Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 345–350

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

NAPHTHALENEBIS[α,α-DIFLUOROMETHYLENEPHOSPHONATES] AS POTENT INHIBITORS OF PROTEIN TYROSINE PHOSPHATASES.

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Received 3 November 1997; accepted 6 January 1998

Abstract: A series of naphthalenebis(difluoromethylenephosphonates) were prepared and compared to their monosubstituted counterparts as inhibitors of the protein phosphatases, PTP1B, CD45 and PP2A. In general, the bissubstituted compounds were better inhibitors than the mono derivatives and some of these are among the most potent, nonpeptidyl inhibitors of protein tyrosine phosphatases, PTP1B and CD45, reported to date.
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Protein tyrosine phosphatases (PTPs) are enzymes that catalyze the removal of phosphate groups from phosphotyrosyl residue in proteins. In conjunction with the protein tyrosine kinases (PTKs), they are responsible for the regulation of a wide variety of important cellular processes such as T-cell activation, cell growth and proliferation, and oncogenic activation.[1a,b] The increasing number of reports implicating the involvement of PTPs in a variety of disease states and as positive signal transducers[2a-e] have made these enzymes attractive targets for therapeutic intervention. One tactic that is often used to obtain inhibitors of enzymes that catalyze the hydrolysis of phosphate esters is to replace the labile phosphate group with a stable, nonhydrolyzable phosphate mimetic. One such phosphate mimetic is the α,α-difluoromethylenephosphonate group, and there are numerous reports describing compounds bearing this moiety that are inhibitors of enzymes that hydrolyze or bind phosphate esters.[3a-e] It has been suggested that the α,α-difluoromethylenephosphonate group is an effective phosphate mimetic because it is isopolar and isosteric to the phosphate group.[4] This moiety has proven to be especially effective for obtaining potent PTP inhibitors. For example, Burke and coworkers have shown that peptides bearing difluorophosphonomethylphenylalanine (F_2Pmp, **1**) are potent PTP inhibitors and over 1000-fold more effective inhibitors than the analogous peptides bearing phosphonomethylphenylalanine (Pmp, **2**).[5a,b] The high affinity of peptides bearing **1** for PTPs has been attributed to a direct interaction of the fluorines with active site residues and not to the lower pK_a values of the phosphonic acid in **1** than in **2**.[5b]

Although peptide-based inhibitors are useful in determining important properties for substrate-PTP interactions, their use as probes for cellular studies and as therapeutics is limited. Thus, Burke and coworkers have examined a series of nonpeptidyl benzylic phosphonates as PTP inhibitors.[6a,b] From this series, some

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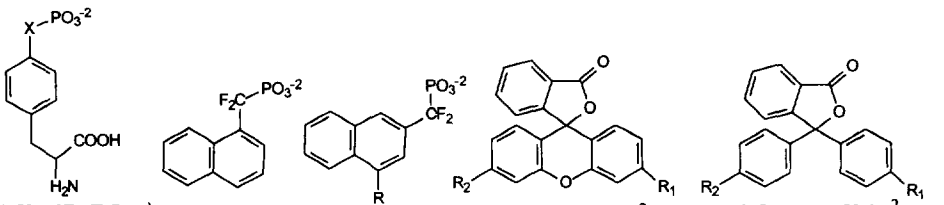
PII: S0960-894X(98)00027-4

naphthalene derivatives bearing the α,α-difluoromethylenephosphonic acid (DFMP) group, **3**, **4** and **5**, were relatively good (K_i = 255 μM for **3**, 197 μM for **4**, and 93 μM for **5**) competitive inhibitors of the PTP, PTP1B but also inhibited the serine/threonine phosphatase, PP2A, and were not examined as inhibitors of other PTPs.

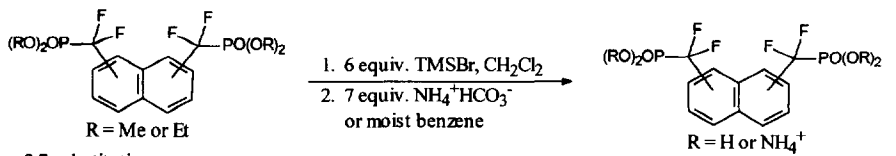


1, X = CF_2 (F_2Pmp) **3** **4**, R = H **6**, $R_1 = R_2 = OPO_3^{-2}$ **8**, $R_1 = R_2 = OPO_3^{-2}$
2, X = CH_2 (Pmp) **5**, R = OH **7**, $R_1 = OPO_3^{-2}$, R_2 = H **9**, $R_1 = OPO_3^{-2}$, R_2 = H

Studies with nonpeptidyl substrates have provided some insight into the structure-activity relationship between PTPs and nonpeptidyl ligands.[7,8] Our earlier studies demonstrated that fluoroesceindiphosphate (FDP), **6**, has a significantly lower K_m and hydrolysis of the first phosphate group occurs much faster than fluoresceinmonophosphate (FMP), **7**, with a number of PTPs.[7] Later, Zhang and coworkers found that a similar phenomenon occurs with phenolphthalein mono and diphosphate, **8** and **9**, and rat PTP1.[8] On the basis of the studies with the naphthalene derivatives[6a,b] and the above bisphosphate substrates,[7,8] we reasoned that incorporation of two DFMP moieties into a naphthalene ring may increase their selectivity and inhibitory potency towards PTPs. In this communication we address the following questions: Does the presence of two DFMP groups on a naphthalene ring increase the selectivity and inhibitory potency relative to their mono-DFMP counterparts? If so, then where on the ring should the DFMP groups be placed in order to achieve maximal inhibition and selectivity? Here we report that a naphthalene ring bearing two DFMP groups does indeed increase the potency of this class of inhibitors relative to those having only one DFMP group and that some of these are among the most potent, reversible, small molecule inhibitors of PTPs reported to date.

We chose two PTPs, human PTP1B and CD45, and one serine/threonine phosphatase, PP2A, for inhibition studies. PTP1B is a cytosolic PTPase consisting of a single catalytic domain. This is one of the most studied of all the PTPs and a crystal structure of it complexed with **4** has recently been reported.[6b,9] In addition, overexpression of this enzyme has been reported in certain cancers [2b] and insulin resistant states.[2a] CD45 is a transmembrane PTP expressed on the surface of hematopoietic cells. It is believed to be responsible for the dephosphorylation of Src PTKs which results in the up-regulation of their catalytic activity that eventually leads to cell activation.[10] PP2A has been implicated in regulating many cellular function[11] and was selected for the purpose of comparing the effects of the inhibitors on other types of phosphatases.

A series of naphthalene derivatives, **10a-10f**, bis-functionalized with a methyl- or ethyl-protected DFMP group, were prepared by electrophilic fluorination of the corresponding phosphonate esters.[12] The methyl or ethyl protecting groups were removed using TMSBr in CH_2Cl_2 to give the silyl esters, which were then treated with an aqueous solution of ammonium bicarbonate followed by repeated lyophilizations or with moist benzene followed by filtration to give the desired bis-DFMP compounds, **11a-11f**, as either the



10a - 2,7 substitution **10d** - 1,3 substitution
10b - 2,6 " **10e** - 1,6 "
10c - 1,5 " **10f** - 1,7 "

11a - 2,7 substitution **11d** - 1,3 substitution
11b - 2,6 " **11e** - 1,6 "
11c - 1,5 " **11f** - 1,7 "

ammonium salts or as the free acids.[13] All of the compounds were found to be stable in neutral aqueous solution and could be stored indefinitely as the solid salt or in neutral aqueous solution without any detectable decomposition.

Rates of PTP-catalyzed[14] dephosphorylation in the presence or absence of inhibitors were determined using FDP as substrate[7] in buffer containing 50 mM Bis-Tris, 2 mM EDTA and 5 mM DMH, pH 6.5 at room temperature. Assay were carried out in 96-well plates with total volume of 200 μL and rates were obtained by continuously monitoring fluorescence from the production of FMP with excitation 440 nm (slit width 20 nm) and emission at 530 nm (slit width 25 nm) using a Cytofluor II plate reader (Perseptive Biosystems). PP2A was assayed using FDP as substrate[15] in 25 mM Tris-HCl, pH, 7.5, containing 5 mM DTT, 0.5 mM $MnCl_2$ and 0.1 mg/ml BSA. Reactions were initiated by the addition of PP2A and the production of FMP was monitored as described above.

An initial assessment of the inhibitory potency of the compounds was performed at a concentration of 500 μM with CD45, PTP1B and PP2A using FDP as substrate at a concentration equal to the K_m value for each enzyme. The results, in terms of percent inhibition, are summarized in Table 1. Consistent with the study by Kole et al,[6a] 500 μM of the monosubstituted compounds, **3** and **4**, inhibited PTP1B relatively well

Table 1. Percent Inhibition of Phosphatases with Naphthyl α,α–Difluoromethylenephosphonic Acids.

	Percent Inhibition[d]		
inhibitor	CD45[a]	PTP1B[b]	PP2A[c]
3	20 ± 20	83 ± 6	52 ± 4
4	5 ± 5	84 ± 1	39 ± 2
11a	64 ± 3	100 ± 0.2	70 ± 0.3
11b	62 ± 3	100 ± 0.2	70 ± 6
11c	26 ± 1	51 ± 8	77 ± 1
11d	78 ± 1	81 ± 3	66 ± 1
11e	70 ± 2	89 ± 2	75 ± 0.9
11f	99 ± 0.1	98 ± 0.3	61 ± 2

[a][FDP] = 20 μM, [b][FDP] = 10 μM, [c][FDP] = 35 μM, [d]mean data ± standard deviation

showing 83% and 84% inhibition, respectively. However, they were less potent with PP2A, in contrast to the report[6a] in which **3** and **4** inhibited PTP1B and PP2A equally well. The difference between our results and Kole et al[6a] may be due to significant differences in assay conditions and substrates used in the PP2A assays. **3** and **4** were very poor inhibitors of CD45. The addition of a second DFMP group had very little effect on PP2A and, consequently, all of the bis-substituted compounds were poor inhibitors of this enzyme. In contrast, the presence of two DFMP groups at certain positions on the ring had a considerable effect on PTP1B

and CD45. For example, 500 μM of the 2,7- (**11a**) and 2,6- (**11b**) derivatives completely inhibited PTP1B and 500 μM of the 1,7-derivative (**11f**) resulted in 98% and 99% inhibition of PTP1B and CD45, respectively.

Compounds **11a**, **11b** and **11f** and the monosubstituted inhibitors, **3** and **4**, were examined in more detail with PTP1B and CD45. IC_{50}'s determination were determined in duplicate at 10 different inhibitor concentrations with FDP at K_m concentration and the results are summarized in Table 2. Compounds **11a**, **11b** and **11f** exhibited IC_{50}s of 26 μM, 29 μM and 118 μM respectively with PTP1B. Thus, **11a** and **11b** have IC_{50} values approximately four-five fold lower than that of the monosubstituted inhibitors, **3** and **4**, with

Table 2. IC_{50} Determination of Selected Naphthyl α,α–Difluoromethylenephosphonic Acids.

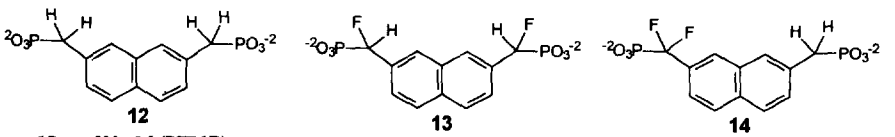
inhibitor	IC_{50} (μM)		
	CD45	PTP1B	PP2A
3	> 1000	112 ± 23	636 ± 152
4	> 1000	95 ± 10	753 ± 144
11a	180 ± 41	26 ± 3	306 ± 28
11b	> 100	29 ± 4	354 ± 32
11f	27 ± 3	118 ± 20	481 ± 34

[a][FDP] = 20 μM, [b][FDP] = 10 μM, [c][FDP] = 35 μM, [d]mean data ± standard deviation

PTP1B. The 1,7-derivative, **11f**, is approximately five-fold more potent with CD45 (IC_{50} = 27 μM) than with PTP1B and the additional DFMP group dramatically increased the inhibitory potency of CD45 relative to the monosubstituted compounds. Compound **11a** is approximately seven-fold more potent with PTP1B than with CD45 and is therefore more selective, in terms of PTP inhibition, than **11f**.

A K_i was determined for **11a**, with PTP1B and **11f**, with CD45. Eight FDP concentrations and six inhibitor concentrations were employed for this experiment and the data points were fit simultaneously to the Michaelis-Menton equation for competitive inhibition using the KaleidaGraph program (version 3.05). This yielded a K_i of 9 ± 1 μM for **11f** with CD45. The K_i with for **11a** with PTP1B was 16 ± 2 μM and one would expect to obtain a similar K_i with **11b**. To our knowledge, *11a, 11b, and 11f are among the most potent, reversible, small molecule inhibitors of PTP1B and CD45 reported to date.*

We also examined whether the fluorines in the 2,7-derivative (**11a**), which was the most selective inhibitor obtained in this study, were necessary for potent and selective inhibition. Thus, the non-fluorinated and monofluorinated 2,7-derivatives, **12** and **13**, were prepared and examined for their ability to inhibit PTP1B and CD45.[16] Both are poorer inhibitors of PTP1B and CD45 than **11a** demonstrating that the fluorines are essential for potent inhibition. However, the effect is small for CD45 (three-fold) but much more significant (twenty-fold) for PTP1B and so selectivity decreases as the number of α-fluorines decreases. One possible explanation for the enhanced affinity of **11a** compared to **12** and **13** is that PTPs require the dianionic form of the phosphonates for binding. It has been shown that the pK_{a2} of phosphonic acids decreases with α-fluorination (CF_2, pK_{a2} ~ 5.4-5.7; CHF, pK_{a2} ~ 6.2-6.5 , CH_2, pK_{a2} ~ 7.5-7.7).[5b,17] Thus, at pH 6-7, **12** and **13** exist only partially as the dianion while **11a** is almost completetly ionized. However, on the basis of data obtained from the crystal structure of PTP1B complexed with **4** and theoretical calculations,[14] it has been



12 IC_{50} > 500 μM (PTP1B) IC_{50} = 500 μM (CD45)

13 IC_{50} = 130 μM (PTP1B) IC_{50} = 279 μM (CD45)

14

suggested that tight binding of **4** to PTP1B is mainly a result of a hydrogen bond between the pro-R fluorine and the amido group of Phe_{182}.[6b] Thus, it is possible that the fluorines in one of the DFMP groups in **11a** (and in the other bis-DFMP inhibitors) enhance binding to PTP1B in a manner similar to that found with **4**, although pK_a effects cannot be ruled out. Nevertheless, whether or not the fluorines contribute via H-bonding or secondary pK_a effect, the presence of the fluorines alpha to at least one of phosphonate groups is necessary for potent inhibition. However, whether α-fluorination on both phosphonate groups is necessary for potent inhibition is less clear. It is possible that the fluorines on the second DFMP groups contribute to enhanced binding via H-bonding and/or pK_a effects. However, PTP1B has a strong preference for acidic residues N-terminal to phosphotyrosine as a result of electrostatic interactions of the acidic residues with certain Arg residues.[9a,9b,18] In addition, Zhang and coworkers have shown that the presence of a $CH_2CO_2^-$ at the para position in phenylphosphate lowers the K_m by approximately 15-fold relative to *p*-ethylphenylphosphate with rat PTP1 which is the rat analogue of human PTP1B.[8] Thus, it is possible that the second DFMP group may enhance binding solely via electrostatic interactions and the presence of the fluorines alpha to the second phosphonate group may not be necessary for this interaction to occur. We are currently exploring methods for synthesizing compound **14** which will allow us to determine whether the fluorines are essential on both phosphonate groups. The crystal structures of PTP1B complexed with **11a** and **11b** are also in progress. In addition, we are also examining methodologies for converting these inhibitors into cell permeable "caged" compounds for cellular studies.

Acknowledgements. We thank Jamie Snider for his help. We thank the Natural Sciences and Engineering Council of Canada for financial support via an operating grant for SDT.

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