Synthesis of Cyclic Hexapeptides Based on the Antibiotic Cyclic Decapeptide Loloatin C by an in situ Indirect Cyclization Method

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Three cyclic hexapeptide units based on the parent loloatin C scaffold have been identified by a 'sliding window' method as part of an expeditious SAR search for the basis of the antibiotic activity of the loloatins. Modified Fmoc-based solid-phase synthesis was used to prepare cyclic(L-valyl-L-ornithyl-D-phenylalanyl-L-asparaginyl-L-aspartyl-L-tryptophanyl) and cyclic(L-valyl-L-ornithyl-L-leucyl-L-tryptophanyl-D-phenylalanyl-L-asparaginyl) in overall yields of 42%–47%. A new solution method combined with an in situ indirect cyclization was specifically developed to prepare cyclic(L-ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-phenylalanyl), involving cyclization of the linear peptide through the amino group in leucine, liberated selectively from the Fmoc-protected amine in situ, with the activated *p*-nitrophenyl ester of ornithine. The method was also effectively used for cyclization of the linear precursors of the first two cyclic hexapeptides. NOE analyses coupled with peptide backbone modelling were used to establish conformations of the target compounds. All have helix-like structures with γ-turns.

Introduction

The problem of bacterial resistance to antibiotics is a major issue in human health today,[1] and the construction of new therapeutic agents to combat micro-organisms resistant to traditional antibiotics is an urgent task. From previously reported bioactivity data, loloatin C (**1**) serves as one possible template for this task. It was originally isolated, together with other cyclic decapeptides, from laboratory cultures of a tropical marine bacterium collected from the Great Barrier Reef off the southern coast of Papua New Guinea, and was reported to display potent antibiotic activity against serial strains of Gram-positive and Gram-negative bacteria.[2] We have synthesized this compound[3] and have established its solution conformation by use of NMR and CD techniques coupled with molecular simulation.[4] In particular, we have established that the solution-state conformation adopted in 70:30 trifluoroethanol/water corresponds to a dumbbell-like shape with an 'intersection' point at Orn^2 and D-Phe^7. All the hydrophobic side chains project upward on one side, forming a hydrophobic surface, while the hydrophilic side chains of Orn^2, Asp^9, and Asn^8 project to the other side, together with most of the carbonyl oxygens, thereby forming a hydrophilic surface (Figure 1). This apparent amphiphilicity, due to lipophilic side chains projecting on one side and hydrophobic chains projecting from the other, is reminiscent of the 'sidedness' displayed by gramicidin S, and it is this feature that appears to play a role in maintaining high levels of antibiotic activity in analogues of gramicidin S.[5] The synthesis and structural examination of analogues of loloatin C are therefore required.

In the first instance, we used this three-dimensional structure of loloatin C to design structurally simpler analogues. The easiest approach to identification of the pharmacophore is to apply the 'sliding window' approach to this peptide. This technique has been successfully used to predict the secondary structures of proteins[6] and to search for genomic regions with special functions.[7–9] It must be appreciated that the method is not rigorous, and because of the restrictions imposed on the overall structure by the smaller sizes of the windowed fragments, it may actually fail in identifying the pharmacophoric unit. In the case of loloatin C, however, the striking dumbbell structure in the TFE/water solvent system (70:30, Figure 1), with its 'waist' at the Orn^2 and D-Phe^7, makes the 'sliding window' analysis rather more attractive. That is, the molecule is subdivided into two hexapeptides, the first incorporating the Orn^2 and D-Phe^7 as the 'southern' face, and the other the Orn^2 and D-Phe^7 as the 'northern face'; in this way, the opposite faces are left relatively intact with respect to the parent cyclic decapeptide. A further subdivision, as dictated by the 'sliding window', places the Orn^2 and D-Phe^7 at each of the

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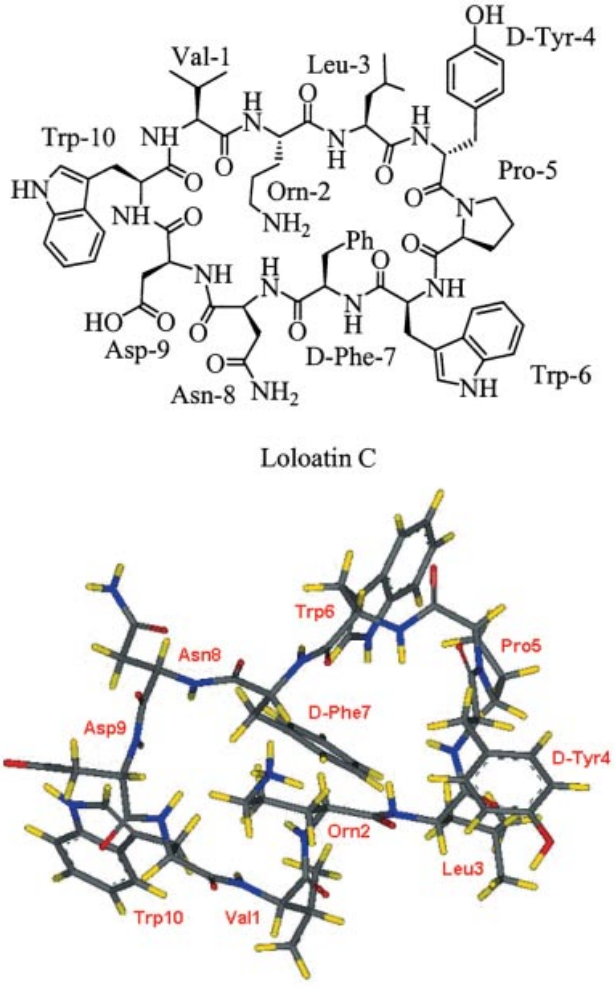


Figure 1. Structure of loloatin C and solution-state conformation in trifluoroethanol/water (70:30) as established by NMR spectroscopy and molecular simulation (ref.[4])

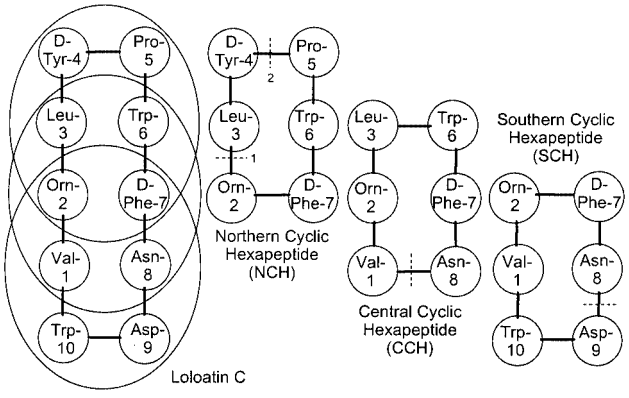


Figure 2. 'Sliding window' analysis of loloatin C to provide three cyclic hexapeptides, and retrosynthetic analysis of the cyclic hexapeptides

'southern' and 'northern' faces (Figure 2). Thus, by the sliding window analysis, the three smaller cyclic hexapeptides generated are the 'northern' cyclic hexapeptide (NCH), cyclic(L-ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-phenylalanyl), the 'southern' cyclic hexapeptide, cyclic(L-valyl-L-ornithyl-D-phenylalanyl-L-asparaginyl-L-aspartyl-L-tryptophanyl) (SCH) and the 'central' cyclic hexapeptide, cyclic(L-valyl-L-ornithyl-L-leucyl-L-tryptophanyl-D-phenylalanyl-L-asparaginyl) (CCH).

It appears likely that the 'northern' face will contribute most to the bioactivity of loloatin C. There are two reasons for this. Firstly, as discussed previously, there is a turn structure (type I β-turn in DMSO, inverse γ-turn in 70:30 TFE/water) in this region, which may contribute to biological activity. Secondly, almost all the aromatic residues lie in this area, and this may be important in relation to binding of the substrate to the active site.

We now describe the synthesis of the cyclic hexapeptides identified by the sliding window operation.

Results and Discussion

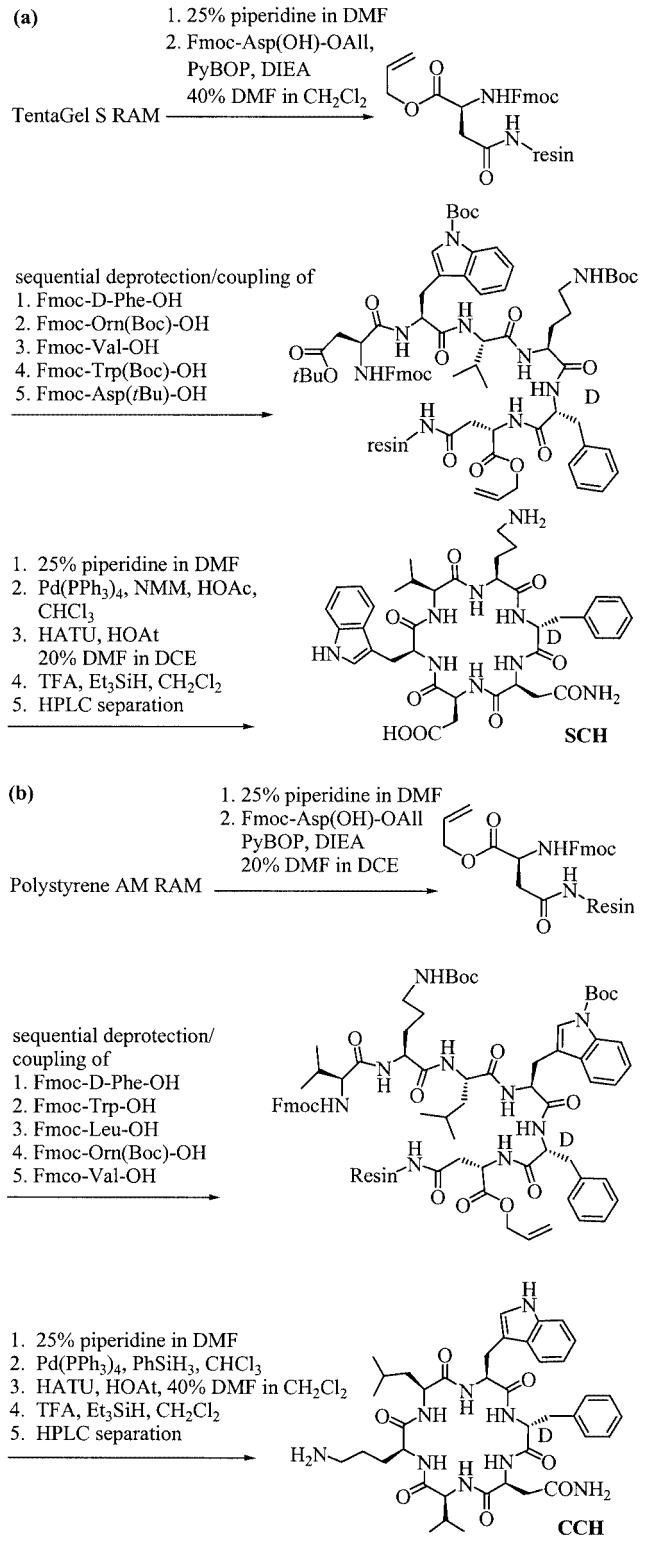
Synthesis

Southern and Central Cyclic Hexapeptides − Solid-Phase Syntheses

The synthesis both of the southern (SCH) and of the central (CCH) cyclic hexapeptides (Figure 2) was successfully carried out by application of the scheme used in the synthesis of the loloatins, reported earlier by our group. Modified Fmoc (fluorenylmethoxycarbonyl)[10] solid-phase peptide synthesis was used. The disconnection is between Asp^9 and Asn^8 (Figure 2) for SCH, and between Val^1 and Asn^8 for CCH. Either TentaGel S RAM or Polystyrene AM RAM were used as solid carriers (Scheme 1). (Benzotriazol-1-yl)oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP[11]) and diisopropylethylamine (DIEA) were used as coupling reagents. Use of a small amount (10 mol %) of 1-hydroxy-7-azabenzotriazole (HOAt)[12] enhances the coupling yield and suppresses racemization. Piperidine (25%) in *N*,*N*-dimethylformamide (DMF) was used to remove the Fmoc groups. DMF (20%) in 1,2-dichloroethane (DCE) was used as solvent for coupling reactions; the effectiveness of this has been noted previously. Alternatively, DMF (40%) in dichloromethane could be used. A new modification was introduced into the synthesis of CCH, with the use of the reagent $Pd(PPh_3)_4/PhSiH_3$ to remove the allyl group. $PhSiH_3$ is a more powerful trapping reagent than *N*-methylmorpholine- (NMM-) acetic acid (HOAc), and is superior to triethylsilane.[13,14] With 12 equivalent of $PhSiH_3$ and a catalytic amount of $Pd(PPh_3)_4$ (10−20 mol %), the deprotection time required for the allyl ester was reduced to around 2 h, whilst over 12 h was required with the usual $Pd(PPh_3)_4$/HOAc/NMM reagent. The overall yields of the solid-phase synthesis were 47% for the southern cyclic hexapeptide (Scheme 1, a) and 42% for the central cyclic hexapeptide (Scheme 1, b).

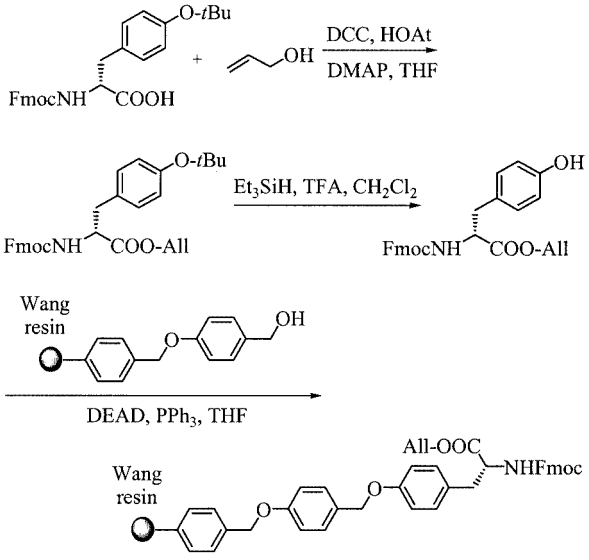
Northern Cyclic Hexapeptide − Solid- and Solution-Phase Syntheses

The synthesis of the northern cyclic hexapeptide, cyclic(L-ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-



Scheme 1. Fmoc-based solid-phase synthesis of: (a) southern cyclic hexapeptide SCH (resin = Tentagel S RAM) and (b) central cyclic hexapeptide CCH (resin = polystyrene AM RAM)

phenylalanyl) (NCH), required a rather different strategy. In the absence of an Asn- or Asp- residue in the sequence (Figure 2), no use can be made of a strategy involving a side chain linked to the resin coupled with an on-resin cyclization, unlike in the previous cases. Modifications in which the side chains of ornithyl or tyrosyl were linked to suitable resins were unsuccessful in the ornithyl case, and only partially successful in the tyrosyl case. Here, the Mitsunobu reaction[15] was used to link the tyrosyl hydroxy group to the Wang resin (Scheme 2); however, only 15% of the hydroxy tags of the resins were occupied by use of this reaction. Nevertheless, the modified resin was used to assemble the linear peptide under the conditions given in Scheme 1.

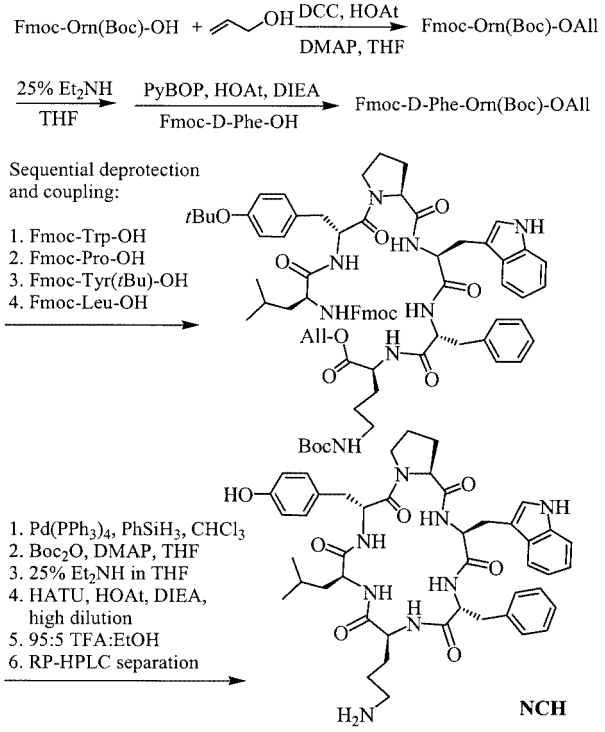


Scheme 2. Modification of Wang resin

The final cyclization was between Tyr[4] and Pro[5] (Figure 2), with an excess of HATU and HOAt in DIEA. After separation and purification by HPLC, however, the northern hexapeptide was obtained only in an overall yield of around 4%. The reasons for the low yield lie not only in the low efficiency of coupling to the resin in the first step, but also in the difficulty of carrying out the cyclization between the *C*-terminal tyrosyl and the secondary *N*-terminal prolyl residues due to steric effects.

The use of a solution method to assemble the linear hexapeptide precursor (Scheme 3) combined with an in situ indirect cyclization (Scheme 4) was therefore developed for the synthesis of the northern cyclic hexapeptide.

As shown in Scheme 3, the *C*-terminus of the first amino acid, ornithine, was protected as the allyl ester, and side chains were protected by *tert*-butyl-based protecting groups. The indole nitrogen of tryptophan was left unprotected, and indeed no side product involving reactions through this centre was found. In all of the coupling reactions, PyBOP in the presence of catalytic amount of HOAt was employed as coupling reagent. Et_2NH (25%) in THF was used to remove the Fmoc group. In the first three couplings, the fulvene-diethylamine adduct was not separated from the mixture, as this did not affect the ensuing coupling reactions. In the next three coupling steps the peptides were separated from the fulvene−diethylamine adducts by column chromatography. Yields in each coupling step were good to excellent, and were generally above 95%.



Scheme 3. Assembly of the linear hexapeptide precursor and its conversion into the northern cyclic hexapeptide NCH by the direct cyclization method

The execution of the intramolecular cyclization in solution was a challenge. The reaction should normally be carried out under highly diluted solutions (10^{-3} to 10^{-4} mol/L) in order to inhibit the intermolecular reaction, due to the entropy problem associated with formation of the macrocycle.[16]

The direct cyclization method[17,18] was used to make the northern cyclic hexapeptide (Scheme 3). It was found that partial loss of the Boc group from the ornithyl δ-amino group took place during chain-elongation, so the peptide was treated with Boc_2O/DMAP to prevent ensuing side reactions. After the removal of the allyl group with $Pd(PPh_3)_4/PhSiH_3$, cyclization was initiated immediately after removal of the Fmoc group by use of 25% Et_2NH in THF. Deprotection of the Boc- and *tert*-butyl-protected groups was then carried out to leave the crude product, which gave the target northern cyclic hexapeptide in 85% yield after purification by semipreparative reversed-phase HPLC.

Northern, Southern, and Central Cyclic Hexapeptides – Indirect in situ Solution-Phase Syntheses

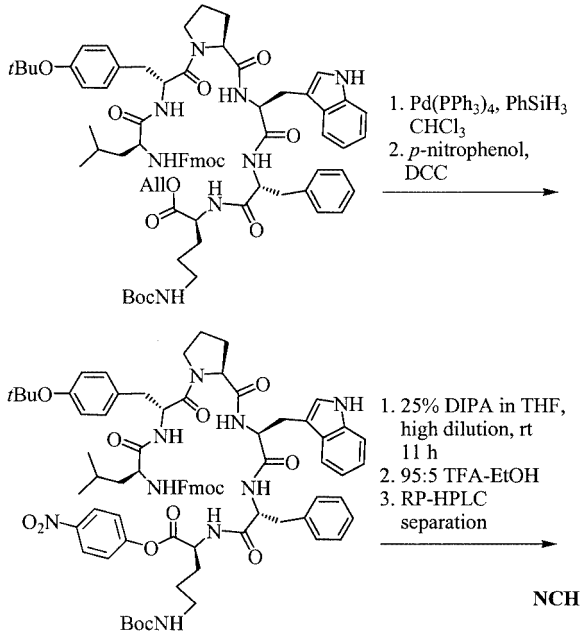
An excess of coupling reagents is usually used for cyclization in the direct cyclization method in solution, with the consequences that side reactions may compete, and purification is rendered difficult. To overcome these problems, we examined the possibility of carrying out a 'indirect' in situ cyclization. This involves selective nucleophile-mediated deprotection of a protected amino group in the presence of an activated ester, and the exposed amino group undergoing intramolecular cyclization by displacement of the group comprising the activated ester, to form the cyclic peptide. Clearly, the nucleophilic reagent used to deprotect the amine must not react with the activated ester.

The general idea of exposing a nucleophilic amino group in the presence of an activated ester in a linear peptide precursor of a cyclic peptide is not new. A robust method involves hydrogenolysis of a Z-protected amino group over a heterogeneous catalyst (Pd-C) in the presence of a pentafluorophenyl ester. Release of the amino group results in cyclization in situ, which is reliant on the presence of the Pd-catalyst surface, upon which the amino group is adsorbed upon its release.[19] A second related method that has been used for construction of cyclic lactams embedded within cyclic peptides relies on the selective acid-mediated deprotection of a 4-methoxytrityl-protected lysine primary ε-amino group in a resin-bound linear peptide containing either a pentafluorophenyl or *p*-nitrophenyl activated ester, followed by basification of the mixture with diisopropylethylamine.[20] The newly released, unhindered, highly nucleophilic primary amino group reacts with the activated ester to form a lactam. The method works very well in the formation of lactams of different ring sizes. In neither method is there a possibility that the reagents used in the deprotection will react with the activated ester.

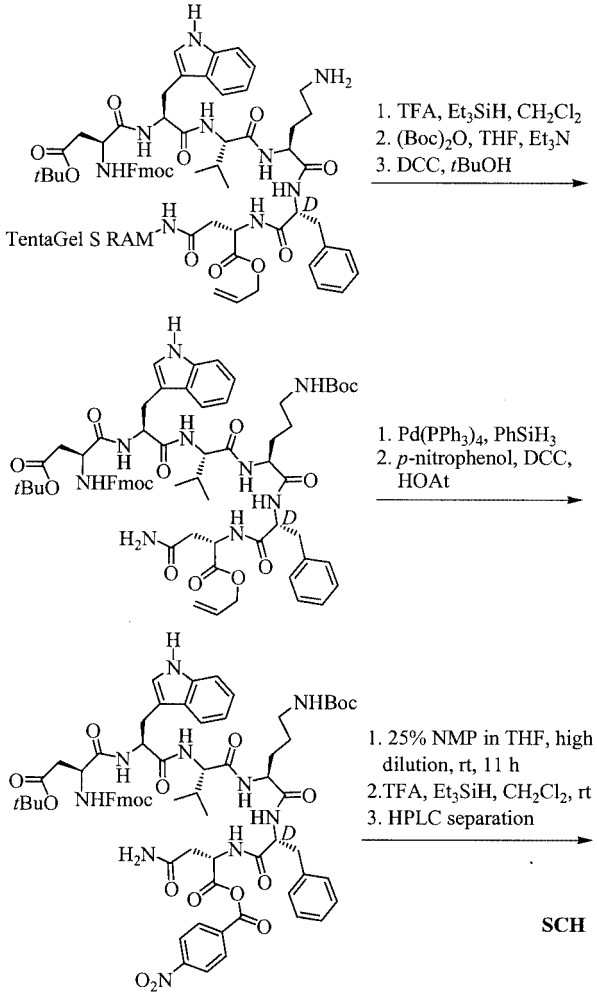
In contrast, our method, to be conducted in solution, was to utilise the hindered, albeit nucleophilic, secondary amine *N*,*N*-diisopropylamine. In principle, the basic reagent *N*-methylpyrrolidine might also be used, as it is also able to remove Fmoc groups,[21] thus exposing the α-amino group in leucine. This group is relatively very hindered, however, since it is attached to a secondary carbon atom bearing an isobutyl group, and so it is not possible to assess a priori how well the planned cyclization would proceed.

The method is shown in Scheme 4. The allyl group was removed from the linear hexapeptide by use of $Pd(PPh_3)_4/PhSiH_3$ as before, and the catalyst and other impurities were removed by chromatography through a short silica gel column. The free *C*-terminus was then converted into the activated *p*-nitrophenyl ester by use of DCC. The mixture was then diluted directly with 25% diisopropylamine (DIPA) in THF at room temperature (11 h). The nucleophilic DIPA induces deprotection of the Fmoc group, and with the release of the free amino group of leucine, intramolecular cyclization is initiated in situ. Because DIPA is a sterically hindered nucleophile, it did not react with the activated ester. The yield of the cyclization was 73%. Given the hindered nature of the amino group, the facility of the intramolecular nucleophilic addition-elimination involving the activated ester to form the cyclic peptide is noteworthy; it proceeds easily at room temperature.

The generality of this indirect in situ cyclization was also demonstrated in the synthesis of the southern and central hexapeptides, in which the linear hexapeptide precursors were made by the solid-phase method (cf. Scheme 1). Assembly of the linear hexapeptide was carried out as described above. For the southern cyclic hexapeptide SCH, the linear peptide was cleaved from the resin with Et_3SiH-



Scheme 4. Synthesis of the northern cyclic hexapeptide NCH by indirect in situ cyclization in solution



Scheme 5. Synthesis of the southern cyclic hexapeptide SCH by indirect in situ cyclization in solution

TFA in dichloromethane (Scheme 5). The free carboxyl moiety of the aspartyl residue and the free amino group of the ornithyl were protected. The allyl group was removed from the protected aspartate with $Pd(PPh_3)_4/PhSiH_3$, and the liberated carboxyl group was converted into the *p*-nitrophenyl ester with DCC. Without further purification, the mixture was brought to high dilution with 25% *N*-methylpyrrolidine (NMP) in THF, such that removal of the Fmoc group and cyclization was spontaneously initiated. The yield of this cyclization method was 80%, and the subsequent steps were carried out as shown in Scheme 5.

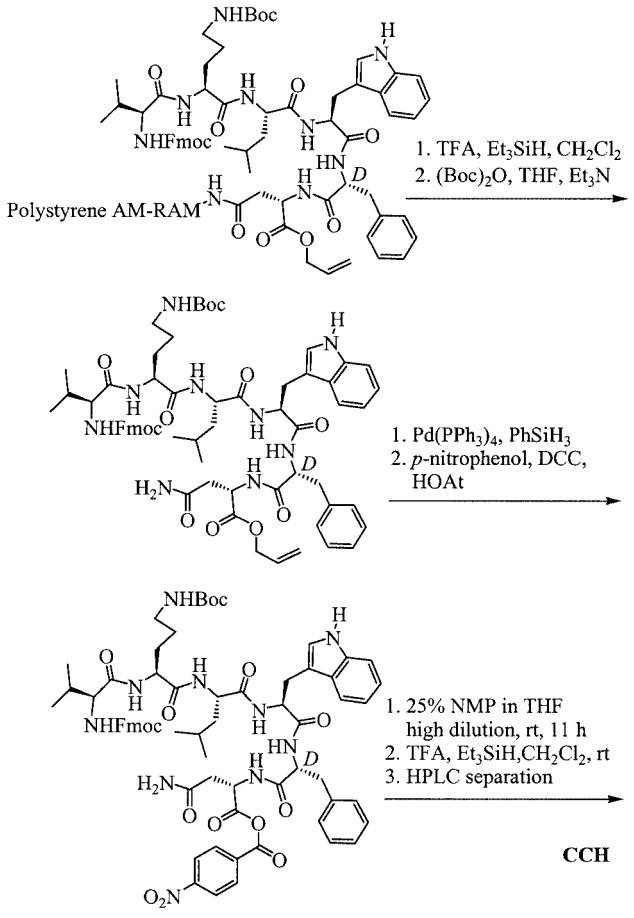
The in situ solution cyclization as applied to the central cyclic peptide CCH was conducted as follows. The linear peptide was cleaved from the resin with Et_3SiH/TFA in dichloromethane (Scheme 6). The ornithyl residues were protected, and the allyl group was removed from the protected aspartate by use of $Pd(PPh_3)_4/PhSiH_3$ as above. The free carboxyl was converted into the *p*-nitrophenyl ester, and the Fmoc group was removed with NMP in THF. Again, the remarkable efficiency of the procedure is apparent. Although the yield for the cyclization reaction (74%) is slightly less than in the above cases, this is probably due to the steric effect caused by the isopropyl group in the cyclization between Val^1 and Asn^8, which is not apparent in the cyclization between Asp^9 and Asn^8 in the previous case.

Preliminary NMR Studies

Through the use of ^1H, ^1H-^1H DQF-COSY, TOCSY, and NOESY experiments it was possible to assign all the spin systems of the three cyclic hexapeptides. Table 1 gives the chemical shift data.

Through analysis of NOEs[22−24] in combination with peptide backbone modelling, preliminary conformational and steric effects in the cyclic hexapeptides in DMSO could be established. As indicated in Figure 3, there are a series of strong NOEs involving αN(*i*, *i*+1), αN(*i*, *i*) in the residues of the southern cyclic hexapeptide. In addition, three NN(*i*, *i*+1)-type NOEs were unambiguously identified. Most of the chemical shifts of the α-methine protons are upfield (−0.1 to −0.3 ppm) of the standard values, except for that of Val, which is close to the standard value.[25] A helix-like conformation combined with a γ-turn secondary structure between Val, Orn and D-Phe is therefore suggested for the southern cyclic hexapeptide.

Important NOEs were also clearly identified in the central cyclic hexapeptide. The characteristic inter-residue NOEs include a series of strong NOEs for αN(*i*, *i*+1), and a weak NOE for αN(6, 1), medium to strong NOEs for αN(*i*, *i*), where the αN(1, 1), αN(2, 2), and αN(5, 5) are medium, whilst the αN(3, 3), αN(4, 4), αN(6, 6) are strong, medium to weak inter-residues NN(*i*, *i*+1) NOEs, where NN(2, 3) and NN(1, 6) are medium, and NN(1, 3) is very weak. The chemical shift of $C^{\alpha}H$ for Val is 0.05 ppm downfield from the standard value, and all other chemical shift values are upfield of the standard values. The differences range between −0.2 to −0.3 ppm. Thus, the conformation



Scheme 6. Synthesis of the central cyclic hexapeptide CCH by non direct in situ cyclization in solution

of the central cyclic hexapeptide in DMSO may be also helix-like, with a γ-turn between Val, Orn, and Leu.

In the case of the northern cyclic hexapeptide, fewer NOEs are found in the spectrum. Only three αN(*i*, *i*+1) NOEs are strong — these are between αN(2, 3), αN(5, 6), and αN(1, 2). The other two are medium NOEs; these are between αN(4, 5) and αN(6, 1), respectively. There are three αN(*i*, *i*) NOEs, of which the αN(1, 1), αN(5, 5) is medium, and the αN(2, 2) is weak. NOEs between amide protons are also observed, the NN(1, 2) and NN(2, 3) being medium, and the NN(5, 3) very weak. The chemical shifts of $C^{\alpha}H$ are, as usual, upfield relative to standard values for a coil structure. The northern cyclic hexapeptide thus adopts a helix-like structure, and a γ-turn structure between Leu, Orn and D-Phe.

Conclusion

We have developed an efficient new in situ cyclization method involving the preparation of activated esters of the free *C*-termini of three linear hexapeptides, followed by selective deprotection of the Fmoc-protected *N*-terminal amino groups with concomitant cyclization to provide three model cyclic hexapeptides based on the parent cyclic decapeptide loloatin C in very good yields. The successful outcome of the in situ cyclization reactions is particularly noteworthy in the case of the northern cyclic hexapeptide, cyclic(L-ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-

Table 1. ^1H NMR spectroscopic data (500 MHz, $[D_6]$DMSO for cyclic hexapeptides)

	SCH	CCH	NCH
Val			
NH	7.73	8.07	
α-CH	4.19	4.23	
β-CH	2.16	2.30	
γ-CH_3	0.83	1.03	
γ -CH_3	0.92	1.03	
Orn			
NH	7.71	8.32	8.10
α-CH	4.05	4.01	4.18
β-CH_2	1.67, 1.48	1.91	1.80, 2.10
γ-CH_2	1.32	1.75	1.52, 1.65
δ-CH_2	2.70, 2.79	2.81, 2.92	2.95, 3.11
δ-NH	7.64	7.90	7.86, 8.46
Leu			
NH		7.90	7.42
α-CH		4.20	4.40
β-CH_2		1.65	1.52
γ-CH		1.65	1.65
δ-CH_3		0.91	0.89
δ-CH_3		1.01	0.89
D-Tyr			
NH			8.23
α-CH			4.56
β-CH_2			2.74, 2.86
o-CH			7.05
m-CH			6.74
p-COH			not observed
Pro			
α-CH			4.20
β-CH_2			2.10
γ-CH_2			1.30
δ-CH_2			3.43
D-Phe			
NH	8.81	8.68	8.18
α-CH	4.49	4.31	4.21
β-CH_2	2.82, 3.29	2.82, 3.08	2.94, 3.04
o-CH	7.31	7.35	7.35
m-CH	7.31	7.35	7.35
p-CH	7.31	7.35	7.35
Asn			
NH	8.70	8.52	
α-CH	4.59	4.59	
β-CH_2	2.72	2.61, 2.79	
NH_2	7.05, 7.61	7.11, 7.58	
Asp			
NH	8.22		
α-CH	4.52		
β-CH_2	2.72, 2.81		
Trp			
NH	8.46	7.95	6.95
α-CH	4.42	4.55	4.52
β-CH_2	3.21	3.09	3.08
2H	7.22	7.09	6.80
4H	7.75	7.65	7.65
5H	7.13	6.95	6.90
6H	7.18	7.12	7.15
7H	7.43	7.46	7.36
NH	10.93	10.91	10.85

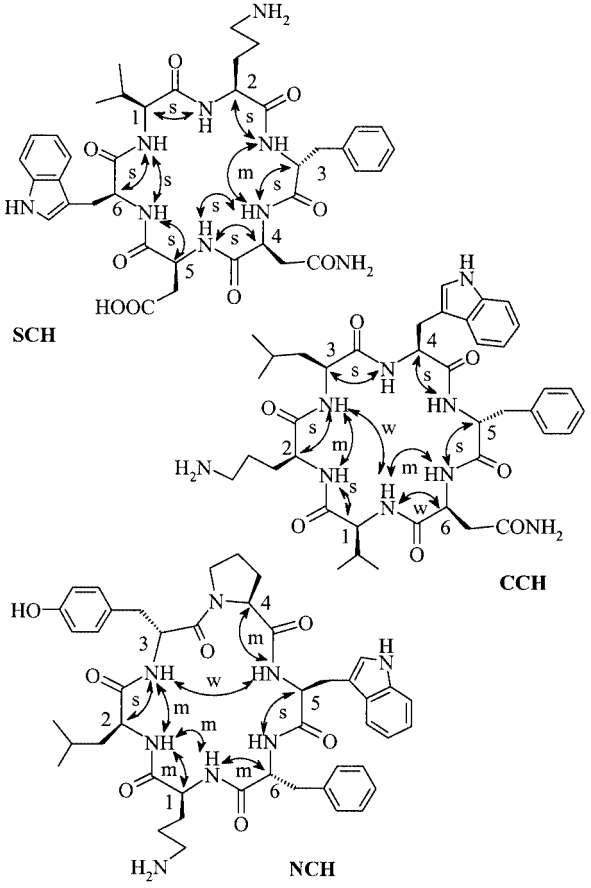


Figure 3. Important NOEs of the southern cyclic hexapeptide (SCH), central cyclic hexapeptide (CCH), and northern cyclic hexapeptide (NCH) (s: strong; m: medium; w: weak)

phenylalanyl), given the hindered nature of the nucleophilic leucine amino group, which undergoes the ring-forming reaction with the activated *p*-nitrophenyl ester of ornithine.

Experimental Section

General Remarks. Peptide Syntheses: *N*,*N*-Dimethylformamide (DMF), peptide synthesis grade (Millipore) was dried over 4Å molecular sieves. Chloroform and dichloromethane were freshly distilled from CaH_2 and stored over 4Å molecular sieves, piperidine was dried with KOH and distilled prior to use, and 1,2-dichloroethane (DCE) was distilled from over phosphorus pentoxide prior to use. Ethyl acetate and *n*-hexane were freshly distilled from anhydrous Na_2SO_4, and THF was freshly distilled from granular sodium. Other commercial reagents and solvents were used as received. Fmoc-protected amino acids were obtained from BACHEM Inc., $(Boc)_2O$, DIPCI, 85% DEAD, DCC, $Pd(PPh_3)_4$, HBTU, HATU, HOAt, and HOBt from Aldrich, PyBOP, TBTU from Nova Biochem, TentaGel S RAM resin (capacity: 0.22−0.25 mmol/g), Polystyrene AM RAM resin (capacity: 0.78 mmol/g) and Wang resin (1.0 mmol/g) from RAPP Polymere Gmbh, Tübingen, Germany. All the resins used in the synthesis were dried at room temperature in vacuo for several hours.

Instrumentation: UV spectra were acquired on a Milton ROY 3000 Spectronic spectrophotometer, and optical rotation values were determined on a Perkin−Elmer 241 Polarimeter. Reversed-phase HPLC was carried out on a Waters 717/600/2996 machine with Xterra™ RP_{18} (4.6 × 250 mm for analysis; 7.8 × 300 mm for semi-preparative scale) by use of isocratic or gradient methanol (A)/0.1% TFA in water (B). Mass spectra were acquired in FAB or CI modes with a Finnigan Mat TSQ 7000 instrument. NMR spectra were acquired either on a Varian Unity INOVA 500-MHz instrument or on a JNM EX-400 instrument.

Southern Cyclic Hexapeptide [Cyclic(L-valyl-L-ornithyl-D-phenylalanyl-L-asparaginyl-L-aspartyl-L-tryptophanyl)] (SCH)

a. Solid-Phase Synthesis: The procedure was similar to that reported previously.[3] The amount of TentaGel S RAM resin was 0.53 gram (capacity 0.22 mmol/g), measured mole quantity was 0.1132 mmol. PyBOP/HOAt/DIEA (0.4 mmol) were employed as coupling reagents in each step, and 25% piperidine in DMF was used to remove the Fmoc groups in each step. Sequential coupling and deprotection was carried out in the sequence Fmoc-Asp(OH)-*O*-All, D-Fmoc-Phe-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, and Fmoc-Asp(*t*Bu)-OH, and 0.3 mmol $Pd(PPh_3)_4$/NMM/HOAc were used to remove the allyl group. HATU/HOAt/DIEA (0.5 mmol) were used for cyclization of the peptide. RP-HPLC purification [eluent: methanol (A), 0.1% TFA in water (B); gradient: 25% A to 65% A within 60 minutes; flow rate: 3.0 mL/min] gave a white solid (41.4 mg), purity 99.2%, (47%). $[\alpha]_D^{25} = -2.34$ (96% ethanol, $c = 0.32$). UV (96% ethanol): λ_{max} (ε) = 219.8 (26 771.84), 283.5 (2997.57), 340.5 (800.97). FAB-MS: m/z = 776.2 (calcd. for M + $H/C_{38}H_{50}N_9O_9$, 776.864). ^1H NMR spectroscopic data are given in Table 1.

b. Chain-Elongation by Solid-Phase Synthesis and Cyclization by the in situ Solution Method: Chain-elongation was carried out as described in a. above by commencing with 0.5 gram TentaGel S RAM resin (capacity 0.22 mmol/g), measured quantity 0.109 mmol. When the chain-assembly was finished, the linear peptide was cleaved from the resin by double application of TFA/Et_3SiH/dichloromethane (95:5:5). The crude linear peptide was purified by RP-HPLC (gradient 10% to 30% methanol/ 0.1% TFA in water within 50 minutes) to give a white solid (80.6 mg), purity 98.3% (71%), FAB-MS 1040.4 (calcd. for M + $H/C_{56}H_{65}N_9O_{11}$, 1040.234). The linear peptide was dissolved in THF (5 mL), $(Boc)_2O$ (30.6 mg, 0.14 mmol) and DMAP (1 mg) were then added, and the solution was stirred at room temperature for 2 h. The THF was removed under vacuum, and the residue was subjected to column chromatography over a short column of silica gel (3 × 10 cm) with $CHCl_3$/methanol (10:1, v/v) to give the protected linear peptide (65.8 mg, 82.5%). A solution of the linear peptide (65.0 mg, 0.063 mmol) in THF (5 mL) was cooled in an ice bath and then treated sequentially with *t*BuOH (14.8 mg, 0.2 mmol), HOAt (0.8 mg, 0.006 mmol), and DMAP (0.4 mg). After that, DCC (23.9 mg, 0.106 mmol) in THF (2 mL) was added by syringe. The resulting mixture was stirred overnight with warming to room temperature. The THF was removed by evaporation under reduced pressure, and the residue was purified by chromatography over silica gel with $CHCl_3$/methanol (20:1) to give the linear protected peptide (60.5 mg, 87%). A solution of the linear peptide (60.5 mg) in chloroform (5 mL) was treated sequentially with $Pd(PPh_3)_4$ (11.78 mg, 0.01 mmol) and $PhSiH_3$ (33.11 mg, 0.31 mmol). The course of the deprotection reaction was followed by TLC (chloroform/methanol 25:1), which indicated that the allyl group had been completely removed after 2 h. The mixture was filtered through silica gel to remove palladium and silicon-containing by-products, and the chloroform was then evaporated under reduced pressure. The residue was dissolved in THF (5 mL), and the solution was treated sequentially with *p*-nitrophenol (14.19 mg, 0.102 mmol),

HOAt (0.7 mg, 0.005 mmol), DMAP (0.3 mg), and DCC (21.0 mg, 0.102 mmol). The resulting mixture was stirred for 2 h, and a solution (100 mL) of 25% *N*-methylpyrrolidine (NMP) in THF (v/v) was then added to initiate the cyclization. The reaction was continued for 11 h. The DIPA and THF were then removed by evaporation in vacuo at room temperature. The residue was dissolved in $CHCl_3$ (50 mL) and filtered to remove DCU. The filtrate was washed with 5% aqueous Na_2CO_3 and water, and the organic layer was then evaporated under reduced pressure. The residue was treated carefully with TFA/Et_3SiH/dichloromethane (95:5:5, 10 mL), and the resulting mixture was stirred for 1.5 h. The TFA and dichloromethane were removed by evaporation under reduced pressure, and the residue was purified by semipreparative RP-HPLC [eluent: methanol (A), 0.1% TFA in water (B); gradient: 25% A to 65% A within 60 minutes; flow rate: 3.0 mL/min] to give the cyclic hexapeptide as a white solid (31.8 mg, 80%), purity 98.6%, FAB-MS: *m*/*z* = 776.5 (calcd. for M + H/$C_{38}H_{50}N_9O_9$, 776.864).

Central Cyclic Hexapeptide [Cyclic(L-Valyl-L-ornithyl-L-leucyl-L-tryptophanyl-D-phenylalanyl-L-asparaginyl)] (CCH)

a. Solid-Phase Synthesis: The procedure was similar to that developed for the synthesis of loloatin C. The amount of polystyrene AM RAM resin (capacity: 0.78 mmol/g) was 0.2047 gram, measured mole quantity 0.16 mmol. PyBOP/HOAt/DIEA (0.4 mmol) was employed as the coupling reagent in each step, and 25% piperidine in DMF was used to remove Fmoc groups in each step. Sequential coupling and deprotection was carried out in the sequence Fmoc-Asp(OH)-*O*-All, Fmoc-D-Phe-OH, Fmoc- Trp(Boc)-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, and Foc-Val-OH. $Pd(PPh_3)_4$ (0.032 mmol) and $PhSiH_3$ (0.96 mmol) were used to remove the allyl group; a reaction time of 2 h was required. HATU/HOAt/DIEA (0.5 mmol) was used to cyclize the peptide. Purification by RP-HPLC [eluent: methanol (A), 0.1% TFA in water (B); gradient: 20% A to 60% A within 60 minutes; flow rate: 3.0 mL/min] gave a white solid, purity 99.7%, 52.5 mg (42%). $[\alpha]_D^{25} = -5.0$ (96% ethanol, $c = 0.35$). UV (96% ethanol) λ_{max} (ε) = 220.2 (19 379.84), 280.9 (2746.40), 373.1 (465.12). FAB-MS: *m*/*z* = 774.3 (calcd. for M + H/$C_{40}H_{56}N_9O_7$, 775.944). ^{1}H NMR spectroscopic data are given in Table 1.

b. Chain-Elongation by Solid-Phase Synthesis and Cyclization by the in situ Solution Method: Chain-elongation was carried out as described in a. above. Polystyrene AM RAM resin (capacity: 0.78 mmol/g), 0.2 g, measured mole quantity 0.15 mmol was used. When the chain-assembly was finished, the linear peptide was cleaved from the resin by double treatment with TFA/Et_3SiH/dichloromethane. The crude linear peptide was purified by semipreparative RP-HPLC (gradient 10% to 30% methanol/0.1% TFA in water within 50 minutes) to give a white solid (113.89 mg, 73%), purity 99.3%. FAB-MS 1038.6 (calcd. for M + H/$C_{58}H_{71}N_9O_9$, 1038.26). A solution of the linear peptide (113.2 mg) in THF (5 mL) was treated sequentially with $(Boc)_2O$ (48.02 mg, 0.22 mmol) and DMAP (1 mg), and the resulting mixture was stirred at room temperature for 2 h. The THF was then removed by evaporation under reduced pressure, and the residue was purified by passage through a short column of silica gel (3 × 15 cm) with $CHCl_3$/methanol (10:1) to give the linear peptide (105.97 mg, 85%). A solution of the linear peptide in $CHCl_3$ (5 mL) was treated sequentially with $Pd(PPh_3)_4$ (23.11 mg, 0.02 mmol) and $PhSiH_3$ (60.6 mg, 0.56 mmol). The course of the ensuing reaction was monitored by TLC in chloroform/methanol (25:1), which indicated that the allyl group had been completely removed after 2 h. The mixture was filtered through silica gel to remove the palladium and silicon-containing by-products. The solvent was evaporated under reduced pressure, and the residue was dissolved in THF (5 mL). The solution was treated sequentially with *p*-nitrophenol (28.38 mg, 0.2 mmol), HOAt (1.4 mg, 0.01 mmol), DMAP (0.6 mg), and DCCI (41.26 mg, 0.2 mmol), and the resulting mixture was stirred for 2 h. *N*-Methylpyrrolidine (NMP) in THF (25%, 100 mL) was then added to initiate the cyclization. The reaction mixture was stirred for 11 h, and the NMP and THF were then removed by evaporation under vacuum at room temperature. The residue was taken up in chloroform (50 mL) and filtered to remove DCU. The filtrate was washed with 5% aqueous Na_2CO_3 and then with water, and the organic layer was separated. The chloroform was removed by evaporation under reduced pressure, and TFA/Et_3SiH/dichloromethane (95:5:5, 10 mL) was then added slowly to the residue. The resulting mixture was stirred for 1.5 h, and the TFA and dichloromethane were removed by evaporation under reduced pressure. The residue was purified by semipreparative RP-HPLC [eluent methanol (A), 0.1% TFA in water (B); gradient 20% A to 60% A within 60 minutes; flow rate 3.0 mL/min] to give the cyclic peptide as a white solid (53.2 mg, 73.8%), purity 97.9%. FAB-MS: 775.2 (calcd. for M + H/$C_{40}H_{56}N_9O_7$, 775.944).

Northern Cyclic Hexapeptide [Cyclic(L-Ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-phenylalanyl)] (NCH)

Solution-Phase Synthesis of Fmoc-Leu-D-Tyr(*t*Bu)-Pro-Trp-D-PheOrn(Boc)-*O*-All

i. Fmoc-Orn(Boc)-*O*-All: Fmoc-Orn(Boc)-OH (0.4545 g, 1.0 mmol), allyl alcohol (69.7 mg, 1.2 mmol), HOAt (11.66 mg, 0.1 mmol) and DMAP (6.1 mg, 0.05 mmol) were dissolved in THF (10 mL). The solution was cooled to 0 °C in an ice bath, and DCC (0.2476 g, 1.2 mmol) in dichloromethane solution was then added slowly by syringe. The solution was stirred overnight, and the THF was then removed by evaporation under reduced pressure. The residue was dissolved in EtOAc (50 mL) and the solution was washed sequentially with water (2 × 15 mL), 5% aqueous $NaHCO_3$ (2 × 15 mL), water, and saturated aqueous $CaCl_2$. The organic layer was then dried with anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography over silica gel with 1:1 EtOAc/*n*-hexane to give the peptide as a white solid (0.481 g, 97.3%), $R_f = 0.59$. $[\alpha]_D^{25} = +3.9$ ($c = 0.74$, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.4−7.8 (m, 4 H, Ar-H), 7.2−7.4 (m, 4 H, Ar-H), 5.88 (1 H, -CH=), 5.53 (t, H), 5.24−5.35 (m, 2 H, $=CH_2$), 4.1−4.63 (m, 5 H), 3.12 (m, 2 H), 1.5−2.0 (m, 4 H), 1.4 (s, 9 H, $-CH_3$).

ii. Fmoc-D-Phe-Orn(Boc)-*O*-All: Fmoc-Orn(Boc)-*O*-All (0.2091 g, 0.45 mmol) was dissolved in 25% diethylamine in THF solution (10 mL) and the system was stirred for 2 h. When analysis by TLC (silica gel; 3:7 EtOAc/*n*-hexane) indicated that the Fmoc group had been completely removed, the THF and excess diethylamine were removed by evaporation under vacuum at room temperature. The residue was kept under vacuum at room temperature for 2 h, and then dissolved in THF (2 mL) for the following reaction. Fmoc-D-Phe-OH and PyBOP (1.2 equivalents each) were dissolved in THF (10 mL), and DIEA was then added. After being stirred for 15 minutes, the mixture was cooled to 0 °C in an ice bath, and was then treated with above amine in THF solution. The ice bath was removed, and the temperature of the reaction mixture was allowed to warm up to room temperature. The mixture was stirred for another 30−60 minutes. The solvent was then removed in vacuo, and the residue was dissolved in chloroform (50 mL). The solution was washed with water (2 × 15 mL), 5% aqueous $NaHCO_3$ (3 × 15 mL), and water (2 × 15 mL), and then dried with anhydrous

Na_2SO_4. After filtration, the chloroform was evaporated under reduced pressure at room temperature, and the residue was purified by flash chromatography with chloroform/methanol (50:1 v:v), to give the protected dipeptide as a white solid (0.2873 g, 99.5%), R_f = 0.48. FAB-MS: *m/z* = 642.2 (20, $[M + H]^+$), 542.2 (100, $[M + H - Boc]^+$). ^1H NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.75 (d, 2 H, Ar-H), 7.38−7.55 (m, 2 H, Ar-H), 7.22−7.32 (m, 9 H, Ar-H), 6.54 (br., 1 H, -NH), 5.81−5.91 (m, 1 H, -CH=), 5.45 (br., 1 H, -NH), 5.22−5.32 (m, 2 H, $=CH_2$), 4.58 (d, 2 H), 4.31−4.51 (m, 5 H), 4.10−4.18 (m, 1 H), 3.03−3.09 (m, 4 H), 1.52−1.71 (m, 4 H), 1.44 (s, 9 H, $-CH_3$).

iii. Fmoc-Trp-D-Phe-Orn(Boc)-*O*-All: Fmoc-D-Phe-Orn(Boc)-*O*-All (0.2873 g, 0.45 mmol) was treated sequentially with 1.2 equivalents each of Fmoc-Trp-OH, PyBOP, and DIEA, according to the procedure in part b above. The product was purified by flash chromatography with chloroform/methanol (25:1) to give the protected tripeptide as a white solid, R_f = 0.51. FAB-MS: *m/z* = 828.1 (68) $[M + H]^+$, 728.0 (100) $[M + H - Boc]^+$. The compound did not dissolve in $CDCl_3$ and no ^1H NMR spectroscopic data were obtained; it was characterized by conversion into the pentapeptide described in section v. below.

iv. Fmoc-Pro-Trp-D-Phe-Orn(Boc)-*O*-All: Fmoc-Trp-D-Phe-Orn-(Boc)-*O*-All (0.2958 g, 0.5 mmol) was treated sequentially with 1.2 equivalents each of Fmoc-ProOH, PyBOP, and DIEA, according to the procedure in part b above. The residue was purified by flash chromatography with chloroform/methanol (10:1) to give the protected tetrapeptide (0.4615 g, 99.8%), R_f = 0.56. FAB-MS: *m/z* = 947.4 (40) $[M + Na]^+$, 924.4 (100) $[M - H]^+$.

v. Fmoc-D-Tyr(*t*Bu)-Pro-Trp-D-Phe-Orn(Boc)-*O*-All: Fmoc-Pro-Trp-D-Phe-Orn(Boc)-*O*-All (0.296 g, 0.32 mmol) was dissolved in 25% Et_2NH in THF (10 mL). The solution was stirred for 2 h, and excess Et_2NH and THF were then removed by evaporation under vacuum at room temperature. The residue was dissolved in chloroform (50 mL), and the solution was washed with water (2 × 15 mL), 5% aqueous K_2CO_3 (2 × 15 mL), and then water (2 × 15 mL). The organic layer was dried with anhydrous Na_2SO_4. After filtration, the chloroform was removed by evaporation under reduced pressure, and the residue was purified by flash chromatography with chloroform/methanol (10:1, with pH value adjusted to 8−9 by triethylamine) to give the partially deprotected tetrapeptide as a white solid (0.2207, 98.1%), R_f = 0.29. FAB-MS: *m/z* = 703.3 (100) $[M + H]^+$, 603.2 (28) $[M + H\text{-Boc}]^+$, calcd. $[M + H]^+$/$C_{38}H_{51}N_6O_7$: 703.86. ^1H NMR (400 MHz, $CDCl_3$): δ [ppm] = 10.16 (sh, 1 H, $N^{in}H$), 8.09 (d, 2 H, -NH-), 7.75 (m, 2 H, Ar-H), 7.54 (d, 1 H, -NH-), 7.35 (d, 1 H, -NH-), 6.90−7.23 (m, 7 H, Ar-H), 6.89 (s, 1 H, -NH-), 5.84−5.92 (m, 2 H), 5.21−5.34 (m, 2 H), 4.36−4.66 (m,5 H), 3.60 (t, 1 H), 2.83−3.07 (m, 9 H), 2.58 (h, 2 H), 1.41−2.0 (m, 5 H), 1.39 (s, 9 H, $-CH_3$).

A solution of Fmoc-D-Tyr(*t*Bu)-OH (0.1654 g, 0.36 mmol) and PyBOP (0.1873 g, 0.36 mmol) in THF (10 mL) was treated with DIEA (0.06 mL, 0.36 mmol). After 15 minutes, the mixture was cooled to 0 °C with an ice bath, and it was then treated dropwise with a solution of the above tetrapeptide (0.2126 g, 0.3 mmol) in THF (3 mL). The reaction was allowed to continue for 2 h, and the mixture was then worked up as described above to give a residue, which was purified by flash chromatography with chloroform/methanol (10:1) to give the protected pentapeptide as a white solid (0.3412 g, 99.4%), R_f = 0.68. FAB-MS: *m/z* = 1145.6 (20) $[M + H]^+$, 1044.5 (100) $[M - Boc]^+$.

vi. Fmoc-Leu-D-Tyr(*t*Bu)-Pro-Trp-D-Phe-Orn(Boc)-*O*-All: The protected pentapeptide (0.3204 g, 0.28 mmol) was dissolved in Et_2NH in THF (25%, 10 mL) to remove the Fmoc group. The details were the same as described in v. above. The compound was purified by flash chromatography with chloroform/methanol (10:1, with pH value adjusted to 8−9 with triethylamine) to give the peptide as a white solid (0.2183 g, 84.6%), R_f = 0.35. FAB-MS: *m/z* = 922.5 (100) $[M]^+$, 822.4 (90) $[M + H - Boc]^+$; calcd. M/$C_{51}H_{67}N_7O_9$: 922.14. ^1H NMR (400 MHz, $CDCl_3$): δ [ppm] = 8.84 (s, 1 H, $N^{in}H$), 7.46−7.52 (m, 2 H, Ar-H), 7.40 (d, 1 H, -NH-), 7.17−7.39 (m, 7 H, Ar-H), 6.85−7.09 (m, 4 H, Ar-H), 6.62 (d, 1 H, $-N^{in}CH$-), 5.88−5.95 (m, 1 H, -CH=), 5.25−5.36 (m, 2 H), 4.75 (br., 1 H), 4.62 (d, 4 H), 4.43−4.46 (m,1 H), 4.07−4.11 (m,1 H), 3.28 (m, 1 H), 2.98−3.25 (m, 10 H), 2.75 (d, 2 H), 2.42 (m, 1 H), 1.74−1.83 (m, 7 H), 1.40 (m, 2 H), 1.33 (s, 9 H, $-CH_3$), 1.28 (s, 9 H, $-CH_3$).

A stirred solution of Fmoc-Leu-OH (99.0 mg, 0.28 mmol) and PyBOP (0.1457 g, 0.28 mmol) in THF (10 mL) was treated with DIEA (0.05 mL) and then with a solution of the above pentapeptide (0.21 g, 0.228 mmol) in THF (4 mL). The reaction was continued for 2 h. A residue was obtained by the usual workup procedure, and was purified by flash chromatography with chloroform/methanol (10:1, with pH value adjusted to 8−9 with triethylamine) to give the protected linear hexapeptide as a white solid (0.2544 g, 88.7%), R_f = 0.57. FAB-MS: *m/z* = 1258.3 (10) $[M + H]^+$, 1157.8 (100) $[M + H - Boc]^+$. It was characterized by cyclization as described below.

vii. In situ Solution Cyclization: The protected hexapeptide Fmoc-Leu-D-Tyr(*t*Bu)-Pro-Trp-D-Phe-Orn(Boc)-*O*-All (0.1258 g, 0.1 mmol) was dissolved in dry chloroform (2 mL, freshly distilled from P_2O_5). Under a nitrogen atmosphere, $Pd(PPh_3)_4$ (11.56 mg, 0.01 mmol) was added to the solution, followed by $PhSiH_3$ (0.074 mL, 0.6 mmol). The course of the reaction was monitored by TLC (chloroform/methanol, 10:1), which indicated that the reaction was complete after 2 h. The chloroform and some of the $PhSiH_3$ were partially removed by evaporation under reduced pressure at room temperature, and the concentrated solution was passed through a short silica gel column (10 × 3 cm) to remove palladium and silicon-containing by-products. The crude product was isolated by evaporation of the eluate, and was used directly without further purification. A solution of the crude product, *p*-nitrophenol (16.7 mg, 0.12 mmol), HOAt (1.4 mg, 0.012 mmol), and DMAP (0.7 mg) in THF (10 mL) was treated with DCC (24.8 mg, 0.12 mmol) in dried THF (10 mL). The reaction mixture was allowed to stand for 4 h, and was then filtered under nitrogen gas. The filtrate was then diluted with 25% diisopropylamine (DIPA) in THF (100 mL) to initiate the cyclization. Monitoring of the cyclization by RP-HPLC indicated that the reaction was complete after 11 h. The excess of DIPA and THF was removed by evaporation under reduced pressure at room temperature, and the residue was dissolved in chloroform (50 mL). The solution was washed successively with water (2 × 15 mL), 5% K_2CO_3 (2 × 15 mL), and water (2 × 15 mL), and the chloroform was then removed under reduced pressure. The residue was purified by RP-HPLC [eluent: methanol (A) and 0.1% TFA in water (B); gradient; from 40% to 80% A within 60 minutes] to give the cyclized protected hexapeptide as a white solid (70.9 mg, 72.6%), purity 97.5%. FAB-MS: *m/z* = 978.3 (25) $[M + H]^+$, 878.2 (100) $[M + H - Boc]^+$, 822.1 (65) $[M + 2H - Boc - t\text{Bu}]^+$; calcd. M + H/$C_{54}H_{72}N_8O_9$: 977.22. A solution of the compound in ethanol (2 mL) at 0 °C was treated with TFA (8 mL). After 10 minutes, the solution was allowed to warm to room temperature, and was then stirred for another 1 h. The excess of TFA and ethanol were removed by evaporation under vacuum at room temperature. The

residues were purified by RP-HPLC [eluent: methanol (A) and 0.1% TFA in water (B); gradient; from 30% to 70% A within 60 minutes] to give the cyclic hexapeptide as a white solid (69.2 mg), purity 98.7%. FAB-MS: *m*/*z* = 837.3 (12) $[M + H_2O + 2H]^+$, 821.3 (100) $[M + H]^+$; calcd. M + H/$C_{45}H_{56}N_8O_7$: 820.992. $[\alpha]_{20}^{D}$ = −45.4 (*c* = 0.41, 96% ethanol). UV (96% ethanol): λ_{max} (ε) = 223.5 (29 305.41), 273.2 (3634.39). ^{1}H NMR spectroscopic data are given in Table 1.

Direct Solution Cyclization: Removal of the allyl group from Fmoc-Leu-D-Tyr(*t*Bu)-Pro-Trp-D-Phe-Orn(Boc)-*O*-All (0.1257 g, 0.1 mmol) was performed as described above. After purification through a short column of silica gel (10 × 3 cm), the residue obtained after evaporation of the solvent was treated with Et_2NH in THF solution (25%, 10 mL) for 2 h to remove the Fmoc group. Excess Et_2NH and THF were removed by evaporation under reduced pressure at room temperature, and the residue was dried under vacuum at room temperature for 4 h. The residue was then dissolved in dried THF (100 mL) under nitrogen gas. The solution was then treated with HOAt (0.1166 g, 1.0 mmol), HATU (0.3803 g, 1.0 mmol) and DIEA (0.15 mL, 1.0 mmol). The resulting mixture was allowed to stand for 11 h, and then worked up in the usual way to give the cyclic hexapeptide as a white solid (83.4 mg, 85.4%), purity 96.8%. FAB-MS: *m*/*z* = 978.3 (40) $[M + H]^+$, 878.2 (100) $[M + H - Boc]^+$, 822.1 (54) $[M + 2H - Boc - tBu]^+$; calcd. M + H/$C_{54}H_{72}N_8O_9$: 977.22.

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