A Backbone Linker for BOC-Based Peptide Synthesis and On-Resin Cyclization: Synthesis of Stylostatin 1[†,§]

Gregory T. Bourne, Wim D. F. Meutermans, Paul F. Alewood, Ross P. McGeary, Martin Scanlon, Andrew A. Watson, and Mark L. Smythe*

Centre for Drug Design and Development, The University of Queensland, St. Lucia 4072, Brisbane, Australia

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We have developed a new 4-alkoxybenzyl-derived linker that anchors the C-terminal amino acid to the resin through the α-nitrogen atom. The linker allows BOC solid-phase peptide assembly and peptide cleavage using standard HF protocols. This linking strategy provides a versatile on-resin route to cyclic peptides and avoids the diketopiperazine formation that is prominent when using FMOC chemistry on backbone linkers. The linker was prepared by forming the aryl ether from 4-hydroxybenzaldehyde and bromovaleric acid. Subsequent reductive amination of the aldehyde with an allyl-protected amino acid ester and acylation of the resulting secondary amine provided the tertiary amide. After linking the amide to the resin, standard BOC SPPS, followed by allyl deprotection, cyclization, and HF cleavage gave cyclic peptides in high purity. To exemplify the strategy, the cytotoxic heptapeptide, stylostatin 1, was synthesized from two linear precursors. For comparison purposes, the yields of the on-resin and solution-phase cyclization were determined and found to be dependent upon the linear precursor. This linker technology provides new solid-phase avenues in accessing libraries of cyclic peptides.

Introduction

The drug lead discovery process has recently undergone dramatic changes through the advent of combinatorial chemistry and high throughput screening.[1−7] Arrays of analogues are accessed simultaneously via solid-supported synthetic strategies.[3−7] Cyclic peptides are appealing targets for combinatorial library development.[8,9] They are excellent tools to examine the conformational requirements of peptide or protein recognition and, provided bioactivity is maintained, serve as models for the design of bioavailable drugs. Some cyclic peptides are drugs in their own right; examples include octreotide[10] and cyclosporin A.[11] To obtain generic access to arrays of small cyclic peptides, we sought to establish solid-phase strategies that allowed on-resin cyclization prior to cleavage/deprotection.

Several approaches to on-resin cyclization have been established using two types of linking strategies (Scheme 1): (a) through electrophilic linkers that are labile to free amines (Method A),[12−15] where cyclization effects cleavage from the solid support and (b) through attachment of an amino acid side chain to the solid support (Method B)[15−17] followed by on-resin cyclization and cleavage/deprotection. With library development in mind, we were seeking to develop a versatile linker that does not require the presence of side chain functionality at the C-terminus and allows on-resin head to tail cyclization. Jensen et al.[18,19] have recently reported a TFA labile backbone

* To whom correspondence should be addressed: Tel: (+61) 7 3365 1271. Fax: (+61) 7 3365 1990. E-mail: m.smythe@mailbox.uq.edu.au.

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§ Abbreviations used as follows; BOC, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate; Bz, benzyl; DAST, diethylaminosulfur trifluoride; DCC 1,3-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ES-MS, electron-spray mass spectrometry; EtOAc, ethyl acetate; FMOC, fluorenyl-methyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HF, anhydrous hydrogen fluoride; HOAc, acetic acid; HPLC, high-perfomance liquid chromatography; MeOH, methanol; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; SPPS, solid-phase peptide synthesis; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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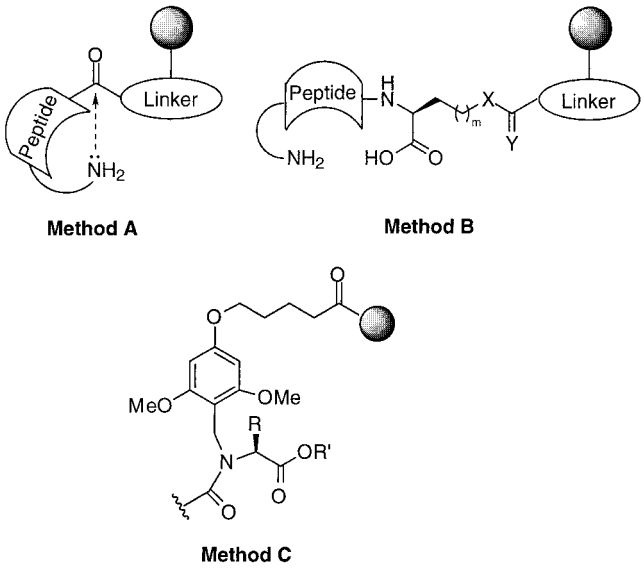
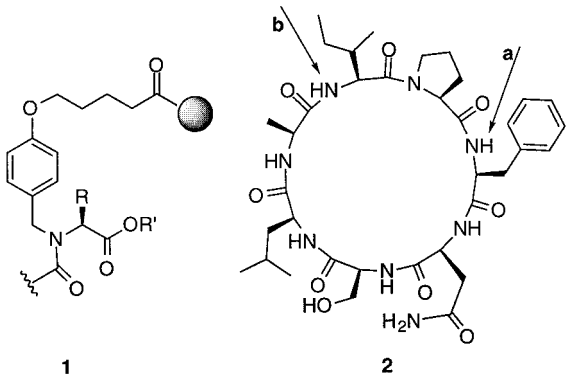
Scheme 1. Methods for Solid-Phase Synthesis of Cyclic Peptides



Scheme 2. Linker Design and Stylostatin 1[a]



[a] Positions a and b indicate the selected sites for linkage to the resin.

linker (Method C) suitable for FMOC SPPS, where the C-terminal residue is linked via its α-nitrogen atom. The strategy is particularly useful for accessing C-terminal modified peptides such as amides, alcohols, or aldehydes. In the case of C-terminal carboxylic acids or esters, however, the strategy suffers from substantial diketopiperazine formation.[20] N-1 amide-substituted dipeptides, when left in the unprotonated form (e.g., during FMOC deprotection), are highly prone to this side reaction. Clearly, alternative protection strategies need to be developed. Here we report on a novel backbone linker based on the original backbone linker of Jensen et al. but designed for BOC solid-phase peptide synthesis (Scheme 2). The α-amine remains protonated after deprotection and thus unreactive prior to the in situ neutralization coupling step,[21] thereby minimizing potential diketopiperazine formation. Such linkers should enable facile solid-phase access to cyclic peptides using readily available BOC-protected amino acids and inexpensive starting materials.

Backbone linking allows one to select, at least in theory, any linear peptide sequence and any position of cyclization (except proline at the C-terminus). It thus provides a means to target diverse libraries of cyclic peptides. Further, it is well known that a different position of cyclization can drastically affect the yield of target product. The ability to include several linear precursors to the same target in a designed library improves the chances of successful cyclization and thus the quality of the target library. In addition, we and others have found that backbone-substituted peptides cyclize more readily then their nonsubstituted analogues.[22] The position of our linker, i.e., on the first backbone nitrogen of the linear peptide, improves the flexibility of the linear peptide by presumably decreasing the cis−trans rotational barrier and therefore increases the propensity for cyclization (as opposed to oligomerization).

We employed the novel linker strategy in the synthesis of the cyclic heptapeptide stylostatin 1 (Scheme 2) that has been isolated from *Stylotella aurantium*.[23] Stylostatin 1 serves as an interesting model to evaluate our linker in the context of library development of small cyclic peptides. It has recently been the focus of a small combinatorial library, which involved the attachment of Asn of stylostatin and related analogues to resin.[24]

Results and Discussion

The backbone linker **1** is attached to the amine of the first residue of the peptide via an alkoxybenzyl group. The linker should have improved acid stability, accommodating standard BOC chemistry protocols, in particular, TFA stablity but very strong acid or HF lability.

We chose to evaluate this linker at two positions in the stylostatin 1 (**2**) backbone. This involved the attachment of the linker to the backbone nitrogens of Phe and Ile, with (a) Phe and (b) Ile at the C-terminus (Scheme 2). The positions of the linker at these two sites were selected for the following reasons. Site a: Pro−Phe is a common motif in a large number of natural cyclic peptides such as hymenistatin,[25] malaysiatin,[26] axinastatin,[26,27] and stylopeptide.[28] Additionally, this dipeptide unit has been widely used in peptide chemistry to introduce conformational turns, e.g., analogues of somatostatin.[29] Consequently, successful attachment of the linker and cyclization at this point would presumably

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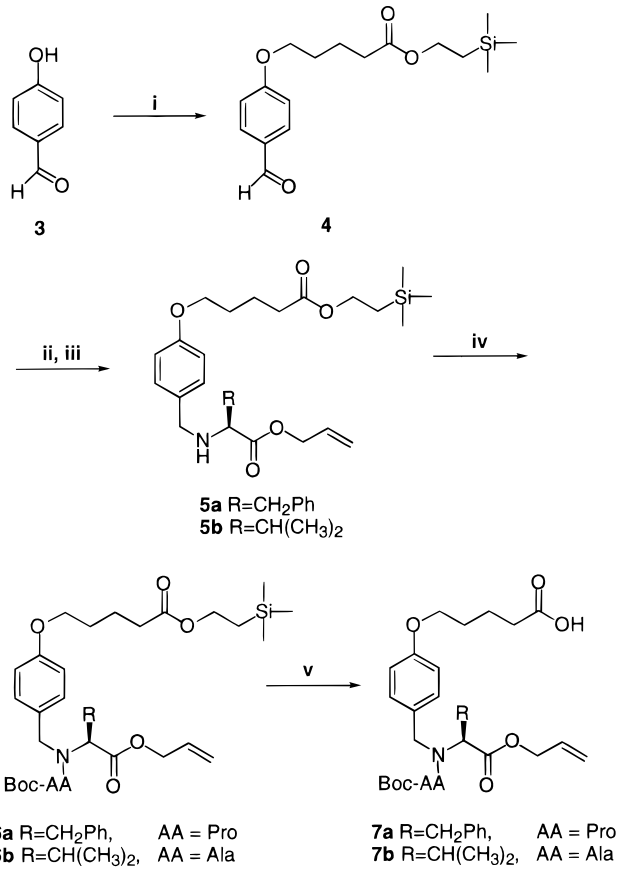
Scheme 3. Synthesis of the Dipeptide−Linker Units 7a and 7b[a]

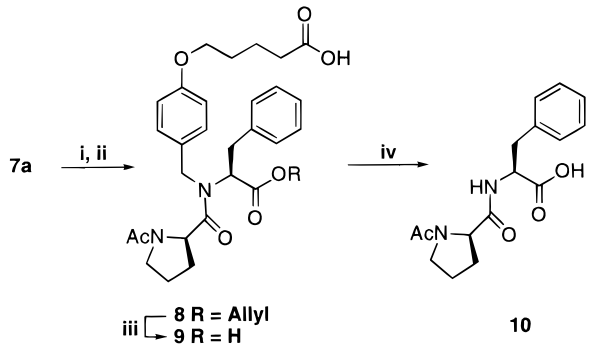


[a] Reagents: (i) $BrCH_2CH_2CH_2CH_2CO_2CH_2CH_2Si(CH_3)_3$, K_2CO_3, acetone, Δ, 16 h; (ii) H-Phe-OAllyl or H-Ile-O-Allyl, $MgSO_4$, CH_2Cl_2, rt, 3 h; (iii) $NaBH_3CN$, MeOH, rt, 2 h; (iv) $(BOC\text{-}Pro)_2$-O, DIEA, EtOAc, rt, 16 h; or BOC-Ala-F, DIEA, THF, rt, 30 min; (v) TBAF, THF, rt, 2 h.

allow access to a wide range of biologically active peptides. Appendage site b was chosen to examine sterically hindered cyclization sites (Ile−Pro in this case), and it allows an investigation of potential difficulties in acylating N-substituted *β*-branched amino acids.

We commenced with the synthesis of the two dipeptide−linker units **7a** and **7b** via solution-phase chemistry as depicted in Scheme 3. This involved reacting the *p*-hydroxybenzaldehyde **3** with 5-bromo trimethylsilylethylvalerate in the presence of K_2CO_3 and acetone. The resulting aldehyde **4** was then condensed with an allyl ester-protected amino acid, (H-Phe-OAllyl or H−Ile-OAllyl), and the corresponding imines were reduced in situ with $NaBH_3CN$.[30,31] Acylation of the secondary amine **5a** with BOC-Pro-OH was accomplished using the symmetrical anhydride procedure.[32] Acylation of the more hindered amine **5b** with $(BOC\text{-}Ala)_2$-O gave low yields of the desired product. However, acylation with BOC-Ala-F[33] proceeded well, leading to 44% yield of isolated product **6b**. The silyl protection group was then

Scheme 4. HF-Induced Cleavage of the Dipeptide Ac-Pro-Phe-OH from the Linker Unit[a]



[a] Reagents: (i) THF, rt, 5 min. (ii) Ac_2O, DIEA, DCM, 2 h; (iii) 0.1 M LiOH, THF/H_2O 1:1, rt, 1 h; (iv) HF/*p*-cresol 10:1, 0 °C, 1 h.

removed with TBAF followed by purification of the dipeptide−linker units **7a** and **7b**.

To examine the efficiency of cleavage of the linker−peptide backbone bond, we initially treated linker unit **7a**, after BOC deprotection, with acetic anhydride to give **8** (Scheme 4). Allyl deprotection was accomplished using LiOH, generating acid **9**. Ester **8** and the acid **9** were both subjected to standard HF deprotection/cleavage conditions (HF/*p*-cresol 9/1, 0 °C, 1 h). After workup, dipeptide **10** was isolated in excellent yield. No starting material (**8** or **9**) could be detected in the crude cleavage product, indicating that the amide linker was cleaved quantitatively under the selected conditions. It should also be noted that the allyl protecting group of **8** was removed quantitatively under the HF conditions. In addition, the linker−dipeptide unit **8** remained unchanged after 2 h in TFA at 25 °C, demonstrating that this backbone linker is ideally suited for in situ neutralization BOC SPPS.

We attached the dipeptide−linker units **7a** and **7b** to aminomethylated resin (Scheme 5). We have noted several times that peptide assembly on resin is more effective when the linker is separated from the resin backbone by a small peptide sequence. We therefore introduced a tripeptide spacer group (Gly−Leu−Leu) on the aminomethylated resin prior to attaching the dipeptide−linker units. The linear peptides **12a** and **12b** were then assembled on the respective resins **11a** and **11b** in a stepwise fashion using in situ neutralization protocols and HBTU activation.[21] Following allyl- and BOC-deprotection of resin **12a**, HF treatment of the resulting resin **13a** produced linear peptide **14a** in 70% yield (after HPLC purification). The analytical HPLC of the crude cleavage product **14a** is shown in Figure 1i. Purified linear peptide **14b** was obtained in the same fashion in 39% yield.

The resin-bound peptides **13a** and **13b** were cyclized with BOP/2,6-lutidine at −10 °C. Following HF cleavage, the crude products were analyzed by HPLC and ES-MS. From comparison of the HPLC profiles it is clear that the two linear precursors, though generated and cyclized on the same resin using the same linker and activation conditions, give significantly different product mixtures. Whereas **13b** cyclizes almost exclusively to stylostatin 1 (Figure 1v, peak b), the precursor **13a** generates stylostatin 1 and the corresponding cyclic dimer in a ratio of 1:1 (Figure 1ii, peaks b and c). The synthetic stylostatin 1 coeluted with native stylostatin 1 in two solvent systems (water/acetonitrile and water/methanol). In

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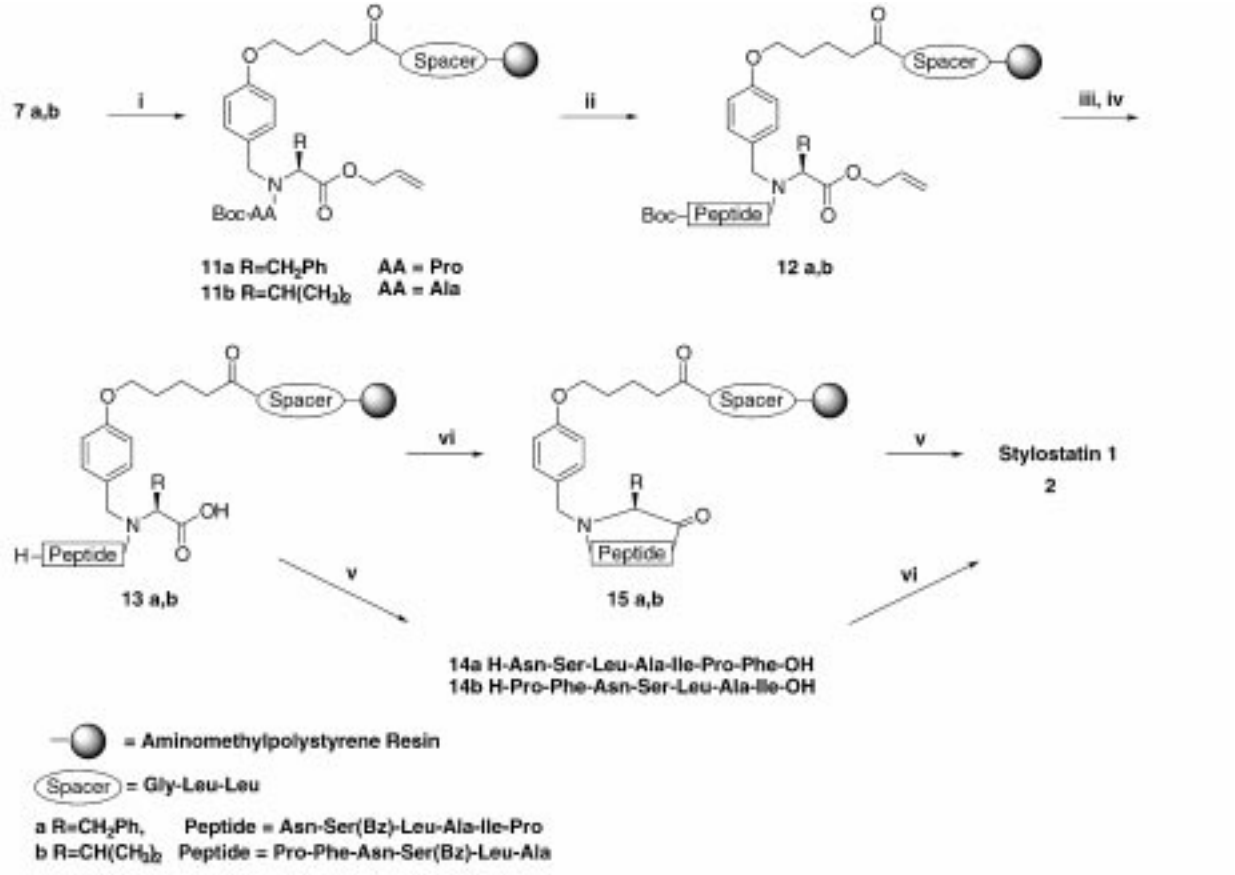
Scheme 5. Synthesis of Stylostatin 1 on Solid Support[a]



[a]Reagents: (i) H-Spacer-◉, HBTU, DIEA, DMF, r.t.; (ii) SPPS; (iii) 3 equiv. $Pd(Ph_3)_4$, CH_3Cl : HOAc : NMM, 37:2:1, r.t, 3 h; (iv) TFA, r.t., 2 x 1 min.; (v) HF : p-cresol, 10:1, -5 °C, 1 h, (vi) 3 equiv. BOP, 2,6-Lutidine or DIEA, -10 °C, 3 h.

DMSO, the ^{1}H NMR data obtained from the synthetic material were identical to those reported for the native product.[34]

To evaluate the effect of resin-bound cyclization on yields and purity of the product, we carried out control solution-phase cyclization of the corresponding linear precursors **14a** and **14b** (see Table 1). For peptide **14b**, cyclization at 10^{-3} M (in DMF) proceeded as expected to yield almost exclusively stylostatin 1. Solution-phase cyclization of the linear peptide **14a** was performed at 10^{-2} and 10^{-3} M (see Table 1). The analytical HPLC of the crude mixtures displayed significant differences in the ratio of cyclic monomer (stylostatin 1) over higher oligomers (Figure 1iii and 1iv, respectively). The monomer:dimer ratio was approximately 2:5 at 10^{-2} M concentration. At 10^{-3} M, this was reversed to 5:2 monomer:dimer.

The product profile of the on-resin cyclization of **13a** (Figure 1ii) compares well with the solution-phase experiment on **14a** at 10^{-2} M (Figure 1iii). The on-resin cyclization was performed on 250 mg of resin-bound peptide (sv 0.16 mmol/g, swollen volume ~ 1 mL) and is in the order of 10^{-2} M. It appears that the on-resin cyclization does not confer any significant benefit via the so-called pseudodilution effect.[35,36] However, it may be that the pseudodilution effect in on-resin chemistry is significant but is counterbalanced by the higher "local" concentrations of linear peptide (i.e., the resin is not homogeneously activated across the bead).

A remaining question that currently complicates synthesis of cyclic peptides is the selection of the optimal position for cyclization. Our method allows relatively fast comparison of cyclization of several linear precursors in a combinatorial fashion, with no intermediate purification required. From a library perspective, this permits one to include several precursors to the same target, thereby increasing the probability of obtaining "pure" cyclic peptide for each target in the library. This is exemplified by the fact that one would predict the Ile−Pro cyclization site to be the most combersome, but of the two selected in this study, it results in the highest yield and purity of cyclic material.

In summary, we have developed a new linker that anchors the C-terminal amino acid to the resin through the α-nitrogen atom and permits standard BOC chemistries to be performed for assembly and cleavage. The linker can be used on all amino acids except proline and thus provides new solid-phase avenues to access libraries of cyclic peptides.

(34) The structure of the synthetic material was calculated on the basis of distance constraints derived from NOE data recorded for the peptide in DMSO. The DMSO solution structure of synthetic stylostatin 1 is very similar to the previously reported crystal structure of native stylostatin 1.[23] Backbone atoms of synthetic stylostatin 1 superimpose on the X-ray structure of the native material with an RMSD of 0.51 ± 0.01 Å.

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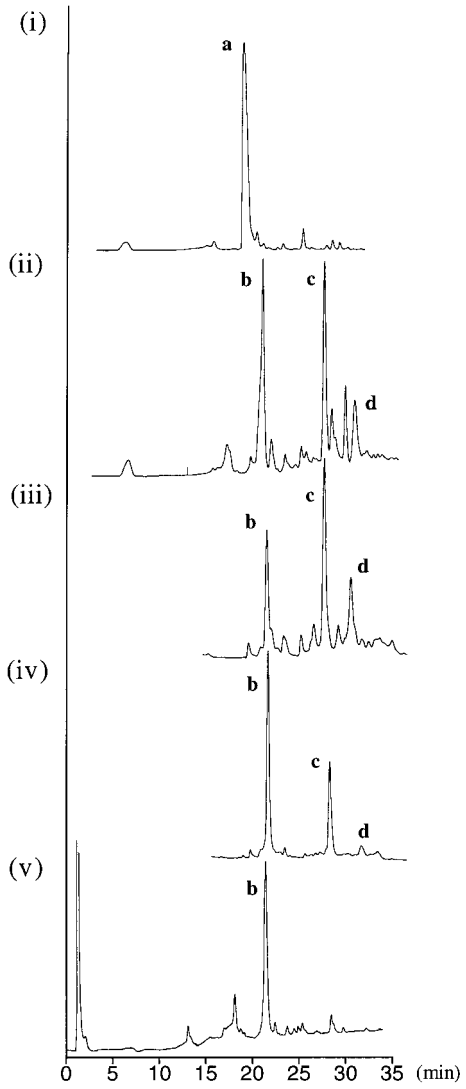

Figure 1. Analytical HPLC of crude mixtures: (i) HF cleavage of **13a**; (ii) on-resin cyclization of **13a** followed by HF cleavage; (iii) solution-phase cyclization of **14a** (1×10^{-2} M); (iv) solution-phase cyclization of **14a** (1×10^{-3} M); (v) on-resin cyclization of **13b** followed by HF cleavage. **a** = H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH (**14a**); **b** = stylostatin 1 (**2**); **c** = cyclic dimer of **14a**; **d** = cyclic trimer of **14a**.

Experimental Section

General Methods. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a 300 MHz spectrometer, and the chemical shifts are reported in δ parts per million downfield from tetramethylsilane. Coupling constants (J) refer to proton−proton coupling ($J_{H,H}$). ^{13}C NMR spectra were also recorded on the spectrometer at 75.5 MHz. TOCSY and ROESY spectra were obtained on a 500 MHz spectrometer. Data analysis and structure calculations were undertaken using standard protocols.[37] Mass spectra were acquired on a triple-quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 μL) were injected into a moving solvent (30 μL/min; 50:50 CH_3CN/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100−200 μm diameter) at a potential of 80 V. Full scan mass spectra were acquired over a range of 200−2000 Da with a scan step size of 0.1 Da. TLC (thin-layer chromatography) was performed on silica gel 60 F254 plates. The chromatograms were viewed under uv light and/or developed with iodine vapor. Preparative column chromatography was effected under pressure, using normal-phase Merck Kieselgel 60. Analytical reverse-phase HPLC was run on a C18 column (0.46 cm × 25 cm); preparative reverse-phase HPLC was run on a C18 column (2.2 cm × 25 cm). Both columns were attached to a HPLC setup fitted with a Holochrome UV detector. Measurements were carried out at λ = 214 nm.

Materials. BOC-L-amino acids and, synthesis grade DMF, TFA, and DIEA were purchased from Auspep (Parkville, Australia). HBTU and BOP were purchased from Richelieu Biotechnologies (Montreal, Canada). AR grade EtOAc, HOAc, MeOH, CH_2Cl_2, $CHCl_3$, hexane, and acetone and HPLC grade CH_3CN were all obtained from Laboratory Supply (Australia); HF was purchased from CIG (Australia). Aminomethylpolystyrene resins with a substitution value of 0.21 mmol/g or 0.26 mmol/g were purchased from Novabiochem. All other reagents were AR grade or better and were obtained from Aldrich or Fluka.

4-[5-Oxy-(trimethylsilylethylvalerate)]benzaldehyde (4). 4-Hydroxybenzaldehyde (**3**) (12.2 g, 100 mmol), 5-bromo-(trimethylsilylethyl)valerate (13.82 g, 200 mmol), and K_2C0_3 (40.0 g, 290 mmol) were refluxed in acetone (250 mL) for 16 h. Solids were filtered and washed with acetone, and the volatiles were removed in vacuo. The product **4** was purified by column chromatography (hexane/EtOAc, 8:1) to yield a colorless oil (28.2 g, 87%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.82 (d, J = 7.0 Hz, 2H), 6.98 (d, J = 7.0 Hz, 2H), 4.20 (t, J = 6.9 Hz, 2H), 4.05 (t, J = 6.0 Hz, 2H), 2.42 (m, 2H), 1.80 (m, 4H), 0.96 (t, J = 6.9 Hz, 2H), 0.10 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.80, 173.45, 164.026, 131.99, 131.99, 129.87, 114.72, 114.72, 67.82, 62.63, 34.00, 28.49, 21.55, 17.35, −1.49; ES-MS M_r 322.4 (calcd 322.2).

***N*-[4-(5-Oxy-(trimethylsilylethylvalerate))benzyl]-L-phenylalanine Allyl Ester (5a).** The aldehyde **4** (16.2 g, 50.2 mmol), phenylalanine allyl ester (20.5 g, 100 mmol), and excess $MgSO_4$ (~40 g) were stirred in CH_2Cl_2 (75 mL) at rt for 16 h. Solids were filtered, and volatiles were removed in vacuo to yield the crude imine as a yellow oil. MeOH (200 mL) and HOAc (3 mL) were added, and the reaction mixture was cooled to 10 °C. $NaBH_3CN$ (6.1 g, 100 mmol) was added portionwise to the stirred solution. The reaction mixture was allowed to warm to room temperature before being stirred for an additional 2 h. Volatiles were removed in vacuo, and the resulting residue was diluted with H_2O and extracted with EtOAc. The combined EtOAc extracts were washed with saturated brine and water before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil was purified by flash chromatography (hexane/EtOAc, 1:1) to yield **5a** as a clear colorless oil (20.2 g, 79%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28 (m, 5H), 7.20 (d, J = 7.0 Hz, 2H), 6.85 (d, J = 7.0 Hz, 2H), 5.80 (m, 1H), 5.28 (dd, J = 12.1 Hz, 1.7 Hz, 1H), 5.23 (dd, J = 10.0 Hz, 1.7 Hz, 1H), 4.55 (d, J = 6.4 Hz, 2H), 4.15 (t, J = 6.9 Hz, 2H), 3.92 (m, 2H), 3.80 (dd, J = 12.2 Hz, 1.2 Hz, 2H), 3.65 (dd, J = 11.7 Hz, 1.2 Hz, 2H), 3.58 (m, 1H), 3.05 (m, 1H), 2.25 (m, 2H), 1.80 (m, 4H), 0.95 (t, J = 6.9 Hz, 2H), 0.10 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.56, 173.00, 158.32, 136.78, 131.96, 130.67, 129.57, 129.57, 129.27, 129.27, 128.39, 128.39, 126.76, 118.77, 114.36, 114.36, 67.33, 66.48, 62.51, 61.60, 51.13, 39.18, 34.08, 28.68, 21.62, 17,32, −1.51; ES-MS M_r 511.1 (calcd 511.3).

BOC-L-Pro-[*N*-(4-(5-oxy-(trimethylsilylethylvalerate))-benzyl)]-L-phenylalanine Allyl Ester (6a). BOC-Pro-OH (8.61 g, 40.0 mmol) was dissolved in EtOAc (30 mL), to which was added DCC (4.12 g, 20.0 mmol). After activation for 10−15 min to form the symmetric anhydride, the mixture was filtered. The filtrate was added to a solution of the amine **5a** (5.11 g, 10.0 mmol) and DIEA (2.67 mL, 15 mmol) in EtOAc (20 mL). The reaction was stirred at rt for 16 h. EtOAc (100 mL) was added, and the reaction mixture was washed with a

(37) Scanlon, M. J.; Naranjo, D.; Thomas, L.; Alewood, P. F.; Lewis, R. J.; Craik, D. J. *Structure* **1997**, *5*, 1585−1597.

Table 1. Yields of Isolated Cyclic Monomer, Dimer and Trimer from On-Resin and Solution-Phase Cyclization of Two Stylostatin 1 Precursors

peptide	cyclization conditions	yield (%) monomer	dimer	trimer
H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH	soln (1×10^{-2} M)	11	43	7
	soln (1×10^{-3} M)	48	21	1
	resin[a]	10	25	5
H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH	soln (1×10^{-3} M)	67	<1	<1
	resin[a]	25	<1	<1

[a] Yields based on weight of isolated material and the original resin substitution value.

10% K_2CO_3 solution, brine, and H_2O before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil was purified by flash chromatography (hexane: Et_2O, 5:1) to yield **6a** as a clear colorless oil (3.55 g, 60%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (m, 7H), 6.85 (d, J = 7.0 Hz, 2H), 5.98 (m, 1H), 5.20 (m, 2H), 4.50 (m, 3H), 4.20 and 4.13 (rotomers, m, 1H), 4.15 (t, J = 6.9 Hz, 2H), 3.92 (m, 2H), 3.71 (m, 2H), 3.31 (m, 4H), 2.25 (m, 2H), 2.05 (m, 4H), 1.80 (m, 4H), 1.48 (br s, 9H), 0.95 (t, J = 6.9 Hz, 2H), 0.10 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ rotomers 173.54 and 173.00, 172.42, rotomers 170.08 and 169.47, rotomers 158.68 and 158.50, rotomers 154.31 and 153.98, rotomers 138.35 and 138.05, rotomers 132.45 and 131.96, 129.40, 129.40, 129.10, 128.91, 128.63, 128.63, 127.52, rotomers 126.75 and 126.62, rotomers 118.26 and 118.06, 114.32, 114.32, rotomers 79.96 and 79.19, 67.34, rotomers 65.96 and 65.80, 62.55, rotomers 60.68 and 60.58, rotomers 57.44 and 56.94, 51.37, rotomers 46.83 and 46.77, rotomers 35.11 and 34.97, 34.07, rotomers 30.84 and 29.78, 28.67, 28.46, rotomers 24.02 and 22.77, 21.68 17.32, −1.50; ES-MS M_r 708.6 (calcd 708.4).

BOC-L-Pro-[N−(4-(5-oxyvaleric acid)benzyl)]-L-phenylalanine Allyl Ester (7a). The ester **6a** (2.0 g, 2.82 mmol) was stirred in THF (20 mL) at rt TBAF (3 mL, 1 M in THF) was added dropwise, and stirring proceeded for an addition 3 h. H_2O (100 mL) and HOAc (3 mL) were added to the reaction mixture. The acid **7a** was extracted into EtOAc and washed with a 10% solution of citric acid followed by H_2O before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil was purified by flash chromatography (CH_2Cl_2/MeOH, 19:1) to yield a white solid (2.54 g, 90%): mp 28−30 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.89 (br s, 1H), 7.20 (m, 7H), 6.75 (dd, J = 7.1 Hz, J = 1.9 Hz, 2H), 5.88 (m, 1H), 5.25 (m, 2H), 4.50 (m, 3H), 4.20 and 4.13 (rotomers, dd, J = 6.9 Hz, 1.9 Hz, 1H), 3.88 (m, 2H), 3.71 (m, 2H), 3.41 (m, 4H), 2.25 (m, 2H), 2.05−1.85 (m, 8H), 1.48 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ rotomers 179.09 and 177.04, 173.05, rotomers 170.08 and 169.48, rotomers 158.64 and 158.44, rotomers 154.28 and 153.96, rotomers 138.31 and 138.02, rotomers 132.43 and 131.94, 129.41, 129.41, 128.99, 128.69, 128.48, 128.48, 127.50, rotomers 126.78 and 126.65, rotomers 118.30 and 118.10, 114.37, 114.37 rotomers 80.17 and 79.38, 67.30, rotomers 65.99 and 65.84, rotomers 60.72 and 60.54, rotomers 57.49 and 57.00, 51.40, rotomers 46.86, rotomers 35.09 and 34.95, 33.56, rotomers 30.83 and 29.78, rotomers 28.46 and 20.76, rotomers 24.00 and 22.78, 21.39; ES-MS M_r 608.3 (calcd 608.3).

***N*-[4-(5-Oxy-(trimethylsilylethylvalerate))benzyl]-L-isoleucine Allyl Ester (5b).** The aldehyde **4** (16.2 g, 50.2 mmol), isoleucine allyl ester (20.5, 100 mmol), and excess $MgSO_4$ (~40 g) were stirred in CH_2Cl_2 (75 mL) at rt for 3 h. Solids were filtered, and volatiles were removed in vacuo to yield the crude imine as a yellow oil. MeOH (200 mL) and HOAc (3 mL) were added, and the reaction mixture was cooled to 10 °C. $NaBH_3CN$ (6.1 g, 100 mmol) was added portionwise to the stirred solution. The reaction mixture was allowed to warm to room temperature before being stirred for an additional 2 h. Volatiles were removed in vacuo, and the resulting residue was diluted with H_2O and extracted with EtOAc. The combined EtOAc extractions were washed with saturated brine and water before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil was purified by flash chromatography (1:1 hexane/EtOAc) to yield **5b** as a clear colorless oil (19.6 g, 82%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 8.0 Hz, 2H), 5.98 (m, 1H), 5.31 (d, J = 27.2 Hz, 1H), 5.27 (dd, J = 13.2 Hz, 1.7 Hz, 1H), 5.10 (dd, J = 11.2 Hz, 1.7 Hz, 1H), 4.65 (m, 2H), 4.15 (t, J = 6.9 Hz, 2H), 3.92 (m, 2H), 3.81 (d, J = 13 Hz, 1H), 3.60 (d, J = 13 Hz, 1H), 3.17 (m, 1H), 2.90 (m, 1H), 2.35 (m, 2H), 1.80 (m, 2H), 1.52 (m, 1H), 1.20 (m, 1H), 0.95 (t, J = 6.9 Hz, 2H), 0.92 (d, J = 7.6 Hz, 3H), 0.90 (t, J = 7.0 Hz, 3H), 0.10 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.55, 174.25, 158.96 132.66, 131.22, 130.48, 130.48, 119.45, 115.02, 115.02, 68.05 65.92, 65.52, 63.20, 52.36, 38.74, 34.78, 29.39, 29.39, 26.35, 22.34, 18.02, 16.23, 12.13, −0.81; ES-MS M_r 477.3 (calcd 477.3).

BOC-L-Ala-[*N*-(4-(5-oxy-(trimethylsilylethylvalerate)-benzyl)]-L-isoleucine Allyl Ester (6b). BOC-Ala-OH (2.89 g, 15.0 mmol) was dissolved in CH_2Cl_2 (30 mL), to which was added DAST (4.12 g, 20.0 mmol). After activation for 10−15 min to form the acid fluoride, the mixture was washed with cold H_2O and dried over $MgSO_4$, and the volatiles were removed in vacuo. The acid fluoride was then added immediately to a solution of the amine **5b** (4.78 g, 10.0 mmol) and DIEA (2.67 mL, 15 mmol) in THF (20 mL). The reaction was stirred at rt for 16 h. EtOAc (100 mL) was added, and the reaction mixture was washed with 10% K_2CO_3 solution, brine, and H_2O before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil was purified by flash chromatography (hexane/diethyl ether, 1:5) to yield **6b** as a clear colorless oil (2.86 g, 44%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 8.0 Hz, 2H), 5.98 (m, 1H), 5.31 (d, J = 14.2 Hz, 1H), 5.23 (d, J = 12.0 Hz, 1H) 4.65 (m, 3H), 4.15 (t, J = 6.9 Hz, 2H), 3.92 (m, 2H), 3.81 (d, J = 13 Hz, 1H), 3.60 (d, J = 13 Hz, 1H), 3.17 (m, 1H), 2.90 (m, 1H), 2.35 (m, 2H), 1.80 (m, 2H), 1.52 (m, 1H), 1.45 (s, 9H), 1.20 (m, 1H), 0.95 (t, J = 6.9 Hz, 2H), 0.97 (s, 3H), 0.92 (d, J = 7.6 Hz, 3H), 0.90 (t, 3H, J = 7.0 Hz), 0.10 (s, 9H); ES-MS M_r 648.5 (calcd 648.4).

BOC-L-Ala-[*N*-(4-(5-oxyvaleric acid)benzyl)]-L-isoleucine Allyl Ester (7b). The ester **6b** (2.0 g, 2.82 mmol) was stirred in THF (20 mL) at rt TBAF (3 mL, 1 M in THF) was added dropwise, and saponification proceeded for 3 h. H_2O (100 mL) and HOAc (3 mL) was added to the reaction mixture. The acid was extracted into EtOAc. The combined EtOAc extractions were washed with saturated brine and water before being dried over $MgSO_4$. Volatiles were removed in vacuo, and the resulting oil purified by semipreparative HPLC (0−60% B over 60 min) to yield the tertiary amide **7b** as a colorless oil (2.54 g, 44%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22 (d, 2H, J = 8.0 Hz), 6.80 (d, J = 8.0 Hz, 2H), 5.91 (m, 1H), 5.21 (d, J = 14.2 Hz, 1H), 5.22 (d, J = 11.0 Hz, 1H), 4.65 (m, 3H), 3.92 (m, 2H), 3.81 (d, J = 13 Hz, 1H), 3.60 (d, J = 13 Hz, 1H), 3.17 (m, 1H), 2.90 (m, 1H), 2.35 (m, 2H), 1.80 (m, 2H), 1.52 (m, 1H), 1.45 (s, 9H), 1.20 (m, 1H), 0.97 (s, 3H), 0.92 (d, 3H, J = 7.6 Hz), 0.90 (t, 3H, J = 7.0 Hz); ES-MS $[M + H]^+$ = 549.1 (expected 549.3).

Ac-(L-Pro)-[*N*-(4-(5-oxyvaleric acid)benzyl)]-L-phenylalanine Allyl Ester (8). The BOC-protected tertiary amide **7a** (61 mg, 0.1 mmol) was treated with TFA (2 mL) for 10 min at rt. TFA was removed under reduced pressure, and the resulting oil was dissolved in DMF (20 mL). Triethylamine (4 mL) was added followed by acetic anhydride (4 mL). The reaction was stirred at rt for 2 h. Volatiles were removed in vacuo, and the resulting oil was dissolved in CH_3CN/H_2O (1:1, 10 mL). The solution was stirred for an additional 2 h. The mixture was then passed through a semipreparative HPLC

column (40−100% B over 60 min) to yield **8** as a white powder (47 mg, 85%): ES-MS M_r 550.7 (calcd 550.3).

Ac-(L-Pro)-[*N*-(4-(5-oxyvaleric acid)benzyl)]-L-phenylalanine (9). The allyl ester **8** (25 mg, 45.5 μmol) was dissolved in THF/H_2O (1:1, 10 mL), and LiOH·H_2O (3.64 mg, 91 μmol) was added portionwise to the solution at rt The solution was stirred for 2 h before HCl (0.01 M) was added until the solution was at pH 5.0. The mixture was then passed through a semipreparative HPLC column (30−90% B over 60 min) to yield the diacid **9** (20 mg, 86%): ES-MS M_r 510.8 (calcd 510.3).

Ac-Pro-Phe-OH (10). Method 1. The peptide **8** (20 mg, 36.2 μmol) was treated with HF/*p*-cresol, (5 mL, 10:1) for 1 h at −5 °C. After removal of the HF under reduced pressure, the crude peptide was dissolved in the HPLC buffer and lyophilized. The acetylated dipeptide Ac-Pro-Phe-OH **10** was purified by semipreparative HPLC (30−90% B over 60 min) to yield a white powder (8.6 mg 78%): ES-MS M_r 304.2 (calcd 304.1).

Method 2. The peptide **9** (20 mg, 39.2 μmol) was treated with HF/*p*-cresol, (5 mL, 10:1) for 1 h at −5 °C. After removal of the HF under reduced pressure, the crude peptide was dissolved in the HPLC buffer and lyophilized. The acetylated dipeptide Ac-Pro-Phe-OH **10** was purified by semipreparative HPLC (30−90% B over 60 min) to yield a white powder (9.7 mg, 82%): ES-MS M_r 304.2 (calcd 304.14).

Solid-Phase Peptide Synthesis. H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH (14a). The peptide was synthesized in a stepwise fashion by established methods using in situ neutralization/HBTU activation protocols for BOC chemistry. The xanthyl protecting group was used for the Asn residue and the benzyl ether for the Ser residue. Coupling reactions were monitored by quantitative ninhydrin assay and were typically >99.9% complete. After chain assembly was complete, the removal of the allyl protecting group was achieved by the addition of tetrakis(triphenylphosphine) palladium [$Pd(PPh_3)_4$] (580 mg, 0.5 μmol, 3 molar equiv) to the resin in a solution of $CHCl_3$/HOAc/NMM (37:2:1), and the solution was stirred for 14 h at rt The resin was washed with a 10% solution of diethyldithiocarbamic acid (sodium salt trihydrate, $(C_2H_5)N_2CS_2Na·3H_2O$) in DMF, DMF, MeOH/CH_2Cl_2 1: 1, and finally CH_2Cl_2. The N^{α}-BOC group was removed with neat TFA (2 × 1 min treatment), and the peptide was cleaved from the resin (200 mg, 0.166 mmol/g) using HF/*p*-cresol (11 mL, 10:1) for 1 h at −5 °C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether, filtered, dissolved in the HPLC buffer, and lyophilized. The peptide *H*-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH **14a** was purified by semipreparative HPLC (30−90% B over 60 min) to yield a white powder (25 mg 78%): ES-MS M_r 760.21 (calcd 760.42).

H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH (14b). A procedure similar to that above was followed, using precursor **7b** (200 mg, 0.180 mmol/g). The peptide H-Pro-Phe-Asn-Ser-Leu-Ala-Ile **14b** was purified by semipreparative HPLC (30−90% B over 60 min) to yield a white powder (10.5 mg, 39%): ES-MS M_r 760.2 (calcd 760.4).

Solution Phase Cyclization. Method 1. Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) (2). The linear peptide H-Asn-Ser-Leu-Ala-Ile-Pro-Phe-OH **14a** (15.0 mg, 0.020 mmol) and BOP (26.1 mg, 0.060 mmol) were stirred in DMF (19.7 mL, 1×10^{-3} M) at −10 °C. DIEA (35 μL, 0.197 mmol) was added dropwise to the solution. After the reaction was left to stir for an additional 2 h at this temperature, all volatiles were removed in vacuo. The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) **2** was isolated by preparative HPLC (30−90% B over 60 min) to yield a white powder (7.0 mg, 48%): ES-MS M_r 742.2 (calcd 742.4). The ^1H and ^{13}C NMR were identical to reported data.[22] Also isolated was the dimer, cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (3 mg, 21%): ES-MS M_r 1485.2 (calcd 1485.8), and the trimer, cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (0.7 mg, 5%): ES-MS M_r 2229.2 (calcd 2229.2).

Method 2. Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) (2). A procedure similar to that above was followed, using precursor **14b** (100 mg, 0.131 mmol), BOP (174 mg, 0.393 mmol), and DIEA (228 μL, 1.31 mmol). The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) **2** was isolated by preparative HPLC (10−70% B over 60 min) to yield a white powder (10.5 mg, 67%): ES-MS M_r 742.2 (calcd 742.4).

On-Resin Cyclization. Method 1. Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) (2). After chain assembly of the linear peptide **13a** (250 mg, 0.167 mmol/g), the allyl protecting group and the N^{α}-BOC group were removed with [$Pd(PPh_3)_4$] (580 mg, 0.5 μmol) and TFA (2 × 1 min treatment), respectively, as described before. The resin in DMF (20 mL) was then cooled to −10 °C, and BOP (221 mg, 0.5 mmol) was added. Next, 2,6-Lutidine (194 μL, 1.66 mmol) was added dropwise, and the reaction was monitored by ninhydrin test. After completion of the reaction (amine <1% by ninhydrin), the resin was filtered, and the cyclic peptide was cleaved from the resin using HF/*p*-cresol (11 mL, 10:1) for 1 h at 0 °C. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether before being dissolved in the HPLC buffer and lyophilized. The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) **2** was isolated by preparative HPLC (30−90% B over 60 min) to yield a white powder (3.1 mg, 10%): ES-MS M_r 742.2 (calcd 742.4). Also isolated was the dimer, cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (7.6 mg, 24.5%): ES-MS M_r 1485.2 (calcd 1485.8), and the trimer, cyclo-(Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe-Asn-Ser-Leu-Ala-Ile-Pro-Phe) (0.4 mg, 1%): ES-MS M_r 2229.4 (calcd 2229.2).

Method 2. Cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) (2). A procedure similar to that above was followed, using precursor **13b** (200 mg, 0.203 mmol/g), BOP (60 mg, 0.136 mmol), and 2,6-lutidene (237 μL, 2.03 mmol). The peptide cyclo-(Pro-Phe-Asn-Ser-Leu-Ala-Ile) **2** was isolated by preparative HPLC (30−90% B over 60 min) to yield a white powder (8.2 mg, 25%): ES-MS M_r 742.2 (calcd 742.4).

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