Development of an 'Overmethylation' Strategy for Corrin Synthesis. Multi-enzyme Preparation of Pyrrocorphins

A. Ian Scott,* Martin J. Warren, Charles A. Roessner, Neal J. Stolowich, and Patricio J. Santander

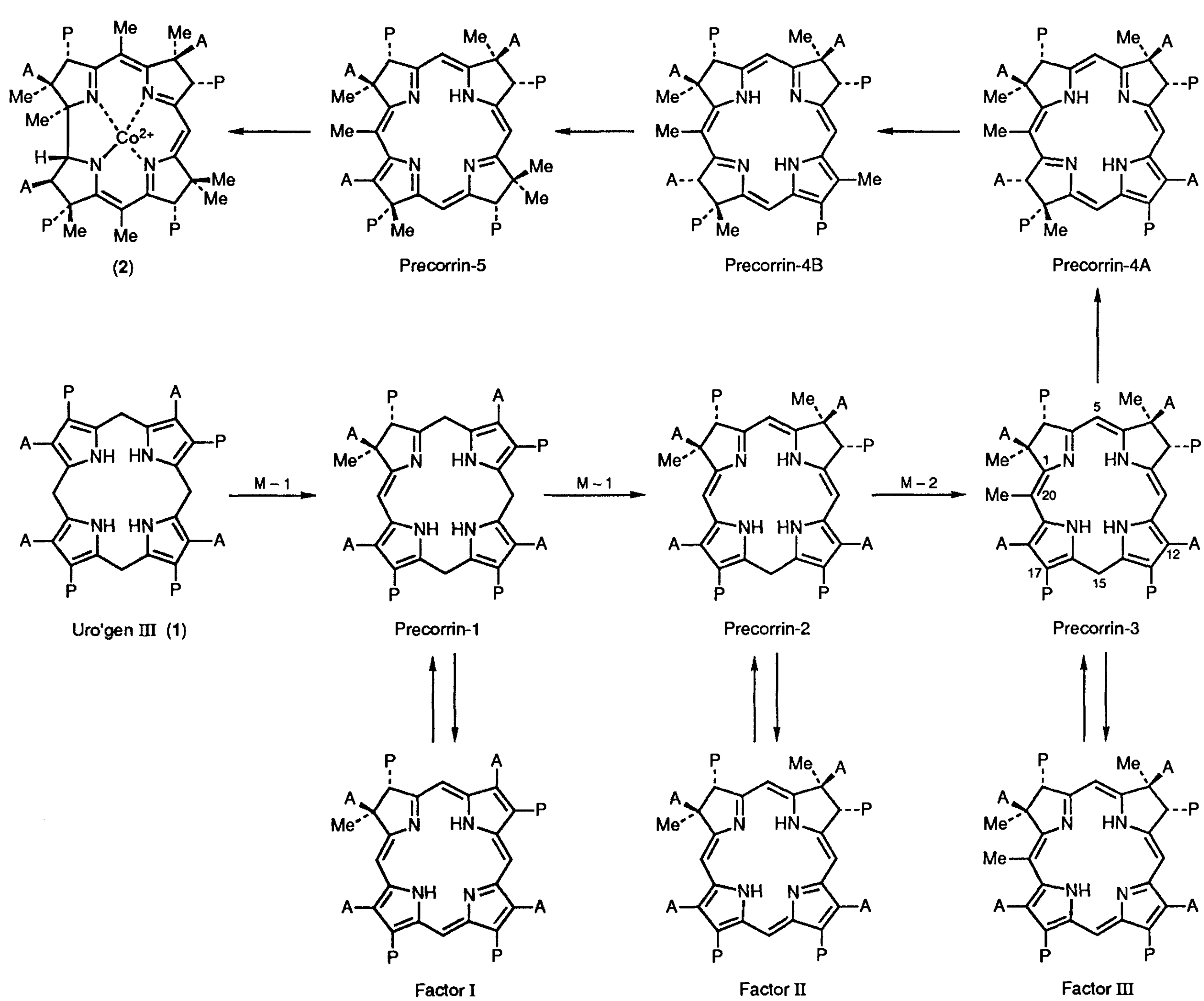
Center for Biological NMR, Department of Chemistry, Texas A & M University, College Station, Texas 77843-3255, U.S.A.

A novel trimethyl pyrrocorphin has been synthesized from 5-aminolevulinic acid (ALA) by the combination of the enzymes ALA dehydratase, porphobilinogen deaminase, uro'gen III synthase, and uro'gen III methylase, leading to a new concept for the synthesis of intermediates of vitamin B_{12} biosynthesis.

The order of the eight discrete *C*-methylation steps which are involved in the biochemical conversion of uro'gen III (**1**) to cobyrinic acid (**2**) has been defined recently,[1,2] and the structures of three intermediates, precorrins-1, -2, and -3 corresponding to the reduced isolates Factors I, II, and III have been deduced,[3,4] as shown in Scheme 1. Thus uro'gen III methylase (M-1)[5]† inserts methyl groups into uro'gen III first at C-2 (→ precorrin-1) and then at C-7 (→ precorrin-2). The latter compound, dihydrosirohydrochlorin, then becomes the substrate for the second methylase (M-2) whose function is to introduce the third methyl group at C-20 to give precorrin-3 (Scheme 1).[2b,6] It is now believed[1c,7,8] that the oxidation level of a hexahydroporphyrin (equivalent to the original substrate uro'gen III) is maintained, not only in these precursors, but throughout the subsequent *C*-methylations which take place on the precorrin-3 template in the sequence C-17 > 12α > 1 > 5 > 15.[1,2]

Implicit in the mechanism of peripheral *C*-methylation of uro'gen III is the biomimetic chemistry pioneered by Eschenmoser[8,9] which involves tautomerism of porphyrinogen to dipyrrocorphinoid, pyrrocorphinoid, and corphinoid forms and the generation of these chromophores as a necessary consequence of the sequential insertion of methyl groups at positions 2, 7, 20, 17, and 12. Thus precorrins-2 and -3 are found to exist at equilibrium as dipyrrocorphins,[1c,3,4] while the hypothetical precorrin-4A (methylation at C-17) is a pyrrocorphin[9] (Scheme 1) and a corphin[10] structure can be predicted for precorrin-5, which has undergone both decarboxylation of the acetate moiety (→ precorrin-4B) and methylation in ring *C* (C-12α). Corphins, first synthesized over 20 years ago,[10] have been found only recently as natural products in *Propionibacterium shermanii*[11] and to date there are no examples of pyrrocorphins, other than those of synthetic provenance,[9] in nature. In this communication we describe a synthesis of

† M-1 was first described by G. Müller[5a] and more recently was obtained in pure form by genetic engineering from *Pseudomonas denitrificans*[5b] and from *E. coli*.[5c]



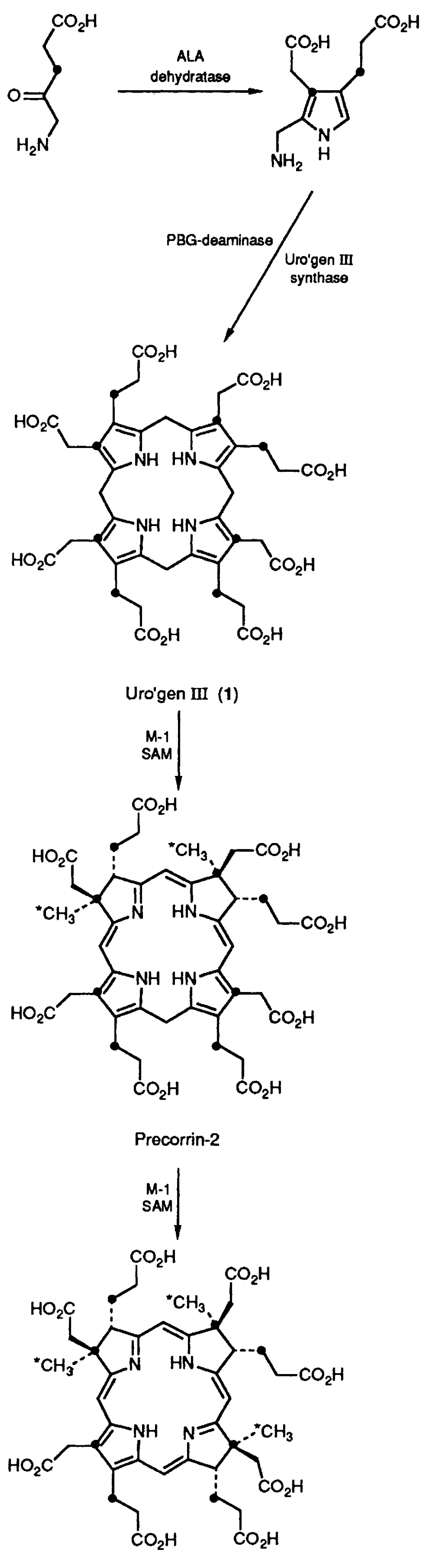
A = CH_2CO_2H, P = $(CH_2)_2CO_2H$

Scheme 1

pyrrocorphins employing a multi-enzyme strategy which holds promise for regio- and stereo-specific synthesis of the 'missing' intermediates of the B_{12} pathway (at the desired hexahydroporphyrinoid level) and for the exploration of the stereoelectronic factors which govern the methyl transferase mechanisms of corrin biosynthesis.

Uro'gen III, the substrate for M-1, was synthesized in the ^{13}C-labelled form shown in Scheme 2 by incubation of [3-^{13}C]-5-aminolevulinic acid ([3-^{13}C]-ALA) with a mixture of the purified enzymes ALA dehydratase, porphobilinogen (PBG) deaminase, and uro'gen III synthase. When purified M-1[5c] (from an overproducing strain of *E. coli*) and [$^{13}CH_3$]-SAM [SAM = (*S*)-adenosyl-L-methionine] were included in the incubation‡ the ^{13}C NMR spectrum of precorrin-2 could be observed after 1 h and provided direct evidence [2 sp^3 (●) doublets; 2 sp^2 (●) singlets] that the dipyrrocorphin tautomer[3] (precorrin-2, Scheme 2) is indeed synthesized by M-1. It was found that by increasing the enzyme concentration from 0.1 to 0.2 mg ml^{-1}, the initial dipyrrocorphin chromophore was quantitatively replaced by a pure pyrrocorphin chromophore, as (**3**), a result which could have been due to prototropic tautomerism. However, the resultant ^{13}C NMR spectrum now showed three doublets in the sp^3 region (δ 47—52) corresponding to $^{13}\overset{*}{C}H_3$–$^{13}\overset{\bullet}{C}$ coupling at the quaternary carbons of three acetate termini confirmed by the presence of three overlapping doublets for three enriched methyl (*) signals at δ 19—22. Under these conditions of high concentration M-1 therefore has carried out a surprising additional *C*-methylation of its normal product, precorrin-2, in either ring *C* or ring *D*. Proof for ring *C* alkylation was achieved by employing a pulse labelling technique used earlier[12,13] to establish the order of assembly (*A*>*B*>*C*>*D*) of the tetrapyrrolic ring system of uro'gen III. Thus, preparation of a mixture of the stable, catalytically competent ^{13}C-enriched ES_2 and ES_3 covalent complexes[14] of [4,6-$^{13}C_2$]PBG with the assembling enzyme, PBG deaminase, followed by addition of unlabelled

‡ *Incubation conditions*: in 5 ml phosphate buffer (pH 8.0, 0.1 M, < 2 p.p.m. O_2) were dissolved ALA dehydratase (50 μg), PBG deaminase (50 μg), uro'gen III synthase (50 μg), M-1 (1 mg), ALA (250 μg), and SAM (1 mg). The solution was kept under argon (glovebox < 2 ppm O_2) for 3—4 h. Overall conversion to (**3**) (from ALA) was 85—90%. NMR spectra were determined directly on this solution. The reaction can be scaled up for multi-milligram synthesis of (**3**) without a loss in yield.



Scheme 2

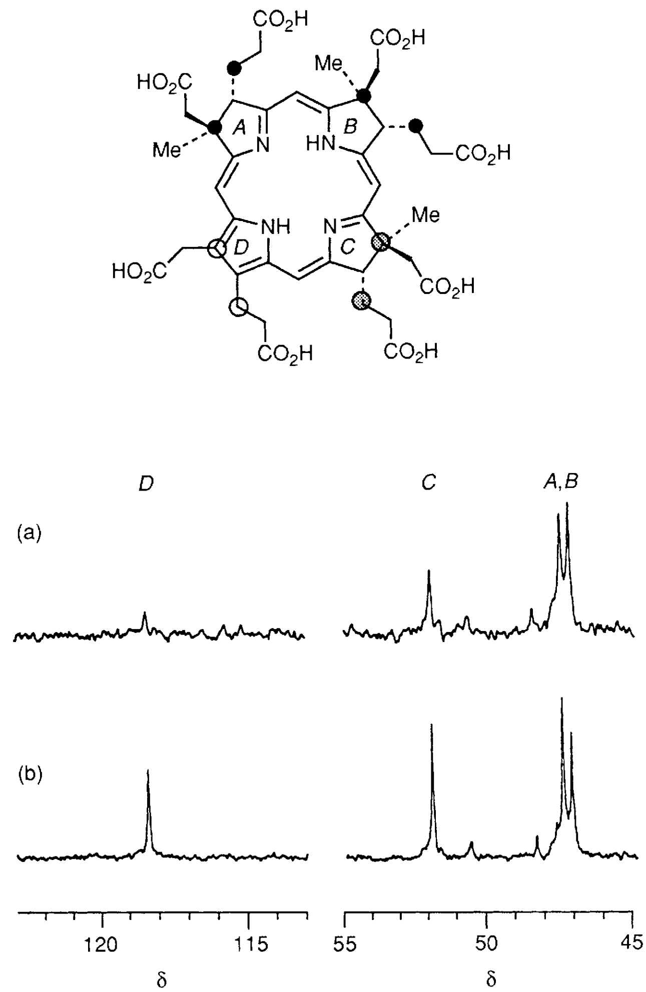
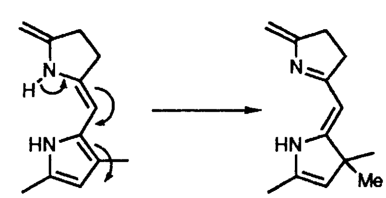
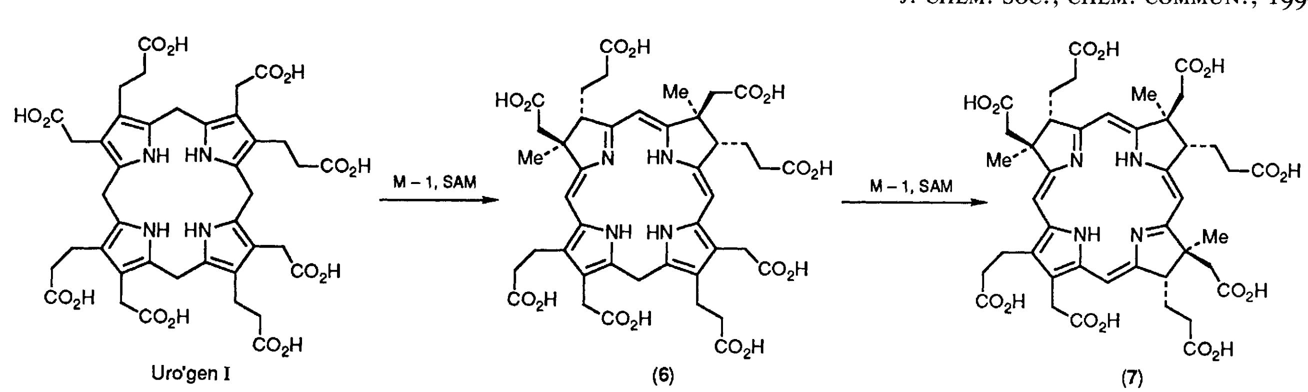


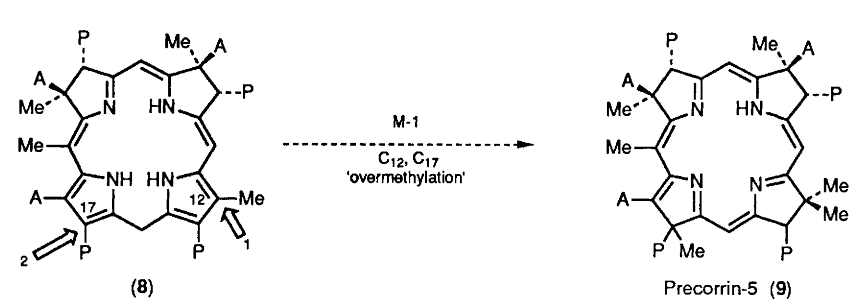
Figure 1. Selected regions of the 75 MHz ^{13}C NMR spectra of trimethylpyrrocorphin (**3**) showing the pyrrolic carbons derived from [4,6-$^{13}C_2$]-PBG (propionate carbons not show). (a) Sample obtained *via* pulse experiment utilizing ^{13}C-enriched PBG deaminase ES_2/ES_3 complexes, showing high (●, ring *A*/*B*), medium (◍, ring *C*), and low (○) enrichment in ring *D* (see text for additional details). (b) Uniformly ^{13}C-enriched control. Pyrrocorphin samples were ~250 µg ml^{-1} in NaCl (2 M) containing 20% D_2O maintained under argon to prevent oxidation. Number of scans: (a) 40000; (b) 42000.



(**4**) (**5**)

PBG and uro'gen III synthase, M-1, and SAM (as earlier) afforded a solution of the pyrrocorphin (**3**) whose NMR spectrum [Figure 1(a)] revealed, on comparison with the fully enriched spectrum derived from a non-pulsed incubation [Figure 1(b)], high enrichment in rings *A* and *B* (δ 47.2, 47.5), medium enrichment in ring *C* (δ 52), and a very small ^{13}C signal above natural abundance at δ 117.3 corresponding to the enriched sp^2 carbon of ring *D*, in full accord with the





derivation of (3) from a 'pulse-derived' specimen of uro'gen III whose ring system is ^{13}C-enriched in the order $A = B > C \gg D$. This technique allowed assignment of the third site of methylation since an enriched sp^3 carbon [δ 52; Figure 1(b)] must be present in ring *C* and can be assigned to C-12 by the regiospecificity of the ^{13}C label. On the assumption (still to be rigorously proved) that this new *C*-alkylation takes place from the lower face of the substrate (as in the case of the first two methyl insertions), together with the knowledge that all three methyl signals are shifted 8—10 ppm upfield due to γ interaction with the *cis*-propionate side-chains, the structural proposal (3) can be made for the trimethyl pyrrocorphin,§ in which only the absolute configuration at C-12 remains to be established.

The high-yielding 'over-methylation' by uro'gen III methylase can be ascribed to the increased (non-physiological) enzyme concentration used in the preparation combined with a lack of substrate specificity, *i.e.* rather than the sequence of acetate and propionate side-chains, the enzyme may recognize the chromophoric transposition which is thought to lie at the heart of Eschenmoser's biomimetic experiments.[8,9] This involves conversion of a vinylogously hydrazinic (destabilizing) system (4) to a stabilizing vinamidinic conjugation (5) in which an NH electron pair is linearly conjugated with an electrophilic ketimine.

In support of this suggestion, it was found that uro'gen I, the symmetrical isomer of uro'gen III, is not only an excellent substrate for M-1, yielding the precorrin-2 analogue (6), but under the same conditions as described above[15] (excess M-1), a third methyl group is inserted into this substrate to give quantitatively the pure trimethyl pyrrocorphin (7) of the type-I family, whose structure was determined exactly as for the type III pyrrocorphin (3) described above. It is emphasized that precorrin-2 and the pyrrocorphins (3) and (7) are extremely air-sensitive and can be isolated only under strict anaerobic conditions (< 5 ppm O_2). Although these new pyrrocorphins are not directly on the B_{12} pathway, their six-step, one-flask synthesis with four enzymes in yields of over 80% from ALA and SAM, together with the knowledge that unnatural porphyrinogens such as uro'gen I can serve as efficient substrates for the methylases of corrin biosynthesis,[15] provides a welcome *entrée* to the synthesis of precorrins-4 and -5 (Scheme 1). For example, ring-*C* decarboxylated precorrin-3 (8) which has already been prepared by non-specific *C*-methylation of 12-decarboxylated uro'gen III using M-1 and M-2,[2b,6] although *not* a direct precursor of cobyrinic acid, can now be tested as a substrate for regiospecific 'over-methylation' by M-1 in rings *C* and *D* to afford the corphinate (9) corresponding to the unknown precorrin-5, thereby providing re-entry to the B_{12} pathway.

We thank N.I.H. (Grant DK32596) and the Robert A. Welch Foundation for financial support and Mr. T. Higuchi,

§ Esterification ($MeOH/H_2SO_4$ < 2 ppm O_2) of a specimen of (3) derived from [$^{13}C_8$]-uro'gen III labelled as shown in Scheme 2, gave the octamethyl ester whose FAB mass spectrum (M^+ 998.5) confirmed the formula $^{12}C_{43}{}^{13}C_8H_{66}O_{16}N_4$ (M 998.4745). The UV-VIS spectrum of (3), $\lambda_{max.}$ (rel. ε) 350 (1.00), 352 (1.00), 376 (0.74), 405 (0.22), 492 (0.15), 530sh (0.13), 575 (0.13), 625 (0.06), corresponds closely with that of a typical pyrrocorphin.[8,9]

Mass Spectrometer Application Lab., JEOL U.S.A., for FAB mass spectra.

Received, 21st December 1989; Com. 9/05455C

References

1 (a) H. C. Uzar and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1982, 1204; (b) 1985, 585; (c) H. C. Uzar, A. R. Battersby, T. A. Carpenter, and F. J. Leeper, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1689. In *Closetridium tetanomorphum* methylation at C-15 precedes that at C-5. The reverse sequence (C-5 > C-15) is found in *Propionibacterium shermanii*. See ref. 2.

2 (a) A. I. Scott, N. E. Mackenzie, P. J. Santander, P. E. Fagerness, G. Müller, E. Schneider, R. Sedlmeier, and G. Wörner, *Bioorg. Chem.*, 1984, **12**, 356; (b) A. I. Scott, H. J. Williams, N. J. Stolowich, P. Karuso, M. D. Gonzalez, G. Müller, K. Hlineny, E. Savvidis, E. Schneider, U. Traub-Eberhard, and G. Wirth, *J. Am. Chem. Soc.*, 1989, **111**, 1897.

3 A. R. Battersby, K. Frobel, F. Hammerschmidt, and C. Jones, *J. Chem. Soc., Chem. Commun.*, 1982, 455; R. D. Brunt, F. J. Leeper, I. Grgurina, and A. R. Battersby, *ibid.*, 1989, 428.

4 M. J. Warren, N. J. Stolowich, P. J. Santander, C. A. Roessner, B. A. Sowa, and A. I. Scott, *FEBS Lett.*, 1990, **261**, 76.

5 (a) G. Müller, R. Deeg, K. D. Gneuss, G. Gunzer, and H.-P. Kriemler, in 'Vitamin B_{12},' eds. B. Zagalak and W. Friedrich, de Gruyter, Berlin, 1979, p. 279; (b) F. Blanche, L. Debussche, D. Thibaut, J. Crouzet, and B. Cameron, *J. Bacteriol.*, 1989, **171**, 4222; (c) M. J. Warren, C. A. Roessner, P. J. Santander, and A. I. Scott, *Biochem. J.*, 1990, **265**, 725.

6 F. Blanche, S. Handa, D. Thibaut, C. L. Gibson, F. J. Leeper, and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1988, 1117.

7 A. I. Scott, *Pure Appl. Chem.*, 1989, **61**, 501.

8 A. Eschenmoser, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 5.

9 C. Leumann, K. Hilpert, J. Schreiber, and A. Eschenmoser, *J. Chem. Soc., Chem. Commun.*, 1983, 1404.

10 A. P. Johnson, P. Wehrli, R. Fletcher, and A. Eschenmoser, *Angew. Chem., Int. Ed. Engl.*, 1968, **7**, 623.

11 G. Müller, J. Schmiedl, E. Schneider, R. Sedlmeier, G. Wörner, A. I. Scott, J. H. Williams, P. J. Santander, N. J. Stolowich, P. E. Fagerness, N. E. Mackenzie, and H.-P. Kriemler, *J. Am. Chem. Soc.*, 1986, **108**, 7875; G. Müller, J. Schmiedl, E. Schneider, L. Savvidis, G. Wirth, A. I. Scott, P. J. Santander, H. J. Williams, N. J. Stolowich, and H.-P. Kriemler, *ibid.*, 1987, **109**, 6902.

12 P. M. Jordan and J. S. Seehra, *FEBS Lett.*, 1979, **104**, 364.

13 A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, and E. McDonald, *J. Chem. Soc., Chem. Commun.*, 1979, 539.

14 A. I. Scott, C. A. Roessner, N. J. Stolowich, P. Karuso, H. J. Williams, S. K. Grant, M. D. Gonzalez, and T. Hoshino, *Biochemistry*, 1988, **27**, 7984. ES_2 and ES_3 are productive covalent complexes of the enzyme PBG deaminase (E) with two and three molecules of the substrate PBG,(S) respectively.

15 The conversion of uro'gen I to a dimethylisobacteriochlorin by the *P. denitrificans* methylase[5b] (SUMT) was described earlier and the stereochemistry of rings *A* and *B* established as in (**6**), but no evidence of overmethylation (to a pyrrocorphin) was found in this study: A. I. Scott, H. J. Williams, N. J. Stolowich, P. Karuso, M. D. Gonzalez, F. Blanche, D. Thibaut, G. Müller, and G. Wörner, *J. Chem. Soc., Chem. Commun.*, 1989, 522.