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Pseudo-Prolines in Cyclic Peptides : Conformational Stabilisation of *cyclo*[Pro-Thr($\Psi^{Me,Me}$pro)-Pro]

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ABSTRACT

Linear peptide H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH containing a preformed *cis*-Pro-Thr($\Psi^{Me,Me}$pro) tertiary amide bond cyclises instantaneously and free of formation of oligomeric structures to the cyclic tripeptide *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro]. Even at concentrations up to 10^{-1} M peptide, no oligomeric structures are detected by mass spectroscopy and HPLC. 2D ^{1}H NMR studies of purified cyclotripeptide reveal the compound to exist in one single conformation with all peptide bonds in the *cis* conformation. These results indicate enhanced cyclisation tendencies of cis-amide bond containing peptides of short chain length.

Keywords: Pseudo-prolines; Cyclic peptides; Peptide analogues/mimetics; *cis*-Amide bonds.

Cyclic peptides play important roles in biology and chemistry. Among the most prominent examples is the undecapeptide cyclosporin — exhibiting a wide variety of biological functions in such different areas as immunosuppression, neurodegenerative diseases and inhibition of HIV-1 propagation [1] — and the antibiotic pentadecapeptide gramicidin, an ionophor [2]. Their cyclic structure — in both cases accompanied by D-amino acids — is not only protective against degradation by proteases, but also restricts the number of possible conformational structures in solution [3]. Cyclisation of biologically important peptides is therefore appreciated as a structure modifying tool in order to overcome rapid degradation *in vivo* and/or enhancing affinity for target receptors by introducing conformational constraint [4]. However, the cyclisation reaction often is accompanied by undesired side reactions such as the formation of oligo- or multimeric structures, and racemisation [5].

Spatially close ends of peptide chains are more likely to form a covalent cross-link; it is thus desirable for a cyclisation reaction that the average end-to-end distance is minimal in order to prevent formation of oligomeric structures [6, 7]. An elegant way of achieving this goal is the introduction of a *cis*-amide bond into the middle of the peptide in analogy of forming a β-turn type VI. This approaches the two ends and thus increases the probability of an *intra*molecular reaction. Within the scope of naturally occurring amino acids, proline is the only representative which energetically allows both the *cis* and the *trans* Xaa-Pro tertiary amide bond. Depending on the sequence, *cis*-contents up to 60% frequently are observed. This observation has been exploited by *Rothe* who reported on the facile cyclisation of *cyclo*-triproline with 88% yield [8]. We have

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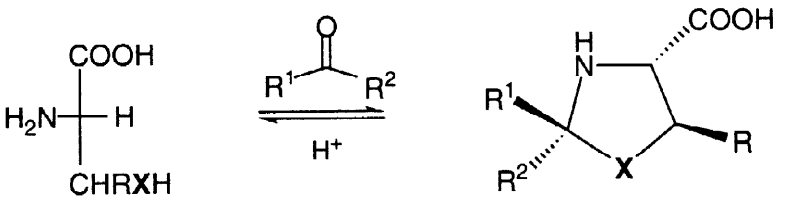
recently introduced a class of Pro mimics referred to as pseudo-prolines (ΨPro, *cf. Scheme 1*) [9]. Their use as a tool to prevent secondary structure formation and self-aggregation of the growing peptide chain has been demonstrated extensively [10]. In addition, ^{1}H NMR analyses [11] and a *ω-cis/trans*-conformationally sensitive recognition assay of ΨPro containing model peptides demonstrated the possibility of quantitative stabilisation of *cis*-amide bonds of type Xaa-ΨPro [12].

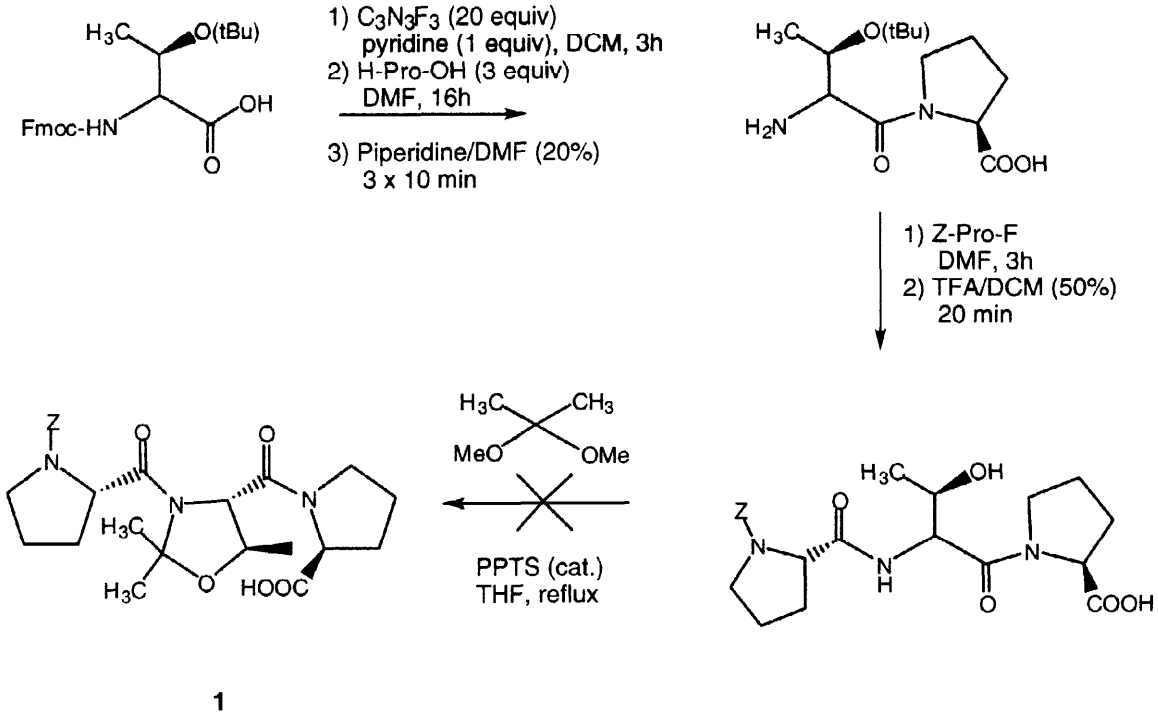


Scheme 1

Pseudo-prolines [Xaa($\Psi^{R1,R2}$pro)]: Xaa = Ser (X = O, R = H) and Thr (X = O, R = CH_3) derived oxazolidines; Xaa = Cys (X = S, R = H) derived thiazolidines. R^1 and R^2 (derived from the aldehyde or ketone used for the reaction with the native amino acid) are the substituents at the 2-C position of the pseudo-proline ring.

In the context of selective introduction of *cis*-amide bonds into peptide backbones, we chose the sequence H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH as a probe to test if (**a**) an overall stabilisation of the tripeptide can be achieved by the conformationally rigid *cis*-Pro-Thr($\Psi^{Me,Me}$pro) unit, (**b**) the cyclisation of the linear sequence to the cyclic compound is facilitated and (**c**) whether at high peptide concentrations up to 10^{-1} M, the preformed shape of the molecule due to the *cis*-Pro-Thr($\Psi^{Me,Me}$pro) peptide bond prevents formation of oligo- and/or multimeric structures. The first synthetic strategy implied the synthesis of linear peptide sequence Z-Pro-Thr-Pro-OH **1** followed by the formation of the oxazolidine (ΨPro) by treating the peptide with dimethoxypropane (DMP, 5 eq.) in presence of the pyridinium salt of *p*-toluene sulfonic acid (PPTS, 0.3 eq., *post*-insertion route, *cf. Scheme 2*).

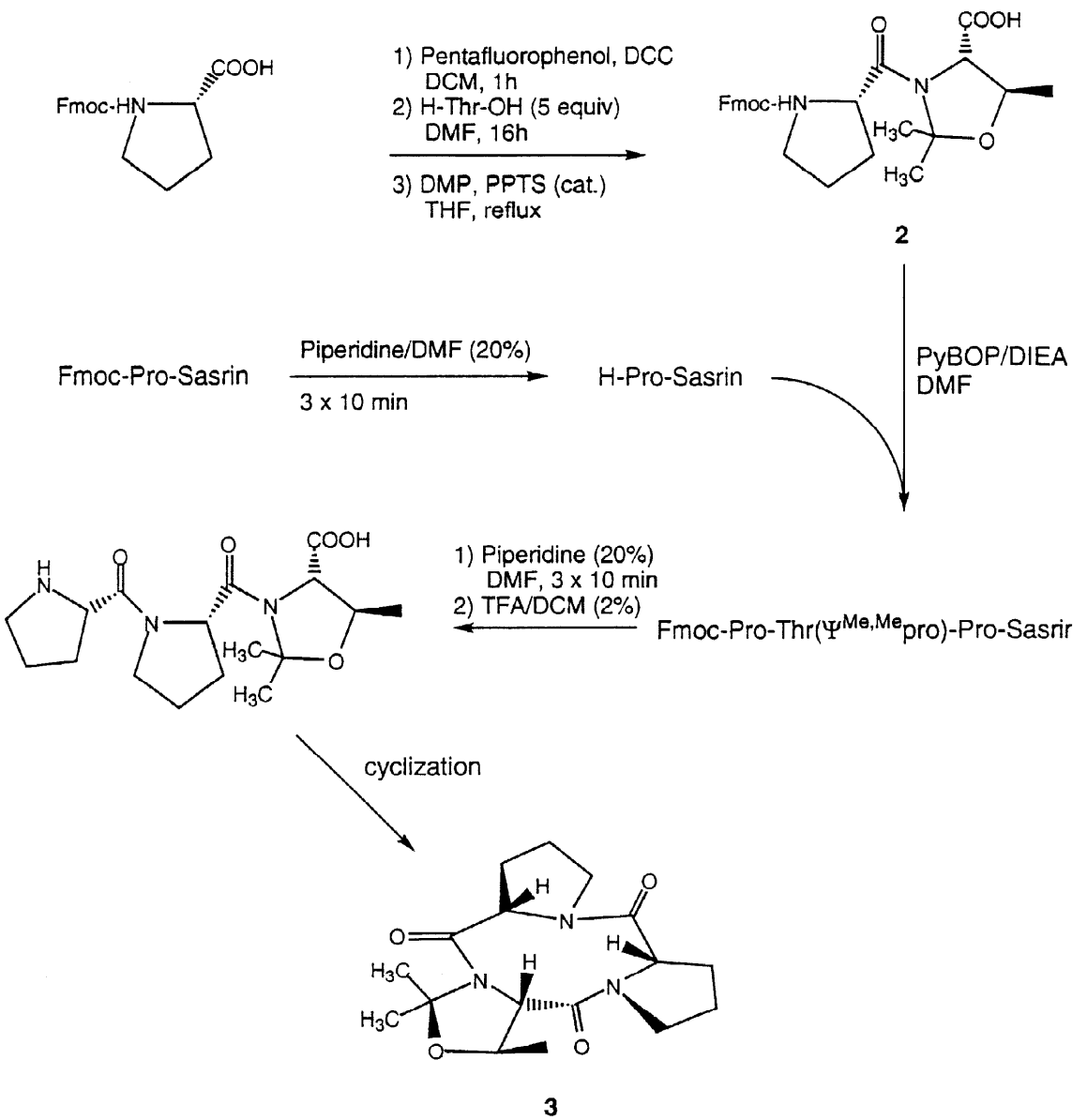
Unexpectedly, this strategy proved to be unsuccessful. Insolubility problems during the coupling between Fmoc-Pro-Thr(*t*Bu)-OH and Pro-*O*Bzl afforded very low coupling yields 5-10%). Further, the *post*-insertion reaction to the 2-C dimethylated oxazolidine (ΨPro) proved unsuccessful and prevented the synthesis of the desired cyclic tripeptide by this approach. These shortcomings could not be overcome by various modifications of the reaction conditions.



Scheme 2

Synthesis of linear tripeptide Z-Pro-Thr-Pro-OH **1** by standard coupling methods using the fluoride and the pentafluorophenylester as reagents to activate the carboxyl groups. No formation of the oxazolidine could be observed using standard conditions for this *post*-insertion reaction.

Therefore, dipeptide Fmoc-Pro-Thr($\Psi^{Me,Me}$pro)-OH **2** was prepared *via* Fmoc-Pro-Thr-OH followed by coupling this ΨPro containing dipeptide building block to Pro. However, various strong activation reagents such as fluoride, mixed anhydride (*tert*-butylchloroformate) and PyBOP resulted in poor reaction yields of the activated dipeptide building block with H-Pro-*O*Bzl. We finally were successful in coupling dipeptide building block Fmoc-Pro-Thr($\Psi^{Me,Me}$pro)-OH **2** to Sasrin-bound proline quantitatively and devoid of side reactions (see Scheme 3).



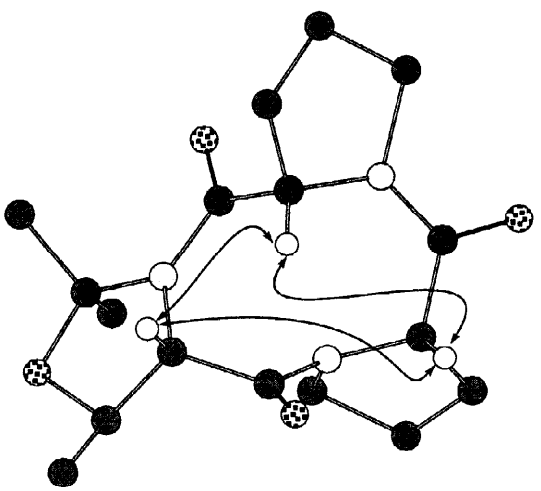
Scheme 3

Successful strategy to synthesize *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3** partially in solution and by solid phase peptide synthesis (SPPS). Fragment condensation of the dipeptide building block to resin bound proline proved quantitative and devoid of side reactions such as diketopiperazine formation.

Cleavage from the resin was achieved using TFA/DCM (2%) and the peptide purified by reversed phase HPLC (C_{18}). Three cyclisation experiments with varying concentrations were carried out with PyBOP as coupling reagent and diisopropylethylamine as base. For each experiment, a different concentration in linear peptide was taken ranging from 10^{-3} to 10^{-1} M. For all three concentrations, immediate formation of *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3** occurred as detected by HPLC and confirmed by both electron spray mass spectrometry and ^{1}H NMR spectroscopy. The yields for each concentration varied from 85% (10^{-3} M) to 80% (10^{-1} M) as

detected by reversed phase HPLC. The cyclisation reaction further was completely devoid of racemisation, which often is a disturbing side reaction of cyclisations. This result can be attributed to the fast kinetics for cyclisation.

Detailed two dimensional ^{1}H NMR measurements in methanol-d_4 and $CDCl_3$ using DQF COSY and ROESY techniques as wells as ^{1}H—^{13}C HMQC permitted the unambiguous assignment of all proton resonances to one single conformation with all peptide bonds in the *cis* form. In deuterated methanol and chloroform, the proton resonances of α-Thr($\Psi^{Me,Me}$pro) appear as singlets, indicating that the α and the β-proton of Thr($\Psi^{Me,Me}$pro) exhibit a torsion angle of about 90°. In the 400 MHz ROESY spectrum, strong NOE connectivities between the α-protons of Thr($\Psi^{Me,Me}$pro) and the two non-discernable α-protons of the two Pro is observed, which previously has been demonstrated to be indicative for the *all cis* form of the amide bonds true for all three amino acids (*cf.* Scheme 4) [13].



Scheme 4

Observed NOE connectivities (*arrows*) in *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3** used to attribute the conformation of the three amide bonds to the all *cis* form.

In conclusion, we have shown for the example of model peptide *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3**, that by introduction of a *cis*-amide bond into the peptide backbone, increased cyclisation kinetics and decreased formation of undesired oligomeric peptide structures are obtained. Furthermore, conformational rigidity due to the presence of at least one favored *cis* amide bond (ΨPro) is achieved. The presence of an energetically favored *cis*-tertiary amide bond can stabilize the other two peptide bonds in their *cis* form, resulting in a cyclotripeptide exhibiting one single conformation in both methanol and chloroform. The preformed shape of linear peptide H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH leads, upon activation, to instantaneous formation of cyclotripeptide in a racemation free cyclisation reaction.

EXPERIMENTAL

Materials. Reagents and solvents were purchased from Fluka (Buchs, Switzerland) unless otherwise stated and used without further purification. Deuterated methanol and chloroform were purchased from Dr. Glaser AG (Basel, Switzerland). Fmoc-Pro-Sasrin was from BACHEM (Bubendorf, Switzerland), Fmoc-Pro-OH and PyBOP from Novabiochem (Läufelfingen, Switzerland). HPLC was performed on a Waters equipment using columns packed with Vydac Nucleosil 300Å/5 m C_{18} particles. Analytical columns (250 × 4.6 mm) were operated at 1mL/min and preparative ones (250 × 21 mm) at 18 mL/min with UV monitoring at 214 nm. Solvent A is water purified on a Milli Q Ion exchange cartridge containing 0.09% TFA, and solvent B is acetonitrile HPLC-*R* (preparative) or HPLC-*S* (analytical; both purchased from Biosolve, Valkenswaard, Netherlands) containing 0.09% TFA. ^{1}H NMR experiments were run on a Bruker DPX-400. Mass spectra were obtained by electron spray ionization (ESI-MS) on a Finnigan LC 710. Abbreviations were used as follows: NMM = *N*-methylmorpholine, THF = tetrahydrofuran, DCM = dichloromethane, DMF = dimethylformamide, PPTS = pyridinium-*p*-toluene sulfonic acid, DCC = dicyclohexylcarbodiimide, DMP = dimethoxypropane.

Fmoc-Pro-Thr($\Psi^{Me,Me}$pro)-OH 2 $C_{24}H_{26}N_2O_6$ = 438.5 g/mol Fmoc-Pro-OH (4.05g, 12 mmol) and pentafluorophenol (2.56 g, 13.9 mmol) were dissolved in DCM (50 mL) before the addition of DCC (2.87 g, 13.9 mmol) and reacted for one hour. A heavy white precipitate developed, which was filtrated over Celite® and all DCM evaporated to obtain quantitatively Fmoc-Pro-*O*pF, which subsequently was dissolved in acetone (150 mL). A solution of H-Thr-OH (7.1 g, 60 mmol) in water (50 mL) containing NMM (6 mL) was added dropwise to give a white suspension, which was stirred for 16 h. All acetone was removed under reduced pressure, the suspension acidified to pH 1 with hydrochloric acid (1 M) to give a heavy white precipitate. The desired dipeptide was extracted with ethyl acetate (5 × 50 mL). The combined organic layers were washed with water (3 × 50 mL), dried over magnesium sulfate and the solvent evaporated to dryness to give 3.14 g (7.16 mmol, 60%) Fmoc-Pro-Thr-OH. HPLC (40—100% B, 15 min) t_R = 7.68 min, >95% purity. ESI-MS (m/z) = 439.1 $[M+H]^+$.

Fmoc-Pro-Thr-OH (2.5 g, 5.7 mmol) and PPTS (0.72 g, 2.85 mmol) were dissolved in dry THF (45 mL) and DMF (4.5 mL) before the addition of DMP (4.45 g, 42.8 mmol) and heated under reflux for 4 h. All solvent was evaporated and replaced by ethyl acetate (50 mL). The organic layer was washed with sodium carbonate (10%, 3 × 50 mL), dried over magnesium sulfate and the solvent removed under *vacuo*. The desired product was purified on a silica column using $CHCl_3$/MeOH/AcOH (100:10:1/v/v/v) as eluent. The product containing fractions were collected, the solvent evaporated after addition of 10 mL toluene to remove AcOH. Fmoc-Pro-Thr($\Psi^{Me,Me}$pro)-OH (2.22 g, 5.14 mmol, 81%) with high purity (>96%) was obtained. HPLC (40—100% B, 15 min) t_R = 11.82 min. ESI-MS (m/z) = 477.1 $[M+H]^+$.

***Cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] 3** $C_{17}H_{25}N_3O_4 = 335.4$ Fmoc-Pro-Sasrin (0.44 mmol/g, 1.5 g, 0.66 mmol) was extensively washed with DCM (30 mL, 30 min) and DMF (30 mL, 30 min) before use. Deblocking of the Fmoc-protecting group was achieved with piperidine in DMF (20%, 20 min, 10 mL). After each deprotection and coupling step, extensive washing with DMF (3 × 20 mL) was undertaken. Coupling reagent was PyBOP (690 mg, 1.33 mmol, 2 eq.), in presence of DIEA (681 μL, 3.98 mmol, 6 eq.) in DMF (15 mL). Coupling time was 90 min. Cleavage of linear H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-Sasrin from the resin was done with TFA/DCM (1%, 5 × 10 min). The crude reaction product was purified on an HPLC preparative column (0—50% B, 40 min) to obtain 137 mg (60%) of H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH. HPLC (0—50% B, 40 min) t_R = 14.56 min. ESI-MS (m/z) = 354.0 $[M+H]^+$.

^{1}H NMR [400 MHz, C*D*Cl$_3$, 300 K] σ (ppm) 5.01 (*m*, 1H, α-Pro), 4.89, (*d*×*d*, 1H, 6.6 Hz, β-Thr(ΨPro)), 4.71 (*s*, 1H, α- Thr(ΨPro)), 3.92 (*m*, 2H, δ–Pro), 3.41 (*m*, 2H, δ-Pro), 2.45 (*m*, 4H, β-Pro/γ-Pro), 1.97 (*m*, 4H, β-Pro/γ-Pro), 1.68 (*s*, 3H, 2-C-CH$_3$-Thr(ΨPro)), 1.59 (*s*, 3H, 2-C-CH$_3$-Thr(ΨPro)), 1.39 (*d*, 3H, 6.6 Hz, β-CH$_3$-Thr(ΨPro)).

^{1}H NMR [400 MHz, MeOH-d_4, 300 K] σ (ppm) 5.48 (*m*, 2H, α–Pro), 5.28 (*s*, 1H, α-Thr(ΨPro)), 4.77 (*d*×*d*, 1H, 6.7 Hz, β-Thr(ΨPro)), 3.91 (*m*, 2H, δ-Pro), 3.31 (*m*, 2H, δ-Pro), 2.42 (*m*, 4H, β-Pro/γ-Pro), 2.04 (*m*, 4H, β-Pro/γ-Pro), 1.69 (*s*, 3H, 2-C-CH$_3$-Thr(ΨPro)), 1.54 (*s*, 3H, 2-C-CH$_3$-Thr(ΨPro)), 1.39 (*d*, 3H, 6.6 Hz, β-CH$_3$-Thr(ΨPro)).

Cyclisation experiments. *General remark: A stock solution of peptide (3.874 × 10^{-2} mmol/mL) for all three experiments was prepared, and aliquots either diluted (a) or the volume reduced in vacuo (b, c) and the residal retaken up in fewer solvent in order to keep consistency for the amount of peptide for all three cases.*

(a) 10^{-3} M A solution of H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH (6.45 × 10^{-3} mmol/mL) in DMF (18 mL) was added dropwise (45 min) to of PyBOP (300 mg, 0.58 mmol) and DIEA (76 mg, 0.58 mmol) in DMF (100 mL). During the 45 minutes of addition of tripeptide, samples were taken and analyzed by mass spectrometry. During the whole cyclisation process, only cyclic tripeptide was detected. After completion of addition, all DMF was evaporated, the residal taken up in few acetonitrile and purified by reversed phase HPLC (0—50% B, 40 min) to obtain 31 mg (80%) pure *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3.** HPLC analytic t_R = 16.18 min. ESI-MS (m/z) = 375.0 $[M+CH_3CN]^+$.

(b) 10^{-2} M A solution of H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH (8.57 × 10^{-2} mmol/mL) in DMF (0.66 mL) was added to a solution of PyBOP (150 mg, 0.28 mmol) and DIEA (48 μL, 0.28 mmol) in DMF (5 mL) over a period of 45 min. During the whole cyclisation process, only cyclic tripeptide was detected by mass spectrometry and HPLC. After completion of addition, all DMF was evaporated, the residual taken up in few acetonitrile and purified by reversed phase HPLC (0—50% B, 40 min) to obtain 17 mg (85%) pure *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] **3.** HPLC analytic t_R = 16.18 min. ESI-MS (m/z) = 375.0 $[M+CH_3CN]^+$.

(c) 10^{-1} M 110 μL of a solution of H-Pro-Thr($\Psi^{Me,Me}$pro)-Pro-OH (28.3×10^{-3} mmol/mL) were added dropwise to a solution of PyBOP (70 mg, 0.14 mmol) and DIEA (24 μL, 0.14 mmol) in DMF (183 μL). Analyses and purification as described above. Yield of **3**: 8 mg, 80%.

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