Influenza A (H_1N_1) Antiviral and Cytotoxic Agents from *Ferula assa-foetida*

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Two new sesquiterpene coumarins, designated 5′-acetoxy-8′-hydroxyumbelliprenin (**1**) and 10′*R*-acetoxy-11′-hydroxyumbelliprenin (**2**), and a new diterpene, 15-hydroxy-6-en-dehydroabietic acid (**3**), along with 27 known compounds, were isolated from a $CHCl_3$-soluble extract of *Ferula assa-foetida* through bioassay-guided fractionation. The structures of the new metabolites **1**−**3** were identified by spectroscopic data interpretation and by the Mosher ester method. Compounds **4** and **6**−**13** showed greater potency against influenza A virus (H_1N_1) (IC_{50} 0.26−0.86 μg/mL) than amantadine (IC_{50} 0.92 μg/mL), and **11** exhibited the best potency (IC_{50} 0.51, 2.6, and 3.4 μg/mL) of these compounds against the HepG2, Hep3B, and MCF-7 cancer cell lines, respectively.

Influenza occurs with seasonal variations and reaches peak prevalence in winter, with many people killed worldwide every year. Until now, only a few organic compounds including amantadine, rimantadine, and ribavirin have been used for influenza therapy. However, drug-resistant influenza viruses are generated quickly. During the course of an anti-influenza and cytotoxicity screening program on natural products, it was found that a $CHCl_3$ extract of *Ferula assa-foetida* was active against H_1N_1 influenza virus (IC_{50} < 3.4 μg/mL) and various human cancer cell lines (IC_{50} < 20 μg/mL). Therefore, the plant was selected as a lead to be investigated.

F. assa-foetida L. (Apiaceae) is distributed mainly in Iran, Afghanistan, and mainland China. Nearly a hundred years ago, the roots of this plant were an important remedy for the well-known "Spanish Flu" (type A influenza, H_1N_1 subtype), which led to 20−100 million people dying at that time.[1] The roots of *F. assa-foetida* have been used for their perceived anthelmintic, anticarcinogenesis, anti-HIV, antimicrobial, antirheumatic, antispasmodic, diuretic, and emmenagogue actions in folk medicine.[2−7] There are 236 natural products, including 129 sesquiterpene coumarins, seven monoterpene coumarins, 49 daucane sesquiterpenes, nine polysulfide derivatives, 10 sesquiterpene phenylpropanoids, 21 simple sesquiterpenes, six simple monoterpenes, and five oleanane triterpenes, reported from the genus *Ferula* to date. While *F. assa-foetida* has had extensive use in folk medicine for the treatment of influenza, it is interesting that the antiviral activities of the isolated compounds from this plant have not been evaluated in a systematic manner. Bioassay-directed chromatographic fractionation of *F. assa-foetida* resin led to the isolation of 30 compounds, including 20 sesquiterpene coumarins, 5′-acetoxy-8′-hydroxyumbelliprenin (**1**), 10′*R*-acetoxy-11′-hydroxyumbelliprenin (**2**), 5′*S*-hydroxyumbelliprenin (**4**),[8] 10′*R*-karatavicinol (**5**),[9] 8′-acetoxy-5′*S*-hydroxyumbelliprenin (**6**),[8,9] methyl galbanate (**7**),[8,10,11] galbanic acid (**8**),[10,11] farnesiferol C (**9**),[10,12,13] farnesiferol A (**10**),[12,13] farnesiferol B,[12,13] assafoetidin,[14] lehmferin,[15] conferol (**11**),[16] feselol,[17] ligupersin A (**12**),[18] *epi*-conferdione (**13**),[9] microlobin,[11] lehmannolone,[19] polyanthinin,[20] and kamolonol;[21] two polysulfide derivatives, foetisulfide A and foetisulfide C;[22] a simple coumarin, umbelliferone; a benzaldehyde, vanillin; a drimane sesquiterpenoid, fetidone B;[23] three diterpenes, 7-oxocallitrisic acid, picealactone C, and 15-hydroxy-6-en-dehydroabietic acid (**3**); a sterol, β-sitosterol; and a fatty acid, oleic acid. Among them, two new sesquiterpene coumarins (**1** and **2**) and a new diterpene (**3**) were identified. Most of these compounds were evaluated in influenza A (H_1N_1) antiviral and cytotoxicity assays, and the results are described herein.

Results and Discussion

A MeOH extract of the resin of *F. assa-foetida* was partitioned between *n*-hexane−MeOH (1:1), and then the MeOH layer was partitioned between $CHCl_3$−H_2O (1:1) to obtain a $CHCl_3$ extract, which showed significant anti-H_1N_1 activity (IC_{50} < 3.4 μg/mL) and cytotoxicity for three cancer cell lines (IC_{50} < 20 μg/mL). Initial fractionation of the $CHCl_3$ extract was carried out by open liquid chromatography on silica gel to give 11 fractions. Chromatographic fractionation of these active subfractions provided two new sesquiterpene coumarins (**1** and **2**), a new diterpene (**3**), and 27 known compounds.

HRESIMS of **1** exhibited a $[M + Na]^+$ ion at *m*/*z* 463.2099 ($C_{26}H_{32}O_6Na$). The IR spectrum showed absorptions for hydroxy (3440 cm^{-1}), acetoxy (1729 cm^{-1}), and aromatic (1613 and 1555 cm^{-1}) functional groups. UV absorptions at 211 and 318 nm also indicated a coumarin nucleus oxygenated at the C-7 position.[12] In the ^{13}C NMR spectrum, compound **1** displayed 26 carbon signals, with nine being typical for an umbelliferone skeleton [δ 101.5 (C-8), 112.5 (C-10), 113.1 (C-3), 114.2 (C-6), 128.7 (C-5), 143.4 (C-4), 155.8 (C-9), 161.2 (C-2), and 161.9 (C-7)] and the remaining 17 signals ascribable to a sesquiterpene moiety [δ 18.0 (C-13′), 13.1 (C-14′), 17.1 (C-15′), 25.9 (C-12′), 34.2 (C-9′), 44.9 (C-4′), 65.2 (C-1′), 68.9 (C-5′), 75.9 (C-8′), 119.5 (C-10′), 122.1 (C-2′), 123.3 (C-6′), 135.5 (C-11′), 137.6 (C-3′), 141.9 (C-7′)] with an acetoxy group (δ 21.2 and 170.3). In the ^1H NMR spectrum, signals for two main moieties, a coumarin and a sesquiterpene, were revealed. The coumarin moiety appeared as five signals [δ_H 6.24 and 7.63 (each 1H, d, J = 9.4 Hz), 7.35 (1H, d, J = 8.4 Hz), 6.82 (1H, dd, J = 2.4, 8.4 Hz), 6.79 (1H, d, J = 2.4 Hz)]. In turn, the sesquiterpene moiety displayed signals for four methyls [δ_H 1.62, (3H, brs), 1.71 (3H, brs), 1.72 (3H, brs), 1.81, (3H, s)], two methylenes [δ_H 2.23 (2H, m), 2.28 (1H, d, J = 6.0 Hz), 2.45 (1H, dd, J = 7.6, 13.6 Hz)], an oxygenated methylene [δ_H 4.57 (2H, d, J = 6.2 Hz)], three olefinic methines [δ_H 5.08 (1H, brt, J = 6.4 Hz), 5.41 (1H, d, J = 8.8 Hz), 5.50 (1H, brt, J = 6.2 Hz)], and two oxygenated methines [δ_H 3.99 (1H, brt, J = 6.4 Hz), 5.70 (1H,

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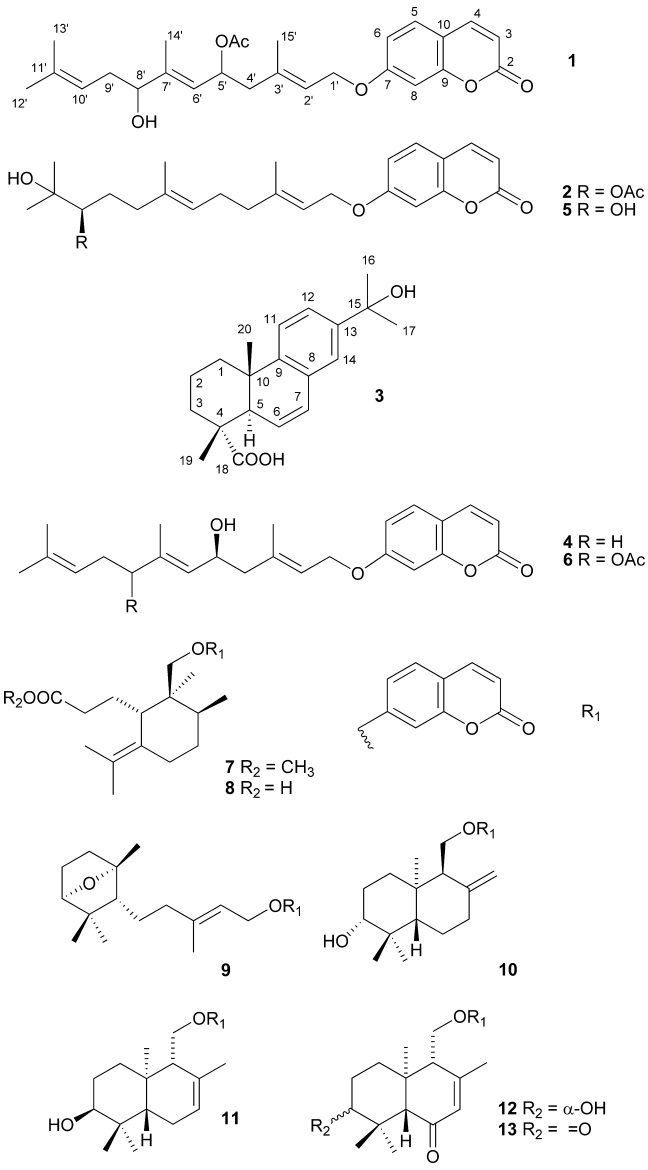


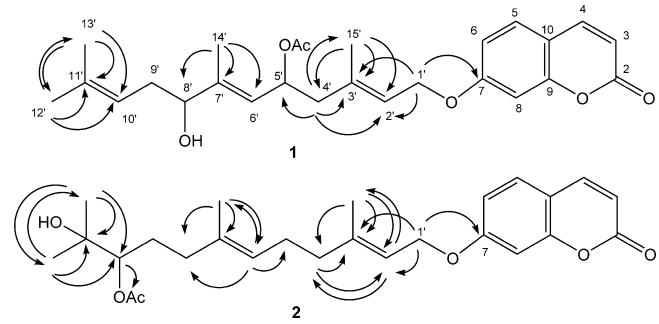


Figure 1. Important HMBC correlations for compounds **1** and **2**.

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** (400 and 100 MHz in $CDCl_3$, δ in ppm, J in Hz)

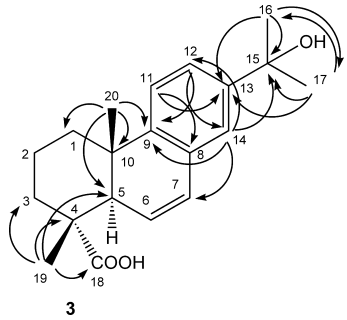
	1		**2**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2		161.2 (s)		161.3 (s)
3	6.24 (d, 9.4)	113.1 (d)	6.23 (d, 9.4)	112.9 (d)
4	7.63 (d, 9.4)	143.4 (d)	7.63 (d, 9.4)	143.5 (d)
5	7.35 (d, 8.4)	128.7 (d)	7.35 (d, 8.4),	128.7 (d)
6	6.82 (dd, 8.4, 2.4)	114.2 (d)	6.82 (dd, 8.4, 2.4)	113.2 (d)
7		161.9 (s)		162.1 (s)
8	6.79 (d, 2.4)	101.5 (d)	6.79 (d, 2.4)	101.5 (d)
9		155.8 (s)		155.8 (s)
10		112.5 (s)		112.4 (s)
1′	4.57 (d, 6.2)	65.2 (t)	4.59 (d, 6.4)	65.5 (t)
2′	5.50 (brt, 6.2)	122.1 (d)	5.45 (brt, 6.4)	118.4 (d)
3′		137.6 (s)		142.2 (s)
4′	2.45 (brdd, 13.6, 7.6) 2.28 (d, 6.0)	44.9 (t)	2.14−2.06 (2H, m)	39.3 (t)
5′	5.70 (m)	68.9 (d)	2.14−2.06 (2H, m)	26.1 (t)
6′	5.41 (d, 8.8)	123.3 (d)	5.09 (brt, 6.2)	124.2 (d)
7′		141.9 (s)		134.5 (s)
8′	3.99 (brt, 6.4)	75.9 (d)	1.93 (2H, m)	36.0 (t)
9′	2.23 (m)	34.2 (t)	1.68 (2H, m)	27.8 (t)
10′	5.08 (brt, 6.4)	119.5 (d)	4.77 (dd, 10.0, 2.8)	79.5 (d)
11′		135.5 (s)		72.4 (s)
12′	1.62 (brs)	25.9 (q)	1.17 (brs)	24.8 (q)
13′	1.71 (brs)	18.0 (q)	1.18 (brs)	26.7 (q)
14′	1.72 (brs)	13.1 (q)	1.58 (brs)	16.0 (q)
15′	1.81 (s)	17.1 (q)	1.74 (brs)	16.7 (q)
OAc	1.99 (s) (C-5′)	21.2 (q)	2.10 (s) (C-10′)	21.1 (q)
		170.3 (s)		171.2 (s)

m)]. Compound **1** gave the same molecular formula, $C_{26}H_{32}O_6$, and similar 1D NMR data to compound **6**, 8′-acetoxy-5′-hydroxyumbelliprenin,[12] with the only difference being due to the placement of an acetoxy group and a hydroxy group. The key HMBC correlations suggested that the acetoxy and hydroxy groups could be positioned at C-5′ and C-8′, respectively (Figure 1). The HMBC correlation of an oxygenated methylene at δ_H 4.57 (H-1′) with a carbon signal at δ_C 161.9 (C-7) indicated that the sesquiterpene unit is attached to C-7 of the coumarin moiety via an ether linkage. The absolute configuration at C-8′ was not determined due to the small amount of **1** available and also because no previous literature values could be used for comparison. The new compound **1** was therefore assigned as 5′-acetoxy-8′-hydroxyumbelliprenin.

A $[M + H]^+$ ion at m/z 443.2433 ($C_{26}H_{35}O_6$) was present in the HRFABMS of **2**. IR absorptions at 3480, 1731, and (1555 and 1613) cm^{-1} supported the presence of hydroxy, acetoxy, and aromatic ring functions, respectively. UV absorptions at 213 and 321 nm indicated a coumarin nucleus oxygenated at the C-7 position.[12] In the 1D NMR spectrum (Table 1), the signals indicated a carbon skeleton with two main moieties, a coumarin and a sesquiterpene. On the basis of HMBC correlations [δ_H 4.59 (H-1′)/δ_C 162.1 (C-7), 118.4 (C-2′), and 142.2 (C-3′); δ_H 4.77 (H-10′)/δ_C 171.2 (O$\underline{C}$OCH$_3$); δ_H 1.17 (H-12′)/δ_C 79.5 (C-10′), 72.4 (C-11′), and 26.7 (C-13′); δ_H 1.18 (H-13′)/δ_C 79.5 (C-10′), 72.4 (C-11′), and 24.8 (C-12′)], the sesquiterpene unit could be attached to C-7 (δ 162.1) of the coumarin moiety via an ether linkage, and the acetoxy and hydroxy groups were positioned at C-10′ (δ 79.5) and C-11′ (δ 72.4), respectively. Compound **2** was elucidated as 10′-acetoxy-11′-hydroxyumbelliprenin.[24]

To determine the only chiral center at C-10′, we speculated that **2** and 10′-karatavicinol (**5**) possess the same biogenetic origin. Consequently, **5** was treated separately with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*R*)- and (*S*)-MTPA-Cl] in the presence of C_5D_5N, to yield the (*S*)- and (*R*)-MTPA esters (**5a** and **5b**), respectively.[25] The MTPA esters were generated successfully at C-10′ as elucidated from the ^{1}H NMR spectra (**5a**, H-8′ δ 2.15, H-9′ δ 2.15, H-10′ δ 5.49, H-12′ δ 1.37, H-13′ δ 1.70; **5b**, H-8′ δ 2.13, H-9′ δ 2.13, H-10′ δ 5.50, H-12′ δ 1.43, H-13′ δ 1.71). The differences between the ^{1}H NMR chemical shifts for **5a** and **5b** (Δ values shown in Figure 3) led to the assignment of the *R*-configuration at C-10′ of **5**. From a comparison of the specific rotation data of **5** ($[\alpha]^{25}_D$ +16.2) and **2** ($[\alpha]^{25}_D$ +11.2), the absolute configuration of **2** at C-10′ could therefore be assigned with an *R*-configuration. Compound **2** was established as 10′*R*-acetoxy-11′-hydroxyumbelliprenin.

Compound **3** gave the molecular formula $C_{20}H_{26}O_3$, as determined by HRESIMS (m/z 337.1784 $[M + Na]^+$), indicating eight degrees of unsaturation. Its IR spectrum showed absorptions attributable to hydroxy (3417 cm^{-1}), carboxylic acid (1696 cm^{-1}), and aromatic ring (1613 and 1514 cm^{-1}) functions. On the basis of its ^{13}C NMR and DEPT data, **3** showed 20 carbon signals,



3

Figure 2. Important HMBC correlations for compound **3**.

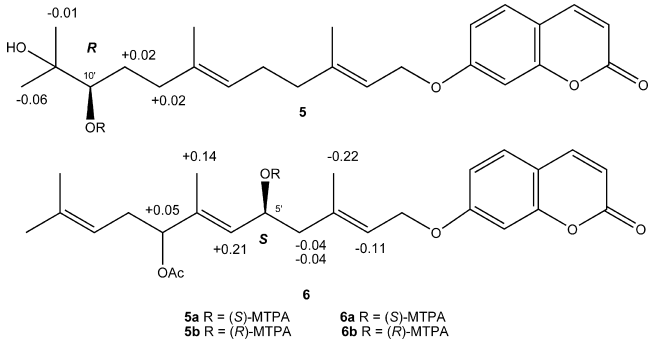


Figure 3. ^{1}H NMR chemical shift differences [$\Delta\delta = \delta$ H$_{(S)\text{-MTPA}} - \delta$ H$_{(R)\text{-MTPA}}$ (ppm)] of the MTPA esters of **5** and **6**.

Table 2. ^{1}H and ^{13}C NMR Data of Compound **3** (400 and 100 MHz in $CDCl_3$, δ in ppm, J in Hz)

position	δ_H (J in Hz)	δ_C
1	2.21 (brd, 11.2)	35.2 (t)
2	1.81 (m)	18.3 (t)
3	1.81 (m)	35.6 (t)
	1.76 (m)	
4		46.0 (s)
5	2.91 (t, 2.8)	46.3 (d)
6	5.81 (dd, 9.6, 2.7)	130.0 (d)
7	6.55 (dd, 9.6, 2.9)	128.3 (d)
8		132.4 (s)
9		145.9 (s)
10		37.1 (s)
11	7.13 (d, 8.4)	121.6 (d)
12	7.30 (dd, 8.4, 2.0)	123.7 (d)
13		146.6 (s)
14	7.17 (d, 2.0)	122.8 (d)
15		72.4 (s)
16	1.57 (brs)	31.6 (q)
17	1.57 (brs)	31.6 (q)
18		183.5 (s)
19	1.40 (s)	17.7 (q)
20	1.08 (s)	20.7 (q)

including four methyls (δ_C 17.7, 20.7, 31.6, 31.6), three methylenes (δ_C 18.3, 35.2, 35.6), six methines (δ_C 46.3, 121.6, 122.8, 123.7, 128.3, 130.0), and seven quaternary (δ_C 37.1, 46.0, 72.4, 132.4, 145.9, 146.6, 183.5) carbons (Table 2). Among the seven quaternary carbons, one was assigned as a carbonyl carbon at δ 183.5. Therefore, the data supported the presence of one carbonyl, four olefins, and three ring moieties to fulfill the eight degrees of apparent unsaturation, and **3** was postulated to be an abietane-type diterpene.[26] The HMBC correlations [δ_H 1.57 (H-16)/δ_C 72.4 (C-15), 31.6 (C-17), and 146.6 (C-13); δ_H 1.57 (H-17)/δ_C 72.4 (C-15), 31.6 (C-16), and 146.6 (C-13); δ_H 1.40 (H-19)/δ_C 35.6 (C-3), 46.0 (C-4), 46.3 (C-5), and 183.5 (C-18)] suggested the hydroxy and carboxylic groups to be located at C-15 (δ 72.4) and C-4 (δ 46.0), respectively (Figure 2). The NOESY correlation between Me-19 and Me-20 as well as the absence of NOESY correlations between H-5/Me-19 and H-5/Me-20 indicated β- and α-orientations, respectively, for Me-20 and H-5 (*trans* A/B ring junction). Compound **3** was therefore elucidated as 15-hydroxy-6-en-dehydroabietic acid. To the best of our knowledge, this is the first report of a diterpene with an abietane skeleton from the genus *Ferula*.

Table 3. Influenza A (H_1N_1) Antiviral Activity for Compounds **2** and **4**−**13**

compound	IC_{50} (μg/mL)	IC_{90} (μg/mL)
2	0.94 ± 0.07	1.98 ± 0.12
4	0.36 ± 0.03	0.62 ± 0.08
5	0.99 ± 0.07	2.05 ± 0.14
6	0.81 ± 0.06	1.56 ± 0.11
7	0.26 ± 0.03	0.44 ± 0.05
8	0.45 ± 0.04	0.88 ± 0.07
9	0.29 ± 0.02	0.50 ± 0.07
10	0.51 ± 0.04	0.64 ± 0.07
11	0.47 ± 0.05	0.83 ± 0.09
12	0.86 ± 0.08	1.75 ± 0.12
13	0.32 ± 0.02	0.37 ± 0.03
amantadine	0.92 ± 0.04	1.73 ± 0.11

On the basis of the work of Nittala et al.,[26] the chiral center of the known 5′-hydroxyumbelliprenin (**4**) is similar to that of 12,13-didehydrofurospongin-1. The absolute configuration, the *S* form, of 12,13-didehydrofurospongin-1 was determined by the Mosher method. From a comparison of their specific rotation data {12,13-didehydrofurospongin-1 ($[\alpha]^{28}_D$ −8); **4** ($[\alpha]^{25}_D$ −3.8)}, the absolute configuration of **4** at C-5′ was assigned the *S* form. Compound **4** is accordingly 5′*S*- hydroxyumbelliprenin.

The absolute configuration of the known 8′-acetoxy-5′-hydroxyumbelliprenin (**6**)[12] at C-5′ was still unknown at the time of the present study. Compound **6** was treated separately with (*R*)- and (*S*)-MTPA-Cl in C_5D_5N to yield the (*S*)- and (*R*)-MTPA esters (**6a** and **6b**), respectively. The MTPA esters were generated successfully at C-5′, as elucidated from the ^{1}H NMR spectra (**6a**, H-2′ δ 5.58, H-4′a δ 2.37, H-4′b δ 2.31, H-5′ δ 6.20, H-6′ δ 5.70, H-8′ δ 5.35, H-14′ δ 1.93, H-15′ δ 1.72; **6b**, H-2′ δ 5.69, H-4′a δ 2.41, H-4′b δ 2.35, H-5′ δ 6.13, H-6′ δ 5.49, H-8′ δ 5.30, H-14′ δ 1.79, H-15′ δ 1.94). The differences between the ^{1}H NMR chemical shifts for **6a** and **6b** (Δ values shown in Figure 3) led to the assignment of the *S*-configuration at C-5′, and **6** was determined as 8′-acetoxy-5′*S*-hydroxyumbelliprenin.

Sesquiterpene coumarin ethers (7-hydroxycoumarin analogues) have been reported as possessing various biological activies, including potent NF-κB inhibition [8′-acetoxy-5′*S*-hydroxyumbelliprenin (**6**)],[22] antibacterial activity [galbanic acid (**8**)],[27] a potential cancer chemopreventive effect [farnesiferol C (**9**)],[28] squalene-hopene cyclase inhibition [karatavicinol (**5**) and farnesiferol C (**9**)],[29] and human rhinovirus coat protein inhibition [karatavicinol (**5**) and farnesiferol **B**].[30] Since 7-hydroxycoumarin analogues showed good anti-HIV activity in previous studies,[3,22] the pure 7-*O*-sesquiterpene coumarins, compounds **2** and **4**−**6** (acyclic), **7**−**9** (monocyclic), and **10**−**13** (bicyclic), were screened in an in vitro anti-influenza A viral (H_1N_1) assay, with amantadine as a positive control (Table 3). Most of these compounds exhibited a higher antiviral potency than amantadine, except for **2**, **5**, and **12**. These results are of interest because the structures of many of these compounds are quite similar. For example, **6** (OH-5′, OAc-8′) has an additional acetoxy group in comparison with **4** (OH-5′), but **6** was much less potent than **4**. Compound **2** (OAc-10′) has the same skeleton as **5** (OH-10′), and these two compounds showed a similar potency to the positive control, amantadine. Between compounds **7** ($COOCH_3$-3′) and **8** (COOH-3′), **7** showed better potency than **8** and, therefore, indicated that methyl esterification of C-3′ enhanced the activity in the bioassay used. For the bicyclic-sesquiterpene coumarins, **12** and **13**, a C-3′-carbonyl afforded greater potency than a C-3′-OH in this kind of skeleton. Overall, the present study has determined that sesquiterpene coumarins from *F. assa-foetida* may serve as

promising lead compounds for new drug development against influenza A (H_1N_1) viral infection. A standardized plant extract of *F. assa-foetida*, a species that has been used to treat influenza for many years, may also be worthy of being further investigated as a new phytomedicine.

Furthermore, the pure compounds were screened in a cytotoxicity assay with doxorubicin as the positive control. The minor compound **11** exhibited the best potency (IC_{50} 0.51, 2.6, and 3.4 μg/mL) against HepG2, Hep3B, and MCF-7 tested cancer cell lines, respectively. Interestingly, compound **11** also showed high potency influenza activity (IC_{50} 0.47 ± 0.05 μg/mL). The remaining compounds were all inactive for all cancer cell lines (IC_{50} > 4 μg/mL; Table S1, Supporting Information).

Experimental Section

General Experimental Procedures. Optical rotations were taken on a JASCO-P-1020 polarimeter (cell length 10 mm). UV spectra were measured on a JASCO V-530 UV/vis spectrophotometer. IR spectra were recorded on a Mattson Genesis II FT-IR spectrophotometer. NMR spectra were recorded on Varian Gemini-20000 (200 MHz), Varian Unity-plus (400 MHz), and Varian Unity-plus (600 MHz) FT-NMR NMR spectrometers. Chemical shift (δ) values are in ppm (parts per million), with $CDCl_3$ as internal standard, and coupling constants (J) are in Hz. HRFABMS, HRESIMS, and ESIMS measurements were performed on JEOL JMS-700, Bruker APEX II, and Finnigan PO-LARISQ mass spectrometers. TLC was performed on Kieselgel 60, F_{254} (0.20 mm, Merck), and spots were viewed under UV light at 254 and 356 nm and/or stained by spraying with 50% H_2SO_4 and heating on a hot plate. For column chromatography, silica gel (Kieselgel 60, 70−230 and 230−400 mesh, Merck) and Sephadex LH-20 were used. The instrumentation for the RP-MPLC experiment was composed of a Supelco VersaFlash flash chromatography apparatus and VersaFlash C-18 cartridges (40 × 150 mm). Further purification of some compounds obtained was achieved by preparative HPLC, using a Shimadzu LC-10AT*vp*/Shimadzu SCL-10A*vp* UV−vis detector, and Thermo columns (analytical: 5 μm, 250 × 4.6 mm; preparative: 8 μm, 250 × 10 mm; C_{18}) were used. For the preparation of Mosher ester derivatives, (*S*)-(+)- and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride were used as the reagents.

Plant Material. *Ferula assa-foetida* resin (3.23 kg) was purchased from a Chinese herb shop in Taipei, Taiwan, in November 2006 and identified by one of the authors (M.H.A.R.). A voucher specimen (FA200611) was deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. *F. assa-foetida* resin (3.23 kg) was extracted four times with MeOH (5 L each) at room temperature to obtain a crude extract (313.8 g). The crude extract was partitioned between *n*-hexane−MeOH (1:1), and then the MeOH extract (120.1 g) was partitioned between $CHCl_3$−H_2O (1:1), to obtain a $CHCl_3$ extract (65.2 g), which showed significant anti-H_1N_1 antiviral and cytotoxic activities. Initial fractionation of the $CHCl_3$ extract (65.2 g) was carried out by open column chromatography on silica gel (230−400 mesh, column: 7 × 30 cm), using gradients of *n*-hexane−$CHCl_3$−MeOH (100:0:0 to 0:80:20), and gave 11 fractions. A precipitation was obtained from the first chromatographic step, using *n*-hexane as the eluent, and was washed with a trace amount of *n*-hexane to afford oleic acid (2.0 g).

Fraction 3 (6.66 g) was fractionated into 15 fractions by silica gel chromatography (70−230 mesh, column: 5 × 23 cm; $CHCl_3$). Subfraction 3-6 (602.06 mg) was subjected to silica chromatography (70−230 mesh, column: 3 × 20 cm; *n*-hexane−EtOAc, 1:1) and purified by preparative TLC ($CHCl_3$) to give **9** (1.9 mg). Subfraction 3-7 (619.90 mg) was purified by solid-phase extraction (SPE) (DSC-18, 60 mL, 10GMS, No. 52609-U; MeOH−H_2O, 80:20) to obtain vanillin (23.5 mg).

Fraction 4 (11.78 g) was separated into eight subfractions by column chromatography on silica gel (70−230 mesh, column: 7 × 24 cm; *n*-hexane−EtOAc, 1:1), and crystalline β-sitosterol (0.30 g) was obtained with MeOH. Subfraction 4-6 was subjected to silica gel chromatography (70−230 mesh, column: 5 × 17 cm; $CHCl_3$−MeOH, 60:1) to give six subfrations. Subfraction 4-6-2 was purified with HPLC (ODS Thermo, 10 × 250 mm; MeOH−H_2O, 78:22; flow rate: 3.0 mL/min) to give lehmannolone (1.7 mg, t_R = 31 min). Subfraction 4-6-4 was purified by preparative TLC ($CHCl_3$) to give **4** (5.7 mg) and lehmferin (2.8 mg). Subfraction 4-6-5 (400 mg) was chromatographed on a silica gel column (70−230 mesh, column: 2.5 × 28 cm), using *n*-hexane−CH_2Cl_2 (2:7) as eluent, and subfraction 4-6-5-1 (253.30 mg) was purified subsequently with *n*-hexane−EtOAc (7:2), to obtain seven subfractions. Subfractions 4-6-5-1-2 (9.5 mg), 4-6-5-1-3 (68.7 mg), and 4-6-5-1-6 (21.9 mg) were purified by RP-HPLC (flow rate: 3.0 mL/min) to give fetidone B (2.2 mg, MeOH−H_2O, 70:30, t_R = 14 min), a farnesiferol B and assafoetidin mixture (16.4 mg, MeOH−H_2O, 63:37, t_R = 123 min), feselol (3.3 mg, MeOH−H_2O, 63:37, t_R = 136 min), and **1** (2.3 mg, MeOH−H_2O 70:30, t_R = 34 min), respectively. Subfractions 4-6-5-2 (43.4 mg) and 4-6-7 (354.7 mg) were purified by RP-HPLC (MeOH−H_2O, 76:24; flow rate: 3.0 mL/min) to give **10** (8.9 mg, t_R = 38 min) and **11** (6.2 mg, t_R = 41 min), and **7** (58.9 mg, t_R = 27 min), **8** (15.7 mg, t_R = 31 min), and 7-oxocallitrisic acid (1.5 mg, t_R = 53 min), respectively. Subfraction 4-7 (12.40 g) was separated by silica gel chromatography (70−230 mesh, column: 5 × 25 cm; $CHCl_3$−MeOH, 60:1) into six subfractions. Subfraction 4-7-4 (746.80 mg) was subjected to silica gel chromatography (70−230 mesh, column: 3 × 20 cm; *n*-hexane−EtOAc, 5:2) and purified by RP-HPLC (MeOH−H_2O, 75:25; flow rate: 3.0 mL/min) to give **2** (19.7 mg, t_R = 24 min), **6** (57.0 mg, t_R = 34 min), and **13** (11.6 mg, t_R = 12 min). The $CHCl_3$/MeOH-insoluble umbelliferone (3.4 mg) was obtained from subfraction 4-8-7 (58.2 mg).

Fraction 5 (11.77 g) was chromatographed over silica gel (70−230 mesh, column: 5 × 30 cm; $CHCl_3$−MeOH, 50:1), with subfraction 5-2 (334.30 mg) then subjected to RP-MPLC (MeOH−H_2O, 65:35), to give five subfractions. Subfraction 5-2-1 was purified by RP-HPLC (MeOH−H_2O, 61:39; flow rate: 3.0 mL/min) to give foetisulfide A (1.5 mg, t_R = 43 min). Subfraction 5-3 (255.2 mg) was subjected to RP-MPLC (MeOH−H_2O, 70:30) and subfraction 5-3-3 (112.3 mg) was purified by silica gel chromatography (70−230 mesh, column: 2.5 × 30 cm; CH_2Cl_2−MeOH, 40:1) to obtain kamolonol (14.3 mg). Subfraction 5-3-4 (351.4 mg) was chromatographed by RP-HPLC (MeOH−H_2O, 65:35; flow rate: 3.0 mL/min) to give microlobin (30.4 mg, t_R = 73 min), and then the mother liquor was purified by RP-HPLC (MeOH−H_2O, 53:47; flow rate: 3.0 mL/min) to give **12** (21.3 mg, t_R = 64 min) and foetisulfide C (3.6 mg, t_R = 50 min). Subfractions 5-3-5 and 5-3-6 were chromatographed over silica gel (70−230 mesh, column: 1.5 × 25 cm; CH_2Cl_2−MeOH, 60:1 and 50:1) to give picealactone C (0.7 mg) and **5** (241.7 mg). Subfraction 5-5 (352.3 mg) was chromatographed by RP-HPLC (MeOH−H_2O, 72:28; flow rate: 3.0 mL/min) to give **3** (7.0 mg, t_R = 16 min).

Fraction 6 (4.33 g) was chromatographed over silica gel (70−230 mesh, column: 7 × 35 cm; CH_2Cl_2−MeOH, 25:1) to give five subfractions. The third subfraction was purified with RP-MPLC (MeOH−H_2O, 55:45) to give polyanthinin (23.4 mg).

5′-Acetoxy-8′-hydroxyumbelliprenin (1): yellow oil; $[\alpha]^{25}_D$ +8.6 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 211 (4.54), 318 (3.95) nm; IR (neat) ν_{max} 3440, 1729, 1613, 1555, 1233, 836 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$, 400 MHz), see Table 1; ESIMS *m/z* 463 $[M + Na]^+$; HRESIMS *m/z* 463.2099 $[M + Na]^+$(calcd for $C_{26}H_{32}O_6Na$, 463.2096).

10′*R*-Acetoxy-11′-hydroxyumbelliprenin (2): yellow oil; $[\alpha]^{25}_D$ +11.2 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 213 (4.77), 321 (4.39) nm; IR (neat) ν_{max} 3480, 1731, 1613, 1555, 1234, 836 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$, 400 MHz), see Table 1; ESIMS *m/z* 465 $[M + Na]^+$; HRFABMS *m/z* 443.2433 $[M + H]^+$ (calcd for $C_{26}H_{34}O_6$ + H, 443.2434).

15-Hydroxy-6-en-dehydroabietic acid (3): yellow oil; $[\alpha]^{25}_D$ −14.6 (*c* 0.16, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 243 (3.95), 271 (3.74) nm; IR (neat) ν_{max} 3417, 1696, 1613, 1514 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$, 400 MHz), see Table 2; ESIMS *m/z* 337 $[M + Na]^+$; HRESIMS *m/z* 337.1784 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_3Na$, 337.1780).

Anti-influenza A Virus (H_1N_1) Bioassay.[31,32] Madin-Darby canine kidney (MDCK) cells (ATCC CCL34) were used as target cells for viral infection in the XTT (tetrazolium hydroxide salt) assay. They were grown as adherent cells in MEM medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. In the antiviral assay, the medium was supplemented with 2% FCS and the above-mentioned antibiotics. Virus titers were determined by the cytopathic effect in MDCK cells and expressed as 50% tissue culture infective dose ($TCID_{50}$) values per mL. All viruses were stored at −70 °C until use. The antiviral activity against influenza A virus (H_1N_1) was evaluated by the XTT method. MDCK cells, treated by trypsin, were seeded onto 96-well

plates with a concentration of 1.0×10^5 cells per mL and a volume of 70 μL per well. After incubation at 35 °C with 5% CO_2 for 24 h, 20 μL of test virus solution was added and incubated for another 1 h. Different concentrations of test substances were then added to culture wells in triplicate. Amantadine was used as a positive control. After incubation at 35 °C with 5% CO_2 for 3 days, XTT reagent was added and incubated for 3 h. The viral inhibition rate (%) was calculated as $[100 - (OD_{492}/OD_{690}) \times 100]\%$. The antiviral concentration of 50% inhibition (IC_{50}) was defined as the concentration achieving 50% cytoprotection against virus infection.

Cytotoxicity Bioassays. Fractions and isolates were tested against lung (A549), breast (MEA-MB-231 and MCF7), liver (HepG2 and Hep3B), and oral (Ca9-22) human cancer cell lines using an established colorimetric MTT (diphenyltetrazolium bromide) assay protocol.[33] Doxorubicin was used as a positive control. In brief, freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000−10 000 cells per well with test compounds added from a DMSO stock solution. After 3 days in culture, the attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using an ELISA reader. The IC_{50} is the concentration of agent that reduced cell growth by 50%, under the experimental conditions used.

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Supporting Information Available: ^1H, ^{13}C, HSQC, HMBC, COSY, and NOESY spectra of compounds **1**−**3** along with cytotoxicity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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