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All-L-Leu-Pro-Leu-Pro: A Challenging Cyclization

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Abstract: In this paper, we report the difficult synthesis of cyclo(Leu-Pro-Leu-Pro). While the cyclization of Leu-Pro-Leu-D-Pro did not cause problems, the all-L-peptide afforded cyclodimer rather than cyclotetrapeptide (cyclomonomer). A first attempt using our reversible backbone substitution methodology failed. However, we were successful in obtaining the desired cyclo(Leu-Pro-Leu-Pro) by decreasing the concentration. The ratio of cyclomonomer to cyclodimer was raised to 1:1.1 using BOP and 1:0.6 using HATU under our high dilution condition. The structures of the cyclopeptides were confidently assigned by electrospray ionization mass spectrometry and NMR. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclotetrapeptides; ring-closure reaction; cyclization; high dilution

Natural cyclic peptides have been extensively studied in recent years, and the literature on cyclopeptide analogues has expanded correspondingly. Peptide cyclization is of great interest for reducing the number of conformations, increasing *in vivo* resistance and enhancing biological properties. Therefore, the development of reliable and convenient methods for the synthesis of cyclic peptides is a continuing concern for peptide chemists.

For the synthesis of such compounds, the yield-limiting step is the cyclization reaction. The various techniques and reagents described in the literature are often unsatisfactory. Unfavourable competition between dimerization and cyclization of the monomer can reduce the yield in cyclomonomer. To decrease unwanted oligomerization, highly dilute conditions are commonly used. However, the slowness of the cyclization reaction increases the *C*-terminal amino acid epimerization-risk, a matter for concern since a peptide cyclization corresponds in that regard to a fragment coupling [1,2]. Sequence permitting, the *C*-activation must be performed at Gly or Pro residues. If all the possible ring-closure sites are chemically equivalent, the choice should be based on other criteria, since it is well known that the cyclization yield depends very much on the cyclization site [3]. The difficulty of obtaining high yields of cyclomonomer is mainly attributable to the planar transoid conformation of the peptide bond, inducing a rigid and extended conformation in the linear precursor. The greatest difficulties are encountered with tetrapeptides which rise to a constrained 12-membered ring. Characteristic structural features can be recognized in many naturally occurring peptides which facilitate cyclization: the presence of D-amino acids (well-known to stabilize turn structures) or *N*-substituted amino acids (Pro or *N*-methyl amino acids), believed to generate a cisoid conformation. In the case of cyclization of all-L-tetrapeptides lacking *N*-substituted amino acids [4,5], the intramolecular cyclization often affords only traces of cyclotetrapeptide, the major product resulting from dimerization. For example, the yield of monocyclization of all-L-tetraphenylalanine is lower than 2% whatever the reagents and conditions used. Some years ago, we proposed the

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introduction of reversible backbone substitutions (RBS) to facilitate the cyclization step by temporary modification of conformational properties [6]. Applying this methodology to tetraphenylalanine, the introduction of a Boc group on the amide bonds improved the yield of cyclization up to 27%. These results corroborate the effect of *N*-substituted amino acids in promoting cyclization. Some differences between peptides containing *N*-methyl amino acid residues and those containing Pro were observed. Whereas tetrasarcosine affords cyclotetrasarcosine in 43% yield (to be compared to a yield of 5% for cyclotetraglycine) [7] cyclotetraproline is not accessible [8]. However, insertion of D-Pro assists the cyclization since cyclo(D-Pro-L-Pro-D-Pro-L-Pro) [9,10] and cyclo(D-Pro-D-Pro-L-Pro-L-Pro) [11] have been synthesized. This is in accordance with the prediction of conformations performed by molecular mechanics calculations [12]. The influence of the *Cα*-configuration in Pro-containing peptides is also illustrated by unsuccessful syntheses [8] of cyclo(Pro-VaL-Pro-Tyr) [13] and the difficult synthesis of cyclo(Pro-Val)$_2$ [14]. Even the attempted synthesis of cyclo(Pro-Gly)$_2$, in which L-Pro residues alternate with Gly residues, which is assumed to promote cyclization, failed [15] since the cyclization of the precursor Gly-Pro-Gly-Pro-*p*-nitrophenyl ester resulted in a mixture of cyclo(Pro-Gly)$_4$ and Pro-Gly diketopiperazine cyclo(Pro-Gly)$_1$. However, when cyclization was performed from Pro-Gly-Pro-Gly-*p*-nitrophenyl ester, few traces of cyclo(Pro-Gly)$_2$ are finally obtained. Indeed the peptide chain fragmentation in diketopiperazine is expected to be minimized by the choice of Pro-Gly sequence instead of Gly-Pro. Pro-Xxx peptide bond is not *cis* allowed whereas the *cis–trans* ratio of the Xxx-Pro peptide bond is strongly impelled by Xxx [16,17]. The relative abundance of *cis*-Pro increases with the steric hindrance of Xxx side chain (i.e. Leu ≫ Ala ≫ Gly). That means that Leu-Pro-Leu-Pro could be expected to be *cis* conformed and hence favourable to intramolecular cyclization. However a cyclization attempt was reported to only afford cyclodimer [8]. This questions previous results [18] claiming the synthesis of cyclo(Leu-Pro)$_2$. In addition, the synthetic compound does not exhibit the cancerostatic activity of the natural product, supporting the doubts raised by Schmidt [8], who imputes the mistake to a wrong mass spectroscopic data interpretation.

Since cyclo(Leu-Pro)$_2$ seems to be a particularly difficult target, we selected this tetrapeptide to test our methodology by reversible backbone substitution to promote cyclization.

THEORETICAL PART

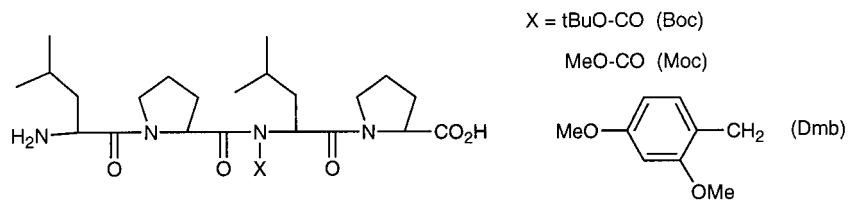
Starting from the linear precursor H-Leu-Pro-Leu-Pro-OH to minimize epimerization, the sole nitrogen open to substitution belongs to the Leu in position 3 (Scheme 1).

Its substitution with a Boc group using the procedure (Boc_2O/DMAP) which succeeded in the case of tetraphenylalanine failed completely. Postulating that the Boc group could be too bulky considering the steric hindrance of the surrounding Pro residues, we shifted from the Boc group to the smaller Moc group. Unfortunately, decarboxylation of the unstable methyle monocarbonate anion occurs instead of the expected reaction (Scheme 2).

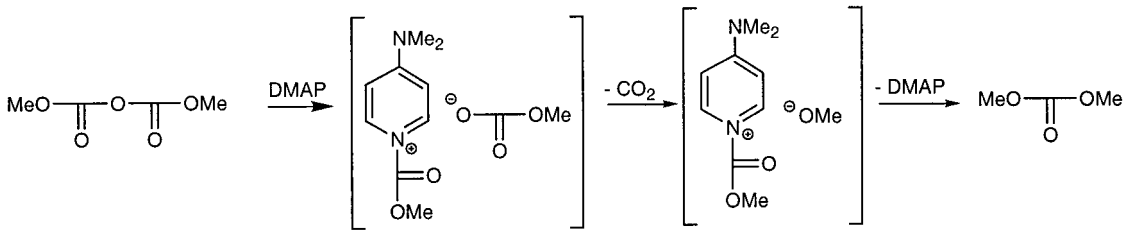
Replacing Moc_2O with methyl chloroformate did not bring any improvement, since no acylated linear peptide was detected. These results are further support to a finding we observed earlier: a peptide bond flanked by two *N*-substituted peptide bonds cannot be alkylated (Cavelier F, Garcia A, Verducci J, unpublished results).

Then we tried to acylate a substituted Leu. We selected the acid-labile and readily accessible (through Schiff base reduction) dimethoxybenzyl group (Dmb) [19].

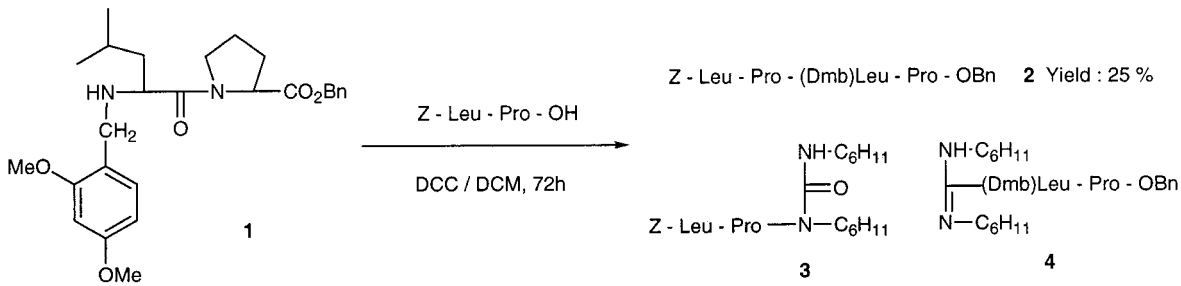
The fragment coupling (Scheme 3) turned out to be especially difficult. Reagents such as BOP, BOP/HOBt or DCC/DMAP did not afford the expected peptide: only acyl chloride activation led to traces.



Scheme 1 *N*-Substitution of H-Leu-Pro-Leu-Pro-OH.



Scheme 2 Decarboxylation of Moc_2O.



Scheme 3 Synthesis of the *N*-substituted linear precursor.

In agreement with the work of Boojamra *et al.* [20], the best result was obtained with DCC without additive, giving rise to the desired peptide **2** (25% yield) in a mixture containing two other products, identified by mass spectroscopy as the *N*-acyl urea **3** and the guanidine **4**.

Cyclization of H-Leu-Pro-(Dmb)Leu-Pro-OH (5)

The terminally deprotected tetrapeptide **5** was obtained by hydrogenolysis of **2**. For the exploration of the cyclization step, we studied the influence of the reagent (BOP, HBTU or DPPA), addition time (from 5 min to 24 h), and reaction temperature (room temperature to 60°C). All cyclization reactions were carried out in DMF with a 10^{-3} M peptide substrate final concentration. Products were investigated by ESI-MS. Cyclodimer and cyclotrimer were induated by the following ions: cyclodimer: $(M+H)^+ = 1141$; $(M+Na)^+ = 1163$; $(M+2H)^{2+} = 571$; $(M+H+Na)^{2+} = 582$; cyclotrimer: $(M+H)^+ = 1711$; $(M+Na)^+ = 1733$; $(M+2H)^{2+} = 856$; $(M+H+Na)^{2+} = 867$.

With the BOP reagent and slow addition of the peptide precursor (24 h), a low intensity ion $(M+Na)^+ = 593$ revealed the presence of *N*-substituted cyclomonomer traces.

Cyclization of H-Leu-Pro-Leu-Pro-OH (7)

To clarify the contribution of the Dmb group, we carried out the cyclization starting from the unsubstituted linear precursor **7**. The first experiment was performed using the BOP reagent, TEA as base, with a rapid addition of **7** to the reaction mixture in DMF at a 10^{-3} M concentration. ESI-MS analysis showed preponderantly the cyclodimer **11**, in agreement with Schmidt's results [8]. The characteristic ions of the trimer and the tetramer were also observed. Since confusion is easily possible [8], a detailed study was necessary to discriminate between monoprotonated and multicharged ions of the same mass as well as monoprotonated ions of aggregates and of cyclopolymers [21]. Cationization with sodium enabled us to differentiate between such ions. A monoprotonated ion exhibited a difference of 22 m/e with the sodium adduct ion, whereas multiprotonated ions and cationized ions were separated by $22/n$ m/e, n being the charge of the ion. Unambiguous characterizations of the cyclopeptides present in the crude cyclization product were achieved by these ion assignments.

Quantification of the various cyclopeptides was carried out by HPLC coupled with mass spectroscopy (LC/ESI-MS). The peak with the shortest retention time, which had a low integration, exhibited the expected mass spectrum expected for the cyclomonomer **10** (Figure 1(a)).

The production of a tiny amount of the cyclomonomer in this preliminary attempt prompted us to investigate other conditions which might favour the intramolecular ring closure. We examined several coupling reagents (BOP, HBTU, HATU) and

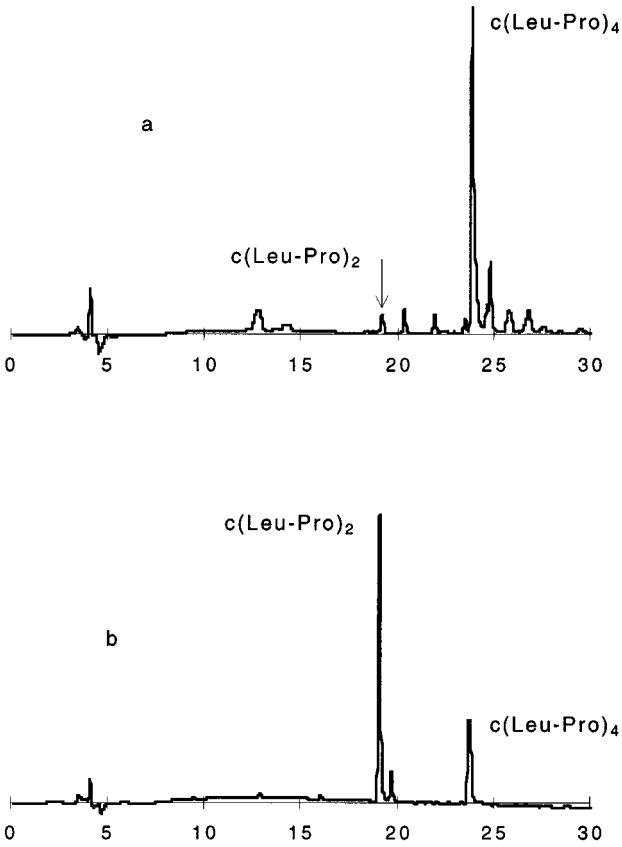


Figure 1 HPLC analyses of the crude cyclization product using the conditions of entry 1 (a) or entry 12 (b) of Table 1.

we added the linear precursor very slowly using a syringe-pump so that the concentration did not exceed 10^{-3} M. Our results are reported in Table 1.

Quite obviously, a 10^{-3} M concentration is not dilute enough to allow monomer formation (entries 1–4). With the BOP reagent, increasing the addition time from 3.5 to 15 h (entries 5–7) raised the monomer:dimer ratio to 1:1.1. Slower addition (entry 8) did not bring further improvement and afforded a complex crude reaction mixture (entry 8). Switching from DMF to DCM as solvent under conditions of entry 7 led to mere traces of cyclomonomer (entry 9).

We had previously observed a rather rapid decomposition of the HBTU reagent under basic conditions. We therefore decided to add the HBTU reagent slowly to preserve the *C*-terminal activation. Using two syringe-pumps to add both the HBTU reagent and the peptide slowly led to a 1:2 ratio (entry 10). Applying these conditions to the BOP reagent (entry 11) resulted in no improvement-compare to entry 7. Finally, the best result was obtained with HATU [22] (entry 12 and Figure 1(b)). This reagent proved to be particularly efficient for ring closure as for the first time the amount of monomer was twice that of the dimer, allowing us to isolate the expected cyclomonomer by preparative HPLC, although the yield was still only 5%.

Table 1 Cyclization Conditions

Entry	Reagent (*n* equivalents)	Time of addition of the peptide	Ratio cyclomonomer:cyclodimer
1	BOP (1.5)	[a]	[b]
2	BOP (10)	[a]	[b]
3	HBTU (1.5)	[a]	[b]
4	HBTU (10)	[a]	[b]
5	BOP (10)	3 h 30	1:3
6	BOP (10)	7 h	1:1.5
7	BOP (10)	15 h	1:1.1
8	BOP (10)	24 h	[e]
9	BOP (10)[c]	15 h	[b]
10	HBTU (10)	15 h[d]	1:2
11	BOP (10)	15 h[d]	1:1.2
12	HATU (10)	15 h[d]	1:0.6

[a] Rapid addition of reagents and peptide.
[b] Cyclomonomer traces.
[c] DCM as solvent.
[d] Prior addition of 3 equivalents of reagent followed by slow addition (15 h) of peptide.and 7 equivalents of reagent using two syringe-pumps.
[e] Reaction mixture too complicated to allow cyclodimer HPLC integration.

Cyclization of H-Leu-Pro-Leu-D-Pro-OH (9)

Although Pro is not easily epimerized, we had to consider this possibility, which would lead to the isomeric cyclo(Leu-Pro-Leu-D-Pro) (**12**), especially as ring closure is preferred between amino acid residues of opposite configuration. This proved to be the case, since cyclization of the H-Leu-Pro-Leu-D-Pro-OH under the conditions of the entry 10 provided the cyclic peptide **12** in a much higher yield (45% after preparative HPLC) than was the case for the all-L-peptide (5% yield). Moreover, the cyclodimer was formed only in very small amount. HPLC analysis of the two epimeric cyclopeptides displayed different retention times and the HPLC profile of the crude reaction proved without doubt that no racemization occurred in the all-L-tetrapeptide cyclization.

NMR Characterization of the Cyclopeptides

Although ESI mass spectroscopy suffered to identify the cyclopeptides, their characterization was completed by NMR.

For cyclo(Leu-Pro-Leu-Pro) (**10**), the NMR spectra (Figure 2) shows the high symmetry of the molecule: signals belonging to the two Leu residues are equivalent, as well as those of the two Pro residues. In particular, only two types of CHα peaks are displayed in the ^{1}H-NMR spectrum, the ^{13}C-NMR spectrum confirmed this symmetry: the carbons of the four peptide bonds gave only two distinct peaks. Replacement of one L-Pro residue with D-Pro (compound **12**) disrupted this symmetry as shown on NMR data (Figure 3). Indeed, the four CHα are individually visible as well as the four carbonyls. The NMR spectra of the cyclodimer **11** (Figure 4) are much more complicated, especially in the CHα region, in agreement with the presence of eight types of CHα as well as different conformers, in contrast to the previous cases.

It is important to note that the chemical shifts of these compounds do not match with those reported by Aracyl *et al.* [18].

Biological Activity Assay

The compounds **10**, **11** and **12** were tested at different concentrations (1, 5, 10, 20, 30, 50, 100 μM) for the antiproliferative effect described by Aracyl *et al.* [18]. Incubation was performed for 24 and 48 h in most cases and once for 66 h, at doses up to 50 μM, on L1210 cells. No antiproliferative effects, even marginal, were observed despite the use of concentrations up to 100 times as great as those previously described [18] to have an antiproliferative effect. Microscopic observation of the cells did not show any modification either in cell shape or size compared to the control cells.

As we proved the structure of compound **10**, we can conclude that the antiproliferative activity observed with the natural extract can not be imputed to it.

In conclusion, cyclization of H-Leu-Pro-Leu-Pro-OH, previously described as not accessible [8], has been investigated. To realize this difficult cyclization, we first tested our methodology by RBS, which proved its efficacy in the synthesis of cyclo(Phe$_4$) using the Boc group [6] and was then successfully applied by Ehrlich *et al.* [4] to a cyclization of a pentapeptide, in which a substitution at position 3 by the Hmb group doubled the yield of cyclomonomer that was obtained. Unfortunately, in the case of H-Leu-Pro-Leu-Pro-OH, substitution with Dmb did not induce cyclization, and the yield of cyclomonomer remained under 1%. This failure could be attributed to conformational problems, which are often directly responsible for the low yield of the ring-closure reaction. As we have previously established [3], the significant factor is the ability of the precursor to adopt a conformation in which cyclization is possible and facile: it appears that in this case *N*-substitution does not help.

However, we were successful in obtaining the desired cyclopeptide, in a mixture along with the cyclodimer, by decreasing the concentration. Because classical highly dilute conditions (10^{-3} M) did not yield the cyclic monomer, we performed slow addition of the precursor by using a syringe-pump. These new conditions, using the BOP reagent, afforded a ratio of cyclomonomer:cyclodimer of 1:1.1. A 5% yield of cyclomonomer was obtained after preparative HPLC. The best result (ratio 1:0.6) was obtained with HATU.

Finally, we prepared the isomeric cyclo(Leu-Pro-Leu-D-Pro) to confirm the chiral integrity of the all-L-cyclopeptide. It was obtained in 45% yield, further evidence for the importance of alternating α-configuration in cyclization.

EXPERIMENTAL PART

Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H-NMR spectra were recorded with a Bruker AC 250 or DRX 400 apparatus. The ESI mass spectra were recorded on a Micromass Platform II quadrapole mass

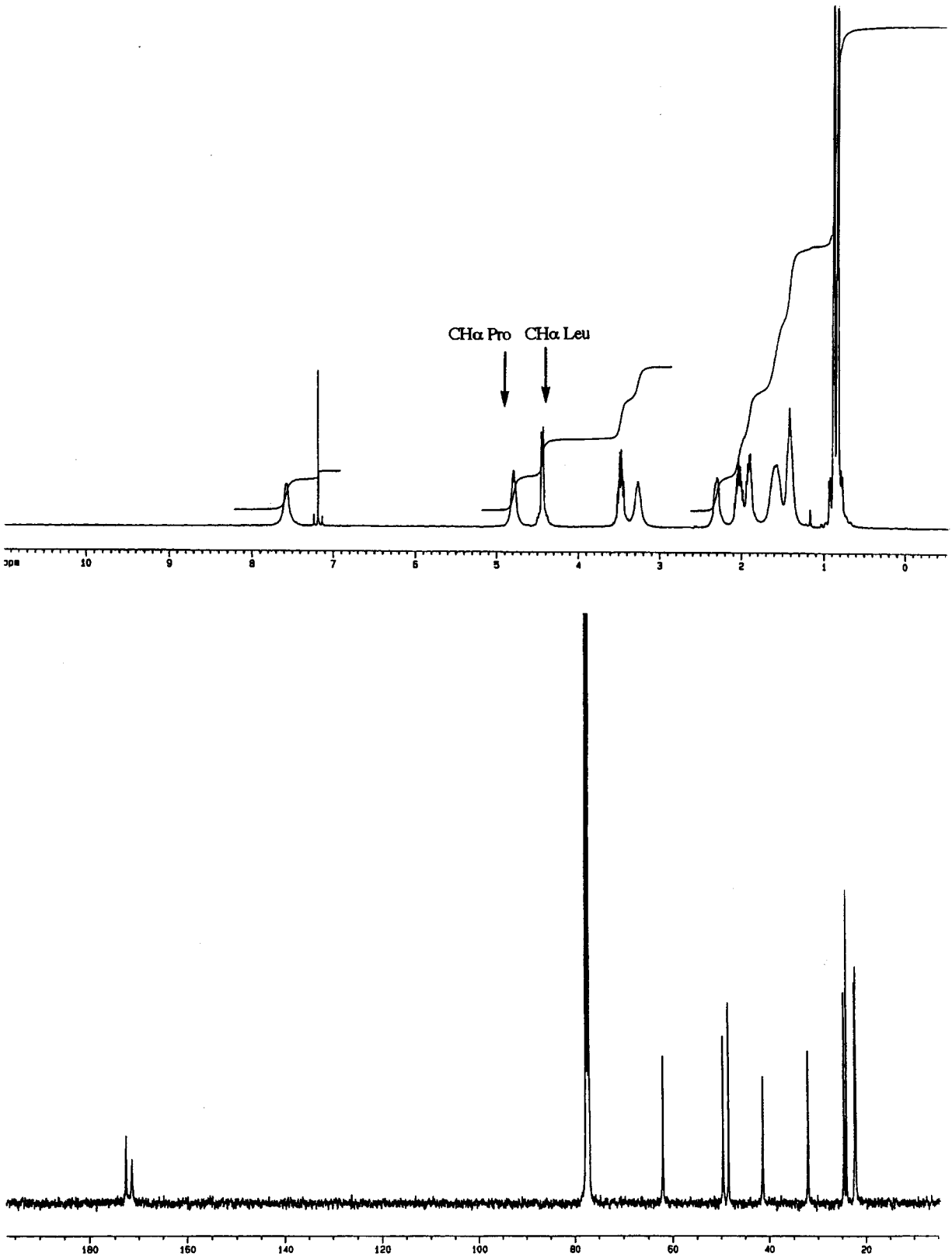


Figure 2 ^{1}H- and ^{13}C-NMR spectra of cyclo(Leu-Pro-Leu-Pro) (**10**).

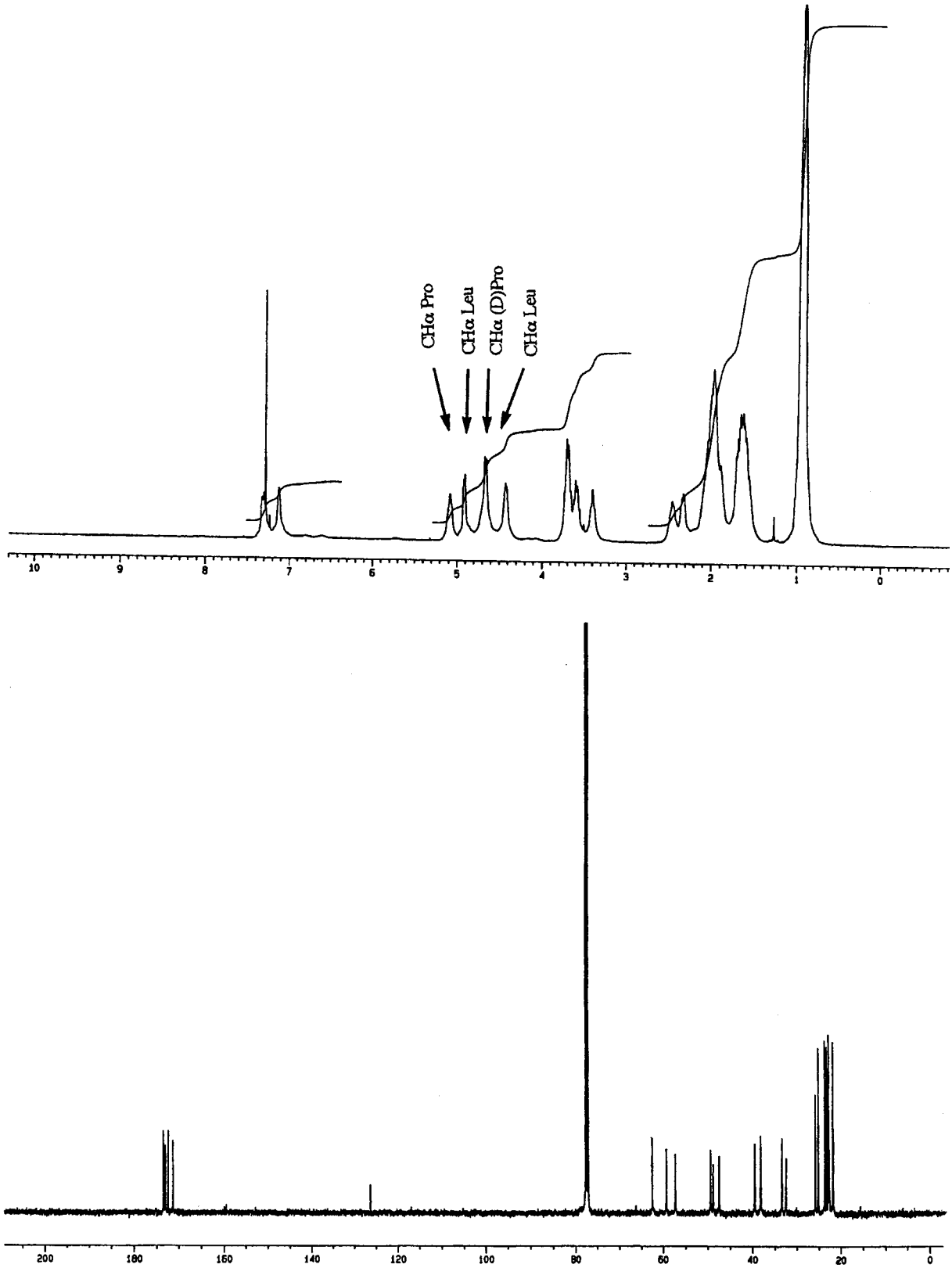


Figure 3 ^{1}H- and ^{13}C-NMR spectra of cyclo(Leu-Pro-Leu-D-Pro) (**12**).

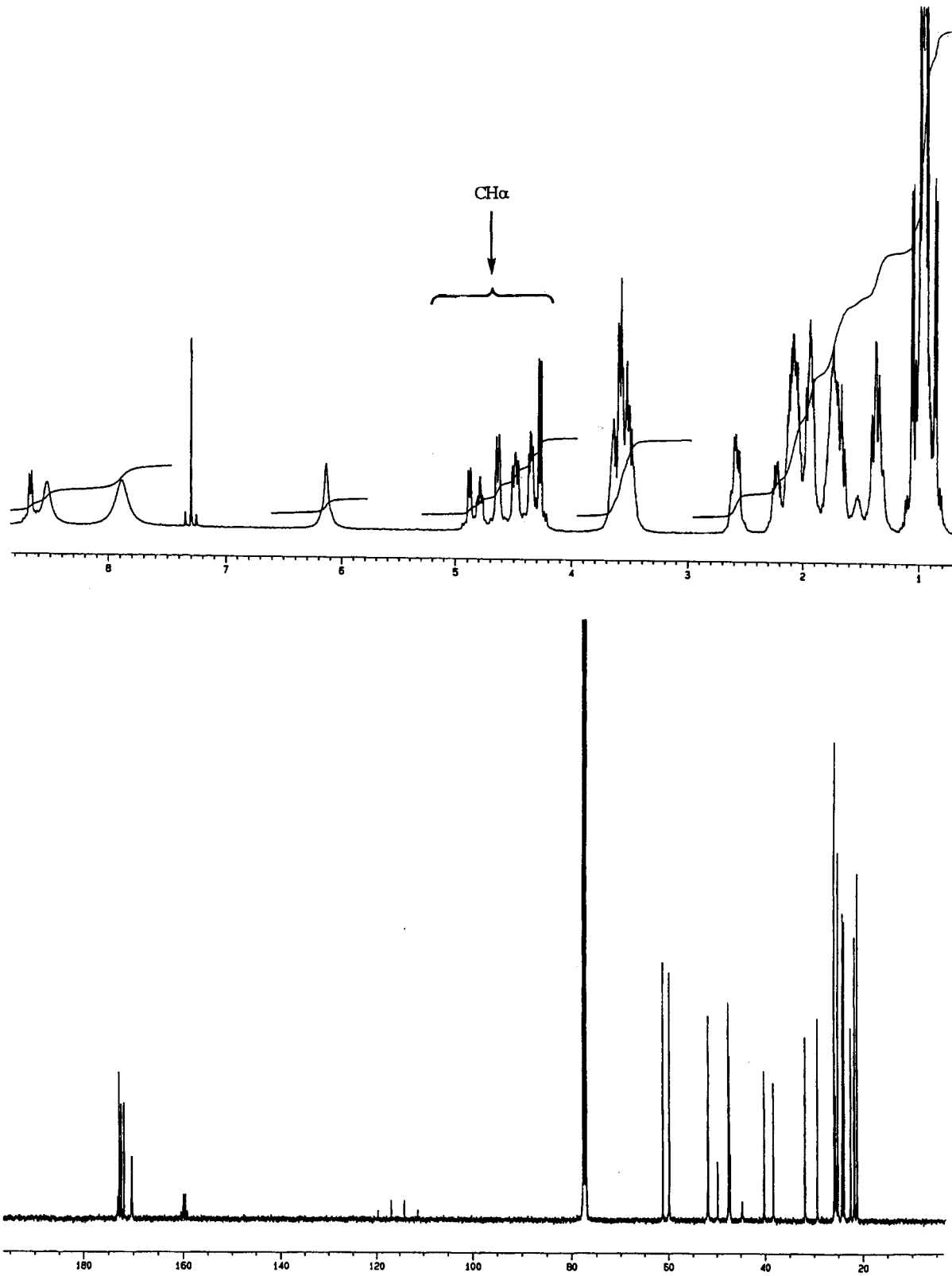


Figure 4 ^{1}H- and ^{13}C-NMR spectra of cyclo(Leu-Pro-Leu-Pro)$_2$ (**11**).

spectrometer (Micromass, Manchester, UK) fitted with an electrospray source coupled with a Waters HPLC. The analytical HPLC analyses were carried out on a Waters Millennium with a Photodiode Array Detector 996, using a reversed phase Nucleosil C_{18}, 5 μ column, i.d. = 4.6 mm, length 250 mm (Macherey and Nagel): the flow rate was 1 ml/min. Preparative HPLC was performed with a Waters PerpLC4000 with a Delta Pack column (40 × 100 mm). The UV 486 detector operated at 214 nm, the flow rate was 50 ml/min. Solvents were: A, water with 0.1% TFA; B, acetonitrile with 0.1% TFA. The gradient conditions were: (I) 90% A, 10% B to 0% A, 100% B in 30 min; (II) 35% A, 65% B to 0% A, 100% B in 35 min; (III) 80% A, 20% B to 45% A, 55% B in 35 min; (IV) 55% A, 45% B to 20% A, 80% B in 35 min.

Water was obtained from a Milli-Q plus system (Millipore), and acetonitrile from Merck. Thin-layer chromatography was performed using Merck silica gel plates 60 F254. Protected amino acids were purchased from Novabiochem.

H-(Dmb)Leu-Pro-OBn (1)

Coupling of Boc-Leu-OH with H-Pro-OBn was performed in DMF using HBTU as reagent and TEA as base. After *N*-deprotection of the dipeptide by 30% TFA in DCM, the crude product (6.2 mmol, 2.5 g) was dissolved in DCM (20 ml). 2,4-Dimethoxybenzaldehyde (6.2 mmol, 1.03 g), $MgSO_4$ (10 g) and TEA (7 mmol, 0.9 ml, pH 8–9) were added. After stirring for 24 h at room temperature then filtration, the organic phase was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. To the residue dissolved in methanol (40 ml) was slowly added $NaBH_4$ (6.8 mmol, 26 mg). After stirring for 30 min at room temperature, water (30 ml) was added and the solution was concentrated under reduced pressure. The resulting aqueous phase was extracted into ethyl acetate, the organic phase was dried over $MgSO_4$ then evaporated to dryness. The residue was dissolved in water (30 ml) and the pH was adjusted to 2 by addition of 6 N HCl. The solution was washed with ether to remove the unreacted aldehyde. To the aqueous solution was added $NaHCO_3$ (pH 8–9): this was followed by ethyl acetate extraction. The organic phase was dried over $MgSO_4$ then concentrated to dryness to give a yellow oil: 1.06 g, yield 55%. R_f (EtOAc) = 0.55. ESI-MS: $(M + H)^+ = 469$. HPLC (gradient I) $t_R = 21.6$ min.

Z-Leu-Pro-(Dmb)Leu-Pro-OBn (2)

To a solution of compound **1** (2.2 mmol, 1 g) and Z-Leu-Pro-OH (2.2 mmol, 0.77 g) (obtained by saponification of the corresponding methyl ester) in DCM (20 ml) was added DCC (2.35 mmol, 0.49 g) at 0°C. The mixture was stirred for 30 min at 0°C then 72 h at room temperature. After filtration to remove the DCU, the solution was concentrated and the residue was dissolved in the minimum amount of ethyl acetate and filtered again. The filtrate was washed successively with 1 N citric acid, water, saturated $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$ then concentrated to dryness. The residue was chromatographed on silica gel (eluent hexane:EtOAc, 2:3).

The tetrapeptide **2** was obtained in 25% yield. M.p. = 65–66°C. ESI-MS $(M + H)^+ = 813$. R_f (EtOAc:hexane, 3:2) = 0.57. HPLC (gradient II) t_R = 16.7 min.

The dipeptide H-Leu(Dmb)-Pro-OBn (**1**) was partially recovered (40% of the starting material) together with small quantities of the *N*-acylurea (**3**) and guanidine (**4**).

TFA, H-Leu-Pro-(Dmb)Leu-Pro-OH (5)

To a solution of compound **2** (1 mmol, 0.812 g) in a *tert*-butanol:water:TFA (3:2:0.01) mixture was added 10% Pd/C (20% by weight) and the suspension was stirred overnight at room temperature under hydrogen at atmospheric pressure. After filtration through celite and concentration, the residue was freeze-dried. The deprotected tetrapeptide **5** was obtained in 98% yield. M.p. = 90–91°C, ESI-MS $(M + H)^+ = 589$. HPLC (gradient I) $t_R = 18.2$ min.

Z-Leu-Pro-Leu-Pro-OBn (6)

To a solution of Z-Leu-Pro-OH (2.7 mmol, 1 g) and TFA, H-Leu-Pro-OBn (2.7 mmol, 1.2 g) in 20 ml of DCM, were added BOP (3.2 mmol, 1.7 g) and TEA (7.1 mmol, 1.2 ml). After stirring for 4 h at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (50 ml) and the solution was washed successively with 1 N citric acid, water, saturated $NaHCO_3$ and water. The organic phase was dried over $MgSO_4$ then concentrated to dryness. The residue was chromatographed on silica gel using ethyl acetate as eluent.

The tetrapeptide **6** was obtained in 78% yield. M.p. = 54–55°C. R_f (EtOAc) = 0.58. ESI-MS: $(M + H)^+ = 663$. HPLC (gradient I) $t_R = 29.4$ min.

TFA, H-Leu-Pro-Leu-Pro-OH (7)

Hydrogenolysis of the protected tetrapeptide under the conditions described for the preparation of **5** afforded the tetrapeptide **7** as its trifluoroacetate salt, in 98% yield. M.p. = 69–70°C. ESI-MS $(M+H)^+ = 439$. HPLC (gradient I) $t_R = 14.2$ min.

Z-Leu-Pro-Leu-D-Pro-OBn (8)

This compound was prepared by analogy with the procedure described for the synthesis of compound **2**. M.p. = 49–50°C. ESI-MS $(M+H)^+ = 663$. R_f (EtOAc) = 0.67. HPLC (gradient I) $t_R = 26.4$ min.

TFA, H-Leu-Pro-Leu-D-Pro-OH (9)

Hydrogenolysis of compound **8** afforded **9** quantitatively. M.p. = 62–63°C. ESI-MS $(M+H)^+ = 439$. HPLC (gradient I) $t_R = 15.5$ min.

Cyclization of 5

The tetrapeptide **5** was added to a DMF solution containing the coupling reagent (final concentration 10^{-3} M). Various reagents were used (BOP, HBTU, DPPA), different stoichiometries (1.5 or 10 equivalents) in various conditions (rapid addition of the peptide or slow addition over period of 2, 6 or 15 h, at room temperature or 60°C). The solvent was then concentrated under reduced pressure and the residue was dissolved in ethyl acetate. After washing (1 N citric acid and saturated $NaHCO_3$) and concentration to dryness, the crude product was analysed by ESI-MS (see results given in the text).

Cyclization of 7

General Procedure: Optimized Conditions. The tetrapeptide **7** (20 mg) was allowed to react with the coupling reagent under the conditions described in Table 1. After concentration, the residue was dissolved in a methanol:water (4:1) mixture (10 ml) and treated for 4 h with mixed-bed resin (Amberlite MB3) (5 g by mmol). After filtration and evaporation, the residue was generally analysed by HPLC and ESI-MS, but sometimes by LC-MS.

Cyclo(Leu-Pro-Leu-Pro) (***10***). R_f (DCM:MeOH, 93:7) = 0.63. HPLC (gradient I) $t_R = 19.7$ min. ESI-MS: $(M+H)^+ = 421$, $(M+Na)^+ = 443$.

Cyclo(Leu-Pro-Leu-Pro)$_2$ (***11***). R_f (DCM:MeOH, 93:7) = 0.48. HPLC (gradient I) $t_R = 24.2$ min. ESI-MS: $(M+H)^+ = 841$, $(M+Na)^+ = 863$.

Preparative Conditions. The tetrapeptide **5** (200 mg) was cyclized following the conditions of entry 10 or entry 1 of Table 1. After mixed-bed resin treatment, the crude product was purified by preparative HPLC.

Conditions of entry 10: 90 mg of crude product were obtained and then purified by preparative HPLC (gradient III), leading to: cyclo(Leu-Pro-Leu-Pro) (**10**), 10 mg (yield 5%); cyclo(Leu-Pro-Leu-Pro)$_2$ (**11**), 20 mg (yield 10%).

Conditions of entry 1: 92 mg of crude product were obtained and then purified by preparative HPLC (gradient IV), leading to: cyclo(Leu-Pro-Leu-Pro)$_2$ (**11**), 32 mg (yield 16%).

Cyclization of 9

Cyclization of compound **9** under the conditions of the entry 10 provided after workup of cyclopeptide **12**. R_f (DCM:MeOH, 93:7) = 0.44. HPLC (gradient I) $t_R = 18.4$ min. ESI-MS: $(M+H)^+ = 421$, $(M+Na)^+ = 443$. Preparative HPLC (gradient III) afforded **12** in 45% yield.

Biological Activity Assay

Cell and Cell Culture. Lymphocytic leukemia cells L1210 were in exponential phase in RPMI medium supplemented with 10% foetal calf serum, 2 mM glutamine, penicillin (200 U/ml) and streptomycin (50 μg/ml) at 37°C in a 5% CO_2 atmosphere. Incubations with the different peptides at the appropriate concentration were performed in a 12-well plate (Nunc). 80000 cells per well in medium culture (2 ml) were used for each experiment. Cells were counted with a cell counter (Coulter Coultronics) after incubation at 37°C for 24 or 48 h. Cell counts were performed twice for each well after homogenization of the culture medium.

Cellular Incubation of Peptides. All peptides were first resuspended in 2:1 H_2O:MeCN to ensure full solubilization before accurate concentration determination for each peptide by amino acid analyses after acidic hydrolysis in 5.6 M HCl for 24 h at 110°C. Amino acid analyses were run on a Beckman amino acid analyser. Results were in agreement with the expected amino acid composition. The required volume of peptide solution was taken and solvent was fully evaporated with a Speed Vac unit at room temperature. Finally, peptides were resuspended in DMSO (25 μl) before the experiment. The appropriate volume (between 0.1 and 10 μl, depending on the required peptide concentration) was taken and adjusted to 10 μl with DMSO. Two millilitres of fresh

culture medium was then added to the peptide solution just prior to incubation with the cells (final DMSO concentration of 0.5% by volume). Full solubilization of the peptides in DMSO was assessed by performing HPLC chromatography. A mock assay was performed by incubating cells with 0.5% DMSO in a fresh culture medium. Experiments were made three times in duplicate for all peptides at each concentration.

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