Three New Taxane Diterpenoids from *Taxus sumatrana*

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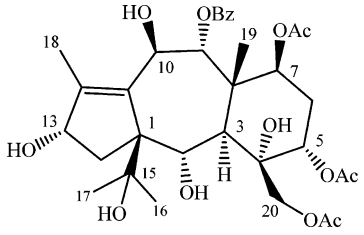
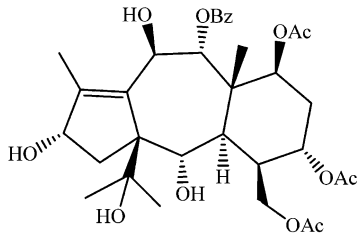
Phytochemical investigation of taxane diterpenoidal content of an acetone extract of the leaves and twigs of *Taxus sumatrana* has resulted in the isolation of three new taxoid compounds, tasumatrols E (**1**), F (**2**), and G (**3**) together with 13 known taxanes (**5**−**16**). The structures of these taxanes as well as their derivatives were established on the basis of spectroscopic analyses, especially 1D and 2D NMR. Compounds **1**, **2**, **12**, and **16** exhibited significant cytotoxicity against human A-498, NCI-H226, A549, and PC-3 tumor cells.

The chemical constituents of *Taxus* spp. have been extensively investigated as a result of the discovery of paclitaxel (Taxol) with its remarkable antitumor activity.[1−3] Paclitaxel and its semisynthetic analogue docetaxel possess a unique antimitotic mechanism of action, acting as inducers for the polymerization of tubulin α,*β*-heterodimers and stabilizers of the resulting microtubule polymer. Both taxoids exhibited antitumor activity against various types of advanced cancer, e.g., ovarian, breast, head-and-neck, and lung solid cancers, which cannot effectively be treated by existing antitumor drugs.[4−6] In continuation of our research on the taxane diterpenoids of endemic *Taxus* species,[7−15] a phytochemical study of taxane diterpenoidal content of *T. sumatrana* was carried out. A meticulous chromatographic fractionation of an acetone extract of the leaves and twigs of the title plant has resulted in the isolation of three new taxoid diterpenes, tasumatrols E (**1**), F (**2**), and G (**3**). During the course of fractionation, 13 known taxanes were identified as taxinin M (**5**), 2α,5α,-dihydroxy-7*β*,9α,10*β*,13α-tetraacetoxy-4(20),11-taxadiene (**6**), 2α-deacetyl-5α-decinnamoyltaxagifin (**7**), wallifoliol (**8**), 10-deacetylbaccatin III (**9**), 10,13-deacetyl-*abeo*-baccatin IV (**10**), 10-deacetyl-13-oxobaccatin III (**11**), 19-hydroxy-13-oxobaccatin III (**12**), 19-hydroxy-10-deacetylbaccatin III (**13**), 19-hydroxybaccatin III (**14**), taxayuntin G (**15**), and 10-deacetyl-10-oxobaccatin III (**16**). The structures of the isolated taxanes as well as their acylated derivatives were established on the basis of spectroscopic analyses, especially 1D and 2D NMR.



1



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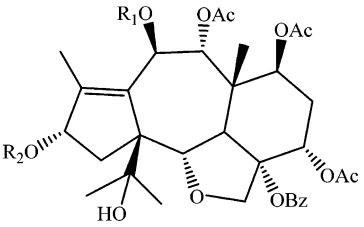

3 $R_1 = R_2 = H$
4 $R_1 = R_2 = Ac$

The FABMS of **1** revealed a molecular ion peak at *m/z* 649 $[M + 1]^+$, consistent with the molecular formula $C_{33}H_{44}O_{13}$ and 12 degrees of unsaturation. The IR spectrum displayed absorption bands diagnostic of hydroxyl, double bonds, and ester groups. Both ^1H and ^{13}C NMR spectrocopic data (Tables 1 and 2) indicated the presence of three *O*-acetyl units at δ_H 2.18, 2.13, and 1.99 (each 3H, s), δ_C 21.5, 21.3, and 20.9, and δ_C 171.1, 170.8, and 170.2. A benzoyl ester was evident from signals resonating at δ_C 165.7, 134.5, 129.6, 128.3, and 133.6 and δ_H 7.99 (d, J = 7.2 Hz), 7.43 (t, J = 7.2 Hz), and 7.56 (d, J = 7.2 Hz). The MS data indicated the existence of a diterpene with three rings and one double bond, as verified by two olefinic carbons at δ_C 139.9 (C-11) and 145.4 (C-12). The ^1H NMR spectrum revealed six oxygenated methines at δ 4.65 (d, J = 10.3 Hz, H-10), 4.71 (d, J = 6.3 Hz, H-2), 4.75 (m, H-13), 5.09 (t, J = 2.5 Hz, H-5), 5.44 (dd, J = 11.5, 5.5 Hz, H-7), and 5.84 (d, J = 10.3 Hz, H-9). The relative low-field chemical shift of the latter three protons implied their acylation that was supported by their HMBC correlations with their respective carbonyl carbons of the attached ester (Figure 1). The COSY experiment displayed connectivities

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Table 1. ^{1}HMR Data ($CDCl_3$, 300 MHz) of Compounds **1**−**3**[a,b]

position	1	2	3
2	4.71 d (6.3)	4.73 d (7.8)	5.22 d (10.5)
3	2.82 d (6.3)	2.68 m	2.74 d (10.5)
4		2.32 m	
5	5.09 t (2.5)	5.06 br s	4.93 br s
6	1.92 m	1.90 m	1.44
7	5.44 t (11.5,5.5)	5.36 t (7.8)	5.27 d (3.9)
9	5.84 d (10.3)	5.74 d (10.2)	5.14 d (4.8)
10	4.65 d (10.3)	4.82 d (10.2)	4.91 d (4.8)
13	4.75 m	4.59 m	4.41 m
14	2.01 m	2.20 m	2.14 m
	1.72 m	1.70 m	1.11 m
CH_3-16	1.50 s	1.45 s	1.04 s
CH_3-17	1.26 s	1.07 s	1.43 s
CH_3-18	1.65 s	1.75 s	1.42 s
CH_3-19	1.27 s	1.13 s	1.49 s
20	4.61 d (10.5)	4.48 d (10.8)	4.63 d (8.1)
	4.20 d (10.5)	4.06 d (10.8)	4.58 d (8.1)
OAc-5	2.18 s	2.17 s	2.05 s
OAc-7	2.13 s	2.08 s	2.11 s
OAc-9			1.93 s
OAc-20	1.99 s	2.10 s	
COC_6H_5			
o-	7.99 d (7.2)	8.01 d (7.5)	8.01 d (7.8)
m-	7.43 t (7.2)	7.43 t (7.5)	7.50 t (7.8)
p-	7.56 d (7.2)	7.56 t (7.5)	7.63 d (7.8)

[a] Chemical shifts in ppm, *J* values in Hz are in parentheses. [b] Assignments were made using HMQC and HMBC techniques.

Table 2. ^{13}C NMR Data ($CDCl_3$, 75 MHz) of Compounds **1**−**3**[a]

carbon	1	2	3
1	69.5 s	68.9 s	65.7 s
2	65.5 d	65.6 d	71.8 d
3	45.1 d	41.0 d	36.4 d
4	77.0 s	41.3 d	81.1 d
5	71.4 d	70.4 d	72.2 d
6	29.0 t	29.6 t	29.1 t
7	69.7 d	69.6 d	69.4 d
8	43.5 s	44.9 s	43.5 s
9	81.0 d	82.1 d	76.4 d
10	67.4 d	68.2 d	67.2 d
11	139.9 s	138.1 s	134.0 s
12	145.4 s	145.4 s	147.6 s
13	76.4 d	77.0 d	75.5 d
14	39.0 t	39.9 t	41.0 t
15	74.5 s	76.5 s	75.8 s
CH_3-16	27.4 q	27.8 q	26.9 q
CH_3-17	28.4 q	28.2 q	27.2 q
CH_3-18	11.6 q	11.2 q	12.1 q
CH_3-19	15.9 q	14.8 q	14.6 q
20	62.6 t	63.9 t	70.2 t
OAc-5	170.2 s	170.2 s	169.3 s
	20.9 q	21.1 q	20.0 q
OAc-7	170.8 s	170.3 s	169.0 s
	21.3 q	21.2 q	20.1 q
OAc-9			169.0 s
			21.4 q
OAc-20	171.1 s	171.7 s	
	21.5 q	21.9 q	
COC_6H_5	165.7 s	167.0 s	164.9 s
i-	134.5 s	134.7 s	134.0 s
o-	129.6 d	129.8 d	129.8 d
m-	128.3 d	128.4d	128.5 d
p-	133.6 d	133.2 d	133.1 d

[a] Assignments were made using HMQC and HMBC techniques.

between H-13/H-14, H-2/H-3, H-5/H-6/H-7, and H-9/H-10. The low-field sp^3 quaternary carbon at δ 77.0, which showed HMBC correlations with H-2, H-5, and H-6, was attributed to the hydroxyl-bearing C-4. The oxygenated methylene protons at δ 4.61 and 4.20 (each 1H, d, $J = 10.5$ Hz) that are attached to C-4 were assigned as H_2-20. These two protons showed HMBC correlations with C-5, C-3, and the acetate carbonyl carbon at δ_C 171.1. The four methyl

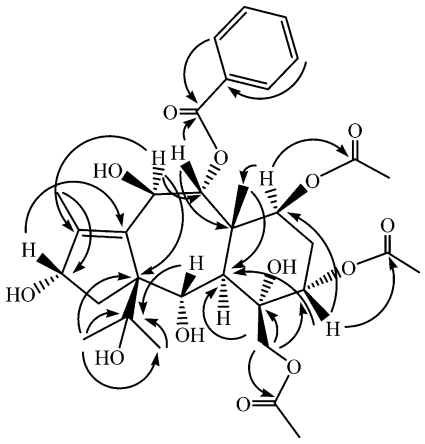

Figure 1. Selected HMBC correlations of **1**.

singlets at δ_H 1.26, 1.27, 1.50, and 1.65 (each 3H) and their corresponding carbon signals at δ_C 11.6, 15.9, 27.4, and 28.4 together with two quaternary carbons at δ_C 69.5 (C-1) and 74.5 (C-15) are characteristic of an 11(15→1)-*abeo*-taxane skeleton.[7,10,16] The presence of an 11(15→1)-*abeo*-taxane skeleton bearing a dimethyl carbinol group at C-1 was confirmed by HMBC correlations between H-17/C-1, C-15, C-16; H-14/C-15 and H-2/C-15 (Figure 1). The relative configuration of **1** was determined through comparing ^{1}H NMR data as well as the optical rotation with those of related taxane diterpenes and from its NOESY spectrum, which showed correlations between H-3α/H-7, H-19β/H-9, H-9/H-2, H-2/H-20, and H-20/H-5. Hence, the structure of **1** was established as tasumatrol E.

The HREIMS of **2** revealed the molecular formula $C_{33}H_{44}O_{12}$, with one oxygen atom less than that of **1**. The ^{13}C NMR of **2** (Table 2) displayed four methyls (δ_C 27.8, 28.2, 11.2, 14.8), two quaternary olefinic carbons (δ_C 138.1 and 145.4, C-11 and C-12), a quaternary oxygenated carbon at δ_C 76.5 (C-15), and an oxygenated CH_2 (δ_C 63.9, C-20) in addition to signals for three acetates and one benzoyl ester. Six oxygenated methines were observed at δ_C 65.6, 70.4, 69.6, 82.1, 68.2, and 77.0, and their corresponding protons at δ_H 4.73, 5.06, 5.36, 5.74, 4.82, and 4.59 assignable to positions 2, 5, 7, 9, 10, and 13 were indicative of an 11(15→1)-*abeo*-taxane. Comparative inspection of the ^{1}H and ^{13}C NMR spectrocopic data of **1** and **2** (Tables 1 and 2) indicated that they are almost identical except for the remarkable upfield shift of a methine signal assigned to C-4 (δ_C 41.3 instead of a quaternary carbon at δ_C 77.0 in the case of **1**) together with a less profound high-field shift of C-3 (δ_C 41.0 instead of δ_C 45.1). This was accompanied by emergence of a proton signal at δ_H 2.32, which showed HMQC correlation to C-4 at δ_C 41.0. The proton at δ_H 2.32 (H-4) exhibited COSY correlations with H-3 and H-5 in addition to HMBC correlations to C-2, C-5, and C-20. On the other hand, the NOESY spectrum of **2** showed cross-peaks between H-19/H-9; H-2/H-9, H-16, H-19; H-3/H-4, H-7; and H-20/H-5, H-19. The spectroscopic data of **2** disclosed the presence of an 11(15→1)-*abeo*-taxane structure similar to that of **1** but devoid of the hydroxyl group at C-4. On the basis of these findings, the structure of **2** was determined as tasumatrol F.

The molecular formula of **3** was determined as $C_{33}H_{42}O_{12}$, implying one double bond/ring more than **2**. The NMR data of **3** were similar to those of **1** (Tables 1 and 2), indicating an 11(15→1)-*abeo*-taxane skeleton with three acetate and one benzoyl esters. The three acetoxy moieties were attached to positions 5, 7, and 9, as verified by HMBC correlations between the CH signals at δ_H 4.93, 5.27, and

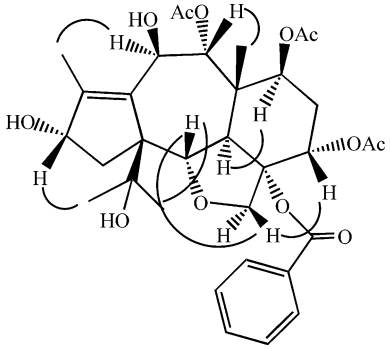

Figure 2. Selected NOESY correlations of **3**.

5.14 with their respective acetyl carbonyls at δ_C 169.3, 169.0 (double intensity). The carbonyl of the benzoyloxy group at δ_C 164.9 did not show any correlation with the protons of the taxane ring system, indicating the attachment of the benzoyloxy group to a quaternary carbon, at either C-4 or C-15. Provided that there was no significant change of the C-15 signal when compared with **1** and **2**, it was deduced that the benzoyl ester was attached to C-4. The two oxygenated geminal protons resonating at δ_H 4.63 and 4.58 (each 1H, d, $J = 8.1$ Hz) attached to the methylene carbon at δ_C 70.2 were assigned to position 20. In the HMBC spectrum of **3**, cross-peaks were observed between H-20 and both C-5 and C-2, suggesting the presence of an oxygen bridge between C-20 and C-2, and hence accounting for a 2(20)-tetrahydrofuran moiety as an additional ring. As reported for related compounds[13,14,17] and inspection of a molecular model, the formation of the latter ring affects orientation of the substituents at both C-9 and C-10 so as to form a dihedral angle of 45° between H-9 and H-10. As a result, an unexpected decrease in the geminal $J_{9,10}$ coupling constant from ca. 11 Hz to 4.8 Hz occurred. This was consistent with the results of NOESY experiments (Figure 2), which showed that H-9 and H-10 retained their β- and α-configurations, respectively. The significantly downfield-shifted quaternary carbon at δ_C 81.1 exhibited long-range correlations with H-2 and H-5; hence it was conveniently assigned to C-4, to which a benzoyloxy group was attached. This was supported by a NOESY correlation between H-2 (δ_H 5.22) and *o*-protons of the benzoyl ester at δ_H 8.01. Acetylation of **3** provided a diacetate **4** whose ^{1}H NMR data (Experimental Section) confirmed the proposed structure. The newly introduced acetates caused a noticeable downfield shift of the signals at δ 4.91 (H-10) and 4.41 (H-13) to δ 5.96 and 5.51, respectively, in addition to the emergence of two *O*-acetyl methyls at δ 2.27 and 1.99. On the basis of these data, the structure of **3** was elucidated as tasumatrol G.

Compounds **1** and **2** belong to taxoids of the taxchin type, which may be biogenetically derived from taxoids of the C-4(20) double bond.[18] Compound **2** is probably generated by the oxidation of **1** at C-4. The occurrence of **1**−**3** may be of great significance in terms of the biosynthetic pathway. The cytotoxicities of the isolated taxoids were tested in vitro against human A549, NCI-H226, A549, and PC-3 tumor cells. Taxol was used as a standard compound. As illustrated in Table 3, compound **1** showed more promising activity than Taxol against these four tumor cell lines. Compound **2** was more active toward A498 and PC-3 tumor cells. In addition, compounds **12** and **16** exhibited significant cytotoxicity against A498, NCI-H226, and PC-3 tumor cells.

Table 3. Results of Cytotoxic Activity (30 μg/mL) of the Isolated Compounds against A498, NCI-H226, A549, and PC-3 Cell Lines[a]

compound	A498	NCI-H226	A549	PC-3
1	100	84.8	91.3	94.7
2	83.0	78.5	72.6	95.0
3	15.3	78.9	24.1	58.9
5	24.3	11.1	14.6	0
8	27.9	29.1	16.7	6.4
9	27.0	5.7	12.2	1.6
10	21.7	19.9	27.1	1.9
11	29.7	49.2	43.9	65.3
12	79.8	84.7	45.4	88.2
13	16.6	32.0	0	2.3
14	26.7	44.6	0	47.7
15	21.2	29.3	0	1.4
16	79.1	97.3	54.7	100
Taxol	98.2	71.2	79.7	91.7

[a] % inhibition against A498 (renal cancer), NCI-H226 (non-small cell lung cancer), A549 (non-small cell lung cancer), and PC-3 (prostate cancer).

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR and UV spectra were measured on Hitachi T-2001 and U-3210 spectrophotometers, respectively. Low-resolution EIMS and FABMS spectra were recorded on a VG Quattro 5022 mass spectrometer. The ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC, and NOESY spectra were recorded on a Bruker FT-300 spectrometer or on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively, using TMS as internal standard. The chemical shifts are given in δ (ppm) and coupling constants in Hz. Silica gel 60 (Merck) was used for column chromatography (CC), and precoated silica gel plates (Merck, Kieselgel 60 F-254, 1 mm) were used for preparative TLC. Sephadex LH-20 (Amersham Pharmacia Biotech AB, Sweden) was used for purification and/or separation.

Plant Material. The leaves and twigs of 7-year-old trees of *Taxus sumatrana* (Miq.) de Laub. were collected from Kaohsiung County, Taiwan, at an altitude of 1000 m in March 2002. This plant was identified by one of the authors (C.-T.C.). A voucher specimen (TPG 8-7) was kept in the Institute of Marine Resources, National Sun Yat-sen University, Kaohsiung, Taiwan.

Extraction and Isolation. Dried leaves and twigs (15.5 kg) were ground and extracted three times with acetone at room temperature. The combined extracts were filtered and concentrated under vacuum to obtain a crude residue (3.05 kg). The residue was stirred with H_2O, and the resulting emulsion was partitioned between EtOAc/H_2O (1:1) to produce an EtOAc extract (173 g) and an aqueous layer that was further extracted with *n*-BuOH to furnish the *n*-BuOH extract (142 g). The EtOAc extract was fractionated on Sephadex LH-20 using MeOH into fractions A (60 g) and B (86 g). Fraction A was chromatographed on an NP-silica gel column using *n*-hexane/CH_2Cl_2/MeOH (100:100:1 to 1:1:1) to yield 10 fractions A-1 to A-10. Part (120 mg) of the obtained A-2 (788 g) was subjected to RP_{18}-preparative TLC using H_2O/MeOH/CH_3-CN (30:65:5) to yield taxinin M (**5**, 27.6 mg). Fraction A-3 (3.1 g) was chromatographed on a RP_{18}-silica gel column using a gradient of H_2O/MeOH/CH_3CN (70:25:5, 60:35:5, 50:45:5, 40:55:5, 30:65:5, and 20:75:5, each 500 mL) to give seven fractions, A-3-B-1 to A-3-B-7. Fraction A-3-B-2 (630 mg) was fractionated on a column packed with Sephadex LH-20 using MeOH to furnish 2α,5α-dihydroxy-7β,9α,10β,13α-tetraacetoxy-4(20),11-taxadiene (**6**, 1.2 mg) and 2α-deacetyl-5α-decinnamoyltaxagifin (**7**, 1.2 mg), while NP-HPLC of A-3-B-6 (280 mg) using *n*-hexane/CH_2Cl_2/MeOH (18:18:1) yielded another amount (2 mg) of taxinin M (**5**). Fraction A-5 was chromatographed on an RP_{18}-silica gel column using the previously mentioned gradient of H_2O/MeOH/CH_3CN in the case of A-3 to give six

fractions, A-5-C-1 to A-5-C-6. Crystallization of fraction A-5-C-3 from MeOH produced needle-shaped crystals of 10,13-deacetyl-*abeo*-baccatin IV (**10**, 21 mg), while crystallization of A-5-C-6 yielded rod-shaped crystals of wallifoliol (**8**, 103 mg). Fraction A-7 was further chromatographed on an RP_{18}-silica gel column using the previously mentioned gradient of H_2O/MeOH/CH_3CN to give six fractions, A-7-D-1 to A-7-D-6. Part (50 mg) of fraction A-7-D-4 (1050 mg) was fractionated on NP-HPLC using *n*-hexane/CH_2Cl_2/MeOH (10:10:1) to afford 19-hydroxybaccatin III (**14**, 30 mg), while treatment of fraction A-7-D-5 in the same way furnished 10-deacetyl-10-oxobaccatin III (**16**, 6 mg) and tasumatrols E (**1**, 7 mg), F (**2**, 8 mg), and G (**3**, 7 mg). Separation of fraction A-8 as mentioned in the case of A-3 yielded six fractions, A-8-E-1 to A-8-E-6. Part (50 mg) of fraction A-8-E-5 (3.96 g) was subjected to NP-HPLC using *n*-hexane/CH_2Cl_2/MeOH (10:10:1) to give 10-deacetylbaccatin III (**9**, 1.8 mg), 10-deacetyl-13-oxobaccatin III (**11**, 2 mg), and 19-hydroxy-13-oxobaccatin III (**12**, 1.5 mg). Crystallization of fraction A-8-E-6 afforded an additional amount of 19-hydroxybaccatin III (**14**, 20 mg), and the supernatant was repeatedly subjected to NP-HPLC using *n*-hexane/CH_2Cl_2/MeOH (10:10:1) to give 19-hydroxy-10-deacetylbaccatin III (**13**, 15 mg). Part (500 mg) of fraction A-9 (7.6 g) was fractionated as mentioned in the case of A-3 to furnish six fractions (A-9-F-1 to A-9-F-6). NP-HPLC using *n*-hexane/CH_2Cl_2/MeOH (10:10:1) of part (100 mg) of fraction A-9-F-3 (1437 mg) followed by crystallization afforded taxayuntin G (**15**, 18 mg).

Tasumatrol E (1): colorless powder, $[\alpha]^{25}_D$ +36° (*c* 0.1, MeOH); IR (CH_2Cl_2) ν_{max} 3441 (O−H st), 2975 (C−H st), 1727 (br s, C=O st of esters), 1246 (C−O st of acetate), 1028 (C−O st), 735, 714 (C=C-H st) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), Table 1; FABMS *m/z* 649 $[M + H]^+$; HREIMS *m/z* 630.2664 $[M - H_2O]^+$ (calcd for $C_{33}H_{44}O_{13}$, 648.2769).

Tasumatrol F (2): colorless powder; $[\alpha]^{25}_D$ +28° (*c* 0.1, MeOH); IR (CH_2Cl_2) ν_{max} 3416 (O−H st), 2976 (C−H st), 1715 (br s, C=O st of esters), 1602 (ar C=C st), 1253 (C−O st of acetate), 1044 (C−O st), 735, 714 (C=C-H st) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$), Table 1; FABMS *m/z* 633 $[M + H]^+$; HREIMS *m/z* 614.2722 $[M - H_2O]^+$ (calcd for $C_{33}H_{44}O_{12}$, 632.2820).

Tasumatrol G (3): colorless powder; $[\alpha]^{25}_D$ −32° (*c* 0.1, MeOH); IR (CH_2Cl_2) ν_{max} 3417 (O−H st), 2975 (C−H st), 1737, 1721, 1714 (C=O st of esters), 1601 (C=C st of aromatic), 1252 (C−O st of acetate), 1114, 1048 (C−O st), 736, 15 (C=C-H st) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$), Table 1; FABMS *m/z* 631 $[M + H]^+$; HRESMS *m/z* 512.2041 $[M - 2OAc]^+$ (calcd for $C_{33}H_{42}O_{12}$, 630.2664).

Tasumatrol G Diacetate (4). A 3 mg sample of **3** was acetylated using Ac_2O/py (1:1) at room temperature for 12 h. After the usual workup of the reaction product it produced 8 mg of **4**. ^1H NMR (300 MHz, $CDCl_3$): δ 5.25 (d, J = 10.5 Hz, H-2), 4.98 (br s, H-5), 5.26 (d, J = 4.2 Hz, H-7), 5.17 (d, J = 5.1 Hz, H-9), 5.96 (d, J = 5.1 Hz, H-10), 5.51 (m, H-13), 1.93, 1.99, 2.05, 2.11, 2.27 (each CH_3, s).

Cytotoxicity Assay.[19] The human A498 (renal), NCI-H226 (non-small cell lung), A549 (non-small cell lung), and PC-3 (prostate) cancer cell lines of the screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40 000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2 , 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition. Experimental drugs were solubilized in DMSO at 400-fold of the final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additional 4-fold, 10-fold, or $^1/_2$ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations. Following drug addition, the plates were incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% HOAc was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% HOAc and the plates were air-dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm.

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