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# Activity-based fluorescent probes that target phosphatases

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**Abstract**—We have successfully designed and synthesized two fluorescently-labeled, activity-based probes, **Probe 1** and **Probe 2**, which were shown to label protein tyrosine phosphatases specifically, as well as other types of phosphatases. The probes were not reactive towards the other non-phosphatase enzymes tested. These probes may find potential applications in large-scale proteomic experiments whereby subclasses of proteins may be selectively identified. 

The human genome project has recently been completed.[1a] It opens up new and exciting possibilities in the understanding of the working of many different human diseases at the cellular and molecular levels. However, genes alone do not tell the whole story of cellular functions. Proteins are ultimately responsible for most processes that take place within the cell. Therefore, emphasis has now shifted from research of the genome to that of the proteome, which aims to understand the function of every protein in an organism.[1b]

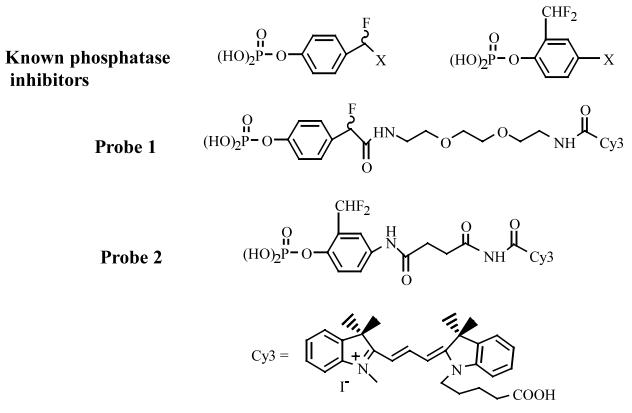


**Figure 1.** Structures of known mechanism-based inhibitors of protein phosphatases, and the two probes synthesized in our studies.

In order to accelerate the functional analysis of a cell's complete protein repertoire, new and rapid techniques capable of genome-wide studies of proteins are essential. High-throughput screening methods, in most cases aided by automation, have enabled scientists to analyze proteins on a global scale quickly and efficiently.[2] Recently, the activity-based profiling of proteins has proven to be a powerful tool in proteomic studies, whereby subclasses of enzymatic proteins could be selectively identified.[3] This strategy takes advantage of mechanism-based probes that react with different classes of enzymes, leading to the formation of covalent probe–protein complexes which are readily distinguished from other non-reactive proteins in a crude proteome mixture. Thus far, a number of research groups have successfully designed and tested a handful of probes against different classes of enzymes.[3] Among them, Cravatt et al. developed fluorophosphonate/fluorophosphate derivatives conjugated to either a biotin moiety or a fluorescent dye, and used them to label serine hydrolases selectively in a crude protein mixture.[3a] The same group developed sulfonate ester-based probes that target proteins having nucleophilic active-site residues.[3b] Bogyo et al. synthesized vinyl-sulfone containing peptides and used them to label eukaryotic proteosomes.[3c] Lo et al. generated *p*-hydroxymandelic acid-containing probes that specifically label protein tyrosine phosphatases in an activity-based fashion.[3d] We recently synthesized an aspartic acid analog that contains a fluoromethylketone (fmk) moiety. By conjugation of this molecule to a fluorescent dye, Cy3, via a simple alkyl linker, the resulting probe was able to label selectively caspases over other enzymes.[3e]

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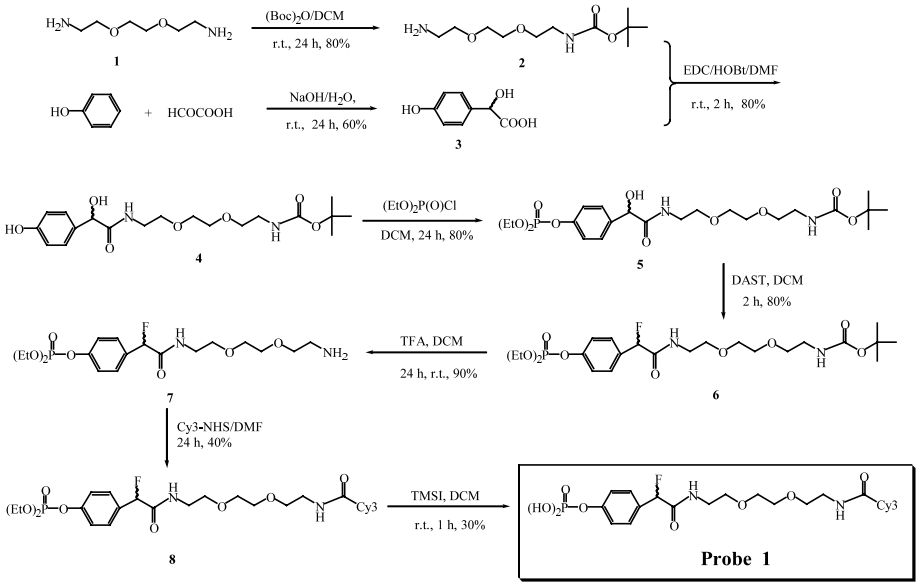
In order to extend the potential of this activity-based approach into studies of other proteins, we would like to develop new activity-based probes capable of detecting other major classes of enzymes, including kinases, phosphatases, and other types of proteases such as metalloproteases and aspartic proteases. We reason that, in order for these probes to be readily applicable in large-scale, high-throughput proteomic experiments, they should preferably be conjugated to a fluorescent dye that not only possesses good fluorescence properties, but also is compatible with most commercially available fluorescence-based gel scanners. Therefore, the cyanine dye, Cy3, is an ideal choice. Herein, we report two Cy3-conjugated, activity-based probes, which are designed to potentially target not only protein tyrosine phosphatases, but also other types of phosphatases. Ultimately, we would like to generate a whole array of probes containing unique reactivity towards different classes of enzymes. These probes, combined with standard gel-based proteomic experiments, may serve as a high-throughput tool to study the human proteome.

The probes were designed based on principles previously described (Fig. 1).[3e] Briefly, the structure of each probe consisted of three units: a Cy3-containing fluorescence unit, a linker and a reactive unit. The fluorescence unit serves as a sensitive means to detect proteins upon labeling with the probe. The reactive unit is made of a mechanism-based group that would covalently react with an enzyme in an activity-dependent fashion, and could be fine tuned to accommodate different enzymes. In this particular study, we were interested in developing probes that specifically target phosphatases. It was shown previously that *p*-hydroxymandelic acid derivatives were good mechanism-based inhibitors of protein tyrosine phosphatases (PTPs).[4] More recently, Lo et al. synthesized dansyl- and biotin-conjugated probes containing these moieties and used them to probe PTPs in a proteomic experiment.[3d] However, their probes were not conjugated to dyes such as Cy3, making them unfeasible for sensitive and quantitative detection of labeled proteins. Furthermore, no experiments were done to assess whether these probes could be used to detect other phosphatases, such as acid/alkaline phosphatases. Therefore, we designed **Probe 1** by conjugation of the *p*-hydroxymandelic acid derivative, shown in Figure 1, to Cy3 and tested its feasibility in detecting different types of phosphatases in a standard activity-based proteomic experiment. We also designed **Probe 2**, by conjugation of Cy3 to a different reactive unit, 2-difluoromethylphenyl phosphate. 2-Difluoromethylphenyl phosphate was shown previously to be a general mechanism-based phosphatase inhibitor against a broad spectrum of different phosphatases, including acid and alkaline phosphatases.[5] However, activity-based probes based on this moiety have not been realized thus far to assess their feasibility in detecting phosphatases in a proteomic experiment.

First, **Probe 1** was synthesized, as shown in Scheme 1, by the modification of procedures reported by Lo et al. in order to accommodate the introduction of the Cy3 dye.[3d] Briefly, Boc-protected diamine **2** was coupled with 4-hydroxylmandelic acid **3**, which was obtained from phenol, to form compound **4** in 80% yield. Phosphorylation of **4** was achieved by treatment with diethyl phosphochloridate to obtain **5** in 80% yield. The hydroxyl group of **5** was converted to fluoride, resulting in **6** in 80% yield using DAST. Following deprotection of the Boc group, compound **7** was directly coupled with the NHS ester of Cy3 to obtain **8** in 40% yield. The final product, **Probe 1**, was obtained by hydrolysis of **8**. Overall, the probe was obtained in seven steps in a yield of ~4.4%.[6]




**Scheme 1.**

**Probe 2** was synthesized from the intermediate **12**, which was prepared from commercially available starting material **9** based on procedures previously reported.[5] Briefly, **10** was obtained by phosphorylation of **9** with diethyl phosphochloridate in 95% yield. Treatment of **10** with DAST afforded **11** in 80% yield, which was then converted to **12** under reductive conditions in 70% yield. Treatment of **12** with succinic anhydride afforded **13** in moderate yield (50%). Compound **14** was obtained by conjugation of **13** with Cy3-containing **15**, in the presence of HOBt and TBTU to form **14**, followed by hydrolysis to generate **Probe 2** (Scheme 2).[7]

Next, the probes were tested for selective labeling of proteins based on their enzymatic activity. We first tested their reactivity towards protein tyrosine phosphatases, as **Probe 1** was designed based on a probe previously shown to label PTPs selectively.[3d] A known protein tyrosine phosphatase from yeast, YBR267W, was used in our experiments.[8] The glutathione-*S*-transferase (GST) fusion of the tyrosine phosphatase was obtained by conventional protein expression protocols,[8] and labeled with both **Probe 1** and **2** (Fig. 2).[9] It was found that 1 h incubation was sufficient for the two probes to label the protein with high efficiency. The labeled proteins were readily separated on a SDS-PAGE gel, and visualized using a commercial fluorescence gel scanner.

We subsequently tested the probes against other types of phosphatases, as well as other non-phosphatase proteins. Many known alkaline phosphatases are known to be involved in a number of critical cellular processes, and have a broad spectrum of substrate specificity, thus making them ideal candidates to test the specificity of our probes. A panel of 10 commercially available proteins was used in our studies; three of them were alkaline phosphatases isolated from different sources. The remainder were non-phosphatase enzymes, including proteases and lipases. Upon incubation with each probe for 1 h (Fig. 3a and b for **Probes 1** and **2**, respectively), the proteins were separated by SDS-PAGE, followed by detection of the labeling reaction with a fluorescence-based gel scanner.[9] It was found that both probes selectively labeled ONLY phosphatases (Lanes 1–3, Fig. 3). Other proteins, including different classes of proteases and lipases, were not labeled by either probe, indicating the high specificity of both probes towards not only protein tyrosine phosphatases (PTPs), but also possibly other types of phosphatases (e.g. alkaline phosphatases). More experiments are underway to test the probes against other classes of phosphatases. The activity-dependent nature of the probes was confirmed by first denaturing the protein samples with heat, followed by treatment with the probes; no labeling of the phosphatase was observed, indicating the enzymatic activity of the phosphatase was a prerequisite for the reaction to occur. Under the same labeling conditions, both probes selectively labeled phosphatases in a similar fashion, with **Probe 1** consistently giving stronger bands on all proteins tested, indicating that the mechanism-based reactive unit (e.g. *p*-hydroxymandelic acid) in this probe may be intrinsically more reactive towards phosphatases.

In conclusion, we have successfully designed, synthesized and tested two fluorescent small molecule probes capable of labeling phosphatases in a highly specific, activity-based fashion. It was found that both probes selectively labeled not only protein tyrosine phosphatases (PTPs), but also other types of phosphatases, such as alkaline phosphatases. Further experiments are underway which involve testing these two probes against a variety of other types of phosphatases, as well as their feasibility in in vivo experiments to profile phosphatase activity in live cells and tissues.

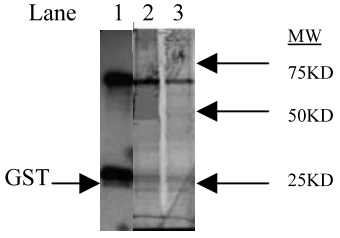
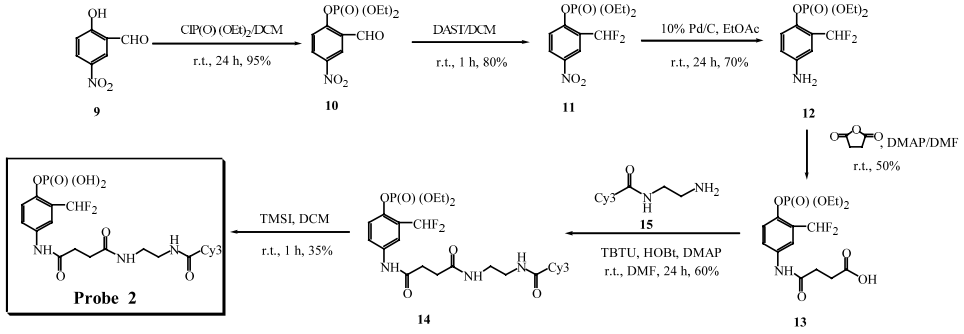



**Figure 2.** Protein tyrosine phosphatase (PTP) from yeast (YBR267W) labeled by the probes. Lane 1: unlabeled protein detected with anti-GST by Western Blotting. Lane 2: protein labeled with **Probe 1**; Lane 3: protein labeled with **Probe 2** followed by detection on a fluorescence gel scanner.[9]




**Scheme 2.**

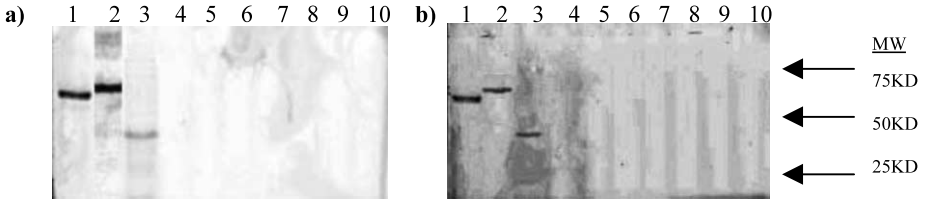



**Figure 3.** Other proteins labeled with (a) **Probe 1** and (b) **Probe 2**. Lane 1: calf alkaline phosphatase (Promega, M182A,); Lane 2: shrimp alkaline phosphatase (Promega, M8201); Lane 3: alkaline phosphatase (Sigma, P-7640); Lane 4: chymopapain (Sigma, C-8526); Lane 5: papain (Sigma, P-4879); Lane 6: α-chymotrypsin (Sigma, C-4129); Lane 7: proteinase K (Sigma, P-6556); Lane 8: proteinase (Sigma, P-5380); Lane 9: lipase (Sigma, L-1754); Lane 10: lipase (Sigma, L-3001). Proteins were detected by a fluorescence gel scanner.[9]

## Acknowledgements

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6. To a cooled solution of **8** (12 mg, 0.012 mmol) in 1 mL of $CH_2Cl_2$ was added TMSI (6 μL, 0.036 mmol). The reaction was allowed to warm to room temperature and stirred further for 1 h. The reaction was stopped by quenching with 10% triethylamine in $H_2O$ (2 mL) and extracted with $CHCl_3$ (3×2 mL). The aqueous layer was separated and concentrated under reduced pressure, followed by purification by flash chromatography ($CH_2Cl_2$/MeOH/TEA; 85/15/5 to 15/85/5 gradients) to afford **Probe 1** (3.3 mg; 30% yield). NMR ($CDCl_3$, TMS): $^1H$, $\delta$ 1.76 (s, 12H), 1.94 (m, 4H), 2.50 (br, 2H), 3.59 (m, 11H), 4.15 (m, 6H), 5.80 (d, $J=48$ Hz, 1H), 7.31 (m, 14H), 8.43 (m, 1H). $^{19}F$ NMR (282.2 MHz, $CDCl_3$, 25°C) $\delta$ −94.89 (d, $J=48.6$ Hz) ppm; ESI-MS: $m/z$ 805.4 $[M-I]^+$.
7. To a cooled solution of **14** (12 mg, 0.012 mmol) in 1 mL of $CH_2Cl_2$ was added TMSI (6 μL, 0.036 mmol). The reaction was allowed to warm to room temperature and stirred further for 1 h. The reaction was stopped by quenching with 10% triethylamine in $H_2O$ (2 mL) and extracted with $CHCl_3$ (3×2 mL). The aqueous layer was separated and concentrated under reduced pressure, followed by purification by flash chromatography ($CH_2Cl_2$/MeOH/TEA; 85/15/5 to 15/85/5 gradients) to afford **Probe 2** (3.4 mg; 35% yield). NMR ($CD_3OD$, TMS): $^1H$, $\delta$ 1.74 (s, 12H), 2.19 (m, 4H), 2.50 (m, 4H), 2.67 (br, 2H), 2.86 (br, 2H), 3.66 (s, 3H), 4.21 (m, 2H), 7.11 (t, $J=54$ Hz, 1H), 7.30 (m, 2H), 7.45 (m, 4H), 7.62 (m, 1H), 7.75 (m, 1H), 7.85 (m, 1H), 8.50 (m, 1H). $^{19}F$ NMR (282.2 MHz, $CD_3OD$, 25°C) $\delta$ −39.16 (d, $J=56.8$ Hz) ppm; ESI-MS: $m/z$ 806.3 $[M-I]^+$.
8. A plasmid containing the GST fusion of the protein tyrosine phosphatase from yeast, YBR267W, was purchased from Invitrogen (San Diego, USA), in the form of a Yeast ExClone™. Yeast cells transformed with the above plasmid were grown in SD-URA-LEU media to $A_{600}=0.8$, as recommended by the supplier, followed by supplementation with 0.5 mM copper sulphate for 2 h to induce expression of the GST-ORF. Cells were harvested, washed with cold water, then lysed by ultrasonication. The resulting crude protein extract was purified with a MicroSpin™ GST column (Amersham, USA) to generate the desired pure GST-fusion protein.
9. SDS-PAGE experiments: 20 μl of the protein (50–500 μg/mL) dissolved in 5 mM Tris (pH 8) was incubated at room temperature with 0.2 μL of each probe (200 μM stocks in DMSO). After 1 h, the reaction was stopped by addition of 6×SDS loading dye, and then heated at 90°C for 5 min. The samples were run on 12% SDS-PAGE gels, then scanned using the Typhoon™ 9200 scanner (Amersham, USA) at $\lambda_{ex}=532$ nm. Western blots were performed using anti-GST monoclonal antibody with the ECL™ kit (Amersham, USA) following the protocol provided by the vendor.