Biosynthesis of Vitamin B_{12}: Synthesis of (±)-[5-^{13}C]Faktor-1 Ester: Determination of the Oxidation State of Precorrin-1

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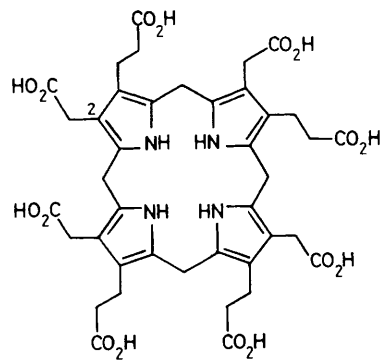
(±)-[5-^{13}C]Faktor-1 ester is synthesised following an east–west disconnection strategy and is used to show by specific enzymic incorporation experiments that the first *C*-methylation product, precorrin-1, on the B_{12}-biosynthetic pathway is a tetrahydrochlorin.

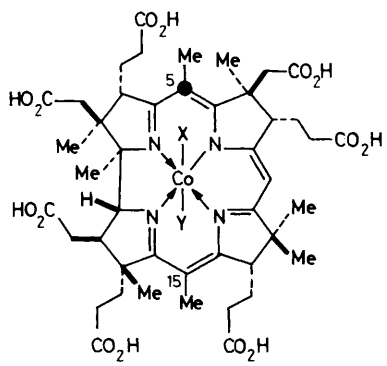
The biosynthesis of vitamin B_{12} from uroporphyrinogen III (uro'gen III) (**1**) goes *via* cobyrinic acid (**2**) and involves, *inter alia*, 8 *C*-methylation steps.[1] Knowledge that the first methyl group is inserted at C-2 came from the isolation of Faktor-1 as its octamethyl ester (**14a**) from the B_{12}-producer *Clostridium tetanomorphum*.[2,3] Faktor-1 (**15a**) itself is not a biosynthetic intermediate for B_{12}, but a reduced form of it, of unknown structure, was incorporated[3] by an appropriate enzyme preparation into cobyrinic acid (**2**). The aim of the present work was to determine the nature of this first methylated intermediate, precorrin-1,[4] and to demonstrate its specific enzymic conversion into cobyrinic acid (**2**).

Progress depended on having available workable quantities of Faktor-1 ester (**14b**) labelled at C-5 with carbon-13; this is not possible at present from natural sources. Accordingly, the synthesis of this material was undertaken. An earlier synthesis[5] of (±)-Faktor-1 ester (as **14a**) relied on a photochemical 18π-electrocyclic process to form the macrocycle and was based on a north–south retro-cleavage of the molecule; see horizontal dashed line on (**14**). A different approach was needed for efficient synthesis of [5-^{13}C]labelled material and was based on the alternative east–west retro-cleavage. The required materials were (**11**) for rings *B* and *C*, and the labile imine (**7b**) for rings *A* and *D*; the latter was produced as required from the stable precursor (**6b**). For simplicity, only one enantiomer is illustrated throughout.

The unlabelled single enantiomer (**6a**) had been synthesised earlier,[6] the later steps involving reaction of the thiolactam (**5**) with di-t-butyl monobromomalonate to give the *S*-malonyl system followed by *S*-extrusion.[7] This same route was followed for the present work but using synthetic racemic imide[8] (**4**) as starting material. The required di-t-butyl monobromo[2-^{13}C]malonate for the step (**5**) → (**6b**) was

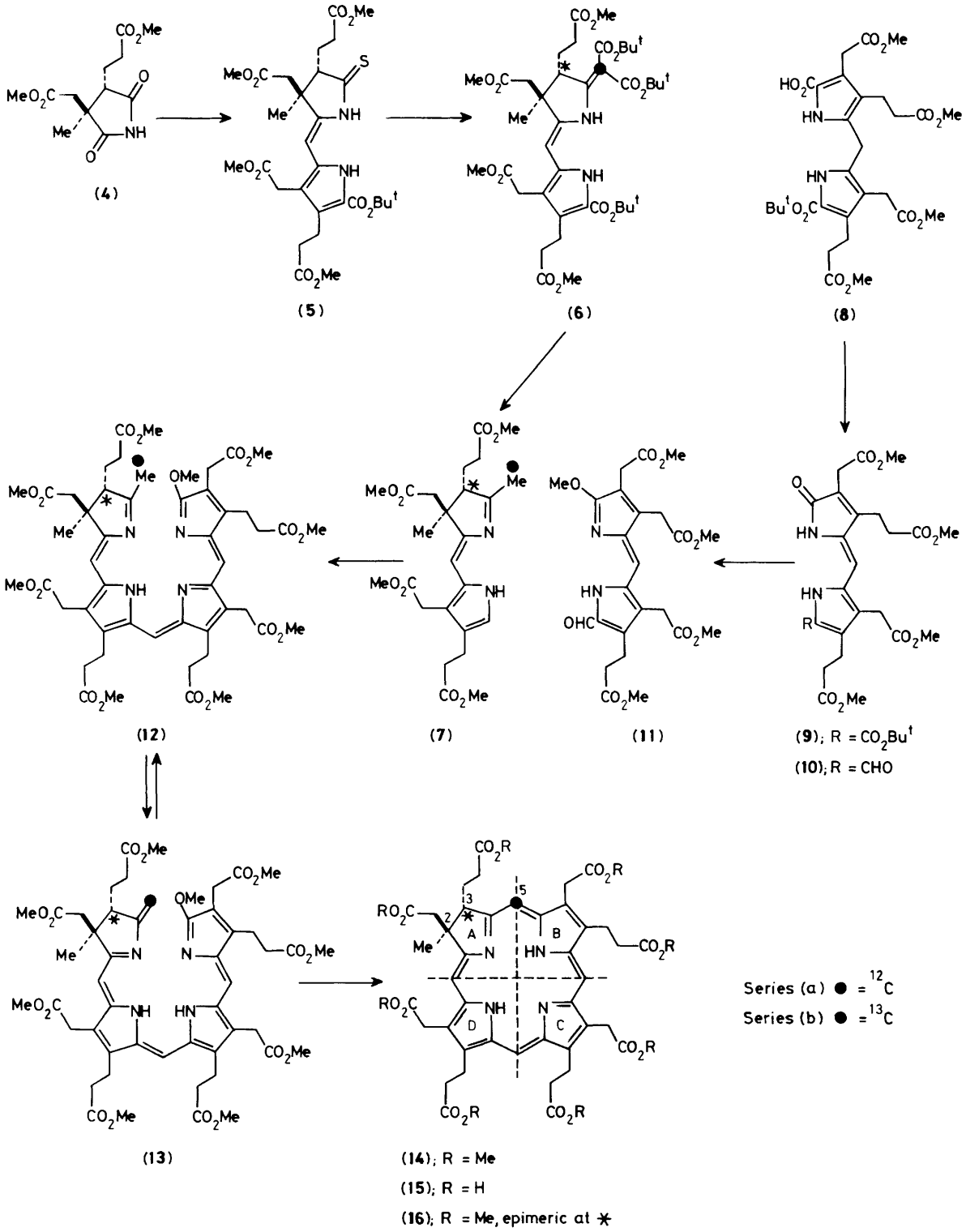


(**1**)



(**2**); X, Y = OH, H_2O; ● = ^{12}C

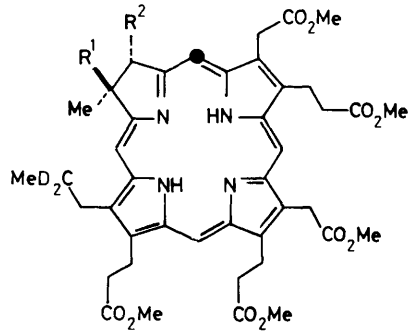
(**3**); X = Y = CN; ● = ^{13}C



prepared from [2-^{13}C]acetic acid, 91 atom % ^{13}C, which was converted[9] into [2-^{13}C]malonic acid, 83%, and the derived[10] di-t-butyl ester, 82%, as its silyl ketene acetal[11] was brominated to yield specifically the monobrominated ester, 94% (overall yield from acetic acid, 64%). The final product was obtained from the *S*-extrusion step, 60%, as a 3.2 : 1 mixture of (**6b**) and its epimer at the *-centre.

The *B*,*C* block (**11**) was obtained starting with the dipyrromethane (**8**), synthesised by standard steps. Oxidative bromination of this acid (**8**) then hydrolysis[12] yielded, 65%, the pyrromethenone (**9**). This was converted by trifluoroacetic acid (TFA) and trimethyl orthoformate into the aldehyde (**10**), 72%, which afforded 50% of the desired imino ether (**11**) on methylation with trimethyloxonium tetrafluoroborate.

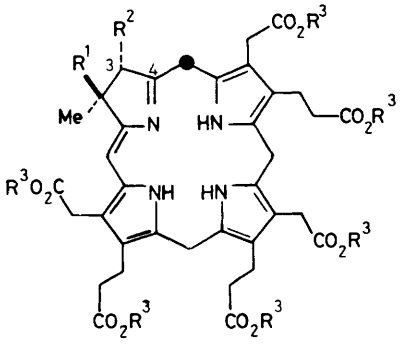
Treatment of the tri-t-butyl ester (**6b**) with TFA cleaved the t-butyl groups and promoted decarboxylation to yield the



(17); R^1 = Me, R^2 = H

(14); R^1 = $CH_2CO_2Me_2$

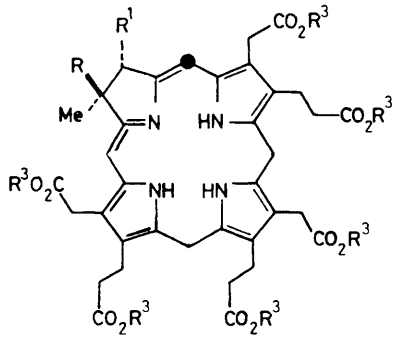
R^2 = $CH_2CH_2CO_2Me$



(18); R^1 = Me, R^2 = H, R^3 = Me

(19); R^1 = CH_2CO_2Me, R^2 = $CH_2CH_2CO_2Me$, R^3 = Me

(20); R^1 = CH_2CO_2H, R^2 = $CH_2CH_2CO_2H$, R^3 = H



(21); R^1 = Me, R^2 = H, R^3 = Me

(22); R^1 = CH_2CO_2Me, R^2 = $CH_2CH_2CO_2Me$, R^3 = Me

(23); R^1 = CH_2CO_2H, R^2 = $CH_2CH_2CO_2H$, R^3 = H

Series (a) ● = ^{12}C

Series (b) ● = ^{13}C

western block (**7b**). This product was immediately condensed with the eastern block (**11**) using TFA to generate the *seco*-system (**12b**) ⇄ (**13b**), M^+ 991 by field desorption mass spectroscopy (FD-m.s.). Extensive study showed that the reproducibility and yield in the next step of photochemical 18π-electrocyclic ring-closure were strongly influenced by added acids or bases. Thus, when the isolated *seco*-system (**12b**) was irradiated in tetrahydrofuran (THF) with or without diethylisopropylamine (Hünig's base), no chlorin was formed. The successful and reproducible method involved photochemical cyclisation of the *seco*-system (**12b**) ⇄ (**13b**) in THF containing a vast excess of pre-prepared Hünig's base-TFA salt. Under these conditions, the yield of (±)-[5-^{13}C]Faktor-1 ester (as **14b**) and its (±)-[5-^{13}C]3-epimer (as **16b**), ratio 3.2:1, was 42%. These were separated, both products were fully characterised (n.m.r., u.v.–visible, and FD-m.s.) and shown to be identical, apart from their racemic and labelled nature, with authentic samples obtained from *Clostridium tetanomorphum*.[13] Sufficient (±)-[5-^{13}C]3-epi-Faktor-1 ester (as **16b**) was available for a complete set of connectivities to be demonstrated around the periphery of the macrocycle [by COSY and nuclear Overhauser enhancement (n.O.e.) difference n.m.r. spectroscopy] and in particular for the *cis*-relationship to be established between the acetate and propionate residues at C-2 and C-3, respectively.

Mono-*C*-methylation of uro'gen III would be expected to lead to tetrahydrochlorins such as (**20a**), (**23a**) or some tautomer of these substances. Accordingly, we sought to prepare tetrahydrochlorins, initially from the model chlorin[14] (**17a**) and its 5-^{13}C-labelled form (**17b**). This was possible by catalytic hydrogenation over platinum but best by reduction with 3% sodium amalgam in dry MeOH–THF. Fractionation of the products in a glove box at <5 p.p.m. O_2 gave a mixture of tetrahydrochlorins, major (**18a**) and minor (**21a**), ratio 5.4:1, 67%, 818 by FD-m.s. ^{1}H N.m.r. spectroscopy (400 MHz) of these products and ^{13}C n.m.r. spectroscopy (100.6 MHz) in the ^{13}C-series (**18b**) and (**21b**) established the illustrated structures and showed that these two isomers were the only significant materials present. They gave a weak blue fluorescence in u.v. light and were highly susceptible to oxidation by air.

Analogous reduction of (±)-[5-^{13}C]Faktor-1 ester (as **14b**)

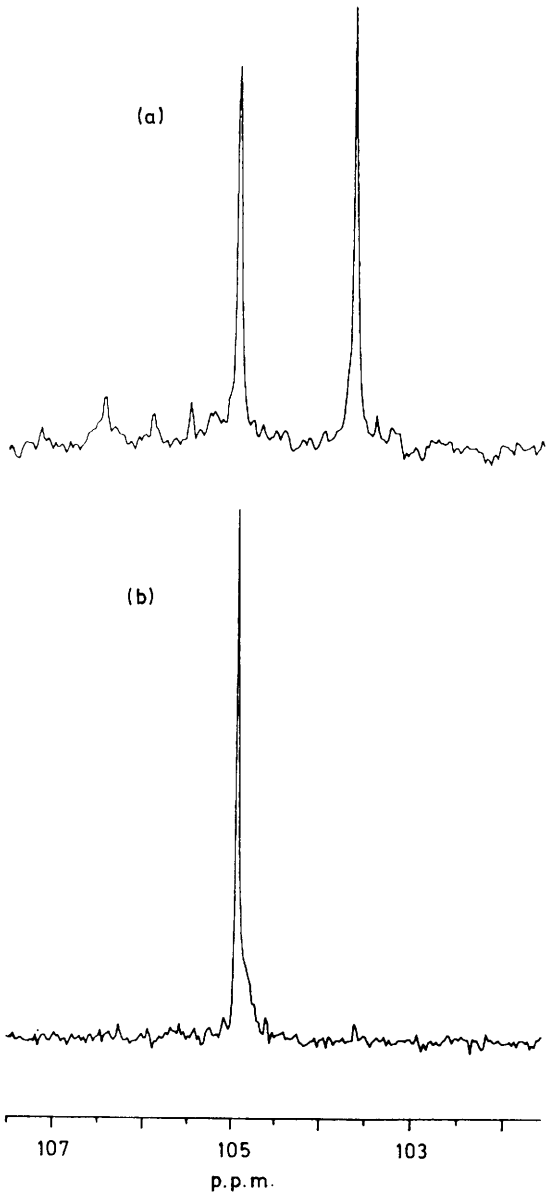


Figure 1. (a) Proton noise decoupled ^{13}C n.m.r. spectrum of cobyrinic acid [(**2**), X = Y = CN] at natural abundance run at 100.6 MHz in D_2O-CD_3OD containing KCN. (b) The equivalent ^{13}C n.m.r. spectrum of cobyrinic acid (**3**) isolated from the enzymic incorporation experiments with the tetrahydrochlorins (**20b**) and (**23b**).

by sodium amalgam gave a mixture of the blue fluorescent tetrahydrochlorins, major (**19b**) and minor (**22b**), ratio 1.4 : 1, 52%, *m/z* 963.4196, $C_{48}{}^{13}CH_{62}N_4O_{16}$ requires 963.4194. ^1H and ^{13}C n.m.r. spectroscopy established the illustrated structures, the major tautomer corresponding in structure to that of the major one in the model series; the 5-^{13}C-signal for major isomer (**19b**) appeared at δ 29.42 and for minor (**22b**) at δ 106.21. Importantly, a 2D COSY spectrum eliminated the possibility that the major isomer possessed a C-3 to C-4 double bond (see **19b**) rather than the illustrated C-4 to N-position (**19b**).

Hydrolysis of the ^{13}C-labelled model tetrahydrochlorins (**18b**) and (**21b**) with 2 M aqueous piperidine (<5 p.p.m. O_2) gave the corresponding hexacarboxylic acid salts; ^{13}C n.m.r. showed that the only skeletal change was in the ratio of tautomers (from 5.4 : 1 at outset to 3.0 : 1 after hydrolysis). The same hydrolysis conditions were then used for the mixture of labelled tetrahydrochlorins (**19b**) and (**22b**). The resultant acids (**20b**) and (**23b**) as their salts were incubated anaerobically with the cell-free enzyme system prepared from *P. shermanii* cells and the cobyrinic acid formed, plus the minimal added carrier cobyrinic acid (unlabelled) was isolated chromatographically[15] as its dicyano complex (**3**).

Figure 1(a) illustrates the δ 102—107 region of the natural abundance ^1H-decoupled ^{13}C n.m.r. spectrum of cobyrinic acid dicyano complex [(**2**) X = Y = CN] which spans only the signals from C-5 and C-15. The ^{13}C-spectrum of the cobyrinic acid (**3**) biosynthesised in the foregoing studies [Figure 1(b)] established that specific incorporation of the 5-^{13}C-labelled precursor had occurred. Conservative estimation of incorporation from signal intensity showed it to be in the 10—15% range.

The foregoing experiments show that (a) the first *C*-methylation product, precorrin-1, on the biosynthetic pathway to vitamin B_{12} is a tetrahydrochlorin. Thus the oxidation level of uro'gen III (**1**) is preserved in the first methylation step as it is at the dimethylated stage[16] of precorrin-2; (b) the structure of precorrin-1 is either (**20a**) or (**23a**) or the product of enzymic *C*-methylation of uro'gen III (**1**) is a mixture (interconverting?) of the two.

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