Antibiotic Indole Sesquiterpene Alkaloid from *Greenwayodendron suaveolens* with a New Natural Product Framework

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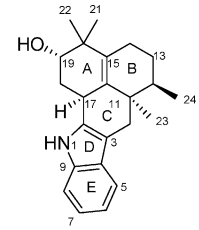
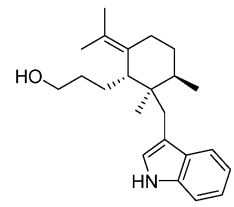
High-throughput natural products chemistry methods have led to the isolation of three new (**1**−**3**) and two known indole sesquiterpene alkaloids (**4**, **5**) from *Greenwayodendron suaveolens*. Their structures were determined using CapNMR and MS. Pentacyclindole (**1**) was determined to possess a new natural product framework. Pentacyclindole (**1**) and polyalthenol (**4**) showed activity against clinical isolates of *Staphylococcus aureus* with polyalthenol (**4**) demonstrating a MIC_{90} of 4 μg/mL.

Recent publications have detailed the lack of diversity among synthetic organic compounds.[1,2] One of these produced particularly shocking results from an investigation of chemical space. Lipkus et al. conducted a quantitative examination of the CAS registry, analyzing the frameworks of each molecule.[1] The framework was defined to be all ring systems and the linkers that connect them. They found a top-heavy distribution, in that a small percentage of frameworks appeared in a large number of the compounds. The 24 282 284 cyclic organic compounds found in the CAS Registry as of June 2007 were represented by 836 708 unique frameworks excluding the positions of elements and bond types (graph level) or 3 380 334 frameworks including the positions of elements and bond types (graph/node/bond level). Of the unique frameworks at the graph level, only 30 frameworks were required to describe 35.7% of those 24 million compounds, and 143 frameworks described 50%. These data demonstrate that a remarkably limited number of frameworks have undergone lead optimization campaigns creating series of analogues around a core framework, or alternatively, vast opportunities exist for synthetic organic chemists to expand around known but neglected frameworks. This is consistent with the analysis performed by Hert and co-workers demonstrating that 83% of the unique scaffolds in the Dictionary of Natural Products are not present in commercially available synthetic libraries.[2] Lipkus and co-workers attributed this concentration of effort within narrow regions of chemical space to the tendency to synthesize what is most economical to produce.[1] Such chemical exploration has led to an increase in the aromatic nature of compounds produced, which agrees with the comparisons of synthetic and natural product libraries that have concluded that natural products contain more stereocenters.[3,4] When one considers, in light of recent reports,[5,6] that increasing the aromatic character of a drug candidate increases its odds of failing in clinical trials, it is no wonder that FDA approval of new molecular entities has been trending down in recent years.[7−10] It is apparent, as the pharmaceutical industry abandons less fruitful research strategies, that the neglected and novel scaffolds provided by natural products must be investigated by a new generation of pioneering medicinal chemists.[11]

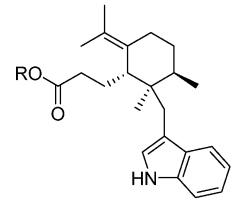
With this perspective we sought to mine our natural product library for bioactive compounds with unique structures. We had previously isolated an unusual indole sesquiterpene alkaloid, suaveolindole (**5**), from an organic extract of the fruits of *Greenwayodendron suaveolens* Verdc. (Annonaceae).[12] The genus *Greenwayodendron* has been lightly studied and shown to produce unique compounds; as such, it appeared to be a good candidate for additional examination.[13−20] Because our initial plant collection did not include the roots, and recognizing that roots may have a unique metabolism compared to the rest of the plant,[21,22] we initiated a collection of *G. suaveolens* roots. Our research herein identifies two compounds active against clinical isolates of *Staphylococcus aureus* and adds a new natural product framework to the CAS Registry.



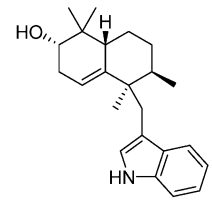
1



2



3 R = CH_3
5 R = H



4

An organic extract of *G. suaveolens* roots was subjected to normal-phase flash chromatography and reversed-phase preparative HPLC to generate the *G. suaveolens* root extract library. The library was screened for activity, and a series of consecutive fractions were identified as hits.[23] An initial semipreparative HPLC collection from these fractions led to the identification of two major compounds, polyalthenol (**4**) and suaveolindole (**5**).[12,24] Polyalthenol (**4**) was first reported in 1976, but it has since been neglected. Only five literature references are available for it, and no bioactivity has been reported.

Further screening and spectroscopic examination led to the identification of three additional minor compounds that were active and showed promise to be new. Two of these (**2**, **3**) were quickly determined to be close analogues of **5**. Compound **1**, however, appeared to merit closer investigation. On the basis of ^{1}H NMR data (Table 1) it appeared to be related to **4**. However, it was also

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Table 1. ^{1}H and ^{13}C NMR Data for **1**−**4** in Methanol-d_4

	1		**2**		**3**		4	
postion	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
2		138.7	7.01 s	125.0	7.01 s	124.6	6.82 s	124.6
3		109.1		111.6		113.8		113.5
4		130.5		131.4		132.5		130.5
5	7.33 d (7.6)	118.7	7.46 d (7.9)	120.7	7.45 d (7.6)	120.7	7.46 d (7.9)	120.3
6	6.94 t (7.6)	119.9	6.97 t (7.0)	119.1	6.95 t (7.6)	119.5	6.94 t (7.3)	119.5
7	7.01 t (7.9)	121.5	7.05 t (7.3)	121.5	7.05 t (7.6)	122.3	7.00 t (7.3)	121.9
8	7.27 d (8.2)	112.1	7.31 d (8.2)	112.5	7.31 d (8.2)	112.5	7.26 d (8.2)	112.1
9		139.2		139.0		139.0		138.1
10	2.43 d (14.7)	30.2	2.63 d[a]	28.0	2.63 d (14.4)	28.2	2.64 d (14.3)	27.7
	2.56 d (14.7)		2.74 d (14.4)		2.74 d (14.4)		2.93 d (14.3)	
11		39.7		41.5		40.4		45.1
12	1.65 m	41.6	1.91 m	38.0	1.88 m	37.6	1.47 m	44.1
13	1.44 m	29.0	1.51[a]	31.8	1.56 m	33.8	1.63 m	32.2
	1.57 m						1.69 m	
14	2.04 m	27.9	2.66[a]	25.5	1.94 m	25.5	1.29 m	29.0
	2.12 m		1.96 m		2.65[a]		1.90 m	
15		134.9		125.9		131.6	2.18 br d (12.6)	46.3
16		134.3	2.64[a]	46.3	2.67[a]	45.9		144.7
17	3.84 m	29.8	1.55[a]	32.6	1.78 m	23.2	4.90 dd (2.4, 2.4)	118.7
18	1.90 ddd (11.6, 11.4, 1.8)	32.2	1.25 m	32.9	2.05 m	33.3	1.82 m	32.6
	2.49 ddd (11.4, 4.7, 1.8)		1.36[a]		2.09 m		1.86 m	
19	3.64 br d (1.8)	75.6	3.46 t (6.2)	63.7		177.4	3.23 dd (9.4, 5.9)	75.6
20		40.2		133.5		126.2		38.6
21	1.11 s	23.9	1.41 s	22.0	1.34 s	21.6	0.71 s	16.5
22	0.98 s	28.2	1.72 s	20.4	1.71 s	20.4	0.98 s	25.9
23	1.13 s	24.3	0.98 s	25.9	1.01 s	25.9	1.00 s	24.3
24	1.10 d (7.0)	16.9	1.01 d (7.0)	16.5	1.02 d[a]	16.5	1.12 d (6.8)	17.3
CH_3O					3.59 s	52.1		

[a] Signal was obscured.

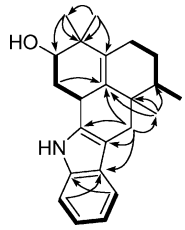

Figure 1. COSY fragments (represented by bold bonds) and key HMBC correlations for **1**.

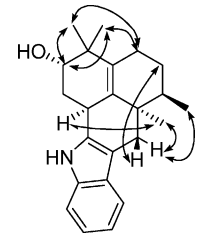

Figure 2. Key ROESY correlations for **1**.

evident that it was further substituted, as it was missing the indole hydrogen singlet and the olefinic hydrogen signal found in **4**. An examination of the literature for compounds in which the indole was further substituted indicated that this was likely a new compound.

Pentacyclindole (**1**) was found to have a molecular formula of $C_{23}H_{29}NO$ based on an HRESIMS ion at m/z 336.2328 $[M + H]^+$. This was 2 mass units less than **4**, indicating that it had either an additional double bond or an additional ring. Analysis of the COSY correlations established the connectivities of H-5, 6, 7, and 8; H-24, 12, 13, and 14; and H-17, 18, and 19 as three COSY fragments (Figure 1). HMBC correlations were used to connect the COSY fragments and assign the quaternary carbons (Figure 1). Specifically, HMBC correlations from the methine hydrogens resonating at δ_H 7.33 (H-5) and 7.27 (H-8) to δ_C 139.2 (C-9) and 130.5 (C-4) completed the aryl portion of the indole moiety. The remainder of the indole fragment was assigned on the basis of HMBC correlations from the methylene hydrogens at C-10 (δ_H 2.43 and 2.56) to δ_C 109.1 (C-3), 138.7 (C-2), and 130.5 (C-4). Further HMBC correlations from the C-10 methylene hydrogens to the C-23 methyl group (δ_C 24.3), δ_C 134.3 (C-16), δ_C 39.7 (C-11), and the C-12 methine hydrogens (δ_C 41.6) connected the indole moiety to the second COSY fragment. No HMBC correlations were observed from the C-13 and C-14 methylene hydrogens that would indicate further connectivity. However, it can be inferred from the chemical shift of H-14 (δ_H 2.04 and 2.12) that it is adjacent to a double bond. HMBC correlations from a geminal dimethyl group (δ_H 0.98, s and δ_H 1.11, s) to δ_C 134.9 (C-15), 40.2 (C-20), and 75.6 (C-19) connected the second and third COSY fragments. The third COSY fragment was further connected to the second through an HMBC correlation from H-18 (δ_H 2.49) to δ_C 134.3 (C-16). The final connection to be assigned was C-17 to C-2 of the indole unit. Despite the lack of supporting HMBC correlations, this assignment was readily made, as C-17 and C-2 were the only two positions left requiring a bond. With the 2-D structure thus assigned, it was apparent that **1** was the result of cyclization of C-2 and C-17 of **4**. The relative configuration was assigned by analysis of the ROESY correlations and comparison to **4** and **5**. A 1,3 diaxial correlation from H-17 to Me-23 confirmed that the C-ring was *cis* fused, while correlations between protons at the remaining stereocenters indicated the same configuration as in **4** (Figure 2).

Compound **2** has a molecular formula of $C_{23}H_{33}NO$ based on a HRESIMS ion at m/z 340.2654 $[M + H]^+$. Analysis of the ^{1}H NMR data (Table 1) indicated that it was an analogue of **5**, with a two-proton triplet at δ 3.46 as the only obvious difference. Analysis of COSY and HMBC correlations confirmed that **2** was the primary alcohol analogue of **5**.

HRESIMS (m/z 368.2596 $[M + H]^+$) indicated **3** had a molecular formula of $C_{24}H_{33}NO_2$. Again the ^{1}H NMR data (Table 1) were similar to those of **5**. In this case the only difference appeared to be the presence of a methyl singlet at δ 3.59 (δ_C 52.1), which had an HMBC correlation to the carbon at δ_C 177.4, indicating that it

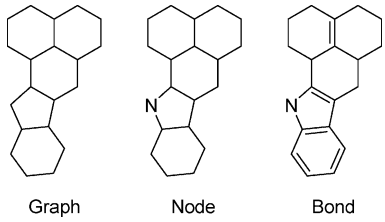

Figure 3. SubScape frameworks for **1** at graph, node, and bond levels.

was the methyl ester of **5**. Further COSY and HMBC correlations were used to confirm the structure of **3** as the methyl ester.

Recognizing that the framework of **1** was not likely to be common, we performed a SubScape analysis. The results indicated that there were 211 compounds with the same framework at the graph level, but only 5 at the node level (heteroframework) (Figure 3). As all of the previously reported compounds had significant aromatic character, there were no examples at the bond level (Figure 3). Of the 211 compounds with the same graph framework, none of them appeared to be natural products, and only 12 were listed as bioactive in SubScape.[25]

Compounds **1**−**4** were screened for antibacterial activity against methicillin-susceptible *Staphylococcus aureus* (MSSA) (ATCC 25923). Compounds **1**, **2**, and **4** showed good inhibitory activity, with MICs of 4, 19, and 4 μg/mL, respectively. Compound **3** was not active. On the basis of their greater activity, compounds **1** and **4** were screened against a series of clinical isolates of methicillin-resistant *Staph. aureus* (MRSA) and MSSA. Both compounds **1** and **4** demonstrated activity against all of the isolates examined (Table 2). The MIC_{90} of compounds **1** and **4** are 8 and 4 μg/mL, respectively.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Jasco P-1010 polarimeter with a 0.1 dm path length cell. NMR spectra were acquired on a Bruker spectrometer at 600 MHz equipped with a 5 μL CapNMR capillary microcoil probe with a 1.5 μL active volume (CapNMR, Magnetic Resonance Microsensors).[26] HRESIMS was performed 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.[26] ACD Structure Elucidator was used to verify the structures of **1** and **4**.

Plant Material. The roots of *Greenwayodendron suaveolens* were collected from the Lopé-Okanda game preserve in Gabon in November 2001. Samples were dried on site in Gabon and shipped to Sequoia Sciences, Inc. They were identified by B. Nziengui. A voucher specimen (Nziengui 440) is deposited at the Herbarium of the Missouri Botanical Garden.

Extraction and Isolation. Dried roots (167 g) were ground and extracted with EtOH/EtOAc (1:1) to obtain 7.5 g of extract. As previously described,[23] 2 g of organic extract was subjected to flash chromatography in 1 g aliquots to generate flash fractions 1 to 5. The hexanes/EtOAc (1:1) fractions (flash fraction 2) from the two flash runs were combined (58 mg), and the flash fraction 2 library was prepared by preparative C_{18} HPLC. The preparative HPLC method employed a 60% to 100% MeCN gradient in H_2O on a Betasil C_{18} column (Thermo Scientific, 21.2 × 100 mm, 5 μm); both solvents contained 0.05% TFA. Preparative HPLC fraction 10 was further purified by semipreparative C_{18} HPLC eluted at 1.5 mL/min with 55% MeCN in H_2O plus 0.05% TFA (Fluophase PFP, Thermo Scientific, 4.6 × 250 mm, 5 μm). Serial collections afforded **5** (270 μg) and **4** (246 μg).

Scale-up to collect minor components was as follows. The remaining extract (5.5 g) was flashed as above and combined to give 1.76 g of flash fraction 2 material. This material was prepared for preparative HPLC with a C_{18} SPE that was eluted with MeCN/H_2O/TFA (85:15:0.05) to remove late eluting material and preserve the HPLC column. Semipreparative C_{18} HPLC was used, as above, to verify that the SPE fraction (1.55 g) contained the peaks of interest. The SPE fraction was further fractionated using preparative C_{18} HPLC employing a 53% to 60% MeCN gradient in H_2O at 20 mL/min on an Aquasil C_{18} column (Thermo Scientific, 21.2 × 100 mm, 5 μm); both solvents contained 0.05% TFA. Serial collections were combined to give fractions 1−5. Fraction 2 was compound **5** (39.4 mg) and fraction 4 was compound **4** (194.0 mg). Fraction 3 (39.8 mg) contained compounds **4** and **5**, as well as a mixture of minor metabolites, and was further purified by semipreparative C_{18} HPLC eluted at 3 mL/min with 83% MeOH in H_2O (Synergi Hydro-RP, Phenomenex, 10 × 250 mm, 4 μm). Serial collections afforded **1** (710 μg), **2** (680 μg), and **3** (980 μg).

Pentacyclindole (1): insufficient material was available to obtain an IR spectrum; $[\alpha]^{22}_D$ +69.4 (*c* 0.31, EtOH); HPLC-UV (aq MeOH) λ_{max} 230, 267, 284, 294 nm; ^{1}H and ^{13}C NMR, see Table 1; LRESIMS *m/z* 336 $[M + H]^+$, 334 $[M - H]^-$; HRESIMS *m/z* 336.2328 ($[M + H]^+$, $C_{23}H_{30}NO$ requires 336.2327, Δ 0.3 ppm).

Compound 2: HPLC-UV (aq MeOH) λ_{max} 224, 282 nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 340.2654 ($[M + H]^+$, $C_{23}H_{34}NO$ requires 340.2640 Δ 4.1 ppm).

Compound 3: HPLC-UV (aq MeOH) λ_{max} 223, 282 nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 368.2596 ($[M + H]^+$, $C_{24}H_{34}NO_2$ requires 368.2590 Δ 1.6 ppm).

Bacterial Assay. Compounds were screened for antibacterial activity using *Staph. aureus* strain 25923 acquired from ATCC. An overnight culture of *Staph. aureus* was diluted 1:100 in media (Trypticase Soy Broth, BD Biosciences) with 0.5% D-glucose and 3% NaCl and incubated at 37 °C until it reached an OD_{600} of 0.4. The resulting culture was diluted 1:10 in media and placed in a 96-well plate (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

Table 2. MIC of **1** and **4** Determined against Clinical Isolates of *Staphylococcus aureus* Using CA-MHB

	virulence gene expression				MIC (μg/mL)	
isolate	PVL	ACME	bsa	Agr	**1**	**4**
cutaneous						
MRSA-105	+	+	B +	1	8	1
MRSA-106	+	+	B +	1	8	2
MRSA-107	+	+	B +	1	8	1
MRSA-108	+	−	A +	1	8	2
MSSA-109	+	−	B +	1	8	1
MRSA-111	+	+	B +	1	8	2
MRSA-148	+	+	B +	1	8	2
MRSA-158	+	+	B +	1	8	4
MSSA-175	+	−	B +	1	8	4
MRSA-295	−	−	B +	1	NT[a]	4
invasive						
MRSA-186	+	−	A +	1	8	4
MRSA-194	+	+	B +	1	NT[a]	2

[a] Not tested due to insufficient material.

inhibition was determined by the change in OD_{600} at the end of the incubation period compared to control wells without compound. Standardized planktonic antibiotic minimum inhibitory concentrations (MIC) were determined by the broth microdilution method outlined by CLSI.[27] Cationic adjusted Mueller−Hinton broth (CA-MHB, BD Biosciences) was used for MIC determination. The initial inoculum was 10^6 cfu/mL. The MIC was defined as the lowest concentration that prevented visible growth of the bacteria after overnight incubation at 37 °C. Visibility was detected at OD_{600} on the Versamax, Molecular Devices. MIC_{90} is the concentration of test compound in which 90% of isolates are inhibited.

The isolates of methicillin-resistant *Staph. aureus* and methicillin-susceptible *Staph. aureus* are described in Table 2. Clinical isolates were obtained from David Hunstad, with the clinical bacteriology laboratory at St. Louis Children's Hospital. The strains were first isolated from patient specimens on 5% sheep blood agar plates (BD Biosciences). Loughman et al. described the genotype of the isolates.[28] In summary, Panton-Valentine leukocidin (PVL) has some pathogenic importance with regulation of the accessory gene regulator (Agr). Agr regulates protein expression during the transition from exponential phase to the stationary phase in the quorum sensing system. An enzyme that encodes for the bacteriocin biosynthesis pathway (bsaB) was also reported along with the arginine catabolic mobile element (ACME), which was originally identified in a USA300 isolate of *Staph. aureus*. For each study overnight broth cultures were inoculated in TSB from frozen stocks stored at −70 °C in TSB + 20% glycerol.

SubScape Analysis. The ring system for **1** was drawn in SciFinder with all of the atoms and bonds described as variable and unspecified, respectively. The ring system was locked out to prevent the return of structures with additional fused rings. The structure was searched as a substructure. To reduce the size of the answer set, the search was performed in narrow molecular weight windows, with a maximum molecular weight of 1000; the individual answer sets were saved and then combined. This combined answer set was then exported as an .akx file and opened with SubScape.

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Supporting Information Available: ^{1}H NMR, COSY, HSQC, HMBC, and ROESY spectra for compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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