Articles

4′-*O*-[2-(2-Fluoromalonyl)]-L-tyrosine: A Phosphotyrosyl Mimic for the Preparation of Signal Transduction Inhibitory Peptides[1]

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Development of phosphotyrosyl (pTyr) mimetics which are stable to protein−tyrosine phosphatases (PTPs), yet can retain biological potency when incorporated into peptides, is an active area of drug development. Since a majority of pTyr mimetics derive their "phosphofunctionality" from phosphorus-containing moieties, such as phosphonates, evolution of new inhibitors and modes of prodrug derivatization have been restricted to chemistries appropriate for phosphorus-containing moieties. A new, nonphosphorus-containing pTyr mimetic has recently been reported, L-*O*-(2-malonyl)tyrosine (OMT, **5**), which can be incorporated into peptides that exhibit good PTP and Src homology 2 (SH2) domain inhibitory potency. For phosphonate-based pTyr mimetics such as phosphonomethyl phenylalanine (Pmp, **2**), introduction of fluorines α to the phosphorus has provided higher affinity pTyr mimetics. This strategy has now been applied to OMT, and herein is reported 4′-*O*-[2-(2-fluoromalonyl)]-L-tyrosine (FOMT, **6**), a new fluorine-containing nonphosphorus pTyr mimetic. Incorporation of FOMT into appropriate peptides results in good inhibition of both PTP and SH2 domains. In an assay measuring the inhibition of PTP 1B-mediated dephosphorylation of phosphorylated insulin receptor, the peptide Ac-D-A-D-E-X-L-amide exhibited a 10-fold enhancement in inhibitory potency for X = FOMT (**19**) (IC_{50} = 1 μM) relative to the unfluorinated peptide, X = OMT (**18**) (IC_{50} = 10 μM). Molecular modeling indicated that this increased affinity may be attributable to new hydrogen-bonding interactions between the fluorine and the enzyme catalytic site, and not due to lowering of pK_a values. In a competition binding assay using the p85 PI 3-kinase C-terminal SH2 domain GST fusion construct, the inhibitory peptide, Ac-D-X-V-P-M-L-amide, showed no enhancement of inhibitory potency for X = FOMT (**22**) (IC_{50} = 18 μM) relative to the unfluorinated peptide, X = OMT (**21**) (IC_{50} = 14 μM). The use of FOMT would therefore appear to have particular potential for the development of PTP inhibitors.

Modulators of cell-signaling pathways promise new approaches toward therapeutic design, with the development of inhibitors of protein−tyrosine kinase (PTK) cascades currently being one important area of investigation.[2,3] Central to signaling through PTK-dependent pathways is the generation (via PTKs), utilization (via Src homology 2 (SH2) domains), and destruction (by protein−tyrosine phosphatases, PTPs) of phosphotyrosyl (pTyr, **1**) residues within key proteins. Additionally, recent reports of pTyr-dependent protein−protein binding not involving SH2 domains[4−7] adds yet another dimension to this signaling scheme. While significant work has been reported on PTK inhibitors,[3,8,9] development of SH2 domain and PTP inhibitors has not been well represented.

Since the pTyr pharmacophore is a defining determinant for both of these interactions, basing the design of SH2 domain and PTP inhibitors on the structure of tyrosine phosphate is one approach toward the development of respective inhibitors, and we have previously reported the synthesis of constrained pTyr mimetics as potential SH2 domain inhibitors. However, one limitation of inhibitors based on either tyrosine phosphate or related phenyl phosphates is their potential susceptibility to phosphorolysis and inactivation by PTPs. To overcome this liability, the use of benzylphosphonate-containing structures, in which the phosphate ester oxygen has been replaced by a chemically and enzymatically stable methylene unit, have been used. In the case of pTyr (**1**), the resulting (phosphonomethyl)-phenylalanine (Pmp; **2**)[10] has been successfully substituted into peptides which are antagonists of both SH2 domain binding[11−13] and PTPs.[14−16] Modification of Pmp by substitution of fluorine at the methylene bridge to give monofluoroPmp (FPmp, **3**)[17] or difluoroPmp (F_2-Pmp, **4**)[17] resulted in enhanced potency against both SH2 domains[12,18,19] and PTPs.[16]

In exploring fresh directions for inhibitor design, nonphosphorus-containing pTyr mimetics are of interest, since they could afford new avenues for prodrug delivery and could potentially display altered biological profiles. We have recently reported one such pTyr mimetic, 4′-

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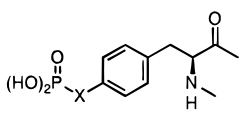
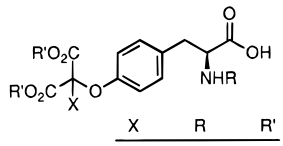


1	pTyr	X = O
2	Pmp	X = CH_2
3	FPmp	X = CHF
4	F_2Pmp	X = CF_2



		X	R	R'
5	L-OMT	H	H	H
6	L-FOMT	F	H	H
7		F	Fmoc	*tert*-butyl

O-(2-malonyl)-L-tyrosine (OMT, **5**), in which the phosphate group has been replaced by a malonate structure.[20] Peptides bearing this new analogue exhibit reasonable inhibitory potency in both SH2 domain[21] and PTP[22] assays. In order to explore structural variants of OMT which may modify its potency or specificity, we took note of the previous dramatic effect on both SH2 domain and PTP inhibition when fluorine was introduced into Pmp. We therefore designed a modified OMT which contains a fluorine at the malonyl α-carbon. Herein we report the synthesis of this analogue, 4′-*O*-[2-(2-fluoromalonyl)]-L-tyrosine (FOMT, **6**), as its N^{α}-Fmoc-*O*,*O*-di-*tert*-butyl derivative (**7**), which is suitably protected for solid-phase peptide synthesis of L-FOMT peptides. (Note: Although these derivatives may be more properly termed, "2-*O*-tyrosinyl)malonate ethers," we have adapted an alternate naming system which is consistent with previous nomenclature applied to the unfluorinated analogues.[21,22]) We include the synthesis of FOMT-containing peptides and examine their inhibitory potency in SH2 domain and PTP assays.

Synthesis

Model Fluorination. The 6-bromo-2-[[2-(2-fluoromalonyl)]oxy]naphthalene (**13**) was prepared as a model compound to examine fluorination conditions. It was also subsequently used for the determination of pK_a values. The 6-bromo substituent of **13** facilitated crystallization of products relative to the unbrominated species. Refluxing a benzene solution of 6-bromo-2-naphthol (**8**) and di-*tert*-butyl α-diazomalonate[23] (**9**) in the presence of rhodium diacetate[24] provided the malonyl adduct **10**, with subsequent TFA-catalyzed hydrolysis of the *tert*-butyl protecting groups affording the free diacid **12** (Scheme 1). Electrophilic fluorination of **10** using sodium bis(trimethylsilyl)amide and *N*-fluorobenzenesulfonimide provided the 2-fluoromalonyl product **11**, which was hydrolyzed to the free diacid **13**.

Fluorination of Protected OMT. Preparation of the final N^{α}-Fmoc-*O*,*O*-di-*tert*-butyl-protected derivative **7** was achieved by initially applying the above outlined electrophilic fluorination on the protected OMT derivative **15** to yield **16** (Scheme 2). Compound **15** was obtained from the corresponding methyl N^{α}-Fmoc-tyrosinate (**14**) as previously described.[20,21] Crucial to the success of the fluorination was the stability of the base-labile *N*-Fmoc protecting group to the strongly alkaline bis(trimethylsilyl)amide. Equally important was the stability of both the Fmoc group and the α-fluoromalonyl ester groups to the lithium hydroxide-catalyzed hydrolysis of the tyrosinate methyl ester to the final free amino acid **7**.[25] The steric bulk afforded by the *tert*-butyl groups contributed to the stability of the α-fluoromalonyl ester groups, as the corresponding *n*-butyl-protected α-fluoromalonyl diester underwent hydrolysis of one ester group under similar conditions.[26]

Results and Discussion

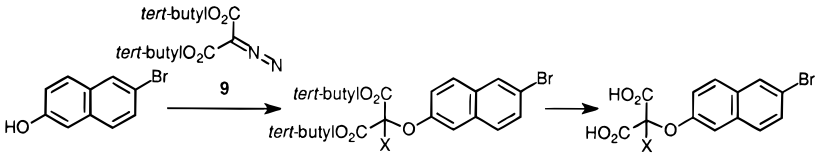
PTP Inhibition. In order to examine the ability of FOMT-containing peptides to inhibit PTPs, the peptide Ac-D-A-D-E-[FOMT]-L-amide (**19**) was prepared, based on the corresponding pTyr-containing sequence, which has been shown to be a high-affinity hexamer substrate of PTP 1.[15] The OMT-substituted peptide **18** has previously been shown to inhibit the PTP 1B-mediated dephosphorylation of endogenously phosphorylated insulin receptor with an IC_{50} value of 10 μM.[22] Under the same assay conditions, FOMT-containing peptide **19** exhibited an IC_{50} value of 1 μM, representing a 10-fold increase in affinity relative to its unfluorinated counterpart **18** (Figure 1).

PTP 1B Inhibitory Peptides	p85 PI 3-Kinase SH2 Domain Inhibitory Peptides
Ac-Asp-Ala-Asp-Glu-X-Leu-amide	Ac-Asp-X-Val-Pro-Met-Leu-amide
18 X = OMT	**20** X = pTyr
19 X = FOMT	**21** X = OMT
	22 X = FOMT

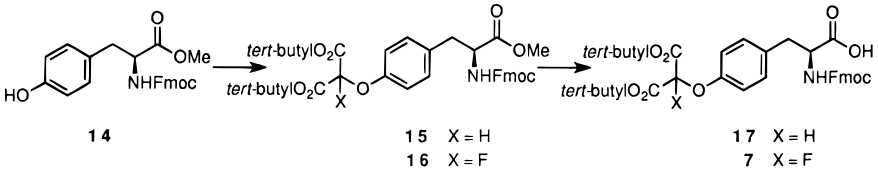
SH2 Domain Inhibition. The "pY-V-P-M-L" sequence has been extensively studied as an inhibitory motif directed against the PI 3-kinase p85 C-terminal SH2 domain, and it has served as a benchmark sequence in our previous studies on the SH2 domain inhibitory potency of both linear[12] and cyclic[18,19] peptides containing phosphonate-based pTyr mimetics. More recently Ac-D-[L-OMT]-V-P-M-L-amide (**21**) has been examined for inhibitory potency in a p85 C-terminal binding assay and has been found to have an IC_{50} value of 14 μM.[21] This is on the order of inhibitory potencies observed for L-OMT-containing peptides directed against the Src SH2 domain (IC_{50} = 25 μM) and the SH-PTP2 SH2 domain (IC_{50} = 22 μM).[21] The present study examined the effect of fluorine substitution in a peptide containing OMT. Analogous fluorine substitution in "pY-V-P-M-L" sequences containing phosphonate-based pTyr mimetics resulted in a doubling of potency for each fluorine added, with the difluorinated "A-D-[L-F_2Pmp]-V-P-M-L-amide" having a potency equivalent to the parent pTyr-containing peptide **20** ($IC_{50} \approx 0.15$ μM).[12] In contrast to the enhancing effect of fluorination on the SH2 domain inhibitory potency of peptides bearing phosphonate-based pTyr mimetics, fluorination of OMT had no effect on inhibitory potency as indicated by p85 C-terminal SH2 domain binding assays using the L-FOMT-containing peptide **20** (IC_{50} = 18.3 μM; Figure 2).

Effect of Fluorination on Ionization Constants. The phosphorus-containing pTyr mimetic, Pmp (**2**), provides two geminal sites for introduction of fluorine at the phosphonate α-methylene bridge. The corresponding difluorinated phosphonate, F_2Pmp (**4**), when incorporated into a peptide, has dramatically enhanced inhibitory potency against the tyrosine phosphatase PTP 1B[16] and appreciably increased binding potency in SH2 domain binding assays.[12] The potentiating effects of fluorine in these cases may be at least partially attributable to two factors: (1) the reintroduction of hydrogen-bonding capability through the fluorine atom at the phosphonate α-methylene which was originally present in the parent phosphate and (2) a reduction in second phosphonate ionization constant (pK_{a_2}).[12] The effect on pK_{a_2} values is of particular note. An aryl phosphate has two ionizable hydroxyls, each with a distinct pK_a value. The first ionization constant is lower

Scheme 1



Scheme 2



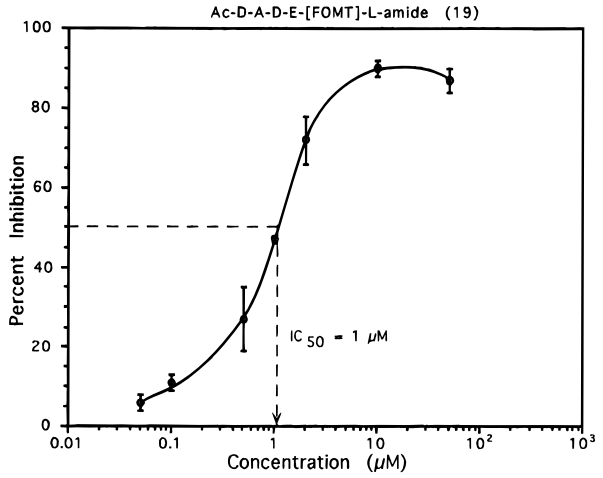


Figure 1. Effect of FOMT-containing peptide **19** on recombinant PTP 1B-mediated dephosphorylation of insulin receptors. Results are the mean ± SE of three separate experiments performed in duplicate.

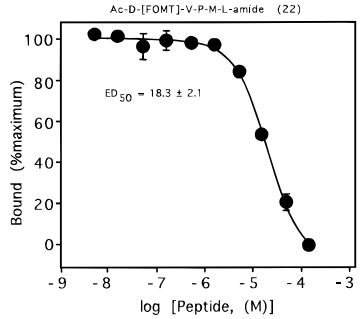
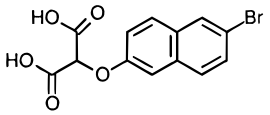
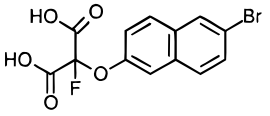


Figure 2. Competitive binding curve for the peptide Ac-D-FOMT-V-P-M-L-amide (**22**) in an assay using the p85 PI 3-kinase C-terminal SH2 domain. Results are the mean ± SE of duplicate experiments.

than the second ionization constant, which occurs at a pK_{a_2} value of approximately 6.2. Therefore at physiological pH the phosphate exists predominately in the di-ionized form. Benzylphosphonic acid has a pK_{a_2} value of approximately 7.7 and therefore has an appreciable population of monoionized species at physiological pH. Substitution of fluorines at the α-methylene of benzylphosphonic acid reduces its pK_{a_2} value by approximately one unit per fluorine added, with the monofluoro- and difluorophosphonates exhibiting pK_{a_2} values of 6.6 and 5.7, respectively.[27] Central to the enhancement of inhibitory potency of benzylphosphonic acid-containing

Table 1. Comparison of pK_{a_2} values

No.	Compound	pKa2
	HO_2C-CH_2-CO_2H	5.78
12		3.91
13		2.84

inhibitors (such as Pmp) by fluorination is the increase in the population of di-ionized species which results because changes in pK_{a_2} values occur at or near physiological pH. In the case of OMT (**5**), a single malonyl α-hydrogen exists, limiting fluorination to the monofluoro species (FOMT, **6**). As with phosphonate-based pTyr mimetics, physiological effects of fluorination could arise from increased hydrogen-bonding interactions and/or from a lowering of the malonate pK_{a_2} value.

To examine the possible contribution changes in pK_a values may have on differential potencies of OMT- and FOMT-containing peptides, pK_a values were determined for compounds **12** and **13**, which represent structurally simplified analogues of OMT and FOMT, respectively (Table 1). Compound **12** exhibits a pK_{a_2} value approximately 2 units lower than unsubstituted malonic acid, with monofluorinated compound **13** showing a reduction in its pK_{a_2} value by yet another unit. For both **12** and **13** however, pK_{a_2} values are greater than 3 log units below physiological pH, and these compounds would exist nearly exclusively as the di-ionized species. For this reason, effects of fluorination on pK_a values would not be expected to affect the biological potency of **13** or similar α-fluoro 2-malonate-containing compounds such as FOMT.

Molecular Modeling. In order to better explain the 10-fold enhancement in the PTP 1B interaction of the FOMT-containing peptide **19** as compared with the nonfluorinated OMT counterpart **18**, a computer-assisted molecular-modeling study were undertaken. For modeling the effect of fluorination on the binding of OMT versus FOMT to the catalytic site of PTP 1B, we utilized the recently solved X-ray structure of PTP 1B bearing a [1,1-difluoro-1-(2-naphthyl)methyl]phosphonic

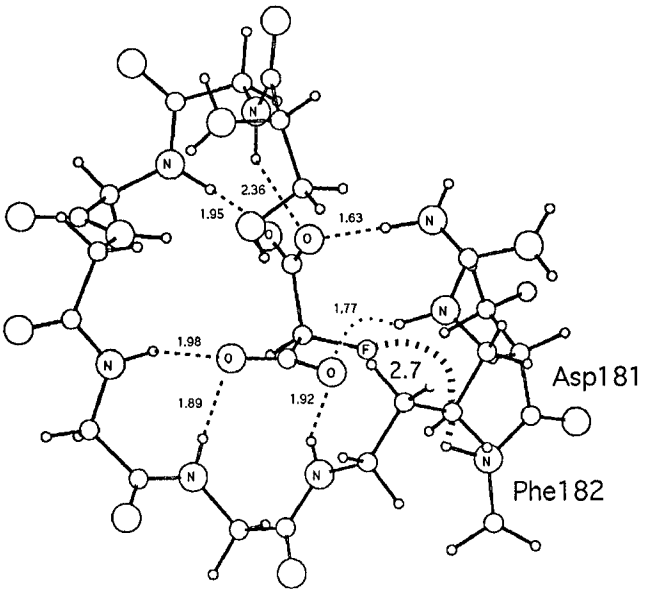


Figure 3. Structure of $CHF(CO_2)_2$ bound within the PTP 1B catalytic site. The overall geometry of binding was based on the X-ray structure of an aryl difluorophosphonate inhibitor complexed to PTP 1B. Key hydrogen bonding interactions are shown by dotted lines. A bold dotted line designates an important F−H interaction with Phe182.

acid ligand bound, which approximates the binding of a tyrosine phosphate moiety.[28] Recently the X-ray structure has also been reported of Ac-D-A-D-E-pY-L-amide bound to a deactivated PTP 1B mutant (the catalytic Cys residue has been substituted with a Ser residue).[29] Replacement of the difluorophosphonate in the X-ray structure with a malonate moiety $[CH_2(CO_2)_2]$ and subsequent minimization showed that although the malonate structure occupies approximately 12% more volume than the phosphonate, it is easily accommodated without significant disruption of the enzyme catalytic site geometry.[22] Introduction of a fluorine atom onto the malonate α-methylene approximates the corresponding structural change in going from OMT to FOMT. As shown in Figure 3, the α-fluoro atom is suitably situated to engage in hydrogen-bonding interactions with the Asp181−Phe182 amide nitrogen. Although the observed distance (2.7 Å) is not optimal for maximum hydrogen bonding, reminimization of the enzyme following introduction of the fluorine would be expected to shorten the distance. The binding energy gained by the F−H hydrogen bonding could contribute to some of the 10-fold enhancement in binding observed for the FOMT-containing peptide **19** relative to the unfluorinated **18**.

Summary. The *O*-(2-malonyl)tyrosyl residue (OMT, **5**) has recently demonstrated usefulness as a pTyr mimetic in peptides which are inhibitory to both PTPs and SH2 domain binding interactions. As a non-phosphorus-based pTyr mimetic, OMT may provide alternative approaches for developing both selective and bioavailable inhibitors of protein−tyrosine kinase-dependent signaling pathways. A first study in preparing modified OMT analogues was undertaken in which a fluorine atom was introduced onto the malonyl α-methylene, providing the new analogue FOMT (**6**). In the phosphonate-based series of pTyr mimetics (i.e., Pmp, **2**), analogous addition of α-fluorines (F_2Pmp, **4**) enhanced both PTP and SH2 domain binding potency. For these latter compounds, this potentiation could be ascribed to the ability of α-fluorines to both reduce the phosphonate pK_{a_2} value and reintroduce hydrogen-bonding interactions similar to the parent tyrosine phosphate (**1**). In studies presented here, we demonstrate that relative to an OMT-containing peptide, the corresponding FOMT-containing peptide exhibited a 10-fold enhancement in inhibitory potency against PTP 1B. Determination of pK_{a_2} values with model compounds indicated that while addition of a fluorine reduced the pK_{a_2} value, any biological effect from this would not be appreciable at physiological pH, where complete ionization of malonyl carboxyls on both OMT and FOMT would occur. However, molecular-modeling studies which simulated the binding of OMT and FOMT to PTP 1B indicated that addition of the fluorine atom could introduce new hydrogen bonding with the catalytic site. Therefore, a hydrogen-bonding effect and not a pK_{a_2} effect could at least partially account for the observed increase in affinity of FOMT-containing **19** relative to unfluorinated **18**.

A study designed to examine the efficacy of FOMT as a pTyr mimetic in SH2 domain-directed peptides was also undertaken. In this assay FOMT-containing peptide **22** showed no increase in binding potency relative to its unfluorinated counterpart **21** using a p85 PI 3-kinase C-terminal binding system. Unlike phosphonate-based pTyr mimetics where α-fluorination increased SH2 domain binding potency, there appears to be no advantage gained by α-fluorination of OMT.

Experimental Section

Synthesis. Petroleum ether was of the boiling range 35−60 °C, and removal of solvents was performed by rotary evaporation under reduced pressure. Silica gel filtration was carried out using TLC grade silica gel (5−25 μm, Aldrich; 6.5 cm diameter × 4 cm high). Melting points were determined on a Mel Temp II melting point apparatus and are uncorrected. Elemental analyses were obtained from Atlantic Microlab Inc., Norcross, GA, and are within 0.4% of theoretical values unless otherwise indicated. Fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. Where indicated, FABMS were run using a matrix of 3-nitrobenzyl alcohol (NBA). ^{1}H NMR data were obtained on Bruker AC250 (250 MHz) or, if indicated, AMX500 (500 MHz) instruments and are reported in ppm relative to TMS and referenced to the solvent in which they were run. Anhydrous solvents were obtained commercially and used without further drying.

6-Bromo-2-[[*O,O′*-di-*tert*-butyl-2-malonyl]oxy]naphthalene (10). A mixture of 6-bromo-2-naphthol (2.23 g, 10.0 mmol), di-*tert*-butyl α-diazomalonate[23] (2.90 g, 12.0 mmol), and rhodium(II) acetate dimer (196 mg, 0.44 mmol) in anhydrous benzene (100 mL) was stirred at reflux overnight. The mixture was cooled to room temperature, filtered through Celite, taken to dryness, and crystallized from petroleum ether (cooling to −78 °C). The resulting solid was triturated with petroleum ether at room temperature and then dried, yielding white crystals (2.85 g) which contained approximately 25% of an impurity (by NMR). The material was recrystallized from hexanes to provide **10** as a white crystalline solid (893 mg, 20%): mp 120 °C soften, 127−128 °C; ^{1}H NMR ($CDCl_3$) δ 7.92 (d, 1H, J = 2 Hz), 7.69 (d, 1H, J = 9.1 Hz), 7.57 (d, 1H, J = 8.7 Hz), 7.50 (dd, 1H, J = 1.8, 8.6 Hz), 7.32 (dd, 1H, J = 2.0, 9.0 Hz), 7.09 (d, 1H, J = 2.5 Hz), 5.12 (s, 1H), 1.50 (2, 18H). Anal. ($C_{21}H_{25}BrO_5$) C,H.

6-Bromo-2-[[*O,O′*-di-*tert*-butyl-2-(2-fluoromalonyl)]oxy]naphthalene (11). To a solution of **10** (180 mg, 0.5 mmol) in anhydrous THF (3 mL) at −78 °C under argon was added sodium bis(trimethylsilyl)amide, 1.0 M in THF (550 μL), and

the mixture was stirred at −78 °C (40 min). A solution of *N*-fluorobenzenesulfonimide (Allied Signal, Inc.) (173 mg, 0.55 mmol) in anhydrous THF (2 mL) was added dropwise, and the reaction was continued first at −78 °C (4 h) and then at ambient temperature (20 min). The resulting white suspension was diluted with saturated aqueous ammonium chloride (50 mL), extracted with $CHCl_3$ (2 × 50 mL), dried ($MgSO_4$), and taken to dryness, yielding a yellow oil (206 mg). Chromatographic purification (4.5 cm diameter × 2.5 cm high column using a stepwise gradient of $CHCl_3$ (0−75%) in petroleum ether) provided pure **11** (TLC $R_f = 0.70$, $CHCl_3$) as a colorless syrup (109 mg, 58%), with additional material being collected as a mixture of **11** and it mono-*tert*-butyl ester (90 mg): ^{1}H NMR ($CDCl_3$) δ 7.90 (d, 1H, $J = 2$ Hz), 7.64 (d, 1H, $J = 9.0$ hz), 7.57 (d, 1H, $J = 8.8$ Hz), 7.51 (br s, 1H), 7.47 (dd, 1H, $J = 2.2$, 9.0 Hz), 7.31 (dd, 1H, $J = 2.3$, 8.9 Hz), 1.32 (s, 18H).

6-Bromo-2-(2-malonyloxy)naphthalene (12). To **10** (180 mg, 0.5 mmol) was added neat TFA (1.0 mL), and the mixture was stirred at room temperature (1 h). The resulting white suspension was diluted with petroleum ether (2 mL) and filtered, and the filter cake was washed with several portions of petroleum ether, providing **12** as white crystals (120 mg, 98%): An analytical sample was obtained by hplc purification (Vydac C_{18} peptide and protein semiprep column; A = 0.05% aqueous TFA, B = 0.5% TFA in acetonitrile; isocratic, 40% B; retention $K' = 1.5$): mp 152 °C (shrink), 215−223 °C (note: if the temperature is increased rapidly, decomposition with evolution of gas occurs at approximately 170 °C); ^{1}H NMR (500 MHz; H_2O:D_2O) δ 8.08 (br s, 1H), 7.84 (dd, 1H, $J = 3.5$, 9.0 Hz), 7.75 (dd, 1H, $J = 4.3$, 8.6 Hz), 7.62 (d, 1H, $J = 8.8$ hz), 7.35 (dd, 1H, $J = 2.1$, 9.0 Hz), 7.22 (br s, 1H), 5.37 (s, 1H); FABMS (NBA, −ve) *m*/*z* 323 $(M - H)^-$. Anal. ($C_{13}H_9BrO_5 \cdot {}^3/_4H_2O$) C,H.

6-Bromo-2-[[2-(2-fluoromalonyl)]oxy]naphthalene (13). A mixture of **11** contaminated with a little of its mono-*tert*-butyl ester (199 mg total, 00.5 mmol theoretical) was stirred at room temperature with 2 mL of 90% TFA (1 h). The mixture was then taken to dryness under high vacuum, and the resulting colorless syrup was purified by silica gel chromatography ($CHCl_3$ followed by EtOAc) to provide a colorless syrup (125 mg), which was crystallized from petroleum ether: $CHCl_3$ to give **13** (TLC $R_f = 0.06$, $CHCl_3$) as white crystals (76 mg, 42%), contaminated with a small amount of impurity. An analytical sample was prepared by recrystallization from $CHCl_3$ to provide crystals, mp 141−142 °C. Further purification by HPLC was performed (Vydac C_{18} peptide and protein semiprep column; A = 0.05% aqueous TFA, B = 0.5% TFA in acetonitrile; linear gradient, 5% B to 95% B over 30 min; retention $K' = 2.3$): ^{1}H NMR (DMSO-d_6) δ 8.23 (d, 1H, $J = 1.9$ Hz), 7.96 (d, 1H, $J = 9.1$ Hz), 7.90 (d, 1H, $J = 8.9$ Hz), 7.65 (dd, 1H, $J = 1.9$, 8.8 Hz), 7.64 (d, 1H, $J = 2.3$ Hz), 7.39 (dd, 1H, $J = 2.3$, 8.9 Hz); FABMS (NBA, −ve) *m*/*z* 341 $(M - H)^-$. Anal. ($C_{13}H_8BrFO_5 \cdot {}^1/_2H_2O$) C,H.

Methyl N^{α}-Fmoc-4-*O*-[*O*′,*O*″-di-*tert*-butyl-2-(2-fluoromalonyl)]-L-tyrosinate (16). To a solution of methyl N^{α}-Fmoc-4-*O*-[*O*′,*O*″-di-*tert*-butyl-2-malonyl]tyrosinate (**15**)[21] (1.26 g, 2.0 mmol) in anhydrous THF (10 mL) at −78 °C under argon was added sodium bis(trimethylsilyl)amide, 1.0 M in THF (2.0 mL, 2.0 mmol), dropwise over 5 min, and then the reaction mixture was stirred at −78 °C (15 min). A solution of *N*-fluorobenzenesulfonimide (Allied Signal Corp.) (630 mg, 2.0 mmol) in anhydrous THF (5 mL) was added dropwise, and then the reaction mixture was stirred at −78 °C (1 h). The reaction was diluted with saturated ammonium chloride (100 mL), extracted with ethyl acetate (2 × 50 mL), washed with saturated ammonium chloride (50 mL), dried ($MgSO_4$), and taken to dryness, yielding a white foam (1.33 g). Purification by silica gel chromatography (ethyl acetate in petroleum ether; 10:90 followed by 20:80) provided **16** (TLC $R_f = 0.78$, ethyl acetate:$CHCl_3$, 1:4) as a white foam (1.0 g, 77%). Crystallization from methanol gave white needles: mp 113−114 °C; ^{1}H NMR ($CDCl_3$) δ 7.71 (d, 2H, $J = 7.4$ Hz), 7.51 (d, 2H $J = 7.3$ Hz), 7.38−7.23 (m, 4H), 7.05 (d, 2H, $J = 8.3$ Hz), 6.92 (d, 2H, $J = 8.4$ Hz), 5.10 (d, 1H, $J = 8.0$ Hz), 4.59−4.44 (m, 1H), 4.43−4.28 (m, 2H), 4.14 (t, 1H, $J = 6.7$ Hz), 3.63 (s, 3H), 3.58−3.52 (m, 1H), 3.00 (d, 2H, $J = 5.5$ Hz), 1.34 (s, 18H). Anal. ($C_{36}H_{40}FNO_9$) C, H, N.

N^{α}-Fmoc-4-*O*-[*O*′,*O*″-di-*tert*-butyl-2-(2-fluoromalonyl)]-L-tyrosine (7). To a solution of **16** (300 mg, 0.46 mmol) in THF (4.6 mL) at 0 °C was added a solution of 0.2 N LiOH (4.6 mL, 0.92 mmol) and the reaction mixture stirred at 0 °C (30 min). An additional portion of ice-cold 0.2 N LiOH (2.3 mL, 0.46 mmol) was added, and the reaction continued at 0 °C (30 min). The mixture was then partitioned between ice-cold 0.2 N HCl (100 mL) and ethyl acetate (2 × 50 mL), dried ($MgSO_4$), and taken to dryness, yielding **7** as a white foam (260 mg, 89%): ^{1}H NMR ($CDCl_3$) δ 7.77 (d, 2H, $J = 7.4$ Hz), 7.56 (d, 2H, $J = 7.3$ Hz), 7.40 (t, 2H, $J = 7.3$ Hz), 7.32 (t, 2H, $J = 7.4$ Hz), 7.12 (d, 2H, $J = 8.3$ Hz), 7.04 (d, 2H, $J = 8.3$ Hz), 5.10 (d, 1H, $J = 8.0$ Hz), 4.69−4.58 (m, 1H), 4.51−4.40 (m, 2H), 4.20 (t, 2H, $J = 6.7$ Hz), 3.73 (m, 1H), 3.21−3.02 (m, 2H), 1.39 (s, 18H); FABMS *m*/*z* 634 $(M + H)^+$. Anal. ($C_{35}H_{38}FNO_9$) C, H, N.

Peptide Synthesis. The pTyr mimetic X = L-FOMT (**6**) was incorporated into peptides **19** and **22** using the amino acid N^{α}-Fmoc-4-*O*-[*O*′,*O*″-di-*tert*-butyl-2-(2-fluoromalonyl)]tyrosine (**7**) and solid-phase synthesis with Fmoc chemistry. Fmoc derivatives of standard amino acids were obtained from Bachem California (Torrence, CA) or from 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,[30] with DIPCDI/HOBT as coupling reagents in DMF (coupling time 1 h, except the FOMT amino acid **7** which was coupled for 39 h (peptide **19**) and for 17 h (peptide **22**)). Fmoc deprotection was conducted using 20% piperidine/DMF (2 min, 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, then 2.5 h at room temperature). To isolate the peptides, two-thirds of the reagent mixture from the deprotection reaction was evaporated under N_2, and the mixture was 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. Mass spectra (fast atom bombardment, 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-Ala-Asp-Glu-(L-FOMT)-Leu-amide (19): RPHPLC retention time 14.7 min (gradient 10−45% B over 25 min); FABMS $(M - H)^-$ 884.4 (calc 884.4); amino acid analysis Asp 1.92 (2), Glu 1.06 (1), Ala (0.94 (1), Leu 1.08 (1). FOMT was not analyzed.

Ac-Asp-(L-FOMT)-Val-Pro-Met-Leu-amide (22): RPHPLC retention time 12.5 min (gradient: 25−45% B over 20 min); FABMS $(M - H)^-$ 896.4 (calc 896.4); amino acid analysis Asp 1.00 (1), Pro 1.00 (1), Val 1.00 (1), Met 0.97 (1), Leu 1.03 (1). FOMT was not analyzed.

PTP Inhibition Assay. The ability of FOMT hexamer **19** to inhibit the PTP 1B-mediated dephosphorylation of phosphorylated insulin receptor was measured as previously described.[22] In summary, Chinese hamster ovary (CHO) cell line transfected with an expression plasmid encoding the normal human insulin receptor [CHO/HIRc] was used in this study and was a generous gift from Dr. Morris F. White, Joslin Diabetes Center, Boston, MA. The cells were maintained in F-12 medium containing 10% fetal bovine serum and were cultured to confluence. Membranes from these cultured CHO/HIRc cells were isolated and solubilized with Triton X-100, essentially as described.[31] Partially purified insulin receptors from solubilized membranes were obtained after passing through a wheat germ agglutin (WGA) column.[32] WGA-purified human insulin receptors were autophosphorylated with [γ-^{32}P]ATP as previously described,[31] and this ^{32}P-labeled

insulin receptor was used as substrate for the assay of PTP 1B activity, essentially following the method described.[16]

SH2 Domain Binding Assays. Details of the SH2 domain competition assay have been published previously.[33] In this study the relative p85 PI 3-kinase C-terminal SH2 domain affinity was determined for the FOMT-containing peptide **22** vs a high-affinity phosphopeptide ligand. The glutathione S-transferase (GST)/SH2 domain fusion protein was paired with [^{125}I]Bolton−Hunter-radiolabeled phosphopeptide corresponding to the sequence derived from IRS-1 pY628, GNGD-pYMPMSPKS,[33] and varying concentrations of unlabeled **20** were added as competitors. 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 **22** were combined in 150 μL total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, 10 mM dithiothreitol, pH 7.4, and vortexed. Glutathione-agarose (50 μL of a 1:10 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.

pK_a Determination. Malonic acid was obtained from Aldrich Chemical Co. and was used as a reference compound. Deionized H_2O (HPLC grade) and a standard solution of NaOH (0.1 N) were purchased from Fischer Scientific. A Beckman Model Φ45 pH meter with a combination epoxy electrode was used to measure pH. The test compounds malonic acid, **12**, and **13** were accurately weighed (approximately 0.01 mmol) and dissolved in 10.0 mL of deionized H_2O using a magnetic stirrer. Each solution was titrated by dispensing 10 μL aliquots of 0.1 N NaOH using a 20 μL pipetman. The pH was measured after each addition. The pH profiles for each analogue were then plotted and used to determine titration equivalents. Titration equivalents are obtained at the maxima of the first derivative of the titration curve. The log of the ionization constant (pK_a) was determined by treating potentiometric titration data according to the method of Noyes. This method is used for compounds containing more than one titratable group with pK_a values differing by less than 2.7 units.[34]

Molecular Modeling. Modeling was conducted as previously described.[22] The structure of a malonate group [CH_2-$(CO_2)_2$], complexed within the catalytic site of the PTP 1B enzyme, was first defined using the geometry of the binding site and mode of binding of a phosphonate derived from X-ray crystallographic data of the difluorophosphonate-containing inhibitor [1,1-difluoro-1-(2-naphthyl)methyl]phosphonic acid bound within the PTP 1B catalytic site.[28] The model was then minimized by an ab initio method using a 3-21G basis set on Cray and CONVEX mainframe computers using GAUSSIAN 92.[35] In minimizing the complex of the PTP 1B with the malonate structure, not only the geometry parameters and position of the malonate were optimized but also the geometry of the enzyme structure within the binding site during the first 50 h of CPU time. In order to examine potential alterations in binding interactions afforded by introduction of a fluorine onto the malonyl α-methylene, one hydrogen was replaced by a fluorine atom, with corresponding changing of the C−F bond length. The results of this modification are shown in Figure 2. From molecular mechanics (CHARMm), binding of benzene−OCF$(CO_2)_2$ to PTP 1B is 2 kcal/mol stronger than benzeneOCF-$(CO_2)_2$.

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