JOURNAL OF BACTERIOLOGY, Sept. 2000, p. 4773–4782 Vol. 182, No. 17
0021-9193/00/$04.00+0


# Native Corrinoids from *Clostridium cochlearium* Are Adeninylcobamides: Spectroscopic Analysis and Identification of Pseudovitamin $B_{12}$ and Factor A

BERND HOFFMANN,[1] MICHAEL OBERHUBER,[1] ERHARD STUPPERICH,[2] HARALD BOTHE,[3] WOLFGANG BUCKEL,[3] ROBERT KONRAT,[1] AND BERNHARD KRÄUTLER[1]*

*Institut für Organische Chemie, Universität Innsbruck, A-6020 Innsbruck, Austria,[1] and Institut für Angewandte Mikrobiologie, Universität Ulm, D-89069 Ulm,[2] and Laboratorium für Mikrobiologie, Fachbereich Biologie, Phillips-Universität Marburg, D-35032 Marburg,[3] Germany*

Received 15 March 1999/Accepted 6 June 2000

**The corrinoids from the obligate anaerobe *Clostridium cochlearium* were extracted as a mixture of $Co_\beta$-cyano derivatives. From 50 g of frozen cells, approximately 2 mg (1.5 μmol) of $B_{12}$ derivatives was obtained as a crystalline sample. Analysis of the corrinoid sample of *C. cochlearium* by a combination of high-pressure liquid chromatography and UV-Vis absorbance spectroscopy revealed the presence of three cyano corrinoids in a ratio of about 3:1:1. The spectroscopic data acquired for the sample indicated the main components to be pseudovitamin $B_{12}$ ($Co_\beta$-cyano-7″-adeninylcobamide) (60%) and factor A ($Co_\beta$-cyano-7″-[2-methyl]adeninylcobamide) (20%). Authentic pseudovitamin $B_{12}$ was prepared by guided biosynthesis from cobinamide and adenine. Both pseudovitamin $B_{12}$ and its homologue, factor A, were subjected to complete spectroscopic analysis by UV-Vis, circular dichroism, mass spectrometry, and by one- and two-dimensional $^1H$, $^{13}C$-, and $^{15}N$ nuclear magnetic resonance (NMR) spectroscopy. The third component was indicated by the mass spectra to be an isomer of factor A and is likely (according to NMR) to be 7″-[$N^6$-methyl]-adeninylcobamide, a previously unknown corrinoid. *C. cochlearium* thus biosynthesizes as its native "complete" $B_{12}$ cofactors the 7″-adeninylcobamides and two homologous corrinoids, in which the nucleotide base is a methylated adenine.**

Besides vitamin $B_{12}$ (cyanocobalamin) itself, a spectrum of corrinoids is provided in nature which differ from vitamin $B_{12}$ by their cobalt-coordinating axial ligands (29). While the corrin moiety of the naturally occurring corrinoids is completely conserved structurally, a rationalized feature of (the catalytic moieties of) essential cofactors (24), the "complete" $B_{12}$ derivatives contain a remarkable variety of aromatic nucleotide functions: benzimidazoles (such as 5-methylbenzimidazole, 5,6-dimethylbenzimidazole [DMB], 5-hydroxybenzimidazole, and 5-methoxybenzimidazole), purine bases (such as adenine or 2-methyladenine) (see Fig. 1) or phenols (phenol and *p*-cresol) (29, 43, 61, 67). In contrast to the situation in the other widespread adenine-containing dinucleotide cofactors, in pseudovitamin $B_{12}$ ($Co_\beta$-cyano-7″-adeninylcobamide) and in factor A ($Co_\beta$-cyano-7″-[2-methyl]adeninylcobamide; Fig. 1), the ribose function is part of an unusual α-nucleotide moiety and binds to N7 of the purine ring. This latter property has been suggested by D. C. Hodgkin to be required in adeninylcobamides in order to enable the purine base to coordinate intramolecularly to the corrin-bound cobalt center via its N9 (30, 31, 41).

Three years after the discovery of vitamin $B_{12}$ (62, 64), pseudovitamin $B_{12}$ was isolated from an incompletely identified microorganism that was obtained from rumen contents (20). In 1958 the coenzyme form of pseudovitamin $B_{12}$ (i.e., pseudocoenzyme $B_{12}$) was discovered and identified by UV and visible (UV-Vis) spectroscopy to be the native cofactor of *Clostridium tetanomorphum* (2). Factor A was the main component of a mixture of corrinoids which were isolated in a crystalline form from pig manure, bovine gut contents, and feces (25, 26, 29). Pseudovitamin $B_{12}$ and factor A often occur in anaerobically fermenting systems, such as ruminal (7) and intestinal (12) contents, feces, and sewage sludge (29). The crystal structure of factor A was determined in 1981 and confirmed the presence of an α-nucleotide and binding of N9 of the purine ring to the corrin-bound cobalt center (41). The different nucleotide bases in factor A and in vitamin $B_{12}$ lead only to small differences in the axial bond lengths, in bond angles, and in the gross three-dimensional (3D) structures of vitamin $B_{12}$ and factor A. The axial bond lengths of factor A [(Co—C) bond length = 1.86 Å and (Co—N) bond length = 2.12 Å] (41) differ only marginally from those of vitamin $B_{12}$ [(Co—C) bond length = 1.86 Å and (Co—N) bond length = 2.01 Å] (45). The longer (Co—N) bond in factor A clearly is the consequence of a weaker coordinating adenine heterocycle compared to the more nucleophilic DMB base of vitamin $B_{12}$.

Pseudovitamin $B_{12}$ and factor A have been found specifically in a variety of microorganisms, e.g., in *Clostridium* (2, 28), *Propionibacterium* (29), and methanogenic (pseudovitamin $B_{12}$) bacteria (66). Remarkably, *Salmonella enterica* serovar Typhimurium synthesizes cobalamins under microaerophilic conditions, while pseudovitamin $B_{12}$ and factor A are found in serovar Typhimurium, when grown strictly anaerobically (39). Whereas aerobic microorganisms have developed a particularly efficient way to biosynthesize the DMB base of their cobalamins from flavine, the biosynthetic access to DMB is now known to be a complex task in obligate anaerobes (60).

Guided biosynthesis opens access to natural but commercially unavailable complete corrinoids and often is also applicable to the synthesis of complete cobamides, whose nucleotide base differs from the known ones, by supplementing the bacterial culture with a suitable nucleotide base precursor (see, for example, reference 29). Early on, the preparation by "guid-

* Corresponding author. Mailing address: Institut fur Organische Chemie, Universitat Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria. Phone: 43-512-507-5200. Fax: 43-512-507-2892. E-mail: bernhard.kraeutler@uibk.ac.at.

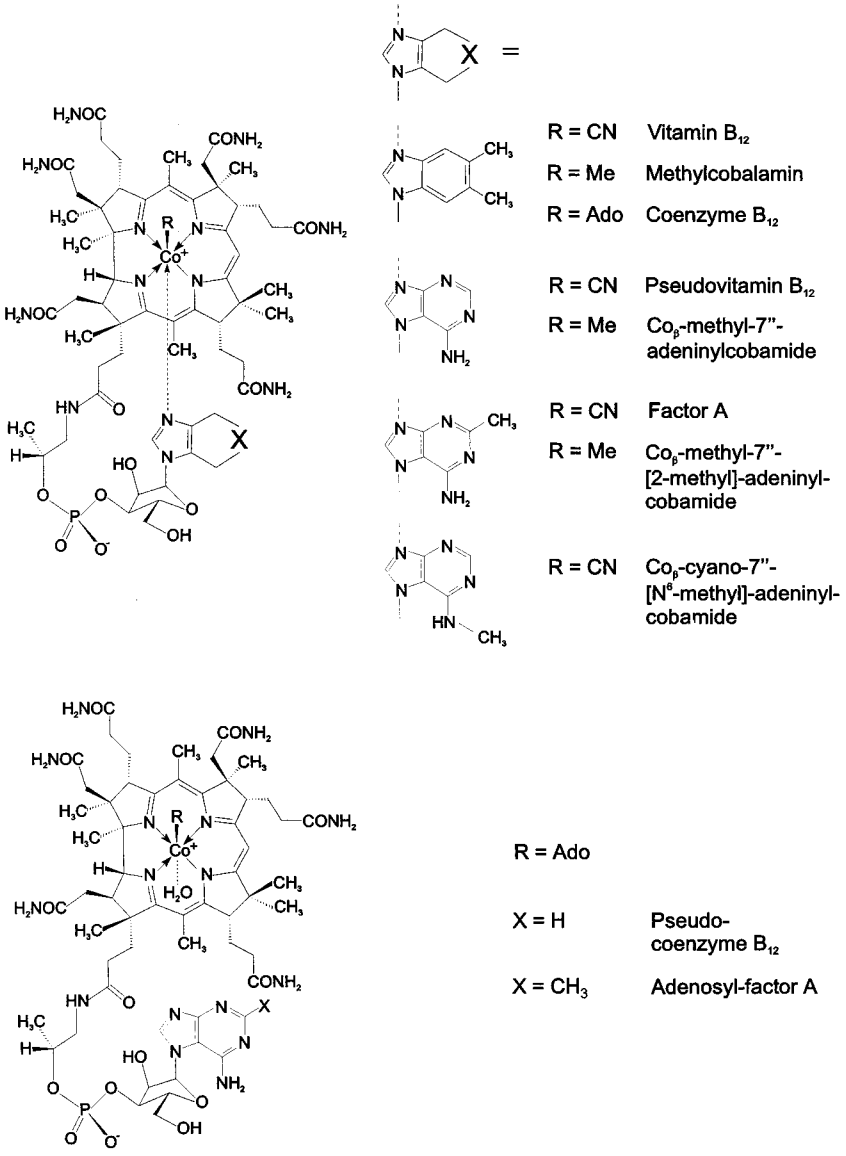


FIG. 1. Structural formulae of vitamin $B_{12}$ derivatives. The general formulae are given on the left; variation of the organic ligand R (Me, methyl; Ado, 5′-deoxy-5′-adenosyl) and/or of the nucleotide base give rise to the different cobamides. (Top) Base-on cobamides vitamin $B_{12}$ (cyanocobalamin), methylcobalamin, coenzyme $B_{12}$ (5′-deoxy-5′-adenosylcobalamin), pseudovitamin $B_{12}$ ($Co_\beta$-cyano-7″-adeninylcobamide), $Co_\beta$-methyl-7″-adeninylcobamide, factor A ($Co_\beta$-cyano-7″-[2-methyl]adeninylcobamide), $Co_\beta$-methyl-7″-[2-methyl]adeninylcobamide, and $Co_\beta$-cyano-7″-[$N^6$-methyl]adeninylcobamide. (Bottom) Base-off cobamides pseudocoenzyme $B_{12}$ (5′-deoxy-5′-adenosyl-7″-adeninylcobamide) and adenosyl factor A (5′-deoxy-5′-adenosyl-7″-[2-methyl]-adeninylcobamide).

ed biosynthesis" of pseudovitamin $B_{12}$ and factor A was explored, e.g., by means of *Escherichia coli*, by adding both cobinamide and adenine or 2-methyladenine to the growth medium (27). A range of related fermentation methods for the production of these purinylcobamides were developed, based on other microorganisms, most notably *Propionibacterium* spp. (13). In line with this, guided biosynthesis has also been employed for the preparation of nonnatural cobamides, such as naphthimidazolyl- and imidazolylcobamides (22, 45, 59, 65), using cultures of *Propionibacterium freudenreichii* and *P. shermanii*.

The nature and the role of the nucleotide base in complete corrinoids has attained renewed interest lately (43): electron spin resonance (ESR) spectroscopic work by Stupperich et al. revealed the coordination of histidine to the protein-bound phenolyl-cobamides in the homoacetogenic *Sporomusa ovata* (67). On the other hand, the bound corrinoid in the corrinoid–iron-sulfur protein involved in the synthesis of acetyl-coenzyme A from *Clostridium thermoaceticum* was indicated by spectroscopic means to be "base-off," i.e., to have the nucleotide base of the corrinoid decoordinated in the protein (57). A pioneering structure analysis by Drennan et al. of the $B_{12}$-binding domain of a methionine synthase from *E. coli* that depends upon methylcobalamin as a cofactor (21, 51) uncovered the "base-off–His-on" form of the protein-bound complete methylcorrinoid, in which a proteinic histidine residue displaces the cobalt-coordinating DMB base of the corrinoid cofactor. The relevance of the base-off–His-on mode of binding of corrinoids in cobamide-dependent enzymes (46, 72) was supported further by two additional crystal structures, of methylmalonyl

coenzyme A mutase (52) and glutamate mutase (58), which both depend upon an adenosylcobamide as a cofactor, such as coenzyme $B_{12}$ (5′-deoxy-5′-adenosylcobalamin). In contrast, the crystal structure of a diol dehydratase showed the bound coenzyme $B_{12}$ in its "classic" base-on constitution (63). Accordingly, questions concerning the effect of the nucleotide base on the binding the $B_{12}$ cofactors by their apoproteins (35, 53, 69) and on the mechanisms of $B_{12}$-dependent enzymatic reactions have received more attention (15, 43).

Ongoing studies in our laboratories concern adenosylcobamide-dependent glutamate mutase from *C. cochlearium* (9, 35), as well as from *C. tetanomorphum* (55, 69), which catalyzes the conversion of L-glutamate to (2*S*,3*S*)-3-methyl aspartate, a particularly intriguing mechanistic puzzle (9, 15). In connection with this work, the crystal structure of inactive recombinant glutamate mutase from *C. cochlearium*, reconstituted with vitamin $B_{12}$ and methylcobalamin, was determined recently (58). Experiments concerning $B_{12}$ binding and enzyme catalysis (6, 10, 17, 37, 54, 72) have been carried out with coenzyme $B_{12}$, although pseudocoenzyme $B_{12}$ is known to be the natural cofactor in *C. tetanomorphum* (2). The identity of the native cofactor(s) of the related *C. cochlearium* was therefore of interest.

We report here the identification of two of the three native corrinoids of *C. cochlearium* in their stable cyano-Co(III) form by means of a comparison of their UV-Vis, circular dichroism (CD), mass, and nuclear-magnetic-resonance (NMR) spectra with the spectra of authentic samples of pseudovitamin $B_{12}$ and factor A. Authentic samples of pseudovitamin $B_{12}$, prepared by guided biosynthesis using *Propionibacterium acidi-propionici*, and of factor A, were both subjected to detailed spectroscopic analysis. In particular, complete NMR signal assignment of both pseudovitamin $B_{12}$ and factor A was carried out to achieve the first thorough spectroscopic analysis of purine analogues of vitamin $B_{12}$. A third corrinoid compound was found, which was tentatively assigned the structure of the novel 7″-[$N^6$-methyl]adeninylcobamide. In this way, the corrinoid sample from *C. cochlearium* was shown to contain three cobamides, pseudovitamin $B_{12}$, factor A, and an unknown isomer of factor A, in a ratio of ca. 3:1:1.

## MATERIALS AND METHODS

**Strains and sources of organisms.** The strains used were *P. acidi-propionici* DSM 20273 and *C. cochlearium* DSM 1285.

**Reagents and solvents.** Crystalline factor A was from the collection of the late W. Friedrich and presumably originated from sewage sludge (P. Renz, personal communication). Cobinamide was prepared by degradation of an aqueous solution of crystalline cyanocobalamin (Hoffmann-LaRoche, Basel, Switzerland) with cerous hydroxide by adaptation of the method described by Renz (59). Separation of cobinamide and α-D-ribazole formed in this reaction was achieved by preparative high-pressure liquid chromatography (HPLC) on an RP-18 column. XAD adsorbent resin (Serdolit AD-4; particle size, 0.1 to 0.2 mm; research grade [Serva, Heidelberg, Germany]) was used. Analytical HPLC was done on an ODS-Hypersil column (5 μm; 250 by 4.6 mm inner diameter, with Phenomenex Security Guard column protection containing two 4-mm C-18 cartridges). The pump used was a Gynkotek model M480G with a vacuum on-line degasser, with a methanol-water (20:80 [vol/vol]) eluent and a flow rate of 1 ml/min. The injection volume was 20 μl, and the detector was a Gynkotek diode array detector UVD340 with detection at 355 nm. All chromatograms were obtained at 18°C, and data were processed by the Gynkotek HPLC data system Gynkosoft 5.50. All other chemicals were of the highest purity and were purchased from Fluka (Neu-Ulm, Germany) Merck (Darmstadt, Germany), or Aldrich (Steinheim, Germany).

**Preparation of pseudovitamin $B_{12}$ by guided biosynthesis.** *P. acidi-propionici* was grown in a 100-liter fermentor which contained 1 kg of casein hydrolysate, 500 g of yeast extract, 170 g of $NaH_2PO_4 \cdot H_2O$, and 180 g of $K_3PO_4 \cdot H_2O$. The solution was sterilized at 120°C for 30 min. The medium was completed by adding autoclaved aqueous solutions of 750 g of glucose (1.5 liters), 60 g of $MgCl_2 \cdot 6H_2O$, 860 mg of $FeCl_2 \cdot 4H_2O$, and 1.7 g of $CoCl_2 \cdot 6H_2O$ (all 50 ml), as well as a sterile filtered solution of 400 mg of Ca-panthotenate, 30 mg of D-biotin, 1 g of cobinamide, and 10 g of adenine. The pH was adjusted to 6 to 7 by the addition of 400-ml batches of a solution containing 80 g of $KHCO_3$ and 20 g of $NaHCO_3$, and the temperature was kept at 37°C. The medium was inoculated with a 2-liter freshly grown culture of *P. acidi-propionici*. To prevent acidification of the batches during cell growth, the medium was further buffered with bicarbonate solution to keep the pH in the range of 6 to 7 and was supplied batchwise with aqueous glucose solution (250 g/500 ml). After 5 days of incubation, approximately 3 kg (wet weight) of cell material was harvested by centrifugation. The cells were resuspended in 5 liters of 0.1 M acetic acid (pH adjusted to 5.0 by NaOH) made 2 mM in cyanide by the addition of KCN and kept in closed half-filled, 1-liter bottles. The suspensions were incubated at 100°C for 20 min. A slightly red-colored cobamide-containing supernatant was obtained after centrifugation. The cell paste was reextracted once as described above. Portions of the pooled supernatants were run over a column of neutral aluminium oxide (3 by 10 cm) and loaded onto an XAD-4 column (3 by 10 cm; XAD grain diameter, 0.1 to 0.2 mm) to adsorb the cobamide. Prior to use, the XAD column was washed with 0.1 M KOH in methanol and then equilibrated with water. The cobamide-loaded column was rinsed with 10 bed volumes of water before eluting the cobamide with 80% methanol. The pooled methanol fractions were flash evaporated to dryness at 40°C. The residue was completely dissolved in approximately 20 ml of water. Further purification was performed by preparative HPLC (65) using an automated gradient controller and an RP-18 column (Nucleosil 120 $C_{18}$; 1 by 25 cm [Marchery & Nagel]). Degassed methanol and 0.1% acetic acid were mixed as solvent components A and B and applied in two different systems at a flow rate of 3 ml/min. Solvent system I (23% A–77% B) led to the elution of pseudovitamin $B_{12}$ after about 15 min; this was observed with a two-wavelength detector operating at 254 and 546 nm. Solvent system II (70% A–30% B, reached by a linear gradient within 10 min) was applied for approximately 30 min in order to elute the matrix. A linear program reversal reestablished the original conditions. The corrinoid-containing HPLC fractions were combined and flash evaporated to dryness. By crystallization from water-acetone, 230 mg (171 μmol) of pure pseudovitamin $B_{12}$ (dried at 2 Pa, 6 h) was obtained. The sample of pseudovitamin $B_{12}$ was subjected to UV-Vis, CD, mass, and NMR spectroscopic analysis as described below.

**Isolation and purification of the corrinoids from *C. cochlearium*.** A culture of *C. cochlearium* was grown anaerobically as reported elsewhere (49). Cells were harvested aerobically and stored at −80°C. The isolation and purification of the corrinoids from *C. cochlearium* in the cyano form were carried out as described above for the isolation of pseudovitamin $B_{12}$. The amounts of solvents used in the purification procedure were adapted to the amount of *C. cochlearium* cell material. From 50 g of frozen cells a sample of approximately 2 mg of crystalline corrinoids was obtained (corresponding to 30 nmol of corrinoids/g [wet weight]). This sample of corrinoids from *C. cochlearium* was subsequently analyzed by HPLC and by UV-Vis, mass, and NMR spectroscopy. HPLC chromatograms of the sample showed three fractions: I (retention time [RT] = 22.96 min, 63% relative integral area [RIA]), II (RT = 24.35 min, 22% RIA), and III (RT = 36.10 min, 15% RIA). All three components had highly similar UV-Vis spectra (200 to 600 nm) which showed no significant difference from those of pseudovitamin $B_{12}$ and of factor A. By peak matching, fraction I was identified with pseudovitamin $B_{12}$ and fraction III was identified with factor A (see Fig. 3).

**Spectroscopic data.** UV-Vis and CD spectra were measured on Hitachi U-3000 and on Jasco-J715 instruments, respectively. Fast-atom-bombardment (FAB) MS spectra were recorded on a Finnigan MAT95 spectrometer (nitrobenzyl alcohol matrix, $Cs^+$ bombardment). NMR spectra were obtained on a Varian 500 Unity Plus spectrometer equipped with field gradient facilities, a 5-mm indirect detection and 5-mm broadband direct detection probe (499.887 MHz, $^1H$; 125.15 MHz, $^{13}C$; and 50.66 MHz, $^{15}N$). NMR solutions had a sample size of 0.7 ml at 26°C. The solvents included 90% 10 mM phosphate buffer (pH 5.2) and 10% $D_2O$. $^1H$, $^{13}C$, and $^{15}N$ signal assignments were from $^1H$ NMR spectra with water presaturation; $^1H$-broad-band-decoupled $^{13}C$ NMR spectra; $^1H$,$^{13}C$ gradient-enhanced heteronuclear single-quantum coherence experiments (PFG-HSQC) (19, 38); $^1H$,$^{15}N$ gradient-enhanced heteronuclear single-quantum coherence experiments (PFG-HSQC) (19, 38), 2D gradient-enhanced heteronuclear multiple-bond coherence experiments (PFG-HMBC) (5, 38), 2D total correlation spectroscopy (watergate-TOCSY) (3, 14, 18, 56), and 2D rotating frame Overhauser enhancement spectroscopy (watergate-ROESY) (4, 11, 56). All 2D NMR experiments were parametrized as described earlier (42). The 2D gradient-enhanced HSQC-TOCSY (PFG-HSQC-TOCSY) experiment (8, 34) was parametrized as described elsewhere (68).

**Pseudovitamin $B_{12}$.** For UV-Vis analyses ($c = 6 \cdot 10^{-4}$ M), with $\lambda_{max}$ expressed in nanometers and where *s* denotes the shoulders, the $\lambda_{max}$ (log ε) values were 276(4.26), 306(3.92), 321(3.89), 343*s*(4.11), 360(4.44), 410(3.53), 479*s*(3.67), 518(3.87), and 548(3.90). For CD analyses ($c = 6 \cdot 10^{-4}$ M), where the wavelengths of the extrema $\lambda_{max}$ and $\lambda_{min}$ and of the zero passages $\lambda_0$ are given in nanometers, the molar decadic CD is indicated by Δε, and *s* denotes the shoulder the $\lambda_{max}/\lambda_{min}(\Delta\varepsilon)$ values were as follows: 578(1.48), 530*s*(−2.29), 488(−6.39), 429(14.13), 362(−11.21), 353(−7.97), 349(−8.38), 333(−4.77), 323(−6.82), 297(−0.35), 284*s*(−1.09), 269(−4.08), 258(−3.23), 247(−6.29), and 235(1.82). The $\lambda_0$ values were 559, 462, 381, 238, and 232. For FAB-MS analyses, the positive-ion spectra, expressed as *m/z* (relative percent intensity) were 1,347.8(8), 1,346.8(29), 1,345.8(76), 1,344.8(100, $MH^+$), 1,343.8(9), 1,342.8(10), 1,320.8(7), 1,319.8(15), 1,318.8(27, $MH^+$-CN), 1,317.8(19),and 1,316.8(23). For NMR analyses ($c$ = 10 mM; see Fig. 4 for a 500-MHz $^1H$ NMR spectrum), a complete

listing of assigned $^1H$, $^{13}C$, and $^{15}N$ signals is given in Tables 1 and 2. For the atom numbering of $B_{12}$ derivatives, see Fig. 2.

**Factor A.** For UV-Vis analyses ($c = 6 \cdot 10^{-4}$ M), the $\lambda_{max}$(log *s*(4.07), 361(4.41), 408(3.50), 480*s*(3.62), 517(3.84), and 548(3.87). For CD analyses ($c = 6 \cdot 10^{-4}$ M), the $\lambda_{max}/\lambda_{min}(\Delta\varepsilon)$ values were 580(0.62), 541(−2.60), 533(−2.44), 492(−5.84), 430(13.18), 387*s*(0.38), 362(−11.66), 352*s*(−7.86), 334(−4.38), 324(−6.78), 313*s*(−4.58), 296(−1.39), 292(−1.43), 284(−0.58), 271(−2.39), 258(−0.96), 247(−3.98), and 235(3.50). The $\lambda_0$ values were 563, 461, 382, 241, and 230. For FAB-MS analyses, the *m/z* (relative percent intensity) values were 1,361.5(8), 1,360.6(25), 1,359.6(77), 1,358.5(100.0, $MH^+$), 1,357.5 (7), 1,333.6(13), 1,332.6(28, $MH^+$-CN), 1,331.6(16), and 1,330.6(16). For NMR analyses ($c = 10$ mM; see Fig. 4 for a 500-MHz $^1H$ NMR spectrum), a complete listing of assigned $^1H$, $^{13}C$, and $^{15}N$ signals is given in Tables 1 and 2. For the atom numbering of $B_{12}$ derivatives, see Fig. 2.

**Sample of cyano corrinoids from *C. cochlearium*.** For HPLC analyses, the three fractions were as follows: I, RT = 23 min (65%); II, RT = 24.4 min (20%); III, RT = 36.1 min (15%). For UV/Vis analyses, the $\lambda_{max}$ values were 276, 305*s*, 321, 360, 410, 517, and 547. For CD analyses, the values were 575, 429, 386*s*, and 236, the $\lambda_{min}$ values were 542, 534, 492, 364, 351, 347, 333, 323, 311*s*, 296, 290, 284, 268, 261, and 248, and the $\lambda_0$ values were 563, 459, 384, 238, and 233. For FAB-MS analyses, 1,361.8(8), 1,360.8(22), 1,359.8(50), 1,358.8(65.5), 1,357.8(13), 1,356.8(15), 1,347.8(9), 1,346.8(30), 1,345.8(70), 1,344.8(100), 1,343.8(11), 1,342.8(14), 1,333.8(10), 1,332.8(16), 1,331.8(15), 1,330.8(19), 1,320.8(6), 1,319.8(14), 1,318.8(23), 1,317.8(19), and 1,316.8(25). For NMR analyses ($c =$ 1.5 mM), see Fig. 4 for a 500-MHz $^1H$ NMR spectrum.

## RESULTS

**Guided biosynthesis of pseudovitamin $B_{12}$.** From about 3 kg of wet cells of a 100-liter *P. acidi-propionici* fermentation, supplemented with approximately 100 mg of adenine/liter of medium, 10 mg of cobinamide/liter, and 17 mg of $CoCl_2 \cdot 6H_2O$/liter, a sample of about 230 mg (171 μmol, 20% yield relative to cobinamide) of bright-red crystalline pseudovitamin $B_{12}$ was obtained and subjected to spectral analysis, as described below.

**Spectroscopic analysis of pseudovitamin $B_{12}$ and of factor A.** Aqueous solutions of pseudovitamin $B_{12}$ and factor A exhibit practically identical UV-Vis and CD spectra. At wavelengths of >300 nm they are similar to the spectrum of vitamin $B_{12}$ (29, 33). The UV-Vis spectra of both purinylcobamides in aqueous solution display typical maxima at 548, 518, and 360 nm (α, β, and γ bands) (33) and at 276 nm (band of the coordinating nucleotide), as described earlier (see, for example, reference 29). The spectra are consistent with the structures of complete base-on corrinoids, with one cyanide ligand bound to the cobalt center.

The FAB mass spectra of the cyano corrins pseudovitamin $B_{12}$ and factor A displayed base peaks at *m/z* = 1,344.8 and 1,358.5, respectively, arising from the intact pseudomolecular ions ($MH^+$). In addition prominent fragments ($MH^+$-CN) due to loss of the cyanide ligand occurred at *m/z* = 1,318.8 for pseudovitamin $B_{12}$ and at *m/z* = 1,332.6 for factor A. The FAB mass spectra confirmed the molecular formulae $C_{59}H_{83}CoN_{17}O_{14}P$ for pseudovitamin $B_{12}$ and $C_{60}H_{85}CoN_{17}O_{14}P$ for factor A.

Very specific information on the structure of pseudovitamin $B_{12}$ and factor A in aqueous solutions was obtained from thorough NMR spectroscopic investigations. Complete assignment of all but three exchange labile hydroxyl and amino protons of the nucleotide moiety and of all carbons was obtained for the spectra of pseudovitamin $B_{12}$ and factor A. Assignment of $^1H$ and $^{13}C$ signals was obtained from heteronuclear ($^1H$,$^{13}C$ PFG-HSQC, $^1H$,$^{13}C$ PFG-HMBC, and $^1H$,$^{13}C$ PFG-HSQC-TOCSY) and homonuclear correlations (watergate-TOCSY and watergate-ROESY). The $^{13}C$ chemical shift of the axially bound cyanide group of pseudovitamin $B_{12}$ could be identified by its broad signal in the 1D proton decoupled $^{13}C$ spectrum. Assignments and chemical shifts of the signals in the $^1H$, $^{13}C$, and $^{15}N$ spectra are listed in Tables 1 and 2; see Fig. 4 for the 500-MHz $^1H$ NMR spectra.

With the exception of resonances originating from the constitutionally different nucleotide bases, the $^1H$ and $^{13}C$ chemical shifts of pseudovitamin $B_{12}$ and factor A differ only slightly. The largest $^1H$ shift difference is observed ($\Delta\delta = 0.08$ ppm) for $H_{re}$(C81); the largest $^{13}C$ shift difference is observed ($\Delta\delta = 1.2$ ppm) for CR3. An unambiguous assignment (pro-R or pro-S) of the signals of all the magnetically nonequivalent methylene hydrogens of pseudovitamin $B_{12}$ and factor A could be derived from analysis of nuclear Overhauser effects (NOEs) involving diastereotopic acetamide and propionamide side chain methylene protons. The $^1H$,$^{15}N$ PFG-HSQC spectra of pseudovitamin $B_{12}$ in combination with watergate-ROESY spectra allowed the assignment of all seven side chain amide nitrogens at natural isotopic ($^{15}N$) abundance, together with their directly bonded amide protons (Table 2). With the exception of the d-side chain, for each amide group the low-field proton signal was assigned to $H_E$ due to the ROESY cross-peaks displayed between the amide protons and side chain methylene protons, since only $H_E$ can be close in space to the α-methylene protons of the carboxamide functions. Such an inverse assignment concerning the d-side chain amide protons has already been reported, e.g., in NMR studies on methylcobalamin (68). This assignment is compatible with a specific shielding effect of the nearby cobalt-coordinated nucleotide base affecting both amide protons and causing highfield shifts of both amide signals. Analysis of the chemical shifts of the $^1H$, $^{15}N$, and $^{13}C$ signals clearly confirmed the constitution and base-on nature of pseudovitamin $B_{12}$ and factor A.

Notable chemical shift differences between the spectrum of vitamin $B_{12}$ on one hand and those of pseudovitamin $B_{12}$ and factor A on the other hand could be observed for signals that were assigned to the constitutionally different nucleotide bases. In the spectra of pseudovitamin $B_{12}$ and factor A, additional $^1H$ upfield or downfield shifts with $|\Delta\delta| > 0.1$ ppm with respect to the signals in the spectrum of vitamin $B_{12}$ are only observed for protons that experience the different anisotropic effects of the cobalt-coordinating nucleotide bases (Table 1): in the spectra of pseudovitamin $B_{12}$ and factor A these are the resonances of H(C3), $H_{re}$(C31), $H_{re}$(C171), $H_{si}$(C172), and H(CR1), and in the spectra of pseudovitamin $B_{12}$ these are the resonances also of H(C51), H(C7A), and $H_{si}$(C81). A comparison of the $^{13}C$ NMR data for pseudovitamin $B_{12}$ and factor A with those for vitamin $B_{12}$ also show similar chemical shifts ($|\Delta\delta| < 2$ ppm) of signals due to the constitutionally corresponding carbons, with the exception of the signal for C1R in the spectrum of pseudovitamin $B_{12}$ and of the signals for C1R and C2R in the spectrum of factor A. The observed NOE intensities were compatible with relevant interproton distances from the crystal structures of vitamin $B_{12}$ and factor A (41, 42). For the spectra of pseudovitamin $B_{12}$ and factor A a strong NOE contact was observed between H(C8N) and the methylene protons H(C131) and the weaker ones of H(C8N) to the methyl groups H(C151) and H(C1A) as well as to the methylene proton $H_{re}$(C172). Furthermore, in the ROESY spectrum of pseudovitamin $B_{12}$, weak contacts between H(C2N) and the methyl groups H(C51) and H(C7A), as well as to the methylene protons $H_{si}$(C31), $H_{re}$(C31), and $H_{re}$(C81), exist. Likewise, the spectrum of factor A exhibits the corresponding contacts of H(C21N) and the methyl groups H(C51) and H(C7A), as well as the methylene protons $H_{si}$(C31), $H_{re}$(C31), and $H_{re}$(C81). All of these NOE contacts indicate the nucleotide base to be cobalt coordinating and suggest a "north-south" orientation with respect to the cobalt-corrin portion, constitutional, and conformational properties of pseudovitamin $B_{12}$ and factor A in aqueous solution, which are also indicated for vitamin $B_{12}$ and factor A by their crystal structures.

TABLE 1. $^{1}H$ and $^{13}C$ NMR chemical shifts and signal assignments for pseudovitamin $B_{12}$, factor A, and vitamin $B_{12}$[a]

| Assignment | Pseudovitamin $B_{12}$[a] | | Factor A[a] | | Vitamin $B_{12}$[b] | |
|---|---|---|---|---|---|---|
| | $\delta(^{1}H)$ (ppm) | $\delta(^{13}C)$[c] (ppm) | $\delta(^{1}H)$ (ppm) | $\delta(^{13}C)$ (ppm) | $\delta(^{1}H)$ (ppm) | $\delta(^{13}C)$ (ppm) |
| β-CN | | 124.2 | | NM | | |
| C1 | | 87.9 | | 88.5 | | 87.9 |
| C1A | 0.33 | 22.2 | 0.34 | 22.8 | 0.34 | 22.2 |
| C2 | | 50.4 | | 50.9 | | 50.1 |
| C21 | 2.27 | 45.9 | 2.27 | 46.2 | 2.29 | 45.6 |
| C22 | | 179.5 | | 180.0 | | 178.6 |
| C2A | 1.29 | 19.8 | 1.29 | 20.0 | 1.29 | 19.6 |
| C3 | 3.94 | 59.5 | 3.96 | 60.2 | 4.06 | 59.2 |
| C31 | 1.86($H_{si}$) | 28.5 | 1.85($H_{si}$) | 29.3 | 1.84 | 28.8 |
| C31 | 1.98($H_{re}$) | 28.5 | 1.95($H_{re}$) | 29.3 | | 28.8 |
| C32 | 2.40 | 38.2 | 2.34($H_{re}$) | 38.7 | 2.39 | 37.8 |
| C32 | 2.40 | 38.2 | 2.42($H_{si}$) | 38.7 | 2.47 | 37.8 |
| C33 | | 182.1 | | 182.1 | | 180.7 |
| C4 | | 183.2 | | 183.7 | | 182.8 |
| C5 | | 111.7 | | 112.4 | | 110.3 |
| C51 | 2.31 | 18.9 | 2.34 | 19.0 | 2.43 | 18.2 |
| C6 | | 167.6 | | 167.9 | | 168.1 |
| C7 | | 53.8 | | 54.6 | | 54.2 |
| C71 | 2.08($H_{re}$) | 46.5 | 2.05($H_{re}$) | 47.1 | 2.09 | 45.9 |
| C71 | 2.44($H_{si}$) | 46.5 | 2.42($H_{si}$) | 47.1 | 2.48 | 45.9 |
| C72 | | 179.4 | | 179.5 | | 177.9 |
| C7A | 1.66 | 22.1 | 1.70 | 22.4 | 1.76 | 21.9 |
| C8 | 3.25 | 59.5 | 3.24 | 59.9 | 3.31 | 58.5 |
| C81 | 0.87($H_{re}$) | 29.1 | 0.95($H_{re}$) | 29.3 | | 28.8 |
| C81 | 1.78($H_{si}$) | 29.1 | 1.85($H_{si}$) | 29.3 | 1.91 | 28.8 |
| C82 | 1.22($H_{re}$) | 35.1 | 1.22($H_{re}$) | 34.9 | 0.91 | 34.6 |
| C82 | 1.72($H_{si}$) | 35.1 | 1.79($H_{si}$) | 34.9 | 1.74 | 34.6 |
| C83 | | 181.9 | | 181.6 | | 180.0 |
| C9 | | 176.9 | | 177.4 | | 176.4 |
| C10 | 5.94 | 97.3 | 5.94 | 97.6 | 5.98 | 97.7 |
| C11 | | 179.3 | | 179.5 | | 179.7 |
| C12 | | 51.0 | | 51.6 | | 50.9 |
| C12A | 1.38 | 22.0 | 1.36 | 22.4 | 1.34 | 22.1 |
| C12B | 1.06 | 34.4 | 1.05 | 34.8 | 1.09 | 34.1 |
| C13 | 3.20 | 57.1 | 3.20 | 57.4 | 3.22 | 56.5 |
| C131 | 1.87 | 31.3 | 1.85 | 31.2 | 1.87 | 30.8 |
| C132 | 2.54 | 37.8 | 2.53 | 38.4 | 2.52 | 37.5 |
| C133 | | 182.1 | | 182.1 | | 181.0 |
| C14 | | 169.6 | | 170.5 | | 168.8 |
| C15 | | 105.9 | | 106.4 | | 106.9 |
| C151 | 2.44 | 18.1 | 2.44 | 18.1 | 2.46 | 18.0 |
| C16 | | 181.8 | | 182.7 | | 181.7 |
| C17 | | 62.4 | | 63.0 | | 62.0 |
| C171 | 1.68($H_{si}$) | 36.0 | 1.69($H_{si}$) | 36.5 | 1.71 | 35.3 |
| C171 | 2.51($H_{re}$) | 36.0 | 2.52($H_{re}$) | 36.5 | 2.40 | 35.3 |
| C172 | 1.92($H_{re}$) | 35.7 | 1.94($H_{re}$) | 35.8 | 2.01 | 34.3 |
| C172 | 2.37($H_{si}$) | 35.7 | 2.40($H_{si}$) | 35.8 | 2.55 | 34.3 |
| C173 | | 178.3 | | 178.9 | | 177.5 |
| C17B | 1.25 | 19.6 | 1.24 | 20.0 | 1.28 | 18.8 |
| C18 | 2.64 | 42.0 | 2.64 | 42.4 | 2.65 | 41.9 |
| C181 | 2.57 | 34.9 | 2.58 | 35.0 | 2.57 | 35.0 |
| C181 | 2.62 | 34.9 | 2.62 | 35.0 | 2.64 | 35.0 |
| C182 | | 179.5 | | 180.1 | | 178.5 |
| C19 | 3.97 | 78.4 | 3.97 | 78.9 | 4.00 | 77.7 |
| C175 | 2.78 | 48.7[1] | 2.77 | 49.0 | 2.85 | 48.3 |
| C175 | 3.48 | 48.7[1] | 3.50 | 49.0 | 3.50 | 48.3 |
| C176 | 4.16 | 76.1[2] | 4.17 | 76.1 | 4.19 | 75.8 |
| C177 | 1.14 | 22.0[3] | 1.14 | 22.3 | 1.15 | 21.8 |
| C1R | 6.40 | 92.2 | 6.37 | 93.0 | 6.25 | 89.8 |
| C2R | 4.14 | 73.3[4] | 4.15 | 74.3 | 4.17 | 71.7 |
| C3R | 4.57 | 75.7[5] | 4.59 | 76.9 | 4.62 | 75.9 |
| C4R | 3.91 | 85.8[6] | 3.92 | 85.9 | 3.93 | 84.9 |
| C5R | 3.62 | 63.3 | 3.63 | 63.5 | 3.64 | 63.3 |
| C5R | 3.81 | 63.3 | 3.81 | 63.5 | 3.82 | 63.3 |
| O2R | 5.61 | | 5.62 | | | |
| C2N | 8.00 | 156.6 | | 166.3 | | 135.8(DMB-C5N) |
| C4N | | 113.1 | | 111.8 | | 139.5(DMB-C9N) |
| C5N | | 160.7 | | 161.5 | | 132.8(DMB-C8N) |
| C6N | | 155.1 | | 155.7 | | 114.3(DMB-C7N) |
| C8N | 7.06 | 147.7 | 7.03 | 147.7 | 7.10 | 144.7(DMB-C2N) |
| C21N | | | 2.26 | 28.4 | 2.16 | 22.8(DMB-C10N) |

[a] $^{1}H$ NMR, δ with $\delta(H_2O)_{int}$ = 4.67 ppm; $^{13}C$ NMR, δ with $\delta(TSP)_{ext}$ = 0.0 ppm. NM, not measured.
[b] All chemical shifts are from Calafat and Marzilli (16). $^{1}H$ chemical shifts are referenced to $H_2O$ by subtracting 0.11 ppm to the reported values (relative to TSP).
[c] $^{31}P$-$^{13}C$ couplings: 1, $J_{CP}$ = 4.8 Hz; 2, $J_{CP}$ = 6.2 Hz; 3, $J_{CP}$ no information because of signal overlap; 4, $J_{CP} < 1$ Hz; 5, $J_{CP}$ = 2.8 Hz; 6, $J_{CP}$ = 7.0 Hz.

TABLE 2. Amide group $^{1}H$ and $^{15}N$ NMR chemical shifts for pseudovitamin $B_{12}$ and factor A

| Assignment | Pseudovitamin $B_{12}$[a] | | Factor A[a] [$\delta(^{1}H)$] (ppm) |
|---|---|---|---|
| | $\delta(^{1}H)$ (ppm) | $\delta(^{15}N)$ (ppm) | |
| N23 | $6.96(H_Z)/7.66(H_E)$ | 110.8 | $6.97(H_Z)/7.67(H_E)$ |
| N34 | $6.79(H_Z)/7.47(H_E)$ | 104.7 | $6.76(H_Z)/7.47(H_E)$ |
| N73 | $6.84(H_Z)/7.27(H_E)$ | 109.5 | $6.89(H_Z)/7.27(H_E)$ |
| N84 | $6.36(H_E)/6.85(H_Z)$ | 102.6 | $6.33(H_E)/6.81(H_Z)$ |
| N134 | $6.87(H_Z)/7.59(H_E)$ | 105.3 | $6.87(H_Z)/7.59(H_E)$ |
| N174 | 8.05 | 113.4 | 8.09 |
| N183 | $6.92(H_Z)/7.78(H_E)$ | 106.3 | $6.92(H_Z)/7.79(H_E)$ |

[a] $^{1}H$ NMR, $\delta$ with $\delta(H_2O)_{int} = 4.67$ ppm; $^{15}N$ NMR, $\delta$ with $\delta[NH_3(l)]_{ext} = 0.0$ ppm.

**Analysis of the corrinoids isolated from *C. cochlearium*.** The sample of crystalline cyano-corrinoids obtained from *C. cochlearium* was analyzed with respect to its composition by HPLC analysis and by UV-Vis, CD, mass, and NMR spectroscopy. These spectra could be examined by comparison with the spectra recorded for pseudovitamin $B_{12}$ and factor A, two well-characterized, authentic reference compounds.

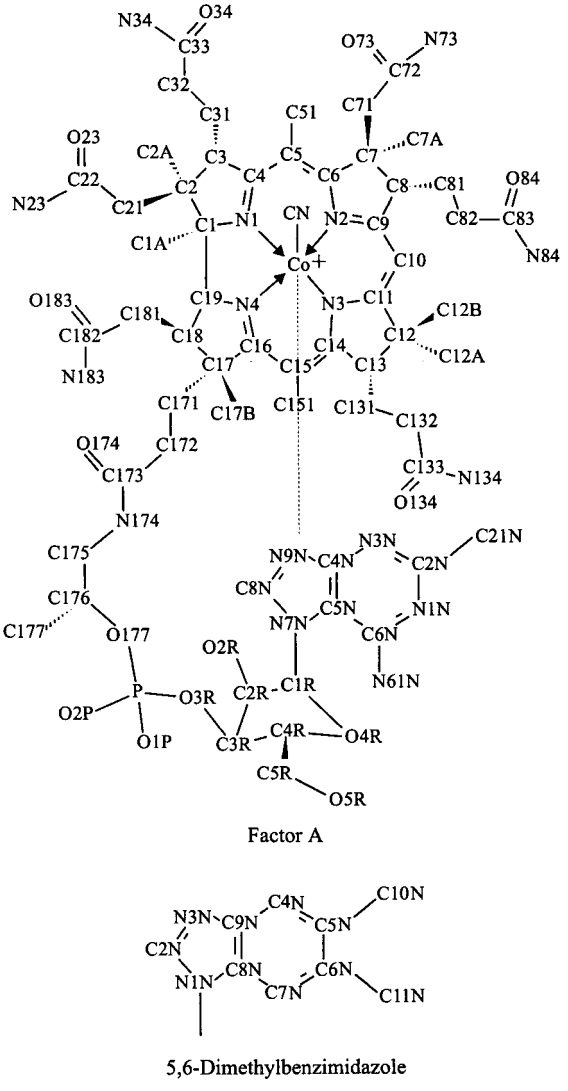


FIG. 2. Atom numbering of pseudovitamin $B_{12}$ (and factor A) used for the description of NMR results (44). The atom numbering for the DMB of vitamin $B_{12}$ is also shown.

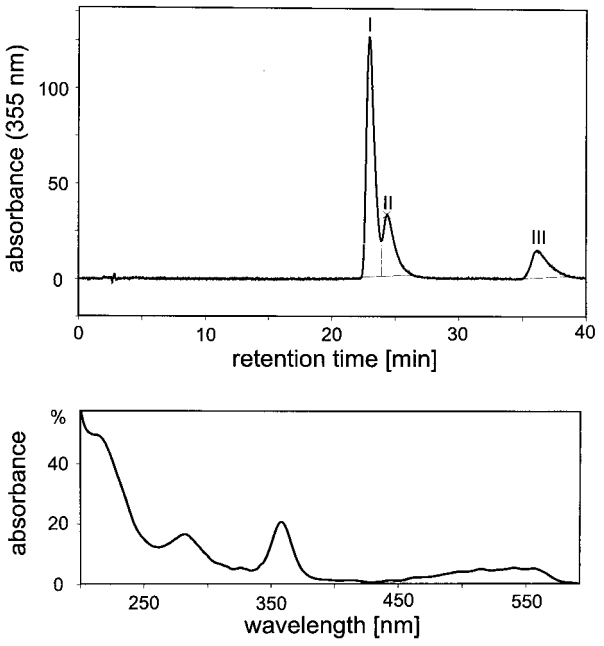


FIG. 3. (Top) HPLC chromatogram of the corrinoid sample isolated from *C. cochlearium*. (Bottom) UV-Vis spectra of the new corrinoid (fraction II of the HPLC chromatogram) (see Materials and Methods for technical details).

By a combination of careful HPLC and UV-Vis spectroscopic analysis, the sample was found to contain three corrinoid components, all having UV-Vis spectral features of $Co_\beta$-cyano-7″-adeninylcobamides (29, 66). (Fig. 3). The most polar, major fraction (RT = 23 min) and the least polar fraction (RT = 36.1 min) had retention times and UV-Vis spectra identical to those of pseudovitamin $B_{12}$ and factor A, respectively. The fraction of intermediate polarity (RT = 23.4 min) had similar UV-Vis absorbance characteristics. The mass spectrum of the corrinoid sample from *C. cochlearium* was consistent with the superposition of the mass spectra of pseudovitamin $B_{12}$ and factor A. The spectrum of the sample from *C. cochlearium* exhibits a base peak at $m/z$ = 1,344.8 and the signal at $m/z$ = 1,358.8 with a relative intensity of 65%. These signals are consistent with the pseudomolecular ions ($MH^+$) of pseudovitamin $B_{12}$ and factor A (or an isomer, thereof), respectively. In addition, signals at $m/z$ = 1,318.8 and $m/z$ = 1,332.8, due to the corresponding fragment ions ($MH^+$-CN), have the same relative intensity ratio. Comparison of the signal pattern in the FAB mass spectrum with the HPLC trace suggests the sample from *C. cochlearium* to contain pseudovitamin $B_{12}$ (representing ca. 60%), factor A, and an isomer of factor A (in total, ca. 40% of the sample).

The 1D $^{1}H$ NMR spectrum of the corrinoid sample from *C. cochlearium* (Fig. 4) also can be deconvoluted mostly as a superposition of spectra of pseudovitamin $B_{12}$ and of factor A. Table 1 and Fig. 4 show that most of the $^{1}H$ (and $^{13}C$) NMR resonances of pseudovitamin $B_{12}$ and factor A superimpose completely or in part. Only some signals experience observable shift differences of >0.01 ppm, due to the constitutionally differing nucleotide bases, and are consequently suitable for securing the existence of both corrinoids in aqueous solution. These signals are the intense methyl singlets of H(C51), H(C7A), H(C12A), and H(C1A) and the resonances of H(C8N),

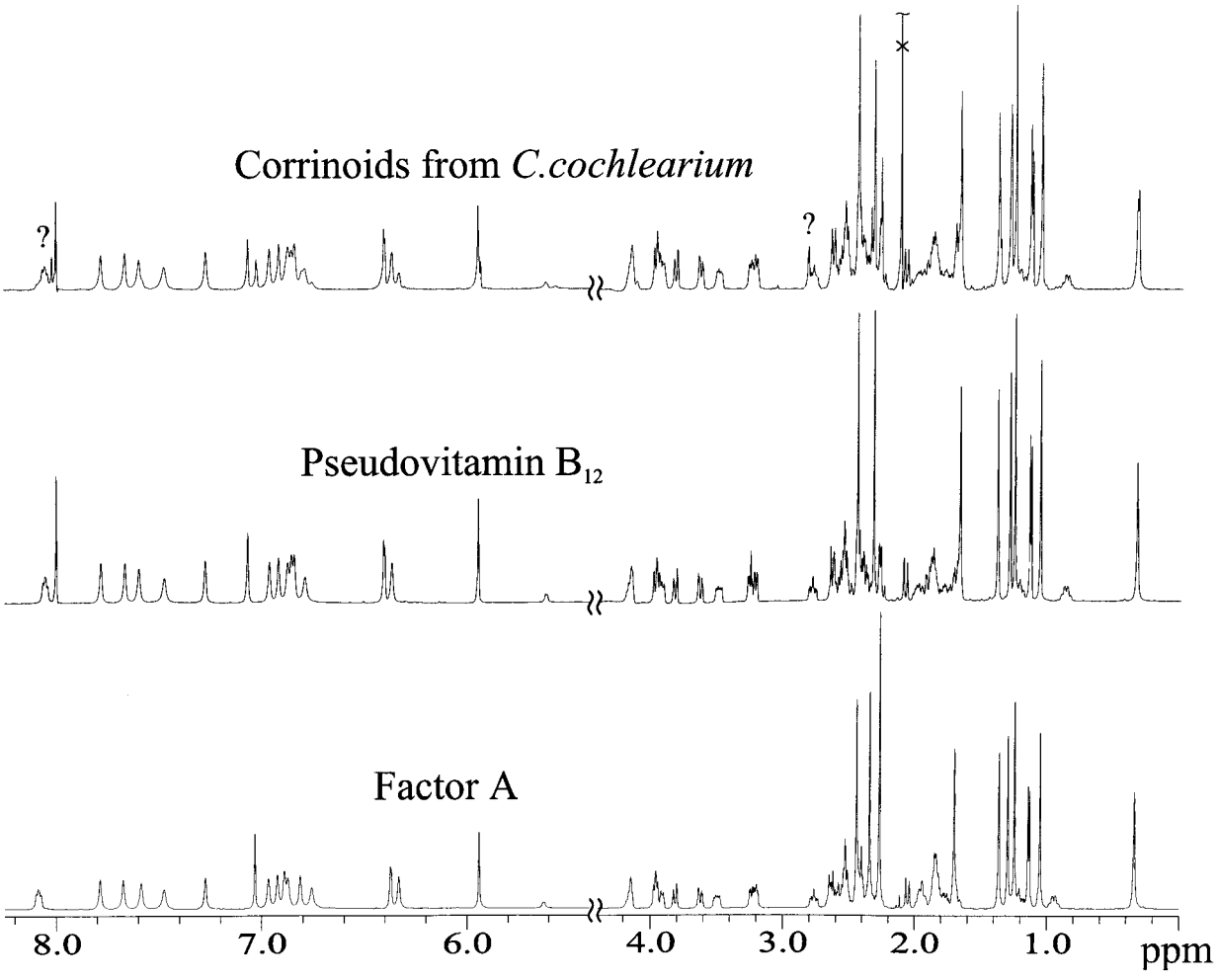


FIG. 4. The 500-MHz $^1$H NMR spectra of aqueous solutions of pseudovitamin $B_{12}$, factor A, and the corrinoid sample isolated from *C. cochlearium* are shown. Two signals, tentatively assigned to 7″-[$N^6$-methyl]adeninylcobamide, are labeled with a question mark.

H(CR1), $H_Z$(N34), $H_E$(N84), $H_Z$(N84), and H(N174) in the low field region. For all of these signals a doubling is observed, a finding consistent with an intensity ratio of approximately 3:2. In addition, the three protons of the methyl group C21N of the 2-methyladenine moiety of factor A have no counterpart in pseudovitamin $B_{12}$, and their signal, a singlet at 2.26 ppm, is a feature of the spectrum of factor A. In the $^1$H NMR spectrum of the sample from *C. cochlearium* the corresponding singlet at 2.26 ppm is clearly identifiable. Due to the low total corrinoid concentration (ca. 1.5 mM) of the sample and lower signal resolution, the 2D $^1$H,$^{13}$C-PFG-HSQC spectrum and the $^1$H,$^{13}$C-PFG-HMBC spectrum of the corrinoid sample of *C. cochlearium* are less informative, but they are also consistent with the presence of pseudovitamin $B_{12}$ and of factor A. A cross-peak with the $^1$H and $^{13}$C coordinates (2.26 and 28.4 ppm [HSQC]) and (2.26 and 166.3 ppm [HMBC]), respectively, can specifically be attributed to the methyl group $H_3$(C21N) of the 2-methyladenine base of factor A. Two further singlets in the $^1$H NMR spectrum at chemical shifts of 8.02 and 2.81 ppm could not be explained by the spectrum of either factor A or pseudovitamin $B_{12}$. Based on their chemical shifts, they were tentatively assigned to H(C2N) of a purinylcobamide and to a methyl group bound to an exocyclic amino function. These tentative assignments of the signals in the $^1$H NMR spectrum were supported by corresponding information from $^1$H,$^{13}$C-PFG-HSQC and $^1$H,$^{13}$C-PFG-HMBC spectra: the signal of 8.02 ppm [assigned to H(C2N)] in the proton dimension correlated with a $^{13}$C signal of 161.0 ppm (HBMC, assigned to C6N), and the one at 2.81 ppm corresponded with a signal at 31.5 ppm (HSQC, of $H_3$C-N61N). In addition, the signal at 2.81 ppm in the $^1$H NMR spectrum produced an NOE to the one at 6.40 ppm, assigned to H(CR1). The data are consistent with the structure of an $N^6$-methyladenine moiety bound via N7N to the ribose unit of the nucleotide function.

## DISCUSSION

Authentic samples of pseudovitamin $B_{12}$ (29), prepared by guided biosynthesis as described here, and of factor A were subjected to an extensive spectroscopic analysis. The most detailed spectroscopic and structural information was obtained from 1D and 2D homo- and heteronuclear NMR spectra in aqueous solution (40). From experiments with $B_{12}$ derivatives (in aqueous medium with a mean deuterium content of 10% or less) signals for nearly all $^1$H, $^{13}$C, and $^{15}$N atoms were detected and could be assigned (a complete listing of assigned $^1$H, $^{13}$C, and $^{15}$N signals is given in Tables 1 and 2; see also Fig. 4 for 500 MHz $^1$H NMR spectra of pseudovitamin $B_{12}$ and

factor A). The close correspondence of the chemical shifts of most $^{1}H$, $^{15}N$, and $^{13}C$ NMR signals in the spectra of the purinylcobamides pseudovitamin $B_{12}$ and factor A on the one hand and of the DMB-cobamide vitamin $B_{12}$ on the other (16) supports a constitutionally and configurationally identical buildup of their cyano-Co(III)-corrin segments. The spectra also indicate attachment at the ribose moiety of the purine base of the cobalt coordinating nucleotide via its N7 in the unique α-configuration. Notable chemical shift differences can be observed only for signals that have been assigned either to the constitutionally different nucleotide bases or to protons that experience the different anisotropic effects of the cobalt-coordinating nucleotide bases. Detailed information on the conformational properties of pseudovitamin $B_{12}$ and factor A in aqueous solution was obtained from the ROESY data. By comparing the observed NOE intensities with the interproton distances from the crystal structures of vitamin $B_{12}$ (45) and of factor A (41), the base-on nature of pseudovitamin $B_{12}$ and factor A in aqueous solution and at pH 5.2 was confirmed, as was the north-south orientation of the cobalt-coordinating purine bases in pseudovitamin $B_{12}$ and factor A.

The corrinoid sample from *C. cochlearium* thus represents a cocrystallisate of pseudovitamin $B_{12}$, factor A, and a third cyanocobamide. The latter is indicated by the mass spectrum to have the same molecular mass as factor A and is tentatively assigned the structure of a $Co_{\beta}$-cyano-7″-[$N^6$-methyl]adeninylcobamide, a previously unknown purinylcobamide. In view of the earlier isolation of pseudovitamin $B_{12}$ and pseudocoenzyme $B_{12}$ from *C. tetanomorphum* (2), the isolation of purinylcobamides from the related *C. cochlearium* is not unexpected. The corrinoids were isolated in their cyano-Co(III) forms, which are unlikely to have a direct cofactor function (23, 43). The presence of purinylcobamides in *C. cochlearium* (and the absence of benzimidazolylcobamides, when grown without the supplement of the corresponding base) can be explained primarily by the difficulty (or even incapacity) of this anaerobe to biosynthesize benzimidazoles. Such a situation is not untypical of anaerobes (see, for example, references 39, 60, and 66). The appearance, besides pseudovitamin $B_{12}$, of its homologue factor A (and of 7″-[$N^6$-methyl]adeninylcobamide), is in line with frequent observations on the coexistence of purinylcobamides in anaerobes (61). While the direct sources of the adeninyl bases are not established, their origin from degradation of oligo(deoxy)nucleotides has been suggested tentatively (28, 61). The appearance of 7″-[$N^6$-methyl]adeninylcobamides would lend further support to this suggestion, since derivatives of $N^6$-methyladenine are ubiquitous natural products of oligonucleotides (50). Clearly, additional methyl groups increase the hydrophobicity of the nucleotide appendage. A related situation is encountered with the naturally occurring benzimidazolylcobamides, which may also differ by the degree of their biosynthetic methylation at the nucleoside base (60).

The determination of two of the cobamides in *C. cochlearium* as the 7″-purinylcobamides pseudovitamin $B_{12}$ and factor A points to the functional relevance in this anaerobe of the corresponding coenzyme forms, pseudocoenzyme $B_{12}$ ($Co_{\beta}$-5′-deoxy-5′-adenosyl-7″-adeninylcobamide) and adenosyl factor A ($Co_{\beta}$-5′-deoxy-5′-adenosyl-7″-[2-methyl]adeninylcobamide). These two adenosylcobamides are known to exist in their base-off constitution mainly in neutral aqueous solution, in contrast to coenzyme $B_{12}$ (47; W. Fieber, B. Hoffmann, H. Bothe, W. Buckel, R. Konrat, and B. Kräutler, unpublished data). It is also likely that the corresponding methyl-Co(III)-corrinoids ($Co_{\beta}$-methyl-7″- adeninylcobamide and $Co_{\beta}$-methyl-7″-[2-methyl]adeninylcobamide) have a functional role in biosynthetic methyl group transfer reactions (e.g., in some methanogens, such as in a variety of *Methanococcales* spp. [66]).

The different constitution of the nucleotide bases of coenzyme $B_{12}$ and of the purinylcobamides could be considered to be of functional relevance in view of the recently established base-off–His-on mode of binding of the corrinoid cofactor to some adenosyl-dependent mutases (55, 58) (see above). The predominance of the base-off form of the purinylcobamides might therefore be considered to be a means in support of a proper preorganization of these "complete" corrinoids and to assist their incorporation in the base-off–His-on mode into the apo form of glutamate mutase. To test this notion, preliminary comparisons were carried out with the adenosylcobamides coenzyme $B_{12}$, pseudocoenzymec $B_{12}$, and adenosyl factor A as cofactors of glutamate mutase from *C. cochlearium*. In contrast to the simple expectations, Michaelis-Menten studies of glutamate mutase revealed coenzyme $B_{12}$ to bind better than its base-off analogues pseudocoenzyme $B_{12}$ and adenosyl factor A and the catalytic activity of the mutase to be similar, irrespective of the adenosyl-corrinoid cofactor used (Fieber et al., unpublished). Accordingly, a direct catalytic advantage for the adenosylcobamide-dependent glutamate mutase from *C. cochlearium* by the use of the purinylcobamides pseudocoenzyme $B_{12}$ and adenosyl-factor A instead of the benzimidazolycobamide coenzyme $B_{12}$ can be excluded.

The purinylcobamides carry a nucleotide base that is able to undergo H bonding with H donors and acceptors and that can become protonated (47), even when coordinated to the corrin-bound cobalt center. This situation is drastically different from that in the benzimidazolylcobamides, where H bonding and protonation of the nucleotide base cannot directly occur in the cobalt-coordinated state. A mechanism for the control of the organometallic reactivity of protein-bound corrinoid cofactors involves weakening of the cobalt coordination of the axial transligand, conveyed (in part) by H bonding it to other protein residues in "regulatory" diads or triads (21, 43, 51, 52). Only in their base-off–His-on form can bound benzimidazolylcobamides be set to participate directly in such H-bonded regulatory units via a coordinated histidine. A possible functional advantage of the presence of a purinyl base in complete corrinoid cofactors in their H-bonding base-on constitution may therefore not be dismissed. Indeed, diol-dehydratase and cobamide-dependent ribonucleotide reductase are two enzymes that depend upon adenosylcobamides and which have been discovered lately to carry their corrinoid cofactors in the base-on form (1, 48, 63).

While two of the native corrinoids of the obligate anaerobes *C. tetanomorphum* and *C. cochlearium* are widely occurring purinylcobamides, *C. tetanomorphum* has been shown to produce benzimidazolylcobamides when supplied with benzimidazoles (70), and coenzyme $B_{12}$ can act as functional corrinoid cofactor of glutamate mutase in both of these clostridia (9, 37, 60). The incapacity of *C. tetanomorphum* and *C. cochlearium* to biosynthesize benzimidazoles parallels the situation encountered in several other anaerobes (29, 39, 66). The native cobamides from *C. cochlearium* are indicative of the biosynthesis of three relevant adeninylcobamides by this anaerobe. Two of these homologous corrinoids (pseudovitamin $B_{12}$ and factor A) are known from other anaerobes also (61). It will be of interest to definitively clarify the structure of the third cobamide of *C. cochlearium* and to examine its occurrence in other anaerobic microorganisms.

## ACKNOWLEDGMENTS

We thank Wolfgang Fieber and Wolfgang Schmidt for communicating their results on organometallic $B_{12}$ derivatives and Iris Schall for

her technical assistance. We are grateful to K. H. Ongania for measuring FAB mass spectra. We thank Paul Renz for providing us with authentic factor A and Hoffmann-LaRoche for a generous gift of vitamin $B_{12}$.

This study was supported by the European Commission (TMR project FMRX.CT96.0018), by the Austrian National Science Foundation (FWF, projects P-13595 and P-12639), by the German Science Foundation (Deutsche Forschungsgemeinschaft), and by the Fonds der Chemischen Industrie.

## REFERENCES

1. **Abend, A., R. Nitsche, V. Banderian, E. Stupperich, and J. Rétey.** 1998. Dioldehydratase binds coenzyme $B_{12}$ in the "base-on" mode: ESR investigations on cob(II)alamin. Angew. Chem. Int. Ed. Engl. **37:**625–627.
2. **Barker, H. A., H. Weissbach, and R. D. Smith.** 1958. A coenzyme containing pseudovitamin $B_{12}$. Proc. Natl. Acad. Sci. USA **44:**1093–1097.
3. **Bax, A., and D. G. Davies.** 1985. Practical aspects of two-dimensional transverse NOE spectroscopy. J. Magn. Reson. **63:**207–213.
4. **Bax, A., and D. G. Davies.** 1985. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. **65:**355–360.
5. **Bax, A., and M. F. Summers.** 1986. $^{1}H$ and $^{13}C$ assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. **108:**2093–2094.
6. **Beatrix, B., O. Zelder, F. Kroll, G. Örlygsson, B. T. Golding, and W. Buckel.** 1995. Evidence for a mechanism involving transient fragmentation in coenzyme-$B_{12}$-dependent carbon skeleton rearrangements. Angew. Chem. Int. Ed. Engl. **34:**2398–2401.
7. **Bigger, G. W., J. M. Elliot, and T. R. Rickard.** 1976. Estimated ruminal production of pseudovitamin $B_{12}$, factor A and factor B in sheep. J. Anim. Sci. **43:**1077–1081.
8. **Bodenhausen, G., and D. Ruben.** 1980. Natural abundance nitrogen-15 NMR by enhanced spectroscopy. Chem. Phys. Lett. **69:**185–189.
9. **Bothe, H., G. Bröker, U. Müller, I. Schall, S. Textor, B. T. Golding, and W. Buckel.** 1998. Mechanisms of coenzyme $B_{12}$-dependent carbon-carbon and carbon-oxygen rearrangements, p. 253–264. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
10. **Bothe, H., D. J. Darley, S. P. Albracht, G. J. Gerfen, B. T. Golding, and W. Buckel.** 1998. Identification of the 4-glutamyl radical as an intermediate in the carbon skeleton rearrangement catalyzed by coenzyme $B_{12}$-dependent glutamate mutase from *Clostridium cochlearium*. Biochemistry **37:**4105–4113.
11. **Bothner-By, A. A., R. L. Stephens, J. Lee, C. D. Warren, and R. W. Jeanloz.** 1984. Structure determination of a tetrasaccharide: transient nuclear Overhauser effects in the rotating frame. J. Am. Chem. Soc. **106:**811–813.
12. **Brandt, L. J., L. H. Bernstein, and A. Wagle.** 1977. Production of vitamin $B_{12}$ analogues in patients with small-bowel overgrowth. Ann. Intern. Med. **87:** 546–551.
13. **Brandt, L. J., L. Goldberg, L. H. Bernstein, and G. Greenberg.** 1979. The effect of bacterially produced vitamin $B_{12}$ analogues (cobamides) on the in vitro absorption of cyanocobalamin. Am. J. Clin. Nutr. **32:**1832–1836.
14. **Braunschweiler, L., and R. R. Ernst.** 1983. Coherence transfer by isotropic mixing: application to proton correlation spectroscopy. J. Magn. Reson. **53:**521–528.
15. **Buckel, W., and B. T. Golding.** 1996. Glutamate and 2-methyleneglutarate mutase: from microbial curiosities to paradigms for coenzyme $B_{12}$-dependent enzymes. Chem. Rev. Soc. **25:**329–337.
16. **Calafat, A. M., and L. G. Marzilli.** 1993. Investigation of $B_{12}$ derivatives with inorganic ligands using 2D NMR spectroscopy. J. Am. Chem. Soc. **115:**9182–9190.
17. **Chen, H. P., and E. N. G. Marsh.** 1997. How enzymes control the reactivity of adenosylcobalamin: effect on coenzyme binding and catalysis of mutations in the conserved histidine-aspartate pair of glutamate mutase. Biochemistry **36:**7884–788932.
18. **Davis, D. G., and A. Bax.** 1985. Assignment of complex $^{1}H$ NMR spectra via 2D-homonuclear Hartmann-Hahn spectroscopy. J. Am. Chem. Soc. **107:** 2820–2821.
19. **Davis, A. L., J. Keeler, E. D. Laue, and D. Moskau.** 1992. Experiments for recording pure-absorption heteronuclear correlation spectra using pulsed field gradients. J. Magn. Reson. **98:**207–216.
20. **Dion, H. W., D. G. Calkins, and J. J. Pfiffner.** 1952. Hydrolysis products of pseudovitamin $B_{12}$. J. Am. Chem. Soc. **74:**1108.
21. **Drennan, C. L., S. Huang, J. T. Drummond, R. G. Matthews, and M. L. Ludwig.** 1994. How a protein binds $B_{12}$: a 3.0 Å X-ray structure of $B_{12}$-binding domains of methionine synthase. Science **266:**1669–1674.
22. **Eberhard, G., H. Schlayer, H. Joseph, E. Fridrich, B. Utz, and O. Müller.** 1988. Untersuchungen zur biologischen Funktion der Nukleotidbase von Vitamin $B_{12}$. Biol. Chem. Hoppe-Seyler **369:**1091–1098.
23. **Ellenbogen, L., and B. A. Cooper.** 1991. Vitamin $B_{12}$, p. 491–536. *In* L. Lawrence and J. Machlin (ed.), Handbook of vitamins. Marcel Dekker, Inc., New York, N.Y.
24. **Eschenmoser, A.** 1988. Vitamin $B_{12}$: Experimente zur Frage nach dem Ursprung seiner molekularen Struktur. Angew. Chem. Int. Ed. Engl. **27:**5–40.
25. **Ford, J. E., and J. W. G. Porter.** 1952. Some properties of vitamin $B_{12}$-like factors from calf faeces. 1. Characteristics of different fractions. Biochem. J. **51:**v–vi.
26. **Ford, J. E., E. S. Holdsworth, S. K. Kon, and J. W. G. Porter.** 1953. Occurrence of the various vitamin $B_{12}$ active compounds. Nature **171:**150.
27. **Ford, J. E., E. S. Holdsworth, and S. K. Kon.** 1955. The biosynthesis of vitamin $B_{12}$-like compounds. Biochem. J. **59:**86–93.
28. **Friedmann, H. C., and L. M. Cagen.** 1970. Microbial biosynthesis of $B_{12}$-like compounds. Annu. Rev. Microbiol. **24:**159–208.
29. **Friedrich, W.** 1975. Vitamin $B_{12}$ und verwandte Corrinoide, p. 173. *In* R. Ammon and W. Dirscherl (ed.), Fermente, Hormone, und Vitamine, vol. III/2. Georg Thieme Verlag, Stuttgart, Germany.
30. **Friedrich, W., and K. Bernhauer.** 1956. Beiträge zur Chemie und Biochemie der "Cobalamine." II. Mitteilung: Über den Abbau der "Cobalamine" mit CerIII-hydroxid. 7α-[D-Ribofuranosido]-adenin, ein Abbauprodukt des Pseudovitamin $B_{12}$. Chem. Ber. **89:**2507–2512.
31. **Friedrich, W., and K. Bernhauer.** 1957. Über den Abbau des Faktors A mit CerIII-hydroxyd. Chem. Ber. **90:**465–470.
32. **Friedrich, W., H. Dellweg, G. Gross, E. Becher, S. Spaude, and K. Bernhauer.** 1957. Vitamin $B_{12}$-ähnliche Faktoren des Faulschlamms, p. 62–72. *In* H. C. Heinrich (ed.), Vitamin $B_{12}$ and intrinsic factor. Enke, Stuttgart, Germany.
33. **Giannotti, C.** 1982. Electronic spectra of $B_{12}$ and related systems, p. 393. *In* D. Dolphin (ed.), $B_{12}$, vol. 1. John Wiley & Sons, New York, N.Y.
34. **Gronenborn, A. M., A. Bax, P. T. Wingfield, and G. M. Clore.** 1989. A powerful method of sequential proton resonance assignment in proteins using relayed $^{15}N$-$^{1}H$ multiple quantum coherence spectroscopy. FEBS Lett. **243:**93–98.
35. **Hoffmann, B., R. Konrat, H. Bothe, W. Buckel, and B. Kräutler.** 1999. Structure and dynamics of the $B_{12}$-binding subunit of glutamate mutase from *Clostridium cochlearium.* Eur. J. Biochem **263:**178–188.
36. **Hollenstein, R., and E. Stupperich.** 1993. Assignment of $^{15}N$-NMR resonances of vitamin $B_{12}$ analogues by 2D-[$^{15}N$,$^{1}H$]long range correlation: fully [$^{15}N$]-labelled co-β-cyano-5-hydroxybenzimidazolylcobamide (factor III) and derivatives. Helv. Chim. Acta **76:**1258–1265.
37. **Holloway, D. E., and E. N. G. Marsh.** 1994. Adenosylcobalamin-dependent glutamate mutase from *Clostridium tetanomorphum*: over-expression in *E. coli*, purification and characterization of the recombinant enzyme. J. Biol. Chem. **269:**20425–20430.
38. **Hurd, R. E., and B. K. John.** 1991. Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy. J. Magn. Reson. **91:**648–653.
39. **Keck, B., and P. Renz.** 2000. *Salmonella typhimurium* forms adenylcobamide and 2-methyladenylcobamide, but no detectable cobalamin during strictly anaerobic growth. Arch. Microbiol. **173:**76–77.
40. **Konrat, R., M. Tollinger, and B. Kräutler.** 1998. New NMR structural and dynamical probes of organometallic $B_{12}$ derivatives, p. 349–368. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
41. **Kopf, J., K. von Deuten, R. Bieganowski, and W. Friedrich.** 1981. X-ray crystal structure analysis of factor A (2-methyladeninyl-cyanocobamide), a native vitamin $B_{12}$-analogue. Z. Naturforsch. **36c:**506–515.
42. **Kratky, C., G. Färber, K. Gruber, K. Wilson, Z. Dauter, H.-F. Nolting, R. Konrat, and B. Kräutler.** 1995. Accurate structural data demystify $B_{12}$: high-resolution solid-state structure of aquocobalamin perchlorate and structure analysis of the aquocobalamin ion in solution. J. Am. Chem. Soc. **117:**4654–4670.
43. **Kräutler, B.** 1998. $B_{12}$-coenzymes, the central theme, p. 3–43. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
44. **Kräutler, B.** 1998. $B_{12}$-nomenclature and a suggested atom-numbering, p. 517–522. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
45. **Kräutler, B., R. Konrat, E. Stupperich, G. Färber, K. Gruber, and C. Kratky.** 1994. Direct evidence for the conformational deformation of the corrin ring by the nucleotide base in vitamin $B_{12}$: synthesis and solution spectroscopic and crystal structure of $Co_{\beta}$-cyanoimidazolylcobamide. Inorganic Chem. **33:** 4128–4139.
46. **Kräutler, B., and C. Kratky.** 1996. Vitamin $B_{12}$: the haze clears. Angew. Chem. **108:**179–181; Angew. Chem. Int. Ed. Engl. **35:**168–170.
47. **Ladd, J. N., H. P. C. Hogenkamp, and H. A. Barker.** 1961. Structure of cobamide coenzymes: influence of pH on absorption spectra and ionophoretic mobilities. J. Biol. Chem. **236:**2114–2118.
48. **Lawrence, C. C., G. J. Gerfen, V. Samano, R. Nitsche, M. J. Robins, J. Rétey, and J.-A. Stubbe.** 1999. Binding of cob(II)alamin to the adenosylcobalamin-dependent ribonucleotide reductase from *Lactobacillus leichmanii*. J. Biol. Chem. **274:**7039–7042.
49. **Leutbecher, U., R. Böcher, D. Linder, and W. Buckel.** 1992. Glutamate mutase from *Clostridium cochlearium*. Purification, cobamide content and stereospecific inhibitors. Eur. J. Biochem. **205:**759–765.

50. **Limbach, P. A., P. F. Crain, and J. A. McCloskey.** 1994. The modified nucleosides of RNA. Nucleic Acids Res. **22:**2183–2196.
51. **Ludwig, M. L., and R. G. Matthews.** 1997. Structure-based perspectives on $B_{12}$-dependent enzymes. Annu. Rev. Biochem. **66:**269–313.
52. **Mancia, F., N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bösecke, O. Diat, and P. R. Evans.** 1996. How coenzyme $B_{12}$ radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. Structure **4:**339–350.
53. **Marsh, E. N. G., and D. E. Holloway.** 1992. Cloning and sequencing of glutamate mutase component S from *Clostridium tetanomorphum*. FEBS Lett. **310:**167–170.
54. **Marsh, E. N. G., and D. P. Ballou.** 1998. Coupling of cobalt-carbon bond homolysis and hydrogen atom abstraction in adenosylcobalamin-dependent glutamate mutase. Biochemistry **37:**11864–11872.
55. **Marsh, E. N. G., D. E. Holloway, and H. P. Chen.** 1998. Glutamate mutase, p. 253–264. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
56. **Piotto, M., V. Sautek, and V. Sklenar.** 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solution. J. Biomol. NMR **2:**661–665.
57. **Ragsdale, S. W., P. A. Lindahl, and E. Münck.** 1987. Mössbauer, EPR and optical studies of the corrinoid/iron-sulfur protein involved in the synthesis of acetyl coenzyme A by *Clostridium thermoaceticum*. J. Biol. Chem. **262:** 14289–14297.
58. **Reitzer, R., K. Gruber, G. Jogl, U. G. Wagner, H. Bothe, W. Buckel, and C. Kratky.** 1999. Glutamate mutase from *Clostridium cochlearium*: the structure of a coenzyme $B_{12}$-dependent enzyme provides new mechanistic insights. Structure **7:**891–902.
59. **Renz, P.** 1972. Some intermediates in the biosynthesis of vitamin $B_{12}$. Methods Enzymol. **18:**85.
60. **Renz, P.** 1998. Investigations of the biosynthesis of the 5,6-dimethylbenzimidazole moiety of vitamin $B_{12}$, p. 119–130. *In* B. Kräutler, B. T. Golding, and D. Arigoni (ed.), Vitamin $B_{12}$ and $B_{12}$-proteins. Verlag Wiley-VCH, Weinheim, Germany.
61. **Renz, P.** 1999. Biosynthesis of the 5,6-dimethylbenzimidazole moiety of cobalamins and of other bases found in natural corrinoids, p. 557–575. *In* R. Banerjee (ed.), Chemistry and Biochemistry of $B_{12}$. John Wiley & Sons, New York, N.Y.
62. **Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers.** 1948. Crystalline vitamin $B_{12}$. Science **107:**396–397.
63. **Shibata, N., J. Masuda, T. Tobimatsu, T. Toraya, K. Suto, Y. Morimoto, and N. Yasuoka.** 1999. A new mode of $B_{12}$ binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. Structure **7:**997–1008.
64. **Smith, E. L., and L. F. J. Parker.** 1948. Purification of anti-pernicious anaemia factor, Biochem. J. **43:**VIII.
65. **Stupperich, E., I. Steiner, and M. Rühlemann.** 1986. Isolation and analysis of bacterial cobamides by high-performance liquid chromatography. Anal. Biochem. **155:**365–370.
66. **Stupperich, E., and B. Kräutler.** 1988. Pseudovitamin $B_{12}$ or 5-hydroxybenzimidazolyl-cobamide are the corrinoids found in methanogenic bacteria. Arch. Microbiol. **149:**268–271.
67. **Stupperich, E., H.-J. Eisinger, and S. Schurr.** 1990. Corrinoids in anaerobic bacteria. FEMS Microbiol. Rev. **87:**355–360.
68. **Tollinger, M., T. Dérer, R. Konrat, and B. Kräutler.** 1997. An efficient method for the preparation of methylcobalamin, nature's organometallic methyl transfer catalyst. J. Mol. Catal. A **116:**147–155.
69. **Tollinger, M., R. Konrat, B. H. Hilbert, E. N. G. Marsh, and B. Kräutler.** 1998. How a protein prepares for $B_{12}$ binding: structure and dynamics of the $B_{12}$-binding subunit of glutamate mutase from *Clostridium tetanomorphum*. Structure **6:**1021–1033.
70. **Weissbach, H., J. Toohey, and H. A. Barker.** 1959. Isolation and properties of $B_{12}$-coenzymes containing benzimidazole or dimethyl-benzimidazole. Proc. Natl. Acad. Sci. USA **45:**521–525.
71. **Zelder, O., B. Beatrix, U. Leutbecher, and W. Buckel.** 1994. Characterization of the coenzyme-$B_{12}$-dependent glutamate mutase from *Clostridium cochlearium* produced in *Escherichia coli*. Eur. J. Biochem. **226:**577–585.
72. **Zelder, O., B. Beatrix, F. Kroll, and W. Buckel.** 1995. Coordination of a histidine residue of the protein component S to the cobalt/Atom in coenzyme $B_{12}$-dependent glutamate mutase from *Clostridium cochlearium*. FEBS Lett. **369:**252–254.