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Highly sensitive peptide-based probes for protein tyrosine phosphatase activity utilizing a fluorogenic mimic of phosphotyrosine

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Abstract—Fluorescent probes that can be incorporated into peptides and proteins are in high demand, with applications ranging from cellular imaging to binding and activity assays. Here, we report the high yielding synthesis of an enantiomerically pure phosphocoumarin-based amino acid and its incorporation into peptides via standard solid-phase peptide synthesis methodologies. Peptides containing this new amino acid serve as highly sensitive fluorogenic probes for protein tyrosine phosphatase activity.

There is a great need for versatile fluorescent probes that can be incorporated into proteins. Such tags are useful not only for visualizing a modified protein or enzyme activity in vivo and in vitro, but also for assaying enzyme activity and determining the substrate specificity of enzymes. For example, fluorogenic and colorimetric substrates are commonly used to assay protease activity and substrate specificity[1] and have been used to a lesser extent to assay the activity of many other enzymes including glycosidases, phosphatases, sulfatases, and esterases.[2,3] With an ever-expanding catalog of post-translational modifications and an analogous library of enzymes catalyzing the corresponding transformations, the need for adaptable fluorogenic substrates is growing.[4]

One example of a family of enzymes whose study would benefit from improved chemical probes is the protein tyrosine phosphatase (PTP) enzyme family. The PTPs are a diverse family of enzymes involved in key signaling pathways and implicated in a number of disease states including cancer and diabetes.[5,6] Currently, PTP activity is followed through the use of small, non-peptidic substrates such as *para*-nitrophenylphosphate (*p*-NPP), 4-methylumbelliferone phosphate (MUP), and difluoromethylumbelliferone phosphate (DiFMUP).[7] Although these substrates are readily hydrolyzed by many PTPs, they do not work with all members of this enzyme family. To measure PTP activity against peptide substrates, the small increase in absorbance or fluorescence upon hydrolysis of a phosphotyrosine residue in a peptide substrate can be followed.[7–9] Alternatively, MS[10] and protease-coupled assays[11] have been reported. Unfortunately, these methods either lack sensitivity or require specialized equipment and expertise, hampering their widespread adoption.

Recent work indicates that many PTPs have distinct substrate specificities mediated both by interactions with residues on either side of the target phosphotyrosine residue and also by interactions of other PTP domains with the substrate.[12] A variety of approaches to defining the substrate sequence specificities of PTPs have been utilized, including substrate-trapping mutants,[13] affinity selection of inhibitory peptides,[14] phosphotyrosine-containing phage libraries,[15] and inverse alanine scanning.[16] A detailed understanding has been slow to emerge because the chemical tools necessary for studying PTP activity against peptide substrates are not widely available.[6,5] To date, there is no method for assaying PTP activity that is highly sensitive, continuous, widely available, and applicable to high-throughput screening of peptide substrate libraries. A well-designed probe based on these criteria would provide crucial information about the similarities and differences in peptide substrates recognized by members of the PTP family of

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enzymes. This detailed knowledge would be invaluable in the design of potent, selective PTP inhibitors for biochemical and therapeutic applications. Furthermore, an appropriate fluorogenic probe for PTP activity would find use in visualizing PTP activity in whole cells.

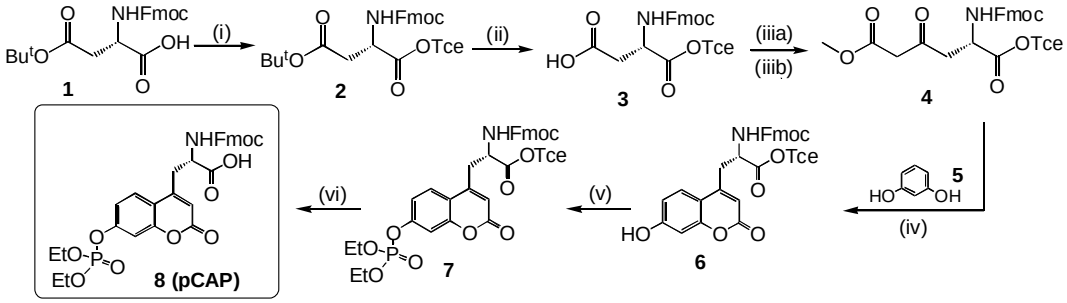
To facilitate the study of PTP activity, substrate specificity, and inhibition, a fluorogenic, peptide-based PTP substrate based on a phosphorylated coumaryl amino acid moiety has been developed. Fluorescent amino acids are highly desirable synthons for a variety of biochemical applications, and a handful of enantioselective syntheses of coumaryl amino acids have been reported in the literature recently.[17–22] In this work, we have synthesized and characterized an appropriately protected, phosphorylated coumaryl amino acid that can be incorporated directly into peptide substrates using standard Fmoc-based solid-phase peptide synthesis (SPPS) methodologies.

Enantiomerically pure *N*-α-Fmoc-L-aspartic acid β-*tert*-butyl ester (**1**) was chosen as the starting point in this synthesis, outlined in Scheme 1. The α-carboxylic acid of (**1**) was first protected as a trichloroethyl (Tce) ester (**2**). The β-carboxylic acid was then deprotected and acylated using isopropenyl chloroformate and Meldrum's acid to obtain the β-ketoester (**4**) in an overall yield of 90% starting from (**1**). Methanesulfonic acid was recently reported to catalyze the condensation between an amino acid derived β-ketoester and resorcinol at room temperature, preserving the chirality of the stereocenter.[14] In our hands, the reaction gave higher yields of the N- and C-protected coumaryl amino acid (**6**) when carried out at 0–4 °C. Phosphorylation of (**6**) with diethylchlorophosphate and diisopropylethylamine generated the phosphorylated coumarin (**7**). Subsequent removal of the Tce group using 50% acetic acid in tetrahydrofuran in the presence of activated zinc dust generated the phosphorylated coumaryl amino acid (pCAP) (**8**) quantitatively from (**7**). As illustrated in Scheme 1, this approach generates an enantiomerically pure, appropriately protected phosphocoumaryl-amino-propionic acid (**8**, pCAP) in high yield via six facile steps from readily available starting materials. Product purification through column chromatography was only necessary for compounds **6** and **7**. Full synthetic details can be found in the Supplementary information available online. This synthesis is superior to other reported syntheses of coumaryl amino acids in its simplicity, yield, and versatility. We anticipate that this new phosphotyrosine mimic will be useful for imaging PTP activity both in vitro and also in vivo.

To demonstrate the utility of pCAP in in vitro PTP assays, we synthesized a series of peptide substrates with the general formula DADE-X-GPAA-NH_2, where X is phosphotyrosine (**9a**), tyrosine (**9b**), pCAP (**10a**) or CAP (**10b**). The coumarin-based amino acids are compatible with standard, Fmoc-based SPPS methodologies.[23] Any standard coupling procedure can be used to extend the growing peptide chain, with the exception of pCAP and CAP addition, which couples more efficiently when PyBOP and HOBt are used as the coupling reagents. Deprotection of the phosphate moiety was accomplished by the addition of trimethylsilyliodide to the peptide prior to the cleavage from the resin. The peptides were then fully deprotected and cleaved from the resin, purified by reverse-phase HPLC, and characterized by mass spectrometry. The peptide sequence DADE-X-GPAA-NH_2 is expected to be a reasonable, though not optimal, substrate for a variety of PTPs.

If the pCAP-containing peptides are to be useful in enzyme activity assays, they must exhibit a large change in fluorescence upon hydrolysis. As anticipated based on the success of MUP in PTP activity assays,[7] we found that a 22 μM solution of peptide **10b** exhibited fluorescence (λ_{ex} = 334 nm, λ_{em} = 460 nm) that is 10^4-fold more intense than a 22 μM solution of peptide **10a** (see inset, Fig. 1). As shown in Figure 1, a large increase in fluorescence is observed when **10a** is hydrolyzed by a PTP. Furthermore, the fluorescence intensity of the peptide **10b** increases linearly in proportion to concentration over a wide concentration range, indicating that the PTP-catalyzed hydrolysis of **10a** to form **10b** should provide a facile, sensitive assay for PTP activity. In addition, no hydrolysis of the fluorogenic peptide substrate **10a** was observed under the conditions of the reaction in the absence of enzymes.

Because the pCAP moiety is slightly larger than phosphotyrosine, we set out to establish the efficiency of



Scheme 1. Reagents and conditions: synthesis of **8** (pCAP) (i) CCl_3CH_2OH, dicyclohexylcarbodiimide, DMAP, 0 °C, 15 h, 97%. (ii) 50% TFA in CH_2Cl_2, rt, 15 min, >99%. (iiia) Isopropenyl chloroformate, Meldrum's acid, DMAP, CH_2Cl_2, 0 °C, 2 h. (iiib) CH_3OH/benzene (4:1), reflux, 12 h, 94%. (iv) CH_3SO_3H, 0 °C, 6 h, 60%. (v) $(EtO)_2POCl$, diisopropylethylamine, $CHCl_3$, rt, 14 h, 85%. (vi) Zn dust (6 equiv) 50% acetic acid in THF, rt, 8 h, 99%.

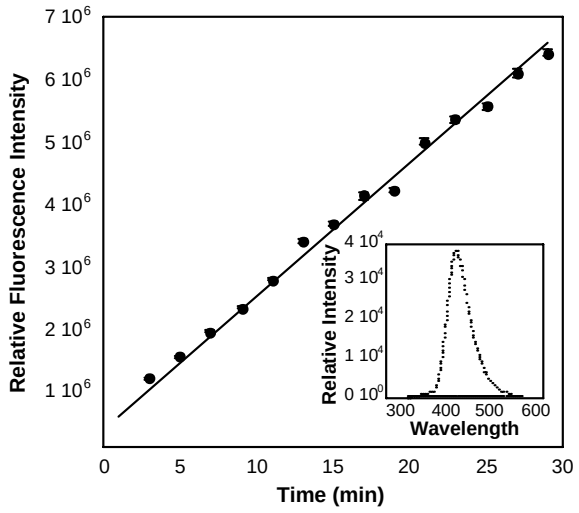


Figure 1. Raw data showing the initial increase in fluorescence over time when a 250 μM solution of **10a** is allowed to react with YOP. Inset: emission spectra of 22 μM solutions of peptides **10a** (solid line) and **10b** (dashed line) with λ_{ex} = 334 nm.

enzymatic turnover of **10a**. As shown in Figure 2A, **10a** is readily dephosphorylated by the PTP from *Yersinia enterocolitica* (YOP). Peptide **10a** (k_{cat}/K_m = 63,000 M^{-1} s^{-1}) is hydrolyzed significantly more efficiently by YOP than MUP (k_{cat}/K_m = 6200 M^{-1} s^{-1}, Supplementary Figure 1) and approximately as efficiently as **9a** (k_{cat}/K_m = 37,000 M^{-1} s^{-1}, Supplementary Figure 2). The turnover rate (k_{cat}) of **10a** by YOP is approxmately 9-fold faster than MUP and about 2-fold slower than **9a**, while the K_m is about the same as MUP and 4-fold lower than **9a**. The *Yersinia* enzyme is one of the most active PTPs known and readily accepts the pCAP moiety as a phosphotyrosine mimic. To determine if these substrates are likely to be broadly applicable, we also tested the activity of human T-cell PTP (TCPTP) against **10a**. Notably, with TCPTP we were unable to obtain satisfactory kinetic data with **9a**, while hydrolysis of **10a** was readily detected, as shown in Figure 2B. The kinetic data are summarized in Table 1.

Table 1. Kinetic data for the hydrolysis of substrates by YOP and TCPTP

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)
YOP	**9a**	100 ± 20	2.7 ± 0.8
	10a	41 ± 2	0.65 ± 0.07
	MUP	5.7 ± 0.5	0.8 ± 0.2
TCPTP	**10a**	35 ± 10	0.8 ± 0.3

In conclusion, we have developed a facile, high-yielding synthesis of a highly fluorescent, phosphorylated amino acid that can be incorporated into peptides using standard SPPS techniques. The resultant substrates are efficiently hydrolyzed by PTPs and exhibit a large increase in fluorescence upon hydrolysis. This work represents a critical step forward in the characterization of PTPs, both because it demonstrates that the pCAP residue can be incorporated into peptides and hydrolyzed by PTPs, and more importantly because it verifies that pCAP is accepted as a phosphotyrosine mimic by both bacterial and human PTPs. The pCAP-containing peptides provide a highly sensitive, continuous assay for PTP activity that is widely accessible and readily incorporated into peptide substrates for high-throughput screening or activity-based assays. Much less enzyme is required for this assay than for others that have been reported previously.[12,13] In principle, CAP could also be derivatized with a variety of other electrophiles to create fluorogenic substrates for aryl sulfatases, glycosidases, etc. Work is currently underway in our laboratory to incorporate CAP and pCAP into combinatorial peptide libraries for assaying enzyme substrate specificities and to synthesize fluorinated derivatives of pCAP (pCAPF and $pCAPF_2$), which may have greater sensitivity, especially at low pH.[7] Furthermore, this series of fluorogenic peptides may aid in the visualization of enzyme activity in vivo.

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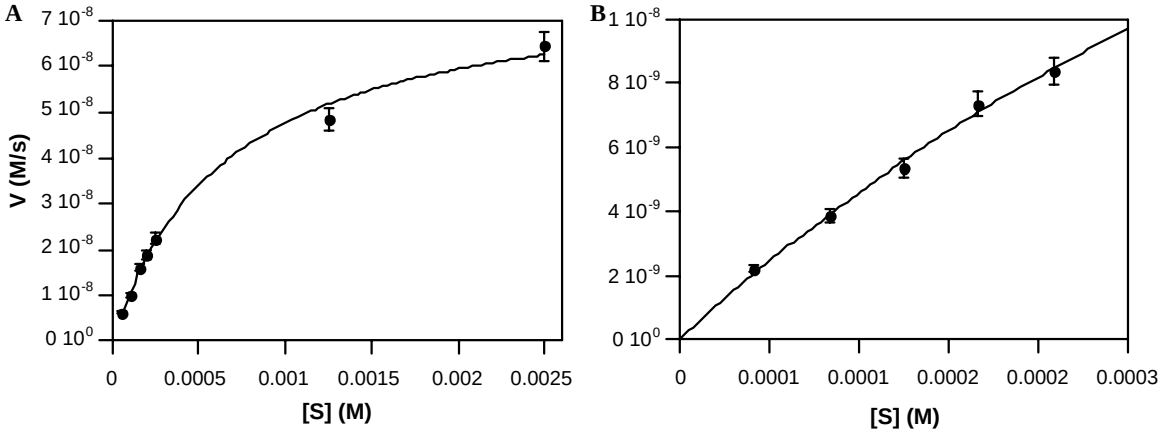

Figure 2. Hydrolysis of **10a** by (A) YOP and (B) TCPTP. The curve represents the best fit of the data to the Michaelis–Menten equation.

Supplementary data

Experimental details for the synthesis and characterization of all compounds and methods used to measure enzyme kinetics. This material is available free of charge on the internet. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.08.054.

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