ICM0201, a New Inhibitor of Osteoclastogenesis from *Cunninghamella* sp. F-1490

**II. Structure Determination and Synthesis**

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ICM0201 (1), a new inhibitor of murine osteoclastogenesis in culture was isolated from a fermentation broth of *Cunninghamella* sp. F-1490. The structure of ICM0201 was determined to be (3S, 10aR)-3, 4a-dihydroxy-2,3,4,4a-tetrahydro-2H-pyrano[3,2-b]benzo[e]morpholine-9-carboxylic acid by spectroscopic analyses and chemical studies. The structure of 1 is unique in that the tricycle ring system is composed of aminol and hemiacetal bonds.

In the course of our screening program for new compounds that inhibit murine osteoclastogenesis in culture, we isolated ICM0201 (1) from a culture filtrate of fungal strain F-1490. In the preceding paper, we reported the taxonomy of producing strain, fermentation, isolation, and biological activities of 1. In this paper, we describe the physico-chemical properties, structure elucidation and the chemical synthesis of 1.

**Results and Discussion**

Physico-chemical Properties of ICM0201 (1)

ICM0201 (1) was isolated as a pale yellow powder with an acidic nature. The physico-chemical properties are summarized in Table 1. The IR spectrum of 1 showed a strong absorption at 1680 cm\(^{-1}\) (\(\alpha,\beta\)-unsaturated carboxylic acid). Compound 1 showed a characteristic UV absorption maxima at 217, 245 and 328 nm in MeOH. Compound 1 is soluble in MeOH, DMSO and H\(_2\)O.

![Fig. 1. Structure of ICM0201 (1).](image)

Structural Elucidation of ICM0201 (1)

The molecular formula of 1 was determined to be C\(_{12}\)H\(_{13}\)N\(_{1}\)O\(_6\) by HRFAB-MS and NMR spectral analyses. The \(^{13}\)C NMR and \(^1\)H NMR spectra of 1 in pyridine-\(d_5\) revealed twelve carbons and nine protons, indicating the presence of four deuterium exchangeable protons in 1. The degree of unsaturation of 1 was estimated to be seven by the molecular formula, C\(_{12}\)H\(_{13}\)N\(_{1}\)O\(_6\). Compound 1 contained six \(sp^2\) carbons consisting of three olefinic methine and three olefinic quaternary carbons, and one carbonyl carbon based on the DEPT spectra. Since the signals of six olefinic carbons and one carbonyl carbon account for four degrees of unsaturation, so the remainder must be due to the three rings of 1. The correlation between H-8 (\(\delta_H\) 8.01), H-7 (\(\delta_H\) 6.76), and H-6 (\(\delta_H\) 7.28) confirmed by \(^1\)H-\(^1\)H COSY showed the presence of 1,2,3-trisubstituted benzene as NMR spectral analyses. The \(^{13}\)C NMR and \(^1\)H NMR spectra of 1 in pyridine-\(d_5\) revealed twelve carbons and nine protons, indicating the presence of four deuterium exchangeable protons in 1. The degree of unsaturation of 1 was estimated to be seven by the molecular formula, C\(_{12}\)H\(_{13}\)N\(_{1}\)O\(_6\). Compound 1 contained six \(sp^2\) carbons consisting of three olefinic methine and three olefinic quaternary carbons, and one carbonyl carbon based on the DEPT spectra. Since the signals of six olefinic carbons and one carbonyl carbon account for four degrees of unsaturation, so the remainder must be due to the three rings of 1. The correlation between H-8 (\(\delta_H\) 8.01), H-7 (\(\delta_H\) 6.76), and H-6 (\(\delta_H\) 7.28) confirmed by \(^1\)H-\(^1\)H COSY showed the presence of 1,2,3-trisubstituted benzene as
shown in Fig. 2. An aromatic proton H-8 showed a long range coupling to a carbonyl carbon (δ_C 171.7), which is assignable to a carboxylic acid carbon based on the chemical shift, IR spectrum and the acidic nature of 1. The sequence from methylene protons H-4 (δ_H 2.45, 3.30) to methylene protons H-2 (δ_H 3.78, 4.40) through a methine proton H-3 (δ_H 4.64) was confirmed by 1H-1H COSY.

According to their 1H and 13C chemical shifts, C-3 (δ_C 64.1) and C-2 (δ_C 72.0) were assigned to oxymethine and oxymethylene, respectively. A long range coupling was observed from the oxymethylene (H-2) to C-10a (δ_C 81.2). Therefore, C-2 and C-10a had to be connected by an ether bond. In addition, long range couplings were observed from the methylene protons H-4 to a quaternary carbon C-4a (δ_C 142.6).
92.3) and the methine C-10a ($\delta_C$ 81.2) resulting in the presence of tetrahydropyrane ring. Furthermore, a singlet methine proton H-10a ($\delta_H$ 5.12) showed a long range coupling to C-9a ($\delta_C$ 135.6). The characteristic low-field ($\delta_C$ 81.2) sp$^3$ carbon was assignable to a aminal carbon$^{2-4)}$. A weak $^4$J$_{CH}$ coupling between one of H-4 protons ($\delta_H$ 2.45) to C-5a ($\delta_C$ 142.6) was observed in HMBC spectrum indicating the connectivity between C-4a ($\delta_C$ 92.3) and C-5a ($\delta_C$ 142.6) through a hemiacetal bond based on the characteristic low-field chemical shifts$^{5,6)}$ of C-4a. Subsequently, C-3 methine should bear the remaining deuterium exchangeable proton to form a hydroxyl group. Thus, the planar structure of 1 except for the stereochemistry was deduced as shown in Fig. 1. Treatment of 1 with 4N-HCl at 80°C for 1 hour gave 3-hydroxyanthranilic acid in a good yield. The result is consistent with the proposed structure. The structure was further confirmed by the total synthesis as described bellow.

Relative Stereochemistry of ICM0201

The relative stereochemistry of tetrahydropyrane moiety was examined by the differential $^1$H decoupling NOE experiments. Apparent NOEs were observed between H-10aax/H-2ax and H-10ax/H-4ax, which would be ascribed to 1,3-diaxial relationships. The large coupling constants ($J_{2ax,3ax}=J_{10ax,4ax}=11$ Hz and $J_{3ax,4eq}=13$ Hz) and the small coupling constants ($J_{2eq,3ax}=J_{3ax,4eq}=5$ Hz) in $^1$H NMR spectrum of 1 are indicative of the chair-conformation. Thus, the configuration of 3-OH and 10a-NH has to be both equatorial. On the other hand, 1 exists in epimeric mixture at C-4a through hemiacetal-ketone tautomerism based on the observation of NMR, MS and HPLC properties.

Synthesis of 1

To clarify the absolute configuration of C-3, 1 and its enantiomer were synthesized as outlined in Scheme 1. The key intermediates 3-deoxy-d-glycero-pentos-2-ulose (3a) and its enantiomer (3b) were obtained from d-(+)- or l-(-)-xylose by the method of H. EL. KHADEM et al.$^{7)}$. Osazone 2a was obtained by refluxing d-(+)-xylose and benzoylhydrazine in the presence of p-toluidine in 35~40% yield. Compound 2a was converted into 3a by transhydrazonation with benzaldehyde in 40~50% yield. Condensation of 3-hydroxy-anthranilic acid with 3a was carried out by stirring in a mixture of MeOH and 1,4-dioxane at room temperature for 20 hours to afford 1a in 40~50% yield. Compound 1b was similarly synthesized from l-(-)-xylose. The physico-chemical properties of both enantiomers were identical in $^1$H and $^{13}$C NMR, IR, and mass spectra with those of natural product 1. The optical rotation value $[\alpha]_D^{22}=-58.3^\circ$ (c 0.9, MeOH) of 1a synthesized from d-(+)-xylose was consistent with $[\alpha]_D^{22}=-57.5^\circ$ (c 0.4, MeOH) of a natural product 1. On the other hand, the optical rotation value $[\alpha]_D^{22}+59.3^\circ$ (c 0.4, MeOH) of 1b synthesized from l-(-)-xylose was opposite to those of 1 and 1a.

Based on the above results, the absolute configuration of ICM0201 was elucidated as (3S,10aR)-configuration.

Experimental

General

Melting points were determined on a Yanagimoto micro melting point apparatus. UV and IR spectra were recorded on a Hitachi 228A spectrometer and a Horiba FT-200
fourier transfer infrared spectrometer, respectively. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectra were measured with a JEOL JNM-A400 spectrometer in pyridine-d$_5$. Chemical shifts were given in ppm using TMS as an internal standard. ESI-MS spectra were measured on a Sciex API-165 mass spectrometer. HRFAB-MS spectra were measured with a VG AutoSpec mass spectrometer. Most chemicals and solvents were analytical grade and used without further purification.

### Acid Hydrolysis of 1 (Preparation of 3-Hydroxy Anthranilic Acid)

Compound 1 (10 mg) was dissolved in 4N HCl (0.5 ml) and the mixture was stirred at 80°C for 1 hour. After the reaction mixture was concentrated in vacuo, the residue was purified by a preparative HPLC (CAPCELL PAK C$_{18}$, 20 mm×250 mm, 30% MeOH containing 0.1% TFA) to give 3-hydroxy-anthranilic acid (3 mg, 52%) as a brownish powder, mp 237–242°C (dec); ESI-MS (positive mode) $m/z$ 154 [M+H]$^+$; $^1$H NMR (pyridine-d$_5$): $\delta$H 6.72 (1H, t, J=8Hz), 7.22 (1H, dd, J=2, 8Hz), 8.10 (1H, dd, J=2, 8Hz); $^{13}$C NMR (pyridine-d$_5$): $\delta$C 112.3, 115.0, 117.5, 122.8, 146.2, 146.2, 172.0.

### 3-Deoxy-d-glycero-pentos-2-ulose Bis(benzoylhydrazone) (2a)

A solution of d-xylose (10.0 g), benzoylhydrazine (12.8 g), and p-toluidine (4.0 g) in ethanol (200 ml) containing 4 ml of acetic acid was boiled under reflux for 3 hours, and cooled. The resulting solid was washed with ethanol and diethyl ether successively to afford 9.4 g (39%) of a yellow powder, mp 228–230°C (lit.$^7$) mp 228–230°C; ESI-MS (positive mode) $m/z$ 391 [M+Na]$^+$, ESI-MS (negative mode) $m/z$ 367 [M−H]$^−$.

### 3-Deoxy-d-glycero-pentos-2-ulose Bis(benzoylhydrazone) (2a)

To a solution of 3-deoxy-d-glycero-pentos-2-ulose bis(benzoylhydrazone) (2a, 4.3 g) in EtOH−H$_2$O (140 ml: 230 ml) were added acetic acid (6 ml) and benzaldehyde (8 ml). After refluxing for 6 hours, ethanol was removed by simultaneous addition of 230 ml of water, and cooled. After filtration, the filtrate was concentrated to 90 ml and washed with diethyl ether (40 ml×6). An aqueous solution was passed through an activated charcoal column (10 ml, Wako Pure Chemical Co. Ltd.). The filtrate was evaporated to afford 770 mg (50%) of yellow syrup. ESI-MS (positive mode) $m/z$ 155 [M+Na]$^+$, 287 [2M+Na]$^+$, (negative mode) $m/z$: 131 [M−H]$^−$, 263 [2M−H]$^−$.

Compound 3a was converted into crystalline bis[(2,4-dinitrophenyl)hydrazone] by the conventional method, mp 257–259°C (dec) (lit.$^7$) mp 258°C).

This product (3a) was used without further purification in the next reaction.

### 3(S,10aR)-3,4a-Dihydroxy-2,3,4,4a-tetrahydro-2H-pyran-3,2b]benzof[e]morpholine-9-carboxylic Acid (1a)

To a solution of 3a (734 mg) in MeOH−1,4-dioxane (10 ml: 10 ml) was added 3-hydroxyanthranilic acid...
(850 mg), and the mixture was stirred for 20 hours at room temperature. The residue was chromatographed on a CAPCELL PAK C18 (20×250 mm, 9 mL/minute) with 18% MeOH containing 0.05% TFA. The fractions (58–71 minutes) containing 1a were applied to a Diaion HP-20 (Mitsubishi Kasei) column equilibrated with H2O. After the column was washed with H2O and 10% MeOH, 1a was eluted with MeOH to afford 752 mg (51%) of pale yellow powder. mp 124–132°C (dec); [α]D 22 +58.3° (c 0.9, MeOH); ESI-MS (positive mode) m/z 268 [M+H]+, 290 [M+Na]+, ESI-MS (negative mode) m/z 266 [M−H]−; 13C NMR (pyridine-d5): δC 45.9 (C-4), 64.2 (C-3), 72.0 (C-2), 81.2 (C-10a), 92.3 (C-4a), 114.8 (C-9), 116.5 (C-7), 120.8 (C-6), 124.9 (C-8), 135.6 (C-9a), 142.6 (C-5a), 172.1 (9-COOH).

Using the same procedures, the following derivatives were obtained.

3-Deoxy-L-glycero-pentos-2-ulose Bis(benzoylhydrazone) (2b)

mp 225–226°C (dec); ESI-MS (positive mode) m/z 391 [M+Na]+, ESI-MS (negative mode) m/z 367 [M−H]−.

Transhydrazonation of 2b afforded 3b: ESI-MS (negative mode) m/z 131 (M−H)−, 263 (2M−H)−.

(3R,10aS)-3,4a-Dihydroxy-2,3,4,4a-tetrahydro-2H-pyran[3,2-b]benz[e]morpholine-9-carboxylic Acid (1b)

mp 125–131°C (dec); [α]D 22 +59.3° (c 0.4, MeOH); ESI-MS (positive mode) m/z 268 [M+H]+, 290 [M+Na]+, ESI-MS (negative mode) m/z 266 [M−H]−; 13C NMR (pyridine-d5): δC 45.9 (C-4), 64.1 (C-3), 72.0 (C-2), 81.1 (C-10a), 92.3 (C-4a), 114.8 (C-9), 116.5 (C-7), 121.1 (C-6), 124.8 (C-8), 135.6 (C-9a), 142.6 (C-5a), 171.7 (9-COOH).

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References