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Antitumor Agents. Part 212: Bucidarasins A–C, Three New Cytotoxic Clerodane Diterpenes from *Bucida buceras*†

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Abstract—As part of a study on antitumor agents from rainforest plants, four new clerodane diterpenes, bucidarasins A–D (**1**–**4**), were isolated from *Bucida buceras*. Their structures were elucidated from detailed 2D NMR analyses. Compounds **1**–**3** showed potent cytotoxicity against human tumor cell lines with IC_{50} values of 0.5–1.9 μM. The potency was retained in drug resistant lines.

In a continuing collaboration with the National Cancer Institute to discover potent antitumor agents from rainforest plants, an extract of *Bucida buceras* (sample number Q65S0650) showed significant in vitro cytotoxicity against various human tumor cell lines. *B. buceras* (Combretaceae) is a widely spreading timber and shade evergreen tree that is found in tropical regions of northern South America and is commonly called a nonedible black-olive tree.[2] Bioassay guided fractionation led to the isolation of four new clerodane diterpenes, bucidarasins A (**1**), B (**2**), C (**3**), and D (**4**); the former three compounds were identified as the cytotoxic active principles.[3–6] Such highly oxygenated clerodane diterpenes have been found previously as constituents of *Caseria* and *Licana* genera.[7–11] However, no secondary metabolites have been reported in the genus *Bucida*; therefore, this is the first report on the constituents of this genus. The structures of the four new compounds were determined by detailed NMR analyses including COSY, HMQC, HMBC, and NOESY techniques. This report deals with the isolation, structure elucidation, and biological activity of **1**–**4**.

Isolation and Structure Elucidation

A crude extract of *B. buceras* was partitioned successively with *n*-hexane and ethyl acetate. The hexane layer showed potent cytotoxic activity and was purified with repeated silica gel column chromatography and reversed-phase HPLC. The fractionation and isolation were guided by cytotoxic activity against the human tumor A549 cell line, and led to the discovery of four new clerodane diterpenes, designated as bucidarasins A–D.[3–6]

Bucidarasin A (**1**), colorless oil, had a molecular formula of $C_{28}H_{40}O_8$ by HR-MS analysis. The IR spectrum of **1** indicated absorptions for hydroxy (3448 cm^{-1}) and two different ester (1753 and 1732 cm^{-1}) groups. Its ^1H NMR spectrum showed two acetomethyl, four olefinic, three doublet methyl, two singlet methyl, two oxymethine, and two acetal methine proton signals. Twenty-eight carbon signals including three ester carbonyl, an *exo*-methylene, six olefinic, and two quarternary carbon signals were observed in the ^{13}C NMR spectrum (Table 1). A diene moiety (C-11–C-16) and an isobutanoyloxy side chain (C-1′–C-3′) were indicated from observed COSY (H-2′/H-3′, H-11/H-12 and H-14/H-15) and HMBC correlations as indicated in Figure 2. An NOE interaction between H-12 and H-14 suggested that the diene had an *E* configuration. The

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Table 1. ^{13}C NMR data of **1**–**4**[a]

Carbon no.	1	2	3	4
1	26.7	26.7	26.1	35.1
2	66.1	66.2	66.3	198.0
3	121.8	121.8	120.4	127.7
4	145.3	145.3	147.0	158.5
5	53.6	53.5	49.1	42.2
6	72.8	72.8	29.1	23.7
7	37.6	37.6	27.4	25.9
8	36.7	36.7	36.5	34.8
9	37.4	37.4	37.4	39.9
10	36.8	36.8	34.7	42.4
11	30.3	30.3	30.4	36.7
12	129.0	129.0	129.3	128.0
13	135.7	135.7	135.6	136.3
14	141.2	141.2	141.3	141.6
15	111.1	111.1	110.8	110.9
16	12.0	12.0	12.0	12.2
17	15.6	15.6	15.7	14.3
18	95.6	95.6	94.5	62.6
19	97.0	97.0	98.8	71.6
20	25.0	24.9	25.7	23.1
1′	176.5	175.9	176.5	
2′	34.1	41.1	34.1	
3′	18.7, 19.1	27.1	18.7, 19.1	
4′		11.6		
5′		16.6		
18-Acetyl Me	21.6	21.5	21.4	20.8
18-CO	169.4	169.4	169.7	170.2
19-Acetyl Me	21.2	21.2	21.2	20.7
19-CO	170.2	170.2	170.3	170.4

[a]All spectra were recorded in $CDCl_3$, 500 MHz NMR, TMS used as internal standard.

two acetoxy groups were located on acetal carbons at C-18,19 on the basis of HMBC correlations between acetal protons and acetocarbonyl carbons. The chemical shift of H-2 (δ^H 5.44) and HMBC correlation between H-2 and C-1′ indicated that the isobutanoyloxy chain was located at C-2. The diene chain was positioned at C-9 based on HMBC correlation between H-11 and C-9. The degree of unsaturation, as determined from the molecular formula, together with the partial structures mentioned above suggested that **1** is a tricylic clerodane diterpene related to compound **5**.[7] This postulate was supported by comparison of the NMR spectral data of **1** with the literature values of **5** (Table 1). The structure of **1** was further confirmed by HMBC correlations as indicated in Figure 2. The detailed NMR assignments of **1** were based on COSY, HMQC and HMBC spectra and are listed in Table 1. The relative stereochemistry of **1** was elucidated by the observed coupling constants and NOESY spectral data. The coupling constant (12.5 Hz) between H-10 and H-1α suggested that both hydrogens have axial configurations. The H-2 proton signals appeared as a broad singlet without large couplings; therefore, the orientation of H-2 is equatorial (α). A NOESY cross peak between H-1α and H-6 indicated that H-6 has an α orientation and the A/B ring junction has a *cis* configuration. The B ring was determined to be in a chair form based on NOE correlations between H-11/H-19 and H-1α/H-6. The orientations of H-8, H-18, H-19 and the methyl group at C-20 are α as determined from the respective NOE correlations (H-19/H-7β, 11, 18 and H-11/H-17). The NOE correlations of **1** are indicated in Figure 2. Thus, the relative stereochemical structure of **1** was determined as shown in Figure 1. Coymbotin E has been reported as the C-2 stereoisomer (H-2 with β- rather than α-orientation) of **1**.[8] However, no physicochemical or NMR data were reported for coymbotin E.

Bucidarasin B (**2**) was obtained as a colorless oil, and its molecular formula was determined to be $C_{29}H_{42}O_8$ by HR-MS analysis. Its ^1H and ^{13}C NMR spectra closely resembled those of **1**, which suggested that **2** is a clerodane diterpene related to **1**. The molecular weight of **2** was 14 mass units (CH_2) larger than that of **1**, and an additional triplet methyl and methylene multiplet were observed in the NMR spectra of **2**, together with disappearance of one doublet methyl observed in **1**. These data indicate that **2** contains a 2-methylbutanoyloxy

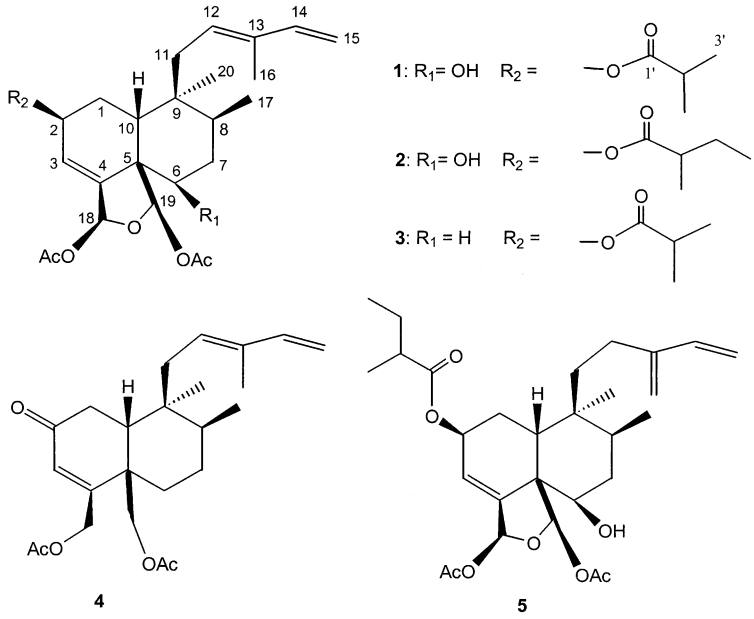

Figure 1. The structures of bucidarasins A (**1**), B (**2**), C (**3**) and D (**4**).

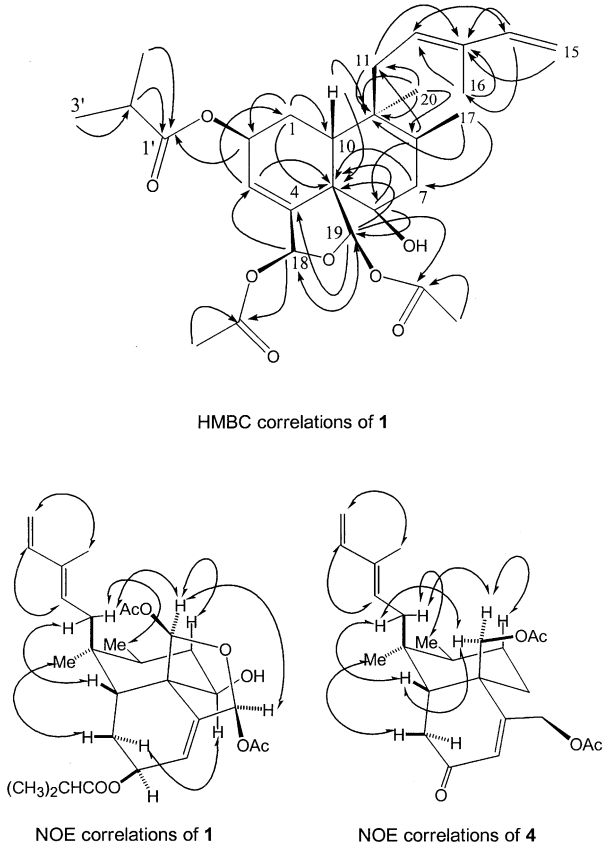

Figure 2. HMBC and NOESY correlations.

group rather than the isobutanoyloxy moiety found in **1**. The presence and location (C-2) of this 2-methylbutanoyloxy chain were further confirmed by COSY (H-2′–H-5′) and HMBC (H-2, H-4′, H-5′/C-1′) correlations. ^{1}H, HMBC, and NOESY spectral data of **1** and **2** showed comparable coupling constants (H-10, H-1, and H-2) and correlations, except for those of the 2-methylbutanoyloxy group. The configuration of the diene chain was again elucidated as the *E* form based on the appropriate NOE interactions (H-12/H-14). The structure of **2** was identical to that of **5**, except for the location of one double bond in the diene side chain (C-12,13 in **2** rather than C-13,16 in **5**). In addition, except for the C-2 substituent, the structure and stereochemistry of **2** were identical to those of **1** and are shown in Figure 1.

Bucidarasin C (**3**), colorless oil, was determined to have a molecular formula of $C_{28}H_{40}O_7$ by HR-MS analysis. The NMR spectra of **3** afforded similar signals to those of **1**, except for the absence of the C-6 hydroxymethine found in **1** and the presence of an additional methylene signal (δ^H 1.73, δ^C 29.1) in **3**. Also, the IR spectrum of **3** showed no hydroxyl absorption. These data suggested that **3** is the C-6 dehydroxylated analogue of **1**. Additional confirmation came from COSY (H-6 to H-17) and HMBC correlations (H-6/C-5, C-19, C-7). The remaining HMBC correlations were comparable to those found in **1**. The relative stereochemistry and configuration of the diene side chain of **3** were established from NOESY data and observed coupling constants. The NOE interactions (H-1α/H-6α; H-11/H-10, 17; H-19/H-7β, 11, 18; H-12/H-14) and the coupling constants (H-2; broad s, H-10/H-1α; 8.4 Hz, axial) of **3** were almost identical to those of **1**. Thus, the relative stereostructure of **3** was determined as shown in Figure 1.

Table 2. Cytotoxicity data for **1**–**4** against parental and drug-resistant human tumor cell lines

Cell line[b]	Compound (IC_{50}, μM)[a]			
	1	**2**	**3**	**4**
KB	0.9	1.1	0.9	>10 (7)[c]
KB-VIN	1.1	1.2	1.0	>10 (22)
KB-7d	0.8	1.1	0.9	>10 (23)
KB-CPT	0.8	1.1	0.9	NA[d]
A549	0.5	1.1	1.1	>10 (6)
IA9	0.5	1.0	0.6	NA
IA9-PTX10	0.5	1.1	0.5	NA
CAKI	1.1	1.9	1.8	NA
HCT-8	0.9	1.3	1.0	NA
MCF-7	0.9	1.8	1.1	>10 (12)
HOS	0.8	1.2	0.9	NA
U87-MG	1.0	1.4	1.2	NA
SK-MEL-2	0.7	1.0	1.0	>10 (7)

[a]IC_{50} = concentration that causes a 50% reduction in absorbance at 562 nm relative to untreated cells using SRB assay.
[b]KB, nasopharyngeal; KB-VIN, vincristine resistant; KB-7d, etoposide resistant, KB-CPT, camptothecin resistant; A549 lung; IA9, ovarian; IA9-PTX10, paclitaxel resistant, CAKI, kidney; HCT-8, ileococal; MCF-7, breast; HO8, bone; U87-MG, gliobastoma; SK-MEL-2, melanoma.
[c]If inhibition <50% at 10 μM, then percent inhibition observed is given as the bracketed value.
[d]NA, not active at 10 μM.

Bucidarasin D (**4**) was isolated as a colorless oil, and its molecular formula was found to be $C_{24}H_{34}O_5$ by HR-MS analysis. The IR spectrum of **4** indicated the presence of ketocarbonyl (1668 cm^{-1}) and ester (1747 cm^{-1}) groups, but no hydroxyl groups were present. The NMR spectrum displayed the characteristic signals of a clerodane diterpene. However, it did not show signals for a butanoyloxy group or acetal moiety as observed in **1**, and instead a ketocarbonyl carbon, an additional methylene, and two oxymethylene signals were found in the NMR spectra of **4**. The disappearance of the C-6 hydroxymethine was also confirmed by the chemical shifts of C-6 (δ^H 1.69, δ^C 23.7) and the COSY correlations from H-6 to H-8. The ketocarbonyl carbon was located at C-2 based on HMBC correlations (H-1, 10, 3/C-2). The connections of the two oxymethylene signals were also established by HMBC correlations (H-19/C-5, 6, 10; 19-acetocarbonyl carbon, H-18/C-3, 4, 5; 18-acetocarbonyl carbon). The remaining HMBC correlations, including those of the diene chain, were identical to those observed in **1**. The stereochemistry of **4** was established by NOE interactions (H-11/H-10, 17; H-19/H-7β, 10, 11; H-12/H-14; H-15/H-16). Thus, the relative stereostructure of **4** was determined as that shown in Figure 1.

Cytotoxic Activity

Compounds were assayed for cytotoxicity using a reported procedure.[5,6] Although bucidarasin D (**4**) was

inactive, bucidarasins A-C (**1**–**3**) were quite potent and had broad spectrum activity as inhibitors of human tumor cell replication. The IC_{50} values ranged from 0.5 to 1.9 μM against nine human tumor cell lines (Table 2). Previous studies have reported that substituents at C-2, -6, and -9 can affect the cytotoxicity of the type of clerodane diterpene.[7–11] The inactivity of **4** suggests that the acetal moiety is essential for cytotoxic activity. The active compounds were not affected by pleiotrophic mechanisms of multi-drug resistance or β-tubulin mutation (Table 2), suggesting that they may have a novel mechanism of action and good potential as clinical candidates.

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References and Notes

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3. Isolation procedure: The twigs of *B. buceras* were collected in Belize in May 1989. Air-dried branches (345 g) were extracted with CH_2Cl_2/MeOH (1:1) and concentrated in vacuo to give a crude extract (15.17 g). This extract was partitioned with *n*-hexane, and the hexane extract was fractionated using silica gel column chromatography (*n*-hexane/EtOAc stepwise gradient) to give 10 fractions. The active fraction 4 was further purified by repeated silica gel column chromatography and ODS HPLC (MeOH/H_2O) to give **3** (29 mg) and **4** (25 mg). Using the same purification method, active fraction 6 afforded **1** (500 mg) and **2** (220 mg).
4. Structural data: Bucidarasin A (**1**), $[\alpha]_D$ +37.7 (*c* 1.4, MeOH), HREIMS *m/z* 445.2589 $[M-AcO]^+$ (calcd for $C_{26}H_{37}O_6$ (Δ 0.0 mmu), IR (neat) 3448, 1753, 1732, 1372, 1230 cm^{-1}, UV (MeOH) λ_{max} 228 nm (log ϵ 4.07), ^1H NMR (500 MHz, $CDCl_3$, δ *J* = Hz) 1.90 (m, H-1αβ), 5.44 (br s, H-2), 5.99 (d, *J* = 3.0, H-3), 3.80 (d, *J* = 9.2, H-6), 1.62, 1.75 (m, H-7αβ), 1.77 (m, H-8), 2.36 (dd, *J* = 5.2, 12.5, H-10), 1.66 (m, H-11), 2.23 (dd, *J* = 8.2, 16.5, H-11), 5.38 (br d, H-12), 6.27 (dd, *J* = 10.5, 17.3, H-14), 4.93 (d, *J* = 10.5, H-15), 5.09 (d, *J* = 17.3, H-15), 1.67 (s, 16-Me), 0.93 (d, *J* = 6.7, 17-Me), 6.73 (t, *J* = 1.5, H-18), 6.52 (s, H-19), 0.81 (s, 20-Me), 2.64 (m, H-2′), 1.21 (d, *J* = 7.0, 3′-Me), 1.22 (d, *J* = 7.0, 3′-Me), 1.95 (s, 18-acetyl Me), 2.10 (s, 19-acetyl Me). Bucidarasin B (**2**), $[\alpha]_D$ +38.7 (*c* 3.2, MeOH), HREIMS *m/z* 459.2744 $[M-AcO]^+$ (calcd for $C_{27}H_{39}O_6$ (Δ −0.2 mmu), IR (neat) 3449, 1754, 1734, 1373, 1229 cm^{-1}, UV (MeOH) λ_{max} 228 nm (log ϵ 4.07), ^1H NMR (500 MHz, $CDCl_3$, δ *J* = Hz) 1.88 (m, H-1αβ), 5.44 (br s, H-2), 6.00 (dd, *J* = 1.8, 4.3, H-3), 3.80 (dd, *J* = 3.7, 12.5, H-6), 1.62, 1.74 (m, H-7αβ), 1.76 (m, H-8), 2.38 (br t, *J* = 8.8, H-10), 1.63 (m, H-11), 2.23 (dd, *J* = 8.0, 16.5, H-11), 5.38 (br d, H-12), 6.27 (dd, *J* = 10.6, 16.6, H-14), 4.93 (d, *J* = 10.6, H-15), 5.10 (d, *J* = 16.6, H-15), 1.66 (s, 16-Me), 0.93 (d, *J* = 7.0, 17-Me), 6.73 (t, *J* = 1.6, H-18), 6.52 (s, H-19), 0.82 (s, 20-Me), 2.44 (m, H-2′), 1.69 (m, H-3′), 0.97 (t, *J* = 7.3, 4′-Me), 1.18 (d, *J* = 6.8, 5′-Me), 1.93 (s, 18-acetyl Me), 2.09 (s, 19-acetyl Me). Bucidarasin C (**3**), $[\alpha]_D$ +14.6 (*c* = 2.9, MeOH), HREIMS *m/z* 429.2640 $[M-AcO]^+$ (calcd for $C_{26}H_{37}O_5$ (Δ 0.0 mmu), IR(neat) 1753, 1732, 1373, 1228 cm^{-1}, UV (MeOH) λ_{max} 227 nm (log ϵ 4.08), ^1H NMR (500 MHz, $CDCl_3$, δ *J* = Hz) 1.90 (m, H-1αβ), 5.40 (br s, H-2), 5.89 (dd, *J* = 1.6, 4.5, H-3), 1.73 (m, H-6αβ), 1.45, 1.50 (m, H-7αβ), 1.63 (m, H-8), 2.23 (br t, *J* = 8.4, H-10), 1.75 (m, H-11), 2.23 (dd, *J* = 8.1, 16.2, H-11), 5.38 (br d, H-12), 6.28 (dd, *J* = 10.7, 17.1, H-14), 4.92 (d, *J* = 10.7, H-15), 5.08 (d, *J* = 17.1, H-15), 1.66 (s, 16-Me), 0.88 (d, *J* = 7.0, 17-Me), 6.73 (t, *J* = 1.5, H-18), 6.36 (s, H-19), 0.83 (s, 20-Me), 2.63 (m, H-2′), 1.20 (d, *J* = 7.0, 3′-Me), 1.22 (d, *J* = 7.0, 3′-Me), 1.94 (s, 18-acetyl Me), 2.10 (s, 19-acetyl Me). Bucidarasin D (**4**), $[\alpha]_D$ −45.5 (*c* 1.4, $CHCl_3$), HREIMS *m/z* 402.2408 $[M]^+$ (calcd for $C_{24}H_{34}O_5$ (Δ +0.2 mmu), IR (neat) 1747, 1668, 1462, 1380, 1225 cm^{-1}, UV (MeOH) λ_{max} 228 nm (log ϵ 4.41), ^1H NMR (500 MHz, $CDCl_3$, δ *J* = Hz) 2.63 (d, *J* = 18.8, H-1β), 2.79 (dd, *J* = 5.6, 18.8, H-1α), 6.17 (s, H-3), 1.69 (m, H-6αβ), 1.40, 1.77 (m, H-7αβ), 1.68 (m, H-8), 2.25 (dd, *J* = 0.8, 5.6, H-10), 1.89 (dd, *J* = 7.8, 15.5, H-11), 2.28 (dd, *J* = 7.9, 15.5, H-11), 5.50 (br t, *J* = 7.0, H-12), 6.36 (dd, *J* = 10.7, 17.8, H-14), 4.94 (d, *J* = 10.7, H-15), 5.09 (d, *J* = 17.8, H-15), 1.73 (s, 16-Me), 0.97 (d, *J* = 7.1, 17-Me), 4.77, 4.90 (dd, *J* = 16.1, 18.0, H-18), 4.00, 4.20 (d, *J* = 11.0, H-19), 0.81 (s, 20-Me), 2.15 (s, 18-acetyl Me), 2.07 (s, 19-acetyl Me).
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6. Cytotoxicity assay: All stock cultures were grown in T-25 flasks (5 mL of RPMI-1640 medium supplemented with 25 mM HEPES, 0.25% sodium bicarbonate, 10% fetal bovine serum, and 100 μg/mL kanamycin). Freshly trypsinized cell suspensions were seeded in 96-well microtitre plates at densities of 1500–7500 cells per well with test compounds from DMSO-diluted stock. After 3 days in culture, cells attached to the plastic substractum were fixed with cold 50% trichloroacetic acid and then stained with 0.4% sulforhodamine B (SRB). The absorbancy at 562 nm was measured using a microplate reader after solubilizing the bound dye. The IC_{50} is the concentration of test compound that reduced cell growth by 50% over a 3-day assay period.
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