Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8795–8799, November 1990
Chemistry

Biosynthesis of vitamin B_{12}: Isolation of precorrin-6x, a metal-free precursor of the corrin macrocycle retaining five *S*-adenosylmethionine-derived peripheral methyl groups

(hydrogenobyrinic acid/cobyrinic acid)

DENIS THIBAUT, LAURENT DEBUSSCHE, AND FRANCIS BLANCHE*

Département de Chimie Analytique, Centre de Recherche de Vitry, Rhône-Poulenc Santé, B.P. 14, 94403 Vitry-sur-Seine Cedex, France

Communicated by J.-M. Lehn, August 20, 1990

ABSTRACT **δ-Aminolevulinic acid and trimethylisobacteriochlorin are converted by cell-free protein preparations from *Pseudomonas denitrificans* into a metal-free pigment, precorrin-6x. This pigment, which accumulates when the cell-free system lacks NADPH, can be enzymically converted in high yield (>50%) into hydrogenobyrinic acid by the complete enzyme preparation. Double-labeling experiments establish that precorrin-6x carries five C-methyl groups, which appear at C-1, C-2, C-7, C-12α, and C-17 of the hydrogenobyrinic acid formed enzymically from precorrin-6x. This precursor of the corrin macrocycle is at the dehydrocorrin level of oxidation, has undergone ring contraction and extrusion of C-20, but still carries the acetic acid side chain at C-12. It is demonstrated that the conversion of precorrin-6x into hydrogenobyrinic acid specifically requires an NADPH-dependent reduction step.**

Cobyrinic acid (structure **5a**), a known precursor of vitamin B_{12}, is biosynthesized from uroporphyrinogen III (structure **1**) by a multistep process including eight C-methylations (Scheme I). Earlier structural work had established that the first three methyl groups are introduced at positions C-2, C-7, and C-20 in that order (1) to form the last known partially methylated intermediate, precorrin-3. This has been isolated, so far, as the octamethyl ester of its aromatized form (structure **2a**) but it is highly probable that precorrin-3 itself has the dihydro structure **3a** (2) and the dihydro form can be generated from added aromatic form in the enzymic incubation mixture. Pulse-labeling experiments (3) revealed that the fourth methyl is introduced at C-17 and extension of this approach showed that C-12 is methylated next followed by C-1 with C-5 and C-15 last (4–7). Finally, the ring contraction steps leading to the corrin macrocycle were found to involve extrusion of C-20 and its attached methyl group, with these appearing, respectively, as the carboxyl and methyl groups of acetic acid (8, 9).

Hydrogenobyrinic acid (structure **4a**), the cobalt-free analogue of cobyrinic acid (structure **5a**), had been thought not to occur in nature (10, 11) but was recently isolated from *Pseudomonas denitrificans* grown in a cobalt-containing medium (12). Labeling studies (12) showed that (*i*) the methylation sequence from precorrin-3 forward to hydrogenobyrinic acid is the same as that outlined above for cobyrinic acid and (*ii*) the methyl group at C-20 of precorrin-3 (structure **3a**) is lost during its conversion into hydrogenobyrinic acid (structure **4a**), presumably as acetic acid. These results clearly indicate that the cobalt-free corrinoid system is constructed by the same sequence of reactions as its cobalt-containing analogue but without cobalt insertion for the whole pathway.

We now outline experiments leading to the isolation of an additional precursor of the corrin macrocycle that carries five peripheral methyl groups.

MATERIALS AND METHODS

Starting Materials. Labeled samples of trimethylisobacteriochlorin (structures **2b** and **2c**) were obtained by incubation of suitably labeled forms of δ-aminolevulinic acid and *S*-adenosylmethionine (SAM) in a cell-free enzyme system prepared from *P. denitrificans* (see below). They were purified chromatographically as the octamethyl ester in the standard way and were then fully hydrolyzed as usual with 2 M aqueous piperidine immediately before use. C^2H_3OH was from Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) and [*methyl*-^{13}C]SAM (>85 atom % ^{13}C) was a generous gift (G. Müller, Institut für Organische Chemie, Biochemie und Isotopenforschung, Stüttgart).

Determination of Radioactivity. Samples were assayed on a Packard Tri-Carb 4430 liquid scintillation counter in Beckman Ready Gel. Quench corrections were carried out by the Spectral Index of the Sample method (Packard) according to the manufacturer's instructions, using [^{3}H]- and [^{14}C]toluene as standards (Amersham).

Preparation of ^{3}H- and ^{14}C-Labeled Pigment x. A crude cell extract of broken cells of *P. denitrificans* SC510 pXL253 was prepared in 0.1 M Tris·HCl, pH 7.7/1 mM EDTA/200 mM KCl (buffer A) and was applied to a column of Sephadex G-25 equilibrated with the same buffer. The protein fraction (≈250 mg of protein) was incubated anaerobically at 30°C for 7 hr with [*methyl*-^{3}H]SAM (5 μmol), the labeled trimethylisobacteriochlorin (200 nmol), NADH (10 μmol), ATP (20 μmol), and $MgCl_2$ (50 μmol) in 25 ml of buffer A. A sample of this preparation was incubated with NADPH (1 mM), to synthesize hydrogenobyrinic acid (structures **4c** and **4e**) from trimethylisobacteriochlorin (structures **2b** and **2c**).

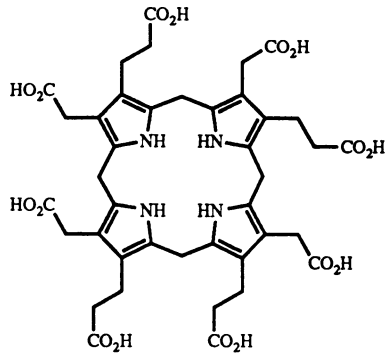
Preparation of [^{13}C]Methyl-Labeled Pigment x. This was obtained by the same method as for the preceding preparation using unlabeled trimethylisobacteriochlorin (structure **2a**) with [*methyl*-^{13}C]SAM on a scale sufficient to obtain 1.2 μmol of purified pigment x. A small amount of [*methyl*-^{14}C]SAM (75 μCi; 1.5 μmol; 1 Ci = 37 GBq) was included in the incubation to trace pigment x during purification and to calculate the incorporation level.

Purification of Pigment x. The incubation mixture was diluted with 4 vol of water and applied to a column of DEAE-Sephadex. After the column was washed with 5 vol of 0.1 M Tris·HCl (pH 7.7), the tetrapyrrolic acids were eluted with 1 M HCl and were separated by HPLC on a μBondapak C-18 column (Waters) using a 0–50% linear gradient of acetonitrile in 0.1 M potassium phosphate buffer (pH 5.8). Pigment x was further purified on a Mono Q HR 5/5 column

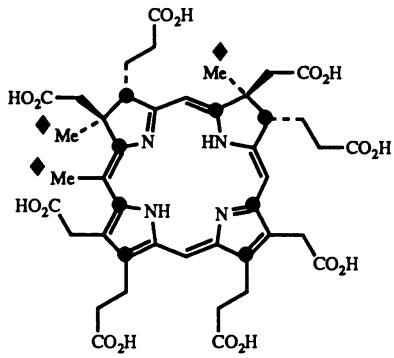
The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: SAM, *S*-adenosylmethionine.

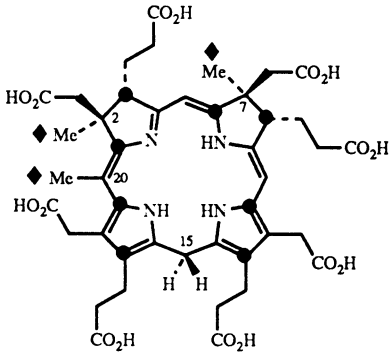
*To whom reprint requests should be addressed.



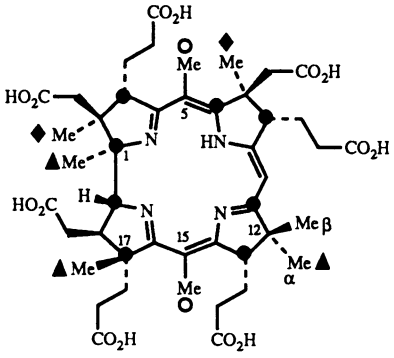
(1)



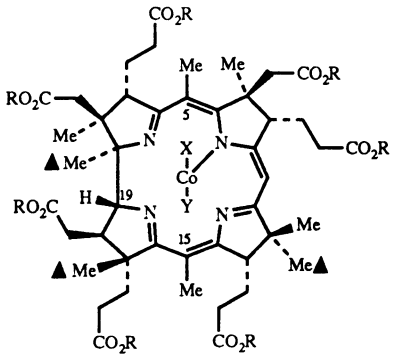
(2a) ● = ◆ = ^{12}C
(2b) ● = ^{14}C, ◆ = ^{12}C
(2c) ● = ^{12}C, ◆ = ^{14}C



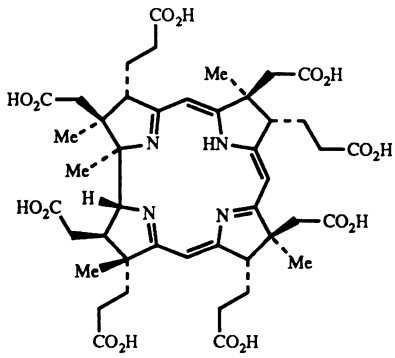
(3a) ● = ◆ = ^{12}C
(3b) ● = ^{14}C, ◆ = ^{12}C
(3c) ● = ^{12}C, ◆ = ^{14}C



(4a) ● = ◆ = ▲ = ^{12}C, ○ = CH_3
(4b) ● = ^{14}C, ◆ = ^{12}C, ▲ = $CH_2{}^3H$, ○ = CH_3
(4c) ● = ^{14}C, ◆ = ^{12}C, ▲ = ○ = $CH_2{}^3H$
(4d) ● = ^{12}C, ◆ = ^{14}C, ▲ = $CH_2{}^3H$, ○ = CH_3
(4e) ● = ^{12}C, ◆ = ^{14}C, Δ = ○ = $CH_2{}^3H$
(4f) ● = ◆ = ^{12}C, ▲ = $^{13}CH_3$, ○ = CH_3



(5a) ▲ = ^{12}C, R = H
(5b) ▲ = ^{13}C, R = H
(5c) ▲ = ^{13}C, R = CH_3



(6)

Scheme I

(Pharmacia) eluted with a linear gradient of 0–1 M KCl in 0.1 M potassium phosphate buffer (pH 6.8). It was finally rechromatographed to constant specific activity (^3H and ^{14}C) on the reversed-phase column described above eluted with a gradient of 5–25% acetonitrile and used directly for biosynthetic experiments. On-line detection of ^{14}C radioactivity during HPLC was achieved with a Berthold LB 505 radioactivity detector equipped with a GT 200 glass scintillator cell.

Biosynthetic Formation of Hydrogenobyrinic Acid from Pigment x. Pigment x (≈60 nmol) was incubated anaerobically for 7 hr at 30°C in 10 ml of buffer A with the protein fraction (≈100 mg of protein) described above from *P. denitrificans*, SAM (5 μmol), and NADPH (20 μmol). The

resultant hydrogenobyrinic acid was isolated as described (12) and rigorously purified by HPLC to constant specific activity and $^3H/^{14}C$ ratio by using the system described for pigment x.

Preparation of Cobester from Hydrogenobyrinic Acid. Cobalt was introduced nonenzymically into previously desalted hydrogenobyrinic acid (structure **4f**) (13), the resultant cobacid was esterified, and the cobester (structure **5c**) was purified by standard methods.

Preparation, Purification, and Subsequent Hydrolysis of the Methyl Ester of Pigment x. ^{14}C-labeled pigment x was prepared as described above from unlabeled trimethylisobacteriochlorin and [*methyl*-^{14}C]SAM. The purified product was desalted with a Sep-Pak C-18 cartridge (Waters) and then esterified under argon with 5% (vol/vol) sulfuric acid in methanol (18 hr at room temperature). Standard workup afforded the ester, which was purified by TLC on silica gel 60 eluting with dichloromethane/methanol (98.3:1.7). Hydrolysis of the ester was achieved in 2 M aqueous piperidine under argon at room temperature for 24 hr.

RESULTS

A cell-free extract was prepared from broken cells of *P. denitrificans* SC510 pXL253 and the partially purified protein fraction was used for the enzymic experiments. When this preparation was incubated with SAM, ATP, $MgCl_2$, NADH, and NADPH, hydrogenobyrinic acid was produced and could be isolated in the pure state by reversed-phase HPLC. The decisive observation was made that when NADPH was omitted from the incubation mixture, hydrogenobyrinic acid was no longer formed and a yellow pigment x was produced in its place. This pigment x could be isolated to purity by HPLC, which enabled the following labeling studies to be carried out.

Experiment 1. The ^{14}C-labeled trimethylisobacteriochlorin (structure **2b**) was produced biosynthetically from δ-amino[4-^{14}C]levulinic acid. This was then converted by way of the dihydrosystem (structure **3b**) using the NADPH-free enzyme system described above with [*methyl*-^3H]SAM into pigment x, the $^3H/^{14}C$ ratio of which showed that three ^3H-labeled methyl groups had been introduced (Table 1). This depended on comparing the $^3H/^{14}C$ ratio for pigment x with the ratio found for hydrogenobyrinic acid (structure **4c**) biosynthesized in parallel from the same two labeled precursors, a ratio that necessarily corresponds to the introduction of five ^3H-labeled methyl groups. The purity of both the hydrogenobyrinic acid and pigment x in the foregoing and following experiments was established by repeated reversed-phase HPLC fractionation to constant specific activity and $^3H/^{14}C$ ratio. When the double-labeled pigment x was incubated with the enzyme system containing NADPH, it was efficiently converted into hydrogenobyrinic acid (structure **4b**; 52% yield) without significant change in the $^3H/^{14}C$ ratio (Table 1). This proves that pigment x is a biosynthetic precursor of hydrogenobyrinic acid (structure **4a**).

Experiment 2. [2,7,20-*methyl*-^{14}C]Trimethylisobacteriochlorin (structure **2c**) as a source of **3c** and [*methyl*-^3H]SAM were used to biosynthesize pigment x, which was isolated to determine its $^3H/^{14}C$ ratio and then was enzymically converted by using unlabeled SAM into hydrogenobyrinic acid (structure **4d**) that had essentially the same $^3H/^{14}C$ ratio (3.02 relative to 3.04; Table 1). This clearly showed that the methyl group at C-20 must already have been extruded at some stage along the pathway to pigment x. Interlocking support for this conclusion comes from the $^3H/^{14}C$ ratio of pigment x, which on the basis of loss of the C-20 methyl, corresponds, in agreement with experiment 1, to the introduction of three ^3H-labeled methyl groups into pigment x [again relative to hydrogenobyrinic acid (structure **4e**) as standard; Table 1]. However, if the calculation assumes the C-20 methyl is still retained in pigment x, the ratio corresponds to the introduction of 4.37 ^3H-labeled methyl groups, which is inconsistent with experiment 1.

The results so far prove that pigment x is a precusor of the corrin macrocycle and that it carries five peripheral methyl groups; namely, the two at C-2 and C-7 of precorrin-3 (structure **3a**), which are retained together with three introduced later from SAM. The sixth SAM-derived methyl group at C-20 of structure **3a** has been extruded. This precursor can therefore be named (6) precorrin-6x. The x will be replaced in the future by a capital letter after it has been determined whether there exist, for example, one (precorrin-6A) or two (precorrins-6A and -6B) biosynthetic intermediate precursors to the present member of the precorrin-6 family.

To gain information about the sites of C-methylation in precorrin-6x, it was biosynthesized from unlabeled trimethylisobacteriochlorin (structure **2a**) and [*methyl*-^{13}C]SAM. After HPLC purification, the ^{13}C-labeled precorrin-6x was enzymically converted in high yield (89%) into hydrogenobyrinic acid (structure **4f**), again isolated in the pure state by HPLC. Nonenzymic insertion of cobalt (II) (13) then gave cobyrinic acid (structure **5b**), which was esterified to yield cobester (structure **5c**). The ^{13}C NMR spectrum of structure **5c** (Fig. 1) showed three ^{13}C-enriched methyl groups at positions C-1, C-12α, and C-17, which confirms that precorrin-6x is an efficient precursor of hydrogenobyrinic acid and that it carries five C-methyl groups.

The methyl ester of precorrin-6x showed by fast atom bombardment MS that $m/z = 1006$, which fits a metal-free macrocycle carrying eight carboxyl groups. Confirmation came by using C^2H_3OH to prepare the ester when the m/z value was 1030, an increment of 24, thus proving the presence of eight carboxyl groups. The octa acid obtained by hydrolysis of the ester with 2 M aqueous piperidine, after HPLC purification, was enzymically converted (as described above) just as efficiently into hydrogenobyrinic acid (structure **4a**) as the freshly isolated precorrin-6x. It follows that no irrevers-

Table 1. Double-labeling experiments with enzyme preparation from *P. denitrificans*

Exp.	Added precursor: Trimethylisobacteriochlorin, μCi,	Added precursor: [*methyl*-^3H]SAM, μCi	Biosynthesis of precorrin-6x (and hydrogenobyrinic acid) from trimethylisobacteriochlorin and [*methyl*-^3H]SAM: $^3H/^{14}C$ ratio of products: Precorrin-6x	Hydrogenobyrinic acid	Added methyls in precorrin-6x*	Conversion of precorrin-6x into hydrogenobyrinic acid: ^3H activity of precorrin-6x, dpm	Incorporation, %	$^3H/^{14}C$ ratio
1	1.06 (**2b**)	15.3	1.76	3.17	2.78	1.2×10^6	52	1.76
2	4.50 (**2c**)	75.0	3.02	5.18	2.92† (4.37)‡	6.0×10^6	51	3.04

*Calculated relative to five methyl groups added for hydrogenobyrinic acid.
†Assuming elimination of C-20 methyl group prior to precorrin-6x formation.
‡Assuming C-20 methyl group still present in precorrin-6x.

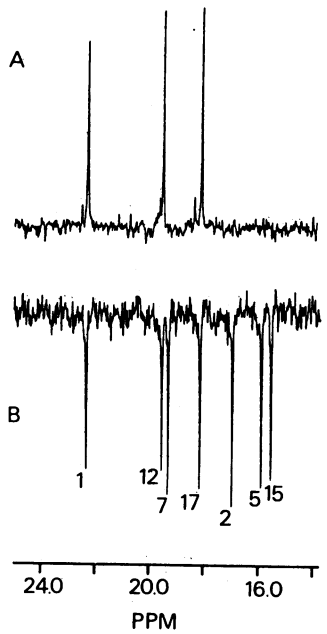


FIG. 1. Proton-noise-decoupled ^{13}C NMR spectra of labeled cobester (**5c**, X = Y = CN) biosynthesized from precorrin-6x (*A*) and unlabeled cobester (natural abundance signals) (*B*).

ible structural changes occur during the esterification and hydrolysis procedures.

The MS result of m/z = 1006 is important. If, to simplify the arithmetic, one considers a possible structure **6** for precorrin-6x related to that of hydrogenobyrinic acid (structure **4a**) with six double bonds, the requirement for the ester would be m/z = 1008, not 1006 as found. Therefore precorrin-6x has seven double bonds and is at the oxidation level of a dehydrocorrin. Reduction is thus needed to reach the final oxidation level of hydrogenobyrinic acid and this is in agreement with the original observation that precorrin-6x accumulates in the absence of NADPH. That NADPH is specifically required for the biosynthetic sequence from precorrin-6x to hydrogenobyrinic acid and cannot be replaced by NADH is demonstrated by the experiments illustrated in Fig. 2. Importantly, the foregoing results on precorrin-6x accumulation and properties were reproduced when δ-aminolevulinic acid was used as precursor rather than the trimethylisobacteriochlorin (structure **2a**). This shows that precorrin-6x is on the normal biosynthetic pathway and eliminates the remote possibility that it is some aberrant product from the aromatic macrocycle **2a**.

DISCUSSION

The isolation of precorrin-6x and knowledge of its formation and properties allows several important conclusions (see below) to be drawn about the biosynthesis of the corrin macrocycle and, hence, of vitamin B_{12} itself. The direct relevance to vitamin B_{12} is certain because the genetically engineered strain SC510 pXL253 overexpresses eight genes involved in the biosynthesis of cobyrinic acid from precorrin-2 (14). When the conclusions below are combined with the structural information presented in the accompanying paper (15), the entire direction of research on B_{12} biosynthesis is changed. Furthermore, they show that the most recently published scheme for corrin biosynthesis (7) and the structures there for precorrin-5, -6, -7, and -8 must be abandoned.

(*i*) It is certain that the pathway to corrins involves an NADPH-dependent reduction step with precorrin-6x as the substrate.

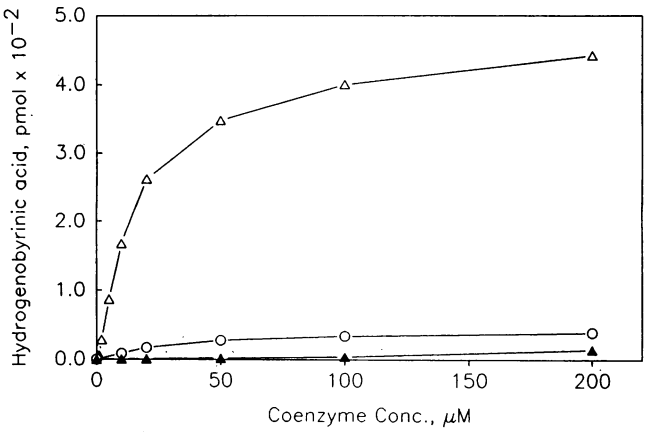


FIG. 2. Requirement for pyridine nucleotide coenzyme for conversion of precorrin-6x into hydrogenobyrinic acid. ^{14}C-labeled precorrin-6x (2 nmol) was incubated at 30°C for 1 hr with 0.75 mg of protein extract and SAM (30 nmol) together with NADPH (△), $NADP^+$ (○), or NADH (▲) in a total vol of 1 ml. The hydrogenobyrinic acid produced was assayed by HPLC.

The results from earlier experiments (16, 17) with *Propionibacterium shermanii* on the possible significance of dehydrocorrins in B_{12} biosynthesis were interpreted as indicating no involvement of biological redox reagents despite there being in both studies a substantially lower level of uptake of label from the medium at C-19 of the corrin system (see structure **5a**) than expected. Two explanations were offered (16, 17) for the result at C-19: (*a*) no reducing cofactor was involved but there was a marked isotope effect on hydrogen uptake from the medium, or (*b*) there was a flavin-mediated exchange of a reducing cofactor with the medium. In the light of conclusion *i*, it is clear that the wrong explanation was selected. Indeed, our biological system possesses transhydrogenase activity since it still converts precorrin-6x into hydrogenobyrinic acid when NADPH is replaced by NADH plus $NADP^+$. All our observations show that rigorous evidence for the NADPH requirement can only be obtained from an optimized enzyme system.

(*ii*) Direct evidence is provided that the methyl groups that appear in hydrogenobyrinic acid (structure **4a**) at C-17, C-12, and C-1 are the next ones to be introduced after those at C-2, C-7, and C-20 (the latter being extruded) and those at C-5 and C-15 are last.

(*iii*) The elimination of the methyl group at C-20 (present in structure **3a**) occurs before methylation at C-5 and C-15.

(*iv*) The most surprising finding is that ring contraction and introduction of the methyl group, which appears at the C-12α position in hydrogenobyrinic acid (structure **4a**), both take place before decarboxylation of the C-12 acetate residue.

The previously held view, based on mechanistic arguments, has been that decarboxylation of the C-12 acetate residue would precede C-12α methylation. This aspect is discussed in the accompanying paper (15), which shows how the problem is overcome in the biological system.

For more than 10 years, a major effort in a number of laboratories has been aimed at isolating biosynthetic intermediates for corrins that carry more than three C-methyl groups, but without success. The isolation of precorrin-6x now changes the scene.

We are grateful for the help of M. Vuilhorgne for the ^{13}C NMR spectra and M. Danzer for the fast atom bombardment mass measurements. J. Lunel, P. E. Bost, and J.-C. Brunie are also warmly thanked for their support as is Prof. A. R. Battersby (Cambridge) for critical reading of the manuscript. This work was supported by Rhône-Poulenc S.A., Health Division.

1. Leeper, F. J. (1985) *Nat. Prod. Rep.* **6,** 171–203.
2. Battersby, A. R. (1986) *Acc. Chem. Res.* **19,** 147–152.
3. Uzar, H. C. & Battersby, A. R. (1982) *J. Chem. Soc. Chem. Commun.*, 1204–1206.
4. Scott, A. I., Mackenzie, N. E., Santander, P. J., Fagerness, P. E., Müller, G., Schneider, E., Sedlmeier, R. & Wörner, G. (1984) *Bioorg. Chem.* **12,** 356–362.
5. Uzar, H. C. & Battersby, A. R. (1985) *J. Chem. Soc. Chem. Commun.*, 585–588.
6. Uzar, H. C., Battersby, A. R., Carpenter, T. A. & Leeper, F. J. (1987) *J. Chem. Soc. Perkin Trans.* **1,** 1689–1696.
7. Scott, A. I., Williams, H. J., Stolowich, N. J., Karuso, P., Gonzalez, M. D., Müller, G., Hlineny, K., Savvidis, E., Schneider, E., Traub-Eberhard, U. & Wirth, G. (1989) *J. Am. Chem. Soc.* **111,** 1897–1900.
8. Mombelli, L., Nussbaumer, C., Weber, H., Müller, G. & Arigoni, D. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 11–12.
9. Battersby, A. R., Bushell, M. J., Jones, C., Lewis, N. G. & Pfenninger, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 13–15.
10. Desow, B., Schlingmann, G., Ernst, L. & Koppenhagen, V. B. (1980) *J. Biol. Chem.* **255,** 7637–7644.
11. Koppenhagen, V. B. (1982) in B_{12}, ed. Dolphin, D. (Wiley, New York), Vol. 2, pp. 105–149.
12. Blanche, F., Thibaut, D., Frechet, D., Vuilhorgne, M., Crouzet, J., Cameron, B., Hlineny, K., Traub-Eberhard, U., Zboron, M. & Müller, G. (1990) *Angew. Chem. Int. Ed. Engl.* **29,** 884–886.
13. Podschun, T. E. & Müller, G. (1985) *Angew. Chem. Int. Ed. Engl.* **24,** 46–47.
14. Crouzet, J., Cameron, B., Cauchois, L., Rigault, S., Rouyez, M.-C., Blanche, F., Thibaut, D. & Debussche, L. (1990) *J. Bacteriol.* **172,** 5980–5990.
15. Thibaut, D., Blanche, F., Debussche, L., Leeper, F. J. & Battersby, A. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8800–8804.
16. Battersby, A. R., Edington, C. & Fookes, C. J. R. (1984) *J. Chem. Soc. Chem. Commun.*, 527–530.
17. Scott, A. I., Kajiwara, M. & Santander, P. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6616–6618.