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Chemistry

Biosynthesis of vitamin B_{12}: Structure of precorrin-6x octamethyl ester

(hydrogenobyrinic acid/cobyrinic acid)

DENIS THIBAUT*, FRANCIS BLANCHE*, LAURENT DEBUSSCHE*, FINIAN J. LEEPER†, AND ALAN R. BATTERSBY†

*Département de Chimie Analytique, Centre de Recherche de Vitry, Rhône-Poulenc Santé, B.P. 14, 94403 Vitry-sur-Seine Cedex, France; and †University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, United Kingdom

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ABSTRACT **^{13}C-labeled precorrin-6x is biosynthesized by cell-free protein preparations from *Pseudomonas denitrificans* in separate experiments using δ-amino[5-^{13}C]levulinic acid and the corresponding δ-amino[4-^{13}C]- and δ-amino[3-^{13}C]levulinic acid-labeled forms in conjunction with *S*-[*methyl*-^{13}C]adenosylmethionine for the latter two experiments. These labeled precorrin-6x samples, as their octamethyl esters, are studied by a range of NMR techniques. In addition, nuclear Overhauser effect difference measurements are made on unlabeled precorrin-6x ester to determine connectivities. The structure 6a so established for precorrin-6x ester (*i*) confirms the results reported in the preceding paper that precorrin-6x has a ring-contracted macrocycle, still carries the C-12 acetate residue, and stands at the oxidation level of a dehydrocorrin; (*ii*) reveals the unexpected methylation at C-11 not C-12, leading to a structure with separated chromophores; and (*iii*) implies that methyl migration from C-11 to C-12 occurs when precorrin-6x is converted into hydrogenobyrinic acid. Proposals for the biosynthesis of the corrin macrocycle of hydrogenobyrinic acid and vitamin B_{12} are made.**

The isolation of precorrin-6x reported in the preceding paper (1) has changed the entire direction of research on vitamin B_{12}, in that views that had almost become dogma (e.g., no involvement of external redox reagents and early decarboxylation of the C-12 acetate before ring contraction) are now swept away. The discovery of this precursor opened the way to further progress in delineating the biosynthetic pathway to hydrogenobyrinic acid (structure **1a**), cobyrinic acid (structure **1b**), and thus to vitamin B_{12} itself. We now report our studies, which have established the unexpected structure **6a** for precorrin-6x octamethyl ester (Scheme I).

Accurate mass measurement (electron impact) on precorrin-6x octamethyl ester proved its composition to be $C_{52}H_{70}N_4O_{16}$ (found: 1006.4786; requires 1006.4724, error 6.2 ppm). This molecular formula corresponds to a ring-contracted macrocycle with seven double bonds, which if conjugated would result in a purple or blue pigment. Yet precorrin-6x ester is only pale yellow and shows λ_{max} (CH_2Cl_2), 361 (log ε 4.30), 371 (sh 4.27), and 430 (sh 3.74) with λ_{min} at 298 nm (3.52); these data prove that the molecule has separated chromophores.

^{13}C-labeled precorrin-6x was produced biosynthetically from ^{13}C-labeled precorrin-3 (structure **3b**), which in turn had been generated via its aromatized form (structure **2b**) as in the preceding paper (1) from δ-amino[5-^{13}C]levulinic acid (ALA) containing 99 atom % ^{13}C ([5-^{13}C]ALA; structure **4b**). The resultant precorrin-6x octamethyl ester (structure **6b**) was examined in $C_6{}^2H_6$ by proton-noise decoupled ^{13}C NMR. Seven signals appeared from ^{13}C-enriched centers (Table 1), thus confirming the loss of C-20 prior to formation of precorrin-6x (1); all the signals were doublets save one, for C-15, which was a triplet, the characteristic pattern (2) for a type III tetrapyrrole macrocycle. This is as expected (1) since the structure of precorrin-6x must accord with its enzymic formation from precorrin-3 (structure **3a**) and its conversion into hydrogenobyrinic acid (structure **1a**). Importantly, one doublet appeared at high field (δ_C 36.2) corresponding to an sp^3 center, shown by the sum of evidence below to arise from C-10. The ^{13}C-coupling partners were established by matching the coupling constants and by a two-dimensional ^{13}C–^{13}C correlation (Table 1). An off-resonance decoupling experiment showed that C-5 and C-15 are methines, while C-10 is a methylene group. Finally, a ^{1}H-detected ^{13}C–^{1}H correlation (3, 4) proved that C-15, identified by giving the only triplet signal, carries the hydrogen at δ_H 5.12 and C-5 carries that at δ_H 4.97, while C-10 carries hydrogens giving signals centered at δ_H 2.06 and δ_H 3.04. Significantly, there was also a weak correlation from C-10 to the methyl group at δ_H 1.36; this relationship is confirmed below.

The next study involved biosynthetic production of **2c** from [4-^{13}C]ALA (structure **4c**) and unlabeled *S*-adenosylmethionine (SAM; structure **5a**); the product was purified and then converted enzymically via **3c** into precorrin-6x as in the preceding paper (1) but, importantly, now using [*methyl*-^{13}C]SAM (structure **5b**). This means that the C-methyl groups at C-2 and C-7 of the precorrin-6x ester (structure **6c**) will be unlabeled, whereas the three added methyl groups will be labeled. This ester was studied by the same NMR techniques used above. Full signal assignments and couplings are given in Table 2 and the following structurally decisive observations can be summarized.

(*i*) Eleven signals were observed, 8 arising from the [4-^{13}C]ALA and 3 from the [*methyl*-^{13}C]SAM. Direct bonding was clear between two carbons of the macrocycle (C-1 and C-19; J = 52 Hz), thus confirming that precorrin-6x has a contracted macrocycle; the chemical shift for C-19 (δ_C 154.1) shows its sp^2 nature.

(*ii*) The signals from the three C-methyl groups were doublets (J = 33–38 Hz), proving that all are directly attached to a ^{13}C atom and a ^{13}C–^{13}C correlation spectrum established the coupling partners. Attachment of the methyls at C-1 and C-17 to carbons derived from C-4 of ALA is as expected (1). However, this important result for the third methyl proves that, although it eventually appears at C-12 of hydrogenobyrinic acid (1) (shown unlabeled; structure **1a**), it must be at C-11 or C-13 in precorrin-6x (structure **6c**).

(*iii*) The ^{13}C–^{1}H correlation demonstrated that only the three labeled C-methyl groups and the C-3 position carry hydrogens; thus, the ^{13}C centers at C-8, C-13, and C-19, which appear as

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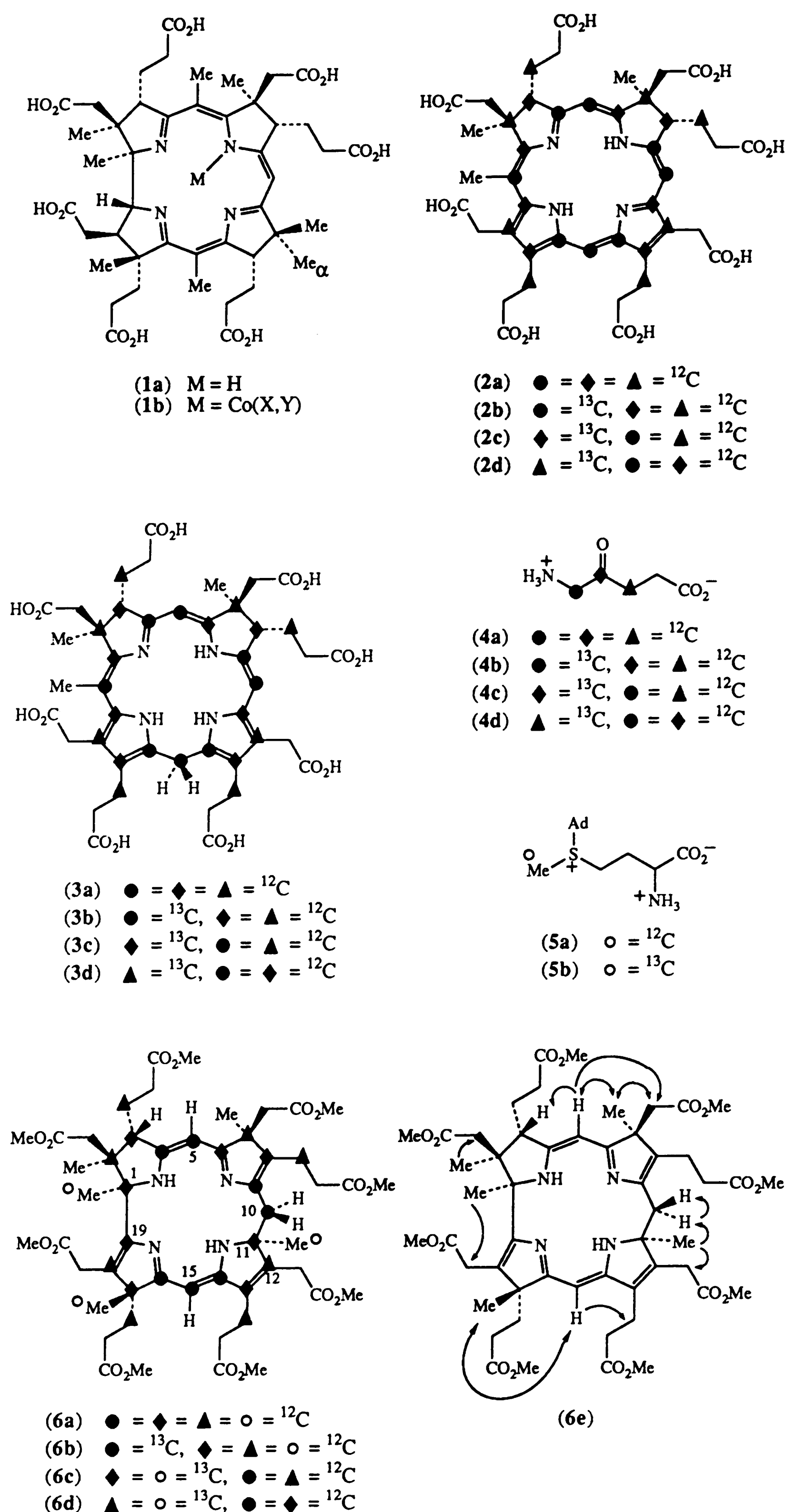
Abbreviations: ALA, δ-aminolevulinic acid; SAM, *S*-adenosylmethionine.



Scheme I

methines in hydrogenobyrinic acid (structure **1a**), carry no hydrogen in precorrin-6x ester (structure **6c**).

The enzymic and NMR studies described above starting from [4-^{13}C]ALA were then repeated but now using [3-^{13}C]ALA (structure **4d**) for the production of structure **2d**; this was enzymically converted first into precorrin-3 (structure **3d**) and then again with [*methyl*-^{13}C]SAM, into precorrin-6x (structure **6d**). Table 3 collects the full data obtained and the following key points are highlighted.

(*i*) The three added C-methyl groups all gave singlets in the ^{13}C spectrum. This result is as expected for those at C-1 and C-17, but it confirms that the methyl that appears at C-12 in hydrogenobyrinic acid (structure **1a**) is not attached to C-12 in precorrin-6x (structure **6d**).

Table 1. NMR data for precorrin-6x octamethyl ester (structure **6b**) derived from [5-^{13}C]ALA

			Chemical shift of coupled protons (δ_H, ppm)	
δ_C	Coupling, Hz	Assignment*	One bond	Two or three bond†
161.5	d, 70.6	C-4[a]	—	3.25 (H-3), 4.97 (H-5)
82.7	dd, 71.1, 6.0	C-5[a]	4.97 (H-5)	—
146.7	dd, 50.4, 6.0	C-9[b]	—	3.04 (H-10)
36.2	d, 50.4	C-10[b]	3.04, 2.06 (H-10)	1.36 (11-Me)
159.9	d, 70.3	C-14[c]	—	—
78.7	t, 69.9	C-15[c,d]	5.12 (H-15)	—
179.8	d, 69.1	C-16[d]	—	1.04 (17-Me), 5.12 (H-15)

*Pairs of carbons marked with the same letter (a–d) were shown to be coupled by two-dimensional ^{13}C–^{13}C correlation.

†In many cases, couplings to hydrogen atoms of the propionate side chains were also seen. These were consistent with structure **6a** but did not assist the assignments (as the ^{1}H signals are not well resolved) and are therefore omitted.

(*ii*) The remaining eight signals were also all singlets (apart from one long-range coupling of ≈3 Hz), of which four appeared at high field and were shown to arise from methylenes (propionate side chains), two at midfield from quaternary centers (C-2 and C-7), and, importantly, two at low field proving that C-12 and C-18 are both sp^2 carbons.

A further ^{1}H–^{13}C correlation experiment (with the delay set to the optimum for couplings of ≈10 Hz) (4) for each of the three ^{13}C-labeled samples of precorrin-6x ester (structures **6b–6d**) described above detected the long-range ^{13}C–^{1}H couplings (through up to three bonds) and the results are given in Tables 1–3. Finally, ^{1}H NMR nuclear Overhauser effect difference spectroscopy (3) established the connectivities illustrated on structure **6e**. These results added the last links that provided for each assignment in Tables 1–3 and on structures **6b–6d**, a set of self-consistent interlocking correlations from all the different spectroscopic approaches. This will now be demonstrated for the structural argument covering half of the macrocycle from C-3 to C-12 of structure **6a**, which serves as an example for those relating to the rest of the molecule of precorrin-6x ester.

[5-^{13}C]ALA Experiment. The triplet at δ_C 78.7 in the ^{13}C NMR spectrum of precorrin-6x ester from [5-^{13}C]ALA is unambiguously from C-15, so δ_C 82.7 must correspond to either C-5 or C-10. Both signals arise from methine groups and δ_H 4.97 is proved to arise from the hydrogen on the carbon at δ_C 82.7. The nuclear Overhauser effect connections (structure **6e**) establish δ_H 4.97 as H-5 and thus δ_C 82.7 as C-5. C-5 is directly bonded to the carbon showing δ_C 161.5 (therefore C-4), which is long-range coupled to δ_H 3.25 (hence H-3). C-5 also shows a long range ^{13}C–^{13}C coupling to δ_C 146.7, which must therefore be C-9 and this is directly bonded to the carbon showing δ_C 36.2, thereby identified as arising from C-10. C-10 is proved to carry two hydrogens demonstrated to give signals at δ_H 2.06 and 3.04. A long-range ^{13}C–^{1}H coupling is observed between C-9 and δ_H 3.04 on C-10.

Importantly, C-10 shows a long-range ^{13}C–^{1}H coupling with the hydrogens of a methyl group at δ_H 1.36. It follows that this methyl is at C-11 and confirmation came from a nuclear Overhauser effect connection from this methyl group to the δ_H 3.04 hydrogen on C-10.

[4-^{13}C]ALA Experiment. All the labeled centers are shown to be quaternary or sp^2 carbons save the [^{13}C]SAM-derived methyl groups and one at δ_C 54.8, which must correspond to C-3 since it is found to carry the H-3 hydrogen at δ_H 3.25. In agreement, C-3 has a long-range coupling to H-5 and to an unlabeled C-methyl group (at C-2). The other unlabeled methyl group (at C-7) and also H-5 have long-range couplings to δ_C 179.9 thus identified at C-6. δ_C 129.0 corresponds to C-8 because it is coupled to the C-7 methyl and also to the δ_H 3.04 at C-10. This latter connection eliminates the possibility that

Table 2. NMR data for precorrin-6x octamethyl ester (structure **6c**) derived from [4-^{13}C]ALA and [*methyl*-^{13}C]SAM

			Chemical shift of coupled protons (δ_H, ppm)	
δ_C	Coupling, Hz	Assignment*	One bond	Two or three bond†
29.5	d, 37.1	1-Me[a]	1.50 (1-Me)	—
73.2	dd, 52.3 36.8	C-1[a,d]	—	1.48 (2-Me), 1.50 (1-Me) 2.97 and 2.50 (2^1-CH$_2$)
54.8	s	C-3	3.25 (H-3)	4.97 (H-5), 1.48 (2-Me) 2.97 and 2.50 (2^1-CH$_2$)
179.9	s	C-6	—	4.97 (H-5), 1.17 (7-Me), 2.32 (7^1-CH$_2$)
129.0	s	C-8	—	1.17 (7-Me), 2.32 (7^1-CH$_2$) 3.04 (H-10)
21.5	d, 38.1	11-Me[b]	1.36 (11-Me)	2.06 (H-10)
71.0	d, 37.7	C-11[b]	—	1.36 (11-Me), 3.32 (12^1-CH$_2$) 3.04 and 2.06 (H-10)
135.8	s	C-13	—	5.12 (H-15), 3.32 (12^1-CH$_2$)
23.5	d, 33.0	17-Me[c]	1.04 (17-Me)	—
61.1	dd, 33.1, ≈4	C-17[c]	—	1.04 (17-Me), 5.12 (H-15) 3.12 and 3.20 (18^1-CH$_2$)
154.1	dd, 51.8, ≈5	C-19[d]	—	1.50 (1-Me), 3.12 and 3.20 (18^1-CH$_2$)

*Pairs of carbons marked with the same latter (a–d) were shown to be coupled by two-dimensional ^{13}C–^{13}C correlation.

†See † footnote in Table 1.

Table 3. NMR data for precorrin-6x octamethyl ester (**6d**) derived from [3-^{13}C]ALA and [*methyl*-^{13}C]SAM

			Chemical shift of coupled protons (δ_H, ppm)	
δ_C	Coupling, Hz	Assignment	One bond*	Two or three bond*
29.5	s	1-Me	1.50 (1-Me)	—
49.2	s	C-2	—	1.48 (2-Me), 1.50 (1-Me) 2.97 and 2.50 (2^1-CH_2)
27.0	s	3^1-CH_2†	—	—
56.6	d, ≈3	C-7	—	1.17 (7-Me), 2.32 (7^1-CH_2)
20.0	d, ≈3	8^1-CH_2	—	—
21.5	s	11-Me	1.36 (11-Me)	2.06 (H-10)
149.7	s	C-12	—	1.36 (11-Me), 3.32 (12^1-CH_2)
20.3	s	13^1-CH_2†	—	—
23.5	s	17-Me	1.04 (17-Me)	—
32.4	s	17^1-CH_2	—	1.04 (17-Me)
116.4	s	C-18	—	3.12 and 3.20 (18^1-CH_2)

*See † footnote in Table 1.

†The assignment of these two signals is based solely on their chemical shifts and may have to be interchanged.

our assignments of the C-2 and C-7 methyl signals are the wrong way around.

A key signal is δ_C 71.0 from a quaternary center, which, because of its coupling to the two hydrogens at C-10, must be C-11. This center carries a labeled methyl group, δ_C 21.5, which correlates with the previously identified methyl signal at δ_H 1.36 and also shows long-range coupling to both hydrogen atoms on C-10. Hence, C-methylation at C-11 of precorrin-6x is further confirmed. Finally, δ_C 135.8 is coupled to H-15 and since it carries no methyl group (and therefore cannot be C-17) must be C-13; it is an sp^2 center.

[3-^{13}C]ALA Experiment. The critical observation here is that no labeled carbon from the ALA carries a labeled methyl group proving, in agreement with the above results, that C-12 is not methylated. Aside from the four labeled methylenes of the propionate side chains, two labeled carbons are quaternary (and must be C-2 and C-7 already methylated in precorrin-3; structure **3d**) and thus C-12 and C-18 are sp^2 carbons that are individually assigned by long-range couplings.

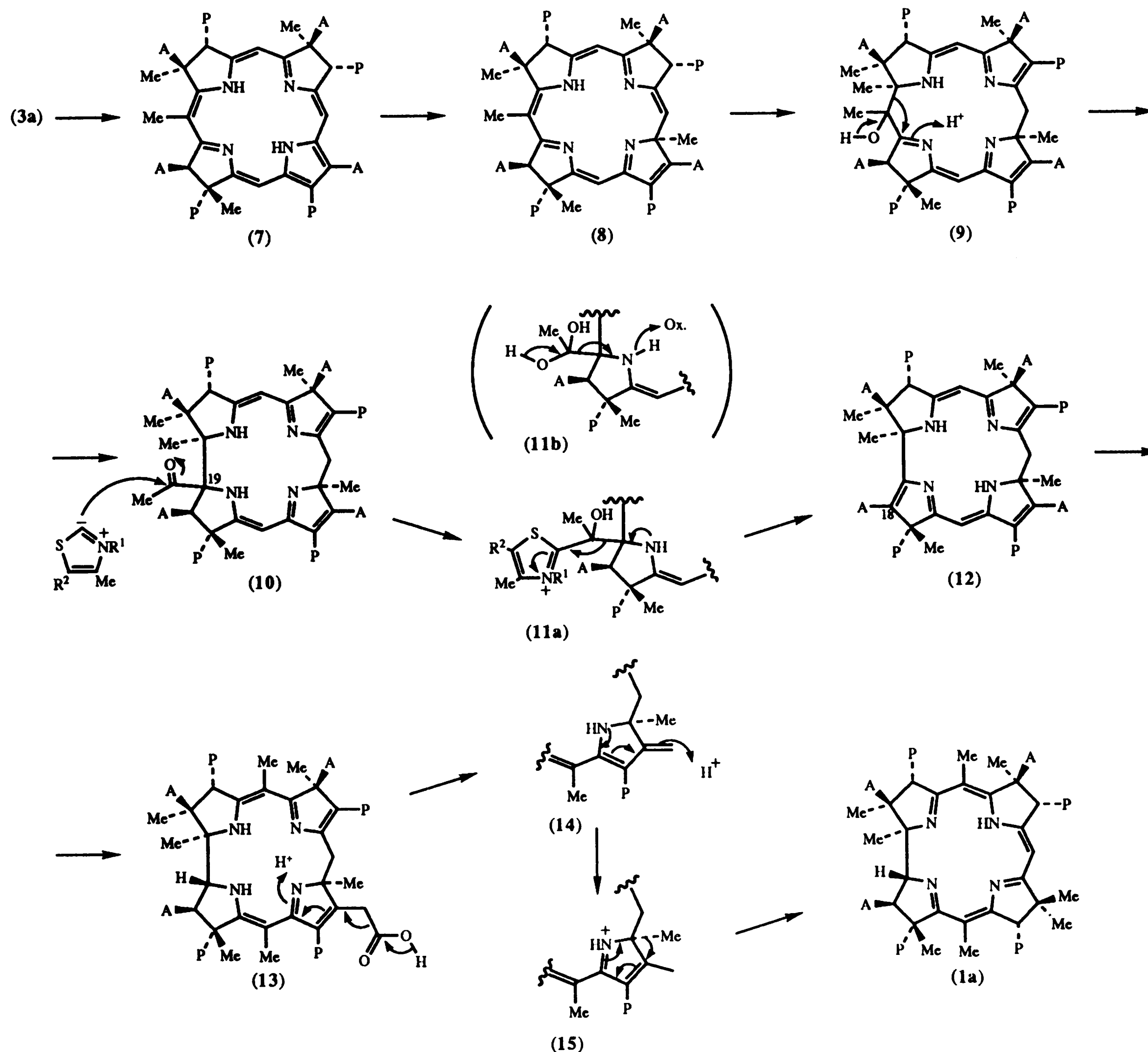
The above arguments establish a large part of the structure of precorrin-6x ester and the connections collected in Tables 1–3 establish in a similar logical stepwise way the remaining features of structure **6a** for precorrin-6x ester. Indeed, the key observations and conclusions, although without every detail, have been summarized in the earlier part of this paper. The configuration at C-11 is based on the conversion of precorrin-6x into hydrogenobyrinic acid (Scheme **II**) and is discussed further below.

The structure **6a** revealed above for precorrin-6x ester is based on the results from the preceding paper (1) combined with those reported here; the two approaches used interlock perfectly. What has been found dramatically changes our view of the biosynthesis of the corrin macrocycle. Precorrin-6x stands close to the middle of the previously unknown section of the pathway to corrins and so is ideally placed to allow a radically different pathway for corrin biosynthesis to be proposed (Scheme **II**). This scheme depends on all the knowledge gained in the preceding paper (1) together with the results reported here.

Methylation of precorrin-3 (structure **3a**) at C-17 as described (5) would afford the tetramethylated pyrrocorphin‡ (structure **7**). The C-methyl group, which eventually appears at C-12 of corrins, is the next to be added (6–8) but this is now known to be inserted at C-11, which leads to structure **8** for the pentamethylated intermediate. Methylation at C-1, the next in sequence, could take place directly at C-1 or first at C-20 followed by a 1,2 shift of the α-methyl group. Either route could generate the hexamethylated macrocycle§ (structure **9**) with a hydroxyl group at C-20 or as a lactone with the C-18 or C-2 acetate side chains. Ring contraction by the type of mechanism considered earlier (9) leads to the precursor **10** of precorrin-6x carrying an acetyl residue at C-19. These two carbon atoms were shown to be eliminated as acetic acid (10, 11) and knowing (1) that a double bond is introduced during the overall process of ring contraction and acetate extrusion, two proposals can be made for what was previously a difficult step to rationalize in an entirely satisfactory way. One, illustrated as part structure **11a** in the main Scheme **II**, involves thiamine in what is the nitrogen equivalent of the transketolase reaction, with oxidative recovery of thiamine cofactor as in oxidative pyruvate decarboxylation, to yield the two-carbon fragment as acetic acid. The other requires oxidative cleavage (see structure **11b**) of the hydrated form of structure **10**. Either sequence, followed by tautomerization, affords precorrin-6x§ (structure **12**) ready for NADPH-mediated reduction (1) of the C-18 protonated form of **12** and methylation at C-5 and C-15 to afford structure **13**. Decarboxylation of the C-12 acetate, by a process that is the C-methylated equivalent of the well-known proton-catalyzed decarboxylation of pyrrole-3-acetic acids, sets the stage (structure **14**) for tautomerization and [1,5] sigmatropic suprafacial rearrangement (structure **15**) followed by tautomerization to the conjugated state to form hydrogenobyrinic acid (structure **1a**). This cobalt-free corrinoid is known (12) to be built by the same sequence of reactions as established (13) for the cobalt-containing cobyrinic acid (structure **1b**). So, although our proposals are illustrated for the cobalt-free series, they refer to the biosynthesis of both corrins **1a** and **1b**. Cobalt insertion is obviously needed for the latter at some presently unknown point on the pathway. The α configuration for the C-11 methyl group in precorrin-6x (structure **12**) allows delivery of this methyl to the α face at C-12, since it is the 12α-methyl group that is derived from SAM (14–16). A key proposal of this scheme is that late decarboxylation of the

‡Precorrin names will not be attached to structures until the intermediates have been isolated and/or structurally characterized.

§One tautomer is illustrated that has C-10 in the sp^3 state as in precorrin-6x ester, but this is an uncertain detail at present. It is clear though, from UV visible spectra and the NMR spectrum of precorrin-6x octa acid derived from [5-^{13}C]ALA, that the acid (as structure **6b**, CO_2H in place of CO_2Me) can exist in at least two tautomeric forms, a major one having C-10 as an sp^3 center. There are several obvious prototropic tautomers for precorrin-6x, but as emphasized in the accompanying paper (1), they are interconvertible in the biological system.



A = CH_2CO_2H, P = $CH_2CH_2CO_2H$

Scheme II

C-12 acetate residue initiates the methyl migration from C-11 to C-12. This aspect and several other key features of the biosynthetic scheme can be studied experimentally.

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