Biosynthesis of Vitamin B_{12}: Isolation of 15,23-Dihydrosirohydrochlorin, a Biosynthetic Intermediate: Structural Studies and Incorporation Experiments

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An enzyme system from the vitamin B_{12} producer *Propionibacterium shermanii* converts uro'gen-III (**2**) into 15,23-dihydrosirohydrochlorin (**7**), the structure being established by ^1H and ^{13}C n.m.r. spectroscopy; this dihydro-macrocycle is enzymically converted into cobyrinic acid (**6**).

Research on the biosynthesis of vitamin B_{12} gained a fresh direction and impetus when it was found that micro-organisms which produce the vitamin could, under controlled conditions, be made to excrete two isobacteriochlorins.[1] One, a di-*C*-methylated system, was first shown to be identical with the known sirohydrochlorin and was then proved[1] to have structure (**3**); the structure of the second new pigment was found to be (**5**), in which the third *C*-methyl group is located at C-20.[1] Labelled forms of the pigments (**3**) and (**5**) were demonstrated to be incorporated specifically into cobyrinic acid (**6**)

when they were incubated with an appropriate enzyme system (*e.g.* prepared from *Propionibacterium shermanii*);[1] cobyrinic acid (**6**) is a late precursor of vitamin B_{12}.

The macrocycles (**3**) and (**5**) are produced by enzymic *C*-methylation of uro'gen-III[1] (**2**) and the conversion (**2**) → (**3**) has been achieved in isolated enzyme systems.[2,3] However, sirohydrochlorin (**3**) has one C=X bond more than uro'gen-III (**2**) so it has seemed possible that the true biosynthetic intermediates on the pathway to vitamin B_{12} are dihydroisobacteriochlorins and that these are converted oxidatively (air?) into the isolated aromatic systems (**3**) and (**5**). If so, a reducing system must be present in the cell-free extract when, *e.g.*, sirohydrochlorin (**3**) is incorporated into cobyrinic acid (**6**) to regenerate from (**3**) the necessary dihydroisobacteriochlorin. Earlier indications[2,3] that the characteristic isobacteriochlorin chromophore appeared only during work-up of enzymic products supported these possibilities but the actual intermediates have remained elusive. Plans for experiments on isolation had to take into account the known extreme oxygen sensitivity of synthetic dihydroisobacteriochlorins[4] and also the minute amounts of material produced in enzymic runs (0.3—0.4 mg).

A cell-free enzyme system[5] derived from cobalt-free *P. shermanii* cells was transferred to an inert atmosphere chamber (<5 p.p.m. O_2) where all subsequent steps were carried out. Uro'gen-III (**2**) was incubated at 33 °C with this system containing *S*-adenosylmethionine, ATP, NADH, reduced glutathione, cysteine, and mercaptoethanol together with sodium dithionite (400 mg) in *ca.* 80 ml of enzyme solution. The products were esterified under mildly basic conditions with $Me_3O^+BF_4^-$ and then fractionated first on silica and then on glucose, both adsorbants rigorously deoxygenated, to yield a yellow fraction, $M^+ + H$ *m/z* 977 (by fast atom bombardment, FAB) which corresponds to a dihydrosirohydrochlorin ester such as (**8**) or an isomer. In support, controlled iodine oxidation of this product gave mainly sirohydrochlorin ester (**4**, 51%), M^+ 974 by field desorption, in admixture with 3-episirohydrochlorin ester[6] [28%, as (**4**) inverted at C-3] and 8-episirohydrochlorin ester[7] [21%, as (**4**) inverted at C-8]. The exact nature of this enzymic product is considered further below.

Catalytic hydrogenation[4] of sirohydrochlorin ester (**4**) gave a single dihydro ester, M^+ 976 by field desorption and $M^+ + H$ 977 by FAB, shown by n.m.r. spectroscopy to have structure (**8**); it was oxidised by air to regenerate the aromatic system (**4**) but in poor yield. The ^1H signal from H-5 of the dihydroproduct (**8**) was assigned by analogous hydrogenation of (**4**) partly deuteriated at C-5.[8] Also, the signal from H-10 of (**8**) showed the familiar allylic coupling[8] to H-8 and the remaining low-field signal (δ_H 5.58) was assigned to H-20 (similar chemical shift to H-10). On this basis, the signal which had moved considerably upfield (see Table 1) arises from H_2-15; it appeared as an AB double doublet, *cf.* ref. 4 (diastereotopic protons). These data support structure (**8**) for the ester of dihydrosirohydrochlorin.

Structure (**8**) was rigorously confirmed by preparing [^{13}C]-sirohydrochlorin ester, specifically ^{13}C-labelled as illustrated,[1] (**4a**), from [5-^{13}C]aminolaevulinic acid (**1**); the enrichment at each labelled site was *ca.* 35 atom %. The unique signal of 5 lines in the proton-decoupled ^{13}C n.m.r. spectrum of this ester (**4a**) can be unambiguously assigned to C-15. The 5 lines arise because the ^{13}C-enrichment is far below 100% so there is a superimposition of a double doublet, a doublet, and a singlet derived from ^{13}C at C-15 being directly bonded, respectively, to two, one, or zero ^{13}C-atoms in the mixture of labelled species present (see **4a**). When this labelled ester (**4a**) was hydrogenated as above, it was the 5-line signal which moved massively upfield [from δ_C 111.6 in (**4a**) to δ_C 22.8 p.p.m. in (**8a**)]; thus C-15 has changed from the sp^2 to the sp^3 state. Moreover, off-resonance decoupling proved that the signal at δ_C 22.8 p.p.m. arises from a CH_2 group.

The dihydroisobacteriochlorin fraction produced enzymically above was then compared by ^1H n.m.r. spectroscopy at 400 MHz with the foregoing 15,23-dihydrosirohydrochlorin ester (**8**); the main enzymic product proved to be identical with the ester (**8**). Smaller amounts of dihydro-3-episirohydrochlorin ester [as (**8**), inverted at C-3] and dihydro-8-episirohydrochlorin ester [as (**8**), inverted at C-8] were present. Authentic samples of these isomers were prepared by catalytic hydrogenation as above of the corresponding aromatic systems [as (**4**), inverted at C-3][6] and [as (**4**), inverted at C-8].[7] The precise origin of these C-3 and C-8 epi-forms requires further examination.

Appropriate blank experiments (*e.g.* omitting only the enzymes) established that dihydrosirohydrochlorin (**7**) is not produced under the foregoing incubation conditions by chemical reduction from sirohydrochlorin (**3**); accordingly,

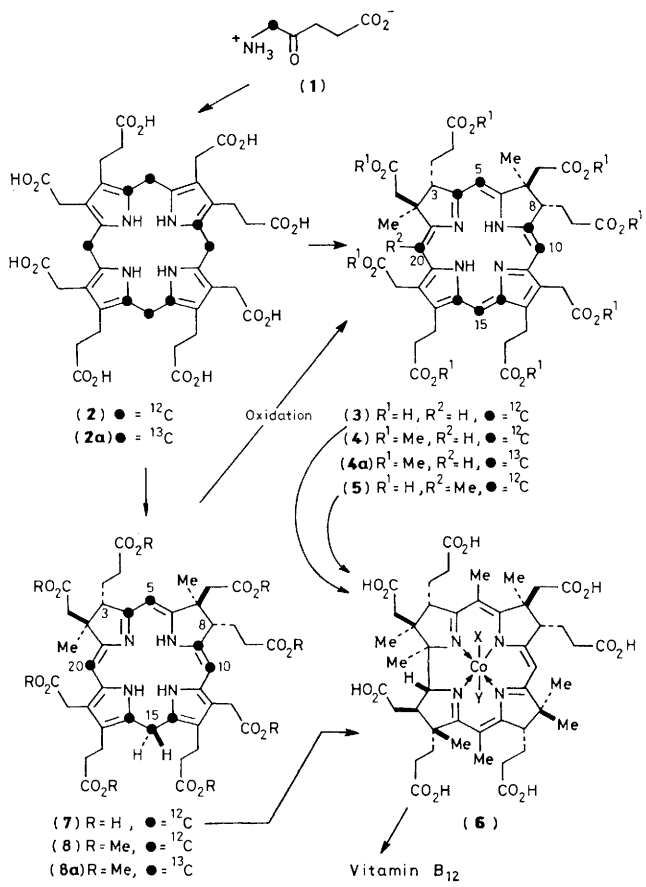

Table 1. ^1H Chemical shifts (δ values) and multiplicity at 400 MHz in C_6D_6.

	H-5	H-10	H-15	H-20
Sirohydrochlorin ester (**4**)	6.78 (s)	7.65 (br s)	8.86 (s)	7.55 (s)
Dihydrosirohydrochlorin ester (**8**)	5.06 (s)	6.07 (br s)	3.81 (d, *J* 17 Hz) 3.66 (d, *J* 17 Hz)	5.81 (s)
Change in δ	1.72	1.58	5.05 5.20	1.74

the biosynthetic intermediate at the natural oxidation level has been isolated as its ester and structurally characterised.

The final phase of the work was a comparison of the incorporations into cobyrinic acid (**6**) of the dihydro-macrocycle (**7**) relative to those of the aromatic system (**3**). These experiments involved difficulties; the extremely labile dihydro-precursor (**7**), obtained by hydrolysis of the ester (**8**), was required in labelled form (*ca.* 0.4 mg) for incubation in an unstable and initially oxygen-containing enzyme system (*ca.* 80 ml). For the initial experiments, 24 h incubations were used which gave incorporations of the dihydro-octa-acid (**7**), ^{14}C-labelled at the *C*-methyl groups, 2.5—3.3 times better into cobyrinic acid (**6**) than strictly parallel runs with the equivalently labelled sirohydrochlorin itself (**3**). However, the results were not totally reproducible; the outcome for one run was that the aromatic system (**3**) was 1.3 times better incorporated than was the dihydro-derivative (**7**). The reproducible method used 3—5 h incubations;† then the incorporation values for (**7**) were 7.4—9.8% which were 1.5—1.6 times higher than strictly parallel experiments using (**3**).

In conclusion, 15,23-dihydrosirohydrochlorin has been isolated as its ester (**8**) from a broken cell preparation from *P. shermanii*, its structure has been established, and the corresponding octa acid (**7**) has been shown to be incorporated by the appropriate enzyme system into cobyrinic acid (**6**) *en route* to vitamin B_{12}.

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† The advantage of using short incubations can be understood by considering the extreme ease of destruction of dihydro-sirohydrochlorin (**7**) relative to the fairly stable aromatic system (**3**) and the ability of the enzyme system gradually to produce the former from the latter.

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