

the experimental data, $\Delta H_f(2) = 71.5 \pm 2.3$ kcal/mol is significantly greater than the thermochemical estimate.²⁵ The discrepancy between the experimental value and the thermochemical estimate of $\Delta H_f(2)$ 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-alkylidene-cyclopentane-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 $\Delta H_f(6) = 48.5$ 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 $\Delta E_{ST} \approx 12.7$ 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/mol³¹ 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.

Acknowledgment. This work was supported by the National Science Foundation (Grant CHE-8713720), the Research Corporation, and the Petroleum Research Fund, as administered by the American Chemical Society.

(34) The rate constant can be evaluated by using the equation $k_{\text{obsd}} = k_0 + k_q[\text{O}_2]$, 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 O₂. The [O₂] 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 .

(35) Engel, P. S.; Melaugh, R. A.; Mansson, M.; Timberlake, J. W.; Garner, A. W.; Rossini, F. D. *J. Chem. Thermodyn.* 1976, 8, 607.

(36) Murov, S. L. *Handbook of Photochemistry*; Marcel Dekker: New York, 1973; p 89.

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

Hideshi Nakamura, Branislav Musicki, and Yoshito Kishi*

Department of Chemistry, Harvard University
Cambridge, Massachusetts 02138

Osamu Shimomura

Marine Biological Laboratory
Woods Hole, Massachusetts 02543

Received November 20, 1987

(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 $\Delta H_{\text{sol}} \sim 0$.

(25) The value for $\Delta H_f(2)$ may be obtained directly from the experimental $\Delta H_f(1 \rightarrow 2)$ value and the known $\Delta H_f(1)$.³⁵ However, we feel the $\Delta H_f(1 \rightarrow 2)$ 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.

(26) Tsang, W. *J. Am. Chem. Soc.* 1985, 107, 2872.

(27) The $\Delta H_f(6)$ value can be obtained in several ways. MM2 calculations give $\Delta H_f(6) = 48.8$ 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 $\Delta H_f(6) = \Delta H_f(3) + \Delta \Delta H_f$, i.e., 47.7 kcal/mol. With use of bond additivities, $\Delta H_f(6) = 53.8$ kcal/mol.

(28) The $\Delta H_f(5)$ is directly related to $\Delta H_f(6)$ or $\Delta H_f(4)$. 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.

(29) (a) Mazur, M. R.; Berson, J. A. *J. Am. Chem. Soc.* 1982, 104, 2217. (b) Sabatelli, A. D.; Salinaro, R. F.; Mondo, J. A.; Berson, J. A. *Tetrahedron Lett.* 1985, 26, 5851.

(30) The $\Delta E_{ST} = \Delta H_f(4 \rightarrow 6) - \Delta H_f(4 \rightarrow 5) + 12.9$. 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 O₂ 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 $\Delta E_{ST} \approx 12.7$ kcal/mol.

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

(1) (a) Bassot, J.-M. *Bull. Soc. Zool. Fr.* 1966, 91, 445. (b) Herring, P. J.; Locket, N. A. *J. Zool. Lond.* 1978, 186, 431.

(2) (a) Shimomura, O.; Johnson, F. H. *Biochemistry* 1967, 2293. (b) Shimomura, O.; Johnson, F. H. *Proc. Natl. Acad. Sci. U.S.A.* 1968, 59, 475.

(3) Dunlap, J. C.; Hastings, J. W.; Shimomura, O. *Proc. Natl. Acad. Sci. U.S.A.* 1980, 77, 1394.

(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.

(5) Dunlap, J. C.; Hastings, J. W.; Shimomura, O. *FEBS Lett.* 1981, 135, 273.

Table I. ^1H and ^{13}C NMR Data for F (1) and oxy-F (2)^a

	1		2	
	^1H NMR ^b	^{13}C NMR ^c	^1H NMR ^b	^{13}C NMR ^{c,d}
1		174.2 (s)		174.3 (s)
2		<i>e</i>		130.8 (s) ^f
2 ¹	1.72 (s)	8.4 (q)	1.72 (s)	8.5 (q)
3		152.1 (s)		152.2 (s) ^g
3 ¹	6.58 (dd, <i>J</i> = 17, 11 Hz)	128.0 (d)	6.61 (dd, <i>J</i> = 18, 11 Hz)	128.1 (d)
3 ²	5.59 (d, <i>J</i> = 11 Hz)	122.6 (t)	5.56 (dd, <i>J</i> = 11, 1 Hz)	122.7 (t)
	5.98 (d, <i>J</i> = 17 Hz)		5.96 (dd, <i>J</i> = 18, 1 Hz)	
4		90.0 (s)		90.1 (s)
5	3.18 (d, <i>J</i> = 15 Hz)	35.6 (t)	3.17 (d, <i>J</i> = 15 Hz)	35.8 (t)
	3.28 (d, <i>J</i> = 15 Hz)		3.21 (d, <i>J</i> = 15 Hz)	
6		<i>e</i>		125.0 (s) ^h
7		<i>e</i>		119.8 (s) ^h
7 ¹	4.21 (d, <i>J</i> = 11 Hz)	55.6 (t)	4.20 (d, <i>J</i> = 12 Hz)	55.8 (t)
	4.40 (d, <i>J</i> = 11 Hz)		4.39 (d, <i>J</i> = 12 Hz)	
8		<i>e</i>		120.7 (s) ^h
8 ¹	2.47	18.5 (t)	2.48 (q, <i>J</i> = 7.5 Hz)	18.5 (t)
8 ²	1.02 (t, <i>J</i> = 7 Hz)	17.4 (q)	1.04 (t, <i>J</i> = 7.5 Hz)	17.5 (q)
9		<i>e</i>		123.6 (s) ^h
10	3.64 (d, <i>J</i> = 16 Hz)	22.2 (t)	3.75 (d, <i>J</i> = 16 Hz)	22.5 (t)
	3.76 (d, <i>J</i> = 16 Hz)		3.85 (d, <i>J</i> = 16 Hz)	
11		<i>e</i>		139.1 (s)
12		<i>e</i>		128.1 (s) ^f
12 ¹	2.12 (s)	9.7 (q)	2.14 (s)	9.6 (q)
13		108 (s)		109.6 (s)
13 ¹		193.4 (s)		197.8 (s)
13 ²	3.11 (d, <i>J</i> = 19 Hz)	45.3 (t)	2.83 (d, <i>J</i> = 18 Hz)	57.4 (t)
	3.28 (d, <i>J</i> = 19 Hz)		3.79 (d, <i>J</i> = 18 Hz)	
14		149 (s)		156.3 (s) ^g
15		88 (s)		74.2 (s)
16		162 (s)		181.0 (s) ⁱ
17	2.49	52.1 (d)	2.73 (dt, <i>J</i> = 13, 3 Hz)	58.1 (d)
17 ¹	1.56 (br t, <i>J</i> = 14 Hz)	29.9 (t)	1.48 (tt, <i>J</i> = 13, 3 Hz)	30.9 (t)
	2.49		2.81 (br t, <i>J</i> = 13 Hz)	
17 ²	2.08 (m)	34.9 (t)	2.32 (ddd, <i>J</i> = 16, 13, 3 Hz)	35.4 (t)
	2.39 (br t, <i>J</i> = 14 Hz)		2.50 (m)	
17 ³		<i>e</i>		181.5 (s) ⁱ
18	2.49	42.2 (d)	2.21 (m)	44.1 (d)
18 ¹	1.18 (d, <i>J</i> = 7 Hz)	23.5 (q)	1.14 (d, <i>J</i> = 7.0 Hz)	23.5 (q)
19	3.59 (d, <i>J</i> = 1 Hz)	69.5 (d)	4.10 (d, <i>J</i> = 4 Hz)	83.8 (d)
20		<i>e</i>		181.2 (s) ⁱ

^aThe spectra were recorded in degassed MeOH-*d*₄ at -5 °C on a Bruker AM 500 instrument at 500 MHz for ^1H and 125 MHz for ^{13}C . The signals of the solvent were used as an internal reference ($\delta_{\text{H}} = 3.3$ ppm, $\delta_{\text{C}} = 49.0$ ppm). ^bThe assignment of signals was made on the basis of homo spin decoupling and 2D-COSY experiments. ^cThe multiplicity of signals was determined by DEPT experiments. ^dThe assignment of signals was made on the basis of proton selective decoupling experiments. ^eThe assignment of this signal was not conclusive because of the poor S/N ratio. ^{f-i}This resonance could be assigned to the carbon or any one of the carbons indicated.

at low temperature under slightly basic conditions. Sixty percent aqueous ethanol extracts of the frozen specimens collected at Victoria, British Columbia,⁶ were purified by alumina column chromatography (Woelm basic alumina, grade I; eluted with 50% ethanol containing 0.6% ammonium hydroxide), followed by DEAE cellulose column chromatography (eluted with a 1:1 mixture of ethanol and 0.02 M Tris buffer containing 0.2 M NaCl, pH 7.5). Compound F was further purified by HPLC (TSK DEAE-5 PW, 85 mM NaCl and 3 mM NaHCO₃ in 40% aqueous acetonitrile) equipped with a degasser (Erma ERC-3000). The eluate showed a clean UV absorption at 388 nm and displayed a single symmetrical peak on analytical HPLC. However, during the procedure of desalting and concentration,⁷ compound F partially decomposed into a complex mixture of degradation products. The purity of compound F thus obtained (ca. 2 mg out of 450 g of the frozen specimens) was usually 60–70%.

Mild chromic acid oxidation of compound F (CrO₃/KHSO₄/H₂O/room temperature) yielded 3-methyl-4-vinylmaleimide (3) and the aldehyde 4, whereas vigorous chromic acid oxidation (CrO₃/2N H₂SO₄/90 °C) gave hematinic acid (5).⁴ Under basic conditions (NaOH/MeOH/65 °C),⁸ compound F

yielded the pyrromethenone 6, the structure of which was unambiguously established by synthesis.⁹

Spectroscopic studies on compound F were hampered by the unavailability of a pure sample. However, we noticed that compound F was slowly converted at -20 °C to a new, relatively stable substance, oxy-F.¹⁰ Fortunately, oxy-F could be isolated in pure form,¹¹ and spectroscopic studies were first carried out on this substance. Oxy-F showed UV absorptions at 237 and 275 nm (shoulder) and no fluorescence, suggesting that some chemical change had taken place on the chromophore of compound F during the transformation. The high-resolution FAB mass spectrum¹²

(8) The major pyrromethenone isolated under these conditions was the corresponding methyl ether, i.e., X = Me in 6. However, we could show that the ether methyl group originated from the solvent.

(9) The complete characterization and unambiguous structure elucidation of 4 and 6 are described in the dissertation of B. Musicki, Harvard University, 1987.

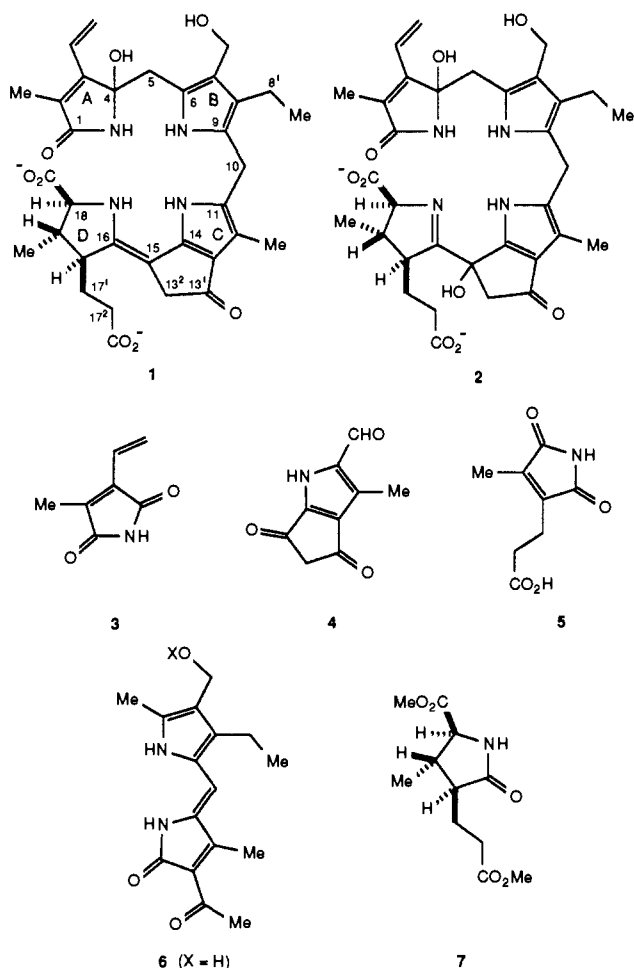
(10) This transformation was best achieved in methanol at -20 °C under an argon atmosphere containing a small amount of oxygen. The reaction was faster in the presence of a large excess of oxygen, but it produced more unidentified byproducts.

(11) HPLC (TSK DEAE-5 PW) equipped with a degasser (Erma ERC-3000) was used for purification. Approximately 3 mg of pure oxy-F was isolated from 2.5 kg of frozen krill.

(12) High-resolution FAB mass measurements were performed on a JEOL JMS-HX110 mass spectrometer by using a dual target sample inlet probe (Xe ionizing gas; glycerol matrix). We thank T. Higuchi, Mass Spectrometer Application Lab., JEOL USA, Inc., for the arrangement of measurements.

(6) *Euphausia pacifica* was collected by towing in Saanich Inlet, and the live specimens were frozen with dry ice. We acknowledge Dr. W. A. Heath, BioOcean Surveys, Ltd., Victoria, British Columbia, Canada, for his help in collecting specimens.

(7) Salts were removed by precipitation with ethanol.



proved the molecular formula of oxy-F to be $C_{33}H_{38}O_9N_4Na_2$ [m/z 703.2363 ($M + Na$)⁺, $\Delta 3.1$ mmu and 681.2483 ($M + H$)⁺, $\Delta 3.0$ mmu], which corresponds to the monooxygenated form of compound F (vide infra). The ¹H and ¹³C NMR data of oxy-F are summarized in Table I. The ¹³C NMR spectrum clarified the nature of all the carbon atoms and suggested, in connection with the ¹H NMR spectrum, the presence of the following groups in oxy-F: two carboxylates (δ_C 181.2 and 181.5),¹³ an amide (δ_C 174.3), a conjugated ketone (δ_C 197.8), a hydroxymethyl [δ_C 55.8, δ_H 4.20 and 4.39 (d, $J = 12$ Hz)], a carbinol amine (δ_C 90.1), and a tertiary alcohol (δ_C 74.2).

On the basis of the chemical shift of a methyl carbon (δ_C 8.5),¹⁴ which was assigned from the long-range coupling ($J < 1$ Hz) observed¹⁵ between the C.2¹ Me and the vinyl protons, and the isolation of 3 on chromic acid oxidation (vide supra), the partial structure from C.1 through C.5 in oxy-F was established.¹⁶ The presence of the C.4 carbinol amine moiety (δ_C 90.1) was consistent with two experimental observations: (1) no ¹⁸O was incorporated into 3-methyl-4-vinylmaleimide (3) in chromic acid oxidation by using H₂¹⁸O and ¹⁸O₂,⁴ and (2) 3 was readily formed when 2 was exposed to silica gel.

The long-range coupling ($J < 1$ Hz) observed¹⁵ between the C.12¹ Me and the C.10 methylene protons, coupled with the formation of the degradation products 4 and 6, clearly established the partial structure from C.5 to C.15. The ring D moiety was concluded on the basis of two experimental facts: (1) 5 was formed on chromic acid oxidation (vide supra) and (2) homonuclear

(13) The high-resolution FAB mass spectrum showed that compound F as isolated was a disodium salt. In addition, its chromatographic behavior is consistent with this conclusion.

(14) Wray, V.; Gossauer, A.; Gruning, B.; Reifensahl, G.; Zilch, H. *J. Chem. Soc., Perkin Trans. 2* 1979, 1558.

(15) This long-range coupling was confirmed by 2D-COSY experiments.

(16) On the basis of these spectroscopic data only, the C.4-C.5 connectivity could not be concluded. However, the isolation of degradation products 4, 6, and 7 established this connectivity unambiguously.

spin-decoupling as well as 2D-COSY experiments revealed the proton connectivity from C.17² to C-19. These results allowed the assignment of the structure 2 for oxy-F.

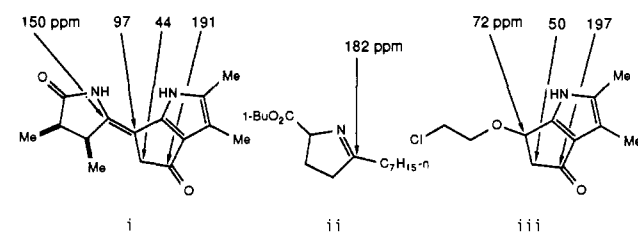
Although compound F has never been isolated in pure form, a comparison of the ¹H and ¹³C NMR spectra of F (Table I) with those of oxy-F provided meaningful structural information for compound F [FAB-MS, m/z 687 ($M + Na$)⁺ and 665 ($M + H$)⁺]. It became clear that the structural difference between F and oxy-F was only in the C.15-C.16-N moiety. The ¹³C chemical shifts observed for the C.15-C.16 carbons of F and oxy-F provided the crucial evidence for assignment of the structure 1 to compound F.¹⁷ In order to confirm the proposed structure, compound F was subjected to ozonolysis, followed by diazomethane treatment. The expected diester 7 was successfully isolated. The relative stereochemistry of 7 was established by a comparison with an authentic sample.¹⁸

On the basis of the experimental evidence described above, euphausiid fluorescent compounds F and oxy-F are represented by the structures 1 and 2, respectively. All the degradation reactions,⁴ the ¹⁸O-incorporation patterns on the chromic acid oxidations,¹⁹ and the spectroscopic data are rationally explained by these structures. However, the stereochemistry of the C.15-C.16 olefinic bond in 1 is not known at this time. Fluorescent compound F (1) is apparently a member of the bile pigments. To the best of our knowledge, however, it is the first naturally occurring bile pigment which is structurally related to chlorophylls rather than to hemes. An additional intriguing structural feature of 1 is the carboxylic acid present at the C.20 position, which may suggest a possibility that an oxidative cleavage of the C.1-C.20 bond might be involved in its biosynthesis.^{20,21} As dinoflagellate luciferin has a close relationship to fluorescent compound F, the structural information gained here should be valuable for understanding the dinoflagellate bioluminescent system as well. We are currently engaged in structural studies on dinoflagellate luciferin.

Acknowledgment. Financial assistance from the National Science Foundation to Harvard University (CHE 86-105050) and to Marine Biological Laboratory (DMB 87-03463) is gratefully acknowledged.

Supplementary Material Available: ¹H NMR, ¹³C NMR, FAB MS, and UV spectra of 1 and 2 and ¹H NMR spectra of 4, 6, and 7 (11 pages). Ordering information is given on any current masthead page.

(17) Compounds i-iii provide examples for the ¹³C chemical shifts relevant to the system present in F and oxy-F. Compound ii is known in the literature (Shiosaki, K.; Rapoport, H. *J. Org. Chem.* 1985, 50, 1229), and compounds i and iii are reported in the dissertation of B. Musicki, Harvard University, 1987.



(18) The authentic sample was synthesized from the lactam obtained on Ti(OOCF₃)₃ oxidation of benzyl 4-(2-methoxycarboxylethyl)-3-methylpyrrole-2-carboxylate. We thank Professor K. M. Smith, University of California, Davis, for the detailed procedure of oxidation and the starting material for the synthesis. The stereochemistry of the side-chains was determined on the basis of NOE experiments. The details of these experiments are described in the dissertation of B. Musicki, Harvard University, 1987.

(19) In order to determine the sequence of the four rings, fluorescent compound F was subjected to CrO₃ oxidation at pH 1.2 in the H₂¹⁸O medium under an ¹⁸O₂ atmosphere to yield 4 with two ¹⁸O atoms incorporated and 3 with no ¹⁸O atoms.⁴

(20) For a recent review on the biosynthesis of hemes, chlorophylls, vitamin B₁₂, and related compounds, see: Leeper, F. *J. Nat. Prod. Rep.* 1985, 2, 19.

(21) If this is the case, then the C.15-C.16 stereochemistry is likely to be that shown in 1.