

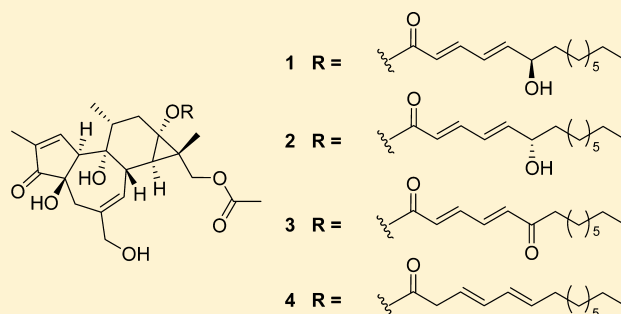
Cytotoxic Diterpenoids from *Sapium insigne*

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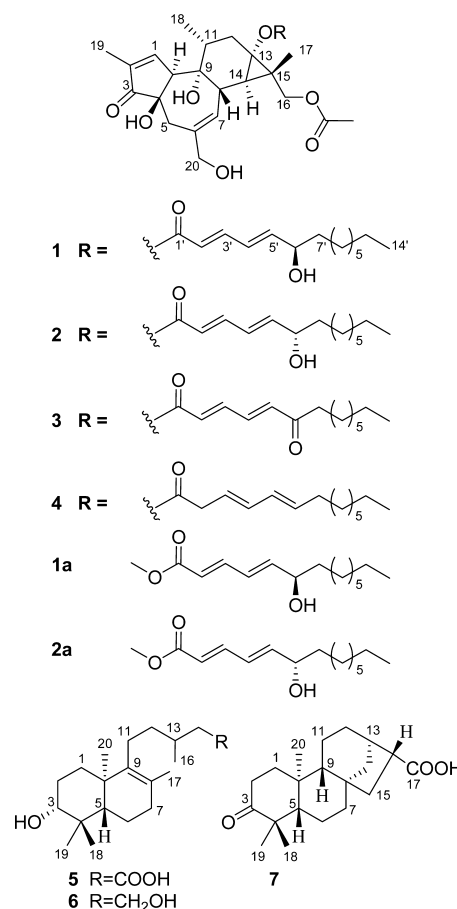
S Supporting Information

ABSTRACT: Chemical investigation into the twigs and leaves of *Sapium insigne* afforded seven new diterpenoids, sapinsignoids A–G (1–7), together with 10 known diterpenoids. The structures of 1–7 were assigned on the basis of detailed spectroscopic analysis and chemical degradation. Compounds 1–4 exhibited significant cytotoxicity against the A-549 tumor cell line (IC_{50} 0.2–1.8 μ M), while compounds 1–3 showed moderate cytotoxicity against the HL-60 cell line (IC_{50} 2.7–6.5 μ M).



Plants of the genus *Sapium* (Euphorbiaceae) are rich sources of antihypertensive phenolic compounds,¹ toxic phorbol esters,² triterpenoids,³ and steroids.⁴ About 120 *Sapium* species are distributed worldwide. *Sapium insigne* (Royle) Benth. ex Hook fil., which is also found in other South Asian countries such as Vietnam, India, and Nepal, is one of the nine native Chinese species.⁵ Although its medicinal virtues have rarely been explored in other countries, the bark juice of *S. insigne* is traditionally used in Nepal to help with wound healing by dispelling worms and killing germs, and the Nepalese also use its bark and leaves as fish poisons.⁶

Previous phytochemical studies of *S. insigne* have led to the isolation and/or identification of fatty acids,⁷ steroids,^{7,8} triterpenoids,^{7,9} phenolic compounds,¹⁰ and phorbol-type diterpenoids.^{10a,11} Recently Indian researchers reported that the methanol extract of the leaves of *S. insigne* showed significant antibacterial activities.¹² Our interest in this plant was due to the previous report of phorbol esters,^{10a,11} as this class of diterpenoids from other *Sapium* species have been reported to be toxic agents,² which may account for its medicinal applications in Nepal. We thus collected the twigs and leaves of *S. insigne* from Jianfengling on Hainan Island in China and carried out an intensive chemical study. In the current study, four new phorbol derivatives, sapinsignoids A–D (1–4), bearing 14-carbon fatty acid chains, two new *ent*-labdane diterpenoids, sapinsignoids E (5) and F (6), and one new *ent*-kaurane diterpenoid, sapinsignoid G (7), along with 10 known diterpenoids, were isolated and characterized. Their structures were elucidated on the basis of spectroscopic data, with the absolute configuration being assigned for the lipid moieties of 1 and 2 by Mosher's method and comparison of their $[\alpha]_D$ values. Bioassay screening showed that sapinsignoids A–D (1–4) were the major cytotoxic compounds of this plant, while the remaining compounds showed no obvious cytotoxic activities against the HL-60 (leukemia) and A-549 (lung cancer) cell lines.



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Table 1. ¹H (400 MHz) and ¹³C (100 MHz) NMR Data for Compounds 1–4 in CDCl₃

no.	1		2		3		4	
	δ_{H} (mult. J in Hz)	δ_{C}	δ_{H} (mult. J in Hz)	δ_{C}	δ_{H} (mult. J in Hz)	δ_{C}	δ_{H} (mult. J in Hz)	δ_{C}
1	7.58, br s	161.0, CH	7.59, br s	161.1, CH	7.59, br s	161.0, CH	7.55, br s	161.1, CH
2		133.0, C		133.0, C		133.0, C		132.9, C
3		209.1, C		209.1, C		209.1, C		209.2, C
4		73.7, C		73.7, C		73.7, C		73.6, C
5	α 2.54, br d (19.2) β 2.47, d (19.2)	38.7, CH ₂	α 2.54, br d (19.1) β 2.47, d (19.1)	38.7, CH ₂	α 2.54, br d (18.6) β 2.47, d (18.6)	38.7, CH ₂	α 2.56, br d (19.1) β 2.45, d (19.1)	38.5, CH ₂
6		140.3, C		140.3, C		140.4, C		140.4, C
7	5.68, br d (5.1)	129.7, CH	5.67, br d (4.9)	129.5, CH	5.65, br d (4.9)	129.3, CH	5.63, m ^g	129.4, CH
8	3.08, dd (5.1, 5.1)	38.5, CH	3.07, dd (4.9, 4.9)	38.5, CH	3.09, dd (4.9, 4.9)	38.5, CH	3.06, m ^h	38.4, CH
9		76.2, C		76.1, C		76.1, C		76.0, C
10	3.27, br s	55.7, CH	3.28, br s	55.7, CH	3.29, br s	55.8, CH	3.25, br s	55.6, CH
11	1.99, m	36.2, CH	1.99, m	36.2, CH	2.01, m	36.2, CH	1.98, m	36.1, CH
12	α 1.64, dd (14.6, 11.6) β 2.12, dd (14.6, 6.9)	31.7, CH ₂	α 1.64, dd (14.5, 11.5) β 2.12, dd (14.5, 7.0)	31.7, CH ₂	α 1.63 ^e β 2.15, dd (13.1, 6.9)	31.6, CH ₂	α 1.58, dd (14.7, 11.3) β 2.11, dd (14.7, 6.9)	31.6, CH ₂
13		63.0, C		63.0, C		63.8, C		63.4, C
14	1.15, d (5.1)	31.0, CH	1.15, d (4.9)	31.1, CH	1.17, d (4.9)	31.0, CH	1.10, d (5.2)	30.9, CH
15		26.3, C		26.3, C		26.5, C		26.2, C
16	4.12, d (11.5) 4.04, d (11.5)	69.6, CH ₂	4.14, d (11.4) 4.04, d (11.4)	69.6, CH ₂	4.13, d (11.4) 4.06, d (11.4)	69.4, CH ₂	4.12, d (11.3) 3.98, d (11.3)	69.5, CH ₂
17	1.17, s	11.2, CH ₃	1.17, s	11.2, CH ₃	1.18, s	11.2, CH ₃	1.15, s	11.2, CH ₃
18	0.90, d (6.9)	18.5, CH ₃	0.90, d (6.0)	18.5, CH ₃	0.90, d (6.5)	18.5, CH ₃	0.88, d (6.4)	18.4, CH ₃
19	1.77, dd (2.7, 1.2)	10.1, CH ₃	1.77, dd (2.6, 1.2)	10.1, CH ₃	1.77, dd (2.8, 1.2)	10.1, CH ₃	1.74, dd (2.8, 1.3)	10.1, CH ₃
20	a 4.04, br d (12.2) b 3.95, br d (12.2)	68.2, CH ₂	a 4.04, br d (12.2) b 3.96, br d (12.2)	68.2, CH ₂	a 4.04, br d (14.1) b 3.98, br d (14.1)	68.2, CH ₂	a 4.02, br d (12.2) b 3.95, br d (12.2)	68.2, CH ₂
16-OAc		171.2, C		171.2, C		171.1, C		171.1, C
	2.11, s	21.1, CH ₃	2.11, s	21.1, CH ₃	2.11, s	21.1, CH ₃	2.08, s	21.0, CH ₃
1'		168.6, C		168.6, C		167.6, C		173.8, C
2'	5.79, d (14.6) ^a	120.2, CH	5.80, d (15.3) ^c	120.2, CH	6.16, d (15.2)	127.5, CH	3.06, d (7.0) ^h	38.2, CH ₂
3'	7.24, dd (14.6, 10.8)	146.1, CH	7.26, dd (15.3, 11.1)	146.1, CH	7.31, dd (15.2, 11.4)	143.5, CH	5.50, dt (14.8, 7.4)	120.8, CH
4'	6.36, dd (15.2, 10.8)	127.1, CH	6.36, dd (15.2, 11.1)	126.9, CH	7.16, dd (15.4, 11.4)	137.5, CH	6.08, dd (14.8, 10.5)	134.9, CH
5'	6.14, dd (15.2, 6.0)	146.9, CH	6.16, dd (15.2, 5.8)	146.9, CH	6.46, d (15.4)	136.6, CH	5.99, dd (14.8, 10.5)	129.1, CH
6'	4.24, td (6.3, 6.0)	72.0, CH	4.24, m	71.9, CH		209.1	5.65, dt (14.8, 6.8) ^g	135.6, CH
7'	1.55, m	36.9, CH ₂	1.55, m	37.0, CH ₂	2.59, 2H, t (7.4)	41.4, CH ₂	2.05, m	32.6, CH ₂
8'	1.35, m	25.3, CH ₂	1.35, m	25.3, CH ₂	1.61, m ^e	24.0, CH ₂	1.36, m	29.2, CH ₂
9'	1.26, m ^b	29.5, CH ₂	1.26, m ^d	29.5, CH ₂	1.28, m ^f	29.2, CH ₂	1.25, m ⁱ	29.4, CH ₂
10'	1.26, m ^b	29.5, CH ₂	1.26, m ^d	29.5, CH ₂	1.28, m ^f	29.4, CH ₂	1.25, m ⁱ	29.2, CH ₂
11'	1.26, m ^b	29.2, CH ₂	1.26, m ^d	29.2, CH ₂	1.28, m ^f	29.2, CH ₂	1.25, m ⁱ	29.2, CH ₂
12'	1.26, m ^b	31.9, CH ₂	1.26, m ^d	31.9, CH ₂	1.26, m ^f	31.8, CH ₂	1.25, m ⁱ	31.8, CH ₂
13'	1.26, m ^b	22.7, CH ₂	1.26, m ^d	22.7, CH ₂	1.28, m ^f	22.6, CH ₂	1.25, m ⁱ	21.0, CH ₂
14'	0.87, t (7.0)	14.1, CH ₃	0.87, t (7.0)	14.1, CH ₃	0.87, t (7.0)	14.1, CH ₃	0.87, t (6.7)	14.1, CH ₃
9-OH	5.81, br s ^a		5.79, br s ^c		5.51, br s		5.41, br s	

^{a–i}Overlapping signals within the same column.

RESULTS AND DISCUSSION

Compound **1** had a molecular formula of C₃₆H₅₂O₉ determined by HR-ESIMS at *m/z* 651.3501 ([M + Na]⁺, calcd 651.3509), indicating 11 degrees of unsaturation. The IR spectrum revealed the presence of hydroxy (3408 cm⁻¹) and carbonyl (1738 and 1697 cm⁻¹) groups. The 1D NMR spectroscopic data (Table 1) displayed signals for three methyl (δ_{H} 0.90 and δ_{C} 18.5, Me-18; δ_{H} 1.17 and δ_{C} 11.2, Me-17; δ_{H} 1.77 and δ_{C} 10.1, Me-19), one trisubstituted double bond (δ_{H} 5.68, H-7; δ_{C} 129.7, C-7; and 140.3, C-6), one α,β -unsaturated carbonyl (δ_{H} 7.58, H-1; δ_{C} 133.0, C-2; 161.0, C-1; and 209.1, C-3), and two oxymethylenes (δ_{H} 4.04 and 3.95 and δ_{C} 68.2, CH₂-20; δ_{H} 4.12 and 4.04 and δ_{C} 69.6, CH₂-16). The above data suggested that compound **1** possessed the 12-deoxy-16-hydroxyphorbol backbone of the tiglane family of diterpenoids,¹³ which was further

supported by ¹H–¹H COSY and HMBC experiments (Figure 1A). Other characteristic resonances, which differentiated the structure of **1** from the known 12-deoxy-16-hydroxyphorbol analogues, included signals for an acetyl (δ_{H} 2.11 and δ_{C} 21.1, 171.2) and a 14-carbon lipid moiety bearing an $\alpha,\beta,\delta,\gamma$ -unsaturated ester unit (δ_{H} 5.79, 7.24, 6.36, and 6.14 for H-2' to H-5'; δ_{C} 168.6, 120.2, 146.1, 127.1, and 146.9 for C-1' to C-5'), in agreement with the molecular formula. The 16-O-acetyl group was evident from the deshielded chemical shifts for H₂-16 (δ_{H} 4.12 and 4.04) and confirmed by HMBC correlations from H₂-16 to the acetyl carbonyl carbon, while the O-acyl chain could be attributed to C-13. The characterization (key COSY, HMBC, and ROESY correlations) of the lipid motif is illustrated in Figure 2A, and the diagnostic ¹H–¹H COSY cross-peak of H-5'/H-6' defined the presence of a 6'-OH group.

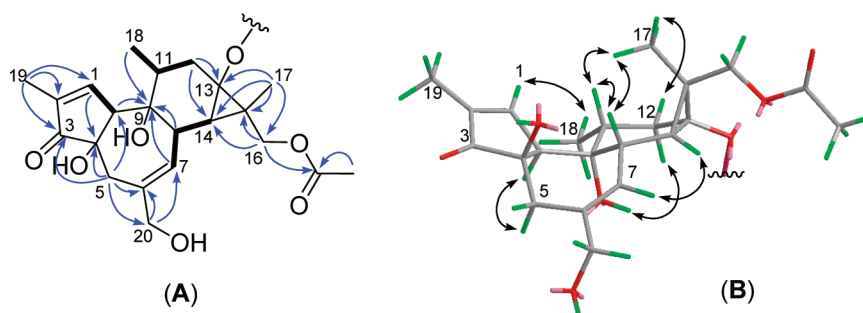


Figure 1. Key ^1H - ^1H COSY (–) and HMBC (→) (A) and ROESY correlations (↔) (B) of the 12-deoxy-16-hydroxyphorbol moiety of 1–4.

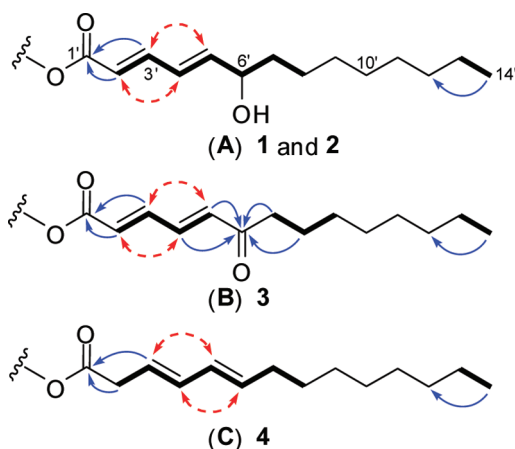


Figure 2. Key ^1H - ^1H COSY (–), HMBC (→), and ROESY correlations (↔) for the lipid moiety of 1 and 2 (A), 3 (B), and 4 (C).

Furthermore, the large coupling constants of $J_{2,3'}$ (14.6 Hz) and $J_{4,5'}$ (15.2 Hz) were indicative of a $2'E,4'E$ -configuration for the conjugated double bonds, and this was supported by the key ROESY correlations of H-2'/4' and H-3'/5'.

The relative configuration of the diterpenoid core for **1** was established, on the basis of ROESY data and NMR data comparison, to be identical with those reported for other 12-deoxy-16-hydroxyphorbol derivatives.¹³ In particular, the ROESY correlations (Figure 1B) of H₃-17/H-8, H₃-17/H-11, and H₃-17/H-12 β indicated that these protons were cofacial and were randomly assigned a β -orientation. Subsequently, the ROESY cross-peaks of 9-OH/H-12 α and H-10/H-5 α revealed that they were α -oriented. Methanolysis of **1** in the presence of NaOH yielded **1a** (colorless gum), which was identified to be the methyl ester of the fatty acid chain. The absolute configuration of C-6 in compound **1a** was determined by the Mosher's method.¹⁴ Negative $\Delta\delta_{S-R}$ values were observed for H-2 (–0.09), H-4 (–0.20), and H-5 (–0.11), while positive $\Delta\delta_{S-R}$ values were observed for Ha-7 (+0.07), Hb-7 (+0.05), and H-8 (+0.12), indicating a $6R$ absolute configuration for **1a** (Figure 3). Therefore, the structure of **1** was determined as shown, and the compound was named sapinsignoid A.

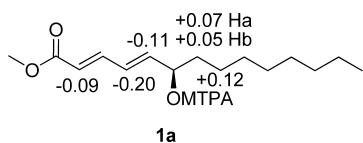


Figure 3. $\Delta\delta_{S-R}$ values of MTPA esters of **1a**.

Compound **2** had the same molecular formula of $\text{C}_{36}\text{H}_{52}\text{O}_9$ as **1** as established by HR-ESIMS at m/z 651.3483 ($[\text{M} + \text{Na}]^+$, calcd 651.3509), indicative of a likely isomeric relationship. The ^1H and ^{13}C NMR data of **2** (Table 1) were similar to those of **1** with only minor deviation in chemical shifts for both carbon (≤ 0.2 ppm) and proton (≤ 0.02 ppm) resonances. Further analysis of the 2D NMR data (HSQC, HMBC, and ROESY) of **2** (see Supporting Information Figures S11 to S13) defined the same diterpenoid core for **2** as that of **1**. HPLC analysis showed two well-resolved UV peaks that indicated two unique compounds (see Supporting Information S3). The above observations, therefore, could only be attributed to the fact that **1** and **2** had different C-6' configurations. Subsequent base-catalyzed methanolysis of **2** yielded **2a**, which was identical in all aspects with those of **1a**, except for showing an opposite specific rotation, $[\alpha]_D^{20}$ (+24.3, MeOH), supportive of a $6'S$ absolute configuration. The structure of **2** (sapinsignoid B) was thus characterized as depicted.

Compound **3** had a molecular formula of $\text{C}_{36}\text{H}_{50}\text{O}_9$ as established by HR-ESIMS at m/z 649.3362 ($[\text{M} + \text{Na}]^+$, calcd 649.3353), which was two mass units less than that of **1**, suggestive of a dihydro analogue of the latter. Analysis of the NMR data (Table 1) for **3** confirmed this hypothesis with diagnostic resonances for an extra carbonyl (δ_{C} 209.1, C-6') replacing the corresponding $-\text{CH}(\text{OH})-$ (δ_{H} 4.24 and δ_{C} 72.0) group in **1**, which was further confirmed by COSY and HMBC data (Figure 2B). The $J_{2,3'}$ (15.2 Hz) and $J_{4,5'}$ (15.4 Hz) values were consistent with E -geometry for the $\Delta^{2'}$ and $\Delta^{4'}$ double bonds. The highly similar NMR data for the diterpenoid core of compounds **3** and **1** indicated that they possessed the same tigliane diterpenoid, which was further confirmed by 2D NMR data (Figure 1). Compound **3** (sapinsignoid C) was thereby identified as the 6'-oxo homologue of **1** and/or **2**.

Compound **4** showed a sodiated molecular ion peak at m/z 635.3581 ($[\text{M} + \text{Na}]^+$, calcd 635.3560) in the HR-ESIMS analysis consistent with a molecular formula of $\text{C}_{36}\text{H}_{52}\text{O}_8$. The ^1H and ^{13}C NMR data (Table 1) revealed that it was an analogue of **1** with the only difference being a different O -acyl chain at C-13. The 2D NMR data (Figure 2C) established this structural fragment as shown by exhibiting an isolated ester carbonyl (δ_{C} 173.8, C-1') connected via a $-\text{CH}_2-$ (δ_{C} 38.2, C-2') to a conjugated diene (δ_{C} 120.8, 134.9, 129.1, and 135.6 for C-3', C-4', C-5', and C-6', respectively). The E -geometry of the conjugated $\Delta^{3'}$ and $\Delta^{5'}$ double bonds was suggested by the large $J_{3,4'}$ (14.8 Hz) and $J_{5,6'}$ (14.8 Hz) values and further supported by ROESY correlations of H-3'/H-5' and H-4'/H-6'. Comparison of the NMR data of compounds **4** and **1** indicated the presence of an identical tigliane diterpenoid core.

Table 2. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data for Compounds 5–7

no.	5^a		6^b		7^a	
	δ_{H} (mult. J in Hz)	δ_{C}	δ_{H} (mult. J in Hz)	δ_{C}	δ_{H} (mult. J in Hz)	δ_{C}
1	α 1.87, m β 1.39, m	35.3, CH ₂	α 1.81, m β 1.29, m	35.0, CH ₂	α 2.00, m β 1.39, m	39.2, CH ₂
2	1.88, m	28.5, CH ₂	1.65, m	27.8, CH ₂	2.47, dd (8.5, 6.4)	34.0, CH ₂
3	3.45, dd (9.9, 6.9)	77.9, CH	3.23, dd (11.6, 4.6)	79.0, CH		218.3, C
4		39.2, C		38.8, C		47.1, C
5	1.13, m	51.2, CH	1.09, dd (12.5, 1.9)	51.1, CH	1.42, m	54.3, CH
6	α 1.48, m β 1.65, m	19.1, CH ₂	α 1.45, m β 1.65, m	18.8, CH ₂	1.47, m	22.0, CH ₂
7	1.97, m	33.9, CH ₂	2.01, m	33.8, CH ₂	1.55, m	39.9, CH ₂
8		125.5, C		125.7, C		44.8, C
9		140.4, C		140.1, C	1.12, br s	54.6, CH
10		39.0, C		38.8, C		38.4, C
11	2.17, m 1.96, m	25.8, CH ₂	2.05, m 1.81, m	25.5, CH ₂	1.60, m	18.9, CH ₂
12	1.62, m 1.44, m	37.5, CH ₂	1.35, m 1.20, m	37.8, CH ₂	1.57, m	30.9, CH ₂
13	2.24, m	31.7, CH	1.54, m	30.6, CH	2.56, br s	41.1, CH
14	2.63, dd (14.7, 5.8) 2.41, dd (14.7, 8.1)	42.3, CH ₂	1.65, m 1.42, m	39.7, CH ₂	1.23, dd (11.5, 4.3) 1.85, d (11.5)	37.6, CH ₂
15		175.6 ^c , C	3.69, m	61.2, CH ₂	1.70, m	44.1, CH ₂
16	1.16, d (6.6)	19.8, CH ₃	0.93, d (6.6)	19.4, CH ₃	2.66, br t (7.4)	45.2, CH
17	1.59, s	19.3, CH ₃	1.55, s	19.4, CH ₃		183.3 ^c , C
18	1.22, s	28.6, CH ₃	0.99, s	28.1, CH ₃	1.07, s	27.3, CH ₃
19	1.04, s	16.2, CH ₃	0.79, s	15.5, CH ₃	1.02, s	21.0, CH ₃
20	1.02, s	20.2, CH ₃	0.93, s	20.1, CH ₃	1.04, s	17.5, CH ₃

^aMeasured in CDCl₃. ^bMeasured in C₅D₅N. ^cConfirmed by the HMBC experiment.

Hence the structure of **4** (sapinsignoid D) was characterized as depicted.

Compound **5** showed a pseudo molecular ion peak at m/z 321.2417 ($[\text{M} - \text{H}]^-$, calcd 321.2430) in the HR-ESIMS spectrum corresponding to a molecular formula of C₂₀H₃₄O₃ incorporating four double-bond equivalents (DBE). The IR spectrum displayed the presence of carbonyl (1684 cm⁻¹) and hydroxy (3315 cm⁻¹) groups. The ^1H and ^{13}C NMR data (Table 2) revealed five methyl, seven methylene, three methine (one oxygenated), and four quaternary carbons (two olefinic). The HMBC correlation from H₂-14 (δ_{H} 2.63 and 2.41) to the signal at δ_{C} 175.6 confirmed the existence of a C-15 hydroxycarbonyl group, which was not detected in the ^{13}C NMR spectrum. These data accounted for two out of the four DBEs, and the remaining two DBEs indicated that compound **5** was bicyclic. The observations suggested that compound **5** possessed an *ent*-labdane skeleton with a terminal C-15 hydroxycarbonyl functionality, an analogue of the coexisting diterpenoid 1-naphthalenepentanoic acid.¹⁵ The only structural difference between these molecules was the presence of a Δ^8 double bond in **5** instead of the $\Delta^{8(17)}$ double bond in 1-naphthalenepentanoic acid, which was determined by the HMBC correlations from Me-17 (δ_{H} 1.59) to C-7 (δ_{C} 33.9), C-8 (δ_{C} 125.5), and C-9 (δ_{C} 140.4). The relative configuration of **5** was assigned by the ROESY spectrum (Supporting Information Figure S48). In particular, the ROESY correlations from H-3 to Me-18, H-5, and H-1 β indicated that they were cofacial and were randomly assigned a β -orientation. Additionally, the ROESY correlations of Me-19/H-2 α and Me-20/H-6 α showed that these protons were α -oriented. The configuration at C-13 in **5** remained undetermined. Thus, the structure of sapinsignoid E (**5**) was assigned as shown.

Compound **6** had a molecular formula of C₂₀H₃₆O₂ as deduced from the HR-EIMS ion at m/z 308.2708 ($[\text{M}]^+$, calcd 308.2715). Analysis of the NMR data (Table 2) showed that it was a reduced analogue of **5** with the only structure difference being the presence of a C-15 hydroxymethylene (δ_{H} 3.69; δ_{C} 61.2) in **6** compared to the C-15 hydroxycarbonyl group (δ_{C} 175.6) in **5**, which was further supported by the HMBC correlations from H₂-14 (δ_{H} 1.65 and 1.42) to C-15. The relative configuration of **6** (excluding C-13) was determined to be the same as that of **5** on the basis of the examination of its ROESY data (Supporting Information Figure S56). Thus, the structure of sapinsignoid F (**6**) was assigned as shown.

Compound **7** had a molecular formula of C₂₀H₃₀O₃ as deduced from the HR-ESIMS ion at m/z 341.2098 ($[\text{M} + \text{Na}]^+$, calcd 341.2093) incorporating six DBEs. The ^1H and ^{13}C NMR data (Table 2) of **7** resolved resonances for three methyl, eight methylene, four methine, and four quaternary carbons (one keto carbonyl), with a carboxylic carbon at δ_{C} 183.3 (C-17) also detected by the HMBC correlations from H₂-15 (δ_{H} 1.70) to H-16 (δ_{H} 2.66). These data accounted for two out of the six DBEs, and the remaining four DBEs required **7** to be tetracyclic. The aforementioned observations suggested that compound **7** was a congener of a coexisting *ent*-17-hydroxy-16 α -kauran-3-one possessing an *ent*-kaurane skeleton.¹⁶ The carbonyl group was allocated at C-3 on the basis of the HMBC correlations from Me-18/19, H₂-2, and H₂-1 to C-3 (δ_{C} 218.3) (Supporting Information Figure S63). The relative configuration of **7** was established by the ROESY spectrum (Supporting Information Figure S65). More specifically, the key correlation between H-16 and H₂-12 indicated that the H-16 of compound **7** was cofacial with CH₂-12. Thus, the

structure of compound 7 (sapinsignoid G) was elucidated as depicted.

Ten known diterpenoids, 1-naphthalenepentanoic acid,¹⁵ *ent*-17-hydroxy-16 α -kauran-3-one,¹⁶ *ent*-16 β ,17-isopropylidenedioxykauran-3-one,¹⁷ abbeokutone,¹⁸ 16-*epi*-abbeokutone,¹⁸ kauranoic acid,¹⁹ dihydroalepterolic acid,²⁰ dihydroaleptero[*ent*-labda-8(17),13*E*-diene-3 α ,15-diol,²¹ and *ent*-16*S*,17-dihydroxyatisan-3-one,²² were also obtained and were identified on the basis of their ¹H NMR, ¹³C NMR, and ESIMS data and comparison with literature values. All the kaurane-, labdane-, and atisane-type diterpenes were determined as members of the *ent*-series through their specific rotations.²³

All compounds were tested against the human tumor cell lines A-549 and HL-60 using SRB²⁴ and MTT²⁵ methods, respectively. Compounds 1–4 showed moderate cytotoxicity against the A-549 cell line with IC₅₀ values of 1.8, 0.2, 0.4, and 0.9 μ M, respectively, and compounds 1–3 displayed weak cytotoxicity against the HL-60 cell line with IC₅₀ values of 3.4, 6.5, and 2.7 μ M, respectively. The *ent*-kaurane, *ent*-labdane, and *ent*-atisane type of known diterpenes showed no significant cytotoxicity at the maximum tested concentration (100 μ M).

In conclusion, our current chemical investigation of *S. insignis* yielded 10 known and seven new diterpenoids and revealed the new phorbol derivatives as the cytotoxic principles from this folk herb, which to a certain extent could explain its traditional applications of dispelling worms and as fish poisons.

EXPERIMENTAL SECTION

General Experimental Procedures. Specific rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were measured on a Shimadzu UV-2550 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 577 spectrometer with KBr disks. NMR spectra were measured on a Varian Mercury-400 spectrometer with TMS as internal standard. EIMS and HREIMS analyses (70 eV) were carried out on a Finnigan MAT 95 mass spectrometer. ESI(\pm)MS and HRESI(+)¹MS were carried out on a Bruker Daltonics Esquire3000plus instrument and a Waters Q-TOF Ultima mass spectrometer, respectively. Silica gel (200–300 mesh) was used for column chromatography (CC), and precoated silica gel GF₂₅₄ plates (Qingdao Haiyang Chemical Plant, Qingdao, P.R. China) were used for TLC. C₁₈ reversed-phased silica gel (150–200 mesh, Merck) was also used for CC. Analytical HPLC was carried out on an Agilent 1100 series LC instrument with a DAD detector and a Symmetry C₁₈ column (3.9 \times 150 mm, 5 μ m). Semipreparative HPLC was performed on a Waters 1525 pump equipped with a Waters 2489 detector and a YMC-Pack ODS-A column (10 \times 250 mm, S-5 μ m, 12 nm). All solvents used for CC were of analytical grade (Shanghai Chemical Reagents Company, Ltd.), and solvents used for HPLC were of HPLC grade (J&K Scientific Ltd.).

Plant Material. The twigs and leaves of *S. insignis* were collected from Jianfengling on Hainan Island, People's Republic of China, in August 2010 and were identified by Prof. Shi-Man Huang, Department of Biology, Hainan University, People's Republic of China. A voucher specimen has been deposited in Shanghai Institute of Materia Medica, Chinese Academy of Sciences (accession number Sin-HS-2010-1Y).

Extraction and Isolation. The air-dried twigs and leaves were ground into a powder (11 kg), which was percolated with 95% aqueous EtOH, and the crude extract (550 g) was subsequently extracted with EtOAc. The EtOAc-soluble fraction (120 g) was separated by a silica gel column (petroleum ether/Me₂CO, 15:1 to 0:1) to give eight fractions (A–H). Fraction C (4.5 g) was separated on a silica gel column eluted with CH₂Cl₂/MeOH (200:1 to 10:1) to give three fractions (C1–C3). Fraction C1 (1.2 g) was applied to an RP-18 column (MeOH/H₂O, 70:30 to 100:0) to give five fractions (C1a–C1e). C1a (200 mg) was purified by semipreparative HPLC (5 mL/min, 20 min 50–80% MeCN/H₂O gradient elution) to yield dihydroalepterolic acid (8 mg, 12.5 min), 1-naphthalenepentanoic acid

(3 mg, 13.5 min), and 5 (4 mg, 13.5 min). C1b was subjected to semipreparative HPLC (4 mL/min, 75% MeCN/H₂O isocratic elution) to give *ent*-17-hydroxy-16 α -kauran-3-one (20 mg, 9.5 min). C1c was subjected to semipreparative HPLC (3 mL/min, 75% MeCN/H₂O isocratic elution) to give dihydroaleptero[*ent*-labda-8(17),13*E*-diene-3 α ,15-diol (33 mg, 12.5 min). C1d was subjected to semipreparative HPLC (4 mL/min, 45% MeCN/H₂O isocratic elution) to give 16-*epi*-abbeokutone (7 mg, 10.5 min). Fraction C3 (0.55 g) was subjected to a silica gel column (petroleum ether/EtOAc, 2:1 to 1:2) to give three fractions (C3a–C3c). C3b was chromatographed on a RP-18 silica gel column (MeOH/H₂O, 80:20 to 100:0) to give *ent*-16 β ,17-isopropylidenedioxykauran-3-one (34 mg). Fraction C3c was subjected to a column of RP-18 silica gel (MeOH/H₂O, 80:20 to 100:0) to obtain three subfractions (C3c1–C3c2). C3c1 afforded abbeokutone (55 mg, 9.5 min) and *ent*-16*S*,17-dihydroxyatisan-3-one (57 mg, 9.5 min) by using semipreparative HPLC (4 mL/min, 45% MeCN/H₂O isocratic elution). Fraction C3c2 was separated on a semipreparative HPLC (4 mL/min, 60% MeCN/H₂O isocratic elution) to obtain 1 (8 mg, 18.0 min) and 2 (7 mg, 18.5 min). Following the same purification procedures, C3c3 afforded compound 4 (14 mg). Fraction D was subjected to CC of silica gel (CH₂Cl₂/MeOH, 200:1 to 20:1) to give four fractions (D1–D4). Fraction D1 was subjected to a column of RP C18 silica gel eluted with MeOH/H₂O (80:20) and purified by semipreparative HPLC (4 mL/min, 60% MeCN/H₂O isocratic elution) to obtain 7 (3 mg, 11.0 min). By the same purification procedures, D2 gave *ent*-labda-8(17),13*E*-diene-3 α ,15-diol (3 mg) and D4 yielded 6 (2 mg). Fraction A was subjected to a column of RP C18 silica gel to obtain six fractions (A1–A6). A2 was further separated by semipreparative HPLC with 85% CH₃CN in H₂O as the mobile phase, to yield 3 (6 mg). Following the same purification procedures, fraction A4 gave kauranoic acid (4 mg).

Sapinsignoid A (1): colorless gum; [α]_D²⁰ +20.5 (*c* 0.2, MeOH); UV (MeOH) λ_{\max} (log ϵ) 264 (4.50) nm; IR (KBr) ν_{\max} 3408, 2927, 2856, 1738, 1697, 1639, 1335, 1273, 1236, 1134, 1103 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; LR-ESI(+)¹MS *m/z* 651.5 [M + Na]⁺, 1279.8 [2 M + Na]⁺; HR-ESI(+)¹MS *m/z* 651.3501 [M + Na]⁺ (calcd for C₃₆H₅₂NaO₉, 651.3509).

Sapinsignoid B (2): colorless gum; [α]_D²⁰ +38.0 (*c* 0.2, MeOH); UV (MeOH) λ_{\max} (log ϵ) 265 (4.39) nm; IR (KBr) ν_{\max} 3410, 2925, 2856, 1739, 1697, 1639, 1335, 1273, 1236, 1134, 1003 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; LR-ESI(+)¹MS *m/z* 651.5 [M + Na]⁺, 1279.8 [2 M + Na]⁺; HR-ESI(+)¹MS *m/z* 651.3483 [M + Na]⁺ (calcd for C₃₆H₅₂NaO₉, 651.3509).

Sapinsignoid C (3): colorless gum; [α]_D²⁰ +54.5 (*c* 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ) 274 (4.73) nm; IR (KBr) ν_{\max} 3425, 2925, 2856, 1742, 1701, 1630, 1595, 1379, 1333, 1240, 1005 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; LR-ESI(+)¹MS *m/z* 649.5 [M + Na]⁺, 1275.9 [2 M + Na]⁺; HR-ESI(+)¹MS *m/z* 649.3362 [M + Na]⁺ (calcd for C₃₆H₅₀NaO₉, 649.3353).

Sapinsignoid D (4): colorless gum; [α]_D²⁰ +39.3 (*c* 0.7, MeOH); UV (MeOH) λ_{\max} (log ϵ) 229 (4.16) nm; IR (KBr) ν_{\max} 3413, 2927, 2856, 1709, 1593, 1458, 1439, 1379, 1333, 1271, 1238, 1173, 1038, 1018 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; LR-ESI(+)¹MS *m/z* 635.5 [M + Na]⁺, 1247.8 [2 M + Na]⁺; HR-ESI(+)¹MS *m/z* 635.3581 [M + Na]⁺ (calcd for C₃₆H₅₂NaO₈, 635.3560).

Sapinsignoid E (5): white powder; [α]_D²⁰ -109.0 (*c* 0.1, MeOH); UV (MeOH) no peak above 200 nm; IR (KBr) ν_{\max} 3315, 2951, 2870, 1684, 1439, 1412, 1296, 1277, 1028, 1005 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; LR-ESI(+)¹MS *m/z* 305.3 [M - H₂O + H]⁺; LR-ESI(-)¹MS *m/z* 321.5 [M - H]⁻; HR-ESI(-)¹MS *m/z* 321.2417 [M - H]⁻ (calcd for C₂₀H₃₃O₃, 321.2430).

Sapinsignoid F (6): white powder; [α]_D²⁰ -66.7 (*c* 0.1, MeOH); UV (MeOH) no peak above 200 nm; IR (KBr) ν_{\max} 3427, 2924, 1645, 1458, 1377, 1338, 1171, 1032, 625 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; EIMS *m/z* 308 (14) [M]⁺, 293 (11), 275 (62), 207 (83), 189 (100), 175 (16), 161 (22), 147 (40), 135 (52), 133 (45), 121 (64), 119 (77), 107 (65), 95 (83), 81 (59); HR-EIMS *m/z* 308.2708 [M]⁺ (calcd for C₂₀H₃₆O₂, 308.2715).

Sapinsignoid G (7): white powder; [α]_D²⁰ -122.6 (*c* 0.5, MeOH); UV (MeOH) λ_{\max} (log ϵ) 278 (2.45) nm; IR (KBr) ν_{\max} 2935, 2663,

1701, 1475, 1460, 1419, 1385, 1300, 1254, 1230, 1198, 1103, 951 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 2; LR-ESI(+)/MS m/z 319.3 $[\text{M} + \text{H}]^+$, 659.4 $[2\text{M} + \text{Na}]^+$; HR-ESI(+)/MS m/z 341.2098 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{20}\text{H}_{30}\text{NaO}_3$, 341.2093).

Base-Catalyzed Methanolysis of Compounds 1 and 2. Compound 1 (3.0 mg) was added to a solution of MeOH (2 mL) in the presence of NaOH (0.25 mg) at room temperature. After stirring for 45 min, the reaction was quenched with a saturated aqueous solution of NH_4Cl (2 mL) and extracted with EtOAc (3×3 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and subjected to silica gel chromatography eluting with petroleum ether/acetone (20:1) to give a product (1a) as a colorless gum (0.8 mg, 66%). By the same method, compound 2a (0.6 mg, 70%) was obtained from 2.

Compound 1a: colorless gum; $[\alpha]_D^{20}$ -23.3 (c 0.03, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 258 (4.37) nm; IR (KBr) ν_{max} 3431, 2924, 2852, 1722, 1647, 1618, 1464, 1435, 1308, 1265, 1232, 1142, 1001 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ_{H} 5.89 (d, $J = 15.4$, H-2), 7.29 (dd, $J = 15.4$, 11.0, H-3), 6.36 (dd, $J = 15.3$, 11.0, H-4), 6.12 (dd, $J = 15.3$, 6.0, H-5), 4.24 (m, H-6), 1.55 (m, H₂-7), 1.37 (m, H₂-8), 1.26 (m, 10H, H₂-9–H₂-13), 0.88 (t, $J = 6.8$, H₃-14), 3.75 (s, H₃-OCH₃); ^{13}C NMR (100 MHz, CDCl_3) δ_{C} 167.4 (C-1), 145.1 (C-5), 144.1 (C-3), 127.3 (C-4), 121.0 (C-2), 72.0 (C-6), 51.6 (–OCH₃), 37.1 (C-7), 25.3 (C-8), 29.2 (C-9), 29.4 (C-10), 29.5 (C-11), 31.8 (C-12), 22.7 (C-13), 14.1 (C-14); EIMS m/z 255 (3) $[\text{M} + \text{H}]^+$, 254 (19), 236 (6), 195 (17), 177 (8), 149 (12), 141 (87), 138 (14), 124 (22), 113 (100), 109 (81), 95 (37), 81 (87), 71 (66), 57 (76); HR-EIMS m/z 254.1875 $[\text{M}]^+$ (calcd for $\text{C}_{15}\text{H}_{26}\text{O}_3$, 254.1882).

Compound 2a: colorless gum; $[\alpha]_D^{20}$ $+24.3$ (c 0.07, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 258 (4.37) nm; IR (KBr) identical with 1a; ^1H NMR data, identical with 1a; EIMS m/z 254 (3) $[\text{M}]^+$, 236 (4), 195 (4), 177 (3), 149 (8), 141 (36), 138 (7), 124 (7), 113 (100), 109 (24), 95 (14), 81 (44), 71 (14), 57 (23); HR-EIMS m/z 254.1880 $[\text{M}]^+$ (calcd for $\text{C}_{15}\text{H}_{26}\text{O}_3$, 254.1882).

Preparation of the (R)- and (S)-MTPA Ester Derivatives of Compound 1a. Compound 1a (0.3 mg) was transferred to an NMR tube, which was dried under vacuum overnight at room temperature, followed by the addition of (S)-(+)- α -methoxy- α -(trifluoromethyl)-phenylacetyl chloride [(S)-MTPA-Cl] (6 μL) in deuterated pyridine (0.5 mL) in a dryer. The reaction tube was immediately sealed and shaken vigorously to ensure even mixing. The reaction was monitored by ^1H NMR acquisition and found to be complete after 8 h. (S)-MTPA ester of 1a (Supporting Information Figure S68): key ^1H NMR data (500 MHz, pyridine- d_5) δ_{H} 6.06 (1H, d, $J = 15.4$ Hz, H-2), 6.44 (dd, $J = 15.3$, 11.1 Hz, H-4), 6.17 (dd, $J = 15.4$, 6.5 Hz, H-5), 5.78 (m, H₂-6), 1.81 (m, Ha-7), 1.71 (m, Hb-7), 1.38 (m, H-8), and 0.86 (t, $J = 7.0$ Hz, H₃-14). The (R)-MTPA ester of 1a was prepared in the same way from compound 1a (0.3 mg) and (R)-MTPA chloride (6.0 μL) (Supporting Information Figure S69): key ^1H NMR data (500 MHz, pyridine- d_5) δ_{H} 6.15 (1H, d, $J = 15.4$ Hz, H-2), 6.64 (dd, $J = 15.2$, 11.1 Hz, H-4), 6.28 (dd, $J = 15.2$, 7.3 Hz, H-5), 5.80 (m, H₂-6), 1.74 (m, Ha-7), 1.66 (m, Hb-7), 1.26 (m, H-8), and 0.86 (t, $J = 7.1$ Hz, H₃-14). The ^1H NMR chemical shifts were assigned on the basis of a COSY experiment, with ambiguous and overlapping signals not used for the calculation of $\Delta\delta_{\text{S-R}}$.

■ ASSOCIATED CONTENT

Supporting Information

IR, HR-MS, and 1D and 2D NMR spectra of compounds 1–7, 1a, and 2a, as well as ^1H NMR spectra of (S)- and (R)-MTPA esters of 1a. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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