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Antiproliferative Alkaloids from *Alangium longiflorum*, an Endangered Tropical Plant Species

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Abstract

Alangium longiflorum is currently in extinction crisis, which will likely severely hamper further phytochemical investigation of this plant species from new collections. A crude extract of leaves of *A. longiflorum* (N33539), collected for the U.S. National Cancer Institute in 1989, showed potent cancer cell line antiproliferative activity. A phytochemical study resulted in the isolation of 17 secondary metabolites, including two new tetrahydroisoquinoline alkaloids, 8-hydroxytubulosine (**1**) and 2'-*O*-*trans*-sinapoylisoalangsides (**2**), as well as a new sinapolyoxylupene derivative (**3**). Using in-house assays and NCI-60 panel screening, compound **1** displayed broad-spectrum inhibitory activity at submicromolar levels against most tested tumor cell lines, except for drug-transporter-overexpressing cells. Compound **1** caused accumulation of sub-G1 cells with no effect on cell cycle progression, suggesting that this substance is an apoptosis inducer.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00411. Structures of all isolates, NCI-60 panel screening data of plant extract, and NMR spectra for 1–3 (PDF)

Graphical Abstract

In the late 1980s and 1990s, the Natural Products Branch (NPB) of the U.S. National Cancer Institute (NCI, Frederick, MD) sponsored the collection of ca. 80 000 plant samples from tropical areas in the Americas, Africa, and Southeast Asia, with samples ranging from leaves to complete plants depending upon the local abundance of the plant. All were collected with the permission of the relevant country authorities, and the initial collections led to the establishment of the NCI's Letter of Collection, first signed with the Malagasy Republic in 1989, three years prior to the Rio Convention. Among them, extracts from ca. 3000 species showed significant cytotoxicity at a concentration of 20 $\mu\text{g/mL}$, and 70% of these active species originated from either rainforests or their adjacent areas.

Rainforests cover only 6% of the earth's total surface, yet more than half of the known plant species occur in these regions.¹ It is well recognized that many useful medicines have originated from diverse plants found in rainforests. Unfortunately, these valuable and important regions are declining due to climate change and disruption by man, which is causing an extinction crisis for many plant species. Consequently, the rainforest plant *Alangium longiflorum* Merr. (Cornaceae) is threatened with extinction and currently on the *Red List of Threatened Species* created by the International Union for Conservation of Nature and Natural Resources (IUCN).

The species *A. longiflorum* is distributed mainly in Borneo, the Philippines, Sumatra, and the Moluccas. Phytochemical research on this species is quite limited, with only a single report published in 2006.² In the present work, a detailed phytochemical investigation was performed on 10 g of a crude organic extract from the leaves of *A. longiflorum* (N33539) provided by NPB, NCI. This extract was found to exhibit broad cytotoxicity with more specific antiproliferative effects against the growth of leukemic cell lines in the NCI-60 human tumor cell line panel (Figure S21, Supporting Information). When tested against five human tumor cell lines, the extract exhibited potent and selective activity against MDA-MB-231 and KB-VIN with IC_{50} values of less than 1 $\mu\text{g/mL}$. Described herein are the isolation and structure elucidation of 17 secondary metabolites, including three new compounds, from the endangered species *A. longiflorum*. Selected compounds were subjected to cytotoxic screening against a panel of cancer cell lines.

RESULTS AND DISCUSSION

The 50% $\text{CH}_3\text{OH}-\text{CH}_2\text{Cl}_2$ extract of the leaves of *A. longiflorum* (N33539) was fractionated with EtOAc and water to provide EtOAc- and water-soluble fractions. The

EtOAc-soluble fraction was separated by a combination of medium-pressure liquid chromatography (MPLC), silica gel column chromatography, preparative TLC, and HPLC techniques to afford two new isoquinoline alkaloids, 8-hydroxytubulosine (**1**) and 2'-*O*-*trans*-sinapoylisoalangsine (**2**), a new lupane-type triterpenoid (**3**), and 14 known compounds, isoalangsine (**4**),³ 2'-*O*-*trans*-sinapoyldemethylalangsine (**5**),⁴ 2'-*O*-*trans*-sinapoyl-3-*O*-demethyl-2-*O*-methylalangsine (**6**),⁴ 2'-*O*-*trans*-sinapoylalangsine (**7**),⁴ alangsine (**8**),³ demethylalangsine (**9**),⁵ 3-*O*-demethyl-2-*O*-methylalangsine (**10**),⁵ 2'-*O*-*trans*-feruloyldemethylalangsine (**11**),⁴ 9-demethyltubulosine (**12**),^{6–8} ankorine (**13**),^{9,10} α -tocopherylquinone (**14**),^{11,12} loganic acid (**15**),^{13,14} methyl pheophorbide a (**16**),^{15,16} and pheophytin a (**17**).^{15,17–19}

The HRFABMS of **1** showed a molecular formula of C₂₉H₃₇N₃O₄, with an ion peak at m/z 492.2860 [M + H]⁺. The IR spectrum displayed absorption bands for hydroxy and amine groups (3310 cm⁻¹), as well as alkane groups (2922 and 2850 cm⁻¹). The ¹H NMR data of **1** (Table 1) indicated signals assignable to an aromatic proton [δ_H 6.40 (1H, s)], an AMX spin-system attributable to three aromatic protons [δ_H 6.49 (1H, dd, J = 8.4, 2.4 Hz), 6.65 (1H, d, J = 2.4 Hz), and 7.02 (1H, d, J = 8.4 Hz)], three protons on heteroatoms exchangeable with D₂O [δ_H 8.47, 8.68, and 10.28 (each 1H, s)], two methoxy groups [δ_H 3.64 and 3.73 (each 3H, s)], and a terminal alkane group [δ_H 0.86 (3H, t, J = 7.2 Hz)]. These data resembled those of the known molecule 9-demethyltubulosine (**12**),⁷ except for the absence of one aromatic proton and the presence of an additional methoxy group [δ_H 3.64 (3H, s)] in **1**. The ¹³C NMR data of **1** (Table 1) were also similar to those of **12**, except for the chemical shifts of C-7, C-7a, C-8, C-9, C-10, C-11, and C-11a. These results suggested that the substituent pattern in ring A of **1** is different from that of **12**. The 2D NMR experiments, including H–H COSY, HMQC, and HMBC, revealed that a hydroxy group (δ_H 8.68) is attached at C-8 (δ_C 147.1) and two methoxy groups (δ_H 3.64 and 3.73) were bound to C-9 (δ_C 134.1) and C-10 (δ_C 150.5), respectively (Figure 1). The ¹³C NMR data for the tetrahydroisoquinoline moiety (rings A/B) of **1** agreed well with those of alangicine,²⁰ in which rings A/B have the same substituent pattern, namely, OH at C-8 and two methoxy groups at C-9 and C-10. Due to difficulties in conducting a NOESY experiment under various conditions, the relative configuration of **1** was determined from the ¹H NMR coupling constants. The splitting pattern and coupling constants of **1** matched well with those of 9-demethyltubulosine (**12**); thus, **1** was assigned the same relative configuration.^{6,7} Since the absolute structure of **12** was verified previously by synthesis,⁸ the absolute configuration of **1** was established by comparison of the experimental electronic circular dichroism (ECD) data and specific optical rotation values of the two compounds. The experimental ECD data of **1** showed the same Cotton effects as those of **12** (Figure S2), and the specific optical rotation values of **1** was almost identical with that of **12**.⁸ Thus, the structure of **1** was determined as 8-hydroxytubulosine.

Compound **2** was isolated as a pale yellow, amorphous solid. The HRFABMS of **2** exhibited a strong [M + H]⁺ peak at m/z 712.2566, suggesting a molecular formula of C₃₆H₄₁NO₁₄. The IR absorption bands at 3359, 1706, and 1655 cm⁻¹ indicated the presence of hydroxy, ester, and amide groups, respectively. The ¹H NMR data of **2** (Table 1) showed signals assignable to two aromatic protons [δ_H 6.49 and 6.63 (each 1H, s)], an olefinic proton [δ_H

7.26 (1H, d, $J = 2.4$ Hz)], a terminal vinyl group [δ_{H} 5.31 (1H, dd, $J = 10.2, 1.8$ Hz), 5.38 (1H, dd, $J = 17.4, 1.8$ Hz), and 5.66 (1H, dt, $J = 17.4, 10.2$ Hz)], an acetal proton [δ_{H} 5.41 (1H, d, $J = 1.8$ Hz)], and a methoxy group [δ_{H} 3.84 (3H, s)]. These data indicated that **2** possesses a similar skeleton to that of the alkaloidal glycosides alangiside (**8**)³ or 3-*O*-demethyl-2-*O*-methylalangiside (**10**).⁵ The location of a methoxy group at C-3 was deduced by a correlation between H-4 and methoxy in the NOESY spectrum of **2** (Figure 2). Furthermore, the ¹H NMR spectrum showed signals for two additional equivalent methoxy groups [δ_{H} 3.94 (6H, s)], a *trans*-olefin [δ_{H} 6.04 and 7.20 (each 1H, d, $J = 15.6$ Hz)], and two equivalent aromatic protons [δ_{H} 6.83 (2H, s)]. Based on the HMBC (Figure 1) and NOESY (Figure 2) spectra, which displayed interactions between the olefinic proton at δ_{H} 7.20 and the aromatic carbons at δ_{C} 107.6/carbonyl carbon at δ_{C} 167.9, as well as the aromatic protons at δ_{H} 6.83 and the methoxy signal at δ_{H} 3.94, these signals were assigned to a *trans*-sinapoyl group. Furthermore, an HMBC correlation between H-2' of the glucose moiety (δ_{H} 4.72) and the carbonyl carbon of the sinapoyl group (δ_{C} 167.9) indicated that the sinapoyl ester is linked to C-2'. The above spectroscopic features indicated a close structural similarity between **2** and 2'-*O*-*trans*-sinapoyl alangiside (**7**).⁴ However, careful inspection of the coupling constants between H₂-13 and H-13a ($J_{13\alpha, 13a} = 4.2$ Hz, $J_{13\beta, 13a} = 4.2$ Hz), as well as the chemical shifts of C-6, C-12a, and C-13, implied that **2** possesses an *S**-configuration at C-13a, rather than an *R**-configuration as in **7**. The *S**-configuration at C-13a in **2** was confirmed by a NOESY correlation (Figure 2) between H-13a (δ_{H} 4.51) and H α -6 (δ_{H} 2.85)/H α -13 (δ_{H} 1.88). Thus, the glucoside **2** was characterized as 2'-*O*-*trans*-sinapoylisoalangiside.

Compound **3** showed a protonated molecular ion at m/z 649.4465 in the HRFABMS, corresponding to the molecular formula C₄₁H₆₀O₆. The IR spectrum of **3** displayed absorption bands for hydroxy groups at 3523–3419 cm⁻¹, an α,β -unsaturated carbonyl ester at 1692 cm⁻¹, and aromatic groups at 1597 and 1514 cm⁻¹. The ¹H NMR data of **3** (Table 1) showed signals assignable to six tertiary methyl groups [δ_{H} 0.81, 0.89, 0.90, 0.92, 1.07, and 1.08 (each 3H, s)], a vinylic methyl [δ_{H} 1.68 (3H, s)], two protons of an isopropenyl group [δ_{H} 4.59 (1H, dd, $J = 2.4, 1.8$ Hz) and 4.71 (1H, d, $J = 1.8$ Hz)], two protons on hydroxylated carbons [δ_{H} 3.62 (1H, dt, $J = 12.6, 4.8$ Hz) and 4.76 (1H, br t, $J = 3.0$ Hz)], and 23 protons on alkyl groups. These data implied that **3** possesses a lupane-type triterpene skeleton. Moreover, the presence of a *trans*-sinapoyl group was suggested by a singlet aromatic peak [δ_{H} 6.79 (2H, s)], two olefinic protons [δ_{H} 6.33 (1H, d, $J = 16.2$ Hz) and 7.59 (1H, d, $J = 16.2$ Hz)], and two methoxy groups [δ_{H} 3.95 (6H, s)] in the ¹H NMR spectrum. The ¹³C NMR spectrum (Table 1) revealed signals assignable to an ester carbonyl carbon (δ_{C} 166.8), 10 double bonds or aromatic carbons [δ_{C} 105.1 (C-2' and C-6'), 109.8, 116.6, 126.3, 137.0, 144.7, 147.2 (C-3' and C-5'), and 149.9], two hydroxylated carbons (δ_{C} 77.0 and 78.4), two methoxy carbons (δ_{C} 56.4), and 26 carbons at high magnetic field. These data also supported a lupane-type skeleton esterified with a *trans*-sinapoyl unit. The HMBC spectrum (Figure 1) showed a correlation between H-3 (δ_{H} 4.76) and an ester carbonyl carbon (δ_{C} 166.8), which suggested that the *trans*-sinapoyl ester group is located at C-3. Further correlations were observed between H-28 (δ_{H} 0.81)/H-15 (δ_{H} 1.58) and the hydroxylated carbon C-16 (δ_{C} 77.0), as well as between H-29 (δ_{H} 4.59, 4.71)/H-30 (δ_{H} 1.68) and C-19 (δ_{C} 47.6). These data were consistent with the presence of a hydroxy group

at C-16 and an isopropenyl group at C-19. The relative configuration of **3** was supported by correlations in the NOESY spectrum (Figure 2) between H-3 (δ_{H} 4.76) and H-24 (δ_{H} 0.92) and between H-16 (δ_{H} 3.62) and H-18 (δ_{H} 1.40)/H-27 (δ_{H} 1.08). Moreover, H-3 (broad triplet, $J = 3.0$ Hz) has an equatorial β -orientation. Thus, compound **3** [fully assigned as 3 α -*trans*-sinapoyloxy-16 β -hydroxylup-20(29)-ene] differs from lupenes with 3 β -*trans*-sinapoyloxy²¹ (H-3 α : dd, $J = 10.7, 5.2$ Hz) and 3 β -*trans*-feruloyloxy²¹ groups but is comparable with 3 α -acetoxylupan-29-oic acid²² (H-3 β : br t, $J = 2.5$ Hz).

Selected compounds (**1**, **3**, **5**, **6**, **8**, **12**, and **13**) were evaluated for antiproliferative activity against five human tumor cell lines including A549 (lung carcinoma), MDA-MB-231 (triple-negative breast cancer), MCF-7 (estrogen receptor-positive and HER2-negative breast cancer), KB (originally isolated from epidermoid carcinoma of the nasopharynx), and KB-VIN (vincristine-resistant KB subline showing MDR phenotype by overexpressing P-gp). Compounds **5** and **6** were nontoxic even at 40 μM , and only compounds **1** and **12**, which are dimeric alkaloids with tetrahydro- β -carboline and tetrahydroisoquinoline units, showed potent antiproliferative activity at submicromolar levels against chemosensitive cell lines (Table 2). The multidrug resistant (MDR) cell line KB-VIN was resistant against **1** and **12** (9-demethyltubulosine), suggesting that these compounds might be substrates of drug transporter(s) such as P-gp. Both alkaloid dimers showed diminished antiproliferative activity when the culture time increased from 24 to 72 h, especially against the KB cell line, while the anticancer drugs doxorubicin (DOX) and paclitaxel (PXL), also known as P-gp substrates, displayed increased activity over time (Table 2). These observations suggested that **1** and **12** were likely detoxified within 48 h in the cultured cells, reversing the antiproliferative effect. Based on these observations, it was predicted that compound **1** could inhibit cell growth by inducing cell cycle arrest while the compound was active. Accordingly, flow cytometric analysis was used to determine whether compound **1** could induce cell cycle arrest (Figure 3). Unexpectedly, compound **1** showed no significant effects on cell cycle progression in MDA-MB-231 cells, even at 10-fold IC₅₀ concentration, while sub-G1 cells accumulated dramatically after 48 h of treatment. These results indicated that compound **1** induced apoptosis in a time- and dose-dependent manner.

Newly isolated **1** was further tested in the NCI-60 screening panel, in which the cells were incubated with the compound for 48 h (Figure S3). Compound **1** showed broad-spectrum inhibitory activity at submicromolar levels (10 to 100 nM) against most tumor types derived from breast, central nervous system, leukemia, melanoma, non-small-cell lung, ovary, prostate, and renal cancers, but was less active against an adriamycin-resistant ovarian line showing an MDR phenotype (NCI/ADR-RES) and also the HCT-15 tumor cell line, both of which express drug transporter(s). These results and those from an in-house assay using KB-VIN demonstrated that compound **1** would likely be a substrate of ABC drug transporter(s), such as P-gp. As the original crude extract showed strong antiproliferative activity against KB-VIN, it may contain an additional bioactive compound or compounds, effective against KB-VIN and/or inhibit the drug transporter(s).

In summary, 17 secondary metabolites were isolated, including three new compounds, 8-hydroxytubulosine (**1**), 2'-*O*-*trans*-sinapoylisoalangsideside (**2**), and 3 α -*trans*-sinapoyloxy-16 β -hydroxylup-20(29)-ene (**3**), from the leaves of *A. longiflorum*, a currently endangered

species. The dimeric alkaloids **1** and **12** (9-demethyltubulosine) displayed potent antiproliferative activities against chemosensitive human tumor cell lines, while they were ineffective against drug-transporter-overexpressing cells.

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotations were determined on a JASCO P-2200 digital polarimeter. ECD spectra were measured on a JASCO J-820 spectrometer. Infrared spectra (IR) were recorded with a Thermo Fisher Scientific Nicolet iS5 FT-TR spectrometer with samples in CH₂Cl₂. NMR spectra were obtained on JEOL JMN-ECA600 NMR spectrometers with tetramethylsilane as an internal standard, and chemical shifts are stated as δ values. HRMS data were recorded on a JMS-SX102A (FAB) or JMS-T100TD (DART) mass spectrometer. Analytical and preparative TLC were performed on precoated silica gel 60F₂₅₄, RP-18F₂₅₄ plates (0.25 or 0.50 mm thickness; Merck) and NH₂ silica gel 60F₂₅₄ plates (0.25 or 0.50 mm thickness; Wako). MPLC was performed on a Combiflash Rf (Teledyne Isco) instrument with silica gel (RediSep Rf Gold High Performance). Preparative HPLC was conducted on a GL Science recycling system using an InertSustain C₁₈ column (5 μ m, 20 $\times$ 250 mm).

Plant Material.

The Natural Products Branch of the Developmental Therapeutics Program of the NCI (Frederick, MD, USA) provided a crude organic CH₃OH–CH₂Cl₂ (1:1) extract (N033539) of *A. longiflorum* leaves collected in Sarawak, Malaysia. The plant material was collected and identified by J.S. Burley in June 1989, and a voucher specimen is housed at the Smithsonian Institution Museum of Natural History (Voucher #Q660–2659).

Extraction and Isolation.

A 50% CH₃OH–CH₂Cl₂ extract of the leaves of *A. longiflorum* (10.0 g) was partitioned between H₂O and EtOAc to yield H₂O-soluble (5.7 g) and EtOAc-soluble (3.9 g) fractions. The EtOAc-soluble fraction was subjected to silica gel column chromatography (CC) eluted with a gradient system [n-hexane–EtOAc, 100:0 (500 mL) $\rightarrow$ 80:20 (500 mL) $\rightarrow$ 67:33 (500 mL) $\rightarrow$ 50:50 (500 mL) $\rightarrow$ 33:67 (500 mL) $\rightarrow$ 0:100 (500 mL) $\rightarrow$ EtOAc–CH₃OH 95:5 (500 mL) $\rightarrow$ 90:10 (500 mL) $\rightarrow$ 80:20 (500 mL) $\rightarrow$ 0:100 (800 mL)] to yield 12 fractions, F1–F12. Fraction F2 (877.3 mg) was purified, in turn, by silica gel CC with *n*-hexanes–EtOAc (9:1/4:1), reversed-phase preparative HPLC with H₂O–acetone (1:9), and reversed-phase preparative TLC with H₂O–acetone (1:10), to afford compound **14** (2.5 mg). Fraction F5 (357.0 mg) was subjected to silica gel MPLC (RediSep Rf Gold 12g) with *n*-hexanes–EtOAc (1:0 to 7:3) and EtOAc, followed by reversed-phase preparative HPLC with H₂O–acetone (1:14), to afford compound **17** (2.8 mg). Fraction F8 (196.9 mg) was subjected to silica gel CC eluted with CH₂Cl₂–EtOAc (1:0/9:1/17:3/4:1/7:3/0:1) followed by EtOAc–CH₃OH (1:1/0:1) to yield five subfractions, 8a–e.

Subfraction 8b (5.2 mg) was purified by reversed-phase preparative HPLC with H₂O–acetone (1:4) to yield compound **16** (0.7 mg). Subfraction 8c (50.4 mg) was purified sequentially by silica gel CC with CH₂Cl₂–acetone (1:0/3:2) followed by acetone–CH₃OH

(1:1/0:1), normal-phase preparative TLC with CH₂Cl₂–CH₃OH (9:1), and reversed-phase preparative HPLC with H₂O–acetone (1:7), to afford compound **3** (3.4 mg). Fraction F11 (290.3 mg) was subjected to silica gel MPLC (RediSep Rf Gold 40g) with CH₂Cl₂–CH₃OH (1:0/9:1/85:15/0:1), followed by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O–CH₃OH (3:2/2:3/1:2/0:1) and reversed-phase preparative HPLC with H₂O–acetone (1:14), to afford compound **11** (0.7 mg). Fraction F12 (664.0 mg) was subjected to silica gel CC, eluted with CH₂Cl₂–CH₃OH (95:5/8:1/ 17:3/1:1/0:1), to yield eight subfractions, 12a–h. Subfraction 12e was purified by silica gel CC with EtOAc–CH₃OH (1:0/8:1/7:3/1:1/0:1) followed by reversed-phase preparative TLC with H₂O–CH₃OH (3:7) to afford compounds **2** (1.7 mg), **6** (1.3 mg), and **7** (0.6 mg). Subfraction 12f was subjected to silica gel CC with *n*-hexanes–EtOAc (4:1/1:1:1:4/0:1) followed by EtOAc–CH₃OH (1:1/0:1) and purified by reversed-phase preparative TLC with H₂O–CH₃OH (2:3) to afford compounds **4** (1.2 mg), **5** (3.3 mg), and **8** (1.0 mg). Subfraction 12h was purified by preparative TLC of NH₂ silica gel with CH₃Cl–CH₃OH (20:1) to yield five subfractions, 12h1–5. Subfractions 12h1 and 12h2 contained pure compounds **13** (1.5 mg) and **1** (7.2 mg), respectively. Subfraction 12h4 was purified again by silica gel CC with CH₃Cl–CH₃OH–TEA (20:80:0.2) to obtain compound **12** (1.1 mg).

A part of the H₂O-soluble extract (4.95 g) was subjected to silica gel MPLC (RediSep Rf Gold 40g) with CH₂Cl₂–CH₃OH (9:1/4:1/7:3/0:1) to yield five fractions, F'1–5. Fraction F'2 (248.5 mg) was purified by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O–CH₃OH (3:2/1:1/0:1) to afford compound **10** (13.0 mg). Fraction F'4 (413.1 mg) was subjected to silica gel MPLC (RediSep Rf Gold 12g) with CH₂Cl₂–CH₃OH (9:1/4:1/7:3/0:1), followed by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O–CH₃OH (3:2/0:1), to obtain compounds **9** (44.4 mg) and **15** (1.5 mg).

8-Hydroxytubulosine (1): yellow, amorphous solid; $[\alpha]_D^{26} -32.8$ (*c* 0.12, pyridine); IR $\nu_{\max}$ (CHCl₃) 3310, 2922, 2850, 1591, 1458, 1118, 748 cm⁻¹; ¹H and ¹³C NMR, see Table 1; HRFABMS *m/z* 492.2860 [M + H]⁺ (calcd for C₂₉H₃₈N₃O₄, 492.2862).

2'-O-trans-Sinapoylisoalangiside (2): pale yellow, amorphous solid; $[\alpha]_D^{24} -191.9$ (*c* 0.003, CH₃OH); IR $\nu_{\max}$ (CH₃OH) 3359, 1706, 1655, 1591, 1514, 899 cm⁻¹; ¹H and ¹³C NMR, see Table 1; HRFABMS *m/z* 712.2566 [M + H]⁺ (calcd for C₃₆H₄₂NO₁₄, 712.2605).

3 α -trans-Sinapoyloxy-16 β -hydroxylup-20(29)-ene (3): pale yellow, amorphous powder; $[\alpha]_D^{26} +18.4$ (*c* 0.125, CH₃OH); IR $\nu_{\max}$ (CHCl₃) 3523–3419, 2941, 2867, 1692, 1597, 1514, 1455, 1283, 1153, 1114, 753 cm⁻¹; ¹H and ¹³C NMR, see Table 2; HRFABMS *m/z* 649.4465 [M + H]⁺ (calcd for C₄₁H₆₁O₆, 649.4468).

Assay for Antiproliferative Activity.

Antiproliferative activity of the compounds was determined by the sulforhodamine B (SRB) assay, as described previously.²³ In brief, freshly prepared cell suspensions were seeded on 96-well microtiter plates at a density of 4000–12 000 cells per well and cultured for 72 h with test compounds. For a time-course study with **1** and **12**, cells were inoculated on 96-well microtiter plates at a density of 4000–12 000 cells per well 24 h prior to treatment with

the compounds for 24, 48, and 72 h. The cells were fixed in 10% trichloroacetic acid and then stained with 0.04% SRB. The absorbance at 515 nm of 10 mM Tris base-solubilized protein-bound dye was measured using a microplate reader (ELx800, BioTek) operated by Gen5 software (BioTek). IC₅₀ data were calculated statistically (MS Excel) from at least three independent experiments performed with duplication ($n = 6$). All human tumor cell lines used in this study, except KB-VIN, were obtained from the Lineberger Comprehensive Cancer Center (UNC-CH) or from ATCC (Manassas, VA, USA). KB-VIN was a generous gift from Professor Y.-C. Cheng of Yale University. The cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 25 mM HEPES (Corning), containing 10% fetal bovine serum (Specialty Media), 100 μ g/mL streptomycin, and 100 IU penicillin (Corning). KB-VIN stock cells were maintained in the presence of 100 nM vincristine. Paclitaxel was used as an experimental control. A list of cell lines and methodology used for NCI-60 screening is available online from NCI.²⁴

Cell Cycle Analysis.

Impact of compound against the cell cycle was evaluated by measurement of cellular DNA content with propidium iodide/RNase (BD Biosciences) as described previously.²⁴ MDA-MB-231 cells were harvested after 24 or 48 h treatment with compounds. Fixed and stained cells were analyzed by flow cytometry (LSRFortessa, BD Biosciences). Experiments were repeated a minimum of two times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Selected HMBC correlations and COSY connectivities (600 MHz) for **1–3**.

Figure 2.
Key NOESY correlations (600 MHz) for **2** and **3**.

Figure 3.

Effects of compound **1** on cell cycle progression. Triple-negative breast cancer MDA-MB-231 cells were treated with **1** for 24 or 48 h, as indicated. DMSO or a tubulin polymerization inhibitor (combretastatin A-4; CA-4) was used at 0.1 μM ($3\times \text{IC}_{50}$) as a vehicle control (CTRL) or a mitotic inhibitor arresting cells at G2/M, respectively. Compound **1** was used based on its IC_{50} at 1- ($1\times \text{IC}_{50}$), 3- ($3\times \text{IC}_{50}$), or 10- ($10\times \text{IC}_{50}$) fold. Cell-cycle distributions of treated cells were analyzed by flow cytometry after staining with propidium iodide (PI) in the presence of RNase.

Chart 1.

Table 1.

¹H NMR and ¹³C NMR Spectroscopic Data of Compounds 1 and 2

Compound 1 (600 MHz, DMSO- <i>d</i> ₆)			
position	δ_{H} (J in Hz)	δ_{C}	
1	2.58 m ^c	36.4	
	1.03 q (12.0)		
2	1.63 m ^d	36.0	
3	1.23 m	41.4	
4	2.97 m ^e	61.1	
	1.95 t (11.4)		
6	2.94 m ^e	51.5	
	2.26 td (10.2, 5.4)		
7	2.56 m ^c	23.7	
7a		115.3	
8		147.1	
9		134.1	
10		150.5	
11	6.40 s	99.9	
11a		134.0	
11b	2.95 m ^e	62.4	
12	1.60 m ^d	22.9	
	1.09 dd (14.8, 7.2)		
13	0.86 t (7.2)	11.0	
1'	4.11 d (12.0)	48.5	
3'	3.10 dt (12.6, 4.8)	41.3	
	2.86 m ^e		
4'	2.48 m	22.5	
4'a		106.2	
4'b		127.8	

Compound 1 (600 MHz, DMSO- d_6)			
position	δ_H (J in Hz)	δ_C	
5'	6.65 d (2.4)	101.6	
6'		150.0	
7'	6.49 dd (8.4, 2.4)	109.9	
8'	7.02 d (8.4)	110.9	
8'a		129.9	
9'a		138.7	
10'	1.81 t (12.0)	37.7	
	1.52 m ^d		
OMe-9	3.64 s	60.1	
OMe-10	3.73 s	55.7	
OH-8	8.68 s		
OH-6'	8.47 s		
NH-9'	10.28 s		
Compound 2 (600 MHz, CD ₃ OD)			
position	δ_H (J in Hz)	δ_C	
1	6.63 s	111.9	
2		146.2	
3		148.0	
4	6.49 s	113.7	
4a		127.8	
5	2.32 m	28.7	
6 β	4.16 ddd (12.6, 6.0, 3.6)	42.9	
6 α	2.85 ddd (12.6, 9.6, 6.0)		
8		166.3	
8a		110.4	
9	7.26 d (2.4)	147.5	
11	5.41d (1.8)	96.7	
12	2.60 ddd (10.2, 6.0, 1.8)	44.5	

Compound 1 (600 MHz, DMSO- <i>d</i> ₆)			
position	<i>J</i> (J in Hz)	<i>H</i> _q	<i>δ</i> _C
12a	2.78 dddd		24.4
	(10.8, 6.0, 6.0, 2.4)		
13β	2.15 ddd		28.8
	(13.8, 6.0, 4.2)		
13α	1.88 ddd		
	(13.8, 10.8, 4.2)		
13a	4.51 br t (4.2)		55.6
13b	-		130.4
14	5.66 dt (17.4, 10.2)		134.0
15	5.38 dd (17.4, 1.8)		120.7
	5.31 dd (10.2, 1.8)		
1'	4.86 overlap		96.7
2'	4.72 dd (9.6, 8.4)		74.4
3'	3.55 dd (9.0, 8.4)		75.9
4'	3.36 m		71.8
5'			78.7
6'	3.68 dd (12.0, 5.4)		62.7
	3.91 dd (12.0, 1.8)		
OMe-3	3.84 s		56.8
C=O	-		167.9
α	6.04 d (15.6)		115.8
β	7.20 d (15.6)		147.0
1''			126.6
2'' 6''	6.83 s		107.6
3'' 5''			149.5
4''			139.2
OMe-3''	3.94 s		57.1
OMe-5''			

^dOverlapping signals.

^bOverlapping signals.

Overlapping signals.

Overlapping signals.

Overlapping signals.

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Table 2.¹H NMR and ¹³C NMR Spectroscopic Data (600 MHz, CDCl₃) of Compound 3

position	δ_{H} (J in Hz)	δ_{C}
1	1.44 m ^a	34.1
	1.19 td (13.8, 6.0)	
2	1.93 m ^b	23.0
	1.67 m ^a	
3	4.76 br t (3.0)	78.4
4		37.2
5	1.28 m ^a	50.5
6	1.49 m ^a	18.2
	1.42 m ^a	
7	1.49 m ^a	34.1
	1.40 m ^a	
8		42.1
9	1.42 m ^a	50.0
10		37.0
11	1.47 m ^a	20.7
	1.25 m ^a	
12	1.66 m ^a	24.8
	1.06 m ^a	
13	1.67 m ^a	37.2
14		44.2
15	1.58 t (12.6)	36.9
	1.32 dd (12.6, 4.8)	
16	3.62 dt (12.6, 4.8)	77.2
17		48.6
18	1.40 m ^a	47.8
19	2.51 td (10.8, 6.0)	47.6
20		149.9
21	1.99 m ^b	29.9
	1.38 m ^a	
22	1.64 m ^a	37.7
	1.28 m ^a	
23	0.90 s	28.0
24	0.92 s	21.8

position	δ_{H} (<i>J</i> in Hz)	δ_{C}
25	0.89 s	15.98
26	1.07 s	16.04
27	1.08 s	16.4
28	0.81 s	11.7
29	4.71 d (1.8)	109.8
	4.59 d (2.4, 1.8)	
30	1.68 s	19.4
C=O		166.8
<i>a</i>	6.33 d (16.2)	116.6
<i>β</i>	7.59 d (16.2)	144.7
1'		126.3
2', 6'	6.79 s	105.1
4'		137.0
3', 5'		147.2
OMe-3', 5'	3.95 s	56.4
OH-16	1.16–1.34 m ^{<i>a</i>}	
OH-4'	5.75 s	

^{*a*}Overlapping signals.

^{*b*}Overlapping signals.

Table 3.

Antiproliferative Activities of Compounds 1 and 12 Depending on Time of Culture

compound	time of culture (h)	cell line ^a (IC ₅₀ μ M $\pm$ SD) ^b				
		A549	MDA-MB-231	MCF-7	KB	KB-VIN
1	24	0.21 $\pm$ 0.01	0.06 $\pm$ 0.02	0.12 $\pm$ 0.04	0.09 $\pm$ 0.02	8.90 $\pm$ 0.38
	48	0.23 $\pm$ 0.02	0.08 $\pm$ 0.01	0.16 $\pm$ 0.03	0.15 $\pm$ 0.05	11.1 $\pm$ 0.66
	72	0.28 $\pm$ 0.06	0.08 $\pm$ 0.01	0.21 $\pm$ 0.01	0.21 $\pm$ 0.02	>10
12	24	0.36 $\pm$ 0.03	0.19 $\pm$ 0.01	0.25 $\pm$ 0.00	0.29 $\pm$ 0.01	>10
	48	0.30 $\pm$ 0.01	0.23 $\pm$ 0.00	0.27 $\pm$ 0.01	0.47 $\pm$ 0.03	>10
	72	0.62 $\pm$ 0.02	0.22 $\pm$ 0.01	0.38 $\pm$ 0.02	0.62 $\pm$ 0.03	>10
DOX ^c	24	0.48 $\pm$ 0.29	0.78 $\pm$ 0.25	0.72 $\pm$ 0.01	0.82 $\pm$ 0.19	>1
	48	0.13 $\pm$ 0.10	0.23 $\pm$ 0.05	0.35 $\pm$ 0.06	0.34 $\pm$ 0.14	>1
	72	0.08 $\pm$ 0.02	0.18 $\pm$ 0.01	0.22 $\pm$ 0.02	0.22 $\pm$ 0.02	>1
PXL ^c (nM)	24	>10	>10	8.37 $\pm$ 0.65	8.21 $\pm$ 2.18	>3000
	48	10.0 $\pm$ 1.51	12.1 $\pm$ 0.37	8.54 $\pm$ 0.64	6.69 $\pm$ 0.82	2879 $\pm$ 91.78
	72	8.76 $\pm$ 0.87	8.51 $\pm$ 0.10	7.52 $\pm$ 0.14	5.71 $\pm$ 0.06	2383 $\pm$ 96.61

^a A549 (lung carcinoma), MDA-MB-231 (triple-negative breast cancer), MCF-7 (estrogen receptor-positive and HER2-negative breast cancer), KB (originally isolated from epidermoid carcinoma of the nasopharynx), KB-VIN (P-gp-overexpressing MDR subline of KB).

^b Antiproliferative activity expressed as IC₅₀ value for each cell line, the concentration of compound that caused 50% reduction relative to untreated cells determined by the SRB assay. SD: Standard deviation ($n = 6$).

^c DOX (doxorubicin) and PXL (paclitaxel).