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Reference Data

^{1}H and ^{13}C NMR Assignments for 3- and 8-Episirohydrochlorin Methyl Esters

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Assignments are provided for the protons and directly bonded carbons of 3- and 8-episirohydrochlorin methyl esters based upon material isolated from *Desulfovibrio vulgaris*. The chemical shifts of the two isomers are highly similar to each other and also to the physiologically relevant authentic sirohydrochlorin, but are sufficiently distinct that NMR can be reliably used to detect their presence in natural isolates.

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INTRODUCTION

It has recently been shown[1] that siroheme (**1a, b**) acting as the prosthetic group in the sulfite reductase of the primitive bacterial genus *Desulfovibrio* is regiospecifically amidated at the 2-acetate substituent, whereas in other species it is not. In the same paper it was noted that an isomer of **1** was isolated from whole cell extracts of *Desulfovibrio*. The stereochemistry of that isolate was not reported, since it was not critical to the spectroscopic characterization of siroamide **1b**. Assignment was complicated by the availability of only limited spectroscopic data in the literature on isomers of **1**, and by peculiarities in the NMR and chromatographic properties of the isomers. Material has now been isolated to permit a firm identification which turned out to be a nearly equimolar mixture of two epimers of **1** at the 3- and 8-positions. The purpose of this paper is to provide sufficiently comprehensive NMR data such that if these isomers are encountered in other studies on natural tetrapyrroles, they may be easily identified.

EXPERIMENTAL

Tetrapyrroles isolated for the bulk of this work came from *Desulfovibrio vulgaris*, but smaller scale isolations from other species of *Desulfovibrio* are highly similar. Natural tetrapyrroles were converted into their ester derivatives by mineral acid esterification as described previously,[2] except that HCl-saturated methanol was used. NMR spectroscopy was performed at 500 MHz on a Bruker AM500 instrument. In our spectrometer, inverse-detected ^{13}C experiments utilize a broadband ^{13}C pulse train to remove $^{1}J(CH)$ during acquisition, and this slightly heats the probe. Therefore, all experiments were performed at 305 K in a temperature-regulated probe. Scalar coupling was determined by two-dimensional HOHAHA experiments.[3] Rotating frame nuclear Overhauser enhancements were determined by a ROESY pulse sequence.[4] Carbons with directly attached protons were assigned by an HMQC experiment.[5] The nomenclature scheme follows IUB–IUPAC conventions for tetrapyrroles. Primed numbers indicate groups attached to the uroporphyrinogen core during *C*-methylation.

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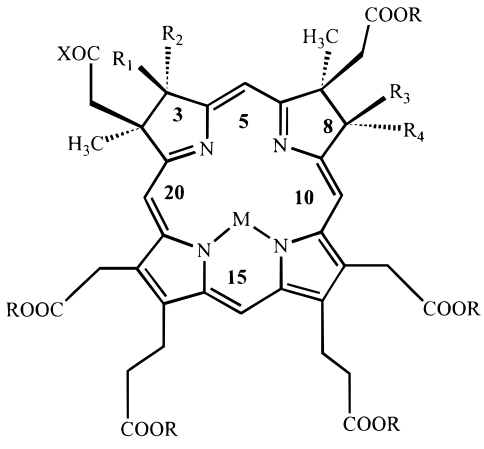
RESULTS AND DISCUSSION

The isomer of **2** with epimerization at the 3-position (**3**) has been observed previously and its stereochemistry assigned.[6] However, only limited resonance assignments were reported. We initially assumed that the isomer isolated from *D. vulgaris* was the same epimer, since its NMR spectrum did have some resonances that matched the previous report. Liquid secondary ion (LSI) mass spectrometry indicated only a parental ion of *m/z* of 975 (corresponding to **2** and its isomers) while the low-mass region showed the usual clutter of daughter fragments. There were additional features in the ^{1}H NMR spectrum corresponding to too many protons for a single compound **3**. Our chromatographic conditions had been optimized to provide baseline separation of **2** from its isomer and other natural tetrapyrroles present such as uroporphyrin III octamethyl ester [HPLC on 5 μm silica, Alltech Econosil, 250 × 4.6 mm i.d., eluted at 1 ml min^{-1} with chloroform–ethyl acetate (95:5) containing 0.025% pyridine; capacity factor k' for **2** is 4.56 and for the isomer isolate 3.75, while uroporphyrin methyl ester elutes with $k' = 4.81$]. Re-chromatography of the isomer under other conditions did not resolve the material into separable fractions. This does not mean that other more skillful workers could not achieve separation, but in our hands we were limited to dealing with what turned out to be a mixture. ^{1}H NMR showed two spin coupled doublets at 7.14 and 6.78 ppm ($J = 8.2$ Hz) with relative areas of 1:1, but non-integer and variable (among different preparations) areas compared with tetrapyrrole resonances. These



1a: siroheme, b: siroamide
M=Fe, R=H, a: X=OH, b: X=NH_2
R_1=R_3=H, R_2=R_4=CH_2CH_2COOH
2: sirohydrochlorin octamethyl ester
M=H,H, R=X=OCH_3
R_1=R_3=H, R_2=R_4=$CH_2CH_2COOCH_3$
3: 3-epimer
M=H,H, R=X=OCH_3
R_2=R_3=H, R_1=R_4=$CH_2CH_2COOCH_3$
4: 8-epimer
M=H,H, R=X=OCH_3,
R_1=R_4=H, R_2=R_3=$CH_2CH_2COOCH_3$
5: 3,8-di-epimer
M=H,H, R=X=OCH_3,
R_2=R_4=H, R_1=R_3=$CH_2CH_2COOCH_3$

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chemical shifts are very close to the aromatic ring protons of tyrosine. One-dimensional or HOHAHA scalar-correlated spectra did not show any resonances near the expected positions of the α- and β-protons of tyrosine or its ester derivative and ROESY spectra did not show that the doublets were dipolar-correlated with any other resonances. Our isomer fraction, derived from a whole cell extract, may contain some disubstituted phenyl compound related to the ring of tyrosine that persistently co-elutes with the isomer. The LSI mass spectrum of the isolate showed too many ions in the low-mass range to provide evidence to aid in further identification.

To the best of our knowledge, the 8-epimer of sirohydrochlorin (**4**) has been only briefly mentioned in the literature,[7] and no report of its NMR properties has been made. Proof that our isomer fraction contained both **3** and **4** was complicated by the fact that the two epimers were present in equivalent amounts and that many of their resonances were coincident at 500 MHz. For example, an apparent singlet is observed at 8.49 ppm in routine ^{1}H NMR spectra, corresponding to the previously assigned 15-*meso* proton of the 3-epimer. In resolution-enhanced spectra (Fig. 1), it is resolved into two peaks of equal area at 8.493 ppm (3-epimer) and 8.489 ppm (8-epimer) with a minor shoulder at 8.482 ppm.

Even though the two epimers were never separated, two-dimensional spectroscopy did permit a reasonably complete assignment of distinctive proton resonances. Attached carbons were also assigned via inverse-detected experiments in order to make a comparison to limited carbon assignments provided earlier based upon isotopically enriched material, and to aid further in resolving spin subsystems. The basic assignment strategy has been described in principle previously.[8] HOHAHA spectra identified scalar coupled systems, while nuclear Overhauser enhancements identified the distinctive pattern of nearest neighbors. The assignments are reported in Table 1.

Some features of the epimer spectra are noteworthy. Sites removed from the chiral centers are indistinguishable, as reflected by the degenerate chemical shifts for C-12 through C-18. Ester methyl resonances overlapped so strongly that individual assignments were not possible. Weak peaks were observed at 8.482 (15), 7.403 (10), 6.676 (5), 1.634 and 1.611 ppm ($2^{1'}$, $7^{1'}$) that could have been due to a small amount of the 3,8 double epimer in the mixture, but their intensity was too weak to prove this conclusively. In the original assignment of the 3-epimer, a γ-effect was noted for C-$2^{1'}$ (a shift to higher frequency for the carbon and to lower frequency for the attached proton, compared with the $7^{1'}$-position, where the methyl group is in a *cis* configuration with respect to the propionate at 8^1). Examination of rows in Table 1 indicates a highly consistent pattern of relative shifts in both isomers for sites 2–8 depending upon the relative orientations at sites 3 and 8. The observed nuclear Overhauser enhancements were consistent with the assigned stereochemistry. For substituents on adjacent β-pyrrole carbons, strong enhancements were seen in each isomer between the *cis* pair, whereas only weak enhancements were seen for the *trans* pair. For example, in the 8-epimer, the $7^{1'}$ methyl gave a strong enhancement to the $8^{1'}$ proton, but a weak enhancement to 8^1 methylene protons. For the 7^1 acetate protons the enhancement magnitudes were reversed. At C-2 and C-3 the situation was reversed again reflecting inversion at C-3. The enhancement patterns are supportive of the relative configurations at C-2 through C-8, but would not establish the absolute stereochemistry. For this we are relying upon the earlier

Table 1. Proton and carbon chemical shifts and assignments of the sirohydrochlorin octamethyl ester epimers[a]

	3-Epimer		8-Epimer		Sirohydrochlorin	
Position	^{1}H[b]	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
5	6.707	89.8	6.623	89.1	6.768	89.3
10	7.440	96.2	7.391	95.4	7.455	95.1
15	8.493	107.9	8.489	107.9	8.530	107.2
20	7.217	92.1	7.319	94.3	7.343	93.2
2^1	3.25, 3.32	38.0	2.656	46.6	2.729, 2.713	46.4
$2^{1'}$	1.56	27.4	1.908	19.5	1.859	20.3
3^1	1.75	25.5	1.94	26.1	2.106, 2.35	26.1
3^2	2.27, 2.13	31.1	2.29, 2.17	31.5	2.460, 2.35	31.9
$3^{1'}$	3.81	55.0	4.03	54.2	4.100	53.5
7^1	2.728	44.5	3.24	38.0	2.745	44.7
$7^{1'}$	1.815	19.5	1.56	27.4	1.818	19.4
8^1	1.88	26.1	1.72	25.5	2.022, 2.35	26.1
8^2	2.48, 2.30	32.0	2.44, 2.28	31.8	2.574	31.9
$8^{1'}$	4.00	50.7	3.78	51.8	4.063	50.3
12^1	4.26	31.5	4.24	31.5	4.282	31.5
13^1	3.71	21.0	3.71	21.0	3.712	20.9
13^2	2.92	36.5	2.92	36.5	2.930	36.5
17^1	3.71	21.0	3.71	36.5	3.712	20.9
17^2	2.92	36.5	2.92	36.5	2.930	36.5
18^1	4.24	31.5	4.26	31.5	4.278	31.5
Ester CH_3	3.6–3.8	52–54	3.6–3.8	52–54	3.58–3.71	52–54

[a] At 305 K in deuterated chloroform, which was used as the internal chemical shift standard at 7.26 and 77.0 ppm.
[b] Chemical shifts reported to three decimal places indicate that these resonances were sufficiently resolved to be identified in normal one-dimensional spectra, whereas shifts reported to two decimal places indicate that they were observed and assigned from cross peaks in two-dimensional spectra.

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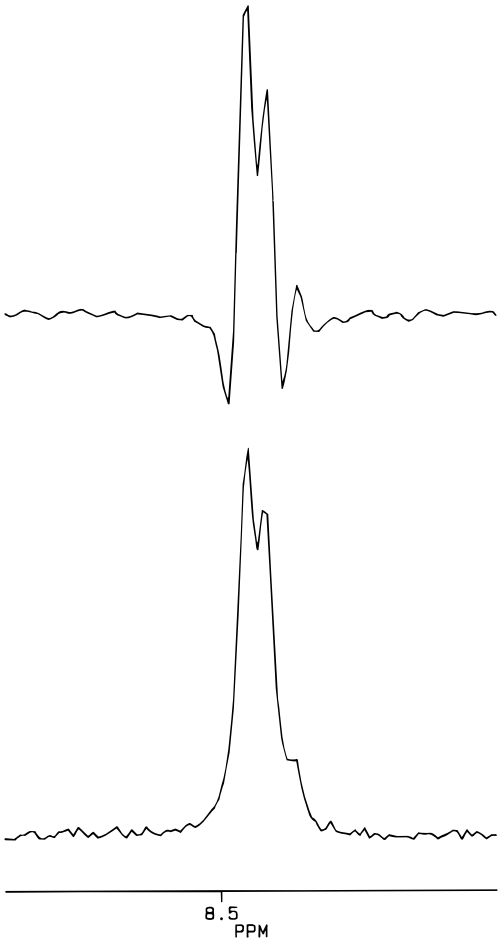

Figure 1. The 15-*meso* resonances of the 3- and 8-epimers of sirohydrochlorin methyl ester. The bottom trace is unapodized, whereas the top trace has been resolution enhanced with a Gaussian apodization scheme with −5 Hz line broadening and a Gaussian maximum at 15% for a free induction decay of 16 384 real plus imaginary data points at 0.55 Hz per data point (Bruker software LB = −5, GB = 0.15).

work not only on the 3-epimer, but also for authentic sirohydrochlorin, and the assumption that *Desulfovibrio* would not be biosynthesizing the enantiomeric series.

The data in Table 1 now make it possible to identify the epimers of sirohydrochlorin methyl ester when they are encountered in other work.

References

1. J. C. Matthews, R. Timkovich, M.-Y. Liu and J. Le Gall, *Biochemistry* **34**, 5248 (1995).
2. A. I. Scott, A. J. Irwin, L. M. Siegel and J. N. Shoolery, *J. Am. Chem. Soc.* **100**, 7987 (1978).
3. A. Bax and D. G. Davis, *J. Magn. Reson.* **65**, 355 (1985).
4. A. Bax and D. G. Davis, *J. Magn. Reson.* **63**, 207 (1985).
5. R. Timkovich and L. L. Bondoc, *Magn. Reson. Chem.* **27**, 1048 (1989).
6. A. R. Battersby, E. McDonald, R. Neier and M. Thompson, *Chem. Commun.* 960 (1979).
7. A. R. Battersby, K. Frobel, F. Hammerschmidt and C. Jones, *Chem. Commun.* 455 (1982).
8. D. M. Arciero, A. B. Hooper, M. Cai and R. Timkovich, *Biochemistry* **32**, 9370 (1993).