APPLICATIONS OF NMR AND MOLECULAR BIOLOGY IN STUDIES OF THE ENZYME MECHANISMS OF VITAMIN B_{12} BIOSYNTHESIS*

A. Ian Scott

Center for Biological NMR, Chemistry Department, Texas A&M University, College Station, Texas 77843, USA

Abstract - The active site of porphobilinogen (PBG) deaminase has been enriched with carbon-13 using cells of genetically engineered *E. coli*. Nmr spectroscopy has uncovered the structure of a novel dipyrromethane cofactor, covalently bound through Cys-242, which acts as a nucleophilic site for the covalent binding of substrate. Based on the results of pulse experiments with ^{13}C-enriched S-adenosylmethionine (SAM), the sequence of methylation in the overall conversion of uro'gen III to cobyrinic acid is $C_2 > C_7 > C_{20} > C_{17} > C_{12\alpha} > C_1 > C_5 > C_{15}$. These results are incorporated into a mechanistic scheme for corrin biosynthesis which also takes into account the discovery of a new series of corphinoids based on the type-I porphyrin template.

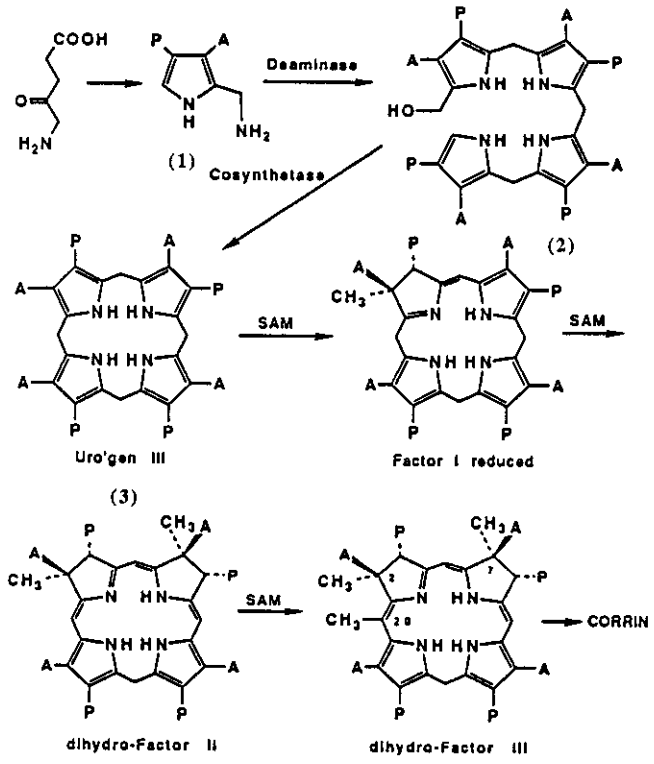
INTRODUCTION

For the past 20 years our laboratory has been engaged in the fascinating process of discovering how Nature synthesizes the corrin structure from δ-aminolevulinic acid (ALA), SAM, and cobalt ion. These two decades of biosynthetic, enzymological and synthetic studies are summarized in Scheme 1 which shows all the known intermediates including PBG, Uro'gen III and the three methylated pre-corrinoids (in their reduced forms) which were unknown at the outset. This review will focus on two specific topics drawn from both "early" and "late" segments of the pathway. The first topic is concerned with mechanistic and structural proposals for the enzyme PBG deaminase which, together with uro'gen III synthase, is responsible for the construction of the type I and III uro'gens. The second theme encompasses the sequence of methylations leading finally to cobyrinic acid, together with a remarkable new series of corphinoids, which result from methylation of uro'gen I.

The Enzymes of Tetrapyrrole Synthesis

PBG deaminase (EC4.3.1.8) catalyzes the tetramerization of PBG (1) to preuro'gen (hydroxymethylbilane, HMB; 2)[1,2] which is cyclized with rearrangement to the unsymmetrical uro'gen III (3) by uro'gen III synthase (EC4.2.1.75) (Scheme 1). In the absence of the latter enzyme, preuro'gen (2) cyclizes to uro'gen I (4),[3] which, as discussed below, is a substrate for the methylases of the vitamin B_{12} pathway.[4,5] We have constructed a plasmid pBG 101 containing the *Escherichia coli hemC* gene[6] for deaminase. *E. coli* (TBI) transformed with this plasmid produces deaminase at levels greater than 200 times those of the wild strain[6] thereby allowing access to substantial quantities of enzyme for detailed study of the catalytic site.

*Dedicated to Sir Derek Barton on the occasion of his 70th birthday.



Scheme 1

Previous work with deaminase (from *Rhodopseudomonas spheroides*) had established that a covalent bond is formed between substrate and enzyme. Application of ^{3}H-nmr spectroscopy to the mono PBG adduct (ES-1) revealed that, in contrast to a claim[7] since withdrawn[8] that the ε-NH_2 of a lysine residue is covalently attached to substrate, the observed (rather broad) ^{3}H chemical shift indicated bond formation with a cysteine thiol group at the active site. Recently, we have found that a novel cofactor, derived from ALA during the biosynthesis of deaminase, is covalently attached to one of the four cysteine residues of the enzyme in the form of a dipyrromethane which, in turn, becomes the site of attachment of the succeeding four moles of substrate during the catalytic cycle. First it was shown that, at pH < 4, PBG deaminase (5) rapidly developed a chromophore (λmax 485nm) diagnostic of a pyrromethene (as 6), whilst reaction with Ehrlich's reagent generated a chromophore typical of a dipyrromethane (λmax 560nm) changing to 490nm after 5 - 10 min. The latter chromophoric interchange was identical with that of the Ehrlich reaction of the synthetic model pyrromethane (7) and can be ascribed to the isomerization shown (Scheme 2) for the model system (7). Incubation of *E. coli* strain SASX41B (transformed with plasmid pBG 101, *hemA*$^{-}$ requiring ALA for growth) with 5-^{13}C-ALA (100 mg/l.; 90 atom ^{13}C %) afforded highly enriched (> 80% ^{13}C) enzyme for nmr studies. At pH8, the enriched carbons of the dipyrromethane (py-CH_2-py) are clearly recognized at 24.0 ppm (py-CH_2-py), 26.7 ppm (py-CH_2-X), 118.3 ppm (α-free pyrrole) and 129.7 ppm (α-substituted pyrrole) (Fig. 1A). The signals are sharpened at pH12 (Fig. 1B) and the CH_2S- resonance is shifted to δ 29.7 in the unfolded enzyme. Comparison with synthetic models reveals that a shift of 26.7 ppm is in the range expected for an α-thiomethylpyrrole (py-CH_2 SR). Confirmation of the dipyrromethane (rather than oligo pyrromethane)[8] came from the ^{13}C INADEQUATE spectrum taken at pH12 (Fig. 1C) which reveals the expected coupling only between py-CH_2-py (δ 24.7) and the adjacent substituted pyrrole carbon (δ 128.5 ppm). When the enriched deaminase was studied by INVERSE INEPT spectroscopy, each of the protons attached to ^{13}C-nuclei were observed as shown in Fig. 1D. The ^{13}C-nmr spectra

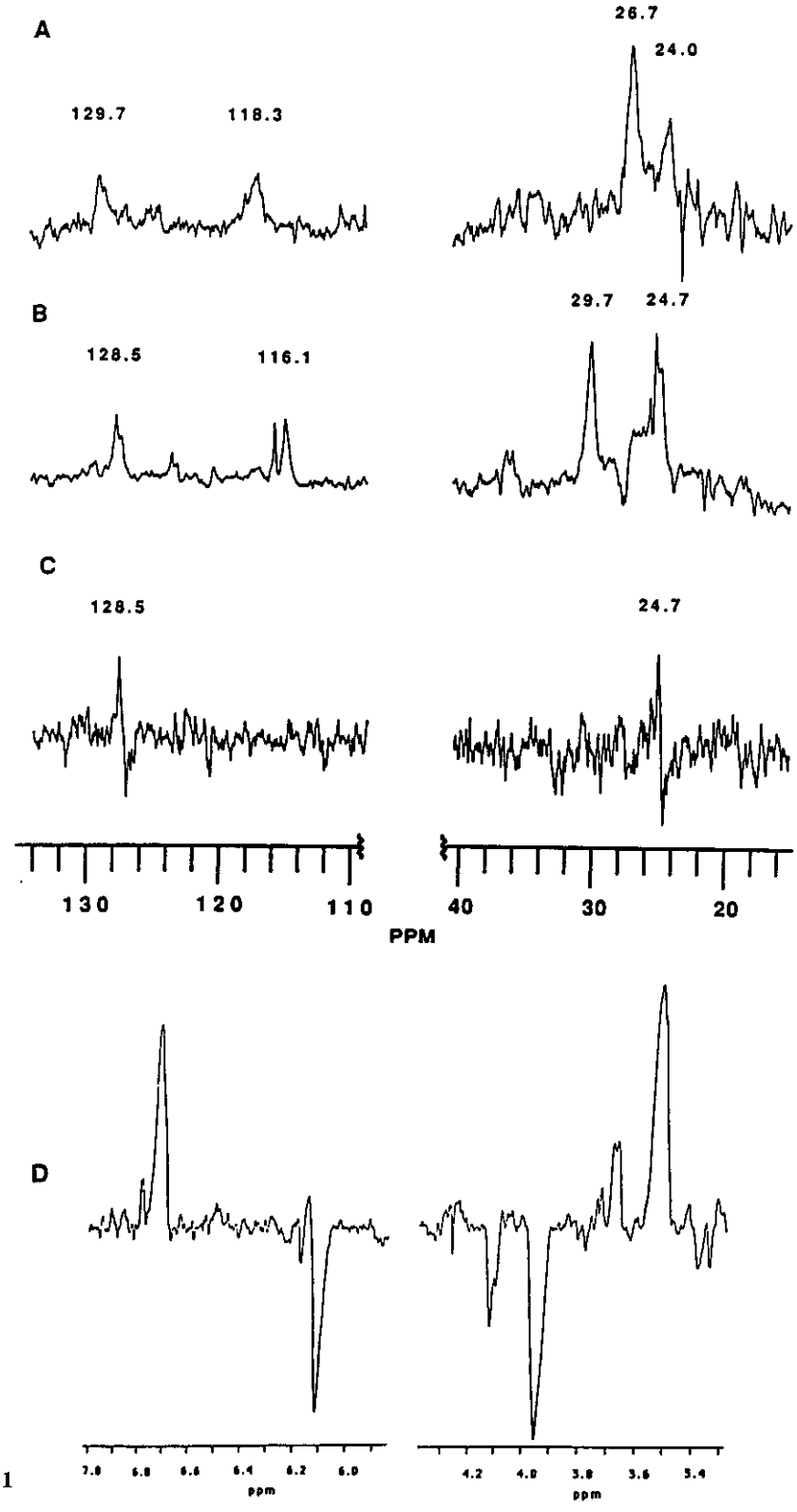
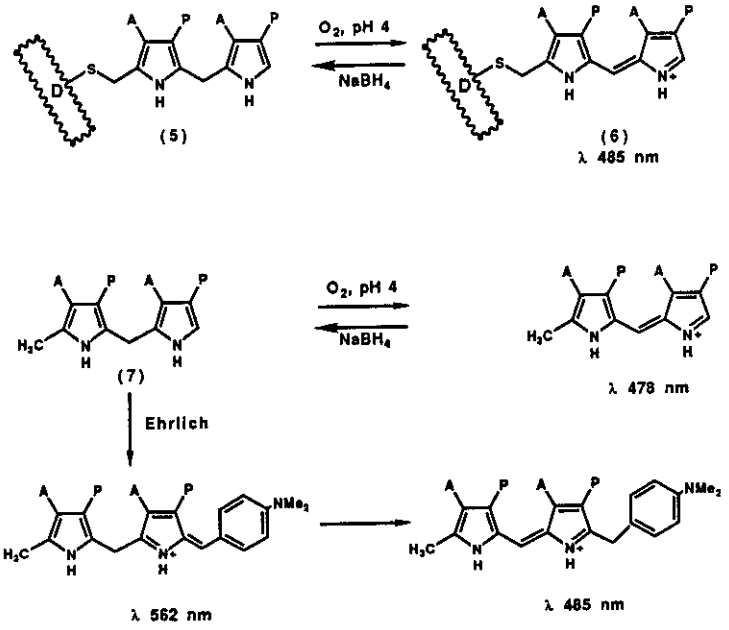
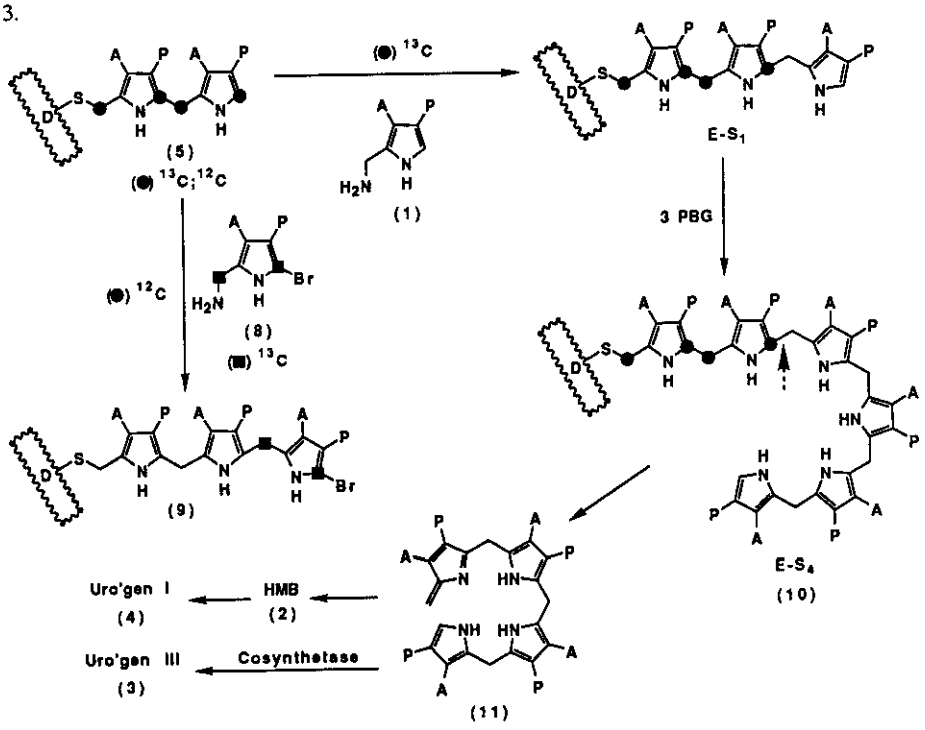

A
129.7
118.3
26.7
24.0
B
128.5
116.1
29.7
24.7
C
128.5
24.7
130
120
110
40
30
20
PPM
D
7.0
6.8
6.6
6.4
6.2
6.0
ppm
4.2
4.0
3.8
3.6
3.4
ppm

Figure 1



Scheme 2

of the ES complexes showed attachment of PBG at the α-free pyrrole of the enzyme. Next, a specimen of deaminase was covalently inhibited with [2,11-$^{13}C_2$]-2-bromo PBG (8, Fig. 2). The ^{13}C-nmr spectrum (pH12) is consistent only with structure 9. The site of covalent attachment of substrate (and inhibitor) is therefore the free α-pyrrole carbon at the terminus of the dipyrromethane in the native enzyme, leading to the structural and mechanistic proposal shown in Scheme 3.



Scheme 3

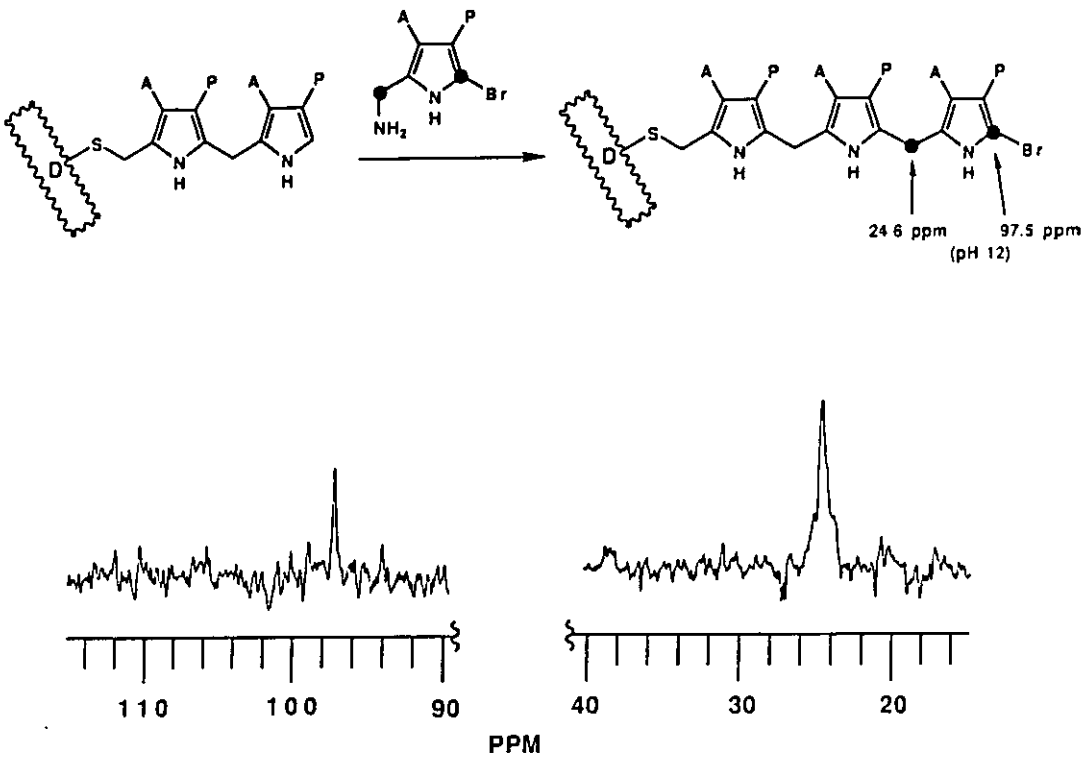
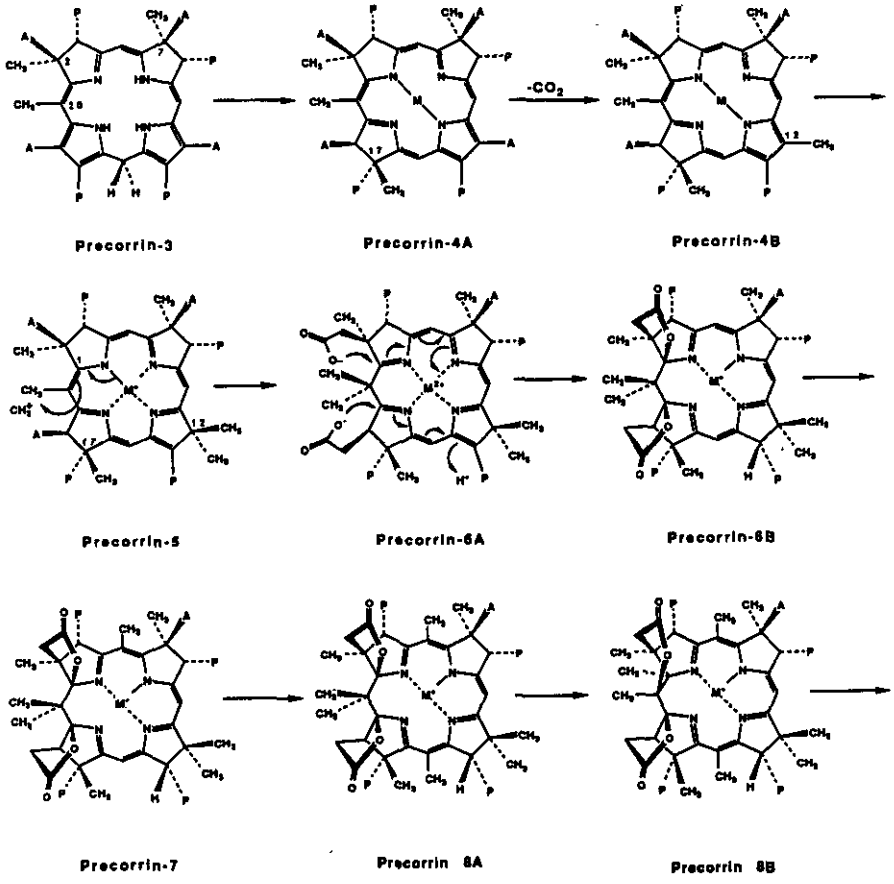


Figure 2

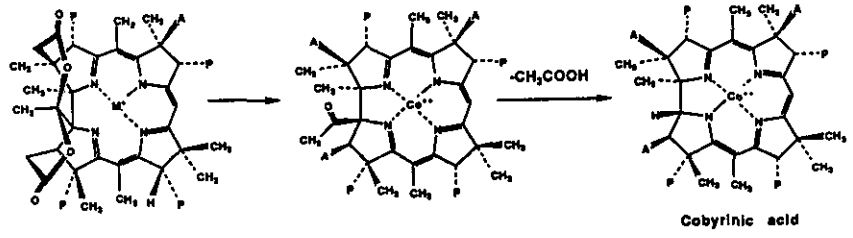
We suggest that PBG is incorporated into the apoenzyme before folding and that the first (kinetic) encounter of PBG deaminase with substrate involves attachment of PBG (with loss of NH_3) to the α-free pyrrole position of the dipyrromethane to form the ES_1 complex (Scheme 3). The process is repeated until the "tetra PBG" (ES_4) adduct (10) is formed. At this juncture site-specific cleavage of the hexapyrrole chain (at →) releases the azafulvene bilane (11) which either becomes the substrate of uro'gen III synthase, or in the absence of the latter enzyme, is stereospecifically hydrated[3] to HMB (2) at pH12, or is cyclized chemically to uro'gen I (4) at pH ≤ 8. Recent independent and complementary work from two other laboratories[8,9] has reached similar conclusions regarding the catalytic site but does not address the question of the covalent linkage to the enzyme or the exact chain length of the oligopyrrolic cofactor. The Texas results define both the number of PBG units (two) attached in a head-to-tail motif to the native enzyme at pH8 as well as revealing the identity of the nucleophilic group (Cys-SH) which anchors the dipyrromethane (and hence the growing oligopyrrolic chain) to the enzyme.[10] Site specific mutagenesis and chemical cleavage have recently revealed Cys-242 as the point of attachment of the cofactor.[11] Thus, replacement of cysteine with serine at residues 99 and 242 (respectively) gave fully active and inactive specimens of enzyme.[11b] Formic acid cleavage and analysis of the fragments confirmed this assignment which has recently been rigorously proved by reconstitution of the holoenzyme from genetically engineered production of apoenzyme.

Temporal Resolution of the Methylation Sequence in Corrin Biosynthesis

Continuing our account of the fate of the uro'gens in B_{12} producing bacteria, investigations of the biosynthetic pathway to vitamin B_{12} in collaboration with Dr. G. Müller (Stuttgart) have recently uncovered the sequence of methylations which mediate the conversion of the precursor uro'gen III (3) to cobyrinic acid (Scheme 4) using cell-free extracts of *Propionibacterium shermanii* and a series of carefully chosen conditions whereby the SAM-derived methyl groups labeled with ^{13}C are "titrated" into the corrin structure by pulse experiments.[3,6a] The complete methylation



Scheme 4



sequence[3,6a,12] is shown in Fig. 3, i.e. C2 > C7 > C20 > C17 > C12α > C1 > C5 > C15 and is confirmed by ^{1}H spectroscopy (Fig. 4) The sequence can be correlated with a set of proposed structures[6] (Scheme 4) which implicates both pyrrocorphin and corphin intermediates.[12] During the ensuing search for intermediates containing four or more methyl groups, the extracts of incubation mixtures containing ^{13}C-labeled ALA and $^{13}CH_3$-SAM have provided four *novel metabolites whose constitutions have been determined by* ^{13}C-nmr spectroscopy on 30 - 100 μg samples. The results of these structural investigations[4,5] are shown in Scheme 5 and rely heavily on the application of ^{13}C-INADEQUATE spectroscopy. Factor S1 (**14**), in common with three further isomers S2-S4 (**12,13,15**), is a tetramethylated version of uro'gen I. Thus, while not on the direct pathway to B12, which requires the unsymmetrical type III template as precursor, these new compounds bear evidence of operation of the first three or four methylases of the B12 pathway on the substrate uro'gen I. The possibility now exists that type I corrinoids may be found in Nature or synthesized using these non-specific enzyme catalyzed methylations.

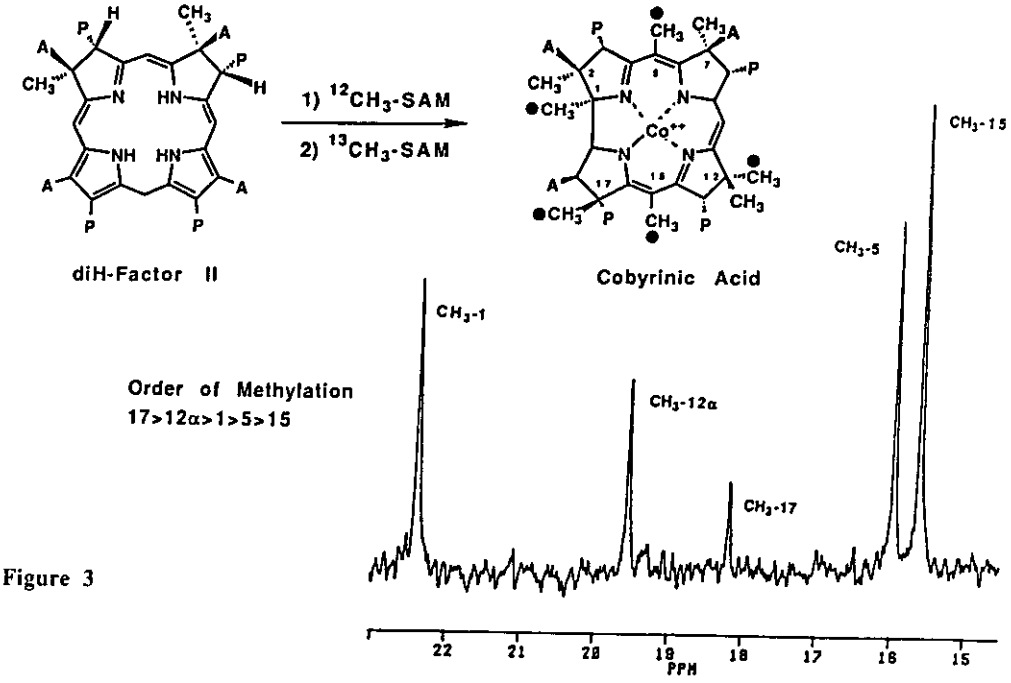


Figure 3

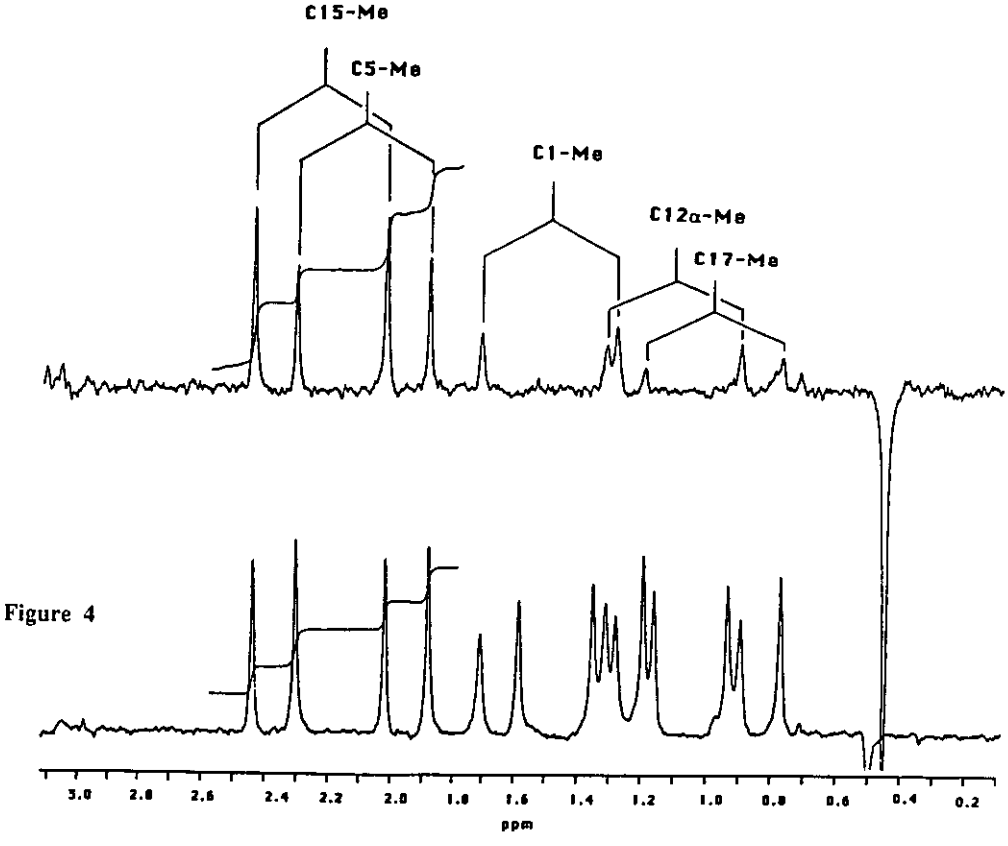
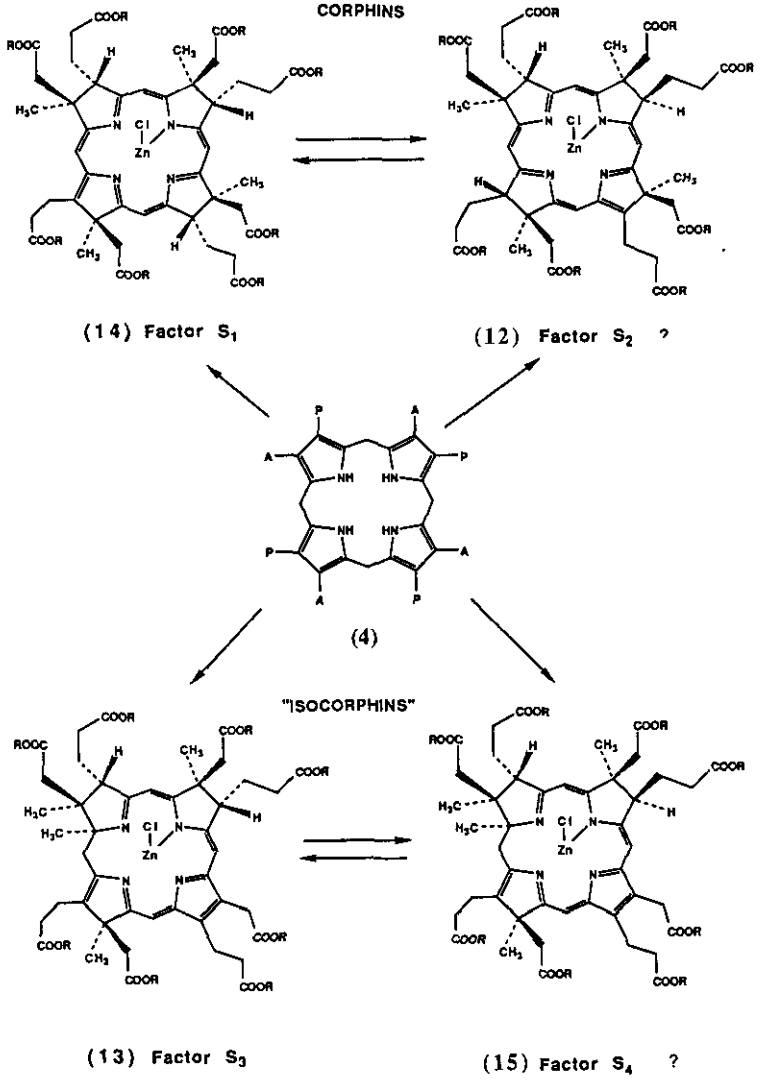


Figure 4

Indirect evidence for the intervention of the 19-acetyl corrin → corrin sequence comes from examination of the ^{13}C-nmr spectrum of B_{12} enriched in both ^{13}C (from ^{13}C-4-ALA) and ^{2}H (from D_2O). The primary deuterium isotope effect of 3.3 for the introduction of ^{2}H at C-19 strongly suggests the kinetic quenching of the carbanion generated uniquely at this position by loss of acetic acid during conversion of the penultimate intermediate to cobyrinic acid.[13] The suggested pathway shown in Scheme 4 relies heavily on the *in vitro* analogies to be found in Eschenmoser's elegant synthetic studies.[14] A recent suggestion by this author uses the device shown in Scheme 4 to hold the rearranging precorrin template together via lactonisation from the ring A acetic acid side chain. A biochemical counterpart for this process would involve the transfer of one of the original oxygens of the participating carboxyl to the acetic acid molecule. Indirect evidence for this process using ^{18}O labeling has very recently been presented.[15] Using the genetic maps for B_{12} synthesis now becoming available it should be possible to determine the structures of the remaining unknown intermediates through the construction of mutants blocked at the appropriate points and we can look forward to rapid progress in this area, as well as in the enzymology of the separated methylase activities where as many as 7 different enzymes may be involved (C_2/C_7, C_{20}, C_{17}, C_{12}, C_1, C_5, C_{15}).



Scheme 5

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