Sir:

In our screening study for new \(\beta\)-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\(^1\) produced by *Micromonospora chalcea* subsp. *izumenensis*, 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\(_4\))\(_2\)SO\(_4\), 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 \(\beta\)-lactamase-inhibiting activity was determined by a plate method\(^2\) against penicillinase obtained from *Escherichia coli* ML2825 using dihydrodotriacolide tetrasodium salt (1,000 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/mg) of the purest sample of dotriacolide tetrasodium salt, mp 118°C (decomp.); \([\alpha]\)\(_D\)\(^{20}\) +10° (c 1, water); UV (water) 211 nm; IR (KBr) 3450, 2940, 2860, 1710, 1655, 1470, 1400, 1250, 1220, 1065, 940 cm\(^{-1}\); \(^1\)H NMR (D\(_2\)O, TMS as an external reference) 0 1.6-3.0 (CH\(_2\), CH), 5.0 (CH-OSO\(_3\) x 4), 5.6 (CH-OCO), 6.3 (d, J = 16 Hz, CO-CH=), 7.5 (dt, J = 16, 8 Hz, C=CH-); positive anisaldehyde - \(\text{H}_2\text{SO}_4\) and phosphomolybdic acid - \(\text{H}_2\text{SO}_4\) 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\(_{32}\)H\(_{30}\)O\(_{19}\)S\(_4\)Na\(_4\) • H\(_2\)O: 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\(^3\). The ID\(_{50}\) values of the tetrasodium salt were 0.61 \(\mu\)g/ml against penicillinase obtained from *E. coli* ML2825 when benzylpenicillin was used as the substrate and 0.15 \(\mu\)g/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 \(\mu\)g/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 the 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. \(^{1}H\) NMR spectroscopy (\(CDCl_3\)) of the peracetylated compound which was derived from the methanalysis compound by acetylation with acetic anhydride in pyridine exhibited two ester methyl signals at \(\delta\) 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/cm\(^2\) overnight gave dihydrodotriacolide (2) tetrasodium salt as the colorless hygroscopic powder, mp 122°C (decomp.), \([\alpha]_D^{21}\) + 12° (c 1, water). Anal. Calcd. for C\(_{40}H_{74}O_{15}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 physico-chemical and biological properties, but showed no UV maximum at a region of 200–400 nm in water and no olefinic proton in the \(^{1}H\) NMR spectrum. The inhibitors 1 and 2 had almost the same biological properties including \(\beta\)-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. Methanalysis of 2 tetrasodium salt with 1.5 N hydrogen chloride in methanol overnight gave dihydrodotriacolide acetate (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\(_{46}H_{82}O_7: C 71.67, H 12.03. Found: C 71.09, H 11.56. The permethylated compound 4, colorless oil, \([\alpha]_D^{14}\) + 14° (c 0.5, chloroform), was prepared by treatment of 3 with methyl iodide and potassium hydride in tetrahydrofuran overnight 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 \(^{1}H\) NMR spectrum (\(CDCl_3\)) in which the terminal methyl protons showed a doublet (\(\delta\) 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 followed by reduction with lithium triethylborohydride in 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).
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 1H NMR spectrum (D2O). 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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References


