Conformationally Stable and Constrained Macrocarbocyclic Pseudopeptide Mimics of β-Hairpin Structures

Stephen Hanessian*[a] and Mauro Angiolini[b]

Abstract: Subjecting a D-Pro-L-Pro template harboring N- and C-terminal ω-alkenyl amino acids to a ring-closure metathesis reaction afforded the corresponding macrocyclic alkenes. A *cis*-alkene analogue crystallized with one molecule each of water and chloroform, which were retained even after heating at 100 °C. By using the reduced macrocyclic product as a template, the metathesis could be repeated twice on newly installed ω-alkenyl amino acids to give three-tiered macrocarbocyclic pseudopeptides as mixtures of conformers. NMR studies revealed the high conformational stability of these motifs.

Keywords: β-sheet · macrocycles · metathesis · peptides

Introduction

The structure and function of proteins are intimately related to their three-dimensional architecture, in which extended strands of amino acid residues can adopt α-helical and β-sheet arrangements.[1] Such motifs define the secondary structures of the protein by virtue of inter-residue H-bonding, which gives specific segments of polypeptide chains an element of topological rigidity and sidedness. Three or more strands with parallel or antiparallel alignments of peptide amide units, connected by short loops consisting of one or two amino acid residues each, can create a β-sheet.[2] The loop region for an antiparallel arrangement requires amino acid residues that adopt a so-called β-turn or β-hairpin shape.[3] These ubiquitous regions and the chiral space provided by the backbone substituents of the polypeptides are important structural and functional elements for recognition by receptors, by DNA, or by small molecules. Consequently, much effort has been devoted to the design and synthesis of mimics of β-hairpin structures that harbor natural and unnatural peptidic appendages.[4] Creative juxtaposition of amide groups has led to the synthesis of artificial β-sheet-like motifs in which H-bonding plays a crucial role in maintaining a compact architecture.[2–4] For example, an imposed proximity of antiparallel residues can be achieved by utilizing proline or related turn-inducing rigid amino acids as loop regions. Nature utilizes disulfide bridges as a means of generating compact cyclic peptides, and "carba" analogues have been devised as synthetic mimetics.[5] The Grubbs ring-closing metathesis reaction is an immensely useful method for C–C bond formation.[6] It can be successfully applied to the synthesis of macrocarbocyclic analogues of cyclic peptides.[7, 8]

Robinson and co-workers[9] have reported on the synthesis of 8- and 10-amino acid residue cyclic peptides incorporating a D-Pro-L-Pro as a template to maintain β-strands in an antiparallel arrangement. We report herein on the design, synthesis, and secondary structural characteristics of novel macrocarbocyclic pseudopeptides as constrained β-hairpin structure mimetics utilizing a D-Pro-L-Pro dipeptide as a loop or turn motif.

Results and Discussion

The synthesis of the tetrapeptide **1** from *S*-butenyl glycine and D-Pro-L-PrOH was accomplished in a straightforward manner.[10] Ring-closing metathesis with the Grubbs catalyst afforded the macrocyclized olefinic products **2** and **3** as a 1:1 mixture of *cis* and *trans* isomers in 92% yield, (Scheme 1).

Slow evaporation from wet hexanes/chloroform yielded X-ray-quality crystals of the *cis* isomer **3**, mp 192 – 195 °C, which contained one molecule of water and another of chloroform in a unique H-bonded arrangement.[11] The unit cell consisted of two conformers, $\mathbf{C_1}$ and $\mathbf{C_2}$, differing only in

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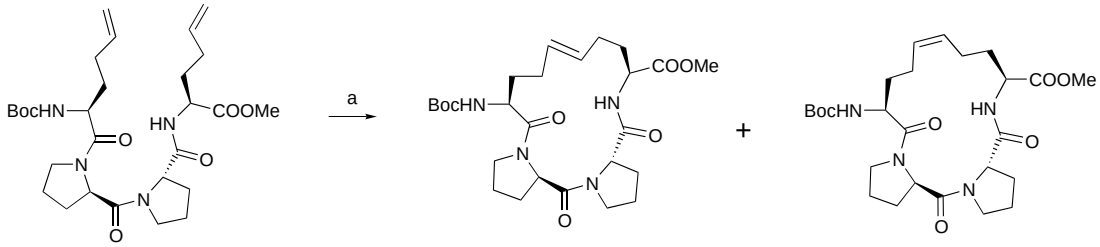


Scheme 1. a) $(PCy_3)_2Cl_2RuCHPh$ (0.1 equiv), CH_2Cl_2, RT, 5 h, 92%.

the torsion angles of specific methylene groups in the hexamethylene bridge, (Figure 1).

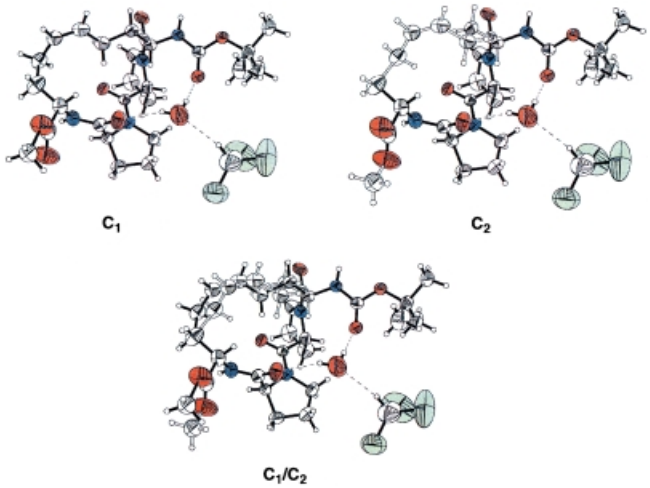
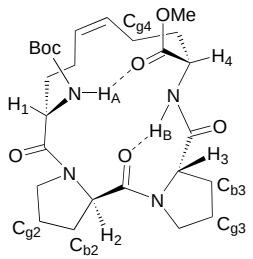


Figure 1. Ortep diagrams for **3**: bridged conformer C_1, bridged conformer C_2, and composite structure C_1/C_2.

Remarkably the crystals retained their three-dimensional structure even at 100 °C, without losing molecules of solvation or changing the ratio of the two hexamethylene bridged conformers. A thermogravimetric analysis confirmed partial loss of solvent at 112 °C.[11] Upon hydration in $CHCl_3$/hexanes, the original structure **3** was restored as a 1:1 mixture of conformers $\mathbf{C_1}$ and $\mathbf{C_2}$. Equally interesting was the conformational study of **3** by ^{1}H NMR spectroscopy at 600 MHz in different solvents.

Spectra in $CDCl_3$ and CD_2Cl_2 showed the presence of different conformers due to *cis*–*trans* tertiary amide-bond isomerism, and several N–H resonances were observed between $\delta = 6.9$ and 8.10; this indicated the existence of intramolecular hydrogen bonds (β or γ turns, λ_{max} in $CDCl_3$ at 3360–3279 cm^{-1}).[12, 13, 14] Only a single conformation was observed in [D_5]pyridine by one-dimensional NMR spectroscopy (^{1}H and ^{13}C); its secondary structure, shown in Scheme 2, was studied by a combination of two-dimensional COSY, TOCSY, NOESY, and ROESY spectra.[11] The geometry of the two tertiary amidic bonds was determined from the carbon chemical shifts of the β and γ carbons of the proline rings.[15] Typical values of δ of 31.03 for $C_{\beta 2}$ and 23.03 for $C_{\gamma 2}$, characteristic for *cis*-Xaa-D-Pro, were observed in the ^{13}C NMR spectra.[11]



Scheme 2. The secondary structure of cyclic peptide **3** in [D_5]pyridine.

A strong NOE effect between the protons H_1 and H_2, and the occurrence of a doublet for H_2 (arising from one large and one small J value between H_2 and vicinal protons) confirmed the *cis*-Xaa-D-Pro geometry.[16, 17] A *trans* geometry for the D-Pro-L-Pro bond was deduced by observing carbon chemical shifts values of $\delta = 29.48$ for $C_{\beta 3}$ and 25.48 for $C_{\gamma 3}$ and the absence of an NOE effect between H_2 and H_3.[16] The presence of a *cis* geometry for the Xaa-D-Pro bond is interesting because D-Pro-L-Pro dipeptides are known to strongly prefer a type II′ β-turn conformation, which cannot be adopted in the case of **3**.[9, 16, 18]

Titration experiments with increasing amounts of DMSO (0–15% *v*/*v*) in [D_5]pyridine showed a similar behavior for the two NHs present in the molecule with $\Delta\delta$(NH) values of -0.24 ppm for NH_A and -0.29 ppm for NH_B, (Figure 2). A large temperature-dependence coefficient of -0.019 ppm K^{-1}

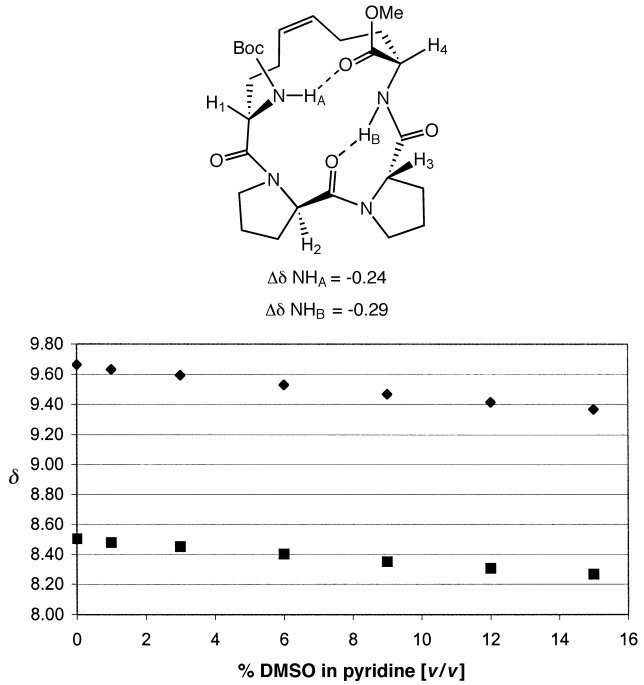
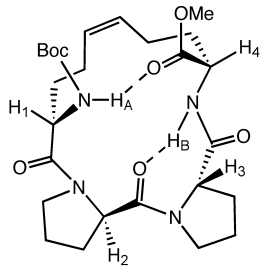


Figure 2. DMSO titration experiments in [D_5]pyridine for **3**. ◆ = NH_B, ■ = NH_A.

0947-6539/02/0801-0112 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 1

for NH_A and of $-0.014\ ppm\,K^{-1}$ for NH_B was observed in variable temperature experiments; this is consistent with the presence of an intramolecular hydrogen bond.[19] The linearity of the temperature dependences for the NHs was a strong indication that major conformational changes were absent in the temperature range 273–323 K,[20] (Figure 3). D_2O exchange experiments and NOE effects allowed the secondary structure of the tetrapeptide **3** to be better understood. Thus NH_A exchanged with 5% D_2O in [D_5]pyridine in 20 hours, while the NH_B took 3 days; this indicates that both the NHs were intramolecularly bonded: NH_A to the carbonyl of the methyl ester to form a 14-membered ring and NH_B to the carbonyl of D-Pro-L-Pro to generate a γ-turn structure, (Scheme 2).



NH_A = -0.019 ppm K^{-1}
NH_B = -0.014 ppm K^{-1}

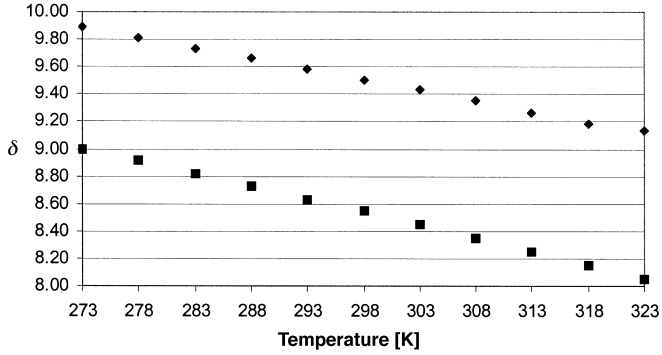


Figure 3. Variable temperature NMR experiments in [D_5]pyridine for **3**. ◆ = NH_B, ■ = NH_A.

That the NH_B proton was internally oriented toward the macrocycle was ascertained from a strong NOE effect between NH_B and H_3, and weak NOE between NH_B and a $C_{\gamma 4}$ proton. The downfield chemical shift for NH_B ($\delta = 9.66$) and for NH_A ($\delta = 8.52$) relative to those of normal amides, and the presence of large values for the homonuclear coupling constants between NH_B and H_4 (8.75 Hz), and NH_A and H_1 (8.45 Hz) are consistent with the presence of the proposed H-bonded array.[21, 22, 23]

Finally the chemical shifts of all four H_α protons of the four amino acid residues were in agreement with a β-hairpin-type structure, showing downfield shifts relative to a random coil: $\delta = 4.70$ for H_1, 5.84 for H_2, 4.86 for H_3, and 5.03 for H_4,[11, 24] (Table 1). Reduction of a mixture consisting of **2** and **3** afforded the saturated macrocycle **4**, whose solution conformation, H-bonding pattern, and NMR characteristics were very similar to the *cis*-olefin **3**, (Table 2). HPLC-MS analysis revealed the presence of two conformers each having exactly the same mass.

Table 1. ^{1}H NMR chemical shifts for peptide **3** at 303 K (1 mM solution in [D_5]pyridine).

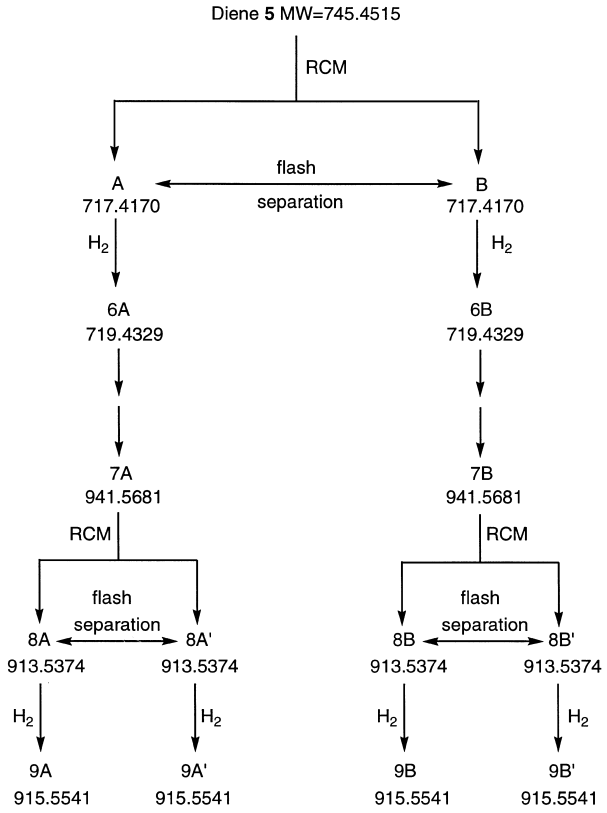
Residue	NH	CH(α)	$CH_2(\beta)$	$CH_2(\gamma)$	$CH_2(\delta)$
1	8.52	4.70	2.50, 2.03	2.50	5.50
2	–	5.84	2.22, 1.95	1.70	3.91, 3.69
3	–	4.86	2.38, 2.07	1.93, 1.74	4.31, 3.47
4	9.66	5.03	1.93, 1.75	2.88, 1.90	5.28

Table 2. ^{1}H NMR chemical shifts for peptide **4** at 303 K (1 mM solution in [D_5]pyridine).

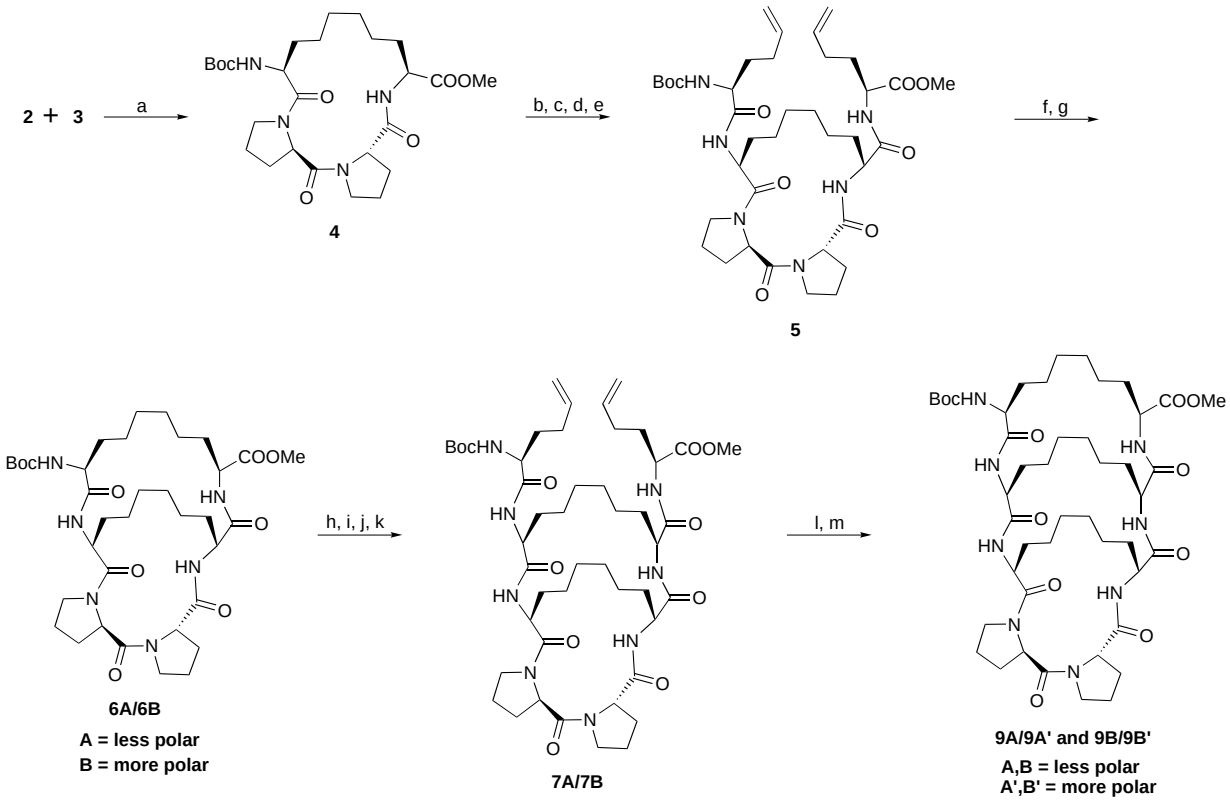
Residue	NH	CH(α)	$CH_2(\beta)$	$CH_2(\gamma)$	$CH_2(\delta)$
1	8.44	4.62	2.30, 1.76	1.68, 1.61	1.35, 1.25
2	–	5.74	2.22, 1.95	1.68	3.90, 3.75
3	–	4.86	2.35, 2.20	1.95, 1.85	4.15, 3.45
4	9.43	5.14	1.93, 1.75	1.67	1.22

A second ring-closing metathesis was successfully accomplished by following the same protocol. Thus, treatment of **5** with the Grubbs catalyst led to two conformers **A** and **B** (with conformer **B** being more polar than **A**), which were separated by column chromatography. Hydrogenation of these products afforded the reduced macrobicyclic pseudopeptides **6A** and **6B** as two conformers, (Schemes 3 and 4). That these were constitutionally the same compounds differing in the conformation of the bridging hexamethylene unit was ascertained by their identical mass spectra.

HPLC analysis of **6A** and **6B** revealed the presence of major peaks that were not common to each. However, heating the solutions of **6A** and **6B** at 120 °C in tetrachloroethane



Scheme 3. Second and third ring-closure metathesis (RCM) products, and their HRMS data $[M+H]^+$.



Scheme 4. a) H_2, Pd-C, MeOH, overnight, 90%; b) TFA, CH_2Cl_2, 5 h, quantitative; c) EDC, HOBt, DIEA, CH_2Cl_2, NHBoc-Hag-OH, 40 h, 90%; d) LiOH 0.2 N, THF, 90%; e) EDC, HOBt, DIEA, CH_2Cl_2, NH_2-Hag-OMe, 40 h, 85%; f) $(PCy_3)_2Cl_2RuCHPh$ (0.2 equiv), CH_2Cl_2, reflux, 5 h, 50%; g) H_2, Pd-C, MeOH, overnight, 90%; h) TFA, CH_2Cl_2, 5 h, quantitative; i) EDC, HOBt, DIEA, CH_2Cl_2, NHBoc-Hag-OH, 40 h, 90%; j) LiOH 0.2 N, THF, 85%; k) EDC, HOBt, DIEA, CH_2Cl_2, NH_2-Hag-OMe, 40 h, 80%; l) $(PCy_3)_2Cl_2RuCHPh$ (0.1 equiv), CH_2Cl_2, reflux, 5 h, 55%; m) H_2, Pd-C, MeOH, overnight, 85%.

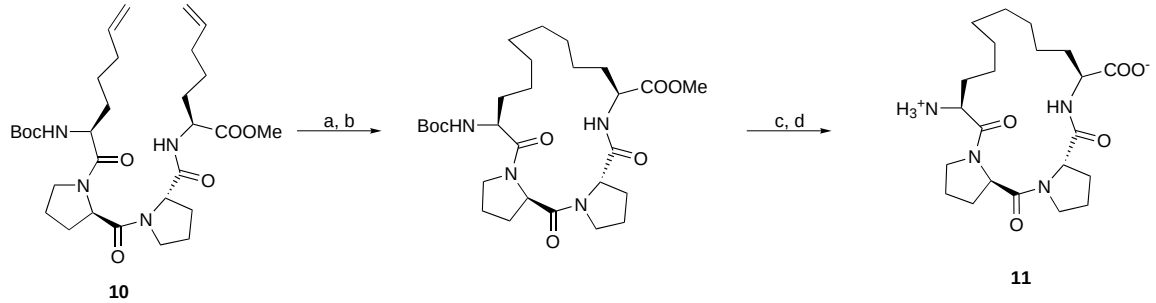
resulted in partial conversion of the major conformers to a common new conformer. The ^{1}H NMR spectra of **6A** and **6B** in [D_5]pyridine and [D_6]DMSO indicated the presence of several conformers, which were also seen with HPLC analysis.[25] Unfortunately, the presence of multiple conformers and amide bond rotamers precluded detailed NMR analysis as in the case of **3** and **4**.

Compounds **6A** and **6B** were converted to the olefinic precursors **7A** and **7B**, respectively, after which a third ring-closing metathesis was done on each one individually (Scheme 4). The mixture of olefinic carbocycles was separated into two new sets of conformers **8A**, **8A′** and **8B**, **8B′** by column chromatography. Final hydrogenation led to four corresponding saturated bridged pseudooctapeptides **9A**, **9A′** and **9B**, **9B′**, which were chromatographically separable into less polar and more polar compounds in each case and had identical mass spectra, (Schemes 3 and 4).[11]

Finally, the versatility of the Grubbs metathesis in the synthesis of macrocyclic carbon-bridged pseudopeptides was demonstrated by the preparation of the crystalline 18-membered analogue **11** starting from the diene **10**, (Scheme 5).

Conclusion

We have described the synthesis and conformational preferences of macrocarbocyclic pseudopeptides that can be elaborated into higher homologues by iterative Grubbs metathesis reactions. The six- and eight-carbon methano-bridges span-



Scheme 5. a) $(PCy_3)_2Cl_2RuCHPh$ (0.1 equiv), CH_2Cl_2, RT, 3.5 h, 85%; b) MeOH, H_2, Pd-C, overnight, 90%; c) LiOH 0.2 N, THF, 2 h, 85%; d) HCOOH, 8 h, quantitative.

0947-6539/02/0801-0114 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 1

ning the peptidic scaffold can adopt highly stable conformations, conferring rigidity on the structures. These can be considered as carbocyclic analogues of β-hairpin and β-sheet model systems.

Experimental Section

General: ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, at room temperature unless mentioned otherwise. ^{1}H and ^{13}C two-dimensional NMR spectra were recorded at 600 MHz (Bruker VMX 600 spectrometer) typically at a peptide concentration of 1 mM. Assignments were made from COSY, TOCSY, NOESY, and ROESY spectra. The NOEs were determined from NOESY and ROESY spectra measured with mixing times of 40, 120, and 250 ms at 303 K. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) with reference to internal solvent. Optical rotations were recorded at 20 °C on a Perkin–Elmer 241 apparatus with a sodium lamp (wavelength of 589 nm). Chromatographic purifications were performed on a column with 230–400 mesh silica gel (Merck 9385) with the indicated solvent system. Dichloromethane was distilled from calcium hydride. Diethyl ether and THF were distilled from sodium metal/benzophenone ketyl. All nonaqueous reactions were performed under an argon atmosphere with oven-dried glassware.

Peptide Synthesis: All the peptides were synthesized by conventional solution-phase methods by using a racemization free fragment condensation strategy. Couplings were mediated by 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC)/1-hydroxybenzotriazole (HOBt). The Boc group was used to protect the N terminus, and the C terminus was protected as a methyl ester. Deprotection of the NBoc group was performed by using 15 equivalents of trifluoroacetic acid (TFA) in CH_2Cl_2, and the methyl ester was removed with 0.2 N LiOH at 0 °C. All intermediates were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy (400 MHz), mass spectroscopy (LRMS and HRMS), TLC, and, if necessary, purified by flash column chromatography on silica gel. Prior to every ring-closing metathesis reaction the dienes were purified by flash column chromatography and fully characterized. Ring-closure metathesis reactions were carried out in dry and degassed CH_2Cl_2 under anhydrous conditions and an argon atmosphere.

General procedure for deprotection of C terminus methyl ester: LiOH solution (6.4 mL 0.2 N, 1.28 mmol, 1.8 equiv) was added dropwise over 10 minutes to a solution of methyl ester (0.71 mmol, 1 equiv) dissolved in THF (10 mL) at 0 °C. The mixture was stirred at 0 °C for 1.5 h. The THF was evaporated in vacuo at 30 °C, and the aqueous solution was acidified to pH 2 with HCl (1 N). The acid was extracted with ethyl acetate, the combined organic layers dried over Na_2SO_4, and the solvent evaporated to afford the product.

General procedure for peptidic coupling: Diisopropylethylamine (DIEA) (1850 µL, 2.5 equiv) and then EDC (5.49 mmol, 1.3 equiv) were added to a solution of acid (4.36 mmol, 1 equiv), amine trifluoroacetate salt (5.49 mmol, 1.3 equiv) and HOBt (5.49 mmol, 1.3 equiv) dissolved in dry CH_2Cl_2 (70 mL) at −15 °C. The mixture was allowed to warm up to room temperature and stirred for 40 h. The organic layer was washed with 5% $KHSO_4$ (aq) (30 mL), saturated $NaHCO_3$ solution (30 mL), and brine (30 mL). The combined organic layers were dried over Na_2SO_4, and the solvent concentrated in vacuo. No purification was done unless otherwise noted.

General procedure for deprotection of N-Boc terminus: TFA (5.2 mL, 15 equiv) was added to a solution of N-Boc derivative (4.50 mmol, 1 equiv) dissolved in CH_2Cl_2 (45 mL), and the solution was stirred at room temperature for 5 h. The solvent was evaporated in vacuo, and the residue was washed several times with diethyl ether. The crude trifluoroacetate salt was then dried under high vacuum for 5 h. The product was used without further purification.

General procedure for ring-closing metathesis: The Grubbs ruthenium catalyst $(PCy_3)_2Cl_2RuCHPh$ (0.073 mmol, 0.1 equiv) was added to a solution of diene (0.73 mmol, 1 equiv) dissolved in dry CH_2Cl_2 (600 mL) under an argon atmosphere at room temperature. Within 15 minutes, the color changed from purple to orange-brown. The solution was stirred for 5 h after which TLC showed full disappearance of starting material. The solution was concentrated to afford a black foam. Usually two purifications by flash column chromatography afforded the cyclic olefin as a white foam. For NMR spectra see Supporting Information.

General procedure for hydrogenation: 10% Pd-C (0.123 mmol, 0.1 equiv) was added to a solution of alkene (1.23 mmol, 1 equiv) dissolved in MeOH (15 mL). The mixture was purged with hydrogen and stirred overnight under 1 atm of hydrogen. The solution was then filtered through a pad of Celite and concentrated to afford the hydrogenated product as a white foam.

2-{{1-[1-(2-*tert*-Butoxycarbonylamino-hex-5-enoyl)pyrrolidine-2-carbonyl]pyrrolidine-2-carbonyl}amino}hex-5-enoic acid methyl ester (1): By using the general procedure for peptidic coupling, peptide **1** was obtained as a colorless oil. Yield: 2.15 g, 90%; $R_f = 0.30$ (ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers): $\delta = 1.46$ (s, 9H), 1.55–2.45 (m, 16H), 3.40–4.20(m, 4H), 3.70, 3.75 (s, 3H), 4.40–4.75 (m, 4H), 4.95–5.15 (m, 4H), 5.83 (m, 2H), 5.95, (d, $J = 8$ Hz, 1H), 7.30, 7.55 (d, $J = 8$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): $\delta = 23.7, 23.9, 25.1, 25.3, 28.1, 29.0, 29.4, 29.7, 30.3, 30.5, 31.8, 32.5, 46.9, 47.1, 51.1, 51.4, 52.0, 58.0, 58.2, 60.1, 60.4, 79.1, 79.3, 114.8, 115.1, 115.4, 115.8, 136.8, 137.0, 137.25, 137.59, 154.8, 155.4, 170.3, 170.5, 170.8, 170.9, 171.2, 172.8$; FAB-HRMS m/z: calcd for $C_{28}H_{45}N_4O_7$ $[M+H]^+$: 549.3288; found: 549.3308.

(*E*)- and (*Z*)-9-*tert*-Butoxycarbonylamino-2,8,18-trioxo-1,7,17-triaza-tricyclo[17.3.0.0^{3,7}]docos-12-ene-16-carboxylic acid methyl esters (2 and 3): By using the general procedure for ring-closing metathesis, the cyclic products **2** and **3** were obtained as an inseparable mixture of *E*/*Z* isomers in 92% yield (1.87 g) as a white foam after two flash chromatography columns. The ratio of olefin isomers was estimated by ^{1}H NMR integration in [D$_5$]pyridine (ca. 55:45 *E*/*Z*). Slow evaporation from a mixture hexanes/chloroform (1:1) yielded X-ray-quality crystals of the *Z* isomer **3**. Crystals contained one molecule of water and another of chloroform. Mp: 192–195 °C; $R_f = 0.30$ (ethyl acetate/methanol 95:5); ^{1}H NMR (600 MHz, [D$_5$]pyridine 1 mM solution concentration, (*Z*)-**3**, one single conformation): $\delta = 1.47$ (s, 9H), 1.49–2.54 (m, 15H), 2.88 (q, $J = 8.5$ Hz, 1H), 3.47 (q, $J = 8$ Hz, 1H), 3.61 (s, 3H), 3.69 (q, $J = 7$ Hz, 1H), 3.91 (dt, $J = 2.4, 7$ Hz, 1H), 4.31 (m, 1H), 4.70 (m, 1H), 4.86 (d, $J = 8.4$ Hz, 1H), 5.03 (t, $J = 8.7$ Hz, 1H), 5.28 (m, 1H), 5.50 (m, 1H), 5.84 (d, $J = 7.5$ Hz, 1H), 8.52 (d, $J = 8.4$ Hz, 1H), 9.66 (d, $J = 8.7$ Hz, 1H); ^{13}C NMR (125 MHz, [D$_5$]pyridine 1 mM solution concentration, (*Z*)-**3**, one single conformation): $\delta = 23.0, 23.9, 24.4, 25.4, 28.7, 29.4, 31.0, 31.9, 33.7, 47.4, 50.8, 52.1, 53.0, 59.3, 60.8, 78.7, 79.9, 128.8, 132.7, 156.8, 172.3, 173.5, 174.1, 175.5$; FAB-HRMS m/z: calcd for $C_{26}H_{41}N_4O_7$ $[M+H]^+$: 521.2975; found: 521.2962.

Crystal structure data for 3: $C_{27}H_{43}N_4O_8 \cdot H_2O \cdot CHCl_3$, $M_r = 658.004$. Crystals were grown by slow evaporation of a solution of **3** in a mixture hexanes/chloroform (1:1). Crystal size: $0.61 \times 0.40 \times 0.30$ mm, orthorhombic, space group $P2_12_12_1$, $a = 13.3813(1)$, $b = 13.5623(1)$, $c = 18.8657(2)$ Å, $V = 3423.77(5)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.2765$ mg m^{-3}, $\mu = 2.839$ mm^{-1}, $4.01 << \theta < 72.93°$; of 41617 reflections collected, 6715 were unique; Bruker AXS, SMART 2 K diffractometer (293 K, $\lambda(Cu_{K\alpha}) = 1.54178$ Å). The structure was solved by direct methods (SHELXS97; G.M. Sheldrick, *Program for the Solution of Crystal Structures*, Universität Göttingen, **1997**) and refined by full matrix least-squares analysis (G. M. Sheldrick, *Program for the Refinement of Crystal Structures*, Universität Göttingen, **1996**). $R(F) = 0.0339$, $wR(F^2) = 0.2503$. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no.CCDC-165298. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

9-*tert*-Butoxycarbonylamino-2,8,18-trioxo-1,7,17-triaza-tricyclo[17.3.0.0^{3,7}]-docosane-16-carboxylic acid methyl ester (4): By using the general procedure for hydrogenation, the saturated peptide **4** was obtained as a white foam. Yield: 1.62 g, 90%; $R_f = 0.30$ (ethyl acetate); ^{1}H NMR (600 MHz, [D$_5$]pyridine 1 mM solution concentration, major conformer): $\delta = 1.52$ (s, 9H), 1.21–2.50 (m, 20H), 3.44 (q, $J = 7.5$ Hz, 1H), 3.65 (s, 3H), 3.71 (m, 1H), 3.90 (m, 1H), 4.14 (m, 1H), 4.61 (m, 1H), 4.87 (d, $J = 7.5$ Hz, 1H), 5.14 (m, 1H), 5.73 (d, $J = 8.6$ Hz, 1H), 8.44 (d, $J = 8.3$ Hz, 1H), 9.43 (d, $J = 9$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): $\delta = 21.7, 22.3, 23.1, 25.5, 26.0, 26.7, 28.6, 29.0, 31.3, 32.7, 47.0, 47.6, 51.8, 52.5, 52.6, 52.7,$

59.2, 61.2, 79.8, 155.6, 171.6, 171.7, 172.0, 173.4; FAB-HRMS *m/z*: calcd for $C_{26}H_{43}N_4O_7$ $[M+H]^+$: 523.3131; found: 523.3151.

2-{[9-(2-*tert*-Butoxycarbonylaminohex-5-enoylamino)-2,8,18-trioxo-1,7,17-triazatricyclo[17.3.0.0^{3,7}]docosane-16-carbonyl]amino}hex-5-enoic acid methyl ester (5): By using the general peptidic-coupling procedure, peptide **5** was obtained as a white foam. Yield: 1.54 g, 85%; $R_f = 0.35$ (ethyl acetate/methanol 95:5); ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers): $\delta =$ 1.45(s, 9H), 1.30–2.50 (m, 28H), 3.70, 3.74 (s, 3H), 3.40–4.80 (m, 10H), 4.90–5.10 (m, 4H), 5.77 (m, 2H), 6.54, 6.75, 6.90, 6.95, 7.1, 7.90 (d, $J = 8$ Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers): $\delta = 22.6$, 23.5, 24.8, 25.6, 26.1, 26.7, 27.3, 28.0, 28.2, 28.7, 29.0, 29.1, 29.2, 29.4, 30.1, 30.6, 31.0, 32.0, 46.7, 46.9, 47.1, 50.3, 51.3, 52.0, 53.8, 57.7, 58.6, 60.1, 60.3, 60.6, 79.4, 115.3, 115.5, 136.4, 136.5, 136.6, 136.9, 155.0, 155.2, 155.5, 169.1, 169.9, 170.3, 170.7, 170.9, 171.1, 171.3, 171.6, 171.9, 172.4; FAB-HRMS *m/z*: calcd for $C_{38}H_{61}N_6O_9$ $[M+H]^+$: 745.4500; found: 745.4515.

***E* and *Z* Cyclic peptides from the second ring closure metathesis**: The general procedure for ring-closing metathesis was modified, and the reaction was carried out under reflux in CH_2Cl_2 for 5 h with 0.2 equivalents of Grubbs catalyst. Starting from peptide **5**, the cyclic peptides **A** and **B** were separated by flash column chromatography (ethyl acetate/methanol 9:1). Each compound was an inseparable mixture of *E*/*Z* isomers. Overall yield: 50%, peptide **A**: 170 mg, peptide **B**: 500 mg; (A/B = 1:3); R_f (**A**) = 0.40 (ethyl acetate/methanol 9:1), R_f (**B**) = 0.30 (ethyl acetate/methanol 9:1). For **A**: FAB-HRMS *m/z*: calcd for $C_{36}H_{57}N_6O_9$ $[M+H]^+$: 717.4187; found: 717.4170. For **B** FAB-HRMS *m/z*: calcd for $C_{36}H_{57}N_6O_9$ $[M+H]^+$: 717.4187; found: 717.4170. ^{1}H NMR spectra are included in the Supporting Information.

Cyclic peptides 6A and 6B: By using the general hydrogenation procedure, the saturated peptides **6A** and **6B** were obtained as white foams starting, respectively, from peptides **A** and **B**. Overall yield: 90%, **6A**: 150 mg, **6B**: 450 mg; R_f (**6A**) = 0.40 (ethyl acetate/methanol 9:1), R_f (**6B**) = 0.30 (ethyl acetate/methanol 9:1); for **6A**: FAB-HRMS *m/z* calcd for $C_{36}H_{59}N_6O_9$ $[M+H]^+$: 719.4343; found: 719.4329, for **6B**: calcd: 719.4343; found: 719.4322. ^{1}H NMR spectra are included in the Supporting Information.

Peptides 7A and 7B: By using the general procedure for peptidic coupling, the peptides **7A** and **7B** were obtained as white foams starting, respectively, from **6A** and **6B**. Overall yield: 80%, **7A**: 70 mg, **7B**: 240 mg; R_f (**7A**) = 0.35 (ethyl acetate/methanol 9:1), R_f (**7B**) = 0.30 (ethyl acetate/methanol 85:15); for **7A** FAB-HRMS *m/z*: calcd for $C_{48}H_{77}N_8O_{11}$ $[M+H]^+$: 941.5711; found: 941.5749, for **7B**: calcd: 941.5711; found: 941.5681. ^{1}H NMR spectra are included in the Supporting Information.

***E* and *Z* Cyclic Peptides from the third ring-closure metathesis of 7A**: By using the general procedure for ring-closing metathesis under reflux, the diene **7A** afforded two cyclic products, **8A** and **8A′**, which were separated by flash column chromatography (ethyl acetate/methanol 9:1). Each compound was an inseparable mixture of *E*/*Z* isomers. Overall yield: 54%, peptide **8A**: 23 mg, peptide **8A′**: 14 mg (**8A**/**8A′** 1.6:1); R_f (**8A**) = 0.35 (ethyl acetate/methanol 9:1), R_f (**8A′**) = 0.30 (ethyl acetate/methanol 9:1); for **8A** FAB-HRMS *m/z*: calcd for $C_{46}H_{73}N_8O_{11}$ $[M+H]^+$: 913.5398; found: 913.5421; for **8A′**: calcd: 913.5398; found: 913.5421. ^{1}H NMR spectra are included in the Supporting Information.

***E* and *Z* Cyclic peptides from the ring closure metathesis of 7B**: By using the general procedure for the ring-closing metathesis reaction under reflux, the diene **7B** afforded two cyclic peptides **8B** and **8B′**, which were separated by flash column chromatography (ethyl acetate/methanol 85:15). Each compound was an inseparable mixture of *E*/*Z* isomers. Overall yield 55%, peptide **8B**: 70 mg, peptide **8B′**: 41 mg. (**8B**/**8B′** 1.7:1); R_f (**8B**) = 0.35 (ethyl acetate/methanol 85:15), R_f (**8B′**) = 0.30 (ethyl acetate/methanol 85:15); for **8B** FAB-HRMS *m/z*: calcd for $C_{46}H_{73}N_8O_{11}$ $[M+H]^+$: 913.5398; found: 913.5374; for **8B′**: calcd: 913.5398; found: 913.5374. ^{1}H NMR spectra are included in the Supporting Information.

Cyclic peptides 9A and 9A′: By using the general hydrogenation procedure, the saturated peptides **9A** and **9A′** were obtained in 85% yield as white foams starting from **8A** and **8A′**, respectively. Peptide **9A**: yield: 20 mg; $R_f = 0.35$ (ethyl acetate/methanol 9:1); FAB-HRMS *m/z*: calcd for $C_{46}H_{75}N_8O_{11}$ $[M+H]^+$: 915.5555; found: 915.5541. Peptide **9A′**: yield: 12 mg; $R_f = 0.30$ (ethyl acetate/methanol 9:1); FAB-HRMS *m/z*: calcd for $C_{46}H_{75}N_8O_{11}$ $[M+H]^+$: 915.5555; found: 915.5541. ^{1}H NMR spectra are included in the Supporting Information.

Cyclic peptides 9B and 9B′: By using the general procedure for hydrogenation, the saturated peptides **9B** and **9B′** were obtained in 85% yield as white foams starting from **8B** and **8B′**, respectively. Peptide **9B**: yield: 60 mg; $R_f = 0.35$ (ethyl acetate/methanol 85:15); FAB-HRMS *m/z*: calcd for $C_{46}H_{75}N_8O_{11}$ $[M+H]^+$: 915.5555; found: 915.5541; elemental analysis calcd for $C_{46}H_{74}N_8O_{11} \cdot 5.5\,H_2O$: C 54.49, H 8.39, N 11.05; found: C 54.91, H 8.26, N 10.64. Peptide **9B′**: yield: 34 mg; $R_f = 0.30$ (ethyl acetate/methanol 85:15); FAB-HRMS *m/z*: calcd for $C_{46}H_{75}N_8O_{11}$ $[M+H]^+$: 915.5555; found: 915.5541; elemental analysis calcd for $C_{46}H_{74}N_8O_{11} \cdot 4\,H_2O$: C 55.98, H 8.31, N 11.35; found: C 56.04, H 8.04, N 10.78. ^{1}H NMR spectra are included in the Supporting Information.

Acknowledgement

We thank the NSERC of Canada for financial support through the Medicinal Chemistry Chair program. We also thank Pharmacia & Upjohn for a sabbatical leave for Mauro Angiolini and generous financial support. We acknowledge the assistance of Michel Simard and Eric Therrien in X-ray structure determinations and computer graphics.

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Received: September 7, 2001 [F3538]