Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 3382–3385
Bioorganic & Medicinal Chemistry Letters

Designing better coumarin-based fluorogenic substrates for PTP1B

Christopher P. Holmes,* Natalie Macher, J. Russell Grove, Larry Jang and Jennifer D. Irvine

Affymax, Inc., Department of Chemistry, 4001 Miranda Avenue, Palo Alto, CA 94304, USA

Received 11 February 2008; revised 5 April 2008; accepted 10 April 2008
Available online 13 April 2008

Abstract—As part of a program to develop better PTP1B fluorogenic substrates that more closely mimic the functionality found in the natural substrate, we have prepared and evaluated nine novel analogs of 4-methylumbelliferone phosphate (MUP) with a variety of additional groups occupying the second phosphate binding pocket.

Inhibition of protein tyrosine phosphatases (PTP) continues to be a popular area of medicinal research due in part to their link to numerous disease states and a surprisingly broad array of acceptable scaffolds.[1–4] The most targeted PTP is PTP1B, the phosphatase responsible for attenuating the signaling of the insulin receptor, with its obvious link to the control of Type II diabetes and obesity. In addition, the recent exciting link between PTP1B and tumor progression will likely fuel more interest in PTP1B inhibitors.[5] Assays for PTP activity frequently employ non-fluorogenic substrates that are rendered fluorogenic after the enzyme-induced hydrolysis of a phosphate bond. Two common substrates have been 3-*O*-methyl-fluorescein phosphate (OMFP) and 4-methylumbelliferone phosphate (MUP) and to a lesser extent difluoroumbelliferone (DiFMUP).[6] What is striking about these substrates is their lack of homology to the natural peptidic substrates except for the immediate phenyl ring bearing the phosphate group (Fig. 1).

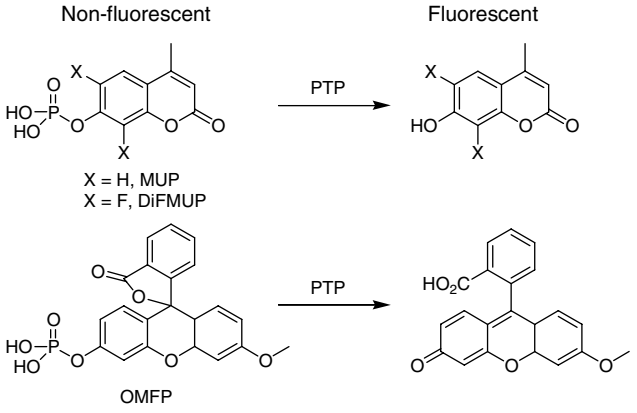


Figure 1. Structures of common fluorogenic PTP substrates.

The natural substrate for PTP1B is the activated insulin receptor, which contains two adjacent phosphotyrosine residues at positions 1162 and 1163, and PTP1B has been shown independently to possess two phosphate binding sites.[7] Thus, tripeptide DY(P)Y(P) represents the natural substrate sequence, with the central phosphate being the one that is removed by PTP1B. The presence of two adjacent phosphotyrosine residues is extremely rare and was believed to be found only in PTP1B and the related T-cell PTP (TCPTP) until the recent discovery that another unrelated PTP, PTPL1, also contained this motif.[8] During the course of our PTP1B screening efforts where we employed either OMFP or MUP substrates,[9,10] we became concerned that it may be possible to have a small molecule selectively bound to the enzyme in the second phosphate site and not extend into the primary site. While binding to either phosphotyrosine pocket is expected to readily block the binding of the natural peptidic substrate and serve as an effective drug, occupying the second site alone may not interfere with the unnatural substrates OMFP or MUP. Thus, we questioned if our screens could be producing false negatives due to our choice of substrate. To address these concerns, we designed a number of easily prepared coumarin-based fluorogenic substrates for

Keywords: PTP1B; Coumarin; Substrate; Fluorogenic.
* Corresponding author. Tel.: +1 650 812 8737; fax: +1 650 424 0832; e-mail: chris_holmes@affymax.com

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.04.021

PTP1B that possess additional substituents intended to occupy more than just the primary phosphotyrosine binding pocket. Evaluation of their kinetic rate constants (k_{cat}) and Michaelis constants (K_m) has revealed interesting substrate requirements for PTP1B. Barrios has described an elegant coumarin-based amino acid that can be incorporated into peptides for substrate screening purposes.[11,12]

Our substrate design uses the commercially available 7-hydroxycoumarinyl-4-acetic acid **1**, which possesses a key carboxylic acid in the same relative position as the natural peptide (Fig. 2). From this versatile scaffold, we have prepared nine new substrates with the chemistry detailed in Scheme 1.[13]

Treatment of **1** with benzyl chloroformate followed by hydrolysis of the carbonate bond affords the benzyl ester of **1**. This could be phosphorylated with carbontetrabromide/diethyl phosphite to form the intermediate diethyl phosphite derivatized coumarin. Deprotection with iodotrimethylsilane (TMSI) in dichloromethane affords substrate **2** as a white solid. Coupling of tyrosine methyl ester (H-Tyr-OMe) with **1** was readily achieved with hydroxybenzotriazole and 1-ethyl-3-(3′dimethylaminopropyl)carbodiimide (EDC). Bis-phosphorylation as before and deprotection afforded the amino acid conjugated compound **4**. Attempts to prepare **5** in an analogous fashion starting from tyrosinamide failed. We found, however, that treatment of **4** with ammonium hydroxide in methanol cleanly produced the desired amide **5** without damaging the phosphate groups.

The remaining novel substrates were prepared from the methyl ester **6**, itself prepared from straightforward esterification of **1** in acidic methanol. Methyl ester **7** was prepared by phosphorylation and deprotection of **6**. Introduction of a propionic acid side chain designed to be the retroamide analog of Asp-phosphoTyr was achieved by displacement of the methyl ester with β-alanine *tert*-butyl ester, followed by phosphorylation as before, TFA deprotection of the *tert*-butyl group, and TMSI treatment to produce **9**. The simple amide **11** was made by displacement of the methyl ester of **6** with 2,4-dimethoxybenzylamine to produce **10**. Phosphorylation and removal of the DMB group with TFA followed by deprotection of the phosphate with TMSI afforded **11** in high yield. The shorter approach of displacing the ester of **6** with ammonia failed at the subsequent phosphorylation step, which led to a complex mixture; we did not attempt treating ester **7** with ammonia at the time. The final substrates were prepared in an analogous fashion from both tyramine and 4-hydroxybenzylamine to produce both **13** and **14**. We found that brief treatment of **12** ($n = 2$) with the phosphorylation conditions employing carbontetrabromide–diethyl phosphite generated a mixture of the mono and bis-phosphate species, which were readily separated by flash chromatography. The monophosphate was deprotected to produce **15**; the structural proof that the phosphate was on the coumarin ring instead of the tryramine ring was obtained from its fluorescent properties: the desired product **15** is non-fluorescent, while the alternative adduct would be highly fluorescent.

The nine new species were evaluated as substrates for PTP1B and the observed kinetic constants are tabulated in Table 1. Because the additional substituents added to the acetic acid side chain in position 4 of the coumarin are not in conjugation with the parent chromophore, we did not evaluate shifts in absorption or emission wavelengths of the new molecules.

Literature results for the natural insulin substrate peptide TRDIY(P)ETDY(P)Y(P)RK are included in Table 1 and serve as benchmark for the evaluation of new substrates. As expected, this substrate possesses the lowest K_m value (8 μM) and highest turnover (11 s^{-1}) which together translates to the best substrate (i.e., highest k_{cat} / K_m value). The kinetic values for MUP reveal that it is about 30-fold worse than the peptide, primarily due to its worse apparent affinity for the enzyme (230 μM).

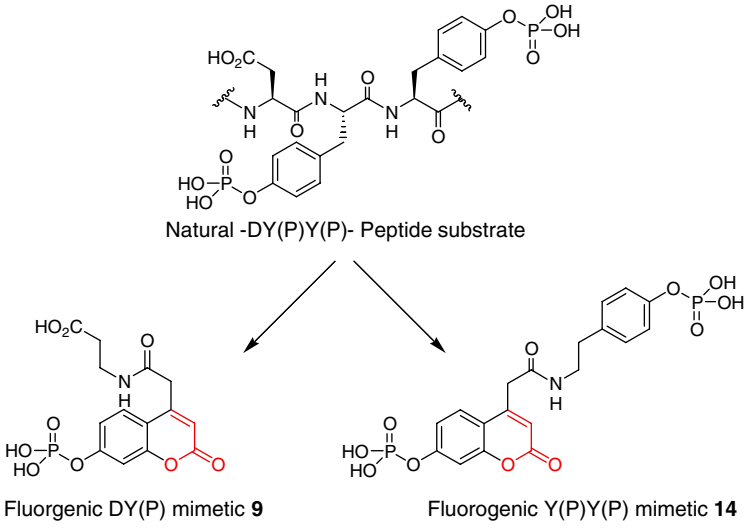
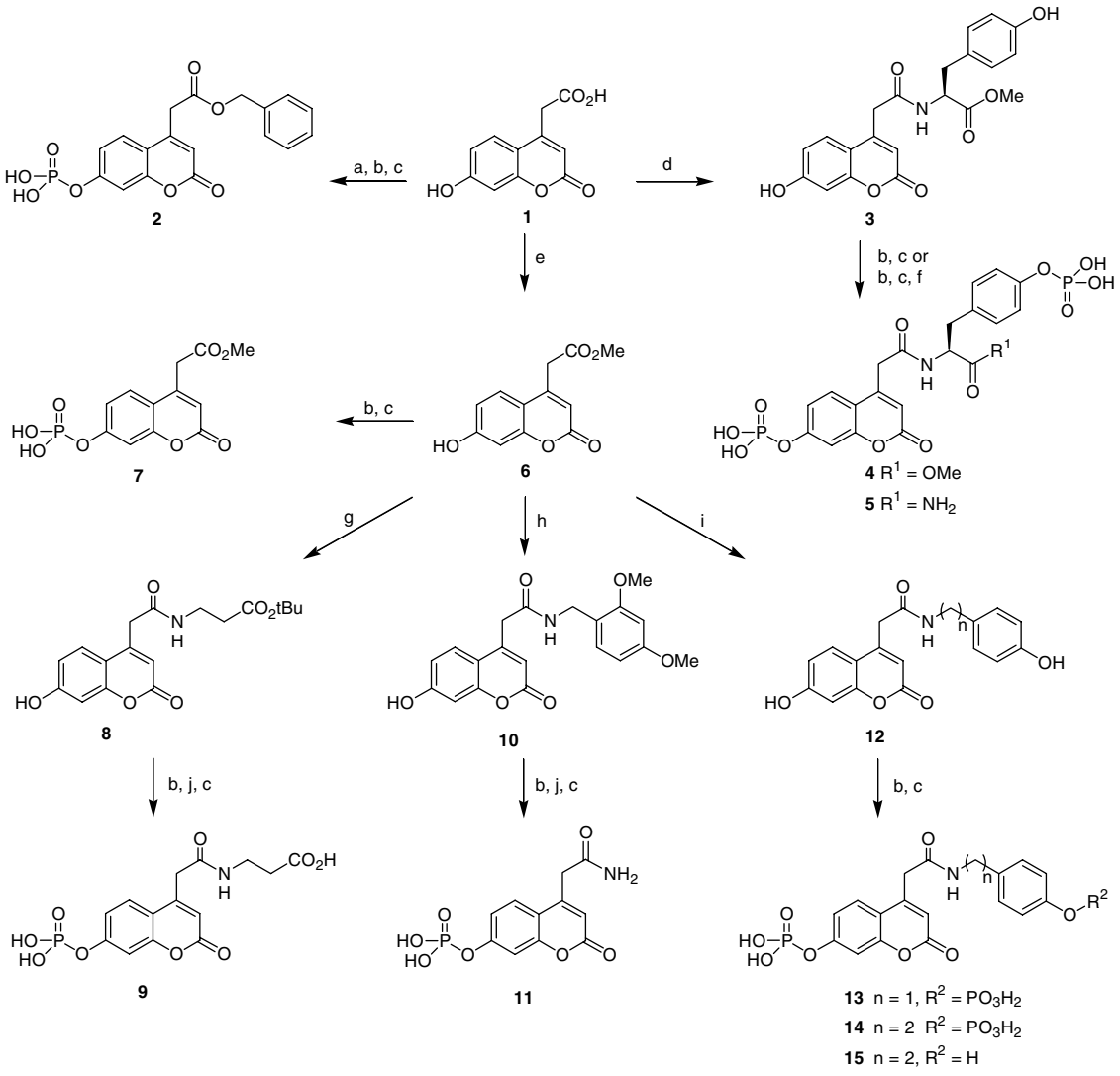


Figure 2. Overlay of natural peptide substrate with fluorogenic substrates **9** and **14** with the additional atoms of coumarin in red.



Scheme 1. Reagents and conditions: (a) $PhCH_2OCOCl$, DIEA, DCM, then K_2CO_3, MeOH, H_2O; (b) CBr_4, $(EtO)_2HPO$, DIEA, THF; (c) TMSI, DCM, then MeOH; (d) HOBt, H-Tyr-OMe, DIEA, EDC; (e) HCl, MeOH; (f) NH_4OH, MeOH; (g) $H_2NCH_2CH_2CO_2t$-Bu,THF, 70 °C; (h) H_2N-2,4-$(MeO)_2$-Ph, THF, 55 °C; (i) $H_2NCH_2CH_2$-4-OH-Ph or H_2NCH_2-4-OH-Ph, THF, 70 °C; (j) TFA, DCM.

Table 1. Kinetic rate constants (k_{cat}), Michaelis constants (K_m), and second order rate constants (k_{cat}/K_m) with PTP1B for fluorogenic substrates

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1}) $\times 10^4$
TRDIY(P)ETDY (P)Y(P)RK[a]	8	11.3	141
MUP	230	9.3	4.12
2	36	10.5	29.9
4	82	3.4	4.33
5	149	3.2	2.05
7	147	8.2	5.54
9	111	5.8	5.67
11	224	5.0	2.25
13	25	5.2	21.1
14	38	7.1	18.9
15	113	4.8	4.77

[a] Data from Ref. 14. Standard deviations for k_{cat} and K_m are ±20%.

Several of the new substrates were subtle variants of the parent MUP material and while not possessing substituents occupying the adjacent phosphotyrosine site, there were designed to explore if simple H-bonding groups would lower the Michaelis constants. Compounds **7** and **11**, representing ester and amide substituents, turned out to be equivalent substrates as MUP itself (k_{cat}/K_m values 2.2–5.5). Evidently, the interaction of Asp_{48} of PTP1B often shown as interacting with the backbone amide group[15] contributes little to the affinity of these two substrates. The simple benzyl ester **2** was found to be a surprisingly good substrate despite the lack of homology with the natural peptidic substrate. Thus, with a k_{cat}/K_m of 30, compound **2** is roughly 7-foldbetter as substrate than MUP and within 5-fold of the peptide. The fact that a simple aromatic ring appears to interact favorably with the enzyme's second phosphate binding site has been noted before with several small molecule inhibitors, most recently by Wilson et al.[16] Presumably, one could explore a variety of esters or amides with the plethora of available benzyl alcohols and amines to discover an even better substrate, but this was beyond the scope of this work.

We envisioned that the dipeptide mimetics **4** and **5** would most closely mirror the kinetics of the peptide, yet the observed kinetic constants were no better than the simple substrates **7** and **11**. It may be that the constrained coumarin ring precludes the second phosphotyrosine ring from matching the native conformation. Alternatively, the carboxymethyl group of **4** or the carboxamide group of **5** might force the second phosphotyrosine group into an unfavorable orientation relative to the natural substrate. Support for this second hypothesis comes from substrate **14**, which lacks the carboxamide group; now the measured k_{cat}/K_m value increases almost 10-fold relative to compound **5**. Interestingly, analog **13** with one fewer methylene in the side chain, and presumable one atom short of matching the natural peptide, is slightly better than **14** as a substrate. We conclude that the additional conformational constraint imposed by the coumarin ring prevents the side chain of our substrates from occupying the second phosphate binding pocket as does the natural substrate. When the second phosphate group is removed to produce **15**, the apparent affinity drops 3-fold and k_{cat}/K_m drops 4-fold, and this novel fluorogenic substrate is now only marginally better than the starting coumarin MUP.

The final substrate explored was the carboxylic acid **9** containing a retroamide linkage between the coumarin and the acid group. This compound did not prove to be a particularly good substrate ($k_{cat}/K_m = 5.67$) and was essentially the same as the simple carboxamide **7**.

With the kinetic evaluation of the nine new substrates complete, we decided to perform inhibitor screens with compounds **2** and **14** as substrates in comparison to OMFP (data not shown).[17] After carefully examining the results of about 100 inhibitor compounds ranging in size from 280 to 750 Da, we have only observed minor qualitative differences in IC_{50} values. This might reflect the fact that our molecules are already large enough to partially fill both sites, or that OMFP itself occupies both sites. Additional work with these new substrates and even smaller potential inhibitors will be required to know if one can discover molecules that occupy the adjacent phosphotyrosine site alone.

In conclusion, we have described the design of more appropriate substrates for high throughput assays of PTP1B. We have found that simple benzyl ester **2** is nearly 7-fold better as substrate than our starting point MUP, and is essentially equal as substrate to the bisphosphates **13** and **14**. While **2**, **13**, and **14** are still 5-fold off the natural peptidic substrate, they nevertheless illustrate how simple fluorogenic substrates can be modified to improve their properties. We anticipate that these principles can be applied to a number of physiological important PTPs.

References and notes

1. Kasibhatla, B.; Wos, J.; Peters, K. G. *Curr. Opin. Investig. Drugs* **2007**, *8*, 805.
2. Lee, S.; Wang, Q. *Med. Res. Rev.* **2007**, *27*, 553.
3. Zhang, S.; Zhang, Z.-Y. *Drug Discovery Today* **2007**, *12*, 373.
4. Pei, Z.; Liu, G.; Lubben, T. H.; Szczepankiewicz, B. G. *Curr. Pharm. Des.* **2004**, *10*, 3481.
5. Julien, S. G.; Dube, N.; Read, M.; Penney, J.; Paquet, M.; Han, Y.; Kennedy, B. P.; Muller, W. J.; Tremblay, M. L. *Nat. Genet.* **2007**, *39*, 338.
6. Montalibet, J.; Skorey, K. I.; Kennedy, B. P. *Methods* **2005**, *35*, 2.
7. Puius, Y. A.; Zhao, Y.; Sullivan, M.; Lawence, D. S.; Almo, S. C.; Zhang, Z.-Y. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13420.
8. Villa, F.; Deak, M.; Bloomberg, G. B.; Alessi, D. R.; van Aalten, D. M. F. *J. Biol. Chem.* **2005**, *280*, 8180.
9. Holmes, C. P.; Li, X.; Pan, P.; Xu, C.; Bhandari, A.; Moody, C. M.; Miguel, J. A.; Ferla, S. W.; De Francisco, M. N.; Frederick, B. T.; Zhou, S.; Macher, N.; Jang, L.; Irvine, J. D.; Grove, J. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2719.
10. Holmes, C. P.; Li, X.; Pan, Y.; Xu, C.; Bhandari, A.; Moody, C. M.; Miguel, J. A.; Ferla, S. W.; De Francisco, N.; Frederick, B. T.; Zhou, S.; Macher, N.; Jang, L.; Irvine, J. D.; Grove, J. R. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4336; Li, X.; Bhandari, A.; Holmes, C. P.; Szardenings, A. K. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4301.
11. Mitra, S.; Barrios, A. M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5142.
12. Mitra, S.; Barrios, A. M. *Anal. Biochem.* **2007**, *370*, 249.
13. Experimental procedure for **13**: To a solution of 2 mL of acetyl chloride in 120 mL of MeOH is added **1** (3.00 g). After 5 min a white ppt begins to form. After stirring for 2 h the reaction mixture is chilled to −15 °C overnight. The mixture is filtered and the white solid collected and dried to give 2.98 g (94% yield) of **6**. A mixture of **6** (0.20 g) and 4-hydroxylbenzyl amine (1.0 g) in 12 mL of THF is heated to 70 °C for 24 h. The reaction mixture is partitioned between EtOAc and 1 N HCl, washed with brine, and dried over $MgSO_4$. The solvent is removed under reduced pressure and the residue chromatographed on flash silica with a step gradient of 1–5–10% MeOH/$CHCl_3$ to afford 0.43 g of **12** as a light yellow solid (62% yield). A mixture of **12** (0.375 g), carbon tetrabromide (1.55 g), diethyl phosphite (0.70 mL), and DIEA (1.00 mL) in 40 mL of THF is stirred at RT for 23 h. The reaction mixture is partitioned between EtOAc and 0.1 N HCl, washed with brine, and dried over $MgSO_4$. The solvent is removed under reduced pressure and the residue chromatographed on flash silica with a step gradient of 5–10% MeOH/$CHCl_3$ to afford 0.62 g of the bis-diethylphosphate intermediate as a colorless oil. To a solution of the bis-diethylphosphate (0.62 g) in 60 mL of DCM is added TMSI (0.80 mL). The reaction mixture is quenched with MeOH after 1 h, and the solvent removed under reduced pressure. The residue is purified by preparative HPLC (C18, ACN/H2O/0.1% TFA) to afford 323 mg of **13** as a white solid.
14. Salmeen, A.; Anderson, J. N.; Myers, M. P.; Tonks, N. K.; Barford, D. *Mol. Cell* **2000**, *6*, 1401.
15. Groves, M. R.; Yao, Z.-J.; Roller, P. P.; Burke, T. R.; Barford, D. *Biochemistry* **1998**, *37*, 17773.
16. Wilson, D. P.; Wan, Z.-K.; Xu, W.-X.; Kirincich, S. J.; Follows, B. C.; Joseph-McCarthy, D.; Foreman, K.; Moretto, A.; Wu, J.; Zhu, M.; Binnun, E.; Zhang, Y.-L.; Tam, M.; Erbe, D. V.; Tobin, J.; Xu, X.; Leung, L.; Shilling, A.; Tam, S. Y.; Mansour, T. S.; Lee, J. *J. Med. Chem.* **2007**, *50*, 4681.
17. We routinely use MUP and OMFP interchangeably in our assays as they produce qualitatively similar results.