SYNTHESIS AND PHARMACOLOGY OF CONFORMATIONALLY-RESTRICTED ANALOGUES OF ENKEPHALIN AND THE N-LONE PAIR EFFECT IN OPIATES.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Chemistry
McGill University
Montreal, Québec
Canada, H3A 2K6

August, 1979

Synthesis and Pharmacology of Conformationally Restricted Analogues of Enkephalin and the N-Lone Pair Effect in Rigid Opiates

John Di Maio PhD Chemistry

ABSTRACT

The molecular parameters responsible for the activity of endogenous opiod peptides were investigated by synthesizing analogues with conformational restrictions imposed on the Tyr-Gly region. The restraint was provided in one case by a 3-(p-hydroxybenzyl)-6-carboxy-2-oxo-piperazine ring (13), (14), and by a 3-(p-hydroxybenzyl)-2-oxo-piperazine-1,N-acetic acid ring (16), (17) in the other. The analgesic activity of the most potent analogues 17-D was shown not to be related to opiate receptors, and the receptor-bound conformation of the tyrosyl region of enkephalin is reviewed in the light of these results.

16α,17-butano morphinan (50-a) was converted to the 16β, 17-butano morphinan (50-b) epimer by a series of oxidation reduction reactions. Single crystal X-ray analysis revealed that the former has its D ring in the boat conformation with the N-lone pair oriented toward the phenolic ring. The latter is characterized by an all chair C,D,E ring system. The 16α epimer is analgesically inactive while the 16β-conformer has an ED_{50} 3±.5 mg/Kg.

A model of the opiate receptor is presented that includes a site for facilitated proton transfer with a suitably oriented N-lone pair.

Synthèse et Pharmacologie d'Analogues Steriquement Empêchés d'Enkephalines. Effet du Doublet Libre de L'Azote dans les Structures Opiacées.

John di Maio PhD, Chimie

RESUME

L'influence des parametres moléculaires impliqués dans l'activité biologique de peptides opiacés endogènes ont été étudiés par la synthèse d'analogues qui possèdent des conformations rigides dans la partie Tyr-Gly. La contrainte stérique a été imposée dans un cas par l'introduction d'un cycle 3-(p-hydroxybenzyl)6-carboxy-2-oxo-piperazine (13),(14) et par un cycle 3-(p-hydroxybenzyl)-2-oxo-piperazine-1,N-acide acétique (16), (17) dans un autre cas.

L'activité analgésique de l'analogue le plus puissant 17-D ne semble pas être reliée aux recepteurs des structures opiacées, et la structure conformationelle de la partie tyrosyle de l'enkephaline impliquée dans la liaison avec le recepteur est analysée à la lumière de ces resultats.

Le composé 16α,17-butanomorphinan (50-a) est converti a l'epimère 16β,17-butanomorphinan (50-b) par une série de réactions d'oxidation et de réduction. Une analyse aux rayons-X revèle que le premier composé possède le cycle D dans une conformation bateau dans laquelle le doublet libre de l'atome d'azote est orienté vers le cycle phenolique, tandisque le

dernier composé est caracterisé par le fait que les cycles C, D, E, sont dans la conformation chaise. L'épimère 16α est du point de vue analgésique inactif tandisque le conformère 16β possède une ED_{50} 3±.5 mg/Kg.

Le modèle de récepteur pour les structures opiacées présenté fait intervenir un site qui possède le doublet libre de l'azote favorablement orienté qui permet de faciliter le transfert d'un proton.

TO MY WIFE AND FAMILY

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ABBREVIATIONS

SAR	structure activity relationship
ID_{50}	inhibitory dose (required to cause 50% inhibition)
ED_{50}	effective dose (required to cause 50% response)
PET	7α-(-phenyl-3T-hydroxybutyl)-6,14-endoethenotetrahydrothebaine
β-LPH	β-liptropin hormone
CSF	cerebral spinal fluid
Tyr	tyrosine
Gly	Glycine
D-Ala	D-alanine
Phe	phenylalanine
Met	methionine
Leu	leucine
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
BOC	t-butoxycarbonyl
Cbz	carbobenzoxy
BOC-ON	2-(t-butoxycarbonyl oximino)-2-phenyl acetonitrile
EtOAc	ethyl acetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
MeOH	methanol
cAMP	cyclic adenosine monophosphate
O-bz	O-benzyl
LHRH	Luteinizing hormone releasing hormone
CT	Chymotrypsin
DCC	dicyclohexyl carbodiimide
AAA	amino acid analysis

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to Dr. Bernard Belleau for allowing me to pursue this research project. I am grateful for the invaluable assistance in the laboratory, with the preparation of this thesis, and for the useful and pleasant conversations during the course of this work.

I gratefully acknowledge the collaboration of Dr. Peter Schiller and his staff, Clinical Research Institute of Montreal. I would also like to acknowledge the collaboration of Dr. A. Pircio, Bristol Labs, for providing the *in vivo* data and Dr. F.R. Ahmed, National Research Council of Canada, for the elucidation of the X-ray structures.

The technical assistance of Bruce Compton is also appreciated. I would like to acknowledge the assistance provided by J.L. Kraus and G. Lajoie and also the useful discussions with members of the laboratory.

I also acknowledge the National Research Council of Canada scholarships 1975-1977, and the Quebec Government scholarships 1977-1979.

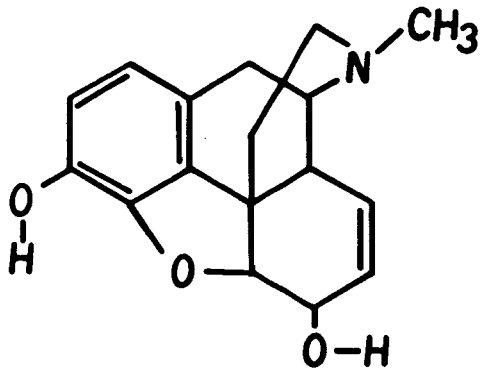
Finally, I would like to extend my gratitude to Miss Olwen Gotts for her skillful typing and perseverance.

INTRODUCTION

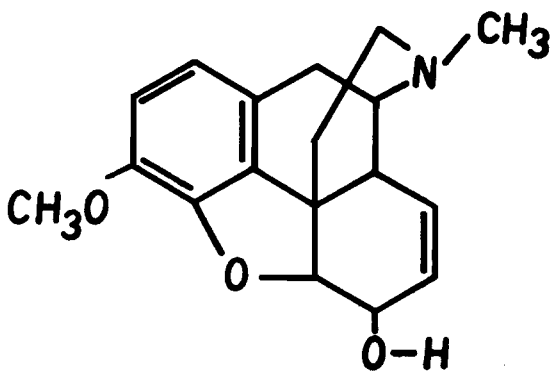
Opioids belong to a class of medicinally important products whose pharmacological actions are similar to those of the prototype narcotic analgesic morphine (figure 1). Their principal therapeutic value lies in their ability to suppress pain and strife.

Opium is the crude dried exudate from the poppy "Papaver Somniferum". Morphine, which constitutes 10% by weight of dried opium powder, was the first opium alkaloid to be characterized as the active ingredient in 1803 by a German pharmacist, Friedrich Surturner[1], while codeine (figure 1) which is present at a concentration of approximately 5% that of morphine was isolated in 1832 by the French pharmacist Jean-Pierre Robiquet.[2] Morphine and codeine are the two most important narcotics belonging to the phenanthrene group of alkaloids in opium.

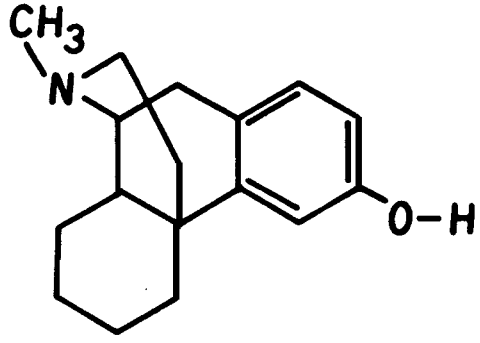
Although it is generally recognized that opioids are beneficial to patients suffering from chronic excruciating pain, they are dangerous and overdosage can be fatal. It is well known that opiates are highly addictive and physical dependence rapidly leads to tolerance. After dependence is established, sudden cessation of drug administration leads to withdrawal symptoms typified by stomach cramps, diarrhea, sleeplessness, vomiting and nervous excitation accompanied by pupillary dilation and gooseflesh. Tolerance develops after repeated administration



MORPHINE



CODEINE



DEXTRORPHAN

FIGURE 1. The chemical structures of naturally occurring narcotic analgesics morphine and codeine. Dextrorphan is the analgetically inactive enantiomer of levorphanol.

of a drug, higher doses being required to evoke the response that was previously elicited by much smaller doses. Although the mechanism of tolerance to the opiates is not clearly understood, the phenomenon is attributable in part to increased metabolism whereby the drug stimulates the synthesis of those enzymes responsible for its inactivation in the liver. Consequently, larger doses are required to achieve critical blood and brain levels. However, no concrete evidence is available that morphine metabolism is increased in tolerant dogs[3] reflecting a cellular tolerance probably involving increased adenyl cyclase synthesis according to Klee and Nirenberg.[4]

Because the opiates exert their effects on both the peripheral and central nervous system they interfere with several bodily functions and cause a wide range of side effects. Therapeutic doses of morphine cause some lowering of the respiratory volume and a lessened response to carbon dioxide without much change in respiratory rate,[5] but larger doses depress the rate of respiration and carbon dioxide retention becomes critical. Indeed, stoppage of respiration is the major cause of death in morphine poisoning. Pupillary constriction is a prominent effect elicited by opiates in man, and respiratory depression with pupillary constriction is virtually diagnostic of morphine consumption.

Morphine also inhibits the coordinated regular intestinal peristalsis and smooth muscle contractions. These effects,

coupled with decreased gastrointestinal secretions, create constipation.[9]

In subanalgesic doses morphine and other opiates possess good therapeutic value as antitussives. However, this effect is also observed with certain enantiomers devoid of opiate-like properties such as dextramethorphan; the enantiomeric drug levorphanol is a strong analgesic but also capable of alleviating cough.

A. CHEMISTRY AND PHARMACOLOGY OF OPIATES

1. Agonist-Antagonist Activities

Despite all the medical problems associated with chronic use of opiates, they still remained until recently the only therapeutic agents capable of alleviating severe pain. The physical dependence induced by opiates has led to serious social disturbances and has served to intensity efforts directed at an improved understanding of the pharmacological mechanisms and has stimulated the search for potent analgesics devoid of dangerous side effects.[10] The determination of the complete structure of morphine by Robinson[11] in 1925 was a landmark and served as a basis for systematic structure-activity relationship studies (SAR). Morphine is a rigid T-shaped molecule with two broad hydrophobic surfaces at right angles to each other. The 3-OH group (meta relative to carbon-12) (figure 2) is essential for

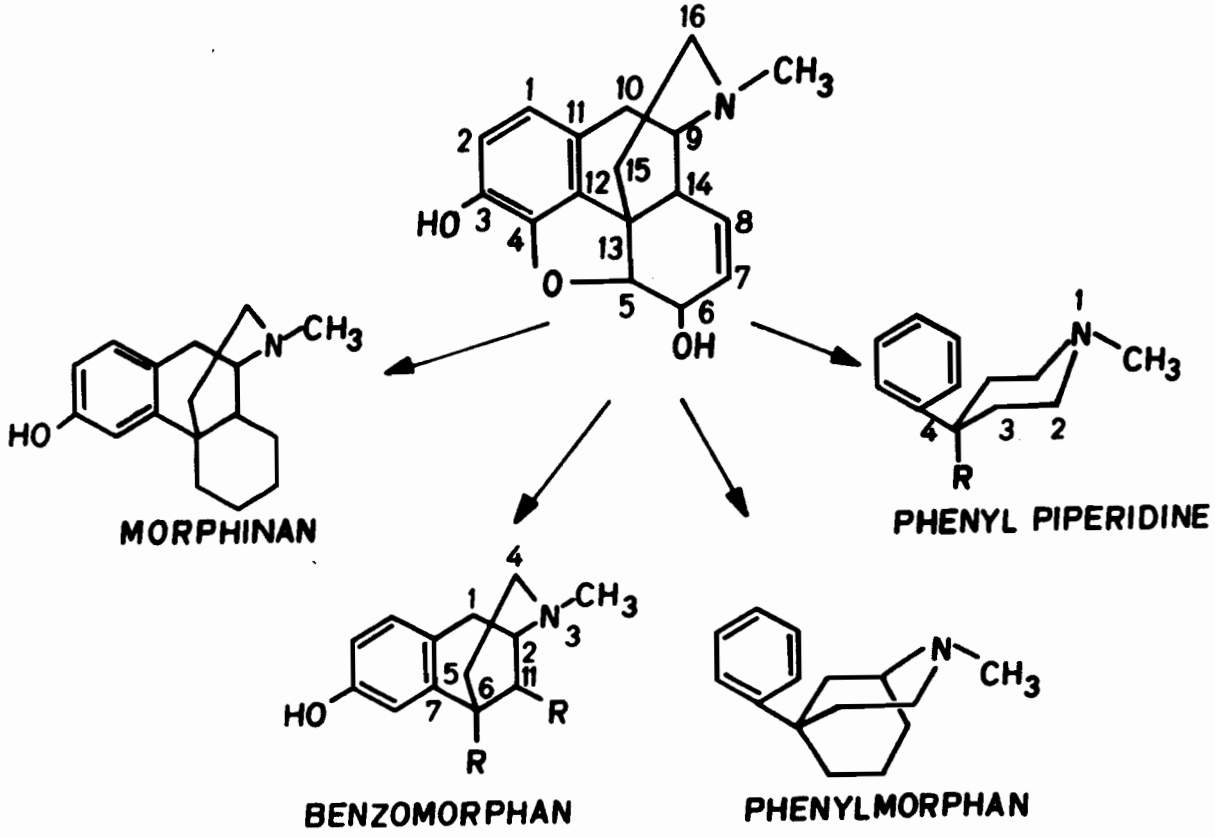


FIGURE 2. Four classes of synthetic analgesics derived from naturally occurring morphine: morphinans, benzomorphans, phenylmorphans, and phenyl piperidines.

high activity; when masked as a methyl ether, one obtains codeine which is only 10% as active as morphine. Even then, abundant evidence is available that codeine may not act directly but only after metabolic conversion through O-demethylation.[12] Thus, although codeine is an effective analgesic when administered intraperitoneally to animals, it has no effect on isolated organs[13] or when administered directly into the brain.[14]

The $N\text{-}CH_3$ substituent of morphine also plays a critical role in conferring analgesic activity. Removal of that N-substituent as in normorphine results in a large drop in anti-nociceptive activity corresponding to 10% that of the parent morphine when it is injected intravenously.[15] But on the guinea pig ileum preparation where the parameter of lipid solubility is less important, the ID_{50} values of morphine and normorphine were found to be 69.2±14.8 nM and 72.8±17.7 nM respectively.[13] Similarly, substituting the N-methyl for an N-ethyl or N-propyl group led to large drop in potency (figure 3) but activity was regained with higher homologous substituents.[16]

The trough on the curve shown in figure 3 is characteristic of a unique class of medicinal agents. It is well known that substituting the N-methyl of morphine, morphinans, and benzomorphans by short alkyl chains such as N-propyl, N-butyl or by N-allyl and N-cyclopropylmethyl confers antagonistic properties to the drugs. In other words these larger substituents lead

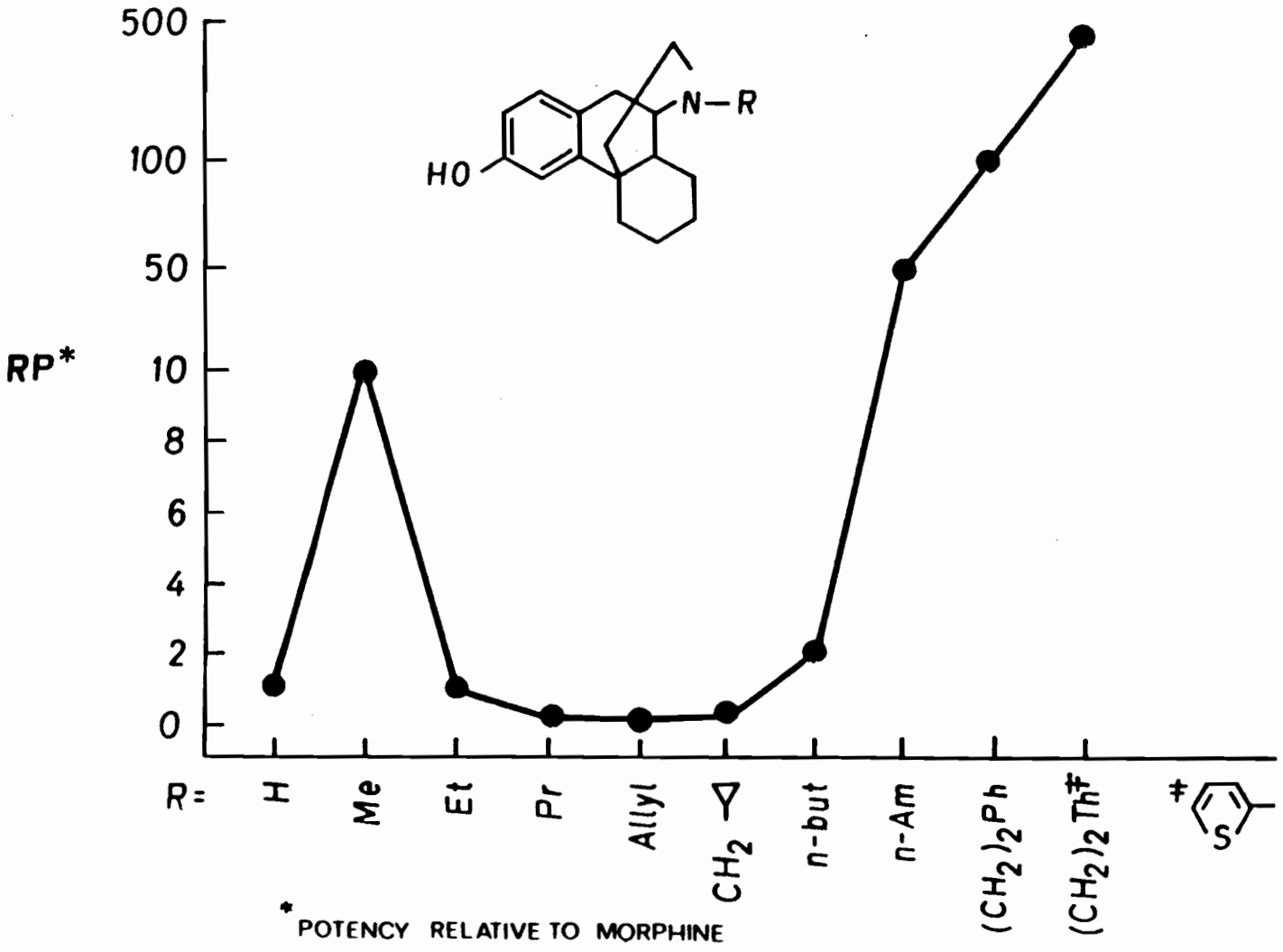
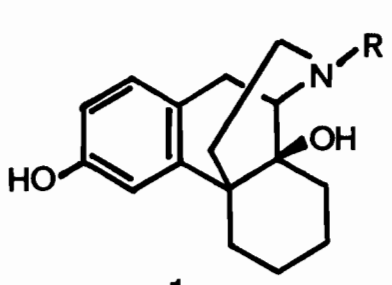
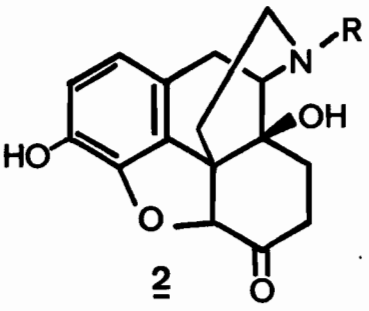


FIGURE 3. Relative potency of morphinan congeners relative to morphine as a function of change in N-substituent (subcutaneous administration).

to a reversal of all the antinociceptive activity as well as all the side effects associated with morphine, while being incapable of inducing narcotic-like effects. The classical example of a pharmacologically "pure" narcotic antagonist is naloxone (figure 4, 2-b) which is the N-allyl analogue of oxymorphone (2-a) itself a potent narcotic analgesic.[17] Interestingly however, N-substitution by methallyl (2-c), cyclopropylmethyl (2-d) or cyclobutylmethyl produces compounds which are either pure antagonists or mixed agonists-antagonists. For example, N-3',3'-dimethallyl noroxymorphone (2-c) is about one-third as active as morphine as an antinociceptive agent and about one-half as active as nalorphine as a narcotic antagonist. On the other hand, N-cyclopropylmethyl noroxymorphone (naltrexone 2-d) is from two to three times more potent than naloxone as an antagonist but exhibits hardly any antinociceptive activity in the rat. In contrast, N-cyclobutylmethyl noroxymorphone (2-e) is an analgesic comparable to morphine but is also a potent narcotic antagonist with one-fifth the activity of naloxone. The first narcotic antagonist (N-allyl norcodeine) was discovered by Pohl[18] in 1915, and 30 years later the superior analogue nalorphine was developed as a medicinal agent for the treatment respiratory depression resulting from morphine overdosage. However, it was subsequently observed that nalorphine possesses strong analgesic properties in man and as such it was classified as a partial agonist. The apparent ambivalence that both



1



2

(a) R = CH_3

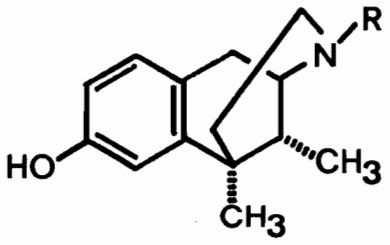
(b) R = $CH_2CH = CH_2$

(c) R = $CH_2CH = C(CH_3)_2$

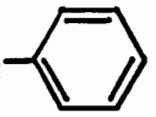
(d) R = CH_2-c-propyl

(e) R = CH_2-c-butyl

FIGURE 4. Synthetic analgesics derived from 3-hydroxy-14-hydroxy normorphinan 1, and noroxymorphone 2.



3

(a) R = CH_2CH_2 

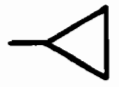
(b) R = CH_2 

FIGURE 5. Chemical structures of phenazocine 3-a and cyclazocine 3-b.

agonist and antagonist properties should be induced by the same molecule remains to be clearly explained. It is now known that opiate agonist-antagonists such as nalorphine are clinically effective but can produce physical dependence and central nervous system disturbances such as hallucinations and disorientation; nevertheless the discovery of nalorphine has opened a new era of analgesic research because it was the first time that significant dissociation of analgesic effectiveness from dependence liability had been observed. Unfortunately its dysphoric and psychotomimetic properties severly limit its clinical usefulness. A clinically desirable analgesic would be one displaying a well-balanced agonist-antagonist potency ratio[19] while being devoid of dysphoric properties.

Although only moderate success has been achieved in terms of bettering activity by chemical modification of the morphine skeleton, most of the advancement from the point of view of dissociating analgesic effectiveness from side effects and addiction has been accomplished in the area of totally synthetic drugs (figure 2) in the benzomorphan group and especially in the morphinan family of analogues where butorphanol[20-22] (figure 4 1-e) occupies a prominent place.

Reasoning that the 14β-hydroxyl of naloxone is the key to its "clean" narcotic antagonist properties, Belleau *et al.*[20-22] incorporated the same functionality into the morphinan molecule. When a cyclobutylmethyl substituent was attached to the nitrogen,

the molecule behaved as a potent agonist endowed with considerable antagonist activity. By comparison, oxilorphan, the N-cyclopropylmethyl analogue of 3-hydroxy,14-hydroxynormorphinan is a weak agonist four times as potent as d,ℓ-cyclazocine and equipotent with naloxone[22] as an antagonist. The clinically effective butorphanol is now commercially available and considered a safe analgesic agent for the treatment of moderate to severe pain. A recent review on butorphanol[23] noted that the major side effect consists of mild sedation in about 37% of patients. Although no evidence has been obtained regarding abuse potential, a definitive conclusion must await the results of widespread use for longer periods of time. It is firmly established, however, that it causes only a slight respiratory depression (10-15%) even at very high doses. Accordingly butorphanol appears as the safest analgesic to date.

One of the first benzomorphans to be brought to general attention was phenazocine[24] (figure 5, 3-a) which is a pure agonist approximately ten times as potent as morphine in the mouse hot plate test. The compound proved to be an orally-effective analgesic with a low order of physical dependence capacity in the monkey but unfortunately the result did not extrapolate to man.[25] Cyclazocine [N-cyclopropylmethyl 5,11-dimethyl-6,7-norbenzomorphan (3-b)][26] is more active than nalorphine as an antagonist and is 40 times more potent than morphine as an agonist. However, the drug evokes psychotomimetic effects and

dysphoria to such an extent as to severely limit its use in man.[27] The drug differs from morphine in several respects and although it will substitute for morphine in addicted subjects, abrupt withdrawal does not produce the psychological effects characteristic of drug-seeking behaviour.[28]

2. The Pharmacodynamics of Analgesia: the Opiate Receptor

The underlying mechanism by which opiates exert a wide range of pharmacological effects remains unknown, nevertheless there exists unanimous agreement that the analgesic response is mediated by the interaction of these drugs with specific sites or receptors located on the external surface of cells in target organs.

Through the use of radioactive agonists or opiate antagonists, Pert and Snyder,[29] Simon *et al.*[30] and Terrenius[31a,b] independently identified stereospecific binding to brain tissue thus confirming the existence of opiate receptors and further establishing that agonists, antagonists, and mixed agonist-antagonists all appear to compete for the same binding sites *in vitro*.[32] In order to ascertain the relevance of such stereospecific binding to the opiate receptor, Pert and Snyder[29] evaluated the ability of a spectrum of agonists and antagonists to displace receptor-bound [^{3}H]-naloxone and found a close parallel between pharmacological potency (either agonistic or antagonistic) and their affinity for the naloxone binding site (table 1).

Table 1

Relative potencies of drugs in displacing stereospecific binding of [3H]-naloxone from rat brain homogenate from ref. 34.

Drug	ED_{50} (nM)[a]
(-)-ETORPHINE	0.3
(-)-ETONITAZINE	0.5
LEVALLORPHAN	1.0
LEVORPHANOL	2.0
(-)-NALORPHINE	3.0
(-)-MORPHINE	7.0
(-)-CYCLIZOCINE	10.0
(-)-NALOXONE	10.0
(-)-HYDROMORPHONE	20
(-)-METHADONE	30
(±)-PENTAZOCINE	50
(+)-METHADONE	300
MEPERIDININE	1,000
DEXTRORPHAN	8,000
(-)-CODEINE	20,000
(-)-OXYCODONE	30,000

[a] ED_{50} is the concentration of drug required to inhibit the binding of [3H]-naloxone by 50%

Until now the absolute chemical nature of the opiate receptor has remained elusive; however, the structural framework of morphine, its relative and absolute stereochemistry, provide valuable information pertaining to the topographical features of the receptor. As early as 1954 Becket and Casey[33] published what is still today an interesting albeit simplistic model of the opiate receptor (figure 6). These authors proposed that the receptor for morphine consists of three basic binding sites comprised of a planar area A that would accommodate the flat aromatic ring, a cavity for inserting the C_{15}-C_{16} moiety, and a complementary anionic site which would attract the basic nitrogen. This receptor model was modified recently by Bentley et al.[35] in order to account for the potent thebaine derivatives known as oripavines, and which include etorphine and PET, analgesics whose potency is 1,000-10,000 times greater than that of morphine (figure 7).

These more complex structures indicate that a more rigid and bulkier framework could increase affinity as well as specificity for the opiate receptor. Molecules of this series are the most potent analgesics known today but they also have a correspondingly high addiction and physical dependence capacity. The new receptor model (figure 8) retains the three essential binding sites suggested by Becket and Casey but incorporates a new lipophilic binding locus D for the additional phenyl ring. This site presumably interacts with the complementary C-19

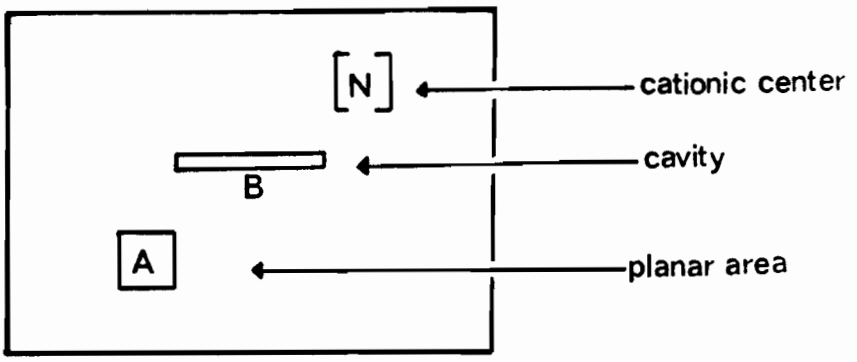


FIGURE 6. A model of the opiate receptor according to Becket and Casey[33] involving a three point attachment of the phenolic ring, piperidine edge, and ammonium cation to A, B and N respectively.

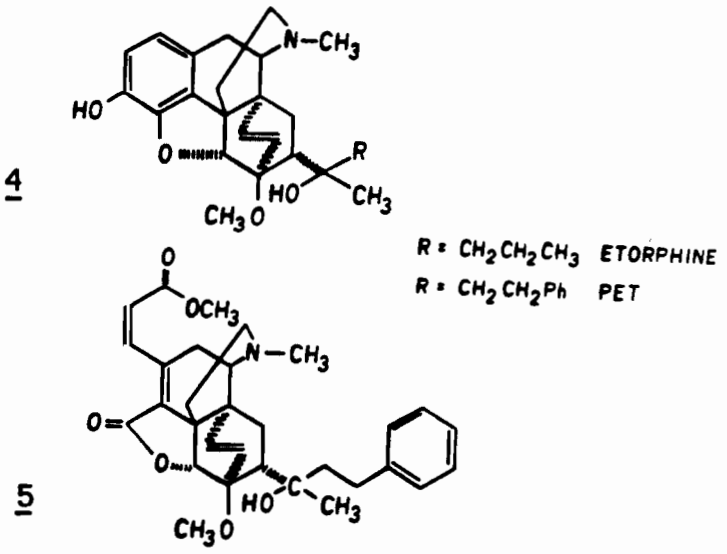
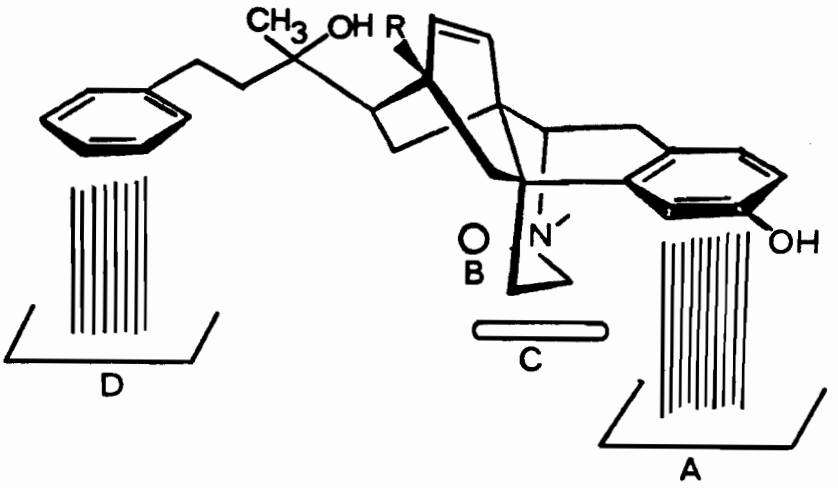


FIGURE 7. Chemical structures of oripavine derivatives including 5 whose aromatic ring is absent.

hydrophobic binding element of the oripavine derivatives. That the forces governing this new interaction are strong indeed was demonstrated by the fact that selective destruction of the aromatic ring A (figure 7(5)) affords analogues as active as morphine. When a similar modification was made in molecules of the benzomorphan group which lack the additional binding element for the D site, all the activity was lost.[36]

The plethora of organic bases that can induce analgesia through an interaction with the opiate receptor defies rationalization of structure-activity relationships especially if one assumes that a single, well-defined binding site is involved. The issue is further complicated by the fact that manipulation of N-substituents can lead to a wide spectrum of compounds ranging from pure agonists such as morphine to pure antagonists (naloxone), and compounds behaving as mixed agonist-antagonists such as butorphanol or cyclazocine.

Recently Pert et al.[37] stumbled upon an unusual phenomenon when they discovered that the sodium ion is a critical variable allowing a distinction between agonist and antagonist binding to rat brain homogenate. The binding of [^{3}H]-agonists was invariably reduced by the presence of sodium at 1 mM whereas [^{3}H]-antagonist binding was favoured under the same conditions. The alteration in receptor affinity was unique to sodium, consequently the effect could not be attributed to changes in ionic strength. On the basis of these results, Pert et al.[37]



A- flat surface for aromatic ring

B- anionic site

C- cavity for c-15 c-16

D- accessory lipopophilic site

R = CH_3O

FIGURE 8. A model of the opiate receptor model by complementarity involving a four point attachment including the accessory lipophilic site D.

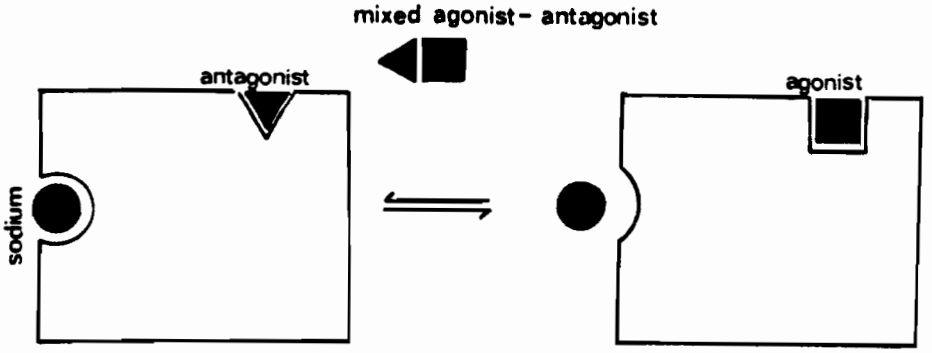


FIGURE 9. A two-state model of the opiate receptor. The transition from the antagonist to agonist state is allosterically modulated by sodium ions.

Pasternack et al.[38] expanded on the classical two-state model (closed and open)[39] for the opiate receptor. The new model incorporates sodium ions which would allosterically modulate the transition between the "sodium" or (antagonist state) and the "no sodium" or (agonist state) (figure 9). Snyder has postulated that tolerance and physical dependence may be associated with a change in receptor population so that it would be less capable of assuming the agonist form. Instead, it would favour the antagonist state.[40]

In a recent communication Fienberg and Snyder[41] advanced a molecular interpretation of the ambivalent (agonist/antagonist) properties of various drugs. They suggested that the antagonist state of the receptor would be stabilized by the interaction of a ligand whose N-Alkyl substituent would be fixed in the equatorial position relative to the piperidine ring. This equatorial orientation would be favoured by the presence of a 14-OH as in naloxone (figure 10(6)) or by an 11β-substituent as in certain benzomorphans (figure 10 (7)). On the other hand, structures like nalorphine (8) which lack a 14-OH or pentazocine, whose equivalent C-11 substituent exists in the α-conformation, behave as partial agonists presumably because their N-substituents would prefer an axial disposition. This model is not only thermodynamically unrealistic but is also self contradictory because it is well known that β-isomers of benzomorphans have accordingly greater antinociceptive activity than their

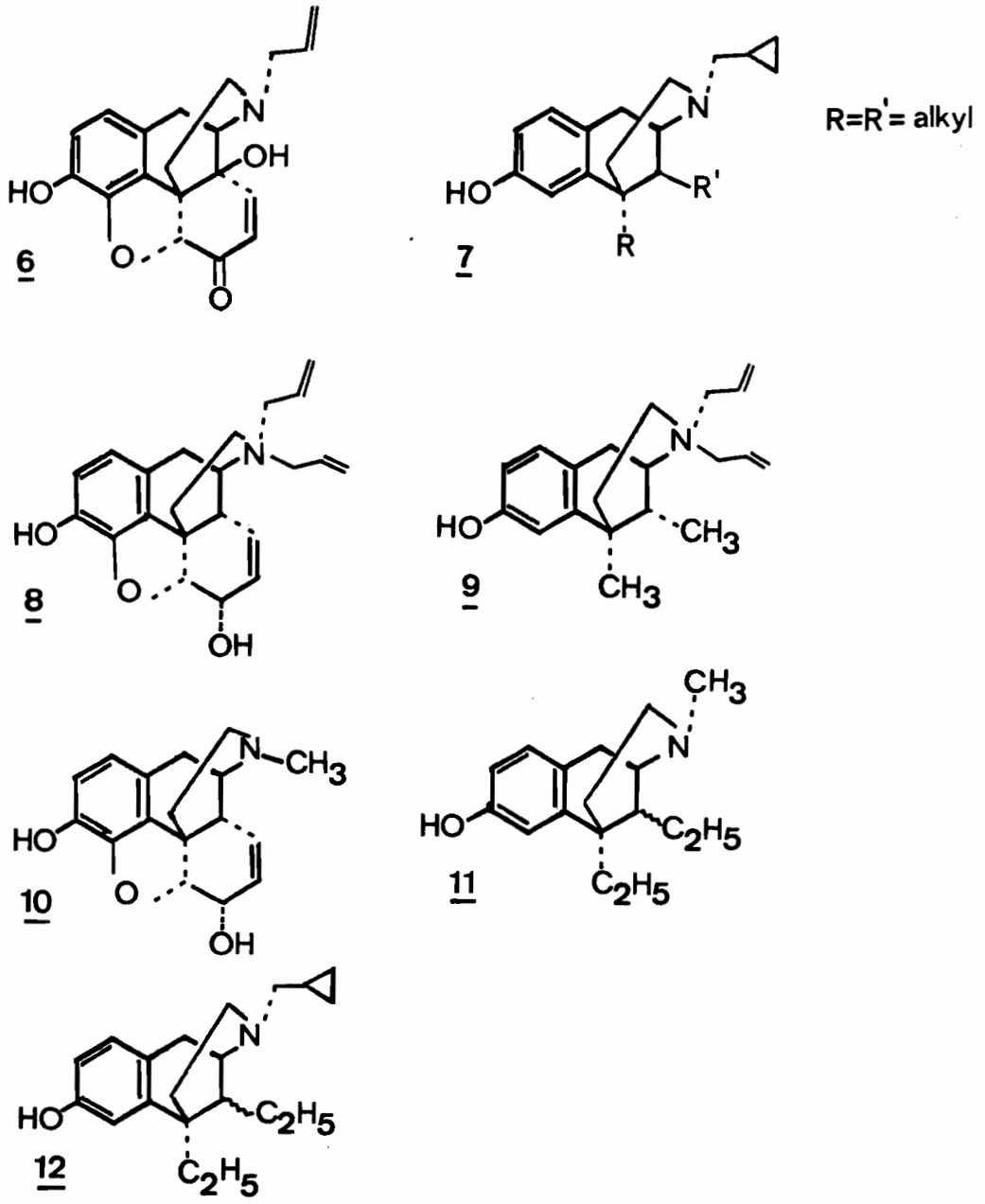


FIGURE 10. Chemical structures of common analgesics showing the possible conformations of the N-substituents.

C_{11}-α epimers.[42] The agonist and antagonist activities of the α and β diastereomers of 5,9-diethyl-2-methyl-2'-hydroxy 6,7-benzomorphan (11) and 5,9-diethyl-2-cyclopropylmethyl-2'-hydroxy 6,7-benzomorphan (12) have been compared[43] and the former (-)C_{11}-β compound (trans Et_5/Et_9) is a much more potent agonist than the (-)C_{11}-α epimer regardless of any possible stabilization of the N-methyl moiety in the equatorial conformation through the interaction with the 11-β substituent. For the latter, the (-)C_{11}-α isomer is five times more active as an antagonist than the corresponding (-)C_{11}-β isomer in the guinea pig ileum preparation contrary to what would be predicted using the above model.

Energy calculations[44] showed that the axial orientation for the morphine N-Methyl (assuming a rigid D ring) is destabilized by 5.7 Kcal/mole relative to the equatorial conformation. For oxymorphone (figure 4 (2-a)) a potent agonist, and naloxone (2-b), a pure antagonist, the equatorial conformations are favoured by 12 and 20 Kcal/mole respectively, making it unlikely that differences in the activity of these two compounds could be due to different equilibrium positions of the N-alkyl axial/equatorial conformations.[45] Furthermore, the model is in direct contradiction with the observation[46,47] that in man (and primates), butorphanol, which possesses a 14-β hydroxyl, is from five to eight times more potent than morphine as an analgesic while behaving as a strong narcotic antagonist devoid

of narcotic properties. Similarly, cyclobutylmethyl noroxymorphone (nalbuphen, figure 4(2-e)), whose bulky N-substituent is in an unfavourable steric proximity to the 14-β OH, has analgesic activity comparable to morphine while displaying about one-fifth the antagonist activity of naloxone.

If morphine is a pure narcotic agonist because its N-Methyl substituent is stabilized in the axial disposition (10) then the N-lone pair would point equatorially relative to the piperidine ring. This stereoelectronic configuration is at variance with the observation[48,49] that the N-lone pair orientation must be axial and project away from the aromatic ring for productive binding in vivo.

B. ENDOGENOUS OPIATES

1. Origin and Activity

In spite of all the efforts to achieve a better understanding of the topographical nature and molecular mechanisms of the opiate receptor, a fundamental question has remained unanswered: Is it coincidental that there should exist a specific receptor for a drug like morphine which is foreign to mammalian species?

Although morphine is not a natural constituent of the brain, it has recently been discovered that extracts of mammalian brain mimic the peripheral effects of opiates;[50] namely the inhibition of electrically induced contractions of the guinea pig myenteric plexus and the mouse vas deferens. Almost

simultaneously other groups demonstrated that brain extracts selectively compete with morphine for opiate receptors.[51-53]

Hughes et al.[54,55] were the first to show that the effects of pig brain extracts on smooth muscle were associated with two related pentapeptides (figure 11) [Methionine^5]-enkephalin and [Leucine^5]-enkephalin which occur in a ratio of approximately 3/1. More recently Simanov and Snyder[56] have isolated the same two peptides from bovine brain but in an inverse ratio to that found in pig brain. Higher molecular weight morphinomimetic peptides have also been isolated from the pituitary[57,58] and the hypothalamus,[59] and these polypeptides have been named "endorphins."* The peptide from camel pituitary glands was isolated and characterized as an untriakontapeptide[60] called β-endorphin while the peptide from porcine hypothalamus which is called α-endorphin, consists of sixteen aminoacid residues.[61] Hughes[50] recognized that the sequence of [Met^5]-enkephalin corresponds to that of Tyr^{61}-Met^{65} of β-lipotropin, a pituitary hormone isolated from sheep,[62,63] bovine,[64] porcine,[65,66] and human[67,68] brain; [Leu^5]-enkephalin would share the sequence Tyr^{61}-Phe^{64}, α-endorphin Tyr^{61}-Thr^{76}, and β-endorphin consists of the entire C-fragment[69] of β-lipotropin.

*The name is derived from endogenous morphine.

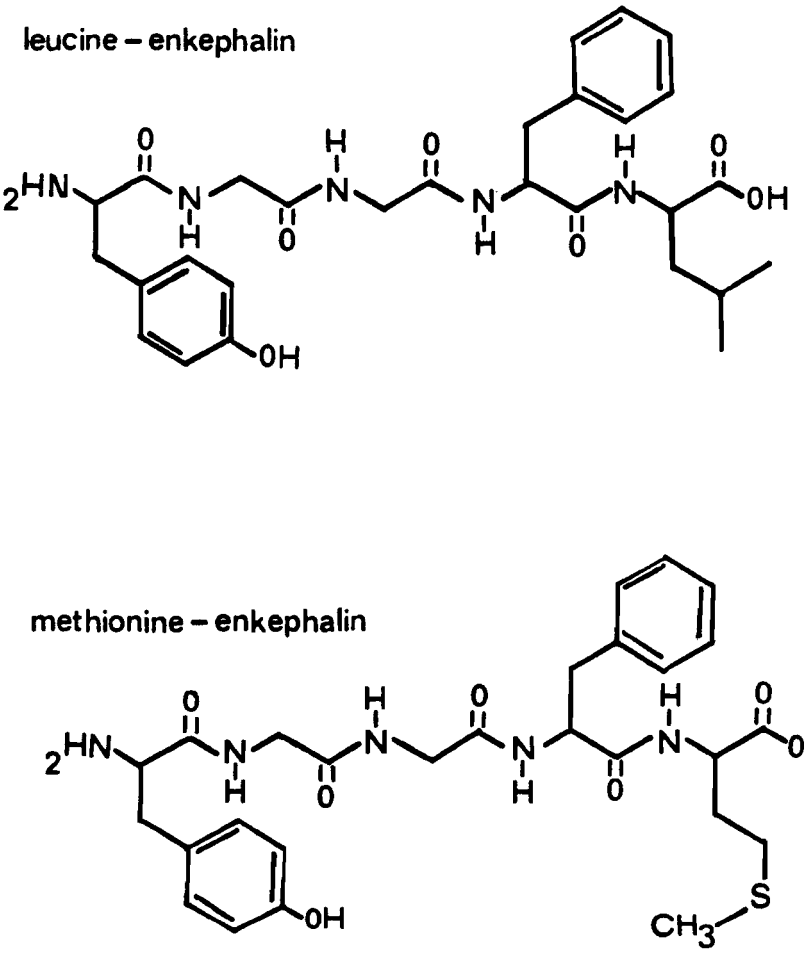


FIGURE 11. Chemical structures of endogenous opioid peptides [Leu^5]-enkephalin and [Met^5]-enkephalin.

Although enkephalin was isolated from brain, β-endorphin from the pituitary, and α-endorphin from the hypothalamus, it is probable that β-lipotropin is a prohormone for the various active fragments. Indeed Lazarus et al.[70] have shown that β-LPH [1-91] possesses no opiate-like activity of its own, but incubation with an aqueous extract of rat brain generates peptide fragments with morphinomimetic activity. Until now the biogenic origin of [Leu^5]-enkephalin has remained uncertain since Leucine is absent in the C-fragment of β-lipotropin. However, another group has reported[71] the isolation of a large polypeptide with the partial sequence Tyr-Gly-Gly-Phe-Leu- but whose remaining primary structure bears no relation to the C-fragment of β-lipotropin.[71]

Guillemin et al.[72] have recently summarized the opioid activity of numerous synthetic β-lipotropin fragments and determined that β-endorphin is 4.5 times more potent than [Met^5]-enkephalin in the guinea pig ileum preparation (table 2). More recently, Li et al.[73] have synthesized β-human endorphin and found it to be 48 times as potent as morphine after intracerebroventricular administration and approximately three times as active upon intravenous injection (table 3). Pretreatment or post administration of naxolone completely reversed analgesia indicating that the analgesic response to the endogenous peptides is mediated through the interactions with the same binding sites that respond to opiate agonists and antagonists. One may speculate that the greater potency of endorphins compared

Table 2. Comparative potencies on an equimolar basis (M) of synthetic peptides (95% fiducial limits) as tested in the MYENTERIC plexus bioassay, from ref. 72.

PEPTIDE	Relation to -LPH	Potency Met- enk = 100
H-tyr-Gly-OH	β-LPH (61-62)	0
H---------Gly-OH	β-LPH (61-63)	0
H----------Phe-OH	β-LPH (61-64)	0.1
H----------Leu-OH	β-LPH (61-65)	37 (24-53)
H----------Met-OH		100
H----------NH_2		302 (179-649)
H----------NHEt		246 (178-335)
(OMe)Tyr----------Met-OH		17 (9-27)
Ac----------OH		Divergence
H-Phe-Gly-Gly-Phe-Met-OH		.2 (0.1-0.3)
H-Tyr-----D-Ala-------OH		4 (2-8)
H---------Gly---------OH		
-Thr-Ser-Glu-Lys-OH	β-LPH (61-69)	60 (38-85)
H---------- ---------Ser- Gln-Thr-Pro-Leu-Val-Thr-Oh	β-LPH (61-76)	36 (19-53)
H---------- ---------- ----------NH_2		76 (47-102)
H----------OH ----------Ser- ---------- Phe-Lys-Asn-Ala-Ile-Val-Lys- Asn-Ala-His-Lys-Lys-Gly-Gln-OH	β-LPH (61-91)	450 (281-966)

Table 3. Median antinociceptive doses (AD_{50}) of synthetic βh-endorphin in mice, from ref. 13.

	AD_{50}	POTENCY RATIO MORPHINE = 1 HUMAN	CAMEL
(A) Intracerebroventricular injection nM/mouse			
Tail-Flick Test	0.026(0.017-0.043)	48.4	33.0
Hot-Plate Test	0.031(0.023-0.01)	32.8	17.5
Writhing Test	0.026(0.017 - 0.4)	17.3	19.5
(B) Intravenous injection μM/mouse			
Tail-Flick Test	3.32 (1.86-5.69)	3.4	3.5

to [Met^5]-enkephalin could be due to interactions with additional binding sites which are otherwise not available to the shorter peptides.[74] However, experimental evidence is available that the high potency of endorphins may be due to their resistance to degradation since in vitro receptor affinity is not enhanced dramatically as the peptide chain is lengthened.

The weak or lack of activity associated with intravenous administration of β-endorphin and enkephalin respectively reflects the vulnerability of these molecules to proteolytic enzymes in the bloodstream. Also, the transient biologic activity

of enkephalin following ICV administration is also the result of rapid deactivation, particularly by aminopeptidases and to a lesser extent by carboxypeptidases. This enzymatic attack of enkephalin at the level of the Tyr^1-Gly^2 peptide bond[75,76] is reflected in a rapid recovery of the mouse vas deferens preparation when compared to normorphine.

The exact physiological role of β-endorphin remains uncertain. Conceivably this pituitary peptide may regulate those gland's functions that are known to be affected by opiates.[77] This notion was substantiated by the finding that psychotic patients invariably possess elevated levels of β-endorphin in their CSF suggesting that this peptide could be involved in schizophrenia.[78] The dysphoric, delusionary, and hallucinatory reactions of narcotic partial agonists such as nalorphine and cyclizocine are similar in many respects to symptoms seen in schizophrenics. Therefore, it would seem logical to assume a direct involvement of β-endorphin in the production of certain cyclozocine-like symptoms as seen in schizophrenic patients.

On the other hand, the enkephalins were considered at an early time as possible natural substrates for the opiate receptor because studies suggested structural analogies to the classical opiates. A summary of these structure-activity relationship studies is given in table 4. A critical requirement for analgesic activity and receptor affinity is the

Table 4. Morphinomimetic activity of enkephalin analogues designed to show the parallelism of the structural requirements between the endogenous substrate and classical opiates.

ANALOG	% Activity	[Met^5]-ENK = 100% Ref	Assay
a. [desamino Tyr^1,Met^5]-ENK	0	80	A,C
b. [N-acetyl Tyr^1,Met^5]-ENK	weak	72	C
c. [O-CH_3 Tyr^1 Met^5]-ENK	0.4	81,82	A,B
d. [Phe^2 Met^5]-ENK	0.2	81,83	A,B,C
e. [o-Acetyl Tyr^1,Met^5]-ENK	divergent	84,72	B

A inhibition of [3H]-naloxone binding

B mouse vas deferens

C guinea pig ileum

presence of a basic nitrogen attached to a phenethyl moiety. This conclusion is supported by the finding that [desamino-Tyr^1,Met^5]-enkephalin (a) or [N-acetyl Tyr^1,Met^5]-enkephalin are devoid of any activity. Furthermore, o-methylation of the phenolic hydroxyl (c) lowers the potency by a factor comparable to the effect of O-methylation of morphine (which yields

codeine).[79] The importance of the phenolic hydroxyl was further corroborated by the observation that substituting Tyr^1 with Phe (d) led to a complete abolishment of antinociceptive activity. This same phenomenon is well documented in the morphine, morphinan, and benzomorphan series. In contrast, o-acetylation of the tyrosyl residue (e) causes full retention of activity analogously to acetylation of morphine which yields heroin.

It has been demonstrated recently that there is a marked inhibition in the basal contractions of the guinea pig ileum when it is stimulated at 10 HZ that has been attributed to the spontaneous release of [Met^5] and [Leu^5]-enkephalin.[85,86] Electrical stimulation of the periaqueductal gray matter of the cat, a region rich in opiate receptors, also produces analgesia and cross tolerance with morphine.[87,88] Accordingly, the structural parallelism between active peptides and opiates taken together with the fact that opiate antagonists reverse the action of peptides, suggest that the enkephalins may play the role of endogenous neurotransmitters at specific neuronal sites which control pain perception.

It becomes apparent that these endogenous effectors share with morphine the property of binding to the same receptor and may therefore be sensitive to structural modifications similar to the classical analgesics. If the assumption is made that the basic productive locus of interaction of the pentapeptide [Met^5]-enkephalin with the opiate receptor involves the tyrosine

terminus it follows that the conformation of that residue in the bound state may control the receptor response since this residue is absolutely essential for activity. It has been proposed that the tyramine portion of the peptide and the corresponding moiety comprised in the piperidine ring of opiates may be made to overlap (figure 12), but that feature is insufficient because the tripeptide Tyr-Gly-Gly is inactive and the tetraepptide Tyr-Gly-Gly-Phe has marginal in vitro activity. The inactivity of these two shorter fragments may have three possible implications:

(1) Secondary binding sites which are not accessible to the rigid opiates are available to the amino acid residues of the pentapeptide sequence and would thus contribute critically to the activity of enkephalins.

(2) The pentapeptide sequence would assume a unique active conformation with some features in common with the rigid opiates.

(3) There may exist more than one kind of opiate receptor capable of accommodating different effectors.[89] Consequently it is not a necessity that the conformation of enkephalin should relate to the structure of morphine since they may not bind to the same receptor.

2. Structure-Activity Relationship Studies

A wide diversity of enkephalin analogues were synthesized in order to map the binding sites and to optimize affinity and

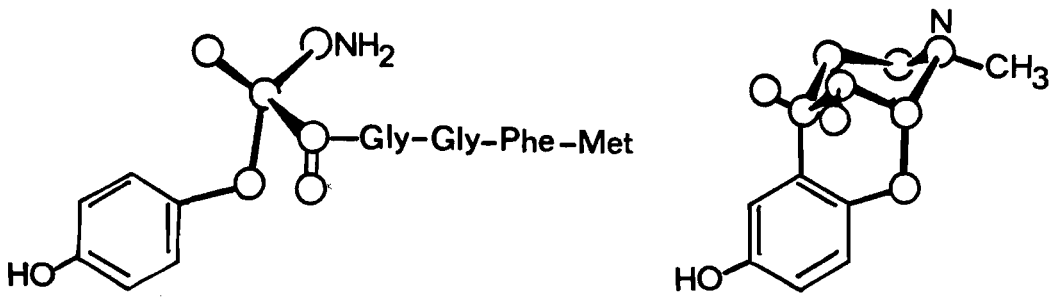


FIGURE 12. Structure of [Met^5]-enkephalin and metazocine showing the possible overlap of the tyramine moieties. This common structural element may be the key to the interaction of Met-enkephalin with opiate receptors.

specificity. Table 5 shows a selection among hundreds of derivatives. It can be seen that the minimum chain-length required for activity comprises four residues as in the tetrapeptide Tyr-Gly-Gly-Phe. Addition of a fifth residue renders the molecule fully active implying that this last amino acid recognizes a discrete binding site on the receptor. Chemical modification of this terminal residue can also cause a large increase in activity. Leaving aside the substitution of D-Ala or D-Met for Gly^2, it can be seen that elimination of the charge on the terminal carboxyl through amidation or reduction to the alcohol enhances both *in vivo* and *in vitro* activity. This suggests that the binding site for the fifth residue may favour an uncharged entity for optimum interaction. Recently Schiller *et al.*[90] synthesized some [Trp^4]-enkephalin analogues and showed that the affinities of these compounds for the opiate receptor are similar to those of the corresponding [Phe^4]-enkephalin effectors; these analogues allowed measurements of the phenol → indole intramolecular distances through fluorescence resonance energy transfer between the aromatic rings. Identical intramolecular distances of 9.3 Å were obtained for [Trp^4, Met^5]-enkephalin, [Trp^4, Leu^5]-enkephalin, and the N-terminal tetrapeptide comprised in the two analogues (table 6). It may be concluded that the presence of the fifth residue has no bearing on the conformational properties of the N-terminal tetrapeptide but by itself contributes strongly to binding.

table 5. MORPHINO MIMETIC ACTIVITY OF SYNTHETIC OPIOID PEPTIDES

PRIMARY STRUCTURE	POTENCY RELATIVE TO MET-ENKEPHALIN=1 OPIATE RECEPTOR	ANALGESIA
H-Tyr-Gly-Gly-OH	0	INACTIVE
H-Tyr-Gly-Gly-Phe-OH	0.01	Na*
H-Tyr-Gly-Gly-Phe-Met-OH	1.0	1.0
H-Tyr-Gly-Gly-Phe-Met-NH_2	3.0	Na
H-Tyr-(D)Ala-Gly-Phe-Met-OH	1.0	100
H-Tyr-(D)Ala-Gly-Phe-PrO-NH_2	Na	1500
H-Tyr-(D)Ala-Gly-Phe-Met-ol	5	1600
H-Tyr-(D)Ala-Gly-Phe-Met(O)-ol	2	9600
H-Tyr-(D)Ala-Gly(Me)Phe-Met(O)-ol	11	28800
β-ENDORPHIN	4	1200
MORPHINE	2	33

*Not available.

Table 6. Intramolecular Tyr-Trp Distances in [Tryptophan]-enkephalin Analogues.

Analogue	r,[a] Å
Tyr-Gly-Gly-Trp-Met	9.3 ± 0.2
Tyr-Gly-Gly-Trp-Leu	9.4 ± 0.1
Tyr-Gly-Gly-Trp	9.3 ± 0.1
Tyr-Gly-Gly-Trp-Met-Thr	10.2 ± 0.2
Tyr-(D)Ala-Gly-Trp-Met	9.5 ± 0.3
Tyr-(L)Ala-Gly-Trp-Met	8.3 ± 0.4
Tyr-Gly-Gly-(D)Trp-Met	9.8 ± 0.1

[a] Solvent is H_2O

It has long been known that introduction of unnatural D-amino acids into polypeptide chains confers resistance to degradative enzymes.[91] This observation has recently been extended to luteinizing hormone releasing hormone (LHRH)[92,93] and successfully applied to enkephalin by substituting Gly^2 with such unnatural amino acids as D-Ala, D-Met[94], and D-Nleu.[95] In all instances the analogues with the unnatural enantiomer at position two had equal or better potency by the intravenous route, and were more active than morphine when they were administered centrally. By far the most impressive of the synthetic

analogues is compound (g) in table 5.[96] With D-Ala in position two, (N-Me)-Phe at position four, and methioninol sulfoxide as the terminal residue, one obtains a compound which is 30,000 times more potent than [Met^5]-enkephalin when injected intracerebroventricularly and 1,000 times more potent than morphine by the intravenous route. Remarkably it is active orally, a property somewhat unexpected for a peptide. Very unexpectedly, when the drug was administered to human volunteers, at very low doses it produced effects totally unlike those of morphine.[97] Since enkephalin and its analogues are known to possess hormone releasing properties,[98] the levels of two pituitary hormones were measured after drug administration and found to be elevated. However, this enhanced hormone secretion could not be prevented by administration of nalorphine suggesting that this compound may not reach the opiate receptor, may bind in a non-specific manner and trigger an avalanche of events leading to side effects masking any manifestation of analgesia at the low doses used.

3. Conformational Analysis

It was mentioned above that receptor-bound enkephalin must assume a unique conformation complementary to the binding site and that adopted conformation may bear some kind of relationship to the morphine molecule. Whereas the geometry of morphine and its analogues is relatively rigid, enkephalin is a rather flexible molecule whose shape is unlike rigid opiates, but whose

elements are more readily adaptable to the geometrical needs of the receptor.

Attempts at demonstrating structural similarities between potent opiates and enkephalin were based primarily on model building. Bradbury et al.[99] originally proposed a conformation consisting of a β-bend[100,101] centered on Gly^2 and Gly^3 with a hydrogen bond between the carbonyl oxygen of tyrosine and the amide nitrogen of Phenylalanine; the tyramine part occupying the same orientation as that same group in morphine. This folded conformation allows the phenethyl moiety of Phenylalanine to coincide with the corresponding phenethyl substituent of the potent morphine derivative, 7α-[1-(R)-hydroxy-1-methyl-3-phenylpropyl]-6,14-endo etheno tetrahydrothebaine[102] (PET) (figure 13). This lipophilic binding element which is absent in morphine could contribute to the tighter receptor-binding and the 10,000 fold increase in in vivo activity of PET, and also account for the strong interaction between enkephalin and the opiate receptor. Even prior to the discovery of endogenous opioid peptides Bentley and Lewis[102] suggested that the higher potency of PET and etorphine would be due to a specific interaction of the C_{19} lipophilic substituent with a unique complementary site on the receptor surface. Snyder et al.[41] expanded this notion in order to account for the uniquely high potency of etonitazine, phenazocine and fentanyl (figure 14) all of which contain an additional aromatic ring (labeled F) in their

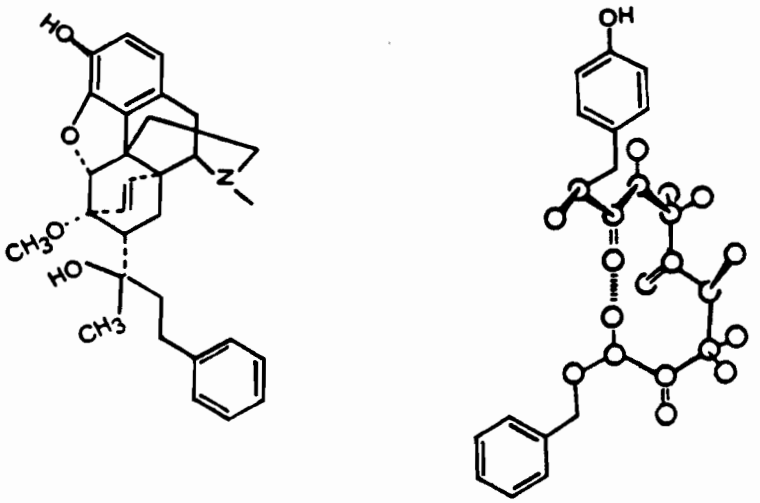


FIGURE 13. The partial structure of enkephalin showing the β-I bend and whose phenylalanine side chain coincides with the C_{19} phenethyl substituent of PET.

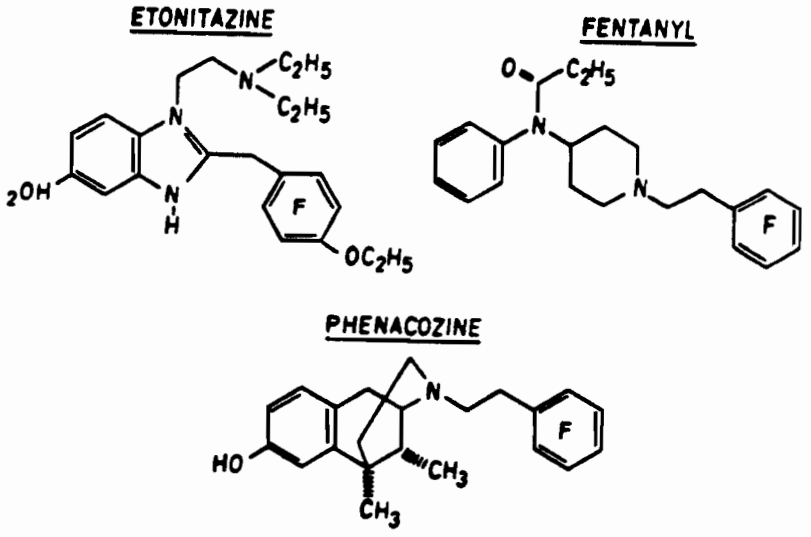


FIGURE 14. Structures of etonitazine, cyclizocine, and fentanyl, all having a common additional binding element (F).

molecular framework.

There is strong evidence that the Phenylalanine side chain does not conform to this model: Firstly, substituting the latter residue with another lipophilic side chain such as in Leucine, which should favour hydrophilic binding on the same site, is detrimental to <u>in vitro</u> receptor affinity.[103] Furthermore, substituting the para position of the Phenylalanine aromatic ring with (OH)[103] or (NH_2)[104] abolishes activity, but it is retained and even enhanced with (p-Cl),[105] (p-NO_2) or (p-N_3)[104] substituents. Unlike enkephalin, morphinans and benzomorphans carrying an N-phenethyl group with or without a para substituent are extremely potent analgesics displaying superior affinity to morphine for the opiate receptor.[106] The activity of these phenethyl analogues is independent of the nature of the para substituent.[107-109] Therefore it would seem that the Phenylalanine side chain may serve a different function than in structurally analogous substituents of rigid opiates.

a. X-ray Crystallography.

The β-bend centered at the Gly^2-Gly^3 level of enkephalin (4 → 1 H-bond) was corroborated by x-ray diffraction on [Leu^5]-enkephalin.[110] The x-ray results also disclosed that the phenol ring is disordered and can occupy two discrete positions with nearly equal probability. The β-bend is stabilized by two anti-parallel hydrogen bonds between the amino nitrogen of Tyrosine and the carbonyl oxygen of Phenylalanine (2.86 Å), and between

the amide nitrogen of Phenylalanine and the carbonyl oxygen of Tyrosine (2.99 Å) respectively. The x-ray structure also revealed that the conformation of the tyramine part of morphine[111] differs from either one of the disordered Tyrosine side chains of [Leu^5]-enkephalin. Furthermore, if the tyramine conformation of the former is superimposed on the latter,[99] the remainder of the peptide chain becomes disoriented and forced away from any region that would be normally occupied by morphine on the receptor. Instead, a good overlap is observed between the 6-hydroxyl and the amino nitrogen of morphine with the Leucine carboxyl and the Tyrosyl amino group. This spatial disposition would orient the Leucine side chain toward the region covered by the C_7-C_8 area of morphine.

One could seriously question the relevance of the solid state conformation to the native receptor topography in view of a variety of arguments against it: Firstly, the presence of an amide N-CH_3 in the analogue [D-Ala^2, N(CH_3)Phe^4]-enkephalin introduces a large bulk into the interior of the β-bend and destroys one of the hydrogen bonds that anchors the bend, yet this analogue is one of the most potent enkephalin derivative known. Secondly, it is well known that both the C_6-hydroxyl of morphine and the entire D ring of morphinans are completely dispensable, and in their absence, the antinociceptive activity is even augmented. Therefore the structural analogy between the Leucine residue and the D ring region of morphine is totally

irrelevant. Thirdly, in solution or in the receptor environment, rotations about the Cα and Cβ bonds may take place causing a total reorientation of the molecule which may be a prerequisite to an induced fit. It was found for example that the optically active R_{biph}-$S_{\alpha\text{-axial}}$ configuration for the 2,2'-bridged biphenyl analogue[112] of benzoyl phenylalanine methyl ester, a chymotrypsin substrate crystallized in the conformation shown in figure 15(a) as established by single crystal x-ray crystallography.[113] In this conformation the molecule is inactive toward CT; however, a solvent-induced isomerization to S_{biph}-$S_{\alpha\text{-eq.}}$ with an equatorial methoxycarbonyl moiety (figure 15(b)) generates the CT active substrate.

b. NMR Studies.

The assumed importance of the solid state conformation of enkephalin is further dwarfed by the abundant evidence based on NMR studies; studies which by themselves are also conflicting and ambiguous. Bleich et al.[114] concluded on the basis of ^{13}C and ^{1}H spin lattice relaxation times in both DMSO-d_6 and D_2O that the tyrosyl side chain of enkephalin exhibits a restricted motion with respect to the main backbone of the molecule. In contrast to the latter findings, Roques et al.[115], Jones et al.[116], and Garbay et al.[117], suggested that the conformation of enkephalin is well defined in both DMSO-d_6 and D_2O. It has been demonstrated previously that the chemical shift temperature independence of an amide proton usually implies that it might

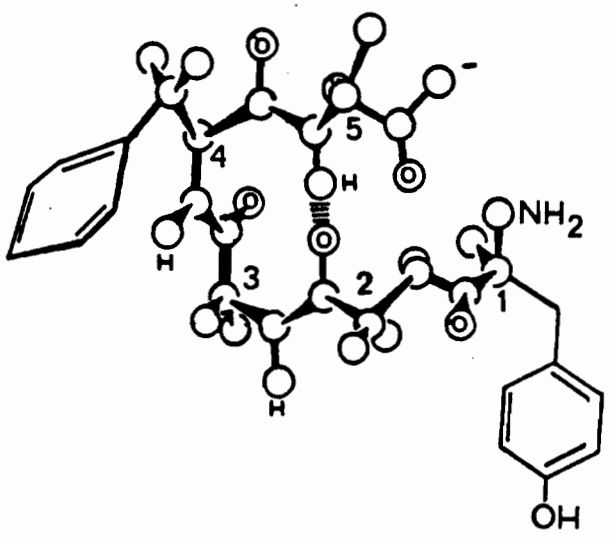


FIGURE 16. The three dimensional structure of Met-enkephalin showing the βI-turn and a 5 → 2 hydrogen bond. The flexibility of the first two residues could be a prerequisite to binding.

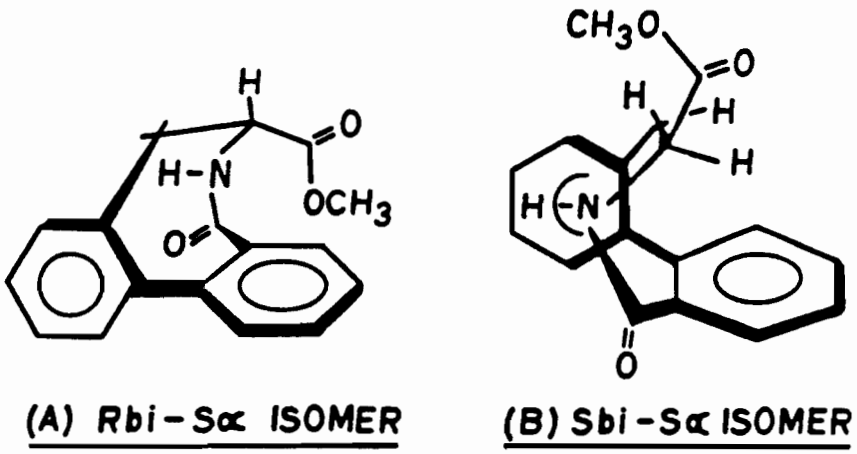


FIGURE 15. The three dimensional structure of the chymotrypsin substrate 2,2'-bridged biphenyl analogue of benzoyl phenylalanine showing the inactive (A) and active (B) conformations.

be involved in a hydrogen bond or may be located in a buried environment.[118] All three groups found such a temperature independence for the amide hydrogen of Methionine, and together with the Phenylalanine coupling constant values ($J_{NH,CH}$), suggested that it might be involved in an intramolecular hydrogen bond within a Gly-Gly-Phe-Met βI-turn as shown in figure 16. Although the resulting conformation does not reveal any concrete structural homologies between [Met^5]-enkephalin and morphine, (except for the common phenethyl binding element discussed previously) it does raise some interesting points: The proposed β-I turn confers upon the $_2$HN-Tyr-Gly^2 moiety a large degree of conformational flexibility and therefore the primary reaction of [Met^5]-enkephalin could conceivably involve the attachment of this group followed by recognition and secondary reaction of the hydrophobic part of the molecule. Although the conformation of the active peptides in the bound state may not be simulated by that in solution, the latter nevertheless may be characterized by a relatively stiff region in the Gly^3-Phe^4-Met^5 region and may be able to tailor its shape through a conformationally-flexible moiety located about the Tyr^1-Gly^2 residues. This model is in agreement with the finding that although the tetrapeptide Tyr-Gly-Gly-Phe bears all the necessary features for biological activity, it will not interact with the opiate receptor. Conceivably, the absence of the 5 → 2 hydrogen bond described above allows other preferential "inactive" conformations to predominate

in solution. Therefore one may ascribe to the fifth residue a primary conformational role[119] although it may subserve as an additional binding element as described by Schiller et al.[82]

More recently Khaled et al.[120] have provided evidence for a concentration-dependent conformation. They suggested that at high concentrations, an associated form of enkephalin predominates in solution consistent with NMR data previously obtained. At concentrations below .01 M (H_2O) a well-defined monomeric form predominates characterized by high rigidity.

C. STATEMENT OF THE PROBLEM

The fundamental problem with such NMR studies is that the microenvironment surrounding the receptor site may not be simulated under solution condition, therefore the carry over from an in vitro conformation to a biologically-intact receptor may not be a valid one. Moreover, in these attempts to uncover overall structural homologies between opioid peptides and their counterparts in the morphine series, the basic assumption that is made is that the two classes of drugs bind to the same receptor sites. Related to this problem are the findings that have made it doubtful that the many synthetic surrogates of morphine, benzomorphans,[121,122] and phenyl piperidines[123] interact with a common receptor or with a homogeneous group of receptors.

The NMR studies also overemphasize gross conformational similarities while overlooking the subtle properties of the substrate

that are an essential ingredient for productive binding on the receptors (i.e. the ability to evoke analgesia). For example, both enkephalin analogs (a) and (b) (figure 17) display high in vitro activity as determined by their ability to displace [^{3}H]-naloxone from rat brain homogenate but (a) was poorly active and (b) was devoid of antinociceptive activity.[124,125]

A remarkable feature of the opiates is that in spite of their diverse chemical constitutions, they are nevertheless highly selective in the type of response induced and are known to interact with opioid receptors in a highly stereoselective manner. This structural diversity was explained partially by the multiple modality model,[123] which supposes an association of different ligands with different recognition loci on a single type or with a group of related but not identical opiate receptors. The basic criterion employed to distinguish multiple from single binding modes is based on the assumption that if the former were involved for two different series of opioids, then constant differences in the rank order potencies of the various congeners would be observed when the N-substituent is varied in an identical manner.[126] This phenomenon is illustrated in table 7 for the phenylmorphans and benzomorphans whose respective aromatic rings are rigidly oriented equatorial and axial relative to the plane of the piperidine ring. It may be readily seen that whereas N-phenethyl substitution in (b) confers a 35 fold increase in agonistic activity, no significant change is observed

H-Lys-Tyr-D-Ser-Gly-Phe-Met-OCH_3

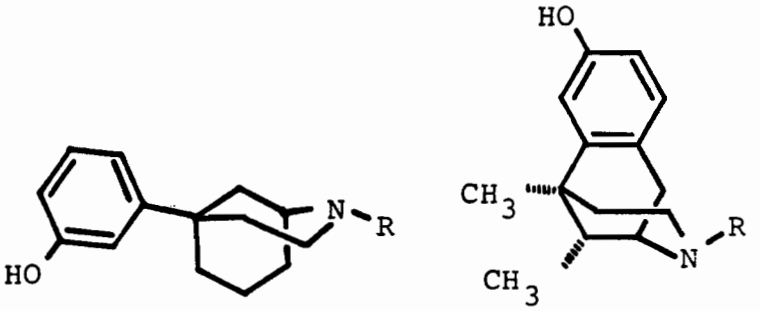
(a)

H-Arg-Tyr-Gly-Gly-Phe-Met-OH

(b)

FIGURE 17. The amino acid sequence of two synthetic hexapeptides with high affinity for opiate receptors. In vivo (a) is moderately active and (b) is completely devoid of analgesic activity.

Table 7. Comparative Analgesic activities of N-substituted Phenylmorphans (a) and Benzomorphans (b), from ref. 126.



a b

R	ED_{50} µmol/Kg	Rel. potency $(CH_3)=1$	ED_{50} µmol/Kg	Rel. potency
CH_3	8.7	1	11.3	1
$(CH_2)_2$	12.5	0.7	0.32	3
$CH_2CH = CH_2$	20.1	0.4	ANTAGONIST	-

for (a). Perhaps the most remarkable distinguishing feature between the pair is that N-allyl substitution, as expected, confers antagonistic properties upon (b) but the same structural change in (a) causes only a decrease in agonistic activity. This divergent behaviour of phenylmorphans and phenyl piperidines will be elaborated on later in the text. It may be inferred that the nitrogen atoms in these two series of compounds

interact with two distinct receptor environments so that the respective N-substituents would probe separate receptor domains.

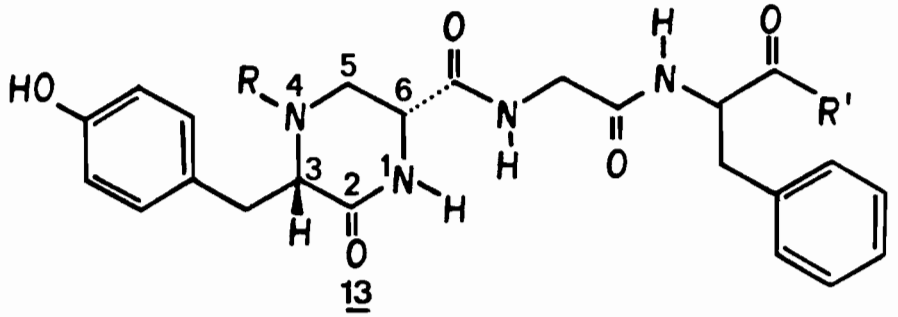
It is well established that the opiate receptor displays absolute chiral specificity toward morphine and its derivatives, morphinans, and benzomorphans, but relatively little toward structurally non-rigid narcotics of the piperidine[126] and methadone[127,128] classes. This may not be surprising since flexible molecules are conformationally adaptable and thus more responsive to the geometric needs of the binding site.[49] [Met^5]-enkephalin and its Leucine analogue may very well be more adaptable to the opiate receptor, but the fact remains that once bound to the active sites they must assume a unique conformation which may or may not bear a resemblance to the rigid opiates.

The active peptides are intolerant to any stereochemical manipulation at the level of Tyr^1, Gly^3, or Phe^4. Furthermore, the receptor does not exhibit stereospecificity at position 5, and tolerates D-Ala but not L-Ala in place of Gly^2. These rigorous stereochemical requirements suggest that enkephalin and its analogues interact unimodally with a unique, well-defined stereospecific receptor surface. However, this does not rule out the existence of multiple receptors. The question of whether or not such a custom fit corresponds to a predetermined molecular shape for the ligand, or whether the substrate undergoes conformational rearrangements in order to maximize overlap with the complementary recognition sites cannot be easily answered.

A clearer insight regarding its receptor-bound conformation may be gained by chemically restricting the conformational freedom of the essential pharmacophores. If the restricted geometry is similar to the one adopted by the natural effector in the bound state, the complementarity of the analogue would be reflected by an increase in the rank order potency. Furthermore, by restricting the conformational mobility of the molecule one would acquire a more reliable understanding of the relative disposition of reactive sites and the conformational analogue may exhibit greater site specificity, since it would be less prone to bind on those accessory receptor sites that are associated with dysphoria and toxicity.

Based on the above considerations it was decided to synthesize and evaluate the pharmacological properties of the enkephalin analogues shown in figures 18 and 19, and relate the presence or absence of analgesic activity to actual binding on opiate receptors.

At the time this work was undertaken, the chemical structures of [Met^5] and [Leu^5]-enkephalin had just been devulged and no structure-activity relationship studies had yet been reported. However, the Tyrosyl part of the endogenous effectors appeared strategic to us because it is this region of the molecules that bears an obvious resemblance to morphine and which later proved to be highly sensitive to chemical modification (table 4). Although it was subsequently shown that maximum

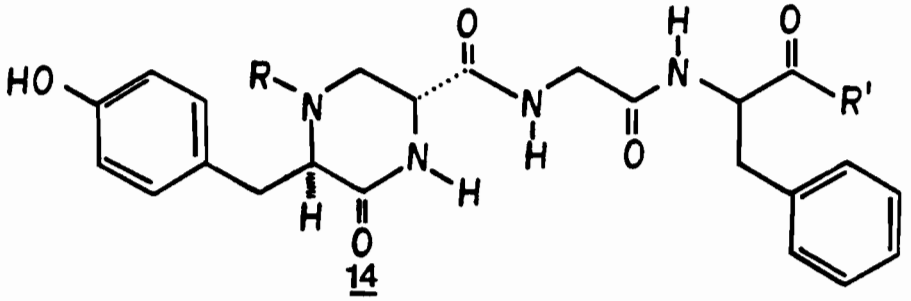


13

(A) R = H R' = $HNCH(CH_2CH(CH_3)_2)COOH$

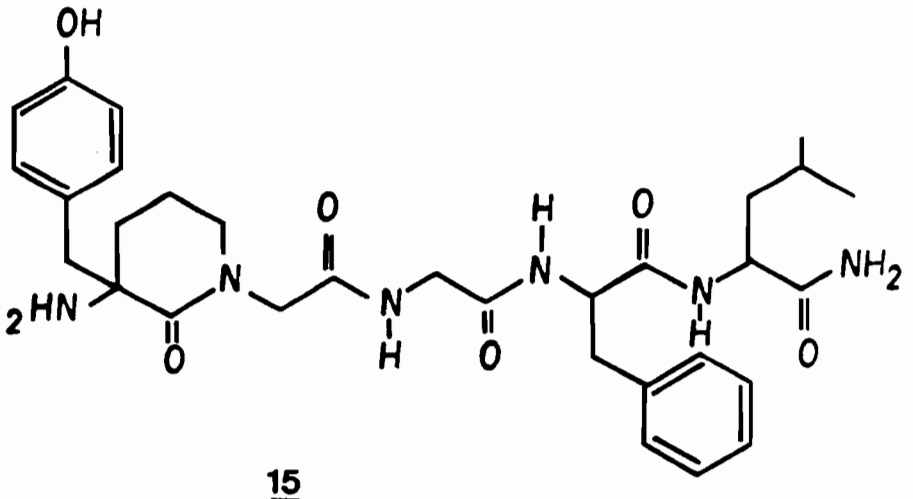
(B) R = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

(C) R = H R' = PROLINE



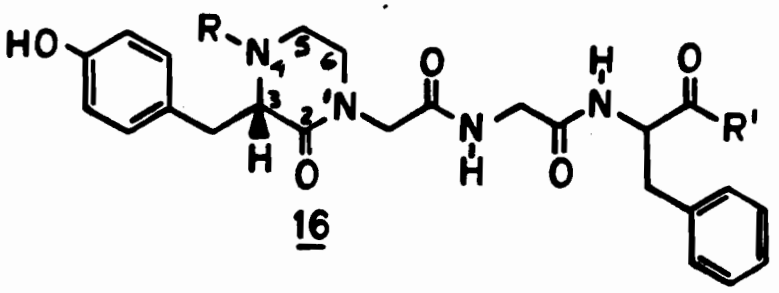
14

(A) R = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$



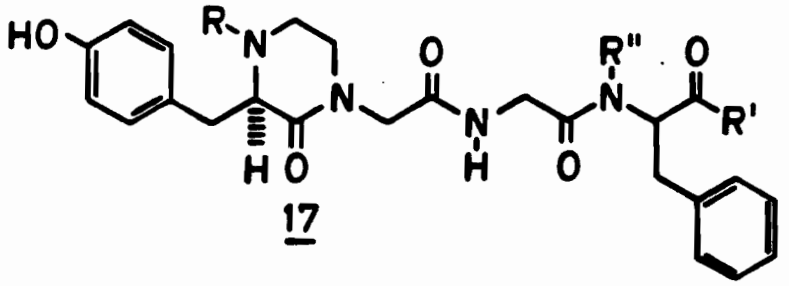
15

FIGURE 18. Some modified enkephalin analogues with conformational restrictions imposed on the tyrosyl-Glycine region.



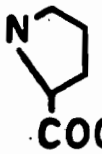
16

(A) R = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$



17

(A) R = H R" = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

(B) R = H R" = H R' = 

(C) R = H R" = H R' = $HNCH(CH_2CH(CH_3)_2)CH_2OH$

(D) R = H R" = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

(E) R = H R" = H R' = $HNCH(CH_2CH_2SCH_3)CH_2OH$

(F) R = H R" = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

(G) R = H R" = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

(H) R = $CH_2CH=CH_2$ R" = H R' = $HNCH(CH_2CH_2CH_2CH_3)COOH$

* CONFIGURATION IS (D)

FIGURE 19. Structures of modified enkephalin analogues with conformational restraint imposed on the tyrosyl residue. The molecules are characterized by a 3-(p-hydroxybenzyl)-2-oxo-piperazine-1, N-acetic acid ring.

interaction with the receptor requires the active participation of all the amino acid residues, it was felt that the sequence of amino acids beyond Gly^2 may be dispensible if the critical Tyrosyl part were imposed a geometry fulfilling the needs of the binding site.

Perhaps the most revealing structural aspect of morphine and its rigid congeners such as levorphanol is the presence of an aromatic ring held axial (at C_4) relative to the piperidine ring and separated from the basic nitrogen by two methylene groups. Although such a spatial disposition of atoms is not visible or even present in enkephalin, it may nevertheless be enforced by the binding site. The peptide nature of enkephalin does not allow for major chemical modifications, but it can be readily seen that the amine function of Tyrosine may be linked to the α-carbon of Gly^2 through a methylene bridge (figure 18 (13) and (14)). The major contrasting structural feature between enkephalin and (13) and (14) is that in the latter two molecules the Gly^2 residue now forms part of a piperazinone ring where the peptide bond is <u>cis</u>. This interesting ring system could potentially mimic the piperidine ring of rigid and semi rigid analgesics (such as the phenyl piperidines); and, owing to the double bond character of the ring peptide bond, severe conformational restrictions are imparted on the molecule. If the phenyl ring of levorphanol (figure 22) is used as a plane of reference, the amine function projects perpendicularly relative to it.

Likewise the corresponding basic nitrogen of the piperazinone rings can potentially assume the same relationship with the plane of its aromatic ring (figure 28). Regardless as to whether the p-hydroxybenzyl part is held axial or equatorial to the plane of the heterocyclic ring, the tyramine moieties present in this structure and in that of levorphanol are stereoelectronically superimposable.

It occurred to us that (13) and (14) could not be valid working models for the interpretation of events at the receptor level because peptide bonds are generally trans whereas the amido linkage of our ring analogues is cis, a geometry which may be incompatible with the receptor sites. Analogous structures (16) and (17) were also conceived (figure 19) where the basic nitrogen of Tyrosine is now linked to the amide nitrogen of Gly^2 through an ethylene bridge. Since Gly^2 remains outside the heterocyclic ring, all peptide bonds can adopt the natural trans[129(a)] conformation. The attempted synthesis of (15) (figure 18) where the basic nitrogen is not part of the ring but attached to it is also described. What distinguishes (13) from (14) and (16) from (17) is the inversed stereochemistry at C_3 and the reason for synthesizing these enantiomorphs as well as (15) will be discussed later.

1. Synthetic Considerations

a. Synthesis of 3-(p-hydroxybenzyl)-6-ethoxycarbonyl-2-oxo-piperazine.

The synthesis of target compounds (13) and (14) was based on the dissection shown in figure 20-A. The final products were obtained by the condensation of the title compound (N-protected with the acid-labile carbobenzoxy group, and as the free carboxylic acid) with the appropriate tripeptide (benzyl ester). All peptides were synthesized by the solution method and peptide couplings were performed with EEDQ[130] unless otherwise noted. The synthetic route toward the title compound is shown in scheme I. Starting with D or L Tyrosine the corresponding methyl ester hydrochloride salt was prepared by dissolving Tyrosine is a solution of thionyl chloride in methanol to give a quantitative yield of (20). The amino group of methyl tyrosinate was protected as the carbobenzoxy derivative using benzyl chloroformate as the reagent. Subsequent hydrolysis of the methyl ester yielded Cbz-Tyrosine (22) quantitatively. Coupling of (22) to ethyl,ββ-diethoxy-α-amino propionate[131] with either DCC or EEDQ gave an excellent yield of dipeptide (24) as a pale yellow oil which could not be crystallized. It is appropriate here to mention that we had to develop a modified procedure for the synthesis of ethyl,ββ-diethoxy-α-amino propionate (23) because countless attempts at reproducing a literature procedure led consistently to yields ranging from

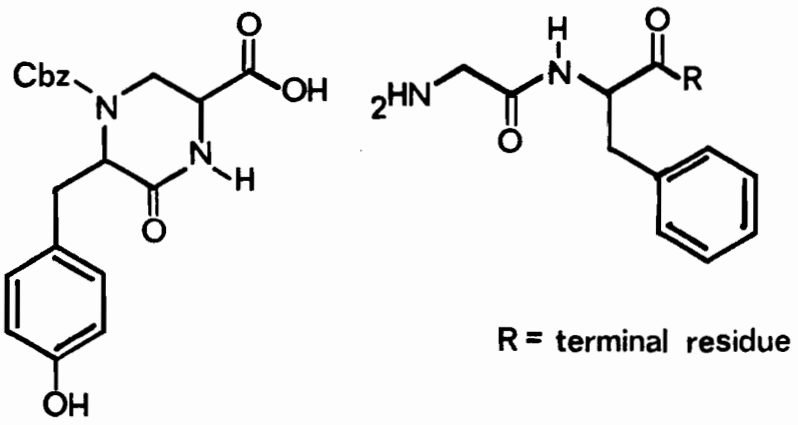


FIGURE 20-A. Representation of the dissection upon which the synthesis of 13 and 14 was based.

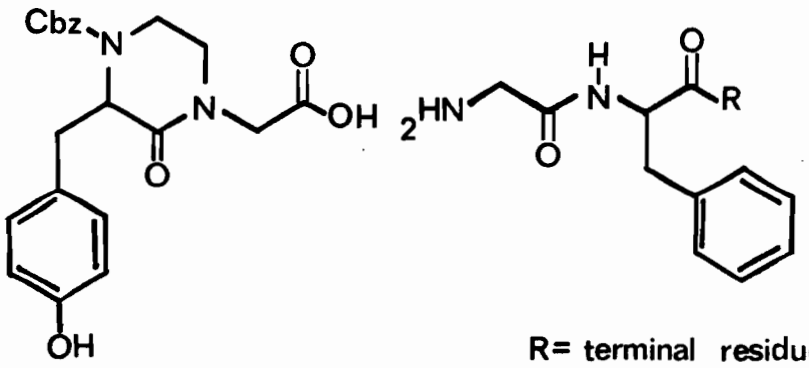
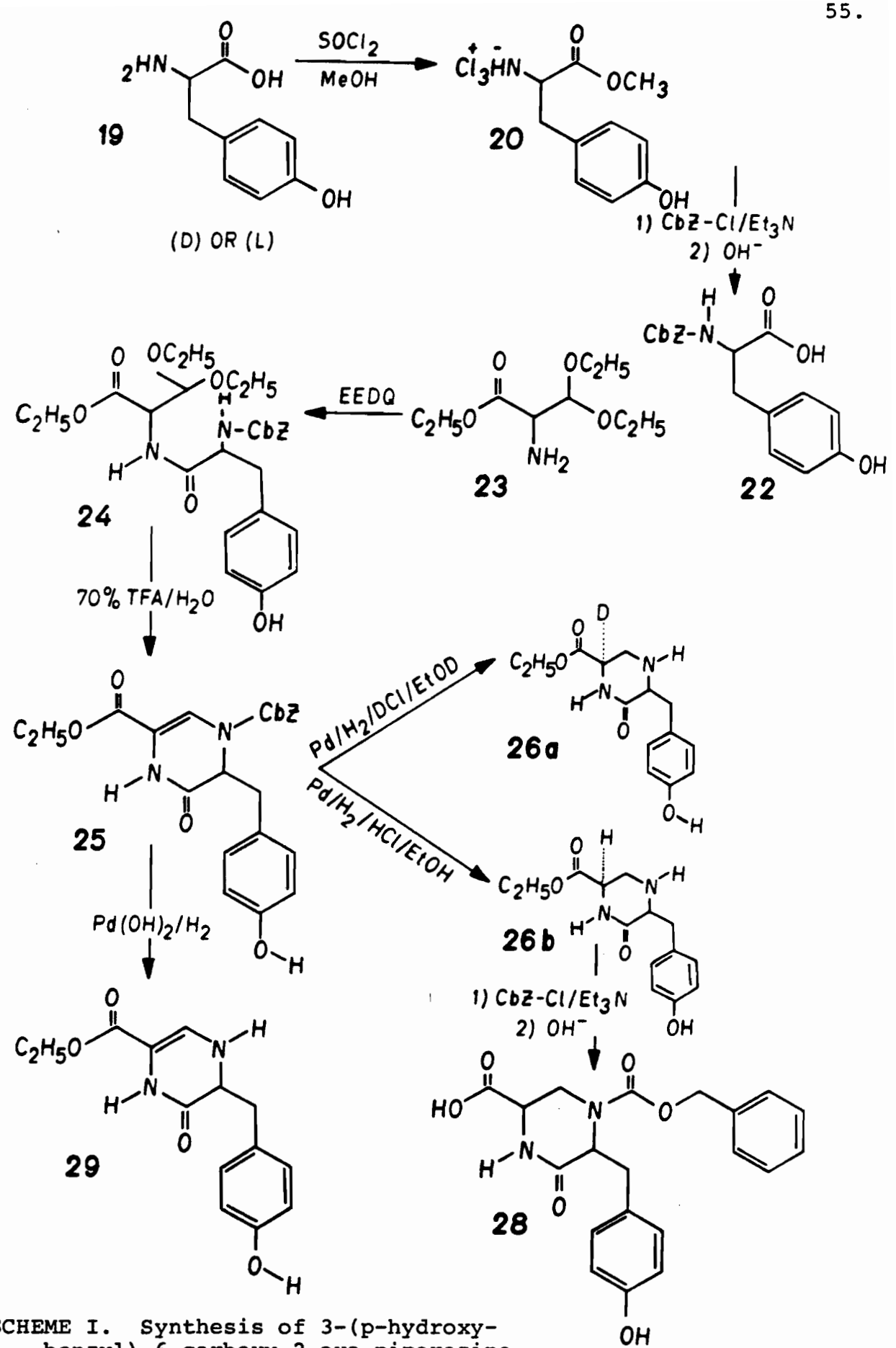
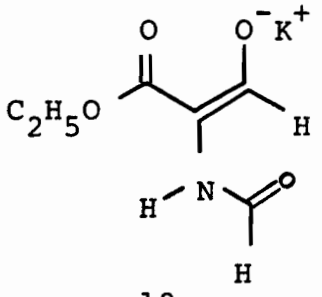


FIGURE 20-B. Representation of the dissection upon which the synthesis of 16 and 17 was based.



SCHEME I. Synthesis of 3-(p-hydroxy-benzyl)-6-carboxy-2-oxo-piperazine-4,N-benzoxycarbonyl.

0 to 5%. The yield of this compound was optimized to better than 70% when sodium ethoxide was substituted by potassium t-butoxide as the base in the reaction between ethyl N-formyl-glycinate and ethyl formate which led to the potassium enolate salt (18). Treatment of (18) with dry gaseous HCl in $EtOH/CH_2Cl_2$



18

for six hours gave excellent yields of the desired compound after vacuum distillation provided that the reaction temperature during the HCl treatment was controlled at dry ice during the entire operation and that the reaction was allowed to reach room temperature only when HCl addition had been stopped.

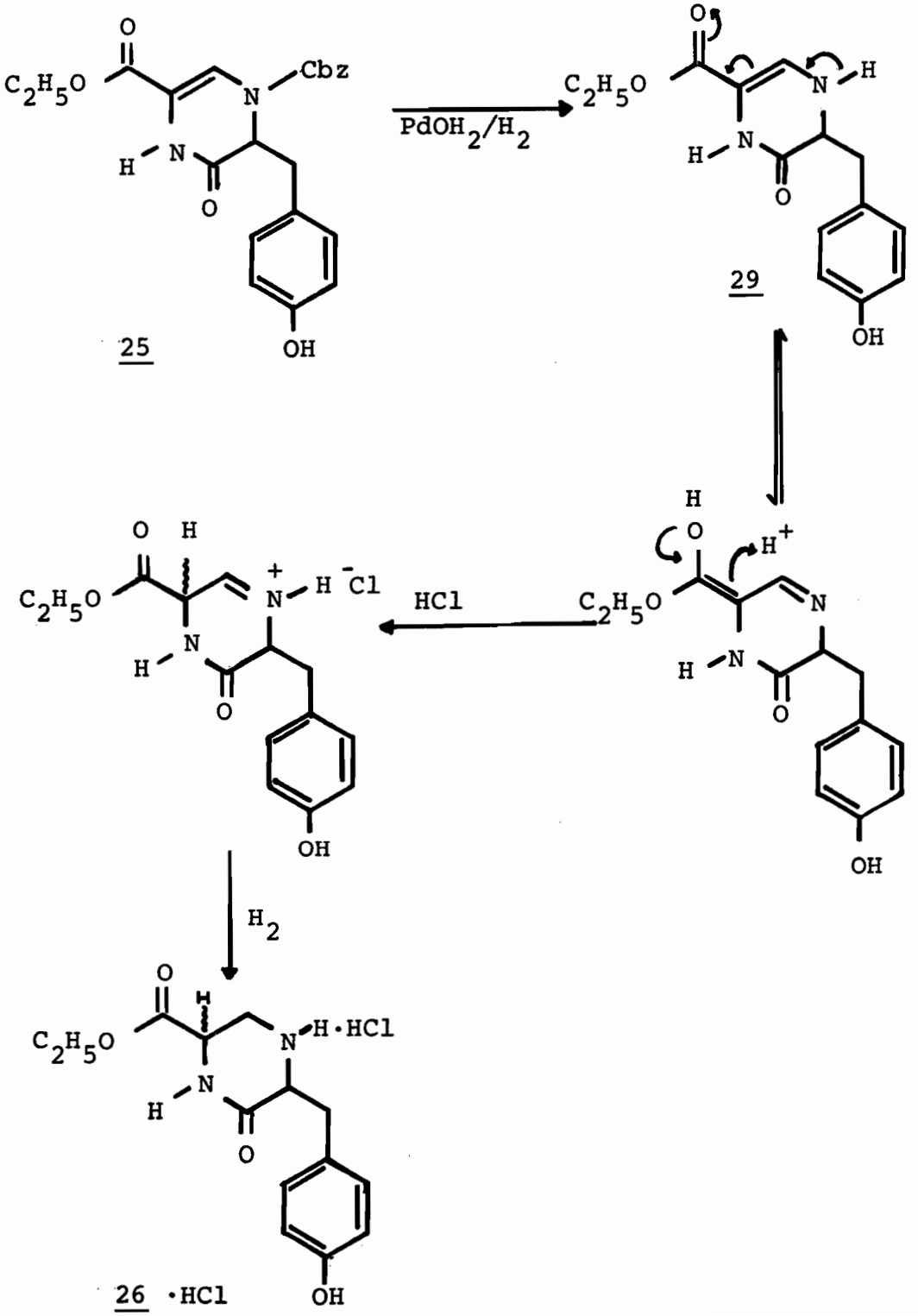
When (24) was treated with 60 to 70% TFA/H_2O (v/v) a dark viscous oil was obtained which showed one major spot on TLC plates (3/1 bz/EtOAc Rf = 0.4). Quick filtration of the product in the same solvent system through a short column of silica CC-7 (Mallinkrodt) afforded a pale yellow oil which crystallized from benzene/hexane. Characterization of this compound by mass spectroscopy, NMR, and UV revealed that it was the vinylogous amide (25). As it turned out, 60 to 70% TFA/H_2O proved to be the best medium for reaction since aqueous AcOH or HCl only

afforded partial hydrolysis of the acetal and no cyclization. Catalytic reduction of (25) with $PdOH_2$[132] in the presence of two to three equivalents of concentrated HCl, gave the amine (26) as the hydrochloride salt which was subsequently converted to the free base and crystallized from methanol to give an overall 60% yield of (26) from (25) as colorless crystals.

At this stage of the synthesis it was anticipated that (26) would be obtained as a diastereomeric mixture since it might be expected that hydrogen would add on both faces of the molecule after removal of the carbobenzoxy group. Unexpectedly only one isomer was obtained and no amount of the other isomer was detected. Further investigation of this phenomenon revealed that if HCl was omitted from the hydrogenation medium, only the conjugated enamine (29) could be isolated with no trace of reduced material being produced.

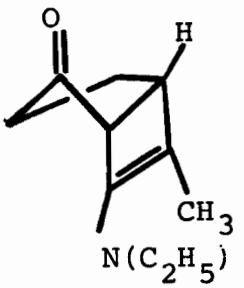
Since it is well known that enamines are susceptible to electrophilic attack at the β-carbon,[133] it was inferred that the reduction of (25) to (26) must initially involve protonation α to the ethoxycarbonyl group followed by reduction of the ammonium cation through the addition of hydrogen as shown in scheme II. This mechanism was confirmed by performing the reaction in EtOD/DCl which resulted in the complete incorporation of one deuterium atom α to the ethoxycarbonyl group. Having established the mechanism for reduction of the vinylogous amide (25), it occurred to us that the intermediate (29) could remain bound to

SCHEME II. Mechanism of reduction of vinylogous amide 25.



the catalyst when the electrophylic attack takes place, thus effectively shielding one face of the molecule and preventing the formation of the second isomer. Accordingly, it was decided to reduce (29) by the method of Borch et al.[134] using $NaCNBH_3$ in methanol in the presence of HCl while maintaining the pH at approximately 3-4, because with this method no adsorption on an active surface is involved. Work up of the product mixture again afforded only one isomer of (26) which was identical to that obtained by catalytic reduction.

A similar stereospecific reduction involving initial protonation of a double bond has been observed with (30) where protonation takes place at the β-carbon specifically from the less hindered side.[135] Dreiding molecular models of (29) show that the



30

molecule is relatively flat and strongly conjugated. In one case (figure 21-A) the benzyl group is equatorial leaving both faces of the molecule equally exposed to electrophiles. In the second (B) the benzyl group is pseudo-axial and effectively overlaps with one face of the heterocyclic ring and precludes interaction

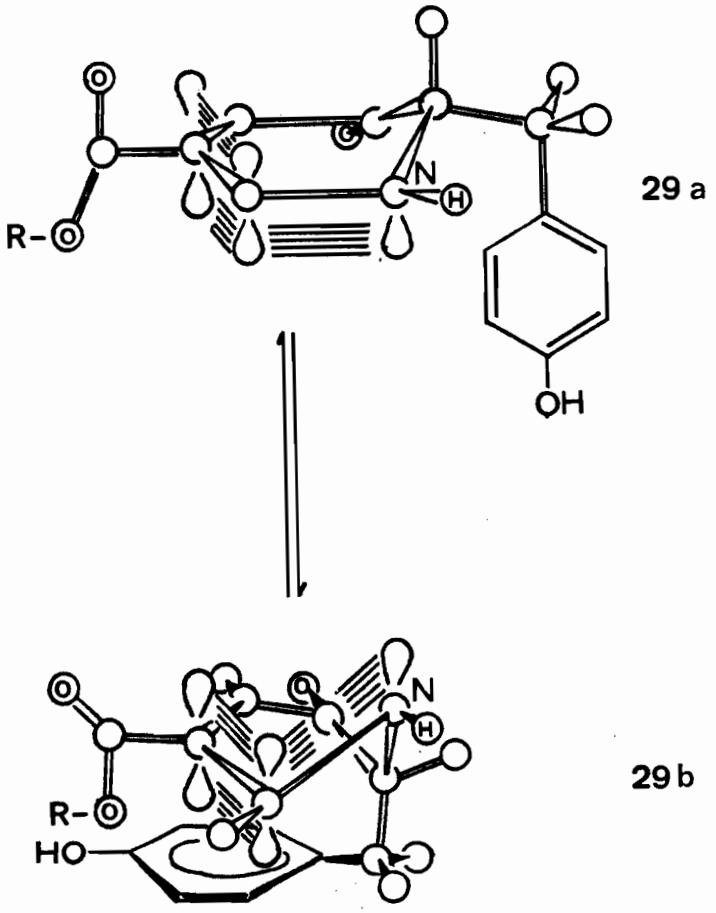


FIGURE 21. Possible conformation of vinylogous amide 29 that may account for the stereospecific reduction.

$R=C_2H_5$

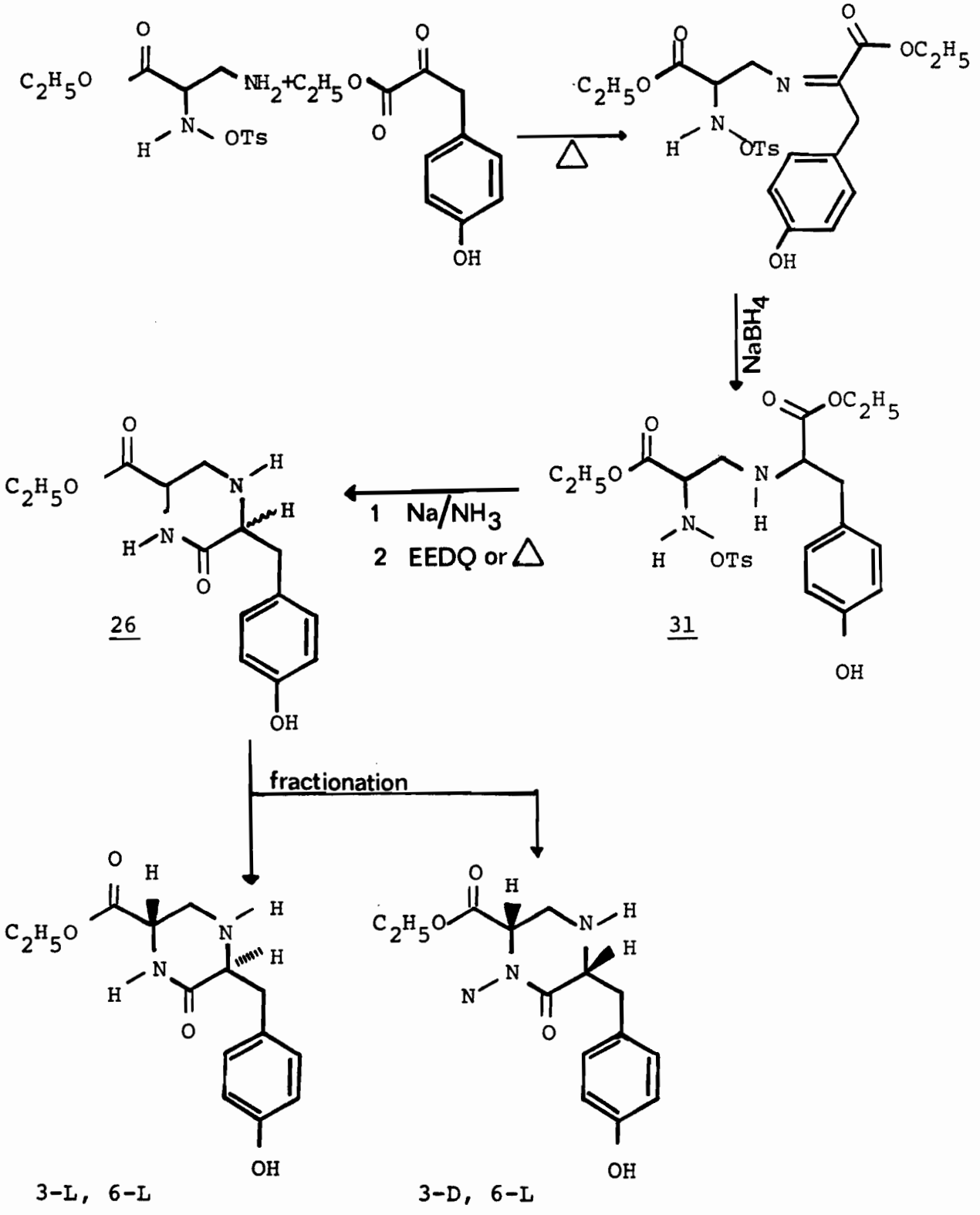
of that face with the catalyst or with a hydride ion donor. This conformational arrangement accounts neatly for the stereospecificity of the protonation step.

The absolute stereochemistry at C_6 of (26) has not yet been determined as we are however, waiting for the results of x-ray analysis. It may be chemically established according to scheme III. Thus, reaction of ethyl, (L)α-N-tosyl,β-amino propionate[136] with p-hydroxyphenyl pyruvic acid and reduction with $NaBH_4$ should yield (31) as a diastereomeric mixture. Deblockage of the α-amino group with Na/NH_3 followed by coupling with EEDQ should yield (26) with an (L) configuration at C_6. Separation of the diastereomeric mixture should afford a 3-(L),6-(L) and a 3-(D),6-(L) isomer. By comparing the physical and optical properties of the former and the latter with those of (26) derived from the hydrogenation reaction, the absolute configuration at C_6 may be confirmed.

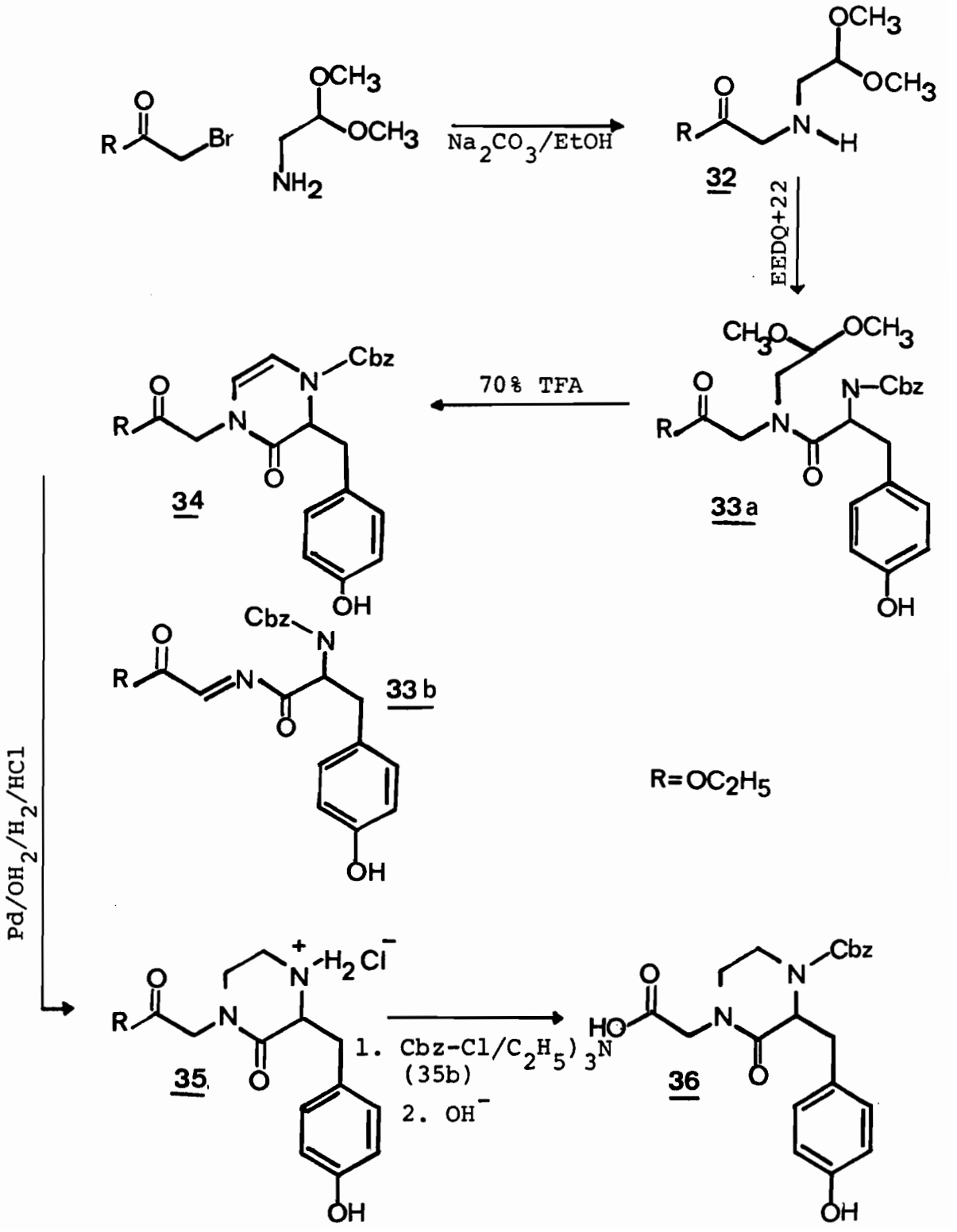
b. Synthesis of 3-(p-hydroxybenzyl)-1,N-ethoxycarbonylmethyl-2-oxo-piperazine hydrochloride.

The synthesis of compounds (16) and (17) was based on the dissection shown in figure 20-B. The final products were obtained by the condensation of the title compound (N-protected with the acid-labile carbobenzoxy group, and as the free carboxylic acid) with the appropriate tripeptide (benzyl ester). The synthetic route toward the title compound is shown in scheme IV. Reaction of aminoacetaldehyde dimethyl acetal with either ethyl bromo-

SCHEME III.



SCHEME IV. Synthesis of 3-(p-hydroxybenzyl)-2-oxo-piperazine-4,N-benzoxycarbonyl-1,N-acetic acid 36.



acetate or ethyl iodoacetate in ethanol in the presence of a large excess of Na_2CO_3 gave a modest yield (30%) of ethyl-N-(dimethoxyethyl)glycinate (32) as a colorless liquid after vacuum distillation. When (32) was coupled to D or L Cbz-Tyrosine in ethyl acetate using EEDQ, the corresponding D or L dipeptide was obtained (33-a) in 80% yield but contaminated with approximately 15% of (33-b). When the reaction was carried out at 5°C for three hours the contaminant was virtually eliminated. The dipeptide was treated with 70% TFA/30% H_2O and after work up and filtration through a short column of Silica CC-7 (Mallinkodt) using 4/1 benzene/ethyl acetate as the eluent, the enamine (34) was obtained as a while powder from ether in 70% yield from (33). Treatment of the enamine (34) in the same way as described for (25) with $PdOH_2$/HCl under H_2 at 40 psi gave the amine (35) as the crystalline hydrochloride salt. However, unlike the enamine (29) the former could be reduced without HCl indicating protonation of the double bond was not a prerequisite.

The basic amine function was protected as the carbobenzoxy derivative by reaction with benzyl chloroformate in chloroform/H_2O using $NaHCO_3$ as the base. The resulting carbamate was a white solid which after hydrolysis of the ester group afforded the piperazinone-1-acetic acid (36).

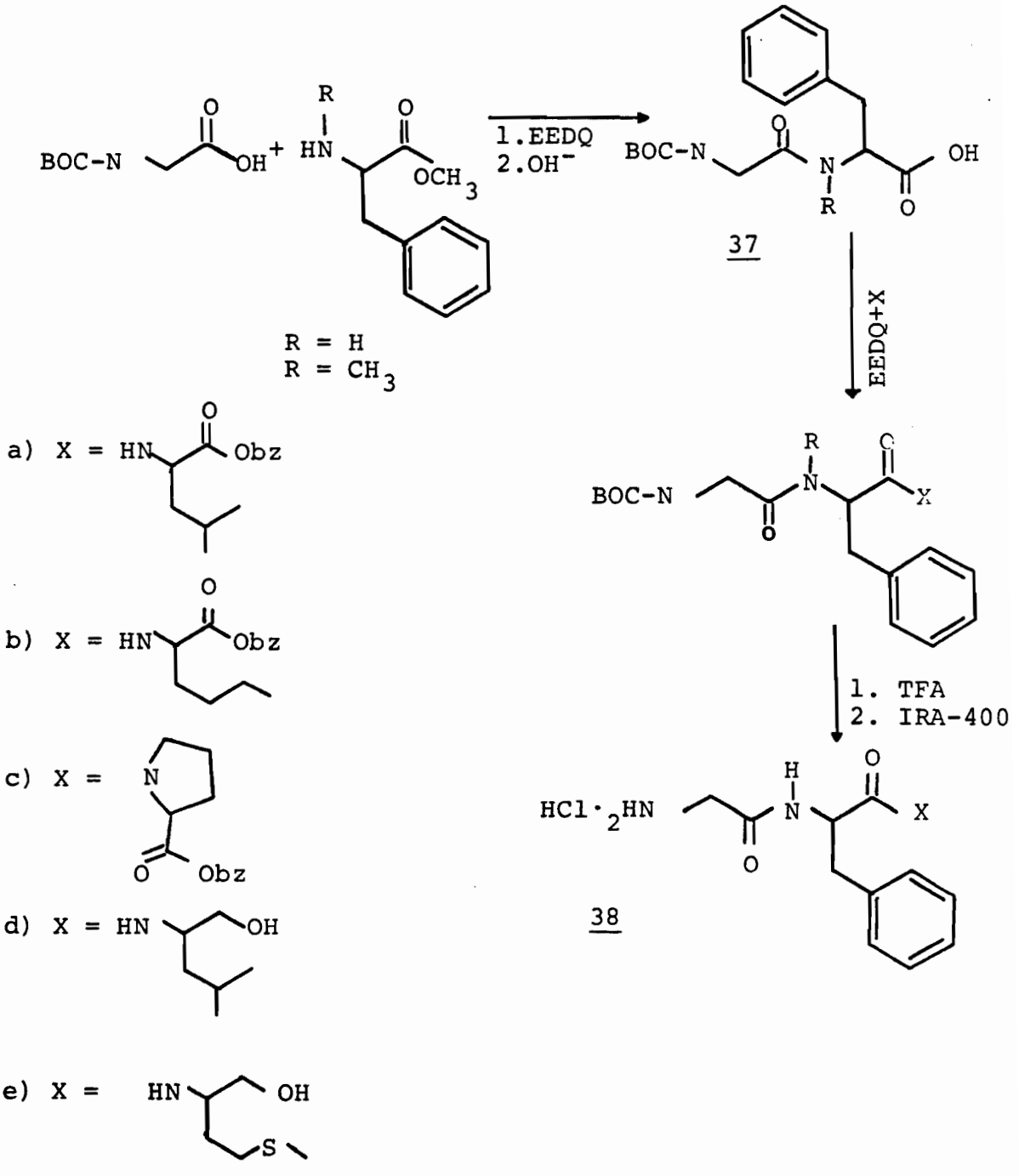
c. Synthesis of tripeptides.

The synthetic routes to the appropriate tripeptides which were subsequently coupled to (28) or (36) are shown in scheme V. Reaction of glycine with BOC-ON[137] in dioxane/H_2O afforded t-BOC-glycine in 95% yield. Coupling of the t-BOC-glycine with phenylalanine methyl ester HCl or N-methyl phenylalanine methyl ester HBr[138] gave the corresponding dipeptide methyl esters which were subsequently soponified to give the corresponding acids (37), the first as a solid and the other as an oil. The dipeptide carboxylic acids were coupled to the appropriate amino acids benzyl esters (prepared by the reaction of the amino acid with thionyl chloride in benzyl alcohol) to give the N-t-BOC, O-bz protected tripeptides. The tripeptides were N-deblocked with anhydrous trifluoroacetic acid to give the trifluoroacetate salts of the corresponding amines which were transformed to the hydrochloride salts (38) after passage through a column of amberlite IRA-400 Cl^-. The products were in turn coupled to (28) or (36) with EEDQ in chloroform to give protected peptides which were deprotected by hydrogenolysis of the N-Cbz and O-bz groups.

d. Purification of peptides.

The deprotected peptides from hydrogenolysis were subjected to a series of purification steps usually involving gel filtration on sephadex,[139] partition chromatogrpahy,[140] and ion exchange chromatography on DEAE- or CM-Sephadex. Product purity was assessed by the shape of the elution profile as monitored at

SCHEME V. Synthesis of tripeptide benzyl esters.

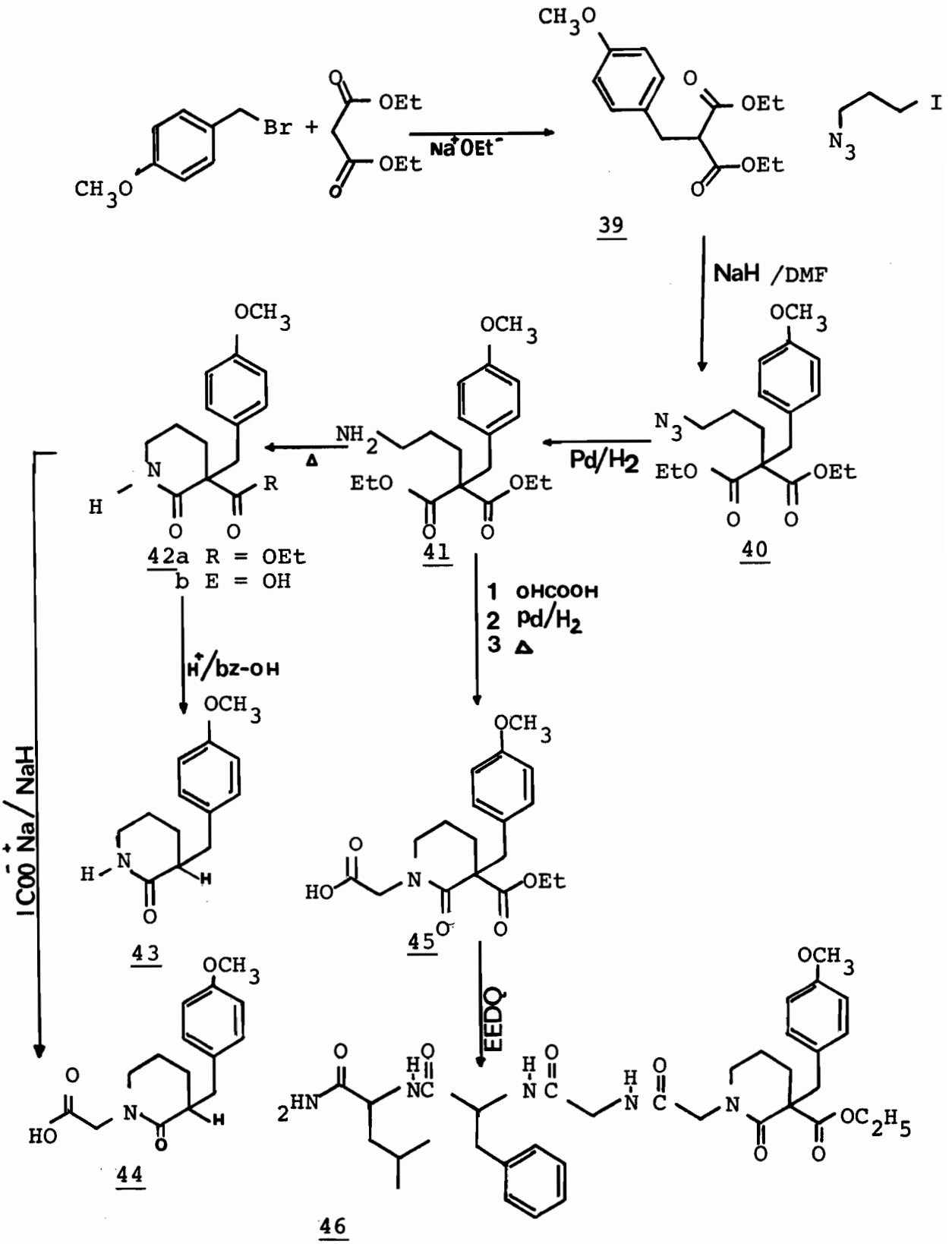


280 nm. The criteria for purity was based on the behaviour of these peptides in four different solvent systems, HPLC, and amino acid analysis (see experimental details). As was expected, the final products could not be crystallized and were obtained as amorphous white powders after lyophilization from 10% aqueous acetic acid.

e. Attempted synthesis of 3-amino-3-(p-methoxybenzyl)-2-oxo-1-piperidineacetic acid.

The reasons underlying the attempted synthesis of this compound will be outlined in detail in the section Results and Discussion. Presently only a describion of the synthetic scheme leading to (15) need be given (scheme VI). Anisyl bromide[141] was reacted with sodio-ethyl malonate in alcohol to give a 60% yield of methoxybenzyl-ethyl amlonate (39) as a clear distillable oil (135-138°C, .15 mm). Reaction of (39) with 3-azido propyl iodide (see Experimental) in DMF using NaH afforded (40) in 90% yield as a clear yellow oil. When (40) was reduced to the amine by hydrogenation over Pd/C, (41) was obtained as the hydrochloride salt and then cyclized to the lactam (42-a) after liberating the base and heating to 150°C (neat) for five hours. Intermediate (42-a), a brown solid, mpt 103-105°C, was saponified to give the carboxylic acid (42-b) as a white precipitate from chloroform/ether. Unfortunately when (42-b) was subjected to standard Fischer esterification conditions, only the decarboxylated lactam (43) was obtained and none of the desired benzyl ester was

SCHEME VI. A possible synthetic route to 15.



produced. Alternatively we decided to leave the ethyl ester function intact and add the required N-acetic acid function first, by reaction of (42-a) with sodium-iodo acetate in DMF using NaH. Unfortunately the hydrolysis of the ester took place and only the decarboxylated N-acetic acid derivative (44) was isolated.

Alternatively, it is felt that the desired peptide (15) containing the exo amine function may be obtained by reacting (41) with glyoxylic acid, reducing the imine by hydrogenation, and cyclizing the product to the 3-ethoxycarbonyl lactam (45). Coupling of (45) to the tripeptide glycyl phenylalanyl leucinamide and hydrolysis of the ester could give the intermediate acid (46). The acid could then be subjected to Curtius conditions forming the exo amine (15).

2. The N-Lone Pair Effect.

a. In rigid opiates.

The absolute chiral specificity of the opiate receptor toward structurally rigid opioids implies strict geometrical requirements for a given response and marked susceptibility of such a response to modulation by substituents. This susceptibility is clearly evidenced through substitution of N-CH_3 of morphine by an allyl group to give nalorphine which is an antagonist with partial agonist properties as discussed previously. Similarly, introduction of a 14-OH group contributes critically

to the "clean" antagonist property of naloxone and the "metagonist" activity[49] of butorphanol. One aspect of structure-activity relationships that has escaped attention until recently concerns the stereoelectronic effects about the basic nitrogen of morphinans as opposed to stereoisomerism about chiral carbons. Perhaps the most dramatic electronic effect in morphinans is associated with a complete loss of *in vivo* activity upon contraction of the six membered piperidine ring of (47) to a five membered ring as in (48) (figure 22). Unexpectedly, the pure racemates of (48-a) and (48-b) exerted no analgesic activity, were totally devoid of antagonistic properties, elicited none of the side effects typical of analgesics, and evoked ataxia in rats at doses of 5-20 mg/kg.

Single crystal x-ray analysis revealed that unlike (47) where the N-lone pair is axial and oriented away from the aromatic ring, (48) has its lone pair directed toward the aromatic ring. Since the D-normorphinan (48) is a tertiary amine which is stereoelectronically superimposable on levorphanol (except for the property described above), and since the relative disposition of the nitrogens in the two molecules is identical, then a *simple* electrostatic mechanism between the protonated cationic species with a complementary anionic site on the receptor[14] should be favoured equally for both structures. In order to account for the lack of analgesic activity of the D-normorphinan (48), Belleau *et al.*[142] concluded that the

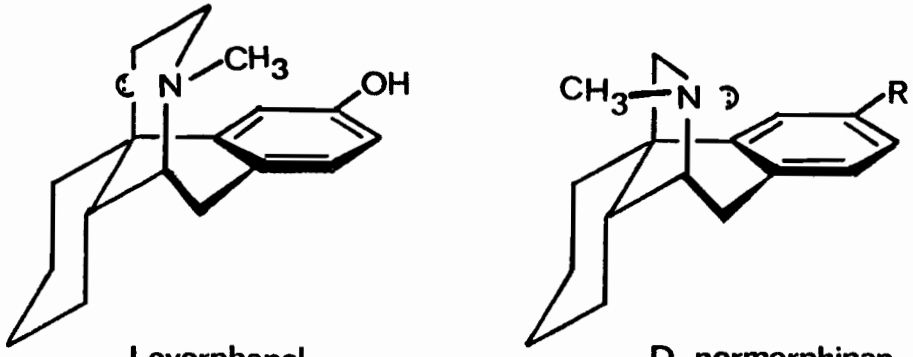


FIGURE 22. Three dimensional structure of levorphanol and ring D-morphinan showing the orientation of the respective N-lone pairs.

orientation of the N-lone pair is a key determinant for productive interaction with the receptor, since in its protonated cationic form it would be expected to bind readily if only electrostatic forces were operative.

This conclusion was misunderstood in the sense that it was taken to mean that levorphanol would bind as the free base through an axial lone pair and therefore the lone pair orientation effect was dismissed as a key parameter for binding on the grounds that the N-dimethyl quaternary iodide analogues of potent agonists are active in the *in vitro* naloxone or etorphine displacement assay.[143] In order to reconcile the differences of interpretations, Di Maio *et al.*[49] emphasized that a proton transfer process at the receptor level would be involved, so that as a potential proton donor the morphinan would bind in the cationic form, but as an acceptor it would have to acquire at least partial cationic character after binding as shown in figure 23.

The quaternary N-methyl derivative of morphine has been reported to elicit analgesia and hypothermia when administered intraventricularly or into the periaqueductal grey matter of the rat.[144] In the guinea pig ileum preparation quaternized morphine has been shown to interact with opiate receptors with a potency of 2.5% that of the parent morphine.[145] In the same preparation, N-methyl quaternary levorphanol had marginal activity which was corroborated by its minimal ability (1.5% that of levorphanol)

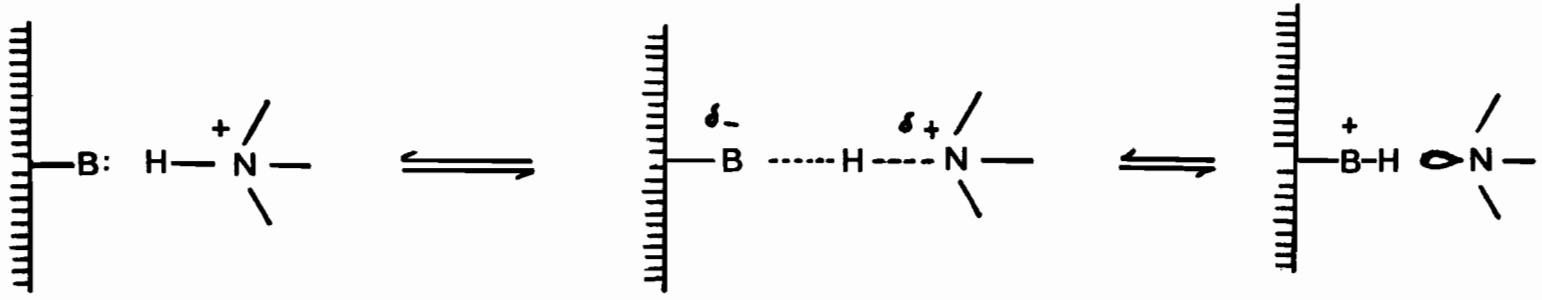
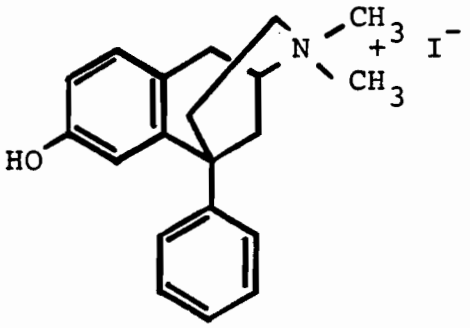


FIGURE 23. Schematic representation of proton transfer between an acceptor base of the opiate receptor and a protonated effector. The position of equilibrium is strongly dependent on relative basicities of acceptor and donor.

to displace [^{3}H]-etorphine from pig brain homogenate.[143] Likewise the quaternary analogue of 5-phenyl benzomorphan[146] (49) displayed very weak activity relative to the tertiary base to the extent of 5% against [^{3}H]-naloxone. More recently



(49)

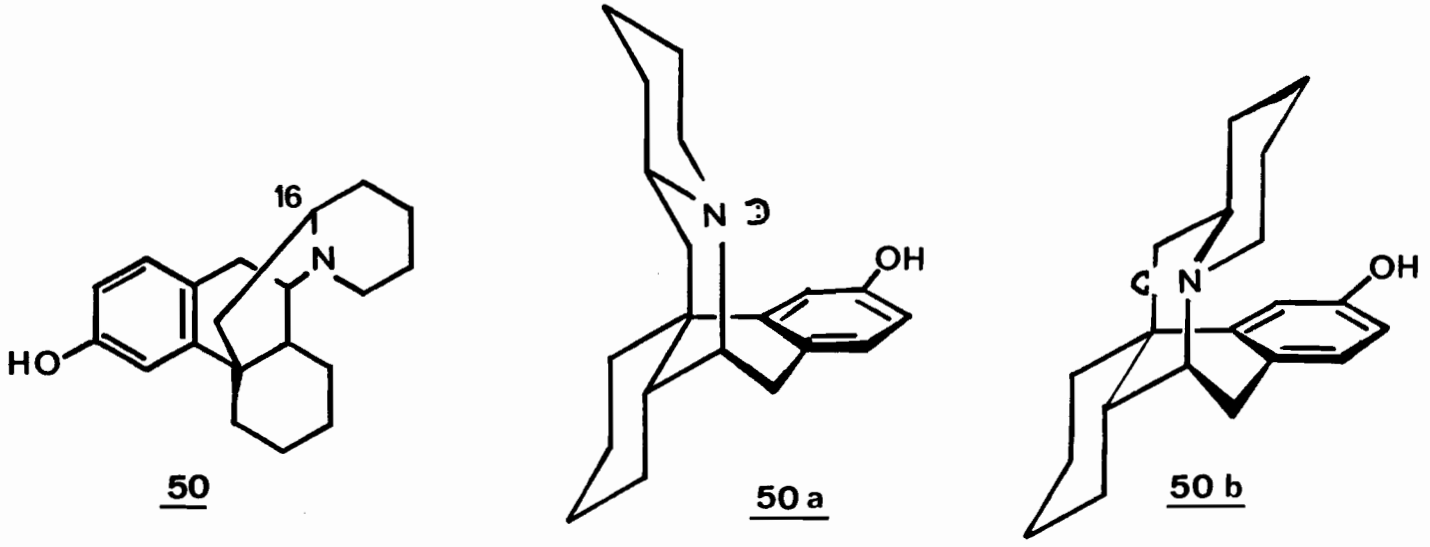
the N-methyl quaternary analogue of morphine has been reported to evoke analgesia when administered intracisternally in the rat and evidence was presented that analgesia was not due to in vivo conversion to morphine.[147] Again the activity in this preparation was only minimal, short lived (1 hour vs. 3 hours for morphine) and the data suffered from large statistical errors.

In order to test for the importance of the N-lone pair effect, we have synthesized the N-methyl quaternary analogue of levorphanol and extended our studies to the N-methyl quaternary salt of the antagonist, levallorphan. The pharmacology of these compounds was assessed using three different assays: (a) [^{3}H]-naloxone displacement from rat brain homogenate, (b) guinea pig ileum assay, (c) ICV administration in mice.

It may be argued that the rigid D-normorphinan (48) is

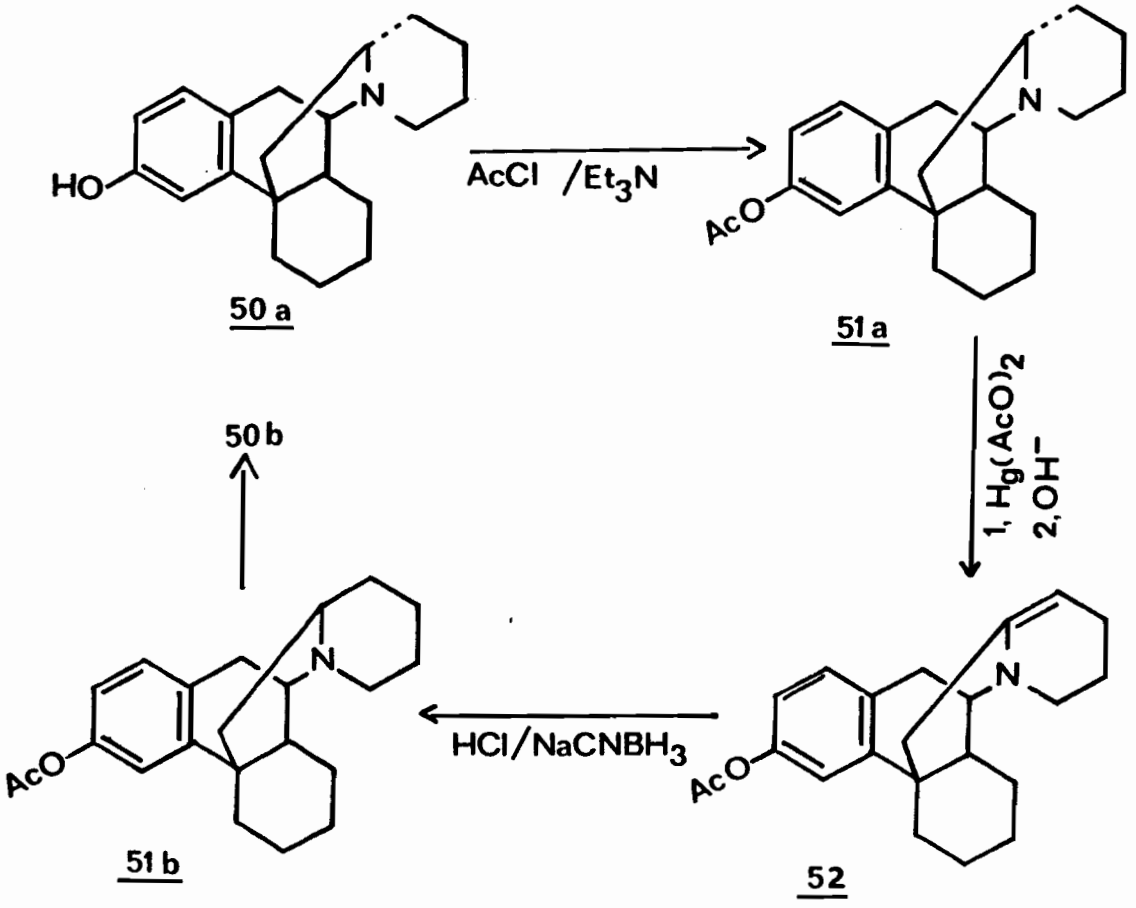
inactive because it is not a true morphinan if such a statement could substitute for a scientific explanation. Nevertheless, as an answer to this rhetorical objection, the following results provide conclusive, unambiguous evidence that the lone pair effect is real and manifests itself in "true" morphinans such as (50) in figure 24.

A few years ago Douglas and Meunier[148] reported the synthesis of one of the two possible epimers of 16,17-butanomorphinan (50) and found it to be inactive both as an agonist or as an antagonist; like the D-normorphinan it produced ataxia. Single crystal x-ray analysis[149] of (50) showed that this epimer has the 16α-configuration which rigidly locks the piperidine ring D in the boat conformation (50-a). Accordingly the N-lone pair projects into the phenyl ring analogously to the N-lone pair of the D-normorphinan, thus accounting for the lack of antinociceptive activity. The 16-α epimer was converted to the thermodynamically more stable 16-β isomer by a series of reactions shown in scheme VII. Thus, treatment of (50-a) with acetyl chloride in chloroform afforded the acetate as a viscous oil (51-a) (Rf = 0.8, acetone). Mercuric acetate oxidation of the tertiary amine[150] afforded the enamine (52) which was reduced with $NaCNBH_3$ in THF/MeOH (pH 4) to give exclusively the o-protected 16-β epimer (51-b). Deprotection by treatment with K_2CO_3 in $EtOH/H_2O$ and solvent extraction gave a powder (50-b) from methylene chloride (Rf =0.3, acetone) (Rf 50-a = 0.5, acetone). The configuration of the new



16-butano morphinan

FIGURE 24. A novel pentacyclic 16,17-butanomorphinan. The orientation of the lone pair is dependent upon the configuration about C_{16}. The α-configuration of 50a forced ring D into a boat conformation. The β-configuration of 50b allows an all chair arrangement.



SCHEME VII. The conversion of 16α-butanomorphinan to the analgesically active 16β-epimer. The key reaction is the $NaCNBH_3$ reduction of enamine 52.

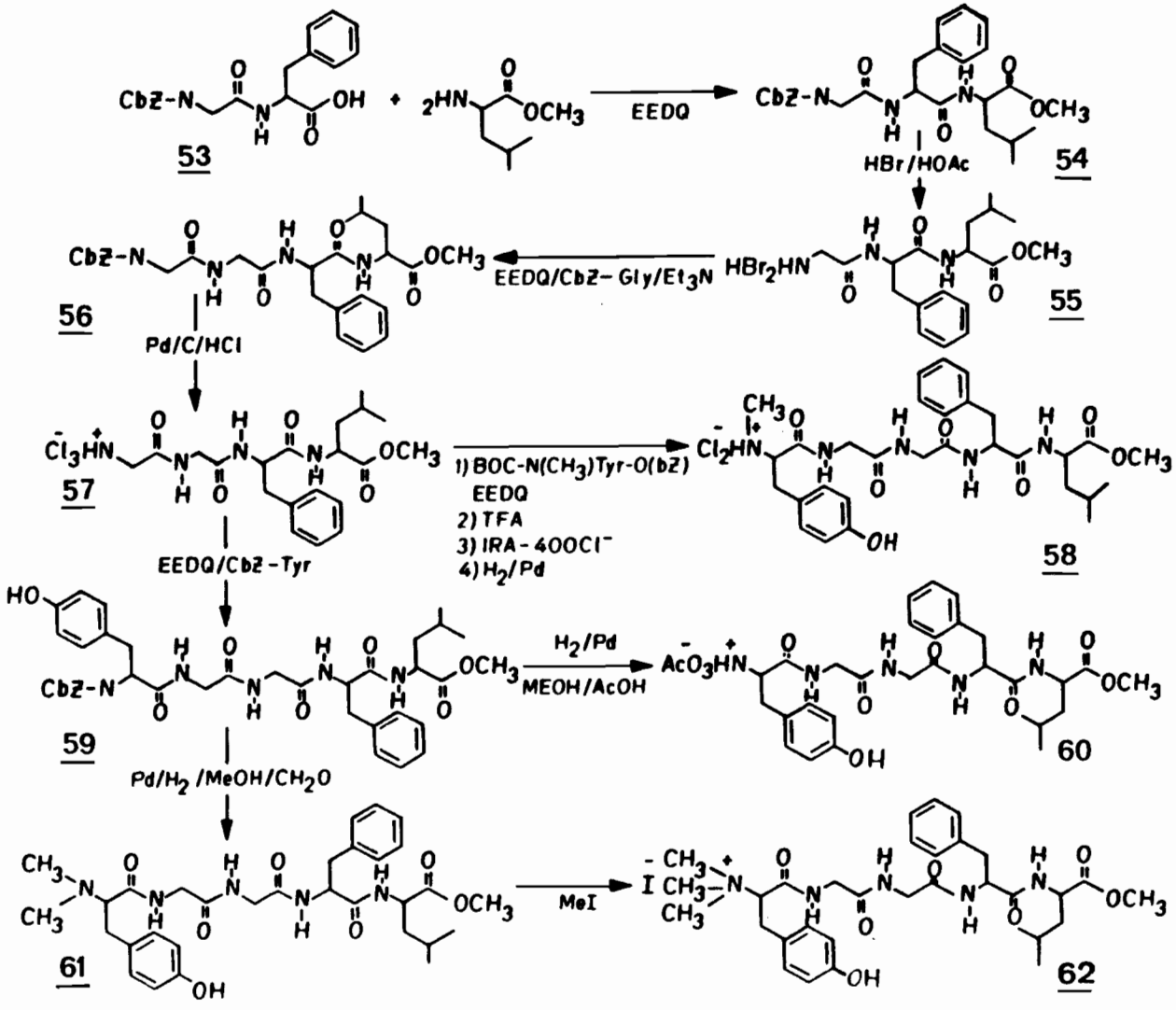
16-β epimer was confirmed by single crystal X-ray analysis of the hydrobromide salt.[149] The 16-β configuration rigidly locks the ring D in the chair conformation which forces the N-lone pair to adopt an orientation exactly opposite to that in the 16-α epimer (pointing away from the aromatic ring. Not surprisingly the new epimer displayed analgesic activity in mice comparable to pentazocine (ED_{50} 3±.5 mg/kg).

b. In enkephalin.

In sharp contrast to morphine and other narcotics the most active of which are tertiary alkyl bases, enkephalin carries a primary amine function. Although this primary amine as supplied by the tyrosine residue may possess significant rotational freedom, alkylation of the nitrogen may introduce some rotational barriers; it follows that inversion and the number of rotational conformers may be reduced owing to unfavourable steric passing interactions. Increased alkylation may conceivably freeze the rotation of the amino group in space so that the lone pair orientation effect, if it applies may be optimized or suppressed. We decided to synthesize the N-methyl, N,N-dimethyl and trimethyl quaternary iodide salt of [Leu-OCH_3^5]-enkephalin and evaluate the morphinomimetic activity <u>in vivo</u> and <u>in vitro</u> with the parent primary amine compound. The synthetic routes to these compounds are shown in scheme VIII.

c. Synthesis of N-methylated analogues of [Leu-OCH_3^5]-Enkephalin.

The commercially-available Cbz-glycyl phenylalanine (53) was coupled to methyl leucinate using EEDQ to give Cbz-glycyl phenylalanyl leucine methyl ester (54) as a clear oil in 95% yield. Treatment of (54) with anhydrous HBr in acetic acid for one hour afforded the hydrobromide salt (55) in 50% yield as an amorphous powder. The latter was in turn coupled to Cbz-glycine to give the tetrapeptide (56) as crystals from ether (mpt. 105-108°C). Deprotection of (56) by hydrogenolysis using Pd/C (HCl) for three hours gave the deprotected tetrapeptide as the hydrochloride salt (mpt. 175-180°C) from ethanol/ether. The tetrapeptide (57) was coupled to N-t-NOC-N-(CH_3)-Tyr(o-bz)-PH and the ensuing product was deblocked sequentially with anhydrous TFA, and with 10% Pd/C/H_2 (10 hours). The pure N-CH_3 analogue (58) was obtained in 30% yield after extensive purification by gel filtration on sephadex G-15, ion exchange chromatography on SP-sephadex, and desalting of G-15 sephadex. The primary amine (60) was obtained by coupling (57) with Chz-Tyr-OH and deblocking of the protected pentapeptide followed by chromatographic purification as described for (58). The dimethyl analogue was obtained as the crystalline free base from the protected peptide (59) (free of paraformaldehyde) using a procedure described previously.[152] In this method, (59) was hydrogenated for 18 hours at 40 psi of H_2 in the presence of



SCHEME VIII. Synthesis of N-methylated analogues of [Leu-OCH_3]-enkephalin.

methanolic formaldehyde to afford (61) as a white powder from ethylacetate (mpt. 104-105°C). The quaternary analogue (62) was prepared by treating a methanolic solution of (61) with a large excess of methyl iodide. The iodide salt was obtained as white crystals from ethanol (mpt. 170-172°C).

EXPERIMENTAL

A. SYNTHETIC PROCEDURES

Pmr spectra were recorded in $CDCl_3$, DMSO-d_6, D_2O, or D_2O/CD_3CO_2D with a Varian T-60, T-60A, or EM-390 spectrometer. All chemical shifts are given in δ values relative to TMS (tetramethyl silane) or TSP (sodium, 3-trimethylsilylpropionate-2,2,3,3-d_4). Resonance signals are described using the following format:

Pmr (solvent), peak multiplicity, chemical shift, (number of protons), coupling constant (Hz), assignment.

Abbreviations, (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet, (b) broad.

Optical rotations were measured on a Perkin Elmer 141 Polarimeter in the solvent and concentration specified. IR spectra were recorded on a Perkin Elmer 257 infrared spectrophotometer using NaCl cells or KBr pellets. Peak intensities are described as (s) strong, (m) medium, or (w) weak.

Mass spectra were recorded on an AEI-MS-902 spectrometer at 70 ev using a direct insertion probe.

HPLC experiments were performed on a Waters Associates LC instrument in 60% H_2O/40% MeOH (.01% H_3PO_4) using a μ-bondapack (RP-18) column at room temperature.

Melting points were determined on an Electrothermal melting point apparatus or a Buchi SMP-20 melting point apparatus and are uncorrected.

Amino acid analyses were performed on a Beckman model 121-M amino acid analyzer coupled to a Beckman system AA computing integrator.

For tlc, precoated silica gel thin-layer sheets (Kodak) with fluorescent indicator were used. Chromatography of the product peptides was performed in the following solvent systems:

(A) 4:1:5 butanol:acetic acid:water (organic phase)

(B) 15:10:3:12 butanol:pyridine:acetic acid:water

(C) 1:1 butanol:water (1.5% pyridine 3.5% acetic acid) (organic phase)

(D) 5/1 sec-butyl alcohol/3% NH_4OH

Purification of the final products was accomplished by gel filtration, ion-exchange, and partition chromatography on Sephadex (Pharmacia). LKB instrumentation was used comprised of a fraction collector, gradient and gradient mixer, UV detector (280 nm) and recorder.

Elemental analyses (C, H and N) were performed by Spang Microanalytical Laboratory (Ann Arbor, Michigan).

1. Synthesis of (D) and (L) trans-amido piperazinones.[129]

Tyrosine methyl ester hydrochloride

To a mixture of 1 gram of tyrosine in 20 ml of absolute methanol chilled to brine temperature, was added 1 ml of thionyl chloride (distilled from triphenyl phosphite) dropwise at such a rate that the reaction temperature did not rise above 0°C. After complete addition, the clear solution was allowed to reach room temperature, stirred overnight, and then concentrated to a volume of 5 ml in vacuo. Addition of anhydrous ether precipitated a white powder that was collected and washed with ether. Recrystallization from methanol/ether afforded crystals corresponding to the methyl ester. Yield 1.0 g (80%).

D-isomer mpt. 189-190°C $[\alpha]_D^{20}$ = +3.6 c 2.26 H_2O

L-isomer mpt. 190-191°C $[\alpha]_D^{20}$ = -3.5 c 2.13 H_2O

lit. mpt.[153] 193-194°C

Pmr (D_2O) (q) 7.1 (4) ring protons; (s) 3.8 (3) methyl ester; (d) 3.2 (2) β-C$\underline{H}_2$; (t) 4.2 (1) α-C$\underline{H}$.

Ethyl-N-(β,β-dimethoxyethyl)glycinate (32)

To a stirred ice-cold mixture of 1.6 g of Na_2CO_3 in 20 ml of ethanol and 1.05 g (.01 mole) of amino acetaldehyde dimethyl acetal was added 2.15 g (.01 mole) of ethyl iodo acetate dropwise over a period of .5 hours. The mixture was stirred vigorously for four hours at 0°C and at room temperature overnight. After this time, the solids were filtered and the filtrate

was evaporated in vacuo. The residue was suspended in water and extracted three times with 30 ml portions of ether. The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo to afford a red oil. Vacuum distillation afforded a clear colorless liquid, 0.6 g. 30% yield. 58-60°C/0.1 mm.

Pmr ($CDCl_3$) (t) 4.5 (1) $C\underline{H}(OCH_3)_2$; (q) 4.3 (2) $CO_2C\underline{H}_2CH_3$; (s) 3.5 (2) $_2OCC\underline{H}_2N$; (s) 3.4 (3) $CH(OC\underline{H}_3)_2$; (d) 2.8 (2) $NC\underline{H}_2CH$; (d) 2.8 (2) $NC\underline{H}_2CH$; (s) 1.8 (1) $N\underline{H}$; (t) 1.3 (3) $CO_2CH_2C\underline{H}_3$.

N-carbobenzoxy tyrosine methyl ester

To an ice-cold mixture of 300 ml of chloroform and 300 ml of water containing 18.4 g (0.22 mole) of $NaHCO_3$ was added 16.88g (73.1 mmol) of methyl tyrosinate HCl all at once and the mixture was stirred for 20 minutes. 10.6 ml (73.3 mmol) of benzyl chloroformate was added dropwise over a period of 45 minutes. The mixture was stirred for 4 hours at 5°C. The phases were separated and the aqueous phase was extracted 2 times with 50 ml portions of chloroform. The combined organic extracts were washed 3 times with 2N HCl and once with water, dried, and filtered. The filtrate was evaporated to yield 22 g of a pale yellow oil. The oil was crystallized from benzene/petroleum ether (30-60°) to afford 21 g of a white solid (87% yield).

D-isomer mpt. 93-95°C $[\alpha]_D^{20}$ = +32.0 c 0.25 DMF

L-isomer mpt. 92-93°C $[\alpha]_D^{20}$ = -33.0 c 0.350 DMF

lit mpt.[156] 92-93°C

Pmr ($CDCl_3$) (s) 7.3 (5) carbamate phenyl; (q) 6.8 (4) tyrosyl aromatic protons; (s) 5.4 (2) carbamate $C\underline{H}_2$; (t) 5.0 (1) α-$C\underline{H}$; (s) 3.7 (3) $CO_2C\underline{H}_3$; (d) 3.0 (2) β-$C\underline{H}_2$.

N-carbobenzoxy-tyrosine (22)

3.289 g (0.01 mol) of methyl-N-Cbz-tyrosinate in 10 ml of methanol was added to 100 ml of 2N NaOH. The mixture was stirred for 3 hours at room temperature, diluted to 200 ml with water and extracted 3 times with 50 ml portions of chloroform. The aqueous phase was cooled and made acidic by dropwise addition of 1N HCl. The milky solution was extracted with chloroform and the combined organic extracts were dried (Na_2SO_4), filtered, and evaporated in vacuo to give 4 grams of an oil. The residue was crystallized from benzene/petroleum ether (30-60°) to afford 2.9 grams (92% yield) of a white solid.

D-isomer mpt. 92-93°C $[\alpha]_D^{20}$ = very small

L-isomer mpt. 92-93°C $[\alpha]_D^{20}$ = very small

lit. mpt.[155] 92-95°C

Pmr ($CDCl_3$/DMSO-d_6) (s) 8.5 (1) COO$\underline{H}$; (s) 7.3 (5) carbamate phenyl; (q) 6.8 (4) tyrosine aromatic; (d) 5.4 (1) carbamate N$\underline{H}$; (s) 5.0 (2) carbamate $C\underline{H}_2$; (q) 4.4 (1) α-$C\underline{H}$; (d) 3.0 (2) β-$C\underline{H}_2$.

N-Cbz-tyrosyl glycine-(N-β,β-dimethoxyethyl)ethyl ester (33-a)

To an ice-cold solution of 3.5 g (18.3 mmol) of ethyl N-β,β-dimethoxyethyl glycinate in 50 ml of ethyl acetate was added 5.9 g (19 mmol) of N-Cbz-tyrosine and 4.6 g (19 mmol) of EEDQ all at once. The solution was stirred at 5°C for 5 hours and subsequently for 24 hours at room temperature.

The yellow solution was chilled and extracted three times with 50 ml portions of ice-cold 1N HCl, three times with 5% $NaHCO_3$ and washed with water. The organic phase was dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to afford 7 g of a yellow oil. Tlc (ether) revealed a major spot with an Rf = .50 and a trace contaminant corresponding to (33-b) with an Rf = .64. One gram of the product was chromatographed on a column of silica gel CC-7 in the solvent system 9/1 benzene/hexane and afforded 850 mg of a gum corresponding to the dipeptide. (70% yield).

The bulk of the product was used directly without further purification.

3-(p-hydroxybenzyl)-4-benzyloxycarbonyl-5^{Δ}-1,N-ethoxycarbonyl-methyl-2-oxo-piperazine (34)

Two grams of the above oil was dissolved in 20 ml of 70% TFA 30% H_2O v/v and the yellow solution was stirred vigorously for four hours. Evaporation in vacuo afforded a dark brown viscous oil which was dissolved in ethyl acetate and extracted

with 5% $NaHCO_3$ until no trace of acid was present. The organic phase was dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to give a dark syrup (1.6 g). The product was applied to a column of silica gel CC-7 (100 g) and eluted with 4/1 benzene/ethyl acetate. Evaporation of the eluent afforded a pale yellow oil (1.2 g) which was dissolved in ether and let stand in the cold. A white solid deposited and collected by filtration and washed with cold ether. 1.0 g (58% yield).

D-isomer mpt. 137-139°C $[\alpha]_D^{20}$ = -137.0 c 1.35 MeOH

L-isomer mpt. 138-141°C $[\alpha]_D^{20}$ = +134.2 c 2.05 MeOH

MS m/e M^+ 424

Pmr ($CDCl_3$) (s) 7.3 (5) carbamate aromatic; (m) 7.2, 6.2 (6) C_3, aromatic ring, O<u>H</u>, C<u>H</u>=CH; (q) 6.6 (1) CH=C<u>H</u>; (m) 5.4, 4.6 (3) carbamate C<u>H</u>$_2$, α-C<u>H</u>; (q) 4.2 (4) $CO_2C\underline{H}_2CH_3$, $_2OCC\underline{H}_2N$; (d) 2.9 (2) β-C<u>H</u>$_2$ Tyr; (t) 1.3 (3) $CO_2CH_2C\underline{H}_3$.

3-(p-hydroxybenzyl)-1,N-ethoxycarbonylmethyl-2-oxo-piperazine hydrochloride (35)

To a solution of 900 mg (2.1 mmole) of (34) in 30 ml of absolute ethanol was added 150 mg of $PdOH_2$ and 0.5 ml of concentrated HCl. The mixture was hydrogenated for 18 hours at 40 psi of hydrogen. The catalyst was filtered over celite and the solid cake was washed with ethanol. The filtrate was concentrated in vacuo. Upon addition of anhydrous ether, a precipitate formed. The solid was collected by filtration, washed

with anhydrous ether and dried. The white powder was recrystallized from absolute ethanol/ether to afford 540 mg (78% yield) of a white powder.

D-isomer mpt. 213-215°C $[\alpha]_D^{20}$ = +91.6 c .592 H_2O

L-isomer mpt. 216-217°C $[\alpha]_D^{20}$ = -93.0 c .688 H_2O

CHN found: 54.60% C 6.33% H 8.51% N

calc.: 54.76% C 6.45% H 8.52% N

ir (KBr), $\nu(NH_2^+)$ = 3000 cm^{-1} (b), $\nu(\overset{O}{\overset{\|}{C}}-O)$ = 1740 cm^{-1} (s), $\nu(\overset{O}{\overset{\|}{C}}-N)$ = 1640 cm^{-1} (s)

Pmr (D_2O) (q) 7.2 (4) aromatic C_3, ring protons; (t) 4.5 (1) $C_3\underline{H}$; (d) 4.3 (2) $_2OCC\underline{H}_2N$; (q) 4.3 (2) $CO_2C\underline{H}_2CH_3$; (m) 3.8, 3.4 (4) $NC\underline{H}_2C\underline{H}_2N$; (m) 3.2 (2) $C\underline{H}_2$Phe; (t) 1.3 (3) $CO_2CH_2C\underline{H}_3$.

3-(p-hydroxybenzyl)-4,N-carbobenzoxy-1,N-ethoxycarbonylmethyl-2-oxo-piperazine (35-b)

To a stirred mixture of 20 ml of chloroform and 10 ml of water containing 240 mg (2 mmoles) of $NaHCO_3$ was added an aqueous 3 ml solution of 330 mg (1 mmole) of (35). The mixture was stirred vigorously over an ice bath for 15 minutes. After this time, a solution of benzyl chloroformate 0.17 ml (1.2 mmoles) in 5 ml of chloroform was added dropwise. When addition was complete, the ice bath was removed and the mixture was stirred at room temperature for 1 hour. The layers were separated and the aqueous phase was extracted once with chloroform. The combined

organic extracts were washed with 2NHCl, dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to afford 400 mg (94% yield) of a clear gum which could not be crystallized.

L-isomer $[\alpha]_D^{20}$ = +62.3 c 0.353 MeOH

tlc 10/1 chloroform/isopropanol 0.7

Pmr ($CDCl_3$) (s) 7.3 (5) carbamate Ph; (q) 6.7 (4) C_3, aromatic protons; (b) 6.7 (1) Ph-O$\underline{H}$; (m) 5.1, 4.5 (3) carbamate C$\underline{H}_2$; (q) 4.1 (2) $CO_2C\underline{H}_2CH_3$; (s) 4.0 (2) C$\underline{H}_2$N; (m) 3.0, 2.6 (6) $NC\underline{H}_2C\underline{H}_2N$, C_3, C$\underline{H}_2$Ph; (t) 1.2 (3) $CO_2CH_2C\underline{H}_3$.

Saponification of (35-b)

(35-b) was dissolved in 5 ml of methanol and to the solution was added 20 ml of cold 2NNaOH. The mixture was stirred at room temperature for one hour and diluted with 50 ml of water. The solution was extracted with 20 ml portions of ether and the organic extracts were discarded. The aqueous phase was chilled in an ice bath and acidified with solid citric acid to pH ≃ 4. The murky solution was extracted with chloroform and the combined organic extracts were dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to give a pale yellow viscous oil that was crystallized from anhydrous ether and afforded 300 mg of a white solid (77% yield). Rf = 0.45 4/1 chloroform/(0.05% Acetic Acid).

D-isomer mpt. 165-167°C $[\alpha]_D^{20} = -5.6$ c = 13.9 MeOH

L-isomer mpt. 165-168°C $[\alpha]_D^{20} = +5.4$ c = 13.9 MeOH

Pmr ($CDCl_3$) (s) 7.3 (6) carbamate Ph, O-H; (q) 6.8 (4) C_3 aromatic protons; (m) 5.0 (2) carbamate $C\underline{H}_2$; (t) 4.7 (1) $C_3\underline{H}$; (d) 4.0 (2) $_2OCC\underline{H}_2N$; (m) 3.6, 2.8; (4) $NC\underline{H}_2C\underline{H}_2N$; (d) 3.1 (2) C_3, $C\underline{H}_2Ph$.

2. Synthesis of (D) and (L) cis-amido piperazinones

Ethyl#β,β-diethoxy-α-amino propionate

To 400 ml of benzene in a 1 liter 3-neck flask cooled in an ice bath, equipped with mechanical stirrer, nitrogen inlet and dropping funnel was added 40 g of potassium t-butoxide. To the mixture was added a solution of 36 g (0.27 moles) of N-formyl-glycine ethyl ester in 100 ml of ethyl formate over a period of 3-4 hours dropwise (caution foaming) and with vigorous stirring. After complete addition, stirring was continued for two more hours. The yellow mixture was allowed to stand at 4°C for 18 hours. The supernatant was decanted and the thick gelatinous deposit was dissolved in 175 ml of absolute ethanol with vigorous stirring. Once solution was complete, 300 ml of methylene chloride was added and the vessel was cooled in a dry ice bath. The yellow solution was subjected to a stream of dry HCl gas for 6 hours. After approximately 1 hour pH ≃ 6 the solution began to gelatinize and the dry ice bath was maintained. After a total of 6 hours bubbling was ceased and solution was allowed to reach ambient temperature and stirred for 24 additional hours. The murky solution was evaporated in vacuo and the residual syrupy liquid was suspended in 500 ml of ether and chilled in ice. The ethereal mixture was treated with a saturated solution of K_2CO_3 until strongly basic (pH = 9). The layers were separated and the

aqueous phase was further extracted with 100 ml portions of ether. The combined ethereal extracts were washed 3 times with 50 ml portions of 5% K_2CO_3 and once with H_2O, dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to yield 50 g of a black oil.

Vacuum distillation (oil pump) afforded 36 g (70%) of a clear colorless oil 68-70°C, .2 Torr.

Pmr ($CDCl_3$) (d) 4.6 (1) α-C<u>H</u>; (q) 4.2 (2) $CO_2C\underline{H}_2CH_3$; (m) 3.9, 3.3 (5) $(CH_3\underline{C}H_2O)_2$-C<u>H</u>; (s) 1.7 (2) N-$\underline{H}_2$; (m) 1.3 (9) $(C\underline{H}_3CH_2O)_3$, $CO_2CH_2C\underline{H}_3$.

Ethyl#β,β-diethoxy-α-amino-N-(N-Cbz-(L)-Tyrosine)-propionate (24)

To an ice-cold solution of ethyl-β,β-diethoxy-propionate 650 mg (3.2 mmoles) in 30 ml of ethyl acetate was added 1 g (3.2 mmoles) of Cbz-(L)-tyrosine and 810 mg (3.3 mmoles) of EEDQ all at once. The ice-bath was removed after two hours and the solution was stirred at room temperature overnight. The yellow solution was cooled in an ice bath and extracted sequentially with cold 1N HCl three times, 5% $NaHCO_3$ three times, and washed with water. The organic phase was dried (Na_2SO_4), filtered and evaporated in vacuo to afford 1.6 g (100%) of a viscous oil. Rf = 0.5 2/1 benzene/ethyl acetate.

Pmr ($CDCl_3$) (s) 7.4 (5) carbamate aromatic; (q) 6.9 (5) Tyrosine aromatic, N-<u>H</u>; (d) 5.6 (1) carbamate N-<u>H</u>; (s) 5.2 (2) carbamate $C\underline{H}_2$; (m) 5.0, 4.3 (4) C<u>H</u> (C_2H_5O),2α-C<u>H</u>; (q) 4.3

(2) $CO_2C\underline{H}_2CH_3$; (m) 3.9, 3.3 (4) $(CH_3C\underline{H}_2O)_2$; (m) 1.3 (9) $(C\underline{H}_3CH_2O)_2$, $CO_2CH_2C\underline{H}_3$.

Vinylogous amide (25)

The above oil (24) was dissolved in 10 ml of 70% TFA/H_2O in a 25 ml flask equipped with a mechanical stirrer. The mixture was stirred vigorously for 3 hours at room temperature. The clear solution was evaporated in vacuo to yield a brown viscous gum. The residue was dissolved in 100 ml of ether and extracted with 30 ml portions of 2N NaOH until neutral. The organic phase was collected and dried (Na_2SO_4). The solution was filtered and the filtrate evaporated in vacuo to afford a red gum. The residue was applied to a column of silica gel CC-7 (Malinkrodt) 100 g and eluted with 3/1 benzene/EtOAc. The combined eluent was evaporated in vacuo to afford a clear oil that crystallized from benzene/petroleum ether (30 - 60°). The crystals were collected, washed with cold petroleum ether and dried. (1 g, 75%).

D-isomer mpt. 130-134°C $[\alpha]_D^{20}$ = -245.5 c 1.05 MeOH

L-isomer mpt. 133-136°C $[\alpha]_D^{20}$ = +244.7 c 0.81 MeOH

MS m/e M^+ 410, 107 (p-hydroxy benzyl)

Pmr ($CDCl_3$) (s) 8.0 (1) C=C$\underline{H}$; (s) 7.5 (1) N-$\underline{H}$; (s) 7.2 (5) carbamate aromatic; (q) 6.6 (4) C_3, aromatic ring; (m) 5.0 (3) carbamate $C\underline{H}_2$, 3-α-C$\underline{H}$; (q) 4.2 (2) CO_3CH_3; (d) 2.9 (2) $C\underline{H}_2$Ph of C_3 benzyl; (t) 1.3 (3) $CO_2CH_2C\underline{H}_3$.

Reduction of (25)

6 g (14.6 mmoles) of (25) was dissolved in 100 ml of absolute ethanol. To the solution was added 2.4 ml of concentrated HCl and 600 mg of $PdOH_2$.[132] The mixture was hydrogenated at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo to give 4.2 g of a brown foam. The residue was crystallized from isopropanol/ether and afforded 4 g of a beige, hygroscopic solid corresponding to the hydrochloride salt of (25). The solid was dissolved in water and extracted three times with methylene chloride. The aqueous phase was made basic by the addition of solid K_2CO_3 and extracted three times with 30 ml portions of methylene chloride. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated in vacuo to give a beige solid. The residue was recrystallized from methanol. The crystals that deposited were collected by filtration and washed with some cold solvent to afford 3.2 g (68% yield) of white needles.

D-isomer mpt. 204-206°C $[\alpha]_D^{20}$ = +140.6 c .572 .1N HCl

L-isomer mpt. 204-206°C $[\alpha]_D^{20}$ = -140.1 c .608 .1N HCl

MS m/e M^+ 278 205 ($-C_2H_5CO_2$) 107 ($^+CH_2PhOH$)

ir (KBr), $\nu(NH_2^+)$ = 3000 cm^{-1} (b), $\nu(\overset{O}{\overset{\|}{C}}-O)$ = 1740 cm^{-1}(s), $\nu(\overset{O}{\overset{\|}{C}}-N)$ = 1650 cm^{-1}(s)

CHN found 60.22% C 6.80% H 10.00% N

calc 60.42% C 6.88% H 10.06% N

Pmr (D_2O/DCl) (q) 7.1 aromatic protons; (t) 5.4 $C_6\underline{H}$; (t) 4.3 $C_3\underline{H}$; (q) 4.2 $CO_2C\underline{H}_2CH_3$; (d) 3.7 $CHC\underline{H}_2N$; (m) 3.3 $C\underline{H}_2$ Ph-OH; (t) 1.3 $CO_2CH_2C\underline{H}_3$

C_6-deutero (26-a)

The procedure for this compound was identical to the one described for (26-b) except that the reaction medium was EtOD and DCl using Pd/C.

Mpt. 205-207°C Yield 65%.

Pmr (D_2O/DCl) (q) 7.0 (4) aromatic ring protons; (t) 4.3 (1) $C_3\underline{H}$; (q) 4.2 (2) $CO_2C\underline{H}_2CH_3$; (d) 3.7 (2) $CHC\underline{H}_2N$; (m) 3.3 (2) $C\underline{H}_2$PhOH; (t) 1.3 (2) $CO_2CH_2C\underline{H}_3$.

(26-b) by reduction with $NaCNBH_3$

1 g (3.6 mmoles) of (25) was suspended in a 30 ml solution of 10/1 v:v of THF/MeOH. The mixture was acidified with 1N methanolic HCl dropwise to pH ≃ 3 (pH paper). A solution of 263 mg (4.2 mmoles) of $NaCNBH_3$ in 5 ml of methanol was added dropwise with vigorous stirring. The pH was monitored and adjusted accordingly so that the solution remained acidic to pH paper. After complete addition, the mixture was stirred for an additional 18 hours. The green solution was evaporated in vacuo and the residual solid was dissolved in water (50 ml) and made strongly acidic with 1N HCl. The aqueous solution was extracted with methylene chloride and the organic extracts were

discarded. The aqueous phase was cooled in an ice bath, rendered basic with solid K_2CO_3 and extracted several times with CH_2Cl_2. The combined organic extracts were dried (Na_2SO_4), filtered, and the filtrate was evaporated in vacuo. The residual solid was dissolved in 20 ml of hot methanol and the solution was allowed to stand in the cold. A crop of crystals formed which were collected by filtration and dried. 690 mg (70% yield) mpt. 204-206°C. $[\alpha]_D^{20}$ = -140.2 c 0.60 .1N HCl. No trace of the diastereomer of (26) was detected in the mother liquor.

C_3(L)-N-carbobenzoxy-26-b

1 g (3.2 mmoles) of (26-b) was dissolved in 3 ml of dry pyridine at brine temperature, and 0.54 g of benzyl chloroformate was added dropwise with a micro syringe. The cold bath was removed after one hour and the yellow solution was stirred vigorously for an additional hour. The clear solution was poured onto 50 g of ice and the precipitate was extracted with ethyl acetate. The combined organic extracts were washed three times with 2N HCl and twice with water. The organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to yield a pale-yellow oil. The residue was dissolved in 20 ml of ether and allowed to stand in the cold. The crop of crystals that developed was collected and washed with cold ether to afford 1 g (90% yield) of a white solid.

mpt. 144-145°C Rf = 0.4 3/1 benzene/ethyl acetate

$[\alpha]_D^{20}$ = +38.7 c 0 2.84 MeOH

Pmr ($CDCl_3$) (s) 7.3 (5) carbamate aromatic; (q) 6.8 (4) $CH_2\underline{Ph}$-OH ring protons; (s) 6.6 (1) O-$\underline{H}$; (b) 6.3 (1) N-$\underline{H}$ amide; (m) 5.2, 4.3 (5) carbamate $C\underline{H}_2$, $C\underline{H}_2N$, $C_3\underline{H}$, $C_6\underline{H}$; (q) 4.2 (2) $CO_2C\underline{H}_2CH_3$; (d) 3.1 (2) $C\underline{H}_2Ph$-OH; (t) 1.2 (3) $CO_2CH_2C\underline{H}_3$

Saponification of (27)

1 g (2.4 mmoles) of (27) was dissolved in 5 ml of methanol and to the solution was added 180 mg of NaOH in 20 ml of water. The mixture was stirred for two hours until the solid had dissolved. The solution was diluted with 20 ml of water and extracted three times with 20 ml portions of chloroform. The aqueous phase was cooled in an ice bath, acidified with solid citric acid and extracted three times with 20 ml portions of chloroform. The combined organic extracts were washed with water, dried (Na_2SO_4), and evaporated in vacuo to give 900 mg of a white foam. The residue was crystallized from ethylacetate/petroleum ether (30-60°) to afford 850 mg (90% yield) of a solid.

D-isomer mpt. 75-77°C (foaming) $[\alpha]_D^{20}$ = -3.9 c 10.2 MeOH

L-isomer mpt. 72-77°C (foaming) $[\alpha]_D^{20}$ = +4.1 c 8.6 MeOH

3-(p-hydroxybenzyl)-4,N-allyl-6-methoxycarbonyl-2-oxo-piperazine

A solution of 150 mg (0.5 mmoles) of (26) in 10 ml of methanol was treated with 60 mg of $NaHCO_3$ and 0.05 ml of allyl bromide. The mixture was stirred at room temperature for 48 hours then evaporated in vacuo. The residue was suspended in ethyl acetate and washed with water. The organic phase was dried (Na_2SO_4), filtered, and concentrated to a volume of 10 ml in vacuo. The concentrate was allowed to stand in the cold and the crystals that deposited were collected and washed with cold solvent to afford 90 mg (60%) of a white solid that corresponded to the methyl ester.

mpt. 160-162°C

MS M/e M^+ 314, 197 (- hydroxybenzyl)

Pmr ($CDCl_3$) 3.7 $CO_2\underline{CH}_3$

CHN	calc	63.07% C	6.69% H	9.23% N
	found	63.10% C	6.61% H	9.21% N

3-(p-hydroxybenzyl)-6-amido-2-oxo-piperazine acetate

150 mg (0.5 mmoles) of (26) was suspended in absolute ethanol and treated with a stream of anhydrous ammonia for 15 minutes. The flask was sealed and the solution was stirred for 48 hours at room temperature. The solvent was evaporated in vacuo to afford a foam that could not be crystallized. The foam was dissolved in water and lyophilized.

Pmr (D_2O) (q) 7.0 (4) aromatic ring protons; (t) 4.2 (1) $C_6\underline{H}$; (m) 3.7 (1) $C_3\underline{H}$; (m) 3.1 (4) $CHC\underline{H}_2N$.

A small analytical sample was prepared by triturating the foam with petroleum ether.

CHN	found	56.31% C	6.40% H	15.65% N
	calc.	57.79% C	6.08% H	16.85% N

The remainder of the product was applied to a column of G-15 sephadex 1.5 x 90 cm eluting with 10% acetic acid (1.5 ml/tube 15 ml/hour). A large symmetrical peak emerged in fractions 70-90. The fractions were pooled and lyophilized to afford 90 mg of a fluffy white powder corresponding to the acetate.

mpt. 90-91°C

$[\alpha]_D^{20} = -213.8$ c 1.22 (75% AcOH 25% H_2O)

3-(p-hydroxybenzyl)-6-carboxy-2-oxo-piperazine

150 mg (0.5 mmoles) of (26-b) was added to 25 ml of 2N NaOH and the solution was stirred at room temperature for 5 hours. After this time, the reaction vessel was cooled in an ice bath and acidified slowly by the dropwise addition of glacial acetic acid to pH 5. The clear solution was allowed to stand at 5°C for 24 hours wherein a crop of crystals deposited. The solid was collected by filtration, washed with water and dried in vacuo to afford 80 mg (70% yield) of a white solid.

mpt. 245-246°C

ir (KBr) ν(O-H) = 3400 cm^{-1} (b), ν(C(=O)-OH) = 3400-3000 cm^{-1} (w), ν(C(=O)-OH) = 1690 cm^{-1} (s)

Pmr (D_2O/DCl) (q) 7.1 (4) aromatic ring protons;

(t) 4.6 (1) J, 2Hz
(t) 4.5 (1) J, 2Hz
} $C_3\underline{H}$, $C_5\underline{H}$;

(d) 3.7 (2) J, 2Hz $CHC\underline{H}_2N$;

(d) 3.3 (2) J, 2Hz $C\underline{H}_2Ph$-OH

3. Synthesis of tripeptides

N-t-BOC-glycyl phenylalanine (37)

4.4 g (20.5 mmoles) of phenylalanine methyl ester·HCl and 4 ml of triethyl amine were mixed in 100 ml of chloroform and stirred vigorously for 15 minutes. After solution was complete 3.6 g of BOC-gly-OH (20.5 mmoles) and EEDQ 7.19 (29 mmoles) were added all at once. The clear solution was stirred at room temperature for 24 hours and extracted sequentially with H_2O, 1N HCl three times, 5% $NaHCO_3$ three times, and washed with water. The organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to give 5.8 g of a pale yellow oil corresponding to 87% yield of the dipeptide methyl ester.

4 grams of the oil was dissolved in 20 ml of methanol and added to 75 ml of 2N NaOH. The cloudy solution was stirred vigorously for 4 hours and diluted with 100 ml of H_2O. The solution was extracted with chloroform and the organic extracts were discarded. The aqueous phase was chilled in an ice bath and acidified with solid citric acid. The milky solution was extracted with chloroform and the combined organic extracts were dried (Na_2SO_4), filtered and evaporated in vacuo. The oily residue was dissolved in ether and allowed to stand in the cold overnight. The solid that deposited was collected by filtration and washed with cold ether to afford 3 g of a white solid, mpt. 142-144°C.

$[\alpha]_D^{20}$ = +12.4 c 1.4 MeOH

Pmr ($CDCl_3$) (a) 7.3 (5) Phe aromatic; (t) 5.7 (1) N-H Gly; (t) 5.7 (1) N-H Gly; (m) 4.9 (1) α-CHPhe; (d) 3.8 (2) α-CH_2Gly; (s) 1.5 (9) $(C\underline{H}_3)_3$

L-N-methyl phenylalanine methyl ester HBr

1 g (3.4 mmoles) of N-Cbz phenylalanine and 3.2 g of Ag_2O in 20 ml of dry DMF were heated to 80°C for 4 hours. The mixture was cooled to 5°C and treated with 1 ml of methyl iodide dissolved in 1 ml of DMF. The mixture was stirred vigorously for 24 hours at room temperature after which another portion of methyl iodide in DMF was added. The procedure was repeated after another 24 hours. After a total of 72 hours the solids were filtered and the solvent was evaporated in vacuo. The residue was suspended in ethyl acetate and filtered. The filtrate was extracted three times with 2N HCl, three times with 5% $NaHCO_3$ and washed with H_2O. The organic phase was dried (Na_2SO_4) and evaporated in vacuo to give 900 mg of a pale yellow oil. The product was dissolved in 10 ml of glacial acetic acid and treated with anhydrous HBr for 1 hour, then treated with 150 ml of anhydrous ether. The murky solution was allowed to stand in the cold, and the crop of crystals that deposited was collected by filtration and washed with cold ether. 500 mg (56% yield).

mpt. 121-122°C

$[\alpha]_D^{20}$ = +16.8 c 0.48 (75% AcOH, 25% H_2O)

Pmr (D_2O) (m) 7.2 (5) ring protons; (t) 4.3 (1) α-C<u>H</u>; (s) 3.7 (3) $CO_2C\underline{H}_3$; (d) 3.2 (2) β-$C\underline{H}_2$; (s) 2.6 (3) N-$C\underline{H}_3$.

N-t-BOC-Glycyl-N(CH_3)-Phenylalanine

To a solution of 400 mg (.15 mmoles) of N(CH_3)-phenylalanine methyl ester·HBr and 0.2 ml of triethyl amine in 25 ml of chloroform was added 233 mg (1.5 mmoles) of N-t-BOC-Gly and 370 mg (1.5 mmoles) of EEDQ all at once. The solution was stirred at room temperature for 18 hours, and then evaporated in vacuo. The residue was dissolved in ethyl acetate, and extracted with cold 1N HCl three times, 5% $NaHCO_3$ three times, and washed with H_2O. The organic phase was dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to afford a thick oil (350 mg) that could not be crystallized. (Rf = 0.6 3/1 benzene/chloroform).

The above oil was dissolved in 5 ml of methanol and treated with 10 ml of 2N NaOH. The turbid solution was stirred vigorously for two hours, then diluted to 50 ml with water. The solution was extracted with chloroform and the organic extracts were discarded. The aqueous phase was cooled in an ice bath, acidified

with solid citric acid and extracted with chloroform. The combined organic extracts were washed with water, dried (Na_2SO_4) and evaporated in vacuo to afford 290 mg (60% yield) of a viscous oil which was chromatographed on a column of silica gel CC-7 in the solvent system 5/1 chloroform methanol to give 190 mg of a clear oil.

tlc Rf = 0.5 3/1 chloroform/methanol

$[\alpha]_D^{20}$ = -4.9 c 0.82 MeOH

Pmr ($CDCl_3$) (b) 9.2 (1) COOH; (m) 7.1 (5) aromatic ring Phe; (b) 5.6 (1) carbamate N-H; (q) 5.2 (1) α-CH Phe; (t) 3.8 (2) α-CH_2 Gly; (s) 2.8 (3) N-CH_3; (s) 1.4 (9) $(CH_3)_3$ C

Glycyl-N(CH_3) phenylalanyl-norleucine benzyl ester·HCl

To a solution of 257 mg (1 mmole) of o-benzyl Norleucinate·HCl in 30 ml of chloroform containing 0.14 ml of triethyl amine was added 330 mg (1 mmole) of N-t-BOC-Gly-N(CH_3)Phe-OH and 247 mg (1 mmole) of EEDQ all at once. The clear solution was stirred at room temperature for 24 hours, then evaporated in vacuo. Work up of the residue as described previously afforded 400 mg of a pale yellow gum. The product was N-deblocked immediately by dissolution in 3 ml of cold anhydrous TFA. The solution was stirred at 5°C for 1 hour under nitrogen and evaporated in vacuo. The residue was passed down a column of AMBERLITE-IRA 400 Cl^- eluting with water. When a negative Cl^- test was

obtained, the eluent was extracted with ether and the organic phase was discarded. The aqueous phase was lyophilized to afford 200 mg (42%) of a fluffy white powder.

Rf = 0.78 10/1 chloroform/methanol (ninhydrin)

mpt. 78-80°C dec

$[\alpha]_D^{20}$ = +31.4 c 0.63 MeOH

Pmr (D_2O) (s) 7.3 (10) carbamate aromatic, Phe aromatic; (m) 5.4 (1) carbamate N-H; (d) 5.2 (2) CO_2 CH_2 Ph; (m) 4.4 (2) α-CH, α-CH_2 (s) 4.0 (2) α-CH_3 Gly; (m) 3.0 (5) N-CH_3, β-CH_2 Phe; (m) 1.8 (2) β-CH_2 Nle; (m) 1.2 (4) γ-CH_2, δ-CH_2 Nle; (m) 0.8 (3) ω-CH_3 Nle

ε-amino caproic acid benzyl ester·HCl

4 ml of thionyl chloride was added dropwise to 25 ml of benzyl alcohol in a dry ice bath over a period of 30 min at such a rate that the temperature did not rise above 5°C. After addition was complete 0.4 g of ε-amino caproic acid was added all at once and stirring was continued for 2 hours at dry ice temperature and overnight at room temperature. The clear solution was treated with 200 ml of anhydrous ether and allowed to stand in the cold. The white solid that deposited was collected by filtration and washed with ether. 7.0 g (90% yield).

mpt. 104-106°C

Pmr (D_2O) (s) 7.4 (5) aromatic ring protons; (s) 5.2 (2) $CO_2C\underline{H}_2$; (t) 3.0 (2) ε-$C\underline{H}_2$; (t) 2.4 (2) α-CH_2; (m) 1.9, 1.2 (6) $C\underline{H}_2C\underline{H}_2C\underline{H}_2$

Norleucine benzyl ester·HCl

The procedure for the synthesis of this compound was the same as that described for ε-amino caproic acid benzyl ester·HCl with the following modification: (The modification was applied because ether did not precipitate the salt.)

The yellow solution containing the product was diluted with 200 ml of ehter and extracted three times with 50 ml portions of water. The aqueous extracts were combined, washed with ether and lyophilized to give a white fluffy powder corresponding to the benzyl ester HCl.

D-isomer $[\alpha]_D^{20} = +17.5$ c 1.38 MeOH

L-isomer $[\alpha]_D^{20} = -17.2$ c 1.50 MeOH

Glycyl Phenylalanyl Leucine benzyl ester·TFA (38-a)

200 mg (0.62 mmoles) of N-t-BOC-Gly-Phe-OH, 0.86 ml of triethylamine and 160 mg (0.62 mmoles) of (L)-Leucine benzyl ester·HCl were mixed in 20 ml of chloroform. After stirring for 15 minutes, EEDQ (200 mg) was added and stirring was continued overnight. Evaporation of the solution in vacuo and work up of the residue as described for (38-b) afforded 130 mg (50%) of a pale yellow oil.

Rf = 0.6 (3/1 benzene/ethyl acetate)

Pmr ($CDCl_3$) (s) 7.4 (5) OCH_2Ph; (s) 7.3 (5) Phe ring protons; (d) 7.1 (1) N-H; (d) 6.8 (1) N-H; (t) 5.4 (1) N-H carbamate; (s) 5.2 (2) $O-CH_2Ph$; (m) 5.0, 4.4 (2) α-CH; (d) 3.8 (2) $\alpha-CH_2Gly$; (d) 3.1 (2) $\beta-CH_2$ Phe; (s) 1.4 (9) $(CH_3)_3$; (m) 1.4 (3) $\beta-CH_2$ Leu, $\gamma-CH_2$ Leu; (d) 0.9 (6) $(CH_3)_2CH$

The above oil was dissolved in 50% anhydrous TFA in CH_2Cl_2 under nitrogen, and the clear solution was stirred at room temperature for one hour. The resulting brown solution was evaporated in vacuo and the residual solid was triturated with anhydrous ether. The solid that formed was collected and recrystallized from ethanol/ether to give 900 mg of the tripeptide TFA salt.

mpt. 75-77°C $[\alpha]_D^{20}$ = +6.5 c 0.462 MeOH

Glycyl Phenylanalyl(D)-Norleucine benzyl ester·TFA (38-b)

To a 30 ml chloroform solution containing D-Norleucine benzyl ester HCl 398 mg (1.6 mmoles) and 0.22 ml of triethylamine was added 500 mg of N-t-BOC glycyl phenylalanine (1.5 mmoles) and 380 mg of EEDQ. The clear solution was stirred at room temperature for 24 hours and then evaporated in vacuo. The residue was dissolved in 75 ml of ethyl acetate and extracted once with water, three times with 30 ml portions of cold 1N HCl,

two times with 5% $NaHCO_3$ and washed with H_2O. The organic phase was dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo. The residual oil was dissolved in anhydrous ether and allowed to stand in the cold overnight. A white solid deposited and was collected. 550 mg (70%). mpt. 150-160°C.

The solid was dissolved in 6 ml of 50% anhydrous TFA in CH_2Cl_2 and the solution was stirred 30 minutes at 5°C and 45 minutes at room temperature. The clear solution was evaporated to dryness and the residual oil was dissolved in ether and allowed to stand in the cold overnight. The crop of crystals that deposited was collected and washed with ether.

Rf = 0.64 (10/1 chloroform/MeOH, ninhydrin)

mpt. 98-100°C $[\alpha]_D^{20}$ = +12.7 c 0.629 MeOH

Pmr ($CDCl_3$) (s) 7.2 (5) carbamate aromatic; (s) 7.1 (5) Phe aromatic; (s) 5.1 (2) $OC\underline{H}_2Ph$; (m) 4.4, 4.9 (2) 2 α-C$\underline{H}$; (s) 3.8 (2) α-$C\underline{H}_2$ gly; (d) 3.0 (2) β-$C\underline{H}_2$ Phe; (m) 0.7, 1.8 (9) α-side chain Nle.

The (L)-Norleucine diastereomer was prepared in the same way. The TFA salt could not be crystallized readily. Therefore the oil was dissolved in isopropanol and applied to a short column of AMBERLITE IRA-400 Cl^- resin so that trifluoroacetate was exchanged for chloride. The compound was eluted with isopropanol and the eluent was monitored with $AgNO_3$. When a

negative chloride test was obtained, the eluent was evaporated in vacuo to yield a viscous colorless oil. The residue was recrystallized from isopropanol/ether and afforded white crystals corresponding to the hydrochloride salt.

mpt. 169-170°C

$[\alpha]_D^{20} = -19.1$ c 0.367 MeOH

The pmr spectrum was essentially the same as the Nleu(D) diastereomer.

Glycyl Phenylalanyl Proline benzyl ester·oxalate salt (38-c)

428 mg (1.8 mmoles) of proline benzyl ester·HCl and 0.26 ml of triethylamine were mixed in 25 ml of chloroform and stirred for 15 minutes. 600 mg of N-t-BOC-gly-Phe-OH and 500 mg of EEDQ was added all at once and the solution was stirred for 24 hours at room temperature. After evaporation in vacuo, the residue was worked up as described for (38-b). An oil was obtained (400 mg 90%) which could not be crystallized and was used directly in the subsequent deblockage.

Pmr ($CDCl_3$) (s) 7.3 (5) aromatic Phe; (s) 7.2 (5) O-Ph; (b) 7.2 (1) COH-H Phe; (s) 5.2 (2) OCH_2 Ph; (b) 5.2 (1) NH carbamate; (m) 4.4 (1) α-CHPhe; (d) 3.8 (2) α-CH_2 Gly; (m) 3.6 (2) N-CH_2 Pro; (d) 3.0 (2) β-CH_2·Phe; (m) 2.0 (4) β-CH_2, γ-CH_2 Pro; (s) 1.5 (9) $(CH_3)_3$

The oil was dissolved in 6 ml of 50% TFA in CH_2Cl_2 and the

solution was stirred at room temperature for 1.5 hours under N_2. The brown solution was evaporated to dryness and the residue was applied to a column of Amberlite IRA-400 1.5 x 20 cm eluting with isopropanol. The eluent was monitored with $AgNO_3$ solution. The Cl^- positive fractions were pooled and separated to afford a viscous gum. Rf = .6, 4/1 chloroform/methanol (ninhydrin spray) 300 mg (85%).

The oxolate salt was prepared by dissolving the free base in 20 ml of ether and adding a solution of oxalic acid in ethylacetate (10 ml). The solid that formed was collected by filtration and washed with cold ether. Mpt. 64-68°C.

Glycyl Phenylalanyl Leucinol·HCl (38-d)

To a cold solution of 306 mg (2.6 mmoles) of (L)-2-amino-4-methyl pentanol (L-leucinol) 0.36 ml of triethylamine in 50 ml of methanol was added 700 mg of EEDQ and 925 mg (2.6 moles) of Cbz-Glyphe-OH all at once. The solution was stirred at room temperature overnight and worked up as described for (38-b). The residual oil 800 mg could not be crystallized, and as such was used directly in the subsequent deblockage step.

The above oil 800 mg (1.8 mmoles) was dissolved in 25 ml of 95% EtOH containing 6 drops of concentrated HCl and 150 mg of 10% Pd/C. The mixture was hydrogenated for 3 hours at 40 psi of H_2. The catalyst was filtered over celite, the cake washed with ethanol and the filtrate was evaporated in vacuo.

The residual foam was crystallized from ethanol/ether to afford 550 mg (85%) of a white solid.

Mpt. 158-160°C Rf = .35 10/1 chloroform/methanol (ninhydrin)

$[\alpha]_D^{20}$ = +2.6 c 0.805 MeOH

Pmr (D_2O) (d) 7.7 (1) unexchangeable proton N-H; (s) 7.3 (5) aromatic Phe; (s) 3.8 (m) 3.8 } (3) α-C$\underline{H}_2$ Gly, α-C$\underline{H}$ (d) 3.3 (2) C$\underline{H}_2$-OH; (d) 3.1 (2) β-C$\underline{H}_2$ Phe; (m) 1.3 (3) β-C$\underline{H}_2$ Leu, γ-C$\underline{H}$ Leu; (q) 9.8 (6) (C$\underline{H}_3$)$_3$CH.

Glycyl Phenylalanyl Methioninol·HCl (38-e)

To a cold solution of 342 mg of (L)-methioninol (2.0 mmoles) and 0.28 ml of triethyl amine in 50 ml of methanol was added 646 mg (2.0 mmoles) of BOC-GlyPhe-OH and 540 mg (2.2 mmoles) of EEDQ all at once. The clear solution was stirred at room temperature overnight and evaporated in vacuo. The residue was dissolved in 75 ml of ethylacetate and worked up as described for (38-b). The residue from evaporation was dissolved in ether and let stand in the cold overnight whereupon a white solid deposited. The solid was filtered and washed with cold ether. 350 mg (80%) mpt. 139-140°C.

The tripeptide 230 mg was added to 3 ml of anhydrous TFA containing 1 ml of anisole and the solution was stirred at 5°C

for 1 hour. After this time it was evaporated in vacuo to afford a brown oil. The oily residue was treated with 50 ml of water and extracted several times with ether. The aqueous phase was concentrated to a volume of 5 ml in vacuo and applied to a column of Amberlite IRA-400·Cl^- 1.5 x 30 cm and eluted with isopropanol. The eluent was evaporated and afforded an oily residue that showed two spots on silica tlc 3/1 benzene/ isopropanol (Eastman). NMR revealed a substantial amount of S-t-butyl. The residue 180 mg was chromatographed on 40 grams of silica CC-7 eluting with 4:1 benzene:isopropanol. The first fraction, Rf = 0.3 1/1 (benzene/isopropanol) corresponded to the right material and was crystallized from isopropanol/ benzene to yield 100 mg (50%) of a hydroscopic solid.

$[\alpha]_D^{20}$ = +5.6 c 0.364 MeOH

Pmr (D_2O) (d) 7.8 (1) unexchangeable proton N-H; (s) 7.3 (5) aromatic ring Phe; (s) 3.8, (m) 3.8 (3) α-$C\underline{H}_2$ Gly, α-$C\underline{H}$ (d) 3.3 (2) $C\underline{H}_2OH$; (d) 3.0 (2) β-CH_2Phe; (t) 2.4 (2) S-$C\underline{H}_2$; (s) 2.1 (3) S-$C\underline{H}_3$; (m) 1.9,1.7 (2) β-$C\underline{H}_2$ Met.

Glycyl Glycyl Phenylalanyl Norleucine benzyl ester·TFA

N-t-BOC-Gly 415 mg (2.6 mmoles) was coupled to methyl, glycyl phenylalaninate·HBr 800 mg (2.5 mmoles) in 50 ml of chloroform with 650 mg (2.6 mmoles) of EEDQ. The clear solution was stirred at room temperature for 48 hours and evaporated in vacuo. The residue was worked up as described for (38-b) to afford 740 mg of a pale yellow oil (75%).

The above oil was dissolved in 10 ml of methanol and to the solution was added 200 mg of NaOH in 30 ml of water. The mixture was stirred for 4 hours and extracted with 20 ml portions of ether. The aqueous phase was cooled in an ice bath and made acidic with solid citric acid. The milky solution was extracted with chloroform, and the combined organic extracts were washed with water, dried (Na_2SO_4), filtered and evaporated in vacuo to afford 600 mg of an oil which was crystallized from chloroform. 520 mg (70%) of a white solid.

Mpt. 142-145°C $[\alpha]_D^{20}$ = +27.8 c 0.46 MeOH

Pmr ($CDCl_3$) (d) 7.5 (1) NH amide; (d) 7.4 (1) NH amide; (s) 7.3 (5) Phe aromatic; (t) 6.0 (1) NH carbamate; (s) 4.7 (1) α-CH Phe; (d) 3.8 (2) α-CH_2 Gly; (d) 3.7 (2) α-CH_2 Gly; (t) 3.1 (2) β-CH_2 Phe; (s) 1.4 (9) $(CH_3)_3$ C.

250 mg of the above solid (0.66 mmoles) and 175 mg (0.71 mmoles) of EEDQ were added to a solution of (L)-benzyl, Norleucinate·HCl 170 mg (0.66 mmoles) and 0.09 ml of triethylamine in 30 ml of chloroform. The clear solution was stirred

at room temperature overnight after which another 50 mg portion of EEDQ was added and stirred for an additional five hours. The solvent was stripped in vacuo and the residue was worked up as described for (44-b). The residual viscous oil from the reaction was crystallized from anhydrous ether to afford 230 mg (60%) of a solid. Mpt. 115-117°C.

The above material was dissolved in 6 ml of 50% TFA in CH_2Cl_2 and stirred at room temperature for 1.5 hours under nitrogen. The yellow solution was evaporated in vacuo and the residual oil was triturated with anhydrous ether. The solid that formed was collected and recrystallized from isopropanol / ether to afford 160 mg (60% yield) of a beige solid.

Mpt. 118-120°C

Rf = 0.6 1/1 benzene/methanol (ninhydrin)

$[\alpha]_D^{20}$ = -12.4 c 0.647 MeOH

4. Synthesis of D and L cis and trans piperazinone enkephalins[129]

[D-trans amido piperazinone]-Gly-N(CH_3)Phe-Nle-OH J0-37

To a solution of 107 mg (0.23 mmoles) of benzyl, glycyl N(CH_3)--Phe phenylalanylnorleucinate·HCl and 0.35 ml of triethylamine in 15 ml of chloroform was added 100 mg of (36) and 67 mg (0.27 mmoles) of EEDQ. The clear solution was stirred at room temperature for 48 hours and evaporated in vacuo. The residue was dissolved in ethyl acetate and worked up as described for (38-b). The organic phase was dried (Na_2SO_4), filtered and evaporated in vacuo to afford 100 mg of an oil that could not be crystallized and was used directly in the subsequent reaction. (55% yield)

The above oil was dissolved in 20 ml of 50% AcOH/H_2O and hydrogenated using 50 mg of 10% Pd/C at 40 psi of hydrogen for 2 hours. The catalyst was filtered over celite and the cake was washed with water. The filtrate was diluted further to a volume of 50 ml with water and extracted twice with 20 ml portions of ether. The aqueous extract was concentrated to a volume of 20 ml in vacuo and lyophilized. The beige fluffy residue was dissolved in 2 ml of 10% AcOH and applied to a column of G-15 Sephadex 1.5 x 90 cm eluting with the same solvent system. Fractions of 1 ml/tube at a rate of 10 ml/hour were collected. Peptide material was monitored continuously by UV at 280 nm and was detected in a series of separable peaks

from tube 70 to 138. Fractions comprising 90-130 were pooled and lyophilized to afford 55 mg of a fluffy powder. This procedure was repeated to ameliorate the purity and fractions 87-113 were collected and lyophilized again to afford 45 mg of a white powder. The powder was subjected to ion exchange chromatography on a column of SP-C25 1.5 x 30 cm eluting with a linear pH gradient of ammonium acetate pH 4.2 to 8.5 (2 ml/tube, 10 ml/hour). Two peaks were detected, the major one, tubes 37-60 was collected and the eluent was concentrated to a volume of 1 ml in vacuo. The concentrate was desalted by passage through a column of G-15 Sephadex 1.5 x 90 cm. A single sharp symmetrical peak was detected in fractions 88-100. The fractions were pooled and lyophilized to afford 30 mg (22%) of a white fluffy powder.

Mpt. 127-130°C dec. $[\alpha]_D^{20}$ = +77.7 c 0.108 (75% AcOH 25% H_2O)

tlc A 0.64 B 0.78 C 0.38 D 0.51

AAA Phe 1.0 Gly 1.0 Nle 1.1

HPLC single peak. Retention time 25 minutes.

Pmr (D_2O/CD_3COOD) (s) 7.3 (5) Phe aromatic ring; (q) 7.0 (4) C_3, aromatic ring; (m) 5.2 (1) α-C$\underline{H}$; (m) 4.5, 4.0 (6) α-C$\underline{H}$, α-C$\underline{H}_2$ Gly^1, α-CH_2 Gly^2; (m) 3.8, 3.3 (4) NC$\underline{H}_2$C$\underline{H}_2$N; (m) 3.3, 2.8 (7) β-C$\underline{H}_2$ Phe, C_3, C$\underline{H}_2$PhOH, NC$\underline{H}_3$ Phe; (m) 1.7 (2) β-C$\underline{H}_2$ Nle; (m) 1.2 (4) γ-C$\underline{H}_2$, δ-C$\underline{H}_2$ Nle; (m) 0.8 (3) ω-C$\underline{H}_3$ Nle.

[4(N)-allyl-trans amidopiperazinone]-Gly-Phe-Nle-OH J0-40

125 ml (0.39 mmoles) of methyl, glycyl phenylalanyl norleucinate·HCl and 0.05 ml of triethyl amine were mixed in 20 ml of methanol. 167 mg (0.42 mmoles) of (36) and 107 mg (0.42 mmoles) of EEDQ were added all at once and stirred at room temperature for 24 hours. The solution was evaporated in vacuo and worked up as described for (38-b) to afford 100 mg of an oil. The oil was dissolved in 20 ml of methanol and added to a mixture of 50 mg of 10% Pd/C and 4 drops of concentrated HCl in 5 ml of isopropanol. The mixture was hydrogenated for 2 hours at 40 psi of H_2. The catalyst was filtered and the filtrate was evaporated in vacuo to afford 80 mg of a foam. The residue was dissolved in 30 ml of water and lyophilized to give 70 mg of a beige powder. Rf = 0.87 methanol/chloroform 1/3.

The above solid was dissolved in 30 ml of anhydrous methanol containing 50 mg of $NaHCO_3$. After stirring for 15 minutes the mixture was treated with 0.03 ml of allyl bromide. The reaction was monitored by the disappearance of the spot corresponding to Rf = 0.36 Chlo/MeOH 10/1 and the appearance of a new one with Rf = 0.74 in the same solvent system. After 48 hours the solution was evaporated in vacuo. The residue was redissolved in ethylacetate and extracted with 5% $NaHCO_3$ and washed with H_2O. The organic phase was dried (Na_2SO_4)

filtered, and evaporated in vacuo to afford a viscous oil.

The oil was dissolved in 2 ml of methanol and treated with 100 mg of NaOH in 20 ml of water. After stirring for 5 hours the solution was diluted to 40 ml with water and extracted with methylene chloride. The aqueous phase was cooled in an ice bath and acidified to pH 4 with 1N HCl and reextracted with methylene chloride. The aqueous phase was lyophilized to give a beige powder. The residue was chromatographed on a column of G-15 Sephadex 1.5 x 90 cm. (1.5 ml/tube, 10 ml/hour). Peptide material was detected in a series of separable peaks from tube 70 to 126. The fractions corresponding to the largest peak (96-126) were pooled and lyophilized. The residue was subjected to partition chromatography on G-15 Sephadex in the solvent system 4/1/5 butanol/acetic acid/water (top phase)(0.8 ml/tube 5 ml/hour). A symmetrical peak emerged in fractions 58-66 followed by a smaller one in tubes 68-75. Fractions corresponding to 58-66 were pooled and evaporated in vacuo. The residue was dissolved in 30 ml of 10% acetic acid and lyophilized to afford 20 mg of a white fluff (10%).

Mpt. 98-102°C dec.

$[\alpha]_D^{20}$ = +21.0 c 0.143 (75% AcOH, 25% H_2O)

tlc A 0.62 B 0.75 C 0.43 D 0.55

AAA Phe 1.0 Gly 1.1 Nle 1.1

HPLC single peak. Retention time 25 minutes.

[4,N(CH_3)-D-trans amido piperazinone]-Gly-Phe-Nle-OH J0-39

To a solution of 191 mg (0.5 mmoles) of Glycylphenylalanyl-norleucine methyl ester·HCl and 0.7 ml of triethylamine in 20 ml of chloroform was added (D)-Cbz-trans piperazinone (36), 200 mg (0.5 mmoles) and EEDQ 12.8 mg (0.52 mmoles) all at once. The solution was stirred at room temperature for 24 hours, then evaporated in vacuo. The residue was worked up as described for (38-b) to give 190 mg of a pale yellow oil which crystallized from ethylacetate/ether. The solid was collected by filtration and washed with ether. Rf = 0.34 chloroform/methanol 12/1, mpt. 98-100°C.

90 mg of the solid was added to a mixture of 100 mg of 10% Pd/C in 30 ml of anhydrous methanol which had been stirring for 24 hours prior (exposed to air). The mixture was hydrogenated for 18 hours, at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo to afford 70 mg of a brown foam. (pmr showed a signal at δ = 2.6 corresponding to N-CH_3). The above compound was dissolved in 5 ml of methanol and to the solution was added 50 mg of NaOH in 5 ml of water. The mixture was stirred vigorously for 2 hours then diluted with 20 ml of H_2O and extracted with 20 ml portions of CH_2Cl_2. The aqueous phase was cooled in an ice bath, made acidic with glacial acetic acid and extracted further with methylene chloride. The organic extracts were discarded and the aqueous phase was concentrated to a volume of 2 ml

in vacuo. The concentrate was applied to a column of G-15 Sephadex 1.5 x 90 cm and eluted with 10% AcOH (1 ml/tube, 10 ml/hr). Peptide material was detected in tubes 80-110. Fractions 82-100 were pooled and lyophilized. The white powder was subjected to partition chromatography in the solvent system (4/1/5 butanol/acetic acid/water) on Sephadex G-15 1.5 x 90 cm previously equilibrated with the aqueous phase of the solvent system (0.6 ml/tube 5ml/hour). A single symmetrical peak emerged in fractions 90-120. Fractions 100-118 were pooled and evaporated in vacuo. The residue was dissolved in 5% AcOH and lyophilized to give 30 mg of a white fluffy powder (50% from the protected peptide).

Mpt. 103-105°C dec.

$[\alpha]_D^{20}$ = +9.0 c 0.11 (75% AcOH, 25% H_2O)
tlc A 0.46 B 0.75 C 0.21 D 0.43
AAA Phe 1.0 Gly 0.95 Nle 0.96
HPLC single peak. Retention time 25 minutes.

Pmr (D_2O/CD_3COOD) (s) 7.3 (5) Phe, aromatic; (q) 7.1 (4) ring aromatic; (t) 4.7 (1) α-C$\underline{H}$; (t) 4.4 (1) α-C$\underline{H}$; (s) 4.3 (2) C$\underline{H}_2$N ring; (s) 4.0 (2) α-C$\underline{H}_2$ Gly; (b) 3.8 (4) NC$\underline{H}_2$C$\underline{H}_2$N; (d) 3.5 (2) C_3, C$\underline{H}_2$ PhOH; (s) 3.2 (3) 4N-C$\underline{H}_3$; (m) 3.2 (2) Phe β C$\underline{H}_2$; (m) 1.8 (2) Nle β C$\underline{H}_2$; (m) 1.3 (4) Nle $CH_3C\underline{H}_2C\underline{H}_2$; (t) 0.9 (3) Nle ω-C$\underline{H}_3$

[D-trans amido-piperazinone]-Gly-Phe-Nle-OH J0-23

To a cold solution of 200 mg (0.43 mmoles) of Glycyl-phenylalanyl norleucine benzyl ester·HCl in 25 ml of chloroform and 0.06 ml of triethyl amine was added 115 mg (0.43 mmoles) of EEDQ and 187 mg (0.44 mmoles) of (D)-Cbz-trans piperazinone (36) all at once. The clear solution was stirred at room temperature for 24 hours then evaporated in vacuo. The residue was worked up as described for (38-b). The oil that remained was crystallized from ethyl acetate/ether to afford a solid which was collected and washed with ether. Mpt. 85-90°C. Rf = 0.70 chloroform/isopropanol 12/1 (200 mg).

The solid was dissolved in 30 ml of 40% AcOH and hydrogenated with 70 mg of 10% Pd/C, at 40 psi of H_2 for 3 hours. The catalyst was filtered and the filtrate was concentrated to a volume of 5 ml, rediluted with 20 ml of water and lyophilized to afford a beige foam. The residue was dissolved in 2 ml of TRIS·HCl buffer pH 10.4 and adjusted to pH 7.7 with .05N HCl. The solution was applied to a column of DEAE-A25 Sephadex 1.5 x 30 mg and eluted with a linear salt gradient 0.05 M to 0.5 M NaCl pH 7.7. A single sharp peak emerged in tubes 104-136 (1 ml/tube, 13 ml/hour). The fractions were pooled and evaporated to dryness. The residual solid was dissolved in 2 ml of 10% AcOH and desalted on a column of G-15 Sephadex 1.5 x 90 cm (1.3 ml/tube, 16 ml/hour). Two peaks emerged: The smaller peak 60-80 was discarded while the fractions

corresponding to 81-109 were pooled at the expense of yield. The eluent was lyophilized to afford 43 mg of a pure fluffy powder. (25%)

Mpt. 148-150°C

$[\alpha]_D^{20}$ = +109.6 c 0.073 (75% AcOH, 25% H_2O)

tlc A 0.65 B 0.80 C 0.40 D 0.45

AAA Phe 1.0 Gly 0.90 Nle 0.90

HPLC Single peak. Retention time 15 minutes.

[L-cis amido-piperazinone]-Gly-Gly-Phe-Nle-OH J0-17

To a solution of 140 mg (0.24 mmoles) of Glycylglycyl phenylalanyl norleucine benzyl ester·TFA and 0.033 ml of triethyl amine in 20 ml of DMF was added 92 mg (0.24 mmoles) of (L)-cis-Cbz-piperazine (28) and 64 mg (0.26 mmoles) of EEDQ. The clear solution was stirred at room temperature for 24 hours, then evaporated to dryness at reduced pressure. The residue was dissolved in ethylacetate and worked up as described for (38-b). The residue was crystallized from ethyl acetate/ether to afford 150 mg of a solid mpt. 100-105°C Rf = 0.12 chloroform/isopropanol 12/1.

150 mg of the above compound (0.18 mmoles) was dissolved in 20 ml of 50% acetic acid containing 70 mg of 5% Pd/C and hydrogenated for 3 hours at 40 psi of hydrogen. The catalyst was filtered and the filtrate extracted with 10 ml portions

of ethyl acetate. The aqueous phase was evaporated in vacuo and the residue was subjected to partition chromatogrpahy on a column of Sephadex G-15 using the solvent system butanol/H_2O 1/1 (water contained 1.5% pyridine 3.5% AcOH). Fractions of 3 ml/tube were collected and peptide material was detected using the Folin Lowry method. Fractions corresponding to correct pepide material were pooled and evaporated in vacuo. The residue was dissolved in 5% AcOH and lyophilized to afford 100 mg of a beige fluff (two spots on TLC).

The solid was dissolved in 2 ml of TRIS·HCl solution pH 10.4 and the pH was adjusted to 7.7 with 0.5N HCl. The beige solution was applied to a column of DEAE-A25 Sephadex 1.5 x 30 cm eluting with a linear salt gradient 0.05M NaCl to 0.5M NaCl (1 ml/tube, 8 ml/hour). A single peak emerged from 86-120 with a broad shoulder overlapping up to fraction 150. Fractions 86-120 were pooled and evaporated in vacuo. The residue was reapplied to another column of DEAE-Sephadex using a convex salt gradient. Two peaks emerged: 20-33 and a major peak comprising fractions 49-74. The appropriate fractions were pooled, concentrated in vacuo and desalted on G-15 Sephadex (1.5 x 90 cm 1.5 ml/tube 10 ml/hour). One single peak emerged in fractions 81-100. The fractions were pooled and lyophilized to afford 80 mg (70%) of a fluffy white powder.

Mpt. 180-183°C dec

$[\alpha]_D^{20}$ = -27.7 c 0.325 (75% AcOH 25% H_2O)

tlc A 0.70 B 0.80 C 0.50 D 0.61

AAA Phe 1.0 Gly 1.9 Nle 1.0

HPLC Single peak. Retention time 12.5 minutes

Pmr (D_2O)/CD_3COOD) (s) 7.3 (5) Phe aromatic ring; (q) 7.2 (4) C_3, aromatic ring; (m) 4.6 (1) α-C<u>H</u>; (m) 4.3 (2) 2, α-CH; (s) 4.0 (2) Gly C<u>H</u>$_2$; (s) 3.9 (2) Gly C<u>H</u>$_2$; (m) 3.8, 3.5 (3) CH C<u>H</u>$_2$-N, C<u>H</u> CH_2-N; (m) 3.4, 3.0 (4) Phe β-C<u>H</u>$_2$, C_3, C<u>H</u>$_2$ Ph; (m) 1.8, 1.5 (2) Nle β-CH_2; (m) 1.5, 1.1 (4) Nle C<u>H</u>$_2$ C<u>H</u>$_2$; (t) 0.9 (3) Nle ω-C<u>H</u>$_3$

<u>[L-cis amido-piperazinone]-Gly-Phe-Leu-OH JO-4</u>

194 mg (0.36 mmoles) of glycyl phenylalanyl leucine benzyl ester·TFA and 0.05 ml of triethyl amine were mixed in chloroform 20 ml, and stirred for 20 minutes. 150 mg (0.36 mmoles) of (L)-cis piperazinone (28) and 98 mg of EEDQ (0.4 mmoles) were added all at once and the clear solution was stirred overnight. After this time it was evaporated in vacuo and worked up as described for (38-b). The residue was triturated with petroleum ether several times but the viscous gum could not be crystallized. Rf = 0.46 $CHCl_3$/isopropanol 12/1, yield 160 mg. The gum was dissolved in 20 ml of 95% ethanol and hydrogenated with 50 mg of 10% Pd/C at 40 psi H_2 for 3 hours. The catalyst

was filtered over celite and the filtrate was evaporated in vacuo to afford a glassy residue which was dissolved in 20 ml of 5% AcOH and lyophilized. The beige fluff was dissolved in 2 ml of TRIS·HCl pH 10.4 and the pH was adjusted to 7.7 with 0.05N HCl. The solution was applied to a column of Sephadex DEAE-A25 1.5 x 30 cm eluting with a linear salt gradient 0.05 M to 0.5 M NaCl pH 7.7. Two peaks emerged: The smaller from 23 to 50 was discarded and the larger at 107-137 was collected and evaporated in vacuo. The residue was dissolved in 2 ml of 10% AcOH and desalted on a column of G-15 Sephadex 1.5 x 90 cm eluting at a rate of 12 ml/hour, 1.5 ml/tube. A large peak emerged from 67-95 flanked by a small peak from 57 to 67. Cut-off was made at 70 to 90 at the expense of yield. The fractions were pooled and freeze dried to give 50 mg of a white fluffy powder (24%) (from the coupling step).

mpt. 148-151°C dec.

$[\alpha]_D^{20}$ = -62.1 c 0.145 (75% AcOH 25% H_2O)

tlc A 0.74 B 0.85 C 0.86 D 0.50

AAA Phe 1.0 Gly 0.92 Leu 1.0

HPLC Single peak. Retention time 13.8 minutes.

Pmr (D_2O/CD_3COOD) (s) 7.3 (5) Phe aromatic ring; (q) 7.0 (4) C_3 $C\underline{H}_2$ Ph; (t) 4.7 (1) α-$C\underline{H}$; (m) 4.3 (3) α-$C\underline{H}$, C_3H, C_6H; (s) 3.9 (2) α-$C\underline{H}_2$ Gly; (m) 2.6 (2) C-$C\underline{H}_2$ N ring; (m) 3.3, 2.9 (4) β-$C\underline{H}_2$ Phe, $C\underline{H}_2$ Ph ring; (m) 1.5 (3) β-$C\underline{H}_2$ Leu, γ-$C\underline{H}$ Leu; (m) 0.8 (6) $(C\underline{H}_3)_2$ CH.

[D-trans amido-piperazinone]-Gly-Phe-Leucinol JO-33

To a solution of 86 mg (0.23 mmoles) of Glycyl phenylalanyl leucinol·HCl and 0.035 ml of triethylamine in 20 ml of chloroform was added 100 mg of (D)-Cbz-trans piperazine (36) and 67 mg (0.26 mmoles) of EEDQ and the solution was stirred at room temperature for 24 hours, then evaporated in vacuo. Work-up as described for (38-b) afforded a viscous oil that was crystallized from ethylacetate/ether to afford 130 mg of a white powder, mpt. 62-66°C, Rf = 0.57 10/1 chlo/MeOH.

The solid was dissolved in 20 ml of 95% ethanol, cooled in an ice bath, and treated with 5 drops of concentrated HCl and 50 mg of 10% Pd/C. The mixture was hydrogenated for 2 hours, at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo to give a foamy residue. The product was dissolved in 30 ml of H_2O, extracted with ethylacetate and the aqueous phase was concentrated in vacuo to a volume of 20 ml and lyophilized. The fluffy powder was dissolved in 1 ml of 10% AcOH and chromatographed on a column of G-15 Sephadex. Fractions of 1 ml/tube at a rate of 10 ml/hour were collected. Peptide material was detected in a series of progressively larger peaks (68-104). Fractions 91-102 were collected, pooled and lyophilized to afford 50 mg of a white fluff, (38%).

Mpt. 78-80°C

$[\alpha]_D^{20}$ = very small

tlc A 0.63 B 0.83 C 0.58 D 0.71

AAA Phe 1.0 Gly 1.1

HPLC Single peak. Retention time 12.5 minutes.

Pmr (s) 7.3 (5) Phe aromatic ring; (q) 7.1 (4) C_3, $C\underline{H}_2$ Ph; (t) 4.6 (1) α-C<u>H</u> Phe; (m) 4.3 (1) α-C<u>H</u> Leu; (s) 4.2 (2) α-$C\underline{H}_2$ Gly; (s) 3.9 (3) $C\underline{H}_2$-N ring, C_3, C<u>H</u>; (m) 3.8, 3.5 (4) $NC\underline{H}_2C\underline{H}_2$ N; (d) 3.4 (2); (m) 3.1 (4) β-CH_2 Phe, $C\underline{H}_2$ Ph; (m) 1.6, 1.1 (3) β-$C\underline{H}_2C\underline{H}$; (q) 0.9 (6) $(C\underline{H}_3)_2CH$

<u>[D-transamido piperazinone -Gly-Phe-(D)-Nle-OH JO-35</u>

100 mg (0.25 mmoles) of D-Cbz-trans piperazinone (36) and 70 mg (0.25 mmoles) of EEDQ were added all at once to a stirring solution of 135 mg (0.25 mmoles) benzyl, Glycyl phenylalanyl(D)-norleucinate·TFA and 0.035 ml of triethyl amine in 25 ml of chloroform. The clear solution was stirred at room temperature for 24 hours then evaporated in vacuo. Work up as described for (38-b) afforded 130 mg of an oil which crystallized from ethyl acetate/ether to give a hygroscopic solid (Rf = 0.78 8/1 chloroform/isopropanol) which was used directly in the following step.

The semisolid obtained above was dissolved in 20 ml of 50% aqueous AcOH containing 70 mg of 10% Pd/C and hydrogenated for 3 hours, at 40 psi of H_2. The catalyst was filtered over

celite and the cake was washed with acetic acid. The filtrate was concentrated in vacuo, rediluted with water to a volume of 30 ml and lyophilized. The fluffy residue was dissolved in 1 ml of 10% AcOH and applied to a column of G-15 Sephadex 1.5 x 90 cm. The peptide was eluted with the same solvent (1 ml/tube, 10 ml/hour). Peptide material emerged in a series of progressively more intense and separable peaks in fractions 68-130. Fractions 90-130 were collected and lyophilized to 90 mg of a fluffy material.

The product was dissolved in 2 ml of TRIS·HCl buffer pH 7.7 and applied to a column of DEAE-A25 Sephadex. A linear gradient of TRIS·HCl buffer pH 7.7, 0.05 M to 0.5 M NaCL was used to elute the peptide (0.8 ml/tube, 10 ml/hour). A single broad peak emerged in fractions 93-138. Fractions 95 to 135 were pooled and concentrated to a volume of 1 ml in vacuo. The concentrate was desalted by passage through a column of G-15 Sephadex 1.45 x 90 cm eluting with 10% aqueous AcOH (10 ml/hour). A single sharp peak emerged in fractions 100-120. The volumes were pooled and lyophilized to afford 40 mg of a white fluffy powder. (30%) from the coupling reaction.

Mpt. 154-156°C dec

$[\alpha]_D^{20}$ = +62.5 c 0.19 (75% AcOH 25% H_2O)

tlc A 0.59 B 0.82 C 0.40 D 0.45

AAA Phe 1.0 Gly 1.1 Nle 1.0

HPLC Single peak (broad). Retention time 42.5 minutes.

Pmr (D_2O/CD_3COOD) (b) 7.3 (5) Phe aromatic; (q) 7.1 (4) C_3, aromatic ring; (t) 4.7 (1) α-C<u>H</u>; (m) 4.3 (2) α-C<u>H</u>, C_3H; (s) 4.3 (2) C<u>H</u>$_2$ N; (s) 4.0 (2) α-C<u>H</u>$_2$ Gly; (m) 3.9, 3.5 (4) NC<u>H</u>$_2$C<u>H</u>$_2$ N ring; (m) 3.5, 2.9 (4) Phe, β-C<u>H</u>$_2$, C_3, C<u>H</u>$_2$Ph; (m) 2.9, 1.5 (2) Nle, β-C<u>H</u>$_2$; (m) 1.5, 1.0 (4) Nle, γ-C<u>H</u>$_2$, δ-C<u>H</u>$_2$; (t) 0.9 (3) ω-C<u>H</u>$_3$

[L-cis amido-piperazinone]-Gly-Phe-Pro-OH JO-10

200 mg (0.395 mmoles) Of benzyl-Glycyl phenylalanyl-prolinate·TFA and 0.055 ml of triethylamine were mixed in 20 ml of chloroform and stirred for 15 minutes. 166 mg (0.395 mmoles) of (L)-Cbz-cis piperazinone (28), 115 mg (0.45 mmoles) of EEDQ were added all at once and the solution was subsequently stirred for 48 hours. The yellow solution was diluted further with 30 ml of chloroform and worked up as described for (38-b). The chloroform was dried (Na_2SO_4), filtered and concentrated to a volume of 2 ml. Addition of anhydrous ether deposited a solid which was collected and washed with ether. 300 mg, mpt. 109-113°C, Rf = 0.70 methanol.

100 mg of the above compound was dissolved in 20 ml of 40% aqueous AcOH containing 50 mg of 10% Pd/C and hydrogenated for 2 hours, at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was concentrated in vacuo, rediluted to 30 ml with H_2O and lyophilized (30 mg beige fluff).

The solid was dissolved in 2 ml of TRIS·HCl buffer pH 10.4 and adjusted to pH 7.7 with 0.5N HCl. The solution was applied to a column of DEAE-A25 Sephadex 1.5 x 30 cm and eluted with a concave salt gradient, from 0.5 M NaCl to 0.5 M NaCl, pH 7.7 (10 ml/hour). A single sharp peak emerged in tubes 50 to 73. The fractions were pooled and evaporated in vacuo. The residue was dissolved in 2 ml of 10% AcOH and applied to a column of G-15 Sephadex 1.5 x 90 cm (2 ml/tube, 10 ml/hour). A major peak appeared flanked by two smaller peaks. Fractions corresponding to 56 to 70 were pooled and lyophilized. Tlc in the solvent systems (A,B,C,D) revealed a minor spot trailing a major one. The compound was subjected to partition chromatography on G-15 Sephadex. Fractions of 0.8 ml/tube at a flow rate of 6 ml/hour were collected. A single peptide peak emerged in tubes 122-136. The fractions were pooled and evaporated to dryness in vacuo. The residue was dissolved in 10% aqueous AcOH and lyophilized to afford 20 mg (28%) of a white fluffy powder.

Mpt. 144-147°C dec

$[\alpha]_D^{20}$ = -74.1 c 0.108 (75% AcOH 25% H_2O)

tlc A 0.55 B 0.75 C 0.35 D 0.38

AAA Phe 1.0 Gly 0.97 Pro 0.73

HPLE Single peak. Retention time 7.5 minutes.

[L-trans amido piperazinone]-Gly-Phe-Nle-OH JO-16

210 mg (0.39 mmoles) of benzyl-Glycyl phenylalanyl norleucinate·TFA and 0.055 ml of triethylamine were mixed in 20 ml of chloroform and stirred for 15 minutes. 160 mg (0.41 mmoles) of (L)-Cbz-trans piperazinone (36) and 100 mg of EEDQ were added all at once. The clear solution was stirred at room temperature for 24 hours then evaporated in vacuo. The residue was worked up as described for (38-b) to afford a solid from ethyl acetate/ether 230 mg mpt. 85-90°C Rf = 0.80 3/1 chloroform/isopropanol.

The above compound was dissolved in 30 ml of 50% aqueous AcOH containing 70 mg of 10% Pd/C and hydrogenated for 3 hours at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was extracted three times with 20 ml portions of ether. The aqueous phase was concentrated in vacuo to a volume of 1 ml and applied to a column of G-10 Sephadex 2.5 x 40 cm. The fractions were monitored by the Folin Lowry method. Peptide material was detected in tubes 27-50. The fractions were pooled and lyophilized to afford 110 mg of a beige fluff. The solid was dissolved in 2 ml of TRIS-HCl buffer pH 10.4 and adjusted to 7.7 with 0.5N HCl. The beige solution was applied to a column of DEAE-A25 Sephadex 1.5 x 30 cm and eluted with a linear salt gradient ranging from 0.05M NaCl (1 ml/tube, 8 ml/hour). A single broad peak emerged in fractions 100-127. The fractions were pooled and evaporated in vacuo. The residue

was dissolved in 2 ml of 10% AcOH and applied to a column of G-15 Sephadex 1.5 x 90 cm and eluted with the same solvent system (1.5 ml/tube, 10 ml/hour). A single peak emerged in fractions 73-90. Tubes 75 to 90 were pooled and lyophilized to afford 60 mg (27%) of a fluffy white powder.

Mpt. 140-142°C

$[\alpha]_D^{20}$ = -41.0 c 0.122 (75% AcOH 25% H_2O)

tlc A 0.66 B 0.85 C 0.43 D 0.47

AAA Phe 1.0 Gly 1.0 Nle 0.96

HPLC Single peak. Retention time 15 minutes.

[D-trans amido piperazinone]-Gly-Phe-Methioninol JO-36

To a mixture of glycylphenylalanyl methioninol·HCl 112 mg (0.3 mmoles) and 0.04 ml of triethylamine in 25 ml of chloroform was added 120 mg (0.3 mmoles) of (36) and 80 mg of EEDQ all at once. The clear solution was stirred at room temperature for 24 hours and then evaporated in vacuo. The residue was worked up as described for (38-b) to afford a solid mpt. 100-102°C in 70% yield (150 mg).

The solid obtained above (120 mg) was treated with approximately 15 ml of liquid HF^{157} in the presence of 0.5 ml of anisole at 0°C for one hour. After complete evaporation of HF the product mixture was dissolved in 50 ml of 10% AcOH and extracted three times with 20 ml protions of ether.

The aqueous phase was lyophilized to afford a beige foamy residue.

The above residue was chromatographed on a column of Sephadex G-15 1.5 x 90 cm (1 ml/tube 6 ml/hour). Fractions corresponding to 74-88 were pooled and lyophilized to afford 70 mg of an off-white powder. The procedure was repeated to afford 50 mg of a white fluff after lyophilization. HPLC of this material (65% H_2O 35% MeOH 1.1% H_3PO_4) revealed two peaks with retention times 6.4 minutes and 15 minutes. The solid obtained after the second chromatography was subjected to partition chromatography on a column of G-15 Sephadex in the organic phase of the solvent system 4:5:1 butanol/ acetic acid/water. Two peaks emerged: The major one in fractions 94-108 was collected and the eluent was evaporated in vacuo. The residue was dissolved in 30 ml of aqueous 10% AcOH and lyophilized to afford 30 mg of a white fluffy solid (16%).

Mpt. 170-175°C dec.

$[\alpha]_D^{20}$ = +62.0 c 0.26 (75% AcOH 25% H_2O)

tlc A 0.60 B 0.86 C 0.51 D 0.77

AAA Phe 1.0 Gly 1.1

HPLC major peak. Retention time 15 minutes.

minor peak. Retention time 6.4 minutes (probably the sulfoxide)

[D-cis amido piperazinone]-Gly-Phe-Nle-OH JO-22

To a cold solution of 200 mg (0.43 mmoles) of benzyl, Glycyl phenylalanyl norleucinate·HCl, and 0.06 ml of triethyl amine in 30 ml of chloroform was added 115 mg (0.45 mmoles) of EEDQ and 160 mg of D-Cbz-cis piperazinone (28) all at once. The clear solution was stirred at room temperature for 18 hours then evaporated in vacuo and worked up as described for (38-b). The oily product was crystallized from ethyl acetate/ether to afford 200 mg of a white solid mpt. 98-105°C Rf = 0.4 chloroform/isopropanol 12/1 (60% yield).

100 mg of the above solid was dissolved in 25 ml of 50% (v/v) acetic acid and hydrogenated for 2 hours using 10% Pd/C at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo to yield 90 mg of a brown foam (Rf = 0.8 5/1 chloroform/methanol). The product was subjected to gel filtration chromatography on G-15 Sephadex 1.5 x 90 cm eluting with 10% AcOH (1.5 ml/tube 20 ml/hour). Peptide material was detected in fractions 80-130. The fractions were pooled and lyophilized. The resulting powder was dissolved in 2 ml of TRIS-HCl buffer pH 7.7 and chromatographed on a column of DEAE-C25 Sephadex using a linear salt gradient 0.05M to 0,5M NaCl. A sharp symmetrical peak emerged in fractions 106-130 (1 ml/tube 10 ml/hour). The fractions were combined, concentrated and desalted on a G-15 Sephadex 1.5 x 90 cm (1.5 ml/tube, 15 ml/hour). Peptide material was

detected in fractions 86-120 flanked by a small peak. The fractions corresponding to 86-100 were pooled and lyophilized to afford 40 mg (55% yield) of a white fluff.

Mpt. 145-147°C dec

$[\alpha]_D^{20}$ = +77.6 c 0.23 (75% AcOH 25% H_2O)

tlc A 0.75 B 0.87 C 0.60 D 0.50

AAA Phe 1.0 Gly 1.1 Nle 1.0

HPLC Single peak. Retention time 16 minutes.

Pmr (D_2O/CD_3COOD) (s) 7.3 (5) aromatic ring Phe; (q) 7.0 (4) C_3, aromatic ring; (t) 4.8 (1) α-C<u>H</u>; (m) 4.3 (3) α-C<u>H</u>, C_3<u>H</u>, C_6<u>H</u>; (b) 3.9 (2) α-C<u>H_2</u> Gly; (m) 3.7 (2) C<u>H_2</u>N; (m) 3.2 (4) β-C<u>H_2</u> Phe, C_3 C<u>H_2</u> PhOH; (m) 1.8 (2) β-C<u>H_2</u> Nle; (m) 1.2 (4) γ-C<u>H_2</u> Nle, δ-C<u>H_2</u> Nle; (t) 0.8 (3) ω-C<u>H_2</u> Nle

<u>[L-cis amido piperazinone]-Gly-Phe-Leu-OH JO-6</u>

100 mg (0.22 mmoles) of benzyl-glycyl phenylalanyl norleucinate·HCl and 0.03 ml of triethyl amine were mixed in 25 ml of methanol and stirred for 15 minutes. To the clear solution was added 100 mg of (28) (0.26 mmoles) and 66 mg (0.26 mmoles) of EEDQ. The solution was stirred at room temperature for 24 hours, then evaporated in vacuo. The residual viscous mass was worked up as described for (38-b).

The oily residue was dissolved in 10 ml of ethyl acetate

and crystallized by slow addition of anhydrous ether. The solid was collected by filtration and washed with cold ether. 80 mg (50%) mpt. 96-110°C Rf = 0.4 12/1 $CHCl_3$/isopropanol.

The solid was dissolved in 10 ml of glacial acetic acid and added to 10 ml of water containing 50 mg of 10% Pd/C. The mixture was hydrogenated for two hours at 40 psi of hydrogen. The catalyst was filtered over celite, the filtrate was extracted with ether and the aqueous phase was lyophilized to afford 40 mg of a beige foam. The solid was chromatographed on a DEAE-A25 ion exchange resin 1.5 x 30 cm eluting with linear salt gradient 0.05M to 0.5M NaCl pH 7.7 TRIS-HCl (1 ml/tube 7 ml/hour). A single sharp symmetrical peak emerged comprising fractions 99-119 was eluted. The eluent was pooled and evaporated in vacuo. The residue was redissolved in 2 ml of 10% AcOH and desalted on a column of G-15 Sephadex 2.5 x 50 cm. A broad peak emerged in tubes 135-167 (1.5 ml/tube 17 ml/hour). The fractions 135-150 were pooled and lyophilized to afford 20 mg of a white fluff (16%) from the coupling reaction.

Mpt. 137-140°C

$[\alpha]_D^{20}$ = -36.0 c 0.14 (75% AcOH 25% H_2O)

tlc A 0.72 B 0.85 C 0.57 D 0.50

HPLC Single peak. Retention time 11 minutes.

[D-Tyr^1, Nle^5]-enkephalin JO-21

100 mg (0.2 mmoles) of benzyl-Glycyl phenylalanyl norleucinate·HCl and 0.03 ml of triethyl amine were mixed in 20 ml of cold chloroform. After stirring for 15 minutes, 63 mg (0.2 mmoles) of D-Cbz-tyrosine and 54 mg of EEDQ were added all at once. The clear solution was stirred at room temperature for 18 hours, diluted to 50 ml with chloroform and worked up as described for (38-b). The residual viscous oil was crystallized from ethylacetate/petroleum ether to give 150 mg of a solid which was collected and washed with cold petroleum ether. mpt. 98-100°C. The solid was dissolved in 20 ml of 40% aqueous AcOH and hydrogenated for 3 hours using 50 mg of 10% Pd/C, 40 psi of H_2. The catalyst was filtered and the filtrate was evaporated in vacuo to yield 100 mg of a beige foam. The material was chromatographed on a column of G-15 Sephadex 2.5 x 60 cm. Peptide material was detected in fractions 90-158 (1 ml/tube 12 ml/hour). The fractions were pooled and lyophilized to yield 70 mg of a white fluff. The powder was dissolved in 2 ml of TRIS-HCl buffer pH 7.7 and chromatographed on a column of DEAE-A25 Sephadex 1.5 x 30 cm ion exchange using a concave salt gradient 0.05 to 0.5M NaCl. A large symmetrical peptide peak emerged in fractions 60-90 (1 ml/tube 10 ml/hour), flanked by a smaller peak from 21-41. The fractions corresponding to 62-88 were pooled, concentrated

to a volume of 2 ml and desalted on G-15 Sephadex 1.5 x 90 cm (2 ml/tube 10 ml/hour). Peptide material emerged in tubes 62-83. Fractions corresponding to 63-72 were pooled and lyophilized to yield 53 mg of a white fluffy material (50% yield).

Mpt. 216-219°C dec

$[\alpha]_D^{20}$ = -16.3 c 0.49 (75% AcOH 25% H_2O)

tlc A 0.70 B 0.85 C 0.57 D 0.46

AAA Phe 1.0 Gly 2.2 Tyr 0.90 Nle 1.1

HPLC Single peak. Retention time 20 minutes.

Pmr (D_2O/CD_3COOD) (s) 7.2 (5) aromatic ring Phe; (q) 7.0 (4) aromatic ring Phe; (t) 4.6 (1) α-C<u>H</u> Phe; (t) 4.3 (2) α-C<u>H</u>, α C<u>H</u>; (s) 3.9 (2) α-C<u>H_2</u> Gly; (s) 3.8 (2) α-CH_2 Gly; (m) 3.2 (4) β-C<u>H_2</u>, β-C<u>H_2</u> Tyr; (m) 2.0, 1.6 (2) β-C<u>H_2</u> Nle; (m) 1.4, 1.0 (4) γ-C<u>H_2</u>, δ-C<u>H_2</u> Nle; (t) 0.8 (3) ω-C<u>H_2</u> Nle.

<u>Tyr-Gly-Phe-Nleu-OH JO-24</u>

To a cold solution of 100 mg (0.19 mmoles) of benzyl-Glycyl phenylalanyl norleucinate·HCl and 0.027 ml of triethylamine in 20 ml of ice-cold chloroform was added 63 mg (0.20 mmoles) of (L)-Cbz-tyrosine and 60 mg of EEDQ. The solution was stirred at room temperature for 24 hours, and then evaporated in vacuo. The residue was partitioned between water and

ethyl acetate and worked up as described for (38-b). The organic phase was concentrated to a volume of 5 ml and allowed to stand in the cold. The crop of crystals that deposited was collected and washed with cold solvent to afford 250 mg of the protected tetrapeptide. The solid was dissolved in 20 ml of glacial acetic acid containing 50 mg of 10% Pd/C. The mixture was hydrogenated for three hours at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo. The residue was dissolved in 2ml of TRIS-HCl buffer pH 7.7 and chromatographed on a column of DEAE-A25 Sephadex (1.5 x 30 cm) eluting with a linear salt gradient 0.05M to 0.5M NaCl. Peptide material was detected in tubes 100-140 and 30-49 (1 ml/tube 15 ml/hour). Fractions corresponding to 105-138 were pooled and evaporated in vacuo. The residue was dissolved in 2 ml of 10% acetic acid and desalted on a column of G-15 Sephadex 1.5 x 90 cm (1.5 ml/tube 10 ml/hour). Peptide material emerged in fractions 90-115. The fractions corresponding to 90-105 were pooled and lyophilized to afford 40 mg of a white fluffy powder (40% yield).

Mpt. 153-154°C dec

$[\alpha]_D^{20}$ = +32.4 c 0.25 (75% AcOH 25% H_2O)

tlc A 0.75 B 0.52 C 0.65 D 0.83

AAA Phe 1.0 Gly 0.92 Tyr 1.0 Nle 0.86

HPLC Single peak. Retention time 11 minutes.

5. Synthesis of N-methylated analogues of [Leu-OCH_3^{5}]-enkephalin

Glycyl Phenylalanyl Leucine methyl ester·HBr (55)

To 80 ml of the chloroform over an ice bath were mixed 1.81 (10 mmoles) of methyl leucinate·HCl and 1.39 ml of triethylamine and the mixture was stirred for 15 minutes. To the clear solution was added 3.56 g (10 mmoles) of Cbz-Gly-Phe-OH and 2.47 g (10 mmoles) of EEDQ all at once. The clear solution was allowed to stir at 5°C for 2 hours and at room temperature for 18 hours. The contents were evaporated in vacuo and the residue was suspended in 50 ml of ethyl acetate. The mixture was extracted with water three times with 1N HCl, two times with 5% $NaHCO_3$, H_2O and dried (Na_2SO_4). The solvent was filtered and evaporated in vacuo to afford 3.5 g (75%) of a pale yellow oil that corresponded to the fully protected tripeptide.

The oil was dissolved in 4 ml of glacial acetic acid and subjected to a gentle stream of anhydrous HBr for approximately 5 minutes. The clear solution was stirred at room temperature for 1.5 hours. After this time, 100 ml of anhydrous ether was added but no precipitate deposited. The solution was extracted with ether several times and the combined organic extracts were discarded. The aqueous phase was concentrated to a volume of 50 ml and lyophilized to afford 1.8 g (60%) of a white fluff. A small quantity was crystallized from isopropanol/

ether to afford white crystals, mpt. 78-79°C.

$[\alpha]_D^{20} = -9.4$ c 0.86 (75% AcOH 25% H_2O)

Pmr (D_2O) (s) 7.3 (5) aromatic ring Phe; (m) 4.8, 4.2 (2) 2α-CH; (s) 3.8 (2) α-$C\underline{H}_2$ Gly; (s) 3.7 (3) O-$C\underline{H}_3$; (d) 3.1 (2) β-$C\underline{H}_2$ Phe; (m) 1.6 (3) β-$C\underline{H}_2$, γ-$C\underline{H}$ Leu; (m) 0.9 (6) $(C\underline{H}_3)_2$ CH

Glycyl Glycyl Phenylalanyl Leucine methyl ester·HCl (57)

To a stirred solution of 1.64 g (4 mmoles) of (55) and 0.55 ml of triethylamine in 30 ml of methanol was added 1.07 g (4.2 mmoles) of EEDQ and 0.88 (4.2 mmoles) of Cbz-Gly. The clear solution was stirred at room temperature overnight and then evaporated in vacuo. The residue was suspended in 100 ml of ethyl acetate and worked up as described for (55). The residual viscous oil was dissolved in 50 ml of ether and allowed to stand in the cold. The solid that deposited was collected by filtration and washed with cold ether. 2.1 g (100%) mpt. 105-8°C.

The solid was dissolved in 100 ml of methanol and added to a mixture of 100 mg of 10% Pd/C and 1.5 ml of concentrated HCl in 10 ml of isopropanol. The mixture was hydrogenated for three hours at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated to dryness. The residue

was crystallized from methanol/ether to give 1.6 g (90%) of white crystals.

Mpt. 175-180°C

$[\alpha]_D^{20} = -6.5$ c 0.40 MeOH

Pmr (D_2O) (s) 7.3 (5) aromatic ring Phe; (t) 4.4 (1) α-CH; (s) 4.0 (2) α-CH_2 Gly; (s) 3.9 (2) α-CH_2 Gly; (s) 3.7 (3) O-CH_3; (d) 3.1 (2) β-CH_2 Phe; (m) 1.6 (3) β-CH_2, γ-CH Leu; (m) 0.9 (6) $(CH_3)_2CH$

[Leu-OCH_3^5]-enkephalin·Acetate JO-28

442 mg (1 mmoles) of (57) and triethyl amine were mixed in 30 ml of chloroform (over an ice bath) and stirred vigorously until solution was complete. 318 mg (1 mmole) of Cbz-Tyr and 260 mg of EEDQ were added and the solution was stirred at room temperature for 18 hours. The solvent was stripped and the residue was worked up as described for (55) to afford a viscous oil (600 mg) that showed two spots on tlc. The oil was chromatographed on silica CC-7 (2 x 40 cm) in the solvent system 12/1 chloroform/isopropanol to give 500 mg of a white solid. Mpt. 108-110°C, Rf = 0.4, 5/1 benzene/isopropanol.

The solid was dissolved in 10 ml of methanol and added to a mixture of 70 mg of 10% Pd/C in 10 ml of glacial acetic acid. The mixture was hydrogenated for 2 hours after which the catalyst was filtered over celite and the filtrate was

evaporated to dryness. 200 mg of the residual foam was applied to a column of G-15 Sephadex 1.5 x 90 cm eluting with 10% acetic acid (10 ml/hour 1 ml/tube). A single large peak emerged in fractions 74-92 flanked by a smaller peak (90-100). Franctions comprising 80-90 were pooled and lyophilized to yield a white powder. The powder was dissolved in 1.5 ml of a 0.1M $Na^{+}AcO^{-}$ pH 5.5 solution and applied to an SP-Sephadex C-25 ion exchange column 1.5 x 30 cm, eluting with a linear $Na^{+}AcO^{-}$ gradient 0.1M to 0.5M (pH 5.5). Two peaks emerged: The major peak consisted of fractions 59-90 while the smaller appeared in tubes 20-39. The fractions corresponding to the former were pooled and evaporated in vacuo. The residue was desalted on a column of G-15 Sephadex 1.5 x 90 cm eluting with 10% AcOH (1 ml/tube, 8 ml/hour). A symmetrical peak appeared in fractions 80-110. Only tubes 85-100 were collected and lyophilized to afford 70 mg (40%) of a white fluffy powder based on the initial coupling reaction.

Mpt. 82-85°C

$[\alpha]_D^{20}$ = +19.2 c 0.47 (75% AcOH 25% H_2O)

tlc A 0.71 B 0.83 C 0.63 D 0.82

AAA Phe 1.0 Gly 2.1 Tyr 0.91 Leu 1.0

HPLC Single peak. Retention time 19 minutes.

Pmr (D_2O) (s) 1.3 (8) aromatic ring Phe; (q) 7.0 (4) aromatic ring Tyr; (m) 4.8, 4.1 (3) α-C<u>H</u>; (d) 3.9 (4) α-C<u>H_2</u> Gly; (s) 3.7 (3) O-C<u>H_3</u>; (m) 3.1 (4) β-C<u>H_2</u> Phe, β-C<u>H_2</u> Tyr; (s) 2.0 (3) C<u>H_3</u>COO^-; (d) 1.8 (3) β-C<u>H_2</u>, γ-C<u>H</u> Leu; (m) 0.9 (6) $(CH_3)_2$CH Leu

[N-CH_3Tyr^1, Leu-OCH_3^{5}]-enkephalin·Acetate JO-29

To a solution of (57) 310 mg (0,7 mmoles) and 0.14 ml of triethyl amine in 30 ml of chloroform was added 286 mg (1 mmole) of N-t-BOC-N(CH_3)-O-bz-Tyr and 260 mg of EEDQ. After stirring for 24 hours the solvent was stripped in vacuo and the residue was worked up as described for (55) to afford 300 mg of an oil (55%) that was used directly.

The oil was dissolved in 2 ml of methylene chloride and added to 3 ml of anhydrous TFA under nitrogen. The clear solution was stirred at room temperature for 1.5 hours and evaporated in vacuo. The oily residue was dissolved in 25 ml of anhydrous ether and allowed to stand in the cold. The crop of crystals that formed was collected and washed with cold ether to afford 270 mg of a beige solid, mpt. 124-126°C.

Trifluoroacetate was exchanged for chloride by passing the solid through a column of Amberlite IRA-400 Cl^- eluting with methanol. The eluent containing the product was concentrated to a volume of 20 ml and added to a mixture of 70 mg

of 10% Pd/C suspended in 10 ml of isopropanol. The mixture was hydrogenated for 12 hours at 40 psi of H_2. The catalyst was filtered over celite and the filtrate was evaporated in vacuo to afford a viscous oil that showed a major spot Rf = 0.1 5/1 benzene/isopropanol and two spots preceeding it. The product from the hydrogenation was chromatographed on a column of silica CC-7 60 g in the same solvent system and the fractions corresponding to Rf = 0.1 were pooled and evaporated in vacuo.

The oily residue was applied to a column of G-15 Sephadex 1.5 x 90 cm eluting with 10% AcOH (1 ml/tube 10 ml/hour). Two separable peaks emerged and, the major peak corresponding to fractions 92-96, was collected and lyophilized to afford 90 mg of a white fluffy powder (20%).

Mpt. 79-82°C dec

$[\alpha]_D^{20}$ = +11.7 c 0.52 (75% AcOH 25% H_2O)

tlc A 0.66 B 0.85 C 0.57 D 0.82

AAA Phe 1.0 Gly 2.1 Leu 1.0 Tyr 0.80[(a)]

HPLC Single peak. Retention time 19 minutes.

[(a)] based on N-CH_3-Tyr

Pmr (D_2O) (m) 7.4 (5) aromatic ring Phe; (q) 7.1 (4) aromatic ring Tyr; (m) 4.8 (1) α-C<u>H</u>; (t) 4.5 (1) α-C<u>H</u>; (t) 4.2 (1) α-C<u>H</u>; (d) 4.0 (4) 2α-C<u>H</u>$_2$ Gly; (s) 3.8 (3) O-C<u>H</u>$_3$; (m) 3.2 (4) β-C<u>H</u>$_2$ Phe, β-C<u>H</u>$_2$ Tyr; (s) 2.9 (3) N-C<u>H</u>$_3$; (s) 1.6 (3) β-C<u>H</u>$_2$, γ-C<u>H</u> Leu; (t) 0.8 (6) (C<u>H</u>$_3$)$_2$CH Leu

<u>Attempted Synthesis of [N(CH$_3$)$_2$Tyr^1, Leu-OCH$_3$^{5}]-enkephalin via EEDQ</u>

To a stirred solution of (57) 200 mg (0.45 mmoles) and 0.06 ml of triethyl amine in 20 ml of methanol was added 100 mg (0.48 mmoles) of N(CH$_3$)$_2$-Tyr (dissolved in 5 ml of H_2O). EEDQ, 118 mg, was added and the mixture was stirred vigorously. Eventually the EEDQ dissolved and the reaction was monitored by tlc (ninhydrin) based on the disappearance of (57). After 20 hours the clear solution was evaporated in vacuo. The residue was dissolved in 20 ml of ethyl acetate and extracted three times with water and 5% $NaHCO_3$. The organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to afford an oil that showed three spots on tlc (one of which was quinoline).

The major product after chromatography on 50 g of silica gel CC-7 (4/1 benzene/isopropanol) proved to be the ethoxy carbamate of Gly-Gly-Phe-Leu-OCH$_3$, and no trace of the N(CH$_3$)$_2$ pentapeptide was detected.

[$N(CH_3)_2$-Tyr^1,Leu-OCH_3]-enkephalin·Acetate (JO-30) via methanolic CH_2O

25 ml of anhydrous methanol was added to a mixture of 200 mg of 10% Pd/C soaked in 5 ml of isopropanol. The mixture was stirred for 24 hours in an open vessel equipped with a calcium chloride drying tube. After this time 300 mg of Cbz-Gly Gly Phe Leu-OCH_3 was added and the mixture was hydrogenated at 40 psi of H_2 for 24 hours. The catalyst was filtered over celite and the filtrate was evaporated in vacuo. The oily residue was dissolved in ethyl acetate and allowed to stand in the cold. The solid that deposited was collected and washed with ether 200 mg (65%), mpt. 104-105°C.

The solid was dissolved in 2 ml of 10% AcOH and applied to a column of G-15 Sephadex 1.5 x 90 cm eluting with the same solvent system (1 ml/tube 10 ml/hour). A single symmetrical peak appeared in fractions 76-98. The fractions were pooled and lyophilized to afford 150 mg of a white fluffy powder.

Mpt. 62-64°C

$[\alpha]_D^{20}$ = +7.2 c 0.73 DMF

tlc A 0.61 B 0.85 C 0.51 D 0.82

AAA Phe 1.0 Gly 2.1 Leu 1.1

HPLC Single peak. Retention time 20 minutes.

Pmr (D_2O) (s) 7.3 (5) aromatic ring Phe; (q) 7.0 (4) aromatic ring Tyr; (m) 4.8, 4,4 (2) 2α-C<u>H</u>; (m) 4.0 (1) α-C<u>H</u>; (s) 3.9 (2) α-C<u>H_2</u> Gly; (s) 3.8 (2) α-C<u>H_2</u> Gly; (s) 3.7 (3) O-C<u>H_3</u>; (m) 3.6, 2.9 (10) N-$(CH_3)_2$, α-C<u>H_2</u> Phe, C<u>H_2</u> Tyr; (s) 2.0 (3) C<u>H_3</u> COO^-; (m) 1.6 (3) β-CH_2, γ-C<u>H</u> Leu; (m) 0.9 (6) $(C\underline{H_3})_2$-CH

<u>[N-$(CH_3)_3^+$ I^--Tyr^1, Leu-OCH_3]-enkephalin JO-31</u>

30 mg of [$N(CH_3)_2Tyr^1$, Leu-OCH_3]-enkephalin was dissolved in 2 ml of absolute methanol and treated with a large excess of methyl iodide. The solution was allowed to stand at room temperature for 48 hours and evaporated in vacuo. The residue was dissolved in 2 ml of absolute ethanol and allowed to stand in the cold. The crop of crystals that deposited was collected by filtration and washed with cold solvent and dried (20 mg, 53%).

Mpt. 170-172°C

CHN	calc	57.38% C	6.89% H	3.19% N
	found	57.51% C	7.04% H	2.95% N

6. α-Amino-α-(p-methoxybenzyl)-δ-lactam-N-acetic acid

Ethyl,p-methoxybenzyl malonate (39)

Sodium 0.58 g (0.025 g-atoms) was dissolved in 100 ml of ethanol under nitrogen. The solution was cooled in an ice bath and 4 g (25 mmoles) of ethyl malonate was added dropwise over a period of 15 minutes. After addition was complete, 5.02 g (22 mmoles) of anisyl bromide was added dropwise (with cooling) over a period of 30 minutes. The mixture was allowed to reach room temperature and was stirred vigorously for 18 hours. The salts were filtered off and the filtrate was evaporated in vacuo. The residual oil was dissolved in ether and extracted 3 times with 50 ml portions of water. The ether phase was dried (Na_2SO_4), filtered and evaporated in vacuo to give a thick yellow oil. Vacuum distillation 130-132°C/0.1 afforded a clear liquid, 3 g (50% yield).

Pmr ($CDCl_3$) (q) 7.0 (4) ring protons; (q) 4.2 (4) $(CO_2C\underline{H}_2CH_3)_2$; (s) 3.7 (3) $C\underline{H}_3O$; (t) 3.5 (1) α-C$\underline{H}$; (d) 3.1 (2) β-$C\underline{H}_2$; (t) 1.2 (6) $(CO_2CH_2C\underline{H}_3)_2$

3-Azido propanol

To a solution of 3-bromo propanol 13.9 g (0.1 mole) in 200 ml of acetone was added a solution of 7.5 g (0.12 moles) of sodium azide in 20 ml of H_2O all at once. The clear solution

was refluxed for 16 hours, cooled, and evaporated in vacuo. The residue was suspended in 150 ml of ether, and extracted three times with 50 ml portions of water. The organic phase was dried (Na_2SO_4), filtered, and the filtrate was evaporated in vacuo. The resulting brown liquid was distilled under vacuum, 78-80°C/15 mm to give 9 g (90% yield) of a clear colorless liquid.

Pmr ($CDCl_3$) (qt) 1.8 (2) $CH_2C\underline{H}_2CH_2$; (b) 2.3 (1) O$\underline{H}$;
(t) 3.3 (2) $C\underline{H}_2OH$; (q) 3.8 (2) $C\underline{H}_2N_3$

1-chloro-3-azido propane

12 g (118 mmoles) of 3-azido propanol was dissolved in 20 ml of benzene and cooled in an ace bath. 12 ml of thionyl chloride was added dropwise with caution (violent evolution of HCl . When addition was complete, the solution was refluxed for one hour, cooled, and evaporated in vacuo. Vacuum distillation of the residual oil 48-50°C/15 mm afforded 9 g (64% yield) of a clear liquid.

ir, ($CHCl_3$), $\bar{\nu}$ (N=N=N) = 2100 cm^{-1} (s)

Pmr ($CDCl_3$) (qt) 2.1 $CH_2C\underline{H}_2CH_2$; (m) 3.5 $C\underline{H}_2N_3$, $C\underline{H}_2Cl$

1-iodo-3-azido propane

The alkylation of p-methoxybenzyl diethyl malonate with 3-azido propyl chloride was not successful in DMF using NaH, or in ethanol using EtO^-Na^+. Consequently the iodo compound was synthesized by dissolving 5 g of 3-azido propyl chloride in 100 ml of reagent acetone containing 6.7 g of sodium iodide. The solution was stirred at room temperature for 18 hours under nitrogen. The resulting brown mixture was evaporated in vacuo. The residue was suspended in 150 ml of ether and extracted three times with 50 ml portions of water. The organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to give 4 g of a red liquid. The product was used directly without further purification.

ethyl-α-(p-methoxybenzyl)-α-(3-azidopropyl)-malonate (40)

To a mixture of 50 mg of NaH (58% oil dispersion previously washed with hexane) in 5 ml of DMF in an ice bath, was added 560 mg (2 mmoles) of p-methoxybenzyl diethyl malonate (with caution). After stirring for 20 minutes at 5°C, a solution of 464 mg (2.2 mmoles) of 3-azido-1-iodo propane in 5 ml of DMF was added dropwise over a period of 15 minutes. The ice bath was removed after one hour and the mixture was subsequently stirred overnight at room temperature. The solvent was evaporated in vacuo, and the residue was dissolved in ether

(50 ml) and extracted with water until the aqueous extracts were colourless. The ether was dried (Na_2SO_4), and evaporated in vacuo to afford 500 mg of a brown viscous gum. The residue was applied to a column of 50 g of silica gel CC-7) and eluted with chloroform. A yellow liquid was obtained 400 mg (55% yield). (Soluble in hexane.)

Pmr ($CDCl_3$) (q) 7.0 (4) aromatic ring protons; (q) 4.3 (4) $(CO_2C\underline{H}_2CH_3)_2$; (s) 3.8 (3) $C\underline{H}_3O$: (s) 3.3 (2) β-$C\underline{H}_2Ph$; (t) 3.3 (2) $C\underline{H}_2N_3$ (m) 2.2, 1.3 (4) $C\underline{H}_2C\underline{H}_2$; (t) 1.3 (6) $(CO_2CH_2CH_3)_2$

The above reaction was repeated on a scale corresponding to 4.8 g (17.4 mmoles) of malonate. The product mixture was suspended in water and extracted with hexane. The combined organic extracts were washed with water and dried (Na_2SO_4). The solvent was filtered and the filtrate was evaporated in vavuo to afford 4 g (65% yield) a pale yellow oil with the identical NMR spectrum as the product obtained from chromatography.

α-ethoxycarbonyl-α-(p-methoxybenzyl)-δ-lactam (42-a)

The oil from the previous reaction 4 g (11 mmoles) was dissolved in 50 ml of absolute ethanol containing 1 ml of concentrated HCl and 300 mg of 10% Pd/C. The mixture was hydrogenated for 10 hours at 40 psi of H_2. The catalyst was filtered over celite and the cake was washed with 20 ml of ethanol. The filtrate was evaporated in vacuo and the residue was suspended in 100 ml of water and extracted twice with 30 ml portions of ether. The aqueous phase was chilled in ice and made strongly basic by addition of solid K_2CO_3. The mixture was extracted with ether, and the combined organic extracts were dried (Na_2SO_4), filtered and evaporated in vacuo. The residue, 3.5 g of a pale yellow oil, showed a positive ninhydrin test.

The oil was heated at 150°C (neat) under nitrogen for five hours. The melt was cooled, dissolved in 30 ml of ether, and allowed to stand in the cold. A crop of beige crystals deposited 2.1 g (55% yield) from the azide.

Mpt. 88-90°C

Ir (KBr), $\bar{\nu}(\overset{O}{\overset{\|}{C}}\text{-N}) = 1650\ cm^{-1}$(s) $\bar{\nu}(\overset{O}{\overset{\|}{C}}\text{-O}) = 1750\ cm^{-1}$(s)

Pmr ($CDCl_3$) (q) 7.0 (4) aromatic ring protons; (b) 6.7 (1) N-H; (q) 4.2 (2) $CO_2\underline{CH_2}CH_3$; (s) 3.8 (3) $\underline{CH_3}O$; (q) 3.3 (2) $NCH_2\underline{CH_2}$; (b) 3.1 (2) $\underline{CH_2}$ Ph; (m) 1.8 (4) $\underline{CH_2}\underline{CH_2}$; (t) 1.3 (3) $CO_2CH_2\underline{CH_3}$

α-carboxy-α-(p-methoxybenzyl)-δ-lactam (42-b)

The solid obtained above was dissolved in 15 ml of ethanol and the clear solution was added to 20 ml of 2N NaOH. The murky solution was stirred vigorously for 18 hours, then diluted to 100 ml with water. The mixture was extracted with chloroform and the organic extracts were discarded. The aqueous phase was cooled in an ice bath and made acidic by dropwise addition of 1N HCl. The mixture was extracted with 30 ml portions of chloroform, and the combined organic extracts were dried (Na_2SO_4), and concentrated in vacuo. The concentrate was diluted with 50 ml of anhydrous ether and allowed to stand in the cold. The solid that deposited was collected by filtration and washed with ether to give 1.5 g (80%) of the carboxylic acid.

Mpt. 103-104°C

ir (KBr), $\bar{\nu}(\overset{O}{\overset{\|}{C}}OH)$ = 3400-2800 cm^{-1}(b), $\bar{\nu}$(COOH) = 1740 cm^{-1}(s)

Pmr ($CDCl_3$) (q) 7.0 (4) aromatic ring protons (b) 6.2 (1) N-H; (s) 3.8 (3) $C\underline{H}_3O$: (q) 3.2 (2) $C\underline{H}_2N$; (b) 3.0 (2) $C\underline{H}_2Ph$; (m) 1.7 (4) $C\underline{H}_2C\underline{H}_2$

α-benzoxycarbonyl - α-(p-methoxybenzyl)-δ-Lactam (attempted synthesis)

(a) 1 g (3.8 mmoles) of the carboxylic acid was suspended in 50 ml of benzene in a flask equipped with a Dean Stark apparatus. To the mixture was added 432 mg (4 mmoles) of benzyl alcohol and one drop of H_2SO_4. The solution was refluxed for 3 hours.

The benzyl ester was not obtained, instead, only decarboxylated δ-lactam was isolated as a white crystalline solid.

Mpt. 84-85° C

ir (KBr) $\nu(\overset{O}{\overset{\|}{C}}\text{-N})$ = 1660 cm^{-1} (s) No COOH

Pmr ($CDCl_3$) (q) 6.9 (5) aromatic ring, N-H; (s) 3.7 (3) $C\underline{H}_3O$; (m) 3.2 (3) $NC\underline{H}_2$, α-C$\underline{H}$; (b) 2.7 (2) $C\underline{H}_2$ Ph; (m) 1.6 (4) $C\underline{H}_2C\underline{H}_2$

(b) An attempt was made to synthesize the acyl chloride by the following procedure: 500 mg (19 mmoles) of the carboxylic acid were suspended in 5 ml of benzene and to the mixture was added 1 ml of thionyl chloride. The solution was stirred at room temperature for 24 hours, and the solvent was evaporated in vacuo. The residue was crystallized from ether to afford a solid that corresponded to the carboxylic acid.

α-ethoxycarbonyl-α-(p-methoxybenzyl)- -lactam-N-acetic acid (attempted synthesis)

To a suspension of 100 mg (50% oil dispersion) in 10 ml of DMF under nitrogen was added (42-a) 291 mg (1 mmole) in 2 ml of DMF dropwise with vigorous stirring. After evolution ceased, 312 mg (1.5 mmoles) of sodium iodo acetate was added. The mixture was stirred at room temperature overnight and evaporated in vacuo. The residue was dissolved in water and the solution was rendered acidic with 1N HCl and extracted with chloroform. The combined organic extracts were washed with water, dried (Na_2SO_4) and evaporated in vacuo to afford 200 mg of a red viscous oil. The product was chromatographed on a column of silica CC-7 (5/1 chloroform/methanol 0.05% AcOH) to afford 140 mg of a pale yellow oil.

Pmr ($CDCl_3$) (b) 9.2 (1) COO$\underline{H}$; (q) 6.9 (5) aromatic ring, N-$\underline{H}$; (s) 4.3 (2) C$\underline{H}_2$COOH; (s) 3.7 (3) C$\underline{H}_3$O; (m) 3.2 (3) NC$\underline{H}_2$, α-CH; (b) 2.7 (2) C$\underline{H}_2$Ph; (m) 1.6 (4) C$\underline{H}_2$C$\underline{H}_2$

7. 16β-butanomorphinan

o-Acetyl-16α-butanomorphinan (48-a)

To a solution of triethylamine 0.22 ml (1.6 mmoles) and 297 mg (1 mmole) of 3-hydroxy-16α-butanomorphinan (50-a) in 30 ml of chloroform in an ice bath was added 0.085 ml (1.2 mmoles) of acetyl chloride dropwise (with a micro syringe) over a period of 10 minutes. The solution was allowed to reach room temperature and stirred for an additional 2 hours. The solution was extracted with 20 ml portions of water, once with 5% NaOH, and washed with water. The organic phase was dried over Na_2SO_4, filtered and evaporated in vacuo to afford a reddish viscous oil 270 mg.

Rf = 0.78 acetone

ir (chloroform) $\bar{\nu}$ ($\overset{O}{\overset{\|}{C}}$-O) = 1750 cm^{-1} (s)

oxidation to enamine (49)

177 mg (0.5 mmoles) of o-acetyl 16α-butano morphinan was dissolved in 10 ml of 10% AcOH and treated with 636 mg (2 mmoles) of Mercuric acetate. The solution was heated at 90°C for 2 hours then cooled in an ice bath. The mercurous acetate that deposited was filtered and washed with water. The filtrate was diluted to 20 ml with 50% AcOH and subjected to a stream of H_2S. The black mixture was centrifuged, the supernatant was decanted and made basic with NH_4OH. The mixture

was extracted with chloroform and the combined organic extracts were washed with water, dried (Na_2SO_4), filtered and the filtrate was evaporated in vacuo to afford 100 mg of a viscous oil which crystallized upon addition of anhydrous ether. The solid corresponding to the enamine was used directly without further purification.

Attempted reduction of (49) by Li/NH_3

Compound (49) suspended in 3 ml of ether was added to 1.2 mg (0.17 mg-atom) of Li wire in 20 ml of NH_3 and the resulting mixture was stirred for 2 hours. NH_4Cl was added portionwise until the blue colour was discharged. The NH_3 was evaporated and the residual white mass was treated with 20 ml of water and 20 ml of methylene chloride. The phases were separated and the aqueous phase was extracted once more with CH_2Cl_2. The combined organic ectracts were washed with water, dried (Na_2SO_4) and evaporated in vacuo to afford 50 mg of a viscous oil. tlc (acetone) revealed three spots. The procedure was abandoned because of its complexity.

Reduction by $NaCNBH_3$

To a solution of enamine (49) 110 mg in 10 ml of 5/1 THF/MeOH was added 1N methanolic HCl dropwise until acidic to pH paper (pH ≃ 3). After stirring for 10 minutes, a solution

of 20 mg of $NaCNBH_3$ in 2 ml of methanol was added dropwise. The pH was monitored and adjusted accordingly with methanolic HCl. After 4 hours, the mixture was evaporated in vacuo and the residue was suspended in 10 ml of water. The mixture was extracted twice with 5 ml portions of chloroform and the organic extracts were discarded. The aqueous phase was cooled in an ice bath, made basic with 5% NH_4OH and extracted with chloroform. The combined organic extracts were dried (Na_2SO_4), filtered, and evaporated in vacuo to afford 25 mg of a viscous oil.

tlc Rf = 0.7 acetone

ir (chloroform) $\bar{\nu}$ (C=O) = 1750 cm^{-1} (s)

16β-butano morphinan·HBr (50-b)

The oil obtained above was dissolved in 3 ml of 20% aqueous ethanol containing 100 mg of K_2CO_3. The solution was stirred at room temperature overnight whereupon a solid deposited. The mixture was diluted to a volume of 10 ml with water and extracted 3 times with 10 ml portions of chloroform. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated in vacuo to afford 20 mg of a solid. (18% yield) from 16α-butano morphinan.

tlc Rf = 0.3 acetone

Mpt. >270°C

The new compound displayed ir spectrum B, p. 166.

The 16α-butano morphinan displayed ir spectrum A, p. 166.

ir (KBr) $\bar{\nu}$ (O-H) = 3400 cm^{-1}

The HBr salt of the new compound was obtained by dissolving 10 mg of the solid in methanol and treating the solution with two drops of 48% HBr. The solution was evaporated in vacuo and the residue was crystallized from ethanol/ether. The crop of crystals was collected and washed with cold ether.

Mpt. 169-170°C dec.

8. Other syntheses

N-methyl levorphanol·iodide

100 mg of levorphanol tartrate was dissolved in 20 ml water and made strongly basic with NH_4OH to pH 9. The solid that precipitated was extracted with 10 ml portions of chloroform. The combined organic extracts were washed with water, dried (Na_2SO_4), filtered and evaporated in vacuo to afford levorphanol, mpt. 251-252°C. The solid was dissolved in 5 ml of ethanol and treated with a large excess of methyl iodide. The solution was allowed to stand at room temperature for 48 hours and evaporated in vacuo. The residue was recrystallized from ethanol to afford 150 mg of white needles (65% from levorphanol).

Mpt. 276-277°C

lit. mpt.[143,156] 268-286°C

CHN found	54.14% C	6.63% H	3.55% N	31.72% I
calc	54.13% C	6.60% H	3.53% N	31.70% I

N-methyl levallorphan·iodide

Levallorphan tartrate was treated in the same manner as described for levorphanol to give the N-methyl iodide salt as white plates from absolute ethanol.

Mpt. 221-222°C

CHN found	56.13% C	6.67% H	3.46% N	29.69% I
calc	57.13% C	7.30% H	3.16% N	29.37% I

B. THE BINDING ASSAY

A modified version of the binding assay described by Pasternak et al.[157] was used for the determination of the opiate receptor affinities. Male Sprague-Dawley rats (Canadian Breeding Laboratories) (250-300 g) were decapitated and after removal of the cerebellum the brains were homogenized in 30 volumes of ice-cold standard buffer (50 mM Tris-HCl, pH 7.7). The combined homogenates were centrifuged at 30'000 x g (30 min at 4°) and the membranes reconstituted in the original volume of standard buffer. After incubation at 37° for 30 min and subsequent centrifugation the pellet was again suspended in the initial volume of the standard buffer to yield the final membrane suspension. Aliquots (2 ml) of the membrane preparation were incubated for 1 hour at 0° (with 1 ml of standard buffer containing the peptide to be tested and [^{3}H]naloxone (17.7 Ci/mmole; New England Nuclear) at a final concentration of 0.5 nm. At the end of the incubation the reaction mixtures were filtered through Whatman GF/B filters under vacuum at 4° and the filters were then washed with two 5 ml-portions of ice-cold standard buffer. Subsequently the filters were transferred to scintillation vials and treated with 1 ml of toluene (Packard) for 30 min, whereupon 0.5 ml of acetic acid and 10 ml of Aquasol (New England Nuclear) were added. After shaking for 30 min the vials were counted at an efficiency of 40-45%. Stereo-

specific binding as determined by displacement of [^{3}H]-naloxane with an excess (10^{-5}M) of cold [Met^{5}]-enkephalin accounted for 70-80% of total binding. Values of half-maximum inhibition (IC_{50}) of the stereospecific binding were obtained graphically from semi-logarithmic plots. Each compound was tested at least three times and [Met^{5}]-enkephalin was included in each determination for comparison.

FIGURE 25. Pmr spectra of some selected compounds

1, 3-(p-hydroxybenzyl)6-ethoxycarbonyl-2-oxo-piperazine

2, 3-(p-hydroxybenzyl)-6-ethoxycarbonyl-6-deutero-2-oxo piperazine

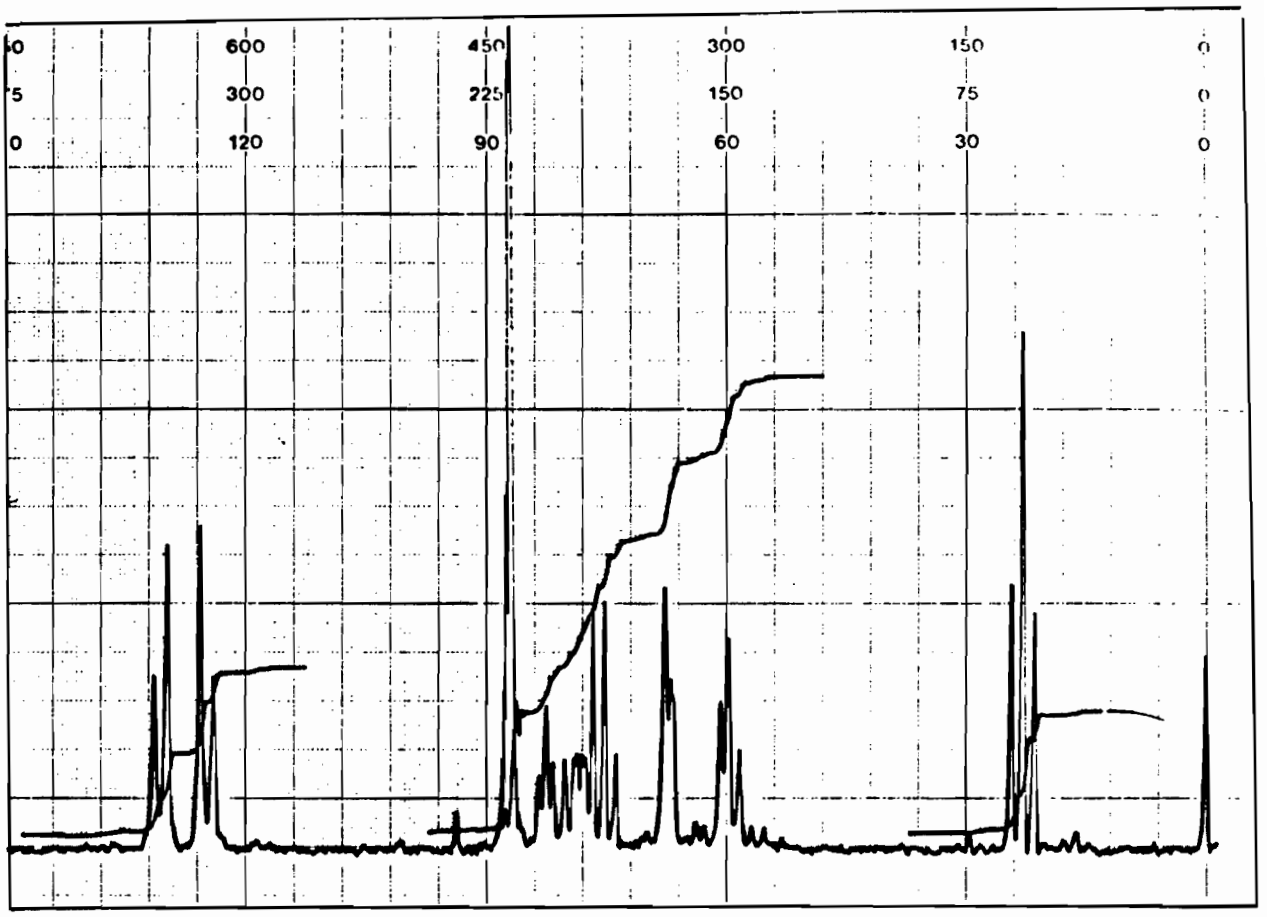
3, 3-(p-hydroxybenzyl)2-oxo-piperazine-1,N-acetic acid·HCl

4, α-(p-methoxybenzyl)-α-ethoxycarbonyl-δ-lactam

5, [$N(CH_3)$-Tyr,Leu-OCH_3^{5}]-enkephalin·Acetate

6, [3D-$N(CH_3)$-trans amido piperazinone]-Phe-Gly-Nle-OH

7, [3L-cis amido piperazinone]-Phe-Gly-Leu-OH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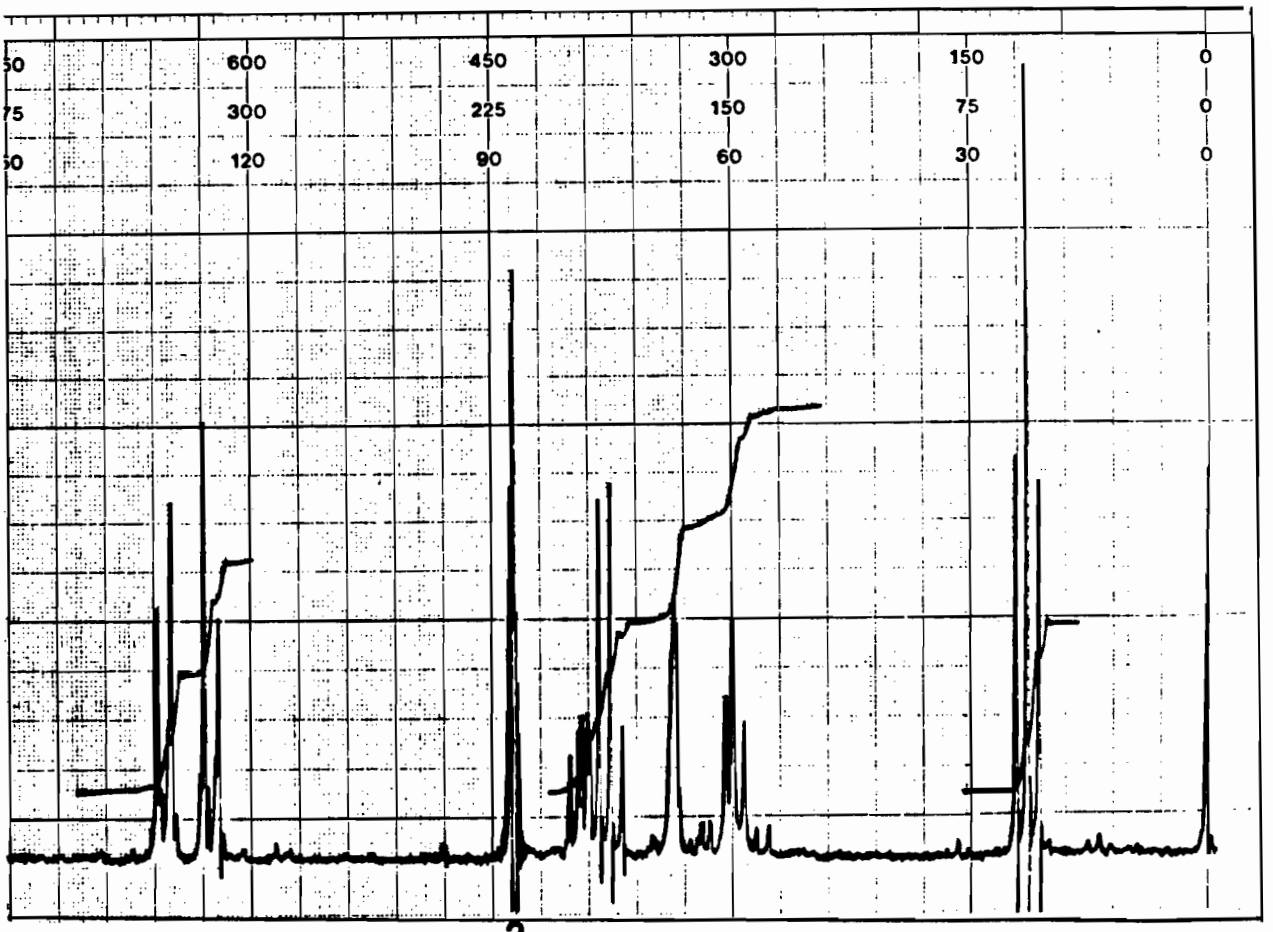
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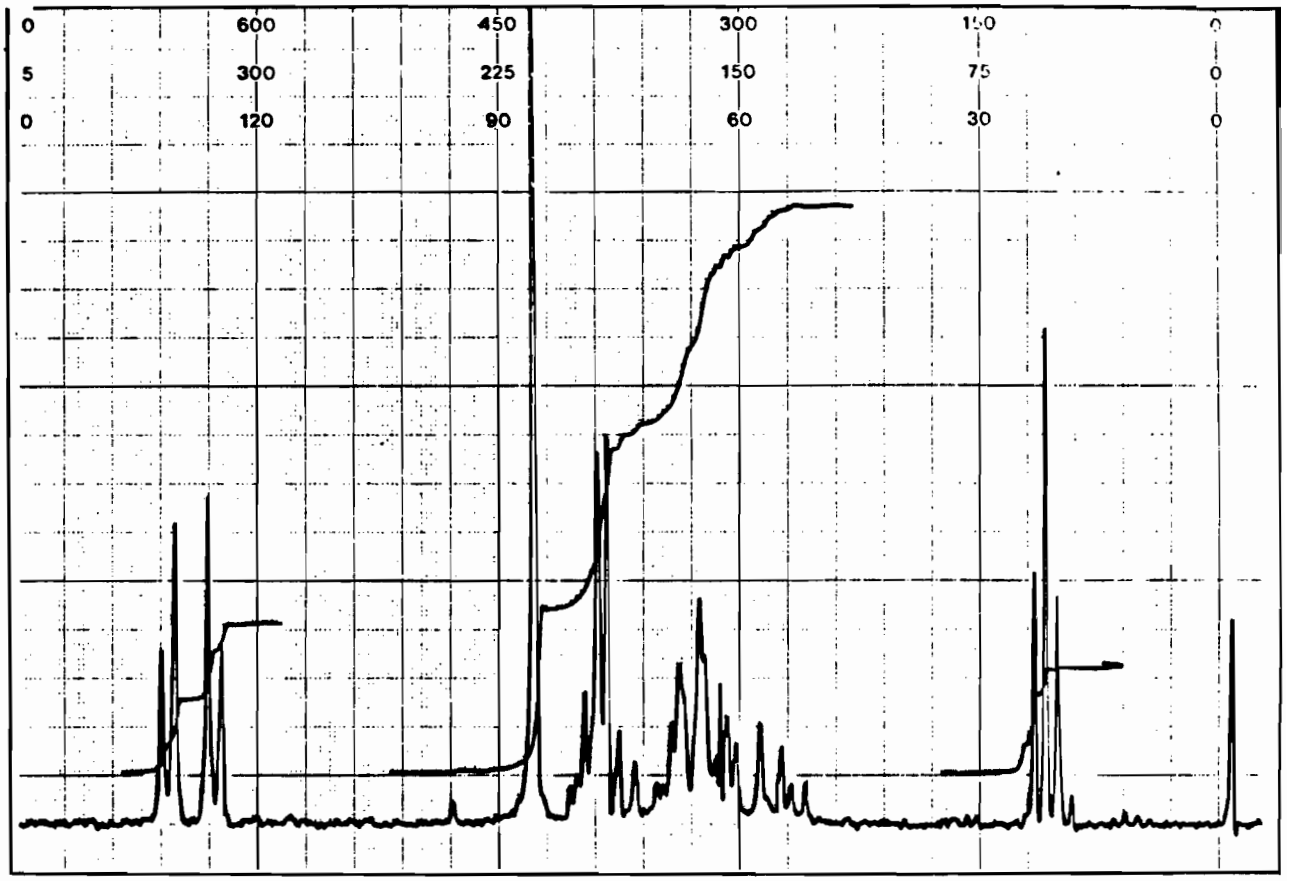
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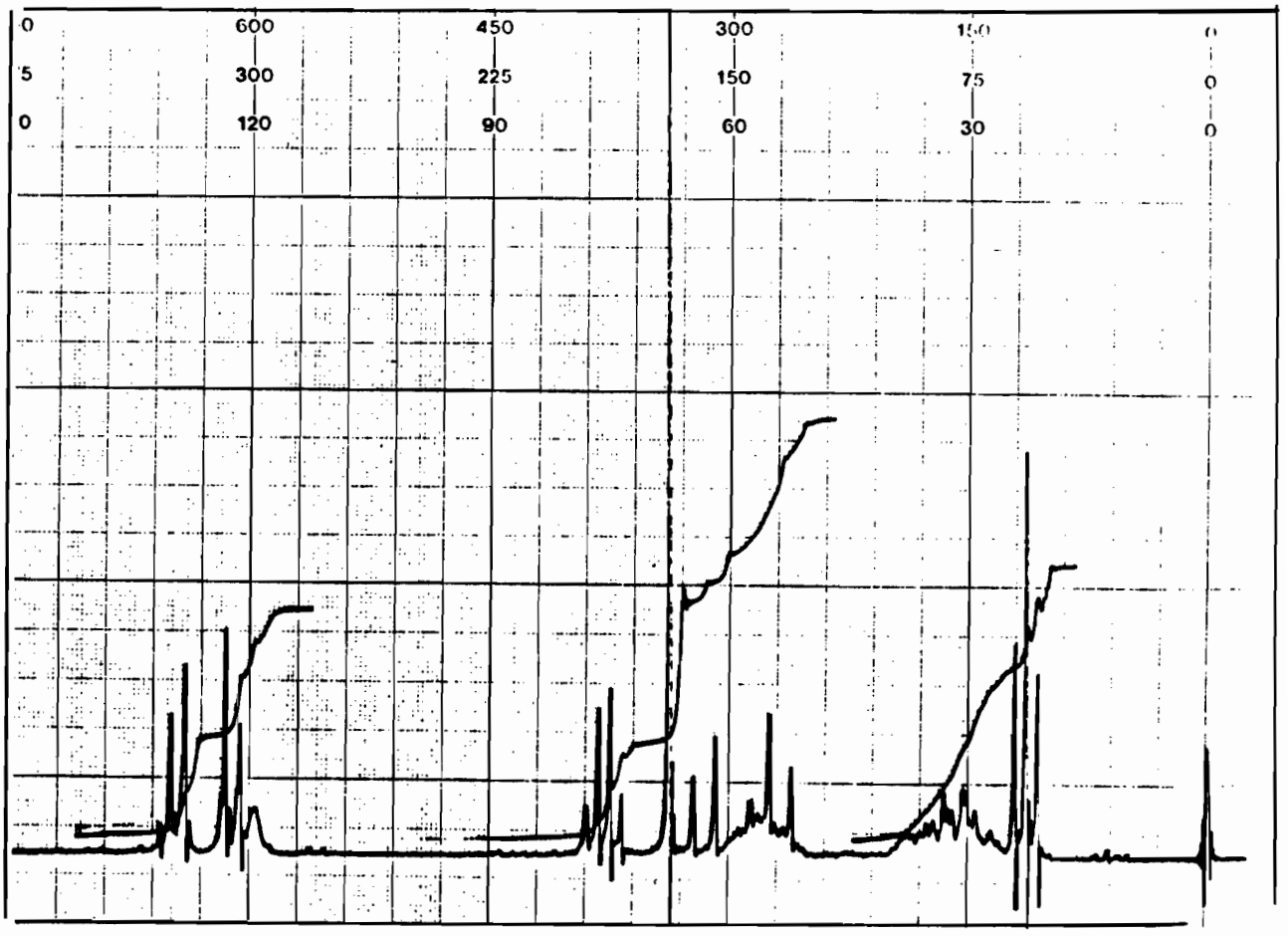
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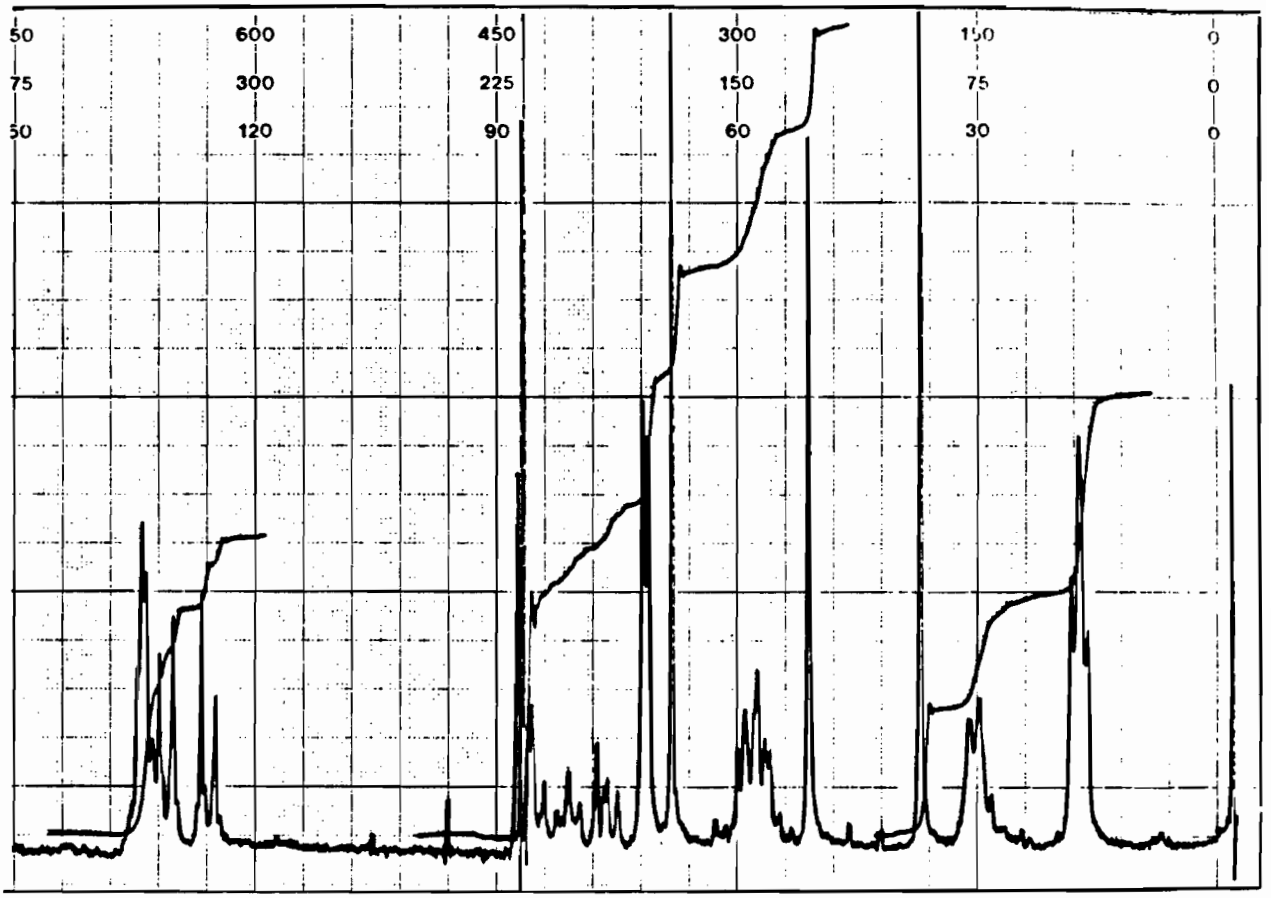
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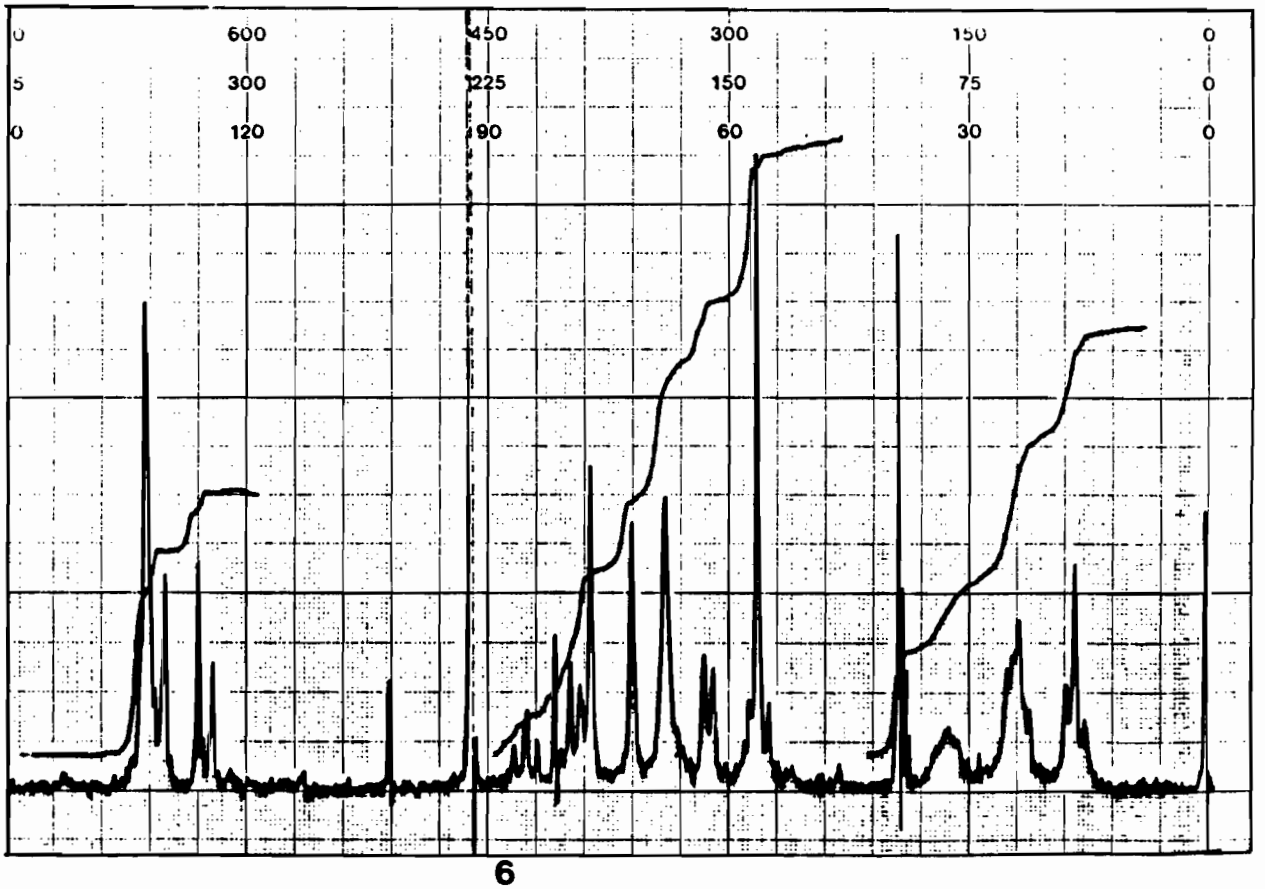
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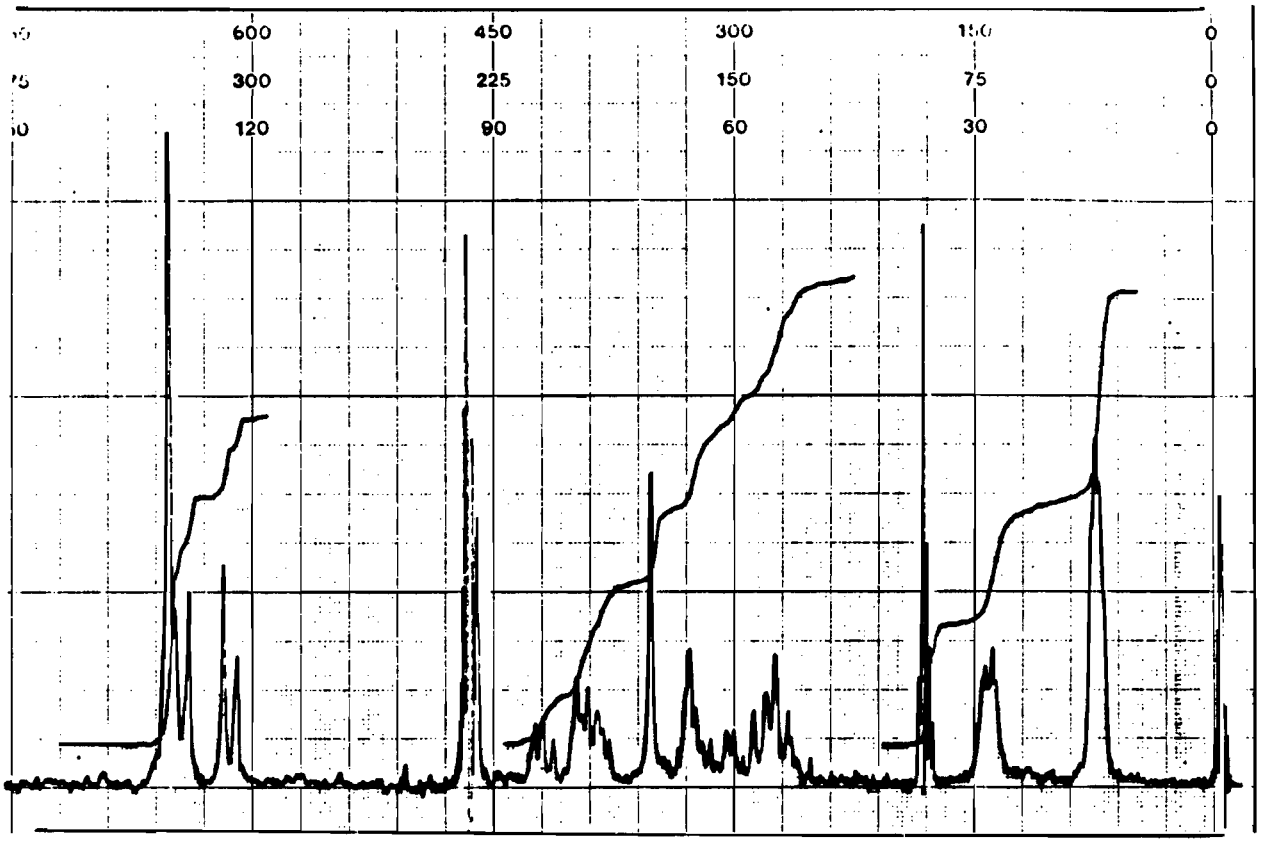

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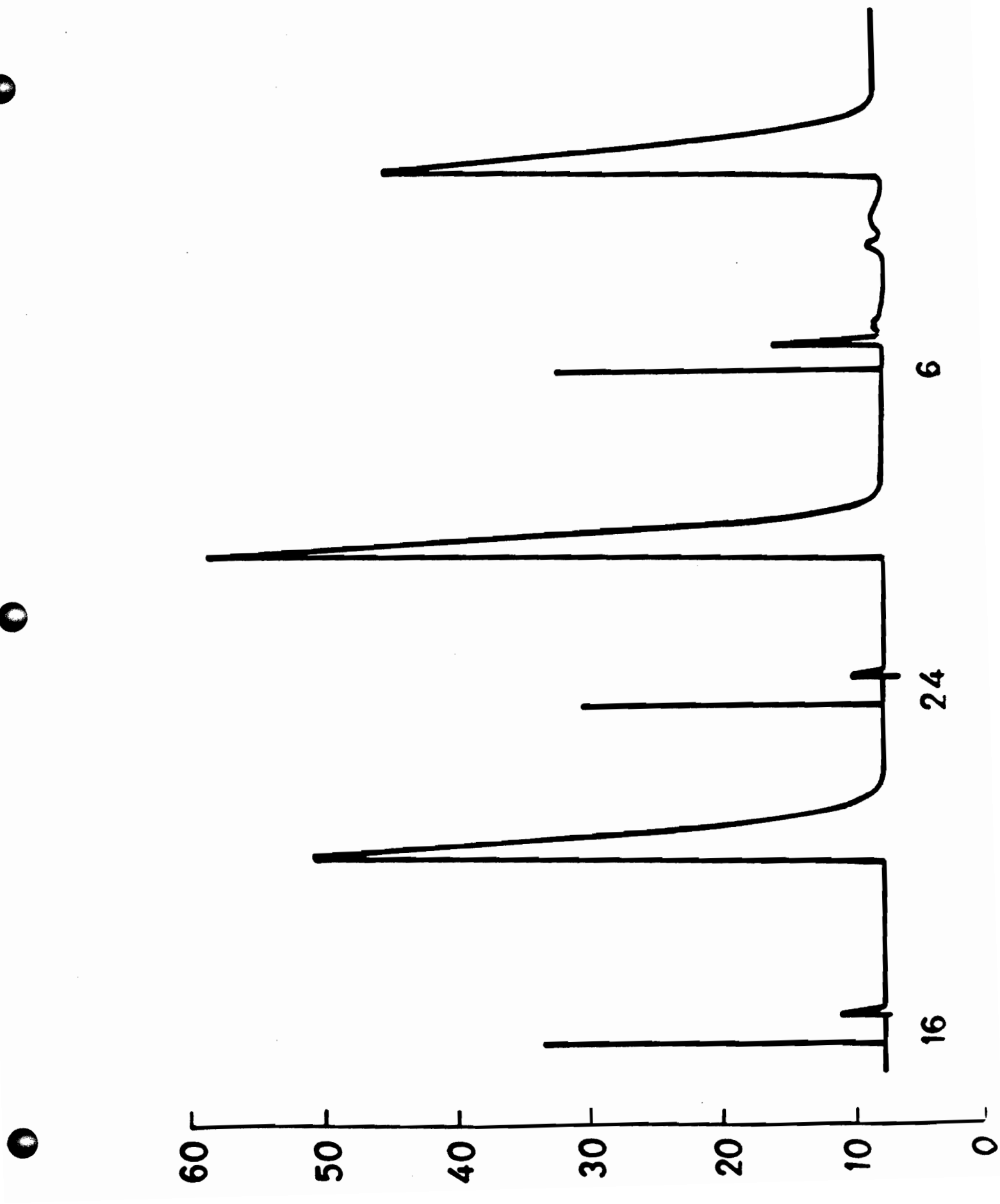
FIGURE 26. Some HPLC elution profiles of final peptide products. Waters μ-bondapack RP-C18 column. 65% H_2O 35% MeOH (0.05% H_3PO_4), 1.5 ml/min, 200 nm.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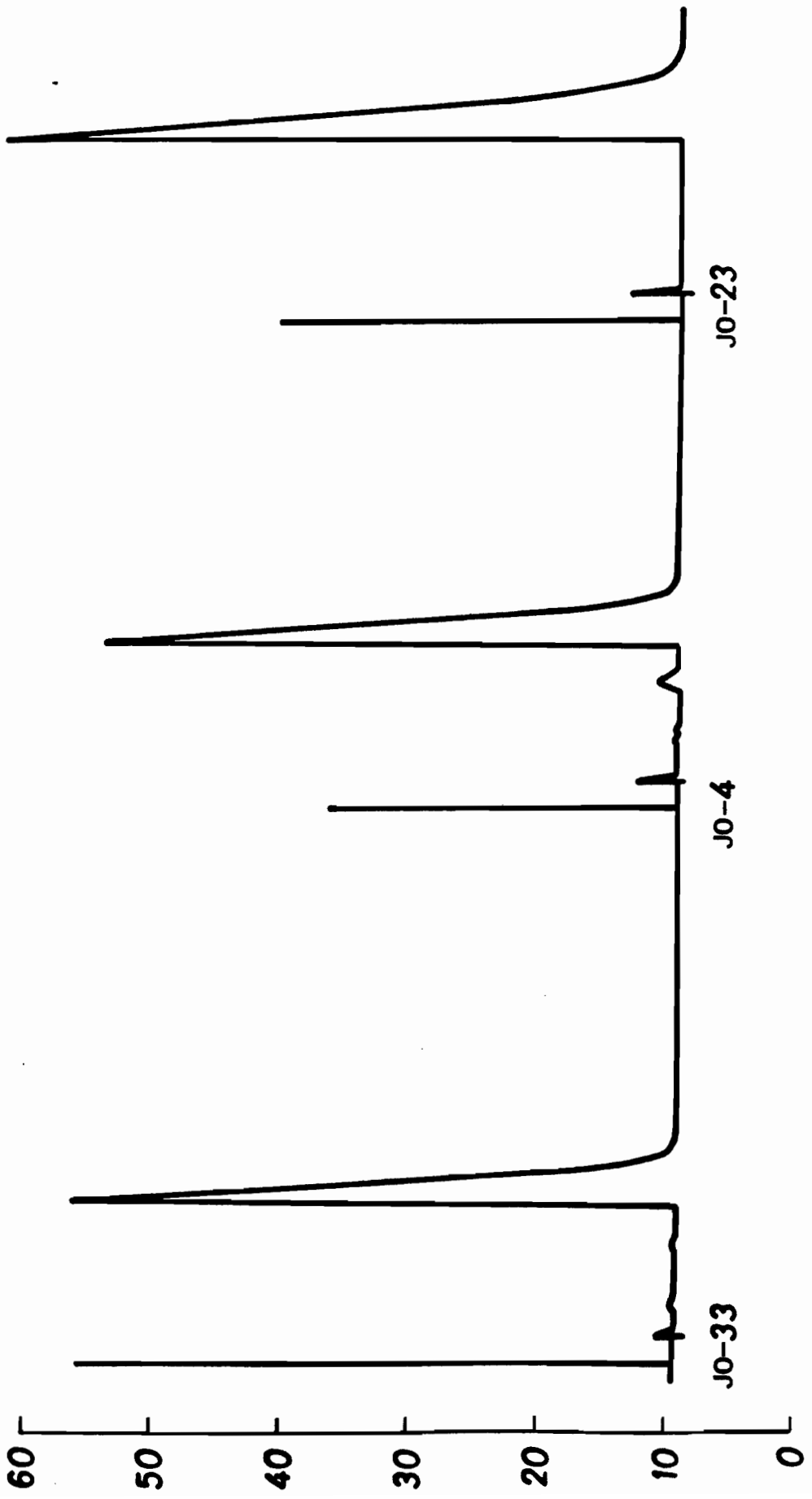
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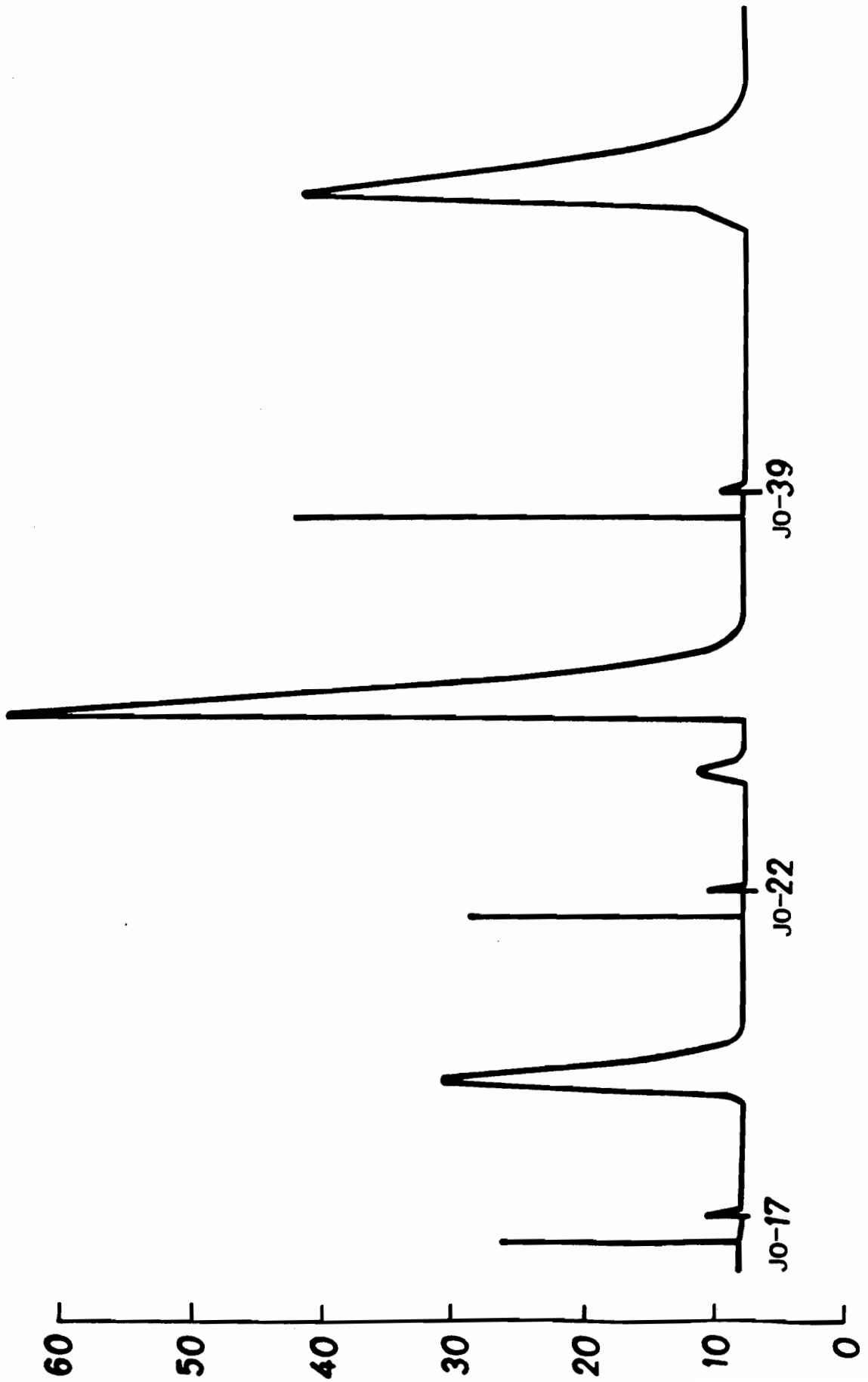
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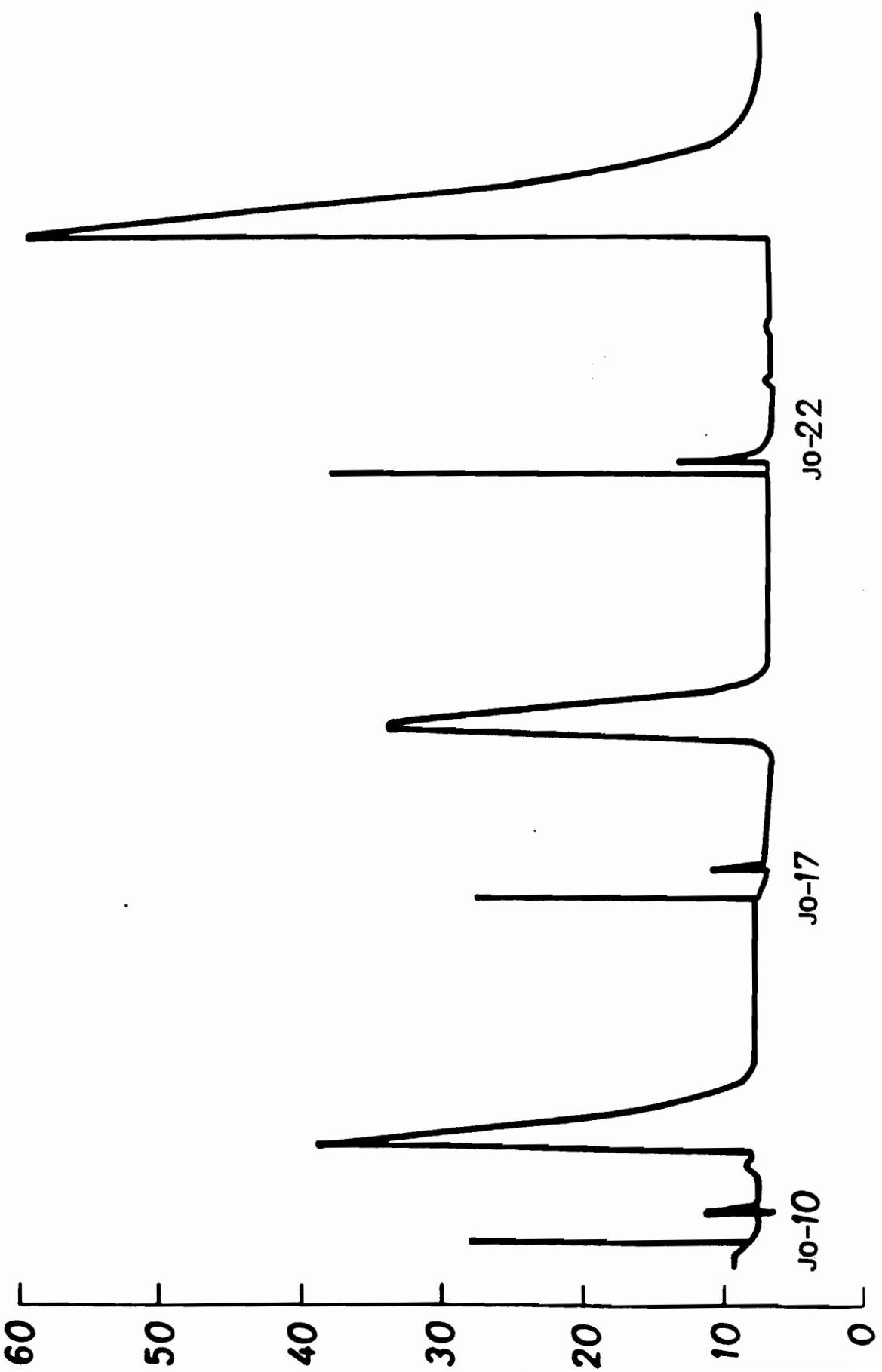
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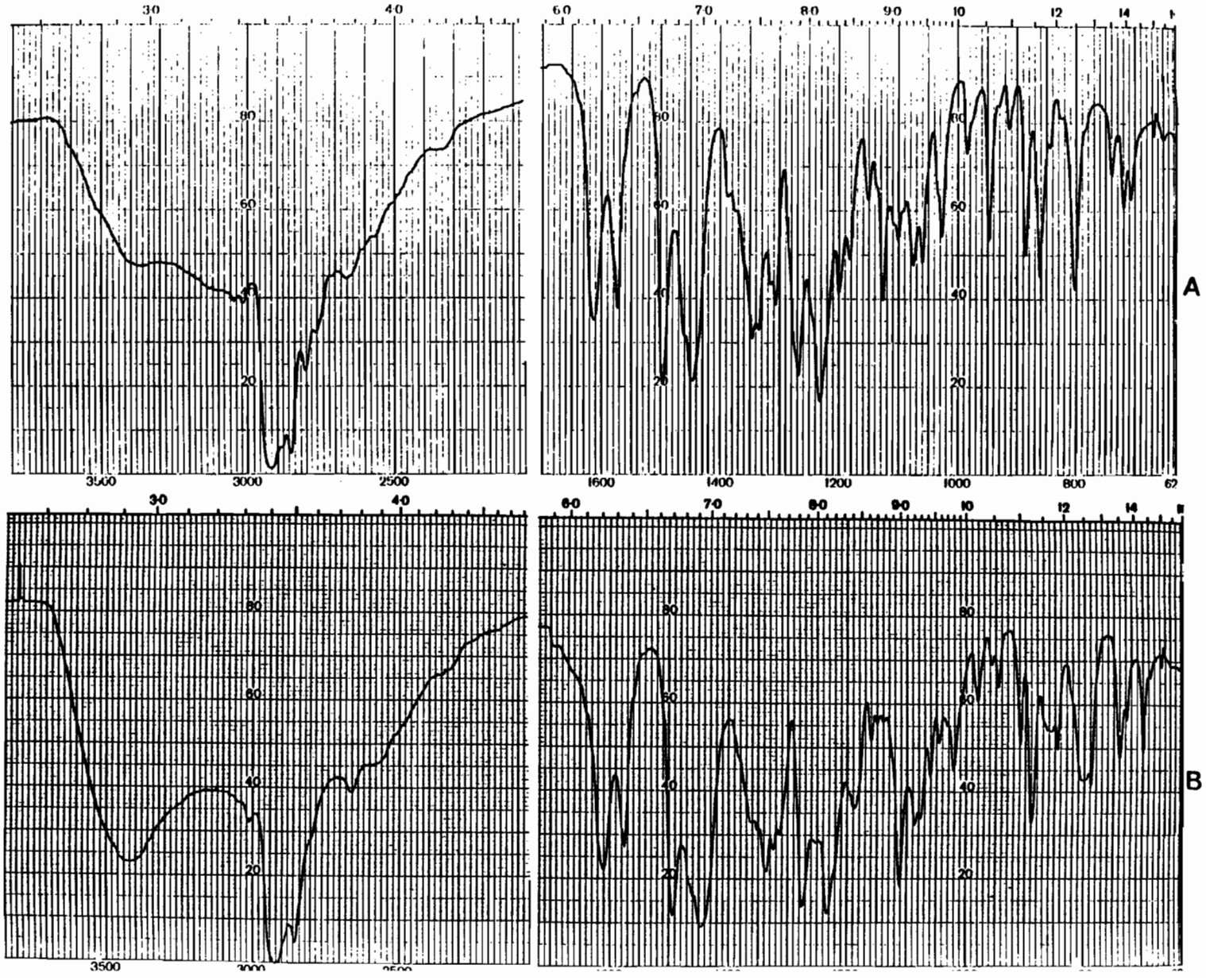
IR Spectra of 16α-butanomorphinan (A), and 16β-butanomorphinan (B)



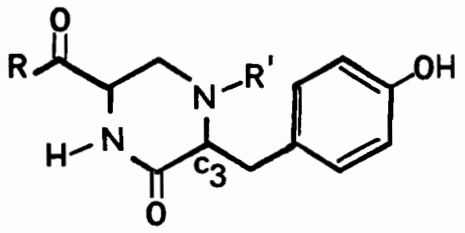
RESULTS AND DISCUSSION

A. IN VIVO AND IN VITRO PHARMACOLOGY OF PIPERAZINONE PEPTIDES

Initially the phenylquinone-induced writhing test[158] was used for assessing analgesia in mice following intracerebroventricular injection. The pharmacological results of some of the "cis amido" piperazinone compounds is shown in Table 8. At first glance it would appear that this series of compounds, especially BE-187 where the remaining peptide chain is absent, are effective antinociceptive analogues of enkephalin. The disclosure that [D-Ala^2]-enkephalin is much more potent that its parent compound appeared to add significance to our results since it seemed possible that the α-methyl group of Ala^2 could mimic the spatial arrangement of the methylene bridge in our BE-series as is shown in Figure 27. However, careful examination of Table 8 reveals that the morphinomimetic activity of this series of compounds is deceptive since it is well known that the minimum chain length required for receptor interaction is the tetrapeptide Tyr-Gly-Gly-Phe. Nevertheless, BE-196 (L)-Tyr-(D)-Ala-NH_2·HBr) elicits a significant degree of analgesic response but elongation of the peptide chain in the cyclic analogues lowers the effectiveness.

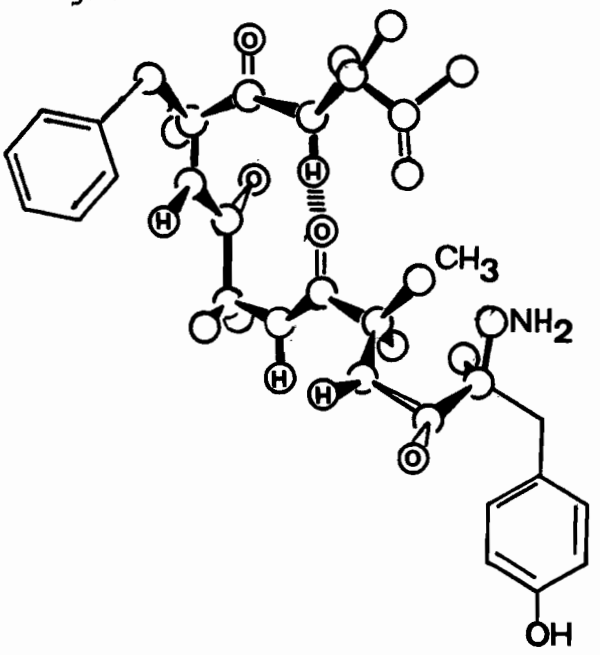
It was decided to test the affinity of these compounds for

Table 8. ED_{50} values of cis-amido piperazinone peptides assessed by the phenylquinone writhing test ICV mice. Values in parenthesis represent 95% confidence limits. Configuration at C_3 is (L).



Compound	R	R'	ED_{50} (95% c.l.) µg/mouse - ICV
BE-185	OEt	H	49(19-269)
BE-186	OH	H	>50
BE-187	NH_2	H	33(24-48)
BE-188	HNGly-Phe-Leu OH	H	∿100
BE-190	HNGly-Phe-Nle OH	H	∿ 41
BE-191	CH_3	$CH_2CH{=}CH_2$	∿100
BE-194	HNGly-Phe-Pro OH	H	∿100
BE-195	HNPhe-NH_2·HBr	H	∿100
BE-196	(L)-Tyr-(D)-AlaNH$_2$·HBr	H	55
βE-197	NH_2	CH_3	61
β-ENDORPHIN			∿0.009 (.005-.011)
MORPHINE-SO_4			0.1
[Met^5]-ENKEPHALIN			∿1000

FIGURE 27. The partial structure of [D-Ala^2]-enkephalin showing the 5 → 2 hydrogen bond and βI-bend. The αCH$_3$ of Ala^2 is in a spatial disposition analogous to the methyl bridge of the "cis-amido" piperazinone rings.



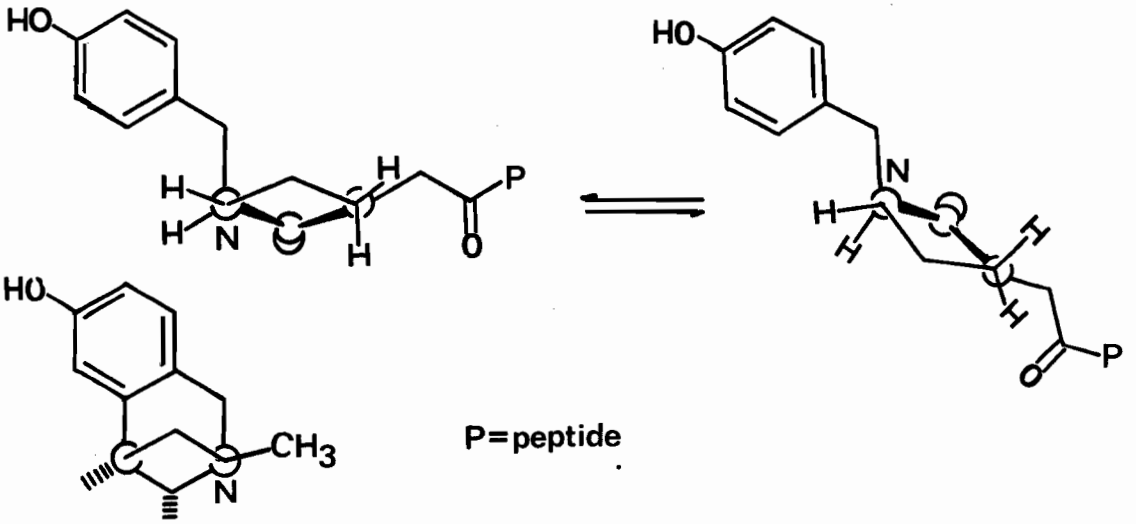
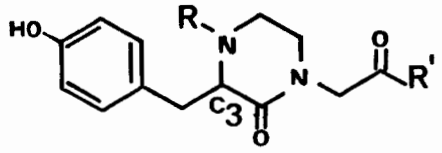


FIGURE 28. The possible conformation of the p-hydroxybenzyl group in the "trans-amido"piperazinone rings. The tyramine moiety may be made to coincide with that same fragment in the representative benzomorphan.

the opiate receptor by evaluating their ability to displace [^{3}H]-naloxone from rat brain homogenate. While [Met^5]-enkephalin had an IC_{50} of approximately 10^{-8} M in this assay, none of the test compounds in Table 8 displaced naloxone at concentrations up to 4×10^{-5} M. In view of the divergence between receptor affinity and the in vivo results, analgesia was measured using the hot plate test:[159] following ICV administration, analgesic activity was assessed by measuring the latency of pain response to thermal stimulation. Again none of the compounds in Table 8 evoked analgesic activity in this test even at ten times the dose applied for [Met^5]-enkephalin. Similarly, the "trans amido piperazinone" compounds JO-14 and JO-16 (Table 9) were also devoid of in vivo activity.

Some important conclusions may be drawn when one attempts to correlate the lack of analgesic activity with the structures of piperazinones BE-185, JO-14 and their respective peptide analogues. It may be seen that when the configuration about the α-carbonyl at C_3 is (L) as depicted in Figure 28 for the "trans-amido" piperazinone JO-14, the p-hydroxybenzyl group and indeed the entire tyramine fragment of the cyclic structures may be made to overlap cleanly on that same fragment of the representative benzomorphan. Therefore the lack of analgesic activity of these cyclic piperazinone peptides is

Table 9. Analgesic activity (ICV mice) and receptor affinity of some "trans amido" piperazinone peptides.



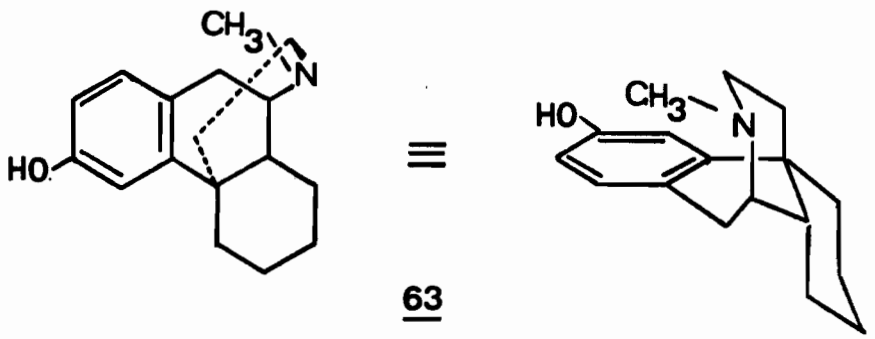
TEST COMPOUND[d]	R	R'	2-MINUTE INTERVALS # OF MICE SHOWING ANALGESIA/# TESTED						OPIATE RECEPTOR
			2	4	6	8	10	15	
[a]JO-14	H	OC_2H_5	INACTIVE						<0.1
[b]JO-20	H	OC_2H_5	INACTIVE						<0.1
[a]JO-16	H	-Gly-Phe-Nle-OH	INACTIVE						0.3
[b]JO-23	H	-Gly-Phe-Nle-OH	6/6	6/6	6/6	4/6	4/6	1/6	
[b]JO-25	H	-Gly-Phe-PrO-OH	INACTIVE						
[b]JO-33	H	-Gly-Phe-Leucinol	2/6	1/6	0/6	0/6	0/6	0/6	
[b]JO-35	H	-Gly-Phe-(D)Nle-OH	1/6	1/6	0/6	0/6	0/6	0/6	
[b]JO-36	H	-Gly-Phe-Methioninol	1/6	1/6	0/6	0/6	0/6	0/6	
[b]JO-37	H	-Gly-(CH_3)Phe-Nle-OH	INACTIVE						
[b]JO-39	CH_3	-Gly-Phe-Nle-OH	2/6	5/6	2/6	2/6	2/6	0/6[c]	
[b]JO-40	ALLYL	-Gly-Phe-Nle-OH	NOT TESTED						
[b][Met^5]-ENK			4/6	1/6	0/6	0/6	0/6	0/6	100

[a]Configuration at C_3 is (L); [b]Configuration at C_3 is (D); [c]Tremors immobility; [d]dose is 50 µg/mouse.

at variance with the hypothesis of Horn and Rodgers[160] who suggested that the morphinomimetic properties of [Met^5] and [Leu^5]-enkephalin is easily understandable since the tyramine moiety of the terminal tyrosine would adopt a conformation at the binding side that is identical to that of the analogous fragment in opiates. Although the tyramine fragment of tyrosine presents itself as an indispensable feature as SAR studies indicate, the results obtained here do not support the conclusion of a common tyramine conformation in agreement with the X-ray results for [Leu^5]-enkephalin.[112] We conclude that the participation of all the amino acid residues is required in order for the backbone interactions to induce a proper alignment of the recognitive binding elements. The bulkiness of the piperazinone ring cannot serve to explain the lack of receptor affinity because a host of amino acids with bulky side chains including methionine and norleucine can be substituted for the second amino acid glycine in enkephalins. In retrospect the opiate receptor demonstrates absolute chiral specificity toward the D-isomers of such analogues of the endogenous effectors and this fact may imply that the second amino acid serves as a point of attachmnet to the binding site. Clearly the second D-amino acid must be unrestricted in space since the conformational restraints as imposed in the "cis amido piperazinones" disorients a critical binding element to the extent that these analogues completely lack activity.

On the other hand, the Gly^2 residue of the "trans amido" piperazinone peptides project away from the heterocyclic ring and consequently this region retains considerable flexibility. Space filling models of JO-16, [L-trans amido piperazinone]-Gly-Phe-Nle-OH, reveal that the $5 \rightarrow 2$ hydrogen bond may still be formed, such that there would be no destruction of the overall conformational integrity of the molecule; therefore the absence of antinociception may be directly related to the disorientation of the tyrosyl residue (or the tyramine fragment) and its immobilization as caused by the $N^1 \rightarrow N^4$ ethylene bridge.

It is interesting to note that the absolute configuration of the α-carbon of the tyrosyl residue in enkephalin is opposite to that of morphine and its congeners. In fact, morphine may be regarded as being derived from D-tyrosine instead of the natural isomer.[161,162] In the configuration shown in Figure 28 (with C_3 being L) the piperazinone rings of the "cis amido" and "trans amido" structures resemble the piperidine rings of inactive enantiomorphs of rigid narcotic opioids such as dextrorphan (63) where ring D projects into



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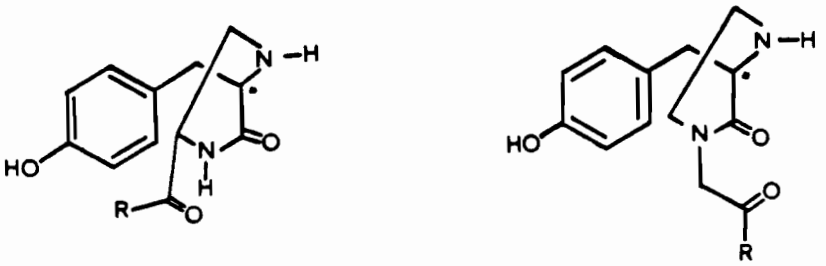
the plane of the paper. It is well known that the opiate receptor displays absolute chiral specificity and fails to bind the d-isomers of rigid agonists such as levorphanol, morphine and antagonists including d(+)-naloxone.[163] It was thought that having made the basic nitrogen of tyrosine part of a ring, the stereochemical preference of the receptor could have been altered. Accordingly, in order to superimpose piperazinone rings of the "cis amido" and "trans amido" structures onto the piperidine ring of levorphanol (while retaining the p-hydroxybenzyl part as a constant element) the configuration about the α-carbonyl at C_3 was inverted to (D) to give the corresponding structures shown in Figure 29. At first, the Norleucine[5] analogues of these stereochemically inverted conformers were synthesized (R'=Nle). Although the Nle[5] analogue of A (Figure 29) with a (D)C_3 was devoid of analgesic activity in the mouse hot plate test, and lacked receptor affinity in the naloxone displacement assay, the corresponding analogue of (B) in Figure 29 displayed significant morphinomimetic properties (JO-23, Table 9).

If the planar aromatic ring is used as a plane of reference, then it may be seen that the basic nitrogen of B (Figure 29) retains the axial relationship which characterizes the relative orientation of that substituent in levorphanol. In contrast, the configuration about the α-C of the tyrosyl



A) cis-amido piperazine

B) trans-amido piperazine

*configuration is D

R= $-HNCH_2CONHCH(CH_2Ph)COR'$ R'= Nle

FIGURE 29. The structures of "cis amido" A and "trans amido" B piperazone peptides with an inverted D configuration at C_3. The piperazinone ring is now superimposable upon the piperidine counterpart in rigid opiates. The tyramine fragment remains constant.

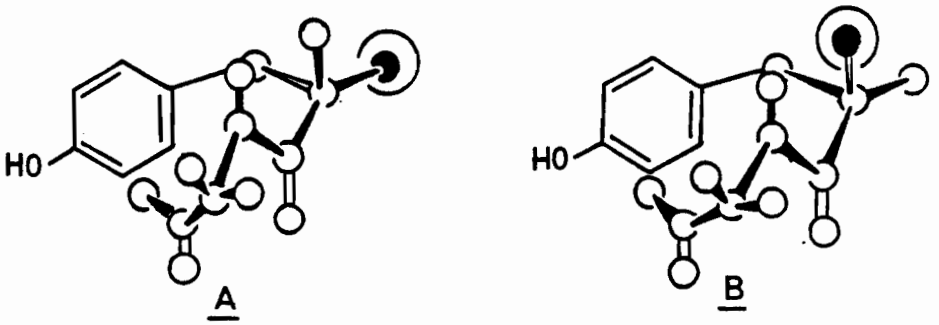


FIGURE 30. Partial structure of Met-enkephalin showing how the amino group acquires an equatorial orientation relative to the phenolic ring. The amino group may gain access to the axial position by inverting the configuration of the C_α of tyrosine. This analogue is inactive.

residue in the endogenous substrate [Met^5]-enkephalin is L; if the same frame of reference is applied, then the basic nitrogen cannot assume a similar axial spatial disposition. Instead, it adopts an equatorial one relative to the aromatic plane (Figure 30-a).

The oddity with JO-23 is that it does not displace bound [^{3}H]-naloxone from rat brain homogenate (Figure 31) nor does it inhibit the electrically-mediated contractions of the guinea pig ileum; in fact, JO-16 the C_3-L isomer of JO-23 is the product that displays some receptor affinity (Figure 31) albeit at very high concentrations ($IC_{50} \sim 5 \times 10^{-6}$). Nevertheless, the antinociception associated with JO-23 is reversed by naloxone and is stereospecific, a basic criterion generally invoked in order to sort out receptor events from spurious non-specific interactions. The onset of analgesia is two minutes, reaches a maximum at approximately four minutes and gradually dissipates after a fifteen minute period. This behaviour is strongly reminiscent of that of [Met^5]-enkephalin which is inactivated by proteolytic enzymes at the level of the Tyr^1-Gly^2 bond. However, unlike enkephalin JO-23 cannot be degraded through initial attack at the N-terminal but it would be expected to be vulnerable to carboxypeptidases and endopeptidases. Substituting D-Leu for L-Leu in [Leu^5]-enkephalin affords an analogue that is slightly more potent than the parent compound presumably because the isomeric

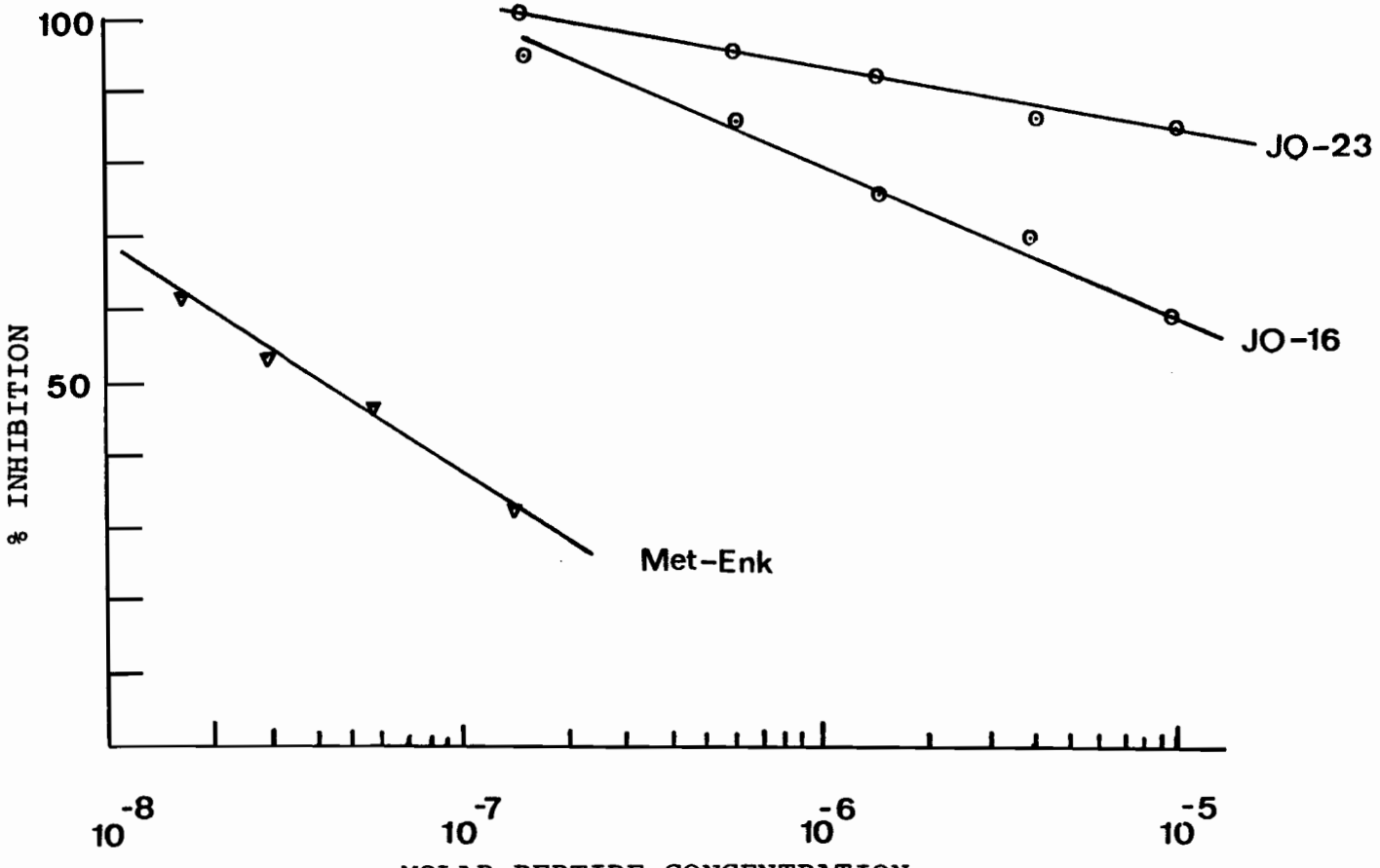


FIGURE 31. Displacement of [^{3}H]-naloxone by JO-16, JO-23, and [Met^5]-enkephalin.

peptide is less susceptible to proteolysis at the carboxyl terminal, yet it can be accommodated at the receptor level. When D-Nle was incorporated as the terminal residue in JO-23 to give JO-35 (Table 9) the compound lost its analgesic effectiveness. Similarly, reduction of the carboxylic acid to give the analogue [D-trans amido piperazinone]-Gly-Phe-Leucinol (JO-33, Table 9) again caused a drop in analgesic activity. The same occurred when methioninol (JO-36) or a proline (JO-25) residue was incorporated in the terminal position of the peptide JO-23. Also, as was mentioned earlier, one of the most potent enkephalin analogues incorporates an N-(CH_3)Phe^4 residue. When this same substitution was inserted into JO-23 to give JO-37, a complete absence of activity was the result.

The question arises at this point as to what is the significance of the dose-dependent analgesia evoked by JO-23 which at the same time displays no affinity for opiate receptors as judged from the results of <u>in vitro</u> [^{3}H]-naloxone displacement. It is not unreasonable to assume that such isolated receptor preparations may not be representative of the biologically intact receptor lattice. This view is supported by the finding that both hexapeptides in Figure 17 display pronounced affinity for opiate receptors <u>in vitro</u> but (A) is moderately active and (B) is devoid of analgesic activity in rodents.

Since the pattern of activity <u>vs</u> substitution for JO-23

does not correlate with analogous substitutions in the endogenous effectors, it must be concluded that this compound is eliciting analgesia indirectly; probably mediated through interactions with some other receptor. Several authors[28,29] have advanced the notion that what the opiate receptor offers to effectors is a network of chemically heterogeneous binding sites for which different ligands have varying affinities. The absence of a relationship between binding assays and isolated organ assays illustrates this point when a comparison is made between the binding affinities and pharmacological properties of enkephalin analogues. For example, [$N(CH_3)$-Tyr,Metamide^5]-enkephalin and [D-Ala^2]-enkephalin show drastic differences in their spectrum of <u>in vitro</u> activities (Table 10). Both compounds display considerable resistance to degradative enzymes so that differences in activities cannot be attributed to enzymatic susceptibility. Therefore the analgesic activity associated with these two compounds may not be exclusively related to the interaction with those binding sites defined by naloxone.[89] The antinociceptive activity which is exclusive to JO-23 may also imply that the molecule interacts with a binding site which is unrelated to naloxone binding. This suspicion was corroborated by the observation that very high concentrations of naloxone are required to reverse the effects of JO-23. Therefore the evidence presented here lends additional support to the notion that the

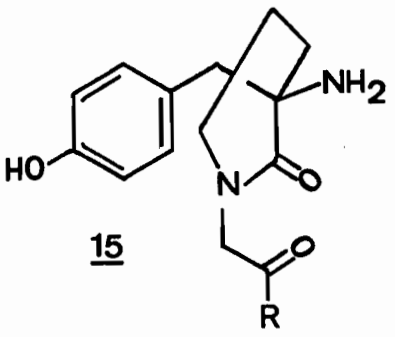
Table 10. Inhibitory potencies of enkephalin analogues on the contractions of guinea pig ileum, mouse vas deferens and on binding of ^{3}H-naloxone and ^{3}H-Leu-enkephalin by brain homogenates, from ref. 89.

Compound	guinea pig ileum	mouse vas deferens	^{3}H-naloxone	^{3}H-Leu-enkephalin
Met-enkephalin	1	1	1	1
[N(CH_3)-Tyr,Metamide^5]-ENK	3.72±0.96	0.33±0.04	1.20±0.10	0.08±0.02
[D-Ala^2,Met^5]-ENK	6.01±0.31	5.60±0.62	0.54±0.13	1.67±0.54

opiate receptors are heterogeneous and exist in multiple forms possessing different affinities for different agonists.

The lack of opiate receptor affinity of the D and L "trans amido" and "cis amido" piperazinones have several implications regarding the mode of interaction of enkephalin. Firstly it would appear that the endogenous pentapeptides and their synthetic analogues do not have a predetermined structural identity that is uniquely complementary to a single binding site. Instead, the picture that emerges is one where enkephalin would bind through an induced fit mechanism that would be governed by the conformational flexibility of the Tyr^1-Gly^2 region. The inactivity of the C_3-L piperazinone peptide analogues where the tyramine parts are at least superimposable on the relevant parts of rigid opiates, as well as the inactivity of C_3-D counterparts where both the A and D rings of morphine are spatially mimicked suggest that the mode of binding of enkephalin is different and likely not related to morphine binding. This conclusion is supported by the fact that whereas the N-allyl group of nalorphine confers antagonistic activity, N-allylation of enkephalins does not do so significantly.[172] Clearly the positioning of the basic nitrogen relative to the phenolic ring emerges as a critical parameter for interaction with the opiate receptor. The results of these studies suggest that in peptide analgesics, the basic nitrogen should be equatorial to the plane of the phenolic group. In order to

test this hypothesis, the synthesis of compound (15) would be a worthwhile undertaking. A workable synthetic approach to



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R = Gly-Phe-LeuNH$_2$

this molecule is outlined in scheme VI. Like enkephalin the structure is characterized by a primary amino group. The p-hydroxybenzyl group and the amine moiety lie in approximately the same plane and this arrangement reproduces what may be a key geometrical feature controlling the binding of the natural analogues.

B. PHARMACOLOGICAL RELEVANCE OF THE N-LONE PAIR ORIENTATION EFFECT

The x-ray structures of the 16α and 16β epimers of 16,17 butanomorphinan (50) are shown in Figure 32. It can be observed that the 16α conformation (50-a) rigidly locks the piperidine ring D into a boat conformation. The chair form is rigorously prohibited as one would have to dismantle the phenyl ring in order to produce it. Accordingly the N-lone pair projects toward the aromatic ring as is the case for the ring D normorphinan (48). In sharp contrast to (50-a) the 16β configuration rigidly locks the piperidine ring in the thermodynamically more stable chair form and fixes the N-lone pair in an orientation exactly opposite to that of the 16α epimer. As was stated earlier, the new 16β epimer displayed analgesic activity comparable to pentazocine [ED_{50} 3±.5 mg/kg ICV in mice). Of considerable importance was the observation that neither epimer displayed any significant binding affinity in the in vitro [^{3}H-naloxone displacement assay (Table 11), a result paralleling that obtained with the peptide JO-23.

This observation reinforces the serious doubts expressed above regarding the actual relevance of displacement activity to in vivo antinociceptive activity. The 16β-butanomorphinan and the peptide JO-23 clearly represent anomalies deserving

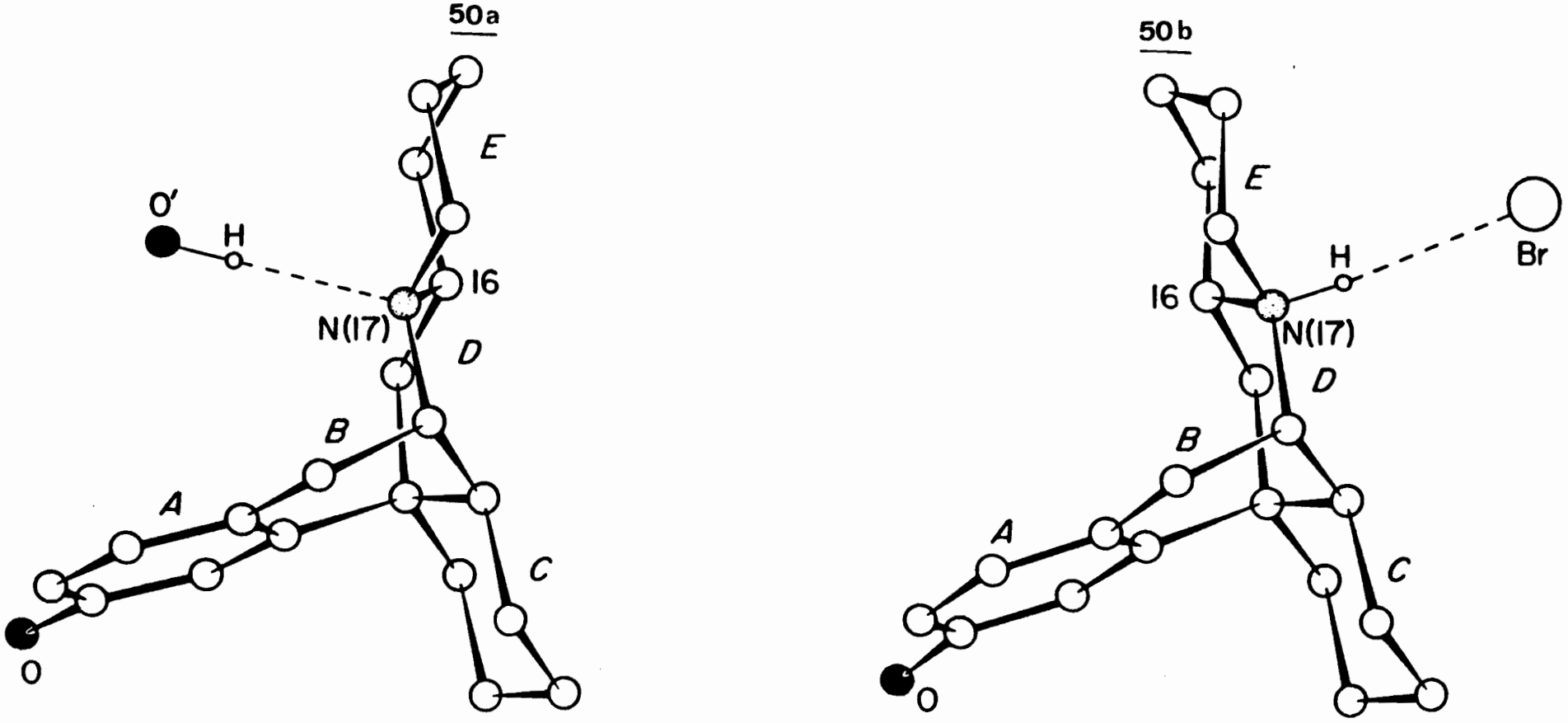


FIGURE 32. The conformations of 16α-50a and 16β-50b butanomorphinan as determined by single crystal x-ray analysis.

Table 11. Displacement of ^{3}H-naloxone from rat brain homogenate relative to Levorphanol = 100%.

Compound	IC_{50}(M)	Relative Affinity Levorphanol = 100%
Naloxone	3.4×10^{-9}	53
16α-butanomorphinan	3.3×10^{-6}	0.05
16β-butanomorphinan	1.2×10^{-6}	0.15

serious attention because their pharmacological properties require explanations if one is to assume the existence of a single well-defined form of the opiate receptor. The [^{3}H]-naloxone displacement assay provides a measure of the relative affinities of narcotic agonists whose binding sites would be shared by the pure antagonist, naloxone. Until now all synthetic opiates that possess this property also induce physical dependence capacity or addiction liability and abuse potential. Those opiates that are clinically desirable owe their success to the dual agonist/antagonist properties that they display; the agonist acitivty reducing the side effects of agonism and minimizing physical dependence and addiction. One may speculate that analgesia without the associated physical dependence may

be associated with the interaction on a different more specific binding site, perhaps physically remote from the naloxone binding site. It must be remembered that naloxone itself is derived from the potent agonist oxymorphone which is not excluded from the problems associated with classical analgesics.

Belleau and Di Maio[49] recently introduced a model for the opiate receptor which calls for the existence of a metagonist state (denoting a state beyond classical agonism). Narcotic analgesic agonists that have abuse potential do not qualify as metagonists but appear to fulfil the basic criteria for potential agonists whose properties are readily accounted for by the classical two-state model of drug receptor interactions. The fundamental concept that emerges from these considerations is that the configuration of the opiate receptor can be stereoelectronically de-controlled (generally perturbed) by agonists to induce analgesia together with narcotic effects (as is the case for morphine) but can be altered by either "pure" antagonists which would stabilize only the closed state (as with naloxone) or metagonists which would stabilize by way of a specific change such a new ordered state that analgesia would be evoked to the exclusion of those effects normally associated with agonist-induced de-controlled states. In other words, the opiate receptor, acting as an ordered molecular cluster, may not only allow a single lattice transition from

an ordered to a relatively disordered state, but would also allow for the existence of thermodynamically-stable allomorphic states as is depicted in Figure 33. Related to this model is the Hypothesis of Martin et al.[28] who identified three distinct syndromes produced by congeners of morphine and postulated the existence of at least three chemically distinct types of opiate receptors: for classical morphine-type drugs (μ-receptor), for certain benzomorphans such as ketocyclizocine which do not substitute for morphine in addicted animals (K-receptor), and for cyclizocine (σ-receptor).

In order to demonstrate these varied responses of the opiate receptors to different durgs, we may turn to the cellular model of Klee and Nirenberg[4] who demonstrated that hybrid glioma-neuroblastoma nerve cells possess well developed opiate receptors which respond to morphine by initially shutting down 3',5'-cyclic AMP production as a result of Adenyl Cyclase inhibition through the breakdown of regulatory links with the receptor. Continued exposure of the cells to morphine leads to compensatory adaptation (tolerance) which manifests itself through a gradual increase in Adenyl Cyclase biosynthesis and restoration of normal cyclic AMP levels. Treatment of such cells with naloxone causes a sudden massive production of cAMP as a result of the de-repression of the Adenyl Cyclase levels. With time, the agonist-stabilized closed state of the receptor

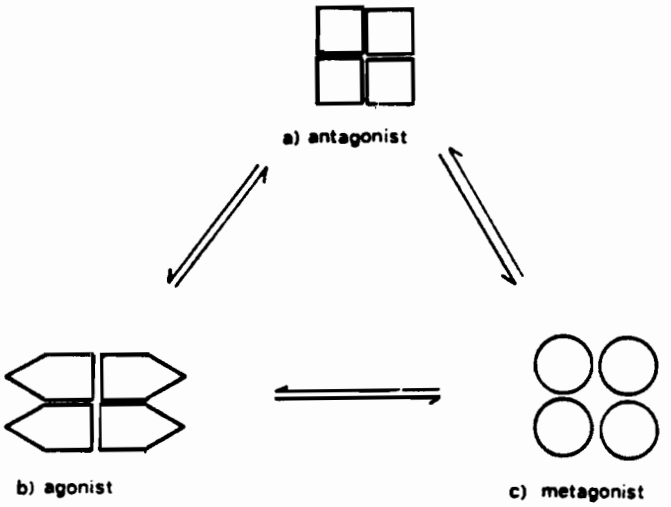


FIGURE 33. Hypothetical conformational states of the opiate receptor. (a) closed state (antagonist) (b) open state (agonist) (c) allotropic state (metagonists).

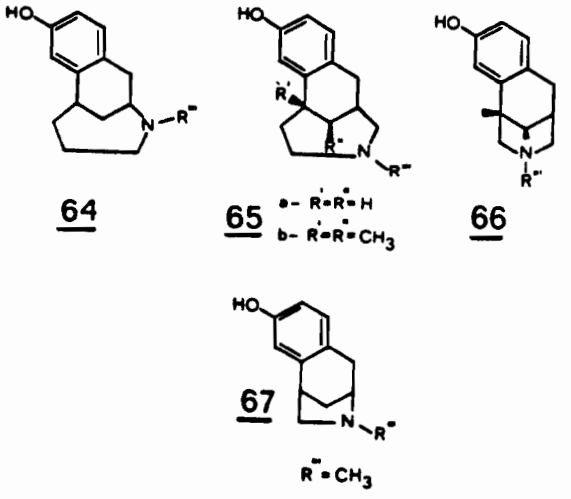
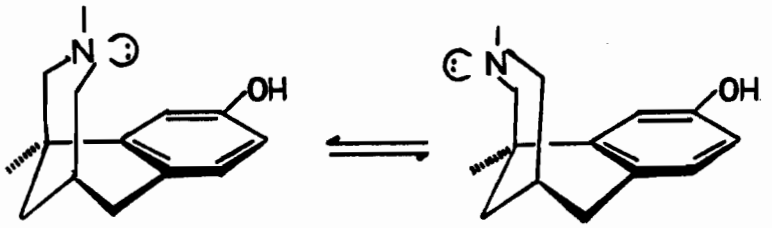


FIGURE 34. N-positional isomers of synthetic benzomorphans, from ref. 164. C-norbenzomorphan is devoid of analgesic activity.

eventually leads to normalization of the cell biochemistry. Therefore this system constitutes a valuable cellular model for addiction and the withdrawal syndrome, and presents itself as a viable tool for evaluating the narcotic effects of other agonists including the metagonist butorphanol and perhaps the 16β-butanomorphinan.

Recently, Shiotani et al.[162] reported the synthesis of C-norbenzomorphan (67, Figure 34) 8-hydroxy-3-methyl-2,3,4,5-tetrahydro-1,4-methano-1,H-benzazepine. Pharmacological evaluation by the method of pressure stimulus on the mouse tail revealed that it possesses negligible activity (ED_{50} 43 mg/Kg or 3% that of morphine). Since this molecule is structurally and stereoelectronically superimposable on D-normorphinan (48), it may be inferred that the N-lone pair phenomenon is operative in benzomorphans as well.[142] These same authors have reported the x-ray crystallographic structures of N-positional isomers of homobenzomorphans (59-a,b) and benzomorphan (60).[163] Previous reports showed that (65-a) and (65-b) are as potent as morphine but (64) has only 25% of its activity whereas (66) is as potent as codeine (10% of morphine). The crystallographic data disclosed that the N-lone pairs of (65-a,b) project outward and away from the aromatic ring, thus accounting for their potent analgesic activity; the corresponding N-electron pairs of (64) and (66) are clearly misaligned. Drieding molecular models reveal that (64) and (65-a,b) are

structurally flexible molecules, and although (64) clearly has its N-lone pair disoriented in the crystal lattice, it may yet undergo umbrella inversion at the receptor level or in solution. For instance, in what may appear to be the most serious case, the N-positional benzomorphan isomer (66) (with codeine-like activity) can assume a conformation in which the piperidine ring is in the boat form (66-b, Figure 40), the free energy demand being no greater than 1.5 to 2 Kcal/ mole. Conceivably the weak activity of (66) may be a reflection of the existence of the active conformer (66-b) at the receptor level.



66b

1. A receptor model.

The N-lone pair effect may serve to account neatly for otherwise anomalous behaviours of other classes of analgesics. For example, in the phenyl piperidine family of opioids, both the equatorial (68-a) and the axial (68-b) phenyl piperidines (Figure 35) are approximately 20 times more potent than morphine. Portoghere and Fries[164] advanced a receptor model where the phenyl rings of (68-a, 68-b) are superimposable. The result of this exercise is that the C_3-C_4-C_5 centers would involve non-identical receptor hemispheres. If we now assume that the complementary binding site for the basic nitrogen is placed in juxtaposition with the differently-depicted amine functions (69), then one can clearly see that the N-lone pair of both structures is critically oriented for facilitated proton transfer and productive interaction with the receptor. Consistent with this view is the report that the azobicycloalkane (70) is six to eight times more potent than pethidine.[165,166]

A special point of interest concerning the 16β-butano morphinan (52-b) is the inactivity of this epimer as an antagonist. It was stated earlier that as a general rule, the presence of short N-alkyl chains invariably confers antagonism to morphinans. In the 16β-epimer such a chain is present but is folded back onto carbon-16 of ring D. It may be inferred on that basis that morphine antagonism is governed

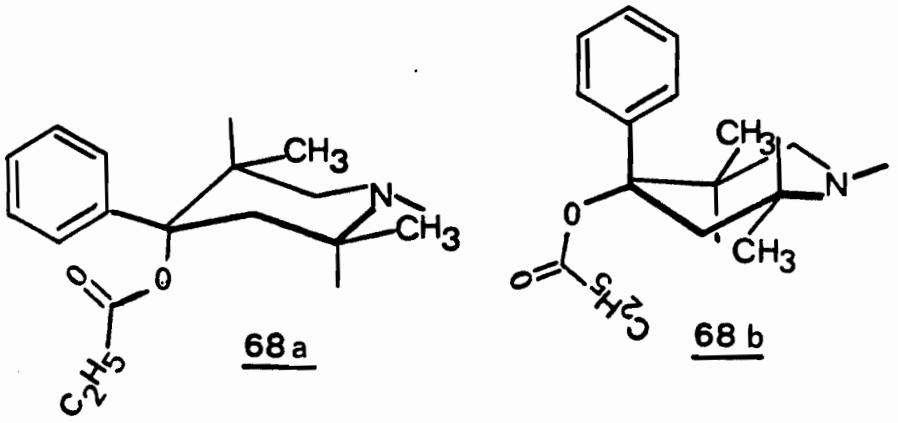


FIGURE 35. Structures of equatorial 68a and axial 68b 4-phenyl piperidines. Both compounds interact equally with opiate receptors.

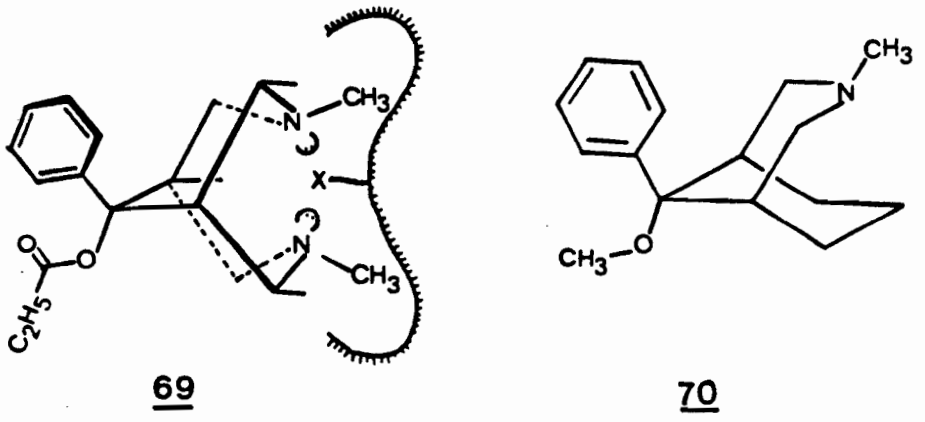
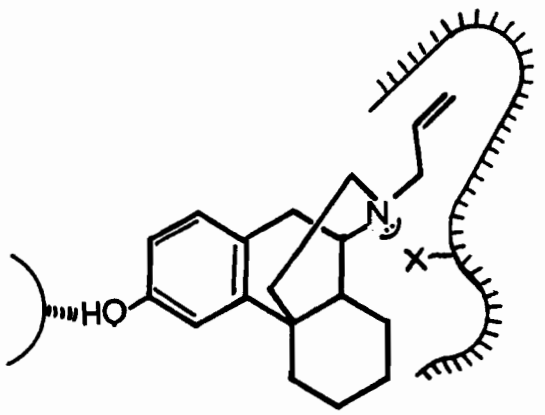


FIGURE 36. A model of the opiate receptor showing the superimposed phenyl rings of 68a and 68b. The nitrogen atoms are located in different receptor hemispheres with the N-lone pairs in the favoured orientation.

by a receptor perturbation generated by the interaction of the N-substituent as long as it projects away from the piperidine ring. It was stated previously that there exists a divergence in activity with N-allyl substituted benzomorphans (axial phenyl at C_4) and phenyl morphans (equatorial phenyl at C_4). If the aromatic ring of the two related structures is made to occupy a common binding locus, then the nitrogen atom and its N-substituent would be located in different receptor hemispheres. In the prototype narcotic



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antagonist levallorphan (71) the N-allyl group may be accommodated in a lipophilic receptor pocket causing receptor perturbation as to preclude agonism even though the N-lone pair is favourably aligned for facilitated proton transfer.

Recently Zimmerman et al.[167] have reported that extremely potent antagonist activity is associated with 4-phenyl piperidines provided that there is a substituent at the C_3 position.

When the N-substituent is $CH_2CH_2C{=}OPh$, the compound acquires antagonistic activity at the level of naloxone (AD_{50} 0.022 mg/Kg rats). It is interesting to note that antagonism is exclusive to the 3β-methyl compounds where the 3-alkyl group is trans to the C_4 methyl substituent and not when it is α as in the cis-3-methyl-4-methyl analogue. This structural specificity may imply that the 4-phenyl ring may be forced into an axial orientation relative to the piperidine ring so that the nitrogen atom and its N-substituent project into the same receptor region as levallorphan.

That the N-lone pair orientation must be an essential component for analgesic activity becomes obvious as judged from the data presented in Table 12 for the N-methylated analogues of [Leu-OCH_3^5]-enkephalin. The receptor binding data in Table 12, shown graphically in Figure 37-a, clearly reveals that whereas the desmethyl and N-methyl compounds are for all practical purposes equipotent, the N,N-dimethyl and the quaternary methiodide salt have 4% and 0.6% affinity respectively. This trend is closely paralleled by the results of the guinea pig ileum assay (Figure 37-b), except for the N-monomethyl analogue which is approximately twice as active as the parent desmethyl compound. If simple electrostatic forces were to govern the interaction of the nitrogen group with a complementary anionic receptor site, then the

Table 12. Relative potencies of methylated [Leu-OCH_3^5]-enkephalin analogues

R-Tyr-Gly-Gly-Phe-Leu-OCH_3 [a]

Compound	R	# MICE SHOWING ANALGESIA[b] # MICE TESTED 2	4	6	8	10	GPI	Opiate Receptor
1	NH_2	0/6	1/6	0/6	0/6	0/6	100	100
2	H-N-CH_3 [c]	1/6	3/6	0/6	0/6	0/6	218±86	107±23
3	$N(CH_3)_2$ [c]	1/6	4/6	3/6	3/6	1/6	8.1±3.2	4.0±0.7
4	$^{+}N(CH_3)_3{}^{-}I$ [c]	INACTIVE					0.2±0.1	0.6±0.3

[a] acetate salts, (1 = 100%); [b] 50 µg/mouse ICV dose; [c] convulsions.

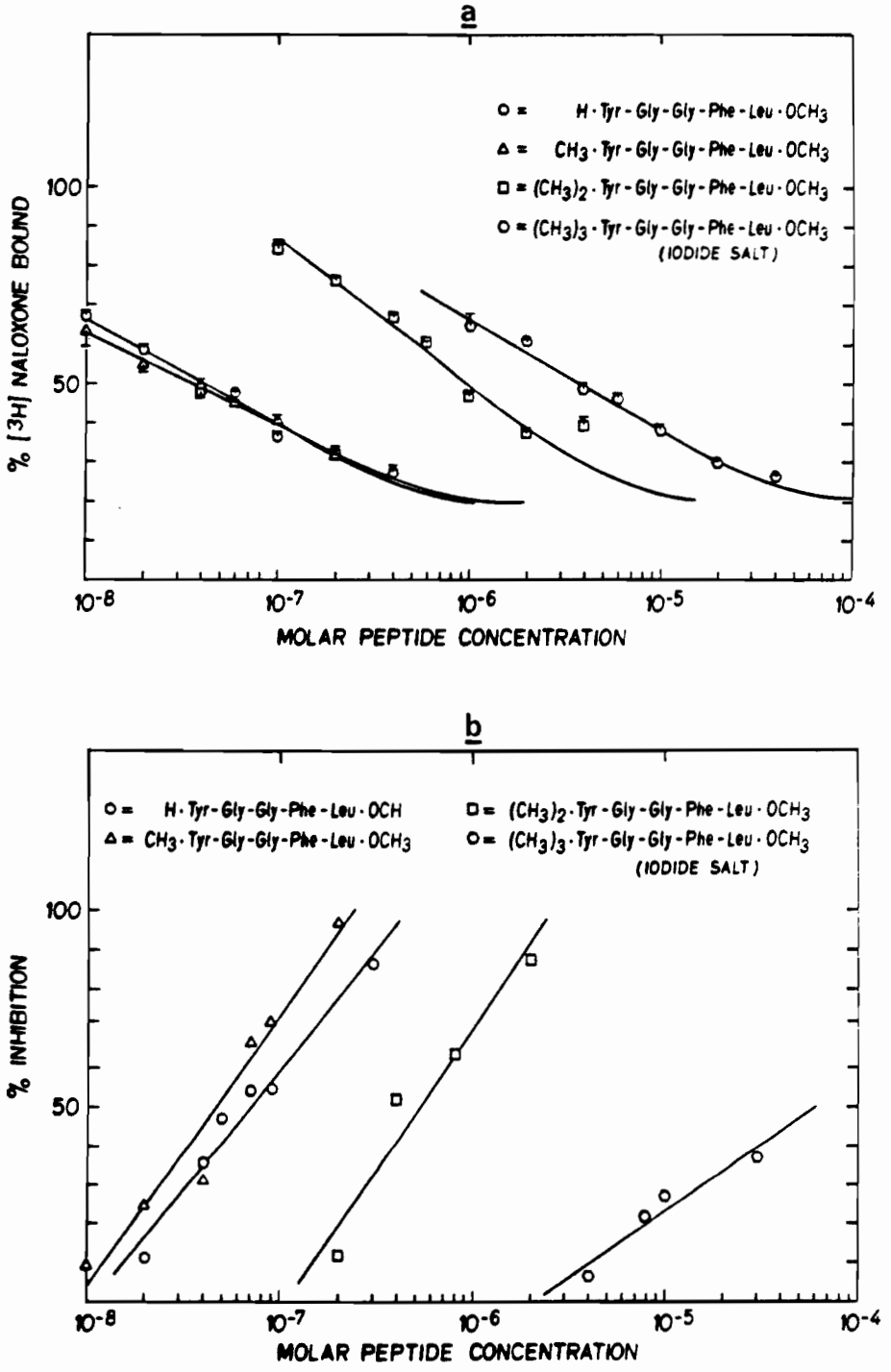
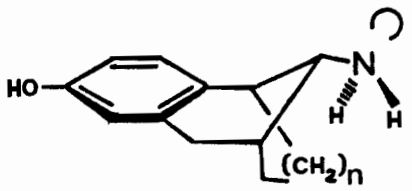


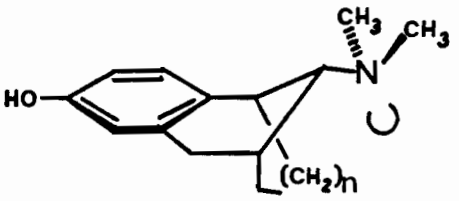
FIGURE 37. The inhibition of [^{3}H]-naloxone from rat brain homogenate, a, and the contractions of the guinea pig ileum, b, by N-methylated analogues of [Leu-OCH_3^5]-enkephalin.

activity would not be expected to be so drastically reduced when going from a mono N-methyl to an N,N-dimethyl derivative.

This situation may very likely apply to a new class of narcotic analgesics that comprises the representative prototype (72-a) of the tricyclic amino tetralins where the primary amino function is rigidly held equatorially to ring B.[168,169]



72 a



72 b

Similar to enkephalin, these novel analgesics are active as primary amines unlike most narcotic analgesics which are tertiary alkyl bases. These compounds are equipotent with morphine and are parenterally active in sharp contrast to normorphine or its surrogates which tend to be inactive parenterally when the nitrogen is secondary.[145] Space filling models clearly show that the hydrocarbon bridge (n=5) envelopes the amino group, thus effectively preventing normal solvation. This "greasy" envelope may account for the ability of these compounds to permeate the blood membrane barrier and gain access to opiate receptors. Furthermore, optimum

activity resides in the β unsubstituted nitrogen epimer and N-methylation abolishes analgesic activity. An N-methyl or an N,N-dimethyl group cannot be accommodated in the hydrocarbon envelope without encountering strong repulsive interactions. If the solvent-exposed orientation of the N-lone pair (72a) is critical for activity, then disubstitution forces it into an inverted alignment (72-b) that is detrimental to activity.

The interpretation of the results for the N-methylated [Leu-OCH_3^5]-enkephalins is complicated by the data obtained for in vivo administration. The data (Table 12) reveal an inverse trend to that obtained with isolated tissues. Thus, successive methylations cause progressive increments in potency but only at doses associated with untoward side effects (tremors, convulsions). The quaternary ammonium salt was, however, totally devoid of morphinomimetic activity and this property may be directly attributed to the absence of a protonated N-lone pair which, as we have already shown, appears to be a critical parameter for productive receptor interaction in the rigid opiate series. One could argue that the N,N-dimethyl analogue is more potent because it is resistant to enzymatic degradation; nonetheless, the N-monomethyl analogue should be equally resistant, yet it is significantly more active than the former in vitro and less active in vivo. It would be of considerable interest to synthesize the [D-Ala^2] analogues of these N-methylated compounds because proteolytic attack would be virtually impossible.

The quaternary N-methiodide salts of levorphanol and levallorphan also exhibited diminished receptor affinities (Table 13), about 10% that of the parent levorphanol and levallorphan respectively in both types of in vitro assays* in agreement with earlier reports.[143] When sodium ions were introduced into the opiate receptor binding medium (100 mM), both levorphanol and its methiodide salt displayed further reduced affinity for the receptors. The affinity ratios (without/with Na^+) were 16 and 20 respectively in good agreement

Table 13. Relative affinities of levorphanol, levallorphan, and their methiodide salts for opiate receptors (Levorphanol = 100%).

Compound	Receptor binding assay[a]	guinea pig ileum assay
Levorphanol	100	100
N-Methyl Levorphanol	10.2 ± 3.3	9.3
Levallorphan	91.0 ±24.8	antagonist
N-Methylevallorphan	9.0 ± 2.3	antagonist

[a]Mean of 3 experiments ± SEM

*N-methyl levellorphan (iodide) was not tested on the guinea pig ileum assay.

with reported values.[170] When levorphanol methiodide was administered ICV in mice, no increase in the latency of pain response was observed at 50 times the active dose of morphine. This observation raises some serious doubts again about the pharmacological relevance of the effects of quaternary salt analogues of narcotics to binding. It is interesting to note that the affinity of the 16α-butano morphinan (52-a) was increased ten fold when it was quaternized to the methiodide salt. This effect indeed could be a reflection of the non-specific and non-productive interactions that do not evoke the desired pharmacological response.

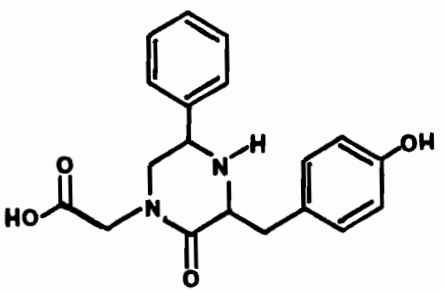
We have shown earlier that analgesia is not intimately associated with interactions with only one receptor on the basis of the results obtained in vivo with the "trans amido" piperazinone JO-23 and the 16β-epimer of butano morphinan (52-b). These results, taken together with those obtained with the N-methyl analogues of [Leu-OCH_3[5]]-enkephalin emphasize the enormous complexity of the opiate receptor network and leads to the conclusion that the use of a single assay system can no longer be considered a reliable method for predicting pharmacological activity of opiate-related compounds.[89]

C. FUTURE RESEARCH

As regards the possibility of further work along the outlines described in this thesis, the following suggestions are offered:

Undoubtedly, the unique pharmacology of the "trans amido" piperazinone peptide (JO-23) where the configuration centered at C_3 is (D) will require further elaboration. The analgesia elicited by this compound may be directly related to receptor events since its activity is highly sensitive to a stereochemical change at position C_3, the JO-16 enantiomer being inactive. In this respect, this compound represents yet another class of CNS agents displaying analgesic properties, although not necessarily related to opiate receptor interaction.

Since the cyclization reaction leading to the 2-oxopiperazines has general applicability, one can readily imagine a host of other analogues of potential interest. For example, amino acetophenone or aminoacetone may be used as starting materials which would serve to introduce substituents at the C_5 position of the heterocyclic ring thus yielding the corresponding 5-phenyl and 5-methyl "trans amido" piperazinones 73 and 74 respectively. The added steric bulk at position 5 may increase the specificity of these molecules toward the relevant



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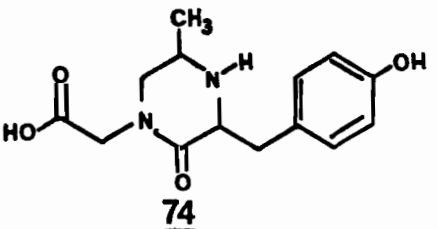

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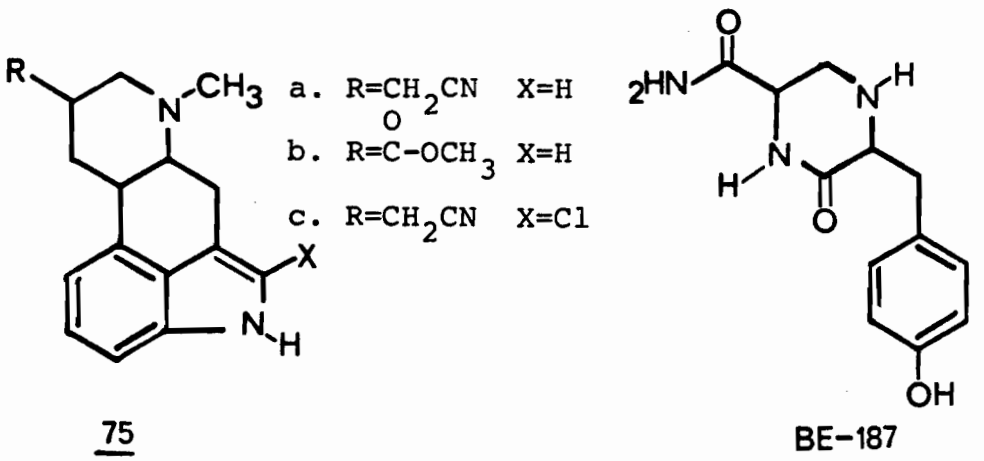
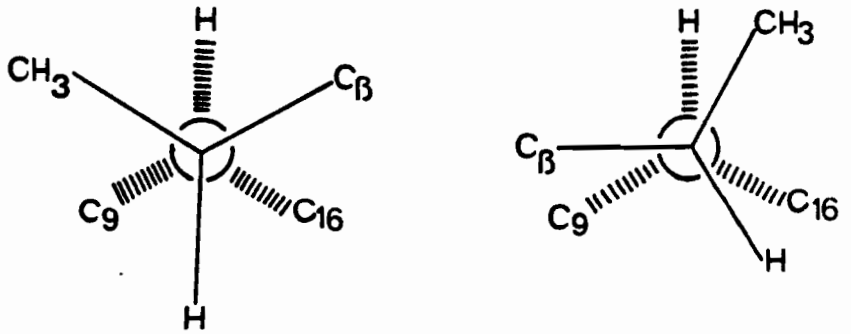


75 BE-187

FIGURE 38. Structural resemblance between the ergot alkaloid 75 and BE-187.



A R isomer B S isomer

FIGURE 39. Schematic representation of chiral N-sec-alkyl substituent on a morphinan skeleton. The quantitative activities of the R isomer and the S isomer may be related to N-alkyl conformational differences.

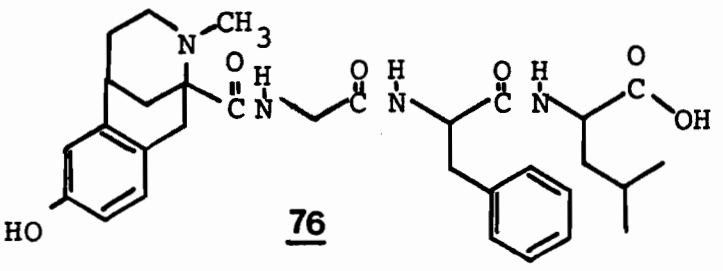
receptor. It may not be unrealistic to assume that the added steric bulk contributed by such substituents could further restrict the ring's flexibility thus making the remaining peptide chain less critical for productive interactions.

In view of the novelty of these piperazinone compounds reported in this thesis, it would be of considerable interest to further explore their chemistry and pharmacology. For instance the 3-(p-hydroxybenzyl)-6-amido-2-oxo-piperazine (BE-187) bears some interesting resemblance to the hallucinogen LSD and other ergoline derivatives such as 75 (Figure 38) which have recently been reported to act as potent inhibitors of pituitary prolactin release.[162] The underlying difference between BE-187 and 75 lies mainly in the nature of the aromatic substituent (phenol vs indole). Nevertheless the described synthesis leading to (BE-187) may well be applied to tryptophan as starting material instead of tyrosine. The function at C_{10} of 75 is responsible for the conformational rigidity of that part of the molecule, and it is felt that this feature may very likely be mimicked by the ring amide bond of BE-187.

The active conformation of enkephalin, especially the relative spatial disposition of the primary amino group and the phenolic ring has remained unresolved. To this end we have proposed the synthesis of compound (15). Like the endogenous effectors, this structure is characterized by a primary amino group. Unlike the above piperazinone compounds, the basic nitrogen of (15) is retained outside the lactam ring.

Space filling models show that the structure permits the possibility of a 5 → 2 hydrogen bond as was proposed on the basis of NMR studies, or a 4 → 1 and 1 → 4 H-bond as deduced from x-ray analysis. Again one may speculate that the unique amino acid sequence of enkephalin serves mainly to favor that conformation of the tyrosine end which is recognized by the receptor. If such a receptor-bound conformational state can be mimicked through structural manipulation of (15) whose structure is rendered inflexible by the $N_1 \rightarrow C_3$ propyl bridge, then the remaining peptide sequence may no longer be a required element.

Repetitious speculations have been offered regarding the possible role of each of the amino acid residues in enkephalin, especially the role of the phenylalanyl unit which is thought to occupy the aromatic binding site of PET on the receptor. For this reason it would be of considerable interest to incorporate the pentapeptide sequence into the benzomorphan skeletal arrangement as shown in structure 76. In this compound, the tyramine part is a common element to both the benzomorphan skeleton and the enkephalin sequence. If the resulting hybrid structure encouvers new binding sites, a significant increase



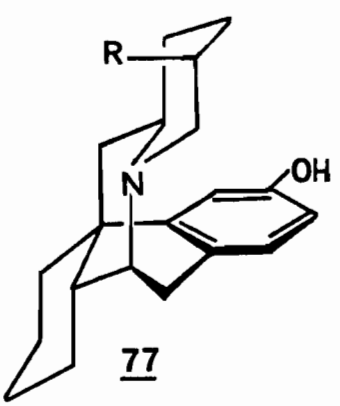
76

in the potency of the parent benzomorphan structure should be observed.

Recently, the N-monomethyl and N,N-dimethyl analogues of [D-Ala^2] have been synthesized and, while the former showed slightly improved activity, the latter was far less potent than the parent desmethyl compound (guinea pig ileum assay) in agreement with our results for the corresponding N-methylated analogues of [Gly^2,Leu-OCH$_3^5$]-enkephalins. Since the presence of the D-Ala^2 residue effectively prevents enzymatic degradation, it would be of interest to evaluate the <u>in vivo</u> activity of these N-methylated [D-Ala^2] analogues including the [$\overset{+}{N}$-(CH$_3$)$_3$-Tyr,D-Ala^2] derivative. It was mentioned above that we found an inverse relationship between the <u>in vitro</u> receptor affinity and <u>in vivo</u> analgesic activity in the N-methylated [Gly^2]-analogues. Since proteolysis may be virtually excluded in the case of [D-Ala^2], any discrepant pharmacological properties would then be attributable only to the effects of methylation.

It was pointed out earlier in the text that the 16β-butano morphinan displays no antagonistic activity despite the presence of an N-alkyl (butyl) substituent, a feature that invariably confers antagonistic properties to structurally-rigid narcotics. This anomaly constitutes valuable information regarding the mechanism underlying the effects of N-substituents. It implies that antagonistic activity is the result of direct receptor perturbation by the specific stereoelectronic features of the

N-alkyl chain since the folded butyl chain of the 16β-butano morphinan is incapable of perturbing the receptor in favor of antagonism. Recently Degraw et al.[171] reported the synthesis and pharmacology of a variety of N-t-butyl and chiral N-Sec-alkyl substituted morphines. Whereas the former derivatives were inactive as analgesics, the latter derivatives exhibited a good balance of agonistic and antagonistic properties. Of particular interest was that the R and S isomers of the N-sec alkyl substituent had quantitatively different properties and the differences were ascribed to different populations of low energy conformers (A) and (B) (Figure 39). Accordingly the higher activity of the S isomer could be rationalized on the basis of different receptor perturbation. It would be of interest then to synthesize and evaluate the effects of substituents at C_{19} of the 16β-butano morphinan on the receptor. The role of such a C_{19} substituent could be unambiguously evaluated due to a well defined orientation (either equatorial or axial) on rigid pentacyclic structures 77 and 78. The dual agonist/antagonist activity of strategically-substituted morphinans and benzomorphans has been suggested as arising from the existence of two types of N-substituent conformations at the receptor level[41] as discussed earlier in the text. The rigid enantiomorphs of 77 and 78 would be valuable tools in studies directed at testing this hypothesis because of the different conformations of the added C_{19} substituent.



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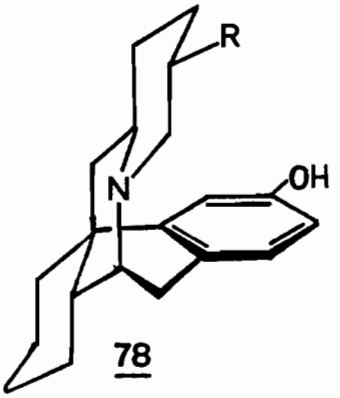

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The low level of receptor affinity of methiodide salts of potent agonists and antagonists and the relevance of this interaction to the opiate receptor remains to be explained. Perhaps further pharmacological screening of diverse quaternary structures could offer a more realistic evaluation of their relevance to opiate receptor chemistry. To this end, it is suggested that bulkier quaternaries by synthesized and tested.

CLAIMS TO ORIGINAL RESEARCH

A number of our main research objectives was attained. It was one of our aims to delineate the conformational features of enkephalin which contribute to its affinity for the opiate receptor. Contrary to expectations and predictions by others, it was shown that the assumed common conformations shared by the tyramine part of enkephalin and rigid opiates is not necessarily the critical ingredient for receptor recognition. Instead, the relative placement of the primary amine and the aromatic phenolic ring as controlled by additional binding elements in the peptide sequence was proposed as a key parameter for enkephalin-like activity.

A new class of analgesics was uncovered whose basic structural features incorporate a piperazinone ring carrying a p-hydroxybenzyl substituent at C_3. This new compound displays unusual pharmacological behaviour in that its pharmacological response is not mediated by the interaction with the same binding site as morphine. In this respect it represents a new drug prototype which may be useful in the study of the structural basis of addiction.

A cyclization reaction leading to a multi-substituted piperazinone ring was devised which appears to be of general applicability. In one case, the presence of a preformed chiral center in a key intermediate allowed the completely stereospecific formation of a second chiral center.

The mono, di, and quaternary methyl analogues of [Leu-OCH_3^5]-enkephalin were synthesized and their *in vivo* analgesic activity shown not to correlate with *in vitro* activity. The quaternary iodide salt of these compounds was shown to be highly toxic and devoid of analgesic properties. This result was explained in terms of the importance of an available N-lone electron pair for productive binding on the receptor. The observation that the N-lone pair of morphinans may be a critical parameter for productive binding was substantiated. New evidence based on crystallographic studies was presented which shows that a known 16,17-butano morphinan analogue is inactive as an analgesic because its N-lone pair has the wrong orientation. Oxidation and reduction of the substance converted it to an active analgesic whose N-lone pair is accessible to a complementary receptor site, thus corroborating the hypothesis that productive binding on opiate receptors depends on the N-lone pair orientation. Accordingly, the nitrogen atom of rigid opiates was implicated in a proton transfer process so that as a porton donor, the ligand would bind in the cationic form, but as a proton acceptor it would acquire at least partial positive charge after binding on the receptor.

Opiate receptor models which incorporate the N-lone pair feature were presented. These receptor models can accommodate the phenyl piperidines and account for the antagonist activity of opioids whose aromatic ring is axial relative to the piperidine ring.

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(b) For purposes of simplification, 3-(p-hydroxybenzyl)-2-oxo-piperazine-1,N-acetic acid and 3-(p-hydroxybenzyl)-2-oxo-6-carboxy-piperazine will be referred to as "trans-amido" and "cis-amido" piperazinones.

The peptide products incorporating these ring systems will be referred to as [D or L-trans amido piperazinone]-peptide and [D or L-cis amido piperazinone]-peptide. The prefixes D or L refer to the configuration at C_3 of the respective rings.

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