Biosynthesis of Corrinoids and Porphyrinoids. IX.[1] Studies on the Origin of Oxygen of Uroporphyrinogen III in *Arthrobacter hyalinus*

Masahiro KAJIWARA,* Ken-ichiro HARA, and Kazuhiko TAKATORI

Department of Medicinal Chemistry, Meiji College of Pharmacy, 1–22–1, Yato-cho, Tanashi, Tokyo 188, Japan.
Received October 4, 1993; accepted December 25, 1993

The biosynthetic origin of carbonyl oxygen of uroporphyrinogen III was investigated. A model experiment using methyl benzoate labeled with ^{13}C and ^{18}O revealed isotope shifts of 0.015 and 0.036 ppm, corresponding to alkoxy-^{18}O- and carbonyl-^{18}O-labeled carbonyl carbons, in the ^{13}C-NMR spectrum. Doubly ^{18}O-labeled methyl benzoate showed an isotope shift of 0.050 ppm, equal to the sum of the two singly ^{18}O-labeled carbonyl carbons. Next, [1-^{13}C, 1,1,4-$^{18}O_3$]5-aminolevulinic acid (ALA) was fed to *Arthrobacter hyalinus*, and the resulting porphyrin was esterified with trimethyloxonium tetrafluoroborate to afford ^{13}C- and ^{18}O-labeled uroporphyrin III octamethyl ester. The ^{13}C-NMR signals exhibited carbonyl carbon ^{18}O-isotope shifts of 0.013, 0.036 and 0.051 ppm, close to those obtained in the model experiment. Thus, the carbonyl oxygens of uroporphyrinogen III and its oxidized product uroporphyrin III originated from ALA. This result shows that the loss of ^{18}O at the acetate carbonyls of the A-ring in vitamin B_{12} does not occur before the formation of uroporphyrinogen III.

Keywords ^{18}O-labeling; uroporphyrin; oxygen origin; biosynthesis; vitamin B_{12}; uroporphyrinogen III

Vitamin B_{12} and other corrinoids and porphyrinoids are biosynthesized from uroporphyrinogen III (uro'gen III, **2**), which is itself derived from eight molecules of 5-aminolevulinic acid (ALA, **1a**) (Fig. 1).[2] In the biosynthetic pathway of vitamin B_{12}, uro'gen III (**2**) is methylated, ring-contracted and decarboxylated to form cobyrinic acid (**4**), which is then converted to vitamin B_{12}. The C20 carbon of uro'gen III (**2**) is removed as acetic acid,[3] and the porphyrin ring is contracted to form the corrin ring. Recently, the ring contraction mechanism has been examined. Uzar and Battersby[4] proposed the lactone intermediate (**3a**) at the D-ring in the process of ring contraction, while Eschenmoser[5] suggested the lactone intermediate at the A-ring (**3b**). These proposals were based on model studies of ring contraction.[6]

We have already reported the result of a feeding experiment of [1-^{13}C, 1,1,4-$^{18}O_3$]ALA (**1b**) to *Propionibacterium shermanii*.[7] The ^{13}C-NMR spectrum of the resulting labeled vitamin B_{12} revealed almost complete exchange from ^{18}O to ^{16}O only at the C27 amide oxygen of one of three acetamides. This result was considered to favor the view that the acetate carbonyl group of the A-ring was converted to the lactone, followed by hydrolysis with the water in the culture to form cobyrinc acid (**4**), as proposed by Eschenmoser. However, it is not clear whether the oxygen exchange occurred before or after the formation of uro'gen III (**2**).

We have already reported that *Arthrobacter hyalinus* produces a large amount of porphyrins.[8] The major component was uroporphyrin III, which is an oxidation product of uro'gen III. If the origin of carbonyl oxygen of uroporphyrin III can be elucidated, the problem of oxygen exchange of uro'gen III in the biosynthesis of vitamin B_{12} would be solved. So we planned to incorporate ^{13}C-, ^{18}O-labeled ALA into uroporphyrin III in *A. hyalinus*.

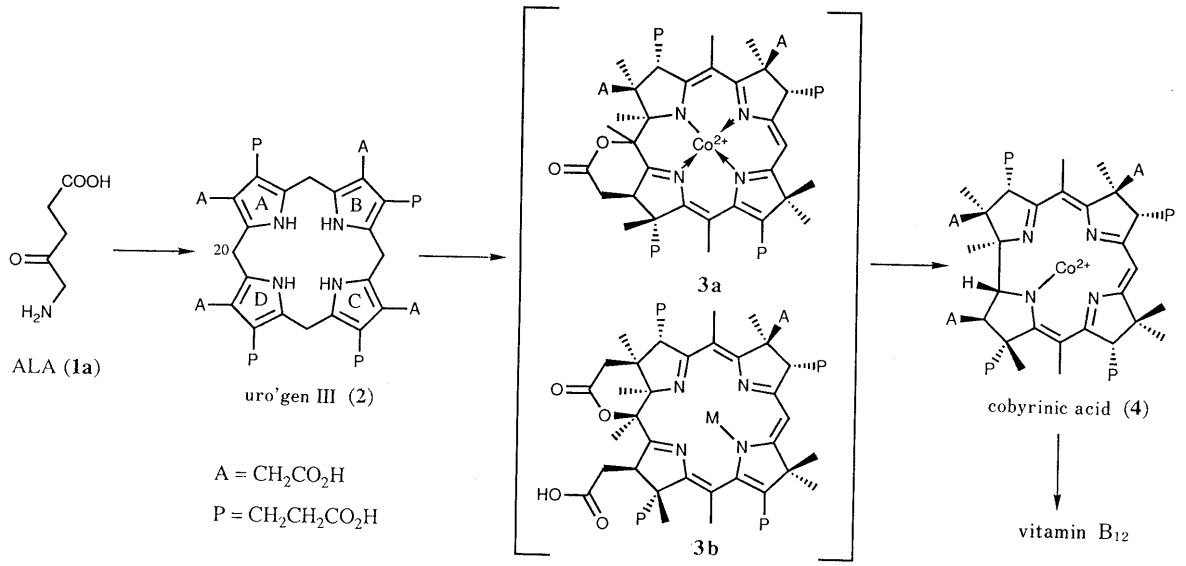

Fig. 1. Proposed Biosynthetic Pathway of Vitamin B_{12}

Results and Discussion

Uroporphyrin III produced by *A. hyalinus* was derived to its methyl ester for easy isolation and purification. If ^{18}O-labeled uroporphyrin is esterified, three types of esters, *i.e.*, alkoxy-oxygen- labeled, carbonyl-oxygen-labeled, and doubly oxygen-labeled esters, may be formed. The carbonyl carbons of these ^{18}O-labeled esters show different ^{18}O-isotope shifts in the ^{13}C-NMR spectrum. Risley and Van Etten[9] showed that the singly ^{18}O-labeled carbonyl carbons were shifted upfield by 0.015 and 0.038 ppm. The sum of these two values was nearly equal to the isotope shift (0.054 ppm) observed for doubly ^{18}O-labeled carbonyl carbon.

Therefore, we conducted a model experiment using [^{13}C, $^{18}O_2$]benzoic acid (**5**). It was prepared from [^{13}C]benzoic acid and [^{18}O]water at 120 °C for 24 h in a sealed tube. [^{13}C, $^{18}O_2$]Benzoic acid (**5**) was converted to the methyl ester by two methods, with methanol–sulfuric acid or with trimethyloxonium tetrafluoroborate (Meerwein reagent).[10] Esterification of **5** with methanol–sulfuric acid afforded [^{13}C, *carbonyl*-^{18}O]methyl benzoate (**6a**) in 88% yield and the mass spectrum indicated 88% enrichment with ^{18}O. Esterification with trimethyloxonium tetrafluoroborate, which does not exchange the carboxylic acid oxygens, gave [^{13}C, $^{18}O_2$]methyl benzoate (**6b**) in 98% yield with 84% enrichment of the two ^{18}O. For comparison, [^{13}C]benzoic acid was also converted to the methyl ester by the same method. The high-resolution 100 MHz ^{13}C-NMR signals of the carbonyl carbon of these methyl esters are shown in Fig. 2. The carbonyl carbon signal of [^{13}C]methyl benzoate was observed at 167.114 ppm, and the other two small peaks were natural abundance peaks of singly ^{18}O-labeled carbonyl carbons. [^{13}C, $^{18}O_2$]Methyl benzoate contained [^{13}C, *alkoxy*-^{18}O]methyl benzoate, which showed an upfield ^{18}O-isotope shift of 0.015 ppm from the carbonyl carbon signal of [^{13}C]methyl benzoate. [^{13}C, *carbonyl*-^{18}O]Methyl benzoate (**6a**) showed an upfield ^{18}O-isotope shift of 0.036 ppm. The doubly ^{18}O-labeled carbonyl carbon of **6b** showed an isotope shift of 0.050 ppm, corresponding to

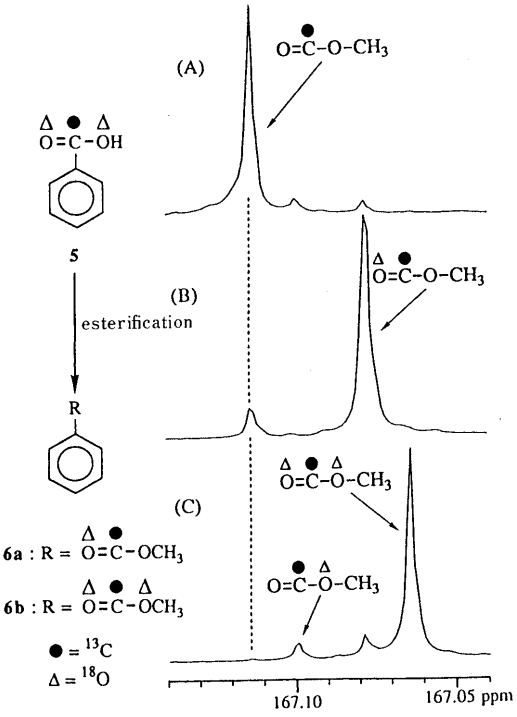

Fig. 2. Synthesis and the ^{13}C-NMR Spectrum of (A) [^{13}C]Methyl Benzoate, (B) [^{13}C, *carbonyl*-^{18}O]Methyl Benzoate, and (C) [^{13}C, $^{18}O_2$]Methyl Benzoate

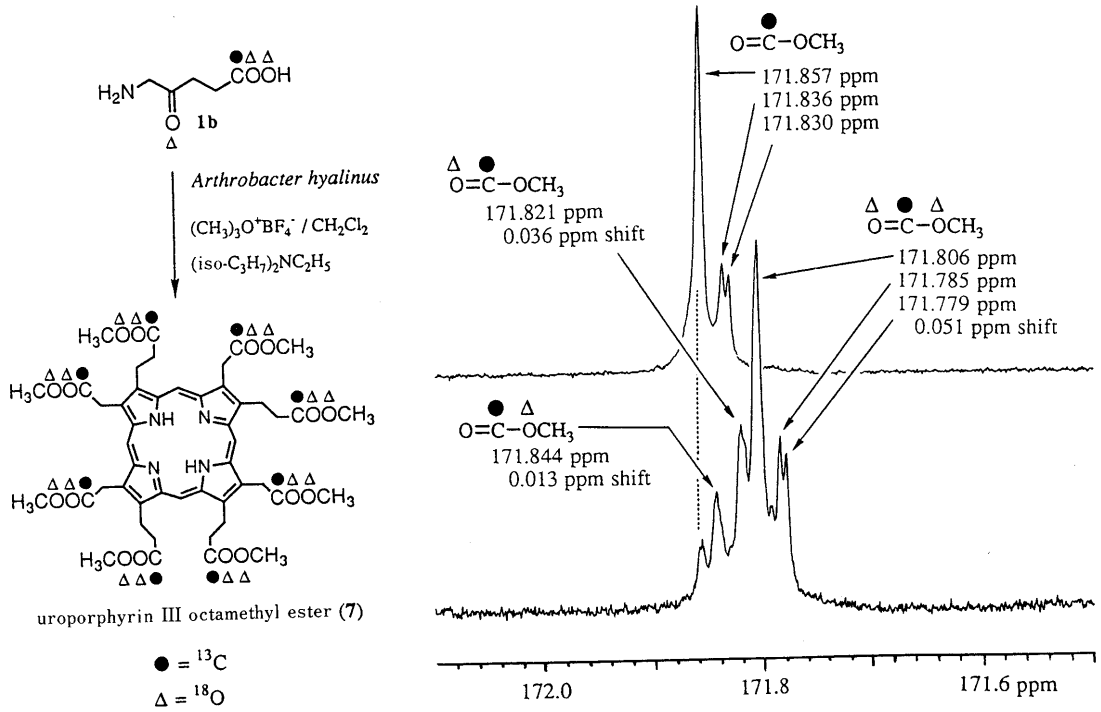

Fig. 3. Feeding Experiment of [1-^{13}C, 1,1,4-$^{18}O_3$]ALA to *A. hyalinus*

The ^{13}C-NMR signals of acetate carbonyls of [1-^{13}C]ALA-incorporated uroporphyrin III octamethyl ester (top) and [1-^{13}C, 1,1,4-$^{18}O_3$]ALA-incorporated uroporphyrin III octamethyl ester (bottom).

the sum of the isotope shifts of singly ^{18}O-labeled methyl benzoates. This experiment made it clear that singly ^{18}O-labeled carbonyl carbon shows two isotope shifts, 0.015 and 0.036 ppm, and their sum is equal to the value of the isotope shift of doubly ^{18}O-labeled carbonyl carbon.

On the basis of the above results, we conducted a feeding experiment with [1-^{13}C, 1,1,4-$^{18}O_3$]ALA (**1b**) in *A. hyalinus*. We prepared [1-^{13}C, 1,1,4-$^{18}O_3$]ALA (**1b**) by heating [1-^{13}C]ALA[11] with [^{18}O]water in a sealed tube, modifying the method of Emery and Akhtar.[12] This method was described in the previous paper.[7] This precursor (**1b**) was fed to *A. hyalinus*. After 4 d, porphyrins were extracted, followed by esterification with trimethyloxonium tetrafluoroborate and *N,N*-diisopropylethylamine in dichloromethane to afford the methyl esters of the porphyrins. The mixture of the methyl esters of porphyrins was separated by column chromatography on silica gel (benzene : ethyl acetate = 5 : 1—2 : 1) to give [^{13}C, 1,1,4-$^{18}O_3$]ALA-incorporated uroporphyrin III octamethyl ester (**7**). At the same time, for comparison, a feeding experiment with [1-^{13}C]ALA was also performed and uroporphyrin III octamethyl ester labeled with ^{13}C at ester carbonyl carbon was obtained. The high-resolution 100 MHz ^{13}C-NMR signals of the acetate carbonyls of these uroporphyrin III octamethyl esters are shown in Fig. 3. The acetate carbonyl signals of the ^{13}C-labeled uroporphyrin III octamethyl ester were observed at δ 171.857, 171.836 and 171.830 ppm. Those of ^{13}C- and ^{18}O-labeled uroporphyrin III octamethyl ester showed upfield ^{18}O-isotope shifts of 0.013, 0.036 and 0.051 ppm. Similarly, the propionate carbonyls showed 0.014, 0.037, and 0.052 ppm ^{18}O-isotope shifts. These values of the isotope shift correspond to alkoxy-^{18}O, carbonyl-^{18}O and doubly ^{18}O-labeled carbonyl carbon, based on the model experiment using methyl benzoate labeled with ^{13}C and ^{18}O. The results show that the all oxygens of uroporphyrin III octamethyl ester were labeled with ^{18}O. Thus, the carbonyl oxygens of uroporphyrin III originated from ALA, and it was concluded that the oxygens of the acetate carbonyls of uro'gen III were transferred from ALA without exchange. It follows that the oxygen exchange occurs during the transformation from uro'gen III to cobyrinic acid in the pathway of biosynthesis of vitamin B_{12}.

Experimental

^{1}H- and ^{13}C-NMR spectra were taken on a JEOL GSX-400 spectrometer (400 and 100 MHz). Chemical shifts are given downfield from tetramethylsilane (TMS) (=0 ppm) in the case of ^{1}H-NMR, and from chloroform-d_1 (=77.0 ppm) as an internal standard in the case of ^{13}C-NMR. The ^{13}C-NMR spectra of ^{18}O-labeled compounds were recorded under the following conditions: a total of 3000 scans, 30° pulse angle, 15105.7 Hz spectrum width, 256 k data points, and an acquisition time of 8.7 s. The digital resolution was 0.12 Hz. An exponential line narrowing of 0.11 Hz was used prior to Fourier transformation. Mass spectra (MS) and fast atom bombardment mass spectra were recorded on a Fisons Instruments VG Analytical VG-autospec spectrometer or a JEOL DX-302 spectrometer.

[^{13}C, $^{18}O_2$]Benzoic Acid (5) [^{13}C]Benzoic acid (10.5 mg, 0.085 mmol) was placed in a sealed tube, and 150 μl of [^{18}O]water (95% atom ^{18}O) and 2 μl of 0.1 N hydrochloric acid were added. The mixture was frozen and sealed in a tube under reduced pressure. The tube was autoclaved (122 °C 1.2 atm) for 24 h. The reaction mixture was cooled to room temperature, and extracted with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate and evaporated to afford **5** as white crystals (9.6 mg, 89%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.46—7.50 (2H, m), 7.60—7.64 (1H, m), 8.11—8.15 (2H, m). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 172.231 ($^{13}\underline{C}^{18}O_2H$). MS *m/z*: 127 ($M^+$), 108 (100%), 77. HRMS Calcd for $C_6{}^{13}C_1H_6{}^{18}O_2$: 127.0486. Found: 127.0485.

[^{13}C, *carbonyl*-^{18}O]Methyl Benzoate (6a) [^{13}C, $^{18}O_2$]Benzoic acid (**5**) (4.8 mg, 0.038 mmol) was dissolved in 0.8 ml of dry methanol, and 40 μl of sulfuric acid was added. The mixture was stirred at room temperature for 36 h. The mixture was diluted with dichloromethane, then washed with saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous magnesium sulfate and evaporated to give **6a** (4.6 mg, 88%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.92 (3H, d, J_{C-H}=3.9 Hz), 7.42—7.46 (2H, m), 7.54—7.58 (1H, m), 8.03—8.06 (2H, m). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.114 ($^{13}\underline{C}^{16}O_2CH_3$), 167.078 (carbonyl-$^{18}O$-labeled $^{13}\underline{C}^{18}O^{16}OCH_3$, 0.036 ppm shift). MS *m/z*: 139 (M^+), 108 (100%), 77. HRMS Calcd for $C_7{}^{13}C_1H_8O_1{}^{18}O_1$: 139.0600. Found: 139.0602.

[^{13}C, $^{18}O_2$]Methyl Benzoate (6b) A stirred solution of [^{13}C, $^{18}O_2$]benzoic acid (**5**) (4.7 mg, 0.037 mmol) in 0.5 ml of dichloromethane was treated with 8.2 mg of trimethyloxonium tetrafluoroborate (Meerwein reagent) and 0.01 ml of *N,N*-diisopropylethylamine at 0 °C. The mixture was stirred at room temperature for 24 h. The mixture was diluted with dichloromethane, then washed with 3 N hydrochloric acid, saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous magnesium sulfate and evaporated to give **6b** (5.1 mg, 98%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.92 (3H, d, J_{C-H}=3.9 Hz), 7.42—7.46 (2H, m), 7.54—7.58 (1H, m), 8.03—8.06 (2H, m). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.099 (alkoxy-^{18}O-labeled $^{13}\underline{C}^{16}O^{18}OCH_3$, 0.015 ppm shift), 167.078 (carbonyl-^{18}O-labeled $^{13}\underline{C}$-$^{18}O^{16}OCH_3$, 0.036 ppm shift), 167.064 ($^{13}\underline{C}^{18}O_2CH_3$, 0.050 ppm shift). MS *m/z*: 141 (M^+), 108 (100%), 77. HRMS Calcd for $C_7{}^{13}C_1H_8{}^{18}O_2$: 141.0642. Found: 141.0639.

Medium for Administration of [1-^{13}C]ALA and [1-^{13}C, 1,1,4-$^{18}O_3$]ALA to *A. hyalinus* The medium consisted of [1-^{13}C]ALA or [1-^{13}C, 1,1,4-$^{18}O_3$]ALA (25 mg), $MgSO_4 \cdot 7H_2O$ (1.0 g), $CaCO_3$ (1.0 g), NH_4NO_3 (0.6 g), peptone (0.6 g), $Na_2HPO_4 \cdot 12H_2O$ (0.3 g), yeast extract (0.2 g), L-cystine (0.12 g), KH_2PO_4 (80 mg), $ZnSO_4 \cdot 7H_2O$ (0.2 mg), $CuSO_4 \cdot 5H_2O$ (10 mg) and MoO_3 (0.2 mg) in a total volume of 200 ml of distilled water.

Incorporation of [1-^{13}C]ALA into Uroporphyrin III Octamethyl Ester [1-^{13}C]ALA·HCl (25 mg × 2) was dissolved in distilled water (15 ml × 2) and the solution was added through a Nalgene disposable filter to 200 ml × 2 of fermentation culture. After 7 d of culture the broth (400 ml) was centrifuged for 20 min at 10000 rpm (15000 × *g*) at 4 °C. Sephadex DEAE A-25 (5.0 g) was added to the supernatant. After 30 min the precipitate was collected by filtration, washed with distilled water and lyophilized. The residue was suspended in 100 ml of methanol : sulfuric acid (95 : 5), and the mixture was allowed to stand at 25 °C for 12 h. Addition of ammonia solution (pH 7.0), followed by extraction of the aqueous layer with dichloromethane (3 × 150 ml) and evaporation of the solvent afforded a mixture of methylated products, which was purified by column chromatography on silica gel (benzene : ethyl acetate = 5 : 1—2 : 1). The resulting ^{13}C-labeled uroporphyrin III octamethyl ester was recrystallized from chloroform–methanol to give 11.5 mg of ^{13}C-labeled uroporphyrin III octamethyl ester. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: −3.77 (2H, br, 2 × NH), 3.37 (8H, m, 4 × $CH_2\underline{CH}_2{}^{13}CO_2$), 3.68, 3.69, 3.69, 3.70 (12H, 4 × s, 4 × $CH_2CH_2{}^{13}CO_2C\underline{H}_3$), 3.79, 3.79, 3.80, 3.80 (12H, 4 × s, 4 × $CH_2CH_2{}^{13}CO_2C\underline{H}_3$), 4.47 (8H, br, 4 × $C\underline{H}_2CH_2{}^{13}CO_2$), 5.15 (8H, br, 4 × $C\underline{H}_2{}^{13}CO_2$), 10.20, 10.21, 10.21, 10.22 (4H, 4 × s, *meso* protons). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.489, 173.484, 173.453 (propionate carbonyls), 171.857, 171.836, 171.830 (acetate carbonyls). FAB-MS *m/z*: 951 ($[M+H]^+$).

Incorporation of [1-^{13}C, 1,1,4-$^{18}O_3$]ALA (1b) into Uroporphyrin III Octamethyl Ester [1-^{13}C, 1,1,4-$^{18}O_3$]ALA·HCl (**1b**) (25 mg × 2) was dissolved in distilled water (15 ml × 2) and the solution was added through a Nalgene disposable filter to 200 ml × 2 of fermentation culture. After 4 d of culture the broth (400 ml) was centrifuged for 20 min at 10000 rpm (15000 × *g*) at 4 °C. The supernatant was acidified to pH 4.0 with acetic acid. The precipitate was collected by centrifugation (10 min, 2500 rpm, 1000 × *g*, room temperature), and washed three times with water (adjusted to pH 4.0 with acetic acid). The precipitated residue was lyophilized. To the residue was added 100 ml of dichloromethane and 4.7 g of trimethyloxonium tetrafluoroborate (Meerwein reagent). The mix-

ture was stirred for 5 min at room temperature, then 7.8 ml of diisopropylethylamine was added dropwise at 0 °C over a period of 15 min. This suspension was vigorously stirred at room temperature. After 2 h an additional 4.7 g of trimethyloxonium tetrafluoroborate and 7.8 ml of diisopropylethylamine were added. The mixture was stirred at room temperature for 12 h. The mixture was neutralized with 1 N hydrochloric acid and diluted with 500 ml of dichloromethane. The organic layer was washed with 1 N hydrochloric acid, saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated to afford a mixture of methylated products, which were purified by column chromatography on silica gel (benzene : ethyl acetate = 5 : 1—2 : 1). The product was recrystallized from chloroform–methanol to afford 3.2 mg of [1-^{13}C, 1,1,4-$^{18}O_3$]ALA-incorporated uroporphyrin III octamethyl ester (**7**). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: −3.77 (2H, br, 2 × NH), 3.37 (8H, m, 4 × $CH_2CH_2{}^{13}C^{18}O_2$), 3.68, 3.69, 3.69, 3.70 (12H, 4 × s, 4 × $CH_2CH_2{}^{13}C^{18}O_2CH_3$), 3.79, 3.79, 3.80, 3.80 (12H, 4 × s, 4 × $CH_2CH_2{}^{13}C^{18}O_2CH_3$), 4.47 (8H, br, 4 × $CH_2CH_2{}^{13}C^{18}O_2$), 5.15 (8H, br, 4 × $CH_2{}^{13}C^{18}O_2$), 10.20, 10.21, 10.21, 10.22 (4H, 4 × s, *meso* protons). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.489, 173.484, 173.453 (propionate carbonyls), 173.475, 173.469, 173.439 (alkoxy-^{18}O-labeled propionate carbonyls, 0.014 ppm shift), 173.452 (shoulder), 173.446, 173.416 (carbonyl-^{18}O-labeled propionate carbonyls, 0.037 ppm shift), 173.437, 173.432, 173.401 (doubly ^{18}O-labeled propionate carbonyls, 0.052 ppm shift), 171.857, 171.836 (shoulder), 171.830 (shoulder) (acetate carbonyls), 171.844, 171.823 (shoulder), 171.817 (shoulder) (alkoxy-^{18}O-labeled acetate carbonyls, 0.013 ppm shift), 171.821, 171.800 (shoulder), 171.794 (carbonyl-^{18}O-labeled acetate carbonyls, 0.036 ppm shift), 171.806, 171.785, 171.779 (doubly ^{18}O-labeled acetate carbonyls, 0.051 ppm shift).

References

1) Part VII: M. Kajiwara, K. Hara, M. Mizutani, M. Kondo, *Chem. Pharm. Bull.*, **40**, 3321 (1992).
2) F. J. Leeper, *Nat. Prod. Rep.*, **4**, 441 (1987).
3) L. Mobelli, C. Nussbaumer, H. Weber, G. Müller, D. Arigoni, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 11 (1981); A. R. Battersby, M. J. Bushell, C. Jones, N.G. Lewis, A. Pfenninger, *ibid.*, **78**, 13 (1981); A. I. Scott, M. Kajiwara, P. J. Santander, *ibid.*, **84**, 6616 (1987).
4) H. C. Uzar, A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, **1985**, 585.
5) A. Eschenmoser, *Angew. Chem. Int. Ed. Engl.*, **27**, 5 (1988).
6) V. Rasetti, A. Pfaltz, C. Kratky, A. Eschenmoser, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 16 (1981).
7) K. Kurumaya, T. Okazaki, M. Kajiwara, *Chem. Pharm. Bull.*, **37**, 1151 (1989); A. I. Scott, N. J. Stolowich, B. Atshaves, P. Karuso, M. J. Warren, H. J. Williams, M. Kajiwara, K. Kurumaya, T. Okazaki, *J. Am. Chem. Soc.*, **113**, 9891 (1991).
8) I. Kojima, K. Maruhashi, Y. Fujiwara, T. Saito, M. Kajiwara, M. Mizutani, *J. Ferment. Bioeng.*, **75**, 353 (1993).
9) J. M. Risley, R. L. Van Etten, *J. Am. Chem. Soc.*, **102**, 6699 (1980); J. C. Vederas, *ibid.*, **102**, 374 (1980).
10) D. J. Raber, P. Gariano, *Tetrahedron Lett.*, **1971**, 4741.
11) K. Kurumaya, T. Okazaki, N. Seido, Y. Akasaka, Y. Kawajiri, M. Kajiwara, *J. Labelled Comp. Radiopharm.*, **27**, 217 (1989).
12) V. C. Emery, M. Akhtar, *J. Chem. Soc., Chem. Commun.*, **1985**, 600.