J. Braz. Chem. Soc., Vol. 21, No. 8, 1446-1450, 2010.
Printed in Brazil - ©2010 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Two Novel Eremophilane Sesquiterpenes from an Endophytic Xylariaceous Fungus Isolated from Leaves of *Cupressus lusitanica*

Luciana S. Amaral and Edson Rodrigues-Filho*

Departamento de Química, Universidade Federal de São Carlos, CP 676, 13565-905 São Carlos-SP, Brazil

Dois novos sesquiterpenos eremofilanos, cupressolideo A e cupressolideo B, além de dois outros conhecidos, foram isolados a partir do extrato AcOEt do meio de cultura de uma espécie de *Xylaria*, isolada como fungo endofítico dos tecidos sadios das folhas de *Cupressus lusitanica*. Estudos espectroscópicos, usando EM e RMN, levaram às estruturas dos dois sesquiterpenos de esqueleto eremofilanos, novos na literatura.

Two new eremophilane sesquiterpenes, cupressolide A and cupressolide B, along with two known sesquiterpenes, has been characterized from the EtOAc extract of a liquid medium where a Xylariaceous fungus, isolated as an endophytic fungus from health tissues of *Cupressus lusitanica* leaves, was cultivated. The structures of the isolated compounds were determined by analyses of their MS and NMR spectroscopic data.

Keywords: *Cupressus lusitanica*, *Xylaria*, eremophilane sesquiterpenes, endophytic fungi

Introduction

Cupressus lusitanica, commonly known as a Mexican Cypress and Portuguese Cypress, belongs to the family Cupressaceae and is usually cultivated as an ornamental tree and in commercial forestry plantation.[1,2] Due to its economic importance, this plant was included in our continuous program established to study the chemistry and biochemistry aspects of plant microorganisms interactions, with emphasis on those apparently symbiotic associations.

Among the endophytic fungi isolated from healthy tissues of *C. lusitanica* leaves, we obtained some strains with macro and micro morphology characteristics of those microorganisms belonging to the genus *Xylaria*. Besides these morphologic characteristics, *Xylaria* species are producers of secondary metabolites, including isocoumarin,[3,4] cytochalasins,[4-6] xanthones,[7] xyloketals,[8-10] sesquiterpenes,[11] that contribute to their classification in this genus. In our study we detected isocoumarins and cytochalasins in the fungus extracts using mass spectrometry, which reinforce the hypothesis of its classification as a Xylareaceous fungus. Among the compounds isolated from the fungus extract, two novel (**1** and **2**) and two known (**3** and **4**) eremophilane sesquiterpenes were identified. The production of these terpenoids corroborated to classify the fungus within Xylariaceae family. Although it is not clear the importance of these compounds as phytotoxic agents, some members of this class of substances have shown remarkable biological activities, such as anti-inflammatory, antihyperglycemic, cytotoxic, HIV-1 integrase inhibitory.[12-15]

*e-mail: edson@dq.ufscar.br

Results and Discussion

The EtOAc extract of liquid medium from endophytic fungi was chromatographed on silica gel columns to give four compounds (**1**-**4**). Compounds **3** and **4** were previously reported in the literature.[16,17]

Compound **1** was obtained as a colorless crystal. The IR spectrum displayed a broad band at 3487 cm^{-1} characteristic of a hydroxyl group and a band at 1745 cm^{-1} attributed to a conjugated γ-lactone. The ^{13}C NMR spectrum exhibited 15 signals which were assigned, by DEPT 135 and HSQC experiments to three methyls, three methylenes, four methines and three sp^2 carbons, one of this being a carbonyl group. Its ESI-MS spectrum contains an ion peak of $[M+H]^+$ at *m/z* 265, consistent with the molecular formula $C_{15}H_{20}O_4$ which also was in accordance with the NMR data. The ^1H NMR spectrum of **1** showed three signals δ_H 1.85, 0.83 and 0.96 attributed to CH_3-13,

CH_3-14 and CH_3-15, respectively. ^{1}H NMR spectrum exhibited the presence of a deshielded signal assignable to an oxymethine hydrogen at δ_H 3.48 (1H, m, H-3); this signal correlated in COSY spectrum with the δ_H 1.89 (1H, m, H-2b); 2.44 (1H, ddd, *J* 15.2; 8.4; 4.4 Hz, H-2a); 1.81 (1H, m, H-4). H-4 showed coupling with the methyl group at δ_H 0.96 (3H, *J* 6.8 Hz, CH_3-15). The presence of an epoxy group was confirmed by the chemical shifts of H-1 (δ 3.06), C-1(δ 58.5), and C-10 (δ 63.2).

The COSY spectrum exhibited correlation peaks among the oxymethine hydrogen at δ_H 4.9 (1H, m, H-8) with hydrogens at δ_H 2.05 (1H, m, H-9a); 1.82 (1H, m, H-9b); 1.85 (3H, t, *J* 1.2 Hz, CH_3-13).

HSQC analysis indicated the presence of one tetrasubstituted double bond which was associated with the carbons at δ_C 159.5; 122.3 (C-7 and C-11, respectively). On the other hand another double bond was observed in the ^{13}C NMR spectrum, with characteristic chemical shifts of a carbonyl group at δ_C 174.6 attributed to C-12.

The HSQC spectrum exhibited correlations among the diasterotopic hydrogens at δ_H 2.23 (1H, br d, *J* 13.6 Hz, H-6b) and δ_H 2.76 (1H, d, *J* 13.6 Hz, H-6b) with the carbon at δ_C 35.1. The HMBC spectrum showed coupling of H-6b with C-5, C-7, C-8, C-10, C-11 and CH_3-14. The COSY and HMBC analysis of **1** led to a partial structure as shown in Figure 1.

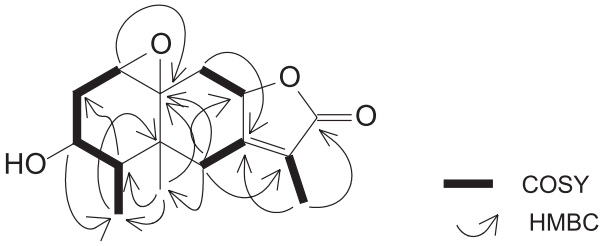

Figure 1. Selected H-H COSY and HMBC correlations for compound **1**.

The relative stereochemistry of **1** was elucidated using nOe and COSY spectroscopy. The β orientation of H-3 was inferred from the nOe correlation with CH_3-14 and CH_3-15. H-8 (δ 4.90) should be α oriented as indicated by the homoallylic coupling with the methyl (CH_3-13, δ 1.85) bounded to C-11 observed in the COSY experiment. This homoallylic coupling requires a 90º dihedral angle of the methyl group at C-11 with H-6α and H-8, witch was in agreement with the nOe experiment. Furthermore, the nOe spectrum showed correlations of H-6α with H-4 (δ 1.81) and of H-6β with CH_3-13, CH_3-14 and CH_3-15. All NMR data are shown in the Table 1.

Compound **2** showed spectroscopic data very similar to those of **1**, indicating the presence of an eremophilane skeleton. The IR spectrum displayed a broad band at 3508 cm^{-1} characteristic of a hydroxyl group. The

Table 1. NMR spectroscopy data for eremophilane sesquiterpene **1**[a]

Position	δ_H, mult, (*J* in Hz)	δ_C	COSY	HMBC (H→C)
1	3.06, d (4.4)	58.5	H2a	C2, C10
2a	2.44, ddd (4.4; 8.4; 15.2)	33.0	H1, H2b, H3	C3, C14
2b	1.89, m		H2a, H3	C1, C10, C3
3	3.48, m	67.4	H2a, H2b, H4	C4, C15
4	1.81, m	40.7	H15, H3	C2, C3
5	-	39.1	-	-
6a	2.23, br d (13.6)	35.1	H6-b, H13, H14	C5, C7, C8, C10, C11, C14
6b	2.76, d (13.6)		H6a	C5, C7, C8, C10, C11, C14
7	-	159.5	-	n.d
8	4.90, m	78.3	H9a, H9b, H13	n.d.
9a	2.05, m	38.0	H9b, H8	C7, C8, C13
9b	1.82, m		H9a, H8	C3, C5,C14, C15
10	-	63.2	-	-
11	-	122.3	-	-
12	-	174.6	-	-
13	1.85, t (1.2)	8.1	H6b, H8, H15	C7, C11, C12, C15
14	0.83, d (1.0)	15.8	H6b	C4, C5, C6, C10, C15
15	0.96, d (6.8)	10.1	H4	C3, C4, C5

[a]The data were acquired at 400 and 100 MHz for ^{1}H and ^{13}C respectively in $CDCl_3$. TMS was used as internal reference.

^{1}H NMR experiment exhibited two olefinic hydrogens, three carbinolic hydrogens and two methyl groups. The EI-MS spectrum of **2** contains an ion peak of M^+ at *m/z* 252, consistent with the molecular formula $C_{15}H_{24}O_3$ and the data observed in the NMR spectrum. The ^{1}H NMR spectrum of compound **2** exhibited deshielded signals due the presence of olefinic hydrogens at δ_H 5.26 (1H, d, *J* 0.8 Hz, H-12a) and δ_H 5.12 (1H, d, *J* 0.8 Hz H-12b). The COSY spectrum showed the coupling of H-12a with the carbinolic hydrogens at δ_H 4.19 (1H, dd, *J* 11.2; 0.8 Hz, H-13a) and δ_H 4.16 (1H, dd, *J* 11.2; 0.8 Hz, H-13b). The peak attributed to H-8 at δ_H 3.88 (1H, ddd, *J* 4.4; 10.0; 15.6 Hz) exhibited correlations in the COSY spectrum with the hydrogens at δ_H 2.43 (1H, ddd, *J* 4.4; 8.0; 13.2 Hz, H-7), δ_H 2.18 (1H, dd, *J* 10.0; 12.4 H-9a) and δ_H 1.38 (1H, m, H-9b).

The presence of a methine group at δ_H 1.71 (1H, m, H-4) was confirmed by COSY correlations at δ_H 1.21 (1H, m, H-3a) and δ_H 1.71 (1H, m, H-3b) and δ_H 0.71 (3H, d, *J* 5.6 Hz, CH_3-15). The signal of CH_2-13 (δ 4.16 and 4.19), CH_3-14 (δ 1.02), CH_3-15 (δ 0.71) and CH_2-12 (δ 5.12 and 5.26) in the ^{1}H NMR spectrum were in agreement with the profile of an eremophilane with a double bound at C-11 and C-12. The epoxide group at C-1 and C-10 was deduced from the chemical shifts of H-1 (δ 2.97), C-1 (δ 59.7), and C-10 (δ 65.8). The COSY analysis of **2** led to a partial structure as shown by bold-faced lines in Figure 2, which were supported by HMBC correlations (Table 2). The relative stereochemistry was based on those determined to compound **2**. All NMR data can be observed in Table 2.

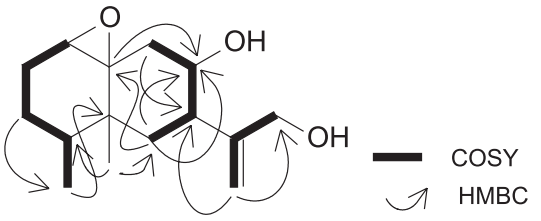

Figure 2. Selected H-H COSY and HMBC correlations for compound **2**.

Conclusions

The genus *Xylaria* is known for being a rich source of structurally diverse natural products including isocoumarin,[3,4] cytochalsins,[4-6] xyloketals,[8-10] sesquiterpenes[11] and others. Among these compounds eremophilane sesquiterpenes stand out for several biological activities.[12-15] One member of this genus reported in the present study showed the notable ability to produce eremophilane sesquiterpenes, including two new in the literature, cupressolide A and cupressolide B. Due to the many biological activities shown

Table 2. NMR spectroscopy data for eremophilane sesquiterpene **2**[a]

Position	δ_H, mult, (*J* in Hz)	δ_C	COSY	HMBC(H→C)
1	2.97, d (6.8)	59.7	H2a/b	n.d.
2a/2b	1.91, m	22.1	H1, H3a/b	n.d.
3a/3b	1.21, m	24.3	H2a/b, H4	C15
4	1.71, m	33.1	H3a/b, H15	n.d.
5	-	35.8	-	C4, C15
6a	1.68, m	39.6	H6b, H7	C7, C8
6b	1.41, m		H6a, H7, H8	C7, C8, C10
7	2.43, ddd, (4.4; 8.0; 13.2)	45.4	H6a, H6b, H8,	n.d.
8	3.88, ddd (4.4; 10.0; 15.6)	71.6	H6b, H7, H9a/b	n.d.
9a	2.18, dd (10.0; 12.4)	38.5	H8, H9b	C8
9b	1.38 (m, 1H)		H9a	C7, C8, C10
10	-	65.8	-	-
11	-	150.1	-	-
12a	5.26, d (0.8)	113.2	12b, H13a, H13b	C7, C13
12b	5.12, d (0.8)		H12a	C7, C13
13a	4.19, dd (11.2; 0.8)	65.8	H12a	C7, C11, C12
13b	4.16, dd (11.2; 0.8)		H12a	C7, C11, C12
14	1.02, s	15.9	n.d.	C4, C5, C6, 10
15	0.71, d (5.6)	14.8	H4	C3, C4, C5

[a]The data were acquired at 400 and 100 MHz for ^{1}H and ^{13}C respectively in $CDCl_3$. TMS was used as internal reference.

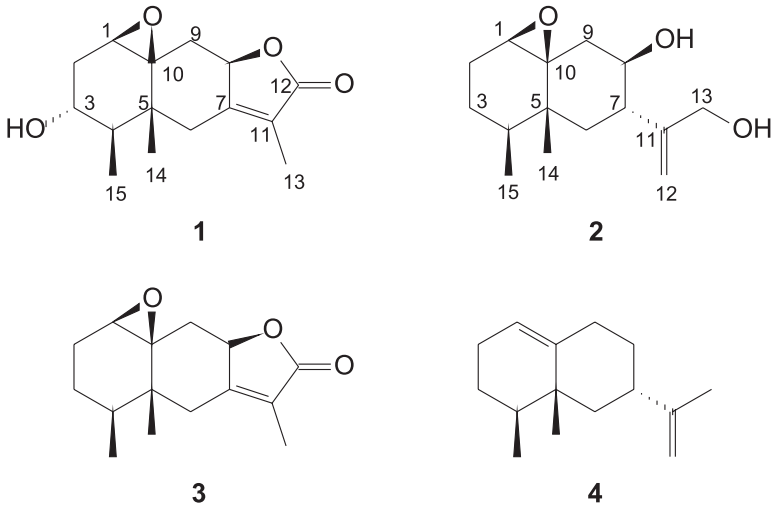

Figure 3. Eremophilane sesquiterpenes produced by *Xylaria* sp., an endophytic fungus isolated from *Cupressus lusitanica* leaves.

by this class of compounds, this *Xylaria* deserves a careful study aiming to sesquiterpene production enhancement.

Experimental

Equipment

IR spectra were run on a Bomen MB102 -IR spectrometer using KBr pellets. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. GC/MS analyses were performed using GC 8000 series Fisons and VG Platform mass spectrometer detector. 1D and 2D NMR spectra were obtained in $CDCl_3$ (Aldrich) on DRX 400 Bruker spectrometer operating at 400 MHz for hydrogen and 100 MHz for ^{13}C and TMS was used as internal standard. MS were acquired in positive ion mode on a triple quadrupole Micromass Quattro LC spectrometer, equipped with an ESI ion source.

Plant material

Health leaves of *Cupressus lusitanica* were collected in São Carlos, São Paulo State, Brazil. A voucher specimen (No. 7281) has been deposited in the Herbarium of the Botanic Department of Universidade Federal de São Carlos, Brazil.

Fungal material

The method of surface sterilization employed in this work was similar to that used by Petrini *et al*.[18] After the collection, the leaves were washed in abundant water (domestic use grade) and then in distilled water. The leaves were surface sterilization by consecutive immersion in 70% ethanol (2 s), sterile distilled water (2 s), 11% aqueous sodium hypochloride for 1-5 min and 70% ethanol (2 s), and then in sterile distilled water. The material was placed in Petri dishes containing PDA medium (potato, dextrose and agar) supplemented with 100 μg mL^{-1} terramicin and incubated at room temperature. Endophytic fungi growing from the plant tissues, were picked and recultured on PDA to determine culture purity. It was deposited at LaBioMMi – Laboratório de Bioquímica Micromolecular de Microorganismos – of the Departamento de Química at Universidade Federal de São Carlos, Brazil. Working stocks were prepared on potato dextrose agar.

Fermentation and extraction

The fungus was grown under static conditions at room temperature for 20 days in 20 Erlenmeyer flasks containing the liquid medium (300 mL *per* flask) composed of glucose (26.7 g L^{-1}), yeast extract (10.0 g L^{-1}), $NaNO_3$ (3.0 g L^{-1}), K_2HPO_4 (1.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KCl (0.5 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}). The mycelium was separated by reduced pressure filtration and the liquid phase was partitioned with ethyl acetate (1,000 mL × 3). The organic solvent was dried with anydrous sodium sulfate, filtered and removed using vaccum to give the crude extract. Crude extract was analyzed by GC/MS. This technique enabled the detection of the eremophilane sesquiterpene valencene (**4**). The extract was chromatographed on silica gel columm (h = 4.5 cm and ∅ = 3.7 cm) eluted with a Hex:CH_2Cl_2 (1:1); CH_2Cl_2:EtOAc (1:1); CH_2Cl_2:EtOAc (1:4); CH_2Cl_2:EtOAc:MeOH (1:4:10%); EtOAc:MeOH (1:1); MeOH (100%) to afford six fractions (A-F). The fractions C and D were rechromatographed on silica gel columm (h = 16 cm and ∅ = 2.5 cm) with Hexane 100% gradient to EtOAc:MeOH (1:1) to give **1** (5.2 mg), **2** (4.8 mg), **3** (2.7 mg).

GC/MS analysis

The extract was submitted to clean-up procedures using solid phase extraction (SPE). The SPE cartridge was activated with 100% Hexane and conditioned with 3 mL of $CHCl_3$. The extract (10 mg) was dissolved in 3 mL of $CHCl_3$ and loaded to the SPE cartridge. Elution of SPE cartridge with $CHCl_3$ produced an apolar fraction. For the GC/MS analysis the injector temperature was kept at 180 ºC and the GC oven temperature was maintained at 70 ºC during 6 min and then increased to 250 ºC at a rate of 6 ºC min^{-1} and finally increased to 325 ºC at 3 ºC min^{-1}. The sample volume injected was 2 μL.

MS data collection

ESI-MS data were colleted from direct introduction of the sample solution 5 μL of compound **1** (5 μg mL^{-1}). The optimal voltages found for the probe and ion source components to produce maximum intensity of the ions $[M+H]^+$ were 3.3 kV for the capillary, 19 V for the sample cone, and 4 V for the extractor cone.

Physical and spectral data

*(1αR,3R,4R,4αR,8αR,9αS)-3-Hydroxy-4,4α,6-trimethyl-1α,2,3,4,4αR,8α,9-octahydro-7H-oxirene[8,8α] naphtho[2,3β] furan-7-one, compound **1**: Cupressolide A*

Colorless crystals (CH_2Cl_2); $[\alpha]_D^{25} = -6.262$ (*c* 0.0001, $CHCl_3$); IR(KBr) ν_{max}/cm^{-1}: 3487, 1745; ESI-MS *m/z* 265 $[M+H]^+$; ESI-MS-MS (20 eV) *m/z* 265 (78%), 247 (17), 229 (39), 201 (50), 183 (100), 173 (70), 147 (60), 119 (55); NMR data see Table 1.

*(1αR,4S,4αR,6S,7R,8αS)-6-[1-(Hydroxymethyl)vinyl]-4,4α-dimethyloctahydro-1αH-naphtho[1, 8αβ]oxiren-7-ol, compound **2**: Cupressolide B*

Colorless crystal (CH_2Cl_2); IR(KBr) ν_{max}/cm^{-1}: 3508; EIMS (70 eV) *m/z* 252 $[M]^+$ (3%), 234 (6), 216 (12), 201 (16), 168 (57), 153 (75), 125 (100); NMR data see Table 2.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

Acknowledgments

The autors are gratefull to the Brazilian institutions FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo, CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico, CAPES – Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior for the financial support.

References

1. Farjon, A.; *Taxon* **1993**, *42*, 81.
2. Graniti, A.; *Annu. Rev. Phytopathol.* **1998**, *36*, 91.
3. Tansuwan, S.; Pornpakakul, S.; Roengsumran, S.; Petsom, A.; Muangsin, N.; Sihanonta, P.; Chaichit N.; *J. Nat. Prod.* **2007**, *10*, 1620.
4. Pongcharoen, W.; Rukachaisrikul, V.; Isaka, M.; Sriklung, K.; *Chem. Pharm. Bull.* **2007**, *55*, 1647.
5. Espada, A.; Rivera-Sagredo, A.; de la Fuente, J. M.; Hueso-Rodríguez, J. A.; Élson, S. W.; *Tetrahedron* **1997**, *53*, 6485.
6. Abate, D.; Abraham, W.; Meyer, H.; *Phytochemistry* **1997**, *44*, 1443.
7. Healy, P. C.; Hocking, A.; Tran-Dinh, N.; Pitt, J. I.; Shivas, R. G.; Mitchell, J. K.; Kotiw, M.; Davis, R. A.; *Phytochemistry* **2004**, *65*, 2373.
8. Lin, Y.; Wu,X.; Feng, S.; Jiang, G.; Luo, J.; Zhou, S.; Vrijmoed, L. L. P.; Jones, E. B. G.; Krohn, K.; Steingröver, K.; Zsila, F.; *J. Org. Chem.* **2001**, *66*, 6252.
9. Xiaobo, Z.; Haiying, W.; Linyu, H.; Yongcheng, L.; Zhongtao, L.; *Process Biochem.* **2006**, *41*, 293.
10. Liu, X.; Xu, F.; Zhang, Y.; Liu, L.; Huang, H.; Cai, X.; Lin, Y.; Chanb W.; *Russ. Chem. Bull.* **2006**, *55*, 1091.
11. McDonald, L. A.; Barbieri, L. R.; Bernan, V. S.; Janso, J.; Lassota, P.; Carter G. T.; *J. Nat. Prod.* **2004**, 67, 1565.
12. Wang, W.; Gao, K.; Jia, Z.; *J. Nat. Prod.* **2002**, *65*, 714.
13. Garduño-Ramírez, M. L.; Trejo, A.; Navarro, V.; Bye, R.; Linares, E.; Delgado, G.; *J. Nat. Prod.* **2001**, *64*, 432.
14. Li, E.-W.; Pan, J.; Gao, K.; Jia, Z.-J.; *Planta Med.* **2005**, *71*, 1140.
15. Zhang, Q. J.; Dou, H.; Zheng, Q. X.; Zhou, C. H.; Xu, Z. J.; Peng, H.; Zhao, Y.; C*hin. Chem. Lett.* **2005**, *16*, 360.
16. Zhao Y.; Schenk, D. J.; Takahashi, S.; Chappell, J.; Coates, R. M.; *J. Org. Chem.* **2004**, *69*, 7428.
17. Prez-Castorena, A. L.; Arciniegas, A.; Guzmn, S. L.; Villaseor, J. L.; de Vivar, A. R.; *J. Nat. Prod.* **2006**, *69,* 1471.
18. Petrini, O.; Sieber, T. N.; Toti, L.; Viret, O.; *Natural Toxins* **1992**, *1*, 185

Received: June 25, 2009
Web Release Date: March 25, 2010

FAPESP has sponsored the publication of this article.