that 66 of the 2424 observed reflections differed by at least 20σ. Furthermore, 22 of these reflections showed a relative difference in $|F_c|^2$ which was at least 40 times the relative standard deviation of the integrated intensity, showing that a change in $|F|^2$ of as little as 2.5% should be measurable. Similarly, 60 reflections were found to be statistically insensitive ($<3\sigma$) to variations in the site occupancies. The strongest ten of these reflections were selected for use as scaling reflections.

Structure factor calculations were made for these 32 reflections at epimeric ratios (expressed as the fraction of the minor epimer) of 0, 0.25, 0.50, 0.75, and 1.00. The "sensitive" reflections showed, without exception, a linear relationship between $|F_c|$ and the epimeric ratio. The "insensitive" reflections showed minor deviations from linearity, but the root mean square deviation from linearity was less than 1.5% in each case. The variation of $|F_c|$ with the epimeric ratio for all 32 reflections is shown in six graphs included in the supplementary material (see paragraph at end of paper).

Triplicate sets of integrated intensity measurements were made for all 32 reflections on three different crystals. Mo Kα radiation was used, and the scan range was set at $1.5 + 0.35 \tan\theta$ degrees. The scan speed was selected on the basis of a preliminary scan at 3°/min so that at least 10 000 net counts could be accumulated, giving a potential precision of 1% in the integrated intensity. The insensitive reflections were weaker, so their precision was slightly poorer, being as high as 3.7% in one case.

Acknowledgment. We wish to express our appreciation to Professor N. L. Allinger for providing us with unpublished data concerning the conformational analysis of the bicyclooctane system.

Supplementary Material Available: A listing of anisotropic and isotropic thermal parameters, comparisons of observed and calculated structure amplitudes, and figures showing the variation of F_c with the epimeric ratio (91 pages). Ordering information is given on any current masthead page.

References and Notes

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Sirohydrochlorin.[1] Prosthetic Group of Sulfite and Nitrite Reductases and Its Role in the Biosynthesis of Vitamin B_{12}

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Abstract: Isolation of metabolites from cobalt-free incubations of *Propionibacterium shermanii* extracts has uncovered a new intermediate ($C_{42}H_{46}N_4O_{16}$) related to the corrin biosynthetic pathway whose physical properties are identical with those of sirohydrochlorin. Structural proposals utilizing ^{13}C NMR and ^1H NMR spectra for this compound, which is the prosthetic group of a number of six-electron reducing enzymes, are discussed and the intermediacy of sirohydrochlorin in corrin biosynthesis is demonstrated in a double-labeling experiment. On the basis of these findings the complete stereostructure (**7**) is proposed for sirohydrochlorin and hence siroheme, the novel iron-containing prosthetic group of *Escherichia coli* NADPH-sulfite reductase (EC 1.8.1.2) and of ferredoxin-nitrite reductase (EC 1.7.7.1) of spinach.

Recent work in these laboratories[2a-c] and independently at Cambridge[3] and Stuttgart[4] has confirmed the role of uro'gen III (**1**) in the biosynthesis of vitamin B_{12} (**2**) in whole cells of *Propionibacterium shermanii*, and of cobyrinic acid (**3**) in cell-free extracts of *P. shermanii* and *Clostridium tetanomorphum* as first suggested by ^{13}C-labeling experiments with the uro'gen I–IV mixed isomers.[5] It has also been shown[2d] that during the bioconversion of both uro'gen III (**1**) and the "ring

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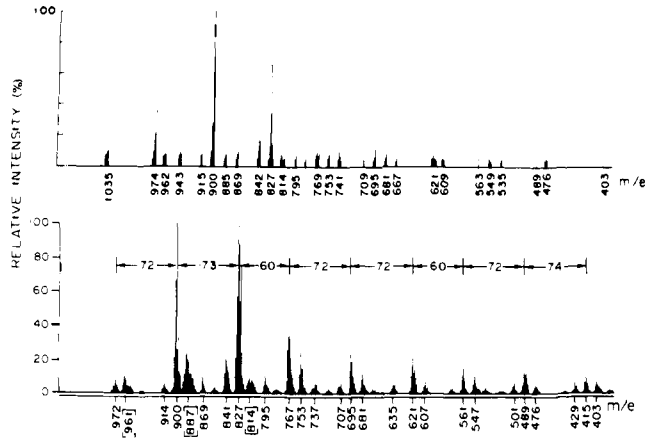


Figure 1. The mass spectra of sirohydrochlorin octamethyl ester from *P. shermanii* (upper) and from *E. coli* NADPH-sulfite reductase (lower).

Table I. Ultraviolet Absorption Spectra of Sirohydrochlorin Methyl Ester from *E. coli* NADPH-Sulfite Reductase (A) and from *P. shermanii* Cell-Free Homogenate (B)

compd		wavelength maxima (λ, nm) and relative peak intensities (R)[a]						
A	λ	362 (sh)	377	482 (sh)	514	546	589	634
	R	2.3	3.5	0.24	0.35	0.63	1.0	0.08
B	λ	363 (sh)	379	482 (sh)	513	547	589	637
	R	2.4	3.5	0.23	0.45	0.70	1.0	0.12

[a] Spectra were determined in chloroform solution.

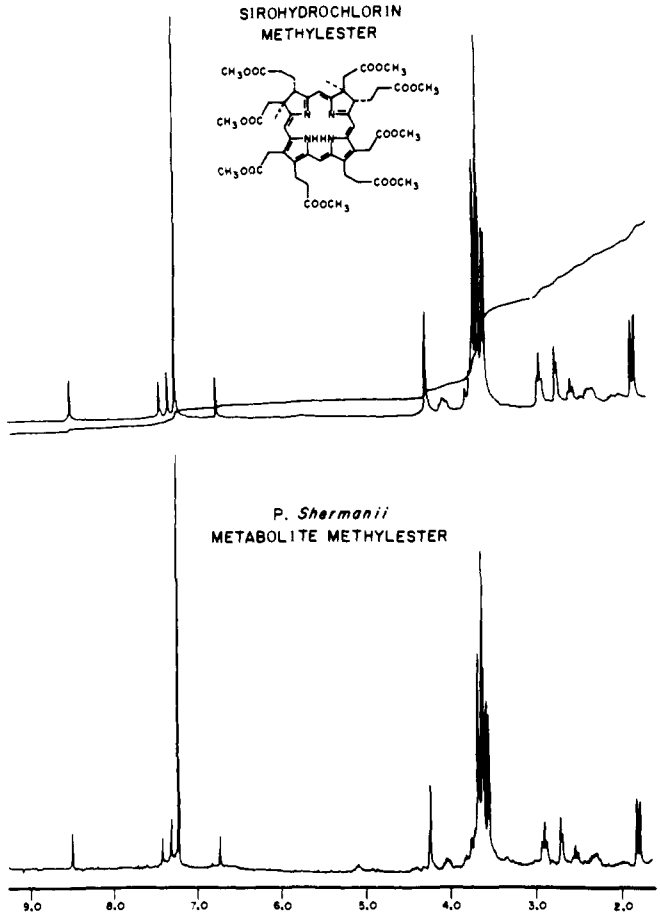
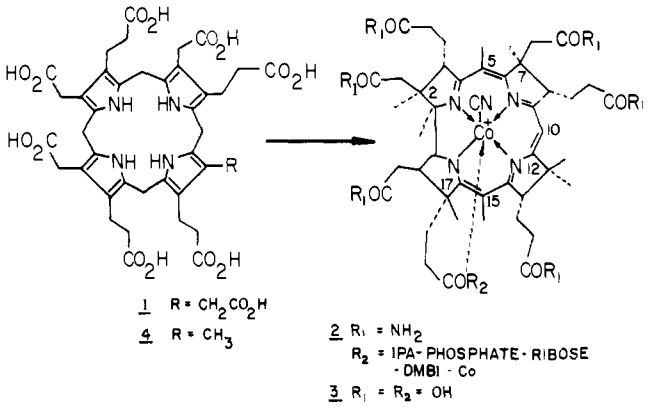


Figure 2. The 270-MHz ^{1}H NMR spectra of sirohydrochlorin octamethyl ester from *P. shermanii* and from *E. coli* NADPH-sulfite reductase in $CDCl_3$.

C heptacarboxylic acid" (**4**) to cobyrinic acid, formaldehyde can be trapped from the δ-meso (C-20) carbon of **1** and **4**. The relatively low but intact conversion of **4** to **3** suggests reassessment of the location of the heptaacid **4** on the metabolic



grid of the porphyrinogen-corrin pathway. In this paper we describe the characterization of a new isolate from a modified version of the *P. shermanii* cell-free system described earlier[6] which has considerable bearing on the porphyrinogen-corrin connection.

A novel heme-like prosthetic group in a rather widespread class of enzymes which catalyze the six-electron reduction of sulfite to sulfide in *E. coli* (EC 1.8.1.2) was first characterized in 1973 and named siroheme.[7] Removal of the iron from this species afforded an orange-fluorescent compound—sirohydrochlorin—which formed octacarboxylic methyl and ethyl esters and displayed a UV spectrum typical of a reduced porphyrin of the isobacteriochlorin class (Table I). High-resolution mass spectrometry permitted assignment of the molecular formula. It was noted by Siegel et al.[8] that sirohydrochlorin was reminiscent of the oxidized version of a doubly β-methylated uro'gen III, and further that "sirohydrochlorin could represent an early intermediate in the biosynthesis of B_{12}." From another quarter[9] came brief reports of a modified (mono?) methylated tetrapyrrole obtained from whole cells of *P. shermanii*, and which appeared to have a similar structure, incorporated radioactive methionine into its methyl group, and furthermore increased B_{12} production in the organism. Siroheme has also been identified as the prosthetic group of ferredoxin-nitrite reductase (EC 1.7.7.1) of spinach.[10]

By modifying the cell-free system developed for corrin biosynthesis it has been possible to isolate from *P. shermanii* almost 1 mg of an orange-fluorescent substance close in R_f in the usual TLC systems to uroporphyrin III. The physical constants of the methyl ester of the new isolate and of sirohydrochlorin octamethyl ester established complete identity as follows.

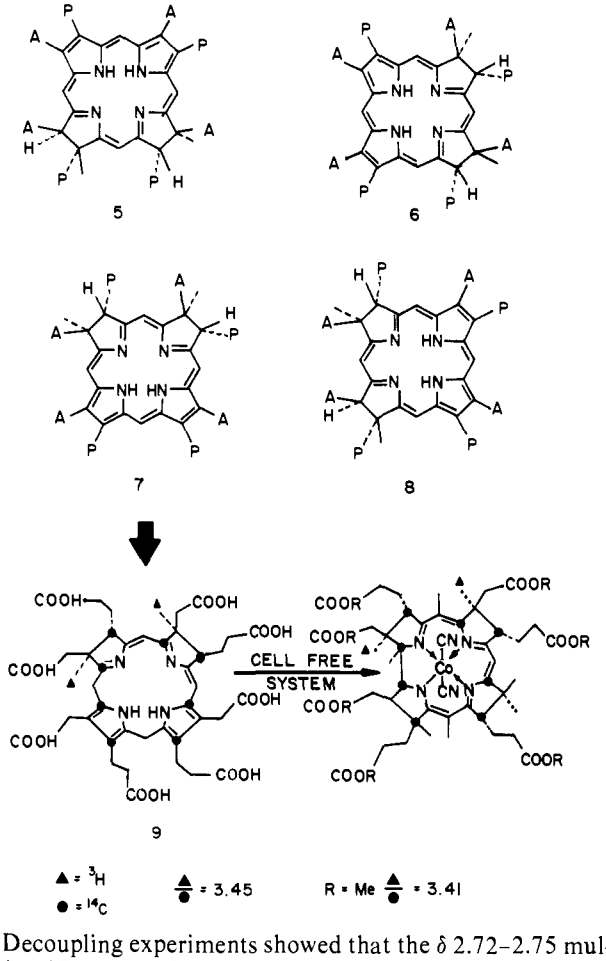
Inspection of the UV (Table I), CD (Table II), mass (Figure 1), and ^{1}H NMR (Figure 2, Table III) spectral data for the methyl ester of the *P. shermanii* metabolite and of sirohydrochlorin from *E. coli* sulfite reductase leaves no doubt that the substances, which also show complete correspondence in TLC R_f values, are identical in every respect. The molecular constitution of the *P. shermanii* metabolite methyl ester was also confirmed by high-resolution mass determination of the molecular ion (974.4167) which revealed the composition $C_{50}H_{62}N_4O_{16}$ (974.4160).

The ^{1}H NMR assignments given in Table III were made as follows. The 3 H singlet resonances at δ 1.87 and 1.83 are consistent with the known chemical shifts of methyl groups on

the reduced ring of a chlorin.[11] Similarly the 2 H multiplet at δ 4.1 also falls within the range of shifts expected for methine hydrogens on such rings.[11-13] The literature model system that is most relevant to the remaining assignments is α-tetrahydrooctaethylporphyrin, which is an isobacteriochlorin.[13] The side-chain chemical shift differences between the latter and octaethylporphyrin should parallel those between uroporphyrin and sirohydrochlorin. The assignments given in Table III followed from this assumption and the peak integrals.

As required by this assignment, irradiation of the partially hidden multiplet at δ 3.77 (C-13a, 17a) resulted in the δ 2.94 triplet (C-13b, 17b) collapsing to a singlet. Additionally, irradiation at frequencies between δ 2.0 and 2.4 simplified the δ 4.1 multiplet and vice versa, thus confirming that the methine hydrogens are coupled to the methylene hydrogens (C-3a, 8a) of the reduced-ring propionate groups. The 2 H triplet at δ 2.50 was found to be coupled to the δ 2.0–2.4 multiplet. It is, therefore, probably one of the methylene groups at C-3b and C-8b which has been shifted downfield from the other by a subtle steric or electronic effect. The 8 H multiplet at δ 2.0–2.4 is assigned to the two NH resonances and the remaining reduced-ring propionate methylene hydrogens (C-3b, 8b). Such a chemical shift would not be unusual as the NH resonances of octaethylisobacteriochlorin were reported as δ 2.96 ($CDCl_3$)[13a] and 1.77 (C_6D_6).[13b]

As pointed out by Siegel et al.,[7,8] the UV spectrum of sirohydrochlorin is diagnostic of an isobacteriochlorin in which the two reduced rings are adjacent. The presence of a methyl group at the β position of two of the pyrrole rings and the intact incorporation of sirohydrochlorin into cobyrinic acid (vide infra) allow only four possible structures, **5–8**, for sirohydrochlorin.



Decoupling experiments showed that the δ 2.72–2.75 multiplet (C-2a, 7a) is not coupled to any other resonances. Structures **5** and **8** are therefore eliminated as they would require coupling between the reduced-ring acetate methylene hydrogens (δ 2.72–2.75) and the neighboring methine hydrogen (δ 4.1). At this stage in the investigation the possible structural proposals for sirohydrochlorin were considerably reduced by a biosynthetic experiment.

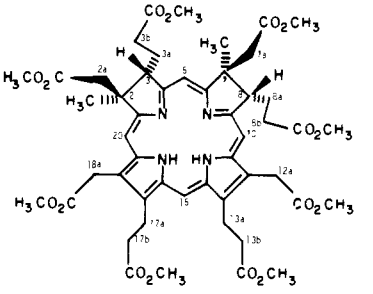
A ^{14}C-labeled specimen of sirohydrochlorin, prepared by incubation of δ-[4-^{14}C]aminolevulinic acid with the crude enzyme mixture normally used for corrin biosynthesis,[6] was converted to the octamethyl ester and chromatographed to

Table II. Circular Dichroism Spectra of Sirohydrochlorin Methyl Ester from *E. coli* NADPH-Sulfite Reductase (A) and from *P. shermanii* Cell-Free Homogenate (B)

compd	wavelength maxima and minima and molecular ellipticity[a]					
A	$[\theta] \times 10^{-3}$	$+45 \pm 11^b$	−8.6	−34	−16	$+3 \pm 1.5$
	λ	385	506	585	544	−625
B	$[\theta] \times 10^{-3}$	$+31 \pm 7^b$	−7.6	−30	−17	
	λ	386	507	584	543	

[a] Wavelength is reported in nm. Molecular ellipticity is reported in deg mol^{-1} cm^3. [b] Very high dilution.

Table III. Proton Chemical Shifts and Assignments of Sirohydrochlorin Methyl Ester from *P. shermanii* Cell-Free Homogenate (A) and from *E. coli* NADPH-Sulfite Reductase (B)[a]



7-A

position[b]	A	B	
15	8.536	8.537	1 H, s
10 or 20	7.461	7.464	1 H, s
10 or 20	7.361	7.359	1 H, s
5	6.777	6.778	1 H, s
18a, 12a	4.284	4.286	4 H, s
3, 8	4.1	4.1	2 H, m
13a, 17a	3.77	3.77	
—CO_2CH_3	3.722 3.716 3.671 3.667 3.651 3.612 3.592	3.722 3.717 3.670 3.668 3.653 3.612 3.591	28 H
13b, 17b	2.968 2.938 2.910	2.964 2.940 2.907	4 H, t
2a, 7a	2.750 2.734 2.724	2.752 2.733 2.725	4 H, m
3b or 8b	2.610 2.583 2.555	2.609 2.580 2.552	2 H, t
3a, 8a, NH and 3b or 8b	2.0–2.4	2.0–2.4	8 H, m
2,7 (CH_3)	1.869 1.828	1.869 1.828	3 H, s 3 H, s

[a] The chemical shifts are quoted in parts per million downfield from Me_4Si. The spectra were obtained at 270 MHz in $CDCl_3$. [b] See structure **7A** for the numbering system.

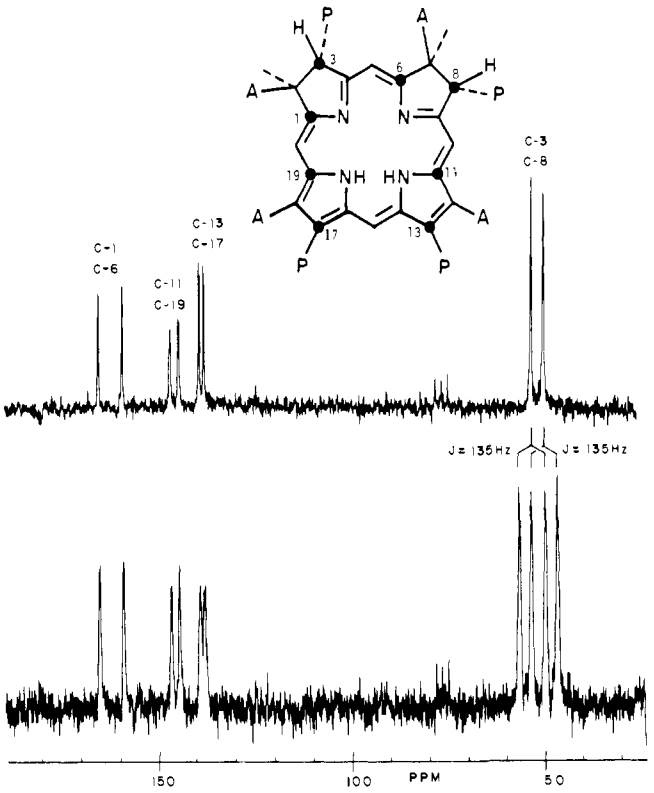


Figure 3. The proton noise decoupled (upper) and proton-coupled (lower) ^{13}C FT spectrum of [4-^{13}C]-ALA-labeled sirohydrochlorin octamethyl ester.

>98% radiochemical purity (Table IV). A sample of this ester was hydrolyzed to the carboxylic acid, reduced with sodium amalgam, and incubated with the corrin synthetase mixture.[6] Radioactive cobyrinic acid was isolated (as cobester) and recrystallized to constant activity to give (experiments 1 and 2) 1.7 and 1.9% incorporation. In order to confirm this preliminary result a doubly labeled sample of sirohydrochlorin methyl ester was prepared by separate incubations with [4-^{14}C]-ALA and [^3H_3C]-*S*-adenosylmethionine. The samples were combined ($^3H/^{14}C$ = 3.45), purified, and refed after hydrolysis and sodium amalgam reduction to generate **9**. The resultant cobester was obtained in 2.4% radiochemical yield with retention of the ratio ($^3H/^{14}C$ = 3.41). The retention of the $^3H/^{14}C$ ratio shows that reduced sirohydrochlorin is incorporated intact into cobyrinic acid and is strong evidence that it lies on the vitamin B_{12} biosynthetic pathway. It also follows that the quaternary methylated carbons of sirohydrochlorin must have the same configuration as the analogous carbons of cobyrinic acid. The same is almost certainly true of the tertiary carbons. Only structures **6** and **7** are consistent with intact incorporation into cobyrinic acid and with the previously described spectral data.

In view of the correspondence of the oxidation level of **9** and of its biosynthetic product cobyrinic acid (**3**), it was surprising to find that incubation of the unreduced species **7** led to equally good (0.3–2.8%) incorporation (Table VI). We discuss below the implication of this finding. At this stage in the investigation Müller[14a] described the isolation and cell-free conversion of a *P. shermanii* metabolite, Faktor II, to cobyrinic acid. Comparison of the reported physical data for Faktor II and sirohydrochlorin leaves little doubt that they are identical.

Two structures, **6** and **7**, are still consistent with the spectroscopic and bioincorporation data mentioned above. However, since it is unlikely that methylation of ring C occurs before decarboxylation of the acetate side chain during B_{12} biosynthesis,[15] structure **7** becomes attractive for sirohydrochlorin.

Table IV. Biosynthesis of [4-^{14}C]-ALA- and [^3H_3C]-SAM-Labeled Sirohydrochlorin by *P. shermanii* Cell-Free Homogenate

expt no.	substrate	amount of radioactivity, μCi	% radiochemical yield[e]
1	[4-^{14}C]-ALA[a]	50	8.1
2	[4-^{14}C]-ALA[a]	100	3.3
3	[4-^{14}C]-ALA[a,b]	2	0.003
4	[$^{14}CH_3$]-SAM[c]	2	8.5
5	[$^{14}CH_3$]-SAM[c]	4	3.5
6	[^3H_3C]-SAM[d]	100	9.2
7	[^3H_3C]-SAM[b,d]	5	0.002

[a] Specific activity 26.6 mCi/mmol. [b] Boiled enzyme. [c] Specific activity 60.5 mCi/mmol. [d] Specific activity 12.25 Ci/mmol. [e] Radiochemical yield = (dpm of substrate × 100)/(dpm of sirohydrochlorin).

Table V. Incorporation of Reduced Radiolabeled Sirohydrochlorin into Cobyrinic Acid by *P. shermanii* Cell-Free System

expt no.	substrate	amount of radioactive substrate	% radiochemical yield of cobester
1	[4-^{14}C]-ALA-labeled sirohydrochlorin	1.2 μCi[a]	1.7
2	as above	1.5 μCi[a]	1.9
3	as above	0.94 μCi	0.4
4	as above[c]	0.99 μCi	0.003
5	sirohydrochlorin $^3H/^{14}C$ = 3.45	6.3 × 10^5 cpm	2.4[b] ($^3H/^{14}C$ = 3.41)

[a] Specific activity 57 mCi/mmol assuming[27] $\epsilon_{588} = 2.4 \times 10^4$ mol^{-1} cm^2. [b] Based on cpm. [c] Boiled enzyme.

The complete stereostructure **7** was confirmed by a series of biosynthetic experiments in which ^{13}C labeling proved to be of diagnostic value in arriving at a unique solution. It was first shown that a natural abundance ^{13}C NMR spectrum could be obtained on a 500-μg sample in which 10^6 transients were collected over 252 h. Next, incubation of 60 mg of [4-^{13}C]-aminolevulinic acid (90% enriched) with the *P. shermanii* cell-free homogenate yielded a purified sample of sirohydrochlorin (400 μg) whose proton-decoupled and proton-coupled spectra are shown in Figure 3. It is clear that only structures **6** and **7** are compatible with the observation of a doublet (J = 135 Hz) for each sp^3-carbon resonance (C-3 and C-8), since structures **5** and **8** would exhibit only one such enriched sp^3-carbon bearing hydrogen. This experiment confirms the same conclusion reached by the previously described ^1H NMR decoupling method.

Although the partial assignments of the sp^2-carbon resonances (Figure 3) are tentative, assignment of the δ 165.6 and 159.3 resonances to C-1 and C-6 is almost certainly correct as the analogous carbon resonances in various chlorins[16] occur at δ 159–173. The C-1, C-6, and C-11 resonances are broadened by multiple, unresolved long-range couplings. However, the C-13, C-17 pair are clearly resolved doublets (J = 5.4 and 5.0 Hz) arising from a single 1,3 coupling in each case (Figure 4).

Finally, incubation with [5-^{13}C]aminolevulinic acid enabled us to show that sirohydrochlorin has structure **7**. The expected labeling pattern is as shown in Figure 5. In the proton decoupled spectrum (Figure 5, spectrum (A)), the C-15 resonance is a triplet (J = 71.1 Hz) due to 1,2 coupling with two adjacent enriched sites. (A lower intensity doublet is also present due to those molecules having only one adjacent enriched site.) The C-5 and C-10 resonances both occur as doublet of doublets due to 1,2 and 1,4 couplings while C-20 shows only 1,4 interactions.

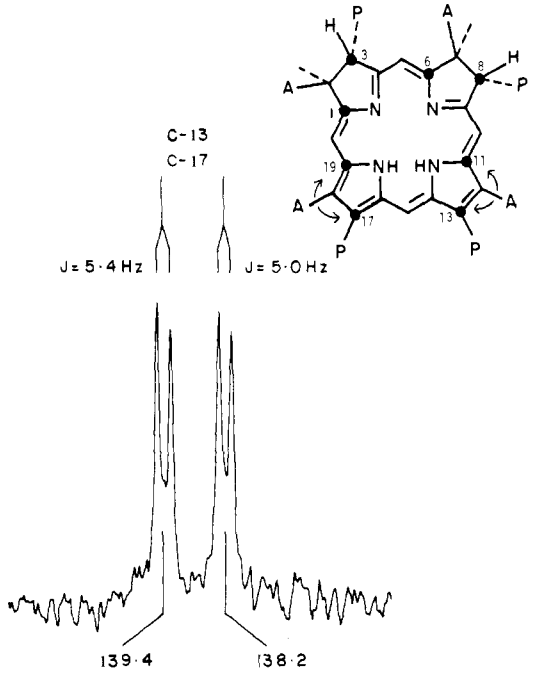


Figure 4. An expansion of the C-13 and C-17 resonances of the upper spectrum in Figure 3.

Table VI. Incorporation of Radiolabeled Sirohydrochlorin into Cobyrinic Acid by *P. shermanii* Cell-Free System

expt no.	substrate	amount of radioactive substrate, μCi	% radiochemical yield of cobester
1	[4-^{14}C]-ALA-labeled sirohydrochlorin	0.94[a]	0.3
2	as above	0.47	1.6
3	as above	0.27	2.8
4	as above[b]	0.94	0.005

[a] Specific activity 57 mCi/mmol. [b] Boiled enzyme.

The four meso-hydrogen ^{1}H NMR resonances occurred as doublets[17] at δ 8.54, 7.46, 7.36, and 6.78 (Figure 6). As discussed by Bonnett et al.,[13a] the upfield meso-hydrogen resonance of an isobacteriochlorin may be assigned to that between the reduced (methylated) rings and the downfield resonance to that between the nonreduced rings. Thus, the δ 8.54 hydrogen would be coupled to C-15 in structure **7** and to C-20 in structure **6**. The former case was confirmed by selective irradiation of the δ 8.54 hydrogen resonance. As can be seen from spectrum (B) in Figure 6, the C-15 resonance at δ 107.4 remained a triplet while the other meso-carbon resonances showed coupling to meso-hydrogen resonances.

The other meso-carbon resonances were assigned as follows. During selective irradiation of the upfield meso-hydrogen resonance at δ 6.78, the upfield meso-carbon resonance at δ 89.4 remained unchanged while the others became more complex. The δ 89.4 resonance may therefore be assigned to C-5 and the other doublet of doublets resonance at δ 95.4 to C-10. The absence of a large coupling to an enriched neighbor identified the triplet resonance ($J \approx 6$ Hz) at δ 93.5 as C-20. Not surprisingly, the distribution of meso-carbon chemical shifts (C-15 > C-10 ≈ C-20 > C-5) parallels that of the meso-hydrogen chemical shifts, being a reflection of the relative charge density at each meso carbon.[13a]

Of the remaining enriched carbon resonances (Figure 7),

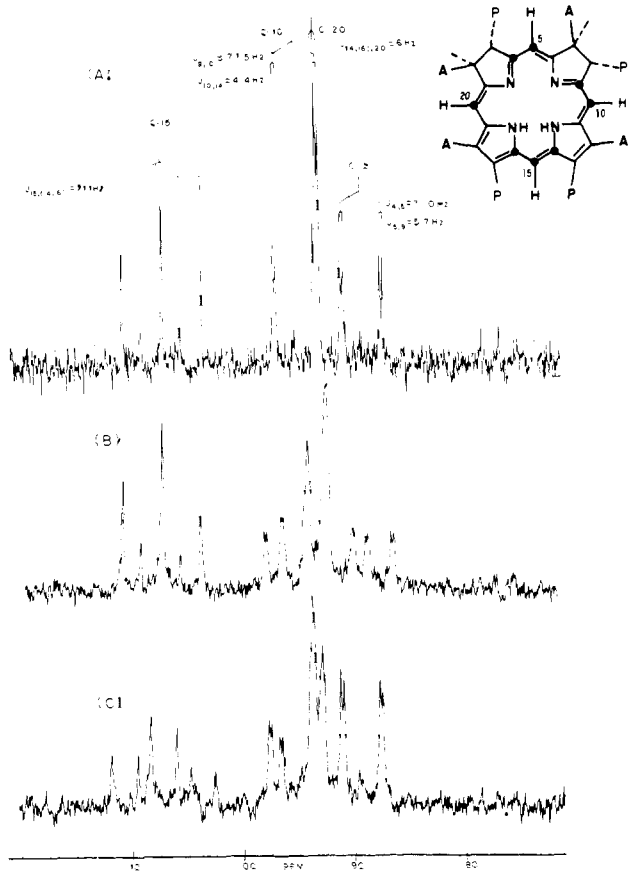


Figure 5. The meso-carbon resonances of the ^{13}C FT spectrum of [5-^{13}C]-ALA-labeled sirohydrochlorin octamethyl ester with proton noise decoupling (spectrum A), selective irradiation of the δ 8.54 meso-hydrogen resonance (spectrum B), and selective irradiation of the δ 6.78 meso-hydrogen resonance (spectrum C).

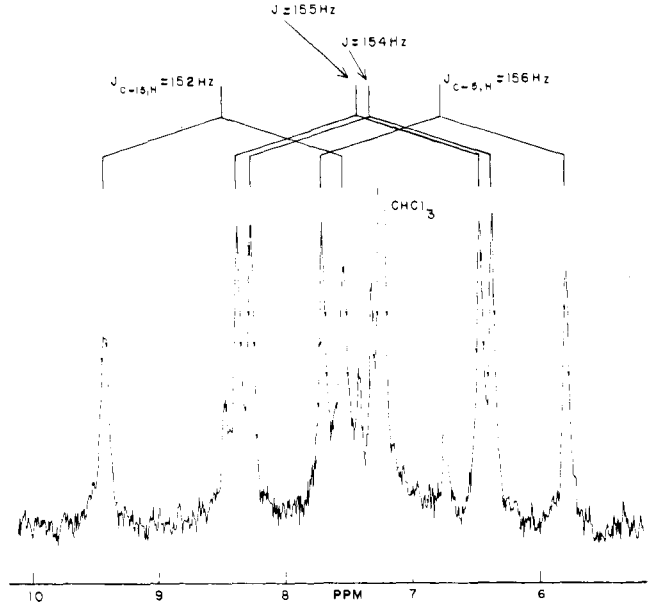


Figure 6. The ^{1}H NMR spectrum of the meso hydrogens in [5-^{13}C]-ALA-labeled sirohydrochlorin octamethyl ester.

C-9 was known to be coupled to C-10 with $J_{9,10}$ = 77.5 Hz. The δ 152.8 resonance with a splitting of 77 Hz may therefore be assigned to C-9. The expected long-range coupling $J_{5,9}$ = 5.7 Hz was incompletely resolved. With the knowledge that the sp^2-carbon resonances of the reduced rings would occur at

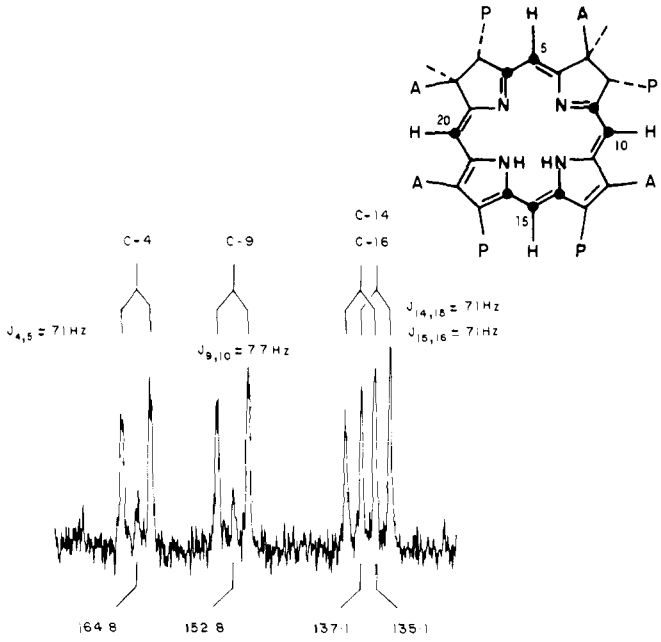


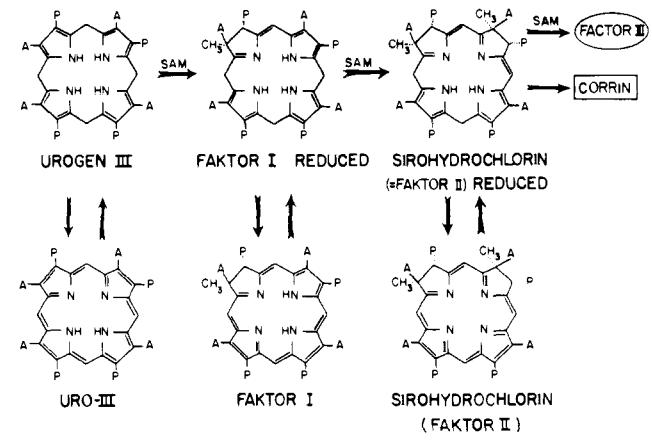
Figure 7. The downfield region of the proton noise decoupled ^{13}C FT spectrum of [5-^{13}C]-ALA-labeled sirohydrochlorin octamethyl ester.

lower field than those of the nonreduced rings,[16] the δ 164.8 doublet (J = 71 Hz) was assigned to C-4. Owing to long-range coupling with C-20, the latter peaks showed an additional splitting of 5.7 Hz. This value agrees within experimental error with the approximate coupling constant of 6 Hz for $J_{4,20}$ shown in Figure 5. As expected from the triplet C-15 resonance (J = 71.1 Hz), the C-14 and C-16 doublets have identical coupling constants of 71 Hz. Broadening of the latter peaks is present but the long-range $J_{16,20}$ and $J_{10,14}$ couplings are not resolved.

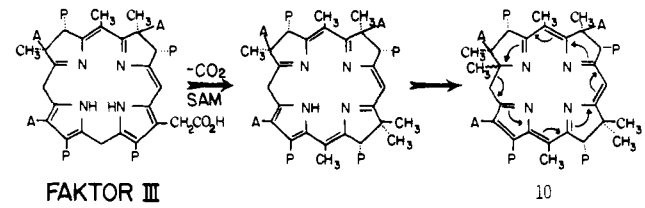
Other workers[14,18] have recently isolated a metabolite from *P. shermanii* with similar UV and mass spectral characteristics to sirohydrochlorin. The Cambridge group postulated structure **7** for their metabolite after assuming that it was on the B_{12} pathway and further that ring C was not methylated.[18c] This paper shows unequivocally that sirohydrochlorin is indeed identical with a *P. shermanii* metabolite, that it has structure **7**, and that it is an intermediate on the corrin pathway. The possibility that siroheme represents a prebiotic sulfate-reducing agent[8] and further that both sirohydrochlorin and vitamin B_{12} producing anaerobic organisms predate the evolution of heme-synthesizing aerobes,[19] suggest that the reductive methylation of reduced porphyrins may be a phenomenon of considerable antiquity (3 billion years).

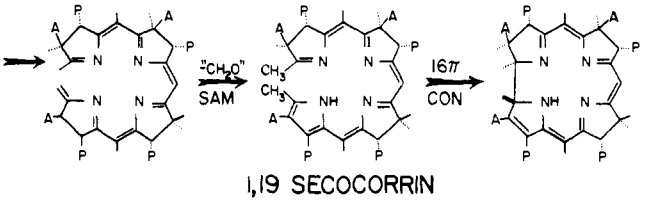
The intermediacy of the dimethylated isobacteriochlorin (**7**) in corrin biosynthesis requires not only reappraisal of the specific incorporation of uro'gen III heptacarboxylic acid (**4**) into cobyrinic acid (**3**) which could be explained by nonspecific enzymatic conversion of a substrate closely related to, but not identical with, the normal metabolic intermediate, but also a significant modification of our present working hypothesis[15] for post-uro'gen III corrin biosynthesis. With the knowledge that **7** is finally established as an intermediate by isolation in cobalt-deficient incubations and by intact specific incorporations into cobyrinic acid (thereby defining the absolute stereochemistry of sirohydrochlorin), the required changes demanded by the sirohydrochlorin–cobyrinic acid pathway are, not necessarily in this order, as follows: (1) introduction of five additional methyl groups from *S*-adenosyl methionine with retention of the methyl protons;[2c] (2) evolution of C-20 and its trapping as formaldehyde[2d] with or without biochemical reduction, e.g., formate → formaldehyde; (3) reclosure of the

Scheme I



Scheme II



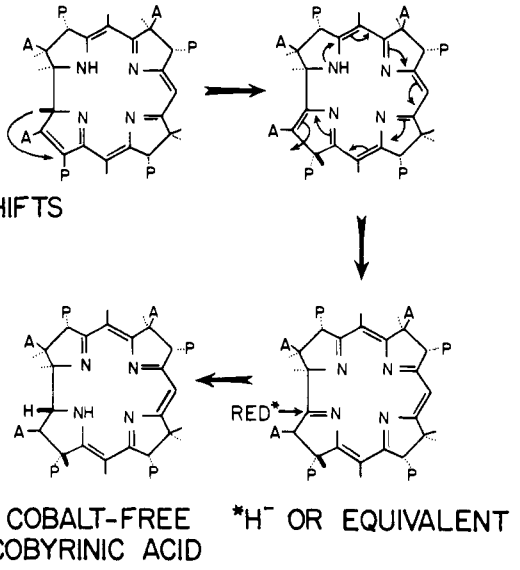


secocorrin system[19] to a dehydrodecobaltocorrinoid; (4) two-electron reduction to corrin; (5) insertion of cobalt with valency change $Co^{2+} \rightarrow Co^{3+}$.

As a guide to future experimentation we offer the following mechanistic rationale of these processes based on the known or presumed chemistry of the reduced porphyrins and of corrins. Many of the ideas embodied in the schemes are presently being tested experimentally and include certain key modifications of a proposal for corrin biosynthesis published several years ago,[15] some of which stem from the recent elegant chemical analogies uncovered in Eschenmoser's[19] several synthetic approaches to corrins.

The hypotheses embodied in Schemes I and II are developed from the known incorporation of sirohydrochlorin (= Faktor II) and the *presumed* intact incorporations of Faktors I and III all taking place at the *reduced* level (Scheme I). The experimental observation of incorporation of Faktors II and III[14] but only the reduced form of Faktor I[14b] indicates the presence of a nonspecific oxido-reductase system which, however, is not capable of reducing either uro III to uro'gen III or Faktor I. Scheme II portrays decarboxylation at ring C and further methylation at C-1, C-7, and C-10. The intermediate formed (**10**) could then lead to cobyrinic acid via transformations analogous to those previously discussed.[15,19] However, in an interesting variant shown in Scheme II, electrocyclic ring opening, loss of "formaldehyde", methylation at C-19, and electrocyclic reclosure would generate a 1,19 *trans*-dimethyldehydrocorrin. The last part of the sequence (Scheme III) portrays the 1,5 shifts of the β-oriented C-19 methyl in ring D to C-17, its final resting place on the upper face of the molecule. A splendid analogy for this step has just been discovered by Johnson.[20] The pathway would then terminate by two-

Scheme III



electron reduction to cobalt-free cobyrinic acid followed by or synchronized with insertion of cobalt.[15,21]

Experimental Section

General. Thin layer chromatographic (TLC) separations were carried out on E. Merck (Darmstadt) precoated silica gel 60 F-254 plates. Ultraviolet-visible (UV) absorption spectra were obtained with a Shimadzu UV-200 double-beam spectrometer, and circular dichroism spectra were obtained with a Cary 60 instrument. High-pressure liquid chromatography (HPLC) was carried out using a Waters Associates ALC/GPC-204. Proton magnetic resonance spectra were obtained with a 270-MHz Bruker HX-270 instrument using "100%" (Merck) $CDCl_3$ distilled from calcium hydride. Carbon-13 FT spectra were obtained in $CDCl_3$ in 1.7-mm tubes with a Varian FT-80 instrument.

Cell disruptions were achieved with a Heat Systems—Ultrasonics, Inc. Model W200R.

Radioactivity determinations were made using a Packard Model 3320 Tri-Carb scintillation spectrometer. Radioactive samples were dissolved in benzene and the sample concentration was determined by UV absorbance. An aliquot of this solution was counted in Bray's solution. To correct the cpm obtained to dpm, efficiency curves were constructed using the channel ratio method. For added accuracy the curve for radioactive cobester was obtained by adding radioinactive cobester to radioactive standards.

Mass spectra were obtained using an Associated Electrical Industries MS902 mass spectrometer, through the courtesy of Dr. A. Gossauer.

Materials. NADH, NADPH, and SAM were supplied by Sigma Chemical Co. DTT was supplied by Calbiochem. δ-[4-^{14}C]Aminolevulinic acid (ALA, specific activity 26.6 mCi/mmol, [^3H_3C]-SAM (specific activity 12.25 Ci/mmol), and [$^{14}CH_3$]-SAM (specific activity 60 mCi/mmol) were supplied by New England Nuclear Co.

Preparation of Cell-Free Homogenate. *Propionibacterium shermanii* cells (ATCC9614) were grown as described previously.[5,22] The freshly harvested wet cells (50 g) were suspended in 0.01 M potassium phosphate buffer (50 mL) at pH 7.6. After the addition of 1 drop of 2-mercaptoethanol the suspension was sonicated for 10 min at 5–10 °C under a stream of nitrogen. The supernatant solution of the centrifuged suspension (37 000 g, 20 min at 0 °C) was used for the following experiments. Protein concentration[23] of the supernatant was 14–18 mg/mL.

Isolation of Sirohydrochlorin Octamethyl Ester from Cell-Free Homogenate. To the crude enzyme solution were added the following ingredients: SAM (25 mg), $MgCl_2 \cdot 6H_2O$ (45 mg), DTT (18 mg), and ALA (10 mg). The pH of the resulting solution was adjusted to 7.6 with potassium phosphate (K_2HPO_4). The flask was then successively evacuated and flushed with nitrogen several times. After the flask was sealed in vacuo it was allowed to stand in the dark at 20–23 °C for 16 h. Air was then admitted and the solution was frozen at −78 °C prior to lyophilization. The freeze-dried residue was suspended in methanol containing 5% sulfuric acid (60 mL) which was stirred overnight under nitrogen. The reaction solution was diluted with chloroform and neutralized with excess saturated aqueous sodium bicarbonate. The aqueous phase was extracted several times with chloroform and the combined organic phases were dried (sodium sulfate) and evaporated. The residue was chromatographed using benzene–ethyl acetate–methanol (80:16:4). A fluorescent-orange band was observed at R_f 0.63 slightly ahead of the fluorescent-red uroporphyrin band. The former was extracted and rechromatographed with multiple elution in the same solvents (85:13:2). Under these conditions the fluorescent-orange sirohydrochlorin methyl ester is readily separated from traces of uroporphyrin methyl ester.

Preparation of ^{13}C-Labeled Sirohydrochlorin Methyl Esters. A. δ-[4-^{13}C]-ALA was prepared by the method of Mitta et al.[24] for [4-^{14}C]-ALA using K^{13}CN (1.5 g, Merck of Canada, 90 atom %) as the source of label. Incubation of δ-[4-^{13}C]-ALA (64 mg) under the conditions described above yielded labeled sirohydrochlorin methyl ester (400 μg) after first TLC and then HPLC purification (C_{18} μ-Bondapak, methanol/water, 80:20).

B. δ-[5-^{13}C]-ALA was prepared by the method of Battersby et al.[25] using K^{13}CN (1.5 g, Merck of Canada, 90 atom %) as the source of label. Incubation of δ-[5-^{13}C]-Ala (80 mg) as described above yielded labeled sirohydrochlorin methyl ester (676 μg).

An HPLC (reverse phase C-18 μ-Bondapak, 80% methanol/water buffered with ammonium carbonate) failed to resolve the sirohydrochlorin band any further and confirmed its purity. Attempts to recrystallize this compound from a variety of mixed solvents gave only an oily solid. Spectroscopic properties are reported in the text.

Preparation of Radiolabeled Sirohydrochlorin Methyl Esters. Incubation and workups were carried out as described above, using firstly [4-^{14}C]-ALA (specific activity 26.6 mCi/mmol, 50–100 μCi) and secondly [^3H_3C]-SAM (specific activity 12.25 Ci/mmol, 100μCi). Cold carrier sirohydrochlorin methyl ester was added after the esterification. In addition to the two TLC purifications reported earlier, three further TLC purifications (two with multiple elution) were employed to ensure radiochemical purity. A radioscanning analysis of a single-elution TLC of the resulting sirohydrochlorin methyl esters showed them to be >98% radiochemically pure. Identical experiments were carried out using boiled enzyme solution.

Samples of [4-^{14}C]-ALA- and [^3H_3C]-SAM-labeled sirohydrochlorin methyl ester were mixed to give a doubly labeled specimen having $^3H/^{14}C = 3.45$.

Incubation of ^{14}C/^{3}H Labeled Reduced Sirohydrochlorin with Cell-Free Homogenate. The labeled sirohydrochlorin methyl ester was dissolved in tetrahydrofuran (2 mL) and stirred with 2 M potassium hydroxide (2 mL) at 20 °C under nitrogen overnight. The aqueous phase was washed with tetrahydrofuran and adjusted to ~pH 8 with dilute hydrochloric acid and 1 M potassium phosphate buffer, pH 7.4. The resulting solution was shaken under nitrogen in the dark with crushed, freshly prepared 3% sodium amalgam until no fluorescence was observable. The solution was filtered under nitrogen into the cell-free homogenate (50 mL) containing the following ingredients: SAM (25 mg), $MgCl_2 \cdot 6H_2O$ (45 mg), DTT (18 mg), NADH (10 mg), NADPH (10 mg), and $CoCl_2 \cdot 6H_2O$ (15 mg). The pH was adjusted to 7.6 with potassium phosphate (K_2HPO_4) and the flask was successively evacuated and flushed with nitrogen several times. After incubation for 16 h in the dark under vacuum at 20 °C air was admitted to the flask. Potassium cyanide (20 mg) was added to the solution which was then frozen at −78 °C and lyophilized. To the residue was added further potassium cyanide (20 mg) and 5% sulfuric acid in methanol (75 mL) which was refluxed for 5 h. After the solution was cooled it was diluted with chloroform and neutralized with saturated aqueous sodium bicarbonate. Potassium cyanide (20 mg) and cold cobester (5 mg) were added to the mixture. The aqueous phase was extracted several times with chloroform and the combined organic phases were dried (sodium sulfate) and evaporated. The residue was chromatographed twice via TLC (silica) using benzene–ethyl acetate–methanol (80:16:4) and then further purified by HPLC on a Waters reverse phase alkylphenyl column (methanol/water, 70:30). The cobester obtained was recrystallized to constant specific activity (see Table V).

Incubation of ^{14}C-Labeled Sirohydrochlorin with Cell-Free Homogenate. The [4-^{14}C]-ALA-labeled sirohydrochlorin octamethyl ester was hydrolyzed and incubated as described above, i.e., without sodium amalgam reduction. The cobyrinic acid product was also purified to constant activity as described above (see Table VI).

Isolation of Sirohydrochlorin Methyl Ester from *E. coli* NADPH-Sulfite Reductase. A solution of *E. coli* NADPH-sulfite reductase (10 mL, 54 mg/mL)[7] in 0.05 M potassium phosphate buffer pH 7.7, 0.1 mM DTA, was frozen at −78 °C and lyophilized. Dry methanol (40 mL) and anhydrous ferrous sulfate (200 mg)[26] were added to the residue. Dry hydrogen chloride was bubbled through the suspension at 0 °C for 2 min. After stirring overnight in the dark at 20 °C the solution was diluted with chloroform, neutralized with saturated aqueous sodium bicarbonate, and worked up as usual. The residue was chromatographed as described above to yield chromatographically pure sirohydrochlorin methyl ester.

The *E. coli* NADPH-sulfite reductase contains 5.47 mg of siroheme per g of enzyme.[27] Assuming an extinction coefficient[27] of 2.4 × 10^4 mol^{-1} cm^2 at 588 nm, the yield of sirohydrochlorin methyl ester was 0.95 μmol, i.e., about 30%. Spectroscopic properties are reported in the text (Tables I-III).

The samples of sirohydrochlorin methyl ester from *E. coli* enzyme and from *P. shermanii* cell-free homogenate migrated with identical R_f in benzene-ethyl acetate-methanol (80:16:4) (85:13:2 with multiple elution) and chloroform-methanol (9:1).

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The Structure of 9,9′-Bitriptycyl

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Abstract: The molecular structure of 9,9′-bitriptycyl has been determined by X-ray crystallography and empirical force field calculations. Agreement is excellent for all bond lengths and angles except for the central bond, where the X-ray distance is 0.031 Å shorter than the calculated value. This discrepancy may be rationalized, in part at least, by invoking rehybridization of the central carbon atoms as a result of strain-induced distortions of valence bond angles; such electronic effects are not taken into account by current empirical force fields.

Introduction

9,9′-Bitriptycyl (**1**) and its derivatives offer the possibility for a number of revealing studies. Molecular models indicate that the opposing inner peri carbon and hydrogen atoms are in very close proximity to each other. The resulting large steric strain should cause appreciable distortion from normal bond distances and angles. It was therefore of interest to carry out an X-ray analysis of **1**, and to compare the results to those of less crowded, but structurally related, compounds.

Also of interest is a comparison of the results of the X-ray analysis and empirical force field (EFF) calculations[2] on **1**. The substantial steric interactions present should pose a challenging test for these calculations.

Because of the close proximity of opposing groups, a large

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