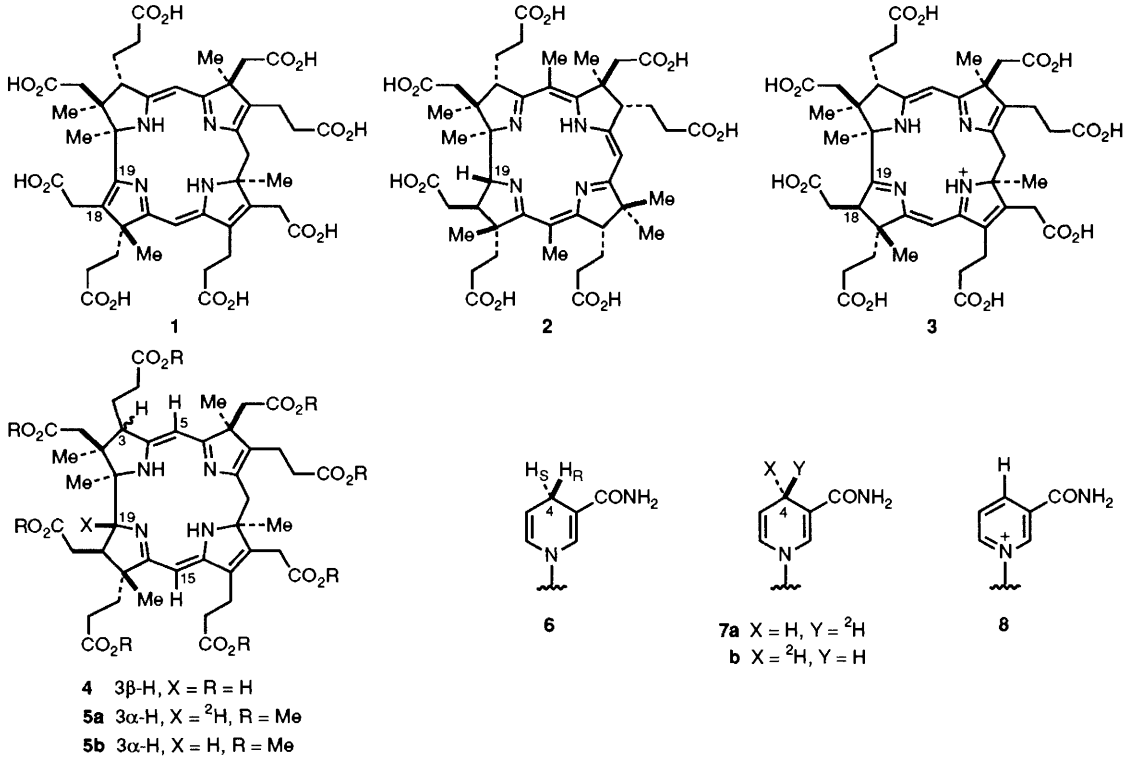
Biosynthesis of Vitamin B_{12}: Stereochemistry of Transfer of a Hydride Equivalent from NADPH by Precorrin-6x Reductase

Fumiyuki Kiuchi,[a] Denis Thibaut,[b] Laurent Debussche,[b] Finian J. Leeper,[a] Francis Blanche*[b] and Alan R. Battersby*[a]

[a] *University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*
[b] *Department Analyse, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer, BP14, F-94403 Vitry-sur-Seine Cedex, France*

Deuterium labelling with NMR analysis demonstrates that the enzyme precorrin-6x reductase transfers H_R from C-4 of the pyridine residue of NADPH as the first step in the sequence leading from precorrin-6x to hydrogenobyrinic acid.

Enzymic conversion of the recently discovered intermediate[1] for corrin biosynthesis, precorrin-6x, of structure[1–3] **1** (or possibly a double-bond tautomer[2,3]) into hydrogenobyrinic acid **2** involves several stages, the first being a reduction step. This reduction specifically depends on NADPH (reduced nicotinamide adenine dinucleotide phosphate) as the reducing cofactor[1] and the corresponding enzyme, precorrin-6x reductase, has been purified, characterised and its structural gene identified.[4] Deuterium labelling experiments proved[5] that the hydride equivalent transferred from NADPH to precorrin-6x



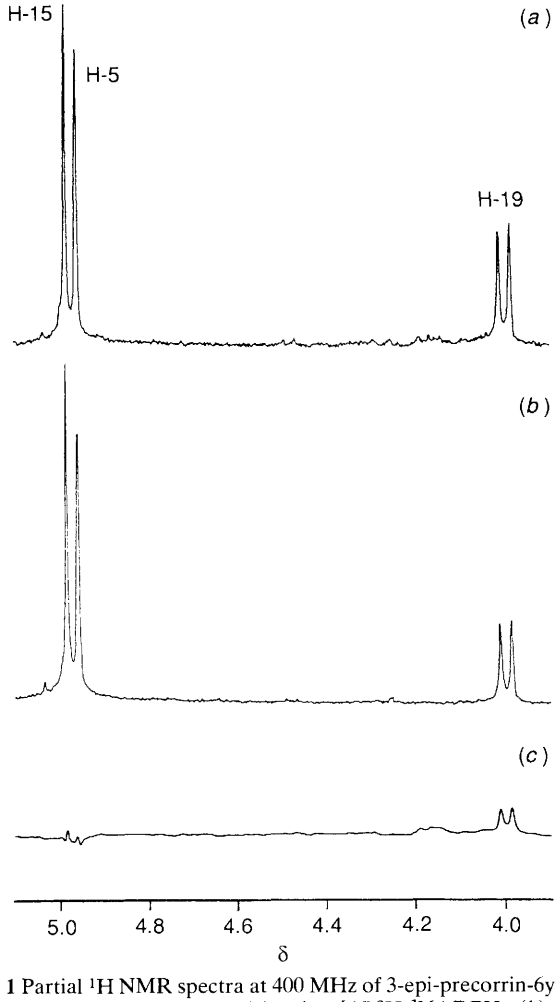


Fig. 1 Partial ^{1}H NMR spectra at 400 MHz of 3-epi-precorrin-6y ester derived from precorrin-6x **1** (*a*) using [4*S*-$^2H_1$]NADPH, (*b*) using [4*R*-$^2H_1$]NADPH, (*c*) difference spectrum (*a*) − (*b*). For the difference spectrum the ratio for subtraction was adjusted to give optimum nulling of the signals for H-5 and H-15.

appears at C-19 in hydrogenobyrinic acid **2** so presumably it is the C-18 protonated form **3** of precorrin-6x to which the transfer of hydride occurs. We now outline experiments which show the stereoselectivity of the reductase enzyme for the diastereotopic hydrogens at C-4 of the pyridine residue in NADPH, part structure **6**.

[4*R*-$^2H_1$]NADPH **7a** was prepared by reducing NADP, part structure **8**, using [$^2H_8$]propan-2-ol and the alcohol dehydrogenase from *Thermoanaerobium brockii*;[6] the 4*R* position was shown to carry 93% ^{2}H by NMR analysis.[7,8] The complementary [4*S*-$^2H_1$]NADPH **7b** was synthesised by first treating NADP **8**, glutathione and ammonium carbonate twice with deuterium oxide to replace all the exchangeable protons for deuterons. The reduction of **8** to afford **7b** was then effected using glutathione reductase from baker's yeast;[6] the product carried 97% ^{2}H at the 4*S* position by NMR.

Precorrin-6x **1** and [4*R*-$^2H_1$]NADPH **7a** were incubated with the protein preparation from *Pseudomonas denitrificans* SC510 Rif^r (pXL 253)[1] which contained an enriched level of precorrin-6x reductase,[4] and the reduction product, precorrin-6y[9] **4** was purified as previously described.[4] The latter was esterified and the two resultant C-3 epimers[9] were separated chromatographically; the major isomer, assigned[9] the 3-epi structure, **5a** was used for NMR analysis. The entire sequence was repeated but now using [4*S*-$^2H_1$]NADPH **7b** to give finally a second sample of 3-epi-precorrin-6y ester **5b**.

The partial epimerisation at C-3 during isolation and esterification procedures (observed previously for earlier intermediates *e.g.* ref. 10) is of no consequence for the current experiments. Either isomer could have been used for NMR analysis and only the major one allowed accurate determination of signal intensities on very small samples (0.3–0.4 mg).

Fig. 1(*a*) shows the ^{1}H-signals from H-19, H-5 and H-15 of 3-epi-precorrin-6y ester **5b** from [4*S*-$^2H_1$]NADPH **7b** and Fig. 1(*b*) shows the same signals from 3-epi-precorrin-6y ester **5a** generated from [4*R*-$^2H_1$]NADPH **7a**. The H-19 signal in Fig. 1(*b*) is clearly diminished as confirmed by the difference spectrum Fig. 1(*c*). Repeated integration (10 times) with averaging of these signal intensities using the signals from H-5 and H-15 as internal standards, showed that ester **5a** derived from precorrin-6x and [4*R*-$^2H_1$]NADPH **7a** was labelled with

deuterium to 33 ± 2% at C-19. The ester **5b** obtained using [4*S*-$^2H_1$]NADPH **7b** was not detectably labelled.

The incorporation of deuterium in the present work is similar to and slightly higher than the 25–30% which was found[5] when [4-$^2H_2$]NADPH was the reducing cofactor. The reason suggested earlier[5] (possible flavin-mediated exchange) for the incorporation being *ca.* 30% despite the [4*R*-$^2H_1$]NADPH being >90% deuteriated also holds here. The two results together indicate that precorrin-6x reductase has essentially complete stereospecificity for the transfer of H_R from NADPH **6**.

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