L-*O*-(2-Malonyl)tyrosine: A New Phosphotyrosyl Mimetic for the Preparation of Src Homology 2 Domain Inhibitory Peptides[1]

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Inhibition of Src homology 2 (SH2) domain-binding interactions affords one potential means of modulating protein-tyrosine kinase-dependent signaling. Small phosphotyrosyl (pTyr)-containing peptides are able to bind to SH2 domains and compete with larger pTyr peptides or native pTyr-containing protein ligands. Such pTyr-containing peptides are limited in their utility as SH2 domain inhibitors *in vivo* due to their hydrolytic lability to protein-tyrosine phosphatases (PTPs) and the poor cellular penetration of the ionized phosphate moiety. An important aspect of SH2 domain inhibitor design is the creation of pTyr mimetics which are stable to PTPs and have reasonable bioavailability. To date, most PTP-resistant pTyr mimetics which bind to SH2 domains are phosphonates such as (phosphonomethyl)phenylalanine (Pmp, **2**), [(monofluorophosphono)methyl]phenylalanine (FPmp, **3**) or [(difluorophosphono)methyl]-phenylalanine (F_2Pmp, **4**). Herein we report the incorporation of a new non-phosphorus-containing pTyr mimetic, L-*O*-(2-malonyl)tyrosine (L-OMT, **5**), into SH2 domain inhibitory peptides using the protected analogue L-N^{α}-Fmoc-*O'*-(*O''*,*O''*-di-*tert*-butyl-2-malonyl)tyrosine (**6**) and solid-phase peptide synthesis techniques. Five OMT-containing peptides were prepared against the following SH2 domains: the PI-3 kinase C-terminal p85 SH2 domain (Ac-D-(L-OMT)-V-P-M-L-amide, **10**, IC_{50} = 14.2 μM), the Src SH2 domain (Ac-Q-(L-OMT)-E-E-I-P-amide, **11**, IC_{50} = 25 μM, and Ac-Q-(L-OMT)-(L-OMT)-E-I-P-amide, **14**, IC_{50} = 23 μM), the Grb2 SH2 domain (Ac-N-(L-OMT)-V-N-I-E-amide, **12**, IC_{50} = 120 μM), and the N-terminal SH-PTP2 SH2 domain (Ac-L-N-(L-OMT)-I-D-L-D-L-V-amide, **13**, IC_{50} = 22.0 μM). These results show that peptides **10**, **11**, **13**, and **14** have reasonable affinity for their respective SH2 domains, with the IC_{50} value for the SH-PTP2 SH2 domain-directed peptide **13** being equivalent to that previously observed for the corresponding F_2Pmp-containing peptide. OMT may afford a new structural starting point for the development of novel and useful SH2 domain inhibitors.

Phosphotyrosyl (pTyr)-dependent processes are central to cellular signal transduction and, when aberrant, can contribute to a number of diseases, including cancers and diabetes.[2] Considerable effort is therefore underway to develop therapeutics by modulating pTyr-dependent pathways.[3–6] Signal transduction by pTyr-dependent mechanisms relies on a complex triad of interactions. This includes protein-tyrosine kinases (PTKs), which generate pTyr residues, frequently in response to external stimuli, such as binding of growth hormones to cell surface receptors.[7] A second signaling component is assumed by protein-tyrosine phosphatases (PTPs), which remove pTyr phosphates and may play either positive or negative roles in the overall signal transduction.[8] The third leg of the triad is assumed by binding of secondary signaling proteins to pTyr residues contained within protein structures. This binding can be mediated by Src homology 2 (SH2) domains[9] or by more recently discovered non-SH2 pTyr-binding domains.[10,11] Because of this triad arrangement, inhibitors of pTyr-dependent signaling could theoretically be directed against PTKs, PTPs, or SH2 domains.

SH2 domain inhibitors are particularly attractive targets since three-dimensional structures have been reported for several SH2 domains, including those with small, high-affinity, pTyr-containing peptide ligands bound.[12–20] Such short (usually 5–6 amino acids) pTyr-containing peptides are able to compete with larger pTyr-containing peptides and protein ligands for binding to SH2 domains and afford starting points for SH2 domain inhibitor design.[21] A key feature of high-affinity SH2 domain binding is the near absolute requirement for a "phosphate-like" moiety on the target tyrosyl residue. When this recognition motif is assumed by a phosphate group, a major limitation of the resulting pTyr-containing peptides is their susceptibility to phosphate hydrolysis by PTPs. This has been addressed by the development of nonhydrolyzable phosphonate-based pTyr mimetics. Among these are (phosphonomethyl)-phenylalanine (Pmp, **2**) and its monofluoro (FPmp, **3**) and difluoro (F_2Pmp, **4**) analogues, which have been shown to retain good SH2 domain-binding potency when substituted into appropriate peptides yet are not hydrolyzed by phosphatases.[22,23] Such F_2Pmp-containing peptides have been successfully used in cell-based systems using either permeabilized cells[24] or microinjection techniques.[25] This reliance on artificial means to introduce the peptides into cells highlights one drawback of phosphonate-containing inhibitors. Although the literature contains several examples of bioreversible protection of phosphates[26] and phosphonates,[27–30] prodrug derivatization is frequently dif-

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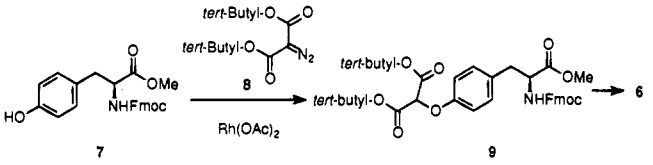
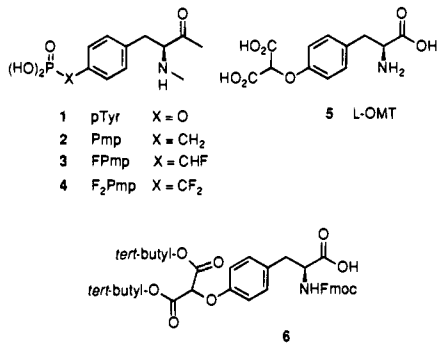
Scheme 1



ficult to accomplish, is not readily applicable to peptides synthesis, and has not yet been extended to F_2Pmp species. A need therefore exists for developing alternative pharmacophores which are recognized by and bound to SH2 domains. The discovery of such ligands could afford fresh avenues for SH2 inhibitor design, including prodrug development.



Along these lines, we set out to develop a nonphosphorus-containing pTyr mimetic. In so doing we utilized the findings of Sikorski et al. that the malonate moiety can mimic a phosphate structure in EPSP (5-(enolpyruvoyl)shikimate-3-phosphate) synthase inhibitors.[31] Accordingly, we designed the new pTyr mimetic, L-*O*-(2-malonyl)tyrosine (L-OMT, **5**), in which a malonate dicarboxylic acid structure replaces the phosphate group of the pTyr residue (**1**).[32] Although the malonate group is structurally quite distinct from a phosphate, preliminary studies showing that L-OMT successfully serves as a pTyr mimetic in a peptide which inhibits the protein-tyrosine phosphatase PTP 1B[33] indicated that this OMT may serve as a pTyr replacement in at least some cell-signaling pathways. We have therefore extended this work, and in this paper we describe how L-OMT can function as a new pTyr mimetic in SH2 domain inhibitory peptides.

Synthesis

Solid-phase synthesis of OMT-containing peptides utilized the L-OMT derivative bearing N^{α}-Fmoc protection and malonyl di-*tert*-butyl ester protection (N^{α}-Fmoc-L-OMT-*O*,*O*-(*tert*-butyl)$_2$, **6**]. The preparation of **6**, which has recently been reported in abbreviated form,[32] employed rhodium diacetate-catalyzed coupling of di-*tert*-butyl α-diazomalonate (**8**)[34] with methyl N^{α}-Fmoc-L-tyrosinate (**7**) to give the methyl OMT **9** (Scheme 1).

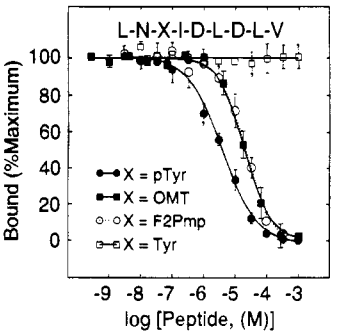


Figure 1. Competitive binding analyses between peptide sequences Ac-L-N-X-I-D-L-D-L-V-amide and the N-terminal SH2 domain of SH-PTP2, where X = pTyr (●), OMT (■), F_2-Pmp (○), or Tyr (□).

Selective methyl ester hydrolysis to yield **6**, without hydrolysis of the malonyl *tert*-butyl esters or removal of the base-labile Fmoc group, was accomplished using ice-cold 0.2 N LiOH similar to our recently reported procedure for the hydrolysis of methyl esters of N^{α}-Fmoc F_2Pmp derivatives.[35]

Results and Discussion

Five L-OMT containing peptides (**10**–**14**) were prepared, with peptides **10**–**13** containing a single OMT residue each and peptide **14** containing two OMT residues. The sequences of peptides **10**–**13** were identical with pTyr- and F_2Pmp-containing peptides which we had previously shown exhibit high affinity to the desired SH2 domain constructs[23,25] and were derived from the following sources: (1) Ac-D-(L-OMT)-V-P-M-L-amide (**10**), Tyr751 of the PDGF receptor for inhibition of the PI 3-kinase p85 C-terminal SH2 domain,[36] (2) Ac-Q-(L-OMT)-E-E-I-P-amide (**11**), Tyr324 of the hamster polyoma virus middle T antigen for inhibition of the Src SH2 domain,[37,38] (3) Ac-N-(L-OMT)-V-N-I-E-amide (**12**), Tyr895 of IRS-1 for inhibition of the Grb2 SH2 domain,[39] and (4) Ac-L-N-(L-OMT)-I-D-L-D-V-amide (**13**), Tyr1172 of IRS-1 for inhibition of the SH-PTP2 (also known as Syp) N-terminal SH2 domain.[39] The Src SH2 domain-directed di-OMT-containing peptide Ac-Ac-Q-(L-OMT)-(L-OMT)-E-I-P-amide (**14**) is identical with peptide **11**, except that a glutamyl residue was replaced with an L-OMT residue. The rationale for this modification was based on the reported retention of Src SH2 domain-binding potency of the related di-pTyr-containing peptide Ac-pTyr-pTyr-E-I-E.[40] Peptides **10**–**14** were examined for their ability to bind to the indicated SH2 domains. As outlined in the Experimental Section, competition assays were employed to determine the relative SH2 domain affinities for the L-OMT-containing peptides vs high-affinity phosphopeptide ligands. The results of these assays are shown in Table 1, with a plot of binding data for the SH-PTP2 L-OMT-containing peptide **13** depicted in Figure 1 as a representative

Table 1. Inhibition Constants of Peptide Inhibitors against Indicated SH2 Domain Constructs

			$IC_{50} \pm SE$ (μM)	
no.	peptide	SH2 domain	X = pTyr	X = L-OMT
10	Ac-D-X-V-P-M-L-amide	p85 (C-terminal)	0.15 ± 0.03[a]	14.2 ± 1.3
11	Ac-Q-X-E-E-I-P-amide	Src	3.2 ± 0.2	25 ± 5.0
12	Ac-N-X-V-N-I-E-amide	Grb2	0.9 ± 0.1[a]	120 ± 31
13	Ac-L-N-X-I-D-L-D-L-V-amide	SH-PTP2 (N-terminal)	1.54 ± 0.1	22.0 ± 1.4
14	Ac-Q-X-X-E-I-P-amide	Src		22.8 ± 4.3

[a] Previously reported.[23]

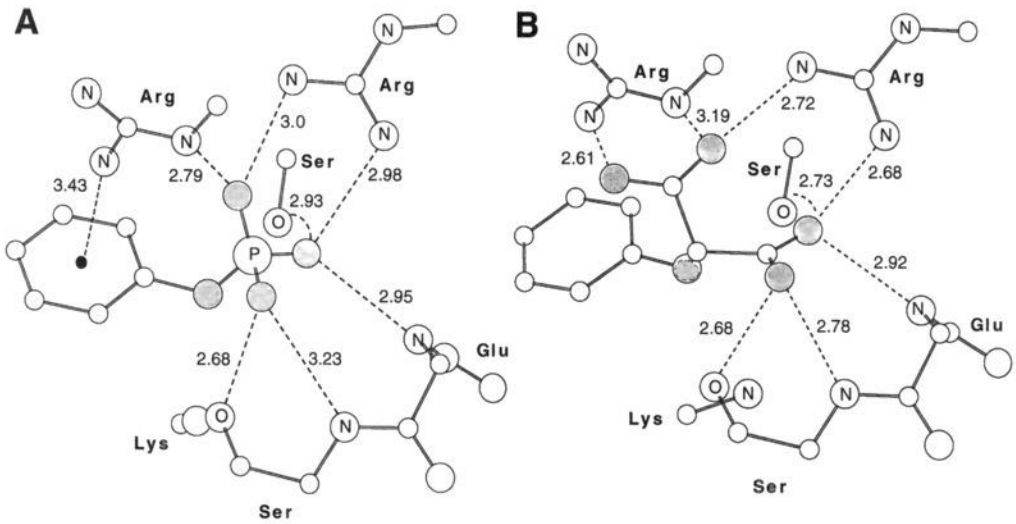


Figure 2. Binding of (A) aryl phosphate and (B) aryl malonate pharmacophores to the p56^{lck} SH2 domain. Phosphate and malonate oxygen atoms are shaded. The structure in panel A was derived from the previously reported X-ray structure of a bound high-affinity pTyr peptide,[16] while the structure in panel B was obtained by molecular modeling as described in the Experimental Section. Dotted lines indicate hydrogen bonds, with hydrogen atoms not shown.

example. Also shown in Table 1 are the inhibition constants of parent pTyr-containing peptides. The data indicate that, with the exception of peptide **12**, all OMT-containing peptides bind to their respective SH2 domains with similar, moderate potencies.

The potency of L-OMT-containing peptide **13** against the SH-PTP2 SH2 domain is noteworthy. While the absolute magnitude of inhibition (IC_{50} = 22 μM) is of the same order as that seen with the p85-directed L-OMT peptide **10**, in this latter example the L-OMT peptide suffered a significant loss of potency relative to either the pTyr or F_2Pmp (IC_{50} = 0.17 μM)[23] peptides. On the other hand, SH-PTP2 peptide **13** shows no loss of potency relative to the corresponding L-F_2Pmp peptide (IC_{50} = 24 μM).[25] This is significant in that we have previously demonstrated the ability of the L-F_2Pmp peptide to block SH-PTP2-mediated mitogenic signaling in rat1 fibroblasts.[25] This strongly suggests that the L-OMT peptide **13** may also possess sufficient potency to elicit a measurable effect in cellular assays.

To compare the SH2 domain interaction of the OMT residue with that of a native pTyr pharmacophore, we performed molecular modeling studies using the previously reported X-ray structure of a high-affinity pTyr peptide bound to the p56^{lck} SH2 domain.[16] Although none of the synthetic OMT-containing peptides in our current study are directed against the p56^{lck} SH2 domain, the binding of pTyr residues is highly homologous among different SH2 domains.[9] OMT modeling using the p56^{lck} data is therefore representative of the general binding interactions of OMT residues with SH2 domains. Complexation of the pTyr phenyl phosphate pharmacophore within this SH2 domain is shown in Figure 2A. Binding of the corresponding OMT pharmacophore is shown in Figure 2B. Although the phosphate and malonate structures are chemically distinct, and the malonate group occupies ca. 18% more volume than the phosphate, their interactions with the SH2 domain are remarkably similar, and both structures can be accommodated while maintaining nearly identical SH2 domain geometries. This supports the interpretation that OMT binds to SH2 domains in a manner similar to pTyr residues. Using a similar modeling approach, the binding of a malonate moiety within the catalytic site of the tyrosine phosphatase PTP 1B was shown to be nearly identical with the binding of a phosphonate structure.[33]

The disparity observed among different SH2 domains in binding potencies of OMT-containing peptides versus their pTyr-containing counterparts may indicate that the OMT residue is bound differently in the pTyr pockets of the respective SH2 domains. Potency differences may also reflect larger discrepancies in the overall mode of binding of peptide ligands. For example, binding of pTyr peptides to SH2 domains of the Src family have been shown to employ pronounced "two-pronged" interactions between the SH2 domain and pTyr and a second hydrophobic pocket located three residues C-terminal to the pTyr residue. It could be anticipated that the high contribution of the pTyr binding to the overall peptide−SH2 domain interaction would amplify any loss of potency brought about by a pTyr mimetic. Alternatively, SH2 domains such as SH-PTP2[18,41] appear to exhibit peptide−SH2 domain-binding interactions distributed over a more extended region. Since the contribution of the pTyr binding to the total binding of the peptide ligand is much less important,[42] loss of affinity at the pTyr binding site may be better tolerated, and a higher retention of potency may be observed for peptides employing pTyr mimetics.

Specificity is a desirable attribute in the development of SH2 domain inhibitors. Generally, specificity for peptide-based inhibitors is determined largely by amino acid sequence. However, we have previously reported that depending on the SH2 domain, peptides bearing the pTyr mimetic F_2Pmp (**4**) can exhibit either enhanced or reduced potency relative to parent pTyr-bearing peptides,[23] indicating that a measure of selectivity may be achieved by differences in binding at the pTyr site itself. Recent X-ray studies on the p56^{lck} SH2 domain complexed to peptides containing the pTyr mimetic Pmp (**2**) suggest a gated mode of binding at the pTyr-binding pocket, which could allow differential recognition of pTyr mimetics.[43] The variation in binding potency of OMT-containing peptides relative to their pTyr counterparts ranges from 5−10-fold (Src and SH-PTP2) to 100-fold

or greater (p85 and Grb2), further supporting the ability of pTyr mimetics to discriminate among different SH2 domains. As a new pTyr motif, OMT may provide a starting point for the design of further structures which can bind to SH2 domains with altered specificity.

Another desired feature of SH2 domain inhibitors is bioavailability. Prodrug delivery of diester-protected OMT peptides through cell membranes may contribute to the development of cell-permeable inhibitors. Work is currently in progress to examine the utility of protected OMT peptides in both SH2 domain and PTP cellular assays.

Experimental Section

Synthesis. Melting points were determined on a Mel-Temp II melting apparatus and are uncorrected. Elemental analysis were obtained from Atlantic Microlab Inc., Norcross, GA. Fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. ^{1}H NMR spectra were obtained using a Bruker AC250 (250 MHz) spectrometer. Optical rotations were measured on a Perkin-Elmer 240 polarimeter, and infrared spectra were acquired on a Perkin-Elmer 1600 series FTIR instrument. Solvent was removed by rotary evaporation under reduced pressure, and silica gel chromatography was performed using Merck silica gel 60 with a particle size of 40–63 um. Anhydrous solvents were obtained commercially and used without further drying.

Methyl N^{α}-Fmoc-L-tyrosinate (7). A solution of L-tyrosine (10.9 g, 60 mmol) in methanolic HCl was stirred at reflux overnight under argon and then taken to dryness to give pure methyl tyrosinate hydrochloride (13.68 g, 98%). To a solution of sodium carbonate (7.74 g, 92.1 mmol) in water (135 mL) was added methyl tyrosinate hydrochloride (7.11 g, 30.7 mmol). After formation of a clear solution, Fmoc-*O*-(*N*-hydroxysuccinimide) (10.4 g, 30.7 mmol) in dioxane (135 mL) was added dropwise, giving a white suspension. The mixture was stirred under argon overnight, adjusted to pH 2 by addition of 6 N HCl, extracted with EtOAc (3 × 200 mL), washed with brine (2 × 50 mL), and dried (Na_2SO_4). Removal of solvent and purification by silica gel chromatography (hexane/EtOAc, 4:1) afforded methyl N^{α}-Fmoc-L-tyrosinate (**7**) (12.7 g, 99%): mp 89 °C; ^{1}H NMR ($CDCl_3$) δ 7.78 (br d, J = 7 Hz, 2H), 7.6 (dd, J = 4, 1 Hz, 2H), 7.38 (t, J = 7 Hz, 2H), 7.3 (dt, J = 4, 5 Hz, 2H), 6.94 (d, J = 8 Hz, 2H), 6.72 (dd, J = 8, 2 Hz, 2H), 5.31 (d, J = 7 Hz, 1H), 4.61 (m, 1H), 4.38 (dd, J = 10, 7 Hz, 1H), 4.2 (dd, J = 10, 7 Hz, 1H), 4.12 (t, J = 7 Hz, 1H), 3.78 (s, 3H), 2.07 (m, 2H).

Methyl N^{α}-Fmoc-*O'*-(*O''*,*O''*-di-*tert*-butyl-2-malonyl)tyrosinate (9). To a stirred solution of **7** (4.31 g, 10.3 mmol) and rhodium diacetate (91 mg, 0.21 mmol) in benzene (450 mL) was added di-*tert*-butyl α-diazomalonate (**8**)[34] (3.0 g, 12.4 mmol) in benzene (20 mL) via cannula under argon. The reaction mixture was heated to reflux for 48 h and then cooled to room temperature and filtered and solvent removed to provide crude **9**. Purification by silica gel chromatography (hexane/EtOAc, 5:1) afforded pure **9** (4.51 g, 70%): mp 76–77 °C; $[\alpha]_D$ = +24° (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 7.4 Hz, 1H), 7.56 (d, J = 7.1 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.1 Hz, 2H), 6.96 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 8.4 Hz, 2H), 5.20 (d, J = 8.1 Hz, 1H), 4.93 (s, 1H), 4.66–4.55 (m, 1H), 4.47–4.36 (m, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.69 (s, 3H), 3.03 (d, J = 5.6 Hz, 2H), 1.47 (s, 18 H); IR ($CHCl_3$) 2978, 2917, 1736, 1713, 1607, 1507, 1231, 837 cm^{-1}; FABMS *m*/*z* 632 (M + H)$^+$. Anal. ($C_{36}H_{41}NO_9$) C, H, N.

N^{α}-Fmoc-*O'*-(*O''*,*O''*-di-*tert*-butyl-2-malonyl)tyrosine (6). To a stirred solution of **9** (2.31 g, 3.66 mmol) in THF (37 mL) at 0 °C was added ice-cold 0.2 N LiOH (33 mL, 6.6 mmol) dropwise over 30 min. The reaction mixture was stirred (40 min), the reaction was quenched with 0.5 N HCl (20 mL), the mixture was extracted with EtOAc (3 × 100 mL), washed with brine (2 × 50 mL), and dried (Na_2SO_4), and the solvent was removed to give crude product **6**. Purification by silica gel chromatography (hexane/EtOAc, 5:1) afforded pure **6** (1.99 g, 88%): mp 58–60 °C; $[\alpha]_D$ = +26° (*c* 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.75 (d, J = 7.3 Hz, 1H), 7.54 (d, J = 7.2 Hz, 2H), 7.39 (t, J = 7.3 Hz, 2H), 7.30 (t, J = 7.2 Hz, 2H), 7.03 (d, J = 8.0 Hz, 2H), 6.87 (d, J = 8.0 Hz, 2H), 5.14 (d, J = 8.0 Hz, 1H), 4.89 (s, 1H), 4.69–4.58 (m, 1H), 4.46–4.37 (m, 2H), 4.19 (t, J = 6.8 Hz, 1H), 3.08 (br m, 2H), 1.47 (s, 18 H); IR ($CHCl_3$) 3447–2780 (br), 2972, 2934, 1758, 1712, 1609, 1510, 1368, 1234, 1050 cm^{-1}; FABMS *m*/*z* 616 (M − H)$^+$. Anal. ($C_{35}H_{39}NO_9 \cdot {}^1/_4H_2O$) C, H, N.

Peptide Synthesis. The pTyr mimetic X = L-OMT (**5**) was incorporated into peptides **10–14** using the amino acid L-N^{α}-Fmoc-*O'*-(*O''*,*O''*-di-*tert*-butyl-2-malonyl)tyrosine (**6**)[44] and solid-phase synthesis with Fmoc chemistry.[32] Fmoc derivatives of standard amino acids were obtained from Bachem California (Torrence, CA) or Millipore/Milligen (Bedford, MA). Side-chain protection was as follows: Asp(tBu) and Glu(tBu), and Asn and Gln were unprotected. The peptides were prepared using PAL amide resin,[45] with DIPCDI/HOBT as coupling reagents in DMF (coupling time 1 h) and 20% piperidine/DMF for Fmoc deprotection (2 min and then 12 min). The resin-bound protected peptide was N-terminally acetylated with 10% 1-acetylimidazole/DMF (2 × 1.5 h room temperature). Resin cleavage and side-chain deprotection was done in one step with TFA containing 5% each (v/v) of ethanedithiol, *m*-cresol, thioanisole, and H_2O (0.5 h at 4 °C and then 2 h at room temperature). To isolate the peptides, two-thirds of the reagent mixture was evaporated under N_2 and the mixture triturated with ice-cold Et_2O. The precipitated crude peptides were purified to homogeneity by reverse-phase HPLC. Conditions: Vydac C_{18} column (10 × 250 mm); solvent gradient, 0.05% TFA in H_2O; B, 0.05% TFA in 90% acetonitrile in H_2O; gradient, as indicated below; flow rate, 2.5 mL/min; UV detector, 220 nm. FABMS (unit resolution, glycerol matrix, positive and/or negative ion mode) were performed on a VG Analytical 7070E-HF mass spectrometer. Amino acid analysis (6 N HCl, 110 °C, 24 h) was carried out at the Protein Structure Laboratory, University of California, Davis, CA.

Ac-Asp-(L-OMT)-Val-Pro-Met-Leu-amide (10): RPHPLC t_R = 15.0 min (gradient, 20–50% B over 20 min); FABMS (M + H)$^+$ 880.4 (calcd 880.4), (M − H)$^-$ 878.2 (calcd 878.4); amino acid analysis Asp 1.02 (1), Val 1.48* (1), Pro 1.04 (1), Met 0.87 (1), Leu 1.07 (1) (asterisk indicates overlapping with L-OMT).

Ac-Gln-(L-OMT)-Glu-Glu-Ile-Pro-amide (11): RPHPLC t_R = 15.4 min (gradient, 10–40% B over 25 min); FABMS (M − H)$^-$ 919.4 (calcd 919.4); amino acid analysis Glu 2.99 (3), Pro 1.03 (1), Ile 0.98 (1).

Ac-Asn-(L-OMT)-Val-Asn-Ile-Glu-amide (12): RPHPLC t_R = 13.8 min (gradient, 10–40% B over 25 min); FABMS (matrix, triethanolamine/glycerol) (M − H)$^-$ 892.3 (calcd 892.3); amino acid analysis Asp 2.00 (2), Glu 1.01 (1), Val <1.73* (1), Ile 0.98 (1).

Ac-Leu-Asn-(L-OMT)-Ile-Asp-Leu-Asp-Leu-Val-amide (13): RPHPLC t_R = 14.0 min (gradient, 30–55% B over 22 min); FABMS (matrix, DMF/glycerol) (M − H)$^-$ 1218.2 (calcd 1218.6); amino acid analysis Asp 3.00 (3), Val <1.73* (1), Ile 0.98 (1), Leu 3.01 (3).

Ac-Gln-(L-OMT)-(L-OMT)-Glu-Ile-Pro-amide (14): RPHPLC t_R = 17.0 min (gradient, 10–40% B over 25 min); FABMS (M − H)$^-$ 1055.3 (calcd 1055.4); amino acid analysis Glu 2.00 (2), Pro 1.02 (1), Ile 0.98 (1).

Molecular Modeling. Figure 2A is derived from the X-ray crystal structure of an 11-mer, high-affinity, pTyr-containing peptide bound to the p56^{lck} SH2 domain.[16,45] Residues important for pTyr binding are shown and include two arginines and one lysine (providing three positive charges), two serines, and one glutamate. The phosphate group is apparently in the diionized form, as all three P–O bonds are short and of similar lengths (1.337, 1.363, and 1.393 Å). Figure 2B depicts a complex between $C_6H_5OCH(COO)_2$ and the same binding site. This was obtained by replacing the PO_3 group of the X-ray structure with the $CH(COO)_2$ group and then minimizing using 3-21G ab initio methods on a Cray mainframe computer using GAUSSIAN92. The $CH(COO)_2$ group is assumed to have two negative charges. During the minimization of the SH2 domain–$CH(COO)_2$ complex, the geometry parameters and the

position of the $C_6H_5OCH(COO)_2$ were optimized. The binding site geometry was fixed, with only distances between binding site substructures being allowed to vary. Figure 2 shows that within the binding cavity, the two complexes have similar interactions between the SH2 domain and the bound ligand. The number of ligand–protein hydrogen bonds is similar with the exception of a single hydrogen bond. In Figure 2A this hydrogen bond occurs between an arginine and the aromatic ring, while in Figure 2B it forms with an oxygen atom of a malonyl COO^- group. The $CH(COO)_2$ group (Figure 2B) also occupies 18% more volume within the binding site than the PO_3 group, consistent with previous calculations.[46]

SH2 Domain-Binding Assays. Details of the SH2 domain competition assay have been published previously.[36] In this study, four distinct assays were used to determine relative SH2 domain affinities for OMT-containing peptides versus high-affinity phosphopeptide ligands. In each assay a glutathione *S*-transferase (GST)/SH2 domain fusion protein was paired with an appropriate high-affinity [^{125}I]Bolton–Hunter-radiolabeled phosphopeptide, and varying concentrations of unlabeled peptides were added as competitors. The C-terminal SH2 domain of PI 3-kinase p85 was paired with IRS-1 pY628, GNGDpYMPMSPKS;[36] the Src SH2 domain was paired with hmT pY324, KEPQpYEEIPIYL;[38] the Grb2 SH2 domain was paired with Shc pY317, ELFDDPSpYVNVQNLDK; and the N-terminal SH2 domain of SH-PTP2 was paired with IRS-1 pY1172, SLNpYIDLDLVK.[25] An underline denotes the position of the [^{125}I]Bolton–Hunter-modified lysine. GST/SH2 domain fusion proteins (0.5–1.0 μM, estimated by Bradford assay), 35 fmol of HPLC-purified, [^{125}I]Bolton–Hunter-treated phosphopeptide (67 nCi), and varying concentrations of pTyr analogues were combined in 200 μL total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, and 10 mM dithiothreitol, pH 7.4, and vortexed. Glutathione-agarose (25 μL of a 1:4 aqueous slurry; Molecular Probes) was added, and the samples were incubated overnight at 22 °C with constant mixing. Following centrifugation for 5 min at 12000*g*, supernatant solutions were removed by aspiration, and ^{125}I radioactivity associated with the unwashed pellets was determined with a γ-counter.

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