Scutiaquinones A and B, Perylenequinones from the Roots of *Scutia myrtina* with Anthelmintic Activity[#]

Sloan Ayers,[†] Deborah L. Zink,[†] Kenneth Mohn,[‡,||] Joanne Staats Powell,[‡] Christine M. Brown,[‡] Terry Murphy,[‡] Robert Brand,[§] Seef Pretorius,[⊥] Dennis Stevenson,[§] Donald Thompson,[‡] and Sheo B. Singh*[,†]

Natural Products Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, Merck and Co., Inc., Branchburg Farm, 203 River Road, Somerville, New Jersey 08876, The New York Botanical Garden, Bronx, New York 10458, and Department of Soil, Crop and Climate Sciences, University of the Free State, P.O. Box 339, Bloemfontein, 9300 South Africa

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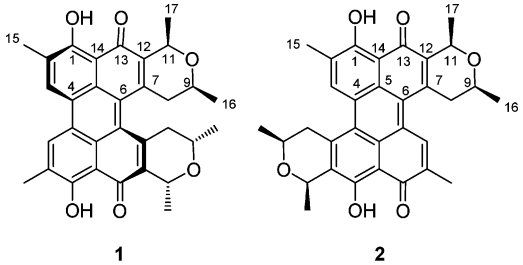
Haemonchus contortus is a worm that causes serious infections in livestock. Two new perylenequinones, scutiaquinone A (**1**) and scutiaquinone B (**2**), have been isolated from a methanol extract of the roots of *Scutia myrtina* by bioassay-guided fractionation, using an assay that kills this parasite in vitro. The structures of compounds **1** and **2** were elucidated by analysis of their 1D- and 2D-NMR and MS spectra. The isolation, structure, and anthelmintic activities of compounds **1** and **2** are reported.

Infection by internal parasites such as *Haemonchus contortus* is one of the greatest impediments to productivity in the livestock industry. Great progress has been made in the development of anthelmintic drugs in the past 50 years. During this time the current classes of synthetic drugs were developed, including the benzimidazoles and imidazothiazoles (such as levamisole). The discovery and development of the macrolactone natural product avermectin was the most significant advance made during the 1980s, leading to the regulatory approval of ivermectin as a treatment for parasitic infections. Ivermectin exhibits excellent broad-spectrum activity as well as superior potency. However, resistance to all of these classes of drugs has been observed, leading to the need for further research to discover new classes of anthelmintics, especially those with novel modes of action.[1]

Our strategy to discover new anthelmintics has been to screen extracts of terrestrial plants and bacterial and fungal fermentations using in vitro activity against *Haemonchus contortus*,[2] which is one of the most prevalent parasitic worms that infect livestock. Evaluation of in vivo activity was accomplished using *Heligmosomoides polygyrus* (a related organism) in mice.[3] The methanol extract of the roots of *Scutia myrtina* (Burm. f.) Kurz. (Rhamnaceae) showed in vitro activity and was selected for further study. Bioassay-guided fractionation using the in vitro *H. contortus* assay led to the isolation of two new perylenequinones, scutiaquinones A (**1**) and B (**2**). The isolation, structure elucidation, and anthelmintic activities of these compounds are described herein.

The methanol extract of the roots was partitioned with hexanes and methylene chloride. The combined methylene chloride and hexanes extract was chromatographed on silica gel, reversed-phase (C_8) preparative HPLC, and finally reversed-phase (C_{18}) semi-preparative HPLC to yield compounds **1** and **2** (see Experimental Section for details).

Scutiaquinone A (**1**) was obtained as a deep red solid. Its molecular formula, $C_{32}H_{30}O_6$, was deduced from the molecular ion at *m*/*z* 511.21195 $[M + H]^+$ by HRESIFTMS. The formula



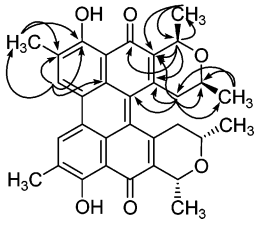


Figure 1. HMBC correlations for scutiaquinone A (**1**).

indicated an index of hydrogen deficiency of 18. The ^{13}C NMR spectrum, however, showed the presence of only 16 carbons, and the ^1H NMR spectrum exhibited 14 proton signals. This indicated that **1** is a symmetrical dimer (with two exchangeable protons). From the DEPT NMR spectrum, each half of **1** contained three methyl groups, one methylene, and three methines, including one aromatic methine. The remaining nine ^{13}C NMR signals were quaternary; seven in the aromatic region at δ_C 108−150 ppm and two in the carbonyl region (δ_C 177.2, δ_C 176.6 ppm), consistent with the high index of hydrogen deficiency. The ^1H NMR spectrum showed methyl doublets at δ_H 1.32 and 1.84 ppm, as well as an aromatic methyl singlet at δ_H 2.55 ppm. The $^1H,^1H$-COSY spectrum showed correlations from the methyl doublet at δ_H 1.84 to a quartet at δ_H 5.26 ppm. The methyl doublet at δ_H 1.32 correlated to a multiplet at δ_H 3.22, which in turn correlated to a multiplet at δ_H 3.46 ppm, and finally correlated to a doublet at δ_H 2.80 ppm. The signals at δ_H 2.80 and 3.46 were nonequivalent methylene protons, according to the HSQC spectrum. These data suggested the presence of a dihydropyran (DHP) ring with methyl groups attached to the carbons adjacent to the oxygen atom. HMBC correlations (Figure 1) suggested that the DHP ring is attached to an aromatic ring. The ROESY correlations of the two methine protons of the DHP ring indicated that these are diaxial (Figure 2).

[#] Dedicated to the late Dr. Kenneth L. Rinehart of the University of Illinois at Urbana−Champaign for his pioneering work on bioactive natural products.

* Corresponding author. Tel: (732) 594-3222. Fax: (732) 594-6880. E-mail: sheo_singh@merck.com.

[†] Natural Products Chemistry, MRL.

[‡] Merck and Co., Branchburg Farm.

[§] New York Botanical Garden.

[⊥] University of the Free State.

[||] Current address: Elanco Animal Health, 2001 W. Main St., P.O. Box 708, Greenfield, IN 46140.

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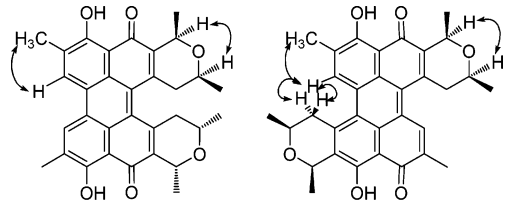

Figure 2. Selected ROESY correlations for scutiaquinones A (**1**) and B (**2**).

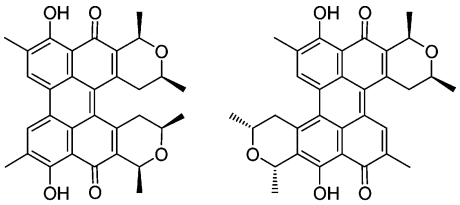

Figure 3. Alternate symmetrical stereoisomers for scutiaquinones A (**1a**) and B (**2a**).

A survey of the literature for natural products from Rhamnaceae indicated a report of pyranonaphthalenes from the genus *Karwinskia* with a dimethyl-branched DHP ring.[4] The MS data showed that compounds **1** and **2** were approximately double the size of the reported pyranonaphthalene, and the HMBC spectrum (Figure 1) confirmed that **1** and **2** are closely related to this class. The nucleus of these dimers would then be a perylenequinone, a number of which have been previously reported from fungi, but only once from a plant source (4,9-dihydroxy-1,2,6,7,11,12-hexamethylperylene-3,10-quinone from *Diospyros natalensis* ssp. *natalensis*).[5] The proposed structures conform to the hydrogen-deficiency index requirement of 18.

ROESY spectroscopic analysis of **1** showed a correlation between the aromatic methyl group and the aromatic proton. The aromatic proton showed no correlation to the methylene protons, indicating that both DHP rings were on the same side of the perylenequinone nucleus, unlike compound **2** (Figure 2).

Numerous symmetrical stereochemical possibilities exist for scutiaquinone A (**1**). All possibilities with *trans*-methyl groups on the DHP rings could be eliminated by the ROESY NMR data. The *cis*-isomers include possibilities with all methyl groups β (mirror-plane symmetry, **1a** in Figure 3) or both methyl groups in one DHP ring α and the other β as in **1** (C_2 symmetry). After considering that the molecule is nonplanar, the C_2 symmetrical structure (**1**) is the only possibility. Another factor to consider is biosynthesis; structure **1** would be favored because it would be biosynthesized by dimerization of a single enantiomer, unlike **1a**. Therefore, structure **1** was proposed for scutiaquinone A.

Scutiaquinone B (**2**) was also obtained as a deep red solid. Its molecular formula, $C_{32}H_{30}O_6$, was identical to **1** and was deduced from the molecular ion at *m*/*z* 511.21078 $[M + H]^+$ by HRESIFT-MS. The ^1H and ^{13}C NMR spectra for **2** were nearly identical to **1** except for small chemical shift differences. The major spectroscopic differences between **1** and **2** were in the ROESY spectra. Unlike compound **1**, the aromatic proton of compound **2** exhibited ROESY correlations to the aromatic methyl and the methylene protons, indicating that the DHP rings were on opposite sides of the perylenequinone nucleus (Figure 2). This is still a symmetrical structure due to tautomerization of the perylenequinone nucleus. The ^{13}C NMR chemical shifts for C-1 and C-13 are consistent with tautomerization. The chemical shift for C-13 is δ_C 176.7, which is upfield compared to the expected shift for an unsaturated ketone, while the reported value for C-1 is δ_C 177.3, which is downfield compared to the expected shift of a phenolic carbon. The fact that these shifts are nearly identical is further evidence of tautomerization.[6] The isolated yield of **2** was less than **1**; however, HPLC analyses of crude fractions containing both **1** and **2** indicated that they are produced in approximately equal amounts.

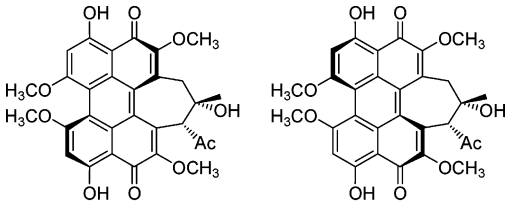

Figure 4. Shiraiachromes A (**3**) and B (and tautomer, hypocrellin A) (**4**).

As with scutiaquinone A (**1**), two symmetrical possibilities existed for scutiaquinone B (**2**). Structure **2** has all methyl groups β (C_2 symmetry), while **2a** (Figure 3) has the methyl groups of one DHP ring α and the other β (*i*, or inversion point, and S_2, or 2-fold improper rotation axis of symmetry). Again from a biosynthetic point of view, structure **2** would be constructed from the same enantiomer, while **2a** has to be constructed from opposite enantiomers. Therefore, structure **2** was proposed for scutiaquinone B.

CD spectra of both **1** and **2** were obtained in 95% EtOH. The magnitude of the molar ellipticities for **1** was approximately 2500 times greater than for **2**, suggesting that the perylenequinone nucleus of **1** is twisted out of plane, while **2** is nearly planar. This is not a surprising result when taking into account the steric crowding of the methylene protons of the two DHP rings for **1**. Compound **2** has no such steric crowding. No direct comparison of the CD data for **1** to literature was possible since no comparable structures exist. However, the CD spectrum for **1** was compared to literature data for other perylenequinones, shiraiachromes A (**3**) and B (and tautomer, hypocrellin A) (**4**), which indicated that the stereochemistry of **1** was consistent with shiraiachrome B (hypocrellin A) (**4**, Figure 4).[7,8] The absolute configuration of the perylenequinone nucleus is therefore known; however, the absolute configuration at C-9 and C-11 is not known.

Compounds **1** and **2** were first tested in the in vitro *H. contortus* assay as described earlier.[2] In vitro activity is reported as "EC_{90}": the concentration at which L3 larval motility is reduced by 90%. Compounds **1** and **2** reduced motility of L3 larvae at approximately the same concentration (EC_{90} = 9.3 and 10.3 μg/mL, respectively), which was much less potent than the ivermectin control (EC_{90} = 0.18 μg/mL). In the in vivo test against *H. polygyrus*-infected mice,[3] both compounds **1** and **2** were ineffective in meaningful reduction of worm counts at 25 and 30 mg/kg, respectively, when dosed intramuscularly. Compound **2** exhibited toxicity at 50 mg/kg. The antibacterial activity and mammalian toxicity of another perylenequinone, the fungal pigment cercosporin, was shown to be due to photosensitization.[9]

In summary, we have reported two new perylenequinones from the roots of *Scutia myrtina*. This class of compound is very rare in plants and showed in vitro anthelmintic activity, but virtually no in vivo activity.

Experimental Section

General Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. IR data were obtained on a Perkin-Elmer Spectrum One. UV/vis spectra were taken on a Perkin-Elmer Lambda 35 UV/vis spectrometer. CD spectra were obtained on a JASCO J-715 spectropolarimeter. NMR spectra were obtained on a Varian Inova 500 MHz spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C nuclei, and solvent shifts were used for internal standard. Low-resolution mass spectra were obtained on an Agilent MSD, and high-resolution mass spectra were obtained on a

Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe.

Plant Material. Roots of *Scutia myrtina* were collected in Humansdorp, East Cape, South Africa, in April 2000. Voucher specimens (accession number 764533) are stored at the New York Botanical Garden.

Extraction and Isolation. Roots from *S. myrtina* (2.86 kg) were extracted with methanol at room temperature, and the methanol was removed under reduced pressure, yielding 71.4 g of gummy material. A 16.8 g portion of the methanol extract was dissolved in 225 mL of 9:1 MeOH/water and extracted with hexanes (3 × 225 mL). The 9:1 MeOH/water layer was diluted with water to 3:2 MeOH/water and extracted with methylene chloride (3 × 325 mL). The methylene chloride and hexanes layers were dried over Na_2SO_4, combined, and evaporated to give 951 mg of a dark red solid. This solid was dissolved in minimal CH_2Cl_2 and applied to a silica gel low-pressure LC column (1 in. diameter, ~250 mL bed volume). The column was eluted with a gradient of CH_2Cl_2/EtOAc (100:0−90:10), followed by 80:10:10 CH_2Cl_2/EtOAc/MeOH. The intense red color eluted in the methanol-containing mobile phase. The active red fractions were combined and evaporated to give 504 mg of a dark red solid. This solid was dissolved in minimal 1:1 CH_2Cl_2/MeOH and injected onto a preparative HPLC column (Zorbax Rx C_8, 250 × 21.2 mm), 95−100% MeCN in H_2O (both with 0.1% TFA) over 40 min, 10 mL/min. Fractions were collected every 0.5 min. Again, the red fractions were pooled and lyophilized to give 44.7 mg of a dark red solid. This material was dissolved in minimal 1:1 CH_2Cl_2/MeOH and injected onto a semi-preparative HPLC column (Zorbax Rx C_{18}, 250 × 9.4 mm), 95−100% MeCN in H_2O (both with 0.1% TFA) over 15 min, 4 mL/min. Compound **1** eluted at 10.3 min (13.3 mg), and **2** eluted at 11.4 min (7.1 mg).

Scutiaquinone A (1): dark red solid; IR (ZnSe film) ν_{max} 3395 (w, br), 2918, 2850, 1619, 1456, 1261 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 208 (4.51), 256 (4.33), 358 (3.45), ~450 (sh, ~4.1), 475 (4.24), 538 (3.73), 578 (3.77) nm; CD (95% EtOH) λ (θ) 234 (−41 200), 265 (61 900), 356 (−13 800), 475 (33 500); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 16.30 (1H, s, −OH), 8.58 (1H, s, H-3), 5.26 (1H, q, J = 6.5 Hz, H-11), 3.46 (1H, ddd, J = 16.0, 9.5, 2.5 Hz, H-8b), 3.22 (1H, m, H-9), 2.80 (1H, d, J = 16.0 Hz, H-8a), 2.55 (3H, s, H-15), 1.84 (3H, d, J = 6.5 Hz, H-17), 1.32 (3H, d, J = 6.5 Hz, H-16); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.2 (C-1), 176.6 (C-13), 145.8 (C-7), 135.8 (C-12), 133.8 (C-2), 132.1 (C-3), 126.1 (C-6), 125.1 (C-5), 123.2 (C-4), 108.9 (C-14), 70.4 (C-11), 70.0 (C-9), 41.7 (C-8), 22.9 (C-17), 21.1 (C-16), 16.4 (C-15); ESIMS m/z 511 $[M + H]^+$ (100); HRESIFTMS m/z 511.21195 $[M + H]^+$ (calcd for $C_{32}H_{30}O_6$ + H, 511.21213).

Scutiaquinone B (2): dark red solid; IR (ZnSe film) ν_{max} 3433 (w, br), 2925, 2854, 1619, 1453, 1261 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 207 (4.42), 255 (4.25), 354 (3.37), ~450 (sh, ~4.0), 471 (4.16), 535 (3.67), 576 (3.74) nm; CD (95% EtOH) λ (θ) 236 (−19), 264 (24), 351 (−6), 473 (14); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 16.83 (1H, s, −OH), 8.60 (1H, s, H-3), 5.34 (1H, q, J = 6.5 Hz, H-11), 3.47 (2H, m, H-8b and H-9), 3.14 (1H, d, J = 15 Hz, H-8a), 2.53 (3H, s, H-15), 1.80 (3H, d, J = 6.5 Hz, H-17), 1.44 (3H, d, J = 6.0 Hz, H-16); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.3 (C-1), 176.7 (C-13), 145.1 (C-7), 136.9 (C-12), 136.5 (C-3), 132.5 (C-2), 126.3 (C-6), 125.3 (C-5), 123.7 (C-4), 108.8 (C-14), 70.9 (C-11), 69.7 (C-9), 42.7 (C-8), 22.4 (C-17), 21.4 (C-16), 16.7 (C-15); ESIMS m/z 511 $[M + H]^+$ (100); HRESIFTMS m/z 511.21078 $[M + H]^+$ (calcd for $C_{32}H_{30}O_6$ + H, 511.21213).

Biological Assays. Both compounds were dissolved in DMSO for bioassays. The in vitro assay against *Haemonchus contortus* was used as described in ref 2. The in vivo mouse assay was modified from ref 3 and performed as follows. Swiss-Webster mice (Vendor Taconic Labs, ca. 30 g) were inoculated with 200−400 L3 *H. polygyrus* larvae. The mice were checked for infection around day 12 post-inoculation, then dosed im (intramuscularly) with test compound in triplicate. On day 3 post-treatment, mice were euthanized and the intestine was collected (from below stomach to above cecum), opened, and placed in 5 mL of sterile water. The mucosa was scraped and rinsed through a 200-mesh screen. The rinsate was then examined for the presence of worms, and the worms were counted. Worm counts for treated mice were then compared to infected, untreated mice as negative controls, and infected mice treated with ivermectin (10 mg/kg) as positive controls.

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