Schizarin B, C, D, and E, Four New Lignans from *Kadsura matsudai* and Their Antihepatitis Activities

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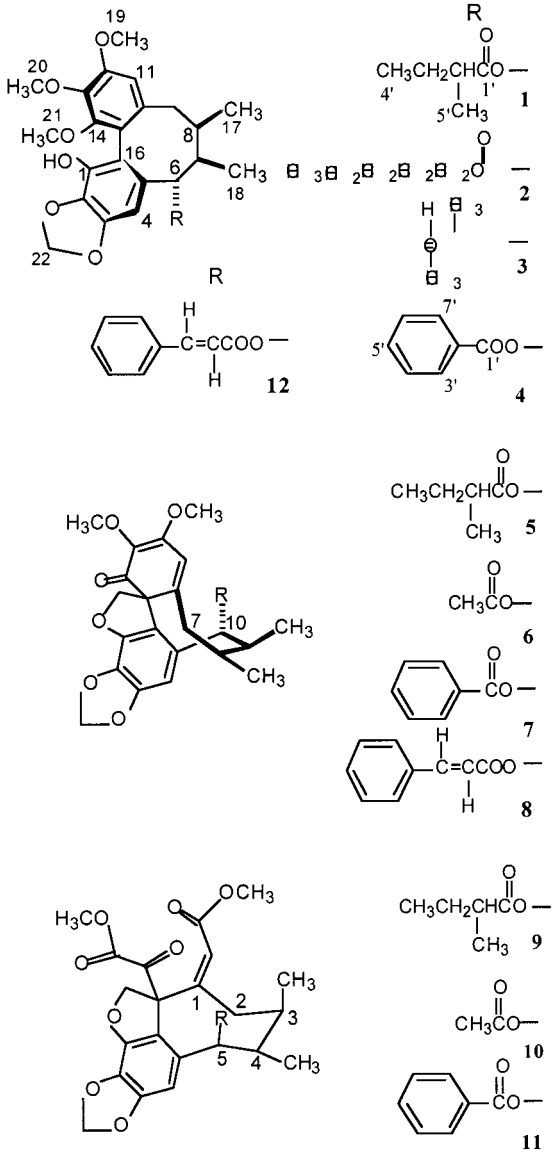
Received May 26, 2000

Bioassay-directed fractionation of ethanolic extract of *Kadsura matsudai* Hayata has resulted in the isolation of four novel C_{18} dibenzocyclooctadiene lignans, schizarin B (**1**), schizarin C (**2**), schizarin D (**3**), and schizarin E (**4**). Schizarin B (**1**) showed moderate to strong activity for antihepatitis in both anti-HBsAg and anti-HBeAg assays, and **3** and **4** also were active in the latter assay. Compounds **1**−**4** were inactive in vitro against HIV replication in H9 lymphocytes. All new structures were elucidated using spectral analysis. Their structural elucidation by spectral and structure−activity relationships is also discussed.

The medicinal fruits *Schizandra chinensis* (Schizandraceae) have been used as a tonic and astringent drug in traditional Chinese medicine. Plants from Schizandraceae have yielded several C_{18} type dibenzocyclooctadiene lignans with pharmacological activity, including antioxidant, antihepatitis, antihepatotoxic, and antilipid peroxidative effects.[1−5] Recently, we reported the isolation of two types of novel C_{19} homolignans, four compounds with 5,4-butano-2,4-cyclohexadienone-6-spiro-3-(2,3-dihydrobenzo[*b*]furan) skeletons [schiarisanrin A (**5**), B (**6**), C (**7**), and D (**8**)], and three compounds with 3,4-{1-[(*Z*)-2-methoxy-2-oxoethylidene]}pentano(2,3-dihydrobenzo[*b*]furan)-3-(2-oxoacetate) skeletons [taiwanschirin A (**9**), taiwanschirin B (**10**), and taiwanschirin C (**11**)], as well as a C_{18} dibenzocyclooctadiene lignan, schizanrin A (**12**) from *Schizandra arisanensis*.[6,7] The C_{19} homolignans **5**−**8** have a substituted cyclohexadienone ring with an oxygenated methylene group, rather than the substituted benzene ring found previously in C_{18} dibenzocyclooctadiene lignans from *Schizandra* spp.[1−5] In the second type of C_{19} homolignans, **9**−**11**, the cyclohexadienone ring has been opened to give keto-ester and α,β-unsaturated ester groups.

This finding motivated our investigation of another Taiwanese Schizandraceae plant, *Kadsura matsudai* Hayata, as its EtOH extract exhibited anti-HBsAg (human type B hepatitis, surface antigen) and anti-HBeAg (human type B hepatitis, e antigen) activity. Bioassay-directed fractionation of this active extract has now led to the isolation and characterization of four novel C_{18} dibenzocyclooctadiene lignans, schizarin B (**1**), C (**2**), D (**3**), and E (**4**), along with the known C_{19} homolignans, schiarisanrin A (**5**), B (**6**), C (**7**), and D (**8**) and taiwanschrins A (**9**) and B (**10**). We report herein on the structural elucidation of new compounds **1**−**4** by 2D NMR techniques including $^1H-^1H$ COSY, $^1H-^{13}C$ heteronuclear COSY, long-range $^1H-^{13}C$ COSY, and NOESY spectra (Tables 1 and 2). Compounds **1**−**4** were tested for the anti-HIV activity. Moreover, we also describe our biological evaluation of these isolated compounds (**1**−**12**) for anti-HBsAg and anti-HBeAg (human type B hepatitis) activity.



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10.1021/np000261m CCC: $20.00
Published on Web 04/10/2001

Table 1. ^{1}H NMR (500 MHz, $CDCl_3$) Data[a] of Schizarins B−E (**1**−**4**)

proton	1	2	3	4
4	6.48 (s)	6.46 (s)	6.53 (s)	6.59 (s)
6	5.58 (br s)	5.53 (br s)	5.56 (br s)	5.84 (br s)
7	2.01 (m)	2.00 (m)	2.08 (m)	2.17 (m)
8	2.03 (m)	2.01 (m)	2.08 (m)	2.17 (m)
9	2.61 (m)	2.60 (m)	2.61 (m)	2.71 (m)
11	6.37 (s)	6.36 (s)	6.36 (s)	6.47 (s)
17	0.90 (d, 6.9)	0.93 (d, 7.0)	0.94 (d, 6.7)	1.06 (d, 6.5)
18	1.06 (d, 6.6)	1.07 (d, 7.2)	1.07 (d, 6.7)	1.13 (d, 6.5)
19	3.88 (s)	3.87 (s)	3.85 (s)	3.93 (s)
20	3.81(s)	3.82(s)	3.83(s)	3.39(s)
21	3.88 (s)	3.88 (s)	3.79 (s)	3.81 (s)
22	5.96, 5.92 (d, 1.2)	5.95, 5.91 (d, 1.2)	5.96, 5.93 (d, 1.2)	5.99, 5.94 (d, 1.2)
2′	1.68 (m)	2.20 (m)		
3′	1.22, 1.34 (m)	1.76 (m)	5.86 (d, 4.3)	7.40 (m)
4′	0.71 (t, 7.2)	1.29 (m)	1.86 (d, 1.3)	7.25 (m)
5′	0.85 (d, 6.9)	1.29 (m)	1.28 (s)	7.40 (m)
6′		0.82 (t, 6.9)		7.25 (m)
7′				7.40 (m)

Table 2. ^{13}C NMR (125 MHz, $CDCl_3$) Data[a] of Schizarins B−E (**1**−**4**)

carbon	**1**	**2**	**3**	**4**	C−H connectivities[b]
1	146.5 (s)	146.6 (s)	146.9 (s)	146.9 (s)	c
2	148.9 (s)	148.9 (s)	148.9 (s)	148.9 (s)	H-22,4
3	136.1 (s)	136.0 (s)	136.1 (s)	136.1 (s)	H-22,4
4	102.9 (d)	102.8 (d)	102.8 (d)	102.8 (d)	H-6
5	135.7 (s)	135.8 (s)	136.1 (s)	135.5 (s)	H-6,4
6	82.3 (d)	82.4 (d)	82.7 (d)	83.4 (d)	H-4
7	41.7 (d)	41.6 (d)	41.7 (d)	41.8 (d)	H-6,17,18
8	34.8 (d)	34.9 (d)	34.7 (d)	34.7 (d)	H-6,9,17,18
9	38.6 (t)	38.6 (t)	38.6 (t)	38.7 (t)	H-11,17
10	133.5 (s)	133.6 (s)	133.5 (s)	133.6 (s)	H-9−11
11	107.2 (d)	107.1 (d)	107.1 (d)	107.0 (d)	H-9
12	150.2 (s)	150.3 (s)	150.4 (s)	150.5 (s)	H-11,19
13	133.4 (s)	133.3 (s)	133.5 (s)	133.6 (s)	H-11,20
14	141.2 (s)	141.2 (s)	141.1 (s)	141.3 (s)	H-21
15	116.9 (s)	116.9 (s)	116.9 (s)	117.0 (s)	H-11,9
16	119.3 (s)	119.1 (s)	119.1 (s)	119.4 (s)	H-6,4
17	14.9 (q)	19.7 (q)	14.8 (q)	15.3 (q)	H-9
18	19.7 (q)	13.8 (q)	19.8 (q)	19.6 (q)	H-6
19	55.8 (q)	55.8 (q)	55.8 (q)	55.9 (q)	*c*
20	60.8 (q)	60.8 (q)	60.4 (q)	60.3 (q)	*c*
21	59.7 (q)	59.7 (q)	59.7 (q)	59.7 (q)	*c*
22	101.2 (t)	101.2 (t)	101.2 (t)	101.2 (t)	*c*
1′	175.9 (s)	172.9 (s)	166.8 (s)	165.9 (s)	H-6
2′	40.4 (d)	33.7 (t)	127.1 (s)	129.7 (s)	*c*
3′	26.7 (t)	24.1 (t)	20.5 (q)	127.9 (d)	*c*
4′	11.4 (q)	22.2 (t)	139.5 (d)	129.5 (d)	*c*
5′	15.6 (q)	31.2 (t)	15.7 (q)	132.5 (d)	*c*
6′		14.8 (q)		129.5 (d)	*c*
7′				127.9 (d)	*c*

[a] Multiplicity was determined from DEPT spectra. [b] ^{1}H−^{13}C long-range correlation (HMBC) corresponded to two- or three-bond bonds. [c] The assignments were explained in the text.

Results and Discussion

An EtOH extract of the dried stems of *Kadsura matsudai* was extracted successively with *n*-hexane, EtOAc, and BuOH. Repeated column chromatography and/or HPLC of EtOAc extract yielded schizarin B (**1**), schizarin C (**2**), schizarin D (**3**), and schizarin E (**4**), together with **5**−**10**.

Schizarin B (**1**) had a molecular weight of 486, corresponding to the molecular formula $C_{27}H_{34}O_8$. The ^{13}C and ^{1}H NMR spectra suggested that **1** might be a dibenzocyclooctadiene lignan with a hydroxyl and an ester substituent. Its functional groups were also deduced from IR spectral bands at 3400 (OH), 1715 (ester), 1610 and 1590 (aromatic) cm^{-1}. The ^{13}C NMR spectrum of **1** clearly indicated the presence of 12 aromatic carbon atoms (δ_C 146.5, 148.9, 136.1, 102.9, 119.3, 135.7 for C-1, -2, -3, -4, -5, and -16, respectively; δ_C 133.5, 107.2, 133.4, 150.2, 141.2, 116.9 for C-10, -11, -12, -13, -14, and -15, respectively), indicative of two aromatic moieties. A butano chain was predicted due to the prominent cross-peaks of H-6 (δ_H 5.58, brs), H-7 (δ_H 2.01, m), H-8 (δ_H 2.03, m), and H-9 (δ_H 2.61, m) in the ^{1}H−^{1}H homonuclear correlation spectroscopy (^{1}H−^{1}H COSY) spectrum. Moreover, in the ^{1}H−^{13}C long-range correlation (HMBC) spectrum, correlations were found between H_b-9 and C-15 and C-11 and between H-6 and C-16 and C-4 of respective aromatic rings. These couplings implied that **1** possesses a substituted butane linkage between C-5 and C-10 and that the two substituted aromatic moieties are linked directly at C-15 and -16. From the above data, the skeleton of **1** was deduced as a substituted dibenzocyclooctadiene lignan, as isolated previously from Schizandraceae plants.[8,9]

The functional groups appearing in the ^{13}C and ^{1}H NMR spectra include three methoxy groups, a methylenedioxy group, and two secondary methyl groups. These groups were assigned on the basis of HMBC and ^{1}H−^{13}C COSY studies. Thus, the cross-peaks of a methylenedioxy proton (H-22) to C-2 and -3, and of two methyl groups (H-17, H-18) to C-9 and -6, respectively, as well as the correlations between three methoxy groups (OCH_3-19, -20, -21) and C-12, -13, and -14, respectively, confirmed these substituted groups' positions undoubtedly. After assigning the proton chemical shifts of three methoxy groups, their carbon chemical shifts were assigned at δ_C 55.8, 60.8, and 59.7 (for C-12, -13, -14, respectively) on the basis of correlations with OCH_3-19, -20, and -21, respectively, in the ^{1}H−^{13}C COSY spectrum. This assignments of methoxy groups are consistent with the rule for the methoxy-carbon shifts, suggesting the *ortho*−*ortho* disubstituted aromatic OCH_3 in the range δ_C 59−62.[10] Moreover, signals characteristic of a 2-methylbutyroxyl group were observed in the ^{1}H and ^{13}C NMR spectra. The mass spectrum of **1** exhibited a molecular ion at *m*/*z* 486 and an intense peak at *m*/*z* 384 [$M^+ - C_4H_9COOH$], reflecting the 1,2-elimination of a pentanoic acid via McLafferty ester rearrangement. From the above evidence, compound **1** and schizarin A (**12**) have similar structures, except for the replacement of a cinnamoxyl group (**12**) with a 2-methylbutanoate group. In a detailed examination of the HMBC spectrum of **1**, cross signals were found between C-1′ and H-6, H-2′, H-3′, and H-5′, between C-2′ and H-3′, H-4′, and H-5′, and between C-3′ and H-2′, H-4′, and H-5′. These results suggested that a 2-methylbutanoate group is located at C-6.

In the MS, schizarin C (**2**) gave *m/z* 500 [M^+], corresponding to $C_{28}H_{36}O_8$. Its ^1H and ^{13}C spectra were similar to those of **1** and indicated the characteristic signals for a substituted dibenzocyclooctadiene lignan. However, proton signals and carbon signals (δ_C 33.7, 24.1, 22.2, 31.2, 14.8, and 172.9) for a caproxyl group (*n*-hexanoic acid, $C_6H_{12}O_2$) rather than a 2-methylbutyroxyl group were observed in the NMR spectra. Moreover, correlation between the carbonyl carbon (C-1′) and H-6 in HMBC spectrum and a corresponding fragment ion at *m/z* 384 [$M^+ - C_6H_{12}O_2$] in the mass spectrum are in good agreement with the hexanoic acid ester at C-6. In addition, the complete chemical shifts of ^1H and ^{13}C NMR of **2** were performed by 2D $^1H-^1H$ COSY and $^1H-^{13}C$ COSY spectra and therefore confirmed the structure of **2** as the C-6 hexanoic acid analogue of **1**.

The IR and NMR spectra of schizarin D (**3**) [$C_{27}H_{32}O_8$] and schizarin E (**4**) [$C_{29}H_{30}O_8$] revealed that, like **1** and **2**, these compounds also have a C_{18} dibenzocyclooctadiene lignan skeleton with one hydroxy, one methylenedioxy, and three methoxy groups. The NMR spectra of **3** and **4** revealed a 2-methyl-2-butenoic acid ester and a benzoic acid ester, respectively, instead of a 2-methylbutyroxyl group found in **1**. Moreover, the mass spectrum of **3** exhibited a molecular ion at *m/z* 484 and an intense peak at *m/z* 384 [M^+ − pentenoic acid], which are further evidence for the presence of a 2-methyl-2-butenoic acid ester in **3**. A molecular ion at *m/z* 506 and a characteristic fragmentation ion at *m/z* 384 [M^+ − benzoic acid] also corresponded to a benzoic acid ester in **4**. The stereochemistry of the double bond in the 2-methyl-2-butenoic acid ester was deduced as an *E* form, based on the correlation between H-3′ and H-5′ in the NOESY spectrum.

This finding was further supported by the long-range correlation (HMBC) NMR spectrum of **3**. Correlations between the carbonyl carbon signal at δ_C 166.8 (C-1′) and the H-6 proton (δ_H 5.56) and olefinic H-3′proton (δ_H5.86) assigned the *E*-2-methyl-2-butenoic acid ester at C-6 and unambiguously determined the structure of schizarin D (**3**). Also, the HMBC spectrum of **4** clearly exhibited a correlation between the carbonyl carbon signal at δ_C 165.9 (benzoxyl C=O) and the proton signal at δ_H 5.84 (H-6), indicating the connectivity between the benzoxyl group and C-6.

On the basis of NOESY spectra of **1**−**4**, three methoxy groups correlated with each other, and the methoxy group at C-12 correlated with H-11, providing further evidence that the three methoxy groups are adjacent in the same aromatic ring. In addition, the correlation between CH_3-17 and H-19, H-11 and between H-6 and H-4, CH_3-18 located these three functional groups at C-6, -7, and -8.

To determine the stereochemistry of these novel compounds, their circular dichroism (CD) absorption values were examined. The CD spectra of **1**−**4** showed a positive Cotton effect around 215−225 and a negative Cotton effect around 225−245 nm, suggesting that these dibenzocyclooctadiene lignans (**1**−**4**) possessed an *S*-biphenyl configuration as gomisin B.[8,9]

Compounds **1**−**4** were tested for in vitro inhibitory effects against HIV replication in H9 lymphocytes. None of these new lignans suppressed HIV replication. However, schizarin E (**4**) demonstrated strong toxicity against the H9 T cells (IC_{50} = 2.08 μg/mL).

The isolated lignans or homolignans (**1**−**14**) were also evaluated in anti-HBsAg and anti-HBeAg assays (human type B hepatitis) (Table 3). In the anti-HBeAg test, **1**, **3**, **4**, **8**, and **9** were active at concentrations of 10 and/or 20 μg/mL. Compounds **5**−**7** and **10**−**12** were inactive (decrease percentage < 25%). Notably, only **1** (schizarin B) was active in the anti-HBsAg assay and exhibited the highest inhibition in both assays (54.9 and 42.1% for anti-HBsAg and anti-HBeAg, respectively). Thus, the C-6 substituent in C_{18} lignans as well as the corresponding C-5 substituent (same relative position but different carbon numbering) in C_{19} homolignans could be significant for bioactivity. Detailed structure−activity relationships of the C_{18} dibenzocyclooctadiene lignans and C_{19} dihydrobenzo[*b*]furan-substituted cyclooctene homolignans are under investigation.

Table 3. Anti-HBsAg and Anti-HBeAg Effects of Schizarins B−E (**1**−**4**), **8**, and **9**

entry	conc, μg/mL (μM)	HBsAg[a] (decrease %)	HBeAg[a] (decrease %)	AST (IU/L)
schizarins B (**1**)	20 (41.1)	54.9	42.1	15.5
	10 (20.5)	42.6	40.7	17.6
schizarins C (**2**)	20 (40.0)	toxic[b]	toxic[b]	35.9
	10 (20.2)	I[c]	I	16.0
schizarins D (**3**)	20 (41.3)	toxic[b]	toxic[b]	47.8
	10 (20.6)	I	25.8	16.8
schizarins E (**4**)	20 (39.5)	toxic[b]	toxic[b]	44.3
	10 (19.7)	I	31.8	20.2
schiarisanrin D (**8**)	20 (37.7)	I	42.9	22.0
	10 (18.8)	I	27.0	20.1
taiwanschirin A (**9**)	20 (38.7)	I	43.0	14.8
	10 (19.3)	I	I	14.1
DMSO	μL/ml	0	0	<25

[a] Active inhibition: decrease 25−35% (moderate inhibition), 35−45% (medium inhibition), >45% (strong inhibition). [b] Toxic and AST were explained in the text (see anti-HBsAg and anti-HBeAg test). [c] I = inactive inhibition: <25% decrease.

Experimental Section

General Experimental Procedures. NMR spectra were measured at 500 MHz for ^1H and 125 MHz for ^{13}C. Heteronuclear long-range correlation (HMBC) spectra were performed by using coupling constants of 8 Hz. Samples for IR spectral measurements were prepared as KBr disks. EIMS were performed in the electron impact mode (20 eV). HPLC was employed by using a semipreparative $5C_{18}$ column.

Plant Material. The stems of *Kadusra matsudai* Hayata were collected in July 1997 in Taichung County, Taiwan. A voucher specimen was deposited at National Research Institute of Chinese Medicine, Taipei, Taiwan, Republic of China.

Extraction and Isolation. The dried stems of *K. matsudai* Hayata were extracted exhaustively with ethanol. The crude ethanol syrup was extracted five times with hexane. The ethanol layer was partitioned with EtOAc− H_2O (1:1) three times to give EtOAc and H_2O layers. After the EtOAc layer was evaporated in vacuo, the residue (82 g) was chromatographed on a Si gel column with *n*-hexane−EtOAc (8:1, 6:1, 4:1, 2:1, 1:1, EtOAc) to give 12 fractions (1−12). Bioactive fraction 3 was further separated by column chromatography on silica gel eluting with CH_2Cl_2−acetone (10:1, 6:1, 4:1, 1:1) to yield 10 fractions (3-1−3-10). Fraction 2 was further rechromatographyed on Si gel with hexanes−EtOH (7:1, 6:1, 5:1, 4:1, 3:1), and 10 fractions (2-1−2-12) were obtained. Fraction 2-4 was further chromatographed using HPLC [$5C_{18}$, 250 × 10 mm, with MeOH−H_2O (3:1) v/v] as the eluent to yield compounds **7** (4.3 mg) and **8** (2.6 mg). Like **7** and **8**, compounds **9** (3.2 mg) and **10** (4.6 mg) were obtained from fraction 2-5 by HPLC using similar conditions. Compounds **5** (5.2 mg) and **6** (5.2 mg) were purified from fractions 2-6 and 2-7 by washing with MeOH, respectively. Fraction 3-3 furnished compound **2** (2.9 mg). Compounds **1** (6.1 mg), **3** (4.5 mg), and **4** (4.2 mg) were obtained from fraction 3-5 by HPLC ($5C_{18}$, 250 × 10 mm, MeOH−H_2O, 80:20).

Schizarin B (1): light yellow, amorphous powder; IR (KBr) 3500 (OH), 1725 (ester), 1625, 1590 (aromatic) cm^{-1}; $[\alpha]_D$ −37.5° ($CHCl_3$, *c* 0.1); EIMS *m/z* (rel int) 486 [$M]^+$ (18), 384-

(100), 335 (24), 329 (7); HRMS *m/z* 486.2258 $[M]^+$ (calcd for $C_{27}H_{34}O_8$, 486.2254); ^1H and $^{13}C-$ NMR, see Tables 1 and 2, respectively.

Schizarin C (2): light yellow, amorphous powder; IR (KBr) 3500 (OH), 1725 (ester), 1625, 1590 (aromatic) cm^{-1}; $[\alpha]_D$ −13.5° ($CHCl_3$, *c* 0.14); EIMS *m/z* (rel int) 500 $[M]^+$ (25), 430 (11), 414 (27), 384(100), 353 (22); ^1H and ^{13}C NMR, see Tables 1 and 2, respectively.

Schizarin D (3): light yellow, amorphous powder; IR (KBr) 3500 (OH), 1720 (ester), 1620, 1590 (aromatic) cm^{-1}; $[\alpha]_D \approx 0°$ ($CHCl_3$, *c* 0.1); EIMS *m/z* (rel int) 484 $[M]^+$ (15), 384(100), 353 (20); HRMS *m/z* 484.2099 $[M]^+$ (calcd for $C_{27}H_{32}O_8$, 484.2097); ^1H and ^{13}C NMR, see Tables 1 and 2, respectively.

Schizarin E (4): light yellow, amorphous powder; IR (KBr) 3500 (OH), 1720 (ester), 1620, 1590 (aromatic) cm^{-1}; $[\alpha]_D$ −325° ($CHCl_3$, *c* 0.1); EIMS *m/z* (rel int) 506 $[M]^+$ (20), 384(100), 353 (12); HRMS *m/z* 506.1944 $[M]^+$ (calcd for $C_{29}H_{30}O_8$, 506.1941); ^1H and ^{13}C NMR, see Tables 1 and 2, respectively.

HIV Inhibition Assay. HIV inhibition was measured as described previously.[11]

Anti-HBsAg and anti-HBeAg Test. The assays for in vitro antiviral activity against hepatitis B virus (HBV) were performed according to our previously described procedure.[12] Briefly, the HBV-producing cell line MS-G2 were plated into 24-well flat-bottomed tissue culture plates at a density of 3×10^5 cells/mL/well. After overnight incubation to ensure that the cells were properly attached, the cell culture was challenged with test compounds. DMSO alone was added to each culture as solvent control. All tested pure compounds were dissolved in DMSO at a concentration of 1, 5, 10, and 20 μg/mL, respectively. The concentration of DMSO in the media was maintained at no more than 2.5 μL/mL. Subsequently, the culture media were collected at 3 days for antiviral assay. Antiviral activity was assessed by analyses of HBsAg and HBeAg values using the ELISA assay (enzyme-linked immunosorbent assay) (instrument: DYNATECH MR 7000 at 490 nm). The percentage inhibition (%) was calculated by comparing with the control group. Inhibition between 25 and 35% was defined as moderate inhibition, 35−45% as medium inhibition, and >45% as strong inhibition, while inhibition below 25% was defined as inactive.

(1) Cell Line and Cell Culture. A HBV DNA integrated HCC cell line, MS-G2, kindly provided by Dr. Max Essex,[12] was established from a hepatoblastoma-derived cell line, HepG2, by transfection with two copies of the entire HBV genome. The MS-G2 cell line secreted HBV containing viral DNA and DNA polymerase activity. The MS-G2 cells were cultured in RPMI-1640 (Gibco, BRL, Grand Island, NY) medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 1% nonessential amino acids, and 2.5 mg/mL amphotericin B. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Under these conditions the plating efficiency was above 95%.

(2) Cytotoxic Assay. Cell damage was tested by an AST (aspartate transaminase) Fuji kit. AST values higher than 25 IU/L served as an indication of cell damage or lysis, as described previously.[12]

Acknowledgment. We would like to thank Mr. Ting-Chuan Chung and Dr. Ge-Hsiung Tsai for plant collecting, and Mr. Shih-Jen Wang, NSC Regional Instrument Center of HSIN-CHU, for measuring the HRMS spectrum. In addition, the authors appreciate the National Science Council, Taiwan, Republic of China, for financial support (NSC 89-2113-M-077-003) to Y.H.K.

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NP000261M