

# Increasing the Kinase Specificity of K252a by Protein Surface Recognition

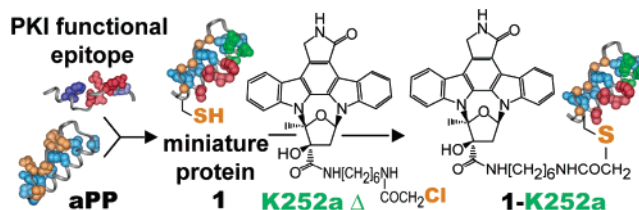
Tanya L. Schneider,<sup>†</sup> Rebecca S. Mathew,<sup>†</sup> Kevin P. Rice,<sup>†</sup> Kazuhiko Tamaki,<sup>†</sup> John L. Wood,<sup>†</sup> and Alanna Schepartz<sup>\*,†,‡</sup>

Department of Chemistry and Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520-8107

alanna.schepartz@yale.edu

Received January 27, 2005

## ABSTRACT



Here we describe a miniature protein (**1**) that presents the cAMP-dependent protein kinase (PKA) recognition epitope found within the heat-stable Protein Kinase Inhibitor protein (PKI) and a miniature protein conjugate (**1-K252a**) in which **1** is joined covalently to the high-affinity but nonselective kinase inhibitor K252a. Miniature protein **1** recognizes PKA with an affinity that rivals that of PKI and, in the context of **1-K252a**, leads to a dramatic increase in kinase specificity.

Protein kinases play fundamental roles defining and maintaining signal transduction pathways, and aberrant kinase activity is linked to a plethora of human disease. Despite intense interest in this area, the design of selective protein kinase inhibitors remains a challenge<sup>1,2</sup> due to the large number of kinases encoded by the human genome<sup>3</sup> and the striking similarity of their active sites.<sup>1,4</sup> We have previously described a strategy for DNA and protein surface recognition in which the  $\alpha$ -helix in the small, well-folded protein avian pancreatic polypeptide (aPP) presents recognition epitopes found within other, larger proteins.<sup>5–13</sup> The miniature proteins

designed in this manner recognize even shallow clefts on protein surfaces with (in many cases) nanomolar affinities and high specificity and inhibit protein–protein interactions.<sup>14,15</sup> Here we describe a miniature protein (**1**) that presents the cAMP-dependent protein kinase (PKA) specificity epitope found within the heat-stable Protein Kinase Inhibitor protein (PKI) and a miniature protein conjugate (**1-K252a**) in which **1** is joined covalently to the high-affinity but otherwise nonselective kinase inhibitor K252a. The indolocarbazole natural product K252a is a potent, active-site-directed inhibitor of many tyrosine and serine/threonine kinases<sup>16–19</sup> and a common starting point for the discovery

<sup>†</sup> Department of Chemistry.

<sup>‡</sup> Department of Molecular, Cellular, and Developmental Biology.

(1) Bridges, A. J. *Chem. Rev.* **2001**, *101*, 2541–2572.

(2) Scapin, G. *Drug. Discuss. Today* **2002**, *7*, 601–611.

(3) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsnam, S. *Science* **2002**, *298*, 1912–1933.

(4) Miller, W. T. *Nat. Struct. Biol.* **2001**, *8*, 16–18.

(5) Chin, J. W.; Schepartz, A. *J. Am. Chem. Soc.* **2001**, *123*, 2929–2930.

(6) Chin, J. W.; Grotzfeld, R. M.; Fabian, M. A.; Schepartz, A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1501–1505.

(7) Chin, J. W.; Schepartz, A. *Angew. Chem., Int. Ed.* **2001**, *20*, 3806–3809.

(8) Montclare, J. K.; Schepartz, A. *J. Am. Chem. Soc.* **2003**, *125*, 3416–3417.

(9) Rutledge, S. E.; Volkmann, H.; Schepartz, A. *J. Am. Chem. Soc.* **2003**, *125*, 14336–14347.

(10) Golemi-Kotra, D.; Mahaffy, R.; Footer, M. J.; Holtzman, J. H.; Pollard, T. D.; Theriot, J. A.; Schepartz, A. *J. Am. Chem. Soc.* **2004**, *126*, 4–5.

(11) Gemperli, A. C.; Rutledge, S. E.; Maranda, A.; Schepartz, A. *J. Am. Chem. Soc.* **2005**, *127*, 1596–1597.

(12) Yang, L.; Schepartz, A. *Biochemistry* **2005**, *44*, in press.

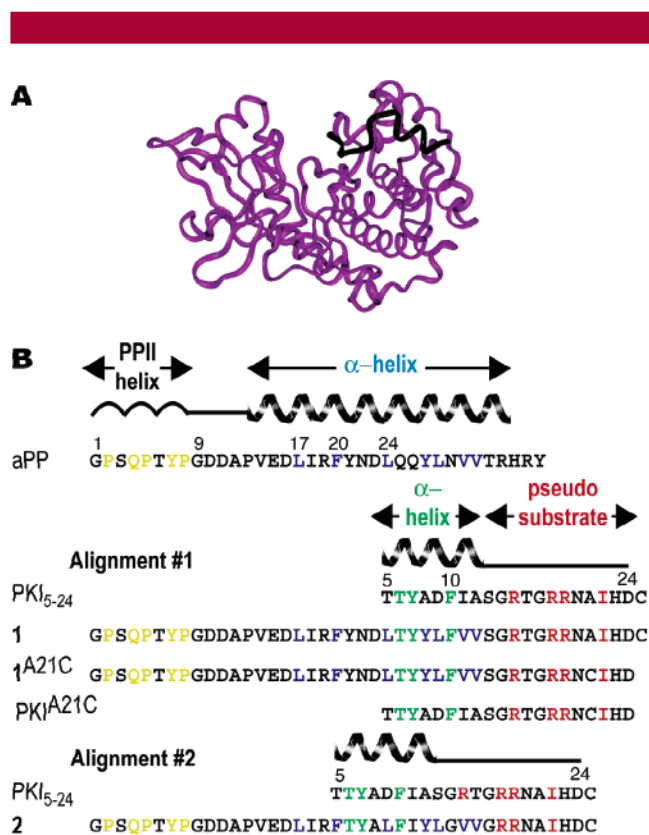
(13) Volkman, H. M.; Rutledge, S. E.; Schepartz, A. *J. Am. Chem. Soc.* **2005**, ASAP (DOI: 10.1021/ja042761v).

(14) Shimba, N.; Nomura, A. M.; Marnett, A. B.; Craik, C. S. *J. Virol.* **2004**, *78*, 6657–6665.

(15) Cobos, E. S.; Pisabarro, M. T.; Vega, M. C.; Lacroix, E.; Serrano, L.; Ruiz-Sanz, J.; Martinez, J. C. *J. Mol. Biol.* **2004**, *342*, 355–365.

38 of specific kinase inhibitors.<sup>1,20</sup> Miniature protein **1** recog-  
 39 nizes PKA selectively with an affinity that rivals that of PKI  
 40 and, in the context of **1-K252a**, leads to a dramatic increase  
 41 in kinase specificity. These results suggest that molecules  
 42 that combine a potent active-site-directed inhibitor with a  
 43 miniature protein specificity element could represent viable  
 44 tools to selectively explore kinase function.<sup>21-27</sup>

45 Our design of miniature protein **1** began with the structure  
 46 of the catalytic subunit of PKA in complex with the active  
 47 portion of PKI (PKI<sub>5-24</sub>, Figure 1A), which selectively



**Figure 1.** (A) Structure of the catalytic subunit of PKA (purple) in complex with PKI<sub>5-24</sub><sup>28</sup> (black). (B) Strategy for miniature protein design. The PPII and  $\alpha$ -helix regions within aPP are identified, as are the  $\alpha$ -helix and pseudosubstrate regions of PKI<sub>5-24</sub>. Residues within aPP that contribute to folding are in blue ( $\alpha$ -helix) or yellow (PPII helix). Residues that comprise the PKI<sub>5-24</sub> pseudosubstrate are in red; residues that comprise the PKI<sub>5-24</sub>  $\alpha$ -helical specificity element are in green.

48 recognizes PKA and inhibits its function. In this complex,

(16) Kase, H.; Iwahashi, K.; Matsuda, Y. *J. Antibiot.* **1986**, *39*, 1059-1065.

(17) Kase, H.; Iwahashi, K.; Nakanishi, S.; Matsuda, Y.; Yamada, K.; Takahashi, M.; Murakata, C.; Sato, A.; Kaneko, M. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 436-440.

(18) Hashimoto, Y.; Nakayama, T.; Teramoto, T.; Kato, H.; Watanabe, T.; Kinoshita, M.; Tsukamoto, K.; Tokunaga, K.; Kurokawa, K.; Nakanishi, S.; Matsuda, Y.; Nonomura, Y. *Biochem. Biophys. Res. Commun.* **1991**, *181*, 423-429.

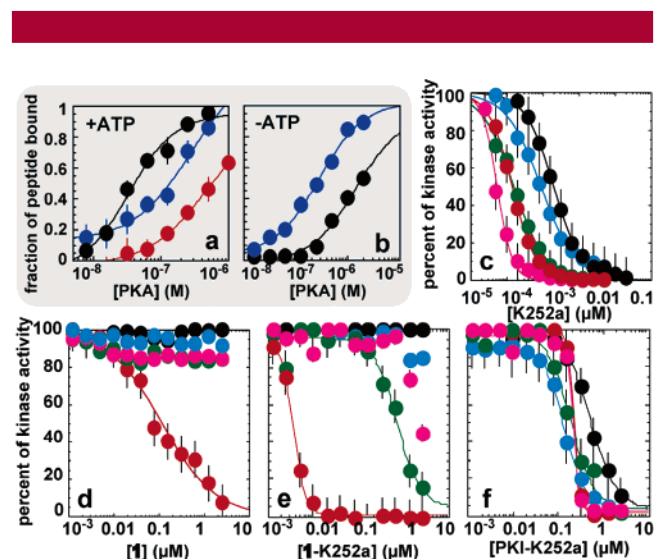
(19) Tapley, P.; Lamballe, F.; Barbacid, M. *Oncogene* **1992**, *7*, 371-381.

(20) Tamaki, K.; Shotwell, J. B.; White, R. D.; Drutu, I.; Petsch, D. T.; Nheu, T. V.; He, H.; Hirokawa, Y.; Maruta, H.; Wood, J. L. *Org. Lett.* **2001**, *3*, 1689-1692.

**B**

the N-terminal  $\alpha$ -helix of PKI<sub>5-24</sub> (residues 5-13) nestles in a shallow hydrophobic groove outside the substrate-binding site of PKA with contacts from F10 and perhaps T6 and Y7 (residues in green in Figure 1B). Located C-terminal to this specificity element is the nonhelical pseudosubstrate region (residues 17-24), which occupies the peptide substrate-binding site of PKA with energetically significant contacts from R18, R19, I22, and R15 (residues in red in Figure 1B). Miniature protein **1** was designed to contain both the PKI<sub>5-24</sub> N-terminal specificity element (Thr at position 25, Tyr at position 26, and Phe at position 30) and the C-terminal pseudosubstrate (Arg at positions 35, 38, and 39 and Ile at position 42), as well as all residues required to maintain the aPP fold. In miniature protein **2**, the specificity and pseudosubstrate elements of PKI<sub>5-24</sub> are located one helical turn closer to the N-terminus compared to their location in miniature protein **1**. Miniature proteins **1** and **2** were synthesized using standard solid-phase methodology; the cysteine residue at the C-terminus of each molecule was modified with 5-iodo-acetamidofluorescein to facilitate fluorescence polarization analysis of PKA affinity.

The relative affinities of **1**<sup>Flu</sup>, **2**<sup>Flu</sup>, and PKI<sub>5-24</sub><sup>Flu</sup> for the catalytic subunit of PKA were measured by fluorescence polarization analysis in the presence and absence of ATP (Figure 2). In the presence of 100  $\mu$ M ATP, the complex



**Figure 2.** Affinity and inhibitory potency of PKA ligands. Fluorescence polarization analysis of the equilibrium affinity of PKI<sup>Flu</sup> (black), **1**<sup>Flu</sup> (blue), and **2**<sup>Flu</sup> (orange) for PKA in the presence (A) and absence (B) of 100  $\mu$ M ATP. Inhibition of the phosphotransferase activity of PKA (red), PKB (black), PKC $\alpha$  (blue), PKG (green), and CamKII (pink) by (C) K252a, (D) **1**, (E) **1-K252a**, and (F) **PKI-K252a**.

between PKA and **1**<sup>Flu</sup> was characterized by an equilibrium

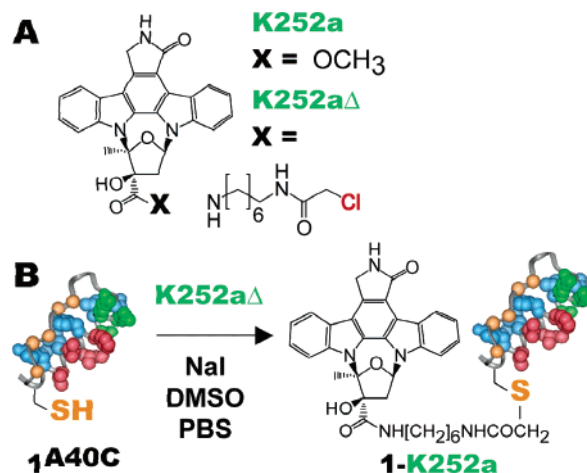
(21) Lashmet, P. R.; Tang, K. C.; Coward, J. K. *Tetrahedron Lett.* **1983**, *24*, 1121-1124.

(22) Medzihradzsky, D.; Chen, S. L.; Kenyon, G. L.; Gibson, B. W. *J. Am. Chem. Soc.* **1994**, *116*, 9413-9419.

(23) Ricouart, A.; Gesquiere, J. C.; Tartar, A.; Sergheraert, C. *J. Med. Chem.* **1991**, *34*, 73-78.

75 dissociation constant ( $K_d$ ) of  $99 \pm 39$  nM. The stability of  
 76  $\text{PKA} \cdot \mathbf{1}^{\text{Flu}}$  was only 3-fold lower than that of  $\text{PKA} \cdot \text{PKI}_{5-24}^{\text{Flu}}$   
 77 under identical conditions ( $K_d = 31 \pm 8$  nM).  $\mathbf{2}^{\text{Flu}}$  bound  
 78 PKA with much lower affinity ( $K_d = 570 \pm 123$  nM),  
 79 perhaps because it lacked R15 and was not considered further  
 80 (Figure 2A). Surprisingly,  $\mathbf{1}^{\text{Flu}}$  retained significant affinity  
 81 for PKA in the absence of ATP ( $K_d = 230 \pm 34$  nM). By  
 82 contrast,  $\text{PKI}_{5-24}^{\text{Flu}}$  bound PKA far more poorly in the  
 83 absence of ATP, as expected,<sup>29</sup> showing a 50-fold decrease  
 84 in affinity ( $K_d = 1.6 \pm 0.4$   $\mu\text{M}$ ) (Figure 2B). Structural and  
 85 biochemical studies have documented the dramatic change  
 86 in PKA conformation induced by the binding of ATP and  
 87 substrate.<sup>30</sup> Whereas the PKA apoenzyme exists in an open  
 88 conformation<sup>31</sup> that maximizes solvent accessibility but binds  
 89 peptide substrate poorly, coordination of ATP rotates the  
 90 large and small enzyme lobes closer together to produce an  
 91 intermediate conformation that is partially preorganized for  
 92 substrate binding.<sup>31,32</sup> Binding of peptide substrate completes  
 93 the conformational change to produce the catalytically active,  
 94 closed conformation seen in several crystal structures.<sup>28,31,33,34</sup>  
 95 Our results suggest that  $\mathbf{1}$  may recognize the open and  
 96 intermediate conformations of PKA with similar affinities  
 97 or, alternatively, that the binding of  $\mathbf{1}$  inhibits the confor-  
 98 mational changes associated with ATP binding.<sup>35</sup>

99 The miniature protein conjugate  $\mathbf{1-K252a}$  was designed  
 100 after examination of the ternary complex of PKA with  
 101  $\text{PKI}_{5-24}$  and the related indolocarbazole natural product  
 102 staurosporine.<sup>36</sup> This examination suggested that an octa-  
 103 methylene chain would appropriately link a C3' amide  
 104 derivative of K252a to the side chain of residue 40 within  
 105  $\mathbf{1}$ . K252a analogues with conservative substitutions at C3'  
 106 retain potency against a range of kinases,<sup>37</sup> suggesting that  
 107 an octamethylene chain at this position would be tolerated.  
 108 Moreover, the  $\text{PKA-PKI}_{5-24}$  structure shows the side chain  
 109 of the corresponding residue of  $\text{PKI}_{5-24}$ , A21, pointing  
 110 directly into the ATP/staurosporine binding pocket.<sup>28</sup> Ac-  
 111 cordingly, we synthesized chloroacetamide K252a $\Delta$  (Figure  
 112 3A) and a derivative of  $\mathbf{1}$  with a cysteine residue in place of



**Figure 3.** (A) Structure of the natural product K252a and K252a $\Delta$ . (B) Reaction of K252a $\Delta$  with  $\mathbf{1}^{\text{A40C}}$  to produce  $\mathbf{1-K252a}$ . Similar conditions were used to produce  $\text{PKI-K252a}$ .

alanine at position 40 (Figure 1B).  $\mathbf{1}^{\text{A40C}}$  was alkylated with  
 K252a $\Delta$  in the presence of NaI, yielding  $\mathbf{1-K252a}$  (Figure  
 3B). K252a $\Delta$  was also used to alkylate  $\text{PKI}^{\text{A21C}}$  to produce  
 $\text{PKI-K252a}$ .<sup>38</sup>

The relative abilities of  $\mathbf{1}$ ,  $\mathbf{1-K252a}$ ,  $\text{PKI-K252a}$ , and  
 K252a itself to inhibit the catalytic activity of PKA were  
 measured using an assay based on streptavidin-matrix capture  
 of biotinylated, [<sup>32</sup>P]-phosphorylated substrates in which ATP  
 and peptide substrate concentrations were fixed below their  
 respective  $K_M$  values.<sup>38,39</sup> As expected, K252a was an  
 excellent PKA inhibitor ( $\text{IC}_{50} = 0.140 \pm 0.003$  nM) (Figure  
 2C) and the inhibitory potency of  $\mathbf{1}$  was similar to its PKA  
 affinity ( $\text{IC}_{50} = 117 \pm 14$  nM) (Figure 2D). The miniature  
 protein conjugate  $\mathbf{1-K252a}$  was 30-fold more active as an  
 inhibitor ( $\text{IC}_{50} = 3.65 \pm 0.13$  nM) than was  $\mathbf{1}$  alone (Figure  
 2E) and only 26-fold less active than K252a itself. Interest-  
 ingly, the analogous molecule  $\text{PKI-K252a}$  was 60-fold less  
 active as an inhibitor of PKA ( $\text{IC}_{50} = 221 \pm 2$  nM) than  
 was  $\mathbf{1-K252a}$  (Figure 2F) and far less active than PKI  
 ( $K_I = 2.3$  nM).<sup>40</sup> Both  $\mathbf{1-K252a}$  and  $\text{PKI-K252a}$  were  
 significantly better inhibitors than were variants of  $\mathbf{1}^{\text{A40C}}$  or  
 $\text{PKI}^{\text{A21C}}$  alkylated with bromoacetamide in place of K252a $\Delta$   
 ( $\text{IC}_{50} > 1$   $\mu\text{M}$ , data not shown). The differential activities  
 of  $\mathbf{1-K252a}$  and  $\text{PKI-K252a}$  may arise from differences in  
 the affinity of  $\mathbf{1}$  and  $\text{PKI}_{5-24}$  for the unique conformation of  
 PKA observed in the ternary complex with  $\text{PKI}_{5-24}$  and  
 staurosporine.<sup>39</sup> Further work will be necessary to character-  
 ize the conformational changes induced in PKA upon the  
 binding of  $\mathbf{1-K252a}$ .<sup>41,42</sup>

To evaluate the extent to which  $\mathbf{1}$  alters the kinase  
 specificity of K252a, the phosphotransferase assay described

(24) Loog, M.; Uri, A.; Raidaru, G.; Järv, J.; Ek, P. *Bio. Med. Chem. Lett.* **1999**, *9*, 1447–1452.

(25) Profit, A. A.; Lee, T. R.; Lawrence, D. S. *J. Am. Chem. Soc.* **1999**, *121*, 280–283.

(26) Parang, K.; Cole, P. A. *Pharmacol. Ther.* **2002**, *93*, 145–157.

(27) Kinase function in vivo can also be probed using unnatural kinase-inhibitor pairs: Bishop, A.; Ubersax, J.; Petsch, D.; Matheos, D.; Gray, N.; Blethrow, J.; Shimizu, E.; Tsien, J.; Schultz, P.; Rose, M.; Wood, J.; Morgan, D.; Shokat, K. *Nature* **2000**, *407*, 395–401.

(28) Zheng, J. H.; Knighton, D. R.; Teneyck, L. F.; Karlsson, R.; Xuong, N. H.; Taylor, S. S.; Sowadski, J. M. *Biochemistry* **1993**, *32*, 2154–2161.

(29) Whitehouse, S.; Walsh, D. A. *J. Biol. Chem.* **1983**, *258*, 3682–3692.

(30) Johnson, D. A.; Akamine, P.; Radzio-Andzelm, E.; Madhusudan, M.; Taylor, S. S. *Chem. Rev.* **2001**, *101*, 2243–70.

(31) Akamine, P.; Madhusudan, W.; Wu, J.; Xuong, N.-H.; Eyck, L. F. T.; Taylor, S. S. *J. Mol. Biol.* **2003**, *327*, 159–171.

(32) Narayana, N.; Cox, S.; Xuong, N.; Ten Eyck, L. F.; Taylor, S. S. *Structure* **1997**, *5*, 921–935.

(33) Bossemeyer, D.; Engh, R. A.; Kinzel, V.; Ponstingl, H.; Huber, R. *Embo J.* **1993**, *12*, 849–859.

(34) Madhusudan, P. A.; Xuong, N. H.; Taylor, S. S. *Nat. Struct. Biol.* **2002**, *9*, 273–277.

(35) Yan, X.; Lawrence, D. S.; Corbin, J. D.; Francis, S. H. *J. Am. Chem. Soc.* **1996**, *118*, 6321–6322.

(36) Prade, L.; Engh, R. A.; Girod, A.; Kinzel, V.; Huber, R.; Bossemeyer, D. *Structure* **1997**, *5*, 1627–1637.

(37) Petsch, D. T. Ph.D. Thesis, Yale University, New Haven, CT, 1999.

(38) Schneider, T. L. Ph.D. Thesis, Yale University, New Haven, CT, 2001.

(39) Goueli, B. S.; Hsiao, K.; Tereba, A.; Goueli, S. A. *Anal. Biochem.* **1995**, *225*, 10–17.

(40) Cheng, H. C.; Kemp, B. E.; Pearson, R. B.; Smith, A. J.; Misconi, L.; Vanpatten, S. M.; Walsh, D. A. *J. Biol. Chem.* **1986**, *261*, 989–992.

(41) Cheng, X.; Shaltiel, S.; Taylor, S. S. *Biochemistry* **1998**, *37*, 14005–14013.

144 above was reconfigured to assay the activities of four distinct  
 145 but related protein kinases. Akt kinase (PKB), protein kinase  
 146 C $\alpha$  (PKC- $\alpha$ ), Ca<sup>++</sup>/calmodulin kinase II (CamKII), and  
 147 cGMP-dependent protein kinase (PKG) are all inhibited by  
 148 K252a<sup>18</sup> (Figure 2C) but not by PKI<sub>5-24</sub>.<sup>40</sup> By contrast, both  
 149 **1** and **1-K252a** showed remarkable specificity for PKA,  
 150 inhibiting no other kinase tested at concentrations as high  
 151 as 100 nM (**1-K252a**) or 5  $\mu$ M (**1**) (Figure 2D,E). The only  
 152 other kinase inhibited by **1-K252a** was PKG (IC<sub>50</sub> = 679  $\pm$   
 153 202 nM), the kinase most similar to PKA.<sup>43</sup> By contrast,  
 154 **PKI-K252a** displayed low specificity, inhibiting all kinases  
 155 tested with IC<sub>50</sub> values within a 4-fold range (Figure 2F). In  
 156 summary, the PKI<sub>5-24</sub> conjugate **PKI-K252a** displayed lower  
 157 activity than K252a and lower specificity than PKI<sub>5-24</sub>,  
 158 whereas the miniature protein conjugate **1-K252a** displayed  
 159 higher specificity than K252a and higher potency than **1**. It  
 160 is possible that the 26-fold lower inhibitory potency of  
 161 **1-K252a** compared to K252a itself could be overcome (and  
 162 perhaps reversed) using in vitro selection methods.<sup>44</sup> Our

(42) Andersen, M. D.; Shaffer, J.; Jennings, P. A.; Adams, J. A. *J. Biol. Chem.* **2001**, *276*, 14204–14211.

(43) Glass, D. B.; Cheng, H.; Kemp, B. E.; Walsh, D. A. *J. Biol. Chem.* **1986**, *261*, 12166–12171.

163 results suggest that molecules such as **1-K252a** that embody  
 164 elements of protein surface recognition, in addition to  
 165 bisubstrate inhibition,<sup>26</sup> could represent a general strategy  
 166 to selectively explore kinase function.<sup>21–26,45–47</sup>

**Acknowledgment.** This work was supported by the NIH  
 (GM 65453 to A.S.), the Yamanouchi Foundation (J.L.W.),  
 and in part by a grant to Yale University, in support of A.S.,  
 from the Howard Hughes Medical Institute.

**Supporting Information Available:** Synthesis and pu-  
 rification of **1**, **2**, PKI<sub>5-24</sub>, **1**<sup>Flu</sup>, **2**<sup>Flu</sup>, **PKI**<sub>5-24</sub><sup>Flu</sup>, **1**<sup>A40C</sup>,  
**PKI**<sub>5-24</sub><sup>A21C</sup>, K252a $\Delta$ , **1**-K252a, and PKI-K252a; analysis  
 of **1**<sup>Flu</sup>, **2**<sup>Flu</sup>, and **PKI**<sub>5-24</sub><sup>Flu</sup> for PKA affinity; inhibition and  
 specificity of **1**, K252a, **1-K252a**, and **PKI-K252**. This  
 material is available free of charge via the Internet at  
<http://pubs.acs.org>.

OL050179O

(44) Li, S.; Roberts, R. W. *Chem. Bio.* **2003**, *10*, 233–239.

(45) Bishop, A. C.; Shah, K.; Liu, Y.; Witucki, L.; Kung, C. Y.; Shokat, K. M. *Curr. Biol.* **1998**, *8*, 257–266.

(46) Shen, K.; Cole, P. A. *J. Am. Chem. Soc.* **2003**, *125*, 16172–16173.

(47) Maly, D. J.; Allen, J. A.; Shokat, K. M. *J. Am. Chem. Soc.* **2004**, *126*, 9106–9161.