Studies on the Metabolism of Gomisin A (TJN-101). II.[1] Structure Determination of Biliary and Urinary Metabolites in Rat

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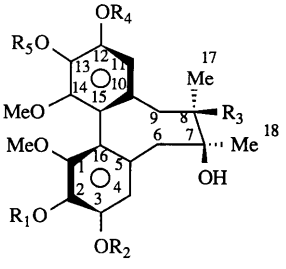
After oral administration of gomisin A (1) to rats, the bile and urine were collected and treated with β-glucuronidase and arylsulfatase. Seven metabolites, met B (2), met A-III (3), met E (4), met D (5), met F (6), met G (7), and met H (8) were isolated from the bile treated with the enzymes. Eight metabolites 2—8, and met A-II (9) were isolated from the urine treated with the enzymes. A major metabolite 2, and two minor metabolites 3 and 9 were identified as met B, met A-III, and met A-II, respectively, which are oxidative products of 1 formed by rat liver S9 mix.[1] The structures of five new metabolites 4—7, and 8 were determined on the basis of chemical and spectral studies.

Keywords *Schizandra chinensis*; gomisin A; TJN-101; lignan; metabolism; metabolite; rat; bile; urine

Gomisin A (TJN-101, **1**)[2] is one of the dibenzocyclooctadiene lignans isolated from the fruits of *Schizandra chinensis* BAILL. (Schizandraceae). Pharmacological studies[3] have revealed that **1** shows an inhibitory effect on some chemical-induced liver injuries, an antihepatotoxic effect and an antitussive effect. We were interested in the metabolism of this bioactive component (**1**), and first studied its metabolites in the rat. In the preceding paper,[1] we reported on five oxidative products of **1** formed by rat liver S9 mix. This paper deals with the structure determination of the biliary and urinary metabolites of **1** treated with β-glucuronidase and arylsulfatase.

After oral administration of **1** to rats, the bile and urine were collected for 48 h. From the results of thin layer chromatography (TLC), it was clear that most of the metabolites existed in bile and urine as their conjugated forms. Therefore, the bile and urine were treated with β-glucuronidase and arylsulfatase, respectively. Seven metabolites, met B (**2**), met A-III (**3**), met E (**4**), met D (**5**), met F (**6**), met G (**7**), and met H (**8**), were isolated from the bile treated with the enzymes, and eight metabolites, **2**—**8**, and met A-II (**9**), were isolated from the urine treated with the enzymes.



1: $R_1=R_2=Me$, $R_3=H$, $R_4+R_5=CH_2$
2: $R_1=R_2=Me$, $R_3=R_4=R_5=H$
3: $R_1=R_3=H$, $R_2=Me$, $R_4+R_5=CH_2$
3a: $R_1=COCH_3$, $R_2=Me$, $R_3=H$, $R_4+R_5=CH_2$
4: $R_1=Me$, $R_2=R_3=H$, $R_4+R_5=CH_2$
4a: $R_1=Me$, $R_2=CH_2C_6H_5$, $R_3=H$, $R_4+R_5=CH_2$
5: $R_1=H$, $R_2=Me$, $R_3=OH$, $R_4+R_5=CH_2$
5a: $R_1=COCH_3$, $R_2=Me$, $R_3=OH$, $R_4+R_5=CH_2$
6: $R_1=R_2=Me$, $R_3=OH$, $R_4=R_5=H$
7: $R_1=Me$, $R_2=H$, $R_3=OH$, $R_4+R_5=CH_2$
8: $R_1=Me$, $R_2=SO_3H$, $R_3=OH$, $R_4+R_5=CH_2$
9: $R_1=R_2=Me$, $R_3=OH$, $R_4+R_5=CH_2$

Chart 1

A major metabolite **2**, and two minor metabolites **3** and **9** were identified as met B, met A-III, and met A-II, respectively, which were obtained by oxidation of **1** with rat liver S9 mix.[1]

Met E (**4**) was obtained as colorless prisms, mp 129—131 °C, $[\alpha]_D$ +45° ($CHCl_3$), and its molecular formula was determined to be $C_{22}H_{26}O_7$ from the high-resolution mass spectrum (MS). The circular dichroism (CD) spectrum of **4** shows a positive Cotton effect at 254 nm ($[\theta]+59000$) and a negative Cotton effect at 220 nm ($[\theta]-50700$), indicating that **4** possesses an *R*-biphenyl configuration.[2] Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table I) is extremely similar to that of **1** except for the functional groups on the aromatic rings. The ^{1}H-NMR spectrum shows that **4** has three methoxyl groups (δ 3.48, 3.79, 3.95), a methylenedioxyl group [δ 5.96, 5.97 (each 1H, d, J=1.5 Hz)], and a phenolic hydroxyl group (δ 5.76) on the aromatic rings, indicating that **4** corresponds to the demethylated derivative of gomisin A. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **4** (Table II), three downfield methoxyl signals are observed at δ 59.7, 60.2, and 61.1, but no upfield methoxyl signal at around δ 56.0, which is assignable to the C-3 methoxyl signal of **1**[4] is observed. This fact suggests that the phenolic hydroxyl group in **4** is located at the C-3 position.

The position of the phenolic hydroxyl group in **4** was determined by measurements of intramolecular nuclear Overhauser effects (NOE) in the benzyl ether (**4a**) of **4** in $CDCl_3$ as shown in Fig. 1. Irradiation of the benzylic methylene signal at δ 5.15 (2H, s) and the C-6β methylene proton signal at δ 2.32 (d, J=13.5 Hz) caused 22% and 19% increases in the integrated intensity of the H-4 signal at δ 6.70. Irradiation of the C-9α methylene proton signal at δ 2.33 (dd, J=14, 7 Hz) and the C-8 methyl signal at δ 0.81 caused 24% and 10% increases in the integrated intensity of the H-11 signal at δ 6.48. These findings indicate that the phenolic hydroxyl group in **4** is linked to C-3. On the basis of the above observations and the J value between the C-9 methylene and the C-8 methine protons ($J_{8,9\alpha}$=8 Hz, $\phi_{8,9\alpha}\fallingdotseq 150°$; $J_{8,9\beta}$=2 Hz, $\phi_{8,9\beta}\fallingdotseq 90°$), the structure of met E was determined as (7*S*,8*S*,*R*-biar)-6,7,8,9-tetrahydro-1,2,14-trimethoxy-12,13-methylenedioxy-7,8-dimethyl-3,7-dibenzo[*a*,*c*]cyclooctenediol (**4**) having a twist-boat-chair conformation[5] of the cyclooctadiene ring.

TABLE I. ^{1}H-NMR Spectral Data for **1**—**9** (δ in $CDCl_3$, 200 MHz)

Compound	H-4, s H-11, s	H-6α (J=Hz)	H-6β (J=Hz)	H-9α (J=Hz)	H-9β (J=Hz)	H–C$_{7(8)}$-Me m	 (J=Hz)	HO[a]–C$_{7(7)}$-Me s	 s	OMe s	OCH_2O (J=Hz)	ArOH[a] br s
1	6.63 6.48	2.69, d (13.5)	2.35, d (13.5)	2.34, dd (14, 7)	2.60, dd (14, 2)	1.86	0.82, d (7)	1.93	1.25	3.52, 3.84 3.91 (×2)	5.96 (2H, s)	
2	6.62 6.65	2.71, d (13.5)	2.39, d (13.5)	2.34, dd (13.5, 7)	2.60, dd (13.5, 1)	1.87	0.82, d (7)	1.87	1.27	3.28, 3.48 3.91 (×2)	—	5.56 (×2)
3	6.63 6.49	2.68, d (13.5)	2.33, d (13.5)	2.35, dd (14, 7)	2.59, dd (14, 2)	1.87	0.82, d (7)	1.60	1.25	3.40, 3.83 3.94	5.97 (2H, s)	5.60
4	6.69 6.49	2.65, d (13.5)	2.31, d (13.5)	2.34, dd (14, 8)	2.64, dd (14, 2)	1.86	0.82, d (7)	1.60	1.24	3.48, 3.79 3.95	5.96 (d, 1.5) 5.97 (d, 1.5)	5.76
5	6.62[b] 6.61[b]	2.77, d (14)	2.52, d (14)	2.37, d (13.5)	2.64, d (13.5)	2.08, s[a] (OH)	1.13, s	1.74	1.31	3.41, 3.84 3.95	5.98 (2H, s)	5.80
6[c]	6.66 6.76	2.80, d (13.7)	2.58, d (13.7)	2.37, d (13.4)	2.66, d (13.4)	2.14, s[a] (OH)	1.13, s	2.14	1.33	3.31, 3.47 3.92, 3.93	—	5.94 (×2)
7[d]	6.70 6.61	2.73, d (13.9)	2.49, d (13.9)	2.36, d (13.6)	2.66, d (13.6)	1.62, s[a] (OH)	1.12, s	1.62	1.29	3.48, 3.82 3.96	5.97 (d, 1.4) 5.98 (d, 1.4)	5.87
8[d,e]	7.26 6.61	2.93, d (13.5)	2.25, d (13.5)	2.50, d (14)	2.62, d (14)	 (OH)	1.06, s	 (OH)	1.24	3.42, 3.77 3.95	5.95 (d, 1.2) 5.96 (d, 1.2)	
9	6.61[b] 6.60[b]	2.78, d (14)	2.53, d (14)	2.36, d (13.5)	2.63, d (13.5)	3.19, s[a] (OH)	1.12, s	2.05	1.30	3.52, 3.85 3.91 (×2)	5.97 (2H, s)	

a) Hydroxyl signals were confirmed on addition of D_2O. *b*) Assignments of these signals may be reversed. *c*) Assignments were based on the ^{1}H–^{1}H correlation spectroscopy (COSY) and NOESY spectra. *d*) These compounds were measured at 500 MHz. *e*) This compound was measured in CD_3OD. *f*) Abbreviations: br = broad, d = doublet, s = singlet.

TABLE II. ^{13}C-NMR Spectral Data for **1**, **3**, **3a**, **4**, **5**, **5a**, **6**, **7**, **8**, and **9** (δ in $CDCl_3$, 50 MHz)

Carbon	**1**	**3**	**3a**	**4**	**5**	**5a**	**6**	**7**	**8**	**9**
1	152.1	145.0	151.4	151.1	145.8	151.6	152.1	151.3	152.7	152.4
2	140.8[b]	137.0	131.7	138.5	137.6	132.2	141.6	139.0	144.8	141.1[b]
3	152.3	146.6	151.0	148.4	146.9	151.2	152.8	148.7	145.8	152.7
4	110.4	110.0	110.5	113.1	109.7	110.3	110.5	113.0	122.5	110.1
5	132.1	127.6	135.1	132.9[b]	126.2	133.9	130.9[b]	131.4	133.9[b]	130.7
6	40.6	40.2	40.9	40.3	40.7	41.1	41.0	40.6	43.3[c]	40.9
7	71.7	71.6	71.8	71.7	75.1	75.1	75.3	75.2	76.4	75.2
8	42.1	42.1	42.0	41.9	72.8	73.0	73.1	72.8	73.8	72.9
9	33.8	33.7	34.0	34.0	42.9	42.8	42.3	42.9	41.8[c]	42.8
10	132.5	132.7	132.7	132.8[b]	132.4	132.4	130.5[b]	132.5	133.8[b]	132.3
11	105.9	106.1	105.9	106.1	106.3	106.2	114.5	106.4	107.4	106.2
12	147.9	148.1	148.2	147.9	148.4	148.4	143.6	148.2	149.7	148.2
13	135.0	135.1	135.1	135.2	135.4	135.4	134.7	135.5	136.8	135.2
14	141.3[b]	141.3	141.4	141.2	141.1	141.2	145.1	141.1	142.4	141.3[b]
15	121.9	121.6	121.5	122.0	121.3	121.1	120.2	121.6	123.3	121.5
16	124.2	123.5	123.9	123.3	123.4	123.7	123.7	123.1	128.6	124.0
17	15.8	15.8	15.9	15.7	22.5	22.7	22.5	22.5	23.2	22.6
18	30.1	30.1	30.0	30.2	26.4	26.6	26.6	26.7	27.3	26.5
OMe										
C-1,14	60.6,59.6	60.2,59.8	60.5,59.8	60.2,59.7	60.3,59.8	60.5,59.8	60.5[c],60.8[c]	60.2,59.7	60.9[d],60.6[d]	60.9,59.6
C-2,13	61.0, —	—, —	—, —	61.1, —	—, —	—, —	61.1, —	61.1, —	61.8, —	61.0, —
C-3,12	56.0, —	56.2, —	56.1, —	—, —	56.3, —	56.2, —	56.2, —	—, —	—, —	56.0, —
OCH_2O	100.8	100.9	100.9	100.9	101.0	101.0	—	101.0	102.3	100.9
CO–CH_3	—	—	168.8,20.6	—	—	168.9,20.6	—	—	—	—

a) This compound was measured in $CDCl_3$. *b*—*d*) Assignments within any column may be reversed.

Met D (**5**) and met G (**7**) were obtained as colorless prisms, mp 128—130 °C, $[\alpha]_D$ +80.5° ($CHCl_3$) and an amorphous powder, $[\alpha]_D$ +61° ($CHCl_3$), respectively. The molecular formulae of both **5** and **7** were determined from the high-resolution MS to be the same $C_{22}H_{26}O_8$. Their CD spectra show a positive Cotton effect at 255 nm and a negative Cotton effect at 226 nm (**5**) or 220 nm (**7**), indicating that both of them possess an *R*-biphenyl configuration. The ^{1}H-NMR spectra of **5** and **7** (Table I) are similar to that of met A-II (**9**) except for the functional groups on the aromatic rings, and show that they have a methylenedioxyl, a phenolic hydroxyl, and three methoxyl groups on the aromatic rings. Methylation of **5** and **7** with dimethylsulfate and potassium carbonate in acetone gave the same monomethyl ether (**9**) which was identical with met A-II on direct comparison (mixed melting point, $[\alpha]_D$, infrared spectrum (IR) and ^{1}H-NMR), indicating that both of them correspond to the demethylated derivative of **9**. The position of the phenolic hydroxyl group in **5** and **7** was determined by ^{13}C-NMR spectral analyses as follows.

On acetylation, **5** afforded a monoacetate (**5a**) as a white amorphous powder, $C_{24}H_{28}O_9$. The C-2 shifts in the ^{13}C-NMR spectra of **5** and **5a** (Table II) show upfield shifts of 3.5 and 8.9 ppm, respectively, compared with the C-2 shift

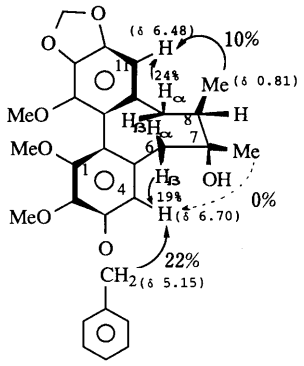


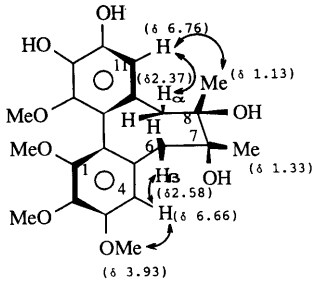
Fig. 1. NOE in **4a** (in $CDCl_3$)

of **9**. These shift values correspond to those in **3**, which is the C-2 demethylated derivative of **1**, and the monoacetate (**3a**)[1] of **3**, respectively, as shown in Table II. Namely, the C-2 shifts of **3** and **3a** show upfield shifts of 3.8 and 9.1 ppm, respectively, compared with the C-2 shift of **1**. The other aromatic carbon signals of **5** appear at the same regions as those of **3** as well as the C-2 signal. The above observations indicate that the phenolic hydroxyl group in **5** is linked to C-2. Thus, met D was identified as (7*S*,8*R*,*R*-biar)-6,7,8,9-tetrahydro-1,3,14-trimethoxy-12,13-methylenedioxy-7,8-dimethyl-2,7,8-dibenzo[*a*,*c*]cyclooctenetriol (**5**).

In the ^{13}C-NMR spectrum of **7** (Table II), three downfield methoxyl signals are observed at δ 59.7, 60.2 and 61.1, but no upfield methoxyl signal at around δ 56.0 which is assigned to the C-3 methoxyl group of **9** is observed. The higher field protonated aromatic carbon signal at δ 106.4 in **7** which appears at the same region as the C-11 signal (δ 106.2) of **9** can be assigned to C-11. The other protonated aromatic carbon signal at δ 113.0 in **7** is consequently assigned to C-4. The C-4 signal of **7** shows a downfield shift of 2.9 ppm compared with that (δ 110.1) of **9**. When a methoxyl group at the *ortho*-position relative to H-4 (or H-11) in the dibenzocyclooctadiene lignan is replaced by a hydroxyl group, the C-4 (or C-11) signal of the hydroxy compound shows a downfield shift of *ca.* 3 ppm.[4] These findings show the presence of a phenolic hydroxyl group at the C-3 position (*ortho*-position relative to H-4) in **7**. Furthermore, the aromatic carbon signals of **7** appear at the same regions as those of met E (**4**) possessing a hydroxyl group at the C-3 position.

On the basis of the above observations, met G was identified as (7*S*,8*R*,*R*-biar)-6,7,8,9-tetrahydro-1,2,14-trimethoxy-12,13-methylenedioxy-7,8-dimethyl-3,7,8-dibenzo[*a*,*c*]cyclooctenetriol (**7**).

Met F (**6**) was obtained as colorless prisms, mp 217.5—220 °C, $[\alpha]_D$ +107° ($CHCl_3$), and its molecular formula was determined to be $C_{22}H_{28}O_8$ from the elemental analysis. Its CD spectrum shows a positive Cotton effect at 251 nm ($[\theta]$+96100) and a negative Cotton effect at 220 nm ($[\theta]$−116000), indicating that **6** possesses an *R*-biphenyl configuration. Its ^{1}H-NMR spectrum (Table I) shows that **6** has four methoxyl and two phenolic hydroxyl [δ 5.94 (2H)] groups on the aromatic rings, and two tertiary methyl (δ 1.13 and 1.33) and two hydroxyl groups on the cyclooctadiene ring. The aromatic carbon signals in the ^{13}C-NMR spectrum of **6** (Table II) appear at the same regions as those



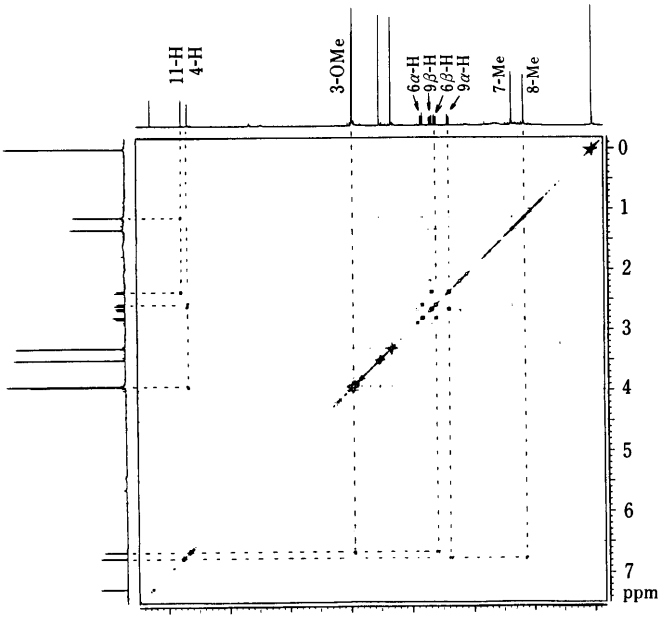


Fig. 2. NOESY Spectrum of **6** in $CDCl_3$.

of **2** possessing two phenolic hydroxyl groups at the C-12 and C-13 positions. A comparison of the ^{1}H- and ^{13}C-NMR spectra of **6** with those of **9** suggested **6** to be the demethylenated derivative of **9**. This was supported by the two-dimensional NOE spectroscopy (NOESY) spectrum of **6** in $CDCl_3$, as shown in Fig. 2. The NOESY spectrum showed appreciable NOE between the C-3 methoxyl signal at δ 3.93 and the higher-field aromatic proton signal (H-4) at δ 6.66, and between the higher-field methyl signal (C-8 methyl) at δ 1.13 and the lower-field aromatic proton signal (H-11) at δ 6.76, but no NOE between the lower-field methyl signal (C-7 methyl) at δ 1.33 and the H-4 signal. These NOESY spectral data indicate that **6** has the *cis*-dimethyl groups as well as **9**.

On methylenation with methylene iodide and potassium carbonate in dry dimethylsulfoxide, **6** afforded a methylenated compound, $C_{23}H_{28}O_8$, which was identified as met A-II (**9**). Thus, met F was identified as (7*S*,8*R*,*R*-biar)-6,7,8,9-tetrahydro-1,2,3,14-tetramethoxy-7,8-dimethyl-7,8,12,13-dibenzocyclo[*a*,*c*]cyclooctenetetraol (**6**).

Met H (**8**) was obtained as a white amorphous powder and its IR spectrum shows bands due to a hydroxyl (3468 cm^{-1}) and a typical sulfate S=O stretching vibration (1230 cm^{-1}).[6] Negative ion fast atom bombardment mass spectral (FAB-MS) data show that **8** has the molecular weight of 498 [*m*/*z* 497 $(M-H)^-$]. The constitution of the significant fragment peak at *m*/*z* 543 [$(M-H+2Na)^+$] in the positive ion FAB-MS of **8** was determined to be $C_{22}H_{25}Na_2O_{11}S$ from the high-resolution FAB-MS. In

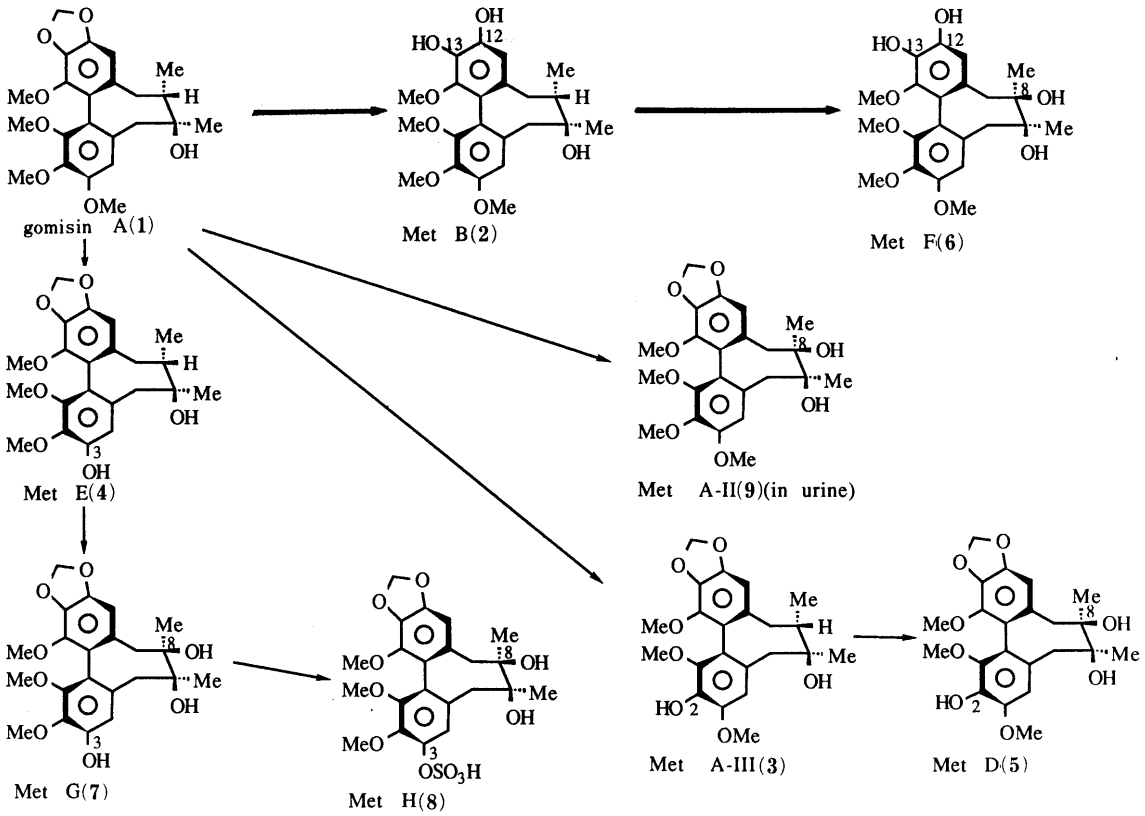


Chart 2. Biliary and Urinary Metabolites of Gomisin A (**1**) in Rat

addition, the significant fragment peak at *m/z* 81 $[(SO_3H)^+]$ in the positive FAB-MS supported the presence of a sulfate group in **8**. These IR and FAB-MS spectral data show that **8** is a sulfated compound possessing the molecular formula $C_{22}H_{26}O_{11}S$.

Its ^{1}H-NMR spectrum (Table I) shows that **8** possesses a methylenedioxyl and three methoxyl groups on the aromatic rings, and two tertiary methyl (δ 1.06 and 1.24) and two methylene groups on the cyclooctadiene ring. Its ^{1}H-NMR spectrum is similar to that of **7** except that the H-4 signal (δ 7.26) of **8** appears at lower field than that (δ 6.70) of **7**. In the ^{13}C-NMR spectra of **7** and **8** (Table II), the C-2, C-4 and C-16 signals of **8** are shifted downfield by 5.8, 9.5 and 5.5 ppm, respectively, compared with those of **7**. The other carbon signals of **8** appear at the same regions as those of **7**. Ragan[7] and Barron and Ibrahim[8] reported on the ^{13}C-NMR spectroscopic investigation of phenol sulfate esters; they found that sulfation of a phenolic hydroxyl group induces downfield shifts of *ortho* (5.4—7.8 ppm) and *para* (5.6—7.4 ppm) carbons to the sulfate group. The C-2, C-4 and C-16 signals in the ^{13}C-NMR spectrum of **8** are shifted downfield by 5.8, 9.5, and 5.5 ppm compared with those of **7**, respectively, and the other carbon signals appear at the same regions as those of **7**. These ^{1}H- and ^{13}C-NMR spectral data suggest that the sulfate group in 8 is linked to the C-3 phenolic hydroxyl group of **7**.

Hydrolysis of **8** with 3% ethanolic potassium hydroxide afforded **7**. From the above results, met H was identified as (7*S*,8*R*,*R*-biar)-6,7,8,9-tetrahydro-7,8-dihydroxy-1,2,14-trimethoxy-12,13-methylenedioxy-7,8-dimethyldibenzo-[*a*,*c*]cycloocten-3-yl hydrogen sulfate (**8**).

The structures of biliary and urinary metabolites of **1** in rats are shown in Chart 2. These metabolites exist in bile and urine as their conjugated forms (sulfates or glucuronides). The major metabolite of **1** found in rat bile and urine was identified as **2**, which is the demethylenated derivative of **1**. From the results of these preliminary studies, the metabolic fate of **1** after oral administration of **1** in rats is suggested to be as follows. First, the demethylenated derivative (**2**) and the *O*-demethylated derivatives (**3** and **4**) of **1** are formed. Next, the 8-hydroxygenated derivatives (**5**, **6** and **7**) of these preliminary metabolites are formed.

Detailed metabolic studies of **1** by using these authentic samples and studies of the general pharmacology of the major metabolite **2** are being undertaken by our coworkers.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus (a hot stage type) and are uncorrected. The ultraviolet (UV) spectra were recorded with a Hitachi U-3200 spectrophotometer and the IR spectra with a Hitachi 270-30 infrared spectrophotometer. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded with Bruker AM-500 and JEOL JNM-FX-200 NMR spectrometers using tetramethylsilane (TMS) as an internal standard. The specific rotations were measured with a JASCO DIP-360 digital polarimeter and CD spectra with a JASCO J-600 spectropolarimeter. The MS were measured with JEOL JMS-DX-300 and JEOL JMS-HX-110 mass spectrometers. For silica gel column chromatography, Kieselgel 60 (Merck) was used. Kieselgel $60F_{254}$ (Merck precoated plate) was used for preparative thin layer chromatography (prep. TLC) and spots were detected under UV (254 nm). For preparative high-pressure liquid chromatography (prep. HPLC), a JASCO BIP-I high-pressure liquid chromatograph coupled with a UVIDEC-100-VI UV spectrophotometer was used.

Isolation of Biliary Metabolites Male SD strain rats weighing about 250 g (n=14) were orally given **1** suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) at 250 mg/kg. The common bile duct was immediately cannulated under ether anesthesia to collect the bile (135 ml) for 48 h. The urine (150 ml) was also collected for 48 h.

TABLE III. Silica Gel Column Chromatography of Biliary Metabolites Fraction

Fraction No.	Solvent	Volume (ml)	Yield (mg)
1	Hexane–EtOAc (40:60)	200	92
2	Hexane–EtOAc (20:80)	200	49
3	EtOAc	400	72
4	$CHCl_3$–MeOH–AcOH (95:5:1)	450	92
5	$CHCl_3$–MeOH–AcOH (70:30:1)	100	87
6	$CHCl_3$–MeOH–AcOH (70:70:1)	100	79
7	$CHCl_3$–MeOH–AcOH (50:50:1)	200	182

TABLE IV. Silica Gel Column Chromatography of Urinary Metabolites Fraction

Fraction No.	Solvent	Volume (ml)	Yield (mg)
1	Hexane–EtOAc (40:60)	250	19
2	Hexane–EtOAc (40:60)	100	11
3	Hexane–EtOAc (20:80)	250	114
4	EtOAc	200	87
5	$CHCl_3$–MeOH (90:10)	350	80
6	$CHCl_3$–MeOH (80:20)	150	102
	$CHCl_3$–MeOH (60:40)	250	
7	MeOH	150	187

The bile was dissolved in 850 ml of 0.1 M acetate buffer (pH 5.0) and β-glucuronidase (from *Helix pomatia*, type H-1, Sigma Chemical Company) (2.46×10^6 units) was added and this solution was incubated at 37 °C for 8 h. The incubated solution was chromatographed on Sepabeads SP207 (Mitsubishi Chemical Industries Limited) (4.5 cm i.d. × 18 cm), developing with H_2O (5 l), 20% MeOH (2 l) and then MeOH (2 l). The MeOH eluate was concentrated to give a residue (0.651 g), which was chromatographed on silica gel (5 cm i.d. × 20 cm) with a mixture of hexane–EtOAc. The details of this chromatography are given in Table III.

Fraction 1 (92 mg) in Table III was purified by prep. TLC [hexane–EtOAc (1:2)] and the zones with *Rf* 0.58, 0.55, 0.5 and 0.46 were extracted with $CHCl_3$–MeOH (4:1). The extract of the zone with *Rf* 0.58 was concentrated to give **1** (2.5 mg). The extract of the zone with *Rf* 0.55 was further purified by prep. TLC [hexane–acetone (3:2), *Rf* 0.47] to give **4** (2.4 mg). The extracts of the zones with *Rf* 0.5 and 0.46 were concentrated to give **3** (7 mg) and **7** (2.1 mg), respectively. Fraction 2 (49 mg) was purified by prep. TLC [hexane–EtOAc (1:2)] to give **3** (*Rf* 0.5, 4 mg, total 11 mg), **2** (*Rf* 0.45, 31 mg) and **5** (*Rf* 0.4, 5 mg). Fraction 3 (72 mg) was purified by prep. TLC [hexane–EtOAc] to give **2** (*Rf* 0.45, 46 mg) and **5** (*Rf* 0.4, 35 mg, total 47 mg). Fraction 4 (92 mg) was purified by prep. TLC [hexane–EtOAc (1:2)] to give **2** (*Rf* 0.45, 43 mg, total 110 mg) and **6** (*Rf* 0.3, 45 mg). Fraction 5 (87 mg) was purified by prep. TLC [hexane–EtOAc (1:2), *Rf* 0.3] to give **6** (8 mg, total 53 mg). Fraction 6 (79 mg) was purified by prep. TLC [$CHCl_3$–MeOH (3:1), *Rf* 0.33] to give **8** (2 mg).

Isolation of Urinary Metabolites The urine collected for 48 h was dissolved in 850 ml of 0.1 M acetate buffer (pH 5.0) and β-glucuronidase (from *Helix pomatia*, type H-1) (1.68×10^6 units) was added. Following incubation at 37 °C for 24 h, the solution was chromatographed on Sepabeads SP207 (4.5 cm i.d. × 18 cm), developing with H_2O (2 l), 20% MeOH (3 l) and then MeOH (2 l). The MeOH eluate was concentrated to give a residue (0.608 g), which was chromatographed on silica gel (4.5 cm i.d. × 17 cm) with a mixture of hexane–EtOAc. The details of this chromatography are given in Table IV. Fraction 1 (19 mg) in Table IV was purified by prep. TLC [hexane–EtOAc (1:2)] to give **1** (*Rf* 0.58, 1.5 mg) and **4** (*Rf* 0.55, 1 mg). Fraction 2 (11 mg) was purified by prep. TLC [hexane–EtOAc (1:2), *Rf* 0.5] to give **3** (1.5 mg). Fraction 3 (114 mg) was purified by prep. TLC [hexane–EtOAc (1:2)] to give **3** (*Rf* 0.5, 2 mg, total 3.5 mg), **9** (*Rf* 0.48, 6 mg), and a mixture of **2** and **7** (*Rf* 0.45, 54 mg). Fraction 4 (87 mg) was purified by prep. TLC [hexane–EtOAc (1:2)] to give **5** (*Rf* 0.4, 21 mg) and a mixture of **2** and **7** (*Rf* 0.45, 35 mg). The mixture of **2** and **7** (total 89 mg) was purified by prep. TLC [hexane–acetone (3:2)] to give **2** (*Rf* 0.56, 80 mg) and **7** (*Rf* 0.65, 2 mg). Fraction 5 (80 mg) was purified by prep. TLC [hexane–EtOAc (1:2), *Rf* 0.30] to give **6** (45 mg). Fraction 6 (102 mg) was purified by repeated prep. TLC [$CHCl_3$–MeOH (3:1), *Rf* 0.33] to give **8** (1 mg).

Compound **1** was obtained as colorless needles (from MeOH), mp 93—94 °C, and identified as gomisin A by direct comparison with an authentic sample (^{1}H-NMR, IR, MS, and mixed melting point).

Met B (2) Compound **2** was obtained as pale brown prisms (from ether), mp 193—195 °C, $[\alpha]_D^{24}$ +88.4° (*c* = 2.75, $CHCl_3$), and identified as met B[1] by direct comparison with an authentic sample (^{1}H-NMR, IR, MS, $[\alpha]_D$, and mixed melting point). *Anal.* Calcd for $C_{22}H_{28}O_7$: C, 65.33; H, 6.98. Found: C, 65.19; H, 7.02.

Met A-III (3) Compound **3** was obtained as a white amorphous powder, $[\alpha]_D^{24}$ +65° (*c* = 0.48, $CHCl_3$), and identified as met A-III[1] by direct comparison with an authentic sample (^{1}H-NMR, IR, MS, and $[\alpha]_D$).

Met E (4) Met E was obtained as colorless needles (from MeOH), mp 129—131 °C, $[\alpha]_D^{24}$ +45° (*c* = 0.20, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3432, 3332 (OH), 1620, 1585 (aromatic ring). UV λ_{max}^{EtOH} nm (log ε): 217 (4.46), 251 (sh 3.88), 278 (sh 3.44). CD (*c* = 0.0096, MeOH) $[\theta]^{21}$ (nm): −50700 (220), 0 (234), +59000 (254), +11900 (279). MS *m/z* (%): 402 (M^+, 100), 384 (37), 327 (26), 299 (80). High-resolution MS, Calcd for $C_{22}H_{26}O_7$(M^+): 402.1679. Found: 402.1726.

Met D (5) Met D was obtained as colorless prisms (from MeOH–H_2O), mp 128—130 °C, $[\alpha]_D^{23}$ +80.5° (*c* = 1.49, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3576, 3432 (OH), 1614 (aromatic ring). UV λ_{max}^{EtOH} nm (log ε): 219 (4.89), 265 (sh 3.89), 295 (sh 3.30). CD (*c* = 0.0101, MeOH) $[\theta]^{21}$ (nm): −108000 (226), 0 (236), +68900 (244), +81800 (255). MS *m/z* (%): 418 (M^+, 15), 400 ($M^+ - H_2O$, 90), 282 (10), 43 (100). High-resolution MS, Calcd for $C_{22}H_{26}O_8$(M^+): 418.1628. Found: 418.1570. *Anal.* Calcd for $C_{22}H_{26}O_8 \cdot 1/2H_2O$: C, 61.85; H, 6.32. Found: C, 61.89; H, 6.50.

Met F (6) Met F was obtained as colorless prisms (from ether), mp 217.5—220 °C, $[\alpha]_D^{23}$ +107° (*c* = 1.10, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3536, 3516, 3304 (OH), 1620, 1594 (aromatic ring). UV λ_{max}^{EtOH} nm (log ε): 216 (4.63), 259 (sh 4.05), 294 (sh 3.40). CD (*c* = 0.0098, MeOH) $[\theta]^{21}$ (nm): −116000 (220), 0 (231), +80200 (239), +96100 (251), +23300 (279). MS *m/z* (%): 420 (M^+, 60), 377 (69), 333 (57), 301 (100), 288 (24). High-resolution MS, Calcd for $C_{22}H_{28}O_8$(M^+): 420.1761. Found: 420.1759. *Anal.* Calcd for $C_{22}H_{28}O_8$: C, 62.84; H, 6.71. Found: C, 62.86; H, 6.63.

Met G (7) Met G was obtained as a white amorphous powder, $[\alpha]_D^{24}$ +61° (*c* = 0.615, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 1620 (aromatic ring). UV λ_{max}^{EtOH} nm (log ε): 218 (4.51), 254 (3.94), 292 (sh 3.38). CD (*c* = 0.00952, MeOH) $[\theta]^{21}$ (nm): −73600 (220), 0 (234), +87600 (255). MS *m/z* (%): 418 (M^+, 39), 375 (100), 331 (40), 316 (22), 301 (72), 300 (34). High-resolution MS, Calcd for $C_{22}H_{26}O_8$(M^+): 418.1627. Found: C, 418.1603.

Met H (8) Met H was obtained as a white amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3468 (OH), 1630, 1568 (aromatic ring), 1230, 1044 (SO_3H). Negative ion FAB-MS *m/z*: 519 ($M-2H+Na)^-$, 497 ($M-H)^-$. Positive ion FAB-MS *m/z*: 543 ($M-H+2Na)^+$, 81 $(SO_3H)^+$. High-resolution FAB-MS, Calcd for $C_{22}H_{26}Na_2O_{11}S$[($M-H+2Na)^+$]: 543.0907. Found: 543.0913.

Met A-II (9) Compound **9** was obtained as colorless needles (from EtOH–H_2O), mp 163—165 °C, $[\alpha]_D^{24}$ +71° (*c* = 0.21, $CHCl_3$), and identified as met A-II[1] by direct comparison with an authentic sample (^{1}H-NMR, MS, $[\alpha]_D$, and mixed melting point). High-resolution MS, Calcd for $C_{23}H_{28}O_8$(M^+): 432.1784. Found: 432.1811.

Benzylation of 4 Benzyl chloride (30 mg) and K_2CO_3 (100 mg) were added to a solution of **4** (5 mg) in a mixture of *N,N*-dimethylformamide and H_2O (100:1) (0.8 ml). The reaction mixture was heated at 90 °C for 2 h, cooled, and diluted with ether. The ethereal solution was washed with H_2O, dried over Na_2SO_4 and concentrated. The residue was purified by prep. TLC [hexane–acetone (3:2)] to give **4a** (5 mg) as a white amorphous powder. MS *m/z* (%): 492 (M^+, 30), 474 (8.5), 401 ($M^+ - C_6H_5CH_2$, 27), 383 (18), 91 (100). High-resolution MS, Calcd for $C_{29}H_{32}O_7$(M^+): 492.2148. Found: 492.2140. ^{1}H-NMR (in $CDCl_3$) δ: 0.81 (3H, d, *J* = 7 Hz, 7-CH_3), 1.23 (3H, s, 8-CH_3), 1.56 (1H, s, OH), 1.80 (1H, m, H-8), 2.32 (1H, d, *J* = 13.5 Hz, H-6β), 2.33 (1H, dd, *J* = 14, 7 Hz, H-9α), 2.59 (1H, dd, *J* = 14, 2 Hz, H-9β), 2.67 (1H, d, *J* = 13.5 Hz, H-6α), 3.55 (3H, s), 3.84 (3H, s), 3.92 (3H, s) (3 × OCH_3), 5.96 (1H, d, *J* = 1.5 Hz), 5.97 (1H, d, *J* = 1.5 Hz) (OCH_2O), 6.48 (1H, s, H-4), 6.70 (1H, s, H-11), 5.15 (2H, s), 7.40 (5H, m) ($C_6H_5CH_2$–).

Methylation of 5 $(CH_3)_2SO_4$ (0.2 ml) and K_2CO_3 (100 mg) were added to a solution of **5** (8 mg) in dry acetone (1 ml). The reaction mixture was stirred at 45 °C for 3 h, and then diluted with ether. The ethereal solution was washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by prep. TLC [hexane–acetone (3:2), *Rf* 0.68] to give the monomethyl ether (7.8 mg) as colorless prisms (from EtOH), mp 163—165 °C, $[\alpha]_D^{24}$ +66° (*c* = 0.39, $CHCl_3$). High-resolution MS, Calcd for $C_{23}H_{28}O_8$(M^+): 432.1772. Found: 432.1771. This compound was identified

as met A-II (**9**) by direct comparison with an authentic sample (^{1}H-NMR, IR, MS, $[\alpha]_D$, and mixed melting point).

Acetylation of 5 A solution of **5** (19 mg) in a mixture of pyridine (0.4 ml) and Ac_2O (0.25 ml) was allowed to stand at room temperature overnight, then diluted with H_2O and extracted with ether. The ethereal extract was washed with 1 N HCl, 5% $NaHCO_3$, then H_2O, dried over Na_2SO_4 and concentrated. The residue was purified by prep. TLC [hexane–acetone (7 : 3)] to give **5a** (15 mg) as a white amorphous powder, $[\alpha]_D^{24}$ +90° (c=0.58, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3524 (OH), 1764 (aromatic ring). MS m/z (%): 460 (M^+, 24), 418 (53), 400 (28), 375 (100), 331 (38), 301 (69). High-resolution MS, Calcd for $C_{24}H_{28}O_9$(M^+): 460.1648. Found: 460.1638. ^{1}H-NMR (in $CDCl_3$) δ: 1.13 (3H, s, 7-CH_3), 1.31 (3H, s, 8-CH_3), 2.35 (3H, s, $COCH_3$), 2.38 (1H, d, J=13.5 Hz, H-9α), 2.57 (1H, d, J=14 Hz, H-6β), 2.63 (1H, d, J=13.5 Hz, H-9β), 2.80 (1H, d, J=14 Hz, H-6α), 3.32 (3H, s), 3.83 (3H, s), 3.87 (3H, s) (3×OCH_3), 5.97 (2H, s, OCH_2), 6.60 (1H, s, H-11), 6.68 (1H, s, H-4).

Methylenation of 6 CH_2I_2 (13.2 mg) and K_2CO_3 (40 mg) were added to a solution of **6** (8.5 mg) in dry dimethylsulfoxide (1 ml). The reaction mixture was stirred at 55 °C for 5 h under argon, then diluted with ether. The ethereal solution was treated as described for the methylation of **5** to give a methylenated compound (3 mg) as colorless prisms (from EtOH), mp 162.5—164 °C, $[\alpha]_D^{24}$ +56° (c=0.18, $CHCl_3$). This compound was identified as met A-II (**9**) by direct comparison with an authentic sample (^{1}H-NMR, MS, IR, $[\alpha]_D$, and mixed melting point).

Methylation of 7 $(CH_3)_2SO_4$ (0.05 ml) and K_2CO_3 (50 mg) were added to a solution of **7** (1.5 mg) in dry acetone (1.5 ml). The reaction mixture was stirred at 45 °C for 3 h, and then treated as described for the methylation of **5** to give a monomethyl ether (1 mg) as a white amorphous powder, $[\alpha]_D^{23}$ +60° (c=0.05, $CHCl_3$). This compound was identified as met A-II (**9**) by direct comparison with an authentic sample (^{1}H-NMR, MS, and $[\alpha]_D$).

Hydrolysis of 8 A solution of **8** (1.5 mg) in 3% ethanolic potassium hydroxide (1 ml) was kept at 60 °C for 5 h, then diluted with H_2O (10 ml). The reaction mixture was chromatographed on Sepabeads SP207 (1 cm i.d. × 3 cm), developing with H_2O (30 ml) and then MeOH (30 ml). The MeOH eluate was concentrated to give a residue, which was purified by prep. TLC [hexane–EtOAc (1 : 2)] to give **7** (0.6 mg) as a white amorphous powder. This compound was identified as met G by direct comparison with an authentic sample (^{1}H-NMR, MS, and TLC).

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