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Destruxin Analogues : Depsi Peptidic Bond Replacement by Amide Bond

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Abstract : In order to determine the importance of the depsi bond present in natural destruxins, we have investigated the replacement of this ester bond by an amide bond, leading to a new family of analogues. Synthesis of six specific members of this new class of compounds is reported. Since none of these cyclopeptides showed any biological activity, we undoubtedly proved that the depside group is a requisite for insecticide effect. Copyright © 1996 Elsevier Science Ltd

As part of a programme designed towards the synthesis of cyclopeptides, we are concerned with the synthesis of natural cyclohexapeptides in which one ester bond takes the place of an amide bond, denominated cyclodepsipeptides. More particularly, we are interested in destruxins, a cyclodepsipeptide family possessing insecticide properties[1-3]. Previously, we outlined a general strategy for the preparation of destruxin analogues, which was then applied to the synthesis of D-Lac-6 destruxin E[4]. Production of analogues is valuable to help the determination of the structural requirements which condition their activity level. A comparative study on insecticide activity and toxic effects of natural destruxins as reference compounds and different analogues should be of assistance in further understanding the features playing an essential role in biological activity. In order to examine the importance of the depsi bond more precisely, we decided to investigate the replacement of this ester bond by an amide bond.

A depside bond adds obstacles to peptide synthesis because :
i - an ester bond is more difficult to form than an amide bond,
ii - an ester bond is chemically more fragile than an amide bond,
iii - the presence of an ester bond restricts the choice of selective protecting group of the terminal carboxylic function for the synthesis strategy,
iv - the availability of optically pure α-hydroxy acids is not as large and immediate as for α-amino acids.

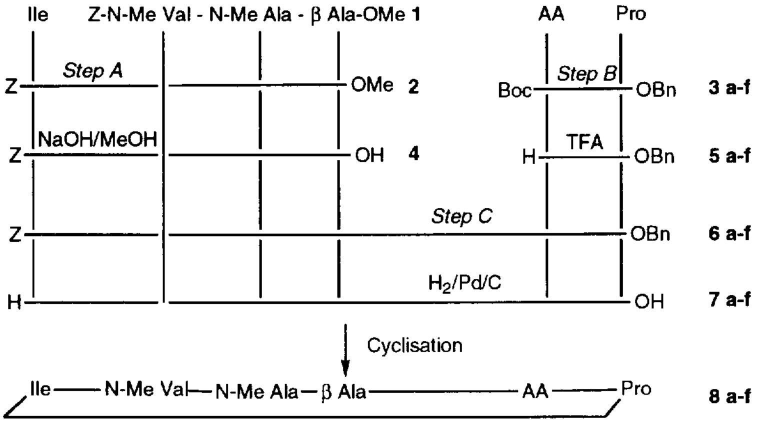
Bearing these considerations in mind, ester replacement implies simplification of synthesis and it appears very important to establish whether or not the depside group is a critical structure required for efficiency and specificity.

N-Disubstituted amide group better mimics ester group because of their decreased double bond character and associated conformational rigidity. Consequently, to leave the conformation of the resulting cyclopeptide as much as possible unaffected, the α-hydroxy acid has to be replaced by a N-Me amino acid.

In order to obtain a meaningful comparison of biological activity, we had to choose the side chain of N-Me amino acid identical to that of a known active destruxin. In the first instance, we selected the N-MeAla to be compared with the active Lac-6 destruxin E[4]. Secondly, we used the N-MeNva, corresponding to the active dihydro destruxin A, which could be obtained by the double bond hydrogenation of the natural destruxin A. In order to investigate the relevance of the N-methylation to the activity, we also scanned the N-unsubstituted Nva. Finally, both amino acids with D and L configuration were used for the synthesis of destruxin analogues to assess the role of the configuration in the biological activity.

Synthesis strategy.

Following our previously reported strategy[4], the tetrapeptide Ile-N-Me Val-N-MeAla-βAla was chosen as the basic starting compound to perform preparation of other six-membered analogues (Scheme 1).



AA	(L) N-Me Ala	(D) N-Me Ala	(L) N-Me Nva	(D)N-Me Nva	(L) Nva	(D) Nva
Number	a	b	c	d	e	f

Scheme 1 : Synthesis strategy

However, when the above tetrapeptide was prepared using the Boc strategy, the acidic conditions (TFA) required for the last cleavage step led to partial degradation of the peptide. Similar degradations have previously been observed[5] and were attributed to the succession of several N-disubstituted amide bonds, which are easily hydrolysable. In our case, since the chain contains two imino acids already, introduction of a third one (N-MeAla or N-MeNva) could increase even more this unwanted hydrolysis. Therefore, we switched from the Boc to the Z protecting group, labile under mild hydrogenolysis conditions.

The use of the Z protecting group provides an additional advantage : the coupling of Z-Ile with the tripeptide **1** (step A) using BroP[6] or BOP-Cl[7-12], occurred in 85% yield, while the bulkier Boc-Ile reacted in 50% yield at best.

The lengthening of the chain to the final hexapeptide linear precursor was effected by fragment condensation. First, the N-methylation of AA was performed according to Benoiton's method[13]. While the reaction was achieved in 80% yield starting from Boc-Ala, steric hindrance of Boc-Nva hampered the complete introduction of the methyl group. Thus, the N-substitution was carried out on Z-Nva following Freidinger's method[14] and the Z group was changed for the Boc group afterwards. This four step-synthesis occurred in higher overall yield (75%). Then, the suitably protected AA-Pro had to be prepared (step B) before being coupled with the tetrapeptide (step C). To obtain the free hexapeptide ready for cyclisation, it is convenient if both terminal protecting groups of the final sequence are cleaved in one step under mild conditions. Consequently, the benzyl group appeared to be the most appropriate for the protection of the C-terminal proline, as it is removable by hydrogenolysis, like the Z protecting group.

Steps optimisation and control of racemisation.

During our previous study[4], we showed that the tripeptide **1** could be obtained without racemisation. In the following steps, we monitored the racemisation rate while optimising the yields by a careful selection of reagents.

Step A. This coupling step between the tripeptide **1** and Z-Ile was achieved using the DCC/DMAP reagent, known to lead to highly racemised products. This allowed us to generate both diastereoisomers ((L) Ile and (D) *allo* Ile containing tetrapeptides) in order to determine the optimum HPLC conditions displaying clearly the two separated compounds. The resulting mixtures from this reaction using other coupling reagents were then analysed under the same optimum HPLC conditions to establish the respective racemisation rate. Results are gathered in Table 1. These results showed that the best compromise between yield and racemisation rate could be obtained with the BOP-Cl reagent.

Table 1 : Optimisation of Coupling Step A

Reagent	Yield %[a](time)	% (D)*allo* Ile[b]
BOP	46 (20h)	undetermined
BroP	86 (16h)	11.6
BOP-Cl	85(16h)	0.7

a) determined after purification by chromatography on silica.

b) absorption coefficients of the two diastereoisomers are supposed to be identical.

Step B. The fragment Boc-AA-Pro-OBn was prepared in good yields using the BroP reagent (Table 2). The absence of racemisation was confirmed by NMR and HPLC, (both L,D and L,L diastereoisomers had been previously prepared to determine the respective retention times).

Table 2 : Synthesis of Boc-AA-Pro-OBn **3a-f**

Compound	Yield %[a,b]	Reaction Time[b]
3a	90	14h
3b	80	24h
3c	75	16h
3d	76	20h
3e	97	20h
3f	96	20h

Table 3 : Synthesis of **6a-f**

Compound	Yield %[a,b]	Reaction Time[b]
6a	71	24h
6b	82	12h
6c	70	16h
6d	73	16h
6e	85	16h
6f	85	16h

a) determined after purification by chromatography on silica.

b) 1.2 equivalents of BroP and 1.2 equivalents of Boc-AA for 1 equivalent of Pro-OBn.

Step C. The coupling of the two peptidic moieties was carried out in good yield with the BroP reagent. Here, no risk of racemisation was present, due to the nature of the involved C-terminal amino acid, *i.e.* the β-alanine. Results are gathered in Table 3.

Cyclisation.

Numerous protocols can be found in the literature, but this survey does not allow to conclude that one reagent is by far superior to the others. The rare comparative studies[15-19] on ring-closure of a single precursor do not lead to results which can be generalised to any peptides. The observed coupling efficiency of different reagent appears to be sequence-dependent and an adaptation of the conditions is often required for each kind of compound. Nevertheless, among all tested reagents, DPPA[20] appears to be of considerable use to address this question of small peptides ring-closure. Hence, we decided to scrutinise in more details cyclisation with DPPA in the case of the destruxin family, and more particularly to examine any temperature effects. Optimization studies were performed on the linear precursor **7b**, in DMF, under high-dilution conditions (10^{-3}M) to prevent cyclodimerization, leading to the cyclopeptide **9b**.

Experimental analysis. Cyclisation yields were determined by HPLC using an internal standard (Boc-Leu-Phe-OMe) which allowed very small scaled experiments. A gradual addition of **7b** within 15h by means of a syringe-pump into a solution containing DPPA, the base and the internal standard, further increase dilution. The reaction is monitored by HPLC, by regularly injecting aliquots of the reaction mixture. Unfortunately, the amount of DMF thus injected masks detection of compounds eluted afterwards. We overcame this problem by using the back-flush technique. Such system involves two reversed phase Nucleosil C18 columns linked together with a valve Rheodyne®. The first column is only 5 cm long (SC) while the second one is 25 cm (LC) as usually. At first, the long column (LC) is isolated and the injection goes through the small column (SC), which retains organic compounds, as cyclic compounds and internal standard, whereas solvents and polar derivatives are eluted. In a second step, both columns are connected and organic compounds are transferred with a back-flush elution from SC to LC, where they could be separated using a more polar solvents mixture. This HPLC technique allows accurate and immediate measurements of yields, consuming tiny amounts of linear peptides, and avoiding tedious purification of resulting cyclopeptides.

Cyclisation with DPPA. While low temperatures (-30 to -20°C) were advised in the first utilisations of DPPA during pre-activation and reaction[21,22], trends tend to moderate temperatures (0 to 5°C)[15,16,23,24]. Some authors claimed good results even at room temperature without detecting Curtius transposition.[25,26]

Therefore, we carried out the same ring-closure reaction, varying the temperature. Results gathered in table 4 indicate that :

- firstly, low temperatures for pre-activation step slow down the reaction, thus favouring intermolecular condensations.
- secondly, increasing temperature both promotes the expected ring-closure reaction and reduces cyclodimerization.

These observations designate the linear peptide activation to be the limiting step, rather than the ring closure reaction. This bimolecular reaction between the peptide and the reagent depends on both temperature and concentration. High dilution aims at separating activated peptidic chains, favouring intramolecular ring-closure. On the other hand, these conditions are prejudicial to the activation. Therefore we tested the ring-closure reaction without the pre-activation step, increasing the reagent excess from 2.5 to 10 eq. to balance dilution effect on the activation rate.

Table 4 : Temperature Effect on Cyclisation Yield

Entry	Temp. (°C)	Temp.(°C)	Yield (%)					
			8b			9b		
	Pre-activation	Cyclisation	2h#	24h#	48h#	2h#	24h#	48h#
1	-30	-15	-	-	15	-	-	15
2	-20	-5	4	14	20	0	12	16
3	-20	20	4	18	28	0	4	10
4	0	40	7	25	30	0	8	10
5	0	60	7	44	52	0	5	7
6	60		42	57	57	0	2	-

after the end of the addition.

Results gathered in table 5 are in agreement with our hypothesis : increasing both temperature and reagent quantity favour the activation reaction, leading to cyclomonomer in good yield. Comparing entries 9 versus 10, we verified that a slow addition of linear precursor avoids cyclodimer formation.

Table 5 : Stoichiometry Variation on Cyclisation Yield

Entry	Temp. (°C)	DPPA	Yield (%)			
			8b		9b	
	Reaction	(eq.)	2h#	24h#	2h#	24h#
7	20	2.5	16	29	3	8
8	20	10	56	57	0	0
6	60	2.5	42	57	0	2
9	60	10	72	73	0	0
10*	60	10	60	61	7	8

after the end of the addition.

*without the syringe-pump, the linear precursor was added at the same time as the reagent and the base, in DMF, previously heated at 60°C.

These conditions are not common with this reagent. Recently, Bailey and Crofts[25] claimed good results using 10 eq. of DPPA at room temperature. But higher temperature are usually employed with active esters[27].

Entries 8 and 9 in table 5 proved the ring-closure reaction to be rapid after the end of the precursor addition, whereas long reaction times are often recommended (3 days[16,23] or even 4-5 days[17,24]).

The resulting cyclopeptide was characterised by mass spectrometry (FAB) and ^1H NMR after HPLC purification. In these conditions, neither side products resulting from Curtius transposition, nor cyclodimer **9b** have been observed, as evidenced by HPLC and MS.

These optimised conditions (10 eq. DPPA, 60°C) were then applied to the ring-closure of linear precursors **7a-f** in preparative quantities. All resulting cyclopeptides **8a-f** were isolated by silica chromatography, then further purified by preparative HPLC and characterised by high resolution mass spectroscopy (FAB) and ^{1}H NMR.

Biological activity.

Finally, the six destruxin analogues thus obtained were used in biological activity tests, performed both with L6 larvae of the lepidopteran insect *Galleria mellonella*[28] and Sf9 insects cells[29]. No insecticide effect was observed, being strong evidence that the depside bond plays a crucial role in the activity.

Conclusion.

Starting from a peptidic linear precursor of destruxin, we optimized the critical cyclisation step. Using 10 eq. of DPPA at 60°C in DMF under high-dilution conditions, the ring-closure reaction occurred in a remarkably high yield (73%). Applying these conditions allowed us to prepare six analogues in which the depside bond is replaced by a primary or secondary amide bond. Unfortunately, none of these cyclopeptides exhibit any biological activity. We therefore proved undoubtedly that the depside group is a requisite for insecticide effect.

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EXPERIMENTAL SECTION

Melting points were determined with a Büchi apparatus and were uncorrected. ^{1}H NMR spectra were recorded with a Bruker AC 250 apparatus. The mass spectra were realized with a Jeol DX 300 apparatus, using xenon in the FAB mode, in glycerol (G), thioglycerol (GT) or nitrobenzyl alcohol (NBA). High resolution mass spectra were determined with a double focusing mass spectrometer Jeol SX102 using glycerol as reference. The HPLC analyses were carried out on a Waters apparatus (two pumps 510, an UV detector model 484 and a Maxima 820 station for data acquisition). The analytical columns were a reversed phase Nucleosil C_{18}, 5μ, i.d.=4.6 mm, length 50 or 250 mm (Société Française de Chromatographie Colonne. The preparative column was a reversed phase Nucleosil C_{18} ,5μ, i.d.=10 mm, length 250 mm (Interchrom). The detector operated at 214 nm; the flow was 1 ml/min under analytical conditions and 3.7 ml/min for preparative runs. Water was obtained from Milli-Q plus system (Millipore), methanol from Fisons FSA and acetonitrile from Merck. Thin-layer chromatography was performed using Merck silica-gel plates 60 F254.

General procedures:

Coupling using BOP or BroP : To a cooled (0°C) solution of N-deprotected peptide (or its trifluoroacetate salt) (10 mmol), N-protected amino acid (12 mmol) and BOP or BroP (12 mmol) in CH_2Cl_2 (10 ml), DIEA (30 mmol, 5.25 ml in the case of amino-free peptide or 40 mmol, 7 ml in the case of peptide salt) were added dropwise. After being stirred for 30 min at 0°C then 20 h at room temperature, the reaction mixture was concentrated under reduced pressure and the resulting residue dissolved in EtOAc (250 ml). If necessary, the solution was filtered, then washed successively with aqueous $KHSO_4$ 0.1N (3x100 ml) and saturated $NaHCO_3$ (3x100 ml). Evaporation of the dried ($MgSO_4$) organic phase gave the expected compound, which was purified by chromatography on silica.

Coupling using BOP-Cl : To a cooled (0°C) solution of the N-protected amino acid (12 mmol) in CH_2Cl_2 (10 ml), DIEA (12 mmol, 2.1 ml) and BOP-Cl (12 mmol) were added. After stirring for 10 min at 0°C, C-protected peptide (or amino acid) (10 mmol) dissolved in CH_2Cl_2 (20 ml) and DIEA (12 mmol, 2.1 ml) (24 mmol in the case of hydrochloride or TFA salt) were added. After being stirred overnight at room temperature, the reaction mixture was washed with 1N aqueous citric acid, saturated aqueous $NaHCO_3$ and water. Evaporation of the dried ($MgSO_4$) organic layer afforded the expected compound, which was purified by chromatography on silica.

Internal standard : Boc-Leu-Phe-OMe. Boc-Leu (15 mmol, 3.74 g) and HCl,Phe-OMe (15 mmol, 3.24 g) were coupled with the BROP reagent according to the general procedure to afford the crude compound, which was purified by chromatography on silica. The relevant fractions yielded the expected dipeptide (5.5 g) in 93% yield as a white solid.

m.p. 83-85 °C (Lit.[30] : 86-88°C). R_f=0.50 (EtOAc/CH_2Cl_2, 10/90). HPLC (CH_3CN/H_2O 70/30) retention time=6.7 min. MS FAB(+) (NBA) $(M+H)^+$=393. ^1H NMR ($CDCl_3$) δ (ppm) 0.9 -1.0 (dd,J=3Hz,J=6Hz, 6H,2CH_3); 1.5 (s,9H,tBu); 1.6-1.8 (m,2H,$CH_2\beta$ Leu); 3.2 (d,J=6Hz,2H,$\underline{CH_2}$-C_6H_5); 3.8 (s,3H,O-CH_3); 4.2-4.3 (m,1H,CHα); 4.8-4.9 (m,1H,CHα); 5.0-5.1 (m,1H,NH); 6.6-6.7 (m,1H,NH); 7.1-7.4 (m,5H,C_6H_5).

Boc-(D)NMeAla or Boc-(L)NMeAla : Benoiton's method[13]. 95% yield. m.p. 92-93 °C (Lit.[13], 93-94); ^1H NMR ($CDCl_3$) δ (ppm) 1.4 (d,J=6Hz,3H,CH_3); 1.5 (s.,9H,tBu); 2.9 (s,3H,N-CH_3); 4.7-4.8 (m,1H,CHα); 10.1 (s,1H,COOH).

Z-(D)NMeNva or Z-(L)NMeNva : A mixture of Z-(D)Nva or Z-(L)Nva (60 mmol, 15 g), paraformaldehyde (14.4 g, 180 mmol) and *p*-toluenesulfonic acid monohydrate (1.5 g, 78 mmol) in toluene (750 ml) was heated to reflux for 1 h with azeotropic elimination of water. After cooling to room temperature, the mixture was washed with 1N NaOH (3x200 ml). Evaporation of the solvent from the dried ($MgSO_4$) organic phase gave the oxazolidinone intermediate. Upon addition of hexane, a colourless oil formed in 85-95 % yield. ^1H NMR ($CDCl_3$) δ (ppm) 0.9-0.95 (m,3H,CH_3); 1.30-1.40 (m,2H,$CH_2\gamma$); 1.80-1.90 (m,2H,$CH_2\beta$); 4.35 (t,J=5Hz,1H,CHα); 5.25 (s,2H,$\underline{CH_2}$-C_6H_5); 5.25 and 5.55 (2d,J=5Hz,2H,N-CH_2-O); 7.45 (s,5H,C_6H_5). To a solution of oxazolidinone (50 mmol, 13.15 g) in a mixture CH_2Cl_2-TFA (1:1) (300 ml), triethylsilane (40 ml, 250 mmol) was added. After stirring for 20 h at room temperature and evaporation of solvents, added CH_2Cl_2 (ca 5ml) was removed *in vacuo* (three times) to afford the title compound as a colourless oil in 90% yield for both L and D isomers. ^1H NMR ($CDCl_3$) δ (ppm) 0.95 (t,J=7Hz,3H,CH_3); 1.25-1.35 (m,2H,$CH_2\gamma$); 1.85-1.95 (m,2H,$CH_2\beta$); 2.95 (s,3H,N-CH_3); 4.75-4.85 (m,1H,CHα); 5.2 (s,2H,$\underline{CH_2}$-C_6H_5); 7.4 (s,5H,C_6H_5).

Boc-(D)NMeNva or Boc-(L)NMeNva : Z-(D)Nva or Z-(L)Nva (30 mmol, 8 g), was dissolved in methanol (1000 ml). Then, ammonium formate (60 mmol, 3.8 g) and Pd/C 10% (30% in weight) were added. The reaction mixture was stirred at room temperature for 3 or 4 h monitored by TLC. After filtration through a pad of Celite®, concentration of the filtrate gave in quantitative yield the amino-free N-MeNva, which was then dissolved in a mixture of 1N NaOH/dioxane 1/1 (100 ml). After addition of Boc_2O (45 mmol, 9.8 g), the reaction mixture was stirred overnight. After removal of dioxane, the residual aqueous solution was washed with EtOAc (3x20 ml), acidified to pH 3 using 1N HCl and then extracted into EtOAc (3x30 ml). The combined organic layers were dried over $MgSO_4$ then evaporated *in vacuo* to afford the expected compound as an oil in 80% yield. ^1H NMR ($CDCl_3$) δ (ppm) 1.0 (t,J=7Hz,3H,CH_3); 1.25-1.35 (m,2H,$CH_2\gamma$); 1.5 (s.,9H,tBu); 1.850-1.90 (m,2H,$CH_2\beta$); 2.80 (s,3H,N-CH_3); 4.70-4.80 (m,1H,CHα).

Z-NMeVal-NMeAla-βAla-OMe (1). Tripeptide **1** was prepared according to the procedures previously described[4].

Z-Ile-NMeVal-NMeAla-βAla-OMe (2) (Step A). The tripeptide **1** (2.3 mmol, 1 g) was dissolved in methanol (10 ml) and cooled to -30°C. Then, ammonium formate (30 mmol, 1.9 g) and Pd/C 10% (30% in weight) were added. The reaction mixture was stirred at room temperature for 3 or 4 h monitored by TLC. After filtration through a pad of Celite®, concentration of the filtrate gave the amino-free peptide, used in the coupling step with Z-Ile (2.8 mmol, 0.75 g) with BroP or BOP according to the general procedure. The residual oil (2.1 g) was purified by chromatography on silica (eluent AcOH/CH_2Cl_2, 35/65 then 50/50). The relevant fractions yielded **(2)** as a colourless viscous oil (1,07 g, 85%). TLC: R_f=0.51 (EtOAc/CH_2Cl_2, 50/50). HPLC (CH_3CN/H_2O 55/45) retention time=9.83 min. MS FAB(+) (GT) $(M+H)^+$=549. ^1H NMR ($CDCl_3$), (*=2 conformers) δ(ppm) 0.78-0.98 (m,12H,4CH_3 Val and Ile); 1.23 and 1.38 (2d*,J=7Hz,3H,CH_3 Ala); 1.4-1.9 (2m,3H,CHβ and CH_2 Ile); 2.3-2.4 (m,1H,CHβ Val); 2.4-2.5 (m,2H,CH_2-CO βAla); 2.75-3.35 (4s*,6H,2N-CH_3); 3.45-3.55 (m,2H,CH_2-N βAla); 3.55 and 3.69 (2s*,3H,OCH_3); 4.48-4.7 (2m*,1H,CHα Ile); 5.0-5.18 (m,3H,CHα Ala and $C\underline{H}_2\text{-}C_6H_5$); 5.19-5.47 (2m*,1H,CHα Val); 6.05 and 6.5 (2d*,J=10Hz,1H,NH Ile); 7.32 and 7.42 (2s*,5H,C_6H_5); 7.35-7.45 and 7.85-7.95 (2m*,1H,NH βAla).

Z-(D,L)Ile-NMeVal-NMeAla-βAla-OMe (2'). The tripeptide **1** (0.2 mmol, 870 mg) was deprotected as described above and dissolved in CH_2Cl_2 (5 ml). To this cooled (0°C) solution, Z-Ile (0.22 mmol, 65 mg) and DCC (0.22 mmol, 46 mg) were added in CH_2Cl_2 (10 ml). After stirring for 72 h at room temperature, the reaction mixture was filtered and the filtrate concentrated under reduced pressure. The residue was taken up with ethyl acetate and stored at 0° for 1 h. The precipitated DCU was filtered and the filtrate was washed with 1N aqueous citric acid, saturated aqueous $NaHCO_3$ and water until neutral pH. Evaporation of the dried ($MgSO_4$) organic layer afforded the title compound, showing one single spot in TLC (R_f=0.51, EtOAc/CH_2Cl_2 50/50) and two picks in HPLC (CH_3CN/H_2O 55/45) retention times=9.83 min and 12.25 min. MS FAB(+) (GT) : $(M+H)^+$=549. ^1H NMR in $CDCl_3$ was very close to that of the single epimer **2**, but the comparison of the shifts of the 4 singlets corresponding to the N-methyl groups permitted the characterisation of the two epimers (Table 6).

Table 6 : N-Me ^1H NMR Chemical Shifts

Isomers	δ s1 (ppm)	δ s2 (ppm)	δ s3 (ppm)	δ s4 (ppm)
L-L-L	3.250	3.080	2.970	2.755
D-L-L	3.250	3.030	2.915	2.780

Boc-AA-Pro-OBn (3a-f) (Step B). The dipeptides were prepared using BroP as the reagent and were purified by chromatography and obtained as yellow oils. Yields are reported in Table 2, according to different Boc-AA used as starting material.

Boc-NMe-(L)Ala-Pro-OBn (3a). R_f= 0.55 (EtOAc/CH_2Cl_2 30/70); HPLC (CH_3CN/H_2O 70/30) retention time=12 min; MS FAB(+) (GT) $(M+H)^+$=391. ^1H NMR ($CDCl_3$), δ(ppm) 1.27 (d,J=7Hz,3H,CH_3 Ala); 1.48 (s,9H,tBu); 1.88-2.25 (m,4H,2CH_2); 2.75 (s,3H,N-CH_3); 3.4-3.75 (m,2H,CH_2-N); 4.45-4.55 (m,1H,CHα Pro); 4.68-4.75 (m,1H,CHα Ala); 5.15-5.25 (m, 2H,$C\underline{H}_2$-C_6H_5), 7.35 (s,5H,C_6H_5).

Boc-NMe-(D)Ala-Pro-OBn (3b). R_f= 0.6 (EtOAc/CH_2Cl_2 30/70); HPLC (CH_3CN/H_2O 70/30) retention time=13.3 min; MS FAB(+) (GT) $(M+H)^+$=391. ^1H NMR ($CDCl_3$), δ(ppm) 1.3 (d,J=7Hz,3H,CH_3 Ala); 1.5 (s,9H,tBu); 1.88-2.5 (m,4H,2CH_2); 2.75 (s,3H,N-CH_3); 3.45-3.85 (m,2H,CH_2-N); 4.6-5.2 (2m,2H,2CHα); 5.25-5.35 (m,2H,$C\underline{H}_2$-C_6H_5); 7.5 (s,5H,C_6H_5).

Boc-NMe-(L)Nva-Pro-OBn (3c). TLC: R_f= 0.66 (EtOAc/CH_2Cl_2 20/80); HPLC (CH_3CN/H_2O 70/30) retention time=9.2 min; MS FAB(+) (GT) $(M+H)^+$=419. ^1H NMR ($CDCl_3$), δ(ppm) 0.90-0.98 (m,3H,CH_3); 1.5 (s,9H,tBu); 1.1-2.2 (m,8H,4CH_2 Nva and Pro); 2.85 (s,3H,N-CH_3); 3.5-3.9 (m,2H,CH_2-N); 4.5-5.0 (m,2H,2CHα); 5.25-5.35 (m,2H,$C\underline{H}_2$-C_6H_5); 7.55 (s,5H,C_6H_5).

Boc-NMe-(D)Nva-Pro-OBn (3d). TLC: R_f= 0.33 (EtOAc/CH_2Cl_2 10/90); HPLC (CH_3CN/H_2O 70/30) retention time=9.4 min; MS FAB(+) (GT) $(M+H)^+$=419. ^1H NMR ($CDCl_3$), δ(ppm) 0.95-1.05 (m,3H,CH_3); 1.5 (s,9H,tBu); 1.2-2.4 (m,8H,4CH_2 Nva and Pro); 2.7 (s,3H,N-CH_3); 3.4-3.7 (m,2H,CH_2-N); 4.4-5.0 (m,2H,2CHα); 5.15-5.25 (m,2H,$C\underline{H}_2$-C_6H_5); 7.45 (s,5H,C_6H_5).

Boc-(L)Nva-Pro-OBn (3e). TLC: R_f= 0.61 (EtOAc/CH_2Cl_2 20/80); HPLC (CH_3CN/H_2O 70/30) retention time=7.16 min; MS FAB(+) (GT) $(M+H)^+$=405. ^1H NMR ($CDCl_3$), δ(ppm) 0.85-0.95 (m,3H,CH_3); 1.5 (s,9H,tBu); 1.2-2.5 (m,8H,4 CH_2 Nva and Pro); 3.5-3.9 (m,2H,CH_2-N); 4.3-4.8 (m,2H,2 CHα); 5.3 (s,2H,$C\underline{H}_2$-C_6H_5); 5.45-5.55 (m,1H,NH); 7.5 (s,5H,C_6H_5).

Boc-(D)Nva-Pro-OBn (3f). TLC: R_f= 0.61 (EtOAc/CH_2Cl_2 25/75); HPLC (CH_3CN/H_2O 70/30) retention time=7.25 min; MS FAB(+) (GT) $(M+H)^+$=405. ^1H NMR ($CDCl_3$), δ(ppm) 0.85-0.95 (m,3H,CH_3); 1.5 (s,9H,tBu); 1.2-2.4 (m,8H,4 CH_2 Nva and Pro); 3.5-3.9 (m,2H,CH_2-N); 4.4-4.6 (m,2H,2CHα); 5.25 (s,2H,$C\underline{H}_2$-C_6H_5); 5.35-5.45 (m,1H,NH); 7.5 (s,5H,C_6H_5).

Z-Ile-NMeVal-NMeAla-βAla-OH (4). A solution of the tetrapeptide **(2)** (10 mmol, 5.48 g) in methanol (50 ml) was cooled to 0°C and a 2N solution of NaOH (10 ml) was then added. After stirring for 2-3 hours at room temperature (monitored by TLC), removal of solvents gave a residue dissolved in water (50 ml) The aqueous solution was acidified to pH 1 using 2N HCl and then extracted with EtOAc (3x20 ml). The combined organic layers were dried over $MgSO_4$ then evaporated *in vacuo* to afford the title compound **(4)** as a white solid (100% yield). m.p. = 136-138°C. TLC: R_f= 0.5 (EtOAc/HCOOH 99/1); HPLC (CH_3CN/H_2O-TFA 0.1% 50/50) retention time=8.5 min; MS FAB(+) (GT) $(M+H)^+$=535. ^1H NMR ($CDCl_3$), (*=2 conformers) δ(ppm) 0.75-0.95 (m,12H,4CH_3 Val and Ile); 1.22 and 1.4 (2d*,J=7Hz,3H,CH_3 Ala); 1.43-1.47 and 1.84-1.88 (2m,3H,CHβ and CH_2 Ile); 2.30-2.35 (m,1H,CHβ Val); 2.50-2.55 (m,2H,CH_2CO); 2.76-3.3 (4s*,6H,2N-CH_3); 3.45-3.50 (m,2H,CH_2-N); 4.47-4.57 (2m*,1H,CHα Ile); 4.95-5.25 (m,2H,CHα Ala and CHα Val); 5.08 (s,2H,$C\underline{H}_2$-C_6H_5); 5.51 and 5.7 (2d*,J=10Hz,1H,NH Ile); 7.03-7.08 and 7.55-7.6 (2m*,1H,NH βAla); 7.31 (s,5H,C_6H_5).

H-AA-Pro-OBn (5a-f). The tripeptide **(3a-f)** (10 mmol) was dissolved in anhydrous ether (50 ml) then gaseous HCl was bubbled for 2 h at room temperature, monitored by TLC. Removal of solvent gave the expected dipeptide as hydrochloride, directly used in the next coupling.

Z-Ile-NMeVal-NMeAla-βAla-AA-Pro-OBn (6a-f) (Step C). The tetrapeptide **(4)** was coupled with dipeptides **(5a-f)** using the BroP reagent, according to the general procedure. Yields obtained for **(6a-f)** after purification by chromatography on silica are reported in Table 3.

Z-Ile-NMeVal-NMeAla-βAla-(L)NMeAla-Pro-OBn (6a): m.p.=48-50°C; TLC: R_f= 0.33 (EtOAc/isopropanol 95/5); HPLC ($MeOH/H_2O$ 75/25) retention time=14.7 min; MS FAB(+) (GT) $(M+H)^+$=807. ^1H NMR ($CDCl_3$), (*=conformers) δ(ppm) 0.75-0.95 (m,12H,$4CH_3$ Val and Ile); 1.15-1.38 (m,6H,$2CH_3$ Ala); 1.4-2.25 (m,7H,$CH\beta$ and CH_2 Ile, $2CH_2$ Pro); 2.25-2.35 (m,1H,$CH\beta$ Val); 2.43-2.53 (m,2H,CH_2CO); 2.6-3.25 (9s*,9H,$3N-CH_3$); 3.35-3.75 (m,4H,$2CH_2$-N Ala and Pro); 4.42-4.55 (m,9H,$5CH\alpha$ and $2C\underline{H}_2-C_6H_5$); 6.12-6.18 and 6.62-6.67 (2m*,1H,NH Ile); 7.3-7.4 (m,10H,$2C_6H_5$); 7.36-7.43 and 7.77-7.82 (2m*,1H,NH βAla).

Z-Ile-NMeVal-NMeAla-βAla-(D)NMeAla-Pro-OBn (6b): m.p.=50-52°C; TLC: R_f= 0.50 (EtOAc/isopropanol 95/5); HPLC ($MeOH/H_2O$ 75/25) retention time=13.7 min; MS FAB(+) (GT) $(M+H)^+$=807. ^1H NMR ($CDCl_3$), (*=conformers) δ(ppm) 0.7-1.6 (m,12H,$4CH_3$ Val and Ile); 1.05-1.4 (m,6H,$2CH_3$ Ala); 1.45-2.65 (m,10H,$CH\beta$ and CH_2 Ile, $2CH_2$ Pro, $CH\beta$ Val and CH_2CO); 2.7-3.22 (5s*,9H,$3N-CH_3$); 3.2-3.65 (m,4H,$2CH_2$-N βAla and Pro); 4.4-4.55 (m,9H,$5CH\alpha$ and $2C\underline{H}_2-C_6H_5$); 6.65-6.72 (m,1H,NH Ile); 7.25-7.4 (m,10H,$2C_6H_5$); 7.78-7.83 (m,1H,NH βAla).

Z-Ile-NMeVal-NMeAla-βAla-(L)NMeNva-Pro-OBn (6c): m.p.=46-48°C; TLC: R_f= 0.5 (EtOAc/isopropanol 96/4); HPLC (CH_3CN/H_2O 70/30) retention time=9.5 min; MS FAB(+) (GT) $(M+H)^+$=835. ^1H NMR ($CDCl_3$), (*=conformers) δ(ppm) 0.7-0.9 (m,15H,$5CH_3$ Nva,Val and Ile); 1.15 and 1.3 (2d*,J=7Hz,3H,CH_3 Ala); 1.0-2.2 (m,11H,$CH\beta$ and CH_2 Ile, $2CH_2$ Pro and $2CH_2$ Nva); 2.22-2.25 (m,1H,$CH\beta$ Val); 2.4-2.5 (m,2H,CH_2CO); 2.6-3.2 (8s*,9H,$3CH_3$); 3.3-3.8 (m,4H,$2CH_2$-N βAla and Pro); 4.4-5.4 (m,9H,$5CH\alpha$ and $2C\underline{H}_2-C_6H_5$); 6.56-6.59 and 7.58-7.63 (2m,2H,2NH Ile and βAla); 7.2-7.35 (m,10H,$2C_6H_5$).

Z-Ile-NMeVal-NMeAla-βAla-(D)NMeNva-Pro-OBn (6d): m.p.=48-52°C; TLC: R_f= 0.66 (EtOAc/isopropanol 95/5); HPLC ($MeOH/H_2O$ 85/15) retention time=6.58 min; MS FAB(+) (GT) $(M+H)^+$=835. ^1H NMR ($CDCl_3$), (*=conformers) δ(ppm) 0.8-1.0 (m,15H,$5CH_3$ Nva,Val and Ile); 1.15-1.4 (3d*,J=7Hz,3H,CH_3 Ala); 1.5-2.3 (m,11H,$CH\beta$ and CH_2 Ile, $2CH_2$ Pro and $2CH_2$ Nva); 2.3-2.4 (m,1H,$CH\beta$ Val); 2.4-2.5 (m,2H,CH_2CO); 2.7-3.2 (8s*,9H,$3CH_3$); 3.3-3.7 (m,4H,$2CH_2$-N βAla and Pro); 4.4-5.5 (m,9H,$5CH\alpha$ and $2C\underline{H}_2-C_6H_5$); 6.58-6.62 and 7.88-7.93 (2m,2H,2NH Ile and βAla); 7.2-7.4 (m,10H,$2C_6H_5$).

Z-Ile-NMeVal-NMeAla-βAla-(L)Nva-Pro-OBn (6e): m.p.=50-54°C; TLC: R_f= 0.53 (EtOAc/isopropanol 96/4); HPLC (CH_3CN/H_2O 70/30) retention time=8.16 min; MS FAB(+) (GT) $(M+H)^+$=821. ^1H NMR ($CDCl_3$), δ(ppm) 0.68-0.9 (m,15H,$5CH_3$ Nva,Val and Ile); 1.2 and 1.35 (2d*,J=7Hz,3H,CH_3 Ala); 1.0-2.0 (m,11H,$CH\beta$ and CH_2 Ile, $2CH_2$ Pro and $2CH_2$ Nva); 2.05-2.3 (m,3H,$CH\beta$ Val and CH_2CO); 2.7-3.02 (4s*,6H,$2N-CH_3$); 3.05-3.8 (m,4H,$2CH_2$-N βAla and Pro); 4.4-5.2 (m,9H,$5CH\alpha$ and $2C\underline{H}_2-C_6H_5$); 5.3 and 5.5 (2d*,J=8Hz,1H,NH Ile); 6.9 and 7.3 (2d*,J=8Hz,1H,NH Nva); 7.2-7.3 (m,10H, $2C_6H_5$); 7.3-7.45 (m,1H,NH βAla).

Z-Ile-NMeVal-NMeAla-βAla-(D)Nva-Pro-OBn (6f): m.p.=52-54°C; TLC: Rf= 0.50 (EtOAc/isopropanol 95/5); HPLC (CH_3CN/H_2O 70/30) retention time=7.9 min; MS FAB(+) (GT) $(M+H)^+$=821. ^1H NMR ($CDCl_3$), (*=conformers) δ(ppm) 0.7-1.0 (m,15H,5CH_3 Nva,Val and Ile); 1.3 and 1.35 (2d*,J=6Hz,3H,CH_3 Ala); 1.2-2.45 (m,14H,CHβ and CH_2 Ile, 2CH_2 Pro, 2CH_2 Nva, CHβ Val and CH_2CO); 2.7-3.3 (4s*,6H,2N-CH_3); 3.5-4.0 (m,4H,2CH_2-N βAla and Pro); 4.3-5.2 (m,9H,5CHα and 2$C\underline{H}_2$-C_6H_5); 5.4 and 6.6 (2d*,J=10Hz,1H,NH Ile); 6.68-6.73 and 7.67-7.72 (2m,2H,2NH Nva and βAla); 7.3-7.4 (m,10H, 2C_6H_5).

H-Ile-NMeVal-NMeAla-βAla-AA-Pro-OH (7a-f). To a cooled (-30°C) solution of **(6a-f)** (10 mmol) in methanol (50 ml), ammonium formate (30 mmol, 1.9 g) and Pd/C 10% (30% in weight) were added. The reaction mixture was stirred overnight at room temperature. After filtration through a pad of Celite®, concentration of the filtrate gave the expected compounds evidenced by mass spectroscopy.

H-Ile-NMeVal-NMeAla-βAla-(L)NMeAla-Pro-OH (7a): m.p.=85-90°C; MS FAB(+) (GT) $(M+H)^+$=583.

H-Ile-NMeVal-NMeAla-βAla-(D)NMeAla-Pro-OH (7b): m.p.=87-90°C; MS FAB(+) (GT) $(M+H)^+$=583.

H-Ile-NMeVal-NMeAla-βAla-(L)NMeNva-Pro-OH (7c): m.p.=120-125°C; MS FAB(+) (GT) $(M+H)^+$=611.

H-Ile-NMeVal-NMeAla-βAla-(D)NMeNva-Pro-OH (7d): m.p.=80-85°C; MS FAB(+) (GT) $(M+H)^+$=611.

H-Ile-NMeVal-NMeAla-βAla-(L)Nva-Pro-OH (7e): m.p.=105-110°C; MS FAB(+) (GT) $(M+H)^+$=597.

H-Ile-NMeVal-NMeAla-βAla-(D)Nva-Pro-OH (7f): m.p.=100-110°C; MS FAB(+) (GT) $(M+H)^+$=597.

Cyclisation.

Optimization conditions. A solution of DPPA, triethylamine and BocLeu-Phe-OMe (10^{-5} mmol, 19.6 mg) in DMF (40 ml) was brought to the required temperature, then the linear precursor **7b** (10^{-5} mmol, 29.1 mg) in solution of DMF (10 ml) was slowly added with a syringe-pump. The reaction was monitored by analytical HPLC.

Table 7 : HPLC Conditions

Time (min)	CH_3CN (%)	H_2O (%)
0	10	90
21*	10	90
25	40	60
71	50	50
72	100	0

* back-flush

In these gradient conditions, the elution order is :

c(Ile-NMeVal-NMeAla-βAla-(D)NMeAla-Pro) **(8b)** (38.9 min) c[(Ile-NMeVal-NMeAla-βAla-(D)NMeAla-Pro)$_2$] **(9b)** (45 min)

BocLeu-Phe-OMe (71.7 min)

Typical preparative procedure. A solution of DPPA (10 mmol, 2.2 ml) and triethylamine (10 mmol, 1.4 ml) in anhydrous DMF (1 l) was heated to 60°C. A solution of **(7a-f)** (1 mmol) in DMF (10 ml) was then added within 15 h using a syringe-pump. After stirring for another 2 h at 60°C, the solvent was evaporated in high *vacuo* and the residue was dissolved in EtOAc. This organic phase was washed with 1N aqueous citric acid, water, saturated aqueous $NaHCO_3$ and water until neutral pH. Evaporation of the dried ($MgSO_4$) organic layer afforded the expected compound, which was purified by chromatography on silica.

c(Ile-NMeVal-NMeAla-βAla-(L)NMeAla-Pro) (8a): m.p.=175-180°C; Yield=68%; TLC: R_f=0.45 ($CHCl_3$/isopropanol 92.5/7.5); HPLC (CH_3OH/H_2O 40/60) retention time=9.4 min; (CH_3CN/H_2O 40/60) retention time=8.2 min; MS FAB(+) (NBA) $(M+H)^+$=565; FAB(-) (G) $(M-H)^-$=563. MS (HR-FAB) : $(M+H)^+$ $C_{28}H_{49}N_6O_6$ calc. 565.3714, found 565.3716.^1H NMR ($CDCl_3$), δ(ppm) 0.75-0.95 (m,12H,4 CH_3 Val and Ile); 1.27 and 1.35 (2d,J=7Hz,6H,2 CH_3 Ala); 1.3-1.5 (m,2H,$CH_2\gamma$ Ile); 1.8-2.15 (m,4H,CHβ Ile, $\underline{H}CH\beta$ and $CH_2\gamma$ Pro); 2.32-2-2.38 (m,1H,CHβ Val); 2.5-2.75 (m,3H,$HC\underline{H}\beta$ Pro and CH_2CO βAla); 2.70 (s,3H,N-CH_3 Ala); 3.0-3.1 and 3.9-4.1 (2m,2H,N-CH_2 βAla); 3.08 (s,3H,N-CH_3 Ala); 3.2 (s,3H,N-CH_3 Val); 3.35-3.45 and 3.6-3.7 (2m,2H,N-CH_2 Pro); 4.38 (m,1H,CHα Pro); 4.45 (q,J=7Hz,1H,CHα Ala); 4.95-5.05 (m,2H,CHα Ile and CHα Val); 5.1 (q,J=7Hz,1H,CHα Ala); 7.8 (d,J=8Hz,1H,NH Ile); 9.2 (d,1H,NH βAla).

c(Ile-NMeVal-NMeAla-βAla-(D)NMeAla-Pro) (8b): m.p.=135-140°C; Yield=70%; TLC: R_f=0.55 ($CHCl_3$/isopropanol 92.5/7.5); HPLC (CH_3CN/H_2O 50/50) retention time=8.6 min; (CH_3CN/H_2O 40/60) retention time=9.2 min; MS FAB(+) (GT) $(M+H)^+$=565; FAB(-) (G) $(M-H)^-$=563. MS (HR-FAB) : $(M+H)^+$ $C_{30}H_{53}N_6O_6$ calc. 565.3714, found 565.3731. ^1H NMR ($CDCl_3$), δ(ppm) 0.74-0.89 (m,12H,4CH_3 Val and Ile); 1.23 and 1.35 (2d,J=7Hz,6H,2CH_3 Ala); 1.25-1.45 (m,2H,$CH_2\gamma$ Ile); 1.82-2.05 (m,4H,CHβ Ile, $\underline{H}CH\beta$ and $CH_2\gamma$ Pro); 2.27 (m,1H,CHβ Val); 2.5-2.65 (m,3H,$HC\underline{H}\beta$ Pro and CH_2CO βAla); 2.68 (s,3H,N-CH_3 Ala); 3.1-3.2 and 3.9-4.1 (2m,2H,N-CH_2 βAla); 3.02 (s,3H,N-CH_3 (D)Ala); 3.18 (s,3H,N-CH_3 Val); 3.40-3.50 and 3.85-3.95 (2m,2H,N-CH_2 Pro); 4.53 (q,J=7Hz,1H,CHα (D)Ala); 4.63 (m,1H,CHα Pro); 4.75-4.85 (m,1H,CHα Ile); 4.97 (d,J=9Hz,1H,CHα Val); 5.18 (q,J=7Hz,1H,CHα Ala); 7.3 (d,J=8Hz,1H,NH Ile); 8.1-8.15 (m,1H,NH βAla).

c(Ile-NMeVal-NMeAla-βAla-(L)NMeNva-Pro) (8c): m.p.=110-120°C; Yield=62%; TLC: R_f=0.41 ($CHCl_3$/isopropanol 92.5/7.5); HPLC (CH_3CN/H_2O 50/50) retention time=8.6 min; (CH_3CN/H_2O 40/60) retention time=13.6; MS FAB(+) (NBA) $(M+H)^+$=593; FAB(-) (G) $(M-H)^-$=591. MS (HR-FAB) : $(M+H)^+$ $C_{30}H_{53}N_6O_6$ calc. 593.4027, found 593.4082. ^1H NMR ($CDCl_3$), δ(ppm) 0.7-1.0 (m,12H,4CH_3 Val and Ile); 1.3 (d,J=7Hz,3H,CH_3 Ala); 1.2-2.1 (m,12H,CHβ and $CH_2\gamma$ Ile, $CH_2\beta$ and $CH_2\gamma$ Pro,$CH_2\gamma$ and $CH_3\delta$ Nva); 2.3-2.34 (m,1H,CHβ Val); 2.5-2.65 (m,2H,CH_2-CO βAla); 2.71 (s,3H,N-CH_3 Ala); 2.95-3.15 and 3.98-4.02 (2m,2H, N-CH_2 βAla); 3.08 (s,3H,N-CH_3 Nva); 3.2 (s,3H,N-CH_3 Val); 3.3-3.5 and 3.9-4.1 (2m,2H,N-CH_2 Pro); 4.4-4.5 (m,2H,2CHα Nva and Pro); 4.95-5.05 (m,2H,CHα Ile and Val); 5.1 (q,J=7Hz,1H,CHα Ala); 7.95 (d,J=9Hz,1H,NH Ile); 9.2 (d,J=9Hz,1H,NH βAla).

c(Ile-NMeVal-NMeAla-βAla-(D)NMeNva-Pro) (8d): m.p.=95-105°C; Yield=60%; TLC: R_f=0.54 ($CHCl_3$/isopropanol 92.5/7.5); HPLC (CH_3CN/H_2O 50/50) retention time=9.0 min; (CH_3CN/H_2O 40/60) retention time=16.5; MS FAB(+) (GT) $(M+H)^+$=593; FAB(-) (G) $(M-H)^-$=591. MS (HR-FAB) : $(M+H)^+$