New Cytotoxic Lupane Triterpenoids from the Twigs of *Coussarea paniculata*

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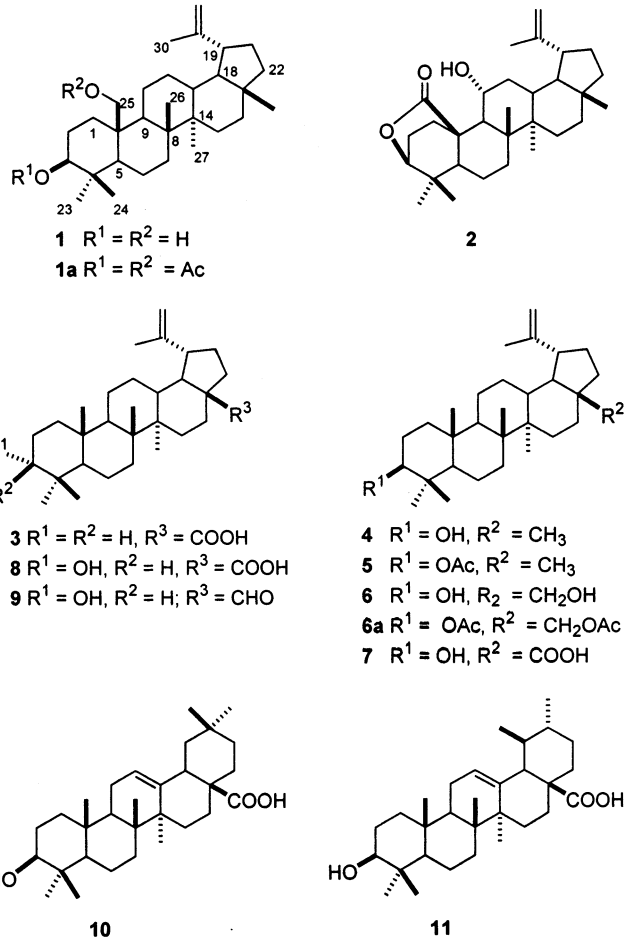
Bioassay-guided fractionation of a CH_2Cl_2−MeOH extract of the twigs of *Coussarea paniculata* using a yeast-based assay for potential DNA-damaging agents resulted in the isolation of three new lupane triterpenoids, **1**−**3**, in addition to eight known triterpenoids, lupeol (**4**), lupeyl acetate (**5**), betulin (**6**), betulinic acid (**7**), 3-*epi*-betulinic acid (**8**), 3-*epi*-betulinaldehyde (**9**), oleanolic acid (**10**), and ursolic acid (**11**). The structures of the new compounds were established as lup-20(29)-en-3β,25-diol (**1**), lup-20(29)-en-11α-ol-25,3β-lactone (**2**), and 3-deoxybetulonic acid (**3**), on the basis of extensive 1D and 2D NMR spectroscopic data interpretation and chemical conversion.

As a part of our ongoing research to identify novel naturally occurring potential anticancer agents,[1−3] a CH_2Cl_2−MeOH (1:1) extract of the twigs of *Coussarea paniculata* Vahl. (Rubiaceae) was initially selected for bioassay-guided fractionation on the basis of its reproducible and selective bioactivity in our yeast assay for DNA-damaging agents,[4] but subsequent experiments failed to confirm this selectivity and indicated a weak cytotoxic activity. Fractionation was thus carried out to isolate the cytotoxic constituents, since there are no reports of any previous phytochemical investigations of this plant, although its taxonomy has been discussed recently as part of a treatment of the subfamily Rubioideae of the Rubiaceae.[5]

The crude extract after extensive chromatography followed by reversed-phase preparative TLC yielded three new lupane triterpenoids (**1**−**3**), in addition to the eight known triterpenoids **4**−**11**. The structures of the eight known compounds were identified as lupeol (**4**), lupeyl acetate (**5**),[6] betulin (**6**), betulinic acid (**7**),[7] 3-*epi*-betulinic acid (**8**),[8] 3-*epi*-betulinaldehyde (**9**),[9] oleanolic acid (**10**),[10] and ursolic acid (**11**),[11] by comparison of their spectral data with values reported in the literature.

Compound **1** was isolated as an inseparable mixture with betulin (**6**), and the mixture was thus acetylated with Ac_2O−pyr. The acetylated products were then separated by reversed-phase preparative TLC using MeOH−H_2O (85:15) to furnish two products, **1a** (1.6 mg) and **6a** (2.3 mg). The structure of **6a** was confirmed as betulin diacetate by comparison of its spectral data with those reported in the literature.[7]

The molecular formula of **1a** was deduced as $C_{34}H_{54}O_4$ by HRFABMS, ^{13}C NMR, and DEPT spectra. It gave a positive Lieberman-Burchard (LB) test for triterpenoids. The mass fragments that were observed at *m*/*z* 466 and 406 in its EIMS indicated the presence of two acetate groups in its structure. The ^1H NMR spectrum of **1a** showed the presence of five methyl singlets at δ 0.82, 0.84, 0.85, 0.96, and 1.02, an oxymethine proton at δ 4.45 (d, J = 11.0, 5.6 Hz), 10 methylenes, four methines, and an acetyl methyl singlet at δ 2.03. These signals are characteristic for the basic skeleton of a 3β-acetylated triterpenoid. The ^1H NMR spectrum also showed the presence of



a primary acetate group [δ 4.24 (1H, d, J = 11.2 Hz), 3.83 (1H, d, J = 11.0 Hz), and 2.06 (3H, s)] and an isopropenyl group, inferred by the presence of a low-field methyl singlet at δ1.67 and two doublets at δ 4.58 (J = 1.8 Hz) and 4.68 (J = 2.1 Hz). The ^{13}C NMR values for all the carbons in **1a** were assigned on the basis of DEPT, HMQC, and HMBC spectra and are given in Table 1. The two sp^2 carbons observed at δ 150.3 and 110.0 in the ^{13}C NMR spectrum of **1a** confirmed the disubstituted alkene of an isopropenyl group. The ^1H NMR spectrum, and especially the presence of an isopropenyl group, suggested that compound **1a** is a pentacyclic triterpene of the lup-20(29)-en-3β-ol or hop-22(29)-en-3β-ol type.[12] The basic skeleton of the hop-22(29)-

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Table 1. NMR Data for Compounds **1**, **1a**, **2**, and **3** ($CDCl_3$)[a]

	1	**1a**		**2**		**3**	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	34.2	1.24 m, 1.40 m	34.0	2.42 d (13.2) 1.92 m	23.8	1.32 m 1.56 m	39.7
2	27.2	1.53 m, 1.88 m	23.7	1.56 m 1.84 m	25.6	1.34 m 2.04 m	18.8
3	79.0	4.45 dd (11.0, 5.6)	81.0	4.52 dd (4.2, 1.6)	81.5	1.76 m	42.2
4	38.9		37.7		38.2		33.1
5	55.4	1.28 m	55.5	1.16 m	34.2	1.26 m	56.3
6	18.2	1.34 m	18.3	1.42 m	21.0	1.34 m	18.7
7	34.1	1.52 m	34.2	1.56 m	34.3	1.49 m	34.2
8	40.8		40.9		40.9		40.8
9	50.4	1.42 m	50.5	1.68 m	54.4	1.46 m	50.0
10	42.6		42.7		49.9		37.1
11	20.8	1.43 m	20.8	3.97 m	68.3	1.38 m	21.0
12	25.3	1.46 m	25.3	1.72 m	27.8	1.46 m	25.6
13	37.9	1.32 m	37.9	1.50 m	38.0	1.30 m	38.5
14	42.8		42.8		42.9		42.5
15	27.2	1.42 m	27.2	1.44 m	27.5	1.62 m	30.6
16	35.3	1.28 m	35.5	1.30 m	35.6	1.32 m, 2.46 m	32.1
17	42.8		42.9		43.1		56.4
18	48.2	1.68 m	48.2	1.74 m	48.3	1.86 m	46.9
19	47.8	2.42 dt (5.2, 11.4)	47.9	2.38 dt (5.8, 11.2)	48.1	2.98 dt (5.4, 11.2)	49.9
20	150.4		150.3		151.0		150.4
21	29.7	1.30 m 1.87 m	29.8	1.28 m 1.84 m	29.8	1.36 m 1.98 m	29.8
22	40.0	1.54 m	40.0	1.54 m	40.0	1.42 m 1.82 m	37.0
23	28.0	0.96 s	28.0	0.95 s	28.1	0.97 s	33.7
24	15.6	0.82 s	15.6	0.78 s	16.8	0.87 s	21.5
25	60.7	3.83 d (11.0) 4.24 d (11.2)	62.9		178.0	0.85 s	16.0
26	16.2	0.85	16.3	0.83 s	16.0	0.99 s	16.1
27	14.8	1.02 s	14.8	1.01 s	14.6	1.07 s	14.7
28	18.1	0.84 s	18.2	0.87 s	18.1		179.5
29	109.9	4.58 d (1.8), 4.68 d (2.1)	110.0	4.56 d (2.5) 4.68 d (2.3)	109.5	4.61 s 4.73 s	109.0
30	19.2	1.67 s	19.3	1.69 s	19.3	1.68 s	19.5
$OCOCH_3$			171.2				
$OCOCH_3$			171.7				
$OCOCH_3$		2.03 s	21.1				
$OCOCH_3$		2.06 s	21.3				

[a] Assignments made on the basis of COSY, HMQC, and HMBC and by comparison with literature data.[7,12]

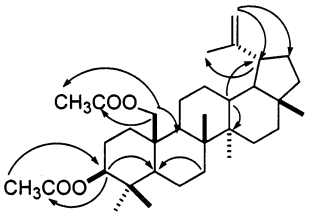

Figure 1. Selected HMBC correlations for **1a**.

en-3β-ol triterpenoid could be ruled out for compound **1a** on the basis of the differences in the ^{13}C NMR values of **1a** with those of hop-22(29)-en-3β-ol derivatives.[12] The lup-20(29)-en-3β-ol skeleton for **1a** was supported by COSY and HMBC (Figure 1) correlations. Assigning the singlet at δ 1.67 to the isopropenyl methyl group at C-20 and the presence of five additional methyl singlets and a primary acetate group in **1a** suggested that one of the six methyl groups at C-23, C-24, C-25, C-26, C-27, and C-28 in lup-20(29)-en-3β-ol must be in the form of an acetyloxymethylene group. A close comparison of the ^{1}H and ^{13}C NMR values of **1a** with those of diacetate derivatives of reported lupane diols, lup-20(29)-en-3β,23-diol,[13] lup-20(29)-en-3β,-24-diol,[14] lup-20(29)-en-3β,27-diol,[15] and lup-20(29)-en-3β,-28-diol (**6**, betulin),[7] indicated that its NMR values did not match with any of them, suggesting the possible placement of the primary acetate group at either C-25 or C-26. The HMBC spectrum of **1a** (Figure 1), in which the acetyloxymethylene group showed correlations to C-1, C-5, C-9, and C-10, confirmed the assignment of the primary acetate to the C-25 position. This was supported by the mass spectrum of **1a**, which had significant peaks at *m*/*z* 240 and 286. The peak at *m*/*z* 240 corresponds to a ring A fragment ion formed by cleavage of the C-5−C-6 and C-9−C-10 bonds, and the peak at *m*/*z* 286 corresponds to $[M - 240]^{+\bullet}$. On the basis of the above spectral data, compound **1a** was assigned as lup-20(29)-en-3β,25-diacetate, and **1** as lup-20(29)-en-3β,25-diol. The ^{13}C NMR spectrum of **1** was deduced by subtraction of the spectrum of **6** from that of the inseparable mixture of **1** and **6** and is shown in Table 1.

The molecular formula of **2** was determined as $C_{30}H_{46}O_3$ by HRFABMS; this molecular composition requires eight degrees of unsaturation. It also gave a positive LB test for triterpenoids. The IR spectrum of **2** showed the presence of carbonyl (1745 cm^{-1}) and hydroxyl (3420 cm^{-1}) groups in its structure. The ^{1}H NMR spectrum showed the presence of five methyl singlets (δ 0.78, 0.83, 0.87, 0.95, and 1.01), an isopropenyl chain [δ 4.56 (1H, d, J = 2.5 Hz), 4.68 (1H, d, J = 2.3 Hz), and 1.69 (3H, s)], and an oxymethine proton as a doublet of doublets at δ 4.52. These data suggested that **2** is a C-3-substituted lup-22(29)-ene

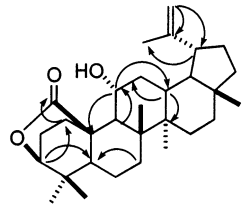

Figure 2. Selected HMBC correlations for **2**.

derivative similar to **1**. A second oxymethine signal was also observed as a one-proton multiplet centered at δ 3.97. The ^{13}C NMR values for all 30 carbons in **2** were assigned on the basis of DEPT, HMQC, and HMBC spectra and are given in Table 1. A close comparison of the ^{13}C NMR values of **2** with those of nepiticin[16] indicated they had identical C, D, and E rings with a secondary hydroxyl group at the C-11 position. This was supported by COSY (H-9/H-11; H-11/H-12) and HMBC (H-9/C-8, C-10, C-11, C-12, C-25; H-12/C-9, C-11, C-13, C-14, C-18) correlations. Since **2** has eight degrees of unsaturation, it must have six rings in addition to the carbonyl and alkene groups. The sixth ring was assigned as a lactone between the C-3 oxygen and the C-25 carbonyl group. This assignment was supported by the key HMBC correlations shown in Figure 2 and by the appearance of the C-3 oxymethine proton at δ 4.52 as a doublet of doublets. Further, the ^{13}C NMR values for carbons C-1 to C-5 of **2** were almost superimposable on those of 22α-hydroxystictano-25,3β-lactone,[17] supporting the presence of a C-3/C-25 lactone in the A ring. The stereochemistry of the oxymethine proton at the C-3 position was assigned as α, like that of 22α-hydroxystictano-25,3β-lactone, on the basis of their almost identical coupling constants; this was supported by its NOESY correlation with the protons of the methyl group at the C-23 position resonating at δ 0.95. Similarly, the relative stereochemistry of the hydroxyl group at C-11 was assigned as α on the basis of the NOESY spectrum of **2**, which showed a correlation between the oxymethine proton at δ 3.97 and the protons of the methyl group at C-26. Thus, compound **2** was assigned as lup-20(29)-en-11α-ol-25,3β-lactone.

Compound **3** was isolated as a colorless optically active solid and was shown to have the molecular formula $C_{30}H_{48}O_2$ from its HRFABMS and ^{13}C NMR spectra. Its IR spectrum showed absorption bands at 3350 and 1695 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups in the structure. The ^{13}C NMR spectrum of **1** showed a signal at δ 179.5, suggesting the presence of a carboxylic acid group, which was supported by the mass spectral fragment observed at *m*/*z* 395 formed by the loss of a COOH group from the molecular ion. The ^{1}H NMR spectrum of **3** was similar to that of **7** and showed the presence of six methyl singlets and an isopropenyl group, suggesting its lup-22(29)-ene terpenoid nature. The ^{13}C NMR values for all the carbons were assigned on the basis of DEPT, HMQC, and HMBC spectra (Table 1), which indicated the presence of seven sp^3 methyls, 11 sp^3 methylenes, four sp^3 methines, five sp^3 quaternary carbons, one sp^2 methylene, one sp^2 quaternary carbon, and a carboxylic acid. This indicated that there are no other characteristic functional groups in **3** except the carboxylic acid group. A search in the literature revealed that the ^{13}C NMR values of compound **3** were almost superimposable on those of lup-22(29)-ene[12] in rings A, B, and C, and on betulinic acid (**7**)[7] in rings D and E, suggesting the presence of the carboxylic acid group at the C-28 position. This was further supported by the key HMBC correlations: H-3/C-1, C-2, C-4, C-23, C-24; H-5/C-3, C-4, C-6; H-6/C-5, C-7, C-8, C-10; H-9/C-8, C-10, C-11, C-12, C-25, H-26; H-13/C-12, C-14, C-17, C-18, C-27; H-16/C-14, C-15, C-17, C-18, C-28; H-19/C-18, C-20, C-21, C-22. The same compound was previously reported as a synthetic product formed by the Wolff−Kishner reduction of betulonic acid,[18] but this is the first report of its occurrence as a natural product; its NMR data have not previously been reported. Thus, **3** was established as 3-deoxybetulonic acid.

Compounds **1a**, **2**−**5**, **6a**, and **7**−**11** were evaluated in the A2780 cytotoxicity assay.[19] The compounds were only very weakly cytotoxic, with IC_{50} values of 20 μg/mL or greater for all compounds except **1a** (9.6), **2** (18.0), **3** (17.4), and **5** (16.0).

Experimental Section

General Experimental Procedures. Melting points were recorded with an Electrothermal digital apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR ($CHCl_3$) and UV (MeOH) spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 spectrometer. The HRFABMS were obtained on a JEOL HX-110 instrument. The chemical shifts are given in ppm (δ) with TMS (tetramethylsilane) as an internal reference, and coupling constants (*J*) are in Hz. Sephadex LH-20 was used for column chromatography.

Plant Material. Twigs of *Coussarea paniculata* Vahl. (Rubiaceae) were collected in Kamana, 10 km north of Orinduik, Guyana, in March 1995 by Dr. Suroojnauth Tiwari of the Institute of Economic Botany, New York Botanical Garden, and were assigned collector number 0CKF888. Herbarium vouchers are deposited in the Smithsonian Institution National Herbarium, Washington DC, and at The New York Botanical Garden, Bronx, NY.

Extraction and Isolation. A CH_2Cl_2−MeOH (1:1) extract of *C. paniculata* was prepared by the National Cancer Institute as previously described[1] and supplied as N086833. The extract (1.5 g) was chromatographed over Sephadex LH-20 using hexane−$CHCl_3$ (100:0 to 0:100) and $CHCl_3$−MeOH (100:0 to 4:6) to yield 10 fractions (A−J), of which fractions D and E were found to be active. The fractions D and E were combined on the basis of their almost identical nature on TLC and ^{1}H NMR spectral data, and the combined residue (0.15 g) was subjected to further column chromatography over Sephadex LH-20 using $CHCl_3$−MeOH (100:0 to 9:1) to furnish nine fractions (DE/1−DE/9), of which fractions DE/1 to DE/4 were found to be the most active. Fraction DE/1 on preparative TLC over RP C_{18} using MeOH−H_2O (90:10) afforded **5** (1.3 mg) and **9** (1.5 mg). Similarly fraction DE/2 on reversed-phase preparative TLC using MeOH−H_2O (85:15) yielded a mixture of two triterpenoids (**1** and **6**, 4.6 mg) and **4** (2.6 mg), **8** (1.2 mg), and **10** (3.2 mg). Fraction DE/3, on preparative TLC over RP C_{18} using MeOH−H_2O (80:20), furnished **2** (1.3 mg) and **3** (1.5 mg). Fraction DE/4 on reversed-phase preparative TLC using MeOH−H_2O (80:20) yielded **7** (2.6 mg) and **11** (2.6 mg).

Acetylation of the Triterpene Mixture of Compounds 1 and 6. Acetylation (Ac_2O−pyr, 1:1, 0.8 mL; room temperature) of the mixture (**1** and **6**, 4.3 mg) and the usual workup gave a product that on purification over reversed-phase preparative TLC using MeOH−H_2O (85:15) furnished two compounds, **1a** (1.6 mg) and **6a** (2.3 mg). Compound **6a** was identified as betulin diacetate by comparison of its spectral data with those reported in the literature.[7]

Lup-20(29)-ene-3β,25-diacetate (1a): viscous oil; $[\alpha]_D^{25}$ +42.5° (*c* 0.62, $CHCl_3$); UV (MeOH) λ_{max} 208 nm (log ϵ 3.24); IR ($CHCl_3$) ν_{max} 2945, 1730, 1640, 1245, 1145 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 526 $[M]^{+\bullet}$ (12), 467 (6), 466 (21), 406 (16), 339 (12), 336 (18), 322 (26), 309 (11), 308 (21), 286 (13), 248 (12), 240 (25), 234 (8), 227 (8), 220 (15), 218 (14), 204 (23), 190 (14), 115 (17), 93 (100); HRFABMS *m*/*z* 526.3991 $[M]^{+\bullet}$ (calcd for $C_{34}H_{54}O_4$ 526.4022).

Lup-20(29)-11α-ol-25,3β-lactone (2): viscous oil; $[\alpha]_D^{25}$ +16.4° (*c* 0.28, $CHCl_3$); UV (MeOH) λ_{max} 220 nm (log ϵ 4.12);

IR ($CHCl_3$) ν_{max} 3420, 2945, 1745, 1640, 1245, 1145 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 454 $[M]^{+\bullet}$ (12), 438 (21), 374 (23), 302 (16), 250 (14), 234 (16), 220 (12), 204 (22), 190 (14), 115 (17), 93 (100); HRFABMS *m*/*z* 454.3452 $[M]^{+\bullet}$ (calcd for $C_{30}H_{46}O_3$ 454.3463).

3-Desoxybetulonic acid (3): white solid; mp 265−268 °C; $[\alpha]_D^{25}$ +21.2° (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} 214 nm (log ϵ 3.24); IR ($CHCl_3$) ν_{max} 3350, 2945, 1695, 1245, 1145 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 440 $[M]^{+\bullet}$ (18), 395 (21), 317 (6), 316 (15), 302 (12), 248 (16), 234 (14), 227 (68), 204 (16), 190 (20), 115 (13), 93 (100); HRFABMS *m*/*z* 440.3644 $[M]^{+\bullet}$ (calcd for $C_{30}H_{48}O_2$ 440.3654).

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Supporting Information Available: ^1H NMR spectral data for compounds **1a**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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