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Fluorescence Probes for Tyrosine Dephosphorylation Based on Coumarin–Proline Conjugates

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Proline-substituted coumarin compounds were prepared and used as environment-sensitive fluorescence probes. Phosphorylation and dephosphorylation of tyrosine derivatives labeled with the coumarin–proline conjugate induced marked changes in fluorescence intensity allowing phosphatase activity to be monitored.

Phosphorylation and dephosphorylation are among the most important and commonly found modifications of hydroxy-containing amino acids (Ser, Thr, and Tyr).[1] These posttranslational modifications are involved in many cell signal transduction pathways and catalyzed by tightly regulated enzyme reactions. To explore these biologically important reactions, many chemical probes have been developed that show fluorescence changes upon the phosphorylation and dephosphorylation of amino acids.[2,3] One of the key issues in the design of fluorescence probes is the proper connection of a fluorophore to the amino acid being modified with optimal sensitivity.

Coumarin derivatives are one of the most widely used fluorophores in biological studies with large Stokes shifts, and we have recently reported the synthesis and conformational properties of a coumarin–proline conjugate (**CP** in Chart 1).[4] In this coumarin derivative, the proline is used as an electron-donating group and can also serve as a rigid linker bridging the coumarin and an amino acid or peptide that can be attached to the proline carboxyl group. Thus, this coumarin–proline conjugate is a potentially useful fluorescence label to monitor the structural modification of neighboring amino acids. In this paper, we report that the phosphorylation and dephosphorylation of **CP**-labeled tyrosine derivatives induce modest changes in fluorescence intensity allowing phosphatase-catalyzed reactions to be monitored.

First, we connected coumarin–proline conjugate **CP** to L-tyrosine methyl ester, and this tyrosine derivative was then converted to the corresponding phosphotyrosine (Scheme 1). After phosphorylation of the tyrosine residue, the fluorescence intensity was increased (Figure 1a) presumably because the aromatic ring of the phosphotyrosine residue is electron-deficient and, therefore, cannot quench the coumarin fluorescence as efficiently as the electron-rich aromatic ring of the tyrosine residue.[5] Phosphorylation of D-**CP-Y** induced relatively large enhancement of emission intensity (ca. 400%) whereas phosphorylation of L-**CP-Y** produced only a marginal intensity change (ca. 50%). According to our previous study on *N*-arylproline amide conformations,[6] the tyrosine residue would be located more closely to the coumarin ring in D-**CP-Y** than in L-**CP-Y** so that more efficient fluorescence quenching is expected for D-**CP-Y**.

To further examine the relationship between the linker structure and the phosphorylation-induced fluorescence change, we prepared coumarin–sarcosine conjugate **CS** where sarcosine

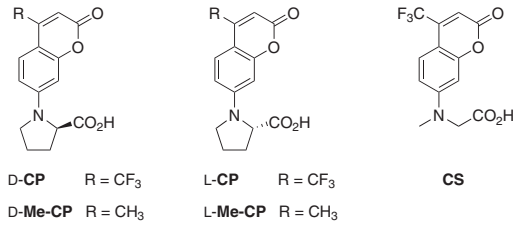
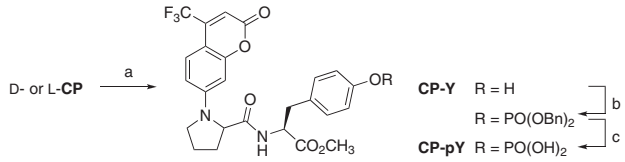


Chart 1.



Scheme 1. Reagents and conditions: (a) L-Tyr-OCH_3, BOP-Cl, *i*-Pr_2EtN, DMF, 95–99%; (b) *i*-$Pr_2NP(OBn)_2$, tetrazole, THF; *m*-Cl-$C_6H_4CO_3H$, CH_2Cl_2, 74–79%; (c) H_2, Pd/C, AcOH, MeOH, 76–78%. BOP-Cl: bis(2-oxo-3-oxazolidinyl)phosphinic chloride.

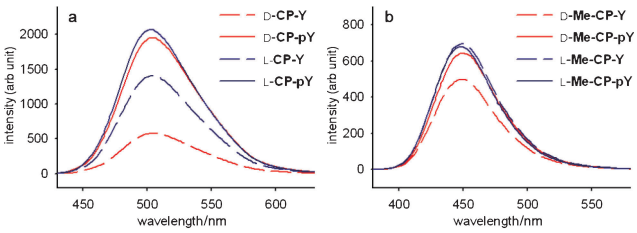
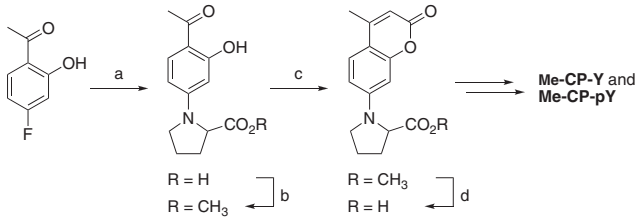


Figure 1. Fluorescence emission spectra of tyrosine and phosphotyrosine compounds with (a) **CP** and (b) **Me-CP** conjugates. [compound] = 1 μM in 10 mM Tris buffer (pH 7.0) with 1% MeOH. λ_{ex} = 390 and 365 nm for (a) and (b), respectively.

serves as a conformationally flexible linker. The tyrosine and phosphotyrosine derivatives of **CS** showed little difference in their emission spectra (data not shown) suggesting that the selection of a rigid linker is important for the connection of the tyrosine modification to the coumarin fluorescence change.[7]

Next, we prepared a second version of coumarin–proline conjugate, **Me-CP**, and compared the fluorescence changes induced by tyrosine phosphorylation. For this CH_3-substituted coumarin conjugate, we devised a short-step synthesis (Scheme 2) starting from the nucleophilic aromatic substitution reaction of 4′-fluoro-2′-hydroxyacetophenone with proline. The coumarin fluorophore was constructed efficiently through the Wittig reaction of the proline-substituted acetophenone followed by intramolecular transesterification.[8] This new coumarin con-



Scheme 2. Reagents and conditions: (a) L- or D-proline, K_2CO_3, DMSO, Δ, 99%; (b) CH_3I, K_2CO_3, DMF, 93%; (c) $Ph_3P{=}CHCO_2CH_3$, Ph_2O, Δ, 78%; (d) LiOH, H_2O, THF, 78%.

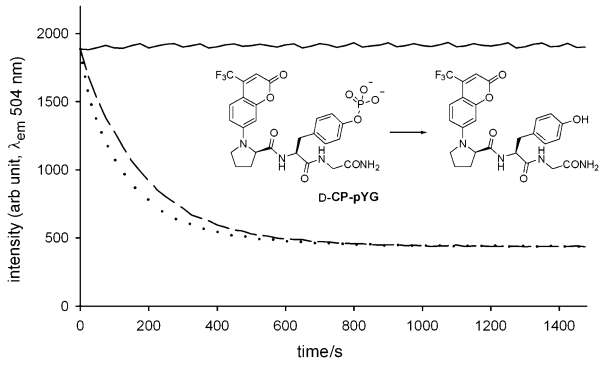


Figure 2. Fluorescence changes during phosphatase-catalyzed reactions (solid, no enzyme; dashed, phosphatase from potato; dotted, phosphatase from wheat germ). [D-**CP-pYG**]$_0 = 1\,\mu M$ and [enzyme] $= 6\,U\,L^{-1}$ in 50 mM citric acid buffer (pH 5.0) with 1% MeOH, $\lambda_{ex} = 390$ nm.

jugate was then converted to its tyrosine and phosphotyrosine derivatives following the same procedure described in Scheme 1. We observed relatively large increase in emission intensity for the coumarin conjugate with D-proline linker (Figure 1b), and this is consistent with the previous result with **CP** conjugates. However, the intensity change is much smaller for the tyrosine–phosphotyrosine pair with **Me-CP** than for the one with **CP** analog, probably due to the difference in the reduction potentials,[9] suggesting that the CF_3-substituted coumarin conjugate is better for the monitoring of tyrosine phosphorylation.

With the effect of the tyrosine modification on the fluorescence at hand, we tried to apply the coumarin–proline conjugate system to enzyme-catalyzed reactions. To avoid possible interference caused by the hydrolysis of carboxylic acid esters, we prepared D-**CP-pYG** as a phosphotyrosine substrate (Figure 2). Dephosphorylation by acid phosphatases proceeded smoothly showing a modest decrease in fluorescence intensity. At the completion of the reaction, the intensity was reduced by 75%, and this change is consistent with the fluorescence difference observed with the tyrosine–phosphotyrosine pair with D-**CP** (Figure 1a).

In summary, we have prepared coumarin–proline conjugates and studied the fluorescence changes induced by the phosphorylation and dephosphorylation of a neighboring tyrosine residue. Two structurally similar coumarin fluorophores showed quite different fluorescence behaviors, and the CF_3-substituted compound with D-proline linker afforded modest changes in emission intensity upon the tyrosine modification. This probe system was successfully used to monitor the tyrosine dephosphorylation catalyzed by phosphatases. We are currently working to apply the coumarin–proline conjugates to study enzyme reactions responsible for the modification of tyrosine and other amino acid residues.

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