Rearranged Diterpenoids from the Biotransformation of *ent*-Trachyloban-18-oic Acid by *Rhizopus arrhizus*

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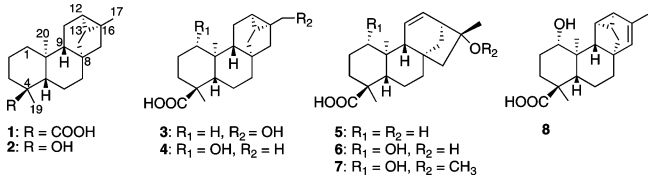
In our search for inhibitors of the antiapoptotic protein Bcl-xL, investigation of *Xylopia caudata* afforded a new diterpenoid, *ent*-trachyloban-4β-ol (**2**), and five known *ent*-trachylobane or *ent*-atisane compounds. Only *ent*-trachyloban-18-oic acid (**1**) exhibited weak binding activity to Bcl-xL. These compounds exhibited cytotoxicity against KB and HCT-116 cell lines with IC_{50} values between 10 and 30 μM. Bioconversion of compound **1** by *Rhizopus arrhizus* afforded new hydroxylated metabolites (**3**−**7**) of the *ent*-trachylobane and *ent*-kaurene type and compound **8**, with a rearranged pentacyclic carbon framework that was named rhizopene. Compounds **3**−**8** were noncytotoxic to the two cancer cell lines, and compounds **3** and **5** exhibited only weak binding affinity for Bcl-xL.

The role of the antiapoptotic protein Bcl-xL, a member of the Bcl-2 family, in the regulation of cell death has been pointed out by many authors. Located in the membrane of the mitochondria of eukaryotic cells, its involvement in apoptosis through activation of the caspase pathway has been widely studied and discussed.[1] Interaction between Bcl-xL and novel ligands appears to be a potentially profitable approach to the discovery of new anticancer drugs.[2] In our search for inhibitors of the antiapoptotic protein Bcl-xL, we used an affinity displacement assay utilizing fluorescence polarization on 1476 ethyl acetate extracts obtained from 670 Malaysian plants as described earlier.[3,4]

Investigation of *Xylopia caudata* Hook. F. & Thomson (Annonaceae) bark extract was motivated by its high-affinity binding to Bcl-xL (31% at 10 μg/mL). Several species of the genus *Xylopia* (Annonaceae) have been reported to contain trachylobane-type diterpenoids[5,6] characterized by a pentacyclic carbon skeleton with a tricyclo[3.2.1.0]octane system. Recently, it was shown that *ent*-trachyloban-3β-ol isolated from the leaves of *Croton zambesicus* possessed cytotoxic effects on HeLa and HL-60 cells and was able to induce apoptosis in human promyelocytic leukemia cells via caspase-3 activation.[7]

In this paper we report the isolation and characterization of six diterpenoids from the bark of *X. caudata* and six new diterpenoids obtained by microbial transformation of *ent*-trachyloban-18-oic acid (**1**). Their binding affinity for Bcl-xL and their cytotoxicity against KB and HCT-116 cell lines were also evaluated.



Results and Discussion

An ethyl acetate extract of *X. caudata* bark was subjected to silica gel chromatography (CC), and subsequent preparative HPLC or preparative TLC afforded *ent*-trachyloban-18-oic acid (**1**), one new diterpenoid (**2**), and four known *ent*-trachylobane or *ent*-atisane compounds. Compound **1** was identified by spectroscopic methods, and its relative configuration was determined by NOESY and X-ray experiments (Figure S17, Supporting Information). The absolute configuration of **1** was deduced from the negative α_D value (−42, *c* 1.0, $CHCl_3$) and from the X-ray analysis of the C-4 epimer reported in the literature.[8]

Table 1. ^{13}C NMR Data (δ) for Compounds **2**−**8**[a]

C	2	3	4	5	6	7	8
1	38.6	39.9	82.3	40.3	82.2	82.8	82.5
2	19.7	18.5	28.9	18.8	28.8	29.4	27.8
3	43.2	38.4	36.6	38.3	36.5	36.6	35.4
4	72.4	48.6	48.5	48.7	48.3	48.8	47.3
5	57.8	51.9	51.3	50.7	50.0	50.5	48.6
6	19.4	24.1	23.9	24.2	23.9	23.9	21.4
7	38.6	38.9	40.6	41.8	42.7	42.7	33.7
8	40.9	42.1	42.9	44.9	45.0	44.9	41.1
9	53.3	55.0	55.6	63.9	64.2	64.5	55.0
10	39.2	39.0	44.5	38.9	45.0	45.2	41.5
11	20.0	20.7	23.7	127.8	132.4	132.9	19.4
12	20.8	20.1	22.2	134.1	132.3	132.1	21.7
13	24.5	23.3	25.7	51.2	51.1	45.6	16.9
14	33.7	34.2	35.1	35.2	36.0	35.4	32.5
15	50.7	47.1	51.9	59.7	59.9	57.0	131.2
16	22.7	30.7	23.9	84.4	83.5	90.3	130.1
17	20.8	67.9	20.9	25.9	26.9	19.7	21.1
18		183.1	183.0	183.1	181.9	183.8	182.0
19	23.2	17.2	17.2	17.2	17.7	17.3	16.8
20	14.4	15.7	12.1	18.4	15.1	14.5	11.4
O-CH_3						49.9	

[a] Spectra recorded in $CDCl_3$ (**2**, **8**), CD_3OD (**3**−**5**, **7**), or pyridine-d_5 (**6**) solution, at 75 MHz (**6**), 125 MHz (**2**, **3**, **5**), or 150 MHz (**4**, **7**, **8**).

Compound **2** was obtained as a white powder. The HREIMS revealed a molecular ion peak at *m/z* 274.2296, compatible with the molecular formula $C_{19}H_{30}O$ and 5 degrees of unsaturation. The IR spectrum of **2** showed an absorption band at 3328 cm^{-1} (OH). The ^{13}C and DEPT spectra revealed 19 signals, indicating three methyl, eight methylene, four methine, and four quaternary carbons, one of which was oxygenated (Table 1). The presence of a cyclopropane moiety was indicated by the chemical shifts for two methine and one quaternary carbon (δ 20.8, 24.5, and 22.7), corresponding to C-12, C-13, and C-16, respectively, consistent with a trachylobane skeleton for compound **2**. The ^{13}C NMR spectrum of **2** was similar to that of **1**, except for C-3 and C-4 (δ 43.2 and 72.4, respectively), indicating the presence of an OH group at C-4. The relative configuration of **2** was established from NOESY correlations between H-5 at δ 1.01 and H-9 and H-6_{eq} (δ 1.15 and

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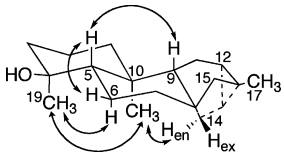

Figure 1. Key NOESY correlations for compound **2**.

1.66, respectively) and between Me-19 at δ_H 1.08 and Me-20 (δ_H 0.88) and H-6_{ax} (δ_H 1.24) (Figure 1), indicating a β-orientation of the OH group at C-4. Compound **1** is likely the precursor of compound **2**, which could be formed by an oxidation at C-4 after decarboxylation of **1**. Taking into account the above and the negative value of α_D (−33, *c* 0.25, $CHCl_3$) for **2**, we assume that **2** belongs to the enantio series. Compound **2** was named *ent*-trachyloban-4β-ol.

Four known diterpenes, *ent*-trachyloban-19-oic acid, *ent*-trachyloban-18-ol, *ent*-trachyloban-19-ol, and *ent*-atisan-16β-ol, were also isolated from the bark extract. Their structures were determined by comparison of their spectroscopic data with those reported in the literature.[9−12] The absolute configurations were suggested by the negative sign of their α_D values. The six diterpenoids were evaluated for their binding affinity to Bcl-xL and for their cytotoxicity in vitro against KB and HCT-116 cancer cell lines. Only *ent*-trachyloban-18-oic acid (**1**) exhibited Bcl-xL binding affinity, though this was weak (IC_{50} of 400 ± 100 μM). Its high concentration in the bark (2.23% w/w) could explain the biological activity found for the crude extract. The six compounds exhibited moderate cytotoxicity on both cancer cell lines with IC_{50}'s ranging from 10 to 30 μM.

Compound **1** was selected as a starting material for further biocatalytically generated chemodiversity. Microbiological transformations of diterpenoids having *ent*-kaurane skeletons[13] have been widely investigated, leading to a large variety of functionalized compounds. In contrast, only a few examples of biotransformation of the *ent*-trachylobane skeleton have been reported.[14,15] Among them, biotransformation of the epimer at C-4 of compound **1**, *ent*-trachyloban-19-oic acid, by *Rhizopus stolonifer*, afforded four hydroxylated compounds, of which two underwent a backbone rearrangement. We screened a range of oxidizing fungi (*Rhizopus arrhizus, Aspergillus terreus, Bauveria bassiana, Mucor plumbeus,* and *Cylindrocarpon radicicola*) for the biotransformation of compound **1**. Bioconversions were monitored by HPLC, and *R. arrhizus*, which gave rise to the highest bioconversion yield and the highest diversity of metabolites, was selected for further analysis.

Compound **1** (125 mg) was incubated with *R. arrhizus* for two weeks. The incubation medium was filtered and extracted with EtOAc and purified by flash chromatography and preparative HPLC, leading to six new hydroxylated products (**3**−**8**), of which one (**8**) presented a rearranged tricyclo[3.2.1.0]octane carbon skeleton with a cyclopropane ring at positions C-11, C-12, and C-13 observed for the first time in nature.

The HRESIMS spectra of compounds **3** and **4** revealed quasi-molecular ion peaks $[M - H]^-$ at *m/z* 317.2144 and 317.2114, respectively, corresponding to the molecular formula $C_{20}H_{30}O_3$ for both compounds with one additional oxygen atom when compared to compound **1**. Their IR spectra showed an absorption band at 3200−3400 cm^{-1} (OH). NMR data of **3** and **4** were similar to those of compound **1** (Tables 1 and 2). The ^{13}C and DEPT NMR spectra of compound **3** revealed the presence of a methylene carbon (δ 67.9) instead of the CH_3-17 group in **1**, which indicated that CH_3-17 was hydroxylated by *R. arrhizus*. This was confirmed in the HMBC spectrum, where cross-peaks between CH_2-17 protons and C-12, C-13, C-15, and C-16 were observed. When compared with **1**, the ^{13}C and DEPT NMR spectra of compound **4** revealed that CH_2-1 in **1** was replaced by a methine carbon at δ 82.3, indicating that it was hydroxylated. The methine proton at δ 3.26, attached to

Table 2. ^1H NMR Spectroscopic Data for Compounds **2**−**8** (δ values in ppm and *J* in Hz)[a]

H	2	3	4	5	6	7	8
1_{ax}	0.73, m	0.87, m	3.26, dd (11.4, 4.2)	1.11, m	3.95, dd (10.8, 4.3)	3.47, dd (11.3, 4.3)	3.41, dd (11.4, 4.1)
1_{eq}	1.44, m	1.59, m		1.81, m			
2_{ax}	1.44, m	1.64, m	1.69, dddd (13.9, 13.9, 11.4, 3.9)	1.67, m	1.95, m	1.73, m	1.71, m
2_{eq}	1.39, m	1.45, m	1.52, m	1.51, m		1.58, m	1.60, m
3_{ax}	1.27, m	1.75, m	1.86, ddd (13.8, 13.8, 4.3)	1.79, m	2.37, td (13.3, 4.1)	1.92, ddd (13.6, 13.6, 4.0)	1.90, ddd (13.6, 13.6, 4.6)
3_{eq}	1.72, m	1.53, m	1.50, m	1.55, m	1.81, m	1.51, m	1.63, m
5	1.01, m	1.65, m	1.59, dd (11.5, 1.7)	1.77, m	2.27, d (10.7)	1.75, d (11.0)	1.71, m
6_{ax}	1.24, m	1.45, m	1.48, m	1.45, m	1.70, m	1.49, m	1.49, m
6_{eq}	1.66, m	1.12, m	1.07, m	1.12, m	1.53, m	1.12, m	1.08, m
7_{ax}	1.40, m	1.41, m[b]	1.42, m	1.62, m[b]	1.89, m	1.60, m	1.49, m
7_{eq}	1.40, m	1.46, m[b]	1.34, m	1.69, m[b]	1.89, m	1.60, m	1.83, br d (8.1)
9	1.15, m	1.28, dd (10.7, 7.3)	1.44, m	1.51, m	2.08, m	1.74, m	0.75, s
11a	1.87, ddd (14.3, 11.6, 2.8)	1.95, ddd (14.3, 11.7, 2.8)	2.24, ddd (15.4, 7.3, 2.4)	5.55, m	7.00, dd (10.0, 3.9)	6.30, dd (10.0, 3.9)	1.82, m
11b	1.64, m	1.74, m	2.10, ddd (15.4, 11.5, 3.5)				
12	0.55, m	0.81, m	0.57, ddd (7.9, 3.5, 2.4)	5.95, m	6.10, dd (10.0, 6.7)	5.80, m	1.46, m (5.9)
13	0.79, dd (7.7, 2.7)	1.06, m (7.9, 3.0)	0.81, dd (7.9, 3.2)	2.19, m	2.54, m	2.44, dd (6.6, 3.0)	1.46, m
14_{en}	2.02, d (11.9)	2.14, d (11.9)	2.06, d (11.8)	1.85, m	2.13, m	1.79, br d (10.5)	1.69, m
14_{ex}	1.14, m	1.17, m	1.15, ddd (11.8, 3.2, 1.7)	1.62, m	2.18, m	1.43, dd (10.5, 3.0)	0.40, d (11.4)
15a	1.37, d (11.4)	1.55, d	1.39, dd (11.3, 0.9)	1.50, s	1.93, m	1.55, d (14.2)	5.28, dd (1.5, 1.5)
15b	1.23, d (11.4)	1.42, d	1.32, d (11.3)	1.50, s	1.78, m	1.40, d (14.2)	
17	1.11, s	3.52, s (2 H)	1.12, s	1.28, s	1.60, s	1.25, s	1.78, d (1.4)
19	1.08, s	1.13, s	1.12, s	1.13, s	1.48, s	1.11, s	1.16, s
20	0.88, s	1.01, s	1.06, s	0.99, s	1.41, s	1.03, s	1.01, s
OCH_3						3.13, s	

[a] Spectra recorded in $CDCl_3$ (**2**, **8**), CD_3OD (**3**−**5**, **7**), or pyridine-d_5 (**6**) solution, at 75 MHz (**6**), 125 MHz (**2**, **3**, **5**), or 150 MHz (**4**, **7**, **8**). [b] Values can be interchanged.

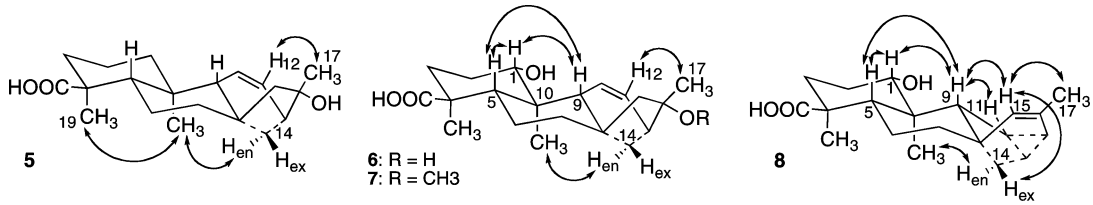

Figure 2. Key NOE correlations for compounds **5**−**8**.

the carbon at δ 82.3, showed cross-peaks with CH_2-2 in the COSY spectrum. In the HMBC spectrum, correlations between this methine proton and C-10 and CH_3-20 confirmed the OH group at C-1. In the NOESY spectrum, correlations between H-1, H-5, and H-9 indicated the α-orientation for OH-1. Additional NMR data confirmed that **3** and **4** were 17-hydroxy-*ent*-trachyloban-18-oic acid and 1α-hydroxy-*ent*-trachyloban-18-oic acid, respectively.

Compound **5** was assigned the molecular formula $C_{20}H_{30}O_3$, on the basis of its HRESIMS, indicating one additional oxygen atom compared to compound **1** and six degrees of unsaturation. Its IR spectrum revealed an absorption band at 3417 cm^{-1} (OH). The NMR data of **5** showed similarities to compound **1**, but the absence of the three signals around δ 20.0−24.5 suggested that the cyclopropane ring was absent in **5**. Instead, a kaurene-type skeleton was suggested by the following evidence. The ^{13}C and DEPT spectra revealed 20 carbon signals: three methyl, seven methylene, five methine (of which two resonated at δ 127.8 and 141.1), and five quaternary carbons. In the COSY spectrum, correlations between two vinylic protons (δ 5.55 and 5.95; H-11 and H-12, respectively) and H-9 and H-13 (δ 1.51 and 2.19, respectively) placed the double bond at position 11. The NMR data of compound **5** were comparable to those of natural *ent*-16α-hydroxykaur-11-en-19-oic acid isolated from *Helianthus rigidus*.[16] HMBC correlations between the methyl protons at δ 1.28 (Me-17) and C-15 and C-16 (at δ 59.7 and 84.4, respectively) placed the OH at position 16. The relative configuration of compound **5** was deduced from cross-peaks between CH_3-17 and H-12 on one hand, leaving the OH-16 group α-oriented, and between CH_3-19 and CH_3-20 with H-14_{endo} (δ_H 1.81) on the other hand, observed in the NOESY spectrum (Figure 2). Compound **5** was named *ent*-16α-hydroxykaur-11-en-18-oic acid.

The HRESIMS of compound **6** displayed a quasi molecular ion peak $[M - H]^-$ at *m*/*z* 333.2080 corresponding to the molecular formula $C_{20}H_{30}O_4$, with one additional oxygen compared to **5**. Its NMR data were similar to those of compound **5**, suggesting a kaurene-type skeleton for **6**. When compared to **5**, CH_2-1 was replaced by a methine at δ_C 82.2 in favor of a secondary OH in structure **6**. The correlations between H-1 at δ_H 3.95 and H_2-2 at δ_H 1.95 in the COSY spectrum and between H-1 and C-9 in the HMBC spectrum allowed placing of the OH at position 1. In the NOESY spectrum, cross-peaks between CH_3-17 and H-12, on one hand, and between H-1, H-5, and H-9, on the other hand, indicated that the OH groups at C-1 and C-16 were both α-oriented (Figure 2). Compound **6** was named *ent*-1α,16α-dihydroxykaur-11-en-18-oic acid.

Compound **7** had the molecular formula $C_{21}H_{32}O_4$ deduced from the HRESIMS. The ^{13}C and DEPT spectra revealed four methyl groups, of which one was an OCH_3 (δ_C 49.9 ppm). NMR data of compounds **6** and **7** are closely comparable (Tables 1 and 2), suggesting that one of the OH groups of compound **6** was methylated. The upfield shift of C-17 at δ_C 19.7 compared to compound **6** (δ_C 26.9) suggested that the OCH_3 group was at position 16. This was confirmed in the HMBC spectrum by a cross-peak between the OCH_3 protons and C-16. NOESY correlations for compound **7** were comparable to those observed for **6**, indicating that **7** had the same relative configuration (Figure 2). Compound **7** was named *ent*-1α-hydroxy-16α-methoxykaur-11-en-18-oic acid.

The HRESIMS of compound **8** indicated the molecular formula

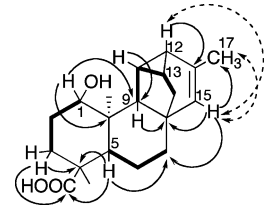

Figure 3. Key 2D NMR correlations of compound **8**: ^3J COSY correlations (bold bonds), ^4J COSY correlations (dashed arrows), and HMBC (solid arrows) correlations.

$C_{20}H_{28}O_3$ and seven degrees of unsaturation. The IR spectrum revealed an OH band at 3294 cm^{-1}. When compared with trachylobanes **3** and **4** and *ent*-kaurenes **5**−**7**, the NMR data of **8** suggested that it was a diterpene having a carbon skeleton characterized by one double bond (two carbons at δ_C 131.2 and 130.1) and a tricyclo[3.2.1.0]octane system with a cyclopropane ring at positions C-11, C-12, and C-13 formed by three methines at δ_C 19.4, 21.7, and 16.9, respectively. The presence of the cyclopropane was supported by the $^1H/^{13}C$ coupling constants observed in the nondecoupled HSQC spectrum at J = 182, 165, and 178 Hz for CH-11, -12, and -13, respectively (see Supporting Information), which are beyond the typical value of 120 Hz for methine groups. The location of the cyclopropane at positions C-11, C-12, and C-13 and the double bond at position C-15 was deduced from the COSY and HMBC correlations (Figure 2). The COSY spectrum revealed the C-9−C-11−C-13−C-14 spin system by observation of correlations between H-9 and H-11, H-11 and H-13, and H-13 and H_2-14. In the HMBC spectrum, correlations between H_2-14 and C-8, C-9, C-11, and C-12 at δ_C 41.1, 55.0, 19.4, and 21.7, respectively, and between the vinyl proton (at δ 5.28) and C-7, C-8, and CH_3-17 supported the tricyclic ring system and indicated that Me-17 was attached to C-16. The ^4J COSY correlations between H-15 and H-12, H-14_{endo}, and CH_3-17 confirmed the double bond at C-15−C-16. Other COSY and HMBC correlations indicated that the OH was at C-1 (Figure 3). The relative configuration of **8** was established from NOESY correlations between H-1, H-5, and H-9, leaving the OH equatorial at C-1, between H-14_{endo} and Me-20, and between H-15 and H-9, H-14_{exo}, and Me-17 at δ_H 1.78, indicating that the cyclopropane ring and the C-8−C-14 bond were α-oriented (Figure 2). Thus, the structure of **8** is established as *ent*-1α-hydroxyrhizop-15-en-18-oic acid, representing a new family of natural pentacyclic diterpenes having a carbon backbone that we have named rhizopene. A compound having a cyclopropane ring at C-11, C-12, and C-13 was reported to have been obtained chemically by R. McCrindle, but no spectroscopic evidence supported this claim.[18] A biogenetic hypothesis of compound **8** is provided in the Supporting Information (S18).

In conclusion, six oxidized compounds were obtained from the microbial transformation of compound **1** by *R. arrhizus*. Compounds **3** and **4** resulted from a direct enzymatic hydroxylation of compound **1** at positions 17 and 1, respectively, whereas a backbone rearrangement prior to the oxidation of *ent*-trachyloban-18-oic acid (**1**) occurred for compounds **5**−**7**. Finally, compound **8**, named *ent*-1α-hydroxyrhizop-15-en-18-oic acid, was a rearranged product. The biotransformation of compound **1** introduced OH groups with high stereo- and regioselectivity. From a biosynthetic point of view, kaur-

11-ene derivatives are isolated only when trachylobane derivatives are also in the plant, indicating that they are rearrangement products of trachylobane diterpenoids.[17]

The six new metabolites were evaluated for their binding affinity to the antiapoptotic Bcl-xL protein and for cytotoxicity against KB and HCT-116 cancer cell lines. Compounds **3** and **5** exhibited weak binding affinity for Bcl-xL comparable to compound **1**, with IC_{50} values of 220 and 380 μM, respectively. The other compounds were inactive. No cytotoxicity was detected against KB or HCT 116 cells for the six products of biotransformation at 10 μg/mL. The introduction of an OH group may be seen as a detoxification process for *R. arrhizus*.

Experimental Section

General Experimental Procedures. Optical rotations were measured at 25 °C on a JASCO P1010 polarimeter. IR spectra were obtained on a Nicolet FTIR 205 spectrophotometer. NMR spectra were recorded on a Bruker 500 MHz instrument (Avance 500) for compounds **2**, **3**, and **5**, on a Bruker 300 MHz instrument for compound **6**, and on a Bruker 600 MHz instrument for compounds **4**, **7**, and **8**, using $CDCl_3$ (**2**, **8**), MeOD (**3**−**5**, **7**), and pyridine-d_5 (**6**) as solvents. HRESIMS were run on a Thermoquest TLM LCQ Deca ion-trap spectrometer and HREIMS on a ThermoFinnigan MAT 95 XL spectrometer. Kromasil analytical, semipreparative, and preparative C_{18} columns (250 × 4.5 mm, 250 × 10 mm, and 250 × 21.2 mm; i.d. 5 μm, Thermo) were used for preparative HPLC separations using a Waters autopurification system equipped with a binary pump (Waters 2525), a UV−vis diode array detector (190−600 nm, Waters 2996), and a PL-ELS 1000 ELSD Polymer Laboratory detector. Silica gel 60 (35−70 μm) and analytical TLC plates (Si gel 60 F 254) were purchased from SDS (France). Prepacked 12 g silica gel Redisep cartridges were used for flash chromatography using a Combiflash-Companion apparatus (Serlabo). All other chemicals and solvents were purchased from SDS (France).

Plant Material. Trunk bark of *Xylopia caudata* was collected in the dense rainy forest of Jelebu, in Negeri Sembilan state (Malaysia), in February 2006, and identified by the botanist T. Leong Eng. A voucher specimen (KL-5188) is deposited at the Herbarium of the Forest Research Institute, Kepong, Malaysia.

Extraction and Isolation. Bark (1.5 kg) was extracted with EtOAc (3 × 1 L) followed by MeOH (3 × 1 L) at 40 °C and 100 bar using a Zippertex automatic extractor. The EtOAc crude extract was concentrated in vacuo at 40 °C to yield 165 g of residue. The EtOAc extract (16 g) was subjected to silica gel CC using a gradient of heptanes−CH_2Cl_2−MeOH (1:0:0 to 0:80:20) of increasing polarity, leading to 30 fractions on the basis of TLC. Fraction 11 (1.4 g; heptanes−CH_2Cl_2, 50:50) was identified as *ent*-trachyloban-18-oic acid (**1**). Fraction 10 (239 mg; heptanes−CH_2Cl_2, 60:40) was subjected to preparative C_{18} CC using the mobile phase MeCN−H_2O (80:20 + 0.1% formic acid) at 21 mL min^{-1} to afford *ent*-trachyloban-18-oic acid (**1**; 140.8 mg) and *ent*-trachyloban-19-oic acid (23.5 mg). Fraction 12 (689.6 mg; heptanes−CH_2Cl_2, 50:50) was purified by flash CC using silica gel and the gradient heptanes−CH_2Cl_2 (20:80 to 0:100) at 20 mL min^{-1} to afford five fractions (12a to 12e). Fraction 12c (176.5 mg; heptanes−CH_2Cl_2, 70:30) was then purified on a preparative C_{18} column using MeCN−H_2O (90:10 + 0.1% formic acid) at 21 mL min^{-1} to afford **1** (43.7 mg) and *ent*-trachyloban-18-ol (49.0 mg). Fraction 14 (100.5 mg; heptanes−CH_2Cl_2, 40:60) was subjected to preparative C_{18} CC using MeOH−H_2O (87:13 + 0.1% formic acid) at 21 mL min^{-1} to afford **1** (56.8 mg), *ent*-atisan-16β-ol (15.1 mg), and *ent*-trachyloban-19-ol (7.8 mg). Fraction 16 (210.8 mg; heptanes−CH_2Cl_2, 30:70) was subjected to preparative C_{18} chromatography using MeCN−H_2O (75:25) at 21 mL min^{-1} to afford three fractions (16a to 16c). Fraction 16b (52.4 mg) was separated by PTLC, using CH_2Cl_2−MeOH (98:2), to give compound **2** (18.4 mg).

Microorganisms and Fermentation Conditions. Five oxidizing fungi were evaluated for the bioconversion of *ent*-trachyloban-18-oic acid (**1**): *Rhizopus arrhizus, Aspergillus terreus, Bauveria bassiana, Mucor plumbeus,* and *Cylindrocarpon radicicola.* The cultivation medium consisted of 10 g of corn steep, 0.5 g of $MgSO_4$, 2 g of $NaNO_3$, 0.5 g of KCl, and 0.02 g of $FeSO_4$. Mixtures were incubated at 27 °C on a rotary shaker (200 rpm). Biomass from 3-day-old cultures was recovered by centrifugation and rinsed with water. The wet biomass (4 g) was introduced into an aqueous suspension of 10 mg of **1**, and the bioconversion progress was followed by HPLC. *R. arrhizus* provided the highest bioconversion yield and diversity of metabolites and was selected for preparative incubation.

Biotransformation of *ent*-Trachyloban-18-oic acid by *R. arrhizus*. Compound **1** (125 mg) was incubated for two weeks with 20 g of *R. arrhizus* cells in 300 mL of water. The aqueous phase was recovered by filtration and extracted with ethyl acetate (3 × 300 mL). The extract was concentrated in vacuo at 40 °C to yield 151 mg of residue. The extract was subjected to flash chromatography using silica gel with the mobile phase CH_2Cl_2−MeOH (100:0 to 95:5 in 60 min, flow rate 15 mL min^{-1}) to give 10 fractions. Fractions 6 and 7 (eluted with CH_2Cl_2−MeOH, 97.5:2.5) were combined (25.2 mg) and subjected to preparative C_{18} CC using MeOH−H_2O (80:20 + 0.1% formic acid) at 4.7 mL min^{-1}, affording compounds **3** (3.5 mg) and **5** (5.3 mg). Using the same method, combined fractions 4 and 5 (12 mg, CH_2Cl_2−MeOH, 97.5:2.5) afforded compound **4** (4.7 mg). Fractions 8 and 9 were combined (55.3 mg; CH_2Cl_2−MeOH, 95:5) and subjected to preparative C_{18} CC using MeOH−H_2O (70:30 + 0.1% formic acid) at 20 mL min^{-1}, affording compounds **6** (12.1 mg) and **7** (2.3 mg) and 3.6 mg of an impure compound (t_R 17.1 min). Separation of the latter using an analytical C_{18} column eluted with MeOH−H_2O (68:32 + 0.1% formic acid) at 1 mL min^{-1} afforded compound **8** (1.3 mg).

***ent*-Trachyloban-18-oic acid (1):** white powder; mp 136−138 °C; $[\alpha]^{25}_D$ −42 (*c* 1.0, $CHCl_3$); IR ν_{max} 2919, 2859, 1690, 1276, 758 cm^{-1}; ^1H NMR and ^{13}C NMR data, see ref 10; HRESIMS *m/z* 301.2181 [M − H]$^-$ (calcd for $C_{20}H_{29}O_2$, 301.2168).

***ent*-Trachyloban-4β-ol (2):** white powder; mp 110−111 °C; $[\alpha]^{25}_D$ −33 (*c* 0.25, $CHCl_3$); IR ν_{max} 3328, 2912, 2851 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HREIMS *m/z* 274.2296 $[M]^{+\bullet}$ (calcd for $C_{19}H_{30}O$, 274.2297).

***ent*-17-Hydroxytrachyloban-18-oic acid (3):** white powder; mp 182−183 °C; $[\alpha]^{25}_D$ −23 (*c* 0.3, CH_3OH); IR ν_{max} 3205, 2929, 2852, 1693, 1599, 1014 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 317.2144 [M − H]$^-$ (calcd for $C_{20}H_{29}O_3$, 317.2117).

***ent*-1α-Hydroxytrachyloban-18-oic acid (4):** white powder; mp 229−232 °C; $[\alpha]^{25}_D$ −31 (*c* 0.45, CH_3OH); IR ν_{max} 3418, 2923, 2856, 1688 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 317.2114 [M − H]$^-$ (calcd for $C_{20}H_{29}O_3$, 317.2117).

***ent*-16α-Hydroxykaur-11-en-18-oic acid (5):** white powder; mp 234−236 °C; $[\alpha]^{25}_D$ −138 (*c* 0.46, CH_3OH); IR ν_{max} 3417, 2924, 2840, 1684, 1261 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 317.2150 [M − H]$^-$ (calcd for $C_{20}H_{29}O_3$, 317.2117).

***ent*-1α,16α-Dihydroxykaur-11-en-18-oic acid (6):** white powder; mp 223−226 °C; $[\alpha]^{25}_D$ −142 (*c* 0.3, pyridine); IR ν_{max} 3304, 2978, 2925, 2868, 1695, 1386, 1241, 1024, 1000, 875 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 333.2080 [M − H]$^-$ (calcd for $C_{20}H_{29}O_4$, 333.2066).

***ent*-1α-Hydroxy-16α-methoxykaur-11-en-18-oic acid (7):** white, amorphous solid; $[\alpha]^{25}_D$ −97 (*c* 0.3, CH_3OH); IR ν_{max} 3343, 2926, 2864, 1541, 1390, 1029 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 347.2213 [M − H]$^-$ (calcd for $C_{21}H_{32}O_4$, 347.2222).

***ent*-1α-Hydroxyrhizop-15-en-18-oic acid:** white, amorphous solid; $[\alpha]^{25}_D$ −57 (*c* 0.1, CH_3Cl); IR ν_{max} 3294, 2935, 2852, 1701, 1157, 993 cm^{-1}; ^1H NMR and ^{13}C NMR data (Tables 1 and 2); HRESIMS *m/z* 315.1972 [M − H]$^-$ (calcd for $C_{20}H_{27}O_3$, 315.1960).

Crystal Data of Compound 1.[19] The X-ray crystallographic data were measured on an Enraf-Nonius kappaCCD diffractometer at 293(2) K using graphite-monochromated Mo Ka radiation (λ = 0.71069 Å). Compound **1** crystallized in the trigonal system with a particularly long cell parameter ($c \approx 54$ Å), suggesting a fine-slicing strategy and to push the detector backward at a distance of 98.7 mm from the sample to prevent overlap of neighboring reflections. In the light of this experimental geometry and the paucity of observed data beyond atomic resolution, data collection was terminated at $q = 20°$. Among the possible space groups allowed by the Laue symmetry and systematic absences, $P3_22_1$ was shown to be the correct choice based on prior knowledge on the enantiomeric species. The structure was solved by conventional direct methods and refined by full-matrix least-squares on F^2. All non-hydrogen atoms were refined anisotropically, whereas hydrogen atoms were located from difference Fourier maps but refined as a riding model with U_{iso} set to 1.5 (methyl and carboxyl) and 1.2 (other carbons) times the equivalent isotropic displacement parameter of the attached atom. The hydrogen atom of the carboxyl group was delocalized over the two oxygen atoms with equal occupancy of 0.5 to allow the formation of a carboxylic acid dimer between the molecule in general position *x*, *y*, *z* and that in *y*−1, *x*+1, 2−*z*,

independently of the crystal symmetry. The crystallographic data are summarized as follows: colorless, massive crystal of dimensions 0.59 × 0.39 × 0.28 mm, $C_{20}H_{30}O_2$, $M_r = 302.44$, trigonal system, space group $P3_221$, $Z = 6$, $a = b = 7.591(2)$ Å, $c = 53.694(10)$ Å, $V = 2679.5(11)$ Å^3, $D_{calcd} = 1.125$ g/cm^3, $F(000) = 996$, $\mu = 0.070$ mm^{-1}, 11 341 collected reflections ($3.10° \leq \theta \leq 19.77°$), $-7 \leq h \leq 7$, $-3 \leq k \leq 3$, $-50 \leq l \leq 49$, 1014 independent reflections ($R_{int} = 0.0256$), goodness-of-fit on F^2: $S = 1.123$, $R_1 = 0.0776$ and $wR_2 = 0.1430$ for all reflections, $R_1 = 0.0566$ and $wR_2 = 0.1236$ for 780 observed reflections [$I > 2\sigma(I)$], refining 203 parameters and no restraints, semiempirical absorption correction from multi *j* and *w*-scans ($T_{min} = 0.735$, $T_{max} = 0.981$), final electron density between −0.250 and 0.204 e Å^{-3}. The programs used for the crystallographic study were as follows: data collection, COLLECT;[20] cell refinement, DENZO[20] and COLLECT; data reduction, SCALEPACK[21] and COLLECT; program used to solve structure, SHELXS97;[22] program used to refine structure, SHELXL97;[22] molecular graphics, ORTEP within PLATON.[23]

Biological Assays. The binding affinity for Bcl-xL was evaluated by competition against a fluorescent-labeled reference compound (fluorescent-tagged BH3 domain of the protein Bak), as described earlier.[3,4] The unlabeled peptide Bak(BH3) was used as a positive control. Results are expressed as binding activity, i.e., percentage of inhibition of the binding of labeled reference compound, or as IC_{50}, the concentration corresponding to 50% of such inhibition.

The cytotoxic activities of the compounds were evaluated against two cancer cell lines: KB (oral epidermoid carcinoma) and HCT-116 (human colon carcinoma). Cytotoxicity assays were performed according to a published procedure.[24] Taxotere was used as a reference compound (IC_{50}'s of 0.15 and 1.2 nM for KB and HCT-116 cells, respectively).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **2**−**8** and ORTEP diagram of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Crystallographic data for the structure of compound **1** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC 765947). Copies of the data can be obtained free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).
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