

A New Antifungal Phenolic Glycoside Derivative, Iridoids and Lignans from *Alibertia sessilis* (Vell.) K. Schum. (Rubiaceae)

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Um novo derivado fenólico glucosilado antifúngico, 3,4,5-trimetoxifenil-1-*O*- β -D-(5-*O*-siringoila)-apiofuranosil-(1 \rightarrow 6)- β -D-glicopiranosídeo (**1**), juntamente com quatro iridóides conhecidos, ácido geniposídico (**2**), geniposídeo (**3**), 6 α -hidroxigeniposídeo (**4**) e 6 β -hidroxigeniposídeo (**5**); duas lignanas, (+)-lioniresinol-3 α -*O*- β -D-glicopiranosídeo (**6**), (-)-lioniresinol-3 α -*O*- β -D-glicopiranosídeo (**7**); e dois ácidos fenólicos, ácido clorogênico (**8**) e salicílico (**9**) e D-manitol (**10**), foram isolados do extrato etanólico dos galhos de *Alibertia sessilis*. As estruturas de **1** e dos compostos conhecidos foram determinadas por análise espectroscópica. Todos os compostos isolados foram avaliados quanto à atividade antifúngica frente aos dois fungos fitopatogênicos *Cladosporium cladosporioides* e *C. sphaerospermum* por bioautografia direta.

A new antifungal phenolic glycoside, 3,4,5-trimethoxyphenyl-1-*O*- β -D-(5-*O*-syringoyl)-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (**1**), together with four known iridoids, geniposidic acid (**2**), geniposide (**3**), 6 α -hydroxygeniposide (**4**) and 6 β -hydroxygeniposide (**5**); two lignans, (+)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (**6**), (-)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (**7**); and two phenolic acids, chlorogenic (**8**) and salicylic acids (**9**) and D-manitol (**10**), were isolated from the ethanolic extract of the stems of *Alibertia sessilis*. Structures of **1** and of the known compounds were determined by spectroscopic analysis. All compounds isolated were evaluated for their antifungal activities against two phytopathogenic fungi strains *Cladosporium cladosporioides* and *C. sphaerospermum* by direct bioautography.

Keywords: Rubiaceae, *Alibertia sessilis*, phenolics, iridoids, antifungal

Introduction

In our continuing search for antifungal agents from plants from the Cerrado and Atlantic Forest in the State of São Paulo, Brazil, we screened several species of the Rubiaceae family, which is recognized as a rich source of bioactive metabolites.¹⁻⁵ We particularly studied plants of the *Alibertia* genus, since relatively few chemical studies have been reported. Our previous investigation with the leaves of *A. macrophylla* resulted in the isolation of antifungal iridoid aglycones, and caffeic acid ester derivatives.^{6,7} Additionally, flavones and coumarin have been reported for *A. myrciifolia*.⁸

This paper reports the bioassay-guided isolation and structure elucidation of the new 3,4,5-trimethoxyphenyl-1-

O- β -D-(5-*O*-syringoyl)-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (**1**) (Figure 1), and the known lignans (+)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (**6**), (-)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (**7**), which are being described for the first time for Rubiaceae. This paper also reports the isolation of iridoids geniposidic acid (**2**), geniposide (**3**), 6 α -hydroxygeniposide (**4**) and 6 β -hydroxygeniposide (**5**), phenolic acids, chlorogenic (**8**) and salicylic acid (**9**) and D-manitol (**10**). The antifungal activity of compound **1** against *Cladosporium sphaerospermum* and *C. cladosporioides* was detected by using direct bioautography.

Results and Discussion

The antifungal ethanol extract from *Alibertia sessilis* stems was partitioned with hexane, ethyl acetate and *n*-

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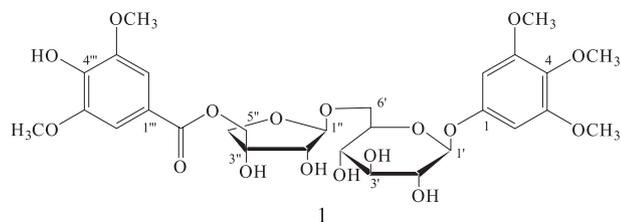


Figure 1. Phenolic glycoside isolated from *Alibertia sessilis*.

butanol. These sub-extracts were tested against *Cladosporium sphaerospermum* and *C. cladosporioides* using direct bioautography showing that only the ethyl acetate fraction was active. This sub-extract was bioassay-guide fractionated to afford a new phenolic derivative **1** along with several known compounds.

Compound **1** was isolated as a brown amorphous powder. The molecular formula was determined as being $C_{29}H_{38}O_{17}$ from the HRESIMS at m/z 657.2065 $[M-H]^-$. The UV spectrum showed an absorption maximum at 279 nm, which is compatible with a phenolic structure. The IR spectrum showed a characteristic absorption band attributable to the following groups: carbonyl (1708 cm^{-1}), hydroxyl (3418 cm^{-1}), and aromatic (1605 and 1509 cm^{-1}), as well as a glycosidic linkage (1063 cm^{-1}). The ^1H and ^{13}C NMR spectra (Table 1) revealed signals for two 1,3,4,5-tetrasubstituted symmetrical aromatic rings. The ^1H NMR exhibited singlet signals corresponding to five aromatic methoxyls at δ_{H} 3.77 (6H, OCH_3 -3,5), 3.68 (3H, OCH_3 -4), and 3.87 (6H, OCH_3 -3''',5''') and two sets of aromatic proton signals at δ_{H} 6.41 (2H, H-2,6) and 7.34 (2H, H-2'',6'') due to a 3,4,5-trimethoxy phenyl derivative and symmetrical 3,5-dimethoxy-4-hydroxy-benzoyl moieties, respectively.⁹ This was supported by the gHMBC spectrum, which showed cross-peaks corresponding to a long-range coupling of hydrogen at δ_{H} 6.41 and carbon signals at δ_{C} 155.9 (C-1), 154.7 (C-3,5), 134.7 (C-4) and 96.5 (C-2,6) and also between hydrogen at δ_{H} 7.34 and carbon signals at δ_{C} 167.9 (C=O), 149.1 (C-3''',5'''), 143.1 (C-4'''), 120.6 (C-1''') and 108.5 (C-2''',6''). Others signals observed in ^1H and ^{13}C NMR spectra are consistent with glycosyl moieties. According to literature,¹⁰ the comparison of the ^{13}C NMR data of the glycosidic moiety of various other analogous compounds, the structure of **1** seems to be composed of a glucosyl [δ_{C} 103.2 (C-1'), 74.9 (C-2'), 77.9 (C-3'), 71.5 (C-4'), 76.9 (C-5') and 68.2 (C-6')] and an apiosyl [δ_{C} 110.4 (C-1''), 78.7 (C-2''), 79.0 (C-3''), 75.0 (C-4'') and 67.9 (C-6'')] moieties. The ^1H , ^{13}C NMR and TOCSY spectra confirmed the presence of these two sugar moieties due to the presence of two anomeric signals at δ_{H} 4.79

(d, J 7.5 Hz)/ δ_{C} 103.2 (CH) for glucose and δ_{H} 5.01 (d, J 2.3 Hz)/ δ_{C} 110.4 (CH) for apiose. Furthermore, the coupling constants J 7.5 Hz of the anomeric proton signal of the D-glucosyl moiety as well as the chemical shift (δ_{C} 110.4) of the anomeric carbon of the D-aposyl moiety, demonstrated that both sugar moieties have β -anomeric configurations. The gHMBC spectrum of **1** revealed cross-peaks corresponding to long-range couplings of hydrogens H-6' of β -glucose at δ_{H} 3.63 (d, J 10.0 Hz) and 4.06 (d, J 10.0 Hz) and carbon C-1'' of apiose at δ_{C} 110.4; in this way, the β -D-aposyl moiety was attached to C-6' of the glucose. The chemical shift of the oxymethylene carbon C-6' (δ_{C} 68.2) was used to confirm the disaccharide linkage as apiofuranosyl-(1 \rightarrow 6)-glucopyranoside.¹⁰ In addition, long-range correlations between H-5'' signals of β -D-apiose [δ_{H} 4.31 (d, J 11.4 Hz) and 4.39 (d, J 11.4 Hz)] and the syringoyl carbonyl group (δ_{C} 167.9) indicated that the 3,5-dimethoxy-4-hydroxy-benzoyl group was attached to C-5'' of the β -D-apiose. Finally, the proton at δ_{H} 4.79 (H-1' of glucose) was correlated with C-1 of the 3,4,5-trimethoxy phenyl group (δ_{C} 155.9), indicating that the β -D-glucosyl moiety was located at C-1. Therefore, the structure of compound **1** was established as being 3,4,5-trimethoxyphenyl-1- O - β -D-(5- O -syringoyl)-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranoside.

The known compounds **2-10** were identified by comparing their ^1H and ^{13}C NMR data with those previously published in literature.¹¹⁻¹⁶

The antifungal activity of compounds **1, 2, 6-10** against *Cladosporium cladosporioides* and *C. sphaerospermum* was evaluated by direct bioautography on a TLC plate.^{17,18} Only compound **1** exhibited moderate activity (MIC of 100 μg), when compared with the standard nystatin (1.0 μg).

Experimental

General experimental procedures

1D- (^1H , ^{13}C , TOCSY) and 2D- (gHMQC and gHMBC) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^1H) and 126 MHz (^{13}C), using adequate solvent with TMS as internal standard. Accurate-mass measurements were performed on a quadrupole-time of flight instrument (UltraTOF-Q, Bruker Daltonics). IR spectrum was obtained on a Nicolet-730 FT-IR spectrometer using KBr discs. UV absorption was measured on a Varian ProStar 330 diode array spectrophotometer. Optical rotation was measured on a Polamat A Carl Zeiss Jena. Preparative HPLC was carried

Table 1. ^1H and ^{13}C NMR (500 and 126 MHz, respectively; J in Hz) and gHMBC data **1** (in CD_3OD)

position	δ_{C}	δ_{H}	gHMBC
1	155.9 (C)	-	
2,6	96.5 (CH)	6.41 s	96.5/134.7/154.7/155.9
3,5	154.7 (C)	-	
4	134.7 (C)	-	
1'	103.2 (CH)	4.79 d (7.5)	155.9
2'	74.9 (CH)	3.43 m	103.2
3'	77.9 (CH)	3.52 m	
4'	71.5 (CH)	3.34 m	77.9
5'	76.9 (CH)	3.60 m	
6'	68.2 (CH_2)	3.63 d (10.0)/4.06 d (10.0)	71.5/110.4
1''	110.4 (CH)	5.01 d (2.3)	68.2/75.0/78.7
2''	78.7 (CH)	3.94 d (2.3)	
3''	79.0 (C)	-	
4''	75.0 (CH_2)	3.85 m/ 4.08 d (9.7)	67.9/ 79.0/ 110.4
5''	67.9 (CH_2)	4.31 d (11.4)/ 4.39 d (11.4)	75.0/ 78.7/ 79.0/167.9
1'''	120.6 (C)	-	
2''',6'''	108.5 (CH)	7.34 s	120.6/143.1/149.1/167.9
3''',5'''	149.1 (C)	-	
4'''	143.1 (C)	-	
C=O	167.9	-	
3,5-OCH ₃	56.7	3.77 s	154.7
4-OCH ₃	61.2	3.68 s	134.7
3''',5'''-OCH ₃	56.9	3.87 s	149.1

out in a Varian HPLC, using a Phenomenex Luna RP18 (2) column (250 × 21.20 mm i.d. × 10 μm).

Plant material

Alibertia sessilis was collected in the Estação Ecológica e Experimental de Mogi-Guaçu (Ecological and Experimental Reserve of Mogi-Guaçu), São Paulo, Brazil in November 2003 by Dr M.C.M. Young and identified by Dr I. Cordeiro. The voucher specimen (SP 370.914) was deposited at the Botanical Institute Herbarium, São Paulo, Brazil.

Extraction and isolation of constituents

The dried and powdered stems (50.0 g) of *A. sessilis* were extracted exhaustively with ethanol, at room temperature, to give a crude material (4.15 g), which was partitioned in hexane, EtOAc and *n*-BuOH. After evaporation, the EtOAc extract (1.30 g) was submitted to column chromatography on Sephadex LH-20 using MeOH as eluent to give 15 fractions after TLC analysis. Fraction 1 (116.2 mg) was composed of a precipitate, which was recrystallized with H_2O to give D-manitol (**10**) (63.7 mg). Fraction 2 (146.4 mg) was subjected to column chromatography on RP-18 using a gradient mixture of $\text{H}_2\text{O}/\text{MeOH}$ and MeOH/EtOAc as eluent to give 11 subfractions. Subfraction 2.1 (80.0 mg) was re-

chromatographed on RP-18, eluted with H_2O with increasing amounts of MeOH to afford geniposidic acid (**2**) (11.8 mg). Subfraction 2.7 (29.6 mg) was also submitted to column chromatography on RP-18, using a gradient solvent system of $\text{H}_2\text{O}/\text{MeOH}$ and MeOH/EtOAc as eluent to yield 7 subfractions after TLC analysis. Subfraction 2.7.6 (18.8 mg) was chromatographed by preparative HPLC (C_{18} column; mobile phase: $\text{MeOH}/\text{H}_2\text{O}$ (3:7 v/v); flow rate: 10 mL min^{-1} ; UV detector: 238 nm) to separate the mixture of the diastereoisomeric lignans (+)-lyoniresinol 3 α -*O*- β -D-glucopyranoside (**6**) (6.5 mg) and (-)-lyoniresinol 3 α -*O*- β -D-glucopyranoside (**7**) (5.8 mg).¹⁹ Subfraction 2.7.7 (6.7 mg) was identified as 3,4,5-trimethoxyphenyl-1-*O*- β -D-(5-*O*-syringoyl)-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (**1**). Fraction 4 (274.2 mg) was fractionated on RP-18 using H_2O with increasing amounts of MeOH as mobile phase to give salicylic acid (**9**) (6.4 mg). Fractions 5 and 6 (104.9 mg) were combined and submitted to column chromatography on silica gel eluting with hexane/EtOAc and EtOAc/MeOH to give 6.9 mg of chlorogenic acid (**8**).

The *n*-butanol extract (0.55 g) was submitted to column chromatography on Sephadex LH-20 using MeOH as eluent to give 7 fractions after TLC analysis. Fraction 3 (180.0 mg) was subjected to column chromatography on RP-18 using a gradient mixture of $\text{H}_2\text{O}/\text{MeOH}$ to give 9 subfractions. Subfraction 3.4 (10.2 mg) was identified as

an iridoid mixture **3-5** and subfraction 3.6 (8.6 mg) as a lignan mixture **6-7**.

3,4,5-trimethoxyphenyl-1-O-β-D-(5-O-syringoyl)-apiofuranosyl-(1→6)-β-D-glucopyranoside (1)

Brown amorphous powder (6.7 mg, 0.16%). $[\alpha]_D^{25}$ -2.36 (c 0.021, MeOH). UV λ_{max} /nm (MeOH) 279. IR (KBr) ν_{max} / cm^{-1} : 3418, 2931, 1708, 1605, 1509, 1461, 1427, 1338, 1228, 1125, 1063, 816, 764. HRESIMS m/z 657.2065 [M - H]⁻ (calcd for C₂₉H₃₇O₁₇, m/z 657.2031 [M - H]⁻). ¹H and ¹³C NMR: see Table 1.

Known compounds isolated

The structures of known compounds were established by ¹H and ¹³C NMR and gHMBC data and by comparing their spectroscopy data with those reported in literature.

Geniposidic acid (2) (0.28%)

The ¹H and ¹³C NMR data, in CD₃OD, were in accordance with those reported in reference 11.

Geniposide (3); 6α-hydroxygeniposide (4); 6β-hydroxygeniposide (5) (0.24%)

The ¹H and ¹³C NMR data of the mixture, in CD₃OD, were in accordance with those reported in references 12 and 13. The compounds were identified in mixture mainly on the basis on their TOCSY spectra.

(+)-Lyoniresinol-3α-O-β-D-glucopyranoside (6) (0.15%) and (-)-lyoniresinol-3α-O-β-D-glucopyranoside (7) (0.14%)

The ¹H and ¹³C NMR data, in CD₃OD, were in accordance with those reported in reference 14.

Chlorogenic acid (8) (0.16%)

The ¹H and ¹³C NMR data, in DMSO-*d*₆, were in accordance with those reported in reference 15.

Salicylic acid (9) (0.15%)

The ¹H and ¹³C NMR data, in CD₃OD, were in accordance with those reported in reference 16.

D-manitol (10) (1.53%)

The ¹H and ¹³C NMR data, in D₂O, were in accordance with those reported in reference 16.

Antifungal Assay

The microorganisms used in the antifungal assays *Cladosporium sphaerospermum* (Penzig) SPC 491 and *C.*

cladosporioides (Fresen) SPC 140 had been kept at the Botanical Institute of São Paulo, Brazil. For the antifungal assay, solutions containing 100, 50, 25, 10, 5 and 1 μg of the test compound were prepared. 10.0 μL of each solution was applied to pre-coated TLC plates eluted with EtOAc/MeOH (7:3 v/v) and dried to completely remove all solvents. The chromatograms were sprayed with a spore suspension of *Cladosporium sphaerospermum* or *C. cladosporioides* in glucose and salt solution and incubated for 72 h in darkness in a moistened chamber at 25 °C. A clear inhibition zone appeared against a dark background indicating the minimal amount of the compound required to eliminate the fungus (detection limits - minimum amount required for the inhibition of fungal growth on TLC plates). Nystatin was used as the positive control (1 μg).^{17,18}

Conclusions

Lignans **6** and **7** are here described for the first time as being found in Rubiaceae and the antifungal phenolic glucoside **1** is being reported for the first time in literature. Compound **10** was previously isolated from *Alibertia myrciifolia*. Phenolics **8** and **9** are here reported for the first time as being found in genus *Alibertia*. Iridoids **2** and **3-5** are in agreement with chemosystematic correlations and botanical occurrence in the Rubiaceae family. This study will contribute significantly to improve knowledge about secondary metabolites and biological activity for one more species from the Brazilian Cerrado.

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Supplementary Information

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

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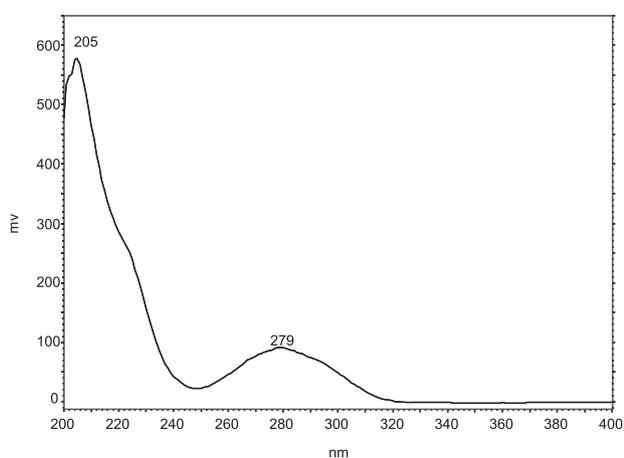


Figure S1. UV spectrum of compound 1 (MeOH).

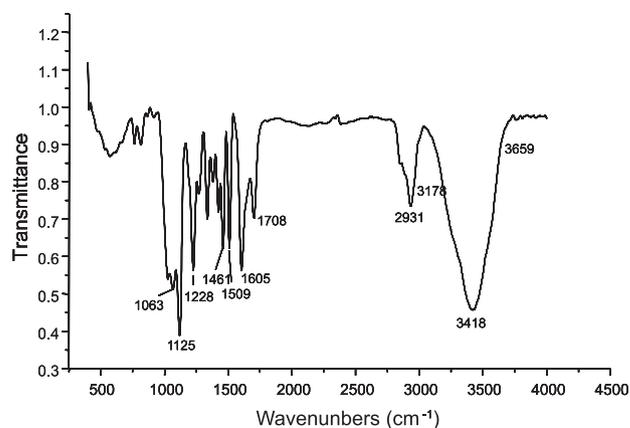


Figure S2. IR spectrum of compound 1 (KBr).

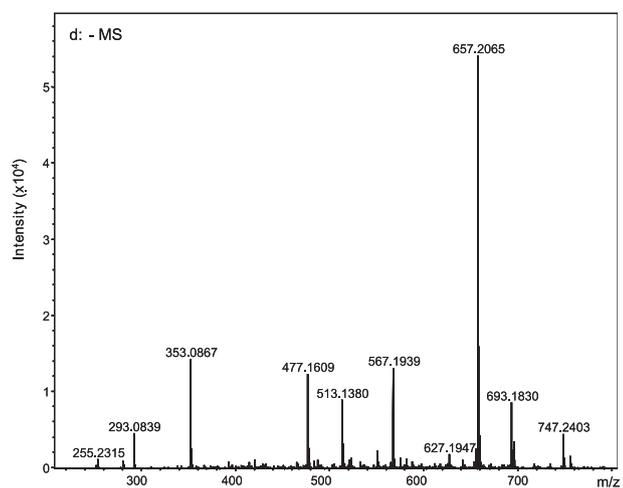


Figure S3. HRESIMS spectrum of compound **1** (negative mode).

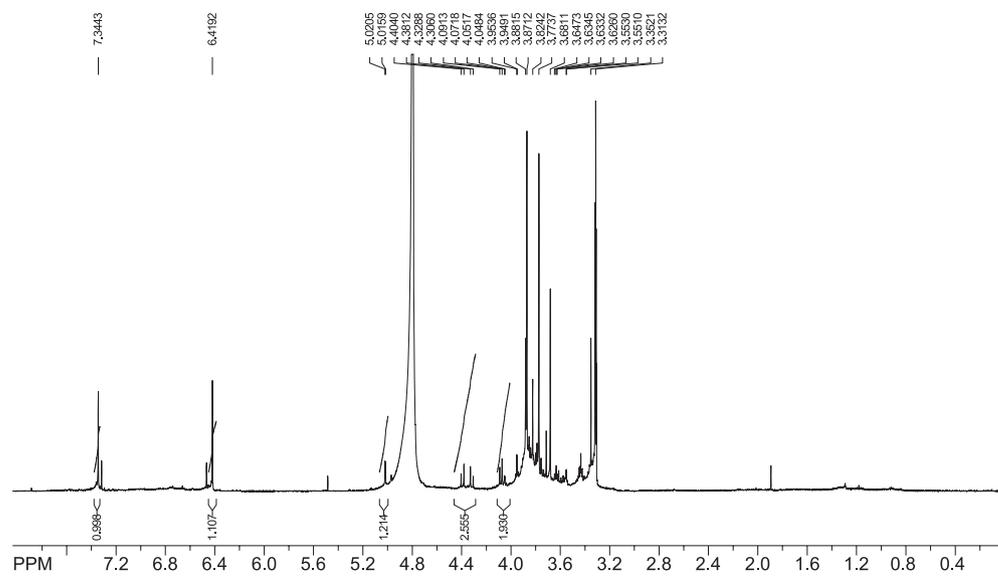


Figure S4. ¹H NMR spectrum of compound **1** (CD₃OD, 500 MHz).

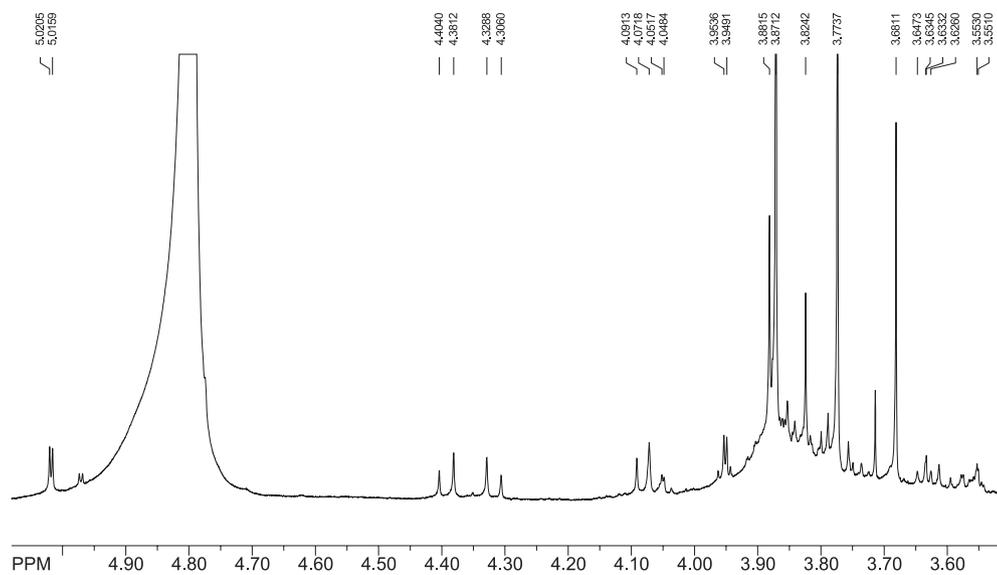


Figure S5. Expansion of ^1H NMR spectrum of compound **1** (CD_3OD , 500 MHz).

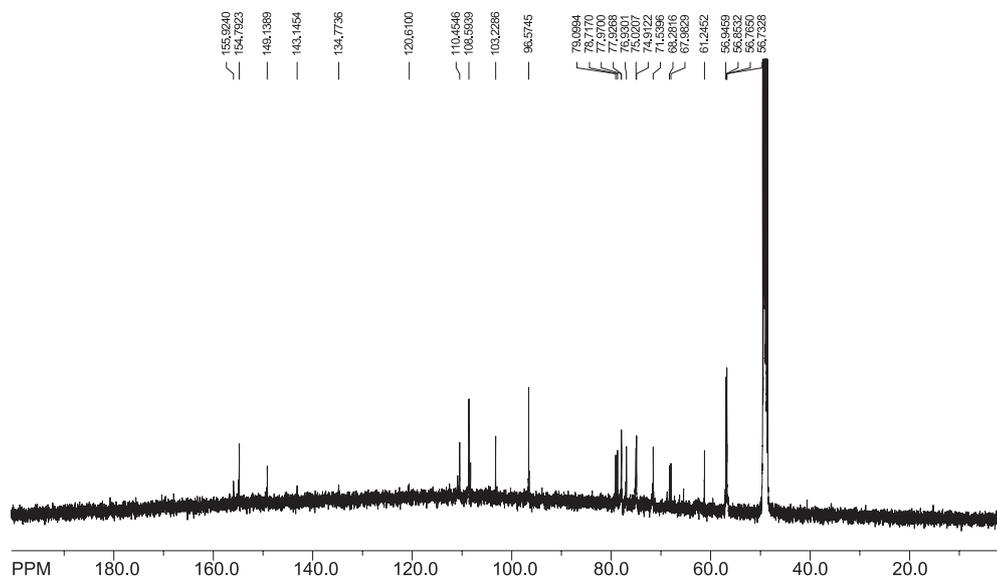


Figure S6. TOCSY spectrum of compound **1** (CD_3OD , 500 MHz).

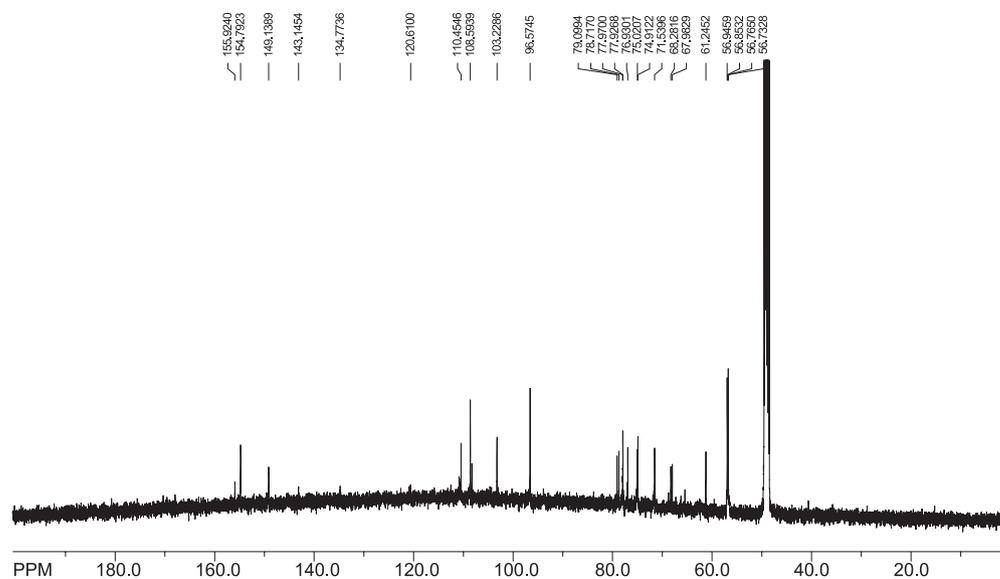


Figure S7. ^{13}C NMR spectrum of compound **1** (CD_3OD , 126 MHz).

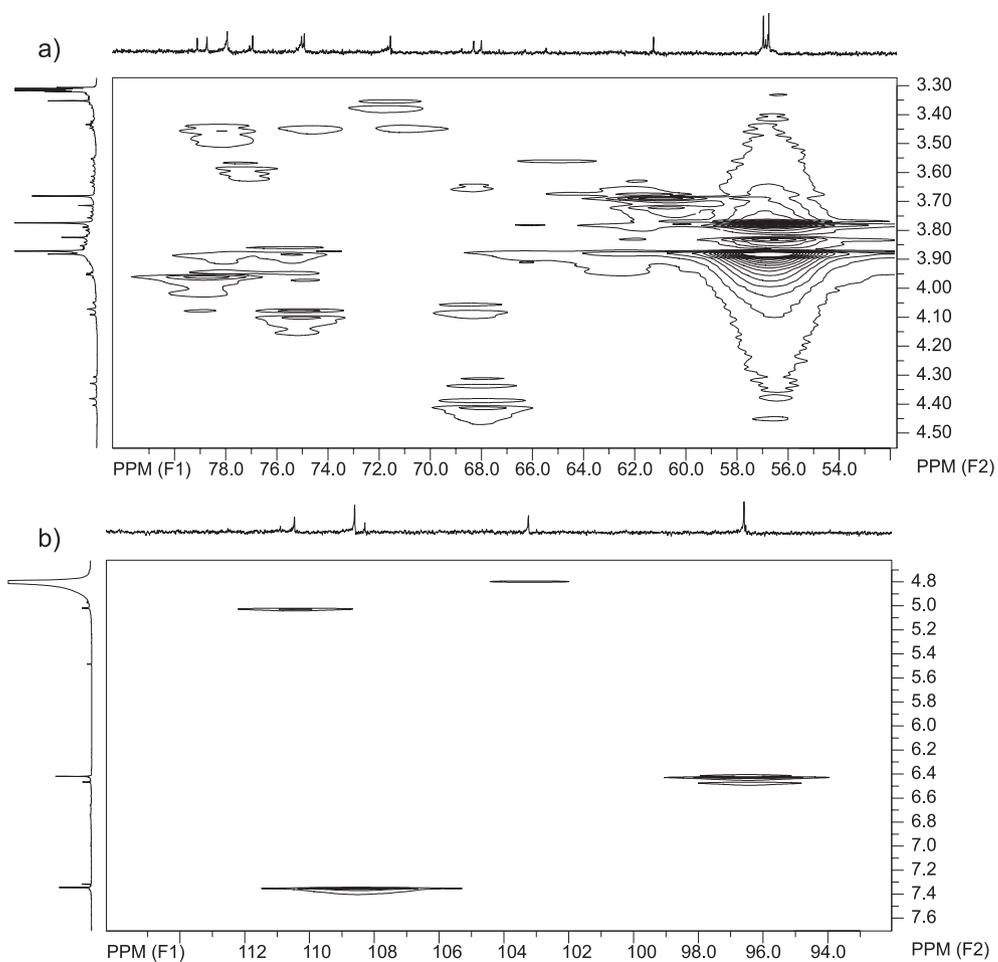


Figure S8. Expansions of the gHMQC spectrum of compound **1** (CD_3OD , 500 MHz).

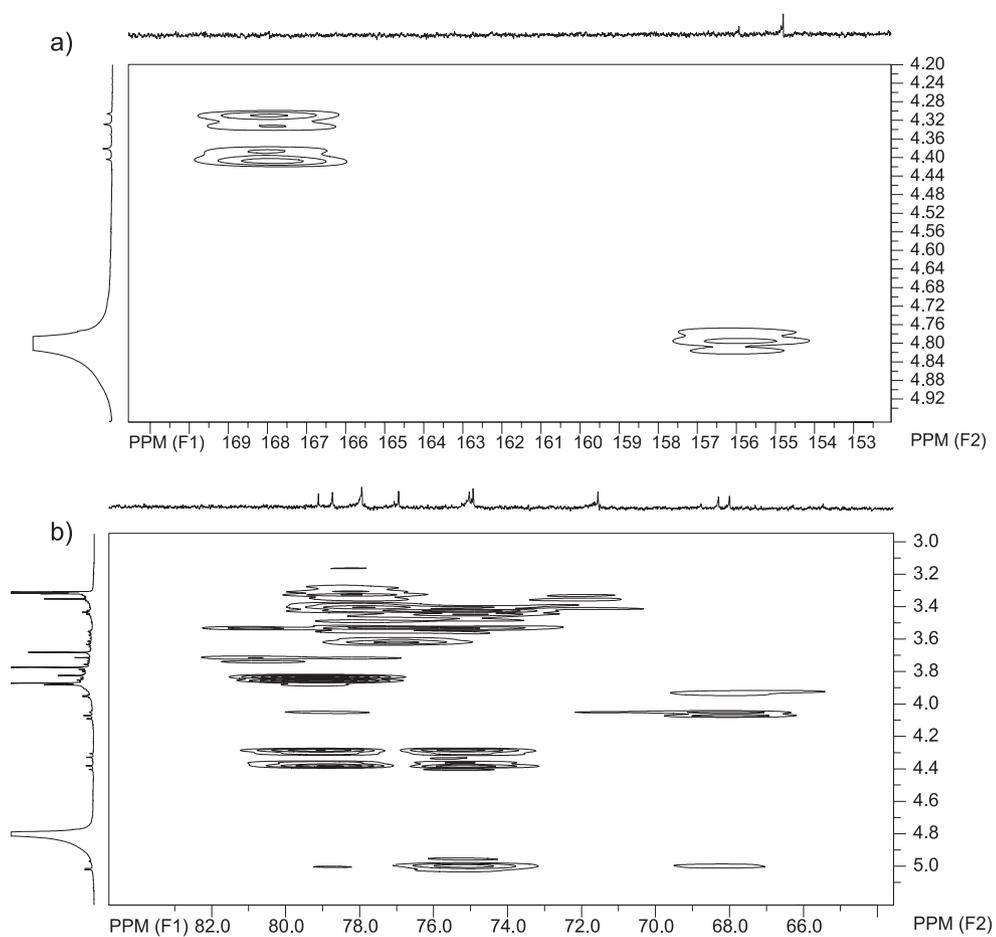


Figure S9. Expansions of the gHMBC spectrum of compound 1 (CD₃OD, 500 MHz).

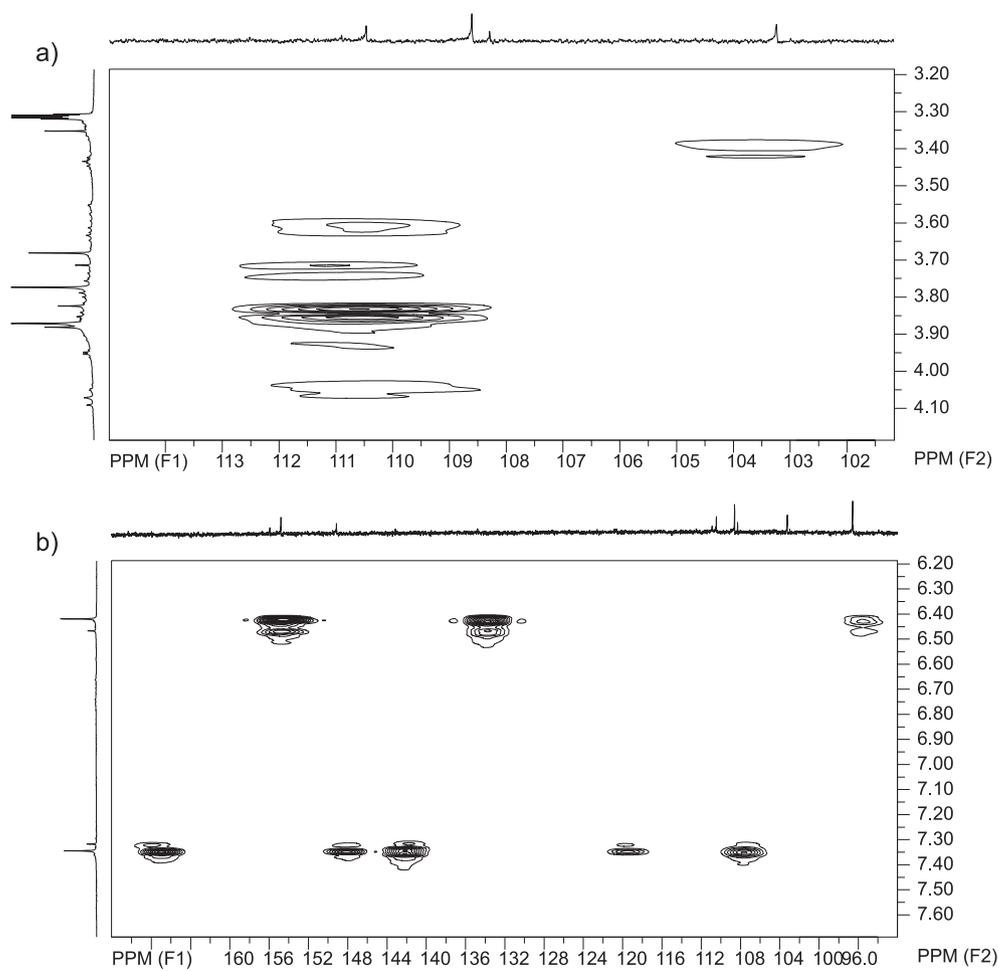


Figure S10. Expansions of the gHMBC spectrum of compound **1** (CD₃OD, 500 MHz).

Table S1. ^1H and ^{13}C NMR data of analogous compounds of **1** in literature

position	1	A	B	C	D	E
1	155.9	153.1	153.6	155.9	156.0	
2,6	96.5/ 6.41 s	103.7/ 6.60 d (2.6) 110.1/ 6.39 dd (2.6, 8.6)	94.0/ 6.26 s	96.7/ 6.43 s	96.4/ 6.45 s	
3,5	154.7	149.9/116.7/ 6.50 d (8.6)	153.0	154.8	154.8	
4	134.7	143.4	132.4	134.8	134.6	
1'	103.2/ 4.79 d (7.5)	102.5/ 4.77 d (7.2)	99.2/ 4.84 d (7.5)	103.3/ 4.79 d (8.0)	103.2/ 4.79 d (7.3)	104.4/ 4.25 d (8.0)
2'	74.9/ 3.43	79.1/ 3.61	77.2/ 3.50	75.0/ 3.44	74.9	75.1/ 3.18
3'	77.9/ 3.52	79.6/ 3.57	75.9/ 3.47	77.9/ 3.44	77.9	78.1/ 3.34
4'	71.5/ 3.34	72.5/ 3.34	70.4/ 3.12	71.5/ 3.35	71.6	71.8/ 3.26
5'	76.9/ 3.60	78.8/ 3.34	77.2/ 3.34	76.9/ 3.56	77.0	76.9/ 3.40
6'	68.2/ 3.63 d (10.0) and 4.06 d (10.0)	63.4/ 3.65 dd (12.2, 5.4) and 3.84 dd (12.2, 2.0)	60.8/ 3.74 m	68.3/ 3.63 dd (11.0, 2.0) and 4.06 dd (11.0, 6.0)	68.8	68.6/ 3.63 dd (10.0, 2.0)
1''	110.4/ 5.01 d (2.3)	111.3/ 5.50 s	108.6/ 5.42 s	110.5/ 5.01 d (2.0)	110.9/ 4.96 d (2.5)	110.8/ 5.05 d (2.0)
2''	78.7/ 3.94 d (2.3)	79.5/ 4.07 s	76.9/ 3.91 s	78.6/ 3.96 d (2.0)	77.9/ 3.87 d (2.5)	78.6/ 4.00 d (2.0)
3''	79.0	80.0	77.5	79.0	80.5	79.1
4''	75.0/ 3.85 m and 4.08 d (9.7)	76.1/ 3.90 m and 4.29 d (9.8)	73.9/ 3.85 d (9.4) and 4.11 d (9.4)	74.9/ 3.86 d (10.0) and 4.08 d (10.0)	74.9/ 3.73 d (9.8) and 3.94 d (9.8)	75.0/ 3.88 d (10.0) and 4.08 d (10.0)
5''	67.9/ 4.31 d (11.4) and 4.39 d (11.4)	68.9/ 4.26 d (11.5) and 4.42 d (11.5)	67.1/ 4.22 d (11.0) and 4.27 d (11.0)	67.6/ 4.31 d (11.5) and 4.36 d (11.5)	65.4/ 3.54 s	68.3/ 4.38 d (11.5) and 4.40 d (11.5)
1'''	120.6	121.8	119.1	123.3		126.4
2''' , 6'''	108.5/ 7.34 s	109.2/ 7.24 s	107.1/ 7.15 s	132.8		108.4/ 7.35 s
3''' , 5'''	149.1	149.6	147.4	114.9		154.5
4'''	143.1	142.9	140.9	165.3		143.9
C=O	167.9	168.5	165.3	167.6		167.4
3,5-	56.7/ 3.77	57.0/ 3.71	55.5/ 3.65	56.7/ 3.77	56.7/ 3.81	56.9
OCH ₃						
4-OCH ₃	61.2/ 3.68		60.0/ 3.53	61.2	61.2/ 3.70	61.2
3''' , 5''' - OCH ₃	56.9/ 3.87	57.7/ 3.84	56.1/ 3.77			

A (CD_3OD) and **B** ($\text{DMSO}-d_6$): ref. 9 - Jung, M. J.; Kang, S. S.; Jung, Y. J.; Choi, J. S.; *Chem. Pharm. Bull.* **2004**, *52*, 1501. **C**, **D** and **E** (CD_3OD): ref. 10 - Warashina, T.; Nagatani, Y.; Noro, T. *Phytochemistry* **2004**, *65*, 2003.

