Design and Synthesis of an Activity-Based Probe Template for Protein Kinases

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Dedicated to Gerry Pattenden in celebration of his 70th birthday

Abstract: A potential activity-based probe for protein kinases was designed around mechanism-based inhibitors of insulin receptor kinase. The probe was synthesized by a chemoselective conjugate addition of ATPγS to an epoxyenone.

Key words: activity-based probes, addition reactions, regioselectivity, enones, epoxides

Proteins with phosphotyrosine, although few (ca. 0.05% of proteins in human cells),[1] play key roles in cellular signaling pathways that regulate cell proliferation, differentiation, adhesion, and motility.[2] Phosphotyrosinyl proteins are the net products of the delicate balance between enzymatic reactions that are catalyzed by protein tyrosine kinases and phosphatases. In order to understand the cellular dynamics of tyrosine phosphorylation, investigations have focused on analyzing the sites of tyrosine phosphorylation across numerous proteins in proteomes, simultaneously, as well as sequentially, over time.[1,3] However, these analyses are indirect measurements of the enzyme activities. Tyrosine kinases and phosphatases that control the phosphorylation and dephosphorylation of proteins have rarely been studied for their activities on a proteome scale.[4]

Small molecules are used in studies of proteome-wide catalytic and binding events in cells. These compounds are often referred to as activity-based probes (ABPs), and analysis of probe–protein adducts, mainly by mass spectrometry, is referred to as activity-based protein profiling or, sometimes, chemical proteomics.[5] This approach provides a means to measure proteome-wide enzyme activity directly. ABPs have a reactive moiety, a linker and a reporter. Some form covalent adducts at enzyme active sites that can be analyzed by mass spectrometry-based proteomics. The design of the reactive moieties takes cues from organic compounds that are used for affinity labeling, suicide inhibition and mechanism-based inhibition of enzymes, where covalent bonds are formed with protein targets.[6] The pool of these molecules has been enriched by new small molecules that have been used in chemical genetics and functional proteomics.[7] ABPs have recently been utilized for profiling the activity of various enzymes in proteomes, including serine proteases,[8] cysteine proteases,[9] metalloproteases,[10] kinases,[11] phosphatases,[4,12] phosphorylases,[13] and carbohydrate-related enzymes.[14]

This work reports the design and synthesis of an activity-based probe (**1**) for protein tyrosine kinases (Figure 1). Our probe was patterned around the potent, mechanism based, bisubstrate inhibitors **2** for insulin receptor kinase.[15] These inhibitors have two elements responsible for high binding affinity. One is the correct distance, defined by the spacing in the dissociative transition state between the ATP unit and the tyrosine unit. The second feature is the hydrogen bonding between Asp-1132 and the amide hydrogen.[14] In probe **1**, the amide NH was changed into an epoxyethylene group in order to target a conserved carboxylate group in the protein tyrosine kinase active site to form covalent adducts between the probe and protein tyrosine kinases. The azide moiety is incorporated for coupling with a reporter group through 'click' chemistry,[16] which can be used for displaying or separating the probe-modified enzymes.

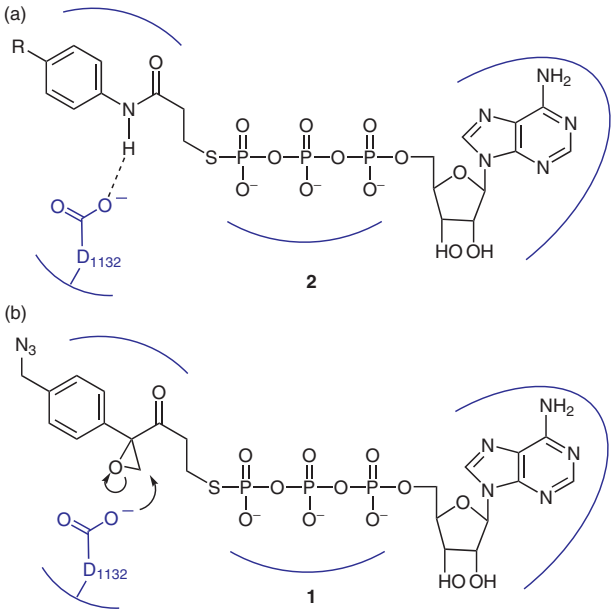

Figure 1 Illustration of protein binding-site interactions with ABPs for protein tyrosine kinases. (a) Interactions between tyrosine kinase and bisubstrate analog inhibitors **2**, drawn based on reference 15; (b) Proposed interactions between tyrosine kinase and ABP **1**. Kinase binding sites are represented with blue lines. D_{1132} represents Asp-1132 on the kinase.

We envisaged that potential ABP **1** could be synthesized by the conjugate addition of ATPγS to epoxyenone **3**

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(Figure 2), which would be prepared from a vinylation/oxidation sequence with epoxyaldehyde **4**. Compound **4** would result from an initial Suzuki coupling of iodopropene **5** and 4-(hydroxymethyl)phenyl boronic acid, followed by routine functional group manipulations.

The synthesis of epoxyenone **3** commenced with Suzuki coupling of known,[17] silyl-protected iodopropene **5** and commercially available 4-(hydroxymethyl)phenyl boronic acid (Scheme 1). Reaction of the coupled product **6** with carbon tetrabromide and triphenylphosphine, followed by treatment of the resultant benzylic bromide with sodium azide, gave **7** in 90% yield over two steps. Variable amounts of desilylated product were observed in the bromination step with reaction times longer than three hours. Intermediate **7** was deprotected using TBAF; epoxidation using dimethyldioxirane (DMDO) and Dess–Martin periodinane oxidation[18] furnished key epoxyaldehyde **4** in 56% yield over three steps. Subsequent nucleophilic addition of vinylmagnesium bromide afforded a mixture (~2:1) of diastereomeric allylic alcohols. The final oxidation to **3** proved to be problematic.

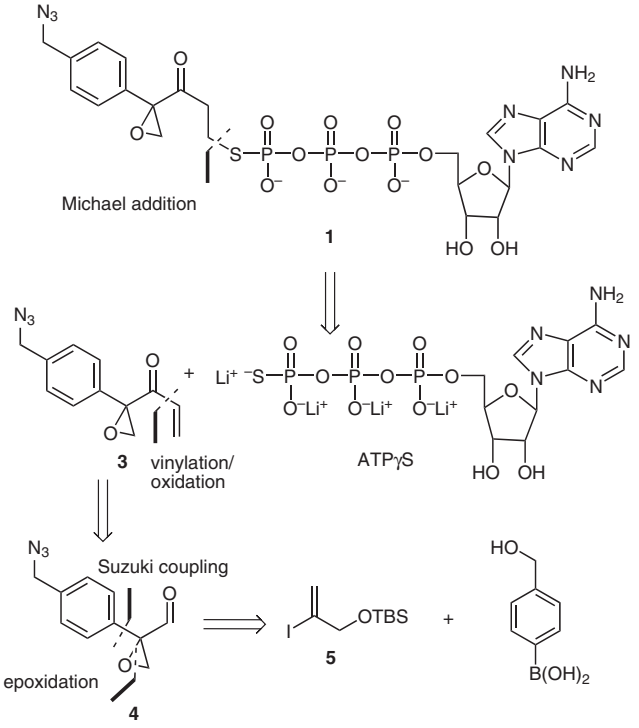
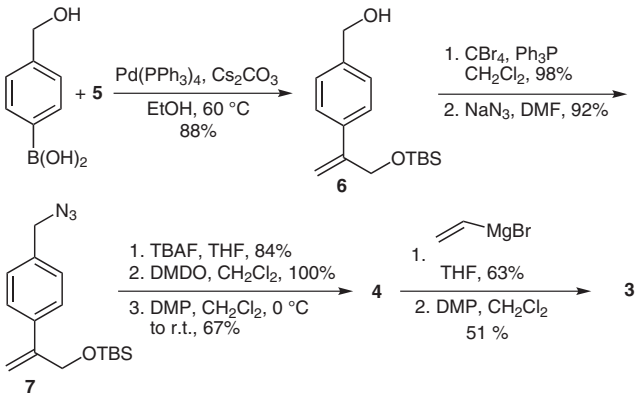


Figure 2 Approach to the synthesis of probe **1**

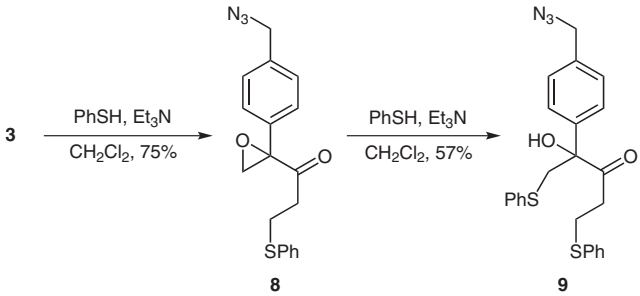
Initial attempts to oxidize allylic alcohol **3** using MnO_2 or standard oxidants (i.e., Dess–Martin periodinane,[18] PCC, PDC, Swern conditions) did not provide clean conversions. Although the alcohol was consumed, if any product was isolated, the yield was low, and the product was not pure. This outcome was not completely unexpected, considering that **3** is a highly reactive, α,β-unsaturated ketone where the carbonyl group is also flanked by a styrene oxide. Indeed, oligomers were observed at ca. 30 °C, and cross-linked polymers, as confirmed by ^{1}H NMR and gel



Scheme 1 Synthesis of epoxyenone **3**

permeation chromatography, were seen at higher temperatures (ca. 45 °C and above). Recognizing the sensitivity of **3** to even slightly elevated temperatures, we returned to Dess–Martin periodinane mediated oxidation with care taken in both the reaction and isolation (via quick flash chromatography) so as not to allow the temperature of the compound to exceed 25 °C. This provided epoxyenone **3** in moderate yields with good purity. Delayed purification resulted in a very low recovery of the product, and extended storage of neat **3**, even at –78 °C, resulted in the formation of oligomers. The lifetime of the epoxyenone could be increased from days to weeks by storing it in CH_2Cl_2 in the freezer.

To access **1** effectively, selective reaction of ATPγS with the enone moiety of **3** would be required. Since epoxyenone **3** has multiple potentially reactive sites, model studies using thiophenol as the nucleophile were conducted (Scheme 2). With one equivalent of thiophenol, compound **3** was consumed within ten minutes, and the conjugate addition adduct **8** was the sole product observed in the ^{1}H NMR spectrum of the crude reaction mixture (75% isolated yield). With two or more equivalents of thiophenol, clean conversion into **9** resulted, although the rate of epoxide opening was considerably slower. These model studies suggested that selective reaction between the enone of **3** and ATPγS should be achievable.



Scheme 2 Model studies of the reactivity of epoxyenone **3**

An initial concern was identifying an appropriate binary solvent system, considering the widely differing polarities of the commercial ATPγS tetralithium salt and epoxyenone **3**. Although such reactions can be conducted under

biphasic conditions,[19] preliminary experiments had shown that, under such conditions, hydrolysis of ATPγS occurred over time. Consequently, we elected to convert the ATPγS into a dichloromethane-soluble form.[20]

The ATPγS lithium salt was converted into the corresponding tetra(tributylammonium) salt by first passing it through an acidic ion-exchange resin, then treating the resultant acid with tributylamine. This salt was reacted with an excess of epoxyenone **3** (3 equiv) in CH_2Cl_2. The consumption of the ATPγS, which occurred within four to six hours, was monitored by LC-MS. MS analysis suggested that product formation was occurring, however, the formation of products of higher molecular weight was also observed. After passing the crude reaction mixture through an acidic resin in order to generate the acid form of the product, LC-MS showed a decrease in the amount of desired product and the generation of new by-products. We therefore decided to avoid acidic conditions in both the reaction and during purification and chose to work with the trilithium salt.

When the reaction was conducted in a mixture of dichloromethane and water, little conversion into the product was observed by LC-MS. Longer reaction times did not produce a significant increase in product formation and resulted in the hydrolysis of ATPγS. Gratifyingly, the use of a combination of THF and water (2:1) as solvent resulted in complete consumption of the ATPγS within six hours with minimal levels of hydrolysis being observed. Over the course of the reaction, LC-MS showed one major product peak with a mass corresponding to target kinase probe **1**.

Probe **1** was purified by spin column chromatography. After purification, a single peak with m/z = 751.045 (theoretical $[M - H]^-$ m/z = 751.050) and m/z = 753.060 (theoretical $[M + H]^+$ m/z = 753.066) was detected in LC-MS analysis. Considering that epoxyenone **3** is racemic, a 1:1 mixture of diastereomers of **1** should result, and this was seen in the ^{1}H NMR spectrum. Each of the expected adenine proton singlets was doubled, although the chemical shift variation of the individual signals was small. There were two sets of doublets for the epoxide protons between δ = 3.0 and 3.2 ppm, which were found in the same region as those corresponding to the hydrogens in **3**. The remaining proton signals were not as well defined as those in the corresponding reactants, which is not unexpected considering the more complex coupling patterns. ^{31}P NMR analysis showed three signals with two of the signals having increased splitting, reflecting the presence of the two diastereomers. The probe was stable in aqueous solution at neutral pH and room temperature for up to six hours. Beyond this time, degradation of the probe was observed by ^{1}H NMR analysis, with an approximate half-life of 12 hours (based on the appearance of a new anomeric proton signal over that time-frame). The probe could be stored in a frozen solution at –80 °C for only a few days.

In summary, the proposed activity-based probe **1** for protein kinases was synthesized. Its preparation took advantage of a chemoselective reaction between ATPγS and the enone of epoxyenone **3**. Probe **1** was found to have limited stability and, consequently, will not be used for the planned proteomic profiling. However, we believe that the array of functionality and distinct reactivity of **3** make it an excellent scaffold for coupling with either an alternative ATP surrogate or with other binding moieties for activity-based profiling.

1-{2-[4-(Azidomethyl)phenyl]oxiran-2-yl}-3-[adenosine-5′-(3-thiotriphosphate)]propan-1-one (**1**): Adenosine-5′-(3-thiotriphosphate)trilithium salt (5 mg, 0.01 mmol) in H_2O (0.8 mL) was added to a solution of 1-{2-[4-(azidomethyl)phenyl]oxiran-2-yl}prop-2-en-1-one (**3**; 11 mg, 0.05 mmol) in THF (1.6 mL). The reaction was monitored by LC-MS. After 6 h, the solvent was removed under vacuum, and H_2O (0.5 mL) and CH_2Cl_2 (0.5 mL) were added to dissolve the resultant solid. The layers were separated and the aqueous solution was washed with CH_2Cl_2 (3 × 0.5 mL) and purified using hydrophilic/lipophilic balanced materials that were packed in-house in a spin column (0.8 mL). Deionized H_2O was used as both the equilibration and washing solution, and MeCN–H_2O (70% v/v) was used as the elution solution. The eluant was lyophilized and further purified using hydrophilic/lipophilic materials packed in-house in a TopTipTM spin column (Glygen, MD; 10–200 μL) using the same equilibration, washing and elution conditions. The eluant was analyzed by LC-MS. The separation was run at 50 μL/min with a binary gradient [solvent A = 1% MeCN in 10 mM aq NH_4OAc (pH 6.86); solvent B = MeCN. Gradient: 1% B at 0 min → 1% B at 5 min → 55% B at 25 min → 70% B at 27 min → 70% B at 30 min → 1% B at 30.1 min → 1% B at 35 min). Pure eluant was lyophilized to yield probe **1** (1:1 mixture of diastereomers) as a white, fluffy solid (2 mg, 30%): ^{1}H NMR (500 MHz, D_2O): δ = 8.53 (s, 0.5 H), 8.52 (s, 0.5 H), 8.27 (s, 0.5 H), 8.26 (s, 0.5 H), 7.42 (m, 4 H), 6.13 (d, J = 5.6 Hz, 1 H), 4.75 (m, 1 H), 4.58 (m, 1 H), 4.44 (s, 1 H), 4.43 (s, 1 H), 4.40 (m, 1 H), 4.29 (m, 2 H), 3.46 (d, J = 4.7 Hz, 0.5 H), 3.45 (d, J = 4.7 Hz, 0.5 H), 3.31 (d, J = 4.7 Hz, 0.5 H), 3.29 (d, J = 4.7 Hz, 0.5 H), 2.97 (m, 4 H); ^{31}P NMR (202 MHz, D_2O): δ = 8.78 (d, J = 29.3 Hz), 8.75 (d, J = 27.6 Hz), –11.48 (d, J = 21.5 Hz), –23.84 (m); HRMS (TOF): m/z $[M - H]^-$ calcd for $C_{22}H_{26}N_8O_{14}P_3S$: 751.050; found: 751.045; HRMS (TOF): m/z $[M + H]^+$ calcd for $C_{22}H_{28}N_8O_{14}P_3S$: 753.066; found: 753.060.

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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This article has been corrected as described in the following Erratum published on April, 15th:

Erratum

Design and Synthesis of an Activity-Based Probe Template for Protein Kinases

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The PDF and the print version of this article contained the wrong publication year (2009 instead of 2010). This mistake has been corrected for the current online PDF version. We apologize profusely for this mistake.