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Pseudoprolines as Removable Turn Inducers: Tools for the Cyclization of Small Peptides

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Received August 31, 2004

The cyclization of small peptides which do not incorporate turn inducers is often difficult. We have developed a method involving the use of removable turn inducers, in the form of pseudoprolines, for the cyclization of difficult peptide sequences. The pseudoprolines induce a *cisoid* amide bond in the peptide backbone which facilitates cyclization. They are then readily removed to yield a cyclic peptide that does not contain any turn inducers.

Introduction

Cyclic peptides are currently attracting the attention of chemists and biochemists alike. Naturally occurring cyclic peptides exhibit a wide range of biological activities, and cyclic peptides generally exhibit improved biological properties when compared to their linear counterparts. Cyclization may also be employed to constrain a bioactive peptide in its active conformation, thereby increasing its potency and/or specificity. As such, the development of efficient and convenient methods for the synthesis of cyclic peptides is of great interest.[1−3]

While it is now commonplace to synthesize linear peptides in high yield and purity, the head-to-tail cyclization of small linear peptides (three to eight amino acids) remains difficult, often resulting in epimerization of the C-terminal amino acid and/or the formation of linear and cyclic oligomers.[4,5] This commonly observed difficulty in cyclization has been attributed to the preference of amide bonds to adopt an *s*-transoid conformation, as a result of their strong π-character. This results in an extended and somewhat rigid linear peptide structure that is highly favored in certain peptide sequences. These compounds can be virtually impossible to cyclize; for example, the cyclization of all-L peptides is notoriously difficult.[5]

Cyclization of a linear peptide is dependent on a number of factors including the peptide sequence. The incorporation of turn-inducing elements (e.g., Gly, Pro, *N*-alkylated, or D-amino acids) into a peptide sequence has been shown to enhance cyclization yields.[4−8] However, the synthesis of cyclic peptides which do not incorporate turn inducers is a more challenging problem. Several methods have recently been developed for the head-to-tail cyclization of small peptides. However, no single method has been found to be generally applicable, and many methods result in only a moderate increase in the yield of the required cyclic product or have only been employed for the synthesis of peptides that already contain some type of turn-inducer.[3,7−10] We now report an approach to the cyclization of small peptides involving the incorporation of turn inducing side chain protecting groups that can be removed once peptide cyclization is complete to give cyclic peptide products that do not contain turn-inducers.

Pseudoprolines (Ψpro's), derived from serine, threonine, and cysteine residues upon cyclocondensation with an aldehyde or ketone, were recently introduced as solubilization aids for solid-phase peptide synthesis,[11,12] and dipeptides containing these modified residues are commercially available.[13] The introduction of these modified residues into a peptide sequence has been found to induce a predominantly *cisoid* conformation about the amide bond immediately preceding the modified amino acid, resulting in the adoption of a type VI β-turn structure.[12] We anticipated that this would enhance both the rate and yield of cyclization of peptides containing these modified amino acid residues.[10] Once cyclization was complete, treatment with acid would result in

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10.1021/jo0484732 CCC: $27.50 © 2004 American Chemical Society
Published on Web 11/12/2004

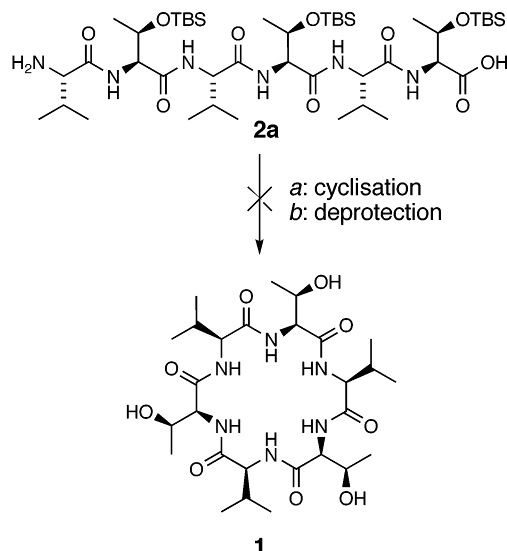
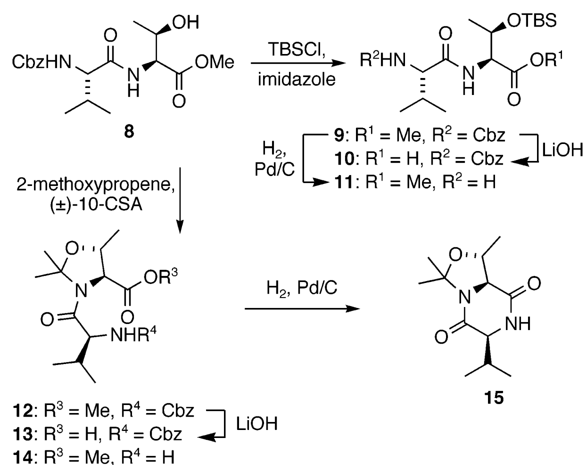


FIGURE 1. Previous attempts to synthesize **1**.[14]

SCHEME 1

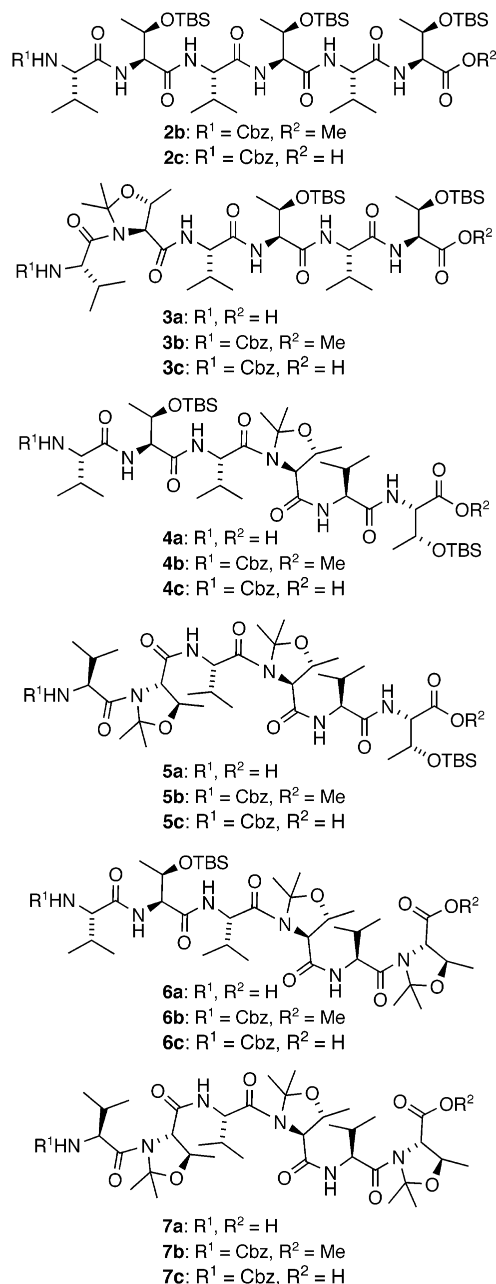


removal of the pseudoprolines to yield cyclic peptides devoid of turn inducers.

Our initial interest in this area stemmed from the desire to prepare derivatives of the hexapeptide cyclo-(Val-Thr)$_3$ (**1**) for molecular recognition studies. Wipf and Miller have previously reported that the TBS side-chain-protected linear peptide H-Val-Thr(TBS)-Val-Thr(TBS)-Val-Thr(TBS)-OH (**2a**) does not cyclize under a wide range of attempted conditions (Figure 1).[14] We therefore investigated the effect that the incorporation of pseudoproline residues had upon the head-to-tail cyclization yield of this hexapeptide sequence.

Results and Discussion

A modular approach to the synthesis of hexapeptides **2a**−**7a** was employed, in which fragment coupling of appropriately protected dipeptides **10**, **11**, and **13** (Scheme 1), using standard solution-phase techniques, gave ready access to the differentially protected hexapeptides **2b**−**7b**. Thus, dipeptide **8** was prepared according to literature procedures.[15] Treatment of **8** with TBSCl and imidazole gave the TBS-protected dipeptide **9** (Scheme 1). Alternatively, the pseudoproline-containing dipeptide **12** was prepared upon treatment of **8** with 2-methoxypropene in the presence of (±)-10-camphorsulfonic acid. Hydrolysis of the methyl esters **9** and **12** was readily achieved upon treatment with LiOH in aqueous methanol to give the carboxylic acids **10** and **13**, respectively, in good yield. Hydrogenolysis of **9** gave the free amine **11**, but when **12** was treated with hydrogen in the presence of a palladium on charcoal catalyst, only the diketopiperazine **15** was isolated.



The hexapeptides **2b**−**7b** were prepared by coupling of these dipeptide fragments using standard solution-phase techniques. While no epimerization was observed

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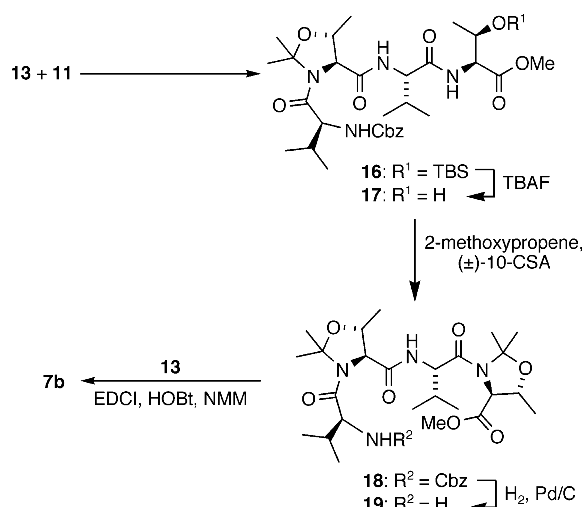
SCHEME 2

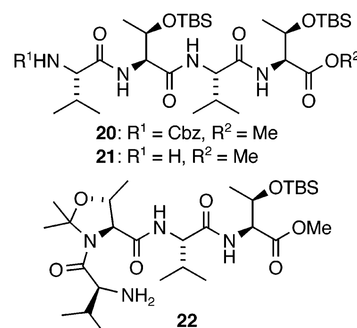


in couplings of the Ψpro-containing acid **13**, some epimerization (up to 30%) was observed during couplings of the TBS-protected acid **10**, as expected for this hindered fragment coupling. Diastereoisomers resulting from epimerization were readily separated by column chromatography. Notably, peptides containing Ψpro residues were easier to handle in terms of both solubility and stability than the corresponding TBS-protected derivatives, indicating that the Ψpro's are useful serine, threonine, and cysteine protecting groups for the preparation of difficult peptide sequences by solution-phase peptide synthesis as well as for solid-phase synthesis.

Since the dipeptide **14** (Scheme 1) was not available for formation of hexapeptides containing a C-terminal pseudoproline moiety, it was necessary to adopt an alternative route to the synthesis of hexapeptides **6b** and **7b** (Scheme 2). In this case, the tetrapeptide **16** was treated with TBAF to give the corresponding tetrapeptide **17** with a free threonine side chain. Subsequent treatment with 2-methoxypropene in a "post-insertion" reaction gave the required tetrapeptide **18** in 82% yield. Deprotection of the N-terminus to give amine **19**, followed by coupling with the carboxylic acids **10** and **13**, respectively, gave the hexapeptides **6b** and **7b** in good yield over the two steps. Hexapeptides **2b**−**5b** were similarly prepared by coupling both acids **10** and **13** with both amines **21** and **22**. Sequential deprotection of the C- and N-terminii of the hexapeptides **2b**−**7b** using the standard conditions described above gave the free peptides **2a**−**7a**, which were immediately subjected to cyclization experiments.

Linear Peptide Conformation. Where possible, the *cis/trans* ratios of the amide bonds in the linear peptides were determined using NMR spectroscopic techniques. In the case of dipeptides **12** and **13**, a major and minor set of resonances are clearly observed in both the ^{1}H and ^{13}C NMR spectra. The major conformer was determined to be that with a *cisoid* amide bond on the basis of the typical NOE cross-peaks observed by 2D NMR ROESY and NOESY experiments (i.e., $\alpha H_{i-1} - \alpha H_i$ and $\alpha H_{i-1} - \beta H_i$ cross-peaks).[16] The minor conformer was assumed to be the *trans* form, although this could not be confirmed by chemical shift or NOE measurements. The *cis/trans* ratios in $CDCl_3$ were determined to be 85:15 and 80:20 for **12** and **13**, respectively, which is in accordance with the ratios observed by Keller et al. for similar Fmoc-protected dipeptides.[17] These ratios did not change significantly with variations in either the solvent ($CDCl_3$, toluene-d_8, DMSO-d_6, CD_3CN, or CD_3OD) or temperature (300−370 K). In contrast, the ^{1}H and ^{13}C NMR spectra of the TBS-protected dipeptide **9** exhibit only a single set of resonances, and the observed $^{1}H-^{15}N$ ^{1}J coupling constant (93 Hz) indicates that **9** is present as a single conformer with *trans* geometry.[18]

While the ^{1}H and ^{13}C NMR spectra for the tetrapeptide **20** contain only a single set of resonances, the ^{1}H and ^{13}C NMR spectra of the tetrapeptide **16** contain two sets of resonances in an 85:15 ratio ($CDCl_3$ and DMSO-d_6). In this case, 2D NMR NOESY and ROESY experiments indicate that the major conformer is that in which the amide bond preceding the pseudoproline residue is *cisoid* with the other amide bonds adopting a *transoid* configuration. The minor conformer is likely to be that in which all amide bonds are *trans*. The ^{1}H and ^{13}C NMR spectra of tetrapeptide **18** (CD_3CN) indicate that this peptide exists as a mixture of 4 conformers in a 12:6:1:1 ratio which interconvert slowly on the NMR time scale. While overlapping of the signals from different rotamers makes it difficult to observe the characteristic NOE cross-peaks for the *cis* rotamers, it can be assumed by analogy with the other peptides discussed here that the major conformer is that in which both pseudoproline residues are preceded by *cis* amide bonds.



The ^{1}H and ^{13}C NMR spectra of the protected linear hexapeptides **3b**−**7b** are either broad (particularly for spectra obtained in $CDCl_3$) or exhibit overlapping resonances for at least two confomers for each peptide (DMSO-d_6, CD_3CN). However, the ^{1}H NMR spectrum of hexapeptide **2b** exhibits a single set of relatively well resolved signals in DMSO-d_6, CD_3CN, and $CDCl_3$, suggesting that this peptide adopts a single conformation in solution. The absence of NOE cross-peaks between the α protons of adjacent Val and Thr residues suggests that this conformer has *all-trans* amide bonds, but this could not be confirmed. Interestingly, ^{1}H and ^{13}C NMR spectra of the N- and C-deprotected hexapeptide **7a** show a highly predominant set of resonances in DMSO-d_6, CD_3CN, and

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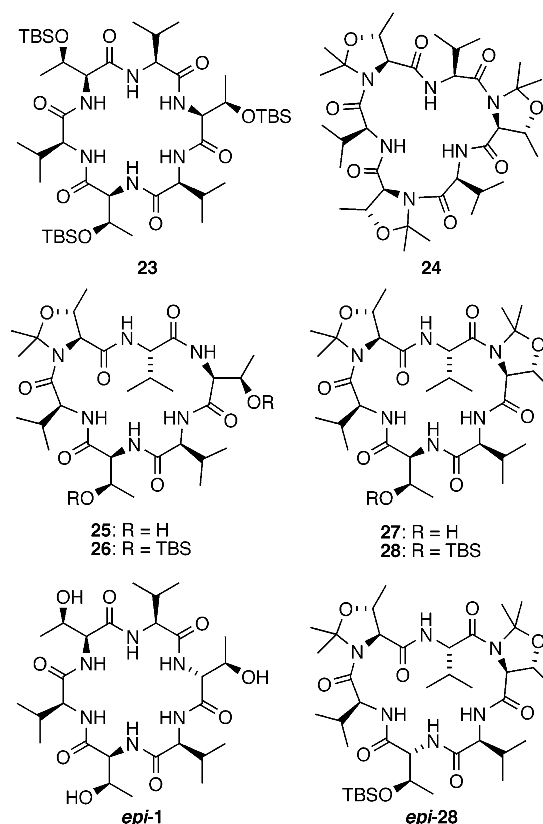
MeOD-d_4, consistent with the presence of a major conformer in solution (a minor set of resonances [<5% as estimated by integration] is also detected). The major conformer was assigned as that in which the three amide bonds preceding the pseudoproline residues are *cisoid*, using 2D NOESY experiments as described above for the dipeptides. An NOE cross-peak between the α protons of the N-terminal and C-terminal amino acid residues of this peptide was also observed, indicating that the terminii of the peptide must be in close proximity.

Cyclization Experiments. Cyclization studies were initially attempted using a variety of coupling reagents, solvents and concentrations. However, pentafluorophenyl diphenylphosphinate (FDPP) was found to give consistently higher yields of cyclic peptide products than the other coupling reagents employed (e.g., HATU, DEPBT) and as such this reagent was used for comparative cyclization experiments. In accordance with previous experiments,[14] peptide **2a** gave no identifiable cyclic products under our optimal cyclization conditions. However, the incorporation of a single Ψpro residue in the center of the linear peptide backbone (hexapeptides **3a** and **4a**) gave trace amounts (<5%) of a cyclic monomer at high dilution (0.005 mM). Subsequent characterization of the cyclic products obtained from these experiments and comparison to an authentic sample (see below) indicated that, in both cases, cyclization proceeded only after epimerisation of the C-terminal amino acid had occurred.

Increasing the number of Ψpro residues present in the peptide backbone led to increased yields of cyclic peptide products. Cyclization of hexapeptide **5a** gave a 40% yield of a single cyclic peptide, but comparison to an authentic sample indicated that only peptide in which the C-terminal residue had epimerized underwent cyclization to yield *epi*-**28**. Since we had observed during the synthesis of the linear peptides that peptides with C-terminal Ψpro residues were less likely to epimerize than the corresponding TBS-protected derivatives, we attempted cyclization of the linear peptides **6a** and **7a**, both of which contain a C-terminal pseudoproline residue. Upon treatment with FDPP, a 0.005 M solution of hexapeptide **6a** gave the corresponding cyclic peptide **28** in 10% yield. In this case, none of the epimeric product was observed. In the case of hexapeptide **7a**, which contains three Ψpro residues, high yields (84−99%) of the corresponding cyclic hexapeptide **24** were consistently obtained, even when the concentration of linear precursor was increased to 0.01 M and no product corresponding to C-terminal epimerisation was observed. The high yield obtained from this cyclization experiment suggests that linear peptide **7a** must be preorganized for cyclization. This is consistent with the end-to-end NOE cross-peak observed for the linear peptide.

Removal of the Ψpro "protecting" group from the cyclic hexapeptide products was achieved upon treatment with 4 M HCl in dioxane or, alternatively, with trifluoroacetic acid. The deprotection is somewhat slow; nevertheless, it proceeds in high yield to give the corresponding cyclic peptide **1**, which does not contain any turn inducers. Intriguingly, deprotection of **24** can be carried out in a stepwise manner to give cyclic peptides **25** and **27** containing one or two Ψpro residues, respectively, or the fully deprotected cyclo(Val-Thr)$_3$, **1**.

Treatment of the partially deprotected cyclic peptides **25** and **27** and the fully deprotected **1** with excess TBS-Cl under standard conditions provided authentic samples of the cyclic peptides (**26**, **28**, and **23**, respectively) that would have been obtained if cyclization of hexapeptides **3a**−**5a** occurred without epimerization. Comparison of these samples (NMR, TLC, α_D) with the products obtained after peptide cyclization should have enabled us to determine if the expected products were obtained. However, the TBS-protected cyclic peptides exist as a mixture of conformers in solution, and the number and ratio of conformers present was found to be very sensitive to the solvent and the presence of impurities, making comparison by NMR difficult. Therefore, to confirm the structures of the cyclized products, they were treated with a 4 M solution of HCl in dioxane to give the fully deprotected cyclic peptides which were then compared to **1**. While **1** exhibits C_3 symmetry in solution (see below), the products obtained after cyclization of **3a**−**5a**, followed by global deprotection, all gave identical ^{1}H NMR spectra in which six signals are clearly observed for the six amide protons present in the now unsymmetrical *epi*-**1**. The product obtained after global deprotection of **28** was identical in all respects to **1**, confirming that the cyclization of **6a** which bears a pseudoproline residue at the C-terminus proceeds without epimerization.



Conformational Studies of the Cyclic Peptides. ^{1}H NMR spectra of **24** indicate that it adopts a C_3-symmetric structure which does not vary with changes in solvent (CD_3CN, $CDCl_3$, CD_3OD), consistent with the presence of a single conformer in solution. NOE cross-peaks observed between the α protons of the Thr$\Psi^{Me,Me}$pro residues and both the α and β protons of the Val residues

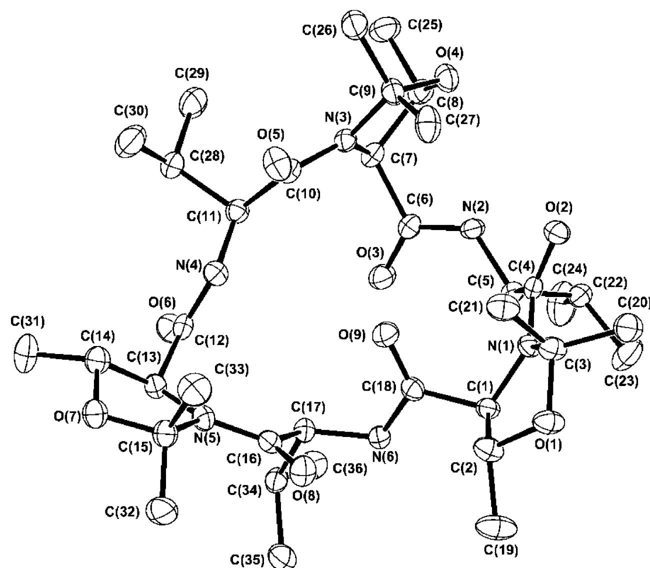


FIGURE 2. ORTEP depiction of **24** with 50% displacement ellipsoids. Water molecules and hydrogen atoms have been omitted for clarity.

indicated that the amide bonds preceding the Ψpro residues adopt a *cisoid* configuration.[16] This was confirmed by a single-crystal X-ray diffraction structure determination for cyclic hexapeptide **24** (Figure 2), which clearly illustrates the alternating arrangement of *cis* and *trans* amide bonds in the cyclic peptide.

The cyclic peptides **25**−**28** containing either one or two pseudoproline residues exhibit multiple conformations in solution, depending on the solvent, temperature, and sample concentration. While the presence of multiple conformers complicates analysis of the structures, the presence of *cisoid* amide bonds preceding the pseudoproline residues in some of the conformers is clear from the presence of the expected $\alpha H_{i-1}-\alpha H_i$ NOE cross-peaks between the Val_{i-1} and $Thr_i[\Psi^{Me,Me}pro]$ α-protons. In contrast, the ^1H and ^{13}C NMR spectra of **1**, in both methanol-d_4 and dimethyl-d_6 sulfoxide at 22 °C, contain a single set of well-resolved valine and threonine signals. However, since the coupling constants and chemical shifts vary between solvents, it is likely that the observed C_3 symmetry is a result of rapid equilibration between different conformers, which precludes the determination of the geometry about the amide bonds.[19] Based on the presence of multiple *cis*−*trans* conformers for the more restricted cyclic peptides (**25**−**28**), together with the absence of any NOE cross-peaks attributable to a *cisoid* amide bond geometry, it can be concluded that the amide bonds of **1** are free to adopt a *transoid* configuration.

Conclusions

The above results indicate that Ψpro derivatives can act as removable turn inducers to induce linear peptide conformations amenable to cyclization. The overall yields of cyclic peptides are still somewhat dependent on peptide sequence, but can be significantly improved if more than one turn inducer is incorporated into the peptide backbone. Previous work by Mutter and co-workers[10] indicates that when other turn inducers (e.g., proline) are present in the peptide, incorporation of a single Ψpro residue in a linear precursor is sufficient to substantially improve the head-to-tail cyclization yield. We have now found that multiple pseudoproline residues can be used to enable the cyclization of peptides that do not contain natural turn inducers. Additionally, we have found that the cyclization of peptides in which the C-terminal amino acid is protected as a Ψpro derivative proceeds without epimerization of this center. This greatly expands the range of linear precursors available for peptide cyclization (currently proline and glycine are the only preferred C-terminal residues). Deprotection of the Ψpro groups can be carried out with concurrent *cis*−*trans* isomerization of the preceding amide bonds to give cyclic peptides devoid of turn inducers. We are currently investigating modifications of this methodology that will permit the synthesis of a wider range of cyclic peptides, including those that lack serine, threonine, or cysteine residues.

Experimental Section

General procedures are available in the Supporting Information.

General Method for *N*-Deprotection. Catalytic Pd/C (10 mol %) was added to a solution of the *Z*-protected peptide in MeOH (20 mL/1 g peptide) in a single-necked, round-bottom flask fitted with a three-way tap attached to a balloon of H_2(g). The flask was evacuated (water aspirator) and then filled with H_2(g), and the process was repeated five times. The reaction mixture was then stirred under a H_2(g) atmosphere for 5 h, after which the contents of the flask were filtered through a pad of Celite, the Celite was washed several times ($CHCl_3$/MeOH/NH_3(aq) 90:9:1), the organic fractions were combined, and the solvent was removed by rotary evaporation with toluene to give the desired free amine in high yield. The free amine was used immediately in subsequent steps, generally without requiring further purification.

General Method for *C*-Deprotection. A solution of LiOH (3 equiv) in water (5 mL/1 g substrate) was added dropwise to a solution of methyl ester protected peptide (1 equiv) in MeOH (15 mL/1 g peptide) and the reaction mixture stirred at rt until the reaction was complete as judged by TLC (typically 6 h). The reaction mixture was then poured into a 10% aqueous solution of citric acid and extracted several times with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4, the solvent was evaporated, and the crude compound was recrystallized to give the desired free carboxylic acid.

General Method for Pseudoproline Formation. 2-Methoxypropene (2.5 equiv) was added to a cooled solution of the threonine-containing peptide (1 equiv) in CH_2Cl_2 (10 mL/1 g peptide at 0 °C), followed by the addition of a catalytic amount of camphorsulfonic acid (10 mol %). Upon addition of the latter, the solution immediately became red. The reaction mixture was stirred at 0 °C for 5 h, diluted with a further 10 mL of CH_2Cl_2, and washed with satd $NaHCO_3$(aq). The organic fraction was dried over $MgSO_4$, filtered, and concentrated and the residue purified by flash chromatography to give the desired pseudoproline-protected peptide as a colorless solid.

General Method for Peptide Coupling. Under anhydrous conditions, NMM (1.1 equiv) was added to a solution of the *Z*-protected peptide carboxylic acid (1 equiv), EDC (1.1 equiv), and HOBt (1.1 equiv) in CH_2Cl_2 (20 mL/1 g substrate) and the mixture stirred at 0 °C for 10 min. A precooled solution of the methyl ester protected free amine peptide (1.1 equiv) and NMM (1.1 equiv) in DMF (8 mL/1 g substrate) was then added and the reaction mixture allowed to warm to rt and stirred overnight. The reaction mixture was then poured into a 10% aqueous solution of citric acid and extracted several times with EtOAc. The combined organic fractions were washed with 10% citric acid (aq), $NaHCO_3$(aq), water, and then

(19) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512.

brine, dried over Na_2SO_4, and filtered, the solvent was evaporated, and the crude compound was purified by flash chromatography to give the desired coupled peptide.

General Method for TBS Protection. Under anhydrous conditions, TBS-Cl (2 equiv per hydroxyl) and imidazole (4 equiv per hydroxyl) were added to a solution of cyclic peptide in DMF (1 mL/100 mg substrate) and the mixture stirred at rt for 36 h. The mixture was partitioned between EtOAc and brine, and then the aqueous layer was extracted with EtOAc (2×). The combined organic phases were dried (Na_2SO_4), and then the solvent was removed under reduced pressure. The residue was purified by flash chromatography to give the desired TBS-protected products.

Cyclo[-Val-Thr($\Psi^{Me.Me}$pro)-Val-Thr($\Psi^{Me.Me}$pro)-Val-Thr-($\Psi^{Me.Me}$pro)-] (24). Under anhydrous conditions, 1.5 equiv of FDPP (154 mg, 0.40 mmol) was added to a solution of the linear hexapeptide **7a** (197 mg, 0.26 mmol) in acetonitrile (54 mL, $C = 0.005$ M), followed by the addition of 3 equiv of DIPEA (0.14 mL, 0.80 mmol). The reaction mixture was then stirred at rt overnight, after which the solvent was removed by rotary evaporation and the residue purified by flash chromatography (silica gel: 1:1 → 2:1 ethyl acetate/hexane) to give the desired cyclized hexapeptide **24** (174 mg, 91% yield) as a colorless solid: mp 234−236 °C; $[\alpha]_D = -65$ (c 1.6, $CHCl_3$). ^1H NMR (CD_3CN/200 MHz) δ 0.83 (d, J 6.8, 9H), 0.92 (d, J 6.8, 9H), 1.31 (d, J 6.2, 9H), 1.47 (s, 9H), 1.50 (s, 9H), 2.06 (dseptet, J 5.9, 6.8, 3H), 4.05 (d, J 5.9, 3H), 4.23 (dq, J 5.9, 6.2, 3H), 4.33 (dd, J 5.9, 6.6, 3H), 6.47 (d, J 6.6 Hz, 3H); ^{13}C NMR (CD_3CN/75 MHz) δ 16.8, 17.9(0), 17.9(1), 24.9, 25.8, 30.3, 57.2, 66.9, 75.0, 96.2, 167.3, 168.0; ESI-MS (positive mode) m/z = 663 (32), 721 ($[MH]^+$, 42), 743 ($[MNa]^+$, 100); HR-ESI-MS m/z = 743.4318 $[MNa]^+$, 743.4314, calcd for $C_{36}H_{60}N_6O_9Na$. Anal. Calcd for $C_{36}H_{60}N_6O_9{\cdot}H_2O$: C, 58.52; H, 8.46; N, 11.37. Found: C, 58.37; H,8.44; N, 11.24.

Cyclo[-Val-Thr-Val-Thr($\Psi^{Me.Me}$pro)-Val-Thr($\Psi^{Me.Me}$pro)-] (27). The pseudoproline-derivatized hexapeptide **24** (149 mg, 0.21 mmol) was treated with 4 M HCl in dioxane (1 mL) and stirred at 0 °C for 1 h after which the reaction mixture was diluted with water (15 mL) and extracted several times with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4, the solvent was evaporated, and the residue was purified by flash chromatography (silica gel: 95:5 EtOAc/MeOH) to give the monounprotected hexapeptide **27** (46 mg, 32% yield) as a colorless solid: mp 148−150 °C; $[\alpha]_D = -140$ (c 1.7, $CHCl_3$); ^1H NMR (CD_3CN/200 MHz) δ 0.84 (m, 15H), 1.05 (d, J 6.3, 3H), 1.10 (d, J 6.9, 3H), 1.33 (d, J 6.3, 3H), 1.40 (d, J 6.1, 3H), 1.43 (s, 6H), 1.52 (s, 3H), 1.54 (s, 3H), 2.12 (dseptet, J 3.7, 6.6, 1H), 2.39 (m, 2H), 3.67 (dd, J 4.9, 7.4, 1H), 3.90 (app. t, J 8.4, 1H), 3.98 (m, 1H), 4.03 (m, 1H), 4.07−4.17 (m, 2H), 4.17−4.25 (m, 2H), 4.35 (br.d, J 4.8, 1H, OH (disappears on D_2O exchange)), 4.49 (dq, J 3.7, J 6.3, 1H), 6.70 (d, J 4.8, 1H), 7.35 (m, 2H), 7.76 (d, J 8.4, 1H); ^{13}C NMR (CD_3CN/75 MHz) δ 15.1, 17.5, 17.8 (3), 17.8 (4), 18.1, 18.9, 19.0, 20.0, 22.4, 25.1, 25.2, 26.8, 27.3, 27.7, 32.4, 32.4, 57.6, 58.2, 60.7, 65.4, 65.5, 66.4, 75.1, 76.0, 95.8, 96.6, 167.7, 168.8, 169.1, 169.6, 170.4, 171.9 (1 signal obscured or overlapping); ESI-MS (positive mode) m/z 681 ($[MH]^+$, 100), 703 ($[MNa]^+$, 37); HR-MS m/z = 703.3960 $[MNa]^+$, 703.4001 calcd for $C_{33}H_{56}N_6O_9Na$.

Cyclo-[-Val-Thr-Val-Thr-Val-Thr($\Psi^{Me.Me}$pro)-] (25). A 5% (v/v) TFA solution was added dropwise to a solution of the pseudoproline-derivatized hexapeptide **24** (194 mg, 0.27 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The reaction mixture was stirred at rt for 3 h and then diluted with water (15 mL) and extracted several times with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4, the solvent was evaporated, and the residue was purified by flash chromatography (silica gel: 95:5 → 80:20 EtOAc/MeOH) to give the di-deprotected hexapeptide **25** (81 mg, 47% yield) as a colorless solid: mp 118−120 °C; $[\alpha]_D = -34$ (c 1.6, MeOH); ^1H NMR ($CDCl_3$/200 MHz) δ 0.91−1.06 (m, 18H), 1.21 (d, J 6.3, 3H), 1.29 (d, J 6.4, 3H), 1.43 (d, J 6.4, 3H), 1.58 (s, 3H), 1.68 (s, 3H), 1.93 (m, 1H), 2.13 (m, 2H), 3.40−3.50 (m, 3H), 3.60 (d, J 6.1, 1H), 3.86 (app t, J 5.8, 1H), 3.93−4.46 (m, 6H), 6.86 (br s, 1H), 6.95 (d, J 9.2, 1H, NH), 7.68 (d, J 7.3, 1H), 8.15 (d, J 6.0, 1H), 8.47 (d, J 8.8, 1H) [minor conformer (<10%) also observed]; ESI-MS (positive mode) m/z = 641 ($[MH]^+$, 30), 663 ($[MNa]^+$, 52), 1303 ($[2M + Na]^+$, 100); HR-MS m/z = 663.3665 $[MNa]^+$, 663.3687 calcd for $C_{30}H_{52}N_6O_9Na$.

Cyclo[-Val-Thr-Val-Thr-Val-Thr-] (1) (Method 1). The pseudoproline-derivatized hexapeptide **24** (183 mg, 0.25 mmol) was treated with 4 M HCl in dioxane (1 mL) at 0 °C, allowed to warm to rt, and then stirred at rt for 3 d. The reaction mixture was diluted with water (15 mL) and extracted several times with EtOAc. The combined organic fractions were washed with brine, dried over Na_2SO_4, and filtered, the solvent was evaporated, and the residue was purified by flash chromatography [silica gel: 80:18:2 $CHCl_3$−MeOH−NH_3(aq)] to give two fractions A (R_f 0.6) and B (R_f 0.5).

Concentration of fraction A gave the di-deprotected hexapeptide **25** (50 mg, 33% yield), which was identical in all respects to the material obtained above.

Concentration of fraction B gave the desired hexapeptide **1** (85 mg, 56% yield) as a colorless solid: mp 188−190 °C; $[\alpha]_D = -98$ (c 0.9, MeOH); ^1H NMR (MeOD/200 MHz) δ 0.90 (d, J 7.0, 9H), 0.93 (d, J 7.0, 9H), 1.13 (d, J 6.4, 9H), 2.30 (dseptet, J 5.6, 7.0, 3H), 4.02 (d, J 5.6, 3H), 4.07 (d, J 3.7, 3H), 4.20 (dq, J 3.7, 6.4, 3H), (NH, OH not observed); ^{13}C NMR (MeOD/75 MHz) δ 19.0, 20.3, 21.2, 31.4, 61.7, 62.3, 68.8, 173.3, 174.5; ESI-MS (positive mode) m/z = 601 ($[MH]^+$, 7), 623 ($[MNa]^+$, 20), 1223 ($[2M + Na]^+$, 100); HR-MS m/z = 623.3380 $[MNa]^+$, 623.3381 calculated for $C_{27}H_{48}N_6O_9Na$.

Cyclo[-Val-Thr(TBS)-Val-Thr(TBS)-Val-Thr(TBS)-] (23). Treatment of **1** (78 mg, 0.13 mmol) according to the general method for TBS protection gave the cyclic hexapeptide **23** as a colorless solid (88 mg, 70%) after flash chromatography (silica gel: 2:1 EtOAc/hexane): mp 170−172 °C; $[\alpha]_D = -18$ (c 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$/200 MHz) δ 0.00 (s, 9H), 0.01 (s, 9H), 0.78 (s, 27 H), 0.87 (d, J 6.8, 9H), 0.91 (d, J 6.8, 9H), 1.03 (d, J 6.3, 9H), 2.38 (m, 3H), 3.73 (dd, J 6.8, 6.8, 3H), 4.12 (dd, J 4.3, 5.6, 3H), 4.46 (dd, J 4.3, 6.3, 1H), 7.18 (d, J 6.8, 3H), 7.35 (d, J 5.6, 3H); ^{13}C NMR ($CDCl_3$/75 MHz) δ −4.5, −4.3, 18.3, 19.5, 19.9, 20.2, 26.2, 29.2, 60.5, 63.3, 67.2, 170.3, 171.3; ESI-MS (positive mode) m/z = 966 ($[MNa]^+$, 100); HR-MS m/z = 965.5927, 965.5970 calcd for $C_{45}H_{90}N_6O_9Si_3Na$.

Acknowledgment. We thank the Australian Research Council for financial support and for the award of a Queen Elizabeth II research fellowship to K.A.J.

Supporting Information Available: Experimental details and characterization data for compounds **1**−**13**, **15**−**22**, and **28** and details of single-crystal X-ray diffraction structure determination for **24**. Copies of ^1H and ^{13}C spectra for compounds **1**, ***epi*-1**, **2b**, **7a**, **8**, **9**, **12**, **13**, **15**−**18**, **23**−**25**, and **27** and NOESY spectra for compounds **7a**, **12**, **16**, and **24**. X-ray crystal structure of **24** (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

JO0484732