ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 10939–10941

www.rsc.org/chemcomm

COMMUNICATION

A self-immobilizing and fluorogenic unnatural amino acid that mimics phosphotyrosine†

Jingyan Ge, Lin Li and Shao Q. Yao*

Received 29th July 2011, Accepted 31st August 2011
DOI: 10.1039/c1cc14653j

Synthesis of the first self-immobilizing, fluorogenic unnatural amino acid that mimics phosphotyrosine (pTyr) is reported. By using solid-phase peptide synthesis, it was subsequently incorporated into peptide-based probes which found applications in bioimaging and fluorescence-activated cell sorting (FACS).

Reversible protein phosphorylation plays a fundamental role in signal transduction. Protein tyrosine phosphatases (PTPs; >100 members in humans), together with protein tyrosine kinases (PTKs), have an essential role in controlling this key cellular process and maintaining the intricate balance of the phosphoproteome network. Defective regulation of these enzymes has significant implications in human diseases.[1] Elevated levels of PTP activities are found in numerous tumor-associated cells and tissues.[2] PTPs dephosphorylate proteins with a high degree of specificity inside the cell, although *in vitro* they have shown only moderate specificity towards synthetic peptide substrates.[3] Consequently, chemical and biological methods that report not only the global PTP activities, but more importantly the precise enzymatic activity of PTPs at single cellular and subcellular levels (and ultimately at the molecular level), may offer unprecedented views on how these enzymes work under physiological settings.[4] Existing chemical tools have so far focused on the detection of PTP activities either *in vitro* or *in situ* using recombinant PTPs or cellular lysates.[5] More recently, a small molecule-based imaging probe capable of detecting PTP activities in live cells has appeared.[6] Notwithstanding, most probes are not able to detect PTP enzymatic activities on the basis of their substrate, organelle or cell specificity.[6] Herein, we have developed the first self-immobilizing and fluorogenic unnatural amino acid, which, upon incorporation into suitable peptide sequences, gave rise to chemical probes suitable for cell-based analysis (*e.g.* imaging and FACS) of endogenous PTP activities, thus successfully addressing some of the above-mentioned issues.

Existing fluorogenic substrates, such as difluoromethylumbelliferone phosphate (DiFMUP; Fig. 1), are widely used to detect PTP activities *in vitro* but are not suitable cell-based reagents due to their tendency to diffuse from the site of the reaction.[7] Activity-based probes of PTPs have been developed with varied degrees of success, but they generally inhibit PTPs irreversibly and do not provide Turn-ON fluorescence read-outs, and thereby are not suitable for cell-based imaging and FACS experiments.[5a–c] An alternative strategy to minimize product diffusion is to, in the probe design, couple fluorescence release to an enzyme–probe covalent linkage.[6] Based on this principle, Withers and co-workers recently reported self-immobilizing fluorogenic imaging agents based on modified derivatives of coumarin glycosides (Fig. 1) for histological and FACS studies of glycosidases.[8] Inspired by this work, our newly developed unnatural amino acid (**2** shown in Fig. 2) is a modified version of the phosphorylated coumaryl amino acid **1** (and the "caged" version **1′**; Fig. 1), which was recently shown by Barrios and Mitra to be a close mimic of phosphotyrosine (pTyr).[5d] We had previously shown in a separate study of another novel pTyr mimic, **2-FMPT** (Fig. 1), that the introduction of a 2-fluoromethyl group in the aromatic ring of pTyr does not disrupt PTP recognition, but upon dephosphorylation generates a highly reactive quinone methide intermediate which subsequently attaches itself covalently to the nearby proteins.[9] **2-FMPT**, however, was not fluorogenic and cannot report PTP activities in real time in cells. Thus, our new pTyr mimic **2** not only is self-immobilizing and fluorogenic (*i.e.* like Wither's sugar probe), it also possesses both N- and C-terminus (as in the case of naturally occurring amino acids), allowing essential PTP-recognizing peptide elements to be introduced when needed (Fig. 2).

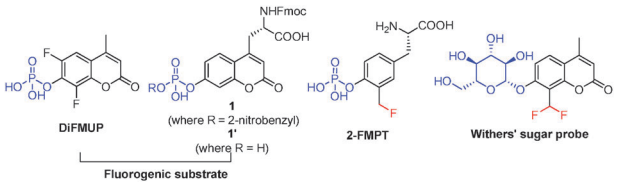

Fig. 1 Representative examples of previously reported fluorogenic PTP substrates, **2-FMPT** and Withers' sugar probe.

Compound **2**, the Fmoc-protected form of the unnatural amino acid, was synthesized in several steps from commercially available *N*-α-Fmoc-L-aspartic acid as shown in Fig. 2b. A 2-nitrobenzyl photolabile protecting group was introduced in **2** to facilitate chemical synthesis and at the same time provide temporal control over PTP-probe recognition.[6,9]

Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore. E-mail: chmyaosq@nus.edu.sg; Fax: +65 67791691; Tel: +65 65162925

† Electronic supplementary information (ESI) available: Detailed experimental procedures and results. See DOI: 10.1039/c1cc14653j

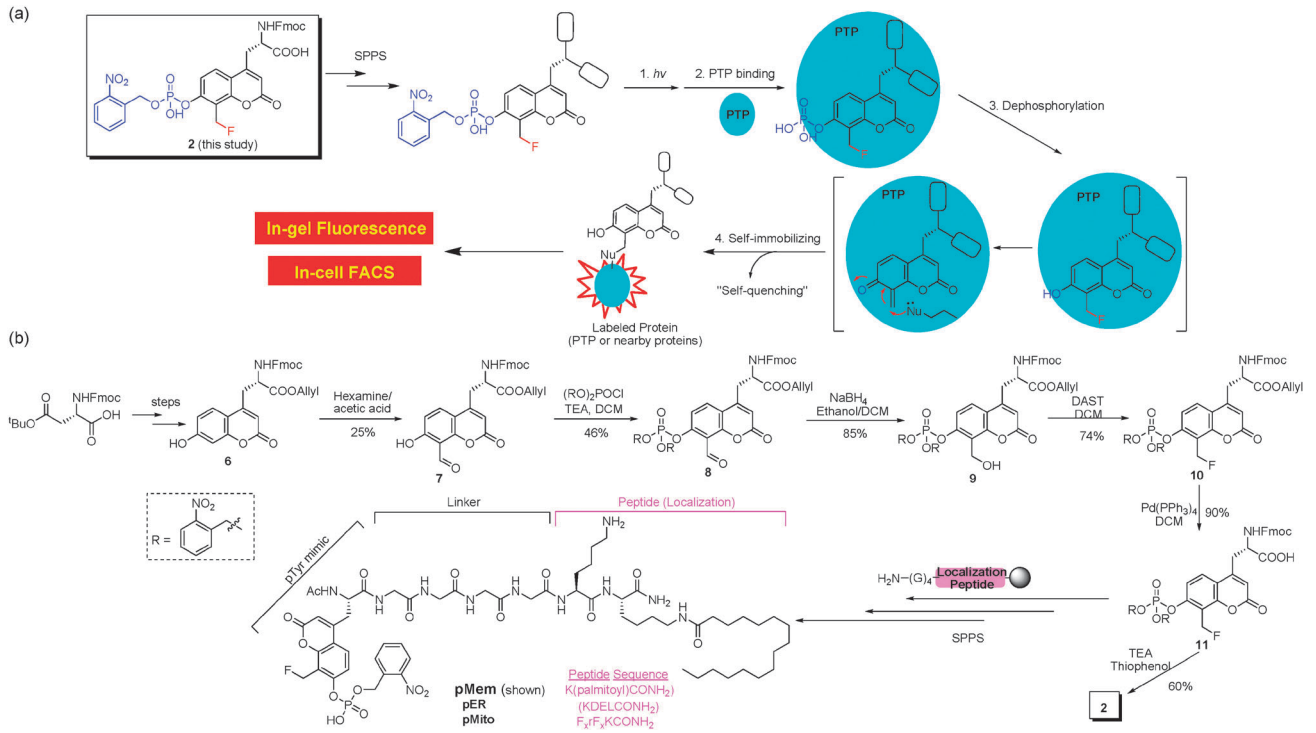

Fig. 2 (a) Overall working principle of the pTyr mimic **2** and its peptide probes. With SPPS, PTP-recognizing elements or CPPs may be introduced into the probe. Upon UV-irradiation to uncage the phosphate, the peptide binds to its intended PTP, and gets dephosphorylated. The ensuring charge delocalization followed by spontaneous elimination of –F generates the highly reactive quinone methide intermediate which either gets quenched by H_2O or diffuses away from the enzyme active site before forming covalent protein–probe complexes (with nearby PTP or other proteins). (b) Chemical synthesis of **2** and Fmoc-based SPPS of the three CPP-containing probes.

Briefly, the first four steps in the synthesis were based on previously reported procedures,[5d] with some modifications, giving the allyl-protected coumaryl amino acid **6**. Subsequently, formylation of **6** by Duff's reaction gave **7** in moderate yield (25%).[10] Next, phosphorylation with di(2-nitrobenzyl) chlorophosphate under basic conditions provided **8** (46% yield), which was reduced with $NaBH_4$ (giving **9**; 85% yield), followed by fluorination with DAST to give **10** (74% yield). Finally, the allyl group in **10** was removed by treatment with $Pd(PPh_3)_4$, giving **11** (90% yield). To obtain the new unnatural amino acid **2**, compound **11** was treated with TEA/thiophenol to remove one of the 2-nitrobenzyl groups. To incorporate the unnatural amino acid into peptide-based probes, the precursor **11** was directly used in standard solid-phase peptide synthesis (SPPS) using Fmoc chemistry (Fig. 2b). We chose to incorporate the self-immobilizing fluorogenic pTyr mimic into three different cell-penetrating peptides (CPPs), giving the resulting peptide-based probes (**pMem**, **pER**, and **pMito**; Fig. 2b). These localization peptides had previously been used to successfully deliver enzyme inhibitors into subcellular organelles of live cells.[11] A flexible $(Gly)_4$ linker was inserted between the CPPs and the unnatural amino acid to minimize potential interferences of PTP recognition by the CPPs. In the present work, we chose to focus on the prospect of these peptide-based probes as agents to study endogenous PTP activities through subcellular imaging and FACS experiments. In future, additional PTP-binding peptide fragments may be similarly introduced, by using the chemistry developed herein, to generate agents that address the substrate specificity of PTPs (Fig. 2).[9]

We first examined the photo- and bio-chemical properties of compound **2** and **pER** peptide (as a representative example). It was established that nearly complete uncaging (>95%) of the 2-nitrobenzyl group, giving the corresponding PTP-responsive adducts, could be achieved under 500 μJ cm^{-2} UV exposure (inset in Fig. 3a for **pER**; see ESI† for **2**). Subsequent addition of recombinant PTP1B (a human PTP involved in diabetes and obesity[1]) or lysates of HeLa cells (a cancer cell line with elevated PTP activities[6]) led to a time-dependent increase in fluorescence (Fig. 3a and ESI†), with a concomitant release of the phosphate group (observed by LC-MS; see ESI†). In a recent study, it was found that the quinone methide generated in live cells underwent both self-quenching (>90%; with aqueous media) and self-immobilizing (<10%; with nearby proteins) pathways,[6] where the former produced a highly fluorescent diffusible dye molecule and the latter generated localized fluorescence. We wondered whether the UV-irradiated **2**, when treated with PTP1B, behaves similarly and produces covalent adducts with proteins nearby (Fig. 2a).[9] As shown in Fig. 3b, the labelling reaction between PTP1B (over-expressed in bacterial lysates) and uncaged **2**, upon SDS separation and in-gel fluorescence scanning, confirmed that, while the majority of **2** was self-quenching (see free probe at the gel front; lane 1), a small amount of PTP1B and other cellular proteins were also labeled (caused by self-immobilization of **2**). Bariros' coumaryl amino acid **1**, which is a non-self-immobilizing analog of **2**, was used as a negative control; no labeled protein was detected (lane 2). Lastly, PTP1B treated with uncaged **2** was further analyzed by mass spectrometry and enzymatic assay; results

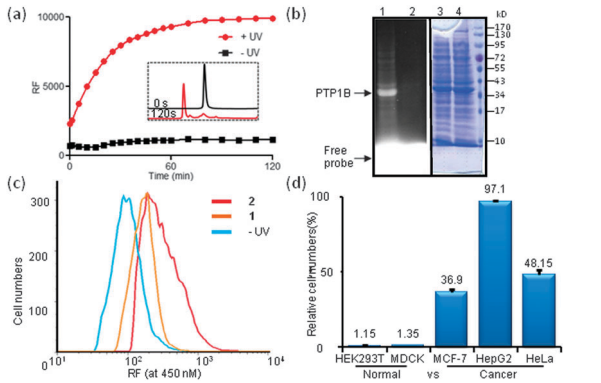

Fig. 3 (a) Time-dependent fluorescence measurements of UV-irradiated **pER** (4 μM) in HeLa cell lysates (λ_{ex} = 360 ± 10 nm; λ_{em} = 460 ± 10 nm). Control (■): non-irradiated **pER** + lysates. Inset: HPLC profiles of 50 μM **pER** before (0 s) and after UV-irradiation (120 s). (b) Fluorescence gel (imaged under a UV transilluminator) of PTP1B-overexpressed bacterial lysates treated with uncaged **2** (1 mM; lane 1) and **1** (0.5 mM; lane 2). The corresponding coomassie gel is shown on the left. A small amount of covalent labelling between **2** and PTP1B and other cellular proteins was observed. Most of **2** was released as the highly fluorescent free probe (arrowed). (c) FACS histogram of HeLa cells treated with 80 μM of uncaged **1** or **2** ("−UV" represents cells treated with non-irradiated **2**, a negative control). (d) Relative numbers of stained cells (by uncaged **2**; 80 μM) from the FACS results of different mammalian cells (Fig. S9 in ESI†).

indicated that most of the PTP1B (>90%) in the reaction was not covalently labeled and still retained its original enzymatic activity (Fig. S6 and S4, ESI†, respectively).

The self-immobilizing Turn-ON fluorescence properties of these new PTP probes make them suitable for FACS experiments.[8] HeLa cells were first incubated with **2** for 30 min, allowing sufficient time for the compound to enter the cells. Subsequently the cells were UV-irradiated, further incubated for 2 h, then sorted. Compound **1** was used as a negative control. As shown in Fig. 3c, we observed a noticeable increase in the number of highly fluorescently labeled cells, over both non-irradiated cells and cells treated with **1**. Repeated washes of **2**-treated cells did not cause significant fluorescence leakage, indicative of a covalent linkage. We next used **2** to detect PTP activities in a cell type-specific manner, in anticipation that most cancers have elevated PTP expression.[2] While UV irradiation itself did not result in any apparent effect on the cells (Fig. S8, ESI†), FACS analysis of 5 common mammalian cells, shown in Fig. 3d, indicated significantly higher fluorescence counts in cancer cells treated with **2** (MCF-7, HeLa & HepG2), possibly caused by their elevated endogenous PTP activities.[2]

Finally, we showed that **2**, upon incorporation into suitable peptide sequences, could be used for subcellular imaging of PTP activities in live cells. **pMem**, **pER**, and **pMito**, each containing a CPP, served to deliver our pTyr mimic to different subcellular organelles (membrane, endoplasmic reticulum and mitochondria, respectively[11]). Upon UV irradiation, the probe-treated HeLa

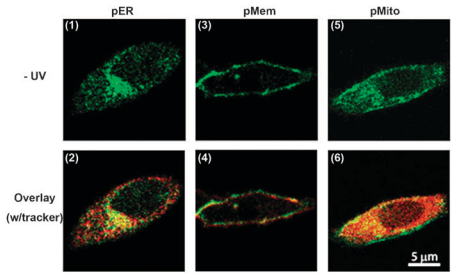

Fig. 4 Fluorescence images of HeLa cells treated with different peptide probes (20 μM, 30–90 min incubation), UV-irradiated, further incubated (1.5 h) then imaged (in coumarin channel). "Overlay": images from the coumarin channel overlaid with the tracker channel. See ESI† for details.

cells were subsequently imaged to detect subcellularly localized, endogenous PTP activities (Fig. 4); all three peptides gave rise to strong fluorescence signals only in their intended organelles, indicating successful delivery and subcellular imaging of PTPs.

In conclusion, we have shown the utility of this newly developed, self-immobilizing and fluorogenic pTyr mimic in studying endogenous PTP activities using FACS and bioimaging experiments. Future work will focus on incorporation of this unnatural amino acid into peptides and proteins, to engineer the corresponding enzyme sensors capable of addressing the substrate specificity of individual PTPs.[3,9]

Funding support was provided by Ministry of Education (R-143-000-394-112) and Agency for Science, Technology and Research (A*Star) of Singapore (R-143-000-391-305).

Notes and references

1 (*a*) N. K. Tonks, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 833–846; (*b*) L. Bialy and H. Waldmann, *Angew. Chem., Int. Ed.*, 2005, **44**, 3814–3839.
2 A. Östman, C. Hellberg and F. D. Böhmer, *Nat. Rev. Cancer*, 2006, **6**, 307–320.
3 Z. Y. Zhang, *Annu. Rev. Pharmacol.*, 2002, **42**, 209–234.
4 I. A. Yudushkin, A. Schleifenbaum, A. Kinkhabwala, B. G. Neel, C. Schultz and P. I. H. Bastiaens, *Science*, 2007, **315**, 115–119.
5 (*a*) L. C. Lo, T. L. Pang, C. H. Kuo, Y. L. Chiang, H. Y. Wang and J. J. Lin, *J. Proteome Res.*, 2002, **1**, 35–40; (*b*) Q. Zhu, X. Huang, G. Y. J. Chen and S. Q. Yao, *Tetrahedron Lett.*, 2003, **44**, 2669–2672; (*c*) S. Kumar, B. Zhou, F. Liang, W.-Q. Wang, Z. Huang and Z.-Y. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 7943–7948; (*d*) S. Mitra and A. M. Barrios, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 5142–5145.
6 M. Hu, L. Li, H. Wu, Y. Su, P.-Y. Yang, M. Uttamchandani, Q. H. Xu and S. Q. Yao, *J. Am. Chem. Soc.*, 2011, **133**, 12009–12020.
7 S. Welte, K.-H. Baringhaus, W. Schmider, G. Müller, S. Petry and N. Tennagels, *Anal. Biochem.*, 2005, **338**, 32–38.
8 D. H. Kwan, H.-M. Chen, K. Ratananikom, S. M. Hancock, Y. Watanabe, P. T. Kongsaeree, A. L. Samuels and S. G. Withers, *Angew. Chem., Int. Ed.*, 2011, **50**, 300–303.
9 K. A. Kalesh, L. P. Tan, K. Liu, L. Gao, J. Wang and S. Q. Yao, *Chem. Commun.*, 2010, **46**, 589–591.
10 R. M. Naik and V. M. Thakor, *J. Org. Chem.*, 1957, **22**, 1626–1629.
11 Y. Loh, H. Shi, M. Hu and S. Q. Yao, *Chem. Commun.*, 2010, **46**, 8407–8409.