Cytotoxic Flavonoids with Isoprenoid Groups from *Morus mongolica*[1]

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A new pyranoflavanone, sanggenol L (**1**), a Diels−Alder type adduct regarded as a cycloaddition product of a dehydrogeranylflavanone and a prenylchalcone, sanggenol M (**2**), along with four new 2-arylbenzofurans with isoprenoid units, mulberrofurans W−Z (**3**−**6**), were isolated together with 10 known flavonoids from Chinese *Morus mongolica*. The structures of these novel compounds were elucidated by spectroscopic methods. All flavanones investigated here showed higher cytotoxicity against human oral tumor cell lines (HSC-2 and HSG) than against normal human gingival fibroblasts (HGF). Among them, the cytotoxicity of compound **2** and the Diels−Alder type flavanone sanggenon C (**7**) isolated from *Morus cathayana* were the most potent. On the other hand, seven 2-arylbenzofurans exhibited lower cytotoxicity and tumor specificity as compared with flavanones.

The mulberry tree has been widely cultivated, and its leaves are used for silkworms in China. We studied the phenolic constituents of cultivated Japanese mulberry tree (*Morus alba*, *M. lhou*, and *M. bombycis*) and isolated several phenols (flavonoids and stilbenes) with 3-methyl-2-butenyl (prenyl) group(s).[2] A variety of bioactivities of these compounds were also reported.[2a,3,4] Furthermore, the main phenolic constituents (sanggenon-type flavanones) of the Chinese crude drug "Sang-bai-pi" (*Morus* root bark)[5,6] were different from those (morusin, kuwanons G and H) isolated from Japanese mulberry tree.[2a,7] Nine *Morus* species and their varieties are distributed in China,[8] and four of these species (*M. alba*, *M. cathayana*, *M. mongolica*, and *M. australis*) have been recorded in books of traditional Chinese herbal medicines as the material from which the drug "Sang-bai-pi" is derived.[5] Previously, Nomura reported that the drug imported from China was derived from *M. mongolica*, judging from the presence of some sanggenons in the plant.[2a] Recently, we isolated these sanggenons from *M. cathayana*.[9] This prompted us to reinvestigate the constituents of wild *M. mongolica* in China. We report here the isolation and structure elucidation of flavonoids including six new compounds from the root bark of Chinese *M. mongolica* (Moraceae), the cytotoxicities of the new compounds, and the structures of flavonoids isolated from Chinese mulberry trees.

Results and Discussion

Sanggenol L (**1**), $C_{25}H_{26}O_6$, $[\alpha]_D$ −18° (MeOH), was isolated as a diastereomeric mixture and gave a positive ferric chloride test. The UV spectrum exhibited maxima at 228, 272, and 359 nm and was similar to that of a linear analogue, kuwanol C (**8**).[10] The ^{1}H NMR spectrum (Table 1) also resembled that of **8** except for the chemical shifts of 5-hydroxyl protons (δ 12.12 and 12.11, each 0.5H, in $CDCl_3$). With the exception of the chemical shift of 5-OH, the ^{1}H and ^{13}C NMR spectra of 6- or 8-isoprenoid-substituted flavanones with the same B- and C-rings are almost identical.[11] Nomura and Fukai reported that structures of 6-prenylflavonoids and 8-prenylflavonoids were differentiated via their 5-OH chemical shifts; that is, the 5-OH signal of 6-prenylflavonoids is more deshielded (0.3−0.4 ppm) than that of 8-prenylflavonoids with the same B- and C-rings.[12−14] A similar shift difference (ca. 0.2 ppm, in $CDCl_3$) was also observed in linear pyranoflavanones and angular pyranoflavanones.[12] The angular structure of **1** was confirmed by comparison of the chemical shifts of 5-OH of **1** and a diastereomeric mixture **8** (δ 12.29 and 12.28, each 0.5H) obtained here.[15] Furthermore, the UV spectrum of **1** showed a bathochromic shift with addition of aluminum chloride, indicating the absence of a bulky group at the 6-position. However, compound **8** resisted the aluminum chloride-induced shift in its UV spectrum due to obstruction of chelation of the aluminum ion to the 5-OH and 4-carbonyl oxygen by the bulky group at the 6-position.[12,16] These observations indicated that sanggenol L is a structural isomer of **8**, and thus sanggenol L is an angular flavanone as shown in formula **1**. (See Chart 1 for structures.)

These flavanones (**1** and **8**) were isolated as C-2 epimers. Their CD spectra showed a positive Cotton effect at 253 and 270 (sh) nm, but Cotton effects on the UV band of the benzoyl chromophore near 300 and 330 nm were not observed. Each stereoisomer of **1** and **8** could be isolated by HPLC (the 5-OH signal of each isolated compound appeared as a single peak in their ^{1}H NMR spectra), but the identification of (2*S*)- and (2*R*)-flavanones failed due to rapid isomerization during the process of CD spectrum measurement in ethanol.[17−19] These observations indicated that **1** is a mixture of (2*S*)- and (2*R*)-sanggenol L, but not the epimers at C-11. The stereochemistry at C-11 remains to be determined.

Sanggenol M (**2**), a pale yellow powder, $[\alpha]_D$ −126° (MeCN), $C_{45}H_{46}O_{11}$, was also isolated as a racemic mixture and gave a positive ferric chloride test. The UV spectrum resembled that of a Diels−Alder type adduct, sanggenon G (**9**).[20] Its ^{1}H NMR spectrum at room temperature in acetone-d_6 showed broad signals, except for the intramolecular hydrogen-bonded hydroxyl protons [δ 12.45 and

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Table 1. NMR Spectral Data for Compound **1** in $CDCl_3$ (25 °C) and **2** in DMSO-d_6 (140 °C)

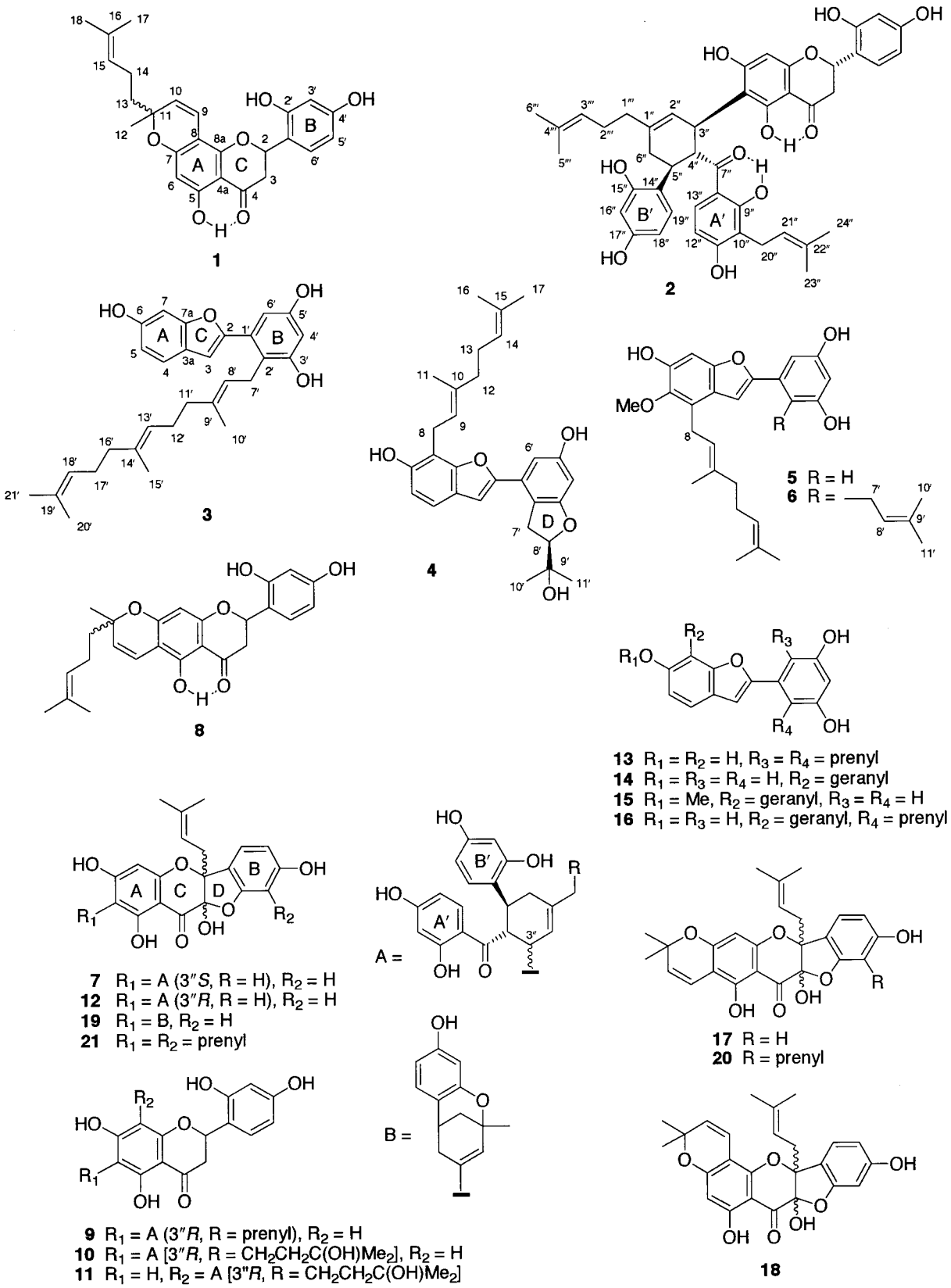
	1			**2**	
C/H no.	^{1}H (δ, J in Hz)[a]	^{13}C[b]	C/H no.	^{1}H (δ, J in Hz)[c,d]	^{13}C
2	5.61 dd (3.1, 13.2)	77.2		5.45, 5.44 (each 0.5H) dd (3.3, 12.5)	73.5 (br)
3$_{(cis)}$	2.86 dd (3.1, 17.2)	41.9		2.58 dd (3.3, 17.0)	40.9, 41.0
3$_{(trans)}$	3.12 dd (13.2, 17.2)			3.01 dd (12.5, 17.0)	
4		196.1			195.6
4a		101.9			101.0
5		164.0			161.3
6	6.01 d (0.7)	97.8			108.5
7		156.2			(163.5)[e]
8		102.8		5.79, 5.78 (each 0.5H) s	94.0
8a		162.5			160.6
1′		116.7			115.5 (br)
2′		155.1			154.9
3′	6.42 d (2.4)	103.9		6.34 br d (2.0)	102.6
4′		157.3			157.9
5′	6.45 dd (2.4, 8.4)	107.9		6.27 dd (2.0, 8.0)	106.3
6′	7.16 d (8.4)	125.8		7.10, 7.09 (each 0.5H) d, (8.0)	127.0 (br)
9	6.55 dd (0.7, 10.3)	115.7	1″		134.8, 134.9
10	5.43 d (10.3)	128.1	2″	5.22 br s	123.4 (br)
11		80.8	3″	4.25 br d (11.0)	36.3, 36.4
Me-12	1.41 s	27.2	4″	4.73 t (11.0)	45.5 (br)
CH_2-13	2.06 m	41.7	5″	3.54 ddd, (5.0, 11.0, 11.0)	35.1 (br)
CH_2-14	2.06 m		6″	2.44 br d (12.7)	38.1 (br)
15	5.07 m	123.7	6″	2.11 m	
16		132.0	7″		207.8
17	1.65 br s	17.6	8″		114.0
18	1.57 br s	25.7	9″		(159.3)
5-OH	12.12, 12.11 (each 0.5H) s		10″		113.0
			11″		(161.9)
			12″	6.13, 6.09 (each 0.5H) d (8.5)	105.6
			13″	7.43, 7.40 (each 0.5H) d (8.5)	128.7
			14″		120.6
			15″		154.9
			16″	6.14 br d (2.0)	102.8
			17″		155.4
			18″	5.99 dd (2.0, 8.0)	105.8
			19″	6.76 d (8.0)	127.8
			CH_2-20″	3.09, 3.08 (each 1H) br d (6.5)	20.6
			21″	5.06, 5.05 (each 0.5H) m	122.1
			22″		129.1
			Me-23″	1.59, 1.57 (each 1.5H) br s	16.7
			Me-24″	1.65, 1.63 (each 1.5H) br s	24.4
			CH_2-1‴	2.02 m	36.1
			CH_2-2‴	2.10 m	25.5
			3‴	5.18 m	123.8
			4‴		129.6
			Me-5‴	1.60 br s	16.7
			Me-6‴	1.68, 1.67 (each 1.5H) br s	24.4
			5-OH	12.41 br s	
			9″-OH	13.00 s	

[a] Digital resolution of the measurement is 0.19 Hz. [b] Temperature of the probe of instrument was 28 °C during the measurement. [c] Digital resolution of the measurement is 0.25 Hz. [d] Hydroxyl groups: δ 10.01, 9.51, 8.97, 8.81, 8.41, and 8.24 (each 1H, br s, 2′-OH, 4′-OH, 7-OH, 11″-OH, 15″-OH, and 17″-OH). [e] The signal in parentheses was observed as a cross-peak in the HMBC spectrum of **2**.

12.87 (each 0.5H, 5-OH), 13.30 (1H, 9″-OH)], due to an equilibrium mixture of conformational isomers (conformer of the cyclohexene ring or rotamer about the C-6−C-3″ or C-5″−C-14″ bond). This was observed for all-*trans* Diels−Alder type adducts with a 3″,4″-*trans*-4″,5″-*trans*-3″,4″,5″-trisubstituted cyclohexene ring from moraceous plants.[2a,21] Thus, NMR was performed at 140 °C in DMSO-d_6 (Table 1). Sharp signals were observed in the ^{1}H NMR spectrum at this temperature, but some protons exhibited different chemical shifts due to their conformational isomers[9b,22] or the presence of the two C-2 diastereoisomers. Thus, H-6′ appeared at δ 7.10 (0.5H) and 7.09 (0.5H) as doublets, and H-8 resonated at δ 5.79 (0.5H, s) and 5.78 (0.5H, s). The NMR spectra (COSY, HMQC, HMBC, etc.) indicated the presence of a 2′,4′,5,7-tetrahydroxyflavanone with a substituent at C-6 or C-8, a 2,4-dihydroxyphenyl group (B′-ring), a 2,4-dihydroxy-3-prenylbenzoyl group (A′-ring), and a cyclohexene ring with a 1″-(4‴-methyl-3‴-pentenyl) group. The above results indicated that sanggenol M was 10″-prenylsanggenon G or a constitutional isomer. The chemical shifts of 5-OH of **2** resembled those of **9** [δ 12.51 and 12.91 (each 0.5H) in acetone-d_6] and sanggenon T (**10**) [δ 12.50 and 12.91 (each 0.5H) in acetone-d_6], but not those of **11** (δ 12.10, 1H, in acetone-d_6),[23] a positional isomer with a cyclohexene ring at C-8. The latter compound was derived from **10** under alkaline conditions. Taken together with the resistance to the $AlCl_3$-induced shift in the UV spectrum of **2**, the chemical shifts of the 5-OH of **2** also confirmed the presence of the cyclohexene ring at C-6.[12,16]

The coupling constants of the cyclohexene ring protons ($J_{H\text{-}3''-H\text{-}4''} = J_{H\text{-}4''-H\text{-}5''} = 11.0$ Hz) indicated that **2** was an all-*trans* type Diels−Alder adduct. Considering the relative configuration of the three chiral centers in the cyclohexene ring and the negative optical rotation value, the absolute configurations of the three chiral centers were assigned as 3″*R*, 4″*R*, and 5″*S*.[2a,23] The similarity of CD curves of **2**

Chart 1



and sanggenon D (**12**) also confirmed this assignment. On the basis of these observations structure **2** was assigned to sanggenol M.

The identification of (2*S*)- and (2*R*)-sanggenol M (**2**) was carried out on the basis of their CD spectra as follows. The differential CD spectrum between the diastereoisomeric mixture of **2** and an isolated isomer (the faster eluting compound in preparative HPLC), the spectrum of the isomer minus that of the mixture [1:1 mixture of (2*S*)- and (2*R*)-flavanones], showed a negative Cotton effect at 296 nm and a positive Cotton effect at 331 nm [(2*S*)-flavanone]. The differential CD spectrum between the diastereoisomeric mixture of **2** and the other isomer (the slower eluting compound in the HPLC) exhibited a positive Cotton effect

Table 2. ^{1}H NMR Spectral Data for Compounds **3**, **4**, **5**, and **6** in Acetone-d_6 (δ, J in Hz)[a]

H no.	**3**[b]	**4**	**5**	**6**
3	6.78 d (0.9)	6.98 s	7.02 d (0.9)	6.80 d (0.7)
4	7.40 d (8.5)	7.26 d (8.5)		
5	6.81 dd (2.1, 8.5)	6.84 d (8.5)		
7	6.97 dd (0.9, 2.1)		6.91 d (0.9)	6.90 d (0.7)
2′			6.83 d (2.1)	
4′	6.49 d (2.5)	6.24 d (2.1)	6.34 t (2.1)	6.47 d (2.5)
6′	6.73 d (2.5)	6.92 d (2.1)	6.83 d (2.1)	6.75 d (2.5)
CH_2-7′	3.51 d (6.1)	3.43 dd (7.9, 15.8)		3.50 br d (6.0)
		3.40 dd (9.5, 15.8)		
8′	5.20 m	4.70 dd (7.9, 9.5)		5.17 m
Me-10′	1.68 br d (1.2)	1.27 s		1.70 br s
CH_2-11′	1.91−2.08 m			
Me-11′		1.26 s		1.66 br s
CH_2-12′	1.91−2.08 m			
13′	5.11 m			
Me-15′	1.61 br d (0.9)			
CH_2-16′	1.91−2.08 m			
CH_2-17′	1.91−2.08 m			
18′	5.04 m			
Me-20′	1.56 br s			
Me-21′	1.53 br s			
CH_2-8		3.65 br d (7.3)	3.59 br d (6.5)	3.58 br d (7.0)
9		5.48 m	5.32 m	5.31 m
Me-11		1.88 br d (0.9)	1.86 br d (0.9)	1.84 br s
CH_2-12		1.97−2.08 m	1.99−2.12 m	2.00−2.04 m
CH_2-13		1.97−2.08 m	1.99−2.12 m	2.00−2.04 m
14		5.04 m	5.05 m	5.05 m
Me-16		1.54 br d (0.9)	1.56 br d (0.9)	1.56 br s
Me-17		1.50 br s	1.52 br d (0.9)	1.52 br s
OMe			3.77 s	3.78 s
OH	8.41 s	8.34 s	8.37 br s	8.31
	8.30 s	8.32 s	8.37 br s	8.13
	8.14 s		8.09 br s	8.02

[a] Digital resolution of the measurement is 0.19 Hz. [b] Cross-peaks on NOESY spectrum of **3**: between H-3 and H_3-10′, H_3-10′ and H_2-7′, H_2-11′ and H-8′, H_3-15′ and H_2-12′, H_2-16′ and H-13′, H_3-20′ and H_2-17′.

at 293 nm and a negative Cotton effect at 334 nm [(2*R*)-flavanone].[17] The other spectral data of the isomers were almost the same except the optical rotation values [(2*S*)-**2** $[\alpha]_D$ −102° (MeCN), (2*R*)-**2** $[\alpha]_D$ −115° (MeCN)]. (2*R*)-Flavanones have always been isolated along with their (2*S*)-isomers[24] except a (2*R*)-naringenin obtained from grapefruit, but the compound was a reaction product by enzymatic hydrolysis of naringin.[17] Therefore, (2*R*)-sanggenol M was considered to be an artifact due to the isolation procedure.[25]

Mulberrofurans W−Z (**3**−**6**) showed a dark blue fluorescence on TLC plates and in solution under UV illumination. The UV spectra of the compounds resembled those of 2-arylbenzofuran derivatives.[26] Laser desorption/ionization time-of-flight (LDI-TOF) MS of the 2-arylbenzofurans (**3**−**6**) indicated a molecular ion ($[M]^{\bullet+}$) rather than a protonated molecule ($[M + H]^+$). It is interesting that the oxidation process (elimination of an electron from the molecule) occurred in the excited state generated by irradiation with the N_2-laser (337 nm). The details of LDI-TOF-MS and matrix-assisted LDI-TOF (MALDI-TOF) MS of 2-arylbenzofuran derivatives and their photoreactions under LDI conditions will be described elsewhere.

The ^{1}H NMR spectrum of mulberrofuran W, **3** ($C_{29}H_{34}O_4$), showed signals of ABX-type aromatic protons (A-ring), an isolated furan proton (H-3), *meta*-coupled aromatic protons (AX-type, B-ring), protons of an (*E*,*E*)-3,7,11-trimethyl-2,6,-10-dodecatrienyl (farnesyl) group, and protons of three hydroxyl groups (Table 2). The presence of the farnesyl group was also confirmed by NOESY and ^{13}C NMR spectra (see Tables 2 and 3). In the ^{13}C NMR spectrum (Table 3), the chemical shifts of the A-ring carbons resembled those of mulberrofuran V (**13**).[9a] The farnesyl group was located at the 2′-position based on the following NOESY and ^{13}C NMR data: H-3 (δ 6.78) was correlated with H-8′ and H_2-7′ by the NOESY spectrum. The chemical shift of C-7′, δ 26.2, indicated that one of the *ortho*-positions of the group was substituted with an oxygen functional group and the other was a carbon functional group.[13] On the basis of these observations, the structure of mulberrofuran W was elucidated as 2′-farnesyl-3′,5′,6-trihydroxy-2-arylbenzofuran (**3**). This is the first naturally occurring 2-arylbenzofuran with a farnesyl group.

Mulberrofuran X (**4**), $[\alpha]_D$ −18° (MeOH), $C_{29}H_{34}O_5$, gave a negative Gibbs test on a TLC plate. The ^{1}H NMR spectrum showed signals of an olefinic proton (H-3), *ortho*-coupled aromatic protons (A-ring), *meta*-coupled aromatic protons (B-ring), protons of a geranyl group, a 2-(1-hydroxy-1-methylethyl)dihydrofuran ring, and two hydroxyl protons (Table 2). The H-3 signal was observed as a singlet, indicating the presence of a substituent at C-7 (absence of a zigzag coupling between H-3 and H-7 as observed in **3**). In the ^{13}C NMR spectrum of **4** (Table 3), oxygenated aromatic carbons appeared between δ 153−163, suggesting that these carbons were located at *meta*-positions relative to each other or at isolated positions, but not *ortho*- or *para*-positions.[27] The chemical shift of C-8, δ 23.4, indicated that the geranyl group was flanked by two *ortho*-oxygen functional groups.[12,13] The A-ring carbons resembled those of mulberrofuran L (**14**) (Table 3). The presence of the geranyl group at C-7 was confirmed by the HMBC spectrum of **4**: The C-6 signal was assigned by the cross-peaks with H-4 and H-5, and C-7a was deduced from the cross-peaks with H-3 and H-4. The cross-peaks between H_2-8 and carbon signals at C-6 and C-7a were observed on the spectrum. Similarity of the NMR data of the second isoprenoid unit (D-ring) and the 2-(1-hydroxy-1-methylethyl)-2,3-dihydrofuran moieties of glyinflanin F[28] and lonchocarpols C and

Table 3. ^{13}C NMR Spectral Data for **3**−**6**, **13**, and **14** in Acetone-d_6 (δ in ppm, 100 MHz)

carbon	3	4	5	6	13	14
2	155.6	154.5	155.5	155.3	155.4	159.5
3	105.5	105.0	103.6	105.3	102.6	105.0
3a	122.6	122.5	122.2	122.2	122.6	122.6
4	121.9	119.1	127.5	127.4	118.9	121.8
5	113.0	113.2	143.9	143.6	113.1	112.9
6	156.6	153.7	149.9	149.8	153.5	156.7
7	98.4	112.1	97.3	97.2	112.2	98.7
7a	156.9	155.1	152.4	152.1	153.5	157.0
1′	132.8	128.5	133.6	132.9		
2′	118.6	115.5	104.0	118.6		
3′	157.5[a]	162.9	160.0	157.1		
4′	103.9	97.6	101.9	103.9		
5′	156.5[a]	158.8	160.0	157.7		
6′	107.8	104.0	104.0	107.7		
MeO			61.8	61.8		
7′	26.2	31.9		26.6		
8′	125.1[b]	90.9		125.7		
9′	135.4[c]	71.6		131.3		
10′	16.4	26.0[a]		18.4		
11′	40.4[d]	25.5[a]		26.1		
12′	27.3[e]					
13′	125.2[b]					
14′	134.8[c]					
15′	16.1					
16′	40.5[d]					
17′	27.4[e]					
18′	125.4[b]					
19′	131.5					
20′	17.8					
21′	25.8					
8		23.4	27.1	27.1		
9		123.1	124.1	124.1		
10		135.8	136.1	136.2		
11		16.5	16.6	16.6		
12		40.4	40.6	40.5		
13		27.4	27.5	27.5		
14		125.0	125.2	125.2		
15		131.7	132.0	132.0		
16		17.6	17.9	17.9		
17		25.7	25.9	25.9		

[a−e] Assignments may be interchangeable.

D[29] confirmed the presence of such a moiety in **4**. The substituent pattern of the B-ring of **4** was deduced from its NOESY spectrum; cross-peaks were observed between H-3 and H_2-7′ (δ 3.43 and 3.40) of the D-ring. The absolute configuration of **4** was tentatively assigned as 8′*R* by comparison of the optical rotation of **4** with those of synthetic (*S*)-(+)- and (*R*)-(−)-2,3-dihydro-2-(2-hydroxyisopropyl)-6-methoxybenzofurans.[30] On the basis of these observations, the structure of mulberrofuran X was established as **4**.

The UV spectrum of mulberrofuran Y (**5**, $C_{25}H_{28}O_5$) resembled that of moracin B (3′,5-dihydroxy-5′,6-dimethoxy-2-arylbenzofuran) rather than that of moracin A (3′,5′-dihydroxy-4,6-dimethoxy-2-arylbenzofuran).[31] The ^1H NMR spectrum (Table 2) exhibited protons of a geranyl, a methoxy, and three hydroxyl groups [δ 8.37 (2H), 8.09 (1H)], *meta*-coupled aromatic protons (A_2M-type, B-ring), an aromatic doublet (J = 0.9 Hz, A-ring), and an olefinic doublet (J = 0.9 Hz, H-3). In the ^{13}C NMR spectrum of **5** (Table 3), oxygenated carbon atoms in the A-ring appeared at δ 152.4, 149.9, and 143.9, indicating *ortho*-oxygenation.[27,31] The chemical shift of the methoxyl group (δ 62.1, in $CDCl_3$) suggested that both *ortho*-positions of the group are substituted.[27] The chemical shift of C-8 (δ 26.3, in $CDCl_3$) indicated that one of the *ortho*-positions flanking the geranyl group was substituted with an oxygen functionality and the other was a carbon functional group.[12,13] On the basis of these observations, the structure of mulberrofuran Y was elucidated as 4-geranyl-5-methoxy-3′,5′,6-trihydroxy-2-arylbenzofuran (**5**).

HR-MS indicated that mulberrofuran Z (**6**, $C_{30}H_{36}O_5$) is a C_5H_8 unit larger than **5**. The ^1H NMR spectrum of **6** exhibited protons of prenyl and geranyl groups (Table 2). In the ^{13}C NMR spectrum (Table 3), the chemical shifts of the A-ring carbons of **6** resembled those of **5**. The B-ring carbons of **6** were similar to those of **3**. The positions of the prenyl and geranyl groups were deduced on the basis of HMBC and NOESY spectra, i.e., from the cross-peaks between H_2-7′ and C-1′/C-3′, H_2-8 and C-5/C-3a, NOE between H-3 and H_2-7′, OMe and H_2-8, etc. On the basis of these results, the structure of mulberrofuran Z was determined as 4-geranyl-5-methoxy-2′-prenyl-3′,5′,6-trihydroxy-2-arylbenzofuran (**6**).

Ten known flavonoids were isolated from the ethanol extract of the root bark of *M. mongolica*. The angular pyranoflavone atalantoflavone[32] was isolated here for the first time from the mulberry tree. Three Diels−Alder type adducts, kuwanons G,[2a] H,[2a] and sanggenon G (**9**),[20] three isoprenoid-substituted flavones, morusin, oxydihydromorusin, and kuwanon C (3,8-diprenyl-2′,4′,5,7-tetrahydroxyflavone),[2a] a linear pyranoflavanone, kuwanol C (**8**),[10] and three geranylated (prenylated) 2-arylbenzofurans, mulberrofurans B (**15**), D (**16**), and L (**14**), were also identified. Except for compound **9**, these flavonoids have previously been isolated from the Japanese mulberry tree.[2a] The known compounds were identified by direct comparison with the relevant authentic samples or by comparing the spectral data of the compounds with published data.[2,7]

The main flavonoids obtained here (kuwanons G and H) were the same as those from the Japanese mulberry tree, but minor flavonoids (**1**−**6**) were different from those of the Japanese plants. Furthermore, the main flavonoid of the Chinese drug "Sang-bai-pi", sanggenon C (**7**) or D (**12**), was not obtained from the Chinese *M. mongolica*. The main phenolic compounds of Chinese *M. alba* are the same as those of the Japanese mulberry trees (*M. alba*, *M. lhou*, and *M. bombycis*).[3c,33] On preliminary examination of Chinese *M. australis* by TLC, the flavonoids **7** and **12** were not detected. Thus, the source of "Sang-bai-pi" on the Japanese market was not *M. mongolica*, *M. alba*, or *M. australis* but presumably *M. cathayana*. However, further systematic studies of Chinese mulberry trees are necessary to confirm the source of Sang-bai-pi, because HPLC analysis demonstrated the presence of compound **7** or **12** in Chinese *M. australis*.[33]

Effects of Flavonoids on Human Oral Tumor Cell Lines. Flavonoids with isoprenoid substituents were first screened for cytotoxic activity against the human oral squamous cell carcinoma cell line HSC-2 (Tables 4 and 5). Sanggenol M [**2**, 50% cytotoxic concentration (CC_{50}) = 0.013 mmol/L (mM)], sanggenon C (**7**, CC_{50} = 0.018 mM), and sanggenon B (**19**, CC_{50} = 0.039 mM) showed higher cytotoxic activities against HSC-2 cells. These compounds are Diels−Alder type adducts with a chalcone and a 6-dehydrogeranyl(prenyl)flavanone and its derivative. Previously, we reported a weaker cytotoxic effect of kuwanon G against these cells.[4a] It is interesting that compounds **2**, **7**, and **19** are Diels−Alder type adducts with a 6-cyclohexene ring and kuwanon G is a Diels−Alder type adduct with an 8-cyclohexene ring. Compounds **2** and **7** also exhibited higher levels of cytotoxicity against the human salivary gland tumor cell line HSG (Table 4). Normal human gingival fibroblasts (HGF) were highly resistant to compounds **2**, **7**, and **19** (Table 4, tumor specificity: B/A ratio = 2.5, 2.3, and 2.5, respectively), suggesting that these

Table 4. Cytotoxicities of Flavanones against Human Oral Squamous Cell Carcinoma (HSC-2), Human Salivary Gland Tumor (HSG), and Normal Human Gingival Fibroblast (HGF) Cells[a]

compound	cytotoxic activity (CC_{50}) in μM (in μg/mL)			tumor specificity (B/A)
	HSC-2 (A)	HSG	HGF (B)	
sanggenol M (**2**)[b]	13 (10)	13 (10)	32 (24)	2.5
sanggenon C (**7**)	18 (13)	23 (16)	42 (30)	2.3
sanggenon D (**12**)	44 (31)	64 (45)	140 (100)	3.2
sanggenon A (**17**)	53 (23)	46 (20)	110 (49)	2.1
sanggenon B (**19**)	39 (22)	47 (27)	98 (56)	2.5
sanggenon B isomer[c]	77 (44)	120 (69)	300 (172)	3.9
sanggenon M (**18**)	48 (21)	53 (23)	110 (49)	2.3
sanggenon O	73 (52)	100 (71)	140 (99)	1.9
sorocein D (**20**)	190 (97)	120 (61)	270 (135)	1.4
sorocein F (**21**)	47 (24)	49 (25)	110 (57)	2.3
2′-hydroxychalcone[d]	58 (13)	49 (11)	110 (25)	1.9

[a] The A_{540} values of control HSC-2, HSG, and HGF cells (viable cells after incubation for 24 h without test compound) were 1.430, 0.520, and 0.278, respectively. [b] Racemic mixture. [c] Structural analysis of the compound is now in progress. [d] Positive control.

Table 5. Cytotoxicities of 2-Arylbenzofurans against HSC-2, HSG, and HGF Cells[a]

compound	cytotoxic activity (CC_{50}) in μM (in μg/mL)			tumor specificity (B/A)
	HSC-2 (A)	HSG	HGF (B)	
mulberrofuran W (**3**)	70 (31)	70 (31)	90 (40)	1.3
mulberrofuran X (**4**)	290 (135)	260 (120)	350 (162)	1.2
mulberrofuran Y (**5**)	110 (46)	140 (55)	190 (78)	1.7
mulberrofuran Z (**6**)	190 (89)	N.D.	N.D.	
mulberrofuran B (**15**)	59 (23)	59 (23)	71 (28)	1.2
mulberrofuran D (**16**)	83 (37)	74 (33)	81 (36)	1.0
mulberrofuran L (**14**)	190 (70)	160 (61)	190 (71)	1.0

[a] The A_{540} values of control HSC-2, HSG, and HGF cells were 1.185, 0.952, and 0.485, respectively. N.D., not determined.

compounds display specific cytotoxic activities against cancer cell lines rather than normal cells. Seven other flavanones studied here also showed similar tumor specificity (B/A ratio = 1.4−3.9, Table 4). Previously, we reported that introduction of a hydrophobic group (prenyl or geranyl group) to flavones, isoflavones, and a 2-arylbenzofuran changed their cytotoxic activities against HSC-2 cells.[4] For example, the cytotoxicity of a monoprenylated 2-arylbenzofuran, moracin C (4′-prenyl-3′,5′,6-trihydroxy-2-arylbenzofuran), was higher than that of moracin M (3′,5′,6-trihydroxy-2-arylbenzofuran).[4b] The cytotoxic activities of more hydrophobic 2-arylbenzofurans (**3**−**6**, **14**−**16**, Table 3) were weaker than that of moracin C and similar to that of moracin M. These seven 2-arylbenzofurans showed only marginal tumor specificity (B/A ratio = 1.0−1.9, Table 5). We have recently found that prenylated flavonoids,[4b] geranylgeraniol, and a vitamin K_2 derivative (MK2), which have an isoprenoid group(s) (ref 34, unpublished data), induced internucleosomal DNA fragmentation in the human promyelocytic leukemic cell line HL-60, but not in the human oral squamous cell carcinoma cell line HSC-2. These results suggested that internucleosomal DNA fragmentation, which has been used as a biochemical hallmark of apoptosis, induced by isoprenoid-substituted compounds is dependent on the type of target cells. The signal transduction pathway might be different between different cell types.

Experimental Section

General Experimental Procedures. Optical rotations and CD spectra were measured with a JASCO DIP-370 digital polarimeter and a JASCO J-720W instrument (Jasco Co., Hachioji, Japan), respectively. The ^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM EX-400, JNM-alpha-500, or JNM-EXP 500 NMR spectrometers (JEOL Ltd., Akishima, Japan). Chemical shifts are reported with respect to acetone-d_6 (δ_H 2.04, δ_C 206.0), $CDCl_3$ (δ_H 7.24, δ_C 77.0), and DMSO-d_6 (δ_H 2.49, δ_C 39.5). EIMS spectra were recorded on a JEOL JMS-AM II 50 spectrometer. MALDI-TOF-MS and LDI-TOF-MS (without matrix) data were obtained on a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA) TOF mass spectrometer equipped with a nitrogen laser (337 nm), TDS 540C 500 MHz digitizing oscilloscope (500 MHz bandwidth, 2G samples/s, Tektronix Inc., Beaverton, OR), PerSeptive Biospectrometry Workstation (Version 3.09), and PerSeptive GRAMS/386 for Microsoft Windows (Version 3.04 Level III, Driver Version 1.00, Galactic Industries Corp., Salem, NH). Sinapinic acid and 4-hydroxy-3-methoxycinnamic acid were used as the matrix. Calibration was performed with two peaks of internal standard(s), mulberrofuran D (**16**, $[M]^+$), albanol B[2a] ($[M + H]^+$), sinapinic acid ($[M + Na]^+$, $[2M + Na]^+$), and angiotensin IV ($[M + H]^+$, $[M + K]^+$). The details of the measurement of HR-MS were previously reported.[35] The absolute mass error of angiotensin II, $[M + H]^+$, was −2.1 mDa, and the 95.5% confidence level ($\pm 2\sigma$) of the ion was ±6.6 mDa. HPLC was carried out on an SSC instrument (Senshu Scientific Co. Ltd., Tokyo, Japan) equipped with a UV detector using the Senshu Pak Silica-4251-N and Pegasil Silica 60-5 columns (1.0 × 25.0 cm) (Senshu Scientific Co. Ltd.). Organic solvents were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan), Godo Solvent Ltd. (Tokyo, Japan), and Isotec Inc. (Miamisburg, OH), and the following chemicals and reagents were obtained from the sources indicated: Chromatorex ODS (100−200 mesh) (Fuji Silysia Chemical, Ltd., Kasugai, Japan); Wakogel C200 and B-5F (Si gel) (Wako Pure Chem. Ind. Ltd.); angiotensin IV (Sigma Chem. Ind., St. Louis, MO); sinapinic acid and 4-hydroxy-3-methoxycinnamic acid (Tokyo Kasei Kogyo, Tokyo, Japan).

Plant Material. The root bark of *M. mongolica* Schneid., *M. cathayana* Hemsl., and *M. australis* Poir. (Moraceae) was collected in Mopan Mt., Tiantai Mt., and Yinghua Mt., Sichuan Province, the People's Republic of China, respectively, in 1995. The materials were identified by one of the authers (W.J.C.). The voucher specimens (*M. mongolica*, TOHO 95001; *M. cathayana*, TOHO 95002; *M. australis*, TOHO 95003) are deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Toho University, Japan.

Extraction and Isolation. The semidried root bark of *M. mongolica* (1.1 kg) was extracted with 95% EtOH, and the extract was then evaporated under reduced pressure, yielding a residue (65 g). This was extracted with Et_2O to give 28 g of residue, which was chromatographed on a Si gel CC with benzene (3 L), $CHCl_3$ (3 L), acetone (2 L), and MeOH (2 L) as the eluents to afford 9, 2.8, 12, and 2 g of residues, respectively. Part of the $CHCl_3$ eluate residue (300 mg) was separated by preparative (p) TLC (*n*-hexane−acetone, 2:1, benzene−acetone−28% ammonia water, 100:20:0.2) to afford mulberrofuran D[2a] (**16**, 150 mg) and atalantoflavone[32] (1.5 mg). The acetone eluate residue (12 g) was subjected to Si gel CC (benzene−MeOH, gradient) to give fractions A−H. Fraction A (2 g) was rechromatographed on Si gel CC (benzene−acetone, gradient) to afford fractions I−VIII. Fraction II (120 mg) was further purified by pTLC (*n*-hexane−acetone, 2:1) and pHPLC (Silica-4251-N**,** *n*-hexanes−Et_2O, 2:1, 2 mL/min, detection at 320 nm) to give mulberrofuran B[2a] (**15**, 1 mg, t_R 46 min), **16** (4 mg, t_R 52 min), and morusin[2a] (6 mg, t_R 76 min), respectively. Fraction IV (300 mg) was separated by pTLC (*n*-hexane−acetone, 2:1, benzene−Et_2O, 1:1) and ODS CC (eluted with 30% MeOH) to give mulberrofuran L[2a] (**14**, 5 mg), mulberrofuran X (**4**, 3 mg), and a mixture (6 mg) that was purified by pHPLC (Pegasil Silica 60-5, *n*-hexanes−Et_2O, 1.4:1, 2 mL/min, detection at 280 nm) to afford kuwanol C[10] (**8**, 2 mg, t_R 74 min) and sanggenol L (**1**, 2 mg, t_R 80 min). Fraction V (310 mg) was fractionated by pTLC (*n*-hexane−acetone, 1:1, $CHCl_3$−acetone, 5:1) and ODS CC (30% MeOH) to give fractions 1 and 2. Mulberrofurans W (**3**, 4.5 mg, t_R 30 min) and Z (**6**, 2.5 mg, t_R 25 min) were isolated from fraction 1 by pHPLC (Pegasil Silica 60-5, 2 mL/min, detection at 320 nm) using *n*-hexanes−EtOAc (7:2), and kuwanon C[2a] (6 mg, t_R 19 min) and mulberrofuran Y (**5**, 40 mg, t_R 23 min) were obtained from fraction 2 using *n*-hexanes−EtOAc (2:1). Sanggenol M (**2**, 15 mg) and kuwanon H[2a] (40 mg) were obtained from fraction B (2 g) by Si gel CC (benzene−EtOAc, 3:2) and ODS CC (25% MeOH). Sanggenon G[20] (**9**, 20 mg) and kuwanon G[2a] (15 mg) were obtained from fraction C in the same way. The separation of racemic mixtures of **1** (t_R 80 and 83 min) and **8** (t_R 74 and 77 min) was carried out by pHPLC as described above. The isolation of (2*S*)- and (3*R*)-sanggenol M (**2**) was performed by pHPLC (Pegasil Silica 60-5, *n*-hexanes−Et_2O, 1:11, 2 mL/min, detection at 280 nm, t_R 59 and 63 min, respectively).

The semidried root bark of *M. cathayana* (4.1 kg) was also extracted with 95% EtOH to afford 160 g of extract. Flavonoids were isolated by a procedure similar to that described above. Six known flavonoids, sanggenons A (**17**, 5 mg), B (**19**, 18 mg), C (**7**, 21 mg), D (**12**, 17 mg), M (**18**, 10 mg), O[14] (15 mg), and sanggenon B isomer (5 mg) were isolated from part of the semipurified fractions of the extract (further isolation of these compounds has not yet been carried out).

Assay for Cytotoxicities. All flavonoid samples were dissolved in DMSO at 20 mg/mL. The final concentration of DMSO in the medium was below 1%. Cell culture and the other procedures were the same as those reported previously.[4,36]

Sanggenol L (**1**, a diastereomeric mixture): pale yellow amorphous solid; $[\alpha]^{22}_D$ −18° (*c* 0.1, MeOH); UV (EtOH) λ_{max} (log ϵ) 203 (4.37), 228 (3.94), 272 (4.31), 359 (3.16) nm, (EtOH+$AlCl_3$) λ_{max} (log ϵ) 203 (4.41), 228 (sh) (3.99), 282 (4.30), 324 (4.00), 414 (3.24) nm; ^{1}H NMR (400 MHz) and ^{13}C NMR, see Table 1; CD (*c* 6.1 × 10^{-5} mol/L, EtOH) $[\theta]^{21}$ (nm) 0 (240), + 530 (253), + 300 (sh) (270), 0 (290); EIMS *m*/*z* 422 $[M]^+$ (3), 404 (7), 339 (47), 321 (46), 307 (6), 257 (7), 256 (7), 229 (8), 203 (100), 83 (41), 69 (50); HRMALDITOFMS *m*/*z* $[M + H]^+$ 423.1830 (calcd for $C_{25}H_{27}O_6$, 423.1808).

Sanggenol M (**2**, a diastereomeric mixture): pale yellow amorphous solid; $[\alpha]^{22}_D$ −126° (*c* 0.1, MeCN); UV (EtOH) λ_{max} (log ϵ) 204 (4.83), 220 (sh) (4.61), 288 (4.38), 330 (sh) (3.92) nm; UV (EtOH+$AlCl_3$) λ_{max} (log ϵ) 204 (4.83), 220 (4.61), 290 (4.38), 330 (sh) (3.92) nm; ^{1}H NMR (500 MHz) and ^{13}C NMR, see Table 1; CD (*c* 2.6 × 10^{-5} mol/L, MeCN) $[\theta]^{21}$ (nm) the diastereomeric mixture **2**, −76 600 (210), −34 800 (240), 0 (275), −22 400 (296), −15 100 (330); (2*S*)-**2** (*c* 2.6 × 10^{-5} mol/L, MeCN) $[\theta]^{21}$ (nm) −71 500 (207), −28 000 (241), 0 (263), +2900 (274), 0 (282), −40 100 (296); (2*R*-**2**) (*c* 2.6 × 10^{-5} mol/L, MeCN) $[\theta]^{21}$ −82 900 (221), 0 (270), +2400 (278), 0 (286), −6100 (303), −15 900 (331); differential CD, [(2*S*)-**2** − the mixture of diastereoisomers **2**], 0 (283), −25 300 (296), 0 (316), +10 300 (331); [(2*R*)-**2** − the mixture of diastereoisomers **2**), 0 (266), +19 000 (293), 0 (315), −6400 (334); HRMALDITOFMS *m*/*z* $[M + Na]^+$ 785.2923 (calcd for $C_{45}H_{46}NaO_{11}$, 785.2938).

Mulberrofuran W (**3**): pale yellow amorphous solid; UV (MeOH) λ_{max} (log ϵ) 207 (4.59), 225 (infl.), 275 (sh) (4.06), 309 (4.31), 330 (infl.) nm; ^{1}H NMR (400 MHz), see Table 2; ^{13}C NMR, see Table 3; HRLDITOFMS *m*/*z* $[M]^+$ 446.2474 (calcd for $C_{29}H_{34}O_4$, 446.2457).

Mulberrofuran X (**4**): pale yellow amorphous solid; $[\alpha]^{22}_D$ −18° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.38), 220 (sh) (4.16), 225 (sh) (3.73), 285 (sh) (3.80), 295 (sh) (3.99), 317 (4.27), 330 (sh) (4.15) nm; ^{1}H NMR (400 MHz), see Table 2; ^{13}C NMR, see Table 3; HRLDITOFMS *m*/*z* $[M]^+$ 462.2389 (calcd for $C_{29}H_{34}O_5$, 462.2406).

Mulberrofuran Y (**5**): pale yellow amorphous solid; UV (MeOH) λ_{max} (log ϵ) 215 (4.52), 255 (sh) (3.79), 285 (sh) (4.04), 295 (sh) (4.20), 320 (4.47), 332 (4.40) nm; ^{1}H NMR (400 MHz), see Table 2; ^{13}C NMR, see Table 3; HRLDITOFMS *m*/*z* $[M]^+$ 408.1964 (calcd for $C_{25}H_{28}O_5$, 408.1937).

Mulberrofuran Z (**6**): pale yellow amorphous solid; UV (MeOH) λ_{max} (log ϵ) 205 (4.49), 215 (sh) (4.38), 273 (4.05), 295 (infl.), 315 (4.14), 330 (sh) (3.98) nm; ^{1}H NMR (400 MHz), see Table 2; ^{13}C NMR, see Table 3; HRLDITOFMS *m*/*z* $[M]^+$ 476.2553 (calcd for $C_{30}H_{36}O_5$, 476.2563).

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(15) When these compounds (**1** and **8**) were measured in acetone-d_6, the 5-OH of **1** appeared at δ 12.281 (0.5H) and 12.277 (0.5H), and those of **8** were observed at δ 12.554 (0.5H) and 12.545 (0.5H).
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