Biosynthesis of Porphyrins and Related Macrocycles. Part 22.[1] Vitamin B_{12}: Studies on the Chlorin, Faktor-I, and the Detection of 3-*epi*-Faktor-I

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The chlorin, Faktor-I, is prepared in greater quantity than previously by enzymic methylation of uro'gen-III using a cell-free preparation from *Clostridium tetanomorphum*. Circular dichroism and ^{1}H n.m.r. spectra are determined on the octamethyl ester of Faktor-I and all the important signals in the n.m.r. spectrum are assigned. An isomeric chlorin is detected in the products from the enzymic runs having properties in accord with its being 3-*epi*-Faktor-I.

The so-called Faktor-I was first obtained [2] by incubating δ-aminolaevulinic acid (1), ALA, with a cell-free enzyme preparation derived from *Clostridium tetanomorphum*, an organism which produces vitamin B_{12}. Faktor-I was shown to be a chlorin and the structure (5) tentatively assigned to it [2] was confirmed by appropriate labelling and degradative experiments.[3] Faktor-I is not itself a precursor of vitamin B_{12} but a reduced form, probably the tetrahydro derivative (3), is incorporated efficiently by appropriate enzyme preparations into cobyrinic acid (4),[3] which is established as a late precursor of vitamin B_{12}.[4] So it is clear that Faktor-I represents in a dehydrogenated form the first *C*-methylation product on the pathway from ALA (1) *via* uroporphyrinogen-III (2), uro'gen-III, to vitamin B_{12} (Scheme). A second and then a third *C*-methyl group are added to the macrocycle (3) as the biosynthesis proceeds further towards cobyrinic acid (4); these dimethylated and trimethylated systems have also been isolated and their structures have been fully established.[4,5]

In the past, Faktor-I has been available only in minute amounts and most of the work summarised above was carried out on a micro-scale with radiolabelled material. No n.m.r. data or chiroptical properties have been reported for Faktor-I but these are essential for future studies, especially for our synthetic work on this chlorin. Accordingly, larger quantities of Faktor-I have been prepared, as outlined below, to allow the appropriate experiments to be carried out.

Preparation of the cell-free extract from *Clostridium tetanomorphum* was modified relative to the original report [2] and the co-factors were also changed. Under the best conditions, with uro'gen-III (2) as the substrate, it was possible to isolate 170—250 μg of the octamethyl ester of Faktor-I from a single enzymic run. Repetition then gave sufficient material for rigorous purification by high pressure liquid chromatography (h.p.l.c.). It was during this phase that a stereoisomer of Faktor-I was detected which will be discussed later.

Faktor-I octamethyl ester (6) showed by field-desorption mass spectroscopy (f.d.–m.s.) a molecular weight of 958.3885 which confirms the elemental composition $C_{49}H_{58}N_4O_{16}$. Circular dichroism of Faktor-I ester (6), kindly studied by Dr. P. M. Scopes (Westfield College), gave a spectrum which is valuable in providing a characteristic finger-print of peaks and troughs (Figure 1).

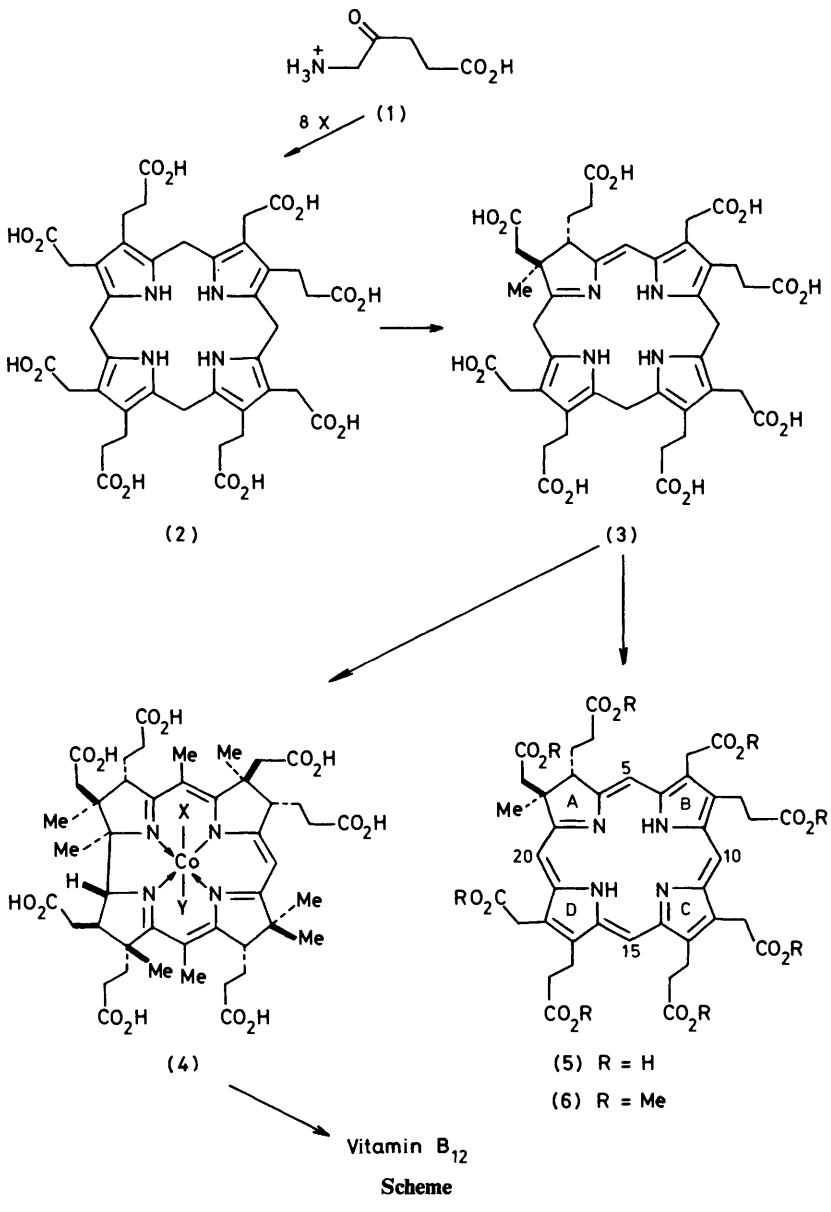
The 400 MHz ^{1}H n.m.r. spectrum of Faktor-I ester (6) is shown as Figure 2. Most of the signals, including all the important ones, were assigned as follows. The four lowfield 1 H singlets arise from the bridge positions (5, 10, 15, and 20) and by chemical shift, the two at higher field (δ 8.91 and 9.04) can be assigned to 5-H and 20-H, adjacent to the reduced ring-A, and the other two at lower field to 10-H and 15-H. Three 2 H signals at *ca.* δ 5.0 correspond to CH_2CO_2Me groups attached to rings B, C, and D, the one at lowest field (δ 5.01) being an AB system, so revealing the diastereotopic nature of these two protons.† A second AB-system (2 H) lies at highfield, *ca.* δ 3.0, and by its chemical shift can be assigned to the acetate methylene group on the reduced ring A. Lastly, the 3H-singlet at δ 2.31 corresponds to the quaternary methyl group at C-2 on ring-A.

Refinement of these assignments depended on n.O.e.-difference spectroscopy [6] which has been found to be particularly helpful in the vitamin B_{12} area.[7] Irradiation at a resonance from a bridge hydrogen at δ 8.91 caused n.O.e. enhancement of the signal from the quaternary methyl group (δ 2.31) and at one of the 2H singlets from a CH_2CO_2Me group (δ 4.99); structure (6a) summarises the n.O.e. experiments. It follows that the irradiated signal (δ 8.91) is from 20-H and the 2H-singlet corresponds to CH_2CO_2Me at C-18. With the signal from 20-H now located, the other highfield bridge signal (δ 9.04) must be from 5-H and irradiation at this resonance caused n.O.e. enhancement of the AB-system at δ 5.01 (2 H). The latter signal thus arises from CH_2CO_2Me at C-7 and its appearance as an AB-system is in keeping with its proximity to the chiral ring-A.

With the resonances from the acetate residues at C-7 and C-18 now located, only δ 4.94 remains for rigorous assignment to the CH_2CO_2Me at C-12. Irradiation at δ 4.94 then located 10-H by n.O.e. enhancement at δ 9.86 so completing all the important assignments which are collected in the Table.

During the purification of Faktor-I ester (6), a second chlorin was detected closely associated with the major chlorin (6) which was found by f.d.–m.s. to be isomeric with it. The methyl esters of Faktor-I and its isomer can be separated by h.p.l.c. but to conserve the very small amount of the isomer (total *ca.* 50 μg), the n.m.r. studies were carried out on enriched material still containing some Faktor-I ester (6). The ^{1}H spectrum at 400 MHz was in keeping with this isomer being 3-*epi*-Faktor-I ester (7) because the 3H-singlet corresponding to the 3-methyl group (δ 1.85) and the 1H-singlet from 20-H (δ 8.86) had both moved upfield relative to the corresponding signals in the spectrum of Faktor-I ester (6); see the Table. These upfield shifts closely match those observed [8] for 3-*epi*-sirohydrochlorin ester (9) relative to sirohydrochlorin ester (8). A complete signal assignment was not possible for the ester of 3-*epi*-Faktor I-(7) because of paucity of material but the following additional signals could be assigned with confidence on grounds of chemical shift: δ 9.04, 9.86, 9.88 to

† The protons on the other two acetic acid residues in this set are also diastereotopic but the protons of each pair fortuitously have the same chemical shift.



Scheme

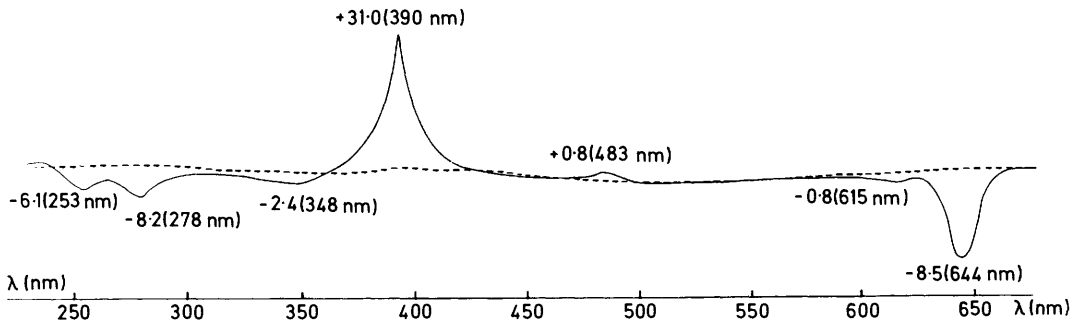


Figure 1. Circular dichroism of Faktor-I octamethyl ester (6) measured in methanol (——); baseline (— — —)

5-H, 10-H, 15-H, respectively, and the double-doublet at δ 4.73 (*J* 3 and 7 Hz) to 3-H.

The detection of 3-*epi*-Faktor-I ester (7) beings the chlorin series into line with the isobacteriochlorins (*e.g.* 8) where both the 3-*epi*- and 8-*epi*-systems have been isolated.[4,8]

Experimental

General directions are given in ref. 9.

Enzymic Formation of Faktor-I (5).—Deep-frozen cells of *C. tetanomorphum* (80 g) were suspended in 0.05M-phosphate

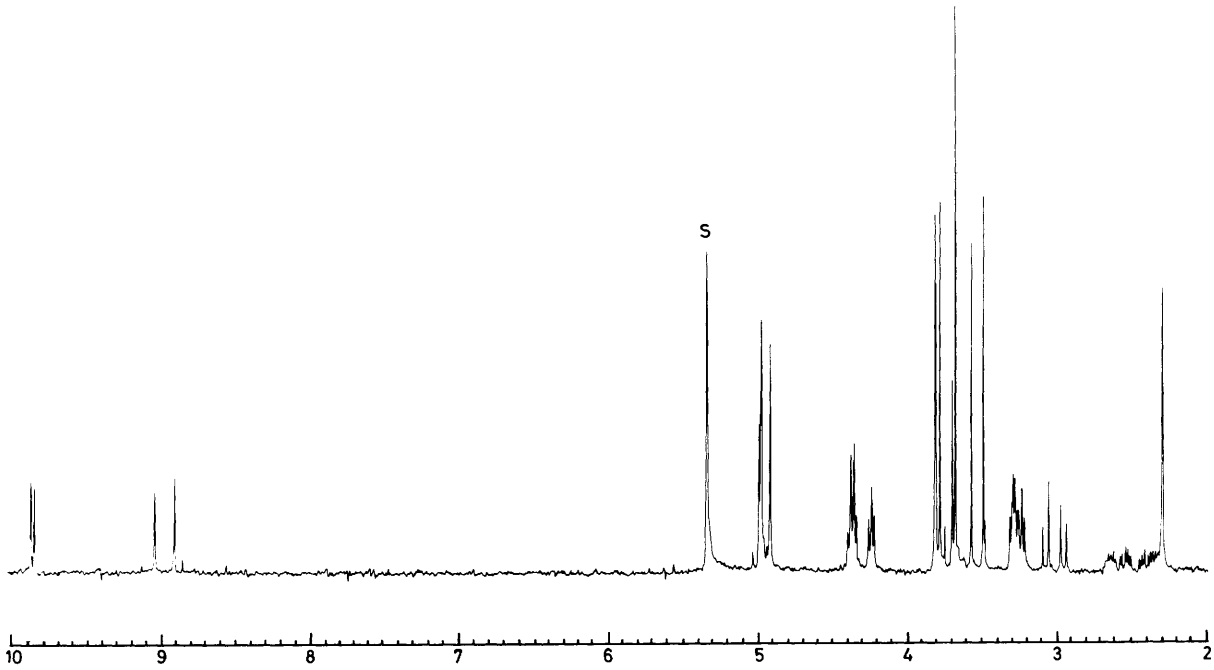


Figure 2. ^{1}H N.m.r. spectrum of Faktor-I octamethyl ester (6) at 400 MHz in CD_2Cl_2 with 264 scans, SW 4 201 Hz, AT 1.95 s and PW 8 μs. S = solvent peak

Table. Assignment of ^{1}H n.m.r. signals from Faktor-I octamethyl ester (6)

δ (multiplicity) [a]	Assignment
9.88 (s)	15-H
9.86 (s)	10-H
9.04 (s)	5-H
8.91 (s)	20-H
5.01 (AB)	7-C*H*$_2$CO$_2$Me
4.99 (s)	18-C*H*$_2$CO$_2$Me
4.94 (s)	12-C*H*$_2$CO$_2$Me
4.39 (t, *J* 8 Hz), 4.37 (t, *J* 8 Hz), 4.25 (t, *J* 8 Hz)	8, 13, 17-C*H*$_2$CH$_2$CO$_2$Me
3.83, 3.82, 3.79, 3.71, 2 × 3.70, 3.59, 3.51 (all s)	8 × CO$_2$Me
3.31 (t, *J* 8 Hz), 3.30 (t, *J* 8 Hz), 3.25 (t, *J* 8 Hz)	8, 13, 17-CH$_2$C*H*$_2$CO$_2$Me
3.09, 2.97 (AB, *J* 15 Hz)	2-C*H*$_2$CO$_2$Me
2.72—2.30 (m)	3-C*H*$_2$C*H*$_2$CO$_2$Me
2.31 (s)	2-CH$_3$

[a] Determined at 400 MHz in CD_2Cl_2.

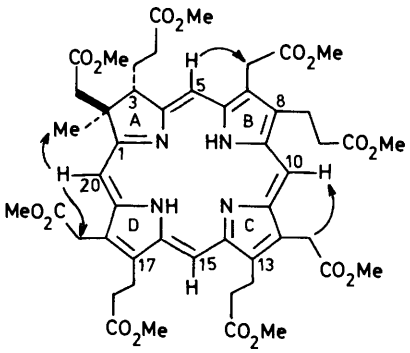
buffer (pH 7.7; 80 ml) and passed three times at 4 °C through a French Press (1 000—1 300 lb in^{-2}) all under nitrogen. The resultant mixture was centrifuged at 18 000 r.p.m. for 1.5 h at 4 °C and the supernatant liquid was passed under a slight pressure of nitrogen through a column of DEAE cellulose (*ca.* 3 × 1 cm of DEAE Sephadex which had been previously swollen in pH 7.7 0.05M-phosphate buffer). The percolate was adjusted to pH 7.0 and then mixed with the following co-factors which had been dissolved in 0.2M-phosphate-EDTA buffer (pH 8; 10 ml): *S*-adenosylmethionine (50 mg), ATP (100 mg), NAD^+ (50 mg), NADH (50 mg), cysteine hydrochloride (16 mg), and glutathione (50 mg).

The preparation of the substrate started in advance of the foregoing work so that enzyme and substrate were available freshly at the same time. Uroporphyrin-III octamethyl ester (12.33 mg) was hydrolysed by being stirred in tetrahydrofuran (8 ml) and aqueous 2M-potassium hydroxide (6 ml) for 16 h at 20 °C. The colourless organic layer was then drawn off using a pipette and the aqueous layer was held at 0.5 mmHg for 10 min to remove all the tetrahydrofuran. Careful addition of the minimum quantity of 10% hydrochloric acid caused uroporphyrin-III to precipitate and 2M-potassium hydroxide was added dropwise until this just redissolved. Pieces of *freshly prepared* 3% sodium amalgam were then added to the stirred solution under nitrogen until it became colourless (*ca.* 30 min); it was then filtered through a cotton plug and adjusted with 0.1M-hydrochloric and to pH 7.5—8.

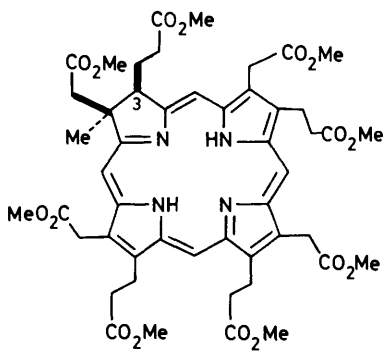
This solution of uro'gen-III was mixed immediately with the foregoing enzyme preparation and the mixture was adjusted to pH 7.3 using saturated aqueous K_3PO_4. The flask was sealed with a septum cap, mercaptoethanol (100 μl) was added *via* the cap by syringe, after which the flask was flushed with nitrogen and incubated at 30 °C for 17 h in the dark.

The resultant pigments were then trapped on DE-52 cellulose by stirring the incubation mixture with 4 consecutive lots (3 g each) of the resin, using 5 min contact each time under nitrogen. The combined DE-52 cellulose was then freeze-dried at 0.5 mmHg for 16 h.

Isolation of Faktor-I Octamethyl Ester (6) *and Detection of* 3-epi-*Faktor-I Octamethyl Ester* (7).—The pigments on the above dry DE-52 cellulose were eluted with 5% (v/v) concentrated H_2SO_4 in dry, degassed methanol (100 ml). Trimethyl orthoformate (5 ml) was added to the eluate which was kept under nitrogen for 16 h at 20 °C in the dark and then poured into a mixture of methylene chloride (50 ml) and water (100 ml); the mixture was then adjusted to *ca.* pH 9 using 10% aqueous ammonium hydroxide. The separated aqueous layer was extracted with methylene chloride (2 × 30 ml) and the combined organic solutions were washed with water (2 × 30 ml), dried, and evaporated. The residue (36 mg) was fractionated on Kieselgel H Type 60 (3 g), the required pigments being eluted with 15—20% methyl acetate in methylene chloride. These were chromatographed on Merck Kieselgel



(6a)



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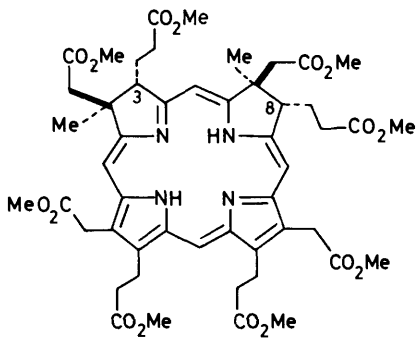

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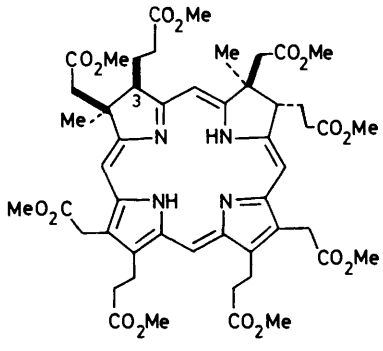

(9)

60 plates with no indicator but with concentration zones using three elutions with methyl acetate–methylene chloride (1 : 8) to give recovered uroporphyrin-III octamethyl ester (8.5 mg), crude Faktor-I ester (0.25 mg), and small amounts of decarboxylation products of uroporphyrin-III which were not examined further. The amount of chlorin was determined spectroscopically using the extinction coefficient reported by Ballantine *et al.*[10]

The crude Faktor-I ester was then fractionated by repetitive p.l.c. using the same plates as above but with chloroform–diethyl ether (1 : 4) as the solvent (4 h runs). 3-*epi*-Faktor-I ester ran at the front of the chlorin band and the repetitive fractionation eventually gave an enriched fraction containing (by n.m.r.) 3-*epi*-Faktor-I ester : Faktor-I ester (5 : 4). The lower part of the chlorin band contained Faktor-I ester.

The following are the characteristics of Faktor-I octamethyl ester. T.l.c.: R_F 0.16 in chloroform–ether (1 : 4); R_F 0.24 in methyl acetate–methylene chloride (1 : 4); R_F 0.25 in acetic acid–methyl acetate–methylene chloride (0.01 : 1 : 4). The corresponding R_F values in these systems for sirohydrochlorin octamethyl ester (8) are 0.19, 0.24, 0.19, respectively; $\lambda_{max.}$ ($CHCl_3$) with relative intensities 646 nm (0.291), 611 (0.026), 588 (0.031), 540 (0.016), 524 (0.02), 498 (0.076), 491 sh (0.074), 398 (1.0), 348 (0.153), and 280 (0.088).

C.d. using 0.2 mg in methanol (3 ml), λ (Δε) 644 (−8.5), 615 (−0.8), 483 (+0.8), 390 (+31.0), 379sh (+9.4), 348 (−2.4), 278 (−8.2), and 253 (−6.1) [Found: (f.d.-m.s.) M^+, 958.3885, $C_{49}H_{58}N_4O_{16}$ requires M, 958.3848].

H.p.l.c. using a Kontron 5μ CN column with 1 ml min^{-1} of n-hexane–toluene–acetonitrile–Hünig's base (300 : 140 : 60 : 1), u.v.-vis. monitor set on 390 nm. Emergence times: 68.8 min for Faktor-I ester, 66 min for 3-*epi*-Faktor-I ester. With a μ-Bondapak CN column under identical conditions, emergence times were Faktor-I ester 40 min, 3-*epi*-Faktor-I ester 38.7 min.

Acknowledgements

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