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Design and synthesis of fluorogenic substrates that target protein phosphatases

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Abstract—We have successfully designed and synthesized new fluorogenic probes that specifically target different classes of protein phosphatases. The fluorescence profiles of the probes have been studied using 12 different phosphatases, and results showed that, besides alkaline and tyrosine phosphatases, our probes were able to detect serine/threonine as well as acid phosphatases.

Protein phosphorylation/dephosphorylation is one of the most critical events in post-translational processes, constituting a significant component of the signal transduction mechanisms by which extracellular signals regulate cell growth, differentiation, and transformation.[1] Two key classes of enzymes, namely protein kinases and protein phosphatases, are intimately involved in catalyzing the opposing activities of phosphorylation and dephosphorylation of proteins, respectively. It is estimated that, more than 10% of all proteins in a typical mammalian cell are phosphorylated. Virtually every human disease is believed to have stemmed from a deficiency in cellular signaling, making kinases and phosphatases valuable targets for novel therapeutics. Much research has thus far been focused on kinases.[2] Relatively, less work has been devoted to the study of protein phosphatases. Our long-term research goal lies in utilizing protein phosphatases as potential therapeutic targets, and developing novel drugs to specifically modulate phosphatase activities in vivo. Herein we report our first steps in achieving this by developing a fluorescence-based assay, which allows sensitive detection of different phosphatase activities in vitro.

Protein phosphatases are classified into a few subfamilies using different criteria.[3] For example, they are classified based on either their dephosphorylating targets (e.g., Tyr and Ser/Thr phosphatases), or their optimum working pH (e.g., acid and alkaline phosphatases). Thus far, protein tyrosine phosphatases (PTPs) have been studied extensively, given the critical roles they play in cell signaling. Standard methods for PTPs detection utilize *p*-nitrophenol phosphate as a chromogenic phosphatase substrate, and have been popular in defining different phosphatase activities. A number of novel approaches have also been developed, making it possible for sensitive detection of PTPs either in a complex proteome,[4a] or in high-throughput microplate formats.[4b] More recently, microarray-based detection of phosphatase activity has also been demonstrated.[5] All these strategies, however, rely on phosphatase substrates that contain a hydrolyzable phosphate group conjugated to aromatic moieties such as phenol and coumarin analogs, which closely mimic the phosphorylated side chains of tyrosine residues in a protein (top of Scheme 1; highlighted in green), inevitably dictating the assay to be biased towards the detection of protein tyrosine phosphatases. Thus, it is imperative that other methods are available, which allow indiscriminative detection of other types of phosphatases (i.e., acid/alkaline and Ser/Thr phosphatases etc.).

In our previous design, a coumarin derivative, conjugated to an enzyme recognition head group (i.e., a phosphate group), was used as a sensitive fluorogenic substrate of PTPs (Probe **1** in Scheme 1).[5b,6] The phosphate-containing coumarin in Probe **1** structurally resembles the phosphorylated tyrosine in a protein, which, upon treatment with a tyrosine phosphatase,

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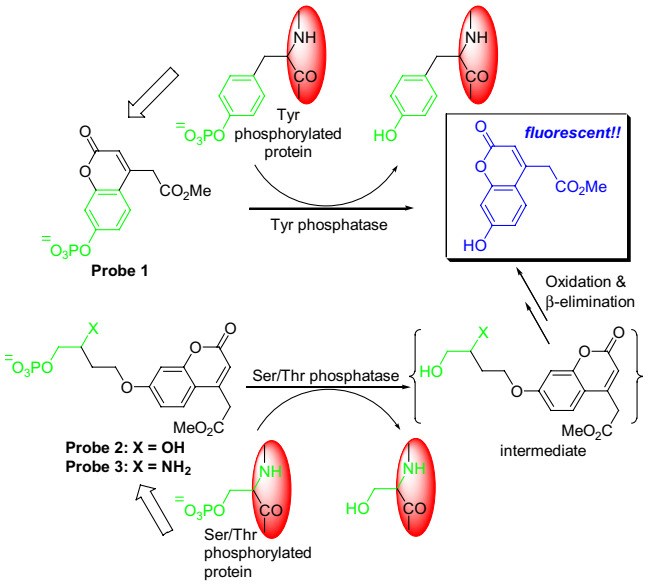
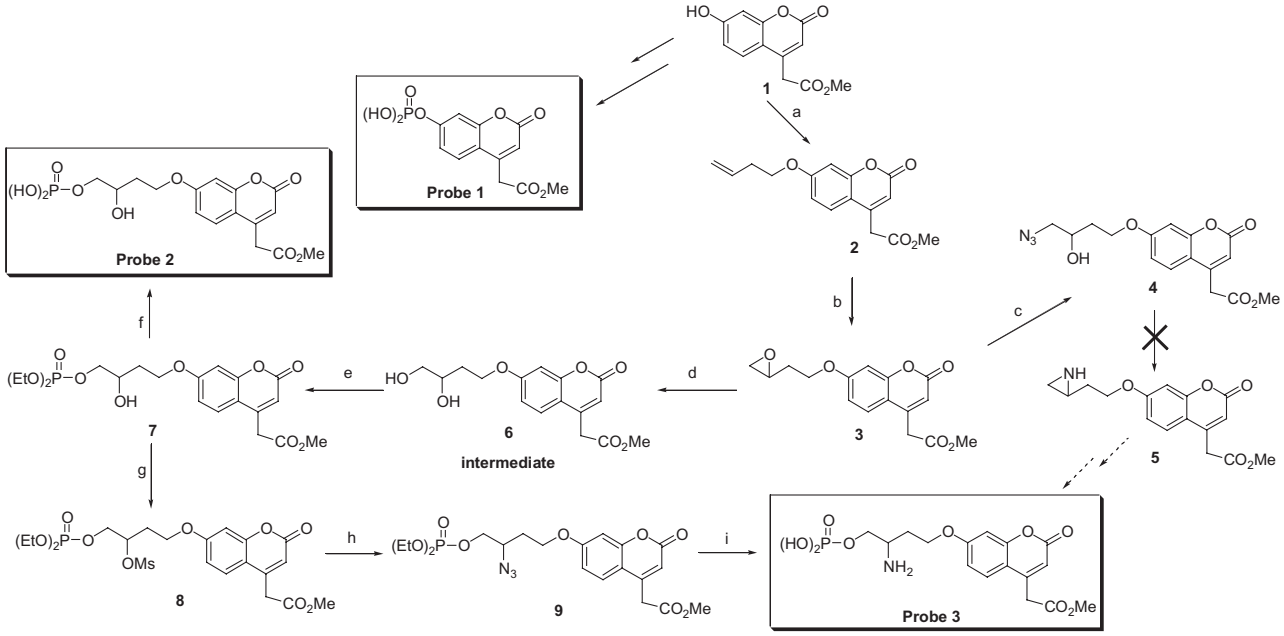


Scheme 1. Design principle of the fluorogenic phosphatase probes.

would undergo dephosphorylation to release the highly fluorescent coumarin, thereby translating the enzymatic activity into a real-time fluorescence readout. To our pleasant surprise, it was found that, in our preliminary screenings, Probe **1** was capable of sensitive detection of alkaline phosphatases, albeit with high specificity toward PTPs.[5b,6] This has prompted us to design the new generation of fluorogenic probes in the present study, in which the aryl phosphate in the original Probe **1** has been replaced with alkyl phosphates to minimize its resemblance to phosphorylated tyrosine (Probes **2** and **3** in Scheme 1). We now report the successful synthesis of the two new probes, as well as detailed enzymatic studies of all three probes against a variety of phosphatases.

In the new design, a 1,2-diol or 1,2-aminoalcohol linker was inserted between the fluorogenic coumarin moiety and the hydrolyzable phosphate group, resulting in Probes **2** and **3**, respectively, in which the enzyme recognition head in Probe **3** (bottom of Scheme 1; highlighted in green) closely resembles the phosphorylated serine/threonine in a protein. Probe **2**, a 1,2-diol analog of Probe **3**, although structurally resembling Ser/Thr-phosphorylated proteins to a lesser extent, also has its phosphate group positioned away from the coumarin unit, and therefore should not be biased towards PTPs as well.[7] Both probes, upon enzymatic removal of the phosphate group, release a geminal aminoalcohol or diol intermediate (Scheme 1), which under appropriate conditions undergoes oxidation followed by spontaneous β-elimination to give rise to the highly fluorescent coumarin adduct.[8]

Probe **1** was prepared from compound **1** according to our previous work.[5b] The synthesis of Probes **2** and **3** is highlighted in Scheme 2. Briefly, alkylation of **1** with 3-butenyl tosylate at 50 °C gave **2** in 67% yield. Epoxidation of **2** with MCPBA gave **3** in nearly quantitative yield (96%). Compound **3** was heated at 60 °C for 12 h in the presence of NaN_3 in DMF to give azide **4** in 60% yield. Numerous attempts to generate aziridine **5**, a key intermediate in the synthesis of Probe **3** in our original synthesis, from **4**, failed.[9] An alternative route was therefore developed. First, epoxide **3** was hydrolyzed under acidic conditions to give diol **6** in 79% yield. Selective phosphorylation of **6** at the primary alcohol



Scheme 2. Synthesis of the fluorogenic phosphatase probes. Reagents and conditions: (a) 3-butenyltosylate, DMF, K_2CO_3, 50 °C, 67%; (b) MCPBA (3 equiv), rt, 96%; (c) NaN_3 (3 equiv), 60 °C, 60%; (d) $HClO_4$, pH = 2, 18 h, 79%; (e) diethyl phosphochloridate, DIEA, DCM, rt, 24 h, 60%; (f) TMSI, DCM, 30 min, 55%; (g) methanesulfonyl chloride (2 equiv), pyridine, 3 h, 57%; (h) NaN_3, DMF, 50 °C, 40%; (i) Pd/C (10%)/H_2/MeOH, followed by TMSI/DCM, rt, 48%.

position was then successfully carried out using diethyl phosphochloridate in DIEA/DCM to afford **7**, which was conveniently converted to Probe **2**[10] by treatment with TMSI in DCM (55% yield). Alternatively, mesylation of **7** with methanesulfonyl chloride in dry pyridine afforded compound **8** in 57% yield. Heating **8** in the presence of NaN_3 in DMF at 50 °C gave **9** (40% yield), which was then converted to the desired product, Probe **3**,[11] under reductive conditions followed by deprotection with TMSI in a 48% overall yield.

We next assessed the enzymatic activities of these probes, as well as intermediate **6**, against a variety of phosphatases using a microplate-based fluorescence assay. A total of 12 different phosphatases were used,[12] of which two were acid phosphatases, five were alkaline phosphatases, one was Ser/Thr phosphatases and four were Tyr phosphatases (Table 1; Enzymes **A** to **B**, **C** to **G**, **H**, and **I** to **L**, respectively). First, the intermediate **6** was tested in different buffers to assess the rate of the release of coumarin in the presence of $NaIO_4$ and BSA, which was shown previously to efficiently catalyze the oxidation/β-elimination reaction.[5b,13] It was found that, as previously reported,[5b] borate buffer (20 mM, pH 8.8) in the presence of $NaIO_4$ (1 mM), and BSA (2 mg/mL) worked the best, efficiently catalyzing the oxidation/β-elimination of the intermediate with a reaction rate of 5.5 units/s, which was much higher than that of the enzyme catalyzed, phosphate release step (see Table 1, columns 3–7), and was therefore chosen for further studies. For enzymatic experiments, 1 μL of the phosphatase was added to solution containing 100 μL borate buffer (20 mM, pH 8.8) in the presence of $NaIO_4$ (1 mM), BSA (2 mg/mL), and 1 μL of each probe. The enzymatic reactions were carried out in a 96-well microtitre plate, and monitored using a fluorescence microplate reader. The results are summarized in Table 1 (Conditions 1). Like other related known phosphatase probes,[6] Probe **1** showed a broad-based activity against almost all the phosphatases tested, including all alkaline, Ser/Thr, and Tyr phosphatases in our experiments. The only exception was the two acid phosphatases, which showed no detectable activity against the probe, even after prolonged incubation. Probe **3** (and Probe **2** to some degree) on the other hand, which was designed to minimize its preference towards Tyr phosphatases and at the same time resemble phosphorylated Ser/Thr residues, showed enzymatic activities only against some alkaline phosphatases (e.g., **F** and **G** in Table 1), but not against the other phosphatases tested, including the Ser/Thr phosphatase (e.g., **H** in Table 1). Furthermore, Probe **2** showed no activity against any of the phosphatases under the same reaction conditions (Table 1, Conditions 1). We suspected that the deficiency in activity of Probes **2** and **3** might be caused by the use of borate/$NaIO_4$/BSA buffer. Therefore, we first incubated the probes with the enzymes, then added $NaIO_4$ and BSA accordingly, before recording the fluorescence of the reaction. First, 1 μL of the probe and the enzyme were added to 10 μL of a suitable buffer. After incubation for 1 h, 90 μL of borate buffer (20 mM, pH 8.8) containing $NaIO_4$ (1 mM), and BSA (2 mg/mL) was introduced, and the fluorescence spectra were collected 1 h thereafter (Fig. 1 and Table 1, Conditions 2). As shown in Figure 1b, Probe **2**, which previously showed no activity against all the phosphatases tested, showed good activity against both acid phosphatases (**A** and **B**) and three of the five alkaline phosphatases (**C**, **D**, and **E**). To the best of our knowledge, this is one of the few known fluorogenic substrates, which allow sensitive detection of acid phosphatases. Probe **3** showed enzymatic activities against all the alkaline phosphatases tested (Fig. 1c and d). Significantly, it also showed small but clearly noticeable activity against the Ser/Thr phosphatase tested (**H**, Fig. 1d). In contrast, Probe **3** showed no appreciable activity against any of the Tyr phosphatases tested (e.g., **I** to **L**), thereby validating our design principle. Work is currently underway to test

Table 1. Rates of the three probes with different phosphatases[14]

Phosphatase type[a]		Apparent reaction rate (unit/s)				
		Conditions 1[b]			Conditions 2[c]	
		Probe **1**	Probe **2**	Probe **3**	Probe **2**	Probe **3**
Acid	**A**	—[d]	—	—	0.21	—
	B	—	—	—	0.94	—
Alkaline	**C**	1.2	—	—	1.1	1.2
	D	1.5	—	—	1.3	1.5
	E	0.4	—	—	1.1	0.26
	F	2.3	—	0.8	—	0.97
	G	0.6	—	0.1	—	0.75
Ser/Thr	**H**	1.0	—	—	—	0.17
Tyr	**I**	1.0	—	—	—	—
	J	0.6	—	—	—	—
	K	0.9	—	—	—	—
	L	1.2	—	—	—	—

[a] All phosphatases were commercially available.[12] The fluorescence was detected at $\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm.
[b] Treated with enzyme and $NaIO_4$/BSA together.
[c] Treated with enzyme first, then with $NaIO_4$/BSA.
[d] '—' indicates no detectable activity.

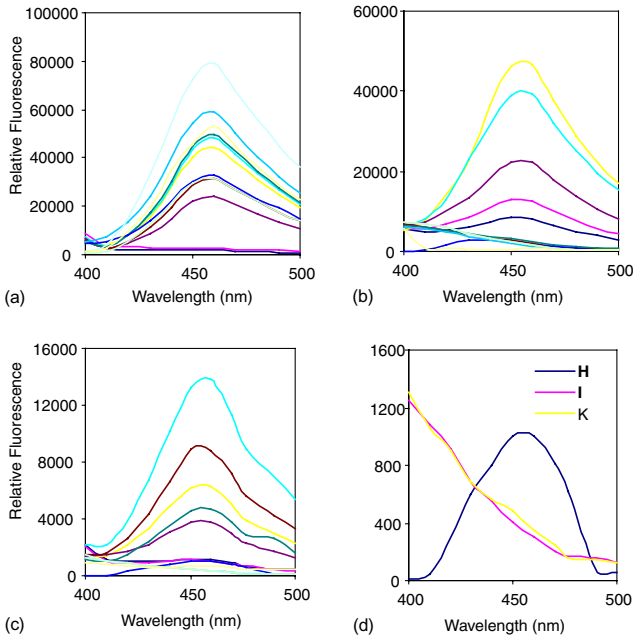


Figure 1. Fluorescence emission spectra of three probes treated with different phosphatases: (a) **Probe 1**; (b) **Probe 2**; (c) **Probe 3**; (d) same as (c) except only spectra of three phosphatases were shown (**H**, **I** & **K**). In each graph, spectra of different phosphatases were shown in different colors: **A** (—); **B** (—); **C** (—); **D** (—); **E** (—); **F** (—); **G** (—); **H** (—); **I** (—); **J** (—); **K** (—); **L** (—). All spectra were collected at $\lambda_{ex} = 360$ nm after 1 hour of enzymatic reactions.

probes against other phosphatases, and full results will be reported in due course.

In conclusion, we have successfully designed, synthesized and tested three fluorogenic substrates of protein phosphatases. All three probes showed good activity against alkaline phosphatases, while each showed some unique properties against different classes of phosphatases: (1) Probe **1** seems to be a broad-based substrate, showing little selectivity over any particular class of phosphatases; (2) Probe **2** shows good activity against acid phosphatases, and (3) Probe **3**, designed to resemble phosphorylated Ser/Thr residues, clearly showed some activity against the Ser/Thr phosphatase but none of the Tyr phosphatases tested. Despite our positive results, further improvement may be made to design better and more selective probes capable of targeting different classes of phosphatases. With potentially a large number of new phosphatases to be identified as a result of the Human Genome Project, we believe our work may help in facilitating the high-throughput characterizations and studies of these enzymes.

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6. Probe **1** to some extent is structurally similar to the two fluorogenic substrates sold by Molecular Probes (Eugene, USA): MUP (4-methylumbelliferone phosphate) and DiFMUP (6,8-difluoro-4-methylumbelliferone phosphate).
7. Coincidently, Probe **2** is structurally similar to the fluorogenic substrate published by Wahler et al., which was shown to be a substrate for alkaline phosphatases. See Refs. 4b,8 for more details.
8. Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J. L. *Angew. Chem., Int. Ed.* **2001**, *40*, 4457–4460.
9. Various conditions (e.g., *p*-toluenesulfonyl chloride/DCM/DIEA or pyridine, methanesulfonyl chloride/DCM/DIEA or pyridine) were attempted. Only trace amounts of the desired product were detected (by TLC and MS). Mostly starting compound, **4**, was recovered.
10. Probe **2**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.61 (d, J = 9 Hz, 1H), 6.97–6.93 (m, 2H), 6.25 (s, 1H), 4.23–4.21 (m, 2H), 3.98–3.94 (m, 3H), 3.70 (s, 2H), 3.30 (s, 3H), 2.11–2.07 (m, 1H), 1.93–1.87 (m, 1H). ^{13}C NMR (75 MHz, D_2O) δ 174.62, 166.50, 164.52, 157.41, 153.11, 128.68, 117.02, 115.97, 115.11, 104.15, 72.23, 70.04, 67.89, 63.66, 55.71, 34.12. ^{31}P NMR (D_2O) δ 1.17. MS (ESI): m/z (relative intensity) 403.0 $[M+1]^+$ (100).
11. Probe **3**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.62 (d, J = 8.9 Hz, 1H), 7.02–6.96 (m, 2H), 6.27 (s, 1H), 4.31–4.28 (m, 4H), 3.91 (s, 2H), 3.78 (m, 1H), 3.71(s, 3H), 2.29–2.27 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 171.32, 163.16, 162.90, 156.59, 151.20, 128.61, 114.44, 114.27, 114.01, 102.90, 66.58, 65.95, 54.04, 53.07, 34.51. ^{31}P NMR (D_2O) δ 0.85. MS (ESI): m/z (relative intensity) 402.0 $[M+1]^+$ (100).
12. **A**: Acid phosphatase from wheat germ (P-3627); **B**: acid phosphatase from potato (P-3752); **C**: alkaline phosphatase from bovine intestinal mucosa (P-7640); **D**: alkaline phosphatase from rabbit intestine (P-2265); **E**: alkaline phosphatase from porcine intestinal mucosa (P-4002); **F**: alkaline phosphate from shrimp (M182A 11302611); **G**: alkaline phosphatase from calf intestinal (M820A 13595209); **H**: PP1 Ser/Thr phosphatase (P0754S); **I**: LAR Tyr phosphatase (P0750S); **J**: Lambda PPase (P0753S); **K**: PTP (P0752S); **L**: YOP (P0751S).
13. Different buffers such as Tris (50 mM, pH 5, 8.8, 12), Borate buffer (20 mM, pH 8.8), $NaHCO_3$ (50 mM, pH 9) in the presence of $NaIO_4$ and BSA were tested.
14. The fluorogenic reaction was carried in a black round-bottom polypropylene 96-well microtitre plates (Nunc, USA). The fluorescence was detected using the SpectraMAX™ Gemini XS fluorescence plate reader (Molecular Devices, USA).