energetics of the proton transfer mechanism. A refinement of the proposed mechanistic descriptions of partial agonism will require the computational simulation and analysis of the protein dynamics related to the binding and activation mechanism, which represents a major challenge for the future of such simulations of receptor mechanisms (see Conclusion).

It thus becomes clear how the exploration of the determinants for the formation of a ligand-receptor complex inside the protein produces information concerning the elements of recognition within the constraints of a protein structure, as well as the conditions necessary to trigger a response. As described earlier (Weinstein et al. 1987), information concerning elements of recognition is essential to understand receptor specificity of agonists and antagonists, while

insight into the activation mechanism describes discriminant molecular determinants of agonists (Weinstein and Osman 1989b, 1989a).

CONCLUSION: IMPLICATIONS FOR RECEPTOR CLASSIFICATION AND DISCRIMINANT DRUG DESIGN

The investigatlon of the binding event between the drug-receptor complex and an effector is severely limited as an approach to the elucidation of the molecular mechanism of receptor function by the lack of a three-dimensional structure of a receptor. However, the elucidation of molecular events that can relate the binding of a ligand to subsequent molecular processes leading to receptor activation is helped by the heuristic approach that we developed (figure 1). The molecular events that ensue as a consequence of the binding of the drug to the receptor constitute the trigger for its activation. The simulations can reveal their relation to efficacy by analyzing the changes induced in the surroundings of the recognition site of the receptor by its interaction with the agonist. Identifying these local consequences of ligand-receptor interaction, as has been done in our work on the 5-HT_{1A} receptor, has led to the definition of molecular correlates of efficacy. Such correlates depend on the mechanistic hypothesis concerning the relationship between the interaction of the drug with the receptor and the manner in which the response is triggered. The mechanistic description of pharmacological efficacy that emerged from the simulation of the receptor mechanism according to this hypothesis is based on a quantitative scale, relating the changes that the ligand produces in the triggering mechanism to the structure of that ligand.

The mechanistic hypothesis and its corollaries can be exploited as a solid basis for the design of ligands with predetermined pharmacological properties. The designs can be tested through simulations with the receptor models to refine the structures according to practical constraints imposed by chemical synthesis. Our findings also showed how the approach is conducive (1) to criteria that can be used to discriminate among such receptor subtypes and (2) to their classification according to mechanistic considerations. These criteria are based on differences in the physicochemical nature of the forces that determine the ligand-receptor interactions and their mechanistic consequences and complement our newly gained insight into receptor mechanisms at the molecular level.

As information on the protein sequences of the authentic 5-HT receptors is rapidly accumulating (Weinstein and Osman 1989b; Fargin et al. 1988; Julius et al. 1988; Strader et al. 1989; Raymond et al. 1989), the exploration of the heuristic approach reviewed here becomes more directly pertinent to the specific receptor system and more desirable. However, it is important to note

that a full implementation of this strategy taxes the extreme frontiers of current theoretical methodology and computational power, because it requires simulations of the electronic mechanisms inside proteins and the dynamic changes in the structures and environments of complex macromolecular systems.

The latter is required to obtain a mechanistic picture of the part of the activation process that is responsible for the propagation of the triggering event into the rest of the protein structure. It is reasonable to expect that an understanding of the mechanism of activation of the receptor for interaction with the effector system will require discrete molecular representations of the specific structural rearrangements that cause an increase in the probability of forming the receptor-effector complex. Such a representation will constitute a molecular description of the functional role played by the ligand-occupied receptor in the generation of the response to ligand-binding and may lead to the design of therapeutic agents of a new kind, that is, those addressing directly the receptor-effector coupling mechanism.

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Computer-Aided Drug Design for the Benzodiazepine Receptor Site

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INTRODUCTION

Benzodiazepine (BDZ) receptor ligands are widely used as therapeutic agents in the treatment of anxiety and related emotional disorders, insomnia, and epilepsies and as a premeditation in anesthesia. All of their uses result from a complex and challenging pharmacology. The first characteristic of the pharmacology of these drugs is that the activation of the BDZ receptor has several behavioral correlates, including muscle relaxation and sedative/ hypnotic, anticonvulsant, amnestic, hyperphagic, and anxiolytic effects. The second unique trait of the BDZ receptor pharmacology is the discovery that some chemical agents could induce functional effects opposite to those of the BDZs by binding to the same receptor site (Poic et al. 1982). Hence, the BDZ receptor ligands are categorized as agonists, inverse agonists, and antagonists. Although agonists have the pharmacological profiles indicated above (i.e., similar to diazepam), inverse agonists induce opposite behavioral effects to those of diazepam. Thus, the pharmacological profile of inverse agonists includes convulsant or proconvulsant, anorectic, anxiogenenic, and procognitive actions. Antagonists are able to block equally the action of agonists and inverse agonists. A third characteristic of these drugs is the number of diverse chemical families that can activate or block the activation of the receptor, among which BDZ, ß-carbolines, imidazobenzodiazepines, pyrazoloquinolines, imidazopyridines, imidazopyrimidines, hydroxiquinolines, and triazoiopyridazines are the most thoroughly studied.

The BDZ receptor is part of the $GABA_A$ receptor /Cl ionophore supercomplex (Schofield et al. 1988; Stephenson 1988). Although initial studies seemed to indicate that BDZ binds to the α-subunit and that GABA binds to the ß-subunit of this complex, recently it has been proposed that the α-,ß-, and γ-subunits contribute to the formation of a functional BDZ site (Pritchett et al. 1989). These three subunits, as well as variants of them, recently have been cloned and sequenced. There is evidence that some differences in the subunits may result

in receptors possessing slightly different pharmacologies (Pritchett et al. 1989), although the importance of these diversities to *in vivo* behavioral endpoints still is undetermined. As this relationship becomes clearer, one strategy for development of BDZ receptor ligands with a subset of behavioral properties would be to design analogs with high affinity for only one receptor subtype, using the known receptor sequence information and a knowledge of the distribution of each subunit within the brain.

Ligands of the BDZ receptor also can differ in their ability to activate the receptor; some have been shown to be partial agonists or partial inverse agonists (Petersen et al. 1984). It has been hypothesized that less total receptor activation is required to achieve a complete anxiolytic effect in comparison to the receptor activation needed to produce sedation or muscle relaxation (Gardner 1989). If this is the case, then it is possible that a partial agonist for these endpoints would be a better anxiolytic therapeutic agent than the traditionally sought full agonist.

One of the major areas of interest in this family of drugs is the design of compounds capable of inducing only one pharmacological action, devoid of secondary actions, through the $GABA_A$/BDZ receptor. Therefore, a rational approach to understanding the mechanisms through which each response is elicited becomes essential. To this end, we have been using the techniques of computational chemistry, together with *in vivo* and *in vitro* pharmacology, to identify stereoelectronic modulators of receptor recognition and activation to specific endpoints. The experimental studies have been carried out to ensure consistency of the data used as the basis for identifying plausible stereoelectronic modulators of receptor recognition and activation.

The innovative iterative approach was used to characterize this receptor and the design of new analogs, schematized in figure 1. In this approach, an initial set of *in vivo* and *in vitro* experimental data is generated, which provides the experimental observations on which a molecular mechanism for receptor recognition and activation is based. In this way, three-dimensional (3D) structural and electronic requirements can be developed. Once a working hypothesis is generated, it is used to identify and design new probes for the characterization of the recognition or activation processes. This step has a dual purpose: It provides a further test of the working hypotheses and also can lead to the identification of clinically promising new analogs not heretofore associated with the $GABA_A$/BDZ receptor. The derivation of the hypotheses and the design of new analogs based on it are carried out using the techniques of computational chemistry.

To profit fully from this scheme, the design and selection of new plausible ligands are essential, should be unbiased by subjective preconceptions, and

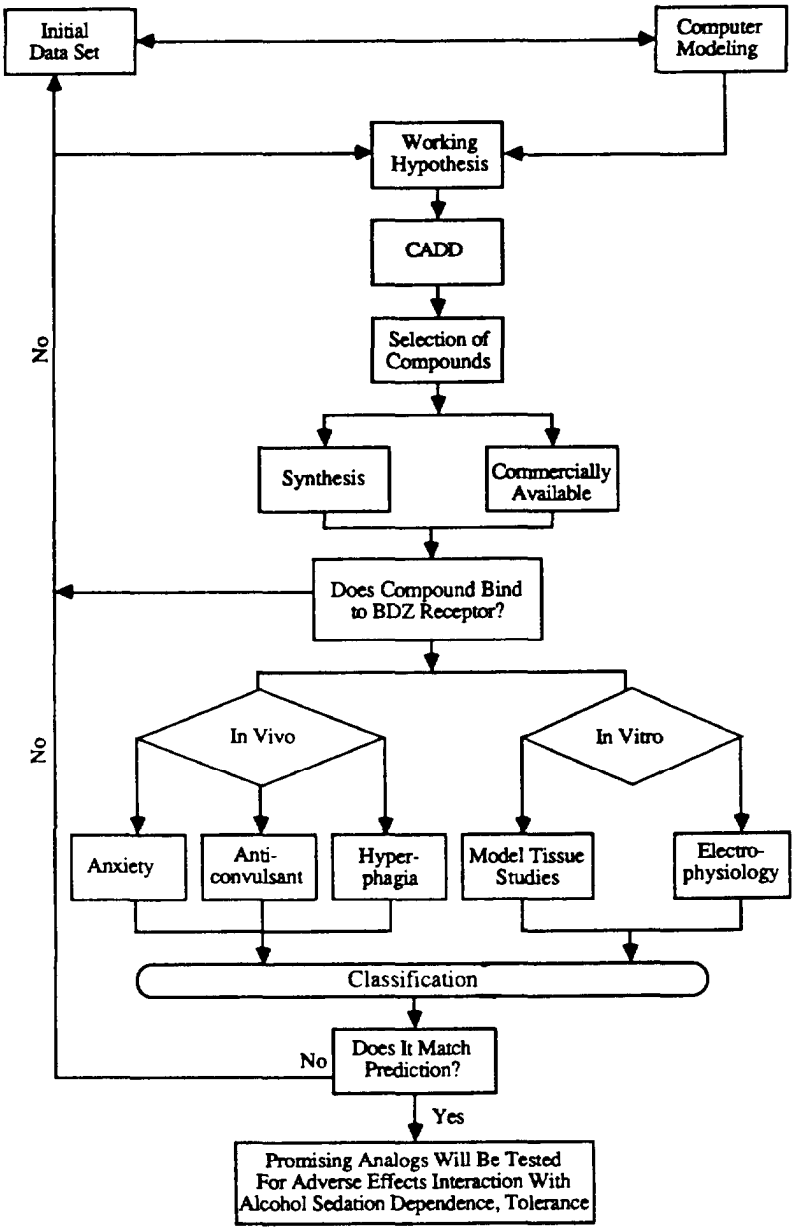


FIGURE 1. *Iterative approach to the selection of new therapeutically useful BDZ receptor ligands*

should cover most chemical families—the next step In this approach to computer-aided drug design (CADD). Promising new analogs are selected in two qualitatively different ways, both using the stereoelectronic properties postulated as modulators of receptor recognition and activation. In the first approach, these criteria are used as the basis for a 3D search of a database of known compounds. This automated, 3D search strategy is the second computational component in the three-component approach to CADD. This particular tool permits the retrieval, from a pharmacological database containing 3D structural information, all compounds fulfilling user-specified 3D stereoelectronic requirements that were developed using computational chemistry. Another way of obtaining new compounds is to use the stereoelectronic criteria identified as the basis for rational modification of known analogs, which then are proposed for synthesis. In either case, whether a compound has been selected from a database or by rational modification of known analogs, a complete theoretical analysis should be performed. This theoretical analysis should be done to verify that the analogs proposed for further testing not only satisfy the steric criteria but also fulfill the electronic criteria for binding and activity.

Successful candidates then either can be acquired, if obtained from the database, or synthesized and subjected to further pharmacological testing. This step also has the dual function of further testing working hypotheses and possibly leading to useful new therapeutic agents. The first pharmacological evaluation is to determine if the therapeutic agents bind to the receptor as expected. If not, they can be used to refine the criteria for receptor recognition. For each compound that has affinity for the receptor, several endpoints for its activation then can be determined. For the BDZ ligands, these behavioral endpoints include anticonvulsant, hyperphagic, and anxioiytic profiles. These responses are used because they allow characterization not only of agonist and antagonist but also of inverse agonist activity. The *in vitro* endpoints include electrophysiological effects on chloride ion channel conductance, because BDZ ligands bind to and modulate the effect of GABA on its chloride ion channel receptor. The endpoints also include the modulation of these ligands on the effect of GABA in model tissues.

All of these pharmacological studies combined should allow a classification of each new compound as agonist, antagonist, or inverse agonist and provide a measure of their efficacy. If the original working hypothesis proves correct and the compounds behave as originally proposed, the procedure should lead to new potentially useful analogs, which should be tested further for detrimental effects (such as alcohol and barbiturates potentiation). Conversely, if a particular compound fails to elicit the predicted pharmacological actions, the

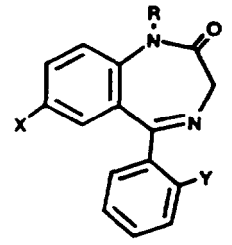
new information should be added to the initial pool of data to refine the existing model or help in producing an entirely new hypothesis.

The stereoelectronic properties of ligands identified as important for recognition and activation also can be used to further probe, by complementarity, the structural characteristics of the receptor-binding site. We have developed a program (RECEPTORSEARCH) that searches the database of proteins with known structure for those with binding site motifs that satisfy these requirements. These proteins can serve as heuristic models for the receptor and allow the explicit characterization of drug-receptor interactions, as well as an evaluation of the role of the protein in them. Thus, this iterative, interdisciplinary approach to rational drug design embodies three closely related computational components: the techniques of theoretical chemistry, 3D searching strategies for ligands, and complementary 3D searching strategies for their macromolecular receptors.

The particular example of this interdisciplinary, three-component computational approach described in this chapter is based on experimental data for the anticonvulsant and binding studies of the BDZ receptor ligands generated in our laboratory. Using these data, we have developed a working hypothesis for the recognition and activation of the receptor in terms of electronic and 3D structural requirements using the techniques of theoretical chemistry (Villar et al. 1989). The development of such hypotheses, central to the approach proposed, is described in the next section. The other two computational tools we have used—3D searching strategies for potential BDZ ligands and for heuristic receptors for them—have been applied for the first time to drugs of abuse. Both techniques are described in the final two sections using the BDZ receptor ligands as an example.

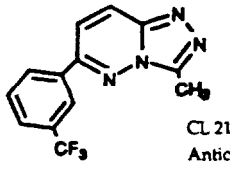
MOLECULAR DETERMINANTS OF BDZ RECEPTOR AFFINITIES AND ANTICONVULSANT ACTIVITIES

We have determined convulsant activity profiles and receptor-binding affinities for 15 compounds from five different chemical families that are known to bind to the BDZ receptor (Villar et al. 1989). These compounds were evaluated to obtain an initial set of consistent pharmacological data for modeling purposes. The analogs selected belong to diverse chemical families; for each of those families, compounds with different pharmacological profiles were included. Their structures and profiles are shown in figure 2. The compounds were studied using the MNDO/H semiempirical technique (Goldblum 1987) and several properties were determined for each molecule.

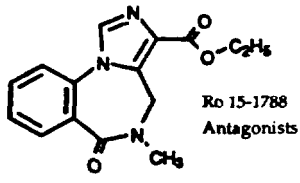


	X	R	Y
Flunitrazepam	NO_2	CH_3	F
Diazepam	Cl	CH_3	H
Prazepam	Cl	$CH_2C_3H_5$	H

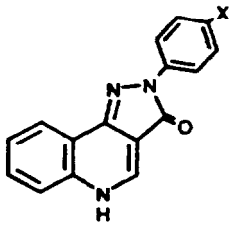
Anticonvulsants



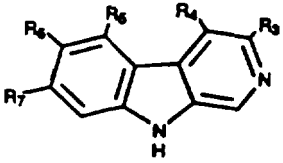
CL 218,872
Anticonvulsant



Ro 15-1788
Antagonists



	X	
CGS 8216	H	Proconvulsant
CGS 9895	OCH_3	Antagonist
CGS 9896	Cl	Anticonvulsant



	R3	R4	R5	R6	R7	
ZK 91926	$COOC_2H_5$	CH_2OCH_3	$OCH_2C_6H_5$	H	H	Anticonvulsant
ZK 93426	$COOC_2H_5$	CH_3	$OCH(CH_3)_2$	H	H	Antagonist
β-CCN	CN	H	H	H	H	Antagonist
β-CCE	$COOC_2H_5$	H	H	H	H	Proconvulsant
β-CCM	$COOCH_3$	H	H	H	H	Proconvulsant
FG 7142	$CONHCH_3$	H	H	H	H	Proconvulsant
DMCM	$COOC_2H_5$	H	H	OCH_3	OCH_3	Convulsant

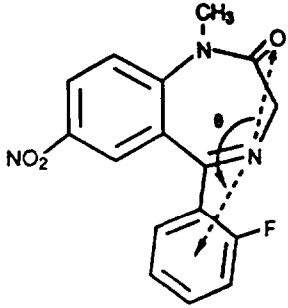
FIGURE 2. *Fifteen analogs that constituted the initial set of compounds to be pharmacologically evaluated and modeled*

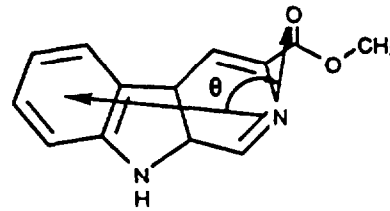
Among the properties calculated and examined for their relevance were polarization volumes and partition coefficients as an indication of the strength of dispersion forces, as a measure of probability of stacking, and as a measure of the extent of the hydrophobic interactions. These properties did not seem to correlate with the experimental results for activation or recognition. In general, ail compounds had large partition coefficients, indicating a highly lipophilic binding site (Villar and Loew 1989).

Heats of protonation at competing sites of each analog also were calculated. These quantities were used as a measure of the strength of the interaction of different proton-accepting atoms in the drug with proton-donating receptor subsites. The analysis of the results for the set of compounds chosen indicated that there were at least two proton-accepting sites at an approximately constant distance of 3.5Å for all analogs. Such consistency in the distance between the two proton-accepting sites suggests that both sites could be involved in receptor recognition. Further plausibility of this hypothesis is provided by the observation that affinity is reduced greatly when one of the proton acceptors is blocked or not present, as in the ß-carboline FG-7142, where the ß-N forms a competing internal hydrogen bond.

A third feature common to all compounds in this initial set is the presence of a highly lipophilic region. Whereas the two proton-accepting sites appear to modulate recognition, the relative orientation of the most lipophilic region of each compound with respect to these two sites seems to modulate activity. As shown in figure 3, the angle formed by the two proton-accepting sites and the lipophilic area clearly discriminates among agonists, antagonists, and inverse agonists. The angle was found to be 140° to 150° for agonists, 105° to 110° for antagonists, and 95° to 100° for inverse agonists. These three different sets of values indicate that when anchored by two common recognition sites, the aromatic rings of all agonists, antagonists, and inverse agonists in the diverse chemical families would occupy unique regions of the receptor according to their pharmacological profile. Thus, the different spatial positions occupied by the lipophilic ring appear to modulate the activation mechanism. This aromatic lipophilic ring wmmon to all high-affinity analogs could participate in stacking interactions, with an aromatic side-chain at the receptor-binding site. The nature and consequences of this interaction could be a determining feature in the pharmacological action of the drug.

These criteria identified for BDZ ligand receptor recognition and activation imply, by complementarity, that the receptor-binding site has two proton-donating amino acids and an aromatic residue in particular spatial orientation. In addition, these results imply a sterically inaccessible region of the receptor. No high-affinity binder to this site, in the classes studied, has lipophilic groups





Agonists	θ	Antagonists	θ	Inverse Agonists	θ
Flunitrazepam	142	ZK 93426	103	β-CCE	95
Diazepam	143	Ro 15-1788	115	CGS 8216	97
CGS 9896	141	CGS 9895	115	β-CCM	95
Prazepam	147	β-CCN	114	FG 7142	99
CL 218, 872	148			DMCM	95
ZK 91296	150				

FIGURE 3. *Position of the most lipophilic ring relative to the two most favorable proton-accepting sires. The angle q is displayed for flunitrazepam and ß-CCM as examples.*

occupying areas of the receptor corresponding to values between 110° and 140° the orientation angle with respect to the two recognition sites. This gap suggests the presence of a sterically inaccessible region of the receptor.

Thus far, our studies agree with our own and other previously published models regarding BDZ receptor recognition (Loew et al. 1984, 1985; Codding and Muir 1985; Fryer et al. 1986; Tebib et al. 1987; Borea et al. 1987). There is widespread agreement in implicating two proton-accepting sites in recognition, but no previous study has characterized them for these diverse families. Additional sites also could be involved in recognition (Alien et al. 1988) although there is no compelling evidence for this idea. Our model also has identified for the first time specific candidate modulators of activity—supporting previous notions that interaction of the ligand with different regions of the receptor somehow should be involved in the activation mechanisms for agonism and inverse agonism. No previous model has explicitly defined semiquantitative

stereochemical requirements for both activation and recognition, as described in the previous paragraph. However, the model obtained was based on certain assumptions, and its validity is subject to them.

The first important assumption is that at physiological temperatures the ligands studied are binding to either a single receptor subtype or to several similar ones with equal affinity and that the anticonvulsant effect observed is elicited by binding to such a site(s). The question of $GABA_A$/BDZ receptor heterogeneity is the subject of current intense pharmacological and molecular biological investigations. Our own and previous receptor-binding studies at 0°C provide strong evidence for receptor heterogeneity (Toll et al. 1984; Sieghart and Karobath 1980; Braestrup and Nielsen 1981). It is interesting to note that receptor-binding studies at higher temperatures consistently indicated that receptor heterogeneity becomes undetectable at more physiologically relevant temperatures (Gee and Yamamura 1982; Gee et al. 1984; Villar et al. 1989). Because only one site was found at 25°C in our competitive binding studies, it was assumed that all the compounds were acting through this site. If the further pharmacological studies that are planned (which include additional analogs) reveal the existence of $GABA_A$/BDZ receptor heterogeneity at physiological temperatures, the current model will be refined to include identification of criteria of recognition and activation at each site. In an intensive effort, the techniques of recombinant DNA are being applied actively to the $GABA_A$/BDZ receptor and have led to the cloning and sequencing of at least 10 variations of subunits that currently are being expressed and tested for functionality in various combinations (Levitan et al. 1988; Schofield et al. 1988). The information from these efforts appears to be consistent with the need for three subunits to produce a functional chloride ion channel that can be modulated by BDZs. However, the evidence for either functionally significant heterogeneity or for the minimum number and nature of the subunits needed is not conclusive.

Another assumption made is that. because no endogenous ligands for this site have been characterized fully, no attempt was made to include its effects in our studies. Current information in this matter is also inconclusive (Davis et al. 1985; Gardner 1989). If endogenous ligands eventually are identified, the behavioral data should be reinterpreted to account for the interplay between the administered drug and the endogenous ligand. The existence of these and other possible reasons for the limitation of our current model is why an iterative approach to the design of new drugs is important. The procedure outlined in figure 1 makes optimum use of the information existing at any one time in the study of receptor-ligand interactions and provides the means of making use of new developments.

in the spirit of this iterative approach, we have estabilshed a set of stereoelectronic requirements for binding and activation of the BDZ receptor. As described in the next sections, these criteria have been used in the identification and design of new analogs for pharmacological evaluation. The new drugs are both suggested modifications of the existing families and more innovative, entirely new classes that have been identified using recently developed computational chemistry tools such as 3D searching strategies and searches for heuristic models for the receptor as described below.

DESIGN OF BDZ RECEPTOR LIGANDS BASED ON 3D SEARCHING

The use of chemical databases is becoming standard in pharmacological and chemical research, but so far their use has been limited mostly to the industrial environment (Martin et al. 1988; Sheridan and Venkataraghavan 1987; Borman 1989; Sheridan et al. 1989). The defining feature of a chemical database is the capability of storing and retrieving information based on a two-dimensional (2D) chemical structure (connectivity) for all the entries. The 2D information ensures that only one representation is associated with each entry. The information stored constitutes the identifier (IDtype) for a given compound. This IDtype then is used to recover all pharmacological and physicochemical information stored as data types. When 3D structural information for each compound is included as an additional data type, a 3D database is generated. The necessary 3D structures could come from a variety of sources because they must be generated in record numbers. Experimentally, techniques such as single-crystal x-ray diffraction structures are widely used. There is also a rapidly growing use of a computer program called CONCORD for the determination of these structures. This program uses artificial intelligence and energy-minimization techniques to produce structures of medium-size molecules in a few seconds. The speed of this program for the determination of such structures makes the generation of 3D databases (Borman 1989) a reasonable endeavor.

The 3D structural information in a 3D chemical database could be used to retrieve the stored structures for structure-activity relationship purposes or as a starting point for quantum mechanical calculations. More creatively, a 3D database could be searched to retrieve all compounds with given structural features; that is, given 3D stereoelectronic requirements for a certain activity or affinity at a given receptor, the information in the database could be searched to retrieve all compounds that fulfill such requirements. The tool required for such purpose is called a 3D searching algorithm.

Systems that combine 3D databases and 3D searching capabilities have been described in the literature. The searching strategy could be different and even

could be based on different criteria such as shape-similarity algorithms. In particular, we have been offered the use of the system developed under the supervision of Dr. Yvonne Martin at Abbott Laboratories. This system consists of the MENTHOR database (Martin et al. 1988) derived from the MedChem software developed by DAYLIGHT information Systems, and a 3D searching algorithm developed at Abbott called ALADDIN (Van Drie et al. 1989). The 3D structures stored in the MENTHOR database were generated using the CONCORD software. The molecular determinants described in the previous section provide the necessary input for a 3D searching strategy.

The 3D searching can be used with two basic purposes: (1) selection of new compounds that fulfill the requirements of the model but have never been tested in relation to the binding site in question or (2) validation of the proposed model pharmacophore.

The first purpose is straightforward. As a result of 3D searching in a reasonable size database, new plausible probes for the characterization of the site could be retrieved. These compounds then should undergo pharmacological screening. The validation of a proposed model can be achieved by its success or failure in (1) finding families of compounds known to bind to the specific receptor but not originally included in the development of the model and (2) finding compounds that fulfill the criteria but do not bind to the receptor.

In these studies, we have applied this automated capability for the first time to drugs of abuse. A 3D search was performed on an extensive database of known compounds using our stereoelectronic criteria for BDZ ligand-receptor rewgnition and activation. Specifically, we have retrieved 200 compounds using the criteria of 3.0 Å to 4.2 Å separation between two proton-acceptor centers, an angle of 140° to 150° with a lipophilic aromatic ring for agonists, and 90° to 120° for antagonists and inverse agonists. Among the compounds retrieved were triazoiam, alprazolam, and brotizoiam, all well-known agonists at the BDZ receptor belonging to chemical families unrelated to the five initially used in the derivation of the model. Furthermore, most of the 1, 4 BDZ and additional compounds from the other families included in the study were obtained as a result of the search. As a further test of our hypothesis, a retrieval of compounds with disallowed angle values between 120° and 140º also was made, resulting in the retrieval of some low-affinity binders. Specifically, these were purine-like compounds, with angles between the two proton acceptors and the lipophilic ring of 132° to 136°. That compounds only with no or low affinity were found (if they contained iipophilic groups forming an angle of 120° to 140° relative to the proton acceptors) reinforces the hypothesis of a sterically hindered region in that area of the receptor. Other families of

compounds retrieved included known antibiotics, which could be ruled out easily because of their highly hydrophilic nature.

Taken together, these findings clearly reinforce our working hypothesis. Hence, this procedure affords an easy first step in the validation of hypothetical requirements for high-affinity binding to a receptor, before starting the more expensive and time-consuming stages of synthesis and evaluation of new analogs. This type of validation procedure also provides an alternative to extensive animal studies, greatly reducing the number of such studies needed to develop a satisfactory model for drug-receptor interaction and activation. Moreover, the large number of chemical families included in a database decreases the possibility of an incorrect hypothesis resulting from a biased initial set of compounds. The 3D-searching procedures used also allowed the identification of six promising new families of compounds not yet considered to be ligands for the BDZ/$GABA_A$ receptor. This process is applicable to any drug-receptor system, provided a well-considered hypothesis about the mechanism of activation and recognition has been developed.

SEARCH FOR A HEURISTIC MODEL FOR DRUG-RECEPTOR INTERACTION

The same criteria developed for ligand requirements for activation or recognition at a receptor can be used, by complementarity, for the purpose of finding a heuristic model for the receptor. The known crystal structures of proteins (mainly soluble enzymes) and ligand-protein complexes could serve as models for other biologically important proteins, including receptors. These models could be used for explicit characterization of ligand-receptor interactions and could help explain the role of the protein structure beyond the binding site in both the recognition and activation processes (Weinstein 1987; Weinstein et al. 1985).

Because multiple subunits of the $GABA_A$/BDZ receptor have been cloned and sequenced, it also should be possible to attempt a completely different approach to building a 3D structure for the binding site of the BDZ ligands. This approach would use current biochemical and pharmacological information about the position and nature of the binding site, as well as information on the different subunits of other ligand-gated ion channel receptors. This information would serve as a guide in the use of the diverse techniques of protein modeling to obtain a verifiable description of the binding site. If validated, this approach could provide a more realistic description of the actual binding site than the one provided by a heuristic receptor model. However, the BDZ receptor ligands require at least three receptor subunits for them to bind and modulate the effects of GABA on the ion channel. This finding indicates that the problem of

modeling the site could involve consideration of more that the interaction of the ligands with a single subunit of the receptor. In collaboration with Dr. Victor Cockcroft (trained in the laboratory of Dr. Barnard), we have begun the first step in such a study; Dr. Cockcroft studies the molecular biology and functionality of the receptor. Specifically, we are beginning to construct and test candidate BDZ-binding regions in the N-terminal extracellular regions of the α-1 and γ-2 subunits of the $GABA_A$ receptor.

RECEPTORSEARCH can search the protein data bank (or any other data bank of known structures of macromolecules) for structures of plausible heuristic receptor models containing a binding site with user-specified criteria suitable for any class of ligands. This searching procedure forms the third component of the CADD approach. It complements the searching procedure, ALADDIN, used to identify new ligands. ALADDIN searches a database of known drugs, trying to find compounds with specific stereoelectronic requirements that are postulated to be important for recognition and/or activation. In the RECEPTORSEARCH program, we use complementarity criteria for the receptor-binding site to search the protein data bank for macromolecules with 3D arrangements of functional groups that could lead to a favorable interaction with the drug and thus serve as models for the binding site.

The features characterized by RECEPTORSEARCH are distances and orientations among proton-donating or -accepting groups, as well as possible centers for stacking, such as aromatic rings. In the case of centers with the capability of docking by hydrogen bonding, the program also checks for directionality (i.e., proper orientation of the hydrogen donor or acceptors in the macromolecule).

For both types of criteria, distance and orientation, a small conformational change in the crystal structure found in the database could make possible an otherwise unfavorable interaction. To allow for such flexibility, a range of acceptable values can be specified for acceptance or rejection of a certain crystal structure as a plausible model.

We have used the RECEPTORSEARCH program to search the protein crystal structures contained in the Brookhaven Databank for a heuristic model for the BDZ receptor. The input data provided to the program were the positions of the two proton-donating sites, as well as that of the center of the aromatic ring of the receptor that could take part in stacking with agonists. Using these strict criteria, deoxymyogiobin (deoxyMb) was the only protein that fulfilled the requirements for the interaction of BDZ agonist ligands. The residues involved in recognition according to this model were HIS 113, ARG 31, and SER 35. To further characterize and test this site, fiunitrazepam was docked in it. As shown in figure 4a, the histidine residue can participate in a stacked complex with the

most lipophilic, fused benzene ring, while the arginine and serine provide the two proton-donating residues necessary for recognition. Other residues, such as LEU 32 and 29, PHE 33, ILE 28, ASP 27, GLN 26, and SER 117, make up the ligand-binding pocket. The interaction of the lipophilic ring with the histidine could constitute the charge-transfer complex that we have implicated in activation. As seen in figure 4b, the lipophilic ring of the antagonist, ZK 93426, docked in that site does not interact with the histidine and could not form such a charge-transfer complex.

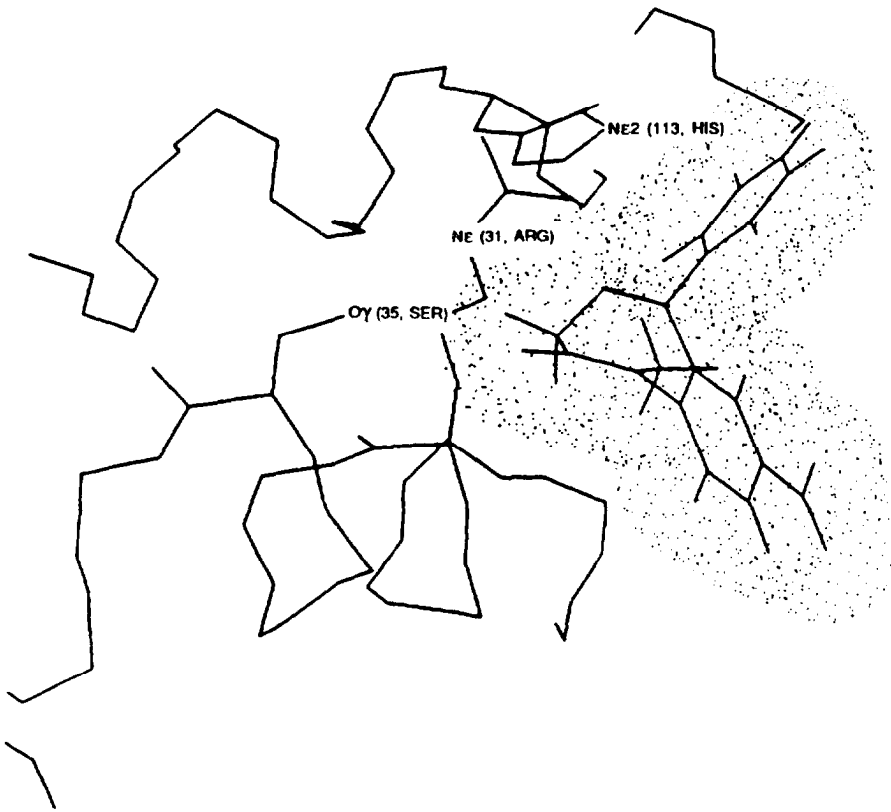


FIGURE 4a. *DeoxyMb provides a heuristic model for the BDZ receptor-ligand binding site. Two proton-donating residues, ARG 31 and SER 35, constitute the common recognition sites. Flunitrazepam is displayed in the cavity. The interaction of its freely rotating ring with an electron-donating residue such as HIS 113 would provide the activation mechanism.*

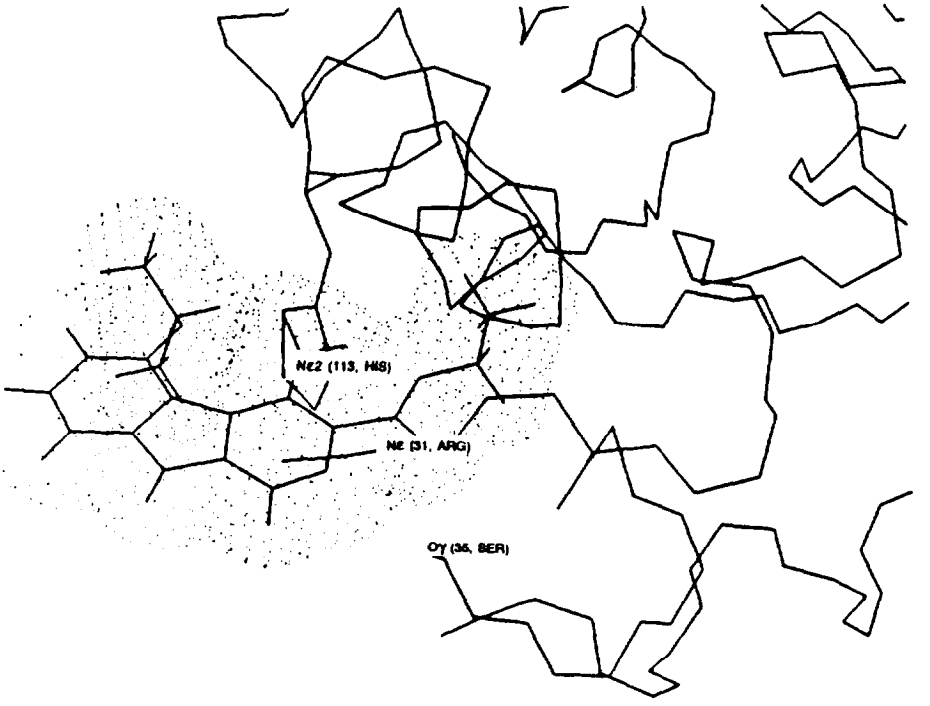


FIGURE 4b. *The antagonist ZK 93426 is represented. In this case, there is no interaction with the HIS 113 residue*

The site found in deoxyMb is in a highly hydrophilic area on the surface of the protein. it is known that BDZ ligands bind to the extracellular N-terminal region of either an α- or γ-subunit of the $GABA_A$ receptor. Thus, it is possible that a region near the binding site is exposed to an aqueous environment near the surface of the receptor. However, most BDZ ligands are highly lipophilic, which is an indication of the nonhydrophilic nature of the binding site of these compounds. Thus, this particular model may not be entirely useful in understanding the changes induced in the macromolecule because of its interaction with the ligand. Nevertheless, this model could provide some insights into the mechanism of interaction.

An additional plausible component of the activation mechanism is that agonists also could induce a change in the conformational state of the macromolecule to allow for stacking between the lipophilic ring and the aromatic residue of the macromolecule. Allowing for this possibility, we have found two additional

heuristic models, an FAB immunoglobin and a tripsinogen with more hydrophobic, buried binding sites. We are in the process of characterizing the docking of diverse ligands to the binding sites identified in these models.

CONCLUSIONS

This chapter describes a model that characterizes plausible molecular modulators of receptor recognition and activation for the BDZ receptor ligands. These modulators are expressed in the form of 3D structural and electronic requirements for interaction as well as activation. The structural requirements of the model then were used to perform 3D searches for other potential BDZ ligands, using a 3D database. Among the retrieved structures were those of known BDZ receptor iigands that were not included in the original set used to develop the working hypothesis. This finding constitutes a validation of the hypothesis developed. The same hypothesis then was used, by complementarity, to search for a heuristic model for the BDZ receptor. To achieve this goal, a program called RECEPTORSEARCH was developed that searches a data bank of protein-crystal structures for macromolecules that satisfy certain user-supplied criteria (i.e., those derived from the working hypothesis). As a result of this search, deoxyMb (and possibly two other proteins) was found to have the necessary features for a favorable interaction with the BDZ ligands. Because the proposed binding site in deoxyMb is on the surface of the enzyme, it may not constitute the most desirable description for the site.

The interdisciplinary mechanistic approach to CADD described includes three computational components embodying the techniques of theoretical chemistry and 3D ligand and macromolecular database searching. This integrated approach has been applied for the first time to a family of drugs of abuse. The methods and protocols used can be applied to any other drugs of abuse or, in general, to the study of therapeutic agents that exert their effect by binding to specific receptors.

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3D-Searching: An Overview of a New Technique for Computer-Assisted Molecular Design

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INTRODUCTION

This chapter provides a rudimentary introduction to the fundamentals of three-dimensional (3D)-searching—a powerful new technique for computer-assisted molecular design (for a more detailed review, see Martin et al. 1990). Following brief remarks relating this technique to other more familiar techniques, there is a brief indication of the essential software components required to implement 3D-searching capabilities. Although the original presentation at the National Institute on Drug Abuse (NIDA) Technical Review in Washington, DC, concluded with an example of an application to drug design, the reader is referred to another chapter in this monograph (Loew et al.) for an interesting example. This chapter closes with a prospective look at the future of 3D-searching and some cautionary comments regarding its use.

3D-SEARCHING—ITS RELATIONSHIP TO OTHER TECHNIQUES

In most cases, the design of novel herbicides, pesticides, or therapeutic agents involves the search for molecular ligands that effectively bind to a biomolecular receptor. Ligand-receptor binding depends on the chemical and geometric compiementarity of the ligand and receptor. For example, if the receptor site contains a hydrogen-bond donor then the iigand should contain a hydrogen-bond acceptor; if the receptor site includes a region of significant positive charge then the ligand should contain a complementary region of negative charge. Moreover, these chemical features of the ligand must be positioned relative to each other in 3-space so that they can all interact with the corresponding features of the receptor in an optimal or near optimal fashion. In the context of drug design, the specific chemical and geometric criteria leading to significant drug-receptor affinity are referred to as the pharmacophore.

Usually, the search for novel bioactive compounds is undertaken after one or more active compounds already is known. Thus, one important aspect of molecular design involves extending known “leads”—modifying and exploring within a class of chemical structures, some of which are already known to possess the desired activity. However, another important aspect of molecular design involves the search for new lead compounds—new chemical classes that also meet the chemical and geometric criteria necessary for significant ligand-receptor binding. Discovery of new leads is usually far more difficult than extension of existing leads.

By performing screening procedures on a computer rather than in the laboratory, computer-assisted molecular design can increase greatly the efficiency of the discovery/design process. Computer-assisted molecular design can be performed at different levels (involving different techniques) depending on the level of knowledge regarding the structure of the particular receptor.

if the 3D coordinates of all atoms in the receptor are known, then the stability and structure of the ligand-receptor complex can be investigated using techniques generally referred to as “molecular modeling.” These techniques involve calculation of ligand-receptor interaction energies and computer graphic display of the ligand-receptor complex. The computer graphic display provides invaluable qualitative insights that can assist the chemist in imagining how modification of the ligand structure might affect ligand-receptor interaction. The calculated interaction energies provide quantitatlve indications of the relative affinities various ligands have for the receptor. Clearly, molecular modeling involves a detailed analysis of the complementarity of ligand and receptor structure. However, this sort of molecular modeling rarely can be applied because the detailed structure of the receptor is usually unknown.

When beginning a molecular design effort with little or no knowledge of the receptor structure, the only option is to attempt to develop empirical quantitative structure-activity relationships (QSARs). QSARs are established by considering the activities of a structurally homologous series of ligands. Although these relationships consider the chemical differences between the substituent groups of the series of ligands, the 3D geometric relationships between the substituent groups are not addressed explicitly. Extrapolation of a given QSAR to proposed ligands dissimilar to those of the original series is not valid. Thus, QSARs may be useful in extending a series but will not be useful for discovering new leads.

Between the two extremes of complete knowledge and no knowledge of the receptor structure lies the increasingly common situation in which the

investigator has acquired limited knowledge of the structure of the receptor. More precisely, at least a crude description of the pharmacophore has been obtained—an indication of the types of substituent groups and the 3D geometric arrangement of those substituent groups leading to favorable ligand-receptor interaction. Under these circumstances, computer-assisted molecular design and discovery can be accomplished by searching large databases of 3D molecular structures for compounds that satisfy the chemical and 3D geometric criteria that constitute the description of the pharmacophore. It is only within the last few years that the pharmaceutical industry has been in a position to explore the utility of 3D-searching; already the results have been most encouraging. In every case, the search not only has returned "hits" (compounds that satisfy the search criteria) similar to known active compounds but also has returned hits that were entirely unexpected, new classes of compounds. Thus, 3D-searching provides an automated mechanism for discovering new lead compounds.

Four essential elements to implement 3D-searching capabilities are discussed below: (1) many interesting 3D molecular structures, (2) a chemical database management system, (3) the 3D-searching software, and (4) rationally determined search criteria.

STRUCTURES FOR 3D-SEARCHING

The tremendous potential of 3D-searching has been recognized by leaders in pharmaceutical research for many years. Perhaps the earliest efforts were those of Peter Gund and his collaborators in the mid-1970s (Gund et al. 1974; Gund 1977). Since then, various groups in academia and industry have reported efforts to develop 3D-searching algorithms (see below), but only within the last few years did 3D-searching become a realistic technique for molecular design. Interestingly, the delay was due primarily to the fact that there were no large databases of interesting 3D structures on which to perform the search.

Until recently, the Cambridge Structural Database (CSD) of x-ray and neutron diffraction results was the only resource for 3D structures. Although the CSD contains structural information for approximately 70,000 compounds, many of these are inorganics or metallo-organics of little or no interest to those searching for new bioactive compounds; furthermore, the low accuracy (resolution) of many of the organic structures in the database renders them of little value for 3D-searching purposes. Many of the remaining organic compounds (approximately 30,000) are of little interest from a pharmaceutical perspective. Building up a large database of interesting 3D structures by performing molecular mechanics or molecular orbital geometry optimizations of crude 3D structures input by some manual technique would take a tremendous amount of time and effort. Such efforts have been undertaken by various

pharmaceutical and chemical companies, but the resulting databases usually contain between 500 and 2,000 structures—not nearly enough to enable profitable 3D-searching.

Several groups have reported on their efforts to develop computer programs for the automatic conversion of two-dimensional (2D) molecular "structures" (i.e., connection tables) to 3D coordinates (Wipke et al. 1972; Wenger and Smith 1982; Wipke and Hahn 1986; Dolata et al. 1987; Cohen et al. 1981; Trindle 1986). However, each of these efforts suffered one or more drawbacks that precluded the use of the program as a generally applicable, production-level software tool. In contrast, the CONCORD program for the rapid generation of high-quality approximate 3D molecular structures (Pearlman 1987a; Pearlman et al. 1987) provides a method offering the required level of reliability, accuracy, speed, and general applicability and has thereby enabled the practical application of 3D-searching methodologies. Several large databases of CONCORD-generated 3D structures are commercially available: the MACCS Drug Data Report (approximately 10,000 compounds) and the Fine Chemicals Directory (approximately 65,000 compounds) from Molecular Design Ltd. (MDL); the POMONA-90C database of approximately 70,000 compounds from Daylight Chemical Information Systems (Daylight); and approximately 4 million structures from the Registry File of Chemical Abstracts Service. Of even greater interest to industrial scientists is the use of CONCORD to convert their corporate databases of proprietary structures (often more than 100,000) from 2D to 3D. Much of the excitement over 3D-searching in industry stems from the fact that companies are finding that compounds they synthesized, recorded, patented, and abandoned during discovery efforts made years ago for other purposes are turning out to be new lead compounds discovered by 3D-searching associated with current discovery efforts.

The CONCORD program is a hybrid of an "expert system" approach and a "pseudomolecular mechanics" approach to structure building (Pearlman 1987b). Bond-lengths are assigned from a very extensive table (Allen et al. 1987). For most acyclic substructures, the bond angles and torsion angles are assigned by performing a rule-based logical analysis that seeks to optimize 1--4 interactions. This logical analysis is at least 1,000 times faster than performing the analogous task using a molecular mechanics approach. However, this approach cannot be applied to rings or ringsystems; minimization of conformational energy is required. Whereas molecular mechanics and molecular orbital geometry optimization procedures minimize energy as a function of many independent atomic coordinates, CONCORD recognizes the correlation of atomic coordinates within a given ring and minimizes a univariate strain function. The overall algorithm is quite fast; Rusinko and colleagues (1989) reported the conversion of a corporate database of approximately

250,000 compounds at the rate of 0.5 seconds per compound on a VAX 8700; of course, faster speeds are being achieved on faster machines.

The minimal input required by CONCORD is the 2D connection table: an indication of which atoms are bonded to one another and the types (e.g., single, double) of the bonds. Because a given 2D connection table may correspond to more than one stereochemical isomer, specific stereochemical information also must be attached to the connection table to ensure that the desired isomer is constructed. The MACCS MOLfile format used by MDL is an extremely common format for this type of information, and CONCORD fully supports the input and output of MDL MOLfiles. CONCORD also supports use of the SMILES chemical line notation (Anderson et al. 1987; Weininger 1988)—an increasingly popular mechanism for providing the same connection table information in a compact format used by the chemical database software tools distributed by Daylight.

The structures generated by CONCORD are usually in excellent agreement with the lowest energy structures optimized using molecular mechanics or molecular orbital methods. However, some limitations should be noted. CONCORD will not generate inorganic or metallo-organic structures. Also, it was not intended for use on highly flexible structures (e.g., peptides, macrocycles, polymers) for which the preferred conformation is ill-defined. The issue of conformational flexibility in the context of 3D-searching is addressed later in this chapter.

CHEMICAL DATABASE MANAGEMENT SOFTWARE

General purpose database management software permits storage and retrieval of information by reference to certain storage keys or identifiers. For example, a corporate personnel database would enable access to a variety of personnel-related data by reference to employee name or employee number. The way in which information is accessed in a general purpose database reflects the perception that information is related to entire objects (e.g., employees, in the foregoing example). Perhaps the most fundamental difference between a general purpose and a chemical database is that a chemical database system reflects the fact that chemical compounds (or structures) are composed of atoms and substructures; the internal parts of these chemical objects are of as much interest as the entire objects.

To access information at the structural and substructural levels, chemical database management software uses the chemical structure to derive the identifiers by which the compound will be identified. The user specifies a structure by specifying its connection table, either by sketching the structure

using a simple light-pen or digitizing tablet or by entering a chemical line notation, which is immediately converted to a connection table. Using modifications of the classic Morgan algorithm (Morgan 1965; Wipke and Dyott 1974; Weininger et al. 1989), the connection table then is reordered in a canonically unique fashion. Because the connection table of an N-atom molecule can be input in at least N! ways, this unique reordering is essential for the task of exact structure matching. It also greatly facilitates the implementation of substructural matching capabilities—finding all compounds in the database that contain the user-specified substructure.

In addition to being searchable by structure and substructure, a chemical database management system also should support the capabilities expected of a general purpose database management system. It is important that the structural information and various property-related information (e.g., experimental biological activity data, physical properties such as partition coefficient, atomic properties such as partial charge) be readily accessible within a single software environment. It is also important that the chemical database management software be tightly interfaced to the molecular modeling software environment.

Several pharmaceutical companies developed "in-house" database management systems to meet these objectives. However, two systems are commercially available: the MACCS-II system distributed by MDL and the Daymenus system distributed by Daylight. Both systems are widely used and accepted.

3D-SEARCH SOFTWARE

The features of the pharmacophore that constitute the 3D-search criteria should be perceived as falling into two categories: 2D chemical substructures and the 3D geometric relationships between the substructures. Then, conceptually, 3D-searching can be perceived as being accomplished in two corresponding phases. In the first phase, the 2D substructural searching capabilities of the chemical database management software are used to screen out those compounds in the database that could not possibly meet the 3D-search criteria because they do not contain the required chemical substructures in any relative positions. This 2D substructural screening takes little time and eliminates the vast majority of compounds in the database. Those compounds that remain then are subjected to geometric screening, which also can be perceived as being accomplished in two phases. It is relatively easy to screen compounds for simple distance-related criteria. In fact, when the 3D structural database is being constructed (or updated), the various types of interatomic separations (i.e., atom types and interatomic distances) contained within each structure are

recorded in much the same way as are the atom types. Different programs implement these distance keys in different ways, but the objective is the same: to screen out compounds that could not possibly meet the 3D-search criteria because they do not contain required atoms separated by required distances. Like the 2D substructural screens, these distance screens require little computer time and further reduce the number of compounds that must be subjected to the final phase of searching.

In the final phase of 3D-searching, the atomic coordinates of each compound are examined in detail to determine whether the required chemical substructures occupy positions in 3-space that will satisfy the 3D geometric criteria. These criteria can involve much more than simple interatomic distance constraints. The atomic coordinates can be used to define various objects, and the search constraints can be formulated in terms of these objects. For example, suppose that two of the substructural features of a particular pharmacophore are a carboxylete group and an aromatic ring. One geometric constraint might be the distance between the carbon of the carboxylate and the centroid of the ring. Another constraint might be the dihedral angle between one of the carbon-oxygen bonds of the carboxylate group and the plane of the aromatic ring. Atomic coordinates also can be used to define points that, in turn, are used to define "forbidden regions"—regions of space around the essential features of the pharmecophore, which, if occupied, would lead to sterically unfavorable interaction between the ligand and receptor.

Several groups have made significant contributions to the development of 3D-searching software (Gund et al. 1974; Ullman 1976; Jekes et al. 1987; Sheridan et al. 1989a; Van Drie et al. 1989, and others). These efforts have resulted in working systems. However, because 3D-searching builds on the searching capabilities of chemical database management software and because only two chemical database management software packages are commercially available, it should not be surprising that only two 3D-searching packages are commercially available. MDL distributes MACCS-3D as an add-on option to its MACCS-II database management system. Daylight now distributes ALADDIN, which, although developed at Abbott Laboratories by John Van Drie working with Yvonne Martin, is based in part on Daylight searching capabilities.

3D-SEARCH CRITERIA

The first three essential elements of 3D-searching—a mechanism for rapid 3D structure generation, software for managing chemical databases, and software for performing the search—might be regarded as "software tools" that can be readily applied to any 3D-searching problem. In contrast, the fourth essential element of 3D-searching—rational 3D-search criteria—must be tailored

specifically for each new discovery problem. Defining the search criteria requires substantial effort on the user's part, whereas performing the search requires relatively little user input.

The approach to computer-assisted molecular design and the approach to developing 3D-search criteria depend on how much is known about the structure of the receptor. If the structure of the receptor is known, it is a relatively straightfomard matter to develop chemical and geometric criteria for complementary ligands that could interact favorably. Most often, however, the structure of the receptor is not known and attempts to formulate the search criteria are based solely on the structures of a few ligands that have been found to exhibit the desired bioactivity.

Inferring 3D-search criteria from known ligand structures is relatively straightforward when the ligands are fairly rigid. However, if the known ligands are flexible structures that could undergo significant conformational distortion during the binding process (i.e., if the bound conformation could be significantly different than the low energy conformation), then establishing rational 3D-search criteria is a far more complex process. Indeed, it may be impossible to determine accurately the geometry of the receptor. It is possible, however, to determine how the "conformation space" accessible to each ligand in the training set compares with the conformation space accessible to all other ligands in the set. More precisely, a search can be undertaken for those regions of space that are conformationally accessible to all ligands in the training set. The geometric constraints derived in this manner may not be particularly specific but are at least guaranteed to include the bound conformations of all ligands in the training set. Techniques for performing this sort of systematic search of the conformation space of a set of compounds were developed by Marshall and coworkers (1979) and Mayer and coworkers (1987) and recently were refined with respect to computational efficiency by Dammkoehler and colleagues (1989). The SYBYL molecular modeling package (Tripos Associates) includes conformational searching features that are useful in this regard. Once the regions of conformational space common to all ligands in the training set have been identified, a description of these regions must be specified in terms that the searching software can understand. As previously indicated, this primarily involves specifying ranges of distances and angles between objects defined in terms of the coordinates of the key features of the pharmacophore. The breadth of the ranges at least should be sufficient to include all active members of the training set.

There are several aspects to the efficiency with which a search is accomplished. For a database of given size, an obvious measure of efficiency is the central processing unit (CPU) time required to complete the search.

Because the 2D substructural screens and distance screens are performed so rapidly, greater specificity regarding these aspects of the search criteria will result in fewer compounds for which more CPU-intensive 3D examination is required. In contrast, as the specificity of the 3D criteria is increased by adding additional geometric constraints, the required CPU time increases proportionally. Another measure of searching efficiency is the ratio of the number of hits to the number of compounds in the database. If the search criteria are not specific, they might be satisfied by large numbers of compounds and the search might result in so many hits as to be of little value. Most of these hits would not possess the correct geometry required for receptor binding. Clearly, the geometric criteria should be as detailed as possible but expressed as concisely as possible. A third aspect of searching efficiency is the ratio of the number of truly active hits to the total number of hits returned by the search. Although this aspect is related to the preceding aspect, it differs in an interesting way. If 3D geometric criteria are formulated by examining a training set composed only of ligands known to bind well with the receptor, then the user has failed to provide information concerning the "forbidden regions," which, if occupied, result in sterically unfavorable ligand-receptor interactions. The resulting hit list might contain many compounds that satisfy all the criteria specified for receptor affinity but contain substructures extending into the unspecified forbidden region(s). Thus, it is important to include active and rationally selected inactive compounds in the process of defining the 3D-search criteria.

It is instructive to contrast 2D- and 3D-searching in terms of the type of information used to formulate the search criteria. Because ligand-receptor interaction depends on the complementarity of two 3D objects, the inability explicitly to include 3D considerations in the specification of 2D substructural search criteria is an obvious disadvantage. However, there is another important distinction to be made. When specifying 2D substructural searching criteria, the user not only specifies the key substructural features identified as essential elements of the pharmacophore but also specifies, to some extent, the chemical environment surrounding these features. Any efforts to specify (albeit crudely) the geometric relationship between the substructural features involves specifying even more of the chemical structure of the hits that the search returns. Therefore, the 2D hit list reflects the structures the user had in mind while formulating the search criteria and rarely contains any surprises. In contrast, 3D-search criteria allow the user to specify the essential pharmacophore features and their geometric relationship in geometric rather than chemical structural terms. The correspondence between 3D-search criteria and 3D-search hits is far less explicit than the correspondence between the criteria and hits of a 2D-search. Thus, the hits returned by a 3D-search are

likely to include more structurally diverse compounds—surprising new leads—that the user did not have in mind when formulating the search criteria.

3D-SEARCH EXAMPLES

As of this writing, the essential elements required for 3D-searching have been available for less than a year. Nevertheless, accounts of successful 3D-searching efforts already are being presented at meetings and are beginning to appear in the literature (Sheridan et al. 1989b; Van Drie et al. 1989). Obviously, other industrial examples are of proprietary interest and cannot be published at this time. Loew and colleagues (this volume) used 3D-searching to identify novel benzodiazepine receptor ligands.

USE OF 3D-SEARCHING AND FUTURE DEVELOPMENTS

The aforementioned examples (and others) leave absolutely no doubt that, even in its current state of development, 3D-searching works. It already is proving to be a useful technique for the extension of existing leads and the discovery of new leads to bioactive compounds; however, some proposed improvements and caveats are discussed below.

As currently implemented, 3D-searching does a poor job in accounting for the possible flexibility of ligands. Let us assume that we have applied the techniques of systematic conformational searching to a training set of flexible ligands and have thereby formulated 3D-search criteria that reflect the required conformation of a ligand once it is bound to the receptor. Let us also assume that we have stored in the database the low-energy conformation of each of these ligands (such as would be generated by CONCORD or some geometry optimization procedure). If the bound conformations are significantly different than the low-energy conformations, it is entirely possible that a search based on these 3D-search criteria would not return the compounds in the training set used to formulate the search criteria. Moreover, other compounds for which the low-energy conformation does not satisfy the search criteria but which could be distorted so as to match the criteria would not be returned as hits. Although this appears to be an undesirable situation, before we consider how it might be remedied, let us consider its positive aspects. There are numerous contributions to the overall free energy of ligand-receptor interaction (which, of course, determines the stability of the ligand-receptor complex). The most obvious contribution is the electronic intermolecular interaction energy that results when the key features of the pharmacophore are placed close to the corresponding features of the receptor. If the 3D-search criteria accurately reflect the ligand-receptor interaction, the intermolecular interaction energy contribution of all hits will be roughly equivalent. Another contribution to the

overall free energy of interaction is the energy required to distort the ligand from its preferred conformation to its bound conformation. This energy will be positive and, therefore, will be an unfavorable contribution to the binding process. Thus, a compound for which the preferred conformation closely approximates the bound conformation would be expected to yield a more stable ligand-receptor complex than compounds that must undergo conformational distortion to bind. Thus, although the aforementioned search example might fail to return flexible compounds that could be distorted so as to match the search criteria, the compounds that *are* returned would be expected to bind to the receptor more effectively. (Relatively rigid compounds satisfying the search criteria might be expected to bind even more effectively because their complexation would not entail as large a decrease in conformational entropy.)

If receptor affinity were the only factor to be considered, then we might be satisfied with a search strategy that returns only compounds for which the prefer red conformation satisfies the search criteria. However, issues such as toxicity, transportability, and “patent-ability” often prompt us to consider ligands for which receptor binding requires conformational distortion. How can the 3D-search procedure be modified so that these types of compounds will be found? One approach is to relax the 3D-search criteria until the list of hits includes the flexible, known-active compounds included in the training set. This may prove satisfactory in some cases but in other cases will result in such vague 3D geometric constraints that the search will offer little advantage over a simple 2D-search. Another approach frequently suggested is to store multiple conformations of each compound in the database. Obviously, storing additional conformations cannot reduce (can only increase) the number of compounds returned by a 3D-search, generating considerable interest in this approach.

However, there are some significant problems that have not received the same degree of attention as have the potential benefits. For simplicity, let us imagine that we only need to consider conformational changes resulting from rotations about single bonds between sp^3-hybridized atoms. Let us begin by assuming that we consider storing just three conformations (dihedral angels of 60, 180, and 300 degrees) per rotatable bond. If there were only three such independently rotatable bonds in a compound, there would be 3^3=27 conformations to be stored. In reality, most compounds in a real database possess at least that many conformational possibilities. Recalling that computer-assisted 3D-searching is only worthwhile when performed on databases containing many thousands of compounds, it is clear that storage of multiple conformations would lead to substantial disk requirements. The CPU time required to perform the search also would increase substantially. These increases might be regarded as worthwhile if this strategy were guaranteed to return a substantially larger hit list. Unfortunately, it does not. Although the

three dihedral angles mentioned above might correspond to local minima in the conformational energy surface of an isolated compound, the conformational energy surface of the same compound interacting with the receptor will be entirely different. Strong, specific intermolecular interactions (such as hydrogen-bonding) can result in a bound conformation near a local maximum of the conformational energy surface of the isolated compound. Decreasing the torsion-angle increment in an attempt to include these sorts of conformations would result in much larger numbers of conformations to be stored per compound.

The 3D geometric phase of current 3D-search algorithms examines each conformation, asking the question, "Does this conformation satisfy the search criteria?" Rather than applying this type of search algorithm to databases containing many conformations of each compound, it is more logical to develop new searching algorithms that examine databases containing only low-energy conformations and that scan the conformational space of potential hits at search time. These new search algorithms will ask, "Could this compound be distorted so as to satisfy the search criteria and, if so, how much conformational energy will be required?" Most of the software technology required for this next generation of search algorithms already exists; such products probably will be available during the coming year.

Another improvement that could and should be made soon is to provide a quantitative indication of how well each compound on the hit list satisfies the search criteria. As previously explained, the search criteria specify ranges of acceptable distances and angles. Current searching software returns a list of compounds that lie somewhere within the acceptable limits. An indication of how close a compound is to the center of the acceptable ranges could be useful in prioritizing the subsequent laboratory testing efforts. This "goodness of hit" concept also could be extended to the chemical aspects of the search criteria. For example, if a hydrogen-bond acceptor was stipulated as a key substructural feature, a basicity index that distinguished carbonyl oxygens from etheric oxygens from amides might prove quite useful.

Thus far, the focus has been on the chemical structural complementarity between the ligand and receptor, that is, the electronic intermolecular interaction energy and the conformational energy contributions to the overall free energy of interaction. However, Amidon and colleagues (1979) pointed out the importance of considering the free energy of desolvation, which contributes to the free energy of intermolecular interaction in solution; desolvation usually is addressed in current molecular modeling efforts. An earlier hypothesis states that if two ligands present to the receptor the same key substructural features in essentially the same geometric disposition, then the electronic intermolecular

interaction between the receptor and the two ligands will be roughly the same. It is presumed that, if the chemical nature of the framework connecting the key substructural features had a significant effect on the stabiltty of the ligand-receptor complex, then aspects of the framework would have been identified as key features of the pharmawphore. However, the free energy required to desolvate the framework of two dissimilar ligands might differ by several kcal/mol. Identifying those portions of hit compounds that must be desolvated and including an estimate of the desolvation energy as part of the aforementioned effort to quantitate goodness of hit might prove useful.

The foregoing discussion pertains to various factors that contribute to ligand-receptor affinity. However, even the simplest models of bioactivity involve at least two processes. First, the compound must be transported to the biophase; then it must interact with the receptor. Both processes depend on the chemical structure of the compound. Therefore, rational molecular design efforts should consider the effect of structure on both of these essential aspects of bioactivity. It does little good to identify compounds that exhibit high *in vitro* receptor affinity but low *in vivo* bioactivity due to poor transport properties. As with other receptor affinity-oriented techniques for computer-assisted molecular design, 3D-searching should be used in conjunction with a method for estimating the permeability of hit compounds through relevant biological barriers. Such a method has been developed by Skell and Pearlman (in preparation).

CONCLUSION

3D-searching for pharmacophores within large databases of 3D chemical structures is proving to be a powerful new technique for computer-assisted molecular design. In particular, it has proven useful not only for the exploration of existing lead compounds but also for the discovery of entirely novel leads to bioactive agents. Four essential elements are available, thereby enabling 3D-searching: a method for the rapid generation of large databases of 3D molecular structures, software for the management of and 2D substructural searching within chemical databases, software to perform the 3D geometric aspects of 3D-searching, and techniques for the formulation of rational 3D-search criteria.

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Protein Structure Determination in Solution Using Nuclear Magnetic Resonance Spectroscopy

Angela M. Gronenborn and G. Marius Clore

INTRODUCTION

Over the past few years, we have witnessed a renewed interest in protein studies, especially those aimed at understanding their structures, functions, and physiological roles. Some of this increased enthusiasm can be attributed to a variety of technological advances in the area of modern molecular biology, in particular, molecular cloning of protein encoding genes. The resulting wealth of data is clearly impressive; however, amino acid sequences are of limited value in understanding protein function. We need to know the three-dimensional structure before we can begin to make progress in analyzing the intricate reactions carried out by proteins such as catalysis, ligand binding, gene regulation, and assembly. The design of modified proteins, rational design of drugs, and attempts at *de novo* design of protein molecules all have to be based on concepts at the atomic level in 3D space, thereby creating an increasing need for detailed structural analysis.

Until recently, the only experimental technique available for determining 3D structures has been single crystal x-ray diffraction, and most of our structural knowledge about proteins is based on those crystal structures. There are approximately 400 coordinate sets available to date, comprising about 120 different protein folds. Analyzing protein structures by crystallography can be a slow and difficult undertaking because the first and possibly hardest task involves growing x-ray, quality-grade crystals, which have to be well ordered to give rise to good diffraction patterns. Even if this task is accomplished, a second hurdle still needs to be overcome, as the phases have to be solved, commonly achieved by collecting data on heavy atom derivatives. Thus, despite spectacular advances in protein crystallography, we are faced with an enormous gap between the available primary sequence data and the tertiary structure data on proteins.

Over the past 10 years, a second method for determining protein structures has been developed and is by now well established. This method makes use of NMR spectroscopy. Unlike crystallography, NMR measurements are carried out in solution under potentially physiological conditions and are therefore not hampered by the ability or inability of a protein to crystallize.

The principal source of information used to solve 3D protein structures by NMR spectroscopy resides in short interproton distances supplemented by torsion angles. The distances are derived from nuclear Overhauser effect (NOE) measurements, because the size of the NOE between two protons is proportional to r^6, where r is the distance between them. Torsion angles are obtained from an analysis of three-bond coupling constants that are related to dihedral angles. An essential prerequisite for obtaining interproton distance restraints and torsion angle restraints is the assignment of the NMR spectrum, that is, the identity of every proton resonance has to be determined. This is not a trivial task considering that the proton spectrum of even a small protein comprising only 80 amino acids contains approximately 650 resonances. All these exhibit several cross-peaks in the 2D spectrum, and the number of cross-peaks whose identity has to be ascertained can easily rearch several thousand. Complete spectral assignment is, therefore, an integral part of the structure determination.

Although it was appreciated relatively early on that NMR could in principle provide the necessary information to obtain 3D structures, only recently has this goal been realized. The reasons for this are threefold: (1) The development of 2D NMR experiments (Jeener 1971; Jeener et al. 1979; Aue et al. 1976) alleviated problems associated with resonance overlap, which for macromolecules prevents any analysis of the traditional one-dimensional spectrum. This is achieved by spreading all the information out in a plane, thereby permitting a detailed interpretation of the pertinent spectral features. This conceptual idea has been extended more recently to 3D NMR (Oschkinat et al. 1988, 1989; Vuister et al. 1988; Fesik and Zuiderweg 1988; Zuiderweg and Fesik 1989; Marion et al. 1989a, 1989b), again relieving problems arising from spectral crowding; and it is the 3D approach in particular that will extend the present limits with respect to the size of the proteins that can be studied. (2) The availability of high-field magnets (500 and 600 MHz) has resulted in spectrometers with a significant increase in the signal-to-noise ratio and better spectral resolution, and continuing development in this area again will extend the limits even further. (3) Suitable mathematical algorithms and computational approaches, which convert the NMR-derived restraints into 3D structures, have been developed (Havel and Wüthrich 1984; Havel et al. 1983; Braun and Go 1985; Clore et al. 1985, 1986; Kaptein et al. 1985; Billeter et al. 1987; Nilges et al. 1988a, 1988b, 1988c; Litharge et al. 1987), and several robust and efficient methods are available.

This chapter outlines the general methodology for protein structure determinations by NMR (figure 1); however, no attempt is made to provide an indepth description of either the general NMR theory or the details of the mathematical algorithms. Emphasis is placed on the application of NMR to structural studies, and two examples from the authors' laboratory are used to illustrate various points.

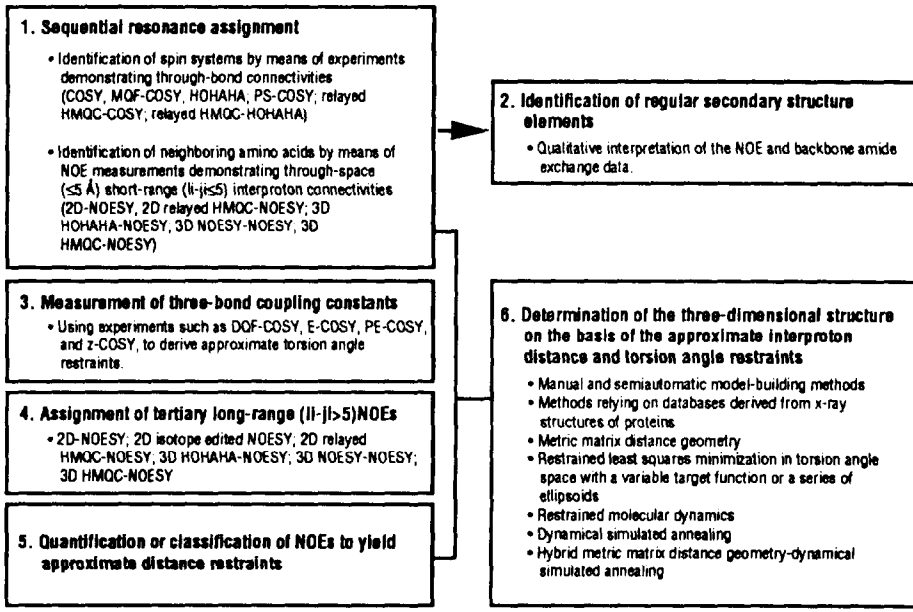
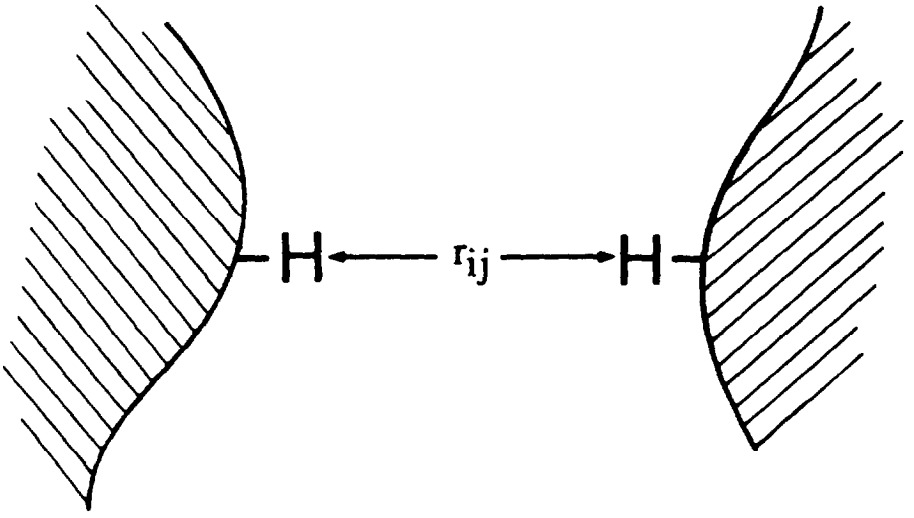


FIGURE 1. *Flow chart of the various steps involved in determining the 3D structure of a protein in solution by NMR*

THE NUCLEAR OVERHAUSER EFFECT

The principles of 2D NMR have been discussed in depth (Ernst et al. 1986) and the authors deliberately refrain from explaining the basic NMR experiment because this would be beyond the scope of the present article. Only the relevant NMR parameters are briefly introduced.

NMR-derived protein structures are mainly based on NOE measurements that can demonstrate the proximity of protons in space and that allow the determination of their approximate separation (Overhauser 1953a, 1953b;



$$N_{ij}(t) \sim \sigma_{ij}\, t$$

$$\sigma_{ij} = \frac{\gamma^4 \hbar^2}{10\, r_{ij}^6} \left(\tau_{app} - \frac{6\tau_{app}}{1 + 4\omega^2 \tau_{app}^2} \right)$$

FIGURE 2. *The basis of the NOE*

r_{ij}=distance between the protons i and j
σ=cross-relaxation rate;
N, NOE, τ_{app}=correlation time

Solomon 1955, Noggle and Schirmer 1971). The principle of the NOE is relatively straightforward and is summarized in figure 2. Consider the simplest system with only two protons, each of which possesses a property known as cross-relaxation. Because the cross-relaxation rates in both directions are equal, the magnetization of the two protons at equilibrium is equal. The

approximate chemical analogy of such a system would be one with two interconverting species with an equilibrium constant of 1. The cross-relaxation rate is proportional to two variables: r^{-6} where r is the distance between the two protons, and τ_{app} the effective correlation time of the interproton vector. It follows that if the magnetization of one of the spins is perturbed, the magnetization of the second spin will change. In the case of macromolecules, the cross-relaxation rates are positive and the leakage rate from the system is small, so that in the limit, the magnetization of the two protons would be equalized. The change in magnetization of proton i upon perturbation of the magnetization of proton j is known as the nuclear Overhauser effect. The initial buildup rate of the NOE is equal to the cross-relaxation rate and, hence, proportional to r^{-6}.

SEQUENTIAL RESONANCE ASSIGNMENT

Sequential resonance assignment of the ^{1}H-NMR spectra of proteins relies on two sorts of experiments: (1) those demonstrating through-bond scaler connectivities, and (2) those demonstratlng through-space (<5 Å) connectivities. The former, which are generally referred to as correlation experiments, serve to group together protons belonging to the same residue. The latter involve the detection of NOEs and serve to connect one residue with its immediate neighbors in the linear sequence of amino acids.

The first step in the assignment procedure lies in identifying spin systems, that is to say, protons belonging to one residue unit in the polypeptide chain. Such experiments have to be carried out in H_2O and D_2O, the former to establish connectivities involving the exchangeable NH protons, and the latter to identify connectivities between nonexchangeable protons. Some spin systems are characteristic of several different amino acids. This is the case for Gly, Ala, Thr, Leu, Ile, and Lys. Others are characteristic of several different amino acids. For example, Asp, Asn, Cys, Ser, and the aliphatic protons of all aromatic amino acids belong to the AMX spin system (i.e., they all have one $C^{a}H$ and two $C^{ß}H$ protons). The experiments used for the identification of amino acid types are collectively known as correlation experiments and comprise COSY (Aue et al. 1976) and COSY-type and HOHAHA (Davis and Bax 1985; Bax and Davis 1985, Bax 1988) experiments.

A schematic representation of cross-peak patterns observed in HOHAHA spectra for the various spin systems is illustrated in figure 3, and an example of a protein HOHAHA spectrum in H_2O is shown in figure 4.

Once a few spin systems have been identified, one can then proceed to identify sequential through-space connectivities involving the NH, $C^{a}H$, and $C^{ß}H$ protons

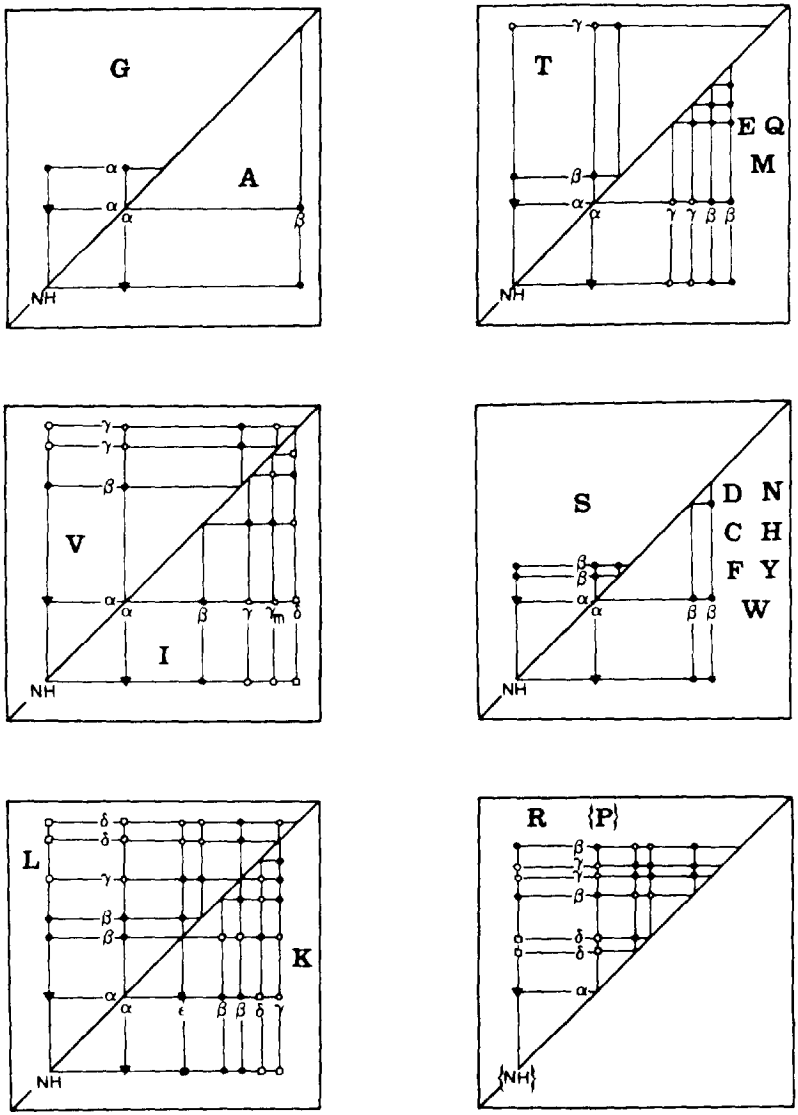


FIGURE 3. *Schematic representation of cross-peak patterns observed in HOHAHA spectra for various spin systems. In a COSY spectrum, only direct connectivities are observed.*

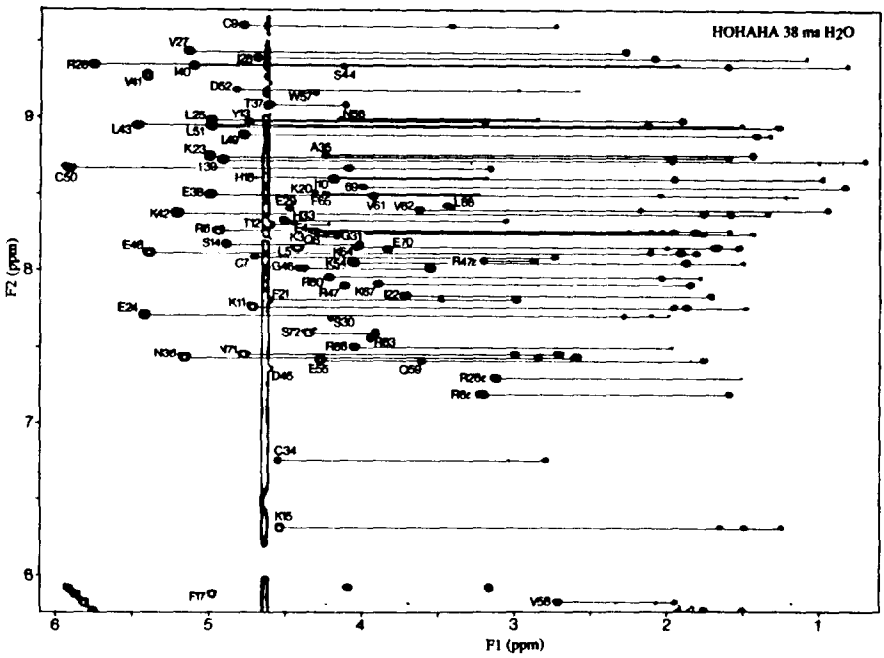


FIGURE 4. *600 MHz HOHAHA spectrum of IL-8 in H_2O showing the NH(F2)-aliphatic (F1) region of the spectrum. Peaks are labeled at the positions of the direct NH-$C^{a}H$ connectivities.*

SOURCE: Clore et al. 1989.

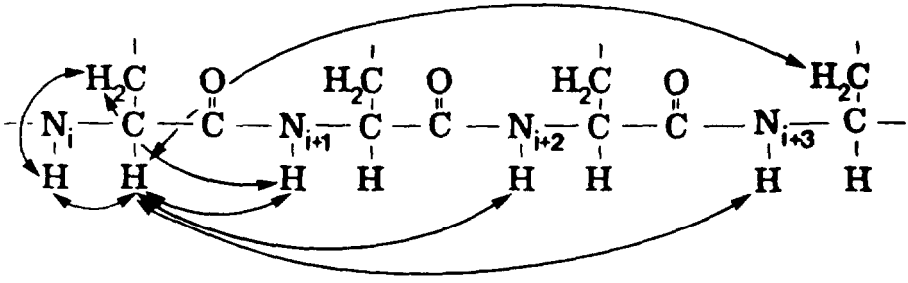


FIGURE 5. *Schematic illustration of the connectivities used for sequential resonance assignment of protein spectra*

by means of 2D NOE spectroscopy (NOESY [Macura and Ernst 1980; Macura et al. 1981]). For the purpose of sequential assignment, the most important connectivities are the $C^{a}H(i)$-NH(i+1,2,3,4), $C^{ß}H(i)$-NH(i+1), NH(i)-NH(i+1), and $C^{a}H(i)$-$C^{ß}H(i+3)$ NOEs. This is illustrated schematically in figure 5, and an example of a NOESY spectrum is shown in figure 6.

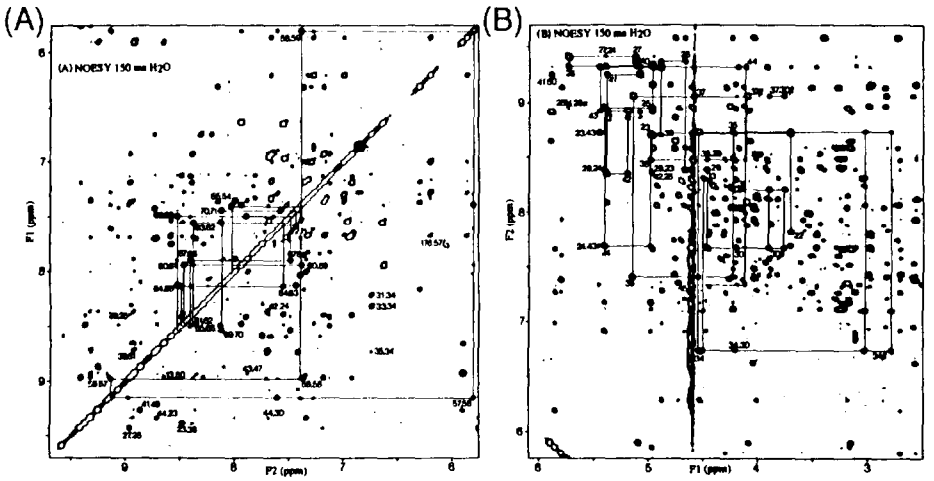


FIGURE 6. *600 MHz NOESY spectrum of IL-8 in H_2O showing the NH(F1)-NH(F2) region (A) and the NH(F2)-$C^{a}H/C^{ß}H$(F1) region (B) of the spectrum. A stretch of NH(i)-NH(i+1) connectivities extending from residues 54 to 72 is indicated in (A) and sequential $C^{a}H(i)$-NH(i+1) connectivities are marked in (B). In addition, several long-range NOEs are labeled.*

SOURCE: Clore et al. 1989.

As proteins get larger, problems associated with chemical shift dispersion become increasingly severe. One approach for alleviating such problems in the sequential assignment of proteins involves correlating proton-proton through-space and through-bond connectivities with the chemical shift of a directly bonded NMR active nucleus, such as ^{15}N or ^{13}C. This experiment can be carried out on uniformly labeled protein samples that can be easily obtained if the gene for the protein under investigation has been cloned and overexpressed.

A further avenue for large proteins involves the application of 3D NMR. For ^{15}N 3D spectra, the normal rules for making sequence specific assignments can be readily applied. The only difference to the normal 2D case is that connections between one residue and the next must be made not only between different sets of peaks but also between different planes of the spectrum. The major advantage going from 2D to 3D is that the distribution of overlapping or closely spaced cross-peaks throughout the entire cube removes most of the ambiguities present in the 2D spectrum.

IDENTIFICATION OF SECONDARY STRUCTURE ELEMENTS

Because each type of secondary structure element is characterized by a particular pattern of short-range (Ii-jI<5) NOEs (Wüthrich 1986; Wüthrich et al. 1982, 1984; Billeter et al. 1982; Wagner and Wüthrich 1982), qualitative interpretation of the sequential NOEs allow the identification of regular secondary structure elements. This is illustrated in figure 7. Thus, for example, helices are characterized by a stretch of strong or medium NH(i)-NH(i+1) NOEs, and medium or weak C^aH(i)-NH(i+3) NOEs, C^aH(i)-$C^ßH$(i+3) NOEs, and C^aH(i)-NH(i+1) NOEs, sometimes supplemented by NH(i)-NH(i+2) and C^aH(i)-NH(i+4) NOEs. On the other hand, strands are characterized by strong C^aH(i)-NH(i+1) NOEs and by the absence of other short-range NOEs involving the NH and C^aH protons. ß-Sheets can be identified and aligned from interstrand NOEs involving the NH, C^aH, and $C^ßH$ protons. It also should be pointed out that the identification of secondary structure elements is aided by (1) NH exchange data in that slowly exchanging NH protons usually are involved in hydrogen bonding and by (2) $^3J_{HNa}$ coupling constant data. Figure 8 illustrates the application of this method to the protein interleukin-8 (IL-8) (Clore et al. 1989). Inspection of the short-range NOE data immediately enables one to identify three ß-strands, several turns, and a long helix at the carboxy terminus. Although very effective, it should be realized that this kind of secondary structure determination can be used only in a qualitative fashion, and accurate positioning of the identified secondary structure elements can be accomplished only after the complete 3D protein structure has been determined.

NMR-DERIVED RESTRAINTS

Because of the $<r^{-6}>$ dependence of the NOE, approximate interproton distance restraints can be derived from the initial slope of the time dependence of the NOE. Because initial slope measurements are not entirely trivial, one generally resorts to a qualitative classification of NOEs. Empirically, the type of classification used is one in which strong, medium, and weak NOEs correspond to distance ranges of approximately 1.8-2.7 Å, 1.8-3.3 Å, and 1.8-5.0 Å, where the lower limit of 1.8 Å corresponds to the sum of the van der Waals radii of two

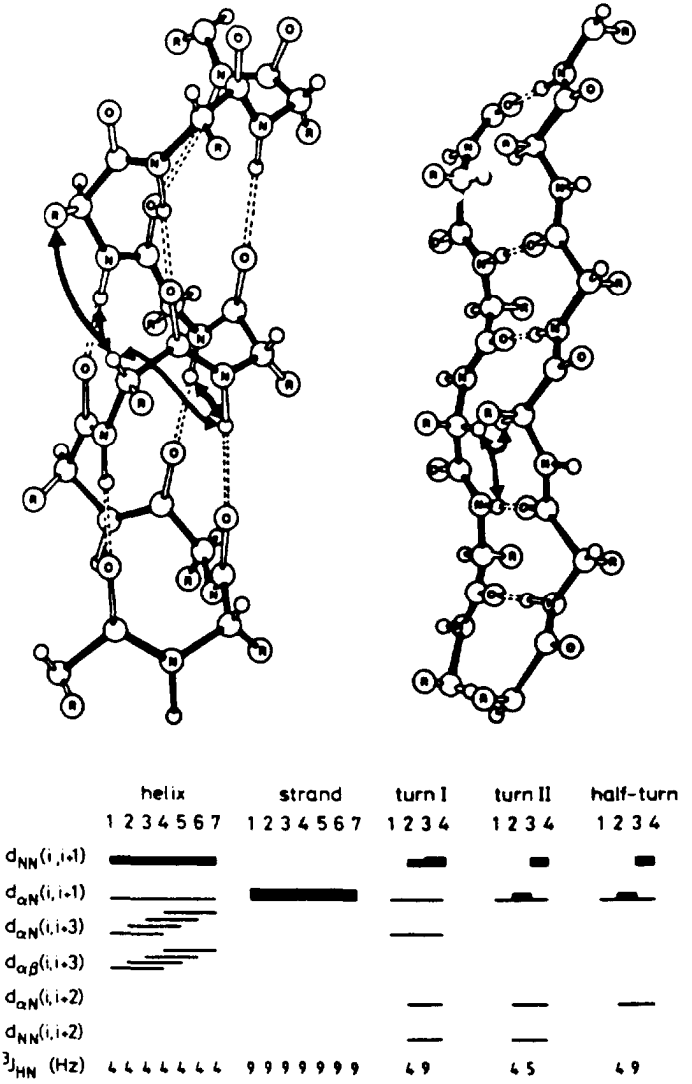


FIGURE 7. *Characteristic patterns of short-range NOEs involving the NH, $C^{a}H$, and $C^{\beta}H$ protons seen in various regular secondary structure elements. The NOEs are classified as strong, medium, and weak, reflected in the thickness of the lines.*

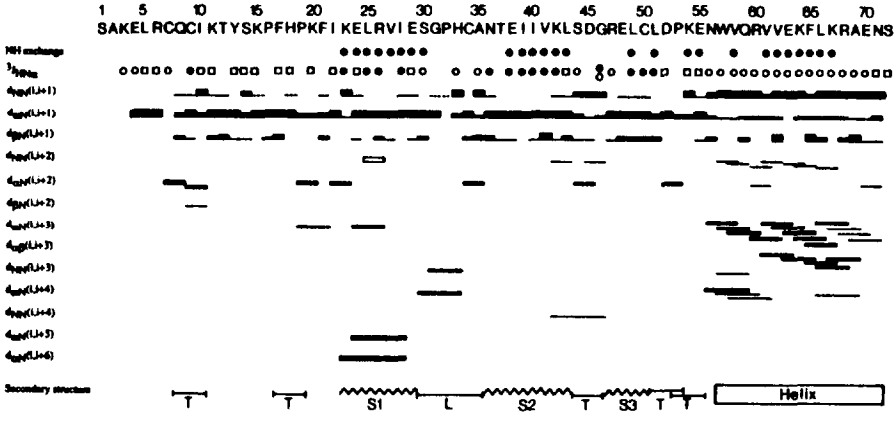


FIGURE 8. *Pattern of short-range NOEs involving the NH, $C^{a}H$, and $C^{\beta}H$ protons, as well as the $C^{\beta}H$ protons, for IL-8. The intensities of the NOEs are indicated by the thickness of the lines. In addition, slowly exchanging backbone amide protons and values of $^{3}J_{HNa}$ are indicated. The secondary structure deduced from these data is shown at the bottom of the figure.*

SOURCE: Clore et al. 1989.

protons. By using such a scheme, variations in effective correlation times do not introduce errors into the distance restraints. Rather, they only result in an increase in the estimated range for a particular interproton distance.

Vicinal spin-spin coupling constants can provide useful information supplementing the interproton distance restraints derived from NOE data. In particular, ranges of torsion angles can be estimated from the size of the coupling constants. The latter may be obtained by analyzing the multiplex patterns in COSY and COSY-like (e.g., DQF-COSY, E-COSY, z-COSY) spectra.

The easiest coupling constants to determine in proteins are the $^{3}J_{HNa}$ coupling constants that can be obtained by measuring the peak-to-peak separation of the antiphase components of the $C^{a}H$-NH COSY cross-peaks. The size of the $^{3}J_{HNa}$ coupling constant is related to the ϕ backbone torsion angle through a Karplus-type relationship (Karplus 1963; Pardl et al. 1984).

x_1 Side-chain torsion angle restraints and stereospecific assignments can be obtained by analyzing the pattern of $^3J_{a\beta}$ coupling constants and the relative intensities of the intraresidue NOEs from the NH and $C^{\alpha}H$ protons on the one hand to the two $C^{\beta}H$ protons on the other, and, in the case of valine, to the $C^{\gamma}H_3$ protons. The $^3J_{a\beta}$ coupling constants are related to the x_1 torsion angle and are best measured from correlation spectra, which yield reduced multiplets such as ß-COSY, E-COSY, P.E.COSY, or z-COSY.

ASSIGNMENT OF LONG-RANGE (|i-j|>5) NOEs IN PROTEINS

In globular proteins, the linear amino acid chain is folded into a tertiary structure such that protons far apart in the sequence may be close together in space. These protons give rise to tertiary NOEs whose identification is essential for determining the polypeptide fold. Once complete assignments have been made, many such long-range NOEs can be identified in a straightforward manner. It is usually the case, however, that the assignment of several long-range NOE cross-peaks remains ambiguous due to resonance overlap. In some cases, this ambiguity can be removed by recording additional spectra. Where ambiguities still remain, it is often possible to resolve them by deriving a low-resolution structure on the basis of the available data (i.e., the secondary structure and the assignment of a subset of all the long-range NOEs) either by model building or by distance geometry calculations. This low-resolution structure then can be used to test possible assignments of certain long-range NOEs. For larger proteins, it becomes increasingly difficult to assign tertiary NOEs because of the associated overlap problems. In these cases, the 3D approach will become a necessity, in particular, ^{13}C 3D experiments because they allow editing with respect to particular side-chain positions for which the individual NOEs then can be extracted. In figure 9, all experimental short- and intermediate-range NOE restraints (A) and all long-range NOE restraints (B) that were measured from the NOESY spectra are shown as dotted lines superimposed on the framework of the finally determined structure of hirudin, illustrating the dense network of distances throughout the protein core.

TERTIARY STRUCTURE DETERMINATION

Several different approaches can be used to determine the 3D structure of a protein from experimental NMR data. The simplest approach, at least conceptually, is model building. This can be carried out either with real models or by means of interactive molecular graphics. It suffers, however, from the disadvantage that no unbiased measure of the size of the conformational space consistent with the NMR data can be obtained. Consequently, there is no guarantee that the modeled structure is the only one consistent with the experimental data. Furthermore, in this way, nothing more than a low-resolution structure can be obtained.

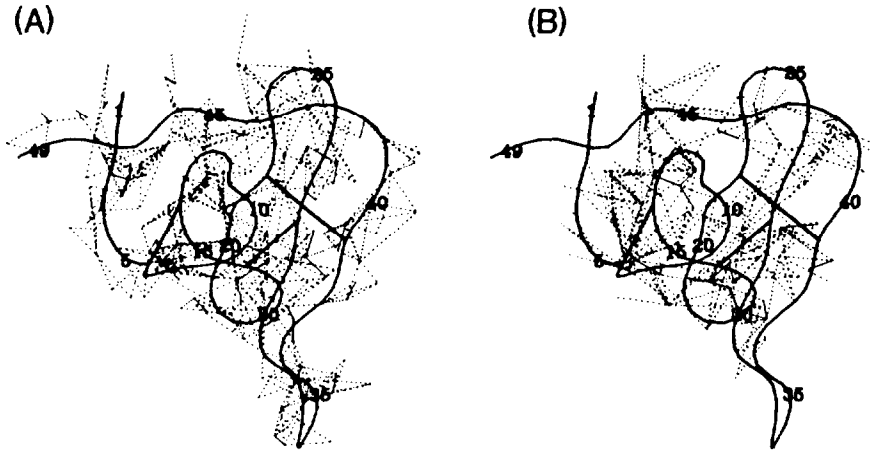


FIGURE 9. *Short-range interresidue and intraresidue (A) and long-range interresidue NOE distance restraints (B) shown as dashed lines superimposed on a framework of the final hirudin structure in a smoothed backbone representation*

The main computational methods for generating structures from NMR data comprise as a common feature a conformational search to locate the global minimum of a target function that is made up of stereochemical and experimental NMR restraints. The descent to the global minimum region is not a simple straightforward path as the target function is characterized by many false local minima that have to be avoided or surmounted by all the methods.

There are essentially two general classes of methods. The first can be termed real-space methods. These include restrained least squares minimization in torsion angle space with either a variable target function (Braun and Go 1985) or a sequence of ellipsoids of constantly decreasing volume, each of which contains the minimum of the target function (Billeter et al. 1987), and restrained molecular dynamics (Clore et al. 1985, 1986; Kaptein et al. 1985) and dynamical simulated annealing (Nilges et al. 1988a, 1988b, 1988c) in Cartesian coordinate space. All real-space methods require initial structures. These can be (1) random structures with correct covalent geometry; (2) structures that are far from the final structure (e.g., a completely extended strand); (3) structures made up of a completely random array of atoms; and (4) structures generated by distance space methods. They should not, however, comprise structures derived by model building, as this inevitably biases the final outcome. Because these methods operate in real space, great care generally has to be taken to

ensure that incorrect folding of the polypeptide chain does not occur. A new real-space approach involving the use of dynamical simulated annealing, however, has succeeded in circumventing this problem (Nilges et al. 1988a). In contrast to the real-space methods, the folding problem does not exist in the second class of methods that operates in distance space and is generally referred to as metric matrix distance geometry (Crippen 1977). Here, the coordinates of the calculated structure are generated by a projection from N(N-1)/2D distance space (where N is the number of atoms) into 3D Cartesian coordinate space by a procedure known as embedding (Crippen and Havel 1988).

A flow chart of the calculational strategy that is generally used to solve protein structures is shown in figure 10. Because a detailed description of all the various methods would go far beyond the scope of this chapter, the interested reader is referred to the references cited above. (A comparison between the different methods is given in Clore and Gronenborn 1989.) All the methods are comparable in convergence power. In general, however, the structures generated by dynamical simulated annealing or refined by restrained molecular dynamics tend to be better in energetic terms than the structures generated by the other methods, particularly with respect to nonbonded contacts and agreement with the experimental NMR data.

To assess the uniqueness and the precision of the structures determined by any of the above methods, it is essential to calculate a reasonable number of structures with the same experimental data set, but with different starting structures or conditions and to examine their atomic rms distribution. If these calculations result in several different folds of the protein while satisfying the experimental restraints, then the data is not sufficient to determine a unique structure, and either more data must be gathered or the structure determination has to be abandoned. If, however, convergence to a single fold is achieved with only small deviations from idealized covalent geometry, exhibiting good nonbonded contacts in addition to satisfying the experimental restraints, then one can be confident that a realistic and accurate picture of the solution structure of the protein has been obtained. The spread observed in the superposition within the family of structures, or a plot of the rms distribution with respect to the mean, allows one to assess the precision associated with different regions of the protein.

EXAMPLES OF NMR SOLUTION STRUCTURES

Hirudin

Hirudin is a small 65-residue protein from the leech and is the most potent natural inhibitor of coagulation known. It acts by interacting specifically with α-

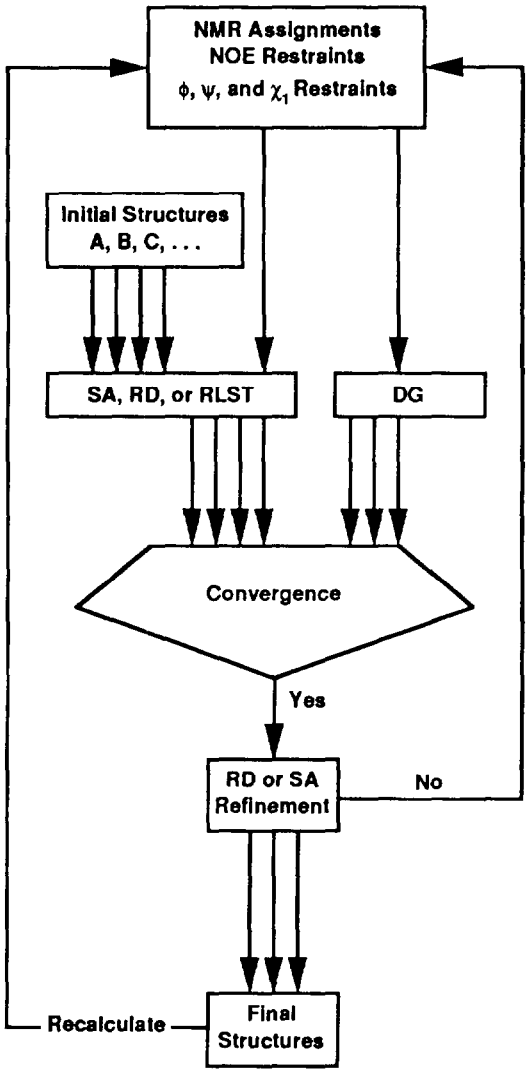


FIGURE 10. *Calculational strategy used to solve 3D structures of macromolecules on the basis of NMR data*

thrombin, thereby preventing the cleavage of fibrinogen. Two recombinant hirudin variants have been examined by NMR: namely, wild-type hirudin and the Lys-47→Glu mutant (Folkers et al. 1988). Analysis of the NMR data indicated that hirudin consists of an N-terminal compact domain (residues 1 to

49) held together by three disulfide bonds and a disordered C-terminal tail (residues 50 to 65). Evidence for the presence of a flexible C-terminal tail was provided by the absence of any intermediate-range or long-range NOEs beyond amino acid 49. Therefore, structure calculations were restricted to the N-terminal domain using the hybrid distance geometry-dynamical simulated annealing method. Experimental input data comprised 701 and 677 approximate interproton distance restraints derived from NOE data for the wild-type and mutant hirudin, respectively, 26 ϕ backbone and 18 x_1 torsion angle restraints derived from NOE and three-bond coupling constant data, and eight backbone hydrogen bonds identified on the basis of NOE and amide exchange data were used as experimental input data. A total of 32 structures were computed for the wild-type and the mutant hirudins (figure 11). The structure of residues 2 to 30 and 37 to 48 constitute the core of the N-terminal domain formed by a triple-stranded antiparallel ß-sheet, and the atomic rms difference between the individual structures and the mean structure is ~0.7 Å for the backbone atoms and ~1 Å for all atoms. The orientation of the exposed finger of antiparallel ß-sheet (residues 31 to 36) with respect to the core could not be determined because no long-range NOEs were observed between the exposed finger and the core. This is easily appreciated from figure 11 because the structures in that region exhibit a large spread. Locally, however, the polypeptide fold of residues 31 to 36 is reasonably well defined.

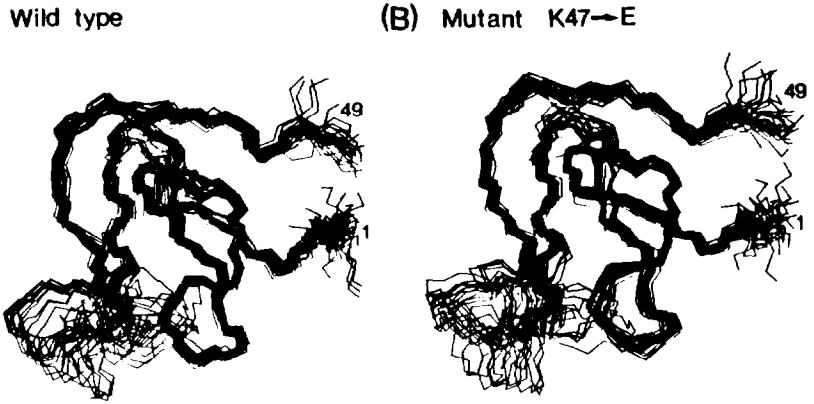


FIGURE 11. *Superposition of the backbone (N, C^a, C) atoms of 32 dynamical simulated annealing structures of wild-type hirudin (A) and the Lys-47→Glu mutant (B) for the first 49 amino acids. The wild-type and mutant structures were calculated on the basis of 701 and 677 interproton distance restraints, respectively, 26 ϕ and 1 18 x_1 torsion angle restraints.*

SOURCE: Folkers et al. 1988.

The first five residues form an irregular strand that leads into a loop closed off at its base by the disulfide bridge between Cys-6 and Cys-14. This is followed by a miniantiparallel ß-sheet formed by residues 14 to 16 (strand I) and 21 to 22 (strand I') connected by a type II turn. This ß-sheet is distorted by a ß-bulge at Cys-16. Strand I' leads into a second antiparallel ß-sheet formed by residues 27 to 31 (strand II) and 36 to 40 (strand II') connected by a ß-turn (residues 32 to 35). In addition, residues 10 to 11 exhibit features of a ß-bulge with the amide of Gly-10 and the carbonyl oxygen atom of Glu-11 hydrogen bonded to the carbonyl and amide groups, respectively, of Cys-28. Finally, strand II' leads into an irregular strand that folds back onto the protein such that residue 47 (Lys in the wild type, Glu in the mutant) is in close proximity to residues in the loop closed off by the disulfide bridge between Cys-6 and Cys-14. Not only the backbone but also many of the side-chain conformations are well defined, especially those in the interior of the protein.

A superposition of the core (residues 1 to 30 and 37 to 49) of the restrained minimized mean structures of the wild-type and mutant hirudin provides a good representation of the differences between the two structures (figure 12, panel A). Regions of noticeable difference can be identified where the atomic rms difference between the two mean structures is larger than the atomic rms distribution of the individual structures about their respective means. This analysis indicates the presence of clear differences for the backbone atoms of residues 3, 5, 8, 11 to 15, 22, 26, and 27. In the mutant, the backbone atoms of residues 3, 4, and 11 to 15 are slightly closer to that of residues 45 to 47 than in the wild type, a change that can be rationalized in terms of the shorter length of the Glu side-chain relative to that of Lys. Concomitantly, the backbone of residue 8 appears to be pushed away in the wild-type structure. Residues 22, 26, and 27 also move in the same direction as residues 11 to 15, but this is secondary to the perturbation of residues 11 to 15 and can be attributed to the presence of numerous contacts between residues 8 to 11 on the one hand and 28 to 30 on the other, including hydrogen bonds between Cys-28 and Gly-10 and between Cys-28 and Glu-11. There seem to be no significant differences, however, with respect to the side-chain positions within the errors of the coordinates, even within the immediate vicinity of residue 47 (figure 12, panel B).

At this point, it may be worth mentioning that NMR can have two important applications with respect to genetically engineered proteins. The structural studies on hirudin were first initiated using natural protein extracted from the whole body of leeches (Clore et al. 1987b). All further work was subsequently carried out on recombinant products, either comprising the wild-type sequence or mutants thereof (Folkers et al. 1988). It was a fast, easy task to assess the structural identity of the recombinant product, because only a comparison of the

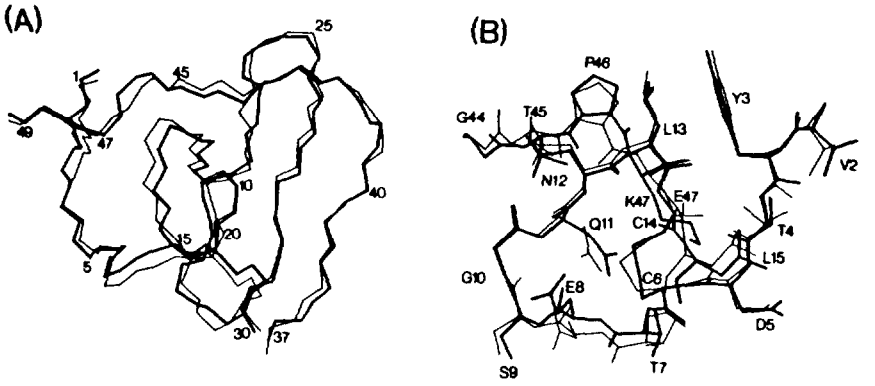


FIGURE 12. *(A) Superposition of the core (residues 1 to 30 and 37 to 49) of the restrained minimized mean structure of wild-type (thick lines) and Lys-47→ Glu mutant (thin lines) hirudin*

SOURCE: Folkers et al. 1989.

(B) View of all atoms around the site of mutation

SOURCE: Folkers et al. 1988.

appropriate 2D spectra had to be carried out. These spectra can be regarded as a fingerprint of a particular protein and, hence, a reflection of the 3D structure in solution. Simple overlay often allows one to ascertain whether the recombinant product is folded indistinguishably from the natural counterpart. In the same way, NMR can establish whether the structure of a mutant is essentially unchanged from the wild-type one, because similar spectra will arise from closely related structures. Analyses of that kind can be carried out without a full assignment of the spectra or a complete structure calculation, thus providing a useful tool for guiding genetic engineering projects.

Interleukin-8

Communications between different cells of the immune system is achieved in part by a complex cascade of interacting proteins known as cytokines, one of which is IL-8, also known as neutrophil activation factor, monocyte-derived neutrophil chemotactic factor (for a review, see Matsushima and Oppenheim 1989). IL-8 is a dimeric protein composed of two identical subunits, each of M_r~8,000, which is released from several cell types, including monocytes, fibroblasts, endothelial cells, and keratinocytes, in response to an inflammatory stimulus.

The pleiotropic properties of IL-8 have important consequences from a medical and clinical perspective. High levels of IL-8 are found in the synovial fluid of rheumatoid arthritis patients and in psoriatic lesions, and the role of IL-8 in several other inflammatory conditions, such as glomerulonephritis, uveities, and asthma, is being investigated (Matsushima and Oppenheim 1989).

The structure of recombinant IL-8 was determined using the hybrid distance geometry-dynamical simulated annealing method (Nilges et al. 1988b, 1988c). The total number of experimental restraints used in the final calculations was 2,242 for the dimer. These comprised a total of 899 interproton distance restraints within each monomer made up of 392 short-range (Ii-j<5) and 185 long-range (Ii-jI>5 interresidue and 270 intraresidue interproton distance restraints and 52 hydrogen bonding restraints, 70 interproton distance and 12 hydrogen bonding restraints between the two monomers, and 181 torsion angle restraints per monomer. The latter comprised 68 ϕ, 61 ψ, and 52x_1 torsion angle restraints. Stereospecific assignments were obtained for 38 of the 55 ß-methylene groups per monomer and for the α-methylene groups of both glycine residues. The latter, and four of the ß-methylene stereospecific assignments, could only be obtained after the initial rounds of calculations.

A total of 30 final simulated annealing (SA) structures were computed. All the structures satisfy the experimental restraints, and there are no interproton distance violations greater than 0.3 Å. The deviations from idealized covalent geometry are small, and the nonbonded contacts as judged by the value of the quartic van der Waals repulsion term and by the Lennard-Jones van der Waals energy are good. Residues 1 to 5 of both subunits are completely ill-determined, because no sequential NOEs were observed for the first three residues and only (i,i+1) sequential NOEs were observed from residues 3 to 6. The remainder of the structure, however, is exceptionally well determined with an overall atomic rms difference between the individual dimer SA structures and the mean coordinate positions of 0.41±0.08 Å for the backbone atoms and 0.90±0.08 Å for all atoms. The average angular rms difference for the ϕ and ψ torsion angles is 8.7±5.9°, and the backbone torsion angles for all nonglycine residues lie in the allowed region of the Ramachandran plot. The majority of side-chain positions are also well defined. However, 18 side chains have atomic rms distributions about the mean coordinate positions larger than 1 Å. All these are highly solvent accessible, and in the case of eight of them, the $^3J_{a\beta}$ coupling constants are indicative of multiple x_1 side-chain conformations.

Three views of the overall polypeptide fold are shown in figure 13. The approximate overall dimensions of the IL-8 dimer (excluding side chains) are 30 Å long, 26 Å wide, and 18 Å deep in the view shown in figure 13, panel A. The structure essentially consists of two antiparallel α-helices lying on top of a

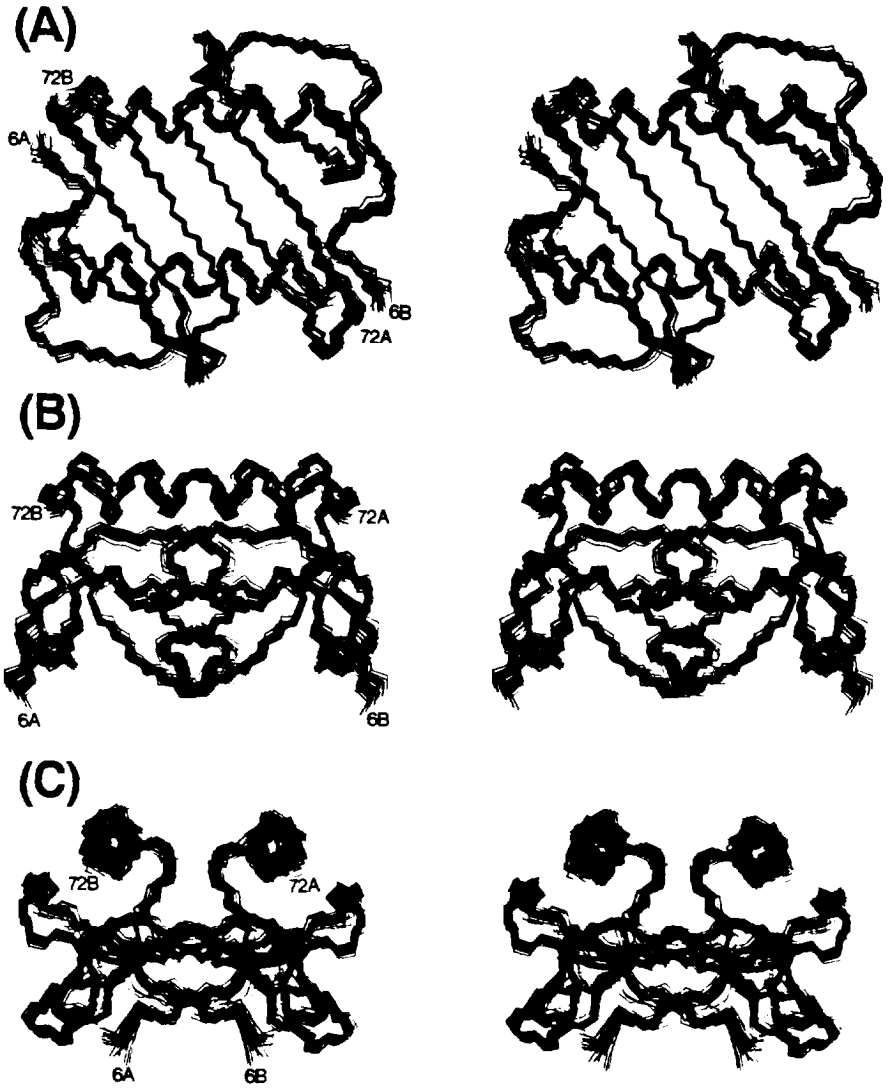


FIGURE 13. *Three stereoviews showing best fit superpositions of the backbone (N, C^a, C) atoms of the 30 SA structures of IL-8. The molecule is viewed down the C_2 symmetry axis in (A), which is located in the center of the ß-sheet halfway between the C^a atom positions of Arg-26 of the two subunits. The views in (B) and (C) are rotations about the C_2 axis, which lies parallel to the plane of the paper.*

SOURCE: Clore et al. 1990.

six-stranded antiparallel ß-sheet. The surface under the ß-sheet is concave and, except for two lie residues, is composed entirely of hydrophilic and charged residues. The two symmetry-related helices are ~24 Å long and separated by a center-to-center distance of ~14 Å. The angle (~172°) between the long axes of the two helices is slightly less than 180° due to the right-handed twist of the ß-sheet below and, in particular, of strand 1 of one subunit and strand 1' of the other. The view of IL-8 shown in figure 13, panel A, is one looking down the C_2 symmetry axis that is located in the center of the ß-sheet halfway between the C^a atom positions of Arg-26 of the two subunits; the views in figure 13, panels B and C, are rotations about the C_2 axis that lies parallel to the plane of the paper. The orientation depicted in figure 13, panel C, demonstrates most strikingly the exposed position of the two α-helices on the top of the molecule.

The overall structure of the IL-8 dimer is similar to that of the AB dimer in the recently determined 3 Å resolution crystal structure of the PF4 tetramer (St. Charles et al. 1989); in particular, the arrangement of the ß-sheet and α-helices is essentially identical.

The orientation of the helix relative to the triple-stranded ß-sheet within each monomer is stabilized by several hydrophobic interactions. The dimer interface is stabilized principally by an antiparallel ß-sheet with six hydrogen bonds between strand 1 from one subunit and strand 1' of the second. In addition to these backbone interactions, there are several other stabilizing forces involving side chains. In particular, there are some hydrophobic contacts and an electrostatic interaction between the two helices. These interactions further serve to maintain the orientation of the C-terminal helices, with respect to each other and to the ß-sheet below.

It is interesting to note that the structure of IL-8 is closely related to the fold of the α1/α2 domains of the class I major histocompatibility (MHC) antigen HLA-A2 determined by x-ray crystallography (Brown et al. 1988) where the sheet is formed by eight ß-strands, four from each domain, with two helices separated by ~18 Å running across them at an angle of ~-45°. Thus, the same kind of general architecture is achieved with two different kinds of ß-pleated sheets in which the strands run almost orthogonal to each other. In the case of HLA-A2, the ß-sheet is formed by strands arising from two domains of a single subunit (i.e., a pseudodimer), whereas in the case of IL-8 and putatively the class II MHC molecules, it is formed by strands originating from two separate subunits (i.e., an intermolecular dimer).

The views of the IL-8 structure presented in figure 13 clearly demonstrate the prominent positioning of the two α-helices. They sit on top of the rather flat ß-

sheet domain, with hydrophobic amino acid side chains forming the central features in the α/β interface; indeed, this α-helix represents an almost idealized amphophilic helix, with hydrophobic residues on the interior face and hydrophilic residues on the solvent-exposed face.

In light of the structural similarities between the α1/α2 domains of HLA-A2 and IL-8, it is tempting to speculate on the functional role of the long helix in IL-8. It may well be that the two long α-helices in IL-8 and other related proteins serve as a major interaction site with the respective receptor. Specific recognition then would be achieved primarily through the different combinations of polar and charged residues on the outside of the helix, which would have their counterparts on the surface of the cellular receptor.

PERSPECTIVE AND CONCLUDING REMARKS

It should be clear from the above discussion that NMR now stands side by side with x-ray crystallography as a powerful method for 3D structure determination. What are the limitations of this approach? At present, the NMR approach is limited to proteins of MW<20,000. Indeed, the largest proteins whose 3D structures have been determined to date are plastocyanin (99 residues) (Moore et al. 1988) the globular domain of histone H5 (79 residues) (Clore et al. 1987a), α-amylases inhibitor (74 residues) (Kline et al. 1986, 1988) and IL-8 (a dimer of 72 residues per monomer) (Clore et al. 1990). Virtually complete assignments, however, have been made for a variety of larger systems, in particular, hen egg white lysozyme (129 residues) (Redfield and Dobson 1988) and the lac repressor headpiece-operator complex (MW~15,000) (Boelens et al. 1987) Staphylococcal nuclease (148 residues) (Torchia et al. 1988), and interleukin-1ß (153 residues) (Driscoll et al. 1990). Further development of novel techniques based on multidimensional NMR in combination with isotopic labeling and the introduction of yet more powerful magnet may make it possible to extend the molecular weight range up to proteins of MW~40,000 in the future. This, however, probably will present a fundamental limit, as the large linewidths of such proteins significantly reduce even the sensitivity of ^{1}H-detected heteronuclear correlation experiments.

At this point, it is appropriate to add a word of caution concerning the practical limits of structure determination by NMR. It is not always the size or the number of residues in a particular protein that determines the feasibility of an NMR structure determination. Other factors play equally important roles, such that the protein is soluble up to millimolar concentrations, nonaggregating, and preferably stable up to at least 40°C, particularly for large proteins. A further consideration is the chemical shift dispersion of the ^{1}H-NMR spectrum. This depends largely on the structure of the protein under investigation. Proteins

that are made up only of α-helices, loops, and turns invariably exhibit a fairly poor proton chemical shift dispersion, while the chemical shift dispersion in ß-sheet proteins is usually good. To a certain degree, such problems associated with chemical shift degeneracy can be overcome by heteronuclear 3D experiments.

Another potential problem may arise from the fact that different regions or domains of a protein may be well defined and, therefore, amenable to an NMR structure determination, while other parts of the same protein may not. This is reflected in the absence of long-range tertiary NOEs for the ill-defined regions and leads to the inability to position these regions with respect to the rest of the protein satisfactorily (e.g., the case of hirudin discussed above). We therefore believe that it is necessary to calculate a reasonable number of structures (ca. 20) with the same experimental data set to obtain a good representation of the NMR structure. Only by analyzing such a family of structures can the local and global definition of a structure be assessed.

X-ray crystallography, of course, also has its limitations, the most obvious being the requirement for a protein to crystallize. Thus, suitable crystals that diffract to high resolution have to be grown, and a successful search for heavy-atom derivatives to solve the phase problem is necessary. Therefore, not every protein will be amenable to NMR and x-ray crystallography. In those cases where this is feasible, the information afforded by NMR and crystallography is clearly complementary and may lead to a deeper understanding of the differences between the solution and crystalline state of the protein.
Finally, it should be stressed that, in addition to being able to determine 3D structures of proteins, NMR has the potential to address other questions, in particular, those concerning the dynamics of the system. This opens the possibility for a variety of different NMR studies, initiated on the basis of an NMR structure, such as the investigation of the dynamics of conformational changes upon ligand binding, unfolding kinetics, conformational equilibria between different conformational states, fast internal dynamics on the nanosecond time scale and below, and slow internal motions on the second and millisecond time scales. The results obtained from these kinetic studies can then be interpreted in the light of the previously determined structure, thus bringing together structure and dynamics of proteins in a unified picture.

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Combined Use of Solid-State Nuclear Magnetic Resonance Spectroscopy, Small-Angle X-Ray Diffraction, and Differential Scanning Calorimetry in Studies of Cannabinoid: Membrane Interactions

Alexandros Makriyannis, De-Ping Yang, and Thomas Mavromoustakos

INTRODUCTION

Because of their complexity and inherent instability, membranes do not lend themselves to a detailed analysis of their structure and dynamics by means of a single physicochemical method. It is, therefore, preferable to combine several experimental methods when seeking to obtain molecular information on the interactions of drugs with membranes. This chapter describes how three biophysical techniques—differential scanning calorimetry (DSC), small-angle x-ray diffraction, and solid-state nuclear magnetic resonance (NMR) spectroscopy—can be combined for such studies. For example, this chapter includes some results from our work with cannabinoids, which appear to induce their physiological effects, in part, by interacting with cellular membranes. Our studies have shown that cannabinoid analogs, which are closely related to each other in structure, can produce strikingly different effects on membrane preparations. These differences are used to explain the respectively large differences in pharmacological potencies among the analogs.

DRUG: MEMBRANE INTERACTIONS

The interactions of many drug molecules with cellular membranes play an important role in determining drug activity. This role can be dual: (1) The drug may induce changes in membrane function through the perturbation of one or more membrane components; and (2) lipophilic drugs may diffuse through the membrane lipid bilayer to reach some specific site(s) of action on a

membrane-associated protein. Thus, it has been argued (Herbette et al. 1986) that for some groups of drugs the location and orientation of the drug molecule in the membrane are critical in determining its ability to reach its site(s) of action in the proper orientation and/or conformation so that it can interact productively with that site.

Evidence indicates that many of the biological properties of cannabinoids can be attributed to their effects on biological membranes (Lawrence and Gill 1975; Bach et al. 1976). These effects may involve amphipathic interactions either with the membrane lipids or with noncatalytic sites in membrane-associated proteins leading to conformational changes at the catalytic sites of those proteins whose functions are affected. Our studies sought to determine the structural requirements for cannabinoid activity by comparing the effects produced on membranes by a group of analogs that are closely related in structure but have different pharmacological potencies (Makriyannis et al. 1986; Kriwacki and Makriyannis 1989). For a more complete understanding of cannabinoid: membrane interactions at the molecular level, we also studied the orientation, conformation, and location of the cannabinoids in the membrane. This chapter compares the psychotropically active (—)-Δ^8-tetrahydrocannabinol (Δ^8-THC) with its inactive O-methyl-ether analog (Me-Δ^8-THC) (Edery et al. 1971; Razdan 1986) and examines the role of the phenolic hydroxyl group in cannabinoid activity.

DSC (Jain and Wu 1977), small-angle x-ray diffraction (Franks and Lieb 1979; Herbette et al. 1983), and solid-state ^{2}H-NMR (Dufourc et al. 1984; Makriyannis et al. 1985, 1986, 1989; Auger et al. 1988) all have been used to study the effects of drugs on membranes. Each method has distinct advantages and limitations and when used independently can give only partial information. However, the three methods can be complementary when combined and can lead to a more detailed understanding of the molecular features of the drug/membrane interaction. DSC is a relatively inexpensive technique that allows quantitative study of the thermotropic properties of membranes and the effects of drugs on these properties. Small-angle x-ray diffraction provides information on the unit cell repeat distance and packing of the membrane bilayer. Solid-state NMR gives the most detailed molecular information on membrane conformation and dynamics and on the effects of drugs on each. NMR also can be used to identify the orientation and conformation of the drug molecule in the membrane.

The first part of this chapter outlines the basic principles for each of the three biophysical methods and presents appropriate examples; the second part demonstrates how these methods can be combined to yield detailed information on drug: membrane interactions.

DIFFERENTIAL SCANNING CALORIMETRY

DSC is a thermodynamic technique suitable for studying phase transitions of various materials. It has been used extensively to investigate the thermotropic properties of membranes and obtain information on the effects of drugs on membranes. In a DSC experiment, a sample and a reference "inert" material are heated simultaneously at identical rates. If there is no phase transition, the differential heat flow between the sample and the reference is zero. During the phase transition, when the sample undergoes a thermally induced event, the control system senses the resulting temperature differential between the sample and reference cells and supplies heat to the sample cell to hold its temperature equal to that of the reference. The term "scanning" signifies that the temperatures of both the sample and the reference are varied in the same programed rate and that the excess specific or differential heat is recorded as a function of temperature. The area under the peak during the thermal event permits calculation of the enthalpy of transition (ΔH) (Ladbrooke and Chapman 1969; Jürgen and Sturtevant 1972; Melchior and Steim 1976).

Certain hydrated phospholipids spontaneously form bilayers that share many conformational and dynamical properties with the natural membranes. Studies with these fully hydrated phospholipids are, therefore, useful because they allow insight into the physical chemistry of lipid interactions in natural membranes in which phospholipids are believed to exist, largely as liquid crystalline bilayers (Steim et al. 1969).

Among membrane phospholipids, phosphatidylcholines are a major component whose phase properties have received much attention. An example for L-α-dipalmitoylphosphatidylcholine (DPPC) is given in figure 1. The thermograms for a fully hydrated DPPC preparation show two endothermic transitions in the temperature range usually used to study membranes: a broad low-enthalpy pretransition (T'_c=35.3°C) and a main transition (T_c=41.3°C). Below the pretransition, the phospholipid molecules are arranged in a one-dimensional lamellar gel phase (L_{β}'); above the main transition they exist in the liquid crystalline phase (L_a). At temperatures between T'_c and T_c, there is a ripple phase (P_{β}'), which, on the basis of solid-state NMR evidence, has been shown to be composed of coexisting gel and liquid crystalline components. These phase transitions also can be detected by small-angle x-ray diffraction and solid-state NMR. However, DSC is a simple and accurate method that can be used to study the gel-to-liquid crystalline phase transitions in phospholipid bilayers and biological membranes (McElhaney 1982).

The effects of more than 100 compounds on the thermotropic behavior of DPPC were investigated by Jain and Wu (1977), who stated that lipophilicity of a

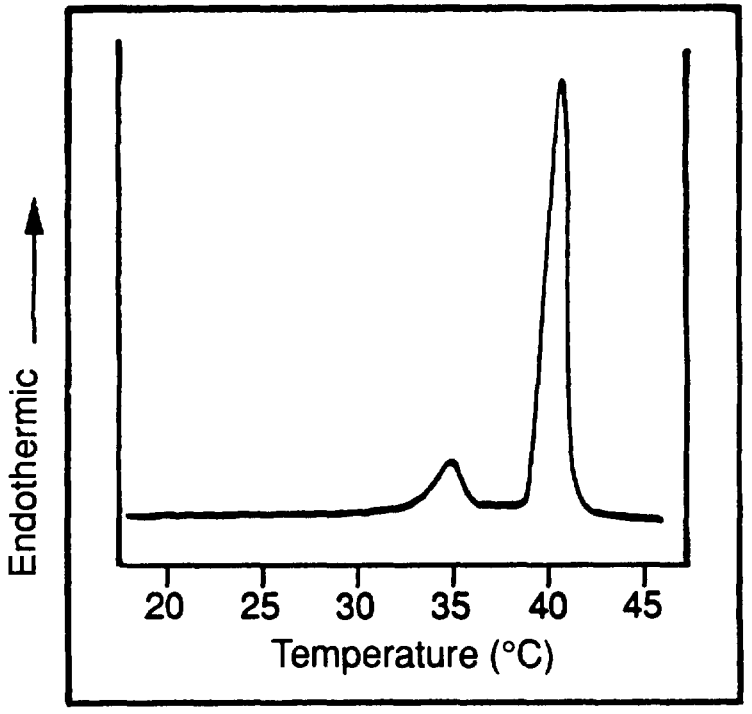

FIGURE 1. *Thermogram of hydrated DPPC bilayers (50 percent w/w) using a scanning rate of 2.5°C/min*

molecule cannot always explain its localization in the membrane. In contrast, the localization of a molecule in a lipid bilayer is determined by the presence of the polar and apolar groups in the compound and by the geometric arrangements of these groups within the molecule.

SMALL-ANGLE X-RAY DIFFRACTION

X-ray diffraction is a useful and direct method for characterizing materials having periodicity in their structures. Lipid bilayers can be packed into a stack of lamellae that give coherent Bragg-like reflections. Thus, information can be obtained about bilayer structures and about the effects of drugs on them (Franks and Levine 1981).

X-rays are produced by bombarding a metal target (almost always copper) with electrons that have been accelerated through a voltage of about 30 to 40 kV. These x-rays consist mainly of a Cu K_a spectral line at 1.54 Å, $K_ß$ at 1.34 Å, and a broad background whose wavelength distribution is determined by the accelerating voltage. Nickel foils are used to eliminate the Cu $K_ß$ line and cause only a tolerable reduction in the intensity of the Cu K_a radiation. The x-rays then are collimated into either a fine line or a point by a system of pinholes or by a combination of reflecting mirrors and crystal monochromators. In the specimen-to-detector path, hellum is used to prevent intensity loss due to air scattering. X-ray diffraction data then can be collected by using either a stack of films or a position-sensitive detector. To prevent overexposure of the films or damage of the detector by the intense main beam, a small beam-stop is required.

Regularly stacked lipid bilayers give various diffraction orders that are approximately equally spaced and follow Bragg's law—$2d \sin\theta = h\lambda$.—where d is the unit cell repeat distance (d-spacing), θ is called the Bragg angle (<15°), h is the order number, and λ is the wavelength of the K_a radiation. Bragg's law reveals the d-spacing from the known wavelength λ and measurable angle θ. To obtain finer details on membrane structure, diffractions at larger angles must be used (Shipley 1973; Silver 1985).

From the diffraction pattern, the electron density profile structure of the membrane can be derived by assuming that the electron density profile is centrosymmetric and the membrane stack has a characteristic time-averaged electron density distribution only in the dimension perpendicular to the membrane plane. This one-dimensional projection is considered as a continuous distribution of electron density that repeats periodically (Franks and Levine 1981). Figure 2 shows examples of diffraction patterns of partially hydrated dimyristoylphosphatidylcholine (DMPC) bilayers at temperatures below and above the phase transition and the corresponding derived electron density profile structures.

The x-ray diffraction pattern shows that the d-spacing of the bilayer decreases when going from the gel to the liquid crystalline state. This is due to an increased *gauche: trans* conformer ratio in the methylene segments of the bilayer chains in the liquid crystalline phase. On this basis, x-ray diffraction experiments can be used to obtain information on the conformational properties of the bilayer chains. On the other hand, d-spacing measurements as a function of temperature provide information about the phase transition of the membrane, including phase transition temperatures and the sharpness of these transitions. In this aspect, x-ray diffraction is complementary to DSC.

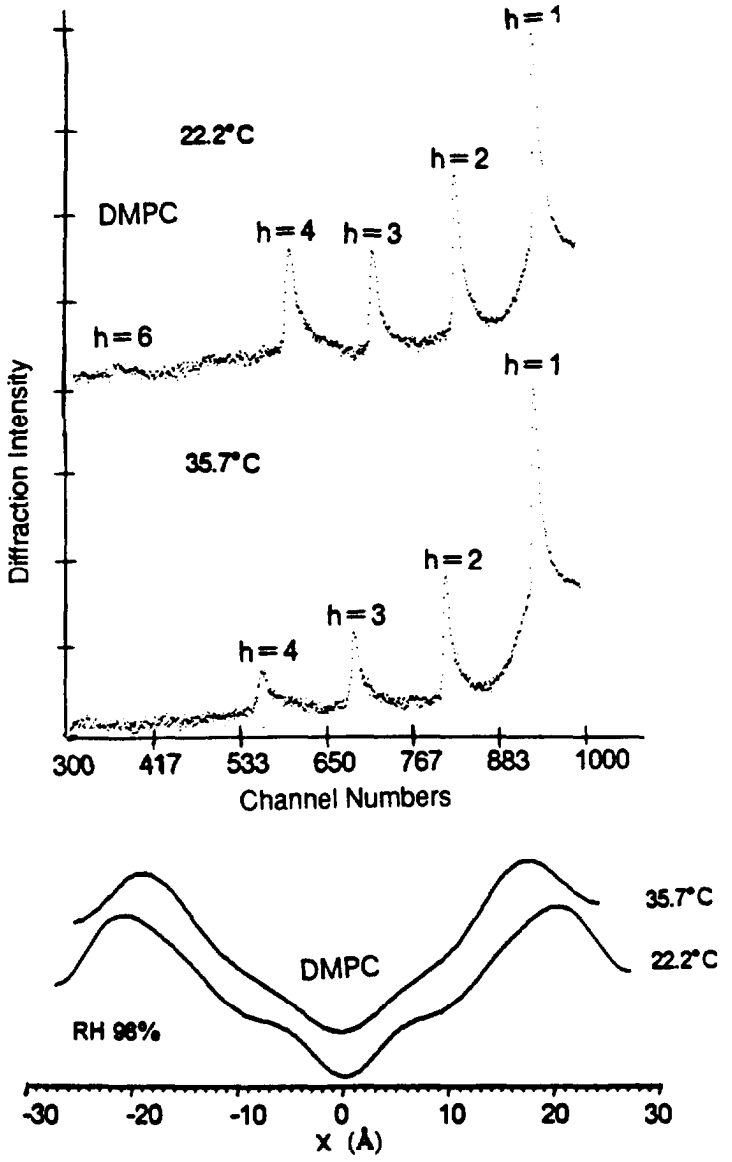

FIGURE 2. *Diffraction patterns of DMPC bilayers at a relative humidity (RH) of 98 percent below and above its phase transition and their corresponding derived electron density profile structures*

Small-angle x-ray diffraction in drug research is useful in obtaining information on the location of a drug molecule in the membrane. This method is based on comparing the electron density profile from a membrane preparation serving as a control with that from an identical membrane preparation into which the drug molecule is incorporated. The observed differences allow determination of the approximate location of the drug in the bilayer (Franks and Lieb 1979; Herbette et al. 1986).

SOLID-STATE NUCLEAR MAGNETIC RESONANCE

Since the early 1960s NMR has found many useful applications in biochemistry and other related fields of research. It is the method of choice for obtaining detailed molecular and dynamic information on the interaction of ligands with functional biopolymers. In this regard, it has been used to study drug: active-site interactions and provide information for rational drug design.

To date, most of this work has made use of high-resolution NMR, which is most successful if both ligand and biopolymer are present in solution. However, in spite of its wide applicability in the study of biological systems, high-resolution NMR suffers from serious limitations. Principal among these is the inability to obtain well-resolved spectra from relatively large biopolymers (molecular weight>25,000) because of severe line broadening. These difficulties can be circumvented with the use of solid-state NMR techniques. During the past decade these methods have led to previously unavailable detailed molecular information on the conformational and dynamic properties of proteins and membranes (Griffin 1981).

The solid-state NMR experiment can be carried out by observing the wide-line spectrum obtained from one or a small number of nuclei in the system under investigation. In such experiments, the sample is stationary and the nucleus under observation either is introduced by isotopic labeling (e.g., ^{2}H, ^{13}C) or is already present in natural abundance in the system (e.g., ^{31}P, ^{14}N). Information from the spectra then is obtained by lineshape analysis, including spectral simulations based on specific conformational and motional models, Alternatively, the spectrum from the solid or semisolid sample can be obtained in high-resolution mode using the magic angle sample spinning experiment (Groot et al. 1988). Such spectra resemble those obtained from solution and can be analyzed using analogous principles.

Stationary solid-state ^{2}H-NMR has been used to obtain information on the conformational and dynamic properties of model and biological membranes (Fyfe 1983; Davis 1983; Smith and Oldfield 1984). The use of solid-state ^{2}H-NMR also has been extended successfully to study drug: membrane

interactions (Smith 1983; Makriyannis et al. 1985, 1986; Auger et al. 1988). This method finds application in systems undergoing anisotropic motions such as with phospholipid multilamellar bilayers or biological membranes (semisolids). In such systems the deuterium nuclei give rise to a "powder spectrum" in which the doublet with the maximum intensity is the ^2H quadrupolar splitting ($\Delta\nu_Q$) and is equal to (Davis 1983)

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD}$$

where e^2qQ/h is the static quadrupolar coupling constant, which for paraffinic C—D bonds is approximately 170 kHz. S_{CD} is the order parameter, which can be used to describe the amount of motional averaging of the C—D bond vector with respect to a fixed symmetry axis and has been used as a measure of membrane "fluidity."

Figure 3 is an example of solid-state ^2H-NMR spectra of aqueous dispersions of DPPC, ^2H-labeled in the 7'-methylene group of the *sn-2* chain, at a temperature range covering three phases. At 42°C, the hydrated 2[7', 7'-$^2H_2$]-DPPC (Makriyannis et al. 1986) is in the liquid crystalline phase and gives rise to a powder pattern similar to that observed in the ^2H spectrum of a solid. However, the quadrupolar splitting is drastically reduced due to motional averaging, including axial diffusion of the chains and *gauche: trans* isomerization in the methylene chain segments. Its spectrum is axially symmetric with sharp parallel and perpendicular edges and has a residual quadrupolar splitting of $\Delta\nu_Q$ =28.1 kHz. As the temperature is raised, the $\Delta\nu_Q$ values decrease, indicating an increase in the *gauche: trans* conformer ratio in the chains. When the preparation exists in the $P_{\beta'}$ phase (36°C), the spectrum is broadened, but it has a flattop appearance and only its perpendicular edges are discernible. This spectral appearance is compatible with the existence of two exchanging components, a gel component and a liquid crystalline-like one. At 17°C, the preparation exists in the gel phase and gives a broadened spectrum having a conical shape with *a* round top.

Solid-state ^2H-NMR also can be used to study the orientation of drug molecules in membrane bilayers. The study requires the strategic introduction of ^2H labels in different positions of the rigid part of the molecule. The spectra due to the ^2H labels on the drug molecule in the liquid crystalline phospholipid bilayer have quadrupolar splittings that depend on the angle between the individual C—D bonds and the director axis. If, as expected, the drug molecule undergoes anisotropic motions in the bilayer, then each deuteron attached to the rigid part will be expected to have an ^2H quadrupolar splitting described by the following equation:

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}\left(\frac{3\cos^2\alpha - 1}{2}\right)\left\langle\frac{3\cos^2\theta - 1}{2}\right\rangle$$

where α is the angle between the C-^{2}H bond in question and the director axis. The last term, which is known as the molecular order parameter (S_{mol}), describes the anisotropic motion of the rigid component. Because S_{mol} is

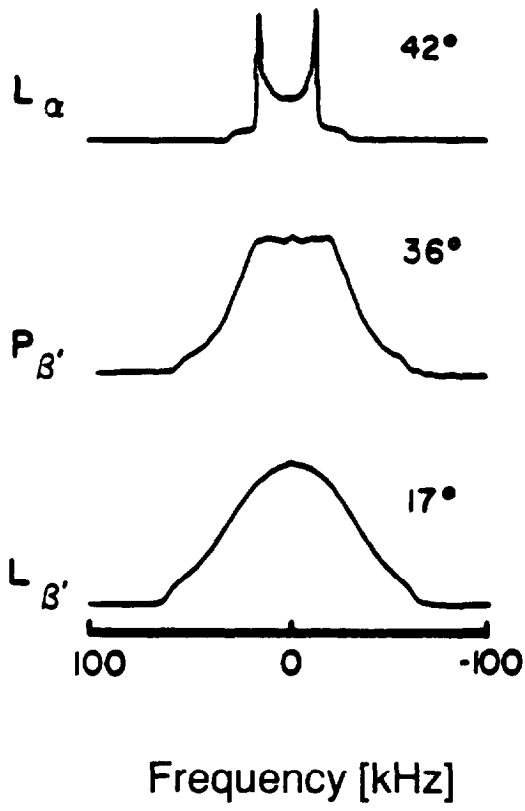

FIGURE 3. *Solid state ^{2}H-NMR spectra representing the three phases for DPPC aqueous dispersions within the temperature range at which studies were conducted*

NOTE: The solid state ^{2}H-NMR spectra due to the 2[7', 7'-$^2H_2$] segment of DPPC was obtained using a $90°_x$-τ-$90°_y$ quadrupolar echo pulse sequence at 45.3 MHz with a 90° pulse width of 2.1 μs; 8,000 echoes were signal averaged.

expected to be identical for all ^2H atoms attached to the rigid part of the molecule, the value of the observed quadrupolar splitting thus can be directly related to α. If the Dv_Q values for a set of deuterons are available, one can then obtain a value for S_{mol} and a set of values for α, which give the orientation of the director axis. Because this axis is assumed to be parallel to the bilayer chains, the orientation of the drug molecule in the bilayer can be determined.

This method was initially introduced for molecules undergoing symmetric axial rotation in the bilayer (Taylor et al. 1981; Ekiel et al. 1988). Recently, we modified this method to include molecules undergoing asymmetric axial motions and, thus, expanded its scope significantly (Makriyannis et al. 1989).

CANNABINOID: MEMBRANE INTERACTIONS

Extensive studies on the structure-function correlation of cannabinoids have shown that removal of the phenolic hydroxyl group or methylation to give the corresponding O-methyl ether results in an analog devoid of biological activity (Edery et al. 1971; Razdan 1986). In this example, the effects of Δ^8-THC on membranes are compared with those of Me-Δ^8-THC.

Differential Scanning Calorimetry

Normalized thermograms of fully hydrated DPPC preparations containing increasing concentrations of Δ^8-THC and Me-Δ^8-THC are shown in figure 4. A low THC: DPPC molar ratio (5:95, or x=0.05) produces elimination of the pretransition and broadening of the main transition. At a higher drug concentration (x=0.10), the main transition broadens and moves down in temperature, while two new broad peaks appear in the thermogram at lower temperatures. When the drug concentration is increased to x=0.20, the middle peak intensifies and becomes the dominant one in the thermogram (table 1). The DSC results of Δ^8-THC: DPPC data closely correspond to those of another active THC isomer, the naturally occurring (—)-Δ^9-tetrahydrocannabinol (Δ^9-THC) (Van der Schyf et al. 1988). In all of the above thermograms, the phase transition enthalpy ΔH remains essentially unchanged.

The thermotropic effects of Me-Δ^8-THC on DPPC bilayers are different from those of Δ^8-THC. At x=0.05 the pretransition is still observable, although broadened. The main transition has the same halfwidth as that of the pure hydrated lipid, with only a small decrease in T_c. At higher drug concentrations (x=0.10, x=0.20), there is still no appreciable change in the onset of the main transition; only a progressive broadening of the transition is observed, and a shoulder appears at the low temperature end when x=0.20.

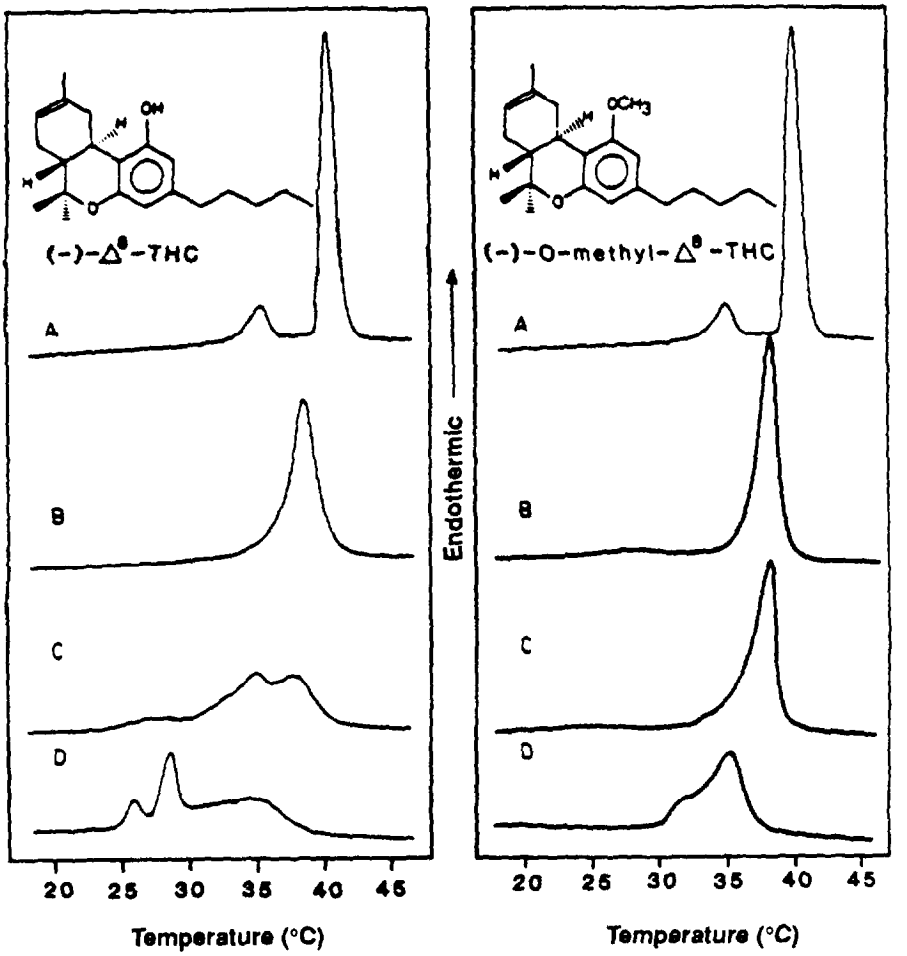

FIGURE 4. *Normalized thermograms of DPPC preparations containing increasing concentrations of Δ⁸-THC and Me-Δ⁸-THC*

KEY: (A) x=0.00; (B) x=0.05; (C) x=0.10; (D) x=0.20

The experiments show that only the active cannabinoid associates or complexes with the phospholipids in the bilayer, in contrast with the inactive one, which does not appear to participate in such interactions. These results indicate that the presence of a free phenolic OH seems to be a necessary requirement for the formation of the Δ⁸-THC: DPPC complex.

TABLE 1. *Effect of (—)-Δ⁸-THC and (—)-O-methyl-Δ⁸-THC on the onset temperature, midpoint transition temperature (T_c), and enthalpy change (ΔH) of hydrated DPPC obtained by DSC*

Cannabinoid	Concentration (x)	Onset Temperature (C)	T_c (C)	DH (cal/g)
(—)-Δ⁸-THC	0.05	37.9	39.6	10.5
	0.10	32.1	27.5,35.0,38.0	9.7
	0.20	27.5	26.5,29.2,35.5	10.9
(—)-O-mathyl-Δ⁸-THC	0.05	38.2	39.9	10.3
	0.10	37.5	39.6	9.9
	0.20	38.4	35.2	10.0

X-Ray Diffraction

Small-angle x-ray diffraction was used to study the location of the biologically active Δ⁸-THC in a partially hydrated model membrane and obtain direct evidence on the topography of the drug in the bilayer. We compared the electron density profiles of DMPC bilayers containing Δ⁸-THC and those with (—)-5'-iodo-Δ⁸-tetrahydrocannabinol (5'-I-Δ⁸-THC), a molecule that contains the heavy atom iodine at the end of the alkyl chain, and obtained an estimate of the position of the iodine atom in the bilayer. To describe the differences in electron density profiles, the centrosymmetric bilayer profile was divided into three regions (figure 5). Region I (from 0 to±9Å) covers the terminal methyl groups and the last 5 to 6 methylene segments of the acyl chains. Region II (from ±9Å to ±20Å) covers the remaining methylene segments and the interface, up to the headgroup region. Region III (from±20Å to *±d/2)* covers the rest of the headgroup and the water region between the bilayers. The electron density is the number of electrons per unit volume and is denoted by *e/v.*

Some representative electron density profiles of pure DMPC, DMPC+Δ⁸-THC, and DMPC+5'-I-Δ⁸-THC at RH 98 percent are shown in figure 6. A careful comparison shows that the presence of the cannabinoid always enhances the *e/v* in region II. This enhancement is centered in a region approximately 13Å from the center of the bilayer and strongly suggests that the drug molecule is located in region II (i.e., at or near the amphipathic interface of the bilayer). Thus, it appears that the cannabinoid may anchor itself to the bilayer interface through its phenolic hydroxyl group, probably by hydrogen bonding with the ether or the carbonyl oxygens of the lipid molecule. When comparing the electron density profiles of DMPC+Δ⁸-THC and DMPC+5'-I-Δ⁸-THC at RH 98 percent, we observed that the *e/v* difference was in region I (below and above

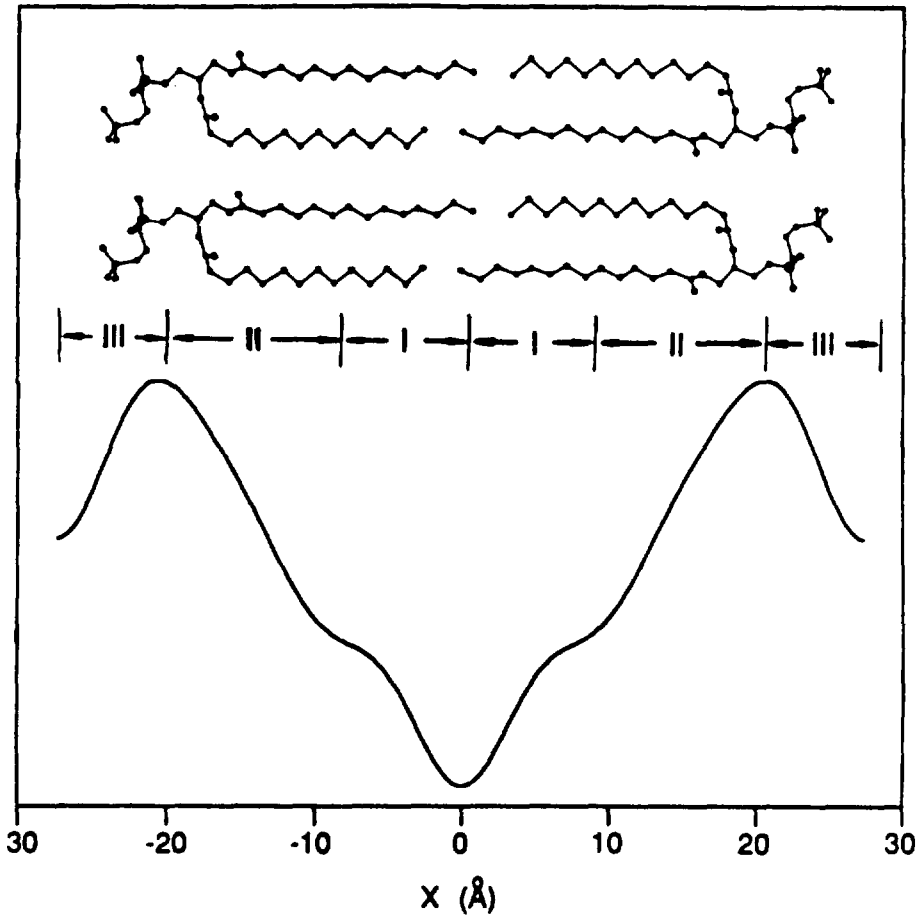

FIGURE 5. *Schematic correlation between an electron density profile and a space filling model of a phospholipid bilayer, with the centrosymmetric bilayer divided into regions I, II, and III*

T_c). This enhancement is centered at approximately 6Å from the center of the bilayer and is due to a contribution from the iodine atom of 5'-I-Δ^8-THC (figure 7). This is evidence that the cannabinoid side chain exists in an extended conformation in the bilayer and runs parallel with the bilayer chains.

Also, the temperature dependence of the *d*-spacing of DMPC bilayers was compared with those containingΔ^8-THC (figure 8). The results show again that

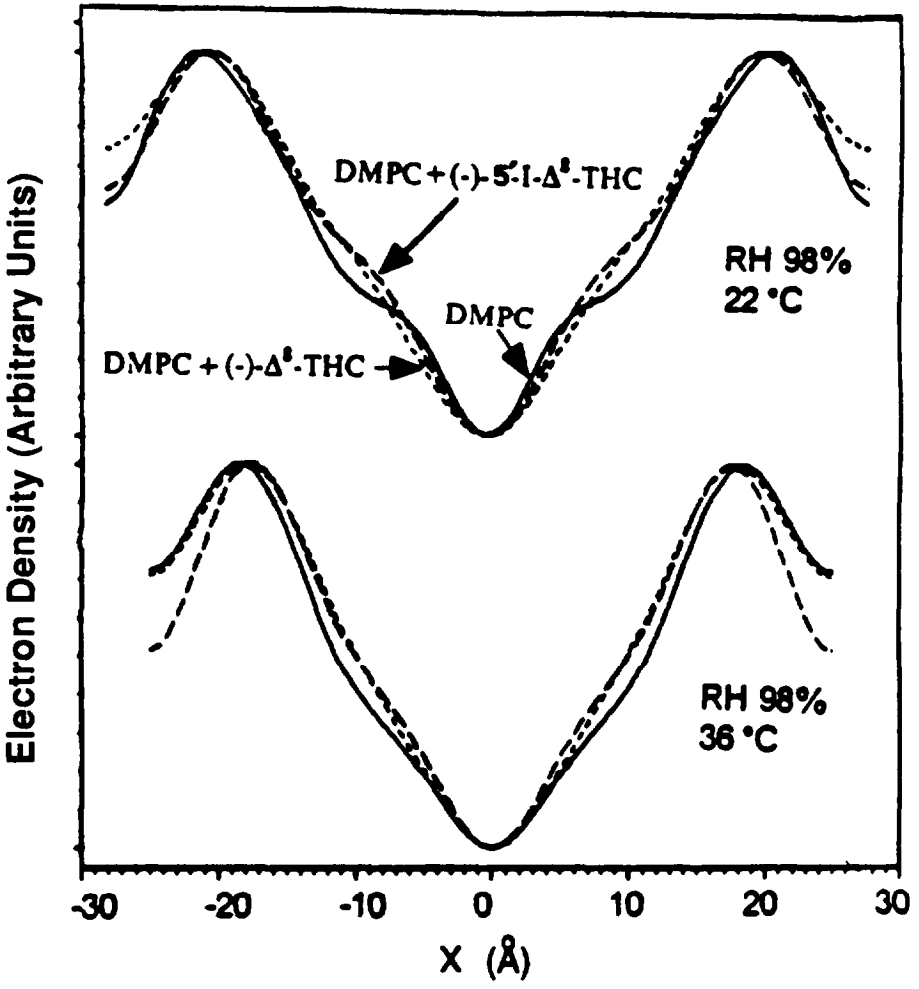

FIGURE 6. *Comparisons of the electron density profiles of DMPC (solid line), DMPC+D^6-THC (x=0.15, short dashed line), and DMPC+5'-I-D^6-THC (x=0.15, long dashed line) at RH 98 percent*

Δ^8-THC induces significant changes in the *d*-spacing of DMPC, whereas the profile of DMPC+Me-Δ^8-THC differs only slightly from that of DMPC. As with DSC, these data provide evidence that the active cannabinoid Δ^8-THC induces severe changes in the conformational and phase properties of the membrane bilayer, whereas the inactive O-methyl analog alters the membrane properties significantly less.

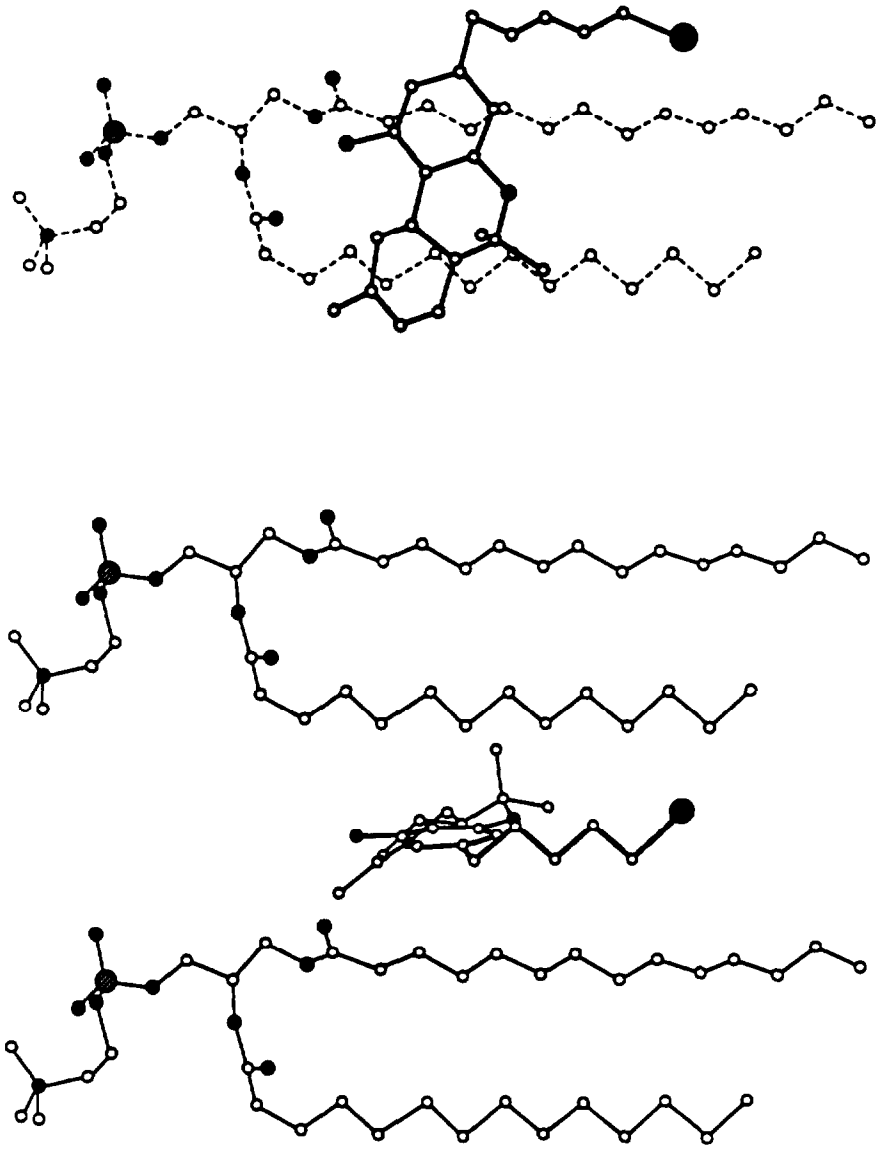

FIGURE 7. *Representation of 5'-l-Δ^9-THC molecule (two different perspectives) in a DMPC bilayer based on results from x-ray diffraction and solid-state ^{2}H-NMR experimental data*

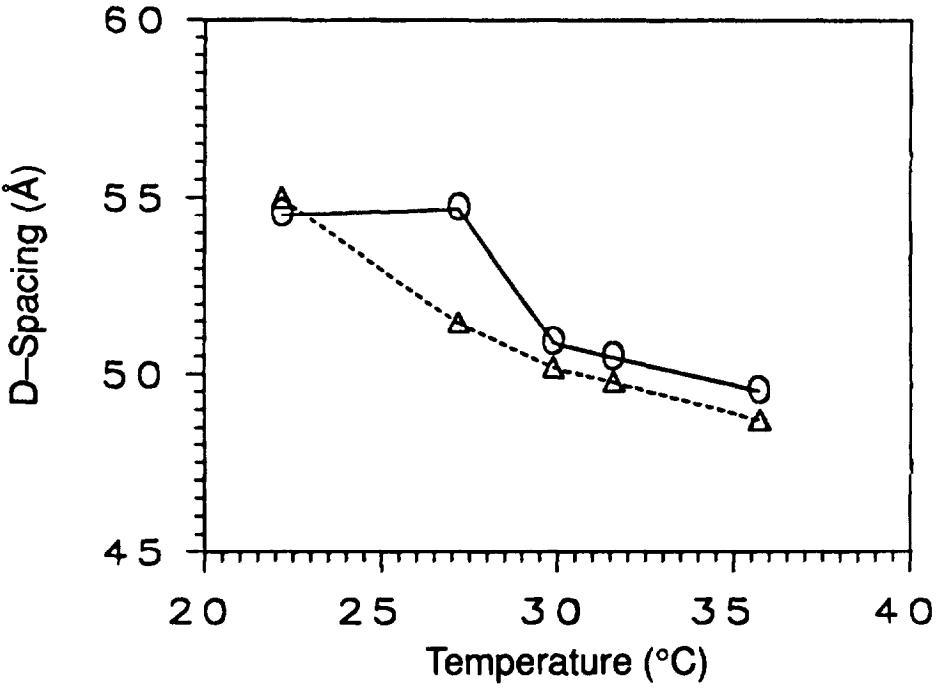

FIGURE 8. *D-spacing as a function of temperature for bilayers of DMPC (circles and solid lines) and DMPC+Δ⁸-THC (x=0.15, triangles and dashed lines) at RH 98 percent*

Solid-State Nuclear Magnetic Resonance

The phase properties of hydrated DPPC preparations as described earlier are reflected in the respective solid-state ^{2}H-NMR spectra from 1,2[2', 2'-$^2H_2$]-DPPC. Figure 9 shows representative spectra obtained from the model membrane preparation in the absence and presence of Δ⁸-THC (x=0.20) or Me-Δ⁸-THC (x=0.20).

In the liquid crystalline phase (45°C), the ^{2}H spectrum of DPPC shows three components represented by three quadrupolar splittings. According to Seelig (1977), the largest splitting corresponds to the two deuterons at the C-2' position of the *sn*-1 chain and the other two splittings to the corresponding deuterons on the *sn*-2 chain. This inequivalence of the spectra due to the *sn*-1 and *sn*-2 α-C$^2H_2$ segments has been attributed to the different average conformations of the chains in the region of the glycerol backbone. At 39°C the

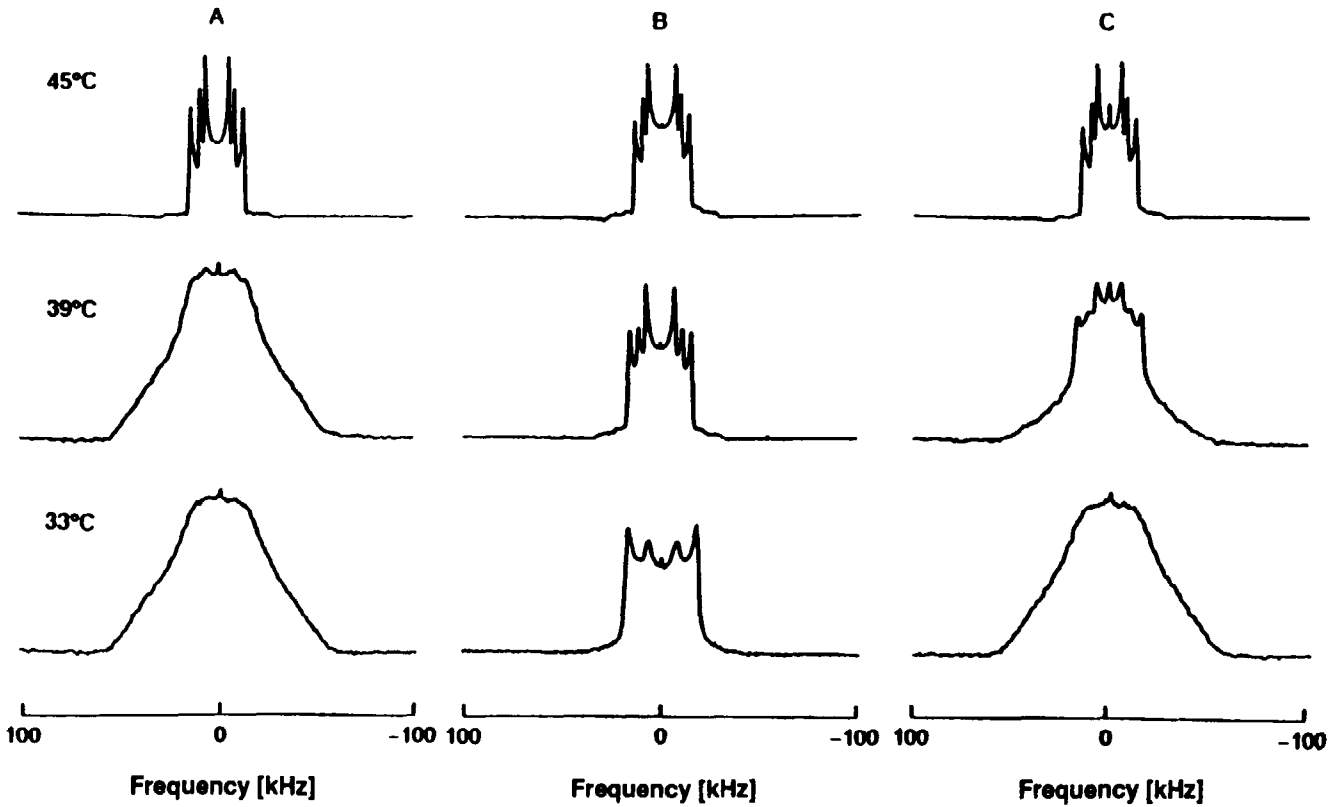

FIGURE 9. *Temperature dependence of solid-state ^{2}H-NMR spectra due to the 1,2[2', 2'-$^2H_2$] segment of DPPC*

KEY: (A) DPPC; (B) DPPC+Δ^8-THC (x=0.20); (C) DPPC+Me-Δ^8-THC (x=0.20)

spectrum from pure DPPC exhibits the flattop lineshape characteristic of the $P_{ß}$, phase. Δ^{8}-THC produces significant changes in the spectra of 1,2[2', 2'-$^{2}H_{2}$]-DPPC, whereas its O-methyl ether analog has little effect. The Δ^{8}-THC/DPPC preparation gives a three-component L_{a}-type spectrum, and Me-Δ^{8}-THC/DPPC gives a spectrum in which the gel and liquid crystalline phases are discernible. At 33°C the spectra from pure DPPC and Me-Δ^{8}-THC/DPPC are virtually identical with no sharp features in their lineshapes. On the other hand, the Δ^{8}-THC/DPPC spectra show the characteristic features described earlier, with sharp perpendicular edges but no parallel edges. Only two of the three components observed in the L_{a} spectrum can be seen. The third intermediate component is invisible, probably due to broadening in the other two. This type of spectrum is evidence for the presence of a mix of gel and liquid crystalline components undergoing intermolecular exchange at an intermediate rate.

As already observed in the DSC and x-ray diffraction experiments, Δ^{8}-THC decreases the main phase transition by 6°C, while Me-Δ^{8}-THC produces a decrease of less than 2°C. The phase transition of DPPC/Δ^{8}-THC is more gradual than that of pure DPPC, and the spectra retain more liquid crystalline features. Although the presence of Δ^{8}-THC produces no qualitative changes in the L_{a} bilayer lineshapes of DPPC, it does increase the quadrupolar splittings in all three spectral components (table 2). The increase is more significant with the two components representing the *sn*-2 chain. This effect may be interpreted to indicate a closer interaction of the drug molecule with the *sn*-2 chain in the bilayer and could be the result of a slight increase in the *trans: gauche* methylene conformer ratio. Alternatively, the increased $\Delta\nu_{Q}$ values may be associated with small drug-induced changes in the orientation of the α-$C^{2}H_{2}$ segment with respect to the bilayer surface. The methyl ether analog produces no significant changes in the quadrupolar splittings of the DPPC preparation. The data point to the significance of the phenolic hydroxyl group in cannabinoids during their interactions with membranes. These effects are almost entirely reversed when hydroxyl hydrogen is substituted with the hydrophobic methyl group.

The above conclusion is further strengthened by results from experiments aimed at determining the orientation of Δ^{8}-THC and Me-Δ^{8}-THC in DPPC multilamellar bilayers. This study required the introduction of ^{2}H labels in different positions of the tricyclic cannabinoid system. For this experiment, we synthesized Δ^{8}-THC and Me-Δ^{8}-THC with specific deuterium labels in the 2, 4, 8, 10α, 10ß, and 10a positions. ^{2}H-NMR spectra were obtained for preparations containing one or more ^{2}H labels that could be unambiguously assigned in the solid-state spectra. Calculations (Makriyannis et al. 1989) showed that Δ^{8}-THC orients with the long axis of its tricyclic structure perpendicular to the bilayer chains (figure 10). Presumably, this “awkward” orientation optimizes the

TABLE 2. *Solid-state ^{2}H-NMR quadrupolar splittings for 1,2[2', 2'-$^2H_2$] DPPC preparations in the absence and presence of drug molecule (x=0.20) at 45°C*

	$\Delta\nu_Q$ (kHz)		
Chain	DPPC	DPPC+Δ^8-THC	DPPC+Me-Δ^8-THC
sn-1	26.8	7.4	26.5
	17.8	9.3	17.7
sn-2	12.0	14.1	12.1

interaction of the cannabinoid OH group with the polar membrane groups. On the other hand, calculations showed that Me-Δ^8-THC assumes an orientation in which the long axis of the tricyclic structure is parallel to the lipid chains (figure 10). From geometrical and mechanical points of view, that the drug's rotation axis coincides with its natural long axis points to the absence of hydrogen bonding between Me-Δ^8-THC and the lipid bilayer. This may explain the inability of this molecule to effectively perturb the membrane at the interface.

The striking difference between the orientations of Δ^8-THC and its O-methyl ether analog in DPPC provides evidence that the anchoring effect by the THC phenolic OH on the membrane determines its orientation in the bilayer. The results also suggest that the orientation of cannabinoids in the amphipathic membrane may play an important role in determining the nature of the drug: membrane interaction and the drug's ability to induce productive membrane perturbations. The above conclusions on the location and orientation of the biologically active (—)-Δ^8-THC also may have some bearing on those cannabinoid effects that may be produced through interactions with specific sites on one or more membrane-associated proteins. Assuming that the cannabinoid preferentially partitions in the membrane bilayer then reaches the protein sites through lateral diffusion, its ability to reach these sites is determined to a large extent by its location and conformation in the bilayer. It is tempting to speculate that the cannabinoid sites on the protein(s) are present at the level of the bilayer interface and that the conformation of (—)-Δ^8-THC in the membrane, as described here, allows it to engage in the productive drug: active-site interactions.

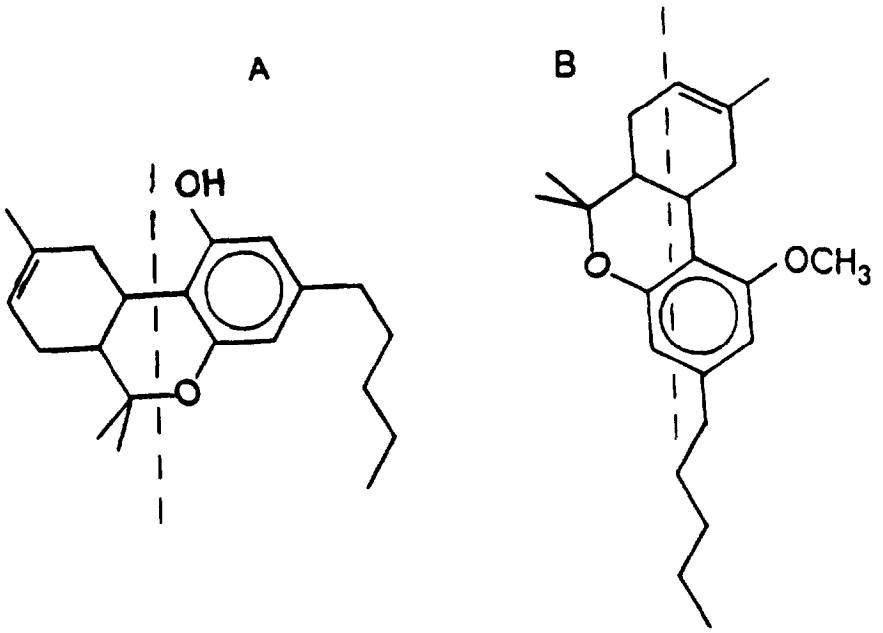

FIGURE 10. *The orientations of Δ^8-THC (A) and Me-Δ^8-THC (B) in hydrated DPPC bilayers as determined by solid-state ^{2}H-NMR*

NOTE: Dashed lines indicate the direction of the lipid chains.

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Characterization and Localization of Cannabinoid Receptors in Brain: An In Vitro Technique Using Slide-Mounted Tissue Sections

Miles Herkenham

INTRODUCTION

Marijuana *(Cannabis sativa)* is one of the oldest and most widely used drugs in the world, with a history of use dating back over 4,000 years (Harris et al. 1977; Mechoulam 1986). It was not until about 20 years ago that the principal psychoactive ingredient of the marijuana plant was isolated and found to be Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam 1964; Mechoulam 1973; Mechoulam et al. 1970). Δ^9-THC and other natural and synthetic cannabinoids produce characteristic behavioral and cognitive effects (Dewey 1986; Hollister 1986), most of which can be attributed to actions on the central nervous system (Martin 1986). However, until recently, very little was known about the cellular mechanisms through which cannabinoids act. The unique spectrum of cannabinoid effects, coupled with evidence of stereoselectivity of action of cannabinoid isomers (see below), strongly suggested the existence of a specific cannabinoid receptor in brain, but attempts to identify and characterize such a recognition site were not successful. As discussed by Devane and colleagues (1988), cannabinoids are extremely hydrophobic and adhere to fitters and other surfaces (Garrett and Hunt 1974). Other obstacles in the early attempts to characterize the receptor were (1) the use of [^{3}H]Δ^9-THC (Harris et al. 1978) or [^{3}H]Δ^9-THC (Roth and Williams 1979), which bind with low affinity and have low specific activities, and (2) the use of [^{3}H]5'-trimethylammonium-Δ^8-THC (Nye et al. 1985), which is water-soluble but does not act like a cannabinoid in animal tests.

Without evidence that cannabinoids act through a specific receptor coupled to a functional effector system, researchers were prone to study the effects of cannabinoids on membrane properties (Hilliard et al. 1985), second messengers, metabolism, and other neurotransmitter systems *in vitro* (Pertwee 1988). Most of the biochemical studies employed concentrations of Δ^9-THC that

were far in excess of physiologically meaningful concentrations that might be found in brain (for a review, see Martin 1986; Pertwee 1988).

In many of these studies, the criterion of structure-activity correlation was not met—that is, the potencies of various cannabinoids in the *in vitro* assays bore no resemblance to their relative potencies in eliciting characteristic behavioral effects. Particularly damaging to the relevance of these *in vitro* studies was the absence of stereoselectivity or enantioselectivity. In contrast, Martin's group had found that the potencies of (-) and (+) forms of each of the cis and trans isomers of Δ^{9}-THC differ by 10- to 100-fold in producing static ataxia in dogs, depressing schedule-controlled responding in monkeys, and producing hypothermia and inhibiting spontaneous activity in mice (Martin et al. 1981). Similarly, Hollister and coworkers (1987) had shown cannabinoid enantioselectivity in human studies using indices of the subjective experience, or "high."

May's group had found enantioselectivity of a series of synthetic compounds in tests of motor depression and analgesia (Wilson and May 1975; Wilson et al. 1976, 1979). One of these compounds, (-)9-nor-9ß-hydroxyhexahydro-cannabinol (ß-HHC), was used as a starting compound by Johnson and Melvin (1986) for the synthesis of a rather large series of structurally novel cannabinoids for studies of their potential use as analgesics (figure 1). The synthetic cannabinoids shared physicochemical properties with the natural cannabinoids and produced many behavioral and physiological effects characteristic of Δ^{9}-THC, but were 5 to 1,000 times more potent and showed high enantioselectivity.

The availability of these Central Pfizer (CP) compounds revolutionized the study of the biochemical basis of cannabinoid activity. Hewlett's group used them in neuroblastoma cell lines to show inhibition of adenylate cyclase activity (Howlett et al. 1988). Such inhibition was enantioselective, and the pharmacological profile correlated well with that observed by Martin's group, which showed similar orders of potencies for the CP compounds in tests of mouse spontaneous activity, catalepsy, body temperature, and analgesia (Little et al. 1988).

One of the compounds, CP55,940, was tritiated and used to identify and fully characterize a unique cannabinoid receptor in membranes from rat brain (Devane et al. 1988). The centrifugation assay showed that the [^{3}H]CP55,940 binding site is saturable, has high affinity and enantioselectivity, and exhibits characteristics expected for a neuromodulator receptor associated with a G protein. The slide-mounted section assay, described in this chapter, is used to

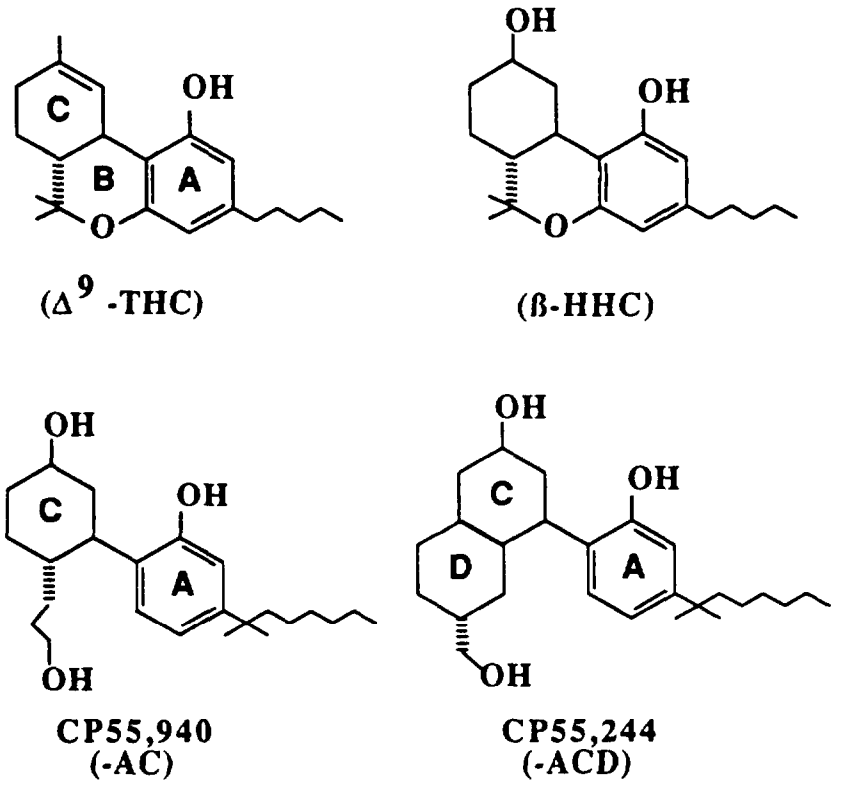

FIGURE 1. *Chemical structures of Δ⁹-THC and three synthetic cannabinoids*

NOTE: According to the nomenclature of Johnson and Melvin (1986), Δ⁹-THC and ß-HHC are defined as members of the ABC-tricyclic cannabinoid class. CP55,940 is a hydroxypropyl analog of a 2-(3-hydroxy-cyclohexyl)phenol, defined as an AC-bicyclic cannabinoid. CP55,244 is an ACD-tricyclic cannabinoid with a rigidly positioned hydroxypropyl moiety.

SOURCE: Herkenham et al. 1990.

characterize and validate the binding of [^{3}H]CP55,940, and the same assay is used to autoradiographically visualize the distribution of cannabinoid receptors.

MATERIALS AND METHODS

To perform exhaustive optimization and characterization studies before autoradiographic analysis, many brain sections of uniform composition and size are required. For these kinetics studies, bound radioactivity is determined by liquid scintillation counting; therefore, heterogeneity of receptor distribution from section to section must be avoided. Relative uniformity of receptor content was created by preparing rat brain "sausage" sections. These sections were prepared by placing three whole rat brains (removed fresh after decapitation) in a petri dish on ice, combining and mincing them with a razor blade (thoroughly, for about 5 minutes), then placing the paste into a tube, briefly spinning at low speed in a centrifuge (1,000 rpm for 1 minute), and freezing it to produce a cylindrical sausage that can be removed from the tube, mounted on a pedestal with embedding matrix, and cryostat-cut to make sections of uniform composition and size (Rothman et al. 1983). Sections (30 µm thick) were thaw-mounted onto gelatin-coated ("subbed") slides, dried briefly on a hot plate at 30°C and stored in airtight boxes at -35°C prior to use (Herkenham 1988).

Cannabinoid and noncannabinoid drugs were stored at -70°C either in solid form or dissolved in absolute ethanol. Cannabinoids were kept under argon to prevent oxidation. Some drugs were dissolved in dimethyl sulfoxide and stored frozen at -70°C. Stock solutions of drugs were kept at 10 mM concentration or lower, depending on their potencies in the assay. [^{3}H]CP55,940 (specific activity 76-79 Ci/mmole after custom preparation [Herkenham et al. 1990]) was found to be unstable unless kept at low concentrations in absolute ethanol. To prevent conversion to a nonradioactive form, it was stored at -80°C in a stock concentration of 1 mCi per 5 mL ethanol under argon.

The assay procedures used were described previously (Herkenham 1988; Herkenham et al. 1990). All optimization, kinetic, and competition studies were carried out using slide-mounted rat brain sausage sections. Incubations were in polyethylene cytomailers, each containing eight sections on four slides in 5 mL of solution. During incubations, cytomailers were held in test tube racks in a water bath. For washes, slides were transferred to cytomailers placed in ice. At the end of the wash period, slides were transferred to stainless steel slide racks (30 slides/rack) and blown dry with slightly warm air from a hair dryer. Slides were scored and broken; the section-laden slide fragments were placed into vials to which 10 mL of detergent-fluor were added. After equilibrating overnight, the radioactivity was counted.

Solutions were prepared in large glass beakers; Tris-HCl and bovine serum albumin (BSA) were added while stirring. [^{3}H]CP55,940 and other drugs were delivered to the source solution and/or individual cytomailers with micropipettes and disposable polyethylene tips. Cannabinoids were drawn up and expelled from the tips several times to saturate binding sites on the plastic tips. Concentration of [^{3}H]CP55,940 was checked by scintillation counting of 100 µL of the solution. For inhibition studies requiring dilutions of unlabeled drug, serial dilutions were achieved by mixing drug in the first cytomailer containing 15 mL of solution and then removing 6 mL for addition to the next in the line, which was prefilled with 9 mL, achieving a 2.5-fold dilution. After thorough mixing by using a 5 mL disposable tip to draw up and expel the solution five to six times, the sequence was repeated. Similar strategies were used to achieve 2-fold and 10-fold dilutions in some assays.

Cannabinoids are extremely hydrophobic (Garrett and Hunt 1974), so preliminary studies were performed to determine how to avoid ligand adherence to glass and plastic surfaces. The disposition of iigand during pipetting and dilution steps was checked in mock assays in which either [^{3}H]CP55,940 or [^{3}H]Δ^9-THC (provided by NIDA) was substituted for cold drug, and the solutions were assayed for radioactivity by scintillation counting. Tests were performed using subbed pipette tips and subbed cytomailers and using plain glass or sialyzed glass test tubes in place of cytomailers. Using solutions prepared according to the optimized assay conditions (see below), no differences were found between calculated and observed radioactivity in the initial pipetting from source vials or throughout the cascade of mock dilutions when using the untreated polyethylene tips and cytomailers; therefore, protective coatings were deemed unnecessary.

RESULTS

Preliminary binding studies were carried out to determine appropriate buffer type and pH, effects of salts and preincubation in various media, optimal incubation time and temperature, and optimal postincubation wash time. Early experiments, carried out in incubations with 2 to 3 nM [^{3}H]CP55,940 at 24°C for 2 hours, revealed that the best binding (around 70 percent specific) was produced by incubation in 50 mM Tris-HCl buffer, pH 7.4, with 5 percent BSA (reagent grade, Sigma, worked as well as fatty acid-free BSA) and by washing at 0°C for 4 hours in Tris buffer with 1 percent BSA. Nonspecific (NS) binding was determined by addition of 10 µM CP55,244. Incubations without BSA gave no specific binding—counts were actually lower in the total than in the NS condition (total=8,900 cpms/section; NS=10,700 cpms/section).

Binding was relatively unaffected by preincubation in Tris buffer with 100 mM NaCl for 30 minutes, at either 0°C or 24°C or by the inclusion of 100 mM NaCl in the incubation medium. Incubations in 10 percent BSA gave lower total and specific binding. Binding was reduced by use of other buffers (KPO_4, HEPES, MOPS) or by increases or decreases in pH or Tris buffer concentration. Binding was eliminated by heat (preincubation for 30 minutes at 60°C in buffer). The optimal postincubation wash condition, to remove nonspecifically bound label, was 4 hours in Tris buffer with 1 percent BSA at 0°C. Longer wash times or wash at 24°C for 1 hour gave lower percent specific binding. Specific binding was reduced by wash with 0.1 percent BSA (higher nonspecific), whereas 1 percent and 5 percent BSA in the wash gave similar results.

The next set of variables to be examined were temperature and time of incubation, optimal concentrations of BSA and [^{3}H]CP55,940 in the incubation, and their interactions. As shown in figure 2, virtually no binding occurred at 0°C; binding at 24°C reached equilibrium at 16 hours of incubation; and binding at 37°C was greater and reached equilibrium faster than at 24°C. Therefore, the next set of parameters was examined at 37°C. As shown in figure 3, incubations in 10 nM [^{3}H]CP55,940 gave much greater binding than at 2.5 nM, and reduction of BSA concentration elevated binding still more. However, a plateau of binding was reached more quickly with 5 percent BSA than with 1 percent BSA (3 hours vs. 4 hours); in all experiments at 37°C, binding fell off at longer incubation times, probably due to degradation of the ligand and/or receptors. Binding of 1 to 20 nM [^{3}H]CP55,940 at 37°C for 2 hours was typically 80 percent to 90 percent specific.

Binding surface analysis (McGonigle et al. 1986; Rothman 1986; Rothman et al. 1988) was next used to determine kinetics of binding using optimized conditions with either 1 percent or 5 percent BSA in the incubation medium (figure 4). To determine binding kinetics, each of two concentrations of [^{3}H]CP55,940 (1 and 10 nM) was competitively inhibited by 6 to 12 concentrations of unlabeled drug. Competitive inhibition curves were subjected to a computerized iterative curve-fitting program for determining best-fit parameter estimates (K_d, K_I, B_{max}) according to one- or two-site competitive binding models. The competitive inhibition curves were best fit by a single-site kinetic model: The affinity (K_d) of CP55,940 in 1 percent BSA was 2.6 nM, and the binding capacity (B_{max}) was 528 fmoles/section (1,158 fmoles/mg protein, since sausage section has 456±26 mg protein/section). With 5 percent BSA, the K_d was 15 nM and the B_{max} was 415 fmoles/section (910 fmoles/mg protein).

A large series of cannabinoid and noncannabinoid drugs was assayed to test for competitive displacement of [^{3}H]CP55,940 (table 1). To ensure that the lipophilic drugs would dissolve at the high concentrations required (at least for

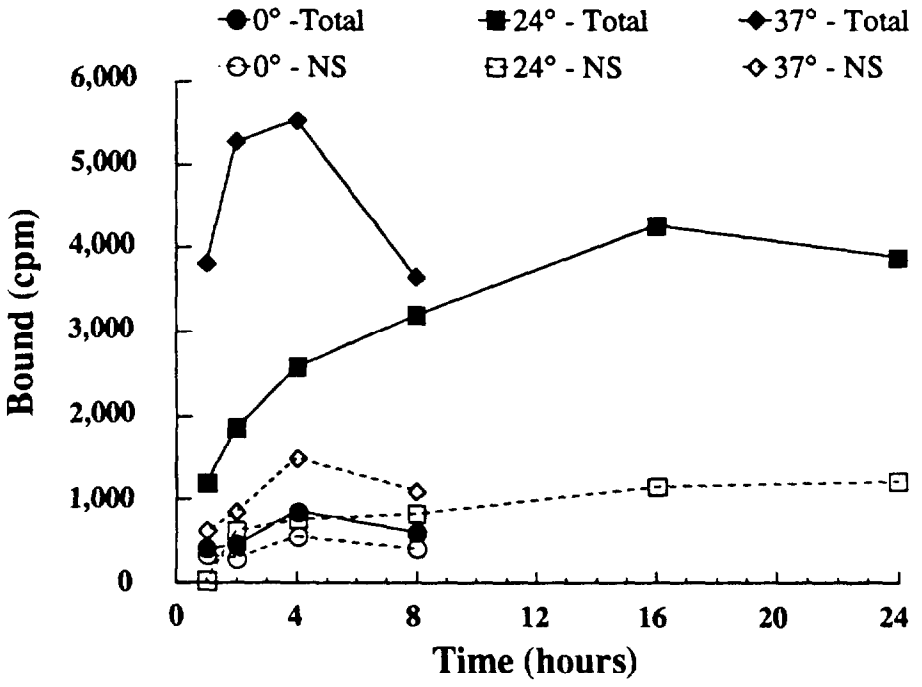

FIGURE 2. *Time course of association of 2.5 nM [^{3}H]CP55,940 at three different temperatures (°C)*

NOTE: Total and NS binding are shown.

the drugs with low potency), all competition studies were performed in the assay with 5 percent BSA. Drug concentrations of 10^{-4} M were achieved, which are well higher than the limit of solubility of cannabinoids in water or buffer without a carrier, such as BSA; Δ^9-THC solubility limit in water at 23°C is 6.8 µM and 1.9 µM in physiological saline (Garrett and Hunt 1974). Solubility of other cannabinoids is similar (Bach et al. 1976). It also was found that variability of binding was reduced in the 5 percent BSA condition as opposed to the 1 percent BSA condition (data not shown).

The competition curves (figure 5) and derived inhibition constants (K_i's) for the natural and synthetic cannabinoids (table 1) provided a test for validation of binding. It was found that highly significant ($p<0.0001$) correlations exist between the K_i's and potencies of the drugs in tests of dog ataxia and human subjective experience, the two most reliable markers of cannabinoid activity

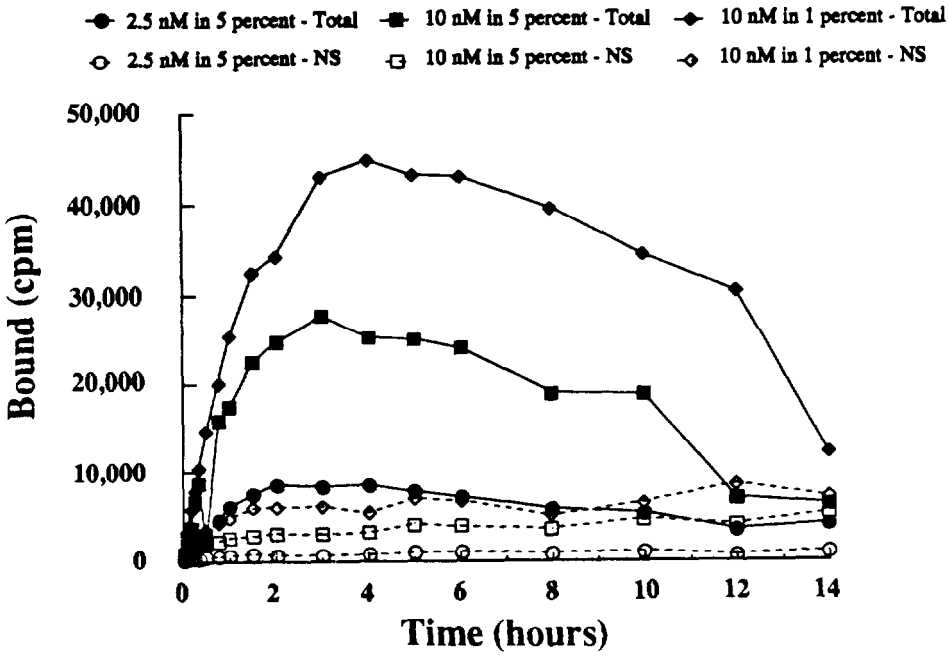

FIGURE 3. *Time course of association of [^{3}H]CP55,940 as a function of its concentration and the concentration of BSA in the incubation medium*

(Dewey 1986; Hollister 1986). The K_i's also correlate very closely with relative potencies in tests of motor function (ataxia, hypokinesia, catalepsy), analgesia, and inhibition of contractions of guinea pig ileum and adenylate cyclase in neuroblastoma cell lines *in vitro* (Herkenham et al. 1990). Enantioselectivity was striking (figure 5; table 1); the (-) and (+) forms of CP55,244 differ by more than 10,000-fold *in vitro,* a separation predicted by the rigid structure of the molecule (figure 1) (Johnson and Melvin 1986) and by potencies *in vivo.* Natural cannabinoids that lack psychoactive properties, such as cannabidiol, showed extremely low potency at the receptor, and all tested noncannabinoid drugs had no potency (table 1).

Because the validation of binding had been performed using the 5 percent BSA condition, and because we believed that this concentration might support preservation of tissue quality, the following binding conditions for autoradiographic visualization of receptor distributions were selected: incubation at 37°C for 2 to 3 hours in 50 mM Tris-HCl buffer, pH 7.4, with 5 percent BSA and 10 nM [^{3}H]CP 55,940, and washing at 0°C for 4 hours in the

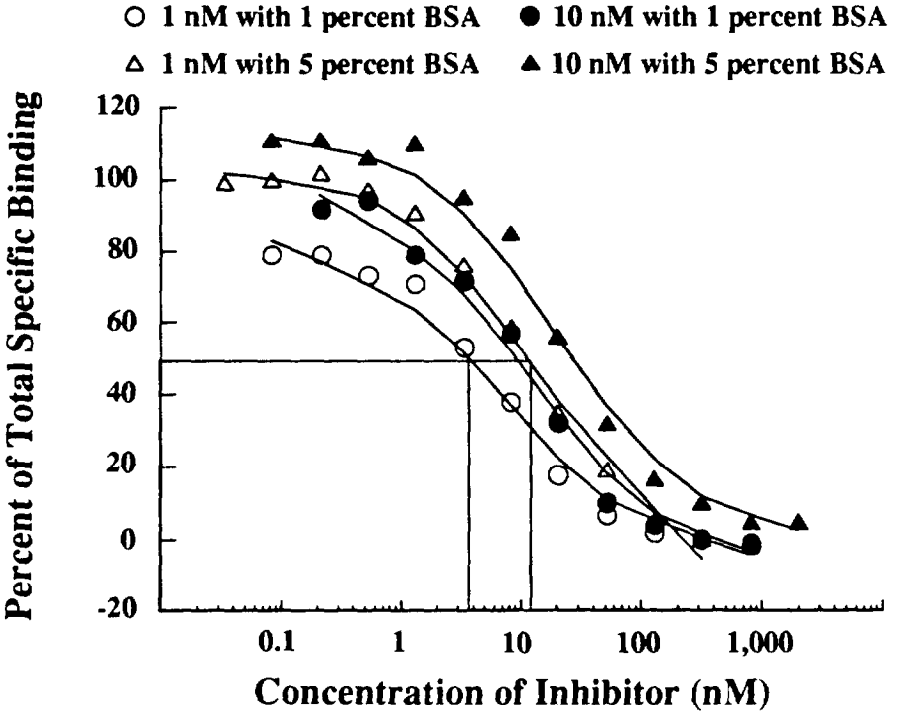

FIGURE 4. *Binding surface analysis of unlabeled CP55,940 displacement of two concentrations (1 and 10 nM) of [^{3}H]CP55,940, with either 1 percent or 5 percent BSA in the incubation*

NOTE: Horizontal and vertical lines mark the concentrations at which 50 percent of the binding of 1 nM [^{3}H]CP55,940 is inhibited (IC_{50}). Compare with K_d's, described in test.

same buffer with 1 percent BSA. Sections were blown dry; slides were placed in x-ray cassettes and exposed to tritium-sensitive film for 3 to 4 weeks before developing.

Autoradiography showed great heterogeneity of binding, with patterns of receptor distribution that correlated closely with cytoarchitecture. Very dense binding was found in the globus pallidus, the substantia nigra pars reticulata, and the molecular layers of the cerebellum and hippocampal dentate gyrus (figure 6). Dense binding also was found in the cerebral cortex, in other parts of the hippocampal formation, and in the striatum. Neocortex had similar levels of

TABLE 1. *Potencies of several cannabinoids in the section-binding assay*

Compound	K_i (nM)
CP55,940 (-AC)	15±3 (K_d)
CP56,667 (+AC)	470 ± 57
CP55,244 (-ACD)	1.4 ± 0.3
CP55,243 (+ACD)	18,000 ± 1,100
CP50,556 (levonantradol)	14 ± 2
CP53,870 (dextronantradol)	26,000 ± 3,500
CP54,939 (desacetyl levonantradol)	14 ± 2
Nabilone	120 ± 13
ß-HHC	124 ± 17
α-HHC	2,590 ± 360
(-)Δ^9-THC	420 ± 51
(+)Δ^9-THC	7,700 ± 2,100
Δ^8-THC	498 ± 52
11-OH-Δ^9-THC	210 ± 56
Trimethylammonium-Δ^8-THC (TMA)	2,300 ± 1,000
8ß-OH-Δ^9-THC	4,200 ± 700
8α-OH-Δ^9-THC	8,700 ± 1,800
11-OH-cannabinol	800 ± 150
Cannabinol	3,200 ± 450
Cannabidiol	53,000 ± 6,700
Cannabigerol	275,000
9-COOH-11-nor-Δ^9-THC	75,000
9-COOH-11-nor-Δ^9-THC	Inactive

NOTE: Structures of CP analogs have been reported by Johnson and Melvin (1986). The first six analogs are enantiomeric pairs, as are (-) and (+)Δ^9-THC. The last two are Δ^9-TCH metabolites. K_i's ±standard deviations are derived from binding surface analysis. Each represents the results from a single experiment. In a separate set of "prediction" experiments (Rothman et al. 1988), each drug was tested for inhibition at the calculated K_i. For CP55,940 itself, the K_d shown represents the mean and standard deviation of three separate determinations. Drugs that at 10 µM concentration show no inhibition of [^{3}H]CP55,940 binding are amphetamine, ß-estradiol, cis-flupenthixol (dopamine receptor ligand), cocaine, corticosterone, cyclohexyl-adenosine, dexamethasone, etorphine (opiate receptor ligand), γ-amino butyric acid, glutamate, leukotriene B_4 and D_4 (both at 1 µM), lysergic acid diethylamide (LSD), phencyclidine (PCP), prostaglandin E^2, and Ro 15-1788 (benzodiazepine receptor ligand).

SOURCE: Herkenham et al. 1990.

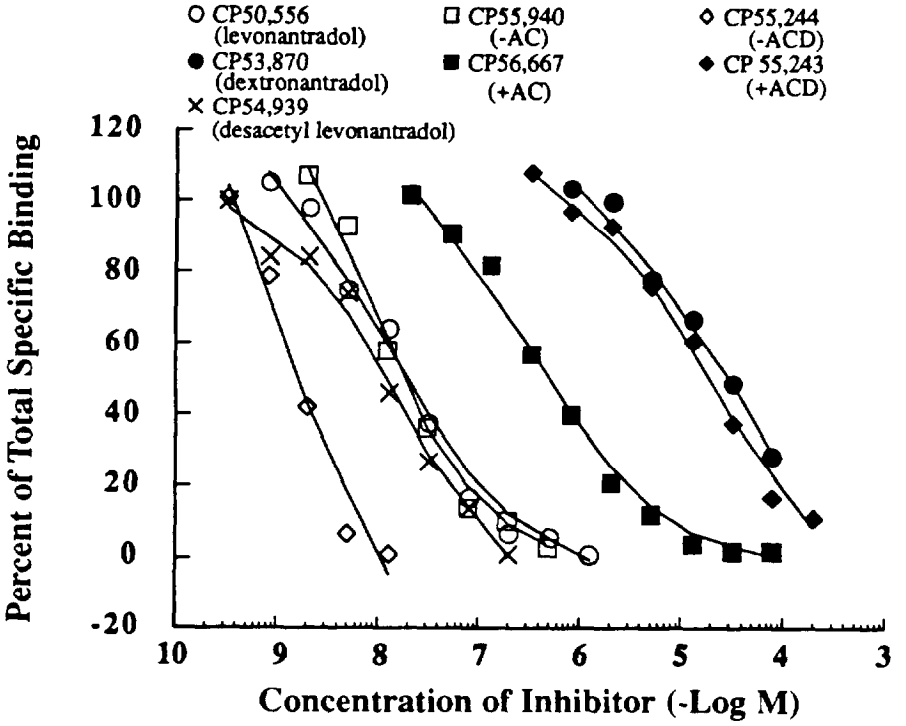

FIGURE 5. *Competition curves for several enantiomers of the CP series of synthetic cannabinoids*

NOTE: Competitive inhibition of 1 nM [^{3}H]CP55,940 binding in whole rat brain sausage sections by various synthetic and natural cannabinoids at the concentrations indicated. The data are normalized to specific binding (total minus nonspecific) in absence of competitors. Nonspecific binding was determined by addition of the 10 μM CP55,244 (the most potent cannabinoid in the CP series) (Johnson and Melvin 1986) and typically represented 10 to 20 percent of total binding at both 1 and 10 nM [^{3}H]CP 55,940. Data points represent means of eight determinations.

SOURCE: Herkenham et al. 1990.

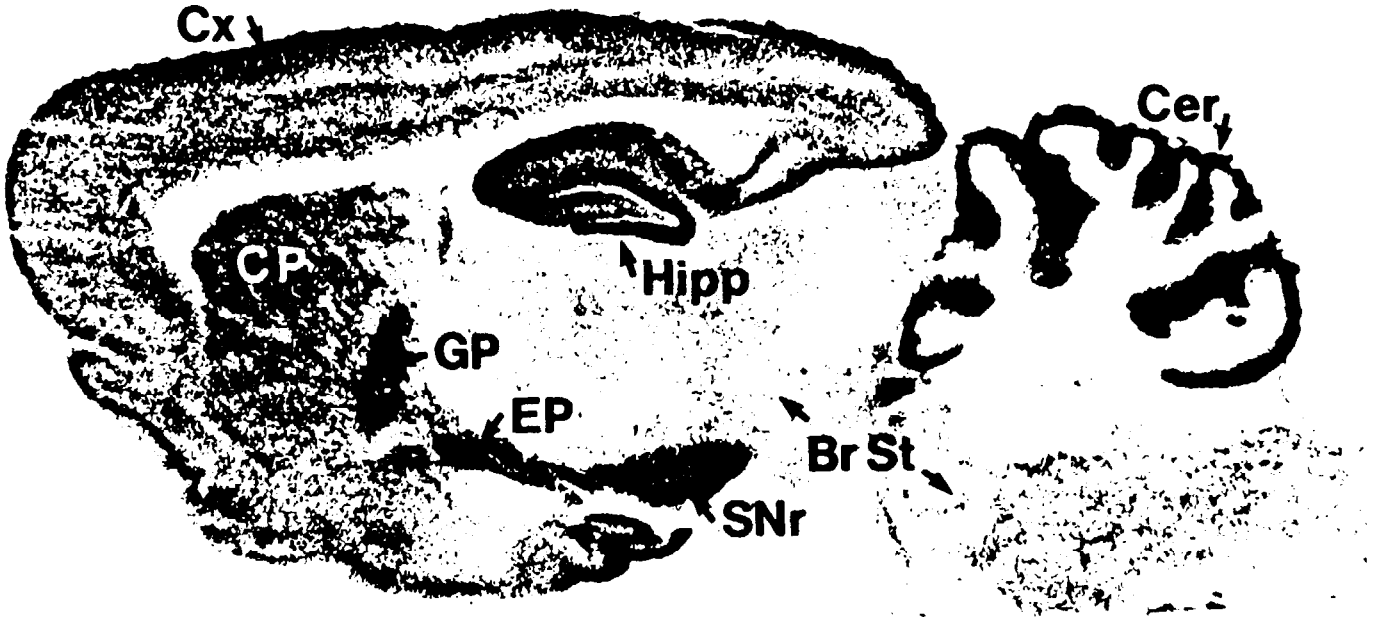

FIGURE 6. *Autoradiography of 10 nM [^{3}H]CP55,940 binding in sagittal section of brain*

NOTE: From left to right, the most densely labeled areas are the globus pallidus (GP), entopeduncular nucleus (EP), substantia nigra pars reticulata (SNr), and cerebellar molecular layer (Cer). The GP, EP, and SNr are all components of the basal ganglia. The basal ganglia and cerebellum are critical centers for the control of movement. In addition, the cerebral cortex (Cx) and caudate-putamen (CP) are well labeled. Within the cerebral cortex, the hippocampus (Hipp) has the densest labeling. The hippocampus is a critical center for the laying down of permanent memory traces and is specifically involved in spatial and temporal aspects of memory. The brain stem (Br St) has very few receptors.

SOURCE: Herkenham et al. 1990.

binding across fields, with peaks in superficial and deep layers. Very low and homogeneous binding characterized the thalamus and most of the brain stem, including all of the monoamine-containing cell groups, as well as the reticular formation; the primary sensory, visceromotor, and cranial motor nuclei; and the area postrema. The exceptions—hypothalamus, basal amygdala, central gray, nucleus of the solitary tract, and laminae I-III and X of the spinal cord—showed slightly higher but still sparse binding (Herkenham et al. 1990).

DISCUSSION

Binding parameters are dependent on the assay used to characterize them. For this reason, optimization, kinetics, and validation must be done on slide-mounted brain sections of the type that will be used for autoradiographic visualization. In the case of cannabinoids, this requirement delivered an additional major dividend when it was discovered that the section assay, by its nature, obviates many of the technical problems associated with membrane binding. The section-binding assay is easy to perform, does not require expensive equipment other than a cryostat, gives highly reproducible data, and shows high sensitivity to manipulations of binding conditions such as the addition of guanine nucleotides (Herkenham et al. 1990). The assay circumvents technical problems inherent in membrane assays, such as adherence of ligand to glass and plastic surfaces and to filters; Devane and colleagues (1988) were unsuccessful at developing an assay based on filtration techniques. In addition, BSA appears to act as a carrier to keep cannabinoids in solution without appreciably affecting binding kinetics. The low nonspecific binding and absence of binding in white matter indicate that the autoradiographic patterns are not affected by ligand lipophilia. The inclusion of BSA in the incubation medium actually may mimic the disposition of cannabinoids administered *in vivo,* as they would quickly complex with serum albumin or other carriers in the blood.

The structure-activity profile suggests that the receptor defined by the binding of [^{3}H]CP55,940 is the same receptor that mediates many of the behavioral and pharmacological effects of cannabinoids (table 1), including the subjective experience termed the human “high.” All other tested psychoactive drugs, neurotransmitters, steroids, and eicosanoids at 10 μM concentrations failed to bind to this receptor, There was no compelling evidence for receptor subtypes from that analysis.

Autoradiography of cannabinoid receptors reveals a heterogeneous distribution pattern that conforms to cytoarchitectural and functional subdivisions in the brain. The distribution is unique—no other pattern of receptors is similar; and it is similar across several mammalian species, including human, suggesting that

cannabinoid receptors are phylogenetically stable and conserved in evolution. Knowing the locations of cannabinoid receptors increases understanding of cannabinoid pharmacology. Generally high densities in forebrain and cerebellum implicate roles for cannabinoids in cognition and movement. Sparse densities in lower brain stem areas that control cardiovascular and respiratory functions may explain why high doses of Δ^9-THC are not lethal.

Accounts of cannabis use in humans stress the loosening of associations, fragmentation of thought, and confusion on attempting to remember recent occurrences (Hollister 1986; Miller 1984). These cognitive effects may be mediated by receptors in the cerebral cortex, especially the receptor-dense hippocampal cortex. The hippocampus "gates" information during memory consolidation and codes spatial and temporal relations among stimuli and responses (Douglas 1967; Eichenbaum and Cohen 1988). Δ^9-THC causes memory "intrusions" (Hooker and Jones 1987), impairs temporal aspects of performance (Schulze et al. 1988), and suppresses hippocampal electrical activity (Campbell et al. 1986).

The localization of cannabinoid receptors in motor areas suggests therapeutic applications. Cannabinoids exacerbate hypokinesia in Parkinson's disease but are beneficial for some forms of dystonia, tremor, and spasticity (Clifford 1983; Dewey 1986; Hollister 1986; Marsden 1981; Petro and Ellenberger 1981). The development of an antagonist could provide additional therapeutic uses of value. The section-binding assay will be helpful in this regard and can be used also to screen novel drugs that have greater potency or that bind irreversibly to aid in the identification of the receptor gene and the putative endogenous ligand.

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New Developments in Radiotracers for Positron Emission Tomography

Joanna S. Fowler and Alfred P. Wolf

INTRODUCTION

Organic molecules labeled with short-lived positron emitters are utilized in tracer kinetic experiments to study complex biochemical processes in living kinetic experiments allowing the study of complex biochemical processes in living animals and humans. A positron emission tomograph is used to determine the absolute amount of radioactivity in small volumes of tissue. Thus, the uptake and retention of labeled organic molecules, which are used as probes, can reflect a particular biochemical process. The development and application of positron emission tomography (PET) is a multidisciplinary and interdisciplinary effort involving the overlap of four major areas (figure 1).

It is the purpose of this chapter to place one of these areas, chemistry, in the perspective of its use in radiotracer design and development and to describe some of the special challenges associated with applying isotopes of short half-lives to problems in biology and medicine.

To understand the PET method, some comment is appropriate on the basic technology that makes the measurement on *in vivo* biochemical processes possible. The following four elements constitute the PET method.

1. Cyclotron or other suitable accelerator for isotope production

2. Chemistry laboratory for the synthesis of labeled tracers and the study of their biodistribution and metabolism

3. Positron emission tomograph for the quantitative measurement of radioactivity concentration *in vivo*

4. Computational facilities for data reconstruction and manipulation

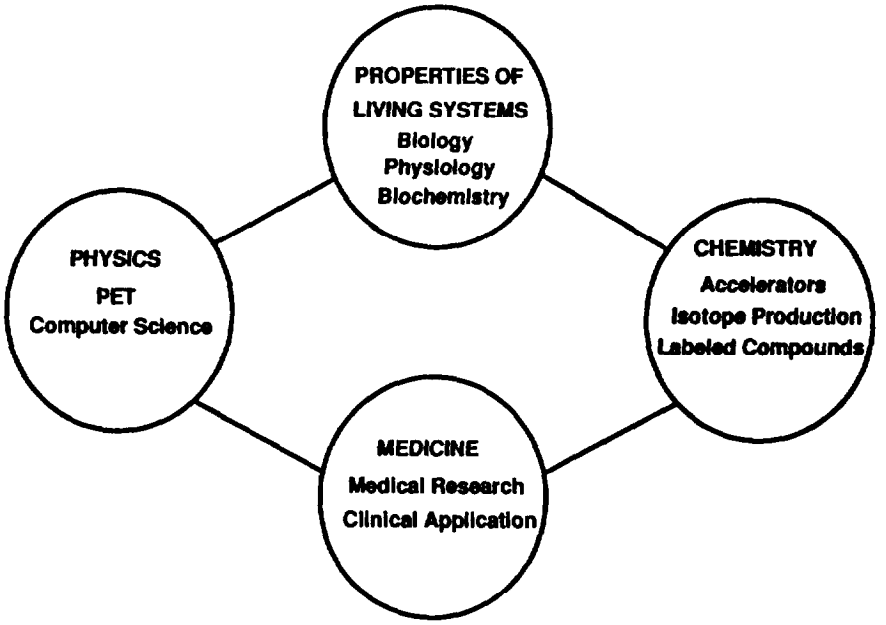


FIGURE 1. *Four major areas of PET research*

ACCELERATORS FOR ISOTOPE PRODUCTION

The four most commonly used radionuclides in PET and some of their properties are listed in table 1. For comparison, the properties of carbon-14 and tritium, the two most commonly used tracers in biochemical research, also are listed. They are not positron emitters.

There are several other positron emitters used in PET work, but they are rarely used in labeling procedures. Generator-produced radionuclides also are used in PET, although these are typically metal ions, which do not have the synthetic flexibility of the four short-lived positron emitters listed in table 1 (Guillaume and Brihaye 1986).

The positron emitters are produced by nuclear reactions most commonly brought about by bombarding appropriate targets with either protons or deuterons. These nuclear reactions are listed in table 2.

The accelerators used to bring about these reactions are most often cyclotrons with proton energies in the 10- to 17-million electron volt (MeV) range (Wolf 1988). The most widely used machines are listed in table 3.

TABLE 1. *Properties of the short-lived positron emitters and carbon-14 and tritium*

Isotope	Half-Life	Specific Activity[a]	Decay Product
Carbon-11	20.4 min	9.2×10^6	Boron-11
Fluorine-18	109.8 min	1.7×10^6	Oxygen-18
Nitrogen-13	10 min	1.9×10^7	Carbon-13
Oxygen-15	2.07 min	9.1×10^7	Nitrogen-15
Carbon-14	5,730 y	0.0624	Nitrogen-14
Tritium	12.4 y	29	Helium-3

[a]Specific activities are in curies/mmol. These specific activities are never achieved because of unavoidable contamination with the corresponding stable isotopes. Dilutions with the stable isotope are usually from 100 to 100,000.

TABLE 2. *Nuclear reactions for the production of positron emitters*

Isotope	Nuclear Reaction
Carbon-11	$^{14}N(p,\alpha')^{11}C$
Fluorine-18	$^{18}O(p,n)^{18}F$ $^{20}Ne(d,\alpha)^{18}F$
Nitrogen-13	$^{13}C(p,n)^{13}N$ $^{16}O(p,\alpha)^{13}N$, $^{12}C(d,n)^{13}N$
Oxygen-15	$^{15}N(p,n)^{15}O$ $^{14}N(d,n)^{15}O$

TABLE 3. *Cyclotrons for PET*

Company[a]	Proton Energy (MeV)	Deuteron Energy (MeV)	Beam Current (μA)
JSW 1710	17	10	10
MC 17F	17.2	8.5	65
CTI-RDS	11	—	50
CGR-MeV	18	10	70

[a]JSW 1710, Japan Steel Works; MC 17F, Scanditronix Corporation; CTI-RDS, Siemens Corporation; CGR-MeV, Sumitomo and the CGR Corporations

The beam currents listed allow a more than adequate supply of the isotope to be made. Because of the recent increase in interest in this field, several new companies are involved with the research and development of small cyclotrons and other accelerators. These so-called “medical cyclotrons” are designed to be relatively simple to operate and serve as a convenient onsite source of the needed isotope.

SYNTHESIS OF LABELED TRACERS

Some of the precursors needed for labeling procedures are produced online in the cyclotron target or are prepared synthetically within a few minutes following the delivery of the isotope from the cyclotron. The major precursors are listed in table 4. Descriptions of methods for preparation of these precursor molecules can be found in Fowler and Wolf (1986), Wolf (1981) and Wolf and Fowler (1985).

TABLE 4. *Precursor molecules*

Isotope	Precursor		
Carbon-11	$[^{11}C]O_2$	$H[^{11}C]N$	$[^{11}C]H_3I$
Nitrogen-13	$[^{13}N]H_3$		
Oxygen-15	$H_2[^{15}O]$	$[^{15}O]CO_2$	$[^{15}O]O_2$
Fluorine-18	$[^{18}F]F_2$	$H[^{18}F]$	

Utilization of these precursors will become more evident in the subsequent sections on a variety of labeled compounds used as biochemical or physiological probes. Some of the more common reactions include carboxylation or reductive carboxylation with $[^{11}C]O_2$, alkylation via $[^{11}C]H_3I$, nucleophilic substitution via ^{11}CN, aliphatic and aromatic nucleophilic substitution with ^{18}F or the use of reactions such as the Schiemann or triazine decomposition, and electrophilic addition and substitution with $[^{18}F]F_2$ (Fowler and Wolf 1982, 1986 and references therein).

One of the key requirements is rapid incorporation into the tracer molecule, a rule of thumb being a maximum of the half-life of the isotope used for synthesis multiplied by three. This time relates to the end of cyclotron bombardment for isotope production. Thus, carbon-11 compounds need to be prepared in less than 1 hour, whereas fluorine-18 compounds require a maximum of about 5 hours for preparation. Of the approximately 900 syntheses reported to date, the average preparation time is reported to be between 20 minutes and 1 hour.

Only a few of these tracers have proven to be useful for *in vivo* PET studies of a specific biochemical process due to factors such as *in vivo* transport to the region of interest, localization rate, metabolism, binding to plasma proteins, nonspecific binding, excess lipophilicity, inability to cross the blood-brain barrier, or entry into metabolic pathways other than the one of interest. The selection of a compound for study of a definable biochemical process usually is made with guidance from the literature of pharmacology and biochemistry, and the validation of the tracer typically is carried out on small animals and nonhuman primates.

Another important factor in studies with positron emitters is the achievable specific activity, which varies with the nature of the study. In receptor-ligand work a true tracer should have a high specific activity so that the animal or human under study will not show a physiology response. However, when tracing the activity of relatively abundant endogenous substances such as glucose, there is greater latitude in choice of specific activity.

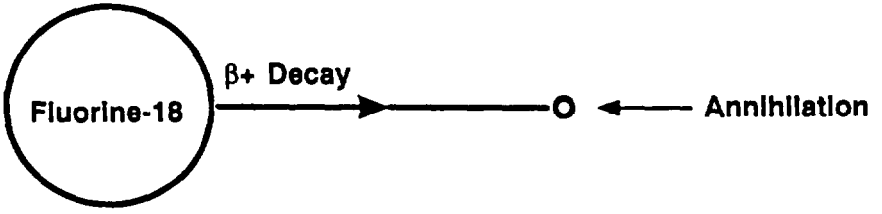
As can be seen from table 1, three of the positron emitters are elements that can be found in every natural biomolecule and in drugs that are used to treat disease. Fluorine, although rarely found in nature in organic combination, frequently is a good substitute for hydrogen when placed in a nonreactive position in the molecule. Thus, PET tracers can be contrasted with other tracers such as iodine or technetium used in single-photon emission computed tomography studies where the molecules used do not always accurately mimic the process being studied because of the gross steric and electronic perturbation of the labeled molecule by the “unnatural” isotope.

Once a synthesis has been developed and the compound has undergone the required analytical control for chemical, radiochemical, and radionuclidic purity in addition to testing of sterility and apyrogenicity, it is ready for testing animals and ultimately for human studies with PET.

The tracer can be used to determine the following parameters: regional distribution in a tissue element, pharmacokinetics of the uptake and clearance of radioactivity, response of the tracer to sensory or pharmacological challenge, mechanism of a biochemical process *in vivo,* and tissue response in pathological conditions and other processes that can shed light on the nature of biochemical processes in the complex milieu of a living organism. Once the uptake and kinetic behavior of a tracer has been determined, a tracer kinetic model may be required to extract more detail from the observations. Good examples of this can be found in such diverse processes as regional glucose metabolism, brain blood flow, and some properties of neurotransmitter receptors (Huang and Phelps 1986 and references therein).

POSITRON EMISSION TOMOGRAPHY

The annihilation radiation (see below) produced on decay of the tracer labeled with a positron emitter (or its labeled metabolites) can be detected in animals and humans using a positron emission tomograph. The unique capability of PET to allow absolute measurement of the concentration of radioactivity in a tissue element—and thus provide quantitative data rather than relative values—stems from the nature of the positron decay process. A nucleus such as that of fluorine-18 emits a positron (figure 2) that travels a finite distance until it collides with an electron, resulting in an annihilation process that yields two photons of equal energy (511 keV) traveling in opposite directions at an angle nearly equal to 180°. Because the positrons are not monoenergetic, a distribution of distances from origin to the point of annihilation is seen from any element undergoing this decay process. The distance of travel before annihilation and slight deviation from the 180° angle of movement limit the ultimate resolution of any positron emission tomograph.



2 Photons at 511 keV at 180°

Maximum distance of travel for ^{18}F positron=2.4 mm

FIGURE 2. *Positron annihilation using the decay of fluorine-18 as an example. Two photons of equal energy (511 keV are produced at an approximate 180° angle when the positron is annihilated with an electron.*

If two radioactivity detectors are directly opposite each other, it can be seen that any set of events detected simultaneously must have occurred in the volume element of space seen by the two detectors (figure 3a). If an array of detectors surrounds the object being measured, the lines of coincidence drawn between each detector will determine the position in space of the decay events (figure 3b). The coincidence detection also allows quantitative measurement of radioactivity coming from any volume element.

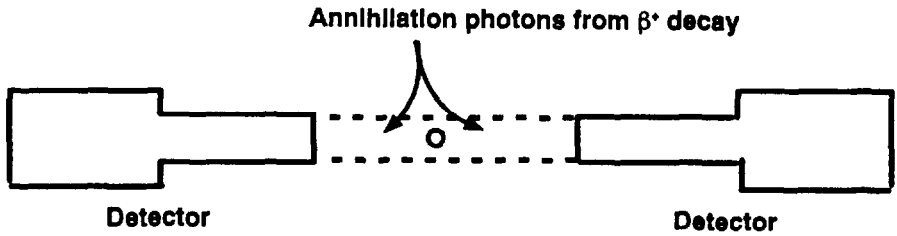


FIGURE 3a. *Coincidence detection of the two photons produced when the positron annihilates with an electron allows the decay event to be positioned in the volume between the detectors.*

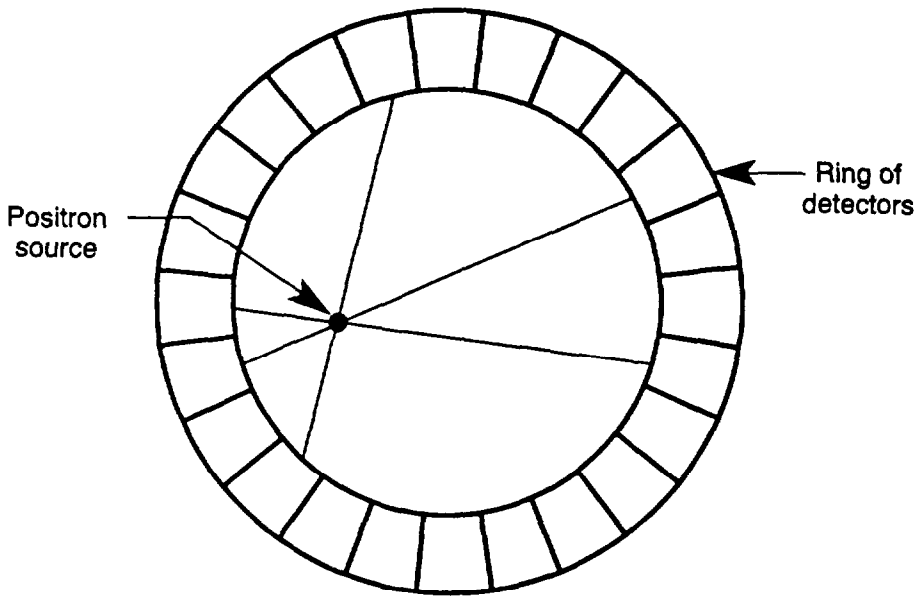


FIGURE 3b. *An array of detectors in coincidence surrounds a source of positron emitters and allows the position in space of the source to be determined.*

PET cannot achieve the ultimate resolution possible with tritium autoradiography; however, as the resolution of positron emission tomographs increases, exceedingly small tissue volume elements can be studied. Table 5 lists the situation as it exists today and indicates what the future holds. In general, however, practical considerations lead to quantitation of a volume

element that is about twice the volume element listed in table 5. Thus, with a current tomograph, the volume element is closer to 0.5 to 0.7 cm^3 rather than 0.25 cm^3.

TABLE 5. *Positron emission tomograph resolution*[a]

Instrument	X Y Z	Volume Element
PET III (circa 1974)	2.0x2.0x2.5 cm	10 cm^3
PET VI and NeuroEcat II	0.8x0.8x1.5 cm	1 cm^3
Commercial Devices (1989)	0.6x0.6x0.7 cm	0.25 cm^3
Ultimate Device (1995)[b]	0.1x0.1x0.1 cm	0.001 cm^3

[a]Although resolution in PET is more complex than this simple table implies (see also Hoffman and Phelps 1986), the values are for full-width, half-maximum resolution commonly quoted.

[b]Resolution below 0.1 cm, although theoretically possible, is of no practical value because of the range of the positron, which results in smearing of the image seen if there were a "true" point source. The annihilation event occurs at a finite distance from the tracer molecule undergoing decay, hence the limitation in what ultimately can be seen with PET.

A PET experiment on a human (e.g., a brain study of the corpus striatum) involves the usual medical requirement of informed consent before the study. The patient receives a medical checkup followed by preparation of a head holder or mask molded individually for each patient so that there is no movement during data acquisition. Arterial and venous catheters then are inserted for blood sampling (assay of blood constituents and metabolites) and tracer injection, and the patient is positioned in the positron emission tomograph. Depending on the isotope used, duration and nature of the study, and other factors, acquisition of data by the positron emission tomograph may involve durations that vary from 1 to 20 minutes. An automated blood sampler for arterial blood usually is used to obtain the blood input function so that the uptake curve can be adjusted for the instantaneous blood concentration of the tracer corrected for the presence of labeled metabolites. At the end of the experiment the data are reconstructed and image analysis takes place. Examples of PET studies follow in the text.

The technology of PET requires the cooperation of diverse professional and technical personnel. Currently, its major power is as a research tool, but increasing interest in clinically useful procedures also is apparent from the

increasing number of hospitals that are setting up cyclotron-PET facilities. Some procedures—for example, measurement of brain glucose metabolism with 2-deoxy-2-[^{18}F]fluoro-D-glucose (^{18}FDG)—have been converted to technician-operated procedures that can be used in daily routine clinical diagnosis. This chapter, however, provides examples of the many areas of biomedical and fundamental biochemical research that can be carried out. The following sections delineate several major areas that are probed with positron-emitting radiotracers. The radiotracer is the key element served by the technology just described.

RADIOTRACERS FOR PET STUDIES OF THE BRAIN

To date, PET research and the development of new radiopharmaceuticals have focused primarily on the brain. This was stimulated, in large part, by the early development of radiotracers and tracer kinetic models for measuring regional brain glucose metabolism (Fowler and Wolf 1986 and references therein: Reivich et al. 1979) and blood flow (Ter-Pogossian and Herscovitch 1985 and references therein). The ^{18}FDG method for measuring regional brain glucose metabolism was developed in the mid-1970s and is now the most widely used radiotracer in PET research. The synthesis of ^{18}FDG has improved continually since it was first developed in 1976 (Ido et al. 1978; Fowler and Wolf 1986 and references therein). Currently, this tracer is prepared in high yield via a displacement reaction (Hamacher et al. 1986). In addition, fully automated synthesis systems are now commercially available.

The development of ^{18}FDG is a classic example of radiotracer design based on a knowledge of the biochemistry of the molecule and the principles of tracer kinetics. The ^{18}FDG method is based on the metabolic trapping of ^{18}FDG-6-phosphate, the product of hexokinase-catalyzed phosphorylation of ^{18}FDG. Because glucose derivatives missing the hydroxyl group on C-2 do not undergo further steps in glycolysis, the radioactivity in tissue after the injection consists only of free ^{18}FDG and ^{18}FDG-6-phosphate, allowing the measurement of glucose metabolism via a tracer kinetic model.

The primary application of ^{18}FDG has been in the measurement of regional glucose metabolism in normal and diseased brain and heart, although it also has been used to measure the effects of drugs and other substances of abuse, cognitive processing, and somatosensory stimulation on brain glucose metabolism (Pheips and Mazziotta 1985). Carbon-11-labeled 2-deoxy-D-glucose has been used in several protocols because its short half-life permits serial studies to be carried out on a single subject on the same day (Reivich et al. 1982). Although carbon-11-labeled glucose also has been developed along with a tracer kinetic model (Raichle et al. 1975), it has neither been applied widely nor validated for the measurement of regional brain glucose metabolism.

Brain blood flow and oxygen utilization are measured most conveniently using oxygen-15-labeled tracers (Ter-Pogossian and Herscovitch 1985). In spite of the 2-minute half-life of oxygen-15, online methods have been developed for its production in the chemical forms required for the measurement of blood flow and oxygen utilization. Its short half-life allows multiple serial studies in a single individual, and there are many examples of its use to study cognitive psychology (Petersen et al. 1990). As in the case of ^{18}FDG, the success of the oxygen-15 methods can be traced to the early development and validation of tracer kinetic models, which allow the quantitative measurement of important biochemical parameters.

Following the early successful use of PET to measure regional brain glucose metabolism and blood flow, an interest developed in applying *in vivo* tracer methodology to the study of neurotransmitters and their receptors. A key development in the application of PET to neurotransmitter studies has been the discovery of synthetic methods for producing high specific activity tracers that also have high affinity and selectivity for the receptor. High specific activity, where the dilution of radioisotope by the naturally occurring element can be in the range of factors from 100 to 10,000, is required to avoid saturation and measurable physiological reactions to the labeled compound.

Radiotracers are under development for studying many neurotransmitters and their receptors: dopamine (Fowler and Wolf 1991 and references therein), opiate (Frost et al. 1985; Luthra et al. 1985), serotonin (Wong et al. 1987; Blin et al. 1988), acetylcholine-muscarine (Mulholland et al. 1988; Mulholland et al. 1989; Dewey et al. 1990; Dannals et al. 1988), benzodiazepine (Persson et al. 1985; Pappata et al. 1988), and N-methyl-D-aspartate (Brady et al. 1989; Wieland et al. 1988; Burns et al. 1989; Monn and Rice 1989). However, the major focus has been on the dopaminergic system stimulated by the importance of dopamine in Parkinson's disease and in schizophrenia.

The dopaminergic system has been studied from several neurochemical perspectives. For example, dopamine metabolism has been probed with 6-[^{18}F]fluoro-DOPA, which crosses the blood-brain barrier and is converted into 6-[^{18}F]fluoro-dopamine, which traces dopamine metabolism in the striatum (Firnau et al. 1986). More recently, fluorine-18-labeled derivatives of metatyrosine (a good substrate for amino acid decarboxylase) have been developed (DeJesus et al. 1989; Gildersleeve et al. 1989; Melega et al. 1989). This tracer does not exhibit some of the metabolic complexities of 6-[^{18}F]fluoro-DOPA and may prove to be superior for PET studies.

Dopamine (D_2) receptor activity also has been examined with PET using positron emitter-labeled antagonists of the D_2 receptor. For example,

N-methylspiroperidol has been labeled with carbon-11 and fluorine-18 and used to study D_2 receptors in normal and diseased brain and to probe D_2 receptor occupancy by antipsychotic drugs (Wagner et al. 1983; Arnett et al. 1986; Wolkin et al. 1989a; Smith et al. 1988b). The benzamide, [^{11}C]raclopride, also has been used in human studies (Farde et al. 1985; Farde et al. 1988). Of current interest are two studies, one reporting the use of [^{11}C]N-methylspiroperidol and the other reporting use of [^{11}C]raclopride to measure D_2 receptor density in schizophrenic patients (Wong et al. 1986; Farde et al. 1987). With [^{11}C]N-methylspiroperidol, a significant elevation in D_2 receptor density was found, a result consistent with *in vitro* measurements made with postmortem human brain tissue (Seeman et al. 1984). In contrast, with [^{11}C]raclopride, no differences were found between the schizophrenic subjects and the normal control population. The factors that may have contributed to this apparent contradiction have been enumerated by a recent panel (Andreasen et al. 1988).

Several tracers that bind to the dopamine reuptake system have been developed, including ^{11}C-nomifensine (Aquilonius et al. 1987), [^{18}F]GBR 13119 (Kilbourn 1988), and [^{11}C]cocaine (Fowler et al. 1989). These tracers are of potential interest in mapping drug-binding sites and probing the loss of dopaminergic neurons in neurodegenerative diseases such as Parkinson's disease.

Protein synthesis and turnover in brain are important biochemical features of brain metabolism, and several positron emitter-labeled amino acids have been investigated as tracers for the quantitative measurement of the regional incorporation of amino acids into protein. The carboxyl-labeled amino acid [^{11}C-carboxyl]L-leucine has been studied (Phelps et al. 1984). The important design feature of this molecule is the position of the label in the carboxyl group so that the products of amino acid metabolism (loss of [^{11}C]O_2) are not labeled, but proteins of interest are labeled. Despite the rational approach to designing this tracer so that the label in *in vivo* tissue could be quantitatively interpreted in terms of protein synthesis, a recent detailed examination of the carbon-14-labeled analog showed that recycling of unlabeled amino acids derived from protein degradation complicated the estimation of precursor pool-specific activity (Smith et al. 1988a). [^{11}C-S-methyl]L-methionine and [1-^{11}C]L-methionine also have been examined as substrates for protein synthesis (Bustany et al. 1982; Ishiwata et al. 1988). A promising new development in the use of PET to measure regional brain protein synthesis is the report that 2-[^{18}F]fluorotyrosine is rapidly incorporated into protein to the exclusion of other metabolic pathways (Coenen et al. 1989).

RADIOTRACERS FOR STUDYING THE BINDING OF THERAPEUTIC DRUGSANDOTHERSUBSTANCESOFABUSE

The use of PET to better understand drug behavior can be approached in the following two ways or by applying a combination of these two approaches: (1) A radiotracer that selectively probes a biochemical transformation (e.g., ^{18}FDG) can be used in serial studies with drug intervention to examine the effect of the drug on a particular biochemical reaction; or (2) the labeled drug can be used to map its binding sites and kinetics.

Among the important parameters that can be measured by PET are the effect of a drug on metabolism and neurotransmitter properties, binding sites and target organs for the drug, absolute brain uptake of the drug, pharmacokinetics, binding mechanisms, drug interactions, duration of action, effect of route of administration on target uptake, and relationship between drug-binding to target receptors and plasma drug concentration.

There are an increasing number of examples of the use of PET to probe the effects of drugs on glucose metabolism and D_2 receptor availability as measured by ^{18}FDG or [^{11}C]2-deoxy-D-glucose or carbon-11- or fluorine-18-labeled neuroleptics (Fowler and Wolf 1991 and references therein).

The use of PET to map drug-binding sites and kinetics is exemplified by studies with [^{11}C]cocaine in human and baboon brains (Fowler et al. 1989). Details on the results of these studies are included in the chapter by Volkow and colleagues (this volume).

In vivo PET studies of the stereoselectivity of cocaine-binding in baboon brain with the behaviorally active- and inactive-labeled enantiomers of cocaine (-)- and (+)- [^{11}C]cocaine with PET (Gatley et al. 1990) provided an important new perspective on the previously observed lack of behavioral activity of the (+)-enantiomer (Spealman et al. 1983). Carbon-11-labeled (-)-cocaine was taken up rapidly and cleared from brain tissue after intravenous (IV) injection. In contrast, carbon-11-labeled (+)-cocaine was excluded from the brain (figure 4). On examination of this unexpected result, it was found that the (+)-enantiomer is metabolized so rapidly in plasma (by butyrylcholinesterase) that there is essentially no tracer left a few seconds after injection (figure 5). Thus, the biological activity of the (+)-enantiomer could be assessed only if the compound could be administered directly and then would be available only if brain enzymatic activity relative to the (+)-enantiomer is slow.

The regional distribution of binding sites for the most widely used antipsychotic drug, haloperidol, also has been determined using [^{18}F]haloperidol and PET

(Wolf et al. 1988). It is known that haloperidol binds to D_2 receptors. Although the structure of haloperidol is similar to N-methylspiroperidol, which is selectively taken up in the striatum, the brain uptake of haloperidol is widespread. This indicates that nonspecific binding or binding to other receptor sites, in addition to D_2 receptors, is operative and may be partially responsible for the therapeutic characteristics of the drug.

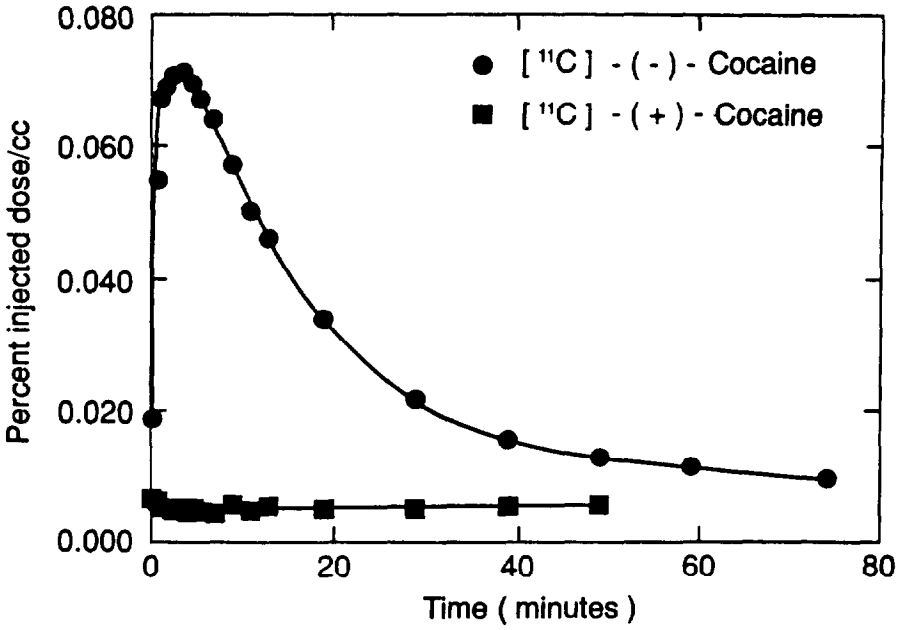


FIGURE 4. *Comparative uptake of [^{11}C]-(-)-cocaine and [^{11}C]-(+)-cocaine in baboon brain showing that the (+)-enantiomer does not penetrate the brain after intravenous injection*

SOURCE: Gatley et al. 1990, copyright 1990, Raven Press.

The question of whether receptor occupancy by haloperidol parallels the concentration of drug in the plasma also was addressed using PET to determine whether plasma drug levels are a good measure of receptor occupancy by antipsychotic drugs (Wolkin et al. 1989a; Smith et al. 1988b) using [^{18}F]N-methylspiroperidol to probe receptor occupancy. Plasma drug levels were measured at the time of each PET study. It was found that receptor occupancy by haloperidol increased with increasing plasma concentrations of

the drug, leveling off at 5 to 15 ng/mL. Interestingly, clinical studies show this to be the initial phase of therapeutic levels for haloperidol. However, at the higher plasma drug concentrations achieved by high haloperidol doses, there was no increase in receptor occupancy. This supports a growing clinical consensus that there is little added benefit in increasing plasma haloperidol levels above 20 ng/mL.

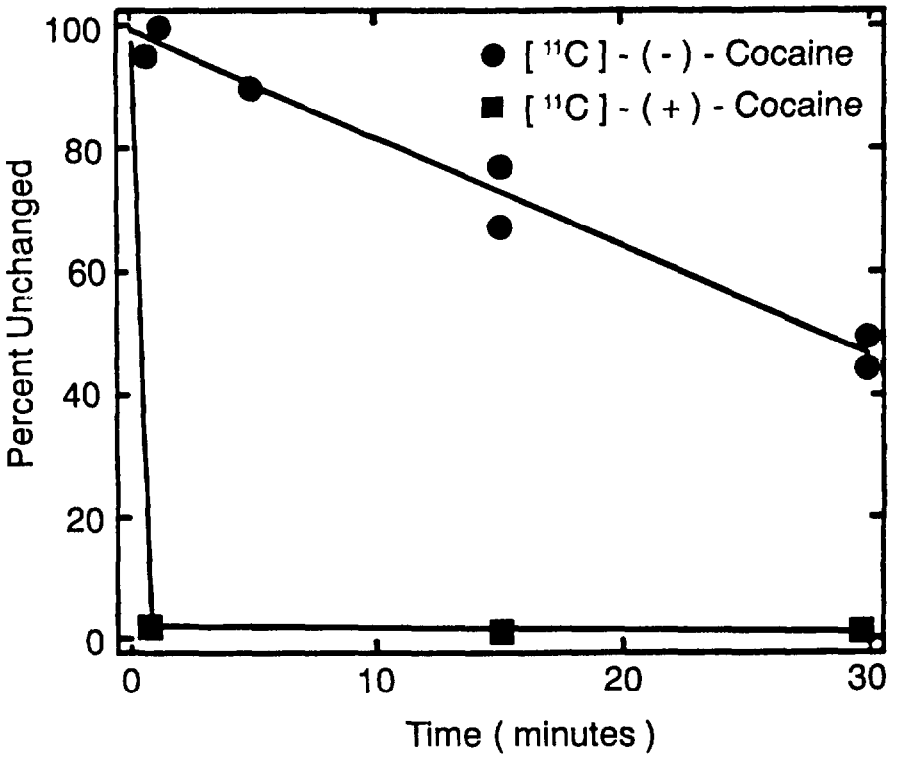


FIGURE 5. *Residual unchanged tracer in baboon plasma after the injection of carbon-11-labeled (-)-cocaine and (+)-cocaine showing the rapid metabolism of the (+)-enantiomer*

SOURCE: Gatley et al. 1990, copyright 1990, Raven Press.

[^{18}F]N-methylspiroperidol also was used to address the question of whether schizophrenic subjects who respond positively to antipsychotic drug therapy have a different receptor occupancy by the drug than those who do not respond

(Wolkin et al. 1989b). The conclusion of this study was that receptor blockade by haloperidol occurs to the same extent in responders and nonresponders and that the treatment of nonresponders with high neuroleptic doses to increase receptor occupancy is not warranted.

The duration of action of drugs directly at their target sites has been measured with PET. One study examined the duration of action of a single dose of (-)-deprenyl, the suicide enzyme inactivator of monoamine oxidase B (MAO B) (Arnett et al. 1987), which is currently used in the therapy of Parkinson's disease. (-)-Deprenyl acts by suicide inactivation with covalent attachment of the activated inhibitor to MAO B. This results in the destruction of the enzyme. The distribution of [^{11}C]L-deprenyl in brain has been shown to parallel MAO B (Fowler et al. 1987) distribution, and its trapping in brain appears to reflect MAO B activity (Fowler et al. 1988). Tracer doses of [^{11}C]L(-) deprenyl were used to probe MAO B activity in baboon brain before and 34 minutes after a single dose of 1 mg/kg of (-)-deprenyl and then at 1, 5, 22, 34, 61, and 111 days following the dose. With PET, it was found that the recovery of the enzyme activity is surprisingly slow, with a half-life of 30 days. This information is important in designing a drug dosage regimen, especially in the case of drugs that act by irreversibly inhibiting a biological substrate such as an enzyme.

Another report addressed the duration of occupancy of μ-opiate receptors by naltrexone, an orally administered opiate antagonist (Lee et al. 1988). Occupancy was probed by the μ-specific ligand [^{11}C]carfentanil and an external detector before naltrexone and at 1, 48, 72, 120, and 168 hours after the oral administration of 50 mg of naltrexone. The half-life of blockade by naltrexone in the brain ranged from 72 to 108 hours, which corresponds to the half-life of the terminal plasma phase of naltrexone clearance. Both studies illustrate the advantage of *in vivo* imaging and detection—to probe not only the target sites for a drug but also the duration of its pharmacological action and to provide a direct assessment of the relevance of measurements of drug concentration in plasma to receptor occupancy by the drug.

PET also has been used to examine the effect of intra-arterial vs. IV administration of the chemotherapeutic drug N,N-bif(2-chlorethyl)-N-nitroso-urea (BCNU) on tumor (Tyler et al. 1988). Intra-arterial administration of BCNU resulted in a 50-fold greater concentration in tumor than IV administration and suggested that the early metabolic trapping of BCNU in tumor correlates with clinical response.

OUTLOOK

As a tool for investigating *in vivo* biochemistry and physiology in normal and pathological states, PET has made great strides since the early 1970s,

especially in the past 10 years (Phelps et al. 1986). This is attributable to the increased sophistication in radiopharmaceutical probes and to rapid advanced in PET instrumentation. The coming years will see increasing clinical application (Coleman et al. 1988; Jacobson 1988a-d), establishment of PET in drug research, and a greater understanding of the biochemistry and functioning of the human brain.

It must be kept in mind that the basis for the PET method is the use of tracer kinetics in the study of human biochemistry and physiology *in vivo.* Thus, any PET study involves a multiplicity of processes, which together result in the PET image and the pharmacokinetics that define that image. PET cannot be used to define a biochemical pathway in a single cell and does not compete with the elegant basic work on the structure and function of single cells or small groups of cells. However, one of the unique capabilities of PET is in the quantitative assessment of the behavior, including the interactions of aggregates of cells in a volume element of tissue *in vivo,* especially in the human brain.

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Use of Positron Emission Tomography To Study Cocaine in the Human Brain

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INTRODUCTION

Positron emission tomography (PET) is a nuclear imaging method that can be used to measure receptor concentration, neurotransmitter activity, brain glucose metabolism, cerebral blood flow, and pharmacokinetics in the living brain (Phelps and Mazziotta 1985). PET is a unique technique to investigate the neurochemical and physiological processes underlying drug reinforcement and addiction. This chapter reviews the different experimental strategies used with PET to investigate the pharmacological actions of cocaine in the human brain. These same experimental strategies can be applied to investigate other drugs of abuse.

THE DRUG

Cocaine is a complex pharmacological agent with local anesthetic properties as well as sympathomimetic effects (Johanson and Fischman 1989), which have been related to its reinforcing and addicting properties (Koob and Bloom 1988; Wise and Bozarth 1984; Dackis and Gold 1985). Cocaine's sympathomimetic effects are due to its ability to inhibit the reuptake of monoamines into the presynaptic terminal, which produces a transient increase in the concentration of the monoamine at the synapse (Hurd and Ungerstedt 1989). Although it has been shown that cocaine can inhibit transport of serotonin (Cunningham et al. 1987), norepinephrine (Banerjee et al. 1979) and dopamine (Ritz et al. 1987) into the presynaptic neuron, it is the drug's ability to block the reuptake of dopamine that has been associated with its reinforcing properties (Ritz et al. 1987). In this respect, it has been shown that destruction of dopamine cells with the neurotoxin 6-hydroxydopamine (Roberts et al. 1977), and blockade of postsynaptic dopamine receptors with neuroleptic (Goeders and Smith 1986) reduces the reinforcing properties of cocaine. Similarly, the addicting properties of cocaine have been related to its effect on the dopamine system (Dackis and Gold 1985). It has been postulated that chronic cocaine use leads to dopamine depletion in the brain, which in turn generates dependence on cocaine. Several

pharmacological treatments that increase the concentration of dopamine in the brain have been explored in the treatment of cocaine abusers (Dackis et al. 1987; Extein et al. 1986; Tennant and Sagherian 1987; Berger et al. 1989).

In the past 10 years the abuse of cocaine has reached epidemiological proportions (Adams 1982; Schnoll et al. 1984; Turner 1988) resulting in increased morbidity and mortality (Gawin and Ellinwood 1988; Lowenstein et al. 1987). Medical complications from cocaine include cardiac, pulmonary, cerebral, renal, gastrointestinal, and gynecobstetrical problems (Johanson and Fischman 1989), and many have been related to the sympathomimetic actions of cocaine. This chapter reviews cocaine studies done with PET in the areas of brain toxicity, addiction, and reinforcement.

TOXICITY OF COCAINE IN THE BRAIN

Measurement of brain dysfunction can be achieved with PET with tracers that monitor glucose metabolism (Reivich et al. 1985) such as 2-deoxy-2[^{18}F] fluoro-18 D-glucose (^{18}FDG) or [1-^{11}C] deoxyglucose (^{11}CDG) or with tracers that monitor cerebral blood flow (CBF) such as $H_2{}^{15}O$ (Raichle et al. 1983).

The effects of chronic cocaine use on brain function have been evaluated with glucose metabolism and with CBF. The study that investigated CBF used $H_2{}^{15}O$ and PET in a group of 24 chronic cocaine abusers (Volkow 1988). The patients for this study had taken cocaine for at least 6 months, with an average dose of 4 g per week. All the subjects came from an inpatient unit for drug detoxification and rehabilitation. Measurement of CBF was performed twice during the hospitalization; the first measurement was done within 1 week of entering the hospital, and the second was done 10 days after the first study. During this 10-day period patients were kept free of any medication; routine urine samples were obtained to ensure that patients did not take any drug during the detoxification period. The measurement of CBF revealed evidence of defective CBF, suggestive of cerebrovascular pathology. Although the defects in isotope accumulation occurred throughout most of the cortical brain areas, they were seen predominantly in the frontal cortex, left temporal cortex, and left parietal cortex (figure 1) (Volkow et al. 1988). The defects in CBF remained after 10 days of cocaine detoxification and were interpreted as reflecting the vasoconstricting actions of cocaine. *In vitro* studies had demonstrated that cocaine can induce vasospasm in cerebral blood vessels (Altura et al. 1985), which in turn decreases CBF. Although the pharmacological half-life of cocaine is short (40 to 60 minutes) (Javaid et al. 1983) the pattern of utilization of cocaine by the cocaine abuser involves repeated administration for periods of 48 to 72 hours (binge), which can lead to prolonged periods of cerebral blood vessel vasospasm. This continuous vasospasm could induce ischemia and necrosis of brain tissue.

FIGURE 1. *Images obtained using $H_2{}^{15}0$ to measure CBF in a chronic cocaine abuser. Note the marked disruptions in uptake of the tracer in the anterior areas of the brain (arrows) suggestive of decreases in regional CBF.*

Under normal physiological conditions CBF and brain glucose metabolism are tightly coupled (Siesjo 1978) and reflect brain function (Sokoloff 1978). However, under certain circumstances, CBF and metabolism can be uncoupled as could be hypothesized to happen when administering a vasoactive agent. In the case of cocaine this is a complex issue because cocaine has vasoactive effects as well as neurochemical effects on brain tissue activity (Pitts and Marwah 1988). To assess whether decreases in CBF secondary to chronic

cocaine use were due to decreased brain metabolism, a subgroup of 10 chronic cocaine abusers were investigated using $H_2{}^{15}O$ for CBF and ^{18}FDG to measure regional brain glucose metabolism. Only four of these patients showed evidence of metabolic defects similar to the flow deficiencies seen using $H_2{}^{15}O$. For the six remaining patients, the metabolic defects were smaller than the CBF effects, suggesting that, for most of the cases, the decrease in CBF had not led to comparable changes in brain metabolism. Nevertheless, most of the patients showed evidence of decreased metabolic activity in the frontal cortex (Volkow et al. 1988). These preliminary studies support the notion that the widespread decreases in CBF in cocaine abusers are due to the vasoactive properties and not to the actions of cocaine on brain tissue activity. However, it is difficult to assess whether the decreased metabolic activity in the frontal cortex is secondary to changes in perfusion or whether it reflects changes directly associated with the actions of cocaine in the dopamine system.

COCAINE ADDICTION

The addicting properties of cocaine have been related to its effect on the dopamine system (Dackis and Gold 1985). The “dopamine depletion hypothesis” suggests that chronic cocaine abuse leads to a depletion of dopamine in the presynaptic neuron. Although there have been several animal experiments designed to document decreases in dopamine function as assessed by measurements of dopamine synthesis, metabolism, and receptor concentrations, the results are controversial, with different groups showing decreases, no change, or increases in dopamine activity (Post et al. 1987; Galloway 1988).

With PET it is feasible to directly evaluate dopamine function in the living human brain. The following different parameters of the dopamine system can be measured:

- Postsynaptic dopamine D-2 receptors using compounds such as ^{11}C- or ^{18}F-labeled N-methylspiroperidol (NMS) (Wagner et al. 1983; Arnett et al. 1985) or ^{11}C-raclopride (Farde et al. 1985).

- Postsynaptic dopamine D-1 receptors using a compound such as ^{11}C-labeled SCH 23390 (Halldin et al. 1986).

- The presynaptic dopamine transporter using compounds such as ^{11}C-nomifensine (Aquilonius et al. 1987), ^{18}F-GBR (Kilbourn 1988) and ^{11}C-cocaine (Fowler et al. 1989).

- Regional brain activity of those areas that either have a high concentration of dopamine receptors or receive afferents from the dopamine system. This can be achieved using ^{18}FDG and/or ^{11}CDG (Reivich et al. 1985).

- Dopamine neurotransmitter synthesis using compounds such as [^{18}F]fluoro-L-Dopa (Baxter et al. 1988).

Preliminary work has been done with PET in measuring the concentration of postsynaptic dopamine receptors in the human brain to assess if chronic cocaine use leads to dopamine depletion. If there is depletion of dopamine, there should be an upregulation of postsynaptic dopamine receptors, which then could be measured with ^{18}F-NMS or ^{11}C-raclopride and PET. A study of 10 chronic cocaine abusers—7 were studied within 1 week of the last administration of the drug, and 3 were studied after 4 weeks of detoxification—showed that the short-term detoxified cocaine abusers had a decrease in binding of ^{18}F-NMS suggestive of a decrease in the number of postsynaptic dopamine receptors (Volkow et al. 1990). In contrast, the patients who had been detoxified for 4 weeks before the study showed values of uptake of ^{18}F-NMS similar to those of normal individuals. This study showed that the changes in postsynaptic dopamine receptors are not fixed and appear to be related to the amount of time that has elapsed after cocaine discontinuation. During active cocaine administration or shortly after its withdrawal, there is downregulation of postsynaptic dopamine receptors to compensate for dopamine overstimulation from cocaine. With protracted cocaine withdrawal, dopamine overstimulation is discontinued with a consequent increase in postsynaptic dopamine receptors.

Effects of chronic cocaine use on dopamine synthesis also have been investigated in humans using PET and [^{18}F]fluoro-L-Dopa. A preliminary study reported that shortly after detoxification from cocaine, there was an overall decrease in uptake of [^{18}F]fluoro-L-Dopa in the brain consistent with decreased dopamine synthesis (Baxter et al. 1988). After 30 days of cocaine detoxification, the uptake of [^{18}F]fluoro-L-Dopa was similar to that seen in normals, suggesting a normalization of dopamine synthesis after discontinuation of the drug.

Studies evaluating the effects of chronic cocaine on glucose metabolism are controversial, with one study reporting a decrease in prefrontal cortical activity in the chronic cocaine abuser (Volkow et al. 1988) and another failing to report abnormalities in glucose metabolism in these patients (Baxter et al. 1988). The study documenting decreased metabolism in the frontal cortex related these effects to the action of cocaine in the dopamine system, which sends projections into the prefrontal cortex.

With PET it is feasible to monitor not only the postsynaptic receptor but also the presynaptic dopamine terminal. Currently, labeling of the dopamine presynaptic terminal can be accomplished using compounds that bind to the dopamine transporter. There have not been any human studies investigating the effects of chronic cocaine on the dopamine transporter or on dopamine D-1 receptors.

COCAINE REINFORCEMENT

Mechanisms of reinforcement of cocaine can be assessed with PET using different experimental strategies.

1. Acute administration of cocaine to monitor sensitivity of different brain areas to actions of cocaine. This in turn can provide information on the brain areas that are responsible for the behavioral actions of cocaine and the neurotransmitters that may be involved with the function of these regions.

2. Measurement of pharmacokinetics of cocaine within the brain. This can be achieved using ^{11}C-cocaine and monitoring its distribution and kinetics in the human brain.

The effects of acute cocaine administration on regional brain glucose metabolism have been investigated with PET and ^{18}FDG. The study was done on a group of chronic cocaine abusers who were given an acute dose of 40 mg of cocaine hydrochloride intravenously. Two minutes after cocaine injection, these patients were injected with ^{18}FDG to monitor for the effects of the drug on regional brain glucose metabolism (London et al. 1990). This study reported an overall decrease in glucose metabolism in the cortex and in the extrapyramidal system after acute cocaine administration. In contrast, the cerebellum failed to show any response to cocaine. The decrease in cortical metabolism after acute cocaine administration was interpreted as reflecting the actions of cocaine on dopamine, because other dopamine agents such as amphetamines also have been shown to decrease cortical glucose metabolism (Wolkin et al. 1987); dopamine antagonists have been shown to increase glucose metabolism (Volkow et al. 1986).

With PET, it is also feasible to directly monitor the pharmacokinetics of a given drug at its site of action. ^{11}C-cocaine was used to monitor the distribution and kinetics of cocaine in the human brain. This study was done in baboons and in humans and demonstrated that cocaine rapidly enters the brain. Maximal accumulation of cocaine in the brain occurred between 4 and 8 minutes after injection. Clearance of ^{11}C-cocaine was rapid; by 20 minutes postinjection, only 50 percent of the radioactivity remained in the brain. The accumulation of

cocaine in the brain was heterogenous, with the basal ganglia showing the highest concentration of ^{11}C-cocaine (figure 2). Binding of cocaine into the basal ganglia appeared to be related to the dopamine transporter because pretreatment with nomifensin (a dopamine transporter inhibitor) decreased binding of ^{11}C-cocaine in the basal ganglia, whereas desipramine (a blocker of

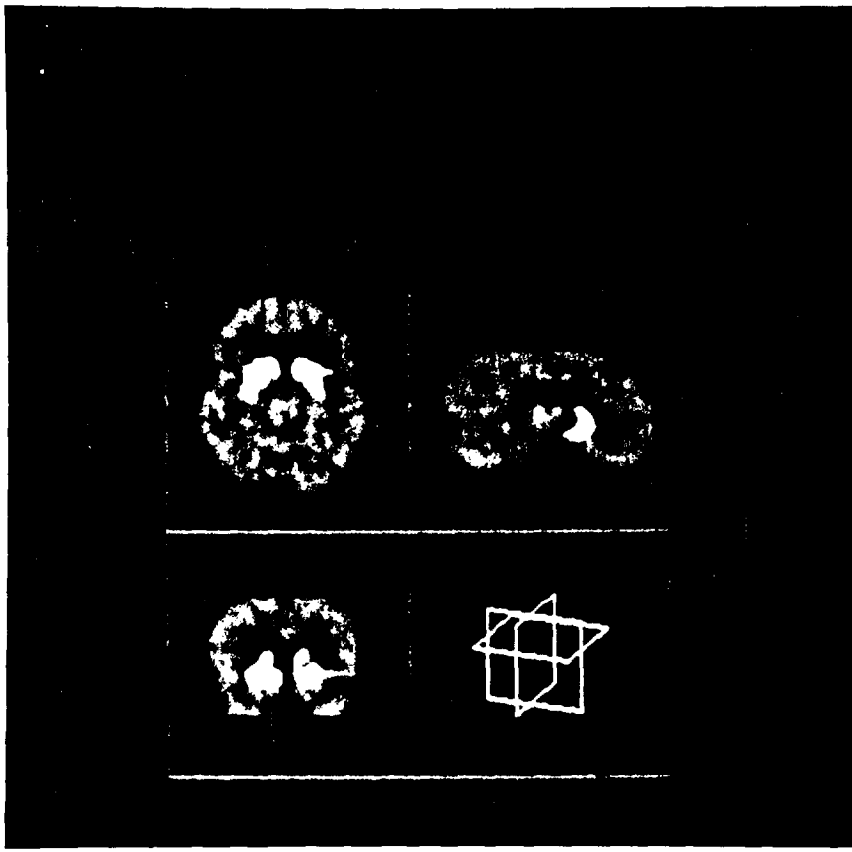

FIGURE 2. *Images obtained 15 minutes after administration of ^{11}C-cocaine in a normal human volunteer. The images represent an axial (upper left), a sagittal (upper right), and a coronal (lower left) plane at the level where the basal ganglia are located. Note that maximal binding of ^{11}C-cocaine occurs in the basal ganglia.*

the serotonin and norepinephrine transporter) failed to affect the binding of cocaine in the basal ganglia (Fowler et al. 1989). Rapid uptake and clearance of cocaine from the brain were compared with the time response to the euphorigenic actions of cocaine when administered intravenously. The data for the euphorigenic effects of cocaine were obtained from Cook and colleagues (1985). In this study cocaine abusers were injected intravenously with 20 mg of cocaine, and their subjective response to the "high" from cocaine was monitored every minute during the first 5 minutes and at 2-minute intervals thereafter for 30 minutes (Perez-Reyes et al. 1982). The values for the time-related response to the euphorigenic actions of cocaine were compared with the kinetics of ^{11}C-cocaine in the striatum in a group of eight normal subjects. Although the two populations of subjects were different, there was a close parallel between the time activity curves of these two groups (figure 3).

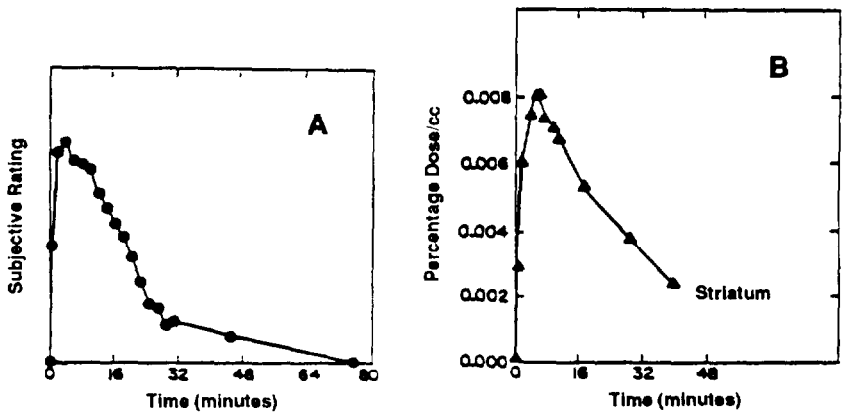

FIGURE 3. *Panel A shows the time course of the mean subjective high after intravenous cocaine (data from Cook et al. 1985). Panel B shows the time course of ^{11}C-cocaine in the human basal ganglia measured by PET (Fowler et al. 1989). Note that the curves parallel one another, suggesting an association between behavioral elevation and cocaine binding in the basal ganglia.*

The parallel between the two time curves suggests that the euphorigenic actions of cocaine are associated with the rapid binding of cocaine into the dopamine transporter and that the short duration of cocaine action also is related to the release of cocaine from its binding site.

SUMMARY

It is feasible to directly image and measure the concentration of different drugs of abuse and their consequences on regional brain function and neurotransmitter activity with PET. Although PET is a relatively new technique, it already is proving to be useful in disclosing the mechanisms of toxicity reinforcement and addiction of drugs of abuse.

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Development of Opioid Peptide Analogs as Pharmacologic Tools and as Potential Drugs: Current Status and Future Directions

Peter W. Schiller

INTRODUCTION

It is generally recognized that opiates and opioid peptides exert their various biological effects through interaction with either central or peripheral receptors, and the existence of at least three different classes of opioid receptors (μ, δ, η) also generally is accepted (Paterson et al. 1984). More recently, several investigators obtained evidence indicating that subtypes of the three major opioid receptor classes may exist; however, the issue of opioid receptor subtypes still remains somewhat controversial and needs to be clarified further. It has not yet been possible to establish clear-cut relationships linking specific opioid receptor types or subtypes to distinct opioid effects. This is mainly due to the fact that, until recently, potent stable opioid agonists and antagonists with high specificity for the various receptor types or subtypes have not been available. Unfortunately, the various endogenous opioid peptides resulting from processing of the three precursor molecules display only limited selectivity toward the various receptor types (HöllIt 1986). On the other hand, various peptides of the (ß-casomorphin family show high μ-receptor selectivity but relatively low receptor affinity (Brantl et al. 1981). The most selective naturally occurring opioid peptides discovered to date are the dermorphins (μ-selective) (Montecucchi et al. 1981) and the deltorphins δ--selective) (Erspamer et al. 1989). Because of their already high-receptor selectivity, the dermorphins and deltorphins represent excellent starting points for the development of ligands with even higher μ- or δ-receptor specificity.

Numerous efforts have been made to obtain more selective opioid receptor ligands through synthesis of opioid peptide analogs that were developed on the basis of the following three design principles:

1. Substitution, deletion, or addition of natural or artificial (nonproteinogenic) amino acids

2. Concept of conformational restriction of opioid peptides through various appropriate cyclizations

3. Design of bivalent compounds containing two opioid receptor ligands separated by a spacer of appropriate length and able to interact simultaneously with two receptor binding sites

The applications of these three design strategies in the opioid peptide field are described in the following sections.

LINEAR ANALOGS OF OPIOID PEPTIDES

Many efforts have been made to develop more selective opioid receptor ligands through synthesis of linear opioid peptide analogs. Hundreds of enkephalin analogs have been prepared, mostly by the classical approach of substituting one or several natural or artificial amino acids in various positions of the peptide sequence. These efforts resulted in several selective compounds, such as H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO; μ-selective) (Handa et al. 1981) and H-Tyr-D-Ser(OtBu)- Gly-Phe-Leu-Thr-OH (DSTBULET; δ-selective) (Fournié-Zaluski et al. 1988). The substituted tripeptide enkephalin analog MeTyr-D-Ala-Gly-N(Et)-CH(CH_2-Phe)CH_2-N(CH_3)$_2$ (LY164929) also shows high μ-selectivity (Rothman et al. 1988). The enkephalin analog (N-allyl)$_2$-Tyr-Gly-ψ[CH_2S]Phe-Leu-OH is a fairly selective but not very potent δ-antagonist (Shaw et al. 1982). Among various analogs derived from peptides of the ß-casomorphin family, the potent agonist PL017 (H-Tyr-Pro-Phe(NMe)- D-Pro-NH_2) is the most μ-selective (Chang et al. 1983).

The most selective μ-agonist developed to date is the dermorphin tetrapeptide analog H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) (Schiller et al. 1989a). DALDA displays high affinity for μ-receptors, and its extraordinary μ-selectivity is a consequence of extremely poor δ-receptor affinity (table 1). The tetrapeptide amide is about 60 times more μ-selective than the parent peptide, dermorphin. Furthermore, the μ-selectivity displayed by DALDA is 11 times higher than that of DAGO and 8 times higher than that of PL017. Its affinity for η-opioid receptors is negligible (K_i 1,000 nM). In the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, DALDA was shown to be a full agonist. As expected, this compound showed extremely low potency (IC50~40 μM) in the bioassay based on inhibition of electrically evoked contractions of the hamster vas deferens, a tissue containing opioid receptors exclusively of the δ-type. Because of its high positive net charge (3+), DALDA most likely will not be able

to cross the blood-brain barrier to any significant extent. Thus, it may be that, aside from its high μ-receptor preference, this analog also may show pronounced selectivity for peripheral vs. central receptors. Therefore, DALDA may be particularly useful as a specific agonist for studying peripheral μ-receptor interactions (see below).

TABLE 1. *Receptor affinities and μ-selectivity of opioid peptide analogs*[a]

Compound	K_i^μ [nM][b]	K_i^δ [nM][b]	K_i^δ/K_i^μ
DALDA	1.69 ± 0.25	19,200 ± 2,000	11,400
Dermorphin	0.839 ± 0.013	160 ± 56	191
DAGO	1.22 ± 0.12	1,280 ± 90	1,050
PLO17	2.89 ± 0.30	4,250 ± 540	1,470

[a]Binding assays based on displacement of [^{3}H]DAGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane preparations (DSLET=H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH).
[b]Mean of three determinations±SEM.

Because peptides with the phenylalanine residue in the 3-position (ß-casomorphins, dermorphins, deltorphins) and peptides with the Phe residue in the 4-position of the peptide sequence (enkephalins, dynorphins, ß-endorphin) are potent opioid agonists, it was of interest to prepare dermorphin-enkephalin hybrids containing a Phe residue in the 3- and 4-positions. The prototype analog H-Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) showed high μ-receptor affinity and selectivity in the rat brain membrane receptor binding assays (table 2) (Schiller et al. 1989a). Introduction of a nitro substituent in *para* position of the Phe aromatic rings produced a potency drop in position 3 and a potency increase in position 4, as it had been observed in the case of opioid peptides with a single Phe residue in either the 3- or 4-position (Schiller et al. 1983), indicating that the two Phe residues in TAPP do interact with the two distinct receptor subsites previously proposed. Unlike the polar analog DALDA, TAPP is a relatively lipophilic molecule. In an effort to obtain even more lipophilic opioid peptide analogs, we substituted bulky, hydrophobic amino acids, such as tryptophan or 1 (or 2)-naphthylalanine (Nap), in positions 3 and 4 of TAPP. The resulting analogs still showed high μ-receptor selectivity and, because of their quite high lipophilicity, may be able to cross the blood-brain barrier to some extent after peripheral administration.

TABLE 2. *Opioid receptor affinities of dermorphin-enkephalin hybrid analogs*[a]

Compound	K_i^μ [nM][b]	K_i^δ [nM][b]	K_i^δ/K_i^μ
H-Tyr-D-Ala-Phe-Phe-NH_2	1.53 ± 0.59	626 ± 162	409
H-Tyr-D-Ala-Phe(pNO_2)-Phe-NH_2	56.80 ± 12.70	1,380 ± 276	24.3
H-Tyr-D-Ala-Phe-Phe(pNO_2)-NH_2	0.509 ± 0.022	65.5 ± 14.0	129
H-Tyr-D-Ala-Trp-Trp-NH_2	0.833 ± 0.071	652 ± 49	783
H-Tyr-D-Ala-1-Nap-1-Nap-NH_2	2.88 ± 0.43	1,180 ± 230	410
H-Tyr-D-Ala-2-Nap-1-Nap-NH_2	5.61 ± 0.60	575 ± 13	102

[a]Binding assays were performed as indicated in table 1.
[b]Mean of three determinations±SEM.

The most selective δ-agonists known to date are the deltorphins, three opioid peptides that have recently been isolated from frog skin (Erspamer et al. 1989). No consensus regarding nomenclature seems to have been reached as yet, but referring to them as deitorphin, [D-Ala^2]deltorphin I, and [D-Ala^2]deltorphin II, as suggested by the discoverers, seems reasonable:

H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (deltorphin)
H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([D-Ala^2]deltorphin I)
H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 ([D-Ala^2]deltorphin II)

Among these three peptides, [D-Ala^2]deltorphin II shows highest δ-selectivity with a reported selectivity ratio (K_i^μ/K_i^δ) of 21,000 (Erspamer et al. 1989), thus apparently being nearly 100 times more δ-selective than the cyclic enkephalin analog [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (Mosberg et al. 1983).

Among the various endogenous opioid peptides, dynorphin A is the most potent at the η-receptor; but it also interacts with μ- and δ-receptors and, therefore, displays only moderate η-selectivity. A relatively limited number of analogs have been prepared in attempts to improve the η-selectivity and the stability of dynorphin A. The dynorphin analog with highest η-selectivity reported to date is (D-Pro^{10}]dynorphin A-(1-11), showing a K_i^μ/K_i^η ratio of 60 and a K_i^δ/K_i^η ratio of 230 (Gairin et al. 1985). Efforts will have to be made to develop dynorphin analogs with further improved general η-selectivity. Such analogs also may be useful for the elucidation of the still controversial issue of η-receptor heterogeneity and may be selective for distinct η-receptor subtypes.

CYCLIC OPIOID PEPTIDE ANALOGS

Small linear opioid peptides, such as the enkephalins, are flexible molecules, as indicated by numerous conformational studies (Schiller 1984). This structural flexibility may be one of the reasons for the lack of receptor selectivity of these peptides, because conformational adaptation to the various receptor binding sites is possible. Because of this, a different approach of analog design based on conformational restriction through peptide cyclization via side chains of appropriately substituted amino acids was taken in the author's laboratory a decade ago (DiMaio and Schiller 1980). The first cyclic enkephalin analog, H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (A_2bu=α,γ-diaminobutyric acid), showed high potency in the GPI assay and moderate μ-receptor selectivity (Schiller and DiMaio 1982). Variation of the side-chain length in the 2-position of this prototype analog through substitution of A_2pr (α,β-diaminopropionic acid), Orn, or Lys resulted in a series of cyclic homologs that were also moderately μ-selective (DiMaio et al. 1982). The cystine-containing cyclic peptides H-Tyr-D-Cys-Gly-Phe-D(or L)-Cys-X are examples of side-chain-to-side-chain cyclized analogs. [D-Cys^2, D(or L)-Cys^5]Enkephalinamides (X=NH_2) were highly potent in the GPI assay but nonselective (Schiller et al. 1981), whereas the corresponding analogs with a free C-terminal carboxyl group (X=OH) were about as moderately δ-receptor selective as the natural enkephalins (Schiller et al. 1985a). Replacement of the two half-cystine residues in these peptides with penicillamine residues resulted in compounds with considerably improved δ-selectivity (H-Tyr-D-Pen-Gly-Phe-Pen-OH [DPLPE] and H-Tyr-D-Pen-Gly-Phe-D-Pen-OH[DPDPE]) (Mosberg et al. 1983).

The receptor selectivities of the cyclic enkephalin analogs described above have been further improved through additional structural modifications. For example, the reversal of two peptide bonds in the ring structure of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] led to a compound, H-Tyr-cyclo[-D-Glu-Gly-gPhe-D-Leu-], which is three times more μ-selective than the cyclic parent peptide (Berman et al. 1983). Furthermore, thioamide ψ[CSNH] peptide bond replacements in H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-](selectivity ratio $K_i^δ/K_i^μ$=13.7) produced two compounds, H-Tyr-cyclo[-D-Lys-Gly ψ(CSNH]Phe-Leu-] ($K_i^δ/K_i^μ$=144) and H-Tyr-cyclo[-D-Lys-Glyψ[[CSNH]Phe-Leuψ [CSN'H]] ($K_i^δ/K_i^μ$=107), showing an approximate tenfold improvement in μ-selectivity (Sherman et al. 1989). Finally, substitution of (S,S)ß-methyl-p-nitrophenylalanine for Phe in position 4 of H-Tyr-D-Pen-Gly-Phe-D-Pen-OH resulted in a compound showing δ- vs. μ-selectivity five times higher than that of the DPDPE parent peptide, as assessed in the GPI and MVD assays (Hruby et al. 1989).

The dermorphin tetrapeptide analog H-Tyr-D-Orn-Phe-Asp-NH_2 is the most selective cyclic opioid peptide analog with μ-agonist properties reported to date

(Kᵢ/Kₑ = 213) (Schiller et al. 1985b). Interestingly, the cyclic tetrapeptide analog H-Tyr-D-Cys-Phe-D-Pen-OH displayed δ-receptor selectivity similar to that of DPDPE, but had about 3.5 times higher δ-receptor affinity than DPDPE (Mosberg et al. 1988). In contrast to most of the cyclic enkephalin analogs described above, these two cyclic opioid peptides with Phe in the 3-position of the peptide sequence contain highly constrained 13- and 11 -membered ring structures, respectively.

In an effort to reduce the conformational flexibility of the κ-receptor-selective opioid peptide dynorphin A, the cyclic analogs [D-Orn^2, Asp^5]dynorphin A-(1-8), [Orn^5, Asp^5]dynorphin A-(1-13), [Orn^5, Asp^{10}]dynorphin A-(1-13), and [Orn^5, Asp^{13}]dynorphin A-(1-13) were recently synthesized (Schiller et al. 1988). All four analogs showed K_o values for naloxone as antagonist below 5 nM in the GPI assay, indicating that they no longer interact significantly with κ-receptors. In the receptor binding assays, [D-Orn^2, Asp^5]dynorphin A-(1-8) displayed extraordinarily high affinity for the μ-receptor, and the determined ratios of the binding inhibition constants (K_i/K_μ) indicated that all four analogs are μ-selective. It was concluded that the performed cyclizations resulted in overall folded conformations that are incompatible with the conformational requirements of the κ-receptor. Further attempts will have to be made to develop cyclic dynorphin analogs that retain the κ-characteristics of the native peptide.

Recently, a cyclic somatostatin-related peptide analog, H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol (SMS 201-995), was shown to possess antagonist properties at μ-opioid receptors (Maurer et al. 1982). Similar somatostatin analogs characterized by additional constraints in the ring structure, such as TCTOP (H-D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (Kazmierski et al. 1988), were subsequently prepared. The latter analogs are the most selective μ-antagonists reported to date and, unlike SMS 201-995, no longer display significant affinity for somatostatin receptors. Aside from representing valuable tools in opioid pharmacology, the various receptor-selective cyclic opioid peptide analogs are of great interest for conformational studies aimed at determining the bioactive conformation of opioid peptides at the μ- or δ-receptor. The conformation(s) of some of the cyclic analogs have been studied by various techniques, including theoretical studies based on the molecular mechanics or molecular dynamics approach, nuclear magnetic resonance (NMR) spectroscopy, and fluorescence spectroscopy (Schiller and Wilkes 1988). These conformational studies have not yet led to a consensus concerning a possible unique bioactive conformation at the receptor. An important finding of these endeavors was the realization that the ring structures in the cyclic enkephalin analogs still retain some flexibility and that the various intramolecular hydrogen bonds observed are constantly formed, broken, and

reformed again, as shown most conclusively in molecular dynamics studies (Mammi et al. 1985). In the case of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-] and H-Tyr-c[-D-Orn-Gly-Phe-Leu-] (Kessler et al. 1985), the results of NMR studies indicated the existence of a hydrogen bond that defines a γ-turn centered on Phe^4 (figure 1). Computer simulations revealed that this hydrogen bond occurs with relatively high frequency on the picosecond time scale (Mammi et al. 1985). In a molecular mechanics study, extensive energy minimization was performed with 10 cyclic tetrapeptides structurally related to H-Tyr-D-Orn-Phe-Asp-NH_2 and displaying considerable diversity in μ-receptor affinity (Wilkes and Schiller 1990). The results of this study suggested that a tilted stacking arrangement of the Tyr^1 and Phe^3 aromatic rings may represent a structural requirement for high μ-receptor affinity of the examined cyclic dermorphin analogs. However, this study also revealed that the exocyclic Tyr^1 residue and the Phe^3 side-chain still enjoy considerable orientational freedom. To obtain more definitive insight into the receptor-bound conformation of the various cyclic opioid peptides described above, it will be necessary to develop analogs in which the Tyr residue and the Phe side-chain also are restricted conformationally.

OPIOID PEPTIDE MIMETICS

In efforts to develop peptide-derived drugs, it often would be desirable to replace parts of the peptide backbone with other appropriate structural elements to eliminate susceptibility to enzymatic degradation and to reduce the relatively polar character of peptide molecules that may prevent them from being well absorbed and from crossing certain barriers, such as the blood-brain barrier. Such compounds generally are referred to as peptide mimetics. It is imperative that the spatial disposition of side chains that are crucial for the interaction of the native peptide with the receptor be retained in the peptide mimetic. Whereas the various modifications of individual peptide bonds described above represent a first step in the direction of developing peptide mimetics, the substitution of structural elements mimicking larger portions of the peptide backbone also has been attempted. The latter approach is particularly applicable to peptides with a relatively well-defined conformation such as the semirigid cyclic analogs discussed above. An example is the peptide mimetic derived from the cyclic enkephalin analog H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] (Huffman et al. 1989), shown in figure 1. In this compound, the γ-turn structure detected in the cyclic parent peptide (see above) is replaced by a γ-turn mimic. One of the peptide bonds in the γ-turn mimic is replaced with a *trans* olefin, and the oxygen and hydrogen atoms of the hydrogen bond (C=O H-N) are replaced with an ethylene moiety. Diastereomers of the bicyclic compound with either R or S configuration at the chiral center of the γ-turn mimic were obtained separately. Both diastereomers essentially were inactive in the GPI and MVD

assays and showed weak affinity for μ- and δ-opioid receptors in the rat brain membrane binding assay. The lack of activity of these peptide mimetics can be explained in several ways. First, it is possible that the γ-turn detected in the NMR and computer simulation studies of the monocyclic parent peptide may not be a structural feature of the receptor-bound conformation. Second, the Gly^3 carbonyl group that is no longer present in the γ-turn mimic may be important for the interaction with the receptor. Third, the ethylene bridge introduced in the mimic may interfere with the receptor binding process either due to unfavorable steric interactions or due to the fact that it produces too much rigidity in the molecule.

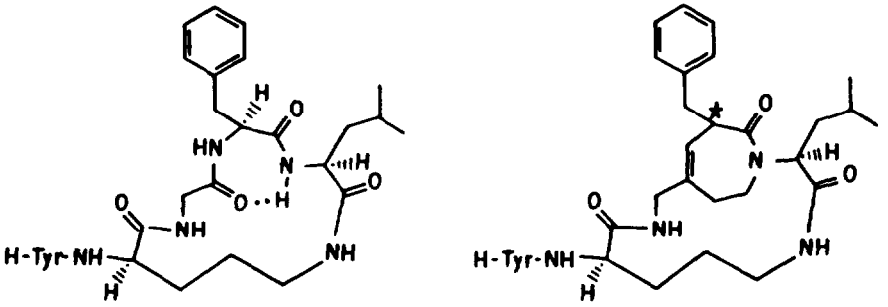

FIGURE 1. *Structural formulas of the cyclic enkephalin analog H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] (left) and of a bicyclic enkephalin analog containing γ-turn mimic (right)*

In contrast to the rational design of peptide mimetics just described, large-scale screening of substances from various sources by means of a receptor binding assay recently also led to the discovery and development of most useful peptide mimetics. The best known example is the discovery of the novel benzodiazepine natural product asperlicin, which is an antagonist of cholecystokinin (CCK) (Freidinger 1989). Using asperlicin as a starting point, a series of structurally related methylbenzodiazepines then was developed by the Merck group. One of these compounds, MK-329, became a potent competitive CCK antagonist with high preference for CCK_A over CCK_B receptors; whereas another, L-365260, was a potent, competitive, and orally active CCK antagonist showing CCK_B receptor selectivity. Another screening discovery is the recently described nonpeptide angiotensin II receptor antagonists, such as the imidazole acetic acid derivative S-8307 (Wong et al. 1988). Even though these compounds have relatively weak receptor affinity ($K_I \sim 10^{-5}$M), they represent

excellent starting points for the development of angiotensin antagonists with therapeutic potential.

BIVALENT OPIOID RECEPTOR LIGANDS

In attempts to bridge two opioid receptor binding sites, various bivalent ligands containing two enkephalin-related peptides linked via their C-terminal carboxyl group through flexible spacers of varying length have been synthesized and pharmacologically tested. It has been observed that, at a certain spacer length, the affinity and selectivity for δ-receptors increased drastically (Shimohigashi et al. 1982). This result was interpreted to indicate that a bivalent ligand with the right spacer length is capable of bridging two δ-binding sites. Analogous dimerization of the tripeptide H-Tyr-D-Ala-Gly-OH via a short spacer resulted in a highly μ-selective compound (Lutz et al. 1985a), which apparently is able to simultaneously bind to two μ-binding sites. These studies with bivalent opioid peptide ligands had been initiated based on the hypothesis that opioid receptors of the δ- or the μ-type might be clustered together and that the distance between two δ-receptors in such clusters might be different from that between two μ-receptors. Therefore, it was thought that variation of the spacer length might produce either δ- or μ-selective ligands, as turned out to be the case. However, on the basis of data obtained from binding experiments in which the opioid receptor density was altered through irreversible labeling with the δ-selective ligand FIT (Lutz et al. 1985b) as well as on the basis of structural considerations, it now seems more likely that these bivalent ligands are able to bridge binding sites on two putative subunits of either the δ- or μ-receptor rather than interacting with two separate receptors. The best bivalent μ-ligand is about three times less selective than morphiceptin, and the most selective bivalent δ-ligand shows δ- vs. μ-selectivity comparable to that of DPDPE. These bivalent opioid peptide ligands represent valuable tools for basic opioid receptor research, but are perhaps less attractive as candidates for peptide drug development because of their increased molecular size.

OPIOID PEPTIDE ANALOGS AS POTENTIAL DRUGS

The two best known enkephalin analogs that have undergone fairly extensive clinical testing so far are the Sandoz compound FK33-824(H-Tyr-D-Ala-Gly-Phe(NMe)-Met(O)-ol) (Von Graffenried et al. 1978) and the Eli Lilly compound metkephamid (H-Tyr-D-Ala-Gly-Phe-Met(NMe)-NH_2) (Frederickson 1986). FK33-824 shows only moderate preference for μ-receptors over δ-receptors, and metkephamid is essentially nonselective toward μ- and δ-receptors (table 3); neither analog has significant affinity for η-receptors. Both FK33-824 and metkephamid exert a potent centrally mediated analgesic effect after systemic administration. However, FK33-824 also produced a number of quite serious

side effects (table 3) and is no longer pursued clinically as an analgesic candidate. Relatively fewer side effects were observed with metkephamid. Some evidence was obtained to indicate that metkephamid may exert its central analgesic effect through interaction with δ-receptors and that it may produce less physical dependence than morphine. Of particular interest was the observation that this analog does not cross the placental barrier to a significant extent in the pregnant sheep model (Frederickson 1986). This finding suggested that metkephamid might be of interest for use in obstetric analgesia. However, metkephamid finally was abandoned as a candidate for obstetric analgesia because it also produced a transient hypotensive effect in the particular obstetric population that had been subjected to a clinical trial. The various side effects observed with FK33-824 and metkephamid led to a certain discouragement with regard to the potential of opioid peptides as viable analgesics. However, it should be realized that the lack of receptor selectivity of these two enkephalin analogs may be responsible for some of the observed side effects. Progress recently made in the development of opioid peptide analogs now permits the preparation of compounds showing greatly improved receptor selectivity and, therefore, showing better potential for application in various types of analgesia.

In recent years, increasing evidence has been obtained to indicate that opioids are able to produce peripherally mediated analgesic effects. Although data on peripheral antinociceptive effects in normal noninflamed tissue are still controversial, effects in hyperalgesic and inflammatory conditions have been well documented (Ferreira and Nakamura 1979; Smith et al. 1982; Stein et al. 1988). The mechanism underlying peripherally mediated, opioid-induced antinociceptive effects is not entirely clear, but they appear to be mediated by inhibition of sensory neurons that would prevent information about noxious events from reaching the spinal cord. The most convincing evidence for the occurrence of peripherally mediated analgesic effects has been obtained from studies with opioid agonists and antagonists that are unable to cross the blood-brain barrier. Thus, it has been demonstrated that N-methylmorphine was able to produce an analgesic effect in the mouse writhing test that could be antagonized with N-methylnalorphine (Smith et al. 1982). Because these quaternized opiates do not have access to the brain, it is obvious that antinociception was induced through interaction with peripheral opioid receptors. Obviously, peripherally acting opioid agonists have potential for clinical applications, because they will not produce centrally mediated side effects such as dependence and respiratory depression. For example, such compounds could be used for the treatment of painful inflammatory conditions. Recently, a relatively polar enkephalin analog, H-Tyr-D-Arg-Gly-Phe(pNO_2)-Pro-NH_2 (BW443C), also has been shown to produce peripherally mediated antinociception in the mouse writhing test at a dose of about 5 mg/kg (Hardy et

TABLE 3. *Clinical testing of enkephalin analogs*

Compound	Selectivity	Analgesia	Side Effects
Tyr-DAla-Qly-Phe(NMe)-Met(O)-ol (FK33-824)	K^{δ}/K^{μ}=3.4	Centrally mediated (systemic administration)	Muscle heaviness Chest oppression Anxiety Bowel sound Increase Chemosis Whole body flush
Tyr-DAla-Gly-Phe-Met(NMe)-NH_2 (metkephamid)	Nonselective	Centrally mediated (systemic administration) Does not cross placental barrier	Heavy sensation in extremities Nasal congestion Emotional detachment Conjunctival infection Hypotension
Tyr-DArg-Qly-Phe(pNO_2)-Pro-NH_2 (BW443c)	Somewhat μ-selective	Peripherally mediated (S.C. administration)	Nasal stuffiness Dry mouth Postural hypotension No central effects (such as sedation, mood change, respiratory depression)

al. 1988). Preliminary clinical studies indicated that side effects of this moderately μ-selective compound given by intravenous (IV) infusions (10 μgkg^{-1} min^{-1} for 20 minutes) were relatively minor (table 3); and there was no evidence for central activity such as sedation, respiratory depression, and mood changes (Posner et al. 1988).

The analgesic activity of the highly μ-selective dermorphin tetrapeptide analog DALDA was compared with that of morphine in the mouse writhing test (table 4) (Schiller et al. 1989b). In this assay, DALDA showed an ED50 comparable to that of morphine 5 minutes after administration (s.c.). The effect of DALDA seems to be of somewhat shorter duration than that of morphine; but nevertheless, it is remarkable that this peptide analog showed a still relatively low ED50 (2.80 mg/kg) when given 60 minutes before the administration of phenyl-1, 4-benzoquinone. The antinociceptive effect of DALDA in the writhing assay could be antagonized with the quaternary opiate antagonist N-methyllevallorphan (10 mg/kg intraperitoneal [IP]), which increased the ED50

(20 minutes) from 0.65 mg/kg *to* 2.98 mg/kg (figure 2). Because N-methyllevallorphan is unable to cross the blood-brain barrier, this observation suggests that DALDA produces antinociception through interaction with peripheral μ-receptors. On the other hand, the antinociceptive effect of morphine (s.c.) was unchanged after IP administration of the same dose of N-methyllevallorphan, as expected in the case of a centrally acting compound. DALDA also showed high potency in the writhing assay after IV administration. On the other hand, in the mouse hot plate test, peripheral administration of DALDA produced an analgesic effect only at high doses (ED50>20 mg/kg), indicating that access of this compound to the central nervous system is limited. In comparison with the peripherally acting enkephalin analog BW443C discussed above, DALDA is more polar, more potent, and much more μ-selective. DALDA also shows considerably higher μ-selectivity than do the dermorphin analogs H-Tyr-D-Arg-Phe-Sar-OH (Sasaki et al. 1985) and H-Tyr-D-Met(O)- Phe-D-Ala-OH (Marastoni et al. 1987), which have been shown to be highly active in various analgesic tests.

TABLE 4. *Mouse writhing test of DALDA and morphine*

Compound	Time[a] (min)	ED50 (mg/kg)
DALDA	5	0.48
	20	0.65
	60	2.80
Morphine	5	0.31
	20	0.43
	80	0.60

[a]Time interval between administration of test compound (s.c.) and phenyl-1,4-benzoquinone (2.5 mg/kg IP).

Aside from peripherally induced analgesia, other peripheral effects of opioid peptides might be exploited for the development of potential drugs. Of particular interest are the various effects of opioid peptides in the gastrointestinal tract. Recently, the enkephalin analog H-Tyr-D-Met(O)-Gly-Phe(pNO_2)-Pro-NH_2 (BW942C) was shown to be a safe and effective agent for controlling diarrhea after cisplatin administration in cancer patients (Kris et al. 1988). Another study performed with the μ-selective enkephalin analog H-Tyr-D-Lys-Gly-Phe-homocysteine-thiolactone (Hoe 825) revealed that this

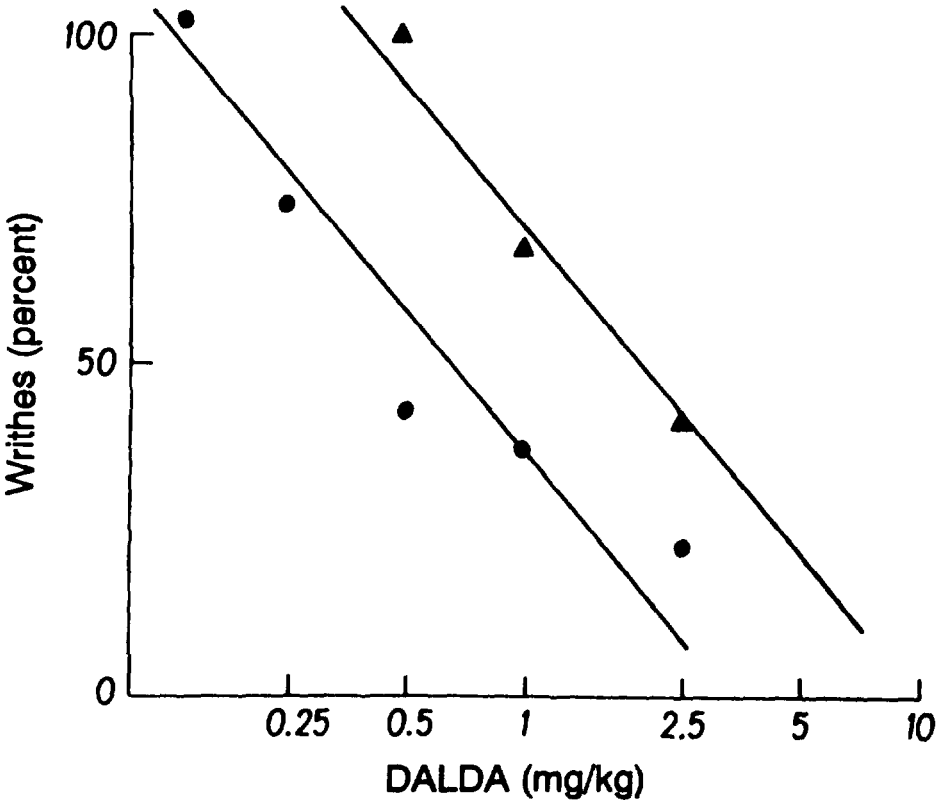

FIGURE 2. *Analgesic effect of DALDA in the mouse writhing test in absence (•) and presence (▲) of N-methyllevallorphan (10 mg/kg IP)*

compound has a powerful gut-stimulating effect and may have therapeutic potential for the management of paralytic ileus or other gut dysfunctions requiring stimulation of motor activity (Bickel et al. 1985).

CONCLUSION

In recent years, substantial progress has been made toward the development of opioid peptide analogs showing high selectivity for μ- or δ-opioid receptors. Further efforts are required to obtain peptide ligands with high preference for η-receptors. Another future goal of great importance is the development of opioid peptide analogs showing selectivity for the putative μ-, δ-, and η-receptor subtypes. Early clinical trials with relatively nonselective enkephalin analogs led

to a certain disappointment because of several serious side effects that were observed with these compounds. Peptide analogs with high specificity for distinct opioid receptor types or subtypes can be expected to produce fewer side effects and should be examined in future clinical trials. Peptides offer the possibility of developing analogs that display great diversity in the relative lipophilicity/hydrophilicity of their structures, such that they may or may not cross certain barriers (blood-brain barrier, placental barrier, etc.). Therefore, analogs can be designed that may act at either central or distinct peripheral sites and that may have potential as selective therapeutic agents in various clinical situations.

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CANADA

Conformational Analysis of Opioid Receptor-Selective Peptides Using Nuclear Magnetic Resonance and Theoretical Calculations

Victor J. Hruby, Om Prakash, Wieslaw Kazmierski, Catherine Gehrig, and Terry O. Matsunaga

INTRODUCTION

It is now generally accepted that there are four different types of opioid receptor: μ, δ, κ, and ε (Rapaka et al. 1986; Udenfriend and Meienhofer 1984); and there is increasing evidence for subtypes of these receptors. Efforts to determine the physiological roles of this multiple receptor system and the individual receptors have been difficult owing to the lack of highly selective ligands (>1,000-fold) for these receptors. Recently, our laboratory (Mosberg et al. 1982, 1983; Hruby 1986) developed a class of cyclic disulfide-containing enkephalin analogs that possess exceptional δ-opioid receptor selectivities as measured in the classical mouse vas deference (MVD)/guinea pig ileum (GPI) bioassays and in binding studies using the plasma membranes from brain tissue and selective radioligands (Akiyama et al. 1985; Vaughn et al. 1989). The prototypical peptide with high δ-receptor selectivity is [D-Pen^2, D-Pen^5]enkephalin (DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen-OH). In parallel studies using a different approach, researchers have succeeded in converting somatostatin, a tetradecapeptide with high potency in inhibition of release of glucagon, growth hormone, and insulin among other activities, into a highly μ-opioid receptor-selective ligand (Pelton et al. 1985a, 1986; Kazmierski et al. 1988). It has virtually no somatostatin-like biological activities, as measured by bioassays and binding studies similar to those used in the evaluation of δ-ligands.

This chapter discusses some of the considerations applied in the design of these compounds and the evaluation of their conformational properties using nuclear magnetic resonance (NMR) and other biophysical methods. Special emphasis is given to the use of conformational constraints and topographical considerations in the design of the peptide ligands the authors have developed.

GENERAL DESIGN PRINCIPLES

Most peptide hormones and neurotransmitters are flexible molecules with multiple conformations accessible under physiological conditions. As such, it perhaps is not surprising that these peptides can adopt multiple conformations and interact with multiple receptors. A basic hypothesis of this approach, which uses conformational (Hruby 1982; Hruby et al. 1990) and topographical (Hruby et al. 1987, 1990; Kezmierski and Hruby 1988) constraints, is that different receptor subtypes will utilize different structural, conformational, and topographical features of the peptide ligand. Imposition of proper conformational and topographical constraints should result in more highly receptor-selective ligands. From this perspective, morphine—a highly constrained ligand (figure 1) with only modest opioid-receptor selectivity—is not a suitable ligand to model the bioactive conformations of opioid-receptor ligands. Rather, one should design conformationally and topographically constrained opioid analogs and then utilize the most selective ones for exploring the differential conformational and topographical requirements of the opioid receptors. In this regard, this research group has been involved primarily in the design of opioid peptides, and some of these efforts are discussed here. Methionine enkephalin (figure 1)—a natural endogenous opioid-receptor ligand possessing modest δ-opioid receptor selectivity (Hruby and Gehrig 1989)—is a particularly suitable starting point. The use of somatostatin-14 (H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) as the starting point for a μ-opioid receptor-selective ligand design begins from a different premise (discussed later in this chapter).

The initial design principles for DPDPE were twofold: (1) pseudoisosteric cyclization (Sawyer et al. 1982) in which the Gly^2 and Met^5 residues and side-chain moieties are replaced by residues D-Cys^2 and D- or L-Cys^5 to give a cyclic 14-membered macrocyclic ring structure and (2) geminai dimethyl substitution on the ß-carbons of the Cys residues (penicillamine=ß,ß-dimethylcysteine). The use of penicillamine in cyclic peptides previously had been shown to have both local conformational and more global topographical effects in peptide disulfide rings (Meraldi et al. 1977). Both of these modifications are necessary for high δ-opfoid receptor selectivity. For example, the authors have found that [D-Cys^2, D-Cys^5]enkephalin, though 10 to 20 times more potent than DPDPE in the MVD and rat brain binding assays, has only modest δ-receptor selectivity as measured by the MVD compared with the GPI bioassays and by rat brain binding studies (unpublished). These studies suggest that conformational and stereostructural properties were of critical importance for the high δ-opioid receptor selectivity of DPDPE.

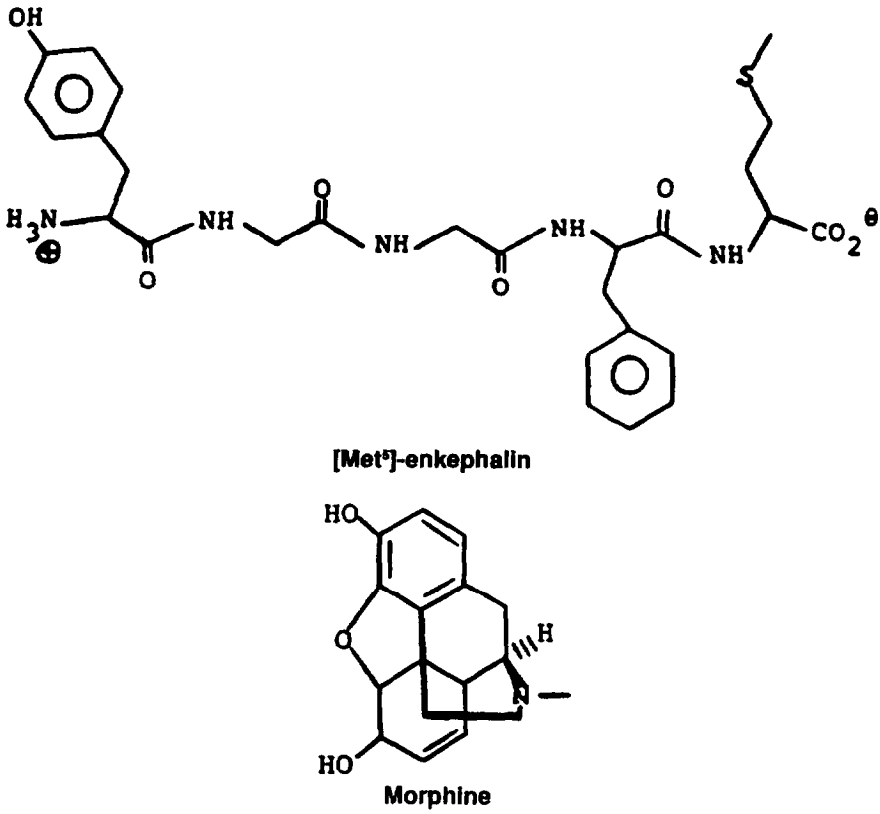

FIGURE 1. *Structure of [Met^5]-enkephalin (top) and of morphine (bottom)*

The redesign of somatostatin-related peptides to be opioid peptides with little or no somatostatin-like activities had different origins. In this case, both biological and conformational considerations were critical. On the biological side, it was observed that somatostatin, albeit at high concentration, had analgesic activities (Terenius 1976; Rezek et al. 1981). Subsequent studies with a series of cyclic octapeptide analogs of somatostatin, especially H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol), which was found to be superpotent in the release of growth hormone and to have antagonist activities on enkephalin-mediated electrophysiological actions (Maurer et al. 1982) gave further evidence of the interaction of somatostatin analogs with opioid receptors. This led to examination of the possibility that appropriately modified analogs of the cyclic octapeptide might lose most of their somatostatin-like activities but, at the same

time, have highly selective and potent opioid-receptor bioactivity. Structure-activity studies (Pelton et al. 1985a, 1985b, 1986) led to the development of the peptide D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2(CTP), which was found to have selective and potent binding at the μ-opioid receptor and to only weakly interact with the somatostatin receptor.

CONFORMATION: TOPOGRAPHICAL/BIOLOGICAL ACTIVITY RELATIONSHIPS OF DPDPE AT THE δ-OPIOID RECEPTOR

The highly δ-opioid receptor selectivity of DPDPE has led to its extensive use by many laboratories to explore the biological properties of this receptor. A summary of some of the biological properties of DPDPE is given in table 1. These properties suggest that highly δ-opioid receptor-selective ligands, such as DPDPE, have exceptional potential for the treatment of a variety of diseases related to opioid receptors. In addition, these ligands may provide a central analgesic that does not have many of the severe side effects of drugs such as morphine and heroin, which have many of their biological effects through interaction with μ-opioid receptors. These findings have led the authors and other researchers to continue developing approaches to obtain more potent and selective δ-opioid peptides.

TABLE 1. *Some biological properties of DPDPE*

1. δ- vs. μ-receptor—100- to 5,000-fold selective for δ-receptor, depending on system
2. δ- vs. κ-receptor—>50,000-fold selective for δ-receptor
3. Potent and prolonged analgesic action
4. Does *not* inhibit gut transit
5. Greatly reduced addiction potential relative to μ-opioids
6. Potent antidiarrheal effects
7. Positive interaction with μ-ligands

As part of our efforts to develop a more rational approach to the design of biologically active peptide ligands (Hruby 1981a, 1981b, 1982, 1984, 1985, 1990; Hruby and Hadley 1986; Hruby et al. 1990), we have been developing a

topographical approach to peptide design. This approach requires a careful analysis of the conformational properties of DPDPE and its analogs to determine what structural changes can maintain the same backbone template conformation. Careful use of two-dimensional (2D) NMR spectroscopy is an important aspect of such an approach. The authors have prepared more than 50 analogs of DPDPE, some of which are more potent and δ-opioid receptor selective than DPDPE (Toth et al. 1990).

Previously, Hruby and colleagues (1988) reported a comprehensive study on the NMR of DPDPE in aqueous solution. The authors have repeated many of these studies in dimethyl sulfoxide (DMSO)-d_6 (e.g., see figure 2, the COSY spectrum of [pCl-Phe^4]DPDPE). The chemical shifts and coupling constants for DPDPE in DMSO-d_6 are given in table 2, and the results are compared with those previously reported for DPDPE in aqueous solution. Although there are chemical shift differences as a result of the solvent change, the chemical shift parameters remain basically the same. The changes in C_aH-$C_ßH$ coupling constants for the Tyr^1 and Phe^4 residues indicate that, in DMSO, these residues are even more strongly biased to the trans and/or gauche(-) side-chain rotamers. These studies, coupled with molecular mechanics calculations similar to those previously reported (Hruby et al. 1988), indicate that, overall, DPDPE analogs modified in the Phe^4 aromatic ring and in the ß-carbon of Phe^4 maintain the same overall conformation. Particularly interesting is [ß,ß-dimethyl-Phe^4]DPDPE, because molecular dynamics calculations indicate that the Phe^4 side-chain conformation is "frozen" in the trans conformation, as shown in figure 3 (Kao and Hruby, unpublished results). Since this compound is about 500 times less potent than DPDPE, but appears to retain δ-receptor selectivity and agonist activity, it might be suggested that the topography associated with the Phe^4 aromatic side-chain group in the x_1 trans conformation is not highly compatible with δ-opioid receptor recognition (binding) but is consistent with transduction. Previous studies (Hruby et al. 1985) have shown that the Tic^4 analog of DPDPE (in which the aromatic side-chain group is exclusively in the gauche(+) conformation) is even less potent at the δ-receptor than is the ß,ß-dimethylPhe^4 analog, but it also is an agonist. It might be suggested, therefore, that δ-opioid receptor recognition prefers the gauche(-) side-chain conformation for receptor recognition, but that any one of the side-chain conformations of that residue may be compatible with transduction of activity at the δ-opioid receptor. Further studies under way are examining these topographical aspects of structure-activity relationships, as are similar studies examining the topographical requirements of the Tyr^1 side-chain group for interaction with the δ-opioid receptor.

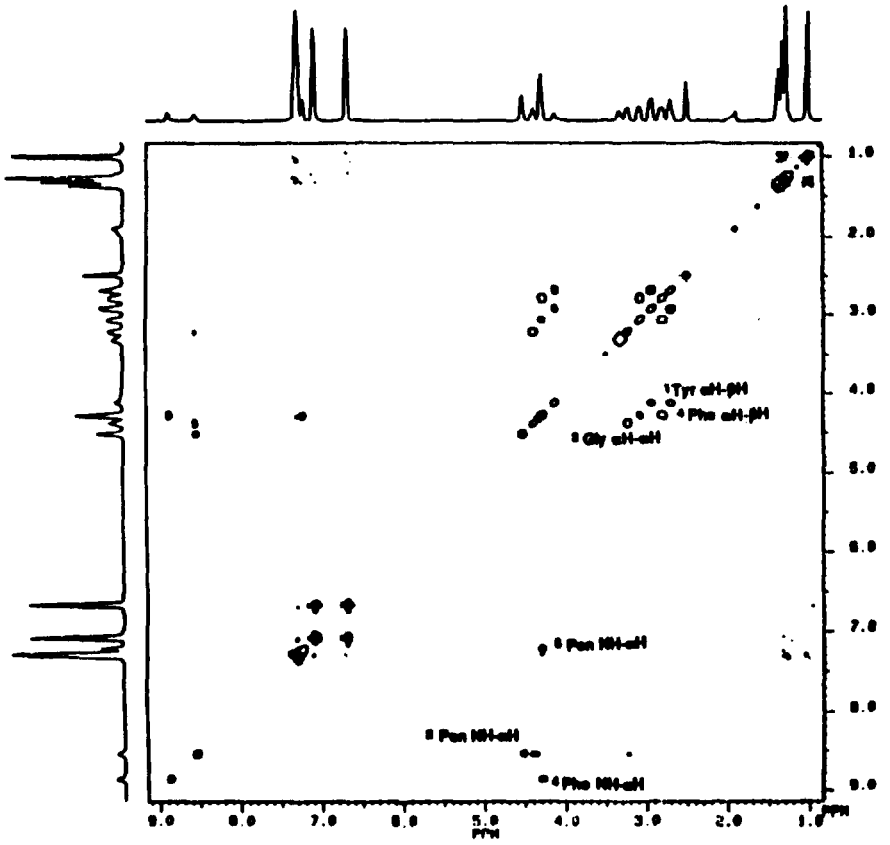

FIGURE 2. *^{1}H-^{1}H COSY spectrum at 500 MHz of [pCl-Phe^4]DPDPE in DMSO-d_6 solution*

Based on studies to date, the following tentative conclusions regarding the structural and topographical requirements of the δ-opioid receptor can be made:

1. DPDPE can serve as a relatively conformationally stable template for examining the stereostructural and topographical requirements for δ- vs. μ- (and κ-) opioid receptors.

2. The overall conformation of the cyclic 14-membered ring of DPDPE can be retained within a closely related conformational family with substitution at the ß-carbon and further removed carbons in residues of DPDPE (table 3) and, possibly at the α-carbons of the D-Pen^5 and Phe^4 residues as well.

TABLE 2. *Proton chemical shifts (ppm) and coupling constants (Hz) for DPDPE in DMSO-d_6*

Residue	δ_α	δ_β	δ_{NH}	δ_{Other}
Tyr[1]	4.28 (m)	2.95 (dd; 13.5, 6.4 Hz) 2.76 (dd; 13.5, 8.1 Hz)	—	7.10 (d; 8.4 Hz) 6.9 (d; 8.4 Hz)
Pen[2]	4.54 (d; 9.1 Hz)	—	8.64 (d; 9.1 Hz)	1.35 (s) 0.98 (s)
Gly[3]	4.38 (dd; 14.3, 9.5 Hz) 3.21 (dd; 14.3, 3.1 Hz)	—	8.58 (dd; 9.5, 3.1 Hz)	—
Phe[4]	4.30 (ddd; 8.0, 3.8, 11.1 Hz)	3.08 (dd; 14.3, 3.8 Hz)	8.85 (d; 8.0 Hz) 2.79 (dd; 14.3, 11.1 Hz)	7.26 (m)
Pen[5]	4.33 (d; 8.5 Hz)	—	7.24 (d; 8.5 Hz)	—

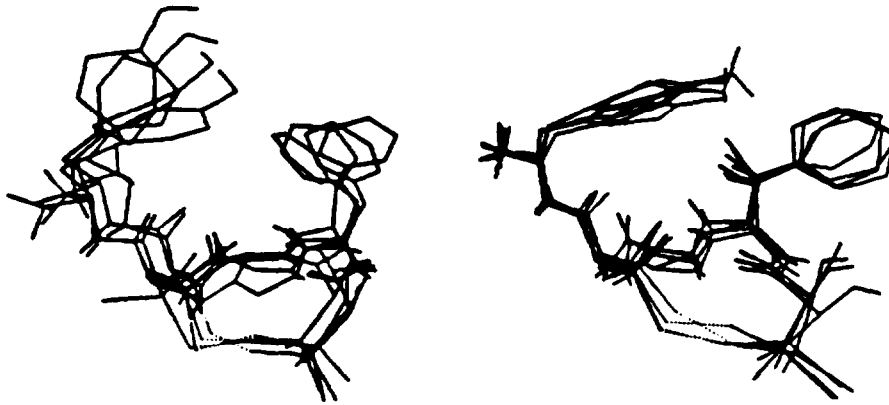

FIGURE 3. *Superimposed molecular dynamics structures of DPDPE (left) and [ß-ß-dimethylphenylalanine[4]]DPDPE (right) at 1000° C showing the relatively rigid conformation of the ßß-Me_2-Phe residue in a trans rotamer conformation*

3. For maximal agonist activity and selectivity, topographical relationships of the aromatic residues are preferred in which the aromatic rings are on the same surface.

4. The paraposition in the aromatic ring of Phe[4] has specific stereoelectronic requirements at the δ-opioid receptor but not at the μ-opioid receptor (Toth et al. 1990).

5. The selectivity of DPDPE for the δ-opioid receptor lies primarily in the topographical relationships of residues 1, 2, and 4.

6. Topographical changes without any large changes in backbone conformation can dramatically affect receptor potency and especially specificity. The approach of topographical constraints is likely to provide a general approach to the design of peptide ligands for a variety of receptor and acceptor molecules, including enzymes, surface receptors, nucleic acids, and other biologically important macromolecules.

TABLE 3. *Proton chemical shifts (ppm) of C_a-H and of the peptide backbone in DPDPE analogs*

Peptide	D-Pen[2] (ppm)		Gly[3] (ppm)		Phe[4] (ppm)			D-Pen[5] (ppm)	
	C_a-H	NH	C_a-H	C_a-H[1]	NH	C_a-H	NH	C_a-H	NH
1. DPDPE	4.54	8.64	4.38	3.21	8.58	4.30	8.85	4.33	7.24
2. p-F-Phe[4]-DP	4.54	8.63	4.38	3.21	8.58	4.27	8.86	4.31	7.22
3. p-Cl-Phe[4]-DP	4.51	8.54	4.38	3.21	8.54	4.28	8.87	4.29	7.22
4. p-Br-Phe[4]-DP	4.52	8.55	4.38	3.22	8.55	4.28	8.85	4.29	7.21
5. p-I-Phe[4]-DP	4.53	8.63	4.34	3.24	8.61	4.28	8.86	4.29	7.23
6. p-NO_2-Phe[4]-DP	4.55	8.63	4.38	3.21	8.60	4.40	8.93	4.31	7.30
7. p-NH_2-Phe[4]-DP	4.54	8.64	4.39	3.21	8.58	4.32	8.76	4.29	7.20
8. [(S,S)ß-MePhe[4]]-DPDPE	4.47	8.40	4.15	3.10	8.42	4.20	8.30	4.18	7.38

CONFORMATION: BIOLOGICAL ACTIVITY RELATIONSHIPS OF HIGHLY μ-OPIOID RECEPTOR-SELECTIVE PEPTIDE ANTAGONISTS

Somatostatin is a tetradecapeptide with the structure H-Ala[1]-Gly-Cys-Lys-Asn[5]-Phe-Phe-Trp-Lys-Thr[10]-Phe-Thr-Ser-Cys[14]-OH. Its primary physical role appears to be in controlling the release of several key hormones, enzymes, and neurotransmitters—especially growth hormone, insulin, and glucagon; as such,

it is widely distributed throughout peripheral tissues and the central nervous system (CNS). These biological activities have been shown to be related to specific structural and conformational properties of the hormone (Veber and Saperstein 1979; Hruby et al. 1984). In particular, via a series of conformational, structural, and structure-activity studies, it has been found that the sequence -Phe-Trp-Lys-Thr- (residues 7-10) in somatostatin, when stabilized In a ß-turn conformation, provides the key structural and conformational properties of the hormone at the receptors related to somatostatin inhibition of growth hormone, glucagon, and insulin release (Freidinger and Veber 1984).

On the other hand, though much higher concentrations were required than were needed to inhibit growth hormone release, it was demonstrated early that somatostatin could interact with the opioid receptor system and, among other effects, stimulate analgesia (Terenius 1976; Rezek et al. 1981). These effects were largely ignored, presumably because they were thought to be of no interest because of the high concentrations required for analgesia. However, in conjunction with appropriate conformational constraints (Hruby 1982, 1984, 1985) and structural considerations, it might be possible to modify the somatostatin structure in such a way as to select for its opioid-like properties while diminishing or even eliminating its classical “somatostatin” effects. Particularly intriguing was the prospect of utilizing the ß-turn structure of somatostatin -Phe-Trp-Lys-Thr- as a “template” on which to design an opioid ligand with high opioid-receptor selectivity. It was anticipated that such studies might be successful for several reasons. First, because somatostatin was only weakly active as an opioid-receptor ligand but had very potent activity as a somatostatin receptor ligand, it seemed reasonable to conclude that the structure-activity relationships at the two different receptor types would be different. Second, analysis of structure-function studies for many peptide hormones and neurotransmitters that have both peripheral and central receptors indicates that, in general, receptors found in the CNS compared with those in the periphery have uniquely different structure-activity relationships, Third, it was observed by Maurer and coworkers (1982) that the cyclic analog D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cis-Thr(ol), a highly potent somatostatin analog at inhibiting growth hormone release, also interacted with brain receptors related to the opioid receptors. These later findings also indicated that truncated somatostatin octapeptide analogs, utilizing the modified somatostatin 5-12 sequence, would be sufficient to explore the design of potent and selective opioid peptides.

The examination began with the synthesis of several of C-terminal carboxylate analogs of somatostatin 5-12 of the type H-D-Phe^{1}-Cys-Phe-D-Trp^{4}-Lys-Thr-Cys-Thr^{8}-OH in which the Cys$^{2\ and\ 7}$ residues were replaced with L-Pen (Pelton

et al. 1985b). It was found that the Pen substitution significantly reduced somatostatin-like binding; if, in addition, L-Pen was substituted for the Cys^7 residue, it increased potency at opioid receptor sites. Interestingly, this modification resulted in a significant increase in μ- vs. δ-opioid receptor selectivity. Subsequently, it was found that substitution of Phe^3 by Tyr, and especially modification of the carboxylate terminal to a carboxamide group, resulted in a profound increase in μ-receptor selectivity with a concomitant decrease in δ-receptor binding. In addition, the somatostatin receptor affinity diminished dramatically (Pelton et al. 1985b). The most favored ligand was D-Phe^1-Cys-Tyr-D-Trp-Lys^5-The-Pen-Thr^6-NH_2 (referred to as CTP for the substitution Cys^2, Tyr^3, and Pen^7) in the basic somatostatin structure. (It was found that a D-Pen^7 substituted analog has weak interaction with the opioid receptor.) Furthermore, it was shown that CTP was highly selective for the μ-vs.δ- (or κ-) opioid receptor (Pelton et al. 1985b).

CONFORMATION: TOPOGRAPHICAL/BIOLOGICAL ACTIVITY RELATIONSHIPS OF CTP AT THE μ-OPIOID RECEPTOR

The highly μ-opioid receptor selectivities that were observed in the binding studies of CTP raised several questions related to its biological and biophysical properties and their relationships to one another. On the biological side, an immediate question was whether CTP was an agonist or antagonist as an opioid receptor ligand. This was examined using classical oploid assays in collaborative biological studies at the University of Arizona (Thomas F. Burks, Henry I. Yamamura, and their coworkers). It was determined that in the *in vitro* GPI assay, the classical preceptor assay, CTP was a potent and selective μ-receptor antagonist; similar results were found for its other μ-opioid receptor activities (Shook et al. 1987a, 1986; Gulya et al. 1986). *In vivo,* CTP also was found to be a potent and selective inhibitor of a variety of actions mediated by μ-opioid receptor ligands such as morphine; by the highly μ-opioid receptor-selective agonists [D-Ala^2, N-$MePhe^4$, $Gly(ol)^s$]enkephalin (DAGO); and by [N-$MePhe^3$, D-Pro^4]morphiceptin (PLO17). For example, it was shown that CTP could block against morphine-induced analgesia and μ-opioid-mediated inhibition of transit and could precipitate withdrawal in morphine-dependent mice (Shook et al. 1987a, 1987b, 1987c, 1987d; Porreca et al. 1987; Wire et al. 1987; Ayers et al. 1988). Interestingly, it was found that, whereas CTP was a highly potent μ-opioid receptor antagonist, it was a weak agonist at the δ-opioid receptor. In addition, the *in vitro* and, especially, the *in vivo* studies uncovered bioactivities of CTP that were not opioid-like and, thus, were assumed to be due to some residual somatostatin-like activities. It was found later that appropriate modification of the structural features of CTP can eliminate this putative somatostatin-like activity. In particular, substitution of the Lys^5 by Arg^5 (but not Orn^5) gave the analog H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP)

(Pelton et al. 1986), which was found to have essentially none of the undesirable side actions of CTP (Kramer et al. 1989). Furthermore, CTAP was found to be more selective than CTP, not only compared with other opioid receptors but also, and especially, compared with the somatostatin receptor. Thus, we recommend CTAP or the D-Tic analog (see below) as the ligand of choice for a prolonged acting, potent, highly μ-oploid receptor-selective antagonist.

From the beginning of these studies we have tried to determine whether it is a valid, initial goat to utilize the ß-turn in somatostatin as a reasonably stable conformational template on which to build an opioid-receptor ligand. This problem has been addressed by extensive 2D NMR spectroscopy studies in conjunction with molecular mechanics and molecular dynamics calculations. Extensive NMR investigations of CTP and related analogs in which the L-Pen^{7} is replaced by D-Pen^{7} or L-Cys^{7} were made in aqueous and DMSO solutions. These studies (Pelton et al. 1988; Sugg et al. 1988; Kazmierski and Hruby 1988) led to several tentative conclusions.

1. The presence of a ß-turn for the sequence -Tyr-D-Trp-Lys-Thr- in the various μ-opioid receptor-selective ligands examined, although in some cases a γ-turn also would be compatible with the NMR data obtained. Strong support for this was seen especially in the nuclear Overhauser effect (NOE) pattern for most of these compounds; for example, the NOESY (NOE spectroscopy) spectrum of [D-Pgl^{1}]CTP (figure 4), is that expected of a molecule with a reverse-turn structure for the residues -Tyr-D-Trp-Lys-Thr- (this reverse-turn conformation also is retained when the Lys residue is replaced by Arg, Orn, and related amino acid residues).

2. The disulfide bridge appears to prefer a negative helicity as determined by NMR (Sugg et al. 1988). When a right-handed helicity was observed as in the D-Pen^{7} analog, the compound was found to have low potency at μ-opioid receptors (Kazmierski et al., unpublished results). These results suggest that interaction of the terminal Thr^{8}-NH_2 and D-Phe^{1} residues with the μ-opioid receptor requires a specific topographical orientation of these residues for strong interaction with the m-opioid receptor to occur.

3. The ß-turn conformation thus obtained obligated the placement of the 3-aromatic side-chain groups (D-Phe^{1}, Tyr^{3}, and D-Trp^{4}) to the same topographical face. The highly lipophilic surface thus obtained would appear to suggest that this arrangement is critical for interaction of these molecules with the μ-opioid receptor in the antagonist conformation of that receptor. These observations and model building studies led to the development of a new approach in the conformational design of peptide

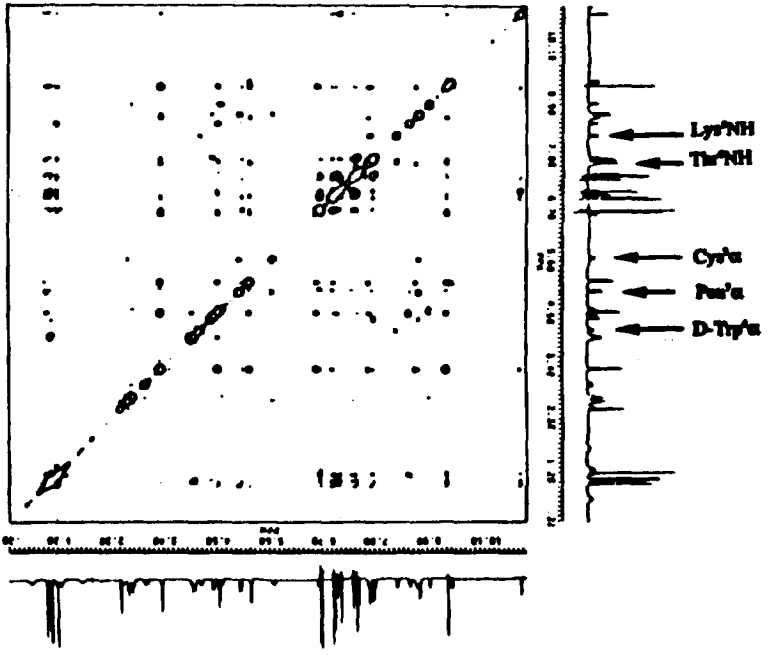

FIGURE 4. *The ^{1}H-^{1}H NOESY spectrum at 500 MHz of [D-Pgl^{4}]CTP in DMSO-d_6 solution*

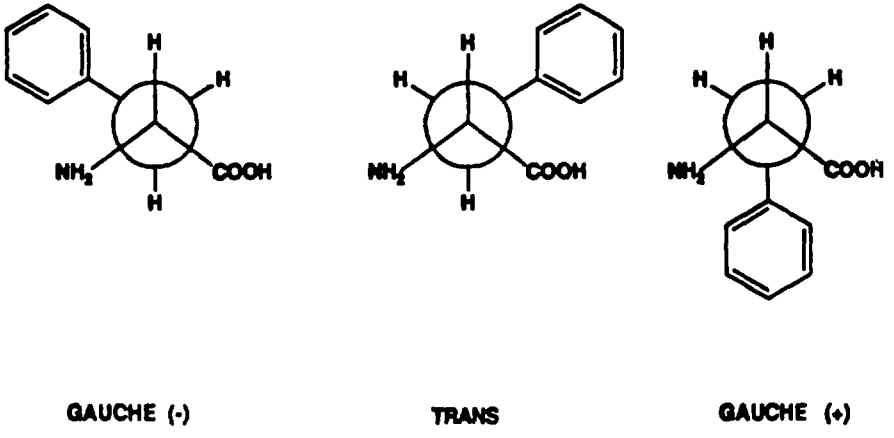

FIGURE 5. *Low-energy side-chain conformations of phenylalanine-gauche(-), trans, and gauche(+)*

ligands. This approach sought to establish the specific topographical properties of the side-chain groups within the context of the same conformational (backbone) template and the ways in which the side-chain conformations might specifically be constrained or biased to one specific side-chain topography (figure 5). Thus, peptide analogs with specific topographical features could be obtained.

DISCUSSION

The authors began this approach by carefully examining the topographical requirements for the D-Phe^1 residue in the CTP-related structures. An important consideration in this approach is the necessity to retain the specific backbone conformation of the original analogs so that any changes in biological activity as the result of a particular substitution will be due to different topographical structural features and *not* due to changes in the backbone conformation of the peptide. In this approach, it is critical that the basic assumption with regard to conformation be carefully checked. Thus, the investigations began by examining the use of the phenylalanine analogs D-phenylglycine (D-Pgl), D-N-methylphenylalanine (D-N-MePhe), and D-tetrahydroisoquinoline carboxylate (D-Tic) (figure 6) as replacements for the D-Phe^1 residue in CTAP or D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2(CTOP). It is expected that the N-methyl substitution on D-Phe will bias the side-chain conformation of D-Phe to the *trans* x_1 angle. On the other hand, D-Tic is so constrained by the cyclization of the N-methyl to the 2' carbon of the aromatic ring in D-Phe that it now can exist only in a gauche(-) or a gauche(+) conformation (figure 7). The D-phenylglycine residue, on the other hand, primarily would be expected to place the aromatic residue as close to the 20-membered disulfide-containing ring to maximize nonbonded van der Waals stabilization.

Preparation of analogs containing these various amino acids led to interesting and important results. In fact, the D-N-MePhe^1 and D-Pgl^1 analogs of CTP and CTOP, respectively, we found to be about 250 times less potent than their parent analogs at the μ-opioid receptor (Kazmierski and Hruby 1988; Kazmierski et al. 1988). Extensive 2D NMR investigations (Kazmierski et al. 1989, 1991) firmly establish that these analogs have the same overall backbone conformation; the only differences are their topographic structures. In both the D-N-MePhe^1 and the D-Pgl^1 analogs, the aromatic ring at position 1 is on the same surface and directed toward the Tyr^3 aromatic ring, whereas in the D-Tic^1 analog, the observed side-chain conformation [gauche(-)] placed the aromatic ring on the same face as the Tyr^3 aromatic ring, but a maximum distance apart (figure 8). These results further support the idea that a maximal separation of the D-Phe^1 and Tyr^3 side-chain groups favors interactions of these analogs with

the μ-opioid receptor in its antagonist conformation. These compounds are the most selective opioid-receptor ligands known and should be of great use in examining the physiological and pharmacological roles of μ-opioid receptors. Furthermore, these results establish the importance of the topographical properties of a peptide ligand for its interactions with its receptor(s). It is believed that the use of topographical considerations in conjunction with topographical constraints provides a new and highly important approach to the design of peptide hormone and neurotransmitter ligands. Several other amino acid derivatives and analogs are being examined that may be utilized for topographical design and constraint and for devising asymmetric syntheses to obtain these compounds. It is anticipated that important new ligands for a variety of hormone and neurotransmitter receptors will emerge from these studies.

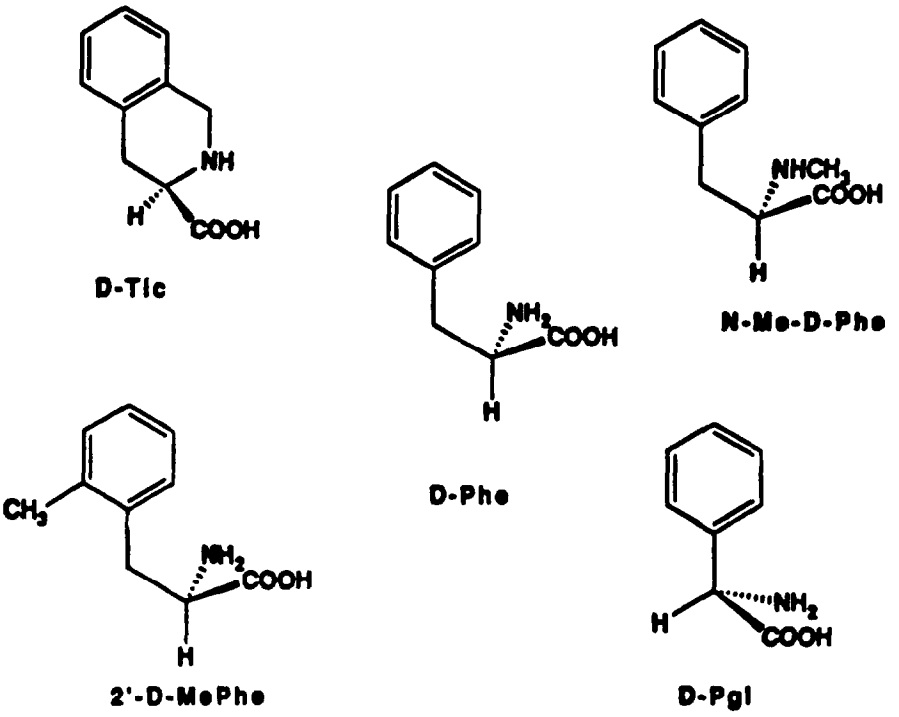

FIGURE 6. *Structures of D-phenylalanine analogs D-Tic, N-Me-D-Phe, D-Pgl, and D-2-MePhe*

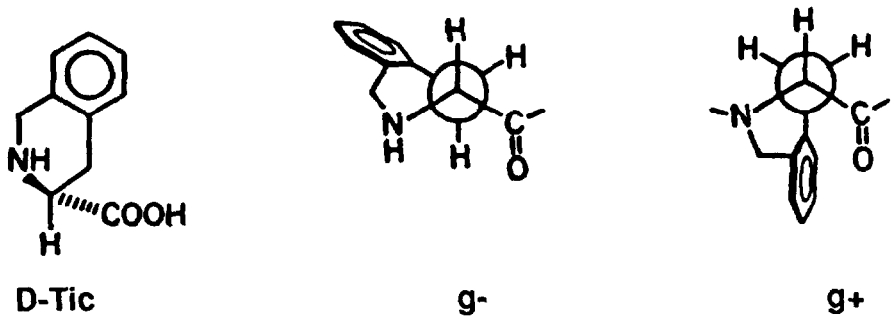

FIGURE 7. *Conformations possible for D-tetrahydroisoquinoline carboxylate (D-Tic)*

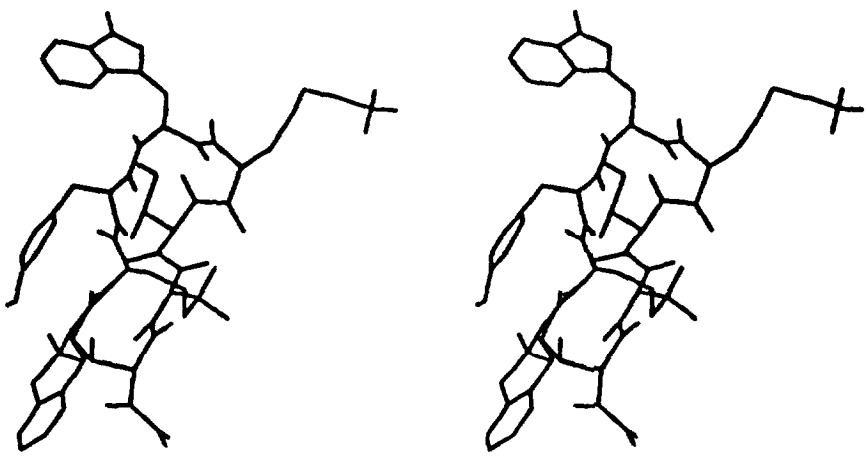

FIGURE 8. *Conformation (stereoview) of [D-Tic[1]]CTP (D-Tic-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2)*

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Peptidomimetic Opioids—Synthesis, Spectroscopy, and Computer Simulations

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INTRODUCTION

Over the past several years, the authors have synthesized many retro-inverso modified enkephalin analogs structurally related to Tyr-c(D-A_2bu-Gly-Phe-Leu], originally designed and synthesized by Schiller and associates (DiMaio et al. 1982). Results from the *in vitro* activities emphasize that the peptide bonds between residues Phe^4-Leu^5 and Leu^5-X^2 (side-chain) can be reversed with maintenance of high activity (Berman and Goodman 1984). The retro-inverso modifications at all the other positions greatly reduced the activities of the analogs in the guinea pig ileum (GPI) and the mouse vas deferens (MVD) assays.

The conformational analysis of four of the stereoisomeric enkephalin analogs, Tyr-c[D-A_2bu-Gly-Phe-(L and D)-Leu] and Tyr-c[D-A_2bu-Gly-gPhe-(S and R)-mLeu], was carried out by using nuclear magnetic resonance (NMR) and computational techniques employing flexible geometry energy minimization and molecular dynamic simulations (Mierke et al. 1987). The results obtained demonstrated that the orientation of the aromatic residues Tyr^1 and Phe^4 and the conformational flexibility of the 14-membered backbone ring are important factors that govern the selective activity of opioid peptides. It was found that, for a specific ligand to be recognized at the μ-opiate receptor, the molecule must adopt a conformation in which a large distance between the aromatic rings at positions 1 and 4 is maintained. The constrained 14-membered backbone rings in Tyr-c[D-A_2bu-Gly-Phe-Leu] and Tyr-c[D-A_2bu-Gly-gPhe-S-mLeu] stabilized by intramolecular hydrogen bonds help maintain this desired conformation. For activity at the μ- and δ-opiate receptors, as observed in Tyr-c[D-A_2bu-Gly-Phe-D-Leu] and Tyr-c[D-A_2bu-Gly-gPhe-R-mLeu], the molecules undergo greater conformational flexibility. This flexibility allows the peptide to adapt to various opioid receptor surfaces (μ, δ) (Goodman and Mierke 1987).

Other examples in the literature are in agreement with the authors' findings. The constrained 13-membered ring analog Tyr-c[D-Orn-Phe-Asp]-NH_2, synthesized by Schiller, turned out to be highly active and μ-receptor selective (Schiller et al. 1985; Schiller and Nguyen 1984). The high selectivity of this compound toward the μ-receptor was the result of its complete inactivity at the δ-opioid receptor. Conformational studies of this analog have been carried out in our laboratories (Mierke 1988). The NMR analysis revealed two intramolecular hydrogen bonds between D-Orn NH (side-chain)-D-Orn CO and Asp NH-D-Orn CO. The hydrogen bonds also were present for a large fraction of the molecular dynamic simulations. No significant backbone mobilities in this analog were detected from the dynamics.

The effect of varying the length of the side-chain at position 2 on opiate activity and selectivity also has been examined by altering the size of the backbone ring from 13 to 15 members by the substitution of different combinations of Asp, Glu, A_2bu, Orn, and Lys at positions 2 and 4. Complete loss of selectivity was observed in the less constrained 15-membered ring dermorphin analog Tyr-c[D-Lys-Phe-Glu]-NH_2 (Goodman and Mierke 1987; Schiller and Nguyen 1984). In the series of cyclic enkephaiins Tyr-c[D-Xxx-Gly-Phe-Leu], the selectivity for the μ-opioid receptor increased with the length of the side-chain of the second residue. The D-Lys containing analog was the most selective, followed by the D-Orn and D-A_2bu analogs (DiMaio et al. 1982). Increasing the size of the ring further to 18-membered, as shown in the synthesis of Tyr-c[D-Lys-Gly-Phe-Glu]-NH_2, yielded a highly potent but nonselective analog (Schiller et al. 1986). These findings indicated that the degree of flexibility of the backbone ring can have a major effect on the selective activity of the various analogs.

To characterize the effect of the phenylalanine residue at position 3 of dermorphins and at position 4 of enkephalins on biological activity, a series of 14-membered cyclic opiate analogs were synthesized that contain phenylalanine at positions 3 and 4 (figure 1). Four of these analogs, Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu] and Tyr-c[D-Glu-Phe-gPhe-(L and D)-rLeu], incorporate retro-inverso modifications between residues 4 and 5 and residues 5 and 2 (side-chain). Few substitutions in the third position of the linear enkephalin have been reported to date. Replacement of Gly with an Ala or Pro resulted in weakly active compounds (Morley 1980). Tetrapeptides containing phenylalanine residues in the 3 and 4 positions, however, have been reported. Among these, H-Tyr-D-Ala-Phe-Phe-NH_2 showed high preference for the μ-receptor over the δ-receptor (Schiller 1988). Compared to Leu-enkephalin, this compound was 75 times more potent in the GPI assay and 70 times less potent in the MVD assay.

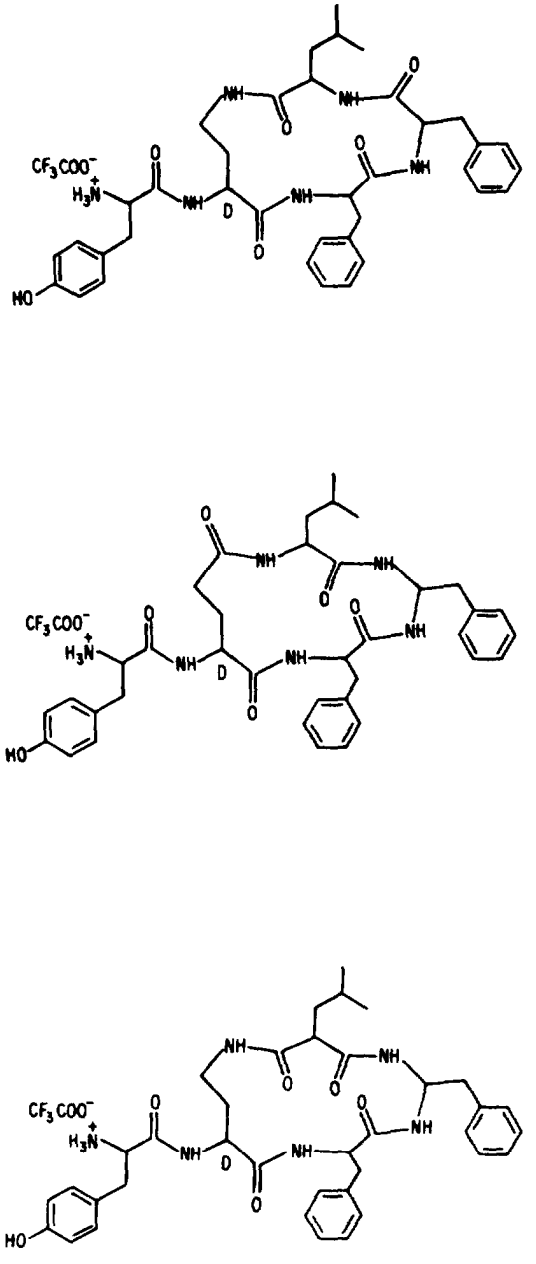


FIGURE 1. *Structure of a series of cyclic molecules related to dermorphin*

This chapter reports the synthesis, biological activities, and preliminary conformational analysis of the six cyclic dermorphin analogs completed to date.

SYNTHESIS

Solid-Phase Synthesis of Tyr-c[D-A_2bu-Phe-Phe-(L and D)-Leu]

The synthesis of the two cyclic parent dermorphin analogs was achieved by a combination of the solid-phase technique and peptide synthesis in solution. The partially protected linear peptides Z-Tyr(Bzl)-D-A_2bu-Phe-Phe-(L and D)-Leu-OH were assembled separately from the C-terminus on the p-alkyloxybenzyl alcohol resin, commonly known as the Wang resin (Wang and Merrifield 1969). The cleavage of the peptides from the resin was followed by cyclization in solution (figure 2).

All couplings were accomplished by preformed N-hydroxybenzotriazole (HOBt) active esters, obtained by allowing the appropriate protected amino acids to react with dicyclohexylcarbodiimide (DCC) and HOBt in CH_2Cl_2 for 30 minutes. After the removal of the insoluble N,N'-dicyclohexylurea (DCU), the filtrate containing the activated form of the urethane protected amino acid derivative was added directly to the reaction vessel. Except for the Tyr residue, the 9-fluorenylmethyloxycarbonyl (Fmoc) group was used for the N^a-protection of all amino acids. Each coupling step gave quantitative yields as measured by spectrophotometric Fmoc-amino acyl resin analysis (Kaiser et al. 1970; Stewart and Young 1984).

To prevent branching in the growing peptide chain the amino group of the α,γ-D-diaminobutyric acid (D-A_2bu) side-chain also was protected with the acid labile tert-butyloxycarbonyl (Boc) group. This protecting group was expected to remain in place during the chain elongation process and to resist the reagent used for the deprotection of α-amino Fmoc groups.

The cleavage of the peptide from the resin and the removal of the side-chain Boc protecting group of the D-A_2bu residue was achieved in one step using a mixture of trifluoroacetic acid (TFA) in CH_2Cl_2 solution in the presence of anisole and thioanisole scavengers for tert-butyl carbocation, which could lead to undesired alkylation of the tyrosine ring.

After recrystallization, the partially protected linear pentapeptides were cyclized using diphenylphosphoryi azide (DPPA) in dimethylformamide (DMF) to generate the fully protected products N^a-(Z)-Tyr(Bzl)-c[D-A_2bu-Phe-Phe-(L and D)-Leu] (Brady et al. 1983). At this stage, the peptides were purified on a mixed bed ion exchange resin to remove any unreacted starting materials or any

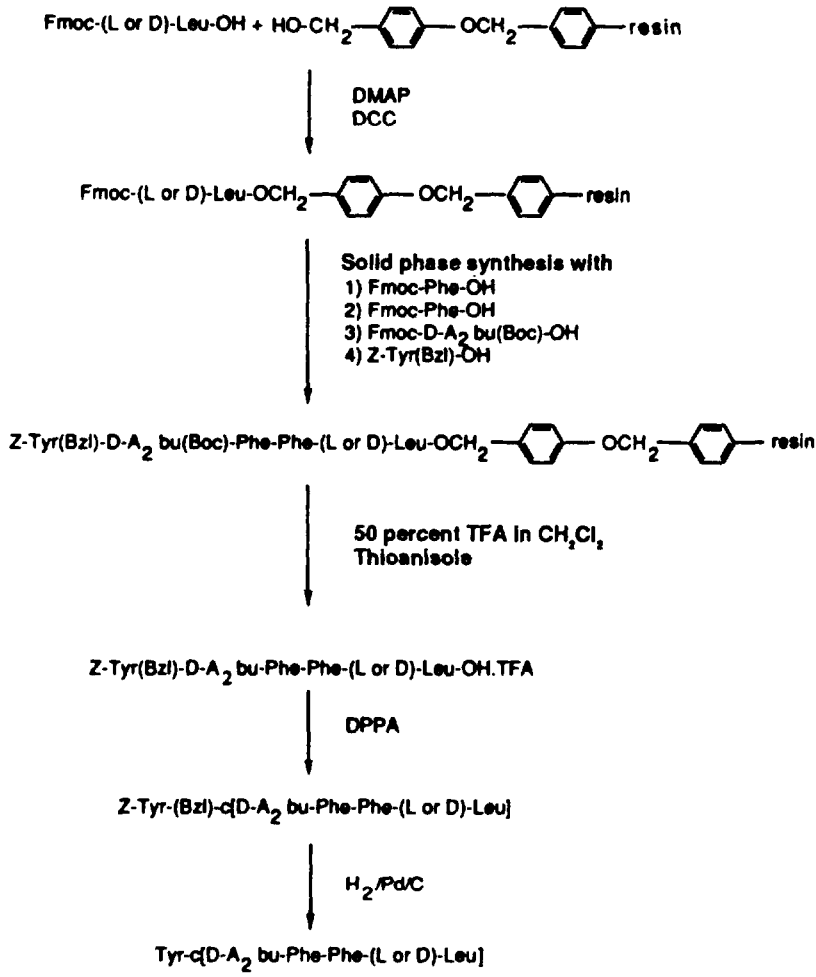


FIGURE 2. *Solid synthesis of parent dermorphin analogs Tyr-c[D-A_2bu-Phe-Phe-(L and D)-Leu]*

undesired dimers formed during the cyclization step. The homogeneous white powders obtained in both cases were subjected to catalytic hydrogenation in the presence of Pd/C in methanol/acetic acid mixture to yield the crude final products. Each peptide was subjected to reverse-phase (RP)/high-pressure liquid chromatography (HPLC) purification in a mixture of acetonitrile/water/TFA using isocratic conditions.

Synthesis of Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]

A representative synthetic scheme for the preparation of the diastereomeric pairs Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu] carried out in solution is shown in figure 3. The synthesis of the Tyr-c[D-Glu-Phe-gPhe-(L and D)-rLeu] follows a similar route and therefore is not included. The gem-diaminoalkyl residue Boc-Phe-gPhe-H•TFA was obtained by the conversion of dipeptide carboxamide Boc-Phe-Phe-NH_2 to the corresponding *gem*-diaminoalkyl dipeptide salt using bis[(trifluoroacetoxy)iodo]benzene (IBTFA). This rearrangement is known to proceed without racemization (Pallai et al. 1983; Pallai and Goodman 1982).

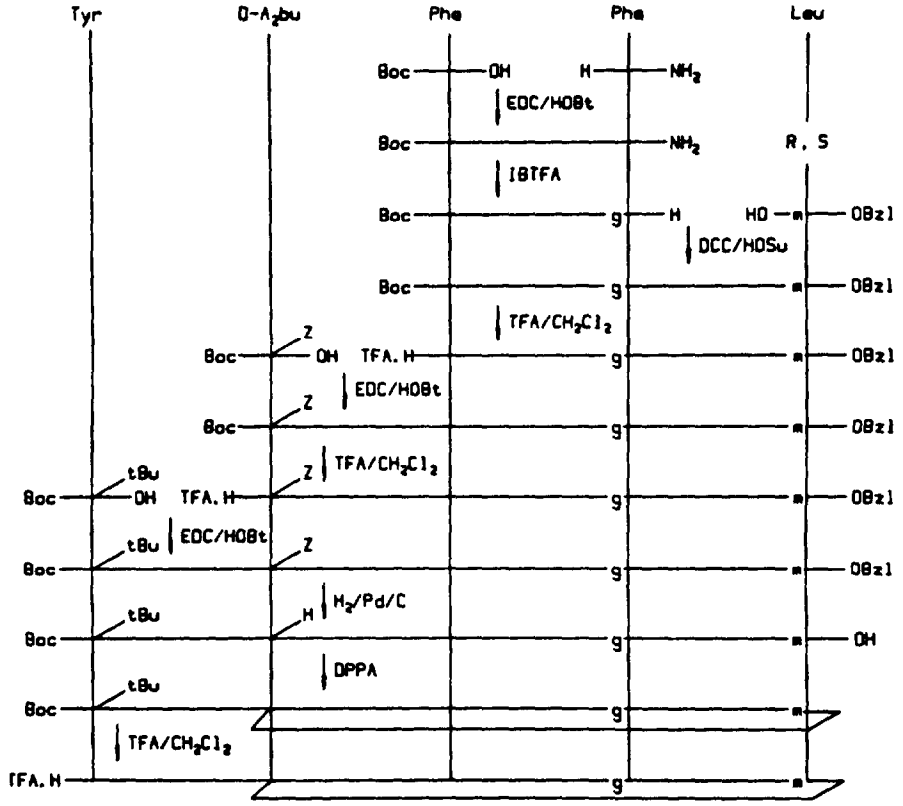


FIGURE 3. *Synthesis of retro-inverso analogs Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]*

The commercially available 2-isobutylmalonic acid diethyl ester was converted to R,S-isobutylmalonic acid monobenzyl ester in one step involving saponification and transesterification. The R,S-isobutylmalonic acid was incorporated into the peptide chain as a racemate, thereby generating a pair of diastereomers.

Stepwise elongation of the peptide chain led to the fully protected pentapeptide N^a-Boc-Tyr(tBu)-N^y(Z)-D-A_2bu-Phe-gPhe-R,S-mLeu-OBzl. Catalytic hydrogenation of this compound simultaneously removed the α-benzyl ester and the N^y-benzyloxycarbonyl (Z) protecting groups to yield the partially protected zwitterionic pentapeptide N^a-Boc-Tyr(tBu)-D-A_2bu-Phe-gPhe-R,S-mLeu-OH. Activation of the malonyl leucine carboxyl group was chosen for ring closure, because the attacking amine, from the side-chain of D-A_2bu was a good nucleophile. In addition, no chirality concerns are involved with the amide formation.

Cyclization was achieved using DPPA as described above. A second technique using the BOP reagent also was employed (Castro et al. 1975; Le-Nguyen et al. 1981). In this case, the linear peptide Boc-Tyr(tBu)-D-A_2bu-Phe-gPhe-(R and S)-mLeu-OH was dissolved in DMF in the presence of diisopropylethylamine and was stirred for 1 day at room temperature. The cyclic protected peptide obtained from each route, Boc-Tyr(tBu)-c[D-A_2bu-Phe-gPhe-(R and S)-mLeu], was purified by treatment with a mixed bed ion exchange resin to remove any starting materials or dimeric linear peptides formed during the cyclization step.

The final diastereomeric mixture was obtained by acidolytic cleavage of the protecting groups on the tyrosine in the presence of anisole and thioanisole, scavengers used to trap the tert-butyl carbocation. It was possible to separate the diastereomeric mixture by RP-HPLC, as shown in figure 4. The chirality at the maionyl leucine center was assigned by carrying out two-dimensional (2D) NMR techniques.

BIOLOGICAL ACTIVITY

All the *in vitro* biological activities were measured in Schiller's laboratories. The observed in vitro biological activities of the dermorphin analogs measured against the GPI and the MVD assays are shown in table 1 along with biological activities of selected enkephaiin analogs for comparison.

Column: VYDAC C18 10μ (1.0x25 cm)

Eluent: 68 percent A (95 percent H_2O, 5 percent CH_3CN, 0.1 percent TFA)
32 percent B (95 percent CH_3CN, 5 percent H_2O, 0.1 percent TFA)

Flow rate: 4 mL/min

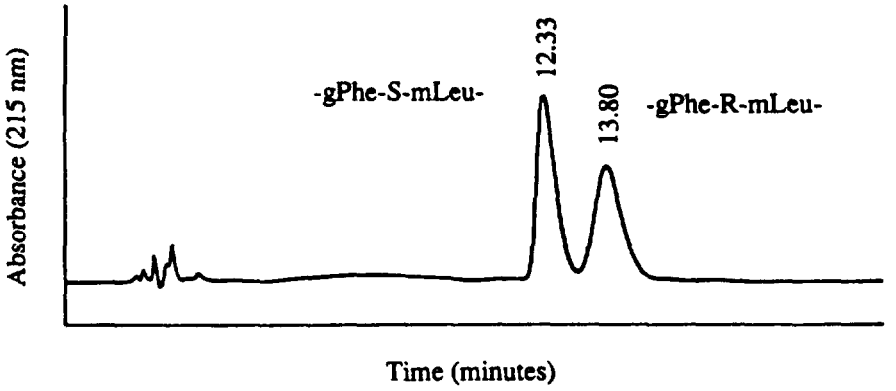


FIGURE 4. *RP-HPLC profile of the final diastereomeric mixture of Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]*

As can be seen in table 1, all the dermorphin analogs were effective at inhibiting the electrically induced contractions in the GPI and MVD assays. All the modified analogs are more potent (relative to Leu^5-enkephalin) in the GPI assay. With the exception of the two analogs Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] and Tyr-c[D-Glu-Phe-gPhe-D-rLeu], which display reduced activity at the δ-opioid receptor, the remaining analogs display equal or higher potencies in the MVD bioassay than does Leu^5-enkephalin. The greatly reduced activity of Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] provides important information about the requirement for selective opiate receptor interactions and is discussed below in conjunction with the results obtained from computer simulations.

All the dermorphin analogs display higher potencies in the GPI and the MVD bioassays than do the enkephalin analogs incorporating a Gly residue at position 3, although the activity profiles of the two analogs Tyr-c[D-A_2bu-Gly-gPhe-S-mLeu] and Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu] are similar. The reason for the increased potencies of the dermorphin analogs containing a Phe at position 3 is not well understood. It can be speculated that the hydrophobic

TABLE 1. *GPI and MVD assays of dermorphin analog in comparison with analogous enkephalin analogs*[a]

Compound	GPI IC_{50}[nM]	MVD IC_{50}[nM]	MVD/GPI IC_{50}-ratio
Tyr-c[D-A_2bu-Phe-Phe-Leu]	1.08 ± 0.10	4.70 ± 0.92	4.35
Tyr-c[D-A_2bu-Phe-Phe-D-Leu]	1.14 ± 0.12	13.9 ± 0.9	12.2
Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu]	0.518 ± 0.099	6.30 ± 1.11	12.2
Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu]	17.6 ± 2.8	117 ± 31	10.1
Tyr-c[D-Glu-Phe-gPhe-rLeu]	39.0 ± 9.3	3.64 ± 0.10	0.09
Tyr-c[D-Glu-Phe-gPhe-rDLeu]	2.75 ± 0.63	49.1 ± 9.9	17.9
Tyr-c[D-A_2bu-Gly-Phe-Leu]	14.1 ± 2.9	81.4 ± 5.8	5.77
Tyr-c[D-A_2bu-Gly-Phe-D-Leu]	66.1 ± 8.4	27.1 ± 1.6	0.409
Tyr-c[D-A_2bu-Gly-gPhe-S-mLeu]	1.51 ± 0.19	7.76 ± 3.17	5.14
Tyr-c[D-A_2bu-Gly-gPhe-R-mLeu]	25.5 ± 2.0	14.9 ± 5.0	0.584
Tyr-c[D-Glu-Gly-gPhe-rDLeu]	19.4 ± 3.4	313 ± 102	16.1
[Leu^5]-Enk	246 ± 39	11.4 ± 1.1	0.0463

[a]Mean of three determinations±SEM.

side-chain of the Phe^3 interacts favorably with a hydrophobic site at the opiate receptor surfaces. The preferred interaction of the opiate receptor with the hydrophobic side-chain of the Phe at position 3 may result in higher ligand-receptor recognition and, consequently, lead to the increased potencies observed.

All the modified dermorphin analogs were found to be highly resistant to proteolytic degradation by rat brain membrane preparations measured in our laboratories (table 2), which rapidly hydrolyzes Leu^5-enkephalin ($t_{½}$ 7 minutes). With the exception of Tyr-c[D-A_2bu-Phe-Phe-(L and D)-Leu], all the modified analogs show no appreciable degradation and prove more enzyme resistant than the enzyme-stable [D-Ala^2-Leu^5]-enkephalinamide.

These results are in full agreement with the authors' previous results obtained with the retro-inverso modified analogs containing a Gly at position 3. It was demonstrated that application of the partial retro-inverso modifications to dermorphins gives rise to opioid peptides that exhibit higher potency and selectivity than their corresponding enkephalin counterparts.

TABLE 2. *Half-lives determined from degradation of peptides against rat brain homogenate*

Analog	$Time_{½}$ (min)
Tyr-c[D-A_2bu-Phe-Phe-Leu]	35
Tyr-c[D-A_2bu-Phe-Phe-D-Leu]	53
Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu]	227
Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu]	265
Tyrc[D-Glu-Phe-gPhe-rLeu]	137
Tyr-c[D-Glu-Phe-gPhe-D-rLeu]	338
[Leu^5]-Enk	7
[D-Ala^2-Leu^5]-Enkephalinamide	62

CONFORMATIONAL ANALYSIS

It is clearly important to understand the conformational consequences of the phenylalanine substitution in partial retro-inverso modified analogs and, ultimately, to relate these to the pharmacological profile of the analogs. The conformational characterization of the molecules was carried out by ^{1}H NMR and nuclear Overhauser effect (NOE)-restrained molecular dynamics and flexible geometry energy minimizations. The proton resonances were assigned using 2D COSY and HOHAHA techniques. The NOEs were measured in the rotating frame (ROESY experiments) at various mixing times, from 75 to 500 ms. The computer simulations were carried out at the San Diego Supercomputer Center using the DISCOVER program.

Nuclear Magnetic Resonance

The temperature coefficients obtained for all six cyclic analogs are shown in table 3. The NOEs obtained from the ROESY experiments are shown in figure 5 for compounds Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]. These NOEs were useful in unambiguously assigning the chirality at the malonyl leucine center (as described below).

Temperature coefficients obtained from one of the two diastereomeric pairs, Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu], show one intramolecular hydrogen bond involving the D-A_2bu side-chain NH. The acceptor of the hydrogen bond was deduced from the NOEs. The amide proton of the D-A_2bu side-chain is directed

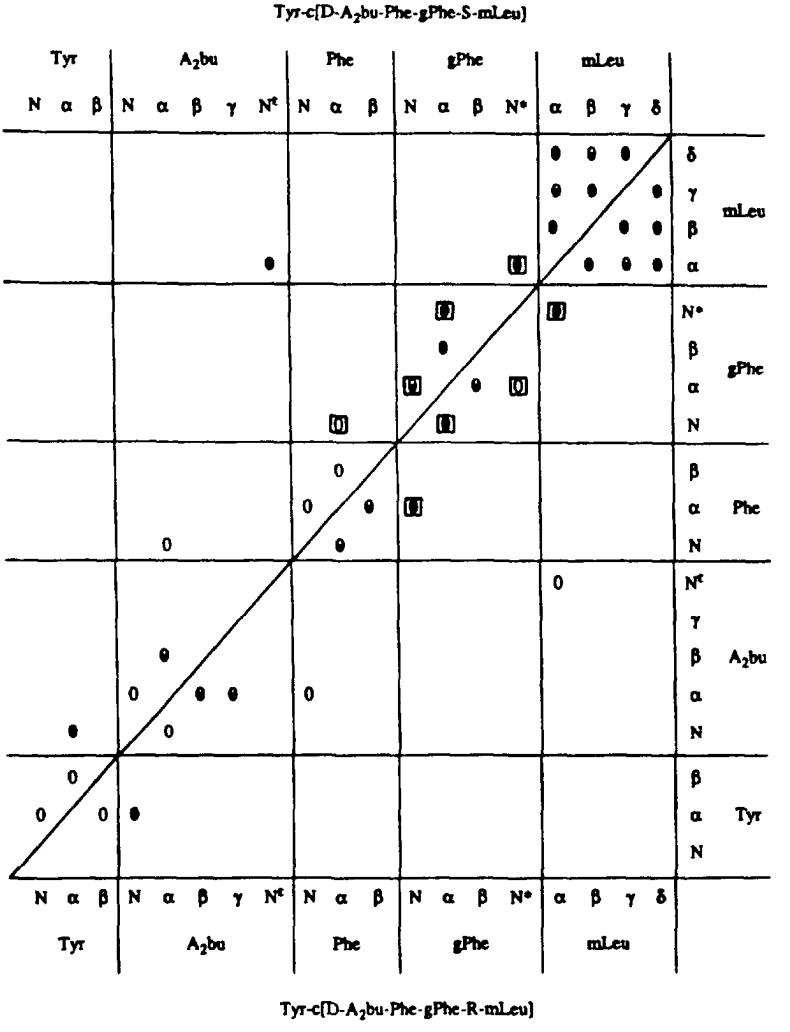
TABLE 3. *Temperature coefficients ($\Delta\delta/\Delta T$ in -ppb/K) of amide protons of cyclic dermorphin analogs containing intramolecular hydrogen bonds*

Amide Protons	[L-Leu^5]	[D-Leu^5]	[S-mLeu^5]	[R-mLeu^5]	[L-rLeu^5]	[D-rLeu^5]
D-A_2bu	3.1	3.3	3.6	2.6	—	—
D-A_2bu (side-chain)	1.5	3.5	3.3	-0.6	—	—
D-Glu	—	—	—	—	-0.2	3.9
Phe^3	4.4	2.9	3.3	3.2	3.4	8.7
Phe^4	4.3	5.3	—	—	—	—
gPhe^4 (normal)*	—	—	5.1	7.1	3.3	5.4
gPhe^4 (new)*	—	—	2.8	7.1	4.1	1.9
Leu	-0.3	1.1	—	—	4.1	3.4

* The amino precursors of the normal and the new amide protons are the α-amino group present in Phe and the amino group generated from the Hofmann rearrangement, respectively.

toward the ring, forming a 7-membered hydrogen bond with the D-A_2bu CO and accounting for a relatively small temperature coefficient of the D-A_2bu NH (side-chain) amide proton. The chirality at the fifth position was assigned in the following way: The analog Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu], which lacks intramolecular hydrogen bonds, shows a weak NOE between Phe NH and Phe C^{a}H and a weak NOE between Phe C^{a}H and gPhe NH. For these NOEs to be fulfilled, the Phe NH and gPhe NH must be projecting downward with respect to the plane of the ring. Medium NOEs between gPhe NH, gPhe C^{a}H and a strong NOE between gPhe C^{a}H-gPhe N*H require that the gPhe N*H and gPhe C^{a}H be pointing downward (figure 6). The strong NOE between gPhe N*H-mLeu C^{a}H protons is possible if the residue at position 5 has an S configuration,

The hydrogen bond between the D-A_2bu CO and the D-A_2bu NH (side-chain) in Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] requires that the D-A_2bu CO be directed in the plane of the ring. The Phe NH, which should be trans to the D-A_2bu CO, must project out of the plane of the ring. A medium NOE between Phe NH-Phe C^{a}H, a strong NOE between Phe C^{a}H-gPhe NH, and a strong NOE between gPhe NH-gPhe C^{a}H are consistent with a geometry in which the Phe C^{a}H, the gPhe NH, and the gPhe C^{a}H are pointing downward with respect to the plane of the 14-membered ring. A weak NOE between gPhe C^{a}H and gPhe N*H requires that the N*H be projecting upward, as shown in figure 6. A strong NOE between gPhe N*H and mLeu C^{a}H at this position requires an R configuration at the mLeu C^{a}H center.



Strong ● Medium ◐ Weak 0

FIGURE 5. *NOE data obtained for Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]*

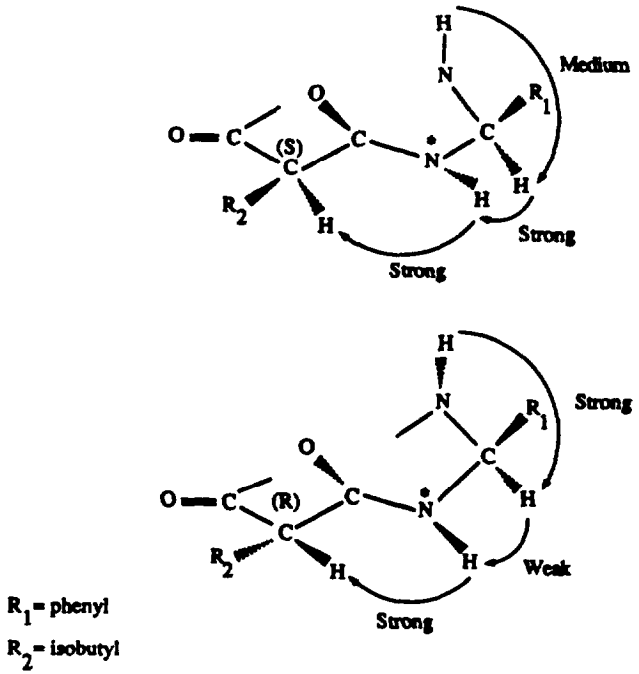


FIGURE 6. *Configurational assignment of the malonyl leucyl center in Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu] (top) and Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] (bottom)*

Observation of Cis-Trans Isomerization In the Dermorphin Analogs

Multiple resonances were observed in the proton spectra of two of the six compounds shown in figure 1. The Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] consists of predominantly *trans* conformer and two minor *cis* conformers accounting for 21 and 13 percent, respectively. The analog Tyr-c[D-Glu-Phe-gPhe-D-retro-Leu] is composed of only 28 percent of all *trans* structure, with two *cis* amide containing isomers accounting for 51 and 21 percent, respectively. We have been able to show with ROESY experiments that the additional resonances arise from *cis/trans* isomerization about unsubstituted amide linkages. Using one-dimensional saturation transfer techniques, the rate of isomerization was measured at various temperatures (Forsen and Hoffman 1964; Grathwohl and Wüthrich 1981; Sanders and Mersh 1982). The results are shown in table 4.

The rates of interconversion are similar to the values reported by Grathwohl and Wüthrich (1981) for proline containing linear peptide sequences, which may be indicative of similar energy barriers for *cis/trans* transitions.

TABLE 4. *Rate constants and free energies of activation for the cis/trans isomerization measured in DMSO at 30°C*

Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu]
Tyr-c[D-Glu-Phe-gPhe-D-rLeu]

Analog	*cis* Bond	*cis* Fraction	Rate (sec^{-1}) c→t	Rate (sec^{-1}) t→c	DG (kJ mol^{-1}) c→t	DG (kJ mol^{-1}) t→c
Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu]	Phe-gPhe	0.21	2.0±0.1	1.6±0.1	72	74
	mLeu-D-A_2bu	0.13	—	—	—	—
Tyr-c[D-Glu-Phe-gPhe-D-rLeu]	D-Glu-Phe	0.51	1.6±0.2	1.4±0.2	74	72
	Phe-gPhe	0.21	—	—	—	—

The assignment of the resonances was carried out with HOHAHA and ROESY experiments (Davis and Bax 1985; Bothner-By et al. 1984; Bax and Davis 1985). From the ROESY spectra, chemical exchange between the different sets of resonances was observed (Mierke et al. 1989). The two *cis* isomers of Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] contain *cis* amide bonds between Phe-gPhe and mleu-D-A_2bu side-chain, accounting for 21 and 13 percent of the population, respectively. The major isomer of Tyr-c[D-Glu-Phe-gPhe-D-retroLeu], accounting for 51 percent of the population, has a *cis* amide linkage about the D-Glu-Phe amide bond. The other *cis* isomer, accounting for 21 percent, has a *cis* arrangement between Phe-gPhe.

The observation of *cis* and *trans* isomers within the series is unexpected. The results from the NMR analysis of the series of enkephalin analogs with a glycine at the third position, Tyr-c[D-A_2bu-Gly-gPhe-(R and S)-mLeu and Tyr-c[D-Glu-Gly-gPhe-rDLeu], containing analogous retro-inverso modifications have been reported (Mammi and Goodman 1986). There was no evidence of multiple resonances or configurational isomers. It therefore must be concluded that the additional constraint from the replacement of phenylalanine is the *source* of the observed configurational isomers.

Computer Simulations

The molecular dynamic simulations of five of the six dermorphin analogs have been completed. These simulations were carried out for 20 picoseconds with the measured NOEs as restraints. A superposition of the structures obtained from the molecular dynamic simulations for the molecules under consideration provided qualitative information about the mobility of the side-chains and backbone in each peptide. These structures are shown in figures 7, 8, and 9. These results indicate that the mobilities of the side-chains of Tyr^1, Phe^3, and Phe^4 are similar in all compounds, with the Tyr side-chain exhibiting the greatest degree of freedom. With the exception of Tyr-c[D-A_2bu-Gly-gPhe-R-mLeu], which displays superactivity at the μ-receptor only, the remaining molecules are superactive at the μ- and the δ-opioid receptors. All the molecules that display activity at both opioid receptors contain flexible backbones. The Tyr-c[D-A_2bu-Gly-gPhe-R-mLeu] analog consisting of two *cis* conformers and one *trans* conformer has a more constrained backbone and displays activity at the μ-receptor only.

The conformational analysis of the dermorphin analogs presented here indicate that the interaction between the side-chains of Tyr^1 and Leu^5 or $mLeu^5$ residues may be important to elicit a proper biological response at the δ-opiate receptor. A strong hydrophobic interaction between the side-chains of Tyr^1 and Leu^5 and Tyr^1 and $mLeu^5$ in Tyr-c[D-A_2bu-Phe-Phe-Leu], Tyr-c[D-A_2bu-Phe-Phe-D-Leu], Tyr-c[D-A_2bu-Phe-gPhe-S-mLeu], and Tyr-c[D-Glu-Phe-gPhe-rLeu] dominate the structures obtained from molecular dynamic simulations and is believed to be important for ligand-receptor interactions at the δ-opiate receptor. On the other hand, the two analogs Tyr-c[D-A_2bu-Phe-gPhe-R-mL/eu] and Tyr-c[D-Glu-Phe-gPhe-D-rLeu] display superactivity at the μ-opioid receptor. The conformational analysis of Tyr-c[D-A_2bu-Phe-gPhe-R-mLeu] carried out to date indicates that there is no interaction between the Tyr^1 and the $mLeu^5$ side-chains.

Although all the 14-membered ring dermorphin analogs in this study are more active than their 14-membered enkephalin counterparts, the exact role of the Phe side-chain at position 3 is not well understood. Future research in the authors' laboratories is aimed at unraveling the mysterious role of the Phe at position 3 on biological activity in the cyclic dermorphin series of analogs. Toward this goal, cyclic enkephalin and dermorphin analogs are being synthesized, incorporating different substitutions at the third position. Some of these target molecules will incorporate a D-Phe at position 3 to study the chirality effect at position 3 on biological activity. Others will incorporate nonaromatic hydrophobic residues such as Ala and cyclohexylalanine at position 3 to understand which of the two interactions—hydrophobic or

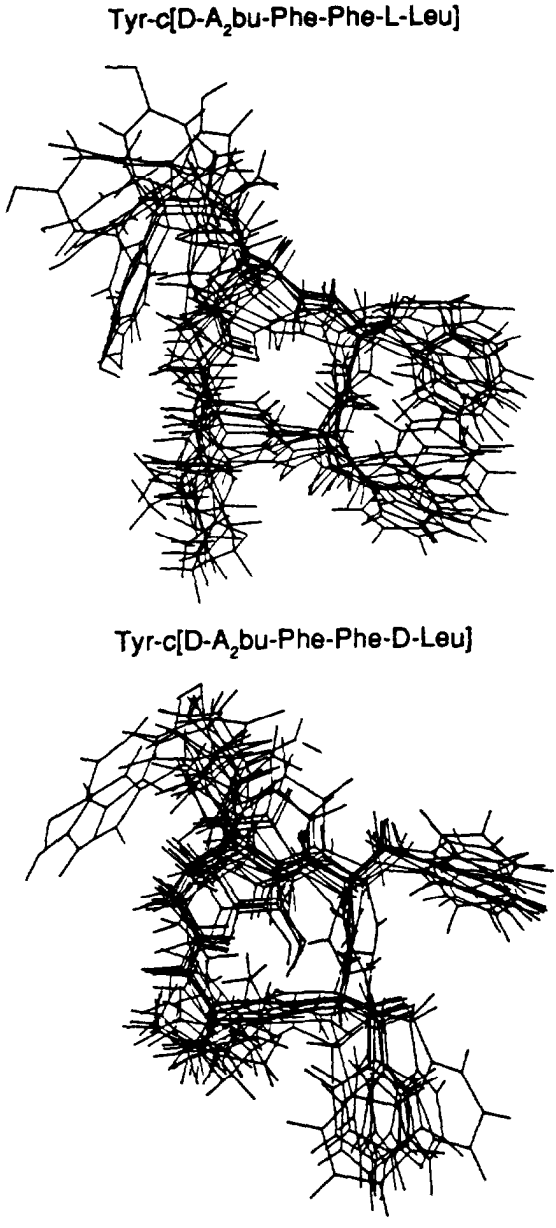


FIGURE 7. *Superposition of structures obtained from the molecular dynamic simulations of Tyr-c[D-A_2bu-Phe-gPhe-(L and D)-Leu]*

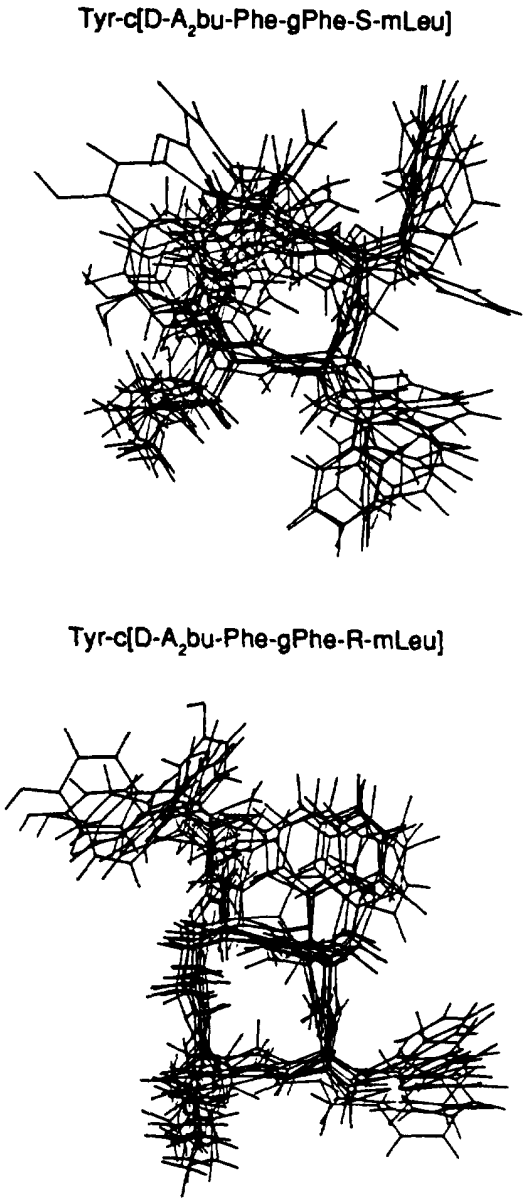


FIGURE 8. *Superposition of structures obtained from the molecular dynamic simulations of Tyr-c[D-A_2bu-Phe-gPhe-(S and R)-mLeu]*

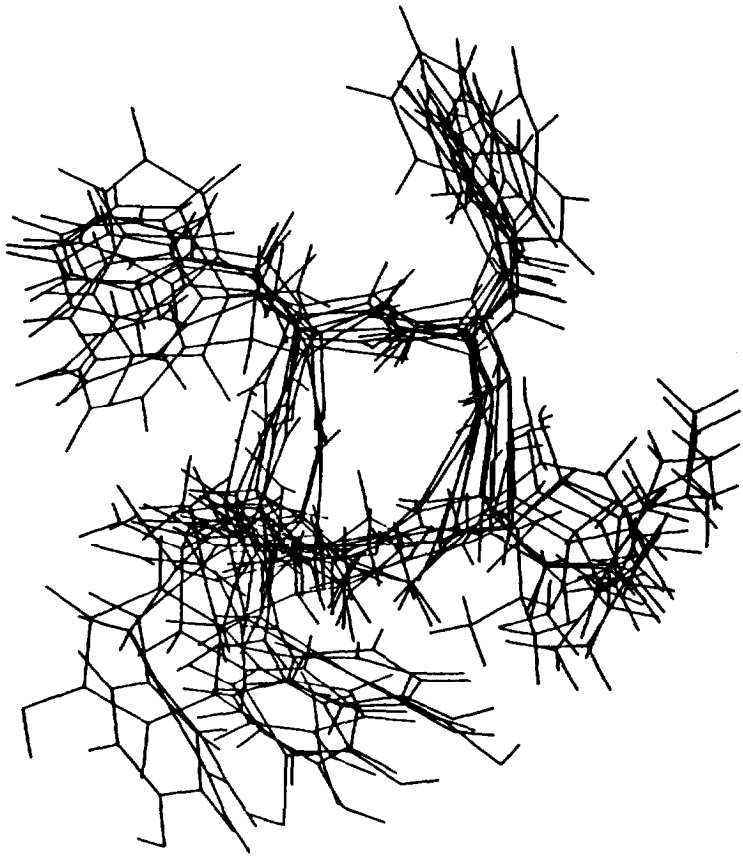


FIGURE 9. *Superposition of structures obtained from the molecular dynamic simulations of Tyr-c[D-Glu-Phe-gPhe-L-Reu]*

electronic—have a greater influence on potency. Novel analogs also will be synthesized that will incorporate side-chain modifications at position 5 to alter the nature of the hydrophobic interactions between Tyr^1 and Leu^5, believed to be necessary for recognition and activity at the δ-opiate receptor.

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CANADA

Modified Solid-Phase Methods for the Rapid Synthesis of Opioid Peptides

Richard A. Houghten, John M. Ostresh, and Suzanne M. Pratt

INTRODUCTION

Since the discovery of endogenous peptides having opiate activity in 1975 by Hughes and Kosterlitz (Hughes et al. 1975) the opioid peptide system has been shown to involve at least a dozen different naturally occurring peptide sequences (Holaday et al. 1987). The resulting investigations have involved not only the preparation of these naturally occurring peptides but also, in almost all cases, the tritiated and ^{125}I-labeled analogs as well. In the course of investigations, the natural opioid peptides have been compared and contrasted to at least a thousand different analogs, using *in vivo* and *in vitro* assays. These have been directed toward the development of analogs having increased potency, length of action, resistance to degradation, and selectivity toward receptor subclasses.

The continually escalating need for synthetic peptides in the neurosciences has far exceeded the supply available to most laboratories. This is also true in molecular biology and immunology in which synthetic peptides are being used for vaccine development (Lerner 1982; Bittle et al. 1982; Wilson et al. 1984; Geysen et al. 1984) and for the detailed study of peptide antigen-antibody interactions (Appel et al. 1990), for the determination of antigens having clinical diagnostic utility (Chen et al. 1984), and for the mapping of protein products of brain-specific genes (Sutcliffe et al. 1983). Researchers in pharmacology, biochemistry, and biology also need numerous peptides in the study of biologically active peptides (Yamashiro and Li 1984) and the investigation of peptide and protein conformation (Hruby 1984) and folding (Shoemaker et al. 1985). In the majority of these studies, in which hundreds to thousands of peptides are needed, the limiting factor has remained the availability and cost of the desired peptides in the necessary amounts and purity. It is possible to produce large numbers of peptides (i.e., hundreds) by conventional manual and automated methods, but the effort is so time-consuming and costly that to carry out this number of syntheses is normally impractical, even in especially well-equipped laboratories. In smaller research facilities or individual laboratories,

the preparation of this number of peptides is virtually impossible. Straightforward synthesis methodologies, which now enable the preparation of large numbers of specific synthetic peptides in milligram to multigram quantities more efficiently and in a much shorter timeframe, will aid all fields utilizing peptides.

MATERIALS AND METHODS

Protected N·α-Boc amino acids were purchased from BACHEM (Torrance, CA). The accuracy of quality control sheets for each amino acid concerning melting point, optical purity, and thin-layer chromatographic behavior was confirmed for each lot received. Major solvents ($CHC1_2$, DMF, and isopropanol) were purchased in bulk (50-gallon drums) from Fisher Scientific. For the past 5 years, these reagent-grade quality solvents have proved acceptable in the author's laboratory. Trifluoracetic acid was purchased from Halocarbon in 5-gallon lots—a quantity that was cost-effective and safe to handle. All resins were purchased from BACHEM. The 4-(oxymethyl)phenylacetamidomethyl resins (Pam resins) were significantly more costly than benzyl ester resins but, in many instances, yielded a higher quality peptide. All peptides needing a C-terminal carboxyl were prepared using Pam resins. C-terminal amides were prepared using p-methyl benzhydrylamine resin.

The technique of simultaneous multiple peptide synthesis (SMPS) (Houghten 1985; Houghten et al. 1968) was used throughout this work for small-scale (50 to 100 mg) and large-scale (50 g) synthesis and is described below. The differences between a standard solid-phase protocol and one involving SMPS, as well as an illustration of a compartmentalized resin packet used in SMPS, are shown in figures 1, 2, and 3.

Standard t-Boc (tertiary butoxycarbonyl) amino acid resin (50 to 100 mg, 100 to 200 mesh, 0.2 to 0.8 meq/g) was contained in tared polypropylene mesh packets (74 u), having approximate dimensions of 20x20 mm. These dimensions were selected after trying various resin sizes (20 to 400 mesh) and mesh materials (polyethylene, teflon, nylon, etc.). Although the example described below uses 100 mg of resin, it was found that at least 5.0 g of resin can be readily accommodated by adjusting the dimensions of the packet size. After placing a number at the top of each unsealed bag with a black graphite-based marking pen, the packet was dosed and the number permanently sealed into the polypropylene, giving an easily readable label for each bag (figure 2). With the appropriate preliminary sieving, no resin was lost from the packet. The resin-filled packets have excellent mechanical stability, and even with violent shaking or stirring, the resin beads are not broken. These resin packets can be used for SMPS—that is, syntheses in which many different peptides are

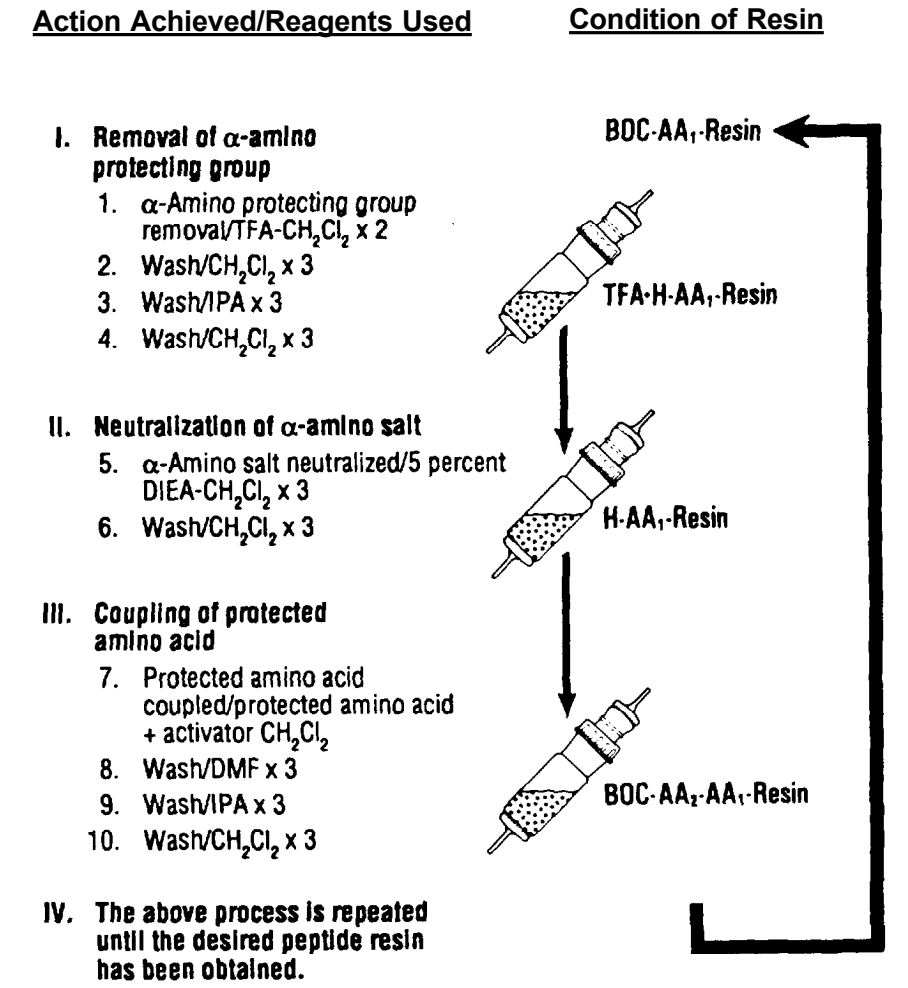

FIGURE 1. *Solid-phase peptide synthesis*

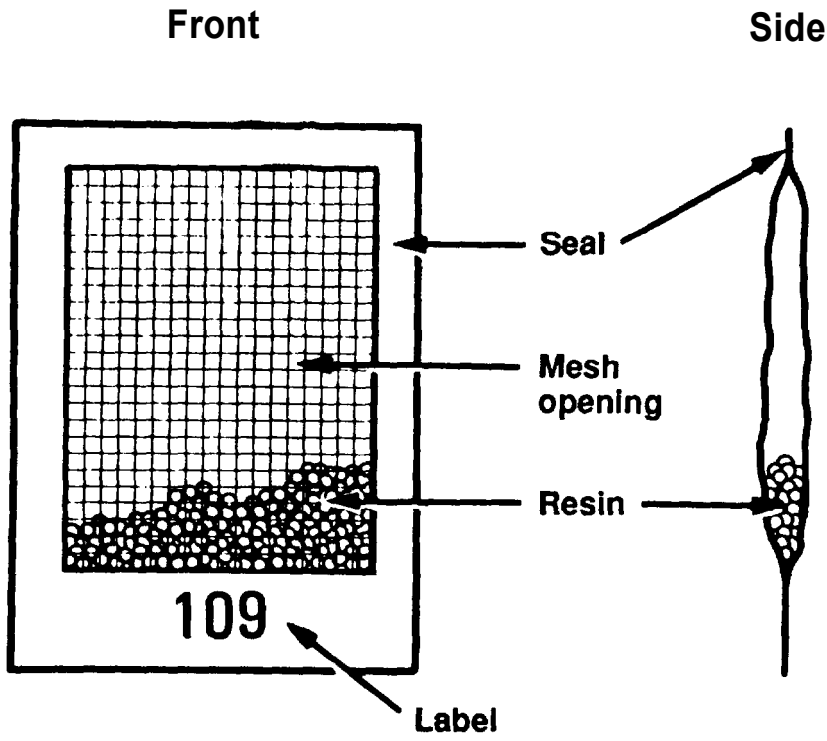

FIGURE 2. *Resin packet*

produced concurrently—or multiple analog peptide synthesis—syntheses in which many analogs of a particular peptide are produced concurrently. Standard deprotecting, neutralization, coupling, and wash protocols used in this laboratory are variations of Merrifield's original solid-phase procedures (1963).

Using completely manual methods, from 1 to as many as 400 individual packets containing the desired starting resin can be carried through their common N-α-Boc removal, washing, and neutralization steps (figure 3). It was determined that vigorous agitation of the resin packets was required to ensure successful completion of the various steps in the synthesis protocol. Three washes were found sufficient for effective removal of the total solvent retained in the resin and polypropylene mesh for packets containing 100 mg of resin. Because the total solvent retained after pouring off the residual wash solvent was approximately

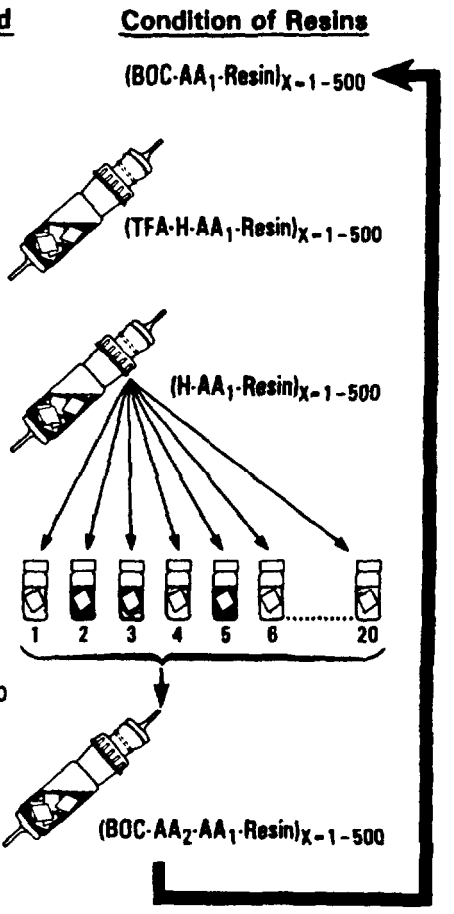

FIGURE 3. *Simultaneous multiple peptide synthesis*

0.50 mL per bag, three 400 mL washes would be expected to remove greater than 99.8 percent of the previous solvent when using 100 resin packets.

While routinely using 100 mg of resin per 20x20 mm packet, it was determined that a range of resin quantities could be successfully accommodated in this size packet. Approximately 250 to 300 mg appears to be the upper limit for this

packet size, although the simple solution of using larger packets enables 10 g or more of resin to be readily utilized.

Following the dichloromethane washes for the removal of excess base (figure 3, step II 6), the packets that contained the neutralized peptide resins were removed from the reaction vessel and added to solutions of the desired activated amino acid. Routinely, either preformed symmetrical anhydrides of the protected amino acids or coupling procedures in which the protected amino acid and activator are added *in situ* are used. The individual coupling steps were carried out for 60 minutes at room temperature while shaking on an Eberbach reciprocating shaker. Each coupling step can be checked for completion by Gisin's picric acid procedure (1972), which was easily carried out on all the resin packets simultaneously. Incompletely coupled resins can be treated again with activated amino acid or they also can be intentionally terminated with acetylimidazole to form the N-acetyl derivative (Markley and Dorman 1970). After completion of the coupling step, the resin packets were returned to the common reaction vessel, and the synthesis was continued through additional cycles of common wash, deprotection, neutralization, and individualized couplings until the syntheses were completed.

Computer programs are necessary to assist in keeping track of the various coupling steps when large numbers (i.e., >50) of individual peptides are being synthesized simultaneously. The programs calculate the amounts and volumes of reagents to be used for each coupling step as well as provide organized checklists for the amino acids being added at each coupling step of the synthesis. Specific variations of analogous peptides, such as residue replacement, omission analogs, or chain-lengthened or -shortened analogs, were easily accomplished by removing the individual, coded packets at the point of variation during the synthesis, carrying out the desired coupling separately, and if appropriate, returning the packet to the common reaction vessel for completion. Using ^{14}C-labeled t-Boc-alanine, cross-contamination was determined to be less than 0.01 percent when recombining all the packets following their coupling.

Following the synthesis of a set of protected peptide resins, the resin-filled packets were washed thoroughly, dried, and weighed to give an initial indication of yield based on the tared weight of starting resin and packet. It is important to note that no resin is lost from the bag if the resin used has been thoroughly sieved before its use. This enables an accurate weight determination of the resulting protected peptide resin to be made at the end of the synthesis. The protected peptide resins, still contained within their packets, were then cleaved using the multivessel apparatus for hydrogen fluoride/anisole described below (Houghten el al. 1986a).

The multivessel hydrogen fluoride (HF) cleavage apparatus (figure 4) consists of three polypropylene plates with a polypropylene-threaded chamber separating the top and middle plates. The bottom plate (not shown) serves as a support for the 24 individual reaction vessels (made from either polypropylene or Kel-F) and is attached through threaded post (point "A") on the middle plate. Alternate configurations having 10 to 80 mL vessels, 4 to 250 mL vessels, or one large 2,000 mL vessel are also available for use. The top plate has two threaded ports, the first of which serves as a vent, whereas the second leads to a polypropylene radial distribution manifold having 24 outlets. Hollow polypropylene tubes are connected to the radial manifold in the central chamber leading through the middle plate and terminating 8 cm from the bottom of each reaction vessel. The bottom safety plate ensures the security of the tubes. Into each of the 24 reaction vessels was added either a free resin (100 to 500 mg) or, more conveniently, a resin contained in its original polypropylene synthesis packet. An amount of anisole equaling 7.5 percent of the expected volume of HF along with a small teflon-coated magnetic stir bar was added to each reaction vessel. After cooling in a dry ice/acetone solution, the vessels were purged with N_2 for 15 minutes, and HF (5 to 10mL) was condensed simultaneously and equally into all 24 reaction chambers. After 1.0 hour at 4°C, the HF was removed rapidly (<15 minutes) with a strong flow of N_2. Nitrogen purging was continued for another 15 minutes; the apparatus then was subjected to an aspirator or mechanical pump vacuum for 15 minutes. The vessels then were removed from the apparatus; the residual anisole was removed with ethyl ether or ethyl acetate, and the peptide was extracted with aqueous acetic acid (5 to 25 percent, depending on the solubility of the peptide).

Initial analytical reverse phase-high-performance liquid chromatography (RP-HPLC), to determine the homogeneity and approximate elution conditions of the peptides produced, was carried out using (1) a Beckman 338 Gradient HPLC system equipped with a Biorad AS-100 autosampler and (2) a Shimadzu CR4A integrator. This system is used routinely to ascertain the homogeneity of all peptides produced using standard pH 3.5 or pH 7.0 buffer systems. The peptides then were purified using a Waters Milliprep 300 preparative HPLC modified with a Gilson Model 232 preparative autosampler and preparative Foxy Fraction collector with a Waters Prep Pak 5 cmx46 cm column. The preliminary RP-HPLC information determined for each peptide enabled an optimized eluting system to be used for each peptide. Fifty percent of these peptides required a second pass through the preparative HPLC to obtain 95 percent or greater purity. The peptides were collected in 3 to 5 fractions. Analytical HPLC chromatograms of each fraction then were run, and finally, fractions of acceptable purity were combined and lyophilized. The average yield of 200 mg of a 15-mer peptide that is 70 percent pure by analytical RP-

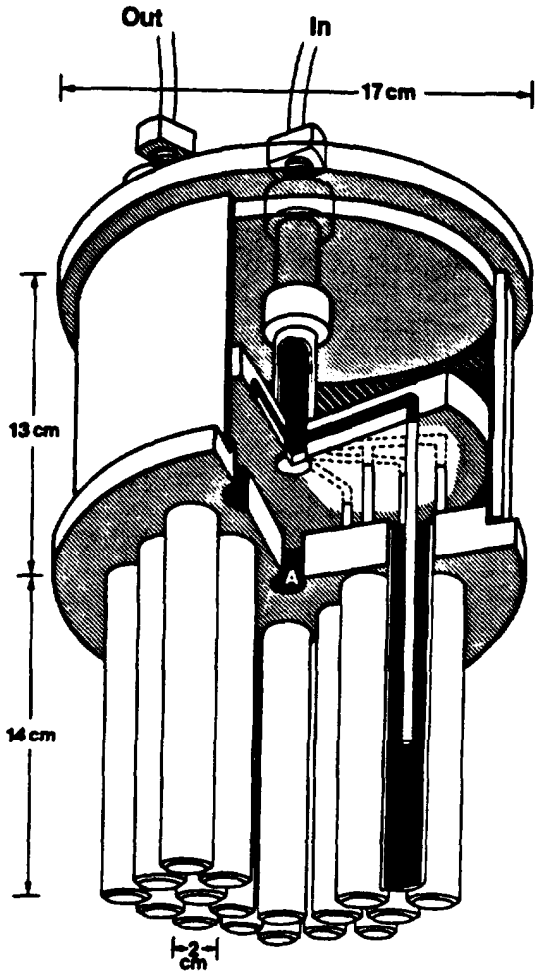

FIGURE 4. *Simultaneous multiple HF cleavage apparatus*

HPLC was approximately 100 mg of purified material after two passes through the preparative HPLC. The losses of desired peptide were mainly from the leading and trailing edge "cuts." Each purified peptide then was characterized by two analytical RP-HPLC systems, three thin-layer chromatography (TLC) systems, mass spectroscopy, amino acid analysis, and sequencing.

DISCUSSION

Although there are several protocols for the preparation of peptides, the most direct is Merrifield's solid-phase method (1963). This approach toward the solid-phase synthesis of peptides has changed little, conceptually, from Merrifield's original presentation in 1963. Synthesis is typically carried out in a reservoir containing a single polymeric solid support to which the initial carboxy-terminal amino acid of the desired peptide is covalently attached. The addition of successive amino acids is a process that uses repetitive washing, deprotection, neutralization, and coupling steps (figure 1). Although all steps except the coupling reaction are identical, only a single peptide is typically prepared at a given time. Thus, if multiple peptides that differ only in their carboxy-terminal amino acid are desired, a separate synthesis for each would be required, even though all the individual steps are identical except the initial coupling of the first amino acid to the resin. Based on this fact, the author and colleagues have developed the SMPS method (Houghten 1985, 1986; Houghten et al. 1968) for use in the rapid production of large numbers of peptides. In this patented procedure (U.S. Patent No. 4,631,211), the individual starting resins for the solid-phase peptide synthesis of various peptides are placed in separate solvent permeable packets (figure 2) which, when used in conjunction with standard solid-phase peptide synthesis protocols, enable the multiple repetitive steps involved to be carried out simultaneously (figure 3). This procedure enables large numbers of discrete protected peptide resins to be prepared rapidly. However, one still would face the formidable task of cleavage and deprotection of many individual resins, one at a time, to generate the desired free peptides. Therefore, we also have developed an apparatus capable of cleaving 24 peptide resins simultaneously with hydrogen fluoride (figure 4) (Houghten et al. 1986a). As a result, we are able to produce useful quantities of large numbers of peptides, as pure as those produced by conventional methods. The overall rate of synthesis is much faster and the use of materials and personnel is more cost-effective. With SMPS, it is possible to accomplish at least two couplings per day in the preparation of 150 peptides having entirely different sequences. This is equivalent to the preparation of 20 different 15-residue peptides per day per technician. SMPS has been used to prepare large numbers of peptides for use in various research areas, including the study of antibody/antigen interactions (Houghten 1985; Houghten et al. 1986b), development of potential synthetic vaccines (Parry et al. 1985), and optimal analog development for biologically relevant peptides (Ruggeri et al. 1986). We are using these methods to prepare 10-g quantities, and specific tritiated analogs, of the peptides listed in table 1. The majority of these are now available from the NIDA Drug Supply System.

TABLE 1. *List of peptides prepared*

1.	DPDPE	H-Tyr-D-Pen-Gly-Phe-D-Pen-OH
2.	^{3}H-DPDPE	H-Tyr($^{3}H_2$)-D-Pen-Gly-Phe-D-Pen-OH
3.	DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
4.	^{3}H-DSLET	H-Tyr($^{3}H_2$)-D-Ser-Gly-Phe-Leu-Thr-OH
5.	DTLET	H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH
6.	DADLE	H-Tyr-D-Ala-Gly-Phe-D-Leu-OH
7.	^{3}H-DADLE	H-Tyr($^{3}H_2$)-D-Ala-Gly-Phe-D-Leu-OH
8.	ICI 174,864	H-Diallyl-Tyr-Aib-Aib-Gly-Phe-Leu-OH
9.	PLO17	H-Tyr-Pro-N-Me-Phe-D-Pro-NH_2
10.	$^{3}H_2$-PLO17	H-Tyr($^{3}H_2$)-Pro-N-Me-Phe-D-Pro-NH_2
11.	DAGO	H-Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol
12.	^{3}H-DAGO	H-Tyr($^{3}H_2$)-D-Ala-Gly-N-Me-Phe-Gly-ol
13.	CTAP	H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
14.	^{3}H-CTAP	H-D-Phe-Cys-Tyr($^{3}H_2$)-D-Trp-Arg-Thr-Pen-Thr-NH_z
15.	Dynotphin A 1-17, porcine	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
16.	Dynorphin A 1-13, porcine	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH
17.	Dynorphin A 1-13 amide, porcine	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH_2
18.	^{3}H-Dynorphin A 1-13 amide porcine	H-Tyr($^{3}H_2$)-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH_2
19.	Dynorphin A 1-11, porcine	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-OH
20.	^{3}H-Dynorphin A 1-11, porcine	H-Tyr($^{3}H_2$)-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pm-Lys-OH
21.	Dynorphin A 1-9, porcine	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH
22.	^{3}H-Dynorphin A 1-9. porcine	H-Tyr($^{3}H_2$)-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH
23.	Dynorphin B	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Vai-Thr-OH
24.	ß-Endorphin	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-IIe-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
25.	^{3}H-ß-Endorphin	H-Tyr($^{3}H_2$)-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
26.	^{125}I-ß-Endorphin	H-Tyr(^{125}I)-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-GIn-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
27.	DALDA	H-Tyr-D-Arg-Phe-Lys-NH_2

The difficulties in peptide synthesis typically occur at the purification stage, not in the assembly of the peptide. Although the two stages are intimately linked, the assembly process always must be carried out with the ultimate necessary purification in mind. Several obvious but essential precautions must be followed to avoid later problems when preparing synthetic peptides. The highest quality starting materials (protected amino acids, solvents, etc.) prevent later difficulty in characterization and purification. All solvents used must be freshly distilled as needed and all the protected amino acids must be of the highest quality available. Currently, several suppliers are available for N-α-tt-butoxycarbonyl amino acids. The highest quality in terms of level of impurities, and the least amount of racemate (typically, <0.05 percent), must be used; and every lot must be checked against each manufacturers specifications.

The use of Merrifield's solid-phase method often has been criticized as unable to generate peptides of extremely high quality. We have found that the solid-phase method, in conjunction with SMPS and standard purification and analysis procedures, permits the preparation of 99+ percent pure peptides. An example of the protocol and results found for dynorphin (1-13) amide are shown in figures 5 and 6. As illustrated, this peptide can be prepared in >99 percent purity multigram quantities in a final overall yield of approximately 53 percent with no detectable impurity, as determined using two different RP-HPLC systems, three TLC systems, amino acid analysis, mass spectral analysis, and sequencing. The largest losses came at the extraction step following the final HF cleavage of the peptide. Further evidence for the overall purity of the peptide was found following the preparation of all the single position omission analogs of the sequence and their examination by RP-HPLC (pH 3.5). As shown in figure 7, all these analogs separate from the desired sequence on analytical RP-HPLC. During preparative runs, the two glycine omission analogs and the proline omission at positions 2, 3, and 10, respectively, would not have been successfully separated. However, the presence of these peptides would be detectable by analytical HPLC. Although clearly the successful separability of individual omission analogs of specific peptides will vary greatly, the majority of the opioid peptides shown in table 1 can be separated successfully from their expected major contaminants.

The ability to prepare synthetic peptides conveniently and rapidly is essential to many areas of biomedical research, including the investigation of the causative mechanisms of drug abuse. The methods utilized in the preparation of the opioid peptides presented here enable large numbers of totally different sequences, or large numbers of analogs of a given sequence, to be prepared quickly and cost-effectively.

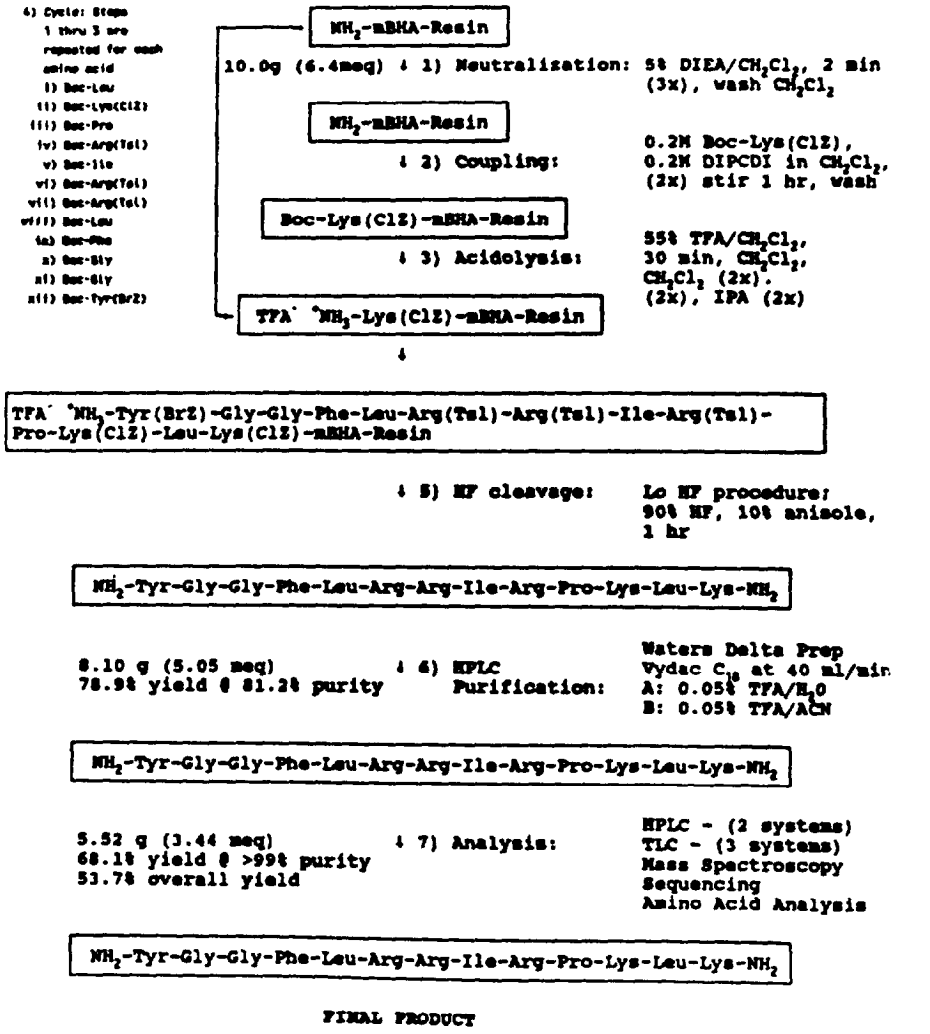

FIGURE 5. *Flow chart—compound 17: dynorphin A (1-13) amide, porcine*

National Institute on Drug Abuse Research Technology Branch Division of Research Peptide Data Sheet	Batch No.: MPSP-17-02 Amt. Supplied: Date Supplied:

Name: Dynorphin A (1-13) amide, Porcine		
Sequence: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH_2		
Molecular Formula: $C_{75}H_{127}N_{25}O_{14}$	Molecular Weight: 1603.2	FAB-MS: confirmed, see attached
Description: White solid	Melting Point: dec 127-143°C	Peptide Content: 87%

Amino Acid Analysis:	Tyr	Gly	Phe	Leu	Arg	Ile	Pro	Lys
Calculated	1.00	2.00	1.00	2.00	3.00	1.00	1.00	2.00
Found	0.93	2.06	0.96	1.90	3.28	1.01	1.01	1.85

Sequencing Data: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
Confirmed, on file

NMR:

TLC: See attached
System 1: single spot, R_f 0.66, on silica gel (E. Merck analytical plate) using BuOH:Pyr:AcOH:H_2O (50:10:3:8)
System 2: single spot, R_f 0.29, on cellulose (EM Reagents analytical plate) using BuOH:AcOH:H_2O (4:1:5) upper phase
System 3: single spot, R_f 0.33, on silica gel (E. Merck analytical plate) using BuOH:Pyr:AcOH:H_2O (42:24:4:20)

HPLC: See attached
System 1: single peak, R_t 15.624 min, on a Vydac C_{18} column with buffer A (pH 3.5): 0.05% (w/v) trifluoroacetic acid/water and buffer B: 0.05% (w/v) trifluoroacetic acid/acetonitrile using a gradient of 5 to 65% B in 30 minutes at 1.0 ml/min with detection at 215 nm.
System 2: single peak, R_t 17.760 min, on a Vydac C_{18} column with buffer A (pH 7.0): 0.05% (w/v) ammonium trifluoroacetate/water and buffer B: 0.05% (w/v) ammonium trifluoroacetate/10% water/90% acetonitrile using a gradient of 5 to 65% B in 30 minutes at 1.0 ml/min with detection at 215 nm.
Comments: Coinjection of this material and a commercial sample of Dynorphin A (1-13) amide, Porcine (Peninsula Lot # 010476) gave a single peak using both systems.

References:

Signature of Principal Investigator:

FIGURE 6. *Data sheet*

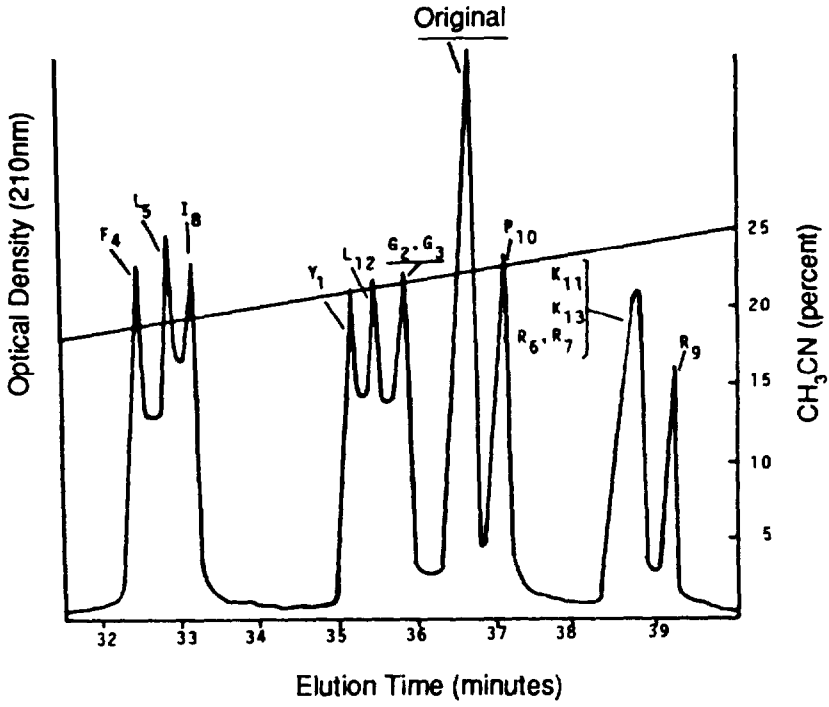

FIGURE 7. *Omission analogs of dynorphin (1-13) amide $Y_1G_2G_3F_4L_5R_6R_7I_8R_9P_{10}K_{11}L_{12}K_{13}$ amide (pH 2.1)*

NOTE

Inquiries about the availability of these peptides for research studies should be addressed to Drug Supply System-NIDA, Research Technology Branch, National Institute on Drug Abuse, Room 10A-13, 5600 Fishers Lane, Rockville, MD 20857; the telephone number is (301) 443-5280.

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Irreversible Ligands as Probes for Drug Receptors

Amy Hauck Newman

INTRODUCTION

The design and synthesis of highly selective chemical probes are critical for progress in the area of drug receptor pharmacology. Drugs of abuse mediate their pharmacological actions and abuse liability via interactions with specific receptor system(s). The preparation and use of selective probes, referred to as irreversible or affinity labels, have proven to be extremely useful in the isolation, purification, and characterization of many receptor systems. By definition, irreversible ligands first recognize a specific binding site and then form a covalent bond on or near that recognition site, resulting in irreversible attachment (Baker 1967; Takemori and Portoghese 1985; Portoghese and Takemori 1986). For the purpose of this review, all ligands discussed possess a reactive chemical moiety that is capable of forming a covalent bond. These groups may be intrinsically active and are generally electrophilic in nature, or they may require a photoactivation step that leads to a reactive species (Takemori and Portoghese 1985). The compounds are classified according to the receptor system they interact with and are further categorized into specific functionalities, beginning with electrophiles and ending with the photoactivated agents.

The successful design of highly receptor-selective irreversible ligands, containing an electrophilic moiety, has been described as being dependent on receptor affinity and selectivity of the ligand, location of the electrophile on the ligand, and the reactivity of the electrophile (Takemori and Portoghese 1985; Portoghese and Takemori 1986). Electrophilic moieties most commonly used include isothiocyanates, Michael acceptors, haloacetamides, aldol esters, and nitrogen mustards. Placement of the electrophilic moiety within the parent ligand generally takes into account ease of chemical synthesis and avoidance of a known site of ligand receptor interaction. Ideally, when the ligand binds to the receptor site, a nucleophile on or near that site will be in a position to covalently bond. Because biological tissues possess a multitude of reactive nucleophiles, the probability is high for nonspecific covalent attachment. This

must be taken into account when analyzing results of experiments with these agents.

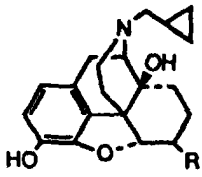
Photoaffinity labels are ligands that have an inherent affinity for a binding site, may function as agonists or antagonists, and also contain a photosensitive functional group that, when photoactivated with light, is capable of forming a covalent bond at or near the binding site (Fedan et al. 1984). Unlike electrophilic affinity labels, the photoaffinity labels' association with the recognition site is reversible until photolysis is initiated (Fedan et al. 1984). Due to the high reactivity of the photoactivated intermediate relative to electrophiles, it is generally easier to design a photoaffinity label, since the location of the photoactivated group is not crucial for covalent attachment to the binding site (Takemori and Portoghese 1985). The chemical mechanism for photoaffinity labeling has been reviewed (Cavalla and Neff 1985). Caution must be taken to assess the possible effects of irradiation on the tissue being studied (Fedan et al. 1984). For example, UV-radiation damage to the opioid receptor has been reported (Capponi and Catt 1980; Glasel and Venn 1981; Smolarsky and Koshland 1980). Another limitation of the photoaffinity labels is that they are only useful under conditions that support photolysis, which, at the very least, eliminates *in vivo* work. Eiectrophilic agents need not be activated and, hence, are amenable to both *in vitro* and *in vivo* pharmacology (Takemori and Portoghese 1985).

OPIOID RECEPTORS

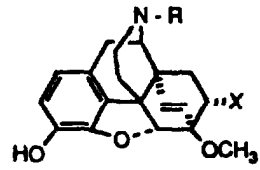
The central nervous system (CNS) opioid receptor subtypes μ, δ, and κ have been characterized with several structurally diverse, irreversible ligands (for review, see Takemori and Portoghese 1985; Portoghese and Takemorl 1986; Zimmerman and Leander 1990). Ligands that bind selectively and irreversibly to particular opioid receptor subtypes can provide insight into the relationship of these subtypes to one another and their physiological roles. A nitrogen mustard derivative of naltrexone, ß-CNA, 1, was the first successful irreversible antagonist for opioid receptors. ß-CNA proved to be a potent affinity label selective for all three opioid receptors, both *in vitro* and *in vivo* (Portoghese et al. 1978, 1979). The lack of selectivity of 1 for a particular opioid receptor appears to be due to the high reactivity and low selectivity of the reactive species, the aziridinium ion (Takemori and Portoghese 1985). Michael acceptors react preferentially with sulfhydryl groups, which are thought to be present on opioid receptors (Simon et al. 1973). These considerations led to the discovery of ß-FNA, 2, having a fumaramate methyl ester function (Portoghese et al. 1980; Takemori et al. 1981). This compound has been reported to interact reversibly with κ- and δ-receptors and irreversibly with μ-receptors (Ward et al. 1985). ß-FNA displays reversible agonlst activity that

appears to be κ-receptor mediated (Takemori et al. 1981). However, selective antagonism of μ-activities has been observed after systemic administration of 2, increasing its usefulness in studying these receptors *in vivo* (for review, see Zimmerman and Leander 1990). The N-methyl-derivatives of ß-CNA and ß-FNA are irreversible ligands with long-lasting agonist properties (Takemori and Portoghese 1985; Larson and Armstrong 1980). Structure-activity relationships have been described for a series of ester and fumaramate morphinan and endoethenooripavine analogs (Burke et al. 1984a; Jacobson et al. 1983; Lessor et al. 1984, 1986; Schoenecker et al. 1986; Portoghese et al. 1986). Halogenated amide derivatives of endoethenooripavine, 3 (Lessor et al. 1986), and α-naltrexamine, 4 (Archer et al. 1985), have also been described as potent irreversible ligands of opioid receptors. CAN, 5, and BAM, 6, are C-14-position derivatives of naltrexone and have been described as covalently bonding to a high-affinity μ-site (Reichman et al. 1986).

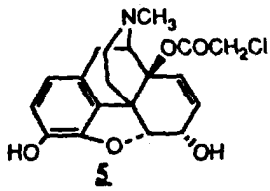
The isothiocyanate function has proven to be highly versatile as an electrophilic moiety for irreversible ligands. The ease of synthesis from primary amines (Assony 1961), the known rapid reactivity with amino and sulfhydryl groups, and the low reactivity toward water and hydroxyl functions (Williams et al. 1980a) have led to successful application of this functionality to affinity ligand preparation and use. Fentanyl isothiocyanate (FIT, 7 is a highly selective acylator of δ-receptors (Rice et al. 1983; Burke et al. 1984a). [^{3}H]FIT (Burke et al. 1984b) was first used to identify and partially purify a glycoprotein subunit, Mr 58,000, from NG-108 neuroblastoma x glioma hybrid cells, which was acylated by this ligand in the presence of dextromethorphan, but not levorphanol, and was presumed to be a component of the opioid receptor (Klee et al. 1982). The (+)- and (-)-*cis*-3-methyl derivatives of FIT were the first enantiomeric pair of irreversible ligands to be described (Burke et al. 1986). The (+)-enantiomer, SUPERFIT, 8, was found to be 10 times more potent than 7 in acyiation of δ-receptors and 50 times more potent than its (-)-enantiomer (Burke et al. 1986). [^{3}H]SUPERFIT labeled the same 58,000-dalton subunit of the opioid receptor as 8 (Burke et al. 1986) and was used to purify this subunit to homogeneity (Simonds et al. 1985). This ligand subsequently has been used to characterize functional coupling of opioid receptors to GTPase (Clark and Medzihradsky 1987). An antibody was generated to the opioid receptor complex from NG-108 cells, and [^{3}H]SUPERFIT was employed to characterize the 58,000-dalton subunit of the immunoaffinity purified receptor (Carr et al. 1987). The *trans*-isomers of 8 were prepared to obtain further insight into the steric requirements of the δ-receptor (Kim et al. 1989). The (+)-*cis*- and (+)-*trans*-isomers demonstrated similar affinity to the high-affinity δ-sites, which was considerably greater than for either of the (-)-isomers, suggesting that optical isomerism plays a more important role than the *cis:trans* configuration. *In vivo,* the (+)-*trans*-isomer was only weakly active (Kim et al. 1989).



1, R=β-N(CH$_2$CH$_2$Cl)$_2$
2, R=β-NHCOCH=CHCOOCH$_3$
4, R=α-NHCO(Cl)=CH$_2$



3, R=CH$_2$-c-propyl, X=NHCOCH$_2$Br
10, R=CH$_3$, X=NHCOCH$_2$-p-phenyl-NCS
11, R=CH$_2$-c-propyl, X=NCS



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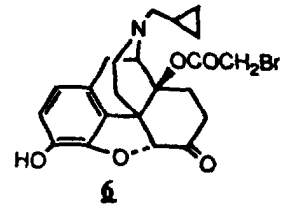

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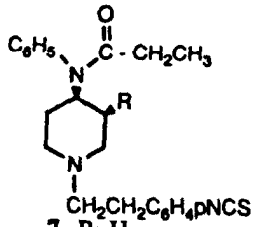
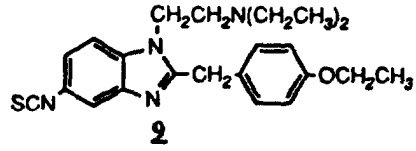


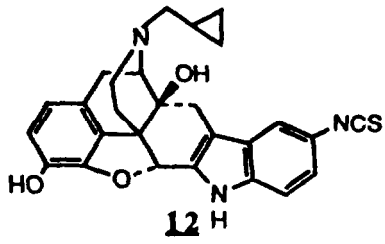
7, R=H
8, R=CH$_3$



9

The benzimidazole isothiocyanate (BIT, 9), related to the extremely potent opioid agonist etonitazene, is a high-affinity irreversible ligand for μ-receptors (Rice et al. 1983; Burke et al. 1984a). Several endoethenooripavine-isothiocyanates 10, 11) also have been reported to have μ- or δ-receptor selectivity (Jacobson et al. 1983; Lessor et al. 1984, 1986). Another new δ-selective ligand, naitrindoie-isothiocyanate, 12, has been preliminarily described (Sultana et al. 1989). Recently, the first κ-receptor selective irreversible ligand was prepared (de Costa et al. 1989a). Compound 13 is an isothiocyanate-derivative of the most active *(1S,2S)* enantiomer of the κ-selective compound U-50, 488 (14). At 1 μM concentration in guinea pig membranes (completely depleted of functional

μ- and δ-receptors with 9 and 7, respectively), compound 13 enantiospecifically and irreversibly reduced the binding of [^{3}H]U-69,593 to 11.2 percent of control without affecting [^{3}H]bremazocine binding, thus providing an independent line of evidence for x-receptor heterogeneity (de Costa et al. 1989a). The racemic mixture UPHIT, 15, and its *1S,2S*- and *1R,2R-trans*-enantiomers, more closely related derivatives of U-50,488, have been reported to be potent displacers of [^{3}H]U-69,593 and weak displacers of [^{3}H]bremazocine, possibly identifying κ-receptor subtypes (de Costa et al. 1989b). A 300-fold enantioselectivity was demonstrated, and the *1S,2S* enantiomer of 15 was marginally more potent than 13 *in vitro.* Racemic 15 was a potent and selective κ-acylator *in vivo* compared to an inactive 13 in this assay, perhaps because of increased reactivity of 15 toward nucleophiles (de Costa et al. 1989b; Band et al. 1989).



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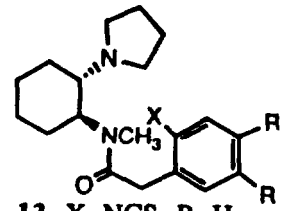
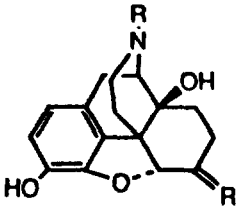
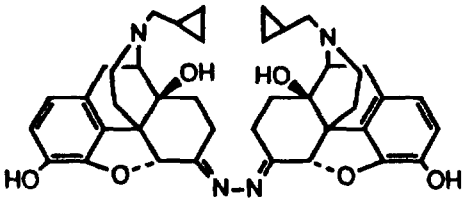


13, X=NCS, R=H
14, X=H, R=Cl
15, X=NCS, R=Cl

Substitution of the C-6 carbonyl of naloxone and naltrexone by a group that has similar spatial configuration to that of a carbonyl group and is electrophilic in nature led to the discovery of naioxazone, 16 and naltrexazone, 17 (Pasternak and Hahn 1980). Both compounds were selective, high-affinity ligands for opioid receptors and caused long-term inhibition of morphine analgesia (Pasternak and Hahn 1980; Pasternak et al. 1980). The irreversible inhibition was characterized as selectively occurring at the high-affinity μ_1-subtype (Pasternak et al. 1980; Hahn et al. 1982). It was later discovered that these hydrazones could be converted to more active azines such as naioxonazine, 18 This compound is a more potent μ_1-ligand, whose mechanism of irreversible binding is not well understood and may not occur through covalent attachment (Hahn et al. 1982; Johnson and Pasternak 1984). Subsequently, a series of phenyl hydrazones were developed to further investigate the mechanism of action of these agents; however, none of these compounds was as subtype selective as 16 (Hahn et al. 1985; Luke et al. 1988; Williams et al. 1988). Comparison of μ_1-selectivity between naloxonazine, 16 and ß-FNA, 2, revealed that ß-FNA is also μ_1-selective *in vitro* but interacts with μ_1 and μ_2 *in vivo* (Recht and Pasternak 1987).





16, R=allyl, X=N-NH_2
17, R=methyl-c-propyl, X=N-NH_2

18

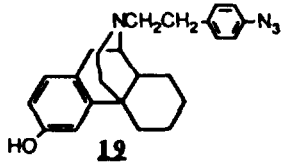
Photoaffinity ligands have not proven to be as successful as electrophilic-type irreversible agents in opioid receptor research, primarily due to the reported UV-inactivation of the receptor (Capponi and Catt 1980; Glasel and Venn 1981; Smoiarsky and Koshiand 1980). The opioid analgesic azido-levorphanol, 19, caused irreversible incorporation of radioactivity into biological material upon photolysis (Winter and Goldstein 1972). However, extensive nonspecific binding resulted, diminishing the usefulness of this compound (Winter and Goldstein 1972). Photoaffinity labels based on fentanyl, (+)-*cis*-3-methylfentanyl, lofentanyl, and sufentanyl were similarly unsuccessful; photodecomposition of opioid receptors was reported (Maryanoff et al. 1982). Azido-derivatives of the opioid peptides DAGO and DTLET have been described as binding to μ- and δ-receptors, respectively (Garbay-Jaureguiberry et al. 1983, 1986). Recently, azido-derivatives of the δ-selective peptide DPDPE and μ-selective peptide CTP have been synthesized (Landis et al. 1989).

NMDA RECEPTORS/PCP BINDING SITES

The bizarre behavioral actions induced by PCP, the abuse liability, and the neuroprotective effects produced by this drug have generated considerable interest in its biochemical and behavioral pharmacology. Saturable and stereoselective PCP binding sites in the brain have been described (Vincent et al. 1979; Zukin and Zukin 1979; Quirion et al. 1981; Hampton et al. 1982). These sites appear to be allosterically coupled to the N-methyl-D-aspartate (NMDA)/L-glutamate macromolecular complex, and PCP and related compounds have been shown to block the increases in neuronal discharges induced by NMDA (Lodge and Anis 1982; Quirion et al. 1987). The probability of interaction between PCP and NMDA receptors is further supported by the striking similarity in distribution of these receptors in the brain (Maragos et al. 1986).

The *meta*-isothiocyanate derivative of PCP, metaphit, 20, was the first electrophilic irreversible ligand to selectively label PCP sites in rat brain

(Rafferty et al. 1985). The metaphit isomer with the isothiocyanate function in the 4-position of the piperidine ring did not irreversibly inhibit [^{3}H]PCP binding, indicating structural specificity for the acylation of these receptors (Rafferty et al. 1985). Metaphit demonstrated long-term inhibition of PCP-induced stereotypy in rats (Contreras et al. 1985) but did not antagonize stereotyped behavior induced by the σ-ligands (-)-cyclazocine or (+)-SKF-10,047, suggesting selective acylation of only the PCP sites (Contreras et al. 1986). However, it has been proposed that 20 prevents PCP-induced locomotor behavior through mechanisms not related to the PCP recognition site (French et al. 1987). In addition, in pigeons and monkeys, 20 induced PCP-like catalepsy, ataxia, and convulsions and did not inhibit these PCP-induced behaviors (Koek et al. 1985, 1986). *In vitro* studies showed 20 caused a concentration-dependent inhibition of acetylcholine release evoked by NMDA, which paralleled loss of [^{3}H]TCP binding in striatal tissue, indicating metaphit as having long-term PCP-like effects (Snell et al. 1987). Furthermore, 20 has been described as a tool for the study of the molecular basis of stimulant action (Schweri et al. 1987) and has been shown to inactivate cocaine binding sites as well as antagonize cocaine-induced locomotor stimulation in rats (Berger et al. 1986). Finally, autoradiographic studies have revealed that metaphit inhibits most of the PCP-induced increases in glucose metabolism in selected areas of the rat cortex (Tamminga et al. 1987); microelectrophoresis studies in rat spinal neurones showed a decrease in NMDA-induced excitation by 20 (Davies et al. 1986).



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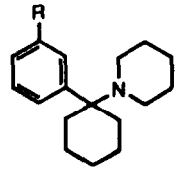
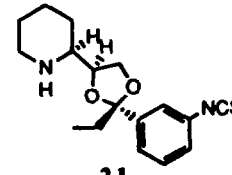


20, R=NCS
22, R=N$_3$



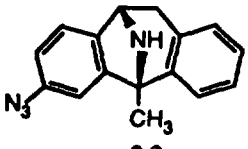
21

Etoxadrol-*meta*-isothiocyanate, 21, has been synthesized and characterized as an irreversible ligand for PCP receptors (Thurkauf et al. 1988). It is the first chiral electrophilic affinity ligand to be described for this site and displaces [^{3}H]TCP, from PCP sites in rat brain, with four times higher affinity than 20 (Thurkauf et al. 1988).

Photoaffinity labeling of rat brain PCP receptors with [^{3}H]azido-PCP, 22, revealed five polypeptides with Mr's 90,000, 62,000, 49,000, 40,000, and 33,000, which were unevenly distributed in the brain (Haring et al. 1985, 1986).

Competition studies with [^{3}H]PCP and dexoxadrol suggest that the high-affinity PCP site correlated best with the 90,000-dalton peptide (Haring et al. 1985, 1986); the 33,000-dalton peptide is suggested to be a low-affinity PCP site (Haring et al. 1986, 1987).

MK 801 potently and selectively inhibits binding of [^{3}H]TCP from PCP sites and weakly inhibits binding of [^{3}H]SKF-10,047 from σ-sites (Loo et al. 1987). (+)-[^{3}H]-7-Azido-MK 801, 23, was preliminarily reported to display reversible binding to PCP sites and a pharmacological profile identical to the parent compound, MK 801 (Sonders et al. 1989). Photoactivation in guinea pig membranes resulted in the labeling of a single radioactive band of 115,000 daltons. This band was absent from lanes that had been preincubated with PCP, suggesting a PCP component of the NMDA-receptor complex (Sonders et al. 1989).



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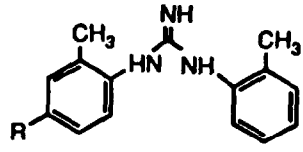


24, R=NCS
25, R=N_3

SIGMA RECEPTORS

The benzomorphans, such as SKF-10,047, represent a class of psychotomimetic drugs that were once believed to interact at a σ-opioid site (Martin et al. 1976). Further studies demonstrated that many actions of these agents could not be blocked by the opioid antagonist naloxone; thus, they were not interacting at an opioid receptor subtype (Tam and Cook 1984). Many compounds that bind with high affinity to σ-sites exert similar behavioral effects to PCP, and there is an overlap in the binding of these agents to both receptor sites (Contreras et al. 1987). The striking similarities between the actions of the PCP and σ-drug classes led to the concept that there was only one receptor system involved. However, radioreceptor binding studies and different rank order of potencies of drugs that interact at both sites implicate two distinct drug recognition sites (Manallack et al. 1986). In addition, autoradiography studies show that the anatomical distribution of these sites is very different in rodent brain (Sonders et al. 1988). Recently, [^{3}H]-(+)-pentazocine has been introduced as a ligand with high specificity for σ-receptors and insignificant affinity for PCP sites, further demonstrating that these receptor sites are not the same (de

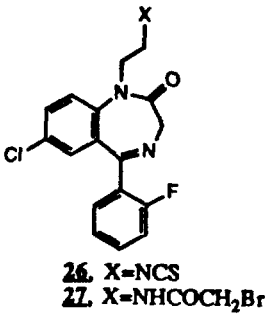
Costa et al. 1989c). σ-Receptors now have been defined as nondopaminergic, nonopioid receptors that are distinct from PCP receptors (de Costa et al. 1990).

Metaphit (20) was shown to displace [^{3}H]DTG and [^{3}H]3-PPP from σ-sites in guinea pig brain, in an irreversible and competitive manner (Bluth et al. 1989). An isothiocyanato-derivative of the selective σ-receptor ligand 1,3-di-ortho-tolyl guanidine (DTG) has been prepared (DIGIT, 24) (Adams et al. 1987). σ-Receptors are selectively and irreversibly modified by 24, whereas PCP receptors are not affected. A radiolabeled azido-derivative of DTG, 25, recently was used to isolate a 29,000-dalton polypeptide that may represent the intact σ-receptor complex (Kavanaugh et al. 1988).

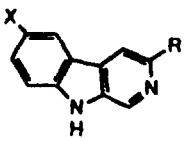
BENZODIAZEPINE RECEPTORS—CENTRAL/GABA AND PERIPHERAL TYPES

The benzodiazepine receptors in the CNS are associated with GABA receptors and a chloride ion channel and are responsible for the mediation of anxiolytic, anticonvulsant, and muscle relaxant properties of this class of drugs (Haefly et al. 1985). Abuse liability and physical dependence have been demonstrated for many of the centrally acting benzodiazepines. The first isothiocyanato-benzodiazepine to demonstrate noncompetitive, irreversible binding to central benzodiazepine sites was irazepine, 26 (Rice et al. 1979). Irazepine, 26, demonstrated long-term protection against pentylenetetrazol-induced seizures when administered intracerebroventricularly, which was accompanied by a decrease in [^{3}H]-diazepam binding in forebrain of treated mice (Williams et al. 1980b). The primary amino-precursor of 26 was used to prepare an affinity column for the purification of anti-benzodiazepine antibodies (Goldman et al. 1986). A related bromoacetamide derivative, kenazepine, 27, demonstrated similar irreversible properties to 26 but, in addition, appeared to interact reversibly with one population of benzodiazepine receptors and irreversibly with another (Williams et al. 1980a, 1980b). More recently, an isothiocyanato-analog of the inverse agonist ß-CCE (28) has been prepared, 29 (Allen et al. 1988).

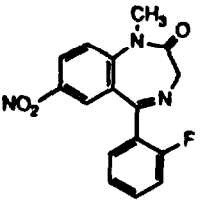
When irradiated, the potent centrally active benzodiazepine flunitrazepam, 30, irreversibly binds to the benzodiazepine receptor (Mohler et al. 1980). The receptor component labeled by 30 is a protein with Mr 50,000; its locations in cerebral and cerebellar cortical slices were visualized with electron microscopic autoradiography (Mohler et al. 1980). In contrast to opioid receptors, the benzodiezepine receptors are not damaged by irradiation (Mohler et al. 1980). Heterogeneity of the benzodiazepine receptors has been reported in subsequent studies using 30, with Mr 51,000 in cerebellum and Mr's 55,000 and 51,000 in hippocampus (Sieghart and Karobath 1980; Eichinger and



26. X=NCS
27. X=NHCOCH$_2$Br



28. R=$CO_2CH_2CH_3$, X=H
29. R=NCS, X=H
33. R=$CO_2CH_2CH_3$, $X=N_3$



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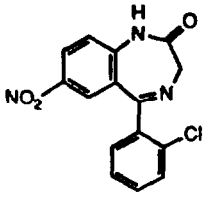

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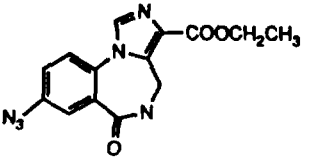

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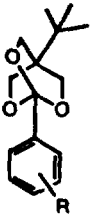


34, m-NCS
35, p-NCS

Sieghart 1984). Shortwave ultraviolet irradiation has been reported to improve the photoaffinity labeling of the benzodiazepine receptors by 30 (Herblin and Mechem 1984). [^{3}H]Clonazepam, 31, can also be photoactivated and binds to benzodiazepine receptors irreversibly, but with fivefold lower specific activity compared to 30 (Sieghart and Mohler 1982; Bowling and DeLorenzo 1987). Another photoaffinity ligand, the partial inverse agonist [^{3}H]Ro 15-4513, 32, ligands the same proteins as flunitrazepam; however, instead of only labeling 25 percent of the benzodiazepine sites, as with 30, compound 32 is reported to photolabel all binding sites (Sieghart et al. 1987; Mohler et al. 1984). The first ß-carboline photoaffinity label, 33, has recently been described as producing a 42-percent decrease in maximal binding of ß-carboline carboxylic propyl ester (ß-CCP) sites, with essentially no effect on flunitrazepam sites (Dellouve-Lourillon et al. 1989). These results provide evidence that the ß-carbolines and the benzodiazepines bind to discrete sites on the central benzodiazepine receptor (Dellouve-Lourillon et al. 1989).

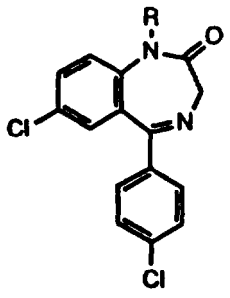
The related GABA-regulated chloride ionophore has been treated with two isothiocyanates, 34 and 35, derived from the cage convulsant TBOB (Lewin et al. 1989). These compounds are specific, site-directed acylating agents for sites on or near GABA-gated chloride channels labeled by [^{3}H]TBOB and [35S]TBPS (Lewin et al. 1989). Compound 34 inhibited binding of the above

radioligands with an affinity comparable to the parent ligand, whereas 35 was more than one order of magnitude less potent (Lewin et al. 1989).

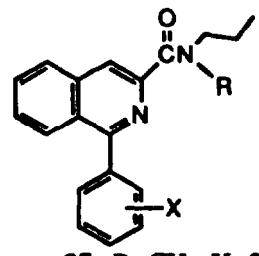
[^{3}H]Muscimol has been described as photolabeling 86 percent of the $GABA_A$ receptors in rat cerebellar synaptosomal membranes (Cavalla and Neff 1985). Ultraviolet irradiation did not adversely affect the GABA receptor, and the two components isolated were proteins with Mr's of more than 250,000 and 52,000 (Cavalla and Neff 1985). [^{3}H]Muscimol and [^{3}H]flunitrazepam have been used in a large-scale purification of the GABA-benzodiazepine receptor protein from rat brain, by affinity chromatography (Stauber et al. 1987). The material recovered was used for structural and immunochemical studies, including the production of antisera against the purified receptor complex protein (Stauber et al. 1987). A series of aryl-diazo compounds based on muscimol also have been described as potential photoaffinity ligands for the GABA receptor (Bouchet et al. 1987).

In comparison to the central-type benzodiazepine receptors, very little is known about the function and molecular organization of recognition sites for benzodiazepines found outside the CNS. The benzodiazepine Ro 5-4864 (36) and the isoquinoline PK 11195 (37) have been used to characterize the peripheral-type benzodiazepine receptors because they have high affinity and selectivity for these sites and do not possess any of the pharmacological characteristics of classical centrally acting benzodiazepines. The ethylisothiocyanato-derivatives of these compounds, AHN 086, 38, and AHN 070, 39, respectively, have proven to be potent, selective, and irreversible ligands for these receptors (Lueddens et al. 1986; Newman et al. 1987). Compound 38 has been used to characterize the association between peripheral benzodiazepine receptors and the calcium channels in guinea pig atria and ileal longitudinal smooth muscle (Bolger et al. 1989), and [^{3}H]AHN 086 has been used to label the peripheral benzodiazepine receptor protein, Mr 30,000, in rat pineal gland (McCabe et al. 1989).

Although the peripheral benzodiazepine receptor sites recognize flunitrazepam, 30, they cannot be photoaffinity-labeled by it (Thomas and Tallman 1981). However, photoaffinity labeling of the peripheral benzodiazepine receptors has been achieved with PK 14105, 40 (Doble et al. 1987a, 1987b; Skowronski et al. 1988; Antkiewicz-Michaluk et al. 1988a, 1988b; Benavides et al. 1989). Photoactivation of 40 results in the covalent coupling of this ligand to an approximately 18,000-dalton protein in cardiac, adrenal, brain, and kidney membranes (Doble et al. 1987a, 1987b; Skowronski et al. 1988). Additional characterization and subcellular distribution studies of the peripheral benzodiazepine sites in rat adrenal mitochondria revealed a protein of approximately 17,000 daltons (Antkiewicz-Michaluk et al. 1988a, 1988b).



36, R=CH_3
38, R=CH_2CH_2NCS



37, R=CH_3, X=2'-Cl
39, R=CH_2CH_2NCS, X=2'Cl
40, R=CH_3, X=2'-F, 5'-NO_2

Recently, 40 has been employed to study the peripheral benzodiazepine sites in immune system organs of rats (Benavides et al. 1989). The distribution of these sites in the immune system organs suggests a preferential labeling of T-cells and monocytic cells consistent with a proposed immunomodulatory role for some peripheral benzodiazepine receptor ligands (Benavides et al. 1989).

DOPAMINE RECEPTORS

Central dopamine receptors have been implicated in the mechanism of action of several drugs of abuse, including LSD and cocaine. As in many of the other receptor systems described, N-chloroethyl-substituted monoamine agonists have been prepared as irreversible ligands for studying dopamine receptors. An early example of this is the N-chloroethyl-derivative of apomorphine, 41 (Neumeyer et al. 1980; Costall et al. 1980). Tritiated 41 has been used to characterize dopamine receptors and, furthermore, has been suggested to bind selectively to the D_2-sites (Guan et al. 1984). The N-chloroethyl derivative, 42, of 3-PPP (described in the σ-receptor section of this chapter) was prepared as another potential alkylating agent for dopamine receptors but was not a good irreversible agent due to its reduced affinity and inability to covalently bond to the receptor as evidenced by facile washout (Williams et al. 1983).

LSD is reported to be a “naturally photoreactive agent” that needs no chemical modification and whose receptor-binding properties already have been well characterized (Mahon and Hartig 1982). LSD covalently binds to bovine caudate membranes, but the fact that this compound binds with high affinity to serotonin and adrenergic receptors in addition to dopamine receptors limits its usefulness as a photoaffinity label (Mahon and Hartig 1982). More selective photoaffinity ligands have since been introduced that bind selectively to either D_1 or D_2 receptors. The D_2 dopamine receptor has many functional correlates with such dopaminergic behaviors as rotation, locomotion, emesis, and

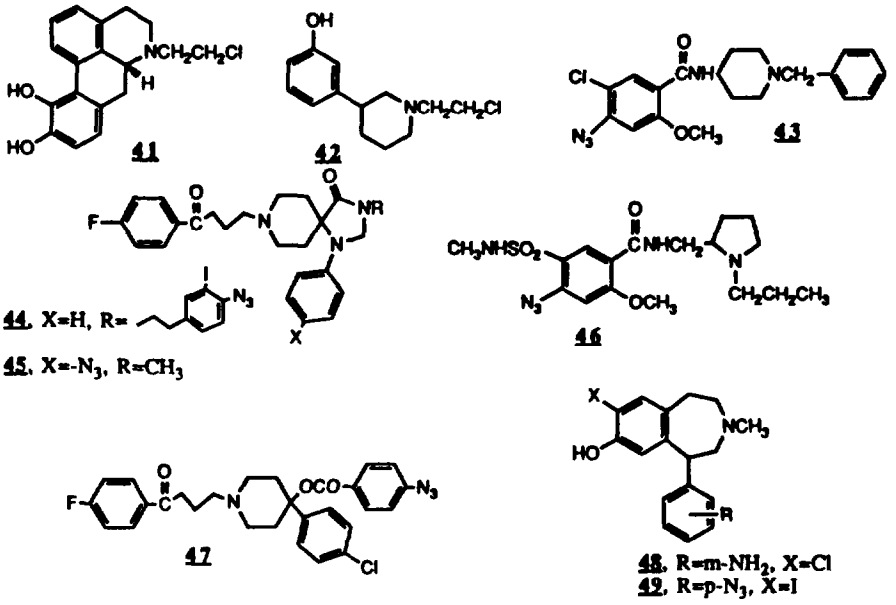
stereotypy (Seeman 1980). The first selective striatal D_2-photoaffinity label was an azido-derivative of clebopride, 43 (Niznik et al. 1984, 1985a). Compound 43 also was found to inhibit [^{3}H]spiperone binding in human striatal membranes irreversibly and with high affinity (Wouters et al. 1984). Replacing the 5-chloro group of 43 with ^{125}I resulted in a D_2-selective photoaffinity label with high specific radioactivity (Neumeyer et al. 1985; Niznik et al. 1985a, 1985b). The [^{125}I]azidoaryl-derivative of spiroperidol, 44, covalently labels a peptide of Mr 94,000 in rat striatal membranes, which has been suggested to be the D_2-receptor (Amlaiky and Caron 1985). An azidomethyl-derivative of spiperone, 45, covalently labels a peptide of Mr 92,000 in canine striatal membranes, although several minor lower molecular weight proteins also were labeled and this agent had a much lower specific activity than 44 (Niznik et al. 1986). Azidosulpride, 46, was used to photolabel D_2-receptors in rat striatal, anterior pituitary, and olfactory bulb tissues (Redouane et al. 1985). The radiolabeled receptors from the three tissues were concentrated in a single band at Mr 85,000, with low nonspecific incorporation (Redouane et al. 1985). Azidohaloperidol, 47, selectively photolabels D_2-receptors in bovine striatum and has been used to identify the photolabled D_2-receptor binding subunit (Mr 94,000) by antibodies specific for haloperidol (Kanety and Fuchs 1988).

Selective tools for the identification of the D_1-receptor protein more recently have become available. After radioiodination of an amino-derivative of the D_1-selective antagonist SCH 23390, 48 a radioactive probe that binds selectively and with high affinity to D_1-receptors resulted (Amlaiky et al. 1987). By crosslinking 48 with the heterobifunctional reagent SANPAH, this compound was covalently incorporated into a peptide of Mr 72,000, representing the D_1-receptor subtype (Amlaiky et al. 1987). A closely related azido-analog, 49, was effectively used to photolabel D_1-receptors in canine, bovine, and porcine striatal membranes and covalently incorporated into a peptide with Mr 75,000, as well as minor bands at Mr's 62,000 and 51,000 (Baindur et al. 1988; Niznik et al. 1988).

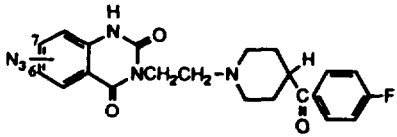
SEROTONIN RECEPTORS

Serotonin receptors have been identified in various areas of the brain and have been classified into three major subtypes: 5-HT_1, 5-HT_2, and 5-HT_3. These subtypes have been further divided into subclassifications but, thus far, irreversible ligands only have been developed for the 5-HT_2 and 5-HT_{1A} subtypes. The 5-HT_2 site has been associated with the mediation of the hallucinogenic action of phenylisopropylamine drugs, such as DOM (Glennon et al. 1984; Glennon and Hauck 1985). The 6- and 7-azido derivatives 50 and 51, respectively, of the 5-HT_2 receptor selective antagonist ketanserin, have been prepared as photoaffinity probes for these and the histamine receptors

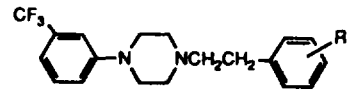
(Wouters et al. 1985a, 1985b). Incorporation of an azido-moiety in either the 6- or 7-position of ketanserin increased the affinity of both compounds for the histamine receptors while retaining equal affinity for the 5-HT_2 receptors; however, compound 51 photolabels both receptors, whereas 50 did not (Wouters et al. 1985b). The 8-[^{125}I]-analog of 51 was recently used to photolabel a 73,000-dalton component of the monoamine transporter of bovine chromaffin granule membranes (Isambert et al. 1989). The previously described metaphit, 20, has also been reported to deplete 5-HT_2 sites as well as PCP binding sites (Nabeshima et al. 1989).



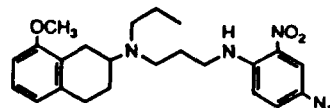
The bromoacetyl derivative of TFMPP, 52, was designed as a 5-HT_1 receptor alkylating agent (Ransom et al. 1985). This compound distinguishes the same two subpopulations of [^{3}H]5-HT binding sites as spiperone, in rat cortex, but receptor alkylation was not achieved (Ransom et al. 1985). However, the *para*-azido derivative of TFMPP, 53, photolabeled a single class of sites in rat hippocampal membranes with high affinity (Ransom et al. 1986). A polypeptide of Mr 55,000 was irreversibly labeled with 53, which is suggested to be the 5-HT_{1A} receptor or a subunit thereof (Ransom et al. 1986). An electrophilic chloroamino-derivative of the 5-HT_{1A} selective agonist, 8-hydroxy-DPAT,



50, 51



52, R=m-NHCOCH$_2$Br
53, R=p-N$_3$



54

displayed irreversible but low-affinity binding to 5-HT$_1$ sites (Hamon et al. 1984; Emerit et al. 1985). The arylazido-derivative of this agent, 54, displayed selective, irreversible blockade of 5HT$_{1A}$ sites in rat hippocampal membranes, in the nanomolar range (Emerit et al. 1986). Tritiated 54 photolabels a P$_1$ protein in hippocampal microsomal membranes with Mr 63,000, which is believed to be the 5-HT$_{1A}$ site (Emerit et al. 1987). A protein of similar molecular weight also has been isolated from human hippocampus and frontal cortex using photoaffinity labeling and immunoprecipitation techniques with the azido-spiperone derivative, 49, previously described (Raymond et al. 1989).

SUMMARY

Electrophilic and photoactivated agents have proven to be useful as receptor-selective irreversible probes. These compounds are generally derivatives of selected ligands that may be chemically modified in such a way as to retain high receptor affinity and selectivity while permitting covalent bonding to the receptor protein. The receptor systems described in this chapter are associated with a variety of classes of abused drugs. These irreversible agents are allowing the isolation and purification of these drug receptors to improve our understanding of their physiological and pharmacological properties and to aid us in better design of agents with therapeutic value without abuse or physical dependence liability. In the opioid field, ß-FNA and SUPERFIT have been successfully used to elucidate structure and function of the μ-, κ-, and δ-receptors, respectively. Recently, the δ- and κ-subtype selective irreversible ligands naltrindol and UPHIT have been introduced for receptor subtype studies that are designed to further clarify the physiological function of these sites. The PCP recognition site on the NMDA receptor complex and the sigma receptors have been characterized in part by the use of irreversible agents. These receptors, although sharing similar pharmacological properties, are clearly different systems and the irreversible agents described will allow their further

characterization. The central-type benzodiazepine receptors have been labeled with irreversible agents; and, now, receptor subsites that differentially recognize ß-carbolines and benzodiazepines have been discovered through studies using a ß-carboline photoaffinity probe. The associated chloride ionophore has been studied with electrophilic irreversible ligands, as well as the pharmacologically distinct peripheral-type benzodiazepine receptors. Physiological function of the latter receptor system has been implicated through the photoaffinity ligand PK 14105, which is highly selective for these sites. Receptor subtype selective irreversible ligands also have been prepared for both the dopamine and serotonin receptors systems in hopes of clarifying their physiological roles in the CNS.

It is clear that more selective irreversible compounds with higher affinity will continue to be in demand for further receptor characterization. In addition, radioligands with higher specific activity will continue to be important for molecular weight determination of receptor proteins and autoradiographic studies key to neuroanatomical localization of these sites.

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Important Compounds in the Cocaine Class: A Synthesis Overview

F.I. Carroll and Anita H. Lewin

INTRODUCTION

Previous reviews on tropane alkaloids have addressed the occurrence, chemistry, and pharmacology of cocaine and its analogs (Lounasmaa 1988; Clarke 1977; Fodor 1960; Holmes 1950). Another review was devoted solely to the chemistry of cocaine and its derivatives (Archer and Hawks 1976). An annotated bibliography of cocaine also has been published (Turner et al. 1988). This chapter presents an overview of the synthesis of cocaine and some of the more important cocaine derivatives and analogs that have been used for biochemical, pharmacological, and analytical studies. The chapter is restricted to compounds possessing the azabicyclo-[3•2•1]octane ring system, even though there is interest, from a biological standpoint, in other structural types. The chapter includes mass-labeled, radiolabeled, and nonlabeled compounds (figure 1).

The *Chemical Abstracts* name for natural (-)-cocaine is methyl [1R-(exo,exo)]-3-benzoyloxy-8-methyl-8-azabicyclo[3•2•1]octane-2-carboxylate; the 1R designation refers to the configuration of the natural cocaine ring structure following the convention of Cahn and colleagues (1966). For simplicity, the more familiar traditional names are used in this review. However, all structures derivable from the natural cocaine ring system are designated by an "R" prefix. Thus, natural cocaine and its three possible stereoisomers are designated (R)-cocaine (1), (R)-pseudococaine (2), (R)-allococaine (3), and (R)-allopseudococaine (4). These isomers differ by the relative configurations of the carbomethoxy and benzoyloxy substituents at C2 and C3, respectively, of the azabicyclo[3•2•1]octane skeleton. There was some confusion in the early literature with regard to the nomenclature of structures 3 and 4 (Findlay 1956; Bainova et al. 1960). Specifically, structure 4 originally was named allococaine and structure 3, identified subsequently, was named allopseudococaine (Findlay 1956). The opposite nomenclature since has been proposed (Bainova et al. 1960, 1964; Sinnema et al. 1968) and now is generally accepted. Table 1 helps to clarify this situation by listing the names and summarizing the

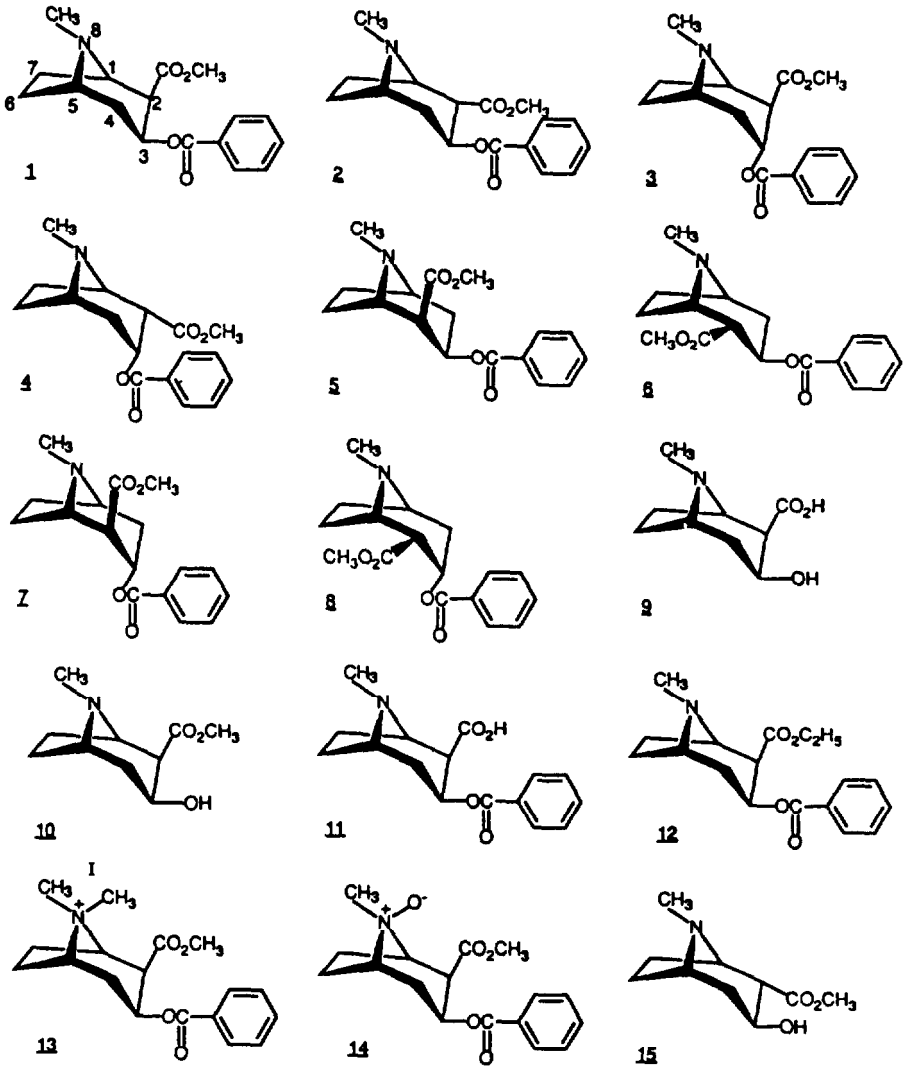


FIGURE 1. *Important compounds in the cocaine class*

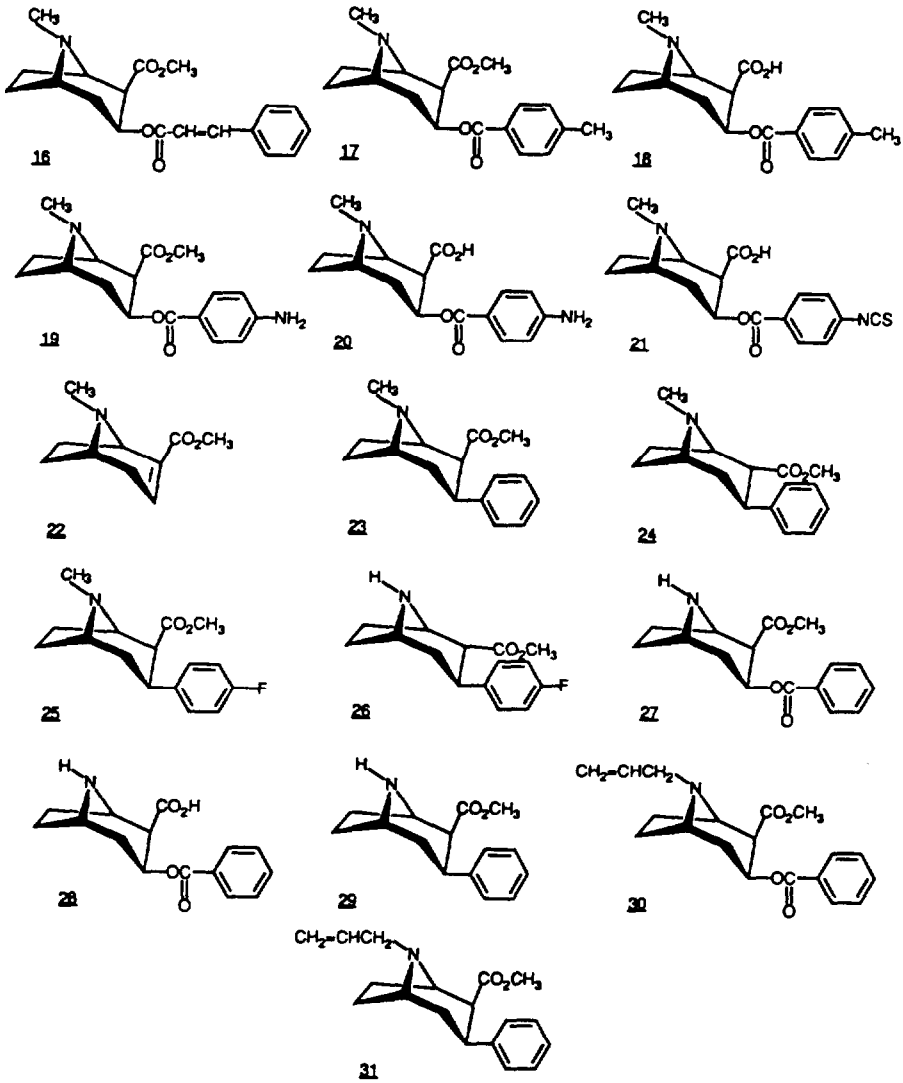


FIGURE 1. *Important compounds in the cocaine class (continued)*

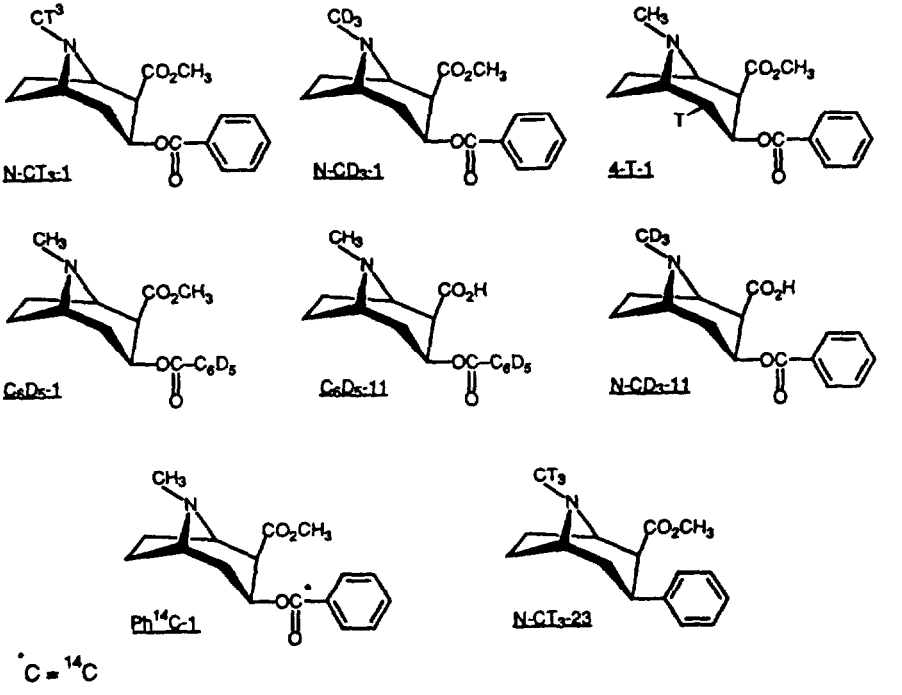


FIGURE 1. *Important compounds in the cocaine class (continued)*

stereochemical positions of the substituents in all eight cocaine isomers, assuming a chairlike conformation for the piperidine ring portion of the molecule. Thus, the prefix "pseudo" identifies equatorial carbomethoxy groups, and the prefix "allo" means that the benzoyloxy group is axial. The converse is true in the absence of a prefix.

COMPOUNDS PREPARED FROM (R)-COCAINE

Many of the (R)-cocaine analogs shown in figure 1 can be derived from commercially available natural (R)-cocaine using the reactions outlined in figure 2. Hydrolysis of (R)-cocaine in 3N hydrochloric acid gives (R)-ecgonine (9) (Bell and Archer 1960). Fisher esteriflcation of 9 using methanol and hydrochloric acid yields (R)-ecgonine methyl ester (10). If cocaine is hydrolyzed in neutral water, (R)-benzoylecgonine (11) is obtained (Findlay 1954). A modified hydrolysis using a 1:1 dioxane:water mixture at 55 to 60°C gave a cleaner reaction (unpublished work from the authors' laboratory). Fisher esterification of 11 using ethanol and hydrochloric acid affords the ethyl ester analog of cocaine 12 (McCurdy 1980). The methiodlde 13 and N-oxide 14 of (R)-cocaine are

TABLE 1. *Stereochemistry and nomenclature of the isomeric cocaines*

Structure	Position of Substituent		Common Name
	Carbomethoxy	Benzoyloxy	
1	Axial	Equatorial	(R)-Cocaine
2	Equatorial	Equatorial	(R)-Pseudococaine
3	Axial	Axial	(R)-Allococaine
4	Equatorial	Axial	(R)-Allopseudococaine
5	Axial	Equatorial	(S)-Cocaine
6	Equatorial	Equatorial	(S)-Pseudococaine
7	Axial	Axial	(S)-Allococaine
8	Equatorial	Axial	(S)-Allopseudococaine

prepared by treating (R)-cocaine with methyl iodide in the dark in acetone or with m-chloroperoxybenzoic acid, respectively (Tessel et al. 1978; Misra et al. 1979).

Because the 2-carbomethoxy group in R-cocaine is in the less stable axial orientation, subjection of (R)-cocaine to hydrolysis conditions using potassium methoxide in anhydrous methanol gives (R)-pseudoecgonine methyl ester 15, which has the 2-carbomethoxy group in the more stable equatorial position (Findlay 1954). Benzoylation of 15 with benzoyl chloride in pyridine gives (R)-pseudococaine (2) (Lewin et al. 1987).

The synthesis of several (R)-cocaine analogs starting with (R)-ecgonine methyl ester (10) is shown in figure 3. Treatment of 10 with trans-cinnamoyl chloride in pyridine gives the (R)-trans-cinnamoylcocaine 16, a cocaine analog that also is found in the *Erythroxylon* coca plant (Turner et al. 1981). Acylation of 10 with p-methylbenzoyl chloride gives the cocaine analog 17, which has been referred to as (R)-p-toluoylecgonine methyl ester, (R)-p-methylcocaine, or (R)-p-toluoylcocaine (Singh et al. 1979; Seyda and Chrupala 1986). Hydrolysis of 17 under conditions similar to those used for the preparation of 11 affords (R)-p-toluoylecgonine (18) (unpublished work from the authors' laboratory). (R)-p-aminococaine (19) is prepared from 10 by acylation with p-nitrobenzoyl chloride followed by catalytic reduction of the nitro function (Soffer and Schneider 1975). Hydrolysis of 19 affords (R)-p-aminobenzoyl-ecgonine (20), which gives the isothiocyanate derivative (21) on treatment with thiophosgene (Soffer and Schneider 1975).

In addition to being a pyrolysis product of cocaine, anhydroecgonine methyl ester (22) is a useful intermediate for the preparation of some important cocaine analogs. These syntheses are outlined in figure 4. Treatment of (R)-ecgonine with phosphorus oxychloride followed by methanol gives 22 (Clarke et al. 1973).

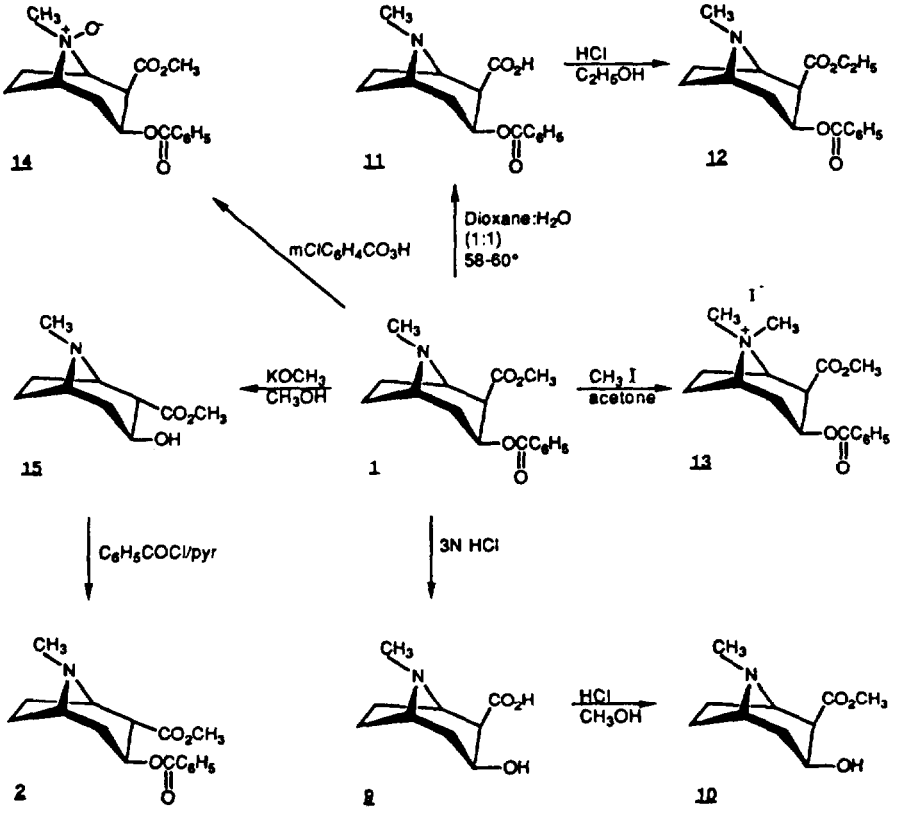


FIGURE 2. *Syntheses starting from cocaine (14)*

When 22 is treated with phenylmagnesium bromide in ether at -20°C a 1:3 mixture of (R)-WIN35,065-2 (23) and (R)-WIN35,140 (24) is obtained; these compounds can be separated chromatographically. If p-flurophenylmagnesium bromide is used in place of phenyimagnesium bromide, (R)-WIN35,428 (25) and the α-isomer 26 (Clarke et al. 1973) are obtained.

(R)-Norcocaine (27) has been prepared by several methods. The best method (figure 5) involves treatment of (R)-cocaine (1) with 2,2,2-trichloroethyl chloroformate to yield a carbamate that, when treated with zinc dust in acetic acid, leads to the desired 27 (Baldwin et al. 1977; Borne et al. 1977). Subjection of (R)-benzoylecgonine (11) and (R)-WIN35,065-2 (23) to a similar sequence provides (R)-norbenzoylecgonine (28) and (R)-WIN35,981 (29),

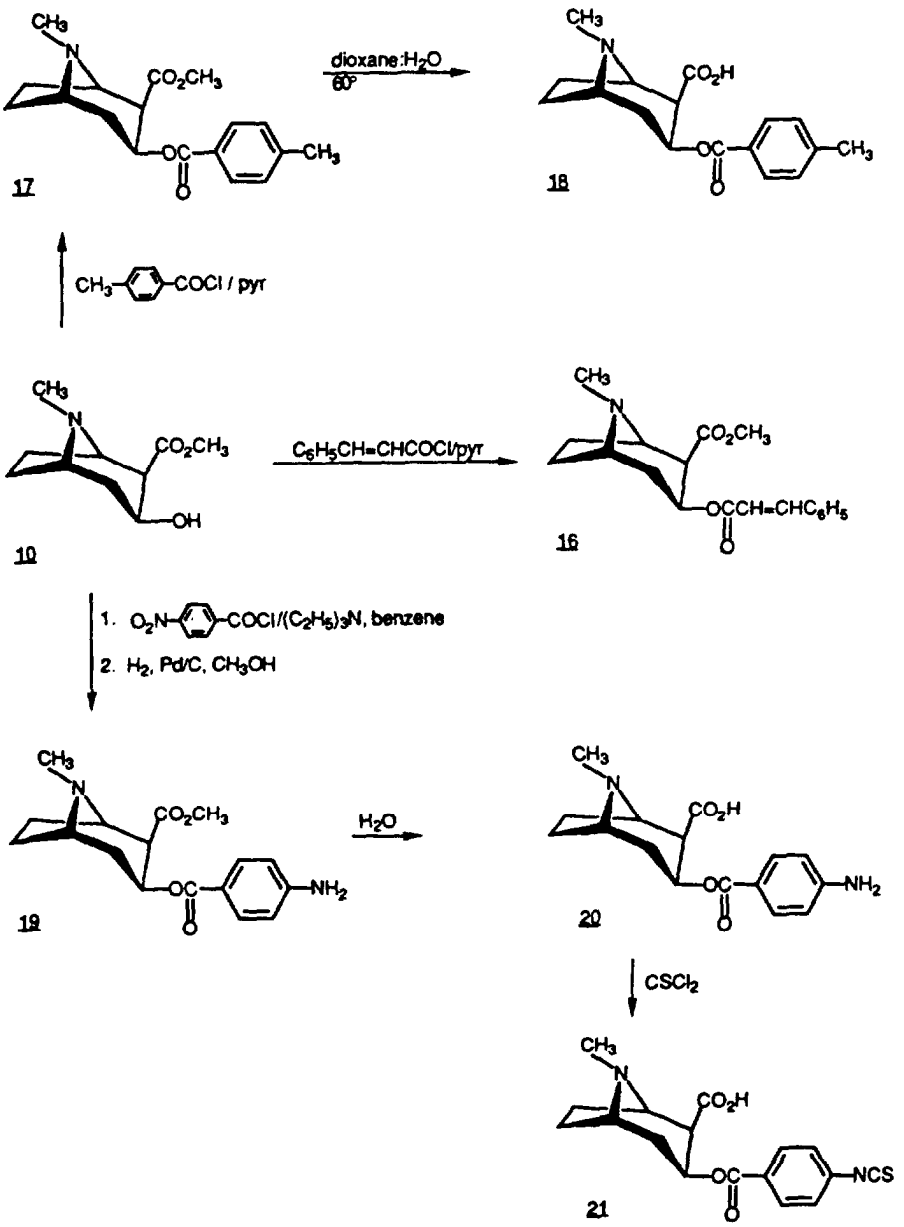


FIGURE 3. *Syntheses starting from ecgonine methyl ester (17)*

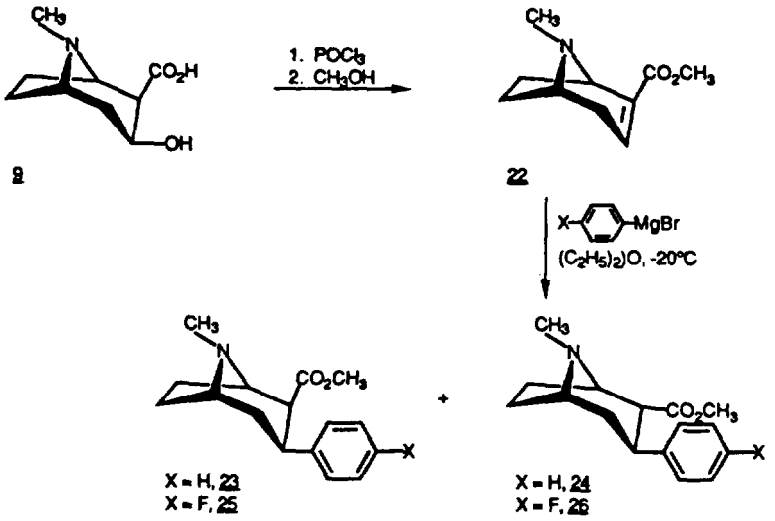


FIGURE 4. *Syntheses starting from ecgonine (9)*

respectively (unpublished work from the authors' laboratory). The latter also has been prepared (Clarke et al. 1973) via the 2-chloroethylcarbamate derivative of WIN35,065-2. Alkylation of 27-29 provides N-alkyl-N-nor derivatives of (R)-cocaine, (R)-benzoylecgonine, and (R)-WIN35,065-2, respectively. For example, alkylation of 27 and 29 with allyl bromide leads to N-allyl-N-norcocaine (30) and the N-allyl derivatives of WIN35,065-2 (31), respectively (Lazer et al. 1978; Kamien et al. 1989). The use of 27-29 for preparing mass-labeled and radiolabeled derivatives is addressed in a later section.

COMPOUNDS PREPARED FROM 3-TROPINONE

The facile condensation of 3-tropinone (32) with dimethyl carbonate using sodium hydride as the base (figure 6) to yield 2-carbomethoxy-3-tropinone (RS)-33 (Carroll et al. 1982) makes 32 a useful starting point for several of the compounds shown in figure 1. The first synthesis of (RS)-33, reported by Wilstatter and colleagues (1923), involved the condensation of acetone-dicarboxylic acid monomethyl ester, methylamine, and succindialdehyde. Several improvements of this synthesis have been reported, the latest by Casale (1987). Resolution of (RS)-33 using (+)- and (-)-tartaric acid provides (R)-2-carbomethoxy-3-tropinone [(R)-33] and (S)-2-carbomethoxy-3-tropinone [(S)-33] (Findlay 1957; Clarke et al. 1973; Casale 1987). Sodium borohydride

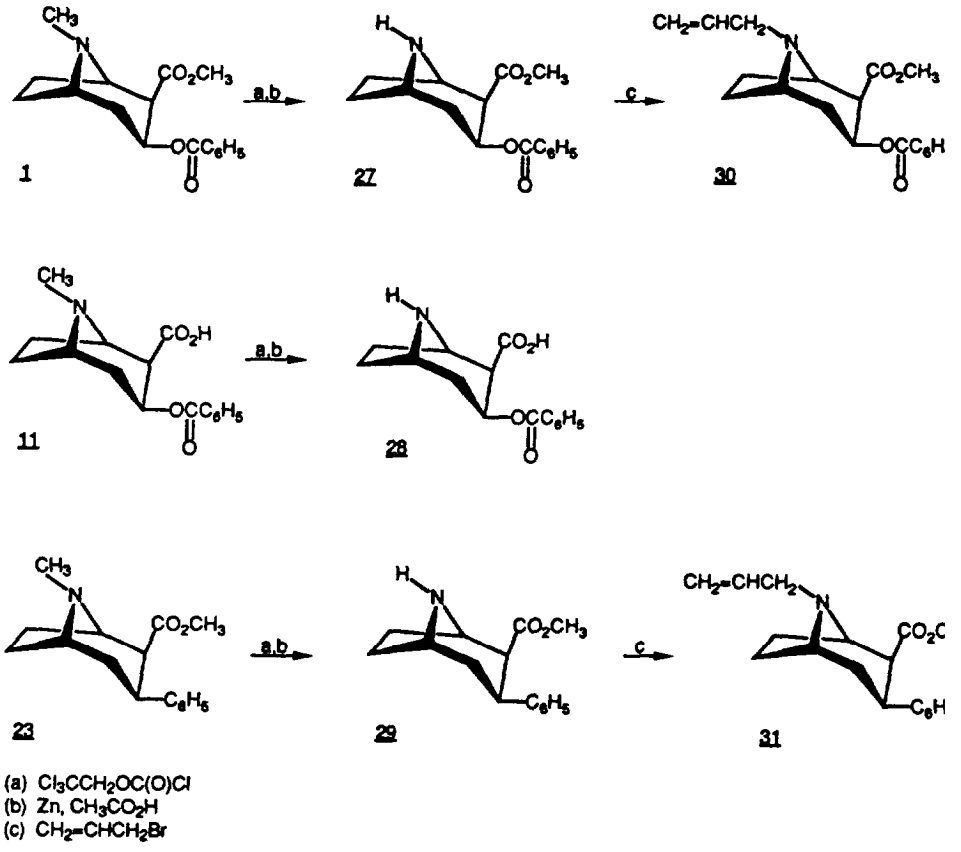


FIGURE 5. *Synthesis of N-norcocaine (27) and N-norcocaine-derived compounds*

reduction of (R)-33 in methanol at -20°C (as described for (RS)-33 in Carroll et al. 1982) provides (R)-allopseudoecgonine methyl ester (34), which on benzoylation gives (R)-allopseudococaine (4) (unpublished work from the authors' laboratory). When 34 is heated in water, isomerization at the 2-position takes place along with hydrolysis to give (R)-alloecgonine (35) (Sinnema et al. 1968). Fisher esterification of 35 using methanol and hydrogen chloride gives (R)-alloecgonine methyl ester (37), which is benzoylated to give (R)-allococaine (3).

(S)-cocaine was first prepared by reduction of (RS)-33 with sodium amalgam to a mixture of (RS)-ecgonine methyl ester and (RS)-pseudoecgonine methyl

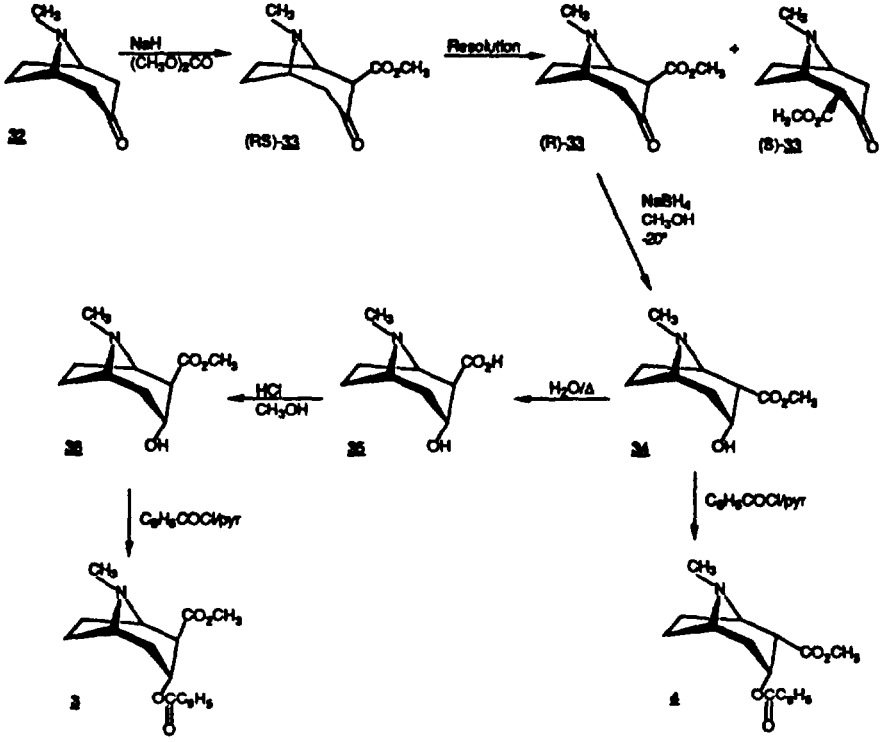


FIGURE 8. *Synthesis of analogs in the alloseries and allopseudoseries*

ester. The latter, being less soluble, was separated by crystallization: the mother liquor yielded (RS)-ecgonine methyl ester, which was benzoylated to give (RS)-cocaine. Resolution via the bitartrate salt gave (S)-cocaine (5) (Wilstatter et al. 1923). A more practical synthesis of (S)-cocaine (5) starts with (S)-33 and incorporates several reported improvements to the Wilstatter synthesis (Lewin et al. 1987). The improved synthesis of (S)-cocaine (5) as well as the synthesis of other (S)-cocaine isomers is outlined in figure 7. Reduction of (S)-33 with sodium amalgam provided (S)-ecgonine methyl ester (37) and (S)-pseudoecgonine methyl ester (38), which were separated by chromatography and benzoylated with benzoyl chloride in pyridine to give (S)-cocaine (5) and (S)-pseudococaine (6), respectively (Lewin et al. 1987). (S)-Allococaine (7) and (S)-allopseudococaine (8) were prepared via intermediates 39-41 by procedures exactly analogous to those described for the (R)-isomer (unpublished work from the authors' laboratory).

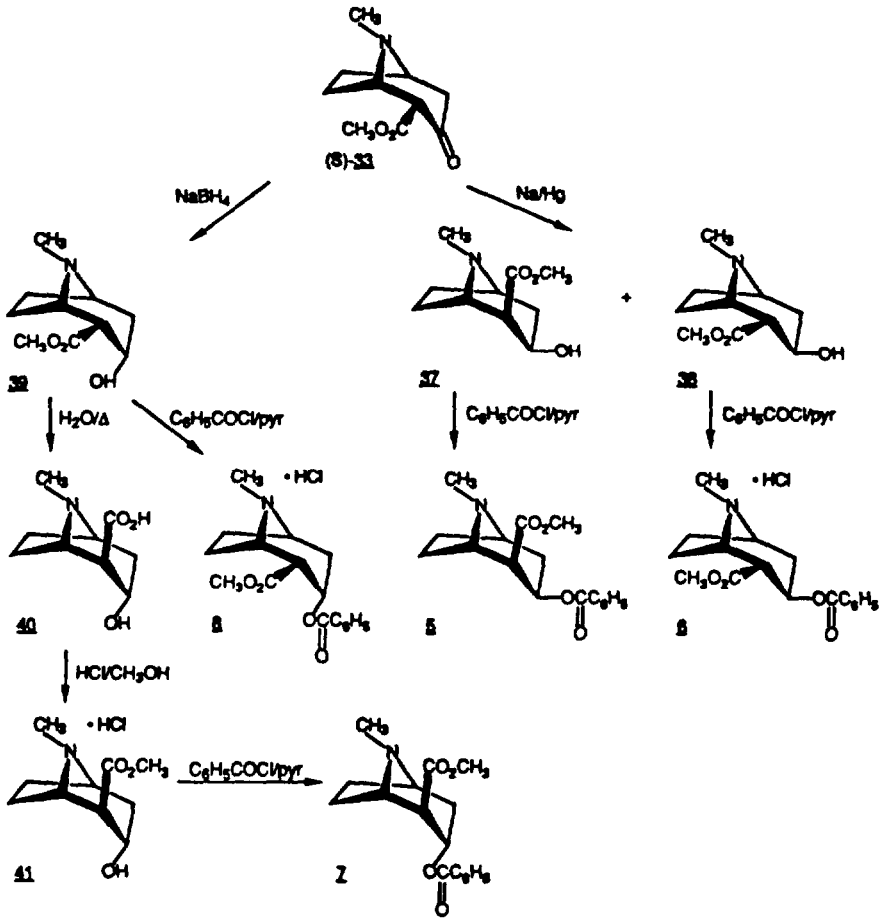
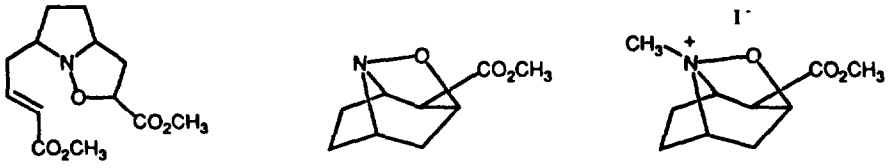


FIGURE 7. *Synthesis of (S)-cocaine and analogs*

Tufariello and coworkers (1979) reported an interesting multistep synthesis of (RS)-cocaine that involved the preparation of hexahydropyrrolo[1,2-b] isoxazole (42) as a key intermediate. When 42 was refluxed in xylene, methyl acrylate was expelled; the resulting nitrone spontaneously cyclized to 43. Methylation of 43 with methyl iodide gave the methiodide 44 that on treatment with activated zinc in aqueous acetic acid provided, stereo-specifically, (RS)-ecgonine methyl ester that could be benzoylated to afford (RS)-cocaine (figure 8).



42 43 44

FIGURE 8. *Intermediates in the Tufariello and colleagues (1979) synthesis*

ISOTOPICALLY LABELED COMPOUNDS

The preparation of isotopically labeled cocaine and derivatives has been reviewed (Archer and Hawks 1976). Compounds prepared since then fall into three categories: (1) N-methyl-labeled, (2) benzoyloxy-labeled, and (3) ring-labeled. The preparation of the isotopically labeled compounds in figure 1 uses the synthetic intermediates and route described above.

N-Methyl-Labeled Compounds

Cocaine deuterated or tritiated in the N-methyl moiety (N-CD_3-1 and N-CT_3-1, respectively) is obtained on treatment of N-norcocaine (27) (figure 5) with labeled iodomethane in the presence of potassium carbonate. Although such alkylation reactions usually are carried out in polar media, such as dimethylformamide and with excess alkylating agent, this alkylation can be performed successfully in toluene (the solvent in which high specific activity tritiated iodomethane is supplied) and with excess substrate (unpublished work from the authors' laboratory). The analogously labeled WIN compound N-CT_3-23 is prepared similarly from the N-nor compound 29 (Naseree et al., in press). Saponification of the carbomethoxy group of N-CD_3-1 under mild conditions affords N-CD_3 benzoylecgonine (N-CP_3-11) (unpublished work from the authors' laboratory).

Benroyloxy-Labeled Compounds

Benzoylation of ecgonine methyl ester (10) with labeled benzoyl chloride in excess pyridine affords the cocaines labeled in the benzoyl moiety, C_6D_5-1 and Ph^{14}C-1 (unpublished work from the authors' laboratory). The required ^{14}C-benzoyl chloride is prepared by carbonation of phenylmagnesium bromide using ^{14}C-barium carbonate as the source for ^{14}C-carbon dioxide followed by treatment of the isolated ^{14}C-benzoic acid with excess oxalyl chloride (unpublished work from the authors' laboratory). Dilution of Ph^{14}C-1 with

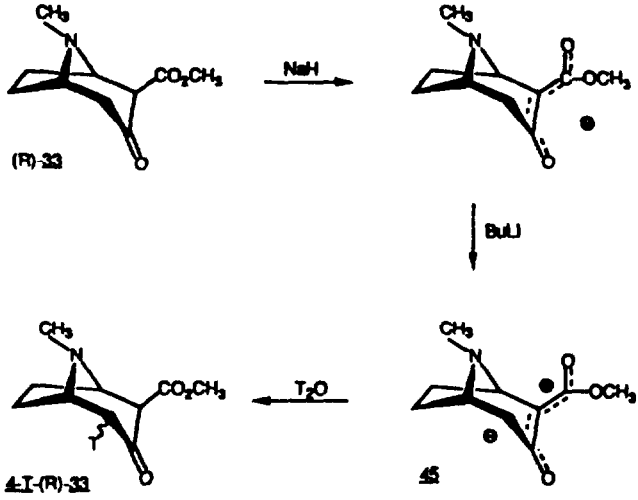


FIGURE 9. *Synthesis of [4-^{3}H]-2carbomethoxy-3-tropinone[4-T-(R)-33]*

unlabeled 1 gives specific activity 5 mCi/mmol, suitable for autoradiography. Mild saponification of the deuterated cocaine C_6D_5-1 gives pentadeutero benzoylecgonine (C_6D_5-11).

Ring-Labeled Compounds

Synthesis of cocaine with an isotopic label at the nonlabile, nonexchangeable 4-position of the backbone makes use of 2-carbomethoxy-3-tropinone [(R)-33], a key intermediate in the total synthesis of cocaine. The label is introduced at the 4-position by quenching of the dianion 45, (figure 9), obtained by treatment of (R)-33 with a single equivalent of sodium hydride followed by less than an equivalent of n-butyl lithium, with tritium oxide. Processing the labeled 2-carbomethoxy-3-tropinone 4-T-(R)-33, as described for the synthesis of (S)-cocaine (5) (figure 7), affords 4-T-1 (Lewin et al. 1983). Use of 0.17 equivalents of tritium oxide gives 4-T-1 with specific activity 3.2 Ci/mmol.

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Immunomodulatory Effects of Drugs of Abuse and the Importance of Structure-Immunomodulatory Activity Studies

Rae S. Rapaka and Charles P. Holberg

The discovery of endogenous ligands for the opioid receptors (Hughes et al. 1976) and the demonstration of receptors on the surface of the cells of the immune system generated an explosive interest in the role of opioid peptides and neuropeptides in the regulation of the immune system. Because of the acquired immunodeficiency syndrome (AIDS) epidemic, interest in the effects of drugs of abuse has increased recently. Even in the absence of AIDS, understanding the mechanisms of modulation of the immune system by neuropeptides, opioid peptides, and drugs of abuse is an important topic (Kind 1988). Clearly, a compromised immune system predisposes the individual to infectious diseases and complicates treatment procedures considerably.

Treatment of addictive disorders usually involves long-term treatment; hence, it is imperative that their effects on the immune system be understood. If new medications are developed, it is conceivable that the new compounds will be structurally related to the drugs of abuse and may be immunologically active. It is particularly important to avoid using immunosuppressive drugs in a patient population that is already immunocompromised. Hence, the immunomodulatory effects of new drugs must be investigated before embarking on extensive studies for the drug approval process. Immune effects of new treatment drugs must be determined; the chemical classes of drugs of abuse to be investigated include neuropeptides, opiates, opioid peptides, marijuana and cannabinoids, phencyclidine and its analogs, cocaine and its analogs, and inhalants.
There are several studies on the use of opiates in animals, particularly morphine (Fuchs et al. 1987; Louria et al. 1967; for reviews, see Donahoe 1988; Kreek 1988; Pham and Rice 1990; Weber 1988; Yahya and Watson 1987). Chronic administration of morphine to mice depressed the responsiveness of lymphocytes to concanavalin A (Con A) stimulation when compared to lymphocytes of nonaddicted mice; this suppressive effect was

somewhat reversible if naloxone was coadministered with morphine. Morphine administration to mice resulted in an enhanced susceptibility to bacterial and fungal infections; this increased susceptibility was due to a decrease in reticuloendothelial system activity and a reduction in the number of phagocytes. Morphine administration reduces the capacity to produce superoxide anion (a parameter to measure macrophage activation), primary antibody response to sheep red blood cell (SRBC), and the ratio of spleen to body weight. Some of the above effects were again naloxone-reversible, suggesting these effects may be mediated via the opiate receptor. Morphine also was shown to reduce natural killer (NK) activity, lymphoid organ weight, and mitogenic response to Con A and interferon titers. In rats chronically treated with morphine, cell-mediated immunity (CMI) is altered, as evidenced by the animals' inability to produce a tuberculin reaction and a lowered ability to produce an inflammatory response to carrageenan.

Studies by Tubaro and colleagues (1983, 1985) showed a severe depression of phagocytosis, NK cell activity, and superoxide anion generation in opiate addicts. McDonough and coworkers (1980) reported a decrease in absolute number of T lymphocytes in the peripheral blood of opiate addicts as measured by the ability to form rosettes with SRBC. Naloxone was able to reverse some of the above effects. Heroin produced similar immunosuppressive effects to morphine. Nair and coworkers (1986) and others (Novick et al. 1986; for reviews, see Donahoe 1988; Weber 1988) reported that NK cell activity is significantly reduced in street heroin addicts. Medical status of heroin addicts entering clinics was studied by Kreek (1988), whose observations included absolute lymphocytosis and elevated serum levels of IgM and IgG. Brown and coworkers (1974) observed abnormalities in T-cell rosette formation and diminished response to mitogens. Several investigators (for a review, see Morley et al. 1987) reported that in heroin addicts the ratio of T-helper to T-suppressor cells was depressed. Evidently, the depressed immune function by the opiates makes the animals more susceptible to bacterial and fungal infections. Several other studies supported the above results. Thus, most of the human studies indicate the immunosuppressive action of opiates.
A few other opiates also were studied. Neither (+) nor (-) naloxone have significant effect on NK cell activity (Ochshorn et al. 1988a). Similarly, (+) and (-) methadone do not appear to have any significant effect on NK activity (Ochshorn et al. 1988b); however, at higher doses a significant reduction of NK cell activity was observed (Kreek 1990).

At present, a preferred treatment modality for opiate addicts is treatment with methadone. Brown and coworkers (1974) evaluated heroin addicts entering clinics and found elevated levels of IgM and IgG. After methadone maintenance treatment, these abnormalities lessened. Cushman and Grieco

(1973), Cushman and coworkers (1977), and several other groups (Kreek 1988; for reviews and references, see Pham and Rice 1990) showed that abnormal T rosetting found in most heroin addicts decreased with time following methadone treatment. Methadone treatment still is not the ideal mode of treatment due to some of its immunomodulatory effects. Donahoe (1988) and other groups (Kreek 1988) reported that the effects of methadone may be dose-dependent and that immunoenhancing effects predominate in the low doses of methadone used for treatment. Thus, new and more efficient treatment modalities are needed not only for the treatment of opiate addiction, but also for problems arising out of usage of other drugs of abuse.

Wybran and coworkers (1979) were the first to provide evidence for the presence of receptors specific for morphine on normal human T lymphocytes, and this observation was confirmed by several other investigators. Opioid receptors later were demonstrated to be present on human polymorphonucleocytes and monocytes and platelets (for a review, see Sibinga and Goldstein 1988). Presently, receptors have been shown to be present on the cells of the immune system for opiates, opioid peptides, phencyclidine, benzodiazepine, and dopamine. Another exciting development in this area is the report by Donahoe (1990) suggesting that opiates modulate the expression of HIV-1 viroreceptor, the CD4 molecule. The presence of receptors for chemical agents on the immune cells presents possibilities for modulation of specific immune function by exogenousiy administered drugs.

However, a factor that complicates the *in vivo* studies is the necessity to discriminate carefully the observed effects as either direct or indirect. The situation is made even more complex because opioid peptides are involved with the modulation of the immune system via the autonomic nervous system and the endocrine system, as well as within the central nervous system.

Several reports from animal studies indicate that opioid peptides enhance the immune function (for a review, see Morley et al. 1987). Several investigators reported that beta-endorphin-enhanced NK activity in *in vitro* and *in vivo* studies (Faith et al. 1984; Mathews et al. 1983). Other studies in animals have shown that opiates are involved with the modulation of tumor growth. Beta-endorphin potentiates Con A and phytohemagglutinin-induced proliferation of T lymphocytes but had no effect on the response to the B-cell mitogen lipopolysaccharide/dextran sulfate (Gilman et al. 1982). On the other hand, McCain and coworkers (1982) reported the beta-endorphin is a potent suppressor of phytohemagglutinin-induced blastogenesis in cultured human T cells. The effect of beta-endorphin on the lymphocyte function appears to be due to the fragment beta-endorphin 10-16 peptide fragment. Studies by Kay and colleagues (1987) indicate that the ability of the endorphins to stimulate NK

cell activity resides in 6-9 sequence peptide fragment, which is part of the helical portion. It is further speculated that the alpha-helical portion is involved in the interaction with the receptor. Beta-endorphin enhances *in vitro* antibody production, NK function, and mitogenesis, while alpha-endorphin, which is a slightly modified analog, is a potent suppressor of *in vitro* antibody response. Recently, Shah and coworkers (1989) reported on the conformational features for interaction at the viroreceptor. Beta-bend conformations of several tetrapeptides related to peptide T were correlated with their activities in CD4-receptor binding assays. It is to be especially noted that enkephalins (for reviews, see Schiller 1984; Rapaka et al. 1986) and dermorphins were shown to exist with beta-turn conformations. It may be speculated that the therapeutic effects of enkephalins in general and Met-enkephalin in particular may be due to beta-bend conformation that might serve as a conformational recognition site for interaction with the CD4 receptor. Research into the conformational analysis only has begun in these peptides, and it is unfortunate that extensive and systematic structure-immunological activity studies have not been undertaken by any research group. Only a few studies have been conducted to date on some of the opioid peptides, and these studies are inadequate to formulate any meaningful structure-activity correlations. Several other studies (Kay et al. 1984; Faith et al. 1984; Mathews et al. 1983; Morley et al. 1987) reported that endogenousopioid peptides are immunoenhancers and Met-enkephalin and beta-endorphin enhance NK cell activity. Several investigators also have reported that opioids are mildly chemotactic. Mandler and coworkers (1986) and Brown (1986) reported that beta-endorphin and Met-enkephalin augment interferon production. It also was reported that beta-endorphin modulates the expression of cell surface antigens. Miller and colleagues (1984) showed that beta-endorphin enhances the expression of HLA-DR antigen in mononuclear cells and reduces expression of T3 and T4 antigen in T lymphocyte.

The subtle differences in structure resulting in alteration of immunomodulatory properties merit further investigation because several such observations cannot yet be explained on structure-activity relationships (SAR) alone. Met-enkephalin increased the percentage of active T-cell rosettes formed by lymphocytes, an effect that is naloxone reversible, whereas the other naturally occurring analog, Leu-enkephalin, lacks the rosette-forming activity. Beta-endorphin and another opioid peptide, dynorphin, stimulate superoxide radical formation (Sharp et al. 1985), and this effect probably is not mediated through the opioid receptor. Met-enkephaiin and Leu-enkephalin significantly increase NK cell activity in isolated human peripheral blood lymphocytes, and Met-enkephalin and beta-endorphin enhance interferon production. An interesting report by Sinha and coworkers (1988) indicates that immunologically active components of IMREG-1 (a low molecular weight subfraction derived from human leukocyte dialysate) are Tyr-Gly and Tyr-Gly-Gly. As these sequences

are present in enkephalins and endorphins, they may function as molecular links between the neuroendocrine and immune systems. Note especially that Tyr-Gly and Tyr-Gly-Gly are metabolites of enkephalins and are devoid of analgesic activity; however, they are active immunologically. Thus, there are only a few observations reported on the SAR of these compounds.

Due to the immunoenhanclng properties of Met-enkephalin, this peptide was tried clinically. Met-enkephalin appears to enhance the immunostatus of the patients with AIDS and AIDS-related complex (Wybran et al. 1987; Bhargava 1990). In Met-enkephalin-treated patients, there was a significant increase in the absolute number of blood CD3 and CD4 lymphocytes without an increase in the absolute number of lymphocytes and a significant increase in NK cell activity, interleukin production, and the phytohemagglutinin response. Although these are only preliminary studies, they provide optimism on the potential use of this compound or its analogs in future and encourage SAR studies and rational drug design.

There are significant differences between the opiates and the opioid peptides in their immunomodulatory properties. For example, morphine depresses E-rosette formation, whereas beta-endorphin and Met-enkephalin enhance it. Morphine decreases superoxide formation, whereas Met-enkephalin and dynorphin increase the formation. Opiates, in general, appear to be suppressive, whereas opioid peptides are immunoenhancers. These contrasting effects are not particularly discouraging because they pave the way for extensive SAR studies to enable medicinal chemists to design new immunoenhancing drugs.

lmmunomodulatory effects of marijuana were reviewed recently by Yahya and Watson (1987). Due to the widespread abuse of marijuana, several investigators focused their attention on the effects of marijuana on cardiac, psychomotor, genetic, and immune functions. Most studies on the immune function were performed with delta-9-tetrahydrocannabinol (THC), the major psychoactive ingredient of marijuana. THC exhibits a wide spectrum of effects in human and animal studies. Studies in animals revealed the immunosuppressive effects of THC in such parameters as phagocytic function, interferon production, and NK cell activity. Several investigators (Petersen et al. 1975; Lefkowitz and Chiang 1975; Zimmerman et al. 1977; Smith et al. 1978; Yahya and Watson 1987) have demonstrated the *in vivo* immunosuppressive effects of THC on humoral immune response of animals by such measurements as plaque-forming cells in spleen and hemagglutinin titers after THC administration.

In *in vitro* tests, THC was shown to produce a depressive response of mouse splenocytes against SRBC and reduced blastogenic response of splenic lymphocytes to E. coli lipopolysaccharide or pokeweed mitogen, and THC was shown to reduce CMI in animals. The immunosuppressive effects of THC on CMI were shown by *in vitro* and in *vivo* tests; the parameters investigated included delayed-type hypersensitivity to SRBC, rosette-forming T-cell numbers, blastogenic responses of lymphocytes to phytohemaggiutinin and Con A, skin graft survival, mixed lymphocyte reaction, and migration inhibition factor activity.

All these studies demonstrated the immunosuppressive effects of THC in animals and showed that animals exposed to THC were more susceptible to infections, both viral and bacterial.

The immunosuppressive effects of THC in humans are less convincing. Some of the effects attributed to marijuana or THC (Armand et al. 1974; Gupta et al. 1974; Petersen et al. 1976; Schwartzfarb et al. 1974; Nahas et al. 1979; Specter et al. 1986) include reduced serum IgG levels in chronic smokers, inhibition of NK cell activity, phagocytic activity, and reduced T-cell numbers and leucocyte migration. Studies from marijuana smokers showed that response of the cells to PHA stimulation was depressed, and lymphocytes isolated from marijuana smokers contained a reduced number of rosette forming T lymphocytes. However, several other investigators have reported that neither THC nor marijuana smoke are immunosuppressive, and only future research might be able to clarify these inconsistencies.

Effects of cocaine and its analogs on the immune function were investigated by a few investigators (Crary et al. 1983; Van Dyke et al. 1986; Harvas et al. 1987). Crary and colleagues (1983) found that cocaine rapidly increases NK cell activity in humans, and it was suggested that this effect might be due to an increase in the number of circulating cells. The increase in NK cell activity after *in vivo* administration probably is not due to metabolism of cocaine, because benzylecgonine, a major cocaine metabolite, had no effect on NK cell numbers or activity. It also is suggested that the *in vivo* effect of cocaine on NK cell activity may be due to its ability to stimulate the release of catechoiamine Into the bloodstream and to diminish the reuptake of norepinephrine by the sympathetic nervous system.

Welch (1983) investigated the effect of cocaine on nonspecific human cellular host defenses such as the oxidative microbicidal activity of polymorphonuclear leukocytes and lymphocyte NK activity. Welch found that such activities were found only at high concentrations (500 times those found in the blood of cocaine users).

Harvas and coworkers (1987) studied the effect of cocaine on three parameters of immune response: antibody production, resistance to infection, and resistance to tumors. Their studies revealed that at low doses cocaine showed no effect; in fact, at high doses it enhanced some immune responses. Finally, note that opiates and some of the other drugs of abuse such as cocaine and alcohol have a common ability to modulate surface molecules on T cells. With opiates the effects are presumably mediated through receptor-ligand interaction, whether it is the T cell acting directly with the opiate or some hormonal product resulting from other systemic and centrally mediated points of opiate-receptor stimulation. With cocaine, a key effect may relate to its anesthetic properties of Na channel blockade that could alter surface-receptor expression within the network in various ways (Donahoe et al. 1986; Donahoe 1990).

Few studies have been done on phencyclidine (PCP) or its analogs. PCP was shown to interact with the cells of the immune system and cause suppression of lymphocyte DNA synthesis *in vitro,* and PCP receptor sites were shown to be present on membranes of lymphoid cells (Khansari and Whitten 1988; Singh and Fudenberg 1988).

Histories from AIDS patients revealed a relatively high use of amyl and butyl nitrites by inhalation (Marmor et al. 1982; for a review, see Haverkos and Dougherty 1988). An evaluation of the immune status of the individuals would be helpful to understand the effects of the inhalants on the immune system, if any.

Laboratory results have shown various conflicting reports. For example, the literature on the direct effects of opioids on NK activity is full of conflicting and sometimes contradicting reports. This is also the case with several immunochemical studies with opioids. For example, different reports suggest that peptide ligands may enhance NK activity, have no effect on NK activity, or may decrease NK activity. Opposite effects of opiates on immune function have been observed among species, as well as varied responses between strains of the same species.

There are obvious differences in the susceptibility of humans and animals to the immunomodulatory effects of marijuana and THC. Animal studies clearly demonstrate immunosuppressive effects, whereas in humans the effects are less clear. These inconclusive results may be due to fewer studies conducted and problems associated with dose.

The conflicting data also indicate how various parameters such as sex, stress, hormonal balance, nutritional status, age, length of exposure to the drug,

species variation, and use of other drugs may influence the results. Although on the surface these apparent complexities seem discouraging, the recognition of the key variables involved in the mechanisms by which drugs of abuse modulate immunological activity will help researchers to understand and manipulate the immune system. It is anticipated that rational drug design ultimately will yield potential therapeutic agents.

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FDA Regulatory Requirements for Investigational New Drugs and New Drug Applications: An Update

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INTRODUCTION

Since our previous article was published in the National Institute on Drug Abuse Research Monograph Series (Hoiberg and Rapaka 1986), several significant regulations have been promulgated by the Food and Drug Administration (FDA) concerning the regulatory requirements for human drug products. This chapter addresses these changes as they pertain to the requirements for the submission of investigational new drug applications (INDs) and new drug applications (NDAs). Other regulatory topics include orphan drug products, the Center for Drug Evaluation and Research (CDER) guidelines, environmental assessments, and two innovative procedures that have been implemented to facilitate and improve the review process (i.e., computer-assisted new drug applications [CANDA] and "NDA Days").

INVESTIGATIONAL NEW DRUG APPLICATIONS

In the 1987 article only the proposed revisions to the IND Rewrite regulations were discussed in detail. Since then, the following major rules or proposals have been published pertaining to the IND process: (1) the final rule for the IND Rewrite, (2) the treatment IND program, (3) subpart E of the IND provisions, and (4) the proposed parallel track protocols. Together, these revisions have altered or will alter significantly the development of investigational new drugs, particularly those administered to patients with serious or life-threatening diseases.

IND Rewrite

The final rule that revised the regulations governing the submission and review of INDs was published in the *Federal Register* (52 FR 87988) on March 19, 1987. The intent of these new regulations, called the "IND Rewrite," was to

facilitate the development of new beneficial drug therapies while ensuring the ability of FDA to monitor carefully the safety of patients participating in clinical investigations. The objectives of the IND Rewrite were to focus FDA's attention on protecting the safety of human test subjects during the early phase of clinical research and to give sponsors greater freedom to design, revise, and implement clinical research studies. IND sponsors now are encouraged to consult with FDA before submitting an IND to help ensure that the design of the major clinical trials will be acceptable. Conferences are suggested at the "end-of-Phase 2" and at the "pre-NDA" stage to facilitate dialog between the sponsor and FDA personnel to expedite NDA approval. As with the "NDA Rewrite," the use of the CDER guidelines is encouraged to provide sponsors with assistance in implementing the "IND Rewrite."

The regulatory requirements for an IND are codified in the IND Rewrite under part 312 of title 21 of the Code of Federal Regulations (CFR). The regulations state that an IND must be filed for all drug products used in clinical investigation that are subject to sections 505 and 507 of the Federal Food, Drug, and Cosmetic (FD&C) Act. Exemptions to these filing requirements now are permitted under 21 CFR 312.2(b), for example, when (1) the investigation of a lawfully U.S.-marketed drug product is not intended to be reported to FDA as a well-controlled study in support of a new indication for use nor is intended to be used to support any other significant change in the labeling of the drug; (2) the investigation does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risk (or decreases the acceptability of the risks) associated with the use of the drug product; and (3) the investigation is conducted in compliance with the requirements for institutional review set forth in 21 CFR 56 and with the requirements for informed consent set forth in 21 CFR 50.

If the exemptions do not apply, the sponsor must file an IND with the agency stating his or her intentions to conduct a clinical investigation with a specified drug product. The investigation of this new drug may commence 30 days [21 CFR 312.40(b)(1)] after the FDA receives an IND, unless the sponsor has been notified by FDA that the IND has been placed on clinical hold. Grounds for a clinical hold are described in 21 CFR 312.42. Examples of a basis for a clinical hold are as follows: (1) The human subjects are or would be exposed to an unreasonable and significant risk of illness or injury and (2) the application does not contain sufficient information required under 21 CFR 312.23 to assess the risks to subjects in the proposed studies. The IND Rewrite also provided for clinical holds during Phases 2 and 3 of the clinical investigation under 21 CFR 312.42(b)(2), for example, if the plan or protocol is clearly deficient in design to meet its stated objectives. The imposition of a clinical hold may be made by telephone or other means of rapid communication or in writing. The responsible

FDA Division Director is to provide the sponsor a written explanation of the basis of the hold in not more than 30 days after its imposition. Once the stated corrections or modifications are made by the sponsor, the investigation may proceed, but only after the FDA Division Director has notified the sponsor, unless stated otherwise in the clinical hold order.

In the IND Rewrite, the recommended content and format for an IND application are described in 21 CFR 312.23. The sponsor is required to submit an original and two copies of the IND to the agency. Each copy of the IND is to contain in the following order: (1) a cover sheet (Form FDA-1571); (2) a table of contents: (3) an introductory statement and general investigational plan; (4) an investigator's brochure; (5) protocols; (6) chemistry, manufacturing, and control information; (7) pharmacology and toxicology information; (8) previous human experience with the investigational drug; and (9) any additional or relevant information. If any of the required data have been submitted previously, the sponsor generally is not required to resubmit this material, but must cite the reference by name, reference number, volume, and page number.

Investigational Process

As described earlier (Hoiberg and Rapaka 1987), the clinical investigation of a previously untested drug generally is divided into three stages (i.e., Phases 1, 2, and 3). These phases (21 CFR 312.21) usually are conducted sequentially but may overlap. In general, a protocol for a Phase 1 study may be less detailed and more flexible than protocols for Phases 2 and 3. The Phase 1 protocol should provide an outline of the investigation and should specify in detail only those elements of the study that are critical to safety. In Phases 2 and 3, detailed protocols describing all aspects of the study should be submitted. The required contents of a protocol are described in 21 CFR 312.23(a)(6)(iii).

In general, the amount of chemistry, manufacturing, and control information required is less in the clinical pharmacology stage (Phase 1) than in the later stages of drug development. The chemistry material required under 21 CFR 312.23(a)(6) should be adequate to ensure the proper identification, quality, purity, and strength of the investigational drug so that subject safety will not be compromised. The application should include descriptions of the general method of preparing the drug substance and of the method of manufacturing and packaging of the drug product. A description of the composition and methods of control of the drug substance and of the drug product should be provided. FDA recognizes that modifications to the method of preparation of the new drug substance and the dosage form may be necessitated as the investigation progresses. Therefore, emphasis should be placed on the identification and control of the raw materials and the new drug substance.

Final specifications for all parameters for the drug substance and drug product are not necessarily expected until the end of the investigational process. The amount of information submitted depends on the scope of the proposed clinical trial. For example, although stability data are required in all phases of the investigation to demonstrate that the new drug substance and drug product are within acceptable chemical and physical limits for the planned duration of the proposed clinical trial, only limited stability data (e.g., accelerated studies) should be submitted if very short-term tests are proposed.

An important guiding principle in the FDA review of INDs is that safety concerns predominate at the beginning of the process to ensure that research subjects are not exposed to unreasonable risks. In later phases, FDA evaluates the scientific merit of the study protocols to ensure that planned clinical studies are capable of producing the valid information on safety and effectiveness that is necessary to obtain marketing approval.

The IND Rewrite also contains regulations (21 CFR 312.110) pertaining to investigational drug products that may be imported or exported for clinical trials. An investigational drug may be imported into the United States if it is subject to an IND that is in effect under 21 CFR 312.40 and if (1) the consignee in the United States is the sponsor of the IND, (2) the consignee is a qualified investigator named in the IND, or (3) the consignee is the domestic agent of a foreign sponsor. Regarding the export of investigational new drugs, the following requirements must be met: (1) An IND is in effect for the drug under 21 CFR 312.40, and each person who receives the drug is an investigator named in the application or (2) the FDA (International Affairs Staff) has authorized shipment of the drug for use in clinical investigation. These requirements do not apply to the export of new drugs approved for export under section 802 of the FD&C Act. Section 802 of the Drug Export Amendment Act of 1986 authorizes the commercial marketing of human drugs not approved for marketing in the United States. One provision under section 802 allows the export of unapproved drugs to 21 listed countries that have sophisticated health care regulatory systems.

The emergency use of an investigational drug was codified in the IND Rewrite under 21 CFR 312.36. These regulations provide for situations in which a physician decides that the emergency use of an investigational drug is required for a single patient and there is not adequate time for the submission of an IND in accordance with 21 CFR 312.23 or 312.34. FDA may authorize shipment of the drug; however, the physician's flrst step should be to contact the manufacturer of the drug and determine whether the physician may be added as an investigator under the manufacturer's IND. If the manufacturer elects not to add the physician to its IND, the physician should contact FDA by telephone

or other rapid communication. Usually, the requesting physician will be placed in contact with an FDA medical officer familiar with the drug who will review the proposed circumstances for its use. If the medical officer is satisfied that the emergency use of the drug is justified, he or she may authorize its shipment and use in advance of any formal written submission to the Agency. Except in extraordinary circumstances, such authorization will be conditional on the sponsor making an appropriate IND submission (Forms FDA 1571 and 1572) as soon as practicable after receiving authorization. If the sponsor fails to complete and submit the requisite forms within 30 days, the "emergency" IND will be deemed to have been canceled before use of the drug and any use of the drug will not have been in compliance with the FD&C Act. The emergency use "of a test article" also must be reported to the responsible Institutional Review Board within 5 working days (21 CFR 56.104[c]), and written, informed patient consent must be obtained.

Treatment IND Program

On May 22, 1987, FDA published a final rule (52 FR 19466) pertaining to treatment use and sale of investigational new drugs. Known as the "treatment IND program," these procedures allow promising drugs to be available to desperately ill patients as early in the drug development process as possible (before general marketing begins) and permit FDA to obtain additional data on the drug's safety and effectiveness. These procedures apply to patients with serious and immediately life-threatening diseases for which no comparable or satisfactory alternative drug or other therapies exist. A treatment IND may be permitted after sufficient data have been collected to show that the drug may be effective and does not have unreasonable risks. In the notice, FDA defined under 21 CFR 312.7 the conditions under which drug manufacturers may charge for investigational new drug products. These procedures are intended to provide sufficient incentives for drug manufacturers to make investigational new drugs available to patients before general marketing begins, but under sufficient safeguard so as to prevent commercialization of the product as well as to ensure the integrity of clinical trials. The sponsor may not charge a price larger than that necessary to recover costs of manufacture, research, development, and handling of the investigational drug. The FDA Commissioner may deny a request for treatment use if the available scientific evidence, taken as a whole, fails to provide a reasonable basis for concluding that the drug (1) may be effective for its intended use in its intended patient population or (2) would not expose the patients to an unreasonable and significant additional risk of illness or injury. To protect the integrity and timeliness of the clinical testing process and to ensure that the distribution under a treatment IND does not become a substitute for obtaining marketing approval, regulations were promulgated so that treatment INDs and protocols may be placed on clinical

hold if the sponsor is not pursuing marketing approval with due diligence. As of June 1, 1990, treatment IND status has been granted for 18 INDs, 6 of these for AIDS-related conditions. Examples of drugs distributed under the treatment IND provisions are (1) selegiline for Parkinson's disease with incomplete response to other agents, (2) pulmonary surfactants for preterm infants with risk of respiratory distress syndrome, and (3) beta-glucocerebrosidase for Type I Gaucher's disease.

Subpart E

On October 21, 1988, an interim rule (53 FR 41516) was published to facilitate the development, evaluation, and marketing of drugs intended to treat life-threatening and severely debilitating illnesses, especially where no satisfactory alternative therapies exist. These procedures, referred to as "Subpart E," focus on the entire drug development and evaluation process from early preclinical and clinical testing, through FDA evaluation of controlled clinical trials and marketing applications, to postmarketing surveillance. By treating the entire process as a coherent whole, the overall efficiency of the process was believed to be significantly increased. These new procedures were codified as part of the IND regulations by adding a new subpart E (21 CFR 312.80 through 312.88) and by adding a conforming amendment to the NDA regulations under a new paragraph, 21 CFR 314.25. A key component of the procedure is early consultation (e.g., pre-IND conferences, end of Phase 1 meetings) between FDA and the drug sponsor to seek agreement on the design of necessary preclinical and clinical studies needed to gain marketing approval. If the preliminary analysis of the test results during clinical investigation appears promising, FDA may ask the sponsor to submit a treatment protocol to be reviewed under the treatment regulations. Such a treatment protocol, if submitted and granted, would serve as a bridge between completion of early stages of clinical trials and the final marketing approval. Once the Phase 2 testing and analysis are completed by the sponsor and a marketing application is submitted, FDA will evaluate the data utilizing a medical risk-benefit analysis. As part of this evaluation, FDA will consider whether the benefits of the drug outweigh the known and potential risks and the need to answer remaining questions (Phase 3 clinical trials) about risks and benefits of the drug, taking into consideration the severity of the disease and the absence of satisfactory alternative therapy. If approval of a drug product is granted, the Agency may seek agreement from the sponsor to conduct certain postmarketing (Phase 4) studies to delineate additional information about the drug's risk, benefit, and optimal use. These procedures were modeled after the highly successful development, evaluation, and approval of zidovudine, the first drug approved to treat patients with AIDS. FDA believes that this program, taken as a whole, establishes a new and innovative approach to stimulating the development of

particularly important drugs, while building on past practices that have proven to be successful.

Parallel Track Protocols

The Public Health Service (PHS) proposed on May 21, 1990 (55 FR 20856), a policy to make promising investigational drugs for AIDS and HIV-related diseases more widely available under "parallel track" protocols, while ensuring that the controlled clinical trials essential to establish the safety and effectiveness of the new drug are carried out. Under the parallel track mechanism, eligible physicians and patients would be able to participate in open, nonconcurrently controlled studies and receive promising investigational drugs at an early stage in the drug development process. In studies where all patients receive the drug and there is no comparison group, the objective is not to provide substantial evidence of effectiveness; rather, it is designed to provide significant safety data, representing a wider patient experience than is usually possible in other, more rigorously designed studies. It is possible under this proposed procedure that investigational drugs being investigated for an HIV-related condition may be made available in a large open study even earlier than under the treatment IND mechanism. The process is referred to as a "parallel track" because drugs will be made available through studies that lack concurrent control groups to monitor drug safety and that are conducted in *parallel* with the principal controlled clinical investigations. Although persons with other life-threatening diseases also might desire to have investigational drugs available through the parallel track mechanism, the current proposal is limited to individuals with AIDS or HIV-related diseases. A similar mechanism previously was implemented to provide investigational drugs to persons with cancer. The FDA and the National Cancer Institute have described a special category of investigational drugs, "Group C" drugs, which may be provided by oncologists to appropriately chosen patients through protocols outside the controlled clinical trials before the drug's approval by FDA.

At the same time the parallel track protocols were announced, FDA proposed on May 21, 1990 (55 FR 20802), that it plans to amend two provisions of its regulations governing INDs. These provisions include additional grounds for placing an investigation on "clinical hold" and for terminating an IND. If adopted, FDA could require a sponsor to cease distributing an experimental drug in an open, nonconcurrently controlled investigation if any of several specified conditions exist. Two examples why FDA could place a proposed or ongoing investigation on clinical hold are: (1) There is reasonable evidence that the investigation that is not designed to be adequate and well controlled is impeding enrollment in, or otherwise interfering with the conduct or completion of, a study that is designed to be an adequate and well-controlled investigation

of the same or another Investigational drug or (2) insufficient quantities of the investigational drug exist to adequately conduct both the investigation not designed to be adequate and well controlled and the investigations that are so designed. These amendments were proposed to help implement the proposed PHS policy on expanded availability of promising investigational new drugs through the parallel track mechanism for people with AIDS or HIV-related diseases.

NEW DRUG APPLICATIONS

A comprehensive revision of the CFR pertaining to the procedures and requirements for the submission, review, and approval of new drugs and antibiotics for human use was published by FDA on February 22, 1985 (50 FR 7542). These regulations were fully discussed in a 1986 article (Hoiberg and Rapaka 1986).

FDA has not proposed any significant changes to these regulations since the CFR revision of 1985. Topics discussed later in this chapter that will impinge on material that should be submitted in an NDA are environmental assessments, CDER guidelines, and computer-assisted NDAs.

Orphan Drug Products

On January 4, 1983, the Orphan Drug Act was signed, which added four new sections to the FD&C Act (sections 525 through 528) to facilitate the development of drugs for rare diseases or conditions. This legislation was in response to the plight of patients with rare diseases who could not obtain potentially useful drugs and medical devices. These products have become known as “orphans.” The purpose of this legislation was to overcome the reluctance of applicants to become sponsors of orphan drugs and undertake the expense of conducting clinical trials and obtaining FDA approval of these drugs. This reluctance was based in part on the limited number of patients with rare diseases, their geographic dispersion, and the nonpatentability of many of these drugs. Section 525 requires FDA to give protocol assistance to sponsors of drugs for rare diseases or conditions; section 526 defines orphan drugs and requires FDA to publicize its designation of orphan drugs; section 527 describes a 7-year period of granting exclusive approval to certain designated orphan drugs; and section 528 facilitates the use of open protocols to permit patients to use orphan drugs for treatment purposes while the drugs are being investigated in clinical trials. The Orphan Drug Act also (1) amended the PHS Act by establishing the Orphan Products Board whose role is to foster action within the Government to expedite the development of orphan products, (2) amended the *Internal Revenue Code* to allow tax credits for qualified clinical testing

expenses, (3) provided for grants to defray costs of clinical testing, and (4) provided patent term extension under certain conditions. On October 30, 1984, the Health Promotion and Disease Prevention Amendments of 1984 were signed, which, among other actions, amended section 526 to specify that a rare disease or condition is one that (1) affects fewer than 200,000 persons in the United States at the time of the date of the request for designation or (2) affects more than 200,000 persons in the United States and for which there is no reasonable expectation that the cost of developing and making it available in this country will be recovered from sales. On August 15, 1985, the Orphan Drug Amendments of 1985 became law. One provision of these amendments provides marketing exclusivity for 7 years to the holder of the first approval of a designated orphan drug whether it is patented or unpatented. The enactment of the Orphan Drug Act has resulted in development and approval of numerous drug products. Examples of drugs that received orphan designation are (1) 1-alpha-acetylmethadol for the treatment of heroin addicts who are suitable for maintenance on opiate agonists, (2) naltrexone HC1 for the blockade of the pharmacological effects of exogenously administered opioids as an adjunct to the maintenance of the opioid-free state in detoxified, formerly opioid-dependent individuals, and (3) oxymorphone HC1 for the relief of severe intractable pain in narcotic-tolerant patients. Copies of the list of current orphan drug designations are available from the Food and Drug Administration, Dockets Management Branch (HFA-305), Room 4-62, 5600 Fishers Lane, Rockville, MD 20857 and from the National information Center for Orphan Drugs and Rare Diseases, P.O. Box 1133, Washington, DC 20013-1133.

CDER GUIDELINES

To assist sponsors of INDs and those filing NDAs in complying with regulatory requirements, FDA has made available guidelines under 21 CFR 10.90(b). Draft guidelines are issued first so that comments from the regulated industry, the medical community, and other members of the public may be obtained before the guidelines are finalized. Guidelines do not impose mandatory requirements; however, they offer guidance on acceptable approaches to meeting regulatory requirements. Different approaches may be followed, but the applicant is encouraged to discuss significant variations in advance with FDA reviewers to preclude spending time and effort in preparing a submission that the Agency later may determine to be unacceptable. The following available guidelines apply to the regulations administered by CDER concerning chemistry, manufacturing, and controls.

- Guideline for the Format and Content of the Chemistry, Manufacturing, and Controls Section of an Application

- Guideline for Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances

- Guideline for Submitting Documentation for the Manufacture of and Controls for Drug Products

- Guideline for Submitting Documentation for Packaging for Human Drugs and Biologics

- Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics

- Guideline for Submitting Samples and Analytical Data for Methods Validation

- Guideline for Drug Master Files (September 1989)

In the notice published on October 7, 1988 (53 FR 39523), FDA announced the availability of the final "Guideline for the Format and Content of the Clinical and Statistical Sections of an Application" to market a new drug. The intent of this guideline is to assist an applicant in presenting the clinical and statistical data required as part of an application under 21 CFR 314.50. It describes an acceptable format for organizing the clinical and statistical sections and presenting the requisite information and documentation obtained in clinical trials.

Additional CDER guidelines currently under development or under consideration will address radiopharmaceutical drug products, sterilization/ sterility requirements, labeling issues, and stereochemistry considerations. The last issue is becoming important because current technological advances now permit the large-scale separation of isomeric mixtures and the synthesis of a single enantiomer. FDA is assessing the clinical, pharmacology/toxicology, chemistry, and other requirements for a drug product proposed for development, which might be a single stereoisomer or several stereoisomers derived from an approved racemic, diastereomeric, optically enriched, or mixture of geometric stereoisomers.

ENVIRONMENTAL ASSESSMENTS

An area of increasing regulatory importance is environmental considerations. On April 26, 1985, FDA published (50 FR 16636) regulations that set forth its policies and supplemental procedures for compliance with the National Environmental Policy Act of 1969 and the Council on Environmental Quality's regulations. IND [21 CFR 312.23(a)(7)(e)] and NDAs [21 CFR 314.50(d)(1)(iii)]

are required to contain either a claim for categorical exclusion under 21 CFR 25.24 or an environmental assessment (EA) under 21 CFR 25.31. An EA is a public document that contains environmental and other pertinent information on a proposed action, providing a basis for FDA's determination whether to prepare an Environmental Impact Statement or a Finding of No Significant Impact. If an EA is required for approval of an FDA-regulated drug, it should be prepared [21 CFR 25.31 (a)] in the following format: (1) date, (2) name of applicant/petitioner, (3) address, (4) description of the proposed action, (5) identification of chemical substances that are the subject of the proposed action, (6) introduction of substances into the environment, (7) fate of the emitted substances into the environment, (8) environmental effects of released substances, (9) use of resources and energy, (10) mitigation measures, (11) alternatives to the proposed action, (12) list of preparers, (13) certification, (14) references, and (15) appendices. If an NDA is for a human drug intended (1) for the prevention, treatment, or diagnosis of a rare disease or for a similarly infrequent use; (2) for ophthalmic or topical application; or (3) for local or general anesthesia, an abbreviated EA may be filed [21 CFR 25.31 a(b)]. Guidelines are being prepared to assist applicants in meeting the regulatory requirements. The purpose of these regulations is to ensure that environmental information is available to the public and the decisionmaker before decisions are made that may significantly affect the quality of the human environment and to ensure that FDA actions are supported by accurate scientific analysis.

CANDAS AND NDA DAYS

On September 15, 1988, FDA published (53 FR 35912) a notice titled "Submission of Drug Applications to the Food and Drug Administration Using Computer Technology." The Agency believes that increased use of computer technology may improve the efficiency of the drug application review process. To facilitate the use of computers, FDA provided guidance on factors that it considers when accepting CANDAs, which are defined as any method using computer technology to improve the transmission, storage, retrieval, and analysis of data submitted to FDA as part of the drug development and marketing approval process. Computer technology may be applied to INDs, NDAs, abbreviated new drug applications, and antibiotic applications. Use of computers should enhance the speed and quality of the drug review program because FDA must handle and evaluate large quantities of data concerning safety and efficacy, which is often burdensome and involves nonessential hard-copy handling. An NDA may include thousands of pages of reports, analyses, tabulations, and case reports. The filing of a CANDA, however, is conditional on the reviewing Division's willingness to accept the application in the CANDA format used by the applicant. To ensure the acceptability of a proposed CANDA, it is important that the applicant discuss with the appropriate FDA

personnel the proposed application well in advance of its filing. The submission of a CANDA will not influence the priority given to an application. At this time, the submission of a CANDA generally will not affect the required submission of an application in paper form.

Another innovative approach being explored by the agency is the "NDA Day." It was introduced by Dr. John Harter and developed jointly with Dr. Temple, both with FDA, to facilitate NDA approval by reducing the institutional review time at the Office level and by coordinating all reviews at the same time. It is a designated day in which a specific NDA is reviewed and evaluated by FDA personnel at the Center, Office, and Division level. Typically, the assigned reviewers make presentations of the submitted data and their interpretations and conclusions. These results and recommendations are discussed intensively by all participants. The NDA applicant, although not in the room, is available in another conference room to handle inquiries. The applicant also usually can communicate by telephone to his or her database to handle any questions FDA may have. At the conclusion of the NDA Day review, there may be extensive followup, and additional information or data may have to be submitted subsequently. To date, few NDAs have been handled by this experimental approach.

CONCLUSION

The involvement of FDA in the new drug development process is lengthy and complex. The Agency's requirements for drug approval are delineated and explained in the CFR and its guidelines. Many previous requirements and procedures have been eliminated or revised in subsequent rewrites to facilitate drug review. Several studies have indicated that the length of time from laboratory synthesis of a new molecular entity to NDA approval is 10 to 12 years at a cost of $125 million to $200 million. The mean total approval time for the 23 new molecular entities approved by FDA in 1989 was 32.5 months. The Agency is engaged in many new innovative programs to further improve the review process.

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