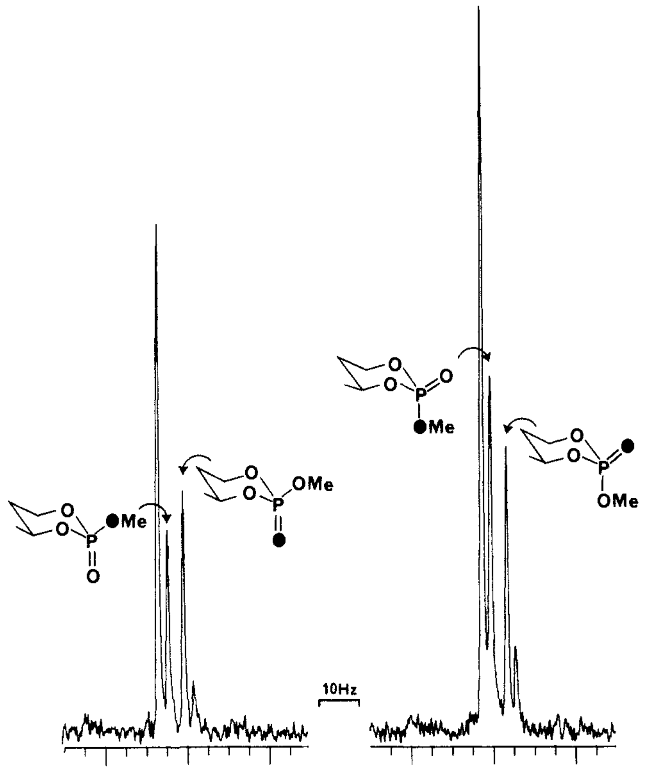

Figure 2. ^{31}P NMR spectrum of the products from the "in-line" ring closure and methylation of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-butane-1,3-diol obtained from *E. coli* alkaline phosphatase catalyzed phospho group transfer with *O*-[^{16}O,^{17}O,^{18}O]phosphotetrapeptide as the phospho group 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.

Acknowledgment. We are grateful to Joseph Vaughn for help with the NMR spectra, Art Cook for samples of chirally labeled ATP, and Sally Freeman for advice. This research was supported in part by USPHS Grants GM32204 (to E.T.K.) and GM21659 (to J.R.K.).

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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).

(17) Buchwald, S. L.; Friedman, J. M.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 4911–4516.

(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).

(20) Schwartz, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *49*, 871–878.

(21) The relative enzymic hydrolysis rates of *p*-nitrophenyl phosphate, *O*-phosphoserine, *O*-phosphotetrapeptide, and *O*-phosphoheptapeptide were estimated by ^{31}P NMR to be approximately 43:6.6:3.7:1 in potassium carbonate buffer, pH 10, and approximately 110:7.4:1.7:1 in the presence of the acceptor butane-1,3-diol.

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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(1) (a) Woodward, R. B.; Hoffmann, R. *The Conservation of Orbital Symmetry*; Verlag: Weinheim/Bergstrasse, 1970. (b) Doering, W. v. E. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5279.

(2) (a) Benson, S. W. *J. Chem. Phys.* **1961**, *34*, 521. (b) Bergman, R. G. In *Free Radicals*; Kochi, J. K., Ed.; Wiley: New York, 1973; Chapter 5. (c) O'Neal, H. E.; Benson, S. W. *Intl. J. Chem. Kinet.* **1970**, *2*, 423. (d) Benson, S. W.; Cruickshank, F. R.; Golden, D. M.; Haugen, G. R.; O'Neal, H. E.; Rogers, A. S.; Shaw, R.; Walsh, R. *Chem. Rev.* **1969**, *69*, 279.

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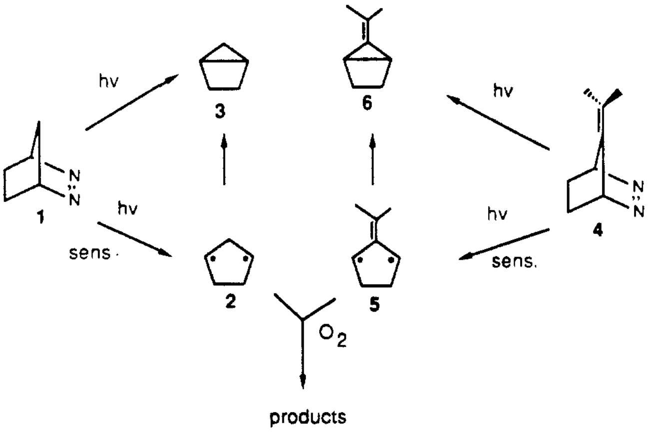
Table I. Deconvolution Parameters For Experimental Waveforms

compd	condns	α_1[e]	$\Delta H_r(1)$[f]	α_2	$\Delta H_r(2)$[f]	τ_2 (ns)
1	*a*	1.24 ± 0.02[d]	−20.4			
	b	0.82 ± 0.02	+14.1	0.51 ± 0.06	−40.0	316 ± 80[g]
	c	0.86 ± 0.07	+11.0	0.87 ± 0.06	−68.2	75 ± 21
4	*a*	1.23 ± 0.03	−19.5			
	b	1.17 ± 0.03	−19.3	0.38 ± 0.14	−43.2	913 ± 196[g,h]
	c	1.11 ± 0.08	−12.5	0.71 ± 0.05	−80.7	122 ± 25

[a] Direct irradiation, 337 nm, argon purged benzene, ~10^{-3} M azo. [b] Sensitized irradiation, 365 nm, argon and purged benzene, 0.1 M azo, ~10^{-3} M benzophenone. [c] Sensitized irradiation, 365 nm, air saturated benzene, 0.1 M azo, ~10^{-3} M benzophenone. [d] Values are from at least four separate runs, the errors are 1σ. [e] τ_1 is defined as 5 ns, which is the limit of the ~5 MHz transducer. [f] Values are the calculated heats of reaction (kcal/mol) as described in the text. [g] The large error is due to the variable concentration of O_2 present under argon purging. [h] The second decay is approaching the long time resolution of the transducer, so the accurate resolution of the second decay is difficult.

Scheme I



Controversy concerning these estimates of enthalpies has emphasized the need to experimentally measure the heats of formation of biradicals.[3] For example, the role of 1,3-biradicals in the stereomutation of cyclopropanes as either a transition state or local minimum on the energy surface is still unclear. Furthermore, the kinetic behavior of simple biradicals is generally unknown. In this context, we now report the use of time-resolved photoacoustic calorimetry (PAC)[4] to directly measure the heats of formation and kinetics of two well-studied triplet biradicals, cyclopentane-1,3-diyl and 2-isopropylidenecyclopentane-1,3-diyl.

Direct irradiation of the diazenes **1** or **4** produces bicyclo[2.1.0]pentane, **3**, or the bicyclic **6**, respectively, via their corresponding short-lived singlet biradicals, Scheme I.[5,6] Triplet sensitization of diazenes **1** or **4** produces the longer lived triplet biradicals **2** or **5**, respectively, which subsequently undergo ring closure or, in the presence of dioxygen, trapping reactions.[7,8]

PAC allows the simultaneous determination of the dynamics and energetics of photoinitiated reactions.[4a,9–12] Deconvolution of the experimental waveforms[4a] measures the magnitude and time evolution of heat deposition. The kinetic, τ, and enthalpic, α, fitting parameters for the experimental waveforms obtained from the direct and sensitized irradiation of diazenes **1** and **4** in benzene are given in Table I.[13–15] In order to obtain acceptable fits, two decays were employed for the sensitized decompositions and one for the direct.

In the sensitized irradiations, the first rate, $\tau_1 \cong 5$ ns, and the second rate, τ_2, correspond to the triplet energy transfer to form the biradicals **2** and **5** and their subsequent decay, respectively.[16] The heats of reaction of these processes, $\Delta H_r(1)$ and $\Delta H_r(2)$, are given by $\Delta H_r(1) = E_{hv}(1 - \alpha_i)/\Phi_i$ and $\Delta H_r(2) = -(E_{hv}\alpha_i)/\Phi_i$, where E_{hv} is the photon energy, Φ_i is the quantum yield, and α_i is the enthalpic weighting parameter for that process.[17] The α_i is defined as the fraction of photon energy released as heat for the process i. In the direct irradiations, the singlet biradical cannot be resolved, $\tau < 10$ ns, and so the heat deposition, α_1, is a measure of the overall transformation of **1** → **3** and **4** → **6**.

The comparison of the experimentally derived heat of formation of the transition state for the ring inversion of bicyclopentane, 76 kcal/mol,[19] with the *thermochemical* estimate of the heat of formation of the biradical, 67 kcal/mol, suggests a barrier of 9 kcal/mol for ring closure.[2,22] This barrier is inconsistent with both the experimental activation energy of 2.3 kcal/mol for ring closure of **2**[23a] and ab initio MO calculations.[23b] The *experimental* heat of formation of **2**, $\Delta H_f(\mathbf{2})$, is given by eq 1

$$\Delta H_f(\mathbf{2}) = \Delta H_f(\mathbf{3}) - \Delta H_r(\mathbf{1} \rightarrow \mathbf{3}) + \Delta H_r(\mathbf{1} \rightarrow \mathbf{2}) \qquad (1)$$

where $\Delta H_f(\mathbf{3})$ is the heat of formation of **3**, and ΔH_r is the appropriate heat of reaction.[24] With $\Delta H_f(\mathbf{3}) = 37.0$ kcal/mol[20] and

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(7) (a) Adam, W.; Hannemann, K.; Wilson, R. M. *J. Am. Chem. Soc.* **1986**, *108*, 929. (b) Wilson, R. M. In *Organic Photochemistry*; Padwa, A.; Ed.; Marcel Dekker: New York, 1983; Vol. 7, p 339.

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(12) LaVilla, J. A.; Goodman, J. L. *Chem. Phys. Lett.* **1987**, *141*, 149.

(13) An ~0.5-MHz PZT transducer was used for signal detection. Similar decon values were obtained by using a 2.25-MHz transducer. The use of xanthone as sensitizer or acetonitrile as solvent gave similar results.

(14) Under the sensitized irradiation conditions, compounds **1** and **4** absorbed <2% and ~15% of the light, respectively. In the case of **4**, a minor correction has been made for the α values based on the values obtained from the direct irradiation.

(15) The volume change for the photochemical reaction, ΔV, may contribute to the observed acoustic wave. In this case, the positive ΔV of reaction would give an apparently large value for α.

(16) The triplet energy transfer rate from benzophenone to azo **1** is ~5 ns based on the interaction rate constant of 2.1×10^9 M^{-1} s^{-1}. The energy transfer rate constant for azo **4** is not known. With use of PAC, we measured the rate constant to be ~1.4×10^9 M^{-1} s^{-1}.

(17) The quantum yield for both the direct and sensitized irradiation of azo **1** is unity.[5] The quantum yield for direct irradiation of azo **4** is ~1.0.[18] Our preliminary quantum yield value for the sensitized irradiation of **4** is ~0.69. This quantum yield determination was done by using the photosensitized decomposition of **1** as the actinometer. The disappearance of **1** and **4** (0.025 M) in degassed benzene-d_6 was followed by ^{1}H NMR integration by using δ 4.6 (br s, 2 H) for **1** and δ 5.0 (br s, 2 H) for **4**. Benzophenone (~0.01 M) was used as the sensitizer. A medium-pressure mercury lamp and bandpass filters were used to isolate wavelengths between 360–370 nm. Dichloroethane was used as the internal standard.

(18) Platz, M. S.; Kelsey, D. R.; Berson, J. A.; Turro, N. J.; Mirbach, M. J. *J. Am. Chem. Soc.* **1977**, *99*, 2009.

(19) This value uses $\Delta H_f(\mathbf{1}) = 37$ kcal/mol,[20] and the energy of activation for ring inversion of 2-methylbicyclopentane, 39 kcal/mol.[21]

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(23) (a) Buchwalter, S. L.; Closs, G. L. *J. Am. Chem. Soc.* **1979**, *101*, 4688. (b) Conrad, M. P.; Pitzer, R. M.; Schaefer, H. F. *J. Am. Chem. Soc.* **1979**, *101*, 2245.

the experimental data, $\Delta H_f(\mathbf{2}) = 71.5 \pm 2.3$ kcal/mol is significantly greater than the thermochemical estimate.[25] The discrepancy between the experimental value and the thermochemical estimate of $\Delta H_f(\mathbf{2})$ adds further impetus for the upward revision in the heats of formation of simple free radicals on which these estimates are based.[1b,26]

The experimental heat of formation of the transition state for the ring closure of **2** is ~74 kcal/mol, which is within experimental error of that of thermal isomerization of **3**. The heat of formation of the singlet biradical **2** is between 71.5 and 73.8 kcal/mol.[23] This suggests that it is either a transition state or a local minimum with a barrier for ring closure significantly smaller than that predicted from thermochemical estimates.

Although much experimental work on the 2-alkylidenecyclopentane-1,3-diyl system has been done,[6] the heats of formation of the singlet and triplet **5**, are not known so no experimental value for the singlet–triplet energy gap, ΔE_{ST}, is available. With use of eq 1 for the analogous reactions of **4**, the experimental data, and $\Delta H_f(\mathbf{6}) = 48.5$ kcal/mol,[27] the experimental heat of formation of triplet biradical **5** is 48.7 ± 3.5 kcal/mol.[28] The ring closure of singlet biradical **5** to the strained bicyclic **6** is exothermic by ~12.9 kcal/mol.[29] Consequently, the heat of formation of singlet **5** is 61.4 kcal/mol and so $\Delta E_{ST} \simeq 12.7$ kcal/mol.[30] This is in reasonable agreement with both the most recent calculations on 2-methylenecyclopentane-1,3-diyl which show a triplet–singlet gap of 15.5 kcal/mol[31] and experimental estimations.[6,29]

The lifetimes of spectroscopically "invisible" biradicals, such as **2** and **5**, can be obtained directly by using PAC. The observed lifetime of **2** is 316 ns in argon purged benzene, which is significantly shorter than the reported value of 720 ns, measured indirectly by using oxygen trapping.[7,32] This decay is primarily due to the ring closure to bicyclic **3**. Without purging, the lifetime of **2** decreases and the heat of reaction increases owing to the increased contribution from the highly exothermic reaction of **2** with dioxygen. To our knowledge, this is the first direct measurement of the lifetime of a nonconjugated biradical in solution. The observed lifetime of biradical **5**, ~900 ns, is significantly longer than **2**.[33] The bimolecular rate constants for the reaction of biradicals **2** and **5** with dioxygen can be estimated to be about 5.3×10^9 and 4.3×10^9 M^{-1} s^{-1},[34] respectively.

In conclusion, we have demonstrated the use of PAC to measure the heats of formation of reactive, nonspectroscopic biradical intermediates. This information details the role of such species in thermal rearrangements. In addition, their kinetics and reactivity can be examined. Further studies will examine the effect of temperature, solvent, and substitution on the dynamics and energetics of these intermediates.

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(24) The equation does not include the differential heat of solvation between the reactants and products, ΔH_{sol}. Given the nonpolar nature of the reaction and the solvent employed, it is assumed $\Delta H_{sol} \sim 0$.

(25) The value for $\Delta H_f(\mathbf{2})$ may be obtained directly from the experimental $\Delta H_r(\mathbf{1} \rightarrow \mathbf{2})$ value and the known $\Delta H_f(\mathbf{1})$.[35] However, we feel the $\Delta H_r(\mathbf{1} \rightarrow \mathbf{2})$ value may be high due to a contribution from the volume change of the chemical reaction. By using the difference between the direct and sensitized heats of reaction of **1**, this contribution is removed.

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(27) The $\Delta H_f(\mathbf{6})$ value can be obtained in several ways. MM2 calculations give $\Delta H_f(\mathbf{6}) = 48.8$ kcal/mol. Alternatively, MM2 gives the heat of formation difference between **1** and **4**, $\Delta\Delta H_f$, to be 10.7 kcal/mol. Given the heats of reaction to produce **3** or **6** are about equal, then $\Delta H_f(\mathbf{6}) = \Delta H_f(\mathbf{3}) + \Delta\Delta H_f$, i.e., 47.7 kcal/mol. With use of bond additivities, $\Delta H_f(\mathbf{6}) = 53.8$ kcal/mol.

(28) The $\Delta H_f(\mathbf{5})$ is directly related to $\Delta H_f(\mathbf{6})$ or $\Delta H_f(\mathbf{4})$. Unfortunately, no experimental value for either is presently available. Consequently, the PAC results can only give the difference in the heats of formation between biradical **5** and bicyclic **6**, $\Delta\Delta H_f$. This difference is small, ~1 kcal/mol.

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(30) The $\Delta E_{ST} = \Delta H_r\ (\mathbf{4} \rightarrow \mathbf{6}) - \Delta H_r\ (\mathbf{4} \rightarrow \mathbf{5}) + 12.9$. The error in our experimental value for ΔE_{ST}, ~4 kcal/mol, is from the uncertainties of the enthalpic weighting factors of the direct and sensitized irradiations of **4**.

(31) Dixon, D. A.; Dunning, T. H.; Eades, R. H.; Kleier, D. A. *J. Am. Chem. Soc.* **1981**, *103*, 2878.

(32) (a) The oxygen-trapping technique requires the knowledge of the rate of oxygen trapping of the biradical. A value of 3.1×10^9 M^{-1} s^{-1} was used for the trapping rate with **2**. Recent experiments on other biradicals have indicated the rate may be substantially faster. Consequently, the lifetime of **2** may be significantly less than 720 ns. See: Adam, W.; Grabowski, S.; Wilson, R. M.; Hannemann, K.; Wirz, J. *J. Am. Chem. Soc.* **1987**, *109*, 7572. (b) Under argon purged conditions, the biradical **2** does undergo some reaction with oxygen in competition with ring closure to **3**. The $\alpha_1 + \alpha_2$ value for the sensitized irradiation of **1** is slightly greater than the α_1 value for the direct irradiation. The values should be equal if no reaction of biradical **2** with O_2 occurred. This, in part, could explain the shorter lifetime of **2**.

(33) The decay of **5**, even under degassed conditions, is probably due to bimolecular reactions and not the unimolecular ring closure to form **6**, which should be slow based on $\Delta E_{ST} \cong 12.7$ kcal/mol.

(34) The rate constant can be evaluated by using the equation $k_{obsd} = k_0 + k_q[O_2]$, where k_{obsd} is the observed rate of decay of the biradical, k_0 is the observed rate under degassed conditions, and k_q is the bimolecular quenching of the triplet by O_2. The $[O_2]$ in air saturated benzene is $\sim 1.91 \times 10^{-3}$ mol/l.[36] The assumption that k_0 is the rate of decay under degassed conditions may not be valid for **5**, however, given its long lifetime in degassed benzene relative to air saturated benzene, k_0 will be small in evaluating k_q.

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Structure of the Light Emitter in Krill (*Euphausia pacifica*) Bioluminescence

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Euphausiid krill comprise a significant proportion of the biomass in the oceans. They form swarms and migrate in response to light, food, temperature, and many other factors of environment. Almost all euphausiid species emit light from well-developed photophores.[1] Shimomura and Johnson have suggested the bioluminescence of the krill *Meganyctiphanes norvegica* involves a new type of light-emitting system wherein a protein P (MW ca. 360 000 dalton) is catalytically oxidized by molecular oxygen in the presence of a highly fluorescent compound F (MW ca. 600).[2] Compound F is the light emitter, as the bioluminescence spectrum (λ_{max} 476 nm) is identical with the fluorescence spectrum of F. It is interesting to note the fact that cross-reaction is observed between euphausiid protein P and dinoflagellate luciferin as well as between euphausiid fluorescent compound F and dinoflagellate luciferase.[3] Preliminary studies have shown that euphausiid fluorescent compound F and dinoflagellate luciferin are structurally related to each other and to bile pigments.[4,5] In this communication we wish to report the structure of fluorescent compound F isolated from the krill *Euphausia pacifica*.

Since compound F is unstable toward both oxygen and acids, isolation and purification were performed in an argon atmosphere

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(4) Early structural studies of compound F were primarily based on degradation reactions and functional group tests. For the structure proposed through these studies, see: Shimomura, O. *FEBS Lett.* **1980**, *116*, 203.

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