In Figure 2B we show a ^{13}C PRFT nmr spectrum of horse heart cyanoferricytochrome *c* in H_2O, recorded using an interval (τ) of 0.5 sec between each 180° radiofrequency pulse and the following 90° pulse. In a PRFT nmr spectrum, a resonance will appear negative if $\tau < T_1 \ln 2$, nulled if $\tau = T_1 \ln 2$, and positive if $\tau > T_1 \ln 2$.[10] Peaks 12–16 are positive in the PRFT nmr spectrum of Figure 2B, while peak 17 is nulled. On this basis, peak 17 is assigned to $C^{\delta 2}$ of Trp-59.

The PRFT nmr spectrum of Figure 2B does not identify the resonance of $C^{\epsilon 2}$ of Trp-59 (one of peaks 6–11). In Figure 2C we show a PRFT nmr spectrum of horse heart cyanoferricytochrome *c* in D_2O, recorded using the same τ value as with the sample in H_2O. Note that peak 9 is nulled while peaks 6–8, 10, and 11 are positive. On this basis peak 9 is assigned to $C^{\epsilon 2}$ of Trp-59. We recorded the normal ^{13}C nmr spectrum of the sample in D_2O, in order to make sure that it did not show anomalies such as a missing peak 9. It is of interest that we noticed deuterium isotope effects on the chemical shifts of some resonances. For example, the two-carbon resonance of C^{ζ} of the two Arg residues (peak 2) moves upfield by about 0.2 ppm when going from H_2O to D_2O solution. Details will be given elsewhere.[14]

In Figure 3A we show the aromatic region of the convolution-difference ^{13}C nmr spectrum of hen egg white lysozyme at pH 3.3. The region 126–131 ppm downfield from tetramethylsilane contains two two-carbon resonances (at 127.0 and 129.0 ppm) and six single-carbon resonances, for a total of ten carbons: C^{γ} of the lone His (residue 15), C^{γ} of the three Tyr residues, and $C^{\delta 2}$ of the six Trp residues.[5] In the PRFT nmr spectrum of Figure 3B five of these peaks (representing a total of six carbons) are nulled, while three peaks (four carbons) are positive. We assign the resonances that are nulled to $C^{\delta 2}$ of the six Trp residues, a result that is consistent with assignments based on other methods.[12]

The γ-carbons of the three Phe residues and the ϵ_2-carbons of the six Trp residues of hen egg white lysozyme give rise to the single-carbon resonance at 136.4 ppm and the cluster of peaks in the range 137.7–139.0 ppm[5] (Figure 3A). The identification of the resonances of $C^{\epsilon 2}$ of the Trp residues will be presented elsewhere.[14]

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References and Notes

(1) P. C. Lauterbur, *Appl. Spectrosc.*, **24**, 450 (1970); A. Allerhand, D. W. Cochran, and D. Doddrell, *Proc. Nat. Acad. Sci. U. S.*, 67, 1093 (1970); J. C. W. Chien and J. F. Brandts, *Nature (London), New Biol.*, **230**, 209 (1971); F. Conti and M. Paci, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 17, 149 (1971); V. Glushko, P. J. Lawson, and F. R. N. Gurd, *J. Biol. Chem.*, **247**, 3176 (1972); R. B. Moon and J. H. Richards, *Proc. Nat. Acad. Sci. U. S.*, **69**, 2193 (1972); A. M. Nigen, P. Keim, R. C. Marshall, V. Glushko, P. J. Lawson, and F. R. N. Gurd, *J. Biol. Chem.*, **248**, 3716 (1973); J. C. W. Chien and W. B. Wise, *Biochemistry*, **12**, 3418 (1973); D. A. Torchia and K. A. Piez, *J. Mol. Biol.*, **76**, 419 (1973); M. H. Freedman, J. R. Lyerla, Jr., I. M. Chaiken, and J. S. Cohen, *Eur. J. Biochem.*, **32**, 215 (1973); N. A. Matwiyoff and B. F. Burnham, *Ann. N. Y. Acad. Sci.*, **206**, 365 (1973); J. H. Bradbury and R. S. Norton, *Biochim. Biophys. Acta*, **328**, 10 (1973); R. B. Visscher and F. R. N. Gurd, *J. Biol. Chem.*, in press.

(2) E. L. Packer, H. Sternlicht, and J. C. Rabinowitz, *Proc. Nat. Acad. Sci. U. S.*, **69**, 3278 (1972); *Ann. N. Y. Acad. Sci.*, **222**, 824 (1973); D. T. Browne, G. L. Kenyon, E. L. Packer, D. M. Wilson, and H. Sternlicht, *Biochem. Biophys. Res. Commun.*, **50**, 42 (1973); D. T. Browne, G. L. Kenyon, E. L. Packer, H. Sternlicht, and D. M. Wilson, *J. Amer. Chem. Soc.*, **95**, 1316 (1973); M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, *J. Biol. Chem.*, **248**, 8306 (1973); M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, *Biochemistry*, **12**, 4732 (1973).

(3) R. B. Moon and J. H. Richards, *J. Amer. Chem. Soc.*, **94**, 5093 (1972); A. M. Nigen, P. Keim, R. C. Marshall, J. S. Morrow, and F. R. N. Gurd, *J. Biol. Chem.*, **247**, 4100 (1972); D. J. Saunders and R. E. Offord, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **26**, 286 (1972); N. A. Matwiyoff, P. J. Vergamini, T. E. Needham, C. T. Gregg, J. A. Volpe, and W. S. Caughey, *J. Amer. Chem. Soc.*, **95**, 4429 (1973); C. H. Fung, A. S. Mildvan, A. Allerhand, R. Komoroski, and M. C. Scrutton, *Biochemistry*, **12**, 620 (1973); A. M. Nigen, P. Keim, R. C. Marshall, J. S. Morrow, R. A. Vigna, and F. R. N. Gurd, *J. Biol. Chem.*, **248**, 3724 (1973); J. S. Morrow, P. Keim, R. B. Visscher, R. C. Marshall, and F. R. N. Gurd, *Proc. Nat. Acad. Sci. U. S.*, **70**, 1414 (1973); P. J. Vergamini, N. A. Matwiyoff, R. C. Wohl, and T. Bradley, *Biochem. Biophys. Res. Commun.*, **55**, 453 (1973); E. Antonini, M. Brunori, F. Contl, and G. Geraci, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **34**, 69 (1973); R. G. Shulman, S. Ogawa, A. Mayer, and C. L. Castillo, *Ann. N. Y. Acad. Sci.*, **222**, 9 (1973); S. H. Koenig, R. D. Brown, T. E. Needham, and N. A. Matwiyoff, *Biochem. Biophys. Res. Commun.*, **53**, 624 (1973); I. M. Chaiken, J. S. Cohen, and E. A. Sokoloski, *J. Amer. Chem. Soc.*, **96**, 4703 (1974); I. M. Chaiken, *J. Biol. Chem.*, **249**, 1247 (1974); R. B. Moon and J. H. Richards, *Biochemistry*, **13**, 3437 (1974); R. B. Moon, M. J. Nelson, J. H. Richards, and D. F. Powars, *Physiol. Chem. Phys.*, 6, 31 (1974); G. Robillard, E. Shaw, and R. G. Shulman, *Proc. Nat. Acad. Sci. U. S.*, **71**, 2623 (1974); H. M. Miziorko and A. S. Mildvan, *J. Biol. Chem.*, **249**, 2743 (1974); D. C. Harris, G. A. Gray, and P. Aisen, *J. Biol. Chem.*, **249**, 5261 (1974); C. F. Brewer, H. Sternlicht, D. M. Marcus, and A. P. Grollman, *Biochemistry*, **12**, 4448 (1973).

(4) A. Allerhand, D. Doddrell, V. Glushko, D. W. Cochran, E. Wenkert, P. J. Lawson, and F. R. N. Gurd, *J. Amer. Chem. Soc.*, **93**, 544 (1971).

(5) A. Allerhand, R. F. Childers, and E. Oldfield, *Biochemistry*, **12**, 1335 (1973).

(6) E. Oldfield and A. Allerhand, *Proc. Nat. Acad. Sci. U. S.*, **70**, 3531 (1973).

(7) A. Allerhand, R. F. Childers, and E. Oldfield, *Ann. N. Y. Acad. Sci.*, **222**, 764 (1973).

(8) A. Allerhand, R. F. Childers, and E. Oldfield, *J. Magn. Resonance*, 11, 272 (1973).

(9) V. G. Glushko, Ph.D. Thesis, Indiana University, 1972.

(10) R. L. Vold, J. S. Waugh, M. P. Klein, and D. E. Phelps, *J. Chem. Phys.*, **48**, 3831 (1968).

(11) A. Allerhand, D. Doddrell, and R. Komoroski, *J. Chem. Phys.*, **55**, 189 (1971).

(12) R. S. Norton, R. F. Childers, and A. Allerhand, unpublished results.

(13) E. Wenkert, A. O. Clouse, D. W. Cochran, and D. Doddrell, *J. Amer. Chem. Soc.*, **91**, 6879 (1969).

(14) E. Oldfield, R. S. Norton, and A. Allerhand, to be submitted for publication.

(15) I. D. Campbell, C. M. Dobson, R. J. P. Williams, and A. V. Xavier, *J. Magn. Resonance*, 11, 172 (1973); *Ann. N. Y. Acad. Sci.*, **222**, 163 (1973).

(16) A. Allerhand and D. Doddrell, *J. Amer. Chem. Soc.*, **93**, 2777 (1971); D. Doddrell and A. Allerhand, *Proc. Nat. Acad. Sci. U. S.*, **68**, 1083 (1971).

(17) "Tables of Interatomic Distances and Configuration in Molecules and Ions," *Chem. Soc., Spec. Publ.*, **No. 11** (1958); Suppl., **No. 18** (1965); R. W. G. Wyckoff, "Crystal Structures," 2nd ed, Vol. 6, Part 1, Interscience, New York, N.Y., 1969.

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A Convenient Preparation of Solutions of Superoxide Anion and the Reaction of Superoxide Anion with a Copper(II) Complex

Sir:

In recent years, metalloenzymes containing copper and zinc,[1,2] manganese,[3-5] and iron[5,6] have been discovered which catalyze the disproportionation of superoxide anion, O_2^-, to molecular oxygen and hydrogen peroxide.[7,8] In attempting to understand the mechanism of these superoxide dismutase enzymes, it would be useful to know how superoxide anion reacts with transition metal complexes but there is little information about this chemistry, probably because of the inconvenience of the electrochemical preparation used previously.[1,9-16]

We wish to report a convenient preparation of solutions of superoxide in aprotic solvents and a reaction of dissolved superoxide with a complex of copper(II) which illustrates the effect of protons in changing the relative redox potentials of oxygen and superoxide. This effect may be important in determining enzymatic mechanisms.

Potassium superoxide, KO_2, is sparingly soluble in dry dimethyl sulfoxide (DMSO).[10,17] A 0.30 *M* solution of dicy-

clohexyl-18-crown-6 in DMSO, however, can be used to prepare pale yellow 0.15 M solutions of KO_2.[18] Solutions of KO_2 prepared in this manner have been shown to contain O_2^- in approximately the same concentration. These solutions give a positive test for superoxide ion (deep blue precipitate upon addition of several drops of the KO_2 solution to a 2.0×10^{-4} M aqueous solution of nitro blue tetrazolium[19]) immediately and 1 week after preparation when stored at room temperature under dry argon. Dilute solutions of KO_2 (10^{-3} and 10^{-4} M) in DMSO–crown ether give electron paramagnetic resonance (epr) spectra at 77°K demonstrating an anisotropic g tensor (g = 2.01, 2.11) similar to spectra observed previously for electrochemically generated superoxide.[14] Addition of dilute aqueous HCl to the yellow solution 1 day after preparation results in evolution of >90% of the gas predicted for a 0.15 M O_2^- solution and bleaching of the spectrum to that of DMSO. The characteristic reduction of cytochrome c_{ox} by superoxide[1] is also observed. Infusion of 0.5 ml of a 5×10^{-4} M KO_2–crown ether solution into a stirred dilute solution of cytochrome c_{ox} (horse heart Sigma type III, λ_{max} 528 nm) in 0.05 M phosphate buffer pH 7.8 in the presence of catalase (to remove H_2O_2) results in reduction to cytochrome c_{red} (λ_{max} 520, 549 nm).[20] The characteristic reduction of tetranitromethane (TNM) by superoxide[1] is also observed but is complicated by what appears to be a slow reaction of TNM with DMSO.

The specificity of the superoxide dismutase enzyme has been used to show that the species in solution is superoxide. A 5×10^{-4} M superoxide solution in DMSO was prepared by 100-fold dilution of a 0.05 M KO_2–0.15 M crown ether solution. Slow infusion of 0.10 ml of this solution into 2.0 ml of 10^{-4} M nitro blue tetrazolium (NBT)–10^{-4} M EDTA in 0.05 M carbonate buffer pH 10.2[19] with rapid stirring results in an absorbance change of 0.26 ± 0.01 at 560 nm. Under the same conditions in the presence of 3×10^{-7} M bovine erythrocyte superoxide dismutase (Sigma), the change in absorbance is 0.04 ± 0.01. Thus at this concentration the enzyme inhibits the NBT reduction by 85% by catalyzing the disproportionation of superoxide.

We chose to study reactions of O_2^- with a complex of copper(II) because the first step (reaction 1) of the proposed mechanism[21,22] of the copper–zinc enzyme (reactions 1–3) is reaction of O_2^- with the copper(II) enzyme.

$$\text{reduction:} \quad \text{Enz–Cu}^{II} + O_2^- \longrightarrow \text{Enz–Cu}^{I} + O_2 \qquad (1)$$

$$\text{oxidation:} \quad \text{Enz–Cu}^{I} + 2H^+ + O_2^- \longrightarrow \text{Enz–Cu}^{II} + H_2O_2 \qquad (2)$$

$$\text{overall:} \quad 2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad (3)$$

Superoxide ion can function either as an oxidizing agent (reaction 4) or a reducing agent (reaction 5). Reaction 4,

$$O_2^- + 2H^+ + e^- \longrightarrow H_2O_2 \qquad (4)$$

$$O_2^- \longrightarrow O_2 + e^- \qquad (5)$$

however, requires protons to stabilize the peroxide dianion O_2^{2-}. We hoped, therefore, to isolate the first step of the proposed enzymatic reaction by carrying out our reaction in an aprotic solvent. The complex we chose to study, $[Cu(phen)_2](ClO_4)_2$ (phen = 1,10-phenanthroline), has heterocyclic nitrogen ligands similar to the histidine imidazole sites thought to be present in the copper–zinc enzyme.[23] Pale blue solutions of $[Cu(phen)_2](ClO_4)_2$ in DMSO react rapidly with superoxide upon mixing with the KO_2 solution to give deep brown solutions of $[Cu(phen)_2](ClO_4)$ (λ_{max} 445 nm). Addition of larger amounts of superoxide results eventually in a further reaction which we believe to involve modification of phenanthroline since the $[Cu(phen)_2]^+$ spectrum can be regenerated by addition of phenanthroline. Large excesses (3 equiv) of superoxide result in immediate precipitation of an insoluble brown material which is also obtained by addition of solid KO_2 to the $[Cu(phen)_2](ClO_4)_2$ solution. Side reactions can be minimized by mixing a 0.03 M solution of $[Cu(phen)_2](ClO_4)_2$ with 2 equiv of the KO_2 solution and immediately quenching the reaction and precipitating the product by addition of water. This results in 46% yield of $[Cu(phen)_2](ClO_4)$.

The reaction of a Cu(II) complex with superoxide is the reverse of the first step of the postulated mechanism for autooxidation of Cu(I) complexes (reaction 6 and 7).[24-26] The

$$Cu^+ + O_2 \rightleftharpoons Cu^{2+}O_2^- \qquad (6)$$

$$Cu^{2+}O_2^- + H^+ \longrightarrow Cu^{2+} + HO_2 \qquad (7)$$

sensitivity to air oxidation of $[Cu(phen)_2]^+$ is dependent upon solvent. Solutions in nitromethane rapidly oxidize to give $[Cu^{II}(phen)_2(CH_2NO_2)](ClO_4)$.[27] In a less acidic solvent, however, such as DMSO, we have found that solutions of $[Cu(phen)_2](ClO_4)$ react relatively slowly when dry oxygen at room temperature is bubbled through the solution. This reaction can be dramatically accelerated by the presence of protons. Addition of an equivalent amount of 1 M $HClO_4$ and treatment of the solution with oxygen results in rapid conversion to $[Cu(phen)_2](ClO_4)_2$. The cupric complex can be precipitated by addition of water and a small amount of $NaClO_4$ and isolated in 83% yield. We conclude, therefore, that the position of equilibrium (reactions 8 and 9) is reversed by the presence of protons.

$$Cu(phen)_2^+ + O_2 \rightleftharpoons Cu(phen)_2^{2+} + O_2^- \qquad (8)$$

$$Cu(phen)_2^+ + O_2 + H^+ \rightleftharpoons Cu(phen)_2^{2+} + HO_2 \qquad (9)$$

Further examples of the inorganic and organic chemistry of dissolved superoxide are under investigation.[28]

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References and Notes

(1) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
(2) B. B. Keele, Jr., J. M. McCord, and I. Fridovich, *J. Biol. Chem.*, **246**, 2875 (1971).
(3) B. B. Keele, Jr., J. M. McCord, and I. Fridovich, *J. Biol. Chem.*, **245**, 6176 (1970).
(4) R. A. Weisiger and I. Fridovich, *J. Biol. Chem.*, **248**, 3582 (1973).
(5) J. J. Villafranca, F. J. Yost, Jr., and I. Fridovich, *J. Biol. Chem.*, **249**, 3532 (1974).
(6) F. J. Yost, Jr., and I. Fridovich, *J. Biol. Chem.*, **248**, 4905 (1973).
(7) I. Fridovich, *Accounts Chem. Res.*, **5**, 321 (1972).
(8) J. M. McCord, C. O. Beauchamp, S. Goscin, H. P. Misra, and I. Fridovich in "Oxidases and Related Redox Systems," Vol. I, T. E. King, H. S. Mason, and M. Morrison, Ed., University Park Press, Baltimore, Md., 1973, p 51.
(9) D. L. Maricle and W. G. Hodgson, *Anal. Chem.*, **37**, 1562 (1965).
(10) E. L. Johnson, K. H. Pool, and R. E. Hamm, *Anal. Chem.*, **38**, 183 (1966).
(11) M. E. Peover and B. S. White, *Electrochim. Acta*, **11**, 1061 (1966).
(12) A. D. Goolsby and D. T. Sawyer, *Anal. Chem.*, **40**, 83 (1968).
(13) R. Dietz, A. E. J. Forno, B. E. Larcomb, and M. E. Peover, *J. Chem. Soc. B*, 816 (1970).
(14) J. A. Fee and P. G. Hildebrand, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **39**, 79 (1974).
(15) J. Ellis, J. M. Pratt, and M. Green, *J. Chem. Soc., Chem. Commun.*, 781 (1973).

(16) H. A. O. Hill, D. R. Turner, and G. Pellizer, *Biochem. Biophys. Res. Commun.*, **56**, 739 (1974).
(17) A. U. Khan, *Science*, **168**, 476 (1970).
(18) The solubilization of potassium salts by complexation of K^+ with crown ethers was reported by Pederson (C. J. Pederson, *J. Amer. Chem. Soc.*, **89**, 7017 (1967); C. J. Pederson and H. K. Frensdorff, *Angew. Chem., Int. Ed. Engl.*, 11, 16 (1972)) and has been used to dissolve many such salts (C. L. Liotta and H. P. Harris, *J. Amer. Chem. Soc.*, **96**, 2250 (1974); D. J. Sam and H. E. Simmons, *ibid.*, **96**, 2252 (1974)).
(19) C. Beauchamp and I. Fridovich, *Anal. Biochem.*, **44**, 276 (1971).
(20) H. A. Harbury and R. H. L. Marks in "Inorganic Biochemistry," Vol. II. G. L. Eichhorn, Ed., Elsevier, Amsterdam, 1973, p 902.
(21) J. A. Fee and B. P. Gaber in "Oxidases and Related Redox Systems," Vol. I, T. E. King, H. S. Mason, and H. Morrison, Ed., University Park Press, Baltimore, Md., 1973, p 77.
(22) D. Klug-Roth, I. Fridovich, and J. Rabani, *J. Amer. Chem. Soc.*, **95**, 2786 (1973).
(23) H. J. Forman, H. J. Evans, R. L. Hill, and I. Fridovich, *Biochemistry*, **12**, 823 (1973).
(24) H. Taube, *J. Gen. Physiol.*, **49** (2), 29 (1965).
(25) S. Fallab, *Angew. Chem., Int. Ed. Engl.*, **6**, 496 (1967).
(26) R. D. Gray, *J. Amer. Chem. Soc.*, **91**, 56 (1969).
(27) J. Zagal, E. Spodine and W. Zamudio, *J. Chem. Soc., Dalton Trans.*, 85 (1974).
(28) J. S. Valentine and J. San Filippo, to be submitted for publication.

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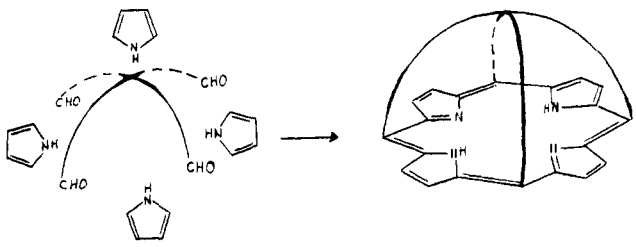
Condensation of Tetraaldehydes with Pyrrole. Direct Synthesis of "Capped" Porphyrins

Sir:

In order to develop simple methods for the synthesis of sterically hindered macrocycles, we have examined the direct condensation of suitable tetraaldehydes with pyrrole, as a route to "capped" porphyrins.[1] We report here the results of these experiments which lead to a method for the synthesis of sterically hindered porphyrins without the need for chromatographic separation of isomers.[2] The strategy behind our approach is exemplified in Figure 1; thus a suitable tetraaldehyde could condense with four molecules of pyrrole, **1**, to provide, in one step, only *one* possible stereoisomer of the tetrameso-substituted porphyrin **2**.

A suitable tetraaldehyde **3** was prepared by alkylation of salicylaldehyde with bromoethanol in aqueous alkali to yield (70%) hydroxyethyl ether **4**,[3] mp 37°, which on acylation with pyromellitoyl chloride in tetrahydrofuran–triethylamine gave the aldehyde **3**: mp 113–114° (30%), ν_{max} ($CHCl_3$) 1690, 1735 cm^{-1}; nmr ($CDCl_3$) δ 4.39 (8 H, m),



1 2

Figure 1.

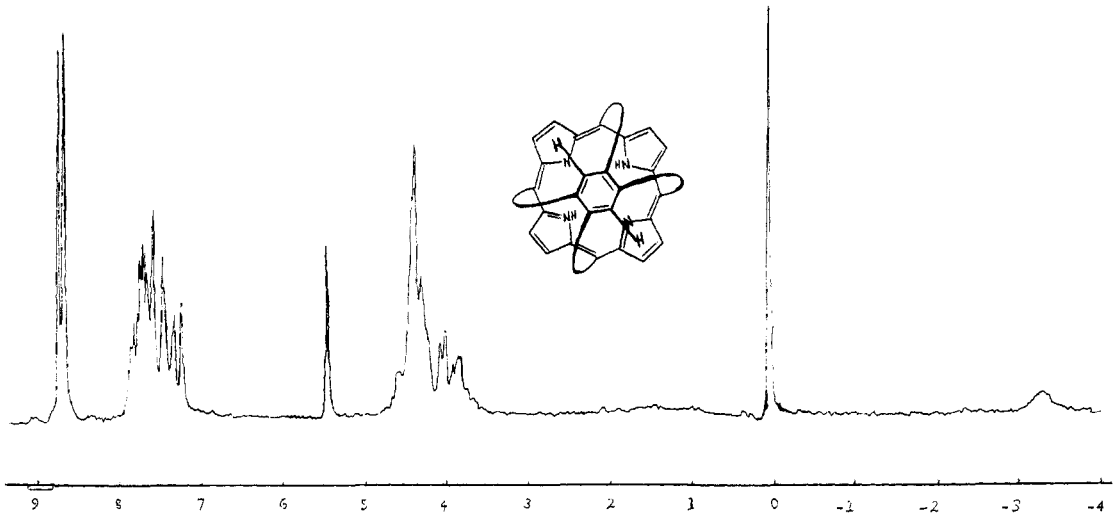
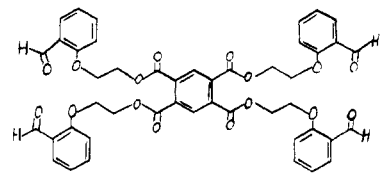


Figure 2.



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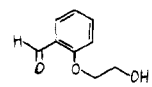

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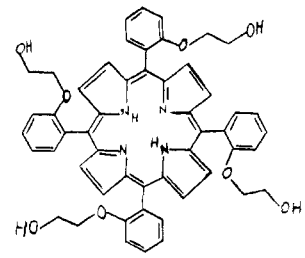

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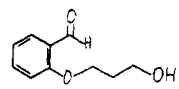

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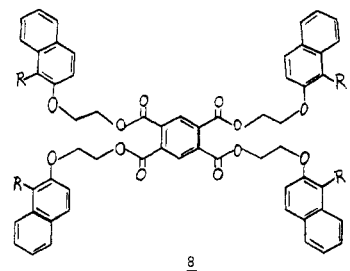

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