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THE STRUCTURE OF FACTOR III

A TRIMETHYL ISOBACTERIOCHLORIN INTERMEDIATE IN THE BIOSYNTHESIS OF VITAMIN B_{12}†

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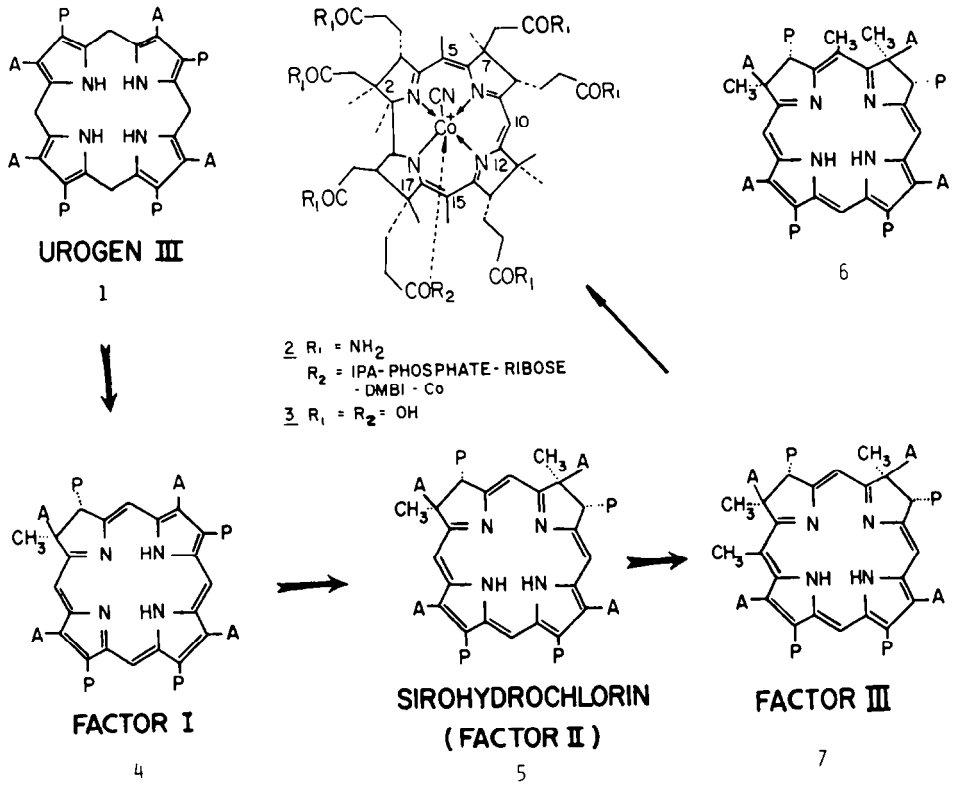
Abstract—Factor III, an intermediate in corrin biosynthesis isolated from *Propionibacterium shermanii*, is shown to be a 3,7,20-trimethylisobacteriochlorin (20-methylsirohydrochlorin) by examination of the CMR spectrum of a biosynthetically enriched specimen derived from [5-^{13}C]-δ-aminolevulinic acid and [$^{13}CH_3$]-L-methionine. In cell-free extracts of *Clostridium tetanomorphum* the conversion of Factor III to cobyrinic acid involves loss of C-20 together with the methionine-derived methyl group attached to this position. The implications of these observations, which cast a new light on the uro'gen-corrin connection, are discussed.

The discovery of a series of methylated isobacteriochlorins from preparations of *P. shermanii* and *C. tetanomorphum* has already led to a deeper understanding of the biochemical processes which connect uro'gen III[1,2] (**1**) with vitamin B_{12} *in vivo* and with cobyrinic acid (**3**) *in vitro*. On the basis of mass and UV-visible spectral analysis and of biochemical evidence, Factors I,[3] II[3,4] and III[4] were assigned the constitutions **4**, **5** and **6**, respectively, i.e. a mono-, di- and tri-methylated isobacteriochlorin, although at the time the full structural and stereochemical details could not be determined. Subsequently, Factor II and sirohydrochlorin, the metal-free prosthetic group of nitrite-sulfite reducing enzyme, siroheme,[5] were shown to be identical and to have the absolute stereochemistry (**5**).[6-9] By analogy and on the basis of the PMR data for the lactonic form of Factor III (= corriphyrin **3**), the 5-methylated isomer (**6**) was preferred[8,10] for the structure of Factor III, especially since a methyl group is present in the 5-position of the natural corrins. Factor I, from the outset presumed to be the ring-A mono-methylated dihydroporphyrin, has recently been assigned[11] this structure and the stereochemistry shown in **4**. The proven biochemical relationships within this series is shown in Scheme 1 where the experimental observation of incorporation of Factors II and III, but only the *reduced* forms of Factor I and of uro'gen III suggest that an oxido-reductase system is present in cell-free extracts which, although incapable of reducing either uro'III to uro'gen-III or Factor I to the tetrahydro level, can effect such reduction on administered samples of Factors II and III. The alternative explanation is that the isobacteriochlorins II and III in fact represent the physiological oxidation level of the incorporated substrates, i.e. the biochemical transformations Factor II → Factor III → corrin take place at the fully oxidised state which corresponds to the structure of these factors as isolated from the bacterial preparations. At present this point remains unresolved but obviously must be settled in order to rationalise the mechanism of the isobacteriochlorin → corrin transformation.

We next describe the evidence for structure **7** for Factor III, which leads to a revision of the earlier postulate.[8,10] Factor III was isolated from δ-aminolevulinic acid (ALA)-supplemented cobalt-free incubations of *P. shermanii* (ATCC 9614) and from a B_{12}-deficient mutant of this organism.[12] High resolution FD mass spectrometry of the octamethyl ester established the formula $C_{51}H_{64}N_4O_{16}$ (988.4281) while the mass spectrum of the octa-ester isolated from incubation with [methyl-$^2H_3$]-L-methionine exhibited peaks at *m/e* 997, 994, 991 and 988 corresponding to enrichment with a maximum of three (M + 9) CD_3 groups. The presence of three such methyl groups was also confirmed by analysis of the ^{1}H NMR spectrum recorded on a sample of Factor III methyl ester (400 μg) prepared from a suspended cell incubation of *P. shermanii* containing [$^{13}CH_3$]-methionine and [5-^{13}C]-ALA followed by esterification of the metabolite and purification by HPLC. The

†Dedicated to the memory of Prof. R. B. Woodward.



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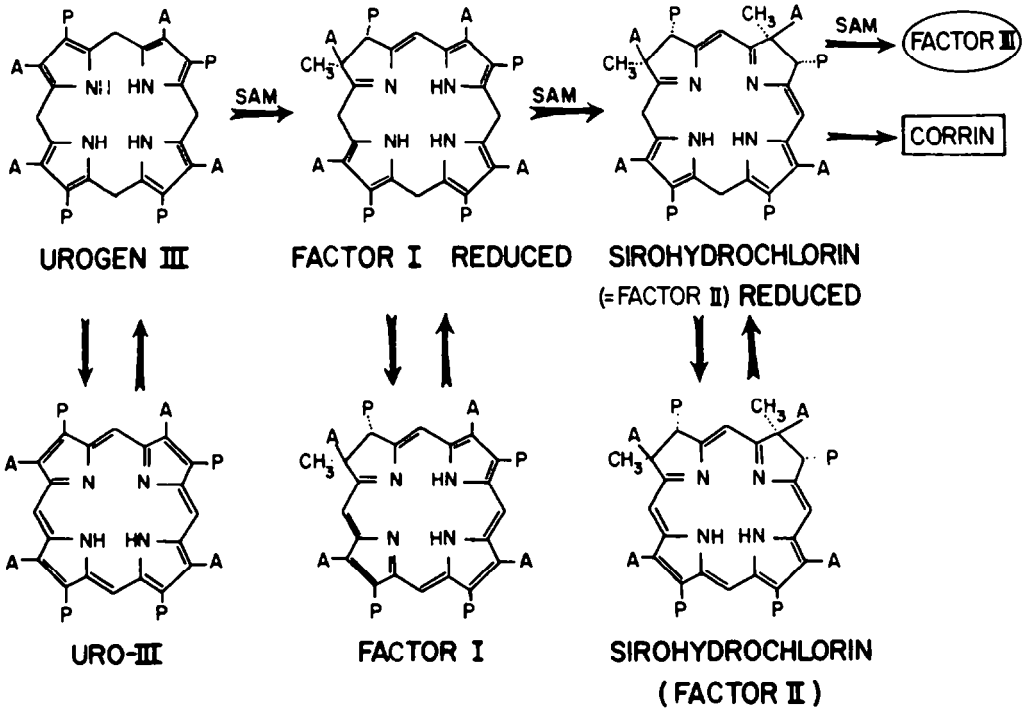


Scheme 1.

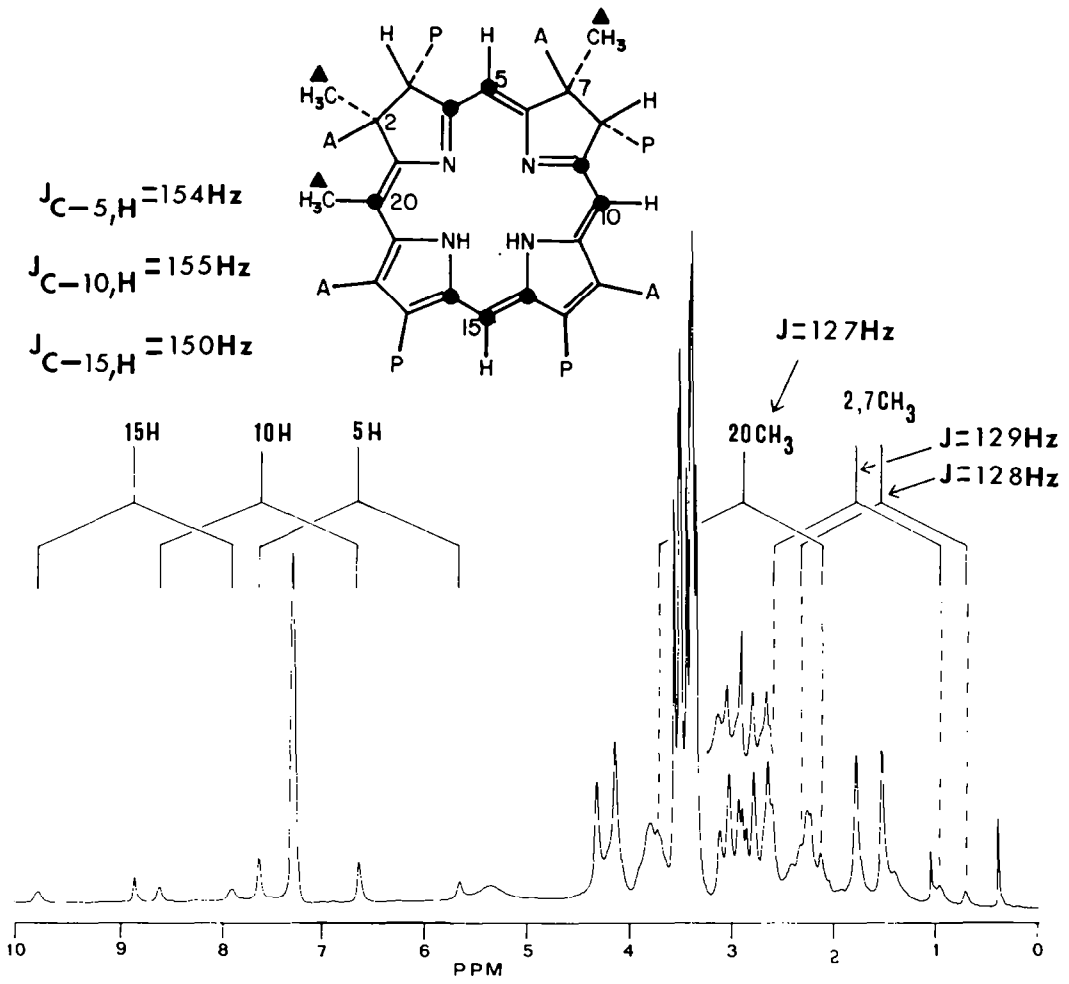


Fig. 1. The 300 MHz ^{1}H NMR spectrum (C_6D_6) of [5-^{13}C]-ALA- and $^{13}CH_3$]-methionine enriched 20-methylsiro-hydrochlorin octamethyl ester. Inset: The 20-methyl proton resonance (δ 2.98 ppm) of 20-methylsirohydrochlorin at natural abundance.

proton spectrum (300 MHz) of this ^{13}C-enriched sample is shown in Fig. 1 where the methyl resonances at 1.5 and 1.8 ppm (CH_3's on sp^3 carbon) are assigned provisionally to the 2 and 7 positions (J_{H-C} = 128 and 129 Hz) whilst a resonance at δ 2.98 (J = 127 Hz), although almost obscured in the ^{13}C-enriched spectrum, is clearly discerned in the natural abundance spectrum (see inset in Fig. 1) and can be assigned to CH_3 attached to a sp^2 center. Aside from clean resolution of the eight methoxy resonances of the octa-ester (δ 3.2–3.6) the most important features of this proton spectrum are signals at δ 6.43, 7.21 and 8.33 ppm, corresponding to only *three* of the *meso*-proton resonances of an isobacteriochlorin. In contrast, Factor II (**5**) exhibits *four* well-defined signals in this region which have been assigned to the four *meso*-protons at C-5, C-10/C-20, and C-15 (δ 6.78, 7.36/7.46 and 8.54, respectively). Factor III must therefore be methylated at C-10 or C-20.

When this ^{13}C-enriched species (as the octamethyl ester) was examined by ^{13}C NMR spectroscopy (Fig. 2), it became possible to deduce the complete structure (**8**). First, the *downfield* position of the C-15 meso-carbon triplet at δ 108.98 (J = 72 Hz) confirms that rings A and B are methylated,[6] and since the *meso*-carbon signals at δ 89.5 and 95.4 each shown ^{13}C-^{13}C coupling to an enriched sp^2 neighbor ($J \approx 70$ Hz), these are assigned to C-5 and C-10, respectively, by analogy with the corresponding resonances in sirohydrochlorin derived by biochemical enrichment with [5-^{13}C]-ALA.[6] Thus, the remaining *meso*-carbon resonance at δ 104.8 consists of a doublet (J = 44.2 Hz) and must correspond to C-20, with additional fine structure due to long-range coupling with C-4 and C-16. That the ^{13}C–^{13}C coupling constant of 44.2 Hz for C-20 is due to substitution by a methionine-derived methyl group is confirmed by inspection of the *methyl* region of the ^{13}C NMR spectrum which displays three enriched species consisting of singlets at δ 20.17 and 19.62 and a doublet at δ 18.79 (J = 44.2 Hz). It can be seen that, owing to different efficiences of incorporation of [^{13}C]-methionine and of [5-^{13}C]-ALA, the enrichments in the methyl groups and in the ALA-derived sp^2 carbons are not identical, the satellite intensities reflecting a greater enrichment in C-20 than in its pendant Me group.

The other sp^2 carbon resonances (Fig. 3) were assigned as follows on the basis of the reciprocity of the

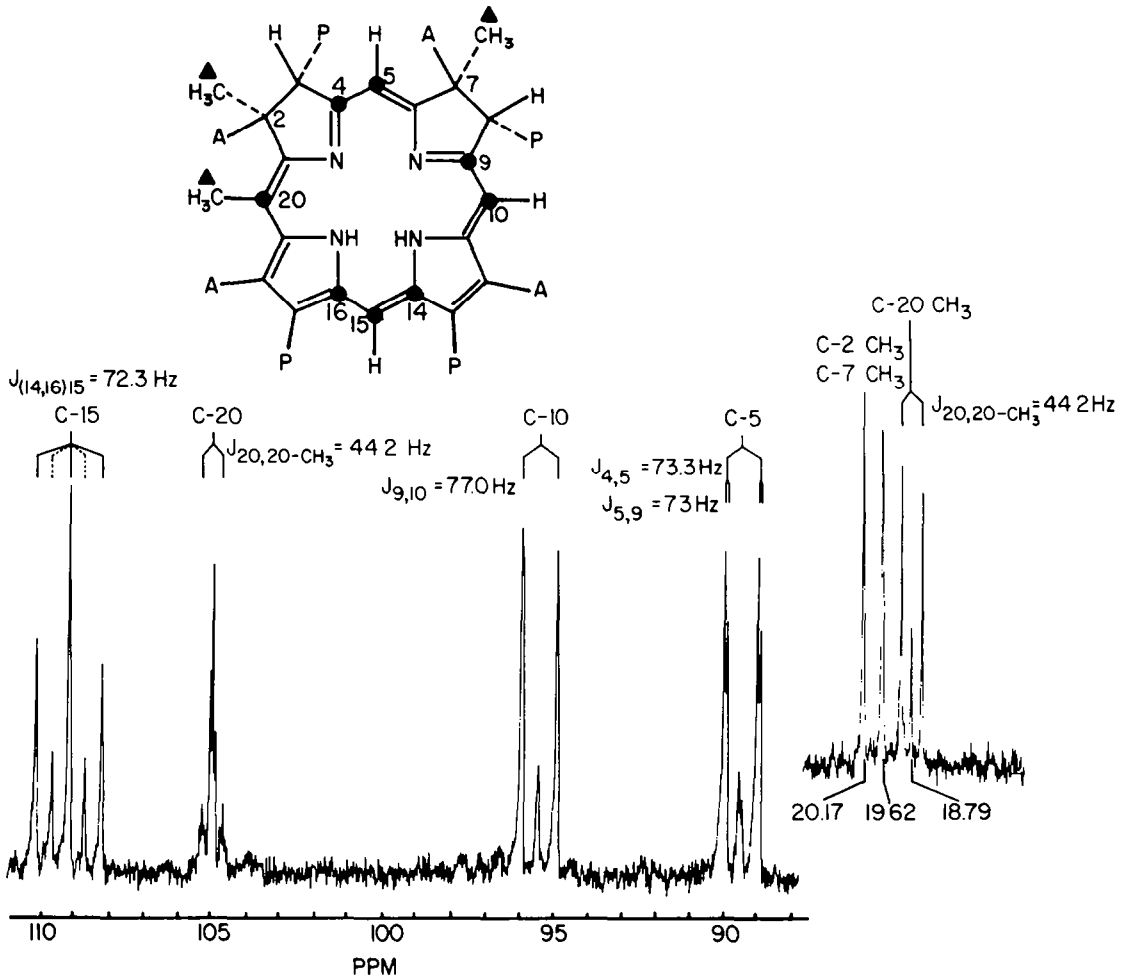


Fig. 2. The *meso*-carbon resonances of the proton noise decoupled ^{13}C-FT spectrum of [5-^{13}C]-ALA- and [$^{13}CH_3$]-methionine labelled 20-methylsirohydrochlorin octamethyl ester. Inset: The methyl carbon resonances of the same sample. The spectrum was obtained in C_6D_6 at 75 MHz.

coupling constants with C-5, 10, 15 and 20 and by analogy with the chemical shift data for sirohydrochlorin, similarly enriched with ^{13}C. Thus C-4 and C-9 resonate at 159.6 and 158.3 ppm, respectively, ($J_{4,5}$ = 73 Hz; $J_{9,10}$ = 77 Hz) with additional long-range coupling to C-20 and C-5 observed ($J_{4,20}$ = 5 Hz; $J_{5,9}$ = 7.3 Hz). A distinction can be made between the (usually) ambiguous assignment of C-14 and C-16 in this spectrum on the basis of the observed long-range coupling between C-16 and C-20 (J = 2.8 Hz) observed for both enriched centers. The long-range coupling between C-10 and C-14 is not resolved. Although C-14 and C-16 have almost identical one-bond coupling constants ($J_{15,16}$ = 73 Hz; $J_{14,15}$ = 72 Hz), they can be assigned to the signals at 136.5 and 132.3 ppm, respectively.

Hence Factor III is **7**, i.e. 20-methylsirohydrochlorin rather than the C-5 methylated isobacteriochlorin (**6**), contrary to what had previously been thought.[8,10] The absolute stereochemistry of Factor III and its relationship to cobyrinic acid was obtained by the following experiments.

Incorporation into cobyrinic acid of Factor III labelled from [4-^{14}C]-ALA was demonstrated in earlier experiments using cell-free extracts of *C. tetanomorphum*,[3,4] but at that time the structural assignment (now shown to be **7**) had not been made. In order to trace the fate of the methyl groups during the conversion of Factor III (**7**) to cobyrinic acid (**3**), specimens of Factor II (**5**) and Factor III (**7**) were prepared from whole-cell incubations of *P. shermanii* using [*methyl*-^{14}C]-L-methionine and [2,3-$^{3}H_4$]-ALA. By this strategem, **7** is labelled as shown in Scheme 1 (● = ^{3}H; ▲ = ^{14}C]. Taking the value of 2.00 for the methyl group number in Factor II (Experiments A: 1a, 2a, 3a, 4a, Table 1), the $^{14}C/^{3}H$ ratios in Factors III and IIIa are in good accord with the presence of three methyl groups in both of these isobacteriochlorins (Experiments A: 1b, 1c, 2b, 3b, 4c, Table 1), while Factor IIa is seen to contain two such methionine-derived methyl groups (Experiment A: 4b, Table 1). The structures of Factors IIa and IIIa, isomeric with Factors II and III, respectively, are under investigation (s Experimental) but their lack of incorporation into cobyrinic acid (Table 1) removes them as serious contenders for intermediacy in the corrin pathway. In this connection, an isomer of Factor II (3-epi-sirohydrochlorin) has recently been

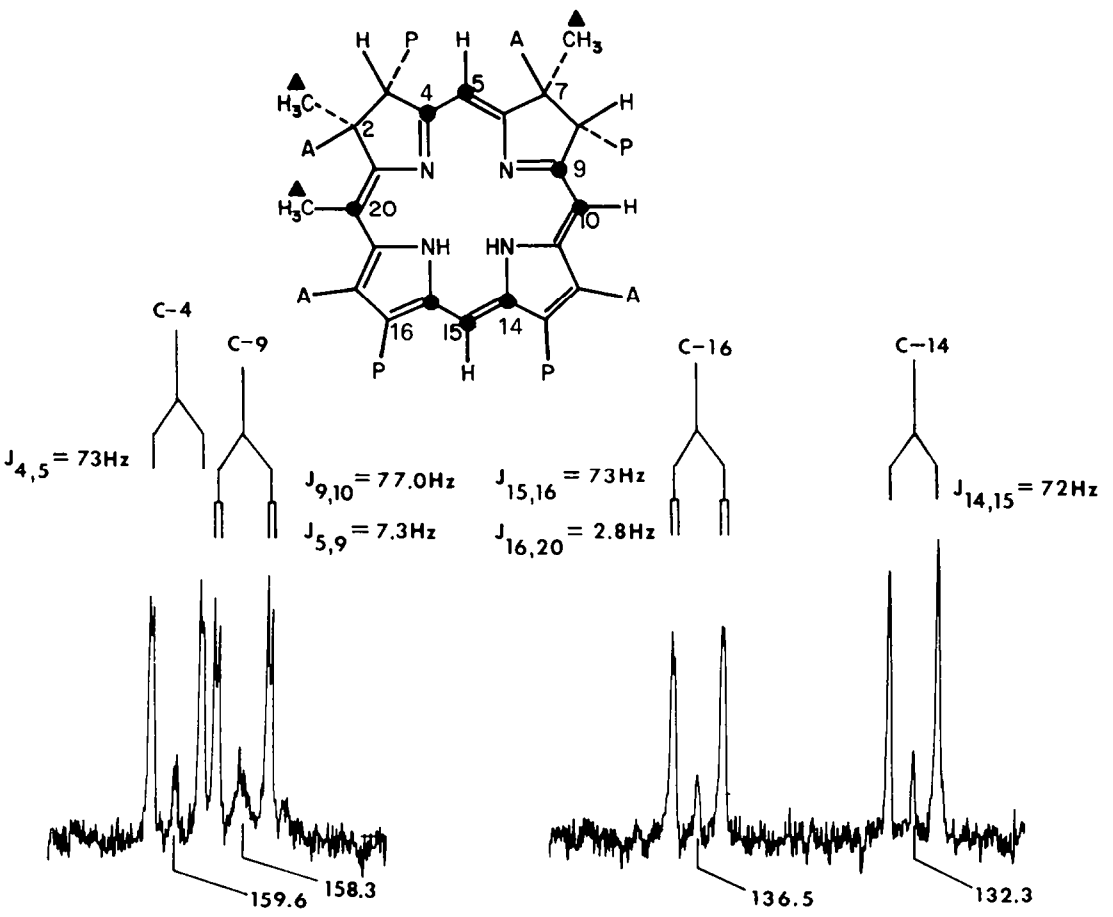


Fig. 3. The proton decoupled downfield portion of the 75 MHz ^{13}C-FT spectrum of 20-methylsirohydrochlorin (C_6D_6) showing the sp^2 resonances associated with C-4, C-9, C-14 and C-16 enriched from [5-^{13}C]-ALA.

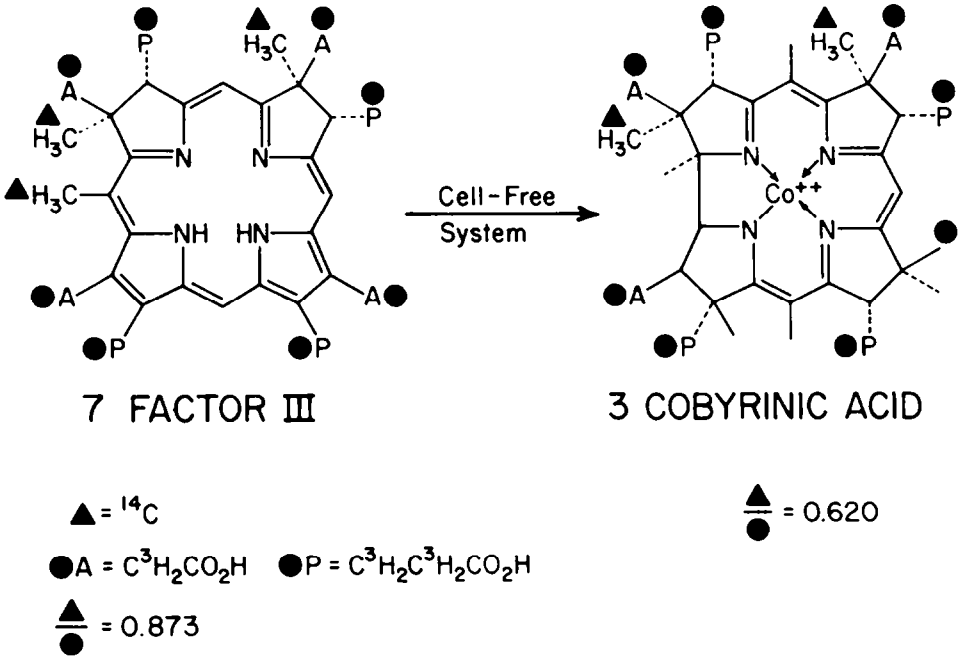
Table 1. Comparative studies on the incorporation of di- and trimethylisobacteriochlorins from *P. shermanii* into cobyrinic acid

	A. Double labeling of cobyrinic acid intermediates					B. Incorporation of intermediates into cobyrinic acid(3)			
				radioactivity ratio of intermediate				radioactivity ratio of cobyrinic acid	
expt	inter-mediate	added substrate	μCi	$^{14}C/^{3}H$	with ref to methyl groups	added ^{3}H-radio-activities of isobacterio-chlorins, dpm	incorpn, %*	$^{14}C/^{3}H$	with ref to methyl groups
1a	5	[methyl-^{14}C]Met [2,3-^{3}H]ALA	350 500	0.600	2.00	1.84×10^6	15.3	0.620	2.00
b	7			0.873	2.91	1.57×10^6	16.1	0.620	2.00
c	7			0.873	2.91	1.65×10^6	14.3	0.660	2.13
2a	5	dto	250	0.092	2.00	1.63×10^6	4.0	0.109	2.00
b	7		1200	0.141	3.06	1.62×10^6	9.9	0.102	1.87
3a	5	dto	250	0.376	2.00	1.27×10^6	4.9	0.400	2.00
b	7		300	0.537	2.86	1.20×10^6	11.5	0.405	2.03
4a	5	dto	250	0.376	2.00	1.59×10^5	9.6		
b	FIIa		300	0.391	2.08	0.95×10^5	0.8		
c	FIIIa			0.543	2.89	1.59×10^5	0.9		

A: Series of incubations with cell suspensions of P. shermanii (1-4) for the preparation of [$^{14}C/^{3}H$]-doubly labelled isobacteriochlorins from [methyl-^{14}C] Met and [2,3-^{3}H] ALA; isolated isobacteriochlorins were methyl esters.

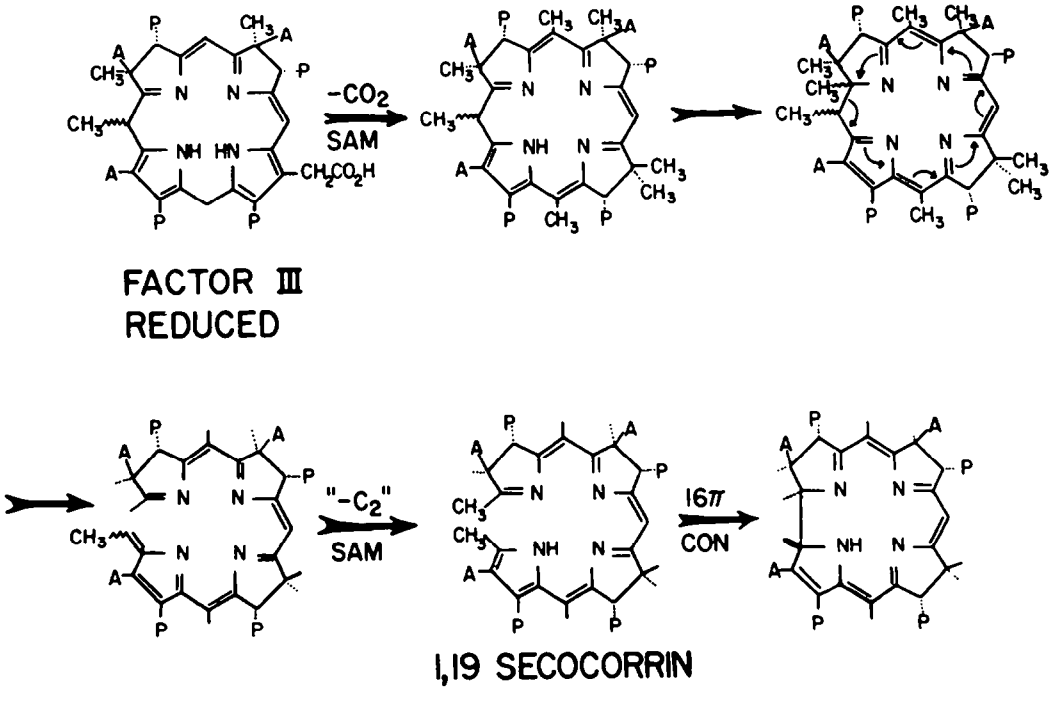
B: Experiments for incorporation of the [$^{14}C/^{3}H$]-labelled isobacteriochlorins (obtained from methyl esters by 2 M piperidine hydrolysis) into cobyrinic acid by tetrapyrrole-free cell extracts from C. tetanomorphum.

* % incorporation: ^{3}H-radioactivity of formed cobyrinic acid/added ^{3}H-radioactivity of substrate x 100.

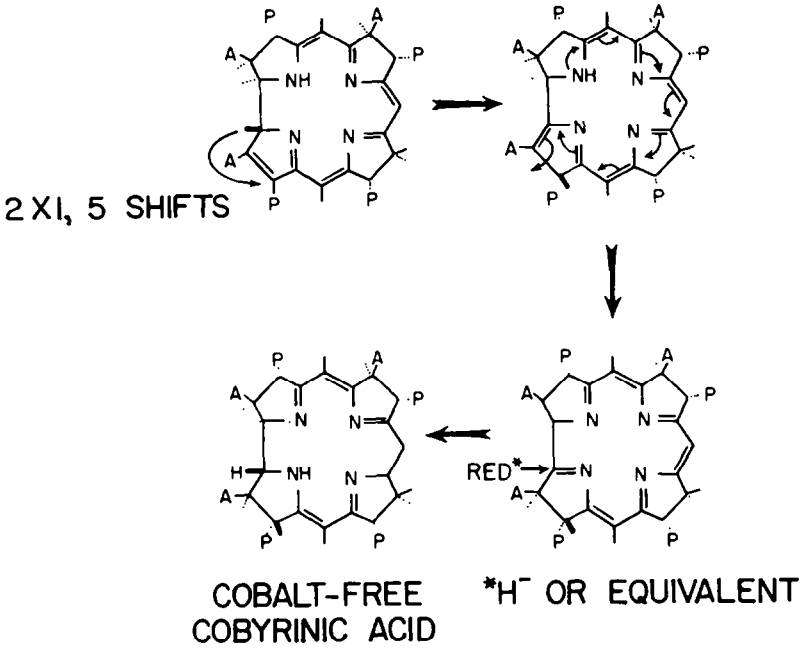


Scheme 2.

characterized by Battersby *et al.*[13] and may indeed correspond to Factor IIa, since it is not a precursor of cobyrinic acid. When Factors II and III doubly labelled in this way (see Table 1) were re-incubated with the *C. tetanomorphum* cell-free system, the derived cobyrinic acid, obtained with excellent overall incorporation (5–16%), contains only *two* methionine-derived methyl groups in each case (Experiments B: 1a–c, Table 1). In order to remove any possibility of cross contamination, separate samples of doubly labelled **5** and **7** were again chromatographed to constant, but *different* radioactivity ratios (Experiments A: 2a, 2b and 3a, 3b, Table 1). Pure Factor III of Experiment 2 was mixed with



Scheme 3.



Scheme 4.

sirohydrochlorin (Factor II) from Experiment 3 (and *vice versa*) and the methyl esters were separated, purified and again administered (after hydrolysis) to the cobyrinic acid synthesizing system (Experiments B: 2a, 2b and 3a, 3b, Table 1). In each experiment the isolated cobyrinic acid contains a $^{14}C/^{3}H$ ratio compatible with only *two* ^{14}C Me groups. These experiments show that (a) there is no possibility of cross-contamination of Factors II and III during isolation, (b) Factor III is converted to cobryinic acid in good radiochemical yield (10–15%) with the loss of C-20 and its attached methyl group (the nature of the "C_2" fragment remains to be determined[14]), and (c) the absolute stereochemistry of Factor III (as 7) follows from its relationship to cobyrinic acid. These conclusions summarized in Scheme 2 have been independently confirmed by the work of the Cambridge group.[17]

It thus appears that, in order to achieve the intercorrin A → D ring junction, the biosynthetic route requires not only the specific formation and subsequent disruption of the type III uro'gen macrocycle but the sacrifice of at least one methionine-derived methyl group and the carbon to which it is attached (C-20), since *both* of these must be excised from the species undergoing (or having undergone) secocorrin → corrin[2] closure. This apparently prodigal series of events can be accommodated within several hypotheses consonant with published data. On the assumption that the incorporation of Factor III occurs at the *dihydro*-level *in vivo* and *in vitro*, i.e. a reductase system is present in the cell-free extracts, one such hypothetical scheme is shown in Scheme 3 where the oxidation level of both the ejected "C_2" unit and the substrate for this cleavage reaction are still unknown. The net result of the 1,19 secocorrin closure is to furnish a 1,19-dimethyl dehydrocorrin by an allowed 16π conrotatory electrocyclic ring closure, thus setting the C-1 and C-19 methyl groups in the correct absolute stereochemistry for eventual 1,5 shifts of C-19 methyl to C-17 as proposed earlier (see Scheme 4). These and related ideas are now under experimental scrutiny.[18]

EXPERIMENTAL

Determination of radioactivity. Water soluble radioactive samples (e.g. cobyrinic acid) were measured with modified Bray's dioxan scintillator (PPO, 5 g; POPOP, 0.1 g; naphthalene, 180 g in dioxan, 1 l) using a Beckman LS250 counter; for all other samples a toluene scintillator was used (PPO, 5 g; POPOP, 0.5 g in toluene, 1 l). For doubly labelled (^{3}H; ^{14}C) samples the screening method of Tykva[19] was employed.

Spectral measurements. UV-visible spectra were recorded with a Beckman ACTA MVI spectrophotometer, photometric determinations of sample concentrations with a Carl Zeiss PMQII instrument and FD mass spectra as described previously.[3,20] PMR spectra and CMR spectra with Varian FT-80 and SC-300 instruments in "100%" C_6D_6 (Merck) distilled from calcium hydride.

Chromatography. HPLC was performed with a Waters Associates ALC/GPC 204 instrument using C-18 μ-Bondapak columns in 80% MeOH/H_2O.

Tlc for purifications and separations of Factors II, IIa and III, IIIa methyl esters were carried out on Merck Silica gel (tlc aluminum sheets, 20 × 20 cm, 0.2 mm thickness) and on precoated cellulose plates (20 × 20 cm, 0.5 mm thickness: Schleicher and Schüll G 1805; 0.1 mm thickness: tlc aluminum sheets, Merck). Heptamethyl cobyrinate was purified using KCN saturated solvents throughout. Column chromatographic (C.C.) purifications were performed on Merck silica gel 60 for column chromatography, 230–400 mesh (usual size: 5 cm × 1.5 cm). The following solvent systems were used as indicated below.

System I	Benzene/Ethyl Acetate/Methanol (60:38.5:1.5; v/v/v)
System II	Hexane/2-Propanol/Methanol (5:2:1; v/v/v)
System III	Hexane/Chloroform/1-Propanol/2-Propanol (94:4:2:0.5; v/v/v/v)
System IV	CH_2Cl_2/Chloroform (2:1; v/v)
System V	Chloroform/Methanol (95:5; v/v)
System VI	Chloroform/Methanol (99:1; v/v)

Paper electrophoresis. Cobyrinic acid was purified on Schleicher and Schull paper Nos. 2043 and 2045 using the following KCN saturated buffers:

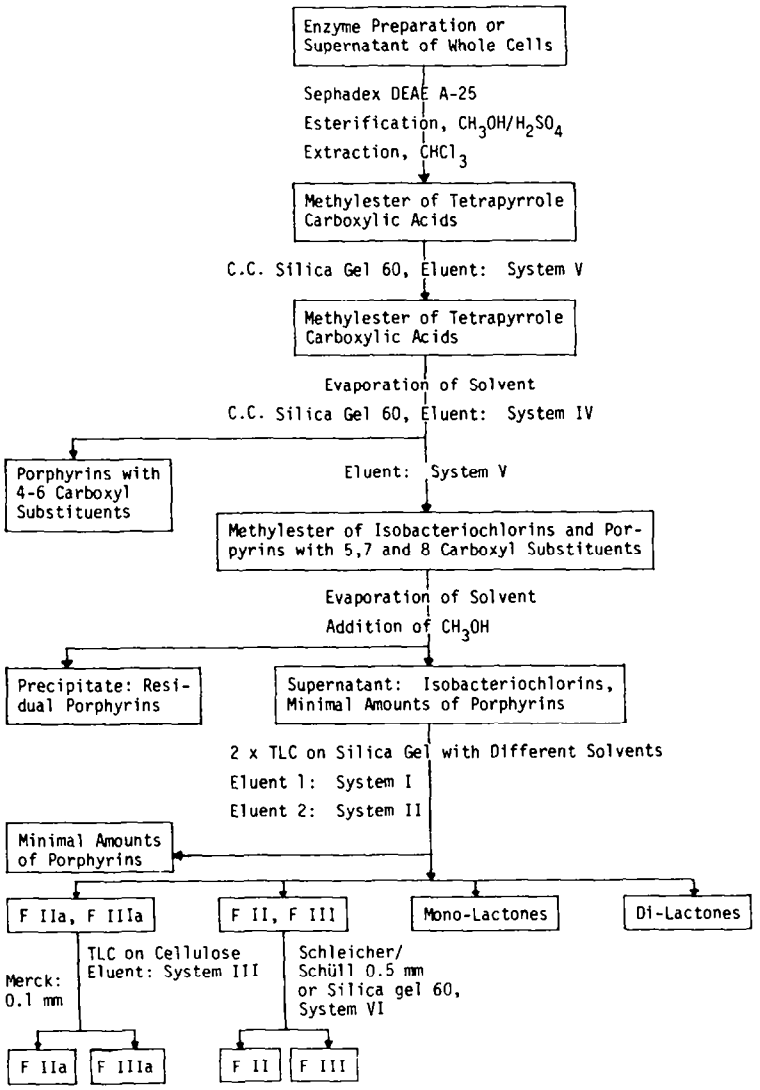
(a) Buffer pH 2.7, 0.5 M acetic acid,

(b) Buffer pH 8.6, 0.01 M veronal

Reagents. Adenosine-5'-triphosphate cryst. di-sodium salt $\times 3H_2O$ (Boehringer, Mannheim); S-Adenosyl-L-methionine $\cdot HSO_4$ (Boehringer, Mannheim); 5-Aminolevulinic acid·HCl (prepared by a method of Zav'yalov *et al.*[21]); [2,3-^{3}H]-5-Aminolevulinic acid (specific activity 28 mCi/μmol) (Isotopendienst West, Frankfurt); [4-^{14}C]-5-Aminolevulinic acid (specific activity 59 μCi/μmol (Isotopendienst West, Frankfurt); L-Cysteine·HCl (Serva Feinbiochemica, Heidelberg); Cobyrinic acid (prepared from acid methanolysis of vitamin B_{12} followed by hydrolysis); L-Glutathione (red) (Serva Feinbiochemica, Heidelberg); L-Methionine (Merck, Darmstadt), [Me-^{14}C]-L-methionine (specific activity 56 μCi/μmol) (Amersham; Buchler, Braunschweig); [Me-$^2H_3$]-D,L-methionine (prepared as described in l.c.[22]); β-nicotinamide-adenine-dinucleotide, reduced form, di-sodium salt (Boehringer, Mannheim); β-nicotinamide-adenine-dinucleotide, oxidized form, free acid (Boehringer, Mannheim); Vitamin B_{12} (Merck, Darmstadt).

Incubations with cell suspensions of P. shermanii. Freshly harvested wet cells (90 g) of *P. shermanii*[3,4,20] were suspended in degassed, nitrogen saturated 52 mM phosphate buffer, pH 7.7 (300 ml). 5-Aminolevulinic acid hydrochloride (20 mg; 0.12 mmole) and L-methionine (4 mg; 27 μmole) were added before and after 20 hr of incubation which was continued for a further 20 hr, with regular pH adjustment (to 7.7).

Isolation of the tetrapyrrolic methyl esters. The pH of the above cell suspension was adjusted to 7.8 to avoid formation of the lactones of Factors II and III and after



Isolation and Purification of Methylated Isobacteriochlorins from P. shermanii or C. tetanomorphum.

Scheme 5.

centrifugation Sephadex DEAE A-25 (previously washed with degassed phosphate buffer pH 7.7 (52 mM) was added to the supernatant. After 2–3 hr the Sephadex was filtered, washed with degassed distilled water and dried with MeOH (200 ml). The residue was freeze-dried and suspended in $MeOH/H_2SO_4$ (95:5; v/v; 100 ml) and allowed to stand (in the dark) at room temp for 24 hr. Addition of $CHCl_3$ (20 ml) and aqueous $NaHCO_3$, followed by extraction of the aqueous layer with $CHCl_3$ (3 × 20 ml), drying (Na_2SO_4) and evaporation of solvent afforded a mixture of tetrapyrrole methyl esters which were purified and separated on small silica gel 60 columns (5 cm × 1.5 cm) and by tlc using solvent systems as shown above. The structures of factors IIa and IIIa are under further investigation.

Isolation and purification of the isobacteriochlorin methyl esters. The procedure is described in Scheme 5 where F II, III, etc. refer to methyl esters of Factors II, III, etc.

Chromatographic separation of the methyl esters of factors II, IIa, III *and* IIIa *and their γ-mono- and di-lactones on silica gel.* The chromatographic steps as well as the R_f values of the single compounds are summarized in Table 2. The methyl esters of the isobacteriochlorins were first separated on precoated silica gel plates using solvent system I and then rechromatographed several times. The bands containing the methyl esters of Factors IIa and IIIa and II and III as well as their γ-mono- and di-lactones were scraped carefully and eluted with chloroform/methanol (99:1; v/v). It is necessary to chromatograph the isobacteriochlorin bands on silica gel using solvent system II in order to separate them from the traces of porphyrin esters. All the above chromatographic steps were carried out in subdued light. The isolated isobacteriochlorin methyl esters are blue-violet in soln and show strong orange fluorescent under long-wave UV light (366 nm).

Separation of factor II *and* III *methyl esters.* Traces of silica gel were separated by solution in CH_2Cl_2 and filtration. The ester mixture was then chromatographed on precoated cellulose plates (Schleicher and Schull, 0.5 mm) using solvent system III and/or on precoated silica gel 60 aluminum sheets (Merck, 0.2 mm) using solvent system VI. In both cases Factor II methyl ester (orange fluorescence) runs just ahead of Factor III methyl ester (red fluorescence).

The pigments were purified by rechromatography on silica gel plates using solvent systems I or II.

Preparation of [^{3}H,^{14}C]*-labelled isobacteriochlorins.* The freshly harvested wet cells (90 g) were suspended in nitrogen phosphate buffer (52 mM) at pH 7.7. 5-Aminolevulinic acid (40 mg; 0.24 mmol) and L-methionine (8 mg; 54 μmol) plus [2,3-^{3}H]-5-aminolevulinic acid and [Me-^{14}C]-L-methionine were added to the suspension (Table 1). The added radioactivities of the labelled substrates are summarized in Table 1; the specific radioactivities are summarized under reagents. The radioactive labelled pigments were purified to constant specific activities using the tlc systems described above.

Incorporation experiments to form cobyrinic acid with porphyrinogen-free cell extracts of C. tetanomorphum. Porphyrinogen-free cell extracts of *C. tetanomorphum*[3,4] (40 ml) were used for each experiment (Table 1). S-adenosyl-L-methionine (5 mg; 10 μmol), glutathione (reduced; 4 mg; 13 μmol), ATP (7 mg; 13.8 μmol), NAD (3 mg; 4.5 μmol), NADH (6 mg; 8.5 μmol) and L-cysteine (1 mg; 5.7 μmol) were dissolved in buffer and added to the soln together with aqueous $Co(NO_3)_2$ soln (0.5 ml; 39 mM). After the addition of the radioactive labelled substrates, obtained from their methyl esters by hydrolysis in 2 ml degassed 2 M piperidine soln (8 hr, 4°), evaporation of the piperidine–water mixture *in vacuo* and dissolution of residue in a few ml distilled and degassed water, the suspension was incubated in the dark at 37°, for 20 hr under N_2.

Isolation and purification of cobyrinic acid. Unlabelled cobyrinic acid (957 μg; 0.92 μmol), KCN soln (1 ml; 1 M; pH 7.8) and glacial AcOH were added to the suspension after incubation. The suspension was allowed to stand for 15 min at 4°. The cobyrinic acid was isolated from the incubation media by DEAE-fixation[4] and esterified with 50 ml $MeOH/HSO_4$ (95:5; v/v). The cobester was extracted with chloroform and purified by the following procedure.

Table 2. Estimated R_f values in solvent systems used for the separation of the methylesters from porphyrins and isobacteriochlorins isolated from incubations with cell suspensions of *P. shermanii*

Methylesters of	TLC on silica gel 60 system I	TLC on silica gel 60 system II	TLC on cellulose system III	TLC on silica gel 60 system VI
Coproporphyrin	0.59	0.75	0.80	0.55
Pentacarboxyporphyrin	0.49	0.61		0.49
Hexacarboxyporphyrin	0.39	0.50		0.44
Heptacarboxyporphyrin	0.30	0.38	0.21	0.39
Uroporphyrin	0.19	0	0	0.34
Factor IIa	0.30	0.48	0.54*	0.31
Factor IIIa	0.30	0.48	0.60*	0.31
Factor II	0.29	0.46	0.40**	0.31
Factor III	0.29	0.46	0.20**	0.25
Factor-II-γ-monolacton	0.25	0.39		0.30
Factor-III-γ-monolacton	0.20	0.31		0.31
Factor-II-γ-monolacton	0.18	0.29	broad badly separated bands from 0.1 to 0.2	0.29
Factor-II-γ-dilacton	0.15	0.24		0.28
Factor-III-γ-dilacton	0.14	0.24		0.25

*Merck, aluminum sheets, 0.1 mm

**Schleicher and Schüll, G 1805, 0.5 mm

(1) Chrom AR sheet 1000 (Malinckrodt), solvent system I. Porphyrin methyl esters and starting substrates were separated from cobester which remained at the origin.

(2) Silica gel tlc plates, solvent system II (saturated with KCN). The R_f value of cobester is 0.4. The cobester band was scraped off, eluted with $CHCl_3$/MeOH (99:1; v/v) and evaporated *in vacuo*. To separate traces of silica gel the cobester was dissolved in CH_2Cl_2 and filtered through a cotton plug. The cobester was then hydrolysed with 2 ml degassed 2 M piperidine soln for 24 hr at 4°. The piperidine–water mixture was evaporated under vacuum and cobyrinic acid dissolved in a small amount of distilled water, containing 5% HCN, and electrophoresis of the cobyrinic acid was carried out at pH 8.6. Thereafter the cobyrinic acid band was eluted with HCN-containing distilled water, and after evaporation of water an electrophoresis at pH 2.7 was carried out.

In order to control the radiochemical purity of the isolated cobyrinic acid, the radioactive distribution was measured along the electropherogram and the specific radioactivity of cobyrinic acid determined after each electrophoresis.[20]

The results of the incorporation data are given in Table 1.

Preparation of ^{13}C-*labelled* 20-*methylsirohydrochlorin.* δ-[5-^{13}C]-aminolevulinic acid (90 atom %, 100 mg) and $^{13}CH_3$-L-methionine (Merck, 90 atom %, 100 mg) were incubated for 50 hr with *P. shermanii* (ATCC 9614). The mixture was then centrifuged (20,000 g, 20 min) and the supernatant was filtered through DEAE cellulose (Whatman DE-23, 10 gm) which had been previously equilibrated with 0.02 M potassium phosphate buffer pH 7.6. The cellulose was freeze-dried and then stirred for 18 hr in the dark under argon with MeOH (250 ml) containing 5% HSO_4. The solution was poured into $CHCl_3$ (250 ml and neutralized with sat $NaHCO_3$ aq. The aqueous phase was extracted several times with $CHCl_3$ and the combined organic layers were dried ($NaSO_4$) and evaporated. The residue was chromatographed (TLC) using benzene-EtOH-MeOH (80:16:4). The fluorescent orange band at R_f 0.6 was eluted (10% MeOH in benzene) and subjected to hplc purification using a Waters C-18 μ-Bondapak column in 80% MeOH/water. The two main fractions consisting of sirohydrochlorin and 20-methyl-sirohydrochlorin octamethyl esters were collected. The latter was rechromatographed on the same column in 70% acetonitrile/H_2O to remove a trace impurity not resolved in the MeOH/water system. Collected fractions were evaporated in the dark at <50° and used directly for the NMR experiments.

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[14]In earlier experiments it was clearly shown[1a,15] that, under carefully controlled conditions, [^{14}C]-formaldehyde could be trapped from the C-20 position of uro'gen III. The data can be interpreted in several ways: (a) methylation at C-20 is followed by loss of a "C_2" unit which is further cleaved to "C_1" units, one of which is trapped as formaldehyde; (b) the formaldehyde is released (under enzymic control[16]) *only* from the uro'gen III molecule and *not* from Factor III; (c) more than one pathway exists for the biotransformation of uro'gen III to cobyrinic acid.

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Note added in press. The "missing" C-2 fragment has been identified as acetic acid by independent work in our laboratory (with A. J. Irwin and I. Ichinose) and in Cambridge, Zürich, and Stuttgart. We thank Professors A. R. Battersby, D. Arigoni and G. Müller for informing us of their work, and Professor A. Eschenmoser for communicating his results on a model system which generates corrins from isobacteriochlorin via "Acetate extrusion."