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Peptaibolin: synthesis, 3D-structure, and membrane modifying properties of the natural antibiotic and selected analogues

Marco Crisma,[a] Alessandra Barazza,[a] Fernando Formaggio,[a] Bernard Kaptein,[b] Quirinus B. Broxterman,[b] Johan Kamphuis[c] and Claudio Toniolo[a,*]

[a]*Department of Organic Chemistry, University of Padova, Biopolymer Research Centre, C.N.R., Via Marzolo 1, 35131 Padova, Italy*
[b]*DSM Research, Organic Chemistry and Biotechnology Section, P.O. Box 18, 6160 MD Geleen, The Netherlands*
[c]*DSM Food Specialties, Nutritional Ingredients, P.O. Box 1, 2600 MA Delft, The Netherlands*

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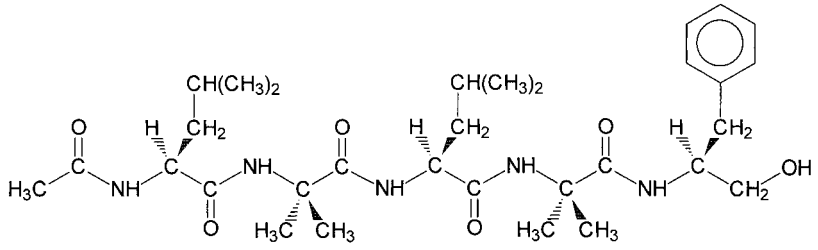
Abstract—We synthesized by solution methods and fully characterized the naturally occurring, tetrapeptide antibiotic peptaibolin and selected analogues with replacements at the N- and C-terminal groups and the C^{α}-tetrasubstituted α-amino acids. The preferred conformation of all of the peptides was assessed in solution by using FT-IR absorption and ^{1}H NMR techniques. Results of the X-ray diffraction analyses of peptaibolin itself and three analogues are also presented. Permeability measurements of such multiple turn forming, very short peptides indicate that peptaibolin is devoid of membrane activity because a lipoyl N-terminal blocking group is an essential requisite.

1. Introduction

Peptaibols[1] are a unique class of membrane active *pept*ides biosynthesized by fungi.[2] These antibiotics are characterized by a high proportion of *Aib* (α-aminoisobutyric acid or $C^{\alpha,\alpha}$-dimethylglycine), a strong $\alpha/3_{10}$-helix[3] supporting C^{α}-tetrasubstituted α-amino acid,[4–7] and a C-terminal 1,2-amino alcoh*ol*. Some peptaibols typically contain Iva (isovaline or C^{α}-methyl, C^{α}-ethylglycine), another representative of the Aib family. An additional common feature is represented by an N^{α}-terminal acyl group (more specifically, an acetyl group in the longest members of the class, while a fatty acyl group of eight or ten carbon atoms in the shortest members). Sequences of these peptides range from 19 amino acids (e.g. in the extensively investigated alamethicin) to as low as six amino acids (in the lipopeptaibol trichodecenin). Peptaibols are known to induce leakage of the cytoplasmic material and eventually to lead to cell death. It has been demonstrated that the long-sequence peptaibols form voltage-dependent channels,[2] whereas the short-sequence (lipo)peptaibols tend to float on the lipid bilayer (carpet-like mechanism).[8]

In the course of a screening for fungal peptides Hülsmann et al.[9] have recently reported on the isolation and primary structure of peptaibolin, an unusual representative of the peptaibol class in that it contains only four amino acids in combination with the small acetyl blocking group at the N-terminus. The sequence of peptaibolin is as follows:



Ac-L-Leu-Aib-L-Leu-Aib-L-Phol

where Ac is acetyl and Phol is the 1,2-amino alcohol phenylalaninol. This compound exhibits antimicrobial activity, albeit moderate, against Gram-positive bacteria and yeasts.

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* Corresponding author. Tel.: +39-049-827-5247; fax: +39-049-827-5239; e-mail: biop02@chor.unipd.it

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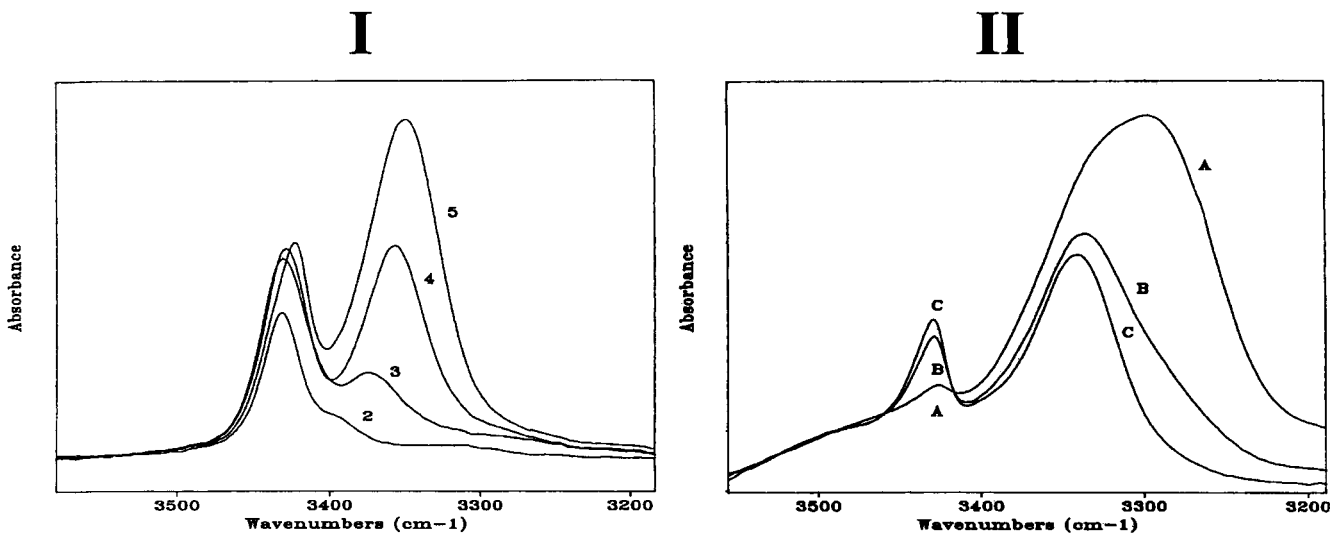

Figure 1. (I) FT-IR absorption spectra (3500–3200 cm^{-1} region) in $CDCl_3$ solution (peptide concentration: 1.0 mM) of Z-Aib-L-Phe-OMe (**2**), Z-L-Leu-Aib-L-Phe-OMe (**3**), Z-Aib-L-Leu-Aib-L-Phe-OMe (**4**), and Z-(L-Leu-Aib)$_2$-L-Phe-OMe (**5**). (II) FT-IR absorption spectra (3500–3200 cm^{-1} region) in $CDCl_3$ solution of Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin) at the peptide concentrations 10 mM (**A**), 1.0 mM (**B**), and 0.1 mM (**C**).

In an attempt to shed light on the relationship between 3D-structure and mechanism of action of peptaibolin we have carried out the total synthesis of this terminally blocked tetrapeptide, assessed its preferred conformation in a structure-supporting solvent ($CDCl_3$) by FT-IR absorption and ^1H NMR techniques and in the crystal state by X-ray diffraction, and analyzed its tendency to disrupt membrane organization. The results obtained are compared with those (also reported in this paper) of carefully selected analogues which include modifications at the N-terminus (from the short native acetyl group to the lipophilic *n*-octanoyl group), the C-terminus (from the native 1,2-amino alcohol to the α-amino acid methyl ester), and the two internal C^{α}-tetrasubstituted α-amino acids (from the native Aib to the slightly more hydrophobic Iva residues).

2. Results and discussion

2.1. Peptide synthesis

For the production of the enantiomerically pure L-Iva we exploited an economically attractive and generally applicable chemo-enzymatic synthesis developed by the DSM Research group a few years ago.[10] It involves a combination of organic syntheses for preparation of the racemic amino acid amide followed by the use of a broadly specific aminopeptidase to achieve optical resolution on a large scale.

Peptide coupling reactions were performed step-by-step in solution beginning from the C-terminal amino acid ester by using the EDC [*N*-ethyl,*N*′-(3-dimethylaminopropyl)-carbodiimide]/HOAt (1-hydroxy,7-aza-1,2,3-benzotriazole) method[11] in CH_2Cl_2 in the presence of *N*-methylmorpholine (NMM). N^{α}-Acetylation and N^{α}-*n*-octanoylation were achieved by treatment of the N^{α}-deprotected peptide with acetic anhydride and *n*-octanoic acid/EDC/HOAt, respectively. In the last step of the synthesis the methyl group was reduced to primary alcohol by using $LiBH_4$.[12] Removal of the benzyloxycarbonyl (Z) N^{α}-protecting group was achieved by catalytic hydrogenation.

The chemical and optical purities of all intermediates and final synthetic compounds were assessed by TLC in three different solvent systems, polarimetry, solid-state IR absorption, analytical HPLC, ^1H NMR, high-resolution mass spectrometry and, in selected cases, by amino acid analysis as well.

2.2. Solution conformational analysis

The conformational preferences of Ac-(L-Leu-Aib)$_2$-L-Phe-OMe (OMe, methoxy) and Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe, and their synthetic precursors Z-(L-Leu-Aib)$_2$-L-Phe-OMe and Z-(L-Leu-L-Iva)$_2$-L-Phe-OMe and short sequences, along with those of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe (*n*-Oct, *n*-octanoyl), *n*-Oct-(L-Leu-L-Iva)$_2$-L-Phe-OMe, Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin), *n*-Oct-(L-Leu-Aib)$_2$-L-Phol, Ac-(L-Leu-L-Iva)$_2$-L-Phol, and *n*-Oct-(L-Leu-L-Iva)$_2$-L-Phol were examined in a solvent of low polarity ($CDCl_3$) at different peptide concentrations by using FT-IR absorption. A more detailed solution conformational analysis of two representative peptides was performed by ^1H NMR.

Fig. 1(I) shows the FT-IR absorption spectra in the N–H stretching region of the Z-protected Aib/Phe-OMe series from di- to pentapeptide. The curves are characterized by two bands at 3428 ± 6 cm^{-1}, assigned to free (solvated) NH groups, and at 3364 ± 14 cm^{-1}, assigned to H-bonded NH groups.[13–15] The intensity of the low-frequency band, relative to the high-frequency band, increases as the peptide main-chain length increases. Concomitantly, the absorption maximum markedly shifts to lower wavenumbers. A remarkably similar trend was observed for the Z-protected L-Iva/Phe-OMe series (results not shown). All of the members of these two peptide series tend to self-associate only marginally in the concentration range examined (10–0.1 mM) (not shown). Therefore, the observed band at

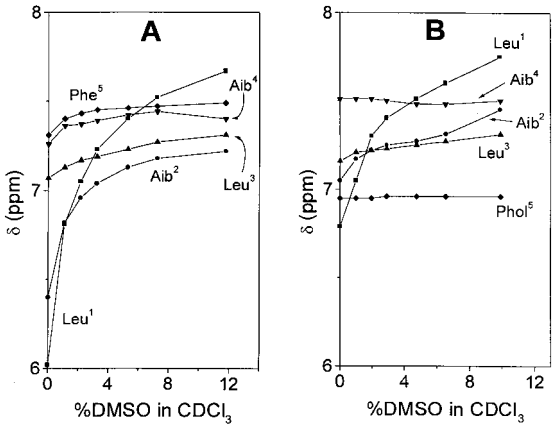

Figure 2. Plot of NH chemical shifts as a function of DMSO added to the $CDCl_3$ solution (v/v) from the ^1H NMR titrations of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe (**A**) and Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin) (**B**). Peptide concentration: 0.7 mM.

$3364\pm14\ cm^{-1}$, quite intense for the longest peptides, should be ascribed almost entirely to intramolecular C═O···H–N interactions. Replacement of the Z urethane N^{α}-protecting group with the *n*-Oct and, in particular, the Ac acyl groups significantly promoted peptide self-association, but only above 1.0 mM concentration (not shown). Remarkably, the Ac-blocked Iva/Phe-OMe peptide is sparingly soluble in $CDCl_3$ solution at 10 mM concentration.

As for the FT-IR spectra of the Phol C-terminating peptides (Fig. 1(II)), an additional broad band was seen at 3460–3450 cm^{-1}, which we assigned to the H-bonded alcoholic OH group.[16] Further, the curves below 3350 cm^{-1} showed that all of the C-terminal Phol peptides tend to self-associate more extensively than their Phe-OMe ester counterparts, again particularly above 1.0 mM concentration. However, besides the contribution of H-bonded NH groups, it is not safe to exclude the absorption of the H-bonded OH group also in that spectral region.[16]

In the 1800–1600 cm^{-1} region ester, urethane, amide and peptide C═O groups are responsible for the observed bands (results not shown).[15,16] Clearly, the band at about 1745 cm^{-1} (ester C═O stretching) was absent in the spectra of the C-terminal Phol peptides. The urethane C═O stretching band (1727–1712 cm^{-1}) was seen only in the Z-protected peptides. The amide and peptide C═O stretching bands (amide I) were observed between 1690 and 1657 cm^{-1}. Additionally, the C-terminal Phol peptides exhibited a shoulder at approximately 1635 cm^{-1} (but only at the highest concentration examined). The intensity of the amide I band increased with increasing peptide main-chain length and its maximum progressively shifted to lower wavenumbers. In the longest peptides the position of this maximum (1663–1657 cm^{-1}) was close to the canonical wavelengths for the 3_{10}-helical (1662 cm^{-1}) and α-helical (1658 cm^{-1}) structures.[17]

From our FT-IR absorption analysis it may be concluded that in the structure-supporting solvent $CDCl_3$ in the absence of self-association the longest peptides examined tend to adopt highly folded structures extensively stabilized by intramolecular C═O···H–N–H bonds. However, this technique alone did not allow us to unambiguously determine the nature of the folded structures that were formed.

To get more detailed, local information on the preferred conformations of the Phe-OMe and Phol C-terminating peptides in $CDCl_3$ solution we performed a 400 MHz ^1H NMR investigation of two representative examples, *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe and Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin). The delineation of inaccessible (or intramolecularly H-bonded) NH groups was carried out by using solvent dependences of NH chemical shifts by adding increasing amounts of the H-bonding acceptor DMSO (dimethylsulphoxide) to the $CDCl_3$ solutions.[18,19] Unambiguous assignments of the NH protons were performed via analysis of their multiplicities and 2D ROESY experiments. In particular, sequential assignments were conducted beginning from the N-terminal amide NH proton, which was identified by means of its NOE effects with either the CH_3CO– protons of the Ac group or the $-CH_2CO$– protons of the *n*-Oct group.

From an analysis of the spectra of the Phe-OMe peptide as a function of concentration (in the range 10–1.0 mM) (not shown) we were able to conclude that dilution induces a significant shift (>0.2 ppm) to higher fields of the N(1)H and N(2)H protons only. More specifically, the N(1)H proton is more sensitive (0.55 ppm) than the N(2)H proton (0.30 ppm). We also found that peptaibolin is much more strongly aggregated than the related Phe-OMe peptide. Indeed, upon dilution the signals of its NH protons were significantly broadened to a concentration as low as 0.7 mM, where only the N(1)H and N(2)H were still somewhat broad (but both clearly visible).

For each peptide in the $CDCl_3$–DMSO solvent mixtures at 0.7 mM concentration, where self-association is of minor significance, two classes of NH protons were observed (Fig. 2). Class (i) (N(1)H and N(2)H protons) included protons whose chemical shifts are sensitive to the addition of DMSO. For both compounds the sensitivity of the N(1)H proton was markedly higher than that of the N(2)H proton. Class (ii) (N(3)H to N(5)H protons) included those displaying a behaviour characteristic of shielded protons (relative insensitivity of chemical shifts to solvent composition).

These 1D-^1H NMR findings, which agree well with the FT-IR absorption data discussed above, allowed us to conclude that in $CDCl_3$ solution self-association of peptaibolin and its *n*-Oct/Phe-OMe analogue is promoted by the N(1)H and N(2)H protons, while the N(3)H to N(5)H protons are involved in the intramolecular H-bonding scheme. However, on the basis of these results alone it was premature to distinguish between a regular 3_{10}-helix[3] (with three consecutive β-turns[20–22]) and a mixed helix formed by the co-existence of β- and α-turns.[21,23–25] This conformational problem was solved for the *n*-Oct/Phe-OMe peptide by a thorough analysis of its ROESY spectrum. Fig. 3 clearly illustrates that, in addition to cross-peaks corresponding to the $d_{\alpha N}(i,i+1)$ and $d_{\alpha N}(i,i+3)$ short distances, a cross-peak corresponding to the $d_{\alpha N}(i,i+2)$ short distance, diagnostic of the 3_{10}-helix,[26] was observed. Conversely, the typical cross-peak for an α-turn (α-helix), $d_{\alpha N}(i,i+4)$,[26] was not seen.

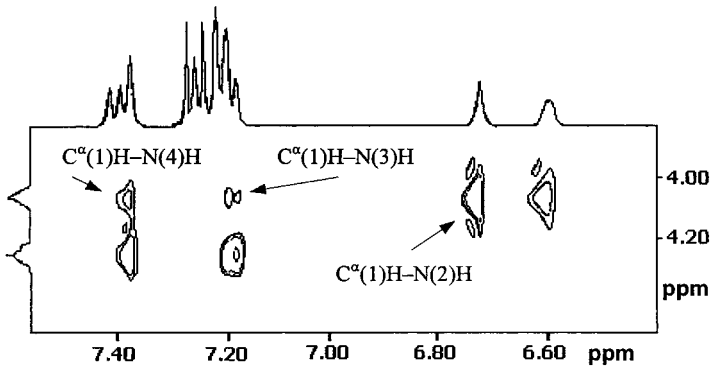

Figure 3. Partial ROESY spectrum of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe in $CDCl_3$ solution.

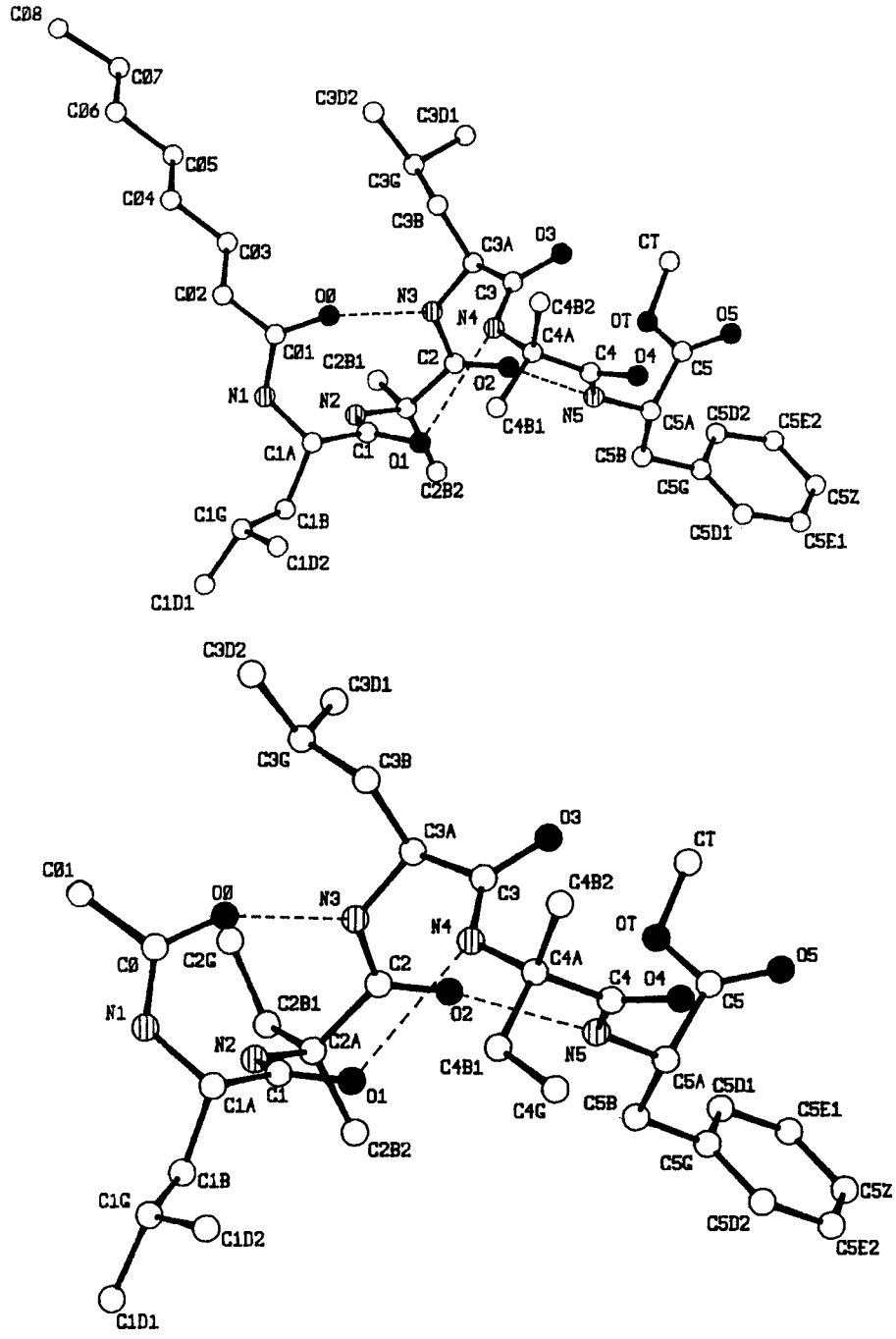

Figure 4. X-Ray diffraction structures of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe (top) and Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe (bottom) with numbering of the atoms. The intramolecular H-bonds are represented by dashed lines.

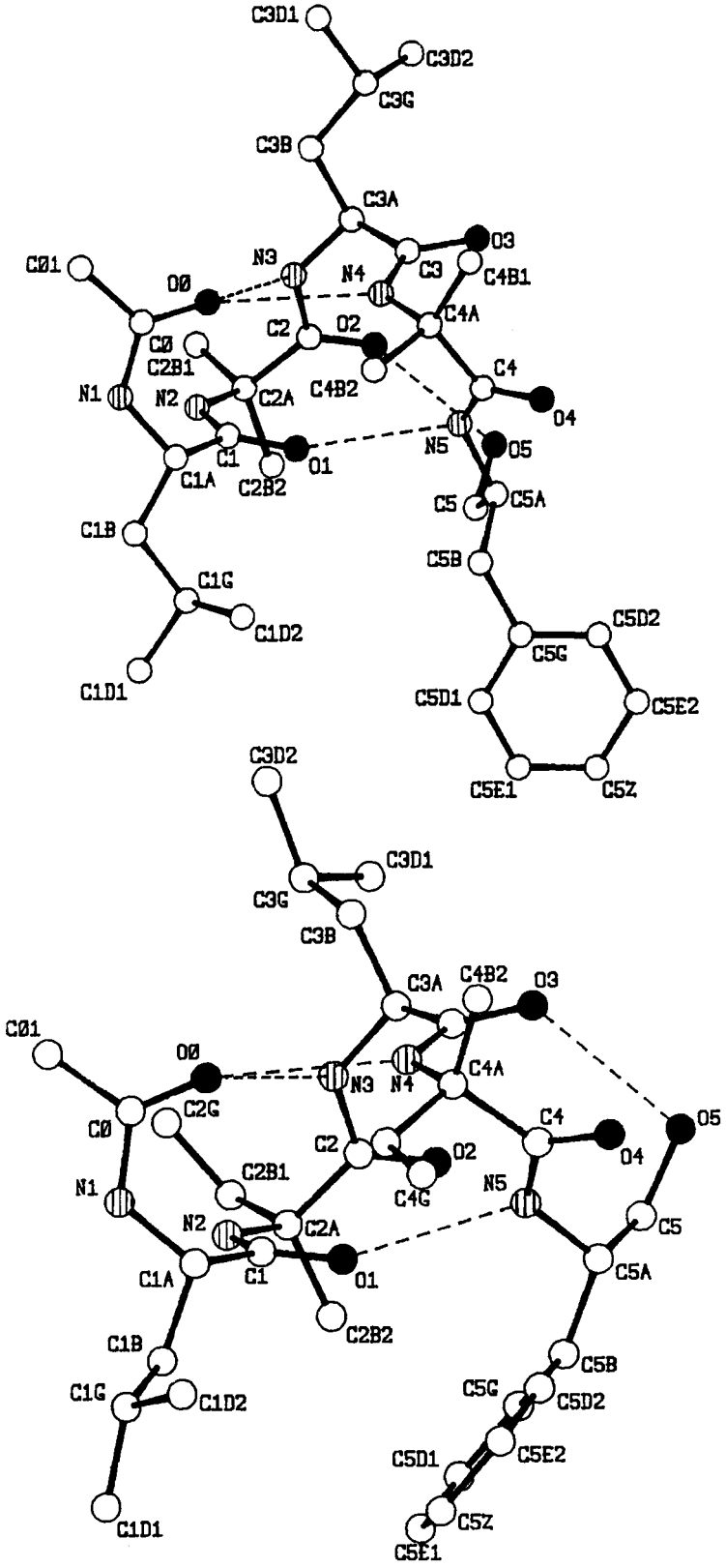

Figure 5. X-Ray diffraction structures of Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin) (top) and Ac-(L-Leu-L-Iva)$_2$-L-Phol (bottom) with numbering of the atoms. The intramolecular H-bonds are represented by dashed lines.

2.3. Crystal-state conformational analysis

The molecular and crystal structures of peptaibolin, Ac-(L-Leu-Aib)$_2$-L-Phol, and the three analogues *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe, Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe and Ac-(L-Leu-L-Iva)$_2$-L-Phol were determined by X-ray diffraction. The molecular structures of the four peptides with the atomic numbering schemes are illustrated in Figs. 4

Table 1. Selected backbone and side-chain torsion angles (the torsion angles for rotation about bonds of the peptide backbone (ϕ,ψ,ω) and side chains (χ) are described in Ref. 27) (deg) for the four peptides investigated

Torsion angle	*n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe	Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe	Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin)	Ac-(L-Leu-L-Iva)$_2$-L-Phol
ω_0	−172.8 (8)	−178.2 (9)	−172.2 (8)	176.9 (7)
ϕ_1	−55.2 (10)	−53.6 (12)	−54.1 (12)	−53.4 (9)
ψ_1	−38.7 (10)	−41.8 (11)	−47.5 (11)	−38.2 (9)
ω_1	−176.1 (6)	−176.4 (7)	−177.9 (8)	−178.4 (6)
ϕ_2	−55.7 (9)	−52.4 (10)	−51.2 (11)	−53.1 (8)
ψ_2	−37.1 (9)	−36.6 (10)	−45.7 (10)	−42.1 (8)
ω_2	−175.6 (6)	−174.2 (7)	−174.8 (8)	−178.9 (6)
ϕ_3	−72.7 (9)	−66.6 (10)	−74.8 (10)	−82.1 (7)
ψ_3	−23.8 (9)	−24.9 (12)	−41.0 (11)	−40.2 (8)
ω_3	−175.4 (5)	−179.8 (8)	−177.4 (9)	177.0 (5)
ϕ_4	−57.2 (8)	−60.8 (11)	−68.1 (11)	−50.1 (7)
ψ_4	−33.0 (9)	−33.2 (11)	−25.9 (11)	−40.4 (7)
ω_4	172.7 (7)	179.7 (8)	−166.8 (8)	175.7 (6)
ϕ_5	−73.2 (8)	−75.2 (11)	−161.3 (8)[a]	−94.2 (8)[a]
ψ_5	−62.1 (7)[b]	−56.4 (10)[b]	56.7 (10)[c]	60.6 (8)[c]
ω_5	176.7 (6)[d]	175.4 (9)[d]	–	–
χ_1^1	−70.8 (8)	−84.8 (14)[e]/−158.5 (13)[e]	−178.5 (9)	−80.2 (11)[e]/−147.6 (13)[e]
$\chi_1^{2,1}$	−74.2 (11)	46(2)[f]/172.5 (12)[f]	59.9 (12)	−46.6 (16)[f]/178.8 (14)[f]
$\chi_1^{2,2}$	163.6 (8)	−62.3 (19)[f]/141.8(8)[f]	−176.3 (11)	52 (3)[f]/−174.3 (10)[f]
χ_2^1	–	66.8 (12)[e]/−74 (2)[e]	–	46.3 (13)[e]/−66(2)[e]
χ_3^1	−76.5 (10)	−63.7 (11)	−176.7 (9)	−61.2 (8)
$\chi_3^{2,1}$	−63.7 (11)	−60.4 (14)	60.0 (14)	−55.3 (10)
$\chi_3^{2,2}$	174.6 (8)	172.6 (10)	−175.7 (9)	−176.0 (8)
χ_4^1	–	179.3 (8)	–	−179.8 (6)
χ_5^1	178.6 (6)	179.6 (8)	−175.6 (9)	−56.7 (8)
$\chi_5^{2,1}$	82.6 (6)	75.0 (9)	59.3 (11)	−25.9 (8)
$\chi_5^{2,2}$	−92.7 (6)	−101.9 (9)	−120.9 (8)	158.0 (5)

[a] C-N-CA-C(sp^3).
[b] N-CA-C-OT.
[c] N-CA-C(sp^3)-O.
[d] CA-C-OT-CT.
[e] The CG atom is disordered over two sites.
[f] The CD atom is disordered over two sites.

and 5. Selected backbone and side-chain torsion angles[27] are given in Table 1. In Table 2, the intra- and intermolecular H-bond parameters are listed.

Bond lengths and bond angles are in general agreement with previously reported values for the geometry of the *n*-octanoyl moiety,[28] the amide,[29] peptide[30,31] and ester[32] groups, the Aib/Iva[33–35] and Leu/Phe[36,37] residues, and the Phol unit.[28]

The backbone of the two N$^{\alpha}$-blocked pentapeptide esters is very similar (Fig. 4) in that both adopt a regular right-handed 3_{10}-helical structure.[3] Peptide groups N3–H to N5–H and O0═C′0 to O2═C′2 participate in three consecutive 1←4 (type-III β-turn[20–22]) C′═O···H–N intramolecular H-bonds, appropriate for a 3_{10}-helix. The range of observed N···O distances is 2.935 (8)–3.290 (8) Å, while that of N–H···O angles is 135.8–148.6°.[38–40] In both structures the C-terminal L-Phe residue is also right-handed helical.

With the exception of the C-terminal part, the backbone of the two N$^{\alpha}$-blocked tetrapeptide amino alcohols is very similar (Fig. 5), but somewhat different from that of the two N$^{\alpha}$-blocked pentapeptide esters discussed above. At the N-terminus the conformation of the peptide chain is characterized by an intramolecular three-centre double H-bond,[41] forming a type-III β-turn (C_{10}-ring structure) fused with an α-turn (C_{13}-ring structure).[21,23–25] The acyl C′0═O0 is the common acceptor of the two H-bonds, with the N3–H and N4–H as the donor groups. This structural motif develops into an additional α-turn (C′1═O1···H–N5), giving rise to an incipient α-helix, an uncommon observation for such short peptides.[42,43] Near the C-terminus both compounds are folded in a unusual oxy-analogue of a turn motif,[21] but peptaibolin is characterized by the oxy-analogue of a wider α-turn,[44] while Ac-(L-Leu-L-Iva)$_2$-L-Phol by the oxy-analogue of a narrower β-turn.[45] The related intramolecular C′2═O2···H–O5 and C′3═O3···H–O5 H-bonds have (carbonyl)O···O(alcohol) distances of 2.745 (9) and 2.875 (7) Å, respectively.[46,47]

In the four peptides investigated only one significant deviation of the ω torsion angles ($|\Delta\omega|>8°$) from the ideal value of the *trans* planar amide, peptide, and ester units (180°) is observed, namely the peptide ω_4 torsion angle of peptaibolin, −166.8 (8)°. In the two pentapeptides the methyl ester conformation with respect to the preceding C$^{\alpha}$–N bond is intermediate between the *anticlinal* and *antiperiplanar* conformations.[48]

The Iva side-chain conformations of both Iva-containing peptides confirm the lack of a clear bias,[35] in that they are g^+ (g^-) in the disordered residue 2, while *t* in residue 4. The absence of the side-chain g^+ rotamer (χ^1 torsion angle) in the Leu, Phe and Phol residues is not unexpected in view of the significant steric hindrance experienced (destabilization due to repulsive interaction of the C$^{\gamma}$ atom with the N and C′

Table 2. Intra- and intermolecular H-bond parameters for the four peptides investigated

Peptide	Donor D–H	Acceptor A	Symmetry operation	Distance (Å) D···A	Distance (Å) H···A	Angle (deg) D–H···A
n-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe	N3–H	O0	x, y, z	2.935 (8)	2.23	138.6
	N4–H	O1	x, y, z	3.082 (7)	2.41	135.8
	N5–H	O2	x, y, z	3.290 (8)	2.61	136.4
	N1–H	O4	$x, y-1, z$	2.879 (8)	2.02	176.2
	N2–H	O5	$x, y-1, z$	2.959 (7)	2.11	169.9
Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe	N3–H	O0	x, y, z	2.947 (10)	2.19	146.8
	N4–H	O1	x, y, z	3.003 (9)	2.24	148.6
	N5–H	O2	x, y, z	3.216 (10)	2.48	143.4
	N1–H	O4	$x+1, y, z$	2.883 (10)	2.04	167.7
	N2–H	O5	$x+1, y, z$	2.989 (9)	2.13	174.6
Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin)	N3–H	O0	x, y, z	3.053 (10)	2.46	126.4
	N4–H	O0	x, y, z	3.062 (9)	2.22	167.3
	N5–H	O1	x, y, z	2.963 (9)	2.33	130.7
	O5–H	O2	x, y, z	2.745 (9)	1.92	178.4
	N1–H	O5	$x-1, y, z-1$	2.955 (9)	2.16	154.1
	N2–H	O4	$x, y, z-1$	2.878 (9)	2.05	161.0
Ac-(L-Leu-L-Iva)$_2$-L-Phol	N3–H	O0	x, y, z	2.975 (7)	2.33	131.8
	N4–H	O0	x, y, z	2.995 (7)	2.14	174.3
	N5–H	O1	x, y, z	2.989 (7)	2.15	164.6
	O5–H	O3	x, y, z	2.875 (7)	2.06	171.7
	N1–H	O4	$x-1, y, z$	2.880 (8)	2.04	167.4
	N2–H	O5	$x-1, y, z$	3.339 (8)	2.49	168.5

atoms).[36,37] The two known overwhelming combinations of side-chain conformers for the Leu residue, g^- (tg^-) and $t(g^+\ t)$,[36] are those exclusively observed in these four peptides. The usual definite preference of the Phe residues for the χ^2 angle $\cong \pm 90°$[36] is observed. The difference of χ^2 from $\pm 90°$ is wider for the Phol residues, in particular when χ^1 is g^- (in the Iva/Phol peptide).

The molecules of the two 3_{10}-helical pentapeptide esters are held together in the crystal in a head-to-tail fashion along the *y*-direction (for the Aib compound) or the *x*-direction (for the Iva compound) in rows stabilized by (amide) N1–H···O4=C′4 (peptide) and (peptide) N2–H···O5=C′5 (ester) intermolecular H-bonds of normal strength.[38–40]

The same intermolecular H-bonding motif as above characterizes the crystal packing mode of the Iva/Phol peptide, with rows along the *z*-direction. The only remarkable variation is given by the nature of the acceptor of the H-bond with the (peptide) N2–H donor, namely the unusual O5–C5 (*alcohol*) group replacing the O5=C′5 (*ester*) group.

In the crystal packing of the Aib/Phol peptide (peptaibolin) the donor and acceptor groups of the two intermolecular H-bonds are the same as those of the Iva/Phol analogue, but they are cross-exchanged: (amide) N1–H···O5–C5 (alcohol) and (peptide) N2–H···O4=C′4 (peptide) H-bonds, generating rows of molecules along the *x*,*z*-direction and the *z*-direction, respectively.

2.4. Membrane permeability measurements

Peptaibols bind to phospholipid bilayers and are able to modify their permeability. Therefore, the permeability properties of peptaibolin and its analogues were investigated by following fluorimetrically the induced carboxyfluorescein (CF) leakage from small unilamellar vesicles (egg phosphatidycholine (PC)/cholesterol 70:30) for different R^{-1}=[peptide]/[lipid] molar ratios.[49,50]

In Fig. 6, the membrane modifying properties of peptaibolin and its derivatives with modifications at either the N- or C-terminus are compared to some of their Iva-containing analogues. It is evident that only the N^{α}-*n*-octanoylated tetrapeptides are significantly active. Neither C-terminal modification nor Aib→Iva double replacement induced any marked variation of membrane activity in the N^{α}-acetylated peptides.

3. Conclusions

In the present work, we have described the first total chemical synthesis of the natural antibiotic peptaibolin, the shortest peptaibol, and some analogues with N^{α}-terminal Ac→*n*-Oct, C-terminal Phe-OMe→Phol, and internal Aib→Iva replacements, using the step-by-step solution approach. The results of the solution and crystal-state conformational analyses, also reported in this paper, strongly favour the conclusion that all of these peptides are highly folded. These 3D-structural conclusions are in excellent agreement with those already published by our and other groups on Aib-[4–7,33] and Iva-rich[35,51] peptides.

Finally, our permeability measurements clearly indicate that peptaibolin is membrane inactive. However, we were able to show that a significantly beneficial effect can be induced by replacing the small N^{α}-blocking group with a fatty acid characterized by a markedly longer (*n*-octanoyl) alkyl chain. Conversely, the effects produced by the C-terminal ester→alcohol modification or by slightly increasing amino

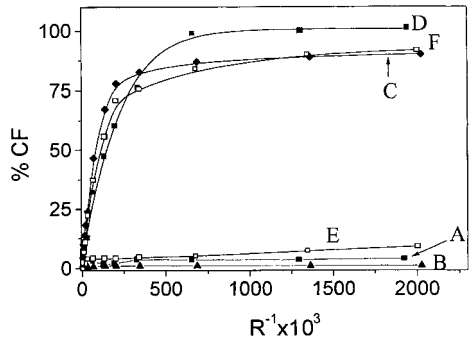

Figure 6. Peptide-induced CF leakage at 20 min for different ratios R^{-1}=[peptide]/[lipid] from egg PC/cholesterol (70:30) unilamellar vesicles: (A) Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin); (B) Ac-(L-Leu-Aib)$_2$-L-Phe-OMe; (C) *n*-Oct-(L-Leu-Aib)$_2$-L-Phol; (D) *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe; (E) Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe; (F) *n*-Oct-(L-Leu-L-Iva)$_2$-L-Phe-OMe.

acid side-chain hydrophobicity (Aib→Iva) were found to be of minor significance. These results agree well with those published in previous studies on longer peptaibols.[50] In conclusion, the moderate antimicrobial activity reported for peptaibolin[9] does not seem to be ascribable to a mechanism involving membrane modification. It remains to be seen whether this activity might be related to the tendency of peptaibolin for self-association, unusually high for a peptide of such limited size.

4. Experimental

4.1. Peptide characterization

Melting points were determined using a Leitz model Laborlux 12 apparatus and are not corrected. Optical rotations were measured using a Perkin–Elmer model 241 polarimeter equipped with a Haake model D thermostat. Thin-layer chromatography was performed on Merck Kieselgel 60/F_{254} precoated plates. The solvent systems used were: I, chloroform/ethanol (9:1); II, 1-butanol/acetic acid/water (3:1:1); III, toluene/ethanol (7:1). The chromatograms were developed by quenching of UV fluorescence, chlorine/starch/potassium iodide or ninhydrin chromatic reaction, as appropriate. Amino acid analyses were performed on a C. Erba model 3A 30 analyzer. The Aib and Iva colour yields with ninhydrin are about 1/15 of those of protein amino acids.[52]

4.1.1. Z-L-Leu-Aib-L-Phe-OMe. To a solution of Z-L-Leu-OH (3.7 g, 14 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (1.7 g, 13 mmol) and EDC·HCl (2.7 g, 14 mmol) were added. After 10 min H-Aib-L-Phe-OMe (obtained by catalytic hydrogenation of the corresponding Z-derivative[53] (5.1 g, 13 mmol) in MeOH) and NMM (2.9 mL, 26 mmol) were added and the reaction was stirred at room temperature for 12 h. Then, EtOAc was added and the solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (98:2)) and crystallized as a white solid from EtOAc/PE (4.99 g, 75%), mp 96–97°C; R_fI 0.95, R_fII 0.95, R_fIII 0.45; $[\alpha]_D^{20}=-7.0$ (*c* 0.5, MeOH); ν_{max} (KBr) 3302, 1740, 1672, 1526 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.33–7.10 (10H, Z and Phe aromatic C*H*), 6.80 (d, 1H, *J*=6.9 Hz, Phe N*H*), 6.53 (s, 1H, Aib N*H*), 5.07 (3H, Leu N*H* and Z CH_2), 4.80 (m, 1H, Phe αC*H*), 4.06 (m, 1H, Leu αC*H*), 3.69 (s, 3H, OMe CH_3), 3.11 (m, 2H, Phe βCH_2), 1.63 (m, 3H, Leu βCH_2 and γC*H*), 1.47 and 1.45 (2s, 6H, Aib βCH_3), 0.92 (m, 6H, Leu δCH_3); HRMS (FAB): M^+, found 512.2762. $C_{28}H_{38}N_3O_6$ requires 512.2761.

4.1.2. Z-Aib-L-Leu-Aib-L-Phe-OMe. To a solution of Z-Aib-OH[54–56] (2.3 g, 10 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (1.2 g, 9 mmol) and EDC·HCl (1.8 g, 10 mmol) were added. After 10 min H-L-Leu-Aib-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (4.4 g, 9 mmol) in MeOH) and NMM (2.0 mL, 17 mmol) were added and the reaction was stirred at room temperature for 24 h. Then, EtOAc was added and the solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: EtOAc/PE (2:1)) and obtained as a white solid (3.70 g, 69%), mp 62–63°C; R_fI 0.90, R_fII 0.95, R_fIII 0.40; $[\alpha]_D^{20}=-23.6$ (*c* 0.5, MeOH); HPLC: R_t 17.55 min (Phenomenex-Rp C_{18}; gradient: 30–80% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3337, 1735, 1703, 1662, 1524 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.35 (m, 5H, Z aromatic C*H*), 7.25–7.13 (6H, Phe aromatic C*H* and Phe N*H*), 7.02 (s, 1H, Aib^3 N*H*), 6.39 (d, 1H, *J*=7.0 Hz, Leu N*H*), 5.22 (s, 1H, Aib^1 N*H*), 5.08 (s, 2H, Z CH_2), 4.80 (m, 1H, Phe αC*H*), 4.21 (m, 1H, Leu αC*H*), 3.67 (s, 3H, OMe CH_3), 3.12 (m, 2H, Phe βCH_2), 1.77–1.57 (3H, Leu βCH_2 and γC*H*), 1.51, 1.48, 1.47, 1.46 (4s, 12H, 2Aib βCH_3), 0.94–0.82 (m, 6H, Leu δCH_3); HRMS (FAB): M^+, found 597.3269. $C_{32}H_{45}N_4O_7$ requires 597.3288.

4.1.3. Z-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe. To a solution of Z-L-Leu-OH (1.4 g, 5.4 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (0.7 g, 4.9 mmol) and EDC·HCl (1.0 g, 5.4 mmol) were added. After 10 min H-Aib-L-Leu-Aib-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (3.0 g, 4.9 mmol) in MeOH) and NMM (1.1 mL, 9.9 mmol) were added and the reaction was stirred at room temperature for 24 h. Then, the solvent was evaporated and the residue dissolved in EtOAc. The solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (98:2)) and crystallized as a white solid from EtOAc/PE (2.54 g, 73%), mp 166–167°C; R_fI 0.85, R_fII 0.95, R_fIII 0.35; $[\alpha]_D^{20}=-8.8$ (*c* 0.5, MeOH); HPLC: R_t 11.31 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3351, 3321, 1742, 1669, 1641, 1529 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.38–7.18 (m, 12H, Phe N*H*, Aib^4 N*H*, and Phe and Z aromatic C*H*), 7.13 (d, 1H, *J*=6.9 Hz, Leu^3 N*H*), 6.59 (s, 1H, Aib^2 N*H*), 5.48 (d, 1H, *J*=4.6 Hz, Leu^1 N*H*), 5.05 (m, 2H, Z CH_2), 4.81 (m, 1H, Phe αC*H*), 4.24 (m, 1H, Leu^3 αC*H*), 3.98 (m, 1H, Leu^1 αC*H*), 3.65 (s, 3H, OMe CH_3), 3.11 (m, 2H, Phe βCH_2), 1.76–1.60 (6H, 2Leu βCH_2 and γC*H*), 1.58, 1.55, 1.52 and 1.44 (4s, 12H, 2Aib βCH_3),

1.00–0.88 (12H, 2Leu δCH_3); HRMS (FAB): M^+, found 710.4097. $C_{38}H_{56}N_5O_8$ requires 710.4129.

4.1.4. Ac-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe. A solution of H-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (0.5 g, 0.7 mmol) in MeOH) in CH_2Cl_2 (2 mL) was treated with an excess of Ac_2O. The reaction was stirred at room temperature for 3 h. Then, the solvent was removed and the residue flushed several times with toluene. The crude title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (92:8)) and crystallized as a white solid from $CHCl_3$/PE (0.41 g, 94%), mp 208–209°C; R_fI 0.60, R_fII 0.90, R_fIII 0.25; $[\alpha]_D^{20}=-18.0$ (*c* 0.5, MeOH); HPLC: R_t 5.03 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3321, 1732, 1666, 1530 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.40 (d, 1H, *J*=10.0 Hz, N*H*), 7.31 (s, 1H, Aib N*H*), 7.21 (m, 5H, Phe aromatic C*H*), 7.03 (d, 1H, *J*=7.5 Hz, N*H*), 6.69 (s, 1H, Aib N*H*), 6.54 (d, 1H, *J*=5.0 Hz, N*H*), 4.76 (m, 1H, αC*H*), 4.23 (m, 1H, αC*H*), 4.05 (m, 1H, αC*H*), 3.63 (s, 3H, OMe CH_3), 3.09 (m, 2H, Phe βCH_2), 1.99 (s, 3H, Ac CH_3), 1.80–1.65 (6H, 2Leu βCH_2 and γC*H*), 1.51, 1.49, 1.44 and 1.41 (4s, 12H, 2Aib βCH_3), 0.98–0.88 (m, 12H, 2Leu δCH_3); Amino acid analysis: Aib 1.88, Leu 2.10, Phe 1.02; HRMS (FAB): M^+, found 618.3906. $C_{32}H_{52}N_5O_7$ requires 618.3867.

4.1.5. *n*-Oct-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe. To a solution of *n*-Oct-OH (0.12 mL, 0.8 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (0.1 g, 0.7 mmol) and EDC·HCl (0.15 g, 0.8 mmol) were added. After 10 min H-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (0.5 g, 0.7 mmol) in MeOH) and NMM (0.16 mL, 1.4 mmol) were added and the reaction was stirred at room temperature for 24 h. Then, the solvent was removed and the residue dissolved in EtOAc. The solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was crystallized as a white solid from $CHCl_3$/PE (0.42 g, 85%), mp 188–189°C; R_fI 0.80, R_fII 0.95, R_fIII 0.30; $[\alpha]_D^{20}=-15.6$ (*c* 0.5, MeOH); HPLC: R_t 16.70 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3330, 1730, 1658, 1532 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.40 (d, *J*=8.1 Hz, 1H, Phe N*H*), 7.37 (s, 1H, Aib^4 N*H*), 7.26–7.18 (m, 5H, Phe aromatic C*H*), 7.17 (d, 1H, *J*=7.6 Hz, Leu^3 N*H*), 6.69 (s, 1H, Aib^2 N*H*), 6.56 (broad s, 1H, Leu^1 N*H*), 4.74 (m, 1H, Phe αC*H*), 4.23 (m, 1H, Leu^3 αC*H*), 4.05 (m, 1H, Leu^1 αC*H*), 3.62 (s, 3H, OMe CH_3), 3.10 (m, 2H, Phe βCH_2), 2.23 (m, 2H, *n*-Oct αCH_2), 1.82–1.55 (6H, 2Leu βCH_2 and γC*H*), 1.53 and 1.39 (2s, 6H, Aib^2 βCH_3), 1.51 and 1.45 (2s, 6H, Aib^4 βCH_3), 1.27 (10H, *n*-Oct (CH_2)$_5$), 0.99–0.84 (m, 15H, 2Leu δCH_3 and *n*-Oct ωCH_3); HRMS (FAB): M^+, found 702.4796. $C_{38}H_{64}N_5O_7$ requires 702.4806.

4.1.6. Ac-L-Leu-Aib-L-Leu-Aib-L-Phol (peptaibolin). To a solution of Ac-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe (0.3 g, 0.5 mmol) in dry THF (freshly distilled over $LiAlH_4$), a solution of $LiBH_4$ in THF (3 mL) was added. The reaction was stirred at room temperature for 20 min. Then, cold EtOAc was added and the excess of $LiBH_4$ was neutralized by addition of 10% $KHSO_4$. The organic layer was washed with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (91:9)) and crystallized as a white solid from $CHCl_3$/PE (0.23 g, 77%), mp 224–225°C; R_fI 0.50, R_fII 0.90, R_fIII 0.10; $[\alpha]_D^{20}=-26.0$ (*c* 0.5, MeOH); HPLC: R_t 4.23 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3326, 1653, 1539 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.91 (d, 1H, *J*=4.5 Hz, Leu^1 N*H*), 7.75 (s, 1H, Aib^2 N*H*), 7.52 (s, 1H, Aib^4 N*H*), 7.39 (d, 1H, *J*=6.3 Hz, Leu^3 N*H*), 7.17 (m, 5H, Phol aromatic C*H*), 6.97 (d, 1H, *J*=8.9 Hz, Phol N*H*), 4.04 (m, 1H, Phol αC*H*), 4.01 (m, 1H, Leu^1 αC*H*), 3.95 (m, 1H, Leu^3 αC*H*), 3.60 (d, 2H, *J*=5.3 Hz, Phol CH_2OH), 2.81 (m, 2H, Phol βCH_2), 2.02 (s, 3H, Ac CH_3), 1.75 (m, 2H, 2Leu γC*H*), 1.57–1.55 (m, 4H, 2Leu βCH_2), 1.51, 1.43, 1.40 and 1.34 (4s, 12H, 2Aib βCH_3), 0.99 (d, 3H, *J*=6.5 Hz, Leu^1 δCH_3), 0.96 (d, 3H, *J*=7.7 Hz, Leu^3 δCH_3), 0.94 (d, 3H, *J*=6.8 Hz, Leu^1 δCH_3), 0.88 (d, 3H, *J*=6.1 Hz, Leu^3 δCH_3); Amino acid analysis: Aib 1.98, Leu 2.02; HRMS (FAB): M^+, found 590.3954. $C_{31}H_{52}N_5O_6$ requires 590.3918.

4.1.7. *n*-Oct-L-Leu-Aib-L-Leu-Aib-L-Phol. To a solution of *n*-Oct-L-Leu-Aib-L-Leu-Aib-L-Phe-OMe (0.4 g, 0.5 mmol) in dry THF (freshly distilled over $LiAlH_4$), a solution of $LiBH_4$ in THF (3 mL) was added. The reaction was stirred at room temperature for 20 min. Then, cold EtOAc was added and the excess of $LiBH_4$ was neutralized by addition of 10% $KHSO_4$. The organic layer was washed with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: CH_2Cl_2/EtOH (93:7)) and crystallized as a white solid from $CHCl_3$/PE (0.29 g, 87%), mp 185–186°C; R_fI 0.65, R_fII 0.95, R_fIII 0.20; $[\alpha]_D^{20}=-19.0$ (*c* 0.5, MeOH); HPLC: R_t 15.40 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3290, 1652, 1537 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.90 (m, 3H, 3N*H*), 7.37 (d, 1H, *J*=5.0 Hz, N*H*), 7.26–7.13 (6H, Phol aromatic C*H* and 1N*H*), 4.30 (m, 1H, αC*H*), 4.13–3.95 (m, 1H, αC*H*), 3.66 (m, 1H, αC*H*), 2.77 (m, 2H, CH_2), 2.37 (m, 2H, CH_2), 1.82–1.63 (8H, 2Leu βCH_2 and γC*H* and *n*-Oct αCH_2), 1.52, 1.48, 1.38 and 1.35 (4s, 12H, 2Aib βCH_3), 1.27–1.18 (m, 10H, *n*-Oct (CH_2)$_5$), 1.00–0.85 (m, 15H, 2Leu δCH_3 and *n*-Oct ωCH_3); HRMS (FAB): M^+, found 674.4860. $C_{37}H_{64}N_5O_6$ requires 674.4857.

4.1.8. Z-L-Iva-L-Phe-OMe. To a solution of Z-L-Iva-OH[35] (3.2 g, 13 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (1.7 g, 13 mmol) and EDC·HCl (2.7 g, 14 mmol) were added. After 10 min HCl·H-L-Phe-OMe (2.8 g, 13 mmol), neutralized with NMM (2.9 mL, 26 mmol), was added and the reaction mixture stirred at room temperature for 12 h. Then, EtOAc was added and the solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: EtOAc/PE (2:3)) and crystallized as a white solid from EtOAc/PE (3.91 g, 73%), mp 95–96°C; R_fI 0.95, R_fII

0.95, R_fIII 0.65; $[\alpha]_D^{20}=-8.2$ (*c* 0.5, MeOH); ν_{max} (KBr) 3319, 1747, 1658, 1535 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.34 (m, 5H, Z aromatic C*H*), 7.25 (m, 5H, Phe aromatic C*H*), 6.46 (d, 1H, *J*=7.7 Hz, Phe N*H*), 5.56 (s, 1H, Iva N*H*), 5.07 (s, 2H, Z CH_2), 4.88 (m, 1H, Phe αC*H*), 3.72 (s, 3H, OMe CH_3), 3.12 (m, 2H, Phe CH_2), 2.08 and 1.70 (2m, 2H, Iva βCH_2), 1.45 (m, 3H, Iva βCH_3), 0.77 (t, 3H, *J*=7.4 Hz, Iva γCH_3); HRMS (FAB): M^+, found 413.2070. $C_{23}H_{29}N_2O_5$ requires 413.2076.

4.1.9. Z-L-Leu-L-Iva-L-Phe-OMe. To a solution of Z-L-Leu-OH (2.6 g, 9.9 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (1.2 g, 9.0 mmol) and EDC·HCl (1.9 g, 9.9 mmol) were added. After 10 min H-L-Iva-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (3.7 g, 9.0 mmol) in MeOH) and NMM (2.0 mL, 18.1 mmol) were added and the reaction was stirred at room temperature for 12 h. Then, EtOAc was added and the solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (99:1)) and crystallized as a white solid from EtOAc/PE (3.45 g, 73%), mp 51–52°C; R_fI 0.95, R_fII 0.95, R_fIII 0.50; $[\alpha]_D^{20}=-26.2$ (*c* 0.5, MeOH); ν_{max} (KBr) 3328, 1737, 1731, 1672, 1651, 1527 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.35 (m, 5H, Z aromatic C*H*), 7.12 (m, 5H, Phe aromatic C*H*), 6.80 (s, 1H, Iva N*H*), 6.52 (d, 1H, *J*=9.2 Hz, Phe N*H*), 5.11 (m, 3H, Leu N*H* and Z CH_2), 4.85 (m, 1H, Phe αC*H*), 4.11 (m, 1H, Leu αC*H*), 3.72 (s, 3H, OMe CH_3), 3.13 (m, 2H, Phe βCH_2), 2.21 and 1.64 (2m, 2H, Iva βCH_2), 1.62–1.44 (m, 3H, Leu βCH_2 and γC*H*), 1.47 (s, 3H, Iva βCH_3), 0.96–0.93 (m, 6H, Leu δCH_3), 0.72 (t, 3H, *J*=9.2 Hz, Iva γCH_3); HRMS (FAB): M^+, found 526.2940. $C_{29}H_{40}N_3O_6$ requires 526.2917.

4.1.10. Z-L-Iva-L-Leu-L-Iva-L-Phe-OMe. To a solution of Z-L-Iva-OH (1.5 g, 6.1 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (0.8 g, 5.5 mmol) and EDC·HCl (1.2 g, 6.1 mmol) were added. After 10 min H-L-Leu-L-Iva-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (2.9 g, 5.5 mmol) in MeOH) and NMM (1.2 mL, 11.1 mmol) were added and the reaction was stirred a room temperature for 24 h. Then, EtOAc was added and the solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (98:2)) and crystallized as a white solid from EtOAc/PE (3.02 g, 88%), mp 60–61°C; R_fI 0.90, R_fII 0.95, R_fIII 0.40; $[\alpha]_D^{20}=-37.6$ (*c* 0.5, MeOH); HPLC: R_t 14.07 min (Phenomenex-Rp C_{18}; gradient: 65–85% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3333, 1733, 1705, 1659, 1523 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.35 (m, 5H, Z aromatic C*H*), 7.25 (m, 5H, Phe aromatic C*H*), 6.99 (m, 2H, Phe N*H* and Iva[3] N*H*), 6.36 (d, 1H, *J*=8.9 Hz, Leu N*H*), 5.29 (s, 1H, Iva[1] N*H*), 5.08 (s, 2H, Z CH_2), 4.84 (m, 1H, Phe αC*H*), 4.30 (m, 1H, Leu αC*H*), 3.68 (s, 3H, OMe CH_3), 3.14 (m, 2H, Phe βCH_2), 1.86–1.39 (m, 7H, 2Iva βCH_2, Leu βCH_2 and γC*H*), 1.52 and 1.44 (2s, 6H, 2Iva βCH_3), 0.90 (m, 9H, Leu δCH_3 and Iva γCH_3), 0.71 (t, 3H, *J*=9.3 Hz, Iva γCH_3); HRMS (FAB): M^+, found 625.3606. $C_{34}H_{49}N_4O_7$ requires 625.3601.

4.1.11. Z-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe. To a solution of Z-L-Leu-OH (1.4 g, 5.3 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (0.7 g, 4.9 mmol) and EDC·HCl (1.0 g, 5.3 mmol) were added. After 10 min H-L-Iva-L-Leu-L-Iva-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (3.0 g, 4.9 mmol) in MeOH) and NMM (1.1 mL, 9.7 mmol) were added and the reaction was stirred at room temperature for 12 h. Then, the solvent was evaporated and the residue dissolved in EtOAc. The solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63; eluant: $CHCl_3$/EtOH (97:3)) and crystallized as a white solid from EtOAc/PE (2.82 g, 78%), mp 156–157°C; R_fI 0.90, R_fII 0.95, R_fIII 0.35; $[\alpha]_D^{20}=-19.2$ (*c* 0.5, MeOH); HPLC: R_t 15.15 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3322, 1743, 1658, 1528 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.34 (m, 5H, Z aromatic C*H*), 7.20 (m, 7H, 2N*H* and Phe aromatic C*H*), 7.07 (d, 1H, *J*=6.9 Hz, N*H*), 6.51 (s, 1H, Iva N*H*), 5.39 (d, 1H, *J*=3.1 Hz, Leu[1] N*H*), 5.08 (m, 2H, Z CH_2), 4.80 (m, 1H, αC*H*), 4.24 (m, 1H, αC*H*), 3.98 (m, 1H, αC*H*), 3.66 (s, 3H, OMe CH_3), 3.09 (m, 2H, Phe βCH_2), 1.92–1.63 (m, 10H, 2Iva βCH_2, 2Leu βCH_2 and γC*H*), 1.52 and 1.46 (2s, 6H, 2Iva βCH_3), 0.99–0.89 (m, 12H, 2Leu δCH_3), 0.81 (t, 3H, *J*=7.4 Hz, Iva γCH_3), 0.71 (t, 6H, *J*=7.5 Hz, Iva γCH_3); HRMS (FAB): M^+, found 738.4456. $C_{40}H_{60}N_5O_8$ requires 738.4442.

4.1.12. Ac-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe. A solution of H-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z derivative (0.6 g, 0.8 mmol) in MeOH) in CH_2Cl_2 (2 mL) was treated with an excess of Ac_2O. The reaction was stirred at room temperature for 3 h. Then, the solvent was removed and the residue flushed several times with toluene. The crude title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: $CHCl_3$/EtOH (97:3)) and crystallized as a white solid from CH_2Cl_2/PE (0.45 g, 87%), mp 215–216°C; R_fI 0.65, R_fII 0.90, R_fIII 0.25; $[\alpha]_D^{20}=-37.6$ (*c* 0.5, MeOH); HPLC: R_t 6.93 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3313, 1732, 1658, 1531 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.22–7.17 (6H, 1 N*H* and Phe aromatic C*H*), 7.11 (s, 1H, Iva N*H*), 6.99 (d, 1H, *J*=7.5 Hz, N*H*), 6.53 (s, 1H, Iva N*H*), 6.23 (d, 1H, *J*=5.0 Hz, N*H*), 4.79 (m, 1H, αC*H*), 4.23 (m, 1H, αC*H*), 4.13 (m, 1H, αC*H*), 3.66 (s, 3H, OMe CH_3), 3.10 (m, 2H, Phe βCH_2), 2.02 (s, 3H, Ac CH_3), 1.96–1.63 (10H, 2Iva βCH_2, 2Leu β-CH_2 and γC*H*), 1.49 and 1.44 (2s, 6H, 2Iva βCH_3), 0.98–0.85 (15H, 2Leu δCH_3 and Iva γCH_3), 0.71 (t, 3H, *J*=7.5 Hz, Iva γCH_3); Amino acid analysis: Iva 2.10, Leu 1.92, Phe 0.95; HRMS (FAB): M^+, found 646.4166. $C_{34}H_{56}N_5O_7$ requires 646.4180.

4.1.13. *n*-Oct-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe. To a solution of *n*-Oct-OH (0.14 mL, 0.9 mmol) in CH_2Cl_2 cooled to 0°C, HOAt (0.1 g, 0.8 mmol) and EDC·HCl (0.12 g, 0.9 mmol) were added. After 10 min H-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe (obtained by catalytic hydrogenation of the above-mentioned Z-derivative (0.6 g,

0.8 mmol) in MeOH) and NMM (0.2 mL, 1.7 mmol) were added and the reaction was stirred at room temperature for 24 h. Then, the solvent was removed and the residue dissolved in EtOAc. The solution was extracted with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and dried over anhydrous Na_2SO_4. The title compound was crystallized as a white solid from $CHCl_3$/PE (0.47 g, 81%), mp 198–199°C; R_fI 0.85, R_fII 0.95, R_fIII 0.30; $[\alpha]_D^{20}=-28.6$ (*c* 0.5, MeOH); HPLC: R_t 14.41 min (Phenomenex-Rp C_{18}; gradient: 80–100% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3305, 1732, 1657, 1532 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.40 (d, 1H, *J*=7.1 Hz, N*H*), 7.37 (s, 1H, Iva N*H*), 7.32 (m, 5H, Phe aromatic C*H*), 7.23 (d, 1H, *J*=2.9 Hz, N*H*), 6.75 (d, 1H, *J*=5.0 Hz, N*H*), 6.73 (s, 1H, Iva N*H*), 4.72 (m, 1H, αC*H*), 4.23 (m, 1H, αC*H*), 4.05 (m, 1H, αC*H*), 3.66 (s, 3H, OMe CH_3), 3.12 (m, 2H, Phe βCH_2), 2.26 (t, 2H, *J*=7.5 Hz, *n*-Oct αCH_2), 2.02–1.63 (10H, 2Iva βCH_2, 2Leu βCH_2 and γC*H*), 1.51 and 1.47 (2s, 6H, 2Iva βCH_3), 1.27 (m, 10H, *n*-Oct (CH_2)$_5$), 0.99–0.86 (m, 18H, 2Leu δCH_3, *n*-Oct ωCH_3 and Iva γCH_3), 0.74 (t, 3H, *J*=7.9 Hz, Iva γCH_3); HRMS (FAB): M^+, found 730.5089. $C_{40}H_{68}N_5O_7$ requires 730.5119.

4.1.14. Ac-L-Leu-L-Iva-L-Leu-L-Iva-L-Phol. To a solution of Ac-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe (0.2 g, 0.3 mmol) in dry THF (freshly distilled over $LiAlH_4$), a solution of $LiBH_4$ in THF (3 mL) was added. The reaction was stirred at room temperature for 20 min. Then, cold EtOAc was added and the excess of $LiBH_4$ was neutralized by addition of 10% $KHSO_4$. The organic layer was washed with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: CH_2Cl_2/EtOH (95:5)) and crystallized as a white solid from CH_2Cl_2/PE (0.14 g, 78%), mp 235–236°C; R_fI 0.50, R_fII 0.90, R_fIII 0.10; $[\alpha]_D^{20}=-31.0$ (*c* 0.5, MeOH); HPLC: R_t 5.90 min (Phenomenex-Rp C_{18}; gradient: 70–90% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3298, 1652, 1537 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 8.17 (d, 1H, *J*=2.5 Hz, N*H*), 7.92 (s, 1H, Iva N*H*), 7.78 (s, 1H, Iva N*H*), 7.38 (d, 1H, *J*=7.5 Hz, N*H*), 7.22 (m, 5H, Phol aromatic C*H*), 7.09 (d, 1H, *J*=10.0 Hz, N*H*), 4.36–3.67 (m, 5H, 3 αC*H* and Phol CH_2OH), 2.75 (m, 2H, Phol βCH_2), 2.12 (s, 3H, Ac CH_3), 1.85–1.60 (10H, 2Iva βCH_2, 2Leu βCH_2 and γC*H*), 1.52 and 1.39 (2s, 6H, 2Iva βCH_3), 0.97–0.87 (12H, 2Leu δCH_3), 0.81 (t, 3H, *J*=7.8 Hz, Iva γCH_3), 0.63 (t, 3H, *J*=7.5 Hz, Iva γCH_3); Amino acid analysis: Iva 2.08, Leu 1.96; HRMS (FAB): M^+, found 618.4221. $C_{33}H_{56}N_5O_6$ requires 618.4231.

4.1.15. *n*-Oct-L-Leu-L-Iva-L-Leu-L-Iva-L-Phol. To a solution of *n*-Oct-L-Leu-L-Iva-L-Leu-L-Iva-L-Phe-OMe (0.3 g, 0.4 mmol) in dry THF (freshly distilled over $LiAlH_4$), a solution of $LiBH_4$ in THF (3 mL) was added. The reaction was stirred at room temperature for 20 min. Then, cold EtOAc was added and the excess of $LiBH_4$ was neutralized by addition of 10% $KHSO_4$. The organic layer was washed with 10% $KHSO_4$, H_2O, 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. The title compound was purified by flash chromatography (ICN silica 32–63, 60A; eluant: CH_2Cl_2/EtOH (97:3)) and crystallized as a white solid from $CHCl_3$/PE (0.23 g, 81%), mp 218–219°C; R_fI 0.60, R_fII 0.95, R_fIII 0.20; $[\alpha]_D^{20}=-25.8$ (*c* 0.5, MeOH); HPLC: R_t 14.01 min (Phenomenex-Rp C_{18}; gradient: 80–100% B in 20 min; eluants: A, 0.05% TFA in H_2O, and B, 0.05% TFA in CH_3CN/H_2O (9:1)); ν_{max} (KBr) 3287, 1650, 1538 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 7.97 (broad s, 1H, N*H*), 7.84 (m, 2H, 2 N*H*), 7.38 (d, 1H, *J*=5.0 Hz, N*H*), 7.22 (m, 5H, Phol aromatic C*H*), 7.05 (d, 1H, *J*=7.7 Hz, N*H*), 4.42–3.60 (m, 5H, 3 αC*H* and Phol CH_2OH), 2.75 (m, 2H, Phol βCH_2), 2.38 (t, 2H, *J*=6.9 Hz, *n*-Oct αCH_2), 1.74–1.68 (10H, 2Leu βCH_2 and γC*H*, 2Iva βCH_2), 1.52 and 1.39 (2s, 6H, Iva βCH_3), 1.27 (m, 10H, *n*-Oct (CH_2)$_5$), 1.00–0.81 (m, 18H, 2Leu δCH_3, *n*-Oct ωCH_3 and Iva γCH_3), 0.60 (t, 3H, *J*=7.3 Hz, Iva γCH_3); HRMS (FAB): M^+, found 702.5188. $C_{39}H_{68}N_5O_6$ requires 702.5170.

4.2. FT-IR absorption

The solid-state infrared absorption spectra (KBr disk technique) were recorded with a Perkin–Elmer model 580 B spectrophotometer equipped with a Perkin–Elmer model 3600 IR data station. The solution IR absorption spectra were recorded using a Perkin–Elmer model 1720 X FT-IR spectrophotometer, nitrogen-flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Solvent (baseline) spectra were obtained under the same conditions. Cell with path lengths of 0.1, 1.0 and 10 mm (with CaF_2 windows) were used. Spectrograde deuterochloroform (99.8% D) was purchased from Fluka.

4.3. Nuclear magnetic resonance

The ^1H NMR spectra were recorded either with a Bruker model AC 250 spectrometer or with a Bruker model AM 400 spectrometer. Measurements were carried out in deuterochloroform (99.96% D; Aldrich) and deuterated DMSO (99.96% D_6; Acros Organics) with tetramethylsilane as the internal standard.

4.4. Mass spectrometry

Fast Atom Bombardment (FAB) mass spectrometry was carried out using a JEOL SX/SX model 102A four-sector tandem mass spectrometer ($B_1E_1B_2E_2$ geometry), coupled to a JEOL MS/MP9021D/UPD data system. The samples were loaded in a nitrobenzyl alcohol solution onto a stainless steel probe and bombarded with xenon atoms with an energy of 8 keV.

During the high-resolution FABMS measurements a resolving power of 5,000–10,000 (10% valley definition) was used. Polyethyleneglycols (PEG) 300 and 600 were used to calibrate the mass spectrometer.

4.5. X-Ray diffraction

Single crystals of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe, Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe, Ac-(L-Leu-Aib)$_2$-L-Phol (peptaibolin), and Ac-(L-Leu-L-Iva)$_2$-L-Phol were obtained by slow evaporation at room temperature from an MeOH/H_2O (the former peptide) or an MeOH (the other three peptides) solution. Intensity data collection was performed

by using a Philips PW 1100 four-circle diffractometer. Graphite-monochromated Cu Kα radiation (λ=1.54178 Å) and $\theta/2\theta$ scan mode were used. Cell parameters were obtained by least-squares refinements of the angular setting of 48 carefully centred high angle reflections. The structures were solved by direct methods (SHELXS 97[57] program). Refinement was carried out by the full-matrix block least-squares procedure on F^2, with all non-H atoms anisotropic, by application of the SHELXL 97[58] program.

The crystals of *n*-Oct-(L-Leu-Aib)$_2$-L-Phe-OMe did not significantly diffract above θ=50° (1.0 Å resolution). The positional parameters and the anisotropic displacements of the non-H atoms were allowed to refine at alternate cycles. H atoms were calculated at idealized positions. During the refinement the H atoms were allowed to ride on their carrying atom, with U_{iso} set equal to 1.2 (or 1.5 for methyl groups) times the $U_{equiv.}$ of the parent atom.

The trial structure of Ac-(L-Leu-L-Iva)$_2$-L-Phe-OMe with the best combined figure of merit allowed the location of 41 (out of 46) non-H atoms. The positions of the remaining non-H atoms were recovered from subsequent difference Fourier maps. The positional parameters and the anisotropic displacements of the non-H atoms were allowed to refine at alternate cycles. The side chain of the Leu(1) residue is disordered. Its C^{γ} and C^{δ} atoms were refined on two sets of positions (atoms C1G, C1D1, C1D2, and atoms C1G′, C1D3, C1D4) with population parameters of 0.65 and 0.35, respectively. A disorder was also observed at the C^{γ} atom of the Iva(2) residue, which was refined on two positions (atoms C2G and C2G′) with population parameters of 0.77 and 0.23, respectively. A number of restraints were applied to the 1–2 and 1–3 interatomic distances involving atoms of the disordered side chains. The anisotropic displacement parameters of the same atoms were also restrained to approach isotropic behaviour. The phenyl ring of the Phe(5) residue was constrained to the idealized geometry. H atoms were calculated at idealized positions and refined by allowing them to ride on their carrying atom, with U_{iso} set equal to 1.2 (or 1.5 for methyl groups) times the $U_{equiv.}$ of the parent atom.

The solution of the structure of Ac-(L-Leu-Aib)$_2$-L-Phol was somewhat complicated by the low diffracting power of the crystal, which in turn has to be ascribed to the reduced crystal thickness (only 0.05 mm). Indeed, the fraction of reflections having I≥4σ(I) was 25% in the resolution range 1.2–1.1 Å, while it was 5% between 1.1 and 1.0 Å. Eventually, the structure was solved by using 422 reflections for the phasing process and 554 reflections (E≥1.1) for the structure expansion with the tangent formula. The trial structure with the best combined figure of merit allowed the location of 23 out of 42 non-H atoms. The remaining non-H atoms were recovered from subsequent difference Fourier maps. The positional parameters and the anisotropic displacements of the non-H atoms were allowed to refine at alternate cycles. The phenyl ring of the Phol residue was constrained to the idealized geometry. Restraints were applied to most of the bond distances, the chiral volume of the carbonyl carbon atoms, and the anisotropic displacement parameters to approach isotropic behaviour. H atoms were calculated at idealized positions. The position of the H atom of the C-terminal alcoholic function was selected as the one able to form the best H-bond among the three possible staggered orientations relative to the C5–O5 bond. During the refinement all H atoms were allowed to ride on their carrying atom, with U_{iso} set equal to 1.2 (or 1.5 for the hydroxyl and methyl groups) times the $U_{equiv.}$ of the parent atom.

The trial structure of Ac-(L-Leu-L-Iva)$_2$-L-Phol having the best combined figure of merit allowed the location of all non-H atoms with the exception of those belonging to the disordered parts of the side chains of Leu(1) and Iva(2) residues. The positions of the latter atoms were recovered from subsequent difference Fourier maps. The positional parameters and the anisotropic displacements of the non-H atoms were allowed to refine at alternate cycles. The disordered C^{γ}, $C^{\delta 1}$ and $C^{\delta 2}$ atoms of the Leu(1) side chain were refined on two sets of positions (atoms C1G, C1D1, C1D2, and atoms C1G′, C1D3, C1D4) with population parameters of 0.65 and 0.35, respectively. The phenyl ring of the Phol residue was constrained to the idealized geometry. Restraints were applied to most of the bond distances, the chiral volume of the carbonyl carbon atoms, and the anisotropic displacement parameters to approach isotropic behaviour. H atoms were calculated at idealized positions. During the refinement they were allowed to ride on their carrying atom, with U_{iso} set equal to 1.2 (or 1.5 for the hydroxyl and methyl groups) times the $U_{equiv.}$ of the parent atom.

4.6. Liposome leakage assay

Peptide-induced leakage from egg PC vesicles was measured at 20°C using the CF-entrapped vesicle technique.[49,50] CF-encapsulated small unilamellar vesicles (egg PC/cholesterol, 70:30) were prepared by sonication in Hepes buffer, pH 7.4. The phospholipid concentration was kept constant (0.06 mM), and increasing [peptide]/[lipid] molar ratios (R^{-1}) were obtained by adding aliquots of MeOH solutions of peptides, keeping the final MeOH concentration below 5% by volume. After rapid and vigorous stirring the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (6 nm band pass) with λ_{exc} 488 nm (3 nm band pass). The percentage of released CF at time t was determined as $(F_t-F_0)/(F_T-F_0)\times 100$, with F_0 the fluorescence intensity of vesicles in the absence of peptide, F_t the fluorescence intensity at time t in the presence of peptide, and F_T the total fluorescence intensity determined by disrupting the vesicles by addition of 50 μL of a 10% Triton X-100 solution. The kinetics were stopped at 20 min.

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