THIOCARBONYL ANALOGUES OF AMINO ACIDS AND PEPTIDES: SYNTHESIS AND BIOLOGICAL PROPERTIES

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THIOCARBONYL ANALOGUES OF AMINO ACIDS AND PEPTIDES:
SYNTHESIS AND BIOLOGICAL PROPERTIES

by

Gilles Lajoie

ABSTRACT

New thionation experimental conditions and new reagents for the synthesis of thioamide analogues of protected amino acids and peptides are presented. The interaction of thiocarbonyl analogues of model substrates of α-chymotrypsin and leucine aminopeptidase were also studied. Optically active dithioester derivatives of protected amino acids were prepared and used as thioacylating agents.

The synthesis of four thioamide-containing analogues of the chemotactic tripeptide f-Met-Leu-Phe was accomplished. The conformational properties of these novel analogues were studied by ^{1}H and ^{13}C NMR spectroscopy. Their biological activity was also evaluated *in vitro* and the results interpreted in terms of their molecular properties.

The regioselectivity of the new thionation methodology allowed for the rapid and efficient synthesis of the four possible monothioamide positional isomers of [Leu^{5}]-enkephalin. Their biological activity was studied both *in vitro* and *in vivo*.

Amidoxime and amidrazide analogues of the peptidic bond were also obtained using thioamides as intermediates.

Chemistry Ph.D.

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SYNTHESE ET PROPRIETES BIOLOGIQUES

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RESUME

De nouvelles conditions de réaction et de nouveaux réactifs pour la synthèse d'analogues thioamidés d'acides aminés et de peptides sont décrits. Les interactions enzymatiques d'analogues thiocarbonylés de substrats modèles pour l'α-chymotrypsine et la leucine aminopeptidase furent etudiées. Des dérivés d'acides aminés optiquement actifs contenant une fonction dithioester ont été preparés et utilisés comme agent thioacylants.

La synthèse de quatre analogues du tripeptide chemotactique f-Met-Leu-Phe contenant une ou deux fonctions thioamides fut aussi réalisée. Les proprietés conformationnelles de ces nouveaux composés furent évaluées par spectroscopie RMN du proton et du carbone. Leur activité biologique fut mesurée par des tests appropriés in vitro.

La régiosélectivité de cette nouvelle méthodologie de thionation fut appliquée à la synthèse d'analogues monothioamidé de la [Leu^5]-encéphaline. Leurs propriétés biologiques furent évaluées in vitro et in vivo.

D'autres fonctions analogues au lien peptidique telles les amidoximes et les amidrazides furent preparées à partir des précurseurs thioamidés.

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ABBREVIATIONS

Ac	Acetyl
AcOH	Acetic acid
Ala	Alanine
Ar	Aryl
Boc	t-butyl oxycarbonyl
bp	Boiling point
br	Broad
bu	butyl
c	Concentration
^{13}C NMR	Carbon magnetic resonance
Calcd	Calculated
CI	Chemical ionisation
d	Doublet
DMAP	Dimethylaminopyridine
DCC	N,N'-Dicyclohexylcarbodiimide
DCU	N,N'-Dicyclohexylurea
DME	Dimethoxyethane
DMF	Dimethylformamide
EEDQ	N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
EI	Electron impact
eq	Equivalent in mole
Et	Ethyl
EtOAc	Ethyl acetate

EtOH	Ethanol
$Eu(fod)_3$	Tris(6,6,7,7,88,-heptafluro-2,2-dimethyl-3,5 octanedionato)europium
$Eu(thc)_3$	Tris [(3-trifluromethylhydroxymethylene)-d-camphorato] europium
EV	Electron volt
f	Formyl
FT	Fourier transform
g	Gram
Gly	Glycine
GPI	Guinea pig ileum
HOBT	Hydroxybenzotriazole
^1H NMR	Proton magnetic resonance
HPLC	High performance liquid chromatography
Hz	Hertz
IR	Infra red
Ile	Isoleucine
LAP	Leucine aminopeptidase
Leu	Leucine
m	Multiplet
M	Molar
max	Maximum
Me	Methyl
MeOH	Methanol
Met	Methionine
MHz	Megahertz
MVD	Mouse vas deferens

μmol	Micromole
mp	Melting point
MS	mass spectrum
N-Ac	N-acetyl
NLe	Norleucine
nm	Nanomole
p	Para
Ph	Phenyl
Phe	Phenylalanine
pyr	Pyridine
^{31}P NMR	Phosphorous magnetic resonance
Pro	Proline
q	Quartet
RT	Room temperature
s	Singlet
sh	Shoulder
t	Triplet
TEA	Triethylanine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
Tyr	Tyrosine
Troc	N-trichloroethyloxycarbonyl
Val	Valine

INTRODUCTION

Natural products are still the most important source of model compounds for the development of new drugs[1]. In the past decade or so, a number of naturally occurring peptides, often consisting of only a few amino acids, have been discovered which are extremely potent regulators of key biological processes. Their diversified roles encompass activities such as hormonal function, neurotransmission and immunological modulation. (Fig 1) Their therapeutic potential could conceivably include the control of pain, the regulation of metabolic diseases, and the stimulation or deactivation of the immune system.

Peptide		Number of amino acids
Hypothalamic releasing factors		
for luteinizing hormones	LRH	10
for thyreotropin	TRH	3
for growth hormone	GRH	10
Pancreatic hormones		
	insulin	51
	glucagon	29
	somatostatin	14
Pituitary hormones		
adrenocorticotropic	ACTH	39
	oxytocin	9
	vasopressin	9
Tissue hormones	bradykinin	9
	angiotensin II	8
Neurotransmitters	substance P	11
	enkephalins	5
	endorphins	>5
Immune hormone	factor thymic seric	9
	thymopoeitin fragment	5

Figure 1 Peptide of quite different size and structure that act as hormone or mediators in many organs of the human body.

However, some of their properties present several disadvantages for practical chemotherapy. Firstly, the high lability of these substances to the degradative peptidases limits their duration of action and contributes to their lack of oral activity. Secondly, the same peptidic structure can mediate different processes in different organs and tissues and thus lacks biological selectivity[2]. Finally, their relative chemical complexity makes their large scale preparation difficult and uneconomical. An important research goal would then be to design simpler compounds which reproduce only those key features of the natural effectors that are essential for selective biological activity[3].

It is widely accepted that peptides transmit their message to the target cells through specific receptors on the cell surface. According to general theories of drug-receptor interactions, the peptide would fit on its receptor (the "lock and key" principle) thus causing a conformational change in the macromolecular complex which in turn would lead to the transduction of a signal into the cell[4].

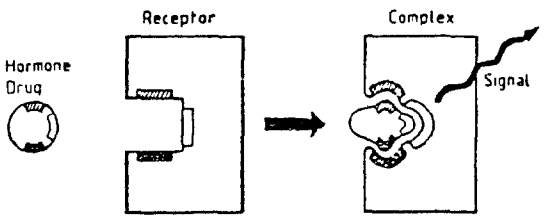


Figure 2 Model of hormone- or drug-receptor complex.

Complex formation and signal transduction are not necessarily interconnected and for this reason compounds that form complexes with the recptor but do not cause signal transduction are often sought in order to antagonize or block the message. Complexation with the receptor can also induce a conformational change in the peptidic effector itself. The concept of induced fit upon binding has in fact been extended so as to encompass oligopeptidic structures[5].

It is also believed that a given family of receptors can exist as subpopulations displaying subtle but specific variations in conformational requirements toward related peptidic modulators. For instance, the μ-subgroup of the opiate receptor in the central nervous system binds preferentially morphine derivatives and endorphins, whereas the δ-subgroup which is more abundant in the gut shows a preference for the enkephalins[6-8].

The exact parameters involved in the binding of oligopepties and signal transduction are still poorly understood. Steric, electrostatic and conformational effects at the receptor level have without doubt a determinant role[9]. There exists no reliable method as yet which permits the direct observation of peptide-receptor interactions. Indirect approaches involving structurally and conformationally induced variations in the dynamic behavior of oligopeptides are generally helpful in unraveling the receptor binding site topography which in turn can serve as a model for

the design of potentially useful drugs. Once identification of an active oligopeptidic fragment has been accomplished and the structural features responsible for its agonist or antagonist activity have been delineated, the major remaining problems are to achieve improved receptor selectivity and greater metabolic stability of the fragment.

In the past these parameters were generally manipulated, more or less indiscriminately, through side chain modifications of the native peptidic structures. These alterations involved the substitution of one or more amino acid residues by another, including enantiomers and the deletion of one or more residues in the sequence[10]. Other chemical changes concerned functional groups such as the O-methylation of tyrosine[11-13], the O-acetylation of serine[14-15], the S-oxidation of methionine[16-17], etc... It is now well-known that the structural requirements for the activity of an oligopeptide are often very strict[10, 18]. For instance, even a single inversion of chirality can produce dramatic changes in potency[19]. Such approaches generally entail the preparation of a large number of analogues before useful information can be obtained. Overall, this research has been rarely successful as regards the development of new therapeutic agents[3,20].

However, new refinements in the rational design of peptide analogues have recently been developed and involve the imposition

of conformational constraints through ring formation between key sites. When the loop freezes the conformation into a favorable geometry, the biological activity is either preserved or enhanced[21-22]. Frequently, cyclization confers resistance against enzymatic degradation[21,23]. This strategy has been most successfully exploited by the Merck group and has led to the discovery of somatostatin analogues of considerable promise in the treatment of juvenile diabetes[24-25].

There have been surprisingly few attempts to replace the amide backbone linkages by isosteric functionalities. This is remarkable in the light of the fact that the amide linkages themselves are the only target of degradative peptidases whose role is to inactivate the oligopeptidic effectors. The reason so few examples of isosteric backbone modifications are available stems from the fact that such isosteres can not readily be incorporated in the peptidic backbone and from the lack of incentive to overcome these synthetic difficulties owing to the general belief that the amide linkages play a minor role in the binding chemistry of oligopeptides at the receptor level. The most important backbone changes that were explored in the past are listed in Fig. 3.

As expected these modifications make the peptide structure resistant to enzymatic cleavage. However this advantage is, in most cases, outweighed by large decreases in biological activity.

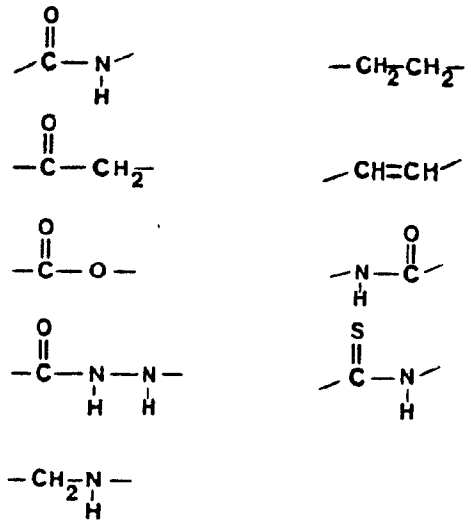


Figure 3 Isosteric Analogues of the Peptide Bond

This is not too surprising since most of these analogues incorporate backbone features that are non-equivalent to amide linkages as regards hydrogen bonding, solvation and geometrical properties. The so-called "retro-invero" modification where the direction of the amide bond is reversed using amino acid residues of opposite configuration has been more successful especially when applied to cyclic analogues, but less so in the case of linear peptides[10,26]. Among these backbone variations, it is the thioamide function that appears to bear the greatest similarity to the amide bond as regards geometrical and electronic properties[27]. At the beginning of our investigations, receptor and enzyme interactions with

oligopeptides carrying thioamide bonds had not been studied. The only example of such a modification involved the incorporation of a thiocarboxamide function at the C-terminal end of oxytocin which resulted in a lower potency[28].

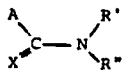
The virtual absence of information on the biological properties of thiopeptidic analogues is undoubtedly due to the lack of an adequate methodology for their preparation. We intuitively expected that backbone thionation of oligopeptides would confer greater resistance to peptidase action and yet introduce only minor changes in the conformational properties. It was our goal to develop a practical and efficient methodology for the synthesis of oligothiopeptides and eventually evaluate their biological properties. It may be worthwhile at first to review some of the more important physico-chemical properties of thioamides in relation to the behavior of the parent amides. The following summary is by no means exhaustive but it will suffice to establish the conceptual basis of our approach.

Physico-chemical Properties of Thioamides

A) Bond Lengths and Angles

X-ray data for several thioamides has revealed that the length of the C=S bond lies between 1.65-1.73 Å [29]. These values fall in between the bond length of a thiocarbonyl (1.60 Å and that of a C-S bonds (1.81 Å) [30,31], thus indicating a marked

electron delocalization in the thioamide functionality. The C-N bond length of the thioamide falls in the vicinity of 1.35 Å, a value close to that of the same bond in amides (Fig. 4).



	Bond lengths (Å)		Bond angles (deg.)			
	C–X	C–N	$\widehat{ACX}$	$\widehat{XCN}$	$\widehat{R'NC}$ / $\widehat{R''NC}$	$\widehat{R'NR''}$
X = O						
(peptide)	1.24	1.32	120	124	120 / 120	120
X = S						
$HCSN^{CH_2Ph}_{CH_3}$	1.66	1.35		123	122 / 119	119
CH_3CSNH_2	1.73	1.324	120.7	117	117 / 116	123

Figure 4 Bond lengths and angles in a peptide bond and in thioamides as determined by X-ray diffraction[29,46].

Thioamides assume a planar geometry similar to that of olefinic and amide bonds. Accordingly, thioamides differ primarily from amides in the length of the C=S linkage vs the C=O (≃1.68 vs 1.25 Å), While bond anles are very similar. This difference may well cause interference with the catalytic process of hydrolytic enzymes. The increase length of the C=S bond of thioamides as well as the larger volume of sulfur would not be expected to affect receptor binding relative to the parent peptide provided that the amide backbone linkages are not directly involved in the binding process.

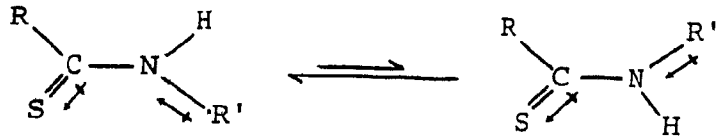
B) Barrier to Rotation and cis-trans Isomerism

Several lines of evidence indicate that thioamides possess

After the completion of this manuscript T. La Tour et al (Int. J. Pept. Prot Res. 22, 509,1983) have confirmed the close geometry of a thiopeptide unit vs the parent peptide. The Cbz-GlyC(S)-Gly-OBzl had the following characteristics: bond lengths : C=S 1.64 Å, C-N 1.33 Å; bond angles: SCN 124 deg., SCC 120 deg., CCN 116 deg., CNC 122 deg.

a higher degree of double bond character than their parent amides which is reflected in the higher barrier to rotation about the C-N bond[29]. On the basis of various NMR analyses, the difference in free energy of activation ($\Delta G^{\ddagger}$) for rotation of a thioamide has been estimated to be approximately 2-5 Kcal/mole higher than for amides[32-38]. A qualitative explanation for the increased barrier is that the more polarizable sulfur atom encourages greater electron delocalization and higher double bond character of the C-N bond in the ground state and destabilization of the transition state which requires a full trigonal character of the C=S for rotation[29,32]. Steric, mesomeric and inductive effects of the substituents are also known to influence the height of the barrier to rotation[33,39,40]. However this higher barrier of rotation may not significantly alter the overall conformational properties of an oligopeptide analogue, but could have an important effect on the mechanism of enzymatic hydrolysis.

The planarity and the higher barrier to rotation of thioamides also imply that geometrical isomers (cis/trans or E/Z of increased stability may be observable[41,43]. Two main factors determine the E/Z ratio: steric repulsion between substituents and intra-molecular electrostatic interactions. The latter can favor the Z isomer in the case of alkyl substituents on account of the partial compensation of bond moments and thus serve to decrease the free energy [44-45]. Except in the case of the thioformyl



(Z) (E)

Figure 5

function (R=H), the greater volume of the sulfur atom does not significantly affect the ratio of isomers[29]. Accordingly, for thioamide analogues a Z configuration should be overwhelmingly predominant as is the case for normal peptides[46].

It should also be noted that the dipole moments of thioamides are considerably higher than those of parent amides, again reflecting the larger electron delocalization in thioamide analogues. For example, N,N-dimethylthioacetamide has a dipole moment of 4.7D which contrasts with the value of 3.86D for the corresponding amide[47].

C) Acidity and Hydrogen Bonding

Thioamides are stronger acids than their parent amides. The difference in pK_a is quite large for compounds that have aromatic substituents ($pK_a \approx 3$) but is smaller for those that have alkyl substituents:

	pK_a	
	X = O	X = S
PhC(X)NHPh	13.7	10.6
$HC(X)NHC_4H_9$	13.8	12.8

Using ^{1}H NMR spectroscopy, it was observed that N-methyl thioacetamide is about 1000 times more reactive towards base-catalyzed NH proton exchange than N-methyl acetamide whereas in the acid-catalyzed process the exchange rate is approximately 10 times slower for thioamides[49].

Similarily to amides, thioamides can form strong hydrogen bonds[50]. They are known to self-associate in solution, although to a lesser extent than amides[51-52]. Investigations by ^{1}H NMR spectroscopy of the hydrogen bonding properties of thioamides revealed that the NH of thioamides is a stronger proton donor, in agreement with its higher acidity, than the NH of amides, whereas the thioamide sulfur is a weaker acceptor than the amide oxygen[53]. These differences may have important consequences in terms of receptor interactions when specific proton acceptor or donor sites are involved.

It is worthwhile noting that thioamides readily engage into complex formations with various metal ions to give adducts having the following composition[54]:

$$M\cdot\left[RC(S)NHR\right],\ M\cdot\left[RC(S)NHR\right]_2,\ \text{and}\ M\cdot\left[RC(S)NHR\right]_4$$

Since many enzymes incorporate metal ions at their catalytic sites, coordination of these metals by thiopeptidic substrate analogues may interfere with the normal catalytic events.

D) Chemical Reactivity and Toxicity

Complete hydrolysis of thioamides yields the corresponding

carboxylic acids, hydrogen sulfide and amines[29,54,55]. No generalization is permitted as regards the relative hydrolytic stability of thioamides under alkaline or acidic conditions as compared to amides[54]. The rate of hydrolysis of thioamides is subject to both substituent and solvent effects and the kinetic analysis of the results is often further complicated by the formation of by-products[54,55]. Hydrolysis proceeds through the formation tetrahedral intermediates, followed by breakdown to products[56].

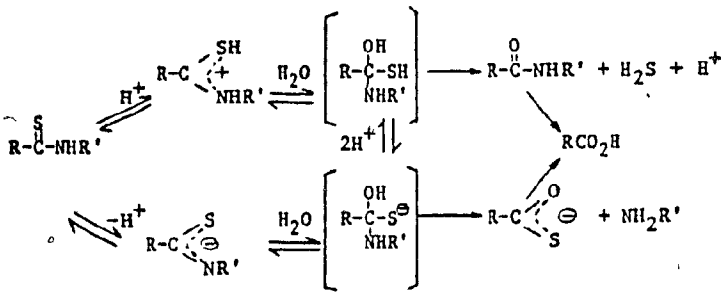


Figure 6

Edward et al[57,58] have shown that under conditions of acid hydrolysis, cleavage of the C-N bond is favored as the concentration of acid increases whereas a rise in temperature accentuates cleavage of the C-S bond. In principle, therefore, enzymatic hydrolysis of thioamides may yield either the amide or the thioacid or both.

As expected, thioamides are much more reactive than amides in alkylation, oxidation and reduction reactions. Each process can be accomplished by several methods[29,59]. It is generally agreed that the well-known toxicity of thioamides stems from oxidative biotransformation products rather than from the thioamide

itself or by the generation of H_2S from their hydrolysis[60]. The flavin-dependent monooxygenase (MFMO; EC 1.14.13.8) present in rat liver microsomes was shown to be responsible for the generation of toxic products from thiobenzamide[61]. A similar mechanism applies to thioacetamide which can cause liver necrosis[62], pulmonary edema, and bone marrow depression[63]. Despite these toxic effects, several thioamide derivatives have been used in the treatment of thyrotoxycosis because of their ability to interfere with thyroxin synthesis by inhibiting the iodination of tyrosine in the thyroid gland[64].

E) Spectroscopic Characteristics

For the purpose of identification and characterization, thioamides differ sharply from amides in some key spectral properties. The C=S group is a strong chromophore in the UV; the $\pi \rightarrow \pi^*$ transition gives rise to a strong absorption band ($\log \varepsilon \approx 4.0$) at wavelengths ranging from 260 to 300 nm[65-66]. A weaker absorption band ($n \rightarrow \pi^*$, $\log \varepsilon$ 1.5-2.5) around 320-420 nm is also observed with thioamides[67].

The IR of thioamides do not display any clear-cut characteristics. The thiocarbonyl (C=S) is weaker than the carbonyl (C=O) bond, and consequently the absorption of the former is not as intense and occurs at lower frequencies (1250-1020 cm^{-1}) and is much more susceptible to coupling effects[68]. Identification by IR spectroscopy is therefore more difficult and uncertain.

However, the N-H stretching frequency of secondary thioamides (Z isomer) occurs at 3400 cm^{-1}, a region which is not of precise diagnostic value.

The NMR resonances for α and β protons of thioamides are shifted downfield relative to those for the parent amides. A downfield shift is also observed for the nitrogen substituents. This latter shift has been attributed to a greater contribution of the polar tautomeric form of the thioamide[69].

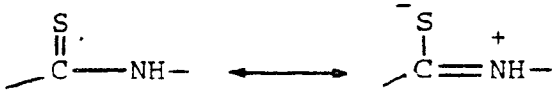


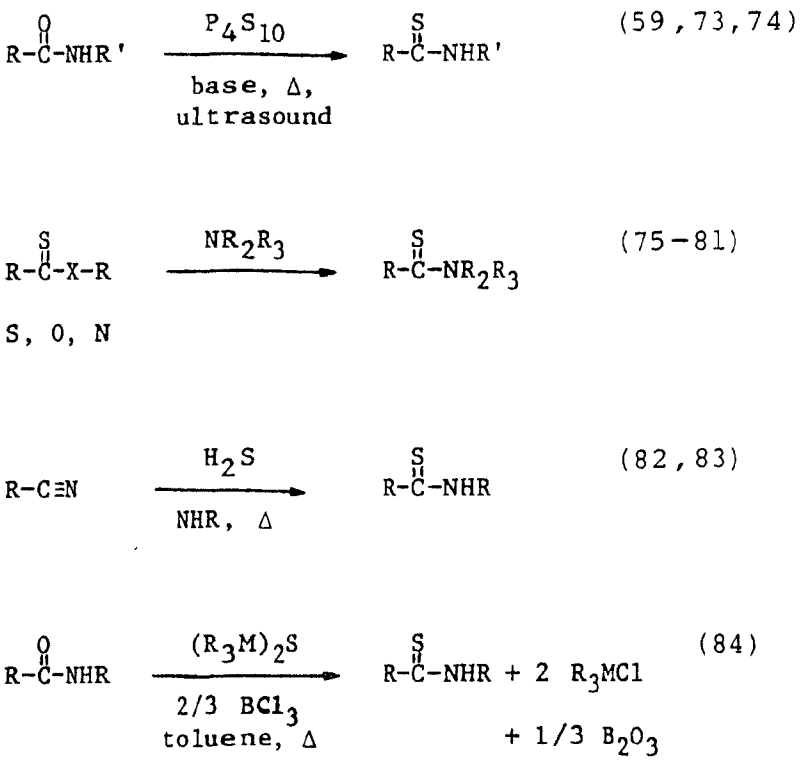
Figure 7

Similarily, the ^{13}C signals for the substituent adjacent to the thioamide function are also shifted downfield[70]. The C=S resonance itself appears at 200 ppm vs 170 ppm for the parent C=O of amide. These shifts have been explained in terms of the different "through bonds" inductive effect exerted by the thioamide group and the "through space" effects created by the larger dipole moment of thioamides[71,72].

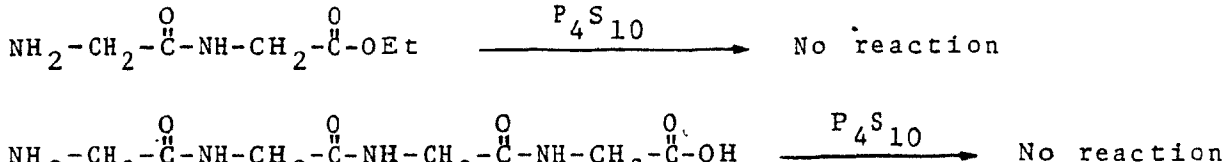
F) Preparation of Thioamides

Although there are several classical methods to prepare thioamides, only very few can be conveniently applied to the synthesis of thiopeptides. These conventional methods are

summarized below:



Direct thionation of amides by means of phosphorous decasulfide was introduced by Hoffman[73] as early as 1878 and to this day remains the most economical method for the preparation of thioamides despite the drastic conditions that are often required. However when this method was applied to peptide substrates, it resulted in failure[85,86].



Prior to the inception of our work, the only succesful synthesis of a thiopeptide was reported by Ried et al[87,88,89]. The linkage was formed in good yield by thioacylation of an amino acid with a thionester derivative of a N-Cbz protected amino acid. The thionester intermediate was prepared from the nitrile analogue of the corresponding amino acid as shown in Fig. 8.

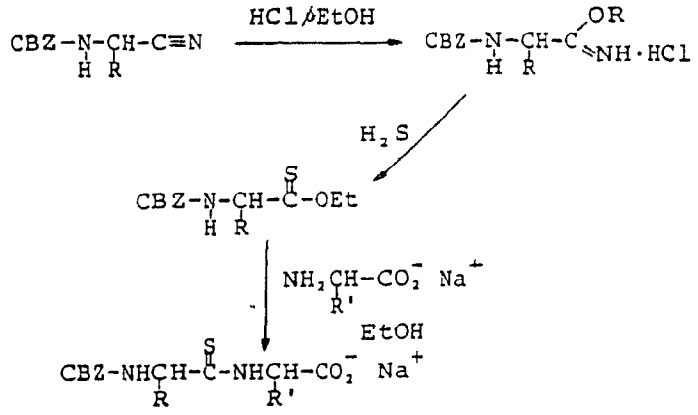


Figure 8

This approach presents several disadvantages. It requires a lengthy preparation of thionester intermediates, the use of strong basic conditions in the thioacylation step which encourages racemization and finally, the use of an unprotected carboxyl function in the coupling reaction which accentuates the solubility and purification problems associated with peptides.

Clearly, new methodologies were required to generate relevant thiopeptide analogues and solutions to this problem were sought as will be described in the following chapter. Subsequent chapters (2 and 3) will include the synthesis and biological evaluations of relevant thiopeptidic analogues of the chemotatic

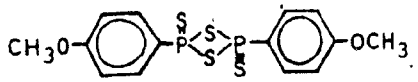
peptide f-Met-Leu-Phe, and the neuroeffector [Leu^5]-enkephalin, Tyr-Gly-Gly-Phe-Leu. Finally, Chapter 4 will be briefly concerned with the potential use of thioamide analogues in the generation of different types of backbone modifications.

CHAPTER 1

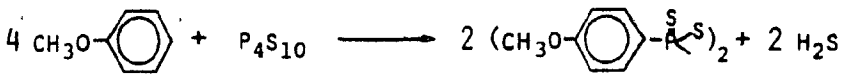
New Approaches for the Synthesis of Thiopeptides and Interaction of Thiocarbonyl Analogues with Peptidases

1.1 Development of New Thionation Reagents

After several attempts in our laboratories to transform amides into thioamides by methods applicable to peptidic substrates, it was decided to evaluate the use of a new thionation reagent as recommended by Lawesson et al[90-95]. These authors claimed that 2,4 bis(4-methoxyphenyl)-1,3,2,4-dithiaphosphetane-2,4-disulfide (1) was most convenient for the conversion of amides to thioamides.



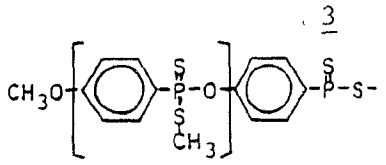
This phosphetane was first described by Lecher et al[96] in the course of their studies on the phosphonation of aromatic compounds with phosphorous decasulfide (P_4S_{10}).



They found that when anisole (10 eq) was heated with P_4S_{10} (1 eq.) to a temperature of 160°C (4-6 h) a yellow solid, possessing the dimeric structure 1, was produced in 80% yield simply upon

cooling the reaction mixture. Compared to other aromatic compounds such as benzene, naphthalene and phenetole, anisole gave the best yield of solid dimer. Different aromatic compounds must each be treated at optimum temperatures within a narrow range. If the temperature is too low no reaction takes place whereas at too high temperatures, polymers (resins) 2 and 3 are formed. In the case of anisole, a side reaction involving cleavage of the methoxy group and polymer formation (4) was observed as evidenced by the evolution of CH_3SH after hydrolysis of the mother liquor[96].

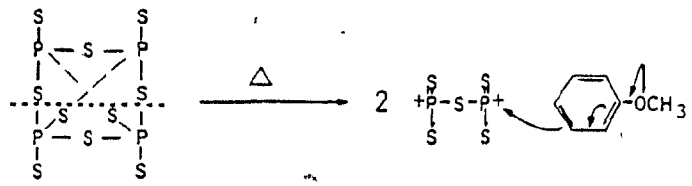
2



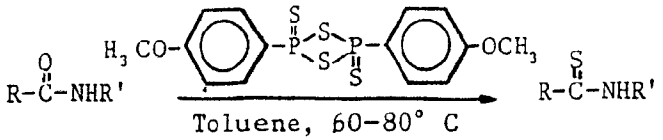
3

4

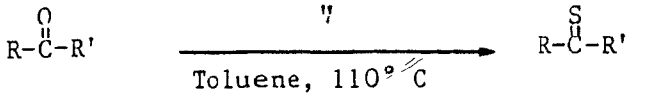
Lecher postulated that the phosphonation reaction proceeded after dissociation of P_4S_{10} into smaller reactive species such as P_2S_5 (6) where semi-polar (P+S) bonds would suffer nucleophilic attack by the electron-donating aromatic ring.



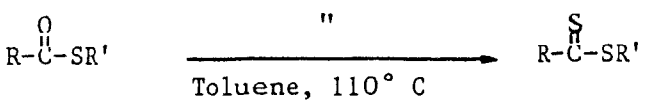
However, the ability of the phosphetane dimer to engage in thionation reactions was not recognized until Lawesson's studies[97-99] of the properties of various phosphorous-sulfur complexes toward appropriate substrates. It was eventually observed that when 1 (0.5 eq) was heated with amide substrates in hot toluene (80°C) for period of 2-3 h, high yields of the corresponding thioamides were obtained[90-93]. Later it was found that other carbonyl compounds can also be transformed into their thiocarbonyl analogues, this process requiring higher temperatures and higher molar ratios of 1 (1-1.5 eq) due to the concomittant formation of phosphetane polymers at higher temperature.



(90-93)



(94)



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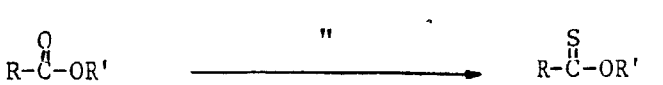
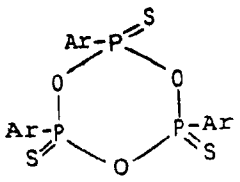


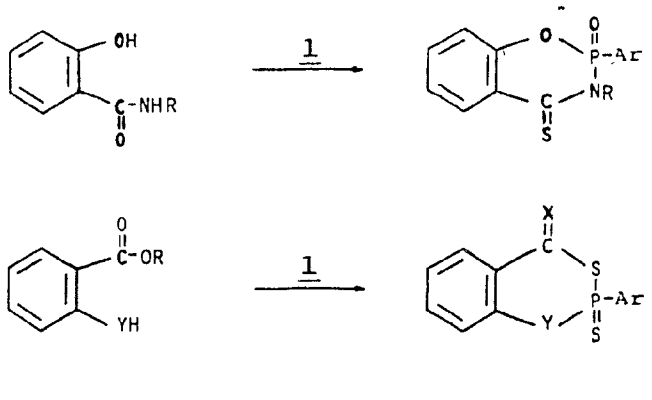
(95)

A by-product of these reactions was identified as the cyclic trimer 7[90,92,95].



7

Subsequent to these preliminiary communications, Lawesson's group[100-101] as well as others[102], reported the preparation of a large number of various heterocyclic derivatives synthesized by reacting this reagent with carbonyl compounds incorporating other nucleophilic functionalities.



X, Y = O, S

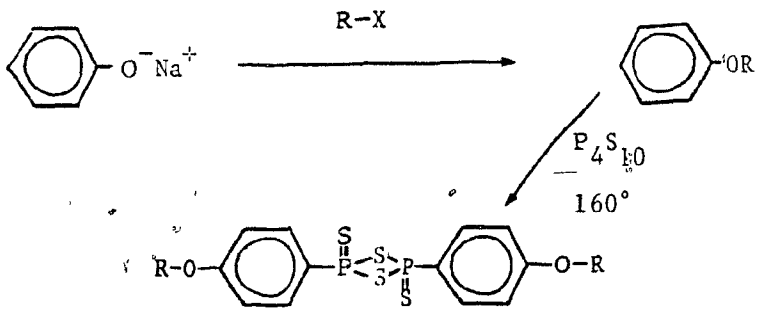
R ≠ H

Although, this thionation reagent was very attractive to us, it was clear at the outset that the reaction conditions described by Lawesson et al[90-93] would not be suitable for the direct, selective synthesis of thioamide analogues of sizeable polypeptides. Firstly, the very limited solubility of reagent 1 and of polypeptides in non-polar solvents and the high reaction temperatures necessary for transformation are undesirably restrictive, especially because of the thermal instability of various protecting groups. Secondly, no regioselectivity with tri- and longer peptides can be

expected under the recommended conditions. These limitations in the use of this reagent were subsequently encountered by others[101-105].

Our first effort was to circumvent at least the solubility problem by generating similar phosphetanes carrying aliphatic substituents larger than methyl on the oxygen of anisole. This change would certainly improve solubility in organic solvents and perhaps promote regioselectivity through steric interactions with the amino acid side chains.

Such O-alkyl analogues of 1 were found to be easily accessible by reacting the appropriate phenolic ether with phosphorous decasulfide at a temperature of 160-170°C for 4-6 h, conditions quite similar to those recommended by Lecher et al[96] for the anisole.



R = $-(CH_2)_3-CH_3$ (8)

$-(CH_2)_4-CH_3$ (9)

$-(CH_2)_3-CH(CH_3)_2$ (10)

The requisite phenyl alkyl ethers were readily prepared by alkylation of the phenoxide anion with the appropriate alkyl bromide and purified by distillation[106]. Crystalline thiono phosphine sulfides were obtained by adding hexane to the phosphonation reaction mixture. In general, the yields were 50-70%, values comparable to that reported by Lecher et al[96] for the case of anisole.

The ^1H NMR spectra (60 MHz, $CDCl_3$) of these thiono phosphine sulfides displayed a distinct downfield shift for the aromatic protons with strong coupling with phosphorous (J_{PCCH}=18 Hz, J_{PCCCH}= 4Hz). In the mass spectrum, the base peak corresponded to that of the monomer.

However, an equivalent product could not be isolated when the O-alkyl side chain incorporated an additional ether function, ($R=CH_2-CH_2-O-CH_2-CH_3$). The aromatic region of the ^1H NMR spectrum (60 MHz, $CDCl_3$) of the crude reaction mixture did not indicate the presence of any desired product. The alkyl region of the spectrum was however significantly changed relative to the starting material: additional signals at 2.85 and 1.1 ppm were now present. This is suggestive that P_4S_{10} reacted with the aliphatic ether rather than with the aromatic ring. Alternatively, it may be that any thiono phosphine sulfides generated under these conditions reacted with the other functions to give polyesters, a side reaction already observed with anisole. It was also observed by others in our laboratory that neither 1,3-di nor 1,3,5-trimethoxybenzene gave recognizable products upon similar treatment

with P_4S_{10}[107].

Eventually, the commercially available phenylether (Dowtherm^R) was found to give good yields (65-75%) of the crystalline dimer 11 when reacted with P_4S_{10} at 160°C for 6 h. This compound could

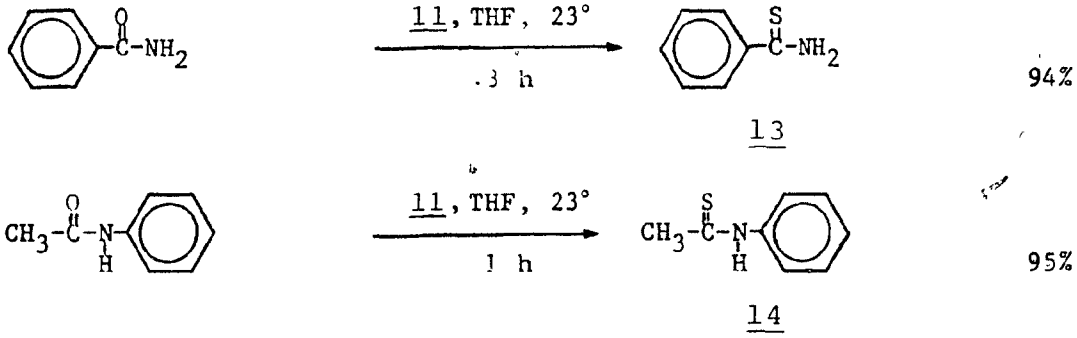
11

be easily purified.by recrystallization (mp 187-190°C) from hot toluene. The ^1H NMR spectrum agreed with the expected structure. All these new p-alkoxy and phenoxy thiono phosphine sulfide dimers were more readily soluble than 1 in a variety of organic solvents such as toluene, chloroform, acetonitrile, and THF. For example, 11 is soluble to the extent of 5% as compared to 0.2% for 1 in dry THF at 23°C and the i-amyl analogue will dissolve to give an 8% concentration in the same solvent.

In dry THF the conversion of simple amides to thioamides by reagent 11 was found to proceed much more rapidly and at a much lower temperature than in the case of 1[90-93]. Thus thioacetamide (13) and N-phenyl thioacetamide (14) were obtained in high yields from their corresponding amides after reaction times of 0.3 h and 1 h respectively, when exposed to 11 (0.6 eq) in THF at room temperature.

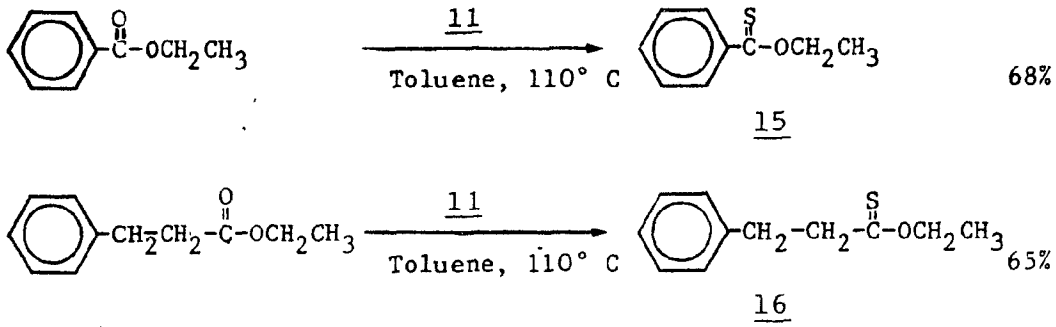
Interestingly, similar reaction rates were observed with thionation reagent 1 in THF under our conditions. However, the rates were decreased when our reagent (11) was reacted at room

temperature in $CHCl_3$ or toluene, solvents recommended by Lawesson et al[90-93].



It is clear then that in addition to the favorable solubility of our new reagents, the nature of the solvent is another key parameter in the kinetics of the reaction. This remarkable solvent effect was never noted before. While our work was in progress Walter et al[108] reported that dimethoxyethane (DME) was a much better solvent than toluene in the transformation of vinylogous carboxamides to their corresponding thiocarboxamides with reagent 1. A temperature of 20° C gave the best results.

We also investigated the conversion of esters into thionesters with our reagent 11. No marked solvent effect was noted for this reaction with simple aryl or alkyl esters. Comparable rates were observed by TLC analysis when the reaction was performed in either boiling dioxane or boiling toluene. In the latter solvent yields of 65-70% were obtained for the thionation of ethyl benzoate and ethyl hydrocinnamate after 24 h in the presence of 1.2 eq of 11.



The absence of a solvent effect on this reaction does not necessarily imply that a transition state of reduced polarity is involved or that a change in mechanism occurs; these thionation reagents may polymerize at enhanced rates in hot polar solvents. In this regard, the phenoxyphenyl reagent 11 which lacks the reactive methoxy group of 1 may be less susceptible to polymerization. This is supported by the observation that a lower temperature (110 °C) is required for thionation of these simple alkyl and aryl esters as compared to the higher temperature (140°C) recommended by Pedersen et al[95].

Although the detailed mechanism for the formation of a thiocarbonyl function by thiono phosphine sulfide reagents is not yet established, the involvement of polar transitions states and/or intermediates is probable in view of the pronounced solvent effect observed in the conversion of amides to thioamides. In fact, polar intermediates have already been postualted in the transformation of amides to thioamides by P_4S_{10} (Fig. 10)[109].

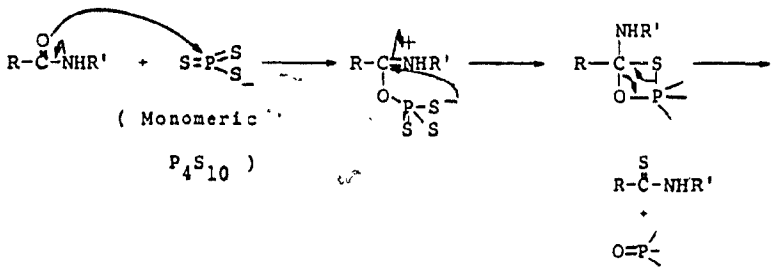


Figure 10 Proposed mechanism for the formation of thioamide with P_4S_{10}.

While it is known that the presence of organic bases like pyridine[110] or triethylamine[111] can accelerate the reaction their exact role has remained conjectural. Recently, Rauschner and Klein[104] have used THF as the solvent and discovered that ultrasound irradiation allowed thionation with P_4S_{10} to proceed at low temperatures (30-40°C) and in the absence of organic bases.

A mechanism such as depicted in Fig. 11 for the transformation of amides into thioamides by thiono phosphine sulfides is therefore plausible in view of the well-known oxophilicity of phosphorous.

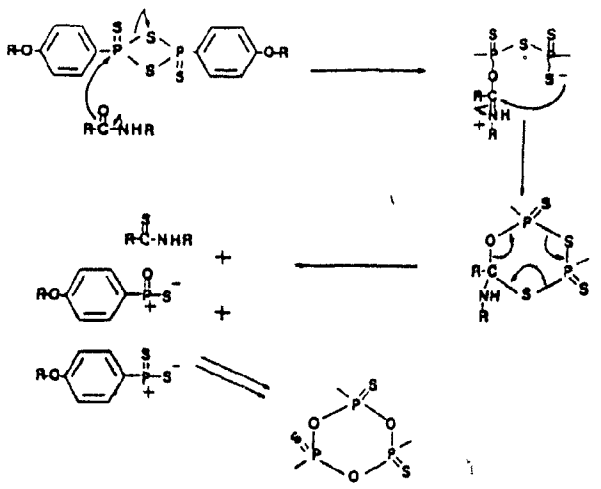


Figure 11 Proposed Mechanism for the formation of thioamide with 1 or 11

Thus, the nucleophilic oxygen of the amide can readily attack the electrophilic phosphorous with concomittant opening of the 4-membered ring to yield an electrostatically stabilized imino-sulfide intermediate. Subsequent attack at the imino carbon by the sulfur anion would then easily generate a cyclic transition state, or intermediate which would spontaneously collapse to the thioamide product and an oxygenated phosphorous compound. This in turn could engage in sulfur exchange reactions leading to another thioamide.

However, it is equally possible that the initial electrophilic species is the monomeric form of the thiono phosphine sulfide reagent (Fig. 12). In this case, the only difference in mechanism centers on the initiation step and thus the reaction pathway is similar to that already proposed for thionation by P_4S_{10}.

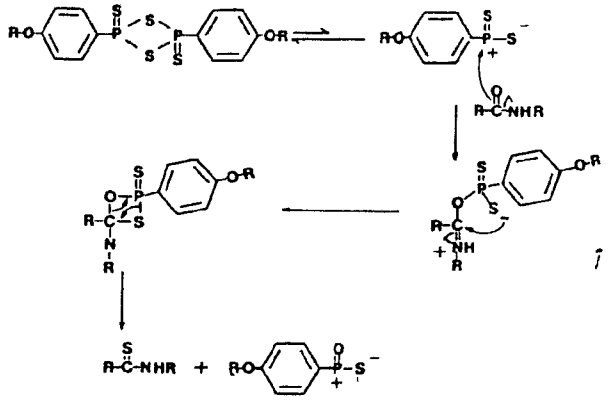


Figure 12

Formation of a 4-membered ring intermediate containing phosphorous has been suggested as a key species in the conversion of epoxides to episulfides[112] and in the Wittig reactions of carbonyl compounds[113-114] It is safe to conclude that the postulated 4-membered ring intermediate

would similarly collapse to thioamide products.

Thionating monomeric species are also possibly ambident and a sulfur anion would thus act as a nucleophile in a mechanism analogous to that proposed for the P_4S_{10}-mediated conversion of ketones to thioketones. In this reaction, a strong solvent effect has been reported[115] and the rate is accelerated by inorganic salts such as $NaHCO_3$ and Na_2S[116]. Under these conditions the conversion of ketones to thioketones is more rapid than that of amides to thioamides which led to the following mechanistic proposal[115]:

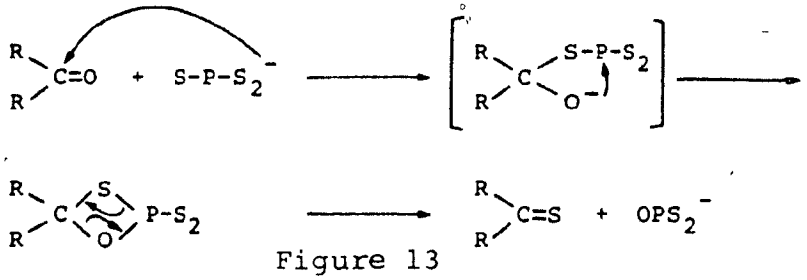


Figure 13

The above mechanism can be applied to the formation of thioketones and thionesters as promoted by the thiono phosphine sulfide reagents. Formation of the reactive monomeric species would also explain the course of some reactions reported by Lawesson et al[117]. For example, it was also observed that the reaction of 1 with β-lactone gave a cyclic phosphorous product, whose formation may be accounted for as follows:

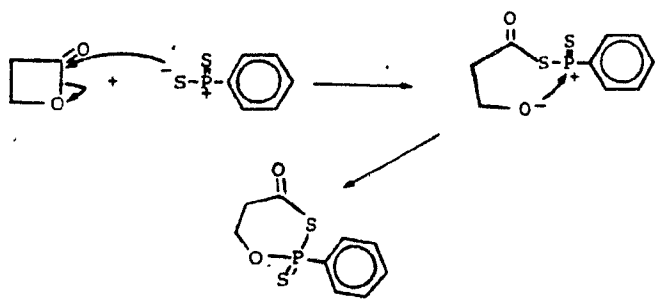
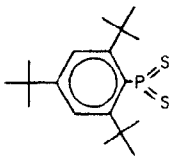


Figure 14

Formation of such dipolar monomeric species is substantiated by the known formation of higher polymers when the thiono phosphine sulfide reagents are heated in high boiling solvents[95-96], and by the isolation of stable metadithiophosphonate whose aryl group bears bulky alkyl substituents[118].



It remains possible of course that both proposed mechanisms are competing under the experimental conditions. Further research is necessary in order to identify the relevant pathway.

Since the temperatures required for the transformation of amides into thioamides are much lower than those required for the attack of esters, the latter functionality should be suitable as a carbonyl protecting group when amide bonds are submitted to thionation reactions. As regards protection of amine groups, it was gratifying to observe that the classical t-butyloxycarbonyl (Boc) function was unaffected by thionation reagent 11 in THF at room temperature over periods of at least 24 h. This was established by exposing Boc-benzylamine to reagent 11 under the relevant conditions.

Earlier observations in our laboratory, indicated that the rate of thioamide formation was influenced by the presence of substituents about the amide bond when reagent 11 was used in dry THF. Accordingly simple amide derivatives of amide acids were prepared in order to quantitatively evaluate the role of the steric

parameters on the reaction rates. Initially, the effect of substitution on the amide nitrogen was studied and to this end the unsubstituted amide (17), the N'-methylamide (19) and piperidide (21) derivatives of Boc-phenylalanine were synthesized. Compounds 17 and 19 were prepared by aminolysis with ammonia or methylamine in MeOH of the corresponding esters[119] while 21 was prepared by coupling piperidine and Boc-Phe with DCC.

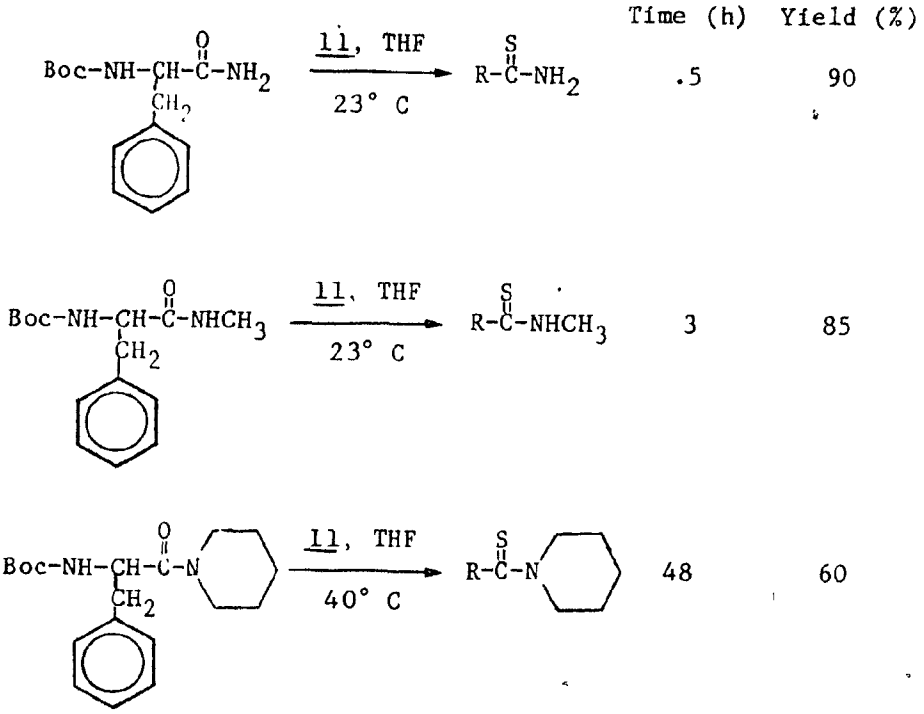
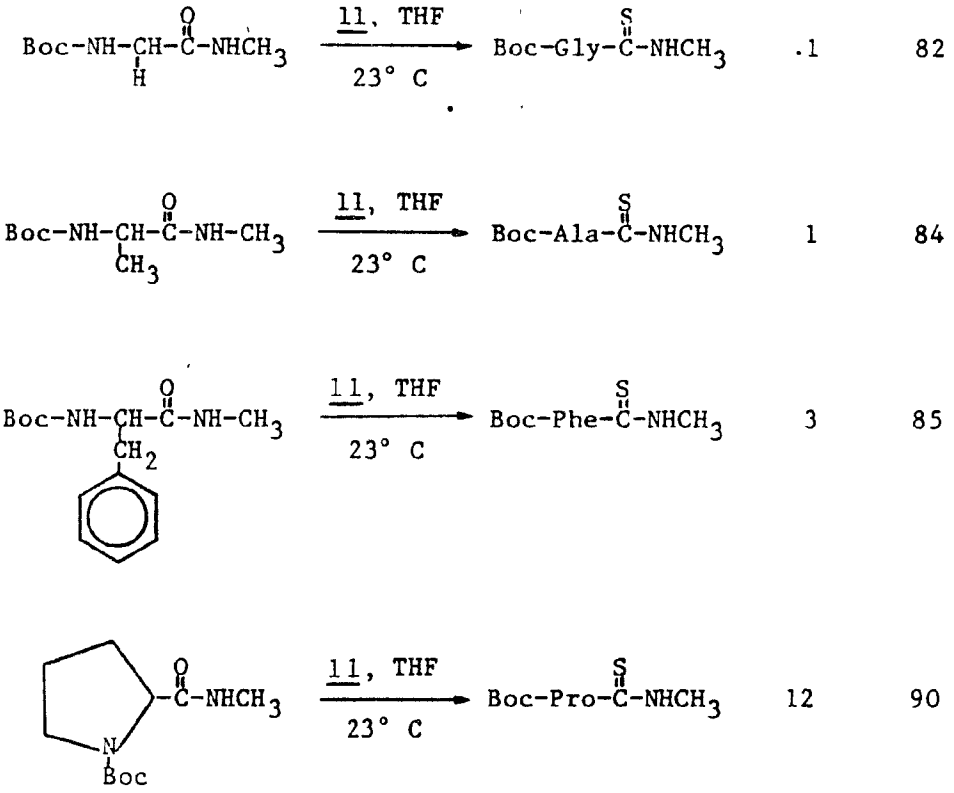


Table 1 Thionation of Boc-Phe amides with reagent 11.

Thionation of these amides using reagent 11 in dry THF at room temperature was performed, and reaction times as monitored by TLC and the isolated yields after chromatography are given in Table 1. The replacement of an amide hydrogen by a methyl group increases the reaction time by a factor of 6 while complete substitution increases the reaction time by a factor of 75, without correction for



Tale 2 Thionation of N'-Methyl N-Boc amino acid derivatives with reagent 11.

the higher reaction temperature. The latter substrate is a valid model for proline residues in peptides and on that basis it was expected that N-acyl proline bonds should suffer thionation more reluctantly than other amide functions. All the new compounds thus generated were identified by ^{1}H NMR, UV and mass spectrometry. We confirmed the previous observation that all thiocarbonyl analogues of amides and esters migrate further than their oxygen analogues on silica gel. This increased mobility on silica gel may well reflect the reduced ability of thiocarbonyl groups to form hydrogen bond complexes.

Next, we examined the effect of substituents on the α-carbon of the amide and the results were equally revealing. Using the same reaction conditions (THF, room temperature, 0.6 eq of 11), thionation of the N'-methyl amide derivatives of Boc-Gly (23), Boc-Ala (25) and Boc-Pro (27) showed that the presence and size of the α-substituent markedly affected the rate of the reaction. In fact, the effect of the substituent on the α-carbon is even more pronounced than that of the N-substituent recorded in Table 2.

Another parameter affecting the rate of amide thionation is the electronegativity of the N-substituent. For example, the p-nitrophenyl amide of Boc-Leu required at least 48 h at 50°C in THF for its conversion to the thioamide analogue. This unfavorable electronegativity effect has also been noted by Baxter et al[120] in the case of the conversion of an ester to the corresponding thionester by reagent 1. However, this parameter is of no importance in

the case of ordinary peptides since electronegativity effects about the amide bond are small and virtually constant. The determinant factor on the course of the thionation reaction as revealed under our experimental conditions is the steric effect of the C-substituent. This observation suggests that regioselective backbone thionation of oligopeptides can be accomplished when relative differences in the steric bulk surrounding the individual peptide bonds are present. We exploited this strategy for the selective synthesis of thioamide analogues of sizeable oligopeptides as illustrated in the following chapters.

The possibility of preparing thioamides by a "one pot" process by reacting unprotected amines and carboxylic acids in the presence of thionation reagent 11, was also considered. We reasoned that the reagent would first activate the carboxyl group followed by acylation of the amine, and the resulting amide bond would subsequently react with more thionation reagent to yield the thioamide in one overall operation.

$$R\text{-}CO_2H + NH_2R' \xrightarrow{11} R\text{-}\overset{O}{\overset{\|}{C}}\text{-}NHR' \xrightarrow{11} R\text{-}\overset{S}{\overset{\|}{C}}\text{-}NHR'$$

Accordingly, Boc-Phe, n-butylamine, and the thiono phosphine sulfide 11 (1 eq) were mixed in THF at room temperature. However, TLC comparison with reference compound indicated that the only significant product formed was the amide, Boc-Phe-$NH(CH_2)_3CH_3$, and

very little of the thioamide was present.

A similar approach has since been described by Blade-Font et al[121] using P_4S_{10}, a primary amine, a carboxylic acid, and pyridine as the solvent. Thioamides were formed but the yields were generally poor (20-40%). In any event, the experimental condition requires high temperatures and thus are unsuitable where oligopeptides are concerned and we decided to postpone our studies along this line. Ironically Pedersen et al[122] very recently reported that their thiono phosphine sulfide reagent 1 is a good carboxyl activating agent allowing ready formation of amide bonds from amines and recommended this method for the synthesis of peptide bonds. In their paper, no mention was made that the amide thus generated will also undergo thionation.

1.2.1 Thiocarbonyl Analogues of Substrates for Chymotrypsin

It was of major importance to us to investigate the susceptibility to enzymatic hydrolysis of relevent thioamide analogues. At the time, no such information was available in the scientific literature. We chose to first pursue such a study with the enzyme α-chymotrypsin. This endopeptidase has a nucleophilic serine residue at its active site and is thus classified as a serine protease[123]. It was first discovered by Kunitz in 1933[124] and its properties have since been thoroughly investigated[125]. Presently, more information is available about its structure and catalytic properties than for most other enzymes. It is synthesized in the pancreas of mammals and is then secreted in

the intestine where it degrades peptides at the carboxyl side of aromatic residues. The cleavage product contains the aromatic residue with a free carboxyl group. The best model substrates for chymotrypsin are the N-acetyl derivative of L-phenylalanine amide[127], ester, or thioester. The D-enantiomers are hydrolyzed at very low rates[128]. Substrate hydrolysis follows saturation (Michaelis-Menten) kinetics commonly described by the following equation[129]:

$$1/v = (1/S)\ K_m/v_{max} + 1/v_{max}$$

The widely accepted mechanism for this process involves attack of the substrate carbonyl by the hydroxyl group of Ser_{195} as catalyzed by His_{57} to give, after breakdown of the tetrahedral intermediate, the corresponding acyl-enzyme derivative. This is then followed by cleavage of the serine ester with a water molecule to regenerate the serine hydroxyl group and liberate the carboxylic acid product (Fig. 15)[130].

It was of interest to verify whether this mechanism would be equally effective with thiocarbonyl analogues of substrates and accordingly, we set about to synthesize such compounds. The unsubstitutued thioamide derivative of N-acetyl-L-phenylalanine 29 was our first goal. The D,L-form of this compound had been synthesized by Peterson and Nieman[131] (by thiolysis of the corresponding nitrile), but its behavior toward enzymes had never been reported. The preparation of Boc-Phe-C(S)NH_2 (18) was described earlier in this chapter. Removal of the Boc group

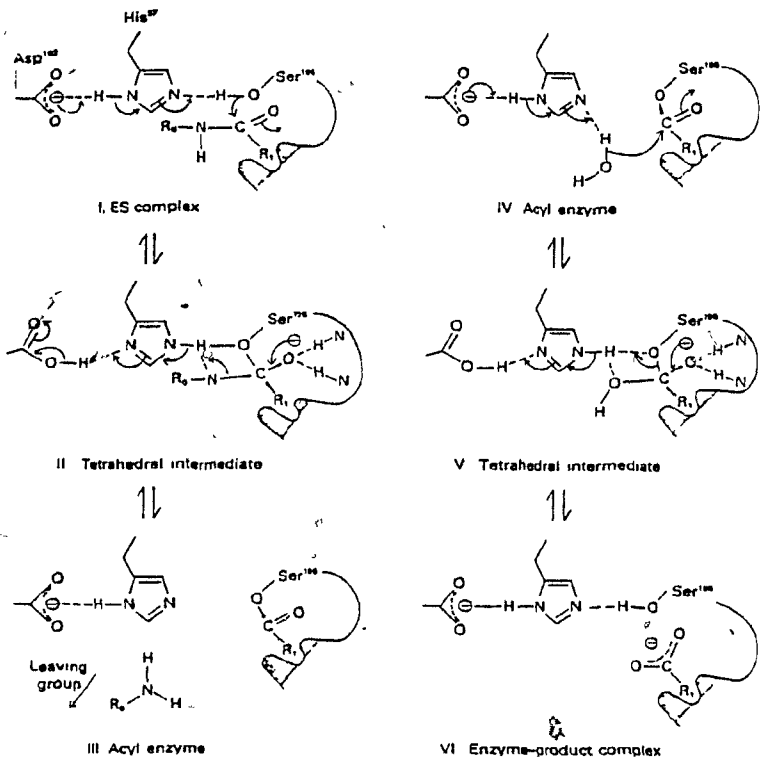


Figure 15 A mechanism for serine protease hydrolysis of peptides or amides. In this representation, the proton shuttle is concerted.

was accomplished by treatment with HCl/ether[132] and the crystalline hydrochloride salt thus obtained was acetylated under standard conditions (Ac_2O/pyridine)[133] to give 29 which was purified by recrystallization (mp 158°-160°C). Surprisingly, this primary thioamide proved to be quite unstable, reverting to the precursor amide after contact with silica gel for 1 to 2 h. It is known that primary thioamides can eliminate H_2S to give the corresponding nitrile when exposed to base[134]. This chemical instability could seriously interfere with the interpretation of enzymatic results

which led us to avoid its use as the only probe for active sites.

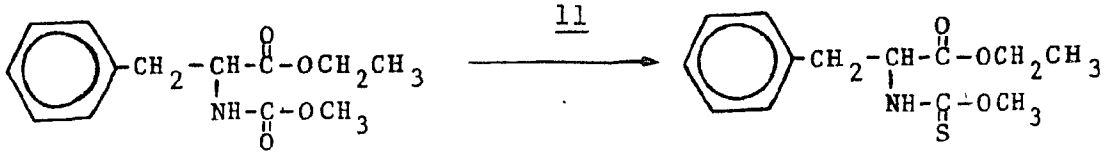
We turned our attention to the N-acetyl derivative of Phe-C(S)NHCH$_3$ (30) which was prepared following a similar reaction sequence. The N'-methyl amide is also a known substrate for α-chymotrypsin[135-136].

As mentioned above, the reactivity of thionester substrate analogues toward hydrolysis was also of interest to us. It is well-known that the ethyl ester of N-acetyl phenylalanine is an excellent substrate for α-chymotrypsin[137-138]. In contrast to enzyme-catalyzed amide hydrolysis, it is the deacylation of the acyl-enzyme intermediate which is the rate limiting step (k_3) for ester hydrolysis[139].

Thus the behavior of N-acetyl-L-Phenylalanine thionester (40) toward the enzyme may provide additional insight into its mechanism of hydrolysis.

The synthesis of thionester 40 was not as straightforward as we had originally anticipated. As noted earlier, high temperature and long reaction times are required in order to directly transform simple esters into thionesters with reagent 1 or 11. Other known methodologies[140] for this conversion were not attractive due to the large number of steps involved and to the racemization induced by the experimental conditions of some intermediates. Instead, we attempted the thionation of Boc-Phe-OEt (32) with thiono phosphine sulfide 11 in toluene at 110°C, but this led to the formation of several compounds as indicated by TLC. Some of these probably resulted from the loss of the

heat-labile Boc group[141] followed by attack of the amine at the phosphorous of the thionation reagent. The same reaction conditions were then applied to the heat-stable methyl carbamate derivative 34.

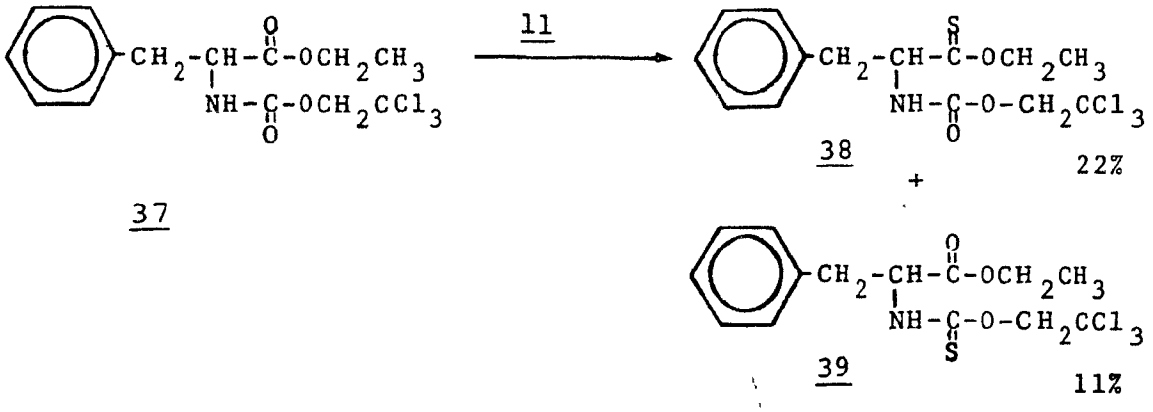


34 35

After 24 h, the reaction was stopped and the mixture analyzed by TLC which revealed the presence of several compounds in addition to starting material. After partial purification, the ^{1}H NMR spectrum of this mixture indicated that both the thiocarbamate 35 and the thionester 36 were produced in 37% and 7% yields, respectively. The thiocarbamate methyl protons were shifted downfield to 4.0 ppm, from 3.6 in the starting material, and the NH was shifted to 6.8 ppm while the ethyl resonances appeared unchanged.

The results indicate that the carbonyl of the methyl carbamate group is more reactive toward reagent 11 than the ester carbonyl, thus emphasizing the necessity of using a carbamate whose carbonyl is deactivated or sterically shielded while displaying stability to high temperatures. The trichloroethyl carbamate (Troc) derivative appeared promising in these respects because the trichloromethyl group is reasonably bulky and sufficiently electronegative to deactivate the carbamate carbonyl[142]. Indeed, after heating the Troc-Phe-OEt (37) with the thionation reagent 11 in

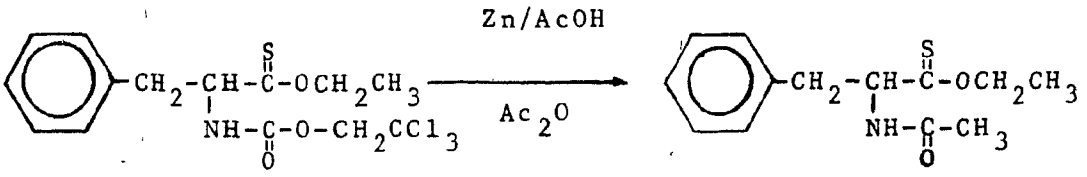
refluxing toluene for 24 h, a large amount of starting material was still present as judged by TLC. Another equivalent of reagent 11 was added and heating was continued for an additional 24 h but no significant change in the TLC profile was produced. Extensive purification of the reaction mixture by flash chromatography on silica gel afforded the desired N-Troc thionester (38) in 22% yield, the carbamate ethyl ester (39) in 11% yield and unreacted starting material (38%).



The identity of these compounds was easily ascertained by ^{1}H NMR spectroscopy owing to the characteristic downfield shift of the protons on carbons adjacent to the thiocarbonyl groups. The spectrum of thionester 38 showed that the methylene protons of the ethyl group were shifted downfield to 4.34 ppm and the CH_α to 4.8 ppm. The ^{1}H NMR spectrum of thiocarbamate (39) displayed a downfield shift of the two doublets at 5.12 and 4.95 ppm attributed to the methylene CH_2 of the trichloroethyl group. In addition, the methylene protons of the ethyl group of thionester (38) appeared as a multiplet and not as the expected quadruplet. Thus the presence

of the thiocarbonyl function makes these two diastereotopic protons non-equivalent leading to more complex coupling patterns. When the methyl group of the ethyl ester was irradiated, the methylene signals appeared as an overlapping doublet of doublets (J=12 Hz), due to geminal coupling.

Although the yield of the desired thionester was disappointing the process could be easily scaled-up to provide sufficient amounts of material (25 mmol) for further transformations. Removal of the trichloroethyl protecting group of 38 was accomplished with zinc in acetic acid[143]. Isolation of the deprotected amino compound was somewhat troublesome because of contamination by other products. However, good yields of the desired N-acetylated product were obtained when excess acetic anhydride was present in the reaction mixture. It proved to be more practical to carry out the



deprotection and acetylation steps in a single operation by stirring a mixture of the thionester 38 with zinc, acetic anhydride and acetic acid for 24 h at room temperature. After filtration of the excess zinc and evaporation of the filtrate in vacuo, the residue was purified by flash chromatography on silica gel and the product recrystallized from ether/hexanes. The N-acetylated derivative (40)

was optically active ($[\alpha]_D^{20}$ +33.0°, c 1.0, EtOH) and its ^{1}H NMR spectrum showed the multiplet characteristic of the two disastereotopic methylene of the ethyl group. The ^{13}C NMR spectrum of this product showed a resonance at 218 ppm which is characteristic of the thiocarbonyl carbon. Finally, its mass spectrum displayed the expected molecular ion ($M^{+\cdot}$) at 251.

The unsubstituted thioamide 29, the N'-methyl thioamide 30 and the thionester 40 were then tested as potential substrates for bovine α-chymotrypsin (Sigma) under standard conditions (25°C, 0.02M Tris buffer, pH 7.8, 0.2M KCl, enzyme 1×10^{-6} M)[144]. Since the thiocarbonyl group of thioamides and thionesters is a strong chromophore in the UV region (ε≈10,000 at 260-275 nm), their hydrolysis can be monitored by measuring decreases in absorption at or near to their characteristic λ_{max} values. No significant hydrolysis was observed for any of the three compounds 29, 30, 40 ($c = 1 \times 10^{-4}$ M) as reflected by no net change in absorbance over 3 h periods. Under the same conditions, N-Cbz-L-Tyr-p-nitro-phenylester was completely hydrolyzed after a few seconds[145].

Moreover, no enzyme-catalyzed hydrolysis was observed using the pH-stat method, for the thionester 40. Preincubation of the thionester with α-chymotrypsin slowed the hydrolysis of N-Ac-L-phenylalanine ethyl ester, indicating that the thionester is bound by the enzyme but in an unproductive manner.

Our preliminary results were recently corroborated by Asboth and Polgar[146] who showed, using the pH-stat method, that

the racemic N-Ac-PheC(S)-OEt is not hydrolyzed by α-chymotrypsin. Using N-Ac-L-Tyr-OEt as the substrate, they showed that the thion ester behaves as a weak competitive inhibitor of the enzyme. Its binding constant (K_s) reached a value of 4.5×10^{-3} M which compares favorably to the value of 7.4×10^{-3} M that is characteristic of the parent oxygen ester[147].

The complete resistance of these compounds, especially the thionester, towards attack by α-chymotrypsin was somewhat surprising to us. Several previous studies[148-149] have shown that the chemical reactivity of oxygen esters and thionesters towards hydroxide ion is very similar and the rates of their hydrolysis are of the same magnitude. In a related study, Campbell and Nashed[150] recently reported a K_{oxy}/K_{thion} ratio of 1.2 for the hydroxide catalyzed hydrolysis of N-Ac-PheC(O)-OCH_3 and N-Ac-PheC(S)-OCH_3. It is also known that the hydrolysis of thionesters proceeds via the formation of tetrahedral intermediates in a pathway similar to that of esters[148-151]. Kaloustian et al[152-155] have recently demonstrated that the breakdown of these tetrahedral intermediates is subject to stereoelectronic control. (Deslongchamps' rules)[156]

The only other example of enzyme catalyzed hydrolysis of thionesters is that of Lowe and Williams who reported that Cbz-GlyC(S)-OEt is hydrolyzed by papain at a rate about 10 times slower than the parent ester[157]. However, direct comparison of α-chymotrypsin and papain is not possible because of the marked differences in their specificity and mechanism of action. A major difference

is that the active site of papain has a thiol (cysteine) as the attacking nucleophile[157].

In the case of α-chymotrypsin, it is clear that the increased bond length of thiocarbonyl vs carbonyl interferes with key steps involved in the hydrolysis. It is possible, as pointed out by Asboth and Polgar[146], that the larger volume of the sulfur atom and the increased length of the C-S bond disrupt the fit within the oxyanion pocket of the enzyme where stabilization of the tetrahedral intermediate involves hydrogen bonding with $NH\text{-}Gly_{193}$ and $NH\text{-}Ser_{195}$[123,158]. The lengths of these hydrogen bonds have been recently estimated to fall in the range of 1.6-1.7 Å[159] and since the C-S bond is longer (1.81 Å vs 1.43 Å for C-O in neutral compound[31]), the sulfur anion of a tetrahedral intermediate may be impossible to accomodate in this enzyme pocket.

We also examined if the reluctance of the thionester to undergo hydrolysis was dependent on the stability of a thioacyl-intermediate $Ser_{195}\text{-}O\text{-}C(S)$ with N-Ac-L-PheC(S)-SCH_3 (57, Sect. 1.3) Formation of a Ser_{195}-O-C(S) bond with this dithioester analogue would cause a strong change in the UV spectrum since the λ_{max} would shift from 310 nm (C(S)-SR) to 245 nm (C(S)-OR).

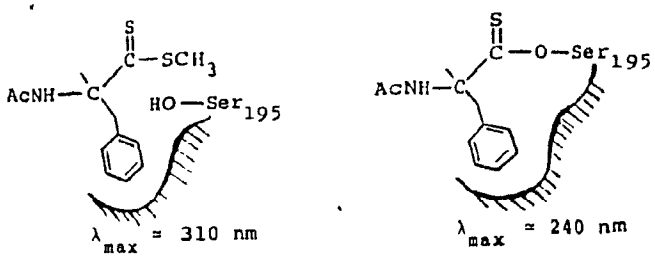


Figure 16

No change in the UV absorbance was observed even at very high enzyme concentrations (1×10^{-5} 1×10^{-4} M) after a period of two hours, thus indicating that no thioacyl-enzyme had formed. This result also indicated that the explanation for the lack of hydrolysis observed for the thiocarbonyl compounds originates prior to the formation of the acyl-enzyme intermediate.

Other types of experiments, such as trapping of the tetrahedral intermediate (by oxidation or alkylation) or X-ray analysis of the enzyme-thionester complex, would be necessary in order to improve our understanding of the parameters responsible for the resistance of the thionester to enzyme-catalyzed hydrolysis. The parallel behaviour of the thioamide analogues toward α-chymotrypsin is likely to originate from the same parameters.

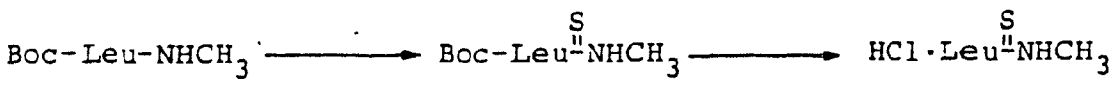
1.2.2 Thioamide Analogues of Leucine Aminopeptidase Substrates

The interaction of thioamide substrate analogue with leucine aminopeptidase (LAP EC 3.4.11.1) was also studied. Although not as well characterized as α-chymotrypsin, this exopeptidase has been shown to inactivate several biologically active peptides through cleavage of the N-terminal amino acids residues[160]. It belongs to a broad class of zinc metallopeptidases but little information is yet available on its mode of catalysis. The enzyme is a hexamer with a molecular weight of 320,000[161] and contains between 4 to 6 atoms of zinc per 300,000 g of protein, which suggests that there is one metal binding site per subunit[162]. Smith and Spackman[163]

showed that the enzyme is activated by Mg^{+2} and Mn^{+2} ions and speculated that these ions are bound at the active site. Recently, Van Hart and Lin[164] showed that activation of the enzyme by Mg^{+2} and Mn^{+2} is due to the binding of one additional mole of these metals per subunit, rather than through their replacement of the catalytic Zn^{+2} atoms.

The action of LAP is not limited to leucine-containing substrates, as its name would suggest. In fact, the rate of hydrolysis of different substrates is primarily determined by the non-polar nature of the residue carrying the free amino group[165]. Because of the known ability of thioamides to form complexes with Zn^{+2}, we speculated that thioamide analogues of substrates of LAP might behave as excellent inhibitors of the enzyme. Incorporation of other good Zn^{+2} coordinators such as thiol, hydroxamate or phosphate groups at appropriate positions of peptidic structures has led to the discovery of inhibitors of certain zinc-dependent enzymes[166-168].

Accordingly, we prepared the thioamide analogue of the substrate N'-methyl L-leucine amide by thionation of the Boc-protected amide 42 with reagent 11, under conditions already described.



41 42 43

The desired thioamide 43 was obtained as a crystalline solid after removal of the Boc group with HCl/ether. It was incubated with LAP (Porcine Kidney, Sigma) under standard conditions (0.5 M Tris buffer, pH 8.5, 5mMol $MgCl_2$)[169] and the possibility of hydrolysis was

monitored spectrophotometrically at 288 nm (which is near the λ_{max} of the thioamide function). No decrease in absorbance was observed even after 1 h of incubation. Using similar conditions, the inhibitory properties of the analogue were evaluated using L-Leu-p-nitro-anilide (Sigma) as the substrate[169]. Product formation was recorded at 405 nm, where absorption increases with time due to the formation of the cleavage product p-nitro-anilide. This catalyzed hydrolysis was monitored for a substrate concentration range of 6.4×10^{-4} M to 5×10^{-3} M in the presence of fixed concentrations, ranging from 1.9 to 7.4×10^{-3} M, of N'-methyl-L-leucine thioamide (43). Double reciprocal plots (1/V vs 1/S)[170] were constructed from the data (Fig. 17) and the results strongly suggest a pattern of competitive inhibition.

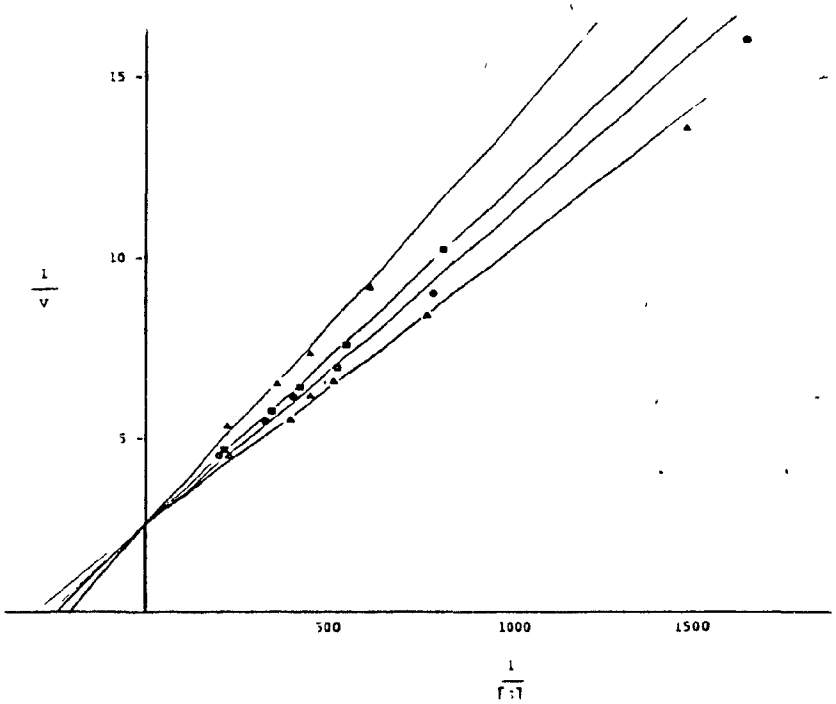
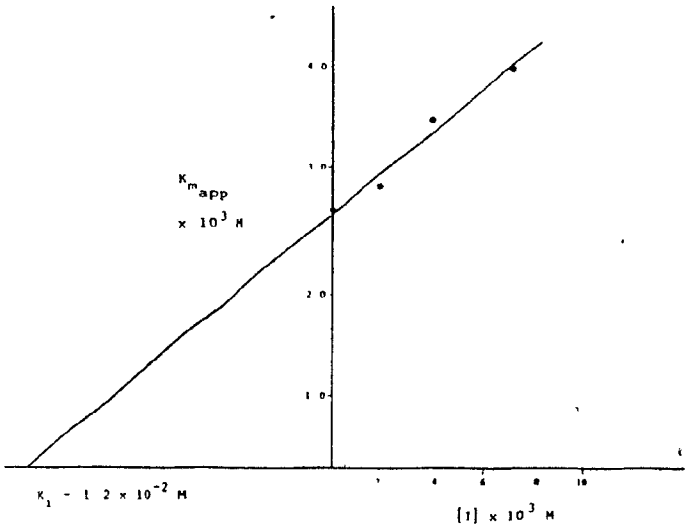


Figure 17 Lineweaver-Burk plot of L-leucine p-nitroanilide hydrolysis in the presence of fixed concentration of N'-methyl L-leucine thioamide as inhibitor: (▲) 0.0 M ; (●) 1.94×10^{-3}M ; (■) 3.72×10^{-3}M ; (▲) 7.20×10^{-3}M

The inhibition constant, K_i, was evaluated by plotting the values of $K_{m_{app}}$ vs inhibitor concentration (Fig. 18). A linear regression analysis of the data provided a K_i value of 1.2×10^{-2} M.



$K_{m_{app}}$ vs [I] for N'-Methyl L-leucinethioamide (L-leucine p-nitroanilide as substrate)

This large K_i value was of course disappointing because it indicates that the thioamide analogue 43 has a very weak affinity for the active site of the enzyme and thus may probably not bind to the zinc atom. In contrast, L-leucine hydroxamate has a high affinity (K_i 3.5×10^{-7} M) for Aeronomas LAP, which presumably results from tight binding through coordination of the hydroxamic acid function with the zinc atom[166]. On the other hand, Nishino and Powers[171] reported that analogues, which incorporated thiosemicarbazides functions (R-NH-NH-C(S)-) into good substrates of thermolysin behaved

as weak inhibitors (K_i 6.7 x 10^{-3} M) of the enzyme, even though it is a zinc dependent protease. It is conceivable that the sulfur atom of thioamides of thiosemicarbazides is not sufficiently electronegative to bind to the zinc atom at the active site. On the other hand, this result might simply indicate that the zinc atom does not coordinate with the oxygen of the carbonyl in normal substrates[172]. The very weak inhibitory activity of our thioamide analogue may also reflect unfavorable steric interaction created by the larger volume of the sulfur atom. It is unlikely that other factors such as difference in the respective pk_a's of the α-amino group of the amide and thioamide ($\Delta pk_a \approx 0.1$)[173] are responsible for the weaker affinity of the thiocarbonyl analogue for the enzyme.

While our work was in progress, three groups reported their results on the interaction of thiopeptide substrate analogues with carboxypeptidase A (CPA, EC 3.4.17.1)[150,174,175]. All three groups reported that thioamide analogues of good substrates of CPA were not readily cleaved by CPA, and behaved as weak competitive inhibitors. The observed K_m values demonstrated that the thioamide analogues have affinities for the active sites similar to those of the amide substrates. Interestingly, Campbell *et al*[150] also observed that a thionester analogue was cleaved at a rate similar to that of the oxygen ester.

Peptides	Rate of hydrolysis vs parent peptide	K_m(oxy) x 10^{-3} M	K_m(thio) x 10^{-4} M	K_i x 10^{-3} M	Ref.
Bz-Gly-Gly-$\overset{S}{\overset{\|}{C}}$-Phe	3%	3.4	4.5	-	174
Cbz-Gly-$\overset{S}{\overset{\|}{C}}$-Phe	10%	2.0	11.0	1.4	175
Bz-Gly-Gly-$\overset{S}{\overset{\|}{C}}$-Phe	.1%	1.0	8.0	-	150

Figure 19 Interaction of CPA with thiopeptides.

On that basis they postulated that the thiopeptide analogues were hydrolyzed more slowly than the parent peptide because of the higher rotational barrier of the thioamide bond. Their results support the Cleland mechanism for CPA-catalyzed hydrolysis which postulates that attack

by the enzyme requires peptide bond rotation a process which would be rate-limiting for hydrolysis[176]. The ratio of their rates of hydrolysis is about 1100[150]. This value corresponds to a 4.1 Kcal/mol difference in free energy, which is in agreement with the difference in activation energy for rotation of thioamide vs amides[31-37].

On the basis of these three examples with different enzymes, one may conclude that the thiopeptide linkage is relatively stable to degradation by peptidases. The fact that thiopeptide analogues retain significant affinity for enzymes with the exception of LAP strongly suggest that similar analogues of larger polypeptidic regulators would display good affinities toward relevant receptors. The resistance of thiopeptides toward endogenous peptidases may be reflected in pharmacological responses of a more prolonged duration relative to the parent peptides.

1.3.1 Thioamides and Peptide Bond Formation

Nucleophilic functionalities present in a number of amino acids must be selectively protected in order to avoid their interference in peptide bond formation[177]. As indicated earlier these same functionalities can also react readily with the thiono phosphine sulfide reagents, and thus require protection when thionation of a peptide bond is performed. In principle the usual protecting groups encountered in modern peptide synthesis may also be used during thiopeptide synthesis, largely because the reaction conditions developed

in our laboratories are remarkably mild. The Boc group, as we have already shown, can be used to protect the α-amino group of amino acids since it remains intact during amide bond thionation, and the conditions for its subsequent removal with mild acid (HCl/ether) or formic acid) are also compatible with the thioamide function. Alkylation of the thioamide function by t-butyl carbocations generated in situ does not readily occur under the conditions for deprotection. However, the strong acidic conditions widely used in routine peptide synthesis should be avoided when dealing with thiopeptides. Alkylation of the methionine sulfur by the t-butyl cation is a significant side reaction when trifluoracetic (TFA) or HF is used to cleave the Boc protecting group[178-179]. Moreover, it is well-known[180] that treatment of an N-terminal thioamide-containing peptide with TFA results in cleavage of the N-terminal amino acid residue as a result of intramolecular attack leading to thiazol-5(4H)-ones (46). An analogous reaction of pehnylthiocarbamates on N-terminal groups forms the basis of the widely known sequential Edman degradation of polypeptides[181-182].

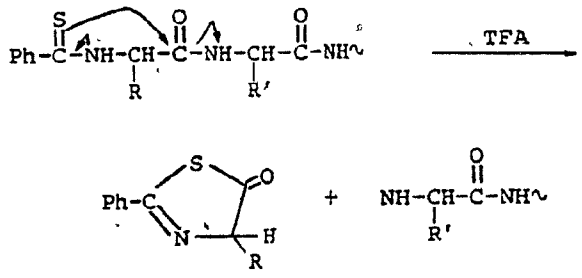


Figure 20

Ried[87-89] has successfully used the carbobenzoxy (Cbz) protecting group for the amine function in the synthesis of thiopeptides. Its subsequent removal by HBr/AcOH was apparently compatible with the thioamide function. These observations were later substantiated by Clausen et al[183]. Du Vigneaud et al[30] used similar conditions in the synthesis of the C-terminal primary thioamide analogue of oxytocin. Protection of the carboxyl group is simply accomplished by ester formation (Me, Et), its regeneration only requiring base-catalyzed hydrolysis under conditions known to leave thioamide bonds intact[184,185]. Several reagents are now known which will readily promote the coupling of amines with carboxyl groups to form peptide bonds[186]. Although the acylation of thioamides can be readily achieved[187-189], it was not expected that this function would effectively compete with a nucleophilic terminal amino group in acylation reactions. In fact, Ried et al[87-89] have already shown that thiodipeptides can be elongated from their C-terminal ends. Moreover, Du Vigneaud et al[28] have demonstrated that primary thioamides are not affected by the coupling reagents DCC-HOBt[189]. Members of our group have successfully used EEDQ[190] as a convenient peptide bond-forming reagent in the presence of thioamide functions.

Coupling from the C-terminal end of a thiodipeptide was not feasible. F. Lépine of our laboratories has shown that treatment of Boc-GlyC(S)-Gly-OH (44) with an amine in the presence of DCC or EEDQ does not lead to amide bond formation[191]. Instead, the thiopeptide is converted to the corresponding thiazlactone

(thiazol-5-4-H-ones) 46 as revealed by NMR spectroscopy. This thiazlactone tautomerized rapidly to 47[192] as demonstrated by proton exchange experiments in D_2O, a process readily monitored by NMR spectroscopy.

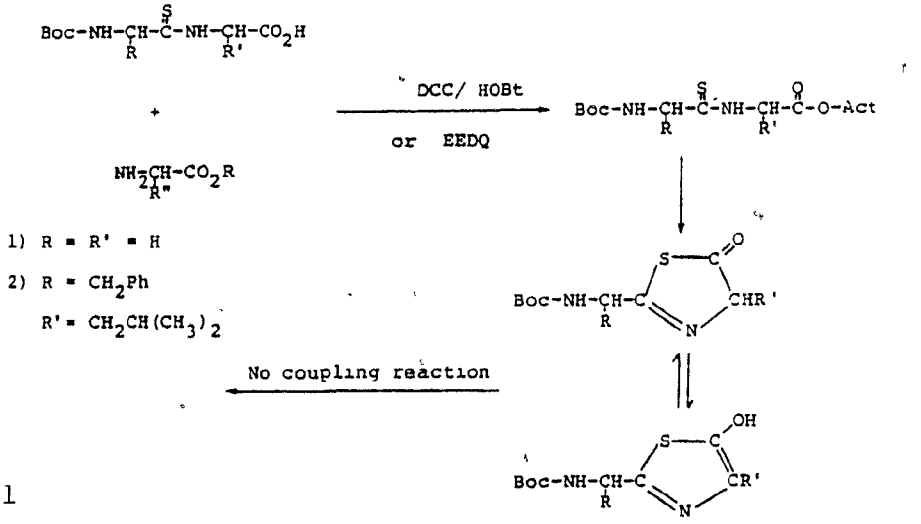


Figure 21

Thiazlactone formation also occurred with thiodipeptides possessing bulkier side chain substituents, as are present in Boc-LeuC(S)-Phe-OH[48]. These lactones were insufficiently reactive to participate in coupling reactions. Aminolysis of such compounds finds precedent in the literature[193-194]. For instance, 4-substituted 2-phenyl-4H-ones have been described as weak acylating agents towards amino acids[193]. However, in our hands, no acylation products were detected under our experimental conditions.

1.3.2 Synthesis of Dithioester Derivatives of Boc-amino acids

The impossibility of lengthening thiodipeptides from the C-terminal end seriously restricts accessibility to various oligo-

thiopeptides especially in those cases where relative size of the amino acids side chain does not favor the thionation of a particular amide bond. Thus it was necessary to investigate the possibility of preparing optically active Boc-protected dithioester derivatives of amino acids, species known to possess good thioacylating properties toward simple amines[140].

Dithioesters can be prepared by thiolysis of alkyl thioimidates[195]. The latter are most often prepared from the corresponding nitriles through addition of an alkyl mercaptan under anyhdrous acidic conditions[196].

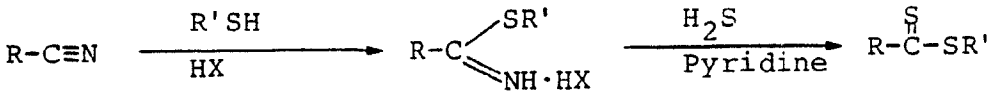


Figure 22

The N-acetyl, N-benzoyl methyl dithioesters of glycine and phenylalanine (as a racemate) were in fact prepared by this method[197]. Obviously, a Boc group would not withstand such strong acid conditions required in the addition step. The thioimidates can be obtained from the corresponding thioamide through alkylation of the sulfur at low temperature in essentially neutral conditions. The resulting thioimidate salt can then be subjected to the thiolysis conditions (H_2S/pyridine) originally described by Marvel et al[195].

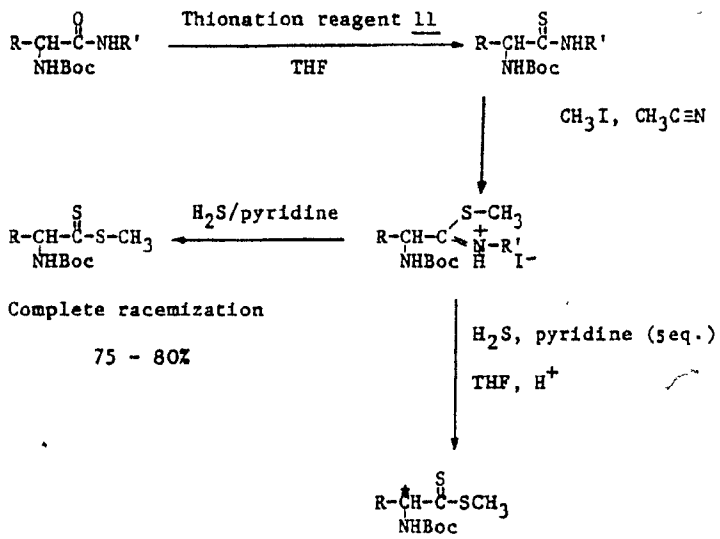


Figure 23

Accordingly, the N'-methyl N-Boc Phenylalanine thioamide 20 was alkylated with methyl iodide (1.5-2.0 eq) in acetonitrile at 30°C for 24 h. After evaporation of the solvent, the resulting thioimidate salt 49 could be recrystallized from CH_3CN/ether. It was sensitive to moisture and regenerated starting material when left in solution for long periods of time. The ^1H NMR spectrum of this compound showed the characteristic resonance for SCH_3 (singlet at 2.58 ppm) and the CH_α proton was now shifted upfield to 5.1 ppm from 5.5 ppm in the thioamide. Interestingly, 47 was optically active ($[\alpha]_D^{20}$ +55.0°, (c 1.0, $CHCl_3$)). The thioimidate salt was treated with H_2S in dry pyridine (saturated solution) at 0°C for 30 minutes. After evaporation of the excess H_2S and pyridine, the resulting solid was purified by flash chromatography on silica gel in order to remove the thioamide precursor, which was the major

contaminant. A bright yellow solid was thus obtained, the color of which is characteristic of aliphatic dithioesters[195]. Its UV spectrum showed the expected absorption with a λ_{max} at 308 nm (log ε 4.1). Its ^1H NMR spectrum (200 MHz, $CDCl_3$) displayed resonances for the S-methyl protons at 2.58 ppm and for the CH_α proton gave a multiplet at 5.1 ppm. Its mass spectrum included the expected molecular ion ($M^{+\cdot}$) at 311, but also an important fragment (18.4%) at 220 resulting from the loss of the C(S)-SCH_3 group. The dithioester 50 displayed no optical activity, thus indicating that the chiral center at position 2 had completely racemized during thiolysis. This loss of optical activity was not too surprising in light of the known acidities of the α-protons of the thioimidates and dithioesters[79,198-199]. Obviously, exposure of these compounds to basic conditions must be minimized if racemization is to be avoided. The reaction was therefore repeated under essentially the same conditions, but work-up of the mixture was carried out rapidly by immediate extraction with a mixture of ether/5% citric acid solution. This proceedure gave a dithioester that had retained some optical activity ($[\alpha]_D^{20}$ +13°, (c 1.0, $CHCl_3$)). Further modifications of the reaction conditions were explored and eventually excellent results obtained by performing the thiolysis reaction at 0°C over a 20 min period in THF saturated with H_2S, followed by rapid washings with aqueous citric acid. The yield of dithioester 50 was slightly decreased but a much higher optical rotation was obtained ($[\alpha]_D^{20}$ 80.5°,c 1.0,$CHCl_3$).

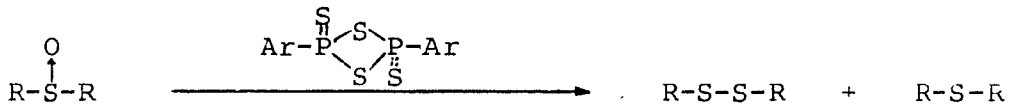
Recrystallization from hexanes did not increase the optical

activity or the melting point. Unfortunately, attempts to determine the optical purity of the product by ^1H NMR spectroscopy in the presence of the shift reagents $Eu(thc)_3$ and $Eu(fod)_3$ were not successful.

With this optically active compound on hand, it was then possible to demonstrate the ease with which such dithioesters racemize in the presence of base. For instance, in dry THF as the solvent, dithioester 50 had an $[\alpha]_D^{20}$ of -17.5°. Triethylamine (1 eq) was then added to that solution; after 5 min the rotation had decreased to -15.4° and after 1.5 h to -11.6°. Racemization was complete ($[\alpha]_D^{20}$ 0°) after 10 h at room temperature. The thioimidate 49 also racemized rapidly: its optical rotation measured in THF at 0°C was +51.2°, but decreased to +21.5° and remained unchanged for 15 minutes following the addition of pyridine (1 eq). This value corresponds to the optical rotation of the free imidate. The subsequent addition of triethylamine (1 eq) caused a rapid decrease of the rotation to +10.3° after 5 minutes. A possible explanation for this anomalous result is that the thioimidate was contaminated by thioamide precursor which does not racemize readily in these conditions. In fact, Boc-Phe-C(S)-$NHCH_3$ (19) failed to racemize significantly at room temperature even after 12 h in the same conditions.

This methodology for dithioester synthesis should be applicable to other amino acids, perhaps with the exception of methionine and cysteine where methylation of sulfur would be expected to occur. However, the thiol group of cysteine can easily be masked by a

variety of protecting groups such as triphenylmethyl thioether[200] or as its corresponding thioester[201] which would reduce its susceptibility to alkylation by methyl iodide. The methyl thioether function of methionine is frequently protected by oxidation to the sulfoxide[202,203]. However, sulfoxides are not compatible with thionation reagents 1 or 11 which convert them to sulfides and disulfides even at room temperature[204].



Since the methionine thioether must compete with the thioamide for methyl iodide in the alkylation reaction, it was believed that the thioamide function may be kinetically favored. The only example of such a competition was given recently by Ireland and Brown[205] who showed that a thioamide sulfur is alkylated preferentially in the presence of a thioether function:

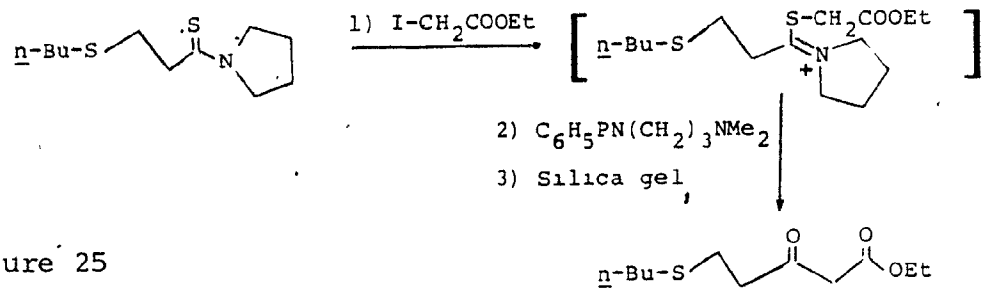


Figure 25

Encouraged by this observation Boc-Met-C(S)-$NHCH_3$ (51) was prepared by the methodology described earlier. Treatment of 49 with methyl iodide under reaction conditions identical to those described for the preparation of Boc-Phe-C(S)-SCH_3 yielded a mixture of products,

several of which proved insoluble in $CHCl_3$. The ^1H NMR spectrum of the soluble fraction showed that part of the mixture contained the product of thioether methylation (≈40%). The crude solid was then subjected to our modified thiolysis conditions (H_2S/THF, pyridine 5 eq, 0°C) to yield after work-up two major products as judged by TLC

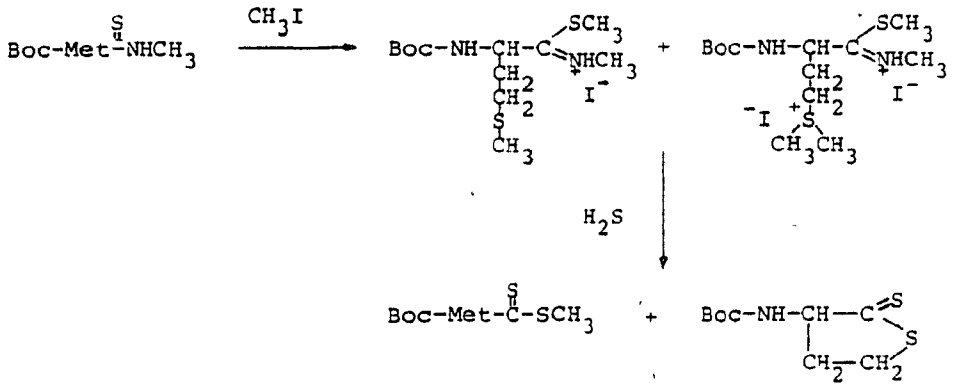


Figure 26

After purification by flash chromatography on silica gel, two yellow compounds were isolated: the most polar compound (R_f 0.3) was identified (^1H NMR, UV, Mass spectrum) as the desired dithioester 54 of Boc-Met (7% yield); the least polar material (R_f 0.4) was found to be cyclic dithioester 55. Its ^1H NMR spectrum displayed complex coupling patterns for all protons. The most striking feature was the difference in chemical shift between the β and β' protons:

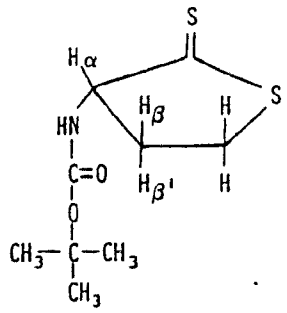
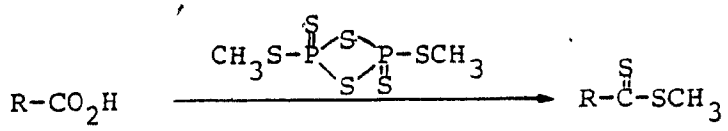


Figure 27

one proton appearing as an octet centered at 2.13 ppm and the other as a complex multiplet centered at 3.12 ppm. These assignments were confirmed by irradiation of the CH_α proton at 4.37 ppm which resulted in simplification of these two resonances. The mass spectrum of the compound included a strong molecular ion at 233. Not surprisingly, it was optically inactive. Its formation resulted from an intramolecular reaction involving attack of the dithioether by a γ-thiol function generated initially by S_N2 displacement of a sulfonium leaving group by an HS-anion or through S-demethylation of the thioether by the same nucleophile. The ratio of 54 to 55 might be altered by using lower concentrations of H_2S, a possibility which was not evaluated.

While this work was under way, Davy[206] described the direct formation of methyl and ethyl dithioesters from carboxylic acids by treatment with a new thiono phosphine disulfide reagent, namely 2, 4-bis-methylthio-1,3,2,4-dithiophosphetane-2,3 disulfide (56).



This process requires too high a temperature for it to be of practical value in dithioester formation from heat sensitive N-protected amino acids. However, we verified that this new thiono phosphine disulfide 56 can also transform amides into thioamides in THF at rates comparable to those observed with our reagent 11. For instance, reagent 56 (0.6 eq) converted Boc-Phe-$NHCH_3$ into the

corresponding thioamide after 2 h (as compared to 3 h with our reagent 11 under similar conditions.) However, 56 is not a choice reagent because of various drawbacks that it offers: it is much less soluble than our reagent in organic solvents, is formed in lower yields from P_4S_{10} and causes very obnoxious odors. It may also be expected to lack regioselectivity towards oligopeptides because of the absence of sterically meaningful substituents in the phosphorous atom.

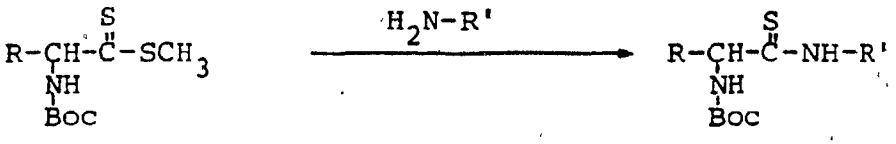
Using the thioimidate route described above, the N-acetyl-phenylalanine dithioester 57 was also prepared and used to study thiocarbonyl substrate analogue interactions with α-chymotrypsin. The Boc group was removed from the dithioester 40 with HCl/ether and the α-amino compound directly obtained in 85% yield as the crystalline hydrochloride salt. Acetylation of the amino group was cleanly accomplished in 3 h using acetic anhydride (1.1 eq) and pyridine (1.1 eq) in CH_2Cl_2 at RT. Higher concentrations of the anhydride/pyridine mixture caused the formation of several other products (TLC analysis). After washing with aqueous citric acid, the N-acetyl derivative was recrystallized from ether/hexanes and obtained as a yellow solid. It displayed the characteristic $\pi \rightarrow \pi^*$ transition in the UV at 308 nm. The ^1H NMR spectrum showed all the expected resonances, and the mass spectrum included the molecular ion, $M^{+\cdot}$, at 253.

1.3.3 Formation of Thiopeptides from Dithioesters

As anticipated, the Boc-Phe-C(S)SCH_3 (50) reacted rapidly with n-butylamine (2 eq) in THF at room temperature to form the corresponding N'-n-butyl thioamide within 10 min. However the reaction time was much slower for the less basic amino acid esters, and in addition steric factors as defined by the amino acid side chain, affect the reaction rate. For instance, the reaction time for gylcine methyl ester (HCl salt; 2 eq TEA) was 6 h whereas 4 days were needed for Leucine methyl ester (HCl salt; 2 eq TEA). In both cases the yields of chromatographically pure thioamides were excellent (>90%).

Unfortunately the reaction conditions caused complete racemization during the course of aminolysis as evidenced by the total absence of optical activity in the thioamide product Boc-Phe-C(S)-Gly-OEt (58). The ^1H NMR spectrum of Boc-Phe-C(S)-Leu-OCH_3 showed two signals of equal intensity for the OCH_3 protons as expected for a 50:50 mixture of two diastereomers. Clearly the basicity of the amine group as well as steric factors strongly affect the rate of formation of the thiopeptide linkage resulting from thioacylation by dithioesters. Other solvents, such as CH_2Cl_2, EtOH, DMF, had no effect on the rate of this reaction. We observed that the reaction is accelerated by triethylamine, imidazole and 4-dimethylamino-pyridine, but not by pyridine (as monitored by TLC). Despite the disadvantages of this approach which include reaction times and racemization, we were nevertheless able to prepare diastereomeric

thioamide analogues of important peptides not available by the thionation route described earlier. Thus Boc-Phe-C(S)-Gly-Gly-OEt (60) could be prepared in 92% yield by reacting Boc-Phe-C(S)-SCH_3 with Gly-Gly-OEt in the presence of TEA (1 eq). This thiotripeptide is a valuable analogue of the well-known tripeptide substrate[207] for Angiotensin converting enzyme (EC 3.4.15.1) and has formed the subject of an investigation by a colleague in our laboratories. (See L. Maziak, M.Sc. thesis).



R = CH_2Ph — Complete racemization

R'	Time	Yield
$(CH_2)_3CH_3$	.2 h	90%
CH_2CO_2Et	6 h	92%
$CH(CH_2CH(CH_3)_2)$	4 days	91%
$CH_2C(O)-NH-CH_2CO_2Et$	12 h	87%

Table 3 Formation of thiopeptides from N-Boc-dithioesters.

Chapter 2

Thioamide Analogues of the Chemotactic Peptide f-Met-Leu-Phe-OCH_3

2.1 Introduction

2.1.1 Definition and Characteristics

Chemotaxis is defined as a reaction by which the direction of locomotion of cells or organisms is determined by substances (chemoattractants) in their environment[208]. In prokaryotic cells, chemotaxis is a means of finding nutrients, whereas in highly organized systems such as man, it is a process by which cells of the immune system become localized at sites of inflammation[209]. In addition chemotaxis is thought to play a significant role in the metastasis of neoplastic cells[210] and in the migration of fibroblasts in wound healing[211]. The importance of leucocyte chemotaxis in the pathogenic cycle of rheumatoid arthritis (RA) is now well established[212-214]. The inflammation and degeneration of the connective tissues is the net result of local concentrations of inflammatory cells such as polymorphonuclear leucocytes (PMN, neutrophils), monocytes and macrophages which discharge chemical mediators (Fig.28). The chemotactic response of the PMN to the activated complement system is responsible for the migration of the cells to sites of injury. Thus, drugs capable of either

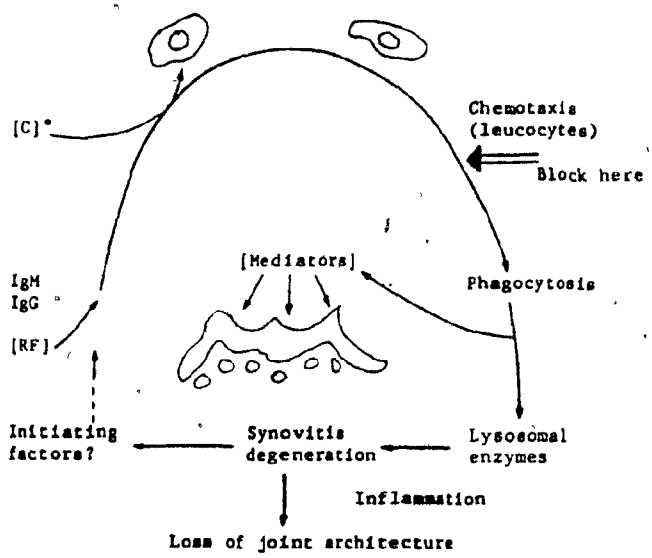


Figure 28 Pathogenesis cycle of rheumatoid arthritis: RF = rheumatoid factor, C = complement. The initiating factor(s) remains to be elucidated.[212]

selectively enhancing or depressing the chemotactic responsiveness of leucocytes and other cells are potentially useful in a wide variety of diseases[215]. For example, Turner et al[216] have recently reported that some anti-arthritic drugs such as gold thiomalate and aspirin at high concentration produce significant inhibition of chemotaxis.

There exist in the body several naturally occurring chemotactic factors which stimulate the cellular immune respones. These include such diverse compounds as: 1) the C_{5a} fragment and its metabolites associated with the complement system[217], 2) diverse lymphokines released by antigens or by mitogen-stimulated lymphocytes[218] and 3) metabolites of the arachidonic acid cascade (5-HETE, leukotriene B (5,12-di-HETE)[219-220] etc...

It was also observed that supernatants from cultures of rapidly growing bacteria contain potent chemoattractants for leucocytes[221]. Since bacteria, but not eukaryotic cells, initiate protein synthesis with an N-formyl methionine residue, it was postulated that peptides carrying that residue might be recognized as chemoattractants by leucocytes[222]. This led to the synthesis and discovery that certain synthetic peptides containing a terminal N-formylated methionine residue were potent chemoattractants for PMN, monocytes and macrophages[223]. Of these peptides, the formyl-L-methionyl-L-leucyl-L-phenylalanine (f-Met-Leu-Phe, f-MLP) was the most potent[224]. This discovery provided a unique tool that was largely responsible for the rapid development of our current understanding of chemotactic phenomena.

2.1.2 Molecular Events Leading to the Chemotactic Response

The chemotactic response of leucocytes differs from that of bacteria which is characterized by a swimming motion[225]. Leucocytes do not swim but crawl along surfaces[226]. Moreover, when leucocytes are exposed to a chemotactic gradient, a number of important biological responses are initiated in the cell and include: increased adherence[227,228], aggregation[229,230], change in cell shape[231,232], directed cellular motility[232], production of O_2^-[233] and arachidonic acid metabolites[234-236], and secretion of lysosomal enzymes[224,237]. This vectorial response to chemical stimuli is believed to be dependent on a complex but integrated

receptor system, which can sense the gradient and transduce the appropriate signal(s) to the inside of the cell leading to the chemolocomotary response[232,239]. The exact mechanistic details of these molecular events are still poorly understood but a large amount of relevant information has appeared in the literature of the past few years.

It is known that rapid changes in the flux of Na^+ and Ca^{++} across the PMN membranes, as well as changes in membrane bound Ca^{++}, are associated with the early events following chemotactic stimulus[239-243]. Other studies have suggested that there is also an early activation of PMN phospholipase A_2 by chemotactic peptides[244,245]. The existence of specific cell surface receptors for the chemotactic peptide f-Met-Leu-Phe has been demonstrated with the aid of radio-labelled ligands[248,249]. It was also shown that the ability of the peptide to bind to the receptor parallels exactly the ability of these peptides to initiate chemotaxis and to secrete lysosomal enzymes[224,250]. Neither the chemotactic peptide C_{5a} nor leukotrienes bind to the f-Met-Leu-Phe receptor[251]. A protein (68,000 dalton) from leucocyte membranes was recently isolated, purified and shown to be a constituent of the receptor for f-Met-Leu-Phe[252].

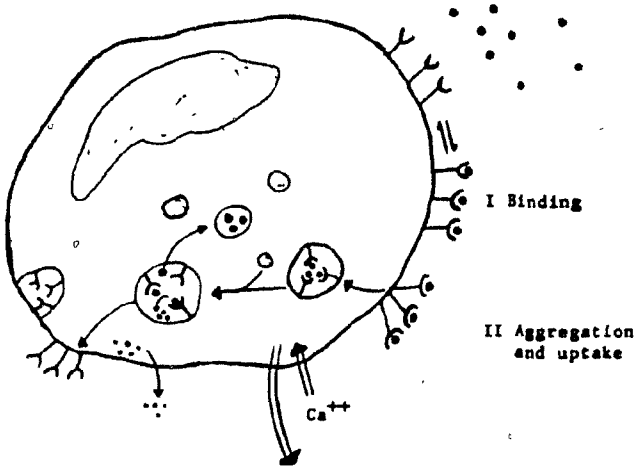
Very fine regulation of the receptors is necessary in order that the cell can migrate directionally in response to extremely small chemotactic gradients (estimated to be as low as 0.1 percent across the membrane surface)[253]. There are several lines of

evidence suggesting that these cell receptors are subject to up and down regulation[208]. Therefore the number, the affinity, and the distribution of the receptors at the cell surface are possible factors controlling cellular sensitivity to chemoattractants. When the leucocytes are exposed to agents that induced degranulation or when the cells are pre-exposed to very small amounts of chemotactic peptide, the subsequent response to chemotactic factors is enhanced[254,255]. The same effect is also observed when the cells are pre-exposed to n-propanol and n-butanol[256-257]. This led to the postulate that additional receptors normally buried in the membrane are available to aid the cells in sensing stronger gradients of chemoattractants[256]. On the other hand, pre-exposure to high concentrations of chemoattractant renders the cells unresponsive to the same agents at concentrations which normally induce a response[258-260].

Other mechanisms are probably involved in the cellular perception of a chemical gradient. It was originally believed that proteases at the membrane surface are available for the degradation and/or inactivation of the chemotactic peptide[261]. The presence of protease inhibitors, especially those of serine protease, prevents chemotaxis[262]. HPLC analysis of peptide catabolism showed a good correlation between the susceptibility of peptides to degradation and their relative potencies as chemoattractants[261]. This early observation was later substantiated by demonstrating that chymotrypsin inhibitors prevent the production of O_2^- by human PMN[263].

Known substrates for chymotrypsin also altered, in a dose dependent fashion, the production of O_2^- through competition with the natural peptide substrate (chemoattractant) for that (those) proteases(s) involved in PMN O_2^- production as a consequence of the chemotactic response[263].

Very recently, it was suggested that myeloperoxidase catalyzed oxidation of the thioether bond of methionine was involved in the inactivation of formyl-methionine containing peptides[264]. It is known that the corresponding sulfoxide or sulfone analogues of f-Met-Leu-Phe are completely devoid of biological acitvity[223]. Clark and Szot even suggested that this inactivation mechanism may promote an anti-inflammatory effect in vivo[265].



III Peptide partitioning and release
Receptor recovery.

- lysosomal enzymes
- arachidonic acid metabolites
- superoxide

In contrast with this evidence is the demonstration, with the aid of fluorescent ligands, that the peptide-receptor complex undergoes internalization by the cell followed by degradation of the peptide and subsequent release of the fragments outside the cell[266,267] (Fig. 12). This conclusion is supported by the fact that radio-labelled peptides were found to reside within the cell, specifically at the level of the Golgi apparatus[268]. The same authors also found however, that large amounts of peptide remain intact within the cell following internalization and that processing occurs later, subsequent to a storage step in the cell[268].

2.1.3 Structure Activity Relationship among f-MLP Analogues

It was realized, very early, that the N-formyl group of these chemotactic peptides was the most stringent requirement for good biological activity[223]. The N-acetylation or the removal of the terminal α-amino group of methionine resulted in a 1000 to 10,000 fold loss in activity[223] (Table 4). Methionine as the first amino acid of the chain gave the most active analogues. Its substitution by norleucine, having a similar chain length and hydrophobicity, resulted in a 10-fold drop in activity[270]. As mentioned above, oxidation of the methionine thioether group to the sulfoxide or the sulfone resulted in completely inactive compounds[223]. Greater flexibility is permitted at the second amino acid position. Hydrophobicity of the leucine side chain is a factor for good

activity, and the exact location of branching is also important as demonstrated by the decreased potency of compounds containing Ile or Val instead of Leu[270]. At the C-terminal of the peptide, phenylalanine confers the best activity. The receptor area for binding of this side chain seems quite restricted as evidenced by the much lower activity of the Tyr and p-chloro-Phe analogues[271]. Also the carbonyl group of Phe seems to be important since the f-Met-Leu-L-phenylethylamine analogue lacking the carbonyl, is less active. However, the carbonyl does not need to be part of a carboxylic acid function: the benzyl ester as well as the benzyl amide analogues are more potent than the parent acid[272]. It was also reported that the methyl ester derivative is a more potent chemoattractant for monocytes by a factor of 10,000[273]. Elongation of the chain by amino acids also resulted in increased activity, thus indicating that this binding area of the receptor can accomodate additional residues[270,274].

On the basis of NMR analysis using DMSO as the solvent, Becker et al[275] (Sect. 2.3) deduced that the tripeptide f-MLP exists in a β-pleated sheet form. Also, the side chains were found to be relatively free to rotate while the peptide backbone was quite rigid. The existence of an ordered, rather than random, conformation was made evident through CD studies in non-polar solvents[276]. A well defined conformation is also supported by the observed specificity of an antibody directed against f-Met-Leu-Phe[277]. It was found that the biological activity of several analogues was proportional to their binding with the antibody. On the basis

Peptides	Lysozyme release ED_{50} (M)
HC(O)-Met-Leu-Phe	3.2×10^{-11}
Ac-Met-Leu-Phe	1.4×10^{-6}
Met-Leu-Phe	8.9×10^{-7}
Desamino-Met-Leu-Phe	1.1×10^{-7}
HC(O)-Nle-Leu-Phe	3.2×10^{-10}
HC(O)-Val-Leu-Phe	1.5×10^{-8}
HC(O)-Cys(Me)-Leu-Phe	8.5×10^{-8}
HC(O)-Met-Val-Phe	1.3×10^{-9}
HC(O)-Met-Ala-Phe	4.5×10^{-8}
HC(O)-Met-Ile-Phe	1.6×10^{-9}
HC(O)-Met-Gly-Phe	2.9×10^{-6}
HC(O)-Met-Leu-Leu	4.8×10^{-8}
HC(O)-Met-Leu-Glu	1.3×10^{-6}
HC(O)-Met-Leu-Arg	3.6×10^{-7}
HC(O)-Met-Leu-Phe-OBz	4.6×10^{-11}
HC(O)-Met-Leu-Phe-NHBz	1.8×10^{-11}
HC(O)-Met-Leu-Phe-Phe	2.7×10^{-11}
HC(O)-Met-Leu-Phe-Nle-Tyr-Lys	4.0×10^{-10}

Table 4 SAR of chemotactic peptides related to f-MLP.

of these structure-activity studies, it has been postulated that the following structural features must be present in a peptide to provide good chemotactic activity:[272,278]

1) the hydrogen of the formyl group, which participates in a weak hydrogen bond at the receptor.
2) the methionine side chain (at position 1), which fits in a hydrophobic pocket. It is believed that this allows the interaction of the electron-rich sulfur with a discrete area of positive charge.
3) the leucine residue, which interacts with a hydrophobic site. A possible role for this residue is the maintenance of a favourable conformation of the peptide.
4) the aromatic ring of Phe (at position 3), which interacts at a specific site of the receptor.
5) the carbonyl group of Phe, which may be present in the form of an amide, an ester or a carboxylic acid. It forms an important hydrogen bond at the receptor.

The question naturally arises as to whether the amide bonds themselves and the N-formyl bond also directly participate in the receptor binding[278]. This question cannot be answered using previous SAR studies as a basis. Nevertheless, one would expect that the aligned hydrogen bond donors or acceptors in the proposed solution conformation for this tripeptide may well provide possible sites of interactions with the receptor[275]. In order to approach this problem, we chose to examine the effect on activity

of replacing the amide bonds by thioamide linkages. The effect of this modification on the solution conformation of the peptide could be evaluated by NMR analysis. Since many molecular events are initiated subsequent to binding of the peptide on the receptors, studies with thiopeptidic analogues should provide new insights into the overall mechanism of action of the parent effectors. The resistance of certain thiopeptides to selective peptidase action, for example, α-chymotrypsin and leucine aminopeptidase, may allow conclusions regarding the importance of catabolism in relation to the generation of a chemotactic response.

2.2 Synthesis of f-Met-Leu-Phe and thioamide analogues

2.2.1 Synthesis of f-Met-Leu-Phe

As mentioned above (2.1), it was our aim to prepare all the monothioamide analogues of this tripeptide, including the N-thioformyl analogue. Firstly, we wished to develop a rapid solution synthesis of f-MLP (65) applicable to the generation of all the thiopeptidic analogues. It was highly desirable to use similar intermediates possessing the same protecting groups, since this would facilitate comparisons of the physical properties of thiopeptide analogues with their amide counterparts. The Boc group was used to protect the amino function because we had established that the conditions for its removal were compatible with the presence of a thioamide functionality. The base labile methyl-ester was chosen as the means to protect the carboxyl end

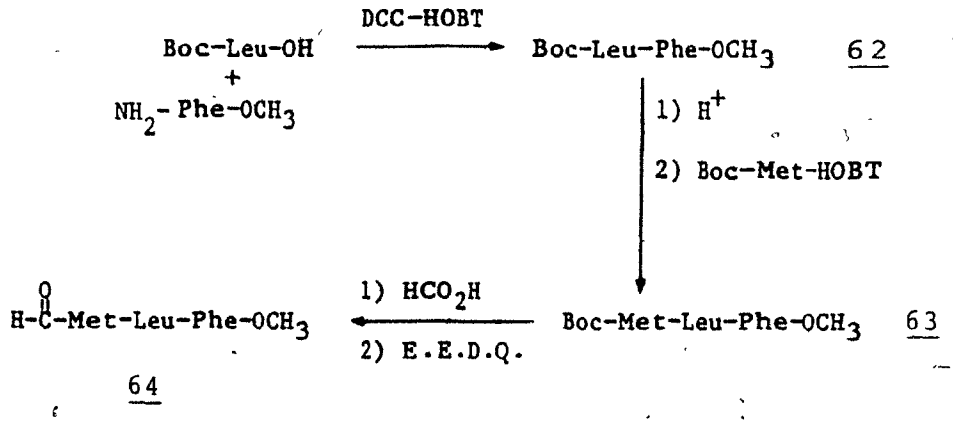
of the peptide. The f-MLP-OCH_3 peptide 64 could be prepared by stepwise coupling using the DCC/HOBt methodology[189]. This coupling proceedure is well documented as regards side reactions and racemization and has been proved to rapidly give good to excellent yields of products, at least in the case of small linear peptides[279-280]. The removal of the Boc group requires very mild acid conditions so as to avoid alkylation of the methionine thioether function. This could be achieved with formic acid (98%) at room temperature[281-282].

L-Phenylalanine methyl ester (61) was prepared by the addition of L-Phenylalanine to a solution of thionyl chloride ($SOCl_2$) in MeOH and the product was crystallized from MeOH/ether[283]. The dipeptide Boc-Leu-Phe-OCH_3 (62) was obtained in 85% yield after recrystallization by DCC-HOBt mediated coupling of Boc-Leu with phenylalanine methyl ester. Formic acid (98%) was used to remove the Boc group of 62 and the resulting formate salt of the dipeptide was added to a solution of Boc-Met-OBt with HOBt and DCC at 0°C. After 24 h, the product was isolated and recrystallization from CH_2Cl_2/hexanes afforded a 65% yield of the Boc-tripeptide 63 (mp 126.5-127.5°C). Removal of the Boc group was accomplished again with formic acid (RT, 1.5 h), leaving the methionine sulfur intact as ascertained by ^1H NMR spectroscopy[179].

Formylation of the terminal amino group of peptides or of amino acids has often been accomplished with DCC and formic acid[284]. However, when this method was applied to the formate salt of

Met-Leu-Phe-OCH_3 in CH_2Cl_2 several products were observed by TLC. Moreover, the dicyclohexylurea (DCU) formed during the reaction was difficult to remove from the formylated tripeptide because of its similar solubility and chromatographic properties. Formylation using the mixed anhydride $HC(O)-O-C(O)CH_3$ generated from formic acid and acetic anhydride is also troublesome, largely due to the fact that both acetylation and formylation occur, thereby making product purification difficult[285,286].

A new method for the clean N-formylation of amino groups was devised. When a chloroform solution of the formate salt tripeptide was treated with N-ethoxycarboxyl-2-ethoxy-1,2 dihydroquinoline (EEDQ)[190] the formation of the N-formyl peptide was complete after 4 h at room temperature.



After evaporation of the solvent and workup using a weak acid (5% aqueous citric acid) to remove the quinoline, the formylated peptide was obtained as a solid (90%), which could be further purified by precipitation from $MeOH/H_2O$. Its melting

point 136-138 °C is comparable to that reported in the literature (132-135°C)[273]. Other physical properties of this tripeptide are given in Table 5. Quantitative amino acid analysis gives a lower molar ratio for methionine as expected for an acid hydrolyzate of a methionine containing peptide when no reducing agent is added during manipulations[285]. Chemical shifts of the relevant protons in the ^1H NMR spectrum of the product are given in Table 6.

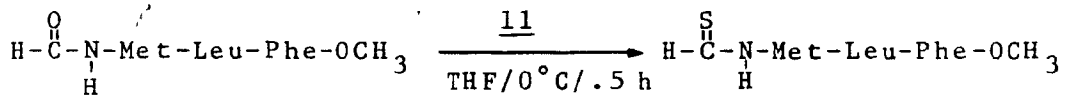
The rapid sequence of removal of the Boc group, evaporation of the excess acid, followed by formylation was most expedient, and constitutes a one pot process. This proved to be equally useful for the synthesis of thioamide and other functionalized analogues of chemotactic peptides[287].

The methyl ester function of (64) was removed by saponification with NaOH in THF/H_2O. However, when the biological potencies of the free acid and the methyl ester of f-MLP were evaluated, the methyl ester was found to be 10 times more potent both as a chemoattractant and as a releaser of lysosomes. Accordingly, f-Met-Leu-Phe-OCH_3 (64) was selected as our standard for purposes of comparison with the thioamide analogues.

2.2.2 Synthesis of H-C(S)-Met-Leu-Phe-OCH_3

The carbonyl of the tripeptide formyl group is by far the least sterically hindered amide carbonyl of the molecule. Thus, the reaction of thionation reagent 11 in THF with the formyl-tripeptide,

under the controlled conditions, was expected to preferentially introduce the sulfur atom at the formyl group. Indeed, when the formyl tripeptide 64 was reacted with reagent 11 in THF at 0°C, in order to maximize regioselectivity, disappearance of the starting material was observed within 30 minutes, the solution giving rise to one major spot when analyzed by TLC.



64 66

After purification by flash chromatography on silica gel ($CHCl_3$/MeOH 9:1) so as to remove the thionation reagent by-products, a white solid was obtained in 78% yield. The product's UV spectrum displayed the characteristic absorption band of a thioamide (λ_{max} 264.3 nm, log ε 4.01). Other physical properties are given in Table 5. The ^1H NMR spectrum (200 MHz, acetone-D_6) of the product exhibited a broad signal at 9.0 ppm corresponding to the amide proton of Met while the CH_α of Met was now shifted downfield to 5.28 ppm. The identification of the other resonances was accomplished by selective decoupling experiments and the results are summarized in Table 6. The ^{13}C NMR spectrum of the thiopeptide (acetone-D_6) showed a peak at 189 ppm which corresponds to the carbon of the thioformyl group. Final assignments of other ^{13}C resonances were made by comparative analysis of model compounds and other thiopeptide analogues are shown in Table 7. The mass spectrum of this monothionated peptide agreed with the expected

structure: a molecular ion peak ($M^{+\cdot}$) at 467 and a fragment at 422, resulting from the loss of $H-C\equiv S^{+\cdot}$ were observed.

Hydrolysis of the methyl ester of 66 with sodium hydroxide proved to be incompatible with the thioformyl functionality and several products were observed by TLC. Attempts to cleave the methyl ester with BBr_3 also resulted in a mixture of products. Under similar conditions the parent peptide methyl ester 64 led to good yields of the expected acid, thus BBr_3 appeared to react with the thiocarbonyl group of 66. Other methods for the cleavage of the methyl ester or its replacement by another labile functionality were not attempted because, as mentioned above, the parent peptide methyl ester itself gave superior results in the biological assays.

2.2.3 Synthesis of H-C(O)-Met-LeuC(S)-Phe-OCH_3

This analogue was synthesized by elongating the appropriate thiodipeptide intermediate from its N-terminal function. Thionation of the dipeptide Boc-Leu-Phe-OCH_3 (62) with reagent 11 (0.6 eq) in THF, at room temperature, for 24 h, gave an 87% yield of the thiodipeptide Boc-Leu-C(S)-Phe-OCH_3 (67) after chromatography on silica gel. Its structure was confirmed by ^1H NMR spectroscopy (200 MHz, $CDCl_3$). Characteristically, the thioamide NH was shifted downfield, relative to the parent peptide to 8.2 ppm while the CH_α of Phe appeared at 5.36 ppm and the CH_α of Leu to 4.42 ppm. Its mass spectrum displayed a molecular ion ($M^{+\cdot}$) at 408.

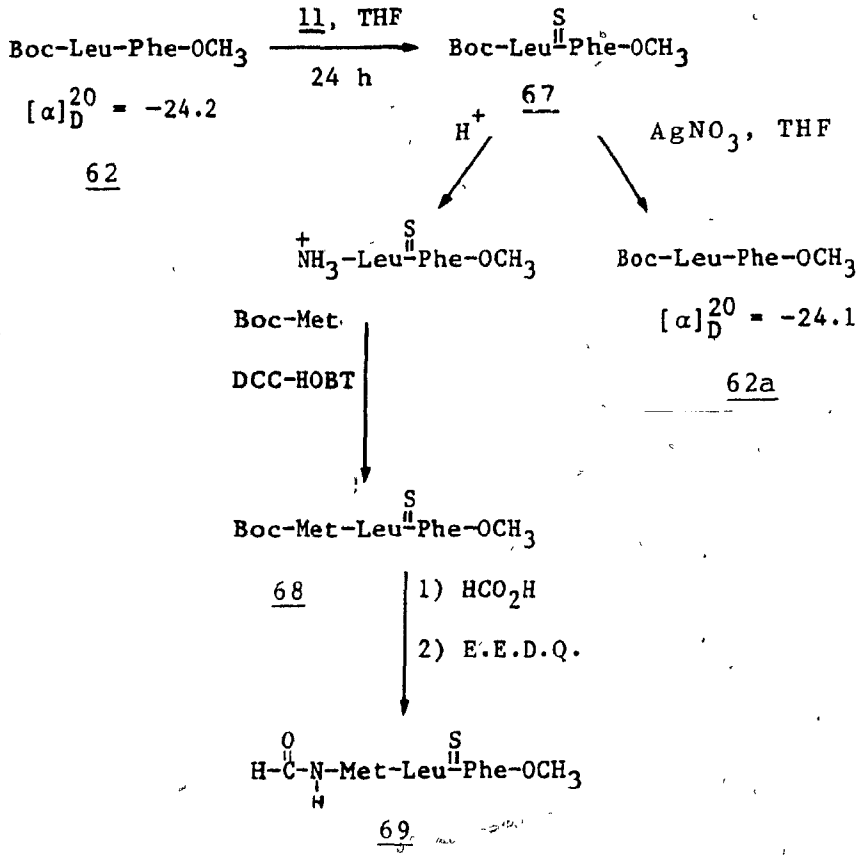


Figure 32

At this stage, we wished to verify whether any racemization occurred during the course of the thionation reaction (Fig. 32). To this end, the thiodipeptide product was reacted with silver nitrate (2 eq) in dry THF[288]. After 1.5 h, no trace of starting material remained, as indicated by TLC. Purification of the product by flash chromatography afforded Boc-Leu-Phe-OCH_3 (62a) in 50% yield. The optical rotation was $[\alpha]_D^{20}$ -24.1° (c 1.1, MeOH)

which corresponds to that of the starting Boc-Leu-Phe-OCH_3 (62) and thus no racemization had occurred at either chiral center of the dipeptide. The relatively poor yield of recovered dipeptide after silver nitrate treatment of the thio analogue is principally due to the loss of the Boc group under the experimental conditions, a side reaction not concerning the chiral centers. Absence of racemization during similar processes was also reported by Clausen et al[183] for the Cbz-protected thiopeptides, although their desulfuration conditions were more rigorous ($AgNO_3$ (3 eq), dioxane, reflux 0.5 h) Their conditions as applied to our Boc-thiodipeptide led to much lower yields of recovered peptide, presumably because of increased losses of the Boc protecting group.

Cleavage of the Boc group from the thiodipeptide 67 was achieved with formic acid (98%) at room temperature over a 2 h period. Removal of excess acid in vacuo gave the formate salt which was used as such without purification. It was added to the HOBt activated ester of Boc-Met followed by the addition of triethylamine (1.0 eq) and the mixture was stirred for 24 h. The resulting tripeptide, Boc-Met-Leu-C(S)-Phe-OCH_3 (68) was thus obtained as a solid (mp 131-133°C) in 73% yield after recrystallization from $CHCl_3$/hexanes. This product displayed all the expected spectral characteristics which are summarized in Tables 6 and 7. The sequence of deprotection-formylation was applied to this thiopeptide as already described for Boc-Met-Leu-Phe-OCH_3 (63). However, the product 69 could be purified by

direct crystallization from CH_2Cl_2/hexanes (81% yield). Its physical and spectroscopic characteristics are given in Tables 5, 6, and 7.

2.2.4 Synthesis of f-MetC(S)-Leu-Phe-OCH_3

Since it was already known that an N-protected thiodipeptide acid cannot be coupled with an amine due to the formation of thiazol-5(4H)-one upon activation of the carboxyl, the desired analogue was initially prepared by the aminolysis of Boc-Met-C(S)-SEt (54) as described before (Sec. 1.3). The formate salt of Leu-Phe-OCH_3 in THF was reacted with Boc-Met-C(S)-SEt in the presence of triethylamine at room temperature for 3 days (Fig. 33).

54 70

Figure 33

The product was purified by flash chromatography on silica gel to give the desired product in 67% yield. The ^1H NMR spectrum of the product (200 MHz, $CDCl_3$) revealed chemical shifts consistant with the expected structure 70 but incorporating two distinct signals for the methyl ester, thus indicating that complete racemization had occurred about the C_α of the methionine residue. Rather than separating these two diastereomers by semipreparative HPLC, another route for the preparation of this regioisomer was sought.

	R_f [a]	MP °C	$[\alpha]_D^{20}$ [b]	UV [c] λ_{max} (log ε)	Elemental analysis C	H	N	S	(Calcd) (Found)
HC(=O)-Met-Leu-Phe-OCH_3 64	.04	157-159	-39.2	---	58.52 58.41	7.37 7.52	9.31 9.29	7.08 6.87	
HC(=S)-Met-Leu-Phe-OCH_3 66	.18	158-160	-60.5	264.3 (4.01)	56.50 56.87	7.11 7.39	8.98 8.84	13.81 13.67	
HC(=O)-Met(=S)-Leu-Phe-OCH_3 74	.11	111-112	-59.3	271.4 (4.05)	56.50 56.50	7.11 7.35	8.98 8.96	13.81 13.71	
HC(=O)-Met-Leu(=S)-Phe-OCH_3 75	.09	142-145	-5.0	269.8 (4.06)	56.50 56.41	7.11 7.31	8.98 8.97	13.81 13.47	
HC(=O)-Met(=S)-Leu(=S)-Phe-OCH_3 76	.48	103-106	+0.6	271.5 (4.27)	54.62 54.42	6.81 6.76	8.68 8.62	19.88 19.47	

a) $CHCl_3$ / EtOAc 3:1

b) C= 1.0, MeOH

c) All spectra were recorded in EtOH at 20°C.

Table 5 Physico-chemical characteristics of f-Met-Leu-Phe-OCH_3 and thioamide analogues.

	MET			LEU			PHE		
	NH	CH_α	CH_β	NH	CH_α	CH_β	NH	CH_α	CH_β
HC(=O)-Met-Leu-Phe-OCH_3 64	7.45	4.60	2.01 1.89	7.45	4.46	1.53 1.65	7.45	4.67	3.01 3.11
HC(=S)-Met-Leu-Phe-OCH_3 66	9.0	5.28	2.05 1.92	7.56	4.50	1.6	7.50	4.69	3.0
HC(=O)-Met(=S)-Leu-Phe-OCH_3 74	7.60	4.95	2.16 1.96	9.37	5.17	1.6	7.60	4.70	3.03 3.12
HC(=O)-Met-Leu(=S)-Phe-OCH_3 75	7.55	4.63	2.11 1.89	7.55	4.85	1.6	9.38	5.33	3.26 3.19
HC(=O)-Met(=S)-Leu(=S)-Phe-OCH_3 76	7.63	4.98	2.19 1.97	9.46	5.53	1.87 1.74	9.57	5.35	3.29 3.20

Table 6 ^1H NMR chemical shifts (ppm) of f-MLP-OCH_3 and thioamide analogues (200 MHz, acetone-D_6, 1.3×10^{-3} M, 20°C).

	MET		LEU		PHE	
	C(S)	C_α	C_β	C_α	C_β	C_α	C_β
HC(=O)-Met-Leu-Phe(=S)-OCH_3	207.1	51.6	33.0	58.6	45.2	59.6	37.0
HC(=O)-Met-Leu(=S)-Phe-OCH_3	207.7	58.0	38.5	57.6	41.5	54	36.2
HC(=O)-Met(=S)-Leu(=S)-Phe-OCH_3	205.1 204.1	57.5	36.9	63.7	44.9	59.8	36.1
HC(=S)-Met-Leu-Phe-OCH_3	189	55.9	38.2	52.4	42.0	54.5	38.2

Table 7 ^{13}C NMR chemical shifts (ppm) of f-MLP-OCH_3 and thioamide analogues (2.0×10^{-1} M, acetone-D_6, 20°C).

Since the bulk of the amino acid side chains surrounding the amide bonds linking Met to Leu and Leu to Phe is essentially the same in the tripeptide Boc-Met-Leu-Phe-OCH_3 (63), it was expected that direct thionation should yield a mixture of monothioamide regioisomers together with some dithionated product.

When a THF solution of the Boc-tripeptide 63 was reacted with 0.6 eq of thionation reagent 11 at room temperature, two new spots with larger R_f's than that of the starting material appeared on TLC.

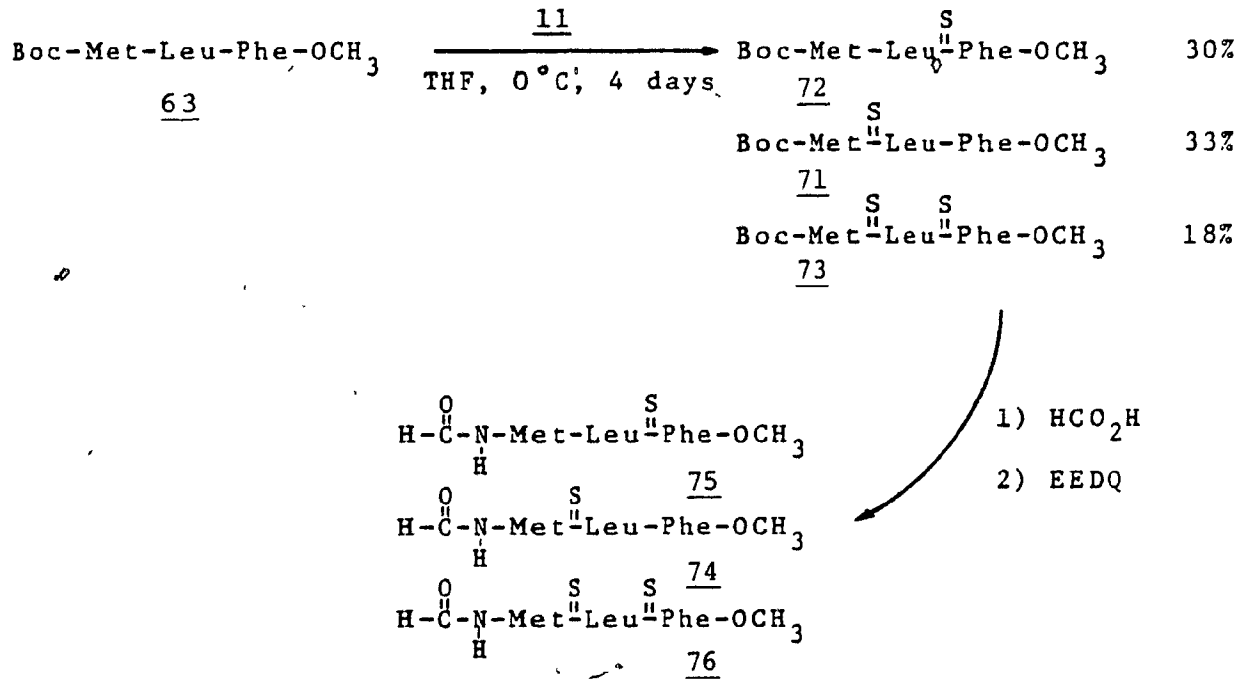


Figure 34

After 24 h, the reaction was stopped and the mixture separated by flash chromatography on silica gel. The least polar compound, not unexpectedly, was the dithioamide derivative 73 as evidenced by the much greater log ε 4.25 at 272 nm than that of the monothionated analogue 68. It's ^{1}H NMR spectrum (200 MHz, $CDCl_3$) showed two thioamide hydrogens at 8.47 and 8.01 ppm and all three CH_α were also shifted downfield to 5.40-4.50 ppm. Also, the mass spectrum of this compound displayed a strong molecular ion ($M^{+\cdot}$) 27% at 569.

The most polar component on TLC consisted of a mixture of these two regioisomers giving a ratio of 45:65 of least polar to most polar compound. The presence of the thioamide functionality in each compound was confirmed by their respective UV absorption spectrum. The identity of each compound was further established by ^{1}H NMR spectroscopy, the more polar compound having characteristics corresponding to those already described with Boc-Met-LeuC(S)-Phe-OCH_3 (68). A complete ^{1}H NMR spectral characterization of Boc-MetC(S)-Leu-Phe-OCH_3 (71) was carried out by selective decoupling experiments (200 MHz, $CDCl_3$).

Formylation of the thiotripeptide 71 was performed as described above to yield HC(O)-Met-LeuC(S)-Phe-OCH_3 whose physical and spectroscopic characteristics are assembled in Tables 5,6 and 7. We were fortunate to find that the N-formyl monothiotripeptides 74 and 75 possessed sufficiently different R_f characteristics to allow their separation by flash chromatography on silica gel. Accordingly for the large-scale preparation of these regioisomers

required for biological testing and for ^{13}C NMR studies, the separation of the monothioamides could be accomplished more efficiently and conveniently after N-formylation.

The reaction conditions required for a maximum yield of the monothioamides 71 and 72 at the expense of dithioamide 73 and which allowed a maximal conversion of the Boc-tripeptide to desired products were as follows: thionation of the tripeptide with 0.75 eq of reagent 11 at 0°C for 4 days. In this way, yields of the thionated regioisomer at position 2 (71, less polar) and at position 3 (72, more polar) were about 30% and 33% respectively, accompanied by an 18% yield of the dithiotripeptide.

Larger amounts of the dithiotripeptide 73 could be obtained simply by reacting Boc-Met-Leu-Phe-OCH_3 with excess reagent (1.2 eq) at room temperature. Under these conditions an 82% yield of 73 was obtained after a reaction time of 24 h. N-formylation of the N-deprotected product was accomplished as described above for the other analogues to give HC(O)-MetC(S)-LeuC(S)-Phe-OCH_3 (76).

2.3 Conformational Analysis

We sought to determine whether a thioamide group at specific positions of the f-Met-Leu-Phe-OCH_3 backbone can impose special conformational constraints on the molecule. As discussed earlier, the increased bond length of the thiocarbonyl relative to the amide carbonyl[29], the higher rotational barrier of thioamides[32-37], as well as other factors such as dipole moment[47] and hydrogen

bonding[51-53] properties may indeed cause important conformational differences, such as increased rigidity which may not allow biologically active conformations. This would be reflected in receptor interaction and in the behaviour of the analogue toward peptidase(s) that are presumably involved in the molecular events leading to a chemotactic response[261-264].

Since bond angles and bond lengths are equal in a amide linkage for all peptides, the conformation of the backbone is expressed in terms of three torsional angles: ϕ, ψ, ω, which describe the rotation around the N-C_α, C_α-C' and C'-N bonds respectively, while the rotation about the side chain, corresponding to C_α-C_β is described by the angle χ[289].

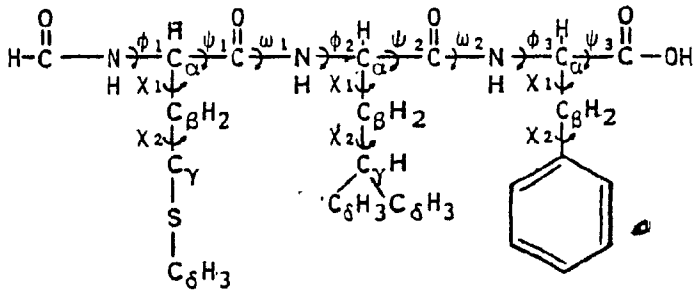


Figure 35 The peptide f-MLP defined in standard bond angle nomenclature[289].

A complete conformational analysis of f-Met-Leu-Phe-OCH_3 and its thiopeptide analogues was not possible because of time limitations. A combination of several spectroscopic (NMR, IR, CD) techniques is necessary to arrive at firm conclusions because of the high flexibility of linear peptides and the numerous parameters that must be considered before preferred solution conformation can be identified[290-291]. Traditionally, X-ray crystallography has provided unique information for the analysis of peptide conformation[292]. However, at the time this thesis was in preparation, crystallographic analyses of relevant analogues (64, 74, 74) were still in progress but as yet no firm results are on hand owing to serious difficulties in the generation of suitable crystals (f-Met-Leu-Phe-OCH_3 itself being the most difficult to crystallize.)

Prior to our studies a detailed analysis of the solution conformation of f-Met-Leu-Phe using ^1H and ^{13}C NMR spectroscopy was reported by Becker et al[275]. We thought that a good evaluation of the occurrence of special conformational changes associated with the presence of thioamide linkages could be made using the various ^1H NMR (200 MHz) spectroscopic techniques available to us and thus allow a comparison of our results with those of Becker and co-workers[275]. Based on ^1H NMR coupling constants (J_{NH,CH_α} $J_{CH_\alpha-CH_\beta}$) and ^1H and ^{13}C relaxation times, they found that the conformation of the backbone of this peptide in the negatively charged form (pH 8) assumes an antiparallel β-sheet arrangement. A stick model of the most populated conformation according to these authors is illustrated in Fig. 36.

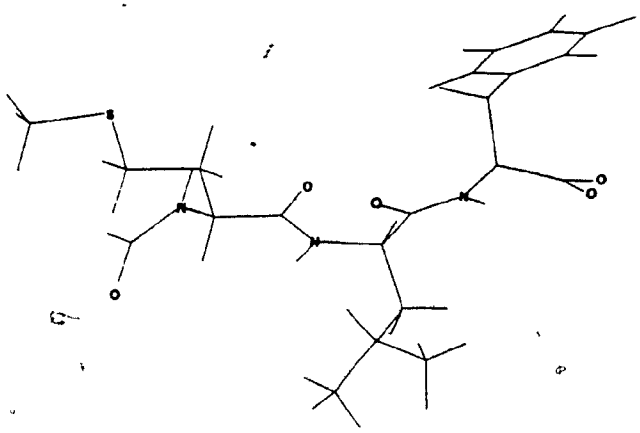


Figure 36 Stick model of the most populated final stucture as described by Becker et al[275].

Our own analysis was performed on the methyl ester derivative rather than the free acid because of its superior biological activity. However, this tripeptide methyl ester and its thioamide analogues are insoluble in H_2O which forced us to use DMSO and acetone as solvents.

For all studies in these deuterated solvents, correct identification of the amide protons was made by selective decoupling of the CH_α protons. Spin simulation was used in order to determine the coupling constants for the leucine side chain but

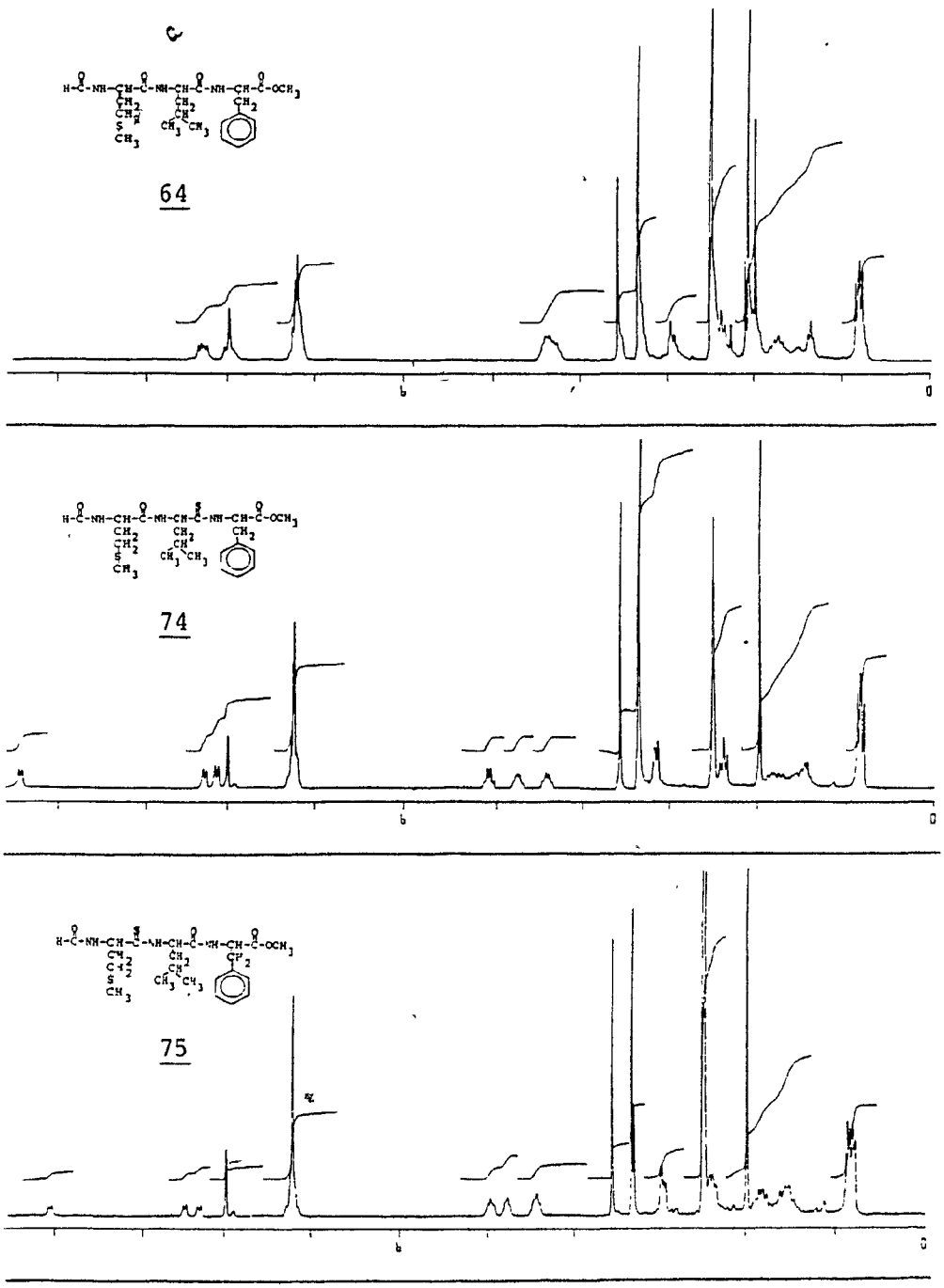


Figure 37 ^{1}H NMR spectra (200 MHz, DMSO) of 65, 74, and 75

the homonuclear 2D-J-resolved NMR technique[293] was used in order to obtain the coupling constants for f-MLP-OCH_3 in acetone-D_6. As it turns out, the backbone thioamide modification separates the CH_α signals owing to the downfield shift of the adjacent protons. (CH_α, CH_β, NH), thus simplifying the assignments and measurement of the coupling constants of the resonances (Fig.37). One can say then, that the thiocarbonyl group acts as an internal shift reagent.

In peptides, a downfield shift of the CH_α resonance is indicative of a change in the orientation of the α protons vis à vis the carbonyl group[294,295]. However, the downfield shift observed for certain protons (e.g. 0.4-0.5 ppm for the proton on the C_α of the thiocarbonyl in acetone-D_6) is associated with the increased anisotropy created by the thiocarbonyl, and not to a conformational change[69,72]. This is substantiated by the observed deshielding seen in the case of simple thioamide containing compounds (Chap. 1).

Since the orientation of the amide linkage in peptide are defined by hydrogen bonding (intra or intermolecular or by interaction with the solvent[296]) it was important to initially determine whether hydrogen bonding differences occur in the methyl ester derivative of f-MLP and its thioamides analogues. Although there are several ways through which such information may be obtained (D_2O exchange, $CDCl_3$ titration of DMSO solutions, etc...[289,296]) the variation of the NH chemical shift vs temperature appeared to be the most reliable procedure[297]. Intermolecular hydrogen bonds and those

to the solvent are readily cleaved with increasing temperature, while intramolecular hydrogen bonds are more stable and experience a smaller chemical shift with increasing temperature. The upfield shift observed is reflective of increased electron density around the hydrogen atom[298].

Generally speaking, temperature gradients in this solvent for the chemical shift of the NH signal in excess of $|4 \times 10^{-3}|$ ppm/°K are considered evidence of external NH orientation where the H is solvent bonded while values under $|3 \times 10^{-3}|$ ppm/°K suggest internal hydrogen bonding (Fig. 38)[297].

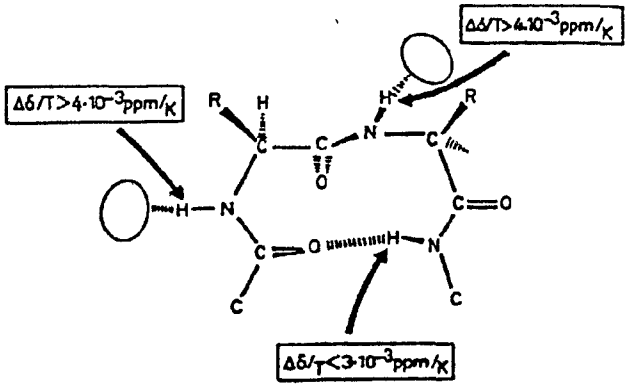


Figure 38 Temperature gradients $\Delta\delta/T$ of the NH signals in DMSO using the example of a β-loop[297].

This experiment was performed with compounds 64, 74, and 75 at identical concentrations in a 60°C temperature range; and the data are plotted in the form of chemical shift vs temperature as shown in Fig. 39.

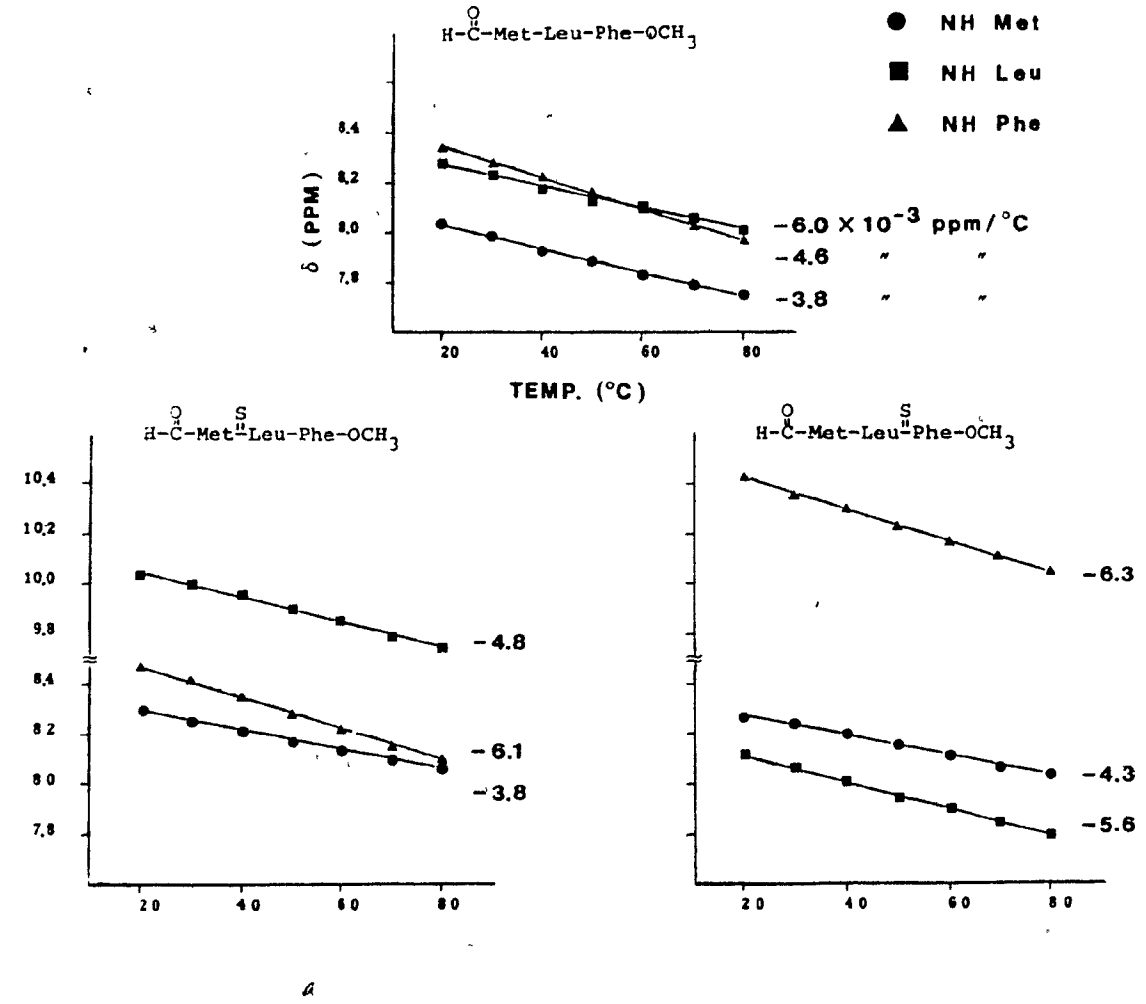


Figure 39 Variation of the NH chemical shift vs temperature for 64, 74, 75.

The corresponding slopes were evaluated by linear regression analysis and are assembled in Table 8 together with values of f-MLP-OH reported by Becker et al[275] at low (2) and high (8) pH.

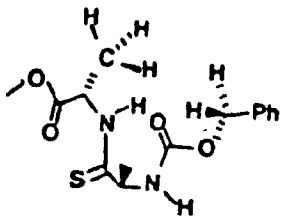
	NH MET	NH LEU	NH PHE	
		X 10^{3} ppm / °K		
HC-Met-Leu-Phe-OCH_3	4.8	4.4	6.4	
HC-Met-Leu-Phe-OCH_3 (64)	3.7	5.0	6.3	
HC-Met-Leu-Phe-OCH_3 (74)	4.0	5.6	6.3	
HC-Met-Leu-Phe-OH (75) pH 2	4.1	4.5	6.1	(275)
pH 8	8.5	8.5	2.6	

Table 8 Variation of chemical shift vs temperature for the NH protons of f-MLP, fMLP-OCH_3 and thiopeptide analogues.

The values obtained by Becker for[275] f-MLP (68) at low pH, which favors the protonated form of the carboxyl group, are nearly identical to those of methyl ester derivative. For all the analogues the values for the NH of Phe varies very little while the values for the Met NH and Leu NH vary maximally by $|1.1 \times 10^{-3}|$ ppm/°K. The only value below $|4.0 \times 10^{-3}|$ ppm/°K is associated with the NH of Met when the thioamide group occupies position 2, perhaps suggesting an involvement of this NH in a partial intramolecular H-bond which is shielded from the solvent in some of the conformers. However, this value of 3.7 or the change in this value (vs 4.8×10^{-3}) is too small to be attributed to a major conformational change in the environment of this bond. On the basis of these

studies, we can conclude that the orientation of the NH group of the thioamide function relative to that of the parent amide is not significantly different.

While this work was in progress, Brown and co-workers[299] reported an example where the different hydrogen bonding properties of thioamides resulted in a conformational change. The thiopeptide Cbz-AlaC(S)-Ala-OMe (77) adopts a conformation in $CDCl_3$ where the thioamide NH engages in H-bonding and in which the alanine methyl group is in close proximity to the methylene of the Cbz group as evidenced by an enhancement of 33% in the NMR signal of this methylene when the alanine methyl group was continously irradiated (Fig. 40).



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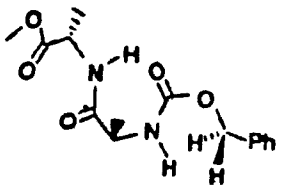


78

Figure 40

No corresponding enhancement effects were noted with the parent peptide (78); instead, a weaker enhancement (14%) of the signal carbamate NH proton was observed when the methylene proton were irradiated. This type of NOE experiment was not performed with our thioamide analogues since no strong intramolecular hydrogen

bond had been detected during the temperature dependence studies.

The temperature dependent experiments also indicate that the CH_α chemical shift do not vary significantly with increasing temperature (the largest shift being .06 ppm over a 60° range for CH_α of Phe in 64 and 74), indicating that no conformational process involving either the peptidic backbone occurred[300]. Consequently a dynamic equilibrium of energetically similar conformational states is reached at room temperature[297].

In agreement with this conclusion, single resonances are observed for all absorbing species in the fully decoupled ^{13}C NMR spectra (20°C) of all the thioamide analogues in acetone-D_6 or $CDCl_3$. When high energy requiring processes are in equilibrium, such as in the case of cis and trans isomerizations, doubling of the resonances or broadening of the peaks are usually observed[301]. No such effect was noted for any of the thioamide analogues.

It is possible to gain substantial information about peptide conformation from J_{NH-CH_α} coupling constant (θ angle) which is dependent on the angle ϕ as illustrated in Fig. 41[302-304].

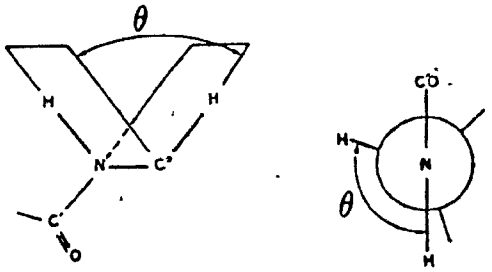
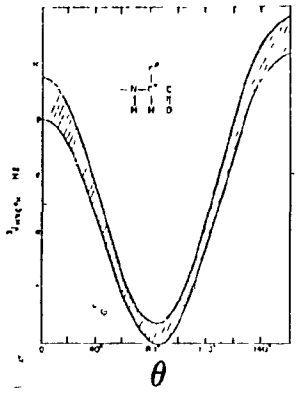


Figure 41

The angle θ can be obtained from a function representing a Karplus curve[305]:



$$^3J = A\cos^2\theta - B\cos\theta + C\sin^2\theta \quad \text{or,}$$

$$^3J = A\cos^2\theta - B\cos\theta + C$$

where A, B and C > 0 and $\theta = |\phi - 60°|$

In our calculations, the coefficients descibed by Brystov et al[306] where A = 9.4, B = 1.1, C = 0.4 as determined for a trans peptide bond were used. In addition, each measured coupling constant J_{NH-CH_α} was corrected so as to take in account the electronegativity of the C_α subsituent as calculated for amide bond of peptides[307].

$$J_{corr} = 1.09\ J_{obs}$$

We also assumed the same electronegativity in both amide and thioamide.

The measured coupling constants (acetone-D_6) for the $NH-CH_\alpha$ and $CH_\alpha-CH_\beta$ are given in Table 9. Using the corrected coupling constants, the θ angles were calculated and these values as well as the corresponding φ values are assembled in Table 10.

	MET		LEU		PHE	
	$NH-CH_\alpha$	$CH_\alpha-CH_\beta$	$NH-CH_\alpha$	$CH_\alpha-CH_\beta$	$NH-CH_\alpha$	$CH_\alpha-CH_\beta$
HC(=O)-Met-Leu-Phe-OCH_3 64	8.15	6.1 8.5	8.20	5.9 9.2	7.7	7.9 5.6
HC(=S)-Met-Leu-Phe-OCH_3 66	--	6.4 6.4	7.1	7.2 7.2	6.8	6.6 6.6
HC(=O)-Met(=S)-Leu-Phe-OCH_3 74	7.0	6.4 7.3	7.9	--	7.0	5.9 7.1
HC(=O)-Met-Leu(=S)-Phe-OCH_3 75	7.5	5.1 7.8	7.5	6.0 8.7	7.3	6.2 7.1
HC(=O)-Met(=S)-Leu(=S)-Phe-OCH_3 76	8.7	5.7 7.6	7.9	--	7.3	6.2 7.1

Table 9 Protons coupling constants (Hz) of f-MLP-OCH_3 and thioamide analogues (200 MHz, acetone-D_6, 1.3×10^{-3} M, 20°C).

	MET		LEU		PHE	
	θ	ϕ_1	θ	ϕ_2	θ	ϕ_3
O HC-Met-Leu-Phe-OCH_3 64	153	-93, -147	154	-86, -146	150 11	-90, -150 71, 49
S HC-Met-Leu-Phe-OCH_3 66	--	---	145 20	-85, -155 80, 40	144 22	-84, -156 82, 38
O S HC-Met-Leu-Phe-OCH_3 74	145 21	-85, -155 81, 39	151 6	-91, -149 66, 54	145 21	-85, -155 81, 39
O S HC-Met-Leu-Phe-OCH_3 75	149 14	-89, -151 74, 46	148	-89, -151	147 18	-87, -155 78, 42
O S S HC-Met-Leu-Phe-OCH_3 76	158	-98, -142	151 6	-91, -149 66, 54	147 18	-87, -153 78, 42
O HC-Met-Leu-Phe-OH 65		-130		-120		-150

Table 10 Calculated θ and ϕ angles (degrees) from ^1H NMR coupling constants J_{NH-CH_α}. Values underlined are those corresponding to the final values obtained by Becker *et al* [275].

After additional refinements using ^{13}C relaxation time measurements, Becker et al further limited the possible ϕ angles to single values of ϕ_1=-130°, ϕ_2=-120°, and ϕ_3=-150° for the tripeptide f-MLP-OH at pH 8 (Table 10). The angles calculated for the corresponding methyl ester differ by 17° for ϕ_1 and 26° for ϕ_2 and are essentially the same for ϕ_3. However, the thioamide analogues gave ϕ values which were in close proximity to those obtained for f-MLP-OCH_3 (±8° for ϕ_1, ±9° for ϕ_2, ±6° for ϕ_3).

It is possible then, according to these results, that the f-MLP-OCH_3 conformation differs somewhat from that reported for f-MLP-OH especially at the Leu and Met residue level. These differences could be due to pH effects[308]. However there is probably little difference between f-MLP-OCH_3 and its thioamide analogues.

The small variations in the angles are probably meaningless for all analogues because even for a fixed conformation, fluctuation of the dihedral angles ϕ around given values present magnitudes which are at least of the order of 10°[309,310]. Accordingly, our results indicate that the ϕ angles in these tripeptides are not altered by the incorporation of a thiocarbonyl group.

Further analysis of the ^1H NMR spectra of our analogues suggests that little variation of the torsional χ angle also occurs. The latter can be derived from the CH_α-CH_β coupling constants. When the amino acid residue includes a C_β methylene group with magnetically non-equivalent H_β and H_β protons, then two vicinal coupling constants can usually be observed in the spectrum: $J_{H_\alpha-H_\beta}$.

and $J_{H_\alpha-H_\beta}$, from which the individual rotamer population can be derived (Fig. 43)[289].

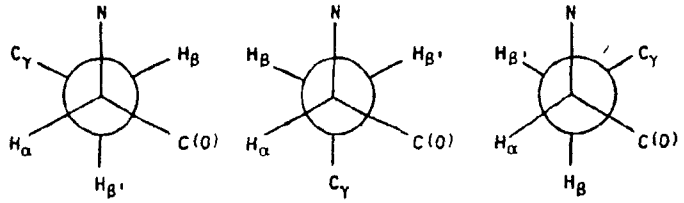


Figure 43

These coupling constants can be analysed in terms of populations of the three classical rotamers P_I (t^-), P_{II} (g^-), P_{III} (g^+) using the equations described by Feeney:[311]

$$J_{H_\alpha-H_\beta} = 4.1\ P_I + 11.7\ P_{II} + 2.9\ P_{III}$$

$$J_{H_\alpha-H_{\beta'}} = 12.0\ P_I + 2.1\ P_{II} + 4.7\ P_{III}$$

$$\text{where } P_I + P_{II} + P_{III} = 1$$

The results obtained from such claculations for f-MLP-OCH_3 and the thioamide analogues (acetone-D_6) are given in Table 11. Systematic errors of ±6%, ±4%, ±8% were obtained in the calculations

	MET			LEU			PHE		
	P_I	P_{II}	P_{III}	P_I	P_{II}	P_{III}	P_I	P_{II}	P_{III}
HC(=O)-Met-Leu-Phe-OCH_3 <u>64</u>	.58	.28	.15	.68	.25	.07	.53	.27	.20
HC(=S)-Met-Leu-Phe-OCH_3 <u>66</u>	.35	.35	.30	.57	.40	.03	.39	.37	.24
HC(=O)-Met(=S)-Leu-Phe-OCH_3 <u>74</u>	.47	.33	.19	--	--	--	.43	.28	.29
HC(=O)-Met-Leu(=S)-Phe-OCH_3 <u>75</u>	.49	.18	.33	.65	.26	.09	.44	.31	.24
HC(=O)-Met(=S)-Leu(=S)-Phe-OCH_3 <u>76</u>	.49	.25	.26	--	--	--	.46	.30	.24

Table 11 Rotamer population (χ_1 angle) of f-MLP-OCH_3 and thioamide analogues in acetone-D_6 at 20°C.

of the t, g^+, and g^- population's when taking into account a probable error of .2 Hz in the measured coupling constant. For the methionine and phenylalanine rotamers, a decrease in the $t(P_I)$ population and a small increase in the $g^+(P_{II})$ and $g^-(P_{III})$ is observed with the thioformyl analogue 66 which suggests that a conformational change in the peptide backbone is induced by the thioformyl functionality. However the change is too small to allow any firm conclusion. The differences in rotamer populations for the other analogues are even smaller, thus indicating that only very minor changes in the side chains mobility are associated with the presence of the thioamide group in the backbone of f-MLP-OCH_3. Further NMR analysis based on ^{13}C and ^{15}N resonances would be necessary in order to define the other substituent angles (ψ,ω) imposed by the thioamide modification[313].

From our preliminary analysis, we can assert that no major conformational restriction is introduced in the formyl tripeptide after thionation of its backbone. If any change has occurred, it must concern only minor conformer population. Accordingly, when interpreting the biological properties of these thiopeptides analogues any observed differences with the parent effector may be expected to originate primarily from the differences in the hydrogen bonding at the receptor level and in the increased bulk of the thiocarbonyl and not to changes in the spacial orientation of the side chains. This latter point is important because recognition process must depend on the three dimensional orientation of the signal residues[9,10].

2.4 Biological Activity

Biological activity of the four thiopeptide analogues was investigated using as the assay the release of lysozyme from human neutrophils[224]. Consistent results were obtained with blood from healthy blood donnors. Showell *et al*[224] have demonstrated that an excellent correlation exists between the chemotactic activity of an effector and its ability to release lysosomal enzymes from responsive cells.

The lysosomal enzyme-inducing activity for each peptide was obtained from the dose-response curve as its ED_{50}, the concentration (nMol/L) of peptide causing 50% of the maximal release of lysozyme Table 12).

Compounds	ED_{50} (nM)	Relative Potency (%)
H-C(=O)-Met-Leu-Phe-OCH_3 (64)	66	100
H-C(=S)-Met-Leu-Phe-OCH_3 (66)	200	33
H-C(=O)-Met-C(=S)-Leu-Phe-OCH_3 (74)	3,000	2
H-C(=O)-Met-Leu-C(=S)-Phe-OCH_3 (75)	not active	-
H-C(=O)-Met-C(=S)-Leu-C(=S)-Phe-OCH_3 (76)	not active	-

Table 12 Lysozyme release assay of f-MLP-OCH_3 and thioamide analogues.

The results are then expressed as percentage values of the activity of the standard f-MLP-OCH_3 (64) which is set at 100%. Details of the assay are given in the experimental section.

The thiopeptides were also tested for their spasmogenic activity as described by Marasco et al[314] using the guinea pig ileum as the test organ. Concentration-response curves were constructed and response and the results are reported as ED_{50} (Table 13).

Compound	ED_{50} (nM)	Relative Potency (%)
H-C(=O)-Met-Leu-Phe-OCH_3 (64)	61.6 ± 1.4	100
H-C(=S)-Met-Leu-Phe-OCH_3 (66)	85.0 ± 2.0	72
H-C(=O)-Met(=S)-Leu-Phe-OCH_3 (74)	81.5 ± 5.5	76
H-C(=O)-Met-Leu(=S)-Phe-OCH_3 (75)	Inactive	0
H-C(=O)-Met(=S)-Leu(=S)-Phe-OCH_3 (76)	Inactive	0

Table 13 Spasmogenic activity of f-MLP-OCH_3 and thioamide analogues.

Two of our analogues were found to be devoid of spasmogenic activity and to act as potent inhibitors of the standard agonist. The data are assembled in Table 14 and indicate that a thioamide linkage at position 3 confers strong antagonistic properties to this peptide.

Compound	Inhibition (%)
75 $H-\overset{O}{\overset{\|}{C}}-Met-\overset{S}{\overset{\|}{Leu}}-Phe-OCH_3$	
(0.5 μM)	56
(0.7 μM)	100
76 $H-\overset{O}{\overset{\|}{C}}-Met-\overset{S}{\overset{\|}{Leu}}-\overset{S}{\overset{\|}{Phe}}-OCH_3$	
(0.5 μM)	40

Agonist: $H-\overset{O}{\overset{\|}{C}}-Met-Leu-Phe-OCH_3$, 0.3 μM

Table 14 Inhibition of the spasmogenic response with 75, and 76.

In the lysozyme release assay, three of the thioamide analogues were found to be virtually inactive; only the thioformyl (66) displayed significant activity (≈33% of the standard.) Both the 3-thioamide regioisomer 75 and the 2,3-bis-thioamide analogue 76 were completely inactive, a result paralleling that of the spasmogenic assay except for analogue 74 which retained about 75% of the spasmogenic activity of the standard. The inhibitory

analogues 75 and 76 probably occupy the same receptor site(s) as the standard because of their close structural similarities.

2.5 Discussion

The structure-activity relationships among the thiopeptide analogues are of high significance. It may be recalled that the only structural difference between one analogue and an other is the presence and position of a thioamide functionality as part of the backbone. As pointed out earlier no evidence of conformational restriction as might be anticipated by thioamide linkages was obtained in the non-polar solvent systems (acetone, DMSO) that were used. Since it is known that the receptor for these peptides is a hydrophobic entity[252], it is reasonable to assume that similar conformations may be present in the receptor environment. Hence, the drastic difference in the activity of these analogues cannot be accounted for solely on the basis of mismatching with the affinity sites of the receptor owing to important alterations in hydrogen bonding or to the larger volume of a thiocarbonyl function. However, more refined structural information about thiopeptides is needed before a detailed understanding of the interaction mechanisms becomes available. It is conjectural at this stage to attempt correlating the results of the lysozyme release assay with those of the spasmogenic assay. It is still unknown whether the action of chemotactic peptides on the guinea pig ileum is the result of a direct interaction with receptors of

this tissue, or is the consequence of a mediated effect involving leucocyte-like cells in the tissue. It is remarkable that the correlation in rank order of potencies as reported by Marasco et al[314] with other peptides was also observed with our thioamide analogues.

It is interesting to note that it is the thioformyl analogue 66 that leads to the greatest similarities of response with the standard 64 in either assay. This constitutes the first structural modification at this position of the tripeptide which preserves a significant level of potency. It is plausible that the decrease in potency for this compound involves reduced receptor binding as a result of the larger bulk of the thiocarbonyl function. This could mean that the thioformyl group does not directly interfere with the biochemical processes normally involved in chemotaxis.

When the thioamide function is between Met and Leu (74), the activity is nearly abolished (2%) in the lysozyme release assay but its activity is similar to that of the thioformyl analogue in the GPI assay. The much lower ability of this analogue to cause the release of lysosomes may well reflect interference with the biochemical events ultimately controlling lysosome extrusion.

Agents capable of inhibiting cellular methylation reactions, like L-homocysteine thiolactone, are known to depress the chemotactic response of monocytes and macrophages[315]. Methylation reactions are themselves believed to be required for the maintenance of the receptor in its high affinity form[244,315].

Inhibition of the myeloperoxidase catalyzed oxidation of

methionine was also recently shown by Clark[264] to reduce the chemotactic activity of neutrophils. It is not inconceivable that thiopeptidic structures may somehow engage in processes of this kind and hence, disfavor chemotactic activity. Thioamides are strong nucleophiles that can be readily S-methylated thereby suggesting that they may interfere with biochemical methylation processes. Moreover, the well-known ease with which the sulfur of thioamides can be oxidized[29] suggests that they may act as efficient scavengers of active oxygen species such as H_2O_2 or O_2^- and thus protect against oxidation by the myeloperoxidase system. However, this can hardly apply to the thioformyl analogue which is quite active in the lysozyme release assay. In vitro inhibition studies of these biochemical systems with suitable thioamide substrates would be required in order to ascertain the possible involvement of these enzymatic parameters.

It is clear, however, that other processes must be invoked to explain the inactivity of analogues 75 and 76 in both the GPI and the lysozyme release assays. Their common structural feature resides in the presence of a thiocarbonyl at position 3 of the tripeptide. It is evident that the integrity of this peptide linkage is of critical importance for chemotactic and spasmogenic activity. Interference by these thioamide analogues with the two aforementioned enzyme systems is certainly possible, but it does not explain the strong antagonistic properties associated with this backbone modification.

Several groups have demonstrated the involvement of a neutral protease (chymotrypsin-like) in the production of H_2O_2 and in the leucocyte chemotactic response[261-263]. It seems therefore logical at present to attribute the inhibitory properties of these analogues to antagonism of such a protease. In vitro studies with bovine chymotrypsin (Sect. 1.2) have indeed shown that the thioamide analogues of normal substrates are completely resistant to hydrolysis. It was also shown by Niedel[262] that α-chymotrypsin is most efficient in causing degradation of f-MLP peptide derivatives.

These observations support the conclusion of Aswanikumar et al[261] who showed that a good correlation exists between the potency of a chemotactic peptide and its ability to be degraded by peptidases of the responding cell. It is plausible then that thiopeptides 75 and 76 behave as antagonists of chemotaxis by virtue of their non-productive binding to peptidase active sites. Accordingly, peptidase action would be a key event allowing the cell to sense a gradient. HC(O)-Met-LecC(S)-Phe-OCH_3 (75) is the closest analogue of f-MLP that can cause strong inhibition of the secretion of lysozomal enzymes. Prior to this finding, Boc-Phe-Leu-Phe-Leu described by Day et al[316], was shown to act as an inhibitor with an ID_{50} of 2.6×10^{-7}M (β-glucoronidase assay). It caused a 20% inhibition of f-MLP at 1μmol in the spasmogenic assay as reported by Marasco et al[314]. Compound 75 is significantly more potent as an antagonist in the spasmogenic activity assay.

Accordingly, this thiopeptide analogue holds much promise as a biochemical tool in the investigation of chemotactic and spasmogenic phenomena. Obviously, additional biological research with these thiopeptide analogues is essential in order to pinpoint the site of blockage. Their influence on $O_2^{\cdot-}$ production, arachidonic acid metabolites, ion flux, etc. will have to be evaluated in relation to their potential application in pathological states.

The identification of the Leu-Phe amide carbonyl as a key center controlling the agonistic activity of f-MLP establishes the fundamental usefulness of thiopeptide analogues in the future elucidation of the interaction mechanisms of oligopeptide effectors with biological receptors. Finally, our results with the thionated analogues of the chemotactic peptide may serve as guidelines in the future development of novel anti-inflammatory agents.

Chapter 3

Thioamide Analogues of [Leu^5]-Enkephalin

3.1 Introduction

3.1.1 Discovery of Enkephalins

Opium is one of the oldest medications known to man and morphine was identified as the major constituent responsible for its pharmacological and medicinal properties early in the nineteenth century[317]. Its principal therapeutic use is the relief of pain. It was also demonstrated that opiate action is mediated via specific receptors[318-320]. Several types of receptors (μ,δ,κ,σ etc...) have been postulated in order to account for the diverse effects of opiates[6,321,322].

Hughes et al[323] were the first to successfully isolate and characterize endogenous substances from pig brain exhibiting opiate-like activity. These substances proved to be two related pentapeptides whose structures corresponded to Tyr-Gly-Gly-Phe-Met ([Met^5]-enkephalin, Met-Enk) and Tyr-Gly-Gly-Phe-Leu ([Leu^5]-enkephalin, Leu-Enk). They occur in a ratio of 4 to 1 as determined by mass spectrometry[323]. They were later postulated to behave as δ agonists in contrast to morphine which is classified as a μ agonist[6,324].

3.1.2 Biosynthesis of the enkephalins

Hughes[323] had noted that the amino acid sequence of enkephalin was present in the larger peptide β-LPH which had been isolated ten years earlier by Li[325]. Close examination of pituitary extracts led to the isolation of other peptides with opiate-like activity, namely the α, β, and γ-endorphins, whose sequences were all present in the larger β-LPH molecule[326,327].

Eventually, a large number of peptides with opiate-like activity were described such as [Met^5, Arg^6]-enkephalin[328], [Met^5, Arg^6, Phe^7]-enkephalin[329], peptide F[330], peptide I[331], dynorphin[332], and others[333] incorporating the amino acid sequence of either [Leu^5]or [Met^5]-enkephalin or both. However, these peptides have sequences that are not structurally related to β-LPH but are related to other oligopeptidic precursors[333].

Very recently, DNA cloning experiments permitted the identification of these enkephalin containing peptides[334-336] (Fig. 44). The nascent protein characteristically contains a signal at its N-terminal which consists of 18-25 hydrophobic amino acid residues[337]. The cleavage of this sequence from the pre-prohormone upon its entry into the Golgi apparatus yields the prohormone. The enkephalin sequences of these proteins are bracketed between basic amino acid residues which are proteolytic sites for the appropriate enzymes[337]. Cleavage at these sites appears to involve a trypsin-like enzyme[338] and a carboxypeptidase B-like enzyme[339]. Such hydrolysis at the level of these basic residues would liberate

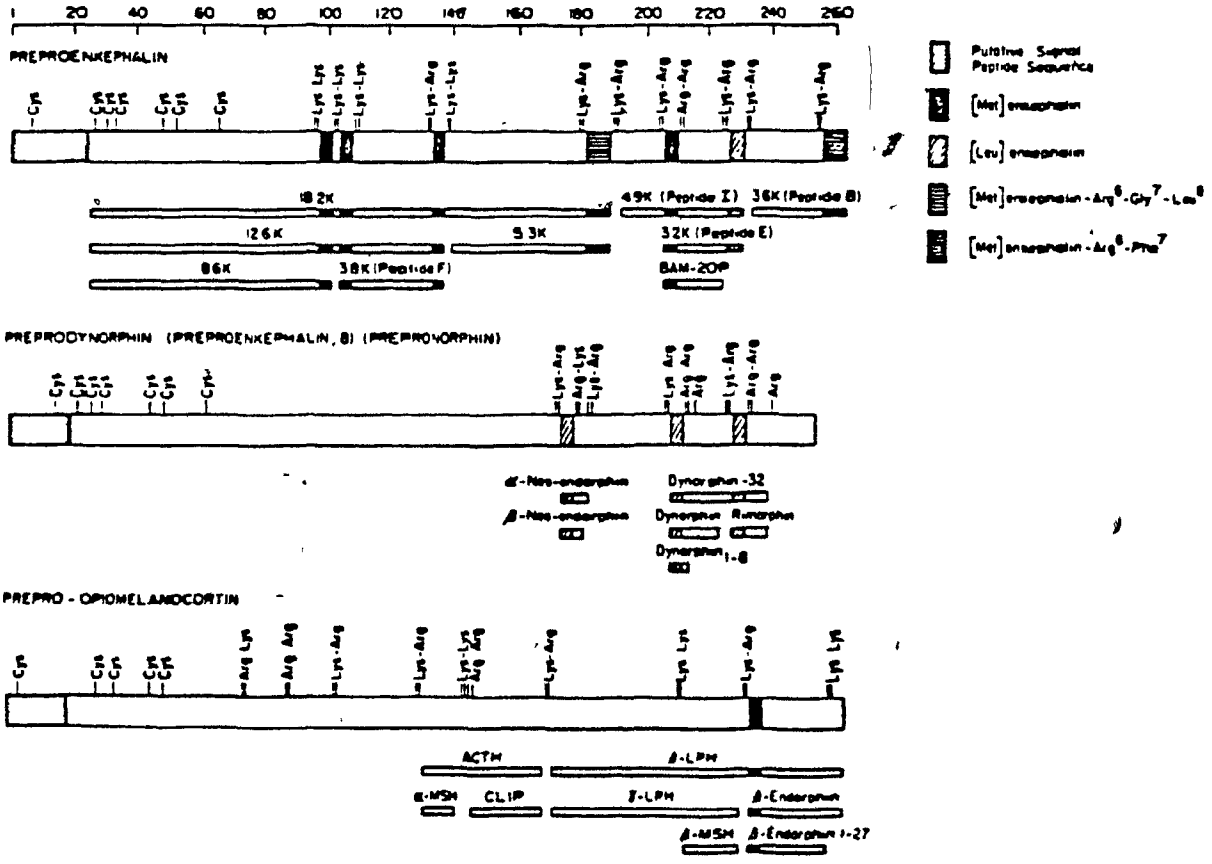


Figure 44 Schematic representation of the three enkephalin-containing peptide gene products.[337]

fragments containing the enkephalin sequence. Other enzymes such as serine and/or thiol proteases might also be involved in the processing of these proteins[310].

There exists also strong evidence that some of these extended enkephalin sequences play a role of their own[341]; for example [Met^5-Arg^6-Phe^7]-enkephalin is believed to be a μ agonist[342], while dynorphin is believed to be a κ agonist[343]. Thus proenkephalin, prodynorphin and β-LPH can yield a variety of peptides which are active on both the endocrine and nervous systems.

3.1.3 Distribution and Role

Phylogenetically, opioid peptides are very widely distributed. One or more have been found in all vertebrate species[344] studied and in some invertebrates such as the earthworm and leech[345,346]. In mammalian tissues, this distribution has been thoroughly investigated by the method of radioimmunoassay and by immunohistochemical procedures[347,348]. Their occurence in the brain and in the spinal chord generally parallels that of opiate receptor binding activity[349,351]. In the brain, the highest levels of binding activity were found in the corpus striatum (globus pallidus), the anterior hypothalamus, the amygdala and periaquaductal gray matter[351,352]. These are all areas of the brain associated with stress and emotions[351].

[Met^5]-enkephalin is invariably found in concentrations that are several fold greater than those of [Leu^5]-enkephalin[347].

Enkephalins were also shown to be present in the intestinal tract, pancreas[353] and other peripheral tissues including the sympathetic ganglia and adrenal medulla[344,348]. It was also demonstrated that synaptic vesicles are subcellular sites of storage for the enkephalins[354]. This is in agreement with the current view that the enkephalins may act as neurotransmitters[344]. In general, their effects are very similar to those of the plant alkaloids. For instance, their pharmacological properties include analgesia, respiratory depression, hypothermia, development of tolerance and physical dependence upon chronic administration together with a number of behavioral changes[355,356]. There is also a clear association between stressful conditions and the release of opioid peptides[357]. Enkephalins were also claimed to be involved in acupuncture analgesia[358] and to play a role in the regulation of pain sensation[359]. The μ receptors are believed to mediate a major portion of the opiate induced analgesia, while the δ receptors are responsible for the behavioral changes induced by opiates[324,360].

However the duration of action of the enkephalin is very short, ranging from a few seconds to a few minutes depending on the tissue preparation and the type of effect monitored[361-363]. The dose required is generally 50-100 times higher than that causing similar responses to morphine, in similar potencies in <u>in vitro</u> receptor binding assays[361].

3.1.4 Metabolism

Several groups have reported that [Met^5]and [Leu^5]-enkephalin were rapidly inactivated in various tissue preparation, including brain homogenates, guinea pig ileum and mouse vas deferens[364-367]. Intracerebral or intravenous administration is also followed by rapid degradation of the enkephalins[368-370]. Cleavage of the Tyr-Gly bond by aminopeptidases was first believed to be responsible for the rapid inactivation of these peptides in <u>in vitro</u> preparations[369-371]. The hydrolysis of this bond can be inhibitied by the antibiotic protease inhibitors bacitracin and puromycin[372]. Substitution of the second residue by D-Ala gave an analogue with greater activity which was ascribed to enzymatic resistance to the aminopeptidase[373].

Both soluble[374-375] and membrane bound (M_I, M_{II})[376-378] aminopeptidases have been in fact purified and characterized. The membrane-bound enzymes were shown to cleave the molecule between the Tyr and Gly residues while leaving the remaining fragment intact[376,378]. These aminopeptidases were inhibited in different degrees by metal chelators, such as EDTA, suggesting that they are indeed metallopeptidases[374,376, 379,380].

However in contrast to the <u>in vitro</u> studies mentioned earlier, it was shown by HPLC analysis of enkephalin metabolites <u>in situ</u>, following intraventricular infusion, that the most rapid enzymatic attack occurs between Gly^3 and Phe^4 and to a smaller extent between the Gly^2 and Gly^3 residues[362,381].

Angiostensin converting enzyme (ACE), a dipeptidyl carboxypeptidase involved in blood pressure regulation, was first believed to degrade the enkephalins between the Gly^3 and Phe^4 residues[382-383]. However a different dipeptidyl carboxypeptidase, enkephalinase A, was later shown to be responsible for cleavage at that position[381,384-386]. Also, its in vivo regional distribution parallels that of the enkephalins and their receptors, thus suggesting that the enzyme may be located at the putative enkephalin synapses[387-390]. On the other hand, it was recently demonstrated that enkephalinase A is not functionally coupled with the opiate receptor, and that the binding of [Met^5]-enkephalin is not a precondition for its degradation by this peptidase[391].

Some recent evidence suggests that enkephalinase A is in fact a neutral metalloendopeptidase which is not specific to the brain but also present in many other tissues, particularly the kidney[392-395]. Accordingly, enkephalinase A appears to be identical to an enzyme previously isolated from kidney brush border by George and Kenney[396] but whose function had remained obscure. The enzyme is inhibited by thiols and by metal chelators such as EDTA and phenantroline[392-395]. It displays a peculiar feature for an endopeptidase in that it has a very good affinity for substrates with a free terminal carboxyl group[395]. Enkephalinase A was shown not to be specific for the enkephalins, since it will also hydrolyze the amide bond on the amine side of hydrophobic amino acid residues of other small peptides either

protected or extended from the C-terminal such as substance P, oxytocin, bradykinin and neurotensin[397-399]. It therefore functions as an endopeptidase.

A dipeptidyl aminopeptidase (enkephalinase B) cleaving at the Gly^2-Gly^3 site was isolated from rat[384] and monkey brain[400] membranes. However, its specificity still remains uncertain although it also appears to be a metal-dependent (probably zinc) peptidase[400].

In brief, of the four bonds that can be cleaved in the enkephalins, specific enzymes for three of the scissile bonds are presently known (Fig. 45). Possible degradation between the Phe^4-(Met^5/Leu^5) residues by a specific carboxypeptidase has not yet been demonstrated, but a non-specific carboxypeptidase has been discovered which cleaves the bond between Phe^4 and Met^5 to release methionine[370].

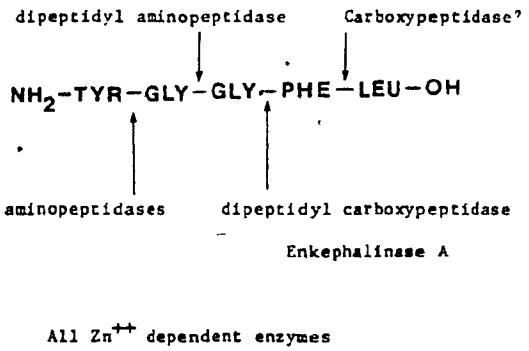


Figure 45

The exact pattern of enkephalin hydrolysis by these enzymes still forms the subject of controversy[401]. Recently the hydrolysis of [^{3}H]-[Met^5]-enkephalin in slices of rat striatum was shown to yield [^{3}H]-Tyr, [^{3}H]-Tyr-Gly and [^{3}H]-Tyr-Gly-Gly in the proportion of 80%, 2% and 18% respectively[402]. When aminopeptidase activity was inhibited by bestatin, the proportion of [^{3}H]-Tyr-Gly-Gly was increased to 60% of the total [^{3}H]metabolites. It was also recently confirmed that aminopeptidase activity was the most important degradative reaction in the guinea pig ileum preparation[403].

Several inhibitors of these enzymes have been designed in the search for possible therapeutic agents. Aminopeptidases and enkephalinase A were shown to be inhibited by amino acid hydrosamates, the most efficent of this serie being Cbz-Leu-NHOH and Cbz-Phe-NHOH[404]. The N-phosphorylated dipeptide K_2PO_3-Leu-Phe[405] and the mercaptoacetylated SCH(O)-Leu-Phe are very potent inhibitors of enkephalinase A[405]. Thiorphan ($SHCH_2$-$CH(CH_2Ph)$-C(O)NH-CH_2-CO_2H) was shown to protect the enkephalins from degradation by enkephalinase A <u>in vitro</u> and <u>in vivo</u>[406]. In addition, thiorphan itself displays antinoceptive activity which is reversed by naloxone[407]. A "retro" version of thiorphan was recently described and shown to be selective for enkephalinase A[408].

Carboxymethyl dipeptides[409-410] also inhibit enkephalinase A action. It was suggested that all these inhibitors, including the naturally occurring inhibitor phosphoramidon, behave as

transition state analogues of enzyme-substrate complexes and that the various functionalites form a tight complex with Zn^{2+} while the amide bond, the free carboxyl group, and the aromatic side chains participate in binding[410] (Fig. 46).

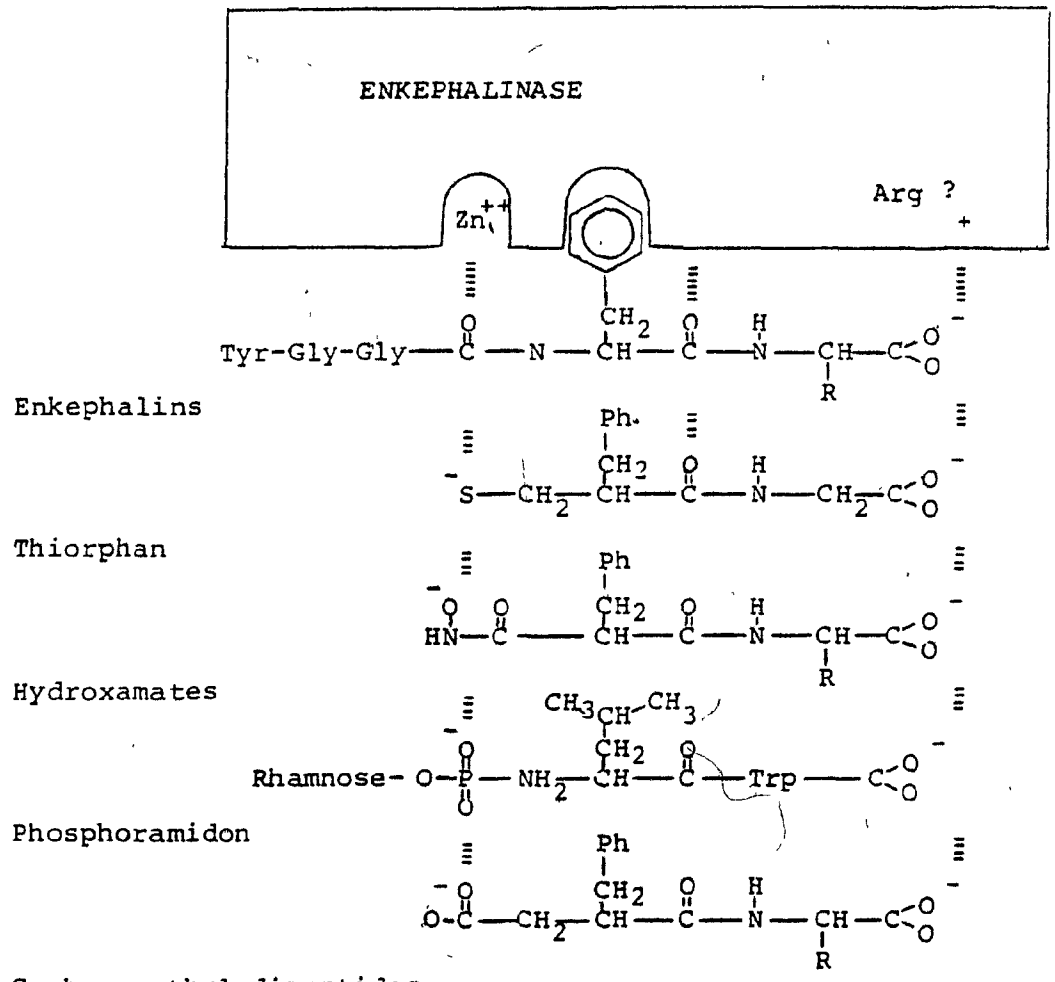


Figure 46 Schematic representation of enkephalinase active site and its interaction with inhibitors.

3.1.5 Structure-Activity Relationships among the Enkephalins

Several hundred enkephalin analogues have been prepared in order to identify the requirements for maximum activity[411-415]. Resistance to peptidases, enhanced binding capacity and more favorable transport properties, such as the ability to cross the blood-brain barrier, constitute the main incentives underlying the research effort.

The smallest fragment retaining activity is Tyr-Gly-Gly-Phe or its des-carboxy analogue Tyr-Gly-Gly-$NH(CH_2)_2Ph$[414]. One current view is that the fifth amino acid residue provides maximal activity by promoting binding and would be responsible, in part, for the μ and δ selectivity[416-417]. The structural requirements at the Tyr^1, Gly^3, and Phe^4 positions decrease in stringency in that order[411]. The only changes about Tyr^1 that will not destroy enkephalin-like activity involve N-methylation and, in some cases, acylation by an amino acid residue[411]. Configurational requirements at that position are also well defined as exemplified by the inactivity of the D-Tyr isomer and the α-Az-Tyr* analogue[418]. Slightly more configurational freedom is permitted at Gly^3 and Phe^4, as exemplified by the activity of the relevant α-aza-analogues[418]. N-methylation of Phe nitrogen causes a small drop in potency[418] and its replacement by Trp gave a compound with significant activity[419]. Para substitution of the phenyl ring by a chlorine, bromine or nitro group affords active analogues[411].

* Az, nitrogen replacing the α-carbon.

Much more latitude is allowed at the two remaining positions (Gly^2 and Met^5/Leu^5). Large increases in potency resulted from the replacement of Gly^2 by D-amino acids such as D-Ala, D-Met or D-Ser[373,420]. At the C-terminal position replacement by other amino acids with the exception of proline generally led to less potent compounds[411,421]. However, amidation of the carboxyl group of Met gave a more active compound in the GPI assay[418] while reduction of the carboxyl group to the corresponding alcohol of either Leu or Met gave less active compounds in the *in vitro* assays but more active *in vivo*[418]. On the other hand substitution by D-Leu gave a more potent compound in the MVD assay[418]. The most potent analogues were obtained when multiple changes of the original structures were made. The $D\text{-}Ala^2$, $N\text{-}Me\text{-}Phe^4$, $Met^5(O)\text{-}ol$ analogue is one of the most active opioids *in vivo*[422] (Table 15).

Modification of the peptidic backbone of the enkephalins has been briefly explored and the effects on activity of the changes are very site-specific. The α-aza-analogues at position 3 and 5 increased potency 2- to 5-fold in the GPI preparation[418], but at position 1 the same modification resulted in a completely inactive analogue[407].

The *trans* carbon-carbon double bond isostere of the Tyr-Gly amide bond has provided a compound which is 3 times more active than the parent compound in the GPI assay[424], while a similar double bond between Gly^2 and Gly^3 gave an analogue with only 0.1 of the activity of the enkephalins in the same assay, suggesting that this

	GPI	In vivo
Morphine	2.2	31
Tyr-Gly-Gly-Phe-Met	1	1
Tyr-Gly-Gly-Phe-Leu	.2	1.6
Tyr-Gly-Gly-Phe	0.01	-
Tyr-(D)-Ala-Gly-Phe-(D)-Leu	3.3	.16
Tyr-(D)-Ala-Gly-(Me)-Phe-Met(O)-OL	21.2	10^3
Tyr-(D)-Ala-Gly-Phe-Met-NH_2	5.0	-
Tyr-(D)-Met-Gly-Phe-Pro-NH_2	9.3	78
Tyr-C(=O)-NH-CH_2-CH_2-CH_2-CH_2-C(=O)-Phe-Leu	-	0.5
Tyr-C(=O)-NH-CH_2-CH=CH-CH_2-C(=O)-Phe-Leu	0.01	-
Tyr-CH=CH-CH_2-C(=O)-Gly-Phe-Leu	3	-
Tyr-(D)-Ala-Gly-NH-CH(-CH_2-Ph)-NH-C(=O)-(D)-Leu	3	-
Tyr-N-(D)-CH(-$(CH_2)_4$-)-C(=O)-Gly-Phe-Leu (cyclic)	4	-
β-Endorphin	3.5	31.5

Table 15 Relative potencies of selected enkephalin analogues.

amide bond of the pentapeptide may be involved in binding[424,425]. Fully "retro" Met enkephalin and its retro enantiomer (D-Met-D-Phe-Gly-Gly-DTyr) were inactive[411]. Retro inverso isomer at the Phe^4- Met^5 bond gave a compound which was longer acting[426].

A growing number of conformationally restricted enkephalin analogues can be witnessed in the recent literature[21,23]. These compounds usually show a greater duration of action which is believed to be associated with the resistance to enzymatic degradation. In some cases, μ[427] or δ[428] selective analogues were obtained.

In brief, SAR studies demonstrate that the presence of the N-terminal amino group, the tyrosine hydroxyl and the correct spatial disposition of the Tyr and Phe aromatic rings are essential for activity, while the peptide bonds themselves would not be involved in binding but would rather promote proper spacing of the residues[411]. The relative spatial arrangement of the aromatic residues was also postulated to be important for discrimination between μ and δ receptors[429].

3.1.6 Conformational Analysis

Cyclic analogues were designed largely on the basis of the assumption that the enkephalins, in spite of their flexible backbone, would exist in a folded conformation. Since their discovery, major efforts have been devoted to structurally relate

the enkephalin active conformation with that of the alkaloid opiates displaying a similar biological profile. On the basis of model studies it was initially postulated that the tyramine part, common to both the enkephalins and the morphinoids, would play the same role in the recognition process at the opiate receptor level[430].

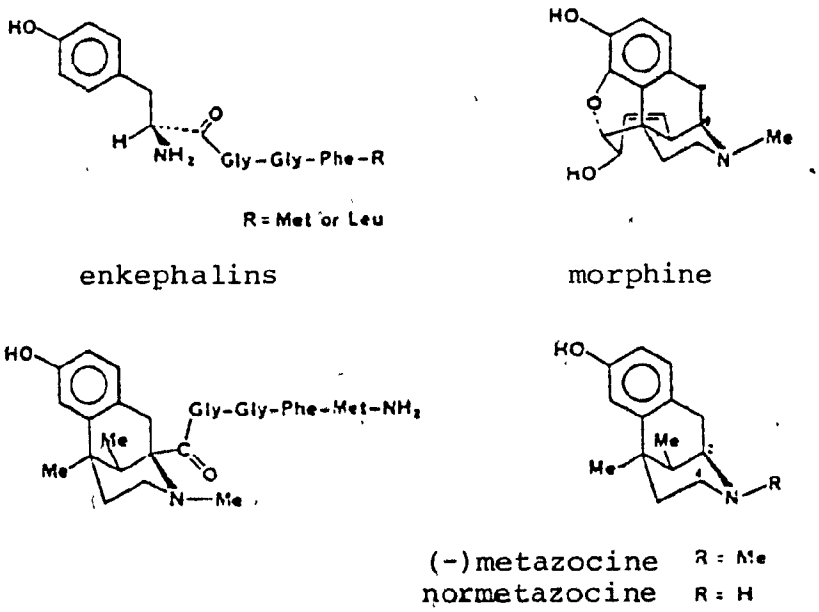


Figure 47 Relationship of the tyramine moiety in opiates and opioid peptides[431].

However since then, Portoghese[431] and Dimaio and Belleau[432,433] have provided evidence showing a non-identical role of the tyramine moiety in these drugs(Fig.47).

X-ray crystal analysis of [Leu^5]-enkephalin[434] confirmed the presence of two intramolecular hydrogen bonds, one between the carboxyl of Tyr^1 and the amide of Phe^4 and the other between the amino nitrogen of Tyr^1 and the carboxyl of Phe, thus leading to

the formation of a β-bend in the sequence Tyr-Gly-Gly-Phe.

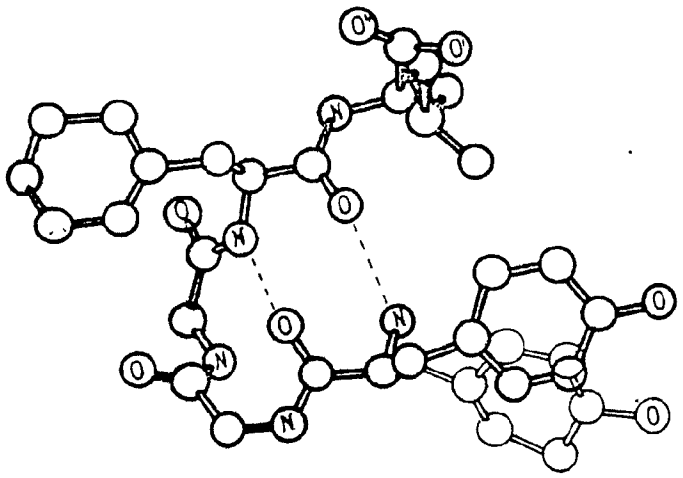


Figure 48 Observed conformation of [Leu^5]-enkephalin. The dashed lines represent the hydrogen bonds[434].

A large number of NMR studies[435-447] and other optical methods[448] have demonstrated that the enkephalins adopt a folded conformation in solution (Fig. 49). The exact nature of this compact conformation still remains unclear. Discrepancies in the location and the type of bend about the backbone, in the extent of motional freedom of the side chain and even in the assignment of some nuclear magnetic resonances have been reported.

Zetta and Cabassi[443] reported that a change in conformation does occur when changing the solvent from DMSO to H_2O, as evidenced by ^1H NMR analysis. This change is manifested by solvent dependent coupling constants $J_{CH_\alpha-CH_\alpha}$, and J_{NH-CH_α} of the Gly^3 residue indicating that this residue participates in backbone folding and unfolding. The authors of this report are, however, very cautious in pinpointing the exact location of the

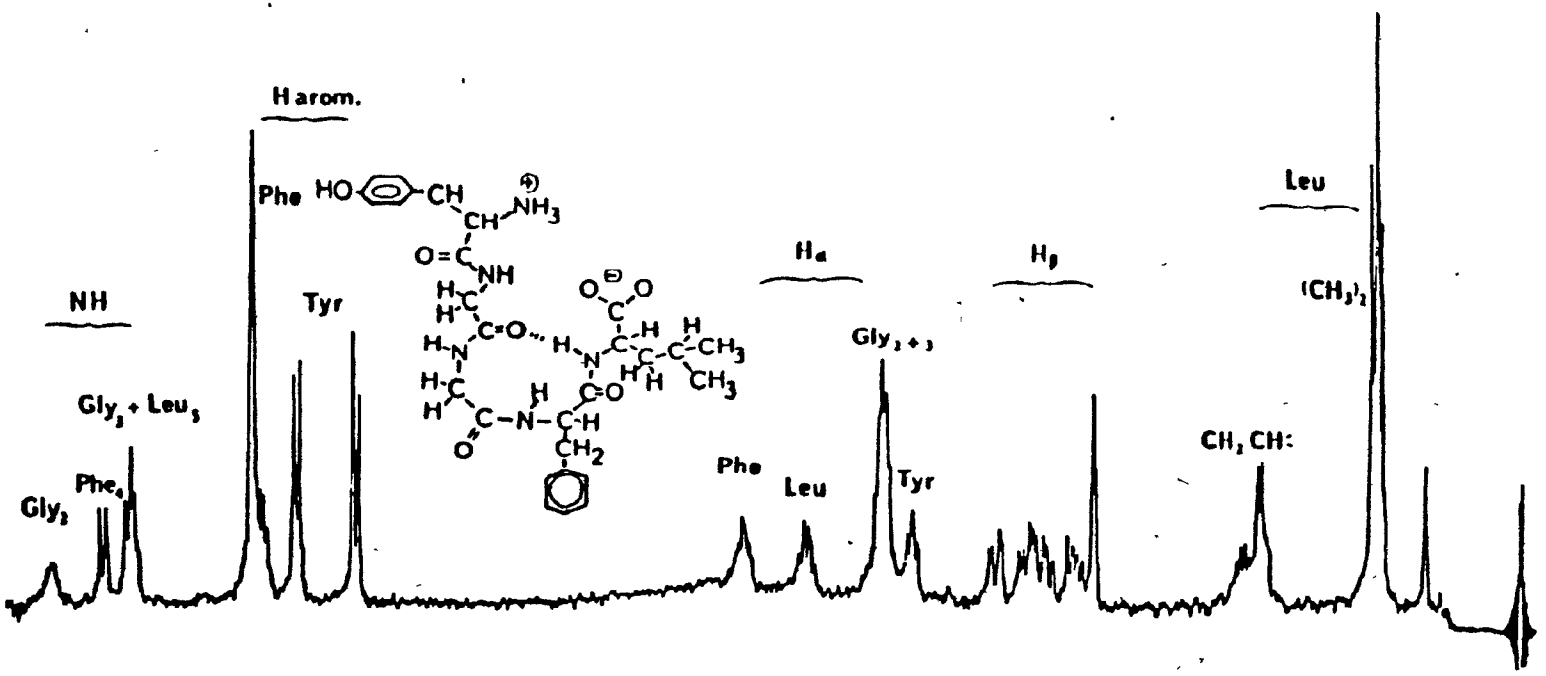


Figure 49 ^{1}H NMR spectrum (250 MHz, DMSO-d_6) of [Leu^5]-enkephalin and the postulated β-turn conformation with a 2←5 hydrogen bond[437].

internal hydrogen bond. Both a 2←5 and a 1←5 hydrogen bond are compatible with their data. Additional interactions of the N-terminal amino group with the carboxyl group are also possible according to their analysis.

Further evidence suggesting that more than one peptide backbone conformation may be recognized by the receptors is provided by the conformational properties of the active analogues [(α-Me)Phe^4, Leu^5]and[D-Ala^2, (N-Me)Phe^4, Leu^5]-enkephalin$^{449-450}$. These compounds cannot possibly adopt the folded conformation seen by X-Ray analysis of Leu enkephalin[6] and neither can they adopt some of the solution conformations proposed for enkephalin.

Another parameter hindering the elucidation of the biologically active conformation of the enkephalins and related peptides concerns the possibility that several different receptors or multiforms of the same receptor may exist and may be available for interaction with the enkephalins and other opiate peptides. These related but different receptors could then accomodate several of the possible conformations of enkephalin and its analogues. Manavalan and Monamy[451] have found, using empirical conformational energy calculations as applied to Leu^5/Met^5 enkephalin and a number of analogues, that several low energy conformations (βII', βI' family) are possible and common to these related peptides. These results are in agreement with previous calculations (Montecarlo) performed by Delmonté et al[452].

The possibility of several conformations, recognizable by different receptor subgroups was further substantiated by Schiller[453], who demonstrated that a sequence common to [Trp^4-Leu^5] enkephalin and [Trp^4]-dynorphin (κ-agonist) assumes markedly different conformations in these two related molecules. It was deduced by fluorescence energy transfer experiments that the distance between the Tyr and Trp residues was large in the dynorphin analogue but was relatively small in the enkephalin analogues. This indicates a predominantly extended conformation for the former at its N-terminal.

Additional conformational alterations may also be imposed by the heterogeneous receptor microenvironment. For example, the presence of metal ions might significantly alter the enkephalin conformation, thus making the analysis of the solution structure more difficult in terms of the relationship between conformations and functions. Such interactions of [Leu^5]-enkephalin analogues with Zn^{2+} ions were recently observed by both ^1H and ^{13}C NMR spectroscopy and this complexation resulted in an increased rigidity of the highly flexible backbone[454].

In conclusion, it can be said that solution analyses do not marry well with SAR findings and accordingly seem unrelated to the conformation adopted by the enkephalins at the receptor. What appears as a highly probable solution conformation may not reflect the conformation of the molecule in the receptor-bound state[455]. All that can be claimed is that a receptor-bound

conformation is not an energetically disfavoured solution conformation.

It was felt that analogues with a monothioamide bond at each position of the peptide chain would serve as probes of the role of the backbone in the molecular properties and biological activity of the enkephalins. Resistance to the metallopeptidases which are responsible for their degradation, can be expected on the basis of our results with leucine aminopeptidase (Chap. 1) as well as recent reports on the inhibition of carboxypeptidase A by thioamide substrates[150,174,175]. This enzymatic parameter should influence the pharmacokinetics of the different analogues, especially in the in vivo assays.

3.2 Synthesis of [Leu^5]-Enkephalin and Thioamide Analogues

We chose to synthesize thioamide analogues of [Leu^5]-enkephalin instead of [Met^5]-enkephalin for simple practical considerations. The methyl thioether of methionine is prone to oxidation during peptide synthesis and/or purification[280,456]. It was also shown to be oxidized in the guinea pig preparation[457,458]. Moreover, methionine can readily be alkylated by the carbonium ions generated during the acid-catalyzed removal of the N-protecting Boc groups[280].

Since we wanted to prepare all the possible monothioamide analogues of the pentapeptide, it was again desirable to generate common intermediates from similar strategies which contained

similar protecting groups. This would facilitate the study of their physico-chemical properties and ensure that potentially complicating factors, such as racemization, would be minimized.

As mentioned in Chapter 1, the hydroxyl group of tyrosine requires protection during thionation of amide bonds. In addition the phenolic group of tyrosine, especially in its ionized form, has been shown to interfere with peptide bond formation when DCC is used as the coupling reagent[459,460]. While a vast number of protecting groups have been described for the phenolic group[461], the incorporation of the sensitive thioamide function into the peptide backbone limits the choice of protecting groups. Reducible protecting groups would be incompatible with thioamides while base labile protecting groups would be incompatible with the carboxylic ester at the C-terminal.

Since tyrosine is the last amino acid to be incorporated in the sequence, a group labile to mild acid would be preferable as it would be removed simultaneously with the N-Boc group. This suggests that an O-Boc functionality would fulfill our requirements. The O-Boc group has only been briefly described in the literature. It was generated by treatment of tyrosine with excess t-butyloxycarbonyl fluoride[462-463]. Its corresponding p-nitrophenyl ester and the dipeptide O,N bis(Boc)-Tyr-Gly-OEt have been described[463]. Also, the O,N bis(Boc)-Tyr-OH derivative has been obtained as a minor side product in the reaction of tyrosine with the Boc-ON reagent[464]. Because of the impracticality of the preparations

of the t-butyloxycarbonyl fluoride reagent, we looked for an alternative more practical methodology to produce the desired O,N-bis(Boc)-Tyr derivative.

It is well-known that dimethylaminopyridine (DMAP) acts a strong bifunctional catalyst and markedly promotes the acetylation of alcohols and phenols[465]. Accordingly, the reaction of tyrosine with 2.2 equivalents of the commercially available di-t-butyl-dicarbonate[466] in THF/H_2O in the presence of DMAP (10%) gave, in good yield (70-80%), the desired O,N bis(Boc)-tyrosine (84) which was recrystallized from Ch_2Cl_2/hexane. This derivative had a rotation of $[\alpha]_D^{20}$ +26.1° (c 1.0, DMF) vs +26.5° and a melting point 96-98°C vs 92-94°C.

3.2.1 Synthesis of [Leu^5] Enkephalin

All protected peptides were prepared by the DCC-HOBt methodology[279]. The synthetic route for the preparation of [Leu^5]-enkephalin is outlined in Fig 50. L-leucine methyl ester hydrocholoride (80) was prepared in nearly quantitative yield by the addition of L-leucine to a solution of thionyl chloride in methanol[283] procedures. Boc-phenylalanine (81) was prepared according to standard using di-t-butyl dicarbonate[466]. The coupling of L-leucine methyl ester with Boc-phenylalanine was accomplished with DCC-HOBt and the resulting dipeptide Boc-Phe-Leu-OCH_3 (82) recrystallized to homogenity (TLC). After removal of the Boc group with formic acid (98%), the resulting formate salt of the dipeptide was added to

a solution of Boc-Gly-Gly-OBt to give, in 93% yield, the tetrapeptide Boc-Gly-Gly-Phe-Leu-OCH_3 (83). This product, after deprotection with formic acid, was in turn coupled with O,N bis(Boc)-tyrosine (84). The resulting pentapeptide 85 was first purified by flash chromatography on silica gel and then by precipitation from THF/ether to remove an impurity with a similar R_f.

^1H NMR (200 MHz) confirmed the identity of the compound. Irradiation of the broad signal (NH) at 7.5 ppm caused a change in the multiplet at 4.00 ppm (CH_2Gly^2, CH_2Gly^3), while irradiation of the NH doublet (NH-Boc) caused a change in the CH_α area at 4.4 ppm ($CH_\alpha Tyr$). Through additional decoupling experiments, the resonance assignments for $CH_\alpha Phe$ (4.9 ppm) and $CH_\alpha Leu$ (4.58 ppm) were made. Other values are summarized in Table 17. Complete assignments of the ^{13}C NMR resonances were facilitated by the use of the APT technique. The relevant data are given in Table 18.

Saponification of the methyl ester was accomplished using sodium hydroxide in THF/H_2O (2:1). After aqueous acid (citric acid) treatment, the product was extracted into EtOAc and the solvent evaporated. Both Boc groups were removed by treatment of the peptide acid with formic acid for 2 h at RT. The resulting greenish powder was purified by reverse phase HPLC and after 3 lyophilizations to ensure complete removal of the volatile buffer, the fluffy powder was recrystallized from EtOH/H_2O to give pure [Leu^5]-enkephalin (86). Its ^1H NMR spectrum (200 MHz, DMSO) (Table 20) and other physical characteristics correspond to those

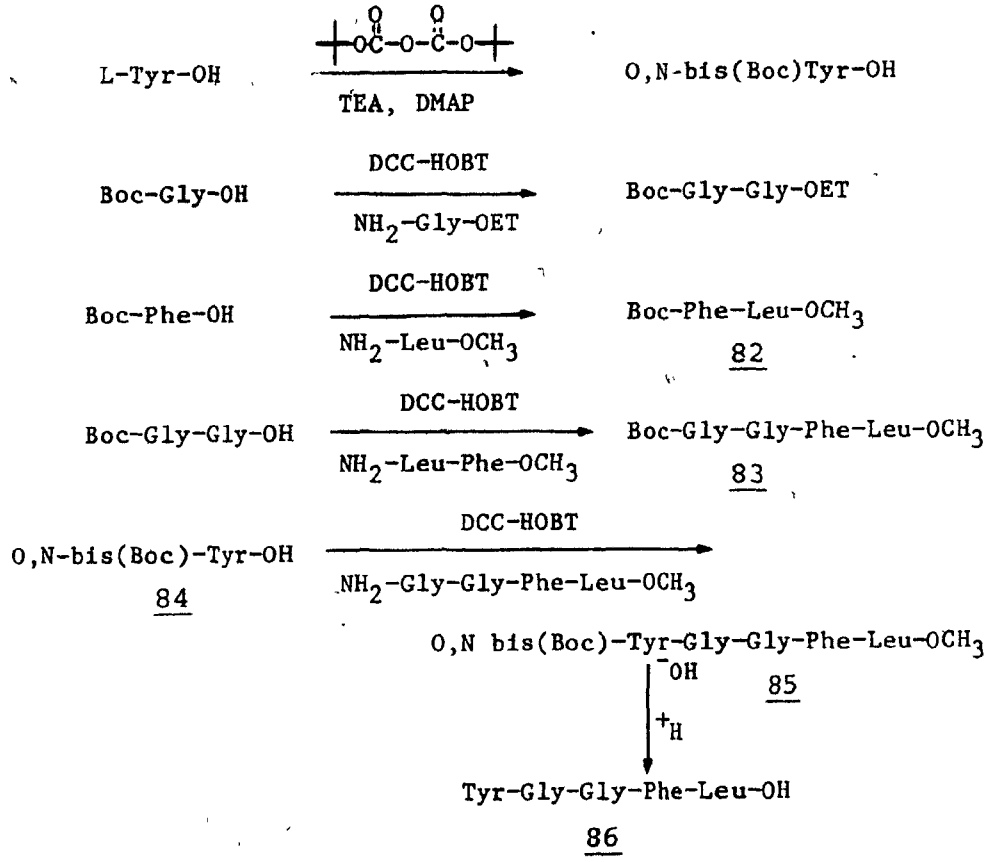


Figure 50 Synthesis of [Leu^5]-ennkephalin.

reported in the literature[442,467].

This modified synthesis of [Leu^5]-enkephalin was rapid and efficient which suggested that a similar scheme could be applied to the synthesis of the desired thioamide analogues.

3.2.2 Synthesis of Tyr-Gly-Gly-PheC(S)-Leu-OH

We have already demonstrated in the previous series of thiopeptide analogues of f-MLP that coupling from the N-terminal end of a dipeptide bearing a thioamide linkage was a feasible process. Accordingly, the synthesis of the title analogue should be straightforward (Fig. 51). Thionation of Boc-Phe-Leu-OCH_3 with excess thionation reagent 11 (.7 eq) gave after 24 h the thiodipeptide 87 in 81% yield. Its structure was confirmed by UV, NMR, and mass spectrometry.

The other tripeptide fragment was prepared by the coupling of O,N bis(Boc)-tyrosine with glycyl-glyciné methyl ester (Sigma) using DCC-HOBt as the coupling reagents to give a homogeneous compound (88) after recrystallization. Saponification of the ethyl ester with sodium hydroxide gave the corresponding Boc-protected tripeptide acid as an amorphous solid.

The fully protected thiopentapeptide was obtained by block condensation of this tripeptide acid 89 and the formate salt of PheC(S)-Leu-OCH_3 with DCC-HOBt. Purification of the product by flash chromatography on silica gel gave a 58% yield of amorphous white solid 90.

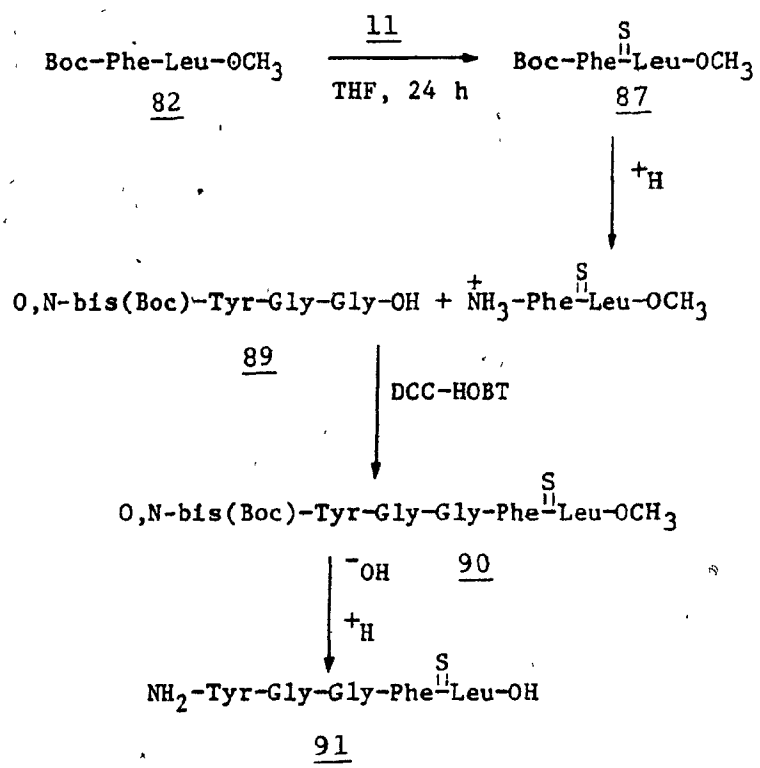


Figure 51

The presence of the the thiocarbonyl function was confirmed by its characteristic UV absorption (λ_{max} 268.3 nm, logε 3.98) and its ^{13}C resonance at 203.9 ppm (Table 18). The ^{1}H NMR spectrum (200 MHz) of the product is consistent with the desired structure and individual NH and CH_{α} proton chemical shifts could be assigned by selective decoupling experiments (Table 17).

We also obtained a two-dimension nitrogen-proton decoupled NMR spectrum (no ^{15}N enrichment) of the pentapeptide analogue(Fig.53)

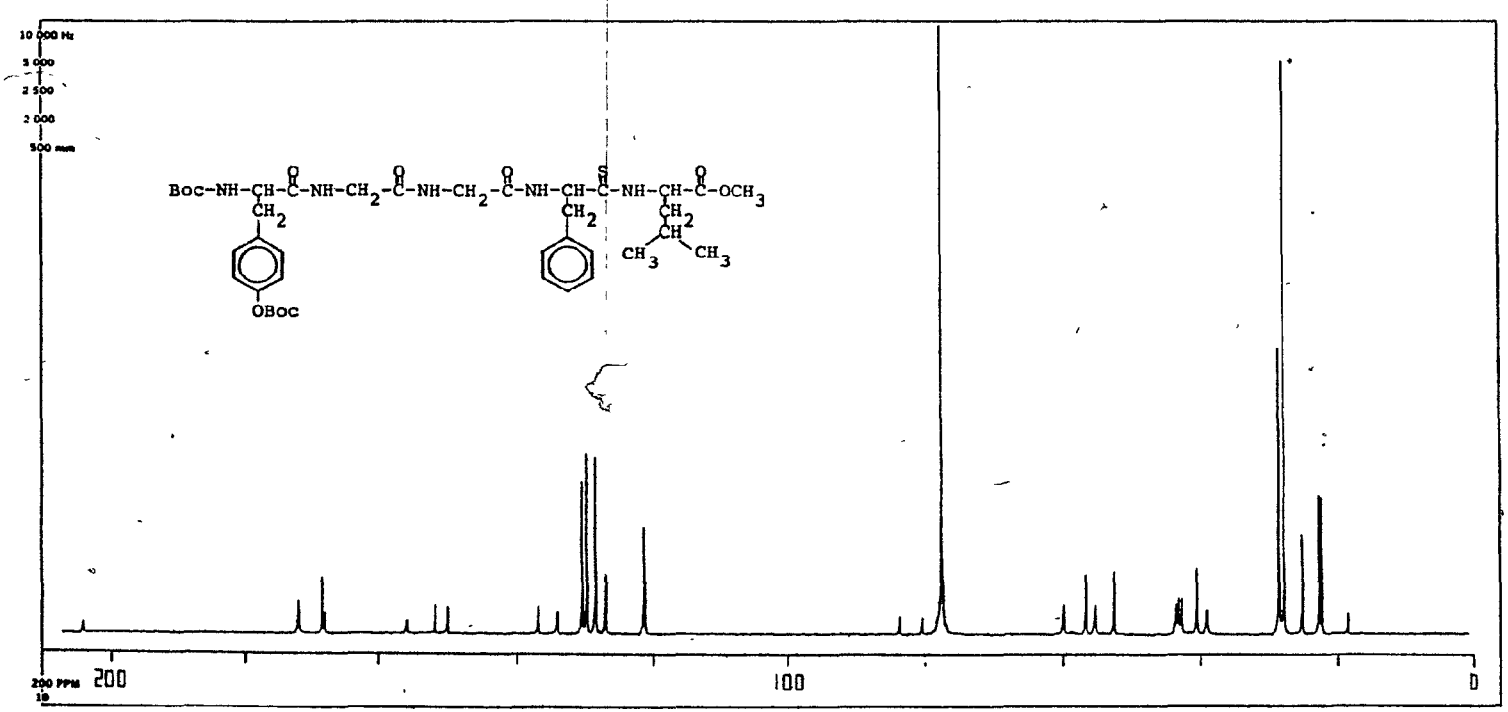


Figure 52 ^{13}C NMR spectrum (300MHz, $CDCl_3$) of thioamide analogue 90.

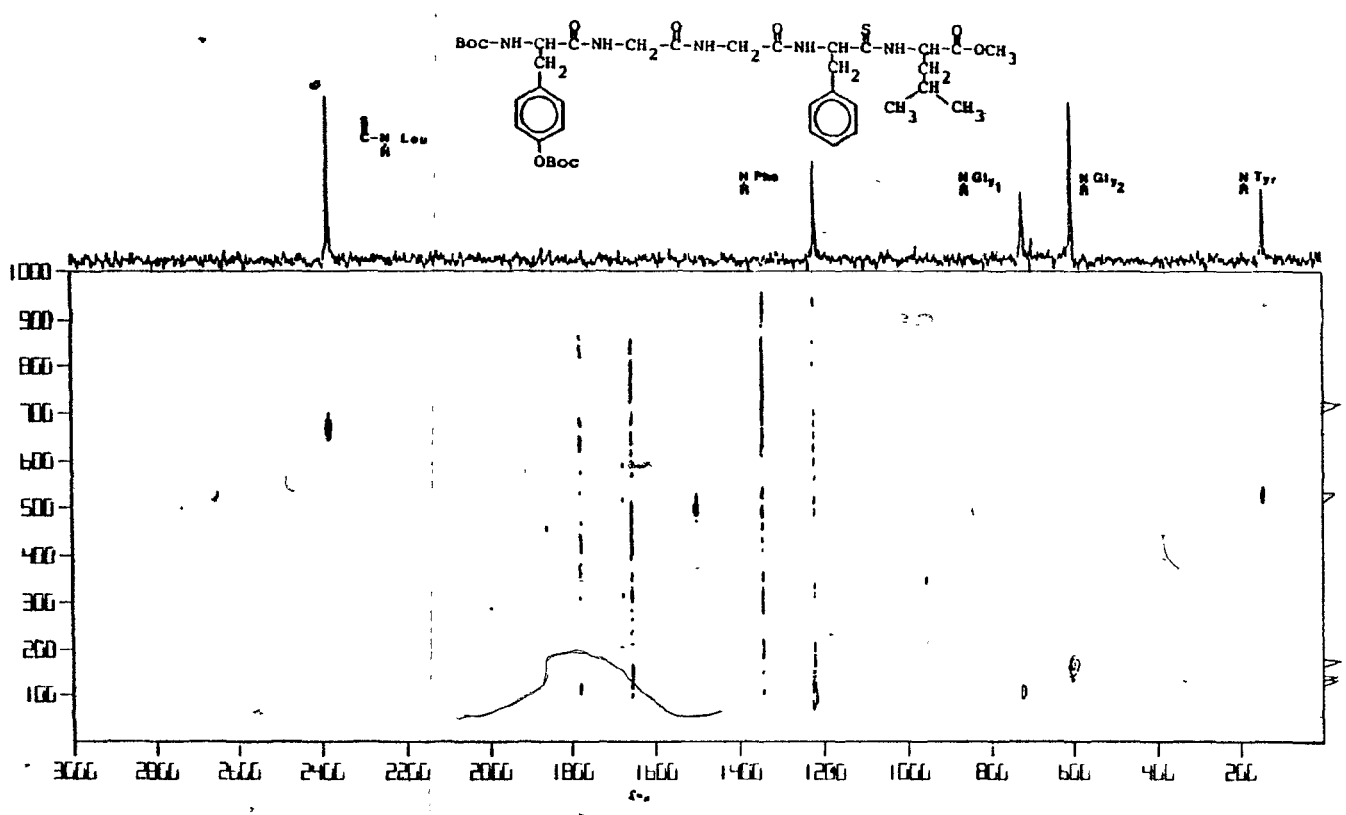


Figure 53 2D (^{15}N-^{1}H) shift correlated NMR spectrum of thioamide analogue 90.

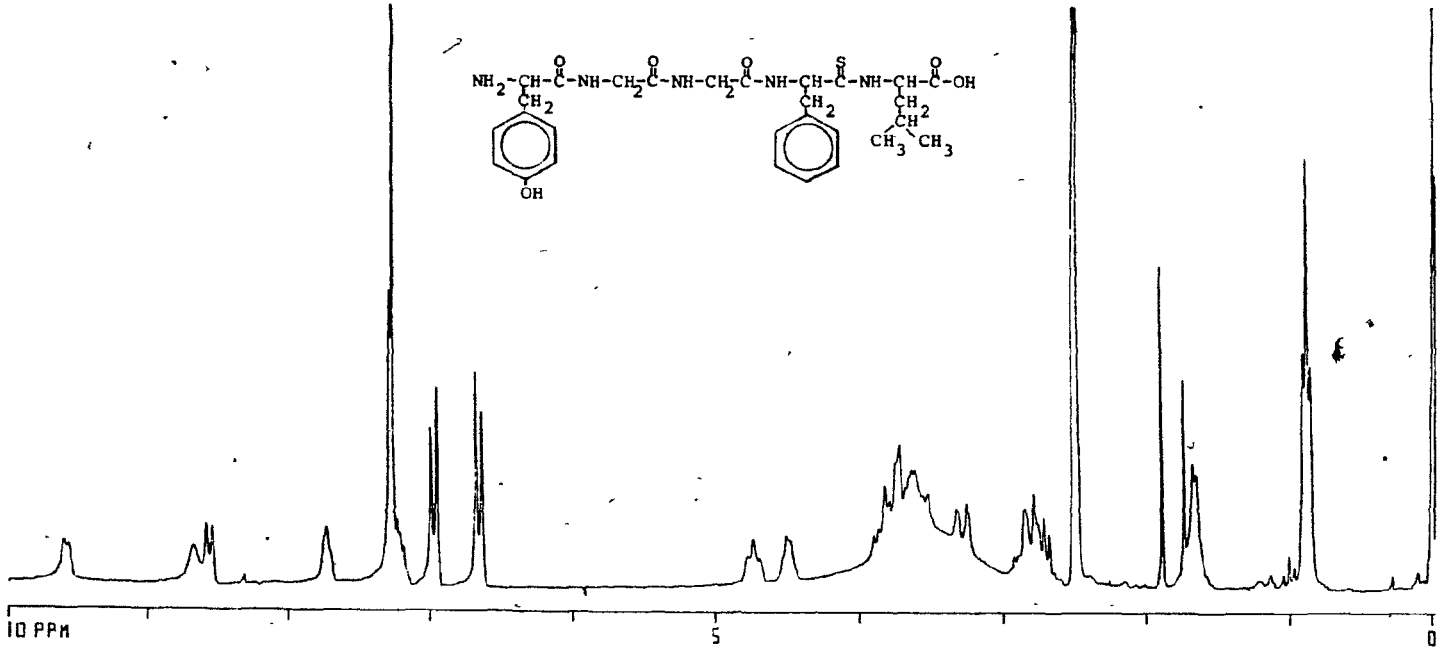


Figure 54 ^{1}H NMR spectrum (200 MHz, DMSO-d_6) of thioamide analogue 91.

The nitrogen of the thioamide is shifted downfield and all the other N-H groups can also be identified individually. The presence of single peaks for the nitrogen atoms indicates that no cis amide bonds are present[468] and that little, if any, racemization has occurred in the synthesis of the thiopentapeptide 90.

Saponification of the methyl ester and removal of both Boc groups with formic acid and purification under the conditions described above for [Leu^5]-enkephalin gave a crude which gave rise to a major peak in the HPLC analysis and several minor peaks corresponding to impurities which were removed by semi-preparative reverse phase HPLC.

The presence of the thioamide function was confirmed by its characteristic UV absorption (λ_{max} 271.2 nm, log ε 4.07). Its ^{1}H NMR spectrum (DMSO) showed all the expected resonances for the amino acid side chains as well as the NH at 9.55 ppm corresponding to the thioamide. Irradiation of this unresolved broad doublet modified the signal at 4.4 ppm corresponding to the CH_α of leucine, while irradiation at 4.71 ppm changed both signals at 8.53 (NH Phe) and the area at 3.4-3.6 attributed to CH_2 of Phe (Table 20). The composition of 91 was verified by amino acid and elemental analysis. Other physical data are summarized in Table 19.

Of interest is the longer HPLC retention time (13 min) of 91 relative to [Leu^5]-enkephalin (8.8 min.), indicating that the thioamide derivative is more hydrophobic. As will be seen, this observation holds true for the other thioamide analogues.

	R_f[a]	U.V.[b] λ_{max} log ε	$[\alpha]_D^{20}$[c]	Amino acid[d] analysis	Elemental analysis[e] C	H	N	S
O,N-bis(Boc)D,L-Tyr"^{S}Gly-Gly-Phe-Leu-OCH_3 **106**	.52	268.3 4.02	-19.0	Tyr .73 Gly 1.58 Phe 1.02 Leu 1.00	59.51	7.07	9.12	4.00
O,N-bis(Boc)Tyr-Gly"^{S}Gly-Phe-Leu-OCH_3 **100**	.51	264.7 4.04	-12.1	Tyr .78 Gly 1.65 Phe .98 Leu 1.00	59.74	7.19	9.01	3.89
O,N-bis(Boc)Tyr-Gly-Gly"^{S}Phe-Leu-OCH_3 **96**	.49	264.8 4.07	-18.9	Tyr .85 Gly 1.78 Phe 1.10 Leu 1.00	59.80	6.69	8.87	4.07
O,N-bis(Boc)Tyr-Gly-Gly-Phe"^{S}Leu-OCH_3 **90**	.47	268.3 4.05	+ 3.6	Tyr .88 Gly 1.86 Phe 1.11 Leu 1.15	59.72	7.15	9.10	4.02

a) $CHCl_3$/MeOH 9:1
b) EtOH, 20°C
d) c 1.0, $CHCl_3$
d) Hydrolysis with 6 N HCl in a sealed tube, 20 h, 110°C.
e) Calculated for analogues: C 59.60, H 7.05, N 8.91, S 4.07.

Table 16 Analalytical data for the protected thioamide analogues of [Leu^5]-enkphalin.

	Tyr^1			Gly^2		Gly^3		Phe^4			Leu^5	
	H_α	H_β	$H_{\beta'}$	H_α	$H_{\alpha'}$	H_α	$H_{\alpha'}$	H_α	H_β	$H_{\beta'}$	H_α	H_β
85 [Leu^5]-enk	4.40	3.10	2.95	3.96	3.96	3.96	3.96	4.94	3.14	3.05	4.56	1.6
90 [Phe^{4}C(S)]-	4.63	3.05	2.96	4.06	4.06	4.10	4.10	5.36	3.18	3.05	5.18	1.7
96 [GLy^{3}C(S)]-	4.40	3.28	2.98	3.97	3.76	4.28	4.18	5.25	3.38	3.22	4.55	1.7
100 [Gly^{2}C(S)]-[Tyr^{1}C(S)]-	4.30	3.10	2.98	4.32	4.32	4.32	4.32	4.94	3.15	3.05	4.58	1.6
106a L-Tyr	4.80	3.26	3.05	4.25	4.25	4.01	3.75	5.00	3.12	3.00	4.69	1.6
106b D-Tyr	4.84	3.28	3.05	4.38	4.11	3.97	3.97	5.04	3.13	3.00	4.58	1.6

Table 17 ^{1}H NMR (200 MHz, $CDCl_3$) chemical shifts of protected [Leu^5]-enkephalin and thioamide analogues.

		Tyr^1		Gly^2	Gly^3	Phe^4		Leu^5	
		C_α	C_β	C_α	C_α	C_α	C_β	C_α	C_β
85	[Leu^5]-enk	55.2	39.3	43.26	43.4	54.0	38.5	50.9	41.2
90	[Phe^4C(S)]-	55.3	38.8	43.32	43.7	60.4	42.8	56.5	40.5
96	[Gly^3C(S)]-	56.3	36.8	43.46	50.5	59.6	36.7	51.2	41.0
100	[Gly^2C(S)]-	56.13	39.5	49.05	48.8	54.3	37.3	51.0	41.2
106	[Tyr^1C(S)]- (D,L)	62.4	41.4	48.4	43.13	54.3	38.9	50.9	39.4

Table 18 ^{13}C NMR chemical shifts (ppm) of [Leu^5]- enkephalin and thioamide analogues. ($CDCl_3$, 20°C)

3.2.3 Synthesis of Tyr-Gly-GlyC(S)-Phe-Leu-OH.

For the synthesis of the regioisomer thionated at the Gly^3 position, we took advantage of the sensitivity of the thionation reaction to the steric environment of amide groups.

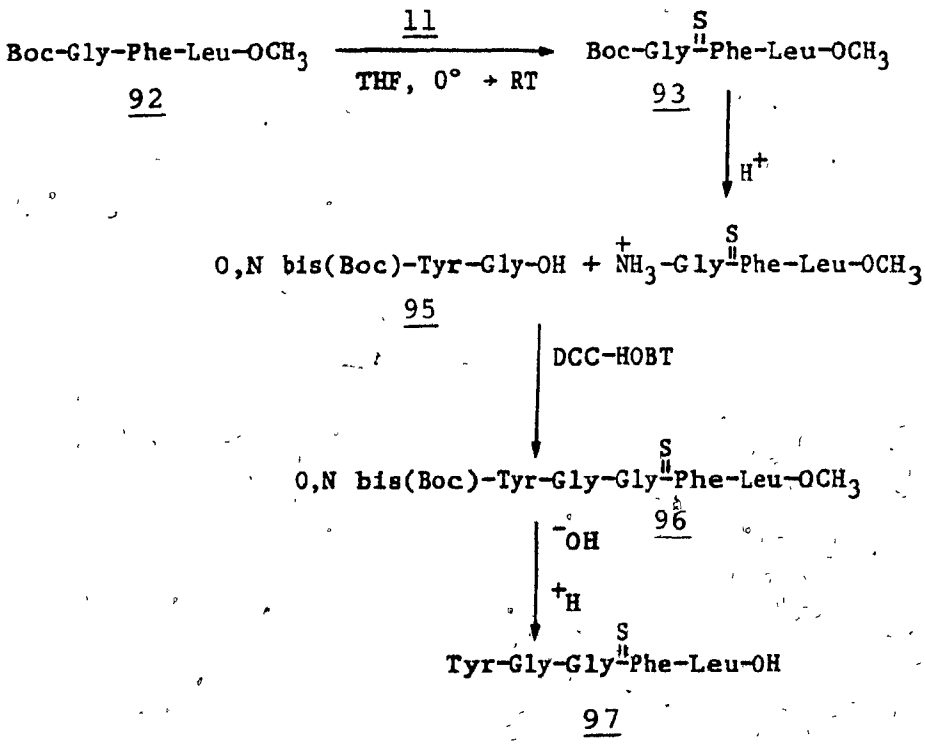


Figure 55

The thioamide could be selectively incorporated at the desired position in the tripeptide fragment, Boc-Gly-Phe-Leu-OMe (92). Elongation from the N-terminal with the appropriate dipeptide fragment could then be accomplished, as previously described (Fig. 55)

The starting tripeptide 92 was prepared in high yield by coupling Boc-Gly with Phe-Leu-OCH_3 using DCC-HOBt as the coupling reagents. Thionation with reagent 11 in THF at 0°C for 0.5 h and at room temperature for 1 h gave, after purification, an 83% yield of the desired Boc-GlyC(S)-Phe-Leu-OCH_3 (93). The ^1H NMR spectrum of the product displayed all the expected resonances. Irradiation of the thioamide proton at 9.64 ppm simplified the multiplet corresponding to the CH_αPhe at 5.2 ppm, confirming that the thioamide linkage is at the correct position.

Removal of the Boc group with formic acid gave the thiotripeptide as its formate salt which was added to the activated ester (DCC-HOBt) of the O,N bis(Boc)-Tyr-Gly-OH (95) which had been previously prepared by condensation of the O,N bis(Boc)-tyrosine with glycine ethyl ester followed by the removal of the ester group with base.

The resulting monothiopentapeptide 96 was obtained in 75% yield after purification by flash chromatography on silica gel. Analytical data are summarized in Table 16. Pertinent ^1H NMR and ^{13}C NMR data for this compound are summarized in Tables 17 and 18. Deprotection and purification of the final product was carried out under conditions identical with those described above for the thioamide analogue 97. The relevant physico-chemical data are given in Tables 19 and 20.

	R_f[a]	HPLC[b] ret. time ± 0.2 min	U.V.[c] λ_{max} log ε	Amino acid[d] analysis	Elemental analysis[e] C	H	N	S
108 (D,L)Tyr$\overset{S}{}$Gly-Gly-Phe-Leu	.67	12.0	269.3 4.06	Tyr .61 Gly 1.69 Phe 1.02 Leu 1.00	An insufficient amount of compound was available.			
101 Tyr-Gly$\overset{S}{}$Gly-Phe-Leu	.66	11.2	267.3 3.97	Tyr .66 Gly 1.57 Phe 1.00 Leu 1.00	"			
97 Tyr-Gly-Gly$\overset{S}{}$Phe-Leu	.68	10.2	269.3 4.02	Tyr .60 Gly 1.85 Phe 1.05 Leu 1.00	58.90	6.54	12.16	5.66
91 Tyr-Gly-Gly-Phe$\overset{S}{}$Leu	.63	13.0	271.7 4.05	Tyr 1.00 Gly 2.0 Phe 1.01 Leu .94	58.65	6.53	12.10	5.66

a) Butanol/acetic acid/ H_2O, 4:1:1, Leu^5-enkephalin: .56

b) C-18 μBondapack (Waters) MeOH/ammonium acetate .1 M, pH7, Leu^5 enk leu^5 - enkephalin 8.8 min.

c) EtOH, 20°C.

d) Hydrolysis with 6 N HCl in a sealed tube, 20 h, 110°C.

e) Calculated for analogues: C 58.82, H 6.52, N 12.25, S 5.59.

Table 19 Analatical data of deprotected [Leu^5]-enkephalin and thioamide analogues.

		Tyr^1	Gly^2		Gly^3		Phe^4		Leu^5	
		CH_α	NH	CH_α	NH	CH_α	NH	CH_α	NH	CH_α
86	[Leu^5]-enk	3.5	8.38	3.5	7.9	3.6	8.2	4.42	7.94	4.07
91	[$Phe^4C(S)$]-	3.4	7.68	3.5	8.66	3.7	8.5	4.71	9.55	4.44
97	[$Gly^3C(S)$]-	3.7	8.45	3.7	8.16	4.1 3.95	9.85	5.15	8.18	4.05
101	[$Gly^2C(S)$]-	3.7	7.85	4.11	9.45	4.06	8.44	4.42	7.85	4.05
108	[$Tyr^1C(S)$]-	3.78	9.4	4.10	8.2	3.6	8.14	4.47	8.06	4.07

Table 20 Relevant ^1H NMR chemical shifts (200 MHz, DMSO-D_6) of [Leu^5]-enkephalin and thioamide analogues.

3.2.4 Synthesis of Tyr-GlyC(S)-Gly-Phe-Leu-OH

The documented regioselectivity of our thionation reaction methodology was again exploited for the synthesis of this analogue. The presence of two glycine residues makes the carboxyl of Gly^2 the least crowded site of the oligopeptide backbone, thus making it the most accessible in principle to the thionation reagent 11 (Fig. 56).

However, thionation at the Gly^3 carboxyl residue can also occur easily as was exemplified with the preparation of the tripeptide 93. In fact, when the tetrapeptide Boc-Gly-Gly-Phe-Leu-OCH_3 (83) was reacted with 0.6 eq of thionation reagent 11 at room temperature in dry THF, a substantial amount (20%) of the dithiotetrapeptide Boc-GlyC(S)-GlyC(S)-Leu-OCH_3 (98) was isolated and

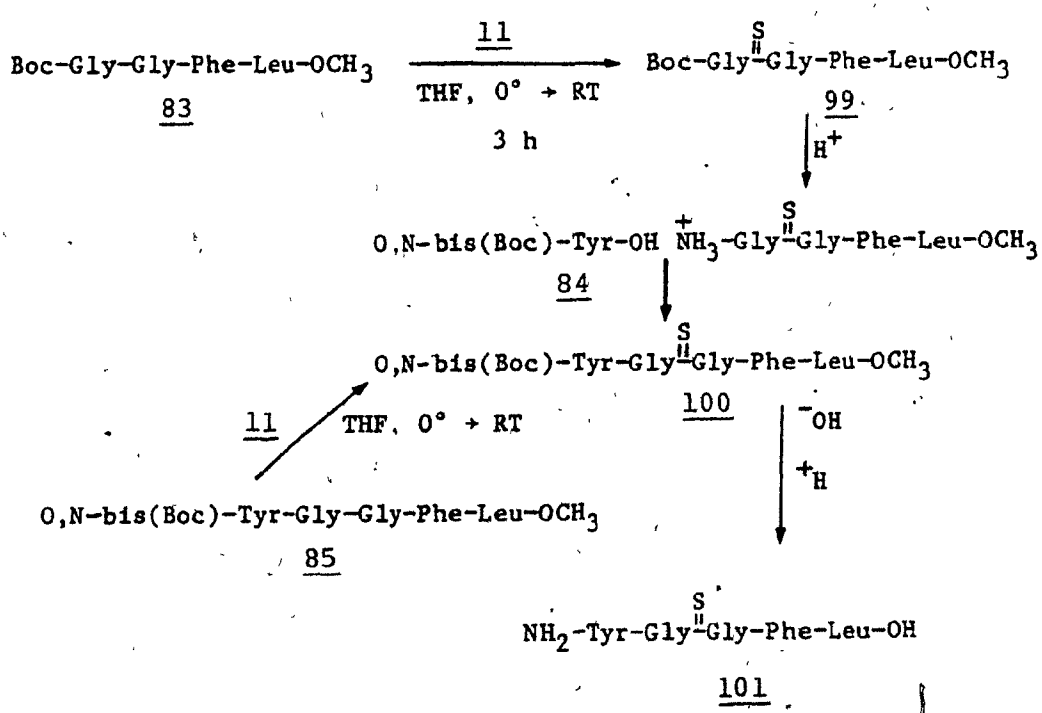


Figure 56

only 45% of the desired monothioamide 99. The ^{1}H NMR spectrum (60 MHz) of the dithioamide analogue 118b showed a downfield shift of 2 NH protons at 9.46-8.66 ppm appearing as two different sets of multiplets. The CH_α of Phe was also shifted downfield to 5.3 ppm indicating the presence of a thiocarbonyl function at the Gly^3 site.

Better control of the same reaction was achieved by slow addition of the thionation reagent 11 (in THF) at 0°C. The reaction mixture was kept at 0°C for 3 h followed by a rise to room temperature for 1 h. In this way the yield of the desired monothioamide analogue 99 was increased to 75-80% after purification by flash chromatography on silica gel and very little (5%, TLC) of dithioamide 98 was produced.

The identity of Boc-GlyC(S)-Gly-Phe-Leu-OCH_3 (99) was ascertained by ^{1}H NMR (200 MHz) analysis. Only one thioamide NH signal appeared downfield (9.1 ppm) and the CH_αPhe multiplet was centered at 5.0 ppm. Irradiation of the C(S) NH at 9.1 ppm reduced the CH_α and $CH_{\alpha'}$ of Gly^3 to two sets of doublets, while irradiation of the Boc NH at 5.7 simplified the doublet for $CH_{\alpha,\alpha'}$ of Gly^2 to a broad singlet.

The ^{13}C NMR spectrum of the compound revealed only one resonance at 200.1 ppm corresponding to the thiocarbonyl function. Its mass spectrum ($M^{+\cdot}$ 522) also confirmed that the product contained a single thioamide group. This conclusion was further strengthened by the results of elemental analysis which confirmed

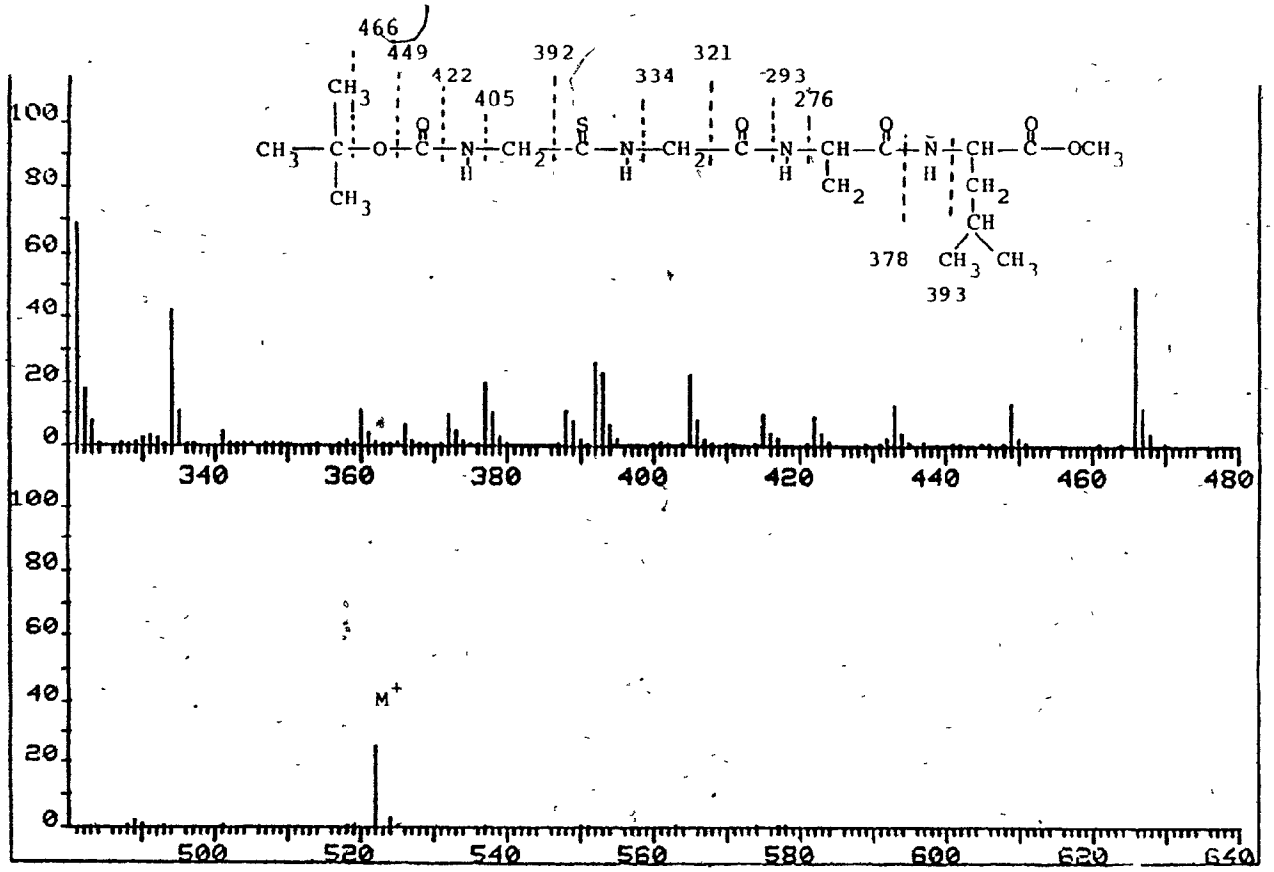


Figure 57. Mass spectrum (70 eV) of the thiotetrapeptide 99

the proposed composition.

Removal of the Boc group of the thiotetrapeptide was performed as previously described and the resulting formate salt was coupled with O,N bis(Boc)-Tyr using DCC-HOBt as coupling reagents. After purification by flash chromatography on silica gel, a waxy solid (75%) yield) corresponding to the thiopentapeptide was obtained and characterized (Tables 16,17 and 18).

The same product was also obtained by thionation with reagent 11 of the fully protected [Leu^5]-enkephalin 85. It was isolated in 70% yield after purification. Obviously, the presence of the tyrosine residue instead of a Boc group did not alter the regioselectivity of the thionation reaction.

The monothiopentapeptide was deprotected and purified using the same conditions already described for previous analogues. The analytical data for this compound are summarized in Tables 19 and 20.

3.2.5 Synthesis of TyrC(S)-Gly-Gly-Phe-Leu-OH

The synthesis of this analogue proved to be more complicated and more difficult. Thionation with reagent 11 (0.6 eq) at room temperature of the tripeptide O,N bis(Boc)-Tyr-Gly-Gly-OEt substituted the Gly^2 carboxyl as expected, and no monothioamide at the site of the Tyr carboxyl was observed. We knew from previous experiments that elongation from the C-terminal end of the preformed thiodipeptide was not feasible (Chapter 1).

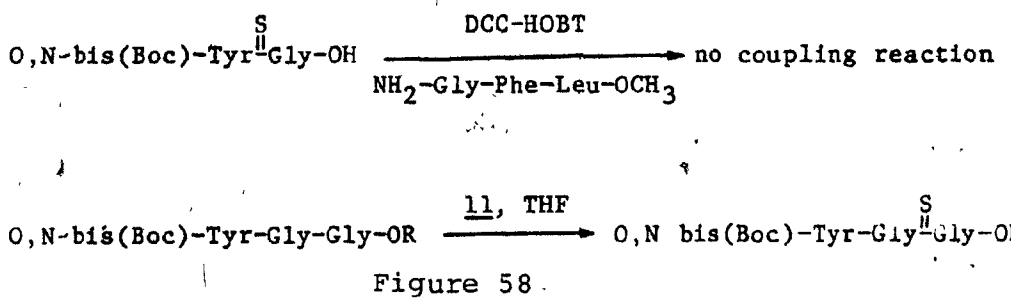


Figure 58

Therefore, we had to resort to the use of a dithioester as an intermediate for the incorporation of the thioamide linkage at that first position. The required O,N bis(Boc)-Tyr-C(S)-SCH_3 was prepared as previously described for the synthesis of the analogous Boc-Phe dithioester (50). The N-methyl amide derivative of O,N bis(Boc)-Tyr-OH was prepared using DCC-HOBt and methylamine hydrochloride as the reagents.

Thionation with reagent 11 of the N'-methyl amide 102 followed by flash chromatography gave the corresponding N'-methyl thioamide 103 in 83% yield. Alkylation with methyl iodide (2 eq, 35°C) in acetonitrile under anhydrous conditions gave, after 24 h, the corresponding methyl imino thioether 104 which was not purified at this stage because of the known sensitivity of thioimidates to moisture and heat[469]. However, the ^1H NMR spectrum (200 MHz) of the resulting product confirmed a structure in which the two Boc groups were intact.

Thiolysis of the crude thioimidate with hydrogen sulfide in THF at 0°C in the presence of pyridine (5 eq) gave the corresponding O,N bis(Boc)-TyrC(S)-SCH_3 105 as a bright yellow solid after purification by flash chromatography on silica gel.

Only, the N'-methyl thioamide 103 was isolated as a side product indicating that both Boc groups were resistant to thiolysis. The structure of the dithioester was confirmed by its ^{1}H NMR spectrum (200 MHz) and mass spectra ($M^{+\cdot}$ 427, and M^+-(C(S)-SCH_3 336).

An optically active derivative ($[\alpha]_D^{20}$ +60.5° c 1.0, $CHCl_3$) was obtained but no attempt was made to determine the optical purity of the product or to optimize the rotation by recrystallisation, since we expected the α-carbon to undergo complete racemization in the next reaction.

Reaction of the dithioester 105 with Gly-Gly-Phe-Leu-OCH_3 in THF, under N_2, at room temperature for 24 h in the presence of imidazole gave the thiopentapeptide 106 in 89% yield (Fig. 59). Its ^{1}H NMR spectrum (200 MHz) displayed two signals of equal intensity for the methyl ester resonance and complex signals in the 4.28-3.94 ppm region corresponding to the CH_2 of Gly^2 and Gly^3. This was indicative that racemization of the Tyr residue had taken place. An optical rotation of -19.0° was measured for the compound. The presence of a diastereomeric mixture was confirmed by HPLC analysis which showed the presence of two proximal peaks of similar intensities. Surprisingly, however, the ^{13}C NMR ($CDCl_3$) spectrum of this mixture gave no evidence of any doubling of the peaks as might be expected for a diastereomeric mixture (Table 18).

As we expected the D-Tyr^1 isomer of the pentapeptide to be inactive (as in the case of D-Tyr^1,[Leu^5]-enkephalin), we chose to

deprotect and purify the diastereomeric mixture and test the resulting thiopentapeptide, as such, for biological activity. However, in contrast to the behavior of the previous analogues towards base-catalyzed hydrolysis of methyl ester function, similar treatment of this thiopeptide led to the development of a very intense yellow color and required a longer reaction time than for any other analogue, as judged by the rate of disappearance of the starting material (TLC). The yellow contaminant thus produced could not be removed from the desired product by flash chromatography on silica gel.

Removal of the Boc group with formic acid was accomplished as before on the crude yellow peptide acid to give a yellow solid which was submitted to the same HPLC purification proceedure previously described for the other thiopeptide analogues of [Leu^5]-enkephalin. A white powder was obtained, but the yield after both deprotection and purification was somewhat lower (10-15%). Its structure was confirmed by UV and ^{1}H NMR (200 MHz) spectroscopy and by quantitative amino acid analysis (Table 19 and 20).

The fully protected analogue was also prepared following another route as outlined in Fig. 60. Condensation of the dithioester 105 with Gly-Gly-OEt gave the corresponding thiotripeptide 107 (90% yield), which again had suffered racemization at the level of the tyrosine residue ($[\alpha]_D^{20}$ +4.5°, c 1.0, $CHCl_3$). After saponification of the ethyl ester with sodium hydroxide in THF/H_2O,

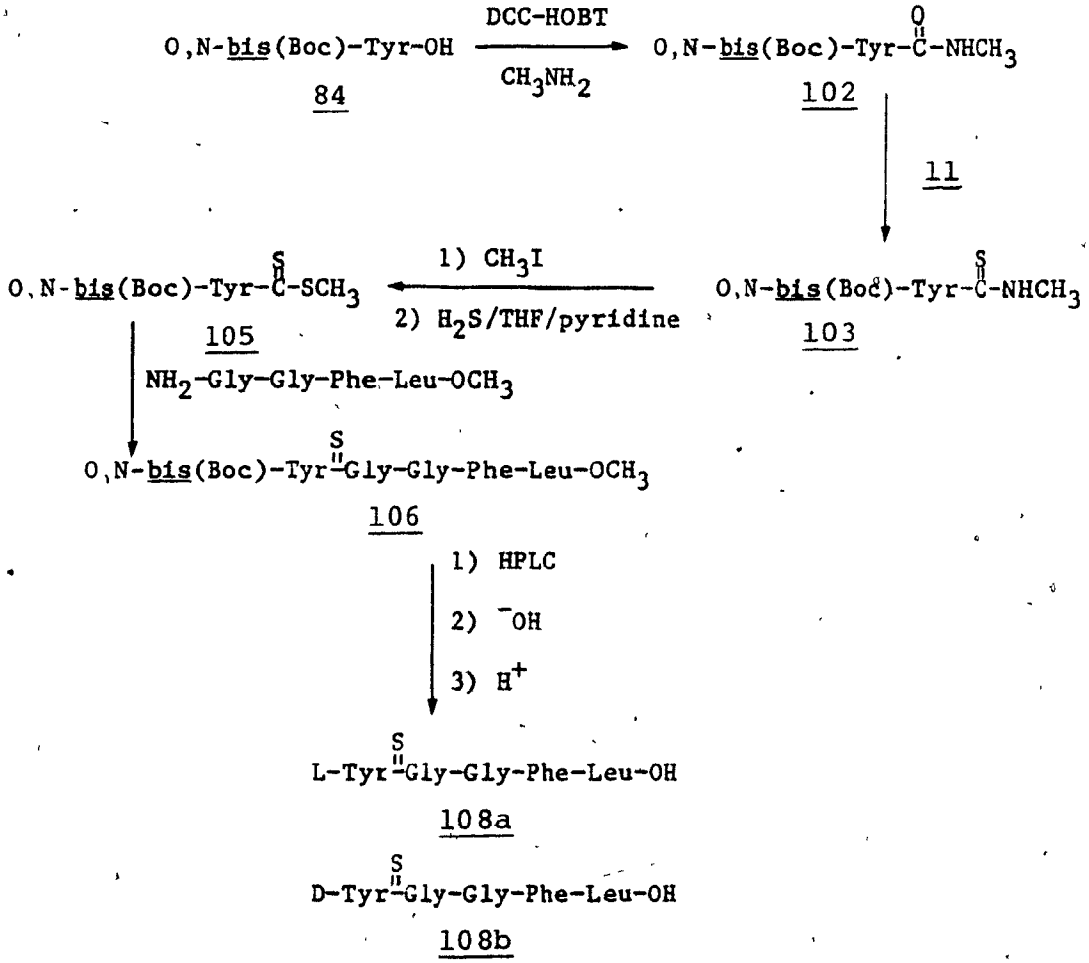


Figure 59 Synthesis of thioamide in position 1 of [Leu^5]-enk.

the thiotripeptide acid was reacted with DCC-HOBt and subsequently with Phe-Leu-OCH_3 to yield the protected thiopentapeptide in 68% yield. The ^1H NMR spectrum was nearly identical to that previously obtained with the diastereomeric mixture of 106, the major difference lying in the intensities of the OCH_3 signals which were in a ratio of 60:40 instead of 50:50. The coupling reaction proceeded normally and without interference from the thioamide because sulfur participation would involve formation of an eight membered ring and this would require the amide bond in the cis configuration.

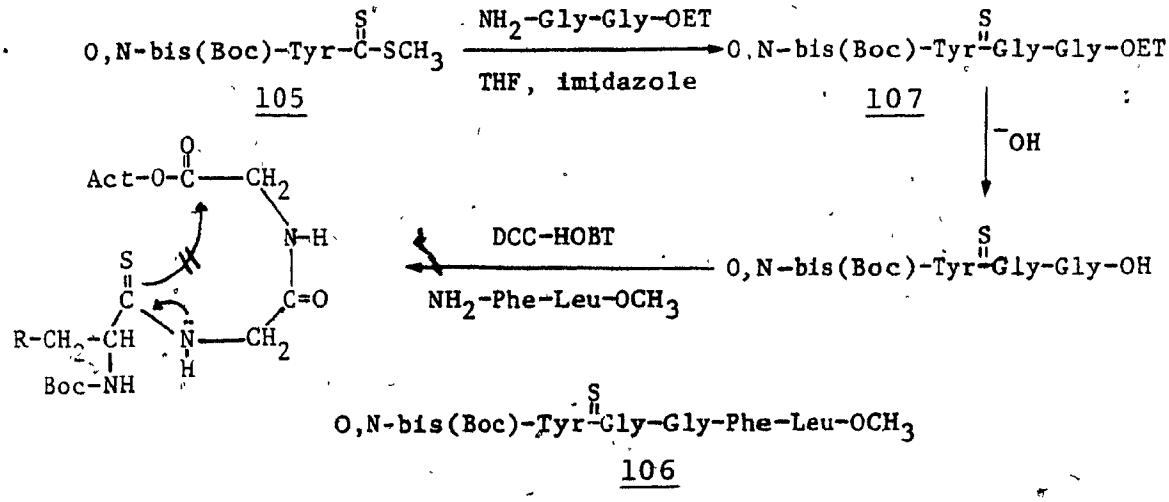


Figure 60

Although this alternative route to 106 offered no synthetic advantage over the preceeding one, it served to illustrate a synthetic sequence that could be very valuable for the preparation of other thiopeptide analogues. For example this route would be valuable if one wished to prepare thio-analogues where only

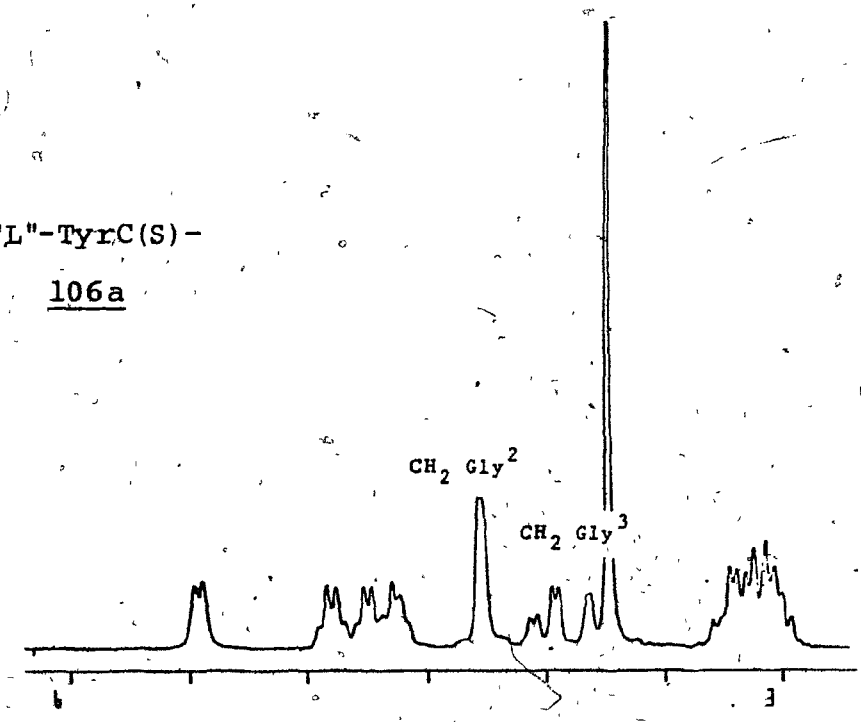
the fourth amino acid residue would be varied (eg. [Gly^2C(S), Met^5]-enkephalin, [Gly^2C(S), Pro^5]-enkephalin, etc...)

Conditions were eventually found for separating the two isomers of the fully protected thiopentapeptide 106 originating from racemization about Tyr^1. Thus, 20 mg of the least polar and 23 mg of the most polar isomer could be separated. The least polar isomer had a rotation of +1.68°, while the most polar exhibited a rotation of -35.6°. Based on an optical activity of +50,5° for O,N bis(Boc)TyrC(S)$NHCH_3$ (103), we tentatively assigned the absolute stereochemistry L for the least polar and D for the most polar compound. We expected this assignment to be confirmed by the biological activity of the deprotected analogues[411].

Of interest is the significant difference in their ^1H NMR spectra. For the "L-Tyr" isomer the CH_2 of Gly^2 gave rise to a broad doublet at 4.25 ppm while the CH_2 of Gly^3 gave two doublets of doublets at 4.01 and 3.75 ppm (ABX pattern). The other isomer which is the most polar ("D-Tyr" isomer) showed a reversed pattern for the coupling constants: two doublets of doublets for the CH_2 of Gly^2 at 4.36 and 4.11 ppm and a broad apparent triplet for the CH_2 of Gly^3 at 3.9 ppm (Fig.61). The difference at the Gly^2 and Gly^3 residues is certainly indicative of different predominant conformations induced by the stereochemistry of the tyrosine residue. A large difference in the chemical shift of the diastereotopic protons of glycine are believed to reflect special conformational requirements about the glycine residue[470]. However, such

"L"-TyrC(S)-

106a



"D"-TyrC(S)-

106b

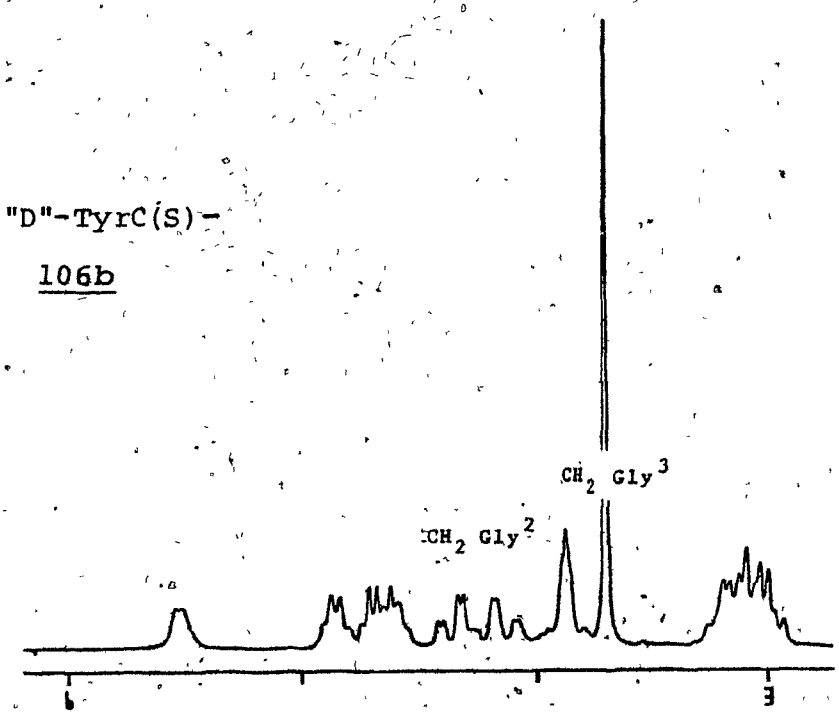


Figure 61 ^{1}H NMR spectra (200MHz, $CDCl_3$) of "L"- and "D"-TyrC(S)-Gly-Gly-Phe-Leu derivatives.

non-equivalence of chemical shifts has been mainly seen with small deprotected peptides where head to tail interactions may occur or with small cyclic peptides, such as substituted diketopiperazine, where conformational mobility is severly restricted[471]. Extrapolation of such observations to the conformational properties of the thiopentapeptide backbone is at best tentative at this time. In these previous studies, it was noted that both the presence of aromatic residues in close proximity to the gylcine residue and their absolute stereochemistry are important factors[471a].

Nevertheless, we can speculate that in one isomer (L-Tyr) the tyrosine aromatic ring is closer to the Gly^2 residue while in the other isomer (D-Tyr) the aromatic ring is in greater proximity to the Gly^3 residue, thus enhancing the H_α-$H_{\alpha'}$ chemical shift difference. In support of this hypothesis is the observation that in model cyclic structures the aromatic ring is preferentially oriented toward the diketopiperazine ring and affects H_α through the aromatic ring current (Fig. 62)[471a].

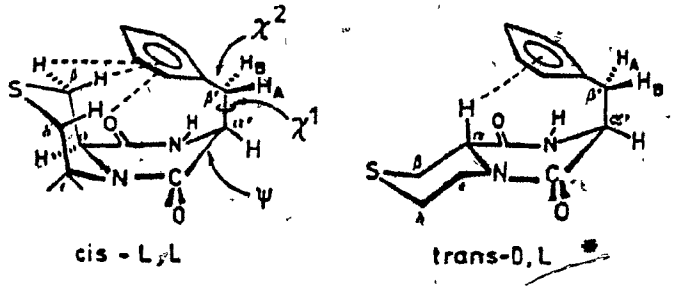


Figure 62

Of course in our peptide, the involvement of the aromatic ring of Phenylalanine instead of Tyrosine can not be ruled out. Obviously, more experimental data would be necessary in order to provide a definitive answer.

Even though these results are incomplete, they clearly illustrate the potential use of thioamide analogues for the purpose of separating the glycine residue signals, thereby making them more amenable to conformational analysis. Such non-equivalence of the Gly^2 and Gly^3 residues was also observed for the analogues with the thioamide function at position 3 (97), but was not observed in the case of protected [Leu^5]-enkephalin.

Deprotection of both the D and L diastereomers by the method previously described also gave a poor yield (10-15%) of the final product, after HPLC purification. The reasons for this poor yield are not immediately apparent. The thioamide in that position is somewhat sterically hindered and thus its potential attack during the hydrolysis of the methyl ester should be no greater than for the more accesible thioamide of O,N-bis(Boc)-Tyr-GlyC(S)-Gly-Phe-Leu-OCH_3 100. It is possible however, that due to a special conformational effect, such as folding of the backbone, the thioamide function of 106 may be more exposed to attack by hydroxide ion.

Due to the difficulty in achieving the separation of the D, L mixture of 106, attempts at generating the pure L-Tyr diastereomer by another route were made. If our starting material

was an appropriately chosen dithiopeptide, which can be readily obtained by thionation with excess thionation reagent, it might then be possible to selectively exchange one of the sulfur atoms for an oxygen through a reaction sensitive to differences in local steric crowding. In order to illustrate this approach, the O,N-bis(Boc)TyrC(S)-GlyC(S)-Gly-OEt was prepared by reacting the precursor peptide with excess thionation reagent 11 (70%). Kochar et al[472] have recently reported the conversion of thioamide to amide by oxidation with mCPBA. It is believed that this reaction proceeds through the rearrangement of a thioamide oxide intermediate as shown:

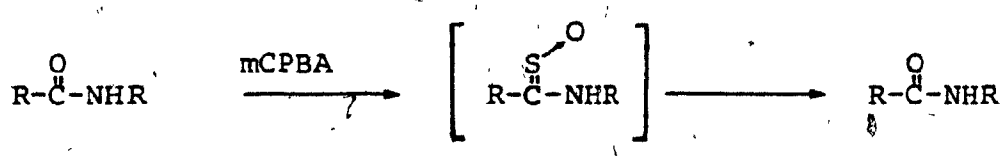
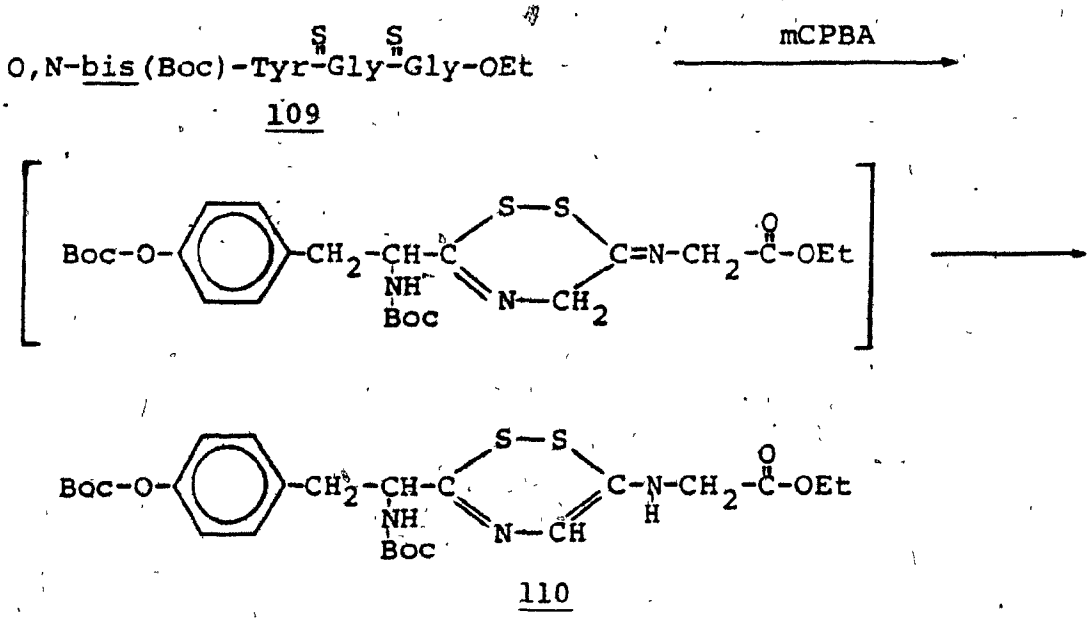


Figure 64

However when this reaction was performed on our dithiotripeptide substrate 109 a variety of products were obtained, none of which had an R_f corresponding to that expected for either of the monothioamide analogues. After purification of the main component by flash chromatography on silica gel, its ^1H NMR spectrum (200 MHz) indicated the formation of an unexpected product which has been tentatively assigned to structure 110 (Fig. 65). Thus, the methylene resonance of Gly^2 disappeared after oxidation while that of Gly^3 was shifted from 4.3 ppm in 104 to 3.83 ppm in the product. A singlet also appeared at

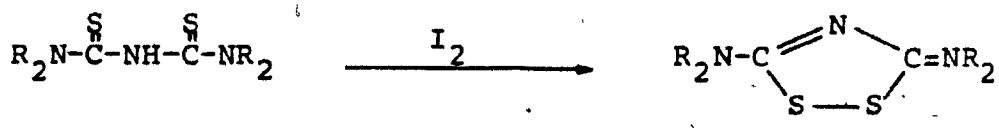


109

110

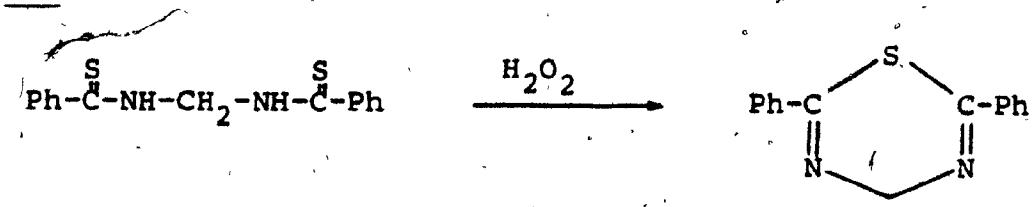
Figure 65

6.80 ppm corresponding to a deshielded proton typical of alkenes or aromatic protons. In addition, there was no signal for the thioamide NH but an exchangeable proton was transformed by D_2O from a broad doublet characteristic of Gly^3 at 3.83 to a singlet. Similar oxidation products of thioamides have been described in the literature. For example, dithioburets 111 undergoes oxidative ring closure to form 112 upon treatment with iodine in pyridine[473].



111 112

Also, when 2,6-diphenyl-1,3,5-thiadiazine 113 is reacted with H_2O_2 114 is formed[474].



113 114

Other reaction conditions, such as lower temperatures or the presence of radical inhibitors (3-t-butyl-4-hydroxy-5-methylphenyl sulfide) were tried, but the same major product was produced.

In any case, we had all the desired analogues on hand and thus there was little incentive to develop this conceptual approach. Other unrelated approaches based on the development of novel thioacylating reagents are being explored by others in our laboratory.

3.3 Biological Activity

All of our thiopeptidic analogues were submitted to standard biological assays. Smooth muscle assays were performed using the guinea pig ileum (GPI) and the mouse vas deferens preparations

(MVD)[475] (Table 21). Binding to the opiate receptors was evaluated with rat brain homogenates by comparing their respective ability to displace [^{3}H]-entorphine[476] (all receptor types) and [^{3}H]-dihydromorphine (μ receptor)[476] (Table 22).

The hot plate test was used to monitor behavorial changes, such as antinociceptive activity, as induced by [Leu^5]-enkephalin with which our thioamide analogues were compared[477] (Table 23). Finally, for [Gly^{2}C(S), Leu^5]-enkephalin, the minimum dose required to significantly enhance the response latencies of animals in the hot plate test was determined (data not shown).

In the GPI assay, thioamide functions at position 4, 3 and 1 (the latter being racemic about Tyr^1) do not significantly alter the activity of [Leu^5]-enkephalin whereas the same function at position 2 caused a moderate drop in activity (26%). In the MVD preparations, a thiopeptide bond at position 4 had little effect on activity. However, thioamide functions at positions 1 and 3 exhibit markedly reduced potencies. On the other hand, the analogue [Gly^{2}C(S), Leu^5]-enkephalin was some 9 times more potent than [Leu^5]-enkephalin in the same preparation.

In the binding assay, the presence of a thioamide group at postion 1 and 3 decreased the binding affinity by 14-71%. as evaluated by the displacement of the radio-labelled ligands of etorphine and dihydromorphine. The analogue with sulfur at position 4 had 32% and 96% the activity of [Leu^5]-enkephalin in the same assays. [Gly^{2}C(S), Leu^5] enkephalin was significantly better than [Leu^5]-enkephalin in the displacement of dihydromorphine

Table 21 Relative inhibitory potencies of Leu-Enk and sulfur-containing analogs on the electrically induced contractions of guinea pig ileum (GPI) and Mouse vas deferens (MVD)

	Synthetic Compound	GPI		MVD	
		ID_{50}[a] (nM)	Rel. potency[b] (%)	ID_{50}[a] (nM)	Rel. potency[b] (%)
86	Leu-Enk	420 ± 130	100	10.0 ± 2.0	100
108	[Tyr^1C(S)]-	760 ± 40	55	400 ± 65	2
101	[Gly^2C(S)]-	570 ± 290	74	1.1 ± 0.9	909
97	[Gly^3C(S)]-	370 ± 65	113	65 ± 15	15
91	[Phe^4C(S)]-	460 ± 180	91	10.4 ± 1.2	96

[a] Concentration which gives half maximal response.

[b] Relative to Leu-Enk

Table 22 Relative inhibitory potencies of Leu-Enk and sulfur-containing analogs on the binding of [^{3}H]-etorphine and [^{3}H]-dihydromorphine to rat brain homogenates.

	Synthetic Compound	[^{3}H]-etorphine		[^{3}H]-dihydromorphine	
		ID_{50}[a] (nM)	Rel. potency[b] (%)	ID_{50}[a] (nM)	Rel. potency[b] (%)
86	Leu-Enk	22 ± 3	100	25 ± 2	100
108	[Tyr^1C(S)]-	115 ± 8	19	80 ± 5	31
101	[Gly^2C(S)]-	37 ± 7	59	11.1 ± 1	227
97	[Gly^3C(S)]-	31 ± 5	71	.50 ± 3	50
91	[Phe^4C(S)]-	68 ± 6	32	26 ± 3	96

[a] Concentration which gives half maximal response.

[b] Relative to Leu-Enk

Table 23 Response latencies (sec) in the hot plate test (54°C)* subsequent to intracerebroventricular administration of Leu-Enk monothionated at positions 2, 3 and 4 respectively.

Compound/Time (min)	0	2	4	6	8	10	15	30	60
Saline	4.8 ±0.4	4.3 ±0.8	4.1 ±0.4	4.3 ±0.5	3.8 ±0.3	3.5 ±0.7	3.1 ±0.4	4.1 ±0.5	4.3 ±0.6
Leu-Enk 86	4.4 ±0.7	12.1† ±2.9	8.3† ±1.1	9.2 ±2.8	6.3 ±0.8	5.6 ±0.8	3.6 ±0.5	4.3 ±0.4	3.7 ±0.7
[$Gly^2C(S)$]- 101	5.1 ±0.8	14.2† ±3.0	10.5† ±1.1	12.3† ±2.5	14.5† ±1.3	10.0† ±2.2	9.2† ±1.3	5.3 ±0.7	5.3 ±0.7
[$Gly^3C(S)$]- 97	5.1 ±1.0	7.8 ±1.3	6.6 ±1.4	6.2 ±0.7	6.2 ±0.7	6.3 ±1.2	4.1 ±0.5	4.8 ±0.6	3.1 ±0.5
[$Phe^4C(S)$]- 91	4.7 ±0.7	7.3 ±0.8	7.0† ±0.7	6.7 ±1.0	6.3 ±1.1	5.6 ±0.9	6.0 ±1.0	4.5 ±0.5	4.1 ±0.4

*Immediately after establishment of baseline values (Time 0), groups of eight animals were administered intraventricularly 10 µl of either 0.9% saline or 360 µg of Leu-Enk, or the same dose of each of the thionated analogs (a single test with D,L-Tyr^1-thio-Leu-Enk indicated an activity in the range of the Gly^2-thio-analog).

†Significant difference between treated groups and saline-injected animals as revealed by Mann-Whitney tests.

while possessing only 60% the activity of enkephalin in the displacement of etorphine.

In the hot plate test, (intracerebroventricular administration; hooded rats) response latencies were obtained with [Leu^5]-enkephalin, [Gly^{2}C(S), Leu^5]-enkephalin and [Phe^{4}C(S), Leu^5]-enkephalin, the analogue with the thioamide function at position 2 displaying enhanced activity. For the analogue with sulfur at position 1, no quantitative data could be obtained because the compound appeared chemically unstable and also not available in sufficient quantities to provide statistically significant data. In preliminary experiments, however, it was shown to have a longer duration exceeding that of positional isomer 2 by an order of magnitude. Further experiments are needed to quantitate this observation.

Finally, it was observed (data not shown) that a positive response in the latencies of animals in the hot plate test was obtained with a dose of 80 µg of the positional thio-isomer 2 whereas a dose of 240 µg of [Leu^5]-enkephalin was required to observe the same effect.

3.4 Discussion

It is problematic to attempt a detailed analysis of these results using accepted literature criteria. For instance, we expected the analogues with sulfur at positions 1 and 3 to be the most active because these bonds are susceptible to the amino-

peptidases and enkephalinases in [Leu^5]-enkephalin. As it turns out, these analogues do not demonstrate enhanced potency in any of the biological assays. It is probable then that their lower activity actually reflects a decreased affinity for the receptors and that their expected resistance to peptidase action is an insignificant parameter in the limitation of the potency not only of such analogues but also of the enkephalins themselves. A similar deduction has been made by others in relation to the interpretation of the biological activity of certain analogues of [Leu^5]-enkephalin[411,414].

When the thioamide function appeared at position 4 (91) little change in potency was expected because it was already known from several studies that the amide bond at this position is not critical for activity[414]. For example, the aminoxy analogue (-C(=O)-NH-O-CH-) at position 4 of [Leu^5]-enkephalin (91) has similar activity while the same replacement at other positions of the backbone give analogues with no activity[478]; parallel results were obtained with a retro-inverso analogue[426]. Moreover, no specific enzyme has yet been detected which cleaves the amide bond at position 4. In most assays with the exception of displacement of [^{3}H]-etorphine, the positional thioisomer 4 91 had a profile almost identical to that of [Leu^5]-enkephalin. Therefore, our results confirm the relative unimportance of this amide bond for intrinsic biological activity, and illustrate the usefulness of thiopeptide analogues in the evaluation of the relevance of the

individual amide linkages of oligopeptides to their biological activity.

The most revealing results, however, were obtained with [Gly^2C(S), Leu^5]-enkephalin[101]. This compound was more active than [Leu^5]-enkephalin in all assays except, again, in its ability to displace [^{3}H]-etorphine from rat brain receptors where its potency was reduced to 60% of that of the normal peptide. Cleavage of that amide linkage by a specific dipeptidyl aminopeptidase was estimated to account for at best 5% of the overall process of inactivation of [Leu^5]-enkephalin. However, our results cannot be used to support the conclusion that cleavage of this amide bond in [Leu^5]-enkephalin has greater importance in the inactivation process as other parameters are probably involved. For instance, inhibition of other enzymes cannot be ruled out. Further biological testing in the presence of known inhibitiors (bestatin; thiorphan) of aminopeptidases and enkephalinase would be necessary in order to evaluate this possibility. Under such conditions, the absence of an increase in activity would mean that these enzymes are inhibited by 101 which could thus explain its enhanced potency.

On the other hand the higher activity exhibited by this analogue may also be attributed to some conformational change induced by the thioamide function. In this regard, several possibilites come to mind. First, the NH of Gly^3, being more acidic, might create a strong internal hydrogen bond and this could stabilize a conformation not yet observed using any of

the forementioned solution analyses. Secondly, the introduction of a thioamide function at position 2 which is normally responsible for the documented flexibility of the enkephalin backbone[414,479], might simply reduce the number of inactive conformations, thereby causing a net increase in the concentration of active species. Obviously, a more complete conformational analysis using NMR and other physico-chemical methods are required in order to test these possibilities.

The greater hydrophobicity of the thioamide analogues, as evidenced by their characteristic retention time on reverse phase chromatography columns, is probably not important enough in itself to account for potency changes. If the hydrophobicity were a determinant factor, all of the monothioamide analogues should uniformly display enhanced activity relative to [Leu^5]-enkephalin. This is contradicted by the facts.

Another interesting aspect is the different behavior of positional thio-isomer 2 in the GPI and MVD assays. It is now well established that these two tissues contain different subpopulations of μ and δ receptors[480,481]. According to the current literature, the ratio IC_{50}GPI/IC_{50}MVD gives a good estimate of the selectivity of opiates for the μ (GPI) and δ (MVD) receptors. The postulated selective analogue [D-Ala^2, D-Leu^5]-enkephalin (DADLE) gives a ratio of 82.5 whereas [D-Ser^2, Leu^5,Thr^6]-enkephalin gave the highest known ratio of 620[482]. Similar analysis for the case of [Gly^{2}C(S), Leu^5]-enkephalin

gives a ratio of 518; this high value appears strongly indicative of a high selectivity of this thiopeptide for δ receptors.

In contradiction with this conclusion, this same analogue is weaker than [Leu^5]-enkephalin in the displacement of [^{3}H]-etorphine (high affinity for all receptor types) from membrane receptors but twice as active in the displacement of [^{3}H]-dihydromorphine (μ receptor)[482]. This suggests, again in agreement with current literature view, that this analogue would be selective for the μ receptors. The increased potency of compound 101 with both μ and δ opiate ligands suggest that it may result from a stronger resistance to proteolytic cleavage rather than any particular affinity for a specific receptor.

Other types of analogues carrying thioamide functions are certainly desirable. Structures such as [D-Ala^{2}C(S), D-Leu^5]-enkephalin and the analogue [D-Ala^{2}C(S), NMe-Phe, Met(O)-ol^5]-enkephalin might yield additional valuable information about the molecular basis of structure activity relationships in this field. Work in this direction is in progress.

CHAPTER 4

Further Modifications of the Thioamide Function and Applications to other Peptide Analogues: Amidoximes and Amidrazides

If the fundamental geometrical properties of thioamides are similar to those of amides, no such assumption can be made regarding their chemical reactivity. It is well-documented that the thioamide is both a better nucleophile, at sulfur and a better electrophile, at carbon than the corresponding amide[29,59]. Having established a general methodology for the introduction of a thioamide function at any position of a given peptide backbone, it became of interest to exploit the richer chemistry of that function and attempt the generation of other types of backbone analogues.

Thioamides are known to react with several strong nucleophiles such as hydroxylamines, hydrazines and amines to yield the corresponding amidoximes, amidrazides and amidines[29]. We chose at first to prepare amidoxime analogues of simple enzyme substrates in order to evaluate their behavior toward relevant enzymes and also to establish reaction conditions that may be compatible with more complex peptidic structures. The amidoxime derivative of N-acetyl phenylalanine was previously described by Peterson et al[131]. It was prepared by the direct addition of hydroxylamine to the corresponding nitrile. These authors showed that this substrate analogue was completely resistant to attack

by α-chymotrypsin, while behaving as a weak competitive inhibior characterized by a K_i of 4.3×10^{-2}M[483].

These observations encouraged us to examine an amidoxime analogue of a Leucine aminopeptidase (LAP) substrate. L-Leucine amidoxime was expected to be resistant to hydrolysis by this enzyme and to behave as a good inhibitor because of the known ability of amidoximes to form coordination complexes with metal ions[484]. Accordingly Boc-LeuC(S)-NH_2 (116), prepared by reacting Boc-Leucine amide with thionation reagent 11, was treated with hydroxylamine (1.2 eq) in the presence of triethylamine (1.5 eq) in THF at RT for 20 h.

Purification of the crude product by flash chromatography afforded a 65% yield of Boc-LeuC(NHOH)-NH_2 (117) and a 12% yield of the corresponding nitrile 152.

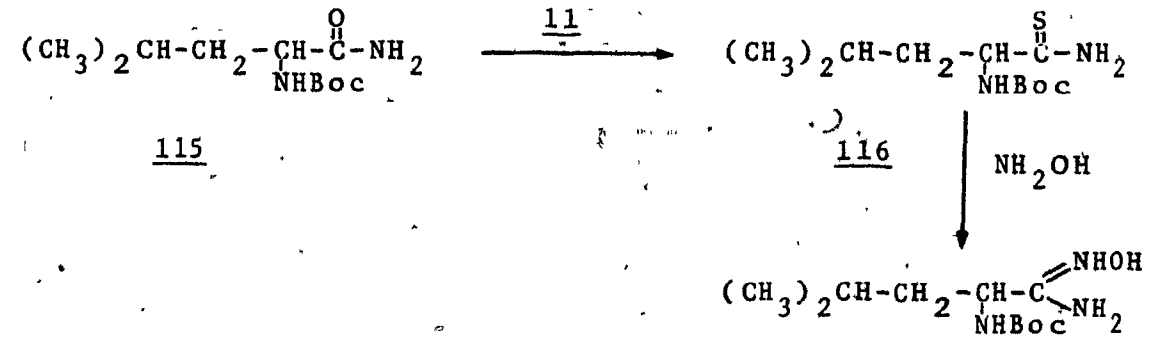


Figure 67

117

The ^{1}H NMR spectrum (60 MHz, $CDCl_3$) of amidoxime 117 displayed all the expected resonances. Proton exchange experiments (D_2O) resulted in the disappearance of the signals at 7.56 (1H)

5.42 (2H) and 4.96 (1H) ppm which correspond to O<u>H</u>, N<u>H</u>$_2$ and Boc-N<u>H</u>, respectively. The compound appeared to be only in the <u>syn</u> configuration as revealed by the single resonance arising from the Boc protons as well as the relatively high field at which the CH_α resonance (4.1 ppm) appeared. The IR spectrum displayed the characteristic absorption of the free OH of primary amidoxime at 3,6000 cm^{-1} [485]. The mass spectrum included the molecular ion at 245 and an abundant fragment at 189, resulting from the loss of the isobutylene from the Boc group.

The Boc protecting group of <u>117</u> was easily removed by treatment with HCl in ether to yield the desired analogue <u>118</u> as a white hydroscopic powder. It was characterized by ^1H NMR (200 MHz) and mass spectroscopy which revealed a characteristic M + 1 peak at 146. However, elemental analysis showed that the compound had crystallized as a dihydrochloride salt in agreement with the known ability of amidoximes to form salts [486].

The behavior of the amidoxime toward LAP was examined using Leu p-nitroanilide as the substrate under conditions similar to those described previously (Section 1.2). Double reciprocal plots (Lineweaver-Burk) indicated that the analogue behaved as a competitive inhibitor whose K_i amounted to 1.5×10^{-3} M (Fig. 68 and 69). The affinity of the amidoxime for the enzyme is therefore rather small which suggests that coordination of the amidoxime function with the zinc atom of the enzyme active site is not significant. Since the amidoxime functionality is not

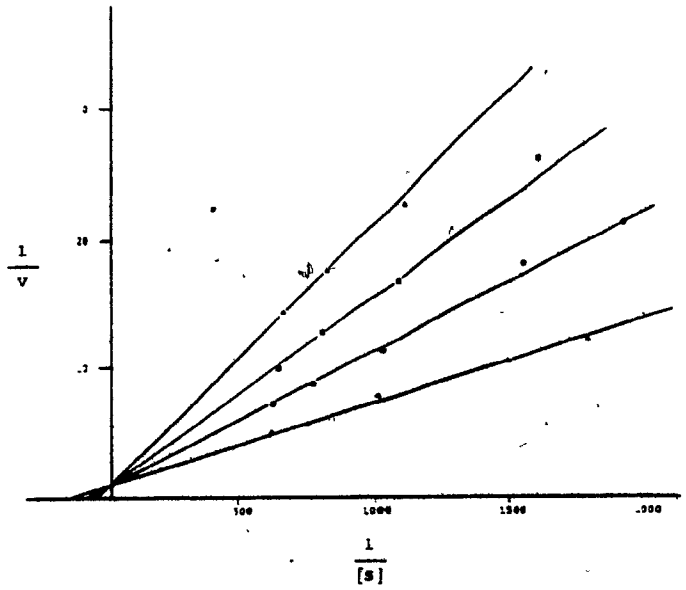


Figure 68 Lineweaver-Burk plot of L-leucine p-nitroanilide hydrolysis in the presence of fixed concentration of L-leucine amidoxime as inhibitor : (▲) 0.0 M ; (●) 2.11 x 10^{-3}M ; (■) 4.08 x 10^{-3}M ; (▲) 5.90 x 10^{-3}M

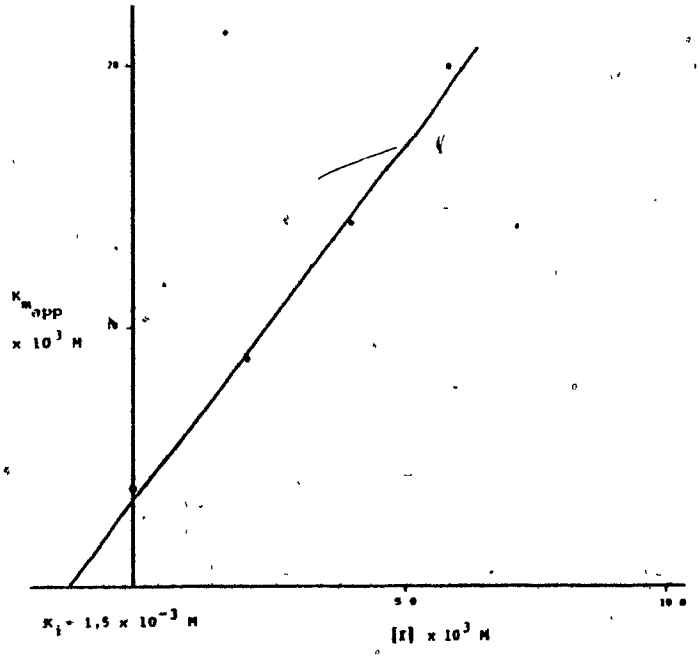
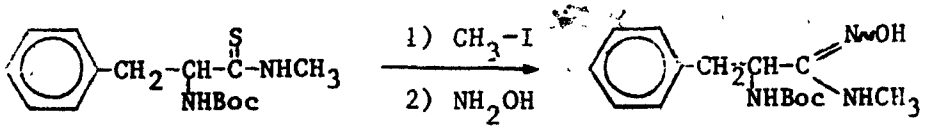


Figure 69 $K_{m_{app}}$ vs [I] for L-leucine amidoxime ((L-leucine p-nitroanilide as substate)

hydrolyzed by either LAP or α-chymotrypsin[483], it would appear to constitute a choice peptidic modification for the purpose of confering backbone resistance to enzymatic attack.

Application of the same chemical methodology for amidoxime formation to a closer model of a peptide backbone such as Boc-PheC(S)-$NHCH_3$ was not successful. It was previously reported that long reaction times (15 h) in refluxing methanol were necessary for amidoxime formation from N-alkyl thioamides[487]. However, we found that hydroxylamine reacted at room temperature with the corresponding thioimidate 47 of Boc-PheC(S)-$NHCH_3$ (20) to give after 24 h, the desired amidoxime 119 in 85% yield after purification by flash chromatography on silica gel.



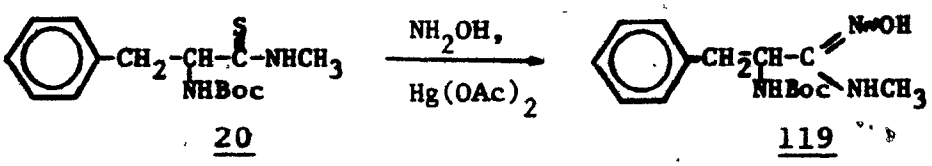
20 119

Figure 70

The mass spectrum of this compound displayed the expected molecular ion at 293 and an equally intense peak corresponding to the M-1 fragment. Its ^1H NMR spectrum (200 MHz, $CDCl_3$) indicated the presence of both the E and Z isomers in a ratio of 5:3 which was deduced from the downfield shift exerted by the hydroxyl group on the N-methyl protons.

The synthesis of the same amidoxime (117) by a one-step process was eventually accomplished more conveniently by treatment

of the N'-methyl thioamide 20 with hydroxylamine in the presence of mercuric acetate (1.2 eq) and triethylamine (3 eq). After stirring the reaction mixture overnight at RT, the mercuric salts were filtered and the crude was washed with mild acid. The desired amidoxime 119 was thus obtained in 70% yield after purification by flash chromatography.



20 119

Figure 71

^1H NMR spectroscopy again revealed an E/Z ratio of 5:3.

These reaction conditions were then applied to the protected thiodipeptide, Boc-LeuC(S)-Phe-OCH_3 (67) (C-terminal end of the chemotactic peptide f-MLP). The reaction was monitored by TLC which revealed that most of the thioamide precursor 20 had reacted after 3 h and that at least two more polar products were formed. After a work up similar to that described above, these more polar products were separated by flash chromatography. The ^1H NMR spectrum (200 MHz, $CDCl_3$) did not include a resonance for the methyl ester protons and was consistent with the novel cyclic structure 120. The mass spectrum of this product did not contain the molecular ion at 375 but indicated fragmentation at the C-C(=N-O-R)N- giving fragments at 189 and 186.

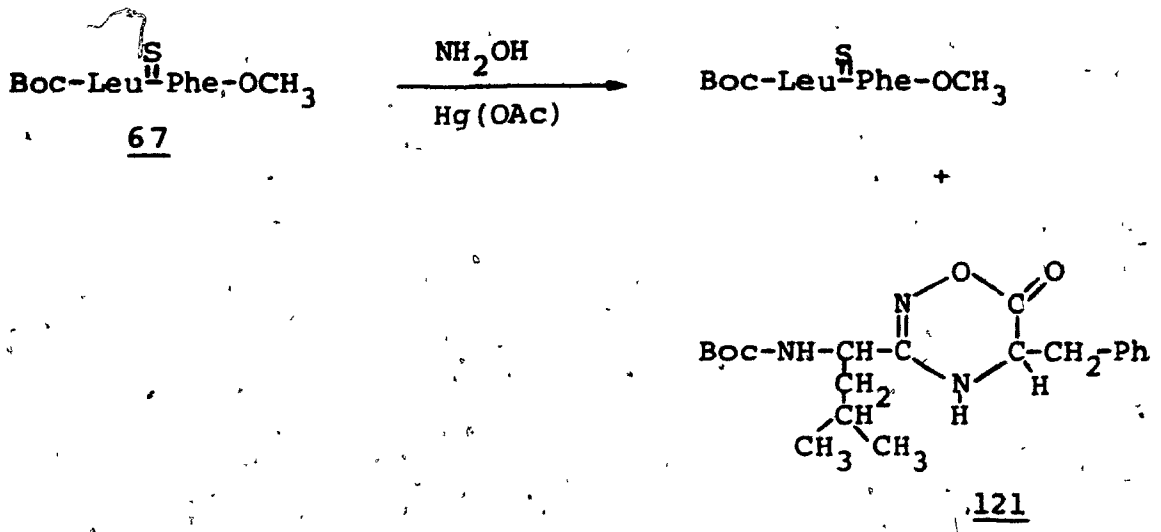


Figure 72

The other product, obtained in 3% yield, was identified as starting material thioamide. Its ^{1}H NMR spectrum displayed the correct resonance for the methyl ester protons at 3.76 ppm and the expected downfield shift to 5.32 ppm for the resonance of the leucine α-proton which is accounted for by the E isomeric form of the product.

Interestingly when hydrazine was substituted for hydroxylamine under similar reaction conditions, improved yields of products were obtained. Thus, the cyclic amidrazide 122 was isolated in 55% yield and the corresponding amide 62, in 10% yield. The mass spectrum of the cyclic compound included the molecular ion at 374, and an important fragment at 258 corresponding to $M^{+\cdot}$ -NHBoc.

Despite the formation of these cyclic products, these preliminary experiments nevertheless clearly illustrate the versa-

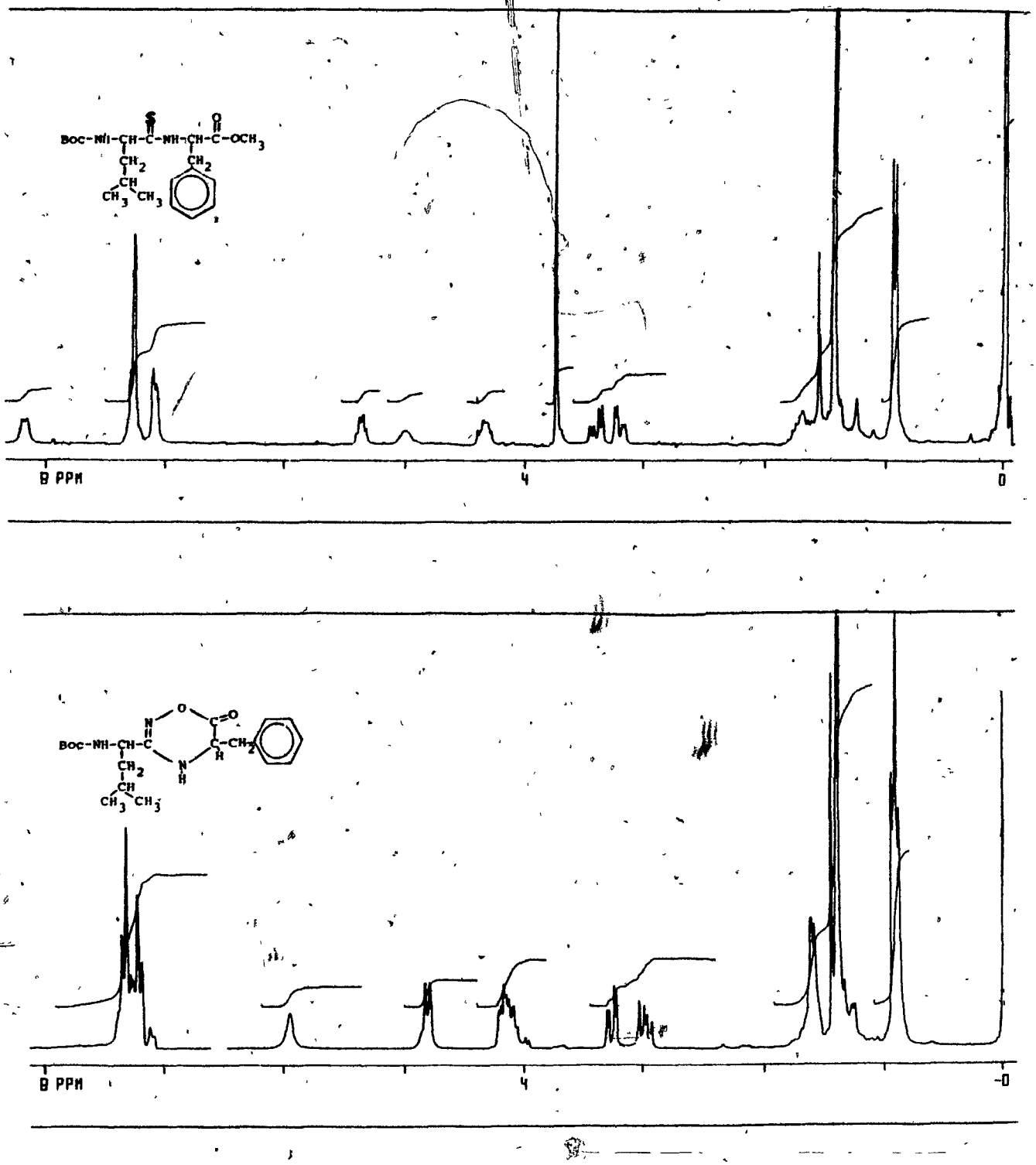


Figure 73 ^{1}H NMR spectra (200 MHz, $CDCl_3$) of 120 and 121.

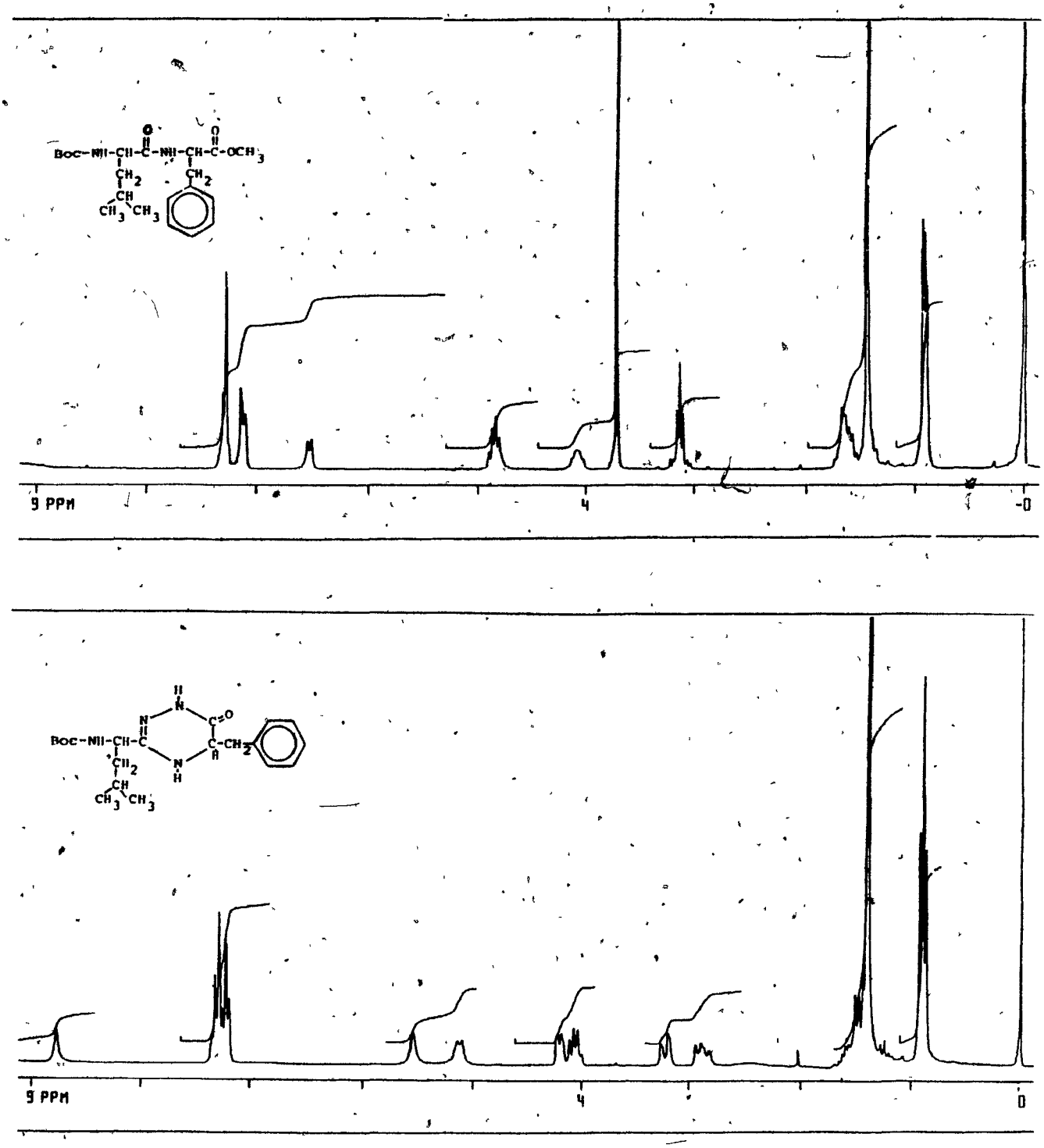


Figure 74 ^{1}H NMR spectra (200MHz, $CDCl_3$) of 122 and 123.

tility of the thioamide function once incorporated into the backbone of peptides.

It should be pointed out that cyclic product formation would not occur when the amidoxime or amidrazide function is at a backbone position removed from the C-terminal ester function. In addition, it should be possible to minimize the observed cyclization through the use of a bulky carboxyl-protecting group, such as a t-butyl group. By selecting suitably substituted nucleophiles it should be possible to generate novel cyclic analogues of oligopeptides. Work along this line is being continued by others in our laboratories.

CONTRIBUTIONS TO KNOWLEDGE

The thionation reaction of amides with thiono phosphine sulfide reagents can proceed at low temperature (RT, 0°C) in dry THF and under these conditions the rate of reaction is markedly affected by the bulk of the amide substituents. This observation permits regioselective thionation of certain polypeptides and thus is critical for the rapid synthesis of thiopeptide analogues.

α-Chymotrypsin and leucine aminopeptidase do not readily cleave the thioamide bond when present in substrate analogues. Optically active dithioester derivatives of amino acids were successfully prepared. These are important for enzyme mechanism studies and moreover can be used as thioacylating agents in the synthesis of thiopeptides.

NMR studies revealed that the solution conformation of relevant thiopeptides is not significantly changed relative to the parent peptides, at least in the case of short peptide sequences. In contrast, we discovered that the thioamide modification can dramatically alter the biological activity of an oligopeptide regulator depending on the site of alteration along the backbone. This was clearly demonstrated with the thioanalogue CH(O)-Met-LeuC(S)-Phe-OCH_3 (75) which causes inhibition of the chemotactic response. Also, the Tyr-GlyC(S)-Gly-Phe-Leu (101) analogue of

[Leu^5]-enkephalin is significantly more potent and possesses a longer duration of action than the parent peptide.

Finally, a general procedure allowing for the further modification of thiopeptide linkage was developed. Amidoxime and amidrazide analogues could thus be readily obtained from the nucleophilic thioamide functionality.

SUGGESTIONS FOR FURTHER STUDY

Having successfully synthesized all the positional isomers of two different peptides, an obvious extension of this approach would involve the synthesis of thioamide analogues (and their derivatives,eg. amidoximes) of other relevant natural peptide regulators such as kyotorphin (Tyr-Arg)[488], tuftsin[489] (Thr-Lys-Pro-Arg), etc... as well as thioamide analogues of [D-Ala, D-Leu^5]-enkephalin[490]. With this in mind the use of easily removable protecting groups such as the F-Moc group and the t-butyl ester function[491] might improve the yields of deprotected products.

In depth conformational analysis of longer thiopeptide analogues should provide valuable information on the effects of backbone thioamide linkages. This task would be greatly facilitated through the use of the recently acquired 2-D NMR facility. The use of other physical methods such as CD and ORD would also considerably help in conformational studies of thionated oligopeptides. In fact the much enhanced chromophoric properties of the thioamide function would allow studies of thiopeptides at very low concentration, thus eliminating the problems associated with aggregation or self-association.

The synthesis of thioamide analogues of peptides using solid phase supports should also be explored. Thionation in the solid phase should be attempted and the search for appropriate

thioacylating agents should be rewarding. Even if racemization happens to be a complicating factor, such methodologies would nevertheless offer the advantage of speed.

Finally, it would be very interesting to compare the interaction of antibodies directed against thiopeptide analogues with those which recognize the parent oligopeptides. The results would be of great value from the point of view of conformation-activity relationship studies.

EXPERIMENTAL

General Experimental

Inorganics were used as purchased from the suppliers: P_4S_{10} (Matheson-Coleman), silver nitrate (A & C American Chemical) mercuric acetate (Fischer), hydroxylamine hydrochloride (Aldrich) hydrazine-dihydrochloride (Fischer).

Reagent grade solvents were used, unless otherwise specified. Dry thetrahydrofuran (THF) and dry dioxane were obtained by refluxing in the presence of sodium and benzophenone. Triethylamine was purified by distillation from barium oxide. Other solvents (acetonitrile, toluene, benzene) were dried by standing over molecular sieves. HPLC grade solvents, and doubly distilled H_2O used in HPLC purification.

All amino acids starting material used were of the L-configuration and used as supplied from the manufactureres (Aldrich, Sigma, and Bachem or Chemalog for the Boc-amino acids). EEDQ (Aldrich) was recrystallized from ether. The DCC (Aldrich) was purified by dilution in ether and filtration of the DCU present, and evaporation of the ether in vacuo for a period of at least 40 h. Formic acid 98% (BDH) was used without purification in the deprotection reaction. Drying of organic solutions during work up was accomplished with magnesium sulfate (Fischer). Solvent evaporation was carried out under reduced pressure (water aspirator) with a

with a bath temperature of 20 to 30°C. Products in aqueous solution were obtained as fluffy powders by lyophilization using a Viatis 10-010 automatic freeze-dryer.

Analytical thin layer chromatography was carried out on aluminum-based sheets, precoated with Kieselgel 60 F_{254}, 0.2 mm thick (Merck Co. Ltd., Darmstadt). Visualization of the plates was done by ultra violet light source (254 mm) or by ninhydrin. Column chromatography was performed by the flash chromatography technique as described by Still and co-workers[493] on 32 to 64 μ (400 to 230 mesh) silica gel (British Drug House, Toronto).

High pressure liquid chromatography (HPLC) separations were accomplished on a column (150 x 10 mm) packed with Spherisorb S-10 W (Technical Marketing Associates, Montreal) fitted to a Waters Associate-400 single pump using fixed wavelength UV detector (254 mm) and a refractive index detector. A semi-preparative C-18 Bondapack (Waters) column was used for reverse phase separations.

Melting points were determined in closed capillary tubes on a Buchi SMP-20 and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 297 spectrophotometer and mass spectra on HP 5984 or LKB 9000 mass spectrometers. The ultra violet UV spectrum were recorded on a Varian carry 210 spectrophotometer, interfaced with an Apple II Plus microcomputer and thermostated, using absolute EtOH as the solvent at 20°C. The optical rotation

$[\alpha]_D$ were obtained with a Perkin Elmer 141 using a thermostated cell (1 decm, 1 mL) at the temperature indicated.

Proton magnetic resonance spectra (^{1}H NMR) were recorded on Varian T-60, T60A or XL-200 spectrometers, using tetramethylsilane as internal standard. The carbon and phosphorous magnetic resonance spectra were recorded on a Brooker WH-90. Chemical shifts are reported on the scale in parts per million (ppm). In the ^{1}H NMR spectra, all apparently simple multiplets are described as they appeared, and are given chemical shifts equal to their central position. Undefined multiplets (m) containing more than one proton signals are described by their ranges of absorbtion.

Elemental analysis were performed at Guelph Laboratories, Guelph, Ontario. Amino acid analysis were performed after acid hydrolysis (6N HCl, 24 h, 110°C) of the peptides at the Institut de recherche clinique by Dr. Lazure and Dr. Schiller or at the Departement de Pharmacologie de L'Univeristé de Sherbrooke by Dr. S. Lemaire.

EXPERIMENTAL

Chapter 1

n-Pentyl phenyl ether

Sodium (11.5 g, 0.50 mol) cut in small pieces and added to EtOH (ABS) (40 mL) at 0°C and stirred until all dissolved. Phenol (Aldrich, 47.0 g, 0.50 mol) was then added all at once, followed by the dropwise addition of 1-bromopentane (105.7 g, 0.70 mol). The reaction mixture was then heated to reflux for 3 h. After filtration of the sodium bromide, most of the ethanol was removed *in vacuo* using a flash evaporator at 25°C. The resulting liquid was washed with 1N NaOH (2x50 mL), H_2O and dried ($MgSO_4$). Distillation of this liquid (bp 105-106°C/ 25 mmHg) afforded 68.5 g (85% yield) of a clean oil ^1H NMR (60 MHz, $CDCl_3$) δ : 7.4-6.8 (m, 4H, ArH), 3.95 (t, 2H, J=7 Hz, O-CH_2, 2.0-0.85 (m, 9H, $(CH_2)_3$-CH_3) ppm.

Ethoxyethyl phenyl ether

This compound was prepared as previously described for n-pentyl phenyl ether with sodium (7.5 g, 0.44 mol) in EtOH (25 mL), phenol (30.7 g, 0.33 mol) and 2-bromoethyl ethyl ether (50 g, 0.33 mol). Distillation of the resulting liquid (bp 98-100°C/25 mmHg) gave a colorless liquid (40.4 g, 75% yield). ^1H NMR (60 MHz, $CDCl_3$) δ : 7.33-6.68 (m, 5H, ArH), 4.26-3.43 (m, 6H, O-CH_2-CH_2-O-CH_2-), 1.3 (t, 3H, J=8 Hz, CH_3) ppm.

2,4-bis(4-phenoxyphenyl)-1,3,2,4-dithiaphosphetane 2,4-disulfide (11

P_4S_{10} (39.0, 0.08 mmol) was added to phebyl ether (Aldrich) 150 g, 0.88 mmol) and the mixture heated to 165-170°C under N_2, for 6 h. After this time, the heating was stopped and the hot solution decanted in an erlenmeyer and hexanes (150 mL) was added slowly. The solution was allowed to crystallize at RT for 10 h and at 0°C for 20 h. The yellow crystals were filtered and washed with cold hexanes, and recrystallized from hot toluene (550 mL). After washing with hexanes and drying in vacuo, 35.0 g (0.066 mol, 75%, based on P_2S_5) of yellow crystals were obtained.
mp 187-190°C; IR (KBr) λ_{max}: 3100, 1588, 1493, 1300 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ : 8.51 (dd, 2H, J_{H-H}=8.3 Hz, J_{P-H}= 15.5 Hz, ArH ortho), 7.50-6.90 (m, 7H, ArH) ppm.
^{31}P NMR (THF) δ (relative to H_3PO_4): 17.7 (s) ppm.
MS (70 ev) m/e: 443 (1), 410 (10), 411 (4), 348 (5), 66 (10), 265 (17), 64 (100); Analysis calcd.: C 54.53, H 3.43, S 24.26, P 11.72; Found: C 54.77, H 3.74, S 24.19, P 12.63.

2,4-bis (4-n-pentyl oxyphenyl)-1,3,2,4-dithiaphosphetane 2,4-disulfide (10)

Prepared as described for 11 with P_4S_{10} (6.78 g, 15 mmol) and n-pentyl phenyl ether (25.0 g, 0.15 mmol) and obtained a yellow solid 9.42 g, (60% yield).
mp 135-137°C; ^1H NMR (60 MHz, $CDCl_3$): 8.15 (dd, 2H, J_{H-H}=9 Hz J_{PH}=18 Hz, ArH ortho), 7.21 (dd, 2H, J_{H-H}=9 Hz, J_{PH}=4Hz, ArH meta), 4.10 (t, 2H, J=7 Hz, $-OCH_2$), 2.0-.95 (m, 9H, $CH_2-CH_2-CH_2-CH_3$) ppm.

Thiobenzamide (13)

Benzamide (Fisher) (200 mg, 1.6 mmol) and the thiono phosphine sulfide reagent 11 (510 mg, 0.96 mmol) were dissolved in dry THF (5 mL) and the mixture stirred at room temperature for 20 min. The reaction mixture was diluted with EtOAc (20 mL) and washed with H_2O, dried ($MgSO_4$) and evaporated in vacuo. The resulting solid was recrystallized from ether to yield 190 mg (94% yield) of a pale yellow solid.

R_f .81 (EtOAc/hexane 2:1), mp 115-117°C; IR (KBr) ν_{max}: 3500 (NH), 3480 (NH), 3050, 1600 cm^{-1}; UV (EtOH) λ_{max}: 238.5 log ε 3.98; ^1H NMR (60 MHz, $CDCl_3$) δ: 8.05-7.00 (br, m, ArH, $C(S)NH_2$) ppm.

N-phenyl thioacetamide (14)

Acetanilide (200 mg, 1.48 mmol) and the thiono phosphine sulfide 11 (420 mg, 0.81 mmol) were dissolved in dry THF and stirred at room temperature for 1 h. The reaction was diluted with ether, and washed with $NaHCO_3$ (10%), dried ($MgSO_4$) and evaporated in vacuo. The oily residue was then recrystallized from EtOAC/pet. ether to yield 190 mg (95% yield) of a white solid.

R_f .51 (EtOAc/pet. ether 5:3); ^1H NMR (60 MHz, $CDCl_3$) δ : 7.85-7.00 (m, 6H, ArH and NH), 2.58 and 2.26 (2 s, in a ratio of 6:4, 3H, CH_3 E and Z respectively). MS (CI): 152 (100, $M^{+\cdot}+1$), 151 (6.2, $M^{+\cdot}$), 136 (1.4, $M^{+\cdot}-CH_3$), 118 (1.4, $M^{+\cdot}-H_2S$).

Ethyl thionebenzoate (15)

Ethyl benzoate (200 mg, 1.3 mmol) and the thiono phosphine sulfide 11 (825 mg, 1.56 mmol) were heated in dry toluene (2mL) at 110°C for 24 h, under an argon atmosphere. After two purifications by flash chromatography on silica gel using hexanes as eluent 150 mg (68% yield) of a yellow oil was obtained.

IR (neat) ν_{max}: 3200, 2990, 1600, 1510, 1280, 1240 (C=S) cm^{-1}; ^1H NMR (60 MHz, $CDCl_3$) δ: 8.28-8.06 (m, 2H, ArH ortho), 7.53-7.10 (m, 3H, ArH para), 4.75 (t, 3H, J 7Hz, $C\underline{H}_2$), 1.5 (t, 3H, CH_3) ppm.

Ethyl 3-phenylethylthiopropionate (16)

Ethyl 3-phenylpropionate (200 mg, 1.1 mmol) and thiono phosphine sulfide 11 (580 mg, 1.3 mmol) in toluene (2 mL) were heated to 110°C for 24 h. After this time, the crude mixture was applied on a silica gel column and eluted with hexanes. After evaporation of the desired fractions 150 mg (75% yield of a pale yellow oil was obtained.

IR (neat) ν_{max}: 3200, 3100, 1600 (arene), 1500, 1460, 1300 (br, C = S), 760 cm^{-1}; ^1H NMR (60 MHz, $CDCl_3$) δ: 7.21 (s, 5H, ArH), 4.5 (q, 2H, J 7Hz, O-$C\underline{H}_2$), 3.03 (s, 4H, $C\underline{H}_2$-$C\underline{H}_2$), 1.33 (t, 3H, J 7Hz, CH_3), MS (CI) m/e: 195 (100, $M^{+\cdot}$+1), 194 (80, $M^{+\cdot}$), 149 (33, $M^{+\cdot}$-OEt), 105 (85, $M^{+\cdot}$-C(S)-OEt), 91 (60, trop. ion).

N-Boc-L-phenylalanine amide (17)

N-Boc-L-phenylalanine ethyl ester (5.0 g, 17 mmol) was added to a saturated (0°C) solution of ammonia in MeOH. The solution was left standing at RT for 48 hrs. The excess ammonia and MeOH were removed in vacuo, and the resulting solid recrystallized from EtOH/H_2O giving 4.0 g (84% yield of a white powder.

mp 179-180; $[\alpha]_D^{20}$+11.7 (c 1.0, EtOH), ^{1}H NMR (60 MHz, $CDCl_3$): 7.33 (s, 5H ArH), 6.49-6.09 (br, 2H, NH_2), 5.19 (brd, 1H, NH Boc), 4.44 (m, 1H, CH_α), 3.09 (d, 2H, J = 7 Hz, $CH_{2\beta}$), 1.40 (s. 9H, t-bu) ppm.

N-Boc-L-phenylalanine thioamide (18)

To N-Boc-L-phenylalanine amide (17) (1.5 g, 5.3 mmol) dissolved dry THF (30 mL) were added the thionation reagent 11 and the mixture was stirred at 23°C, under a nitrogen atmosphere for 35 minutes after which time the starting material was all consumed (TLC). Evaporation of the solvent, and purification by flash chromatography on silica gel (CH_2Cl_2) gave 1.35 g (90%) of a white powder which was recrystallized from EtOAC/hexanes.

mp 152-154°C; UV (EtOH: λ_{max}: 268.8 log ε 4.11 $[\alpha]_D^{20}$+29.0° (c 1.0, MeOH); ^{1}H NMR (60 MHz, $CDCl_3$) δ: 7.91-7.41 (br, 2H, NH_2), 7.24 (s. 5H, ArH), 5.47 (br d, 1H, J = 8 Hz, NH Boc), 4.8 (m, 1H, CH_α), 3.11 (d, 2H, J = 7 Hz) 1.40 (s, 9H, t-bu) ppm.

MS (70 eV, 115°C) m/e: 280 (25, $M^{+\cdot}$ - NH_2), 224 (48, $M^{+\cdot}$ - t-bu), 220 (39, $M^{+\cdot}$ - $C(S)NH_2$), 190 (30), 163 (263, $M^{+\cdot}$ - Boc-NH_2), 149 (240) 120 (650).

Synthesis of N-Boc amino acid N'-methyl amide (23, 25, 27, 41, 51a)

The Boc-amino acid (Bachem) and HOBt (1.1 eq) were dissolved in CH_2Cl_2 and temperature of the solution cooled to 0°C. DCC (1.05 eq) was added and the mixture stirred at 0°C for 30 min. A 1% methyl amine (1.1 eq) solution in THF was then added to 0°C and stirred for 1 h at that temperature and 20-24 h at RT. The dicyclohexylurea (DCU) was filtered off, the solution diluted with CH_2Cl_2 and washed with citric acid (5%), $NaHCO_3$ (5%) and brine, dried ($MgSO_4$) and evaporated in vacuo. Isolated yields and physico-chemical properties are given in Table 24.

Thionation of N'-methyl N-Boc amino acid amide (24, 26, 28, 42, 51)

To the appropriate N'-methyl N-Boc amino acid amide (1 to 10 mmol) in dry THF (10% solution) was added the thionation reagent 11 (0.6 eq) all at once and the mixture stirred at 23 ± 2°C under a nitrogen atmosphere. The reaction progress was monitored by TLC and time of reaction were obtained when no starting material could be detected (ninhydrin). All these compounds were purified by flash chromatography on silica gel. Time of reaction and yields are given in the text in Table 2. Their physico-chemical properties are summarized in Table 25.

	Yield (%)	R_f [a]	mp °C	^1H NMR [b]						
				O CNH	NHBoc	CH_α	CH_β	Me	t-bu	others
Boc-Gly-$NHCH_3$ 23	73	.19	75-76	6.73	5.63	3.78	-	2.83	1.46	
Boc-Ala-$NHCH_3$ 25	74	.24	115-116	6.30	5.13	4.20	1.40	2.78	1.43	
Boc-Phe-$NHCH_3$ 19	80	.39	138-139	6.0	5.16	4.13	3.05	2.73	1.43	7.23(s) ArH
Boc-Pro-$NHCH_3$ 27	90	.19	93-94	6.46	-	4.16	2.30	2.8	1.43	3.36(t) CH_δ 1.8 (m) CH_γ
Boc-Leu-$NHCH_3$ 41	75	.42	119-121	6.50	5.16	4.15	1.5	2.75	1.43	1.75(m) CH_γ 0.88(d) CH_δ
Boc-Met-$NHCH_3$ 51a	75	.31	-	6.50	5.3	4.26	2.13	2.78	1.43	2.5 (q) CH_γ 2.05(s) SCH_3

a) EtOAc/ Hexanes 2:1

b) 60 MHz, $CDCl_3$

Table 24 Physico-chemical characteristtics of N'-Methyl N-Boc amino acids.

	R_f [a]	mp °C	UV [b]	^{1}H NMR [c]						
				S CNH	NHBoc	CH_α	CH_β	Me	t-bu	others
Boc-Gly-NHCH$_3$ (24)	.54	109-110	261.5	8.33	5.60	4.16	-	3.70	1.48	
Boc-Ala-NHCH$_3$ (26)	.59	125-126	262.9	8.66	5.56	4.59	1.46	3.13	1.46	
Boc-Phe-NHCH$_3$ (20)	.74	112-113	264.4	8.23	5.86	4.70	3.16	3.16	1.40	7.23(bs) ArH
Boc-Pro-NHCH$_3$ (28)	.59	182-183	263.2	8.30	-	4.66	2.10	3.16	1.46	3.46(t) CH_δ 1.49(m) CH_γ
Boc-Leu-NHCH$_3$ (42)	.69	106-107	262.9	8.83	5.60	4.49	2.0	3.16	1.43	1.70(m) CH_γ 0.90(d) CH_δ
Boc-Met-NHCH$_3$ (51)	.70	°oil	263.8	8.90	5.90	4.66	2.2	3.13	1.43	2.50(t) CH_γ 2.11(s) SCH_3

a) EtOAc/Hexanes 2:1
b) EtOH, 1×10^{-4} M
c) 60 MHz, $CDCl_3$

Table 25 Physico-chemical characteristics of N'-Methyl N-Boc amino acids thioamide.

N'-methyl N-acetyl phenylalanine

The corresponding Boc derivative (600 mg, 2.1 mmol) was dissolved in a HCl/ether solution and left standing at 23°C for 12 h. The hydrochloride crystallized from the solution; it was filtered and washed with anhydrous ether yielding 399 mg (86%) of a white powder. This hydrochloride (370 mg, 1.7 mmol) salt was then acetelated with acetic anhydride and pyridine as described previously for __ and the crude product recrystallized to give 210 mg (56% yield) of white crystals.

mp 204-205°C; $[\alpha]_D^{20}$+22.0° (c 1.0, MeOH); ^{1}H NMR (60 MHz, $CDCl_3$) δ : 7.18 (s, 5H, ArH), 6.4 (b, 1H, NH), 5.75 (br, 1H, NH), 4.50 (m, 1H, CH_α), 3.03 (d, 2H, J, 6 Hz, $2CH_\beta$), 2.70 (d, 3H, $NHCH_3$), 1.98 (s, 3H, CH_3) ppm.

N'-methyl N-acetyl L-phenylalaninethioamide (30)

The Boc group of (18) (300 mg, 1.02 mmol) was removed with 2N HCl/ether and the hydrochloride acetylated with acetic anhydride pyridine as described previously for (29). The crude compound was recrystallized from hexane-ether to yield (1.85 mg, 77%) of white solid.

mp 110-111°C; $[\alpha]_D^{20}$+45.0° (c 1.0, MeOH), UV (EtOH) : λ_{max} 267.8 log ε 4.03; ^{1}H NMR (200 MHz, $CDCl_3$) δ :8.2 (b, 1H, NH), 7.36-7.10 (m, 5H, ArH), 6.60 (brd, 1H, NH Boc, 4.92 (m, 1H, CH_α), 3.22-2.98 (m, 2H, $CH_{2\beta}$), 2.94 (d, 3H, J 4Hz), 1.96 (s, 9H, t-bu) ppm; MS (70 eV) m/e: 294 (66, $M^{+\cdot}$), 238 (70, $M^{+\cdot}$- t-bu), 220 (81, $M^{+\cdot}$-C(S) $NHCH_3$), 194 ($M^{+\cdot}$-Boc), 177 (575), 120 (910).

N-acetyl-L-phenylalanine thioamide (29)

Boc-L-phenylalanine thioamide (18) (770 mg, 2.5 mmol) was added to a 2N HCl solution in dry ether and the mixture was left standing at 23°C for 12 h. The solvent was evaporated to dryness and the white solid recrystallized from EtOH/EtOAC. After filtration and drying in vacuo 490 mg (87%) of white powder was obtained. mp 210-212°C; UV (EtOH) : λ_{max} 269.4 log ϵ 4.03. This hydrochloride salt (370 mg, 1.8 mmol) was dissolved in CH_2Cl_2 (10 mL). Acetic anhydride (191 mg, 1.8 mmol) and pyridine (297 mg, 3.7 mmol) were then added and the mixture stirred at 23°C, for 3 h. The reaction mixture was transferred to a separatory funnel and washed successively with citric acid (5%), brine, dried ($MgSO_4$) and evaporated. The resulting solid was recrystallized from EtOAC/hexanes affording 250 mg (68% yield) of the acetylated derivative. mp 156.5-160°C; $[\alpha]_D^{20}$+40.2° (c 1.0, MeOH); UV EtOH): λ_{max} 269.8 log ϵ 4.07; ^1H NMR (60 MHz, $CDCl_3$) δ: 8.0-7.5 (b, 2H, NH_2), 6.5 (b, 1H, NH), 7.21 (S, 5H, ArH), 5.0 (m, 1H, CH), 3.15 (d, 1H, J 8 Hz), 2.03 (S, 3H, CH_3) ppm.

N-trichloroethyl L-phenylalanine ethyl ester (37)

To L-phenylalanine ethyl ester hydrochloride (12.0 g, 5.2 mmol) suspended in CH_2Cl_2 (200 mL) was added pyridine (8.25 g, 10.4 mmol) and 2,2,2 trichloroethyl chloroformate (Aldrich) (11.51 g, 5.2 mmol) and the mixture was stirred at

23°C overnight. The reaction mixture was then transferred to a separatory funnel and washed with .1 N HCl (2x25 mL), Na_2CO_3 (2x25 mL) and brine. The organic layer was dried ($MgSO_4$) and evaporated in vacuo to give 18.25 g (95% of a clear oil. $[\alpha]_D^{20}$-19.9° (c 1.0, EtOH), IR ($CHCl_3$) λ_{max}: 3400 (NH), 3005 (ArH), 1735 (C=O) 1510, 1210 cm^{-1}, ^1H NMR (60 MHz, $CDCl_3$) δ: 7.13 (m, 5H, ArH), 5.79 (br d, 1H, J=8 Hz, NH TCE), 4.89-4.40 (multiplet containing doublet, 3H, CH_α and CH_2 TCE), 4.16 (q, 2H, J=8 Hz, CH_2-CH_3), 3.01 (d, 2H, J=7 Hz, $CH_{2\beta}$), 1.19 (t, 3H, J=8 Hz, CH_2-CH_3).

N-trichloroethyl L-phenylalanine thione ethyl ester (38)

N-TCE-L-phenylalanine ethyl ester (9.9 g, 24 mmol) and thionation reagent 11 (15.2 g, .29 mmol) were heated at 110°C in dry toluene for 36 h. The solvent was evapored in vacuo, the residue taken in $CHCl_3$ (5 mL) and applied to a silica gel column for flash chromatography. Elution with hexanes/EtOAC (9:1) gave 4.28 g of a mixture of thionoester and thionocarbamate and 3.78 g of unreacted starting material. Further purification by flash chromatography with a gradient system, hexane/EtOAC 20:1→9:1 of the mixture afforded pure thionoester 38 (2.06 g, 22.5%), R_f .35 (EtOAc/hexane 1:6) as a pale yellow oil and 1.26 g (13.4%) R_f .31 (EtOAc/hexane 1:6) of the thiocarbamate 39. a) thionoester 38: $[\alpha]_D^{20}$+70.0° (c 0.45, $CHCl_3$), UV (EtOH) λ_{max}: 250.5, log ε 3.97, IR ($CHCl_3$) ν_{max}: 3400 (NH), 3050 (ArH), 1745 (CO), 1510, 1220 ^1H NMR ($CDCl_3$, 200 MHz) δ :7.3-71 (m, 5H,

ArH), 5.56 (d, 1H, J 8.5 Hz, NH TCE), 4.85-4.45 (multiplet containing 2 doublets centered at 4.76 and 4.64, 3H J_{gem}= 6.8 Hz, CH_2), 1.24 (t, 3H, J=7.05 Hz, CH_3) ppm.

MS (70 eV, 30) m/e : 383 (1.3, $M^{+\cdot}$), 384 (.3, $M^{+\cdot}$+1), 385 (1.3 $M^{+\cdot}$+2), 386 (.3, $M^{+\cdot}$+3) 387 (.5, $M^{+\cdot}$+4), 294 (41.7, $M^{+\cdot}$-C(S)OEt), 190 (94.1), 91 (100).

b) thiocarbamate 39: $[\alpha]_D^{20}$+71.0° (c 1.0, $CHCl_3$), IR ($CHCl_3$) ν_{max}: 3.370 (NH), 3010 (ArH), 1735 (C(O)), 1505, 1400, 1200, 1170 ^1H NMR (200 MHz, $CDCl_3$) : 7.4-7.0 (m, 5H, ArH), 6.98 (d, 1H, J=8 Hz, NH), 5.2-4.95 (multiplet containing 2 doublets centered at 5.14 and 4.96, J=11.7 Hz, 3H, 2CH TROC, CH_α), 4.14 (q, 2H, J=7.2 Hz, CH_2-CH_3), 3.30 (dd, 1H, J=6.4 Hz, J=15.1 Hz, CH_β), 3.20 (dd, 1H, J=5.0 Hz, J=15 Hz, CH_β'), 1.18 (t, 3H, J=7.2 Hz) ppm.

N-acetyl L-phenylalanine thione ethyl ester (40)

N-Troc L-phenylalanine thione ethyl ester (530 mg, 1.37 mmol) was dissolved in AcOH (10 mL) and acetic anhydride (4.6 g, 45 mmol) and zinc dust (Fisher) (1.0 g, 15 mmol) and the mixture stirred at 23°C for 24 h. The zinc dust was then filtered through celite. Evaporation of the solvents, and purification by flash chromatography on silica gel ($CHCl_3$/EtOAc 3:1) yielded an oil which was recrystallized from hexanes. After two additional recrystallizations 204 mg, (58% yield) of white needles were obtained.

R_f .41 ($CHCl_3$/EtOAc 3:1); mp 79-80°C; UV λ_{max} 243.8 log ε 3.96 $[\alpha]_D^{20}$+33.3° (c 1.0, EtOH), IR (KBr) ν_{max}: 3120, 1655, 1540, 1380, 1285 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ : 7.37-7.05 (m, 5H, ArH), 7.33 (bd, 1H, J 7.0 Hz, NH-C(O)), 5.03 (m, 1H, CH_α), 4.39 (dq, 1H, J 7.1 Hz, J=11.0 Hz, O-CH_2CH_3) 4.37 (dq, 1H, J=7.1 Hz, J=11.0 Hz, O-CH_2-CH_3), 3.16 (dd, 1H, J=6.1 Hz, J=13.4 Hz, CH_β), 3.05 (dd, 1H, J=6.9 Hz, J=13.4 Hz, CH_β), 1.25 (t, 3H, J=7.1 Hz, CH_2-CH_3) ppm. ^{13}C NMR ($CDCl_3$) :218.03 (C=S), 169.1 (C=O), 136, 129, 128, 127, (C Ar), 68.89 (O-CH_2), 60.7 (C_α), 41.6 (C_β), 34.3 ($C(O)CH_3$), 13.3 (CH_2-CH_3); MS (70 eV, 31°C) m/e: 251 (5.1, $M^{+\cdot}$), 192 (68.4) M^+ -NH_2-$C(O)CH_3$, 120 (100, C_6H_5-CH_2,-CH NH), Analysis: Calcd. C 62.12, H 6.81, N 5.49, S 12.75, Found: C 62.06, H 7.02, N 5.49, S 12.63.

Boc-LeuC(S)-Phe-OH (48)

Boc-LeuC(S)-Phe-OCH_3 (67) (400 mg, 0.96 mmol) was dissolved in a mixture of THF/H_2O (2:1, 5 mL) and 1N sodium hydroxide (1.15 mL) (1.2 eq.) was added and the mixture was stirred at room temperature for 1 h. After evaporation of the THF in vacuo, the residue was diluted with H_2O (10 mL) and acidified with citric acid (5%) and extracted twice with EtOAc and the organic layer was washed with H_2O, dried ($MgSO_4$) and evaporated in vacuo to yield 170 mg (85% yield) of a foamy solid.

R_f .36 ($CHCl_3$/MeOH 9:1); UV (ETOH) : λ_{max} 267.3 log ε 3.95; $[\alpha]_D^{20}$+67.3° (c 1.0, MeOH); ^1H NMR (200 MHz, $CDCl_3$) δ : 8.95

(br d, 1H, NH Phe), 7.30-7.08 (m, 5H, ArH, 5.44-5.14 (m, 2H, Ch_{α} Phe, NH Boc), 4.66 (m, 1H, CH_{β} Phe), 3.02 (m, 1H, CH_{β}' Phe), 1.80-1.20 (multiplet containing a singlet at 1.46, 12H, CH_{γ}, $CH_{2\beta}$ Leu, t-bu), 0.90 (br s, 6H, $2CH_3$ δ Leu) ppm.

Attempts to prepare Boc-LeuC(S)-Phe-Phe OCH_2CH_3 from the thiopeptide acid (48)

Boc-LeuC(S)-Phe-OH (100 mg, 1.257 mmol), triethylamine (25 mg, 0.25 mmol) and HOBt (35 mg, 0.25 mmol) were dissolved in CH_2Cl_2 (5 mL) and cooled to 0°C. DCC (53 mg, 0.25 mmol) was added all at once and the mixture stirred at 0°C for 30 min. TLC analysis indicated the disappearance of the starting material and the formation of two new products: Major product R_f .47 (hexane/EtOAC 5:2) and minor product R_f .49. The reaction mixture was diluted with CH_2Cl_2 (10 mL), washed with citric acid (5%) H_2O and the resulting products were purified partially on silica gel chromatography. The product was obtained as an oil and rapidly turned bright yellow on standing.

^1H NMR (200 MHz, $CDCl_3$) δ : 7.36-7.06 (m, 5H, ArH), 4.96-4.10 (m, 3H, CH_{α} Phe, CH_{α} Leu, NH Boc), 3.44-2.80 (m, 2H, $CH_{2\beta}$ Phe), 1.70-1.24 (multiplet containing a singlet at 1.42, 12H, CH_{γ} $CH_{2\beta}$ Leu, t-bu), 0.90 (m, 6H, $2CH_3$ Leu) ppm.

N'-methyl S-methyl N-Boc-L-phenylalanine thioimidate

To the thioamide 18 (500 mg, 1.7 mmol) dissolved in dry acetonitrile (10 mL) was added methyl iodide (482 mg, 3.4 mmol) and the mixture stirred under a nitrogen atmosphere at 35°C for 28 h. The solvent and excess methyl iodide were removed in vacuo to yield a brownish oily residue. Addition of anhydrous ether yielded a hydroscopic yellow solid which was filtered rapidly and washed with anhyrous ether to give 595 mg (80%) of the thioimidate 49 which was used without further purification.

mp 124-127°C; $[\alpha]_D^{20}$+55.0° (c 1.0, $CHCl_3$), ^1H NMR (60 MHz, $CDCl_3/DMSO_{D6}$ 1:1) δ : 8.0-7.5 (b, 1H, NH), 7.21 (s, 5H, ArH), 6.1-5.9 (b, 1H, NHBoc), 4.8-4.4 (m, 1H, CH_α), 3.3-3.0 (m, 5H, $CH_{2\beta}$, CH_3), 2.30 (s, 3H, S-CH_3), 1.45 (s, 9H, t-bu) ppm.
MS (70 eV, 55°C) m/e: 295 (2, $M^{+\cdot}-CH_3$), 220 ($M^{+\cdot}-C(S)NHCH_3$), 205 (19), 164 (226), 120 (682).

N-Boc-S-methyl-L-phenylalanine methyl dithioester (50)

The thioimidate (49) (2.0 g, 4.58 mmol) was added all at once to a saturated solution of H_2S in dry THF (25 mL) at 0°C, followed by the addition of dry pyridine (1.8 g, 22.9 mmol). H_2S was introduced in the reaction mixture for an additional 20 min. After this was flushed out of the solution with a stream of nitrogen into traps containing lead (II) acetate and sodium hydroxide. The reaction mixture was then transferred

to a separatory funnel diluted with ether (100 mL), washed with 5% citric acid (2x50 mL), 5% $NaHCO_3$ (2x50 mL, brine, dried ($MgSO_4$) and evaporated in vacuo. Flash chromatography on silica gel using hexanes/EtOAc (5:2) as eluent gave a yellow solid which was recrystallized from hexanes and filtered at 0°C to give 943 mg (66% yield) of bright yellow crystals. mp 79-81°C; $[\alpha]_D^{20}$+84.5° (c 1.0, $CHCl_3$); UV (EtOH) λ_{max}: 307.3 log ε 4.13 ^1H NMR (200 MHz, $CDCl_3$) 7.40-7.14 (m, 5H, ArH), 5.44-5.30 (bd, 1H, NHBoc), 5.2-5.0 (m, 1H, CH_α), 3.30-3.05 (m, 2H, $CH_{2\beta}$), 2.58 (s, 3H, SCH_3), 1.38 (s, 9H, t-bu) ppm. MS (70 eV, 20°C) m/e: 311 (1.5, $M^{+\cdot}$), 200 (18.4, $M^{+\cdot}$-C(S)-SCH_3), 164 (21.4, $M^{+\cdot}$-C(S)-SCH_3), 120 (51.2, C_6H_5-CH_2=$CH^{+\cdot}$), 91 (35.0), 57 (100).

Elemental analysis calcd: C 57.84, H 6.79, N 4.49, S 20.55. Found: C 57.73, H 6.84, N 4.45, S 20.41.

N-Boc-methionine ethyl dithioester (54)

N'-methyl Boc methioninethioamide (51) (781 mg, 2.8 mmol) was dissolved in dry acetonitrile (2 mL) and methyliodide (400 mg, 5.6 mmol) was added and the mixture heated at 30°C for 24 h. The solvent was evaporated and yielded an insoluble mixture of products. This crude mixture was added to a THF solution saturated with H_2S at 0°C. H_2S was then added for an additional 20 minutes and the reaction mixture worked up as described for the dithioester derivative 50. The two products present were separated by flash chromatography on silica gel

using ether/hexanes (5:2) as eluent and yield 200 mg (40%) of the least polar component: R_f .4 (hexanes/EtOAc 5:2), and 40 mg (7%) of the most polar component R_f .3 hexane (EtOAc 5:2) as a yellow oil corresponding to the desired Boc-Met-C(S)-SCH_3.

$[\alpha]_D^{20}$+12.0° (c 1.0, $CHCl_3$); UV (EtOH λ_{max}: 306 log ε 4.06, ^1H NMR (60 MHz, $CDCl_3$) δ : 5.66-5.20 (m, 1H, NHBoc), 5.19-4.85 (m, 1H, CH_α), 2.80-2.33 (multiplet containing a singlet at 2.60, 7H, (C(S)-SCH_3, CH_2-S), 2.30-1.95 (multiplet containing a singlet at 2.09, (5H, S-CH_3,CH_β), 1.50 (s, 9H, t-bu) ppm. MS (70 eV) m/e: 295 ($M^{+\cdot}$, 55), 239 ($M^{+\cdot}$-SCH_3), 204 ($M^{+\cdot}$-C(S)-SCH_3), 191 (55), 148 (339, 61 (526). Least polar identified as N-Boc dithioester 55. mp 86-89°C decomp; $[\alpha]_D^{20}$ 0.0 (c 1.0, $CHCl_3$); UV (EtOH) λ_{max} 3.0.3. log ε 4.11, ^1H NMR (200 MHz, $CDCl_3$) δ : 5.30 (br, 1H, NHBoc), 4.40 (m, 1H, CH_α), 3.50 (m, 1H, CH_β), 3.30 (m, 1H, CH_β'), 2.04 (octet, 1H, J=7.1 Hz, J=12.4 Hz, J=24.7 Hz, CH_γ), 1.44 (S, 9H, NHBoc) ppm.
MS (70 eV) m/e: 233 ($M^{+\cdot}$, 19), 133 (284), 101 (697).

Boc-DL-PheC(S)-Gly-OEt (58)

The dithioester of Boc-Phe (50) (200 mg, 0.67 mmol) was dissolved in dry THF (5 mL) together with glycine ethylester hydrochloride (108 mg, 0.77 mmol), imidazole (48 mg, 0.70 mmol) and triethylamine (80 mg, 0.77 mmol). After 6 h of stirring at room temperature the solvent was removed in vacuo. The

residue was taken in EtOAc, washed with citric acid (5%), brine, dried ($MgSO_4$) and evaporated to give 205 mg (92%) of pale yellow powder, which was recrystallized from $CHCl_3$/hexane. R_f .36 (hexanes/EtOAc 5:2)
mp 127-131°C; $[\alpha]_D^{20}$ 0.0 (c 1.0 $CHCl_3$); UV (ETOH) : λ_{max} 266.0 log ε 4.02; ^1H NMR (200 MHz, $CDCl_3$) δ : 9.0 (br, 1H, C(S)N<u>H</u>), 8.40-8.14 (m, 5H, ArH), 5.32 (br, 1H, N<u>H</u>Boc), 4.6 (m, 1H, CH_α Phe) 4.34-4.12 (m, 4H, CH_2 Gly, CH_2-CH_3), 3.16 (d, 2H, J=4.5 Hz, $CH_{2\beta}$Phe), 1.40 (s, 9H, <u>t</u>-bu), 1.26 (t, 3H, J=7.6 Hz, CH_2-CH_3) ppm.

<u>Boc-DL-PheC(S)-Leu-OCH$_3$ (59)</u>

Boc-PheC(S)-SCH_3 (<u>50</u>) (50 mg, 0.16 mmol) dissolved in dry THF (2 mL) with L-leucine methyl ester hydrochloride (32 mg, 0.17 mmol) triethylamine (0.17 mmol) and imidazole (11 mg, 0.17 mmol) and the mixture stirred at room temperature for 4 days. After evaporation and purification by flash chromatography on silica gel (hexanes/EtOAc 5:2) 59 mg (91%) of white powder was obtained. R_f .44 (hexanes/EtOAc 5:2)
UV (ETOH) : λ_{max} 268.1 log ε 4.20, $[\alpha]_D^{20}$ -13.1° (c 0.95, $CHCl_3$); ^1H NMR (60 MHz, $CDCl_3$) δ : 7.85 (br, 1H, N<u>H</u> Leu), 5.38-4.85 (m, 2H, N<u>H</u>Boc, CH_αPhe), 4.85-4.35 (m, 1H, CH_αLeu), 3.66-3.68 (2S, 3H, OCH_3), 3.16 (d, 2H, J=7 Hz, CH_βPhe), 1.66-1.16 (multiplet containing a singlet at 1.40, 12H, CH_β, CH_γLeu, <u>t</u>-bu), 1.05-0.85 (m, 6H, $2CH_3$ δ Leu) ppm.

Boc-DL-PheC(S)-Gly-Gly-OEt (60)

This compound was prepared as described for Boc-PheC(S)-Gly-OEt 58 with Boc-PheC(S)-SCH_3 (200 mg, 0.67 mmol), glycylglycine ethyl ester hydrochloride (Sigma) (139 mg, 0.73 mmol), imidazole (48 mg, 0.70 mmol) and triethylamine (80 mg, 0.77 mmol). The reaction was stopped after 12 h. After identical work up as for 58 the product was purified by flash chromatography on silica gel (hexane/EtOAc 1:1) to give 248 mg (87% yield) of a waxy solid.

R_f .44 (hexanes/EtOAc 1:1); $[\alpha]_D^{20}$ 0.0°, (c 1.0, $CDCl_3$) UV (ETOH) λ_{max} 267.3 log ε 4.04, ^1H NMR (200 MHz, $CDCl_3$) δ : 8.9 (br, 1H, NH Gly_1) 7.40-7.12 (m, 5H, ArH), 7.06 (br, 1H, NH Gly_2), 5.70 (br, 1H, NHBoc), 4.70 (m, 1H, CH_α Phe), 4.40-4.24 (m, 2H, CH_2 Gly_1), 4.18 (q, 2H, J=7.6 Hz, CH_2-CH_3), 5.08-4.86 (m, 2H, CH_2 Gly_2), 3.20 (m, 1H, CH_β Phe), 3.04 (m, 1H, CH_β, Phe), 1.36 (s, 9H, t-bu), 1.27 (t, 3H, J=7.6 Hz, CH_2-CH_3) ppm.

MS (70 eV) m/e: 423 (6.1, $M^{+\cdot}$), 367 (7.6, $M^{+\cdot}$ - isobutylene), 349 (10.8), 306 (47.6), 273 (19.0), 215 (11.5), 120 (51.3).

N'-Methyl leucinethioamide hydrochloride (43)

N'-Methyl N-Boc leucinethioamide (300 mg, 1.15 mmol) was dissolved in anhydrous ether (5 mL). This solution was then added to a HCl/ether solution (2N) and left standing at room temperature overnight. The solvent was evaporated and anhydrous ether was added and a white precipitate formed. This powder was collected by filtration and washed with ether to yield 215 mg (95% yield) of a white powder.
mp 197-198°C; UV (EtOH) λ_{max} 264.8 log ε 4.02; $[\alpha]_D^{20}$+43.4° (c 1.0, MeOH); ^{1}H NMR (60 MHz, DMSO) δ : 8.8-8.2 (br, 3H, NH_2, NH), 4.4-4.0 (m, 1H, CH_α), 3.0 (s, 3H, NH $\underline{CH_3}$), 1.83-1.40 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.1-0.86 (bd, 6H, 2 $\underline{CH_{3\delta}}$) ppm. MS (70 ev) m/e: 160 (343, $M^{+\cdot}$), 161 (27, $M^{+\cdot}$+1), 144 (10, $M^{+\cdot}$-CH_3), 104 (557, $M^{+\cdot}$- isobutylene), 86 (100).

N-Acetyl-L-phenylalanine methyl dithioester (57)

The Boc-PheC(S)-SCH_3 50 ($[\alpha]_D^{20}$+80.5° (c 1.0 $CHCl_3$)) (160 mg, 0.51 mmol) was dissolved in a HCl/ether solution (2N) and left standing at room temperature for 2 h and 0°C overnight. The resulting yellow crystals were filtered and washed with anhydrous ether and dried in vacuo to give 120 mg (90% yield) of bright yellow crystals (mp 168-170°C). This hydrochloride salt (100 mg, 0.4 mmol) was dissolved in CH_2Cl_2. Acetic anhydride (43 mg, 0.4 mmol) and pyridine (32 mg, 0.4 mmol) were then added, and the mixture was stirred at room temperature

for 3 h. The reaction mixture was then diluted with CH_2Cl_2 (10 mL) and transferred to a separatory funnel, washed with citric acid (5%) and H_2O, dried ($MgSO_4$). Evaporation of the solvent gave an oil which was crystallized from ether/hexane to yield 72 mg (75% yield) of bright yellow compound.

mp 134-135°C; $[\alpha]_D^{20}$+76.8° (c 0.5, $CHCl_3$); UV (EtOH): λ_{max} 310.3 log ε 3.98; ^1H NMR (200 MHz, $CDCl_3$) δ : 7.34-7.08 (m, 5H, ArH), 6.40-6.24 (m, 1H, C(O)NH), 4.54-4.39 (m, 1H, CH_α), 3.24-3.03 (m, 2H, $CH_{2\beta}$), 2.57 (s, 3H, SCH_3), 1.98 (s, 3H, C(O)-CH_3) ppm. MS (70 ev) m/e: 253 (137, $M^{+\cdot}$), 220 (55, $M^{+\cdot}$-HS), 205 (133, $M^{+\cdot}$-CH_3-SH), 194 (122, $M^{+\cdot}$- NH_2-C(O)CH_3), 162 (359, $M^{+\cdot}$-C(S)-SCH_3) 120 (100).

Chapter 2

Boc-Leu-Phe-OCH_3 (62)

Boc-Leu (Bachem) (5.0 g, 23 mmol), phenylalanine methyl ester hydrochloride (4.96 g, 23 mmol), triethylamine (2.32 g, 23 mmol) and HOBt. (3.10 g, 23 mmol) were dissolved in CH_2Cl_2 (50 mL) and cooled to 0°C. DCC (4.75 g, 23 mmol) was then added and the reaction mixture stirred at 0°C overnight. The solvent was removed *in vacuo*, and the residue suspended in EtOAc (100 mL) and filtered. The filtrate was transferred into a separatory funnel and washed successively with citric acid (5%) 2x25 mL), $NaHCO_3$ (2x25 mL) and brine (2x25 mL). The organic layer was dried ($MgSO_4$) and evaporated *in vacuo*. The oily residue was crystallized from EtOAc/hexanes giving 7.2 g (86% yield) of a white solid.

R_f .51 ($CHCl_3$/EtOAc 3:1); mp 83-85°C; $[\alpha]_D^{20}$-24.2° (c 1.0, MeOH); ^1H NMR (60 MHz, $CDCl_3$) δ : 7.53 (d, 1H, J=7.0 Hz, C(O)N<u>H</u>), 7.6-7.0 (m, 5H, ArH), 5.13-4.66 (m, 2H, CH_α Phe and N<u>H</u>Boc), 4.16 (m, 1H, CH_α Leu), 3.72 (s, 3H, OCH_3), 3.05 (d, 2H, J=8 Hz), $CH_{2\beta}$ Phe), 2.1-1.64 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.40 (s, 9H, NHBoc), .9 (d, 6H, J=7.0 Hz, $2CH_{3\delta}$ Leu) ppm.

Boc-Met-Leu-Phe-OCH_3 (63)

Boc-Phe-Leu-OCH_3 (3.0 g, 7.6 mmol) was dissolved in formic acid 98% (25 mL) and stirred at room temperature for 2 h. The

excess acid was removed *in vacuo* at 30°C. The resulting formate salt is then neutralized by the addition of $NaHCO_3$ (5%) (20 mL) and extracted with CH_2Cl_2 (2x50 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to a volume of approximately 10 mL. To a solution of Boc-methionine (Bachem) (1.89 g, 7.6 mmol) and HOBt (1.14 g, 8.4 mmol) in CH_2Cl_2 (20 mL) and DMF (5 mL) at 0°C was added DCC (1.65 g, 8 mmol) and the mixture stirred at 0°C for 1 h. After this time, the solution of Leu-Phe-OCH_3 previously cooled at 0°C was added and the resulting mixture stirred at 0°C for 1 h, and room temperature for 15 h. The solvents were evaporated *in vacuo*, and the residue suspended in EtOAc and filtered. The filtrate was washed with citric acid (5%), $NaHCO_3$ (5%) and brine. After drying ($MgSO_4$) and evaporation, the resulting solid was recrystallized from EtOAc-hexanes giving 2.6 g (65% yield) of a white powder homogenous by TLC.

R_f .26 ($CHCl_3$/ETOAc 3:1); mp 126.5-127.5°C; $[\alpha]_D^{20}$ -39.2° (c 1.0, MeOH); ^1H NMR (60 MHz, $CDCl_3$) δ : 7.3-71 (m, 5H, ArH), 6.56 (d, 1H, J=7 Hz, C(O)NH), 6.43 (d, 1H, J=6.2 Hz, C(O)NH), 5.14 (d, 1H, J=7 Hz, NHBoc), 4.81 (m, 1H, CH_α Phe), 4.38 (m, 1H, CH_α Met), 4.18 (m, 1H, Ch_α Leu), 3.67 (s, 3H, OCH_3), 3.09 (d, 2H, J=8 Hz), 2.54 (t, 2H, J=7.4 $CH_{2\gamma}$ Met), 2.09 (s, 3H, S-CH_3), 2.07-1.83 (m, 2H, $CH_{2\beta}$ Met), 1.70-1.52 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.45 (s, 9H, NHBoc), .88 (dd, 6H, J=3.9 Hz, J=4.3 Hz, $2CH_{3\delta}$ Leu) ppm.

HC(O)-Met-Leu-Phe-OCH_3 (64)

Boc-Met-Leu-Phe-OCH_3 (1.0 g, 1.9 mmol) was dissolved in formic acid 98% (20 mL) for 3 h. Evaporation of formic acid *in vacuo* gave an oil which was dissolved in $CHCl_3$ (25 mL), EEDQ (505 mg, 20 mmol) was then added. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated *in vacuo*. The residue was dissolved in EtOAc (100 mL), washed with citric acid (5%) (2x25 mL), 5% $NaHCO_3$ (2x25 mL), brine (2x25 mL). The organic layer was dried ($MgSO_4$) and filtered. Evaporation of the solvent gave a solid which was precipitated from MeOH-H_2O yielding 800 mg (95% yield) of a white powder homogenous by TLC.

Physico-chemical data are given in Table 5.

^1H NMR chemical shift and coupling constants (200 MHz, acetone-D_6 in Tables 6 and 9.

HC(S)-Met-Leu-Phe-OCH_3 (66)

HC(O)-Met-Leu-Phe-OCH_3 (300 mg, 0.66 mmol) was dissolved in dry THF (10 mL) and the solution cooled to 0°C. The thionation reagent 11 (175 mg, 0.33 mmol) was added all at once and the mixture was stirred at 0°C for 0.5 h. The solvent was evaporated *in vacuo*, and the residue dissolved in $CHCl_3$ (2 mL) and applied on a silica gel column. Rapid elution using $CHCl_3$/MeOH (9:1) as eluent afforded a solid which was recrystallized from EtOAc/hexanes giving 220 mg (73% yield) of a white powder.

Physico-chemical characteristics are given in Table 5. ^{1}H NMR chemical shift and coupling constants (acetone-D_6) are given in Tables 6 and 9. ^{13}C NMR chemical shift (acetone-D_6) are given in Table 7.

MS (70 ev) m/e: 468 (.8, $M^{+\cdot}$), 422 (.3, $M^{+\cdot}$-HC≡S), 318 (2.5), 303 (70, $M^{+\cdot}$-Phe OCH_3), 162 (14.1), 120 (42.7), 86 (30.4), 61 (26.4).

Boc-LeuC(S)-Phe-OCH_3 (67)

The thionation reagent 11 (1.3 g, 1.75 mmol) was added to a solution of Boc-Leu-Phe-OCH_3 (1.0 g, 2.5 mmol) in dry THF (25 mL) and stirred under nitrogen for 24 h. The solvent was evaporated in vacuo. The oily residue was dissolved in $CHCl_3$ (2 mL) and purified by flash chromatography on silica gel with hexanes/EtOAc 5:1 as eluent. After evaporation of the desired fractions, 890 mg (87% yield) of an oil, which solidifies on standing, was obtained.

R_f .49 (EtOAc/hexanes 2:5). mp 73-76°C; $[\alpha]_D^{20}$+39.3° (c 1.0, MeOH); UV (EtOH) : λ_{max} 267.4 log ε 4.08; ^{1}H NMR (200 MHz, $CDCl_3$) δ : 8.2 (d, 1H, J=7.4, C(S)NH), 7.3-7.06 (m, 5H, ArH), 5.36 (ddd, 1H, J=6.1 Hz, J=4.8 Hz, J=7.3 Hz, CH_α Phe), 5.01 (br s, 1H, NHBoc), 4.42-4.32 (m, 1H, CH_α Leu), 3.74 (s, 3H, OMe), 3.41 (dd, 1H, J=6.13 Hz, J=13.9, CH_β Phe), 3.2 (dd, 1H, J=4.66 Hz, J=13.9 CH_β, Phe), 1.68-1.58 (m, 3H, $CH_{2\beta}$-CH_γ Leu), 0.92 (d, 6H, J=6.1 Hz), $2CH_{3\delta}$ Leu) ppm.

MS (70 ev), m/e: 408 (22, $M^{+\cdot}$), 352 (71, $M^{+\cdot}$ - isobutylene), 335 (27), 223 (161), 162 (198), 130 (212), 120 (297), 91 (581).

Desulfuration experiment of Boc-LeuC(S)-Phe-OCH_3 (67)

To Boc-LeuC(S)-Phe-OCH_3 (88 mg, 0.21 mmol) in dry THF (2 mL) was added silver nitrate (81 mg, 0.47 mmol) and the mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc and filtered through celite. The solvents were evaporated and the product purified by flash chromatography on silica get using EtOAc/hexanes (5:2) to yield 43 mg (50%) of a white powder which had identical properties to those previously described Boc-Leu-Phe-OCH_3.
R_f .51 ($CHCl_3$/EtOAc 3:1); $[\alpha]_D^{20}$ -24.1° (c 1.1, MeOH).

Boc-Met-LeuC(S)-Phe-OCH_3 (68) Method A.

Boc-LeuC(S)-Phe-OCH_3 (500 mg, 1.22 mmol) was dissolved in formic acid 98% (5 mL) and left standing at room temperature for 2 h. The excess formic acid was removed in vacuo. To the oily residue was added $NaHCO_3$ (5%, 10 mL) and the aqueous solution immediately extracted with EtOAc (2x25 mL) and the organic layer dried with $MgSO_4$ and evaporated in vacuo. The resulting oil was dissolved in CH_2Cl_2 (5 mL) and added to a solution of Boc-Met-HOBt at 0°C. This solution had been prepared from Boc-methionine (304 mg, 1.22 mmol) HOBt (181 mg, 1.3 mmol) and DCC (251 mg, 1.22 mmol) in CH_2Cl_2 (5 mL), DMF (2mL) at 0°C as for 64.

The resulting mixture was stirred at 0°C for 1 h, and at room temperature for 4 h. The DCU was filtered and the filtrate washed successively with citric acid (5%) (2x25 mL), $NaHCO_3$ (5%) (2x25 mL), and brine. The organic layer was dried ($MgSO_4$) and evaporated *in vacuo* to yield a solid which was recrystallized from $CHCl_3$/hexanes giving 376 mg (73% yield) of white crystals.

R_f .23 (Hexanes/EtOAc 5:2), mp 131-133°C; $[\alpha]_D^{20}$ -17.4 (c 1.0, MeOH); UV (ETOH): λ_{max} 268.4 log ε 4.01; ^1H NMR (200 MHz, $CDCl_3$) δ : 8.32 (br d, 1H, J=6.4 Hz, C(S)NH), 7.3-7.1 (m, 5H, ArH), 6.80 (d, 1H, J=8.0 Hz, C(O)NH Leu), 5.36 (m, 1H, CH_α Phe), 5.22 (d, 1H, J=8.0 Hz, NHBoc), 4.70 (m, 1H, CH_α Leu), 4.25 (m, 1H, CH_α Met), 3.73 (s, 3H, OCH_3), 3.34 (dd, 1H, J=6.2 Hz, J=14.8 Hz, CH_β Phe), 3.19 (dd, 1H, J=5.3 Hz, J=14.8 Hz, CH_β' Phe), 2.54 (t, 2H, J=7.21 Hz, $CH_{2\gamma}$ Met), 2.10-1.80 (multiplet containing a singlet at 2.10, 5H, S-CH_3, CH_β Met), 2.76-2.50 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.44 (s, 9H, t-bu), .91-.88 (2d, 6H, J=2.6 Hz, $2CH_{3\delta}$ Leu) ppm.

HC(O)-Met-LeuC(S)-Phe-OCH_3 (69)

Boc-Met-LeuC(S)-Phe-OCH_3 (200 mg, 0.37 mmol) was dissolved in formic acid (10 mL) and left standing at room temperature for 2 h. The formic acid was removed *in vacuo*, and the residue was dissolved in $CHCl_3$ (5mL) and EEDQ (100 mg, 0.040 mmol) was added and the mixture was stirred overnight at room temperature. Evaporation of the solvent yielded a solid which was recrystal-

lized with $CHCl_3$/hexanes to give 140 mg (81% yield) of a white powder.

Physico-chemical characteristics are summarized in Table 5. ^1H NMR chemical shifts and coupling constants are given in Table 6 and Table 9. ^{13}C NMR chemical shifts are given in Table 7.

MS (70 eV) m/e: 467 (7.3, $M^{+\cdot}$), 411 (79.8, $M^{+\cdot}$ - isobutylene), 344 (2.9), 291 (14.8), 248 (11.7), 180 (19.0), 120 (6.6), 104 (14.4), 91 (13.0), 86 (100), 61 (59.6).

Boc-DL-MetC(S)-Leu-Phe-OCH_3 (70) (via the dithioester)

Boc-MetC(S)-SCH_3 (54) (30 mg, 0.11 mmol) was dissolved in THF (2 mL) with HCl.Leu-Phe-OCH_3 (38 mg, 0.12 mmol) triethylamine (12 mg, 0.12 mmol) and imidazole (7 mg, 0.12 mmol) and the mixture stirred at room temperature for 3 days. The solid was filtered and the filtrate evaporated in vacuo to yield an oily residue which was purified by flash chromatography on silica gel (hexanes/EtOAc 5:2) 38 mg of an oil (67% yield). $[\alpha]_D^{20}$ -56.5° (c 0.72, $CHCl_3$); UV (EtOH): λ_{max} 268.8 log ε 4.01; ^1H NMR (200 MHz, $CDCl_3$) δ : 9.40-9.20 (m, 1H, C(S)NH), 7.30-7.00 (m, 5H, ArH), 6.64 (m, 5H, C(O)NH), 6.48 (m, 5H, C(O)NH, ArH), 5.40 (m, 1H, NHBoc), 5.0 (m, 1H, CH_α Leu), 4.78 (m, 1H, CH_α Leu), 4.52 (m, 1H, CH_α Met), 3.64 and 3.66 (2s, 3H, OCH_3), 3.14-2.98 (m, 2H, CH_2 Phe), 2.58-2.34 (m, 2H, CH_γ Met), 3.20-2.74 (multiplet containing 2 singlets at 2.08 and 2.06, 5H, SCH_3, $CH_{2\beta}$ Met,

2.78-2.46 (m, 3H, CH_β CH_γ Leu), 1.38 (s, 9H, t-bu), .92-0.84 (m, 6H, $2CH_{3\delta}$ Leu) ppm.

Boc-MetC(S)-LeuC(S)-Phe-OCH_3 (73)

To Boc-Met-Leu-Phe-OCH_3 (600 mg, 1.14 mmol) dissolved in dry THF (10 mL) was added the thionation reagent 11 (720 mg, 1.36 mmol) and the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated in vacuo and the resulting oil dissolved in $CHCl_3$ (2mL) and purified by flash chromatography on silica get using hexanes/EtOAc (5:2) as eluent to give a white solid 518 mg (82% yield).

R_f .55 (hexanes/EtOAc 5:2); $[\alpha]_D^{20}$-36.5° (c 1.0, $CHCl_3$); UV EtOH): λ_{max} 272.2 log ε 4.27; H^1 NMR (200 MHz, $CDCl_3$) δ : 8.47 (d, 1H, J=7.9, C(S)NH Leu), 8.01 (d, 1H, J=6.75 Hz, C(S)NH Phe), 7.32-7.18 (m, 5H, ArH), 5.42 (m, 3H, NHBoc, CH_α Phe, CH_α Leu), 4.54 (m, 1H, CH_α Met), 3.75 (s, 3H, OMe), 3.32 (dd, 1H, J=6.1 Hz, J=13.2 Hz, CH_β Phe), 3.23 (dd, 1H, J=5.3 Hz, J=13.2 Hz, CH_β, Phe), 2.53 (m, 2H, $CH_{2\gamma}$ Met), 2.1 (s, 3H, SCH_3 Met), 2.05-1.98 (m, $CH_{2\beta}$ Met), 1.85-1.5 (m, 3H, CH_γ $CH_{2\beta}$ Leu), 1.44 (s, 9H, t-bu), 0.90 (dd, 6H, J=6.4 Hz, J=4.7 Hz, $2CH_{3\delta}$ Leu) ppm.

MS (70 ev) on ethyl ester derivative m/e: 569 (27.0, $m^{+\cdot}$), 496 (6.1), 480 (39.9), 419 (73.7), 333 (19.1), 306 (13.8), 264 (15.5), 263 (16.7), 262 (19.3), 194 (6.8) 120 (18.0), 104 (28.4), 86 (12), 61 (57).

HC(O)-MetC(S)-LeuC(S)-Phe-OCH_3 (76)

Boc-MetC(S)-LeuC(S)-Phe-O-CH_3 (300 mg, 0.54 mmol) was dissolved in formic acid 98% (10 mL) and stirred at room temperature for 3.5 h. The excess formic acid was removed in vacuo to give an oil which solidified on standing. This formate salt was dissolved in $CHCl_3$ (10 mL) and EEDQ (151 mg, 0.61 mmol) was added. After stirring at room temperature for 4 h, the solvent was removed in vacuo and the resulting oil purified by flash chromatography on silica gel. An oil was obtained from evaporation of the desired fractions. This oil was precipitated from MeOH-H_2O giving 198 mg (76% yield for both steps) of a white powder.

The physico-chemical characteristics are given in Table 5. ^1H NMR (200 MHz) chemical shifts and coupling constants are given in Table 6 and Table 9. ^{13}C NMR chemical shifts are given in Table 7.

MS (70 ev) m/e: 483 (24.6, $M^{+\cdot}$), 449 (100, $M^{+\cdot}$ - H_2S), 405 (55.4, $M^{+\cdot}$ - H_2S - HC(O)NH), 388 (17.8), 360 (74.0), 346 (11.8), 319 (6.8, $M^{+\cdot}$ - Ph-CH_2-CH-C(O)OCH_3), 155 (3.1), 122 (3.8), 91 (16.7).

Simultaneous preparation of monothioamide 71 and 72 and dithioamide 73 from Boc-Met-Leu-Phe-OCH_3 (64)

Boc-Met-Leu-Phe-OCH_3 (2.0 g, 3.8 mmol) was dissolved in dry THF (60 mL) and cooled to 0°C. The thionation reagent 11

(1.51 g, 2.8 mmol) was added and the reaction mixture stirred in a sealed flask at 0°C for 3 days. The solvent was removed in vacuo. Flash chromatography on silica gel using a gradient solvent system hexanes/EtOAc 5:1 → 5:2 gave after evaporation of the different fractions 360 mg (18% yield) of the least polar component Boc-MetC(S)-LeuC(S)-Phe-OCH_3 and 1.350 g (66% yield) of the most polar component and 100 mg of unreacted starting material.

HPLC (silica gel, hexanes/$CHCl_3$/MeOH 800:50:4) was used to separate the mixture of monothio Boc-tripeptide and these were found to be in a ratio 65:45 least polar/most polar

a) Boc-MetC(S)-Leu-Phe-OCH_3 71 (least polar) HPLC retention time 9.0 min, oil, UV (ETOH): λ_{max} 268.8 log ε 4.00; $[\alpha]_D^{20}$ -47.4° (c 1.0, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ : 8.13 (br d, 1H, J=7.60 Hz, C(S)NH), 7.31-7.1 (m, 5H, ArH), 6.4 (br d, 1H, J=7.3 Hz, C(O)NH Phe), 5.4 (br d, 1H, J=7.8 Hz, NHBoc), 5.02 (m, 1H, CH_α Leu), 4.82 (m, 1H, CH_α Phe), 4.52 (m, 1H, CH_α Met), 3.72 (s, 3H, OCH_3), 3.12 (d, 1H, J=2.8 Hz, CH_β Phe), 3.09 (d, 1H, J=3.1 Hz, CH_β' Phe), 2.4 (m, 2H, $CH_{2\gamma}$ Met), 2.10 (s, 3H, S-CH_3), 2.05-1.97 (m, 2H, $CH_{2\beta}$ Met), 1.43 (s, 9H, t-bu), 1.8-1.6 (m, 3H, $CH_{2\beta}$, $CH_{2\gamma}$ Leu), 0.90 (dd, 6H, $2CH_{3\delta}$ Leu) ppm.

MS (of the corresponding ethylester) (70 eV) m/e: 553 (10.9, $M^{+\cdot}$), 520 (3.0), 300 (14.3), 194 (100), 186 (21.0) 120 (33.9), 104 (27.4), 57 (67).

b) most polar

retention time 9.5 min.

physico chemical properties identical to those described previously for 68.

HC(O)-MetC(S)-Leu-Phe-OCH_3 (74) and HC(O)-Met-LeuC(S)-Phe-OCH_3 (75)

The mixture of Boc monothiotripeptide 71 and 72 (830 mg, 15.9 mmol) were dissolved in formic acid 98% (20 mL) and left standing at room temperature for 2 hr. After evaporation of the formic acid in vacuo, $CHCl_3$ (20mL) and EEDQ (598 mg, 16.3 mmol) were added and the mixture was stirred at room temperature for 4 h. The solvent was evaporated in vacuo and the products were isolated by flash chromatography on silica gel using a gradient elution (EtOAc/hexanes 1:1 → 2:1) to give 374 mg (50% yield) of least polar 74 and 270 mg (36% yield) of the most polar 75. Their physico-chemical characterictics are summarized in Table 5; ^1H NMR chemical shifts and coupling constants are given in Table 6 and Table 9; ^{13}C NMR chemical shifts are given in Table 7.

^{1}H NMR EXPERIMENTS

A) Observed NH chemical shift (ppm) vs temperature (DMSO-d_6).

#	T (°C)	NH Met	NH Leu	NH Phe
64				
	20	8.03	8.27	8.34
	30	7.98	8.23	8.27
	40	7.93	8.18	8.22
	50	7.88	8.13	8.16
	60	7.83	8.09	8.10
	70	7.79	8.06	8.03
	80	7.74	8.00	7.96
74				
	20	8.30	10.04	8.47
	30	8.25	10.00	8.41
	40	8.21	9.95	8.35
	50	8.17	9.90	8.28
	60	8.14	9.85	8.23
	70	8.11	9.79	8.15
	80	8.07	9.75	8.10
75				
	20	8.27	8.12	10.42
	30	8.24	8.07	10.35
	40	8.20	8.01	10.29
	50	8.16	7.95	10.22
	60	8.12	7.90	10.16
	70	8.07	7.84	10.10
	80	8.04	7.79	10.04

B) Angles θ and ϕ calculation:

e.g.: $J_{NHCH_\alpha} = 8.8$ Hz

$$8.8 = 9.4 \cos^2 \theta - 1.4 \cos \theta + 0.4$$

$$0.936 = 1.0 \cos^2 \theta - .117 \cos \theta + 0.42$$

$$0 = 1.0 \cos^2 \theta - .117 \cos \theta - 0.894$$

$$= \frac{0.117 \pm (0.117)^2 - 4(1-0.894)}{2}$$

$$\theta = 153°$$

$$\theta = |\phi - 60°|$$

$$\phi = -93°, -147° (213)$$

C) Rotamer population analyses:

e.g.: $J_{CH_\alpha - CH_\beta} = 6.1$ Hz

$J_{CH_\alpha - CH_\beta'} = 8.1$ Hz

$$6.1 = 2.6\ P_I + 11.8\ P_{II} + 3.3\ P_{III}$$

$$8.1 = 11.8\ P_I + 2.6\ P_{II} + 3.0\ P_{III}$$

$$P_I = 1 - P_{II} - P_{III}$$

$$6.1 = 2.6 + 9.2\ P_{II} + 0.7 P_{III}$$

$$8.1 = 11.8 - 9.2\ P_{II} - 8.8\ P_{III}$$

$$P_{II} = 0.38 - 9.2(0.38 - 0.076\ P_{III}) - 8.8\ P_{III}$$

$$P_{III} = 0.28$$

$$P_{II} = 0.36$$

$$P_I = 0.36$$

Chapter 3

Boc-Phe-Leu-OCH_3 (82)

DCC (3.73 g, 18 mmol) was added to a mixture of Boc-Phe-OH (4.38 g, 16.5 mmol), leucine methyl ester hydrochloride (3.0 g, 16.5 mmol) and triethylamine (1.66 g, 16.5 mmol) in CH_2Cl_2 (25 mL) at 0°C. The reaction mixture was stirred at 0°C for 1 h and at room temperature for 4 h. The solvent was removed *in vacuo* and the residue suspended in EtOAc (100 mL) and filtered. The filtrate was washed successively with citric acid (5%), $NaHCO_3$ (5%), and brine. After drying ($MgSO_4$) the solvent was removed to yield a solid which was recrystallized from EtOAc/hexanes giving 5.3 g (83%) of white powder.
R_f .29 (EtOAc/hexanes (5:2)); mp 105-107°C; $[\alpha]_D^{20}$-25.0° (c 2.0, MeOH), ^1H NMR (60 MHz, $CDCl_3$) δ : 7.26 (s, 5H, ArH), 6.29 (bd, 1H, J=8.0 Hz, C(O)N<u>H</u>), 5.06 (bd, 1H, J=9.0 Hz, N<u>H</u>Boc), 4.79-4.15 (m, 2H, CH_α Phe, CH_α Leu), 3.68 (s, 3H, OCH_3), 3.06 (d, 2H, J=7.0 Hz, $CH_{2\beta}$ Phe), 1.75-1.18 (multiplet containing a singlet at 1.42, 12H, N<u>H</u>Boc, $CHCH_2$ Leu), .90 (d, 6H, J=5.0 Hz, $CH_{3\delta}$ Leu) ppm.

Boc-Gly-Gly-OH

Di-*tert*-butyl dicarbonate (9.07 g, 41 mmol) was added to glycyl-glycine (5.0 g, 38 mmol), dissolved in THF/H_2O 1:1

(40 mL) and followed by the addition of triethylamine (7.63 g, 75 mmol). The mixture was stirred for 3 h at room temperature. After removal of the THF in vacuo, the aqueous phase was washed with ether. The organic layer was extracted with 1N NaOH solution (5 mL) and the aqueous phase then acidified with 0.1N HCl to pH 3.0. The aqueous layer was extracted several times with EtOAc and the combined organic extracts were washed with brine (3x), dried ($MgSO_4$), filtered and the solvent removed in vacuo to yield 6.33 g (71.5%) of a white solid.

mp 135-136°C decomp.

^1H NMR (60 MHz, DMSO) δ : 8.05-7.75 (b, 1H, $CO_2\underline{H}$), 7.0-6.5 (b, 1H, $N\underline{H}$ Gly_2), 6.5-5.8 (b, 1H, $N\underline{H}$Boc), 3.90-3.20 (b, 4H, CH_2 Gly, CH_2 Gly_2), 1.3 (bs, 9H, t-bu) ppm.

Boc-Gly-Gly-Phe-Leu-OCH_3 (83)

Boc-Phe-Leu-OCH_3 was deprotected by treatment with formic acid for 1.5 h followed by evaporation of the excess acid in vacuo. Boc-Gly-Gly-OH (1.0 g, 4.3 mmol) and HOBt (640 mg, 4.7 mmol) were dissolved in a mixture of CH_2Cl_2 (10 mL) and DMF (3 mL) and cooled to 0°C. DCC (887 mg, 4.3 mmol) and triethylamine (434 mg, 4.3 mmol) were added. The mixture is stirred overnight at 0°C and RT for 2 h. Evaporation of the solvents and usual work up gave a solid which was crystallized from THF/ether giving 1.98 g (91.5%) of a white powder which was homogeneous by TLC.

R_f .34 (MeOH/$CHCl_3$ 1:9); mp 134-136°C; $[\alpha]_D^{22}$ -19.7° (c 2.0, MeOH), ^1H NMR (200 MHz, $CDCl_3$) δ: 7.37 (bs, 1H, C(O)-NH, 7.28-7.06 (m, 7H, ArH (Phe), 2C(O)-NH), 5.58 (m, 1H, NHBoc), 4.91 (m, 1H, CH_α Phe), 4.57 (m, 1H, CH_α Leu), 4.1-3.8 (m, 4H, CH_2 Gly_2, CH_2 Gly_3), 3.70 (s, 3H, OCH_3), 3.12 (dd, 1H, J=5.86, J=13.6, CH_β, Phe), 3.96 (dd, 1H, J=7.51, J=13.6, CH_β, Phe), 2.70-2.40 (m, containing a singlet at 1.45, 12H, NHBoc, CH_γ $CH_{2\beta}$ Leu), 0.88 (d, 6H, J=5.86, $CH_{3\delta}$ Leu) ppm.

O,N-bis-(Boc)-Tyr-OH (84)

Di-tert-butyl dicarbonate (13.2 g, 59 mmol), triethylamine (5.95 g, 59 mmol) and dimethylaminopyridine (320 mg, 2.7 mmol) were added to L-tyrosine (5.0 g, 27 mmol) in THF/H_2O (1:1) (60 mL) and the resulting solution stirred at room temperature for 3 h. After removal of the solvent the residue was dissolved in EtOAc (100 mL) and washed with citric acid (5%) and H_2O. After drying ($MgSO_4$) the solvent was removed in vacuo to yield a foamy solid which was crystallized from CH_2Cl_2/hexanes to give 7.23 g (70%) of a white solid.

R_f .51 ($CHCl_3$/MeOH (9:1)); mp 96-98°C; litt[463] 92-94°C $[\alpha]_D^{20}$+10.5° (c 2.0, MeOH), ^1H NMR (60 MHz, $CDCl_3$) δ : 9.6 (bs, 1H, CO_2H), 7.38-6.9 (m, 4H, ArH), 5.24-4.83 (m, 1H, NHBoc), 4.80-4.73 (m, 1H, CH_α), 3.30-2.96 (m, 2H, CH_β), 1.56 (s, 9H, O-Boc), 1.36 (s, 9H, NH-Boc).

O,N-bis-(Boc)-Tyr-Gly-Gly-Phe-Leu-OCH_3 (85)

Boc-Gly-Gly-Phe-Leu-OCH_3 (1.0 g, 1.9 mmol) was deprotected by treatment with formic acid (98%) (20 mL) at room temperature for 1.5 h. The excess acid was removed in vacuo. DCC (432 mg, 2.1 mmol) was added to a mixture of O,N-bis-Tyr-OH (724 mg, 1.9 mmol) and HOBt (282 mg, 2.1 mmol) in CH_2Cl_2 (5 mL) and dry THF (2 mL) at 0°C. The reaction mixture was stirred at 0°C. After 1 h, a solution of the formate salt in CH_2Cl_2 (5 mL) containing triethylamine (191 mg, 19 mmol) was added and the mixture was stirred at 0°C for 18 h and room temperature for 4 h. The solvents were evaporated, the residue suspended in $CHCl_3$ and filtered. The filtrate was washed with citric acid (5%), H_2O, the organic layer was dried with $MgSO_4$ and the solvent removed in vacuo. The residue was dissolved in $CHCl_3$ (5 mL) and filtered. After removal of the $CHCl_3$, the resulting oil was precipitated from THF/ether yielding 950 mg of a solid with two minor impurities R_f .58, and .20. These were removed by flash chromatography on silica gel with $CHCl_3$/MeOH 9:1 as eluent. The combined fractions were evaporated and the residue crystallized from THF/ether to give 700 mg (61%) of white powder. R_f .45 ($CHCl_3$/MeOH 9:1); mp 136-138°C; $[\alpha]_D^{20}$ -12.1° (c 1.0 $CHCl_3$). δ : ^1H NMR (200 MHz, $CDCl_3$) resonances are given in Table 17. ^{13}C NMR ($CDCl_3$) chemical shifts are given in Table 18.

Boc-PheC(S)-Leu-OCH_3 (87)

Thionation reagent 11 (1.34 g, 2.5 mmol) was added to Boc-Phe-Leu-OCH_3 (2.0, 51 mmol) in dry THF and the solution stirred at room temperature under a nitrogen atmosphere for 24 h. The solvent was evaporated, the residual oil taken in $CHCl_3$ (2 mL) and applied on a silica gel column. Elution with hexanes/EtOAc 5:2 gave after evaporation of the combined desired fractions 1.68 g (81%) of hygroscopic white solid. R_f .54 (hexanes/EtOAc 5:2); $[\alpha]_D^{20}$+9.0 (c 2.0, MeOH); UV (ETOH): λ_{max}: 268.1 log ε 4.02 ^1H NMR (200 MHz, $CDCl_3$) δ : 8.68 (bd, 1H, C(S) NH), 7.22 (bs, 5H, ArH Phe), 5.54 (bs, 1H, NHBoc), 5.04 (m, 1H, CH Leu), 4.75 (m, 1H, CH_α Phe), 3.68 (s, 3H, OCH_3), 3.14 (dd, 1H, J=5.8 Hz, J=15.5 Hz, CH_β Phe), 3.04 (dd, 1H, J=7.8 Hz, J=15.5 Hz, CH_β, Phe), 1.74-1.56 (m, 3H, CH $CH_{2\beta}$ Leu), 1.40 (s, 9H, t-bu), .91 (d, 3H, J=3.8 Hz, $CH_{3\delta}$ Leu), .88 (d, 3H, J=4.0 Hz, $CH_{3\delta}$ Leu).
MS (70 ev) m/e: 408 (45, $M^{+\cdot}$), 352 (28, $M^{+\cdot}$ - isobutylene), 335 (34.7, $M^{+\cdot}$ - O-$C(CH_3)_3$), 296 (33.8), 235 (38.1), 16 (13.8), 120 (34.3), 91 (16.7), 57 (100).

O,N-bis-(Boc)-Tyr-Gly-Gly-OEt (88)

DCC (2.11 g, 10.5 mmol) was added with stirring to a solution of O,N-bis-(Boc)-Tyr-OH (4.0 g, 10.5 mmol) and HOBt (1.43 g, 10.5 mmol) in CH_2Cl_2 (25 mL) and THF (15 mL) at 0°C. After stirring for .5 h at 0°C, glycylglycine ethyl ester

hydrochloride (2.04 g, 10.4 mmol) and triethylamine (726 mg, 10.4 mmol) were added. The reaction mixture was stirred at 0°C for 18 h and 1 h at room temperature. The solvents were evaporated, the residue suspended in EtOAc and filtered. The filtrate was washed successively with citric acid (5%), $NaHCHO_3$ (5%) and H_2O. After drying ($MgSO_4$) and removal of EtOAc in vacuo a solid (5.19 g) was obtained which contained a minor impurity (R_f .12). Recrystallization from hexanes/EtOAc gave 4.3 (80%) of a white powder homogenous by TLC.
R_f .45 ($CHCl_3$/MeOH 9:1); mp 140-142°C; $[\alpha]_D^{20}$+7.65° (c 2.0, MeOH), ^1H NMR (60 MHz, $CDCl_3$) δ : 7.5-6.75 (m, 6H, ArH Tyr and 2C(O)NH, 5.3 (bd, 2H, J=7.0 Hz, CH_2-CH_3), 4.6-4.0 (m, 5H, CH_α Tyr, CH_2 Gly_2, CH_2 Gly_3), 3.15-3.0 (m, 2H, CH_β Tyr), 1.66-1.0 multiplet containing 2 singlets at 1.56 and 1.36, 21H, OBoc, NBoc, CH_2-CH_3) ppm.

O,N-bis-(Boc)-Tyr-Gly-Gly-PheC(S)-Leu-OCH_3 (90)

The Boc group of Boc-PheC(S)-Leu-OCH_3 (1.68 g, 4.1 mmol) was deprotected using formic acid (98%) (10 mL) for 2 h at room temperature. Evaporation in vacuo of the excess acid yielded the formate salt which was used without any purification. O,N-bis-(Boc)-Tyr-Gly-Gly-OH (1.78 g, 3.6 mmol) was mixed with HOBt (741 mg, 5.4 mmol) in CH_2Cl_2 (10 mL) and THF (5 mL). The solution was cooled to 0°C and DCC (740 mg, 3.6 mmol was added. After stirring at 0°C for 1 h, the formate salt of PheC(S)-Leu-

OCH_3 (1.23 g, 3.6 mmol) and triethylamine (364 mg, 3.6 mmol) were added and the resulting solution stirred at 0°C for 1 h and room temperature for 20 h. The solvents were evaporated, the residue taken up in EtOAc (50 mL) and filtered. The filtrate was washed successively with citric acid (5%), $NaHO_3$ (5%) and brine, dried ($MgSO_4$), filtered and evaporated. The solution is concentrated by removal of EtOAc in vacuo and addition of ether gave a white precipitate 1.88 g (66% yield) which contained a small contaminant R_f .64. Purification by flash chromatography on silica gel with EtOAc/hexanes (4:1) gave 1.63 g (58%) of a white powder.
mp 168-169°C decomp. Other physico-chemical characteristics are given in Table 16. ^1H NMR resonances are given in Table 17, and ^{13}C NMR resonances summarized in Table 18.

Boc-Gly-Phe-Leu-OCH_3 (92)

The formate salt of Phe-Leu-OCH_3 was obtained as described previously. To a solution of Boc-Gly-OH (1.33 g, 7.65 mmol) and HOBt (1.13 g, 8.4 mmol) in a mixture of CH_2Cl_2 (10 mL) and THF (5 mL) cooled to 0°C, DCC (1.73 g, 8.4 mmol) was added and the mixture stirred at 0°C for 12 h and 4 h at room temperature. After evaporation of the solvent in vacuo, suspension of the residue in EtOAc (50 mL) followed by filtration, the filtrate was washed successively with citric acid (5%), $NaHCO_3$ (5%) and brine. After drying ($MgSO_4$) and evaporation of the solvent in vacuo, the oily residue is crystallized from CH_2Cl_2/hexanes

to give 3.20 g (93%) of a pinkish powder homogeneous by TLC. R_f .40 ($CHCl_3$/MeOH 4:1); mp 104.5-106.5°C; $[\alpha]_D^{20}$-21.8° (c 1.0, $CHCl_3$) ^1H NMR (60 MHz, $CDCl_3$) δ : 7.21 (s, 5H, ArH Phe), 6.77 (d, 1H, J=8.0 Hz, C(O)NH), 6.95 (d, 1H, J=8.0 Hz, C(O)NH), 5.16 (t, 1H, J=6 Hz, NHBoc), 4.95-4.15 (multiplet containing a quartet at 4H, CH_α Phe, CH_α Leu, OCH_3), 3.82-3.69 (multiplet containing a singlet at 3.70, m, 5H, CH_2 Gly, OCH_3), 3.10 (d, 2H, J=7.0 Hz, $CH_{2\beta}$ Phe), 1.66-1.33 (multiplet containing a singlet at 1.12, 12H, t-bu and CH_β-$CH_{2\gamma}$ Leu), .89 (d, 6H, J=5.0 Hz, 2 $CH_{3\delta}$ Leu).

Boc-GlyC(S)-Phe-Leu-OCH_3 (93)

To Boc-Gly-Phe-Leu-OCH_3 (1.0 g, 2.2 mmol) in dry THF (15 mL) at 0°C was added thionation reagent 11 (754 mg, 1.32 mmol) and the solution was stirred under N_2 for 30 min. at 0°C and an additional 1 h after warming to room temperature. After evaporation of solvent the remaining oil was purified by flash chromatography on silica gel with EtOAc/hexanes 2:1 as eluent and yielded 835 mg (83%) of a colorless oil.

$[\alpha]_D^{20}$-38.8° (c 1.0, $CHCl_3$) UV (ETOH): λ_{max} 265.3 log ε 4.01; ^1H NMR (200 MHz, $CDCl_3$): 9.64 (bd, 1H, J=7.5 Hz, C(S)NH Phe), 7.38-7.1 (m, 5H, C(O)NH Leu, ArH Phe), 5.90 (bd, 1H, J=6.9 Hz), 5.14 (m, 1H, CH_α Phe), 4.46 (m, 1H, CH_α Leu), 4.16 (bd, 2H, J=5.8 Hz, CH_2 Gly) 3.68 (s, 3H, OCH_3), 3.36 (dd, 1H, J=6.6 Hz, J=13.8 Hz, CH_β Phe), 3.04 (dd, 1H, J=8.6 Hz, J=13.8 Hz, CH_β, Phe), 1.66-1.30 (multiplet containing a singlet at 1.40, 12H, CH_γ $CH_{2\beta}$ Leu and

t-bu), 0.88 (d, 6H, J=6.0 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ : 199.58 (C(S)), 172 (C(O)-O, 169 (C(O)NH), 155.9 (C(O)Boc), 135.8, 129.4, 128.2, 127.2 (C-Arom), 80.8 (O-C Boc), 59.25 (C_α Phe), 52.2 (C_α Gly, OCH_3), 51.2 (C_α Leu), 41.2 (C_β Leu), 36.9 (C_β Phe), 28.1 (CH_3 Boc), 24.6 (C_γ Leu), 22.6 and 21.8 (C_δ Leu) ppm.

O,N-(bis)-Boc-Tyr-Gly-OEt (94)

To O,N-bis-(Boc)-Tyr-OH (3.0 g, 7.8 mmol) and HOBt (1.11 g, 8.6 mmol) in CH_2Cl_2 (15 mL) and THF (5 mL) at 0°C was added DCC (1.77 g, 8.6 mmol). The resulting solution was stirred at 0°C for 1 h. Glycine ethyl ester hydrochloride (1.14 g, 8.2 mmol) was added and stirred for 2 h at 0°C and 4 h at room temperature. The solvents were evaporated, the residue was suspended in EtOAc (100 mL) and filtered. The filtrate was washed successively with citric acid (5%), $NaHCO_3$ (5%) and brine. Evaporation of EtOAc in vacuo gave an oil which was purified by flash chromatography on silica gel (EtOAc/hexanes 1:1) to give 3.17 g of a hygroscopic solid.

R_f .70 ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{23}$ -8.3 (c=1.0, DMF), litt [463] $[\alpha]_D^{23}$ -7.8° (c 1.0, DMF); ^1H NMR (60 MHz, $CDCl_3$), 7.33-6.92 (m, 4H, ArH Tyr), 6.72-6.33 (m, 4H, C(O)NH Gly), 5.1 (d, 1H, J=7.5 Hz, NHBoc), 4.42 (m, 1H, CH_α Tyr), 4.20 (q, 2H, CH_2-CH_3), 3.98 (d, 2H, J=5.6 Hz), 3.05 (d, 2H, CH_2 Tyr), 1.36-1.0 (multiplet. containing 2 singlets at 1.52 and at 1.40 and parts of a triplet, 21 H, O-Boc NHBoc and CH_2CH_3).

O,N-bis-(Boc)-Tyr-Gly-GlyC(S)-Phe-Leu-OCH_3 (95)

Boc-GlyC(S)-Phe-Leu-OCH_3 (900 mg, 1.94 mmol) was dissolved in formic acid (98%) (10 mL) and left standing at room temperature for 2 h. The excess acid was removed in vacuo to yield the formate salt which was used without any further purification. To a mixture of O,N-bis-(Boc)-Tyr-Gly-OH (850 mg, 1.9 mmol) and HOBt (288 mg, 2.1 mmol) in CH_2Cl_2 (10 mL) and THF (5 mL) at 0°C, was added DCC (433 mg, 2.1 mmol) and the mixture was stirred at 0°C for 1 h. After this time, the formate salt obtained previously dissolved in CH_2Cl_2 (5 mL) containing triethylamine (1.96 g, 19.4 mmol) was added. The mixture was stirred at 0°C for 2 h and at room temperature for 18 h. The solvents were evaporated, and the residue suspended in EtOAc (50 mL) and filtered. The filtrate was washed successively with citric acid (5%), $NaHCO_3$ (5%), and brine. The solvent was evaporated and the residue purified by flash chromatography ($CHCl_3$/MeOH 9:1) giving 1.0 g (75%) of a waxy solid.

Physico-chemical characteristics are given in Table 16. ^1H NMR resonances are summarized in Table 17 and the ^{13}C NMR resonances given in Table 18.

Boc-GlyC(S)-Gly-Phe-Leu-OCH_3 (99)

Boc-Gly-Gly-Phe-Leu-OCH_3 (462 mg, 0.91 mmol) was dissolved in dry THF (25 mL) and the temperature lowered to 0°C. To this solution, was added dropwise a solution of thionation reagent 11 (286 mg, 0.53 mmol) in 15 mL of dry THF. After stirring 3 h

at 0°C, the reaction mixture was allowed to warm to room temperature and stirred and an additional 1 h (no starting material by TLC). The solvent was evaporated, the residue dissolved in $CHCl_3$ (2 mL). Purification by silica gel chromatography EtOAc/hexanes (3:1) yielded an amorphous solid (373 mg, 80%).

R_f .49 ($CHCl_3$/MeOH 9:1); mp 134-136°C; $[\alpha]_D^{20}$-26.5° (c 1.0, MeOH), UV (ETOH): λ_{max} 263.7, log ε 3.99, ^1H NMR (200 MHz, $CDCl_3$ δ : 9.1 (bs, 1H, C(S)NH Gly_2), 9.4-7.1 (m, 7H, 5 ArH, Phe, 2 C(O)NH, Phe, Leu), 5.0-4.84 (m, 1H, NHBoc), 5.12-4.94 (m, 1H, CH_α Phe), 4.72-4.52 (m, 1H, CH_α Leu), 4.38 (dd, 1H, J=5.6 Hz, J=16 Hz, CH_2 Gly_3), 4.20-4.10 (multiplet containing part of dd and a doublet, 3H, J=5.6 Hz, CH Gly_3, J=5:1 Hz, CH_2 Gly_2), 3.71 (s, 3H, OCH_3), 3.10 (dd, 1H, J=5.37 Hz, J=15.2 Hz, CH_β Phe), 2.9 (dd, 1H, J=8.0 Hz, J=15.2 Hz, CH_β' Phe), 1.74-1.7 (multiplet containing a singlet at 1.47, 12H, CH_γ $CH_{2\beta}$ Leu O-Boc, CH_γ $CH_{2\beta}$ Leu), .89 (d, 6H, J=5.67, 2 $CH_{3\delta}$ Leu). MS (70 ev), m/e: 522 (25.2, $M^{+\cdot}$), 466 (50.0, $M^{+\cdot}$- isobutylene) 405 (22.6), 334 (41.9), 321 (68.9), 293 (78), 219 (52.3), 146 (37.2), 120 (100). Amino acid analysis: Gly 1.88, Phe .97, Leu 1.00; Elemental analysis: Calcd: C 57.45, H 7.33, N 10.7, S 6.12. Found: C 57.41, H 7.23, N 10.41, S 6.18

O,N-bis-(Boc)-Tyr-GlyC(S)-Gly-Phe-Leu-OCH_3 (100)

Boc-GlyC(S)-Gly-Phe-Leu-OCH_3 (273 mg, 0.54 mmol) was dissolved in formic acid (98%, 10 mL) and left standing at room temperature for 2 h. Excess formic acid was removed in vacuo to yield the formate salt. DCC (127 mg, 0.61 mmol) was added to a solution of O,N-bis-(Boc)-Tyr-OH (228 mg, 0.159 mmol) and HOBt (80 mg, 0.65 mmol) in CH_2Cl_2 (2 mL) and THF (1 mL) at 0°C and the resulting solution was stirred at 0°C for 5 h. After this time, the formate salt and triethylamine (54 mg, 0.54 mmol) were added and the resulting solution stirred at 0°C for 1 h and room temperature for 24 h. The solvents were evaporated, the residue suspended in EtOAc (20 mL). After filtration, the filtrate was washed successively with citric acid (5%) $NaCHO_3$ (5%) and brine. Evaporation of EtOAc, and purification by flash chromatography on silica gel with EtOAc/hexanes 3:1 gave 423 mg (81%) of a waxy solid.

Physico-chemical data are given in Table 16, ^1H NMR resonances are summarized in Table 17, ^{13}C NMR resonances are given in Table 18.

O,N-bis-(Boc)-Tyr-GlyC(S)-Gly-Phe-Leu-OCH_3 (100)
(thionation of the protected pentapeptide)

Thionation reagent 11 (60 mg, 0.11 mmol) was added to O,N-bis-(Boc)-Tyr-Gly-Gly-Phe-Leu-OCH_3 (150 mg, 0.19 mmol) in dry THF (10 mL) at 0°C. The reaction mixture was stirred at 0°C

for 1.5 h, after which time the solution was allowed to warm up to room temperature and was stirred an additional 1 h. Evaporation of the solvent followed by flash chromatography on silica gel using a gradient elution system (EtOAc/hexanes 3:1 → 5:1) yielded 112 mg (75%) of the waxy solid.
R_f .51 ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$-14.3° (c 1.95, $CHCl_3$), ^1H NMR 200 MHz) identical with sample prepared by the coupling reaction.

Deprotection of [Leu^5] enkephalin and thioamide analogues. Synthesis of 86, 91, 97, 101, 108

The O,N-bis-(Boc) pentapeptide ester (100 mg) was dissolved in a mixture of THF/H_2O (2:1, 3 mL) and 1N NaOH (0.15 mL) was added and the mixture was stirred under nitrogen at room temperature until disappearance of the starting material on TLC (1.5 - 3 h), except for analogue 106 where a 20 h period was required. A citric acid solution (5%, 10 mL) was then added to the reaction mixture and the solution extracted with EtOAc (2x20 mL), washed with H_2O (2x10 mL), dried ($MgSO_4$) and evaporated to yield a foamy solid in all cases. ^1H NMR ($CDCl_3$, 60 MHz) confirmed the disappearance of the methyl ester.

This solid was then dissolved in formic acid and allowed to stand at room temperature for 2 h. After removal of the excess formic acid in vacuo, the mixture was purified by semi-preparative HPLC using a μBondapak column (Waters Associates) with an isocratic elution using a mixture of MeOH/H_2O buffered with ammonium acetate 0.1M, ph 7. Relevant fractions were

collected and extensively lyophilized. Overall yields of 45-55% were obtained for 86, 91, 97 and 101 and 10-15% for 108. Analytical data for these deprotected enkephalins are given in Table 19 and the ^{1}H NMR (200 MHz, DMSO) resonances summarized in Table 20.

O,N-bis-(Boc)-Tyr-NHCH$_3$ (102)

DCC (1.74 g, 5.2 mmol) was added to a stirred solution of O,N-bis-(Boc)-Tyr-OH (2.0 g, 5.2 mmol) and HOBt (283 mg, 5.7 mmol) in CH_2Cl_2 (10 mL) and THF (5 mL) at 0°C. After stirring for 1 h at 0°C, methylamine hydrochloride (394 mg, 5.7 mmol) and triethylamine (570 mg, 5.7 mmol) were added and the mixture stirred at 0°C for 1 h and then at room temperature for 20 h. The solvent was evaporated and the residue suspended in EtOAc (100 mL). The organic layer was washed with citric acid (5%), $NaHCO_3$ (5%), brine, dried ($MgSO_4$) and the solvent removed in vacuo. The residue was crystallized from ether/hexanes affording 1.24 g (61%) of a white powder.

R_f .63 ($CHCl_3$/MeOH (9:1)); mp 130-132°C; $[\alpha]_D^{20}$+13.0 (c 2.0, $CHCl_3$); ^{1}H NMR (60 MHz, $CHCl_3$) δ : 7.33-7.0 (m, 4H, ArH), 5.82 (b, 1H, C(O)NH), 5.13 (bd, 1H, 8 Hz, NHBoc), 4.45-4.06 (m, 1H, CH_α), 3.06 (d, 2H, 7.0 Hz, $CH_{2\beta}$), 2.66 (d, 3H, 5.0 Hz, CH_3), 1.56 (s, 9H, NHBoc).

O,N-bis-(Boc)-TyrC(S)-$NHCH_3$ (103)

Thionation reagent 11 (1.15 g, 2.1 mmol) was added to a solution of O,N-bis-(Boc)-Tyr-C(=O)-$NHCH_3$ (1.4 g, 3.6 mmol) in dry THF (30 mL) and the mixture stirred at room temperature for 3.5 h. Solvent evaporation followed by flash chromatography on silica gel using hexanes/EtOAc (3:1) as solvent gave 1.27 g (85%) of a white solid.

R_f .26 (EtOAc/hexanes 5:2), R_f .81 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$+50.5 (c 1.0, $CHCl_3$); UV (EtOH): λ_{max} 264.4 log ε 4.11, ^{1}H NMR (200 MHz, $CDCl_3$): 8.66 (bs, 1H, C(S)NH), 7.2 (d, 2H, 8.3 Hz, ArH); 7.06 (d, 2H, 8.3 Hz, ArH), 5.72 (d, 1H, 8.7 Hz, NHBoc); 4.65 (m, 1H, CH), 3.14-2.84 (m, 5H, CH_2 , CH_3), 1.54 (s, 9H, OBoc), 1.35 (s, 9H, NHBoc); MS (70 eV) m/e: 410 (9.3, $M^{+\cdot}$), 354 (1.8), 293 (4.7), 193 (22.1), 107 (15.4), 57 (100).

O,N-bis-(Boc-TyrC(SCH_3)=$NHCH_3$·HI (104)

Methyl iodide (692 mg, 4.8 mmol) was added to a solution of the preceding O,N-bis-(Boc)-TyrC(S)-$NHCH_3$ (IV) in acetonitrile (10 mL) and the mixture heated to 35°C under N_2 for 24 hr. The solvent was evaporated in vacuo to give a foamy yellow solid which was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$) δ : 7.34 (d, 2H, 8.2 Hz, ArH), 7.1 (d, 2H, 8.2 Hz, ArH), 6.74 (d, 1H, 7.8 Hz, NHBoc), 4.15 (m, 1H, CH_α), 3.6-3.4 (m, 2H, CH_β), 3.29 (s, 3H, NCH_3), 2.84 (s, 3H, SCH_3), 1.55 (s, 9H, OBoc), 1.39 (s, 9H, NBoc).

O,N-bis-(Boc)-TyrC(S)-SCH_3 (105)

A THF solution saturated with H_2S at 0°C (20 mL) was added to the preceding S-methyl, N'-methyl imidate salt of the protected tyrosine (980 mg, 1.75 mmol). Following the addition of dry pyridine (692 mg, 8.7 mmol) the mixture was stirred at 0°C for 20 min. Citric acid (1.68 g, 8.7 mmol) was then added and excess H_2S was flushed out with a stream of N_2 (5 min) at 0°C. The solution was placed in a separatory funnel containing ether (25 mL) and washed with citric acid 5%, H_2O and with brine. After drying ($MgSO_4$), the solvent was removed in vacuo and the residue flash chromatographed on silica gel (EtOAc/hexane 5:2 as eluent) to yield a bright yellow solid 500 mg (67%) and 125 mg of thioamide precursor. The dithioester was recrystallized from hexanes. Additional recrystallization did not increase its mp or rotation.

R_f .56 (hexane/EtOAc 5:2) mp 109.5-111°C; $[\alpha]_D^{20}$ = + 60.5 (c, 1.0, $CDCl_3$), UV (EtOH) λ_{max}: 308.3 log ε 4.10; ^1H NMR (200 MHz, $CDCl_3$) δ : 7.17 (d, 2H, J=8.7 Hz, ArH), 7.07 (d, 2H, J=8.7 Hz), 5.40 (d, 1H, J=8.0 Hz, NHBoc), 5.25-5.05 (m, 1H, CH_α), 3.28-2.97 (m, 2H, $CH_{2\beta}$), 2.58 (s, 3H SCH_3), 1.55 (s, 9H, OBoc), 1.39 (s, 9H, NHBoc), MS (70 eV) m/e: 427 (3.5, $M^{+\cdot}$), 336 (21.0), 280 (9.4), 236 (10.8).

O,N-bis-(Boc)-D,L-TyrC(S)-Gly-Gly-Phe-Leu-OCH_3 (106)

Removal of the Boc group from 83 (284 mg, 0.56 mmol) was accomplished with formic acid 98% (5 mL). After standing

at room temperature for 2 h, the excess acid was removed in vacuo to yield the formate salt as an oil. It (83a) was dissolved in DMF (5 mL) and after addition of triethylamine (56 mg, 0.56 mmol) the mixture was stirred for 30 min. To this was added O,N-bis-(Boc)-TyrC(S)SCH_3 (200 mg, 0.46 mmol) and imidazole (33 mg, 0.46 mmol). After stirring for 13 h at room temperature, the solvent was evaporated, the residue suspended in EtOAc and the organic layer washed with citric acid (5%), $NaHCO_3$ (5%) and brine. The organic layer was dried ($MgSO_4$), the solvent removed in vacuo and the residue purified by flash chromatography on silica gel using a solvent gradient system (EtOAc/hexane (2:1 → 5:1)) to give 328 mg (89%) of product. Analytical HPLC (silica column, 3 mL/min, EtOAc/hexane 3:1) of this latter material showed 2 peaks of equal intensity with retention times of 13.7 and 14.3 min, respectively.

Mixture 106 (D,L Tyr): $[\alpha]_D^{20}$ - 20.0° (c 1.0, $CDCl_3$). Other physico-chemical data are given in Table 16, ^{13}C NMR ($CDCl_3$) are summarized in Table 18.

a) Least polar 106a (L-Tyr): $[\alpha]_D^{20}$ +1.68° (c 1.25, $CHCl_3$) ^1H NMR (200 MHz, $CDCl_3$) δ : 9.90 (b, 1H, C(S)NH Gly_2), 7.36-6.90 (m, 12H, ArH Phe, ArH Tyr, 3 C(O)NH Phe, Gly_3, Leu), 5.44 (b, 1H, NHBoc), 4.88 (m, 1H, CH_α Tyr), 4.70 (m, 1H, CH_α Phe), 4.58 (m, 1H, CH_α Leu), 4.25 (bs, 2H, CH_2 Gly_2), 4.01 (dd, 1H, J=5.3 Hz, J=17 Hz, CH_α Gly_3), 3.80-3.70 (multiplet 3H, CH_α Gly_3, OCH_3) 3.30-2.90 (m, 4H, CH_α Tyr, CH_α Phe), 1.86-1.46 (multiplet containing a singlet at 1.44, 12H, NHBoc, $CH_\gamma CH_{2\beta}$ Leu), 1.34 (s, 9H, O-Boc), .88 (d, 6H, J=6 Hz, $CH_{3\delta}$ Leu) ppm.

b) Most polar 106b (D-Tyr): $[\alpha]_D^{20}$ -35.6° (c 1.15, $CDCl_3$) ^1H NMR (200 MHz, $CDCl_3$) δ : 9.92 (bs, 1H, C(S)NH), 7.44-6.98 (m, 12H, ArH Phe, ArH Tyr, 3 C(O)NH, Gly_3, Phe, Leu), 5.42 (b, 1H, NHBoc), 4.88 (m, 1H, CH_α Tyr), 4.76 (m, 1H, CH_α Phe), 4.58 (m, 1H, CH_α Leu), 4.38 (dd, 1H, J=5.15 Hz, J=17.7 Hz, CH_α Gly_2), 4.11 (dd, 1H, J=4.06, J=17.7 Hz, CH_α, Gly_3), 3.32-2.90 (m, 4H, CH_β Tyr, CH_β Phe), 1.70-1.46 (multiplet containing a singlet at 1.48, 12H, NHBoc, CH_γ $CH_{2\beta}$ Leu), 1.36 (s, 9H, OBoc), 0.88 (bd, 6H, 2 $CH_{3\delta}$ Leu) ppm.

O,N-bis-(Boc)-TyrC(S)-Gly-Gly-OEt (107)

To a solution of O,N-bis-(Boc)-TyrC(S)-SCH_3 (600 mg, 1.4 mmol) in dry THF was added ethyl glycylglycinate hydrochloride (412 mg, 2.1 mmol) followed by triethylamine (212 mg, 2.1 mmol) and imidazole (95 mg, 1.4 mmol). The reaction mixture was stirred under N_2 for 4.5 h. The solvent was evaporated, the residue dissolved in EtOAc and washed with citric acid (5%) and H_2O. After evaporation of the solvent, the crude product was purified by flash chromatography on silica gel (EtOAc/hexanes 1:1) giving 680 mg (90% yield) of a white solid.

R_f .38 (EtOAc/hexanes 1:1); $[\alpha]_D^{20}$=0.0° (c 1.0, $CHCl_3$) UV (ETOH): λ_{max} 267.4 log ε 4.02, ^1H NMR (200 MHz, $CDCl_3$) δ : 8.8 (bs, 1H, C(S)NH), 7.17 (d, 2H, J=8.5 Hz, ArH Tyr), 7.17-7.00 (multiplet containing a doublet J=8.6 Hz, ArH Tyr, C(O)NH Gly_2), 5.45-5.55 (bs, 1H, NHBoc), 4.85-4.75 (m, 1H, CH_α Tyr), 4.4-4.1 (multiplet

containing a quadruplet J=7.2 Hz, 4H, $C\underline{H}_2$-CH_3, $C\underline{H}_2$ Gly_2), 3.94 (bs, 2H, CH_2 Gly_3), 3.18 (dd, 1H, J=6.0 Hz, J=14.0 Hz, CH_β Tyr), 3.0 (dd, 1H, J=5.24, J=14.0 Hz, CH_β, Tyr), 1.49 (s, 9H, OBoc), 1.31 (s, 9H, NBoc) ppm. MS (70 ev) m/e: 439 (5.4, $M^{+\cdot}$ - C(O)-O-$C(CH_3)_3$), 322 (100, $M^{+\cdot}$ - OBoc - C(O)-O-$C(CH_3)_3$), 289 (59.8), 215 (18.7), 136 (25.0), 107 (30.2), 57 (79.8).

O,N-bis-(Boc)-TyrC(S)-Gly-Gly-OH (107b)

O,N-bis-(Boc)-TyrC(S)-Gly-Gly-OEt (500 mg, 0.92 mmol) was dissolved in a mixture of THF/H_2O (2:1, 4 mL). A solution of 1N NaOH (9.1 mL) was then added and the reaction mixture was stirred under N_2 for 50 min. The mixture was diluted with EtOAc (50 mL) and washed with citric acid (5%, 2x25 mL) and brine. The organic layer was dried ($MgSO_4$) and evaporated to yield 423 mg (85% yield) of a foamy solid.
^1H NMR (60 MHz, $CDCl_3$): 9.14 (b, 1H, C(S)N$\underline{H}$), 8.80 (b, 2H, C(O)N$\underline{H}$, $CO_2\underline{H}$), 7.22 (d, 2H, J=8.2 Hz, ArH Tyr), 7.04 (d, 2H, J=8.2 Hz, ArH), 5.60 (b, 1H, N$\underline{H}$Boc), 4.72 (b, 1H, CH_α Tyr), 4.34-4.18 (m, 2H, $C\underline{H}_2$ Gly_2), 4.06-3.96 (m, 2H, $C\underline{H}_2$ Gly_3), 3.28-2.74 (m, 2H, CH_β Tyr), 1.56 (s, 9H, OBoc), 1.24 (bs, 9H, NHBoc) ppm.

O,N-bis-(Boc)-Tyr-GlyC(S)-GlyC(S)-OEt (109)

O,N-bis-(Boc)-Tyr-Gly-Gly-OET (500 mg, 0.95 mmol) was dissolved in THF (15 mL) and the thionation reagent 11 (606 mg, 1.15 mmol) was added. After stirring overnight, the solvent

was evaporated and the residue purified by flash chromatography on silica gel with hexanes/EtOAc 3:2. Evaporation of the fraction gave 369 mg (74% yield) of a pale yellow oil.
R_f .44 (hexanes/EtOAc 3:2); UV (ETOH) λ_{max} 267.5 log ε 4.25
^1H NMR (200 MHz, $CDCl_3$) δ : 8.80 (bt, 1H, C(S)NH), 8.42 (bt, 1H, C(S)NH), 7.14 (d, 2H, J=8.4 Hz, ArH), 7.00 (d, 2H, J=8.4 Hz, ArH), 5.21 (bd, 1H, NHBoc), 4.62-4.43 (m, 3H, CH_α Tyr, CH_2 Gly_2), 4.42-4.19 (m, 2H, CH_2 Gly_3), 4.18-4.09 (q, 2H, J=7.1 Hz, CH_2-CH_3), 3.15 (dd, 1H, J=6.0 Hz, J=15.0 Hz, CH_β Tyr), 3.0 (dd, 1H, J=7.5 Hz, J=15.0 Hz, CH_β, Tyr), 1.45 (s, 9H, O-Boc), 1.29 (s, 9H, NHBoc), 1.17 (t, 3H, J=7.1 Hz, CH_2-CH_3) ppm.

Attempts to prepare O,N-bis-(Boc)-Tyr-GlyC(S)-Gly-OEt from oxidative desulfuration of 109.

To the dithiotripeptide 109 (87 mg, 0.16 mmol) dissolved in CH_2Cl_2 (3 mL) at 0°C was added a solution of meta-chloroperbenzoic acid (33 mg, 0.19 mmol) in CH_2Cl_2 (5 mL). All starting material was consumed after 5 min following the addition. Several spots appeared on TLC. One component (R_f .37 hexanes/EtOAc 3:2) was purified by flash chromatography on silica gel to yield a pale yellow oil (74% yield) which decomposed rapidly (turning bright yellow) when exposed to air at room temperature.
^1H NMR (200 MHz, $CDCl_3$) δ : 7.24-7.0 (m, 1H, ArH), 6.80 (s, 1H, NH from Gly_2), 5.27-5.00 (m, 2H, NHBoc, NH Gly_3), 4.43-4.09 (multiplet containing a quadruplet, 3H, J=7.1 Hz, CH_2-CH_3, CH_α Tyr),

3.78 (bs, 2H, CH_2 Gly_3, sharpens with addition of D_2O), 3.32-3.06 (m, 2H, $CH_{2\beta}$ Tyr), 1.51 (s, 9H, NHBoc), 1.37 (s, 9H, O-Boc), 1.27 (t, 3H, J=7.1 Hz, CH_2-CH_3) ppm.

Chapter 4

Boc-L-leucineamide (115)

To L-leucineamide hydrochloride (Sigma) (1.50 g, 9.0 mmol) dissolved in H_2O/dioxane (1:2, 15 mL) was added di-t-butyl-dicarbonate (Aldrich) (2.22 g, 9.9 mmol), followed by triethylamine (2.26 g, 22.5 mmol). The mixture was stirred at room temperature for 3 h. Most of the solvent was evaporated in vacuo and the residue taken in ether (100 mL), washed with citric acid (5%, 2x25 mL), brine (2x25 mL), dried ($MgSO_4$) and evaporated in vacuo. Recrystallization of the resulting solid in ether/hexane gave 1.49 g (72% yield) of a white powder. mp 144-146°C, $[\alpha]_D^{20}$-9.7° (c 1.0, MeOH); IR (KBr) ν_{max}: 3400 (NH), 3380 (NH), 3200, 2960, 1680 (C(O)Boc), 1650 (C(O)NH), 1540 cm^{-1} ^1H NMR (60 MHz, $CDCl_3$) δ :6.49-6.19 (br, 1H, C(O)NH), 6.00-5.61 (br, 1H, C(O)NH), 5.09 (br d, 1H, J=7.5 Hz, NHBoc), 4.29-4.0 (m, 1H, CH_α), 2.79-2.46 multiplet containing a singlet at 1.46, 12H, $CH_{2\beta}$ CH_γ, t-bu), 0.92 (d, 6H, J=5.6 Hz, 2 $CH_{3\delta}$ Leu) ppm.

Boc-L-leucinethioamide (116)

Boc-L-Leucineamide (115) (1.40 g, 6.08 mmol) was dissolved in dry THF (40 mL) followed by the addition of the thionation reagent 11 (1.92 g, 3.6 mmol) and left stirring for 40 min. After evaporation of the solvent, flash chromatography on silica gel using $CHCl_3$/EtOAc (3:1) as eluent gave 1.3 g (87 yield) of a colorless oil.

R_f .69 (EtOAc/hexane 2:1), $[\ ]_D^{20}$= -51.2° (c 2.5, $CHCl_3$), ^1H NMR (60 MHz, $CDCl_3$) δ : 8.79-8.46 (br, 1H, C(S)NH), 8.39-8.16 (br, 1H, C(S)NH), 5.52 (bd, 1H, NHBoc), 4.79-4.36 (m, 1H, CH_α), 1.92-1.39 (multiplet containing a singlet at 1.42, 12H, $CH_{2\beta}$ CH_γ and t-bu), 0.95 (d, 6H, $CH_{3\delta}$) ppm. MS (70 ev) m/e: 246 (10, $M^{+\cdot}$), 190 (119, $M^{+\cdot}$ - isobutylene), 186 ($M^{+\cdot}$ - $C(S)NH_2$), 154 (20), 134 (571), 119 (663), 91 (309), 86 (282).

Boc-LeuC(NHOH)-NH_2 (117)

Boc-L-leucinethioamide (115) (1.19 g, 4.9 mmol) was dissolved in $CHCl_3$ (15 mL) and hydroxylamine hydrochloride (386 mg, 5.6 mmol) followed by triethylamine (561 mg, 56 mmol) were added. The mixture was stirred at room temperature for 20 h. The reaction mixture was evaporated to dryness, taken up in ether and filtered. After evaporation of the solvent *in vacuo*, the residue was purified by flash chromatography on silica gel with a gradient elution ($CHCl_3$/EtOAc 3:1 → 1:1). The amidoxime was obtained as a pale yellow oil (745 mg, 62% yield). The other component was identified as the corresponding nitrile

a) amidoxime 117

R_f .17 $CHCl_3$/EtOAc 1:3; mp 56-58°C; $[\alpha]_D^{20}$-63.3° (c 0.75, $CHCl_3$); IR ($CHCl_3$) ν_{max}: 3600 (OH), 3500, 3440, 3400-3100, 3020, 2880, 1720 (C(O)), 1670, 1580, 1520. ^1H NMR (60 MHz, $CDCl_3$) δ : 7.56 (br, 1H, OH), 7.52 (bd, 1H, NHBoc), 4.96 (br, 2H, NH_2), 4.39-3.92 (m, 1H, CH_α), 1.69-1.39 (multiplet containing a

singlet at 1.46, 12H, CH_β $CH_{2\gamma}$, t-bu), 1.92 (d, 6H, J=5.6 Hz, $2CH_{3\delta}$) ppm. Resonances at 7.56, 5.42 and 4.96 disappeared with the addition of D_2O. MS (70 ev) m/e: 245 (.2, $M^{+\cdot}$), 189 (17.8, $M^{+\cdot}$ - isobutylene - OH), 133 (94.6), 116 (48.2), 57 (100).

Elemental analysis Calcd: C 53.85 H 9.45 N 17.12

Found: C 53.84 H 9.72 N 16.97

b) nitrile 117b

R_f .74 ($CHCl_3$/EtOAc, 1:1), IR ($CHCl_3$) ν_{max}: 3420 (NHBoc), 2280, 2260, 1730, 1500. ^1H NMR (60 MHz, $CDCl_3$) δ : 5.0-4.33 (m, 2H, NHBoc, CH_α), 2.12-1.10 (multiplet containing a singlet at 1.50, 12H, CH_β $CH_{2\gamma}$, t-bu), 0.98 (d, 6H, $2CH_{3\delta}$) ppm.

L-Leucineamidoxime hydrochloride (118)

N-Boc-leucineamidoxime (600 mg, 2.44 mmol) was added to a HCl solution in ether (2N, 10 mL). After 3 h, the crystals were filtered and washed with small portions of anhydrous ether, and dried in vacuo yielding 452 mg (93%) of a hygroscopic white solid.

$[\alpha]_D^{20}$+21.9 (c 1.0, H_2O), ^1H NMR (200 MH_z, D_2O) δ : 4.15 (dd, 1H, J=6.45 Hz, J=6.56 Hz, CH), 2.04-1.74 (m, 2H, CH2), 1.70-1.40 (m, 1H, CH), 0.96 (d, 6H, J=6 Hz, $2CH_{3\delta}$) ppm. MS (70 ev) m/e: 146 (3.9, $M^{+\cdot}$+1), 145 (1.5, $M^{+\cdot}$), 129 (1.6), 88 (51.8), 89 (32.3), 86 (86).

Elemental analysis: Calcd for $C_6H_{17}N_3Cl_2$: C 33.03 H 7.8 N 19.2 Cl 32.49, Found C 35.52 H 8.95 N 19.62 Cl 32.49.

Boc-PheC(NHOH)-$NHCH_3$ (119a)

Hydroxylamine hydrochloride (22 mg, 0.32 mmol) and triethylamine (64 mg, 0.64 mmol) were added to the thioimidate of Boc Phe (49) (140 mg, 0.32 mmol) and the mixture was stirred under a N_2 atmosphere for 24 h. The reaction mixture was filtered and the filtrate evaporated in vacuo. Flash chromatography (EtOAc/hexanes 3:1) gave 81 mg (88% yield) of a white powder.

mp 115-116°C, $[\alpha]_D^{20}$+49.5 (c 1.0, $CHCl_3$), IR (KBr) ν_{max}: 3440, 3420, 1670, 1650; ^1H NMR (200 MHz, $CDCl_3$) δ: 7.4-7.1 (s, 5H, ArH), 5.60 (bd, 1H, NH), 5.26-4.54 (m, 3H, CH_α, NH), 3.20-2.84 (m, 2H, $CH_{2\beta}$), 2.80 and 2.74 (2d, 3H, ratio 2:3, J=5.3 Hz, J=5.0 Hz, N-CH_3) ppm. MS (70 ev) m/e: 293 (4.3, $M^{+\cdot}$), 237 (11.3, $M^{+\cdot}$ - isolutylene), 220 (9.6), 177 (10.3), 164 (5.6), 120 (17).

Boc-PheC(NHOH)-$NHCH_3$ (119b)

To Boc-PheC(S)-$NHCH_3$ (150 mg, 0.51 mmol) dissolved in dry THF (5 mL) was added hydroxylamine hydrochloride (42 mg, 0.61 mmol) and TEA (154 mg, 7.05 mmol). After 1 h at room temperature no reaction occurred as indicated by TLC analysis. Mercuric acetate (195 mg, 0.61 mmol) was then added and the mixture stirred overnight at room temperature under N_2 atmosphere. The reaction mixture was diluted with EtOAc (10 mL) and filtered through celite. The solvents were removed in vacuo and the residue dissolved in EtOAc (20 mL), washed with citric acid (5% solution) and brine. The solution was dried ($MgSO_4$) to

give 150 mg (75%) of the amidoxime derivative. This compound had the same characteristics (including the E/Z ratio) as previously described in 119a.

Cyclic amidoxime 121

Boc-LeuC(S)-Phe-OCH_3 (67) (100 mg, 0.24 mmol) was dissolved in THF (5 mL) and hydroxylamine hydrochloride (20 mg, 0.29 mol) and triethylamine (73 mg, 0.73 mmol) and mercuric acetate (94 mg, 0.29 mmol) were added in that order. After stirring under N_2 for 2 h, the reaction mixture is diluted with EtOAc (10 mL) and filtered through celite to yield 90 mg of a crude solid. Purification by flash chromatography on silica gel (EtOAc/hexanes 2:3) yielded two products

least polar 67 : (3 mg, 3% yield) : R_f .68 (EtOAc/hexanes 1:1), ^1H NMR (200 MHz, $CDCl_3$) δ : 8.14 (bd, 1H, NH Phe), 7.34-7.0 (m, 5H, ArH), 5.4 (m, 1H, CH_α Leu), 5.0 (br, 1H, NHBoc), 4.32 (m, 1H, CH_α Phe), 3.73 (s, 3H, OCH_3), 3.40 (dd, 1H, CH_β Phe), 3.20 (dd, 1H, CH_β' Phe), 1.78-1.11 (multiplet containing a singlet at 1.39, CH_γ $CH_{2\beta}$ Leu, t-bu), 0.90 (d, 6H, $2CH_{3\delta}$) ppm.

most polar 121: 44 mg (44% yield): R_f .57 (EtOAc/hexanes 1:1) ^1H NMR (200 MHz, $CDCl_3$) δ : 7.41-7.0 (m, 5H, ArH), 5.96 (b, 1H, NH Phe), 4.81 (bd, 1H, J=5 Hz, NHBoc), 4.26-3.98 (m, 2H, CH_α Phe, CH_α Leu), 3.26 (dd, 1H, CH_β Phe), 2.91 (dd, 1H, CH_β' Phe), 1.77-1.43 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.34 (s, 9H, t-bu), 0.84 (m, 6H, $2CH_{3\delta}$ Leu) ppm. MS (70 ev) m/e: 319 (13, $M^{+\cdot}$ - isobutylene),

279 (27), 259 (44, $M^{+\cdot}$ - NHBoc), 241 (62), 189 (15, $M^{+\cdot}$ - Boc-Leu), 186 (41, Boc-$Leu^{+\cdot}$), 129 (500), 120 (229), 91 (283), 86 (1000).

Cyclic amidrazide 123

Boc-LeuC(S)-Phe-OCH_3 (67) (200 mg, 0.5 mmol) was dissolved in dry THF (10 mL) followed by the addition of hydrazine hydrochloride (62 mg, 0.52 mmol), triethylamine (168 mg, 1.7 mmol) and mercuric acetate (171 mg, 0.51 mmol) and the mixture stirred at room temperature for 2 h. After filtration through celite and evaporation of the solvent, the residue was purified by flash chromatography on silica gel to give two products.

least polar 62 : 21 mg (10% yield) R_f .34 (EtOAc/hexanes 5:2), IR (KBr) ν_{max}: 3440, 3400, 2800, 1740, 1680, 1660, 1530 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$) δ : 7.34-7.06 (m, 5H, ArH), 6.52 (bd, 1H, NH Phe), 4.92-4.74 (m, 2H, CH_α Phe, NHBoc), 4.06 (m, 1H, CH_α Leu), 3.70 (s, 3H, OCH_3), 3.22-3.0 (m, 2H, CH_β Phe), 1.76-1.26 (m, 3H, $CH_{2\beta}$ CH_γ Leu), 1.42 (s, 9H, t-bu), 0.92 (m, 6H, $2CH_{3\delta}$ Leu). MS (70 ev, 250°C) m/e: 392 (.5, $M^{+\cdot}$-15), 336 (8.3), 319 (9.28), 305 (1.0, $M^{+\cdot}$ - C(O)-$OC(CH_3)_3$), 259 (6.33), 186 (11.4), 130 (100), 120 (28.7).

most polar 123: 110 mg (55% yield) R_f .18 (EtOAc/hexanes), IR (KBr) ν_{max}: 3400-3150 (br, NH), 2880, 1720, 1680, 1640, 1500 cm^{-1}, ^1H NMR (200 MHz, $CDCl_3$) δ : 8.65 (br, 1H, NH Phe), 7.38-7.14 (m, 5H, ArH), 5.42 (br, 1H, NH-NC(O)), 5.1 (bd, 1H, J=5 Hz, NHBoc), 4.28-4.00 (m, 2H, CH_α Phe, CH_α Leu), 3.22 (m, 1H, CH_β Phe), 2.86 (m, 1H, CH_β Phe), 2.74-2.20 (multiplet

containing a singlet at 1.40, $CH_{2\beta}$ CH_{γ} Leu), 0.88 (2d, 6H, $2CH_{3\delta}$ Leu) ppm. MS (70 ev) m/e: 374 (15.6, $M^{+\cdot}$), 318 (8, $M^{+\cdot}$ - isobutylene), 301 (15.1, $M^{+\cdot}$ - O-t-bu), 283 (10.8), 258 ($M^{+\cdot}$ - NHBoc), 262 (8.3), 227 (87.3), 183 (44.5), 166 (100).

ENZYME ASSAYS

A) General

α-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas (3X recrystallized) and leucine aminopeptidase (E.C. 3.4.11.1) from porcine kidney (chromatographically purified, suspension in .75 M saturated ammonium sulfate, 0.1 M TRIS, 0.0005 M $MgCl_2$, pH 8) were purchased from Sigma. L-leucine p-nitroanilide was purchased from Sigma and N-Cbz-L-Tyr-p-nitrophenylester was prepared as described by Martin et al[494].

Spectrophotometric assays were performed on a Varian Cary 210 interfaced with an Apple II plus microcomputer. Thermostated cuvettes of either 1.35 mL, or 2.5 mL were used. All enzymes runs were blanked with the corresponding buffer solution.

B) Assay with α-Chymotrypsin

Stock solutions of α-chymotrypsin (4×10^{-6} M) were prepared by dissolving the enzyme in a THAM·Hcl buffer (0.02 M) at pH 7.9 containing KCl (0.2 M) and maintained at 0°C until used. The thiocarbonyls 30, 40, and 57 were initially dissolved in acetonitrile (1 mL). Aliquots (100 µl) of these solutions were added to the enzyme solutions to bring the final concentration of these derivatives to $\simeq 1 \times 10^{-4}$ M. These experiments were done in thermostated cells (quartz 2.5 mL) at 25.0 ±.2°C.

Hydrolysis of these thiocarbonyls substrates was monitored at the following wavelength λ:

270	thioamide	30
253	thionester	40
310	dithioester	57

Under these conditions no change in absorbance was seen when the assay period was extended to 3 h. Similar incubation of Cbz-L-Tyr-p-nitrophenyl ester (1×10^{-4} M) resulted in complete hydrolysis within seconds as monitored by an increase in absorbance at 400 nm which is proportional to the formation of the p-nitrophenolate anion.

Inhibition studies using Cbz-L-Tyr-p-nitrophenylester as substrate and the thionester as inhibitor were attempted, but hydrolysis of the substrate was too rapid and thus did not give reproducible results.

C) Assays with Leucine Aminopeptidase (LAP)

The leucine aminopeptidase solutions were prepared by dissolving 2.5 mL of the commercially available suspension of leucine aminopeptidase (Sigma) in 0.05 M Tris buffer (100 mL) containg 5 mM $MgCl_2$ at pH 8.5. This solution was kept at 4°C overnight to allow full activation of the enzyme by $MgCl_2$. Different batches of the commercial enzyme were used for the study of the thioamide and amidoxime derivatives.

Both thioamide 43 and amidoxime 118 derivatives of L-leucine were initially dissolved in acetonitrile and added to the ~~enzyme~~ solutions.

To evaluate the behavior of the thioamide compound 43 as a substrate, a 100 µL aliquot (final concentration ≈ 1×10^{-4} M) of 43 was added to 2.5 mL of the enzyme buffer solution in a 1 cm quartz cuvette and allowed to incubate at 30 °C for 1 h. The reaction was monitored spectrophotometrically at 288 nm; no significant change in absorbance was detected in these conditions.

Inhibition studies were performed using L-leucine p-nitroanilide as substrate. The rate of enzyme-catalysed hydrolysis was measured spectrophotometrically by recording the rate of p-nitroanilide formation at λ=405 nm. A typical assay for the studies comprised the incubation of 100 µL of inhibitor (43), and 2.5 mL of the enzyme-buffer solution at 30°C for 20 min in a 1 cm cuvette followed by addition of 10 µL of L-leucine p-nitroanilide. The velocity was derived in units of absorbance per minute for the period of 4-10 min to ensure linearity. In all the runs, no more than 10% of the substrate was hydrolyzed. The reaction was followed for several different substrate concentrations at each of the three inhibitor concentration. The data are summarized in Table 26 and 27 for the thioamide 43 and amidoxime 118 respectively. Reciprocal plots according to Lineweaver-Burk were constructed for both compounds and are illustrated in the text. Plots of $K_{m_{app}}$ vs inhibitor concentration were made and are also shown in the text. The K_i values were estimated from these plots.

[S] x 10^3	[I] x 10^3	v (Δa/min)	1/[S]	1/v	K_m x 10^3
5.36	-	.082	1865	12.2	
6.7	-	.098	1492	10.2	
10.0	-	.128	1000	7.8	
13.4	-	.174	746	5.7	
16.75	-	.200	611	5.0	3.5
5.16	2.11	.047	1937	21.27	
6.45	2.11	.055	1550	18.18	
9.37	2.11	.089	1034	11.23	
12.9	2.11	.112	775	8.92	
16.1	2.11	.140	621	7.14	8.9
3.11	4.08	.021	3215	50.1	
6.22	4.08	.038	1510	23.5	
9.32	4.08	.060	1073	16.7	
12.44	4.08	.078	806	12.8	
15.0	4.08	.101	645	10.0	15.0
4.8	5.90	.026	2083	38.5	
9.0	5.90	.044	1111	22.7	
12.0	5.90	.056	833	17.8	
15.0	5.90	.070	666	14.2	20.0

Table 27 Velocities (Abs/min) and K_m (M) of LAP-catalyzed hydrolysis of L-leucine p-nitroanilide in the presence of fixed concentration of L-leucine amodoxime (118).

[S] x 10^3	[I] x 10^3	v (Δa/min)	1/[S]	1/v	K_m
0.65	-	.073	1504	13.63	
1.32	-	.119	758	8.40	
1.98	-	.155	505	6.45	
2.30	-	.167	434	5.99	
2.63	-	.183	380	5.44	
4.54	-	.225	220	4.43	2.57
0.64	1.94	.061	1564	16.10	
1.27	1.94	.113	785	8.85	
1.90	1.94	.147	525	6.76	
2.50	1.94	.167	396	5.98	
3.15	1.94	.498	317	5.18	
4.98	1.94	.233	200	4.30	2.79
1.28	3.72	.098	815	10.22	
1.83	3.72	.135	546	7.39	
2.44	3.72	.162	411	6.17	
3.03	3.72	.180	330	5.55	
4.80	3.72	.229	208	4.37	3.50
1.13	7.20	.080	882	12.50	
1.69	7.20	.108	590	9.20	
2.20	7.20	.137	444	7.31	
2.80	7.20	.157	356	6.42	
4.41	7.20	.189	225	5.31	4.00

Table 26 Velocities (Abs/min) and K_m (M) values of LAP-catalyzed hydrolysis of L-leucine p-nitroanilide in the presence of fixed concentration of N'-methyl L-leucinethioamide (43).

LYSOZYME RELEASE ASSAY [495]

Blood from healthy human donors was drawn by venipuncture into 10 mL vacutainer tubes with 143 U.S.P. units sodium heparin and was used within 2 h of collection. The blood in 3-3.5 mL aliquots was layered over 3 mL of Mono-Poly Resolving Medium (Flow Laboratories, Virginia) in 16 x 102 mm posyallomer tubes (Beakman). The tubes were then centrifuged at 300 x g for 30 min in a swinging bucket rotor at room temperature (15-30°C). From this point on, all manipulations were carried on at 4°C unless specified otherwise. The PMN layer was drawn off with a pasteur pipette and washed once in Gey's solution with .2% bovine serum albumin (Gey's BSA). This was centrifuged at 275 x g for 10 min and resuspended for 30 min in erythrocyte lysing solution (8.29 g/L NH_4Cl, .37 g/L Na_2EDTA, 1g/L $KHCO_3$), centrifuged for 10 min, and washed twice with Gey's BSA. The cells were resuspended with Gey's BSA to 10^7/mL and used within 1 h. The cell suspension was preincubated with cytochalasin B (5 µg/mL (Sigma Chem. Co., St. Louis, Mo.) at 37°C for 5 min. Reaction mixtures contained 100 µL of the treated cell suspension (treated cells) and 100 µL of the appropriate compounds, in sterile 5 mL polypropylene tubes (Sarstedt, St. Laurent, Quebec). Controls were run daily containing treated cells and Gey's BSA. Total lysozyme release was obtained by incubating treated cells with 1% Triton X-100 (Calbiochem, La Jolla, Ca.) in .067 M phosphate buffer pH 6.25.

ysozyme was measured in supernatants of reaction mixtures after 15 min. of incubation at 37°C.

The assays for lysozyme were conducted at room temperature and all runs were performed 3 times in duplicate. The substrate used was freeze-dried Micrococcus Lysodeikticus (Sigma Chem. Co., St. Louis, Mo.), which was diluted in .067 M phosphate buffer pH 6.25 (10 mg/100 mL) to give an $A_{450}nm$ between .6 and .7 against a buffer reference. The solution was discarded after use. The reaction mixture consisted of:

.9 mL substrate
.05 mL 1% Triton X-100
.05 mL enzyme supernatant/standard

in a 1.5 mL quartz cuvette with a light path of 1 cm. The cuvette was stoppered and inverted 2-3 times and the change in absorbance was read immediately for 3 min. Three concentrations of egg white lysozyme standard solution (Sigma Chem. Co., St. Louis, Mo.) were run daily to ensure that the standard curve did not vary greatly from day to day.

The lysozyme-inducing activity for each peptide was obtained from the dose-response curve as its ED_{50}, the molar concentration of peptide causing 50% of the maximal release of lysozyme. In order to correct for changes in the responsiveness of different cells, the dose response curve of the standard peptide, f-MLP-OCH_3 (64) was also measured so that the activity or potency relative to the standard could be also calculated.

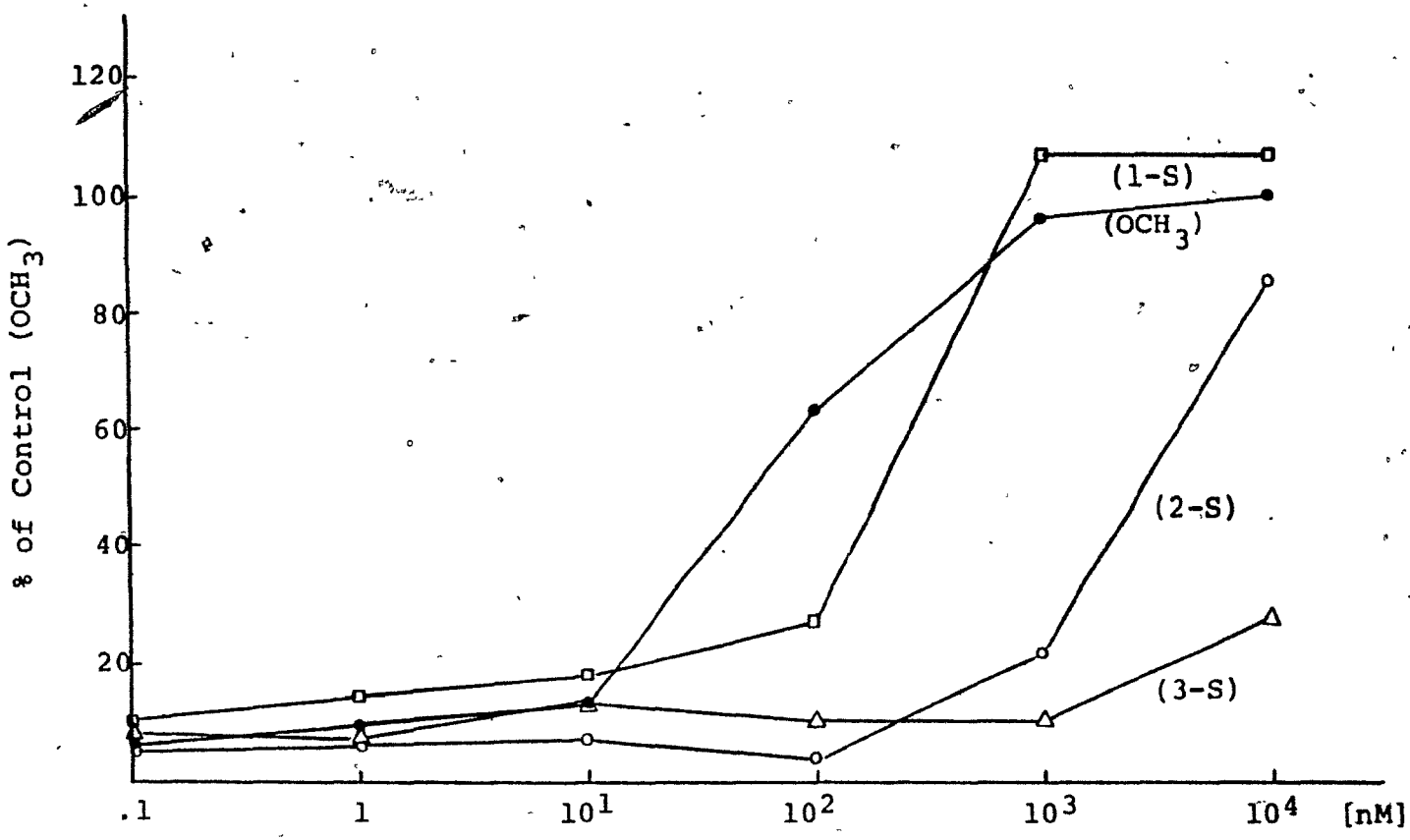


Figure 75 Dose response curve for the release of lysozyme by f-MLP-OCH_3 and thioamide analogues: OCH_3=64, 1-S=66, 2-S=74, 3-S=75.

Evaluation of the Biological Activity of the Enkephalin Analogues[496]

A) Smooth muscle assays

The depression of electrically-induced contractions of the GIP and MVD was measured as described (475). Guinea pigs (200-300 g) and mice (20-30 g Swiss Webster) were obtained from the Canadian Breeding Farm (St-Constant, Québec). They were sacrificed by a blow in the neck. The tissues were rapidly dissected and mounted on a 10-ml double jacketed organ bath in tyrode solution (pH 7.4) at 37°C. Tensions of 0.15 g (vas deferns) and 1.0 g (ileum) were applied and contractions were recorded with a force displacement transducer (FT03C) that was coupled to a Grass polygraph. The parameters for the electrical stimulation were as follows: 10 ms delay, 80 volts, 0.1 Hz, 1 ms duration and double pulsation for the vas deferns; 10 ms delay, 30 volts, 0.1 Hz, 1 ms duration and monophasic pulsation for the ileum. Dose-response curves were effected with the synthetic peptides and the concentration of the opiate that caused 50% inhibition (IC_{50}) was obtained from log-probit plots of six increasing concentrations, each representing the mean ± SEM of 6 different tissues.

B) Opiate binding assays

The opiate binding assays were based on the ability of the synthetic peptides to inhibit [^{3}H]-etorphine or [^{3}H]-dihydromorphine binding to rat brain homogenates as described (476). Assays were performed

at room temperature (22°) for 30 min with 2 mL aliquots of the tissue homogenate (1 mg, wet weight) and 0.7 nmole of ^{3}H -ligand (40-50 Ci/mmol, New England Nuclear, Boston, MA). The bound ligand was separated from free by filtration through glass filters (GF/B, Whatman, England). The IC_{50} were derived from log-probit plots of six concentrations of the compounds, each representing the mean ± SEM of 3 sets of duplicated data.

C) Behavorial Testing

Male hooded rats obtained from Canadian Breeding Farm (St-Constant, Québec) and weighing approximately 300 g were used. They were housed in a temperature-controlled room having a 12 hr light/dark cycle. Food (Purina rat chow) and water were available ad libitum. Under pentobarbital anesthesia, animals were implanted with an indwelling stainless steel cannula into the left cerebral ventricle according to a previously published procedure (477). Following surgery, animals were allowed at least 72 hrs recovery before initiation of experimental procedures. On test day, reactivity of animals to a noxious stimulus was examined by means of a hot plate (35 x 35 cm) with a surface temperature of 54°C. Latencies in seconds for responses to occur, either paw licking or leaving the plate, were recorded. Animals were tested immediately prior to and at 2, 4, 6, 8, 10, 15, 30 and 60 min following intracerebroventricular injections. Individual groups of animals (n=8) were administered 360 ug of either Leu-Enk, [Gly^{2}-C(S)]-, [Gly^{3}-C(S)]

or [Phe^4-C(S)]-Leu-Enk. All peptides were dissolved in 0.9% NaCl and injected in a volume of 10 ul over a 30 sec period. Another group of eight control animals received the same volume of 0.9% NaCl. Following this first experiment, additional groups were administered increasing doses of either Leu-Enk or [Gly^2-C(S)]-Leu-Enk in order to find for each of these peptides, the minimal dose capable of significantly enhancing response latencies of animals on the hot plate.

APPENDIX A

NOTE ON NOMENCLATURE

In an effort to clearly indicate the position of the thiocarbonyl in the various analogues of amino acids peptides in this thesis, we have found it advantageous to employ the two following symbolisms:

a) C(S) succeeding the name of the amino acyl residue bearing the thiocarbonyl function as in:

Tyr-Gly-GlyC(S)-Phe-Leu

b) $\overset{S}{\overset{\|}{\ }}$ between the two amino acyl residues joined by a thioamide as in:

Tyr-Gly-Gly$\overset{S}{\overset{\|}{\ }}$Phe-Leu

These thiocarbonyl analogues can also be referred to by the specific peptide name preceeded by the modified residue in square brackets as:

[Gly^3C(S), Leu^5]-enkephalin

The previous nomenclature for thiopeptide, also referred to as "endothiopeptides", consisted of writing a small "t" at the residue bearing the thiocarbonyl function[497], e.g.:

Tyr-Gly-Glyt-Phe-Leu

Another nomenclature used to describe backbone modification of peptides has been recently introduced by Morley[498] and Spatola[499]. The analogues in which the CO-NH group is replaced by another functionnality are written by placing a psi (ψ) with the substitutuion in square brackets before the name of the peptide. The ψ is placed between superscripts indicating the modified residues. Thus, the same compound as above would be named:

$$[^{3}\psi^{4}, \text{CSNH}]\ \text{Leu-enkephalin}$$

and written:

$$\text{Tyr-Gly-Gly-}\psi[\text{CSNH}]\text{-Phe-Leu}$$

After completion of this thesis this latter nomenclature has been recently recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature[500].

APPENDIX B

A) Derivation of the Lineweaver-Burk Equation.[125,170]

The central feature of the Michealis-Menten kinetic model for enzymatic catalysis is based on the premise that an enzyme (E) interacts with a substrate (S) to form the non-covalent ES complex, which can either undergo chemical reaction to products (P) or physical dissociation.

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} EP \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} E + P$$

Under initial velocity conditions, that is before a significant amount of P has accumulated, the back reaction can be ignored. Thus, the actual reaction under consideration becomes:

$$E + S \underset{K\text{-}1}{\overset{K_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

In rapid equilibrium conditions, where E, S and ES equilibrate rapidly in comparison to the rate at which ES breaks down to E + P, the instantaneous velocity at any time depends on the concentration of ES:

$$v = k_{cat}[ES]$$

where k_{cat} is the catalytic rate constant. The total enzyme is distributed between E and ES as follows:

$$[E]_T = [E] + [ES]$$

Dividing the velocity-dependence equation by $[E]_T$ where [E] + [S] is used on the right-hand side reduces the equation to:

$$\frac{v}{[E]_T} = \frac{k_{cat}[ES]}{[E] + [ES]}$$

Because of the equilibrium assumption, [ES] can be expressed in terms of [S], [E] and K_S where K_S is the dissociation constant of the ES complex:

$$K_S = \frac{[E][S]}{[ES]} = \frac{K_{+1}}{K_{-1}}, \qquad [ES] = \frac{[S][E]}{K_S}$$

Substituting for [ES]:

$$\frac{v}{[E]_T} = \frac{k_{cat}\frac{[S]}{K_S}[E]}{[E] + \frac{[S]}{K_S}[E]}$$

Cross multiplying by k_{cat} and cancelling:

$$\frac{v}{k_{cat}[E]_T} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S}}$$

If $v = k_{cat}[ES]$, the $k_{cat}[E]_T = v_{max}$ which is the maxiamal velocity that would be observed when all the enzyme is present as ES.

$$\frac{v}{v_{max}} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S}}$$

This equation can be rearranged to yield the more familiar Henri-Michealis-Menten equation:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S + [S]}$$

The equation gives the initial velocities relative to V_{max} at a given substrate concentration. The equation is valid only if v is measured over a short enough time period so that [S] remains essentially constant. This requires that no more than 5% of the substrate be utilized over the assay period.

Using the steady state approach (Briggs and Haldane) in which the concentration of ES remains essentially constant with time, the following equations apply:

$$\frac{d[ES]}{dt} = 0$$

$$K_1[E][S] = (K_{-1} + k_{cat})[ES]$$

$$[ES] = \frac{K_1[E][S]}{(K_{-1} + k_{cat})}$$

The group of three rate constants can be defined as a single Michealis constant, K_m:

$$K_m = \frac{K_{-1} + k_{cat}}{K_m} ,$$

which upon substitution into the velocity dependence equation yields:

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_m}}{1 + \frac{[S]}{K_m}} \quad \text{or} \quad \frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

This last equation can be rewritten in the more familiar form, as follows:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

Thus, by plotting 1/v versus 1/[S], the slope of the line is K_m/V_{max} and the intercept on the y axis is $1/V_{max}$. When 1/v is equal to 0, then 1/[S] equals $-1/K_m$.

B) Competitive Inhibition.

A competitive inhibitor is a substrate that combines with free enzyme in a manner that prevents substrate binding. This signifies that the inhibitor and the substrate are mutually exclusive, and thus compete for the same site on the enzyme. This enzymic reaction can be described in the following manner:

$$\begin{array}{ccc} E + S & \rightleftharpoons & ES \xrightarrow{k_{cat}} E + P \\ + & & \\ I & & \\ \Updownarrow K_I & & K_I = \dfrac{[E][I]}{[EI]} \\ EI & & \end{array}$$

The kinetic expression for the above reaction is:

$$v = k_{cat}[ES] \quad , \quad \frac{v}{[E]_T} = \frac{k_{cat}[ES]}{[E] + [ES] + [EI]}$$

$$[ES] = \frac{[S][E]}{K_S} \quad , \quad [EI] = \frac{[I][E]}{K_I}$$

$$\frac{v}{k_{cat}[E]_T} = \frac{\frac{[S]}{K_S}[E]}{[E] + \frac{[S]}{K_S}[E] + \frac{[I]}{K_I}[E]}$$

or $$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I}}$$

The velocity equation for competitive inhibition in reciprocal form is:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

The increased apparent K_m (or $K_{m_{app}}$) results from the distribution of available enzyme. The factor $(1 + [I]/K_I)$ may be considered as an inhibitor concentration-dependent statistical factor describing the distribution of enzyme between E and EI forms. When plotting $1/v$ vs $1/[S]$, the slope is increased by the factor $(1 + [I]/K_I)$ which is a multiple of the original K_m yet the y-intercept is unchanged. The apparent K_m value is a linear function of inhibitor concentration. This relationship is described by the following equation:

$$K_{m_{app}} = \frac{K_m}{K_I}[I] + K_m$$

The absolute value of K_I can be obtained from the graph of this equation by reading the x-coordinate when $K_{m_{app}}$ equals 0.

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