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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 (3*S*,10a*R*)-3,4a-dihydroxy-2,3,4,4a-tetrahydro-2*H*-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 aminal 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[1], 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} (α,β-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_2O.

Structural Elucidation of ICM0201 (1)

The molecular formula of **1** was determined to be $C_{12}H_{13}N_1O_6$ by HRFAB-MS and NMR spectral analyses. The structure determination of **1** was examined by various 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, $C_{12}H_{13}N_1O_6$. 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 (δ_H 8.01), H-7 (δ_H 6.76), and H-6 (δ_H 7.28) confirmed by ^1H-^1H COSY showed the presence of 1,2,3-trisubstituted benzene as

Fig. 1. Structure of ICM0201 (1).

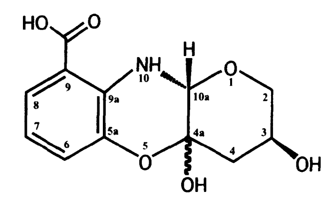

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Table 1. Physico-chemical properties of **1**.

Appearance	Pale yellow powder
Mp	125~131°C (dec)
$[\alpha]_D^{22}$ (MeOH)	-57.5° (*c* 0.4)
Molecular formula	$C_{12}H_{13}N_1O_6$
ESI-MS (*m/z*)	
Positive	268 $[M+H]^+$, 290 $[M+Na]^+$
Negative	266 $[M-H]^-$
HRFAB-MS (*m/z*)	
Calcd:	268.0821 for $C_{12}H_{14}N_1O_6$
Found:	268.0861 $(M+H)^+$
UV λ_{max} nm (ε) in MeOH	217 (27,500), 245 (sh, 6,800), 328 (5,000)
IR ν (KBr) cm^{-1}	3380, 1680, 1620, 1500, 1440, 1375, 1260, 1090, 1035

Table 2. ^1H and ^{13}C NMR chemical sifts of **1**.

	δ_C	δ_H
2	72.0	3.78 (1H, dd, *J*=11, 11Hz), 4.40 (1H, ddd, *J*=2, 5, 11 Hz)
3	64.1	4.64 (1H, dddd, *J*=5, 5, 11, 11 Hz)
4	45.9	2.45 (1H, dd, *J*=11, 13 Hz), 3.30 (1H, ddd, *J*=2, 5, 13 Hz)
4a	92.3	
5a	142.6	
6	121.1	7.28 (1H, dd, *J*=1, 8 Hz)
7	116.6	6.76 (1H, t, *J*=8 Hz)
8	124.8	8.01 (1H, dd, *J*=1, 8 Hz)
9	114.1	
9a	135.6	
10a	81.2	5.12 (1H, s)
9-COOH	171.7	

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 ^{13}C 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 quaternaty carbon C-4a (δ_C

Fig. 2. Structure of **1** elucidated by ^{1}H-^{1}H COSY (—) and HMBC (→) experiments.

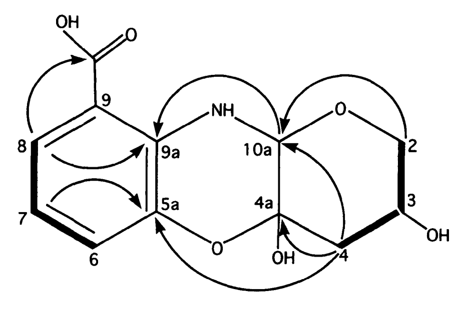
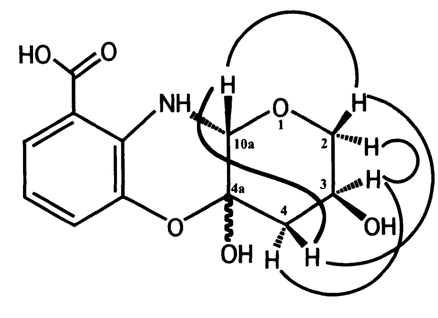


Fig. 3. NOE correlations of **1**.



92.3) and the methine C-10a (δ_C 81.2) resulting in the presence of tetrahydropyrane ring. Furthermore, a singlet methine proton H-10a (δ_H 5.12) showed a long range coupling to C-9a (δ_C 135.6). The characteristic low-field (δ_C 81.2) *sp*^{3} carbon was assignable to a aminal carbon[2~4]. A weak $^4J_{C\text{-}H}$ coupling between one of H-4 protons (δ_H 2.45) to C-5a (δ_C 142.6) was observed in HMBC spectrum indicating the connectivity between C-4a (δ_C 92.3) and C-5a (δ_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 4 N-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-10a$_{ax}$/H-2$_{ax}$ and H-10a$_{ax}$/H-4$_{ax}$, which would be ascribed to 1,3-*diaxial* relationships. The large coupling constants ($J_{2ax,3ax}=J_{3ax,4ax}=11$ Hz and $J_{4ax,4eq}=13$ 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° (*c* 0.9, MeOH) of **1a** synthesized from D-(+)-xylose was consistent with $[\alpha]_D^{22}$ −57.5° (*c* 0.4, MeOH) of a natural product **1**. On the other hand, the optical rotation value $[\alpha]_D^{22}$ +59.3° (*c* 0.4, MeOH) of **1b** synthesized from L-(−)-xylose was opposite to those of **1** and **1a**.

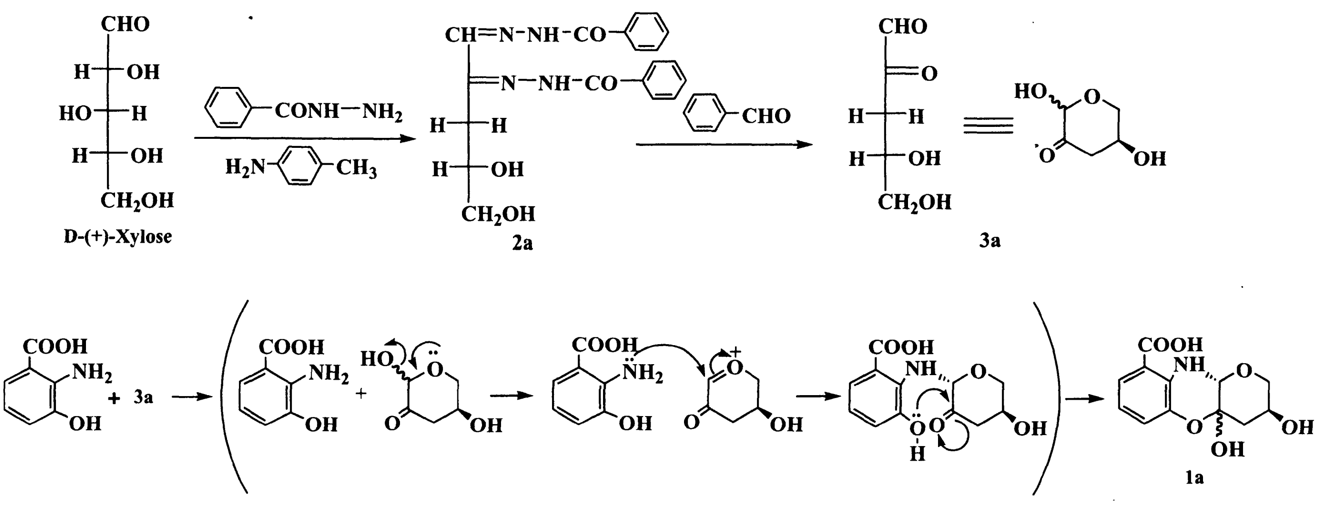
Based on the above results, the absolute configuration of ICM0201 was elucidated as (3*S*,10a*R*)-configuration.

Experimental

General

Melting points were determinred on a Yanagimoto micro melting point apparatus. UV and IR spectra were recorded on a Hitachi 228A spectrometer and a Horiba FT-200

Scheme 1



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 4 N 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): δ_H 6.72 (1H, t, J=8 Hz), 7.22 (1H, dd, J=2, 8 Hz), 8.10 (1H, dd, J=2, 8 Hz); ^{13}C NMR (pyridine-d_5): δ_C 112.3, 115.0, 117.5, 122.8, 142.9, 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 (**3a**)

To a solution of 3-deoxy-D-*glycero*-pentos-2-ulose bis(benzoylhydrazone) (**2a**, 4.3 g) in EtOH - H_2O (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*,10a*R*)-3,4a-Dihydroxy-2,3,4,4a-tetrahydro-2*H* pyrano[3,2*b*]benzo[*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 C_{18} (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 equibrated with H_2O. After the column was washed with H_2O and 10% MeOH, **1a** was eluted with MeOH to afford 752 mg (51%) of pale yellow powder. mp 124~132°C (dec); $[\alpha]_D^{22}$ −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 $[M-H]^-$; ^{13}C NMR (pyridine-d_5): δ_C 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 $(2M-H)^-$.

(3*R*,10a*S*)-3,4a-Dihydroxy-2,3,4,4a-tetrahydro-2*H*-pyrano[3,2*b*]benzo[*e*]morpholine-9-carboxylic Acid (**1b**)

mp 125~131°C (dec); $[\alpha]_D^{22}$ +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 $[M-H]^-$; ^{13}C NMR (pyridine-d_5): δ_C 45.9 (C-4), 64.1 (C-3), 72.0 (C-2), 81.1 (C-10a), 92.2 (C-4a), 113.8 (C-9), 116.6 (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

1) Inoue, H.; H. Kumagai, M. Osono, M. Matsufuji, T. Sameshima, N. Kawamura, T. Someno, M. Ishizuka & T. Takeuchi: ICM0201, a new inhibitor of osteoclastogenesis from *Cunninghamella* sp. F-1490. I. Taxonomy, fermentation, isolation and biological activity. J. Antibiotics 56: 209~213, 2003
2) Kondo, K.; H. Adachi, E. Shitara, F. Kojima & Y. Nishimura: Glycosidase inhibitors of *gem*-diamine 1-*N*-iminosugars: Structures in media of enzyme assays. Bioorg. Med. Chem. 9: 1091~1095, 2001
3) Argoudelis, A. D.; F. Reusser, S. A. Mizsak & L. Baczynskyj: Antibiotics produced by *Streptomyces ficellus*. II. Feldamycin and nojirimycin. J. Antibiotics 29: 1007~1014, 1976
4) Ueno, M.; T. Someno, R. Sawa, H. Iinuma, H. Naganawa, M. Ishizuka & T. Takeuchi: Delaminomycins, novel extracellular matrix receptor antagonist. III. Physico-chemical properties and structure elucidation of delaminomycins B and C. J. Antibiotics 46: 1020~1023, 1993
5) Nakajima, H.; S. Takase, H. Terano, & H. Tanaka: New antitumor substance, FR901465. III. Structure of FR901463, FR901464 and FR901465. J. Antibiotics 50: 96~99, 1997
6) Funayama, S. & S. Nozoe: Isolation and structure of a new polyether antibiotic, octacyclomycin. J. Antibiotics 45: 1686~1691, 1992
7) Khadem, H. El.; D. Horton, M. H. Meshreki & M. A. Nashed: New route for the synthesis of 3-deoxyaldos-2-uloses. Carbohyd. Res. 17: 183~192, 1971