Cytotoxic Arylnaphthalene Lignans from a Vietnamese Acanthaceae, *Justicia patentiflora*

Sophie Susplugas,[†] Nguyen Van Hung,[‡] Jérôme Bignon,[†] Odile Thoison,[†] Anna Kruczynski,[§] Thierry Sévenet,[†] and Françoise Guéritte*,[†]

Institut de Chimie des Substances Naturelles, CNRS, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France, Institute of Chemistry, NCST, Nghia Do, Cau Giay, Hanoi, Vietnam, and Institut de Recherche Pierre Fabre, Centre de Recherche en Oncologie Expérimentale, Parc Technologique du Canal, 3 Rue des Satellites, 31400, Toulouse, France

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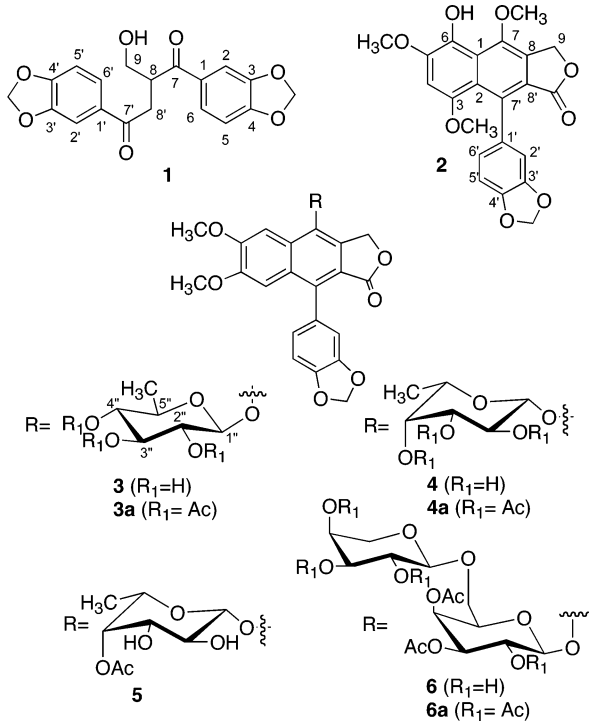
One new norlignan (**1**) and five new lignans (**2**−**6**) were isolated from the leaves and stems of *Justicia patentiflora* by a bioassay-guided purification. Five known compounds, carinatone, diphyllin, justicidin A, taiwanin E, and tuberculatin, were also found in *J. patentiflora*. Most of the new compounds display significant activity in in vitro cytotoxic assays against KB, HCT116, and MCF-7 cancer cell lines and arrest the cell cycle in the G_0/G_1 phase.

The genus *Justicia* (Acanthaceae) consists of about six hundred species, and several of them contain lignans displaying various biological activities.[1] In the course of our ongoing search to investigate bioactive plants from Vietnam, *Justicia patentiflora* Hemsl. (syn. *Mananthes patentiflora* Bremek.) was collected in Tram Täu, Yen Bai Province, in North Vietnam.[2] We selected this plant for a phytochemical study due to its high cytotoxicity against KB cells. Thus, the EtOAc extract of leaves and stems of *J. patentiflora*, respectively, produced 64% and 76% inhibition of cell growth at 0.1 μg mL^{-1}. Bioassay-directed fractionation of the extracts provided six new molecules named justiflorinol (**1**), justicinol (**2**), patentiflorin A (**3**), patentiflorin B (**4**), 4″-*O*-acetylpatentiflorin B (**5**), and 4″-*O*-acetylmananthoside B (**6**). In this paper, we report the structural elucidation of these compounds together with their cytotoxic evaluation as well as cell cycle and in vivo studies. We also describe the isolation of five known compounds: carinatone, diphyllin, justicidin A, taiwanin E, and tuberculatin. At the beginning of this study, a paper was published by Chen et al., who reported the isolation from *Mananthes patentiflora* (considered to be identical to *J. patentiflora*) of two other diphyllin glycosides, mananthosides A and B, that also demonstrated cytotoxicity.[3]

Chart 1. Structures of Compounds **1**−**6**



Results and Discussion

An EtOAc extract of the dried leaves of *J. patentiflora* was chromatographed on a silica gel column and further purified by HPLC to afford justicidin A,[4,5] diphyllin,[4] taiwanin E,[6] tuberculatin,[7] carinatone,[8] and the new compounds **1** and **2**. The air-dried stems of *J. patentiflora* were extracted with EtOAc and MeOH. Purification of the EtOAc and MeOH extracts by chromatography over silica gel followed by HPLC or preparative TLC resulted in the isolation of four new lignan glycosides (**3**−**6**). The structures of the known compounds were determined by comparison with literature data.

Compound **1** exhibited the molecular formula $C_{19}H_{16}O_7$, as deduced from its HRESIMS, which gave an ion ([M + Na]$^+$) at *m/z* 379.0745. A McLafferty rearrangement led to an ion at *m/z* 325 $[C_{18}H_{13}O_6]^+$ after loss of a hydroxymethylene group and to a fragment at *m/z* 149 $[C_8H_5O_3]^+$ corresponding to the methylene dioxyphenylacylium ion. The ^{1}H NMR spectrum of **1** displayed signals for two phenyl units with an ABX system (δ 6.89, 1H, d, J = 8.0 Hz; 7.51, 1H, J = 1.7 Hz; 7.70, 1H, dd, J = 8.0, 1.7 Hz and 6.85, 1H, d, J = 8.0 Hz; 7.44, 1H, d, J = 1.7 Hz; 7.61, 1H, dd, J = 8.0, 1.7 Hz) and two methylenedioxy groups at δ 6.02 and 6.03. The connectivity between H-8, H-8′, and H-9 was deduced from the COSY spectrum. Moreover, the HMBC experiment revealed the correlations between H-2′ (δ 7.51) and H-6′ (δ 7.70) of the aromatic ring and C-7′ (δ 200.7) and also between H-2 (δ 7.44), H-6 (δ 7.61) and C-7 (δ 196.2). All these data indicated the presence of two piperonyl units linked by a butadione spacer. Thus, compound **1** was characterized as a new 9′-norlignan named justiflorinol. Unfortunately, the limited amount available of this compound did not allow the elucidation of its absolute configuration.

* To whom correspondence should be addressed. Tel: 33 1 69 82 45 80. Fax: 33 1 69 07 72 47. E-mail: gueritte@icsn.cnrs-gif.fr.
† CNRS.
‡ NCST, Hanoi.
§ IRPF, Toulouse.

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Table 1. ^{1}H NMR Data (δ (J Hz)) of Compounds **3**, **4**, and **5** in $CDCl_3$ and **6** in CD_3OD

position	**3**	**4**	**5**	**6**
3	7.11 s	7.11 s	7.09 s	7.04 s
6	7.92 s	7.98 s	7.99 s	8.14 s
9	5.53 d (15)	5.46 d (15)	5.46 dd (15.5, 1)	5.71, d (15)
	5.63 d (15)	5.55 d (15)	5.54 d (15.5)	5.47, d (15)
2′	6.85 d (1.0)	6.85 d (1.0)	6.84 d (1.4)	6.82, d (1.5)
5′	7.00 d (7.8)	6.97 d (7.8)	6.97 d (7.9)	6.96, d (8.2)
6′	6.81 dd (7.8, 1.0)	6.82 dd (7.8, 1.0)	6.81 dd (7.9, 1.4)	6.76, dd (8.2, 1.5)
4-OMe	3.80 s	3.82 s	3.77 s	3.97, s
5-OMe	4.05 s	4.06 s	4.06 s	3.68, s
OCH_2O	6.07 s, 6.11 s	6.06 s, 6.11 s	6.06 s, 6.10 s	6.04, s
1″	4.84 d (7.8)	4.80 d (7.8)	4.82 d (7.8)	4.98, d (7.8)
2″	3.87 dd (7.8, 9.1)	4.03 m	4.07 dd (7.8, 9.7)	4.09, dd (7.8, 10.2)
3″	3.57 dd (9.1, 9.1)	3.66 m	3.86 dd (9.7, 3.5)	4.44, dd (3.4, 10.2)
4″	3.38 m	3.80 m	5.17 brd (3.5, 1)	5.44, d (3.4)
5″	3.35 m	3.64 m	3.72 brd (6.5)	4.10, dd (7.8, 10.1)
6″	1.35 d (7.9)	1.38 d (6.2)	1.25 d (6.5)	3.80, d (10.1), 3.7, m
C3″COCH_3				2.06, s
C4″COCH_3			2.28, s	2.20, s
1‴				4.16, d (6.4)
2‴				3.40, m
3‴				3.40, m
4‴				3.70, m
5‴				3.46, d (12.4)
				3.77, d (12.4)

The HRESIMS (positive-ion mode) of compound **2** gave the quasimolecular ion peak ($[M + Na]^+$) at *m/z* 433,0906, corresponding to the formula $C_{22}H_{18}O_8Na$. In the IR spectrum, absorptions for an aromatic γ-lactone at 1762 cm^{-1} and a methylenedioxy group at 933 cm^{-1} are observed. The ^{1}H NMR spectrum of **2** showed the ABX pattern of one piperonyl unit at δ 6.77 (d, J = 1.5 Hz), 6.78 (dd, J = 7.6, 1.5 Hz), and 6.96 (d, J = 7.6 Hz). The singlet at δ 5.51 assigned to the methylene group of the γ-lactone suggested the presence of a 1-aryl-2,3-naphthalide type.[7] The position of the three *O*-methyls on C-3, C-5, and C-7 at δ 3.77, 4.08, and 4.17 was deduced from the HMBC experiment. The slight doubling of the methylenedioxy signal at δ 6.05 and 6.09 (d, J = 1.4 Hz) may be due to atropoisomerism, as the methoxyl in the C-3 position could hinder the rotation of the aryl along the C-7′/C-1′ bond.[9] From these data, the structure of **2** was established as a new arylnaphthalene lignan named justicinol.

Compounds **3**−**6** have in common in their ESI mass spectrum a fragment at *m/z* 403 $[aglycone + Na]^+$, which could correspond to a diphyllin molecule resulting from the loss of a sugar moiety. Examination of the NMR spectra of the compounds (Tables 1 and 2) indicates that compounds **3**, **4**, and **5** are monoglycosylated, whereas **6** is a diglycosylated lignan. The ^{1}H NMR spectrum of the four compounds (Table 1) confirmed the presence of a 1-aryl-2,3-naphthalide unit owing to the shift of the γ-lactone methylene group included in the range between 5.53 and 5.71 ppm. The HMBC spectrum of compounds **3**−**6** gave the linking of the sugar to the diphyllin moiety with a C-7/H-1″ correlation. The HRESIMS of compounds **3** and **4** afforded a quasimolecular peak ($[M + Na]^+$) at *m/z* 549.1364 and 549.1401, respectively, which both correspond to the formula $C_{27}H_{26}O_{11}Na$. The ^{1}H NMR and ^{13}C NMR data of **3** and **4** (Tables 1 and 2) revealed the presence of a sugar moiety that possesses a methyl group at C-5″ with protons resonating at δ 1.35 and 1.38, respectively. The structural determination of both deoxysugars was deduced from the NMR data of the acetylated derivatives **3a** and **4a**. The coupling constants of the sugar moiety for compound **3a** show that the vicinal protons are diaxially oriented with coupling constants varying from 6.0 to 9.7 Hz. These data together with the shift of the anomeric proton at 4.84 ppm indicate that the sugar is a 6-β-deoxyglucose (β-quinovose).

Table 2. ^{13}C NMR Data (δ) of **3**−**5** in $CDCl_3$ and **6** in CD_3OD

position	**3**	**4**	**5**	**6**
1	132.2	131.2	131.5	132.0
2	129.0	127	127.5	128.9
3	106.9	106.6	106.5	107.1
4	151.7	150.0	152.2	152.0
5	153.3	152.2	152.5	153.5
6	102.8	101.3	101.2	102.8
7	146.4	147.0	144.7	146.2
8	131.9	131.6	131.8	132.0
9	69.1	67.7	67.5	69.4
1′	130.0	128.0	128.0	130.5
2′	112.0	111.2	111.1	111.9
3′	149.0	147.0	147.6	149.1
4′	149.0	147.0	147.6	149.1
5′	109.0	108.7	108.1	109.1
6′	125.0	124.5	123.6	124.8
7′	137.6	136.0	136.7	137.9
8′	119.9	120.0	120.0	120.0
4-OMe	56.4	55.9	55.8	56.1
5-OMe	56.7	56.5	56.2	56.8
OCH_2O	102.6	101.7	101.2	102.7
C=O	172.2	170.0	171.0	172.2
1″	106.7	105.5	105.4	106.8
2″	74.6	72.2	71.9	70.4
3″	76.8	74.3	73.1	69.6
4″	72.6	71.4	72.6	75.0
5″	75.5	71.2	69.2	73.8
6″	17.8	16.5	16.5	68.6
C3″O*C*OCH_3				172.2
C3″CO*C*H_3				20.8
C4″O*C*OCH_3			172.5	172.4
C4″CO*C*H_3			20.9	20.8
1‴				105.1
2‴				74.3
3‴				73.8
4‴				69.6
5‴				67.0

This was confirmed by acid hydrolysis of **3** leading to diphyllin and a sugar identified with an authentic sample of D-quinovose. Therefore, the structure of **3** was established as 7-*O*-β-D-quinovopyranosyldiphyllin, which was named patentiflorin A.

The ^{1}H NMR spectra of compounds **4** and **4a** were very similar to those of **3** and **3a**, respectively, except for the coupling constant between H-3″ and H-4″, which in **4a** corresponds to an equatorial−axial relationship ($J_{H-3'',H-4''}$

= 3.4 Hz). Thus, the structure of **4** was established as 7-*O*-β-L-fucopyranosyldiphyllin, which was called patentiflorin B.

The HRESIMS spectrum of compound **5** gave a quasi-molecular ion peak at *m/z* 591.1487 ([M + Na]$^+$) that corresponded to the molecular formula [$C_{29}H_{28}O_{12}$+Na]$^+$. The ^{1}H NMR and ^{13}C NMR data of **5** (Tables 1 and 2) revealed the presence of one *O*-acetyl group in addition to signals corresponding to the glycosylated arylnaphthalene lignan **4**. The COSY cross-peaks and the value of the vicinal coupling constants confirmed that the sugar moiety is identical to that of compound **4**. The C-4″ position of the *O*-acetyl group was deduced from the HMBC correlations and from the shift of the H-4″ to lower field than for the equivalent proton of compound **4**. Therefore the structure of compound **5** was assigned as 4″-*O*-acetyl-7-*O*-β-L-fucopyranosyldiphyllin and named 4″-*O*-acetylpatentiflorin B.

HRESIMS measurement of compound **6** gave a quasi-molecular ion peak at *m/z* 781.1932 ([M + Na]$^+$) corresponding to the molecular formula [$C_{36}H_{38}O_{18}$+Na]$^+$. The MS^n experiment of the [M + Na]$^+$ ion gave fragments at *m/z* 649.1 [M + Na − 132]$^+$ and *m/z* 402.6 [M + Na − 132 − 246]$^+$ attributed to the losses of a pentose and a diacetylhexose. Three other fragments appear at *m/z* 401.2 [M + Na − genin]$^+$, 341.0 [M + Na − genin − 60]$^+$, and 281.0 [M + Na − genin − 60 − 60]$^+$ corresponding to the diglycoside chain, which lost twice one acetic acid moiety. The ^{1}H NMR spectrum (Table 2) confirmed the presence of two sugar units with signals for the anomeric protons at 4.98 ($J_{H-1'',H-2''}$ = 7.8 Hz) and 4.16 ppm ($J_{H-1''',H-2'''}$ = 6.4 Hz), which give rise in the ^{13}C NMR spectrum to signals at δ 106.8 and 105.1, respectively. A COSY experiment gave the connection of the protons of the first sugar, and the coupling constants between the protons of the first sugar linked to diphyllin showed that all the protons are in an axial position except the hydrogen at position 4″. These data were compatible with the presence of β-D-galactopyranose. Acetylation of **6** to **6a** was performed to elucidate the structure of the second sugar. A COSY experiment, measurement of the vicinal coupling constants, and the chemical shift of the anomeric proton at δ 4.16 ppm suggest that this second sugar is α-L-arabinopyranose. This was confirmed first by the ROESY experiment of **6** showing correlations of H-3″ with H-4″ and H-5″ and of H-3‴ with H-4‴ and second by comparison of the acidic hydrolysate of **6** with authentic samples of L-arabinose and D-galactose on TLC. Consequently the structure of compound **6** was established as 7-*O*-[α-L-arabinopyranosyl-(1‴→6″)-β-D-galactopyranosyl]diphyllin, for which we proposed the trivial name of 4″-*O*-acetylmananthoside B, the acetylated derivative of mananthoside B previously isolated from *Mananthes patentiflora*.[3]

The isolated compounds were screened for their in vitro cytotoxic activity against a panel of cancer cell lines (Table 3) and compared to the cytotoxicity of docetaxel and doxorubicin. The lignans **3** to **6** and their acetylated derivatives exhibited significant cytotoxicities. The most active compounds was found to be patentiflorin A (**3**), inhibiting the growth of KB and MCF 7 cell lines with an IC_{50} value in the nanomolar range. Interestingly, compounds **3**, **3a**, and **5** present significant IC_{50}'s against MCF-7 sensitive and resistant cell lines.

To determine the mechanism of growth arrest by patentiflorin A (**3**), we assayed the changes in the cell cycle profile (Figure 1). Treatment of asynchronously growing HCT 116 cells with 10^{-8} M **3** caused a G1 arrest. The proportion of cells in the S phase fell from 70 to 41%, while the G1 fraction rose from 21 to 42% by 48 h. The same results were obtained on MCF7 cells. Thus, patentiflorin A (**3**) inhibits the cell cycle progression at the G_0/G_1 phase. We also observed that compounds **3a**, **5**, **6**, and **6a** showed the same G_0/G_1 arrest of both cell lines. The mechanism of action of these compounds is thus different from that of the aryltetralin lignans podophyllotoxin and etoposide, which are mitotic and G2-specific arrest agents, respectively.[10] It has to be noted that a natural cytotoxic arylnaphthalene analogue, cleistanthine A, isolated from an Euphorbiaceae, *Cleistanthus collinus*, was found to arrest cells in the G_1 phase as observed for patentiflorin A (**3**) and was described as an antitumor compound causing complete regression of ascites tumors in mice.[11,12] Compound **3** was thus selected for an in vivo study and tested in P388-bearing mice. The P388 leukemia model was selected for

Table 3. Evaluation of the Cytotoxic Activity of Compounds **1**−**6** and **3a**, **4a**, and **6a**

	cell line IC_{50} (μMol/L)			
compound	KB[a]	HCT 116[b]	MCF7-S[c]	MCF7-R[c]
1	>10			
2	0.30		0.36	
3	0.004	0.012	0.003	0.040
4	0.006	0.25	0.30	
5	0.024	0.030	0.012	0.10
6)	0.50	1.20	0.26	2.20
3a	0.014	0.016	0.002	0.065
4a	0.020	0.035	0.045	
6a	0.18	0.26	0.080	8.0
docetaxel	0.002	0.004	0.020	
doxo[d]			0.17	50

[a] Human epidermoid carcinoma. [b] Human colon carcinoma. [c] Human breast carcinoma. [d] Doxorubicin.

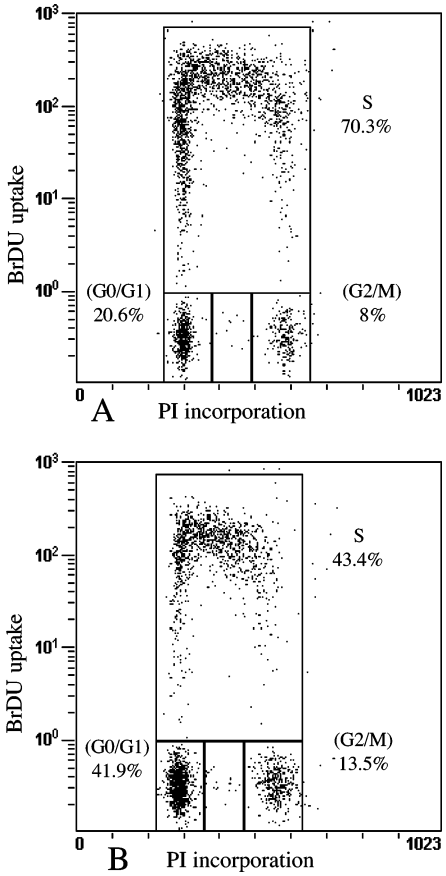

Figure 1. Cell cycle distribution, measured by bromodeoxyuridine (BrdU) uptake and propidium iodide (PI) staining, of HCT116 cells (control, A) and HCT116 cells treated with 10^{-8} M of **3** for 48 h (B).

this preliminary in vivo evaluation since it is a highly reproducible model.[13] Furthermore, it is also a rapid proliferating tumor model, therefore allowing an in vivo evaluation in a short time.[13] Two doses of patentiflorin A (**3**) were injected intraperitoneally 1 day after the tumor graft. The dose 5 mg/kg was shown to be toxic for the animal, quickly inducing its death. However, the dose of 2.5 mg/kg under these conditions induces no effect on tumor mass and no significant reduction of the body weight. Although test compounds with unknown mechanism of action are usually given i.p., as a single dose in preliminary studies, repeated administrations might be required to reveal an antitumor activity. Therefore, in the present study, additional in vivo evaluations of compound **3** have to be performed to determine whether it has antitumor activity. A repeated injection-based schedule should be tried in the P388 model. Furthermore, the effects of compound **3** in a solid tumor model, exhibiting different pharmacological properties (tumor growth rate and overall chemosensitivity), should also be investigated.

Experimental Section

General Experimental Procedures. Optical rotations were measured at 20 °C on a Jasco P1010 polarimeter, and ORD spectra were recorded on a Jobin Yvon CD6 dichrograph. UV spectra were recorded in CH_2Cl_2 or MeOH on a Varian Cary 100 spectrophotometer, and IR spectra on a Perkin-Elmer Spectrum BX FT-IR spectrometer. Mass spectra for MS^n were carried out using an LCQ-Deca Thermofinnigan ion trap mass spectrometer (Finnigan MAT, San Jose, CA). Mass spectra for MS were carried out using an LCT-Waters TOF mass spectrometer (Micromass, 2002, Manchester, UK) equipped with an ESI source and operated in positive polarity. An LC Alliance HT 2795 equipped with an autosampler was used for the infusion of compounds dissolved in MeOH. EI were recorded on an AutoMass Multi (Thermo-Finnigan). Measurements of exact mass were obtained using a ZAB-SEQ mass spectrometer. The NMR spectra were recorded in $CDCl_3$, $(CD_3)_2CO$, or CD_3OD. ^1H NMR and ^{13}C NMR spectra were recorded on an Aspect AC 250 or AC 300 or a Bruker AMX 400 or AMX 600 spectrometer. Chemical shifts (relative to TMS) are in ppm, and coupling constants (in brackets) in Hz. Column chromatography (CC) was performed using silica gel Merck H60. Preparative plates (PLC) [silica gel 60 F_{254}] were used for purification. Preparative HPLC was performed on a Waters PrepPak cartridge (SymmetryPrep C-18, 7 μm, 19 × 300 mm) at 15 mL/min using a Waters 600 multisolvent delivery system apparatus. Analytical HPLC was performed on a Waters Alliance 2690 (Symmetry C-18, 5 μm, 4.6 × 250 mm) with a Waters 600^E system controller.

Plant Material. *Justicia patentiflora* was collected in March 2001 at Tram Tau, Vietnam, with the collaboration of Dao Dinh Cuong of the National Center of Sciences and Technology of Hanoi (NCST). The plant was identified by J. Munzinger (Museum National d'Histoire Naturelle (MNHN), France). A voucher herbarium specimen VN 801 is kept at the herbarium of the Institute of Ecology (NCST-Hanoi) and at the Laboratoire de Phanérogamie, Museum National d'Histoire Naturelle (MNHN), Paris, France.

Extraction and Isolation. The air-dried leaves of *J. patentiflora* were extracted with EtOAc at room temperature. The extract (27.5 g) was chromatographed on a Si gel column with heptane−EtOAc (4:6) to yield 11 fractions (A−K), of which fractions H−K were found to be cytotoxic on KB human cancer cells. Fraction H (1.4 g) was chromatographed on silica gel (CH_2Cl_2−MeOH, 99:1) to yield carinatone (4 mg) and taiwanine E (6 mg). Fraction I (0.700 g) was separated by repeated CC on silica gel (CH_2Cl_2) and HPLC with the mobile phase H_2O−CH_3CN (7:3 or 5:5) to yield justicidin A (4.5 mg), justiflorinol **1** (8 mg), justicinol **2** (8 mg), and diphyllin (6 mg). Fraction K, by CC on silica gel (CH_2Cl_2−MeOH, 90:10), led to a fraction that was again chromatographed on silica gel (EtOAc−MeOH, 90:10) to afford tuberculatin (140 mg).

The air-dried stems of *J. patentiflora* were extracted with EtOAc, then MeOH. The EtOAc extract (11.8 g) was chromatographed to give 13 fractions on a silica gel column using heptane−AcOEt (5:5) as eluent. The most polar fractions by reversed-phase chromatography (H_2O−CH_3CN, 8:2) yielded the new compounds patentiflorin A (**3**, 8 mg), patentiflorin B (**4**, 5 mg), and 4″-*O*-acetylpatentiflorin B (**5**, 7 mg). The methanolic extract (5 g) was first partitioned with EtOAc and H_2O. The EtOAc extract (1.7 g) was then chromatographed on a silica gel column using methylene chloride as eluent. The new lignan 4″-*O*-acetylmananthoside B (**6**, 11 mg) was obtained after purification on preparative TLC plates using a mixture of MEK (methyl ethyl ketone)−EtOAc−HCOOH−H_2O, 3:5:0.1:0.1, as eluent.

Justiflorinol (1): white powder; $[\alpha]_D^{25}$ −75.6° (*c* 0.17, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 225 (3.22), 269 (2.85), 305 (2.94) nm; IR (KBr) ν_{max} 3441, 1667, 931 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.70 (1H, dd, J = 1.7, 8 Hz, H-6′), 7.61 (1H, dd, J = 1.7, 8 Hz, H-6), 7.51 (1H, J = 1.7 Hz, H-2′), 7.44 (1H, J = 1.7 Hz, H-2), 6.89 (1H, d, J = 8 Hz, H-5′), 6.85 (1H, d, J = 8 Hz, H-5), 6.03 (2H, s, OCH_2O), 6.02 (2H, s, OCH_2O), 4.24 (1H, m, H-8), 3.89 (2H, dd, J = 5.2, 6.8 Hz, H-9), 3.44 (2H, dd, J = 6.2, 18 Hz, H-8′); ^{13}C NMR (CD_3Cl_3, 75 MHz) δ 200.7 (C-7′), 196.2 (C-7), 152.0−152.1 (C-4, C-4′), 148.1−148.4 (C-3, C-3′), 130.1−131.3 (C-1, C-1′), 125.1 (C-6′), 124.6 (C-6), 108.3 (C-5′), 107.8−108.0 (C-5, C-2, C-2′), 101.9 (−OCH_2O−), 63.4 (C-9), 43.5 (C-8), 37.6 (C-8′); EIMS *m/z* 356 $[M]^+$, 325, 189, 149, 121; HRMS (ESI^+) *m/z* 379.0745 (calcd for $C_{19}H_{16}O_7Na$, 379.0794).

Justicinol (2): white powder; UV (MeOH) λ_{max} (log ϵ) 202.5 (4.46), 217.5 (4.23), 231 (4.25), 268 (4.48), 310 (3.69), 362 (3.49) nm; IR (KBr) ν_{max} 2918, 1762 (CO), 933 (OCH_2O) cm^{-1}; ^1H NMR (CD_3Cl_3, 300 MHz) δ 9.32 (1H, s, OH), 6.96 (1H, d, J = 7.6 Hz, H-5′), 6.78 (1H, s, H-4), 6.78 (1H, dd, J = 1.5, 7.6 Hz, H-6′), 6.77 (1H, dd, J = 1.5 Hz, H-2′), 6.09 and 6.05 (2H, 2d, J = 1.4 Hz, OCH_2O), 5.51 (2H, s, H-9), 4.17 (3H, s, 7-OMe), 4.08 (3H, s, 5-OMe), 3.77 (3H, s, 3-OMe); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.0 (CO), 153.9 (C-5), 148.9 (C-7), 147.6 (C-4′), 147.7 (C-3′), 146.5 (C-3), 135.9 (C-7′), 133.1 (C-8), 128.2 (C-1), 124.4 (C-1′), 123.6 (C-6′), 123.5 (C-2), 120.6 (C-8′), 115.9 (C-6), 110.5 (C-2′), 108.3 (C-5′), 101.3 (OCH_2O), 99.5 (C-7), 66.3 (C-9), 61.2 (7-OMe), 60.9 (3-OMe), 55.7 (5-OMe); HRMS (ESI^+) *m/z* 433.0906 (calcd for $C_{22}H_{18}O_8Na$, 433.0899).

Patentiflorin A (3): white powder: $[\alpha]_D^{25}$ −54.3° (*c* 1.03, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 204.5 (4.69), 224 (4.51), 261 (4.8) nm; IR (CH_2Cl_2) ν_{max} 3611 (OH), 1758 (CO), 895 (OCH_2O) cm^{-1}; HRMS (ESI^+) *m/z* 549.1364 (calcd for $C_{27}H_{26}O_{11}Na$, 549.1373); ^1H NMR (CD_3Cl_3, 300 MHz) and ^{13}C NMR (CD_3Cl_3, 75 MHz), see Tables 1 and 2.

Acetylation of 3. Patentiflorin A (**3**) was dissolved in pyridine and treated with an excess of acetic anhydride. The mixture was kept for 20 h at 25 °C. The solvent and reagent were evaporated under high vacuum. The residual material was purified by preparative TLC with appropriate solvent (CH_2Cl_2−MeOH, 90:10). Compound **3a**: $[\alpha]_D^{25}$ −128° (*c* 0.3, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 262.5 (5.02), 294.5 (4.41), 312 (4.41), 349 (4.07) nm; IR (CH_2Cl_2) ν_{max} 3691 (OH), 1749 (CO), 1550, 977 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.55 (1H, d, J = 1.4 Hz, H-6), 7.09 (1H, s, H-3), 6.97 (1H, d, J = 7.8 Hz, H-5′), 6.83 (1H, d, J = 1.5 Hz, H-2′), 6.81 (1H, dd, J = 7.8, 1.5 Hz, H-6′), 6.11 and 6.06 (2H, 2d, J = 1.4 Hz, OCH_2O), 5.49 (1H, dd, 9.7, 8 Hz, H-2″), 5.45 (1H, d, J = 14.1 Hz, H-9), 5.40 (1H, dd, J = 14.1, 2.2 Hz, H-9), 5.29 (1H, dd, J = 9.7, 9.7 Hz, H-3″), 5.13 (1H, d, J = 8 Hz, H-1″), 5.01 (1H, dd, J = 9.7, 9.7 Hz, H-4″), 4.07 (3H, s, 5-OMe), 3.82 (3H, s, 4-OMe), 3.63 (1H, qd, J = 9.7, 6.0 Hz, H-5″), 2.13 (3H, s, 3″-OAc), 2.07 (6H, 2s, 2″-OAc and 4″-OAc), 1.29 (3H, d, J = 6.0 Hz, 6″-Me); HRMS (ESI^+) *m/z* 675.1674 (calcd for $C_{33}H_{32}O_{14}Na$, 675.1690).

Patentiflorin B (4): white powder: $[\alpha]_D^{25}$ −28.3° (*c* 1.16, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 3612 (OH), 1743 (CO), 931 (OCH_2O) cm^{-1}; HRMS (ESI^+) *m/z* 549.1340 (calcd for $C_{27}H_{26}O_{11}Na$, 549.1373); ^1H NMR (CD_3Cl_3, 300 MHz) and ^{13}C NMR (CD_3Cl_3, 75 MHz), see Tables 1 and 2.

Acetylation of 4. Using the same procedure as described above, **4** was acetylated to give **4a**: $[\alpha]_D^{25}$ −100° (*c* 0.3, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 263 (4.55), 296 (3.87), 312 (3.86), 340 (3.57) nm; IR (CH_2Cl_2) ν_{max} cm^{-1}; 3691 (OH), 1775 (CO), 1421, 1272; HRMS (ESI^+) *m/z* 675.1824 (calcd for $C_{33}H_{32}O_{14}Na$, 675.1690); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.58 (1H, s, H-6), 7.08 (1H, s, H-3), 7.00 (1H, s, H-5′), 6.84 (1H, d, *J* = 1.5 Hz, H-2′), 6.81 (1H, dd, *J* = 7.8, 1.5 Hz, H-6′), 6.11 and 6.07 (2H, d, *J* = 1.0 Hz, OCH_2O), 5.69 (1H, dd, *J* = 9.7, 8 Hz, H-2″), 5.51 (1H, d, *J* = 14.6 Hz, H-9), 5.41 (1H, dd, *J* = 14.6, 1.3 Hz, H-9), 5.34 (1H, d, *J* = 3.4 Hz, H-4″), 5.15 (1H, dd, *J* = 10.3, 3.4 Hz, H-3″), 5.14 (1H, d, *J* = 7.8 Hz, H-1″), 4.08 (3H, s, 5-OMe), 3.90 (1H, d, *J* = 6.3 Hz, H-5″), 3.77 (3H, s, 4-OMe), 2.28 (3H, s, 4″-OAc), 2.12 (3H, 1s, 3″-OAc), 2.07 (3H, 1s, 2″-OAc), 1.31 (3H, d, *J* = 6.2 Hz, 6″-Me); HRMS (ESI^+) *m/z* 675.1674 (calcd for $C_{33}H_{32}O_{14}Na$, 675.1690).

4″-*O*-Acetylpatentiflorin B (5): white powder: $[\alpha]^{25}_D$ −62.5° (*c* 1.12, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 203 (4.56), 261 (4.49), 293 (3.96), 306 (3.43) nm; IR (CH_2Cl_2) ν_{max} 3612 (OH), 1756 (CO), 1506, 930 (OCH_2O) cm^{-1}; HRMS (ESI^+) *m/z* 591.1487 (calcd for $C_{29}H_{28}O_{12}Na$, 591.1478); ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Tables 1 and 2.

4″-*O*-Acetylmananthoside B (6): white colorless powder; $[\alpha]^{25}_D$ −74.3° (*c* 0.95; CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 262.5 (4.73), 312 (4.04), 340 (3.72) nm; IR (CH_2Cl_2) ν_{max} 3586 (OH), 1752 (CO) cm^{-1}; MS/MS (ESI^+) *m/z* 781.1 ($[C_{36}H_{38}O_{18} + Na]^+$), 649.1 ($[C_{31}H_{30}O_{14} + Na]^+$), 402.7 ($[C_{21}H_{16}O_7 + Na]^+$), 401.2 ($[C_{15}H_{22}O_{11}+Na]^+$), 341.0 ($[C_{13}H_{21}O_{10}+Na]^+$), 281.0 ($[C_{11}H_{14}O_7 + Na]^+$); HRMS ($ESI^+$) *m/z* 781.1932 (calcd for $C_{36}H_{38}O_{18}Na$, 781.1956); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 3.

Acetylation of 6. Using the same procedure as described above, **6** was acetylated to give **6a**: $[\alpha]_D^{25}$ −2° (*c* 0.95; CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 228 (4.40), 263 (4.76), 295 (4.12), 312 (4.23), 349 (3.75); IR (CH_2Cl_2) ν_{max} cm^{-1} 3054, 1751 (CO); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.57 (1H, s, H-6), 7.08 (1H, s, H-3), 6.96 (1H, d, *J* = 8 Hz, H-5′), 6.82 (1H, d, *J* = 1.5 Hz, H-2′), 6.81 (1H, dd, *J* = 8, 1.5 Hz, H-6′), 6.11 and 6.06 (2H, s, OCH_2O), 5.65 (1H, dd, *J* = 10.5, 7.8 Hz, H-2″), 5.60 (1H, d, *J* = 14.8 Hz, H-9), 5.51 (1H, m, H-4″), 5.40 (1H, d, *J* = 14.8, H-9), 5.21 (1H, m, H-4‴), 5.12 (1H, d, *J* = 7.8 Hz, H-1″), 5.10 (1H, dd, *J* = 10.5, 3.3 Hz, H-3″), 5.09 (1H, ddd, *J* = 9.4, 6.2, 1.5 Hz, H-2‴), 4.95 (1H, dd, *J* = 9.4, 3.4 Hz, H-3‴), 4.40 (1H, t, *J* = 6.2 Hz, H-1‴), 4.09 (3H, s, 5-OMe), 4.03 (1H, m, H-5″), 3.96 (1H, dd, *J* = 13.0, 3.6 Hz, H-5‴), 3.83 (3H, s, 4-OMe), 3.75−3.85 (2H, m, H-6″), 3.58 (1H, dd, *J* = 13.0, 3.6 Hz, H-5‴), 2.02−2.23 (18H, s, 2″-OAc, 3″-OAc, 4″-OAc, 2‴-OAc, 3‴-OAc, and 4‴-OAc); HRMS (ESI^+) *m/z* 949.2370 (calcd for $C_{44}H_{46}O_{22}Na$, 949.2378).

Cell Culture and Assay for Cytotoxicity Activity. The human tumor cell lines KB (mouth epidermoid carcinoma), HCT116 (colon cancer), and sensitive MCF-7 (breast cancer) were originally obtained from the ATCC. Resistant MCF-7 cell lines were obtained by prolonged treatment by doxorubicine. The cytotoxicity assays were performed according to a published procedure.[14]

Flow Cytometry. The cell cycle distribution of HCT116 and MCF-7 was evaluated by flow cytometry as described previously.[15]

In Vivo Assays. Mice and Tumor Model. Female DBA/2 (DBA/2JIco, Iffa Credo, L'Arbresle, France) and hybrid CDF1 (CD2F1/CrlBR, Charles River, St Aubin-les-Elbeuf, France) mice were used for tumor model propagation and experimental chemotherapy, respectively. Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the European Directive EEC/86/609, under the supervision of authorized investigators. The P388 murine leukemia was obtained from the Division of Cancer Treatment, Tumor Repository of the National Cancer Institute, NCI, (Frederick, MD).

Experimental Chemotherapy. All experiments were conducted in compliance with French regulations and ethical committee guidelines, based on the UKCCCR guidelines for the welfare of animals in experimental neoplasia, as detailed previously.[16] 10^6 P388 or P388/F 11782 cells were implanted i.v. into CDF1 mice on day zero. Two doses (2.5 and 5 mg/kg) of compound **3** were intraperitonealy administered in CDF1 mice[16] as a single dose, the day following tumor implantation. Mice were weighed twice weekly during treatment and once weekly thereafter.

Evaluation of Antitumor Activity. The increase of lifespan was the criteria for antitumor activity evaluation. An increase of life span (ILS) was defined as follows: ILS (%) = T/C − 100, with T/C (%) ratio being (the median survival of treated mice/median survival of control mice) × 100. According to NCI criteria for the P388 model, 20% ≤ ILS < 75% is judged as being the minimum level for activity, and an ILS ≥ 75% is judged as corresponding to a high level of antileukemic activity.[17]

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