Conformational Control by Thiazole and Oxazoline Rings in Cyclic Octapeptides of Marine Origin. Novel Macrocyclic Chair and Boat Conformations

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Abstract: A comparison of a closely related set of cyclic octapeptides demonstrates how Nature has adapted two common amino acid building blocks (Thr, Cys) as conformational ring constraints (oxazoline, thiazole) to regulate the three-dimensional structures and reactivities of marine macrocycles. A 2D NMR spectroscopic study shows that conversion of two Cys residues in the flexible cyclic octapeptide **1**, c[Ile-Thr-D-Val-Cys-Ile-Thr-D-Val-Cys-], to 5-membered thiazole rings (Thz) leads to the formation of a novel pseudochair conformation in **2,** c[Ile-Thr-D-(Val)-Thz-Ile-Thr-D-(Val)Thz-]. The conformational flexibility of **2** is significantly restricted by three intramolecular hydrogen bonds induced by the D-(Val)Thz components, resulting in a single solution conformation with non-C_2 symmetric side chains. Additional modification, through conversion of the two threonine side chains to 5-membered oxazoline rings (Oxn), produces a highly constrained pseudoboat or saddle-shaped macrocycle, c[(Ile)Oxn-D-(Val)-Thz-(Ile)Oxn-D-(Val)Thz-] (**7**), having C_2 symmetric side chains. Acid hydrolysis of **7**, previously isolated from the ascidian *Lissoclinum patella*, selectively opens the two oxazoline rings with further conformational rearrangement to a novel cyclic octapeptide (**8**) possessing a shallower pseudoboat conformation. The comparison reveals that oxazoline and thiazole rings impose severe conformational restrictions upon these cyclic octapeptides, creating unusual shapes and clefts with varying capacities to capture organic or metal ion guests (e.g. **10**). Such dramatic changes in macrocycle shape may be related to the differential antitumour and metal-binding properties of this class of molecule.

Introduction

Novel cyclic peptides containing unusual amino acids are frequently isolated from bacteria, fungi, plants, and marine organisms and many are potent inhibitors of cellular functions.[1−5] While the macrocycle itself restricts conformational freedom in peptides, this becomes less restrictive as the cycle increases in size, and additional constraints become necessary to control shape. Nature provides these constraints in the form of unnatural amino acids by nonribosomal syntheses involving activation as thiol esters using multienzyme complexes. Precisely how these components influence macrocyclic shape and reactivity is still unknown, despite the promise that many of these compounds show as drug leads or probes of biological processes.[1−5] Here we compare the conformations of a set of cyclic octapeptides related to ascidiacyclamide[5] from the ascidian *Lissoclinum patella* found on the Great Barrier Reef. They demonstrate how Nature has utilized two common amino acid building blocks (Cys, Thr) as conformational ring constraints (thiazole, Thz; oxazoline, Oxn) to control the three-dimensional structures and reactivities of the macrocycles.

Results and Discussion

Molecular modeling studies indicate that the cyclic octapeptide **1**, c[Ile-Thr-D-Val-Cys-Ile-Thr-D-Val-Cys-] is very flexible, adopting many low-energy structures.[6] To gauge the conformational effect of transforming each Cys in **1** to the planar 5-membered thiazole (Thz) ring, the derivative **2**, c[Ile-Thr-D-(Val)Thz-Ile-Thr-D-(Val)Thz-], was synthesized (Scheme 1).

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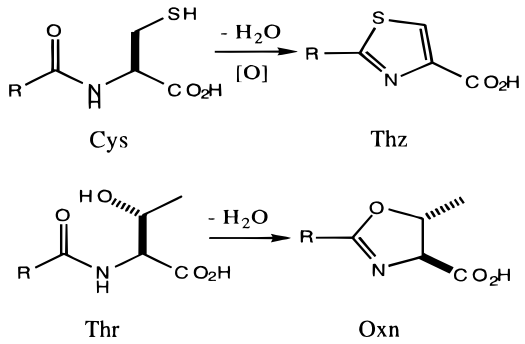
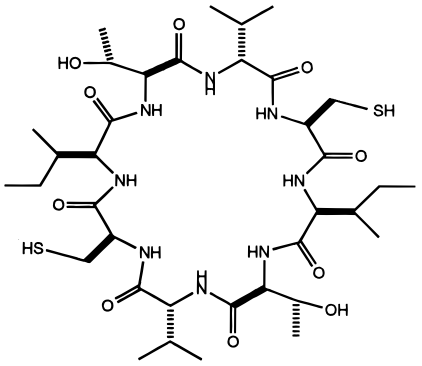


The N-protected D-Val-OH (**3**) was converted in a two-step process[7] to the aldehyde (**4**), which was condensed with cysteine



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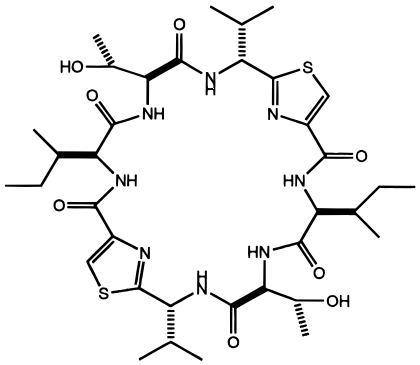

2

methyl ester, followed by oxidation with MnO_2 and deesterification, giving N-protected D-(Val)Thz-OOH (**5**).[8] Components of **2** were assembled by solid phase synthesis on Boc-Ile-PAM resin. Boc-D-(Val)Thz-OH, Boc-Thr(Bzl)-OH, Boc-Ile-OH, Boc-D-(Val)Thz-OH, and Boc-Thr(Bzl)-OH were sequentially coupled to the resin using HBTU in DIPEA/DMF. Cleavage from the resin (HF/*p*-cresol) gave the acyclic peptide H_2N-Thr-D-(Val)Thz-Ile-Thr-D-(Val)Thz-Ile-OH (**6**, yield 52%), which was cyclized (10^{-4} M in DMF) with HATU (2 equiv) and DIPEA (3 equiv) at 20 °C to produce **2** (39%).

The 1D ^{1}H-NMR spectrum for **2** in acetone-d_6 (4−37 °C) or $CDCl_3$ (24 °C) has six separate α-CH proton resonances (Table 1), consistent with one asymmetric conformation for **2** under these conditions. By contrast, the C_2-symmetrical cyclic peptide **7** shows just three α-CH proton resonances under the same conditions.[5a] The asymmetry of **2** is shown by unique proton

(7) Fehrenz, J.; Castro, B. *Synthesis* **1983**, 676−8.

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Scheme 1. Synthesis of Cyclic Octapeptide **2**

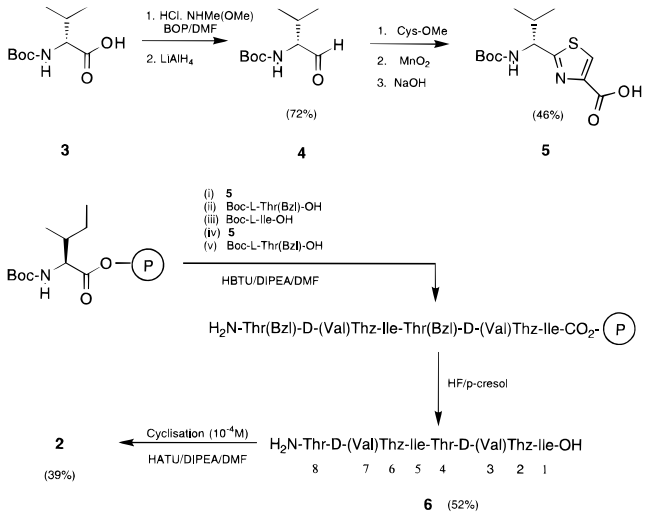


Table 1. ^{1}H-NMR Resonance Assignments[a] for **2** in d_6-Acetone

residue	HN	Hα	Hβ	Hγ
Thr1	7.92	4.40	4.25	1.19
D-Val2	8.11	5.14	2.24	0.93
Ile4	8.21	4.61	2.14	1.11 (Me)[b]
Thr5	8.07	4.29	4.45	1.19
D-Val6	7.95	5.25	2.30	1.01
Ile8	8.26	4.40	1.83	0.49 (Me)[c]

[a] Referenced to residual acetone (2.05 ppm). The chemical shift for the thiazole proton of each ring not specifically assigned (8.04, 8.15 ppm). [b] 1.32, 1.61 (CH_2), 0.93 (Hδ). [c] 0.87, 1.38 (CH_2), 0.64 (Hδ).

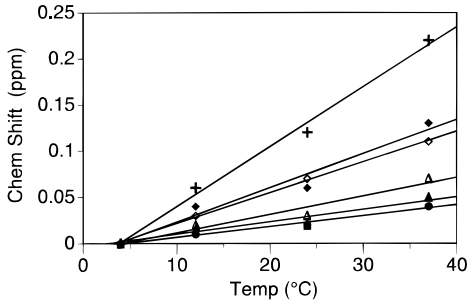


Figure 1. Temperature dependence of chemical shift ($\Delta\delta$) for amide NH protons of **2** in acetone-d_6 at 4, 12, 24, and 37 °C (Thr1 (◆), D-Val2 (◇), Ile4 (Δ), Thr5 (+), D-Val6 (●), Ile8 (▲)).

resonances for each amino acid component of the cycle (Table 1). To understand the origin of this asymmetry, we examined the molecule for intramolecular hydrogen bonds by monitoring the temperature dependence of chemical shifts for the amide NH protons (Figure 1). The chemical shifts for three amide NH protons (D-Val6, Ile4, Ile8) in **2** were independent of temperature ($\Delta\delta/T$ 1 ppb, 4−37 °C), whereas the others (Thr1, Thr5, D-Val4) shifted significantly upfield ($\Delta\delta/T$ 3−7 ppb) with increasing temperature. Furthermore, addition of CD_3OD exchanged both Thr-NH protons within minutes, while the D-Val and Ile-NH proton resonances remained after 2 h. Together these results are consistent with participation of two Ile and at least one D-Val amide proton in intramolecular hydrogen bonds.

A series of 2D ^{1}H-NMR spectra were measured for **2** at 24 °C in acetone-d_6 to determine the three-dimensional structure. TOCSY and DFCOSY experiments were used to identify residue types while sequential assignments were made from analysis of nuclear Overhauser effect spectroscopy (NOESY). A series of 50 structures which satisfied the distance constraints were subjected to restrained molecular dynamics (200−500 K) and energy minimized. Nine calculated structures with root

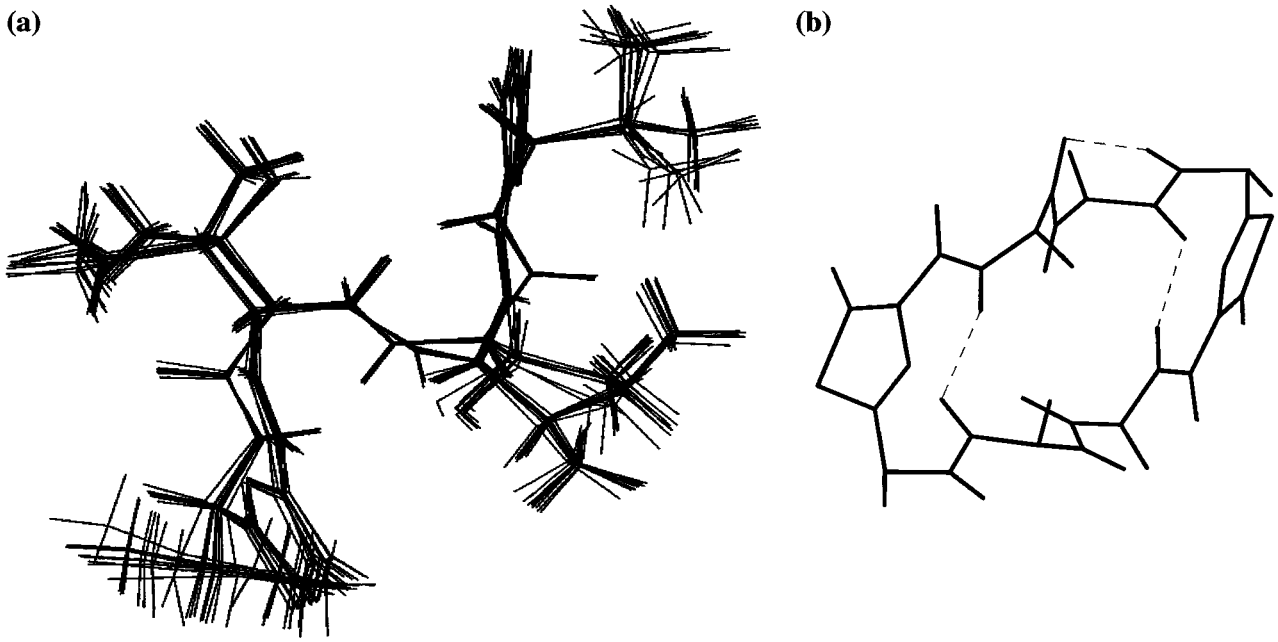


Figure 2. (a) Nine lowest energy minimized NMR structures of **2** in acetone-d_6 (24 °C). (b) Top view for one of the structures in (a) showing hydrogen-bonding.

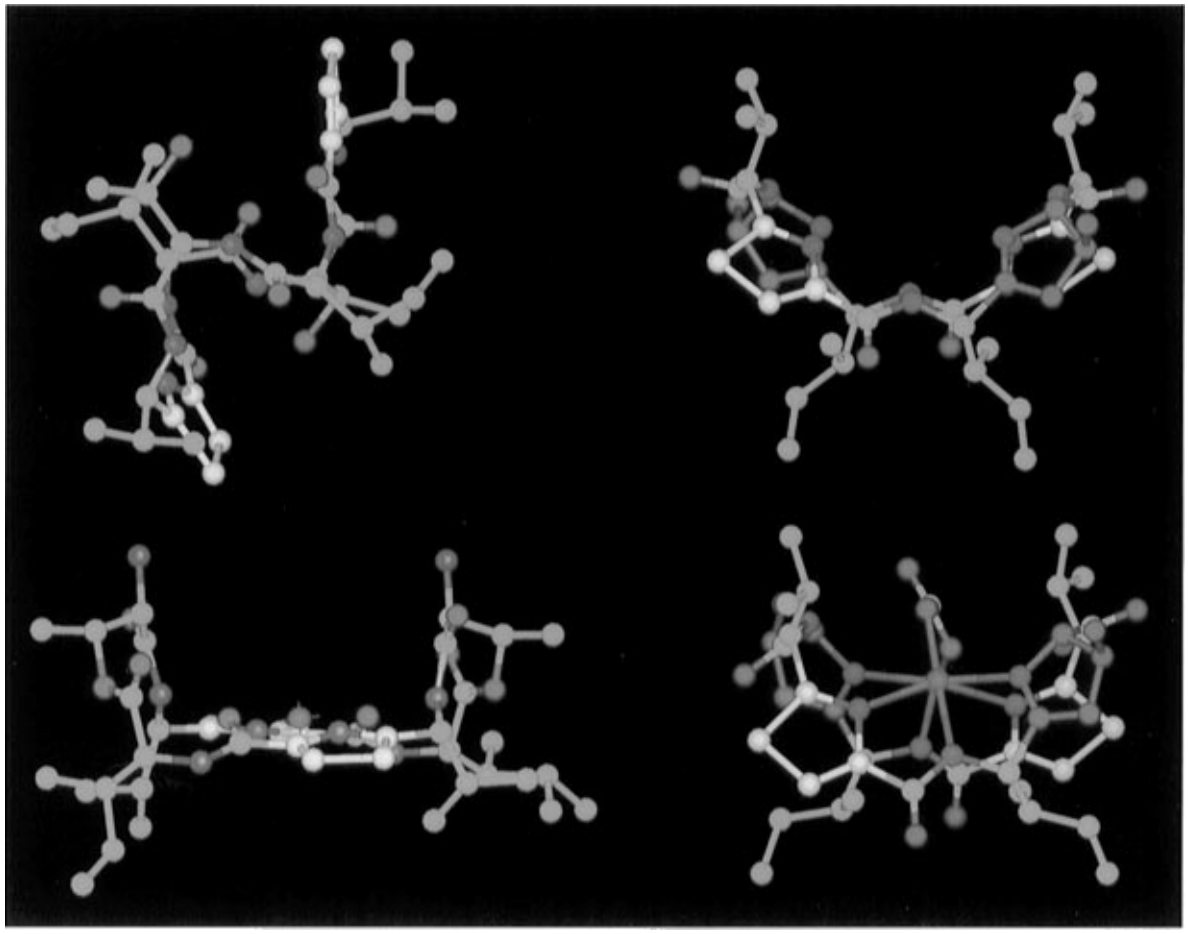


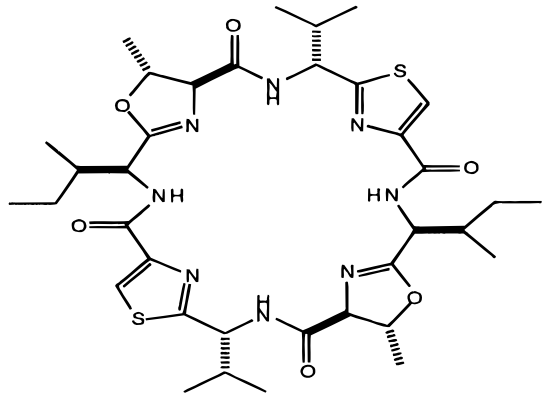
Figure 3. Structures of **2**, **7**, **8**, and **10** showing thiazole rings (yellow), oxazoline rings (red), Ile and D-Val side chains (green), and all nitrogens (blue) and oxygens (red). (a, top left) the pseudochair conformation of **2** (NMR structure), (b, top right) the pseudoboat conformation of **7** (X-ray structure);[5] (c, bottom left) the shallow pseudoboat conformation of **8** and the K^+ ion in purple (X-ray structure),[9] (d, bottom right) the pseudoboat conformation of **10** with two superimposed Cu^{2+} ions (purple) bridged by carbonate (X-ray structure).[10a]

mean square deviation (rmsd) < 0.2 Å are superimposed in Figure 2a; the hydrogen bonds are shown in Figure 2b.

The NMR solution structure for **2** shows a single pseudochair conformation (Figure 3a), with Thz rings at apical positions, a D-Val, Ile, and Thr in pseudoaxial positions, and the other residues in pseudoequatorial positions. There are two transannular hydrogen bonds (both 3.3 Å, N−H···O angles ∼ 160°) between the Ile-NH and Thr-CO groups creating two β-turns, while a third intramolecular hydrogen bond (2.9 Å, N−H···O angle ∼ 160°) links a D-Val-NH with a Ile-CO in a γ-turn that

renders the molecule asymmetric. The two Ile residues show very different γ-Me resonances (δ 1.11, 0.49), arising from positioning of one Ile side chain over a Thz ring. The planarity of the thiazole rings and adjacent (planar) amide bonds encourages formation of the hydrogen bonds which in turn fix the conformation.

Further introduction of two additional pseudoplanar ring constraints by replacing both threonines of **2** with oxazoline rings causes another pronounced conformational change, from the chair (Figure 3a) to the boat conformation for **7** (Figure 3b), as evidenced by both NMR and X-ray crystal structures.[5] The planar thiazole and oxazoline rings in **7** lie at the four corners of the macrocycle rather than at apical positions as in **2**. Nonbonding distances that particularly highlight differences between these structures (Table 2) are between the thiazole nitrogens and between the two Val-Cα atoms, each ~2.2 Å further apart in **2** than in the more compact **7**. Also the Ile-Cα atoms are ~4.5 Å closer in the more rectangular **2** than in **7**.



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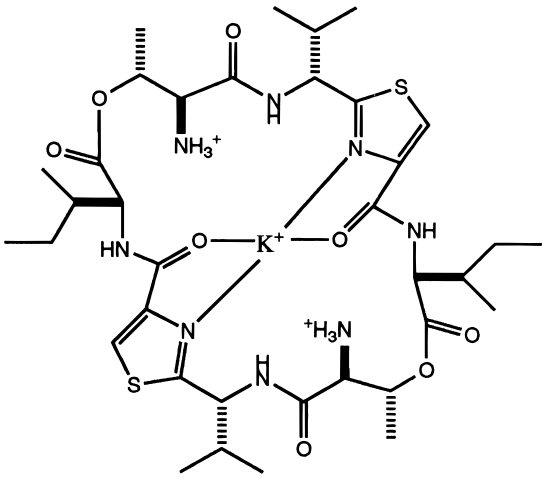

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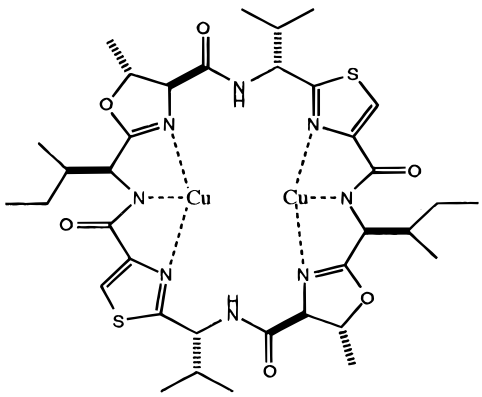

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Table 2. Comparison of Selected Nonbonding Distances for Structures of **2**, **7**, **8**, and **10**

	2[a]	**7**[b]	**8**[c]	**10**[d]
N(Thz)-N(Thz)	7.21	4.92	5.76	6.04
N(Oxn)-N(Oxn)	5.35	7.55[e]	6.42	6.37
Cα(D-Val)-Cα(D-Val)	10.30	8.10	8.51	7.73
Cα(Ile)-Cα(D-Ile)	5.12	9.67	6.32	7.72

[a] This work; **2**·H_2O in acetone-d_6. [b] X-ray structure of **7**·C_6H_6[5]. [c] X-ray structure.[9] [d] X-ray structure of **10** [Cu_2(ascid)(η^2-CO_3^{2-})].[10a] [e] Amine nitrogens.

We have shown[9] that hydrolysis of **7** in acidic methanol opens both Oxn rings, with formation of an ester rather than an amide, producing **8**. An X-ray crystal structure of the potassium salt of **8** shows another boat conformation (Figure 3c). The different conformations for **2** and **8**, which both contain thiazoles but no oxazolines, are attributed to greater flexibility in **8** arising from two less amide bonds. This permits the macrocycle to be folded into a new cleft by a potassium ion, which binds to the two thiazole rings, and a perchlorate anion (not shown, trapped between the amine nitrogens).[9] Cycle **8** exhibits very different affinities for and modes of binding to biologicaly relevant metal ions.[9]

Nature utilizes a number of devices to restrict conformational freedom in peptides, including disulfide bonds, hydrogen bonds, salt bridges, and, as shown here, cyclization, molecular constraints, and metal ions.[1] This structural control may be responsible for the differential biological and metal-binding properties of cyclic peptides like **7**. For example, analogues of **7** are cytotoxic with antitumour properties[2,5] that are reported to be critically dependent upon the presence of oxazoline rings[4f] which are shown above to induce a boat-shaped cleft in **7**. This cleft is known to bind small molecules (e.g. benzene, ethanol, water)[5] and could conceivably bind larger receptors. Similarly we find[10a] that **7** binds to two Cu^{2+} ions (e.g. **9**) which trap CO_3^{2-} between them as the symmetrical **10** [Cu_2(ascid)(η^2-CO_3^{2-})] (Figure 3d). Incorporation of the two copper ions and the carbonate bridge separates the Thz nitrogens by a further ~1 Å and brings the two Ile-Cα closer together by ~2 Å (Table 2). This binding to copper and carbonate is dependent upon the presence of the cleft in **7** and may be important in biological transport of metal ions and mobilization or activation of small molecules (e.g. CO_2 fixation).[10a]

Finally we note the ease of formation and hydrolysis of the critical oxazoline rings, raising an interesting possibility of these macrocycles switching conformations in their natural environment. In view of their promising protein-binding[1−4] and metal-binding properties,[9−11] further systematic structural studies on this class of macrocyclic peptides containing unusual amino

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acids or other conformational constraints seem warranted and should provide important structure−reactivity information.

Experimental Section

Abbreviations: TFA = trifluoroacetic acid; DIPEA = diisopropylethylamine; HBTU = *O*-benzotriazolyl-*N*′,*N*′,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HATU = *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMF = dimethylformamide; PAM = phenylacetamidomethyl; SV = substitution value of the resin; TOCSY = total correlation spectroscopy; DFCOSY = double quantum correlation spectroscopy.

General Methods. Materials obtained commercially were reagent grade unless otherwise stated. ^{1}H NMR spectra were recorded on either Bruker ARX 500 M Hz or Varian Unity 400 spectrometers. Proton assignments were determined by 2D NMR experiments (DFCOSY, TOCSY, NOESY). Preparative scale reverse phase HPLC separations were performed on Waters Delta-Pak PrepPak C_{18} 40 mm × 100 mm cartridges (100 Å) and analytical reverse phase HPLC on Waters Delta-Pak Radial-Pak C_{18} 8 mm × 100 mm cartridges (100 Å) using gradient mixtures of water/0.1% TFA and 10% water/90% acetonitrile/0.1% TFA.

Mass spectra were obtained on a triple quadrupole mass spectrometer (PE SCIEX API III) equipped with an Ionspray (pneumatically assisted electrospray) atmospheric pressure ionization source (ISMS). Solutions of compounds in 9:1 acetonitrile/0.1% aqueous trifluoroacetic acid were injected by syringe infusion pump at micro- to picomolar concentrations and flow rates of 20−50 μL/min into the spectrometer. Molecular ions, $\{[M + nH]^{n+}\}/n$, were generated by ion evaporation and focused into the analyzer of the spectrometer through a 100 mm sampling orifice. Full scan data were acquired by scanning quadrupole from *m*/*z* 100 to 900 with a scan step of 0.1 Da and a dwell time of 2 ms.

Synthesis. The synthesis of compounds **7**,[5] **8**,[9] and **10**[10a] have been reported previously.

H_2N-Thr-D-(Val)thiazole-Ile-Thr-D-(Val)Thiazole-Ile-OH (6). Boc-Ile-PAM resin (0.33 g, 0.25 mmol, SV = 0.76 mequiv/g) was deprotected with TFA (5 mL, 2 × 1 min), washed with DMF, and reacted with a solution of Boc-D-(Val)Thiazole-OH (0.30 g, 1 mmol, 2 equiv), HBTU (0.5 M in DMF, 2 mL, 1 mmol), and DIPEA (0.35 mL, 2 mmol). The resin was shaken with this solution for 10 min and monitored by the negative ninhydrin test (>99.6% coupling). The resin was washed with DMF and TFA (2 × 10 mL, 1 min each). The synthesis was completed by coupling Boc-Thr(OBz)-OH (4 equiv), Boc-Ile-OH (4 equiv), Boc-D-(Val)Thz-OH (2 equiv), and finally Boc-Thr-(OBz) (4 equiv) using the same procedure. The peptide was cleaved from the resin (0.45 g, 0.25 mmol) with HF, lyophilized, treated with a 0.1 M ammonium carbonate solution at room temperature for 15 min, and relyophilized. The crude powder was purified by HPLC [gradient, water/0.1% TFA to 50% (90% acetonitrile/10% water/0.1% TFA)/50% (water/0.1% TFA) over 60 min] to give **6** as the trifluoroacetate salt (0.10 g, 52% overall yield from Boc-Ile-resin). HPLC analysis: [gradient, water/0.1% TFA to 50% (90% acetonitrile/10% water/0.1% TFA)/50% (water/0.1% TFA) over 30 min], one peak, retention time = 26.2 min. ^{1}H NMR (H_2O/D_2O, 8:2, 293 K): δ 9.07 (d, 1H, J = 10 Hz, Val7-NH), 8.69 (d, 1H, J = 10 Hz,Val3-NH), 8.26 (m, 2H, Ile-NH, Thr4-NH), 8.19 (d, 1H, J = 5 Hz, Ile-NH) 7.99 (s, 1H, Thz-H), 7.96 (s, 1H, Thz-H), 4.90 (m, 1H, Val7-αH), 4.82 (m, 1H, Val3-αH), 4.29 (m, 2H, Ile1-αH, Thr4-αH), 4.22 (m, 1H, Ile5-αH), 4.08 (m, 1H, Thr8-βH), 4.04 (m, 1H, Thr4-βH), 3.79 (d, 1H, J = 5.7 Hz, Thr8-αH), 2.18 (m, 2H, Val3-βH, Val7-βH), 1.9 (m, 1H, Ile1-βH), 1.73 (m, 1H, Ile5-βH), 1.38 (m, 1H, Ile1-γH), 1.28 (m, 1H, Ile5-γH), 1.21 (d, 3H, J = 5 Hz, Thr8-γCH_3), 1.14 (m, 1H, Ile1-γH), 1.07 (d, 3H, J = 5 Hz, Thr4-γCH_3), 0.93 (m, 1H, Ile5-γH), 0.83 (m, 9H, Ile1-γCH_3,-Val3-γCH_3,Val7-γCH_3), 0.75 (m, 9H, Ile1-δCH_3,Val3-γCH_3,Val7-γCH_3), 0.62 (m, 6H, Ile5-γCH_3, Ile5-δCH_3). ISMS: *m*/*z* 811 $[M + H]^+$ (100).

Cyclo-[Thr-D-(Val)Thz-Ile-Thr-D-(Val)Thz-Ile-] (2). The linear peptide **6** (40 mg, 49 μmol) was dissolved in DMF (500 mL, $C = 1 \times 10^{-4}$ M); HATU reagent[12] (38 mg, 98 μmol) and DIPEA (25 μL, 0.15 mmol) were added; and the solution was stirred at room temperature for 1 h. The reaction mixture was neutralized with 1 M HCl and evaporated *in vacuo*, and the residue was redissolved in a water/acetonitrile (1:1, 20 mL) and purified by reverse phase HPLC (gradient, water/0.1% TFA to 100% (10% water/90% acetonitrile/0.1% TFA) over 60 min) to give a pure sample of **2** (15 mg, 39%). Analytical HPLC: (gradient, water/0.1% TFA to 100% (10% water/90% acetonitrile/0.1% TFA) over 30 min) retention time = 28 min. ISMS: *m*/*z* 792 $[M + H]^+$ (100). HRMS (EI^+): *m*/*e* 792.3621, M^+ calculated for $C_{36}H_{56}N_8O_8S_2$ *m*/*e* 792.3662. ^{1}H-NMR: chemical shift data and assignments are compiled in Table 1.

NMR Structure Determination. ^{1}H-NMR spectra were recorded for macrocycle **2** (2 mg in 750 μL acetone-d_6, δ 2.05) referenced to solvent on a Varian Unity 400 spectrometer at 24 °C. Two-dimensional ^{1}H NMR NOESY (relaxation delay 2.0 s, mixing time 200−500 ms), DFCOSY, and TOCSY (mixing time 75 ms) spectra were acquired and recorded in the phase sensitive mode. Acquisition times = 0.130 s, spectral width = 5259 Hz, number of complex points (t_1 dimension) = 786 for the NOESY experiments and 512 for the DFCOSY and TOCSY. Data were zero-filled and Fourier transformed to 1024 real points in both dimensions.

Data were processed using TRIAD 6.2 software (Tripos Inc.) on a Silicon Graphics Indy work station. 2D NOE cross peaks were integrated and characterized as strong (1.8−2.5 Å), medium (2.3−3.5 Å), and weak (3.3−5.0 Å). Three-dimensional structures were calculated from upper and lower distance limit files using cycles of Diana 2.8 (64 distance constraints), redundant dihedral angle constraints (REDAC), and MARDIGRAS. All Diana structures were subjected to restrained molecular dynamics (RMD) and energy minimization (REM). Initially, REM consisted of a 50-step steepest descent followed by 100-step conjugate gradient minimization. Hydrogen-bonding information from the 1D ^{1}H-NMR spectrum was then incorporated into the 50 calculated structures before simulated heating to 300 K for 1 ps, followed by 500 K for 1 ps. The temperature was gradually lowered to 300 K over 2 ps and finally held for 2 ps at 200 K. REM was performed again with a 50-step steepest descent and 200-step conjugate gradient followed by a 300-step Powell minimization. The final structures were examined to obtain a mean pairwise root mean square difference over the backbone heavy atoms (N, Cα, and C). Nine of the 50 structures had a mean rmsd < 0.2 Å for all backbone atoms (O, N, C, S).

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