Supplementary Material Available: Spectral and other analytical data for **7** and **8** and a listing of spectral data for related compounds (3 pages). Ordering information is given on any current masthead page.

An Affinity Label of Absolute Peptidic Origin

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A large number of peptide-based active site directed irreversible inhibitors (affinity labels) have been reported for enzymes that act upon protein substrates.[1] These inhibitors are an amalgam of two components. The peptidic portion is designed to resemble the substrate and therefore specifically binds to the active site. It serves as the carrier for highly reactive non-proteinoid electrophilic appendages, such as chloromethyl ketones. These electrophilic groups irreversibly modify an active site nucleophile resulting in the concomitant inactivation of the target enzyme. In this communication, we describe a purely peptidic affinity label for the cAMP-dependent protein kinase ("A-kinase"). *In contrast to previously described peptide-based inhibitors, this species contains only functionality present in naturally occurring proteins.*

The A-kinase catalyzes phosphoryl transfer from MgATP to the hydroxyl groups of serine and threonine residues in a vast array of proteins.[2] In addition, a number of peptide-based substrates have been reported for this enzyme, including kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly).[3] The arginine dyad is known to be crucial for substrate recognition and therefore would, by necessity, comprise an essential portion of any active site directed inhibitor of the A-kinase. We synthesized[4] the heptapeptide Leu-Arg-Arg-Cys-Cys-Leu-Gly and subsequently oxidized[5] it to the intramolecular disulfide analogue, Leu-Arg-Arg-**Cys⇔Cys**-Leu-Gly (**1**)[6] (where Cys⇔Cys represents a Cys-Cys dyad connected via both a peptide and disulfide bond). The intramolecular disulfide is a potent electrophile, resulting in the rapid inactivation of the cAMP-dependent protein kinase.[7]

Incubation of the A-kinase[4] with Leu-Arg-Arg-Cys⇔Cys-Leu-Gly under standard conditions[8] resulted in a time-dependent

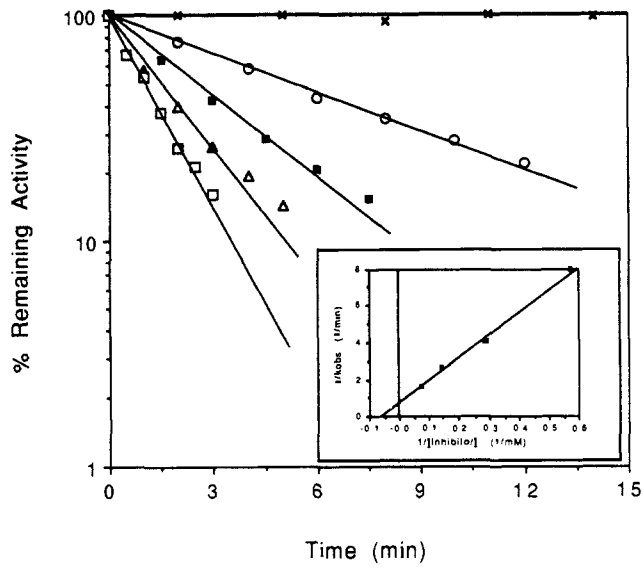
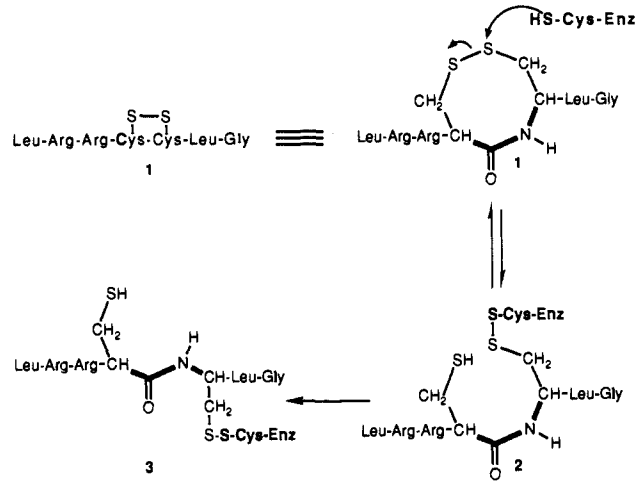


Figure 1. Time-dependent inactivation of the cAMP-dependent protein kinase by **1** at the following concentrations: 14 mM (□), 7 mM (△), 3.5 mM (■), 1.75 mM (○), and 0 mM (×). A double reciprocal plot of rate constant versus inhibitor concentration yields K_i = 17.7 ± 0.9 mM and k_2 = 1.42 ± 0.1 min^{-1}.

Scheme I



pseudo-first-order inactivation of kinase activity (Figure 1). Saturation kinetics is observed, suggesting that the affinity label is active site directed. The double reciprocal plot of $1/k_{obs}$ versus $1/[I]$ (Figure 1, inset) yields a K_i = 17.7 ± 0.9 mM and a k_2 = 1.42 ± 0.1 min^{-1} (unimolecular rate constant for modification). Modification is covalent, since dialysis of the inactivated enzyme against buffer did not restore activity. However, treatment of the covalently modified enzyme with dithiothreitol did reestablish its ability to catalyze phosphoryl transfer, suggesting that it is an active site cysteine that has undergone modification (the A-kinase contains a cysteine residue in the active site[9,10]). In addition, MgATP (150 μM) completely protected the enzyme against inactivation by the affinity label.[11] It has been previously proposed

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(1) *Methods Enzymol.* **1977**, *46*.

(2) Carlson, G. M.; Bechtel, P. J.; Graves, D. J. *Adv. Enzymol.* **1979**, *50*, 41.

(3) Bramson, H. N.; Kaiser, E. T.; Mildvan, A. S. *CRC Crit. Rev. Biochem.* **1983**, *15*, 93.

(4) The peptides were synthesized and the cAMP-dependent protein kinase was purified as previously described: Prorok, M.; Lawrence, D. S. *J. Biochem. Biophys. Methods* **1989**, *18*, 167.

(5) Zhang, R.; Snyder, G. H. *J. Biol. Chem.* **1989**, *264*, 18472.

(6) The structure **1** was confirmed by fast atom bombardment mass spectrometry and two-dimensional correlated and nuclear Overhauser enhancement NMR spectroscopies.

(7) Peptide-based enzyme inhibitors containing disulfide groups composed of nonpeptidic functionality have been described. For example, see: (a) Bramson, H. N.; Thomas, N.; Matsueda, R.; Nelson, N. C.; Taylor, S. S.; Kaiser, E. T. *J. Biol. Chem.* **1982**, *257*, 10575. (b) Evans, B.; Shaw, E. *J. Biol. Chem.* **1983**, *257*, 10227.

(8) Inactivation reactions (total volume 60 μL, pH 7.1, 150 mM KCl, 100 mM MOPS, and 0.125 mg/mL BSA) were carried out by incubating the A-kinase (100 nM) with peptide **1** (0–14 mM) at 6 °C. At selected times, aliquots were removed and diluted 20-, 25-, or 30-fold into an ice-cold assay mixture containing 100 mM MOPS, 150 μM [γ-^{32}P]ATP (200 cpm/pmol), 12.5 mM $MgCl_2$, 150 mM KCl, and 0.125 mg/mL BSA (total volume 100 μL, pH 7.1). No further inactivation occurred after dilution of the enzyme-inactivator solution. After incubation at 30 °C, the kinase reactions were initiated by addition of kemptide to a final concentration of 50 μM. After 1.5 min, the assays were quenched by spotting 25-μL aliquots onto phosphocellulose paper followed quickly by immersion into 10% acetic acid. After exhaustive washings with 5 mM H_3PO_4, the disks were dried and scintillation counted for radioactivity.

(9) First, E. A.; Johnson, D. A.; Taylor, S. S. *Biochemistry* **1989**, *28*, 3606 and references cited therein.

(10) The A-kinase contains two cysteine residues, one of which is in the active site. We have found that [1-^{14}C]Ac-Leu-Arg-Arg-Cys⇔Cys-Leu-Gly labels the enzyme only once (1.02 ± 0.04 equiv of label/mol of enzyme; performed in triplicate). Modification results in complete enzymatic inactivation. In marked contrast, a nonselective reagent, such as Ellman's reagent, labels both cysteine residues. See: Armstrong, R. N.; Kaiser, E. T. *Biochemistry* **1978**, *17*, 2840.

(11) Kemptide (5.0 mM), in the absence of ATP, confers approximately 65% protection against inactivation.

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that the γ-phosphate of ATP is positioned within the vicinity of the active site cysteine.[12]

The high K_i (17.7 mM) for **1** is somewhat surprising, especially since even the simple dipeptide Arg-Arg exhibits a K_i of 3.8 mM as a reversible inhibitor of the A-kinase.[13] It occurred to us that the disulfide-containing peptide might be obliged to bind in a less than optimal fashion in order to modify the active site cysteine residue.[14] This notion is consistent with our observation that the K_i obtained for peptide **1** from inactivation kinetics (17.7 mM) is larger than that acquired from competitive inhibition kinetics (970 ± 26 μM).[15] The latter value is reasonable for peptides of this size.[13,16] These results suggest that more potent inhibitors may be accessible via positional isomers of Leu-Arg-Arg-Cys⇔Cys-Leu-Gly. These experiments are currently in progress.

One possible pathway for inactivation is depicted in Scheme I. Theoretical studies have predicted that the peptide bond within a Cys⇔Cys dyad should occupy the cis configuration.[17,18] We note that for peptide bonds in general, the trans arrangement is the preferred structure. In species **2**,[19] the peptide bond is now free to isomerize to the trans isomer **3**. The release of the strain inherent in a Cys⇔Cys dyad may very well explain why disulfide exchange back to the unmodified A-kinase and Leu-Arg-Arg-Cys⇔Cys-Leu-Gly is not observed.

In summary, we have described the inhibitory activity of a disulfide-containing peptide. This prototype of a potentially valuable class of affinity labels contains several attractive features. First, the preparation of the intramolecular disulfide bond is straightforward since oxidation is readily accomplished in aqueous solution. Second, the synthesis of this class of affinity labels does not require modification of a peptide by some external reagent. Therefore, even extremely long peptides, containing the natural array of nucleophilic side chains, can be converted into reagents that will covalently modify the appropriate target enzyme. Third, this class of affinity labels contains only functional groups that are present in proteins. Consequently, this suggests that it may be possible to construct *protein*-based affinity labels via site-directed mutagenesis. We note that modification is most likely specific for sulfhydryl groups. The active site cysteine-199 in the A-kinase is conserved in other protein kinases as well, including other members of the cyclic nucleotide-dependent subfamily (e.g. cGMP dependent), the calcium/phospholipid-dependent subfamily (i.e. protein kinase C), and the calcium/calmodulin-dependent subfamily (e.g. human HeLa cell serine kinase).[20] In addition, other enzymes that act upon protein substrates, such as cathepsin B (a cysteine proteinase),[21] cyclophilin (a peptidyl-prolyl-cis-trans isomerase),[22] and Factor XIIIa (a transglutaminase),[23] appear to be potential targets as well.

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Supplementary Material Available: A Lineweaver-Burk plot of **1** as a competitive inhibitor versus [kemptide] and a replot of the slopes of the reciprocal plot versus [**1**] (2 pages). Ordering information is given on any current masthead page.

(12) Jimenez, J. S.; Kupfer, A.; Gani, V.; Shaltiel, S. *Biochemistry* **1982**, *21*, 1623.
(13) Feramisco, J. R.; Krebs, E. G. *J. Biol. Chem.* **1978**, *253*, 8968.
(14) Plapp, B. V. *Methods Enzymol.* **1982**, *87*, 469.
(15) Covalent modification of the enzyme is precluded under the assay conditions employed to assess the K_i of **1** via competitive inhibition kinetics (i.e. in the presence of MgATP): Assays contained 100 mM MOPS, 150 μM [γ-^{32}P]ATP (200 cpm/pmol), 12.5 mM $MgCl_2$, 150 mM KCl, 0.125 mg/mL BSA, kemptide (10-40 μM), and peptide **1** (0-1200 μM) in a total volume of 80 μL at pH 7.1 and 30 °C. Kinase reactions were initiated by addition of the A-kinase to a final concentration of 5 nM. Reactions were terminated as described in ref 8.
(16) It should be possible to enhance the affinity of this peptide for the enzyme-active site by addition of appropriate amino acids on the N-terminus. See: Glass, D. B.; Cheng, G.-H.; Mende-Mueller, L.; Reed, J.; Walsh, D. A. *J. Biol. Chem.* **1989**, *264*, 8802.
(17) Chandrasekaran, R.; Balasubramanian, R. *Biochem. Biophys. Res. Commun.* **1968**, *188*, 1.
(18) We have recently determined that compound **1** exists as both the cis and trans isomers in solution (based on NOESY NMR experiments, Sukumaran, D. K.; Prorok, M.; Lawrence, D. S., manuscript submitted for publication). The trans isomer constitutes a sizable percentage (35%) of the total cis/trans population. Consequently, it appears likely that this species also contributes to the inactivation of the A-kinase.
(19) Compound **2** is the result of only one of two possible modes of nucleophilic attack. Experiments are in progress to determine the relative ratios of the two potential products.
(20) (a) Hanks, S. K. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 388. (b) Hanks, S. K.; Quinn, A. M.; Hunter, T. *Science* **1988**, *241*, 42.
(21) Rich, D. H. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, Eds.; Elsevier: New York, 1986; p 153.
(22) Although the active site cysteine in cyclophilin does not appear to be essential for catalysis, modification of this residue does result in the elimination of enzymatic activity. See: (a) Fisher, G.; Wittman-Liebold, B.; Lang, K.; Kiefhaber, T.; Schmid, F. X. *Nature* **1989**, *337*, 476. (b) Harrison, R. K.; Stein, R. L. *Biochemistry* **1990**, *29*, 1684. (c) Liu, J.; Albers, M. W.; Chen, C.-M.; Schreiber, S. L.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2304.
(23) Folk, J. E. *Adv. Enzymol.* **1983**, *54*, 1.

Synthesis and X-ray Crystal Structure of a Highly Strained Anti-Bredt Olefin/Anti-Bredt Lactam. *exo*-2-Carbomethoxy-1-aza-8-oxobicyclo[3.3.1]non-4-ene

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Bridgehead double bonds experience topologic constraints that can result in nonplanar olefin geometries.[1] Such distortions can also profoundly influence chemical reactivity. These anti-Bredt olefins may be isolated only when the trans double bond is contained in a ring of eight or more atoms. We report the synthesis and X-ray crystal structure of *exo*-2-carbomethoxy-1-aza-8-oxobicyclo[3.3.1]non-4-ene (**1**), a molecule that contains both a bridgehead olefin and a bridgehead amide in an eight-membered ring. *This is the first X-ray structure of a bridgehead olefin containing a trans-cyclooctene ring,*[2] *and it represents one of the most highly strained isolable anti-Bredt molecules prepared to date.*

Anti-Bredt olefin **1** was prepared by a type 2 intramolecular Diels-Alder cycloaddition.[3] Condensation of diene amide **2** with methyl glyoxalate followed by acetylation of the resulting methylol according to the procedure of Weinreb[4] afforded diene acetate **3**, the Diels-Alder precursor (Scheme I). In situ thermolytic elimination of acetic acid generated the *N*-acylimine intermediate **4**, which cyclized to give **1**. The cycloadduct was produced in optimum yield (35%) by heating dilute solutions of **3** (0.01 M,

(1) Bridgehead olefin reviews: (a) Warner, P. M. *Chem. Rev.* **1989**, *89*, 1067-1093. (b) Wentrup, C. *Reactive Molecules*; John Wiley and Sons: New York, 1984; pp 265-308. (c) Szeimies, G. In *Reactive Intermediates*; Abramovitch, R. A., Ed; Plenum: New York, 1983; Vol. 3, pp 299-366. (d) Shea, K. J. *Tetrahedron* **1980**, *36*, 1683-1715.
(2) For several recent examples of X-ray crystal structures of transition-metal complexes of strained olefins see: (a) Bly, R. S.; Hossain, M. M.; Lebioda, L. *J. Am. Chem. Soc.* **1985**, *107*, 5549-5550. (b) Kumar, A.; Lichtenhan, J. D.; Critchlow, S. C.; Eichinger, B. E.; Borden, W. T. *J. Am. Chem. Soc.* **1990**, *112*, 5633-5634. (c) For the X-ray crystal structure of a 1-azabicyclo[3.3.1]nonan-2-one derivative see: Buchanan, G. L.; Kitson, D. H.; Mallinson, P. R.; Sim, G. A.; White, D. N. J.; Cox, P. J. *J. Chem. Soc., Perkin Trans. II* **1983**, 1709-1712.
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