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Mycoleptones A–C and Polyketides from the Endophyte *Mycoleptodiscus indicus*

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ABSTRACT

Three new azaphilones with an unusual methylene bridge, named mycoleptones A, B and C (**2**, **4** and **5**), were isolated from cultures of *Mycoleptodiscus indicus*, a fungus associated with the South American medicinal plant *Borreria verticillata* (Rubiaceae). Additionally, four known polyketides, austdiol (**1**), eugenitin (**3**), 6-methoxyeugenin (**6**) and 9-hydroxyeugenin (**7**), were also isolated. The structural characterization of compounds was carried out by nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry (HRMS), electronic circular dichroism (ECD) spectroscopy, time-dependent density functional theory (TD-DFT) calculations and X-ray crystallography. Compounds **1–9** were weakly active when tested in anti-leishmanial and cytotoxicity assays.

INTRODUCTION

Endophytic microorganisms live inside the tissues of host plants without apparently harming them, and are a promising source of bioactive compounds.¹⁻⁴ Species of *Mycoleptodiscus*, such as *M. indicus*, *M. terrestris* and *M. sphericus* are commonly isolated as endophytes; they are probably latently phytopathogenic and may become phytopathogenic when the host plant is subjected to stress.⁵ *M. indicus*, a tropical-to-subtropical species, occurs in leaves of different host plants, mainly monocotyledons. *M. indicus* is associated with large spreading lesions on leaves of *Zamia* spp., an American cycad, and other monocotyledonous plants.⁶ *M. indicus* has occasionally been reported to infect humans and canines, causing septic arthritis and skin infections.⁷

In this paper, the isolation, structural characterization and biological activity of secondary metabolites from cultures of *Mycoleptodiscus indicus*, an endophytic fungus isolated from the leaves from *Borreria verticillata*, are reported. Three new azaphilones, named mycoleptones A, B and C (**2**, **4** and **5**), were isolated and fully characterized; in addition, four known polyketides, namely austdiol (**1**), eugenitin (**3**), 6-methoxyeugenin (**6**), 9-hydroxyeugenin (**7**), were isolated and identified, and the acetylated derivatives of austdiol (**8**) and mycoleptone A (**9**) were synthesized (Figure 1).

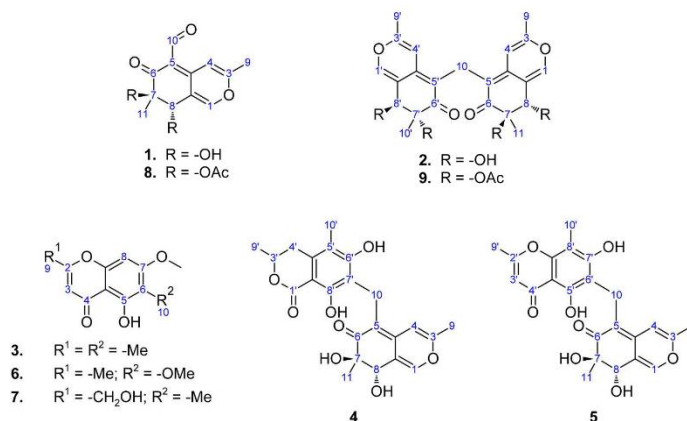


Figure 1. Mycoleptones A–C and polyketides from *Mycoleptodiscus indicus*.

RESULTS AND DISCUSSION

Seven secondary metabolites of *Mycoleptodiscus indicus* were isolated, four of which were identified as austdiol, eugenitin, 6-methoxyeugenin and 9-hydroxyeugenin by comparison of experimental spectroscopic data with literature values.^{8,9,10,11,12,13} Austdiol ((7*R*,8*S*)-7,8-dihydroxy-3,7-dimethyl-6-oxo-7,8-dihydro-6*H*-isochromene-5-carbaldehyde) is the main toxic component of a mixture of compounds produced in mouldy maize meal by *Aspergillus ustus* and belongs to a class of compounds known generically as azaphilones.^{14,15} Azaphilones are yellow or orange pigments produced by fungi: their pyran oxygen atoms are easily exchanged to nitrogen atoms with ammonia, and the name is derived from the ready reaction of these metabolites with ammonia to yield vinylogous γ -pyridones.¹⁶ The skeleton of austdiol is derived from a single pentaketide chain, composed of head-to-tail acetate units, and possesses two C₁

units introduced from *S*-adenosyl-methionine.¹⁷ A wide range of interesting biological activities of azaphilones, such as antimicrobial, antifungal, antiviral, antioxidant, cytotoxic, nematocidal and anti-inflammatory, have been reported in the literature;^{14,17} their non-selective biological activities may be related to the production of vinylogous γ -pyridones.¹⁸

Three of the isolated metabolites are previously unreported azaphilones and were named mycoleptones A, B and C (**2**, **4** and **5**). Mycoleptone A (**2**) was isolated as a yellow powder, and the complete structural characterization was carried out by nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry (HRMS), electronic circular dichroism (ECD) spectroscopy, time-dependent density functional theory (TD-DFT) calculations and single-crystal X-ray diffraction studies (Figure 2).

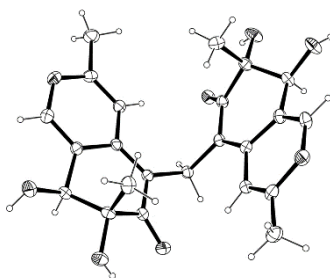


Figure 2. ORTEP representation of mycoleptone A (atom displacement at 30% of probability). Hydrogen atoms are represented by spheres of arbitrary radius.

The UV spectrum of **2** displays maximum absorptions at 254 nm ($\log \epsilon$ 2.90) and 361 nm ($\log \epsilon$ 2.83), revealing the presence of an extended conjugated system, characteristic of azaphilones. Compound **2** displays similar ^1H and ^{13}C NMR spectra to austdiol,^{8,9} with the replacement of the aldehyde signals (δ_{H} 9.99 and δ_{C} 189.3) by methylene signals (δ_{H} 3.26 and δ_{C} 18.5) at position C-5. The ^1H NMR spectrum exhibits two signals at δ_{H} 7.30 and 6.25 due to hydrogen atoms on the pyran ring, two hydroxyl hydrogens at δ_{H} 5.66 (OH, d, $J = 4.6$ Hz) and 4.93 (OH, s), a methine signal at δ_{H} 4.22 (1H, d, $J = 4.6$ Hz), a methylene signal at δ_{H} 3.26 and two methyl signals at δ_{H} 2.07 and 0.91. The ^{13}C NMR spectrum displays signals for twelve carbon atoms, eleven of which refer to two atoms each: two signals due to methyl carbon atoms (δ_{C} 19.2 e 18.8), five signals from the skeleton of the pyran rings (δ_{C} 157.8; 143.5; 141.0; 120.7 and 104.6), and four signals from the skeleton of the cyclohexenone, with one signal due to ketone carbonyl groups (δ_{C} 197.8; IR ν_{max} 1667 cm^{-1}), one due to quaternary unsaturated carbon atoms (δ_{C} 112.7) and two due to oxygenated carbon atoms (δ_{C} 75.6 and 71.0). The remaining signal refers to a methylene group (δ_{C} 18.5). The heteronuclear multiple-bond correlation (HMBC) spectrum shows key correlations of CH_2 -10 to C-4a, C-5, C-6, C-4a', C-5' and C-6', revealing the attachment of two azaphilone units through a methylene bridge at position C-5 (Table 1). Furthermore, the presence of four hydroxyl groups was confirmed by acetylation with pyridine/ Ac_2O to afford the tetraacetylated derivative (**9**). NMR spectroscopy identifies the structure of **2** as being formed by two austdiol units linked by a methylene group, generating a compound with molecular formula $\text{C}_{23}\text{H}_{24}\text{O}_8$ as determined by HRMS ($[\text{M}+\text{H}]^+$ at m/z 429.1543). The chemical structure explains the simplicity of the ^1H and ^{13}C NMR spectra, in which the signal intensities are doubled, except for the methylene group.

Table 1. NMR spectroscopic data (400 MHz) for mycoleptones A–C.

Position	Mycoleptone A (2) ^a			Mycoleptone B (4) ^b			Mycoleptone C (5) ^a		
	δ_C mult.	δ_H (J in Hz)	HMBC ^c	δ_C mult.	δ_H (J in Hz)	HMBC ^c	δ_C mult.	δ_H (J in Hz)	HMBC ^c
1	143.5, CH	7.30, s	3, 4a, 8, 8a	144.8, CH	7.43, d (1.8)	3, 4a, 8, 8a	145.7, CH	7.52, s	3, 4a, 8, 8a
2									
3	157.8, C			160.1, C			160.5, C		
4	104.6, CH	6.25, s	3, 4a, 5, 8a, 9	104.4, CH	6.63, s	3, 5, 8a, 9	105.5, CH	7.03, s	3, 8a
4a	141.0, C			143.7, C			144.3, C		
5	112.7, C			112.8, C			112.1, C		
6	197.8, C			199.3, C			200.5, C		
7	75.6, C	4.93, s (OH)		76.1, C			75.4, C		
8	71.0, CH	4.22, d (4.6); 5.66, d (OH) (4.6)	1, 6, 7, 8a, 10	71.5, CH	4.42, d (1.8)	7, 8a, 11	70.7, CH	4.25, s	1, 6, 7, 8a
8a	120.7, C			121.1, C			121.3, C		
9	19.2, CH ₃	2.07, s	3, 4	18.3, CH ₃	2.22, s	3, 4	19.5, CH ₃	2.25, s	3, 4
10	18.5, CH ₂	3.26, s	4a, 5, 6, 4a', 5', 6'	20.7, CH ₂	3.53, 3.60, d (15.0)	4a, 5, 6, 6', 7', 8'	18.6, CH ₂	3.74, 3.46, d (15.0)	4a, 5, 6, 5', 6', 7'
11	18.8, CH ₃	0.91, s	6, 7, 8	17.5, CH ₃	1.10, s	6, 7	19.0, CH ₃	1.00, s	6, 7, 8
1'	143.5, CH	7.30, s	3', 4a', 8', 8a'	171.2, C					
2'							167.2, C		
3'	157.8, C			75.0, CH	4.55, m		107.9, CH	6.13, s	2', 4a', 9'
4'	104.6, CH	6.25, s	3', 4a', 5', 8a', 9'	32.2, CH ₂	3.17, dd (16.0, 3.0) 2.60, dd (16.0, 11.0)	5', 8a' 3', 5', 8a', 9'	181.8, C		
4a'	141.0, C			110.1, C			103.1, C		
5'	112.7, C			136.8, C			156.6, C	13.06, s (OH)	4a', 5', 6'
6'	197.8, C			160.2, C			107.8, C		
7'	75.6, C	4.93, s (OH)		116.5, C			161.1, C		
8'	71.0, CH	4.22, d (4.6); 5.66, d (OH) (4.6)	1', 6', 7', 8a', 10'	160.9, C			105.0, C		
8a'	120.7, C			100.1, C			153.5, C		
9'	19.2, CH ₃	2.07, s	3', 4'	19.6, CH ₃	1.44, d (6.2)	3', 4'	20.2, CH ₃	2.37, s	2', 3'
10'	18.8, CH ₃	0.91, s	6', 7', 8'	6.9, CH ₃	2.06, s	4a', 5', 6'	8.5, CH ₃	2.00, s	7', 8', 8a'

a: In DMSO-*d*₆. **b:** In CD₃OD. **c:** HMBC correlations are from hydrogen(s) stated to the indicated carbon.

Compound **2** is a chiral molecule having four asymmetric carbons and a formal binary symmetry axis. An accurate analysis of the eight enantiomeric pairs of **2** reveals that two pairs are formed by non-optimally active *meso* structures and two more pairs are repeated twice; as a result, four pairs of enantiomers and two *meso* structures are possible. Since the reliable assessment of the absolute configuration of a compound is achieved when the theoretical chiroptical properties¹⁹ of all the possible optically-active diastereomers are determined,²⁰ quantum-mechanical (QM) calculations were performed on four diastereomers of **2** (Figure 3): (7*R*,8*S*,7'*R*,8'*S*)-**2** (**2A**), (7*R*,8*S*,7'*R*,8'*R*)-**2** (**2B**), (7*R*,8*S*,7'*S*,8'*S*)-**2** (**2C**) and (7*S*,8*S*,7'*S*,8'*S*)-**2** (**2D**).

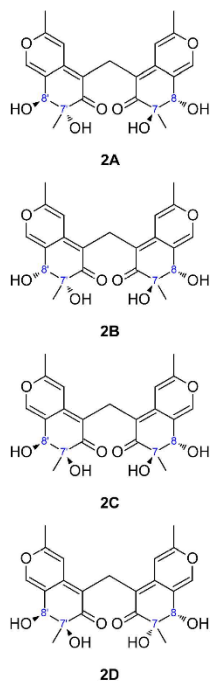


Figure 3. Diastereomers of mycoleptone A.

QM calculations were carried out using density functional theory (DFT) and its time-dependent extension (TD-DFT); detailed results are reported in the Supporting Information. DFT geometry optimization (Table 2) yielded two equilibrium conformers having $\Delta E_{\text{QM}} \leq 2 \text{ kcal mol}^{-1}$ for diastereomers **2A**, **2B** and **2C**, while a single equilibrium conformer within the same threshold energy was found for diastereomer **2D**. The most populated conformers of **2A** (Figure 4) display similar geometric features: the azaphilone units are oriented in an “open folder” conformation, the pyran oxygen atoms are pointing toward opposite directions, all the hydroxyl groups are equatorial and form intramolecular hydrogen bonds with each other and with the ketone carbonyl groups. The main difference lies in the direction of the axial methyl groups on C-7 and C-7', which point outside the molecular cavity for **2A-e** and inside for **2A-b**.

Table 2. Geometric parameters, energy values and fractional equilibrium populations for the most populated equilibrium conformers of the diastereomers of mycoleptone A, as obtained after DFT geometry optimization at the B97D/6-311++G(2d,2p)/IEFPCM(MeOH) level.

Conformer		α_1 (deg) ^a	α_2 (deg) ^b	β (deg) ^c	d (Å) ^d	E_{QM} (Ha)	ΔE_{QM} (kcal mol ⁻¹)	χ_{QM}^e
2A	2A-e	-57.348	-57.348	-95.326	7.453	-1492.14731609	0.000	0.5934
	2A-b	66.788	66.788	101.136	7.806	-1492.14692907	0.243	0.3939
2B	2B-f	-56.583	-55.703	-92.428	7.308	-1492.14939496	0.000	0.6540
	2B-b	68.871	69.671	105.736	8.045	-1492.14875818	0.400	0.3332
2C	2C-e	62.947	57.866	94.503	7.444	-1492.14938642	0.000	0.5886
	2C-f	-60.999	-66.239	-101.552	7.806	-1492.14901068	0.236	0.3953
2D	2D-b	-68.275	-68.275	-104.848	8.007	-1492.15018572	0.000	0.9709

a: C-10 – C-5 – C-12 – C-5' dihedral angle. **b:** C-10' – C-5' – C-12 – C-5 dihedral angle. **c:** O-2 – C-5 – C-5' – O-2' dihedral angle. **d:** O-2 – O-2' distance. **e:** calculated using Boltzmann statistics at 298.15 K and 1 atm.

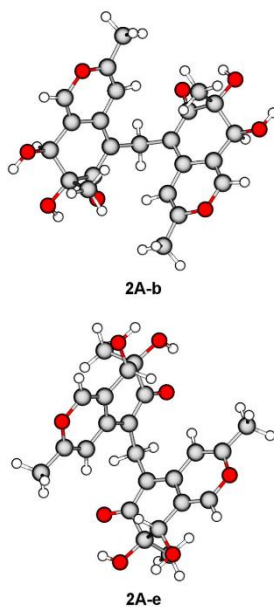


Figure 4. Most populated equilibrium conformers of **2A**.

The experimental ECD spectrum of **2** in methanol (Figure 5) shows four bands. The broad positive band centered at 372 nm has a strong contribution from the $n \rightarrow \pi^*$ transitions of the ketone carbonyls, which are affected by the chiral environment around these groups. The positive band at 311 nm with a shoulder around 285 nm, the sharp negative peak at 252 nm and the positive peak at 228 nm are due to $\pi \rightarrow \pi^*$ transitions of the conjugated system, which are affected by the chirality of the rings and by the mutual orientation of the azaphilone units.

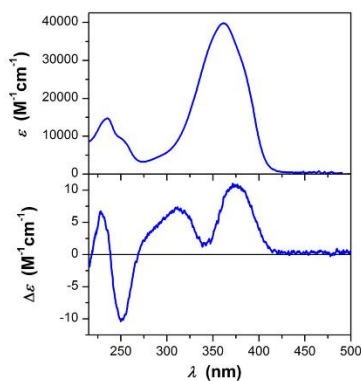


Figure 5. Experimental UV and ECD spectra of mycoleptone A (19 μM in MeOH, 1 cm pathlength).

The theoretical ECD spectra of the diastereomers of **2** (Figure 6) displayed different patterns, reflecting their different stereochemistry. The comparison between the calculated spectrum of **2A** and the experimental spectrum of **2** (Figure 7) shows that TD-DFT calculations are able to reproduce the experimental pattern of the transitions in the high-energy region, considering that transition energies are usually underestimated by PBE0.²¹ The transitions in the low-energy region are not reproduced, suggesting that QM calculations are unable to describe the chiral environment of the ketone moieties, in particular hydrogen bonding interactions with hydroxyl groups and solvent molecules, with adequate accuracy. On the other hand, the theoretical ECD spectra of the remaining diastereomers (reported in the Supporting Information) are unable to reproduce the experimental ECD pattern in the high-energy region: on this basis, a (7*R*,8*S*,7'*R*,8'*S*) absolute configuration can be predicted for **2**.

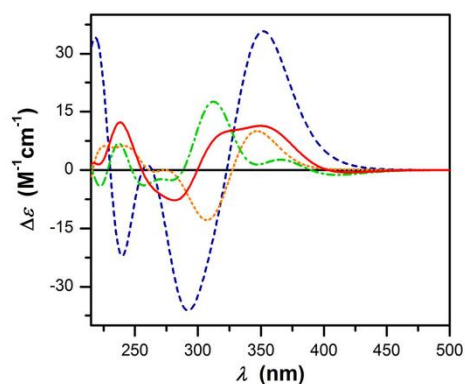


Figure 6. Theoretical ECD spectra of the diastereomers of mycoleptone A at the PBE0/6-311++G(2*d*,2*p*)/IEFPCM(MeOH)//B97D/6-311++G(2*d*,2*p*)/IEFPCM(MeOH) level (χ_{QM} -based conformational averaging, $\Delta\sigma = 0.3$ eV). *Solid*, **2A**. *Dash-dotted*, **2B**. *Dotted*, **2C**. *Dashed*, **2D**.

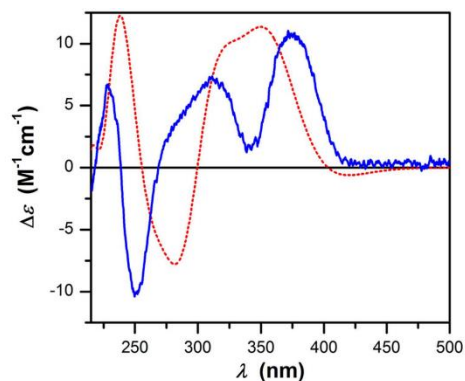


Figure 7. Theoretical ECD spectrum of **2A** and comparison with experimental data. *Solid*, experimental ECD spectrum of **2** (19 μ M in MeOH, 1 cm pathlength). *Dotted*, theoretical ECD spectrum of **2A** at the PBE0/6-311++G(2d,2p)/IEFPCM(MeOH)//B97D/6-311++G(2d,2p)/IEFPCM(MeOH) level (χ_{QM} -based conformational averaging, $\Delta\sigma = 0.3$ eV).

Compound **2** crystallizes with two independent molecules in the asymmetric unit (Figure 2); the X-ray geometric parameters selected for comparison with the precursor austdiol are listed in Table 3. The C-5 – C-10 distance was elongated upon change of hybridization to sp^3 for atom C-10 and the C-5 – C-4a distance was shortened, increasing the double bond character. The pyran rings are almost planar with RMSD from the mean plane of 0.03 Å, while the cyclohexenone rings adopt a twisted envelope conformation with atoms C-7 and C-7' as flap atoms. The X-ray structure of **2** shows that the azaphilone units have the same absolute configuration as austdiol,²² retaining the stereochemistry of the chiral centers as (7*R*,8*S*); moreover, DFT conformer **2A-b** shows a close resemblance to the crystallographic structure of **2**. On the basis of the foregoing evidence, the structure of mycoleptone A (**2**) was therefore elucidated as (7*R*,8*S*,7'*R*,8'*S*)-5-[(7',8'-dihydroxy-3',7'-dimethyl-6'-oxo-7',8'-dihydro-6*H*-isochromen-5'-yl)methyl]-7,8-dihydroxy-3,7-dimethyl-7,8-dihydro-6*H*-isochromen-6-one.

Table 3. Selected bond distances and angles for mycoleptone A and austdiol, as determined by X-ray crystallography.

Parameter	Mycoleptone A (2)	Austdiol (1)
C-1 – C-8a	1.337 Å (8)	1.338 Å (3)
C-3 – C-4a	1.341 Å (8)	1.345 Å (3)
C-5 – C-4a	1.373 Å (7)	1.399 Å (2)
C-5 – C-6	1.433 Å (8)	1.432 Å (3)
C-5 – C-10	1.533 Å (7)	1.455 Å (2)
C-4a – C-5 – C-6	118.6 deg (5)	124.3 deg (2)

The molecular formula of mycoleptone B (**4**) was determined as C₂₃H₂₄O₈ by a combination of HRMS, ¹H and ¹³C NMR, homonuclear correlation (COSY), heteronuclear multiple-quantum correlation (HMQC) and HMBC spectroscopies. The ¹H NMR spectrum contains five doublets, four singlets, two double doublets and one multiplet;

some similarities with the ^1H NMR spectrum of **2** are observed, with the presence of two hydrogen atoms on the pyran ring at δ_{H} 7.43 (1H, d, $J = 1.8$ Hz) and 6.63 (1H, s), an oxygenated sp^3 methine group at δ_{H} 4.42 (1H, d, $J = 1.8$ Hz), a CH_2 group at δ_{H} 3.60 (1H, d, $J_{\text{gem}} = 15.0$ Hz) and 3.53 (1H, d, $J_{\text{gem}} = 15.0$ Hz) due to the methylene bridge at position C-10 connecting the azaphilone unit to a dihydroisocoumarin moiety, and two methyl signals at δ_{H} 2.22 and 1.10. For the dihydroisocoumarin moiety, the ^1H NMR spectrum displays an oxygen-bearing methine hydrogen at δ_{H} 4.55 (1H, m), a methylene group at δ_{H} 3.17 (1H, dd, $J = 16.0, 3.0$ Hz) and 2.60 (1H, dd, $J = 16.0, 11.0$ Hz), and two methyl groups at δ_{H} 2.06 (3H, s) and 1.44 (3H, d, $J = 6.2$ Hz). The ^{13}C NMR spectrum of **4** displays signals for twenty-three carbon atoms: one signal due to the methylene bridge (δ_{C} 20.7), eleven signals belonging to the azaphilone moiety as in **2**, and eleven signals belonging to the dihydroisocoumarin moiety. The ^{13}C NMR data reveal six resonances for sp^2 carbon atoms being part of a benzene ring, two of them characterized by a downfield shift due to the hydroxyl substituents (C-6' at δ_{C} 160.2; C-8' at δ_{C} 160.9) and the presence of a carbonyl group at δ_{C} 171.2 (C-1'; IR ν_{max} 1652 cm^{-1}). The structural features of the lactone ring were determined from the $^1\text{H} - ^1\text{H}$ COSY spectrum based on the couplings between H₂-4' and H-3', as well as between H-3' and H₃-9'. The HMBC correlations of CH_2 -4' to δ_{C} 136.8 (C-5'), δ_{C} 100.1 (C-8a'), δ_{C} 75.0 (C-3') and δ_{C} 19.6 (C-9') proved the connection of the two units. In addition, the HMBC spectrum displays relevant correlations between CH_2 -10 (δ_{H} 3.60, 3.53) to δ_{C} 199.3 (C-6), 143.7 (C-4a), and 112.8 (C-5) from the azaphilone moiety, and to δ_{C} 160.9 (C-8'), 160.2 (C-6') and 116.5 (C-7') from the dihydroisocoumarin moiety, characterizing the connection between the two units by a methylene bridge to afford the compound (7*R*,8*S*)-5-[(6',8'-dihydroxy-3',5'-dimethyl-1'-oxoisochroman-7'-yl)methyl]-7,8-dihydroxy-3,7-dimethyl-7,8-dihydro-6*H*-isochromen-6-one.

The molecular formula of mycoleptone C (**5**) was deduced as $\text{C}_{23}\text{H}_{22}\text{O}_8$ by HRMS analysis in combination with ^1H and ^{13}C NMR spectroscopies. The ^1H NMR spectrum displays nine singlets and two doublets; as in compounds **2** and **4**, the methylene group at δ_{H} 3.74 (1H, d, $J = 15.0$ Hz) and 3.46 (1H, d, $J = 15.0$ Hz) connects two moieties, the azaphilone and chromone units. The azaphilone moiety shows five uncoupled hydrogen atoms at δ_{H} 7.52 (H-1), 7.03 (H-4), 4.25 (H-8), 2.25 (H-9) and 1.00 (H-10); the chromone moiety displays four uncoupled hydrogen atoms at δ_{H} 13.06 (H-5'), 6.13 (H-3'), 2.37 (H-9') and 2.00 (H-10'), the sharp singlet at δ_{H} 13.06 being interpreted as a hydrogen-bonded phenol hydroxyl group. The ^{13}C NMR spectrum confirms twenty-three carbon signals, namely four methyl, one methylene, four methine and fourteen quaternary carbon signals. The chromone moiety displays 181.8; IR ν_{max} 1690 cm^{-1}), four quaternary aromatic (δ_{C} 153.5, 107.8, 105.0 and 103.1), two oxygenated aromatic (δ_{C} 161.1 and 156.6), two unsaturated (δ_{C} 167.2 and 107.9), and two methyl (δ_{C} 20.2 and 8.5) carbon atoms. The structure of the chromone portion was determined on consideration of the molecular formula, double bond equivalents, and the downfield shifts of the carbon signals at C-2' and C-8a' (δ_{C} 167.2 and 153.5). The HMBC spectrum of **5** revealed relevant correlations of CH_2 -10 (δ_{H} 3.74, 3.46) to δ_{C} 200.5 (C-6), 144.3 (C-4a) and 112.1 (C-5) from the azaphilone moiety and to δ_{C} 161.1 (C-7'), 156.6 (C-5') and 107.8 (C-6') from the chromone moiety, revealing the connection of both skeletons and featuring **5** as (7*R*,8*S*)-5-[(5',7'-dihydroxy-2',8'-dimethyl-4'-oxo-4'*H*-chromen-6'-yl)methyl]-7,8-dihydroxy-3,7-dimethyl-7,8-dihydro-6*H*-isochromen-6-one.

The presence of a methylene bridge is very uncommon among natural compounds, and only few azaphilone dimers have been reported showing such a feature.¹⁵ Dicitrinones A–C²³ were isolated from *Penicillium citrinum*, a volcano ash-derived fungus: the presence of a single *sp*³ carbon bridge, either a methine or methylene group, was proposed to derive from the carboxyl group of citrinin through decarboxylation, reduction and Friedel–Crafts alkylation mediated by a polyketide biosynthetic pathway. The methylene bridge in aspergilone B,²⁴ an azaphilone dimer which was isolated from a marine-derived fungus of the *Aspergillus* species, was hypothesized to derive from a formaldehyde biosynthetic pathway; the same hypothesis was proposed for the biosynthesis of xyloketal F,²⁵ a dimer isolated from a mangrove fungus of the *Xylaria* species.

The condensation of two austdiol units to form mycoleptone A (**2**) is consistent with a possible biosynthetic pathway similar to the one proposed for dicitrinone C²³ (Figure 8). One austdiol unit may undergo decarbonylation, while the aldehyde group of the second austdiol unit may be reduced to a reactive carbocation species: the two units may then react by Friedel–Crafts alkylation. Similar pathways may also explain the condensation of the dihydroisocoumarin and chromone units with an austdiol unit in mycoleptones B (**4**) and C (**5**), respectively. Due to the limited number of methylene-bridged azaphilones discovered to date, however, a general biosynthetic pathway cannot be postulated, and the biologically-mediated mechanisms of such reactions are unknown.

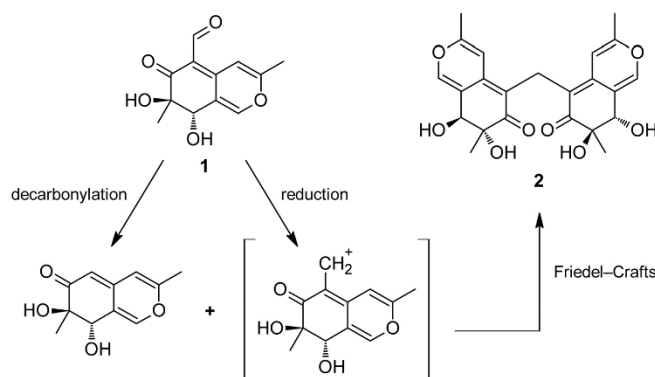


Figure 8. Possible pathway for the biosynthesis of mycoleptone A.

The results of anti-leishmanial and cytotoxicity tests on the isolated compounds are shown in Table 4. Compounds **1–9** exhibit no significant activity against *Leishmania donovani* and *Leishmania major* compared to the standard anti-leishmanial drugs geneticin (*L. major*), amphotericin B and pentamidine (*L. donovani*). Mycoleptone B (**4**) was the most cytotoxic metabolite (PC3 cells: IC₅₀ = 7.1 ± 3.8 μM), but its activity was lower than that of doxorubicin, the reference compound for cytotoxicity assays.

Table 4. Biological activities of compounds **1–9**.

Compound	<i>L. major</i> (LD ₅₀ , μM)	<i>L. donovani</i> (IC ₅₀ , μM)	Cytotoxicity (IC ₅₀ , μM)					
			HCT-8	HL-60	MDA-MB435	PC3	SF-295	Human lymphocyte
Austdiol (1)	20.5	80.5	12.90 ± 3.51	38.80 ± 9.32	34.30 ± 4.36	ND	28.00 ± 7.16	> 113
Mycoleptone A (2)	28.5	> 93.4	> 58	> 58	> 58	10.00 ± 6.54	> 58	> 58
Eugenitin (3)	39.9	> 181	> 113	> 113	> 113	> 113	> 113	> 113
Mycoleptone B (4)	21.7	ND	> 58	> 58	> 58	7.05 ± 3.78	> 58	> 58
Mycoleptone C (5)	ND	ND	ND	ND	ND	ND	ND	ND
6-Methoxyeugenin (6)	34.5	> 169	> 105	> 105	> 105	20.50 ± 11.9	> 105	> 105
9-Hydroxyeugenin (7)	ND	ND	> 105	> 105	> 105	> 105	> 105	> 105
Austdiol diacetate (8)	19.8	59.3	13.90 ± 2.56	22.10 ± 2.56	26.90 ± 0.31	ND	26.30 ± 1.18	> 78
Mycoleptone A tetraacetate (9)	ND	ND	> 42	> 42	> 42	33.30 ± 29.00	> 42	> 42
Geneticin (G418) ^a	3.43	-	-	-	-	-	-	-
Pentamidine ^b	-	0.0041	-	-	-	-	-	-
Amphotericin B ^b	-	0.140	-	-	-	-	-	-
Doxorubicin ^c	-	-	0.10 ± 0.01	0.05 ± 0.01	1.51 ± 0.20	0.41 ± 0.05	0.70 ± 0.20	0.70 ± 0.20

a: standard anti-leishmanial drug against *L. major*. **b:** standard anti-leishmanial drugs against *L. donovani*. **c:** reference compound for cytotoxicity assays. ND: not determined.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in CHCl_3 , MeOH or DMSO using a Jasco DIP-370 digital polarimeter at room temperature; IR spectra were recorded with KBr discs using a Bruker Tensor 27 FTIR spectrometer. UV spectra were measured in CHCl_3 , MeOH and DMSO on a Varian Cary 50 Bio UV-visible spectrophotometer. The HPLC system consisted of a Shimadzu SCL-10Avp multisolvent delivery system, a SPD-M10Avp photodiode array detector, an Intel Celeron computer for analytical system control, data collection and processing, and a Shim-pack CLC-ODS(M) reversed-phase column (250×4.5 mm I.D.; 5 μm particle size) protected by a PELLIGUARD™ LC-18 cartridge. The NMR spectra were acquired on a Bruker Avance DRX-400 spectrometer operating at 400 MHz for ^1H NMR, and 100 MHz for ^{13}C NMR in CDCl_3 , CD_3OD or $\text{DMSO}-d_6$; multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC and HMBC) were obtained using standard Bruker pulse programs. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. HRMS were obtained by direct injection using a Bruker Bioapex FTMS spectrometer with electro-spray ionization (ESI). UV and ECD spectra of **2** in MeOH (sample concentration: 19 μM) were recorded in the 500–215 nm spectral range on a Jasco J-810 spectropolarimeter, using a 1 cm pathlength cell at room temperature: measurements were carried out at 0.2 nm intervals using a 1 nm spectral bandwidth, a 20 nm min^{-1} scan rate and a 4 sec time constant.

Fungal material. The fungus was isolated as an endophyte from the medicinal plant *Borreria verticillata* (L.) G. F. W. Meyer, belonging to the Rubiaceae family, and is native to South America. Five specimens of *B. verticillata* with a healthy appearance were collected from the campus of the Federal University of Pernambuco (UFPE) ($34^\circ 56' 57''$ W; $8^\circ 2' 53''$ S) in January 2006 with approximately 43 cm in height and bearing flowers. Then, the samples were taken to the laboratory and processed in 24 hours. A voucher specimen of the plant (N° 42.234/UFPE) was identified and deposited at the Herbarium Professor Geraldo Mariz – UFP, Department of Botany, UFPE, Recife, Brazil. The isolate was identified as *Mycoleptodiscus indicus* based on sequence analysis of the ITS region of the rDNA (GenBank Accession number GU220382.1).

Cultivation, Extraction and Isolation. *M. indicus* was grown on potato dextrose agar (PDA) plates for 7 days at 30°C . Then, 10 plugs were transferred to five Erlenmeyer flasks (500 mL), each containing 100 mL of potato dextrose broth (PDB) prepared with distilled water. Flasks were shaken on rotary shaker at 30°C and 120 RPM for 48 hours. Next, 10 mL were transferred to each of 50 flasks containing 90 g of solid medium (rice-oat), which were grown for 30 days. On day 30, the mycelial mass was macerated with ethanol overnight and filtered. The filtrate was concentrated under vacuum to obtain a crude ethanol extract (22 g) that was partitioned with three times equal volumes of hexane and CH_2Cl_2 , respectively, yielding hexane (6 g) and dichloromethane fractions (6.9 g). The CH_2Cl_2 fraction was suspended in MeOH and centrifuged at 3000 RPM for 3 min furnishing a precipitate fraction (3870 mg) and a soluble fraction (3030 mg). The precipitate fraction was subjected to chromatography over a silica gel column (30×3.5 cm I.D.) using a MeOH- CH_2Cl_2 gradient to yield sixty-seven fractions, which were combined on the basis of their

TLC profiles into seven fractions: A (19 mg), B (33 mg), C (2968 mg), D (100 mg), E (210 mg), F (285 mg) and G (109 mg). Fraction C (2968 mg) yielded austdiol (2830 mg), after crystallization in MeOH, and fraction E afforded mycolectone A (**2**, 200 mg) by precipitation. The soluble fraction (3030 mg) was subjected to gel filtration on a Sephadex LH-20 column (50 × 3 cm I.D.) using MeOH as the mobile phase furnishing 10 fractions (S1–S10). Fraction S6 (280 mg) was washed with MeOH yielding eugenitin (50 mg). The soluble portion was subjected to chromatography over a silica gel column (25 × 2 cm I.D.) using a MeOH-CH₂Cl₂ gradient. Forty-two fractions were collected and combined on the basis of their TLC profiles to yield 9 fractions. The fourth fraction (S6.4, 28 mg) was purified by preparative silica gel TLC using hexane-ethyl acetate (70:30, v/v) as eluent to furnish mycolectone B (**4**, 10 mg) and mycolectone C (**5**, 2 mg). Fraction S5 (461 mg) was subjected to chromatography over silica gel column (25 × 2 cm I.D.) eluted with gradient of MeOH in CH₂Cl₂. Thirty fractions were collected and combined on the basis of their TLC profiles to yield 10 fractions. The fourth fraction (S5.4, 60 mg) was purified by preparative silica gel TLC using hexane-ethyl acetate (70:30, v/v) as eluent to provide 6-methoxyeugenin (14 mg). The fifth fraction (S5.5, 4 mg) was subjected to reversed-phase HPLC using CH₃CN-H₂O (10:90 to 100:0, v/v) as mobile phase at a flow rate of 1.0 mL/min over 23 minutes, yielding 9-hydroxyeugenin (2mg).

Acetylation of austdiol and mycolectone A (2). A solution containing austdiol (80 mg), pyridine (2 mL), and Ac₂O (3 mL) was stirred at room temperature for 8 hours. H₂O (6 mL) was added into the reaction mixture, which was subsequently extracted with CH₂Cl₂. The organic layer was evaporated to give a yellow amorphous solid (140 mg), which was purified by preparative TLC eluted with a mixture of CH₂Cl₂-MeOH (98:2, v/v), to afford the diacetylated derivative (**8**, 80.7 mg). Acetylation of mycolectone A (**2**, 25 mg) was performed in a similar manner, yielding the tetraacetylated derivative (**9**, 20 mg).

Quantum mechanical calculations. Conformational analysis on the selected diastereomers of **2** was performed in two steps. In the first step, a preliminary conformer distribution was determined by molecular mechanics (MM) calculations at the MMFF94s²⁶ level using the Spartan'02 software.²⁷ In the second step, DFT^{28,29} geometry optimizations were performed on the MM conformers having relative energies (ΔE_{MM}) within a threshold value of 3 kcal mol⁻¹. DFT calculations were carried out at the B97D/6-311++G(2d,2p) level^{30,31,32,33} using the Gaussian 09 software package.³⁴ The Boltzmann distribution of conformers at 298.15 K and 1 atm was then calculated from the relative electronic energies (ΔE_{QM}). TD-DFT³⁵ calculations were carried out at the PBE0/6-311++G(2d,2p) level^{31,32,33,36,37,38} using the Gaussian 09 software package.³⁴ All calculations were performed using the IEFPCM continuum solvation model^{39,40} for MeOH. Rotational strengths (R_j) and excitation wavelengths (λ_j) were calculated for the lowest 50 excited states on the optimized geometries having $\Delta E_{QM} \leq 2$ kcal mol⁻¹. Theoretical ECD spectra were then obtained by approximation of R_j values to Gaussian functions with a half-bandwidth at $\Delta \epsilon_{max}/e$ ($\Delta \sigma$) of 0.3 eV, summation over all excited states and conformational averaging, according to the Boltzmann distribution of conformers (χ_{QM}).⁴¹

Crystallographic Structure. Crystals of **2** suitable for X-ray analysis were obtained by crystallization from a

methanol solution. A colorless prism crystal of approximate dimensions of $0.3 \times 0.2 \times 0.2 \text{ mm}^3$ was used for the experiment. X-ray data were measured on an Enraf-Nonius CAD-4 diffractometer employing graphite-monochromated Cu-K α radiation ($\lambda = 1.5418 \text{ \AA}$) at 298 K and operating in the φ - ω scan mode. Crystal data: C₂₃H₂₄O₈, $M = 428.42$, triclinic, space group $P1$, $a = 10.473(6) \text{ \AA}$, $b = 11.223(3) \text{ \AA}$, $c = 11.429(2) \text{ \AA}$, $\alpha = 60.65(2)^\circ$, $\beta = 89.83(3)^\circ$, $\gamma = 67.75(4)^\circ$, $V = 1055.5(9) \text{ \AA}^3$, $Z = 2$, $D_c = 1.348 \text{ g/cm}^3$, $F(000) = 452$, and $\mu(\text{Cu K}\alpha) = 0.86 \text{ mm}^{-1}$. All tested crystal samples showed gemination, and the sample used for data collection was visually homogenous but it showed pseudo-merohedral twinning, which caused partial superposition of structure factors leading to high residuals during the structure refinements. A total of 8384 reflections were collected (4273 unique plus 96.2% of Friedel mates) in the θ range 4.56° to 73.92° and index ranges h from 15 to -15 , k from -24 to 25 and l from -15 to 15. Cell refinement and data reduction were performed with XCAD4 software suite. The structure was solved and refined using the WingX with SHELX97 suite.^{42,43} Structure refinement was performed on F^2 by full-matrix least-squares calculations. Non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were placed in idealized coordinates and refined as riding atoms with the relative isotropic parameters to their parent atoms. The final refinement residuals were $R1 = 0.091$ ($wR2 = 0.32$) for 6964 observed reflections with $I > 2\sigma(I)$, 575 variable parameters and three restraints, being $R1 = 0.11$ ($wR2 = 0.32$) for all unique reflections with $\text{GoF} = 1.41$. The absolute structure Flack parameter was 0.1(3), indicating that the chiral sense was correctly chosen, although this number was not reliable, given the large standard deviation, in the absence of strong anomalous scatterers.

Biological Assays. The microplate Alamar-Blue assay (MABA) was used to determine growth inhibition against *L. donovani*.⁴⁴ Anti-leishmanial (*L. major*) and cytotoxicity assays against human colon cancer (HCT-8) cells, human leukemia cancer (HL-60) cells, human melanoma cancer (MDA-MB435) cells, human prostate cancer (PC3) cells, human glioblastoma cancer (SF-95) cells and human lymphocyte cells were assessed employing a colorimetric method.⁴⁵

Austdiol (1): yellow crystal; mp 255–257 °C; $[\alpha]_D^{25} +223$ (c 1.0, DMSO); UV (MeOH) λ_{max} ($\log \epsilon$) 212 (2.64), 255 (2.57), 333 (2.62) nm; IR (KBr) ν_{max} 3470, 3373, 3100, 1679, 1604, 1471 cm^{-1} ; ¹H NMR and ¹³C NMR reported in the Supporting Information; HRMS m/z 237.0759 $[\text{M}+\text{H}]^+$ (calcd for C₁₂H₁₂O₅ + H⁺, 237.0757).

Mycoleptone A (2): yellow crystal; mp 307–308 °C; $[\alpha]_D^{25} +351$ (c 1.0, DMSO); UV (MeOH) λ_{max} ($\log \epsilon$) 254 (2.90), 361 (2.83) nm; IR (KBr) ν_{max} 3360, 1667, 1581, 1526, 1229, 1197, 1090, 878 cm^{-1} ; ¹H and ¹³C NMR reported in Table 1; HRMS m/z 429.1543 $[\text{M}+\text{H}]^+$ (calcd for C₂₃H₂₄O₈ + H⁺, 429.1544).

Eugenitin (3): white solid powder; mp 153–156 °C; $[\alpha]_D^{25} 0$ (c 0.3, CHCl₃); UV (MeOH) λ_{max} ($\log \epsilon$) 267 (2.53), 307 (2.55), 338 (2.54) nm; IR (KBr) ν_{max} 1652, 1586, 1435, 1338, 1179 cm^{-1} ; ¹H NMR and ¹³C NMR reported in the Supporting Information; HRMS m/z 221.0810 $[\text{M}+\text{H}]^+$ (calcd for C₁₂H₁₂O₄ + H⁺, 221.0808).

Mycoleptone B (4): colorless amorphous solid; $[\alpha]_D^{25} +71$ (c 0.2, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 237 (2.83),

299 (2.83), 317 (2.90) nm; IR (KBr) ν_{\max} 3391, 2925, 1652, 1615, 1289, 1127 cm^{-1} ; ^1H and ^{13}C NMR reported in Table 1; HRMS m/z 451.1363 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{23}\text{H}_{24}\text{O}_8 + \text{Na}^+$, 451.1363).

Mycolectone C (5): brown amorphous solid; $[\alpha]_D^{25} +95$ (c 0.2, DMSO); UV (MeOH) λ_{\max} ($\log \epsilon$) 224 (3.17), 297 (3.15), 321 (3.17) nm; IR (KBr) ν_{\max} 2920, 2360, 1690, 1573, 1478 cm^{-1} ; ^1H and ^{13}C NMR reported in Table 1; HRMS m/z 427.1387 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{23}\text{H}_{22}\text{O}_8 + \text{H}^+$, 427.1387).

6-Methoxyeugenin (6): white solid powder; mp 155–158 $^{\circ}\text{C}$; $[\alpha]_D^{25}$ 0 (c 0.3, CHCl_3); UV (MeOH) λ_{\max} ($\log \epsilon$) 292 (2.85), 307 (2.89), 334 (2.88) nm; IR (KBr) ν_{\max} 3404, 2923, 1657, 1617, 1570, 1492, 1169 cm^{-1} ; ^1H NMR and ^{13}C NMR reported in the Supporting Information; HRMS m/z 259.0577 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{12}\text{H}_{12}\text{O}_5 + \text{Na}^+$, 259.0577).

9-Hydroxyeugenin (7): white amorphous solid; $[\alpha]_D^{25}$ 0 (c 0.3, CHCl_3); UV (MeOH) λ 279 (2.87), 319 (2.91), 335 (2.92) nm; IR (KBr) ν_{\max} 3217, 2360, 1662, 1573, 1497, 1330, 1138 cm^{-1} ; ^1H NMR and ^{13}C NMR reported in the Supporting Information; HRMS m/z 237.0711 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{12}\text{H}_{12}\text{O}_5 + \text{H}^+$, 237.0757).

Austdiol diacetate (8): yellow crystal; mp 255–257 $^{\circ}\text{C}$; $[\alpha]_D^{25} +39$ (c 1.0, CDCl_3); UV (CHCl_3) λ_{\max} ($\log \epsilon$) 262 (2.99), 281 (2.98), 362 (3.01) nm; IR (KBr) ν_{\max} 2946, 1746, 1628, 1500, 1241 cm^{-1} ; ^1H NMR and ^{13}C NMR reported in the Supporting Information; HRMS m/z 321.0970 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{16}\text{H}_{16}\text{O}_7 + \text{H}^+$, 321.0969).

Mycolectone A tetraacetate (9): white crystal; mp 260–263 $^{\circ}\text{C}$; $[\alpha]_D^{25} +159$ (c 1.0, CDCl_3); UV (MeOH) λ_{\max} ($\log \epsilon$) 285 (3.04), 328 (2.98), 343 (3.03) nm; IR (KBr) ν_{\max} 3470, 3373, 3100, 1679, 1604 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 6.95 (1H, *d*, H-1), 6.71 (1H, *d*, H-8), 6.27 (1H, *s*, H-4), 3.48 (2H, *s*, H-10), 2.20 (3H, *s*, 7- COOCH_3), 2.18 (3H, *s*, H-9), 2.04 (3H, *s*, 8- COOCH_3), 1.32 (3H, *s*, H-11); ^{13}C NMR (CDCl_3 , 100 MHz) δ 190.5 (C, CO), 169.7 (C, 7- CO_2Me), 169.5 (C, 8- CO_2Me), 160.0 (C, C-3), 142.6 (CH, C-1), 142.3 (C, C-4a), 117.3 (C, C-8a), 114.6 (C, C-5), 106.1 (CH, C-4), 82.5 (C, C-7), 69.1 (CH, C-8), 19.5 (CH_3 , C-9), 20.8 (CH_3 , 7- CO_2CH_3), 21.4 (CH_3 , 8- CO_2CH_3), 17.2 (CH_2 , C-10); HRMS m/z 597.1966 (calcd for $\text{C}_{31}\text{H}_{32}\text{O}_{12} + \text{H}^+$, 597.1967).

ASSOCIATED CONTENT

NMR and HRMS spectra; results for the computational study on mycolectone A. This material is available free of charge via the Internet at <http://pubs.acs.org>. Crystallographic data, excluding structure factors, have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 938460. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: C44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation (FAPESP) grants # 04/07935-6 and 07/58650-0, CAPES, CNPq, the PRIN 2008 project 2008LYSEBR_005 (MIUR, Italy) and the University of Bologna for financial support.

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