Trachyspic Acid, a New Metabolite Produced by *Talaromyces trachyspermus*, that Inhibits Tumor Cell Heparanase: Taxonomy of the Producing Strain, Fermentation, Isolation, Structural Elucidation, and Biological Activity

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Trachyspic acid, a new metabolite that inhibited heparanase, was isolated from the culture broth of *Talaromyces trachyspermus* SANK 12191. Its structure was deduced from NMR spectral analyses and chemical reactions as a tricarboxylic acid derivative containing a spiroketal. The IC_{50} value of trachyspic acid against heparanase was 36 μM.

Basement membranes are a tissue barrier that tumor cells must penetrate in the process of invasion and metastasis. Therefore, their degradation is an important step in pathogenesis, and extensive efforts have been paid to clarify the role of proteases and glycosidases in this step.[1] Among them is heparanase an endo-β-glucuronidase that was originally identified in murine B16 melanoma. It degrades heparan sulfate (HS), one of the major components of basement membranes. Reportedly, heparanase activities are correlated with the metastatic potential of malignant cells.[2,3] HS also provides a storage site for heparin-binding growth factors, such as basic fibroblast growth factor (bFGF). When HS is degraded by heparanase, bFGF is released from HS, to stimulate endothelial cell proliferation and capillary cell growth.[4] It is, therefore, suggested that heparanase participates in both tumor invasion and angiogenesis.

Heparanase is inhibited by heparin, heparin derivatives, and suramin. In fact, they inhibit pulmonary metastasis of B16 melanoma.[5~7] In spite of these studies, little is known to date about the structural requirement of low-molecular-weight heparanase inhibitors. In this sense, studies on the natural compounds that inhibit this glycosidase will be informative.

In the course of our screening for heparanase inhibitors, we found a new metabolite from the culture broth of *Talaromyces trachyspermus* SANK 12191, and named it trachyspic acid. In this paper, we report the taxonomy of the producing strain, fermentation, isolation, structural elucidation, and some biological characteristics of trachyspic acid.

Experimental

Heparanase Assay

B16-BL6 was kindly supplied by Dr. FIDLER, Univ. of Texas, M. D. Anderson Cancer Center. Heparin-Sepharose was purchased from Pharmacia-LKB. Heparan sulfate was purchased from Seikagaku Kogyo, Japan. Heparanase was extracted and partially purified from B16-BL6 melanoma cells using heparin-Sepharose according to the method of NAKAJIMA *et al.*[6] HS was radiolabeled with [^{3}H] acetic anhydride.[8] The solid-phase radiolabeled substrate was prepared based on the method of NAKAJIMA *et al.*[8] with slight modifications, and was used for the enzyme assay. A suspension of the [^{3}H] acetyl HS immobilized to gel beads was mixed with the partially purified enzyme and incubated in a total volume of 400 μl of assay mixture, consisting of 0.2 M sodium acetate (pH 5.0) and 20 mM D-saccharic acid 1,4-lactone, at 37°C for 3 hours with vigorous shaking. The enzyme reaction was terminated by adding 40 μl of 50% trichloroacetic acid. The mixture was then centrifuged at 9,800 $\times g$ for 5 minutes, and radioactivity in a 200-μl aliquot of the supernatant was determined in a

scintillation counter.

Taxonomic Studies

The strain SANK 12191 was isolated from soil collected at Kagoshima Pref., Japan, in April 1989. Taxonomic studies and identification were conducted according to the procedures described by PITT.[9] Color hues are described according to the method of KORNERUP and WANSCHER.[10]

Fermentation

A slant culture of the strain SANK 12191 was used to inoculate into a 500-ml Erlenmeyer flask containing 100 ml of GPMY medium (glycerol 50 g, fresh potato 50 g, yeast extract 5 g, malt extract 5 g, tap water 1,000 ml). The flask was shaken on a rotary shaker for 5 days at 26°C. Two ml of the seed culture was transferred into respective 500-ml Erlenmeyer flasks containing the same medium described above, and fermentation in 20 flasks was carried out at 26°C for 6 days.

Trimethyl Ester (2)

Trachyspic acid (224 mg) was dissolved in 5 ml of MeOH, followed by addition of excessive diazomethane ether solution. After reaction for 5 minutes at 0°C, the reaction mixture was concentrated under reduced pressure. The obtained residue was purified with preparative HPLC (Senshu pak ODS H-4251 (Senshu Scientific Co., Ltd.), 10 × 250 mm, eluent: 75% CH_3CN), to give 78 mg of **2**: MS (EI) *m/z* 454 (M^+), 455 ($M+H^+$), 243 (base peak); IR ($CHCl_3$) cm^{-1} 1740, 1610; ^1H NMR (400 MHz, DMSO-d_6) δ 2.88 (1H, d, J=16.9 Hz, 2-H_a), 2.94 (1H, d, J=16.9 Hz, 2-H_b), 3.82 (1H, dd, J=7.3, 12.5 Hz, 4-H), 2.44 (2H, m, 5-H), 8.48 (1H, s, 9-H), 2.02 (2H, m, 10-H), 1.41 (2H, m, 11-H), 1.24 (12H, br, from 12- to 17-H), 0.86 (3H, t, J=7.0 Hz, 18-H), 3.57 (3H, s), 3.65 (3H, s), 3.72 (3H, s).

Reduction of Trachyspic Acid

Trachyspic acid (290 mg) was dissolved in 15 ml of MeOH, and 300 mg of 10% Pd-C was added to the solution. After reduction under H_2 gas atmosphere, the reaction mixture was filtered, to remove the catalyst, followed by concentration under reduced pressure. The obtained residue was applied on a Sephadex LH-20 (Pharmacia) column (25 × 200 mm), and the column was developed with a mixture of EtOAc - CH_2Cl_2 - MeOH (19 : 19 : 2). The obtained material was further purified with preparative HPLC (Senshu pak ODS H-4251, 10 × 250 mm, eluent: 55% CH_3CN containing 0.1% trifluoroacetic acid), to give 10.5, 17.6, 68.5, and 26.8 mg of reduced compounds **3**, **4**, **5**, and **6**, respectively.

3: MS (FAB) *m/z* 415 ($M+H^+$); ^1H NMR (400 MHz, CD_3OD) δ 2.71 (1H, d, J=16.4 Hz, 2-H_a), 3.08 (1H, d, J=16.4 Hz, 2-H_b), 3.01 (1H, dd, J=2.8, 10.2 Hz, 4-H), 1.67 (1H, m, 5-H_a), 2.16 (1H, m, 5-H_b), 4.46 (1H, dd, J=5.6, 8.3 Hz, 6-H), 8.21 (1H, s, 9-H), 2.10 (2H, m, 10-H), 1.46 (2H, m, 11-H), 1.29 (12H, br, from 12- to 17-H), 0.89 (3H, t, J=6.7 Hz, 18-H).

4: MS (FAB) *m/z* 417 ($M+H^+$); ^1H NMR (400 MHz, CD_3OD) δ 2.68 (1H, d, J=16.5 Hz, 2-H_a), 3.07 (1H, d, J=16.5 Hz, 2-H_b), 2.89 (1H, dd, J=2.3, 10.9 Hz, 4-H), 1.82 (1H, ddd, J=2.3, 5.1, 14.1 Hz, 5-H_a), 2.07 (1H, ddd, J=9.4, 10.9, 14.1 Hz, 5-H_b), 3.87 (1H, dd, J=5.1, 9.4 Hz, 6-H), 2.51 (1H, m, 8-H), 3.82 (1H, dd, J=7.4, 9.3 Hz, 9-H_a), 4.22 (1H, t, J=8.7 Hz, 9-H_b), 1.39 (1H, m, 10-H_a), 1.67 (1H, m, 10-H_b), 1.29 (14H, br, from 11- to 17-H), 0.90 (3H, t, J=6.9 Hz, 18-H); ^{13}C NMR (90 MHz, DMSO-d_6) δ 174.5 (s, C-1), 40.5 (t, C-2), 74.9 (s, C-3), 50.4 (d, C-4), 27.4 (t, C-5), 78.4 (d, C-6), 217.1 (s, C-7), 46.0 (d, C-8), 68.7 (t, C-9), 28.0 (t, C-10), 22.0, 26.7, 28.6, 28.7, 28.8 × 2 and 31.2 (t, from C-11 to C-17), 13.9 (q, C-18), 171.3 (s, C-19), 172.8 (s, C-20). The assignment of C-1 and C-19 is interchangeable.

5: MS (FAB) *m/z* 439 (positive) ($M+Na^+$), 415 (negative) ($M-H^-$); The ^1H (400 MHz, CD_3OD) and ^{13}C (90 MHz, CD_3OD) NMR spectral data are summarized in Table 1.

6: MS (FAB) *m/z* 415 ($M+H^+$); ^1H NMR (400 MHz, CD_3OD) δ 2.82 (1H, d, J=16.7 Hz, 2-H_a), 2.96 (1H, d, J=16.7 Hz, 2-H_b), 3.63 (1H, dd, J=7.6, 12.2 Hz, 4-H), 2.19 (1H, dd, J=7.6, 13.3 Hz, 5-H_a), 2.61 (1H, t, J=12.7 Hz, 5-H_b), 2.51 (1H, m, 8-H), 3.99 (1H, t, J=9.0 Hz, 9-H_a), 4.37 (1H, t, J=8.8 Hz, 9-H_b), 1.42 (1H, m, 10-H_a), 1.75 (1H, m, 10-H_b), 1.29 (14H, br, from 11- to 17-H), 0.90 (3H, t, J=6.8 Hz, 18-H).

Results and Discussion

Taxonomy of the Strain SANK 12191

The mycological characteristics of the strain SANK 12191 are described below. Colonies on CYA (Czapek Yeast Extract Agar) were 21 mm after 7 days at 25°C. The surfaces were floccose, forming a thick mycelial mat at the center, white all over, later becoming dull green (25D3) due to the production of conidia. Conidia were very sparsely produced. The reverses of the colonies were brownish orange (5C4). Colonies on MEA (Malt Extract Agar) were 25 mm after 7 days at 25°C. The surfaces were plane and floccose, forming a thick mycelial mat, white all over. The reverses of the colonies were greyish yellow (4B4). Conidia were sparsely produced. Ascomata were not produced during 7 days at 25°C. Conidia germinated neither at 5°C nor on G25N (25% Glycerol Nitrate Agar). Colonies on CYA were 30 mm after 7 days at 37°C. They were similar to those at 25°C, except for forming more condensed mycelia. Cleistothecia were not produced during 7 days of incubation on either CYA or MEA. Cleistothecia could be produced with prolonged incubation, longer than one month. They were globose, 300 ~ 500 μm in diameter, and pale yellow. The ascus were eight-spored, globose to subglobose, and 8 μm in

diameter. Ascospores were ellipsoid with a finely rough surface, and 3.5~4.5 μm in diameter. The anamorph, which was rarely observed, was *Penicillium*. Conidiophores were short, smooth-walled. Penicilli were mono-verticillate, and partly irregularly branched. Phialides were acicular with long necks, and 11~13 × 1.5 μm. Conidia were ellipsoid, smooth-walled, and formed in chains on phialides. From the characteristics stated above, the present strain was identified as *Talaromyces trachyspermus* (Shear) Stolk and Samson.

Isolation of Trachyspic Acid

After the fermentation, an equivalent volume of acetone was added to 1.6 liters of culture broth obtained, and the mixture was filtered with the aid of Celite 545 (Johns Manville). The filtrate was adjusted to pH 2.0 with HCl, followed by two extractions, each with 2 liters of ethyl acetate. The extract was concentrated to 400 ml under reduced pressure, and the concentrated organic layer was extracted twice, each time with 200 ml of 50 mM $NaHCO_3$ aqueous solution. The aqueous alkaline layer was adjusted to pH 2.0, and then re-extracted with an equivalent volume of ethyl acetate. The extract was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to dryness. The residue (2.8 g) was applied on a Sephadex LH-20 column (40 × 430 mm), and the column was developed with a mixture of EtOAc-CH_2Cl_2-MeOH (9:9:1). The eluted fractions were assayed for the activity, and active fractions were pooled and concentrated. Trachyspic acid was finally purified with preparative HPLC (Senshu pak ODS H-5251 (Senshu Scientific Co., Ltd.), 20 × 250 mm, eluent; 60% CH_3CN containing 0.1% trifluoroacetic acid, flow rate; 15 ml/minute). A peak eluted from 7.8 to 10.2 minutes was collected, concentrated under reduced pressure, and lyophilized, to give 370 mg of a hygroscopic powder. The obtained trachyspic acid showed a single peak on HPLC (Senshu pak ODS H-2151 (Senshu Scientific Co., Ltd.), 6 × 150 mm, eluent; 55% CH_3CN containing 0.1% trifluoroacetic acid, flow rate; 1.5 ml/minute, retention time; 7.5 minutes) and a single spot on TLC (silica gel plate (Merck Art. 5719), developed with *n*-BuOH-AcOH-H_2O (4:2:1), colored with hot H_2SO_4, Rf 0.59).

Physico-chemical Properties of Trachyspic Acid

Trachyspic acid (**1**) was obtained as a hygroscopic and acidic white powder. **1** was soluble in methanol, ethanol, ethyl acetate, chloroform, DMSO, and alkaline water. Based on the elementary analysis (Found: C 56.21, H 7.18, Calcd for $C_{20}H_{28}O_9 \cdot H_2O$: C 55.81, H 7.02) and HR-FAB-MS (matrix: *m*-nitrobenzylalcohol, QM^-: *m/z* Found: 411.16505, Calcd: 411.16551), the molecular formula was established to be $C_{20}H_{28}O_9$ (MW: 412). **1** showed $[\alpha]_D^{25}$ +3.1° (*c* 1.0, MeOH). The UV spectra showed maxima at 202 nm (ε 1,100) and 280 (2,600) in MeOH, and 205 (5,000) and 284 (1,900) in alkaline MeOH. The IR spectrum (KBr) showed the presence of carbonyl groups at 1723 cm^{-1} and a C=C double bond at 1607 cm^{-1}.

Structural Elucidation of Trachyspic Acid

Reaction of **1** with diazomethane afforded a trimethyl ester (**2**, $C_{23}H_{34}O_9$, MW: 454). The IR spectrum of **2** did not show the presence of OH groups. Therefore, nine oxygen atoms consistent with the molecular formula of **1** were classified into three carboxylic, one ketone (198.1 ppm in the ^{13}C NMR spectrum), and two ether groups. The ^1H and ^{13}C NMR spectral data of **1** are summarized in Table 1.

The connectivity of proton and carbon atoms was confirmed with the ^1H-^{13}C COSY spectrum, as shown in Table 1. Analysis of the ^1H-^1H COSY spectrum revealed the presence of an *n*-nonyl group, an isolated olefinic methine, a geminally coupled isolated methylene, and an ABX proton spin system. ^1H-^{13}C long range couplings of ^2J and ^3J observed in the COLOC

Fig. 1. Structures of trachyspic acid and its reduced products.

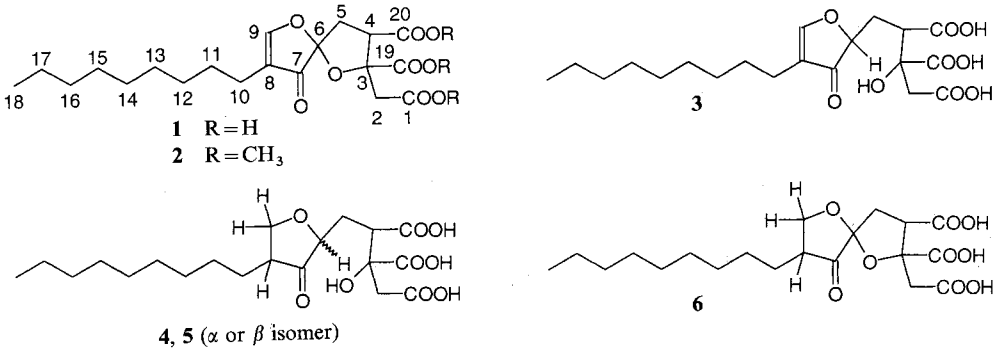


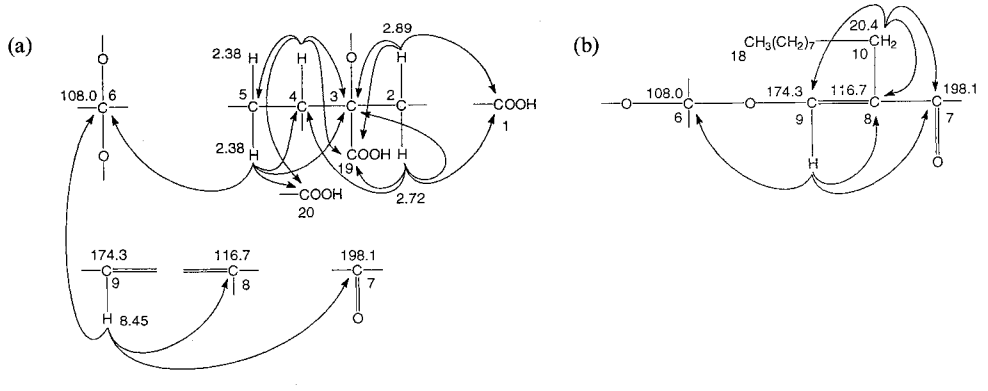
Table 1. NMR spectral data for **1** (trachyspic acid) and **5**.

	1 (DMSO-d_6)			**5** (CD_3OD)	
Position	δ_C	δ_H (intensity, multiplicity, J)	Position	δ_C	δ_H (intensity, multiplicity, J)
1	170.0		1	176.5[b]	
2	38.7	2.72 (1H, d, J=16.8 Hz), 2.89 (1H, d, J=16.8 Hz)	2	42.4	2.74 (1H, d, J=16.7 Hz), 3.07 (1H, d, J=16.7 Hz)
3	86.5		3	76.7	
4	48.4	3.57 (1H, dd, J=7.7, 12.0 Hz)	4	50.9	2.87 (1H, dd, J=2.3, 12.2 Hz)
5	37.4	2.38 (2H, m)	5	29.1	1.61 (1H, ddd, J=2.3, 10.2, 14.0 Hz), 2.17 (1H, ddd, J=3.2, 12.2, 14.0 Hz)
6	108.0		6	79.1	3.80 (1H, dd, J=3.2, 10.2 Hz)
7	198.1		7	218.6	
8	116.7		8	47.8	2.49 (1H, m)
9	174.3	8.45 (1H, s)	9	70.8	3.86 (1H, dd, J=6.0, 9.3 Hz), 4.24 (1H, dd, J=7.8, 9.3 Hz)
10	20.4	2.04 (1H, t, J=7.5 Hz)	10	30.4	1.39 (1H, m), 1.65 (1H, m)
11	27.4	1.40 (2H, m)	11	28.6	[c]
12	28.5	[a]	12, 13, 14, 15	30.5, 30.6, 30.7×2	1.30 (14H, br)[c]
13, 14, 15	28.6×2, 28.8	1.26 (12H, br)[a]			
16	31.2	[a]	16	33.1	[c]
17	22.0	[a]	17	23.7	[c]
18	13.8	0.86 (3H, t, J=6.8 Hz)	18	14.5	0.90 (3H, t, J=6.9 Hz)
19	171.2		19	173.9[b]	
20	170.5		20	174.9	

[a] The ^1H signals from 12-H to 17-H of **1** were observed as envelopes from 1.16 ppm to 1.32 ppm (total 12H).
[b] The assignments of C-1 and C-19 of **5** may be interchangeable.
[c] The ^1H signals from 11-H to 17-H of **5** were observed as envelopes from 1.24 ppm to 1.41 ppm (total 14H).

Fig. 2. Informative ^1H-^{13}C long range couplings (arrows) and established partial structures of trachyspic acid (**1**).

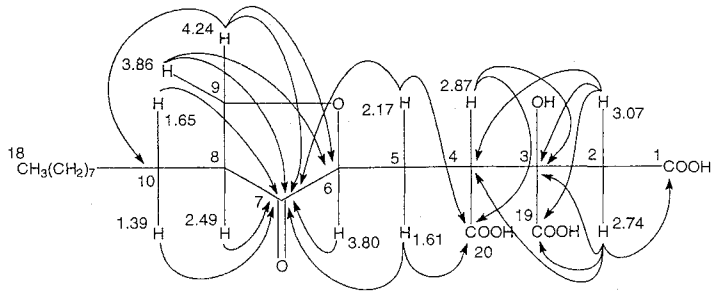
(a) ^1H-^{13}C long range couplings obtained from the COLOC experiment. (b) ^1H-^{13}C long range couplings obtained from the HMBC experiment.



(correlation spectroscopy *via* long-range coupling)[11] spectrum are shown in Fig. 2a. Cross peaks from 5-H to C-3, C-4, C-6, and C-20, and from 4-H to C-3, C-5, C-19, and C-20, and from 2-H to C-1, C-3, C-4, and C-19 suggested that three carboxylic groups were placed in a citric acid structure, although other connectivities (for example, from C-5 to C-20, and from C-4 to C-6) could not be excluded. Cross peaks from 9-H to C-6, C-7, and C-8, and the carbon chemical shifts of δ 108.0, 198.1, 116.7, and 174.3 for C-6, C-7, C-8, and C-9, respectively, suggested the presence of a β-*O*-substituted α,β-unsaturated ketone skeleton. The UV maximum at 280 nm also required this chromophore. This partial structure could be extended to an α-*n*-nonyl-β-*O*-substituted α,β-unsaturated ketone skeleton from additional ^1H-^{13}C long range couplings, from 10-H to C-7, C-8, and C-9, observed in the HMBC (heteronuclear multiple bond correlation)[12] spectrum, as shown in Fig. 2b.

Catalytic reduction of **1** with 10% Pd-C in MeOH yielded four compounds (**3**, **4**, **5**, and **6**). The molecular formula of the main product (**5**) was ascertained to be

Fig. 3. Informative ^{1}H-^{13}C long range couplings (arrows) obtained from the field gradient HMBC experiment on **5** (the assignment of C-1 and C-19 is interchangeable).



$C_{20}H_{32}O_9$ (MW: 416), from FAB-MS. From this molecular formula, hydrogenation of 2 moles occurred in the reduction. The ^{1}H and ^{13}C NMR spectral data of **5** are summarized in Table 1. The UV maximum at 280 nm disappeared in **5**, and the ketone carbon (C-7) was shifted to a lower field at 218.6 ppm, which indicated that the C=C double bond of the α,β-unsaturated ketone group was reduced. The olefinic methine proton (9-H) at 8.45 ppm disappeared, and newly appeared ABX type of oxymethylene protons (9-H) at 3.86 and 4.24 ppm was coupled with an X-proton (8-H) at 2.49 ppm, which was further coupled with the *n*-nonyl protons. These observations confirmed the connectivity (Fig. 2b) between the *n*-nonyl group and the α,β-unsaturated ketone group in **1**. The ketal carbon (C-6) at 108.0 ppm in **1** was changed to an oxymethine carbon at 79.1 ppm in **5**, indicating hydrogenolysis of an ether ring in the reduction. A newly appeared oxymethine proton (6-H) at 3.80 ppm, occurring from the hydrogenolysis, was coupled with methylene protons (5-H) at 1.61 and 2.17 ppm. Therefore, the linkage between C-5 and C-6 of **1** was established. Accordingly, the linkages between C-4 and C-20, and between C-1 and C-2, of **1** were now established with the cross peaks from 4-H and 5-H to C-20, and from 2-H to C-1, respectively (Fig. 2a). Thus the citric acid skeleton of **1** was confirmed. The structure of **5** was finally established from ^{1}H-^{13}C long range couplings observed in the field gradient HMBC spectrum, as shown in Fig. 3. The linkage between C-6 and C-7 was established by cross peaks from 5-H and 6-H to C-7. The linkage between C-7 and C-8 was also confirmed by cross peaks from 8-H, 9-H, and 10-H to C-7. The ether bond between C-6 and C-9 was supported by cross peaks from 9-H to C-6.

Table 2. IC_{50} values of trachyspic acid and its reduced compounds for heparanase.

Compound	IC_{50} (μM)
Trachyspic acid (**1**)	36
3	36
4	99
5	91
6	145

Based on the structure of reduction product **5**, the structure of trachyspic acid was deduced as **1** (Fig. 1), since the remaining bond linkage of C-3 to C-6 *via* an oxygen was suggested by the molecular formula of **1**. Trachyspic acid possesses a long alkyl (*n*-nonyl) chain, an α,β-unsaturated ketone, a spiroketal, and a polycarboxylic acid (citric acid skeleton) moiety.

Reduction product **4** showed the same molecular formula and a similar ^{1}H NMR spectrum to those of **5**. The coupling pattern between the oxymethine proton (6-H) at 3.87 ppm and the methylene protons (5-H) at 1.82 and 2.07 ppm of **4** was different from that of **5**, and these proton signals were shifted from those of **5**. These observations suggested that **5** was the isomer of **4** at the C-6 position, occurring from the hydrogenolysis of the ether bond of **1**. Reduction product **3** ($C_{20}H_{30}O_9$, MW: 414), having the UV maximum at 280 nm, showed a similar ^{1}H NMR spectrum to that of **5**, except the α,β-unsaturated ketone moiety. An olefinic methine proton (9-H) at 8.21 ppm and an oxymethine proton (6-H) at 4.46 ppm were observed. These observations indicated that only hydrogenolysis of the ether ring occurred in the reduction of **1** to **3**. Reduction product **6** ($C_{20}H_{30}O_9$, MW: 414) showed no UV maxima at 280 nm, and a similar ^{1}H NMR spectrum to that of **5**, except at the oxymethine (6-H). These observations indicated that only the C=C double bond of the α,β-unsaturated ketone was reduced in **6**.

Biological Activity

The IC_{50} values of trachyspic acid and its reduced

compounds for heparanase are summarized in Table 2. Trachyspic acid inhibited heparanase in a dose-dependent manner with an IC_{50} value of 36 μM (15 μg/ml). On the other hand, trachyspic acid did not show any inhibitory activity towards bovine liver β-glucuronidase, at the concentration of 100 μg/ml, suggesting its specificity to heparanase. Trachyspic acid has a hydrophobic and a hydrophilic moiety within the molecule. It should be noted, however, that the activity of trachyspic acid was not due to the detergent-like influence on the enzyme assay, as Triton X-100 did not affect the assay system, up to the concentration of 0.5%.

The reduced compounds that lost the spiroketal or the C=C double bond of the α,β-unsaturated ketone also showed the inhibitory activities at the same concentration range. Citric acid, whose structure is contained in trachyspic acid, did not inhibit heparanase *per se*, up to the concentration of 1 mM.

It has been reported that *T. trachyspermus*, originally described as *Penicillium spiculisporum*, produces decylcitric acid[13,14] and spiculisporic acid.[15] They also have a long alkyl chain and a polycarboxylic acid moiety, but they have not been reported to have inhibitory activity against heparanase. Further study of the structure-activity relationship of these compounds on the inhibition of heparanase will be informative for the development of new inhibitors of heparanase.

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