Constrained Derivatives of Stylostatin 1. 1. Synthesis and Biological Evaluation as Potential Anticancer Agents

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Received July 1, 2003

Hydroxyaminolactams have been used as constrained surrogates of the Ser-Leu dipeptide in the synthesis of analogues of the cycloheptapeptide stylostatin 1 (**2**). The rate of cyclization through formation of the Ile-Pro amide bond allowed us to prove that the valerolactams used induced a turn in the linear precursor. Ring closure at the Pro-Phe amide bond was much quicker and provided access to larger amounts of the target structures, with high purity. The conformation of ψ-stylostatin **4** was compared to that of native stylostatin 1 using NMR analysis. The ability of three ψ-stylostatins and the native stylostatin 1 to inhibit growth of cancer cell lines was tested. None of the compounds showed activity below 1 μM. A possible relationship between the decrease in activity and the presence of the piperidone Ser-Leu surrogate is considered.

Introduction

Cyclic peptides are of biological and chemico-pharmaceutical interest.[1] Structurally their mobility is more restricted than that of their linear counterparts so that they adopt fewer conformations and, as a result, show higher specificity for the target receptors.[2] In addition they are often more stable in vivo. They therefore represent promising drug candidates.

Cyclic L−D peptides usually show a strong tendency to self-assemble in tubular arrays which are held together by parallel or antiparallel β-sheet interactions.[3] This property, related to their diverse biological activities, depends on the ring size of the peptide. In this regard, the mode of action of small cyclopeptides remains to be properly explained.

The characteristic of small cyclic peptides that interests us is the tendency of their linear precursors to exhibit preorganization, which facilitates their cyclization to give specific compounds.[4] We intended to test the properties of 3-aminohydroxylactams type **1**[5] as constrained surrogates of the Ser-Leu dipeptide (Figure 1). In general lactams of type **1** induce a β-turn[6] in linear peptides. Therefore, the construction of a cyclic peptide from a linear precursor that contains the aminopiperidone should be faster than that of the native cyclopeptide. The cycloheptapeptide stylostatin 1 (Figure 1) was isolated from the marine sponge *Stylotella sp.*[7] It showed a cell growth inhibition ED_{50} of 0.8 μg/mL against P388 lymphocytic leukemia cells, and the 3D-structure, elucidated by the authors,[7] showed two β-turns centered at the Ser-Leu and Ile-Pro residues. Both its biological properties and its structure were appropriate for our studies.

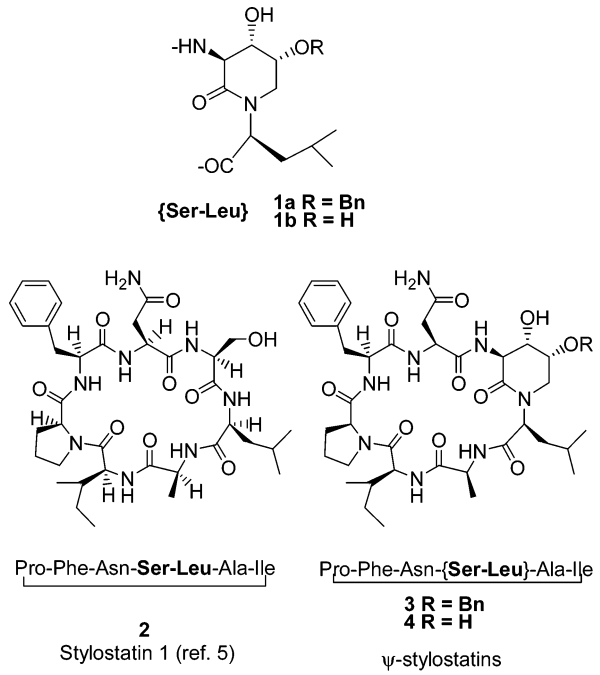

Figure 1. Chemical structures of {Ser-Leu} pseudodipeptide, native stylostatin 1, and proposed Ψ-stylostatins.

We planned to compare the synthesis of native stylostatin 1 and ψ-stylostatins **3** and **4** in order to evaluate the cyclization rate. We also intended to study possible conformational variations and the differences in anticancer activity.

Results and Discussion

Synthesis via Ile-Pro Amide Bond Formation. We planned to obtain the cyclopeptides by solid-phase

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10.1021/jm030943h CCC: $25.00
Published on Web 11/21/2003

Scheme 1

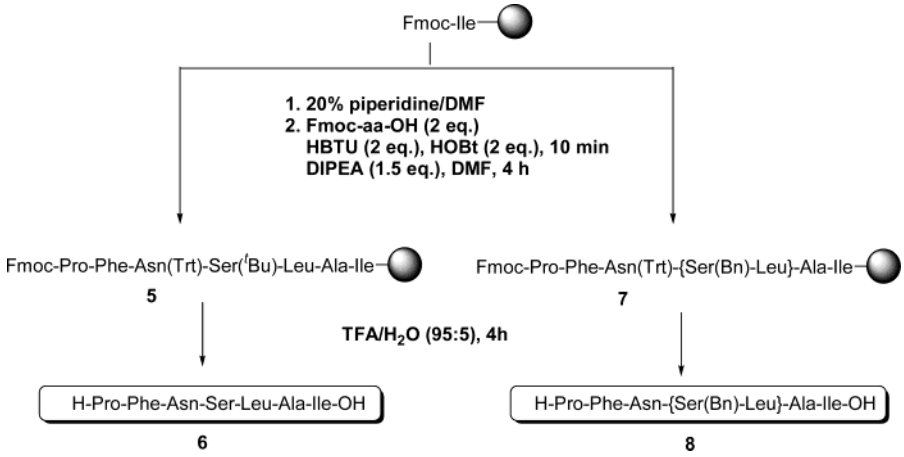
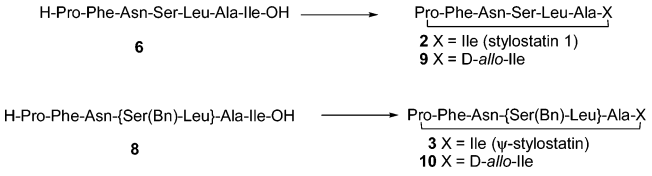


Table 1. Conditions Assayed for the Cyclization of Compounds **6** and **8**

entry	reagents	solvent	concentration, M	temperature, °C	time, h	**2**:**9**[a]	**3**:**10**[a]
1	BOP (3 equiv) DIPEA (10 equiv)	DMF	10^{-3}	−10	2	1:0 reported (67%) **1:4**	- - **1:1.25**
2	BOP (3 equiv) DIPEA (10 equiv)	DMF	10^{-3}	−10	24	1:9	- -
3	PyBOP (3 equiv) DIPEA (10 equiv)	DMF	10^{-3}	−10	21	1:3	1.5:1
4	PyBOP (3 equiv) DIPEA (10 equiv)	DMF	10^{-3}	rt	3	1:1.6	1:1
5	**PyBOP (3** equiv) **DIPEA (10** equiv)	**DCM/DMF (97:3)**	**7.8×10^{-4}**	**rt**	**5**	**1:1**	**3.5:1**
6	PyBOP (3 equiv) DIPEA (10 equiv)	ACN	7.7×10^{-4}	rt	6.5	_	1.3:1

[a] Ratio obtained from analytical HPLC.

Scheme 2



synthesis of the linear precursors followed by cyclization in solution. In this way we would be able to follow the kinetics of the cyclization step by HPLC analysis. Bourne et al.[8] have reported syntheses of stylostatin 1 through formation of either the Ile-Pro or the Phe-Asn amide bond and found that the former was more efficient, leading to stylostatin 1 as a single compound in 67% yield. Following this first report, we prepared the linear heptapeptides **6** and **8**,[9] starting from commercial Fmoc-Ile Wang resin in DMF and using a mixture of HBTU, HOBt, and DIPEA as the activating agent (Scheme 1).[10] Final deprotection and cleavage were performed simultaneously by treatment of resins **5** and **7** with 95% TFA in water. The linear peptides **6** and **8** (>85% pure by HPLC) were identified by ES-MS and by amino acid analysis.

We then tested the cyclization conditions (Scheme 2), using 5 mg samples of compound **6**. Treatment of **6** with BOP/DIPEA in DMF at −10 °C[8] gave a mixture of two cyclic compounds in a 1:4 ratio (Table 1, entry 1). Prolonging reaction times, increasing the temperature, or changing the activating reagent to PyBOP did not modify the results. The best results were obtained when DCM:DMF (97:3) was used as the solvent (Table 1, entry 5). In this case an equimolar mixture of **2** and **9** was obtained.

After isolation by semipreparative HPLC, compounds **2** and **9** showed the same molecular peak by ES-MS (743 Da). Amino acid analysis showed the presence of Ile in the minor isomer, which was thus identified as native stylostatin 1 (**2**), whereas compound **9** contained D-*allo*-Ile instead (see Supporting Information). Therefore, epimerization on the α-carbon of Ile had taken place during the cyclization.[11]

Cyclization of compound **8** was also optimized. As above, we obtained the best results using DCM:DMF (97:3) as the solvent, but here the nonepimerized product **3** was the major product (Table 1, entry 5), as shown by amino acid analysis following HPLC purification.

Synthesis via Pro-Phe Amide Bond Formation. To avoid epimerization of Ile and to try to improve the synthesis of stylostatin 1[12,13] and its analogues, we performed the head to tail cyclization through the Pro-Phe amide bond. The required linear peptides **11** and **12** were synthesized in solid phase, using Fmoc-Pro anchored to commercial Cl-TrtCl resin[14] (Scheme 3) and the HBTU/HOBt/DIPEA system in DMF. Peptides **11** and **12** were obtained >85% pure (HPLC) in 55 and 52% chemical yield, respectively, and were identified by ES-MS and amino acid analysis.

The cyclization of compounds **11** and **12** was performed both in DMF and in DCM:DMF, using PyBOP as the activating agent[15] in the presence of DIPEA (Scheme 4). In all cases the cyclization was complete in 5 min. The expected cyclopeptides, stylostatin **2** and

Scheme 3

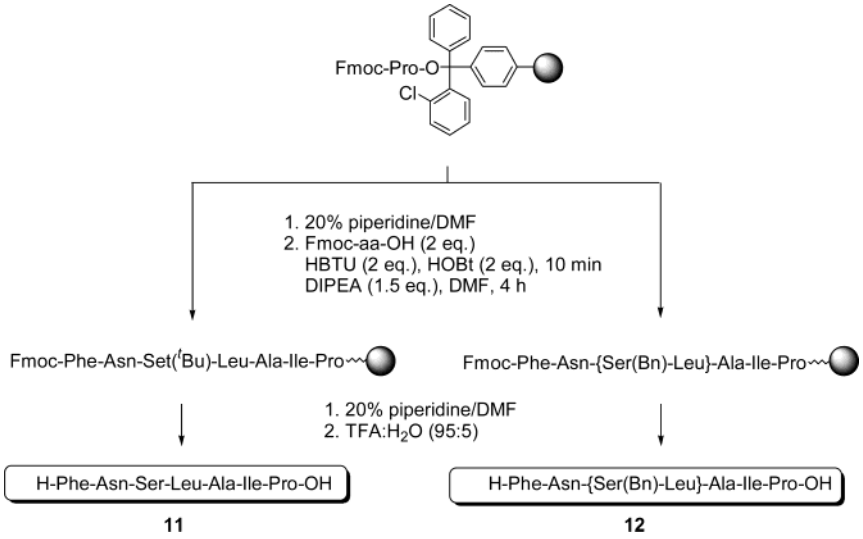
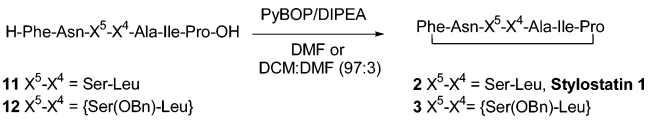


Table 2. Comparative Cyclization of Compounds **6** and **8**

time, h	**6:2:9**	**8:3:10**
2	6.4:1.2:2.4	0:7.8:2.2
28	0:3.6:6.4	- -

Scheme 4



ψ-stylostatin **3**, were obtained very pure (>95% by HPLC) as the only reaction products, and in good yields. Neither epimerization nor dimerization was observed.

Once the cyclization had been optimized, we hydrogenolyzed compounds **3** and **10** using Pd/C as the catalyst to obtain ψ-stylostatins **4** and **13**, which were identified by MS and amino acid analysis (Scheme 5).

Kinetic Studies. To study the influence of the constrained Ser-Leu surrogate **1a** on the cyclization process, we followed the kinetics of the transformation of compounds **6** and **8** by analytical HPLC (Scheme 2, Table 2).[16] The reaction was carried out on 40 mg samples in DCM/DMF using PyBOP/DIPEA. The reaction of compound **8** was complete after 2 h, whereas **6** required 28 h to be fully transformed. Thus, the introduction of the pseudopeptide {Ser(Bn)-Leu} **1a** increased the cyclization rate 14-fold, suggesting that the restricted pseudopeptides may induce a bent conformation of the linear precursor.

Structural NMR Studies. As expected, the NMR data of compound **2** in DMSO-d_6 were in agreement with those reported for native stylostatin 1.[7] However, the ^{1}H NMR spectra of Ψ-cyclopeptides **3** and **4** showed two sets of signals (Table 3), indicating the presence of two conformers in DMSO-d_6. We considered that this slow interconversion could be due to Ile-Pro amide bond rotamers. A characteristic feature of cis X-Pro dipeptides, compared to the corresponding trans X-Pro isomers, is the chemical shift difference between the β and γ carbon atoms of Pro: $\Delta\delta^{\beta\gamma} \geq 8$ ppm for the cis isomers, whereas $\Delta\delta^{\beta\gamma} < 6$ ppm for the trans.[1b] For native stylostatin 1, $\Delta\delta^{\beta\gamma} = 9$ ppm, which indicates a cis conformation of the Ile-Pro amide bond. This is in agreement with the reported X-ray diffraction structure,[7] thus validating the method for determining the conformation of pseudostylostatins. Comparison of the $\Delta\delta^{\beta\gamma}$ values of the two isomers of pseudostylostatins **3** and **4** made it clear that they correspond to the expected cis and trans isomers (Table 3). In both cases the cis isomer is the major one.

Interestingly, *epi*-stylostatin **9** and *epi*-ψ-stylostatins **10** and **13** showed only the trans Ile-Pro conformation in DMSO-d_6 ($\Delta\delta^{\beta\gamma} < 6$ ppm).

Solvent Addition Experiment (%DMSO). We performed additional NMR experiments in order to probe further the conformational differences between stylostatin 1 (**2**) and Ψ-stylostatin **4**. We first wanted to know which amide protons were involved in hydrogen bonding or were not accessible to the solvent, since this would indicate orientation toward the inside of the macrocycle. To ensure that the hydrogen bonds were intramolecular, we first ran the spectrum at various concentrations of the compounds in DMSO (0.25−16 mM) and in $CDCl_3$ + 0.5% DMSO (0.25−1 mM).[17] No chemical shift differences were observed by changing concentration, which confirmed that the results would not be influenced by intermolecular interactions.

We then compared the chemical shifts of the amide NH protons in a gradient of DMSO in $CDCl_3$. Protons that are non-hydrogen-bonded, and totally accessible to the solvent, should quickly establish a hydrogen bond with DMSO so that their signal shifts downfield, whereas protons that do not shift significantly may be either hydrogen-bonded or not accessible to the solvent. The spectra of stylostatin 1 (**2**) showed that the side chain Asn amide protons and Ile-NH are totally accessible to the solvent, but that most of the other NH protons appear to be involved in hydrogen bonds (Figure 2a). The Ser-NH signal shift started at 8% DMSO, suggesting limited access to solvent or weak hydrogen bonding.

It was interesting that the spectrum of ψ-stylostatin **4** in $CDCl_3$ showed very broad signals, especially for the amide protons. On addition of DMSO the signals gained resolution, reaching a maximum at 8% DMSO. In this range the chemical shifts do not vary, and only a cis

Scheme 5

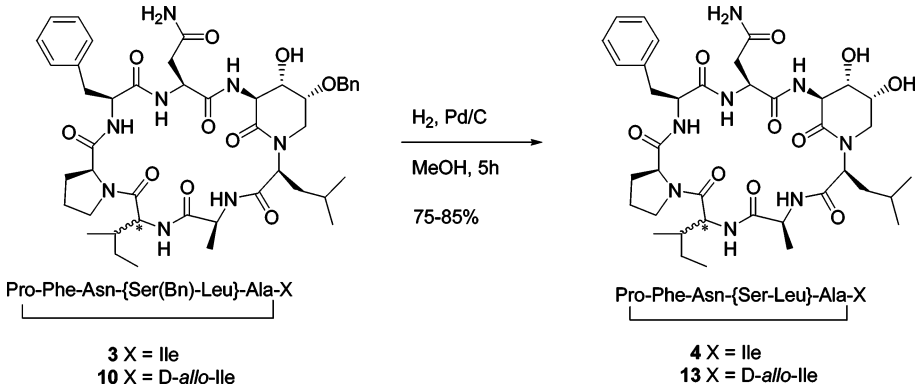


Table 3. Proline Relevant ^{13}C NMR Chemical Shifts in DMSO-d_6

	2	**3**	**4**	**9**	**10**	**13**
$\delta_{C\beta}$	29.7	29.0/27.9	29.2/28.0	28.8	28.4	28.5
$\delta_{C\gamma}$	20.7	21.0/24.9	21.0/25.1	24.3	24.5	24.4
Dd^{bg}	9	8/3	8.2 /2.9	4.5	3.9	4.1
Ile-Pro conformation	cis	cis:trans = 1.6:1	cis:trans = 1.8:1	trans	trans	trans

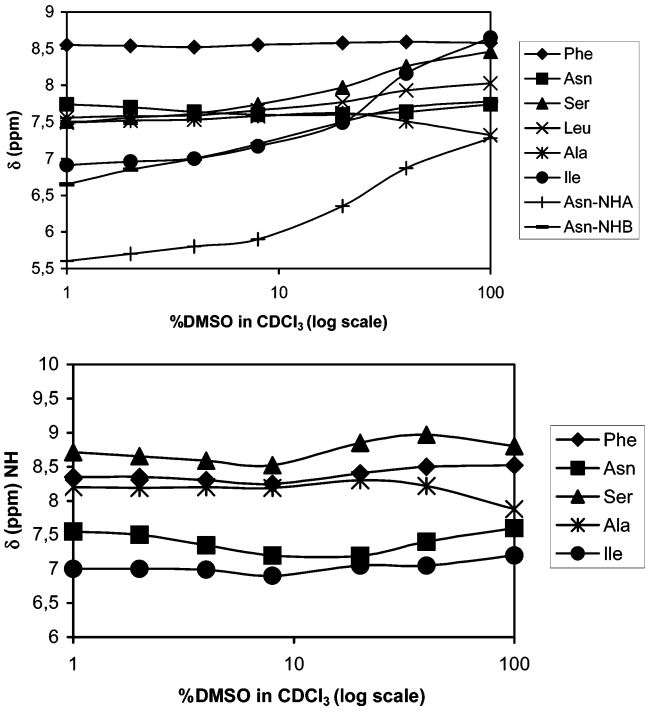


Figure 2. (a) ^1H NMR chemical shifts of NH protons of stylostatin 1 (**2**) at different proportions of DMSO-d_6 in $CDCl_3$ (3 mM, 25 °C). (b) ^1H NMR chemical shifts of NH protons of ψ-stylostatin 1 (*cis*-**4**) at different proportions of DMSO-d_6 in $CDCl_3$ (3 mM, 25 °C).

Ile-Pro conformation is observed (Pro $\Delta\delta^{\beta\gamma} = 9$ ppm, in 8% DMSO). With continued addition of DMSO, a second set of signals, corresponding to the trans Ile-Pro conformer (Pro $\Delta\delta^{\beta\gamma} = 2.9$ ppm, in DMSO), appears, which increases with the proportion of DMSO up to a 1.9:1 cis:trans proportion in DMSO.

The chemical shifts of the amide protons in the solvent gradient show no major variations (Figure 2b), and the fact that they are deshielded ($\delta > 7.5$) suggests that they are mostly accessible to the solvent or hydrogen bonded.

This experiment showed that the replacement of the Ser-Leu dipeptide by the piperidone surrogate affects the conformational response of the cyclopeptide to solvent variation. The lack of the Leu NH may contribute to this behavior, and the fact that the Ile-Pro trans conformation is visible for ψ-stylostatin **4** indicates an increased structural flexibility with respect to compound **2**. In addition, the trans isomer becomes significant in the presence of DMSO. This suggests that in the trans isomer the amide protons are oriented toward the external side of the cycle, and that hydrogen bonding with the solvent may stabilize the conformation.

Temperature Coefficients ($\Delta\delta/\delta T$). Our second experiment consisted of calculating the temperature coefficients of the amide protons in DMSO solution in order to confirm their hydrogen-bonding state.[18] In general, absolute values above 4 ppb/K indicate that the proton is non-hydrogen-bonded and fully accessible to the solvent, whereas coefficients below 3 ppb/K indicate a hydrogen-bonded state. Intermediate values would indicate that the proton is in equilibrium between a hydrogen-bonded and a non-hydrogen-bonded state.

The spectrum of stylostatin 1 (**2**) in DMSO showed that only the Ser and Ile-NH amide protons are free (Table 4). This corresponds to the conformation that Pettit et al. observed by X-ray diffraction,[7] in which the hydrogen bonds of the two β-turns involve the Phe-NH and the Ala-NH. The authors also indicate a third hydrogen bond between the Leu-NH and the carbonyl of the side chain of Asn. The fourth hydrogen bond is not apparent from the X-ray diffraction analysis, but the orientation of the Asn-NH toward the inner part of the cycle is clear.

For ψ-stylostatin **4** (Table 4), the first observation was that increasing temperature led to a predominance of the cis Ile-Pro conformer, and a dramatic loss in resolution of signals corresponding to the Ile-Pro trans

Table 4. Temperature Coefficients ($|\Delta\delta/\Delta T|$ in ppb/K) in DMSO

	stylostatin 1 (**2**)	ψ-stylostatin (*cis*-**4**)
Phe	2.3	3.5
Asn	0.3	2
Ser	5.4	4.8
Leu	2.5	- -
Ala	0.9	1
Ile	6.5	4.7
$Asn\text{-}NH_A$	2.9	3.2
$Asn\text{-}NH_B$	3.9	- -[a]

[a] The signals were masked in the 7.05−7.20 region.

Table 5. Growth Inhibition Effect (GI_{50} in μM) Found for the Compounds Tested

		2	**4**	**9**	**13**
prostate tumors	DU-145	8.44	10.6	3.55	9.82
	LN-caP	2.44	3.49	2.60	3.13
	SKOV-3	3.77	4.99	3.04	4.03
	IGROV	3.35	3.33	2.58	3.48
ovarian tumors	IGROV-ET	4.29	4.97	3.30	5.02
	SK−BR-3	4.16	3.86	4.39	4.06
melanoma	MEL-28	4.64	6.69	3.06	5.55
NSCL	A-549	4.28	5.20	3.46	4.32
pancreas	PANC-1	4.97	5.10	5.03	4.94
	HT-29	7.13	9.31	6.85	7.22
colon	LOVO	11.5	8.83	4.55	12.7
	LOVO-DOX	8.52	8.84	6.77	12.4
leukemia	K-652	3.42	3.59	1.94	4.32

conformer, to the point that only the data for the cis conformer were reliable.

Of the four hydrogen bonds that stabilize the Ile-Pro cis conformation of the native stylostatin 1 (**2**), ψ-stylostatin *cis*-**4** lacks that of the Leu-NH, the Phe-NH appears to be in equilibrium between a hydrogen-bonded and a non hydrogen-bonded state, the Asn-NH hydrogen bond becomes weaker, and only the Ala-NH hydrogen bond is maintained. These data clearly indicate that the cis conformer of ψ-stylostatin **4** is less stable than that of the native peptide **2**. This lower degree of structuring implies a greater flexibility for the molecule. In turn, this would allow the slow equilibrium with the Ile-Pro trans conformer to take place.

From the NMR experiments, we can conclude that on introducing the piperidone surrogate the turn centered on the Ser-Leu dipeptide is maintained but that the other stabilizing hydrogen bonds are significantly modified, with the result that the molecule becomes more flexible, allowing for the onset Ile-Pro trans-conformation in DMSO.

Upon heating of the sample,[19] the proportion of the Ile-Pro trans isomer of **4** diminishes with respect to *cis*-**4**. However, we did not observe a coalescence of the signals to an intermediate shift, as it is usual in simple amide rotamers. The fact that the two conformers showed two independent sets of signals indicates that *cis*-**4** and *trans*-**4** are in a very slow equilibrium.

It is noteworthy that this X-Pro trans conformation is the only one observed in the three "epi" analogues **9**, **10**, and **13**. This is understandable, since the Ile-NH amide proton stabilizes the second β-turn of the native stylostatin 1 (**2**) structure. The different orientation of the substituents about the α-carbon of D-*allo*-Ile probably exerts a steric effect that favors the D-*allo*-Ile-Pro trans conformer.

Bioassay Data. Since stylostatin 1 (**2**) had been reported as cytostatic against P388 lymphocyte leukemia cells (ED_{50} of 0.8 μg/mL),[7] we tested the ability of compounds **2**, **4**, and their epi analogues **9** and **13** to inhibit the growth of a series of cancer cell lines (see Experimental Section and Supporting Information). The colorimetric tests showed that the most active compound is *epi*-stylostatin **9**, which shows a GI_{50} of 1.94 μM against the K-652 leukemia cell line. However, none of these compounds is active below 1 μM (GI_{50} shown in Table 5).

Despite the lack of growth inhibitory activity, a comparison of the GI_{50} of **2** vs **4** and of **9** vs **13**, shows that the presence of the Ser-Leu piperidone surrogate slightly decreases the activity of the compounds. The conformational alteration is possibly responsible for this difference, although this explanation would not account for the observation that the epi analogue **9** (only trans) is more active than the others.

Conclusion

We have obtained five derivatives of the cycloheptapeptide stylostatin 1 (**2**), and we have established a new and improved synthetic route for the synthesis of stylostatin 1 (**2**). We have shown that the piperidone surrogate {Ser-Leu} induces a turn in the linear peptide which facilitates the cyclization.

The NMR data showed that the effect of introducing the piperidone ring in the structure was much more dramatic than expected. The hydrogen bonding pattern of the molecule **4** is completely altered. As a result, the molecule is more flexible and establishes a slow equilibrium between cis and trans Ile-Pro amide bond conformations, which is favored by the hydrogen bonding solvent DMSO. We have evaluated the anticancer activity of compounds **2**, **4**, **9**, and **13**. None of them is active (GI_{50}) below 1 μM, but the differences observed in activity suggest that the piperidone ring can cause a loss of activity.

Experimental Section

Abbreviations. Abbreviations used for amino acids and peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature.[20] The following additional abbreviations are used: BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium ; Cl-TrtCl-resin, 2-chlorotrityl chloride resin; DCM, dichlorometane; DHB, 2,5-dihydroxybenzoic acid; DIPEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ES-MS, electrospray mass spectrometry; EtOAc, ethyl acetate; Fmoc-OSu, 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide; HPLC, high performance liquid chromatography; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium ; HOBt, 1-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MeOH, methanol; NMR, nuclear magnetic resonance; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium ; SPS, solid-phase synthesis; SRB, sulforhodamine B; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UV, ultraviolet. Amino acid symbols denote the L-configuration unless stated otherwise.

General. Solid-phase syntheses were carried out at 25 °C in polypropylene syringes (5/10 mL) fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings were performed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) using 10 mL of solvent per gram of resin for each wash. Flash chromatography was eluted through SiO_2 (silica gel 60 A CC, 230−240 mesh ASTM, SDS). TLC analysis was done on SiO_2 plates (F254, Macherey-Nagel) and developed with the solvent described in each case for flash chromatography. The spots were located by UV light and K_2MnO_4 reagent. Purification of reagents and solvents was effected according to standard methods.[21] Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. MALDI-TOF and ES-MS analyses of peptide samples were performed in a Perspective Biosystems Voyager DE RP, using DHB matrixes, and in a Micromass VG-quattro spectrometer.

^1H and ^{13}C NMR spectra of compounds **1a** and **1b** were recorded on a Varian Gemini-200, in $CDCl_3$. NMR experiments of the peptides were carried out using a Bruker 600-MHz NMR spectrometer. The samples were approximately 3 mM in DMSO-d_6. Chemical shifts were reported in δ (ppm) relative to the undeuterated fraction of DMSO-d_6 and to TMS. Amino

acid analyses were performed on a Beckman system Gold 6300 at the Serveis Científico-Tècnics (UB).

(3*R*,4*S*,5*R*)-5-Benzyloxy-*N*-[(1*S*)-1-*tert*-butoxycarbonyl-3-methylbutyl]-3-fluorenyloxycarbonylamino-4-hydroxypiperidin-2-one (Fmoc-{Ser(Bn)-Leu}-O^{t}Bu). To a solution of amine **1a**[5b] (282 mg, 0.69 mmol) in acetone (3 mL) were added Fmoc-OSu (299 mg, 0.88 mmol) and $NaHCO_3$ (185 mg, 2.21 mmol). The mixture was stirred at room temperature for 8 h. The solvent was evaporated, and the residue was partitioned in brine and CH_2Cl_2. The aqueous phase was extracted with CH_2Cl_2, and the combined organic layers, dried and evaporated, gave an oil. Purification by flash chromatography (hexane:EtOAc, 3:1) yielded the Fmoc-protected aminopiperidone (412 mg, 98%) as an oil: $[\alpha]^{20}_D$ −21° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) 0.88 (d, *J* = 6.3 Hz, 3H, CH(C*H*$_3$)$_2$), 0.92 (d, *J* = 6.3 Hz, 3H, CH(C*H*$_3$)$_2$′), 1.44 (s, 9H, C(C*H*$_3$)$_3$), 1.5−1.7 (m, 3H, C*H*(CH$_3$)$_2$, C*H*$_2$), 3.34 (dd, *J* = 13.2 and 3.0 Hz, 1H, H-6), 3.42 (dd, *J* = 13.1 and 2.4 Hz, 1H, H-6′), 4.0−4.2 (m, 2H, H-4, H-5), 4.24 (t, *J* = 6.9 Hz, 1H, CH-Fmoc), 4.40 (d, *J* = 6.7 Hz, 2H, CH$_2$-Fmoc), 4.60 (dd, *J* = 9.0 and 4.8 Hz, 1H, H-3), 4.70 (d, *J* = 12.3 Hz, 1H, C*H*$_2$Ph), 4.86 (d, *J* = 12.3 Hz, 1H, C*H*$_2$Ph), 5.16 (t, *J* = 7.8 Hz, 1H, NC*H*), 6.00 (d, *J* = 4.0 Hz, 1H, NH), 7.25−7.50 (m, 9H, Ph, H2 and H3-Fmoc), 7.61 (d, *J* = 11.2 Hz, 2H, H1-Fmoc), 7.78 (d, *J* = 8.2 Hz, 2H, H4-Fmoc); ^{13}C NMR ($CDCl_3$) 21.0 (CH(*C*H$_3$)$_2$), 23.2 (CH(*C*H$_3$)$_2$′), 24.3 (*C*H(CH$_3$)$_2$), 27.9 (C(*C*H$_3$)$_3$), 36.6 (*C*H$_2$), 44.1 (C6), 46.9 (CH-Fmoc), 54.7 (N*C*H), 55.6 (C3), 67.6 (CH$_2$-Fmoc), 72.8 (C5), 72.9 (*C*H$_2$Ph), 73.5 (C4), 81.9 (*C*(CH$_3$)$_3$), 120.3 (C4-Fmoc), 126.9 (C1-Fmoc), 128.0−128.5 (Ph-C2, Ph-C3, Ph-C4, C2 and C3-Fmoc), 137.7 (Ph-C1), 141.1 (C5-Fmoc), 143.5 (C6-Fmoc), 158.7 (CO carbamate), 167.8 (CO), 170.1 (CO); MS *m*/*z* 628 (M^+, 2%). Anal. Calcd for $C_{37}H_{44}N_2O_7$: C, 70.68; H, 7.05; N, 4.46. Found: C, 70.65; H, 7.08; N, 4.43.

2(*S*)-2-[(3*R*, 4*S*,5*R*)-5-Benzyloxy-3-(fluorenyloxycarbonylamino)-4-hydroxy-2-oxo-1-piperidinyl]isohexanoic Acid (Fmoc-{Ser(Bn)-Leu}-OH). A mixture of Fmoc-{Ser(Bn)-Leu}O^{t}Bu (500 mg, 0.82 mmol), CH_2Cl_2 (3 mL), and TFA (3 mL) was stirred at room temperature for 2.5 h. After evaporation of the solvent, the solid residue was triturated several times with cold Et_2O to provide Fmoc-{Ser(Bn)-Leu}-OH (380 mg, 80%) as a white solid. Mp 197 °C (Et_2O). $[\alpha]^{20}_D$ −41° (*c* 0.5, MeOH); ^{1}H NMR (CD_3OD) 0.94 (d, *J* = 6.6 Hz, 6H, CH(C*H*$_3$)$_2$), 1.6−2.0 (m, 3H, C*H*(CH$_3$)$_2$, C*H*$_2$), 3.5−3.6 (m, 2H, H-6), 4.1−4.2 (m, 2H, H-4, H-5), 4.34 (t, *J* = 6.6 Hz, 1H, CH-Fmoc), 4.42 (d, *J* = 6.6 Hz, 2H, CH$_2$-Fmoc), 4.50 (d, *J* = 9.6 Hz, 1H, H-3), 4.79 (d, *J* = 12 Hz, 1H, C*H*$_2$Ph), 4.86 (d, *J* = 12 Hz, 1H, C*H*$_2$Ph), 5.31 (dd, *J* = 10.8 and 3.9 Hz, 1H, NC*H*), 7.25−7.30 (m, 5H, Ph), 7.40 (t, *J* = 6 Hz, 2H, H2-Fmoc), 7.47 (t, *J* = 7.2 Hz, 2H, H3-Fmoc), 7.79 (d, *J* = 7.2 Hz, 2H, H1-Fmoc), 7.89 (d, *J* = 7.2 Hz, 2H, H4-Fmoc); ^{13}C NMR (CD_3OD) 21.4 (CH(*C*H$_3$)$_2$), 23.7 (CH(*C*H$_3$)$_2$′), 25.2 (*C*H(CH$_3$)$_2$), 37.8 (*C*H$_2$), 44.6 (C6), 48.1 (CH-Fmoc), 56.8 (C3, N*C*H), 68.3 (CH$_2$-Fmoc), 71.1 (C5), 73.3 (*C*H$_2$Ph), 75.7 (C4), 120.8 (C4-Fmoc), 126. 4 (C1-Fmoc), 128.5−129.5 (Ph-C2, Ph-C3, Ph-C4, C2 and C3-Fmoc), 139.6 (Ph-C1), 142.5 (C5-Fmoc), 145.3 (C6-Fmoc), 159.3 (CO carbamate), 171.2 (CO); MS *m*/*z* 572 (M^+, 2%).

General Method for the Solid-Phase Peptide Synthesis. The synthesis of linear peptides was accomplished manually by the Fmoc methodology using either the Fmoc-Ile Wang resin or the Cl-Trt-Cl resin as solid supports. The *N*-Fmoc-amino acids were activated using HBTU (2 equiv), HOBt (2 equiv), and DIPEA (1.5 equiv) in DMF for 10 min, before their addition to the resin. The coupling reactions were maintained for 4 h, and their completion was verified by the ninhydrin (Kaiser) test. The coupling process was repeated under the same conditions in case of positive ninhydrin test. Removal of the Fmoc group was performed by incubation in 20% piperidine in DMF (v/v) (2 × 1 min and 2 × 10 min). After the final deprotection step, the obtained peptide resin was dried in vacuum and treated with TFA/H_2O (95/5, v/v, 15 mL/g resin) for 4 h at room temperature to cleave the peptide from the resin and remove the side chain protecting groups. The solid support was filtered off and washed with TFA several times. The solvent was evaporated and the obtained oily products were suspended in *tert*-butyl methyl ether and precipitated by centrifugation. The peptide was then purified and characterized according to the following general protocol.

General Method for Purification and Characterization of the Peptides. The compounds were purified on a Waters HPLC system using a SymmetryPrep C_{18} reversed-phase preparative column (7.8 × 300 mm) with 7 μm packing material. The crude peptides (50−100 mg) were dissolved in water/acetonitrile; 1/2 (1 mL), filtered and injected through a Rheodyne injector with 1 mL sample loop. The mobile phases used were A: 0.05% aqueous TFA and B: 0.05% TFA in acetonitrile, with a linear gradient from 10%B to 45%B over 40 min (3 mL/min). Fractions were manually collected at 1 min intervals. Elution of the peptide was determined simultaneously from the absorbances at 220 and 254 nm (Waters 2487). Fractions were reinjected to assess purity on analytical reversed-phase HPLC (Waters NovaPak C_{18}, 3.9 × 150 mm, 4 μm). In this case, we used a linear gradient from 0%B to 100%B over 30 min for peptides characterization. Pure fractions were combined and lyophilized. Each peptide showed [M + H^+] and/or [M + Na^+] peaks in its mass spectrum, and each was >90% pure. The structure was confirmed by amino acid analysis and NMR. The NMR signal assignments required 2D-HSQC, 2D-TOCSY, COSY (H,H), and 2D-NOESY experiments. The samples for NMR were prepared approximately 3 mM in DMSO-d_6.

Stylostatin 1 Precursor (6). Operating as in the general method, using Fmoc-Ile Wang resin (600 mg, 0.57 mmol/g), we obtained compound **6** (H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH). The required Fmoc-amino acids were Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(O^{t}Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Phe-OH, and Fmoc-Pro-OH. Peptide **6** (195 mg, 75%) was obtained as a white solid with a purity of >85% after HPLC purification (t_R 14.4 min, characterization conditions). ES-MS calcd for $C_{36}H_{56}N_8O_{10}$ 760.9, found *m*/*z* 761.1 [M + H^+].

Stylostatin 1 Precursor (11). Attachment of the First Amino Acid. A solution of Fmoc-Pro-OH (81 mg, 0.24 mmol, 0.5 equiv) and DIPEA (84 μL, 0.48 mmol, 2 equiv) in DCM (1 mL) was added to the Cl-TrtCl-resin (300 mg, 1.6 mmol/g), and the reaction mixture was gently stirred for 1 h. MeOH (0.8 mL) was then added, and after stirring for 10 min, the Fmoc-Pro-O-TrtCl resin (0.8 mmol/g) was sequentially washed with DCM and DMF. Operating as in the general method for synthesis and purification, from the Fmoc-Pro-O-TrtCl resin, and Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(O^{t}Bu)-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Phe-OH, peptide **11** (100 mg, 55%) was obtained as a white solid with a purity of >85% as determined by HPLC (t_R 14.1 min, characterization conditions). ES-MS calcd for $C_{36}H_{56}N_8O_{10}$ 760.9, found *m*/*z* 761.3 [M + H^+].

ψ-Stylostatin Precursor (8). Operating as in the general method for the synthesis and purification, from Fmoc-Ile Wang resin, and Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, and Fmoc-{Ser-Leu}-OH, we obtained compound **8** (H-Pro-Phe-Asn-{Ser-Leu}-Ala-Ile-OH). The linear ψ-peptide **8** (231 mg, 76%) was obtained as a white solid and >85% pure, as determined by HPLC (t_R 18.2 min, characterization conditions). ES-MS calcd for $C_{45}H_{64}N_8O_{11}$ 892.4, found *m*/*z* 893.2 [M + H^+].

ψ-Stylostatin Precursor (12). Operating as in the general method for the synthesis and purification, from Fmoc-Pro-O-TrtCl resin (0.8 mmol/g, obtained as for the preparation of **11**), Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-{Ser(Bn)Leu}-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Phe-OH, we obtained ψ-peptide **12** (H-Phe-Asn-{Ser(Bn)-Leu}-Ala-Ile-Pro-OH). The linear γ-peptide **12** (94.5 mg, 52%) was obtained as a white solid >85% pure, as determined by HPLC (t_R 17.6 min, characterization conditions). ES-MS calcd for $C_{45}H_{64}N_8O_{11}$ 892.4, found *m*/*z* 892.9 [M + H^+].

General Procedure for Cyclization. To a solution of the linear heptapeptides in DMF at room temperature were added BOP or PyBOP and DIPEA. The cyclization was monitored by analytical reversed-phase HPLC. When cyclization was completed, the solvent was removed under reduced pressure

to yield a light yellow oil, which was purified by semipreparative RP-HPLC. After lyophilization of the pure cyclopeptides they were white solids.

Cyclization of 6 To Give Stylostatin 1 (2) and *epi*-Stylostatin (9). Following the general procedure for cyclization, from compound **6** (20 mg, 0.026 mmol), PyBOP (16.2 mg, 0.031 mmol), and DIPEA (11 μL, 0.063 mmol), the cyclization was completed after 28 h, as determined by analytical reversed-phase HPLC. The crude product was purified by semipreparative RP-HPLC and lyophilized to yield stylostatin 1 (**2**) (4.5 mg, 23%) and its Ile epimer **9** (7.7 mg, 40%) as white solids. **Stylostatin 1 (2)**: Analytical HPLC (t_R 15.6 min, characterization conditions); ES-MS calcd for $C_{36}H_{54}N_8O_9$ 742.4, found *m*/*z* 743.4 [M + H$^+$]; NMR (DMSO-d_6) **serine** unit, ^{1}H 3.67−3.69 (m, 2H, H-3), 3.85 (dd, J = 9.2 and 4 Hz, 1H, H-2), 8.46 (d, J = 3.6 Hz, 1H, NH); ^{13}C 58.8 (C-2), 60.4 (C-3); **alanine** unit, ^{1}H 1.13 (d, J = 6.5 Hz, 3H, H-3), 4.44−4.46 (m, 1H, H-2), 7.32 (d, J = 7.2 Hz, 1H, NH); ^{13}C 15.9 (C-3), 47.4 (C-2); **isoleucine** unit, ^{1}H 0.77−0.82 (m, 9H, C(3)−CH$_3$, H-5), 1.22−1.30 (m, 1H, H-4), 1.50−1.55 (m, 1H, H-4), 1.71 (m, 1H, H-3), 4.02 (dd, J = 8.7 and 5 Hz, 1H, H-2), 8.65 (d, J = 4.8 Hz, 1H, NH); ^{13}C 10.5 (C-5), 14.4 (C(3)−CH$_3$), 24.4 (C-4), 35.2 (C-3), 56.6 (C-2); **proline** unit, ^{1}H 0.68−0.77 (m, 1H, H-4), 1.49−1.60 (m, 2H, H-4 and H-3), 2.15 (dd, J = 12.4 and 7.1 Hz, 1H, H-3), 2.6 (t, J = 10.6 Hz, 1H, H-5), 3.23 (dt, J = 10.6 and 7.9 Hz, 1H, H-5), 4.42 (d, J = 7.1 Hz, 1H, H-2); ^{13}C 20.7 (C-4), 29.7 (C-3), 45.1 (C-5), 60.1 (C-2); **phenylalanine** unit, ^{1}H 3.02 (under the H$_2$O signal, 1H, H-3), 3.14 (dd, J = 14 and 4 Hz, 1H, H-3), 4.15 (ddd, J = 12, 7.8 and 4.4 Hz, 1H, H-2), 7.12 (d, J = 7.1 Hz, 2H, H-*o*Ph), 7.20 (t, J = 7.3 Hz, 1H, H-*p*Ph), 7.27 (d, J = 7.3 Hz, 2H, H-*m*Ph), 8.58 (d, J = 7.8 Hz, 1H, NH); ^{13}C 36.8 (C-3), 57.5 (C-2), 126.3 (C-*p*Ph), 128.2 (C-*m*Ph), 128.4 (C-*o*Ph); **leucine** unit, ^{1}H 0.77−0.82 (m, 3H, H-5), 0.83 (d, J = 6.2 Hz, 3H, H-5), 1.48−1.52 (m, 1H, H-4), 1.53−1.63 (m, 2H, H-3), 4.27−4.31 (m, 1H, H-2), 8.03 (d, J = 9.6 Hz, 1H, NH); ^{13}C 21.0 and 22.9 (C-5), 24.2 (C-4), 39.9 (C-3), 51.3 (C-2); **asparagine** unit, ^{1}H 3.04−3.08 (m, 2H, H-3), 4.27−4.31 (m, 1H, H-2), 7.26−7.30 (m, 1H, CONH$_2$), 7.74 (d, J = 5.4 Hz, 1H, NH), 7.78 (sa, 1H, CONH$_2$); ^{13}C 35.7 (C-3), 49.5 (C-2). ***epi*-Stylostatin (9)**: Analytical HPLC (t_R 16.9 min, characterization conditions); ES-MS calcd for $C_{36}H_{54}N_8O_9$ 742.9, found *m*/*z* 743.9 [M + H]$^+$; NMR (DMSO-d_6) **serine** unit, ^{1}H 3.72 (ddd, J = 11.6, 4.8 and 3 Hz, 1H, H-3), 3.96−4.03 (m, 1H, H-3), 4.17−4.25 (m, 1H, H-2), 5.13 (m, 1H, OH), 7.22−7.28 (m, 1H, NH); ^{13}C 55.5 (C-2), 62.2 (C-3); **alanine** unit, ^{1}H 1.23 (d, J = 7.3 Hz, 3H, H-3), 4.25−4.27 (m, 1H, H-2), 7.22−7.27 (m, 1H, NH); ^{13}C 18.1 (C-3), 48.7 (C-2); **D-*allo*-isoleucine** unit, ^{1}H 0.73 (d, J = 7 Hz, 3H, C(3)−CH$_3$), 0.81−0.86 (m, 3H, H-5), 0.91−0.97 (m, 1H, H-4), 1.27−1.33 (m, 1H, H-4), 1.84−1.90 (m, 1H, H-3), 4.37 (t, J = 9 Hz, 1H, H-2), 7.46 (br s, 1H, NH); ^{13}C 11.2 (C-5), 14.0 (C(3)−CH$_3$), 26.0 (C-4), 34.8 (C-3), 53.6 (C-2); **proline** unit, ^{1}H 1.54−1.64 (m, 1H, H-3), 1.74−1.80 (m, 2H, H-4), 1.8−1.91 (m, 1H, H-3), 3.3 (under the H$_2$O signal, 1H, H-5), 3.48 (dt, J = 10.2 and 5.8 Hz, 1H, H-5), 4.08 (t, J = 7.2 Hz, 1H, H-2); ^{13}C 24.3 (C-4), 28.8 (C-3), 46.8 (C-5), 59.6 (C-2); **phenylalanine** unit, ^{1}H 3.09 (br t, J = 14 Hz, 1H, H-3), 3.16 (dd, J = 14 and 4.4 Hz, 1H, H-3), 3.92−4.00 (m, 1H, H-2), 7.16 (d, J = 7.1 Hz, 2H, H-*o*Ph), 7.20 (t, J = 7.3 Hz, 1H, H-*p*Ph), 7.28 (t, J = 7.5 Hz, 2H, H-*m*Ph), 8.69 (br s, 1H, NH); ^{13}C 34.3 (C-3), 56.3 (C-2), 126.3 (C-*p*Ph), 128.2 (C-*m*Ph), 128.4 (C-*o*Ph); **leucine** unit, ^{1}H 0.81−0.86 (m, 3H, H-5), 0.89 (d, J = 6.8 Hz, 3H, H-5), 1.45 (ddd, J = 14, 9.2 and 4.6 Hz, 1H, H-3), 1.54−1.64 (m, 1H, H-3), 1.65−1.73 (m, 1H, H-4), 3.92−4.00 (m, 1H, H-2), 8.34 (br s, 1H, NH); ^{13}C 21.0 and 22.7 (C-5), 24.0 (C-4), 39.3 (C-3), 52.0 (C-2); **asparagine** unit, ^{1}H 2.66 (dd, J = 15.4 and 7 Hz, 1H, H-3), 2.75 (br d, J = 15.4 Hz, 1H, H-3), 4.23−4.31 (m, 1H, H-2), 6.91 (br s, 1H, CONH$_2$), 7.46 (br s, 1H, CONH$_2$), 8.10 (br s, 1H, NH); ^{13}C 35.5 (C-3), 50.5 (C-2).

Cyclization of Peptide 11 To Give Stylostatin 1 (2). Operating as in the general method, from peptide **11** (20 mg, 0.026 mmol), PyBOP (16.2 mg, 0.031 mmol), and DIPEA (11 μL, 0.063 mmol), the cyclization was completed after 5 min. The crude product was purified by semipreparative RP-HPLC as in the general method to yield stylostatin 1 (**2**, 9.6 mg, 50%) as a white solid.

Cyclization of Peptide 8 To Give Benzyl-ψ-stylostatin (3) and *epi*-Benzyl-ψ-stylostatin (10). Following the general procedure, from linear peptide **8** (20 mg, 0.022 mmol), PyBOP (14 mg, 0.027 mmol), and DIPEA (9.2 μL, 0.053 mmol), cyclization was completed after 2 h as determined by analytical reversed-phase HPLC. The crude product was purified by semipreparative RP-HPLC and lyophilized to yield ψ-stylostatin **3** (6.2 mg, 32%) and its Ile epimer **10** (3.7 mg, 19%) as white solids. **Benzyl-ψ-stylostatin (3)**: Analytical HPLC (t_R 19.5 min, characterization conditions); ES-MS calcd for $C_{45}H_{62}N_8O_{10}$ 874.4, found *m*/*z* 875.5 [M + H$^+$]. **Trans-3 rotamer**: NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.00−3.05 (m, 1H, H-5), 3.32−3.42 (m, 1H, H-5), 3.65 (br t, J = 7.3 Hz, 1H, H-2), 4.03−4.06 (m, 1H, H-4), 4.12 (br d, J = 8.4 Hz, 1H, H-3), 4.61 (d, J = 12 Hz, 1H, CH$_A$Ph), 4.67 (d, J = 12 Hz, 1H, CH$_B$Ph), 5.10−5.20 (br s, 1H, OH), 7.10−7.34 (m, 5H, H-Ph), 9.07 (br s, 1H, NH); ^{13}C 41.6 (C-5), 55.3 (C-2), 67.7 (C-3), 70.7 (OCH$_2$Ph), 73.3 (C-4), 125.9−128.6 (C-Ph); **alanine** unit, ^{1}H 1.30 (d, J = 7 Hz, 3H, H-3), 4.25−4.34 (m, 1H, H-2), 7.71 (br s, 1H, NH); ^{13}C 18.6 (C-3), 50.2 (C-2); **isoleucine** unit, ^{1}H 0.92 (d, J = 6.7 Hz, 3H, H-5), 0.79−0.86 (m, 3H, C(3)−CH$_3$), 1.36−1.45 (m, 2H, H-4), 1.92−1.95 (m, 1H, H-3), 4.50−4.56 (m, 1H, H-2), 7.16−7.20 (m, 1H, NH); ^{13}C 11.2 (C-5), 16.0 (C(3)−CH$_3$), 22.9 (C-4), 37.2 (C-3), 54.4 (C-2); **proline** unit, ^{1}H 1.60−1.65 (m, 1H, H-4), 1.65−1.70 (m, 1H, H-3), 1.77−1.83 (m, 1H, H-3), 1.90−2.00 (m, 1H, H-4), 3.33−3.50 (m, 1H, H-5), 3.70−3.75 (m, 1H, H-5), 3.90−3.96 (m, 1H, H-2); ^{13}C 24.9 (C-4), 27.9 (C-3), 46.8 (C-5), 61.4 (C-2); **phenylalanine** unit, ^{1}H 3.26−3.32 (m, 1H, H-3), 3.75−3.80 (m, 1H, H-3), 3.75−3.80 (m, 1H, H-2), 7.10−7.34 (m, 5H, H-Ph), 8.43 (br s, 1H, NH); ^{13}C 33.6 (C-3), 56.4 (C-2), 125.9−128.9 (C-Ph); **leucine** unit, ^{1}H 0.74 (d, J = 6.2 Hz, 3H, H-5), 0.79−0.86 (m, 3H, H-5), 1.36−1.42 (m, 1H, H-4), 1.55−1.60 (m, 2H, H-3), 5.03−5.08 (m, 1H, H-2); ^{13}C 20.3 and 23.0 (C-5), 23.0 (C-4), 34.1 (C-3), 52.8 (C-2); **asparagine** unit, ^{1}H 2.50−2.60 (m, 1H, H-3), 2.27 (dd, J = 15 and 5.2 Hz, 1H, H-3), 4.70−4.76 (m, 1H, H-2), 6.92 (br s, 1H, CONH$_2$), 7.16−7.20 (m, 1H, CONH$_2$), 8.15 (d, J = 8.8 Hz, 1H, NH); ^{13}C 37.5 (C-3), 50.0 (C-2). ***Cis*-3 rotamer**: NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.05 (br d, J = 10.5 Hz, 1H, H-5), 3.32−3.42 (m, 1H, H-5), 3.65 (br t, J = 7.3 Hz, 1H, H-2), 3.96−3.99 (m, 1H, H-4), 4.03−4.06 (m, 1H, H-3), 4.59 (d, J = 12 Hz, 1H, CH$_A$Ph), 4.66 (d, J = 12 Hz, 1H, CH$_B$Ph), 5.10−5.20 (br s, 1H, OH), 7.10−7.34 (m, 5H, H-Ph), 8.96 (d, J = 6.1 Hz, 1H, NH); ^{13}C 42.3 (C-5), 56.2 (C-2), 68.5 (C-3), 70.7 (OCH$_2$Ph), 73.5 (C-4), 125.9−128.6 (C-Ph); **alanine** unit, ^{1}H 1.30 (d, J = 7 Hz, 3H, H-3), 4.50−5.56 (m, 1H, H-2), 7.94 (d, J = 8.8 Hz, 1H, NH); ^{13}C 16.2 (C-3), 47.2 (C-2); **isoleucine** unit, ^{1}H 0.75 (d, J = 6.4 Hz, 3H, H-5), 0.79−0.86 (m, 3H, C(3)−CH$_3$), 1.10−1.17 (m, 1H, H-4), 1.48−1.53 (m, 1H, H-4), 1.60−1.65 (m, 1H, H-3), 4.08 (dd, J = 8.4 and 6 Hz, 1H, H-2), 7.99 (d, J = 6 Hz, 1H, NH); ^{13}C 10.8 (C-5), 14.2 (C(3)−CH$_3$), 24.2 (C-4), 36.2 (C-3), 55.2 (C-2); **proline** unit, ^{1}H 1.07−1.16 (m, 1H, H-4), 1.54−1.60 (m, 1H, H-4), 1.65−1.70 (m, 1H, H-3), 2.05 (dd, J = 12 and 6.5 Hz, 1H, H-3), 2.78 (br t, J = 10.3 Hz, 1H, H-5), 3.24−3.30 (m, 1H, H-5), 4.56−4.62 (m, 1H, H-2); ^{13}C 21.0 (C-4), 29.0 (C-3), 44.9 (C-5), 59.9 (C-2); **phenylalanine** unit, ^{1}H 2.90 (dd, J = 13.8 and 10.7 Hz, 1H, H-3), 3.17 (dd, J = 13.8 and 4.5 Hz, 1H, H-3), 4.36 (ddd, J = 10.8, 7.5 and 4.5 Hz, 1H, H-2), 7.10−7.34 (m, 5H, H-Ph), 8.52 (d, J = 7.5 Hz, 1H, NH); ^{13}C 37.0 (C-3), 55.2 (C-2), 125.9−128.6 (C-Ph); **leucine** unit, ^{1}H 0.76 (d, J = 6.2 Hz, 3H, H-5), 0.79−0.86 (m, 3H, H-5), 1.42−1.47 (m, 1H, H-4), 1.60−1.65 (m, 2H, H-3), 5.10 (dd, J = 10.7 and 5.5 Hz, 1H, H-2); ^{13}C 20.4 and 23.0 (C-5), 22.9 (C-4), 34.2 (C-3), 53.0 (C-2); **asparagine** unit, ^{1}H 2.50−2.60 (m, 1H, H-3), 4.45 (dd, J = 13 and 6 Hz, 1H, H-2), 6.98 (br s, 1H, CONH$_2$), 7.44 (s, 1H, CONH$_2$), 7.56 (d, J = 7.3 Hz, 1H, NH); ^{13}C 37.2 (C-3), 49.6 (C-2).

***epi*-Benzyl-ψ-stylostatin 10**: Analytical HPLC (t_R 20.5 min, characterization conditions); ES-MS calcd for $C_{45}H_{62}N_8O_{10}$ 874.4, found *m*/*z* 875.6 [M + H$^+$]; NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.05 (dd, J = 12.5 and 2.3 Hz, 1H, H-5), 3.36 (dd, J = 12.5 and 5.6 Hz, 1H, H-5), 3.55 (t, J = 6 Hz, 1H, H-3), 3.99−

4.02 (m, 1H, H-4), 4.00−4.04 (m, 1H, H-3), 4.60 (d, J = 12 Hz, 1H, CH_APh), 4.69 (dd, J = 12 Hz, 1H, CH_BPh), 5.41 (br s, 1H, OH), 7.25−7.35 (m, 5H, H-Ph), 9.2 (d, J = 6 Hz, 1H, NH); ^{13}C 42.1 (C-5), 56.6 (C-2), 69.2 (C-4), 71.2 (OCH_2Ph), 73.4 (C-3), 127.1, 127.2, 127.9 (C-Ph); **alanine** unit, ^{1}H 1.35 (d, J = 7.6 Hz, 3H, H-3), 4.23 (m, 1H, H-2), 8.395 (d, J = 8.5 Hz, 1H, NH); ^{13}C 16.0 (C-3), 49.0 (C-2); **D-*allo*-isoleucine** unit, ^{1}H 0.65 (d, J = 6.7 Hz, 3H, C(3)−CH_3), 0.88 (t, J = 7.1 Hz, 3H, H-5), 0.97−1.05 (m, 1H, H-4), 1.19−1.25 (m, 1H, H-4), 1.59−1.65 (m, 1H, H-3), 4.67−4.72 (m, 1H, H-2), 6.77 (d, J = 8.5 Hz, 1H, NH); ^{13}C 11.5 (C-5), 13.0 (C(3)−CH_3), 26.5 (C-4), 37.5 (C-3), 52.0 (C-2); **proline** unit, ^{1}H 1.45−1.54 (m, 1H, H-3), 1.77−1.84 (m, 2H, H-4), 1.85−1.89 (m, 1H, H-3), 3.45 (dt, J = 9.6 and 6.4 Hz, 1H, H-5), 3.58 (dt, J = 9.6 and 7 Hz, 1H, H-5), 3.90 (dd, J = 8 and 5 Hz, 1H, H-2); ^{13}C 24.5 (C-4), 28.4 (C-3), 46.8 (C-5), 60.4 (C-2); **phenylalanine** unit, ^{1}H 3.02−3.08 (m, 1H, H-3), 3.28−3.34 (under water, 1H, H-3), 3.94 (br s, 1H, H-2), 7.13 (d, J = 7 Hz, 2H, H-*o*Ph), 7.19 (t, J = 7 Hz, 1H, H-*p*Ph), 7.27 (d, J = 7 Hz, 2H, H-*m*Ph), 8.44 (br s, 1H, NH); ^{13}C 34.5 (C-3), 55.6 (C-2), 126.0 (C-*p*Ph), 128.0 (C-*m*Ph), 128.8 (C-*o*Ph); **leucine** unit, ^{1}H 0.71 (d, J = 6.6 Hz, 3H, H-5), 0.83 (d, J = 6.7 Hz, 3H, H-5), 1.45−1.54 (m, 1H, H-4), 1.59−1.64 (m, 2H, H-3), 1.73 (ddd, J = 15, 11 and 4 Hz, 1H, H-3), 5.08 (dd, J = 12 and 4 Hz, 1H, H-2); ^{13}C 20.5 and 23 (C-5), 23.0 (C-4), 34 (C-3), 53.5 (C-2); **asparagine** unit, ^{1}H 2.14 (br d, J = 15.6 Hz, 1H, H-3), 2.45 (dd, J = 15.6 and 9.5 Hz, 1H, H-3), 4.77 (m, 1H, H-2), 6.72 (br s, 1H, $CONH_2$), 7.26−7.30 (br s, 1H, $CONH_2$), 7.60 (d, J = 6 Hz, 1H, NH); ^{13}C 36.5 (C-3), 49.3 (C-2).

Cyclization of Linear Peptide 12 To Give Benzyl-ψ-stylostatin (3). From linear peptide **12** (20 mg, 0.022 mmol), PyBOP (14 mg, 0.027 mmol), and DIPEA (9.2 μL, 0.053 mmol), the cyclization was completed after 5 min. Purification and lyophilization of the reaction product yielded benzyl-ψ-stylostatin (**3**, 11.7 mg, 61%) as a white solid.

Hydrogenation of Benzyl-ψ-stylostatin (3) To Give ψ-Stylostatin (4). A solution of the cyclic pseudopeptide **3** (15.4 mg, 0.018 mmol) in MeOH (5 mL), was hydrogenated at room temperature and P_{at} in the presence of Pd−C (10%, 5 mg). The reaction, monitored by analytical HPLC of filtered samples, was completed after 5 h. The catalyst was filtered off, the solvent was evaporated, and the crude was purified by semipreparative RP-HPLC (purification conditions) to yield ψ-stylostatin **4** (10.5 mg, 81%). Analytical HPLC (t_R 15.9 min, characterization conditions); ES-MS calcd for $C_{38}H_{56}N_8O_{10}$ 784.4, found m/z 785.4 [M + H$^+$]. **Trans-4 rotamer**: NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.02−3.10 (m, 2H, H-5), 3.61 (t, J = 6 Hz, 1H, H-2), 3.87 (ddd, J = 7.3, 5.2 and 2 Hz, 1H, H-3), 3.95−4.00 (m, 1H, H-4), 5.05 (d, J = 5.2 Hz, 1H, C(4)−OH), 5.13 (d, J = 4 Hz, 1H, C(3)−OH), 9.05 (br s, 1H, NH); ^{13}C 45.9 (C-5), 55.9 (C-2), 65.3 (C-4), 69.0 (C-3); **alanine** unit, ^{1}H 1.32 (d, J = 6.8 Hz, 3H, H-3), 4.25−4.33 (m, 1H, H-2), 7.68 (br s, 1H, NH); ^{13}C 18.6 (C-3), 50.3 (C-2); **isoleucine** unit, ^{1}H 0.80−0.90 (m, 3H, C(3)−CH_3), 0.92 (d, J = 6.4 Hz, 3H, H-5), 1.40−1.46 (m, 2H, H-4), 1.91−1.96 (m, 1H, H-3), 4.50−4.55 (m, 1H, H-2); ^{13}C 11.3 (C-5), 16.1 (C(3)−CH_3), 21 (C-4), 37.3 (C-3), 54.5 (C-2); **proline** unit, ^{1}H 1.65−1.70 (m, 1H, H-3), 1.71−1.78 (m, 1H, H-4), 1.78−1.84 (m, 1H, H-3), 1.95−2.00 (m, 2H, H-4), 3.42−3.46 (m, 1H, H-5), 3.70−3.75 (m, 1H, H-5), 3.91−3.95 (m, 1H, H-2); ^{13}C 25.1 (C-4), 28.0 (C-3), 46.8 (C-5), 61.5 (C-2); **phenylalanine** unit, ^{1}H 3.30−3.40 (under DMSO signal, 2H, H-3), 3.74−3.80 (m, 1H, H-2), 7.10−7.30 (m, 5H, H-Ph), 8.43 (br s, 1H, NH); ^{13}C 33.7 (C-3), 56.5 (C-2), 125.8 (C-*p*Ph), 127.9 (C-*m*Ph), 128.6 (C-*o*Ph); **leucine** unit, ^{1}H 0.76−0.90 (m, 9H, H-5), 1.40−1.46 (m, 2H, H-4), 1.50−1.70 (m, 1H, H-3), 5.00−5.04 (m, 1H, H-2); ^{13}C 20.8 and 23.2 (C-5), 23 (C-4), 34.3 (C-3), 52.8 (C-2); **asparagine** unit, ^{1}H 2.26 (d, J = 5.2 Hz, 1H, H-3), 2.28 (d, J = 5.2 Hz, 1H, H-3), 4.70−4.78 (m, 1H, H-2), 6.93 (br s, 1H, $CONH_2$), 7.16−7.22 (br s, 1H, $CONH_2$), 8.15 (d, J = 9 Hz, 1H, NH); ^{13}C 37.5 (C-3), 50.0 (C-2). **Cis-4 rotamer**: NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.02−3.10 (m, 1H, H-5), 3.12−3.20 (m, 1H, H-5), 3.63 (m, 1H, H-2), 3.95−4.00 (m, 1H, H-3), 4.05−4.10 (m, 1H, H-4), 5.01 (br s, 1H, C(4)−OH), 5.26 (d, J = 4 Hz, 1H, C(3)−OH), 8.82 (d, J = 6 Hz, 1H, NH); ^{13}C 44.4 (C-5), 54.9 (C-2), 65.0 (C-4), 67.8 (C-3); **alanine** unit, ^{1}H 1.31 (d, J = 6.8 Hz, 3H, H-3), 4.49−4.56 (m, 1H, H-2), 7.85 (d, J = 8.7 Hz, 1H, NH); ^{13}C 16.3 (C-3), 47.2 (C-2); **isoleucine** unit, ^{1}H 0.75−0.80 (m, 9H, H-5 and C(3)−CH_3), 1.06−1.15 (m, 1H, H-4), 1.50−1.54 (m, 1H, H-4), 1.54−1.65 (m, 1H, H-3), 4.05−4.10 (m, 1H, H-2); ^{13}C 10.9 (C-5), 14.3 (C(3)−CH_3), 24.2 (C-4), 36.2 (C-3), 55.3 (C-2); **proline** unit, ^{1}H 1.05−1.15 (m, 1H, H-4), 1.52.1.58 (m, 1H, H-4), 1.65−1.70 (m, 1H, H-3), 2.05 (dd, J = 12 and 6 Hz, 1H, H-4), 2.78 (br t, J = 10 Hz, 1H, H-5), 3.24−3.30 (m, 1H, H-5), 4.58 (d, J = 7.3 Hz, 1H, H-2); ^{13}C 24.2 (C-4), 29.2 (C-3), 45.1 (C-5), 60.0 (C-2); **phenylalanine** unit, ^{1}H 2.89 (dd, J = 13.8 and 10.8 Hz, 1H, H-3), 3.12−3.20 (m, 1H, H-3), ^{1}H 4.36 (ddd, J = 12.3, 7.5 and 4.6 Hz, 1H, H-2), 7.10−7.30 (m, 5H, H-Ph), 8.51 (d, J = 7.74 Hz, 1H, NH); ^{13}C 36.5 (C-3), 55.3 (C-2), 125.8 (C-*p*Ph), 127.9 (C-*m*Ph), 128.6 (C-*o*Ph); **leucine** unit, ^{1}H 0.76−0.90 (m, 9H, H-5), 1.55−1.65 (m, 2H, H-4), 1.60−1.70 (m, 1H, H-3), 5.08 (dd, J = 11 and 4.6 Hz, 1H, H-2); ^{13}C 20.8 and 23.2 (C-5), 22.8 (C-4), 34.3 (C-3), 53.0 (C-2); **asparagine** unit, ^{1}H 2.51 (dd, J = 15 and 5.5 Hz, 1H, H-3), 2.57 (dd, J = 15 and 7 Hz, 1H, H-3), 4.45 (dd, J = 13 and 6.5 Hz, 1H, H-2), 6.95 (br s, 1H, $CONH_2$), 7.42 (br s, 1H, $CONH_2$), 7.60 (d, J = 7.3 Hz, 1H, NH); ^{13}C 37.1 (C-3), 49.5 (C-2).

Hydrogenation of *epi*-Benzyl-ψ-stylostatin (10) To Yield *epi*-ψ-Stylostatin (13). Operating as above, from compound **10** (9.4 mg, 0.015 mmol) we obtained ψ-stylostatin **13** (6 mg, 73%). Analytical HPLC (t_R 16.9 min, characterization conditions); ES-MS calcd for $C_{38}H_{56}N_8O_{10}$ 784.4, found m/z 785.4 [M + H$^+$]; NMR (DMSO-d_6) {**serine**} unit, ^{1}H 3.03−3.10 (m, 1H, H-5), 3.17 (dd, J = 12 and 5 Hz, 1H, H-5), 3.50−3.52 (m, 1H, H-2), 3.85 (br s, 1H, H-3), 4.02 (br s, 1H, H-4), 5.16 (d, J = 4 Hz, 1H, C(5)−OH), 5.20 (br s, 1H, C(4)−OH), 9.15 (br s, 1H, NH); ^{13}C 44.5 (C-5), 56.2 (C-2), 65.0 (C-4), 69.8 (C-3); **alanine** unit, ^{1}H 1.37 (d, J = 7.5 Hz, 3H, H-3), 4.24 (q, J = 7.5 Hz, 1H, H-2), 8.32 (d, J = 8.6 Hz, 1H, NH); ^{13}C 16.2 (C-3), 49.0 (C-2); D-***allo*-isoleucine** unit, ^{1}H 0.65 (d, J = 6.7 Hz, 3H, C(3)−CH_3), 0.88 (d, J = 7.3 Hz, 3H, H-5), 1.00 (q, J = 7 Hz, 1H, H-4), 1.20 (br s, 1H, H-4), 1.59−1.64 (m, 1H, H-3), 4.69 (d, J = 7 Hz, 1H, H-2); ^{13}C 11.5 (C-5), 13.2 (C(3)−CH_3), 22.8 (C-4), 37.4 (C-3), 51.8 (C-2); **proline** unit, ^{1}H 1.53 (br s, 1H, H-3), 1.78−1.83 (m, 1H, H-4), 1.82−1.90 (m, 2H, H-3 and H-4), 3.42−3.50 (m, 1H, H-5), 3.55−3.62 (m, 1H, H-5), 3.89−3.92 (m, 1H, H-2); ^{13}C 24.4 (C-4), 28.5 (C-3), 46.8 (C-5), 60.2 (C-2); **phenylalanine** unit, ^{1}H 3.03−3.10 (m, 1H, H-3), 3.25−3.40 (under the H_2O signal, 1H, H-3), 3.87−3.92 (m, 1H, H-2), 7.13 (d, J = 7.1 Hz, 2H, H-*o*Ph), 7.19 (t, J = 7.5 Hz, 1H, H-*p*Ph), 7.27 (t, J = 7.5 Hz, 2H, H-*m*Ph), 8.48 (br s, 1H, NH); ^{13}C 34.7 (C-3), 55.8 (C-2), 125.8 (C-*p*Ph), 127.9 (C-*m*Ph), 128.7 (C-*o*Ph); **leucine** unit, ^{1}H 0.74 (d, J = 6.3 Hz, 3H, H-5), 0.87 (d, J = 7.5 Hz, 3H, H-5), 1.60−1.70 (m, 2H, H-4 and H-3), 1.75 (ddd, J = 15, 11.6 and 3.5 Hz, 1H, H-4), 5.05 (dd, J = 11.8 and 3.8 Hz, 1H, H-2); ^{13}C 20.3 and 23.2 (C-5), 22.8 (C-4), 34.3 (C-3), 53.6 (C-2); **asparagine** unit, ^{1}H 2.06−2.12 (m, 1H, H-3), 2.42 (dd, J = 15.6 and 9.5 Hz, 1H, H-3), 4.77 (br s, 1H, H-2), 6.67 (br s, 1H, $CONH_2$), 7.23 (br s, 1H, $CONH_2$), 7.65 (d, J = 6 Hz, 1H, NH); ^{13}C 36.5 (C-3), 49.2 (C-2).

Inhibition of Cell Growth by Colorimetric Assay.[22] A colorimetric type of assay, using the SRB reaction, has been adapted for a quantitative measurement of cell growth and viability, following the technique described by Skehan et al.:[23] cells are seeded in 96-well microtiter plates, at 5 × 10^3 cells per well in aliquots of 195 μL of RPMI medium, and they are allowed to attach to the plate surface by growing in drug free medium for 18 h. Afterward, samples are added in aliquots of 5 μL (dissolved in DMSO/H_2O 3:7). After 48−72 h exposure, the antitumor effect is measured by the SRB methodology: cells are fixed by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid and incubating for 60 min at 4 °C. Plates are washed with deionized water and dried; 100 μL of SRB solution (0.4wt %/vol in 1% acetic acid) is added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB is removed by washing with 1% AcOH. Plates are air-dried, and bound stain is solubilized with Tris buffer. Optical densities are read on an automated spectrophotometer plate

reader at a single wavelength of 490 nm. Data analyses are generated automatically by LIMS implementation at Pharma Mar and some parameters for cellular responses are calculated.

Using control OD values (C), test OD values (T) and time zero OD values (T_0), calculations are made for the following parameters: GI_{50} = concentration that causes 50% **G**rowth **I**nhibition, means the growth inhibition effect, calculated from: 100 x $[(T - T_0)/C - T_0)] = 50$; TGI = total growth inhibition, signifies **a** cytostatic effect: 100 x $[(T - T_0)/C - T_0)] = 0$; LC_{50} = concentration that causes 50% cell killing, means the cytotoxic effect calculated from: $100 \times [(T - T_0)/T_0)] = -50$.

Acknowledgment. Support for this research was provided by the CIRIT (Generalitat de Catalunya) through grants 1999SGR-00077 and 2001SGR-00082, and by the MCYT (Ministerio de Ciencia y Tecnología, Spain) through grants PB97-0976, 2FD97-0293, and BQU2001-3228.

Supporting Information Available: Amino acid analysis of stylostatin 1 (**2**) and epistylostatin **9**. HPLC monitorization of the comparative cyclization of linear peptides **6** and **8**. NMR spectra of ψ-stylostatin **4**: (a) full set in DMSO (cis and trans conformers); (b) full set in $CDCl_3$:DMSO (92:8) (only cis conformer); (c) comparative figure of variable temperature NMR spectra (6.6−9.2 ppm). Full bioassay results. This information is available free of charge via the Internet at http://pubs.acs.org.

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(10) Pseudodipeptide Fmoc-{Ser(Bn)-Leu}-OH showed no coupling/deprotection peculiarities.

(11) In contrast to what had previously been reported,[8] no dimerization products were detected.

(12) Cyclization through the Phe-Asn[8] and through the Leu-Ala[13] amide bonds have been reported as less efficient than by formation of Ile-Pro.[8]

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JM030943H