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Design and Synthesis of a Fluorescent Reporter of Protein Kinase Activity

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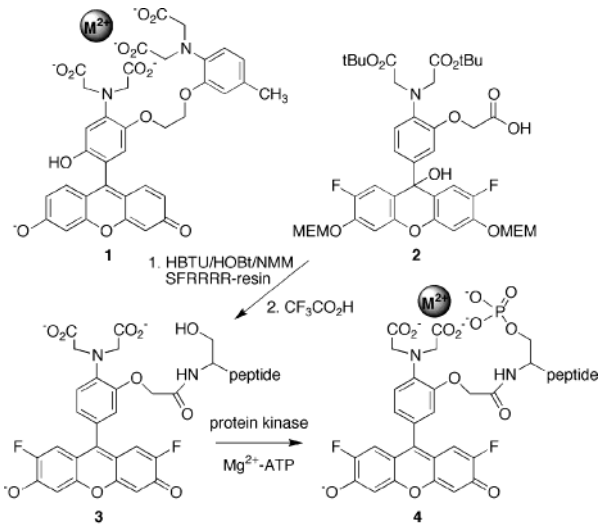
Cells recognize and respond to environmental stimuli via activation of intracellular biochemical pathways primarily composed of protein kinases. These enzymes catalyze the phosphorylation of serine, threonine, or tyrosine residues on protein substrates. Members of this large enzyme family have been the objects of intense scientific scrutiny due to their role in disease onset and progression. In addition, they participate in the pathways that drive a variety of important processes that range from cell division[1] to apoptosis.[2] Consequently, there has been widespread interest in developing sensors of protein kinase activity, species that could furnish a visual readout of both where and when specific intracellular kinases are activated in response to a stimulus. A variety of approaches have been described,[3] including small peptide substrates that possess an appended fluorophore positioned near the site of phosphorylation.[4] Unfortunately, the phosphorylation-induced change in fluorescence intensity for these peptides is modest, at best (~20%). We have developed a strategically different approach for the construction of a fluorescent reporter of protein kinase activity.



Perhaps the preeminent example of a fluorescent sensor that samples biologically relevant processes is the family of Ca^{2+} indicators (e.g., **1**) developed by Tsien and his colleagues.[5] Formation of the Ca^{2+}−fluorophore complex, via coordination to the two iminodiacetic acid moieties, is manifested by a dramatic fluorescence change. Tsien has proposed that Ca^{2+} coordination induces a twist about the arylamine bond, altering the coupling between the nitrogen lone pair and the aromatic ring system.[5] We have designed a peptide-based species (**3**) that contains some of the structural features present in **1**. Specifically, phosphorylation of **3** should generate a M^{2+} receptor site composed of two carboxylates and the newly introduced phosphate (**4**). Upon divalent metal ion coordination, a fluorescence change should transpire via a mechanism analogous to that described for the Ca^{2+} indicators.

The strategy outlined above is made possible by the observation that protein kinases will phosphorylate alcohol-containing residues attached to the N- or C-terminus of appropriately designed peptides.[6,7] This structural feature allows the fluorophore to be directly attached to the phosphorylatable residue (e.g., **3**). With the latter in mind, our initial synthetic target was compound **2**, which contains the requisite functionality in protected form, along with a free carboxylate that can be activated and condensed with the N-terminus of the peptide H_2N-Ser-Phe-Arg-Arg-Arg-Arg−resin. The latter sequence serves as a substrate for protein kinase C (PKC),[7] an enzyme family implicated in a variety of physiologically critical processes, including cell division.[8]

The xanthene half of **2** was synthesized from the xanthone precursor **7** (Scheme 1). The latter was prepared via the Friedel−Crafts acylation of **6** [9] with **5**. The product was subsequently heated in a sealed tube to furnish the xanthone **8** and the phenol moieties then protected as 2-methoxyethoxymethyl (MEM)[10] ethers (**9**). The aromatic precursor (**10**) to the "northern" half of compound **2** was prepared in three steps from commercially available *o*-aminophenol (see Supporting Information). Compound **10** contains a doubly protected iminodiacetic acid moiety and a benzylated phenol. The latter will ultimately be debenzylated so that it can serve as the attachment site for the peptide (vide infra). Compound **10** was lithiated at −105 °C and coupled to **9**, to furnish adduct **11** in 69% yield. The benzylated phenol in **11** was transformed in three steps to the desired carboxylic acid (**2**) and then coupled to H_2N-Ser-(*O*-tBu)-Phe-[(Arg)Mtr]$_4$−resin (prepared via standard Fmoc solid-phase peptide synthesis on the Rink resin). Finally, exposure of the resin-appended fluorophore−peptide to 95% CF_3CO_2H resulted in the simultaneous cleavage of the peptide from the resin, MEM ether deprotection, and complete aromatization of the tricyclic nucleus via loss of the tertiary hydroxyl moiety to yield **3**.

As expected, peptide **3** serves as a substrate for the Ca^{2+}-dependent PKCα. **3** displays a 140% increase in fluorescence intensity upon phosphorylation, nearly an order of magnitude greater than that previously described in protein kinase monitoring systems.[3,4] In addition, the difluorofluorescein moiety in **3** is an extremely bright fluorophore ($\epsilon = 78\,000\ cm^{-1}\ M^{-1}$ and $\Phi = 0.60$) and, thus, is easily an order of magnitude more sensitive than fluorophores that have been previously used to observe protein kinase activity.[9] Although the K_m value (26.5 μM) for the PKCα-catalyzed phosphorylation of **3** is quite good, the corresponding V_{max} (0.32 μmol/min·mg) is an order of magnitude less than ideal. The large, negatively charged fluorophore, which is positioned adjacent to the site of phosphorylation, might interfere with the ability of the PKC active site to accommodate the serine moiety.

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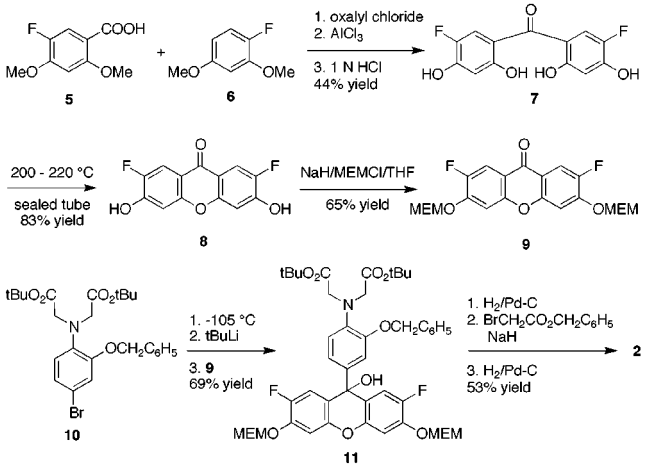
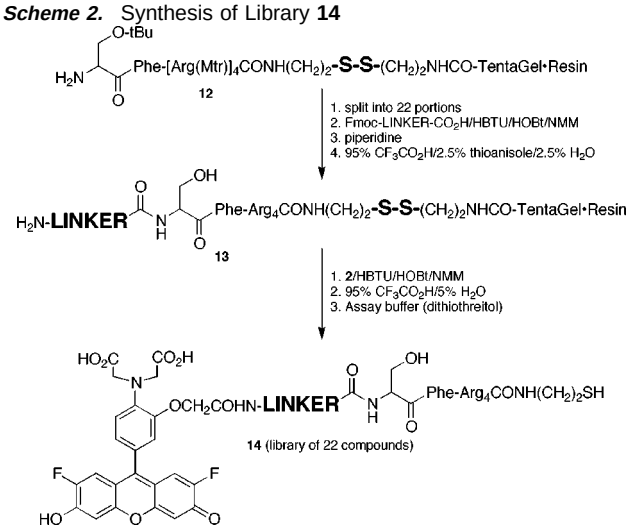
Scheme 1. Synthesis of the Fluorescein Precursor **2**

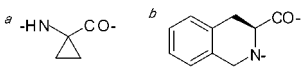


Scheme 2. Synthesis of Library **14**



We addressed the possibility of fluorophore-mediated disruption of the kinase-catalyzed reaction by preparing a small library of 22 derivatives of **3** using the synthetic strategy outlined in Scheme 2. A series of turn-promoting/metal chelating LINKERs was inserted between the peptide and the fluorophore (**14**), which might allow the serine moiety to be more optimally accommodated within the active site. Following phosphorylation, the turn-inducing/chelating ability of the LINKER should enable the iminodiacetic acid carboxylates to assume a position that promotes metal coordination. The library of 22 compounds was prepared on a cystamine-derivatized TentaGel resin,[11] which contains a disulfide bridge between the peptide and the resin (**12**). The side-chain protected peptide **12** was split into 22 portions of 10 mg each and added to a solvent-resistant multiwell filter plate. Twenty-two Fmoc-amino acids ("LINKER"s, see Supporting Information) were added to individual wells and condensed with **12**. The Fmoc group was removed (**13**) and the product coupled to compound **2**. CF_3CO_2H (95%) was subsequently employed to simultaneously deprotect the phenol and carboxylic acid moieties and transform the xanthene nucleus into the fluorescein derivative. Finally, all 22 compounds were cleaved from the Tentagel resin with PKC assay buffer, which contains dithiothreitol. The library members (**14**) were filtered into a receiving plate and then assayed under standard conditions with monitoring for both the magnitude and rate of fluorescence change. Several fluorophore−LINKER−peptide analogues were identified that display promising enzymological and photophysical properties (Table 1). *N*-methyl glycine serves as the LINKER in the lead protein kinase substrate. Phosphorylation of the latter generates a 264% enhancement in fluorescence intensity and proceeds with a V_{max} of 8.5 μmol/min·mg and a K_m of 20.5 μM. Indeed, the V_{max} is more than an order of magnitude greater than that displayed by compound **3**, which lacks a LINKER residue between the fluorophore and the peptide.

Table 1. V_{max}, K_m, and Fluorescence Change Associated with the PKC-Catalyzed Phosphorylation of Peptides **14** (five different linkers), **3**, and AcSFRRRRK[7]



LINKER	V_{max} (μmol/min·mg)	K_m (μM)	% change fluorescence
L-proline	1.9	63.0	150%
D-proline	1.0	23.5	156%
N-Me glycine	8.5	20.5	264%
a	1.7	25.0	164%
b	2.2	24.9	157%
peptide **3**	0.32	26.5	140%
AcSFRRRRK	24	10	−

Saturating [Ca^{2+}] produces 1.2- and 2.0-fold fluorescence enhancements in **3** and **14** (N-Me Gly), respectively. By contrast, enhancements of 5- and 23-fold were observed with the chemically synthesized *phosphorylated* analogues of **3** and **14**, respectively. However, the large metal-induced fluorescence change in the phosphorylated species appears to be partly offset by a reduction in the inherent (i.e., metal-free) fluorescence of the phosphopeptides (Supporting Information). In summary, we have prepared, via rational design and library synthesis, a new family of protein kinase substrates that respond to phosphoryation in a fluorescently sensitive fashion. The ability of compounds **3** and **14** to sample PKC activity in a variety of cell-based systems is under investigation.

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Supporting Information Available: Experimental details of the synthesis, characterization, and enzymology of compound **3**/library **14** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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