Tetrahedron 72 (2016) 1024–1030

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Peptaibolin analogues by incorporation of α,α-dialkylglycines: synthesis and study of their membrane permeating ability

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ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form 28 December 2015
Accepted 29 December 2015
Available online 31 December 2015

Keywords:
α,α-Dialkylglycines
Peptaibolin
Membrane permeating peptides
Model membranes
Fluorescence spectroscopy

ABSTRACT

Analogues of Peptaibolin, a peptaibol with antibiotic activity, incorporating α,α-dialkylglycines (Deg, Dpg, and Ac_6c) at selected positions were synthesised by MW-SPPS and fully characterized. A control analogue incorporating L-alanine was also prepared. The native peptide and the analogues were studied by fluorescence spectroscopy for their membrane permeating activity. Small unilamellar vesicles (SUVs) of egg phosphatidylcholine/cholesterol (70:30) containing an encapsulated fluorescence probe (6-carboxyfluorescein) were used as membrane models. The assays of carboxyfluorescein release from SUVs upon peptide addition showed that Peptaibolin-Dpg and Peptaibolin-Ac_6c are the most active peptides. These results indicate that the structure of the α,α-dialkylglycines is crucial for the membrane permeating ability of these Peptaibolin analogues.

1. Introduction

Microbial resistance to classical antibiotics is a serious worldwide problem and as such there is a fast-growing interest in the design and synthesis of new therapeutic agents as alternatives to the established drugs. To address this issue the scientific community has been focussing on natural antimicrobial peptides (AMPs), which are ubiquitous components of the innate defence mechanisms of animals, plants and microorganisms, known to be active against bacteria, fungi and protozoans.[1,2] AMPs present a large variety of sizes, amino acid composition and secondary structures,[3,4] and can interfere with several intercellular processes.[2,5] Nevertheless, most AMPs present a cationic and hydrophobic nature that allows them to adopt amphipathic conformations. This enables their interaction with microbial membranes and leads to the disruption of membrane integrity either by pore formation that allows the leakage of cell contents, or by acting in a detergent like manner.[6–11] Despite the fact that some microorganisms are known to resist AMPs action,[12,13] due to their broad spectrum of action AMPs are considered lead compounds for the development of a new class of antibiotic pharmaceuticals.[14–16]

Among the various classes of peptides with recognized antibiotic activity one can find peptaibols. This family of naturally occurring AMPs isolated from soil fungi has interesting structural features: they all bear a C-terminal amino alcohol and a variable number of α,α-dialkylglycines in their composition.[17,18] The major α,α-dialkylglycine present is α-aminoisobutyric acid (Aib) but isovaline (Iva) and α,α-diethylglycine (Deg) have also been detected.[19,20] This class of amino acids is frequently used in the construction of peptides with pharmacological interest since they are not recognized by hydrolytic enzymes, thus rendering peptides more resistant to biodegradation and increasing their bioavailability.[21,22] Another remarkable feature about α,α-dialkylglycines is the tetrasubstitution at the central carbon atom that results in the restriction of the conformational space available around peptide bonds, thus yielding peptides with more defined conformations.[23–28] Albeit interesting this is also responsible for the fact that the synthesis of peptide analogues bearing these amino acids is usually a major synthetic challenge that can only be overcome by taking advantage of synthetic methodologies more recently used in peptide chemistry.[29–32]

Peptaibolin (Ac-Leu-Aib-Leu-Aib-Phol) is the smallest member of the peptaibols family. This five-residue peptide was first isolated by Hülsmann et al. from *Sepedonium* sp. and *Sepedonium ampullosporum*[33] and later synthesized using solution[34] or solid phase strategies.[30] Although initial findings suggested that Peptaibolin and its Iva analogue (with Iva at the position of Aib) possessed no affinity towards membranes,[34] recent *in silico* studies suggest that membrane affinity might be increased by the substitution of the

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http://dx.doi.org/10.1016/j.tet.2015.12.079
0040-4020/

Aib residues by more structurally constrained and more hydrophobic α,α-dialkylglycines.[27]

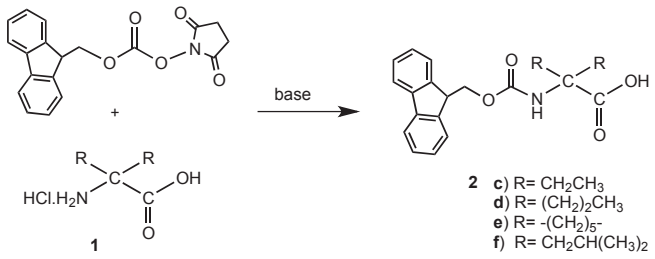
Bearing the above facts in mind and our interest in the synthesis and application of α,α-dialkylglycines,[35–37] herein we report for the first time the synthesis of several Peptaibolin analogues bearing different α,α-dialkylglycines at the positions of the Aib residues and the evaluation of their membrane permeating ability by carboxyfluorescein leakage assays from model membranes (small unilamellar vesicles of egg phosphatidylcholine/cholesterol, 70:30).

2. Results and discussion

2.1. Synthesis of native Peptaibolin and Peptaibolin analogues

The synthesis of Peptaibolin analogues **3c**–**e** by substitution of the native Aib residues by other α,α-dialkylglycines was envisaged in order to obtain information about the influence of the different side chains in terms of length and bulk on the membrane permeating activity of the resulting peptides. The native Peptaibolin, Ac-Leu-Aib-Leu-Aib-Phol, **3a** was synthesized to act as a positive control for the subsequent membrane permeation studies and the Peptaibolin analogue bearing an alanine instead of Aib, Ac-Leu-Ala-Leu-Ala-Phol **3b** was thought as the negative control (Fig. 1). The synthesis of the peptides was carried out by a microwave-assisted solid phase peptide synthesis (MW-SPPS) protocol.

*2.1.1. Synthesis of Fmoc-α,α-dialkylglycines **2c**–**f**.* Fmoc protected α,α-dialkylglycines **2c**–**f**, necessary for the Fmoc-based MW-SPPS protocol, were prepared except for Fmoc-α-aminoisobutyric acid **2a** (Fmoc-Aib-OH) and Fmoc-L-alanine **2b** (Fmoc-Ala-OH), used as commercially acquired. α,α-Dialkylglycines **1c**–**f**, obtained previously through a methodology based on an Ugi multicomponent reaction,[29,35,37] were reacted with *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu). This reaction was carried out in two different solvent mixtures, namely 5% sodium carbonate aqueous solution with 1,4-dioxane or acetone, depending on the solubility properties of the initial α,α-dialkylglycines **1c**–**f**. N-protection was achieved in good to excellent yields (66–98%) (Scheme 1) and the prepared amino acid derivatives were characterized by NMR spectroscopy and were in accordance with previously reported data.[38–40]



Scheme 1. Preparation of Fmoc-α,α-dialkylglycines **2c**–**f**.

*2.1.2. MW-SPP synthesis of peptides **3a**–**e**.* Peptides **3a**–**e** were synthesized on a manual microwave-assisted peptide synthesizer (CEM Discover). The amino acid coupling reactions were based on the Fmoc protocol and a preloaded Wang resin was used, namely Fmoc-Phe-Wang (with a functionalization degree of about 0.5–0.6 mmol/g). The preloaded and all other Fmoc protecting groups were removed with 20% piperidine in DMF solution using a standard MW protocol (30 s+3 min, 28 W, 75 °C). All Fmoc amino acids were dissolved in dry DMF containing *N,N′*-diisopropylcarbodiimide (DIC) and 6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt), before they were added to the Wang resin (a 5 equiv excess was used), and coupled using a standard MW protocol (5 min, 28 W, 75 °C). To ensure complete activation of the α,α-dialkylglycines, considering their stereochemical constraints, the activation mixture was allowed to react for 1.5–2 h, before being added to the resin and subjected to microwave irradiation. This ensures that the active esters are transformed into the corresponding oxazolinones before coupling.[41,42]

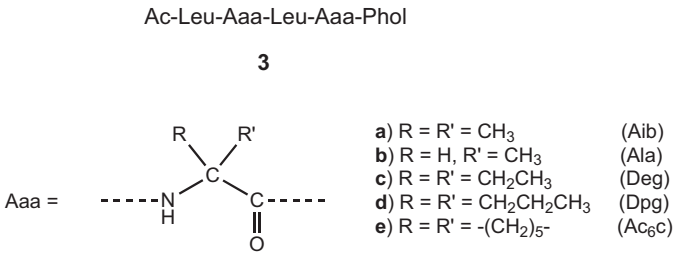
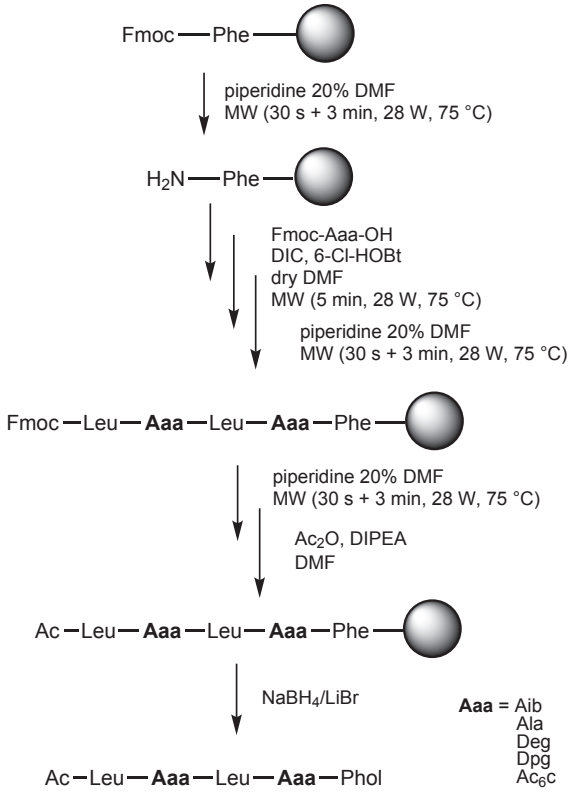


Fig. 1. Structure of Peptaibolin **3a** and its analogues **3b**–**e**.

After removal of the Fmoc group from the last amino acid in the sequence, the N-terminal was acetylated by treatment with acetic anhydride in the presence of a base (*N,N*-diisopropylethylamine, DIPEA). Finally, the peptide was cleaved from the resin by a reductive cleavage protocol using *in situ* generated lithium borohydride to yield the required C-terminal amino alcohol (phenylalaninol, Phol) (Scheme 2).



Scheme 2. MW-SPPS of Peptaibolin **3a** and its analogues **3b**–**e**.

The purity of the peptides was checked by analytical HPLC and samples of each peptide were purified by semi-preparative HPLC using a Europa Peptide C18 column and ACN/water mixtures (1:1 or 2:1) as eluent. The peptides were obtained in fair to excellent yields and characterized by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry (Table 1). In general, as α,α-dialkylglycines side chain length increases, a decrease in the yield of peptide obtained can be observed, except for Peptaibolin-Deg **3c**

Table 1
Synthesis and characterization data of Peptaibolin **3a** and analogues **3b**–**e**

Peptide		Yield (%)[a]	^{1}H and ^{13}C NMR		
			α-C Aaa[b] (ppm)	α-C*H* Phol (ppm)	C*H*$_2$OH (ppm)
3a	Ac-Leu-Aib-Leu-Aib-Phol	89	56.42, 56.88	4.23–4.34	3.60–3.72
3b	Ac-Leu-Ala-Leu-Ala-Phol	87	48.40	4.13–4.26	3.25–3.32
3c	Ac-Leu-Deg-Leu-Deg-Phol	22	63.85	4.01–4.12	3.25–3.33
3d	Ac-Leu-Dpg-Leu-Dpg-Phol	60	62.76, 62.87	4.72	3.20–3.30
3e	Ac-Leu-Ac$_6$c-Leu-Ac$_6$c-Phol	32	59.00, 59.36	4.12–4.18	3.21–3.33

[a] Relative to the resin initial loading and after preparative HPLC purification.
[b] Aaa refers to Ala or α,α-dialkylglycines at the native Aib positions.

whose lower yield is due to poor solubility properties. An attempt to use Fmoc-Dibg-OH in the synthesis of the corresponding Peptaibolin-Dibg derivative was made but only the dipeptide Ac-Dibg-Phol **3f** was isolated.

2.2. Permeation of model membranes by peptides 3a–e

Peptaibols are known to interact with phospholipid bilayers, by permeating its structure and allowing the leakage of the contents of the vesicle. Therefore, the permeating properties of Peptaibolin and its analogues were studied through the release of an entrapped fluorophore, carboxyfluorescein (CF), in small unilamellar vesicles (SUVs) of egg phosphatidylcholine/cholesterol 70:30, used as membrane models.[43,44] The percentage of CF release with increasing [peptide]/[lipid] ratio (until ca. 80), for the synthesized peptides **3a**–**e**, is shown in Fig. 2.

It can be observed that the analogues bearing 1-amino-1-cyclohexane carboxylic acid (Peptaibolin-Ac$_6$c) **3e** and α,α-dipropylglycine (Peptaibolin-Dpg) **3d** are the ones with larger permeation ability, that is always higher than for the native Peptaibolin **3a**, and is significant even at low [peptide] to [lipid] ratio. At high [peptide] to [lipid] ratio (above 80), the peptide containing α,α-diethylglycine (Peptaibolin-Deg) **3c** also becomes very active. As expected, a very low membrane permeating ability was detected for the analogue **3b** with L-alanine.

Recent molecular dynamics simulations[27] showed that membrane affinity might be increased by the substitution of the Aib residues by more structurally constrained and more hydrophobic α,α-dialkylglycines. In particular, it was shown that Peptaibolin-Ac$_6$c and also a peptide bearing α,α-dihexylglycine, its related acyclic side chain counterpart, were able to induce α-helix-type secondary structures of Peptaibolin in water, which are not present in the native structure, despite no apparent correlation between increased helicity and membrane permeating ability being found.[27]

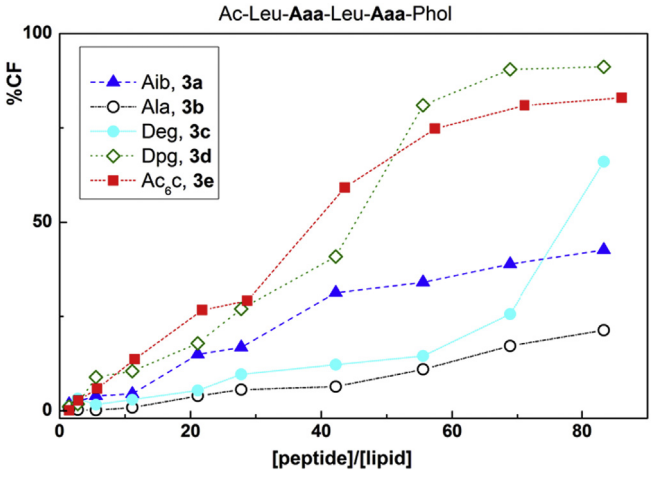


Fig. 2. Peptide-induced CF leakage at 20 min for different [peptide]/[lipid] ratios from egg phosphatidylcholine/cholesterol (70:30) vesicles.

At [peptide]/[lipid]≈80 (Fig. 2), where near full membrane permeation is achieved for the most active peptides, the permeating ability follows the sequence Peptabolin-Dpg **3d**>Peptabolin-Ac$_6$c **3e**>Peptabolin-Deg **3c**>Peptabolin-Aib **3a**>Peptabolin-Ala **3b**, pointing to a correlation between the length and bulk of the side chain of the unnatural α,α-dialkylglycines and the ability of the corresponding peptide to permeate the model membranes.

At low [peptide]/[lipid] ratios (below 30), the permeation ability follows the order Peptaibolin-Ac$_6$c **3e**≥Peptabolin-Dpg **3d**>Peptabolin-Aib **3a**>Peptabolin-Deg **3c**>Peptabolin-Ala **3b**. It should be noted that the peptides containing Dpg and Ac$_6$c have a similar behaviour throughout the range of [peptide]/[lipid] ratios used, with several intersections between the corresponding curves (Fig. 2). Although very active at high [peptide]/[lipid] ratios, Peptaibolin-Deg **3c** presents a low permeating ability at [peptide]/[lipid]<60.

The influence of the composition of model membranes in the permeability cannot be discarded and may justify the negligible permeating ability in 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phosphocholine (POPC) membranes predicted for the native Peptaibolin by molecular dynamics simulations,[27] contrarily to what is observed in Fig. 2. It is also expected that the packing of the hydrophobic phospholipid chains in membrane, determining membrane fluidity, play a major role in the permeation ability. Phosphatidylcholine from egg yolk is a natural mixture of different phosphatidylcholines (PC), varying in chain length and degree of unsaturation. The main components are PC 16:0, PC 18:0 (both saturated and rigid at room temperature) and PC 18:1 (unsaturated and fluid).[45] Variations in composition can affect the membrane fluidity and the size of phosphatidylcholine SUVs.[46]

Another important factor is the amount of cholesterol, the latter acting as modulator of the bilayer fluidity. Significant differences in permeation of phosphatidylcholine/cholesterol 70:30 and 80:20 model membranes were found for short-sequence peptaibols (Harzianins HC).[44] The role of these factors in the permeating ability of the Peptaibolin analogues is being investigated and the results will be presented in the near future.

3. Conclusions

The microwave-assisted solid phase synthesis of Peptaibolin and a series of analogues by substitution of the native Aib residue by L-alanine or different α,α-dialkylglycines was reported for the first time, in fair to excellent yields. The native peptide, the negative control analogue and the dialkylglycine-bearing analogues were studied for their ability to interact with model membranes (phosphatidylcholine/cholesterol 70:30 vesicles) containing encapsulated carboxyfluorescein. By addition of peptides at different peptide to lipid molar ratios, it was possible to follow the release of the entrapped fluorescent probe, monitoring the increase in its fluorescence intensity over time, due to the decrease of the self-quenching effect. The obtained results revealed that the Peptaibolin analogues bearing Ac$_6$c and Deg are the peptides with higher permeating ability, evidencing a correlation between the length

and bulk of the α,α-dialkylglycines side chain and the ability of the corresponding peptides to permeate the membranes.

The successful synthesis of a series of Peptaibolin analogues with enhanced membrane permeating capability may represent a step forward in the improvement of the antimicrobial properties of this type of AMPs.

4. Experimental Section

4.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. Chromatography on silica gel was carried out on Merck Kieselgel (230–240 mesh). UV/visible absorption spectra (200–700 nm) were obtained using a Shimadzu UV-2501PC spectrophotometer. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using Me_4Si as reference and *J* values are given in Hz. Assignments were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the 'C.A.C.T.I.—Unidad de Espectrometria de Masas', at University of Vigo, Spain. Microwave-assisted solid phase peptide synthesis was carried out on a CEM Discover SPS equipment. Peptide analysis and purification was carried out with analytical HPLC using a Licrospher 100 RP18 (5 μm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software, and semipreparative HPLC with a Shimadzu LC-8A, UV/Vis JASCO 875-UV detector and a Shimadzu C-RGA Chromatopac register on a Europa Peptide 120 C18 (5 μm) column using ACN/water mixtures (1:1 or 2:1) with 0.1% TFA (λ_{det}=215 nm). Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer (Horiba—Jobin-Yvon), equipped with double monochromators in both excitation and emission and a temperature-controlled cuvette holder. Fluorescence spectra were corrected for the instrumental response of the system.

Fmoc-Aib-OH **2a**, Fmoc-L-Leu-OH, *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide (Fmoc-OSu), Fmoc-Phe-Wang resin, *N,N′*-diisopropylcarbodiimide (DIC), 6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt) and *N,N*-diisopropylethylamine (DIPEA) were purchased from AAPPTec and ACROS Organics and used as received. L-α-Phosphatidylcholine from egg yolk, cholesterol, 6-carboxyfluorescein, Triton® X-100 and Sephadex® G-50 were purchased from Sigma–Aldrich and used as received.

4.2. General method for the N-protection of α,α-dialkylglycines 2c–f

4.2.1. Method A. The amino acid hydrochloride **1** (2 mmol) was dissolved in a mixture of aqueous 5% Na_2CO_3 solution/1,4-dioxane (5:2). The mixture was placed in an ice bath and Fmoc-OSu (1.1–2 equiv) dissolved in 1,4-dioxane (4 mL) was added drop wise. The final pH was adjusted to 8 with aqueous 5% Na_2CO_3 solution, if necessary. The mixture was stirred at room temperature overnight. Water (15 mL) was added and the mixture was extracted with diethyl ether/petroleum ether (1:3) (3×15 mL). The aqueous layer was acidified to pH 3 with aqueous 10% citric acid solution and extracted with ethyl acetate (3×15 mL). The organic layer was washed with aqueous 5% citric acid solution (2×10 mL), followed by washing with water as many times necessary until neutral. The organic layer was dried with anhydrous $MgSO_4$, filtered and evaporated to dryness in a rotary evaporator.

4.2.2. Method B. The amino acid hydrochloride **1** (1 equiv) was dissolved in a mixture of acetone (40 mL) and a solution of Na_2CO_3 (5 equiv) in water (50 mL). Fmoc-OSu (1.1–2.0 equiv), dissolved in acetone (10 mL), was added drop wise and the mixture was stirred at room temperature overnight. The acetone was evaporated and the aqueous phase washed with ethyl acetate (3×40 mL). The aqueous phase was acidified to pH 3 with aqueous 10% citric acid solution and kept in the cold to form a precipitate. The solid was filtered, washed with cold water and dissolved in ethyl acetate, followed by drying with anhydrous $MgSO_4$ and evaporation of the solvent to dryness in a rotary evaporator.

*4.2.3. N-(9-Fluorenylmethyloxycarbonyl)-α,α-diethylglycine, Fmoc-Deg-OH (**2c**).* By reaction of α,α-diethylglycine hydrochloride **1c** (1.023 g, 5.95 mmol) and Fmoc-OSu (2.412 g, 7.15 mmol, 1.2 equiv) using method A, compound **2c** was obtained as colourless oil (1.023 g, 72%) and the characterization in accordance to previously reported data.[38] Mp=98.2–102.2 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H=0.74 (t, *J*=6.8 Hz, 6H, 2×CH_3 Deg), 1.74–1.80 (m, 2H, CH_2 Deg), 2.22–2.28 (m, 2H, CH_2 Deg), 4.14 (t, *J*=6.8 Hz, 1H, H-9 Fmoc), 4.32 (d, *J*=6.4 Hz, 2H, CH_2 Fmoc), 5.56 (s, 1H, NH), 7.23 (dt, *J*=1.1 and 6.4 Hz, 2H, H-2 and H-7 Fmoc), 7.32 (t, *J*=7.4 Hz, 2H, H-3 and H-6 Fmoc), 7.52 (d, *J*=7.2 Hz, 2H, H-4 and H-5 Fmoc), 7.68 (d, *J*=7.2 Hz, 2H, H-1 and H-8 Fmoc). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C=8.29 (2×CH_3 Deg), 28.16 (2×CH_2 Deg), 47.26 (C-9 Fmoc), 65.03 (α-C Deg), 66.28 (CH_2 Fmoc), 119.96 (C-1 and C-8 Fmoc), 124.98 (C-4 and C-5 Fmoc), 127.03 (C-2 and C-7 Fmoc), 127.66 (C-3 and C-6 Fmoc), 141.31 (C-4a and C-4b Fmoc), 143.83 (C-1a and C-8a Fmoc), 154.41 (C=O Fmoc), 178.27 (C=O acid).

*4.2.4. N-(9-Fluorenylmethyloxycarbonyl)-α,α-dipropylglycine, Fmoc-Dpg-OH (**2d**).* By reaction of α,α-dipropylglycine hydrochloride **1d** (0.420 g, 2.1 mmol) and Fmoc-OSu (0.708 g, 1.1 equiv, 2.31 mmol) using method A, after recrystallization from dichloromethane/petroleum ether, compound **2d** was obtained as a colourless solid (0.532 g, 66%) and the characterization in accordance to previously reported data.[38] Mp=143.3–145.5 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H=0.91 (t, *J*=6.4 and 7.2 Hz, 6H, 2×CH_3 Dpg), 1.10–1.13 (m, 2H, γ-CH_2 Dpg), 1.28–1.34 (m, 2H, γ-CH_2 Dpg), 1.74–1.81 (m, 2H, β-CH_2 Dpg), 2.27–2.35 (m, 2H, β-CH_2 Dpg), 4.23 (t, *J*=6.6 Hz, 1H, H-9 Fmoc), 4.42 (d, *J*=6.4 Hz, 2H, CH_2 Fmoc), 5.75 (s, 1H, NH), 7.33 (dt, *J*=1.0 and 7.4 Hz, 2H, H-2 and H-7 Fmoc), 7.41 (t, *J*=7.6 Hz, 2H, H-3 and H-6 Fmoc), 7.58–7.64 (m, 2H, H-4 and H-5 Fmoc), 7.78 (d, *J*=7.6 Hz, 2H, H-1 and H-8 Fmoc). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C=13.93 (4×CH_3 Dpg), 17.32 (2×γ-CH_2 Dpg), 37.63 (2×β-CH_2 Dpg), 47.33 (C-9 Fmoc), 63.94 (α-C Dpg), 66.12 (CH_2 Fmoc), 120.20 (C-1 and C-8 Fmoc), 124.98 (C-4 and C-5 Fmoc), 127.60 (C-2 and C-7 Fmoc), 127.65 (C-3 and C-6 Fmoc), 141.33 (C-4a and C-4b Fmoc), 143.85 (C-1a and C-8a Fmoc), 154.00 (C=O Fmoc), 178.84 (C=O acid).

*4.2.5. 1-(9-Fluorenylmethyloxycarbonylamino)-cyclohexyl-1-carboxylic acid, Fmoc-Ac$_6$c-OH (**2e**).* By reaction of 1-amino-1-cyclohexane carboxylic acid hydrochloride **1e** (0.973 g, 6.42 mmol) and Fmoc-OSu (2.597 g, 1.2 equiv, 7.70 mmol) using method B, after recrystallization from dichloromethane and petroleum ether, compound **2e** was obtained as a light orange solid (1.638 g, 70%) and the characterization in accordance to previously reported data.[39,40] Mp=172.9–173.8 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H=1.37–1.51 (m, 2H, H-3′ or H-5′ Ac_6c), 1.55–1.71 (m, 4H, H-3′or H-5′ and H-4′ Ac_6c), 1.78–1.90 (m, 2H, H-2′ or H-6′ Ac_6c), 2.0–2.2 (m, 2H, H-2′ or H-6′ Ac_6c), 4.23 (t, *J*=6.0 Hz, 1H, H-9 Fmoc), 4.43 (d, *J*=4.0 Hz, 2H, CH_2 Fmoc), 5.04 (s, 1H, NH), 7.31 (t, *J*=7.2 Hz, 2H, H-2 and H-7 Fmoc), 7.39 (t, *J*=7.2 Hz, 2H, H-3 and H-6 Fmoc), 7.59 (d, *J*=7.6 Hz, 2H, H-4 and H-5 Fmoc), 7.76 (d, *J*=7.6 Hz, 2H, H-1 and H-8 Fmoc). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C=21.09 (C-3′ and C-5′ Ac_6c), 25.03 (C-4′ Ac_6c), 32.24 (C-2′ and C-6′ Ac_6c), 47.20 (C-9 Fmoc), 58.97 (C-1′ Ac_6c), 66.76 (CH_2 Fmoc), 119.90 (C-1 and C-8 Fmoc), 125.17 (C-

4 and C-5 Fmoc), 127.04 (C-2 and C-7 Fmoc), 127.63 (C-3 and C-6 Fmoc), 141.29 (C-4a and C-4b Fmoc), 143.79 (C-1a and C-8a Fmoc), 155.58 (C=O Fmoc), 179.22 (C=O acid).

*4.2.6. N-(9-Fluorenylmethyloxycarbonyl)-α,α-diisobutylglycine, Fmoc-Dibg-OH (**2f**).* By reaction of α,α-diisobutylglycine hydrochloride **1f** (1.718 g, 7.68 mmol) and Fmoc-OSu (5.194 g, 2.0 equiv, 15.4 mmol) using method A, after column chromatography on silica gel with mixtures of dichloromethane and methanol, compound **2f** was obtained as a colourless solid (3.085 g, 98%). Mp=143.3–145.5 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ=0.83 (d, J=6.4 Hz, 6H, 2×CH_3 Dibg), 0.89 (d, J=6.4 Hz, 6H, 2×CH_3 Dibg), 1.53–1.63 (m, 2H, 2×γ-CH Dibg), 1.66–1.71 (m, 2H, β-CH_2 Dibg), 2.40 (dd, J=5.8 and 8.4 Hz, 2H, β-CH_2 Dibg), 4.21 (t, J=7.0 Hz, 1H, H-9 Fmoc), 4.40 (d, J=6.8 Hz, 2H, CH_2 Fmoc), 5.94 (s, 1H, NH), 7.32 (dt, J=1.1 and 6.4 Hz, 2H, H-2 and H-7 Fmoc), 7.41 (t, J=7.4 Hz, 2H, H-3 and H-6 Fmoc), 7.61 (d, J=7.2 Hz, 2H, H-4 and H-5 Fmoc), 7.6 (d, J=7.6 Hz, 2H, H-1 and H-8 Fmoc). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ=22.86 (2×CH_3 Dibg), 23.69 (2×CH_3 Dibg), 24.61 (2×γ-CH Dibg), 44.82 (2×β-CH_2 Dibg), 47.37 (C-9 Fmoc), 62.87 (α-C Dibg), 66.15 (CH_2 Fmoc), 119.95 (C-1 and C-8 Fmoc), 125.01 (C-4 and C-5 Fmoc), 127.02 (C-2 and C-7 Fmoc), 127.64 (C-3 and C-6 Fmoc), 141.33 (C-4a and C-4b Fmoc), 143.92 (C-1a and C-8a Fmoc), 153.83 (C=O Fmoc), 179.85 (C=O acid). HRMS (ESI): calcd for $C_{25}H_{32}NO_4$ [M^++H]: 410.23326; found: 410.23321.

4.3. General procedure of the synthesis of peptides 3a–e by MW-SPPS

4.3.1. Preparation for coupling of preloaded Fmoc-Phe-Wang resin. After swelling of the resin in DMF for 15 min, the resin was subjected to an initial deprotection to remove the Fmoc group using a solution of 20% piperidine in DMF (7 mL/g resin) for 30 s, under microwave irradiation (power=28 W, temperature=75 °C). The resin was washed with DMF (2×10 mL), MeOH (2×10 mL) and DMF. A fresh solution of 20% piperidine in DMF was added and allowed to react for 3 min under microwave irradiation (power=28 W, temperature=75 °C). The resin was washed with DMF (2×10 mL) and MeOH (2×10 mL) and this washing cycle was repeated five times.

4.3.2. Coupling of Fmoc-amino acids. The Fmoc-amino acid **2** (5 equiv on the degree of functionalization of the resin) was dissolved in dry DMF (6 mL/g resin) and *N,N′*-diisopropylcarbodiimide (DIC) (5 equiv) and 6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt) (5 equiv) dissolved in dry DMF (2 mL/g) were added. The reaction mixture was allowed to stir at room temperature for 1.5–2 h, for the α,α-dialkylglycines, and 20 min for the remaining amino acids, before being added to the resin and subjected to microwave irradiation. The suspension was then subjected to microwave irradiation for 5 min (power=25 W, temperature=75 °C). The resin was washed with DMF (2×10 mL) followed by MeOH (2×10 mL) and this washing cycle was repeated three times. The N-terminal Fmoc group was removed as described above for the preparation of the resin.

4.3.3. N-terminal acetylation of the peptides. The resin with the peptide was suspended in dry DMF (7 mL/g of resin) and DIPEA (14 equiv) and acetic anhydride (7 equiv) were added. The mixture was allowed to stir at room temperature for 1.5 h. The resin was washed with DMF (2×10 mL), MeOH (2×10 mL), DCM (3×10 mL) and diethyl ether (3×10 mL). The resin was dried in a vacuum oven at room temperature.

4.3.4. Reductive separation of the peptides from Wang resin. Sodium borohydride (5 equiv) was suspended in dry THF (16 mL/g of resin) under a nitrogen atmosphere, in an ice bath. Then, lithium bromide (5 equiv) suspended in dry ethanol (4 mL/g of resin) was added. The mixture was stirred vigorously for 15 min on ice and 15 min at room temperature. The dried resin was added to this mixture and allowed to stir at room temperature for 24 h. The organic solvent mixture was separated from the resin by vacuum filtration and the resin was washed with THF (2×15 mL), ethanol (2×15 mL) and ethyl acetate (2×15 mL). The solvents were combined and a small amount of acetic acid was added, just enough to decompose borohydride in excess. The solution was concentrated almost to dryness on a rotary evaporator without heating and water was added (25 mL). The resulting aqueous solution was extracted with ethyl acetate (3×25 mL), and the combined organic extracts were washed with water (15 mL) and saturated NaCl solution (2×15 mL). The organic layer was dried with anhydrous $MgSO_4$ and the solvent evaporated to dryness without heating.

4.3.5. Purification of the peptides by HPLC. The purity of the peptides was checked by analytical HPLC and a sample of each peptide was purified by preparative HPLC.

*4.3.6. Peptaibolin, Ac-L-Leu-Aib-L-Leu-Aib-L-Phol (**3a**).* Starting from Fmoc-Phe-Wang resin (1.5 g, 0.51 mmol/g), following the above general methods, by sequential coupling of Fmoc-Aib-OH **2a** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, the crude peptide was obtained as a colourless solid (0.5727 g). A sample of the crude product (0.0502 g) was purified by preparative HPLC yielding the pure peptide as a colourless solid (0.0347 g, purification yield 70%, global yield 89%). Mp=209–211 °C (224–225 °C[34]). ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H=0.89–1.01 (m, 12H, 4×CH_3 Leu), 1.36 (s, 3H, CH_3 Aib), 1.38 (s, 3H, CH_3 Aib), 1.47 (s, 3H, CH_3 Aib), 1.54 (s, 3H, CH_3 Aib), 1.58–1.63 (m, 2H, β-CH_2 Leu), 1.66–1.75 (m, 3H, β-CH_2 and γ-CH Leu), 1.76–1.90 (m, 1H, γ-CH Leu), 2.13 (s, 3H, CH_3 Ac), 2.72–2.82 (m, 2H, β-CH_2 Phol), 3.60–3.72 (m, 2H, CH_2OH), 3.92–3.98 (m, 1H, α-CH Leu), 4.01–4.07 (m, 1H, α-CH Leu), 4.23–4.34 (m, 1H, α-CH Phol), 7.15–7.17 (m, 1H, NH), 7.21–7.25 (m, 5H, Ph-H Phol), 7.28–7.30 (m, 1H, NH Aib), 7.85 (br s, 1H, NH Leu), 8.11 (br s, 1H, NH) 8.20 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C=21.39 (CH_3 Leu), 22.02 (CH_3 Leu), 22.41 (CH_3 Leu), 22.81 (CH_3 Ac), 22.84 (CH_3 Aib), 22.89 (CH_3 Leu), 23.13 (CH_3 Aib), 24.79 (γ-CH Leu), 25.24 (γ-CH Leu) 26.81 (CH_3 Aib), 27.36 (CH_3 Aib), 36.90 (β-CH_2 Phol), 39.34 (β-CH_2 Leu), 39.61 (β-CH_2 Leu), 53.23 (α-CH Phol), 55.10 (α-CH Leu), 56.03 (α-CH Leu), 56.42 (α-C Aib), 56.88 (α-C Aib), 65.00 (CH_2OH), 126.22 (C-4 Phol), 128.19 (C-3 and C-5 Phol), 129.10 (C-2 and C-6 Phol), 138.38 (C-1 Phol), 173.48 (C=O Ac), 174.44 (C=O Leu), 174.71 (C=O Leu), 175.39 (C=O Aib), 176.72 (C=O Aib). HRMS (ESI): calcd for $C_{31}H_{52}N_5O_6$ [M^++H]: 590.39121; found: 590.39114.

*4.3.7. Peptaibolin-Ala, Ac-L-Leu-L-Ala-L-Leu-L-Ala-L-Phol (**3b**).* Starting from Fmoc-Phe-Wang resin (2.0 g, 0.51 mmol/g), following the above general methods, by sequential coupling of Fmoc-L-Ala-OH **2b** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, the crude peptide was obtained as a colourless solid (0.9450 g). A sample of the crude product (0.0403 g) was purified by preparative HPLC yielding the pure peptide as a white solid (0.0385 g), purification yield 90%, global yield 87%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H=0.79–0.86 (m, 12H, 4×CH_3 Leu), 1.13 (d, J=7.2 Hz, 3H, CH_3 Ala), 1.18 (d, J=7.2 Hz, 3H, CH_3 Ala), 1.37–1.44 (m, 4H, 2×β-CH_2 Leu), 1.55–1.57 (m, 2H, 2×γ-CH Leu), 1.82 (s, 3H, CH_3 Ac), 2.55–2.83 (m, 2H, β-CH_2 Phol), 3.25–3.32 (m, 2H, CH_2OH), 3.80–3.84 (m, 1H, α-CH Phol), 4.13–4.26 (m, 1H, CH_2OH), 7.13–7.26 (m, 5H, Ph-H Phol), 7.53 (d, J=8.4 Hz, 1H, NH Phol), 7.78 (d, J=8.0 Hz, 1H, NH Ala), 7.79 (d, J=7.6 Hz, 1H, NH Leu), 8.01 (d, J=8.0 Hz, 1H, NH Leu), 8.10 (d, J=6.8 Hz, 1H, NH Ala). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C=17.58 (CH_3 Ala), 18.37 (CH_3 Ala), 21.55 (CH_3 Leu), 21.68 (CH_3 Leu), 22.54 (CH_3 Ac), 23.10 (CH_3 Leu), 23.19 (CH_3 Leu), 24.18

(γ-CH Leu), 24.25 (γ-CH Leu), 36.48 (β-CH_2 Phol), 40.57 (β-CH_2 Leu), 40.79 (β-CH_2 Leu), 48.40 (2×α-CH Ala), 51.26 (α-CH Leu), 52.39 (α-CH Leu), 52.37 (α-CH Phol), 62.31 (CH_2OH), 126.02 (C-4 Phol), 128.19 (C-3 and C-5 Phol), 129.22 (C-2 and C-6 Phol), 138.00 (C-1 Phol), 169.73 (C=O Ac), 171.56 (C=O Leu), 171.72 (C=O Leu), 172.36 (C=O Ala), 172.43 (C=O Ala). HRMS (ESI): calcd for $C_{29}H_{48}N_5O_6$ [M^++H]: 562.36074; found: 562.35978.

*4.3.8. Peptaibolin-Deg, Ac-L-Leu-Deg-L-Leu-Deg-L-Phol (**3c**).* Starting from Fmoc-Phe-Wang resin (1.5 g, 0.60 mmol/g), following the above general methods, by sequential coupling of Fmoc-Deg-OH **2c** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, the crude peptide was obtained as a colourless solid (0.3593 g). A sample of the crude product (0.1042 g) was purified by preparative HPLC yielding the pure peptide as colourless solid (0.0318 g, purification yield 30%, global yield 22%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H=0.12 (t, *J*=8.0 Hz, 3H, CH_3 Deg), 0.50–0.56 (m, 9H, 3×CH_3 Deg), 0.75–0.88 (m, 12H, 4×CH_3 Leu), 1.37–1.49 (m, 5H, β-CH_2, γ-CH Leu and β-CH_2 Deg), 1.51–1.66 (m, 3H, β-CH_2 and γ-CH Leu), 1.74–1.90 (m, 2H, β-CH_2 Deg), 1.87 (s, 3H, CH_3 Ac), 2.08–2.20 (m, 4H, 2×β-CH_2 Deg), 2.55–2.61 (m, 1H, β-CH_2 Phol), 2.89 (dd, *J*=4.4 and 9.2 Hz, 1H, β-CH_2 Phol), 3.25–3.33 (m, 2H, CH_2OH), 4.01–4.12 (m, 3H, 2×α-CH Leu and α-CH Phol), 7.11–7.12 (m, 1H, H-4 Phol), 7.16–7.19 (m, 4H, H-2, H-3, H-5 and H-6 Phol), 7.34 (s, 1H, NH Deg), 7.48 (d, *J*=8.8 Hz, 1H, NH Leu), 7.55 (s, 1H, NH Deg), 7.98 (d, *J*=7.6 Hz, 1H, NH Leu) 8.21 (d, *J*=6.8 Hz, 1H, NH Phol). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C=7.61 (CH_3 Deg), 7.72 (CH_3 Deg), 7.86 (2×CH_3 Deg), 20.82 (CH_3 Ac), 20.90 (CH_3 Leu), 21.11 (β-CH_2 Leu), 21.37 (CH_3 Leu), 23.00 (CH_3 Leu), 23.22 (CH_3 Leu), 24.11 (γ-CH Leu), 24.35 (γ-CH Leu), 26.50 (β-CH_2 Deg), 26.71 (β-CH_2 Deg), 27.03 (β-CH_2 Deg), 27.11 (β-CH_2 Deg), 36.30 (β-CH_2 Phol), 39.91 (β-CH_2 Leu), 40.12 (β-CH_2 Leu), 52.58 (α-CH Leu), 52.77 (α-CH Phol), 52.95 (α-CH Leu), 63.42 (CH_2OH), 63.85 (2×α-C Deg), 125.84 (C-4 Phol), 128.06 (C-3 and C-5 Phol), 128.96 (C-2 and C-6 Phol), 139.31 (C-1 Phol), 169.95 (C=O Leu) 170.60 (C=O Ac), 171.03 (C=O Leu), 171.80 (C=O Deg), 173.10 (C=O Deg). HRMS (ESI): calcd for $C_{35}H_{60}N_5O_6$ [M^++H]: 646.4538; found: 646.4533.

*4.3.9. Peptaibolin-Dpg, Ac-L-Leu-Dpg-L-Leu-Dpg-L-Phol (**3d**).* Starting from Fmoc-Phe-Wang resin (1.5 g, 0.51 mmol/g), following the above general methods, by sequential coupling of Fmoc-Dpg-OH **2d** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, the crude peptide was obtained as a colourless solid (0.4427 g). A sample of the crude product (0.0370 g) was purified by preparative HPLC yielding the pure peptide as colourless solid (0.0318 g, purification yield 76%, global yield 60%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H=0.58 (t, *J*=7.2 Hz, 3H, CH_3 Leu), 0.64–0.78 (m, 12H, 3×CH_3 Leu and CH_3 Dpg) 0.79–0.91 (m, 12H, 3×CH_3 Dpg and γ-CH_2 Dpg), 0.92–1.20 (m, 6H, 3×γ-CH_2 Dpg), 1.33–1.81 (m, 4H, 2×β-CH_2 Dpg and 2×γ-CH Leu), 1.84 (s, 3H, CH_3 Ac), 1.98–2.20 (m, 4H, 2×β-CH_2 Dpg), 2.57–2.62 (m, 1H, β-CH_2 Phol), 2.89–2.94 (m, 1H, β-CH_2 Phol), 3.20–3.30 (m, 4H, 2×γ-CH Leu and *C*H_2OH), 3.98–4.13 (m, 3H, 2×α-CH Leu and α-CH Phol), 4.72 (br s, 1H, CH_2O*H*), 7.10–7.20 (m, 5H, Ph-H Phol), 7.39 (s, 1H, NH Dpg), 7.47 (d, *J*=8.4 Hz, 1H, NH), 7.61 (s, 1H, NH Dpg), 7.93 (d, *J*=7.6 Hz, 1H, NH) 8.18 (d, *J*=7.2 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C=13.83 (CH_3 Leu), 13.95 (CH_3 Leu), 14.13 (CH_3 Leu), 14.22 (CH_3 Leu), 15.87 (γ-CH_2 Dpg), 16.27 (γ-CH_2 Dpg), 16.36 (2×γ-CH_2 Dpg), 20.87 (CH_3 Dpg), 21.46 (CH_3 Dpg), 22.33 (CH_3 Ac), 22.87 (CH_3 Dpg), 23.14 (CH_3 Dpg), 23.96 (γ-CH Leu), 24.25 (γ-CH Leu), 35.96 (β-CH_2 Phol), 36.06 (β-CH_2 Dpg), 36.39 (β-CH_2 Dpg), 36.64 (β-CH_2 Dpg), 36.72 (β-CH_2 Dpg), 39.92 (β-CH_2 Leu), 40.02 (β-CH_2 Leu), 52.48 (α-CH Leu), 52.71 (α-CH Leu), 53.04 (α-CH Phol), 62.76 (α-C Dpg), 62.87 (α-C Dpg), 63.30 (CH_2OH), 125.70 (C-4 Phol), 127.89 (C-3 and C-5 Phol), 128.80 (C-2 and C-6 Phol), 139.32 (C-1 Phol), 168.78 (C=O Ac), 170.44 (C=O Dpg), 170.85 (C=O Leu), 172.03 (C=O Dpg) 173.17 (C=O Leu). HRMS (ESI): calcd for $C_{39}H_{68}N_5O_6$ [M^++H]: 702.51641; found: 702.51662.

*4.3.10. Peptaibolin-Ac$_6$c, Ac-L-Leu-Ac$_6$c-L-Leu-Ac$_6$c-L-Phol (**3e**).* Starting from Fmoc-Phe-Wang resin (1.5 g, 0.51 mmol/g), following the above general methods, by sequential coupling of Fmoc-Ac$_6$c-OH **2e** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, the crude peptide was obtained as colourless oil (0.3840 g). A sample of the crude product (0.0370 g) was purified by preparative HPLC yielding the pure peptide as a colourless solid (0.0341 g, purification yield 43%, global yield 32%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H=0.81–0.94 (m, 12H, 4×CH_3 Leu), 1.08–1.74 (m, 22H, 8×CH_2 Ac$_6$c, 2×γ-CH and 2×β-CH_2 Leu), 1.89 (s, 3H, CH_3 Ac), 1.96–2.08 (m, 4H, 2×CH_2 Ac$_6$c), 2.53–2.59 (m, 1H, β-CH_2 Phol), 2.84 (dd, *J*=4.8 and 8.8 Hz, 1H, β-CH_2 Phol), 3.21–3.33 (m, 2H, CH_2OH), 3.81–3.89 (m, 2H, 2×α-CH Leu), 4.12–4.18 (m, 1H, α-CH Phol), 6.94 (d, *J*=9.2 Hz, 1H, NH Leu or Phol), 7.10–7.14 (m, 1H, H-4 Phol), 7.15 (s, 1H, NH Ac$_6$c), 7.17–7.20 (m, 4H, H-2, H-3, H-5 and H-6 Phol), 7.64 (d, *J*=6.0 Hz, 1H, NH Leu or Phol), 7.96 (s, 1H, NH Ac$_6$c) 8.31 (d, *J*=5.6 Hz, 1H, NH Leu). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C=21.04 (4×CH_2 Ac$_6$c), 21.33 (CH_3 Leu), 22.01 (CH_3 Leu), 22.35 (CH_3 Ac), 22.46 (CH_3 Leu), 22.95 (CH_3 Leu), 24.16 (γ-CH Leu), 24 31 (γ-CH Leu), 24.87 (CH_2 Ac$_6$c), 25.01 (CH_2 Ac$_6$c), 30.78 (CH_2 Ac$_6$c), 31.14 (CH_2 Ac$_6$c), 31.47 (2×CH_2 Ac$_6$c), 36.50 (β-CH_2 Phol), 38.87 (β-CH_2 Leu), 39.08 (β-CH_2 Leu), 52.62 (α-CH Leu), 52.83 (α-CH Phol), 53.30 (α-CH Leu), 59.00 (α-C Ac$_6$c), 59.36 (α-C Ac$_6$c), 63.02 (CH_2OH), 125.70 (C-4 Phol), 127.83 (C-3 and C-5 Phol), 129.16 (C-2 and C-6 Phol), 139.35 (C-1 Phol), 171.13 (C=O Ac), 171.83 (C=O Ac$_6$c), 173.28 (C=O Ac$_6$c), 173.66 (C=O Leu) 175.45 (C=O Leu). HRMS (ESI): calcd for $C_{37}H_{60}N_5O_6$ [M^++H]: 670.45381; found: 670.45324.

*4.3.11. Attempted synthesis of Peptaibolin-Dibg: isolation of Ac-Dibg-L-Phol (**3f**).* Starting from Fmoc-Phe-Wang resin (1.5 g, 0.60 mmol/g), following the above general methods, by sequential coupling of Fmoc-Dibg-OH **2f** and Fmoc-L-Leu-OH, after reductive cleavage from the resin, only the dipeptide Ac-Dibg-Phol was isolated as off-white solid (0.0123 g). ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H=0.37 (d, *J*=6.8 Hz, 3H, CH_3 Dibg), 0.63 (d, *J*=6.4 Hz, 3H, CH_3 Dibg), 0.73–0.78 (m, 6H, 2×CH_3 Dibg), 1.04–1.11 (m, 1H, γ-CH Dibg), 1.35–1.45 (m, 2H, β-CH_2 Dibg), 1.55–1.61 (m, 1H, γ-CH Dibg), 1.81 (s, 3H, CH_3 Ac), 2.07–2.21 (m, 2H, β-CH_2 Dibg), 2.65–2.71 (m, 1H, β-CH_2 Phol), 2.89–2.94 (m, 1H, β-CH_2 Phol), 3.20–3.25 (m, 1H, CH_2OH), 3.39–3.45 (m, 1H, CH_2OH), 3.98–4.04 (m, 1H, α-CH Phol), 4.75 (br s, 1H, CH_2O*H*), 7.13–7.16 (m, 1H, H-4 Phol), 7.21–7.26 (m, 5H, H-2, H-3, H-5 and H-6 Phol and NH Dibg), 7.59 (d, *J*=8.0 Hz, 1H, NH Phol). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C=23.03 (CH_3 Dibg), 21.13 (CH_3 Dibg), 23.50 (CH_3 Ac), 23.46 (γ-CH Dibg), 23.88 (CH_3 Dibg), 23.86 (γ-CH Dibg), 23.88 (CH_3 Dibg), 36.07 (β-CH_2 Phol), 43.20 (β-CH_2 Dibg), 43.45 (β-CH_2 Dibg), 53.45 (α-CH Phol), 62.43 (CH_2OH), 125.85 (C-4 Phol), 128.10 (C-3 and C-5 Phol), 128.94 (C-2 and C-6 Phol), 139.32 (C-1 Phol), 167.94 (C=O Ac), 173.22 (C=O Dibg). HRMS (ESI): calcd for $C_{21}H_{35}N_2O_3$ [M^++H]: 363.26485; found: 363.26512.

4.4. Membrane permeation studies

The membrane permeation ability of Peptaibolin and its analogues was monitored at 22 °C using the carboxyfluorescein-entrapped vesicle technique.[43,44]

4.4.1. Vesicles preparation. The small unilamellar vesicles (SUVs) were composed of egg phosphatidylcholine and cholesterol (70:30). The SUVs preparation was carried out in a concentrated solution of 6-carboxyfluorescein (CF) in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH=7.4). An ethanolic solution of the lipid mixture was injected in the CF solution under vortexing (ethanolic injection method),[47] followed by sonication for 10 min (with pauses of 1 min) at 71 W and 5.5 J in

a Q-Sonica Q125 sonicator, to obtain the SUVs. The vesicles with entrapped fluorophore were separated from non-encapsulated CF by size exclusion chromatography, using Sephadex® G-50 as gel filtration medium and HEPES buffer (pH=7.4) as eluent. The fractions containing the vesicles with entrapped CF were identified by measuring the absorbance (λ_{abs}=461 nm) and fluorescence intensity (λ_{exc}=488 nm; λ_{em}=520 nm), these fractions corresponding to the ones with simultaneously higher absorption (higher CF concentration) and lower emission (higher CF self-quenching).

4.4.2. Peptide-induced CF leakage monitored by fluorescence emission. In the CF leakage assays, the phospholipid concentration was kept constant (0.6 mM). Increasing [peptide]/[lipid] molar ratios were obtained by adding successive aliquots of methanolic solutions of peptides, keeping the final methanol concentration below 5% by volume. After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF release was recorded at λ_{em}=520 nm (1.0 nm bandpass) with λ_{exc}=488 nm (1.0 nm bandpass). The percentage of released CF (%CF) at time *t* due to peptide addition was determined using Eq. 1:

$$\%CF = \frac{(F_t - F_0)}{(F_T - F_0)} \times 100 \quad (1)$$

where F_0 is the fluorescence intensity in the absence of peptide, F_t is the fluorescence intensity in the presence of peptide, and F_T is the fluorescence intensity with total membrane rupture and CF release (obtained by addition of 50 μL of a 10% Triton X-100 solution). The fluorescence intensity increased over time, confirming the rupture of the vesicles and consequent release of CF. The time course for each peptide/membrane interaction assay was 20 min.

Acknowledgements

The authors acknowledge *Fundação para a Ciência e a Tecnologia* (Portugal) and FEDER-COMPETE-QREN-EU for financial support through projects PTDC/QUI-BIQ/118389/2010 (FCOMP-01-0124-FEDER-020906), PEst-C/QUI/UI0686/2013 (F-COMP-01-0124-FEDER-037302), PEst-C/FIS/UI0607/2013 (F-COMP-01-0124-FEDER-022711) and the Portuguese NMR network (PTNMR, Bruker Avance III 400-Univ. Minho).

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