DOTRIACOLIDE, A NEW β-LACTAMASE INHIBITOR

Sir:

In our screening study for new β-lactamase inhibitors, we have found a new inhibitor and named it dotriacolide. This is produced by *Micromonospora echinospora* MG299-fF35 together with its dihydro derivative. The strain was isolated from a soil sample collected at Tokachigawa Hot Springs in Hokkaido, Japan. Dotriacolide resembles to izumenolide[1~4] produced by *Micromonospora chalcea* subsp. *izumensis*, but differs in the number of the *O*-sulfate groups and the ring size of the lactone.

The fermentation was carried out at 27°C for 7 days under aeration at a rate of 7.5 liters per minute and agitation at 300 rpm in a 30-liter jar fermentor containing 15 liters of a production medium (2.0% galactose, 1.0% soy peptone, 0.5% corn steep liquor, 0.2% $(NH_4)_2SO_4$ and 0.2% $CaCO_3$, adjusted to pH 7.4). The fermentation was inoculated with 1.3% (volume) of a seed culture prepared as follows. The strain was first cultured for 3 days at 30°C on a reciprocal shaker (120 strokes per minute) in a Sakaguchi flask containing 120 ml of a seed medium (1.0% glucose, 1.0% glycerol, 1.0% sucrose, 2.0% soybean meal, 1.0% dry yeast, 0.5% oat meal, 0.5% Casamino acids (Difco) and 0.2% $CaCO_3$, adjusted to pH 7.4) and the culture was then used to inoculate (3% by volume) 110 ml of the production medium described above in a 500-ml baffled Erlenmeyer flask and cultured for 4 days at 27°C on a rotatory shaker (180 rpm).

The β-lactamase-inhibiting activity was determined by a plate method[5] against penicillinase obtained from *Escherichia coli* ML2825 using dihydrodotriacolide tetrasodium salt (1,000 μg/mg) as the assay standard.

The fermentation broth was harvested from three jar fermentors and centrifuged to obtain 39.5 liters of the supernatant (pH 7.3, 360 μg/ml). The inhibitors in the supernatant were adsorbed on a column of Diaion HP-20 (a macroreticular resin, Mitsubishi Chemical Industries Ltd., 5 liters) and eluted with a 1:1 mixture of acetone and 0.005 M sodium phosphate buffer (pH 8.5). The active eluate was concentrated to dryness and a brownish powder (60 g, 225 μg/ml) was obtained. An aqueous solution of the powder (3.3 g/330 ml) was washed twice with 330 ml of 1-butanol at pH 7 and then concentrated to give 2.4 g of a crude powder. The crude powder in 22 ml of water was purified by column chromatography on cellulose powder (Avicel, 2.5 liters) developed with a 9:1 mixture and thereafter with a 7:3 mixture of 2-propanol and water to give 1.1 g of a yellowish powder (potency 315 μg/mg). The powder (542 mg) was chromatographed successively on two columns of silica gel (Wakogel C-200, Wako Pure Chemical Industries, Ltd.) developed with a mixture (4:1) of acetonitrile and water, and with a mixture (8:1:2) of 1-butanol, methanol and water (98.3 mg, 890 μg/mg). The inhibitors were further purified by Sephadex LH-20 column chromatography developed with 80% aqueous methanol, yielding a colorless hygroscopic powder (82.5 mg, 1,000 μg/mg) of the purest sample of dotriacolide tetrasodium salt, mp 118°C (decomp.); $[\alpha]_D^{22}+10°$ (*c* 1, water); UV (water) 211 nm; IR(KBr) 3450, 2940, 2860, 1710, 1655, 1470, 1400, 1250, 1220, 1065, 940 cm^{-1}; ^1H NMR (D_2O, TMS as an external reference) δ 1.6~3.0 (CH_3, CH_2), 5.0 (CH–OSO_3 ×4), 5.6 (CH–OCO), 6.3 (d, J= 16 Hz, CO–CH=), 7.5 (dt, J=16, 8 Hz, C= CH–); positive anisaldehyde - H_2SO_4 and phosphomolybdic acid - H_2SO_4 reactions; soluble in water and methanol; insoluble or almost insoluble in ethanol and other organic solvents; TLC (silica gel with a 4:1:2 mixture of 1-butanol, methanol and water) a single spot at Rf 0.30. *Anal.* Calcd. for $C_{40}H_{72}O_{18}S_4Na_4 \cdot H_2O$: C 44.52, H 6.91, O 28.17, S 11.88. Found: C 44.20, H 6.89, O 27.83, S 12.45.

Dotriacolide showed a marked inhibitory activity not only against penicillinase but also against cephalosporinase when the activity was determined by UV spectrophotometric method[5]. The ID_{50} values of the tetrasodium salt were 0.61 μg/ml against penicillinase obtained from *E. coli* ML2825 when benzylpenicillin was used as the substrate and 0.15 μg/ml against cephalosporinase of *Citrobacter freundii* GN346 when cephaloridine was used. Dotriacolide tetrasodium salt did not inhibit any test organisms belonging to Gram-positive and -negative bacteria at 100 μg/ml. Acute LD_{50} values in mice were 0.18~0.35 mg/kg intravenously and more than 250 mg/kg orally.

MS spectrometry of the permethylated compound which was obtained by *O*-methylation of a methanolysis compound of the purest sample of

dotriacolide showed the $(M+1)^+$ peak at *m/z* 755 together with the peak at *m/z* 757, suggesting the coexistence with a dihydro derivative. ^1H NMR spectroscopy ($CDCl_3$) of the peracetylated compound which was derived from the methanolysis compound by acetylation with acetic anhydride in pyridine exhibited two ester methyl signals at δ 3.66 and 3.73, and the former signal was superimposed on that of the peracetylated compound derived from dihydrodotriacolide. From the strength of both signals, it was calculated that about 30% of dihydrodotriacolide tetrasodium salt was contained in the purest sample of dotriacolide tetrasodium salt.

Catalytic hydrogenation of dotriacolide (**1**) tetrasodium salt in water with platinum dioxide in a Parr apparatus at 3.7 kg/cm² overnight gave dihydrodotriacolide (**2**) tetrasodium salt as the colorless hygroscopic powder, mp 122°C (decomp.), $[\alpha]_D^{22}+12°$ (*c* 1, water). *Anal.* Calcd. for $C_{40}H_{74}O_{18}S_4Na_4 \cdot 2H_2O$: C 43.71, H 7.15, O 29.11, S 11.67. Found: C 43.88, H 7.60, O 29.19, S 11.70. It was very similar to **1** in their physicochemical and biological properties, but showed no UV maximum at a region of 200~400 nm in water and no olefinic proton in the ^1H NMR spectrum. The inhibitors **1** and **2** had almost the same biological properties including β-lactamase-inhibiting activity. These biological properties will be reported elsewhere.

The structures of **1** and **2** were elucidated by the following chemical and spectrometric studies. Methanolysis of **2** tetrasodium salt with 1.5 N hydrogen chloride in methanol overnight at 80°C in a sealed tube gave methyl 15,17,29,31,39-pentahydroxytetracontanoate (**3**) as the waxy solid in 67% yield, mp 103~104°C, $[\alpha]_D^{23}+3°$ (*c* 0.4, 1:1 of chloroform-methanol). *Anal.* Calcd. for $C_{41}H_{82}O_7$: C 71.67, H 12.03. Found: C 71.09, H 11.56. The permethylated compound **4**, colorless oil, $[\alpha]_D^{23}+14°$ (*c* 0.5, chloroform), was prepared by treatment of **3** with methyl iodide and potassium hydride in tetrahydrofuran overnight at room temperature in 35% yield. By the MS analysis of **4** (Fig. 2), the positions of four methoxy groups were confirmed to be 15, 17, 29 and 31. The 39-methoxy group was determined by the ^1H NMR spectrum ($CDCl_3$) of **4**, in which the terminal methyl protons showed a doublet (δ 1.11, J=6 Hz).

In order to determine the ring size of the lactone in **1** and **2**, **2** was converted into methyl 31-oxotetracontanoate (Fig. 2) by a 7-step modification, that is alkaline hydrolysis with a mixture of 1 N NaOH and methanol (5:3) at 60°C for 4.5 hours, oxidation with ruthenium tetroxide in a mixture of chloroform and water (2:3) at 0°C for 2 hours, acid hydrolysis with a mixture of 1 N HCl and dioxane (1:1) at 97°C for 30 minutes, esterification with diazomethane in a mixture of chloroform-methanol-ether (2:2:3) at room temperature for 30 minutes, *O*-mesylation with methanesulfonyl chloride in pyridine at room temperature for 2 hours, iodination with sodium iodide in *N,N*-dimethylformamide at 90°C for 3 days, and then hydrogenolysis with RANEY Ni in dioxane overnight in a Parr aparatus (3.7 kg/cm²). The 32-membered lactone was also confirmed by the method of PARKER *et al.* Reduction of **2** with lithium triethylborohydride in a mixture of dimethyl sulfoxide and tetrahydrofuran, *O*-methylation with methyl iodide and sodium hydride in *N,N*-dimethylformamide, acid hydrolysis in 0.1 N HCl, *O*-tosylation with *p*-toluenesulfonyl chloride in pyridine followed by reduction with lithium triethylborohydride in tetrahydrofuran gave 1,31-dimethoxytetracontane (**6**) which was confirmed by the MS analysis (Fig. 2).

Fig. 1. Structures of dotriacolide (**1**) and dihydrodotriacolide (**2**).

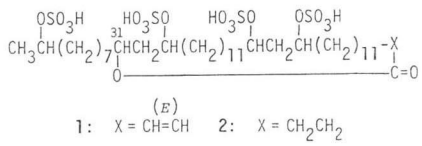
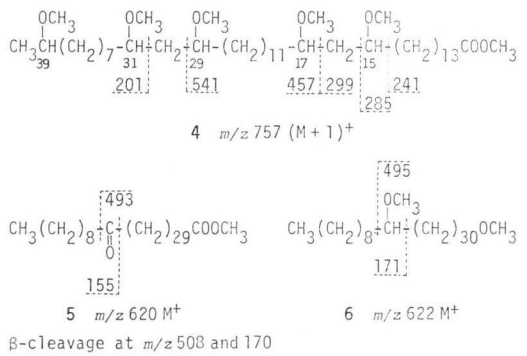


Fig. 2. Significant MS fragments of compounds **4**, **5** and **6**.



From the foregoing results, the structure of sodium salt of dihydrodotriacolide (**2**) was determined to be tetrasodium 15,17,29,31,39-pentahydroxytetracontanoic 1,31-lactone tetrasulfate. In case of **1**, the presence of an *E*-olefin (δ 6.32 and 7.50, J=16 Hz) conjugated with the carbonyl group was shown by the ^{1}H NMR spectrum (D_2O). Therefore, the structure of sodium salt of **1** can be proposed to be (*E*)-tetrasodium 15,17,29,31,39-pentahydroxy-2-tetracontenoic 1,31-lactone tetrasulfate.

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