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Potent Inhibition of Protein-Tyrosine Phosphatase by Phosphotyrosine-Mimic Containing Cyclic Peptides

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Abstract—In an effort to derive potent and bioavailable protein-tyrosine phosphatase inhibitors, we have previously reported hexameric peptides based on the epidermal growth factor receptor sequence $EGFR_{988-993}$ (Asp-Ala-Asp-Glu-**Xxx**-Leu, where **Xxx** = Tyr), in which the tyrosyl residue has been replaced by the non-hydrolyzable phosphotyrosyl mimics phosphonomethylphenylalanine (Pmp), difluorophosphonomethylphenylalanine (F_2Pmp) and *O*-malonyltyrosine (OMT). Inhibitory potencies (IC_{50} values) of these peptides against the tyrosine phosphatase PTP 1B were 200, 0.2 and 10 μM, respectively. Since cellular penetration of peptides containing highly charged phosphonate residues is compromised, and good bioreversible protection strategies for the F_2Pmp residue have not yet been reported, the OMT residue is of particular interest in that it affords potential new prodrug approaches. In the current study we have prepared cyclized versions of the OMT-containing $EGFR_{988-993}$ peptide in order to increase its proteolytic stability and restrain conformational flexibility. Three different cyclic analogues were synthesized. Two of these were cyclized through the peptide backbone ('head to tail') using in one case a single glycine spacer (heptamer peptide) and in the second instance, two glycines (octamer peptide). In a PTP1-based assay the cyclic heptamer experienced a two-fold loss of potency ($K_i = 25.2 \pm 3.9$ μM) relative to the linear hexamer parent ($K_i = 13 \pm 0.9$ μM), while the cyclic octamer demonstrated a five-fold increase in potency ($K_i = 2.60 \pm 0.11$ μM).The third peptide was cyclized by means of a sulfide bridge between the side chain of a C-terminally added cysteine residue and the β-carbon of a N-terminal acetyl residue. Although the overall size of this ring was identical to that exhibited by the preceding backbone-cyclized octamer, it displayed a three-fold enhancement in potency ($K_i = 0.73 \pm 0.03$ μM). The structural basis for the observed results are discussed. Conformational restrictions induced by cyclization could aid in defining geometries for peptidomimetic design. Finally, it can be speculated that cyclization of other linear PTP-inhibitory peptides, such as the F_2Pmp-containing hexamer, may also increase their potency. Published by Elsevier Science Ltd

Introduction

Aberrations in cellular signaling contribute to a variety of disease processes. For this reason considerable attention is being focused on developing signal transduction modulators as potential therapeutic agents.[1] Protein-tyrosine kinase (PTK)-dependent signaling occupies a position of particular importance within the general scheme of intracellular signaling. PTK-dependent signaling is composed of a triad of interacting components; (1) the protein-tyrosine kinase itself, which generates the phosphotyrosyl (pTyr) pharmacophore, (2) protein-tyrosine phosphatases (PTPs), which dephosphorylate pTyr residues, thereby reversing the action of PTKs and (3) Src homology 2 (SH2) domains, which are protein modules that recognize and bind to pTyr residues contained within peptide or protein sequences. To date, while considerable work has been reported on developing PTK[2–4] and SH2 domain[5,6] inhibitors, much less has appeared in the area of PTP inhibitors. This is in spite of the potential value of PTP inhibitors may have as therapeutics and as tools for studying signaling pathways, particularly since an increasing number of examples are being found, where PTPs serve as positive signal transducers.[7]

We have recently become interested in devising novel PTP inhibitors. As our first step we took note that decamer peptides in which the pTyr reside (**1**) had been replaced by the nonhydrolyzable pTyr mimetic, phosphonomethyl phenylalanine (Pmp, **2**) can bind to PTPs and function as competitive inhibitors with IC_{50} values in the 10–30 μM range.[8,9] In the context of SH2 domain inhibitors, we had demonstrated that introduction of two fluorines on the phosphonate α-methylene bridge of Pmp, providing difluorophosphonomethyl phenylalanine (F_2Pmp, **3**),[10–12] results in a nonhydrolyzable pTyr mimetic which more closely approximates the parent pTyr residue. Using the linear peptide sequence, Asp-Ala-Asp-Glu-pTyr-Leu, which corresponds to one of the autophosphorylation sites of the EGF receptor ($EGFR_{988-993}$), and which has recently been shown to be a good substrate for several tyrosine phosphatases, including PTP1,[9] we compared the inhibitory potencies of the corresponding Pmp and F_2Pmp-substituted peptides.[13] While the Pmp-containing peptide Ac-Asp-Ala-Asp-Glu-(D/L-Pmp)-Leu-amide (**7**) exhibited an IC_{50} value of 200 μM against

Key words: phosphatase, inhibitor, signal transduction, mimic, cyclic peptide.

PTP1B-mediated dephosphorylation of phosphorylated insulin receptor, the identical peptide containing L-F_2Pmp (**8**) was 1000-fold more potent, giving an IC_{50} value of 200 nM.[13] The very high PTP affinity of the difluorophosphonate moiety was further demonstrated by small non-peptide based aryl difluorophosphonates which also exhibit good PTP inhibition.[14] We have suggested that the reason F_2Pmp is a better inhibitory motif than Pmp is likely due to the ability of these fluorine atoms to interact specifically with amino acid side chains in the PTP1 active site.[15]

Unfortunately, a significant drawback associated with difluorophosphonate-based inhibitors is poor cellular penetration of highly charged phosphonate structures, and the lack of suitable bioreversible protection strategies for them. In response to this problem, we recently reported the development of the non-phosphonate containing pTyr mimetic, *O*-malonyltyrosine (OMT, **4**), which utilizes the malonate structure to mimic the phosphate portion of pTyr.[16,17] As a dicarboxylic acid, the OMT residue offers prodrug protection strategies which differ from phosphonate protection protocols. The OMT was synthesized as its N^{α}-Fmoc derivative **5** having the malonyl carboxyl groups protected as their *tert*-butyl esters. As previously described,[16,17] a key step in the synthesis of this analogue is the rhodium diacetate-catalyzed 4′-*O*-alkylation of protected L-tyrosine with di-(*tert*-butyl) α-diazomalonate. We next examined the PTP1B inhibitory potency of Ac-Asp-Ala-Asp-Glu-(L-OMT)-Leu-amide (**9**), prepared using this derivative. It was found that **9** had an IC_{50} value of 10 μM, which was 20-fold more potent than the corresponding (D/L)-Pmp-containing peptide (**7**).[18] Furthermore, introduction of a fluorine onto the malonate methylene, giving fluoro-OMT (FOMT, **6**), gave an additional 10-fold increase in potency (IC_{50} value of 1 μM) when incorporated into the same sequence (**10**).[19] Having examined changes in the pTyr structure in order to derive peptide-based PTP inhibitors, we next sought to explore modifications of the peptide itself in order to enhance biological efficacy.

The small peptides which comprised our initial inhibitors exhibit high degrees of conformational flexibility which can result in decreased biological potency due to entropy penalties associated with achieving the relevant active configurations. As a first approximation towards increasing the potency of small peptides, one of the initial modifications frequently undertaken is to cyclize the peptides. By decreasing conformational flexibility, cyclization can lead to increased potency and/or selectivity, and can provide valuable information for the further design of peptide mimetics.[20,21] Applying these considerations to our parent L-OMT-containing $EGFR_{988-993}$ sequence, Asp-Ala-Asp-Glu-(L-OMT)-Leu (**9**), three cyclic derivatives were designed. Peptides **11** and **12** were cyclized head to tail, and utilized either one (**11**) or two (**12**) glycine linkers. Peptide **13** employed a thioether linkage between a N-terminal acetyl residue and the side chain of a C-terminal cysteine amide. Presented herein is the preparation of these peptides and evaluation of their inhibitory potency in a PTP1 assay system.

Syntheses

Two types of OMT-containing cyclic peptides were prepared using Fmoc based chemistry and various solid phase resin based approaches. In one approach, producing peptides **11** and **12**, a C-terminally extended peptide was synthesized on the acid sensitive Rink Resin.[22] Using this resin, the fully side-chain-protected peptide intermediates could be obtained in solution by treatment of the resin for a 5 min period with 1% TFA in CH_2Cl_2. Backbone cyclization through the free N- and C-terminal groups of the protected peptide was effected using the uronium salt of 1-hydroxy-7-azabenzotriazole (HATU) in the presence of an equivalent of 1-hydroxy-7-azabenzotriazole (HOAt).[23] These reagents are currently considered the most efficient coupling agents for the preparation of cyclic peptides.[24] Incorporation of C-terminal glycine in these peptides prevents the possibility of racemization. The cyclic peptide **11** obtained in this manner was HPLC purified, and the amino acid side-chain protective groups, including the di-*t*-Butyl groups of the OMT-residue, were removed with 95% aqueous TFA. In a similar manner, the eight residue OMT-containing peptide **12**, which was also cyclized through the peptide backbone, and its tyrosine analogue **14** were prepared. For this purpose, the linear precursor intermediates were constructed with both N- and C-terminal glycines, extending the $EGFR_{988-993}$ sequence.

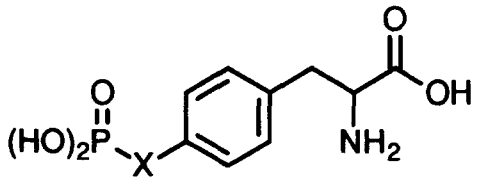
An alternative strategy was employed to cyclize the OMT-containing peptide **13** through the side chain of an added cysteine. This relied on intramolecular attack of the cysteine sulfhydryl on a N-terminal haloacetylated residue.[25] The precursor to this heptameric peptide was synthesized on PAL amide resin[26] with the incorporation of a C-terminal cysteine. All carboxyl side-chains in the constituent amino acids were protected with TFA-cleavable *tert*-butyl groups, whereas the cystein side-chain was protected with the HF-cleavable 4-methoxybenzyl (Mbzl) group. This resin-bound, side-chain-protected peptide was then N-terminally chloroacetylated. After side-chain deprotection and removal from the resin by consecutive treatments with TFA and HF, the resulting peptide was allowed to cyclize by dehydrohalogenation, through the nucleophilic displacement of the N-terminal chloride atom with the cysteine side-chain thiol moiety, under weakly basic conditions. The designed cyclic OMT-containing peptide **13** was obtained in good yield. It was noted in the synthesis of peptide **13** that when the cysteine was protected with the TFA-labile trityl group, very poor yields were achieved (data not shown). The tyrosine-containing cysteine-cyclized analogue **15** was synthesized using *t*-Boc methodology, with amino acids, including (Mbzl)cystein, assembled on an automated instrument, followed by N-chloroacetylation, HF cleavage and cyclization. These latter peptides (**13** and

15) have the same number of bonds in the ring as the above described cyclic octamers **12** and **14**.

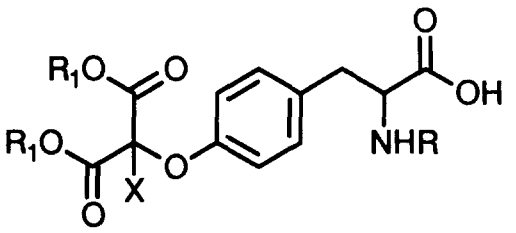
We have found that the four OMT-containing peptides elute as sharp single HPLC peaks by reversed-phase chromatography. However, in principle, malonyl groups are potentially sensitive to decarboxylation. Fast atom bombardment mass spectrometry was therefore employed, which indicated intense protonated molecular ions. At the same time, the mass spectra of these peptides, and all others we have synthesized previously,[17-19] indicate fragment ions corresponding to $[MH^+ - CO_2]$ and $[MH^+ - CH(C_2O_4H)]$, as exemplified for peptide **11** (see Experimental). The latter ion, *m/z* 764.2, would correspond to a protonated tyrosine-containing peptide, presumably arising through gas phase ionization-induced decomposition of the molecular ion. In fact, amino acid analysis of peptide **11**, and of other OMT-containing peptides, confirmed the absence of tyrosine content. Analogously, the $[MH^+ - CO_2]$ ions are generated most probably through ionization-induced fragmentation. In addition, the integrity of the OMT moiety is also confirmed, for example in the case of peptide **11**, by the presence of a singlet at δ 5.09 in the 1H NMR spectrum, corresponding to the malonyl 2-methine proton.

Results and Discussion

This study was undertaken as the continuation of a larger effort to derive potent bioavailable PTP inhibitors.[19] The starting point for the present work was our previous finding that linear OMT-containing peptide **9**, which is based on the $EGFR_{988-993}$ sequence,[9] exhibited good inhibitory potency (an IC_{50} value of 10 μM against PTP1B dephosphorylation of phosphorylated insulin receptor[18]). The present study prepared cyclized analogues in order to constrain conformational flexibility. Under the assay conditions presented herein (all results are shown in Table 1), linear OMT-containing



1 pTyr X = O
2 Pmp X = CH_2
3 F_2Pmp X = CF_2

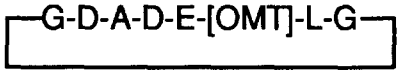


4 OMT X = H; R = H; R_1 = H
5 Fmoc-OMT(*t*-Bu)$_2$-OH X = H; R = Fmoc; R_1 = *t*-Bu
6 FOMT X = F; R = H; R_1 = H

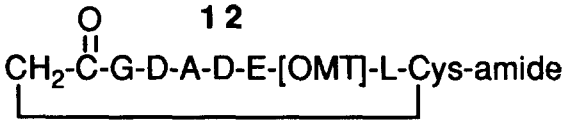
Ac-D-A-D-E-**X**-L-amide

7 X = Pmp
8 X = F_2Pmp
9 X = OMT
10 X = FOMT

11

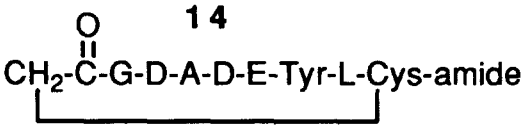


12



13

14



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Structures 1.

peptide **9** exhibited a K_i value of 13 μM, in good agreement with the previously reported IC_{50} value obtained under different conditions.[18] Head to tail cyclization of this peptide through an added Gly residue (heptamer OMT peptide **11**) resulted in a two-fold loss of inhibitory potency (K_i value = 25.2 μM), while expanding the ring size by addition of a second Gly residue resulted in a 10-fold increase in potency (peptide **12**, K_i value = 2.60 μM), thereby providing a net fivefold increase in potency relative to linear parent **9**. Addition of this second Gly residue represents a three atom enlargement of the ring backbone size. When a peptide containing the same number of ring bonds was constructed using a ring closure via a sulfide linkage (peptide **13**), a further threefold enhancement in potency was observed (K_i value of 0.73 μM) relative to the non-sulfur containing **12**, and an overall 18-fold increase in potency relative to **9**. To eliminate the possibility that the cyclo-peptides themselves bind to PTP1, we also measured the effect of control peptides (**14** and **15**) which lack the *O*-malonyl moiety. Peptides **14** and **15** did not show observable inhibition at up to 0.8 mM concentration (Table 1). With a K_i value of 0.73 μM, OMT peptide **13** is among the most potent PTP inhibitors yet reported. It begins to approach the level of potency of our previously reported F_2Pmp containing peptide, Ac-Asp-Ala-Asp-Glu-F_2Pmp-Leu-amide (**8**) (IC_{50} = 0.2 μM),[13] yet does not contain a phosphonate pharmacophore. Since cyclization of the linear OMT-containing peptide **9** in the fashion represented by **12** resulted in a significant enhancement of affinity, it could be speculated that similar cyclization of linear F_2Pmp-containing peptide **8** may also enhance its affinity, thereby resulting in an inhibitory potency greater than the 0.2 μM value observed with the linear peptide.

Table 1. Structures of OMT-containing peptides and their associated inhibition constants as determined in a PTP1 assay system as described in the Experimental

	K_i (μM)
Asp-Ala-Asp-Glu-OMT-Leu **9**	13 ± 0.9
Asp-Ala-Asp-Glu-OMT-Leu **11**	25.2 ± 3.9
Asp-Ala-Asp-Glu-OMT-Leu **12**	2.60 ± 0.11
Asp-Ala-Asp-Glu-OMT-Leu **13**	0.73 ± 0.03
Asp-Ala-Asp-Glu-Tyr-Leu **14**	>800
Asp-Ala-Asp-Glu-Tyr-Leu **15**	>800

The structural basis for the differences in potency exhibited by peptides **9–13** is not known. Variations in potency could reflect cyclization-induced alterations in both the orientation of the OMT residue within the pTyr binding pocket and/or in interactions of the remainder of the peptide with the enzyme outside of this pocket. X-ray studies on a catalytically inert Cys^{215}→Ser mutant PTP1B complexed to the hexapeptide, Asp-Ala-Asp-Glu-pTyr-Leu-amide, have demonstrated the important contribution played by the pTyr residue in overall peptide binding, with the pTyr residue contributing over 50% of the peptide's solvent accessible surface area buried as a result of peptide binding.[27] Consistent with this, we have previously shown that PTPs do not bind tyrosine-bearing peptides that lack the phosphate functionality.[28] In the current study, the inactivity of control peptides **14** and **15**, which had underivatized Tyr residues in place of OMT residues, also indicates that binding of the peptides is highly dependent on the pTyr mimic. The critical role played by this binding, previously led us to examine the manner in which the OMT residue may be bound. Using molecular modeling techniques we determined that the OMT malonyl structure, while occupying a slightly larger volume, could nonetheless duplicate nearly identically the binding interactions of the parent tyrosine phosphate.[18]

Changes in the larger peptide structure through cyclization, could potentially alter overall binding affinity by modifying the orientation of the malonyl pharmacophore within the pTyr binding pocket. Since binding of the parent linear OMT peptide **9** may not allow optimal orientation of the OMT residue within its binding pocket, some of the enhancement in potency shown by **12** and **13** may be due to conformationally induced better fit of the OMT residue itself. Alternatively, the loss of potency shown by **11** may be at least partially attributable to poorer binding of the OMT residue.

Cyclization-induced variations in enzyme interactions with other portions of the peptide besides the OMT residue, may also contribute to differences in potency for peptides **9–13**. Kinetic studies on the $EGFR_{988-993}$ sequence have shown that adding residues to the core 'Asp-Ala-Asp-Glu-pTyr' increases binding affinity little.[9] The previously mentioned X-ray structure of the hexapeptide, Asp-Ala-Asp-Glu-pTyr-Leu-amide bound to the Cys^{215}→Ser mutant PTP1B,[27] defines the major binding interactions of linear pTyr-containing peptides based on the $EGFR_{988-993}$ sequence. Although the X-ray data may not reflect the optimum binding for

these or similar peptides, based on this data linear peptide **9** is probably long enough to span the major peptide binding domain. Differences between the binding of the OMT and pTyr residues within the catalytic pocket may result in somewhat different overall modes of binding for linear OMT peptide **9** relative to analogous pTyr-containing peptides such as Asp-Ala-Asp-Glu-pTyr-Leu-amide. This is supported by our recent photoaffinity labeling experiments which indicate that multiple modes of substrate recognition exist for PTP1.[29] The loss of potency of peptide **11** may reflect conformational restriction to less favorable binding orientations, whereas the enhanced potencies of **12** and **13**, which are conformationally more flexible, may be attributable to their freedom to assume higher affinity conformations.

The high potency of peptide **13** warrants further studies on the possible application of cyclic peptides for the development of PTP inhibitors. Besides potential gains in inhibitory potency, cyclic inhibitors would be proteolytically more stable than their linear counterparts. Finally, conformational restrictions induced by cyclization could aid in defining geometries for peptidomimetic design.

Experimental

Solid-phase peptide synthesis was carried out in the manual mode using Fmoc chemistry. Fmoc-OMT (t-Bu)$_2$-OH (**5**) was synthesized according to our published method,[16,17] and the synthesis of Ac-Asp-Ala-Asp-Glu-(OMT)-Leu-amide (**9**) was described earlier.[18] The acid sensitive 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)-phenoxy 'Rink Resin'[22] was purchased from Bachem California Inc. (Torrence, CA) and 'PAL amide resin'[26] and HOAt and its uronium salt, HATU, were purchased from Millipore/Milligen (Bedford, MA). Fmoc derivatives of standard amino acids were obtained from Bachem California or from Millipore/Milligen, with Fmoc-Cys having 4-methoxybenzyl (Mbzl) side chain protection. Trifluoroacetic acid (TFA), *N*-methylmorpholine (NMM) and anisol were purchased from Aldrich Chem. Co. (Milwaukee, WI), and piperidine, diisopropylcarbodiimide (DIPCDI) and chloroacetic acid were obtained from Fluka Chem. Co. (Ronkonkoma, NY). FAB-mass spectra were run on a VG-7070E-HF mass spectrometer, using a Xenon atom gun and glycerol as sample matrix. ^{1}H NMR spectra were run in D_2O on a Bruker AC-250 instrument. Amino acid analyses (aaa) were performed at Peptide Technologies Co. (Gaithersburg, MD). The OMT amino acid could not be analyzed in the peptides. Both analytical and preoperative HPLC was performed on an LKB Model 2150 solvent gradient system, using an LKB Model 2140 Diode Array Spectral detector. HPLC conditions: Vydac 5C$_{18}$ (10 × 250 mm) column; solvent gradient, A: 0.05% TFA in water, B: 0.05% TFA in 90% acetonitrile in water with gradients as indicated below and a flow rate of 2.5 mL/min; UV detector, 220 nm. Amino acids are of L-configuration unless otherwise indicated.

Synthesis of cyclo(Asp-Ala-Asp-Glu-[L-OMT]-Leu-Gly) (11). The first amino acid, Fmoc-Gly-OH (505 mg, 1.7 mmol) was attached to the Rink Resin (500 mg, 0.17 mmol) using DIPCDI (1.7 mmol) and DMAP (0.17 mmol) in 5 mL of DMF (coupling time 2 h), followed by Fmoc deprotection with 20% piperidine:DMF (2 min, then 12 min). Fmoc-Leu-OH (300 mg, 0.85 mmol) was condensed to the Gly–Rink Resin using DIPCDI:HOBT (0.85 mmol each) in DMF (coupling time 1 h), followed by Fmoc deprotection using 20% piperidine/DMF. The subsequent amino acids, Fmoc-OMT(t-Bu)$_2$-OH (**5**), Fmoc-Glu(tBu)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Ala-OH and Fmoc-Asp(tBu)-OH, were sequentially condensed in a similar manner (standard coupling time, 1 h; coupling with **5**, 1.5 h). The protected peptide was cleaved from the Rink Resin (400 mg, 0.10 mmol) with 25 mL of 1% TFA:CH_2Cl_2 (5 min, RT). The solution was collected by filtration and neutralized with NMM in an ice bath. The resin was treated similarly twice more with 1% TFA:CH_2Cl_2, and the combined neutralized solution (75 mL) was then evapd in vacuo at 25 °C. For cyclization, the crude isolate was dissolved in DMF (200 mL), and DIEA (0.30 mmol) and HATU/HOAt (0.15 mmol each) were added with slow stirring in an ice bath. After 24 h the reaction mixture was neutralized with AcOH and evapd in vacuo at 40 °C. The residual material was dissolved in conc AcOH and purified to homogeneity by reversed-phase HPLC (conditions, vide supra; gradient: 60–90% B over 20 min, retention time 20.0 min), providing purified peptide in 53% yield. This side-chain protected cyclic peptide (45 mg) was treated with TFA containing 5% H_2O (1 h, RT). To isolate the peptide, two-thirds of the deprotected solution from the reaction mixture was evapd under N_2, and the mixture was triturated with ice-cold Et_2O. The pptd crude peptide was purified to homogeneity by reversed-phase HPLC (conditions, vide supra; gradient: 10–40% over 25 min; retention time 17.7 min), providing purified **11** in 56% yield. FAB-MS$^+$ 866.2 (calc 866.3) [100% $(M+H)^+$], 822.3 [25% $(M+H-CO_2)^+$], 764.2 [75% $(M+H-CH(C_2O_4H))^+$]. FAB-MS$^-$ 864.5 (calc 664.3) [86% $(M-H)^-$], 820.6 [66% $(M-H-CO_2)^-$], 762.4 [100% $(M-H-CHC_2O_4H)^-$]; ^{1}H NMR (D_2O): δ 5.09 [-OC<u>H</u>(COOH)$_2$]. aaa Asp 1.85 (2), Glu 1.03 (1), Gly 1.07 (1), Ala 1.02 (1), Leu 1.03 (1).

Synthesis of cyclo(Gly-Asp-Ala-Asp-Glu-[L-OMT]-Leu-Gly) (12). Synthesis of **12** was achieved using methodology similar to that described above for the preparation of **11**. The yield of purified intermediate *tert*-butyl-protected peptide was 25% [HPLC gradient, 60–90% B over 20 min, retention time, 18.9 min. MS $(M+H)^+$ 1203.6 (calc 1203.8)]. The *tert*-butyl-protected cyclic peptide was deprotected using TFA containing 5% H_2O according to conditions described above, providing peptide final **12** in 57% yield after HPLC purification (HPLC gradient, 10–40% B over 25 min, retention time: 17.9 min). MS $(M+H)^+$ 923.5 (calc 923.3), $(M-H)^-$ 921.6 (calc 921.3); aaa Asp 1.97

(2), Glu 1.00 (1), Gly 2.00 (2), Ala 1.02 (1), Leu 1.01 (1).

Synthesis of cyclo(Gly-Asp-Ala-Asp-Glu-Tyr-Leu-Gly) (14). Synthesis of **14** was achieved using methodology similar to that described above for the preparation of OMT containing analogues, except that the Fmoc-Tyr(*t*-Bu)-OH was incorporated at position 6. Synthesis was performed using 500 mg (0.17 mmol) of Rink Resin, providing the intermediate side chain-protected cyclic peptide in 17% yield following HPLC purification [gradient, 60–90% B over 20 min retention time, 15.5 min MS $(M+H)^+$ 1045.5 (calc 1045.6)]. After side-chain deprotection using 5% H_2O in TFA (1 h), final product **14** was obtained in 89% yield after HPLC purification (gradient, 20–100% B over 30 min, retention time, 11.0 min). MS $(M+H)^+$ 821.2 (calc 821.3); aaa Asp 1.96 (2), glu 1.12 (1), Gly 2.19 (2), Ala 0.97 (1), Leu 0.98 (1), Tyr 0.78 (1).

Synthesis of cyclo[CH_2CO-Asp-Ala-Asp-Glu-[L-OMT]-Ley-Cys]-amide* (13). (*Structure as shown in Table 1). Preparation of **13** was performed using manual synthesis. The first amino acid, (Fmoc-Cys(MBzl)-OH (352 mg, 0.76 mmol)) was attached to the PAL amide resin (300 mg, 0.11 mmol) with DIPCDI:HOBT (0.76 mmol each) in DMF (3 mL, coupling time 4 h), followed by Fmoc deprotection using 20% piperidine: DMF (2 min, then 12 min). Fmoc-Leu-OH (191 mg, 0.54 mmol) was condensed to the Cys(MBzl)–PAL resin using DIPCDI:HOBt (0.54 mmol) in DMF (3 mL, coupling time 1 h), followed by Fmoc deprotection using 20% piperidine:DMF. The resin-bound protected peptide was N-terminally chloroacetylated using 0.5 M $ClCH_2COOH:CH_2Cl_2$ (5 mL) and DIPCDI (500 mL, 3.2 mmol) (5 min, RT). Cleavage from the resin and removal of all protecting groups except the cysteine MBzl, was done in one step with TFA containing 5% H_2O (0.5 h, RT). The deprotection of the MBzl was accomplished with HF containing 5% anisole. After removal of HF the product was extracted into 50% AcOH and lyophilized. The resulting crude peptide,
Cl-CH_2CO-Asp-Ala-Asp-Glu-[OMT]-Leu-Cys-amide, was purified to homogeneity by HPLC (gradient, 20–50% over 20 min; retention time, 12.3 min). The solution of linear chloroacetylated peptide in the $H_2O:CH_3CN$ (0.05% TFA) HPLC eluent was immediately diluted with 300 mL of H_2O, and adjusted to pH 7–8 with aq NH_3. Under these basic conditions the linear peptide spontaneously cyclizes with concomitant dehydrochlorination. After 12 h solvent was removed by lyophilization and the crude product purified by HPLC (gradient 20–50% B over 20 min; retention time, 10.4 min) to provide **13** in 36% yield. MS $(M+H)^+$ 969.3 (calc 969.3), $(M-H)^-$ 967.5 (967.3); aaa Asp 2.01 (2), Ala 1.03 (1), Leu 1.00 (1), Glu 1.78 (1). (Note: Glu co-elutes with *S*-carboxymethylcysteine).

Synthesis of cyclo[CH_2CO-Asp-Ala-Asp-Glu-Tyr-Leu-Cys]-amide* (15). (*Structure as shown in Table 1). Synthesis was carried out using solid-phase methodology based on tBoc strategy using a Biosearch Model 9600 automated peptide synthesizer. Boc-Cys(MBzl)-OH and MBHA amide resin (Novabiochem, 500 mg, 0.26 mmol) were employed. The protected resin-bound peptide was N-terminally chloroacetylated using 0.5 M $ClCH_2COOH:CH_2Cl_2$ (5 mL) and DIPCDI (500 μL, 3.2 mmol) for 5 min at RT. Cleavage from the resin with concomitant side-chain deprotection was achieved using HF containing 5% anisole. After evaporation of HF, the product was extracted with 50% AcOH, lyophilized and purified by HPLC (gradient of 25–55% B over 20 min), providing a major product with a retention time of 11.2 min. The $H_2O:CH_3CN$ (0.05% TFA) eluate containing the linear chloroacetylated peptide was diluted with 300 mL of H_2O, and adjusted to pH 7–8 by addition of aq NH_3. After 12 h the solution was lyophilized and the resulting residue purified by HPLC (gradient 25–55% B over 20 min; retention time, 9.7 min) to provide product **15** in 23% yield. MS $(M+H)^+$ 867.3 (calc 867.3); aaa Asp 1.91 (2), Ala 0.98 (1), Tyr 1.15 (1), Leu 0.95 (1), Glu 1.61 (1) (Note: Glu co-elutes with *S*-carboxymethylcysteine).

Biological

Recombinant enzymes. The recombinant catalytic domain of PTP1 was created by inserting a stop codon at residue 323, yielding PTP1U323, in order to eliminate the hydrophobic membrane 'targeting' domain of the molecule. Homogeneous recombinant catalytic domain PTP1U323, from here on referred to PTP1, was purified as described.[30]

Enzyme assay. The PTPase activity was assayed at 30 °C in a reaction mixture (0.2 mL) containing appropriate concentrations of *p*-nitrophenyl phosphate as substrate. The buffer used was pH 7.0, 50 mM 3,3-dimethylglutarate, 1 mM EDTA. The ionic strength of the solution was kept at 0.15 M using NaCl. The reaction was initiated by addition of PTP1 and quenched after 2–3 min by addition of 1 mL of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18,000 M^{-1} cm^{-1}. Michaelis–Menten kinetic parameters were determined from a direct fit of the v versus $[S]$ data to the Michaelis–Menten equation using the nonlinear regression program GraFit (Erithacus Software).

Inhibition by the OMT-containing peptides. The inhibition constants for the OMT-containing peptides were determined for homogeneous PTP1 in the following manner. At various fixed concentrations of inhibitors, the initial rate of various *p*-nitrophenyl phosphate concentrations was measured as described.[31] The inhibition was competitive with respect to the substrate. The data were fit to the equation $v = V_{max}S/(K_m[1+K_i]+S)$ using KINETASYST (IntelliKinetics,

State College, PA) to obtain the inhibition constant (K_i).

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