Antimicrobial Activities of a New Schizozygane Indoline Alkaloid from *Schizozygia coffaeoides* and the Revised Structure of Isoschizogaline

R. M. Kariba,[†] P. J. Houghton,*[,‡] and Abiy Yenesew[§]

Department of Botany and Department of Chemistry, University of Nairobi. P.O. Box 30197, Nairobi, Kenya, and Department of Pharmacy, Kings College, University of London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN, U.K.

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Extracts from *Schizozygia coffaeoides* showed antimicrobial activity against fungal and bacterial species. Alkaloids isolated using bioassay-guided fractionation were isoschizogaline, schizogynine, and a new indoline alkaloid, 7,8-dehydro-19β-hydroxyschizozygine, shown to be the most active antifungal compound. The structure of isoschizagaline, the only active antibacterial, is revised on the basis of NMR analysis.

Fungal infections are common causes of skin diseases in Kenya and other countries. In the recent past there has been an increase in the occurrence of opportunistic systemic mycoses, especially in immunocompromised patients, and an increase of microbial resistance to antibiotics. This has occasioned more efforts in the search for novel antimicrobial agents. The monotypic shrub *Schizozygia coffaeoides* (Boj.) Baill. (Apocynaceae) is one of the plants used in Kenyan traditional medicine for treatment of skin diseases.[1] We have recently shown that the extracts obtained from the leaves of *S. coffaeoides* have very high antifungal activity.[2]

Previous phytochemical studies on the leaves of *S. coffaeoides* have resulted in the isolation of five schizozygane, a small group of hexacyclic *N*-acyl indoline alkaloids.[3,4] Here we report the results of a bioassay-guided fractionation of the leaves and roots of this plant which resulted in the isolation and characterization of a new antifungal schizozygane alkaloid and a known antibacterial schizozygane alkaloid, isoschizogaline. The structure of isoschizogaline was revised on the basis of NMR evidence.

The major compound from the roots of *S. coffaeoides* was readily identified as schizozygine (**1**),[3,4] on the basis of its spectroscopic features. **1** has earlier been reported from the leaves[3,4] of this plant, but this is the first report on its occurrence in the roots.

The roots of this plant also afforded a minor antibacterial alkaloid, **2** ($C_{20}H_{22}O_2N_2$), whose NMR spectral features (Tables 1 and 2) are closely related to those reported for the revised structure of isoschizogamine (**3**) by Haajicek and Budesinsinsky.[5] The only difference between the NMR data of these two compounds was the absence of a second methoxyl group in the aromatic ring of **2**. Thus, the presence of an AXY aromatic spin system (8.45, d, $J = 2.6$ Hz for H-12; 7.00, d, $J = 8.3$ Hz for H-9 and 6.66, dd, $J =$ 2.6, 8.3 Hz for H-10) in **2** is consistent with the methoxy group being at C-11. This was supported from the NOESY spectrum, which showed an NOE interaction between the C-11 methoxy signal and the deshielded proton at C-12.

A five-membered (as in **3**) rather than a six-membered lactam ring (as in **1**) is suggested for compound **2**, on the basis of the chemical shift value of the carbonyl (δ174.4) and C-21 (δ 84.6, quaternary).[5] In the ^{1}H,^{1}H COSY spectrum, a correlation between the signals at δ 3.23 (H-7) and 2.31 (H-2) is in agreement with a five-membered rather than six-membered lactam (where C-2 would be quaternary, as in **1**). Hence, structure **2** is assigned to this compound. An isomeric structure with a six-membered lactam ring (**2a**), named isoschizogaline, had earlier been reported from this plant[4] along with isoschizogamine (**3a**). However, the authors[4] had put some doubts on the correctness of these two structures (**2a** and **3a**), and the structure of the latter has since been revised from **3a** to **3** on the basis of NMR evidence[5] and, similarly, the structure of isoschizogaline is revised here from **2a** to **2**.

HRMS analysis of the antifungal compound, **4**, isolated from the leaves, gave a molecular ion peak at *m/z* 350.17 ($C_{20}H_{18}O_2N_2$). Comparison of the ^{1}H (Table 1) and ^{13}C (Table 2) NMR data of this compound with those of **1** suggested that it should have an identical hexacyclic *N*-acyl indole alkaloid skeleton with a six-membered lactam ring but also having a hydroxyl substituent (3432 cm^{-1}, in IR;

Table 1. ^{1}H NMR Spectral Data of Compounds **2** and **4** ($CDCl_3$, 400 MHz)

proton	**2**	**4**[a]
3	3.28 (ddd, 17.2, 4.7, 1.7)	2.91 (ddd, 16.8, 2.7, 2.1)
	3.44 (ddd, 17.2, 2.7, 1.9)	3.50 (ddd, 16.8, 4.6, 1.8)
5	2.52 (ddd, 14.7, 4.5, 1.6)	3.01 (dd, 16.3, 2.2)
	2.93 (ddd, 14.7, 13.2, 3.6)	3.62 (dd, 16.3, 4.8)
6	1.21 (m)	5.27 (dd, 4.8, 2.3)
	2.21 (m)	
7	3.23 (m)	
9	7.00 (d, 8.3)	6.52 (s)
10	6.66 (dd, 8.3, 2.6)	
12	8.45 (d, 2.6)	8.44 (s)
14	5.59 (ddd, 9.9, 4.3, 1.9)	5.75 (ddd, 10.1, 4.6, 1.8)
15	5.73 (ddd, 9.9, 4.3, 2.2)	6.08 (ddd, 10.1, 2.7, 1.8)
16	1.19 (m)	2.40 (m)
	1.67 (m)	2.12 (m)
17	1.83 (m)	2.44 (m)
	1.76 (m)	1.88 (m)
19	2.51 (d, 18)	4. 05 (d, 1.8)
	2.85 (bd, 18)	
21	2.31 (ddd, 12.1, 6.9, 2.5)	2.58 (s)
OH		7.22 (bs)
OMe	3.83 (s)	
OCH_2O		5.92 (d, 1.4)
		5.94 (d, 1.4)

[a] Run at 500 MHz. Coupling constants (*J*) are given in Hz. Assignments were based on HMBC and HMQC experiments.

* Corresponding author. E-mail: peter.houghton@kcl.ac.uk. Tel: 44 20 7848 4775. Fax: 44 20 78484800.
[†] Department of Botany, University of Nairobi.
[‡] University of London.
[§] Department of Chemistry, University of Nairobi.

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Table 2. ^{13}C NMR Spectral Data of Compounds **2** and **4** ($CDCl_3$, 100 MHz)

carbon	**2**	**4**[a]
2	34.72	64.34
3	47.87	52.24
5	44.25	52.19
6	26.85	100.16
7	37.32	144.77
8	118.08	116.65
9	129.03	102.77
10	110.43	142.35
11	158.75	144.69
12	102.99	98.22
13	138.69	138.37
14	120.18	123.33
15	130.89	127.20
16	24.75	38.13
17	36.40	29.70
18	174.39	170.46
19	46.05	75.96
20	44.27	46.61
21	84.63	67.94
OMe	55.39	
OCH_2O		101.71

[a] Run at 125 MHz. Assignments were based on HMBC and HMQC experiments.

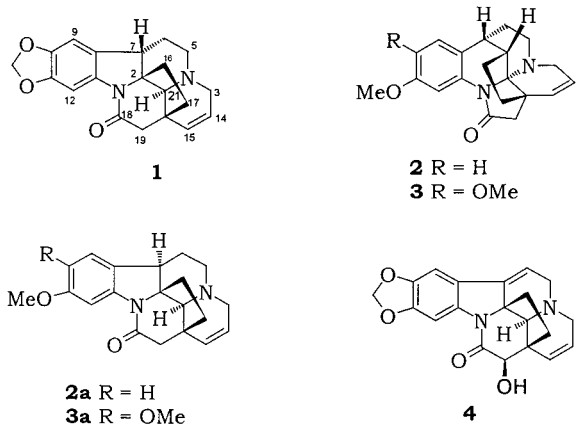

Figure 1.

δ 4.05 (d) for carbinol H in ^1H NMR and δ 75.9 (CH) in the ^{13}C NMR spectra) and an additional olefinic group (δ 5.27, dd J = 4.8, 2.3 Hz, in ^1H NMR and δ 100.2 (CH) and 144.77 (quaternary C) in the ^{13}C NMR spectra).

In the HMBC spectrum of **4**, the signal for the hydroxymethine proton (δ 4.05) correlates with those of the amidic carbonyl (δ 170.5), C-20 (δ 46.6), and C-17 (δ 29.7), which would place the hydroxyl group at C-19 adjacent to the C-18 carbonyl. The long-range (W) coupling (J = 1.8 Hz) seen between H-19 (δ 4.05) and H-17 (δ 1.88) requires that the H-19 is in the α-configuration,[5] thus requiring the 19-OH to have a β-orientation. This assignment is confirmed by the observations of the NOESY spectrum, which shows a clear correlation between H-19 (δ 4.05) and H-21-(δ 2.58) and the OH-19 and the CH_2-17 (Figure 2), the H-21 known to be in the α-configuration in schizogynine.[5] The CD spectra showed that the configurations of **1** and **4** were the same. Similarly, correlation between the signals for the methylene protons at C-5 (δ 3.01 and 3.62) and the olefinic carbon atoms (δ 100 and 144.8) would place the second double bond between C-6 and C-7. This was supported by the ^1H,^1H COSY spectrum, which showed a correlation between the olefinic proton at δ 5.27 and the signals for the C-5 protons (δ 3.01 and 3.62). Hence this new antifungal compound should have structure **4**, for which the trivial name 6,7-dehydro-19β-hydroxyschizozygine is suggested, by relating it to schizozygine (**1**).

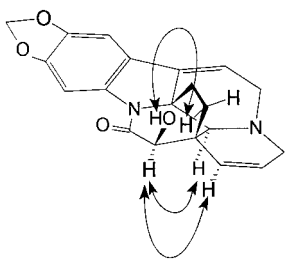

Figure 2. Significant NOE relationships observed in the NOESY spectrum for **4** to show the configuration of H-19.

Antimicrobial Activities of Crude Extracts and Pure Compounds. Preliminary antifungal tests on extracts obtained from the leaves of *S. coffaeoides* using the disk diffusion method had shown that the dichloromethane extract was the most active.[2] The activities of dichloromethane extracts of the leaves and root bark against eight fungal (*Trichophyton interdigitale* (EQ 4115), *T. mentagrophytes* (NCPF 224), *T. tonsurans* (EQ 4329), *Epidermophyton floccossum*, *Microsporum gypseum* (NCPF 40), *Cladosporium cladosporioides* (IMI 299104), *C. harbarum* (Lab stock), and *Candida albicans* (NCPF 3179)) and four bacterial pathogens (*Escherichia coli* (NCTC 9002), *Bacillus subtilis* (NCTC 10073), *Staphyloccoccus aureas* (NCIMB 9515), and *Pseudomonas aeruginosa* (NCIMB 10421)) were tested using the microdilution method.[6] None of the organisms were inhibited from growing in the presence of DMSO alone. These extracts showed activities with MIC = 500 μg/mL against all the organisms,while MICs of both extracts against *Epidermophyton floccossum* and *T. tonsurans* were 250 μg/mL. This shows that the dichloromethane extracts of both the leaves and root bark have marginal activities against dermatophytes and plant pathogenic fungi as well as Gram positive and Gram negative pathogenic bacteria.

The crude dichloromethane extracts of the leaves and the roots of this plant were then subjected to a bioassay-guided fractionation using the sensitive fungal species (*Microsporum gypseum* and *Cladosporium cladosporioide*) and the bacteria species shown in Table 3. This led to the isolation of compounds **2** (antibacterial) and **4** (antifungal) as the active principles. These two compounds as well as the major alkaloid, both in the leaves and the roots of this plant, **1**, were tested against all the test organisms. The Minimum inhibitory concentration (MIC) was determined for each and compared against those of standard antifungal and antibacterial drugs (Table 3). Compound **4** was the most active against these tested fungi and was even significantly more active that the standard drug ketoconazole. Among these test organisms, the dermatophytic fungi were the most inhibited, with MIC values of less than 1.95 μg/mL against *Trichophyton mentagrophytes*. Interestingly, the related alkaloids **1** and **2** were not fungitoxic up to a concentration of 500 μg/mL. This probably suggests the importance of the hydroxyl functional group at C-19 and/or the double bond at C-6 for fungitoxic activity.

On the other hand, compound **2** was weakly active, with an MIC of 62.5 μg/mL for *Bacillus subtilis* and 125 μg/mL for *Staphylococcus aureas*, while **4** showed minimal activity against *E. coli*. The major alkaloid **1** had no antibacterial activity up to a concentration of 500 μg/mL. Prior to this work, there has been no report on the antimicrobial activities for any of the compounds isolated from this plant. It would be worthwhile to test the antifungal activities of

Table 3. Antimicrobial Activities of the Isolated Compounds and Standard Drugs

tested organisms	MIC (μg/mL)				
	1	**2**	**4**	ketoconazole	ampicillin
fungi					
Trichophyton mentagrophytes	>500	>500	<1.95	6.25	N.T.
Microsporum gypseum	>500	>500	1.95	6.25	N.T.
Epidermophyton floccossum	>500	>500	>1.95	25	N.T.
Trichophyton tonsurans	>500	>500	3.9	50	N.T.
Trichophyton interdigitale	>500	>500	<3.9	25	N.T.
Cladosporium cladosporioides	>500	>500	7.8	25	N.T.
Cladosporium harbarum	>500	>500	15.6	50	N.T.
Candida albicans	>500	>500	7.8	25	N.T.
bacteria					
Escherichia coli	>500	>500	>250	N.T.	20
Staphyloccoccus aureas	>500	125	>500	N.T.	12.5
Bacillus subtilis	>500	62.5	>500	N.T.	12.5
Pseudomonas aeruginosa	>500	>500	>500	N.T.	25

[a] N.T. = not tested.

other hexacyclic *N*-acyl indoline alkaloids and their synthetic analogues, especially of compound **4**, as potential leads for clinically useful products.

Experimental Section

Plant Material. Leaves and root bark of *Schizozygia coffaeoides* were collected from Simba Hills Kenya in March 2000. The plant was authenticated by Mr. S. Mathenge of the Botany Department, University of Nairobi, where a voucher specimen (Rkariba 2/22) is deposited.

Extraction and Isolation. The leaves and root bark (500 g of each) were dried separately at room temperature and coarsely powdered. These were then extracted with dichloromethane (2 L) in a Soxhlet apparatus for 10 h. The extracts were concentrated under reduced pressure and were then subjected to bioactivity testing on TLC to determine the relative locations of the bioactive compounds. To achieve this, the extracts were spotted on silica gel $60GF_{254}$ (Merck, 0.25 mm thickness) and developed with hexane/dichloromethane/ethyl acetate (2:2:1). The plates were dried and sprayed with spores of *Cladosporium cladosporioides* in liquid media. Clear zones without any fungal growth after an incubation period of 4 days indicated the presence of antifungal compounds. Preliminary phytochemical analysis indicated the presence of alkaloids in the active zones. The alkaloid fractions were therefore separated. Preparative silica gel TLC (solvent system, hexane/dichloromethane/ethyl acetate, 1:1:1) of the alkaloid fractions obtained from the leaves gave **1** (93 mg, R_f 0.2) and **4** (12 mg, R_f 0.4). In the same way preparative TLC (chloroform/acetone, 4:1) of the alkaloid fraction of the root bark gave **1** (123 mg), R_f 0.6) and **3** (29 mg, R_f 0.4). It might be expected that **4** would have a lower R_f value than **1** since, from the structures, it is more polar, but intramolecular hydrogen bonding between the C-18 carbonyl and OH group at C-19 might reduce the affinity of **4** to the silica stationary phase.

Spectroscopic Techniques. NMR Spectroscopic Studies. ^{1}H NMR, ^{13}C NMR, ^{1}H−^{13}C short- and long-range correlation HETCOR and HMBC spectra were obtained at 400/100 MHz on an AMX 400 NMR spectrometer using $CDCl_3$ as solvent with TMS as internal standard. Standard programs from the library ($^nJ_{C-H}$ = 7 Hz) were used for the long-range experiments. CD spectra were obtained using a AVIV 17DS and Applied Photophysics Ltd π*180 spectrometer with 2 nm bandwidth and 0.2 nm step size and employing 48 000 samples per data point with a 0.2 cm cell path length. The compounds were dissolved in spectroscopic grade chloroform.

Physical and Spectral Data of the Isolated Compounds. Schizozygine (1): white crystals (MeOH); mp 191−193 °C; CD spectrum λ nm ($\Delta\epsilon$; M^{-1} cm^{-1}) 248 max (+ 4.3); 278 min (−0.12); 320 max (+1.3); EIMS *m*/*z* 336 [M^+] (100).[3]

Isoschizogaline (2): amorphous powder; CD spectrum λ nm ($\Delta\epsilon$; M^{-1} cm^{-1}) 248 max (+3.8); 276 min (−0.11); 320 max (+1.2); ^{1}H NMR (Table 1); ^{13}C NMR (Table 2); EIMS *m*/*z* (rel int) 322 [M^+] (100).[5]

6,7-Dehydro-19β-hydroxyschizozygine (4): amorphous powder; mp 212−214 °C; IR ν_{max} (Nujol) 3432 (OH), 2922, 1655 (amidic C=O), 1481, 1368, 1224, 1211, 1189, 1037 cm^{-1} ; UV ($CHCl_3$) λ_{max} (log ϵ) 250 (3.78), 254 (4.03), 260 (4.12), 263 (4.12), 272 (3.81), 320 (3.03); CD spectrum λ nm ($\Delta\epsilon$; M^{-1} cm^{-1}) 252 (max) (+2.6); 282 min (−0.10); 291 max (−0.1); 320 min (−0.3); ^{1}H NMR (Table 1); ^{13}C NMR (Table 2); EIMS *m*/*z* (rel int) 350 [$M]^+$ (100); HREIMS *m*/*z* 350.1263 [$M]^+$ (calcd for $C_{20}H_{18}N_2O_4$, 350.1267).

Test Organisms for Bioassays. The bacterial cultures *Escherichia coli* (NCTC 9002), *Bacillus subtilis* (NCTC 10073), *Staphyloccoccus aureas* (NCIMB 9515), and *Pseudomonas aeruginosa* (NCIMB 10421) were obtained from National Collection of Type Cultures, Central Public Health Laboratories, London. The human fungal pathogens (*Trichophyton interdigitale* (EQ 4115), *T. mentagrophytes* (NCPF 224), *T. tonsurans* (EQ 4329), *Epidermophyton floccossum*, and *Microsporum gypseum* (NCPF 40)) were clinical isolates obtained from the Department of Dermatology, St. Thomas Hospital, London SE1, UK. The phytopathogenic fungi *Cladosporium cladosporioides* (IMI 299104) and *Cladosporium herbarum* (lab stock) were obtained from the culture collection of the Department of Pharmacy, Kings College, London.

Antimicrobial Assay Procedures. The yeasts and dermatophytes were grown on Sabouraud's dextrose agar and incubated at 30 °C for 24 h and 26 °C for 10 days, respectively. Plant pathogenic fungi *Cladosporium cladosporioides* and *Cladosporium harbarum* were cultured in potato dextrose agar and incubated at 26 °C for 10 days, while bacteria were grown in nutrient agar and incubated at 37 °C for 24 h. The microdilution technique was used for the determination of antimicrobial activity. Fresh suspensions were prepared for each assay. The standardization of test inocula was done following the method used by Mensah et al.[6] The suspensions were standardized so that 100 μL would contain 10^4 and 10^5 colony-forming units (CFU) for fungi and bacteria, respectively.

DMSO was used to dissolve the extracts for the microdilution method as previously reported,[6] an initial concentration of 500 μg/mL being used. Ketoconazole and ampicillin were used as positive antifungal and antibacterial agents, respectively, and DMSO was used alone at the concentration used to dissolve the compounds to check that it did not have antimicrobial activity.

One-hundred microliter suspensions of the test organisms were added to each well and the plates incubated. For *Candida albicans* and the yeasts, the plates were incubated at 30 °C for 24 h and the filamentous fungi were maintained at 26 °C for 10 days. The plates were examined visually each day throughout the incubation period for growth. Activity was determined by the lowest concentration showing absence of growth. Each experiment was repeated three times.

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