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Lasionectrin, a Naphthopyrone from a *Lasionectria* sp.

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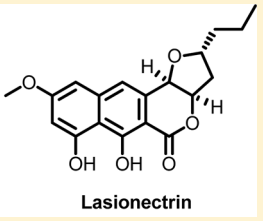
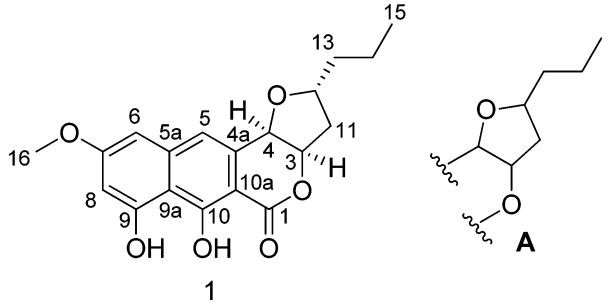
Supporting Information

ABSTRACT: A new naphthopyrone derivative, lasionectrin (**1**), was isolated from fermentations of an *Acremonium*-like fungus provisionally identified as a *Lasionectria* sp. (Ascomycota, Hypocreales) and isolated from forest leaf litter from Equatorial Guinea. Its structure was determined by a combination of spectroscopic techniques, including UV, (+)-HRESIMS, and 1D and 2D NMR spectroscopy, and comparison with published data for related fungal metabolites. Compound **1** inhibited the growth of *Plasmodium falciparum* with an IC_{50} value of 11 μM.



The discovery and development of new drugs for the treatment of malaria is of major importance. Current treatments for this infectious disease include the use of chloroquine and other aminoquinolines, the naphthoquinone atovaquone, part of the successful prophylactic Malarone, and the artemisins, sesquiterpene endoperoxides isolated from the leaves of *Artemisia annua*.[1] The emergence of drug resistance in strains of *Plasmodium* is becoming a major problem, and the cheapest and safest malaria drug, chloroquine, is rapidly losing its effectiveness. Additionally, in some parts of the world, malaria is becoming resistant to several leading frontline drugs. For instance, resistance of *P. falciparum* to artemisins has recently been observed at the Cambodia−Thailand border.[2] New drugs with unique structures based on different modes of action are therefore urgently required to treat sensitive and drug-resistant strains of the parasite.

Full genomic sequences of the fungi in the Hypocreales have revealed that the exceptional chemical diversity and the biological functions of these fungi's metabolites are only beginning to be understood.[3,4] In our ongoing screening for biologically active fungal secondary metabolites, we investigated metabolites produced by a strain tentatively identified as a species of *Lasionectria* (F-176,994), isolated from fern leaf litter from a forest of the Monte Alen National Park, Equatorial Guinea. Acetone extracts of this fungus grown in a buffered mannitol medium displayed *in vitro* antiplasmodial activity against *P. falciparum Pf* 3D7. Bioassay-guided chromatographic fractionation of these extracts on SP-207ss resin followed by repeated semipreparative HPLC yielded a new naphthopyrone derivative, lasionectrin (**1**), as the compound responsible for the biological activity.



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A pseudomolecular ion at m/z 345.1335 by (+)-ESI-TOFMS and the presence of 19 signals in the ^{13}C NMR spectrum established a molecular formula of $C_{19}H_{20}O_6$ for compound **1**. The UV spectrum displayed absorptions at 216, 260, 306, and 363 nm characteristic of a 3,4-dihydro-9,10-dihydroxy-7-methoxynaphtho[2,3-*c*]pyran-1-one moiety (naphthopyrone) and similar to those observed in the structurally related compounds semivioxanthin,[5] vioxanthin,[6] and lichenicolin A.[7] The ^1H NMR spectrum (Table 1) contained signals due to the presence of three aromatic protons at δ_H 7.23, 6.80, and 6.51 ppm (the two latter *m*-coupled), three oxygenated methines (δ_H 4.33, 4.96, and 5.24 ppm), three aliphatic methylenes, one aromatic methoxyl group (δ_H 3.89 ppm), and one aliphatic methyl group (δ_H 0.97 ppm).

The ^{13}C, HSQC, and HMBC data confirmed the presence of the naphthopyrone moiety in the molecule and established the position of the substituents. Thus, proton H-6 showed strong HMBC correlations ($^3J_{CH}$) to carbons C-5, C-8, and C-9a and a

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Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) for Compound 1

	δ_C, type	δ_H, mult. (J in Hz)	COSY	HMBC (H to C)
1	171.2, C			
3	84.2, CH	5.24, m	4, 11	4, 12
4	75.0, CH	4.93, d (1.6)	3	3, 4a, 5, 10, 10a
4a	131.9, C			
5	120.7, CH	7.23, s		1, 4, 5a, 6, 9a, 10, 10a
5a	142.1, C			
6	101.1, CH	6.80, d (2.1)	8	5, 7, 8, 9, 9a
7	164.4, C			
8	103.5, CH	6.51, d (2.1)	6	6, 7, 9, 9a
9	159.5, C			
9a	110.3, C			
10	163.6, C			
10a	98.5, C			
11	40.8, CH_2	2.58, dd, (13.9, 6.2)	3, 11b, 12	3, 4, 4a, 13
		2.12, ddd, (13.9, 9.6, 3.9)	3, 11a, 12	12, 13
12	80.4, CH	4.33, m	11a, 11b, 13a, 13b	4, 11, 14
13	39.4, CH_2	1.70, m	12, 13b, 14a, 14b	11, 12, 14, 15
		1.57, m	12, 13a, 14a, 14b	11, 12, 14, 15
14	20.3, CH_2	1.49, m	13a, 13b, 14b, 14b	12, 13, 15
		1.39, m	13a, 13b, 14a, 15	12, 13, 15
15	14.4, CH_3	0.97, t (7.3), 3H	14	13, 14
16	56.0, CH_3	3.89, s, 3H		7

weak correlation ($^2J_{CH}$) to C-7. Proton H-8 displayed $^3J_{CH}$ correlations to C-6 and C-9a and $^2J_{CH}$ correlations to C-7 and C-9. Finally, proton H-5 showed intense cross-peaks to carbons C-4, C-6, C9a, and C-10a and weak correlations ($^4J_{CH}$) to carbons C-1 and C-10. Cross-peaks observed in the COSY spectrum established the sequence H-4/H-3/H-11 to H-15, and the existence of HMBC correlations between H-4 at δ_H 4.93 ppm and C-12 at δ_C 80.4 ppm and between H-12 at δ_H 4.33 ppm and C-4 at δ_C 75.0 ppm indicated the presence in the molecule of the substructure A, identical to that found in monocerin.[8] This substructure was connected to the aromatic rings of the naphthopyrone through correlations observed in the HMBC spectrum. Thus, H-4 showed cross-peaks to carbons C-4a, C-5, and C-10a and a weak correlation ($^4J_{CH}$) to C-10, whereas H-5 displayed an intense correlation to C-4. No HMBC correlation was observed between H-3 and C-1, but the chemical shift observed for H-3 (5.24 ppm) and the number of unsaturations deduced from the molecular formula clearly pointed to the existence of a lactone bridge between C-1 and C-3. The location of the methoxy group was unambiguously determined via an HMBC correlation between H_3-16 at δ_H 3.89 ppm and C-7 at δ_C 164.4 ppm. In addition, H_3-16 displayed NOESY correlations to both H-6 and H-8, clearly positioning the methoxy group at C-7.

Correlations observed in the NOESY spectrum of compound **1** (Figure 1) determined its relative stereochemistry. Nuclear Overhausser effects were observed between H-3 and H-4, indicating a *cis* junction of the furane and the α-pyrone moieties. Additionally, correlations from H-4 to one of the H-11 protons at 2.12 ppm and from H-12 to the other H-11 proton at 2.58 ppm clearly determined that protons H-4 and H-12 were *trans* oriented.

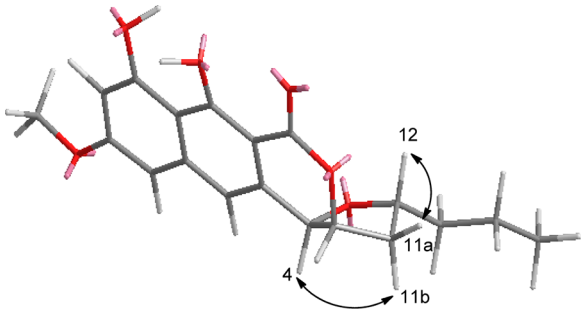

Figure 1. Key NOESY correlations observed in the spectrum of **1**.

Lasionectrin exhibited activity against *P. falciparum Pf*3D7 using the previously described LDH *in vitro* assay[9] with an IC_{50} of 11.0 ± 0.9 μM. Chloroquine gave an IC_{50} of 5 nM when tested under the same conditions. The potency was approximately one-fifteenth that of monocerin,[10] a structurally related compound, suggesting that the number of aromatic rings or the stereochemistry of subunit A has a significant influence on the bioactivity of this structural class.

In conclusion, we have isolated a new naphthopyrone with antiplasmodial properties from fermentation broths of a *Lasionectria* species. To the best of our knowledge, this note constitutes the first account on the isolation of a natural product from fungi of this genus.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined using a Perkin-Elmer 343 polarimeter. UV spectra were obtained with an Agilent 1100 DAD. NMR spectra were recorded on a Varian "INOVA 500" spectrometer at 500/125 MHz ($^1H/^{13}C$). Chemical shifts were reported in ppm using residual CD_3OD (δ 3.31 for ^1H and 49.0 for ^{13}C) as internal reference. HMBC experiments were optimized for a $^3J_{CH}$ of 8 Hz. (+)-ESI-TOFMS was performed on a Bruker maXis spectrometer.

Producing Fungus and Its Characterization. The producing microorganism (F-176,994) was isolated from fern litter collected in Monte Alen National Park, Equatorial Guinea, using a method for plating of washed particles of plant litter.[11] Frozen stock cultures in 10% glycerol (−80 °C) are maintained in the collection of Fundación MEDINA. Total genomic DNA was extracted from mycelia grown on YM agar. The 28S rDNA fragments, containing D1−D2 and ITS regions, were PCR amplified, and sequence alignments[12] and phylogenetic analyses were performed as previously described.[13] In culture, the fungus produced pale pink to peach-colored aerial mycelium (Figure S10A). Simple to sparsely branched phialides arose from the agar surface and aerial hyphae (Figure S10B−D). The phialides produce simple subglobose conidia accumulating in moist drops adhering to the conidiogenesis loci (Figure S10B−D). On the basis of morphology, the fungus could be assigned to the form genus *Acremonium*. However, a revision of generic concepts among fungi classified as *Acremonium* has confirmed the long-recognized phylogenetic heterogeneity of the genus and has designated *A. alternatum* as the type species for the genus.[14] Sequence homology searches in public databases (www.fungalbarcoding.org, NCBI) indicated that the fungus was unrelated to *A. alternatum* but instead exhibited affinities to various genera in the family Bionectriaceae of the Hypocreales, including *Bionectria*, *Ochronectria*, *Lasionectria*, *Protocreopsis*, and *Verrucostoma*. Therefore to seek a more natural classification of F-176,994, an alignment of 28S rDNA sequences of *Acremonium*-like species associated with Bionectriaceae[14,15] was compiled. The most similar fungus based on alignment and Bayesian analysis of the 28s rDNA was a strain of *Lasionectria vulpina* (CBS

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69493). Bayesian analysis of the 28S alignment placed strain F-176,994 in a statistically supported clade with a strain of *Lasionectria vulpina*, *Protocreopsis pertusa*, and *A. longisporum* (Figure S11). The *Acremonium*-like morphology of F-176,994 (Figure S10) was consistent with *Acremonium*-like states of *L. vulpina* and many other fungi of the Bionectriaceae.[15,16] Although this fungus was not congeneric with the type species of *Lasionectria* (*L. mantuana*), provisionally we are calling it a *Lasionectria* sp.

Fermentation. *Lasionectria* sp. (F-176,994) was fermented by inoculating four mycelial agar plugs into SMYA medium (Bacto neopeptone 10 g; maltose 40 g; yeast extract 10 g; agar 3 g; H_2O 1 L) into an Erlenmeyer flask (50 mL of medium in a 250 mL flask). The flask was incubated on a rotary shaker at 220 rpm at 22 °C with 70% relative humidity. After growing the seed stage for 7 days, a 4 mL aliquot was used to inoculate each flask of the production medium XLA (mannitol 75 g; yeast extract 1 g; oat flour 1 g; 2-(*N*-morpholino)ethanesulfonic acid 16.2 g; NH_4Cl 3 g; $MgSO_4 \cdot 7H_2O$ 0.58 g; H_2O 1 L). Eight flasks (150 mL of medium per 500 mL Erlenmeyer flask) were incubated at 22 °C with 70% relative humidity on a rotary shaker at 220 rpm for 14 days.

Extraction and Isolation. The fermentations of the *Lasionectria* sp. (8 × 150 mL) were extracted by addition of an equal volume of acetone, shaking for 1 h, centrifugation, filtration, and evaporation of the organic solvent under a nitrogen stream. The aqueous residue was loaded onto a SP207ss column (65 g, 32 × 100 mm) that was eluted with a gradient of acetone in water (0 to 100% acetone in 15 min + 100% acetone for 15 min, 8 mL/min, 18 mL/fraction). Bioactive fraction 9 from this chromatography was subjected to preparative HPLC (Zorbax SB-C_8, 21.2 × 250 mm, gradient H_2O–CH_3CN from 5% to 100% in 37 min, 20 mL/min, UV detection at 210 and 280 nm) to afford 2.8 mg of compound **1** eluting at 26.8 min.

Lasionectrin (1): white solid; $[\alpha]^{25}_D$ −43.8 (*c* 0.17, MeOH); UV (HPLC-DAD) ν_{max} 216, 260, 306, 363 nm; ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)-ESI-TOFMS *m*/*z* 345.1335 $[M + H]^+$ (calcd for $C_{19}H_{21}O_6$, 345.1333).

Biological Activity. Parasites of the *P. falciparum* strain 3D7 were grown in fresh group 0 positive human erythrocytes, obtained from the Centro Regional de Transfusión Sanguínea-SAS (Granada, Spain), and suspended at 5% hematocrit in RPMI 1640 containing 2% human serum, 0.2% $NaHCO_3$, 0.5% Albumax II, 150 μM hypoxanthine, and 12.5 μg/mL gentamicin. Flasks were incubated at 37 °C, under a 5% CO_2 and 95% air mixture. Stock cultures were synchronized with 5% sorbitol, and 96 h later parasites were mostly late ring stages and early trophozoites. The stock culture was then diluted with complete medium and nonparasitized erythrocytes to yield a hematocrit of 2% and a parasitemia of 0.25%.

The extracts, fractions, and pure compounds were evaluated in 384-well plates. Each plate also included negative (no additions) and positive controls with 100 nM chloroquine. Parasite growth inhibition assays and 50% inhibitory concentration (IC_{50}) determinations were measured using the LDH assay as previously described.[9]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR, (+)-ESI-TOF, and UV spectra for compound **1** and phylogenetic and morphological analysis of the fungus are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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