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Discovering Nature's Diverse Pathways to Vitamin B_{12}: A 35-Year Odyssey

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The chronology of the discoveries along the pathway of vitamin B_{12} biosynthesis is reviewed from a personal perspective, including discussion of the most recent finding that two pathways to B_{12} exist—one aerobic and one anaerobic—which differ mainly in the ring contraction mechanisms that convert porphyrin to corrin.

Introduction

When I was informed of the award of the Nakanishi Prize, I was reminded of the magnitude of the debt which natural product chemists owe to Koji Nakanishi's leadership in applying all facets of spectroscopy to solving structural and stereochemical problems. It is therefore with a sense of gratitude for his lifelong inspiration that I have tried to trace the history of my own adventures in the application of NMR spectroscopy to the solution of biosynthetic mechanisms, illustrated by the complex and, at times, diabolical puzzles embodied in Nature's machinery for the synthesis of its most beautiful cofactor,[1] vitamin B_{12}. We begin with a chronological summary of the discoveries along the pathway to corrins.[2,3] The intersection of our researches with those of the other major laboratories working in the area are clearly referenced, so as to set the historical perspective in place.

The Early Days

From my first encounter with the beautiful structure of vitamin B_{12}, the study of vitamin B_{12} biosynthesis became my major interest beginning in 1968. At that time, the only information about the biosynthesis of corrin was Shemin's observation that 5-aminolevulinic acid (ALA) was the source of the tetrapyrrolic template and that methionine supplied 6 (or was it 7?) of the C-methyl groups, the exact number being difficult to estimate by radiolabeling, the only method available at the time.[2] The involvement of ALA indicated that the first segment of the well-known pathway to the tetrapyrroles of nature was operative, involving first the dehydratase which converts 2 mol of ALA to porphobilinogen (PBG) and thence via the combined actions of deaminase and uro'gen III synthase to uro'gen III (Scheme 1), the unsymmetrical tetrapyrrolic macrocycle, already known to be a precursor of heme and chlorophyll.

We now entered a new world at Yale in 1968—the combination of ^{13}C NMR and biochemistry—to solve not only the nature of the building blocks involved in the architecture of corrin but also their sequence of assembly. By feeding the labeled precursors to whole cells, we found that B_{12} is indeed derived from PBG and most importantly, uro'gen III (Scheme 1), despite earlier literature reports to the contrary. Also, we could count[5] the number of methyl groups (seven) derived from *S*-adenosyl methionine (SAM) with confidence, thereby dispelling any previous notion that the C-1 methyl came from a cyclopropane intermediate associated with a preceding ring contraction—yet another attractive concept that had to be abandoned in the face of experimental evidence.

These early NMR results were, for us, spectacular—no more tedious degradations were necessary to find the site and extent of radiolabeling—once the ^{13}C NMR spectrum of B_{12} was assigned, using the sets of ^{13}C isotopomers of ALA as they found their way into the vitamin.[6,7]

The First Set of New Intermediates

The next breakthrough came with the first cell free system from *P. shermanii*[8] which would incorporate our ^{13}C-enriched building blocks so efficiently that we could label uro'gen III in a unique way and thus determine the fate of each ^{13}C center as it finally appeared in cobyrinic acid—the end product of the cell free system. Next came a major advance when my colleague, Gerhard Müller in Stuttgart, by withholding cobalt from the cell free system, isolated the new intermediate, factor II (= oxidized precorrin-2; Scheme 1), which remarkably corresponded with the structure of sirohydrochlorin, the metal-free

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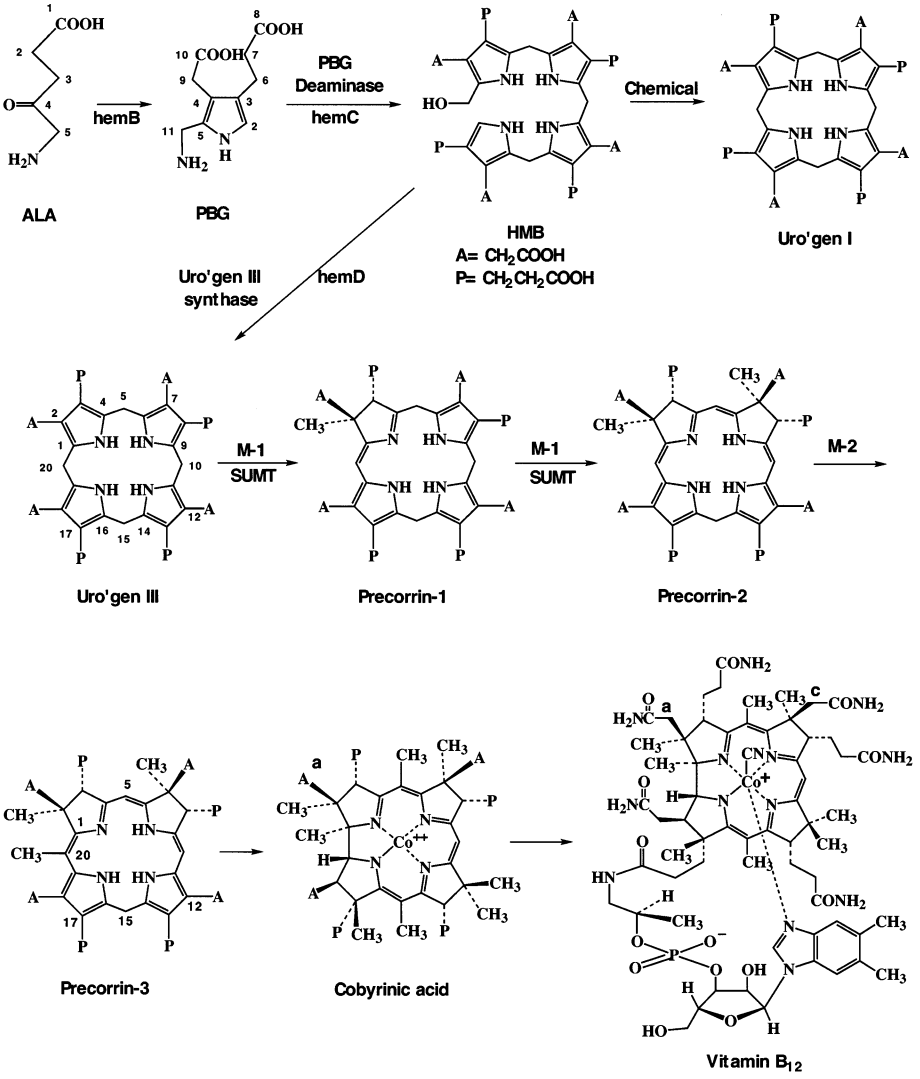
SCHEME 1



version of siroheme.[9,10] By now, in the late 1970s, we realized that in order to solve the complexities of B_{12} biosynthesis we would have to build a new laboratory combining organic chemistry, enzymology, molecular biology, and NMR spectroscopy, hence our move to Texas A&M University. There, we first discovered[11] the enzyme-free product HMB (Scheme 1) discharged from the enzyme PBG deaminase, which became the substrate for the rearranging enzyme, uro'gen III synthase, using NMR spectroscopy, and were able to solve the structure of precorrin-3[12] (in its oxidized form, factor III), which provided the first clue that Nature inserts a C-methyl at C-20 in precorrin-2 (Scheme 1) only to be subsequently lost as a two-carbon species, during (or after) ring contraction, later thought to be acetic acid.[12−14]

The Long Wait

Now came the long wait (1979−1990) where except for some "derailed" metrabolites,[15,16] no new intermediates emerged, despite processing hundreds of liters of B_{12}-producing lysates incubated with (and without) cobalt. During this period, we learned by using pulse-labeling techniques developed earlier in Cambridge[20] that the order of insertion of the C-methyl groups was $C_2 > C_7 > C_{20} > C_{17} > C_{12} > C_1 > C_5 > C_{15}$.[17−21] Although we obtained a "distant" view of what kinds of intermediates must be involved, it still proved impossible to isolate any of those species corresponding to the methylation sequence, perhaps due to their instability toward air and/or low concentration. Realizing that no further intermediates were going to be discovered by the "needle in a haystack" approach, we began in 1987 to assemble the entire repertoire of biosynthetic gene products for B_{12} biosynthesis and started with the "Heme Box" of *E. coli* (Figure 1), whose genetics were well studied, and which has to carry out the synthesis of precorrin-2 necessary for the source of the iron-containing cofactor, siroheme, as part of the machinery of the giant enzyme, sulfite reductase, our earlier source of factor II.

Fortunately, the *hem* gene cluster of *E. coli* had already been cloned and we were able to assemble and overexpress the genes *hemB*, *-C*, and *-D* as well as *cysG* (M-1; Scheme 1), which as we suspected, had the necessary methyl transferase activity to convert uro'gen III into precorrin-2. With ample supplies of these biosynthetic enzymes in hand,[22] we studied the mechanism of PBG

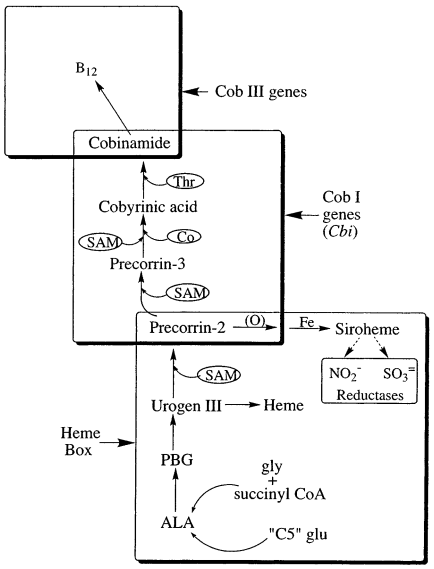


FIGURE 1.

deaminase (hemC) and found that the polymerizing enzyme of tetrapyrrole biosynthesis harbored a remarkable dipyrromethane cofactor (**5**), made from 2 mol of its own substrate PBG (**1**), which was covalently anchored to a cysteine residue (cys 242) in the 33 kD protein (Figure 2). Again, the ^{13}C NMR technique was the unique way to study the assembly of the growing, head-to-tail, tetrapyrrole chain, and we could see each of the new events including inhibition (**9**) or derailment (**12**) signaled by a change in the NMR spectrum and later confirmed by electrospray mass spectrometry.[23] As has happened so often in scientific discovery, we were not alone in this search, and two groups[24,25] in the UK published on the dipyrromethane cofactor within the same 5-month period. We next found that the apoenzyme reconstitutes the holoform autocatalytically from the substrate PBG,[23] and when the right number of PBG units (four) has been added to the cofactor (see structure **10**), the linear tetrapyrrole is discharged as azafulvene (**11**) ready for the next cycle. It was indeed gratifying when our NMR results were confirmed by the 3-D X-ray structure[26] of PBG deaminase. The azafulvene (II) may also be an intermediate in the formation of urogen III since it can lead to the "spiro" compound (**13**) in the presence of the enzyme cosynthetase.

With the capability of making substantial quantities of the pivotal precursor uro'gen III with overexpressed *hem C* and *hem D* (Scheme 1) now established in our laboratory, we could move forward into the chiral world of precorrins, one enzyme at a time, beginning with C-methylation by SAM mediated by CysG (M-1), which allowed us to study the exact double-bond arrangement in precorrin-2.[27] Almost every variant of the arrangement of acetate (A) and propionate (P) side chains were tolerated in the macrocycle when challenged with the methyl transferase enzyme (including the type I and IV urogens), and we observed that CysG could also "overmethylate" precorrin-2 at the unusual position 12, i.e., out of order from the natural methylation sequence to B_{12}, reactions which, as indicated in Scheme 2, could be studied directly in the NMR tube.[28]

The Search for the Biosynthetic Genes

At this juncture, we undertook yet another major new thrust, the cloning and overexpression of the *Salmonella cbi* genes,[29] generously provided by John Roth[30] (Utah) who had found that this organism can produce B_{12} anaerobically. We also engineered the necessary genes from a wild strain of *P. denitrificans* into *E. coli* and expressed the biosynthetic gene products, ready to proceed, one enzyme at a time, into the unknown territory beyond precorrin-2 (Figure 3). Both C-20 (CbiL) and C-11 (CbiF) methyl transferase activities were found by the ^{13}C NMR assay.[29] But by now competition had arisen from Rhone-Poulenc in Paris, who had committed a major effort to engineer their production strain of the aerobic organism *P. denitrificans* to harbor an 8-gene cluster (*cobF-M*), able to synthesize the cobalt free corrinold, hydrogenobyrinic acid (HBA) from factor III (oxidized precorrin-3). By withholding NADPH or by mutating several of the *cob* genes, one at a time, it was possible for the French workers to extend the library of intermediates downstream from precorrin-3 to find[32−34] and, in association with the Cambridge group,[35−37] to deduce the structures of the ring-contracted precorrin-6x, its reduction product, 6y (obtained by restoring NADPH), and an advanced, fully methylated precorrin-8x, lacking only one step[38] (11 → 12 methyl shift) to reach corrin (HBA) in a cell free system from the engineered bacterium. (Figure 4). Although we were never to have access to their industrial strain, the genes encoding each enzyme in the pathway were available from our "academic" gene bank and would in principle allow us to proceed, step by step, into the unknown beyond precorrin-3.

Another Long Wait!

By late 1972, we had the full sets of enzymes for both anaerobic (*Salmonella*; nine enzymes) and aerobic (*P. denitrificans*; eight enzymes) pathways in hand (Figure 5) and felt certain of success, yet despite many months, night and day in front of the NMR console, we could see no change in the ^{13}C spectrum of precorrin-3 when challenged with each of the 17 enzymes in turn. We realized that some vital cofactor was missing from our carefully controlled anaerobic incubations carried out in the glovebox (<0.5 ppm O_2). We had also learned how to combine as many as five biosynthetic enzymes to prepare substantial amounts of the precursor, precorrin-3 in good yield, endowed with ^{13}C labels ready to explore the ring contraction mechanism[39] (Figure 6). It was a frustrating period until factor IV, the oxidized version of (the still unknown) precorrin-4 from a CobM mutant (which could not process the pathway beyond the tetramethylated level), was described by Rhone-Poulenc.[40] From inspection of the structure of factor IV it was at once apparent that the missing cofactor was the very one which we and the French group had carefully tried to exclude from all of the incubations − oxygen!

Now at last when precorrin-3 was incubated with the prime enzymatic candidate for the ring contraction (CobG), this time in the presence of O_2, yet another surprise was in store, for the NMR spectrum changed dramatically not to a ring-contracted product but to that of a 20-hydroxy lactone, precorrin-3x,[41] formally derived

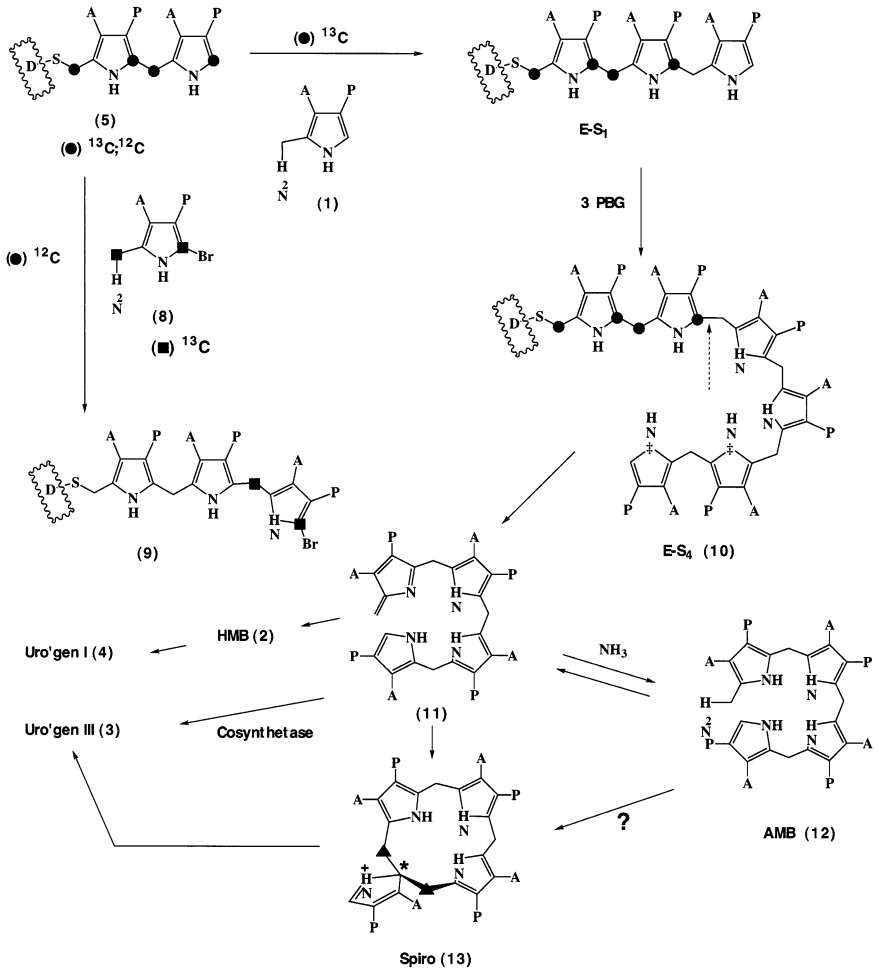


FIGURE 2.

from precorrin-3 by hydroxylation, followed by participation of the ring A acetate carboxyl in γ-lactone formation (Figure 7). All fell quickly into place using $^{18}O_2$ gas to trace these events—as we realized that the function of CobG is not to ring contract but rather to install a beautifully designed mechanism for this very purpose, **which only comes into play in the presence of the next methyl transferase.** This turned out to be CobJ, a bifunctional enzyme which methylates ring D at C-17 and then (and only then) catalyzes the ring-shrinking process to leave a new methyl ketone function at C-1, corresponding to precorrin-4,[42] which was analyzed in its biochemically active, reduced form shown in Figure 7. At the same time, the French group also isolated precorrin-3x, naming it precorrin-3B.[41b] We view the ring contraction process as an acyloin-like rearrangement since we could trace the fate of the original ^{18}O at in the C-20 hydroxyl in −3x as it found its way in to the carbonyl-carbon bearing ^{18}O (Figure 7). By the same isotopic method, we showed that a $^{13}C^{18}O_2H$ label in the ring A acetate was retained beyond ring contraction and indeed all the way to HBA,[43,44] in sharp contrast to the fate of this same acetate function, which undergoes exchange with the medium in the anaerobe, *P. shermanii*,[45,46] and realized for the first time that there are two distinct pathways to the complete corrinoid structure of B_{12}, depending on the type of organism, aerobic or anaerobic.[47] The elucidation of the anaerobic pathway has now become our new challenge, as described later.

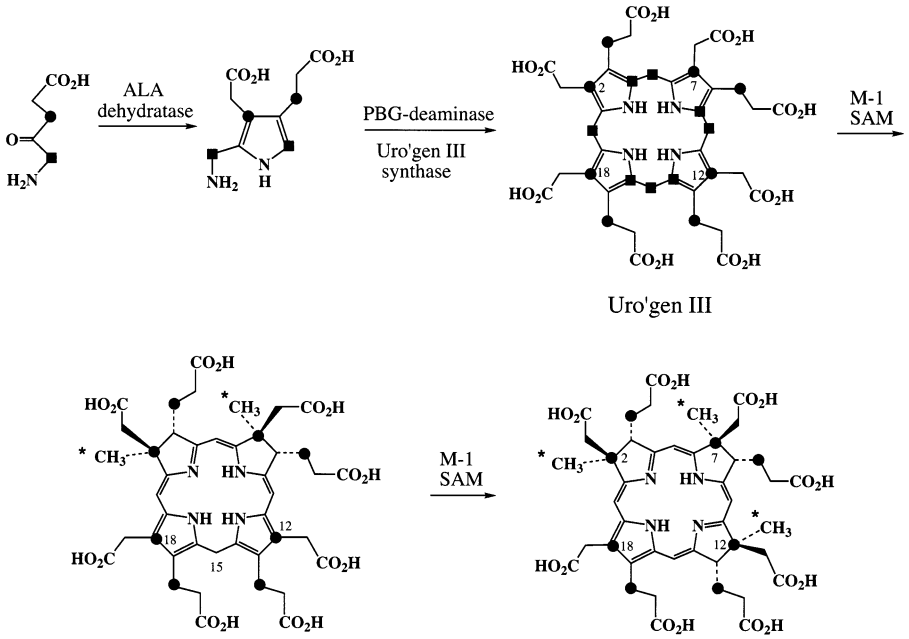
Having reached precorrin-4, only one intermediate remained to be discovered, and this had to involve yet another methylation, this time at C-11. Now everything became clear, for when the gene product, CobM, was included in the preparation, we could go directly from precorrin-3 to the long sought precorrin-5,[42] the necessary NMR analysis providing the structure shown in Figure 8.

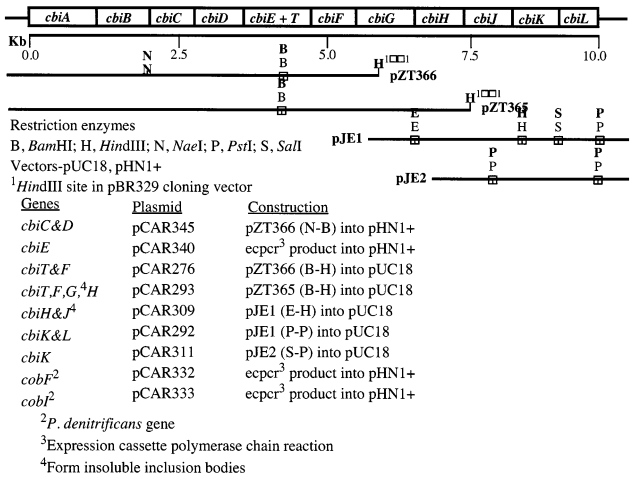
By adding our last unassigned enzyme, CobF, we finally reached, by deacylation and methylation at C-1, the known intermediate precorrin-6x (Figure 9) whose further transformation had been studied genetically [32−34] and chemically[35−37] in Paris and Cambridge, respectively (see Figure 4). Now the pathway in all its complexity was revealed—17 discrete steps from ALA mediated by 12 enzymes—a joy to behold!

Genetically Engineered Multi-Enzyme Synthesis of Corrin

Having uncovered the structures of these new intermediates, we next decided to reconstruct the entire pathway shown in Scheme 3 by incubating ALA with the necessary cofactors SAM, NADPH, and of course, oxygen in a single vessel followed by isolation of the final target,

SCHEME 2





Genes	Plasmid	Construction
cbiC&D	pCAR345	pZT366 (N-B) into pHN1+
cbiE	pCAR340	ecpcr[3] product into pHN1+
cbiT&F	pCAR276	pZT366 (B-H) into pUC18
cbiT,F,G,[4]*H*	pCAR293	pZT365 (B-H) into pUC18
cbiH&J[4]	pCAR309	pJE1 (E-H) into pUC18
cbiK&L	pCAR292	pJE1 (P-P) into pUC18
cbiK	pCAR311	pJE2 (S-P) into pUC18
cobF[2]	pCAR332	ecpcr[3] product into pHN1+
cobI[2]	pCAR333	ecpcr[3] product into pHN1+

[2]*P. denitrificans* gene
[3]Expression cassette polymerase chain reaction
[4]Form insoluble inclusion bodies

FIGURE 3.

HBA, and in 1994 were ready to assemble the ex vivo synthetic machine. Simply by growing 12 1-L batches of *E. coli* bearing 3 *hem* and 9 *cob* genes, combining the lysates from each with ALA and the above cofactors in buffer (pH7) and, after 12 h, isolating the corrinoid product, an overall yield of 20% of HBA from ALA could be reproduced even without optimizing the conditions.[48] Complete identity with natural material was established by ^{1}H and ^{13}C NMR, FAB-MS, UV−vis, CD, and finally by chemical conversion to cobester (Scheme 3; step 13). We had now reached the link with B_{12} itself since the cyanomethyl ester had already been converted by Eschenmoser[4] to the complete structure of the vitamin in a remarkable self-assembly process (Scheme 3; steps 14−16). Thus, of the seven possible sites of attachment only the ring D propionate ester of cobyrinic acid undergoes reaction with activated nucleotide to add the nucleotide loop at the "natural" position 17. We believe that, most fortunately for the success of the 12-enzyme, 17-step synthesis of HBA, the last of these enzymes, CobH, has such a remarkable affinity for HBA that the product can be recovered from protein after simple heat treatment (70 °C/10 min), uncontaminated with any other porphyrinoid impurity.

So we had come full circle and once again returned to synthetic chemistry, but now using the three-dimensional catalysts of nature, and instead of the years required for chemical synthesis, the time for such a genetically engineered synthesis is measured in hours. This is, of course, an oversimplification, since many person-years had first to be spent in finding and expressing the required biosynthetic genes. It is important at this stage for the general reader to understand the difference between combining several (or many) pure enzymes dedicated solely to reaching a synthetic target in a single flask[49,50] and the construction of genetically engineered strains of bacteria harboring the genes for a biosynthetic pathway, which can either be mutated to accumulate intermediates or used to produce the final target, since the lysates contain the complete metabolic machinery of the cell and can divert the substrates into the major arteries of primary metabolism rather than processing the substrate directly to the target.

The Anaerobic Pathway: More Surprises in Store!

Up to precorrin-2, the aerobic and anaerobic pathways are virtually indistinguishable, but a major divergence occurs at this point. In the aerobic pathway, cobalt insertion takes place at a fairly late stage into HBA *a,c*-diamide (see Scheme 3). The cobaltochelatase catalyzing the insertion is encoded by the *cobN*, *cobS*, and *cobT* genes in *P. denitrificans*.[51] However, studies involving the incorporation of ^{14}C- or ^{60}Co-labeled precursors into

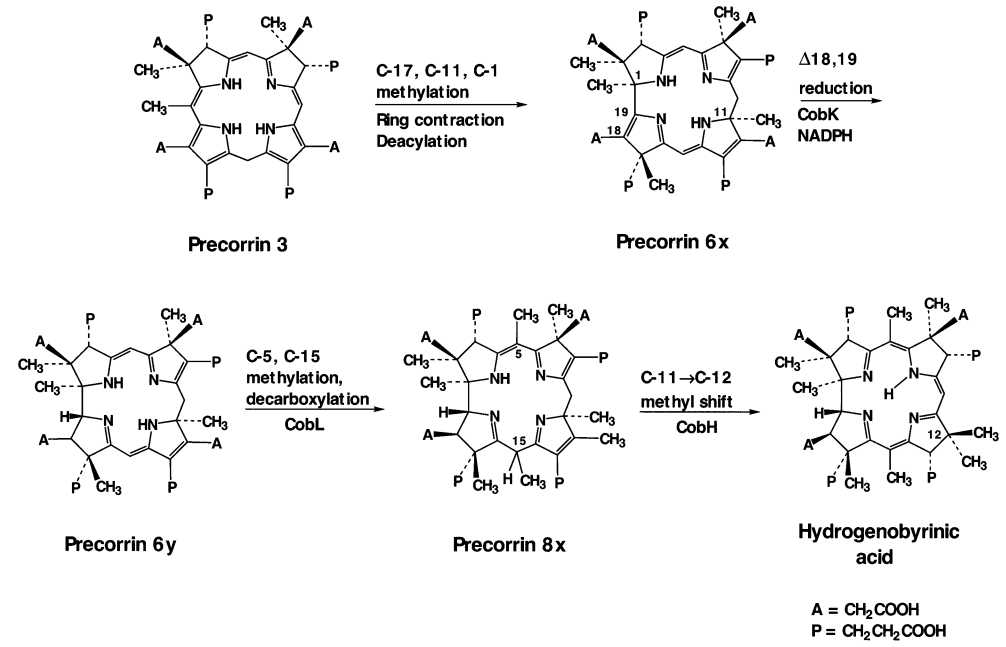


FIGURE 4.

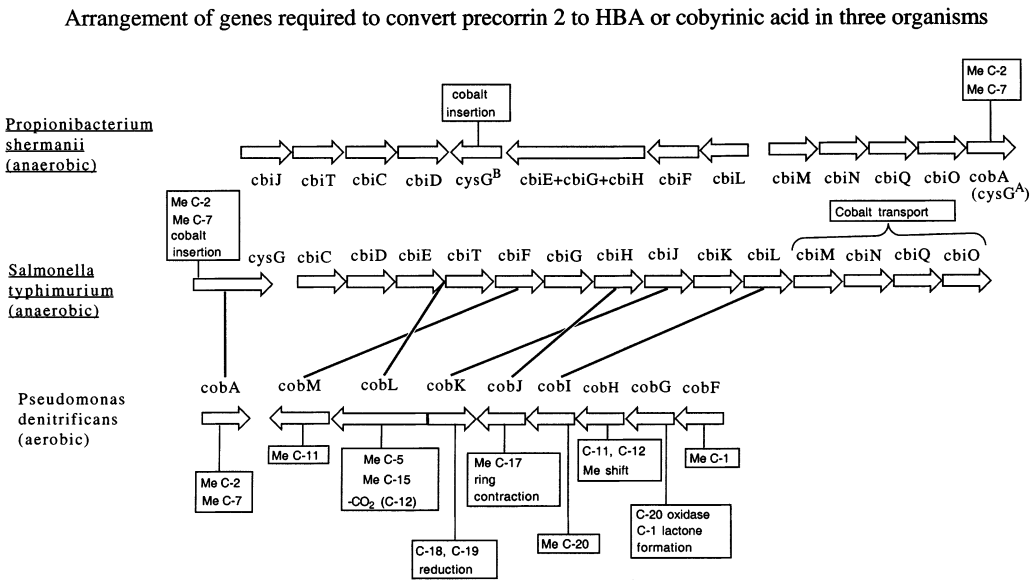


FIGURE 5.

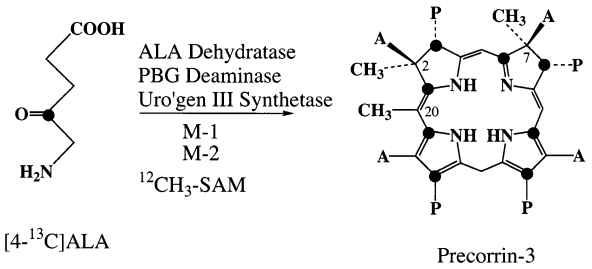


FIGURE 6.

cobyrinic acid[52a,b] have demonstrated that cobalt insertion in the anaerobic pathway occurs as early as precorrin-2. Since organisms that make B_{12} anaerobically do not have genes homologous to *cobN*, *S*, and *T*, cobalt insertion must be catalyzed by some other chelatase in anaerobes. It has been proposed[53,54] that, *in S. typhimurium*, the methyl transferase CysG is able to insert iron into siroheme and also may be a cobalt-inserting enzyme in the B_{12} pathway. While the direct biochemical evidence for this additional activity for CysG is still inconclusive, it is strongly supported by genetic evidence. *S. typhimurium cysG* mutants have been isolated that cannot synthesize B_{12} but can still synthesize siroheme.[55] Some of these mutations can be reversed by higher cobalt concentrations, suggesting that the mutant enzyme has normal iron affinity but a reduced affinity for cobalt, which can be overcome by increased concentrations of cobalt. On the other hand, it has been shown that CbiK from *Salmonella* acts as a cobalt chelatase.[55b] The complete role of Cys G is thus still in question.

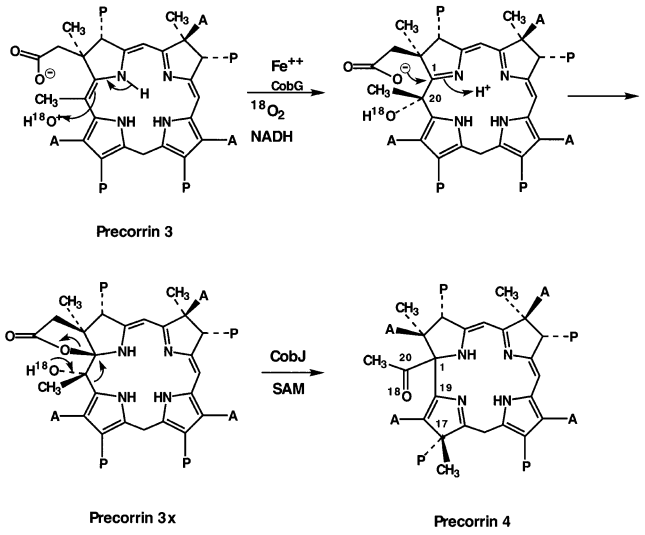


FIGURE 7.

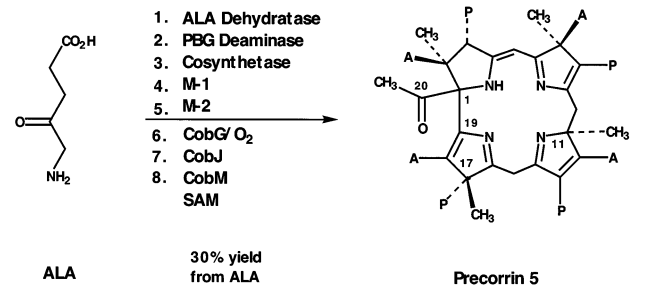


FIGURE 8.

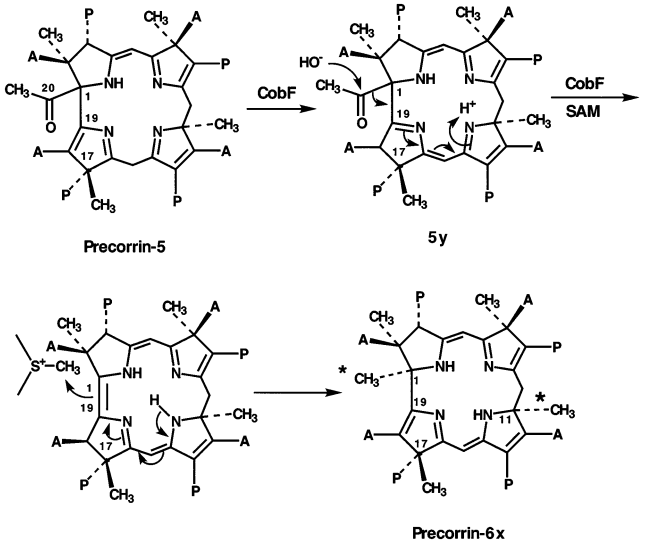


FIGURE 9.

In the aerobic pathway, precorrin-2 methyltransferase, encoded by the *cobI* gene,[56] catalyzes the SAM-dependent methylation of precorrin-2 at C-20 to yield precorrin-3 (Scheme 3). Since precorrin-2 is also the precursor of siroheme, the biosynthesis of precorrin-3 by this enzyme is the first step committed solely to the vitamin B_{12} pathway. In the anaerobic pathway, C-20 methylation is catalyzed by CbiL[57] but only when the cobalt complex of precorrin-2 is used as substrate.

Perhaps the most significant difference between the aerobic and anaerobic pathways lies in the mechanism of contraction of the macrocycle resulting in precorrin-4 and cobalt-precorrin-4, respectively. In the aerobic pathway, two steps and two different enzymes are required to convert precorrin-3 to the ring-contracted product, whereas in the anaerobic pathway a single enzyme CbiH converts cobalt-precorrin-3 to cobalt-precorrin-4.

Thus, CbiH catalyzes not only the insertion of a methyl group at C-17 of cobalt-precorrin-3 but also triggers ring contraction to form cobalt-precorrin-4, which features a δ-lactone involving the acetate of ring A and C-20. This structure suggests a possible mechanism for ring contraction that incorporates the original ideas of Eschenmoser[4] involving the δ-lactone and a cyclopropane intermediate as shown in Figure 10. The cobalt may participate in the process by one- or two-electron chemistry. The intermediacy of cobalt-precorrin-4 in the anaerobic B_{12} pathway has been established by its isolation (in its oxidized form) from *P. shermanii*[58] and by its conversion to cobyrinic acid by a cell free lysate of *P. shermanii*.[59]

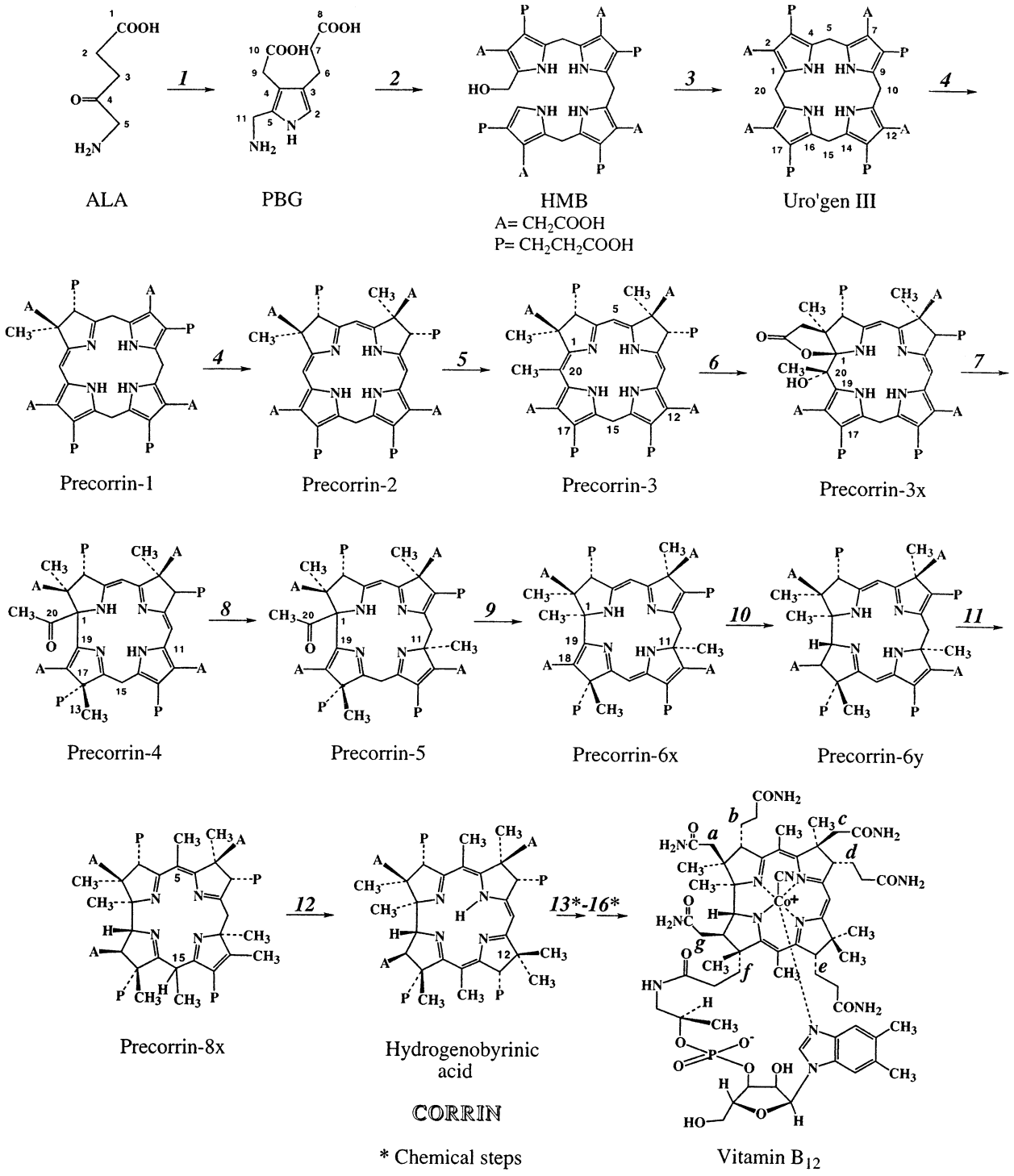
Finally, another major difference that has been observed between the two pathways is that the oxygens of the ring A acetate undergo extensive exchange with water from the medium in the anaerobic pathway but not in the aerobic pathway. Additionally, in the former pathway, C-20 and its attached methyl group are extruded as acetaldehyde,[60] whose oxygen is derived from the carboxyl of the ring A acetate,[61] whereas the oxygen-dependent route produces acetic acid directly with no involvement of the ring A carboxyl. These differences must reflect dissimilarity in the step required to convert precorrin-5 to precorrin-6 in the aerobic pathway and cobalt-precorrin-5 to cobalt-precorrin-6 in the anaerobic pathway. The latter requires opening of the δ-lactone ring, extrusion of acetaldehyde, and C-1 methylation. In the scenario shown in Figure 11, exhange with water in the carboxyl of the ring A acetate results from hydration during opening of the δ-lactone, and the oxygen of the acetaldehyde is concomitantly derived from the ring A acetate during this process.[61,62] The unassigned gene CbiG may encode an enzyme catalyzing the opening of the δ-lactone.[62b]

The conversion of cobalt precorrin 4 to the next intermediate cobalt precorrin 5, believed to have the structure shown in Figure 11, is thought to involve the C-11 methyl transferase CbiF, homologous to CobM in the aerobic pathway, but this has still to be proved. From cobalt precorrin 6 onward the pathways are thought to be in parallel, but considerable work lies ahead in defining each of the anaerobic intermediates that are air-sensitive and difficult to characterize. Now that all of the *P. shermanii* biosynthetic genes have been characterized,[62] we can expect rapid progress.

What of the Future?

With the techniques firmly established, the approach of genetically engineered synthesis should be a general one, for we believe that the most rewarding part of this excursion from synthesis into molecular biology, and back again, is still to come. What if the methods developed form B_{12} synthesis could be applied in a general way to all natural products, regardless of origin? In other words,

SCHEME 3[a]



[a] Key: (1) ALA dehydratase (*hemB*); (2) PBG deaminase (*HemC*); (3) uro'gen III synthase [cosynthase] (*hemD*); (4) uro'gen III methylase [M-1] (*cysG*/*cobA*); (5) M-2 (*cobI*, *cbiL*); (6) precorrin-3x synthase (*cobG*); (7) ring contractase/17 methyl transferase [M-3] (*cobj*); (8) M-4 (*cobM*); (9) M-5 (*cobF*); (10) reductase (*cobK*); (11) precorrin-8x synthase (M-6/decarboxylase) (*cobL*); (12) [1,5]-sigmatropic shiftase [hydrogenobyrinic acid synthase] (*cobH*); (13) insert Co; (14) esterify; (15) add nucleotide; (16) ammonolysis.

can the concept be extended to plant, fungal, and even human metabolites of potential value as chemotherapeutic agents and exploited to prepare rare natural products with interesting biological activities?

As we enter this most exciting phase of the work, I am certain that the answer must be **yes**, for we and others have been able to express the genes for indole alkaloid synthesis in *E. coli*, and at this juncture the literature is beginning to show the first signs of success.[49] Already, heterologous gene transfer using cDNA rather than genomic libraries is starting to uncover the activity of biosynthetic enzymes for secondary metabolites. The central problem, still to be solved, is an analytical one. In other words, in a cloned cDNA library, obtained

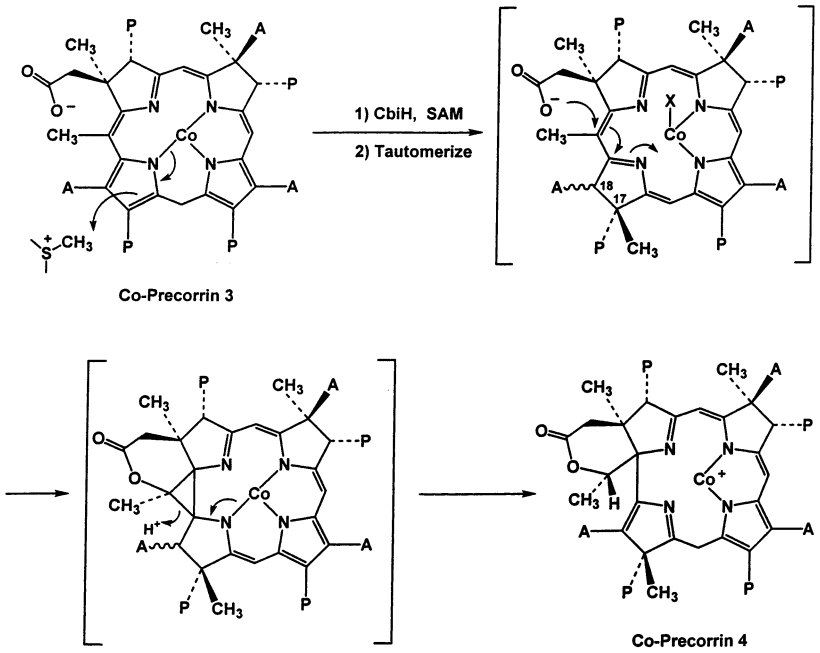


FIGURE 10.

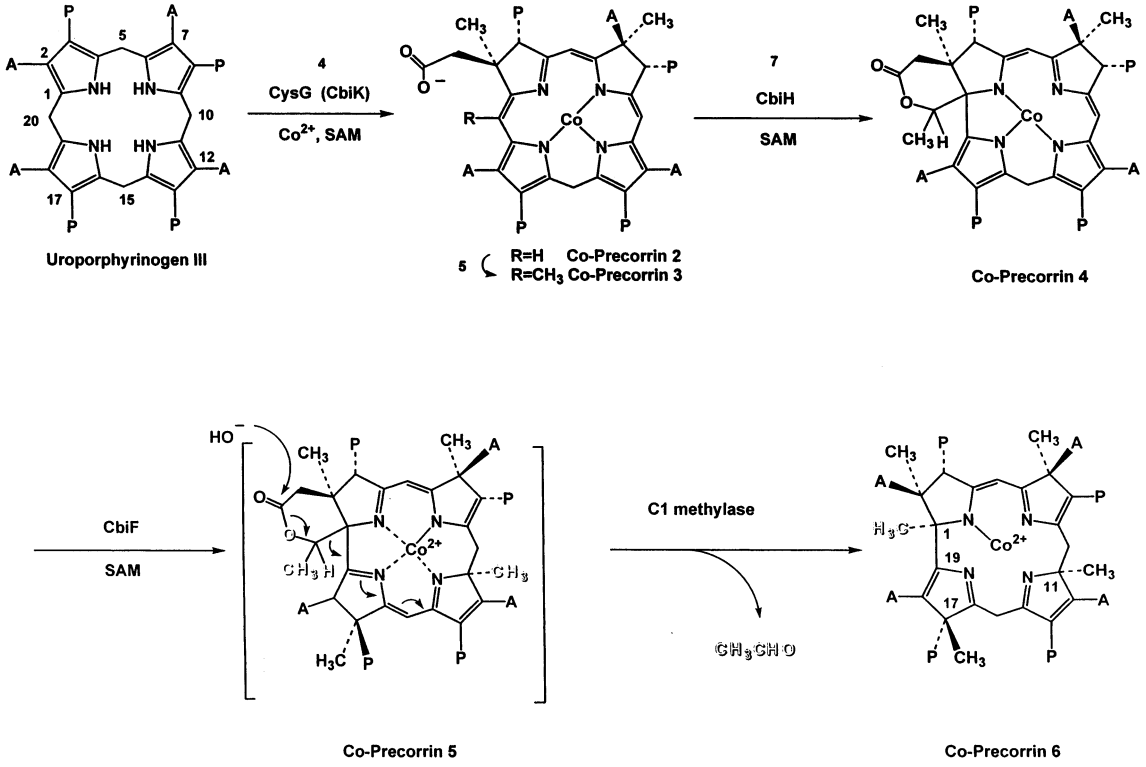


FIGURE 11.

from the action of reverse transcriptase on the isolated m-RNA, the biosynthetic factory is usually spread over some 40000−50000 plaques, of which only ∼20−30 harbor the genes necessary for the synthesis of the target—the breakthrough must come when sensitive assays can be devised to pull the 20 "needles" out of the haystack. When these biosynthetic genes are recombined we will be able to reconstruct the pathway just as was done for B_{12}. Thus, we have shown that three of the "early" genes of Taxol biosynthesis, when engineered into *E. coli*, can produce the key hydrocarbon intermediate, taxadiene,[63] ready for elaboration into the natural anticancer drug. A panoramic summary of our results in this new endeavor can be found on the cover of this issue. We are living in exciting times, since the barrier between chemistry and biology has by now almost disappeared, and from my perspective as a bioorganic chemist, the possibilities presented by combining genetic engineering with organic chemistry are almost limitless. Despite our recent success with harnessing the "gene machine" to

synthesize B_{12}, I still feel humble in the face of Nature's synthetic route, for, just as noted 500 years ago,[64] "Human subtlety will never devise an invention more beautiful, more simple or more direct than does nature, because in her inventions nothing is lacking and nothing is superfluous."

Acknowledgment. It has been my good fortune throughout my career to have had many generations of students, postdoctorals, and technical assistants whose hard work and perseverance reduced so many of my ideas into practicality. The names of all those who have given so much over the last 30 years to overseeing and performing the daily (and nightly) experiments involving cloning, enzymology, organic chemistry, and NMR spectroscopy, which are now our standard tools for solving the mysteries of biosynthesis, will be found in the many references covering the period from 1968 onward. Our research on B_{12} has been supported by the NIH, The Robert A. Welch Foundation, The Texas Advanced Technology and Research Program (TATRP), and Aventis.

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