Identification of Tetrapyrrole Compounds Excreted by *Rhodobacter sphaeroides* and Sources of the Methyl Hydrogens of Bacteriochlorophyll *a* Biosynthesized by *R. sphaeroides*, Based on ^{13}C-NMR Spectral Analysis of Coproporphyrin III Tetramethyl Ester

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Red-fluorescent tetrapyrrole compounds excreted by *Rhodobacter sphaeroides* into the culture broth were concluded to be coproporphyrinogen (Copro'gen) III and uroporphyrinogen (Uro'gen) I, based on the ^{13}C-NMR spectral identification of coproporphyrin (Copro) III tetramethyl ester and uroproporphyrin (Uro) I octamethyl ester. The sources of the methyl hydrogens of bacteriochlorophyll *a* were established by analysis of the ^{13}C-NMR spectra of ^{2}H,^{13}C-Copro III tetramethyl ester chemically derived from ^{2}H,^{13}C-Copro'gen III biosynthesized through the feeding of δ-amino[2-^{13}C]levulinic acid (ALA) to *R. sphaeroides* in medium containing 50% $^{2}H_2O$. We confirmed the previous finding that one of the methyl hydrogens was derived from water in the medium during decarboxylation of four acetyl side chains of Uro'gen III to generate Copro'gen III. It was further shown that the other hydrogen atoms, previously reported to be derived from methylene hydrogens at C-2 of ALA, had been exchanged with hydrogen of water in the medium in the biosynthetic pathways leading from ALA to Copro'gen III.

Key words *Rhodobacter sphaeroides*; bacteriochlorophyll *a*; coproporphyrin III; ^{13}C-NMR; δ-amino[2-^{13}C]levulinic acid (ALA); $^{2}H_2O$

Analysis of the ^{13}C-NMR and broad-band deuterium and proton-decoupled ^{13}C-NMR (^{13}C-{^{1}H}{^{2}H}NMR) spectra of ^{2}H,^{13}C-bacteriochlorophyll *a* (after conversion to methyl ^{2}H,^{13}C-bacteriopheophorbide *a* with CH_3OH/H_2SO_4) derived from our previous two feeding experiments with δ-amino[2-^{13}C]levulinic acid (ALA) (ALA, a biosynthetic intermediate of tetrapyrrole) in medium containing 50% $^{2}H_2O$ and [2,2-$^{2}H_2$,2-^{13}C]ALA to *Rhodobacter sphaeroides* led us to propose a mechanism involving decarboxylation of the four acetyl side chains of uroporphyrinogen (Uro'gen) III in the biosynthesis of coproporphyrinogen (Copro'gen) III (Fig. 1).[1] As regards the sources of the methyl hydrogens of bacteriochlorophyll *a*, we concluded that two of the hydrogens of each methyl group were retained from ALA and the other one was derived from water in the medium. However, in a [2,2-$^{2}H_2$,2-^{13}C]ALA feeding experiment, signals influenced by none and by only one deuterium atom, which could not be interpreted by the proposed decarboxylation mechanism, were observed in the ^{13}C-NMR spectra of ^{2}H,^{13}C-methyl bacteriopheophorbide *a*. These signals could be assigned to the four methyl carbons of ^{2}H,^{13}C-methyl bacteriopheophorbide *a* derived from [2-^{13}C]ALA and [2-^{2}H,2-^{13}C]ALA contained in [2,2-$^{2}H_2$,2-^{13}C]ALA synthesized by our method.[1,2] These results suggested that the deuterium atom, which was derived from [2,2-$^{2}H_2$,2-^{13}C]ALA, had been exchanged with hydrogen of water in the medium in the biosynthetic steps from ALA to bacteriochlorophyll *a* in *R. sphaeroides*. The [2-^{13}C]ALA feeding experiment in medium containing 50% $^{2}H_2O$ resulted in low incorporation of labeled compounds under the influence of $^{2}H_2O$, and the signals of the four methyl carbons influenced by at least two deuterium atoms could not be clearly observed in ^{13}C-NMR spectra of ^{2}H,^{13}C-methyl bacteriopheophorbide *a*. However, we observed a red fluorescence in the culture broth of *R. sphaeroides*, which produces bacteriochlorophylls intracellularly.

Therefore, we tried to identify the compounds having red fluorescence in the culture broth, and utilized them to re-investigate the biosynthetic sources of the methyl hydrogens of bacteriochlorophyll *a* by repeating the [2-^{13}C]ALA feeding experiment in medium containing 50% $^{2}H_2O$ in *R. sphaeroides*.

Experimental

Chemicals and Instruments [2-^{13}C]ALA was synthesized by our method[2] from sodium acetate (99 atom% ^{13}C), which was purchased from Cambridge Isotope Laboratories. $^{2}H_2O$ (98 atom% ^{2}H) was purchased from Shoko Co., Ltd. All other chemicals were of analytical grade. All ^{1}H-NMR (300 MHz) spectra were recorded on a Varian Gemini-300 spectrometer, and the signal of TMS (0 ppm) was used as an internal standard. All ^{13}C-NMR (150 MHz) spectra were recorded on a JEOL JNM-ECA 600 spectrometer with a cold probe for a solution of ^{2}H,^{13}C-Copro III tetramethyl ester in C^2HCl_3; the signal of C^2HCl_3 (77.0 ppm) was used as an internal standard. All UV spectra were recorded on a Jasco UVIDEC-610C spectrometer. All FAB-MS spectra were recorded on a JEOL DX302 with the aid of 3-nitrobenzyl alcohol (3-NOBA).

Isolation of Compounds Having Red Fluorescence from the Culture Broth of *R. sphaeroides* The culture of *R. sphaeroides* IFO 12203 was carried out by a modification of the method described in our previous paper.[1] The cultures were anaerobically grown under illumination (2400 lux) in seed culture medium (60 ml), which consisted of yeast extract (2.0 g·l^{-1}), DL-malic acid (2.7 g·l^{-1}), KH_2PO_4 (0.5 g·l^{-1}), K_2HPO_4 (0.5 g·l^{-1}), $(NH_4)_2HPO_4$ (0.8 g·l^{-1}), $MgSO_4$ (0.2 g·l^{-1}), EDTA (2.5 mg·l^{-1}), $ZnCl_2$ (2.5 mg·l^{-1}), $FeSO_4 \cdot 7H_2O$ (1.0 mg·l^{-1}), $MnCl_2 \cdot 4H_2O$ (0.5 mg·l^{-1}), $CuCl_2 \cdot 2H_2O$ (0.1 mg·l^{-1}), $CoCl_2 \cdot 6H_2O$ (0.5 mg·l^{-1}) and H_3BO_4 (0.02 mg·l^{-1}) at pH 6.8 (adjusted with saturated $NaHCO_3$ solution), in a 60 ml test tube at 27 °C. Seed culture (60 ml) incubated for 7 d and a sterilized solution of ALA (60 mg) in H_2O (10 ml), which had been filtered through a membrane filter (0.2 μm), were added to fermentation culture medium (1 l), which had the same composition as the seed culture medium, in a 1 l fermentation bottle. The cultures of *R. sphaeroides* in two 1 l fermentation bottle were continuously grown photosynthetically (2400 lux) at 27 °C for 7 d. Sephadex DEAE A-25 (3.0 g) was added to the supernatant obtained by centrifugation of the culture broth for 20 min at 12300 ***g***, and the mixture was stirred. After 30 min, the Sephadex was collected by filtration, lyophilized and suspended in a mixture of CH_3OH/H_2SO_4 (95 : 5, v/v). The mixture was left for 24 h at room tempera-

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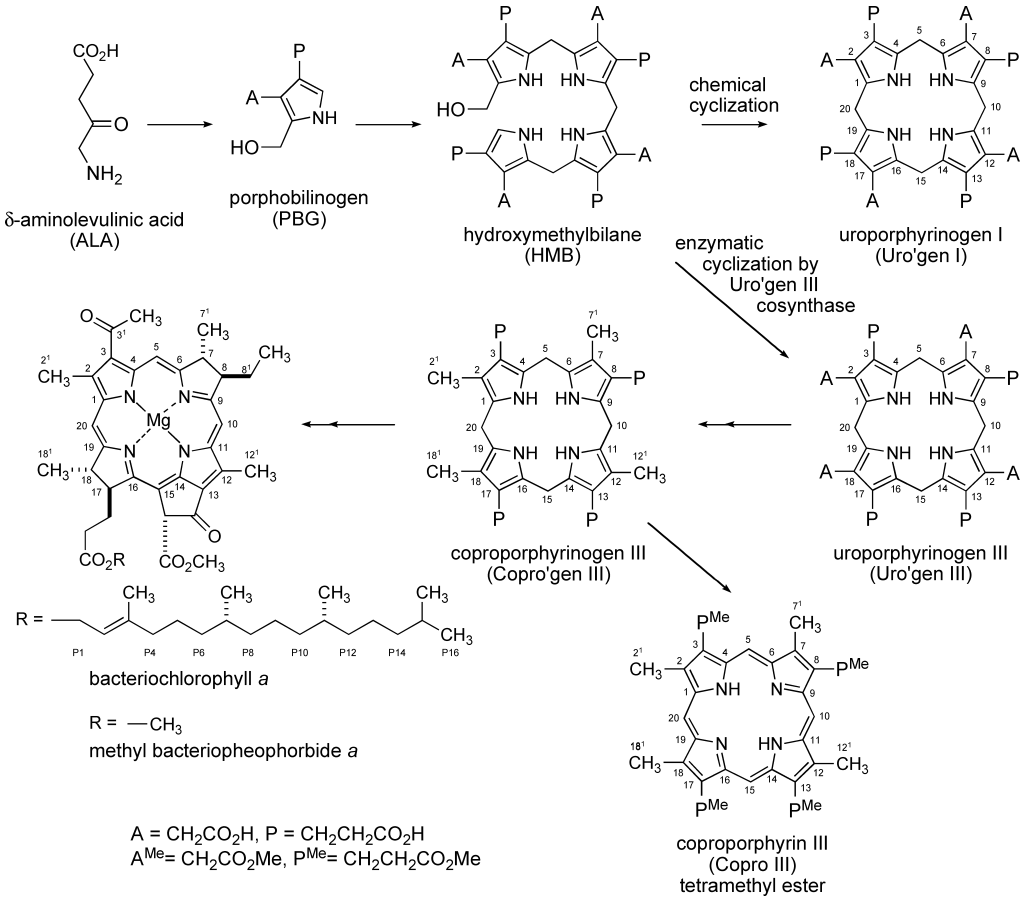

Fig. 1. Biosynthetic Pathways to Bacteriochlorophyll *a* from ALA *via* Uroporphyrinogen (Uro'gen) III and Coproporphyrinogen (Copro'gen) III in *R. sphaeroides*, and Structures of Chemically Derived Methyl Bacteriopheophorbide *a* and Coproporphyrin (Copro) III Tetramethyl Ester

ture. Methanol-insoluble material was removed, then the solution was neutralized with saturated $NaHCO_3$ solution and evaporated. The residue was dissolved in ion-exchanged water and the solution was extracted three times with CH_2Cl_2. The combined organic layer was washed with water. The organic layer was dried over anhydrous sulfate and evaporated. The resulting mixture was purified by column chromatography on silica gel with benzene/ethyl acetate (10 : 1—8 : 1, v/v) to give Copro III tetramethyl ester (11 mg), and with benzene/ethyl acetate (2 : 1, v/v)–$CHCl_3$ to give Uro I octamethyl ester (24 mg); Copro III tetramethyl ester; FAB-MS (3-NOBA): Calcd for $C_{40}H_{46}O_8N_4$; 710.83, Found; 711 (MH^+), UV λ_{max} ($CHCl_3$) nm: 401.2, 500.0, 532.4, 569.6, 623.2, ^{1}H-NMR (300 MHz, $CDCl_3$) δ: −3.81 (2H, br, =NH), 3.28 (8H, m, $-CH_2\underline{CH_2}CO_2-$), 3.61, 3.63, 3.64, 3.65 (12H, 4×s, $-CH_2CH_2CO_2\underline{CH_3}$), 3.67, 3.68, 3.69. 3.70 (12H, 4×s, $-\underline{CH_3}$), 4.40 (8H, m, $-\underline{CH_2}CH_2CO-$), 10.06, 10.07, 10.08, 10.09 (4H, 4×s, meso proton), Uro I octamethylester; FAB-MS (3-NOBA): Calcd for $C_{48}H_{54}O_{16}N_4$; 942.98, Found; 943 (MH^+), UV λ_{max} ($CHCl_3$) nm: 405.6, 501.6, 536.4, 573.2, 626.0, ^{1}H-NMR (300 MHz, $CDCl_3$) δ: −3.63 (2H, br, =NH), 3.38 (8H, t, J=7.7 Hz, $-CH_2\underline{CH_2}CO_2-$), 3.40 (12H, s, $-CH_2CH_2CO_2\underline{CH_3}$), 3.79 (12H, s, $-CH_2CO_2\underline{CH_3}$), 4.47 (8H, t, J=7.7 Hz, $-\underline{CH_2}CH_2CO_2-$), 5.15 (8H, s, $-\underline{CH_2}CO_2-$), 10.21 (4H, s, meso protons).

Feeding of [2-^{13}C]ALA to *R. sphaeroides* in Medium Containing 50% (v/v) ^2H_2O The above-mentioned seed culture (60 ml) and a sterilized solution of [2-^{13}C]ALA (60 mg, 4.5 mmol·l^{-1}) in 50% (v/v) ^2H_2O (10 ml), which had been filtered through a membrane filter (0.2 μm), were added to 50% (v/v) ^2H_2O fermentation culture medium (1 l), which had the same composition as the seed culture medium, in a 1 l fermentation bottle. The cultures of *R. sphaeroides* were continuously grown photosynthetically (2400 lux) at 27 °C for 7 d. ^{2}H,^{13}C-Copro III tetramethyl ester (5 mg) was isolated from the supernatant obtained by centrifugation of the culture broth as described above.

Results and Discussion

Two tetrapyrrole compounds having red fluorescence in the culture broth of *R. sphaeroides* were identified as Copro'gen III and Uro'gen I on the basis of the identification of their derivatives Copro III tetramethyl ester and Uro I octamethyl ester by analysis of the FAB-MS, UV and ^{1}H-NMR spectra and comparison of our previous studies.[3—6] Copro III tetramethyl ester derived from Copro'gen III, a biosynthetic intermediate of bacteriochlorophyll *a*, is more stable than bacteriochlorophyll *a*. Therefore, Copro III tetramethyl ester isolated from the culture broth of *R. sphaeroides* can be used to determine the sources of the methyl hydrogens of bacteriochlorophyll *a* biosynthesized by *R. sphaeroides*.

^{2}H,^{13}C-Copro III tetramethyl ester was isolated from the supernatant obtained by centrifugation of the culture broth containing 50% ^2H_2O of *R. sphaeroides* in the presence of [2-^{13}C]ALA. The magnified ^{13}C-NMR and ^{13}C-{^{1}H}{^{2}H}NMR spectra for the methyl region of ^{2}H,^{13}C-Copro III tetramethyl ester are shown in Figs. 2a and b, respectively. In Fig. 2a, four singlet signals (11.5325, 11.5708, 11.6259, 11.6642 ppm) and four triplet signals (11.2860, 11.3219, 11.3745, 11.4152 ppm) can be observed. In Fig. 2b, there are three groups of four singlet signals. The chemical shifts of the four most downfield singlet signals were 11.5301, 11.5708, 11.6259 and 11.6642 ppm. The chemical shifts of the four singlet signals of the center group were 11.2836, 11.3219, 11.3745 and 11.4176 ppm, and those of the four most upfield singlet signals were 11.0346, 11.0682, 11.1208 and 11.1663 ppm.

By comparison of Fig. 2a with Fig. 2b, the four most

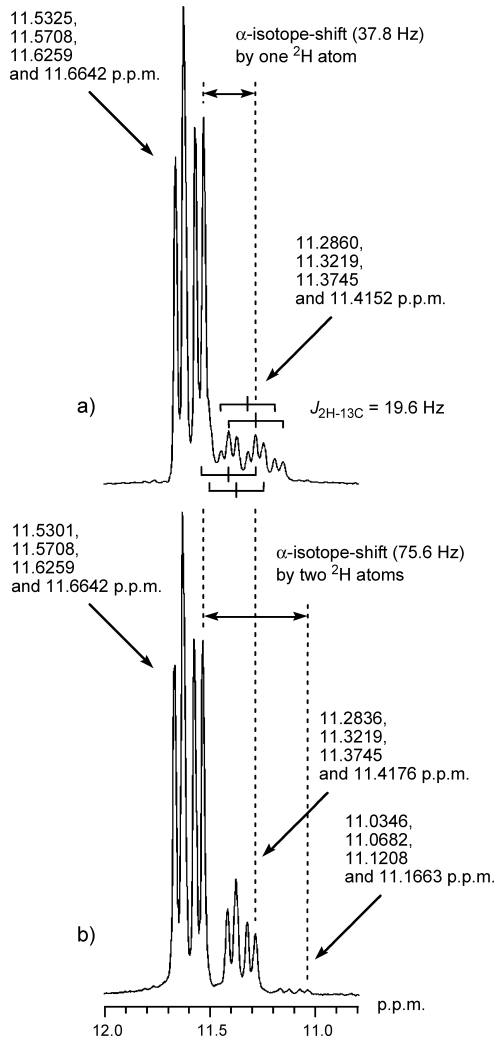

Fig. 2. Magnified (a) ^{13}C-NMR Spectrum and (b) ^{13}C-{^{1}H}{^{2}H}NMR Spectrum of ^{2}H,^{13}C-Coproporphyrin (Copro) III Tetramethyl Ester in the Methyl Region

downfield singlet signals of Figs. 2a and b can be assigned to four ^{13}C-methyl carbons (C-2^{1}, C-7^{1}, C-12^{1} and C-19^{1}), which were derived from the ^{13}C-carbon of [2-^{13}C]ALA, in ^{2}H,^{13}C-Copro III tetramethyl ester. The four triplet signals (11.2860, 11.3219, 11.3745, 11.4152 ppm) in Fig. 2a are transformed to the four singlet signals (11.2836, 11.3219, 11.3745, 11.4176 ppm) in Fig. 2b by additional deuterium decoupling. Therefore, these four triplet signals were derived from the four singlet signals, which were split into triplets owing to ^{2}H–^{13}C spin coupling of 19.6 Hz (J_{2H-13C}) and shifted upfield by 37.8 Hz owing to one deuterium α-isotope effect, so they can be assigned to four ^{13}C-methyl carbons (C-2^{1}, C-7^{1}, C-12^{1} and C-19^{1}, respectively) bearing one deuterium atom. Thus, these results confirmed that one hydrogen atom in these ^{13}C-methyl groups was introduced from deuterium of ^{2}H-water in the medium by the previous proposed decarboxylation mechanism when the four methyl groups of Copro'gen III were generated from the four acetyl side chains of Uro'gen III in *R. sphaeroides*. The small singlet signals (11.0346, 11.0682, 11.1208, 11.1663 ppm), which did not appear in Fig. 2a, appeared after additional deuterium decoupling (Fig. 2b). These four singlet signals are α-isotope-shifted (75.6 Hz) by two deuterium atoms, indicating that two hydrogen atoms of the ^{13}C-methyl hydrogens at C-2^{1}, C-7^{1}, C-12^{1} and C-19^{1} were exchanged with deuterium of ^{2}H-water in the medium (one deuterium atom was introduced into the methyl group during decarboxylation, as mentioned above). These results show that the hydrogen atoms derived from the methylene hydrogens at C-2 of ALA were exchanged with the deuterium of ^{2}H-water in the medium during the biosynthetic steps from ALA to Copro'gen III in *R. sphaeroides*.

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