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THE STRUCTURE OF M-GTFI, AN INHIBITOR OF GLUCOSYLTRANSFERASE FROM *S. MUTANS*, AND ITS INHIBITORY RELATIONSHIP WITH OTHER SULFATE ESTER-CONTAINING INHIBITORS*.

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M-GTFI, an inhibitor of glucosyltransferase from *S. mutans* was produced by *Micromonospora narashinoensis* strain No. 731. The isolation procedure for M-GTFI was improved and established for spectroscopic analyses, and some properties of the inhibitor were investigated. The structure of M-GTFI was shown to be trisodium [2-sulphonato-(*E*)-9-undecenyll-oxacyclotriacont-(*E*)-3-en-2-one, 16, 18-bis sulphonate. The chemical structure of M-GTFI was therefore similar to that of izumenolide which is a β-lactamase inhibitor containing sulfate ester groups in its molecule.

The inhibitory characteristics of M-GTFI were parallel to that of other inhibitory compounds containing sulphate esters but the spectrum of activity was wider.

KEY WORDS: Glucosyltransferase inhibitor, inhibitors, M-GTFI, *Streptococcus mutans*, dental caries, sulfate ester-containing inhibitor

INTRODUCTION

The prospect of preventing dental caries led to the search for inhibitors of the glucosyltransferase (GTF) of *S. mutans* K1-R (serotype; g). M-GTFI, an inhibitor of *S. mutans* GTF was produced by *Micromonospora narashinoensis* strain No. 731 which was recently isolated in our laboratory.[1] In previous papers,[1,2] we reported the isolation of M-GFTI, its biochemical properties and its inhibitory activity. This inhibitor affects the GTF (I-GTF) that produces the water insoluble glucan. In addition to I-GTF, it inhibited α-glucosidase, β-glucosidase, β-amylase, and to a lesser extent, β-glucuronidase. Structural analyses of M-GTFI showed it to contain sulfate esters in its molecule and these, it is suggested, play an important role in the inhibition process.[2]

M-GTFI was precipitated as the barium salt from culture filtrates by addition of barium acetate. This method improved the recently established method of isolation.

This paper reports the structure of M-GTFI elucidated by degradative and spectroscopic methods, and its inhibitory relationship with other inhibitors containing sulphate esters.

* Studies on *S. mutans* glucosyltransferase inhibitor produced by *Micromonospora narashinoensis* strain No. 731. Part III (see references 1 and 2 for Part I and II, respectively).

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EXPERIMENTAL

Materials

Glucosyltransferase (GTF) and trehalase were purified in our laboratory from *Streptococcus mutans* K1-R (serotype; g),[1] or bakers' yeast, respectively. α-Glucosidase from yeast, DNase II from bovine spleen and neuraminidase from *Clostridium perfringens* were purchased from Sigma Co. DNase I from bovine pancreas, nuclease S1 from *Aspergillus oryzae* and β-lactamase from *Escherichia coli* 205 TEM R^+ were purchased from Boehringer Mannheim.

General

NMR spectra were obtained on a JEOL GX-400 spectrometer. Chemical shifts are expressed in δ values (ppm) with Me_4Si as an internal standard; however, when D_2O was used as solvent, in the ^{1}H-NMR spectra HOD was used as the internal reference which was adjusted to 4.70 ppm. In the ^{13}C-NMR spectra dioxane was used as the internal reference which was adjusted to 67.4 ppm. A JEOL-O1SG mass spectrometer was used for mass spectra. Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded on a Hitachi 270-30 spectrophotometer, UV spectra on a Hitachi U-2000 spectrophotometer. Optical rotations were measured with a Jasco DIP-360 digital polarimeter. Elemental analyses were performed with a Yanagimoto MT-3 CHN coder and Takeda Chemical Industries Ltd. kindly determind sulphur in the compound.

Analytical

TLC was performed on Silica gel 60 F_{254} plates (Merck, 0.2 mm). Phosphomolybdic acid-H_2SO_4 or 10% H_2SO_4 were used for detection. For column chromatography, Silica gel 60 (Merck, 0.063–0.200 mm), Sephadex G-75 (Pharmacia) and Diaion HP-20 SS (Mitsubishi Chemical Industries Ltd., 75-150 μm) were used.

Inhibitory Activity

Inhibitory activity of M-GTFI was determined by using glucosyltransferase from *S. mutans* strain K1-R and α-glucosidase from yeast as marker enzymes. The assay methods have been described in previous papers.[1,2]

Isolation and Purification of M-GTFI

The culture broth of *Micromonospora narashinoensis* strain No. 731 was centrifuged to obtain a clear supernatant. This solution was passed through columns of Amberlite IR-120B [H^+] and in turn, Amberlite IRA-410 [OH^-]. To the eluate was added $Ba(CH_3COO)_2$ solution (final concn. 1%) and 10 % EtOH. The mixture was stored overnight at 5°C, and the resulting precipitate was collected by centrifugation, to give 5.18 g of crude M-GTFI as a pale brownish powder from 3 l of the culture broth. 1.5 g of this precipitate was dissolved in 1 N NaOH and neutralized by dialysis against water. The dialyzate was mixed with "celite" and concentrated *in vacuo* to dryness. This was applied to a silica gel column (4 × 40 cm) and eluted with n-BuOH-MeOH-

H_2O (5:1:2). The active fractions were combined and concentrated *in vacuo,* and subsequently precipitated with EtOH. The precipitate was collected by centrifugation, to give 582 mg solid. This was dissolved in water, and the solution was adjusted to pH 2 with 1 N HCl. The solution was distributed with n-BuOH and the aqueous layer was neutralized with 1 N KOH, and then concentrated *in vacuo*. To the concentrate was added 2% KCl solution, and the mixture stored overnight, the resulting precipitate was collected by filtration, dissolved in hot water and passed through a Dowex 50 X-2 [H^+] column to remove potassium ions. The eluate was neutralized with 1 N NaOH, concentrated *in vacuo* and precipitated with MeOH, to give 300 mg of powder. The precipitate was dissolved in 0.02 M sodium phosphate buffer (pH 7.0) and applied to a Sephadex G-75 column (2.0 × 80 cm) and then eluted with the same buffer. The active fractions were combined and dialysed against water overnight to remove any salt, concentrated *in vacuo,* and precipitated with MeOH to give 198 mg of purified M-GTFI (**1**), trisodium salt, as a colorless amorphous powder. The numbering system as lactone is shown in structure **1** in Chart 1. This numbering is not formal, but was used for comparison with other inhibitors.

Acid Hydrolysis of ***1***

1 (359.1 mg) was dissolved in 20 ml of 0.1 N HCl and the solution was heated in an open tube at 100°C for 2 h. The resulting mixture was extracted with $CHCl_3$. The extract was washed with water, dried ($MgSO_4$), and filtered. The filtrate was concentrated *in vacuo,* to give 200.5 mg of **2** as a colorless solid: m.p. 75–78°C; $[\alpha]_D^{20}$ + 2.6°(c 1.00, $CHCl_3$); R_f 0.39 ($CHCl_3$-MeOH, 9:1); IR (KBr) cm^{-1} 3420, 2924, 2856, 1710, 1656, 1470; ^{1}H-NMR ($CDCl_3$) δ 1.26 (br s, $-(CH_2)_n-$), 1.40–2.33 (m, $-CH_3$, $-CH_2-$), 3.83 (3H, br s, 3 × CH–OH), 5.11 (1H, m, CH–OCO), 5.40 (2H, m, CH=CH), 5,82 (< 1 H, d, J=15.8 Hz, CH=CH–CO), 7.01 (< 1 H, dt, J=15.4, 7.0 Hz, CH=CH–CO); ^{13}C-NMR ($CDCl_3$) δ17.9 (q, C-40), 25.2, 25.4, 25.5, 25.9 (t, C-13, 19, 27, 33), 26.8 (C-37^1), 28.0 (C-6), 29.0, 29.2, 29.24, 29.4, 29.6 (t, C-7–12, 20–26, 34, 35), 32.3, 32.5 (C-5, 36), 34.6, 35.0 (t, C-4, 37), 37.0 (C-28), 38.3 (t, C-14, 18, 32), 42.9, 43.1 (t, C-16, 30), 67.2 (d, C-29), 71.6 (d, C-31), 73.2 (d, C-15, 17), 123.1 (d, C-39^1), 124.6 (d, C-39), 130.2 (d, C-38^1), 131.6 (d, C-38), 150.6 (d, C-3), 168.0 (s, C-1), 175.3 (s, C—1″); EI–MS m/z 582, 580 (M^+ – 3 × H_2O), 431, 429, 239, 237.

Found: C 74.33; H 11.64. $C_{40}H_{74}O_5$ requires C, 75.71; H 11.67%.

The aqueous phase described above was acidified with excess of conc. HCl and 10% $BaCl_2$ solution, to give 263.7 mg of $BaSO_4$ (calculated: 266.0 mg).

Base Hydrolysis of ***1***

A solution of 302.6 mg of **1** in 6 ml 1 N NaOH was heated in an open tube at 100°C for 5 h. The resulting mixture was applied to a Diaion HP-20 SS column (1.8 × 20 cm) and washed with water until the pH of the effluent was neutral. The product was then eluted stepwise with 30–100% aqueous MeOH; The 40–50% aqueous methanolic eluate was concentrated *in vacuo*, to give 227.9 mg of **3** as a colorless solid: m.p. 155–157°C; $[\alpha]_D^{20}$ –8.2° (c 1.00, H_2O); R_f 0.64 (2-BuOH–AcOH–H_2O, 3:1:1); IR (KBr) cm^{-1} 3484, 2926, 2854, 1656, 1572, 1416, 1242, 1065; ^{1}H-NMR (CD_3OD) δ1.29 (br s, $-(CH_2)-$), 1.43–2.23 (m, $-CH_2-$), 3.85 (1H, br s, CH–OH), 4.52 (3H, quintet-like, 3 × CH–OSO_3), 5.40 (2H, m, CH=CH), 5.80 (< 1H,

d, J= 15.4 Hz, CH=CH–COO), 6.79 (< 1 H, dt, J=15.8, 7.0 Hz, CH=CH–COO); ^{13}C-NMR (CD_3OD) δ 18.1 (q, C-40), 25.9, 26.0 (t, C-13, 19, 33), 26.7 (C-37′), 27.0 (C-27), 29.5 (C-6), 30.2, 30.3, 30.5, 30.6, 30.7, 30.9 (t, C-7–12, 20–26, 34, 35), 33.1, 33.6 (C-5, 36), 35.5 (t, C-4, 37), 36.7 (t, C-14, 18, 32), 38.5 (t, C-28), 40.1 (t, C-16), 43.7 (t, C-30), 68.6 (d, C-29), 78.2 (d, C-15, 17, 31), 124.6 (d, C-39′), 125.6 (d, C-2), 128.3 (d, C-39), 132.4 (d, C-38′), 132.7 (d, C-38), 147.9 (d, C-3), 180.0 (s, C-1).

Acid Hydrolysis of ***3***

3 (100 mg) was dissolved in 1.25 ml of MeOH and 7.5 ml of 0.1 N HCl. The mixture was heated at 100°C for 1.5 h and the product was extracted as described for **2**, to give 40 mg residue. The residue was chromatographed on a silica gel column (1.2 × 24 cm) eluting with benzene-acetone (85:15), to give 3.6 mg of **4** as a colorless powder: R_f 0.58 (benzene-acetone, 7:3); IR ($CHCl_3$) cm^{-1} 3448, 2932, 2856, 1728, 1628, 1456; ^{1}H-NMR ($CDCl_3$) δ 1.27 (br s, $-(CH_2)_n-$), 1.27–2.19 (m, $-CH_3$, $-CH_2-$), 2.30 (t, J=7.0 Hz, CH_2–COOMe), 3.67 (s, CH_2–COOMe), 3.72 (s, CH=CH–COOMe), 3.84 (2H, m, 2 × CH–OH), 3.93 (2H, m, 2 × CH–OH), 5.41 (2H, m, CH=CH), 5.82 (< 1H, d, J=15.8 Hz, CH=CH–COO), 6.97 (< 1 H, dt J=15.8, 7.0 Hz, CH=CH–COO).

Trimethylsilylation of ***4***

4 (3.1 mg) was treated with a mixture of bis-O, N-(trimethylsilyl) trifluoroacetamide and N-trimethylsilylimidazole and left for 1 h at room temperature. The product was extracted with n-hexane and the extract was washed with water. The n-hexane was evaporated with N_2 gas, to give 3.5 mg of **5** as a colorless solid; EI-MS m/z see Figure 5.

Hydrogenation of ***1***

A solution of 801.1 mg of **1** in 80% aqueous MeOH was hydrogenated in the presense of 2 g of 5% Pd/C at room temperature under an initial pressure of 2 atm. After 5 h, the mixture was filtered, and the filtrate was concentrated *in vacuo*, to give 754 mg of **6** as a colorless powder: m.p. 175–180°C (dec.); $[\alpha]_D^{20}$ –8.8° (c 0.50, H_2O); R_f indistinguishable from that of **1**; IR (KBr) cm^{-1} 2924, 2856, 1726, 1638, 1468, 1386, 1236, 1064; UV (H_2O) weak end absorption; ^{1}H-NMR (D_2O) δ 0.91 (3H, t, J=7.0 Hz, $-CH_3$), 1.30 (br s, $-(CH_2)_n-$), 1.41–2.33 (m, $-CH_2-$), 4.37, 4.44 (3H, each br s, 3 × CH–OSO_3), 5.04 (1H, m, CH–OCO).

Base Hydrolysis of ***6***

A solution of 754 mg of **6** in 7 ml 1 N NaOH was heated at 100°C for 5 h and the product isolated as described for **3**, to give 412 mg of **7** as a colorless solid: m.p. 141–143°C (dec.); R_f 0.64 (2-BuOH–AcOH–H_2O, 3:1:1); IR (KBr) cm^{-1} 3496, 2924, 2852, 1570, 1470, 1240, 1066.

Methylation of ***7***

First, 2 g of NaH (*ca.* 60% in oil) was washed in a reaction vessel with dry ether to

remove oil. The solvent was evaporated with N_2 gas, then 25 ml of dry DMSO was added and the mixture stirred under N_2 in an oil bath at 60–70°C for 2 h. **7** (400 mg) was dissolved in 5 ml of dry DMSO and poured into the reaction vessel. The mixture was stirred at 60–70°C for 3 h. 10 ml of MeI was added and stirring was continued for a further 30 min, 10 ml of MeI was then added, and the reaction mixture stirred at room temperature overnight. After the excess MeI was evaporated, water was added and the mixture was applied to a Diaion HP-20 SS Column (1.8 × 17.5 cm) and washed with water (5 times the bed volume). Then the methylate was eluted stepwise with 40–100% aqueous MeOH. The 50–60% aqueous methanolic eluate was concentrated *in vacuo*, to give 238.1 mg of **8** as a pale yellowish solid: R_f 0.63 (2-BuOH–AcOH–H_2O, 3:1:1); ^{1}H-NMR (DMSO-d_6) δ 0.86 (3H, t, J=7.0 Hz, CH_3), 1.24 (br s, -$(CH_2)_n$-), 1.37–1.53 (m, -CH_2-), 2.28 (2H, t, J=7.3 Hz, CH_2-COOMe), 3.21 (3H, s, OMe), 3.38 (1 H, m, CH-OMe), 3.57 (3 H, s, COOMe), 4.15 (3 H, m, 3 × CH-OSO_3).

Acid Hydrolysis of ***8***

8 (238.1 mg) was dissolved in 4.5 ml MeOH and 3 ml of 0.1 N HCl. The mixture was heated at 100°C for 1.5 h and the product was extracted as described for **2**, to give 176.1 mg as a pale yellowish solid. This material was applied to a silica gel column (1.2 × 24 cm) and eluted with benzene–acetone (11:1), to give 61.6 mg of **9** as a colorless crystalline solid: R_f 0.67 (benzene–acetone, 7:3); IR ($CHCl_3$) cm^{-1} 3512, 2932, 2856, 1734, 1456, 1092; ^{1}H-NMR ($CDCl_3$) δ 0.88 (3 H, t, J=7.0 Hz,-CH_3), 1.26 (br s, -$(CH_2)_n$-), 1.41–1.62 (m, -CH_2-), 2.30 (2 H, t, J=7.3 Hz, CH_2-COOMe), 3.36 (3 H, s, OMe), 3.45 (1 H, m, CH-OMe), 3.66 (3 H, s, COOMe), 3.85 (3 H, m, 3 × CH-OH); EI-MS (trimethylsilylated derivative) m/z 900 (M^+), 868, 810, 778, 720, 688, 657, 630, 616, 599, 567, 543, 477, 453, 445, 421, 363, 343, 253, 229.

Tosylation of ***9***

A solution of 52 mg of **9** and 224.5 mg of TsCl in 1.5 ml pyridine was left under N_2 for 17 h at room temperature. To the resulting mixture was added 1.5 ml of water, and mixed with 1 ml of 1 N HCl, and then extracted four times with 2 ml ether. The extract was washed in turn with 1 N HCl, saturated aqueous $NaHCO_3$ and saturated aqueous NaCl. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*, to give 82.9 mg residue. This was chromatographed on a silica gel column (1.2 × 20 cm). The column was eluted with n-hexane–AcOEt (9:1), to give 60.7 mg of **10** as a colorless waxy solid: R_f 0.25 (n-hexane–AcOEt, 8:2); IR ($CHCl_3$) cm^{-1} 2928, 2856, 1732, 1600, 1496, 1464, 1360, 1098; ^{1}H-NMR ($CDCl_3$) δ 0.88 (3 H, t, J=7.0 Hz, -CH_3), 1.02–2.02 (m, -CH_2-), 2.30 (2 H, t, J=7.7 Hz, CH_2-COOMe), 2.44 (3 H, s, Ar-Me), 2.45 (6 H, s, 2 × Ar-Me), 3.19 (1 H, m, CH-OMe), 3.21 (3 H, s, OMe), 3.66 (3 H, s, COOMe), 4.51 (2 H, m, 2 × CH-OTs), 4.82 (1 H, m, CH-OTs), 7.33 (6 H, d, J=8.1 Hz, Ar-H), 7.77 (4 H, d, J=8.1 Hz, Ar-H), 7.80 (2 H, d, J=8.1 Hz, Ar-H).

Lithium Triethylborohydride Reduction of ***10***

A solution of 33.9 mg of **10** in 2 ml 1 M $LiBEt_3H$ in THF was stirred under N_2 at room temperature for 65 h. 2 ml of water was added and the product was extracted 5 times

with 2 ml $CHCl_3$ The extract was concentrated *in vacuo*, to give 29.6 mg residue. The residue was chromatographed on a silica gel column (1.2 × 20 cm) eluting with n–hexane–AcOEt (10:1), to give 13.3 mg of **11** as a colorless crystalline solid: m.p. 76–77°C; $[\alpha]_D^{20}$ 0°(c 1.00, $CHCl_3$); R_f 0.38 (n–hexane–AcOEt, 8:2); Ir ($CHCl_3$) cm^{-1}

Fermentation broth
↓ centrufuged at 8,000 rpm, 10 min
Culture filtrate
↓ Amberlite IR-120B[H^+] column
↓ Amberlite IRA-410[OH^-] column
Pass-through
↓ 25% $Ba(CH_3COO)_2$ soln. added (final concn. 1%)
↓ 1/10 vol. of EtOH added
Precipitate (Crude M-GTFI)
↓ dissolved in 1 N NaOH
↓ dialysed against water, 24 h
↓ conc. in vacuo
↓ applied on a silica gel column
↓ eluted with n-BuOH-MeOH-H_2O (5:1:2)
Active fraction
↓ conc. in vacuo, and precipitated with EtOH
Precipitate (Fraction I)
↓ dissolved in water
↓ mixed with n-BuOH at pH 2
Aqueous layer
↓ neutralized with 1 N KOH
↓ conc. in vacuo
↓ 2% KCl soln. added (final concn. 1%)
↓ stood at 4°C
Precipitate
↓ dissolved in hot water
↓ Dowex 50W-X2[H^+] column
Pass-through
↓ neutralized with 1 N NaOH
↓ conc. in vacuo, and precipitated with MeOH
Precipitate (Fraction II)
↓ applied on a Sephadex G-75 column
↓ eluted with 0.02 M sodium phosphate buffer (pH 7.0)
Active fraction
↓ dialysed against water
↓ conc. in vacuo, and precipitated with MeOH
M-GTFI (Sodium salt)

FIGURE 1. Isolation procedure for M-GTFI.

3634, 2932, 2854, 1467, 1371, 1206, 1089; ^{1}H-NMR ($CDCl_3$)δ 0.88 (3 H, t, J=7.0 Hz, $-CH_3$), 1.25 (br s, $-(CH_2)_n-$), 1.44 (m, $-CH_2-$), 1.57 (2 H, m, CH_2CH_2-OH), 1.60 (1 H, s, CH_2-OH), 3.12 (1 H, quintet, J=5.9 Hz, CH–OMe), 3.31 (3 H, s, OMe) 3.64 (2 H, t, J=6.6 Hz, CH_2-OH); EI-MS m/z 608 (M^+), 453, 199. Found : C 79.61; H 13.45. $C_{41}H_{84}O_2$ requires C 80.92; H 13.82%.

RESULTS AND DISCUSSION

Fermentation and Isolation

Micromonospora narashinoensis strain No. 731, a producing strain of M-GTFI, was cultured by the same methods as previously described.[1] The isolation procedure for M-GTFI from culture filtrate of *M. narashinoensis* strain No. 731 is shown in Figure 1. M-GTFI passed through not only Amberlite IR-120B [H^+] but also Amberlite IRA-410 [OH^-]. It is considered that M-GTFI forms micelles in water, as will be discussed later. M-GTFI contains sulfate ester groups in the molecule and consequently M-GTFI can be precipitated as the barium salt by addition of barium acetate. This precipitate is insoluble in water, but soluble in 1 N NaOH. Crude M-GTFI wsa further purified according to Figure 1, to give a colorless amorphous powder. This procedure is much easier than the previously described method,[1,2] and leads to a better yield (Table I).

TABLE I
Summary of Purification Steps of M-GTFI.

	Volume or Weight	Inhibitory activity[a] (unit/ml,mg)	Total inhibitory activity (unit × 10^{-3})	Recovery (%)
Culture filtrate	2640.0 ml	1279.8	3378.7	100.0
IR-120 B P.T.	2550.0 ml	1268.7	3235.2	95.8
IRA-410 P.T.	2850.0 ml	965.1	2750.5	81.4
Crude M-GTFI	5184.0 mg	498.4	2583.7	76.5
Fraction I	2011.8 mg	1086.2	2185.2	64.7
Fraction II	1036.7 mg	1370.1	1420.4	42.0
M-GTFI	683.7 mg	1894.7	1295.4	38.3

[a] Inhibitory activity against glucosyltransferase.

Physicochemical Characteristics of M-GTFI

The physicochemical properties of M-GTFI are summarized in Table II. M-GTFI is soluble in water, slightly soluble in MeOH, and insoluble in EtOH, n-BuOH and $CHCl_3$. It is unstable in the acidic pH range 1–5.[2]

The IR spectrum (Figure 2) exibits characteristic absorption bands in the region of sulfate ester (1226 cm^{-1}). The ^{1}H-NMRspectrum and the ^{13}C-NMR spectrum are shown in Figures 3 and 4, respectively.

Judging from these results, it was thought that M-GTFI resembles izumenolide,[3] which is a β-lactamase inhibitor produced by *Micromonospora chalcea* subsp. *izumensis*, or dotriacolide[4] which is also a β-lactamase inhibitor produced by *Micromonospora echinospora* MG 299-fF 35. The structure of **1** was elucidated by reference to the method of Parker *et al.*[5]

TABLE II
Physicochemical Properties of M-GTFI (Sodium salt).

Appearance	white powder
Nature	acidic
Melting point	135°C (decomp.)
$[\alpha]_D^{22}$	-8.2° (c = 0.50, H_2O)
Elementary analysis (%)	C, 47.27; H, 7.91; S, 9.37; Na, 6.7%
$UV\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	213 (97.3)
R_f value	0.43 (n-BuOH-MeOH-H_2O, 4:1:2)
(silica gel plate)	0.53 (n-BuOH-AcOH-H_2O, 2:1:1)
Color reaction	
Positive	Phosphomolybdic acid-H_2SO_4
	Phenol-H_2SO_4
Negative	Lowry, Ninhydrin
Solubility	
Soluble	H_2O
Slightly soluble	MeOH
Insoluble	EtOH, n-BuOH, $CHCl_3$

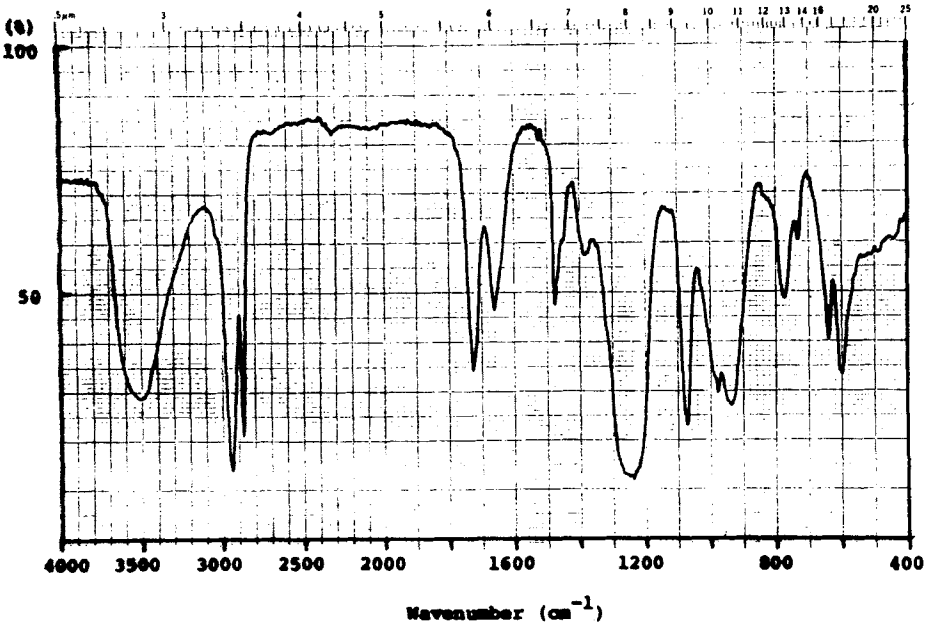


FIGURE 2. IR spectrum of M-GTFI (KBr).

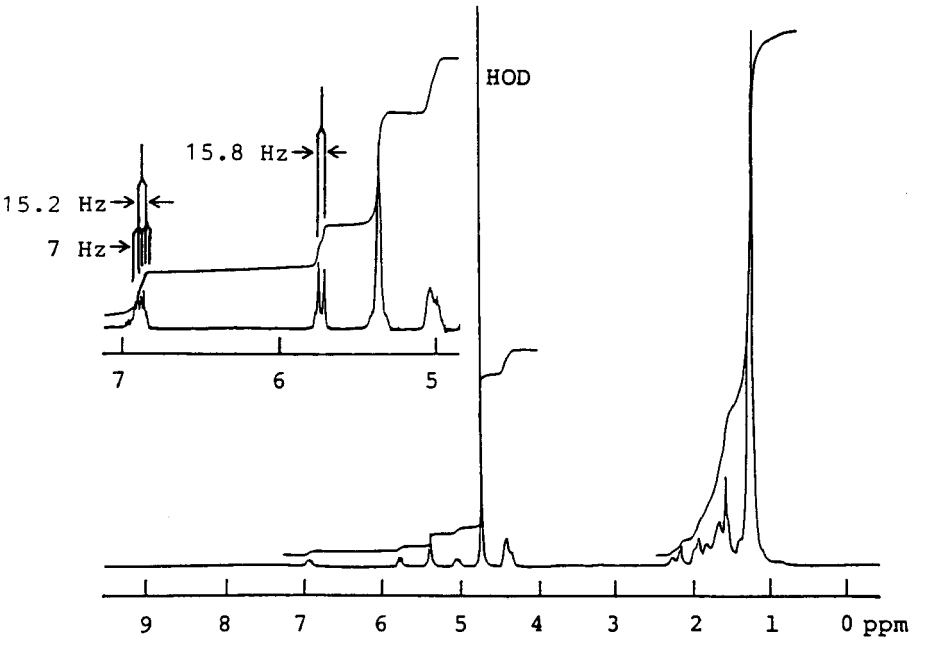


FIGURE 3. ^{1}H–NMR spectrum of M–GTFI (400 MHz, D_2O).

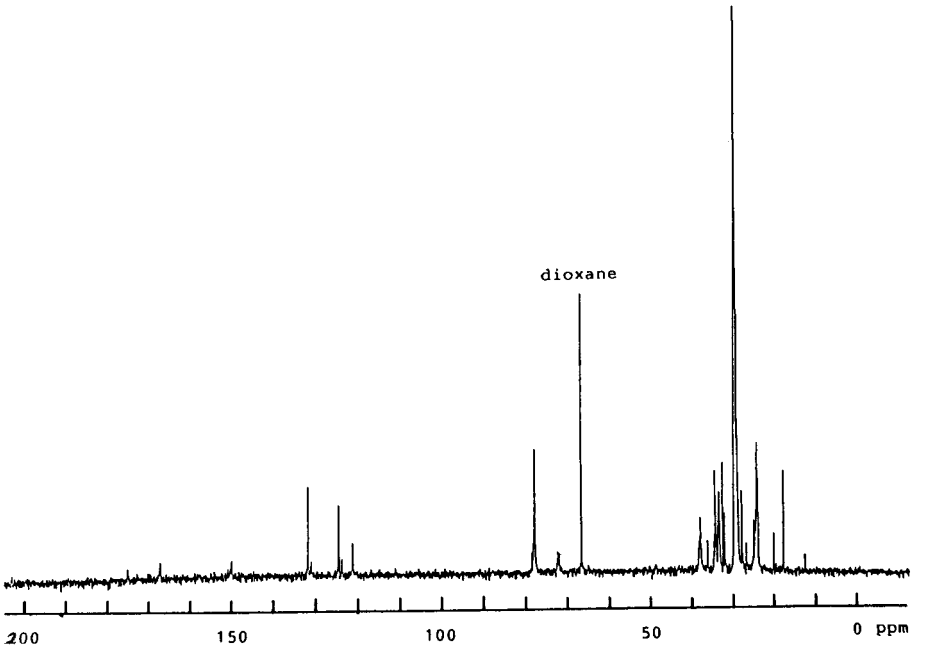


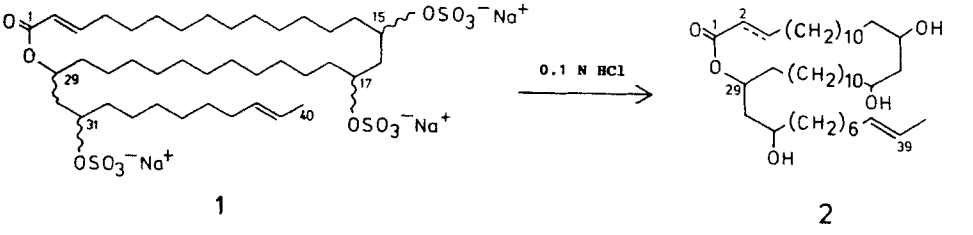
FIGURE 4. ^{13}C–NMR spectrum of M–GTFI (400 MHz, D_2O).

Elucidation of Structure

Acid hydrolysis of M-GTFI (359.1 mg) gave an acid hydrolysate (**2**) and inorganic sulfate ions[2] (Chart 1). After the hydrolysate was extracted with $CHCl_3$, $BaCl_2$ was added to the aqueous layer, to give 263.7 mg of $BaSO_4$. Calculation using the molecular weight (see later) showed that one mole of M-GTFI gave three moles of sulfate ions. Therefore, the molecule contained three sulphate ester groups — these are very important for the inhibitory activity of M-GTFI.[2]

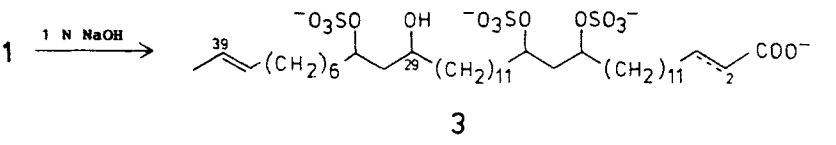
The molecular weight was determined by the sequence in Chart 2. Base hydrolysis of **1** gave **3** in which the lactonic ester was hydrolyzed. Hydrolytic removal of the sulfate ester groups from **3** gave **4** which was converted into the trimethylsilyl derivative **5**. The EI-MS spectrum of **5** yielded a molecular ion at m/z 954 and typical key fragment ions of **5**, shown in Figure 5.

Calculation of the molecular weight (MW) of M-GTFI (**1**) from the TMS-derivative (**5**) is as follows; MW of M-GTFI = MW of TMS-derivative-(4 × TMS + OMe) + 3 SO_3Na. From these results, the molecular weight of com-



1 2

Chart 1



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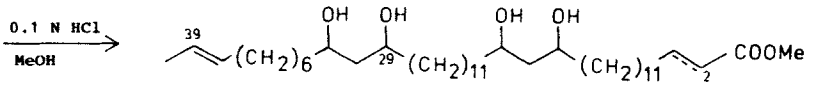
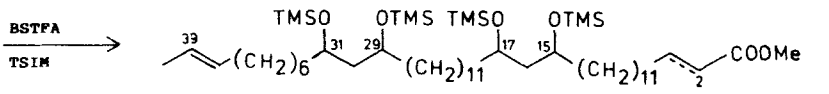


4



5

Chart 2

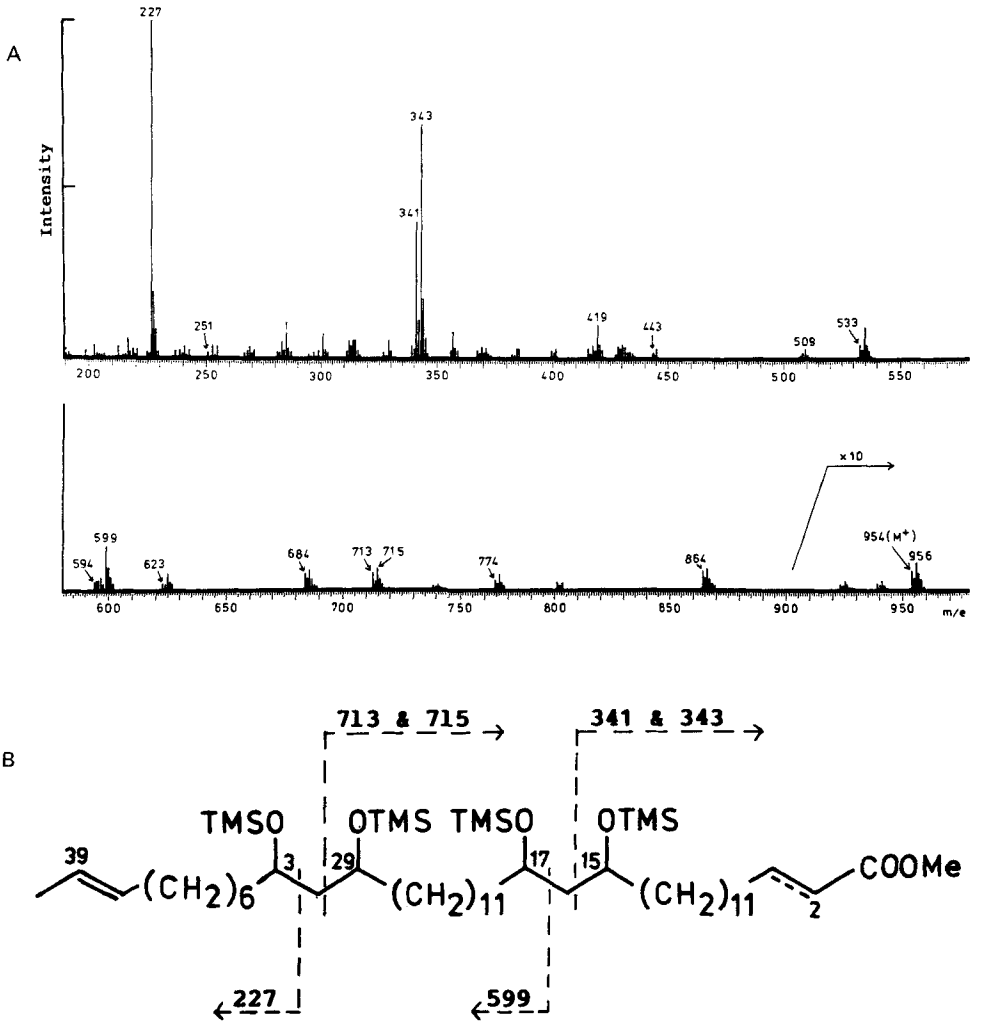


FIGURE 5. (A) EI–MS spectrum and (B) fragmentation pattern of **5**.

pound **1** was determined to be 941 (trisodium salt) and the position of the oxygenated carbons were confirmed to be 1, 15, 17, 29 and 31. However, additional ions were observed at m/z 343 and 715. These facts suggested the presence of a component in which the C–2, C–3 linkage is saturated. This was also demonstrated in the ^{1}H–NMR spectrum of **4**, (Figure 6) which exhibited two carboxymethyl signals at δ3.67 and 3.72. The former signal was assigned to the carboxymethyl of the 2, 3–dihydro component, the latter signal was assigned to the carboxymethyl of the 2, 3–unsaturated one (this was also confirmed in the ^{1}H–NMR spectrum of **8**). From the strength of both signals, the ratio of the 2, 3–dihydro component to the 2, 3–unsaturated one was estimated to be 2:1. Therefore it is considered that the purest

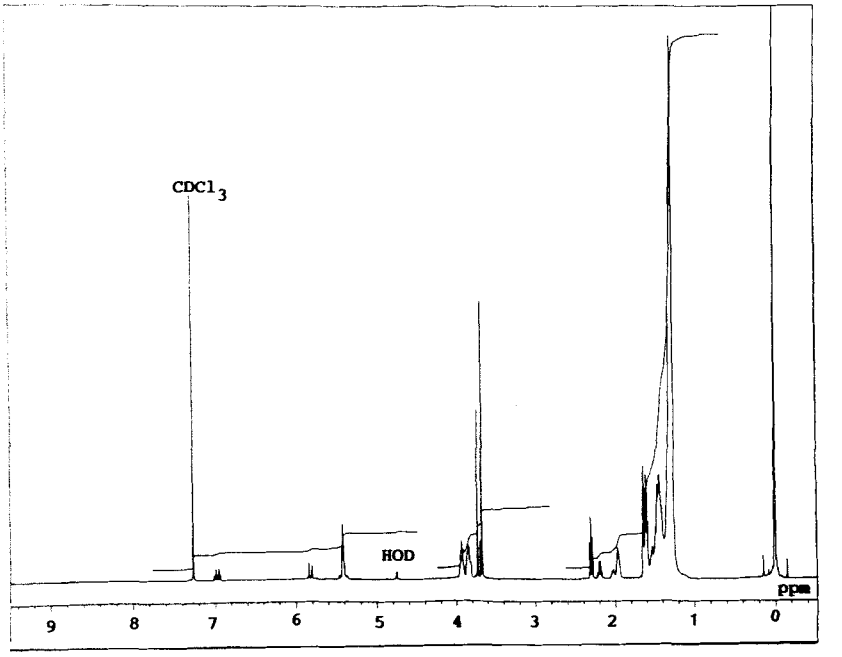


FIGURE 6. ^{1}H-NMR spectrum of **4** (400 MHz, $CDCl_3$ + D_2O).

preparation of M-GTFI contains the 2, 3-dihydro component. It was assumed that an α, β-unsaturated ester was indicated by peaks in the IR spectrum of **1** at 1718 (C = O) and 1652 cm^{-1} (C = C), by a max at 213 nm in the UV spectrum and in the ^{13}C-NMR of **1** (δ121.1, 150.0 and 166.7). The *E*-configuration of C-2 and C-3 was indicated by absorption in the ^{1}H-NMR spectrum at 5.74 (d, J=15,8 Hz) and 6.91 (d.t, J=15.2, 7.0 Hz), respectively. The *E*-configuration of C-38 and C-39 was assigned from the chemical shifts in the ^{13}C-NMR spectrum of **1** of the olefinic carbons and the terminal methyl group.

The ring size of the lactone **1** was determined by the degradative sequence as shown in Chart 3. Hydrogenation of **1** gave dihydro M-GTFI (**6**). **6** was hydrolyzed in 1 N NaOH to open the lactone ring. Base hydrolysate (**7**) was methylated by Hakomori's

TABLE III
Inhibitory Spectrum of M-GTF.

Enzyme	ID_{50}	(μg/ml)
DNase I	10	(106)
DNaseII	35	(74)
Nuclease S_1	>200	(>200)
Neuraminidase	50	(ND)
β-Lactamase	0.25	(>100)
Trehalase	32	(ND)

ID_{50}: Amount of M-GTFI required to give 50% inhibition.
ID_{50} by SDS are shown in parenthesis.
ND = not done

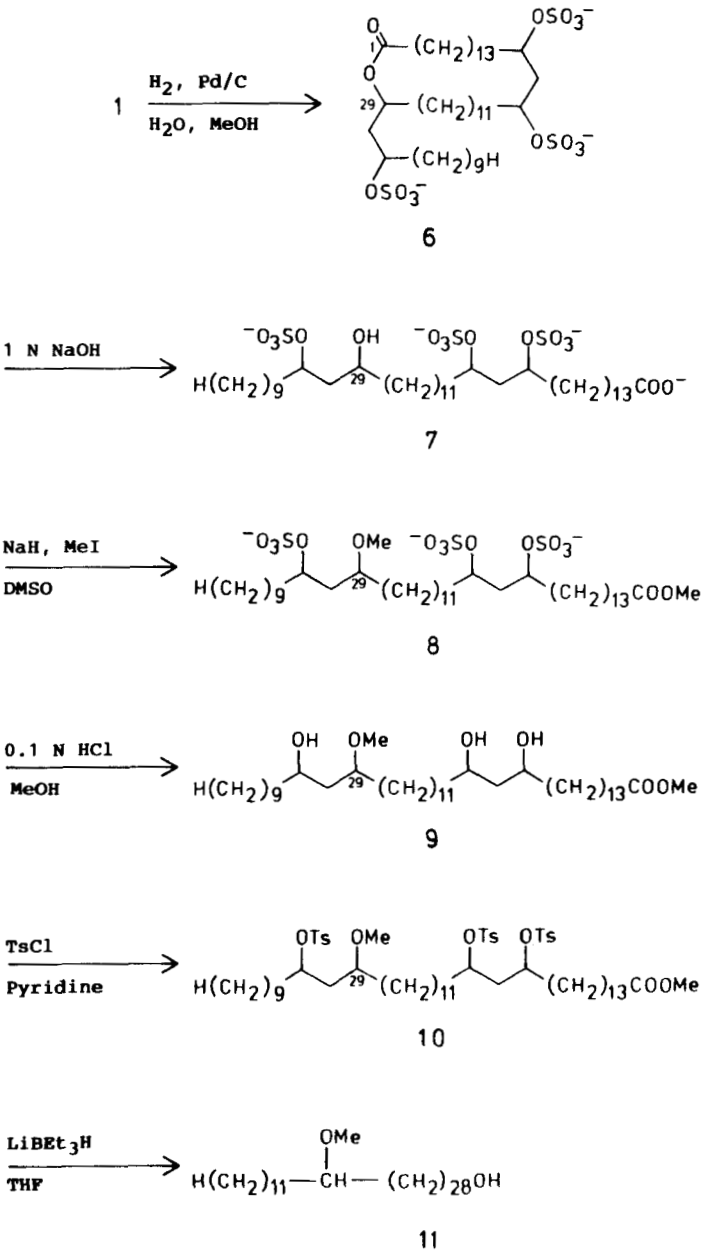


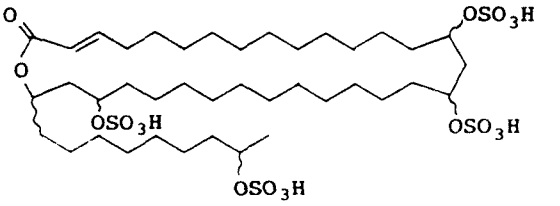
Chart 3

TABLE IV
Inhibitory Spectra of Related Inhibitors.

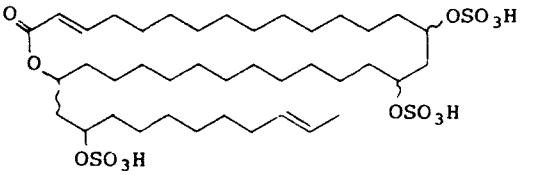
Enzyme	M-GTFI	DOT	IZU	PANO	OLIV	SDS
GTF	+*		+			+
α-Glucosidase	+		+			+
DNase I	+	+				+
DNase II	+	+				+
Nuclease S_1	−					−
Trehalase	+					
Neuraminidase	+			+*		
β-Lactamase	+	+*	+*		+*	−

+, inhibition; −, no inhibition; blanks, not tested; *, marker enzyme originally screened.

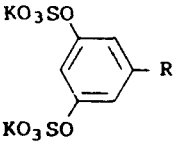
Dotriacolide (DOT)



Izumenolide (IZU)

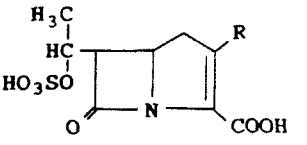


Panosialin (PANO)



R=-(CH2)12CH(CH3)2
-(CH2)14CH3
-(CH2)13CH(CH3)2

Olivanic acid (OLIV)



R=-S-CH=CH-NHCOCH3
-S-CH=CH-NHCOCH3
-S-CH2CH2NHCOCH3

method[6] to give **8**. Acid hydrolysis of **8** followed by tosylation of **9**, gave the tritosylate (**10**). Reduction of **10** with $LiBEt_3H$ in THF gave 12-methoxy-1-tetracontanol (**11**). The EI-MS spectrum of **11** gave fragment ions at m/z 199 and 453, shown in structure **11**. Thus it was determined that M-GTFI is a 30-membered lactone.

From the results described above, the structure of sodium salt of M-GTFI (**1**) was determined to be trisodium [2-sulphonato-(*E*)-9-undecenyl]-oxacyclotriacont-(*E*)-3-en-2-one, 16, 18-bis sulphonate. The chemical structure of M-GTFI is similar to that of izumenolide.

At the beginning of the study, M-GTFI was thought to be a macromolecular compound[1,2] because it did not appear to dialyze through Visking tube which allows materials having molecular weight less than 12,000 to pass. Its molecular weight was estimated as *ca.* 5,700 by a molecular weight estimation on a Sephadex G-75 column. Experimental work described here shows the molecular weight to be 941. The macromolecular behavior of the inhibitor therefore is thought to be due to the polyanionic aggregation of inhibitor molecules as noted with panosialin[7] and izumenolide[3].

Further Examination of Inhibitory Spectrum of M-GTFI

Structural analyses revealed that M-GTFI has 3 sulfate esters in its molecule. A review of "sulfate ester-containing" inhibitors in the literature showed that the group of β-lactamase inhibitors, olivanic acid[8] (one sulfate ester-containing), izumenolide[3] (three ester-containing) and dotriacolide[4] (four ester-containing) occur. A two sulfate ester-containing inhibitor, panosialin[7] was reported as a neuraminidase inhibitor. Also two sulfate ester-containing A-32724A,[9] A-32724B,[9] PF1057B-2 Na[10] and PF1057D-2 Na[10] were reported as glucosyl-transferase inhibitors. Dotriacolide, a β-lactamase inhibitor, was later reported to inhibit even DNase activity[11]. Taking these facts into consideration, we examined whether M-GTFI inhibits β-lactamase, DNase I or II, and neuraminidase *etc.* As shown in Table III, M-GTFI was found to inhibit these enzymes.

Its inhibitory relationship with other sulfate ester-containing inhibitors is summarized in Table IV. A new finding in this work is that izumenolide inhibits glucosyltransferase from *S. mutans*.

As a result, M-GTFI turned out to inhibit various enzymes which "sulfatre ester-containing" inhibitors also inhibit, and showed a wider inhibitory spectrum than that reported in our previous paper.[1]

Acknowledgements

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