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Conformational studies on a medium size cyclopseudopeptide containing the oxazolidin-2-one moiety†

Gaetano Angelici[b], Nicola Castellucci[a] and Claudia Tomasini[a]*

An efficient synthesis of a 24 pseudopeptide membered ring in solution is reported in good yield. The cycle contains four units, all formed by an α-amino acid (Xaa) and a 4-carboxy-5-methyl-oxazolidin-2-one group (4-carboxy-5-methyl-oxazolidin-2-one = Oxd) in the same configuration (L or D). In the final sequence, the four LL or DD units are alternated so that, after cyclization, *cyclo*-(L-Xaa-L-Oxd-D-Xaa-D-Oxd-L-Xaa-L-Oxd-D-Xaa-D-Oxd) is formed. The conformational analysis of this compound has been performed by means of infrared and ^{1}H NMR spectroscopy and shows that the CHα hydrogens of the α-amino acids retain the very deshielded chemical shifts recorded in the spectra of the free precursors. Furthermore, the NH hydrogen have a weak tendency to form cross peaks, thus suggesting that the cycle lies in a large circle able to host small molecules, thus this compound is a promising candidate for drug delivery studies. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: conformational analysis; cyclization; cyclopseudopeptide; medium size ring; peptide coupling; ^{1}H NMR spectroscopy

INTRODUCTION

The incorporation of chosen, nonamino acids into cyclopeptides is a promising approach to designs capable of performing specific tasks.[1] Although such hybrid cyclopeptides are common in the microbial world,[2,3] some groups of synthetic organic chemists have only recently began to explore such designs for creating architecturally beautiful and functionally useful macrocyclic hybrid peptides.[4–7]

Because the synthesis of linear peptides generally proceeds well, the key step for the chemical synthesis of cyclic peptides is usually the cyclization reaction. Incorporation of turn-inducing elements such as glycine, proline, D-amino acids and *N*-alkylated amino acids into the peptide backbone is known to improve cyclization yields.[8,9] Although conformational constraints are usually introduced into peptides through cyclization, cyclic peptides can still possess a remarkable flexibility.[10–12]

We want to show here the synthesis and the conformational analysis of a medium-sized ring containing four units, all formed by an α-amino acid (Xaa) and a 4-carboxy-5-methyl-oxazolidin-2-one group (4-carboxy-5-methyl-oxazolidin-2-one = Oxd) in the same configuration (L or D). In the final sequence, the four LL or DD units are alternated so that, after cyclization, *cyclo*-(L-Xaa-L-Oxd-D-Xaa-D-Oxd-L-Xaa-L-Oxd-D-Xaa-D-Oxd) is formed. The introduction of the Oxd moiety can be very useful in the preparation of rigid cyclopeptides, as the oxazolidin-2-one endocyclic carbonyl imparts rigidity to any pseudopeptide chain.[13–16] This effect can be spotted by checking the CHα proton chemical shift of the nearby amino acid, that is always very deshielded with a chemical shift that is above 5 ppm, while the normal position is between 4 and 4.5 ppm (Fig. 1).[17]

We have recently reported the synthesis of six cyclopseudopeptides all having general formula *cyclo*-(L-Xaa-D-Oxd)$_n$ (with $n = 3$ or 4).[18] In these compounds, the CHα proton chemical shift of the nearby amino acid is less deshielded with a chemical shift below 5 ppm, probably because of the cycle constraint, also confirmed by the low cyclization yields. We show here the synthesis of a 24-membered ring cycle, made of four LL or DD alternated units, which should provide a better conformation.

The synthesis of the cycle has been obtained in solution by coupling four residues containing one Oxd moiety and one α-amino acid each (the four amino acids are all different): two of them are Boc-L-Xaa-L-Oxd-OBn, while the other two are Boc-D-Xaa-D-Oxd-OBn (in one residue glycine was used). They have been coupled in an alternated way so that the heterocycle has general structure *cyclo*-(L-Xaa-L-Oxd-D-Xaa-D-Oxd-L-Xaa-L-Oxd-L-Xaa-L-Oxd) as shown on Fig. 2.

RESULTS AND DISCUSSION

Synthesis

Compound **11** has been synthesized in the liquid phase and in a few steps, all obtained with satisfactory yields (Scheme 1). First of

* *Correspondence to: C. Tomasini, Dipartimento di Chimica 'G. Ciamician', Università di Bologna, Via Selmi 2, I–40126 Bologna, Italy. E-mail: claudia.tomasini@unibo.it*

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a *N. Castellucci, C. Tomasini*
Dipartimento di Chimica 'G. Ciamician', Università di Bologna, Via Selmi 2 I–40126 Bologna, Italy

b *G. Angelici*
Department of Chemistry, University of Basel, St. Johanns-Ring 19 CH-4056 Basel, Switzerland

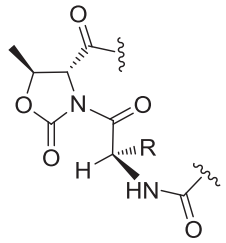


Figure 1. Preferred conformation for a D-Xaa-D-Oxd unit with the preferential *trans* conformation of the imide moiety, which accounts for the anomalous chemical shift of the amino acid CHα proton

all, the four units shown in Fig. 1 have been synthesized in solution by reaction of a Boc-Xaa-OH with H-Oxd-OBn, in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU)[19,20] and an excess of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). They all have the general formula Boc-Xaa-Oxd-OBn, and contain four different amino acids [valine, Val; alanine, Ala; *cyclo*hexyl-aspartic acid, Asp(O*c*Hex); glycine, Gly] to have information on the cycle preferred conformation. The four compounds Boc-L-Val-L-Oxd-OBn **1**, Boc-D-Ala-D-Oxd-OBn **2**, Boc-L-Asp(O*c*Hex)-L-Oxd-OBn **3** and Boc-Gly-D-Oxd-OBn **4** (Fig. 3) have all been obtained in high yield, after purification by silica gel chromatography.

The synthesis of the open chain Boc-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(OcHex)-L-Oxd-Gly-D-Oxd-OBn **9** was obtained in three steps, by coupling the Boc-Xaa-Oxd-OBn units and alternating the L and the D moieties (Scheme 1). In any case one moiety was treated with trifluoroacetic acid in dichloromethane to remove the Boc moiety and the other one with H_2 and Pd/C to obtain the free carboxy unit, then the two compounds were used without any purification and coupled in the presence of HBTU and triethylamine (TEA). Following this protocol, we prepared the tetramer **5**, then the hexamer **7** and finally the octamer **9**. In any case a flash chromatography purification was required after each coupling step. The yields were generally good, ranging between 75% and 80%.

The cyclization step proved to be more difficult, because a large ring with 24 atoms was prepared. Therefore, first of all we removed the protective benzyl group by reaction with H_2 in the presence of Pd/C to obtain the free acid **10** in quantitative yield and then the Boc moiety by reaction with trifluoroacetic acid to prepare the corresponding amine as its triflouroacetic salt. This compound was directly cyclised in acetonitrile in low concentration (1 mM) to promote the ring formation. By reaction with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HATU) and an excess of TEA, desired cycle

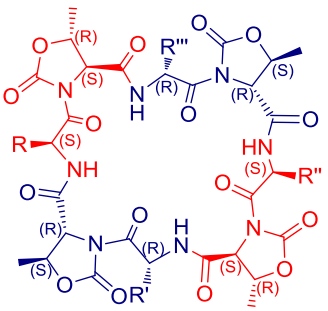
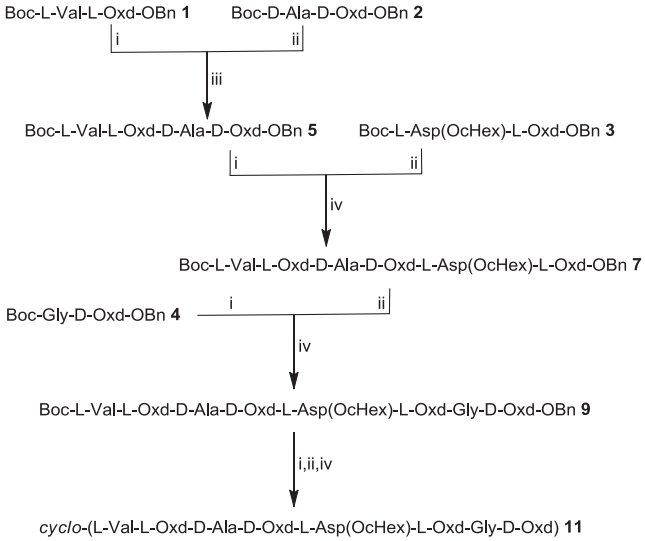


Figure 2. General structure of the compound **11**, a 24-membered ring containing the alternated L-Xaa-L-Oxd and D-Xaa-D-Oxd units



Scheme 1. Reagents and conditions: (i) H_2, Pd/C (10%), MeOH, r.t., 2 h; (ii) TFA (18 equiv.), dry CH_2Cl_2, r.t., 3 h; (iii) HBTU (1 equiv.), DBU (2 equiv.), dry CH_3CN, r.t., 3 h; (iv) HATU (1 equiv.), TEA (3 equiv.), dry CH_3CN, r.t., 45 min

11 was prepared. Because it is very polar and water soluble, it could not be purified by silica gel chromatography, so it was first washed with little amounts of acetonitrile, then purified with high-performance liquid chromatography (HPLC) (H_2O/acetonitrile 80 : 20 → 70:30 as eluant) and obtained pure in 62% yield from **9**.

CONFORMATIONAL ANALYSIS

Infrared spectroscopy

The infrared (IR) spectrum of cycle **11** was obtained as a 3 mM solution in dichloromethane to avoid possible self-aggregation. The analysis of the N–H stretching regions (3600–3200 cm^{-1}) helps one to detect if intramolecular N–H···O = C hydrogen bonds

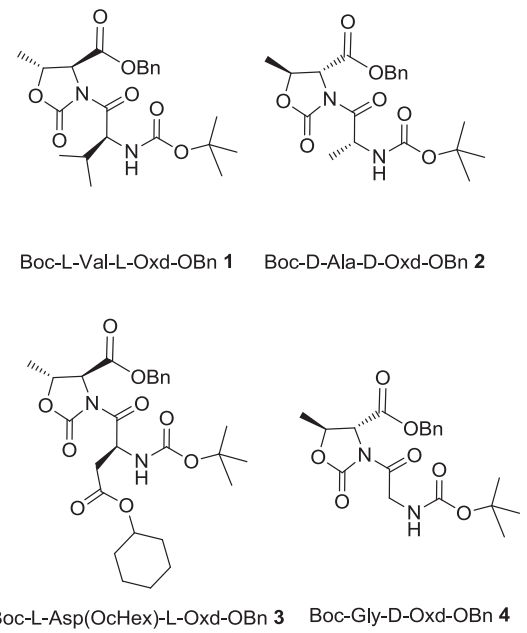


Figure 3. Building blocks utilized in this work

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are formed, because nonhydrogen-bonded amide NH bonds exhibit a stretching signal above 3400 cm^{-1}, while hydrogen-bonded amide NH bonds[21–25] produce a stretching band below 3400 cm^{-1}. The formation of a stretching band at 3409 cm^{-1} (see Experimental) suggests that no hydrogen bond is formed.

^{1}H NMR spectroscopy

The cycle **11** is a 24-membered ring made of four moieties and contains four α-amino acids and four Oxd groups that impart a local constraint to the oligomers. After cyclization, the molecule contains some more constraints because of the cycle formation. The preferential conformation of this very flexible ring was studied by ^{1}H NMR analysis, with the main target of checking if the NH hydrogens are close to one another or if a large hollow is retained after cyclization. All the nuclear magnetic resonance (NMR) spectra were recorded in DMSO,d_6, because of its low solubility in $CDCl_3$. First we recorded ^{1}H NMR and *g*COSY spectra to assign all the chemical shifts, mainly to the CHα and to the NH hydrogens, to check if the rigid *trans* conformation of the imido group is retained (Fig. 4 and Table 1), is in contrast with the results obtained for the *cyclo*-(L-Xaa-D-Oxd)$_n$ reported in.[8]

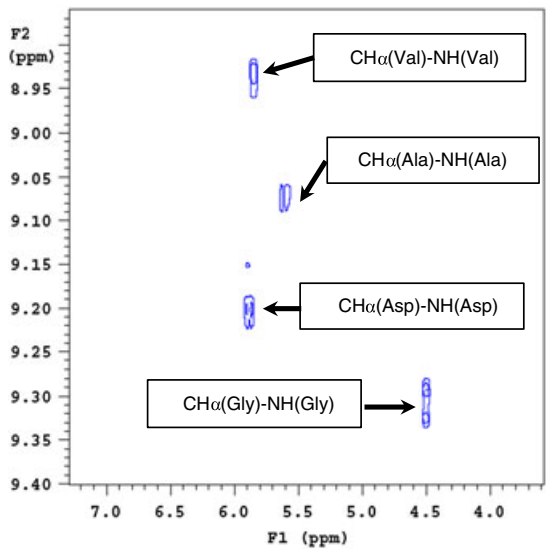

Figure 4. Enlargement of a *g*COSY ^{1}H NMR spectrum of **11**, showing the coupling between NH and the vicinal CHα of the four amino acids

Table 1. Chemical shift of the NH and CHα hydrogens of **11**, compared with the CHα hydrogen of **1–4**

Amino acid	δ (NH)[a]	δ (CHα)[a]	δ (CHα)-**1-4** [b]
L-Val	8.95	5.84	5.46
D-Ala	9.09	5.59	5.35
L-Asp(OcHex)	9.21	5.85	5.76
Gly	9.30	4.50	4.74

[a]The spectrum is recorded in DMSO,d_6.
[b]These chemical shifts are referred to the spectra of the precursors **1–4** and are recorded in $CDCl_3$.

As previously reported, the chemical shifts of the four CHα hydrogens are very deshielded compared with the normal chemical shifts of an α-amino acid, and retain more or less the same position that they have in the four building blocks **1–4**, that are totally free of any constraint. This outcome is a confirmation that the local constraint typical of the 4-carboxy-oxazolidin-2-ones is a strong effect, present also in medium-sized rings.

To have information on the preferential conformation of the cycle **11**, we recorded a NOESY 2D spectrum on a 10 mM of it in DMSO,d_6. In Fig. 5 the most significative nuclear Overhauser effect (NOE) enhancements are reported. The NH groups show a weak tendency to form cross peaks, because only one cross peak between NH(Val) and NH(Ala) is visible (Fig. 5(a)). In Fig. 5(b) three cross peaks, which may be ascribed to the interaction between NH hydrogens and three methyl groups (Oxd and Ala methyl groups), are recorded.

In Fig. 6 we have summarized the significative NOE enhancements of **11** in DMSO,d_6. To have a confirmation that these enhancements are due to the formation of the cycle, we also recorded the same experiment on the open chain **9** to make a comparison between the two results. In blue we reported the signal that the two compounds share, while in red we reported the interaction that are present only in the cycle and not in the open chain. From these experiments, we can gather that the open chain presumably lies in an open form, while the cycle **11** does not.

CONCLUSIONS

We have shown an efficient synthesis of a 24 pseudopeptide membered ring containing four units, all formed by an α-amino acid (Xaa) and a 4-carboxy-5-methyl-oxazolidin-2-one group (4-carboxy-5-methyl-oxazolidin-2-one = Oxd) in the same configuration (L or D). In the final sequence, the four LL or DD units are alternated so that, after cyclization, *cyclo*-(L-Xaa-L-Oxd-D-Xaa-D-Oxd-L-Xaa-L-Oxd-D-Xaa-D-Oxd) is formed.

The conformational analysis of this compound has been performed by means of IR and ^{1}H NMR spectroscopy and shows that no N–H···O = C hydrogen bond is formed within the cycle and that only the NH-Val and NH-Ala have a cross peak. Those outcomes both suggest that the cycle lies in a large circle able to host small molecules. This compound is a good candidate to behave as ionophore or to carry small molecules within the cell membrane. Further studies are currently ongoing on its application to drug delivery.

EXPERIMENTAL SECTION

Routine NMR spectra were recorded with spectrometers at 400 MHz (^{1}H NMR) and at 100 MHz (^{13}C NMR). Chemical shifts are reported in δ values relative to the solvent peak of $CHCl_3$, set at 7.27 ppm. Measurements were carried out in $CDCl_3$, DMSO,d_6, CD_3OD. Proton signals were assigned by COSY spectra.

Infrared spectra were recorded with a Fourier transform-IR spectrometer. High quality infrared spectra (64 scans) were obtained at 2 cm^{-1} resolution using a 1 mm NaCl solution cell. All compounds were dried *in vacuo* and all the sample preparations were performed in a nitrogen atmosphere. Melting points were determined in open capillaries and are uncorrected.

Purification of **11** was accomplished on an Agilent 1100 liquid chromatograph equipped with a variable wavelength UV detector (deuterium lamp 190–600 nm), using a Zorbax Eclipse XDB-C18 PrepHT column (21.2 × 150 mm, 7 μm) (Agilent Technologies, Santa Clara, California, United States).

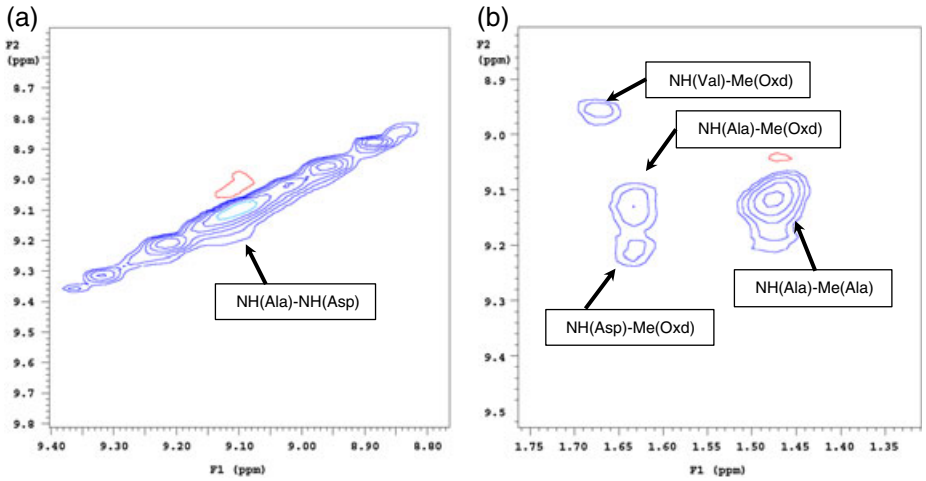

Figure 5. (a) and (b) enlargement of a NOESY 2D experiment recorded on a 10 mM solution of **11** in DMSO,d_6 and utilizing a mixing time of 700 ms

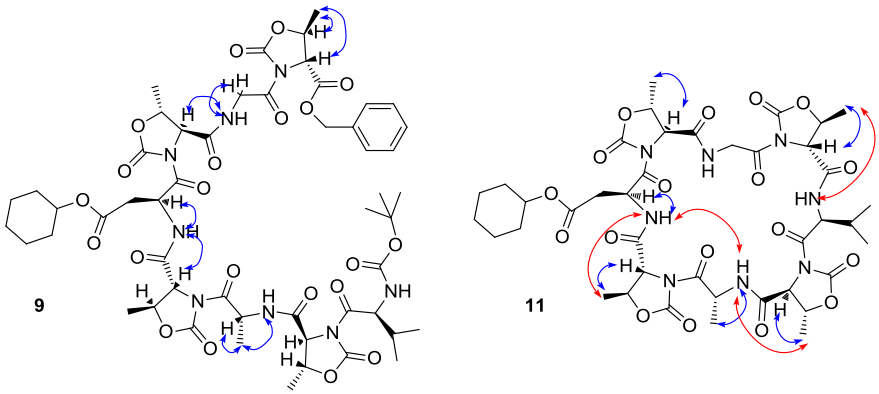

Figure 6. NOE enhancements as gathered from the NOESY 2D spectrum of **9** (left) (10 mM solution in DMSO,d_6, mixing time 900 ms) and **11** (right) (10 mM solution in DMSO,d_6, mixing time 700 ms)

Acetonitrile CHROMASOLV (Saint Louis, Missouri, United States) for HPLC was purchased by Riedel-de Haën and was used as the eluting solvent in a mixture with water.

General method for the synthesis of Boc-Xaa-Oxd-OBn

To a stirred solution of Boc-Xaa-OH (2.5 mmol) in acetonitrile (20 mL) were added HBTU (0.98 g, 2.6 mmol), then D- or L-Oxd-OBn (0.59 g, 2.5 mmol) and lastly DBU (0.75 mL, 5 mmol). The mixture was stirred 1 h, then acetonitrile was removed under reduced pressure and was replaced with ethyl acetate. The mixture was washed with brine, 1 N aqueous HCl (3 × 30 mL) and with 5% aqueous $NaHCO_3$ (1 × 30 mL), dried over sodium sulphate and concentrated *in vacuo*. The product was obtained pure after silica gel chromatography (cyclohexane/ethyl acetate 9 : 1 as eluant).

Boc-L-Val-L-Oxd-OBn 1

Yield = 90%; waxy solid; $[\alpha]_D$ = -16.0 (c 0.1,CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3442, 1793, 1753, 1708 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.80 (d, 3 H, *J* = 7 Hz, Me Val), 1.08 (d, 3 H, *J* = 7 Hz, Me Val), 1.41 (s, 9 H, *t*Bu), 1.57 (d, 3 H, *J* = 5.4 Hz, Me Oxd), 2.25 (bs, 1 H, $CH(CH_3)_2$), 4.58 (m, 2 H, CHN + CHO Oxd), 5.07 (d, 1 H, J = 8.6 Hz, NH), 5.21 (m, 2 H, OCH_2Ph), 5.46 (dd, 1 H, *J* = 3.4, 9.6 Hz, CH_α Val), 7.25–7.48 (m, 5 H, Ph); ^{13}C NMR ($CDCl_3$): δ 15.9, 20.0, 21.3, 28.6, 30.8, 57.4, 62.0, 68.3, 73.8, 80.1, 128.7, 129.1, 134.8, 151.5, 156.1, 167.9. Anal. Calcd. for $C_{22}H_{30}N_2O_7$: C, 60.82; H, 6.96; N, 6.45. Found: C, 60.80; H, 6.99; N, 6.43.

Boc-D-Ala-D-Oxd-OBn 2

Yield = 81%; waxy solid; $[\alpha]_D$ = +44.1 (c 1.0, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3439, 1794, 1755, 1718 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.31 (d, 3 H, *J* = 7.6 Hz), 1.42 (s, 9 H, *t*Bu), 1.51 (d, 3 H, *J* = 6.4 Hz, Me Oxd), 4.50–4.58 (m, 2 H, CHN + CHO Oxd), 5.14 (bs, 1 H), 5.17 (AB, 2 H, *J* = 12.0 Hz), 5.35 (dq, 1 H, *J* = 7.6 Hz, CH_α Ala), 7.25–7.40 (m, 5 H, Ph); ^{13}C NMR ($CDCl_3$): δ 17.8, 20.9, 28.1, 48.8, 61.4, 67.9, 73.5, 79.7, 128.2, 128.4, 128.7, 134.3, 151.1, 154.9, 167.4, 173.9. Anal. Calcd. for $C_{20}H_{26}N_2O_7$: C, 59.10; H, 6.45; N, 6.89. Found: C, 59.08; H, 6.47; N, 6.93.

Boc-L-Asp(OcHex)-L-Oxd-OBn 3

Yield = 70%; M.p. = 110 °C; $[\alpha]_D$ = −20.9 (c 1.0, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3408, 2959, 1794, 1707, 1364 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.48–1.67 (m, 15 H, 9 H *t*Bu + 6 H *c*Hex), 1.72 (d, 3 H, *J* = 4.7 Hz, Me Oxd), 1.89 (bs, 2 H, CH_2 *c*Hex), 1.99 (bs, 2 H, CH_2 *c*Hex), 2.85 (m, 1 H CH*H* Asp), 3.11 (m, 1 H, *CH*H Asp), 4.74 (m, 2 H CHN + CHO Oxd), 4.98 (bs, 1 H, NH), 5.37 (m, 2 H, OCH_2Ph), 5.76 (m, 1 H, CH_α Asp), 7.49–7.58 (m, 5 H, Ph); ^{13}C NMR ($CDCl_3$): δ 21.5, 24.0, 25.8, 28.7, 31.77, 36.7, 50.8, 62.0, 68.5, 73.9, 74.3, 128.8, 129.3, 135.0, 151.8, 167.9, 171.9. Anal. Calcd. for $C_{27}H_{36}N_2O_9$: C, 60.89; H, 6.81; N, 5.26. Found: C, 60.93; H, 6.78; N, 5.29.

Boc-Gly-D-Oxd-OBn 4

Yield = 78%; waxy solid; $[\alpha]_D$ = +37.0 (c 0.1, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3442, 1795, 1757, 1712, 1605, 1511 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.47 (s,

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9 H, *t*Bu), 1.57 (d, 3 H, *J* = 6.6 Hz, Me Oxd), 4.42 (dd, 1 H, *J* = 4.5, 19.5 Hz, CH_α Gly), 4.55 (d, 1 H, *J* = 4.2 Hz CHN Oxd), 4.62 (dq, 1 H, *J* = 6.3, 10.8 Hz, *CH*O Oxd), 4.74 (dd, 1 H, *J* = 6.6, 19.5 Hz, CH_α Gly), 5.10 (bs, 1 H, NH), 5.26 (m, 2 H, OCH_2Ph), 7.34–7.46 (m, 5 H, Ph); ^{13}C NMR ($CDCl_3$): δ 21.1, 26.8, 28.2, 30.8, 44.7, 61.4, 68.0, 74.1, 128.3, 128.7, 134.5, 151.7, 167.5, 170.0. Anal. Calcd. for $C_{19}H_{24}N_2O_7$: C, 58.16; H, 6.16; N, 7.14. Found: C, 58.13; H, 6.20; N, 7.11.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-OBn 5

To a stirred solution of Boc-L-Ala-L-Oxd-OH (0.60 mmol, 200 mg) and HBTU (0.60 mmol, 228 mg) in dry acetonitrile (10 mL) under inert atmosphere was added a mixture of CF_3COO^- $^+H_3$N-D-Ala-D-Oxd-OBn (0.60 mmol, 250 mg) and TEA (1.8 mmol, 0.25 mL) in dry acetonitrile (5 mL) at room temperature. The solution was stirred 45 min under inert atmosphere, then acetonitrile was removed under reduced pressure and replaced with ethyl acetate (30 mL). The mixture was washed with brine (30 mL), 1 M HCl (20 mL) and $NaHCO_3$ 5% (20 mL) and then dried over Na_2SO_4, then ethyl acetate was eliminated under reduced pressure. The product was obtained pure after flash chromatography (eluant: cyclohexane/ethyl acetate 8:2) in 80% yield. M.p. = 175 °C; $[\alpha]_D$ = + 41.5 (c 0.1, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3420, 1791, 1702, 1503, 1362 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.80 (d, 3 H, J = 7 Hz, Me Val), 1.08 (d, 3 H, *J* = 7 Hz, Me Val), 1.41 (m, 12 H, 9 H, *t*Bu + 3 H Me Ala), 1.57 (m, 6 H, 2 Me Oxd), 2.25 (bs, 1 H, *CH*$(CH_3)_2$), 4.41 (d, 1 H, *J* = 4.8 Hz, *CH*N Oxd), 4.5–4.7 (m, 2 H, *CH*N Oxd + *CH*O Oxd), 4.83 (m, 1 H, *CH*O Oxd), 5.07 (bs, 1 H, NH Val), 5.21 (m, 2 H, OCH_2Ph), 5.40 (m, 1 H, CH_α Val), 5.60 (m, 1 H, CH_α Ala), 6.80 (bs, 1 H, NH Ala), 7.25–7.48 (m, 5 H, Ph); ^{13}C NMR ($CDCl_3$): δ 14.5, 16.3, 18.1, 20.1, 20.9, 21.5, 28.6, 30.9, 48.7, 60.7, 61.7, 63.0, 68.5, 74.2, 80.1, 128.8, 129.1, 129.2. 134.6, 151.4, 166.9, 167.7, 172.7, 174.3. Anal. Calcd. for $C_{30}H_{40}N_4O_{11}$: C, 56.95; H, 6.37; N, 8.86. Found: C, 56.92; H, 6.36; N, 8.86.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-OH 6

To a stirred solution of Boc-L-Val-L-Oxd-D-Ala-D-Oxd-OBn **5** (0.3 mmol, 190 mg) in ethyl acetate (5 mL) 10% palladium on charcoal (3 mg) was added. The mixture was stirred under hydrogen atmosphere for 2 h. Then the catalyst was filtered on a celite pad and the mixture was concentrated. The product **6** was obtained pure in 87% yield without any further purification. M.p. = 180 °C; $[\alpha]_D$ = + 29.0 (c 0.1, CH_2Cl_2); IR (nujol): ν = 3397, 1792, 1691, 1462, 1372 cm^{-1}; ^{1}H NMR (CD_3OD): δ 0.85 (d, 3 H, *J* = 7 Hz, Me Val), 1.04 (d, 3 H, *J* = 6.8 Hz, Me Val), 1.45–1.58 (m, 18 H, 9 H, *t*Bu + 6 H 2Me Oxd + 3 H Me Ala), 2.18 (bs, 1 H, CH$(CH_3)_2$), 4.43 (d, 1 H, J = 4 Hz, CHN Oxd), 4.58–4.74 (m, 3 H, 2 CHO Oxd + CHN Oxd), 5.35 (m, 1 H, CH_α Val), 5.54 (m, 1 H, CH_α Ala), 6.71 (d, 1 H, J = 8.8 Hz, NH Val), 8.91 (d, 1 H, *J* = 7.4 Hz, NH Ala); ^{13}C NMR ($CDCl_3$): δ 15.7, 16.3, 18.8, 19.6, 20.2, 27.6, 30.3, 57.5, 62,8, 75.0, 75.3, 79.5, 152.8, 169.0, 172.5, 173.2. Anal. Calcd. for $C_{23}H_{34}N_4O_{11}$: C, 50.92; H, 6.32; N, 10.33. Found: C, 50.89; H, 6.34; N, 10.32.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(OcHex)-L-Oxd-OBn 7

To a stirred solution of Boc-L-Val-L-Oxd-D-Ala-D-Oxd-OH (0.096 mmol, 52 mg) and HATU (0.096 mmol, 36 mg) in dry acetonitrile (10 mL) under inert atmosphere was added a mixture of CF_3COO^- $^+H_3$N-L-Asp(O*c*Hex)-L-Oxd-OBn (0.096 mmol, 52 mg) and TEA (1.8 mmol, 0.25 mL) in dry acetonitrile (5 mL) at room temperature. The solution was stirred 45 min under inert atmosphere, then acetonitrile was removed under reduced pressure and replaced with ethyl acetate (30 mL). The mixture was washed with brine (30 mL), 1 M HCl (20 mL) and $NaHCO_3$ 5% (20 mL) and then dried over Na_2SO_4, then ethyl acetate was eliminated under reduced pressure. Yield = 65%; M.p. = 150 °C; $[\alpha]_D$ = + 6 (c 0.1, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3409, 2859, 1794, 1708, 1512, 1363 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.81 (d, 3 H, *J* = 6.8 Hz, Me Val), 1.06 (d, 3 H, *J* = 6.8 Hz, Me Val), 1.42–1.64 (m, 27 H, 9 H, *t*Bu + 9 H 3Me Oxd + 3 H Me Ala + 6 H *c*Hex), 1.72 (bs, 2 H, CH_2 *c*Hex), 1.81 (bs, 2 H, CH_2 *c*Hex), 2.30 (bs, 1 H, *CH*$(CH_3)_2$), 2.76 (dd, 1 H, *J* = 6.8, 16.4 Hz, *CH*H Asp), 2.88 (m, 1 H, OCH *c*Hex), 2.96 (dd, 1 H, *J* = 4.8, 16.8 Hz, CH*H* Asp), 4.38 (m, 2 H, 2 *CH*N Oxd), 4.57 (m, 2 H, CHN Oxd + CHO Oxd), 4.78 (m, 2 H, CHO Oxd), 5.07 (bs, 1 H, NH Val), 5.20 (m, 2 H, CH_2Ph), 5.39 (bs, 1 H, CH_α Val), 5.58 (m, 1 H, CH_α Ala), 5.78 (m, 1 H, CH_α Asp), 6.77 (bs, 1 H, NH Ala), 7.22 (bs, 1 H, NH Asp); ^{13}C NMR ($CDCl_3$): δ 16.2, 20.1, 21.0, 21.4, 24.0, 25.5, 28.6, 31.2, 31.7, 35.8, 48.8, 49.7, 61.7, 62.8, 68.5, 74.4, 128.7, 128.8, 129.2, 134.7, 151.7, 156.2, 166.9, 167.5, 169.6. Anal. Calcd. for $C_{45}H_{60}N_6O_{17}$: C, 56.48; H, 6.32; N, 8.78. Found: C, 56.49; H, 6.36; N, 8.82.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(OcHex)-L-Oxd-OH 8

For the synthetic details, see above the preparation of **6**, starting from **7**. Yield 86%; M.p. = 184 °C; $[\alpha]_D$ = + 21.0 (c 0.1, CH_2Cl_2); IR (nujol): ν = 3394, 2364, 1790, 1693, 1461, 1374 cm^{-1}; ^{1}H NMR (CD_3OD): δ 0.86 (d, 3 H, *J* = 6.9 Hz Me Val), 1.06 (d, 3 H, *J* = 6.9 Hz, Me Val), 1.37–1.59 (m, 27 H, 9 H, *t*Bu + 9 H 3Me Oxd + 3 H Me Ala + 6 H *c*Hex), 1.78 (bs, 2 H, CH_2 *c*Hex), 1.87 (bs, 2 H, CH_2 *c*Hex), 2.28 (bs, 1 H, *CH*$(CH_3)_2$), 2.63 (m, 1 H, *CH*H Asp), 3.10 (dd, 1 H, *J* = 3.6, 16.8 Hz, CH*H* Asp), 4.48–4.65 (m, 4 H, 3CHN Oxd + CHO Oxd), 4.73–4.82 (m, 2 H, 2CHO Oxd), 5.35 (m, 1 H, CH_α Val), 5.51 (m, 1 H, CH_α Ala), 5.90 (m, 1 H, CH_α Asp), 6.72 (d, 1 H, *J* = 9 Hz, NH Val), 8.92 (d, 1 H, *J* = 6.6 Hz, NH Ala), 9.10 (d, 1 H, *J* = 8.1 Hz, NH Asp); ^{13}C NMR (CD_3OD): δ 15.7, 16.2, 18.9, 19.5, 20.1, 23.5, 25.3, 27.5, 30.3, 31.4, 35.7, 62.5, 62.7, 73.6, 75.2, 78.2, 78.6, 79.5, 152.6, 155.4, 169.0, 169.5, 170.3, 173.1. Anal. Calcd. for $C_{38}H_{54}N_6O_{17}$: C, 52.65; H, 6.28; N, 9.69. Found: C, 52.63; H, 6.31; N, 9.69.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(OcHex)-L-Oxd-Gly-D-Oxd-OBn 9

For the synthetic details, see above the preparation of **7**, starting from **4** and **8**. Yield = 40%; M.p. = 184 °C; $[\alpha]_D$ = + 34.0 (c 0.1, CH_2Cl_2); IR (CH_2Cl_2, 3 mM): ν = 3410, 2937, 1795, 1701, 1507, 1394 cm^{-1}; ^{1}H NMR (DMSO, d_6): δ 0.76 (d, 3 H, *J* = 6.4 Hz, Me Val); 0.90 (d, 3 H, *J* = 6.4 Hz, Me Val), 1.26 (d, 3 H, *J* = 6.8 Hz, Me Ala), 1.35 (m, 21 H, 9 H *t*Bu + 6 H 2Me Oxd + 6 H *c*Hex), 1.42 (d, 3 H, *J* = 6 Hz, Me Oxd), 1.47 (d, 3 H, *J* = 6 Hz, Me Oxd), 1.63 (bs, 2 H, CH_2 *c*Hex), 1.73 (bs, 2 H, CH_2 *c*Hex), 2.11 (m, 1 H, *CH*$(CH_3)_2$), 2.45 (m, 1 H, *CH*H Asp), 2.88 (m, 1 H, CH*H* Asp), 4.38–4.70 (m, 9 H, 4CHN Oxd + 3CHO Oxd + $CH_{2\alpha}$ Gly), 4.83 (m, 1 H, CHO Oxd), 5.13–5.24 (m, 3 H, OCH_2Ph + CH_α Val),5.40 (m,1 H, CH_α Ala), 5.70 (m, 1 H, CH_α Asp), 6.86 (d, 1 H, J = 7.6 Hz, NH Val), 8.80 (m, 1 H, NH Gly), 8.89 (d, 1 H, *J* = 8 Hz, NH Ala), 9.09 (d, 1 H, *J* = 7.6 Hz, NH Asp); ^{13}C NMR (DMSO, d_6): δ = 15.9, 17.7, 19.9, 20.9, 21.1, 23.7, 25.6, 28.9, 30.3, 31.6, 61.6, 62.4, 65.6, 67.9, 73.2, 74.8, 78.9, 79.7, 80.1, 128.7, 129.0, 129.2, 135.9, 152.9, 156.4, 168.0, 168.6, 169.2, 172.7, 173.4. Anal. Calcd. for $C_{52}H_{68}N_8O_{21}$: C, 54.73; H, 6.01; N, 9.82. Found: C, 54.72; H, 6.03; N, 9.80.

Boc-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(OcHex)-L-Oxd-Gly-D-Oxd-OH 10

For the synthetic details, see above the preparation of **6**, starting from **9**. M.p. = 190 °C; $[\alpha]_D$ = + 27.0 (c 0.1, CH_2Cl_2); IR (nujol): ν = 3371, 1793, 1691, 1540, 1458, 1377 cm^{-1}; ^{1}H NMR (CD_3OD): δ 0.86 (d, 3 H, *J* = 6.9 Hz Me Val), 1.06 (d, 3 H, *J* = 6.9 Hz, Me Val), 1.32–1.61 (m, 27 H, 9 H *t*Bu + 12 H 4Me Oxd + 6 H *c*Hex), 1.78 (bs, 2 H, CH_2 *c*Hex), 1.85 (bs, 2 H, CH_2 *c*Hex), 2.23 (bs, 1 H, *CH*$(CH_3)_2$), 2.64 (m, 1 H, *CH*H Asp), 3.07 (m, 1 H, *CH*H Asp), 4.50–5.0 (m, 10 H, 4CHN Oxd + 4CHO Oxd + $CH_{2\alpha}$ Gly), 5.36 (m, 1 H, CH_α Val), 5.50 (m, 1 H, CH_α Ala), 5.85 (m, 1 H, CH_α Asp), 8.90 (d, 1 H, *J* = 5.7 Hz, NH Ala), 9.09 (d, 1 H, *J* = 6.9 Hz, NH Asp); ^{13}C NMR (CD_3OD): δ 15.7, 16.2, 18.8, 19.5, 19.6, 20.1, 23.4, 25.3, 27.5, 31.3, 35.7, 43.3, 49.6, 57.5, 61.9, 62.5, 62.7, 73.7, 75.0, 75.2, 75.6, 79.5, 152.5, 153.0, 168.8, 169.7, 170.3, 173.1. Anal. Calcd. for $C_{45}H_{62}N_8O_{21}$: C, 51.42; H, 5.95; N, 10.66. Found: C, 51.38; H, 5.92; N, 10.69.

cyclo-(L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp(O*c*Hex)-L-Oxd-Gly-D-Oxd) 11

A solution of **10** (0.075 mmol, 79 mg) and TFA (1.35 mmol, 0.1 mL) in dry methylene chloride (10 mL) was stirred 4 h at room temperature, then the volatiles were removed under reduced pressure and the product was obtained pure in quantitative yield without any further purification.

To a stirred solution of $CF_3CO_2^-$ $^+NH_3$-L-Val-L-Oxd-D-Ala-D-Oxd-L-Asp (OcHex)-L-Oxd-Gly-D-Oxd-OH, (0.075 mmol, 80 mg) in dry acetonitrile (80 mL) under inert atmosphere were added HATU (0.082 mmol, 32 mg) and then TEA (0.225 mmol, 0.054 mL) at room temperature. The solution was stirred 45 min under inert atmosphere, then acetonitrile was removed under reduced pressure. The crude was washed with acetonitrile to remove all the by-products. **11** was obtained as a white solid in 62% yield after HPLC (H_2O/acetonitrile 80:20 → 70:30 as eluant). M.p. = 187 °C; $[\alpha]_D = +$ 21 (c 0.1,CH_2Cl_2);); IR (CH_2Cl_2, 1 mM): $\nu = 3409$, 2862, 1800, 1703, 1513, 1356 cm^{-1}; ^{1}H NMR (DMSO, d_6): δ 0.96 (d, 3 H, $J = 7.2$ Hz Me Val), 1.09 (d, 3 H, $J = 6.8$ Hz Me Val), 1.38–1.73 (m, 18 H, 4Me Oxd + 6 H *c*Hex), 1.84 (bs, 2 H, CH_2 *c*Hex), 1.92 (bs, 2 H, CH_2 *c*Hex), 2.43 (bs, 1 H, $CH(CH_3)_2$), 3.07 (m, 2 H, CH_2 Asp), 4.27–4.87 (m, 10 H, 4CHN Oxd + 4CHO Oxd + $CH_{2\alpha}$ Gly), 5.59 (m, 1 H, CH_α Ala), 5.84 (m, 1 H, CH_α Val), 5.85 (m, 1 H, CH_α Asp), 8.95 (bs, 1 H, NH Val), 9.09 (bs, 1 H, NH Ala), 9.21 (bs, 1 H, NH Asp), 9.30 (bs, 1 H, NH Gly); ^{13}C NMR (DMSO, d_6): δ 17.6, 17.8, 18.4, 20.0, 20.5, 23.8, 25.7, 29.0, 30.5, 31.6, 31.8, 36.6, 48.1, 49.5, 49.7, 62.5, 73.4, 75.4, 75.7, 79.1. Anal. Calcd. for $C_{40}H_{52}N_8O_{18}$: C, 51.50; H, 5.62; N, 12.01. Found: C, 51.47; H, 5.63; N, 12.00.

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