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Synthesis of Phosphonomethyl-phenylalanine and Phosphotyrosine Containing Cyclic Peptides as Inhibitors of Protein Tyrosine Kinase / SH2 Interactions.

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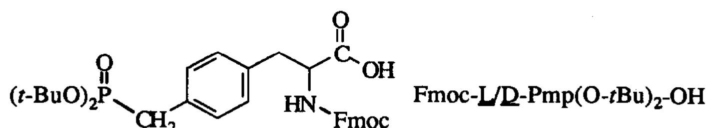
Abstract: Linear and cyclic hexameric peptides were synthesized with the amino acid sequence Gly-Tyr-Val-Pro-Met-Leu, which corresponds to the autophosphorylation segment around Y751 of PDGF receptor ß subunit. The natural L-tyrosine (position 2) was also substituted in peptide analogs with D-Tyr, L-tyrosine phosphate, and L- and D-4-phosphonomethyl-phenylalanine (Pmp). Fmoc chemistry-based SPPS methodology, and Rink resin support were used with diphenylphosphoryl azide as the cyclizing agent. The linear and cyclic peptides were characterized by circular dichroism spectroscopy. The peptides described were designed as inhibitors of receptor tyrosine kinase / src homology region 2 interactions that mediate mitogenic signal transduction pathways.

INTRODUCTION AND RATIONALE

Protein phosphorylation is a primary mechanism of cellular signal transduction. Upon ligand binding, a number of cell surface receptors undergo phosphorylation on specific tyrosyl residues within their intracellular domains. The phosphorylated segments in turn participate in subsequent signal transduction through physical association with specific peptide regions of secondary messenger molecules, now designated as src homology region 2 (SH2) domains.[1] For example, on ligand binding the platelet derived growth factor (PDGF) receptor is autophosphorylated at several specific tyrosines, including Tyr-751, which are known to then bind to phosphatidylinositol 3-kinase (PI 3-kinase),[2] as well as other proteins that function in the mitogenic signaling of cells.[3,4] The disruption of these multiprotein interactions has the potential of inhibiting cellular proliferation, and may provide an approach for antitumor therapy. A phosphotyrosyl-containing pentameric peptide based on the sequence surrounding the PDGF receptor Tyr-751 autophosphorylation site, has previously been shown to effectively bind to the PI 3-kinase p85 subunit SH2 domain.[2] Here we report the synthesis of linear and cyclic hexameric analogs of this sequence which incorporate both L- and D-tyrosine as well as L-tyrosine phosphate. In our design, an N-terminal glycine has been added to the sequence to obviate the presence of a basic amino group adjacent to the tyrosine phosphate, and also to facilitate the synthesis of the cyclic peptide analogs. Additionally, the synthesis of a number of phosphatase resistant tyrosine phosphate amino acid analogs have been reported from our laboratory,[5-7] and others.[8-10] We have accordingly prepared cyclic hexapeptides containing both the L- and D-forms of one of these analogs, 4-phosphonomethyl phenylalanine (Pmp). The cyclic peptides serve as conformationally constrained analogs of potential inhibitors of SH2 domain dependent growth factor signalling, and as such, facilitate better understanding of the structural requirements of effective agents and the design of non-peptide analogs.

RESULTS AND DISCUSSION

Hexameric linear peptides and precursors to cyclic peptides, consisting of the amino acid sequence Gly-Tyr-Val-Pro-Met-Leu and its analogs at the tyrosine position, were synthesized using manual solid-phase peptide synthesis (SPPS) methodologies with Fmoc-protected amino acids, and 1,3-diisopropylcarbodiimide (DIPCDI) N-hydroxybenzotriazole (HOBT) condensation. For synthesis of the side chain protected Pmp and tyrosine phosphate analogs, Fmoc-L/D-Pmp(O-*t*Bu)$_2$-OH[5] and Fmoc- or Boc-Tyr(O-PO_3Me_2)-OH were incorporated into the above sequence. It was beneficial to have the reactive side chains protected during the peptide cyclization steps. Thus, in order to retain the protective side chains during resin cleavage, in particular the moderately acid sensitive *t*-Bu groups on Pmp, the general strategy employed the super acid sensitive Rink resin.[11] Indeed, as described below, different deprotection conditions were found useful for obtaining the fully deprotected linear peptides from the resin, or the linear side chain protected peptides intended for subsequent cyclization.



The first amino acid (Fmoc-Leu-OH) was attached to the Rink resin with DIPCDI in the presence of N,N-dimethyl-4-aminopyridine (DMAP). The subsequent 4 amino acids were condensed in sequence to the Leu-Rink resin using standard Fmoc chemistry methodology, with DIPCDI/HOBT in N,N-dimethylformamide (DMF), and Fmoc deprotection with 20% piperidine/DMF. Finally, the N-terminal glycine was condensed using pentafluorophenyl Fmoc-glycinate in DMF solvent, followed by Fmoc deprotection. In this manner, the resin bound side-chain protected hexameric peptides **1**, **2**, **3** and **4**, were obtained containing L-Tyr(O-*t*Bu), D-Tyr(O-*t*Bu), L/D-Pmp(O-*t*Bu)$_2$ and L-Tyr(O-PO_3HMe)[12, 13] amino acids, respectively.

Synthesis and characterization of linear peptides. The tyrosine phosphate mimetic amino acid analog, Pmp, was incorporated into peptides as a racemic mixture,[5] thereby producing a diastereomeric pair of peptides.[14] For purposes of structural comparison it was also of value to prepare authentic L-Tyr and D-Tyr containing peptides, as well as the authentic L-Tyr phosphate containing analog. In order to effect both protective group removal and resin cleavage for peptides containing these latter amino acids, the resin bound protected peptides **1**, **2**, and **3** were treated separately with trifluoroacetic acid (TFA)-thianisole (9:1, v/v) in the presence of *m*-cresol and ethanedithiol (EDT). The products were purified by reverse phase (RP) HPLC, yielding homogeneous linear peptides L-Tyr-hexamer **5**, and D-Tyr-hexamer **6** from precursors **1** and **2**, respectively. The hexameric L/D-Pmp containing peptide mixture, resulting from precursor resin-peptide **3**, could be separated by RP-HPLC into two diastereomeric components: the early eluting L-Pmp-hexamer **7** (el. time 12.6 min), and the later eluting D-Pmp-hexamer **8** (el. time 13.5 min). The stereochemical identity of Pmp amino acids in these two peptides was determined by analysis of their relative proteolytic susceptibility to amino peptidase M digestion, and by comparison of their circular dichroism (CD) spectra to those of authentic linear L-Tyr-peptide **5**, and D-Tyr-peptide **6**.

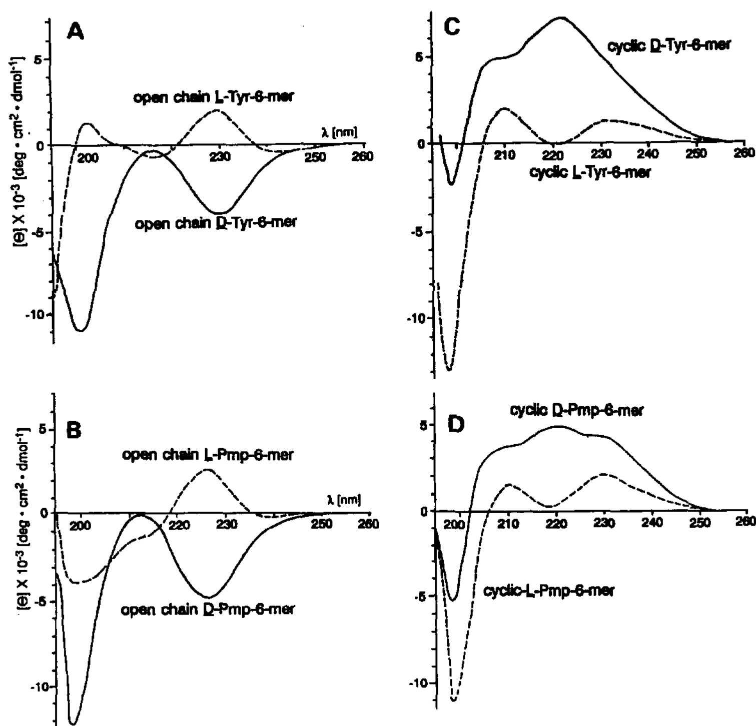


Figure 1. CD spectra of open chain and cyclic peptides in methanol (240 μM): **A**, open chain L- and D-Tyr hexamers, **5** and **6**; **B**, open chain L- and D-Pmp hexamers **7** and **8**; C, cyclic L- and D-Tyr hexamers, **14** and **15**; and **D**, cyclic L- and D-Pmp hexamers **18** and **19**.

Aminopeptidase M acts on the N-terminal end of peptides and proteins and its specificity for cleaving only L-amino acids is well documented.[15] When the L-Tyr hexamer **5** and the faster eluting Pmp-hexamer **7** were treated with this enzyme at 37 °C for 20 hr, HPLC analysis indicated complete consumption of both peptides. Similar enzyme treatment of D-Tyr hexamer **6** and the slower eluting Pmp hexamer **8** left both peptides unchanged, as determined by HPLC and by FAB-MS analysis of the recovered peptides. The correctness of the mass spectrometrically determined molecular weight also confirmed that the N-terminal glycine is resistant to proteolysis when the C-terminal proximate amino acid has the D-configuration. A prominent feature observed in the CD spectrum of L-Tyr hexamer **5** in methanol is the positive ellipticity peak at 229 nm (Figure 1A). This is attributable to the interactions of the tyrosyl and amide chromophores,[16,17]and as such, is dependent on the configuration of the tyrosyl moiety. Appropriately, the D-Tyr hexamer **6** exhibited a significant negative ellipticity peak 229 nm. In comparison, the Pmp hexamer **6**, which was assigned the L-Pmp configuration, also exhibited the prominent positive ellipticity maximum at 227 nm, while the D-Pmp containing peptide **8** showed a near mirror image negative peak at 227 nm (Figure 1B).

In the synthesis of Gly-L-Tyr(O-PO_3H_2)-Val-Pro-Met-Leu, **9**, stronger acid conditions were required for the removal of the methyl protective group from the phosphate side chain.[18,19] Two methods were explored for this purpose.[20] The resin bound monomethyl protected L-Tyr-phosphate containing peptide **4** was treated with 1M trifluoromethanesulfonic acid (TFMSA)-1M thioanisole in TFA, in presence of *m*-cresol and EDT, providing the fully deprotected linear phosphopeptide **9** in 69% yield. In an alternate procedure we used 1M trimethylsilyl bromide (TMSBr)-1M thioanisole in presence of *m*-cresol and EDT. The latter TMSBr reagent based procedure provided the fully deprotected identical product **9** in a higher yield (80%) after HPLC purification. The TMSBr reagent based deprotection procedure produced less side products, based on analytical HPLC (not shown), and therefore this second procedure is favored. The CD spectrum of phosphopeptide **9** revealed a prominent positive ellipticity maximum at 224 nm (Experimental), similar to features found in the spectra of the L-Tyr and L-Pmp containing peptides. The lower wavelength region in the spectrum (190-215 nm) more closely resembled the spectrum of the L-Pmp peptide than that of the L-Tyr-hexamer.

Synthesis and characterization of cyclic peptides. (Figures 2 and 3) The general strategy employed here involved incorporation of protected tyrosine, Pmp or tyrosine phosphate amino acids into peptides, followed by selective cleavage of peptides from the resin under mild conditions. The side chain protected peptides were then purified, and in the case of Pmp containing peptide the diastereomeric components were separated at this stage. Each such peptide was cyclized and side chain deprotected.

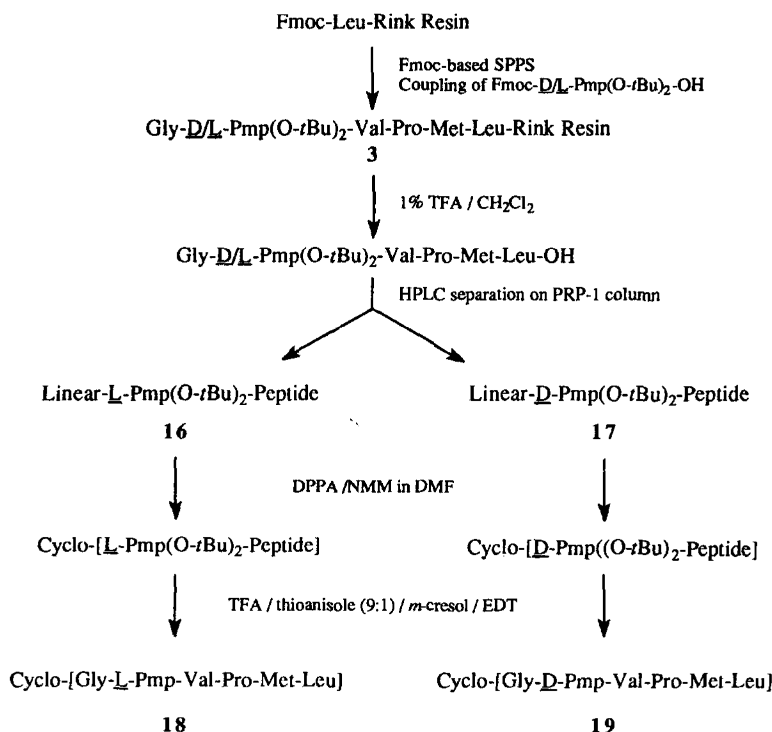


Figure 2. Synthesis of cylic L- and D-Pmp hexamer peptides.

Diphenylphosphoryl azide (DPPA) was chosen for backbone cyclization of the hexamers. This reagent has been applied to the cyclization of a wide variety of peptides under mild conditions,[21,22] with low degree of racemization,[21,23] and is particularly applicable to hexamer peptides with turn inducing amino acids, such as Gly, Pro, and D configuration amino acids.[24,25] It is to be noted, however, that other more versatile cyclizing reagents have been reported recently.[26]

For the synthesis of the tyrosine containing peptides, protected peptide resins **1** and **2** were therefore cleaved from the resin under mild conditions with 1% TFA in CH_2Cl_2, and HPLC purified to produce L-Tyr(O-tBu)-hexamer **10** and the D-Tyr analog **11**. Cyclization of the latter two peptides was accomplished with DPPA in presence of the non-nucleophilic base N-methylmorpholine (NMM), at ice temperature over 48 hr, with a yield of 26-28%. Side chain deprotection was accomplished with TFA-thioanisole (9:1, v/v) in presence of antioxidants to produce cyclic peptides **14** and **15**. Similarly, the L/D-Pmp(O-*t*Bu)$_2$ containing peptide resin **3** was cleaved from the Rink resin with 1% TFA in CH_2Cl_2. The diastereomeric pair of *t*-butyl protected open chain phosphonopeptides were preparatively separated on a polymer based RP column (PRP-1) to yield a 1:1 ratio of peptides **16** and **17**, assigned to the L-Pmp and D-Pmp hexamers, respectively. The stereochemical assignments are based on confirmation of HPLC identity of side chain deprotected **16** and **17** with the fully deprotected open chain peptides **7** and **8**, and by comparison of the CD spectra of the cyclic Tyr and Pmp analogs (Figure 1C and 1D)(vide infra). Cyclization of the open chain Pmp protected peptides **16** and **17** was affected in dilute solutions of DMF with 5x molar excess of DPPA, in the presence of NMM for 48 hr. The unpurified products were treated with side chain deprotecing agents, TFA-thioanisole (9:1, v/v) and antioxidants, to produce separately, after HPLC purification, the cyclic L-Pmp peptide **18**, and the D-configuration analog **19** in 46-49% yield. The stereochemical assignment was also confirmed by the observation that the CD spectrum of cyclic L-Pmp peptide **18** (Figure 1D) is essentially identical to that of cyclic L-Tyr peptide **14** (Figure 1C), where the latter peptide was prepared from authentic L-tyrosine. These low intensity spectra are similar to that of cyclo[Gly$_5$-L-Tyr] reported earlier,[16,27] and suggest a less rigid conformational state, or the presence of several conformers, at least in the methanol solvent used here. Also, there is a close similarity between the spectra of cyclic D-Tyr peptide **15** and the D-Pmp analog **19**. The stronger intensity spectra of the D amino acid containing cyclic peptides express a higher conformational integrity, compared to the all-L configuration analogs.[28]

For the synthesis of L-tyrosine phosphate containing peptide, the strategy involved the Fmoc method of SPPS, while the dimethyl protected L-tyrosine phosphate was attached in its N$^{\alpha}$-Boc protected form as the very last amino acid in the sequence (Figure 3). In this manner the peptide could be removed from the resin simultaneously with Boc deprotection under the acidic conditions (95% TFA) to produce the phosphate protected linear peptide **20**, and without the need for using basic reagents that might remove the protective groups from the tyrosine phosphate in the peptide.[13] Cyclization was affected with DPPA and the crude product submitted to side chain deprotection with 1M TMSBr-1M thioanisole in TFA to provide the desired cyclo[Gly-L-Tyr-(OPO_3H_2)-Val-Pro-Met-Leu], **21** in 26% yield. The CD spectrum of this peptide (Experimental) is qualitatively similar to that of cyclic L-Pmp and L-Tyr containing peptides.

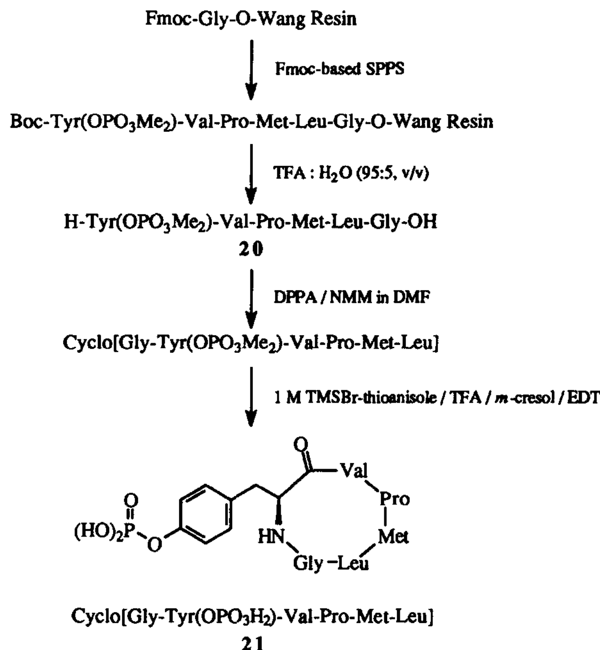


Figure 3. Synthesis of L-tyrosine phosphate containing cyclic peptide.

The sequence of the synthetic peptides described here derives from the autophosphorylation site of PDGF receptor ß subunit which functions in its receptor activated phosphorylated form to bind to and activate various enzymes involved in the cellular signal transduction machinery. The phosphatase resistant Pmp analogs of tyrosine phosphates in peptides compete effectively with the natural sequences in protein binding.[29] In binding assays to the SH2 domain of target proteins the linear and cyclic L-Tyr(OPO_3H_2) and L-Pmp peptides described here showed good binding affinities, the D-Tyr(OPO_3H_2) and D-Pmp analogs were less effective, while the Tyr containing peptides peptides were found to be inactive.[30] Structure-activity studies with the conformationally more restricted molecules are expected to lead to the development of more effective pharmacologic agents.

EXPERIMENTAL

Solid-phase peptide synthesis was carried out in the manual mode using Fmoc chemistries with the acid sensitive 4-(2',4'-dimethoxyphenyl-hydroxymethyl)-phenoxy Rink Resin[11] (source: Bachem Inc., Torrence, CA) or the *p*-alkoxybenzyl alcohol (Wang) Resin. The amino acid derivatives were purchased from Bachem Inc. Fmoc- and Boc-Tyr(OPO_3Me_2)-OH were purchased from Peninsula Lab. Inc. (Belmont, CA). DIPCDI, DMAP, TFA, NMM, TFMSA and TMSBr were purchased from Aldrich Chem. Co.(Milwaukee, WI), and

piperidine and DPPA were from Fluka Chem. Co. (Ronkonkoma, NY). Fmoc-L/D-Pmp(O-*t*Bu)$_2$-OH was synthesized according to methodology developed in our laboratory.[5] FAB mass spectra were run on a VG-7070E-HF mass spectrometer, using a Xenon atom gun and glycerol as sample matrix. CD spectra were recorded on a Jasco Model J500A/DP501N CD spectropolarimeter, in Hellma cells with a path length of 1 mm. Both UV and CD spectra were run in methanol solutions. Amino acid analysis (aaa) was performed at the Protein Structure Laboratory, University of California, Davis, CA. Both analytical and preparative HPLC were performed on an LKB Model 2150 titanium lined solvent gradient system, using an LKB Model 2140 Diode Array Rapid Spectral Detector. HPLC conditions (unless indicated otherwise), Method A: Vydac 5C18 (10 x 250 mm) column; Solvent gradient, A: 0.05% TFA in water: B: 0.05% TFA in 90% acetonitrile in water: gradient (B%) : 20 - 80% over 30 min; flow rate, 2.5 mL/min; UV detector, 225 nm. Amino acids are of L-configuration unless otherwise indicated.

Fmoc-Leu-Rink Resin. The attachment of the first amino acid to the resin was accomplished by incubating the Rink Resin[11] (2.0 g, 0.35 mmol/g), Fmoc-Leu-OH (2.47 g, 7.0 mmol), DIPCDI (1.1 mL, 7.0 mmol), and DMAP (85 mg, 0.70 mmol) in DMF (25 ml) at 25 °C for 2 hr. The resin was rinsed with DMF and CH_2Cl_2 (20 mL, 5 times each) and dried in vacuo (yield 2.14 g).

H-Gly-X-Val-Pro-Met-Leu-Rink Resins [X: L-Tyr(O-*t*Bu)(1), D-Tyr(O-tBu)(2), L/D-Pmp(O-*t*Bu)$_2$ (3), L-Tyr(O-PO$_3$Me$_2$)(4)]. Fmoc-Leu-Rink Resin (1.07 g, 0.34 mmol/g) was deprotected with 20% piperidine in DMF (25 mL, 15 min) and rinsed with DMF (5x20 mL), and the Fmoc protected Met, Pro, Val, and Tyr(O-*t*Bu) or Pmp(O-*t*Bu)$_2$ or Tyr(O-PO$_3$Me$_2$) amino acids were condensed in sequence using a 5-fold molar excess each of amino acid, DIPCDI, and HOBt in DMF (20 mL) per cycle (1 hr, shaking). DMF was used in the washing cycles (5x), and 20% piperidine in DMF (25 mL) was used for Fmoc deprotection (15 min). Coupling of the N-terminal Gly was achieved using 5 equivalents of pentafluorophenyl Fmoc-glycinate (Fmoc-Gly-OPfp) in DMF over a period of 3 hr. Yield of the resin bound side-chain protected peptides: **1**, 1.23 g; **2**, 1.26 g; **3**, 1.20 g; **4**, 1.28 g.

H-Gly-X-Val-Pro-Met-Leu-OH [X: L-Tyr (5), D-Tyr (6), L-Pmp (7), D-Pmp (8)]. The peptide resins **1, 2,** and **3** (approx 200 mg each, 0.28 mmol/g) were treated separately with TFA-thioanisole (1.8 mL: 0.2 mL) in presence of *m*-cresol (50 μL) and EDT (50 μL) at 4 °C. After 1 hr the resin was removed by filtration and the crude peptides were precipitated from the filtrate by addition of petroleum ether (50 mL). Solvent was removed by decantation and the residue was triturated with Et_2O, collected by centrifugation, and then lyophilized from 20% AcOH (10 mL). The peptides were redissolved in 20% AcOH and purified by chromatography (HPLC Method A). **L-Tyr Hexamer, 5:** 30.4 mg (80%); HPLC ret. time, 16.0 min; FAB-MS (M+H)+ 679.4(calc 679.3); UV λ_{max} 278 nm (ε 1,600), 284 (sh); CD Figure 1A; aaa Gly(1.05), Tyr(1.05), Val(1.02), Pro(0.98), Met(0.84), Leu(1.05). **D-Tyr Hexamer, 6:** 29.6 mg (78%); HPLC ret. time, 16.9 min; FAB-MS (M+H)+ 679.3(calc 679.3); UV λ_{max} 278 nm (ε 1,580), 284 (sh); CD Figure 1A; aaa Gly(1.06), Tyr(1.04), Val(1.03), Pro(1.00), Met(0.80), Leu(1.06). CD (MeOH) Figure 1A. The diastereomeric peptide pair **D/L-Pmp Hexamer, 7/8**, was separated by HPLC (Method A) into 2 components. **L-Pmp Hexamer,**

7: 16.3 mg (40%); HPLC ret. time, 12.6 min; FAB-MS (M-H)$^-$ 755.2(calc 755.3); UV λ_{max} 264 nm (ε 350), 258 (sh), 272 (sh); CD Figure 1B; aaa Gly(1.07), Val(1.03), Pro(1.04), Met(0.79), Leu(1.07). **D-Pmp Hexamer, 8:** 16.9 mg (41%); HPLC ret. time, 13.5 min; FAB-MS (M-H)$^-$ 755.3(calc 755.3); UV λ_{max} 264 nm (ε 330), 258 (sh), 272 (sh); CD Figure 1B; aaa Gly(1.06), Val(1.05), Pro(1.04), Met(0.77), Leu(1.08).

Assignment of the absolute configuration of Pmp amino acids in peptides 7 and 8. The relative hydrolysis rates of peptides in presence of aminopeptidase-M were measured. Enzyme digestion: Peptides **7** and **8** (100 μg in 200 μL 50 mM Na-phosphate buffer, pH 7.0) were separately incubated with aminopeptidase-M (Boehringer-Mannheim, EC 3.4.11.2)(10 μL, 0.2 units) at 37°C for 20 hr. The reaction was quenched with 10% AcOH (100 μL) and analysed by HPLC. The D-Pmp containing hexamer **8** was completely resistant to proteolysis, as confirmed by FAB-MS and HPLC analysis. Under such conditions the L-Pmp hexamer, assigned to compound **7**, was completely proteolysed. Enzymatic digest of authentic L- and D-Tyr hexamers **5** and **6** served in control experiments. Comparative analysis of the CD spectra of authentic L- and D-Tyr containing hexapeptides to the Pmp analogs confirmed the stereochemical assignments.

H-Gly-L-Tyr(O-PO$_3$H$_2$)-Val-Pro-Met-Leu-OH (9). Method 1: The peptide resin **4** (150 mg, 0.27 mmol/g) was treated with 1M TFMSA-1M thioanisole/TFA (5 mL) in presence of *m*-cresol (50 μL) and EDT (50 μL) at 4°C for 1 hr, then filtered. Cold petroleum ether (50 mL) was added to the solution to precipitate the desired peptide and the solvent decanted. The solid was triturated with diethyl ether (50 mL). The resulting powder was collected by centrifugation, dissolved in aq. AcOH and HPLC purified (HPLC Method A) to provide peptide **9**: 21 mg (69%); HPLC ret.time, 12.3 min; FAB-MS (M-H)$^-$ 757.3 (calc 757.3); UV λ_{max} 267 nm (ε 670), 272 (sh); CD (MeOH) 224 nm ([θ], +3,260), 237 (-670); aaa Gly(1.01), Tyr(0.99), Val(1.00), Pro(1.02), Met(0.97), Leu(1.02). Method 2: The peptide resin **4** (210 mg, 0.27 mmol/g) was treated with 1M TMSBr-1M thioanisole (5 mL) in presence of *m*-cresol (50 μL) and EDT (50 μL) at 25 °C for 3 hr, then filtered. The desired peptide was isolated and purified as above providing peptide **9**, 34.5 mg (80%), with identical physicochemical properties.

H-Gly-L-Tyr(O-*t*Bu)-Val-Pro-Met-Leu-OH (10), and H-Gly-D-Tyr(O-*t*Bu)-Val-Pro-Met-Leu-OH (11). The resin bound protected peptides **1** and **2** (1.0 g, 0.27 mmol/g) were treated separately with 1% TFA/CH$_2$Cl$_2$ (50 mL) at 25 °C for 5 min with shaking. The solution was filtered off and neutralized with NMM in an ice bath. The resin was treated similarly 3 more times with 50 mL 1% TFA/CH$_2$Cl$_2$. The combined neutralized solution (200 mL) was evaporated at 25 °C in vacuo, the residue extracted with 30% aqueous CH$_3$CN (10 mL) and the solvent evaporated. The product was purified by HPLC (Method A). **L-Tyr(O-*t*Bu) Hexamer, 10:** 98 mg (49%); HPLC ret. time, 19.0 min.; FAB-MS (M-H)$^-$ 733.4(calc 733.4); aaa Gly(1.00), Tyr(1.02), Val(1.06), Pro(1.04), Met(0.82), Leu(1.06); **D-Tyr(O-*t*Bu) Hexamer, 11:** 92 mg (46%); HPLC ret. time, 20.5 min; FAB-MS (M-H)$^-$ 733.4(calc 733.4); aaa Gly(1.00), Tyr(1.00), Val(1.03), Pro(1.03), Met(0.90), Leu(1.05).

Cyclo[Gly-L-Tyr(O-*t*Bu)-Val-Pro-Met-Leu] (12), and cyclo[Gly-D-Tyr(O-*t*Bu)-Val-Pro-Met-Leu] (13). Separately, the side chain protected hexamers **9** and **10** (20 mg, 27 μmol) were dissolved in DMF (200 mL), and NMM (6 μL, 54 μmol) and DPPA (29 μL, 136 μmol) was added while stirring in an ice bath. After 48 hr, the reaction mixture was neutralized with AcOH and evaporated in vacuo at 40°C. The residue was triturated with petroleum ether (2 x 100 mL), then dissolved in 50% aq. AcOH (10 mL), followed by HPLC purification (Method A). Peptide **12**: 5.1 mg (26%); HPLC ret. time, 28.6 min.; FAB-MS $(M-H)^-$ 715.2(calc 715.4); aaa Gly(1.02), Tyr(1.01), Val(1.04), Pro(1.04), Met(0.84), Leu(1.05); Peptide **13**: 5.4 mg (28%); HPLC ret.time, 26.8 min.; FAB-MS $(M-H)^-$ 715.3(calc 715.4); aaa Gly(1.01), Tyr(1.00), Val(1.03), Pro(1.04), Met(0.88), Leu(1.05).

Cyclo[Gly-L-Tyr-Val-Pro-Met-Leu] (14), and cyclo[Gly-D-Tyr-Val-Pro-Met-Leu] (15). The above protected cyclic peptides **12** and **13** (5.1 mg and 5.3 mg, respectively) were treated separately with TFA-thioanisole (1.8 mL: 0.2 mL) in presence of *m*-cresol (50 μL) and EDT (50 μL) at 4 °C. After 1 hr the product was precipitated by addition of petroleum ether (100 mL), the solvent was decanted and petroleum ether treatment repeated one more time. The solid residue was dissolved in 50% AcOH (10 mL) for HPLC purification. Peptide **14**: 2.2 mg (47%); HPLC ret.time, 20.0 min; FAB-MS $(M-H)^-$ 661.4(calc 661.3); UV λ_{max} 278 nm (ε 1,630), 284 (sh); CD Figure 1C; aaa Gly(1.03), Tyr(1.05), Val(1.02), Pro(1.01), Met(0.82), Leu(1.06). Peptide **15**: 2.6 mg (53%); HPLC ret.time, 20.9 min; FAB-MS $(M-H)^-$ 661.4 (calc 661.3); UV λ_{max} 278 nm (ε 1,650), 284 (sh); CD Figure 1C; aaa Gly(1.05), Tyr(1.04), Pro(1.03), Met(0.80), Leu (1.06).

H-Gly-L-Pmp(O-*t*Bu)$_2$-Val-Pro-Met-Leu-OH (16), and H-Gly-D-Pmp(O-*t*Bu)$_2$-Val-Pro-Met-Leu-OH (17). The protected peptide resin **3** (1.0 g, 0.27 mmol/g) in 1% TFA/CH_2Cl_2 (50 mL) was stirred at 25 °C for 5 min. The cleaved peptide in the TFA solution was filtered off and neutralized with NMM at ice bath temperature. TFA/CH_2Cl_2 treatment of the resin was repeated 3 more times. The solvent was evaporated from the combined neutralized solution at 25 °C in vacuo, and the resulting residue was extracted with 30% aqueous CH_3CN (10 mL). The diastereomeric peptides were separated and purified by preparative HPLC on a PRP-1 (Hamilton) column (20 x 250 mm) using gradient elution water - CH_3CN (5 - 50% CH_3CN, 30 min), flow rate 20 mL/min: Peak A, ret.time 22.6 min, and peak B, ret.time 23.3 min.

Peptide **16**: HPLC peak **A**: 63 mg (27%); ret.time (Method A), 20.1 min; FAB-MS $(M-H)^-$ 867.5(calc 867.4); aaa Gly(1.00), Val(1.05), Pro(1.05), Met(0.84), Leu(1.06). Peptide **17**: HPLC peak **B**: 61 mg (26%); ret.time (Method A), 21.0 min, FAB-MS $(M-H)^-$ 867.5(calc 867.4); aaa Gly(0.98), Val(1.03), Pro(1.04), Met(0.88), Leu(1.06). To ascertain the stereochemistry of Pmp substituents in **16** and **17**, the two peptides were side-chain deprotected (TFA - thioanisole (9:1, v/v), 2.5% *m*-cresol, 2.5% EDT), and identity of HPLC mobility with **7** and **8** was confirmed.

Cyclo[Gly-L-Pmp-Val-Pro-Met-Leu] (18), and cyclo[Gly-D-Pmp-Val-Pro-Met-Leu] (19). Separately, the linear protected peptides **15** and **16** (15 mg each, 17.3 μmol) were dissolved in DMF (150 mL), cooled to ice bath temperature, followed by slow addition of NMM (4 μL, 35 μmol) and DPPA (19 μmol, 87

μmol). After 48 hr the mixture was neutralized with AcOH, and the solvent evaporated in vacuo at 40 °C. The residue was triturated with petroleum ether (2 x 50 mL). To ensure complete removal of the *t*-butyl protecting groups the crude oily product was treated with TFA-thioanisole (0.9 mL : 0.1 mL) in presence of *m*-cresol (50 μL) and EDT (50 μL) at 4°C for 1 hr. Petroleum ether (100 mL) was added to precipitate the peptide, and the washing solvent was decanted (x2). The residue was dissolved in 50% aq. AcOH (10 mL) and HPLC purified on a Vydac 5C18 column (Method A). Cyclic peptide **18**: 2.5 mg (46%); HPLC ret.time, 17.0 min; FAB-MS $(M-H)^-$ 737.3 (calc 737.3), $(M+H)^+$ 739.4 (calc 739.3); UV λ_{max} 264 nm (ε 640), 258 (sh), 272 (sh); CD Figure 1D; aaa Gly(1.00), Val(0.98), Pro(1.02), Met(0.54), Leu(1.00), Pmp not analyzed. Cyclic peptide **19**: 3.3 mg (49%); HPLC ret. time 17.2 min; FAB-MS $(M-H)^-$ 737.3 (calc 737.3), $(M+H)^+$ 739.4 (calc 739.3); UV λ_{max} 264 nm (ε 710), 258 (sh), 272 (sh); CD Figure 1D; aaa Gly(1.01), Val(0.98), Pro(1.00), Met(0.50), Leu(1.01), Pmp not analyzed.

H-L-Tyr-(OPO_3Me_2)-Val-Pro-Met-Leu-Gly-OH (20). A mixture of *p*-alkoxybenzyl alcohol resin (0.95g, 0.6 mmol), Fmoc-Gly-OH (0.89 g, 3.0 mmol), DIPCDI (0.47 mL, 3.0 mmol), and DMAP (72 mg, 0.6 mmol) in DMF (25 mL) was shaken at 25 °C for 2 hr. The resin was rinsed with DMF (3 x 25 mL). Fmoc deprotection was achieved using 20% piperidine in DMF (25 mL) for 10 min. The Fmoc protected Leu, Met, Pro, and Val amino acids were then sequentially condensed (2 hr each) using a 5-fold molar excess of amino acid, DIPCDI and HOBt, in DMF (25 mL), per cycle. Coupling of the terminal Tyr(OPO_3Me_2) was achieved using Boc-Tyr(OPO_3Me_2)-OH (5 equivalents). The peptide was N-terminally deprotected and cleaved from the resin simultaneously by treatment with TFA-H_2O (95:5, 20 mL) for 1 hr. Under these conditions the Me groups on the Tyr(OPO_3Me_2) residue remained intact. The resin was removed by filtration and the solvent removed from the filtrate solution in vacuo at 25 °C. The residue was redissolved in 20% aq. AcOH for HPLC purification on a Vydac 5C18 column, solvent gradient (B%): 20 - 36% over 16 min, to produce peptide **20**: 182 mg(46%); HPLC ret.time, 14.4 min; FAB-MS $(M-H)^-$ 785.5 (calc 785.3), also found 25% rel. intensity ion at m/z 771.5 assigned to minor amount of L-Tyr(OPO_3HMe)-6-mer peptide analog; aaa Tyr(1.04), Val(1.10), Pro(1.09), Met(0.52), Leu(1.14), Gly(1.11).

Cyclo[Gly-L-Tyr-(OPO_3H_2)-Val-Pro-Met-Leu] (21). Cyclization of the linear peptide **20** (25 mg, 0.032 mmol) was accomplished with DPPA (69 μL, 0.32 mmol), and NMM (53 μL, 0.48 mmol) in DMF (40 mL) at 25 °C. After 48 hr AcOH (1 mL) was added and the solvent removed by evaporation in vacuo at 30 °C. The residue was treated with 1M TMSBr-1M thioanisole/TFA (5 mL) in presence of *m*-cresol (200 μL) and EDT (200 μL) at 25 °C for 3 hr, then 90% of the solvent/reagents were removed in vacuo at 25 °C. Addition of cold diethyl ether (150 mL) produced a white precipitate of the crude cyclic peptide. The product was collected by centrifugation. After dissolution in 20% aq. AcOH (10 mL) HPLC purification on Vydac 5C18 column, solvent gradient (B%): 30 - 50% over 15 min, provided the pure cyclic peptide **21**: 6.1 mg (26%); HPLC ret.time, 11.0 min; FAB-MS $(M-H)^-$ 739.2 (calc 739.3); UV λ_{max} 267 nm (ε 670), 272 (sh); CD (MeOH) 198 nm ([θ],-18,530), 209 (+930), 217 (-1,390), 229 (+1,540); aaa Gly(1.01), Tyr(0.99), Val(1.03), Pro(1.03), Met(0.91), Leu(1.05).

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