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Article

A New Eremophilane-type Sesquiterpene from the Phytopatogen Fungus *Lasiodiplodia theobromae* (Sphaeropsidaceae)

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O fungo fitopatogênico *Lasiodiplodia theobromae*, isolado de goiaba, foi cultivado em arroz por 32 dias à temperatura ambiente. Extração com CH_2Cl_2:MeOH (3:7), seguido de fracionamento cromatográfico do extrato forneceu o esteróide ergosterol. Da cultura fúngica em meio de Czapeck por 40 dias à temperatura ambiente, foram isolados a isocumarina *cis*-4-hidroximeleína e um sesquiterpeno do tipo eremofilano. O sesquiterpeno eremofilano está sendo descrito pela primeira vez na literatura. Este é o primeiro relato do isolamento de um sesquiterpeno eremofilano para o gênero *Lasiodiplodia*.

The phytopatogenic fungus *Lasiodiplodia theobromae*, isolated from guava, was cultivated in rice for 32 days at room temperature. Extraction with CH_2Cl_2:MeOH (3:7), followed by chromatography fractionation of the extract provided ergosterol. From the fungus culture in Czapeck medium for 40 days at room temperature, were isolated isocoumarin *cis*-4-hydroxymeleine and an eremophilane-type sesquiterpene. The latter compound is being reported for the first time in the literature. Also, this is the first time that an eremophilane sesquiterpene is described for *Lasiodiplodia* genus.

Keywords: fungus, *Lasiodiplodia theobromae*, ergosterol, isocoumarin, eremophilane-type sesquiterpene

Introduction

Microorganisms represent a promising source of biologically active compounds; despite this, only a small portion of the microbial diversity has been chemically investigated. Because of the short life cycle and easy adaptability to external media, fungi can be manipulated for the production of secondary metabolites of biological interest.[1] About 1500 secondary metabolites from fungi were reported in the literature from 1993 to 2001, and more than 50% of these compounds showed antibacterial, antifungal and antitumoral activities.[2] Chemical investigation of phytopatogen fungi, especially those associated with serious agricultural problems, was recently begun.

Lasiodiplodia theobromae (Patouillard) Griffon & Maublanc (Sphaeropsidaceae) is a phytopathogen fungus found in more than 280 different genera of host plants from tropical and subtropical regions of the world.[3] In Brazil, this fungus is considered a serious problem to agriculture since it is associated with several diseases of tropical fruits.[4] *L. theobromae* is the anamorphous form (asexual state) of *Botryosphaeria rhodina* (Berkeley & Curtis) von Arx and, as mitosporic fungus, it belongs to Dothideomycetes class. Although the fungus is also reported as *Botryodiplodia*

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theobromae in the literature, this synonym is falling into disuse.[5] The chemical investigation of strains of this fungus is reported in the literature.[6-14] Jasmonic acid and thirteen derivatives,[6-10] eight hydroxylasiodiplodins,[10-12] two cyclohexene derivatives[13, 14] and two isocoumarins[8, 10] were isolated from *L. theobromae*.

This work reports the isolation of a new eremophilane-type sesquiterpene (**3),** in addition to the known compounds ergosterol (**1**) and isocoumarin *cis*-4-hydroxymelein (**2**). The presence of an eremophilane-type sesquiterpene in *L. theobromae* is being reported for the first time in the literature. The structural elucidation of these compounds was established on the basis of 1D and 2D NMR spectroscopic techniques.

Results and Discussion

Successive chromatography procedures of the CH_2Cl_2:MeOH (3:7) extract of *L. theobromae* cultivated in rice provided ergosterol (**1**). When cultivated in Czapeck broth, this fungus provided isocoumarin 4-hydroxymelein (**2**) and a new eremophilane-type sesquiterpene (**3**) after column chromatography of the EtOAc and n-BuOH fractions obtained by partition of the liquid medium (Figure 1).

The structure of compound **1** was established after analysis of its spectroscopic data (IR, ^{1}H and ^{13}C NMR) and comparison with literature data.[15] Until now, this is the first report of the isolation of ergosterol (**1**) from *L. theobromae*, although the detection of **1** in maize grains has been associated with the presence of this fungus as a contaminant.[16] It should be mentioned that TLC analyses of the extracts from the control flasks did not show the presence of compound **1**.

Compound **2** was identified as the isocoumarin *cis*-4-hydroxymelein by IR, MS and ^{1}H and ^{13}C NMR techniques and by comparison with literature data.[17] This secondary metabolite was previously isolated from several fungi species, including *L. theobromae*.[10]

The molecular formula of compound **3**, $C_{23}H_{32}O_4$, was suggested by ^{1}H and ^{13}C NMR. The IR spectrum displayed a broad band at 3299 cm^{-1}characteristic of a hydroxyl group and bands associated with α,β-unsaturated ketone (1643 cm^{-1}) and α,β-unsaturated-ester (1708 cm^{-1}). The analysis of hydrogen broad band decoupled (HBBD) and DEPT 135° ^{13}C NMR spectra revealed the presence of six methyl groups, two methylene carbons, nine methine carbons and six non-hydrogenated carbons, two of which were associated with carbonyl groups, characteristic of an α,β-unsaturated ketone and another of carbonyl of an ester function at δ_C 181.5 and 167.9, respectively. The ^{1}H NMR spectrum of **3** exhibited the presence of a deshielded signal assignable to an acylated oxymethine proton at δ_H 5.48 (1H, t, *J* 5.0 Hz, H-3). After analysis of the HSQC spectrum, three trisubstituted double bonds presented olefinic hydrogen signals at δ_H 6.56 (1H, dq, *J* 1.4 and 10.0 Hz, H-3'), 6.21(1H, br s, H-9) and 6.30 (1H, d, *J* 0.4 Hz, H-6) which were associated with carbons at δ_C 149.6, 122.7 and 121.0, respectively. The two last signals, together with the signal at δ_H 6.36 (br s, 7-OH) and the carbonyl group signal at δ_C 181.5, are in perfect agreement with the presence of a α-hydroxydienone ring, with an enol suggesting a diosphenol group. In addition, there were ^{1}H NMR signals for one dissubstituted double bond at δ 6.45 (1H, br dd, *J* 0.6 and 9.8 Hz, H-1) and 6.24 (1H, dd, *J* 5.0 and 9.8 Hz, H-2). The presence of an angular methyl was deduced from the observation of one singlet at δ 1.43 (3H, H-11). Additional methyl groups were observed by four doublets integrating to 3H each at δ 1.19 (d, *J* 7.0 Hz, H-12), 1.00 (d, *J* 6.6 Hz, H-10'), 0.84 (d, *J* 6.2 Hz, H-11'), and 1.88 (d, *J* 1.4 Hz, H-9'), the last coherent with a vinyl methyl group. These data, in combination with the four multiplets between δ_H 2.63

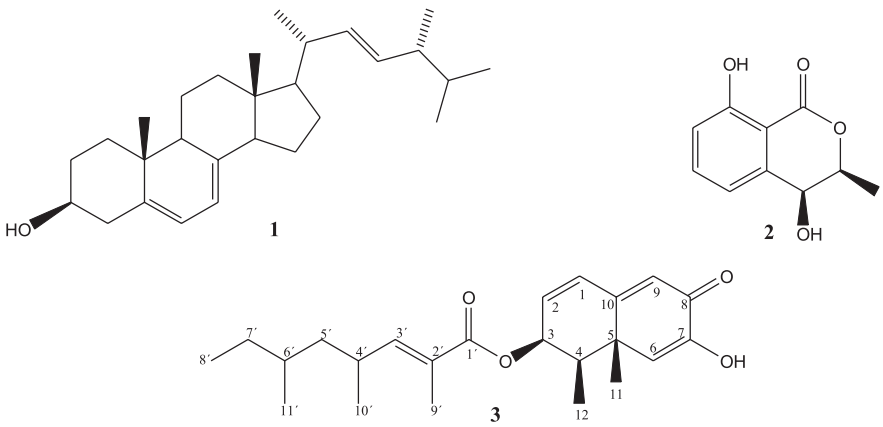

Figure 1. Isolated compounds from *L. theobromae*.

and 1.10, integrating to six protons and the oxyacyl group, δ_C 167.9, suggested that compound **3** had the presence of an unsaturated fatty acid side chain with 11 carbons.

The bicyclic moiety of the postulated structure for **3** was found to be an eremophilane-type sesquiterpene. This class of compound is reported as fungal metabolite and presents a branched unsaturated fatty acid chain bonded at C1 or C3.[18-21] The placement of the unsaturated fatty acid chain, deduced by previous discussion, at C-3 was readily established from the HMBC experiment. Thus, the HMBC spectrum of **3** exhibited correlation peaks among the acylated oxymethine hydrogen at δ_H 5.48 (H-3) with the carbons at δ_C 38.5 (C-4), 131.9 (C-2), 11.7 (C-12), 41.3 (C-5), 130.0 (C-1) and the carbonyl carbon δ_C 167.9 (C-1'). Moreover, correlations were also observed for the methine hydrogen at δ_H 6.24 (H-2) with the carbons at δ_C 69.8 (C-3), 130.0 (C-1), 38.5 (C-4) and with the carbonyl carbon at δ_C 167.9 (C-1'). Likewise, the signal for methyl hydrogens at δ_H 1.88 (H-9') showed long range coupling with the carbons at δ_C 125.7 (C-2'), 167.9 (C-1'), and 149.6 (C-3'), indicating the location of this group. Furthermore, the following correlations of the other hydrogen methyl groups were also observed: δ_H 0.84 (H-8' and H-11') with the carbons located at δ_C 30.0 (C-7'), 32.4 (C-6'), and 44.1 (C-5'); 1.00 (H-10') with the carbons located at δ_C 31.0 (C-4'), 44.1 (C-5') and 149.6 (C-3'). These correlations corroborate the fatty acid moiety with 11 carbons attached at position C-3, similar to that found in eremoxylarin B, an eremophilane sesquiterpene isolated from the xylariaceous endophytic fungus YUA-026.[21]

The long-range correlations observed in the HMBC spectrum of **3** allowed the unambiguous assignment of all carbons and hydrogens from the bicyclic ring of an eremophilane-type skeleton. Correlations were observed among the hydrogen of the hydroxyl group at δ_H 6.36 (OH-7) with the carbons at δ_C 146.5 (C-7), 121.0 (C-6) and 181.5 (C-8). This spectrum also revealed the cross-peak among the vinyl hydrogens at δ_H 6.21 (H-9) with the carbons at δ_C 163.8 (C-10), 41.3 (C-5), 130.0 (C-1) and 146.5 (C-7). Furthermore, the correlation peaks were observed among H-1 (δ_H 6.45) with the carbons at δ_C 131.9 (C-2), 163.8, (C-10), 41.3 (C-5), 69.8 (C-3) as well as the carbon δ_C 122.7 (C-9). Additionally, hydrogen at δ_H 6.30 (H-6) showed cross-peak with the carbons at δ_C 41.3 (C-5), 146.5 (C-7), 38.5 (C-4), 163.8 (C-10), 181.5 (C-8), as well as with the carbon of the angular methyl atδ_C 24.0 (C-11). Indeed, the HMBC spectrum also exhibited the correlation peaks between the methyl hydrogens signals (δ_H 1.43, H-11) and C-5 (δ_C 41.3), the allyl quaternary carbon that bears the methyl group. All the above observations were consistent with the cross-peak correlations observed in the HMQC and ^{1}H, ^{1}H- COSY experiments.

The relative stereochemistry of **3** was elucidated using nOe difference spectroscopy with the aid of geometry optimization using computational calculations. Thus, irradiation of H-3 at δ_H 5.48 enhanced the H-2 signal (δ_H 6.24), H-4 (δ_H 2.11), and 3H at C12 (δ_H 1.19); and when the signal at δ_H 6.30 (H-6) is irradiated, the resonances at δ_H 1.43 (3H at C11) and 1.19 (3H at C12) are increased, which indicates these groups of hydrogen are close in space. The optimized molecular geometry shows the methyl groups 3H at C11 and C12 almost equidistant to H-6 (Figure 2). Finally, irradiation of the H-4 signal (δ_H 2.11) produced an enhancement of the resonances at δ_H 5.48 (H-3) and 1.19 (3H at C12). All of these nOe effects observed are in agreement with the results of the computational calculations used to optimize the molecular geometry. As expected, the atoms that form the diosphenol substructure are all co-planar and the *p*-orbitals at double-bound $\Delta^{1,2}$ parallels those at $\Delta^{9,10}$. The present suggested relative stereochemistries at C-3, C-4 and C-5 are also in agreement with the molecular structure of other fungi eremophilane sesquiterpenes.[18, 22, 23] The stereochemistry of C-4´and C-6´ at the octanoate ester was not determined in this work, since it is not directly deduced from nOe measurements.

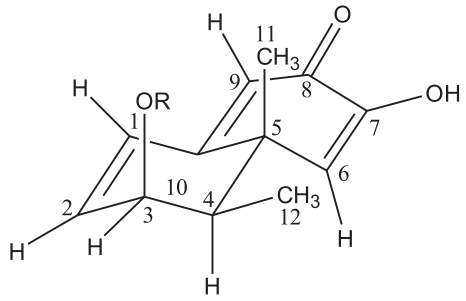

Figure 2. Three dimensional perspective suggested for compound **3** based on geometry optimization, using the software HyperChem (from ref. 23), and on ^{1}H-^{1}H nOe experiments.

These findings revealed **3** as a new eremophilane-type sesquiterpene with a branched unsaturated fatty acid attached to the C-3 position, named 2,4,6-trimethyloct-2-enoic acid, 1,2,6,8a-tetrahydro-7-hydroxy-1,8a-dimethyl-6-oxo-2-naphtalenyl ester. APCIMS spectrum of this compound showed the peak *m/z* 373 $[M+1]^+$ which is in accordance with the molecular formula $C_{23}H_{33}O_4$.

Experimental

General procedure

NMR spectra were recorded on BRUKER spectrometers: DRX 500 for **1**, ARX 200 for **2** and DRX-400 for **3** with

$CDCl_3$ as solvent and TMS as internal standard. IR spectra were run on a Perkin-Elmer 1000 FT-IR spectrometer using KBr pellets. Melting points were determined on a Mettler FP5 apparatus and are uncorrected. Gravity column chromatography was performed on Merck Kieselgel 60 (70-230 mesh). Low-resolution APCIMS data were acquired in positive ion mode, using a MICROMASS QUATTRO-LC instrument equipped with an ESI/APCI "Z-spray" ion source. Molecular modeling of the sesquiterpene was conducted following the MM+ minimum energy optimization routines using the HyperChem[24] for Windows (Release 3) program from Autodesk, Inc (Sausalito, CA).

Fungus material

L. theobromae (strain # 009) was isolated from infected guava in the Laboratory of Phytopathology from Embrapa Agroindústria Tropical, Ceará State, Brazil.

*Fungus culture in rice and isolation of **1***

Twenty seven Erlenmeyer flasks (250 mL), containing 100 g of rice ("*Uncle Ben's*") and 84 mL of distilled water per flask, were autoclaved twice at 121 °C for 60 min. Small discs of the PDA medium from the Petri dish containing mycelium of *L. theobromae* was transferred under sterile conditions to 24 Erlenmeyer flasks containing sterilized rice and three flasks were kept as control. After 32 days of growth at 25 °C, 100 mL of CH_2Cl_2:MeOH (3:7) was added to each flask and allowed to stand for 24 h. Blending of the material followed by filtration under reduced pressure provided 45.5 g of extract after solvent distillation. Vacuum chromatography of the extract on silica gel provided twelve fractions after elution with gradient mixture of hexane, CH_2Cl_2, EtOAc and MeOH. The fraction eluted with CH_2Cl_2/EtOAc 10% (512.0 mg) was chromatographed on silica gel by elution with Hexane/EtOAc 10% and provided 66.8 mg of **1**.

*Fungus culture in Czapeck broth and isolation of **2** and **3***

Small discs were cut from Petri dishes containing mycelium of *L. theobromae* in PDA medium and transferred under sterile conditions to 25 Erlenmeyer flasks (1000 mL), containing 300 mL of Czapeck medium per flask. Both broth and flask were previously autoclaved. Two flasks with no fungus were kept for control purposes. After 40 days of growth at 25 °C under static conditions, the liquid medium was separated from the mycelium by

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR data for **3** in $CDCl_3$

C	^1H	^{13}C	^2J	^3J
1	6.45 (1H, br dd, *J* 0.6 e 9.8)	130.0	131.9; 163.8	41.3; 69.8; 122.7
2	6.24 (1H, dd, *J* 5.0 e 9.8)	131.9	69.8; 130.0	38.5; 163.8
3	5.48 (1H, t, *J* 5.0)	69.8	38.5; 131.9	11.7; 41.3; 130.0; 167.9
4	2.11 (1H, dq, *J* 5.0 e 7.0)	38.5	11.7	24.0; 41.3; 121.0; 163.8
5		41.3		
6	6.30 (1H, d, *J* 0.4)	121.0	41.3; 146.5	24.0; 38.5; 163.8; 181.4
7		146.5		
8		181.5		
9	6.21 (1H, br s)	122.7	163.8	41.3; 130.0; 146.5
10		163.8		
11	1.43 (3H, s)	24.0	41.3	38.5; 121.0; 163.8
12	1.19 (3H, d, *J* 7.0)	11.7	38.5	41.3; 68.9
1'		167.9		
2'		125.7		
3'	6.56 (1H, dq, *J* = 1.4 e 10.0)	149.6	31.0; 125.7	12.5; 20.4; 44.1; 167.9
4'	2.63 (1H, m)	31.0	20.4; 44.1	125.7; 149.6
5'	1.40-1.31/1.17-1.10 (1H, m)	44.1	31.0; 32.4	20.4; 149.6
6'	1.28-1.32 (1H, m)	32.4	19.2; 44.1	
7'	1.34-1.29/1.17-1.10 (2H, m)	30.0	32.4	19.2
8'	0.84 (3H, t, *J* 7.1 Hz)	11.2		30.0; 32.4; 44.1
9'	1.88 (3H, d, *J* 1.4 Hz)	12.5	125.7	149.6; 167.9
10'	1.00 (3H, d, *J* 6.6 Hz)	20.4	31.0	44.1; 149.6
11'	0.84 (3H, d, *J* 6.2 Hz)	19.2		30.0; 32.4; 44.1
7-OH	6.36 (1H, br s)		146.5	121.0; 181.4

vacuum filtration. Liquid-liquid partition of the liquid medium with EtOAc and n-BuOH provided 1.0 (LMA) and 1.5 g (LMB) of extract, respectively. Extraction of mycelium with EtOH yielded 28.3 g of extract (ME). After TLC analysis, extracts LMA and ME were grouped and subjected to vacuum chromatography on silica gel by elution with gradient mixture of Hexane, CH_2Cl_2, EtOAc and MeOH. The fractions eluted with CH_2Cl_2/EtOAc 30%, CH_2Cl_2/EtOAc 50%, CH_2Cl_2/EtOAc 70% and EtOAc were grouped providing 910.0 mg of material which was chromatographed on silica gel by elution with a gradient mixture of Hexane, EtOAc and MeOH. Seventeen fractions (F1-F17) were obtained and F4 (90.4 mg), eluted with Hexane/EtOAc 10%, was purified on silica gel column after elution with gradient Hexane/Acetone mixture, providing 6.9 mg of **3**. Fractions F8 and F9, obtained by elution with Hexane/EtOAc 30%, were grouped (33.6mg) and chromatographed on silica gel with gradient mixture of Hexane/Acetone as eluent. This procedure provided 4.0 mg of compound **2**.

*Physical and spectral data of **3***

2,4,6-trimethyloct-2-enoic acid, 1,2,6,8a-tetrahydro-7-hydroxy-1,8a-dimethyl-6-oxo-2-naphtalenyl ester (**3**). Amorphous solid; mp 115.7-117.3 °C; $[\alpha]_D$ = +0.246 (*c* 0.05, $CHCl_3$); IR ν_{max}/cm^{-1}: 3299, 1708, 1643, 1211(KBr), APCIMS (Daughter ions, 10 eV): *m/z* 373 $[M+1]^+$ (11%), 189 (100%), 171 (60%), 167 (58%); ^{1}H and ^{13}C NMR: see Table 1.

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Supplementary Information

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

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A New Eremophilane-type Sesquiterpene from the Phytopatogen Fungus *Lasiodiplodia theobromae* (Sphaeropsidaceae)

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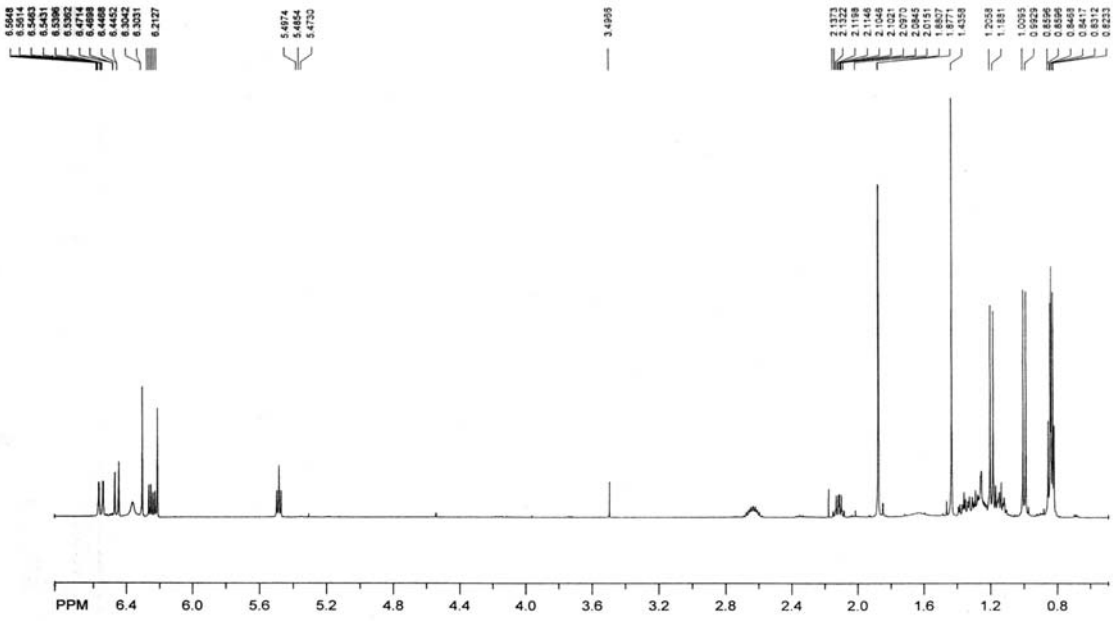

Figure S1. ^{1}H NMR spectrum of **1** (400 MHz, $CDCl_3$).

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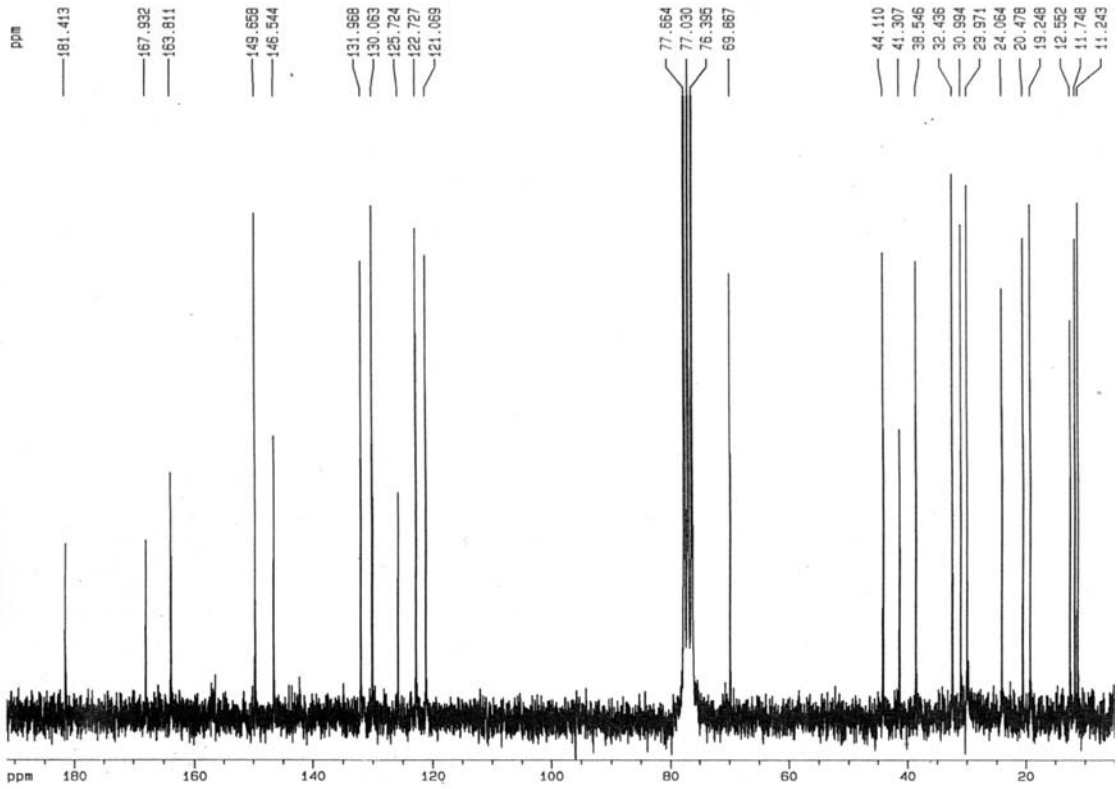

Figure S2. ^{13}C NMR spectrum of **1** (50 MHz, $CDCl_3$).

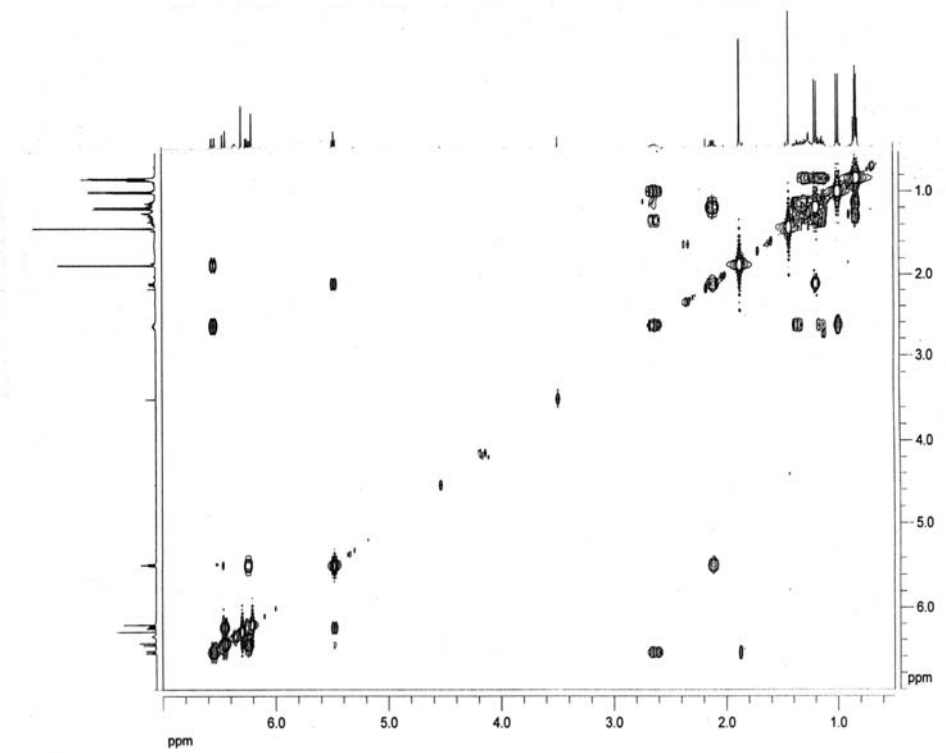

Figure S3. ^{1}H-^{1}H COSY NMR correlation spectroscopy 2D NMR spectrum of **1** (400 MHz, $CDCl_3$).

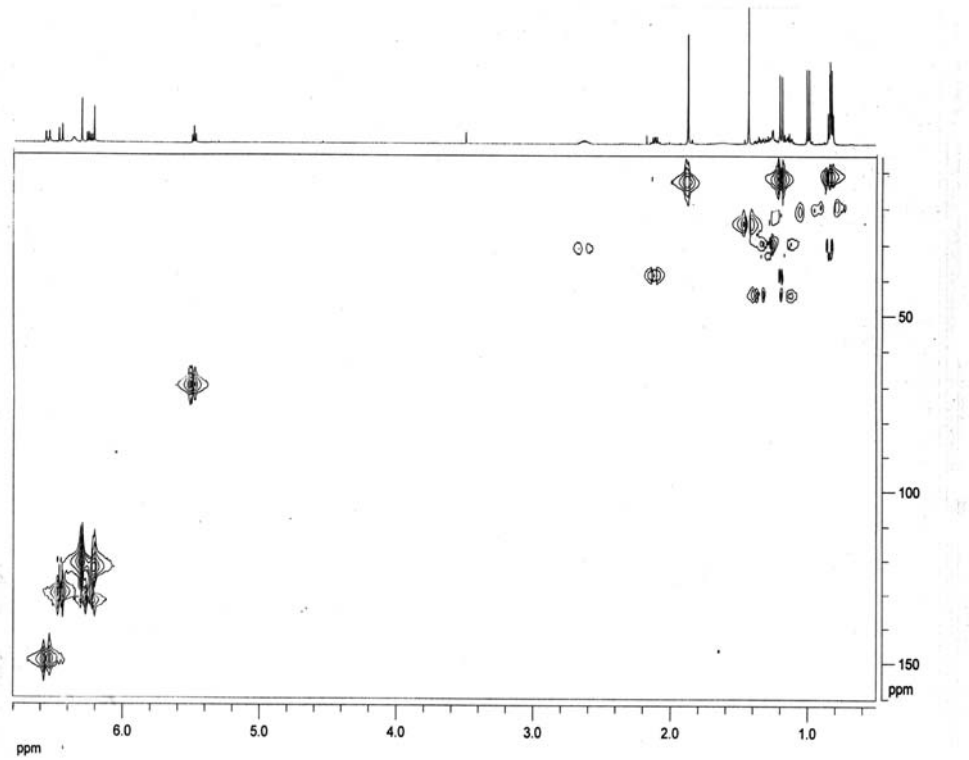

Figure S4. ^{1}H-^{13}C HSQC 2D NMR correlation spectroscopy of **1** (400 MHz/100 MHz, $CDCl_3$).

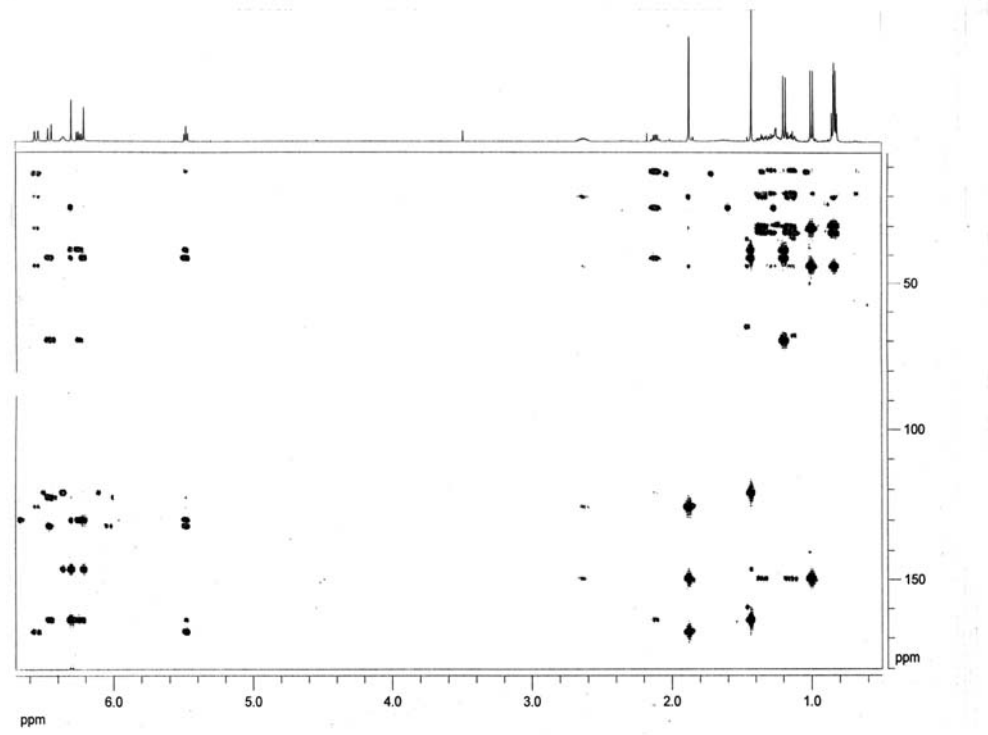

Figure S5. ^{1}H-^{13}C HMBC 2D NMR correlation spectroscopy of **1** (400 MHz/100 MHz, $CDCl_3$).

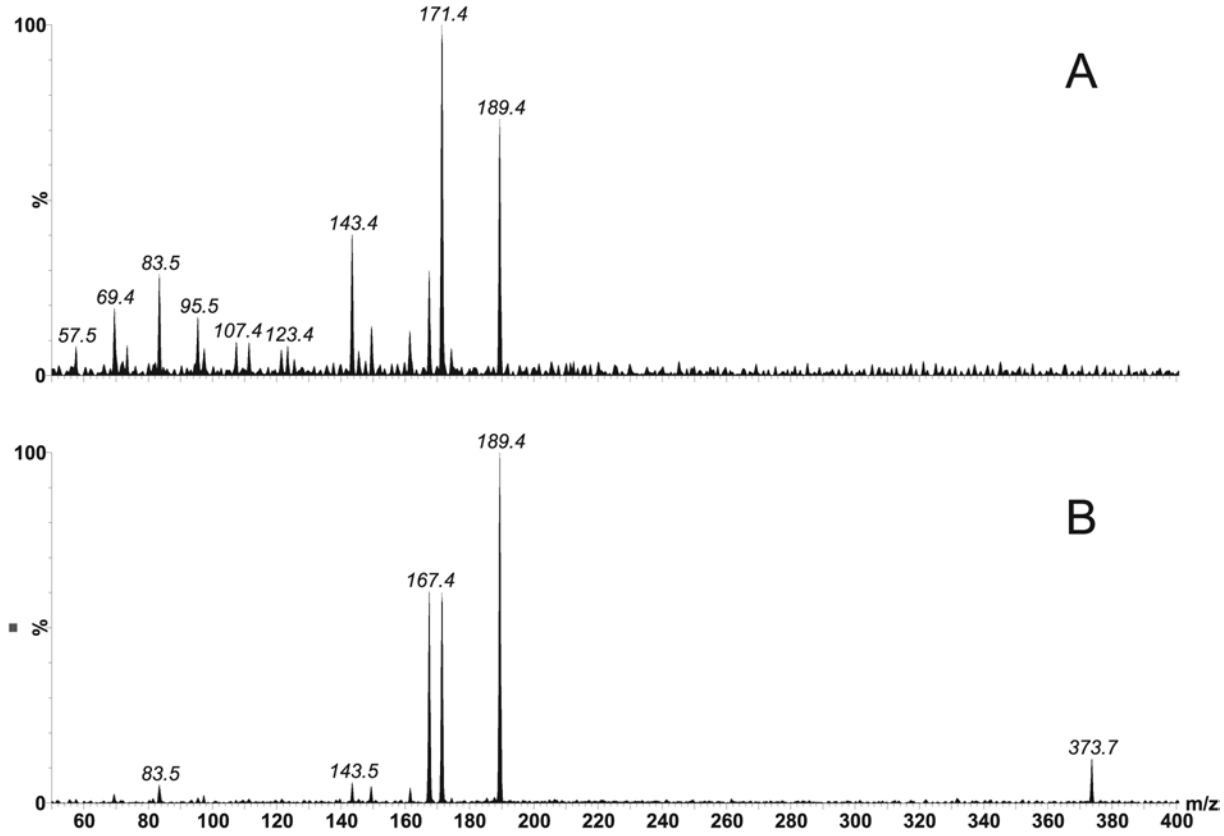

Figure S6. APCIMS [M+H]+ daugther ions spectrum of **1**: (A) 20 eV (B) 10 eV.

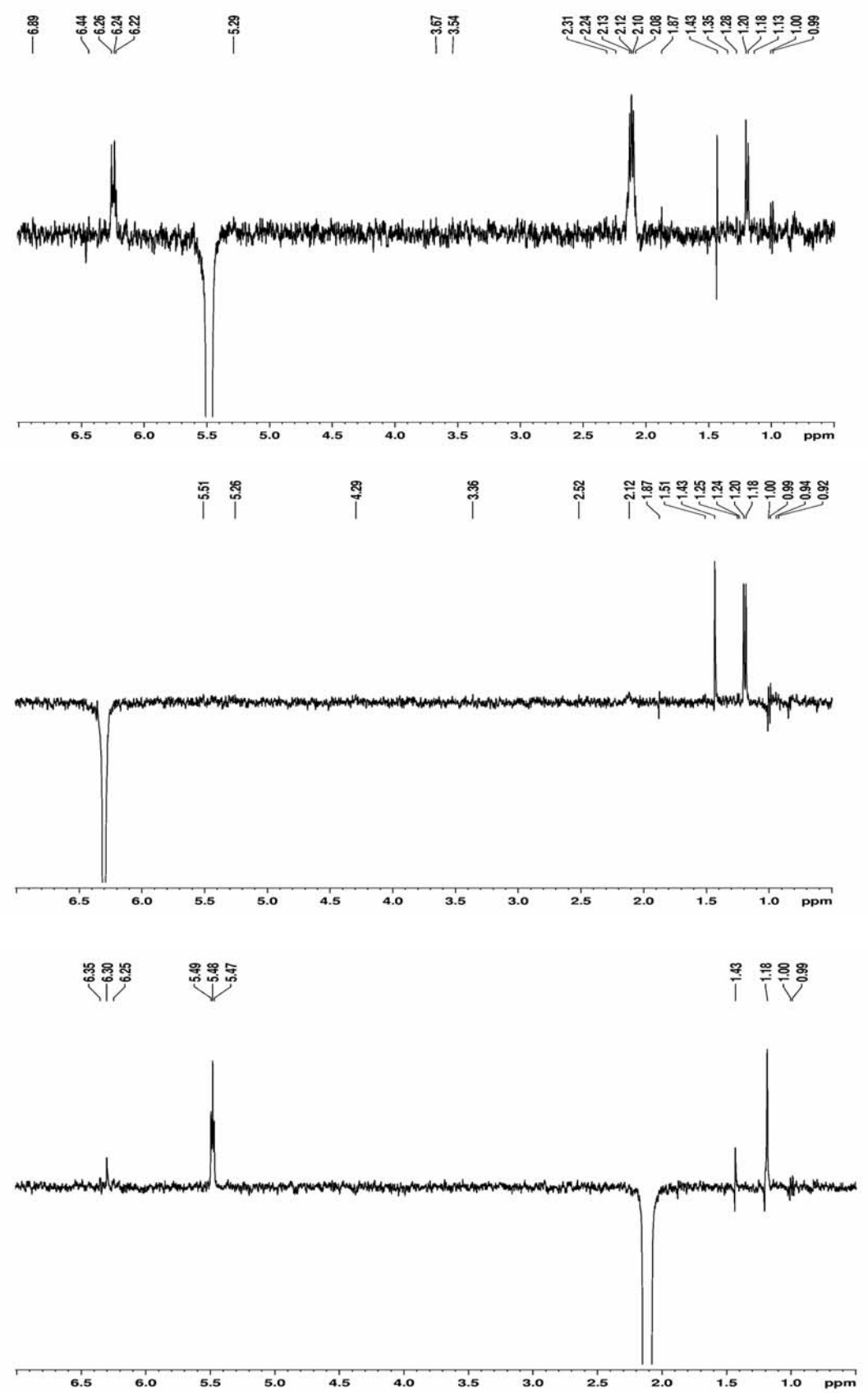

Figure S7. nOe difference spectra for H-3, H-6 and H-4 in **1** (400 MHz, $CDCl_3$).