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Synthesis of fluorescent substrates for protein tyrosine phosphatase assays

Takumi Watanabe†, Masaya Imoto‡, Hiroki Taniguchi‡, Kazuhiko Kinoshita, Jr.§, and Kazuo Umezawa*,‡

†*Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan*

‡*Department of Applied Chemistry, and* §*Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan*

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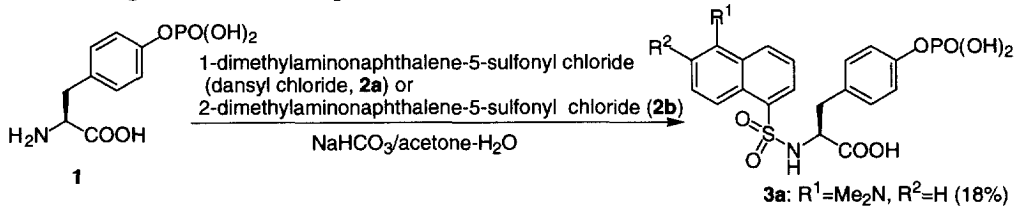
Abstract: Two fluorescent substrates for protein tyrosine phosphatase (PTPase) reaction were prepared by conjugation of commercially available *O*-phosphotyrosine and dansyl chlorides. They were hydrolyzed by CD45 tyrosine phosphatase, and proved to be useful for PTPase assay.

Keywords: Amino acids and derivatives; Enzyme inhibitors; Fluorescence

Intracellular tyrosine phosphorylation should be regulated by both protein tyrosine kinases and protein tyrosine phosphatases (PTPase). PTPase activity can be assayed by the method of Geladopoulos *et al* [1]. In this assay *O*-phospho-L-tyrosine is used as the substrate, and after the reaction, liberated inorganic phosphate is measured with a coloring reagent, malachite green. This method is simple and quick, but interference may occur when foreign phophate is present in the reaction mixture. In this report we prepared fluorescent substrates for the PTPase reaction, and developed new assay system for measuring the fluorescent product after the reaction.



Scheme 1

A fluorescent substrates were prepared as shown in Scheme 1. Because the starting material, *O*-phospho-L-tyrosine (Sigma), was highly soluble in H_2O, the dansyl group could be introduced to the amino group by standard procedures with dansyl chloride **2a** (Sigma) and $NaHCO_3$ in acetone-H_2O to give fluorescent substrate **3a**. Compound **3a** could be purified by preparative TLC developed with $CHCl_3$:MeOH (1:1) and LH-20 eluted with MeOH. The moderate yield came from inevitable decomposition of dansyl chloride in an aqueous solvent and loss during the purification. This method was also

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applicable to introduction of 2-dimethylaminonaphthalene-5-sulfonyl group using the corresponding chloride **2b** (Sigma) to give another fluorescent substrate **3b**.

Crude CD45 PTPase [2] was prepared from the Jurkat cell membrane fraction [3] and solubilized. The enzyme solution (10 μl, 1.5 μg protein) was added to 30 μl of the assay buffer containing 100 mM sodium acetate adjusted to pH 6.0, 1mM EDTA, and 5 μl of a test sample solution with MeOH. The reaction was started by the addition of 10 μl of the fluorescent substrate in assay buffer to give 1 mM. After the reaction at 37 °C, the mixture was diluted with 50 μl of H_2O and extracted with 100 μl of BuOH. The BuOH extract was evaporated, and the residue was dissolved in 10 ml MeOH for application to silica gel TLC. The TLC plate was developed with $CHCl_3$:MeOH:AcOH (15:4:1). For quantitative analysis, the UV spot on TLC was marked and scraped off. The silica was eluted with 2.5 ml of MeOH, and centrifuged at 1,000 rpm for 5 min. The supernatant (2 ml) was used for fluorometric analysis at 330nm/520nm. CD45 PTPase clearly catalyzed the dephosphorylation of **3a** to give dansyl-L-tyrosine. The dephosphorylated product (*Rf*, 0.57) and the substrate **3a** (*Rf*, 0.21) were completely separated by TLC. No spontaneous dephosphorylation of **3a** was observed during the reaction in the absence of the enzyme.

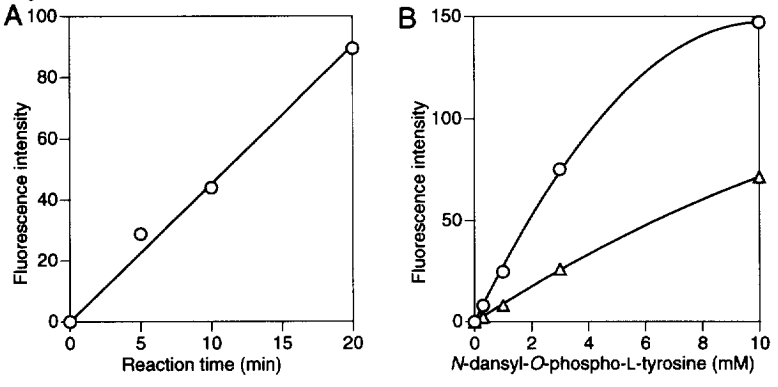

Fig. 1 Quantification of PTPase reaction with **3a**. After development of authentic dephosphrylated sample on TLC plate, the product was scrapped off and eluted with MeOH, then measured for fluorescence intensity in a fluorometer. (A) Time course of the reaction. The reaction was carried out with 10 mM **3a** and 1.8 μg protein of enzyme preparation. (B) The Michaelis-Menten plot for PTPase reaction with **3a**. The reaction was carried out for 15 min with 1.8 (○) or 0.9 (△) μg protein of the enzyme preparation.

The product formation can be quantified by measuring the fluorescent intensity of the spot as shown in Fig. 1. The enzyme reaction was inhibited by PTPase inhibitors, 3,4-dihydroxy analogue of dephostatin [4] and sodium vanadate [5]. Compound **3b** was also hydrolyzed by CD45, and the product detected in the same way as **3a**.

In summary, we have developed new assay system for PTPase with fluorescent substrates.

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