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Cytotoxic and Antibacterial Beilschmiedic Acids from a Gabonese Species of *Beilschmiedia*

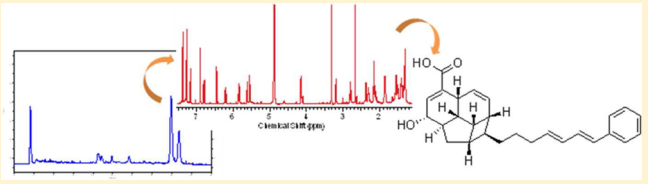
Russell B. Williams,*[,†] Steven M. Martin,[†] Jin-Feng Hu,[†] Vanessa L. Norman,[†] Matt G. Goering,[†] Sandra Loss,[‡] Mark O'Neil-Johnson,[†] Gary R. Eldridge,[†] and Courtney M. Starks[†]

[†]Lead Discovery and Rapid Structure Elucidation Group, Sequoia Sciences, Inc., 1912 Innerbelt Business Center Drive, St. Louis, Missouri 63114, United States

[‡]Bruker BioSpin AG, Industriestrasse 26, CH-8117 Fällanden, Switzerland

Supporting Information

ABSTRACT: High-throughput natural products chemistry methods have facilitated the isolation of eight new (**1**–**8**) and two known (**9** and **10**) beilschmiedic acid derivatives from the leaves of a Gabonese species of *Beilschmiedia*. Compounds **3**–**10** were isolated in microgram quantities, and the NMR data for structure elucidation and dereplication were acquired utilizing a Bruker BioSpin TCI 1.7 mm MicroCryoProbe. All of the compounds were screened for cytotoxic and antibacterial activity against NCI-H460 human lung cancer cells and a clinical isolate of methicillin-resistant *Staphylococcus aureus*, respectively. This is the first report of cytotoxic activity for the endiandric/beilschmiedic acid class of compounds.



A few species of *Beilschmiedia* and one species of *Endiandra* have been found to produce a remarkable series of polycyclic fatty acids known as beilschmiedic and endiandric acids.[1−13] These complex fatty acids, typically isolated as racemic mixtures, were proposed by Black to be the result of a series of consecutive nonenzymatic cyclizations (8πe, 6πe, and Diels−Alder) of a polyunsaturated fatty acid precurser.[14] Support for such a mode of biosynthesis was provided by a biomimetic synthesis carried out by Nicolaou.[15] To date, 29 of these compounds have been reported in the literature as natural products, with 31 additional compounds resulting from derivatization and synthesis. Recent publications have reported antibacterial and anti-inflammatory activity.[9,10,13] Additionally, one analogue was the subject of a patent that claimed it was useful in the treatment of asthma.[7] Given the unique chemical space occupied by these compounds and the lack of biological activity reported, we checked our plant library to see if any material was available that could yield similar compounds. We found that our library contained two collections of *Beilschmiedia* from Gabon. One had previously been screened for cytotoxic activity and was found to be active. Further examination of that hit resulted in the isolation of the known beilschmiedic acids A (**9**) and C (**10**),[9] as well as the new beilschmiedic acids H−O (**1**−**8**). Compounds **4**, **5**, **7**, and **8** represent new frameworks as described by Lipkus et al.[16,17] Several of the compounds demonstrated moderate cytotoxic activity in the NCI-H460 human lung cancer cell line. The second collection contained no evidence of beilschmiedic acids, as indicated by thorough HPLC and NMR analysis.

RESULTS AND DISCUSSION

The two Gabonese collections of *Beilschmiedia* were obtained from the Tchimbele region in November and from the Kwassa region in May by the Missouri Botanical Garden. Both were flowering at the time of collection and identified to the genus level. Unfortunately, determination to the species level was not possible, as the African *Beilschmiedia* are in need of revision.[18] The collection from the Tchimbele region was used to generate a preparative HPLC library that was screened for cytotoxicity. Isolation of the components of the active fractions yielded 10 compounds. The structures of these compounds were determined from microgram amounts using Bruker BioSpin TCI 1.7 mm MicroCryoProbe NMR technology, ESIMS, and comparison to data found in the literature. Investigation of the collection from the Kwassa region contained no evidence of beilschmiedic acids.

Compound **1** was isolated as an optically inactive yellow oil. A molecular formula of $C_{27}H_{30}O_3$ was suggested by HRESIMS. Comparison of the ^{1}H NMR spectrum (Table 1) of **1** to that of beilschmiedic acids A (**9**) and C (**10**) indicated that the tetracyclic portion of **1** matched that reported for beilschmiedic acid C (**10**). Specifically, the chemical shifts for H-4 (δ_H 4.14, d 10 Hz) and H-5 (δ_H 6.91, brs) indicated the presence of an α-OH and an α/β-unsaturated carbonyl.[9] The ^{1}H NMR spectrum also indicated that the tail portion of compound **1** consisted of a monosubstituted phenyl (δ_H 7.38, 2H m; δ_H 7.27, 2H m; δ_H 7.16, 1H m) with two conjugated *trans* double bonds (δ_H 6.44, d 16 Hz; δ_H 6.78, dd 10, 16 Hz; δ_H 6.21, dd 10, 15 Hz; δ_H 5.83,

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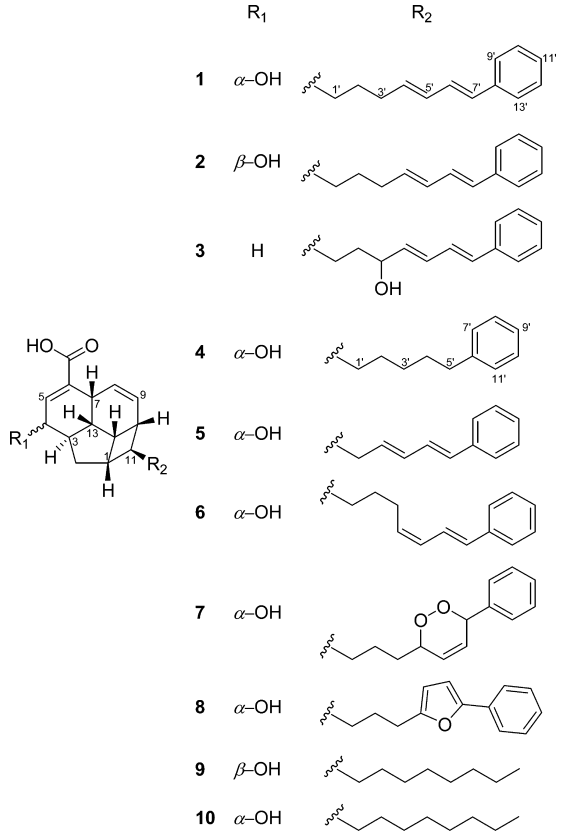

dt 7, 15 Hz). Starting with H-5 (δ_H 6.91), COSY correlations could be followed through the entirety of compound **1** (Figure 1) with the only exceptions being the three quaternary carbons. Those were assigned on the basis of HMBC correlations (Figure 1). In particular, HMBC correlations from H-5 (δ_H 6.91) to CO_2H (δ_C 170.4) and from H-4 (δ_H 4.14) to C-6 (δ_C 136.5) confirmed the presence of the α/β-unsaturated carboxylic acid. Likewise, HMBC correlations from H-6′ (δ_H 6.78) to C-8′ (δ_C 139.8) and from H-9′/13′ (δ_H 7.38) to C-7′ (δ_C 131.3) confirmed the location of the phenyl moiety. Finally, HMBC correlations from H-11 (δ_H 1.49) to C-1′ (δ_C 38.0) and from H-3′ (δ_H 2.14) to C-1′ (δ_C 38.0) and C-2′ (δ_C 28.2) were useful in confirming the connectivity of the side chain to the tetracycle. ROESY correlations were used to confirm the relative configuration of compound **1** (Figure 2). The plausibility of the ROESY correlations was verified by measurement of interatomic distances in an energy-minimized structure (Figure 3). On the basis of the observed ROESY correlations, compound **1** was proposed to have the same relative configuration as beilschmiedic acid C (**10**), and the lack of optical activity for the sample indicated that it was a mixture of enantiomers. In keeping with the previously established convention, compound **1** has been given the trivial name beilschmiedic acid H.

Compound **2** was also isolated as an optically inactive yellow oil, and HRESIMS again indicated a molecular formula of $C_{27}H_{30}O_3$. The ^{1}H NMR spectrum (Table 1) of **2** was very similar to the ^{1}H NMR spectrum of **1**. The most obvious differences were the chemical shifts of H-4 (δ_H 4.30, dd 4, 5 Hz) and H-5 (δ_H 7.07, d 5 Hz). Further comparison of the ^{1}H NMR spectrum of **2** to that of beilschmiedic acids A (**9**) and C (**10**) indicated that the tetracyclic portion of **2** matched that reported for beilschmiedic acid A (**9**).[9] Thus for the tetracyclic portion of **2** the β-OH analogue was indicated. The chemical shifts for the side chain were nearly identical to those found in compound **1**. Analysis of the 2D NMR data (Supporting Information) confirmed that compound **2** was the β-OH analogue of compound **1**. Compound **2** has been given the trivial name beilschmiedic acid I.

As was the case for compounds **1** and **2**, HRESIMS of compound **3** indicated a molecular formula of $C_{27}H_{30}O_3$. The ^{1}H NMR spectrum (Table 1) of **3** was also similar to those of compounds **1** and **2**. Analysis of the COSY correlations (Supporting Information) was again used to make most of the assignments. The COSY correlations for the tetracyclic portion of compound **3** led to the assignment of chemical shifts that were very similar to those of compound **1**. The one exception was that the correlation from H-5 (δ_H 7.13, brd 5 Hz) to H-4 (δ_H 2.48, m) indicated that, unlike compounds **1** and **2**, compound **3** was not oxygenated at the 4 position. Subsequent analysis of the COSY correlations for the side chain placed the OH at the 3′ position on the basis of correlations from H-4′ (δ_H 5.82, dd 7, 15) to H-3′ (δ_H 4.12, m) and from H-3′ (δ_H 4.12) to H-2′ (δ_H 1.47/1.53). Attempts to obtain a complete HMBC data set using the 5 μL CapNMR capillary microcoil probe were not successful (no correlations were observed to C-6 or to CO_2H). The sample was therefore sent to Bruker, where a complete HMBC was obtained using a Bruker BioSpin TCI 1.7 mm MicroCryoProbe. Those HMBC correlations (Supporting Information) made it possible to assign the quaternary carbons and confirm the structure. It was not possible to assign the configuration of the 3′ OH due to the flexibility of the side chain and lack of ROESY correlations. Also, due to the limited amount of material, derivatization experiments were not possible. Compound **3** has been given the trivial name beilschmiedic acid J.

HRESIMS of compound **4** indicated a molecular formula of $C_{25}H_{30}O_3$. The ^{1}H NMR spectrum (Table 1) of **4** indicated the same tetracyclic moiety with an α-OH (H-4 δ_H 4.13, d 9 Hz). It also presented signals for the phenyl group, but there were no signals observed for double bonds in the side chain. A two-proton triplet observed at δ_H 2.60 (H-5′) was found to be adjacent to the phenyl ring based on an HMBC correlation (Supporting Information) from H-7′/11′ (δ_H 7.16, m) to C-5′ (δ_C 36.9). The molecular formula indicated that, in addition to the phenyl ring, the side chain contained five methylenes, two carbons fewer than compounds **1**−**3**. HMBC correlations (Supporting Information) from H-1′ (δ_H 1.50, m) to C-2′ (δ_C 28.3) and C-3′ (δ_C 30.7) and from H-5′ (δ_H 2.60, t 8 Hz) to C-3′ (δ_C 30.7) and C-4′ (δ_C 32.6) were used to assign the side chain methylenes. Compound **4** has been given the trivial name beilschmiedic acid K.

HRESIMS of compound **5** indicated a molecular formula of $C_{25}H_{26}O_3$, four protons fewer than compound **4**. The ^{1}H NMR spectrum (Table 1) of **5** was very similar to that of compound **1**. It contained signals for the tetracycle, the phenyl group, and the two double bonds in the side chain. It was assigned as an α-OH analogue on the basis of the resonance for H-4 (δ_H 4.15, d 10 Hz). COSY correlations (Supporting Information) could be followed from H-11 in the tetracycle to H-5′ in the side chain. Thus the side chain was determined to be of the same length as that found in compound **4**, but was unsaturated like the side chain found in compound **1**. The HMBC spectrum (Supporting Information) also supported this assignment, with correlations from H-2′ (δ_H 5.78, dt 15, 7 Hz) to C-11 (δ_C 46.7) and C-1′ (δ_C 41.2). Compound **5** has been given the trivial name beilschmiedic acid L.

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Table 1. ^{1}H and ^{13}C NMR Data[a] for 1−6 in Methanol-d_4

	1		2		3		4		5		6	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	2.35 m	42.7	2.34 m	42.0	2.34 m	42.7	2.33 m	42.7	2.45 m	42.4	2.37 m	42.4
2	1.30[b]	36.9	1.42[b]	32.7	1.27[b]	38.4	1.29[b]	36.6	1.34[b]	36.1	1.32[b]	36.5
	1.84[b]		1.64 ddd (6, 12, 18)		1.62[b]		1.82 dd (5, 12)		1.86[b]		1.86[b]	
3	2.11 m	45.1	2.09 m	43.1	2.09 m	37.6	2.09 m	45.2	2.12 m	45.1	2.12 m	45.1
4	4.14 brd (10)	74.5	4.30 dd (4, 5)	65.9	2.04 m 2.48 m	33.7	4.13 brd (9)	74.2	4.15 brd (10)	74.1	4.15 brd (9)	74.1
5	6.91 brs	145.8	7.07 brd (5)	141.5	7.13 brd (5)	143.4	6.90 brs	145.9	6.91 brs	145.8	6.91 brs	145.8
6		136.5		138.0		136.0		137.0		137.0		137.1
7	3.19 brs	34.9	3.22 brs	34.9	3.23 brs	34.5	3.18 brs	34.6	3.20 brs	34.5	3.19 brs	34.5
8	5.55 brd (10)	128.5	5.46 brd (10)	126.0	5.56 brd (11)	128.7	5.54 brd (11)	128.2	5.56 brd (11)	128.1	5.53[b]	128.5
9	5.61 m	128.9	5.64 m	129.3	5.58 m	128.2	5.58 m	128.6	5.61 m	128.5	5.61 m	128.9
10	2.29 m	35.7	2.30 m	35.3	2.30 m	36.1	2.27 m	35.8	2.39 m	35.3	2.31[b]	35.7
11	1.49 m	47.1	1.43[b]	47.8	1.46[b]	47.1	1.46 m	47.1	1.61 m	46.7	1.54[b]	47.1
12	2.78 m	35.3	2.75 m	34.1	2.78 m	34.9	2.77 m	35.0	2.81 m	34.9	2.79 m	35.1
13	1.84[b]	42.0	2.02 m	36.1	1.69 m	43.9	1.84 m	42.4	1.87[b]	42.4	1.86[b]	42.4
1′	1.53 m	38.0	1.55 m	37.6	1.54[b]	34.2	1.50 m	38.5	2.35 t (7)	41.2	1.58[b]	37.7
2′	1.39 m	28.2	1.40[b]	27.9	1.47[b] 1.53[b]	36.5	1.30[b]	28.3	5.78 dt (7, 15)	134.0	1.42[b]	28.3
3′	2.14 q (7)	34.1	2.14 q (7)	34.1	4.12 m	73.3	1.32[b]	30.7	6.27 dd (11, 15)	133.2	2.31[b]	28.6
4′	5.83 dt (7, 15)	136.8	5.83 dt (7, 15)	136.4	5.82 dd (7, 15)	138.3	1.62 p (7)	32.6	6.80 dd (11, 16)	130.5	5.52[b]	134.0
5′	6.21 dd (10, 15)	132.5	6.21 dd (11, 15)	132.1	6.38 dd (11, 15)	131.7	2.60 t (8)	36.9	6.46 d (16)	131.7	6.17 dd (11, 11)	130.5
6′	6.78 dd (10, 16)	130.9	6.78 dd (11, 16)	130.9	6.84 dd (11, 16)	130.1		144.7		139.8	7.11 dd (11, 16)	125.4
7′	6.44 d (16)	131.3	6.44 d (16)	131.3	6.56 d (16)	133.6	7.16 m	129.8	7.39 m	127.4	6.53 d (16)	133.6
8′		139.8		139.8		138.9	7.24 m	129.4	7.28 m	129.7		139.0
9′	7.38 m	127.4	7.37 m	127.4	7.41 m	127.4	7.13 m	127.1	7.17 m	128.5	7.42 m	127.8
10′	7.27 m	129.7	7.27 m	129.7	7.29 m	129.7	7.24 m	129.4	7.28 m	129.7	7.29 m	129.7
11′	7.16 m	128.5	7.16 m	128.5	7.19 m	128.5	7.16 m	129.8	7.39 m	127.4	7.19 m	128.9
12′	7.27 m	129.7	7.27 m	129.7	7.29 m	129.7					7.29 m	129.7
13′	7.38 m	127.4	7.37 m	127.4	7.41 m	127.4					7.42 m	127.8
CO_2H		170.4		171.5		170.8		171.0		171.0		170.7

[a] ^{13}C NMR chemical shifts were obtained from HSQC and HMBC experiments. [b] Signal was obscured.

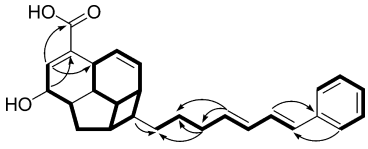

Figure 1. Key COSY correlations (bold) and HMBC correlations (arrows) for compound **1**.

HRESIMS of compound **6** indicated the molecular formula $C_{27}H_{30}O_3$, which was the same as compounds **1** and **2**. The ^{1}H NMR spectrum (Table 1) of **6** indicated that the tetracyclic portion was the α-OH analogue (H-4, δ_H 4.15, d 9 Hz). The ^{1}H

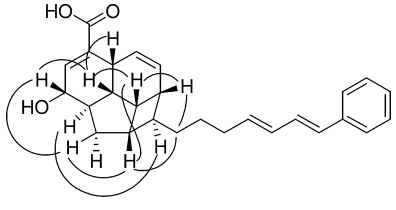

Figure 2. Key ROESY correlations for compound **1**.

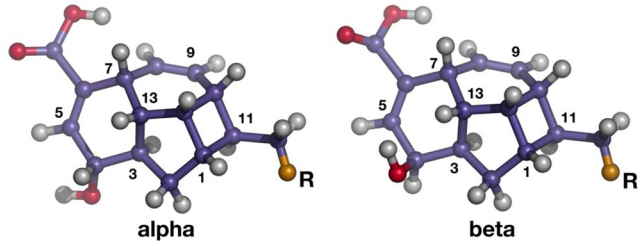

Figure 3. Three-dimensional energy-minimized structures demonstrating the interproton distances and unique chemical space occupied by the beilschmiedic acids. The side chain (**R**) was removed after energy minimization.

NMR signals for the side chain appeared to contain the same number of signals as those found in compound **1**, but the chemical shifts and coupling constants were different. Starting with the doublet at H-7′, and using COSY correlations (Supporting Information), it was possible to assign the side chain as follows: H-7′ (δ_H 6.53, d 16 Hz), H-6′ (δ_H 7.11, dd 11, 16 Hz), H-5′ (δ_H 6.17, dd 11, 11 Hz), H-4′ (δ_H 5.52). On the basis of the coupling constants of H-5′ (both 11 Hz) the C-4′,5′ double bond was *cis* configured, while the coupling constant for

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H-7′ (16 Hz) indicated that the C-6′,7′ double bond was *trans* configured. Analysis of the remaining COSY and HMBC correlations (Supporting Information) completed the assignment of compound **6**. Compound **6** has been given the trivial name beilschmiedic acid M.

HRESIMS of compound **7** indicated a molecular formula of $C_{27}H_{30}O_5$, two more oxygens than the previously isolated beilschmiedic acids. The ^{1}H NMR spectrum (Table 2) indicated that the tetracyclic portion was an α-OH analogue (H-4, δ_H 4.14, d 10 Hz). The ^{1}H NMR signals for the side chain were different from those previously observed. The side chain contained a monosubstituted phenyl group based on resonances for five protons centered at δ_H 7.35 (δ_C 129.2). An HMBC correlation (Supporting Information) was observed from the phenyl (H-9′/13′) to an oxymethine (H-7′, δ_H 5.49 brs; δ_C 81.1). From H-7′, HMBC correlations were observed to C-6′ (δ_C 127.4) and C-5′ (δ_C 129.5). H-6′ (δ_H 6.08, m) and H-5′ (δ_H 6.15, m) had HMBC correlations to C-4′ (δ_C 79.5). COSY correlations (Supporting Information) from H-4′ (δ_H 4.55, m) to H-3′ (δ_H 1.66/1.75) and H-2′ (δ_H 1.42/1.47) were used to assign all but H-1′. Due to overlap it was not possible to use COSY correlations to complete the assignment. HMBC correlations, however, provided the remaining correlations, specifically, correlations from H-1′ (δ_H 1.54) to C-11 (δ_C 46.7) and C-2′ (δ_C 23.8). The final question to answer was the relationship of the oxymethines to each other. The molecular formula indicated that an additional degree of unsaturation was required. This supported the presence of a six-membered endoperoxide. The chemical shifts for a similar endoperoxide phenyl moiety found in plakortoperoxide A strongly supported the presence of the same *cis*-configured moiety in compound **7**.[19] Unfortunately, while the chemical shifts support a *cis*-configured endoperoxide, there are no ROESY correlations or other evidence to suggest the relative configuration of the endoperoxide with respect to the tetracyclic moiety. Also, due to the limited amount of material, it was not possible to perform derivatization experiments. As with plakortoperoxide A, it is possible that compound **7** was formed during the isolation process from compound **1** or compound **6** through Diels–Alder addition of singlet oxygen.[19] Compound **7** has been given the trivial name beilschmiedic acid N.

Table 2. ^{1}H and ^{13}C NMR Data[a] for 7 and 8 in Methanol-d_4

	7		8	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	2.36 m	42.0	2.37 m	42.4
2	1.32[b]	36.1	1.32[b]	36.1
	1.84[b]		1.86[b]	
3	2.10 m	44.7	2.13 m	44.9
4	4.14 brd (10)	73.8	4.14 brd (10)	74.0
5	6.91 brs	145.2	6.90 brs	145.4
6		136.2		136.4
7	3.18 brs	34.1	3.19 brs	34.3
8	5.54 brd (11)	127.8	5.55 brd (10)	128.0
9	5.61 m	128.2	5.63 m	128.4
10	2.29 m	35.1	2.31 m	35.3
11	1.48[b]	46.7	1.54 m	46.7
12	2.78 m	34.5	2.79 m	34.9
13	1.85[b]	42.0	1.86[b]	42.3
1′	1.54 m	37.8	1.60 m	37.5
2′	1.42 m	23.8	1.66 m	26.7
	1.47[b]			
3′	1.66 m	33.8	2.69 t (7)	28.7
	1.75 m			
4′	4.55 m	79.5		156.9
5′	6.15 m	129.5	6.11 d (3)	108.4
6′	6.08 m	127.4	6.62 d (3)	106.5
7′	5.49 brs	81.1		153.3
8′		139.4		132.3
9′	7.35[b]	129.2	7.61 m	124.1
10′	7.35[b]	129.2	7.34 m	129.5
11′	7.35[b]	129.2	7.20 m	127.6
12′	7.35[b]	129.2	7.34 m	129.5
13′	7.35[b]	129.2	7.61 m	124.1
CO_2H		170.2		170.0

[a]^{13}C NMR chemical shifts were obtained from HSQC and HMBC experiments. [b]Signal was obscured.

HRESIMS of compound **8** indicated a molecular formula of $C_{27}H_{28}O_4$. The ^{1}H NMR spectrum (Table 2) for the tetracyclic portion indicated an α-OH moiety (δ_H 4.14, d 10 Hz). Signals for the side chain contained signals for a phenyl group (δ_H 7.61, 2H m; δ_H 7.34, 2H m; δ_H 7.20, 1H m). The remainder of the side chain was assigned with a combination of COSY and HMBC correlations (Supporting Information). An HMBC correlation was observed from H-9′/13′ in the phenyl ring (δ_H 7.61) to C-7′ (δ_C 153.3). The C-7′ signal was assigned as part of a furan moiety based on COSY and HMBC correlations. Briefly, an HMBC correlation from H-6′ (δ_H 6.62, d 3 Hz) to C-7′ (δ_C 153.3), a COSY correlation from H-6′ to H-5′ (δ_H 6.11, d 3 Hz), and an HMBC correlation from H-5′ to C-4′ (δ_C 156.9) allowed assignment of the furan. H-3′ resonated as a triplet (δ_H 2.69, t 7 Hz) and correlated to C-4′ (δ_C 156.9) in the HMBC spectrum. COSY correlations connected H-3′ (δ_H 2.69), H-2′ (δ_H 1.66), and H-1′ (δ_H 1.60). Finally, the side chain was connected to the tetracyclic portion by an HMBC correlation from H-1′ (δ_H 2.69) to C-11 (δ_C 46.7). Compound **8** has been given the trivial name beilschmiedic acid O.

Compounds **1**, **2**, and **4**–**10** were screened for cytotoxic activity against NCI-H460 large cell lung carcinoma, PC-3 prostate adenocarcinoma, and M14 amelanotic melanoma cell lines using an MTT-based assay. Several were found to have moderate cytotoxic activity in the NCI-H460 cell line (Table 3), but were inactive in the other two cell lines. No reports of cytotoxic activity for any of the endiandric/beilschmiedic acid class of compounds were found in the literature. On the basis of

Table 3. Cytotoxic and Antibacterial Activity for 1, 2, and 4–10

compound	NCI-H460 IC_{50}, μM	MRSA MIC, μg/mL
1	NA[a]	NA
2	5.5	12
4	5.9	11
5	4.4	11
6	8.7	12
7	19	13
8	NA	13
9	6.1	10
10	NA	NA
camptothecin	0.003	
vancomycin		2

[a]NA not active at the highest dose (30 μM).

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previous reports of antibacterial activity, the compounds were also screened for their ability to inhibit methicillin-resistant *Staphylococcus aureus* (clinical isolate MRSA 108) (Table 3). Compounds showing activity in the NCI-H460 human lung cancer cell line assay were also found to exhibit moderate activity against *S. aureus*. Compound **3** was not available for screening, as it had been shipped to Bruker for NMR analysis prior to obtaining a Bruker BioSpin TCI 1.7 mm MicroCryoProbe.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, and therefore, its extracellular detection is an indication of cell membrane damage. To determine whether the compounds described here act by inducing membrane disruption, compounds **1**, **2**, **4**, **5**, **6**, **8**, and **10** were screened for their ability to cause the release of LDH in NCI-H460 cells. Compounds **2**, **4**, **5**, and **6** were tested at 3 to 5 times their IC_{50}, as listed in Table 3. Compounds **1**, **8**, and **10** were tested at 20 μM as negative controls because they did not demonstrate cytotoxicity at the concentrations tested. No LDH release above background was detected for any of these compounds, indicating that the cell membranes of the NCI-H460 cells remained intact during the assay even at 3 to 5 times the IC_{50} of the compounds examined. Having established that the compounds did not disrupt the cell membrane, the possibility that they induce apoptosis was examined via caspase 3/7 activation. At 3 times the IC_{50} (26.1 μM), only compound **6** resulted in caspase 3/7 activation.

Given their lipophilic nature, one might initially attribute the cytotoxic activity of these compounds to nonspecific cell membrane disruption. However, considering the unique three-dimensional space (Figure 3) that these compounds occupy, it is reasonable that they could act by a more specific mechanism. Moreover, even simple lipid compounds have demonstrated bioactivity against specific targets that responded to minor structural changes such as chain length and double-bond orientation and position.[20−22] The absence of membrane disruption, based on no LHD release, coupled with the activation of caspase 3/7 by compound **6** supports the possibility that the structural features of these compounds may inhibit a specific mechanism or biological target instead of simply disrupting membranes.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired at Sequoia Sciences, Inc., on a Bruker 600 MHz spectrometer equipped with either a Bruker BioSpin TCI 1.7 mm MicroCryoProbe or a 5 μL CapNMR capillary microcoil probe containing a 1.5 μL active volume (CapNMR: Magnetic Resonance Microsensors).[23] The HMBC spectrum for **3** was acquired at Bruker BioSpin AG on a Bruker 600 MHz spectrometer equipped with a Bruker BioSpin TCI 1.7 mm MicroCryoProbe. HRESIMS was done on an LCT time-of-flight mass spectrometer with an electrospray interface (Waters). Semipreparative HPLC isolation was performed on a single-channel Beckman HPLC system composed of a Beckman 168 diode array UV detector, Alltech 800 ELSD detector, and Gilson FC-204 fraction collector. A splitter was used to split the flow in a 10:90 ratio to the ELSD and fraction collector, respectively. Compounds were quantitated by ELSD as previously described.[23] Optical rotation was measured on a Jasco P-1010 polarimeter using a 100 μL cell with a 0.1 dm path length.

Plant Material. The leaves of a *Beilschmiedia* sp. were collected from Tchimbele, Gabon (N 00 36.59 E 10 24.42) in November 2000 by the Missouri Botanical Garden. Samples were dried on site in Gabon and shipped to Sequoia Sciences, Inc. They were identified by J. Stone of the Missouri Botanical Garden. A voucher specimen (Stone 3198) is deposited at the Herbarium of the Missouri Botanical Garden.

Extraction and Isolation. Dried leaves were ground and extracted with EtOH/EtOAc (1:1) to obtain 5.8 g of extract. As previously described, 1 g aliquots were subjected to flash chromatography to generate flash fractions 1 to 6. The flash fractions were then further separated using preparative HPLC (in 50 mg aliquots) to produce the *Beilschmiedia* compound and screening libraries.[24] Fractions from the hexanes/EtOAc (1:1) fraction (flash fraction 2) and the EtOAc fraction (flash fraction 3) were identified as active. The flash fraction 2 library was collected using a preparative HPLC method that employed a 60% to 100% (2−32 min) MeCN gradient in H_2O on a Betasil C_{18} column (Thermo Scientific, 21.2 × 100 mm, 5 μm). The flash fraction 3 library was collected using a preparative HPLC method that employed a 30% to 70% (2−32 min) MeCN gradient in H_2O followed by 100% MeCN flush (32−40 min) on a Betasil C_{18} column (Thermo Scientific, 21.2 × 100 mm, 5 μm). Fractions for both methods were collected at a rate of 1 tube per minute (0−40 min). Flash fraction 3 preparative HPLC tube 34 (active in the library screening) was further separated using semipreparative C_{18} HPLC eluted at 1.5 mL/min with a gradient of 71−77% MeCN in H_2O plus 0.05% TFA from 5 to 35 min (Synergi Hydro, Phenomenex 250 × 4.6 mm, 4 μm). Serial collections afforded **10** (t_R 14.4 min, 54 μg) and **9** (t_R 17.4 min, 18 μg) along with minor components. Additional flash fraction 3 material was prepared as above to obtain minor components and separated using semipreparative C_{18} HPLC eluted at 3 mL/min isocratically with 58% MeCN in H_2O plus 0.05% TFA (Hypersil Gold, Thermo Scientific 250 × 10 mm, 5 μm). Serial collections afforded **5** (t_R 20.8 min, 220 μg), **10** (t_R 28.7 min, 97 μg), **4** (t_R 33.5 min, 208 μg), **6** (t_R 35.0 min, 55 μg), and **1** (t_R 38.7 min, 166 μg). Flash fraction 2 preparative HPLC tube 10 (found to be chromatographically similar to flash fraction 3 preparative HPLC tube 34) was further separated using semipreparative C_{18} HPLC eluted at 3 mL/min isocratically with 58% MeCN in H_2O plus 0.05% TFA (Hypersil Gold, Thermo Scientific 250 × 10 mm, 5 μm). Serial collections afforded **3** (t_R 24.9 min, 88 μg), **4** (t_R 34.5 min, 26 μg), **1** (t_R 40.0 min, 1.3 mg), and **2** (t_R 41.9 min, 1.1 mg). Additional flash fraction 2 material was prepared as above to obtain further analogues and separated using semipreparative C_{18} HPLC eluted at 3 mL/min isocratically with 58% MeCN in H_2O plus 0.05% TFA (Hypersil Gold, Thermo Scientific 250 × 10 mm, 5 μm). Serial collections afforded a mixture of **7** and **8** (t_R 15.3 min, 708 μg). From that mixture, **7** (t_R 6.7 min, 136 μg) and **8** (t_R 10.1 min, 148 μg) were isolated using semipreparative HPLC eluted at 1.5 mL/min isocratically with 55% MeCN in H_2O plus 0.05% TFA (Fluophase PFP, Thermo Scientific 250 × 4.6 mm, 5 μm).

*Beilschmiedic acid H (**1**):* yellowish oil; $[\alpha]^{22}_D$ ±0 (*c* 0.3, MeOH); HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 212, 222, 279, 290 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 401 $[M - H]^-$; HRESIMS *m/z* 401.2135 ($[M - H]^-$, $C_{27}H_{29}O_3$ requires 401.2117).

*Beilschmiedic acid I (**2**):* yellowish oil; $[\alpha]^{25}_D$ ±0 (*c* 0.3, MeOH); HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 212, 288 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 401 $[M - H]^-$; HRESIMS *m/z* 401.2127 ($[M - H]^-$, $C_{27}H_{29}O_3$ requires 401.2117).

*Beilschmiedic acid J (**3**):* HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 209, 287 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 401 $[M - H]^-$; HRESIMS *m/z* 401.2092 ($[M - H]^-$, $C_{27}H_{29}O_3$ requires 401.2117).

*Beilschmiedic acid K (**4**):* HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 286 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 377 $[M - H]^-$; HRESIMS *m/z* 377.2108 ($[M - H]^-$, $C_{25}H_{29}O_3$ requires 377.2117).

*Beilschmiedic acid L (**5**):* HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 212, 289 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 375 $[M + H]^+$, 392 $[M + NH_4]^+$, 397 $[M + Na]^+$; HRESIMS *m/z* 392.2215 ($[M + NH_4]^+$, $C_{25}H_{30}NO_3$ requires 392.2226).

*Beilschmiedic acid M (**6**):* HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 208, 290 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 401 $[M - H]^-$; HRESIMS *m/z* 401.2084 ($[M - H]^-$, $C_{27}H_{29}O_3$ requires 401.2117).

Beilschmiedic acid N (7): HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 216, 280 sh nm; ^{1}H and ^{13}C NMR, see Table 2; LRESIMS *m/z* 433 $[M - H]^-$; HRESIMS *m/z* 433.2000 ($[M - H]^-$, $C_{27}H_{29}O_5$ requires 433.2015).

Beilschmiedic acid O (8): HPLC-UV (aq MeCN w/0.05% TFA) λ_{max} 220, 280, 294 nm; ^{1}H and ^{13}C NMR, see Table 2; LRESIMS *m/z* 417 $[M + H]^+$, 434 $[M + NH_4]^+$, 439 $[M + Na]^+$; HRESIMS *m/z* 417.2105 ($[M + H]^+$, $C_{27}H_{29}O_4$ requires 417.2066).

Caspase 3/7 and LDH Assay. Caspase 3/7 activity and LDH release were measured using the Promega Caspase-Glo 3/7 assay system and the Promega CytoTox 96 non-radioactive cytotoxicity assay, respectively, following the manufacturer's instructions. The caspase 3/7 assay measures total activity of caspases 3 and 7, but does not distinguish between the two. Briefly, cells were plated at 1×10^4 cells/well in a white-walled 96-well tissue culture plate in 100 μL of RPMI-1640 with 10% fetal calf serum. Cells were allowed to attach overnight, and then compound (or DMSO vehicle/negative control) was added in an additional 100 μL of RPMI-1640. Plates were incubated for either 24 or 72 h, at which point cells and supernatant were assayed. For the LDH assay, supernatant (50 μL) from each test well was transferred to a new plate, and LDH release into the supernatant was detected with the CytoTox 96 assay, with absorbance measured at 490 nm on a Molecular Devices VersaMax microplate reader. The cells remaining in the white-walled plate were tested for caspase 3/7 activity, with luminescence measured as relative light units on a Promega GloMax Multi luminometer. Camptothecin (purity ≥98%; LKT Laboratories, Inc.) was used as a positive control for the caspase 3/7 assay at a concentration of 10 μg/mL. The positive control for the LDH assay was supplied with the kit as lysis solution (10×). Compounds were considered active for the induction of apoptosis if they produced an increase in the level of caspase 3/7 that was greater than background plus two times the standard deviation of the negative control without release of LDH compared to the negative control.

Cytotoxicity Assay. NCI-H460 (large cell lung carcinoma) and PC-3 (prostate adenocarcinoma) cells were obtained from ATCC. M14 (amelanotic melanoma) cells were obtained from the National Cancer Institute. Cells were grown in RPMI-1640 with 10% FBS supplemented with L-glutamine and HEPES. Cells were seeded into 96-well plates at 5×10^2 to 5×10^4 cells/well and allowed to adhere overnight; the medium was then removed. A stock solution of test compound in DMSO was diluted in medium to generate a series of working solutions. Aliquots (100 μL) of the working solutions were added to the appropriate test wells to expose cells to the final concentrations of compound in a total volume of 100 μL. Eight different concentrations were tested, with 2−5 wells per concentration. Camptothecin was used as a positive control; wells containing vehicle without compound were used as negative controls. Plates were kept for 72 h in a 37 °C, 5% CO_2 incubator. After incubation, viable cells were detected with the CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (Promega). Dose−response curves were generated and IC_{50} values were determined using GraphPad Prism 5 software.

Bacterial Assay. Compounds were screened for antibacterial activity using methicillin-resistant *Staphylococcus aureus* (clinical isolate MRSA 108) obtained from David Hunstad with the clinical bacteriology laboratory at St. Louis Children's Hospital. The strain was first isolated from a patient specimen on 5% sheep blood agar plates (BD Biosciences). Loughman et al. have previously described the genotype of the isolate.[25] Standardized planktonic antibiotic minimum inhibitory concentrations (MIC) were determined by the broth microdilution method outlined by CLSI.[26] Briefly, an overnight culture of MRSA 108 in trypticase soy broth was diluted 1:100 in cation-adjusted Mueller−Hinton broth and incubated at 37 °C until it reached an OD_{600} of 0.2. The resulting culture was diluted 1:100 in media and placed in a 96-well plate (10^6 cfu/mL, 100 μL/well). Test compounds were dissolved in DMSO and then diluted in media and added to the wells at a series of concentrations (100 μL/well, 3−6 wells per concentration per compound). Vancomycin was used as a positive control. The plates were incubated for 24 h at 37 °C, and growth inhibition was determined by the change in OD_{600} at the end of the incubation period compared to control wells without compound. The MIC was defined as the lowest concentration that prevented visible growth of the bacteria after overnight incubation at 37 °C.

Energy-Minimized Structures. The structures of compounds **1** and **2** were drawn separately in Chem3D (CambridgeSoft). The MM2 force field was used to minimize the energy of both structures. Following energy minimization the interatomic distances between protons were compared to correlations observed in the ROESY spectrum. Distances less than 3 Å were expected to result in correlations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra for compounds **1**−**8** as well as figures depicting key COSY and HMBC correlations for compounds **2**−**8** are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1-314-373-5181. Fax: +1-314-373-5186. E-mail: rwilliams@sequoiasciences.com.

Notes

The authors declare no competing financial interest.

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