Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 457–460

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Characterization of a Novel Class of Protein Tyrosine Phosphatase Inhibitors

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Received 23 November 1999; accepted 28 December 1999

Abstract—Nonpeptidyl aryloxymethylphosphonates were prepared and evaluated as protein tyrosine phosphatase inhibitors. The results suggest that aryloxymethylphosphonates are effective nonhydrolyzable phosphotyrosine surrogates and provide further insight into the molecular mechanisms by which phosphate mimics inhibit phosphatase function.

Protein tyrosine phosphatases (PTPs), in conjunction with protein tyrosine kinases (PTKs), regulate a myriad of critical signaling events including proliferation, differentiation, motility, development and metabolism.[1] While PTKs have been extensively studied, the characterization of the equally important PTPs has only recently been undertaken.[2] The precise roles of PTPs in many of these signaling pathways are beginning to be elucidated. For example, PTP1B has been implicated as a negative regulator in the insulin-stimulated signaling pathway.[3,4] In addition, mice lacking PTP1B activity exhibit increased sensitivity towards insulin and are resistant to obesity.[5] The design of specific nonpeptidyl PTP1B inhibitors may provide a powerful tool in understanding the in vivo role of this protein in signal transduction. This inhibitor class may also prove therapeutically beneficial in the treatment of Type II diabetes mellitus.

An effective strategy towards the design of PTP inhibitors is the replacement of the labile phosphotyrosine (pTyr) with a nonhydrolyzable analogue. The most commonly used phosphorus-based pTyr analogues are phosphonomethyl phenylalanine (Pmp, **1a**, X = $CH_2CH(NH_2)CO_2H$)[6,7] and phosphonodifluoromethyl phenylalanine (F_2Pmp, **1b**, X = $CH_2CH(NH_2)CO_2H$).[8–10] An interesting observation is that peptides bearing F_2Pmp are over 1000 times more potent PTP inhibitors than the analogous peptides containing Pmp.[9,10] This has been attributed to a direct interaction between the fluorine atoms and PTP active site residues rather than the lowering of the pK_a value of the phosphonic acid.[10] Indeed, specific interactions between one of the fluorine atoms in difluoronaphthylmethyl phosphonic acid and PTP1B have been observed.[11]

Biochemical studies indicate that the phenolic oxygen in the substrate receives a proton from the general acid (Asp181 in PTP1B) in the transition state.[12] Moreover, X-ray crystal structure of PTP1B/C215S in complex with a pTyr-containing substrate reveals a hydrogen bond between the phenolic oxygen and Asp181.[13] It is possible that the enhanced affinity of F_2Pmp over Pmp arises from the ability of the fluorine atoms in F_2Pmp to interact with the PTP active site residues in a fashion analogous to that involving the phenolic oxygen and side chains in the active site of PTPs, which is lost in the Pmp-containing molecules. As part of an ongoing effort to identify effective and specific PTP inhibitors, we have synthesized a class of compounds, aryloxymethylphosphonates **3**. We reasoned that the hydrogen bonding interactions of PTP active site residues with the phenolic oxygen in substrates **1c** would be similarly maintained in analogues **3**. These phosphonate derivatives allow us to test the rationale that the phenolic oxygen plays a central role in enzyme–substrate interactions and to explore other structural features that are important for high affinity binding to PTPs.

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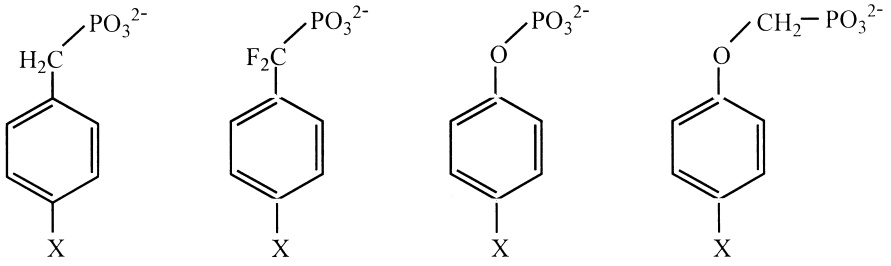
0960-894X/00/$ - see front matter
PII: S0960-894X(00)00019-6



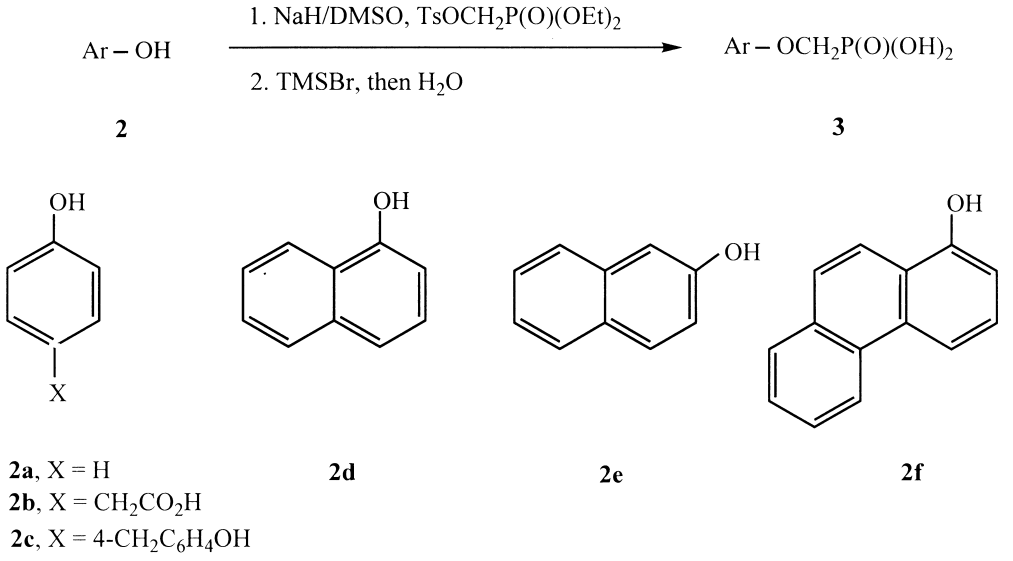
1a **1b** **1c** **3**

Synthesis

Compounds of type **3** were prepared via facile two-pot syntheses from readily available phenol derivatives (Scheme 1). Compounds of type **2** were treated with the corresponding equivalent of NaH (60% in oil; 1.1 equiv for **2a**, **2d–f**; 2.2 equiv for **2b–c**) in DMSO for 30 min to give dark red solutions. Diethyl *p*-tolylsulfonyloxymethyl phosphonate (1.1 equiv for **2a–b**, **2d–f**; 2.2 equiv for **2c**), prepared according to the literature procedure,[14] was then added and the mixture was stirred overnight.[15] Alternatively, diethyl *p*-chlorophenylsulfonyloxymethyl phosphonate can be used to give the desired products with slightly increased yields.[14] Cleavage of the phosphonate diesters followed a modified procedure.[16] After addition of excess trimethylsilyl bromide (TMSBr) to the diester intermediates, the mixture was stirred for 1 h. TMSBr was then evaporated under reduced pressure and followed by the stepwise addition of EtOAc and deionized water to the reaction mixture. The two layers were subsequently mixed followed by removal of the organic layer. The EtOAc wash was repeated two additional times. Final products **3** were then concentrated by the removal of water under reduced pressure. No further purification was needed.[17] Isolated yields for all four steps ranged from 71 to 87%.

Biochemical Characterization

The effect of the aryloxymethylphosphonates on the PTP1B and the dual specificity phosphatase VHR catalyzed *p*-nitrophenyl phosphate hydrolysis reaction was examined at 30 °C and pH 7.0.[18] The aryloxymethylphosphonates were not hydrolyzed by the PTPs and inhibited the PTP reaction reversibly and competitively with respect to the substrate (data not shown). The K_i values are summarized in Table 1. It is interesting to note that the K_i value for **3a** is similar to that of difluorobenzyl phosphonate (**1b**, X = H).[19] In addition, the K_i value of **3a** is also similar to the K_m of phenyl phosphate.[20] Thus insertion of a methylene group between the phenolic oxygen and the phosphonate group does not disrupt the hydrogen bonding interactions between the phenolic oxygen and the enzyme active site. This also suggests that the additional spacer of the methlylene group in **3a** has no adverse effects on the ability of the phosphate group and the aromatic moiety to interact with the enzyme. Thus, aryloxymethylphosphonates, like F_2Pmp, are effective nonhydrolyzable pTyr mimics that when attached to an appropriate peptide template should yield high affinity PTP inhibitors.



2a, X = H
2b, X = CH_2CO_2H
2c, X = 4-$CH_2C_6H_4OH$

2d **2e** **2f**

Scheme 1. Synthesis of aryloxymethylphosphonates.

Table 1. Inhibition constants of PTP1B and VHR with aryloxymethylphosphonates

Compound	PTP1B K_i (mM)	VHR K_i (mM)
3a	3.3 ± 0.2	26 ± 5
3b	1.1 ± 0.1	2.6 ± 0.4
3c	0.047 ± 0.005	1.3 ± 0.2
3d	1.3 ± 0.2	4.8 ± 0.5
3e	1.3 ± 0.1	5.7 ± 0.5
3f	0.41 ± 0.09	0.57 ± 0.08

It is also possible that potent and selective non-peptidyl low molecular weight inhibitors of PTP1B can be obtained when a properly functionalized phosphate surrogate is attached to an appropriate aromatic framework, which effectively occupies the pTyr pocket and interacts with the immediate surroundings beyond the catalytic site, thereby enhancing both inhibitor affinity and specificity. As shown in Table 1, addition of a propanoic acid (**3b**) or an aromatic ring(s) to **3a** (**3d**–**f**) increases the affinity for both PTP1B and VHR. Moreover, it appears that both PTP1B and VHR do not discriminate between **3d** and **3e**, consistent with an early study with difluronaphthalenylmethyl phosphonic acids.[21] These results indicate that there is an inherent plasticity in PTP active sites to accommodate compounds significantly larger than pTyr. Remarkably, compound **3c** bearing two aryloxymethylphosphonates exhibited a K_i value that was 70-fold more potent than that of the mono derivative (**3a**). Compound **3c** was also nearly 30-fold more selective toward PTP1B than VHR. The high affinity of **3c** for PTP1B may be the result of additional binding interactions between the second aryloxymethylphosphonate and the second non-catalytic aryl phosphate binding site in PTP1B.[22] Alternatively, the second aryloxymethylphosphonate in **3c** may also form salt bridges with Arg47 in PTP1B as the acidic amino acids at P-1 and P-2 do.[13] The fact that the K_i for **3c** is comparable to those of bis-diflurobenzyl phosphonate methane[23] and other *bis*-aryldifluorophosphonates[24] is consistent with the conclusion that aryloxymethylphosphonates are effective nonhydrolyzable phosphotyrosine surrogates.

Conclusion

In summary, we have described the synthesis and characterization of a novel class of PTP inhibitors, aryloxymethylphosphonates, which act as nonhydrolyzable pTyr surrogates. The ability of the aryloxymethlyphosphonates to inhibit PTPs as well as the difluoromethylenephenyl phosphonates implies that the active site of PTPs may interact with the ether oxygen in aryloxymethylphosphonates in a manner analogous to that involving the phenolic oxygen of substrates. We have also demonstrated that potent and selective small molecule PTP inhibitors can be obtained with properly functionalized aryloxymethylphosphonates. These molecules effectively occupy the pTyr pocket and interact with the immediate surroundings beyond the catalytic site, thereby enhancing both inhibitor affinity and specificity. Because of their relative ease of preparation without loss of inhibitor potency, the aryloxymethylphosphonates provide an excellent alternative to difluoromethylenephosphonates as nonhydrozable pTyr surrogates. Moreover, the ability of aryloxymethylphosphonates to serve as nonhydrolyzable pTyr mimetics should make them very useful in studying the role of SH2 domains, in addition to PTPs, in cell signaling. Currently, we are exploring methods to incorporate this moiety into appropriate peptide templates.

Acknowledgements

We thank Mr. Nduka M. Amankular for his excellent assistance in the synthesis of compounds **3a**–**3f**. We also thank Ms. Saba Al-Zabin for her assistance in the preparation of this manuscript. This work was supported in part by a Research Award from the American Diabetes Association (Z.-Y. Z.). O.I. is a recipient of the MSTP fellowship administered by the NIH. K. Z. acknowledges the donors of the Petroleum Research Fund, administered by the American Chemical Society, and NSF Faculty Early Career Development Program for their financial support.

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17. All intermediates and final compounds had satisfactory ^{1}H, ^{13}C NMR spectra.

18. The PTP activity was assayed at 30 °C in a reaction mixture (0.2 mL) containing appropriate concentrations of *p*NPP 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 enzyme 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}. Inhibition constants for the PTP inhibitors was determined in the following manner. The initial rate at eight different *p*NPP concentrations (0.2–5 K_m) was measured at three different fixed inhibitor concentrations. The inhibition constant and inhibition pattern were evaluated using a direct curve-fitting program KINETASYST (IntelliKinetics, State College, PA).

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