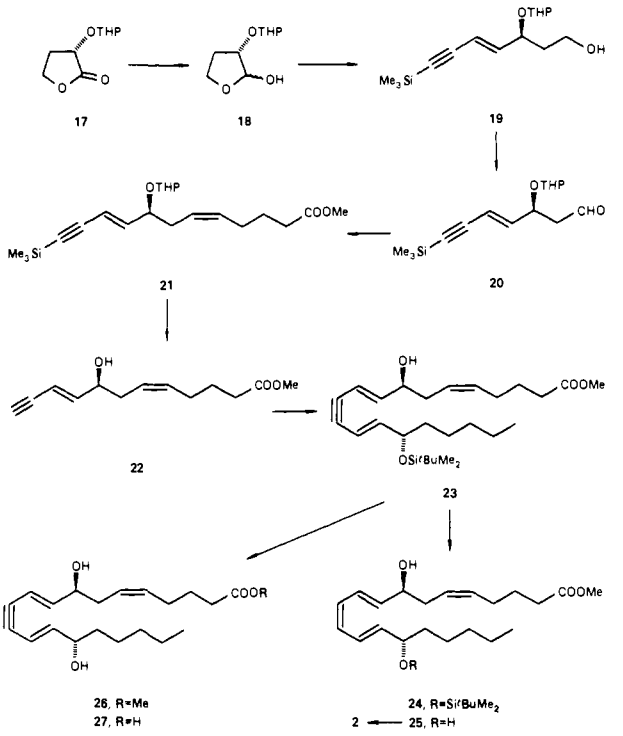
Scheme III. Total Synthesis of 8,15-DiHETE (2)



76% overall yield and with exclusive *E* geometry at the newly generated double bond.

Selective hydrogenation (Lindlar catalyst, H_2, hexane, 25 °C)[14] of the acetylene groupings of **12** proceeded smoothly, furnishing the 5,15-DiHETE derivative **13** in 80% yield. Desilylation of this intermediate with excess HF·pyr (THF, 25 °C)[15] led to the methyl ester of 5,15-DiHETE (**14**) in 80% yield. Finally, alkaline hydrolysis (3.0 equiv of LiOH, THF–H_2O, 1:1, 25 °C) of **14** furnished 5,15-DiHETE (**1**) in essentially quantitative yield. Both synthetic **1** and its methyl ester **14** were spectroscopically (IR, MS, ^1H NMR, UV) and chromatographically (TLC, HPLC) identical with naturally derived samples.[16] This synthetic route also provided the novel diacetylenic analogues **15** and **16** by desilylation and saponification of **12** as described above.

The construction of 8,15-DiHETE (**2**) proceeded along Scheme III. Lactone **17**, readily available from (*S*)-malic acid according to Still[17] and Ohfune,[18] was reduced with DIBAL (1.1 equiv of, CH_2Cl_2, −78 °C) to afford lactol **18** (95% yield). Upon reaction (THF, −78 → 25 °C) with excess ylide derived from $Me_3SiC{\equiv}CCH_2P^+Ph_3Br^-$ and *n*-BuLi (3.0 equiv of each, THF, −78 °C),[19] lactol **18** furnished hydroxy enyne **19** in 60% yield (R_f 0.29, silica, 70% ether in petroleum ether) together with its cis isomer (25%, R_f 0.50 silica, 70% ether in petroleum ether). Oxidation of **19** with PCC (1.5 equiv of CH_2Cl_2, 25 °C) led to aldehyde **20** (82% yield), which reacted (DME, −10 → 25 °C) with the ylide derived from (4-carboxybutyl)triphenylphonium bromide (3.0 equiv) and $NaN(SiMe_3)_2$ (6.0 equiv (DME, −10 °C) furnishing, after diazomethane treatment and chromatography, methyl ester **21** (80% yield) in essentially pure geometrical form.[20] Removal of the tetrahydropyranyl ether protecting group (0.1 equiv of PPTS, MeOH, 50 °C) followed by desilylation (1.1 equiv of *n*-Bu_4NF, THF, 0 °C) led to the key intermediate terminal acetylene **22** in 95% overall yield. Coupling of **22** with vinyl bromide **8** (Scheme II) (1.0 equiv) under carefully controlled conditions [0.04 equiv of $(Ph_3P)_4Pd$, 1.2 equiv of $PrNH_2$, 0.05 equiv of CuI, benzene, 25 °C][8] proceeded smoothly to furnish product **23** in 80% yield and with complete preservation of geometry. Selective hydrogenation of **23** (Lindlar catalyst, H_2, hexane)[14] afforded smoothly compound **24** (87% yield based on ca. 50% conversion). Finally, desilylation of **24** (excess HF·pyr, THF, 25 °C)[15] furnished, in essentially quantitative yield, the methyl ester **25**, which exhibited the expected properties [^1H NMR, MS, IR, UV, $[\alpha]_D$] for natural 8,15-DiHETE methyl ester.[1,3] Free 8,15-DiHETE (**2**) was prepared by alkaline hydrolysis (1.5 equiv of LiOH, THF–H_2O, 3:1, 25 °C, 95% yield) of its methyl ester (**25**). Furthermore, desilylation and saponification of the acetylenic derivative **23** afforded the novel analogues **26** and **27**.

With 5,15- and 8,15-DiHETE's and analogues synthetically available, extensive biological investigations in this area are now possible. Furthermore, these short and stereocontrolled total syntheses demonstrate the generality and efficiency of the developed Pd/Cu-based technology for the construction of linear eicosanoids as well as other, tailored-made biological tools of similar structures. Other applications are currently in progress.[21,22]

Acknowledgment. We express our many thanks to Dr. George Furst and Margaret Davis of this Department for their excellent spectroscopic assistance and helpful comments. Financial support for this work was generously provided by Merck Sharp and Dohme, Smith Kline and French, the Camille and Henry Dreyfus Foundation, and the National Institutes of Health.

Supplementary Material Available: Listing of ^1H NMR and IR spectral data for compounds **12–15** and **22–25** (4 pages). Ordering information is given on any current masthead page.

(14) We thank Dr. John Partridge of Hoffmann-La Roche Co., Nutley, NJ, for a generous gift of a superior Lindlar catalyst.
(15) Nicolaou, K. C.; Seitz, S. P.; Pavia, M. R.; Petasis, N. A. *J. Org. Chem.* **1979**, *44*, 4011.
(16) We thank Professor Alan Brash of the Department of Pharmacology and Medicine, Vanderbilt University School of Medicine, Nashville, TN, for an authentic sample of 5,15-DiHETE (**1**).
(17) Still, W. C.; McDonald, J. H., III; Collum, D. B. *J. Am. Chem. Soc.* **1980**, *102*, 2117.
(18) Ohfune, Y.; Masako, T.; Kyosuke, N. *J. Am. Chem. Soc.* **1981**, *103*, 2409.
(19) Jones, E. R. H.; Ahmed, M.; Barley, G.; Hearn, M. T. W.; Thaller, V.; Yates, J. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1981.
(20) In some runs, considerable desilylation was observed in which case the material was taken through the next two steps as a mixture.
(21) All new compounds were characterized by full spectroscopic and analytical and/or exact mass data. Yields refer to spectroscopically and chromatographically homogeneous materials.
(22) For an application of the present strategy to the synthesis of LTB_4, see ref 5.

Cytochrome Oxidase Heme–Protein Dynamics: A Transient Raman Study of Carbon Monoxide Photolysis from Cytochrome a_3

E. W. Findsen and M. R. Ondrias*

Department of Chemistry, University of New Mexico Albuquerque, New Mexico 87131

Received June 11, 1984

The characterization of the local structures about the redox centers of mammalian cytochrome oxidase has recently been the focus of extensive investigations.[1,2] A variety of physical techniques such as electron paramagnetic resonance (EPR), resonance Raman, and x-ray absorption fine structure (XAFS) have been employed in these studies, and significant structural insights concerning the protein configurations about the active sites in the equilibrium forms of the protein have been obtained.[3-14]

(1) For a good general review, see: Wikström, M.; Krab, K.; Saraste, M. "Cytochrome Oxidase: A Synthesis"; Academic Press: New York, 1981.
(2) Wilson, D. F.; Erecinska, M. In "The Porphyrins"; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 3, pp 1-70.
(3) Brudvig, G. W.; Stevens, T. H.; Morse, R. H.; Chan, S. I. *Biochemistry* **1981**, *20*, 3912-3921.
(4) Dunham, W. R.; Sands, R. H.; Shaw, R. W.; Beinert, H. *Biochim. Biophys. Acta* **1983**, *748*, 73-85.
(5) Hartzell, C. R.; Hansen, R. E.; Beinert, H. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2477-2481.

0002-7863/84/1506-5736$01.50/0

Nonetheless, the molecular mechanisms by which this complex protein functions remain unclear.

Here we report initial data from our efforts to probe the mechanisms of cytochrome oxidase function by coupling a highly specific structural technique, resonance Raman spectroscopy, with time-resolved capabilities. Time-resolved investigations of heme–protein dynamics are desirable since the relevant energies and structural alterations associated with protein modulation of the heme active sites may ultimately be delocalized and not accessible to equilibrium structural determinations. However, in the species generated during ligand binding or electron transfer these changes must be manifested, at least transiently, at the heme–protein interface. Spectra of the transient species of cytochrome a_3 generated within 10 ns of carbon monoxide photolysis indicate that significant structural differences exist between it and the equilibrium geometry of ferrous cytochrome a_3. The most significant of these is a shift of approximately 10 cm^{-1} to higher frequency of the Fe–histidine mode in the transient species.

Time-resolved Raman spectroscopy has proven to be a reliable and informative technique when applied to photolytic transients of human hemoglobin (Hb) and myoglobin (Mb).[15] In particular, the work of Friedman et al.[16-19] has demonstrated that a correlation exists between the dynamic behavior of heme–protein interactions and the overall ligand affinity for a wide variety of hemoglobins. Our data demonstrates that photolytic transients of cytochrome oxidase can indeed be generated via analogous techniques.

Beef heart cytochrome oxidase was supplied to us by Dr. G. T. Babcock and was prepared using a modified Hartzell-Bienert preparation.[20] Samples of reduced oxidase were prepared by gentle degassing under an N_2 atmosphere of ~50-μM samples in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) (Calbiochem Behring) and 0.5% Brij 35 (poly(oxyethylene) 23-lauryl ether) (Sigma Chemical) pH 7.1 followed by the addition of slight excess of sodium dithionite. Carbon monoxide oxidase samples were prepared from anaerobically reduced samples by flushing the cuvette with CO gas. Absorption spectra and pH of the samples were monitored and each was found to be identical before and after Raman spectroscopy.

Resonance Raman spectra were obtained via a spectrometer system described elsewhere.[21] Laser excitation was obtained via a Molectron DL-14 dye laser pumped by a Molectron UV 24 nitrogen laser (nominal pulse width, 10 ns) operating at 10–15 Hz. Average laser power was 0.5 mJ/pulse and a cylindrical lens was used to focus the beam upon the sample. A backscattering geometry was used.

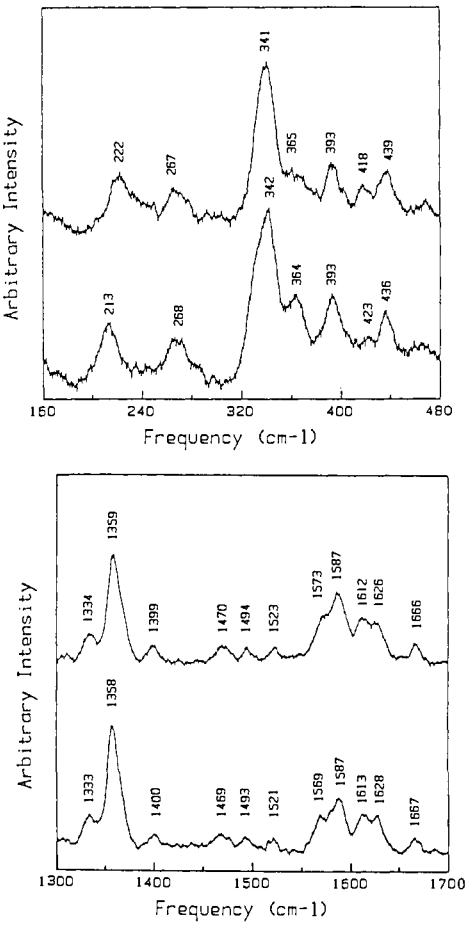


Figure 1. Upper panel: Comparison of low-frequency spectra of equilibrium fully reduced cytochrome oxidase (lower trace) and the transient species obtained within 10 ns of CO photolysis (upper trace). Both samples were ~40 μM in protein with 0.05 M HEPES (pH 7.1) and 0.5% Brij. Spectra were obtained with 440-nm excitation and are the unsmoothed sum of 10 scans. Spectral slit width was 8–10 cm^{-1} and spectra were scanned at 25 cm^{-1}/min. Lower panel: Comparison of the high-frequency spectra of equilibrium (lower trace) and transient (upper trace) cytochrome oxidase obtained under the same conditions except that only four scans were averaged.

The spectra of the fully reduced, steady-state and photolytically induced transient cytochrome oxidase species were obtained under nearly identical conditions. The congruence of our steady-state reduced cytochrome oxidase spectra with those previously published[7-12] demonstrate that the higher peak powers of our pulsed-laser system have no measurable effect on the spectra obtained from the equilibrium species. At the laser powers and concentrations employed the cytochrome oxidase–CO sample was almost completely photolyzed during the laser pulse. Only scant evidence of the liganded heme a_3 modes at 1374 (as a shoulder of the 1358 peak) and 240 cm^{-1} are seen.

Figure 1 compares the spectra of steady-state unliganded cytochrome oxidase to the transient cytochrome oxidase species generated within 10 ns of ligand photolysis. On this time scale we anticipate that any differences in heme–protein interactions between these species are localized at the heme a_3–Cu_B site.

Of particular interest is the behavior of the mode at 213 cm^{-1} in the equilibrium spectrum, which has been previously assigned as the Fe–His stretching mode.[6,11] This mode shifts dramatically to higher frequency (222 cm^{-1}) in the photolyzed transient. Modes assigned to the heme *a* moiety[6,11] at 266, 340, 393, and 437 cm^{-1} remain unchanged. An additional heme a_3 mode[8,11] at 364 cm^{-1} does not change frequency but displays a significantly reduced intensity. Changes in the mode at 422 cm^{-1} implicate it as a heme a_3 mode. The magnitude of the observed transient shift in the Fe–His frequency of heme a_3, unlike the behavior of Hb transients, is dependent upon laser power. At higher powers (achieved by

(6) Babcock, G. T.; Callahan, P. M.; Ondrias, M. R.; Salmeen, I. *Biochemistry* **1981**, *20*, 959–966.
(7) (a) Callahan, P. M.; Babcock, G. T. *Biochemistry* **1981**, *20*, 952–959. (b) Callahan, P. M.; Babcock, G. T. *Biochemistry* **1983**, *22*, 452–461.
(8) Salmeen, I.; Ramai, L.; Babcock, G. T. *Biochemistry* **1978**, *17*, 800–806.
(9) Bocian, D. F.; Lemley, A. T.; Peterson, N. O.; Brudvig, G. W.; Chan, S. I. *Biochemistry* **1979**, *18*, 4396–4402.
(10) Woodruff, W. H.; Dallinger, R. F.; Antalis, T. M.; Palmer, G. *Biochemistry* **1981**, *20*, 1332–1338.
(11) Choi, S.; Lee, J. J.; Wei, Y. H.; Spiro, T. G. *J. Am. Chem. Soc.* **1983**, *105*, 3692–3707.
(12) Carter, K. R.; Antalis, T. M.; Palmer, G.; Ferris, N. S.; Woodruff, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1652–1655.
(13) Argade, P. V.; Ching, Y. C.; Rousseau, D. L. *Science (Washington, D.C.)*, in press.
(14) Powers, L.; Chance, B.; Ching, Y.; Angiolillo, P. *Biophys. J.* **1981**, *34*, 465–498.
(15) For review, see: Friedman, J. M.; Rousseau, D. L.; Ondrias, M. R. *Annu. Rev. Phys. Chem.* **1982**, *33*, 471–491.
(16) Friedman, J. M.; Stepnoski, R. A.; Stavola, M.; Ondrias, M. R.; Cone, R. *Biochemistry* **1982**, *21*, 2022–2028.
(17) Friedman, J. M.; Rousseau, D. L.; Ondrias, M. R.; Stepnoski, R. A. *Science (Washington D.C.)* **1982**, *218*, 1244–1247.
(18) Ondrias, M. R.; Friedman, J. M.; Rousseau, D. L. *Science (Washington, D.C.)* **1983**, *220*, 615–617.
(19) Friedman, J. M.; Scott, T. W.; Stepnoski, R. A.; Ikeda-Saito, M.; Yonetani, T. *J. Biol. Chem.* **1983**, *258*, 10564–10572.
(20) Babcock, G. T.; Vickery, L. E.; Palmer, G. *J. Biol. Chem.* **1976**, *251*, 7904–7919.
(21) Ondrias, M. R.; Findsen, E. W., manuscript in preparation.

a tighter beam focus), we observed much smaller shifts (+2 to +5 cm^{-1}). No dependence upon protein concentration or laser repetition rate (over a 2-20-Hz range) was detected in any of our spectra.

Only minimal changes are evident in the comparison of the high-frequency spectra of steady-state and transient cytochrome oxidase. Changes in ν_4[27] (from 1358 to 1359 cm^{-1}) and the heme a_3 ν_2 mode (from 1569 to 1573 cm^{-1}) may be indicative of a decrease in both porphyrin π^* electron density and core size, respectively, in the transient heme a_3 species. However, the former speculation is tenuous since the certainty of a 1-cm^{-1} shift is questionable at our spectrometer resolution. Also noteworthy is the lack of a shift in the frequency of the formyl mode (at 1667 cm^{-1}) in the transient spectra.

Our present data indicate the heme a_3 site of cytochrome oxidase remains in a transient conformation for at least 10 ns after ligand photolysis. This is most clearly demonstrated in the behavior of the Fe-His vibrational mode in cytochrome oxidase transients. It shifts to higher frequency in a manner analogous to Hb transients.[16-19] This increase can be interpreted as resulting from a proximal perturbation induced by ligand binding. The Fe-His behavior of photolytic transients of Hb can be assigned to geometric constraints in the distal pocket (i.e., heme-histidine tilt) by correlating crystallographic and transient Raman data.[17] Recent resonance Raman data obtained by Rousseau et al.[13] from a steady-state carbon monoxide cytochrome oxidase indicates that the heme a_3 Fe-His bond is highly strained in both five- and six-coordinate geometries. Thus it is not surprising that we find significant reorganization of the heme a_3 proximal pocket subsequent to CO photolysis. We believe that such behavior is a generic property of the dynamics of the low-spin, in-plane to high-spin, out-of-plane transition that occurs subsequent to ligand photolysis. However, variability in the energetics involved in this process may provide an important means of regulating ligand affinity in a large class of proteins.

The possible changes observed in the high-frequency spectra may indicate specific differences between Hb and cytochrome oxidase photolytic transients. While the ν_2 mode of the heme a_3 photolytic transient is clearly at a high-spin value within 10 ns of ligand photolysis, it appears at a frequency indicative of a contracted porphyrin core relative to the heme a_3 equilibrium. This contrasts with the expanded core found in picosecond hemoglobin photolytic transients and the lack of a difference in core size between steady-state deoxyhemoglobin and nanosecond hemoglobin photolytic transients.[15] Furthermore, the position of ν_4 has been found to inversely correlate with the Fe-His mode frequency in a variety of hemoglobin photolytic transient species[19] and steady-state, deoxyhemoglobins.[22] Our cytochrome oxidase photolytic transient spectra indicate either no change in ν_4 frequency or a shift to higher frequency with increasing Fe-His frequency. The lack of a difference in the formyl frequency between steady-state and transient cytochrome oxidase species is, at first, surprising in view of the mechanistic importance ascribed to the formyl group. However, Rosseau et al.[23] have found only a small ($\leq$3 cm^{-1}) difference in the frequency of this mode between unliganded and CO liganded cytochrome a_3. Evidently even this difference has dissipated within 10 ns of CO photolysis.

The absence of any distinct bands assignable to the CO liganded heme a_3 site in transient spectra generated with moderate laser power ($\leq$0.5 mJ/pulse) contrasts with the behavior of HbCO under similar conditions, where the rapid geminate recombination of CO produces a significant amount of HbCO within 10 ns of photolysis.[24] The heme-carbon monoxide geminate recombination rate in cytochrome oxidase is evidently slow on a 10-ns time scale. This behavior may be a direct result of either the specific geometric constraints imposed upon the bound CO by the distal heme a_3 pocket[13] or a secondary binding of the photolyzed CO to the Cu_B site[25] and is not totally unexpected in view of the lower overall CO affinity of cytochrome oxidase relative to HbA.[26]

In conclusion, we believe that this preliminary investigation has established the following: (1) Time-resolved resonance Raman investigation of the transient heme species generated by ligand photolysis is a viable technique for the study of heme-ligand dynamics in proteins other than hemoglobin. (2) A transient proximal geometry leading to a strengthened Fe-His bond is present in cytochrome oxidase photolytic transients. (3) The interplay of porphyrin core size, π^* electron density, and Fe-His bonding as modulated by heme-protein dynamics is different for the ligand binding sites of Hb and cytochrome oxidase. An extensive series of investigations utilizing extended time resolution and a variety of cytochrome oxidase species has been initiated in our laboratory in order to expand upon these promising initial results.

Acknowledgment. We thank Drs. G. T. Babcock, D. L. Rousseau, J. M. Friedman, and P. V. Argade for useful discussions and communication of results before publication. A special thanks to R. Ingle for his technical expertise in the oxidase isolation. We acknowledge the support of the NIH (1 R01 GM 33330-1), BRSG (NIH PHS 2-507-RR07185-04), Research Corporation, the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the University Research program of Sandia National Labs (to M.R.O.).

Registry No. Cytochrome a_3, 72841-18-0; heme a_3, 58916-42-0; carbon monoxide, 630-08-0; cytochrome oxidase, 9001-16-5.

(22) Ondrias, M. R.; Rousseau, D. L.; Shelnutt, J. A.; Simon, S. R. *Biochemistry* **1982**, *21*, 3428-3437.
(23) Rousseau, D. L.; Argade, P. V.; Ching, Y. C., unpublished results.
(24) Ondrias, M. R.; Scott, T. W.; Friedman, J. M.; MacDonald, V. W. *Chem. Phys. Lett.*, submitted for publication.
(25) Fiamingo, F. G.; Altschuld, R. A.; Moh, P. P.; Alben, J. O. *J. Biol. Chem.* **1982**, *257*, 1639-1650.
(26) Gibson, Q. H.; Greenwood, C. *Biochem. J.* **1963**, *86*, 541-547.
(27) The designation of porphyrin skeletal modes follows that of Abe et al.: *J. Chem. Phys.* **1978**, *69*, 4526-4532.

Biosynthesis of Porphyrins and Corrins. Direct Observation of an Enzyme-Substrate Complex by Tritium NMR Spectroscopy

Jeremy N. S. Evans, Paul E. Fagerness, Neil E. Mackenzie, and A. Ian Scott*

Center for Biological NMR, Department of Chemistry Texas A&M University, College Station, Texas 77843

Received May 22, 1984

Recent studies from this laboratory[1-5] have demonstrated the power of CMR spectroscopy in defining the structure and stereochemistry of enzyme-inhibitor and enzyme-substrate complexes, using proteases of modest molecular weight (<30 000). In this communication we describe NMR experiments with porphobilinogen (PBG) deaminase (EC 4.3.1.8) which catalyzes the head-to-tail condensation of 4 mol of PBG (**1**) to pre-uro'gen, whose release and stabilization as the (hydroxymethyl)bilane (HMB, **7**) has been the subject of extensive investigation.[6-10]

(1) Malthouse, J. P. G.; Gamcsik, M. P.; Boyd, A. S. F.; Mackenzie, N. E.; Scott, A. I. *J. Am. Chem. Soc.* **1982**, *104*, 6811.
(2) Malthouse, J. P. G.; Mackenzie, N. E.; Boyd, A. S. F.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 1685.
(3) Gamcsik, M. P.; Malthouse, J. P. G.; Primrose, W. U.; Mackenzie, N. E.; Boyd, A. S. F.; Russell, R. A.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 6324.
(4) Mackenzie, N. E.; Malthouse, J. P. G.; Scott, A. I. *Biochem. J.* **1984**, *219*, 437.
(5) Reviewed by: Mackenzie, N. E.; Malthouse, J. P. G.; Scott, A. I. *Science (Washington, D.C)*, in press.
(6) Burton, G.; Fagerness, P. E.; Hosozawa, S.; Jordan, P. M.; Scott, A. I. *J. Chem. Soc., Chem. Commun.* **1979**, 202.
(7) Jordan, P. M.; Burton, G.; Nordlöv, H.; Schneider, M. M.; Pryde, L.; Scott, A. I. *J. Chem. Soc., Chem. Commun.* **1979**, 204.
(8) Battersby, A. R.; Fookes, C. J. R.; Matcham, G. W. J.; McDonald, E.; (in part) Gustafson-Potter, K. E. *J. Chem. Soc., Chem. Commun.* **1979**, 316.
(9) Battersby, A. R.; Fookes, C. J. R.; McDonald, E.; Matcham, G. W. J. *Bioorg. Chem.* **1979**, *8*, 451.

0002-7863/84/1506-5738$01.50/0