A Novel Family of Minimal PMRI Cyclic Peptides as Versatile Scaffolds for Generating New Molecular Topology

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Abstract. The surface loops of proteins and active peptides are implicated in the activation of biological responses upon recognition by enzymes and receptors. Obviously, it is of interest to investigate these loops as potential leads for drug discovery. Currently, there is an urgent need for novel, general, and conformationally definite cyclic peptidomimetic scaffolds capable to mimic small portions of the protein surface. In this respect, 13-membered ring peptidomimetics can be considered privileged structures, since they represent the smallest possible systems that can retain all of the features of organized protein structures, such as single H-bonded alfa-helix loops and different kind of turns.

In the present work, we report a novel family of 13-membered ring cyclic peptidomimetics based on a minimal PMRI (partially modified retro-inverse) peptide strategy; in particular, we describe the synthesis and the conformational analysis of a representative member of the family. These scaffolds have been designed to permit easy introduction in a combinatorial fashion of a range of pharmacophores that possess a diversity of structure, function, and 3D disposition.

Key Words: PMRI peptide, cyclic peptide, peptidomimetic, scaffold, molecular diversity.

INTRODUCTION

Physiological processes are regulated to a large extent by interactions between peptides with receptors or other proteins. Unfortunately, native peptides and small proteins can not be used as pharmaceutical agents as they stand, due to their scarce ability to penetrate biological barriers [1,2], and the rapid degradation *in vivo* [3]. To circumvent these limitations, diverse strategies have been adopted to modify peptide properties [4]. Since many peptides and proteins exert their biological activity through relatively small regions of their folded surfaces, their actions can be reproduced by much smaller molecules that retain these localised bioactive surfaces but have potentially improved pharmacokinetic or dynamic properties. In several cases, such mimetics have been found to possess much higher biological activity than that expected on the simple basis of binding studies or tissue bio-assays [5].

However, the design of synthetic structures that mimic specific protein or peptide surface regions constitutes a tricky goal. While there has been some success in the field of small mimetics that reproduce extended conformations or β-turns [6], much less progress has been made in the search for structures that mimic α-helix loops. This is remarkable, given the ubiquitous role of α-helical regions in mediating protein-protein interactions.

Peptidomimetic scaffolds proved to be useful tools for exploring the diversity represented by the amino acid side chains on the design of ligands toward a wide range of targets [7]. In this respect, 13-membered ring scaffolds constitute privileged structures; indeed, they represent the smallest possible systems which can retain (in principle) all of the features of H-bonded peptide structures, including a whole α-helix loop. In addition, while cyclic 12-membered ring tetrapeptides as minimalist turn mimetics have limited uses due to inefficient synthesis, instability, and conformational heterogeneity, 13-membered ring tetrapeptidomimetics proved to be easier to synthesize, chemically more stable, and conformationally homogeneous [8]. Nevertheless, a few biologically active 13-membered ring peptidomimetics have been reported in literature (for selected examples see: [8-10]).

One of the most important issues to pursue in the field of bioactive peptide analogues is the search for well-defined 3D structures. Indeed, the rules governing conformational preferences in natural, flexible peptides are poorly understood, and consequently, structure-activity relationships in these molecules can be difficult to define. On the other hand, by incorporating rigid scaffolds that fix in an unambiguous way peptide geometries, synthetic molecules can be devised to mimic the localised elements of protein structure that constitute bioactive surfaces. This is a promising growth area of medicinal chemistry that could impact significantly on biology and medicine.

Among the diverse classes of peptidomimetics, partially modified retro-inverse (PMRI) peptides [11] constitute a noteworthy family, Fig. (**1**), since they found applications in a wide range of biologically active compounds, such as diagnostic reagents, immunogens, immunomodulators, immunostimulators, anti-inflammatories, antimicrobials, membrane-penetrating vectors, etc. [12].

In this paper, the potential of minimalistic cyclic PMRI (*c*-PMRI) peptides [13] as versatile platforms for the design of peptidomimetic libraries is addressed (Fig. **2**) [14]. In the

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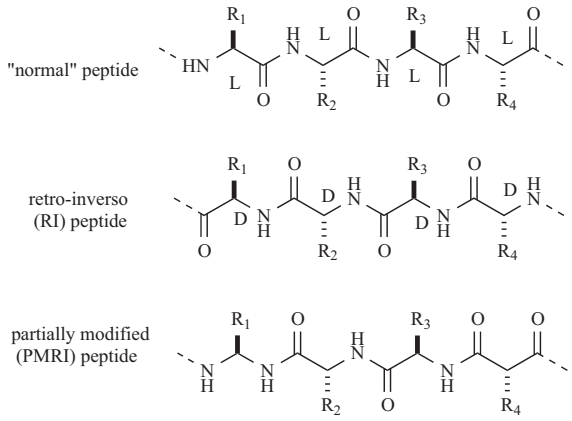


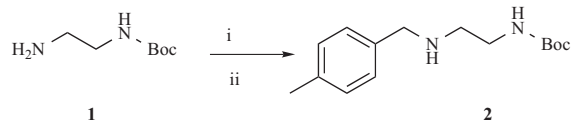
Fig. (1). The RI and PMRI peptide concept.

following sections, the synthesis in solution and the conformation analysis of a prototypic 13-membered ring *c*-PMRI tetrapeptide is described in detail.

RESULTS AND DISCUSSION

In order to reach the critical 13-membered ring size, we planned to introduce a β-amino acid-mimetic diamine, followed by a couple of eventually functionalized amino acids, and a diacid (Fig. **2**). A variety of functionalized diacids [11] is available from the literature, as well as optically pure, differently functionalized diamines, which can be prepared from amino acids, amino alcohols, alkenes, unsaturated amines, dienes, aziridines, imines, diols, dihalides, nitroalkenes, amino ketones, etc. [15].

The N-functionalized diamine **2** was readily obtained from the mono-protected ethylenediamine **1** [16], by reaction with *p*-tolualdehyde and reduction of the resulting imine, not isolated, with $NaBH_4$.



Scheme (1). Reagents: i: *p*-tolualdehyde, $MgSO_4$; ii: $NaBH_4$.

The diamine was coupled under standard conditions with Cbz-Leu-OH, giving the partially peptoid-like compound **3**. Boc group was removed under acidic conditions, and the resulting free amine-TFA salt was coupled with Fmoc-Asp(O*t*-Bu)-OH. The tripeptide mimetic **4** was treated with DMA in THF to remove Fmoc group. Coupling with malonic acid mono-ester gave the PMRI peptide **5**, which was subjected to catalytic hydrogenation. The deprotected **6** was treated with DPPA affording cyclic **7** in satisfactory yield, without any trace of octapeptide cyclodimer. The purification of peptidomimetics **3**, **4**, **5**, and **7** was easily performed by flash chromatography.

After synthesis and isolation, we performed the conformational analysis of the *c*-PMRI peptide **7** by 2D NOESY spectroscopy and Molecular Modeling computations. The ^{1}H-NMR spectrum in C_6D_6 showed a single resonance set [17], suggesting the occurrence of a single conformer. The unambiguous assignment of NMR signals was performed by COSY and HMBC analysis (heteronuclear multiple bond correlation).

VT-NMR (variable temperature) experiments could not unambiguously confirm the presence of any H-bond [18], therefore we examined also the dependence of the ^{1}H-NMR chemical shifts on the addition of small amounts of a competitive solvent; resonances were considerably modified by the addition of DMSO-d_6, suggesting the absence of H-bonds [19].

Finally, 2D-NOESY in C_6D_6 gave, apart from the obvious correlations, several diagnostic cross-peaks, Fig. (**3**)

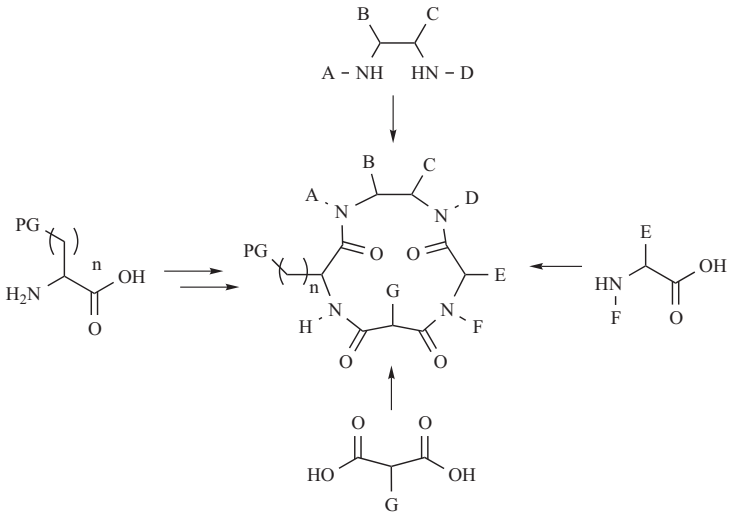
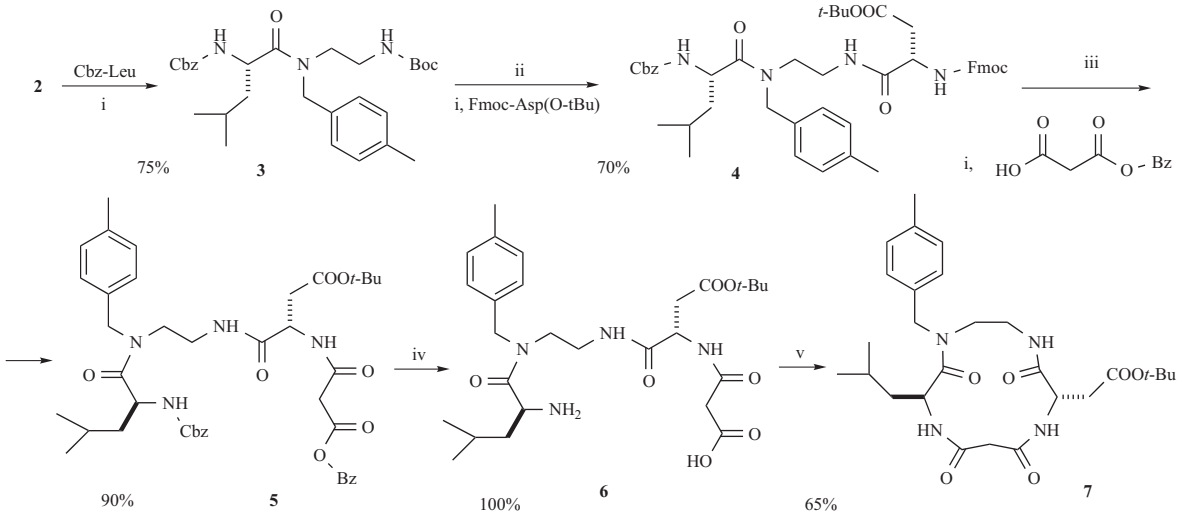


Fig. (2). Design of 13 membered ring *c*-PMRI tetrapeptide scaffolds for combinatorial approach (A-H: different substituents; PG: protecting group).



Scheme (2). Reagents: i: HATU, DIPEA; ii: TFA; iii: DMA; iv: H_2/Pd; v: DPPA.

and Table **1**, which allowed us to determine the approximate distances between side chains.

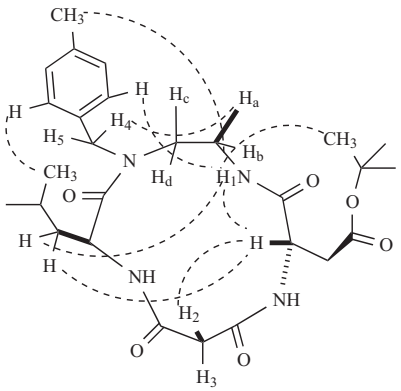


Fig. (3). NOESY analysis; only some selected, non-obvious correlations are shown (see also Table **1**).

Conformational analysis was conducted by Restrained Molecular Dynamics, using non-geminal interproton distances obtained from NOESY as constraints. Initial structures indicated a cis ω bond between Asp and malonyl group, consistent with a strong αAsp -αmalonyl NOESY cross peak, characteristic of cis peptide bond. The Leu-diamine ω bond was set at either 0 or 180°, while the remaining peptide bonds were restrained at 180°. Of the 150 calculated conformations, only structures showing a cis Leu-diamine ω bond showed no significant violations of NOE constraints. A representative structure is shown in Fig. (**4**). The analysis of $^3J_{NH\text{-}H\alpha}$ and $^3J_{H\alpha\text{-}H\beta}$ coupling constants was also used to confirm the conformational features of **7** [20,21].

To summarize, the conformation of **7** deduced from spectroscopic and computational analysis showed a rigid backbone conformation, carrying the side chains on the same side of the molecule.

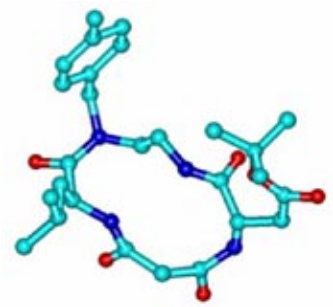


Fig. (4). Low-energy structure of **7** consistent with NOE data.

CONCLUSIONS

In this paper, we have illustrated the synthesis of a novel 13-membered ring *c*-PMRI peptide, and we have reported the conformational analysis. The introduction of a peptoid-like diamine as a turn-inducing residue, and the presence of a further *cis*-amide in the peptide framework, facilitated cyclization and induced conformational rigidity. Indeed, the *c*-PMRI scaffold backbone adopted a well-defined 3D structure, showing the amino acid side chains projected on the same side of the molecular plane. Promising applications for scaffolds of this kind can be found in the design of entities for rapid screening of a number of potentially active peptidomimetics: integrin inhibitors, agonists or antagonists of opioid receptors, somatostatin analogues, metal-chelating scaffolds as antibiotics or as catalysts for enantiomeric synthesis, regulatory peptides, etc. [4,22,23]. In particular, work is in progress in our laboratory for the development of inhibitors of α4β1 integrins based on mimetics of the sequence Leu-Asp-Val (LDV) [24], and of αvβ3 integrin inhibitors based on the sequence Arg-Gly-Asp (RGD) [25].

Finally, it is worthwhile to mention that the easy introduction of an amino acid carrying a functionalized side chain (such as Asp) within the ring framework, could be useful for linking to a polymer support (for selected examples: [26,27]), making it possible the design of a general scaffold suitable for solid phase-based combinatorial chemistry.

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EXPERIMENTAL SECTION

General Methods

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Flash chromatography was performed on Merck silica gel 60 (230-400 mesh), and solvents were simply distilled. NMR Spectra were recorded with a Varian Inova 600. Analytical RP-HPLC was performed on a HP Series 1100, with a HP Hypersil ODS column, (4.6-μm particle size, 100 Å. pore diameter, 250 mm), and with a solvent system: A = water, B = CH_3CN, gradient from 70% A to 80% B in 8 min. ES-MS was performed with an HP 1100MSD.

Conformational NMR Analysis of 7

NMR Spectra were recorded using five-millimeter tubes, using 0.01 M **7** in C_6D_6, at 600 MHz (^{1}H NMR) and at 75 MHz (^{13}C NMR) at room temperature. Chemical shifts are reported as δ values relative to the solvent peak. gCOSY experiments were recorded with a proton spectral width of 9595.8 Hz. gHMBC experiments were recorded with a proton spectral width of 9595.8 Hz and a carbon spectral width of 36199.1 Hz, selecting a spin coupling constant of 8 Hz. VT-NMR experiments were performed over the range of 295.7-325.7 °K. NOESY experiments were recorded with a 300 ms mixing time with a proton spectral width of 3087.8 Hz. NOESY intensities were correlated to distances according to a calibration against the intensity of geminal protons (Table **1**). Geminal couplings ad other obvious correlations were not used.

Table 1. NOESY Experiments Performed on 7 in C_6D_6

Cross peak[a]	Intensity (%)	Distance (Å)
NHLeu - HαLeu	0.6	3.0
NHAsp - HαAsp	0.9	2.7
NH_1 – Ha	0.7	3.0
NH_1 – Hb	1.2	2.6
Ha – Hd	2.3	2.3
Ha – Hc	1.9	2.4
Hb – Hc	3.3	2.2
NH_1 - HαAsp	2.7	2.2
NH_1 – CH_3 (Leu)	0.6	3.1
NH_1 – CH_3 (*t*-Bu)	1.7	2.4
NH_1 – Hc	0.9	2.7
NH_1 – Hd	0.4	3.4
NH_1 – ArH	2.0	2.4
NH_1 - CH_3 (PhMe)	0.9	2.7
NH_1 - NHLeu	0.2	4.1
NH_1 – H_2	0.5	3.2
NH_1- H_3	0.5	3.2
NH_1 - NHAsp	0.7	2.9
NHLeu - HαAsp	0.3	3.7
NH Leu - CH_2 (Leu)	0.4	3.4
Hc – H_4	0.7	2.9
Ha - H_4	2.7	2.3
Hb - H_4	0.4	3.4
Hd - H_4	0.7	2.9
ArH - CH_2 (Leu)	0.5	3.2
HαLeu – CH_3 (Leu)	0.4	3.4
HαLeu – CH (Leu)	0.0	>4.0
HαAsp – H_2	1.6	2.4
HαAsp – CH_2 (Leu)	0.3	3.7
HβAsp – H_3	0.5	3.2

[a] Geminal cross peaks are omitted.

Computational Analysis

Restrained Molecular Dynamics simulations [28] were performed using AMBER [29]. The ω bond between Asp and malonyl group was set at 0°; the Leu-diamine ω bond was set at either 0 or 180°; the remaining peptide bonds were set at 180°. All simulations were conducted in vacuo. Energy minimization was carried out without a nonbonded cutoff to a convergence of 0.001 in the gradient and a distance-dependent dielectric constant of 4*r*. A simulated annealing protocol was used to obtain NMR-derived structures. The NOE-derived distances were restrained with a force constant value of 7 kcal/mol Å^2, while omega bonds were restrained with a force constant of 16 kcal/mol Å^2. A set of 150 randomly generated conformations was subjected to a 20-ps protocol during which the temperature of the system was increased in 0.1 ps from 100 °K to 1200 °K and then cooled to 100°K in 0.3 ps.

[2-(4-Methyl-benzylamino)-ethyl]-carbamic acid *tert*-butyl Ester (2)

To a stirred solution of **1** [16] (0.65 g, 4.1 mmol) and $MgSO_4$ (0.98 g, 8.1 mmol) in DCM (5 mL), *p*-toluenaldehyde (0.58 mL, 4.9 mmol) was added at rt. After 4 h, the mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was diluted with EtOH (10 mL) and $NaBH_4$ (276 mg, 7.47 mmol) was added at rt while stirring. After 0.5 h, the reaction was quenched with 1 M HCl (15 mL), and the mixture was extracted twice with DCM (2 x 20 mL). 3 M NaOH was added to the aqueous layer until pH was around 10. The mixture was extracted three times with EtOAc (3 x 20 mL). The collected organic layers were dried over Na_2SO_4. Solvent was removed under reduced pressure, giving **2** (0.87g, 82 %) which was used without further purification. ^{1}H-NMR ($CDCl_3$): δ = 1.45 (s, 9H), 1.70 (s, 1H), 2.36 (s, 3H), 2.70 (t, 2H, J = 6.5 Hz), 3.15 (q, 2H, J = 6.5 Hz), 3.70 (s, 2H), 5.00 (bs, 1H), 7.10 (d, 2H, J = 7.8 Hz), 7.15 (d, 2H, J = 7.8 Hz); ES-MS *m/z*: 265.1 [M+1].

{(*S*)-1-[(2-*tert*-Butoxycarbonylamino-ethyl)-(4-methyl-benzyl)-carbamoyl]-3-methyl-butyl}-carbamic Acid Benzyl Ester (3)

To a stirred solution of **2** (0.87 g, 3.3 mmol) in DCM (10 mL), a solution of Cbz-Leu (1.05 g, 4.0 mmol) and HATU (1.51 g, 3.96 mmol) in DMF (3 mL) was added, at rt. After that, DIPEA (0.67 mL, 7.3 mmol) was added and the mixture was stirred for 6h. The mixture was diluted with EtOAc (30 mL) and washed with 0.1 M HCl (5 mL), sat. $NaHCO_3$ (5 mL), and brine (5 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel (35:65 EtOAc:cyclohexane), affording **3** (1.50 g, 86%) as a waxy solid. ^{1}H-NMR ($CDCl_3$): δ = 0.80 – 1.00 (m, 6H), 1.43 (s, 9H), 1.40 – 1.80 (m, 3H), 2.36 (s, 3H), 3.10 – 3.50 (m, 4H), 4.10 – 4.20 (m, 1H), 4.40 – 4.60 (m, 2H), 5.00 - 5.25 (m, 3H), 5.48 (d, 1H, J = 8.8 Hz), 7.15 (d, J = 8.5 Hz, 2H), 7.20 – 7.40 (m, 7H); ES-MS: *m/z* 512.2 [M+1].

(*S*)-*N*-{2-[((S)-2-Benzyloxycarbonylamino-4-methyl-pentanoyl)-(4-methyl-benzyl)-amino]-ethyl}-3-(9*H*-fluoren-9-ylmethoxycarbonylamino)-succinamic acid *tert*-butyl Ester (4)

A stirred solution of **3** (0.75 g, 1.42 mmol) in DCM (10 mL) was treated with TFA (2 mL). After 40 min, the mixture was concentrated under reduced pressure. The residue was diluted with DCM (10 mL), and a solution of Fmoc-Asp(O*t*-Bu) (0.70 g, 1.70 mmol) and HATU (0.648, 1.70 mmol) in DCM (4 mL) and DMF (4 mL) was added while stirring at rt. After that, DIPEA (0.85 mL, 4.97 mmol) was added. After 6 h, the reaction was concentrated under reduced pressure, the residue was diluted with EtOAc (30 mL), and the organic layer was washed with 0.1 M HCl (5 mL), sat. $NaHCO_3$ (5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4, concentrated under reduced pressure, and the residue was purified by flash chromatography over silica gel (50:50 EtOAc:cyclohexane), giving **4** (0.857g, 75%) as a waxy solid. ^{1}H-NMR ($CDCl_3$): δ = 0.80 – 1.00 (m, 6H), 1.43 (s, 9H), 1.60 (m, 3H), 2.1 (s, 3H), 2.70 (m, 1H), 3.10 (dd, 1H, J = 16.2, 9.0 Hz), 3.20 – 3.65 (m, 4H), 4.20 – 4.3 (m, 2H), 4.40 – 4.50 (m, 3H), 4.50 – 4.70 (m, 2H), 5.1 (m, 2H), 5.43 (d, 1H, J = 8 Hz), 5.93 (d, 1H, J= 10 Hz), 6.9 (bs, 1H), 7.10 (s, 4H), 7.20 – 7.50 (m, 9H), 7.60 – 7.80 (m, 4H); ES-MS [M+1]: 805.4, Calcd. 805.4

(*S*)-3-(2-Benzyloxycarbonyl-acetylamino)-*N*-{2-[((S)-2-benzyloxycarbonylamino-4-methyl-pentanoyl)-(4-methyl-benzyl)-amino]-ethyl}-succinamic acid *tert*-butyl Ester (5)

4 (0.402 g, 0.50 mmol) was diluted with 2N DMA in THF (5mL). The mixture was stirred for 40 min and concentrated under reduced pressure. The residue was diluted with DCM (5 mL), and a solution of malonic acid benzyl ester (0.13 g, 1.30 mmol) and HATU (0.49 g, 1.30 mmol) in DCM (4 mL) and DMF (1 mL) was added while stirring. DIPEA (0.10 mL, 0.70 mmol) was added, and the mixture was stirred for 6 h. The reaction was concentrated under reduced pressure, and the residue was diluted with EtOAc (30 mL). The organic layer was washed with 0.1 M HCl (5 mL), sat. $NaHCO_3$ (5 mL), and brine (5 mL). The organic layer was dried over Na_2SO_4, concentrated under reduced pressure, and the residue was purified by flash chromatography over silica gel (70:30 AcOEt:cyclohexane), affording **5** (0.265 g, 70%). ^{1}H-NMR ($CDCl_3$): δ = 0.70 – 1.00 (m, 6H), 1.41 (s, 9H), 1.40 – 1.60 (m, 2H), 1.79 (m, 1H), 2.30 (s, 3H), 2.85 (dd, 2H, J = 12.0, 5.0 Hz), 2.85 – 3.05 (m, 5H), 3.80 – 3.92 (m, 1H), 4.00 (d, 1H, J = 9.0 Hz), 4.60 – 4.85 (m, 2H), 4.85 – 5.30 (m, 3H), 5.38 (d, 1H, J = 9.0 Hz), 5.38 – 5.50 (d, 1H, J = 6 Hz), 7.00 – 7.20 (m, 4H), 7.20 – 7.50 (m, 10H), 7.80 – 7.95 (m, 1H), 8.00 – 8.10 (d, 1H, J = 9 Hz); ES-MS: *m/z* 759.5 [M+1].

(*S*)-*N*-{2-[((S)-2-Amino-4-methyl-pentanoyl)-(4-methyl-benzyl)-amino]-ethyl}-3-(2-carboxy-acetylamino)-succinamic Acid *tert*-butyl Ester (6)

A stirred suspension of catalytic Pd/C and **5** (0.12 g, 0.15 mmol) in EtOH (10 mL) was treated with H_2 (1 atm) at rt. After 2 h, the suspension was filtered, and the filtrate was concentrated under reduced pressure, giving **6** (0.069 g, 100%) without further purification. ES-MS: *m/z* 535.2 [M+1].

[(2*S*,9*S*)-9-Isobutyl-7-(4-methyl-benzyl)-3,8,11,13-tetraoxo-1,4,7,10-tetraaza-cyclotridec-2-yl]-acetic Acid *tert*-butyl Ester (7)

To a stirred solution of **6** (0.080 g, 0.15 mmol) in DMF (6 mL), DPPA (0.12 mg, 0.10 mmol) and $NaHCO_3$ (0.19 g, 2.25 mmol) were added at rt. After 48 h, the solvent was distilled under reduced pressure, the residue was diluted with EtOAc (20 mL), and the mixture was washed with water (5 mL). The organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. The residue was purified by flash chromatography over silica gel (98:2 AcOEt:MeOH), giving **7** (0.051 g, 65%, 96% pure by HPLC analysis). ^{1}H-NMR (C_6D_6 for proton numbering/lettering, see also Fig. **3**): δ = 0.68 (d, 3H, J = 6.6 Hz, *i*-Pr), 0.86 (d, 3H, J = 6.6 Hz, *i*-Pr), 1.20 – 1.28 (m, 1H, HβLeu), 1.40 (s, 9H, *t*-Bu), 1.70 – 1.80 (m, 1H, C*H*$(CH_3)_2$), 2.07 (s, 3H, Ph*Me*),

2.10 – 2.17 (m, 1H, HβLeu), 2.33 (dd, 1H, J = 13.8, 3.5 Hz, Hc), 2.70 (m, 2H, Hd, HβAsp), 3.09 (d, 1H, J = 12.6 Hz, H2), 3.10 (dd, 1H, J = 16.2, 9.0 Hz, HβAsp), 3.55 (d, 1H, J = 12.6 Hz, H3), 4.14 – 4.26 (m, 1H, Ha), 4.37 (d, 1H, J = 17.4 Hz, H4), 4.67 (dt, 1H, J =13.2, 4.2 Hz, Hb), 5.29 (dt, 1H, J = 10.2, 4.2 Hz, H_{α}Leu), 5.36 (dt, 1H, J = 9.6, 4.8 Hz, H_{α}Asp), 5.39 (d, 1H, J = 17.4 Hz, H5), 6.93 (d, 2H, J = 7.8 Hz, ArH), 6.97 (d, 2H, J = 7.8 Hz, ArH), 7.77 (d, 1H, J = 9.6 Hz, NHAsp), 8.12 (dd, 1H, J = 9.0, 0.1 Hz, NH_1), 8.75 (d, 1H, J = 9.6 Hz, NHLeu); ^{13}C-NMR-APT (C_6D_6): δ = 20.9(-), 21.5(-), 23.5(-), 24.8(-), 28.0(-), 31.6(-), 36.0(+), 37.2(+), 38.5(+), 40.2(+), 43.6(+), 46.1(+), 47.9(-), 49.0(+), 50.4(-), 59.9(+), 80.3(+), 126.6(-), 129.6(-), 135.1(+), 137.0(+), 166.3(+), 169.3(+), 170.0(+), 171.0(+), 175.0(+); ES-MS: *m/z* 517.3 [M+1].

ABBREVIATIONS

Cbz	=	Carbobenzyloxy
Fmoc	=	Fluorenylmethoxycarbonyl
Boc	=	Tert-butoxycarbonyl
Bz	=	Benzyl
TEA	=	Triethylamine
DPPA	=	Diphenylphosphorylazide
TFA	=	Trifluoracetic acid
DIPEA	=	N,N-diisopropylethylamine
DC	=	Dichloromethane
HATU	=	2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium
^{13}C-NMR-APT	=	^{13}C-NMR attached proton test, quaternary and secondary carbons: positive (+), tertiary and primary carbons: negative (-)

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