Studies on the Biosynthesis of Corrinoids and Porphyrinoids. I. The Labeling of Oxygen of Vitamin B_{12}

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Recently the amide-oxygen has been suggested to participate in the formation of the corrin ring of vitamin B_{12}. To confirm this hypothesis, ^{17}O-labeled aminolevulinic acid (ALA) was prepared and administered to *Propionibacterium shermanii*. The isolated vitamin B_{12} showed only broad ^{17}O signals in the oxygen-17 nuclear magnetic resonance (^{17}O-NMR) spectrum. However, distinct isotope-shifted peaks were observed in the ^{13}C-NMR spectrum of vitamin B_{12} isolated after incorporation of [1-^{13}C:1,4-$^{18}O_2$]ALA. Of these shifted peaks, one peak (C_{27}) showed very low intensity. This indicates that dilution of ^{18}O occurred at the acetyl chain of the A ring of vitamin B_{12}. This result supports the assumption that the lactone formation of the A ring promotes the ring contraction, as proposed by Eschenmoser.

Keywords vitamin B_{12}; ^{17}O-NMR; ^{17}O-labeled ALA; isotope shift; ^{18}O-labeling; lactone intermediate; A-ring

Introduction

The biosynthetic pathway of vitamin B_{12}, the anti-pernicious anemia vitamin, has been intensively studied. Recently, a review was published.[1] Knowledge of the formation of the carbon backbone is almost complete. First, two molecules of 5-amino-levulinic acid (ALA) condense to form porphobilinogen (PBG), which is converted to uroporphyrinogen III. From this intermediate, chlorophyll-a and protoheme are derived. Uroporphyrinogen III is methylated, ring-contracted and decarboxylated to form cobyrinic acid, which is the late corrinoid precursor of vitamin B_{12}. Most of these sequences are completely established, but the mechanism of ring contraction remains unresolved. It has been proved that the C_{20} carbon is excluded as acetic acid in the pathway from uroporphyrinogen III to cobyrinic acid.[2] We have demonstrated that the corrin protons at the 3, 8, 13, 18, and 19 positions of vitamin B_{12} are derived from the water in the culture, which suggests the formation of the carbanion at C_{19}, generated by the expulsion of acetic acid.[3] Recently Eschenmoser suggested a lactam intermediate model at the A ring (**1**) in the process of ringcontraction from porphyrin into corrin.[4] Uzar and Battersby also proposed the lactone intermediate (**2**) at the D ring in that process (Fig. 1).[5] These proposals are based on model studies of ringcontraction.[6] Thus, the flanking carboxyl oxygen should play an important role in the mechanism of ringcontraction. If so, the labeling of oxygen of the precursor might provide useful information concerning these hypothetical intermediates. So we planned to incorporate the ^{17}O-labeled ALA into vitamin B_{12}.

Results and Discussion

We prepared ^{17}O-labeled ALA by heating ALA with $H_2{}^{17}O$ in a sealed tube, modifying the method of Emery and Akhtar,[7] and fed it to *Propionibacterium shermanii*. Figure 2 shows the oxygen-17 nuclear magnetic resonance (^{17}O-NMR) spectrum of [1,1,4-$^{17}O_3$]ALA (**3**). From the signal intensities, the carboxyl group is more labeled with ^{17}O than the carbonyl group. Figure 3 shows the ^{17}O-NMR spectrum of vitamin B_{12} (**4**), obtained after administration of **3** to *P. shermanii*. Though they are overlapping, obvious ^{17}O peaks were seen at 200—340 ppm. Because of the signal overlaps, the intensity of each peak could not be resolved. It is evident that the carboxyl oxygens of ALA are incorporated into the amide oxygens of vitamin B_{12}.

To examine the oxygens of vitamin B_{12} in more detail, we planned to use ^{18}O, ^{13}C double labeling. We prepared [1-^{13}C]ALA (**5**) from sodium [1-^{13}C]acetate,[8] and heated it in $H_2{}^{18}O$ in a sealed tube to obtain [1-^{13}C,1,1,4-^{18}O]ALA (**6**) in the same way as for **3** (Fig. 4). The ratio of ^{16}O:^{18}O in the carboxyl oxygen was 3:7 from the peak intensity. ^{18}O,^{13}C-Doubly labeled ALA thus obtained was incorporated into *P. shermanii*, then vitamin B_{12} labeled with ^{13}C and ^{18}O was isolated. At the same time, for comparison, feeding

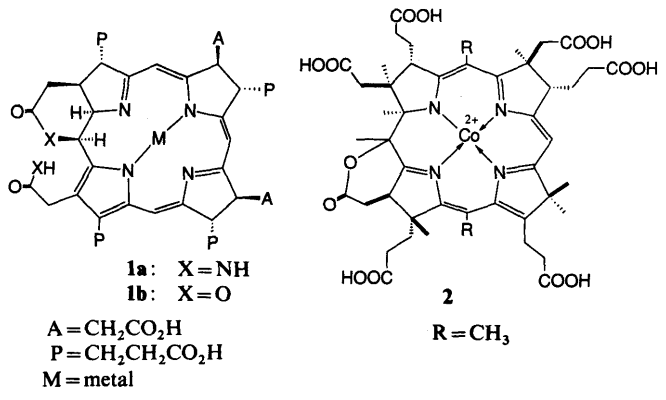

Fig. 1. The Hypothetical Intermediates in the Process of Ring Contraction: **1** (Eschenmoser[4]) and **2**: (Battersby[5])

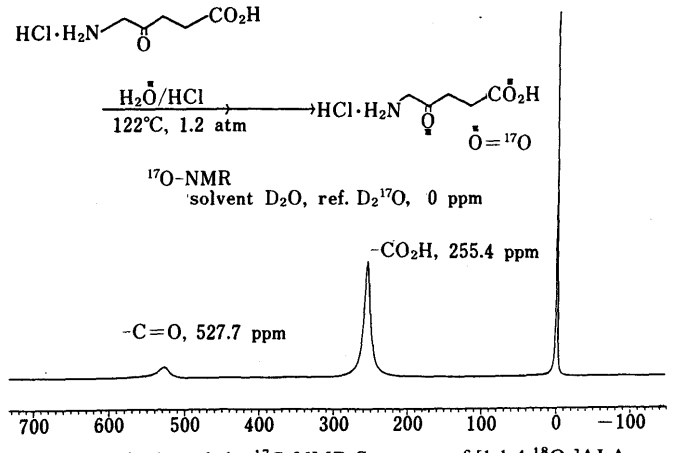

Fig. 2. Synthesis and the ^{17}O-NMR Spectrum of [1,1,4-$^{18}O_3$]ALA

experiment of [1-^{13}C]ALA was also performed, and the vitamin B_{12} (**7**) labeled with ^{13}C at amide carbons was obtained.

Figure 5A shows the ^{13}C-NMR spectrum of the isolated vitamin B_{12} (**7**), after incorporation of [1-^{13}C]ALA (**5**). Seven intense signals were observed in the downfield region. They correspond to ^{13}C-enriched amide carbon signals of the side chains of the corrin ring.[9] Figure 5B shows the ^{13}C-NMR spectrum of the vitamin B_{12} (**8**) incorporated with [1-^{13}C:1,4-$^{18}O_2$]ALA (**6**). In contrast with Fig. 5A, all signals of **8** are split. Most of the signals are more intense at the upper-field signal, which corresponds to the isotope-shifted peak of ^{13}C-enriched amide carbon, bearing ^{18}O. The lower-field signals are those of the ^{13}C-labeled amide carbons of **7**. The isotope shifts are between 2.3—3.8 Hz.

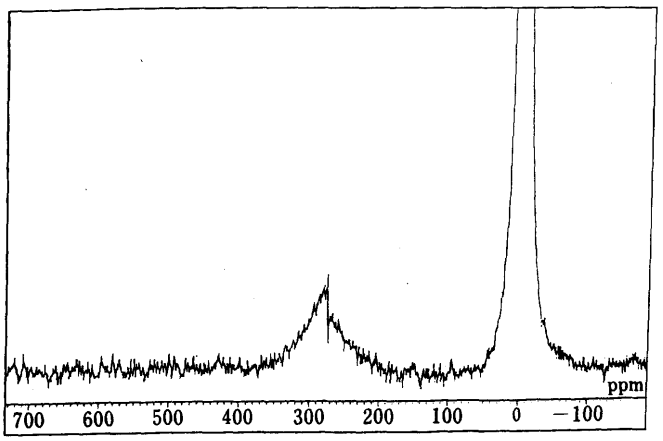

Fig. 3. ^{17}O-NMR Spectrum of [1,1,4-$^{17}O_3$]ALA-Incorporated Vitamin B_{12}

From the peak intensity ratio (2.6:7.4—2.2:7.8), the carboxyl groups were transformed to amides without oxygen exchange with water. Interestingly, in one pair (signal d) the original signal is stronger than the upper-field signal. These are the signals of the acetamide carbon of ring A of vitamin B_{12}. The fact that the intensity of the isotope shifted peak is very weak at this carbon indicates that dilution of ^{18}O occurred at this carbon. We assume the mechanism of this dilution to be as follows (Fig. 6). It is well known that uroporphyrinogen III is methylated to form factor III.[10] We assume that factor III is methylated to form a lactone (**9**). Methylation at C-1 is believed to occur after de-

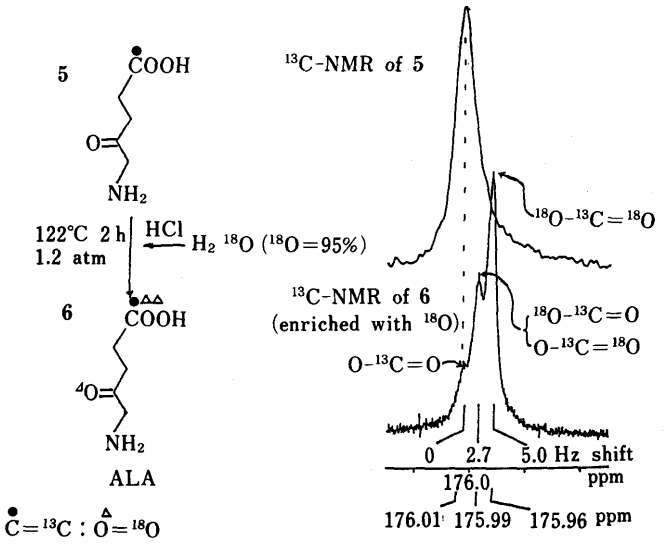

Fig. 4. Synthesis and the ^{13}C-NMR Spectrum of [1-^{13}C: 1,1,4-$^{18}O_3$]ALA

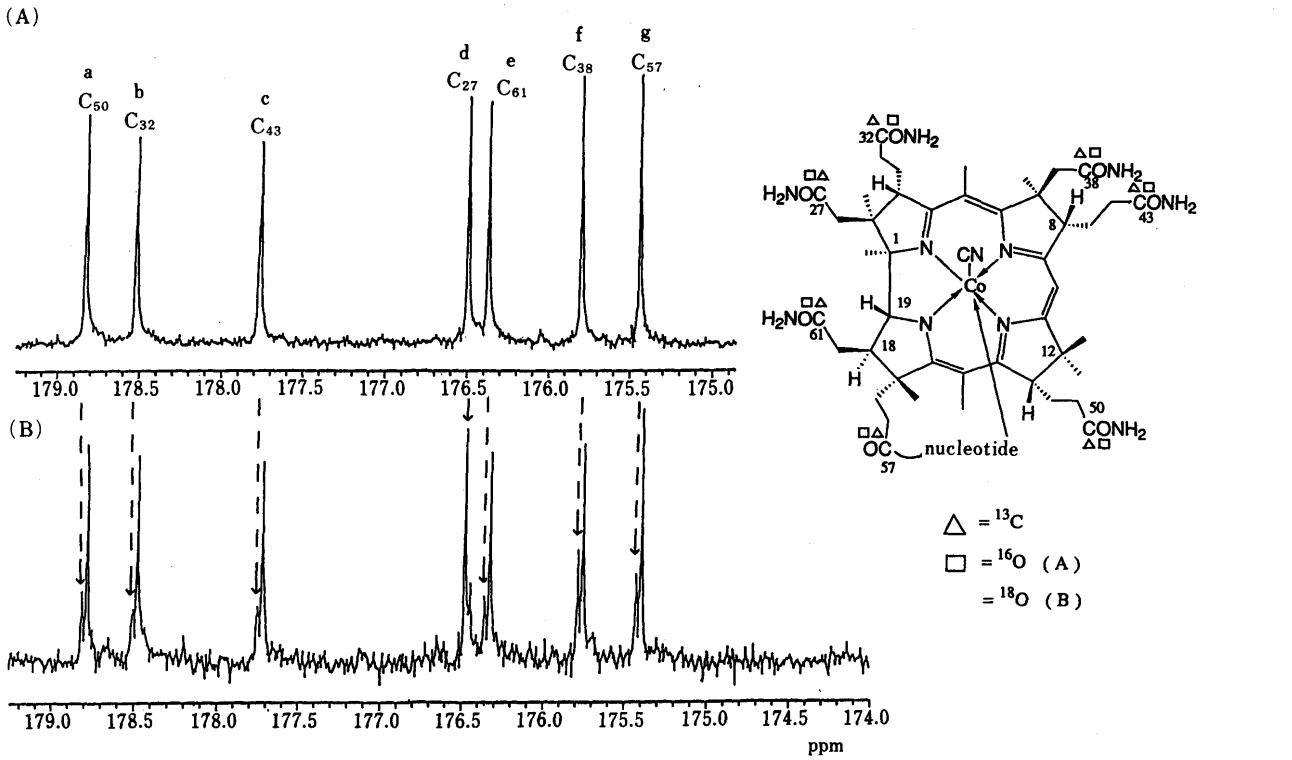

Fig. 5. Comparison of the ^{13}C-NMR Spectrum of [1-^{13}C]ALA-Incorporated Vitamin B_{12} (A) and That of [1-^{13}C:1,1,4-$^{18}O_3$]ALA-Incorporated Vitamin B_{12} (B)

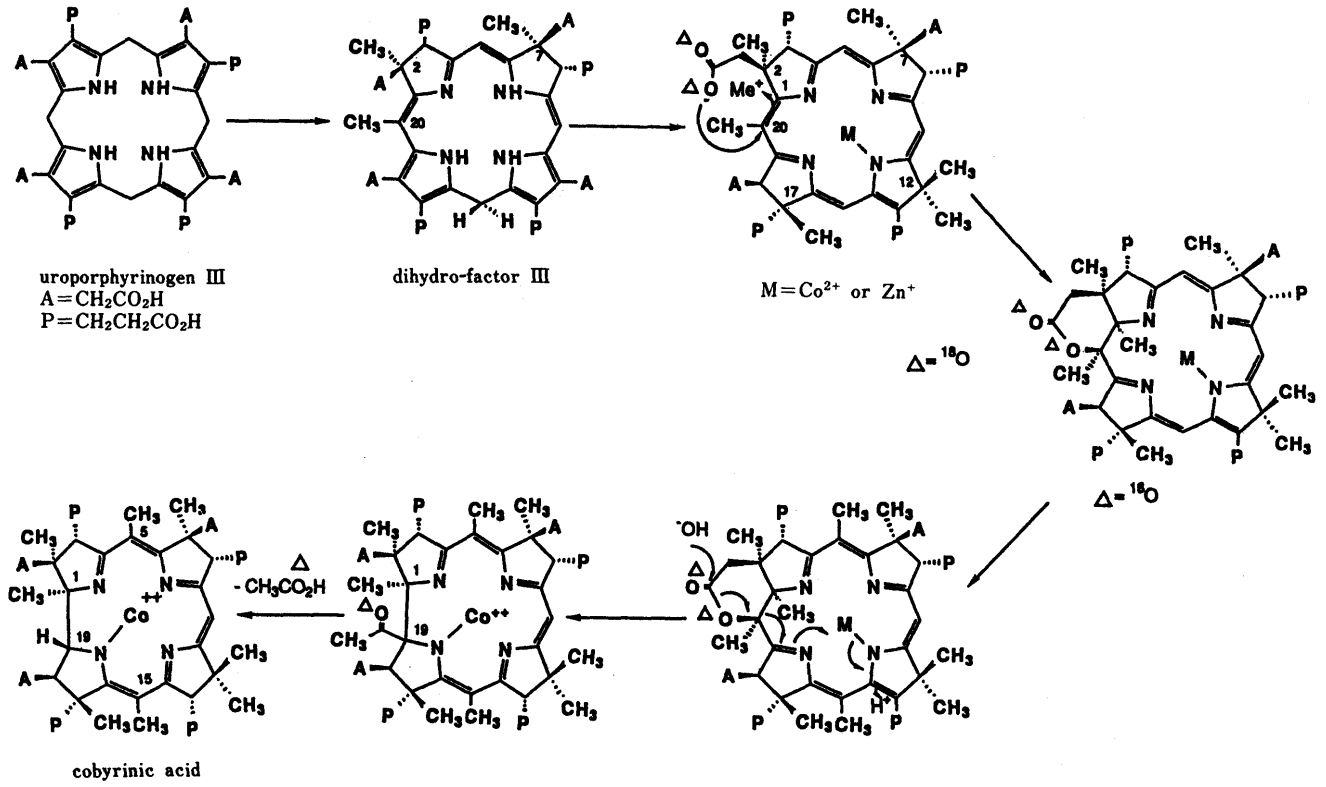

Fig. 6. Hypothetical Scheme for the Dilution of ^{18}O of Amide at C_{27}

carboxylation and methylation at C_{12}.[11] The resulting cation at C_{20} will be attacked by the neighboring carboxylic oxygen of A-ring. This will form a lactone as illustrated, which will be then hydrolyzed. At least half of the ^{18}O will be lost at this stage. If this lactone formation from A ring and hydrolysis actually occur, the isotope shifted peak of the amide carbon (C_{18}) would decrease considerably in intensity. Eschenmoser proposed that the methyl analogue of the lactone intermediate underwent ringcontraction *via* the dilactone (or lactam).[4] Our result does not conflict with his hypothetical monolactone intermediate, nor with the dilactone one. Recently porphobilinogen deaminase bearing dipyrromethane as a co-factor was isolated.[12] If in this enzyme or in subsequent enzymes (cosynthetase, methylase, decarboxylase), binding took place at the acetamide chain, the exchange of oxygen might occur at those stages. This possibility can be excluded only when all the enzymes have been isolated and the binding sites identified. However, this explanation cannot be applied to the case of PBG deaminase. Our study of the labeling of oxygen of vitamin B_{12} supports the possibility of the existence of an A-ring lactone intermediate. The isolation of various key intermediates of biosynthesis labeled with oxygen isotope should be possible, and should enable us to elucidate the mechanism of ringcontraction of vitamin B_{12}.

Experimental

Infrared spectra (IR) were recorded on a Jasco DS-701G spectrometer. ^1H-NMR spectra were taken on a Hitachi R24B instrument (60 MHz), and ^{13}C- and ^{17}O-NMR spectra were taken on a JEOL GSX-400 spectrometer (100, 54 MHz). Chemical shifts are given downfield from DOH (=4.70 ppm) in the case of ^1H-NMR, from tetramethylsilane (TMS) or dioxane (=67.40 ppm) as an internal standard in the case of ^{13}C-NMR, and from D_2O (=0 ppm) as an internal standard in the case of ^{17}O-NMR. Fast atom bombardment mass spectra (FAB-MS) were recorded on a JEOL DX-302 spectrometer equipped with a JMA DA-5000 data system. Ultraviolet (UV) spectra were recorded on a Jasco UVIDEC-610C spectrometer.

Medium for Administration of [1,1,4-$^{17}O_3$]ALA (3), [1-^{13}C]ALA (5) and [1-^{13}C,1,1,4-$^{18}O_3$]ALA (6) to *P. shermanii* The medium consisted of $CoCl_2 \cdot 6H_2O$ (1 mg), labeled ALA (15 mg), methionine (30 mg), 50% glucose (3.2 ml), dimethylbenzimidazole (25 mg), 1/15 M phosphate buffer (K_2HPO_4–KH_2PO_4, pH=7.60) (100 ml).

[1,1,4-$^{17}O_3$]ALA·HCl (3) ALA·HCl (110 mg, 0.65 mmol) was dissolved in 200 μl of [^{17}O]water (50% atm ^{17}O) and 6 μl of 0.33 M hydrochloric acid. The mixture was transferred into a glass tube, which was frozen, and sealed in a vacuum. The glass tube was autoclaved (122 °C, 1.2 atm) for 2 h. The reaction mixture was taken out and concentrated. The residue was transferred to another glass tube and the same procedure was repeated to give **5** as yellow crystals (110.3 mg, 99.5%). ^1H-NMR (60 MHz, D_2O) δ: 2.82 (4H, m, $-COCH_2CH_2COO$), 4.13 (2H, s, $-NCH_2CO$). ^{17}O-NMR (54 MHz, D_2O) δ: 255.4 ($-CO_2H$), 527.7 (CH_2COCH_2). IR (KBr) cm^{-1}: 3420 (m, N–H), 1735 (sh, C=O), 1725, 1710, 1690 (s, C=O). FAB-MS *m/z*: 134 ($M^+ + 1 - HCl$).

Incorporation of [1,1,4-$^{17}O_3$]ALA·HCl (3) into Vitamin B_{12} (4) *P. shermanii* ATCC 9614 was cultivated in 12 l of casein medium for 7 d under nitrogen, with adjustment of the pH to 7.0 every day. The cells were harvested, and washed, then 90 mg of [1,1,4-$^{17}O_3$]ALA·HCl (3) was added and the cells were incubated for 3 d. The weight of wet cells was 163 g. After the general procedure, 1.6 mg of **6** was obtained. UV λ_{max} nm: 550.0, 358.4. ^{17}O-NMR (54 MHz, D_2O) δ: 200—340 ($C=\underline{O}^{17}NH$).

[1-^{13}C,1,1,4-$^{18}O_3$]Aminolevulinic Acid Hydrochloride (ALA·HCl) (6) [1-^{13}C]ALA·HCl[4] (5) (110 mg, 0.65 mmol) was dissolved in 200 μl of [^{18}O] water (95% atm ^{18}O) and 6 μl of 0.33 M hydrochloric acid. The mixture was transferred into a glass tube, which was frozen, and sealed in a vacuum. The tube was autoclaved (122 °C, 1.2 atm) for 2 h, then the reaction mixture was taken out and concentrated. The residue was transferred to another glass tube, and the same procedure was repeated to give **6** as yellow crystals (102 mg, 89.6%). ^1H-NMR (60 MHz, D_2O) δ: 2.75 (4H, m, $-COCH_2CH_2COO$), 4.09 (2H, s, $-NCH_2CO$). ^{13}C-NMR (100 MHz, CD_3OD, TMS) δ: 176.01 ($^{16}O-^{13}C=^{16}O$), 175.99 (2.7 Hz shift, $^{18}O-^{13}C=^{16}O$, $^{16}O-^{13}C=^{18}O$), 175.96 (5.0 Hz shift, $^{18}O^{13}C=^{18}O$). IR (KBr) cm^{-1}: 3420 (m, N–H), 1735 (sh, C=O), 1725, 1680, 1650 (s, C=O). FAB-MS *m/z*: 137 ($M^+ + 1 - HCl$).

Incorporation of [1-^{13}C]ALA (5) into Vitamin B_{12} (7) *P. shermanii* was

incubated for 7 d in 9 l of casein I-B medium under a nitrogen atmosphere, with adjustment of the pH to 7.0 every day, and then collected by centrifugation at 8000 cpm. The cells were washed with brine, and divided into 6 batches, each of which was placed in a 500 ml sterilized flask containing the above feeding medium. They were incubated at room temperature for 3 d under the same conditions. The cells were gathered, washed with brine, and disrupted twice with an ultrasonicator (NIC US-300) at 0 °C for 15 min in 400 ml of 80% methanol solution, containing 0.1% potassium cyanide. The suspension was centrifuged at 8000 cpm at 4 °C for 30 min. The weight of wet cells was 98 g. The supernatant was evaporated to dryness. The residue was extracted with 500 ml of methanol, and the extract was evaporated, diluted with 40 ml of water, and extracted with 1 : 1 phenol–chloroform (40 ml × 2). The extract was washed with water (50 ml × 3), diluted with 1000 ml of ether, and re-extracted with water (50 ml × 3). The extract was washed with 50 ml of chloroform, then with 50 ml of ether. It was evaporated, and purified by column chromatography (SiO_2, methanol) three times (30 g, 2.8 i.d. × 20 cm; 3.0 g, 0.8 i.d. × 9 cm twice). The red fraction (Rf=0.2) was collected and evaporated. The residue was recrystallized from water–acetone (1 : 8) to give 1.2 mg of **3** as needle crystals. UV λ_{max} nm: 550.0, 358.4. ^{13}C-NMR (100 MHz, D_2O, dioxane) δ: 178.8 (C_{50}), 178.5 (C_{32}), 177.8 (C_{43}), 176.5 (C_{27}), 176.4 (C_{61}), 175.8 (C_{38}), 175.5 (C_{57}).

Incorporation of [1-^{13}C,1,1,4-$^{18}O_3$]ALA (6) into Vitamin B_{12} (8) *P. shermanii* ATCC 9614 was cultivated in 12 l of casein medium for 7 d under nitrogen, with adjustment of the pH to 7.0 every day. The cells were harvested, and washed, then 90 mg of [1-^{13}C,1,1,4-$^{18}O_3$]ALA·HCl (**6**) was added and the cells were incubated for 3 d. The weight of wet cells was 136 g. After the same procedure as described above, 1.4 mg of **8** was obtained. UV λ_{max} nm: 550.0, 358.4. ^{13}C-NMR (100 MHz, D_2O, dioxane) δ: 178.82 ($C_{50}=^{16}O$), 178.79 ($C_{50}=^{18}O$, 3.1 Hz shift), 178.50 ($C_{32}=^{16}O$), 178.48 ($C_{32}=^{18}O$, 2.3 Hz shift), 177.75 ($C_{43}=^{16}O$), 177.72 ($C_{43}=^{18}O$, 3.1 Hz shift), 176.48 ($C_{27}=^{16}O$), 176.45 ($C_{27}=^{18}O$, 3.1 Hz shift), 176.37 ($C_{61}=^{16}O$), 176.33 ($C_{61}=^{18}O$, 3.8 Hz shift), 175.79 ($C_{38}=^{16}O$), 175.76 ($C_{38}=^{18}O$, 3.1 Hz shift), 175.44 ($C_{57}=^{16}O$), 175.41 ($C_{57}=^{18}O$, 3.1 Hz shift).

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