Dysoxylins A−D, Tetranortriterpenoids with Potent Anti-RSV Activity from *Dysoxylum gaudichaudianum*

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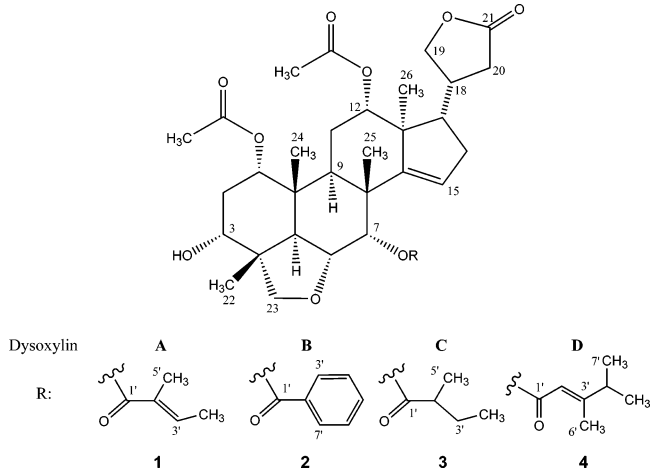
Four new compunds, belonging to the tetranortriterpenoid family, named dysoxylins A−D (**1**−**4**), isolated from *Dysoxylum gaudichaudianum*, were found to exhibit potent antiviral activity against respiratory syncytial virus (RSV). These structures were determined by NMR spectroscopy and mass spectrometry and were shown to have anti-RSV EC_{50} activities in the range 1.0−4.0 μg/mL in cytopathic effect inhibition and plaque reduction assays.

In the course of our studies to discover treatments for respiratory syncytial virus (RSV) infections,[1−3] we evaluated the medicinal plant *Dysoxylum gaudichaudianum* Miq. (Meliaceae) collected in Papua New Guinea. The leaves and bark of *Dysoxylum* spp. are used as a medicine by the indigenous people for treating rigid limbs, facial distortion in children, lumps under the skin, and other irritations, and as a remedy for sexually transmitted diseases.[4] They are also reportedly used as a remedy for fish poisoning and for convulsions.[5] A liquid drink made by adding boiling water to the chopped leaves is considered to be a cure for most aches and pains[6] and is used for lung hemorrhage.[7]

Both aqueous (water or 1:1 water−2-propanol) and organic (1:1 methylene chloride−2-propanol) extracts of *D. gaudichaudianum* bark showed inhibitory activity against the RSV strain A2 in in vitro and in vivo assays. Using respiratory syncytial viral CPE inhibition and plaque reduction assays to guide bioactivity-directed fractionation, the active fraction was found to be present in the more lipophilic phase, following liquid−liquid partition (chloroform/aqueous ethanol). NMR spectroscopic analysis of this fraction indicated the presence of complex structures. Reversed-phase chromatography of the organic extract led to the complete separation and isolation of four structurally related new tetranortriterpenoids, which were named dysoxylins A−D (**1**−**4**). These new compounds showed significant anti-RSV activity in both the cytopathic effect (CPE) inhibition and plaque reduction assays. In this paper, we describe the isolation, structure elucidation, and the anti-RSV activities of dysoxylins A−D (**1**−**4**).

The 1:1 2-propanol−methylene chloride extract of the dried bark of *D. gaudichaudianum*, which showed initial anti-RSV activity (EC_{50} 45 μg/mL, IC_{50} 440 μg/mL) in the CPE assay, was subjected to vacuum-flash chromatography on C_{18} silica gel using a solvent gradient of increasing methanol percentages in water, resulting in the separation of an enriched triterpenoid fraction, which also contained 4′-hydroxyacetophenone, which was subsequently separated out, tested separately, and found inactive in this bioassay. Using reversed-phase HPLC with a C_{18} column (detailed in the Experimental Section), four new compounds, **1**−**4**, were isolated from this bioactive fraction.

Compound **1** was obtained as a white, amorphous powder, mp 131−133 °C. The molecular formula was established as $C_{35}H_{48}O_{10}$



by HREIMS (M^+ *m*/*z* 628.3234, calcd 628.3248). The ^{13}C NMR spectrum accounted for a total of 35 carbon signals. Of these signals, the DEPT experiment revealed that there were eight methyl (CH_3), six methylene (CH_2), and 11 methine (CH) carbons, and therefore 10 quaternary (C) carbons ($C_{35}H_{47}$) in the molecule. One proton not accounted for from the DEPT data is exchangeable and not directly connected to a carbon atom. Two methyl carbons at δ 21.3 and 21.6 were shown to be connected to singlet proton signals at δ 2.16 and 2.13, respectively, in the HETCOR/HMQC experiments. These CH_3 proton signals were shown to have long-range coupling to carbonyl carbons at δ 168.9 and 170.2 in a HMBC study, indicating the presence of two acetyl units in the molecule. A tigloyl unit was also revealed by NMR data, especially from the $^1H-^1H$ COSY and HMBC studies. Thus, the olefinic proton at δ 6.91 (H-3′, m) was shown to correlate to a methyl proton signal at δ 1.80 (H-4′, d) in a COSY spectroscopic study and to carbons at δ 166.6 (C-1′) and 12.06 (C-5′) from the HMBC spectroscopic analysis.

Detailed NMR spectroscopic data analyses including $^1H-^1H$ COSY, HMQC/HETCOR, and HMBC experiments were compared with data for trichilinin E,[8] leading to the frame of the tetranortriterpenoid, 12-hydroxyvilasinin.[9−12] A furan ring in trichilinin E is absent in compound **1**; the data suggested the presence of a γ-lactone in **1** at C-17. Thus, the oxygenated methylene CH_2O signals at δ 4.42 and 3.69 both connected to the carbon at δ 73.1 in the HMQC spectrum and show a geminal coupling in the $^1H-^1H$ COSY spectrum. The proton at δ 4.42 also coupled to a methine proton signal at δ 2.65, and this CH proton signal coupled to another nonoxygenated CH_2 proton signal at δ 2.48 and 2.21; these two protons had geminal coupling in the COSY spectrum. These data analyses resulted in the spin system of a γ-lactone substructure for compound **1**.

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Table 1. NMR Data of Dysoxylins A−D (**1**−**4**) in $CDCl_3$

	δ_C (100 MHz)				δ_H (*J* in Hz, 400 MHz)			
position	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	74.0	73.9	73.9	74.0	4.88 dd (2.6)	4.85 dd (2.8)	4.87 dd (2.6)	4.89 dd (2.8)
2	30.1	30.0	30.3	30.3	2.22,2.05 m	2.21,2.07 m	2.27,2.01 m	2.28,2.01 m
3	70.9	70.6	71.0	71.1	3.86 dd (4.0,2.6)	3.83 dd (4.0,2.8)	3.85 dd (4.0,2.6)	3.85 dd (4.0,2.8)
4	43.3	43.3	43.4	43.4				
5	39.9	40.0	40.1	40.3	2.54 d (12.4)	2.72 d (12.8)	2.40 d (12.4)	2.46 d (12.4)
6	72.4	72.4	72.2	72.2	4.16 dd (14.0,3.0)	4.23 dd (12.8,3.2)	4.19 dd (13.8,3.2)	4.19 dd (12.4,3.2)
7	73.7	74.4	73.4	73.1	5.65 d (3.0)	5.77 d (3.2)	5.57 d 3.2)	5.63 d (3.2)
8	44.2	44.2	44.2	44.5				
9	35.3	35.2	35.4	35.3	2.72 dd (9.7,6.8)	2.85 dd (12.8,6.4)	2.62 dd (12.0,7.0)	2.65 dd (13.2,6.0)
10	39.3	39.3	39.3	39.3				
11	25.0	25.0	25.1	25.4	2.25,1.15 m	2.31,1.09 m	2.25,1.07 m	2.26,1.12 m
12	77.6	77.6	77.6	77.8	4.75 dd (8.6,6.0)	4.71 dd (8.4,6.0)	4.74 dd (8.6,5.6)	4.73 dd (8.8,5.2)
13	51.1	51.1	51.2	51.1				
14	154.9	154.8	155.2	154.9				
15	123.0	123.1	122.9	123.4	5.64 dd (1.2)	5.66 dd (1.4)	5.58 dd (1.2)	5.60 dd (1.6)
16	35.3	35.2	35.3	35.4	2.22 dd (10.6,9.2)	2.18 dd (11.0,9.0)	2.29 dd (10.8,9.2)	2.23 dd (11.2,9.4)
					2.05 dd (10.6,8.4)	1.92 dd (11.0,8.4)	2.04 dd (10.8,8.2)	2.03 dd (11.2,7.6)
17	56.0	55.9	56.1	56.3	1.87 ddd (9.2,8.4)	1.79 ddd (9.0,8.4)	1.87 ddd (9.2,8.2)	1.82 ddd (9.4,7.6)
18	37.6	37.5	37.7	37.7	2.65 m	2.52 m	2.66 m	2.64 m
19	73.1	72.9	73.1	73.0	4.42 dd (10.0,8.4)	4.33 dd (8.4,8.0)	4.42 dd (10.2,8.4)	4.40 dd (9.0,7.6)
					3.69 dd (10.0,8.8)	3.62 dd (10.0,9.2)	3.68 dd (10.0,9.0)	3.68 dd 10.4,9.0)
20	34.0	33.8	34.0	34.0	2.48 dd (17.0,7.8)	2.34 dd (17.0,7.8)	2.50 dd (17.0,8.0)	2.49 dd (17.2,8.0)
					2.21 dd (17.0,4.6)	2.11 dd (17.0,4.6)	2.20 dd (17.0,4.6)	2.24 dd (17.2,4.8)
21	176.3	176.2	176.2	176.2				
22	19.6	19.5	19.8	19.6	1.12 s	1.10 s	1.12 s	1.12 s
23	77.9	77.9	77.8	78.0	3.94 d(7.4) 3.56 d(7.4)	3.79 d (7.8)	3.94 d (7.4)	3.94 d (7.4)
						3.46 d (7.8)	3.55 d (7.4)	3.56 d (7.4)
24	15.0	14.9	15.1	15.1	0.99 s	1.01 s	0.99 s	0.99 s
25	26.6	26.4	26.7	26.5	1.15 s	1.18 s	1.15 s	1.15 s
26	15.3	15.4	15.1	15.1	1.07 s	0.94 s	1.10 s	1.07 s
OAc-1	21.26	21.27	21.32	21.35	2.16 s	2.21 s	2.13 s	2.13 s
	168.9	169.0	168.9	168.9				
OAc-12	21.6	21.5	21.6	21.5	2.13 s	2.09 s	2.15 s	2.16 s
	170.2	170.1	170.2	170.2				
1′	165.6	164.9	171.6	165.9				
2′	128.5	130.3	25.6	113.7			2.07 ddq (7.4,6.8)	5.59 dd (2.0,1.2)
3′	137.5	129.3	44.0	165.0	6.91 m (7.2)	8.02 dd (8.4,1.2)	2.20 ddq (12.4,6.8)	
							1.70 ddq (12.4,6.8)	
4′	14.4	128.2	22.6	38.1	1.80 d (7.2)	7.39 dd (8.4,6.8)	0.99 dd (6.8)	2.38 m (6.8)
5′	12.1	132.8	22.5	20.8	1.87 s	7.53 dd (6.8,1.2)	1.12 d(7.4)	1.06 dd (6.8,2.0)
6′		128.2		16.6		7.39 dd (8.4,6.8)		2.14 d (1.2)
7′		129.3		21.0		8.02 dd (8.4,1.2)		1.06 dd (6.8,2.0)

The other J_{HH} correlations in the COSY spectrum of **1** and especially $^2J_{CH}/^3J_{CH}$ correlations found in the HMBC experiment were established as being consistent with a 12-hydroxyvilasinin lactone structure. For example, HMBC data indicated that H-15 at δ 5.64 correlated to C-13, C-14, C-16, and C-17 at δ 51.1, 154.9, 35.3, and 56.0, respectively. The H-7 signal at δ 5.65 correlated to C-5, C-6, and C-8 at δ 39.9, 72.4, and 44.2, respectively, while the H-22 signal at δ 1.12 correlated to C-3, C-4, C-5, and C-23 at δ 70.9, 43.3, 39.9, and 77.9. The positions of the acetyl and tigloyl units were established by $^3J_{CH}$ correlations between H-1 (δ 4.88), H-12 (δ 4.75), and H-7 (δ 5.65) and the carbonyl carbons at δ 168.9, 170.2, and 166.6 in the HMBC spectrum. Therefore compound **1** was determined to be 12-acetoxy-1-acetyl-7-tigloylvilasinin-17-γ-lactone, as shown.

Compounds **2**−**4** were assigned molecular formulas of $C_{37}H_{46}O_{10}$ (M^+ *m*/*z* 650.3050, calcd 650.3091), $C_{35}H_{50}O_{10}$ (M^+ *m*/*z* 630.3446, calcd 630.3404), and $C_{37}H_{52}O_{10}$ (M^+ *m*/*z* 656.3621, calcd 656.3560), respectively, by HREIMS. The mass spectra of compounds **1**−**4** were shown to have fragment ion peaks $[M - CH_3COOH]^+$ at *m*/*z* 568, 590, 570, and 596, respectively. Diagnostic fragment ion peaks were also found at *m*/*z* 468 in the mass spectra for compound **1** $[M - CH_3COOH - C_5H_8O_2]^+$, for **2** $[M - CH_3COOH - C_7H_6O_2]^+$, for **3** $[M - CH_3COOH - C_5H_{10}O_2]^+$, and for **4** $[M - CH_3COOH - C_7H_{12}O_2]^+$, respectively.

The NMR spectroscopic data indicated that compounds **1**−**4** are all similar tetranortriterpenes possessing the same 12-acetoxy-1-acetylvilasinin lactone moiety. The only significant differences between these compounds were observed at the site of a C-7 ester substitution. For example, NMR signals for the tiglate unit of **1** were missing from **2**, but in its place the NMR spectra of **2** showed signals for a monosubstituted benzene ring. Thus, in place of a 7-tiglate moiety for compound **1**, a 7-benzoate was found for **2**, as indicated by the correlations between the C-1′ carbonyl at δ 164.9 and protons H-7 and H-3′/7′ at δ 5.77 and 8.02 in the HMBC spectrum. Similar comparisons of the NMR spectroscopic data revealed a 7-(2-methylbutate) substitution for **3** and a 7-(3,4-dimethylpent-2-enate) linkage in **4**. Detailed 2D NMR spectral data analyses led to complete assignments of ^1H and ^{13}C signals of compounds **1**−**4**, as shown in Table 1.

Compared to 12-hydroxyvilasinin, in place of the furan ring at C-17, there is a γ-lactone in dysoxylins A−D (**1**−**4**). The stereochemistry of **1**−**4** is proposed as shown by comparison of NMR data with 12-hydroxyvilasinin[8−12] and other known compounds, as well as selective NOE studies. The geometry of the double bond of the 3,4-dimethylpent-2-enate unit in dysoxylin D (**4**) was established by comparison of NMR data with those of bruceanol E.[13] Thus, for example, the ^{13}C NMR chemical shift assignments of the 3,4-dimethylpent-2-enoyl units in **4** were δ 166.2 (C-1′), 113.5 (C-2′), 164.8 (C-3′), 38.1 (C-4′), 20.7 (C-5′), 16.7 (C-6′), and 20.7 (C-7′) as compared to those of bruceanol E, δ 165.9 (C-1′), 113.7 (C-2′), 165.0 (C-3′), 38.1 (C-4′), 21.0 (C-5′),

Table 2. Anti-RSV Activities of Dysoxylins A−D (**1**−**4**)

	CPE inhibition		plaque reduction	
compound	EC_{50} (μg/mL)	IC_{50} (μg/mL)	EC_{50} (μg/mL)	IC_{50} (μg/mL)
1	2	150	150	112
2	4	16	30	7
3	not tested	not tested	146	52
4	1	>200	40	42
ribavirin	3.6	180	20	460

16.6 (C-6′), and 21.0 (C-7′). The observation of NOE correlations between H-2′ and H-4′ and between H-2′ and H-7′ confirmed the assignments.

Limonoids are widely distributed in plants of the Meliaceae.[8−15] Although many tetranortriterpenoids have been reported, to the best of our knowledge compounds **1**−**4** are new structures, and we name these compounds dysoxylins A−D, although Jogia and Andersen have used the name "dysoxylin" for a limonoid compound from *D. richii* in 1987.[16,17] These new compounds, **1**−**4**, showed antiviral activities against RSV in a CPE assay when compared to ribavirin, as presented in Table 2.

The potent antiviral activity of these compounds warrants further consideration.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. Optical rotations were taken on a JASCO polarimeter; UV spectra on a Perkin-Elmer UV−vis photospectrometer; and IR spectra recorded as KBr pellets on a Perkin-Elmer FT1600 IR spectrometer. All NMR spectra were recorded at 400 MHz for ^{1}H and at 100 MHz for ^{13}C on a Varian Unity-Plus 400 using TMS as an internal reference. H−C one-bond connectivities and carbon multiplicities were determined by HMQC/HETCOR and DEPT experiments. Mass spectra were obtained on a Kratos MS-50 mass spectrometer. All solvents used were HPLC grade. TLC was performed on EM Science silica gel ($60F_{254}$) plates and detection of compounds using an H_2SO_4/vanillin stain. A Primesphere C_{18}-(HC) 10 μm column (50 × 250 mm) was used for HPLC on a Hitachi Model D-6500 equipped with L-4500A photodiode array and Sedex 75 evaporative light scattering detectors.

Plant Material. The bark of *Dysoxylum gaudichaudianum* (Meliaceae) was collected from a village in Papua New Guinea and identified by Drs. Michael J. Balick and Weerachai Nanakorn. Voucher specimens are deposited in the reference collection, Department of Ethnobotany and Conservation, Shaman Pharmaceuticals, Inc.

Antiviral and Cytotoxicity Assays. The antiviral activities and cytotoxic effects of the described compounds and control antiviral compound (ribavirin) were determined using the respiratory syncytial viral CPE and plaque reduction assays. Respiratory syncytial viral (RSV), strain A2 (American Type Culture Collection, Rockville, MD), was propagated in embryonic African green monkey kidney cells (MA-104 cells; BioWhittaker Inc., Walkersville, MD). The MA-104 cells were routinely passaged in minimal essential medium (MEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 9% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 0.1% $NaHCO_3$. The procedures used for the antiviral and cytotoxicity assays were previously described.[1,18−23] Plaque reduction assays were done using MA-104 cells and 200 plaque forming units (PFU) virus/well of RSV A2. Virus was adsorbed to cells in MEM without serum for 2.5 h at 37 °C and then removed and overlaid with agarose. Each dilution of compound was plated in duplicate. After 4 days, plaques in each well were counted and then expressed as percentages of control, calculated by regression analysis of the average percents of control at each concentration. The anti-RSV activity of each sample was expressed in μg/mL as 50% effective concentration (EC_{50}), and cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}).

Extraction and Isolation. Dried, ground bark of *D. gaudichaudianum* (5.0 kg) was placed in 1:1 methylene chloride−2-propanol (50 L) in a high-density polypropylene tank equipped with an air-driven agitator (Grovhac, Inc.). The extraction was performed at room temperature for 48 h while mixing vigorously. The extract was then filtered through Celite, the plant marc was soaked with 12 L of 1:1 methylene chloride−2-propanol, and then the marc extract was filtered through Celite. The combined organic extracts were concentrated under reduced pressure at 30 °C to yield 350 g of extract (7.0%). A 50 g portion of the organic extract was subjected to short column liquid chromatography under a vacuum on ∼45 g of C_{18} silica gel packed in 25% methanol−water (30 × 100 cm). The column was eluted with 10 L each of 25, 50, 75, and 100% methanol−water at a flow rate of 50 mL/min. The 75% methanol−water eluent was then concentrated under reduced pressure at 40 °C in a rotary evaporator to yield ∼10 g (1.7%) of an enriched fraction that contained a triterpenoid fraction consisting of dysoxylins A−D (**1**−**4**). Further separation and final purification by preparative HPLC using a Primesphere C_{18} column (10 μm, 50 × 250 mm, isocratic or gradient with 40−65% CH_3CN−H_2O, flow rate at 40 mL/min, monitoring UV wavelengths of λ 205 and 230 nm, evaporative light scattering, sample concentration 500 mg/mL; injection volume 500 μL) gave dysoxylins A (**1**, 0.64 g, 0.11%), B (**2**, 0.25 g, 0.043%), C (**3**, 0.22 g, 0.038%), and D (**4**, 0.48 g, 0.082%).

Dysoxylin A (1): white, amorphous powder, mp 131−133 °C, $[\alpha]^{26}_D$ −19.6 (*c* 0.29, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 203 (4.52) 220 (sh) (3.12) nm; IR (KBr) ν_{max} 3507, 2966, 2931, 1778, 1725, 1643, 1372, 1243, 1167, 1026, 726 cm^{-1}; see Table 1 for ^{1}H and ^{13}C NMR data in $CDCl_3$; HREIMS *m*/*z* 628.3234 [M^+], calcd for $C_{35}H_{48}O_{10}$ 628.3248, 568 [$M - CH_3COOH]^+$, 468 [$M - CH_3COOH - C_5H_8O_2]^+$.

Dysoxylin B (2): see Table 1 for ^{1}H and ^{13}C NMR data in $CDCl_3$; HREIMS *m*/*z* 650.3050 [M^+], calcd for $C_{37}H_{46}O_{10}$ 650.3091, 590 [$M - CH_3COOH]^+$, 468 [$M - CH_3COOH - C_7H_6O_2]^+$.

Dysoxylin C (3): see Table 1 for ^{1}H and ^{13}C NMR data in $CDCl_3$; HREIMS *m*/*z* 630.3446 [M^+], calcd for $C_{35}H_{50}O_{10}$ 630.3404, 570 [$M - CH_3COOH]^+$, 468 [$M - CH_3COOH - C_5H_{10}O_2]^+$.

Dysoxylin D (4): see Table 1 for ^{1}H and ^{13}C NMR data in $CDCl_3$; HREIMS *m*/*z* 656.3621 [M^+], calcd for $C_{37}H_{52}O_{10}$ 656.3560, 596 [$M - CH_3COOH]^+$, 468 [$M - CH_3COOH - C_7H_{12}O_2]^+$.

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