will describe the generalization of this scheme to other enones and the synthetic utilization of the strained products taking advantage of the great versatility of the phenylthio group.

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Supplementary Material Available: Experimental procedures and spectral data for **1** (R = H and R = CH_3), **4**, **4d**, and **6–9** (3 pages). Ordering information is given on any current masthead page.

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Stereochemical Course of the Phospho Group Transfer Catalyzed by cAMP-Dependent Protein Kinase

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Protein phosphorylation by cyclic 3′,5′-adenosine monophosphate (cAMP)-dependent protein kinase (E.C. 2.7.1.37) is the major pathway by which cAMP regulates cellular metabolism. The activation of the enzyme by cAMP is known to occur in a cAMP-promoted dissociation of the holoenzyme into two catalytic subunits and a dimer of regulatory subunits.[1] The catalytic subunit catalyzes the transfer of a phospho group from adenosine triphosphate (ATP) to serine or threonine residues of appropriate peptide and protein substrates.

The mechanism of action of cAMP-dependent protein kinase has been extensively studied and reviewed.[2] Two mechanistic pathways have been considered: (i) a double displacement mechanism involving a phospho-enzyme intermediate and (ii) a single displacement mechanism in which the phospho group is transferred directly between bound substrates. The available experimental data do not, however, distinguish unambiguously between these mechanisms. Thus the presence of a low ATPase activity in cAMP-dependent protein kinase[3] and the relatively long distance between the γ-phosphorus of an enzyme-bound ATP analogue and the hydroxyl oxygen of a bound acceptor peptide substrate, estimated by NMR,[4] are both consistent with a mechanism involving a phospho-enzyme intermediate. Yet no direct evidence for such a phospho-enzyme has ever been obtained. Moreover, the steady-state kinetic behavior of the enzyme,[2,5] the apparent lack of an ATP/ADP exchange reaction in the absence of an acceptor substrate,[6] and the failure of the enzyme to catalyze positional isotope exchange of labeled ATP[7] are all consistent with a direct transfer pathway.

To clarify the reaction mechanism of the cAMP-dependent protein kinase, we have determined the stereochemical course of the phospho group transfer reaction by the use of chiral [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP.[8] For a double displacement mechanism,

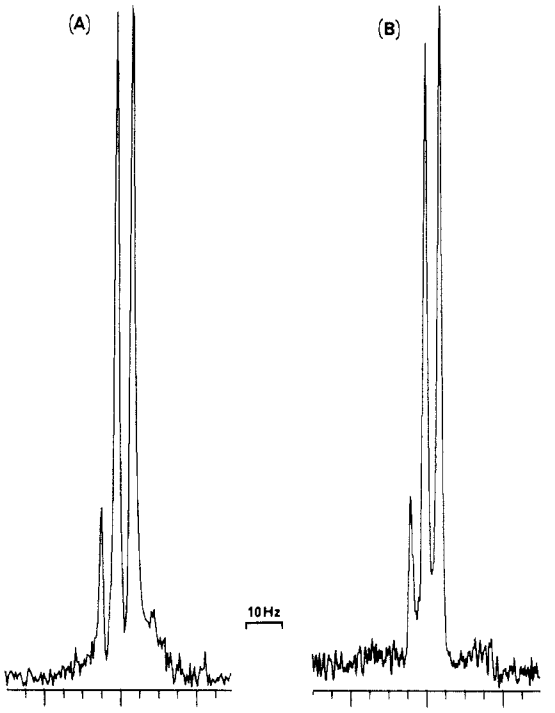

Figure 1. ^{31}P NMR spectra of (A) *O*-[^{16}O,^{17}O,^{18}O]phosphoheptapeptide (180 mg/2.5 mL) and (B) *O*-[^{16}O,^{17}O,^{18}O]phosphotetrapeptide (35 mg/2.5 mL) in D_2O. The spectra shown were recorded on a Nicolet 360 instrument at 146.1 MHz in a bilevel broad band decoupling mode. The spectrum of unreacted phosphotetrapeptide recovered after incubation with alkaline phosphatase was essentially unchanged.

overall retention of the configuration at phosphorus is expected, while the single displacement pathway is predicted to result in inversion of the configuration at phosphorus.[9] We chose the most efficient acceptor peptide substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly,[10] and used the purified catalytic subunit of the protein kinase in the presence of ATP chiral at the γ-phospho group by virtue of the three stable isotopes of oxygen.[11,12] The configuration of the resulting [^{16}O,^{17}O,^{18}O]phosphopeptide was then to be established by the transfer of the chiral phospho group to (*S*)-butane-1,3-diol catalyzed by *E. coli* alkaline phophatase, followed by the determination of the absolute configuration at phosphorus.[8] We found, however, that the phosphoheptapeptide is an unsuitable substrate[13] for alkaline phosphatase in the transfer reaction. Indeed, it is known that although cationic amino alcohols such as ethanolamine or tris(hydroxymethyl)aminomethane are good phosphate acceptors, their *O*-phosphate esters are relatively poor substrates for alkaline phosphatase.[14] We suspected, therefore, that the two guanidinium groups of the heptapeptide could be the reason why the phosphoheptapeptide is such a poor phospho group donor in this reaction.

We therefore synthesized the truncated phosphotetrapeptide, Ala-Ser-[OP(O)(OH)$_2$]Leu-Gly,[15] and found it to be a much

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(10) Kemp, B. E.; Graves, D. J.; Benjamini, E.; Krebs, E. G. *J. Biol. Chem.* **1977**, *252*, 4888–4893. The heptapeptide was prepared either by the solid-phase method on an oxime resin or in solution as Cbz-Leu-Arg-(NO$_2$)-Arg-(NO$_2$)-Ala-Ser(OBn)-Leu-Gly-OBn which was then hydrogenolyzed in acetic acid with Pd/C as catalyst.
(11) Blättler, W. A.; Knowles, J. R. *Biochemistry* **1979**, *18*, 3927–3933.
(12) The reaction mixture containing 3.2 mM peptide, 12.5 mM chiral ATP, 10 mM $MgCl_2$, and 250 nM enzyme was allowed to stand overnight at room temperature.
(13) The progress of hydrolysis was monitored by ^{31}P NMR. The reaction became sluggish after 10% of the substrate was consumed. The release of additional P_i was detected only immediately after addition of fresh enzyme. HPLC studies showed that at pH values above 10, elimination of P_i occurs.
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(15) Phosphotetrapeptide was synthesized in solution as *t*-Boc-Ala-Ser-[OP(O)(OPh)$_2$]-Leu-Gly-OBn, which was hydrogenolyzed in trifluoroacetic acid with PtO_2 as catalyst.

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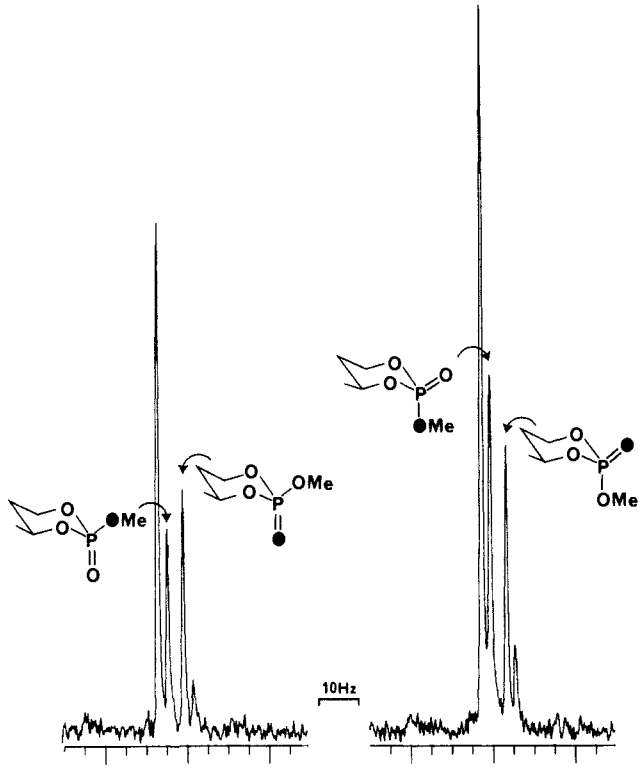

Figure 2. ^{31}P NMR spectrum of the products from the "in-line" ring closure and methylation of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-butane-1,3-diol obtained from *E. coli* alkaline phosphatase catalyzed phospho group transfer with *O*-[^{16}O,^{17}O,^{18}O]phosphotetrapeptide as the phospho group donor. ● represents ^{18}O. The spectrum was taken in DMSO-d_6 and processed with Gaussian broadening of 0.3 Hz and line broadening of −0.4 Hz. The downfield multiplet corresponds to the syn isomers, and the upfield multiplet corresponds to the anti isomers. The isotopically labeled species that provide stereochemical information are marked. The downfield signal in each quartet is from the unlabeled triester and the upfield signal in each quartet is from the $^{18}O_2$ triester.

better substrate than the phosphoheptapeptide for alkaline phosphatase in the presence of butane-1,3-diol.[16,17] The phosphoheptapeptide was therefore exhaustively digested with trypsin to yield the labeled [^{16}O,^{17}O,^{18}O]phosphotetrapeptide in 82% yield.[18] No isotopic label loss occurred during this treatment, as shown by the ^{31}P NMR spectra of the two phosphopeptides (see Figure 1). The labeled phosphotetrapeptide was then subjected to stereochemical analysis[16,17] with (*S*)-butane-1,3-diol as acceptor. The recovered phosphotetrapeptide[19] was then resubjected to the transfer reaction, and the combined phosphobutanediol product,[19] as the bis(tri-*n*-octylammonium) salt, was used in the ring closure and methylation sequence previously described.[8,17]

The ^{31}P NMR spectra of the cyclic triester deriving from the phosphobutanediol is shown in Figure 2. The relative intensities of the stereochemically informative resonances (the middle pair of each quartet) clearly show that the phosphorus of the phosphobutanediol is predominantly *R*. Since alkaline phosphatase catalyzes phospho group transfer with overall retention,[8] the phosphorus of the labeled phosphotetrapeptide is also predominantly *R*. This establishes that the phospho group transfer by cAMP-dependent protein kinase proceeds with *inversion* of the configuration at phosphorus.

It is evident from Figures 1 and 2 that considerable loss of isotopic label occurs in the phospho group transfer reaction and/or in the subsequent steps of the stereochemical analysis. This loss makes quantitation of the enantiomeric excess at phosphorus somewhat unreliable. Since earlier work has demonstrated that little label loss occurs during the ring closure and methylation of phosphobutanediol,[8,17] the observed label washout most likely occurs during the alkaline phosphatase catalyzed phospho group transfer. Although the rate of inorganic phosphate turnover (determined by ^{18}O exchange between H_2O and [^{18}O]PO_4^{3-}) is much slower than the hydrolysis of most phosphoester substrates at high pH,[20] the rate of phosphotetrapeptide hydrolysis is at least 10 times slower at pH 10, and 65 times slower under the phospho group transfer conditions[8,16] in the presence of butane-1,3-diol, than the rate of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase.[21] It therefore seems likely that when a relatively poor substrate such as the phosphopeptide is used, isotopic washout from enzyme-bound phospho groups can become significant.

The stereochemical course of phospho group transfer by cAMP-dependent protein kinase elucidated here eliminates the possibility of a double displacement mechanism. We can conclude that cAMP protein kinase catalyzes the phosphorylation of substrate heptapeptide with inversion of the configuration, which is consistent with a single displacement pathway for this enzyme.

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(16) The phosphopeptide (500–600 μmol) was dissolved in D_2O (4 mL) and titrated with solid K_2CO_3 to pH 9.0. This solution was then mixed with a solution (50 μL) containing 0.3 M $Mg(OAc)_2$ and 3 mM $Zn(OAc)_2$ and (*S*)-butane-1,3-diol (3 mL), before addition of *E. coli* alkaline phosphatase (80 units).

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(18) Phosphoheptapeptide was incubated in 50 mN $NaHCO_3$ buffer (10 mL), pH 8.2, containing trypsin (30 mg) at room temperature for 24 h.

(19) Phosphobutanediol and inorganic phosphate were eluted from Dowex-1 by a gradient of triethylammonium bicarbonate buffer.[17] Phosphotetrapeptide was eluted by a gradient of NaCl solution (0–1 M) and was then desalted on a Sephadex G-15 column. The yield of 1-phospho-(*S*)-butane-1,3-diol was about 8%, after 40% of the phosphotetrapeptide has been consumed. The amount of combined 1-phospho-(*S*)-butane-1,3-diol (60 μmol estimated) was determined by a spectrophotometric method (Ames, B. *Methods Enzymol.* **1966**, *8*, 115–118).

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The Experimental Heats of Formation and Kinetics of 1,3-Biradicals Using Time-Resolved Photoacoustic Calorimetry

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The enthalpic criterion for distinguishing between concerted and nonconcerted thermal rearrangements usually involves a comparison between two enthalpies of activation, one experimental and the other hypothetical, attributable to a nonconcerted model.[1] Commonly, models involving two noninteracting free radicals (biradicals) are discussed, and the knowledge of their enthalpies of formation details their role on the reaction pathway. Unfortunately, only estimates of these enthalpies are currently available.[2]

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