Preparation of Optically Active Epiderstatin and Its Stereoisomers—Epiderstatin is Not a Real Inhibitor of the Mitogenic Activity Induced by Epidermal Growth Factor—

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Epiderstatin (**1a**) was isolated from a culture broth of *Streptomyces pulveraceus* as an inhibitor of mitogenic activity induced by epidermal growth factor (EGF)[1,2] and was also reported to reverse the morphology of *src*^{ts}-NRK cells from the transformed phenotype to the normal phenotype at the permissive temperature (32°C).[3] Using NMR, X-ray crystallographic analysis, COSMIC (Computation and Structure Manipulation In Chemistry) force field calculation and circular dichroism, we established the absolute structure of epiderstatin (Fig. 1).[4] Since it was isolated in very minute quantity, we undertook a total synthesis of (±)-epiderstatin.[5]

In the course of our study on structure-activity relationship within epiderstatin analogues,[6] we needed to prepare optically active epiderstatin as an authentic sample. Enough amounts of (±)-epiderstatin and its C_3-epimer for optical resolution were prepared according to a practical method reported by Dow *et al.*[7] Optical resolution of each analogue was carried out by chiral HPLC to give optically pure epiderstatin (**1a**) and its stereoisomers, (**1b**), (**2a**), (**2b**).

The absolute configurations of synthetic epiderstatin and its stereoisomers, **1a**, **1b**, **2a**, **2b**, were determined by NMR and CD analyses (Fig. 1). The absolute configurations of C_3/C_5 *trans* isomers, **1a** and **1b** were determined by the comparison of their Cotton effects, $\lambda_{287}+1.78$ ($\Delta\varepsilon$) for **1a** and $\lambda_{287}-2.15$ ($\Delta\varepsilon$) for **1b** with the data ($\lambda_{287}+1.78$) of natural epiderstatin[4]. The absolute configurations of *cis* isomers, **2a**, **2b**, could be determined by their Cotton effects ($\lambda_{288}+2.60$ ($\Delta\varepsilon$) for **2a**, $\lambda_{288}-2.20$ ($\Delta\varepsilon$) for **2b**) and the application of allylic axial chirality approach[4] at C_5 axial protons which were deducible from the large coupling constant value (12.7 Hz) between 4-H and 5-H for each isomer (Fig. 2).

After separation and characterization of each isomer, we examined the flat reversion activities on *src*^{ts}-NRK cells, the cell cycle inhibitory activities on tsFT-210 cells, effects on [^{3}H]thymidine uptake into EGF-stimulated Balb/MK cells and antifungal activities. Surprisingly, any isomer including synthetic epiderstatin did not induce the flat reversion of the *src*^{ts}-NRK cells.

To elucidate the reason why optically pure synthetic epiderstatin did not induce the flat reversion of *src*^{ts}-NRK cells, we inspected the original ^{1}H NMR chart of natural epiderstatin[1] and found tiny signals due to a secondary methyl (δ 1.02, d, $J=6.9$ Hz) and a tertiary methyl (δ 2.0, s) groups, which could be assigned respectively to the C_3-methyl and C_5-acetyl methyl protons of acetoxycycloheximide.[8,9] From the integration of the signals, it was considered that approximately 10% of acetoxycycloheximide were contaminated in the sample. This was further supported by the result of chiral HPLC analysis followed by biological examinations. In the chiral HPLC, the contaminant, acetoxycycloheximide, was also clearly detected and after separation by the same way, natural epiderstatin lost the strong activity on the morphology reversion. On the other hand, the synthetic epiderstatin including 10% of acetoxycycloheximide showed activity on flat reversion of *src*^{ts}-NRK cells. However the synthetic epiderstatin did not show any synergistic effect on acetoxycycloheximide (Table 1). All stereoisomers including synthetic epiderstatin did not inhibit [^{3}H]thymidine uptake into EGF-stimulated Balb/MK cells at the dose below that showed cytotoxicity. Also, synthetic epiderstatin and its stereoisomers, **1b**, **2a** and **2b** did not show any effects on the cell cycle of the tsFT-210 cells and antifungal activities against *Piricularia oryzae*.

From the results above mentioned, all the effects of epiderstatin previously reported such as inhibition of the signal transduction of EGF stimulated BALB/MK cells,[1] induction of the flat reversion activity of *src*^{ts}-NRK cells,[3] inhibition of the blastogenesis of mouse spleen cells,[10] overexpression of c-*fos* and the suppression of c-*myc* transcription in EGF stimulated BALB/

Fig. 1. Structures of epiderstatin and its stereoisomers.

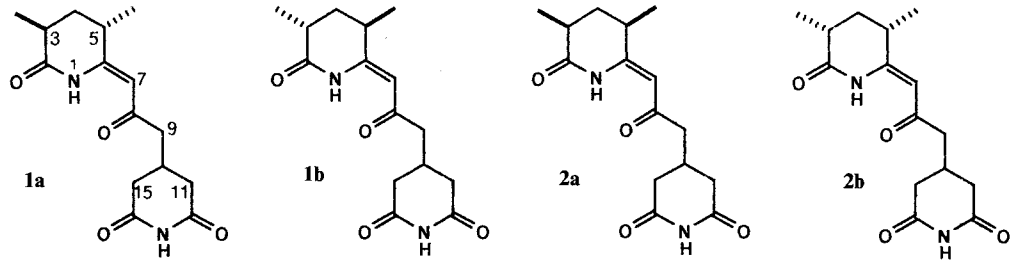

Fig. 2. Molecular conformation of **2a** calculated on Nemesis program.

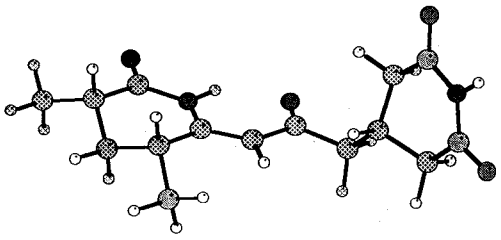

Table 1. Effects of epiderstatin analogues on the flat reversion of *src*^{ts}-NRK cells.

Compound	Minimal effective value (μg/ml)
1a (synthetic)	>50
1a (natural)	0.003*
1a (natural, after chiral HPLC)	5
ACHI	0.005
1a (syn): ACHI 10:1)	0.05
1b	>50
2a	>50
2b	>50

* Reported value.[3] The assay were done as a previously described method.[3] ACHI: acetoxycycloheximide.

MK cells,[11] could be ascribed to the presence of acetoxycycloheximide as a contaminant.

Experimental

General Methods

Melting points were obtained using a Yanagimoto micro-melting point apparatus and were uncorrected. Optical rotations were taken on a JASCO DIP-370 polarimeter. IR spectra were recorded on a Shimadzu FTIR-8100M fourier transform infrared spectrophotometer. CD spectra were measured on a JASCO J-270 spectropolarimeter. ^{1}H and ^{13}C NMR were measured on JEOL EX-270, α-400 and GSX-500 instruments. MS were measured on Hitachi M-80 and JEOL HX-110 mass spectrometers.

Analytical HPLC was carried out on a Waters 600 multisolvent delivery HPLC system equipped with a Waters 990J photodiode array detector. Preparative HPLC was carried out on a HPLC system equipped with a Hitachi L-6000 pump, a SSC UV detector and a SSC-2100 oven.

Epiderstatin (**1a**), (3*R*,5*R*)-isomer (**1b**), (3*S*,5*R*)-isomer (**2a**), (3*R*,5*S*)-isomer (**2b**)

Optical resolution of (±)-epiderstatin which was prepared by a previously described method[7] was carried out by chiral HPLC (CHIRALPAK AS, 10×250 mm; hexane-EtOH (55:45), Flow rate 2.3 ml/minute; detector wave length, 295 nm; temperature, 25°C); retention time (Rt) for **1a**, 23.6 minutes; Rt for **1b**, 31.1 minutes. Compound **1a**: MP 168~169°C; $[\alpha]_D^{24}$ −7.5° (*c* 0.85, CH_2Cl_2); EI-MS *m/z* 292 (M^+); ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 1.24 (3H, d, *J*=6.8 Hz, 3-CH_3), 1.29 (3H, d, *J*=7.3 Hz, 5-CH_3), 1.82 (2H, m, H-4), 2.22 (2H, dd, *J*=17.3, 10.5 Hz, H-11ax and H-15ax), 2.48 (1H, d, *J*=6.3 Hz, H-9), 2.69 (1H, dd, *J*=17.3, 6.3 Hz, H-15eq), 2.60~2.75 (3H, m, H-5, H-3 and H-10), 2.70 (1H, dd, *J*=17.3, 4.4 Hz, H-11eq), 5.24 (1H, s, H-7), 8.20 (1H, br s, 13-NH), 11.54 (1H, br s, 1-NH). Compound **1b**: MP 153~154°C; $[\alpha]_D^{24}$ +7.1° (*c* 0.86, CH_2Cl_2). Optical resolution of C_3-epimer of (±)-epiderstatin which was prepared by a previously described method[6] was carried out by chiral HPLC (CHIRALPAK AS, 10×250 mm; hexane-EtOH (60:40), Flow rate 2.4 ml/minute; detector wave length, 295 nm; temperature, 25°C); Rt for **2a**, 30.1 minutes; Rt for **2b**, 37.3 minutes. Compound **2a**: MP 211~218°C; $[\alpha]_D^{28}$ −3.2° (*c* 1.0, CH_2Cl_2); EI-MS *m/z* 292 (M^+); UV λ_{max} nm (log ε) 295 (4.13); ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 1.21 (3H, d, *J*=6.8 Hz, 3-CH_3), 1.22 (3H, d, *J*=6.8 Hz, 5-CH_3), 1.51 (1H, dt, *J*=13.2, 12.7 Hz, H-4ax), 1.95 (1H, ddd, *J*=13.2, 4.9, 4.3 Hz, H-4eq), 2.32 (2H, dd, *J*=17.8, 11.0 Hz, H-11ax and H-15ax), 2.50 (1H, d, *J*=6.3 Hz, H-9), 2.63~2.75 (4H, m, H-11eq, H-15eq, H-3 and H-5), 2.50 (1H, d, *J*=6.3 Hz, H-9), 2.54 (1H, m, H-10), 5.29 (1H, s, H-7), 8.24 (1H, br s, 13-NH), 11.76 (1H, br s, 1-NH). Compound **2b**: MP 202~204°C; $[\alpha]_D^{28}$ +2.1° (*c* 0.86, CH_2Cl_2).

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