Four New C19 Homolignans, Schiarisanrins A, B, and D and Cytotoxic Schiarisanrin C, from *Schizandra arisanensis*

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Four new C_{19} homolignans with a 5,4′-butano-2,4-cyclohexadienone-6-spiro-3′-(2′,3′-dihydrobenzo-[*b*]furan) skeleton, schiarisanrin A, schiarisanrin B, schiarisanrin D, and cytotoxic schiarisanrin C, were isolated from *Schizandra arisanensis*. Their structure and stereochemistry have been established by spectral and single-crystal X-ray analysis. Schiarisanrin C has cytotoxic activity with ED_{50} values of 0.36, 7.1, 4.9, and 5.7 μg/mL, respectively, against KB epidermoid carcinoma of nasopharynx, COLO-205 colon carcinoma, HEPA hepatoma, and HELA cervix tumor cells.

Introduction

The fruit of *Schizandra chinensis* (*Schizandraceae*) is used in Chinese medicine as an antitussive, an astringent, and a tonic. Several dibenzocyclooctadiene lignans from *S. chinensis* have recently been found to have some beneficial pharmacological effects including antihepatitis activity and antioxidant activity and to be involved in nervous system regulation.[1−3] In our continuing investigation of the antitumor potential of Taiwanese plants,[4−7] we found that an EtOH extract of the stems of *Schizandra arisanensis* (known as "Hong Ku Shern"; *Schizandraceae*) was useful as an antirheumatic and exhibited cytotoxicity against nasopharynx carcinoma cells *in vitro*. Bioassay-directed fractionation of this extract led to the isolation and characterization of four unique C_{19} homolignans with a 5,4′-butano-2,4-cyclohexadienone-6-spiro-3′-(2′,3′-dihydrobenzo[*b*]furan) skeleton: schiarisanrin A (**1**), schiarisanrin B (**2**), schiarisanrin C (**3**), and schiarisanrin D (**4**). The structures of these compound were elucidated by 2D NMR studies, including $^1H-^{13}C$ heteronuclear COSY and long-range $^1H-^{13}C$ COSY for complete assignment of the ^1H (Table 1) and ^{13}C NMR spectra (Table 2). Furthermore, biological evaluation of these new compounds revealed that schiarisanrin C (**3**) demonstrates cytotoxicity against human epidermoid carcinoma of nasopharynx (KB), colon carcinoma (COLO-205), hepatoma (HEPA), and cervix (HELA) tumor cells.

Results and Discussion

An EtOH extract of the dried stems of *S. arisanensis* was extracted successively with *n*-hexane, $CHCl_3$, and BuOH. Repeated column chromatography of the $CHCl_3$ extract yielded schiarisanrins A (**1**) and C (**3**), and the BuOH extract yielded schiarisanrins B (**2**) and D (**4**).

Schiarisanrin A (**1**) [*m/z* 484.2105 (M^+); mp 175−177 °C; $[\alpha]_D$ −133 (*c* 0.3, $CHCl_3$); infrared (IR) υ_{max} cm^{-1} 1725 (C═O), 1650 (C═CC═CC═O)] has a molecular formula of $C_{27}H_{32}O_8$, based on HREIMS. The IR spectrum revealed the presence of an ester and α,*β*,*γ*,*δ*-dienone groups but the absence of a hydroxyl group. In the ^{13}C- and ^1H-NMR spectra, the signals at δ_C 195.5, 156.7, 132.3, 120.4, 147.5, 64.7 ppm (C-1, -2, -3, -4, -5, and -6, respectively), together with the heteronuclear long-range correlation (HMBC) spectrum, which showed coupling between an olefinic proton (H-4, δ_H 6.09, J = 1.9 Hz) and three double-bond carbons at C-5, -3, and -2 (δ_C 147.5, 132.3, and 156.7, respectively), revealed the presence of a cyclohexadienone moiety in **1**. The other aromatic signals at δ_C 128.5, 101.3, 130.2, 150.3, 144.2, and 122.7 were assigned to C-4′, -5′, -6′, -7′, -8′, and -9′, respectively, on the basis of long-range correlations. In the HMBC spectrum of **1**, cross signals between C-8′ and oxygenated methylene protons (δ_H 4.27, 4.54 ppm, d, J = 8.8 Hz, H-2′) and between C-6′ and -7′ and a methylenedioxy moiety (δ_H 5.95, 5.96, ABq, J = 17.6 Hz, H-10′) indicated the presence of an oxygenated benzene moiety. Correlations were also found between H-2′ and C-3′, C-9′ (δ_C 122.66), and C-8′ (δ_C 144.16), all of which suggested a dihydrofuran linkage with $C_{3'}-C_{9'}-C_{8'}-O_{1'}-C_{2'}-C_{3'}$, which together with the above-mentioned oxygenated benzene suggested a 2′,3′-dihydrobenzo[*b*]furan moiety. In addition, the proton signals of H-7 (δ_H 2.51, m, H-7_a; δ_H 2.16, dd, J = 11.3, 3.1 Hz, H-7_b), 8 (δ_H 1.76, m), 9 (δ_H 1.93, m) and 10 (δ_H 5.94, d, J = 6.7 Hz) were unequivocally assigned to a butano moiety on the basis of spin−spin

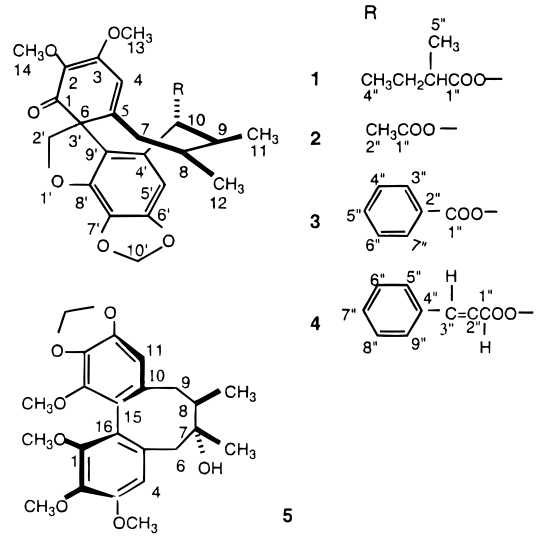

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Table 1. ^{1}H NMR (300 MHz, $CDCl_3$) Data[a] for Compounds 1−4

proton	1	2	3	4
4	6.09 (d, 1.9)	6.06 (d, 2.0)	6.12 (d, 1.9)	6.103 (d, 2.4)
7a	2.51 (m)	2.53 (m)	2.58 (m)	2.58 (m)
7b	2.16 (dd, 11.3, 3.1)	2.14 (dd, 11.8, 3.6)	2.31 (dd, 11.2, 2.6)	2.20 (dd, 11.4, 2.8)
8	1.76 (m)	1.72 (m)	1.90 (m)	1.81 (m)
9	1.93 (m)	1.89 (m)	2.03 (m)	1.97 (m)
10	5.94 (d, 6.7)	5.94 (d, 6.6)	5.79 (d, 6.7)	6.03 (d, 6.8)
11	0.87 (d, 7.3)	0.88 (d, 7.2)	1.10 (d, 7.2)	0.93 (d, 7.2)
12	0.84 (d, 7.3)	0.81 (d, 6.7)	0.90 (d, 7.2)	0.85 (d, 6.7)
13	3.72 (s)	3.72 (s)	2.96 (s)	3.66 (s)
14	4.02 (s)	4.02 (s)	3.82 (s)	3.98 (s)
2′	4.27, 4.54 (ABq, 8.8)	4.27, 4.54 (ABq, 8.8)	4.24, 4.46 (ABq, 8.8)	4.29, 4.57 (ABq, 8.8)
5′	6.29 (s)	6.26 (s)	6.45 (s)	6.33 (s)
10′	5.95, 5.96 (ABq, 17.6)	5.93, 5.96 (ABq, 16.2)	5.97 (d, 17.8)	5.97 (d, 17.8)
2″	2.07 (m)	1.78 (s)		6.15 (ABq, 16.0)
3″	1.54, 1.33 (m)		7.78 (d, 7.6)	7.56 (ABq, 16.0)
4″	0.89 (t, 7.5)		7.33 (t, 7.6)	
5″	0.93 (d, 7.0)		7.47 (t, 7.6)	7.51 (m)
6″			7.33 (t, 7.6)	7.33 (m)
7″			7.78 (d, 7.6)	7.33 (m)
8″				7.33 (m)
9″				7.53 (m)

[a] All assignments are based on 1D and 2D NMR experiments, including COSY90, HETCOR, HMBC, and NOESY spectra.

Table 2. ^{13}C NMR (75.5 MHz, $CDCl_3$) Data[a] for Compounds 1−4

carbon	1	2	3	4	C−H connectivities[b]
1	195.48 s	195.84 s	194.80 s	195.46 s	H-2′
2	156.72 s	157.15 s	156.25 s	157.08 s	H-14
3	132.32 s	132.04 s	132.61 s	132.15 s	H-4, 13
4	120.43 d	120.71 d	121.36 d	121.08 d	H-7
5	147.51 s	147.90 s	146.31 s	147.55 s	H-7, 2′
6 (3′)	64.72 s	64.56 s	64.43 s	64.59 s	H-7, 4
7	40.27 t	40.23 t	40.04 t	40.19 t	H-11, 12
8	31.66 d	31.79 d	31.50 d	31.89 d	H-10, 9, 7, 11, 12
9	42.59 d	42.66 d	42.79 d	43.08 d	H-10, 7, 11, 12
10	76.67 d	76.83 d	78.37 d	76.58 d	H-5′, 12
11	8.96 q	8.70 q	9.60 q	8.95 q	H-9, 11
12	21.49 q	21.39 q	21.57 q	21.65 q	H-7
13	59.29 q	59.38 q	59.27 q	59.50 q	
14	58.40 q	58.65 q	58.43 q	58.75 q	
2′	77.96 t	77.96 t	79.55 t	78.21 t	
4′	128.50 s	128.28 s	128.57 s	128.31 s	H-10
5′	101.34 d	101.09 d	101.32 d	101.15 d	H-10
6′	130.16 s	130.17 s	130.63 s	130.13 s	H-5′, 10′
7′	150.30 s	150.15 s	150.30 s	150.24 s	H-5′, 10′
8′	144.16 s	144.18 s	146.31 s	144.28 s	H-2′
9′	122.66 s	122.75 s	122.13 s	122.92 s	H-5′, 10, 2′
10′	101.94 t	101.93 t	101.99 t	101.98 t	H-6′
1″	176.42 s	170.08 s	166.54 s	166.38 s	H-10, 2″,[c] 3″,[c] 5″ [c]
2″	40.45 d	20.40 q	130.15 s	117.58 d	H-3″,[c] 4″,[c] 5″ [c]
3″	27.00 t		128.01 d	145.52 d	H-2″,[c] 3″,[c] 5″ [c]
4″	11.51 q		129.85 d	134.58 s	H-2″,[c] 3″ [c]
5″	15.92 q		132.61 d	128.31 d	H-2″,[c] 3″ [c]
6″			129.85 d	129.85 d	
7″			128.01 d	128.66 d	
8″				129.85 d	
9″				128.31 d	

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

decoupling and COSY90 studies. A cross peak (H−H long-range coupling) between H-4 and H-7 in the ^{1}H−^{1}H COSY spectrum would explain that the signal of H-4 is a doublet. Moreover, long-range correlations (HMBC) between H-7 and C-5, -6, and -4 of cyclohexandienone and between H-10 and C-4′, -5′, and -9′ of dihydrobenzo[*b*]furan imply that **1** possesses substituted dihydrobenzo[*b*]furan, substituted cyclohexadienone, and substituted 5−4′ butano moieties, in contrast to dibenzocyclooctadiene lignans from Schizandracece plants.[8,9] After all of the proton and carbon resonances were correlated to the results from ^{1}H−^{13}C COSY, the complete chemical shifts of **1** could be deduced from the heteronuclear long-range correlation (HMBC) spectrum. The most important finding was that an atypical aliphatic quaternary carbon signal at δ_C 64.72 was correlated with H-2′ (δ_H 4.27, 4.54, ABq, J = 8.8 Hz), H-4 (δ_H 6.09, d, J = 1.9 Hz) and H-7. Therefore, this signal was assigned to C-6 (=C-3′). In addition, the adjacent carbonyl carbon (δ_C 195.5, C-1) and a conjugated quaternary carbon (C-5) were correlated to H-2′, which was consistent with the cyclohexadienone neighboring the 2′, 3′-dihydrobenzo[*b*]furan through a spiro configuration (6-spiro-3′). Next, the two remaining methoxy groups (δ_H 3.71and 4.02, s, H-13 and 14, respectively) at C-3 and -2 could be predicted from the HMBC spectrum. The mass spectrum of **1** exhibits a molecular ion at *m*/*z* 484 and an intense peak at *m*/*z* 382. This corresponds to the 1,2-elimination of a pentanoic acid (C_4H_9COOH) via McLafferty rearrangement of esters. Thus, **1** contained an ester group of the type −O(C=O)C_4H_9. To clarify the nature of the ester group, the heteronuclear long-range correlation NMR spectrum was examined. The spectrum exhibited cross signals between C-1″ and H-10, 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″. All of these findings suggested that the ester group is a 2-methylbutanoic acid ester group. On the basis of this corroboration, compound **1** must have a 5,4′-butano-2,4-cyclohexadienone-6-spiro-3′-(2′,3′-dihydrobenzo[*b*]furan) skeleton with a 2-methylbutyroxyl group at C-10 (Figure 1).

Single-crystal X-ray analysis on a Nonius (CAD4) diffractometer unequivocally confirmed the complete structure and stereochemistry of **1**. Crystal data: $C_{27}H_{32}O_8$, M = 484.54, monoclinic, space group $C2$, a = 21.039(5) Å, b = 8.914(14) Å, c = 14.798(4) Å, b = 116.27°, V = 2488.5 Å^3, Z = 4, D_{calcd} = 1.293 g cm^{-3}, $F(000)$ = 1035, m = 1.542 cm^{-1}. The stereochemistry of **1** was compared with that reported for the biphenyl lignan gomisin A (**5**).[8,9] Both **1** and **5** have a cyclic (6.8.6)

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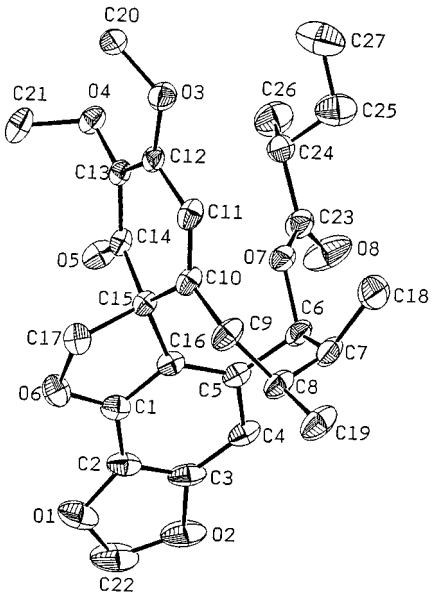

Figure 1. Computer-generated perspective drawing of Schiarisanrin A (**1**). (The tentative numberings are different from the text described, and hydrogens are omitted for clarity.)

skeleton. However, **1** has a substituted cyclohexadienone moiety with an oxygenated methylene group, instead of the substituted benzene moiety found in **5**. In biphenyl lignans, the cyclooctadiene moiety generally exhibits either a boat or chair conformation.[8−13] In contrast, a careful examination of the data obtained by X-ray analysis showed that the cyclooctene moiety of **1** has a unique boatlike shape.

The IR and NMR spectra of schiarisanrin B (**2**) [$C_{24}H_{26}O_8$, mp 98−100 °C; $[\alpha]_D$ −173 (*c* 0.3, $CHCl_3$)] revealed that this compound also has a 5,4′-butano-2,4-cyclohexadienone-6-spiro-3′-(2′,3′-dihydrobenzo[*b*]furan) skeleton with an oxygenated methylene group, as in **1**. The NMR spectra of **2** showed that it possesses an acetoxyl group at C-10 instead of a 2-methylbutyroxyl group. This finding was further supported by the long-range correlation (HMBC) NMR spectrum of **2**. The spectrum showed cross signals between the carbonyl carbon signals at δ_C 170.1 and the signals for the methyl group at δ_H 1.78 (H-2″), and H-10 at δ_H 5.94, revealing that the acetoxyl group is located at C-10. The mass spectrum of **2** showed a molecular ion at *m*/*z* 442 and an intense peak at *m*/*z* 382 ($M^+ - CH_3COOH$), indicating the presence of an acetoxyl group in the compound. The ion at *m*/*z* 382 reflected the 1,2-elimination of acetic acid via McLafferty rearrangement involving the acetoxyl group.

Schiarisanrin C (**3**) showed a molecular ion at *m*/*z* 504, which is consistent with the molecular formula of $C_{29}H_{28}O_8$. The ^1H and ^{13}C NMR spectra of **3** were similar to those of **1**, except for the absence of a 2-methylbutanoate group. The molecular formula of **3** corresponds to the replacement of a 2-methylbutanoate group ($C_5H_9O_2$) with a benzoate group ($C_7H_5O_2$). The NMR spectra of **3** showed benzoate signals, including aromatic protons at δ_H 7.78, 7.33, and 7.47, corresponding to the carbon signals at δC 128.01 (C-3″ and 7″), 129.85 (C-4″ and 6″), and 132.61 (C-5″) ppm, respectively, as well as a quaternary carbon at δ_C 130.15 (C-2″). The benzoyl group was determined to be at C-10 on the basis of the correlation between the carbonyl carbon (C-1″) and H-10 found in the long-range correlation (HMBC) spectra. Moreover, the 2D NOE (NOESY) spectrum revealed a correlation between H-5″ of the benzoxyl group and H-13 of the methoxyl group, which implies that the methoxyl (H-13) and benzoxyl groups have the same orientation and are close to each other. This is in good agreement with the shifting of the methoxyl (H-13) resonance to a higher field (δ_H 2.96) in **3** compared to that in **1** (δ_H 3.72) because of the shielding effect of the benzene ring. This finding further suggests that the cyclooctene moiety in **3** has a boatlike form, as in **1** and **2**. The mass spectrum of **3** exhibited a molecular ion at *m*/*z* 504 and an intense peak at *m*/*z* 382 [M^+ − benzoic acid]. The molecular ion undergoes the elimination of benzoic acid via McLafferty rearrangement involving the benzoxyl group. This is further evidence for the presence of a benzoxyl group in **3**. Thus, the structure of schiarisanrin C (**3**) was determined unambiguously.

Schiarisanrin D (**4**) has a molecular formula of $C_{31}H_{30}O_8$ (*m*/*z* 530 [M^+]). The ^1H NMR and mass spectral data of **4** and **3** reveal that the former is a derivative of **3** in which a cinnamoxyl group has replaced the benzoxyl group at C-6. A cinnamoxyl group was detected by proton signals at δ_H 6.15 and 7.56 (H-2″ and 3″, ABq, J = 16.0 Hz, respectively), which correspond to the signals of the trans double bond and aromatic protons at δ_H 7.33 and 7.51 ppm (H-6″, -7″, -8″ and -5″, -9″, m, respectively) in the ^1H NMR spectrum. Also, aromatic carbon signals at δ_C 128.3 (C-9″, -5″), and 128.7 (C-6″, -7″, -8″), olefinic carbon signals at δ_C 117.6 and 145.5, a quaternary carbon signal at δ_C 134.6, and a carbonyl carbon signal at δ_C 166.4 were seen in the ^{13}C NMR spectrum. In addition, a molecular ion at *m*/*z* 530 and a characteristic fragmentation ion at *m*/*z* 382 (530 − cinnamic acid) also corresponded to a *trans*-cinnamic acid ester in **4**. Moreover, the HMBC spectrum of **4** clearly showed a correlation between the carbonyl carbon signal at δ_C 166.4 (cinnamoyl C=O) and the proton signal at δ_H 6.03 (H-10), indicating a connectivity between the cinnamoxyl group and C-10.

Since schiarisanrins A (**1**), B (**2**), C (**3**), and D (**4**) have an additional 2′-C, they represent a new class of C_{19} lignans with a 5,4′-butano-2,4-cyclohexadienone-6-spiro-3′-(2′,3′-dihydrobenzo[*b*]furan) skeleton. This class of lignans differs from the C_{18} lignans with a dibenzocyclooctadiene skeleton, which are isolated from plants of the Schizandriaceae family. Therefore, we propose the term homolignan for the new class of C_{19} lignans described in this paper. Furthermore, those new homolignans, **1**−**4**, were also assayed for cytotoxicity in four cancer cell lines: KB, COLO-205, HEPA-3B, and HELA. Schiarisanrin C (**3**) exhibited cytotoxicity against all four cell lines, including nasopharynx carcinoma (KB, ED_{50} = 0.36 μg/mL), colon carcinoma (COLO-205, ED_{50} = 7.1 μg/mL), hepatoma (HEPA-2B, ED_{50} = 4.9 μg/mL), and cervical carcinoma (HELA, ED_{50} = 5.7 μg/mL). In contrast, the ED_{50} values for compounds **1**, **2**, and **4** all exceeded 10 μg/mL. Additional studies are needed to further evaluate this bioactivity and to elucidate detailed structure−cytotoxicity relationships.

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Experimental Section

General Experimental Procedures. NMR spectra were obtained at 300 MHz for ^{1}H and 75 MHz for ^{13}C. For the heteronuclear correlation, HMBC spectra were performed using coupling constants of 8 Hz. Samples for IR spectral measurements were prepared as KBr disks. EIMS were performed in the electron impact mode (70 eV). HPLC was accomplished by using the semipreparative Si gel and $5C_{18}$ columns.

Plant Material. The stems of *S. arisanensis* were collected in July 1993 in Taipei County, Taiwan. A voucher specimen was deposited at the National Research Institute of Chinese Medicine, Taipei, Taiwan, R.O.C.

Extraction and Isolation. Dried stems (5.2 Kg) of *S. arisanensis* were exhaustively extracted with ethanol. A crude ethanol extract (280 g) was partitioned with $CHCl_3-H_2O$ (1:1 v/v) three times to give $CHCl_3$ and aqueous layers. The aqueous layer was further partitioned with BuOH (1:1 v/v) three times to give H_2O and BuOH layers. After the $CHCl_3$ layer was evaporated in vacuo, its extract (67g) was chromatographed on silica gel with *n*-hexane containing increasing portions of EtOAc as the eluent; 10 fractions were obtained. Fraction 6 was further separated repeatedly by HPLC (Si gel, 250 × 10 mm; CH_2Cl_2-EtOAc =1:3) to give **1** (36 mg). Fraction 2 was further rechromatographed over Si gel with hexane−EtOAc (3:1) as the eluent to yield **3** (12 mg). The condensed BuOH layer was chromatographyed on Si gel with $CHCl_3-$MeOH (20:1, 10:1, 5:1, 1:1 v/v), and seven fractions were obtained. Fraction 3 was further chromatographed using HPLC ($5C_{18}$, 250 × 10 mm) with $MeOH-H_2O$ (3:1 v/v) as the eluent to isolate compounds **2** (16 mg) and **4** (8 mg).

Schiarisanrin A (**1**): colorless prisms; mp 175−177 °C; IR (KBr) 1725 (C=O), 1650, 1590, 715 (aromatic) cm^{-1}; $[\alpha]_D$ −133 ($CHCl_3$, *c* 0.3); EIMS *m/z* (rel intensity) 486 (5), 485 (23), 484 (M^+, 77), 383 (32), 382 (100), 368 (10), 367 (35), 340 (14), 339 (10), 326 (12), 325 (11); HREIMS *m/z* 484.2105 (M^+, calcd $C_{27}H_{32}O_8$, 0.7 mmu); ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively.

Schiarisanrin B (**2**): colorless prisms; mp 98−100 °C; IR (KBr) 1720 (C=O), 1645, 1590, 720 cm^{-1}; $[\alpha]_D$ −173 ($CHCl_3$, *c* 0.3); EIMS *m/z* (rel intensity) 442 (M^+, 100), 383 (27), 382 (91), 367 (35), 326 (16), 325 (18); HREIMS *m/z* 442.1606 (M^+, calcd $C_{24}H_{26}O_8$,1.1 mmu); ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively.

Schiarisanrin C (**3**): yellow amorphous power; mp 130−133 °C; IR (KBr) 1720 (C=O), 1650, 1590, 720 cm^{-1}; $[\alpha]_D$ −146 ($CHCl_3$, *c* 0.3); EIMS *m/z* (rel intensity) 506 (4), 505 (20), 504 (M^+, 62), 383 (23), 382 (87), 367 (12), 325 (11); HREIMS *m/z* 504.1780 (M^+, calcd $C_{29}H_{28}O_8$, 0.6 mmu); ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively.

Schiarisanrin D (**4**): yellow amorphous power; mp 133−135 °C; IR (KBr) 1720 (C=O), 1650, 1590, 720 cm^{-1}; $[\alpha]_D$ −126 ($CHCl_3$, *c* 0.3); EIMS *m/z* (rel intensity) 532 (3), 531 (15), 530 (M^+, 45), 383 (17), 382 (62), 367 (15), 326 (8); HREIMS *m/z* 530.1947 (M^+, calcd $C_{31}H_{30}O_8$, 1.2 mmu); ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively.

Cytotoxicity Assay. The *in vitro* cytotoxicity assay was performed according to procedures described by Geran et al[14] and Ferguson et al.[15] The assay against KB (nasal pharynegeal carcinoma), HEPA-2B (hepatoma), HELA (cervix carcinoma), and COLO-205 (colon carcinoma) tumor cells was mainly based on a method reported by Chen.[16] These cells, except for KB, were kindly provided by the Cell Bank of the Veterans General Hospital, Taipei, R.O.C. The KB cells were purchased from the American Type Culture Collection. The cells for bioassay were cultured in RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. The cytotoxicity assay by the methylene blue dying method was performed, employing a modification of published procedures.[17,18] The assay depends on binding the methylene blue to the fixed monolayer at pH 8.5 and, after washing the monolayer, releasing dye by lowering the pH. In summary, test samples and control standard agents (antidrug) were prepared at concentrations of 1, 5, 10, 40, and 100 μg/mL. After 2880 cells/well were seeded in 96-well microplate for 3 h, 20 mL of sample or standard agents was placed in each well and incubated at 37 °C for 3 days. After the medium was removed from the microplates, the cells were fixed by 10% formal saline for 30 min and then dyed by 1% (w/v) methylene blue in 0.01 M borated-buffer 100 μL/well for 30 min. The 96-well tray was dipped into a 0.01 M borated-buffer solution four times in order to remove the dye. Then, 100 μL/well ethanol−0.1 M HCl (1/1 v/v) was added as a dye-eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The ED_{50} was defined during a comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

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