IMPROVED METHODOLOGY FOR THE SYNTHESIS OF BICYCLIC OCTAPEPTIDES: AMATOXINS

by

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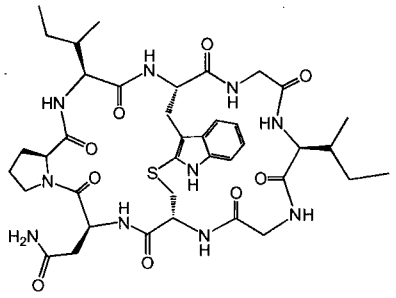
ABSTRACT

Rigidity is an important property of drugs and small molecule toxins that enhances the affinity for their targets. Amatoxins are highly rigid bicyclic peptides that bind to and consequently inhibit RNA Polymerase II. In this thesis we present an improved methodology for the synthesis of a tryptathionine linkage, which constitutes the rigidifying bridge that is a distinguishing feature of amatoxins and related phallotoxins. The Savige-Fontana reaction was used to form 3a-hydroxyhexahydropyrroloindoles (Hpi), critical intermediates for substitution at the 2-position of the tryptophan indole. Our strategy consisted of forming Hpi as a dipeptide by oxidizing with dimethyldioxirane various Tr-Trp-X-OMe compounds (with X=Ser, Val, Lys(Boc), Leu). Good yields were obtained (75 – 92%), except for leucine due to concurrent side-chain oxidation. Our strategy was applied to the inter- or intramolecular formation of a linkage between cysteine and tryptophan, which gave rise to compounds **2**, **3** and **4** and finally to the synthesis of the amatoxin Pro^2,Ile^3-deoxo-amaninamide **1**. After formation of the linear octapeptide Tr-Hpi-Gly-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OtBu using the "rapid continuous peptide synthesis" method, TFA treatment gave rise to the corresponding unprotected monocyclic peptide **64** bearing a tryptathionine bridge. A macrolactamization with PyBOP as coupling reagent gave rise to a mixture of the target **1** and its inactive atropisomer. Compound **1** was isolated and characterized.



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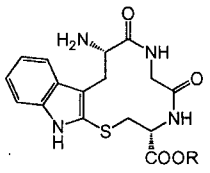
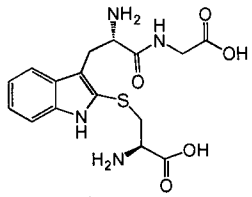


R=H : **2**
R=Me : **3**



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List of abbreviations

Asn	Asparagine
AcOEt	Ethyl acetate
AcOH	Acetic acid
Bn	Benzoyl
Boc	t-butyloxycarbonyl
bs	broad singlet
Cbz	benzyloxycarbonyl
CD	Circular dichroism
CH_2Cl_2	Dichloromethane
COSY	Correlation spectroscopy
Cys	Cysteine
d	doublet
DAST	Diethylaminosulfur trifluoride
DCC	Dicyclohexylcarbodiimide
DCU	*N,N'*-Dicyclohexylurea
dd	doublet of doublets
DMA	*N,N'*-Dimethylacetamide
DMDO	3,3-Dimethyldioxirane
DMF	*N,N'*-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dt	Doublet of triplets
EDTA	Ethylenediamine tetraacetate
ESI	Electro-Spray Ionization
Et	ethyl
Et_2O	Diethyl ether
Fmoc	9-fluorenylmethyloxycarbonyl

Gly	Glycine
HATU	*N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methyl-methanaminium hexafluorophosphate N-oxide
HCl	Hydrochloric acid
His	Histidine
HMBC	^{1}H-detected mutiple bond heteronuclear multiple quantum coherence
HMQC	^{1}H-detected heteronuclear multiple quantum coherence
HOBt	1-Hydroxybenzotriazole
Hpi	3a-Hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]-indolo-2-carboxylic acid
HPLC	High performance Liquid Chromatography
HRMS	High resolution mass spectrometry
Ile	Isoleucine
Leu	Leucine
LRMS	Low resolution mass spectrometry
Lys	Lysine
m	multiplet
Me	methyl
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry.
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser enhancement
NOESY	Nuclear Overhauser enhancement spectroscopy
Ph	phenyl
Pol II	RNA Polymerase II
ppm	part per million
Pro	Proline
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidophosphonium hexafluorophosphate
q	quartet
R_f	retention factor or ratio to front

RNA	Ribonucleic acid
RT	Room temperature
s	singlet
t	triplet
TAEA	Tris(aminoethyl)amine
tBu	*tert*-butyl
TLC	Thin-layer chromatograpy
TOCSY	Total correlation spectroscopy
Tr	trityl, triphenylmethyl
Trp	Tryptophan
Fmoc-OSu	9-Fluorenylmethoxycarbonyloxysuccinimide
TFA	Trifluoroacetic acid
δ	chemical shift

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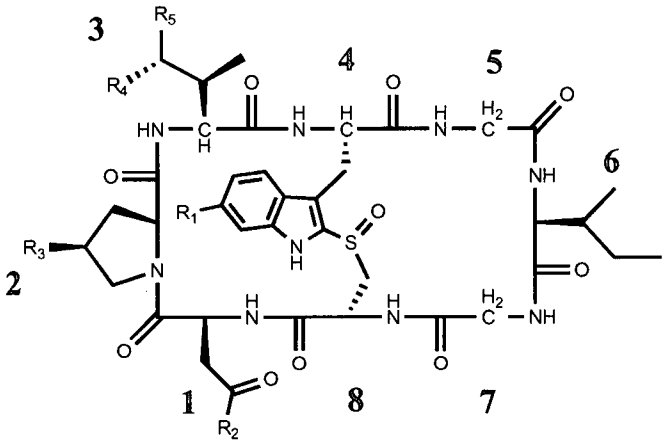
Chapter 1

Introduction

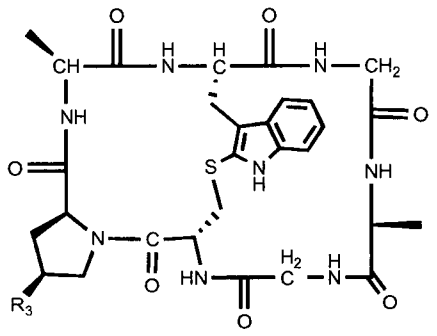
1 Amatoxins and phallotoxins, two families of natural products

1.1 Structure of amatoxins and phallotoxins

Amatoxins and phallotoxins are two families of bicyclic peptides isolated from various toxic fungi of the *Amanita* family such as *Amanita Phalloides* and *Amanita Virosa.*[1] Whereas amatoxins are octapeptides, phallotoxins are similarly structured bicyclic *hepta*peptides, composed of only seven amino acids. Amatoxins and phallotoxins share similar structural features (Figure 1): they are head-to-tail cyclized peptides and contain an additional transannular bridge between the side chains of a tryptophan residue and a cysteine residue. For amatoxins this bridge is further modified as a sulfoxide linkage, whereas for phallotoxins it is a thioether linkage: this thioether linkage forms what is called a tryptathionine moiety. Another distinctive feature of these peptides is the presence of non-proteinogenic amino acids such as 4-hydroxyproline, 6-hydroxytryptophan, 4,5-dihydroxyisoleucine. The 9 natural amatoxins and the 6 natural phallotoxins are described in the table in Figure 1; they are present in variable amounts in fungi, the most abundant of them being α-amanitin. The term "amatoxin" does not only refer to these 9 natural products; with the advent of synthetic endeavors it has been extended to other synthetic derivatives based on the same skeleton.



Name	R1	R2	R3	R4	R5
α-amanitin	OH	NH2	OH	OH	CH2OH
β-amanitin	OH	OH	OH	OH	CH2OH
γ-amanitin	OH	NH2	OH	OH	CH3
ε-amanitin	OH	OH	OH	OH	CH3
Amanin	H	OH	OH	OH	CH2OH
Amaninamide	H	NH2	OH	OH	CH2OH
Amanullin	OH	NH2	OH	H	CH3
Amanullinic acid	OH	OH	OH	H	CH3
Proamanullin	OH	NH2	H	H	CH3



Name	R1	R2	R3	R4	R5
phalloin	OH	NH2	OH	OH	CH2OH
phalloidin	OH	OH	OH	OH	CH2OH
phallisin	OH	NH2	OH	OH	CH3
prophalloin	OH	OH	OH	OH	CH3
phallacin	H	OH	OH	OH	CH2OH
phallacidin	H	NH2	OH	OH	CH2OH
phallisacin	OH	NH2	OH	H	CH3

Figure 1: Natural amatoxins and phallotoxins

1.2 Biological activity of amatoxins and phallotoxins

Both amatoxins and phallotoxins are toxic to mammals. As for phallotoxins, their toxicity is due to their strong affinity towards F-actin, a ubiquitous protein in smooth muscles. The binding of phallotoxins prevents depolymerization of F-actin to G-actin, shifting the natural equilibrium and reducing the G-actin to an intolerable amount for the cell.[2] Amatoxins are of more interest to us because they act as inhibitors of transcription in eukaryotic cells. This strong inhibition places amatoxins among the most toxic of natural substances known; their estimated oral LD_{50} is 0.1 mg/kg. Transcription is a fundamental and highly regulated process occurring in any living cell whereby the information encoded by DNA is revealed: RNA polymerase separates the DNA strands and carries out the synthesis of complementary messenger RNA. Without this step, the synthesis of proteins (that occurs via translation of the messenger RNA) is arrested.

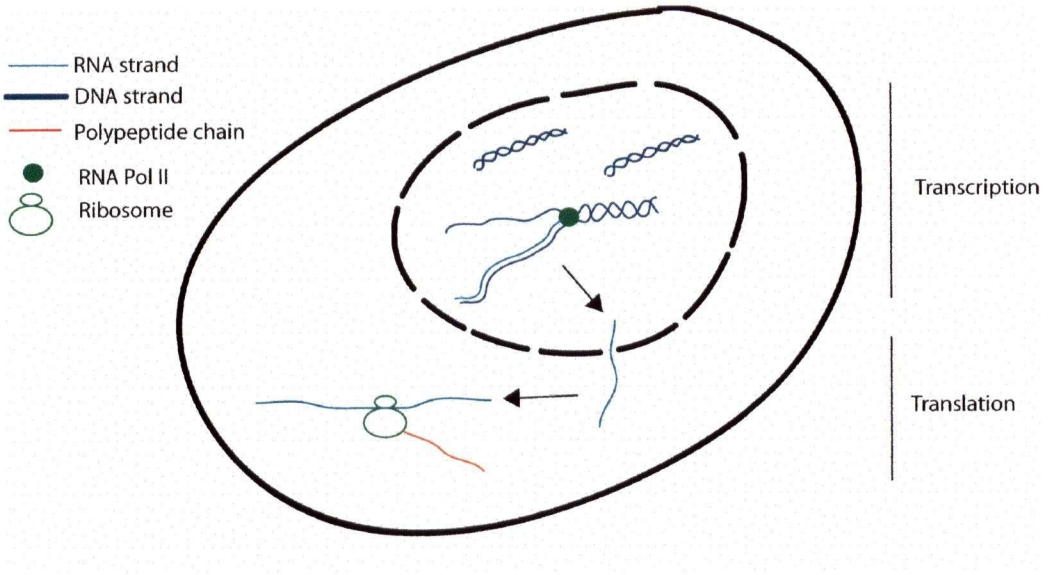


Figure 2: Cartoon representation of a eukaryotic cell and the 2 fundamental processes in polypeptide synthesis, transcription and translation

RNA Polymerase II (Pol II; there are three distinct polymerases: I, II, and III) is the primary enzyme responsible for transcription of mRNA that encodes for proteins. In the course of RNA synthesis, Pol II interacts simultaneously with several components: the DNA strand to be transcribed, which acts as a template, and the ribonucleotides, which are the monomers to be polymerized into RNA. The enzyme moves along the template DNA strand, "reads" it, and incorporates successively into RNA the ribonucleotides that match the template at each position. It has been shown that in most eukaryotes α-amanitin binds with a high affinity to RNA Pol II when Pol II is itself, bound to DNA and presumably engaged in mRNA synthesis.[1] Once bound, α-amanitin prevents Pol II from moving along this strand,[3] thus blocking the enzyme. The activity of α-amanitin, as for any reversible noncompetitive enzyme inhibitor, can be measured by its K_i (the dissociation constant of the enzyme-inhibitor complex). For α-amanitin the K_i, when measured on polymerases purified from various eukaryotic species, ranges from 10^{-8} to 10^{-10} M, and is characteristic of very tight binding. However, little is known on how the amatoxins exactly block the enzyme. One might ask, at which step of transcription does it exactly interfere with the cell machinery? Does the α-amanitin binding induce any conformation change in the enzyme structure? Such questions have not yet been answered. Beyond the "static" inhibitory effects on Pol II is a "dynamic"

effect that has been observed whereby amatoxins not only inhibit Pol II but also trigger its degradation through an unknown process.[4,5] This triggered degradation, that appears to be mediated by a ubiquitination pathway, has also been observed with various chemotherapeutic agents such as cis-platin which induce lesions on DNA and thereby arrest transcription.[5] The degradation of Pol II reaches far beyond the mere inhibition, and may be a general phenomenon linked to cancer and cell division. Thus there is a lot to learn from mechanistic studies on the action of amatoxins.

2 Our goals in the study of amatoxins:

Our three main purposes in studying amatoxins are the following:

1) Structure-activity relationship studies on RNA Polymerase II.

2) Building a library of amatoxins to be screened against a variety of protein targets.

3) Developing robust methodologies for generating bicylic peptides characterized by the tryptophanylated cysteine.

Our first goal consists of synthetically creating small modifications on amatoxins and studying their effect on RNA Polymerase II inhibition. This will provide essential information on the nature of the interaction of amatoxins with their target and on their mechanism of action. Through these modifications we wish to use amatoxin derivatives to target transcription. But we might ask why should we be interested in inhibiting a process essential in all living bodies? In fact, the transcription machinery is not identical in all living bodies. The RNA Pol II enzymes, although showing an important secondary structural homology between all eukaryotic life forms, display some variability on a limited number of amino acid residues. Thus natural amatoxins are not toxic to all living bodies, for example α–amanitin is not toxic to trypanosomes and all prokaryotes that comprise bacteria. We believe that synthetic chemistry could be directed to this natural product to discover a modified amatoxin that would be active only against a pathogenic RNA polymerase and not that of the human host. Such a molecule might have utility as a

drug. This synthetically modified amatoxin would be achieved through engineering of the amatoxin structure and by relying on recently reported x-ray crystal structure coordinates that position the amatoxin near the active site of Pol II (Figure 3).[6]

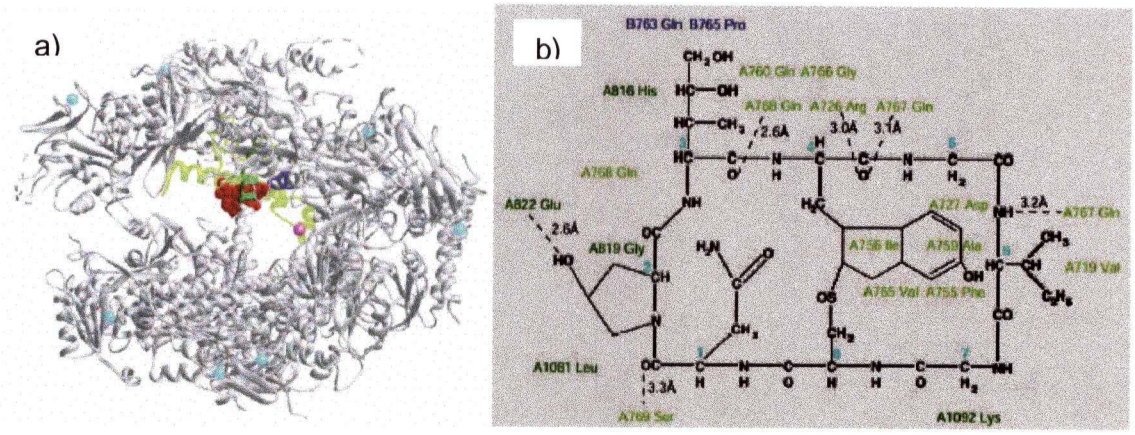
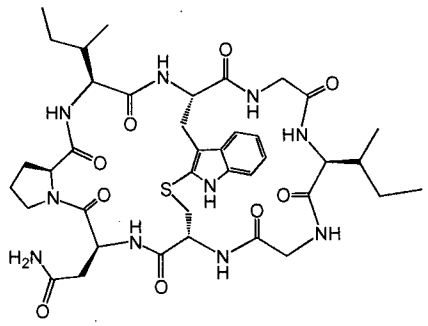


Figure 3: a) Ribbons representation of the complex RNA Pol II-α-amanitin; α-amanitin is in red, the regions of Pol II close to α-amanitin are in green ; b) α-amanitin with residues of Pol II that lie within 4 Å. (from ref. 6)

This crystal structure suggests a strong hydrogen bond between the hydroxyl group of the hydroxyproline residue of α-amanitin and Glu822 of Pol II. It is known from previous structure-activity studies that an amatoxin lacking this hydroxyl group loses about 3 orders of magnitude in activity.[7,8] A single hydrogen bond probably does not account for such a dramatic loss of activity without a concomitant conformation change; therefore it would be interesting to probe this interaction between the proline residue of α-amanitin and Glu822 of Pol II and see how the enzyme structure reacts to modification on this proline ring. It should also be noted from Figure 3b that no specific interaction has been observed between Pol II and the sulfoxide, the tryptophan 6-hydroxyl group or the dihydroxyisoleucine hydroxyl groups of α-amanitin. These functional groups therefore are not critical for activity and need not be included in an initial synthesis of amatoxins. This is why we chose Pro^2,Ile^3-S-deoxo-amaninamide (**1**) as our first amatoxin target: it includes non-hydroxylated tryptophan, isoleucine and proline.



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Besides this specific structure-activity study, it is also of note that both amatoxins and phallotoxins, although very closely related in terms of structure, target very different proteins and operate via very different mechanisms. This very important observation suggests that such bicyclic peptides provide effective templates for small molecule-protein interactions, and thus might form the basis of an interesting scaffold for combinatorial chemistry. Thus our goals also include the synthesis of a combinatorial library of amatoxins by varying all amino acids in the sequence, except for the tryptophan and cysteine residues, which are necessary to form the crosslink. This library will be available for screening against a wide range of protein targets.

3 Bicyclic peptides

3.1 The advantages of a bicyclic peptide structure

As evidenced by Nature's choice of the tryptathionine-based bicylic peptide structure that distinguishes amatoxins and phallotoxins, it is clear that bicyclic peptides are particularly well suited for protein recognition (Pol II, Actin for example). Cyclic structures present several advantages over linear ones. Firstly, in contrast to linear peptides, cyclic peptides do not have any polar primary amino and carboxylate termini, and they usually have a high proportion of cis amide bonds; this accords them with a greater resistance to proteolytic enzyme and therefore a higher stability *in vivo*.[9,10] In addition their decreased polarity improves the permeability of greasy cell membranes to those molecules, which enhanced their bioavailability.

Lastly, the main advantage of cyclic structures lies in their rigidity. Reduced conformational freedom often results in higher affinity to the target for entropic reasons: indeed the molecule is blocked in a conformation close to the active conformation. This pre-organization reduces unfavorable entropic effects and thus increases the affinity dramatically. For all these reasons, rigid cyclic structures are much more potent leads for drugs than linear peptides. For the same reasons, the *bi*cyclic structure would suggest an even stronger rigidity that should further enhance the potency of the corresponding molecule as a drug. Thus cyclic peptides have become increasingly popular in medicinal chemistry. Numerous libraries of cyclic peptides have been synthesized over the years.[11] For the same reasons as cyclic peptides, bicyclic peptides to an even greater extent may hold great promise in the field of drug discovery, for unrivalled *in vivo* stability and affinity for protein targets.

3.2 The formation of cyclic or bicyclic peptides – a brief review

The formation of a rigid peptide structure has been achieved through a variety of approachs, be it in nature or via synthetic attempts. In nature, the most common means of cyclizing peptides are peptide bonds and disulfide bridges. The cyclization through peptide bonds can be achieved in two ways: 1) Head-to-tail cyclization: N- and C-termini are simply connected through a peptide bond, forming a circular molecule, and 2) Side-chain-to-backbone cyclization: in general the N-terminus forms a peptide bond with the carboxylic acid function of a glutamate or aspartate residue; this linkage forms a lariat-shaped molecule.[12] Nature has provided several extraordinary strategies for bolstering peptide rigidity. For instance a very uncharacteristic rigid structure has been recently described simultaneously by two groups[13] in the antibacterial peptide microcin J25: this 21 amino acid peptide includes a side-chain-to-backbone peptide bond between the N-terminus (residue number 1 in the chain) and a glutamate residue (number 8) that forms a lariat structure; the tail of this lariat (residues 19 to 21) passes through the ring and is kept in place by sterical interactions (Figure 4). This is an interesting example of rigidification by noncovalent interactions.

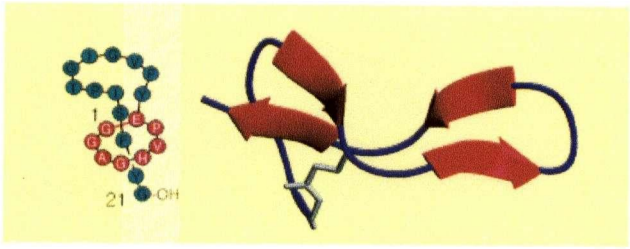
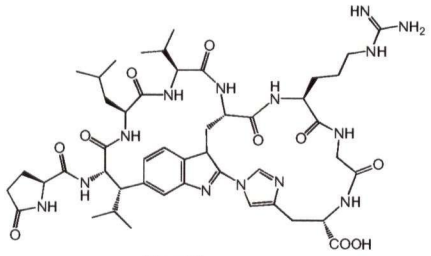


Figure 4: The lasso structure of Microcin 25 (from ref. 13)

It should be noted that the tryptathionine-based amatoxins and phallotoxins are not the only bicyclic peptides in nature: moroidin, isolated from the seeds of *Celosia argentea*,[14] is a bicyclic peptide with a bond between N_1 of a histidine imidazole and the indole C_2 position, and exhibits anti-mitotic activity.



Moroidin

On the synthetic side, most efforts towards rigid peptides use peptide bonds to form a ring. However macrolactamizations are never trivial, they require expensive coupling reagents such as PyBOP[15] or HATU and specific structural features in the peptide to be cyclized, such as the presence of turn-inducing proline residues. Besides the formation of dimers or oligomers is always a side reaction. An alternative to the classical peptide bond formation via dehydrative coupling utilizes the Staudinger ligation. This methodology developed independently by Bertozzi[16] and Raines[17] for very different purposes has indeed been applied to macrolactamization: the C-terminus of a peptide, transformed into a phosphanyl thioester, is allowed to react with a N-terminal azide to form a peptide bond after rearrangement (Figure 5a).[18]

Due to the difficulty to form large cyclic peptides several methodologies have been developed to synthesize rigid structures that mimic cyclic peptides.[19] One noteworthy synthetic crosslinking strategy involves ring-closing metathesis, a very reliable method due to the ability of Grubbs catalyst[20] to achieve macrocyclizations. This can be applied to "side-chain-to-side-chain" cyclization,[21] if C-C double bonds

are introduced in the amino acid side-chains, or to "backbone-to-backbone methods, as in Fig. 5b.[22] Another synthetic method successfully applied to cyclic peptide formation is the nucleophilic aromatic substitution, used for example in Burgess' strategy to form β-turns mimics[24] (Figure 5d): these methods use phenyl rings bearing a fluorine, to serve as leaving group, and a nitro substituent as activating group.

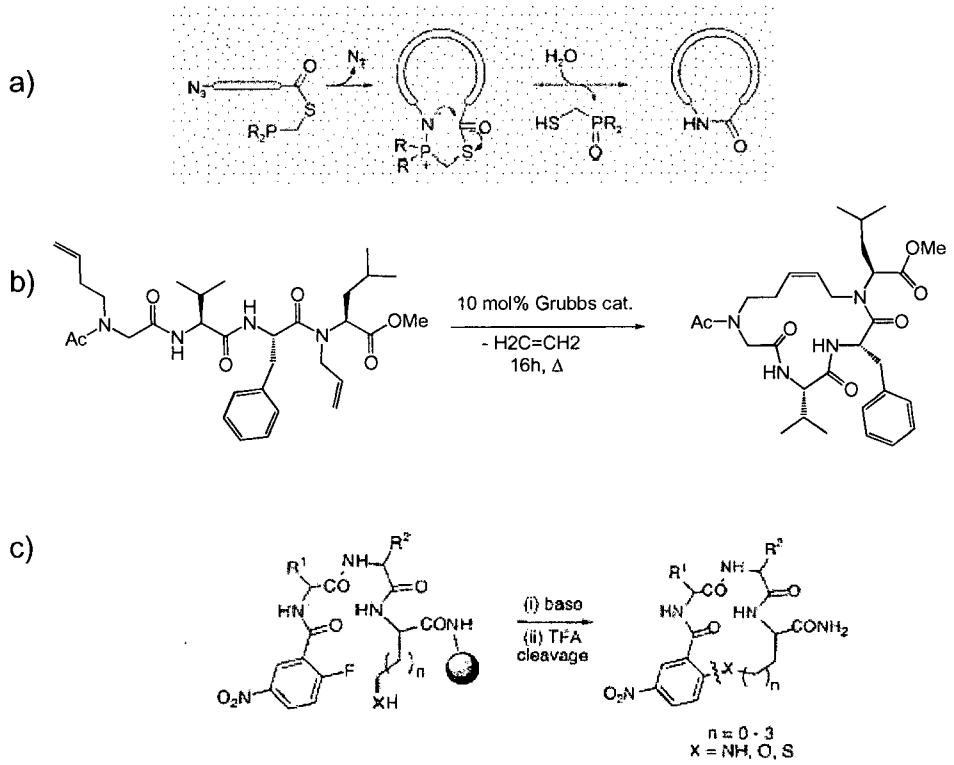


Figure 5: Different approaches to the cyclization of peptides.

Among all the possible rigidification strategies, we have remained focused on the amatoxins' tryptathionine bridge because Nature herself has proven its efficiency in small molecule-protein interactions. However the synthesis of such a crosslink may prove useful to medicinal/peptide chemists because it is *not* based on methodologies such as ring-closing metathesis or nucleophilic aromatic substitution which rely on expensive, toxic and difficult-to-remove metals or on potentially immunogenic nitroaromatic rings. Admittedly the head-to-tail peptide bond is not unheard of, but the development of a robust methodology for the formation of the

carbon-sulfur bond between tryptophan and cysteine remains a challenge. To learn how to achieve this crosslink between tryptophan and cysteine, we have to take a closer look into indole chemistry.

4 Synthesizing tryptathionine

4.1 Aspects of indole chemistry

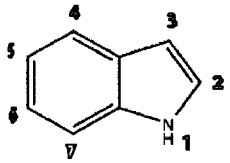
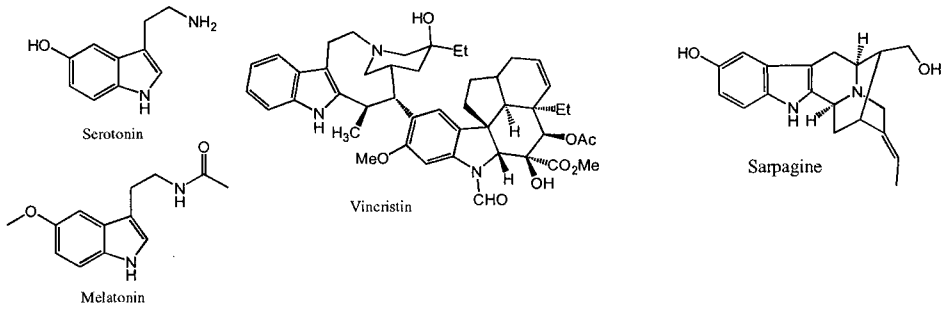


Figure 6: Numbering of the indole ring

Indoles are arguably the most widespread precursors of heterocycles in nature. They are present in important metabolic agents (such as neurotransmitters serotonin and melatonin, or of course tryptophan) as well as in a large number of more complex natural products, such as the important anticancer alkaloid drug vincristin, or the indole alkaloid sarpagine.



This prevalence of the indole in natural products makes it a privileged target for medicinal chemistry and has prompted considerable research effort on new methodologies for the synthesis of indoles and indole-derived synthons. For example the use of cyclophane **5** and inverse-electron-demand Diels-Alder reaction

has also been recently reported[25] for the synthesis of the indole-containing pentacyclic amine **6**, which allows a short formal synthesis of strychnine[26] **7** and related alkaloids from the readily available tryptamine.

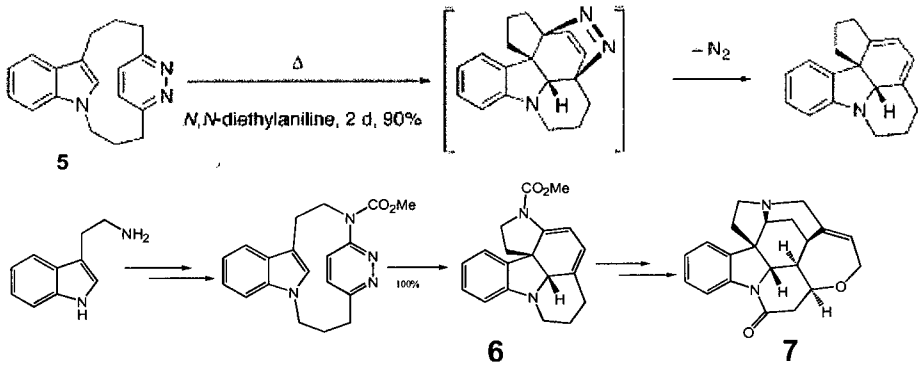
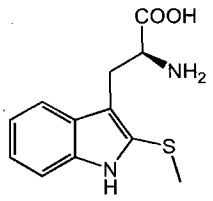


Figure 7: An example of recent synthetic methodology based on the indole ring.

4.2 Synthesis of the tryptathionine moiety



Tryptathionine

The challenge of tryptathionine synthesis consists of forming a carbon-sulfur bond between the weakly activated C_2 position of a tryptophan indole and the thiol of cysteine. The methodology employed should be simple and versatile enough for ultimate application in a combinatorial approach. The reaction must also be achieved in mild conditions to retain the various functional groups present on other amino acids of the peptide as well as avoid racemization at C_α. Finally it is desirable to avoid the use of transition metals, which are expensive, and can be particularly difficult to remove from the resultant bicyclic peptide. When not properly removed they can display inhibitory and toxic effects if present during library screening.

Considering our goal we will briefly pay tribute to literature precedents, which describe approaches for tryptathionine synthesis. Theoretically this linkage can involve either nucleophilic attack by tryptophan on an electrophilic sulfur atom, or a nucleophilic attack by the sulfur atom of Cys on the 2-position of a properly activated electrophilic tryptophan.

4.2.1 Use of electrophilic sulfur

With indole being considered an electron-rich aromatic heterocycle, its chemistry has been dominated by the electrophilic aromatic substitution reactions.[27] As shown in Figure 8, electrophilic attack occurs preferentially at the 3-position of the ring however in the case of 3-substituted indoles (e.g. tryptophan), substitution can also occur at the 2-position.

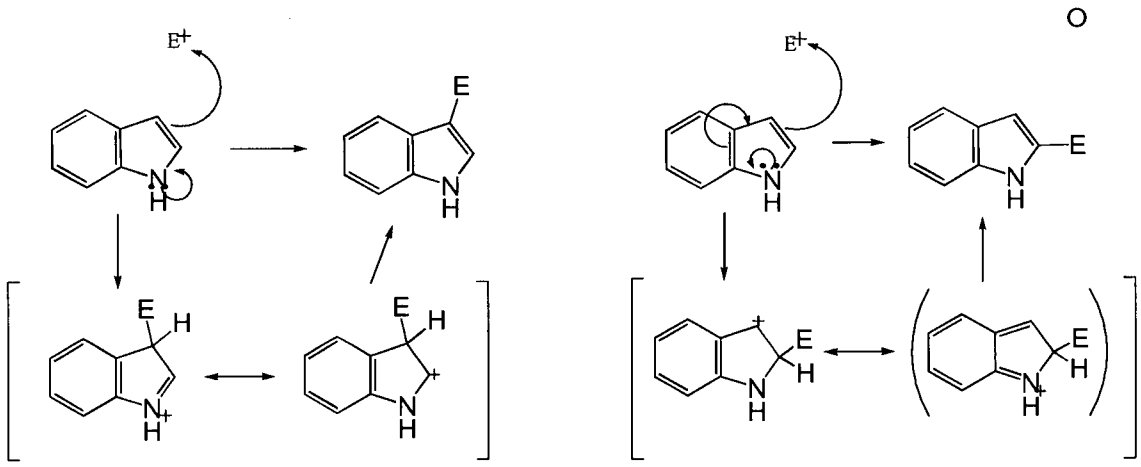
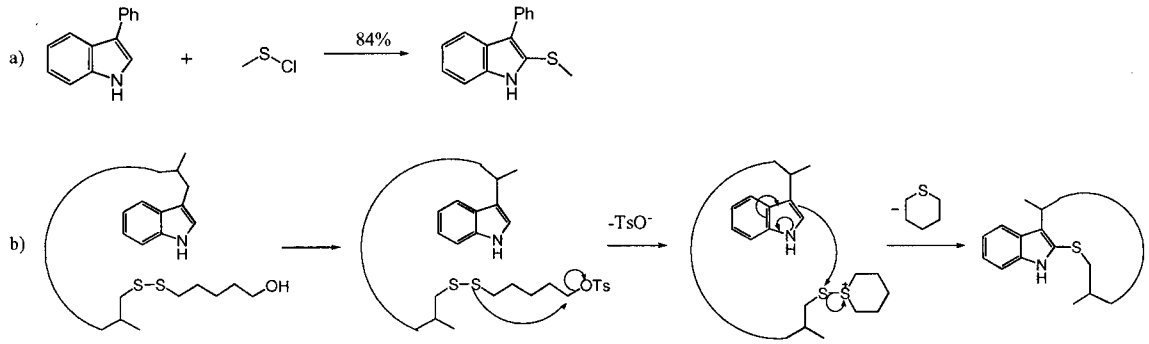


Figure 8: Intermediates in the electrophilic aromatic substitution of indole. The Wheland intermediate is more stabilized in the case of the attack at the 2-position of indole.

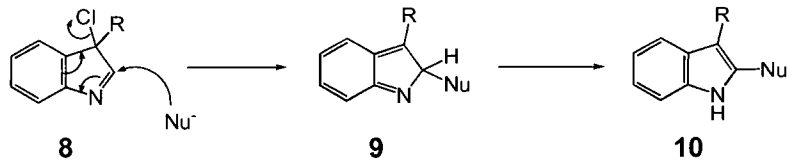
In recognition of the nucleophilic properties of indoles, electrophilic sulfur was used in the first tryptathionine synthesis which involved sulfenyl chloride (Scheme 1a).[28] Another approach that could be applied to the problem might be a disulfide functionality that could be converted into an electrophilic sulfur species (Scheme 1b):



Scheme 1: Strategies towards tryptathionine involving an electrophilic sulfur.

4.2.1 Use of nucleophilic sulfur.

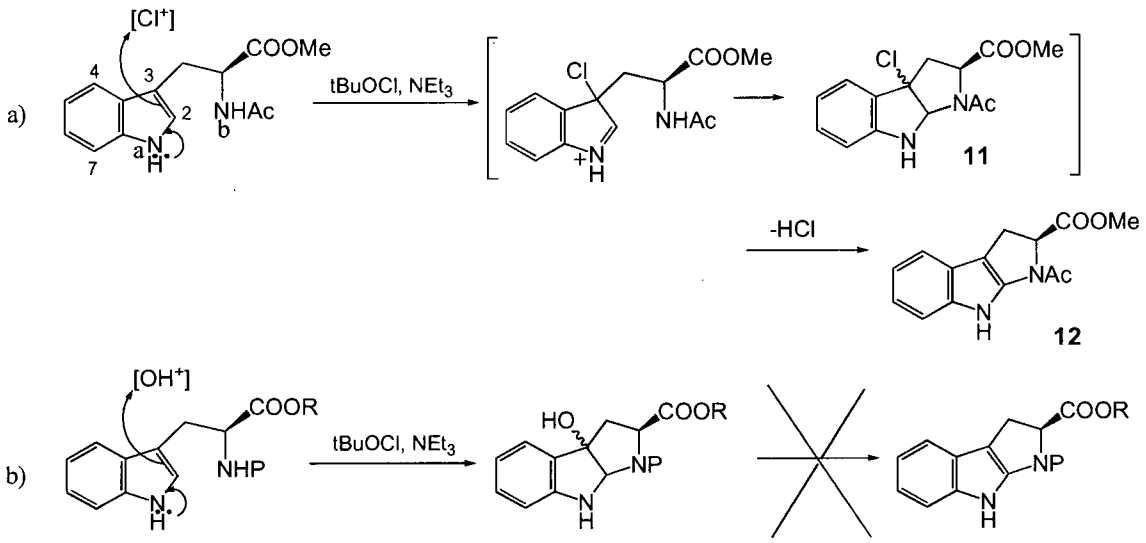
As noted above, indoles are not particularly prone to nucleophilic substitution. However nucleophilic attack at the 2-position of the indole ring can be favored by first introducing an electron-withdrawing group on the ring, an operation that can formally be considered as oxidative. For example, a 3-chloroindolenine derivative of tryptophan such as **8** can undergo nucleophilic attack at the 2-position, yielding the intermediate **9,** which tautomerizes to **10** (Scheme 2).



8 **9** **10**

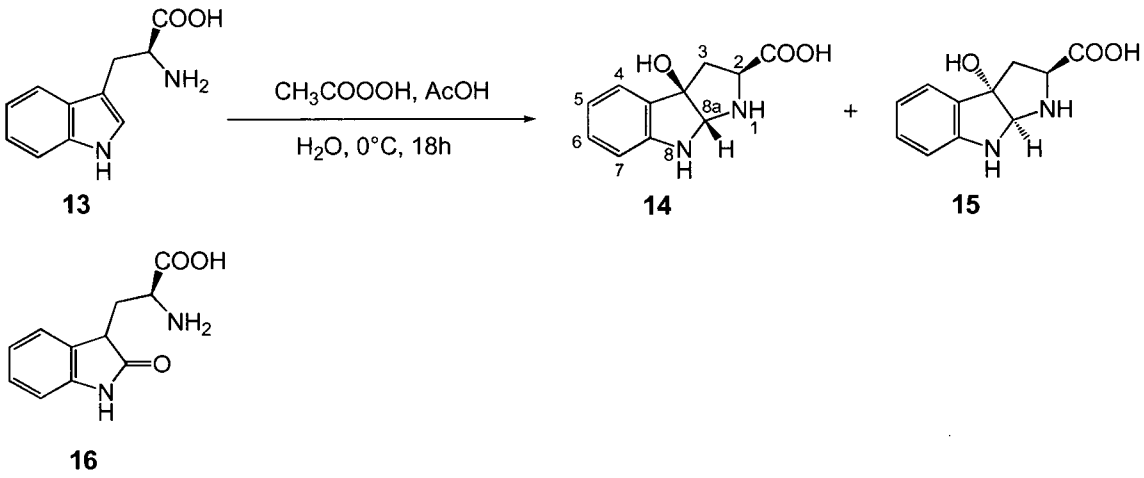
Scheme 2: Activation of the indole ring by a chlorine atom in 3-chloroindolenine

3-Chloroindolenines however, are very reactive species that are not sufficiently stable to survive peptide elongation steps, and their formation requires the use of a strong oxidizing agent such as *tert*-butylhypochlorite (*t*BuOCl).[29] This reagent would also oxidize thiols and thioethers that will necessarily be present in the molecule for tryptathionine synthesis. Alternatively, one can consider "trapping" the activated indole in the form of a more stable intermediate that retains the electrophilic character of the 2-position. The N_b (see Scheme 3 for notation) of tryptophan can be used for such a purpose; indeed, it is possible to induce oxidative cyclization of tryptophan as shown by the work of Witkop:[30]



Scheme 3: Activation of the indole ring by oxidation.

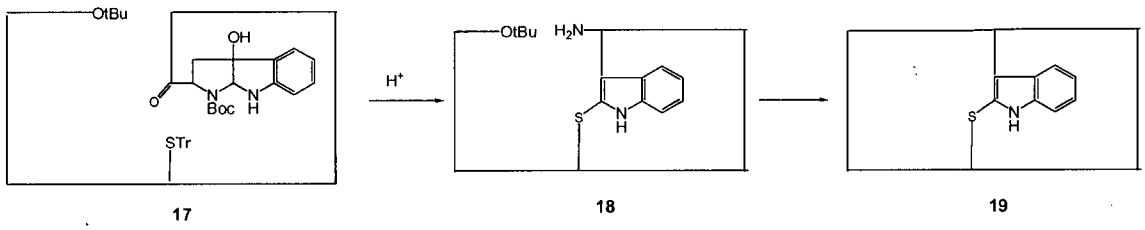
In this case, the very reactive intermediate **11** rearomatizes rapidly and uncontrollably via dehydrohalogenation to give **12,** a stable indolopyrrolidine, which does not undergo attack by a nucleophile (the cysteine thiol for instance). Ideal for our purpose would be a similar oxidative cyclization of N_b of tryptophan, but with concomitant hydroxylation at C_3 via the addition of a formal hydroxenium ion (OH^+)[29] (Scheme 3b). In this case the hydroxyl at the 3-position would be sufficiently stable to allow incorporation into a peptide bond but labile enough to be discharged under acidic conditions to generate an electrophilic indole precursor. Indeed this approach, first described by Savige and Fontana in 1976,[31,32] forms the basis for the strategy we chose. The authors treated tryptophan with peroxyacetic acid and obtained two isomers of L-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]-indolo-2-carboxylic acid (Hpi) **14** and **15** (Scheme 4) with a reported yield of 45%. This Hpi species could be attacked at its 8a position by several thiol nucleophiles to give tryptathionine analogues.



13 14 15 16

Scheme 4: Formation of Hpi by oxidation of tryptophan

The synthesis of natural and non-natural amatoxins by Zanotti et al.[33-35] was based on this method, following the sequence described in Scheme 5: after synthesis of the Hpi-containing linear octapeptide **17**, the Savige-Fontana reaction gave rise to monocyclic product **18**, which after a macrolactamization yielded amatoxins.



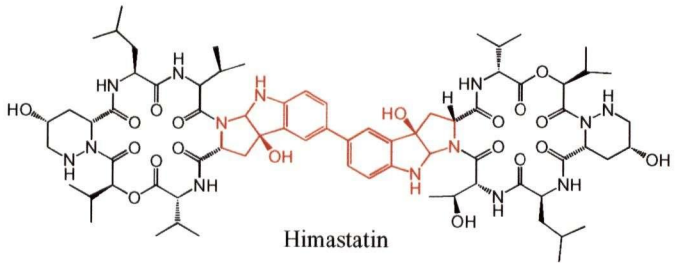
17 18 19

Scheme 5: Savige's Synthetic strategy towards amatoxins

4.3 Our first attempts

We tried to reproduce Savige and Fontana's results, but in our hands very low yields of Hpi were obtained when oxidizing tryptophan with m-chloroperoxybenzoic acid.[36] We then turned to another, milder oxidizing agent, dimethyldioxirane (DMDO).[37] Again no Hpi was obtained when treating Trp or its methyl ester. Instead the main product formed was the corresponding oxindole **16**. We then realized there

is a real need, in our objectives towards amanitin, for a robust method for the formation of Hpi compounds. Danishefsky's synthesis of several Hpi analogues, en route to the natural product himastatin, proved inspiring in that regard.[38]



Himastatin

Having introduced our goals and with this overview of indole chemistry as related to tryptathionine formation, the reader's attention is directed to the following chapters.

Chapter 2

Results and Discussion

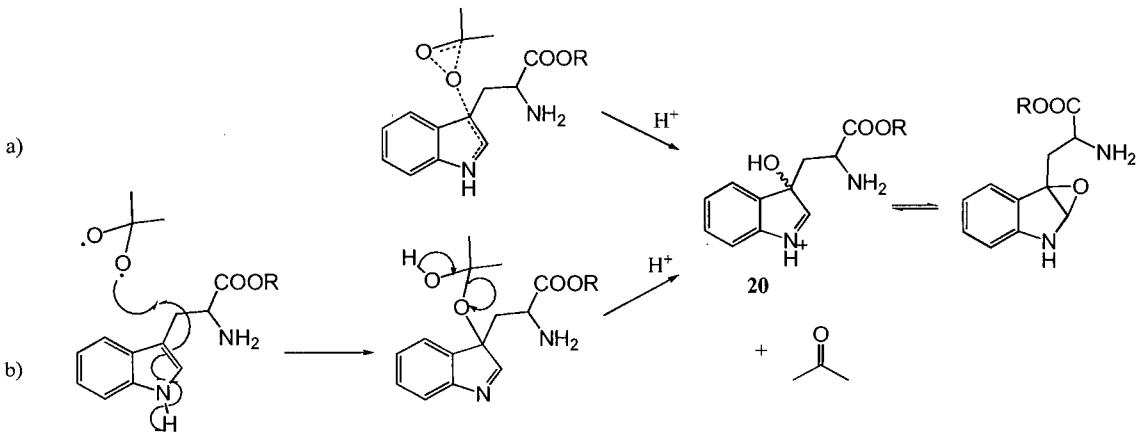
1 Synthesis of derivatives of Hexahydropyrroloindole (Hpi)

1.1 Synthesis of Tr-Hpi-OtBu

We were interested in preparing, by oxidation of the tryptophan indole, an analogue of hexahydropyrroloindole (Hpi, see Scheme 4) **14** that could be stably and reproducibly incorporated into peptide synthesis. Our previous unsuccessful attempts on tryptophan and results found in the literature[38] prompted us to attempt introducing protecting groups on tryptophan before oxidation by dimethyldioxirane.

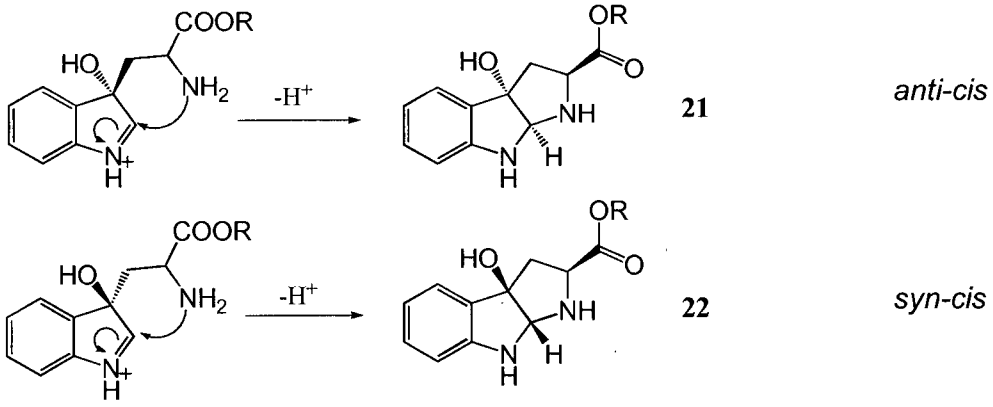
1.1.1 Mechanism of tryptophan oxidation by dimethyldioxirane.

The mechanism of oxidation by dimethyldioxirane (DMDO) is still subject to discussion.[39] Several reports support one of the two alternatives: a concerted mechanism, or a radical, stepwise mechanism. These two possible mechanisms are shown in Scheme 6 in the reaction of DMDO with tryptophan.



Scheme 6: Possible mechanisms for the reaction of DMDO with tryptophan: a: concerted mechanism; b: radical mechanism.

Both mechanisms lead to the oxidized transient intermediate **20**. In **20** attack by the nitrogen atom at C2 can occur only on the opposite face of the newly formed hydroxyl group, thus giving rise to just two diastereomers, the "*anti-cis*" isomer **21** and the "*syn-cis*" isomer **22**.

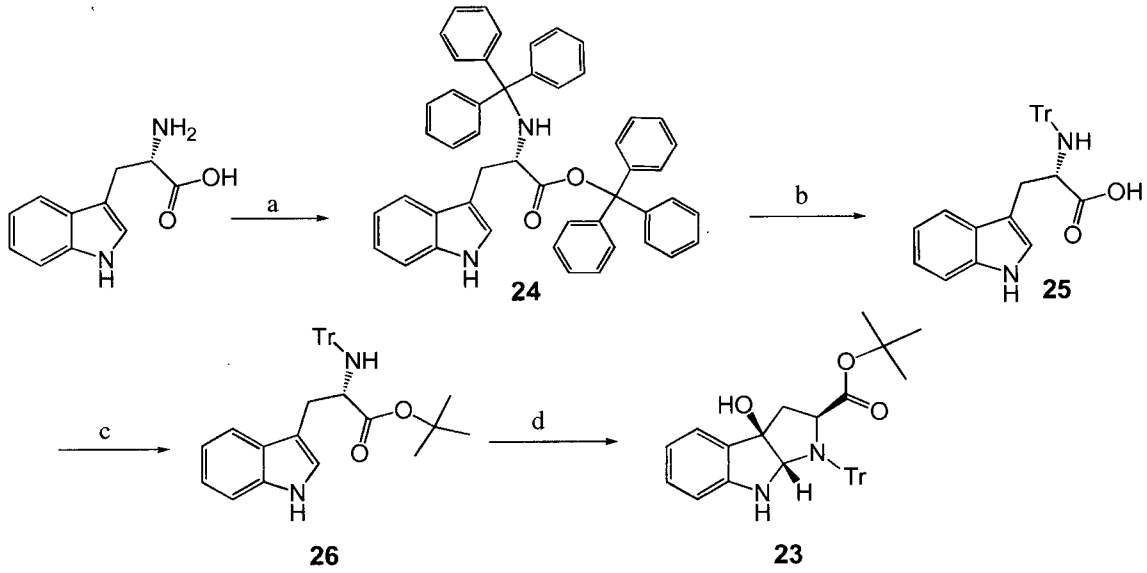


Scheme 7: Formation of the 2 diastereomers of Hpi

1.1.2 Synthesis of Tr-Trp-OtBu

Tr-Trp-OtBu (N-trityl-L-tryptophan *t*-butyl ester) has been reported by Danishefsky et al.[38] to undergo smooth DMDO oxidation and to give rise to the corresponding Hpi. Besides, this choice of protecting groups produced only a single diastereomer, the *syn-cis* isomer. We attempted the synthesis of this compound,

believing this approach would allow us to become familiar with DMDO oxidation methodology and obtain analytical reference without potentially lengthy diastereomer separation. The synthesis of Tr-Trp-OtBu **26** using Danishefsky's method is outlined in Scheme 8.

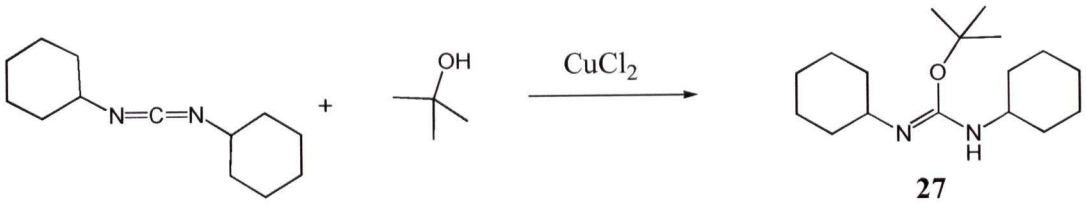


Scheme 8: Synthesis of Tr-Hpi-OtBu **23**. Reagents and conditions: a) TrCl (2.2 eq.), NEt_3, CH_2Cl_2; b) MeOH, reflux, 4h, 68% (2 steps); c) *tert*-butylisourea (10 eq), CH_2Cl_2, reflux, 18h, 62%; d) DMDO, CH_2Cl_2, -78°C, 15 min, 34%.

The trityl protection of the tryptophan primary amine is complicated by the presence in the molecule of the nucleophilic carboxylic acid function. Several methods have been described for the synthesis of N-trityl-protected amino acids.[40,41] The method we chose here[41] uses two equivalents of trityl chloride to form the fully protected N-trityl-tryptophan trityl ester **24**, followed by selective cleavage of the trityl ester by methanol reflux.

Compound **25** was then converted to its *t*-butyl ester, a difficult esterification reaction[42] due to the steric hindrance of the *t*-butyl group. The most common method for *t*-butyl ester formation involves the use of isobutylene in sulfuric acid. This method is not compatible with the acid-sensitive trityl protecting group present in **25** and could therefore not be used. We instead followed Danishefsky's procedure[38] and prepared **26** using *N,N'*-dicyclohexyl-*O*-*t*-butyl-isourea **27**. *O*-Alkylisoureas have been reported as mild, convenient reagents for the alkylation of thiols, amines, phenols, and above all carboxylic acids.[43,44] The alkylation is driven

by the formation of the corresponding urea. *N,N'*-dicyclohexyl-*O*-*t*-butyl-isourea was prepared from *N,N'*-dicyclohexylcarbodiimide (DCC), *t*-butyl alcohol and catalytic $CuCl_2$ (Scheme 9) according to a known procedure.[43,45]



27

Scheme 9: Formation of *O*-*t*-butyl-dicyclohexylisourea **27**.

The reaction can be followed by IR spectroscopy by observing the disappearance of the DCC band at 2120 cm^{-1} and the appearance of the isourea band at 1670 cm^{-1} (see Figure 9). Once the isourea has been formed, the esterification occurs smoothly under reflux for 18 hours. The copper ions, conserved from the isourea formation step, probably intervene to catalyze the esterification reaction.

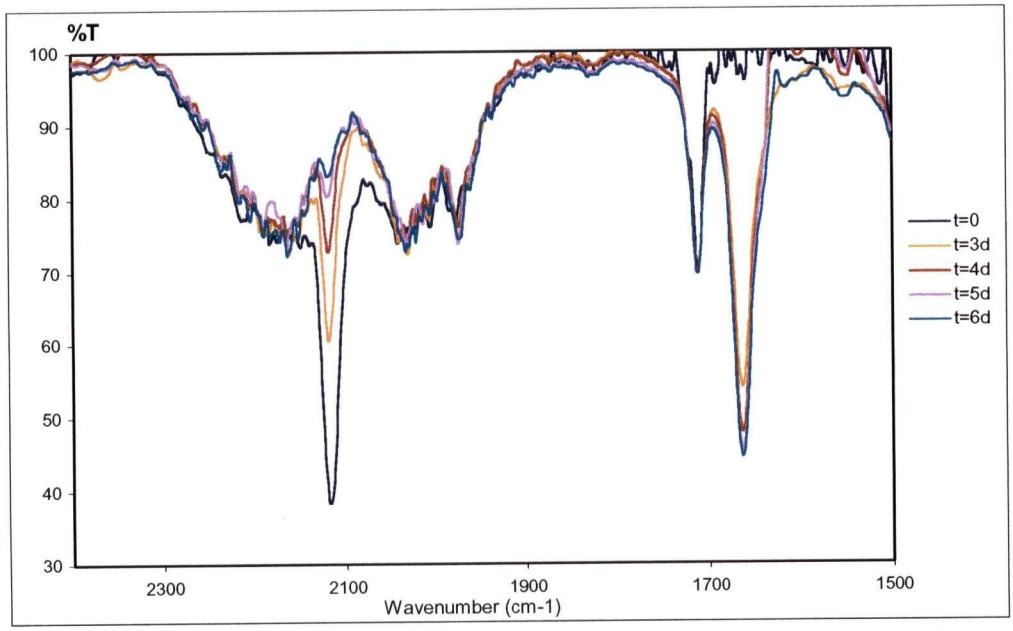
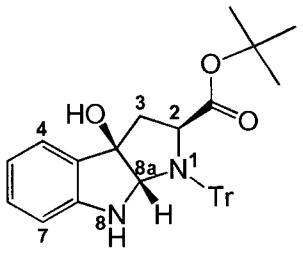


Figure 9: Monitoring of *N,N'*-dicyclohexyl-*O*-*t*-butyl-isourea formation. DCC absorbs at 2120 cm^{-1}, the isourea at 1670 cm^{-1}

1.1.3 Formation of Tr-Hpi-OtBu

1.1.3 Formation of Tr-Hpi-OtBu



23

To synthesize product **23** as reported by Danishefsky[29] the suitably protected tryptophan **26** was reacted with DMDO for 15 minutes at –78°C (see Scheme 9). The NMR spectrum of **23** was found to be consistent with that previously reported[38], which confirmed that a single diastereomer was obtained and not a mixture. Characteristic of the Hpi moiety is the signal for the proton at the 8a-position, which forms a sharp doublet at about 5.4 ppm (see Figure 10). This peak located in a region that is generally void in most peptide NMR spectra, providing a convenient reference for further studies on Hpi.

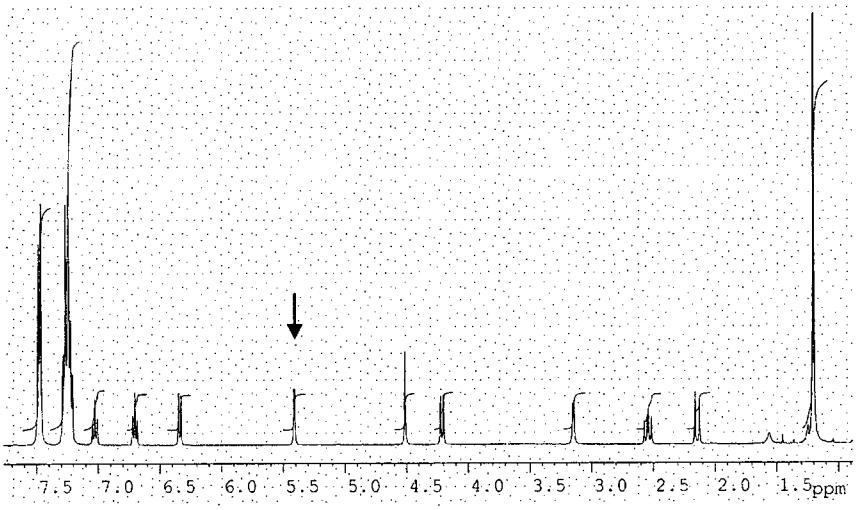


Figure 10: ^{1}H NMR spectrum of **23** (300 MHz, $CDCl_3$). The arrow shows the characteristic signal at 5.4 ppm for H_{8a}.

The protected tryptophan **26** had been optimized to provide a single diastereomer upon DMDO treatment, which simplifies product isolation and avoids a potentially difficult separation. However for our purpose the presence of a mixture of diastereomers would not pose a serious problem; indeed both diastereomers will

1.2 Synthesis of Tr-Hpi-Gly-OMe and Tr-Hpi-Gly-OH

1.2.1 Necessity of an alternative protecting group strategy.

After the synthesis of Hpi derivative **23**, the next step consisted of attaching it to a short cysteine-containing peptide chain at its C-terminus and attempting to form a tryptathionine bridge. It became obvious that the synthesized Tr-Hpi-OtBu would not be adequate for this purpose: cleavage of the *t*-butyl ester to the acid for eventual peptide coupling requires an acid treatment,[42] which would also affect the acid-labile N-terminal trityl group. Moreover acid treatment indeed resulted in general decomposition and gave rise to isolable amounts of oxindole. A different protecting group strategy had to be adopted.

One alternative was to form the *t*-butyl thioester instead of the *t*-butyl ester. This thioester would have a double function: on the one hand it acts as an effective protecting group for carboxylic acids;[42] on the other hand it is an activated form of the acid, which can be used directly in peptide coupling. However there was a good chance that this thioester would be sensitive to oxidation by DMDO, this option was therefore abandoned.

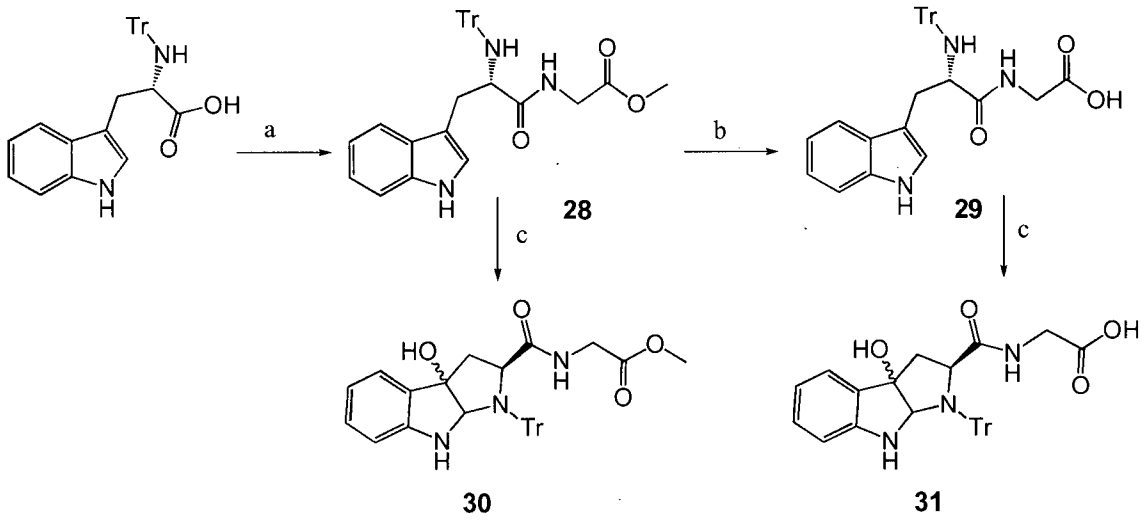
Instead a different approach was adopted: we reasoned that glycine could be used as "C-terminal protecting group", since it was the next amino acid in the amatoxin sequence. The carboxylic acid is thus protected as an amide, a much less nucleophilic function. Yet, a tryptophanylglycine compound would be compatible with the peptide elongation steps that would be necessary to complete the amatoxin ring.

As for the N-terminal protecting group, we chose to stick to the trityl group. Trityl has been shown to be an effective N-terminal protecting group for racemization-free peptide synthesis.[46] A trityl-protected amine may also have a higher nucleophilicity than if protected as a carbamate, which may facilitate the last step of Hpi formation and possibly improve the yield of this reaction.

1.2.2 Preparation of Tr-Hpi-Gly-OMe and Tr-Hpi-Gly-OH

1.2.2 Preparation of Tr-Hpi-Gly-OMe and Tr-Hpi-Gly-OH

The synthesis of Hpi-containing dipeptides **30** and **31** is outlined in scheme 10. A standard DCC/HOBt coupling procedure was adopted for coupling between Tr-Trp **25** and glycine methyl ester. Hydroxybenzotriazole (HOBt) is used as an additive for the activating agent DCC to reduce the extent of racemization in peptide coupling. The obtained dipeptide was saponified to the dipeptide acid **31** by lithium hydroxide in a mixture of dioxane and water.



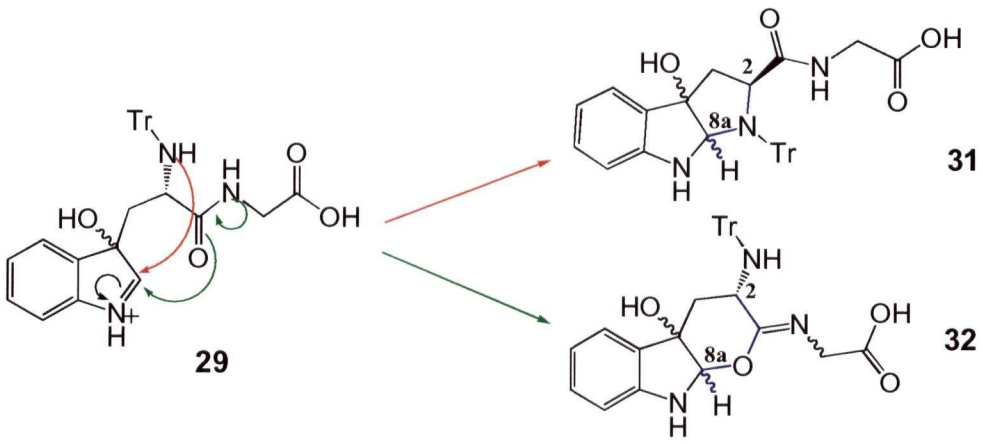
Scheme 10: Synthesis of Tr-Hpi-Gly-OMe and Tr-Hpi-Gly-OH. Reagents and conditions: a) H-Gly-OMe, DCC, HOBt, NEt_3, CH_2Cl_2, 80%; b) LiOH, dioxane/water, 89%; c) DMDO, CH_2Cl_2, -78°C, 15 min, 30%.

Both dipeptides were then subjected to DMDO oxidation. The reaction reached completion within 15 minutes in both cases; however the crude mixture looked much cleaner by TLC analysis in the case of the acid **31** than in the case of the ester **30**.

It should be noted that when carried out on a multi-gram scale this DMDO oxidation gave a low yield (10%). The reaction being very exothermic, it is possible that heat dissipation is difficult when large amounts of reagents are involved, creating elevation of temperature within the solution even if the flask is kept at –78°C. The higher temperature may favor cleavage of the trityl group for example, thus reducing the yield.

1.2.3 Characterisation of the products of oxidation of 28 and 29.

The main spot observed by TLC was isolated and for both **30** and **31** the NMR spectrum contained too many peaks to be consistent with a single product. Every peak seemed to be doubled, whereas MS showed the presence of a single mass: this strongly suggests that 2 isomers of the desired products have been formed. The two products could be simply the *syn-cis* and *trans-cis* diastereomers of Hpi previously described. But we also entertained the possibility of a side-reaction, made possible now that a peptide bond is present in the molecule: the attack by the oxygen atom of the amide linkage, which displays dramatically enhanced nucleophilicity compared to the corresponding acid or ester, due to the donating effect of the amide nitrogen. This would allow formation of a new series of diastereomers (**32**), as shown in scheme 11.



Scheme 11: Possible reactions upon oxidation of dipeptide **29**.

This hypothesis was reasonable, but NMR analysis allowed us to discriminate between the two constitutional isomers **31** and **32**. In isomer **31** three bonds separate the hydrogen atom at the 8a-position and C_2 of Hpi (in blue in scheme 13), whereas in isomer **32** there are four bonds between them. Correlation by HMBC (a two-dimensional NMR experiment that reveals couplings between carbon atoms and hydrogens 2 and 3 bonds away) was well suited to solve this problem. One would expect to observe the scalar coupling between H_{8a} and C_α of Hpi in isomer **31** but not in isomer **32**. As shown in figure 11, HMBC carried out on compound **31a**

(see p.27 for notation) shows a strong crosspeak between H_{8a} and C_2 of Hpi; this piece of evidence rules out isomers **32**, and proves that the two observed compounds are the two diastereomers **31**.

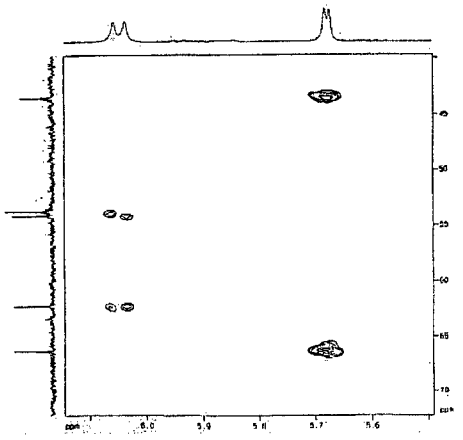


Figure 11: Portion of the HMBC spectrum of compound **31a**. The Hpi 8a proton (5.68 ppm) shows coupling with C_2 (66 ppm) and C_3 (44 ppm) of Hpi. The others spots show coupling of the amide proton (6.02 ppm) with C_α and C_β of serine.

1.2.4 Separation of the diastereomers of Hpi compound 31.

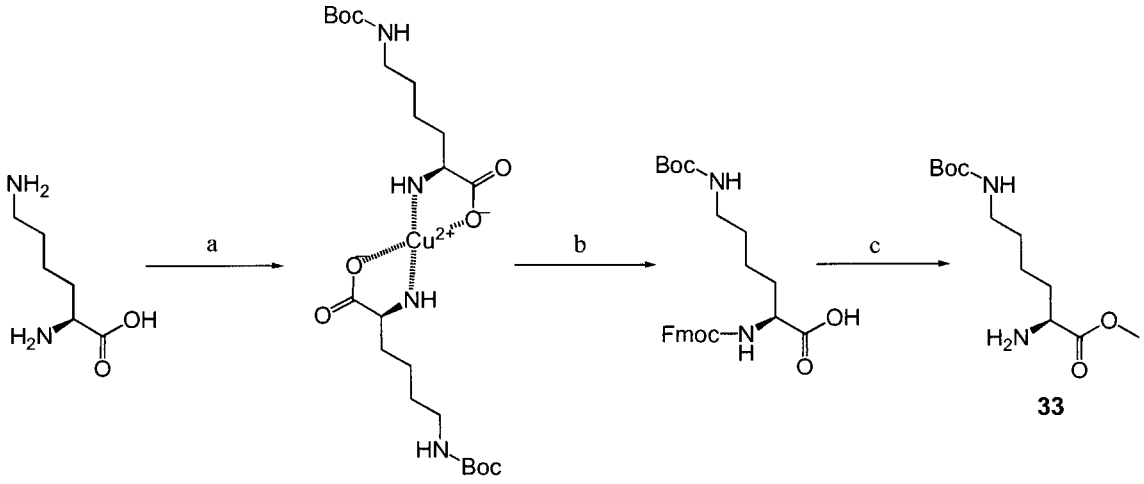
The separation of the two obtained diastereomers is desirable because carrying two compounds along during peptide elongation steps would further complicate the product characterizations. Therefore separation by silica chromatography was attempted. TLC carried out on base-treated TLC plates to avoid acidic cleavage of the trityl protecting group, were found to be ineffective. However, the two diastereomers were separable on non-treated plates. When chromatography was attempted on non-treated silica gel, only the fast isomer was obtained, the slow isomer apparently decomposed on the column. The weak acidity of the silica gel seemed to be strong enough to cleave the trityl protecting group. This may be a limitation to the use of the trityl group as N-terminal protecting group, if silica is to be used for purification.

The NMR spectrum of the faster-migrating isomer displays the characteristic peak at 5.1 ppm for H_{8a} of Hpi. Thus the use of glycine as a C-terminal protecting group for tryptophan is an effective alternative and does not prevent the formation of the desired Hpi compound.

1.3 Synthesis and characterization of other Hpi-containing dipeptides

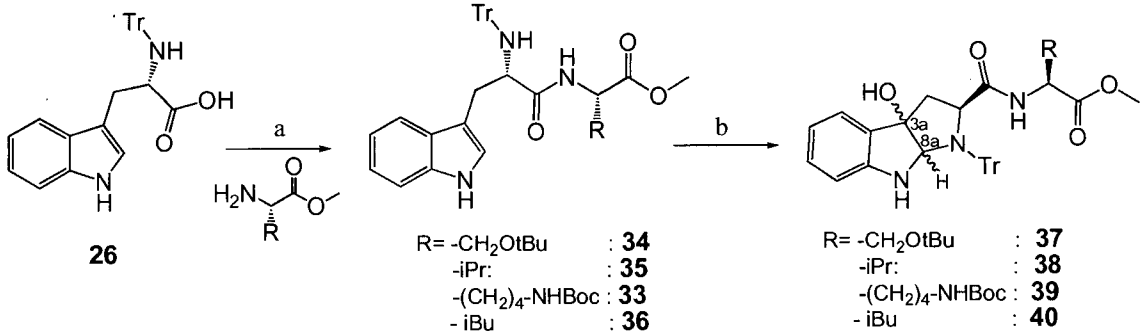
1.3.1 Synthesis of Tr-Hpi-Ser(tBu)-OMe, Tr-Hpi-Val-OMe, Tr-Hpi-Lys(Boc)-OMe, Tr-Hpi-Leu-OMe

We then tried to validate our approach by repeating the experiment on other dipeptides, to verify that amino acid side-chains are not affected by DMDO oxidation. The same reaction scheme was applied to the methyl esters of serine, valine, lysine, and leucine, with Ser and Lys bearing suitably protected side-chains. For side-chain protection of the primary alcohol of serine and of the primary amine of lysine the acid-labile *t*-butyl and Boc protecting groups were respectively chosen. We used commercial *O*-*t*-butyl-serine methyl ester and prepared N_{ω}-Boc-lysine methyl ester **33** according to standard procedures[65] (Scheme 12). The carboxylate and the α-amine of lysine were simultaneously protected using copper sulfate, which was removed with EDTA after the *t*-butyl group had been installed on the side-chain amine. Then the α-amino group was protected with Fmoc, the carboxylate esterified with (trimethylsilyl)diazomethane. Finally removal of the Fmoc protecting group with tris(aminoethyl)amine gave compound **33**.[47]



Scheme 12: Synthesis of N_{ω}-*t*-butyl-lysine methyl ester **33**. Reagents and conditions: a) 1. $CuSO_4$, NaOH; 2. Boc_2O; b) 1. EDTA, NaOH; 2. Fmoc-OSu; c) 1. $Me_3SiCH_2N_2$; 2. TAEA.

Each protected amino acid was coupled with N_b-trityl-tryptophan and then oxidized with DMDO oxidation (Scheme 13) as described previously. Hpi formation occurred at the same rate as before, and it was extremely clean for Ser, Val and Lys, as indicated by high yields for these reactions (Table 1).



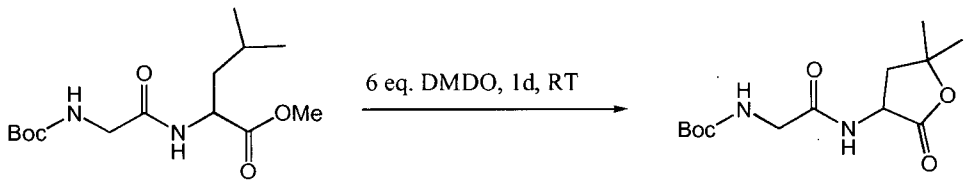
Scheme 13: Synthesis of Hpi dipeptides **37-40**. Reagents and conditions: a) DCC, HOBt, NEt_3, CH_2Cl_2; b) DMDO, CH_2Cl_2, -78°C, 15 min.

Remarkably, two major, well-separated spots were observed. The introduction of an additional chiral center was presumably helping differentiate the R_fs of the two diastereomers. In the following we will designate the fast-migrating diastereomer by the letter "**a**" and the slow-migrating isomer by the letter "**b**".

Table 1: Yields of formation of the Hpi dipeptides.

Dipeptide	Total yield (a+b)	a/b ratio
Hpi-Ser	82%	~1:1
Hpi-Val	92%	~1:1
Hpi-Lys	75%	~1:1
Hpi-Leu	40%	~1:1

Oxidation of Hpi-Leu dipeptide did not occur as smoothly as for the other dipeptides: by-products were observed and a lower yield (40%) was observed. A report by Saladino *et al.*[48] shows that leucine side-chain can be hydroxylated at its tertiary center when subjected to excess DMDO for 1 day at room temperature (Scheme 14). Our results show that even in the milder conditions we used (1 equivalent DMDO, 10 minutes, -78°C), leucine oxidation is not negligible.



Scheme 14: Saladino's DMDO oxidation of leucine

1.3.2 Structure determination for 31a, 37-40.

After separation by chromatography each diastereomer was analyzed by ^{1}H NMR in order to determine the configuration (*syn-cis* or *trans-cis*) of isomers **a** and **b**. Chemical shifts for representative protons are shown in Table 2.

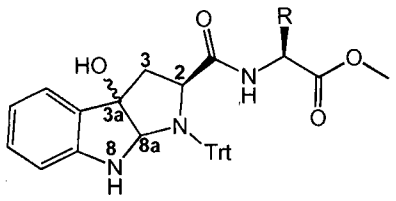


Table 2: Chemical shifts (ppm) for Tr-Hpi-X-OMe dipeptides. (n/d: not determined)

	X	C_{8a}-H	Hpi C_2-H	Hpi C_3-H	Hpi N_8-H	Hpi O-H	Amide N-H
23	n/a	5.40	4.20	2.53, 2.13	3.14	4.50	n/a
31a	Gly	5.31	4.02	2.40, 0.95	n/d	n/d	9.1
37a	Ser(OtBu)	5.68	3.48	2.26, 2.14	2.94	6.27	6.05
37b	Ser(OtBu)	5.27	4.12	2.34, 1.20	n/d	1.20	9.17
38a	Val	5.62	3.59	2.30, 2.14	2.93	5.62	5.78
38b	Val	5.31	4.19	2.36, 1.46	4.76	O.86	8.97
39a	Lys(Boc)	5.54	3.81	2.41, 2.17	2.97	5.40	6.08
39b	Lys(Boc)	5.28	4.13	2.32, 1.33	4.79	0.97	9.13
40a	Leu	5.54	3.81	2.41, 2.17	2.97	5.40	6.08
40b	Leu	5.28	4.13	2.32, 1.33	4.79	0.97	9.13

Table 2 shows that **37a**, **38a**, **39a** and **40a** share a very similar chemical shift pattern, as do **37b**, **38b**, **39b** and **40b**. The patterns for the "**a**" and the "**b**" isomers are very distinct, due to the two configurations (*anti-cis* or *syn-cis*). The most striking differences are the chemical shifts of the amide proton, the Hpi β protons, and the Hpi OH proton. Amide protons usually have chemical shifts between 6.5 and 9.5 ppm, it was therefore surprising to observe amide protons near 6.0 ppm. Similarly amino acid β protons at about 1.3 ppm and OH protons at about 1.0 ppm are quite unusual. By comparison it appears that **31a** is probably in the same configuration as the slow isomers **37b-40b**.

It would be interesting to use the observed NMR patterns to determine the absolute configuration of C_{3a} and C_{8a} of each isomer (see Scheme 13 for notation). We already know from the literature that **23** is *syn-cis*, but **31** does not seem to follow the chemical shift trend observed for the dipeptides, so comparison is impossible. One explanation for the unusual chemical shifts observed in the dipeptides may involve the ring current of the phenyl ring of Hpi.[49] To verify this hypothesis models of both *syn-cis* and *anti-cis* isomers of Hpi containing dipeptides were generated (Figure 12). These models suggest that in the *anti-cis* isomers, a

part of the molecule is folded over the phenyl ring. In that structure it is possible that the amide proton is in the shielding cone of the phenyl ring, which would explain why it is shifted upfield. This would then suggest that the "**a**" isomers (which display low chemical shifts for amide protons) are *anti-cis*. However, the models fail to explain the variation of chemical shifts for β or OH protons between "**a**" and "**b**" isomers, so no conclusion can be drawn.

It may also be necessary to include in the models the ring currents of the trityl protecting groups, which are not shown on these models, but those effects are harder to predict since they are not directionally fixed. Alternatively, phenomena such as solvent effects or hydrogen bonding should be considered to explain the unusual chemical shifts we observed and to assign a configuration to each of the isomers.[50]

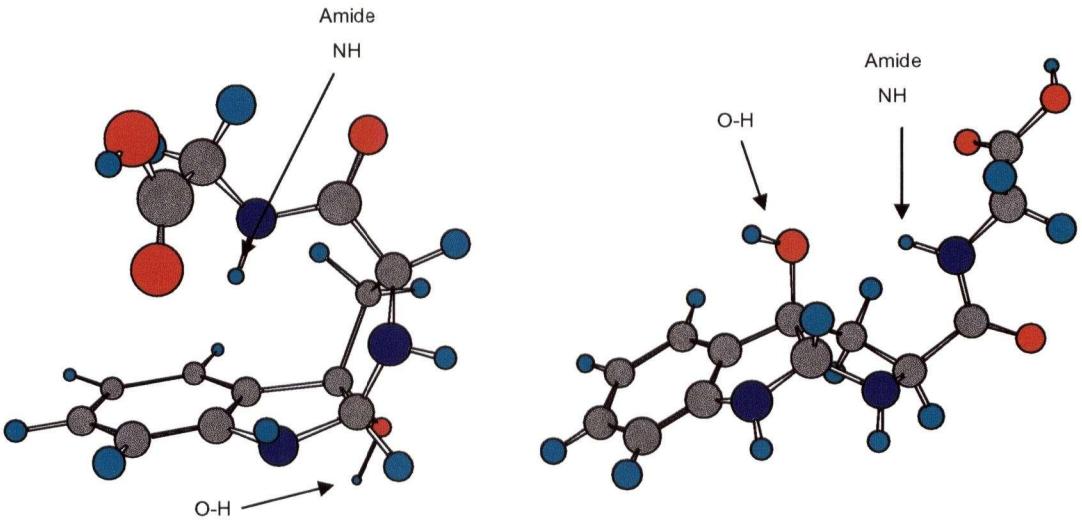


Figure 12: Models of Hpi-Gly **31a** and **31b**, in the *anti-cis* (left) and *syn-cis* (right) forms. The trityl protecting groups were omitted on the figure for clarity. These models were generated by a simple energy minimization using MOPAC.

Nuclear Overhauser Enhancement (NOE) NMR experiments were carried out but they were inconclusive. Indeed no NOE was observed between H_{8a} and H_2 of Hpi in *any* of the considered isomers.

1.4 Conclusion

Four Hpi containing dipeptides were prepared, three of them in high yield (75-92%), as an easily separable mixture of diastereomers. Our methodology, consisting of forming Hpi from dipeptides instead of a protected tryptophan, allows us to form Hpi analogues without protection-deprotection of tryptophan. The obtained dipeptides can be easily saponified for incorporation into peptide synthesis. Studies are under way in our laboratory to further extend the scope of this methodology and confirm the compatibility of other dipeptides with the DMDO oxidation.

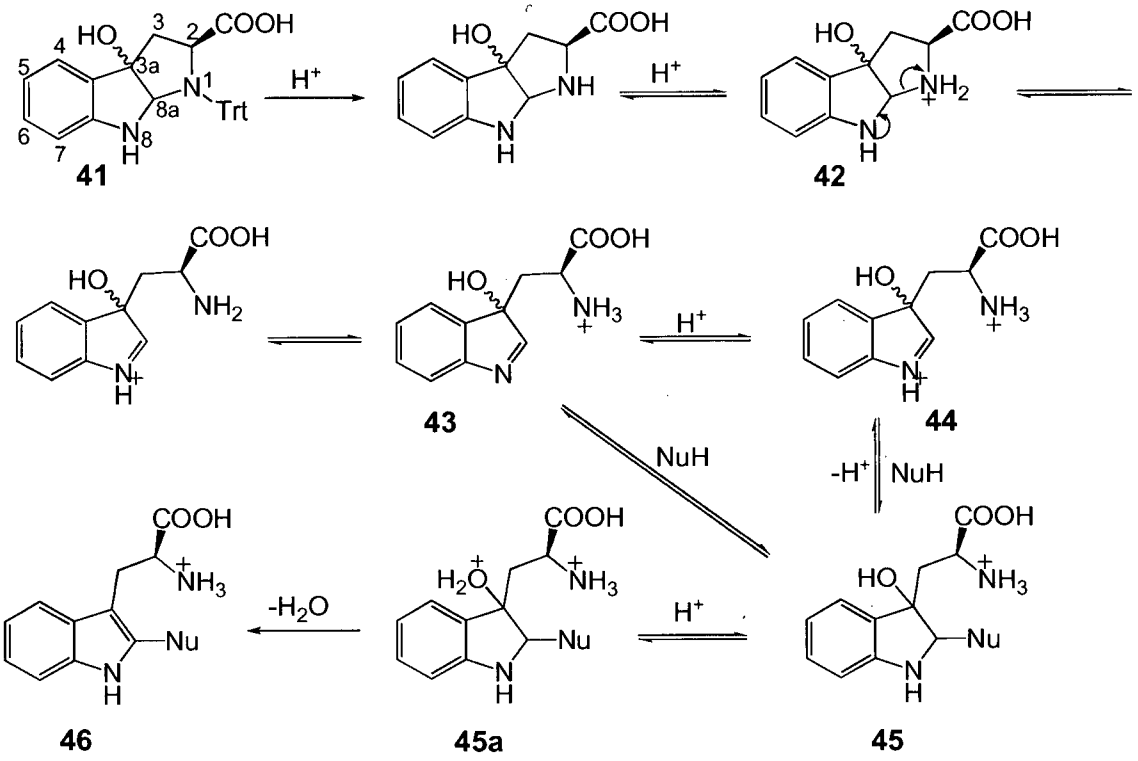
Our approach has a limitation: it prevents the synthesis of Hpi-Trp or Hpi-Cys dipeptides, because tryptophan and cysteine are sensitive to oxidation by DMDO even at low temperature. This limits the range of possible peptides available for the ultimate target of solid-phase combinatorial synthesis.

2 Reactions of Hpi derivatives with nucleophiles

Having demonstrated the efficient formation of Hpi derivatives, we then investigated their utility for tryptophan modification through attack by various nucleophiles.

2.1 Mechanism of attack of nucleophiles on Hpi

The postulated mechanism for the reaction of Hpi with nucleophiles is outlined in scheme 15. This reaction is essentially a nucleophilic substitution at the 8a position of Hpi, with nitrogen N_1 as the leaving group. This observation draws two consequences. First the 8a position of Hpi is a tertiary center, therefore an S_N2 mechanism is probably forbidden; instead the leaving group has to leave before the nucleophile approaches, in an S_N1-like mechanism. Second, a neutral nitrogen atom constitutes a poor leaving group, and must be protonated before leaving, therefore the reaction requires acidic conditions. The pKa of a tertiary amine such as N_1 (~10) is much higher than that of an aniline amine such as N_8 (~5), so in acidic conditions N_1 will be protonated first to form cation **42**. Cleavage of the C_{8a}-N_1 bond is assisted by lone pair donation by nitrogen to give **43**. At this point it is not clear whether the imine is a good enough electrophile, or if it has to undergo a second protonation to **44**; the iminium ion of **44** is admittedly more activated than the imine of **43** but the second protonation requires harsher acidic conditions. Once the electrophilic species (**43** or **44**) is formed, nucleophilic attack can occur and form indoline **45**, which spontaneously eliminates water to re-aromatize and give rise to the desired product. This dehydration may also be facilitated by strong acid that promotes E1 elimination of –OH, through intermediate **45a**. It should be noted that the first equivalent of acid will be used to cleave the protecting group at N_1.

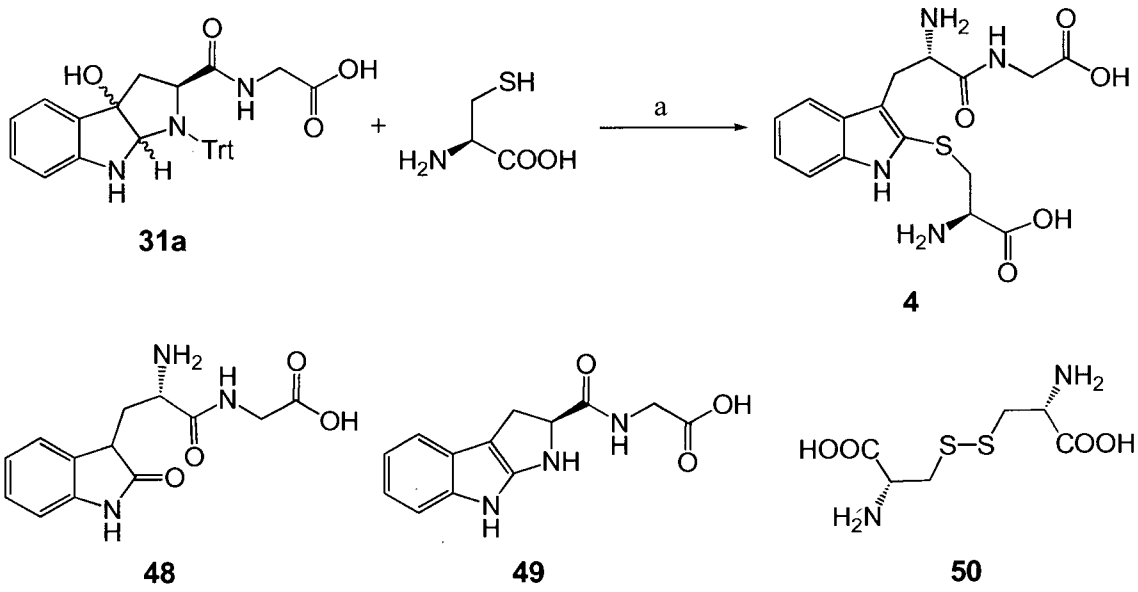


Scheme 15: Mechanism for the attack of nucleophiles on the 2-position of the indole ring

2.2 Intermolecular reaction of Hpi with cysteine

To verify the reactivity of our Hpi derivatives towards nucleophiles the most obvious experiment consists of submitting these compounds to the nucleophile we will eventually use, namely cysteine. This intermolecular reaction is certainly not as favoured as an intramolecular reaction and will likely give a lower yield, but it is an interesting test that can be carried out directly.

Thus the Hpi dipeptide **31a** was submitted to 10 equivalents of cysteine in a 1:3 mixture of trifluoroacetic acid and water (see Scheme 16). After reacting for 2 days a mixture of products was obtained.



Scheme 16: Synthesis of tryptathionine compound **4**. Reagents and conditions: a) TFA/water 1:3, 2d, RT, 12%.

The tryptathionine moiety of the product displays a very characteristic UV absorption at 290 nm, hence UV analysis was usually used to rapidly locate the desired product on the TLC plate. Product **4** was found to co-elute with cysteine, which complicated purification considering the large excess of cysteine introduced in the reaction mixture. However on standing most cysteine precipitated out as the dimer cystine **50** due to oxidation by air oxygen, when left in solution in methanol.

Attempts to purify the crude material by chromatography on silica or on the non-acidic LH-20 medium failed to provide a satisfying separation of the desired product **4**. Eventually a good resolution was obtained by carrying out a chromatography on a short, reverse-phase C18 column with detection of the product by UV spectroscopy at 290 nm. Impurities found in the mixture include oxindole **48** (the product of a putative nucleophilic attack of water on Hpi), as well as the dihydropyrroloindole **49**, the product resulting from dehydration of **4**. The UV spectrum of the isolated product displays the absorbance maximum at 290 nm, with a shoulder on either side at about 282 nm and 299 nm, described by Zanotti et al[34]. Figure 13 shows that this absorbance is characteristic of the tryptathionine moiety since neither the indole compounds (absorbance maxima at 272, 281, 290 nm) nor the Hpi starting material (maximum at 300 nm) share the same pattern. This UV

spectrum also rules out the presence of oxindole, which has an absorption maximum at 250 nm.

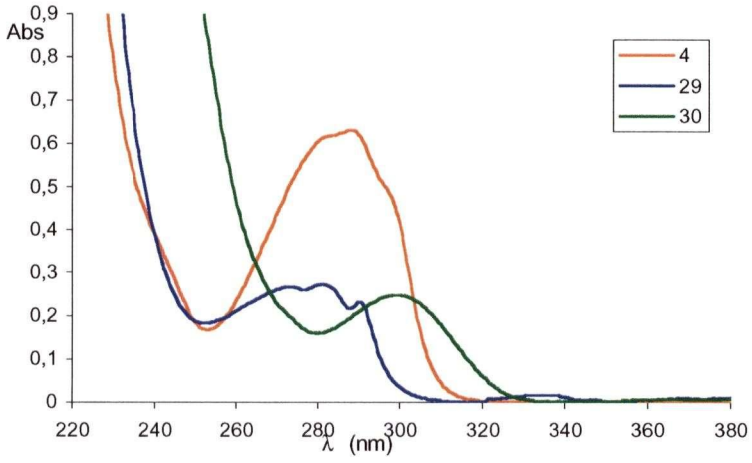


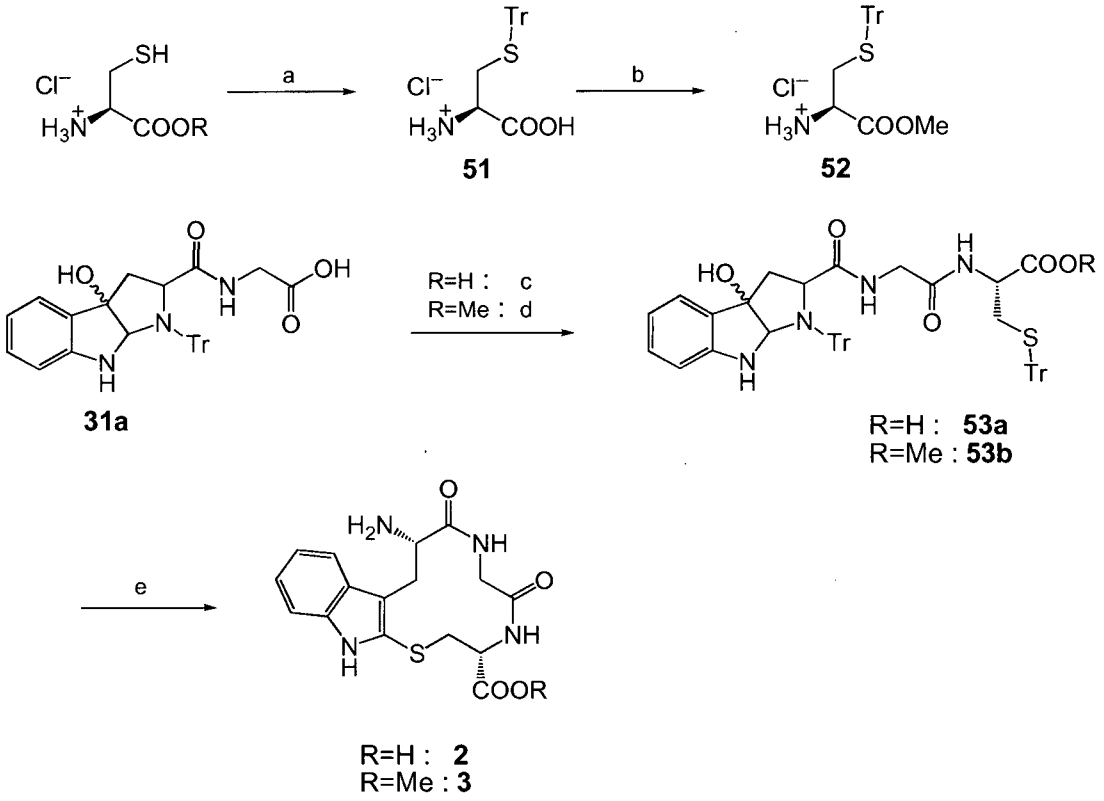
Figure 13: UV Spectra of tryptathionine product **4** and compounds **29** and **30**

The ^{1}H NMR spectrum of the isolated product displays all characteristic peaks for amino acids Trp, Gly and Cys, except that only 4 protons are found in the tryptophan aromatic region, which is consistent with substitution at C_2 of tryptophan.

Based on this initial characterization, we were inclined to conclude that our first attempt to prepare a tryptathionine product was successful.

2.3 Intramolecular reaction of Hpi with cysteine.

The formation of tryptathionine compound **4** confirmed the ability of the Hpi-containing dipeptide **31a** to undergo electrophilic attack. The next step would consist of applying this reaction to the intramolecular case to synthesize a cyclic tripeptide. The synthesis of cyclic tripeptides **2** and **3** is outlined in Scheme 17.



Scheme 17: Synthesis of cyclic tryptathionine compounds **2** and **3**. Reagents and conditions: a) TrCl, DMF, 2d; b) $SOCl_2$, MeOH, 0°C->reflux, 4h, 40% (2 steps); c) 1. isobutyl chloroformate, NEt_3,, CH_2Cl_2; 2. **51**, NEt_3; d) **52**, DCC, HOBt, NEt_3, CH_2Cl_2, 30%; e) TFA, 3h, 24%.

2.3.1 Coupling of cysteine to the dipeptide Hpi-Gly 31a.

The thiol function of cysteine, if left unprotected, does not prevent peptide coupling, on the contrary: upon reaction with a carboxylate function, the thioester, being the kinetic product, rearranges quickly to the desired amide. This reaction has been utilized for coupling of large peptide fragments, in a technique called native chemical ligation.[51] However, we chose to use S-protected cysteine to avoid problems related to disulfide bond formation.

Protection of the cysteine thiol (Scheme 17) was achieved by reacting the unprotected cysteine hydrochloride with trityl chloride in the absence of base. Under these conditions both the acid and the amine of cysteine are in their protonated form and are unreactive towards trityl chloride. The esterification of the resulting S-trityl

cysteine is carried out with thionyl chloride in methanol, which shows the resistance of the S-trityl protecting group to relatively high concentration of HCl.

Our first approach to tripeptide **53** involved the condensation of the free acid form **51** of S-trityl cysteine via the mixed anhydride method: free acid **31a** is first reacted with isobutyl chloroformate to form a mixed anhydride *in situ*, which amine **51** subsequently attacks. The tripeptide **53a** was obtained in poor yield. The reaction was then repeated using the ester **52** and a DCC/HOBt coupling; surprisingly the coupling was still sluggish and the yield obtained was slightly higher. No particular reason was found for the unusual slowliness of this reaction, except that maybe an intramolecular or intermolecular esterification side-reaction with Hpi alcohol. This is unlikely however, because the pendant OH on Hpi alcohol is tertiary and this reaction would form an unfavourable 8-membered ring. The desired peptide **53** could simply constitute a "difficult sequence": it forms intermolecular β-sheet structures that prevent solvent and reagent access.[52] Improving yields remains an important objective.

2.3.2 Cyclization of tripeptides 53a and 53b

To trigger peptide cyclization by tryptathionine formation, tripeptides **53a** and **53b** were reacted in TFA for 3 hours under high dilution conditions, typically at 1 mM concentration. The high dilution is essential to avoid intermolecular reaction of the bifunctional precursor **53**.

Product **3** was purified by chromatography on silica, whereas the more polar product **2** was purified on a reverse-phase C18 column. Both products displayed the expected UV absorption maximum at 290 nm (see Fig 14), characteristic of tryptathionine. In both compounds the NMR analysis showed only 4 aromatic protons and a C-H coupling between the H_β of Cys and C_2 of indole (observed through a HMBC experiment), which proves that the cysteine-tryptophan coupling occurred (see synthesis of **4** above). The high-resolution mass spectra of the isolated products were consistent with the intramolecular cyclization products and not the dimers **55a** or **55b**.

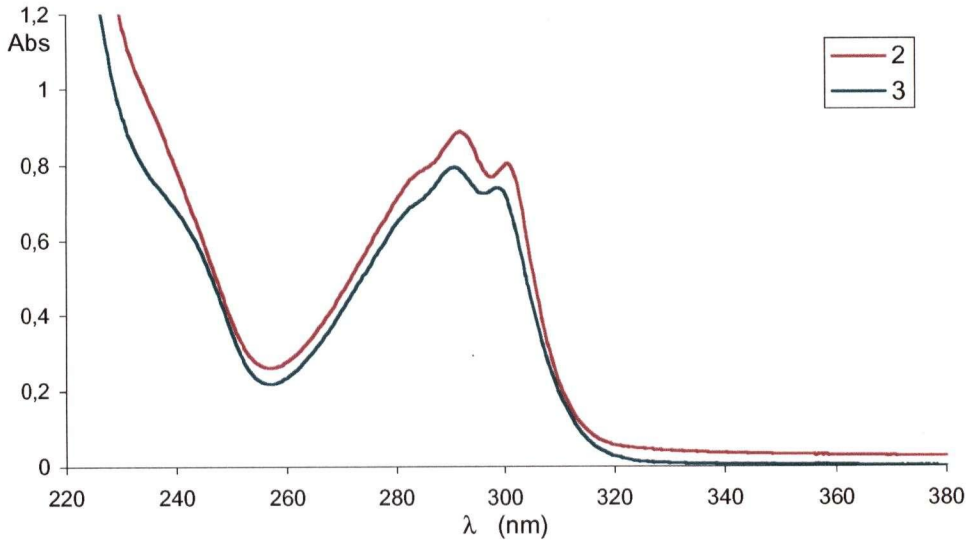
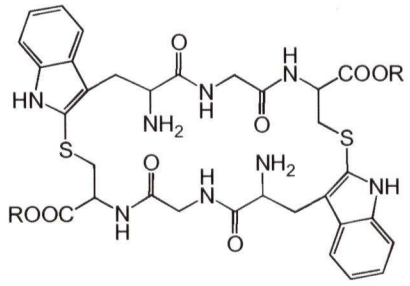


Figure 14: UV spectra or cyclic tripeptides 54a and 54b

In addition, no other compound absorbing at 290 nm was detected by HPLC in the crude mixture, therefore no dimer such as **55a** or **55b** was formed during the reaction.

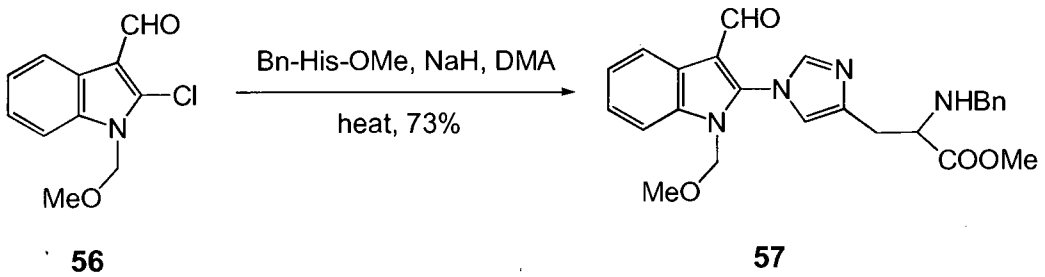


R=H : **55a**
R=Me : **55b**

2.4 Reaction of Hpi with imidazole.

This result demonstrated the possibility of synthesizing amatoxins. However to further generalize the approach and expand the scope of the reaction it would be interesting to know if alternative nucleophiles, besides thiols, can be attached to the 2-position of tryptophan. In that approach our most obvious target was the 2-(1-imidazole)-indole moiety of moroidin (see p.8 for structure). Synthesis of 2-(1-imidazoyl)indoles has been reported. For instance Moody *et al.*[53] used 2-

chloroindole-3-carboxaldehyde **56** as substrate, and subjected it to histidine to form adduct **57** in 73% yield.

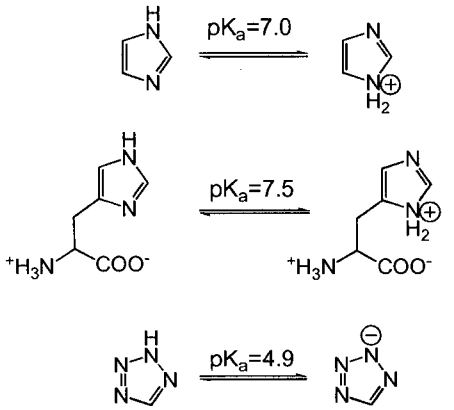


56 **57**

Scheme 18: Moody's synthesis of a 2(1-imidazolyl)indole derivative

But in Moody's procedure the indole is activated by a strong withdrawing group: the aldehyde at the 3-position, and the reaction is carried out in the presence of strong base, two important elements that we cannot transpose to our case.

Coupling an imidazole to tryptophan is clearly more challenging than coupling a thiol, due to the weaker nucleophilicity of the imidazole ring. It remains unclear, and even doubtful, if the Hpi can be used to couple an imidazole to the indole. Indeed, Savige *et al.* [31] reported submitting Hpi to a mixture of all 20 natural α-amino acids in TFA, or even to a whole enzyme (RNAse A), to address the possibility that other nucleophiles may react. Nevertheless only cysteine adducts were observed. Savige's finding is not overly surprising. A strongly acidic medium does not favor nucleophilic attack by nitrogen nucleophiles, and it is normal that in TFA only the most nucleophilic side-chain, i.e. that of cysteine, will be reactive. Admittedly imidazole is a significantly weaker nucleophile than thiol, and it is virtually unreactive when protonated. Nevertheless we felt that it would be interesting to study its behavior in less acidic conditions, when a reasonable amount of imidazole is deprotonated.



Scheme 19: Protonation equilibria of imidazole, histidine and tetrazole.

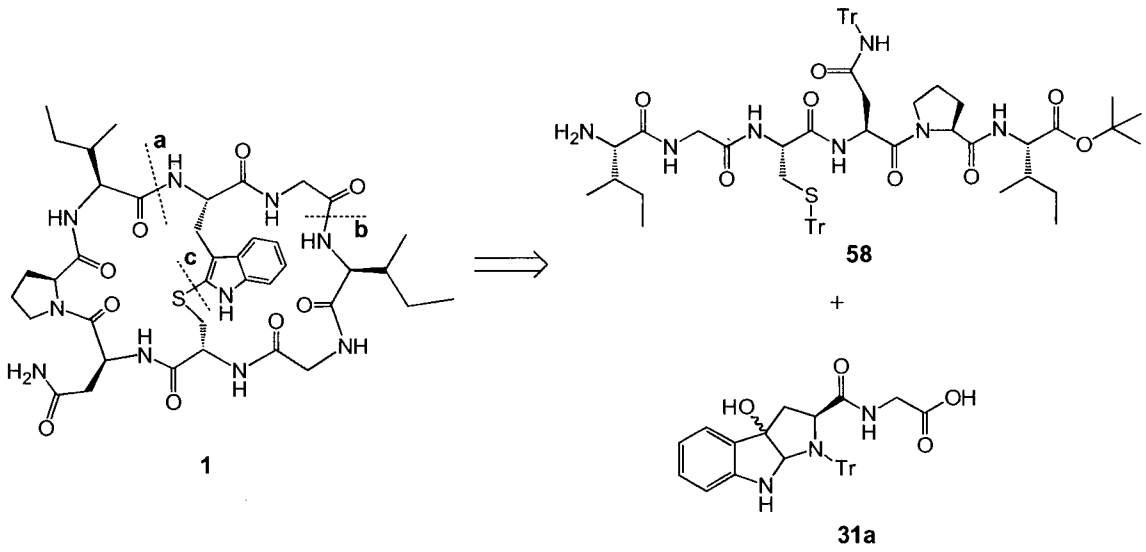
We decided to carry out a series of experiments, and to submit Hpi to imidazole at 4 pH values close to the pKa of imidazole, in two different polar solvents: water and DMF. Imidazole buffers at pH = 5, 6, 7, 8 were prepared. To a solution of de-tritylated Hpi was added a thousand-fold excess of imidazole buffer, in water, or in DMF, and the mixture reacted for several days. The crude mixtures were then analyzed by mass spectrometry, and no evidence of reaction with imidazole was observed. However other products that remain to be identified were observed.

Other experiments will be carried out in our laboratory to verify the reactivity of this type of nucleophiles on Hpi. First, an intramolecular reaction should be attempted, by submitting the dipeptide Hpi-His to TFA. Determination of the pKa of Hpi would certainly help finding what is the optimal pH for the imidazole attack on Hpi. Such pK_a determination could be achieved through simple potentiometric titration or NMR studies as it has been reported for the determination of amino acid side-chains pK_a values in proteins.[54]

If imidazole is unsuccessful it may be replaced by tetrazole, an isosteric heterocycle that displays a much lower pKa (4.9). Tetrazole is thus anionic at neutral pH (Scheme 19) and has more chance to perform nucleophilic attack at low pH.

3 Synthesis of the amatoxin analogue Pro^2-Ile^3-*S*-deoxo-amaninamide 1

The synthesis of **1** primarily followed the Zanotti synthesis of amanin[34]. The retrosynthetic scheme for this synthesis is shown in scheme 20. Disconnection **c** corresponds to tryptathionine formation; disconnections **a** and **b** were chosen as compound **31a** became available to us. Moreover, based on the aforementioned work we wished to maximize the size of the second peptide fragment to be synthesized to give a convergent synthesis of the amatoxin.



Scheme 20: Retrosynthetic scheme for the synthesis of bicyclic octapeptide **1**.

Thus our first target in the synthesis of 1 is the C-protected hexapeptide H-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OtBu **58**.

3.1 Synthesis of hexapeptide 58

3.1.1 Solution-phase vs. Solid-phase synthesis

The choice of solid-phase peptide synthesis is supported by strong arguments such as ease of purification and no solubility issue.[55] On the other hand peptide synthesis in solution allows for the isolation and characterization of

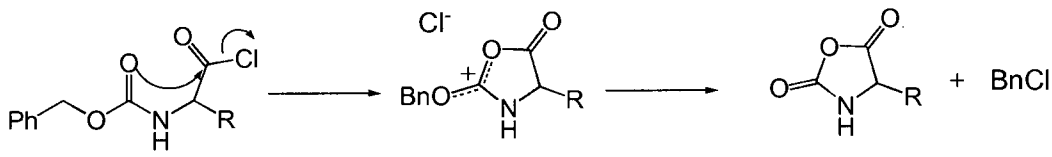
intermediates after every step. For a peptide as short as **58** we decided to use solution-phase synthesis because it does not require any unusual reagent or equipment.

3.1.2 Protection and coupling strategy

As far as solution-phase methods are concerned, the convergent approach is attractive because it is synthetically more efficient, giving a higher overall yield. The protection scheme required is fairly complicated however: it is quite difficult to combine acidic N-terminal and basic C-terminal deprotections with acid-labile side-chain protecting group, such a synthesis would require an elaborated protecting group strategy and would be quite involved. Therefore we turned to a more straightforward method, the "rapid continuous peptide synthesis".

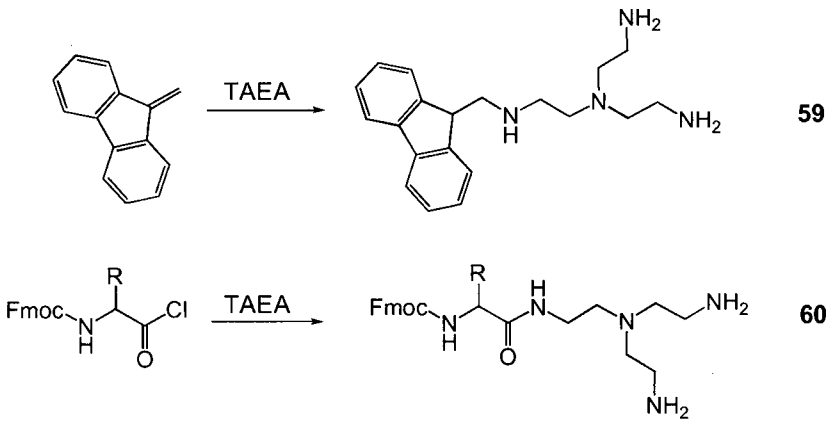
3.1.3 The "rapid continuous peptide synthesis" method.

This solution-phase method, developed by Carpino, has been optimized to speed peptide synthesis, by 1) reducing coupling time; 2) allowing fast purification with a simple water extraction.[56,57] It uses a linear approach, each amino acid is incorporated sequentially as an N-Fmoc-protected acid chloride, allowing a very fast coupling: couplings are complete within 10 to 15 minutes, instead of about 12 hours for standard DCC couplings. Acid chlorides have been used previously for peptide chemistry; they were actually one of the first activated form of amino acids known, applied to peptide synthesis by Emil Fischer as early as 1903, but without the appropriate protecting group they display a very low stability, even in dry conditions.[58] This is due to the strong activation of the acid chloride and the intramolecular reactions it can undergo with protecting groups (see Scheme 21).



Scheme 21: Mechanism of decomposition of Cbz-amino acid chlorides

Their low stability made amino acid chlorides unpractical for peptide synthesis, until the invention of the 9-fluorenylmethylcarbonyloxy (Fmoc) protecting group by Carpino[59,60]. Fmoc is orthogonal to most other protecting groups because it is base-labile. It is very stable to acid, easily deblocked by a solution of secondary amine, and liberates amines as free bases.[60] Due to the particular reactivity of the Fmoc group, Fmoc-acid chlorides cannot undergo intramolecular acylation contrary to Boc- and Cbz-acid chlorides, and are stable indefinitely in anhydrous conditions. After coupling, deblocking of Fmoc is achieved with tris(aminoethyl)amine (TAEA), a particular base specially designed to make all impurities water-soluble. TAEA forms adduct **59** and **60** with the liberated dibenzofulvene and with the acid chloride in excess (Scheme 22), which allows removal of these impurities by a simple water extraction at pH 5.5.



Scheme 22:Formation of water-soluble compounds by reaction of TAEA with impurities.

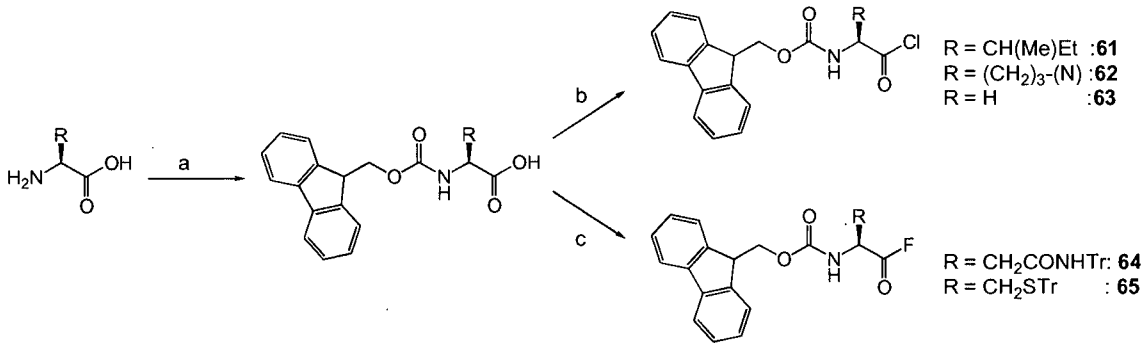
3.1.4 Protecting group strategy.

As far as the C-terminal protecting group strategies are concerned, we chose the *t*-butyl group, cleavable in TFA, over the benzyl group, which requires hydrofluoric acid for cleavage. The only side-chains that need protection are the Cys and Asn side-chains. Protection of the asparagine side-chain amide is not a requirement, but it has several advantages.[61] It increases solubility in organic solvent; in our methodology good solubility in chloroform is required to avoid loss of product during the water extraction. Asparagine protection also rules out aggregation issues and side-reactions such as aspartimide formation or dehydration

of amide to nitrile[55]. For both Cys and Asn the protecting group of choice is the trityl group, which is cleavable in TFA.

3.1.5 Synthesis of *N*-Fmoc-amino acid halides

For the synthesis of Fmoc-amino acids *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (FmocOSu) was preferred over 9-fluorenylmethyl chloroformate (FmocCl) because the latter, as with any chloroformate, can act as a coupling reagent and cause formation of some amount of dipeptide[57]. The reaction was carried out in water/acetonitrile in the presence of triethylamine.



Scheme 23: Synthesis of *N*-Fmoc-amino acid halides. Reagents and conditions: a) Fmoc-OSu, NEt_3, acetonitrile/water, 15 min, RT, 75-89%; b)$SOCl_2$, CH_2Cl_2, 0°C→RT, 30 min, 82-88%; c) DAST, CH_2Cl_2, O°C, 10 min, 76-83%.

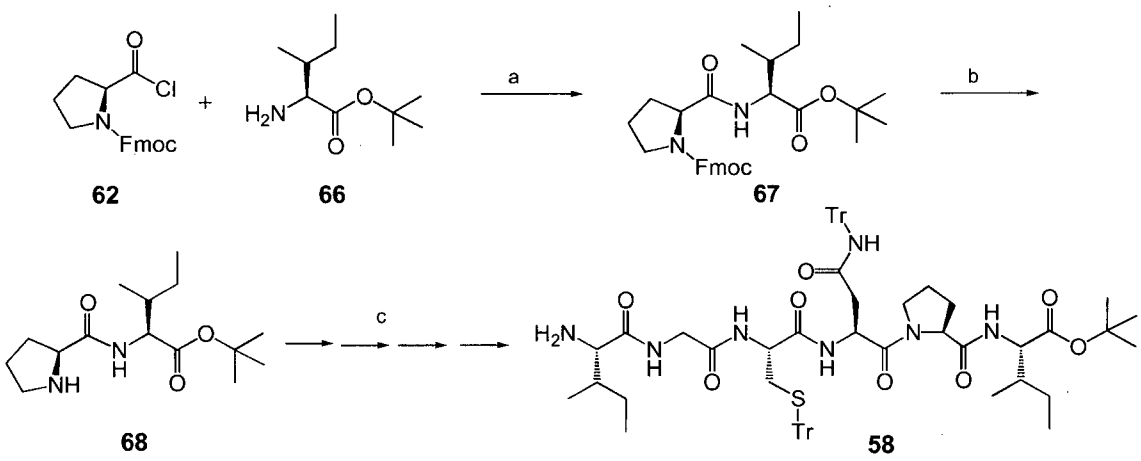
We then had to form the corresponding activated amino acids. The amino acids bearing a protecting group, especially N-trityl-asparagine, are acid-sensitive and will likely not resist thionyl chloride treatment, which releases HCl in the solution. To solve this problem, Carpino generalized the method by replacing acid chlorides with acid fluorides: these reagents are formed in milder conditions, and their lower reactivity make them compatible with Boc and Cbz protecting groups.[57] Acid fluorides are also thought to undergo peptide bond formation in the absence of base, due to the basicity of fluoride ions. This represents a significant advantage because the absence of base rules out the risk of racemization. On the other hand acid fluorides are slightly slower to couple than acid chlorides: coupling time is about 45 minutes instead of 15 minutes for the chlorides. We will therefore use acid

chlorides for stable Fmoc-amino acids: Gly, Ile, Pro, and acid fluorides for sensitive ones: side-chain protected Asn and Cys.

N-Fmoc-amino acid chlorides and fluorides were formed from the corresponding free acid with either thionyl chloride or with diethylaminosulfur trifluoride (DAST) respectively (Scheme 23)

3.1.6 Synthesis of hexapeptide 58.

The reaction sequence for the synthesis of **58** is displayed in Scheme 24.



Scheme 24: Synthesis of Hexapeptide **58**. Reagents and conditions : a) CH_2Cl_2, H_2O, $NaHCO_3$, 15 min, RT ; b) TAEA, 30 min, RT ; c) repeat a) and b) with **64**, **65**, **63**, **61** successively.

H-Ile-OtBu **66** was prepared by using *O-t*-butyl-dicyclohexylisourea as described before. It was then submitted to the activated Fmoc-Pro-Cl in a biphasic system made of chloroform and an aqueous 5% solution of sodium carbonate. The reaction was found to be complete after 15 minutes, and no capping step was carried out. The chloroform phase was then treated with tris(aminoethyl)amine, to remove the Fmoc protecting group and quench the excess Fmoc-Pro-Cl. The deprotection by-products, as well as the unreacted Fmoc-Pro-Cl, were removed by repeated extractions with a phosphate buffer at pH 6.5. It is important to ensure that all deprotection go to completion, since incomplete deprotection of amino acid n will result in deletion of AA n+1. The process described above was repeated four times

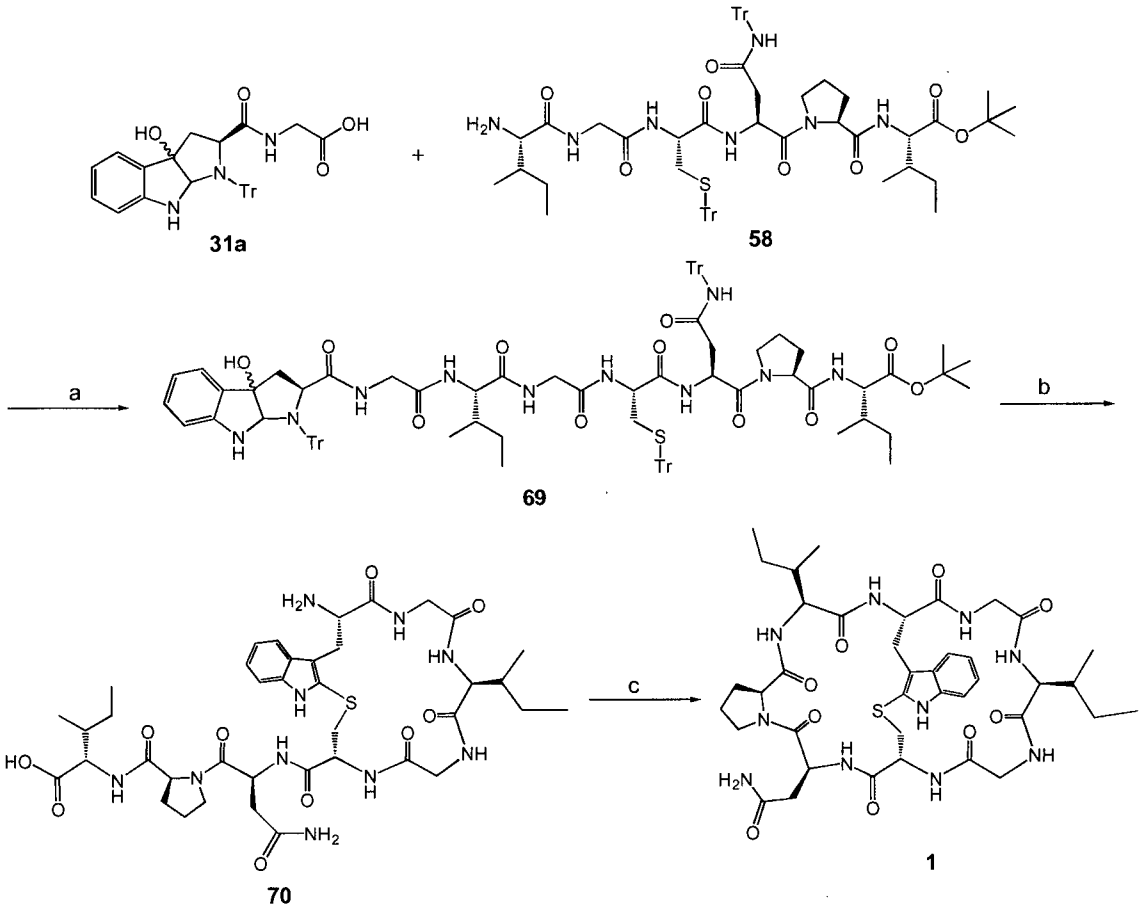
to couple the remaining four amino acids. The hexapeptide was obtained in 48% yield over 5 steps. The purification of the product was achieved by flash chromatography, no HPLC purification was required.

The methodology described by Carpino is efficient, but it is not as rapid as it seems. In our hands The TAEA-dibenzofulvene adduct was not always soluble enough in water: the extraction was not complete, there is some amine left in the mixture. This obliged us to carry out filtrations on silica to remove the polar free amine, and this twice during the synthesis of **58**. Besides, there is sometimes loss of a significant amount of peptide in the slightly acidic aqueous buffer, that requires lengthy back-extraction into chloroform. Finally, contrary to what the authors claim, acid chloride coupling does not go to completion in the absence of base, even after reacting it for 3 hours. It is complete within 10 minutes after addition of base.

Overall the "rapid continuous peptide synthesis" does provide significantly faster couplings, and provides a product with satisfying yield and purity.

3.2 Synthesis of amatoxin analogue 1

With hexapeptide **58** in hand, we carried out the synthesis of amatoxin analogue **1** according to Zanotti's method, which involved formation of a linear octapeptide precursor that in the presence of TFA underwent tryptophanylation at cysteine (Scheme 25).



Scheme 25: Synthesis of compound **1**. Reagents and conditions: a) PyBOP, HOBt, CH_2Cl_2, RT, 15h, 66% total; b) TFA, 3h, 38%; c) PyBOP, HOBt, CH_2Cl_2, RT, 15h, 48%

3.2.1 Synthesis of octapeptide 69.

The coupling of C-protected hexapeptide **55** with N-protected dipeptide **28a** was achieved using PyBOP as the coupling reagent, with HOBt as an additive in excess. The yield (66%) was reasonable for a coupling of this type between two peptide fragments. After work-up two products were observed, with one being a detritylated analogue of **69**. This product was not characterized, it was subjected to the next step and combined with compound **70**. Fully protected octapeptide **69** was obtained pure after chromatography on silica gel, but the NMR spectrum showed only broad peaks and was hard to analyze. This result can be explained by the presence of several conformations or secondary structures of **69** in solution.

3.2.2 Synthesis of cyclic compound 70.

The formation of the tryptathionine bridge between tryptophan and cysteine was carried out in TFA in high dilution conditions as described above for compounds **54a** and **54b**. At the same time the TFA treatment removes the two trityl side-chain protecting groups, as well as the N- and C-terminal protecting groups, leaving both ends free for coupling in the next step. The product was purified by chromatography on a C18 column. It displayed the same spectroscopic features as compound **54a**, namely strong UV absorption at 290 nm, and characteristic NMR pattern. The high resolution mass spectrum was consistent with compound **70** and no trace of dimer was observed.

3.2.3 Synthesis of amatoxin analogue 1.

The second cyclization was carried out in the same conditions as for the formation of octapeptide **69** but this time in high dilution, again to avoid dimerization or oligomerization of bifunctional compound **70**.

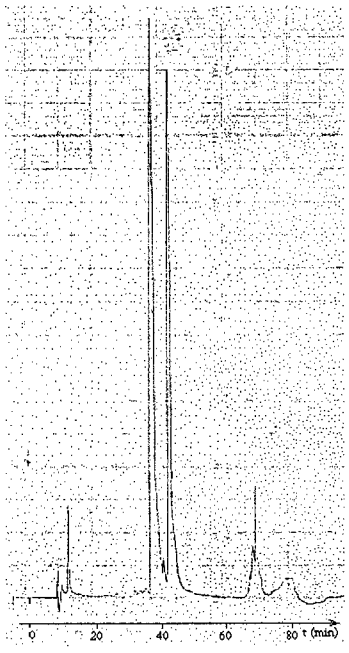


Figure 15:HPLC elution profile of the product of cyclization of **70** after silica gel chromatography. Conditions: C18 column (see experimental), gradient of water(+0.1% TFA)/acetonitrile(+0.05% TFA) from 80/20 to 55/45.

After purification by silica chromatography a single spot was observed by TLC. Further purification by HPLC allowed to separate *two* compounds (Figure 15), present in the purified product and co-eluting by TLC.

3.2.4 Characterization of the bicyclic products.

Both products had the same high-resolution mass and a similar UV spectrum (Figure 18), with an absorption maximum at 290 nm. Their NMR spectra were quite different however, which proved that they are really two distinct compounds. We concluded that the two products are the two possible atropisomers of compound **1**. Two atropisomers have been reported for other amatoxins analogues,[8] but only in amatoxins containing D-amino acids in their sequence. Two isomers can be formed because the carbonyl function of Ile-3 can approach the amine of Trp-4 (see Scheme 25 for residue numbering in **1**) from above or below the plane formed by the existing ring (Figure 16). One isomer will have the tryptathionine function on the same side of the octapeptide ring as the amino acid side chains (we will call it **1**), the other isomer will have it on the opposite side of the ring (we will call it **iso-1**).

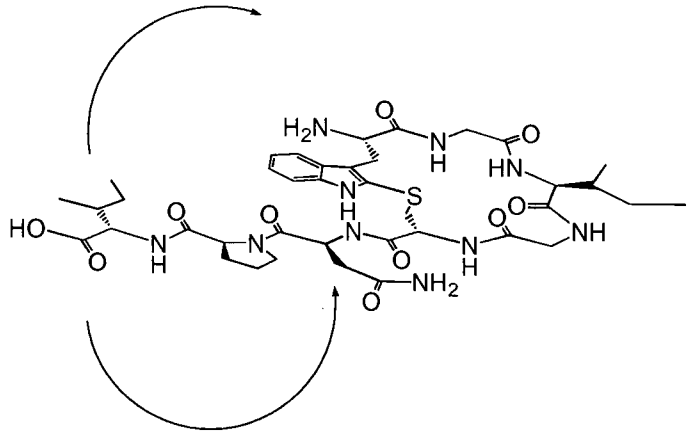


Figure 16: The 2 possible approaches for the terminal carbonyl group (the arrows do not represent a movement of electrons)

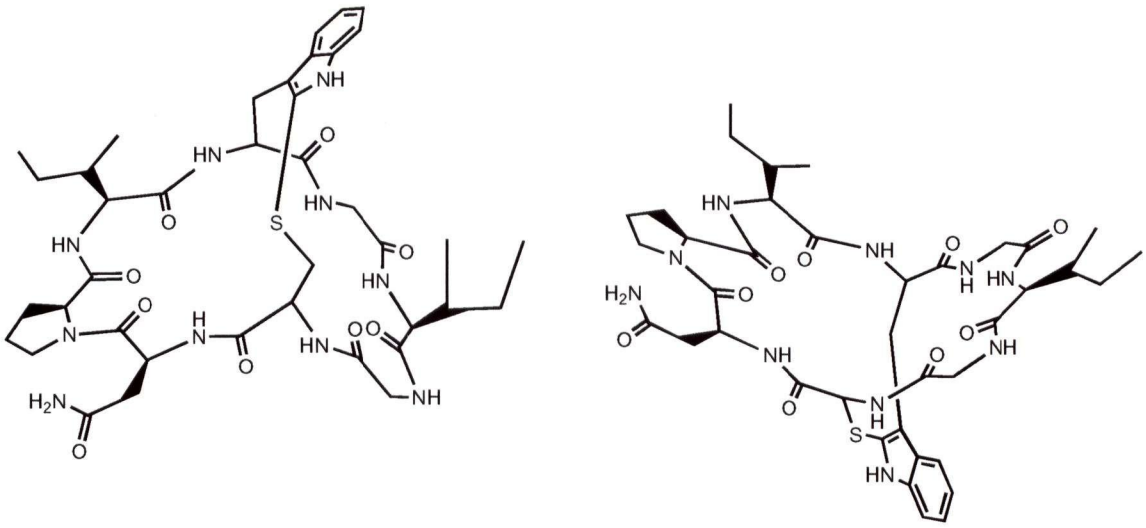


Figure 17: Simplified view of compounds **1** (left) and **iso-1** (right).

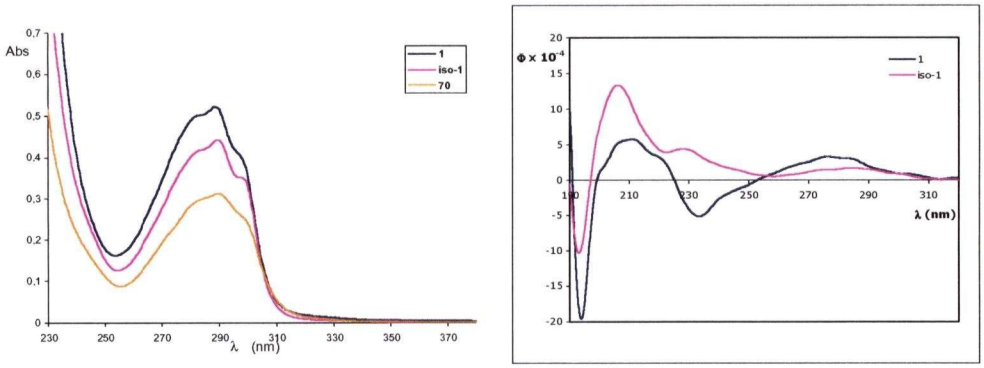


Figure 18: UV spectra of compounds **1**, **iso-1**, **70** and CD spectra of **1** and **iso-1**

The very different CD spectra obtained for the 2 isomers are consistent with this conclusion. Most importantly, CD spectra allow us to assign which of the 2 isomers is likely to be the active one. The slow-eluting isomer (in HPLC) displays a negative Cotton effect at 230 nm, therefore by comparison with the literature[8] it is isomer **1**, which is the active atropisomer. **Iso-1** displays a positive Cotton effect at 230 nm.

The MS/MS spectra of both isomers were found to show the same fragmentation pattern,[61] with different abundances. The MS/MS spectrum of **1** is shown in Figure 19. Analysis of the fragments also enables us to verify that the sequence of the octapeptides is correct. MS peaks in the spectra of **1** and **Iso-1** can be grouped by three: for each expected fragment in addition to the parent $[M+H]^+$

peak we observe a M+H-17 and a M+H-28 peak, the last two corresponding probably to the loss of NH_3 and CO, respectively.

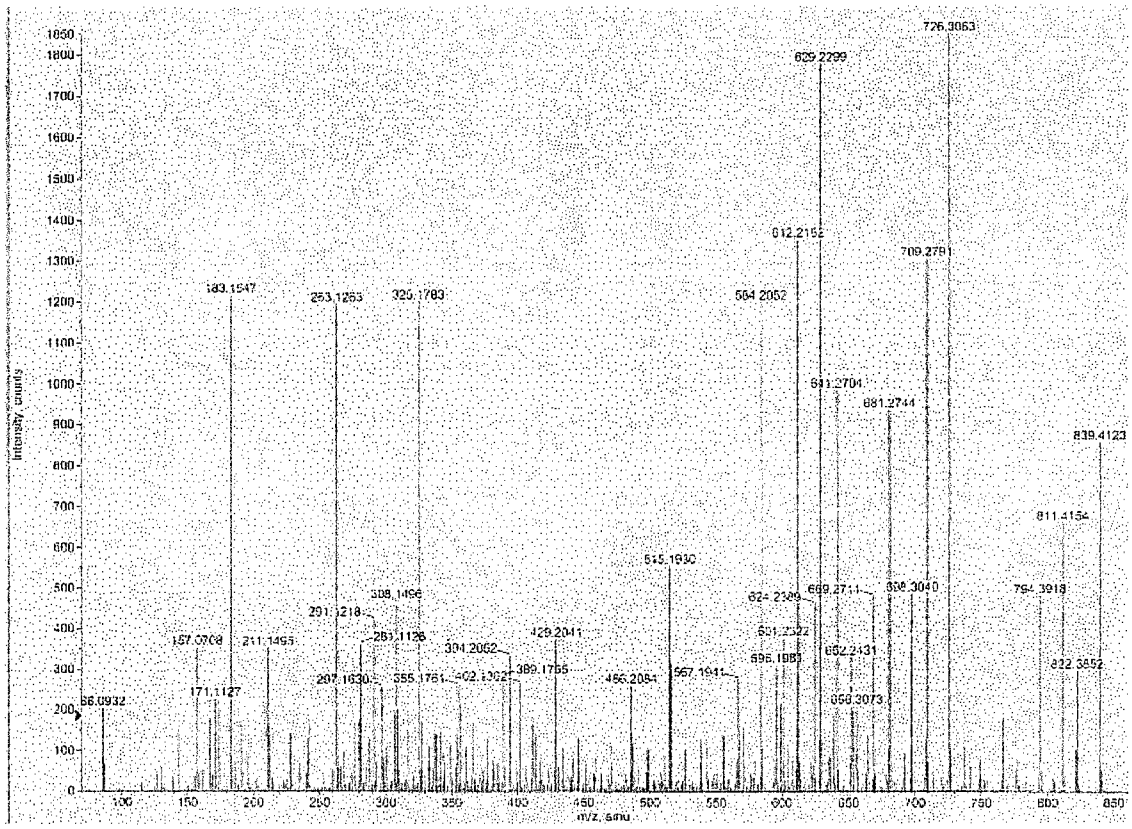


Figure 19: MS/MS spectrum of compound **1**.

Most major peaks can be assigned to an expected fragment (Figure 20), this helps confirm that the sequence of the peptide is correct.

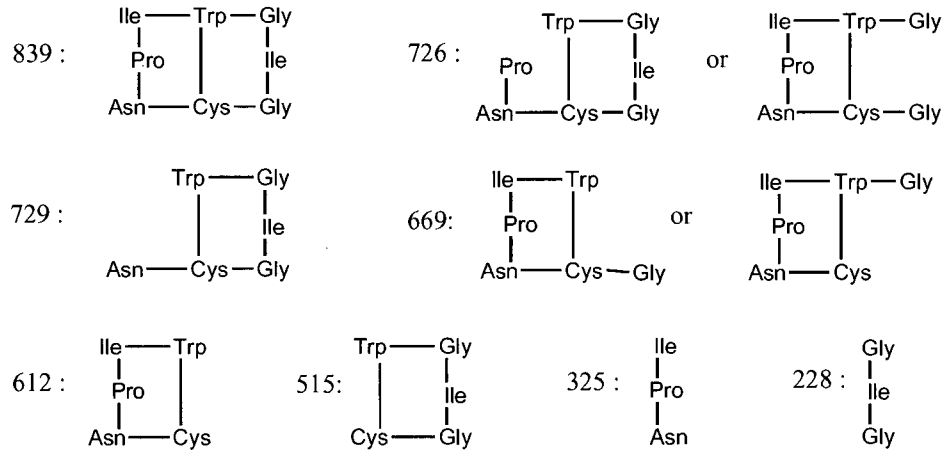


Figure 20: Assignment of masses observed on the MS/MS spectrum of compound **1**.

The assignment of NMR peaks for **1** was achieved using the 2D NMR experiments COSY, HMQC, HMBC, and TOCSY. The latter is especially useful for the assignment of proton NMR peaks in peptides and proteins. A TOCSY experiment shows correlations between all protons within a spin system. It thus allows assignment of protons for complicated amino acids, which in TOCSY display a characteristic pattern. In our case the TOCSY spectrum (Figure 21) was useful to assign the protons of proline and isoleucine. A NOESY spectrum was attempted but gave no result because of the small amount of sample available.

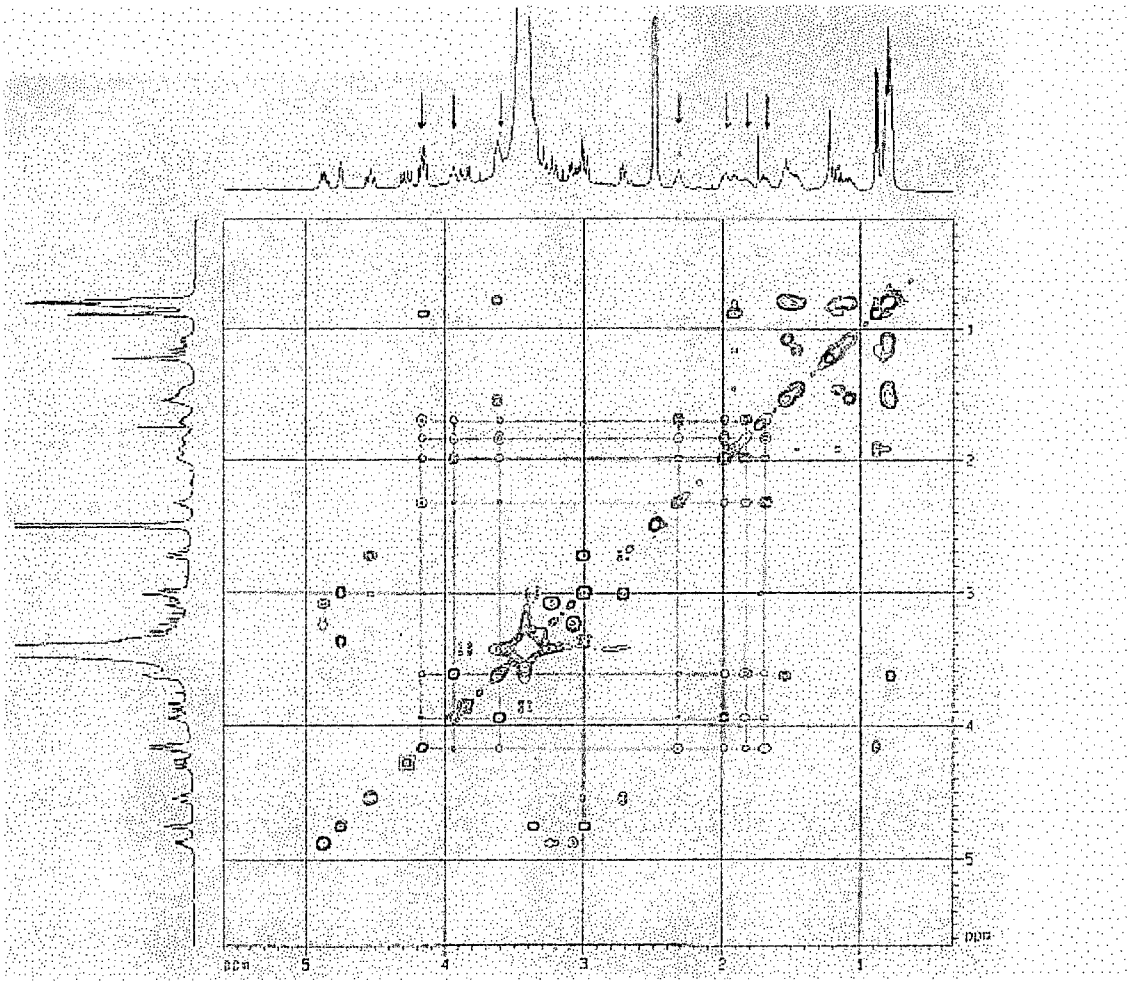


Figure 21: TOCSY spectrum of compound 1; the arrows show the position of the proline protons.

The data available thus confirms that we obtained the desired compound **1**.

4 Conclusion

In this work we have shown that Hpi could be synthesized from dipeptides containing tryptophan at their N terminal. This approach gives good yields and limits the use of protecting groups.

The synthesis of amatoxin analogue **1** has been achieved and is available for crystallographic studies as a complex with its target enzyme, RNA Pol II. This compound is the first of a series of compounds, containing modifications on the proline ring, aimed structure-activity relationship studies. Synthesis of such compounds is being carried out in our laboratory. Studies are also under way for the application of this reaction sequence to solid-phase synthesis.

Chapter 3

Experimental

1 General

All reactions were performed under nitrogen atmosphere in flame-dried glassware unless otherwise stated. Elevated temperature reactions were performed using a mineral oil bath and a temperature controlled hot plate (IKA Ceramag Midi equipped with an IKA ETS-D4 Fuzzy thermometer). Low temperature reactions were performed in an ice/water bath (0°C), an ice/water/NaCl bath (-10°C) or a dry ice/acetone bath (-78°C).

Solvents were purchased from Fisher, except for tetrahydrofuran and *tert*-butyl alcohol, which were purchased from EMD. Except for dimethylformamide, dried over 4Å molecular sieves (three times), anhydrous solvents were obtained by distillation. Tetrahydrofuran and diethyl ether were distilled from sodium in presence of benzophenone. Triethylamine, pyridine and dichloromethane were distilled from calcium hydride. Methanol was distilled from magnesium. *tert*-Butyl alcohol was dried over 4Å molecular sieves. Reagents were purified according to literature procedures[63] or used asprovided by the manufacturer. Unless otherwise indicated, all reagents were purchased from Aldrich Chemicals.

The concentration or removal of solvents under reduced pressure was achieved using a Büchi rotary evaporator. The freeze-drying of water or dimethylsulfoxide solutions refers to the removal of these solvents by sublimation at 50 to 100 mTorr after freezing the solution in a dry ice/acetone bath. A brine solution refers to a saturated sodium chloride solution.

Thin-layer chromatography (TLC) was carried out using silica gel 60 F254 precoated aluminium plates from EM Science. TLC analysis of acid sensitive samples was carried out on plates prerun in a 5% solution of triethylamine in hexanes. In the following procedures R_f refers to a ratio-to-front measured on native

pates, whereas R_f* refers to a ratio-to-front measured on base-treated plates. Detection of TLC spots was effected by UV lamp at 254 and 365 nm or after visualization by ninhydrin. For ninhydrin visualization a solution of ninhydrin (150 mg) in 50 mL n-butanol and 1.5 mL acetic acid was used. Flash column chromatography[64] was performed using silica gel 230-400 mesh from SiliCycle (Quebec). Reverse phase column chromatography was performed on 10 g Waters Sep-Pak prepacked C18 cartridges; pressure was applied manually using a syringe. Analytical high performance liquid chromatography (HPLC) was performed on a Phenomenex Synergi 4m Hydro-RP 250 x 4.6 mm column Preparative HPLC was carried out on a Phenomenex Selectosil 10 C18 250 x 22.5 column. Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter. UV spectra were recorded on a Beckman Coulter DU800 spectrophotometer. Low-resolution mass spectra (LRMS) in electrospray ionization (ESI) mode were determined on a Bruker Esquire spectrometer. High-resolution mass spectra (HRMS) in ESI mode were recorded on a Micromass LCT time-of-flight spectrometer. Tandem mass spectra (MS/MS) were recorded on an Applied Biosystems I API QSTAR Pulsar I spectrometer. Nuclear magnetic resonance spectra were recorded in deuterated solvents (chloroform, water, dimethylsulfoxide, methanol or acetone) supplied by Cambridge Isotope Laboratory, Inc. Proton (^{1}H NMR) spectra were recorded using either a Bruker AC-200 (200 MHz), a Bruker AV-300 (300 MHz), a Bruker AV-400 (400 MHz) or a Bruker AMX-500 (500 MHz) spectrometer. Carbon nuclear magnetic resonance (^{13}C NMR) proton decoupled spectra were recorded using either a Bruker AV-300 (75 MHz) or a Bruker AV-400 (100 MHz) spectrometer. 2D NMR spectra (COSY, HMQC, HMBC, TOCSY) were recorded with 4096 data points in *t*2 and 128 or 256 data points in *t*1. FIDs were extended by forward linear prediction to 1024 data points, zero filled to 2048 data points, and multiplied by sinebell weighting functions in both dimensions prior to Fourier transformation. Chemical shifts for all spectra were reported in parts per million and referenced to the solvent peak.

2 Preparation of HPLC samples

The sample to be purified was dissolved in water or in a water/acetonitrile mixture, and filtered through an Acrodisc syringe filter. The sample was loaded in 5mg fractions dissolved in as much as 4 mL of solvent. The eluent was a gradient of water (containing 0.1% TFA) and acetonitrile (containing 0.05% TFA). The absorbance at 219 nm was recorded, and peaks of interest were manually collected.

3 Reverse phase chromatography

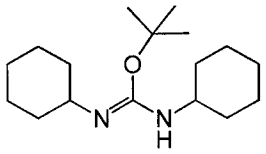
250 mL methanol were passed through a dry SepPak column to swell the stationary phase, followed by 250 mL water to equilibrate the column. The sample (10 to 60 mg) in solution in a minimum amount of water was then loaded, and eluted with increasingly strong methanol/water mixtures, starting with 1% methanol in water, following with 5%, then 10%, 20%, 40% and 60% methanol in water (25 mL of each mixture). 5 mL fractions were collected and analyzed by TLC or UV spectroscopy. When necessary the separation was reiterated with an adjusted methanol/water gradient.

4 Dimethyldioxirane titration[65]

A 0.25 M solution of thioanisole was prepared by mixing 30 µL of thioanisole with 1mL acetone-d6. 0.4 mL of this solution (0.10 mmol) were mixed in an NMR tube at 0°C with 0.5 mL of the DMDO solution to be titrated. A ^{1}H NMR spectrum was recorded, focusing on the region between 7.0 and 8.0 ppm. Traces of sulfone were usually observed, and the peaks from sulfone and sulfoxide were partially overlapping. The integration of the phenyl peaks allows determining the ratios of sulfide, sulfoxide and sulfone present in solution, from which the concentration of the DMDO solution was extracted.

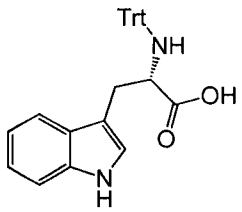
5 Chemical Methods

N,N'-Dicyclohexyl-*O-tert*-butyl-isourea 27



A solution of *N,N'*-dicyclohexylcarbodiimide (2.06 g, 10 mmol) in 10 mL *tert*-butanol was added to a flask containing 33 mg of copper (II) chloride under argon, and stirred at room temperature for while following the reaction by IR spectroscopy. After 5 days, the resulting green slurry was thoroughly concentrated under reduced pressure at room temperature, yielding 2.3 g of a thick green semi-solid that was used without further characterization.

N_b-Trityl-L-tryptophan 25[38]



To a solution of trityl chloride (24.1 g, 86.2 mmol, 2.2 eq.) in 250 mL of a chloroform/DMF 2:1 mixture was added L-tryptophan (8.1 g, 39.17 mmol, 1 eq.), and the resulting slurry was stirred for 1h at room temperature until most of the tryptophan dissolved. Triethylamine (22 mL, 158 mmol, 4 eq.) was then added dropwise over 20 minutes while checking that the temperature remained constant, and the mixture was allowed to stir at room temperature for 5h (R_f for the intermediate ditritylated product: 0.3 (Hexanes/AcOEt 4:1)). 200 mL of methanol were then added, and the reaction mixture was stirred at 50°C for another 5h. 400 mL diethyl ether were then added, and the remaining slurry was washed with a 5% aqueous citric acid solution (3 x 200 mL), and brine (3 x 150mL). The organic phase was dried over magnesium sulfate, concentrated to 200 mL *in vacuo*, and 4 mL (40 mmol) of diethylamine were added. After a few minutes a white precipitate appeared, and the crystallization was allowed to continue overnight. The solid was then filtered, washed with ether; this yielded the diethylammonium salt of the title compound. This solid was then resuspended in a mixture of ether (250 mL) and

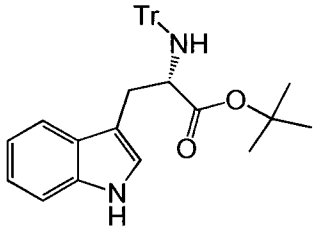
washed with 150 mL fractions of 5% aqueous citric acid solution until it dissolved. The ether phase was washed with brine (2 x 150 mL), dried over magnesium sulfate, and concentrated to dryness, to yield **25** as a tan solid (11.9 g, 68%).

Rf: 0.4 (hexanes/AcOEt 1:1)

Rf*: 0.2 (CH_2Cl_2/MeOH 95:5)

MS (ESI) *m/z:* calculated for $C_{30}H_{27}N_2O_2$ $[M+H]^+$: 447.19, found: 447.32

N_b-Trityl-L-tryptophan *t*-butyl ester 26[38]

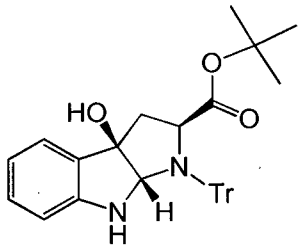


500mg of N_b-trityl-L-tryptophan **25** (1.12 mmol) were dissolved in 2mL dry CH_2Cl_2, this solution was transferred into a flask containing 1.7 g (11 mmol, 10 eq.) of isourea **27** diluted with 4 mL dry CH_2Cl_2, and the mixture was refluxed for 18h. The reaction mixture was then concentrated to dryness under reduced pressure, resuspended in AcOEt and filtered using a sintered glass filter to remove most of the DCU and copper salts. The filtrate was concentrated and purified by chromatography on silica gel (Hexanes/AcOEt 85:15) to yield **26** as a pinkish foam (385 mg, 62%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.95 (s, 1H), 7. 55 (d, J = 8.0 Hz, 1H), 7.4-7.0 (m, 18H), 3.64 (bs, 1H), 3.05 (m, 2H), 2.55 (bs, 1H), 2.14 (d, J = 13.9 Hz, 1H), 0.95 (s, 9H).

MS (ESI) *m/z:* calculated for $C_{34}H_{35}N_2O_2$ $[M+H]^+$: 503.3, found: 503.9.

N_3-Trityl-pyrroloindoline *t*-butyl ester 23



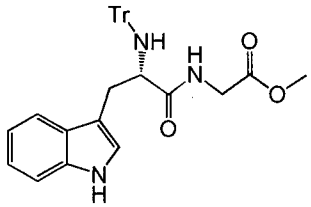
To a solution of N_b-trityl-L-tryptophan *t*-butyl ester **26** (345 mg, 0.687 mmol) in CH_2Cl_2 (5 mL) at –78°C was added 9 mL of a 0.084 M solution of dimethyldioxirane (DMDO) in acetone (0.756 mmol, 1.1 eq.). The solution turned yellow rapidly, and after 15 minutes TLC showed the reaction was not complete. 3 mL of DMDO solution were added, and after 10 minutes the mixture was concentrated to dryness under reduced pressure at room temperature. The crude material was purified by chromatography on pretreated silica gel (Hexanes/EtOAc 95:5) to give **23** (120 mg, 34%).

R_f*: 0.2 (Hexanes/ AcOEt 95:5)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.55 (d, *J* = 6.5 Hz, 6H), 7.25 (m, 10H), 7.05 (t, *J* = 6.9 Hz, 1H), 6.75 (t, *J* = 7.3 Hz, 1H), 6.35 (dd, *J* = Hz, 1H), 5.42 (d, *J* = 3.6 Hz, 1H), 4.51 (s, 1H), 4.18 (d, *J* = 9.3 Hz, 1H), 3.13 (d, *J* = 3.4 Hz, 1H), 2.55 (dd, *J* = 9.5, 13.8 Hz, 1H), 2.14 (d, *J* = 13.9 Hz, 1H), 1.22 (s, 9H).

HRMS (ESI) *m/z:* calculated for $C_{34}H_{35}N_2O_3$ [*M*+Na]$^+$: 519.2648, found: 519.2632.

N_b-Trityl-L-tryptophylglycine methyl ester 28.



A suspension of N_b-trityl-L-tryptophan **25** (7.00 g, 15.6 mmol), L-glycine methyl ester hydrochloride (2.17 g, 17.2 mmol, 1.1 eq.) and hydroxybenzotriazole hydrate (HOBt) (2.33 g, 17.2 mmol, 1.1 eq.) in 80 mL dry dichloromethane with 1.74 g (17.2 mmol, 1.1 eq.) dry triethylamine was put at 0°C, and *N,N'*-dicyclohexylcarbodiimide (3.55 g, 17.2 g, 1.1 eq) was slowly added. After 10 minutes the ice bath was removed and the reaction allowed to react at RT for 14h. The reaction mixture was

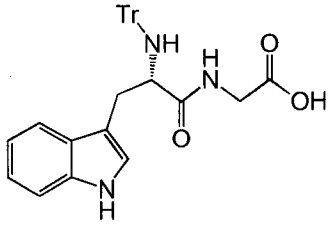
then evaporated to dryness, redissolved in AcOEt and filtered to remove *N,N'*-dicyclohexylurea (operation repeated once). The combined filtrates were washed with 5% aqueous citric acid solution (3 x 100 mL), saturated sodium bicarbonate solution (3 x 100 mL) and brine (2 x 100 mL), dried over magnesium sulfate, and concentrated to dryness. The resulting brown solid was recristallized from ethanol/water. The solid originally filtered out (containing DCU) showed presence of target product by TLC. This solid was suspended in AcOEt (50 mL), washed with brine (2 x 50 mL), the organic phase filtered to remove DCU, dried over magnesium sulfate, concentrated to dryness. The combined fractions of product amounted to 6.465 g (80%).

R_f = 0.4 (Hexanes/AcOEt 1:1)

^{1}H NMR spectrum (300 MHz, $CDCl_3$): δ = 8.03 (s, 1H), 7.55 (d, J = 8.3 Hz, 1H), 7.30-7.00 (m, 19H), 3.63 (s, 3H), 3.45 (m, 3H), 3.12 (dd, *J* = 6.2, 14.7 Hz, 1H), 2.55 (dd, *J* = 5.5, 14.5 Hz, 1H), 2.41 (m, 2H), 1.55 (s, 9H), 1.53 (dd, J = 5.6, 13.1 Hz, 1H).

MS (ESI) *m/z:* calculated for $C_{33}H_{31}N_3O_4Na$ $[M+Na]^+$: 556.3, found: 556.5

N_b-Trityl-L-tryptophylglycine 29



To a solution of N_b-trityl-L-tryptophylglycine methyl ester **28** (2.000 g, 3.86 mmol) in water (10 mL) and 1,4-dioxane (20 mL) was added lithium hydroxide (0.926 g, 38.6 mmol, 10 eq.) and the biphasic solution was stirred at room temperature. After 1h, 20 mL of concentrated HCl were added to the solution to reach pH=7. Upon addition of 30 mL of CH_2Cl_2 and 30 mL of saturated sodium bicarbonate solution, the product precipitates slowly as a jelly (the sodium salt of the product). After letting the precipitation proceed overnight, the solid was filtered, suspended in 40 mL AcOEt and washed with a 5% citric acid solution until it dissolves. The organic phase was then washed with a 5% aqueous citric acid solution (1 x 30 mL), and brine (2 x 40

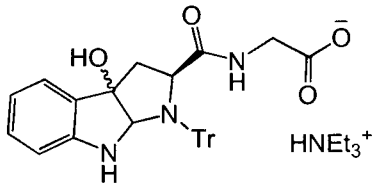
mL), dried over magnesium sulfate and concentrated to dryness under reduced pressure, to give **29** (1.726g, 89%).

R_f *: 0.5 (CH_2Cl_2/MeOH)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.32 (bs, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.25-6.95 (m, 16H), 6.95-6.75 (m, 2H), 3.58 (t, *J* = 4.0 Hz, 1H), 3.36 (d, *J* = 5.2 Hz , 2H), 3.11 (dd, *J* = 5.6, 14.5 Hz, 1H), 2.68 (dd, *J* = 5.9, 14.5 Hz, 1H).

MS (ESI) *m/z:* calculated for $C_{32}H_{29}N_3O_4Na$ [*M*+Na]$^+$: 542.3, found: 542.8.

N_3-Trityl-pyrroloindoline-glycine triethylammonium salt 31a



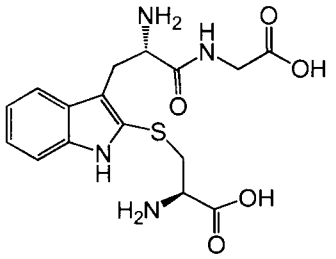
To a solution of N_b-trityl-L-tryptophylglycine **29** (1.673 mg, 3.33 mmol) in CH_2Cl_2 (25 mL) at –78°C was added by 10 mL fractions a 0.059 M solution of dimethyldioxirane in acetone while monitoring the course of the reaction by TLC. After addition of 90 mL of this solution the mixture was concentrated to dryness under reduced pressure at room temperature. The crude material was purified by chromatography on pretreated silica gel (CH_2Cl_2/MeOH/NEt_3 95:5:1, then CH_2Cl_2/MeOH/NEt_3 90:10:1) to give 424 mg of a mixture of diastereomer. The product is homogeneous by TLC on treated plates (R_f = 0.55 in CH_2Cl_2/MeOH 9:1) but displays two distinct spots on non-treated plates (R_f = 0.4 and 0.5 in CH_2Cl_2/MeOH 9:1). This mixture was then purified by chromatography on non-treated silica gel (CH_2Cl_2/MeOH 95:5) to give a colorless oil. This oil was diluted with chloroform (10 mL) and triethylamine (1 mL, large excess) was added. After stirring the mixture for a few minutes, solvents were thoroughly evaporated, to yield **31a** as its triethylamine salt (395 mg, 20%).

R_f*: 0.55 (CH_2Cl_2/MeOH 9:1)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.07 (d, *J* = 4.3 Hz, 1H), 7.70 (d, *J* = 7.6 Hz, 6H), 7.30-7.09 (m, 11H), 7.03-6.95 (m, 2H), 6.63 (t, *J* = 7.4 Hz, 1H), 6.51 (d, *J* = 7.8 Hz), 5.23 (s, 1H), 3.99 (d, *J* = 9.5 Hz, 1H), 3.89 (dd, *J* = 6.69, 17.8 Hz, 1H), , 3.01 (q, *J* = 7.3 Hz, 6H), 2.97 (dd, *J* = 2.3 Hz, 1H), 2.34 (d, *J* = 13.2 Hz, 1H), 1.27 (t, *J* = 7.3 Hz, 9H), 0.95 (dd, *J* = 9.80, 13.2 Hz, 1H).

MS (ESI) *m/z:* calculated for $C_{32}H_{30}N_3O_4$ $[M+H]^+$: 520.2, found: 520.3.

Tryptathionine compound 4



N_3-Trityl-pyrroloindoline-glycine **31a** (30 mg, 0.056 mmol) and cysteine hydrochloride hydrate (100 mg, 0.56 mmol, 10 eq.) were dissolved in 1.5 mL of TFA/water 1:3 and allowed to react at room temperature for 2 days. (The suspension dissolved completely over time). Methanol (10 mL) was then added to the mixture and evaporated under reduced pressure, this procedure was repeated several times until a dry brown residue was obtained. The crude residue was triturated in a mixture water/diethyl ether, the insoluble material, possibly cystine, was filtered out. The filtrate was evaporated to an oil, and re-suspended in 1% MeOH in water. The resulting blurry solution was purified by chromatography on a C18 SepPak cartridge (MeOH 1%, then 2%, then 5% in water), to give after lyophilization **4** as a light white solid (6 mg, 30%).

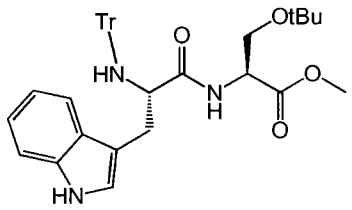
R_f : 0.15 (n-butanol/AcOH/water 4:1:1).

^{1}H NMR spectrum (300 MHz, D_2O): δ = 7.43 (d, *J* = 8.0 Hz, 1H), 7.33 (d, *J* = 8.2 Hz, 1H), 7.16 (t, *J* = 7.5 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 4.09 (t, *J* = 6.8 Hz,1H), 3.71 (dd, *J* = 4.3, 6.7 Hz, 1H), 3.61 (d, *J* = 17.3 Hz,1H), 3.15-3.30 (m, 4H), 3.19 (d, *J* = 17.2 Hz , 1H).

^{13}C NMR spectrum (300 MHz, D_2O): δ = 175.7, 171.9, 169.2, 136.8, 126.7, 125.6, 123.4, 120.0, 118.2, 113.0, 111.5, 43.3, 36.6, 26.5.

HRMS (ESI) *m/z:* calculated for $C_{16}H_{21}N_4O_5S$ $[M+H]^+$: 381.1233, found: 381.1231

N_b-Trityl-L-tryptophyl-*O*-*t*Bu-L-serine methyl ester 34.



To a suspension of N_b-trityl-L-tryptophan **25** (200 mg, 0.385 mmol) and hydroxybenzotriazole hydrate (HOBt) (57 mg, 0.424 mmol, 1.1 eq.) in dry CH_2Cl_2 (2 mL) put in an ice/water bath were added *N,N'*-dicyclohexylcarbodiimide (DCC) (578 µL of a 1M solution in CH_2Cl_2, 0.578 mmol, 1.5 eq.) and triethylamine (161 µL, 1.156 mmol, 3 eq.) and the mixture was allowed to react at 0°C for 15 minutes. *O*-*t*Bu-L-serine methyl ester hydrochloride (from Bachem AG) (90 mg, 0.484 mmol, 1.1 eq.) was then added, as well as 2 mL of dry CH_2Cl_2, and the mixture was allowed to react for 15 minutes at 0°C, then at room temperature for 15h. The slurry was then concentrated to dryness under reduced pressure, 5 mL AcOEt were added, and the resulting slurry was filtered to remove DCU. The filtrate was redissolved in AcOEt, and filtered again. This operation was repeated until the crude mixture was a clear solution in AcOEt. The AcOEt solution (15 mL) was then washed with 5% aqueous citric acid solution (2 x 15 mL), saturated sodium bicarbonate solution (2 x 15 mL) and brine (2 x 15 mL), dried over magnesium sulfate, and concentrated to dryness to give a yellowish solid. The crude material was purified by chromatography on pre-treated silica gel (Hexanes/AcOEt 3:1, then Hexanes/AcOEt 1:1), to give **34** (170 mg, 73%)

R_f : 0.5 (Hexanes/AcOEt 1:1)

^{1}H NMR spectrum (300 MHz, $CDCl_3$): δ = 8.03 (d, *J* = 8.1 Hz, 1H), 7.85 (s, 1H), 7.60-7.10 (m, 19H), 6.95 (t, J = 8.3 Hz, 1H), 6.62 (d, J = 2.2 Hz, 1H), 4.45 (m, 1H), 3.58 (m, 1H), 3.51 (dd, *J* = 2.1, 8.9 Hz , 1H), 3.15 (dd, *J* = 3.3, 14.3 Hz, 1H), 2.41 (m, 2H), 1.55 (s, 9H), 1.53 (dd, J = 5.6, 13.1 Hz, 1H).

MS (ESI) *m/z:* calculated for $C_{38}H_{42}N_3O_4$ $[M+H]^+$: 604.3, found: 604.7.

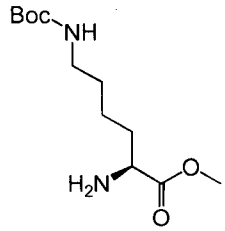
N_b-Trityl-L-tryptophyl-L-valine methyl ester 35.

The same procedure as for **34** was used, with the same amounts of reagents. No chromatography was required for purification. Yield: 155 mg, 72%.

R_f: 0.5 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{36}H_{38}N_3O_3$ $[M+H]^+$: 560.3, found: 560.8.

N_ω-Boc-L-lysine methyl ester 33



To a solution of L-lysine monohydrochloride (2.002 g, 10.95 mmol) in water (12 mL) were added sodium hydroxide (0.876 g, 21.9 mmol, 2 eq.) and a solution of copper sulfate pentahydrate (1.368 g, 5.48 mmol, 0.5 eq.) in water (6 mL). The blue solution was heated to 80°c and allowed to cool down to room temperature. A solution of Boc anhydride (4.782 g, 21.9 mmol, 2 eq.) in 15 mL dioxane was then added, and after bringing the solution from pH 7 to pH~9 by adding about 3 ml of a 2N sodium hydroxide solution, a blue precipitate started to appear. After stirring for 48h the blue precipitate was filtered, rinsed with water, dried under vacuum to give 1.53 g of chelate. This blue solid (2.76 mmol) was suspended in 24 mL of water, and 30 mL of a 0.141 M ethylenediamine tetraacetate (EDTA) solution (made by dissolving 4.128 g EDTA and 2.26 g sodium hydroxide in 100 mL of water) was added and allowed to stir for 3h (the blue solid dissolved after 1h). The resulting blue solution was put at 0°C and treated with 9-fluorenylmethoxycarbonyloxysuccinimide (FmocOSu) (2.046 g, 6.072 mmol, 2.2 eq.), sodium carbonate until pH 9 (1.6 g) and dioxane (10 mL). After stirring for 12h, the solution was put to neutral pH by adding concentrated HCl, and the product extracted into AcOEt (3 x 50 mL). The combined organic phases were dried over magnesium sulfate and concentrated to an oil. This oil was purified by filtration through a silica pad (Hexanes/AcOEt/AcOH 20:10:1), concentrated to obtain a yellowish solid, which was treated with (trimethylsilyl)diazomethane (2 eq.) in 20 mL methanol for 1h and purified by chromatography on silica gel to give **33** as a yellowish solid (310 mg, 11% over 6 steps).

N_b-Trityl-L-tryptophyl-(N_ω-Boc-L-lysine) methyl ester 33

The procedure was identical to formation of **34** and the same amounts of reagents were used.

Yield: 189 mg, 71%

R_f: : 0.5 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{42}H_{43}N_4O_3$ $[M+H]^+$: 689.3, found: 689.6.

N_b-Trityl-L-tryptophyl-L-leucine methyl ester 36

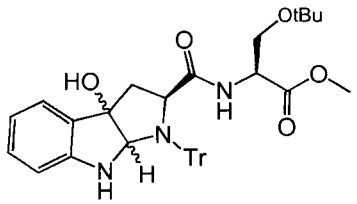
The procedure was identical to formation of **34**, and the same amounts of reagents were used.

Yield : 170 mg, 77%

R_f: : 0.55 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{37}H_{39}N_3O_3$ $[M+H]^+$: 574.3, found: 574.9.

N_3-Trityl-pyrroloindoline-*O*-*t*Bu-L-serine methyl ester 37.



To a solution of N_b-trityl-L-tryptophyl-*O*-*t*Bu-L-serine methyl ester **34** (60 mg, 0.1 mmol) in dry CH_2Cl_2 (1 mL) at –78°C was added 1.9 mL of a 0.66 M solution of DMDO (0.11 mmol, 1.1 eq.). After 15 minutes the mixture was concentrated to dryness under reduced pressure at room temperature. The crude material was purified by chromatography on pretreated silica gel (Hexanes/AcOEt/NEt_3 80:20:1, then Hexanes/AcOEt/NEt_3 60:30:1) to give a fast-running product **37a** (26 mg, 42%) and a slow-running product **37b** (25 mg, 40%).

a : R_f*: : 0.65 (Hexanes/AcOEt 1:1)

HRMS (ESI) *m/z:* calculated for $C_{38}H_{41}N_3O_5Na$ $[M+Na]^+$: 642.2944, found: 642.2930.

b: R_f*: : 0.3 (Hexanes/AcOEt 1:1)

HRMS (ESI) *m/z:* calculated for $C_{38}H_{42}N_3O_5$ $[M+H]^+$: 620.3124, found: 620.3124.

N_3-Trityl-pyrroloindoline-L-valine methyl ester 38.

The procedure was identical to formation of **37**, and the same amounts of reagents were used.

Yield : **a** : 28 mg, 48%; **b** : 25 mg, 44%.

a : R_f*: : 0.6 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{36}H_{38}N_3O_4$ $[M+H]^+$: 576.3, found: 576.2.

b: R_f*: : 0.4 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{36}H_{38}N_3O_4$ $[M+H]^+$: 576.3, found: 576.5.

N_b-Trityl-pyrroloindoline-N_ω-Boc-L-lysine methyl ester 39.

The procedure was identical to formation of **37**, and the same amounts of reagents were used.

Yield : **a** : 25 mg, 35%; **b** : 28 mg, 40%.

a : R_f*: : 0.6 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{49}H_{43}N_4O_4$ $[M+H]^+$: 705.3, found: 705.4.

b: R_f*: : 0.4 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{49}H_{43}N_4O_4$ $[M+H]^+$: 705.3, found: 705.5

N_3-Trityl-pyrroloindoline- L-leucine methyl ester 40.

The procedure was identical to formation of **37**, and the same amounts of reagents were used.

Yield : **a** : 13 mg, 22% **b** : 11 mg, 18%.

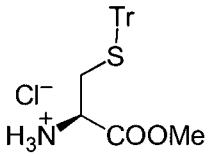
a : R_f*: : 0.6 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{37}H_{39}N_3O_4$ $[M+H]^+$: 590.3, found: 590.7.

b: R_f*: : 0.4 (Hexanes/AcOEt 1:1)

MS (ESI) *m/z:* calculated for $C_{37}H_{39}N_3O_4$ $[M+H]^+$: 590.3, found: 590.6.

S-Trityl-L-cysteine methyl ester hydrochloride 52[9].



L-Cysteine hydrochloride (1.200 g, 6.83 mmol) and trityl chloride (4.757 g, 17.03 mmol, 2.5 eq.) were stirred in 5 mL dry DMF for 2 days at room temperature. A 10% sodium acetate solution was then added (50 mL), a white solid precipitated immediately that was filtered and rinsed with water. The white solid was stirred in acetone at 50°C for 30 minutes, filtered, rinsed with little acetone, and dried under vacuum to give **51** as a white solid (1.854 g, 75%).

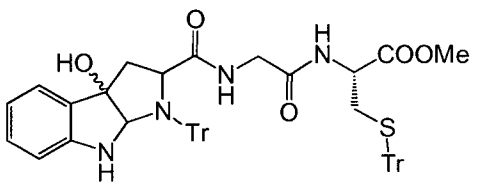
This product was used directly for the esterification: 500 mg (1.37 mmol) of *S*-trityl-L-cysteine was suspended in 10 mL of methanol at 0°C, and thionyl chloride (750 mL, 10.30 mmol, 7.5 eq.) was added dropwise. After 10 minutes, the mixture was allowed to warm up to room temperature, then was stirred under reflux for 4h. The reaction mixture was then evaporated to dryness under reduced pressure, and purified by chromatography on silica gel (Hexanes/AcOEt 4:1, then Hexanes/AcOEt 1:1) to give a yellow oil (265 mg). This oil was treated with 0.7 mL of a 1N HCl solution in methanol to form the hydrochloride salt, and then evaporated to dryness to give **52** as a tan foam (305 mg, 40% over 2 steps).

R_f*: : 0.5 (CH_2Cl_2/MeOH 98:2)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.62 (s, 3H), 7.42 (d, *J* = 7.5 Hz, 6H), 7.3-7.1 (m, 14H), 3.60 (s, 3H), 3.25 (t, *J* = 5.5 Hz, 1H), 2.9 (d, *J* = 5.5 Hz, 2H).

MS (ESI) *m/z:* calculated for $C_{23}H_{23}NO_2SNa$ $[M+Na]^+$: 386.3, found: 386.5.

N_3-Trityl-pyrroloindoline-glycyl-(*S*-trityl-L-cysteine) methyl ester 53b



To a suspension of N_3-trityl-pyrroloindoline-glycine **31a** (150 mg, 0.289 mmol) and hydroxybenzotriazole hydrate (HOBt) (43 mg, 0.318 mmol, 1.1 eq.) in dry CH_2Cl_2 (2 mL) at 0°C were added DCC (433 µL of a 1M solution in CH_2Cl_2, 0.433

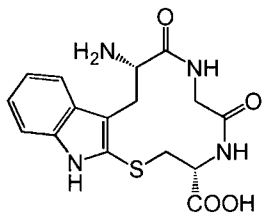
mmol, 1.5 eq.) and triethylamine (120 µL, 0.867 mmol, 3 eq.) and the mixture was allowed to react at 0°C for 15 minutes. *S*-Trityl-L-cysteine methyl ester hydrochloride **52** (131 mg, 0.318 mmol, 1.1 eq.) was dissolved in 1 mL of dry CH_2Cl_2 and 80 µL triethylamine, and this solution was transferred into the reaction flask. The mixture was allowed to react for 15 minutes at 0°C, then at room temperature for 21h. The reaction was not complete, more DCC was added (290 µL of a 1M solution in CH_2Cl_2, 0.290 mmol, 1 eq.) at 0°C, and the mixture allowed to react another 15h. The slurry was then concentrated to dryness under reduced pressure, to give a brown oil, which was redissolved in 10 mL AcOEt, and filtered to remove DCU. The AcOEt solution (15 mL) was then washed with 5% aqueous citric acid solution (2 x 15 mL), saturated sodium bicarbonate solution (2 x 15 mL) and brine (2 x 15 mL), dried over magnesium sulfate, and concentrated to dryness to give a brown foam. The crude material was purified by chromatography on pre-treated silica gel (Hexanes/AcOEt/NEt_3 60:30:1, then Hexanes/AcOEt/NEt_3 60:40:1), to give **53b** (61 mg, 24%)

R_f *: 0.2 (Hexanes/AcOEt 1:1)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.2 (bs, 1H), 7.63 (d; *J* = 7.8 Hz, 6H), 7.1-7.5 (m, 24H), 6.98 (m, *J* = 2H), 6.62 (t, *J* = 5.8 Hz, 1H), 6.53 (d, *J* = 7.8, 1H), 6.31 (d, *J* = 7.7 Hz, 1H), 5.17 (d, *J* = 4.2 Hz, 1H), 4.79 (d, *J* = 4.5 Hz, 1H), 4.51 (q, *J* = 6.8 Hz, 1H), 4.06 (d, *J* = 9.7, 1H), 3.81 (dd, *J* = 6.7, 16.7 Hz, 1H), 3.71 (s, 3H), 3.03 (dd, *J* = 3.7, 16.7 Hz, 1H), 2.66 (d, *J* = 5.2 Hz, 2H), 2.29 (d, *J* = 13.4 Hz, 1H), 1.01 (dd, *J* = 10.1, 13.4 Hz, 1H).

MS (ESI) *m/z:* calculated for $C_{54}H_{49}N_4O_5$ $[M+H]^+$: 879.4, found: 879.7.

Cyclic tripeptide 2[33]



To a solution of N_3-trityl-pyrroloindoline-glycine **31a** (75 mg, 0.14 mmol) in a mixture of 1 mL of dry DMF and 1 mL of dry CH_2Cl_2 at –10°C were added isobutyl chloroformate (iBuCCl) (21 µL, 0.154 mmol, 1.1 eq.) and dry triethylamine (22 mL,

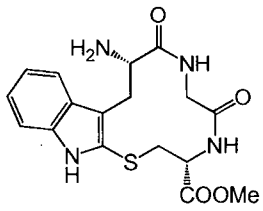
0.154 mmol, 1.1 eq.) and the mixture was stirred at –10°C for 30 minutes. *S*-Trityl-L-cysteine hydrochloride **51** (120 mg, 0.29 mmol, 2 eq.) was dissolved in 1 mL DMF and 100 µL dry triethylamine, and this solution was transferred to the reaction vessel. The mixture was allowed to react at –5°C for 30 minutes, then at room temperature for 15h, after which the reaction was found to have reached completion. 50 mL of AcOEt were then added, the resulting light precipitate filtered, and the filtrate was washed with brine (1 x 50 mL), 5% aqueous citric acid solution (2 x 50 mL), saturated sodium bicarbonate solution (2 x 50 mL) and brine (2 x 50 mL), dried over magnesium sulfate, and concentrated to dryness to give 125 mg of crude material. This crude was dissolved in 2 mL TFA and the bright yellow solution was allowed to react at room temperature for 2h. Methanol (10 mL) was added to the mixture and evaporated under reduced pressure, this procedure was repeated several times until obtaining a dry brown residue (140 mg). The crude was triturated in water, centrifuged to decant the solution, which was evaporated to dryness. The 32 mg remaining were purified by chromatography on a C18 SepPak cartridge (MeOH 1%, then 5%, then 10% in water), to give after lyophilization **2** as a light white solid (2 mg, 5% over 2 steps).

R_f : 0.2 (*n*-Butanol/AcOH/water 8:1:1):

^{1}H NMR (300 MHz, D_2O): δ = 7.67 (d, *J* = 7.8 Hz, 1H), 7.46 (d, *J* = 8.1 Hz, 1H), 7.29 (t, *J* = 7.7 Hz, 1H), 7.20 (t, *J* = 7.5 Hz, 1H), 4.53 (dd, *J* = 2.8, 3.8 Hz, 1H), 4.25 (d, *J* = 15.4 Hz, 1H), 4.13 (m, 1H), 3.69 (dd, *J* = 2.6, 14.3 Hz, 1H), 3.42 (dd, *J* = 4.3, 14.4 Hz, 1H), 3.37 (dd, *J* = 11.5, 13.5 Hz, 1H), 3.21 (dd, *J* = 4.5, 13.8 Hz, 1H), 3.17 (d, *J* = 15.5 Hz, 1H).

HRMS (ESI) *m/z:* calculated for $C_{16}H_{18}N_4O_4S$ $[M+H]^+$: 363.1127, found: 363.1112

Cyclic tripeptide 3[33]



N$_3$-Trityl-pyrroloindoline-glycyl-(*S*-trityl-L-cysteine) methyl ester **53b** (58 mg, 0.067 mmol) was dissolved in trifluoroacetic acid (13 mL) and the bright yellow

solution was allowed to react at room temperature for 3h. Methanol (20 mL) was added to the mixture and evaporated under reduced pressure, this procedure was repeated several times (once with toluene instead of methanol) until obtaining a dry brown residue. This residue was suspended in a mixture of water and diethyl ether, stirred until the entire solid has dissolved. The ether phase was washed with water, the combined water phases were brought to pH 8 and extracted with 6 x 50 mL AcOEt, then with 4 x 75 mL of chloroform. The combined organic phases were dried over magnesium sulfate, concentrated to dryness under reduced pressure to give 10 mg of a yellowish solid. This solid was purified by chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give **3** (4 mg, 16%)

Rf: 0.4 (CH_2Cl_2/MeOH 9:1)

^{1}H NMR (300 MHz, CD_3OD): δ = 7.60 (d, *J* = 7.7 Hz, 1H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 1H), 7.08 (t, *J* = 7.5 Hz, 1H), 4.87 (m, 1H), 4.25 (d, *J* = 15.1 Hz, 1H), 3.99 (d, *J* = 9.9 Hz, 1H), 3.82 (s, 3H), 3.64 (dd, *J* = 2.6, 14.5 Hz, 1H), 3.47 (dd, *J* = 4.5, 14.3 Hz, 1H), 3.39 (dd, *J* = 12.0, 13.5 Hz, 1H), 3.12 (d, *J* = 15.0 Hz, 1H) (dd, *J* = 2.6, 13.8 Hz, 1H).

^{13}C NMR (75 MHz, CD_3OD): δ = 173.0, 172.9, 141.0, 130.1, 129.9, 125.2, 122.2, 121.0, 114.7, 113.5, 56.5, 55.2, 54.8, 46.4, 39.3, 33.6.

HRMS (ESI) *m/z:* calculated for $C_{17}H_{21}N_4O_4S$ $[M+H]^+$: 377.1284, found: 377.1292.

N_α-Fmoc-N_δ-trityl-L-asparagine

To a solution of triphenylmethanol (13.0 g, 50 mmol, 2 eq.), in acetic acid (75 mL) was added L-asparagine (3.3 g, 25 mmol) and acetic anhydride (5 mL, 50 mmol, 2 eq.); the mixture was a slurry. Upon addition of concentrated sulfuric acid (1.53 g, 27.5 mmol, 1.1 eq.) the slurry turned into a brown solution. The mixture was kept at 60°C for 90 minutes, then cooled down and poured slowly over 150 mL of cold water. A white precipitate immediately appeared, that turned into a sticky thick oil. The pH was adjusted to 6 by slow addition of 100 mL of 10 N sodium hydroxide, the mixture became a finely dispersed solid and got very warm. After cooling down in the fridge for 2h, the solid was thoroughly washed with water and toluene and dried on high vacuum. This product was used without further purification.

General procedure for the formation of *N*-Fmoc-L-amino acids:

To a solution of the free amino acid (4mmol) in water (5 mL) with 1 equivalent of triethylamine was added a solution of Fmoc-OSu (3.6 mmol, 0.9 eq.) in acetonitrile (5 mL). The pH of the solution was brought to 8.5-9.0 with triethylamine and maintained until no further drop of pH was observable over 15 minutes. The reaction mixture was then filtered and evaporated under reduced pressure to a thick oil that was slowly added to stirred 1.5 N HCl. The resulting solid or oil was treated with ethyl acetate (20 mL), and the organic phase was washed with 20 mL volumes of 1.5 N HCl, water and brine before drying over magnesium sulfate and solvent evaporation. The resulting solid was recristallized in CH_2Cl_2-petroleum ether (in the case of Pro, Ile, Cys(Tr), Asn(Tr)) or AcOEt -petroleum ether (in the case of Gly).

Yields:

Pro : 1.050 g, 86%

Gly : 0.956g, 89%

Ile: 1.045g, 75%

Cys(Tr): 1.754g, 83%

Asn(Tr): 1.632 g, 76%

General procedure for the formation of *N*-Fmoc- L-amino acid chlorides:

The *N*-Fmoc- L-amino acid was dissolved in CH_2Cl_2 (10 mL) and treated with 10 equivalents of thionyl chloride at 0°C. The reaction was complete after 30 minutes. The reaction mixture was then thoroughly evaporated.

Yields:

Ile: 82%

Pro: 86%

Gly: 88%

General procedure for the formation of *N*-Fmoc- L-amino acid fluorides:

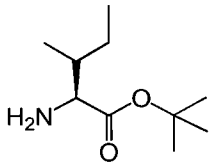
The *N*-Fmoc- L-amino acid was dissolved in CH_2Cl_2 (25 mL) and treated with 1.2 equivalents of diethylaminosulfur trifluoride (DAST) at 0°C. The reaction was complete after 10 minutes. 25 mL of ice water were added onto the mixture, the phases were separated, the organic phase was dried over magnesium sulfate, concentrated to dryness, and recristallized in CH_2Cl_2-petroleum ether.

Yields:

Cys(Tr): 83%

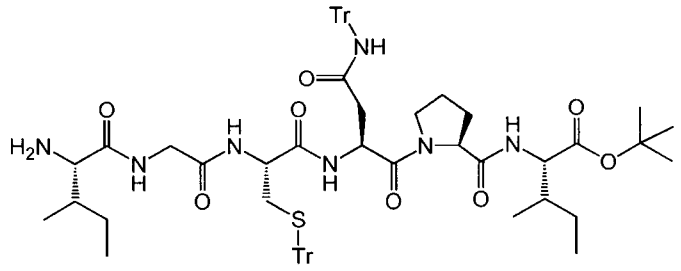
Asn(Tr): 76%.

Isoleucine *t*-butyl ester 66



To a solution of *N*-Fmoc-L-isoleucine *t*-butyl ester (890 mg, 2.18 mmol) in CH_2Cl_2 (20 mL) was added tris(aminoethyl)amine (TAEA, 16 mL, 108 mmol, 50eq.) and the mixture was stirred for 1h, during which time a white precipitate appeared. 100 mL of CH_2Cl_2 and 40 mL of brine were added, the organic phase was separated and further washed with 50 mL of brine, then with 4x50 mL of phosphate buffer (prepared from 90 g of $NaH_2PO_4.H_20$ and 32.7 g of Na_2HPO_4 in 500 mL distilled water, adjusted to pH 5.5 with concentrated hydrochloric acid). The combined aqueous phases were washed with CH_2Cl_2 (50 mL). This last organic phase was then re-washed with 50 mL of brine and 50 mL of buffer at pH 5.5, all organic phases were combined and dried over magnesium sulfate, filtered and concentrated under reduced pressure. The thin yellow oil obtained was dissolved in CH_2Cl_2 (1 mL) and filtered on a silica pad (4x2 cm, elution with CH_2Cl_2, then with 95:5 CH_2Cl_2/MeOH). The resulting yellowish thin oil was treated with 1.5 mL of a 1N HCl solution in ethanol (prepared by adding acetyl chloride (360 mL, 5mmol) to dry ethanol (5 mL) at 0°C), and then concentrated under reduced pressure to give **66** (310 mg, 67% from N-Fmoc-L-isoleucine) as a tan solid. The product was used without further characterization.

Linear Hexapeptide H-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OtBu 58[57]



In a 25 mL round-bottom- flask **66** (111 mg, 0.5 mmol) was suspended in a stirring solution of 5% aqueous sodium bicarbonate solution (10 mL). CH_2Cl_2 (5 mL) was then added to form a clear biphasic solution. **62** (1.2 eq.) was added by small portions to the strongly stirring biphasic solution. After 10 minutes TLC analysis (chloroform/EtOH 10:1 and CH_2Cl_2/MeOH 98:2) showed that the reaction was complete. The phases were separated, the aqueous phase washed with CH_2Cl_2 (5 mL), and the combined organic phases were treated with tris(aminoethyl)amine (3.8 mL, 25 mmol, 50 eq.). After 1 minute a white precipitate appears. After 30 minutes TLC analysis (chloroform/EtOH 10:1, Ninhydrin stain) showed that the deprotection was complete. The reaction mixture was transferred to a 125 mL separatory funnel, and washed with brine (2 x 5 mL) and pH 5.5 phosphate buffer (6 x 10 mL). During washes emulsions form, that are very slow to settle. After TLC analysis (AcOEt/pyridine/AcOH/H_2O 30:20:6:11), the combined aqueous phases are washed with CH_2Cl_2 (2x5 mL). The combined organic phases were concentrated under reduced pressure to a volume of 5 mL, and the same procedure was repeated for the coupling of Fmoc-Asn(Tr)-Cl. After coupling of asparagine the tripeptide H-Asn(Tr)-Pro-Ile-OtBu was purified by filtration on a silica pad (4 x 2 cm, CH_2Cl_2 then CH_2Cl_2/MeOH 95:5). The resulting yellowish solid (314 mg) was dissolved in 15 mL of CH_2Cl_2 and the coupling procedure was repeated with Fmoc-Cys(Tr)-Cl. The tetrapeptide H-Cys(Tr)-Asn(Tr)-Pro-Ile-OtBu was purified by filtration on a silica pad (triethylamine-treated silica, 4 x 2 cm, elution: AcOEt/hexanes, then AcOEt). The tan foam (320 mg) obtained was submitted to H-Gly-Cl and then H-Ile-Cl according to the procedure described above, and after the last deprotection the crude hexapeptide was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 96:4) to give **58** (278 mg, 48%) as a tan foam that was homogeneous by TLC analysis.

R_f = 0.5 (Chloroform/EtOH 10:1)

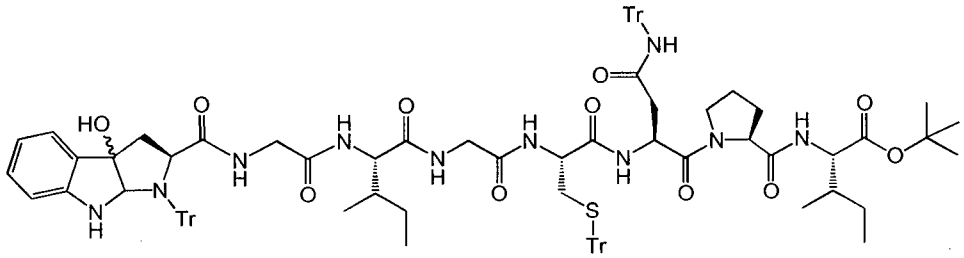
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.72 (bs, 2H), 7.4-7.1 (m, 31H), 6.41 (bs, 1H), 4.66 (q, *J* = 6.8, 13.2 Hz, 1H), 4.42 (dd, *J* = 2.3, 6.1 Hz, 1H), 4.15 (t, *J* = 6.7, 1H), 4.07 (q, *J* = 7.3, 12.8 Hz, 1H), 3.61 (dd, *J* = 5.4, 16.1 Hz, 1H), 3.47 (dd, *J* = 4.3, 16.0 Hz, 1H), 3.35 (q, *J* = 8.5, 16.6 Hz, 1H), 3.05 (d, *J* = 4.0 Hz, 1H), 3.04 (bs, 1H), 2.80 (dd, *J* = 7.2, 12.9 Hz, 1H), 2.69 (m, 2H), 2.51 (dd, *J* = 5.0, 12.9 Hz, 1H), 2.03 (bs, 1H), 1.80 (bs, 2H), 1.70 (bs, 2H), 1.60 (bs, 2H), 1.44 (s, 9H),

1.35 (m, 2H), 1.15 (m, 2H), 1.00 (m, 2H), 0.84 (m, 6H), 0.71 (d, J = 7.0 Hz, 3H), 0.65 (t, J = 7.2 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.8, 171.0, 170.6, 169.8, 169.4, 168.9, 144.3, 129.5, 128.8, 128.0, 127.7, 126.8, 81.0, 70.5, 67.0, 60.3, 59.7, 57.1, 52.0, 48.5, 47.1, 43.2, 39.7, 38.0, 36.3, 33.0, 28.7, 28.0, 25.1, 24.6, 23.8, 16.0, 15.3, 11.8, 10.9.

HRMS (ESI) *m/z:* calculated for $C_{68}H_{82}N_7O_8S$ $[M+H]^+$: 1156.5946, found: 1156.5946.

Linear octapeptide 69



To a suspension of hexapeptide **58** (197 mg, 0.17 mmol) and HOBt (23 mg, 0.17 mmol, 1 eq.) in CH_2Cl_2 (2 mL) were added dipeptide **31a** (106 mg, 0.17 mmol, 1 eq.) and triethylamine (26 μL, 0.19 mmol, 1.1 eq.) and the slurry was stirred in an ice/water bath for 10 min. PyBOP (98 mg, 0.19 mmol, 1.1 eq.) was then added, the ice bath was removed after 10 min and the clear yellow solution was allowed to react at room temperature for 20h. The solution was diluted with 20 mL of CH_2Cl_2, and washed with 5% citric acid solution (2 x 20 mL), saturated sodium bicarbonate solution (2 x 20 mL) and brine (2 x 20 mL). The resulting CH_2Cl_2 was filtered under vacuum to remove a resilient suspension, dried over magnesium sulfate, and concentrated to dryness under reduced pressure, to give 350 mg of crude material. TLC (CH_2Cl_2:MeOH 98:2) shows two close spots, one of them having appeared during work-up: it probably is de-tritylated product. The crude material was purified by chromatography on triethylamine-treated silica gel (CH_2Cl_2/NEt_3 100:1, then CH_2Cl_2/MeOH 90:10) to give 89 mg of the title compound (32%) and 82 mg of de-tritylated product (34%).

R_f: 0.7 (CH_2Cl_2/MeOH 9:1)

R_f*: 0.4 (CH_2Cl_2/MeOH 95:5)

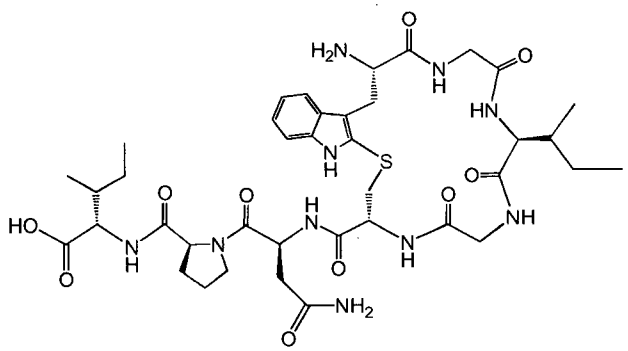
^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ = 9.2 (bs, 1H), 6.5-7.8 (m, 49H), 5.35 (bs, 1H), 4.7 (bs, 1H), 4.3-4.0 (m, 7H), 3.9 (bs, 1H), 3.3-3.6 (m, 3H), 2.6 (m, 7H), 2.0-1.6 (m, 6H), 1.4 (s, 9H), 1.3-0.5 (m, 17H).

HRMS (ESI) *m/z:* calculated for $C_{100}H_{108}N_{10}O_{11}SNa$ $[M+Na]^+$: 1679.7817, found: 1679.7797.

De-tritylated compound:

HRMS (ESI) *m/z:* calculated for $C_{81}H_{95}N_{10}O_{11}S$ $[M+H]^+$: 1415.6903, found: 1415.6913.

Monocyclic octapeptide 70[34]



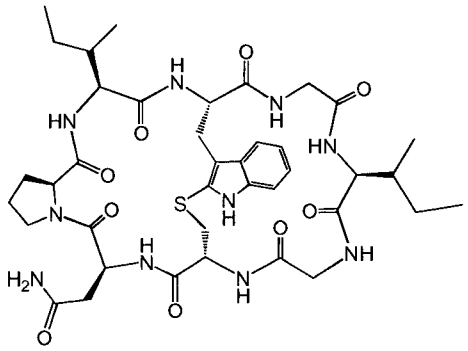
Linear octapeptide **69** (87mg of fully protected **69**, 0.052 mmol + 80 mg of de-tritylated product, 0.056 mmol) was dissolved in trifluoroacetic acid (40 mL) and the bright yellow solution was allowed to react at room temperature for 5h. Methanol (20 mL) was added to the mixture and evaporated under reduced pressure, this procedure was repeated several times until obtaining a dry brown residue. This residue was triturated in Et_2O and centrifuged to isolate the solid phase, which was dissolved in MeOH/water and purified by chromatography on a C18 SepPak column (MeOH/water 1:99, then 5:95, then 10:90, then 20:80, then 40:60, then 60:40) to give (35 mg, 38%). 10 mg of this compound were further purified by preparative HPLC to obtain an analytical sample.

R_f = 0.4 (*n*-BuOH/AcOH/water 4:1:1)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.72 (bs, 2H), 7.4-7.1 (m, 35H), 6.41 (bs, 1H), 4.66 (q, *J* = 6.8, 13.1 Hz, 1H), 4.42 (dd, *J* = Hz, 1H), 4.15 (t, *J* = , 1H), 4.07 (q, *J* = , 1H), 3.61 (dd, *J* = Hz, 1H), 3.47 (dd, *J* = Hz, 1H), 3.35 (q, *J* = Hz, 1H), 3.05 (d, *J* = Hz, 1H), 3.04 (bs, 1H), 2.80 (dd, *J* = Hz, 1H), 2, 69 (m, 2H), 2.51 (dd, *J* = Hz, 1H), 2.03 (bs, 1H), 1.80 (bs, 2H), 1.70 (bs, 2H), 1.60 (bs, 2H), 1.44 (s, 9H), 1.35 (m, 2H), 1.15 (m, 2H), 1.00 (m, 2H), 0.84 (m, 6H), 0.71 (d, *J* = 7.0 Hz, 3H), 0.65 (t, *J* = 7.2 Hz, 3H).

HRMS (ESI) *m/z:* calculated for $C_{39}H_{57}N_{10}O_{10}S$ $[M+H]^+$: 857.3980, found: 857.3987.

Pro^2, Ile^3-S-deoxo-amaninamide 1



To a suspension of linear octapeptide **1** (20 mg, 0.023 mmol) and HOBt (9 mg, 0.075 mmol, 3 eq.) in dry DMF (23 mL) was added triethylamine (20 µL, 0.14 mmol, 6 eq.) and the solution was stirred in an ice/water bath for 10 min. PyBOP (37 mg, 0.07 mmol, 3 eq.) was then added, the ice bath was removed after 10 min and the yellow solution was allowed to react at room temperature overnight. Methanol was added and evaporated under reduced pressure (3 times), the toluene (3 x 50 mL) was added and evaporated until all the DMF is removed. The crude material was purified by chromatography on silica gel (CH_2Cl_2/MeOH/water 90:13:1) and then further purified by preparative HPLC, to give **1** (3 mg, 16%) and **iso-1** (6 mg, 32%)

R_f : 0.5 ($CHCl_3$/MeOH/water 90:13:1)

1 : ^{1}H NMR (500 MHz, DMSO-d^6): δ = 11.21 (bs, 1H, NH(indole)), 8.80 (t, *J* = 6.2 Hz, 1H, NH(Gly)), 8.48 (s, 1H, NH(Asn or Cys)), 8.43 (d, *J* = 3.8 Hz, 1H, NH(Ile)), 8.22 (s, 1H, N_δH(Asn)), 8.08-8.00 (m, 4H, NH(Gly+Ile+Trp+Asn or Cys)), 7.58 (d, *J* = 7.9 Hz, 1H, H(indole)), 7.49 (s, 1H, N_δH(Asn)), 7.23 (d, *J* = 8.2 Hz, 1H, H(indole)), 7.09 (t, *J* = 7.4 Hz, 1H, H(indole)), 6.99 (t, *J* = 7.5 Hz, 1H, H(indole)), 4.88 (m, 1H, H_α(Trp)), 4.75 (d, *J* = 3.2 Hz, 1H, H_α(Cys or Asn)), 4.54 (m, 1H, H_α(Cys or Asn)), 4.28 (dd, *J* = 8.8, 18.5 Hz, 1H, H_α(Gly)), 4.16 (m, 2H, H_α(Pro)+H_α(Ile)), 3.94 (t, *J* = 8.3 Hz, 1H, H_δ(Pro)), 3.85 (dd, *J* = 6.9, 16.9 Hz, 1H, H_α(Gly)), 3.65-3.58 (m, 2H, H_δ(Pro)+H_α(Ile)), 3.4 (m, 1H, H_α(Gly)), 3.35 (m, 1H, H_β(Cys or Asn)), 3.31 (m, 1H, H_α(Gly)), 3.23 (t, *J* = 13.9 Hz, 1H, H_β(Trp)), 3.07 (m, 1H, H_β(Trp)), 3.03-2.98 (m, 2H, 2H_β(Asn or Cys)), 2.72 (dd, *J* = 8.5, 9.6 Hz, 1H, H_β(Asn or Cys)), 2.33 (m, 1H, H_β(Pro)), 1.98 (m, 1H, H_γ(Pro)), 1.92 (m, 1H, H_β(Ile)), 1.83 (m, 1H, H_γ(Pro)), 1.69 (m, 1H, H_β(Pro)), 1.55-1.43 (m, 3H, H_β+H_γ(Ile)), 1.22-1.05 (m, 2H), 0.88 (d, *J* = 6.8 Hz, 3H, $CH3_\gamma$(Ile)), 0.85-0.75 (m, 9H, $CH_{3\gamma}$+2$CH_{3\delta}$(Ile)).

^{13}C NMR (75 MHz, DMSO-d^6): δ = 174.5, 173.7, 172.7, 172.3, 172.3, 171.8, 171.8, 170.14, 169.5, 138.4, 128.9, 126.4, 124.1, 122.3, 120.5, 117.8, 113.0, 65.28, 61.0, 59.8, 55.4, 54.6, 52.6, 44.3, 41.4, 37.5, 36.4, 35.7, 31.8, 27.1, 26.9, 17.7, 16.7, 12.5.

HRMS (ESI) *m/z:* calculated for $C_{39}H_{54}N_{10}O_9SNa$ [*M*+Na]$^+$: 861.3696, found: 861.3694.

Iso-1: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.21 (s, 1H), 8.81 (t, *J* = 6.2, 1H), 8.53 (d, *J* = 5.5 Hz, 1H), 8.42 (d, *J* = 9.4 Hz, 1H), 8.32 (bs, 1H), 8.13 (m, 2H), 7.68 (m, 1H), 7.51 (m, 2H), 7.24 (m, 2H), 7.11 (dt, *J* = 0.9, 7.1 Hz, 1H), 7.02 (t, *J* = 7.6 Hz, 1H), 6.86 (bs, 1H), 4.90-4.84 (m, 2H), 4.62-4.56 (m, 2H), 4.43 (dd, *J* = 3.5, 9.3 Hz, 2H), 4.02-3.85 (m, 3H), 3.80-3.70 (m, 4H), 3.64-3.51 (m, 4H), 3.22 (m, 1H), 2.99 (dd, *J* = 2.5, 11.5 Hz, 1H), 2.79 (dd, *J* = 5.2, 14.0 Hz, 1H), 2.66 (dd, *J* = 7.1, 16.4 Hz, 1H), 2.20-2.12 (m, 2H), 2.12-2.05 (m, 1H), 1.95-1.45 (m, 6H), 1.25-1.10 (m, 3H), 0.89 (d, *J* = 6.4 Hz, 3H), 0.85-0.75 (m, 9H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.0, 173.4, 172.4, 172.1, 171.7, 171.5, 171.4, 170.2, 169.6, 138.6, 129.1, 127.2, 124.7, 124.1, 120.7, 117.4, 112.9, 62.1, 61.0, 56.0, 54.7, 51.3, 44.0, 36.2, 27.9, 27.1, 23.9, 23.9, 16.9, 16.4, 13.4, 12.4.

HRMS (ESI) *m/z:* calculated for $C_{39}H_{54}N_{10}O_9SNa$ $[M+Na]^+$: 861.3694, found: 861.3681.

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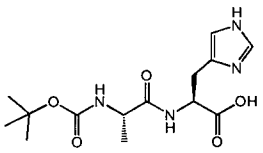
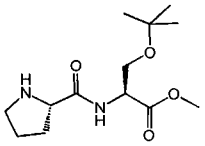
Appendices

Appendix 1 : Peptide nomenclature

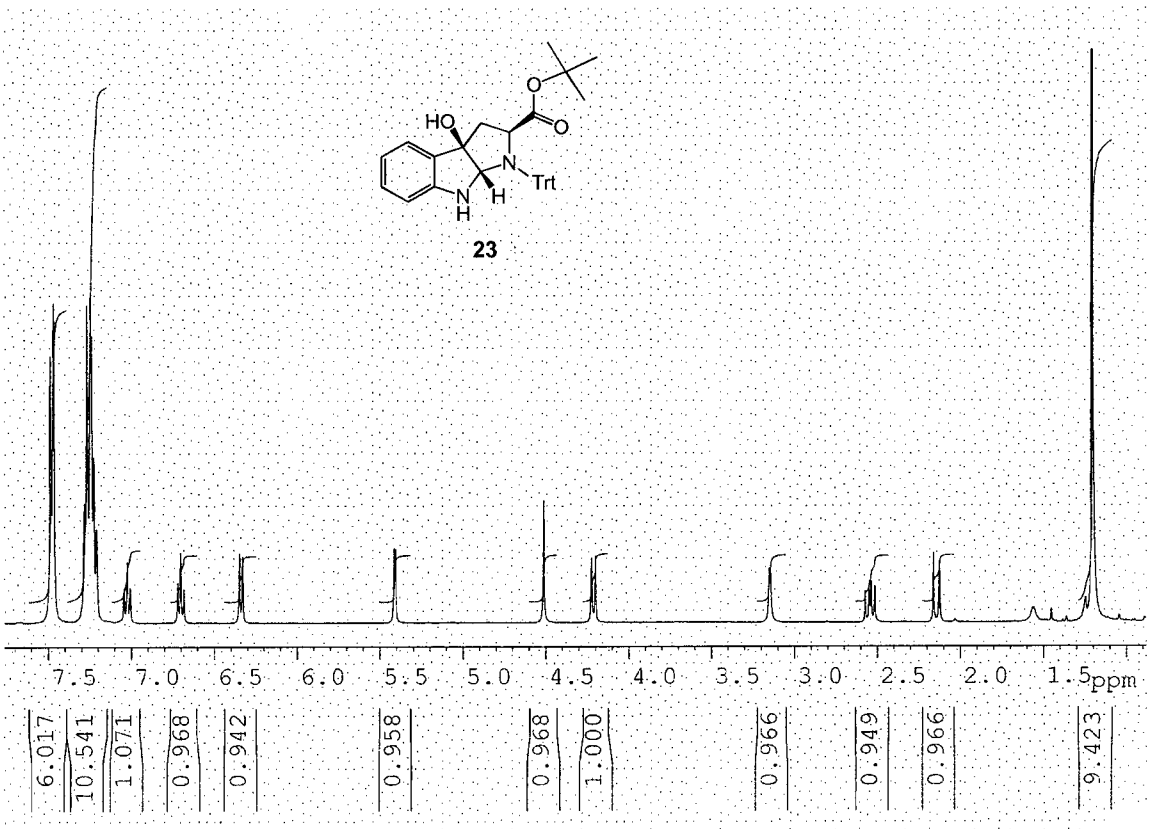
In this work peptides are identified with the standard short notation: a peptide is read from N-terminal to C-terminal, each amino acid is designated by its 3-letter code followed if necessary by the side-chain protecting group between brackets; the terminal protecting groups are also mentioned at each end; if unprotected, the N-terminus is written as "H-", the C-terminal as "-OH",

e.g. Boc-Ala-His-OH designates: 

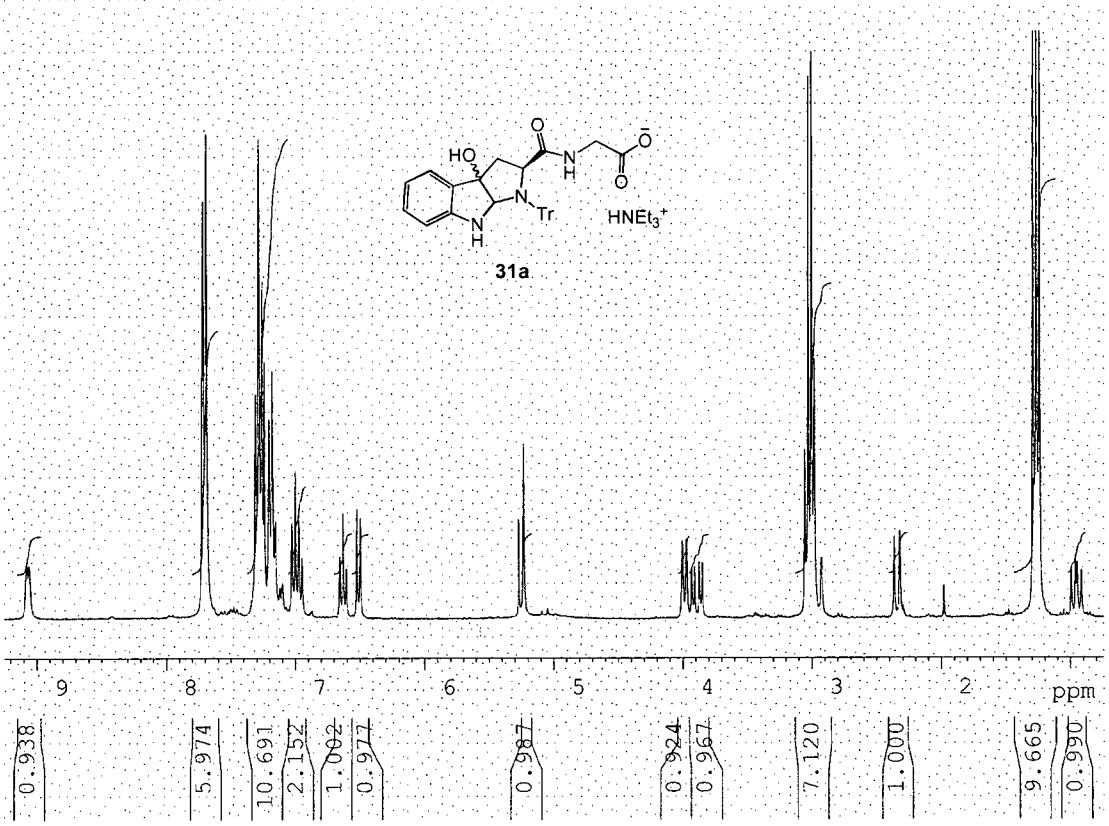
H-Pro-Ser(tBu)-OMe designates 

In the following all amino acids are in the L configuration, therefore the "L" notation will be omitted.

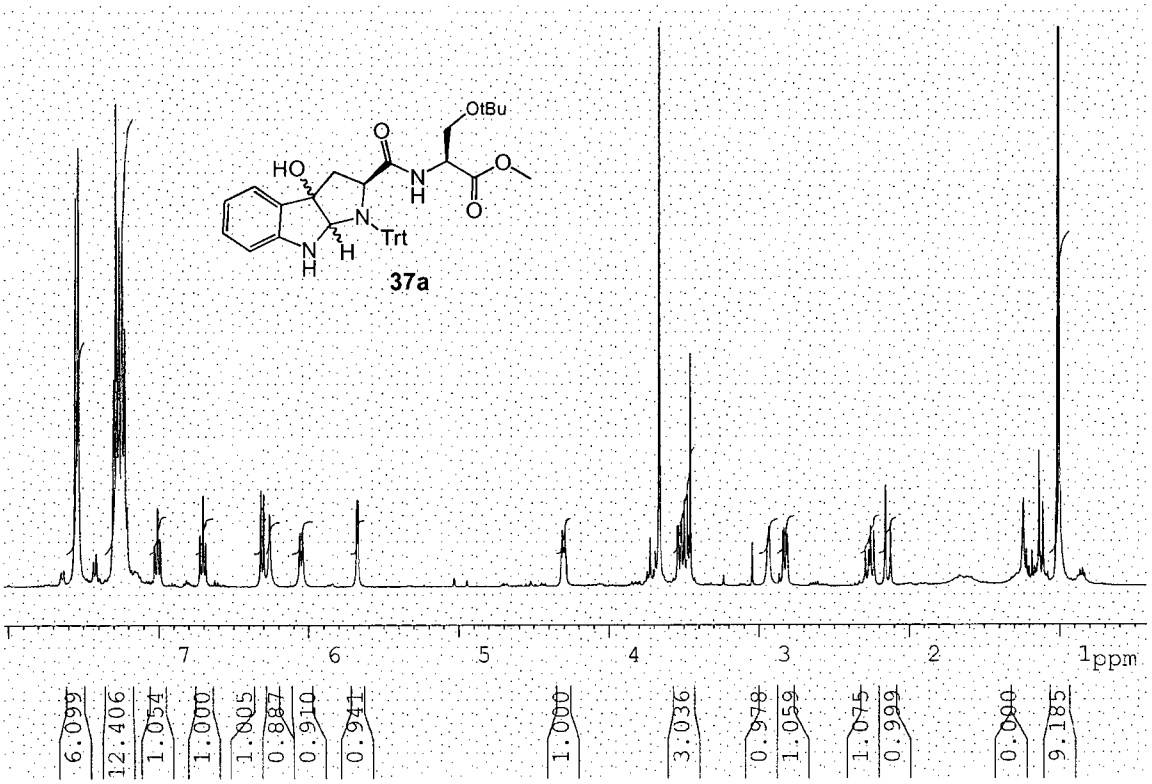
Appendix 2 : Representative chromatograms

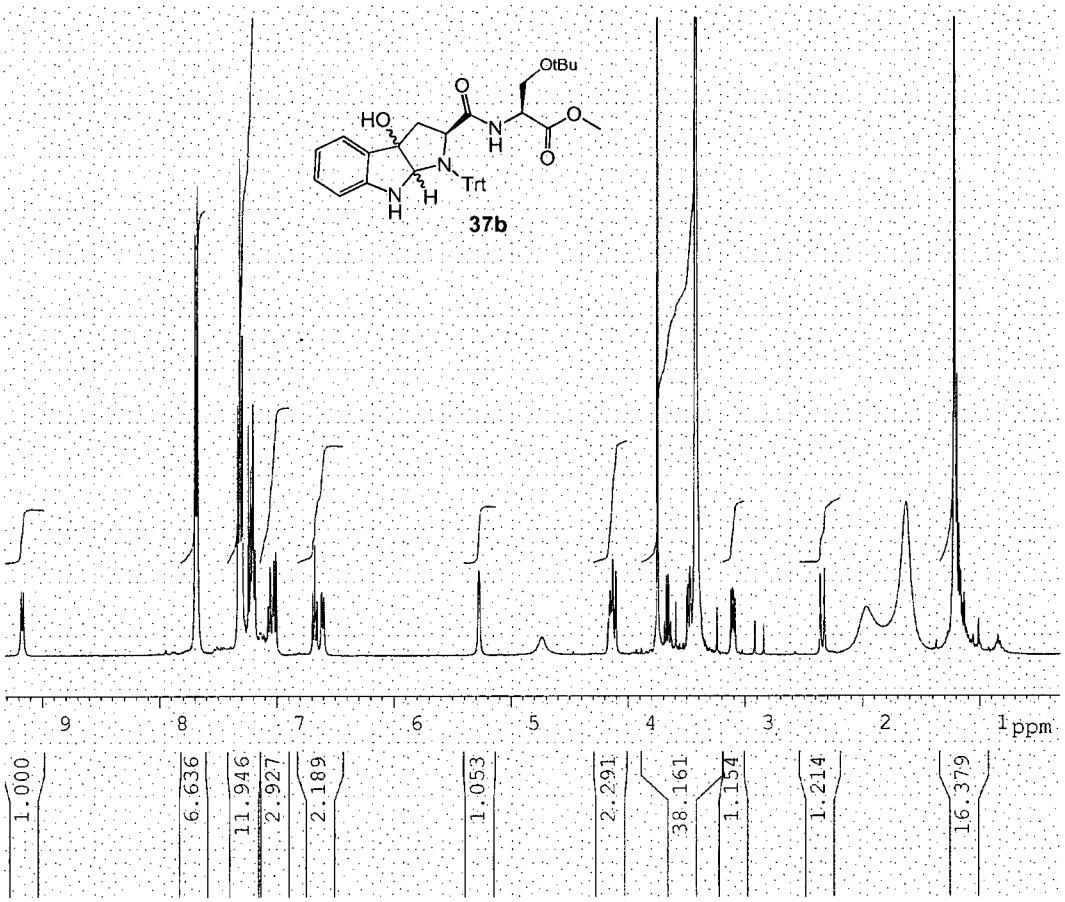

HO
O
O
N
Trt
N
H
H
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5ppm
6.017
10.541
1.071
0.968
0.942
0.958
0.968
1.000
0.966
0.949
0.966
9.423

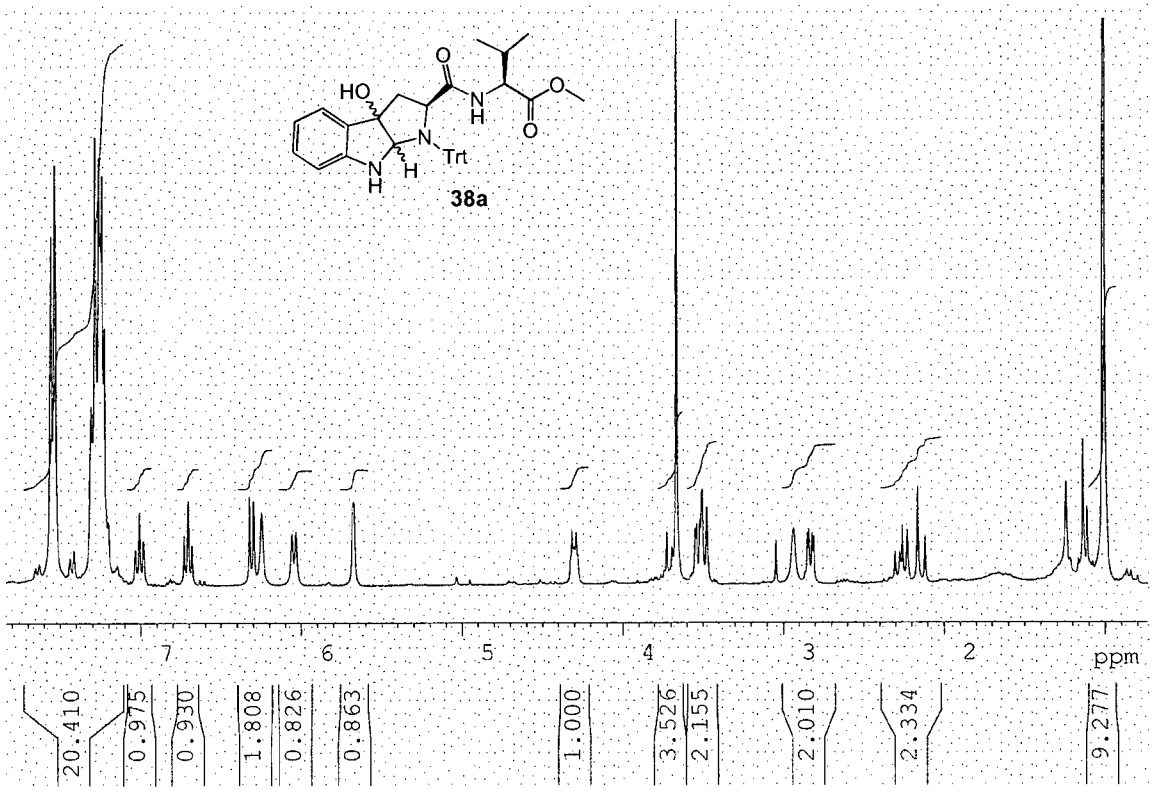
23

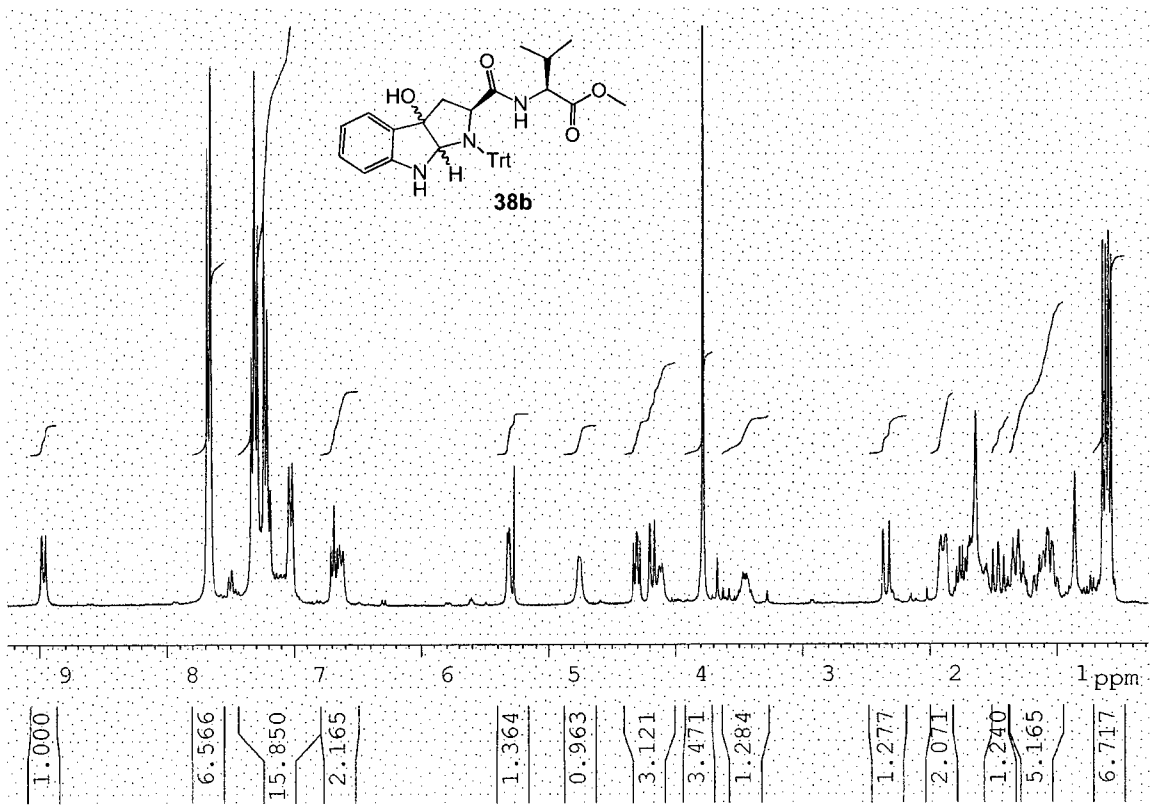

HO
O
N
H
O
O
N
Tr
HNEt3+
9 8 7 6 5 4 3 2 ppm
0.938
5.974
10.691
2.152
1.002
0.977
0.987
0.924
0.967
7.120
1.000
9.665
0.990

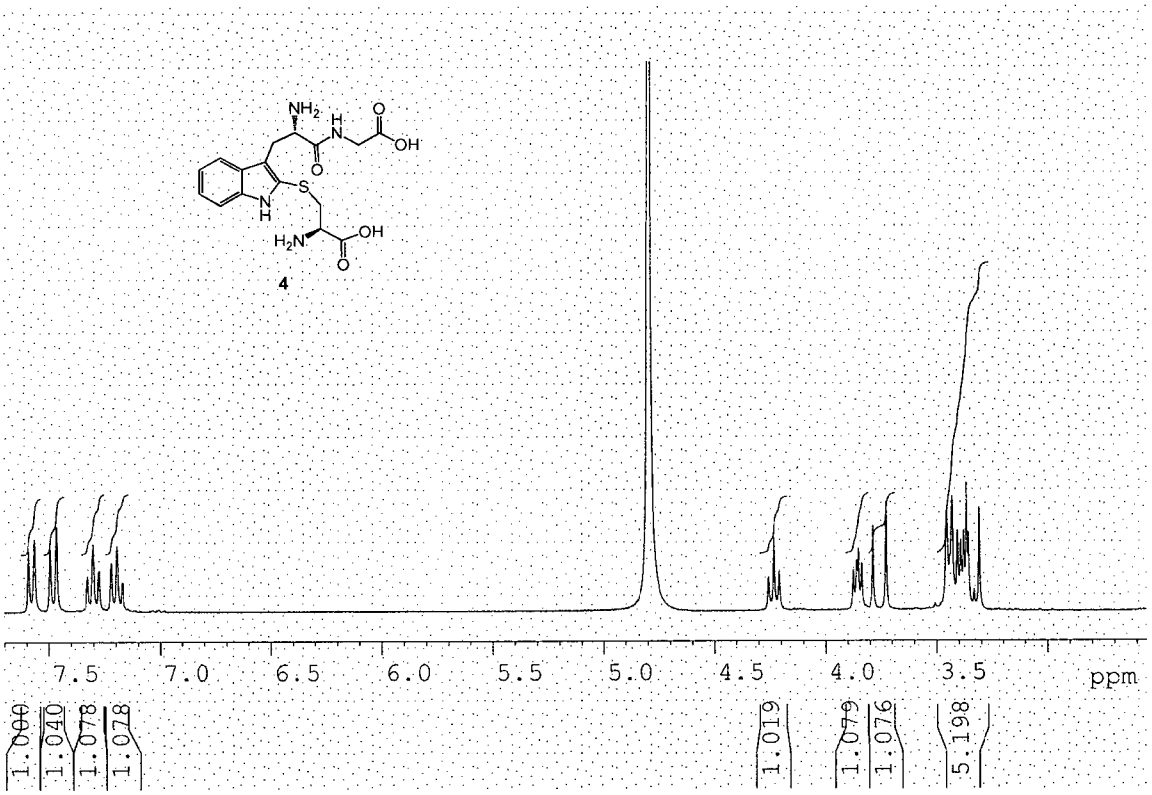
31a

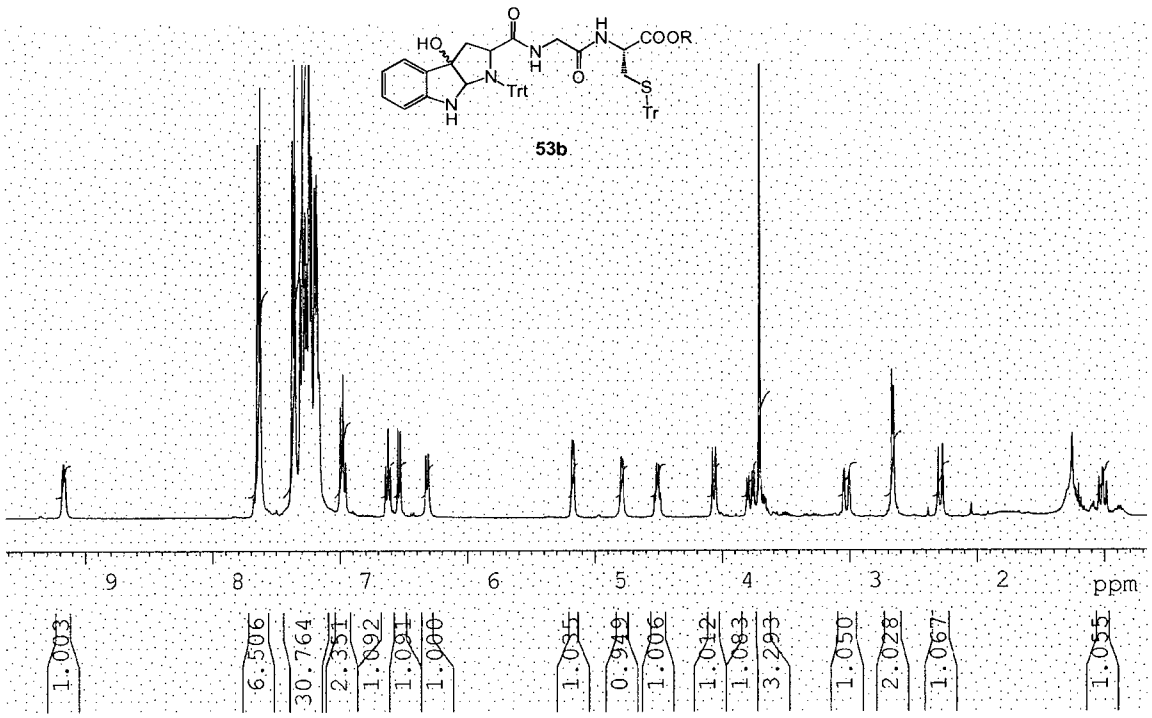

OtBu
HO
O
N
H
O
O
N
Trt
N
H
H
37a
7
6
5
4
3
2
1ppm
6.099
12.406
1.054
1.000
1.005
0.887
0.910
0.941
1.000
3.036
0.978
1.059
1.075
0.999
0.000
9.185

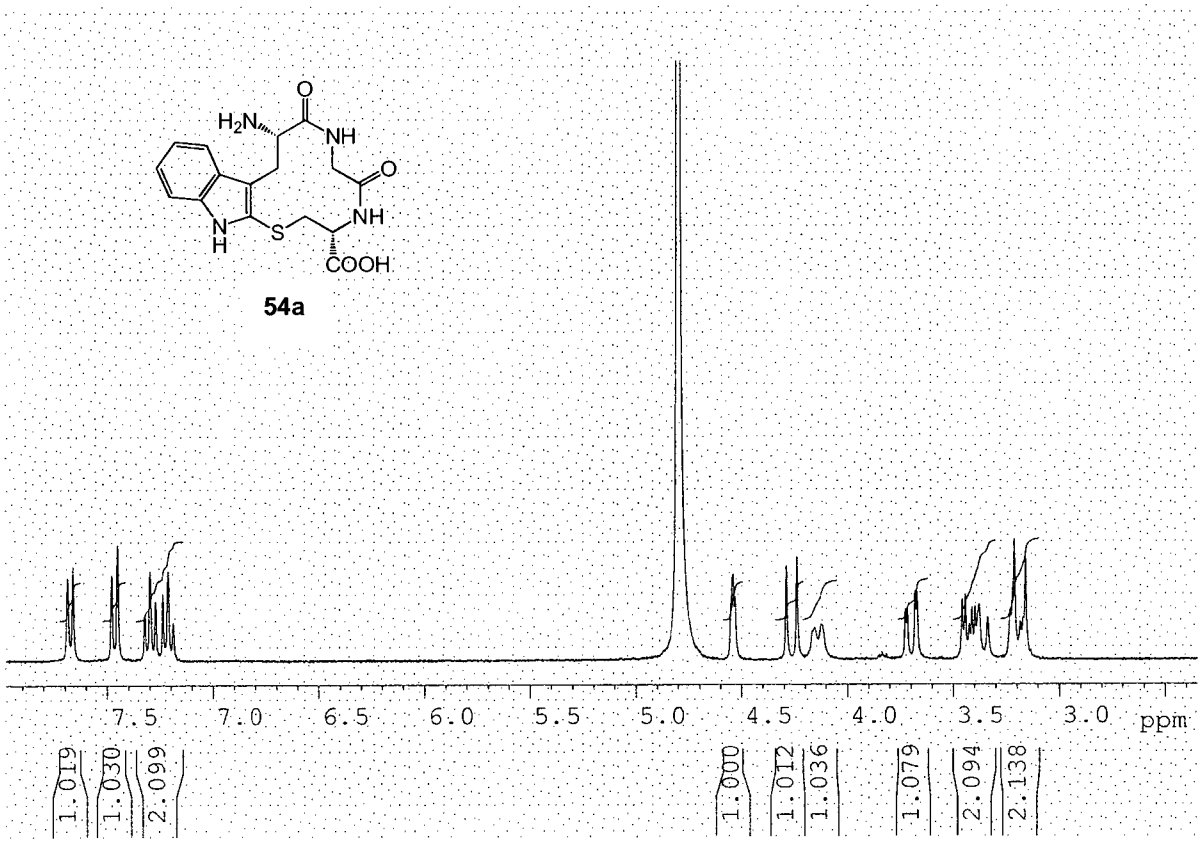

OtBu
HO
O
N
H
O
O
N
Trt
N
H
H
37b
9
8
7
6
5
4
3
2
1ppm
1.000
6.636
11.946
2.927
2.189
1.053
2.291
38.161
1.154
1.214
16.379


HO
O
N
H
O
O
N
Trt
N
H
H
38a
7
6
5
4
3
2
ppm
20.410
0.975
0.930
1.808
0.826
0.863
1.000
3.526
2.155
2.010
2.334
9.277

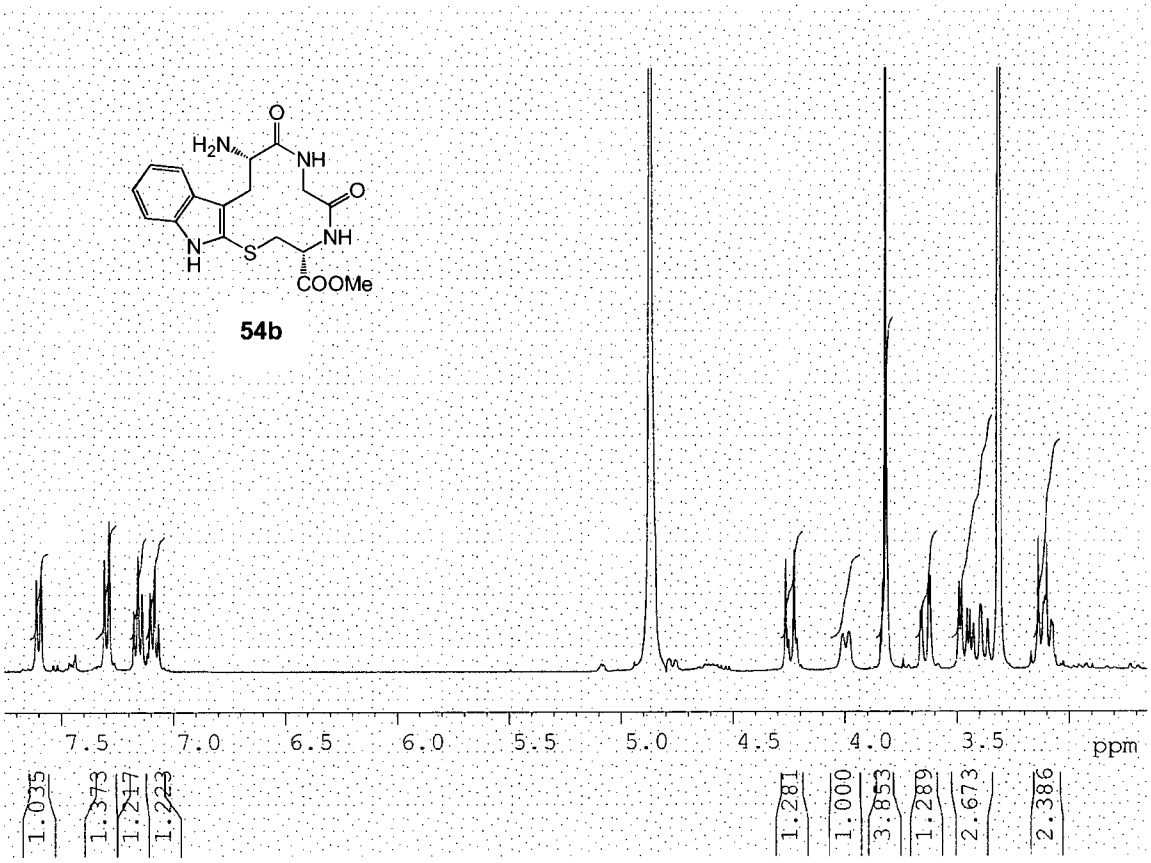

HO
O
N
H
O
O
N
Trt
N
H
H
38b
9
8
7
6
5
4
3
2
1
ppm
1.000
6.566
15.850
2.165
1.364
0.963
3.121
3.471
1.284
1.277
2.071
1.240
5.165
6.717


4
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm
1.000
1.040
1.078
1.078
1.019
1.079
1.076
5.198

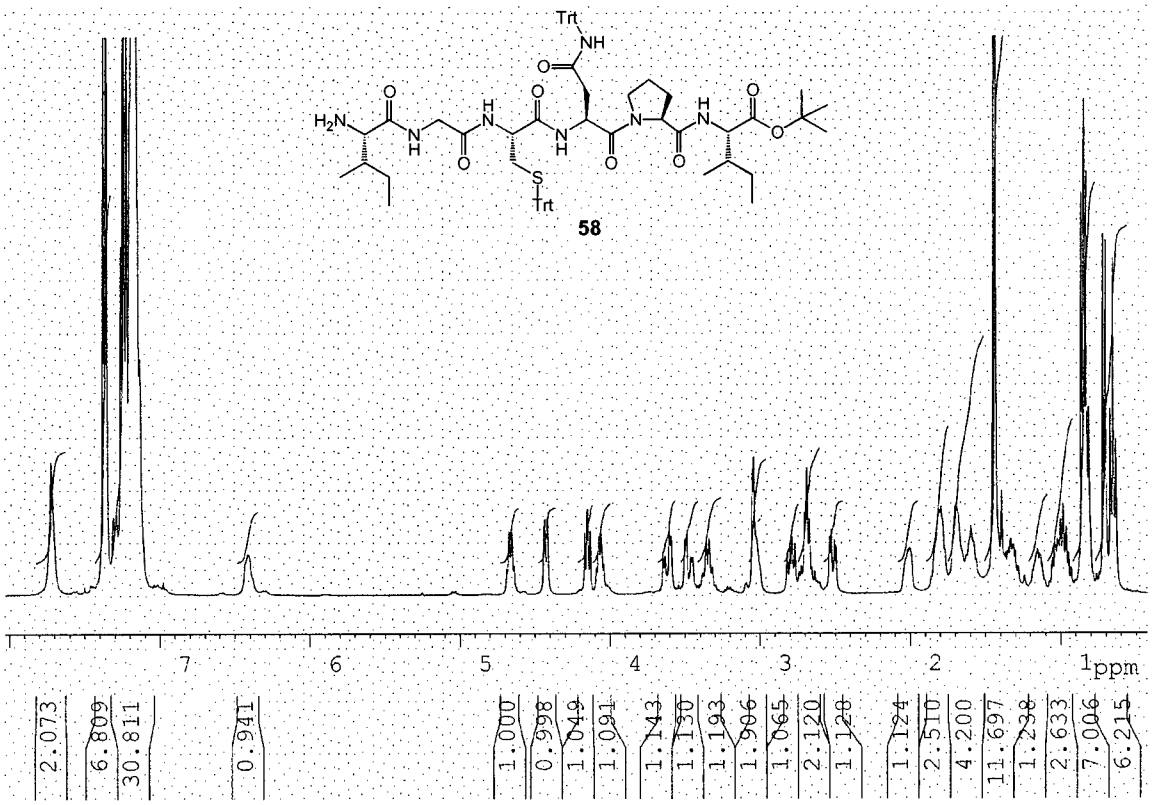

53b
9
8
7
6
5
4
3
2
ppm
1.003
6.506
30.764
2.351
1.092
1.081
1.000
1.035
0.974
1.006
1.012
1.083
3.203
1.050
2.028
1.067
1.055

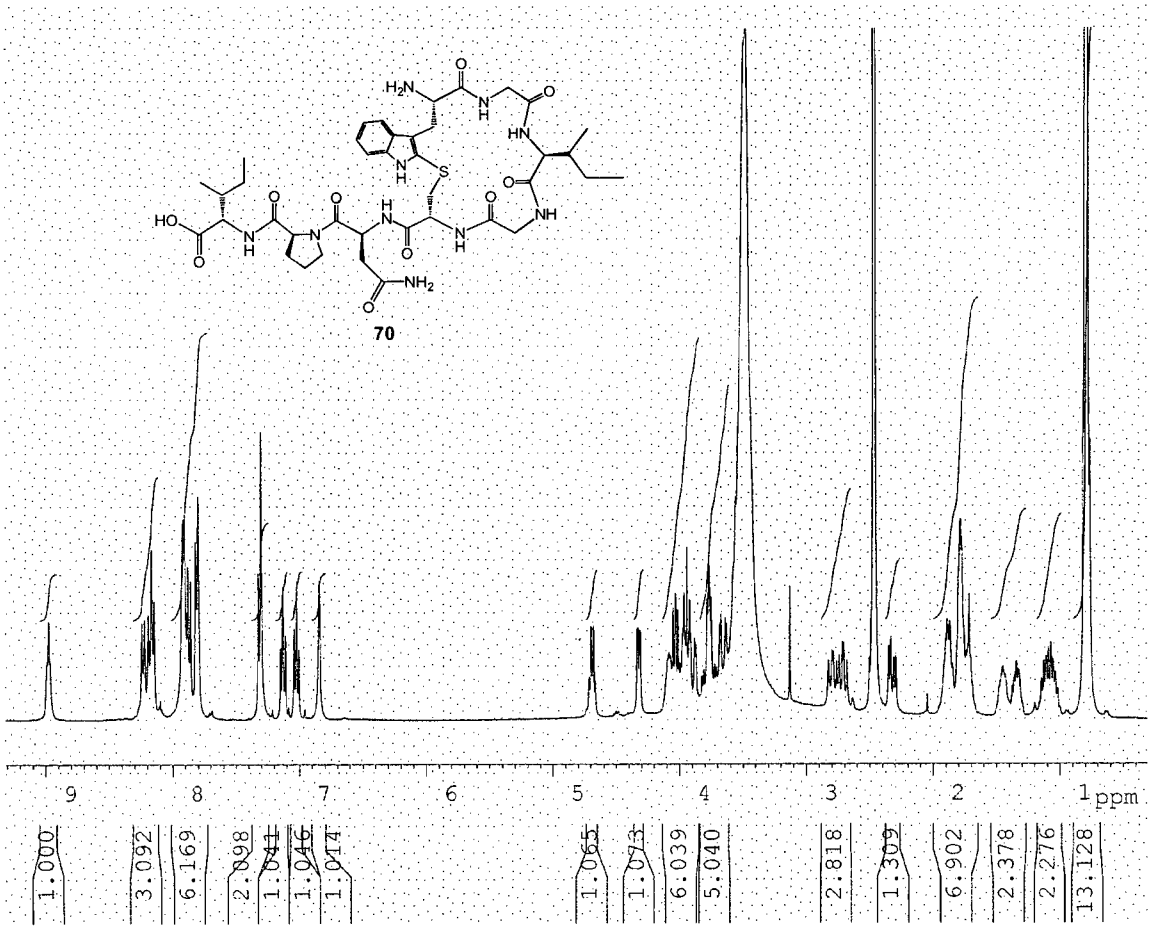

O
H2N
NH
O
NH
N
H
S
COOH
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm
1.019
1.030
2.099
1.000
1.012
1.036
1.079
2.094
2.138

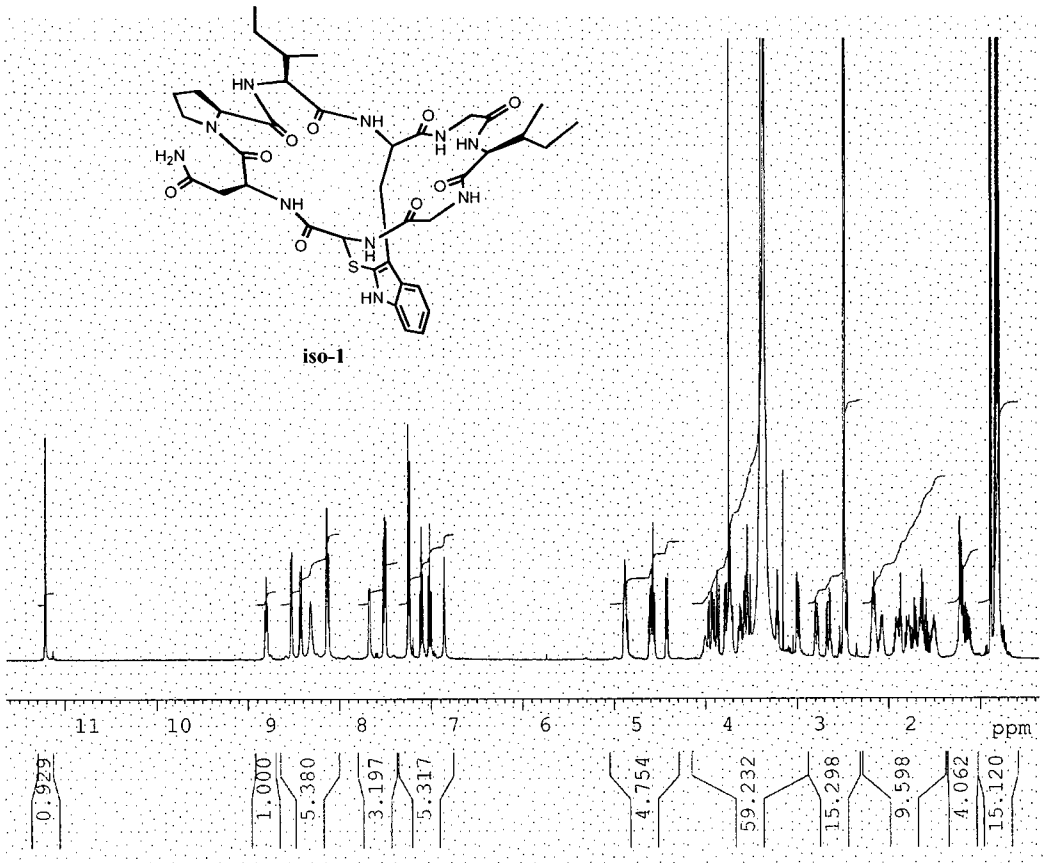
54a

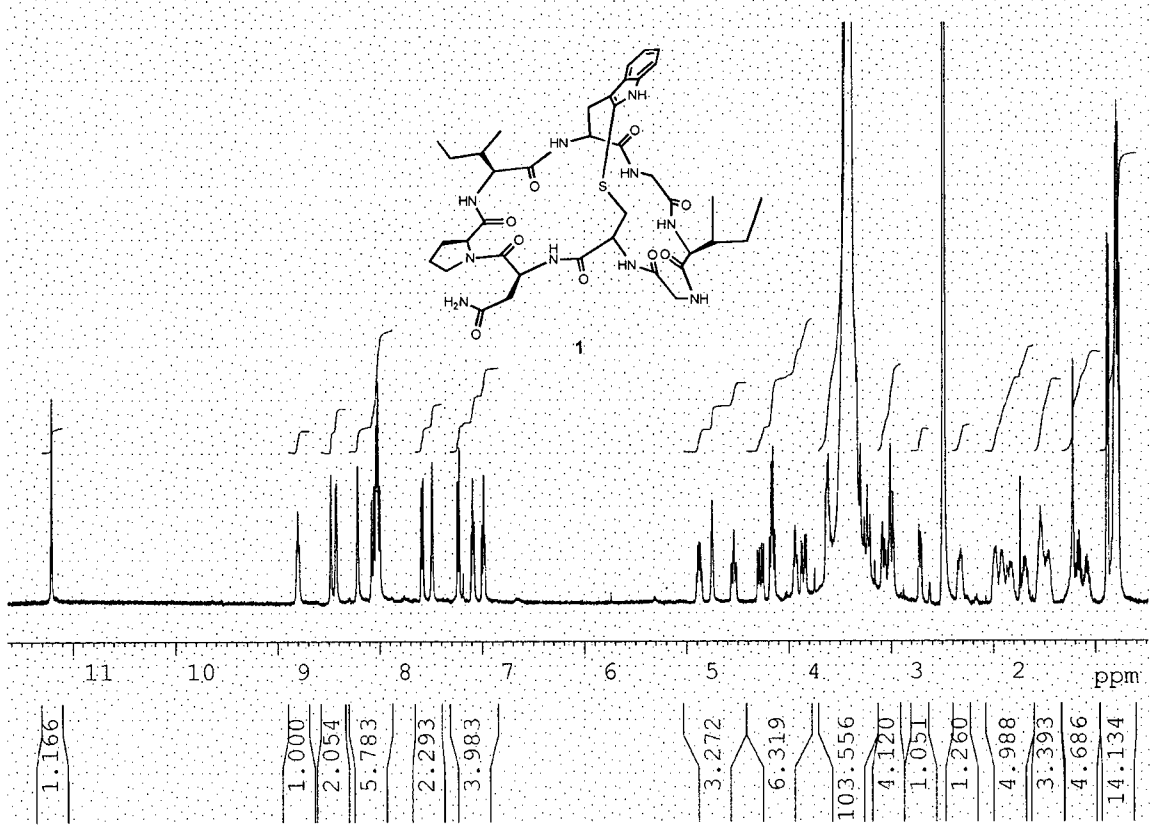

O
H2N
NH
O
N
H
S
NH
COOMe
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm
1.035
1.373
1.217
1.223
1.281
1.000
3.853
1.289
2.673
2.386

54b


Trt
NH
H2N
O
N
H
S
Trt
58
7
6
5
4
3
2
1ppm
2.073
6.809
30.811
0.941
1.000
0.998
1.049
1.091
1.143
1.130
1.193
1.906
1.065
2.120
1.120
1.124
2.510
4.200
11.697
1.238
2.633
7.006
6.215


H2N
HO
NH2
NH
70
9
8
7
6
5
4
3
2
1ppm
1.000
3.092
6.169
2.098
1.041
1.046
1.014
1.065
1.073
6.039
5.040
2.818
1.309
6.902
2.378
2.276
13.128


iso-1
11
10
9
8
7
6
5
4
3
2
ppm
0.929
1.000
5.380
3.197
5.317
4.754
59.232
15.298
9.598
4.062
15.120


1
11
10
9
8
7
6
5
4
3
2
ppm
1.166
1.000
2.054
5.783
2.293
3.983
3.272
6.319
103.556
4.120
1.051
1.260
4.988
3.393
4.686
14.134