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^{1}H and ^{13}C NMR Studies of Sirohydrochlorin (Factor II) and its 20-Methyl Derivative (Factor III)

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Abstract: The complete assignment of the ^{1}H and ^{13}C NMR spectra of sirohydrochlorin octamethylester (**7**) and its 20-methyl derivative (**8**) was accomplished by the use of NOESY, COSY, DEPT, CH correlation and HMBC experiments. Evidence from ^{13}C NMR spectroscopy and molecular mechanics calculations indicates that the methyl group at position 20 affects the relative stability of the tautomeric forms of these isobacteriochlorins.

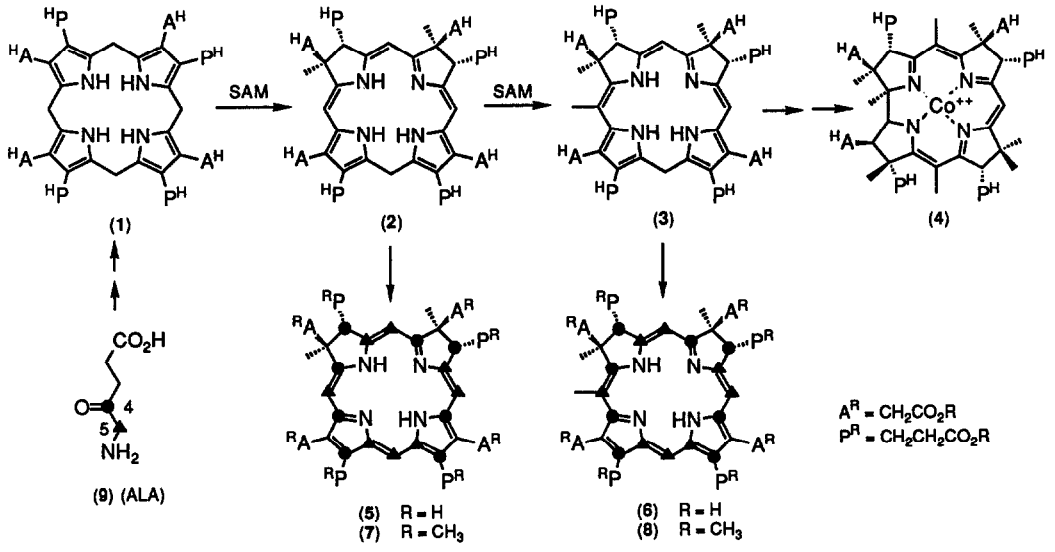
INTRODUCTION

The biosynthesis of cobyrinic acid (**4**) from uroporphyrinogen III (**1**) requires eight methylations, decarboxylation of the acetate side chain at position 12, ring contraction (extrusion of C-20 as acetic acid) and insertion of cobalt, although few intermediates are known[1,2] (Scheme 1). The second and third intermediates in this sequence, precorrin 2 (**2**) and precorrin 3 (**3**) were isolated as their oxidized forms, factor II (**5**)[3] and factor III (**6**).[4] Compound (**5**) possesses the same chemical structure as sirohydrochlorin, the iron free prosthetic group of nitrite and sulfite reductases.[5,6] The S-adenosylmethionine (SAM) dependent enzyme responsible for the first two methylation steps in the transformation of (**1**) has been overexpressed from different sources[7,8] and has been used to prepare several analogs of (**5**),[9,10] and more recently the enzyme that transfers a methyl group from SAM to (**2**) has also been overexpressed.[11]

^{1}H and ^{13}C NMR spectroscopy (sometimes utilizing ^{13}C labeled material) has been very useful in the structure elucidation of (**5**)[3] and (**6**),[4] although unambiguous signal assignment of these spectra has not yet been published. This report presents the complete ^{1}H and ^{13}C NMR spectral assignment of sirohydrochlorin and 20-methyl sirohydrochlorin octamethylesters, (**7**) and (**8**), made possible by the use of modern spectroscopic techniques and the availability of increased amounts of these compounds, obtained from *P. shermanii* by an improved incubation and isolation protocol.

The functional role of sirohydrochlorin as the ligand for one of the iron centers in *E. coli* sulfite reductase has yet to be defined and a complete spectral assignment will be useful for structural studies with the purified enzyme.

During the spectral assignment, the presence of different tautomeric forms of the polycyclic system become evident. The effect of the methyl group at position 20 on such equilibria will be discussed.

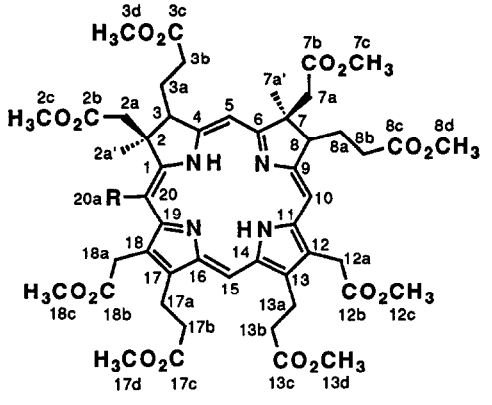


Scheme 1. Biosynthesis of cobyrinic acid.

RESULTS AND DISCUSSION

^1H NMR Spectra.

Compounds (**7**) and (**8**) can be divided into two well defined regions, conveniently designated north and south. The ^1H NMR spectra of the south regions reflect their more aromatic nature, with signals at relative lower field than their northern counterparts. The north regions are characterized by the increased complexity of the signals due to the reduction of the two heterocyclic rings. The ^1H chemical shift (δ) values and assignment for (**7**) and (**8**) are listed in Table 1 (for numbering see Scheme 2).



Scheme 2. Numbering of factors II and III octamethylesters.

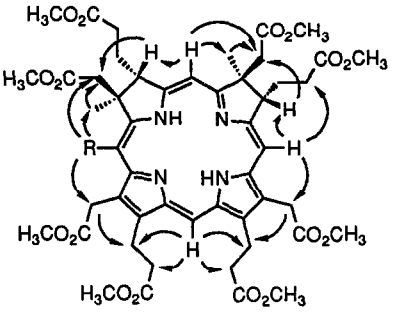
Table 1. ^{1}H NMR Data for Sirohydrochlorin (**7**) and 20-Methyl Sirohydrochlorin (**8**) Octamethylesters.

	(7)		(8)		
Assignment	δ	Type[a]	δ	Type[a]	Δδ[b]
H-5	6.78	s	6.51	s	-0.27
H-10	7.64	s	7.49	s	-0.15
H-15	8.84	s	8.71	s	-0.13
H-20	7.54	s	-	-	-
H_3-20a'	-	-	2.77	s	-
H_3-2a'	1.76	s	1.66	s	-0.10
H_3-7a'	1.61	s	1.41	s	-0.20
H_2-2a	2.55	d (15)	2.50	d (15)	-0.05
	2.63	d (15)	2.54	d (15)	-0.09
H_2-7a	2.52	d (15)	2.65	d (15)	0.13
	2.56	d (15)	2.68	d (15)	0.12
H-3	4.11	t (7)	4.04	t (7)	-0.07
H-8	4.08	dd (9-5)	4.08	dd (8-6)	0
H_2-3a	1.94	m	1.69	m	-0.25
	2.22	m	2.20	m	-0.02
H_2-8a	1.92	m	2.14	m	0.22
	2.27	m	2.28	m	0.01
H_2-3b	2.26	m	2.10	m	-0.16
	2.33	m			-0.23
H_2-8b	2.48	t (8)	2.55	t (8)	0.07
H_2-12a	4.12	d (16)	4.00	d (15)	-0.12
	4.16	d (16)	4.04	d (15)	-0.12
H_2-18a	4.16	s	4.17	d (17)	0.01
			4.24	d (17)	0.08
H_2-13a	3.75	dt (14-7)	3.65[d]	dt (15-7)	-0.10
	3.79	dt (14-7)	3.71[e]	dt (15-7)	-0.08
H_2-17a	3.79	t (7)	3.66[d]	dt (15-7)	-0.13
			3.72[e]	dt (15-7)	-0.07
H_2-13b	2.98[c]	t (7)	2.90	t (7)	-0.08
H_2-17b	2.98[c]	t (7)	2.90	dt (15-7)	-0.08
			2.94	dt (15-7)	-0.04
H_3-2c,7c	3.16	s	3.22	s	0.06
	3.24	s	3.24	s	0
H_3-12c,18c	3.39	s	3.39	s	0
	3.43	s	3.44	s	0.01
H_3-3d,8d,13d,17d	3.27	s	3.26	s	-0.01
	3.27	s	3.28	s	0.01
	3.34	s	3.32	s	-0.02
	3.35	s	3.37	s	0.02

[a] multiplicity (J value). [b] δ(**8**)-δ(**7**).

[c] coincident. [d,e] assignment could be interchanged.

Sirohydrochlorin octamethylester (7). The ^{1}H NMR signals from the meso-hydrogens are an excellent starting point for the assignment of the whole spectrum due to their distinctive chemical shift and location in a "window" portion of the spectrum. The signal at lowest field corresponds to H-15, located in the totally conjugated southern region, while the one at highest field was assigned to H-5, in the reduced northern region, with signals of H-10 and H-20 occupying intermediate positions.[12] These assignments were confirmed by nuclear Overhauser effect (nOe) experiments (Scheme 3). The signals for H-10 and H-20 could be differentiated because H-20 showed nOe with a methyl group (H_3-2a').

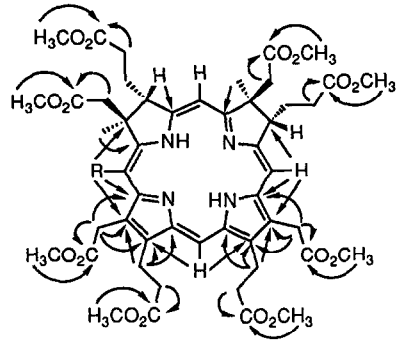


(7) R = H
(8) R = CH_3

Scheme 3. Observed nOe's of interest

The CH_3 groups at positions 2 and 7, as well as the AB quartets corresponding to H_2-2a and H_2-7a, could be differentiated by their nOe's with H-20 and H-5 respectively. NOe experiments utilizing H-5 and H-10, together with information from the COSY spectrum, allowed the complete assignment of the signals corresponding to H-3, H-8 and the propionate side chains at positions 3 and 8. The same approach starting from H-10 and H-20 identified the signals for the acetate side chains at positions 12 and 18. NOe interactions from these substituents to the adjacent propionates differentiated the signals corresponding to H_2-13a and H_2-17a, which in turn showed coupling to a triplet at higher field (H_2-13b,17b). Similar experiments also confirmed the presence of the acetate side chains at positions 2 and 7 oriented to the same face of the molecule as H-3 and H-8.

Finally, partial discrimination of the methylester signals was possible by the analysis of the Heteronuclear Multiple Bond Correlation (HMBC) spectrum[13] (Scheme 4)(see further discussion of this spectrum below).



(7) R = H
(8) R = CH_3

Scheme 4. Observed long range CH interactions of interest

20-Methyl sirohydrochlorin octamethylester (8). The same set of experiments as above were performed with a sample of (**8**), which resulted in the complete assignment of its spectrum (Table 1). The main difference between this spectrum and that obtained from (**7**) was the disappearance of one signal at low field (meso region), which was replaced by a singlet at much higher field, correspond to the substitution of H-20 by a methyl group. More subtle differences were noted for many signals in the spectrum. Examples of such changes are a 0.22 ppm shift 10 atoms distant from the methyl (H_2-8a), and the differentiation of the two hydrogen atoms in CH_2 groups (H_2-17a, H_2-17b and H_2-18a).

Abraham, Smith and coworkers studied the effect of meso substituents on the δ of meso-hydrogen and β-substituents in porphyrins.[14] They explained the high field shifts observed throughout the molecule using a ring-current model based on the double dipole approximation,[15] and proposed that a 10 % decrease in the main macrocyclic ring current has taken place. The perturbation of protons in the vicinity of the substitution through direct magnetic anisotropic effect was also considered. They also extended their model into the chlorin system (7,8-dihydroporphyrin),[16] in which one of the pyrrole dipoles was missing.

The general high field shift in the spectrum of (**8**) can be explained in part using the above concepts. All α- and β-CH_2 group signals (but H_2-18a) in the southern region (the region more similar to porphyrins) were shifted ca. -0.10 ppm and -0.06 ppm respectively, in accordance with the model. Unfortunately, a ring current reduction cannot explain why all the CH_2 groups in ring B (H_2-7a, H_2-8a and H_2-8b) were shifted to low field, while the methyl group in that region (CH_3-7a') and all the signals corresponding to the ring A substituents (H_3-2a', H_2-2a, H_2-3a and H_2-3b) are at higher field. A possible explanation for these findings will be proposed following the analysis of the ^{13}C NMR spectra.

Finally, while meso substitution in porphyrins exerts the strongest effect in the meso-hydrogen "opposite" to the substituent,[14] in (**8**) the more shifted meso-hydrogen signal was H-5. A possible explanation could be that a larger influence of the macrocyclic ring current is expected in the northern region where no pyrrole ring currents are centered.

^{13}C NMR Spectrum.

The ^{13}C δ values and assignment for (**7**) and (**8**) are listed in Table 2 (for numbering see Scheme 2).

Sirohydrochlorin octamethylester (7). The CH correlation spectrum was a very important tool in the assignment of all protonated carbon signals, due to the complete assignment of the ^{1}H NMR spectrum described above. It made possible differentiation between the two methyl signals at positions 2 and 7 (C-2a'/C-7a'), as well as the CH_2 groups of the acetate side chains at the same positions (C-2a/C-7a). The same spectrum allows identification of the signals corresponding to the two aliphatic monoprotonated positions (C-3/C-8), the four meso carbons (C-5/C-10/C-15/C-20), the pair C-3a/C-8a, and the coincident signals for C-13b/C-17b. The remaining six CH_2 signals could only be differentiated in pairs (C-3b,8b/C-12a,18a/C-13a,17a) due to the small differences in δ between them (Δδ=0.04-0.06 ppm). Each of these pairs collapsed into a single signal upon dilution.

The assignment of the nonprotonated carbon signals was accomplished by analysis of the long range CH interactions (Scheme 4) shown in the HMBC spectrum. The cross section at H-10 showed two strong signals at 50.3 ppm and 124.5 ppm. The first signal has already been assigned above (CH correlation) to C-8, confirming that in aromatic systems the stronger observed interactions occur through three rather than two bonds. For that reason the low field signal was assigned to C-12. By the same arguments the cross section at H-20 identified the signals corresponding to C-2 and C-18. The remaining signal in the aliphatic region was then assigned to C-7. Each of these cross sections contained a smaller signal (two bond interaction) that could be assigned to C-9 or C-11 and C-1 or C-19 respectively. These two signals appeared also on the cross sections corresponding to the south acetate protons (three bonds interactions) indicating their final assignment to C-11 and C-19. The main signals in these sections correspond to the two bond interactions with C-12 and C-18 respectively. The same cross sections showed small signals which can be assigned to C-13 and C-17.

Table 2. ^{13}C NMR Data for Sirohydrochlorin (7) and 20-Methyl Sirohydrochlorin (8) Octamethylesters.

Assignment	(7)	(8)	Δδ[a]	Assignment	(7)	(8)	Δδ[a]
C-2	48.5	50.5	2.0	C-1	159.5	153.1	- 6.4
C-7	50.7	51.7	1.0	C-9	152.8	158.3	5.5
CH-3	54.0	55.4	1.4	C-4	164.9	159.5	- 5.4
CH-8	50.6	50.4	- 0.2	C-6	165.7	169.8	4.1
CH_2-2a	46.3	41.8	- 4.5	C-11	147.8	143.8	- 4.0
CH_2-7a	44.2	44.1	- 0.1	C-19	145.0	152.4	7.4
CH_2-3a	26.4	25.1	- 1.3	C-12	124.9	122.0	- 2.9
CH_2-8a	26.0	25.5	- 0.5	C-18	123.6	124.3	0.7
CH_2-	32.12	32.6[d]	0.5	C-13	140.0	138.1	- 1.9
(3,8)b	32.17	32.6[d]	0.4	C-17	138.6	142.3	3.7
CH_2-12a	31.69[b]	31.4	- 0.3	C-14	138.0	132.2	- 5.8
CH_2-18a	31.73[b]	33.9	2.2	C-16	135.7	136.5	0.8
CH_2-13a	21.25[c]	21.3	0	(CH,C)-20	94.8	104.9	10.1
CH_2-17a	21.31[c]	21.1	- 0.2	CH-5	89.6	89.6	0
CH_2-13b	36.8[d]	36.68[f]	- 0.1	CH-10	95.9	95.5	- 0.4
CH_2-17b	36.8[d]	36.74[f]	- 0.1	CH-15	108.5	109.0	0.5
CH_3-2a'	20.1	19.9	- 0.2	CO_2-2b	171.0	171.2	0.2
CH_3-7a'	19.5	20.5	1.0	CO_2-7b	171.3	171.5[h]	0.2
CH_3-20a	-	19.1	-	CO_2-12b	171.69[g]	171.6[h]	- 0.1
OCH_3-	50.93	51.03[d]	0.1	CO_2-18b	171.72[g]	172.2	0.5
(2,7)c	51.00	51.03[d]	0	CO_2-	173.0	173.0	0
OCH_3-	51.59	51.64[d]	0	(3,8,13,17)c	173.1	173.18	0.1
(12,18)c	51.64	51.64[d]	0		173.2[d]	173.24[d]	0
OCH_3-	51.05	51.06	0		173.2[d]	173.24[d]	0
(3,8,13,17)d	51.12[e]	51.13[e]	0				

[a] δ(8)-δ(7)

[b,c,f,g,h] assignment may be interchanged.

[d] denotes two coincident signals.

[e] denotes three coincident signals.

The remaining sp^2 signals were assigned through the study of the cross sections at H_3-2a', H_3-7a', H-3, H_2-13a and H_2-17a.

After this analysis the only ambiguity left was in the assignment of the signals around 138 ppm (C-14/C-17), solved by reference to the published spectra of ^{13}C labeled (7) prepared from 4-^{13}C-ALA (9, ●) and 5-^{13}C-ALA (9, ▲)(Scheme 1).[3] The spectrum of (7) derived from 4-^{13}C-ALA showed a signal at 138.6 ppm while in the 5-^{13}C-ALA derived specimen a signal was observed at 138.0 ppm. Analysis of the labelling pattern for each labeled specimen (Scheme 1) indicated that the resonance at 138.6 ppm must be due to C-17 (●) while the one at 138.0 ppm must be C-14 (▲).

Finally a partial assignment of the carbonyl groups was accomplished by analysis of the HMBC spectrum. These assignments served as the starting point for the ^1H assignment (HMBC) of the OCH_3 groups mentioned above, which in turn were used for the partial assignment (CH correlation) of the OCH_3 carbon signals indicated in Table 2.

The HMBC spectrum showed an array of other long range CH interactions that confirmed the assignment of the protonated carbons, made possible by the analysis of the one bond CH correlation spectrum, thus showing the complementarity of the two techniques.

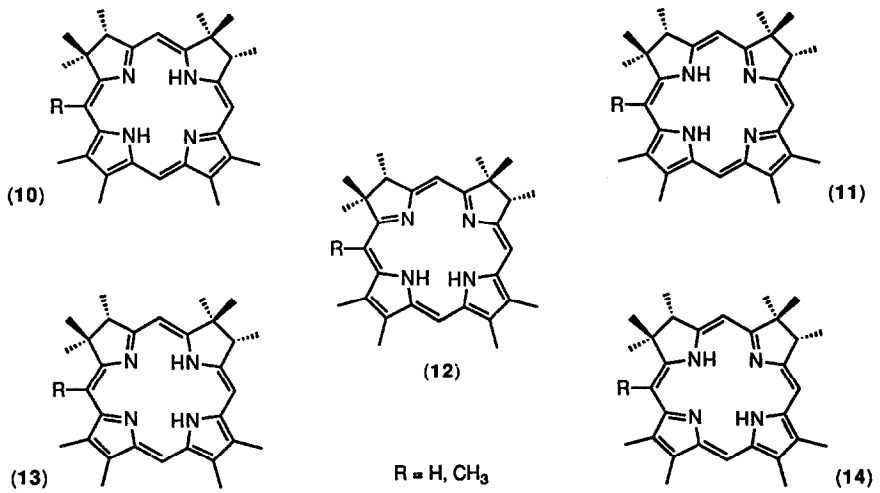
An interesting feature of the spectrum was the considerable broadening of the signals corresponding to C-4/C-6, C-11/C-19 and, in less extent C-14/C-16. This may indicate that the observed signals are the averaged values from different species in equilibrium. The broadening increased until the complete disappearance of the signals when the temperature was slowly lowered (from 20°C to -55°C in toluene-*d8*). Unfortunately, solubility problems precluded further decrease of temperature to observe the signals corresponding to the individual species.

20-Methyl sirohydrochlorin octamethylester (8). The same strategy used for (7) was followed in the assignment of (8). As in the proton spectrum the main differences between (7) and (8) are related to the introduction of a methyl group in position 20, i.e. the appearance of a new high field methyl signal, and a ca. 10 ppm shift of the meso signal corresponding to C-20 on methylation. Smaller but significant changes were also observed for many signals, especially for the macrocyclic ring signals.

The methyl substitution in position 20 exerted little or no influence on acetate, propionate and methyl side chain δ (CH_3, CH_2, CO_2 and OCH_3), except in the case of CH_2-2a and CH_2-18a, the two side chains closest to the substitution. The rest of the aliphatic carbons (C-2,7 and CH-3,8) were also affected to variable extents. The analysis of the meso signals indicate almost no effect of the 20-methylation in the remaining CH-meso carbons.

For the study of the remaining sp^2 signal it is interesting to divide the macrocyclic ring with a plane across C-5 and C-10, which should be a symmetry plane if the side chains and the position of the double bonds are not considered. The division results in the generation of pairs of carbons with similar relative position in respect to the plane (C-1/C-9, C-4/C-6, C-11/C-19, C-12/C-18, C-13/C-17 and C-14/C-16). Table 2 shows that, upon 20-methylation, one signal in each pair is shifted to higher field (carbons in rings A and C), while the other is shifted to lower field (carbons in rings B and D), resulting in the "crossing" of the signals (except for C-4/C-6). Another interesting result of such shifts is that in almost all cases the difference in δ between the two carbons in each pair is increased (except C-1/C-9). The difference change is particularly important in the pairs C-4/C-6 (from 0.8 to 10.3 ppm) and C-11/C-19 (from 2.8 to 8.6 ppm). Finally, no broadening was observed in any signal in the spectrum of (8).

The explanation for these results when considered together with the broadening of signals in the spectrum of (7) lies in the possibility of different tautomers in the macrocyclic ring (Scheme 5), structure (10) being the most frequent representation of (7) and (8) in the literature.



Scheme 5. Theoretical tautomeric forms of sirohydrochlorins.

Line broadening in the sp^2 signals in (**7**) indicates its existence as more than one of these tautomers of similar stability. On the other hand, the lack of such broadening in the spectrum of (**8**) indicates that one of the possible tautomers is more stable in the latter molecule. The "freezing" of one double bond pattern would result also in a greater differentiation of the signals on each of the above mentioned pairs, while equilibration would result in their time averaging in the NMR experiment. This would be especially important where, as in the pairs C-4/C-6 and C-11/C-19, one of the carbons is double bonded to a nitrogen (>C=N-) and therefore shifted to lower field, while the other is attached to nitrogen through a single bond (=C-N<). The analysis of the spectrum of (**8**) indicates that its main tautomer is (**14**, R = CH_3), as C-6 and C-19 δ values are shifted toward lower field (>C=N-). In the case of (**7**) a mixture of tautomers of similar energy is present. The same behavior was observed in samples of ^{13}C labeled (**2**) and (**3**).[11]

The different degrees of tautomerism in (**7**) and (**8**) may be also responsible for the proton signal shifts observed, as conformational and chemical anisotropy effects should be different. For example the presence of a "fixed" double bond >C=N- in ring B and the disappearance of a "partial" double bond in ring A upon methylation will affect the δ of all the substituents on those rings.

Molecular mechanics calculations.

The different tautomers of (**7**) and (**8**) were optimized to their minimum energy conformations and the results shown in Table 3. The ring conformations obtained were very similar to those reported for a model isobacteriochlorin studied by X-ray crystallography.[17] For example rings C and D were shown to be very flat with a torsion angle Cα-Cβ-Cβ-Cα of ca. 1°, while rings A and B were twisted 22-29°.

Table 3. Molecular Mechanics Calculations on the Tautomeric Forms of (**7**) (R = H) and (**8**) (R = CH_3).[a]

R	(**10**)[b]	(**11**)[b]	(**12**)[b]	(**13**)[b]	(**14**)[b]
H	0.00[c]	5.33	1.87	5.10	0.63
CH_3	1.95	8.65	2.21	2.64	0.00[d]

[a] For structures see Scheme 5. [b] Relative Energy (Kcal/mol).
[c] MM2 final E_S = 39.98 Kcal/mol. [d] MM2 final E_S = 45.81 Kcal/mol.

The results clearly showed the existence of two tautomeric forms [(**10**) and (**14**)] of similar energy in the case of sirohydrochlorin (R = H), which could be interconverted through structure (**12**). In contrast for the 20-methyl derivative, structure (**14**) has considerably more stability than the other possibilities, confirming the proposed preference of this form as the main tautomer. Optimizations of the different tautomers of the macrocyclic ring with no substituents except the methyl at position 20, also suggested a preference (1.53 Kcal/mol) for structure (**14**) over (**10**), indicating that the energy difference is not due to the particular substitution pattern but rather is intrinsic in the 20-substituted isobacteriochlorin skeleton.

The calculated structures are also useful in understanding some characteristics of the NMR spectra. The different preferred orientation of the side chains may account for the changes in δ and multiplicity of the CH_2 groups, as they are affected to different extents by ring current, depending on their spatial position. As ring currents are also affected by the substitution, a very complex effect is observed for each proton in the molecule. A clear example is the ^1H-NMR behavior of the propionate side chains in the northern region. CH_2-3b and CH_2-8b were expected to generate signals at very similar δ due to the similarity in their substitution. Surprisingly the CH_2-8b signal appeared at lower field (ca. 0.2 ppm). The explanation comes from the models: while the propionate at position 3 is located below the macrocyclic ring [(C-2)-(C-3)-(C-3a)-(C-3b) dihedral angle - 68°], the propionate at position 8 is directed away from it [(C-7)-(C-8)-(C-8a)-(C-8b) dihedral angle 153°] thus receiving a different ring current effect. The different side chain orientation also resulted in different behavior in nOe experiments (nOe enhancement between H_2-8b and H-10, but no observed interaction between H_2-3b and H-5).

CONCLUSIONS

The ^{1}H and ^{13}C NMR spectra of sirohydrochlorin octamethylester (**7**) and its 20-methyl derivative (**8**) were assigned in detail. These assignments were consistent with and complement the published data for ^{13}C-labeled (**7**) and (**8**).[3,4] The spectral information will be of importance for the identification of novel, related materials obtained from the recently isolated methyl transferase enzymes, as well as in the interpretation of the data obtained from the burgeoning use of NMR spectroscopy in the direct observation of biological processes.

Again the power of modern spectroscopic techniques was demonstrated by the assignment of the signals from ca. fifty carbon and sixty hydrogen atoms using only 10 μMols of pure compound.

The ^{13}C NMR spectra (shape and $\Delta\delta$) provided evidence for the existence of one preferred tautomeric form for (**8**), while (**7**) should be in equilibrium between forms of similar energy. Molecular mechanics calculations confirmed that the preferred tautomer of factor III is (**14**). This discovery could be an essential clue in the understanding of the enzymatic processes involved in the biosynthesis of cobyrinic acid, considering the subtle modulation of regio and stereoselectivity via electronic effects on introduction of both meso methyl substituents and coordinating metals proposed by Eschenmoser.[18]

This work also shows that a careful molecular analysis is necessary before any spectral interpretation based on general ring-current models,[15] as the presence of tautomers of different stability will generate local perturbations that cannot be predicted by such models.

EXPERIMENTAL

Materials.

Compounds (**5**) and (**6**) were obtained from *Propionibacterium shermanii* (whole cell suspensions) inoculated with ALA and methionine.[4] The esterified products (**7**) and (**8**) (CH_3OH/H_2SO_4), purified by flash chromatography and HPLC,[7] were characterized by UV, FAB-MS,[19] ^{1}H and ^{13}C NMR.

NMR spectroscopy.

NMR spectra were recorded in benzene-*d6* (99.96 % D), or toluene-*d8* (99 % D) in 5 mm tubes at a concentration of ca. 10 mg (**7**) or (**8**) in 400 μl of solvent. High resolution ^{1}H and ^{13}C NMR, COSY (90°, 45°, phase sensitive), DEPT (90°, 135°) and CH correlation experiments were performed on a Bruker AM500 500 MHz NMR spectrometer. NOe (nOe difference, NOESY; evolution time of 300 ms), HMBC and low temperature (20°, 0°, - 20°, - 40°, - 55° C) experiments were performed on a Bruker WM300 300 MHz NMR spectrometer.

Molecular mechanics calculations

Calculations were performed on a MicroVax II VMS 5.3 computer system using the program MacroModels V2.0[20] with Allinger's MM2 force field parameters and Block diagonal Newton Raphson minimization (BDNR) optimization procedure.

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