Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 575–591
Published online 13 March 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.754

JOURNAL OF PEPTIDE SCIENCE

Synthesis, conformational analysis and CB_1 binding affinity of hairpin-like anandamide pseudopeptide mimetics

MARIA DI MARZO,[a] AGOSTINO CASAPULLO,[a] GIUSEPPE BIFULCO,[a] PAOLA CIMINO,[a] ALESSIA LIGRESTI,[b] VINCENZO DI MARZO,[b] RAFFAELE RICCIO[a] and LUIGI GOMEZ-PALOMA[a]*

[a] Dipartimento di Scienze Farmaceutiche, Università di Salerno, 84084 Fisciano, Salerno, Italy
[b] Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, CNR, 80078, Pozzuoli, NA, Italy

Received 15 March 2005; *Revised 20 December 2005*; *Accepted 9 January 2006*

Abstract: We have designed, synthesized and evaluated the CB_1 binding affinity of a number of new conformationally restricted lipopeptides (**1**–**17**). All of them present some of the AEA key structural elements incorporated in a hairpinlike peptide framework. Among them, compounds **1**–**3** and **8** showed CB_1 affinities in competitive binding assays with K_i values in the micromolar range (K_i of AEA = 0.8 μM in the same assay). The remaining pseudopeptides showed little binding to the CB_1 receptor (with K_i values $\geq$ 50 μM). Conformational analysis on two representative compounds, performed by a combination of NMR studies, restrained molecular dynamics and QM calculations, allowed us to shed light on the structure-activity relationships (SAR), pointing to a correlation between the predominance of the hairpinlike structural motif and the CB_1 binding affinity. In a more general context, the present study may also prove useful in gaining additional insight into the biological relevance of the various AEA conformations. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: anandamide; cannabinoids; conformational analysis; solid-phase peptide synthesis

INTRODUCTION

The identification in 1964 of the major active component of Indian hemp, Δ^9-THC [1], and the isolation in 1992 of the first endogenous cannabinoid from porcine brain, AEA [2], set the starting point of a number of pharmacological, biochemical and chemical studies [3–7] aimed at the comprehension of the pathways involved in the activation of the cannabinoid subtype receptors CB_1 and CB_2 and their involvement in nervous and immune disorders, cardiovascular diseases, pain, inflammation and cancer processes [8–13], and at the development of new potential drugs for the treatment of all these diseases [14–19]. In particular, AEA (Figure 1), a selective and strong CB_1 ligand, was the subject of detailed chemical investigations: a large number of analogues have been synthesized, leading to a wealth of knowledge on structure–activity relationships (SAR) [20]. Although several studies were devoted to the investigation of the conformations accessible to the long, flexible, carbon chain of AEA [21–23], no conclusive information was possible to be obtained on its biologically relevant conformation(s) when interacting with the CB_1 receptor. U-like (hairpin), J-like (hook) and helical conformations appear to be the most plausible candidates as the bioactive shapes of AEA. Likewise, the difficulty in characterizing the 3D structure of the receptor, a transmembrane protein constituted by seven helixes, limited the information available on the active site(s) and the basis for the interaction with its ligands. Instructive of this intricate ground is the fact that more than one scheme was put forward for the alignment of the AEA molecule into the rigid THC polycyclic framework [21–23], and even more striking is that presently there is no compelling evidence that the two molecules hit the same binding site. Accordingly, although much progress has been achieved in very recent years, a real rational design of innovative CB ligands was hampered by the lack of in-depth structural knowledge both on the ligands and the receptor. Therefore, most of the newly described CB active molecules are either variations on the theme of THC and AEA systems and their derivatives, or the result of extensive screening of large libraries, identified, in other words, by serendipity. In the frame of SAR investigations on AEA, we envisaged that potential CB_1 ligands could be based on a mixed lipopeptidic skeleton. Moreover, we reasoned that conformationally restricted mimetics of AEA of a pseudopeptidic nature might represent a useful means to investigate the biological effect of chemical entities with defined molecular shapes. The choice of embedding AEA molecular determinants into peptide-like molecules is justified on the basis of two main considerations: first of all, peptides

Abbreviations: AEA, anandamide; QM, quantum mechanical; Δ-THC, tetrahydrocannabinol; SSPS, solid-phase peptide synthesis; NMP, N-Methylpyrrolidine; HMPA, p-hydroxymethylphenoxyacetic; Acc; cis-2-aminocyclohexanecarboxylic acid; MM, molecular mechanics; GIAO, guage including atomic orbitals, DFT, density functional theory; IMS, isotropic magnetic shielding; CVFF, consistent-valence force field.

*Correspondence to: L. Gomez-Paloma, Dipartimento di Scienze Farmaceutiche, Università di Salerno, 84084 Fisciano, Salerno, Italy; e-mail: gomez@unisa.it

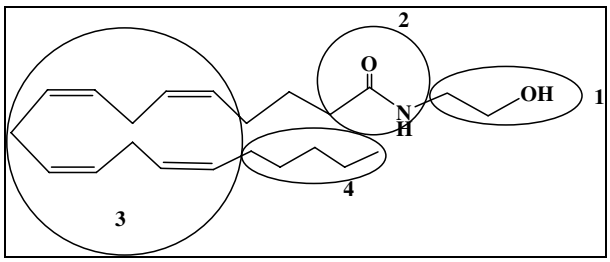

Figure 1 Chemical structure and main features of anandamide (AEA).

and proteins are the main regulatory constituents of living organisms, being implicated in almost all of the biochemical pathways. In addition, a peptide-like framework was appealing also in light of the progress in the field of peptide chemistry, allowing rapid and automated synthetic approaches [24] and structural characterization to be carried out by a variety of efficient spectroscopic techniques and theoretical calculation methods.

Our experimental work can be conveniently divided into three main steps: (i) design of the pseudopeptide skeleton on the basis of the SAR and conformational studies reported in the literature on AEA; (ii) synthesis of three main groups of anandamide-mimetic pseudo peptides and bioassay-guided synthetic modification of the parent compounds; (iii) interpretation of the CB_1 affinity results in terms of conformational analysis and theoretical calculations.

Design, Synthesis and Biological Activity

The first step in the design process was the selection of a particular AEA shape mimic among the U-like, J-like and helical systems, all of them in principle possible candidates for the bioactive 3D form(s). Our choice to investigate hairpin-shaped AEA mimics reflected the ease to generate such 3D profiles by using amino acids as building blocks. Therefore, we tried to implant on a lipopeptide skeleton the main AEA molecular determinants for bioactivity, closely following the SAR studies on endocannabinoids. The main structural features of our designed molecules can be handily examined in reference to the four portions of AEA depicted in Figure 1. Common features are a low-polarity, flexible long chain functionalized by a β-hydroxyethyl head, like in AEA, or characterized by a propyl moiety (area 1), an amide or inverse-amide moiety (area 2), and a terminal alkyl chain, usually five or six carbon long (area 4). Moreover, in an attempt to reproduce the peculiar conformational arrangements due to the presence of four *cis* C=C bonds in AEA (area 3), which also provide a π-electron-rich system, one or two rigid building blocks with ability to induce a turn in the chain, such as anthranilic acid, proline, or *cis*-2-aminocicloesane carboxylic acid (**1**–**7**, Chart 1), maleic acid (derivatives **8**–**17**, Chart 2), have been introduced. The presence, nature, and positioning of such rigid, shape-determining residues proved crucial in promoting the adoption of a hairpin, U-like, 3D profile in our molecules.

The parent compounds of the two groups described above (Charts 1–2, derivatives **1** and **8**, respectively) have been designed and synthesized, and on the basis of CB_1 affinity tests, the descendant compounds, afforded by iterative modifications.

Chemically, compounds **1**–**17** are constituted mainly by non-coded amino acid residues, and were prepared by employing SPPS techniques. The *C*-terminal amino acid residue was anchored to the resin and Fmoc (9-fluorenylmethyloxycarbonyl) was used as the protective group for the α-nitrogen atom. All Fmoc-protected amino acids were activated for the coupling step by the HOBt/HBTU (1-hydroxybenzotriazole/*O*-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) method in the presence of *N*-methylmorpholine (NMM), and the progress of the reactions was monitored by the Kaiser (ninhydrin) test. Fmoc deprotection was obtained by treatment with a 20% piperidine solution in *N*, *N*-dimethylformamide (DMF). The pseudopeptides obtained by cleavage from the resin with 95% aqueous trifluoroacetic acid (TFA) carrying the free carboxylic terminus were submitted to a final synthetic step in solution in order to install the head amide functionality, by coupling their carboxylic group previously activated with HATU (*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) with the required amines. Finally, the crude compounds were purified by RP-HPLC and analyzed by electrospray ionization mass spectrometry (ESI-MS) and NMR spectroscopy.

The biological activity (Table 1) displayed by compound **1** (Chart 1) suggested the design and synthesis of modified derivatives. In particular, compound **2** derives from the substitution of the hydroxy ethyl head of **1** with an *n*-propyl moiety. Together with this modification, compound **3** displays the substitution of the anthranilic acid residue with a 3-amminomethylbenzoic acid. Since such modifications allowed the maintenance of the biological activity (Table 1), a set of the isosteric derivatives **4**–**7** were synthesized. Unfortunately, such derivatives did not show any significant activity, suggesting the important role of the rigid unsaturated turn-inducing building block in the tail region of the molecule. On the basis of these results, a second set of molecules (**8**–**17**, Chart 2) were designed and synthesized, choosing compound **8** as the reference compound. This molecule preserves the hydroxy ethyl head of **1** and presents a maleic residue as a rigid unsaturated moiety that substitutes the anthranilic acid. The encouraging biological results displayed by **8** (Table 1) prompted us to synthesize a large set of

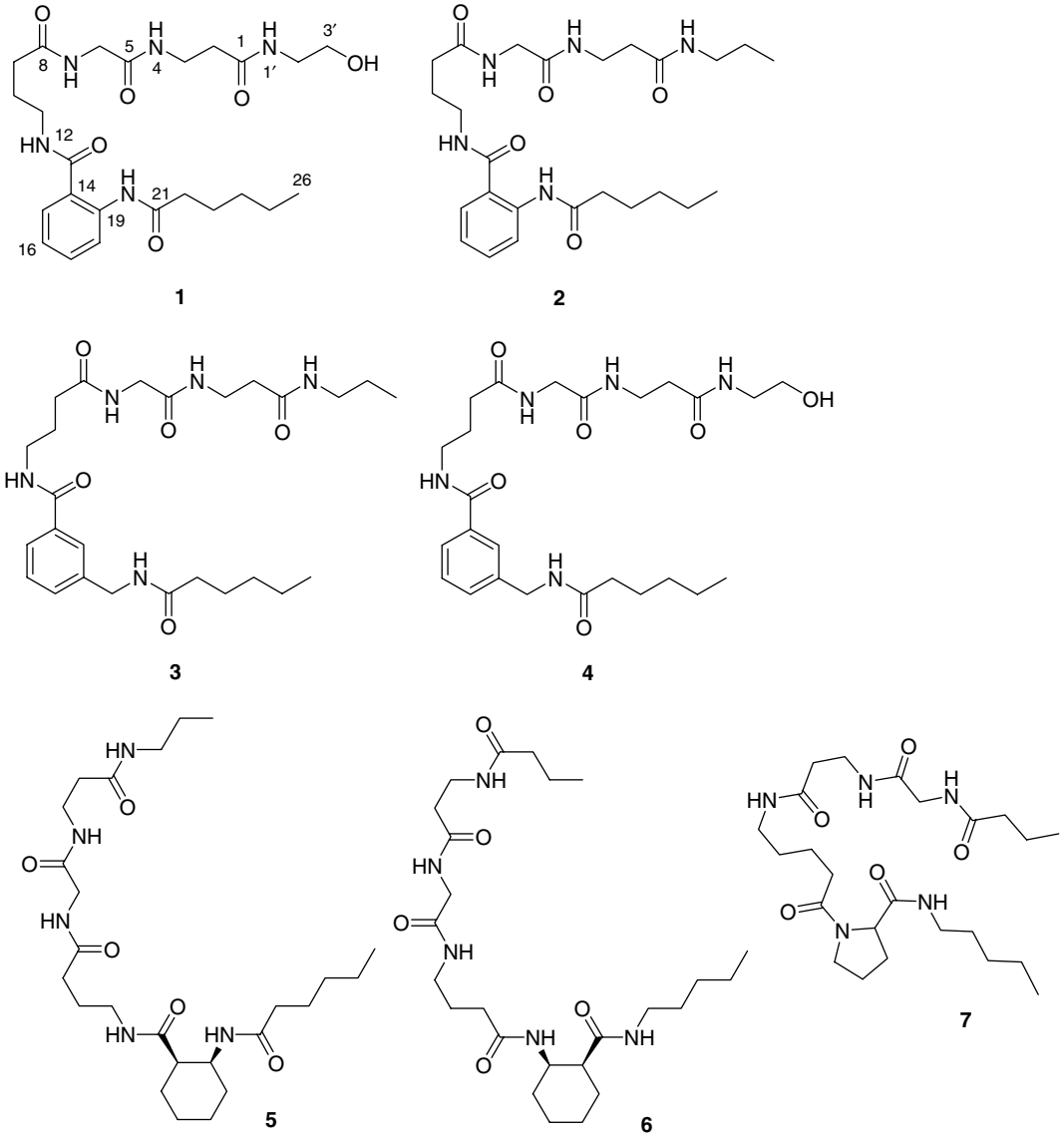

Chart 1 First group of anandamide analogues (**1**–**7**). In analogue **1** the numbering of atoms is shown.

derivatives (**9**–**17**) containing, together with the introduction of the maleic residue, further modification, introduced in order to evaluate the possible contribution to the CB_1 affinity. Compound **9**, in analogy with compound **2**, was synthesized by replacing a hydroxymethyl head with an *n*-propyl moiety. In **10**, a peptide bond was substituted by a flexible portion, and **11** and **12** contained a shorter tail portion and a shift of the maleic acid along the skeleton, respectively. Compound **13** shows the shift of the maleic acid and the lack of a peptidic bond in the tail portion. The skeleton of compounds **14**–**17** reproduces, together with the *n*-propyl head group as in **2**, a double methylation of the Cα-atom at the carboxamide head. This feature was investigated owing to its capability to improve the resistance to enzymatic cleavage in some AEA analogues. [16] Moreover, compounds **15** and **17** were designed to contain one peptide linkage less, in order to investigate the effect of a more flexible chain on binding. Then, the role of the unsaturated portion of the maleic residue was explored by shifting its position along the chain, **16** and **17** or by inserting an additional one, **14**. Unfortunately, the activity displayed by **8** was not reproduced by any of the derivatives **9**–**17**.

Compound **1** contains the anthranilic acid moiety in place of the *cis*-Δ^{14} double bond of AEA. The full synthesis of **1** was performed on solid phase using a resin functionalized with the 2-chlorotrityl function, a useful linker for the hydroxyl group. The anchoring on the solid support was achieved with Fmoc-β-hydroxyethylamine, followed, after Fmoc deprotection, by coupling/Fmoc deprotection cycles with Fmoc-β-Ala-OH, followed by Fmoc-Gly-OH, Fmoc-Gaba-OH, Fmoc-anthranilic acid and hexanoic anhydride. The chain was cleaved from the resin and purified, leading to **1** as a solid.

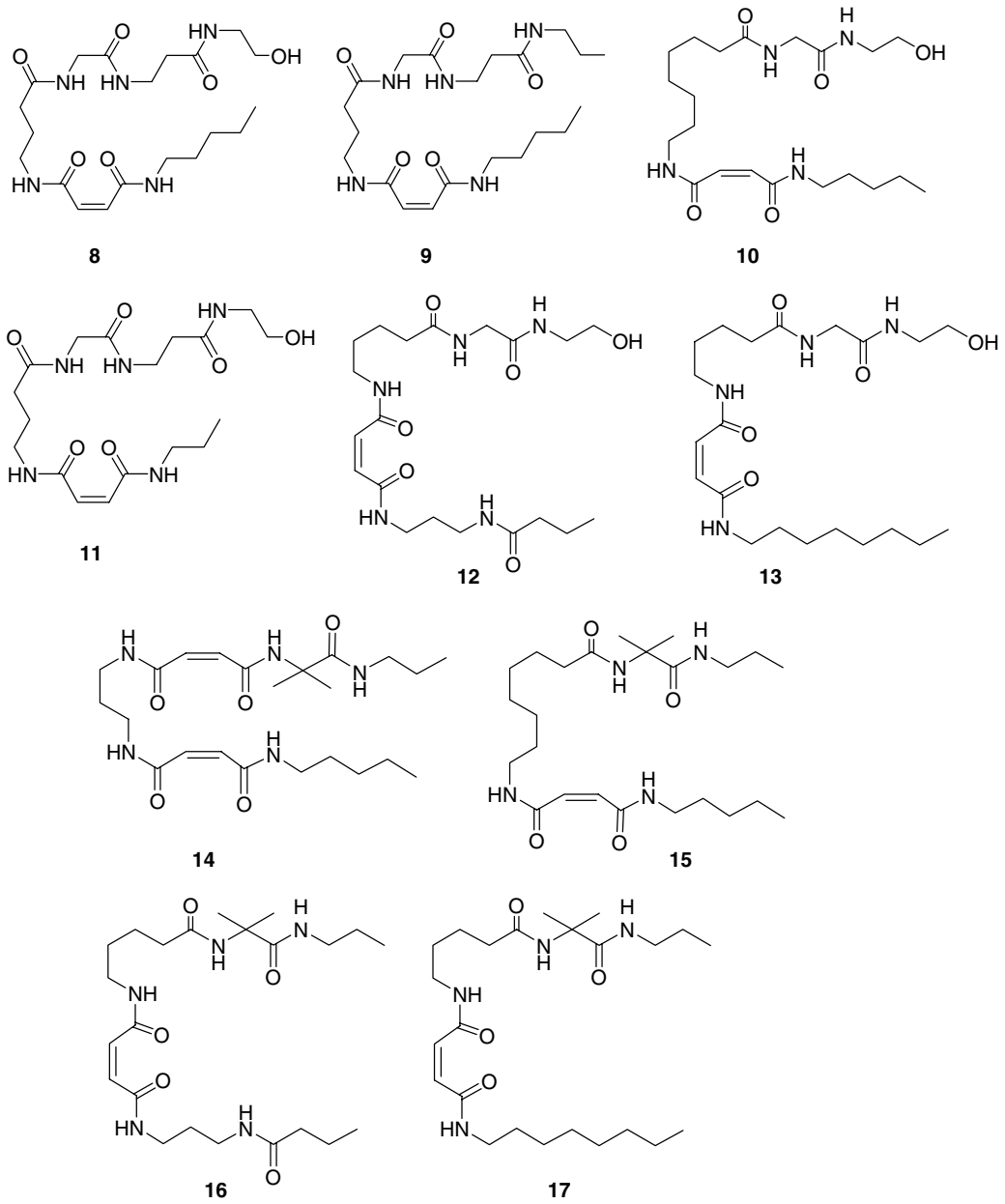

Chart 2 Second group of anandamide analogues (**8**–**17**).

Compound **2** was synthesized after the anchoring of the *C*-terminal-protected amino acid Fmoc-β-Ala-OH in its anhydride form to the HMPA functionalized resin. The sequential coupling steps gave good yields except for the last one, the coupling of the anthranilic residue with hexanoic anhydride. The low yield of this reaction is probably due to steric hindrance of the anthranilic acid and/or to the low nucleophilicity of its amino group. Improvement of the reaction yield was achieved by repeating three times the coupling by dissolving the anhydride in the so-called *magic mixture* [25] (1 : 1 : 1 DMF–DCM (dichloromethane)–NMP +1% Triton X) in presence of LiCl, a chaotropic salt. Compounds **3** and **4** have the same skeleton as **2** and **1**, respectively, except for the substitution of the anthranilic acid residue with the 3-aminomethylbenzoic acid residue. The syntheses of **3** and **4** were achieved with the same procedures already described for **2** and **1**, respectively. Notably, the insertion of the 3-aminomethylbenzoic residue during the synthesis of **3** led to a high yield of the subsequent coupling reactions. **3** and **4** were isolated as pure solids and were characterized spectroscopically.

Compounds **5** and **6**, both based on Acc as the rigidifying element, show the same structural features but with an opposite backbone orientation due to the presence of an amide and an inverse-amide function, respectively. The first step of the synthetic route to compound **5** was the anchoring of Fmoc-β-Ala-OH to the resin, followed by the successive solid-phase Fmoc deprotection/coupling cycles with Fmoc-Gly-OH,

Table 1 CB_1 binding results for compounds **1**–**17**

Ligand	K_i[a](μM)	% displacement at 50 μM
AEA	0.8 ± 0.2	—
1	42.0 ± 5.5	—
2	37.5 ± 3.2	—
3	39.0 ± 3.1	—
4	—	38.3 ± 3.0
5	—	10.0 ± 1.2
6	—	11.0 ± 1.3
7	—	10.0 ± 1.2
8	20.0 ± 1.9	—
9	—	18.8 ± 1.7
10	—	19.2 ± 1.1
11	—	14.5 ± 1.5
12	—	18.8 ± 1.3
13	—	31.5 ± 2.9
14	—	9.9 ± 0.6
15	—	15.5 ± 2.0
16	—	24.0 ± 1.9
17	—	10.3 ± 1.0

[a] K_i values (μM) were calculated only for compounds that were able to cause at least a 50% displacement of labelled ^{3}H-SR141716A from CB_1 receptors. Data are mean ± SEM of $n = 3$ determinations.

Fmoc-Gaba-OH and Fmoc-Acc-OH, and then with *n*-hexanoic anhydride and the final step in solution with *n*-propyl amine. Compound **6**, containing an inverse-amide function, was synthesized proceeding from the opposite direction. Therefore, the resin functionalized with Fmoc-Acc-OH was then subjected to the coupling steps with Fmoc-Gaba-OH, Fmoc-Gly-OH and Fmoc-β-Ala-OH, propanoic anhydride and the final step in solution with *n*-pentylamine. Both compounds **5** and **6**, after purification by HPLC, were obtained in high yields as oils.

Compound **7**, the only one making use of a proteinogenic turn-inducing residue (L-Pro), was synthesized according to the usual procedure by anchoring the Fmoc-L-Pro-OH residue to the resin. The following couplings proceeded with Fmoc-Apa-OH (Fmoc-4-aminopentanoic acid), Fmoc-β-Ala-OH, Fmoc-Gly-OH and butanoic anhydride, respectively, and were followed by cleavage from the solid support and reaction with *n*-pentylamine in solution, leading, after purification, to compound **7** as pure solid.

Compounds **8** and **9** were characterized by the presence of a Δ^{14} C=C bond functionality, similarly to AEA, introduced using a maleic acid residue. However, they had to be synthesized by different strategies owing to the presence in **8** of a β-hydroxyethyl functionality in the head portion. The synthesis of **8**, similar to **1**, was achieved by the use of the 2-chloro-trityl linker functionalized with Fmoc-β-hydroxyethylamine. All the couplings were carried out on solid phase, starting with Fmoc-β-Ala-OH, Fmoc-Gly-OH, Fmoc-Gaba-OH, maleic anhydride and *n*-pentylamine, respectively. After cleavage from the resin the peptide was purified by RP-HPLC and obtained as a solid. Synthesis of **9** proceeded with the same first three steps used for **5**. Then, the maleic acid residue was introduced through its anhydride followed by coupling with *n*-pentylamine. After cleaving from the resin the precursor of **9** as free acid, the last reaction was carried out in solution with *n*-propylamine, affording, after HPLC purification, compound **9** as solid in good yields.

The general synthetic procedures described above for compounds **1**–**9** were similarly applied to the preparation of **10**–**17**. The syntheses of **10**–**13** were completed on the solid support, using the 2-chloro-trityl linker, initially functionalized with Fmoc-β-hydroxyethylamine, as in **8**. Compound **10**, containing one less peptide linkage such as **13**, was obtained after coupling with Fmoc-Gly-OH, Fmoc-Ao-OH, maleic anhydride and *n*-pentylamine. The synthesis of **11** was achieved by starting the couplings with Fmoc-β-Ala-OH, followed by Fmoc-Gly-OH, Fmoc-Gaba-OH, maleic anhydride and *n*-propylamine. The chain was then cleaved from the resin and purified by HPLC. Compounds **12** and **13** shared the same synthetic pathway except for the last coupling on the resin. The common initial functionalization of the linker by Fmoc-β-aminoethanol was followed by Fmoc deprotection and coupling with Fmoc-Gly-OH, Fmoc-Ap-OH and then with maleic anhydride. Half of the native chain was then submitted to reaction with Fmoc-1,3-diaminopropane and then with butyric anhydride, yielding, after cleavage from the solid support and HPLC purification, compound **12** as a colourless oil.

Compound **13** was obtained, as for **12**, from the second half of the resin containing its tripeptide precursor. The last step with *n*-octylamine was followed by cleavage and HPLC purification leading to a colourless oil.

The same kind of solid support functionalization was employed for compounds **14**–**17**, by anchoring to the linker Fmoc-2-methyl-Ala-OH (Fmoc-Aib). Then, the syntheses of the different compounds diverged. Compound **14** is characterized by the presence of two maleic acid residues in the chain. This peculiarity involved a double inversion of the directionality of peptide formation. Therefore, the first mono-coupling with maleic anhydride was followed by reaction with Fmoc-1,3-diaminopropane, which permitted the insertion of the second maleic acid residue. Then, the final step with *n*-pentylamine was followed, as usual, by reaction of the cleaved peptide with *n*-propylamine. Compound **14** was isolated as a colourless oil and spectroscopically characterized. The synthetic route to compound **15** proceeded with the insertion of the 8-aminooctanoyl residue, followed by coupling with maleic anhydride and *n*-pentylamine. A last coupling

with *n*-propylamine occurred in solution, after cleavage of the chain from the solid support, yielding, after chromatographic purification, an oily, colourless liquid.

Compounds **16** and **17** were synthesized, after the common initial functionalization of the linker by Fmoc-Aib, following the same sequential coupling steps already described for **9** and **10**. Then, after cleavage from the resin, the free peptides were allowed to react with *n*-propylamine in solution and purified by HPLC leading to **16** and **17** as pure compounds.

Conformational Analysis and 3D Structure–Activity Relationships

A detailed conformational analysis was carried out on two reference compounds, i.e. **2** and **9**, to compare their 3D structures with the active conformations reported in the literature for the AEA [21], and to obtain a deeper insight into the structural requirements of potential ligands for the CB_1 receptor. The choice of **2** and **9** as reference molecules for the conformational analysis follows the need to investigate both an active and an inactive molecule. After a step of restrained molecular mechanics and dynamics (MD) calculations using NMR-derived distance constraints, we then performed a QM based geometry optimization and GIAO ^{13}C chemical shift calculations to assess the reliability of our structures.

NMR Spectroscopy

An extensive NMR analysis was carried out on compounds **2** and **9** to obtain the sequence-specific assignment of the ^{1}H and ^{13}C resonances of both peptides using a set of homonuclear and heteronuclear two-dimensional experiments. Subsequently, to collect a set of experimental restraints to be used in the course of the MM and MD calculations, a careful analysis of the ROESY data was performed on the basis of a distance calibration (r^{-6}, two-spin approximation) to convert the most intense and significant ROESY cross-peak volumes into inter-proton distances.

Molecular Mechanics and Molecular Dynamics Calculations

A 3D-structural characterization of **2** and **9** is intrinsically complicated by the fact that both peptides are flexible compounds. For this reason, a preliminary round of MM and MD calculations was run at 500 K in order to properly monitor the conformational space. Though the following MM and MD were driven by the restraints collected during the NMR analysis, the final structures may be correctly represented as an ensemble of equilibrium conformations (Figures 2 and 3).

DFT Geometry Optimization and ^{13}C Chemical Shift Calculations

To obtain a Boltzmann distribution of the ensemble of structures obtained by MM and MD studies, DFT

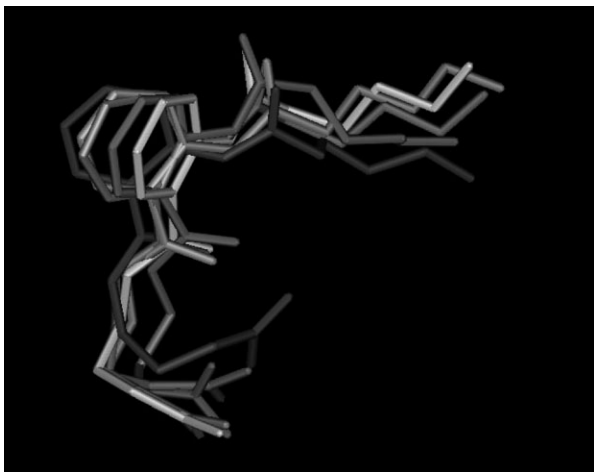

Figure 2 NMR structure found for compound **2** by restrained MD and MM calculations.

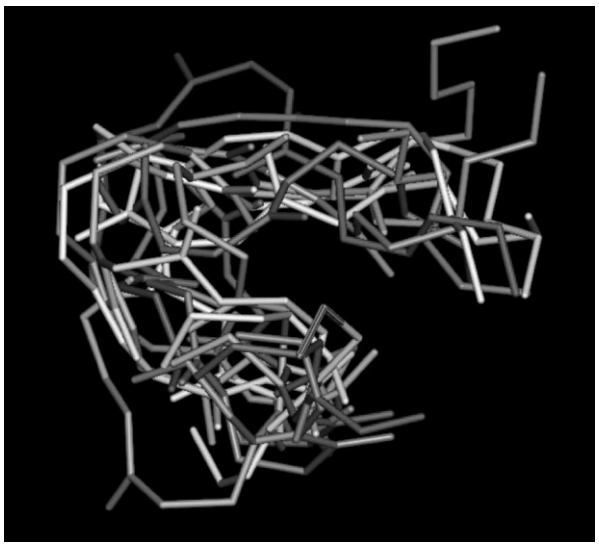

Figure 3 NMR structure found for compound **9** by restrained MD and MM calculations.

thermochemical calculations using the mPW1PW91 method and the 6–31g(d) basis set in the harmonic approximation of the vibration modes were employed for the evaluation of the standard Gibbs free energy of the conformers at 298.15 K. Subsequently, GIAO ^{13}C chemical shift (cs) calculations were performed at the mPW1PW91/6–31g(d,p) level on each set of conformers for both **2** and **9**. All calculations were carried out using the Gaussian 98 W program package. For each peptide, the ^{13}C NMR chemical shift of a given carbon atom was obtained as the weighted average chemical shift value of the same atom in all conformers sampled by the initial conformational search, and, following recent procedures on the structure validation of organic compounds, the calculated ^{13}C NMR cs were compared to the experimental values (see Materials and Methods). An analysis of the fundamental parameters listed in Table 2 offers a useful means for the evaluation of the

ensembles of structures found for **2** and **9**. Moreover, a visual examination of the correlation plots (Figures 4 and 5) of the calculated *versus* the experimental data provides a sound indication of the quality of the ensembles of structures proposed for both **2** and **9**.

Structure–Activity Relationships for 2 and 9

Our synthesis of a series of peptides that may mimic the structural features of AEA had the main goal of determining which conformations of the peptides had affinity for the CB_1 receptor. Ultimately, our results aimed at suggesting, together with the data already reported in the literature, the conformational features that are required and/or are involved in the binding mode to the CB_1 receptor.

Compound **2**, with respect to **9**, shows a more restricted ensemble of equilibrium conformations (Figure 2). By analogy with the classification reported for the conformations characterizing arachidonic

Table 2 Experimental and theoretical chemical shifts (cs), R^2, mean absolute error, error and mean error for analogues **2** and **9**

Compound **2**			Compound **9**		
	^{13}C Chemical shift (cs/ppm)			^{13}C Chemical shift (cs/ppm)	
Carbon atom	Experimental	Theoretical	Carbon atom	Experimental	Theoretical
1	169.80	161.03	1	170.35	161,96
2	34.91	37.81	2	35.21	37,33
3	35.00	35.47	3	35.27	38,04
5	167.90	163.82	5	168.86	161,11
6	41.77	45.62	6	42.02	46,62
8	172.00	164.77	8	172.09	169,43
9	32.31	28.88	9	32.52	36,14
10	24.60	24.55	10	24.98	28,33
11	38.61	36.69	11	38.19	40,78
13	168.00	164.27	13	164.39	159,65
14	120.00	114.42	14	131.52	138,03
15	127.61	120.66	15	131.83	125,66
16	122.06	115.20	16	164.75	157,89
17	131.51	129.33	18	38.66	39,25
18	120.11	118.21	19	28.42	31,00
19	138.90	139.56	20	28.62	29,78
21	170.80	165.64	21	21.77	24,65
22	37.27	40.58	22	13.87	15,42
23	24.33	27.73	2′	41.41	42,18
24	30.58	31.56	3′	59.83	59,77
25	21.51	24.55	—	—	—
26	13.64	16.22	—	—	—
2′	40.10	41.47	—	—	—
3′	22.02	21.70	—	—	—
4′	11.00	12.58	—	—	—
1	169.80	161.03	—	—	—
2	34.91	37.81	—	—	—
3	35.00	35.47	—	—	—
R^2	0.9984[a]	—	R^2	0.9977[a]	—
MAE[b]	3.29	—	MAE[b]	3.59	—
ME[c]	1.36	—	ME[c]	0.08	—

The calculated ^{13}C chemical shift values were obtained by subtracting its ^{13}C isotropic magnetic shielding (the IMS calculated by the GIAO method) from the average ^{13}C (IMS) of the tetramethylsilane (TMS): $\delta_{calcd} = |IMS_{TMS} - IMS_{xi}|$, where xi is referred to as the ith carbon atom considered. The ^{13}C chemical shift values, calculated (δ_{calcd}) for the optimized structures of the compounds were plotted *versus* the corresponding experimental ^{13}C chemical shifts values (δ_{exp}). The least-squares fit values of intercept (*b*), slope (*a*) and linear correlation coefficient (R^2) have been obtained by using the following linear equation: $\delta_{calcd} = a\delta_{exp} + b$.

[a] Correlation coefficient obtained by linear fitting of calculated (δ_{calcd}) *versus* experimental (δ_{exp}) ^{13}C NMR chemical shifts.

[b] MAE = mean absolute error = $\Sigma[|(\delta_{exp} - \delta_{calcd})|]/n$, sum of errors of experimental values from calculated ones divided by the number of carbon atoms ($n = 225$).

[c] ME, mean error = $\Sigma[(\delta_{exp} - \delta_{calcd})]/n$, sum of errors of experimental values from calculated ones divided by the number of carbon atoms ($n = 225$).

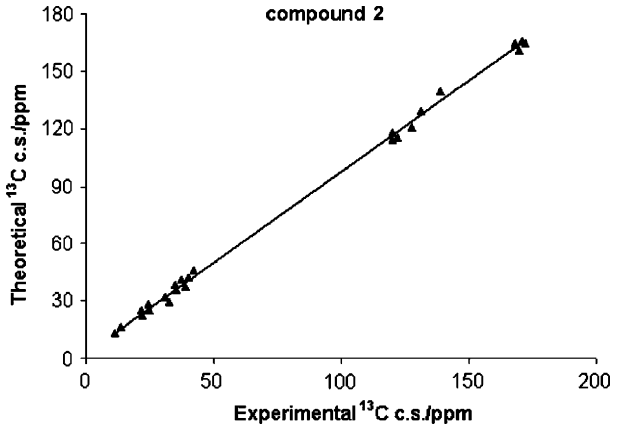

Figure 4 Correlation plot of calculated *versus* experimental ^{13}C NMR chemical shift (cs) for analogue **2**, at the mPW1PW91/6–31G(d,p)//mPW1PW91/6–31G(d) level.

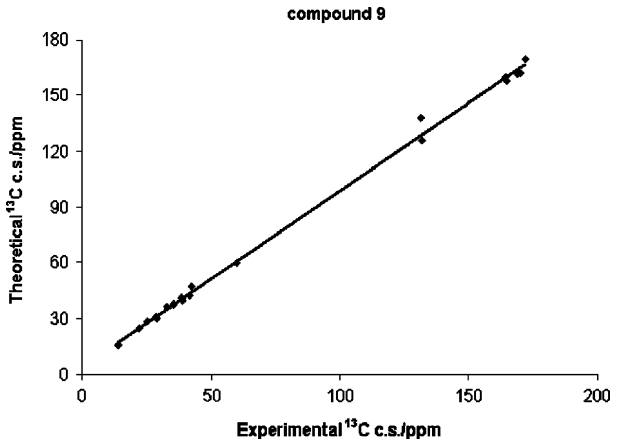

Figure 5 Correlation plot of calculated *versus* experimental ^{13}C NMR chemical shift (cs) for analogue **9**, at the mPW1PW91/6–31G(d,p)//mPW1PW91/6–31G(d) level.

acid and AEA, NMR data and restrained MD and QM calculations all point to a prevalent hairpin (U) molecular shape for **2**. With regard to the observed affinity for the CB_1 receptor of **2** (and of the other micromolar affinity ligands presented here), a definitive conclusion on the biological relevance of the U conformation for targeting this receptor is difficult to draw. Indeed, on the one hand, all compounds are bound to the CB_1 receptor less efficiently than AEA, suggesting that the endogenous cannabinoid may adopt other conformation(s) that allow an optimal fit to its binding site. On the other hand, the micromolar affinity displayed by some of these hairpin-like molecules supports the view that a specific ligand–protein molecular recognition must undoubtedly occur also for these substances. The loss in affinity with respect to AEA could then be explained in several ways: (i) The presence of amide bonds along the chain of these lipopeptides may partially disrupt, by virtue of their polarity, a purely hydrophobic interaction that, perhaps, is a necessary pre-requisite in some region of the ligand–protein contact molecular surface. (ii) The presence of peptide bonds associated with one or two *rigid*, turn-inducing residues may simply render these molecules too rigidified for optimal CB_1 recognition. In this view, flexibility may represent an advantage for AEA–CB_1 affinity due to the lower energetic cost required to fold the endocannabinoid in a more compact 3D form upon binding; one may even imagine that more than one AEA conformation could be biologically relevant. (iii) The presence of a significantly larger number of hydrogen-bond acceptors and donors in our lipopeptides may cause them to be trapped in interactions with regions of the CB_1 receptor different from the active site (i.e. the site capable of transducing the signal), thereby wasting binding energy in unfruitful recognition processes.

Conversely, the analysis of the structural ensemble of **9**, which is not as defined as **2**, seems to indicate that derivatives lacking any preferential conformation hardly bind the CB_1 receptor. Actually, this may well be the main problem for most of the other lipopeptides showing weak CB_1 affinity, except in the case of **7** (the proline-containing lipopeptide), for which molecular modelling calculations suggest too tight a turn in correspondence of the Pro residue, causing the molecule to assume a shape too distant from those considered to be compatible with AEA.

MATERIALS AND METHODS

Unless specified otherwise, solvents were of reagent grade; they were purchased from Aldrich, Fluka or Labscan and were used without further purification. DCM and DMF used for the solid-phase reactions were of synthesis grade (dried over 4 Å molecular sieves); water (A) and MeCN (acetonitrile) (B) for product purifications were HPLC grade. We used three kinds of linkers on polystyrene–divinylbenzene (1%) support: HMPA (loading capacity: 0.24 mol g^{-1}); 2-chlorotrityl chloride (loading capacity: 1.04 mol g^{-1}); glycinol-2-chlorotrityl (loading capacity: 1.10 mol g^{-1}); they were all purchased from Novabiochem. The building blocks (Fmoc-amino acids, Fmoc-amines, anhydrides and amines) and the coupling reagents (HOBt, HBTU, HATU) were supplied by Novabiochem, Neosystem and Aldrich, respectively, and used without further purification. Solid-phase reactions were carried out in batches, on a glass reaction vessel or in a polypropylene column Isolute (on a Vac Master system purchased from Stepbio). For quantification of Fmoc-amino acids on the resin, absorbance at 301 nm was read employing a Shimadzu UV 2101 PC. An LCQ Finnigan mass spectrometer was used to record the ESI-MS spectra. The ^{1}H and ^{13}C (^{1}H–^{1}H and ^{1}H–^{13}C) spectra were recorded using a Bruker DRX 600 MHz spectrometer with a deuterated solvent ($CDCl_3$ or DMSO-d_6). All 2D NMR spectra (^{1}H, HMBC, HSQC, TOCSY, COSY, ROESY 200 ms) were recorded on a Bruker DRX 600 spectrometer at $T = 298$ K. For conformational analysis, the two peptides (**1** and **5**) were dissolved in 0.5 ml of 99.95% DMSO-d_6. The NMR data were processed on a Silicon Graphics Indigo2 workstation using UXNMR software.

Conformational Studies

A conformational search was performed for both peptides by (i) a step of free MD at high temperature (500 K, 5.0 ps); (ii) a round of restrained MD and minimization at high temperature (500 K, 5.0 ps) (the restraints were obtained using a distance calibration (r^{-6}, two-spin approximation) to convert the most intense and significant ROESY cross-peak volumes into inter-proton distances) and (iii) a final step of restrained MD and minimization at 300 K (5.0 ps). During the simulations, the CVFF [26] force field was applied. The effect of the solvent (DMSO) was incorporated in the calculations by considering it as a continuous dielectric medium, characterized by a dielectric constant. Minimizations and MD simulations were performed in the Discover module of Insight II [27].

Standard *ab initio* molecular orbital calculations were carried out with the program Gaussian 98W [28]. The structures (five conformers of **2** and of the eleven conformers of **9**) and energies of all compounds were first optimized at AM1 level; a final optimization at MPW1PW91 level of theory using the 6–31G(d) basis set was then performed. Finally, the chemical shift calculations for the structures optimized at MPW1PW91/6–31G(d) level were performed using the same functional and the 6–31G(d,p) basis set. The absolute chemical shift values obtained by subtracting the GIAO [29–33] calculated ^{13}C IMS of any carbon atom from the average GIAO ^{13}C magnetic shielding of TMS (tetramethylsilane, calculated always at same level of theory, $IMS_{TMS} = 195.987$ ppm): $CS_i = IMS_{TMS} - IMS_i$. The theoretical chemical shift values of the two peptides were determined by using the Boltzmann distribution function that allowed, on the basis of the Gibbs free energy, calculation of the contribution of each conformer to the total population. Equation (1) was used to calculate the cs value:

$$cs_i = \sum_{i}^{N} \frac{cs_i \times e^{-\frac{\Delta G_i^\circ}{RT}}}{\sum_{i}^{N} e^{-\frac{\Delta G_i^\circ}{RT}}} \qquad (1)$$

where N is the number of the conformers found for each peptide (compounds **2** and **9**), R is the ideal gas constant, T the absolute temperature and ΔG°_i the standard free energy value of the i^{th} conformer relative to the energy of the most stable conformer. For compound **9** two conformers have been eliminated in view of their low contribution to the total population ($<10^{-5}$%).

General SPPS Procedures

(a) Anchoring of the first Fmoc-amino acid to the HMPA linker. The polymeric support (600.0 mg, 0.144 mmol) was placed in a 50-ml reaction glass vessel or in a polypropylene column Isolute and allowed to swell for 1 h with DMF (6 ml). During this time the symmetric anhydride of the first Fmoc-amino acid (10 equiv.) was prepared by dissolving it in DCM (7 ml) and DMF (a few drops). N,N-dicyclohexylcarbodiimide (DCC) (5 equiv.) was added at 0 °C and the mixture was stirred for 20 min. The reaction mixture was filtered and the filtrate evaporated under reduced pressure to give the product as a solid. It was dissolved in DMF (4 ml) and added to the swelled support. 4-dimethylaminopyridine (DCC) (17.6 mg, 0.144 mmol) was added and the suspension was left reacting for 4 h under stirring. The support was washed with DMF (3 ml × 3 ml), DCM (3 ml × 3 ml) and Et_2O (3 ml × 3 ml) and then dried *in vacuo*.

(a_1) Anchoring of Fmoc-β-hydroxyethylamine to the 2-chlorotrityl chloride linker. Materials and reagents were dried under vacuum over KOH for one day. The polymeric support (200 mg, 0.208 mmol) was placed in a polypropylene column Isolute and allowed to swell for 2 h with DMF (3 ml). Fmoc-β-hydroxyethylamine (82 mg, 0.291 mmol) was dissolved in DCM/DMF (1.50 ml/0.12 ml) and added to the support with N,N-diisopropylethylamine (DIEA) (76.8 µl, 0.437 mmol), and the resin was left to react under stirring for 24 h. The support was then washed wih DMF (3 × 2.5 ml), DMF/H_2O 1: 1 (4 × 2.5 ml), H_2O (4 × 2.5 ml), MeOH (4 × 2.5 ml), DCM (4 × 2.5 ml) and Et_2O (3 × 2.5 ml) and dried *in vacuo* over KOH for 2 h.

(b) Determination of substitution level (spectrophotometric analysis of the Fmoc chromophore). The assay was performed on duplicate samples. 0.4 ml of piperidine and 0.4 ml of DCM were added to two dried samples of the resin (~5 mg) in two 10-ml volumetric flasks. The reaction was allowed to proceed for 30 min at RT in the sealed flasks. Then, 1.6 ml of MeOH was added and the solutions were diluted to 10 ml volume with DCM. A reference solution was prepared in a 10-ml volumetric flask using 0.4 ml of piperidine, 1.6 ml of MeOH and DCM to volume. The solutions were shaken and the absorbance of the samples *versus* the reference solution was measured at 301 nm. The substitution level (in mmol of amino acid/g of resin) was calculated from the equation: mmol/g = $(A_{301}/7800) \times (10\ mlg^{-1}$ of resin).

(c) Fmoc deprotection. This was carried out in the following steps: 20% piperidine in DMF (3 ml), 1.5 min; 20% piperidine in DMF (3 ml), 10 min; washings (1.5 min each): DMF (2 × 3 ml), DCM (3 × 3 ml), DMF (2 × 3 ml).

(d) Conditions for couplings with Fmoc-amino acids. Fmoc-AA-OH (3 equiv.), HOBt (3 equiv.), HBTU (3 equiv.), NMM (4 equiv.) in DMF (2.5 ml). Washings (1.5 min each) in DMF (3 × 3 ml) and DCM (3 × 3 ml). (d_1) The coupling with maleic anhydride (3 equiv.) and NMM (4 equiv.), 1.30 h, was followed by capping with acetic anhydride (5 equiv.) and DIEA (5 equiv), 45 min. (d_2) Couplings with amines: HATU (1 equiv.), R-NH_2 (1 equiv.), NMM (2 equiv.), 1.30 h. (d_3) Couplings with anhydrides: anhydride (10 equiv.), DIEA (15 equiv.), 1.30 h.

(e) Mini cleavage conditions. After drying the resin-bound peptide *in vacuo* for 1 h, a small aliquot (8–10 mg) was removed and treated with a solution of TFA/H_2O (95 : 5; 100 ml ×1 mg of resin) for 1 h. The mixture was filtered through a small plug of cotton wool in a Pasteur pipette, rinsed with a small volume of 95% TFA and collected in a falcon tube. The filtrate was reduced to minimum volume (~500 µl) with a nitrogen stream, and cold diethyl ether (20 ml ×1 ml of filtrate) was added. The white precipitate was kept at 0 °C for 2 h. The product was collected by centrifugation, washed with cold diethyl ether, suspended in HPLC (Milli-Q) water and lyophilized. Then, it was analyzed by RP-HPLC (5–100% B in 31 min) and ESI-MS to determine the success of the solid-phase synthetic steps.

(f) Cleavage conditions. The support-bound peptide was washed with DMF, DCM, Et_2O, dried *in vacuo* and then treated

with TFA/H_2O (95 : 5; 10 µl × 1 mg of resin) for 1 h under stirring. The cleavage mixture was filtered off and the resin was washed three times with 95% TFA. The filtrate volume was reduced under a nitrogen steam and then added with a 20-fold excess of cold diethyl ether. A white precipitate appeared soon after the diethyl ether addition and the ether peptide mix was kept at 0 °C for 2 h. The precipitated compound was collected by filtration through a 0.45-µm PTFE membrane filter (PALL Corporation/Gelman Laboratory) installed on a 47-mm glass filter holder with a vacuum aspirator. The precipitate was washed with cold diethyl ether. The filter was transferred to a glass vessel, the solid removed by HPLC-grade water (with some drops of glacial acetic acid) and ultrasound and the solution or suspension was lyophilized. (f_1) As in most cases precipitation by addition of cold diethyl ether was absent or not complete, we proceeded also to the evaporation of the acid/ether filtrate under reduced pressure; the residue was utilized for the following coupling in solution without further purification.

(g) Coupling in solution. To a solution in DMF of the crude cleavage product were added HATU (1.0 equiv.), Pra (*n*-propylamine) (1.0 equiv.) and NMM (2.0 equiv.). The reaction mixture was stirred at RT and monitored by analytical RP-HPLC. Then, the solvent was evaporated and the residue purified by semi-preparative RP-HPLC with a linear gradient of A (H_2O + 0.1% TFA) and B (MeCN + 0.1% TFA). (g_1) To a suspension in DMF or in the magic mixture (DMF:DCM:NMP = 1 : 1 : 1 + 1% of Triton × 100) of crude cleavage product were added HATU (1.0 equiv.), Pra (1.0 equiv.) and NMM (2.0 equiv.). The reaction mixture was sonicated at RT and monitored by analytical RP-HPLC. After solvent evaporation the residue was purified by semi-preparative RP-HPLC.

Synthesis of Fmoc-Acc-OH

2-Amino-1-cyclohexanecarboxylic acid (Acc, 100 mg, 0.70 mmol) was dissolved in a 9% Na_2CO_3 solution (1 ml), and a solution of Fmoc-OSu (Fmoc-succinimido ester) (236 mg, 0.70 mmol) in dioxane (2 ml) was added dropwise over 10 min under stirring. The reaction mixture was sonicated for 20 min and the reaction monitorated by thin layer chromatography ($CHCl_3$: EtOH (ethanol) 9: 3). The reaction mixture was then diluted with water (15 ml), acidified to pH 2 with HCl 6 N at 0 °C and extracted with ethyl acetate (1 × 5 ml; 7 × 3 ml). The organic layer was dried over Na_2SO_4 and the solvent was removed to dryness. Yield was 97%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 1.40 (br m, 2H, CH_2); 1.51 (br s, 2H, CH_2); 1.69 (br m, 2H, CH_2), 1.79 (q, J = 9.69 Hz, 2H, CH_2); 2.91 (br s, 1H, CH); 3.93 (br s, 1H, CH); 4.22 (br m, 1H, CH); 4.37 (t, J = 6.06 Hz, 2H, CH_2); 4.50 (br s, 1H, CH); 5.66 (d, J = 8.07 Hz, 1H, NH); 7.30 (t, J = 7.27 Hz, 2H, CH arom.); 7.39 (t, J = 7.27 Hz, 2H, CH arom.); 7.58 (br m, J = 7.27 Hz, 2H, CH arom.); 7.75 (t, J = 7.27 Hz, 2H, CH arom.). ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 22.51; 24.21; 27.31; 29.76; 44.64; 47.42; 49.87; 66.99; 67.22; 120.12; 125.26; 127.21; 127.83; 141.48; 144.08; 156.02; 179.33. ESI-MS m/z: 366.1 $[M + H]^+$.

Compound **1 (Hex-Ant-Gaba-Gly-β-Ala-Ae)**. The glycinol-2-chlorotrityl support (100 mg, 0.11 mmol) was swollen for 1 h in DMF (3 ml) and the resin was subsequently submitted to the following series of coupling-deprotection cycles (according to general procedure (d)): (i) Fmoc-β-Ala-OH (102.7 mg, 0.329 mmol), HOBt (50.5 mg, 0.329 mmol), HBTU (125.2 mg, 0.329 mmol), NMM (48.4 µl, 0.439 mmol), 1.0 h; (ii) Fmoc-Gly-OH (98.1 mg, 0.329 equiv.), HOBt (50.5 mg, 0.329 mmol), HBTU (125.2 mg, 0.329 mmol), NMM (48.4 µl, 0.439 mmol), 1.0 h; (iii) Fmoc-Gaba-OH (107.4 mg, 0.329 mmol), HOBt (50.5 mg, 0.329 mmol), HBTU (125.2 mg, 0.329 mmol), NMM (48.4 µl, 0.439 mmol), 1.0 h; (iv) Fmoc-Ant-OH (118.6 mg, 0.329 mmol), HOBt (50.5 mg, 0.329 mmol), HBTU (125.2 mg, 0.329 mmol), NMM (48.4 µl, 0.439 mmol), 1.0 h; (v) hexanoic anhydride (254.6 µl, 1.10 mmol), DIEA (211.2 µl, 1.21 mmol), 1.30 h. A small aliquot of resin (5.0 mg) was used for a mini cleavage according to general procedure (e): RP-HPLC (15–85% B in 35 min, λ = 230 nm) and MS analysis revealed the presence of a product missing the hexanoic acid residue. The last coupling (v) was then repeated with the same reagent quantities for 3 h. Then, cleavage was performed according to general procedures (f) and (f_1). Purification by RP-HPLC: 15–85% of B in 35 min, λ = 250 nm. Yield after HPLC purification: 37%; ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.83 (t, J = 5.3 Hz, 1H, NH-1′); 3.10 (q, J = 5.5 Hz, 2H, CH_2-2′); 3.38 (t, J = 5.7 Hz, 2H, CH_2-3′); 2.24 (t, J = 7.2 Hz, 2H, CH_2-2); 3.25 (q, J = 5.9 Hz, 2H, CH_2-3); 7.83 (t, J = 5.3 Hz, 1H, NH-4); 3.60 (d, J = 5.3 Hz, 1H, CH_2-6); 8.07 (t, J = 5.3 Hz, 1H, NH-7); 2.21 (t, J = 7.2 Hz, 2H, CH_2-9); 1.77 (qq, J = 7.0 Hz, 2H, CH_2-10); 3.26 (q, J = 5.9 Hz, 2H, CH_2-11); 8.73 (t, J = 4.8 Hz, 1H, NH-12); 7.71 (d, J = 7.9 Hz, 1H, CH-15); 7.12 (t, J = 7.4 Hz, 1H, CH-16); 7.46 (t, J = 7.7 Hz, 1H, CH-17); 8.39 (d, J = 8.3 Hz, 1H, CH-18); 11.26 (s, 1H, NH-20); 2.30 (t, J = 7.4 Hz, 2H, CH_2-22); 1.60 (qq, J = 6.8 Hz, 2H, CH_2-23); 1.28 (br m, 2H, CH_2-24); 1.29 (br m, 2H, CH_2-25); 0.86 (t, J = 5.9 Hz, 3H, CH_3-26). ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 41.30 (C-2′); 59.41 (C-3′); 161.78 (C-1); 35.09 (C-2); 34.78 (C-3); 160.19 (C-5); 42.22 (C-6); 164.03 (C-8); 32.52 (C-9); 24.51 (C-10); 38.57 (C-11); 168.02 (C-13); 168.81 (C-14); 119.57 (C-15); 113.71 (C-16); 123.45 (C-17); 112.13 (C-18); 130.91 (C-19); 162.66 (C-21); 37.11 (C-22); 24.52 (C-23); 30.68 (C-24); 21.30 (C-25); 13.51 (C-26). ESI-MS m/z: 492.1 $[M + H]^+$.

Compound **2 (Hex-Ant-Gaba-Gly-β-Ala-Pra)** (Hex, hexanoic acid; Ant, anthranilic acid). Fmoc-β-Ala-OH (448.3 mg, 1.440 mmol) was anchored to the HMPA support according to general procedure (a) (substitution level: 88%) and Fmoc deprotected according to procedure (c). The resin was subsequently submitted to the following series of coupling–deprotection cycles (couplings according to general procedure (d)): (i) Fmoc-Gly-OH (128.4 mg, 0.432 mmol), HOBt (66.1 mg, 0.432 mmol), HBTU (163.8 mg, 0.432 mmol), NMM (63.3 µl, 0.576 mmol), 1.45 h; (ii) Fmoc-Gaba-OH (140.6 mg, 0.432 mmol), HOBt (66.1 mg, 0.432 mmol), HBTU (163.8 mg, 0.432 mmol), NMM (63.3 µl, 0.576 mmol), 2 h; (iii) Fmoc-Ant-OH (155.3 mg, 0.432 mmol), HOBt (66.1 mg, 0.432 mmol), HBTU (163.8 mg, 0.432 mmol), NMM (63.3 µl, 0.576 mmol), 2 h; (iv) Hexanoic anhydride (333.0 µl, 1.440 mmol), DIEA (377.1 µl, 2.160 mmol); 1.30 h; (iv) Hexanoic anhydride (333.0 µl, 1.440 mmol), DIEA (377.1 µl, 2.160 mmol), in magic mixture, 1.30 h. After this coupling, Kaiser test gave a false negative, therefore a small aliquot of resin (12.0 mg) was utilized for a mini cleavage according to general procedure (e): RP-HPLC and ESI-MS analysis revealed the presence of a free *N*-terminus (missing the hexanoic acid residue). The last coupling (iv) was repeated with the same reagent quantities and with a 2 M LiCl solution in DMF for 5.30 h. Another mini cleavage was carried out on 6 mg of resin and the subsequent HPLC analysis showed the success of the reaction. A double cleavage

(1 h each) was performed according to general procedure (f_1). The liquid phase coupling was then carried out according to general procedure (g): crude peptide (94.5 mg, 0.210 mmol) in DMF (1.0 ml), HATU (80.2 mg, 0.210 mmol), Pra (17.3 µl, 0.210 mmol), NMM (46.4 µl, 0.420 mmol), 1 h. Purification by RP-HPLC: 10–60% B in 27 min, $\lambda = 310$ nm, $R_t = 20$ min. Yield after HPLC purification: 32%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.81 (t, $J = 5.5$ Hz, 1H, N*H*-1′); 2.98 (q, $J = 6.4$ Hz, 2H, CH_2-2′); 1.37 (ss, $J = 7.2$ Hz, 2H, CH_2-3′); 0.82 (t, $J = 6.8$ Hz, 3H, CH_3-4′); 2.22 (br m, 2H, CH_2-2); 3.25 (q, $J = 6.4$ Hz, 2H, CH_2-3); 7.84 (t, $J = 5.7$ Hz, 1H N*H*-4); 3.60 (d, $J = 5.7$ Hz, 2H, CH_2-6); 8.08 (t, $J = 5.7$ Hz, 1H, N*H*-7); 2.21 (br m, 2H, CH_2-9); 1.77 (qq, $J = 7.0$ Hz, 2H, CH_2-10); 3.27 (qq, $J = 6.4$ Hz, 2H, CH_2-11); 8.73 (t, $J = 5.5$ Hz, N*H*-12); 7.70 (d, $J = 7.9$ Hz, 1H, C*H*-15); 7.10 (t, $J = 7.7$ Hz, 1H, C*H*-16); 7.46 (t, $J = 7.7$ Hz, 1H, C*H*-17); 8.40 (d, $J = 8.1$ Hz, 1H, C*H*-18); 11.27 (s, 1H, N*H*-20); 2.30 (t, $J = 7.4$ Hz, 2H, CH_2-22); 1.60 (qq, $J = 7.0$ Hz, 2H, CH_2-23); 1.29 (br m, 2H, CH_2-24); 1.29 (br m, 2H, CH_2-25); 0.86 (t, $J = 7.2$ Hz, 3H, CH_3-26). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.01$ (C-2′); 22.02 (C-3′); 11.21 (C-4′); 169.79 (C-1); 35.02 (C-2); 35.02 (C-3); 167.90 (C-5); 41.81 (C-6); 172.01 (C-8); 32.42 (C-9); 24.59 (C-10); 38.61 (C-11); 168.01 (C-13); 120.00 (C-14); 127.69 (C-15); 121.99 (C-16); 131.49 (C-17); 120.09 (C-18); 138.90 (C-19); 162.52 (C-21); 37.18 (C-22); 24.31 (C-23); 30.47 (C-24); 21.49 (C-25); 13.62 (C-26). ESI-MS m/z: 490.2 $[M + H]^+$.

Compound **3 (Hex-Amb-Gaba-Gly-β-Ala-Pra)** (Amb, 3-aminomethylbenzoic acid). The first Fmoc-amino acid (Fmoc-β-Ala-OH, 448.3 mg, 1.440 mmol) was anchored to the HMPA support according to general procedure (a) (substitution level: 92%), and Fmoc residue was deprotected according to procedure (c). The resin was subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-Gly-OH (128.4 mg, 0.429 mmol), HOBt (66.1 mg, 0.429 mmol), HBTU (163.8 mg, 0.429 mmol), NMM (63.3 µl, 0.576 mmol), 1.30 h; (ii) Fmoc-Gaba-OH (140.6 mg, 0.429 mmol), HOBt (66.1 mg, 0.429 mmol), HBTU (163.8 mg, 0.429 mmol), NMM (63.3 µl, 0.576 mmol), 1.30 h; (iii) Fmoc-Amb-OH (161.3 mg, 0.429 mmol), HOBt (66.1 mg, 0.429 mmol), HBTU (163.8 mg, 0.429 mmol), NMM (63.3 µl, 0.576 mmol), 1.15 h; hexanoic anhydride (333 µl, 1.44 mmol), DIEA (377.1 µl, 2.16 mmol), 20 min. A small aliquot of resin (8.0 mg) was used for a mini cleavage according to general procedure (e): RP-HPLC (15–85% B in 35 min, $\lambda = 230$ nm) and MS analysis revealed the presence of the expected product. Then, cleavage was performed according to general procedure (f_1) and solution coupling according to the general procedure (g): crude peptide (69.1 mg, 0.149 mmol), in DMF (1.0 ml), HATU (58.6 mg, 0.149 mmol), Pra (12.7 µl, 0.149 mmol) and NMM (33.9 µl, 0.298 mmol), 2 h. Purification by RP-HPLC: 15–65% B in 100 min, $\lambda = 280$ nm, $R_t = 26$ min. Yield after HPLC purification: 30%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.82 (t, $J = 5.3$ Hz, 1H, N*H*-1′); 2.98 (q, $J = 6.4$ Hz, 2H, CH_2-2′); 1.38 (ss, $J = 7.0$ Hz, 2H, CH_2-3′); 0.82 (t, $J = 7.4$ Hz, 1H, CH_3-4′); 2.23 (t, $J = 7.2$ Hz, 2H, CH_2-2); 3.24 (br m, 2H, CH_2-3); 7.84 (t, $J = 5.3$ Hz, 1H, N*H*-4); 3.63 (d, $J = 5.3$ Hz, 2H, CH_2-6); 8.06 (t, $J = 5.3$ Hz, 1H, N*H*-7); 2.19 (br m, 2H, CH_2-9); 1.75 (qq, $J = 6.8$ Hz, 2H, CH_2-10); 3.26 (br m, 2H, CH_2-11); 8.45 (t, $J = 4.8$ Hz, 1H, N*H*-12); 7.69 (br d, 1H, C*H*-15); 7.39 (t, $J = 7.4$ Hz, 1H, C*H*-16); 7.37 (br, 1H, C*H*-17); 7.72 (s, 1H, C*H*-19); 4.29 (d, $J = 5.5$ Hz, 2H, CH_2-20); 8.32 (t, $J = 4.8$ Hz, 1H, N*H*-21); 2.13 (d, $J = 7.2$ Hz, 2H, CH_2-23); 1.52 (qq, $J = 6.8$ Hz, 2H, CH_2-24); 1.14–1.31 (br m, 4H: 1.21 (2H, CH_2-25), 1.25 (2H, CH_2-26)) 0.85 (t, $J = 6.8$ Hz, 1H, CH_3-27). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 41.01$ (C-2′); 22.89 (C-3′); 11.88 (C-4′); 170.78 (C-1); 36.12 (C-2); 36.03 (C-3); 169.48 (C-5); 42.73 (C-6); 172.86 (C-8); 33.21 (C-9); 26.03 (C-10); 39.58 (C-11); 166.81 (C-13); 135.08 (C-14); 126.04 (C-15); 130.62 (C-16); 128.89 (C-17); 140.67 (C-18); 127.05 (C-19); 41.60 (C-20); 172.81 (C-22); 36.10 (C-23); 25.68 (C-24); 31.49 (C-25); 22.63 (C-26); 14.41 (C-27). ESI-MS m/z: 504.2 $[M + H]^+$.

Compound **4 (Hex-Amb-Gaba-Gly-β-Ala-Ae)**. The glycinol-2-chlorotrityl support (200 mg, 0.11 mmol to involve 50% of anchoring sites) was swollen for 1 h in DMF (3 ml) and the resin was subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-β-Ala-OH (102.7 mg, 0.329 mmol), HOBt (50.5 mg, 0.329 mmol), HBTU (125.2 mg, 0.329 mmol), NMM (48.4 µl, 0.439 mmol), 1.0 h (substitution level: 0.71 mmol, 65%); (ii) capping with acetic anhydride (51.9 µl, 0.55 mmol) and DIEA (96 µl, 0.55 mmol), 40 min; (iii) twice: Fmoc-Gly-OH (126.7 mg, 0.426 mmol), HOBt (65.2 mg, 0.426 mmol), HBTU (161.9 mg, 0.426 mmol), NMM (62.5 µl, 0.568 mmol), 1.30 h; (iv) Fmoc-Gaba-OH (138.6 mg, 0.426 mmol), HOBt (65.2 mg, 0.426 mmol), HBTU (161.9 mg, 0.426 mmol), NMM (62.5 µl, 0.568 mmol), 1.30 h; (v) Fmoc-Amb-OH (162.4 mg, 0.426 mmol), HOBt (65.2 mg, 0.426 mmol), HBTU (161.9 mg, 0.426 mmol), NMM (62.5 µl, 0.568 mmol), 1.15 h; (vi) hexanoic anhydride (324.0 µl, 1.40 mmol), DIEA (268.9 µl, 1.54 mmol), 20 min. A small aliquot of resin (5.0 mg) was used for a mini cleavage according to the general procedure (e): RP-HPLC (15–85% B in 35 min, $\lambda = 230$ nm) and MS analysis revealed the presence of the complete product. Then, cleavage was performed according to general procedure (f_1). Purification by RP-HPLC: 19–69% B in 71 min, $\lambda = 270$ nm, $R_t = 11.4$ min. Yield after HPLC purification: 40%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.90 (t, $J = 4.8$ Hz, 1H, N*H*-1′); 3.09 (q, $J = 5.7$ Hz, 2H, CH_2-2′); 3.37 (br m, 2H, CH_2-3′); 4.68 (br t, 1H, O*H*-4′); 2.23 (t, $J = 7.0$ Hz, 2H, CH_2-2); 3.23 (t, $J = 7.4$ Hz, 2H, CH_2-3); 7.91 (t, $J = 4.8$ Hz, 1H, N*H*-4); 3.62 (d, $J = 5.5$ Hz, 2H, CH_2-6); 8.11 (t, $J = 5.5$ Hz, 1H, N*H*-7); 2.18 (br m, 2H, CH_2-9); 1.74 (qq, $J = 7.0$ Hz, 2H, CH_2-10); 3.25 (br m, 2H, CH_2-11); 8.50 (t, $J = 4.8$ Hz, 1H, N*H*-12); 7.68 (br d, 1H, C*H*-15); 7.39 (t, $J = 7.4$ Hz, 1H, C*H*-16); 7.37 (br d, 1H, C*H*-17); 7.72 (br d, 1H, C*H*-19); 4.29 (d, $J = 5.9$ Hz, 2H, CH_2-20); 8.36 (t, $J = 5.5$ Hz, 1H, N*H*-21); 2.13 (t, $J = 7.4$ Hz, 2H, CH_2-23); 1.51 (qq, $J = 6.8$ Hz, 2H, CH_2-24); 1.22 (br m, 2H, CH_2-25); 1.25 (br m, 2H, CH_2-26) 0.84 (t, $J = 6.8$ Hz, 3H, CH_3-27). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 41.21$ (C-2′); 59.48 (C-3′); 170.10 (C-1); 35.02 (C-2); 35.13 (C-3); 168.59 (C-5); 41.83 (C-6); 172.02 (C-8); 32.37 (C-9); 25.01 (C-10); 38.58 (C-11); 165.88 (C-13); 134.17 (C-14); 125.11 (C-15); 128.05 (C-16); 129.53 (C-17); 139.78 (C-18); 126.02 (C-19); 41.64 (C-20); 171.90 (C-22); 35.20 (C-23); 24.69 (C-24); 30.73 (C-25); 21.61 (C-26); 13.69 (C-27). ESI-MS m/z: 506.2 $[M + H]^+$.

Compound **5 (Hex-Acc-Gaba-Gly-β-Ala-Pra)**. The first Fmoc-amino acid (Fmoc-β-Ala-OH, 747.1 mg, 2.40 mmol) was anchored to the HMPA support according to general procedure (a) (substitution level: 92%) and Fmoc deprotected according to procedure (c). The resin was subsequently submitted to the following series of coupling-deprotection cycles (couplings according to general procedure (d)): (i) Fmoc-Gly-OH (214.0 mg, 0.72 mmol), HOBt (110.2 mg, 0.72 mmol), HBTU (273.0 mg, 0.72 mmol), NMM (105.5 µl, 0.96 mmol), 2 h; (ii) Fmoc-Gaba-OH (234.3 mg, 0.72 mmol), HOBt (110.2 mg, 0.72 mmol), HBTU (273.0 mg, 0.72 mmol), NMM (105.5 µl,

0.96 mmol), 2.30 h; the resin (1.06 g) was then divided in two parts and one of them (500 mg, 0.12 mmol) was utilized for the synthesis of compound **5** (Pa-Mal-Gaba-Gly-β-Ala-Pra) (Pa, pentylamine); (iii) Fmoc-Acc-OH (131.4 mg, 0.36 mmol), HOBt (55.0 mg, 0.36 mmol), HBTU (136.5 mg, 0.36 mmol), NMM (52.7 µl, 0.48 mmol); (iv) hexanoic anhydride (277.7 µl, 1.20 mmol), DIEA (314.3 µl, 1.80 mmol). A mini cleavage was performed on 11 mg of resin as in (e): RP-HPLC (5–100% di B in 31 min, $\lambda = 210$ nm) and MS analysis revealed the presence of the expected product. Then, a cleavage was performed according to general procedures (f) and (f_1); the residue of the evaporation of the acid/diethyl ether filtrate was utilized for the solution coupling according to general procedure (g): crude peptide (53 mg, 0.12 mmol) in magic mixture (2.0 ml), HATU (44.3 mg, 0.12 mmol), Pra (9.5 µl, 0.12 mmol), NMM (25.5 µl, 0.24 mmol), 1 h. The product obtained by precipitation with diethyl ether (33.7 mg, 0.07 mmol) reacted according to general procedure (g_1): crude (33.7 mg, 0.07 mmol) suspended in the magic mixture (2 ml), HATU (28.1 mg, 0.07 mmol), Pra (6.1 µl, 0.07 mmol), NMM (16.3 µl, 0.14 mmol), 1.30 h. Purification by RP-HPLC: 20–32% B in 30 min, $\lambda = 210$ nm, $R_t = 21.0$ min. Yield after HPLC purification: 53%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.79 (t, $J = 5.7$ Hz, 1H, N*H*-1′); 2.95 (q, $J = 6.8$ Hz, 2H, CH_2-2′); 1.35 (br m, 2H, CH_2-3′); 0.81 (t, $J = 4.2$ Hz, 3H, CH_3-4′); 2.18 (t, $J = 7.2$ Hz, 2H, CH_2-2); 3.21 (q, $J = 6.6$ Hz, 2H, CH_2-3); 7.83 (t, $J = 5.7$ Hz, 1H, N*H*-4); 3.58 (d, $J = 4.8$ Hz, 2H, CH_2-6); 7.96 (t, $J = 5.7$ Hz, 1H, N*H*-7); 2.04 (t, $J = 7.2$ Hz, 2H, CH_2-9); 1.53 (qq, $J = 7.0$ Hz, 2H, CH_2-10); 2.95 (q, $J = 6.8$ Hz, 2H, CH_2-11); 7.66 (t, $J = 5.7$ Hz, 1H, N*H*-12); 2.39 (m, $J = 4.4$ Hz, 1H, C*H*-14); 1.41–1.75 (m, 2H, CH_2-15); 1.32–1.48 (m, 2H, CH_2-16); 1.34–1.36 (m, 2H, CH_2-17); 1.33–1.78 (m, 2H, CH_2-18); 4.05 (br m, 1H, C*H*-19); 7.41 (d, $J = 8.3$ Hz, 1H, N*H*-20); 2.01 (t, $J = 7.4$ Hz, 2H, CH_2-22); 1.55 (m, 2H, CH_2-23); 1.17 (m, 2H, CH_2-24); 1.21 (m, 2H, CH_2-25); 0.78 (t, $J = 4.0$ Hz, 3H, CH_3-26). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 39.19$ (C-2′); 21.34 (C-3′); 12.74 (C-4′); 170.09 (C-1); 34.29 (C-2); 34.30 (C-3); 167.79 (C-5); 41.01 (C-6); 171.35 (C-8); 31.53 (C-9); 24.24 (C-10); 36.71 (C-11); 171.89 (C-13); 34.47 (C-14); 23.65 (C-15); 20.54 (C-16); 21.24 (C-17); 28.62 (C-18); 45.82 (C-19); 170.69 (C-21); 34.41 (C-22); 22.22 (C-23); 29.75 (C-24); 20.92 (C-25); 10.34 (C-26). ESI-MS m/z: 496.5 $[M + H]^+$.

Compound **6 (Bu-β-Ala-Gly-Gaba-Acc-Pa)** (Bu, butanoic acid). Fmoc-Acc-OH (438.0 mg, 1.20 mmol) was anchored to the HMPA support (500.0 mg, 0.12 mmol) according to general procedure (a) (substitution level: 78%) and Fmoc deprotected according to procedure (c). The resin was subsequently submitted to the following series of coupling-deprotection cycles (couplings according to general procedure (d)): (i) Fmoc-Gaba-OH (117.1 mg, 0.36 mmol), HOBt (55.1 mg, 0.36 mmol), HBTU (136.5 mg, 0.36 mmol), NMM (52.7 µl, 0.48 mmol), 2.30 h; (ii) Fmoc-Gly-OH (107.0 mg, 0.36 mmol), HOBt (55.1 mg, 0.36 mmol), HBTU (136.5 mg, 0.36 mmol), NMM (52.7 µl, 0.48 mmol), 1.30 h; (iii) Fmoc-β-Ala-OH (112.0 mg, 0.36 mmol), HOBt (55.1 mg, 0.36 mmol), HBTU (136.5 mg, 0.36 mmol), NMM (52.7 µl, 0.48 mmol); *N*-butanoic anhydride (196.3 µl, 1.20 mmol), DIEA (314.3 µl, 1.80 mmol), 1.30 h. A mini cleavage was performed on an aliquot of resin (9.5 mg) according to procedure (e); RP-HPLC (20–35% di B in 25 min, $\lambda = 210$ nm) and MS analysis revealed the presence of the expected product ($R_t = 6.8$ min; $m/z = 427$ $[M + H]^+$, ESI-MS). Cleavage was performed on the resin as in procedure (f_1) and the crude peptide obtained (71.9 mg, 0.17 mmol) was reacted in DMF (1.4 ml) according to procedure (g_1): HATU (64.2 mg, 0.17 mmol), Pa (19.5 µl, 0.17 mmol), NMM (37.1 µl, 0.34 mmol), 45 min. Purification by RP-HPLC: 20–30% B in 40 min, $\lambda = 210$ nm, $R_t = 23.5$ min. Yield after HPLC purification: 34%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.62 (t, $J = 5.7$ Hz, 1H, N*H*-1′); 2.95 (m, 2H, CH_2-2′); 1.32 (m, 2H, CH_2-3′); 1.18 (m, 2H, CH_2-4′); 1.24 (m, 2H, CH_2-5′); 0.84 (t, $J = 7.0$ Hz, 3H, CH_3-6′); 2.42 (m, 1H, C*H*-2); 1.48–1.77 (br m, 2H, CH_2-3); 1.32–1.49 (m, 2H, CH_2-4); 1.24–1.58 (m, 2H, CH_2-5); 1.34–1.82 (m, 2H, CH_2-6); 4.08 (br, 1H, C*H*-7); 7.46 (d, $J = 8.3$ Hz, 1H, N*H*-8); 2.06 (m, 2H, CH_2-10); 1.57 (qq, $J = 7.4$ Hz, 2H, CH_2-11); 3.02 (m, 2H, CH_2-12); 7.80 (t, $J = 5.3$ Hz, 1H, N*H*-13); 3.64 (d, $J = 5.7$ Hz, 2H, CH_2-15); 8.08 (t, $J = 5.7$ Hz, 1H, N*H*-16); 2.29 ($J = 7.0$ Hz, 2H, CH_2-18); 3.24 (q, $J = 6.6$ Hz, 2H, CH_2-19); 7.79 (t, $J = 5.7$ Hz, 1H, N*H*-20); 2.01 (m, 2H, CH_2-22); 1.48 (m, 2H, CH_2-23); 0.83 (t, $J = 7.4$ Hz, 3H, CH_3-24). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 38.19$ (C-2′); 28.76 (C-3′); 28.51 (C-4′); 21.83 (C-5′); 13.87 (C-6′); 172.69 (C-1); 44.41 (C-2); 24.56 (C-3); 21.43 (C-4); 23.25 (C-5); 29.69 (C-6); 46.91(C-7); 171.13 (C-9); 32.87 (C-10); 25.57 (C-11); 38.14 (C-12); 168.76 (C-14); 40.03 (C-15); 170.82 (C-17); 35.43 (C-18); 35.13 (C-19); 171.96 (C-21); 37.28 (C-22); 18.62 (C-23); 13.57 (C-24). ESI-MS m/z: 496.6 $[M + H]^+$.

Compound **7 (Bu-Gly-β-Ala-Ap-Pro-Pa)** (5-aminopentanoic acid).Fmoc-L-Pro-OH (485.9 mg, 1.44 mmol) was anchored to the HMPA support according to general procedure (a) (substitution level: 71%). The reaction was repeated with the same quantities of reagents to reach a substitution level of 83% and the resin was Fmoc-deprotected according to procedure (c). The resin was subsequently submitted to the following series of coupling–deprotection cycles (couplings according to general procedure (d)): (i) Fmoc-Ap-OH (146.6 mg, 0.43 mmol), HOBt (66.1 mg, 0.43 mmol), HBTU (163.8 mg, 0.43 mmol), NMM (63.3 µl, 0.57 mmol), 1.50 h; (ii) Fmoc-β-Ala-OH (134.5 mg, 0.43 mmol), HOBt (66.1 mg, 0.43 mmol), HBTU (163.8 mg, 0.43 mmol), NMM (63.3 µl, 0.57 mmol), 2 h; (iii) Fmoc-Gly-OH (128.4 mg, 0.43 mmol), HOBt (66.1 mg, 0.43 mmol), HBTU (163.8 mg, 0.43 mmol), NMM (63.3 µl, 0.57 mmol), 2 h; (iv) Butanoic anhydride (235.6 µl, 1.44 mmol), DIEA (377.1 µl, 2.16 mmol). The cleavage was then performed as in (f_1): RP-HPLC and ESI-MS analysis revealed the presence of the complete chain ($R_t = 10.5$ min, $m/z = 413$ $[M + H]^+$). The crude (51.5 mg, 0.12 mmol) was submitted to the solution coupling as in (g): DMF (2.5 ml); HATU (47.5 mg, 0.12 mmol), Pa (14.5 µl, 0.12 mmol), NMM (27.5 µl, 0.24 mmol), $t = 1$ h. Purification by RP-HPLC: 10–40% B in 40 min; $\lambda = 230$ nm; $R_t = 26$ min. Yield after HPLC purification: 35%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.70 (t, $J = 5.6$ Hz, 1H, N*H*-1′); 3.00 (m, 2H, CH_2-2′); 1.36 (m, 2H, CH_2-3′); 1.24 (m, 2H, CH_2-4′); 1.26 (m, 2H, CH_2-5′); 0.85 (t, $J = 7.2$ Hz, 3H, CH_3-6′); 4.21 (br m, 1H, C*H*-2); 1.77, 1.97 (m, 2H, CH_2-3); 1.82, 1.87 (m, 2H, CH_2-4); 3.42, 3.53 (m, 2H, CH_2-5); 2.27 (m, 2H, CH_2-8); 1.46 (qq, $J = 7.0$ Hz, 2H, CH_2-9); 1.41 (m, 2H, CH_2-10); 3.03 (m, 2H, CH_2-11); 7.85 (t, $J = 5.8$ Hz, 1H, NH-12); 2.22 (t, $J = 7.4$ Hz, 2H, CH_2-14); 3.24 (m, 2H, CH_2-15); 7.80 (t, $J = 5.8$ Hz, 1H, N*H*-16); 3.62 (d, $J = 5.9$ Hz, 2H, CH_2-18); 8.0 (t, $J = 5.8$ Hz, 1H, N*H*-19); 2.10 (t, $J = 7.3$ Hz, 2H, CH_2-21); 1.50 (ss, $J = 7.4$ Hz, 2H, CH_2-22); 0.85 (t, $J = 7.2$ Hz, 3H, CH_3-23). ^{13}C-NMR (600 MHz, DMSO-d_6): $\delta = 38.80$ (C-2′); 29.42 (C-3′); 29.42 (C-4′); 22.29 (C-5′); 14.18 (C-6′); 172.23 (C-1); 60.31 (C-2); 30.27 (C-3); 24.78 (C-4); 47.33 (C-5); 34.10 (C-8); 22.03 (C-9); 29.41

(C-10); 38.82 (C-11); 170.59 (C-13); 36.10 (C-14); 35.81 (C-15); 169.58 (C-17); 42.45 (C-18); 173.62 (C-20); 37.91 (C-21); 19.12 (C-22); 14.21 (C-23). ESI-MS m/z: 482.4 $[M+H]^+$.

Compound **8 (Pa-Mal-Gaba-Gly-β-Ala-Ae)** (Ae, 2-aminoethanol). The 2-chlorotrityl chloride support was functionalized according to general procedure (a_1) (substitution level: 45%, 0.47 mmol/g, 0.094 mmol for 0.20 g) and the Fmoc-β-aminoethanol was sequentially deprotected as in procedure (c). The resin was subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-β-Ala-OH (87.8 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); 1.30 h; (ii) Fmoc-Gly-OH (83.8 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); 1.30 h; (iii) Fmoc-Gaba-OH (91.7 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); 1.30 h; (iv) maleic anhydride (45.1 mg, 0.47 mmol), NMM (62.0 µl, 0.56 mmol); 1.30 h. Because of a false positive result of the Kaiser test, we performed another colorimetric test (Green Malachite) based on the detection of free -COOH groups, which gave a positive result. (v) Capping with acetic anhydride (44.4 µl, 0.47 mmol), DIEA (82.0 µl, 0.47 mmol); 45 min; (vi) HATU (35.7 mg, 0.09 mmol), Pa (10.9 µl, 0.09 mmol), NMM (20.7 µl, 0.19 mmol), 1.30 h; Green Malachite test was negative. A mini cleavage was performed on a small aliquot of resin (5.9 mg): RP-HPLC and MS analysis revealed the presence of the expected product. Then, cleavage was performed according to general procedures (f) and (f_1). Purification by RP-HPLC: 10 to 50% B in 40 min, $\lambda = 280$ nm. Yield after HPLC purification: 26%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.86 (t, $J = 5.2$ Hz, 1H, N*H*-1′); 3.08 (m, 2H, CH_2-2′); 3.37 (m, 2H, CH_2-3′); 4.66 (t, $J = 4.8$ Hz, 1H,O*H*-4′); 2.23 (t, $J = 6.9$ Hz, 2H, CH_2-2); 3.23 (q, $J = 6.5$ Hz, 2H, CH_2-3); 7.87 (t, $J = 5.2$ Hz, 1H, N*H*-4); 3.61 (d, $J = 5.6$ Hz, 2H, CH_2-6); 8.07 (t, $J = 5.2$ Hz, 1H, N*H*-7); 2.16 (t, $J = 6.9$ Hz, 2H, CH_2-9); 1.67 (qq, $J = 6.9$ Hz, 2H, CH_2-10); 3.11 (m, 2H, CH_2-11); 9.13 (t, $J = 4.8$ Hz, 1H, N*H*-12); 6.10 (br s, 1H, C*H*-14); 6.10 (br s, 1H, C*H*-15); 9.13 (t, $J = 4.8$ Hz, 1H, N*H*-17); 3.11 (m, 2H, CH_2-18); 1.44 (qq, $J = 6.9$ Hz, 2H, CH_2-19); 1.26 (br m, 2H, CH_2-20); 1.28 (br m, 2H, CH_2-21); 0.86 (t, $J = 6.5$ Hz, 3H, CH_3-22).^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 41.41$ (C-2′); 59.83 (C-3′); 170.35 (C-1); 35.21 (C-2); 35.27 (C-3); 168.86 (C-5); 42.02 (C-6); 172.09 (C-8); 32.52 (C-9); 24.98 (C-10); 38.19 (C-11); 164.75–164.39 (C-13, 16); 131.52–131.83 (C-14, 15); 38.66 (C-18); 28.42 (C-19); 28.60 (C-20); 21.77 (C-21); 13.87 (C-22). ESI-MS m/z: 442.3 $[M+H]^+$.

Compound **9 (Pa-Mal-Gaba-Gly-β-Ala-Pra)** (Mal, maleic acid). The second part of functionalized support (see compound **2**) (560 mg, 0.134 mmol) was submitted to Fmoc deprotection (procedure (c)) and subsequently to the following series of coupling–deprotection cycles (couplings according to general procedure (d)): (i) maleic anhydride (35.9 mg, 0.40 mmol), NMM (59.1 µl, 0.54 mmol), 2 h; (i)′ maleic anhydride (66.7 mg, 0.67 mmol), NMM (88.3 µl, 0.80 mmol), 2 h. Because of a false positive result of the Kaiser test (coloured resin) the coupling was repeated twice: (i) maleic anhydride (66.7 mg, 0.67 mmol), DIEA (234 µl, 1.34 mmol), 2 h. A small aliquot of resin (17.6 mg) was utilized for a mini cleavage according to general procedure (e): RP-HPLC (5–100% di B in 31 min, $\lambda = 210$ nm) and MS analysis revealed the presence of the expected product. The following steps were carried out only on a part of resin (82 mg, 0.02 mmol): (ii) capping with acetic anhydride (9.3 µl, 0.10 mmol), DIEA (17.2 µl, 0.10 mmol), 40 min; (iii) HATU (7.5 mg, 0.02 mmol), Pa (2.3 µl, 0.02 mmol), NMM (4.3 µl, 0.04 mmol), 2 h. A mini cleavage was performed on 20.0 mg of resin: RP-HPLC (5–100% B in 31 min, $\lambda = 210$ nm) and MS analysis revealed the presence of an incomplete chain (without the Pa residue). Coupling (iii) was repeated on the remaining aliquot of resin (62 mg, 0.015 mmol): (iii) HATU (11.3 mg, 0.03 mmol), Pa (3.4 µl, 0.03 mmol), NMM (4.9 µl, 0.04 mmol), 3 h. A second mini cleavage on an aliquot of resin (19.0 mg) revealed the quantitative success of last step ($m/z = 399$ $[M+H]^+$ ESI-MS). Synthesis was completed on the whole resin (478 mg, 0.11 mmol): (ii)′ capping with acetic anhydride (54.3 µl, 0.55 mmol), DIEA (100.0 µl, 0.55 mmol), 1 h; (ii)″ acetic anhydride (54.3 µl, 0.55 mmol), DIEA (100.0 µl, 0.55 mmol), 1 h; (iii)″ HATU (87.4 mg, 0.22 mmol), Pa (26.7 µl, 0.22 mmol), NMM (38 µl, 0.33 mmol), 4.20 h. A mini cleavage on an aliquot of resin (19.0 mg) revealed the quantitative success of the last coupling. Then, cleavage was performed according to the general procedure (f) for 1.30 h. Coupling in solution was performed according to general procedure (g): precipitated product (34.5 mg, 0.09 mmol) in DMF (2.9 ml), HATU (32.9 mg, 0.09 mmol), Pra (7.1 µl, 0.09 mmol) and NMM (18.8 µl, 0.17 mmol), 1 h. The solvent was evaporated and to a solution of the residue in 95: 5 TFA: H_2O (1 ml) was added cold diethyl ether (10 ml): the suspension obtained was treated according to the procedure (f). Purification by RP-HPLC: 20–35% di B in 25 min, $\lambda = 230$ nm, $R_t = 12.4$ min. Yield after HPLC purification: 40%. ^{1}H-NMR (600 MHz, DMSO-d_6)δ (ppm) = 7.81 (t, $J = 4.8$ Hz, 1H, N*H*-1′); 2.98 (q, $J = 6.6$ Hz, 2H, CH_2-2′); 1.37 (m, 2H, CH_2-3′); 0.82 (t, $J = 7.4$ Hz, 3H, CH_3-4′); 2.22 (t, $J = 7.0$ Hz, 2H, CH_2-2); 3.24 (q, $J = 6.1$ Hz, 2H, CH_2-3); 7.84 (t, $J = 4.8$ Hz, 1H, N*H*-4); 3.61 (d, $J = 5.7$ Hz, 2H, CH_2-6); 8.05 (t, $J = 5.7$ Hz, 1H, N*H*-7); 2.16 (t, $J = 7.0$ Hz, 2H, CH_2-9); 1.66 (q, $J = 7.0$ Hz, 2H, CH_2-10); 3.10 (m, 2H, CH_2-11); 9.13 (t, $J = 4.8$ Hz, 1H, N*H*-12); 6.1 (s, 1H, C*H*-14); 6.1 (s, 1H, C*H*-15); 9.13 (t, $J = 4.8$ Hz, 1H, N*H*-17); 3.09 (m, 2H, CH_2-18); 1.42 (m, 2H, CH_2-19); 1.27 (br m, 2H, CH_2-20); 1.25 (br m, 2H, CH_2-21); 0.86 (t, $J = 7.0$ Hz, 3H, CH_3). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.38$ (C-2′); 22.31(C-3′); 11.27 (C-4′); 170.10 (C-1); 35.19 (C-2); 35.50 (C-3); 169.29 (C-5); 42.09 (C-6); 172.17 (C-8); 32.50 (C-9); 25.02 (C-10); 38.58 (C-11); 164.78 (C-13); 131.59 (C-14); 131.59 (C-15); 164.78 (C-16); 38.81 (C-18); 25.10 (C-19); 28.79 (C-20); 21.82 (C-21); 13.65 (C-22). ESI-MS m/z: 440.1 $[M+H]^+$.

Compound **10 (Pa-Mal-Ao-Gly-Ae)**. The glycinol-2-chlorotrityl support (100 mg, 0.11 mmol) was swollen for 1 h with DMF (3 ml) and then submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-Gly-OH (98.1 mg, 0.33 mmol), HOBt (50.5 mg, 0.33 mmol), HBTU (125.2 mg, 0.33 mmol), NMM (48.4 µl, 0.44 mmol), 1.0 h; (ii) Fmoc-Ao-OH (127.1 mg, 0.33 mmol), HOBt (50.5 mg, 0.33 mmol), HBTU (125.2 mg, 0.33 mmol), NMM (48.4 µl, 0.44 mmol), 1 h; (iii) maleic anhydride (31.7 mg, 0.33 mmol), NMM (48.4 µl, 0.44 mmol), 1 h. A double colorimetric test was performed, giving a negative result for the Kaiser test and positive result for the Green Malachite test. (iv) Capping with acetic anhydride (51.9 µl, 0.55 mmol), DIEA (96.0 µl, 0.55 mmol); 1.50 h; (v) HATU (41.8 mg, 0.11 mmol), Pa (12.7 µl, 0.11 mmol), NMM (24.2 µl, 0.22 mmol), 1.30 h; Green Malachite test negative. Then cleavage was performed according to procedures (f) and (f_1). Purification by RP-HPLC: 15–70% B in 55 min, $\lambda = 280$ nm; $R_t = 26.8$ min. Yield after HPLC purification: 24%. An amount of deleted product

(8.9 mg, 0.025 mmol) was isolated during HPLC purification and submitted to the coupling with the lacking Pa in solution: Pa (2.9 µl, 0.025 mmol); HATU (9.5 mg, 0.025 mmol); NMM (5.5 µl, 0.025 mmol), 1 h. After solvent evaporation and RP-HPLC purification, on additional 8% of product was recovered. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.76 (t, J = 4.8 Hz, 1H, N*H*-1′); 3.11 (m, 2H, CH_2-2′); 3.38 (t, J = 6.1 Hz, 2H, CH_2-3′); 3.64 (d, J = 5.3 Hz, 2H, CH_2-2); 7.96 (t, J = 4.8 Hz, 1H, N*H*-3); 2.11 (t, J = 7.4 Hz, 2H, CH_2-5); 1.48 (br m, 2H, CH_2-6); 1.25 (br m, 6H, CH_2-7, 8, 9); 1.43 (br m, 2H, CH_2-10); 3.10 (m, 2H, CH_2-11); 9.21 (br, 1H, N*H*-12); 6.09 (s, 1H, C*H*-14); 6.09 (s, 1H, C*H*-15); 9.21 (br, 1H, N*H*-17); 3.10 (m, 2H, CH_2-18); 1.43 (br m, 2H, CH_2-19); 1.26 (br m, 2H, CH_2-20); 1.28 (br m, 2H, CH_2-21); 0.86 (t, J = 6.5 Hz, 3H, CH_3-22). ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 41.14 (C-2′); 59.40 (C-3′); 168.80 (C-1); 41.67 (C-2); 171.90 (C-4); 34.83 (C-5); 24.87 (C-6); 26.01 (C-7); 28.60 (C-8); 26.41 (C-9); 28.35 (C-10); 38.38 (C-11); 164.48 (C-13); 131.75 (C-14); 131.75 (C-15); 164.48 (C-16); 38.38 (C-18); 28.35 (C-19); 28.31 (C-20); 21.48 (C-21); 13.63 (C-22). ESI-MS m/z: 427.3 $[M + H]^+$.

Compound **11 (Pra-Mal-Gaba-Gly-β-Ala-Ae)**. The 2-chlorotrityl chloride support was functionalized according to general procedure (a_1) (substitution level: 45%, 0.47 mmol/g, 0.094 mmol for 0.20 g) and the Fmoc-β-aminoethanol was deprotected as in procedure (c). The resin was subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-β-Ala-OH (87.8 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); 1.30 h; (ii) Fmoc-Gly-OH (83.8 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); t = 1.30 h; (iii) Fmoc-Gaba-OH (91.7 mg, 0.28 mmol), HOBt (43.2 mg, 0.28 mmol), HBTU (106.9 mg, 0.28 mmol), NMM (41.3 µl, 0.37 mmol); 1.30 h; (iv) maleic anhydride (45.1 mg, 0.47 mmol), NMM (62.0 µl, 0.56 mmol) for 1.30 h; Green Malachite test was positive; (v) capping with acetic anhydride (44.4 µl, 0.47 mmol), DIEA (82.0 µl, 0.47 mmol); 45 min; (vi) HATU (35.7 mg, 0.09 mmol), Pra (7.40 µl, 0.09 mmol), NMM (20.7 µl, 0.19 mmol), 1.30 h; Green Malachite test was negative. A mini cleavage was carried out on a small aliquot of resin (5.9 mg): ESI-MS analysis on the crude revealed the presence of the expected product (m/z = 427 $[M + H]^+$). Then, cleavage was performed according to procedures (f) and (f_1). Purification by RP-HPLC: 10–50% B in 40 min, λ = 280 nm; R_t = 7.5 min. Yield after HPLC purification: 29%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.86 (t, J = 5.8 Hz, 1H, N*H*-1′); 3.08 (q, J = 6.9 Hz, 2H, CH_2-2′); 3.37 (q, J = 5.8 Hz, 2H, CH_2-3′); 4.63 (t, J = 5.4 Hz, 1H, O*H*-4′); 2.23 (q, J = 7.1 Hz, 2H, CH_2-2); 3.24 (q, J = 6.9 Hz, 2H, CH_2-3); 7.86 (t, J = 5.4 Hz, 1H, N*H*-4); 3.62 (d, J = 5.8 Hz, 2H, CH_2-6); 8.04 (t, J = 5.8 Hz, 1H, N*H*-7); 2.16 (q, J = 7.3 Hz, 2H, CH_2-9); 1.67 (qq, J = 7.1 Hz, 2H, CH_2-10); 3.11 (q, J = 6.5 Hz, 2H, CH_2-11); 9.13 (t, J = 5.4 Hz, 1H, N*H*-12); 6.10 (s, 1H, C*H*-14); 6.10 (s, 1H, C*H*-15); 9.13 (t, J = 5.4 Hz, 1H, N*H*-17); 3.11 (q, J = 6.5 Hz, 2H, CH_2-18); 1.44 (ss, J = 7.3 Hz, 2H, CH_2-19); 0.86 (t, J = 7.5 Hz, 3H, CH_3-20). ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 40.35 (C-2′); 59.55 (C-3′); 170.18 (C-1); 34.82 (C-2); 34.87 (C-3); 168.65 (C-5); 41.65 (C-6); 171.98 (C-8); 32.20 (C-9); 24.55 (C-10); 40.35 (C-11); 164.41 (C-13); 131.31 (C-14); 131.31 (C-15); 164.41 (C-16); 40.35 (C-18); 21.59 (C-19); 10.99 (C-20). ESI-MS m/z: 414.3 $[M + H]^+$.

Compound **12 (Bu-Dap-Mal-Ap-Gly-Ae)**. The 2-chlorotrityl chloride support (400 mg, 0.42 mmol) was functionalized according to general procedure (a_1), with the following variations: Fmoc-β-aminoethanol (235.7 mg, 0.83 mmol) in DCM/DMF (4.40 ml/0.34 ml) and DIEA (217.9 µl, 1.25 mmol) were allowed to react with the resin for 95 h. The support was then washed with: DMF (5 × 3 ml), DCM (5 × 3 ml), Et_2O (3 × 3 ml); (substitution level about 40%). The resin was submitted to the capping procedure with MeOH (3 ml), DIEA (25.5 µl) and to the following series of coupling–deprotection cycles (couplings according to general procedure (d)) (we considered a reference substitution value of 50%, 0.50 mmol/g, 0.20 mmol for 0.40 g): (i) Fmoc-Gly-OH (178.4 mg, 0.60 mmol), HOBt (91.9 mg, 0.60 mmol), HBTU (227.6 mg, 0.60 mmol), NMM (87.9 µl, 0.4 mmol); 1 h; (ii) Fmoc-Ap-OH (203.6 mg, 0.6 mmol), HOBt (91.9 mg, 0.60 mmol), HBTU (227.6 mg, 0.60 mmol), NMM (87.9 µl, 0.40 mmol); 1 h; (iii) maleic anhydride (57.6 mg, 0.60 mmol), NMM (87.9 µl, 0.40 mmol); 1 h; Green Malachite test was positive; (iv) capping with acetic anhydride (94.5 µl, 1.00 mmol), DIEA (174.6 µl, 1.00 mmol); 1.50 h. The resin was then dried and divided in two parts; one of them, 227.0 mg (0.11 mmol), was submitted to the following series of coupling–deprotection cycles to complete the synthesis of **16**: (iv) Fmoc-Dap-NH_2 · NH_2 (112.8 mg, 0.34 mmol), HATU (42.9 mg, 0.11 mmol), NMM (62.1 µl, 0.56 mmol) for 1 h; Green Malachite test was negative; (v) Butanoic anhydride (184.9 µl, 1.13 mmol), DIEA (217 µl, 1.24 mmol) for 1.30 h. A mini cleavage was carried out on a small aliquot of resin (5.2 mg): RP-HPLC and ESI-MS analysis revealed the presence of the expected product (10–50% B in 40 min, λ = 230 nm, R_t = 20.3 min; m/z = 442 $[M + H]^+$). Then, cleavage was performed according to procedure (f_1). Purification by RP-HPLC: 15–55% B in 80 min; λ = 280 nm; R_t = 22.5 min. Yield after HPLC purification: 45%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.78 (t, J = 5.3 Hz, 1H, N*H*-1′); 3.09 (m, 2H, CH_2-2′); 3.38 (t, J = 5.9 Hz, 2H, CH_2-3′); 4.48 (br s, 1H, O*H*-4′); 3.65 (d, J = 5.7 Hz, 2H, CH_2-2); 8.00 (t, J = 5.0 Hz, 1H, N*H*-3); 2.14 (t, J = 6.8 Hz, 2H, CH_2-5); 1.49 (m, 2H, CH_2-6); 1.44 (m, 2H, CH_2-7); 3.10 (m, 2H, CH_2-8); 9.15 (br m, 1H, N*H*-9); 6.10 (s, 1H, C*H*-11); 6.10 (s, 1H, C*H*-12); 9.13 (br m, 1H, N*H*-14); 3.11 (m, 2H, CH_2-15); 1.56 (m, 2H, CH_2-16); 3.06 (m, 2H, CH_2-17); 7.78 (t, J = 5.3 Hz, 1H, N*H*-18); 2.02 (t, J = 7.2 Hz, 2H, CH_2-20); 1.50 (m, 2H, CH_2-21); 0.84 (t, J = 7.4 Hz, 3H, CH_3-22). ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 41.12 (C-2′); 59.56 (C-3′); 168.88 (C-1); 41.71 (C-2); 172.31 (C-4); too weak (C-5); 22.37 (C-6); 28.08 (C-7); 38.23 (C-8); 164.57 (C-10); 131.50 (C-11); 131.50 (C-12); 164.57 (C-13); 36.43 (C-15); 28.73 (C-16); 36.08 (C-17); 171.89 (C-19); 37.12 (C-20); 18.51 (C-21); 13.38 (C-22). ESI-MS m/z: 442.4 $[M + H]^+$.

Compound **13 (Oa-Mal-Ap-Gly-Ae)**. The second part (227 mg, 0.11 mmol) of the functionalized support (see compound **16**) was submitted to the following couplings: (i) Oa (18.7 µl, 0.11 mmol), HATU (42.9 mg, 0.11 mmol), NMM (24.8 µl, 0.22 mmol) for 1 h; Green Malachite test negative. A mini cleavage was performed on a small amount of resin (5.2 mg). RP-HPLC and ESI-MS analyses revealed the presence of the expected product (m/z = 427 $[M + H]^+$). Then, cleavage was performed according to procedure (f_1). Purification by RP-HPLC: 35–65% B in 60 min; λ = 280 nm; R_t = 12.5 min. Yield after HPLC purification: 50%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.76 (t, J = 5.3 Hz, 1H, N*H*-1′); 3.09 (m, 2H, CH_2-2′); 3.38 (t, J = 5.3 Hz, 2H, CH_2-3′); 4.64 (t, J = 4.6 Hz, 1H, O*H*-4′); 3.65 (d, J = 5.5 Hz, 2H, CH_2-2); 7.97 (t, J = 5.3 Hz, 1H, N*H*-3); 2.14 (t, J = 6.8 Hz, 2H, CH_2-5); 1.49 (m, 2H, CH_2-6);

1.42 (m, 2H, CH_2-7); 3.13 (m, 2H, CH_2-8); 9.19 (br s, 1H, NH-9); 6.09 (H-11); 6.09 (H-12); 9.20 (br s, 1H, NH-14); 3.13 (m, 2H, CH_2-15); 1.42 (m, 2H, CH_2-16); 1.25 (br m, 6H, CH_2-17, 18, 19); 1.24 (br m, 2H, CH_2-20); 1.25 (br m, 2H, CH_2-21); 0.85 (t, $J = 6.4$ Hz, 3H, CH_3-22). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 38.22$ (C-2′); 59.42 (C-3′); 168.65 (C-1); 41.60 (C-2); 171.92 (C-4); 34.42 (C-5); 22.25 (C-6); 28.20 (C-7); 41.08 (C-18); 164.01 (C-10); 131.48 (C-11); 131.48 (C-12); 164.01 (C-13); 41.08 (C-15); 28.20 (C-16); 28.30, 26.05 (C-17, 18, 19); 30.85 (C-20); 21.72 (C-21); 13.60 (C-22). ESI-MS m/z: 427.2 $[M + H]^+$.

Compound **14 (Pa-Mal-Dap-Mal-Aib-Pra)** (Dap, 1,3-diaminopropane; Aib, 2-amino-2-methylpropanoic acid). The second part of the functionalized support (see compound **7**) (500 mg, 0.12 mmol) was Fmoc deprotected as in procedure (c) and subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) maleic anhydride (48.0 mg, 0.50 mmol), NMM (68.7 µl, 0.62 mmol), 1.30 h; (ii) capping with acetic anhydride (59.1 µl, 0.62 mmol), DIEA (109.1 µl, 0.62 mmol), 40 min; (iii) Fmoc-Dap-$NH_2 \cdot$ HCl (124.8 mg, 0.37 mmol), HATU (47.5 mg, 0.12 mmol), NMM (68.7 µl, 0.62 mmol), 1.30 h; (iv) twice: maleic anhydride (48.0 mg, 0.50 mmol), NMM (68.7 µl, 0.62 mmol), 1.30 h; Kaiser test was positive; a mini cleavage was carried out on a small aliquot of resin (10.0 mg), as in procedure (e): RP-HPLC and MS analysis revealed the presence of the expected product; coupling (iv) was repeated with the same quantities of reagents and the mixture DMF: DCM: NMP = 1 : 1 : 1 + 1% di Triton X as solvent for 1.30 h; (v) capping with acetic anhydride (59.1 µl, 0.62 mmol), DIEA (109.1 µl, 0.62 mmol), 40 min; (vi) HATU (47.5 mg, 0.12 mmol), Pa (29.0 µl, 0.25 mmol), NMM (41.2 µl, 0.37 mmol), 1.30 h; (vii) HATU (47.5 mg, 0.12 mmol), Pa (14.5 µl, 0.12 mmol), NMM (27.5 µl, 0.25 mmol), 2.0 h. Then, cleavage was performed according to procedure (f_1) and the crude product (55.0 mg, 0.13 mmol) was submitted to the coupling in DMF (0.9 ml) solution as described in procedure (g): HATU (49.4 mg, 0.13 mmol), Pra (10.7 µl, 0.13 mmol), NMM (28.6 µl, 0.26 mmol) for 2 h. Purification by RP-HPLC: 20–50% B in 60 min, $\lambda = 290$ nm; $R_t = 18.3$ min. Yield after HPLC purification: 14%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.52 (t, $^3J = 5.8$ Hz, 1H, NH-1′); 2.96 (q, $J = 6.4$ Hz, 2H, CH_2-2′); 1.35 (m, 2H, CH_2-3′); 0.78 (t, $J = 7.5$ Hz, 3H, CH_3-4′); 1.30 (s, 3H, CH_3-3); 1.30 (s, 3H, CH_3-4); 8.33 (s, 1H, NH-5); 6.74 (d, $J = 15.2$ Hz, 1H, CH-7); 6.89 (d, $J = 15.2$ Hz, 1H, CH-8); 8.37 (t, $J = 5.5$ Hz, 1H, NH-10); 3.16 (m, 2H, CH_2-11); 1.61 (q, $J = 6.9$ Hz, 2H, CH_2-12); 3.16 (m, 2H, CH_2-13); 9.15 (t, $J = 5.5$ Hz, 1H, NH-14); 6.10 (s, 1H, CH-16); 6.10 (s, 1H, CH-17); 9.09 (t, $J = 5.5$ Hz, 1H, NH-19); 3.10 (m, 2H, CH_2-20); 1.43 (q, $J = 6.9$ Hz, 2H, CH_2-21); 1.27 (m, 2H, CH_2-22); 1.27 (m, 2H, CH_2-23); 0.86 (t, $J = 6.6$ Hz, 3H, CH_3-24). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.06$ (C-2′); 21.85 (C-3′); 10.86 (C-4′); 173.23 (C-1); 55.73 (C-2); 24.90 (C-3); 24.90 (C-4); 162.92 (C-6); 133.04 (C-7); 131.19 (C-8); 163.78 (C-9); 36.42 (C-11); 28.34 (C-12); 36.42 (C-13); 163.16 (C-15); 131.19 (C-16); 131.19 (C-17); 163.98 (C-18); 38.32 (C-20); 28.09 (C-21); 28.26 (C-22); 21.35 (C-23); 13.56 (C-24). ESI-MS m/z: 466.1 $[M + H]^+$.

Compound **15 (Pa-Mal-Ao-Aib-Pra)** (Ao, 8-aminooctanoic acid). Fmoc-Aib-OH (1.56 g, 4.80 mmol) was anchored to the HMPA support (1.00 g, 0.24 mmol) according to general procedure (a) (substitution level: 80%) and then deprotected as described in procedure (c). The resin was divided in two parts and one of them (500 mg, 0.12 mmol) was submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-Ao-OH (137.3 mg, 0.36 mmol), HOBt (55.1 mg, 0.36 mmol), HBTU (136.5 mg, 0.36 mmol), NMM (52.8 µl, 0.48 mmol), 1.30 h; (ii) maleic anhydride (46.1 mg, 0.48 mmol), NMM (66.0 µl, 0.60 mmol), 1.30 h; (iii) capping with acetic anhydride (56.7 µl, 0.60 mmol), DIEA (104.7 µl, 0.60 mmol), 40 min; (iv) HATU (45.6 mg, 0.12 mmol), Pa (27.8 µl, 0.24 mmol), NMM (39.6 µl, 0.36 mmol), 1.30 h. Cleavage was performed twice according to procedure (f_1). The crude product (61.5 mg, 0.15 mmol) was submitted to the solution coupling (g_1) procedure: DMF (0.9 ml), HATU (85.4 mg, 0.22 mmol), Pra (49.0 µl, 0.60 mmol), NMM (98.2 µl, 0. 90 mmol), 5 h. Purification by RP-HPLC: 25–65% B in 40 min, $\lambda = 280$ nm, $R_t = 28.7$ min. Yield after HPLC purification: 25%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.38 (t, $J = 5.5$ Hz, 1H, NH-1′); 2.96 (q, $J = 6.4$ Hz, 2H, CH_2-2′); 1.35 (ss, $J = 7.2$ Hz, 2H, CH_2-3′); 0.79 (t, $J = 7.4$ Hz, 3H, CH_3-4′); 1.30 (br s, 3H, CH_3-3); 1.30 (br s, 3H, CH_3-4); 7.67 (s, 1H, NH-5); 2.06 (t, $J = 7.2$ Hz, 2H, CH_2-7); 1.45 (m, 2H, CH_2-8); 1.21–1.28 (br m, H-9, 10, 11, 22, 23); 1.42 (m, 2H, CH_2-12); 3.10 (q, $J = 6.4$ Hz, 2H, CH_2-13); 9.23 (t, $J = 4.8$ Hz, 1H, NH-14); 6.09 (s, 1H, CH-16); 6.09 (s, 1H, CH-17); 9.23 (t, $J = 4.8$ Hz, 1H, NH-19); 3.10 (q, $J = 6.4$ Hz, 2H, CH_2-20); 1.42 (m, 2H, CH_2-21); 0.86 (t, $J = 7.0$ Hz, 3H, CH_3-24). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.47$ (C-2′); 22.31 (C-3′); 11.18 (C-4′); 173.79 (C-1); 55.68 (C-2); 25.20 (C-3, 4); 171.46 (C-6); 35.03 (C-7); 25.01 (C-8); 28.73 (C-9); 22.01, 22.48, 26.39, 28.33, 28.6, 31.20 (C-10, 11, 12, 21, 22, 23); 38.52 (C-13); 164.48 (C-15); 131.81 (C-16); 131.81 (C-17); 164.48 (C-18); 38.52 (C-20); 13.90 (C-24). ESI-MS m/z: 453.4 $[M + H]^+$.

Compound **16 (Bu-Dap-Mal-Ap-Aib-Pra)**. Fmoc-Aib-OH (781 mg, 2.40 mmol) was anchored to the HMPA support (1.00 g, 0.24 mmol) according to general procedure (a) (substitution level: 39%); the reaction was repeated with a larger excess of reagents (Fmoc-Aib-OH: 1.09 g, 3.36 mmol; substitution level: 79%). The support was Fmoc deprotected according to procedure (c), and then submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) Fmoc-Ap-OH (244.4 mg, 0.72 mmol), HOBt (110.2 mg, 0.72 mmol), HBTU (273.1 mg, 0.72 mmol), NMM (105.5 µl, 0.96 mmol); (ii) maleic anhydride (69.2 mg, 0.72 mmol), NMM (105.5 µl, 0.96 mmol), 1.30 h. A mini cleavage was carried out on a small aliquot of resin (10.0 mg). RP-HPLC and MS analysis revealed the presence of the expected product ($R_t = 11.0$ min, $m/z = 301$ $[M + H]^+$, ESI-MS). The resin was then dried and divided in two parts; one of them (500 mg, 0.12 mmol) was submitted to the following series of coupling–deprotection cycles to complete the synthesis of **9**: (iii) capping with acetic anhydride (56.7 µl, 0.60 mmol), DIEA (104.7 µl, 0.60 mmol); 1.30 h; (iv) Fmoc-Dap-$NH_2 \cdot$ HCl (160.0 mg, 0.48 mmol), CIP (2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate) (133.8 mg, 0.48 mmol), HOBt (32.7 mg, 0.24 mmol), DIEA (293.3 µl, 1.68 mmol), 1.40 h; (v) Butanoic anhydride (196.3 µl, 1.20 mmol), DIEA (314.3 µl, 1.8 mmol), 1.40 h. Then cleavage was performed according to procedure (f_1) ($t = 1.30$ h $\times 2$): RP-HPLC and ESI-MS analysis revealed the presence of the expected product ($R_t = 11.3$ min, $m/z = 427$ $[M + H]^+$). The crude (62.0 mg, 0.14 mmol) mixture was submitted to the solution coupling: DMF (0.85 ml), HATU (55.3 mg, 0.14 mmol), Pra (11.9 µl, 0.14 mmol), NMM (31.9 µl, 0.29 mmol) for 4 h. Purification by RP-HPLC: 10–30% B in 66 min, $\lambda = 260$ nm;

$R_t = 23.2$ min. Yield after HPLC purification: 24%. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.39 (t, $J = 5.7$ Hz, 1H, N*H*-1′); 2.96 (q, $J = 6.5$ Hz, 2H, CH_2-2′); 1.35 (ss, $J = 7.4$ Hz, 2H, CH_2-3′); 0.78 (t, $J = 7.4$ Hz, 3H, CH_3-4′); 1.30 (s, 3H, CH_3-3); 1.30 (s, 3H, CH_3-4); 7.71 (s, 1H, N*H*-5); 2.10 (t, $J = 7.4$ Hz, 2H, CH_2-7); 1.49 (m, 2H, CH_2-8); 1.41 (qq, $J = 7.4$ Hz, 2H, CH_2-9); 3.10 (m, 2H, CH_2-10); 9.14 (br, 1H, N*H*-11); 6.10 (s, 1H, C*H*-13); 6.10 (s, 1H, C*H*, 14); 9.13 (br, 1H, N*H*-16); 3.12 (m, 2H, CH_2-17); 1.56 (qq, $J = 7.0$ Hz, 2H, CH_2-18); 3.06 (q, $J = 6.5$ Hz, 2H, CH_2-19); 7.76 (t, $J = 4.8$ Hz, 1H, N*H*-20); 2.02 (t, $J = 7.4$ Hz, 2H, CH_2-22); 1.50 (m, 2H, CH_2-23); 0.84 (t, $J = 7.4$ Hz, 3H, CH_3-24). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.50$ (C-2′); 22.29 (C-3′); 11.18 (C-4′); 173.77 (C-1); 55.69 (C-2); 25.21 (C-3, 4); 171.54 (C-6); 35.03 (C-7); 22.47 (C-8); 28.29 (C-9); 38.50 (C-10); 164.45 (C-12); 131.81 (C-13); 131.81(C-14); 164.45 (C-15); 36.58 (C-17); 28.89 (C-18); 36.19 (C-19); 171.88 (C-21); 37.27 (C-22); 18.68 (C-23); 13.59 (C-24). ESI-MS m/z: 468.2 $[M + H]^+$.

Compound 17 (Oa-Mal-Ap-Aib-Pra) (Oa, *N*-octylamine). The second part of functionalized support (see compound **9**) (500 mg, 0.12 mmol) was Fmoc-deprotected and subsequently submitted to the following series of coupling–deprotection cycles (according to general procedure (d)): (i) *N*-octylamine (79.3 µl, 0.48 mmol), CIP (133.8 mg, 0.48 mmol), HOBt (32.7 mg, 0.24 mmol), DIEA (251.4 µl, 1.44 mmol), 1.45 h. A mini cleavage was carried out on a small aliquot of resin (12.5 mg): RP-HPLC and MS analysis revealed the presence of the expected product ($R_t = 20.3$ min, $m/z = 412$ $[M + H]^+$, ESI-MS). Then, cleavage was performed according to procedure (f_1) ($t = 1.0$ h $\times$ 2 times) and the crude (55.3 mg, 0.13 mmol) mixture was suspended in DMF/DCM (1.75 ml/0.20 ml) for the last coupling, as in the (g_1) procedure: HATU (51.1 mg, 0.13 mmol), Pra (33.0 µl, 0.40 mmol), NMM (103.1 µl, 0.94 mmol) for 4 h. Purification by RP-HPLC: 35–75% B in 40 min, $\lambda = 280$ nm; $R_t = 25.1$ min. Yield after HPLC purification: 25% as a colorless oil. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.39 (t, $J = 5.7$ Hz, 1H, N*H*-1′); 2.96 (q, $J = 6.1$ Hz, 2H, CH_2-2′); 1.35 (ss, $J = 7.0$ Hz, 2H, CH_2-3′); 0.78 (t, $J = 7.4$ Hz, 3H, CH_3-4′); 1.30 (t, $J = 7.4$ Hz, 3H, CH_3-3); 1.30 (t, $J = 7.4$ Hz, 3H, CH_3-4); 7.71 (s, 1H, N*H*-5); 2.10 (H-7); 1.49 (qq, $J = 7.4$ Hz, 2H, CH_2-8); 1.42 (m, 2H, CH_2-9); 3.10 (br m, 2H, CH_2-10); 9.24 (H-11); 6.09 (H-13); 6.09 (H-14); 9.24 (H-16); 3.10 (br m, 2H, CH_2-17); 1.42 (m, 2H, CH_2-18); 1.22–1.28 (br m., H-19, 20, 21, 22, 23); 0.85 (t, $J = 7.0$ Hz, 3H, CH_3-24). ^{13}C-NMR (150 MHz, DMSO-d_6): $\delta = 40.51$ (C-2′); 22.40 (C-3′); 11.27 (C-4′); 174.01 (C-1); 55.82 (C-2); 25.43 (C-3, 4); 171.68 (C-6); 34.78 (C-7); 22.56 (C-8); 28.62 (C-9); 38.45 (C-10); 164.42 (C-12); 131.89 (C-13); 131.89 (C-14); 164.42 (C-15); 38.20 (C-17); 28.56 (C-18); 22.31–26.72-31.41-28.78 (C-19-20-21-22-23); 14.22 (C-24). ESI-MS m/z: 453.3 $[M + H]^+$.

CB_1 Binding Assays

Displacement assays for CB_1 receptors were carried out by using [^{3}H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham) as the high-affinity ligand and the filtration technique described previously [34] on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Wilmington, MA) and in the presence of 100 µM *p*-hydroxy-mercuribenzoate as an inhibitor of ligand enzymatic hydrolysis. Specific binding, calculated with 1 µM SR141716A, was 81.0%. Data are expressed as the K_i, calculated using the Cheng–Prusoff equation from the concentration exerting 50% inhibition of [^{3}H]SR141716A specific binding (IC_{50}).

Acknowledgements

We gratefully acknowledge the financial support from The University of Salerno and National Research Council (CNR, Rome) through the Agenzia 2000 Program. The useful suggestions about solid-phase synthesis by Prof. P. Rovero, University of Firenze, Italy, are gratefully acknowledged.

REFERENCES

1. Gaoni Y, Mechoulam R, Hashish III. Isolation, structure, and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 1964; **86**: 1646–1647.
2. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992; **258**: 1946–1949.
3. Martin BR. Cellular effects of cannabinoids. *Pharmacol. Rev.* 1986; **38**: 45–74.
4. Dewey WL. Cannabinoid pharmacology. *Pharmacol. Rev.* 1986; **38**: 151–178.
5. Razdan RK. The total synthesis of cannabinoids. *Total Syntheses in Natural Products*, Apsimon J (ed), Wiley VCH, New York, 1981; **4** 185–262.
6. Razdan RK. Structure-activity relationships in cannabinoids. *Pharmacol. Rev.* 1986; **38**: 75–149.
7. Khanolkar AD, Makriyannis A. Structure-activity relationships of anandamide, an endogenous cannabinoid ligand. *Life Sci.* 1999; **65**: 607–616.
8. Bisogno T, Maurelli S, Melck D, De Petrocellis L, Di Marzo V. Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J. Biol. Chem.* 1997; **272**: 3315–3323.
9. Di Marzo V, De Petrocellis L, Bisogno T, Maurelli S. The endogenous cannabimimetic eicosanoid anandamide induces arachidonate release in J774 mouse macrophages. *Adv. Exp. Med. Biol.* 1997; **407**: 341–346.
10. Cabral GA, Toney DM, Fisher-Stenger K, Harrison MP, Marciano-Cabral F. Anandamide inhibits macrophage-mediated killing of tumor necrosis factor-sensitive cells. *Life Sci.* 1995; **56**: 2065–2072.
11. Schwarz H, Blanco FJ, Lotz M. Anandamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis. *J. Neurochem.* 1994; **55**: 107–115.
12. Ishac EJ, Jiang L, Lake KD, Varga K, Abood ME, Kunos G. Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br. J. Pharmacol.* 1996; **118**: 2023–2028.
13. Pugh G Jr, Smith PB, Dombrowski DS, Welch SP. The role of endogenous opioids in enhancing the antinociception produced by the combination of delta 9-tetrahydrocannabinol and morphine in the spinal cord. *J. Pharmacol. Exp. Ther.* 1996; **279**: 608–616.
14. Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R, Ward SJ. Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacol., Biochem. Behav.* 1991; **40**: 471–478.
15. Compton DR, Johnson MR, Melvin LS, Martin BR. Pharmacological profile of a series of bicyclic cannabinoid analogs: classification

as cannabimimetic agents. *J. Pharmacol. Exp. Ther.* 1992; **260**: 201–209.
16. Adams IB, Ryan W, Singer M, Razdan RK, Compton DR, Martin BR. Pharmacological and behavioral evaluation of alkylated anandamide analogs. *Life Sci.* 1995; **56**: 2041–2048.
17. Khanolkar AD, Abadji V, Lin S, Hill WA, Taha G, Abouzid K, Meng Z, Fan P, Makriyannis A. Head group analogs of arachidonylethanolamide, the endogenous cannabinoid ligand. *J. Med. Chem.* 1996; **39**: 4515–4519.
18. Sheskin T, Hanus L, Slager J, Vogel Z, Mechoulam R. Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. *J. Med. Chem.* 1997; **40**: 659–667.
19. Lin S, Khanolkar AD, Fan P, Goutopoulos A, Qin C, Papahadjis D, Makriyannis A. Novel analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 cannabinoid receptors and metabolic stability. *J. Med. Chem.* 1998; **41**: 5353–5361.
20. Khanolkar AD, Makriyannis A. Structure-activity relationships of anandamide, an endogenous cannabinoid ligand. *Life Sci.* 1999; **65**: 607–616.
21. Thomas BF, Adams IB, Mascarella SW, Martin BR, Razdan RK. Structure-activity analysis of anandamide analogs: relationship to a cannabinoid pharmacophore. *J. Med. Chem.* 1996; **39**: 471–479.
22. Tong W, Collantes ER, Welsh WJ, Berglund BA, Howlett AC. Derivation of a pharmacophore model for anandamide using constrained conformational searching and comparative molecular field analysis. *J. Med. Chem.* 1998; **41**: 4207–4215.
23. Barnett-Norris J, Guarnieri F, Hurst DP, Reggio PH. Exploration of biologically relevant conformations of anandamide, 2-arachidonylglycerol, and their analogues using conformational memories. *J. Med. Chem.* 1998; **41**: 4861–7482.
24. Kent SBH, Alewood D, Alewood P, Baca M, Jones A, Schnolzer M. Total chemical synthesis of proteins: evolution of solid-phase synthetic methods illustrated by total chemical synthesis of the HIV-1 protease. In *Innovations and Perspectives in Solid Phase Synthesis*, Epton R (ed.). Intercept: Andover, 1992; 1–22.
25. Zhang L, Goldammer C, Henkel B, Zühl F, Panhaus G, Jung G, Bayer E. "Magic Mixture, a powerful solvent system for solid-phase synthesis of difficult sequences". In *Innovations and Perspectives in Solid Phase Synthesis*, Epton R (ed.). Mayflower Worldwide: Birmimgham, 1994; 711–716.
26. Dauber-Osguthorpe P, Roberts VA, Osguthorpe DJ, Wolff J, Genest M, Hagler AT. Structure and energetics of ligand binding to proteins: *Escherichia coli* dihydrofolate reductase-trimethoprim, a drug-receptor system. *Proteins: Struct., Funct., Genet.* 1998; **4**: 31–47.
27. Accelerys: San Diego, CA, 92121–3752.
28. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Zakrzewski VG, Montgomery JA, Stratmann RE, Burant JC, Dapprich S, Millam JM, Daniels AD, Kudin KN, Strain MC, Farkas O, Tomasi J, Barone V, Cossi M, Cammi R, Mennucci B, Pomelli C, Adamo C, Clifford S, Ochterski J, Petersson GA, Ayala PY, Cui Q, Morokuma K, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Cioslowski J, Ortiz JV, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Gomperts R, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Gonzalez C, Challacombe M, Gill PMV, Johnson BG, Chen W, Won MW, Andres JL, Head-Gordon M, Replogle ES, Pople JA. *Gaussian 98, Revision A.6*. Gaussian: Pittsburgh, PA, 1998.
29. Ditchfield R. Self-consistent perturbation theory of diamagnetism. I. a gauge-invariant LCAO(linear combination of atomic orbitals) method for NMR chemical shifts. *Mol. Phys.* 1974; **27**: 789–807.
30. Rohlfing CM, Allen LC, Ditchfield R. Proton and carbon-13 chemical shifts: comparison between theory and experiment. *Chem. Phys.* 1984; **87**: 9–15.
31. Wolinski K, Hinton JF, Pulay P. Efficient implementation of the gauge-independent atomic orbital method for NMR chemical shift calculations. *J. Am. Chem. Soc.* 1990; **112**: 8251–8260.
32. Barone G, Duca D, Silvestri A, Gomez-Paloma L, Riccio R, Bifulco G. Determination of the relative stereochemistry of flexible organic compounds by *ab initio* methods: conformational analysis and Boltzmann averaged GIAO ^{13}C NMR chemical shifts. *Chem. Eur. J.* 2002; **8**(14): 3240–3245.
33. Cimino P, Duca D, Gomez-Paloma L, Riccio R, Bifulco G. Comparison of different theory models and basis sets in the calculation of ^{13}C NMR chemical shifts of natural products. *Magn. Reson. Chem.* 2004; **42**: S26–S33.
34. Melck D, Bisogno T, De Petrocellis L, Chuang H, Julius D, Bifulco M, Di Marzo V. Unsaturated long-chain N-acyl-vanillyl-amides (N-AVAMs): vanilloid receptor ligands that inhibit anandamide-facilitated transport and bind to CB1 cannabinoid receptors. *Biochem. Biophys. Res. Commun.* 1999; **262**: 275–284.

