Biosynthesis of Corrinoids and Porphyrinoids. VII.[1] Uroporphyrins from *Saccharopolyspora erythraea*

Masahiro KAJIWARA,*,[a] Ken-ichiro HARA,[a] Minoru MIZUTANI,[a] and Masao KONDO[b]

Department of Medicinal Chemistry, Meiji College of Pharmacy,[a] 1–22–1 Yato-cho, Tanashi-shi, Tokyo 188, Japan and Department of Nutrition and Biochemistry, The Institute of Public Health,[b] 4–6–1 Siroganedai, Minato-ku, Tokyo 108, Japan. *Received March 30, 1992*

Cultured broth of *Saccharopolyspora erythraea* shows a strong red fluorescence. The main fluorescent component was identified as uroporphyrin I by FAB-MS and ^{1}H-NMR. This was confirmed by a feeding experiment using [5-^{13}C]aminolevulinic acid.

Keywords *Saccharopolyspora erythraea*; uroporphyrin I; [5-^{13}C]aminolevulinic acid; ^{13}C-NMR

Uroporphyrinogen III[2] (**1**) is the precursor of natural porphyrins such as heme (**7**) and chlorophyll (**8**), while uroporphyrinogen I (**4**) is not an intermediate in the biosynthetic pathway of these natural porphyrins. Uroporphyrinogen I (**4**) is found only in its oxidized form in the excreta of humans and animals under pathological conditions. Both uroporphyrinogen I (**4**) and III (**1**) are biosynthesized from δ-aminolevulinic acid (ALA) (**9**). Uroporphyrinogen I (**4**) and III (**1**) are spontaneously oxidized to uroporphyrin I (**5**) and III (**2**), respectively (Fig. 1). Porphyrins are very important biologically; for example, porphyria[3] is considered a congenital abnormality. Industrial workers with lead poisoning excrete porphyrin compounds in their urine,[4] and high-performance liquid chromatography (HPLC) using standard porphyrins is a diagnostic aid in such cases. At present, however, chemical synthesis of porphyrins is difficult and porphyrins are expensive.

When *Saccharopolyspora erythraea* was used to produce erythromycin,[5] we observed a strong red fluorescence in the culture medium. In this paper, we described the identification of the major component responsible for this fluorescence. After 10 d of culture the medium was centrifuged. DEAE-Sephadex was added to the supernatant, then collected by filtration and freeze-dried. The residue with strong red fluorescence was methylated with dry methanol and concentrated sulfuric acid (95:5). The methylated products were purified by silica gel column chromatography, the fraction with red fluorescence was evaporated, and the residue was crystallized from methanol–chloroform. Comparison of the isolated compound with authentic uroporphyrin I octamethylester (**6**)

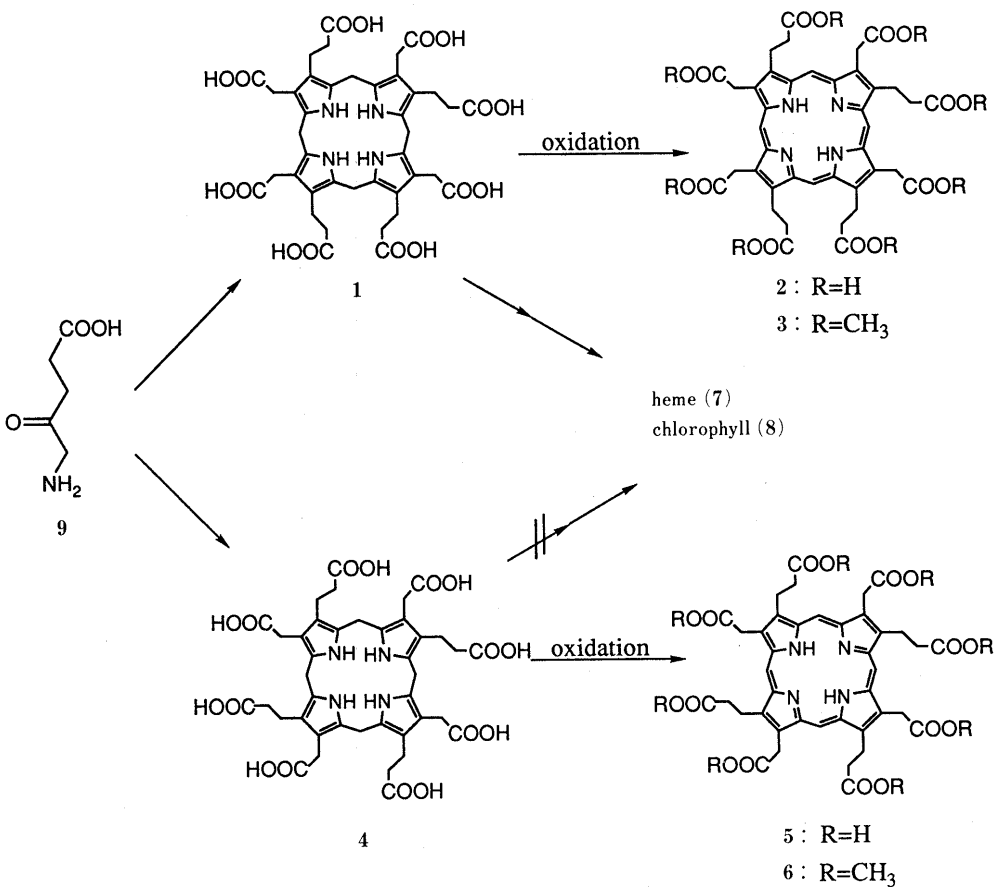

Fig. 1. Structures of Uroporphyrinogens I, III and Uroporphyrins I, III

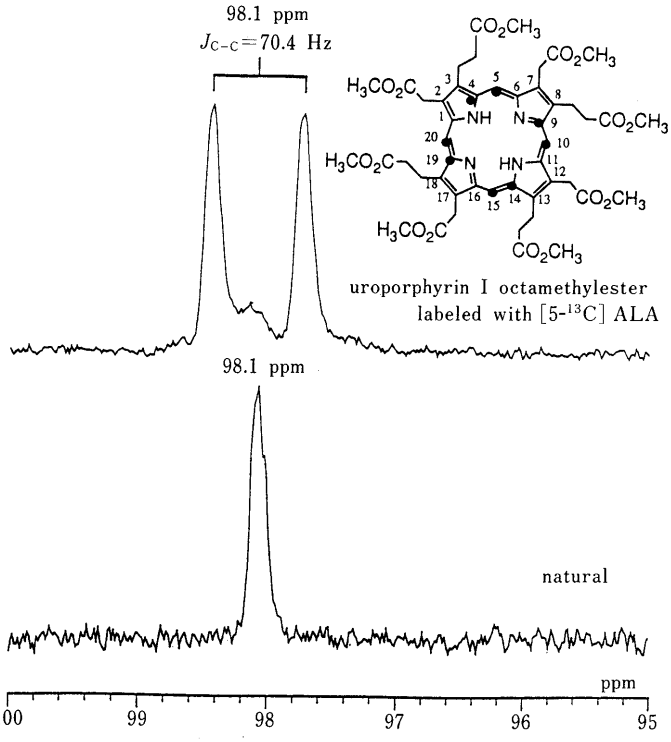

Fig. 2. Comparison of the ^{13}C-NMR Spectrum of Uroporphyrin I Octamethylester with That of [5-^{13}C]ALA-Incorporated Uroporphyrin I Octamethylester

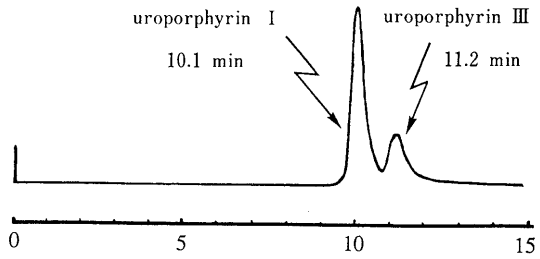

Fig. 3. Separation of Uroporphyrin I and Uroporphyrin III from *S. erythraea* by HPLC

by FAB-MS and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy showed that most of the isolated material was uroporphyrin I octamethylester (**6**).

Furthermore, [5-^{13}C]ALA[6] was fed to *S. erythraea* to confirm this result. The red fluorescent product was isolated and methylated as above, and its proton-decoupled carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum was measured. The meso carbons gave a doublet signal at 98.1 ppm (J_{C-C}=70.4 Hz); the 70.4 Hz coupling was clearly due to the directly bonded ^{13}C atoms and was similar in size to that found for directly bonded sp^2-hydridized ^{13}C atoms in other systems.[7] This confirmed that the major product is uroporphyrin I octamethylester[8] (**6**) (Fig. 2).

We examined whether other methylesters of porphyrins exist in addition to uroporphyrin I octamethylester. The methylated compounds that were purified by silica gel column chromatography were hydrolyzed with 2 N potassium hydroxide. HPLC analysis[9] revealed a ratio of uroporphyrin I (**5**) to uroporphyrin III (**2**) of 70:30 (Fig. 3).

Experimental

Instruments MS were recorded on a VG Analytical Auto Spec-Q. The matrix used for FAB-MS was 3-nitrobenzyl alcohol (3-NOBA). Liquid chromatography-MS (LC-MS) were recorded on JEOL JMSLX 1000 and JEOL JMS-505W instruments. Ultraviolet (UV) spectra were recorded on a JASCO UVIDEC-610C spectrometer, fluorescence spectra on a JASCO FP-777 spectrometer, and infrared (IR) spectra on a JASCO FT-IR 5000 spectrometer. ^{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) or sodium 3-trimethylsilylpropionate-d_4 (TSP) for ^{1}H-NMR, and from chloroform-d_1 (=77.0) for ^{13}C-NMR. HPLC was performed using a JASCO LC-800 chromatography system.

Culture Conditions The slant medium consisted of agar (12.0 g), starch (10.0 g), NZ amine type A (3.0 g), $CaCO_3$ (3.0 g), yeast extract (1.0 g) and meat extract (1.0 g) in a total of 1000 ml of distilled water, adjusted to pH 7.0 and autoclaved at 120 °C for 20 min.

The fermentation medium consisted of $MgSO_4 \cdot 7H_2O$ (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 μg) and MoO_3 (0.2 μg) in 200 ml of distilled water, and was autoclaved at 120 °C for 20 min in a 500 ml Erlenmeyer flask. The culture was incubated for 10 d at 210 rpm and 30 °C.

Isolation of Compound with Strong Red Fluorescence from Fermentation Broth Ten day culture broth (400 ml) was centrifuged for 20 min at 10000 rpm (15,876 g) at 4 °C. Sephadex DEAE A-25 (5.0 g) was added to the supernatant. After 2—3 h the Sephadex DEAE A-25 was collected by filtration, washed with distilled water, freeze-dried, suspended in CH_3OH/H_2SO_4 (95:5) and allowed to stand at room temperature for 12 h. Addition of ammonia solution (pH 7.0), followed by extraction of the aqueous layer with CH_2Cl_2 (3×150 ml) and evaporation of the solvent afforded methylated products, which were purified by silica gel chromatography. The fraction with red fluorescence was collected and evaporated, and the residue was recrystallized from $CHCl_3$–CH_3OH (7.3 mg). FAB-HRMS (3-NOBA): Calcd for $C_{48}H_{54}N_4O_{16}$; 942.3613, Found; 943.3602 (M^++H). UV $\lambda_{max}^{CHCl_3}$ nm: 406.4, 501.6, 536.0, 572.8, 626.0. Fluorescence ($CHCl_3$): Ex λ 408 nm, Em λ 632 nm. IR cm^{-1} (KBr): 1160, 1200 (–CO–O–), 1430 (–CH_2–CO–), 1720 (–CO_2), 3410 (–NH). ^{1}H-NMR ($CDCl_3$) δ: −3.82 (2H, br, 2×NH), 3.38 (8H, t, J=7.81 Hz, 4×–$CH_2CH_2CO_2$–), 3.69 (12H, s, 4×–$CH_2CH_2CO_2CH_3$), 3.79 (12H, s, 4×–$CH_2CO_2CH_3$), 4.46 (8H, t, J=7.81 Hz, 4×–$CH_2CH_2CO_2$–), 5.15 (8H, s, 4×–CH_2CO_2–), 10.22 (4H, s, meso protons). ^{13}C-NMR ($CDCl_3$) δ: 21.89 (4×–$CH_2CH_2CO_2$–), 32.65 (4×–$CH_2CO_2CH_3$), 37.13 (4×–$CH_2CH_2CO_2$–), 51.81 (4×–$CH_2CH_2CO_2CH_3$), 52.41 (4×–$CH_2CO_2CH_3$), 98.06 (meso carbons C-5, C-10, C-15, C-20), 133.10 (C-2, C-7, C-12, C-18), 141.13 (C-3, C-8, C-13, C-17), 171.85 (4×–$CH_2CO_2CH_3$), 173.45 (4×–$CH_2CH_2CO_2CH_3$).

Incorporation of [5-^{13}C]ALA [5-^{13}C]ALA (99.2 atom % ^{13}C, 50 mg) was dissolved in distilled water (30 ml) and added through a Nalgene disposable filter to 400 ml of fermentation culture. At the conclusion of the normal 10-d fermentation period, a mixture of the labeled uroporphyrins was isolated and purified as the methyl ester in the usual manner (7.0 mg). FAB-MS m/z: 951 (M^++H). ^{1}H-NMR ($CDCl_3$) δ: −3.82 (2H, br, 2×NH), 3.38 (8H, t, J=7.81 Hz, 4×–$CH_2CH_2CO_2$–), 3.69 (12H, s, 4×–$CH_2CH_2CO_2CH_3$), 3.79 (12H, s, 4×–$CH_2CO_2CH_3$), 4.46 (8H, t, 4×–$CH_2CH_2CO_2$–), 5.15 (8H, s, 4×–CH_2CO_2–), 10.22 (4H, s, J_{C-H}=156.3 Hz, meso protons). ^{13}C-NMR ($CDCl_3$) δ: 98.06 (J_{C-C}=70.4 Hz, meso carbons C-5, C-10, C-15, C-20), 143.88 (br, C-4, C-9, C-14, C-16).

Conversion of Uroporphyrin Octamethylester to Uroporphyrin Carboxylic Acid A suspension of uroporphyrin octamethylester (10.7 mg) in aqueous 2 N potassium hydroxide (2 ml) and tetrahydrofuran (2 ml) was stirred at room temperature in the dark under argon for 24 h. The organic layer was then removed, and the aqueous solution was washed twice with tetrahydrofuran (30 ml each), adjusted to pH 4.0 with acetic acid and kept at room temperature overnight. The precipitated porphyrin was collected by centrifugation as an amorphous, dark red solid and washed three times with cold water (adjusted to pH 4.0 with acetic acid). The product was freeze-dried to give a mixture of uroporphyrins (9.0 mg, 95% yield). LC-MS m/z: 831 (M^++1). UV λ_{max} nm: 406.0, 551.6 (br), 593.2 (br). Fluorescence (1 N HCl): Ex λ 408 nm, Em λ 597.5, 652.5 nm. ^{1}H-NMR (uroporphyrin I 3.7 mg in 0.5 cm^3 of 99.8% D_2O adjusted to pH 13 with NaOH) δ: 3.04 (8H, s, 4×–CH_2CH_2CO), 4.31 (8H, s, 4×–CH_2CHCO), 5.02 (8H, s, 4×–CH_2CO), 10.35 (4H, br, meso protons).

Separation of Uroporphyrin I and Uroporphyrin III by HPLC HPLC analysis of uroporphyrin I was performed on a 4.6 mm i.d. × 250 mm column packed with Capcell PAC C_{18} AG 120 at 38 °C. The elution solvent was 12% acetonitrile/1 M ammonium acetate/EDTA (100 mg/l)/ pH 5.14. A fluorescence detector was used with the excitation and emission wavelengths set at 404 and 620 nm. The flow rate was 1.5 ml/min.

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