TLC) with the authentic sample[11] derived from natural gephyrotoxin.

Having confirmed the stereochemical assignment, all that remained was the introduction of the *cis*-enyne unit. The alcohol **16** was oxidized to the aldehyde **18**[5b] (PCC/CH_2Cl_2/room temperature; NMR ($CDCl_3$) δ 9.69 (1 H, br s), 3.62 (2 H br t, J = 6.0 Hz)). Wittig reaction of **18** ($EtOCH{=}CHP^+(C_6H_5)_3Br^-$/NaOEt/room temperature)[12] followed by acid hydrolysis (*p*-TSA/acetone/H_2O/0 °C) yielded the unstable cis-unsaturated aldehyde **19**[5b,13] (NMR ($CDCl_3$) δ 10.02 (1 H, d, J = 8.0 Hz), 6.56 (1 H, m), 6.02 (1 H, dd, J = 10.0, 8.0 Hz), 3.61 (2 H, t, J = 6.0 Hz)). The Corey method to convert *cis*-enals to *cis*-enynes [(1) $ClCH_2P^+(C_6H_5)_3Cl^-$/BuLi/THF; (2) MeLi/THF, Me_3SiCl; (3) $Bu_4N^+F^-$/DMF][14] was applied to **19** to give synthetic (±)-gephyrotoxin (**1**) in about 45% overall yield from **16**. The synthetic substance was found to be identical with natural gephyrotoxin[13,15] by comparison of ^1H NMR (C_6D_6) and mass spectra as well as TLC behavior (Merck Al_2O_3 (1:4 acetone–hexane); Merck silica gel (22:1:0.15 chloroform–2-propanol–aqueous ammonia)).

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Supplementary Material Available: Spectra (NMR, IR, MS) of new compounds described in this paper (33 pages). Ordering information is given on any current masthead page.

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Development of a Convenient Spectrophotometric Assay for Peptide Phosphorylation Catalyzed by Adenosine 3′,5′-Monophosphate Dependent Protein Kinase

Sir:

Bovine cardiac muscle adenosine 3′,5′-monophosphate dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) catalyzes the transfer of the terminal phosphoryl group from adenosine 5′-triphosphate to the serine hydroxyl of peptide or protein substrates.[1] Currently, protein kinase is assayed by using adenosine 5′-[γ-^{32}P]triphosphate and quantitating the transfer of the labeled phosphoryl residue to a peptide or protein substrate. This method is inconvenient since (1) it does not permit continuous assay and requires removal of unreacted nucleotide before the amount of radioactive material transferred is determined for each time point; (2) adenosine 5′-[γ-^{32}P]triphosphate is expensive and has a limited shelf life; (3) the use of radioactive materials requires special handling. If a reactive peptide substrate for protein kinase could be prepared which would undergo a significant spectral change upon phosphorylation, kinetic and mechanistic studies of the enzyme would be greatly facilitated. In the present report we wish to describe our finding that phosphorylation of the Ser residue in the reporter group labeled heptapeptide Leu-Arg-Arg-(*o*-NO_2)Tyr-Ser-Leu-Gly (**1**) catalyzed by the catalytic subunit of protein kinase at pH 7.5 causes a spectral change at 430 nm which permits us to monitor this reaction continuously.

The synthesis of peptide **1** was carried out by the solid-phase method employing polystyrene-bound *p*-nitrobenzophenone oxime as the support.[2] Starting with BocLeu resin (substitution level 0.5 mmol/g), the symmetric anhydrides of BocSer(Bzl), Boc(*o*-NO_2)Tyr(Bzl),[3] BocN^gTosArg, and BocLeu were coupled in threefold excess, resulting in BocLeu-(Tos)Arg-(Tos)Arg-(*o*-NO_2)Tyr(Bzl)-Ser(Bzl)-Leu-oxime polymer. The fully protected heptapeptide was obtained by using GlyOBzl·HOAc to displace hexapeptide from the oxime resin and was purified by chromatography on LH 20. Removal of all protecting groups by treatment with HF[4] followed by chromatography on Sephadex G-15 and CM Sephadex C-25 gave peptide **1** in 28% yield, based on the initial substitution level on the resin. The amino acid analysis (Arg (2.0), Gly (1.0), Leu (2.0), Ser (0.91), and (*o*-NO_2)Tyr (1.0)), the 270-MHz NMR spectrum, and the UV-visible spectrum of peptide **1** were consistent with the structure postulated. No impurities were detected by thin-layer chromatography.

The protein kinase catalyzed phosphorylation of peptide **1** was carried out at 30.0 °C in 50 mM Tris buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.15 M KCl, 0.2 mM dithiothreitol, and 0.2 mg/mL bovine serum albumin. Typically, 6.54 nM catalytic subunit, 2.00 mM ATP, and between 25 and 200 μM peptide **1** were employed in the spectrophotometric assays which were performed by using a Cary 219 spectrophotometer. A decrease in ϵ_{430} of 210 M^{-1} was measured when peptide **1** was phosphorylated, and the entire time course of reaction was monitored in the spectrophotometric experiments. To check the validity of the spectrophotometric assays, rates for the transfer of the γ-phosphoryl group from adenosine 5′-[γ-^{32}P]triphosphate to peptide **1** were measured as described previously,[5] except that the entire course was observed. The kinetics of peptide phosphorylation catalyzed by bovine cardiac muscle catalytic subunit have been shown to be consistent with the sequential mechanism[6] illustrated in eq 1.[7] Since the phosphopeptide product is not inhibitory and

$$\begin{aligned} E + MgATP \rightleftharpoons E{\cdot}MgATP + peptide \rightleftharpoons \\ E{\cdot}MgATP{\cdot}peptide \rightarrow E{\cdot}MgADP + phosphopeptide \rightleftharpoons \\ E + MgADP \end{aligned} \quad (1)$$

$$v = \frac{k_{cat}[E]_0[peptide]}{[peptide] + K_{m,peptide}} \quad (2)$$

MgATP is present in large excess, the kinetics of the phosphorylation of peptide **1** measured under the conditions of our experiments can be analyzed by using eq 2. For those reactions where the entire time course was monitored, values for k_{cat} and $K_{m,peptide}$ were obtained by using an iterative curve fitting program (by B. Blumenstein of Emory University) for an IBM 370 com-

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puter. We found that $K_{m,peptide} = (40 \pm 10) \times 10^{-6}$ M, as measured spectrophotometrically, and $(30 \pm 10) \times 10^{-6}$ M, as determined from rate measurements employing adenosine 5′-[γ-^{32}P]triphosphate, while a value of $k_{cat} = 3000 \pm 200$ min^{-1} was obtained in both types of experiments. For analytical purposes, peptide **1** was enzymatically phosphorylated on a preparative scale by a procedure similar to that described elsewhere for Leu-Arg-Arg-Ala-Ser-Leu-Gly.[8] The final phosphorylated product gave an amino acid analysis of Arg (2.0), Gly (1.0), Leu (2.0), Ser (0.88), and (o-NO_2)Tyr (1.0) on acid hydrolysis. The phosphate content was determined to be 1.10 mol/mol of peptide.[9]

In a recent paper we reported that the bovine cardiac muscle protein kinase shows a marked preference for the Mg(II) complex of the A isomer of ATPβS.[7] In order to monitor the transfer of the γ-phosphoryl group to the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly, it was necessary to prepare each of the diastereomers of ATPβS in a radioactively labeled form (γ-^{32}P label).[10] Now that we have in hand a peptide substrate, **1**, which undergoes a significant spectral change on phosphorylation, it is possible to study the stereochemical preference of protein kinase for metal–nucleotide complexes without having to resort to radioactive labeling of the nucleotide. For example, a brief investigation of the action of protein kinase on the A and B isomers of ATPαS was undertaken by using **1** as the peptide substrate.[11] Under conditions identical with those given above for the reaction with ATP, with **1** at a concentration of 100 μM, and ATPαS B at a concentration of 2 mM, the phosphorylation reaction proceeded to completion at 13% of the rate observed at [ATP] = 2 mM. When ATPαS A isomer (2 mM) was employed, however, only 15% of the peptide appeared to be phosphorylated and the rate of the reaction observed was 3% of that seen for ATP under comparable conditions. We believe that this result is due to contamination of ATPαS A with a very small amount (~0.75%) of another nucleotide, possible ATP. Indeed, when an amount of ATP corresponding to the putative contaminant was added to the solution of ATPαS A, phosphorylation of 30% of the peptide was observed to occur, proceeding at a rate which was 4% of that seen with 2 mM ATP. In line with these experiments, when the concentration of ATPαS A was reduced to 50 μM, no phosphorylation of the peptide was detected spectrophotometrically. At a concentration of 50 μM, the ATPαS B isomer reacted at a rate at least 300 times greater than the minimum we would have detected. Thus, our results demonstrate that protein kinase has a definite preference for the Mg(II) complex of the B isomer of ATPαS. While the Mg(II) ion presumably is coordinated to the β,γ-positions of the triphosphate moiety of ATP when the metal–nucleotide complex is bound to the enzyme, our findings may indicate that coordination of the metal ion to the α position is important in the transition state for γ-phosphoryl transfer reactions catalyzed by protein kinase, but additional studies with other metal ions will be needed to test this possibility. Further studies on the metal ion dependence and stereochemical preferences of the catalytic subunit of the bovine cardiac muscle enzyme are currently under way with peptide **1** as the substrate.

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Biomimetic 8α Functionalization of Riboflavin

Sir:

Over the last 20 years flavins bound covalently to flavoenzymes[1] have been isolated from sources ranging from mammalian brain tissue to soil bacteria. Generally, the linkage is through the 8α-methyl group of the isoalloxazine nucleus (**1**) to an imidazole nitrogen of histidine[2] or sulfur of cysteine.[3] A recent hypothesis forwarded by Walsh[4] predicts the genesis of the 8α-peptidyl flavins via the quinone methide tautomer (see **2**) of riboflavin (see tetraisobutyryl derivative **1**, Scheme I). Herein we report model studies which establish chemical precedent for facile tautomerization of flavins and interception of the resulting quinone methide, **2**, by nucleophiles.

During the course of our investigations of flavin monooxygenase activity,[5] we discovered that certain cyclic tertiary amine *N*-oxides, **6a–c** (Table I), catalyze the conversion of tetraisobutyrylriboflavin (**1**) into a brilliant red, crystalline dimer, **4a**[6] (field-desorption MS, M^+ *m/e* 1311). This conversion is also effected by the tertiary amine base 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU, **7**). Potassium phenolates **8a,b** (Table I) convert **1** into a mixture of tautomeric red and orange dimers, **4a** and **5a**,[7,8] respectively. Dimer **4a** upon hydrolysis of the isobutyryl esters is converted into **4b**, previously reported by Hemmerich.[9a,10] In contrast to the

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(6) Data for **4a**: ^{1}H NMR (60 MHz, dimethyl-d_6 sulfoxide) (Me_4Si) δ 0.6–1.2 (m, 48 H), 1.9–2.9 (m, 8 H, partially obscured by solvent), 2.7 (s, 6 H), 3.3 (s, exchangeable H plus H_2O), 4.0–5.6 (br m, 14 H), 7.9 (s, 2 H), 8.1 (s, 2 H), 8.3 (s, 2 H), 11.6 (s, 2 H); ^{13}C NMR (15 MHz, dimethyl-d_6 sulfoxide) (Me_4Si) δ 174.9–175.5 (multiple lines), 170.1, 159.5, 155.0, 150.8, 142.4, 137.7, 135.1, 134.8, 131.4, 112.7 (low intensity, two lines?), 69.4 (multiple lines), 61.6 (multiple lines), 33.2 (multiple lines), 18.4 (multiple lines); IR (KBr) 2980, 1740, 1575, 1535, 1470, 1400, 1350, 1250, 1185, 1150, 830, 810, 750, 680, 475 cm^{-1}; UV (HCOOH) λ_{max} 500 (ε 6.0×10^4), 484 shoulder (ε 5.6×10^4), 262 (ε 6.4×10^4), 280 nm shoulder (ε 3.6×10^4). Anal. Calcd for $C_{66}H_{86}N_8O_{20}$: C, 60.43; H, 6.62; N, 8.54. Found: C, 60.51; H, 6.49; N, 8.33. Field-desorption MS, see text.
(7) Data for **5a**: (a) ^{1}H NMR (90 MHz, $CDCl_3$) (Me_4Si) δ 0.8–1.2 (m, 48 H), 2.5 (s, 6 H), 2.2–2.8 (m, 8 H), 3.3 (s, 4 H), 4.5–5.6 (br m, 14 H), 7.7 (s, 2 H), 8.1 (s, 2 H), 8.9 (s, 2 H); ^{13}C NMR (62.9 MHz, $CDCl_3$) (Me_4Si) δ 176.0–176.6 (multiple lines), 159.3, 154.7, 150.7, 150.0 (2 lines), 136.5, 134.5, 133.8, 131.2, 114.8, 70.4, 69.0 (two lines), 61.8, 44.5, 33.7–34.1 (multiple lines), 18.3–19.0 (multiple lines); IR (KBr) 2980, 1740, 1700, 1580, 1540, 1460, 1380, 1340, 1240, 1180, 1140 cm^{-1}; UV (HCOOH) λ_{max} 444 (ε 6.7×10^3), 372 (ε 5.5×10^3), 252 (ε 2.8×10^4), 274 nm shoulder (ε 1.8×10^4). Field-desorption MS, M^+ *m/e* 1311. (b) The structural assignment for dimer **5a** is based on the measured parent ion *m/e* and the similarity of the ^{1}H, ^{13}C NMR, and UV spectra with those measured for flavin **1**: ^{1}H NMR (60 MHz, $CDCl_3$) (Me_4Si) δ 0.7–1.3 (m, 24 H), 2.5 (s, 3 H), 2.6 (s, 3 H), 2.0–2.9 (m, 4 H), 4.2–5.8 (b m, 7 H), 7.7 (s, 1 H), 8.1 (s, 1 H), 9.2 (s, 1 H); ^{13}C NMR (62.9 MHz) (Me_4Si) δ 175.6–176.6 (multiple lines), 159.5, 154.7, 150.6, 148.0, 136.9, 135.9, 134.4, 132.5, 131.3, 115.9, 69.9, 68.8 (two lines), 61.6, 44.2, 33.9, 33.8, 33.6, 33.5, 21.2, 18.0–19.2 (multiple lines); UV (HCOOH) λ_{max} 442 (ε 9.4×10^3), 380 (ε 1.2×10^4), 272 nm (ε 2.9×10^4).
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