J. Braz. Chem. Soc., Vol. 22, No. 5, 993-996, 2011.
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0103 - 5053 $6.00+0.00

Short Report

Novel Anthraquinone Derivatives Produced by *Pestalotiopsis guepinii*, an Endophytic of the Medicinal Plant *Virola michelii* (Myristicaceae)

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Um novo derivado de antraquinona, denominado guepinone (**1**), juntamente com as conhecidas substâncias isossulocrina (**2**) e cloroissosulocrina (**3**) foram isolados de uma cultura em arroz de *Pestalotiopsis guepinii*, um fungo endofitico de *Virola michelii*. Os compostos foram identificados pela análise de seus dados espectrométricos de RMN 1D e 2D e EM. A atividade antimicrobiana dos compostos isolados foi avaliada e a cloroisossulcrina (**3**) foi a mais ativa.

A new anthraquinone derivative, named guepinone (**1**), along with the known substances isosulochrin (**2**) and chloroisosulochrin (**3**), were isolated from a rice culture of *Pestalotiopsis guepinii*, an endophytic fungus of *Virola michelii*. The compounds were identified by analysis of 1D and 2D NMR and MS spectral data. The antimicrobial activity of these compounds was evaluated and chloroisosulchrin (**3**) was the most active.

Keywords: *Virola michelii, Pestalotiopsis guepinii*, anthraquinone derivatives, endophytic fungus

Introduction

Endophytic fungi have been collected from nearly all plants studied. They colonize the plants without causing visible disease symptoms. The function of the host plant is not yet clear and is supposed to depend on the organ-fungus interaction of each plant indicating that there are no neutral interactions but a balanced antagonism between the plant and the endophytic fungi.[1]

Endophytic fungi are very useful in agriculture and industry. They can be used as a tool for introduction of genes of interest into the plants,[2,3] like plague and pathogen inhibitors.[4,5] They are also a source of primary and secondary metabolites of interest.

In spite of the several reports concerning the production of substances by endophytic fungi that show biological activities, such as fungicide, herbicide and algicide, among others,[6] there are not many studies concerning the chemical composition of endophytic microorganisms, especially when we analize the wide fungic biodiversity and the specificity in the colonization of plants by fungi.

This paper deals with the study of the metabolites produced by the fungus *Pestalotiopsis guepinii* isolated from *Virola michelii.*

V. michelii belongs to the family Myristicaceae and it is used in folk medicine in the treatement of diseases caused by fungi and in the treatment of infections of the skin.[7] Phytochemical study, anti-inflammatory and allelopathic activities of the leaves of the *V. michelii* were reported.[8-11]

There have been described 205 species of *Pestalotiopsis*,[12] some of these produce secondary metabolites with important activities, like phytotoxic, antifungic, antioxidant and anticancer activities.[13] Studies with *P. guepinii* lead

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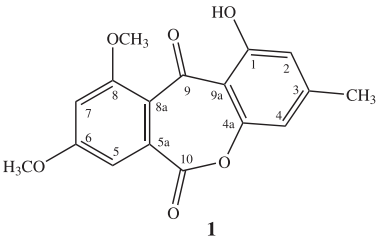
to the isolation of the diterpenoid taxol, an important anticancer drug.[14]

Pestalotiopsis also produce sesquiterpenes, other diterpenes, phenolic derivatives and anthraquinone derivatives.[14-16]

This paper reports the isolation and structural elucidation of three anthraquinone derivatives, a new lactone (**1**) named guepinone, isosulochrin (**2**) and chloroisosulochrin (**3**),[17] Figure 1. In addition, this is the first report of *P. guepinii* as an endophyte from *V. michelii* and its production of anthraquinones derivatives.



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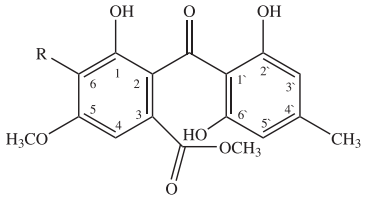

2 R = H
3 R = Cl

Figure 1. Chemical structure of three anthraquinone derivatives: guepinone (**1**), isosulochrin (**2**) and chloroisosulochrin (**3**).

Experimental

General experimental procedures

UV spectra were obtained in MeOH solution on a Hewlett Packard 8452-A spectrophotometer, and IR spectra were measured with a Bomem MB-102 spectrophotometer in KBr pellets. High-resolution mass spectra were measured in a QTOF I (quadrupole-hexapole-TOF) mass spectrometer with an orthogonal Z-spray-electrospray interface (Micromass, Manchester, UK). Low resolution ESI-MS data were acquired in the negative ion mode, using a Quattro-LC instrument (Micromass, Manchester, UK) equipped with an ESI/APCI "Z-spray" ion source. HPLC was carried out in a semi-preparative LC-8A Shimadzu system with SPD-10AV Shimadzu UV detector (Tokyo, Japan) using a RP sinergy fusion column (250 mm × 10 mm, 5 μm), isocratic systems of H_2O:MeCN (40:60) and H_2O:MeCN:MeOH (55:20:25), and a flow rate of 4.7 mL min^{-1}. Detection was performed at 270 nm. All solvents were filtered through a 0.45 mm membrane filter prior to use. Absorbance measurements were recorded on a Spectrum UV SPD-20A spectrophotometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Mercury 300, in $CDCl_3$ and CD_3OD, with residual solvent peak as internal reference.

Plant material

Leaves of *Virola michelii* were collected in Belém, state of Pará, Brazil and a voucher specimen (No. 180621) has been deposited at the herbarium of the Empresa Brasileira de Pesquisa Agropecuária - Embrapa - (Botanic Division in Belém, State of Pará, Brazil).

Microorganism

The general procedures adopted for isolation of the microorganism followed the methodology described by Pereira.[18] After collected, healthy leaves of *Virola michelii* were washed with water and the surface sterilized by immersion in 70% aqueous ethanol (1 min), followed by 5% aqueous sodium hypochlorite (4 min), and finally with 70% aqueous ethanol (30 s). After these procedures, the leaves were rinsed with sterilized water. This latter water was incubated in Petri dishes to guarantee the elimination of all epiphytic microorganisms. Small pieces of the leaves were excised and placed in Petri dishes containing PDA medium at 30 °C. Individual hyphal tips of the emerging fungi were removed and replaced on PDA.

The pgfvm-04 strain was identified as *Pestalotiopsis guepinii* by Francisco das Chagas de Oliveira Freire, Embrapa, State of Ceará, Brazil. A voucher specimen (LISB 68) has been deposited at the Laboratório de Investigação Sistemática em Biotecnologia, LABISISBIO, UFPA, Brazil.

Rice culture of Pestalotiopsis guepinii and isolation of the anthraquinones

The fungus was statically cultured in 45 erlenmeyer flasks (500 mL) containing 100 g of rice ("Uncle Ben's"-parboiled) and 30 mL of distilled water per flask, and autoclaved at 121 °C for 45 min. A small disc of the PDA medium from the Petri dish containing mycelium of *P. guepinii* was transferred under sterile conditions to each erlenmeyer flasks. Three flasks were kept for control purposes. After 30 days of growth at 28 °C, methanol (300 mL) was added to each flask and allowed to stand for 5 h, and then it was filtered by gravity. The methanol was evaporated under reduced pressure to afford 157.3 g of a dark residue. This residue was suspended in 500 mL MeOH:H_2O (1:3) solution. The suspension was extracted

successively with hexane (3 × 1000 mL), dichlorometane (3 × 1000 mL) and EtOAc (3 × 1000 mL) and was further concentrated under vacuum. The hexane fraction (1.03 g) was separated by column chromatography on silica gel using as eluents mixtures of hexane, ethyl acetate and methanol of increasing polarities. After analysis by NMR of the sub-fractions, the hexane:EtOAc (5:1) fraction was submitted to semi-preparative reversed phase HPLC (250/10 synergi fusion C_{18}, H_2O:MeCN 40:60, flow rate 4.7 mL min^{-1}, 215 nm) to yield **1** (32 mg). The dichloromethane fraction (1.04 g) was submitted to the same procedure and two sub-fractions were obtained (hexane:EtOAc 40% and hexane:EtOAc 60%). These fractions were submitted to semi-preparative reversed phase HPLC (250/10 synergi fusion C_{18}, H_2O:MeCN:MeOH 55:20:25, flow rate 4.7 mL min^{-1}, 215 nm) to yield **2** (32 mg) and **3** (16 mg)**.**

*Guepinone (**1**)*

White amorphous solid; mp 162.0-167.0 °C; UV λ_{max}/nm (MeOH): 216, 255 and 313; IR (KBr) ν_{max}/cm^{-1} 3000, 1712, 1653, 1618-1600; HRESIMS $[M+Na]^+$ Found: 337.0691. Calc. for $C_{17}H_{14}O_6Na$: 337.0688; ^1H NMR and ^{13}C NMR spectral data: see Table 1.

Table 1. ^1H and ^{13}C NMR chemical shift (δ in ppm) assignments for compound **1** in $CDCl_3$[a]

Number	^1H NMR δ_H	^{13}C NMR δ_C
1		161.4
2	6.60 (dd, *J* 1.5 and 0.6)[b]	111.7
3		148.6
4	6.68 (dd, *J* 1.5 and 0.6)	107.1
5	6.88 (d, 2.4)	101.4
6		164.7
7	6.86 (d, 2.4)	112.1
8		169.3
9		179.7
10		158.0
4a		155.7
5a		135.0
8a		111.3
9a		106.6
Me	2.41 (s)	22.5
OMe-8	4.01 (s)	53.1
OMe-6	3.93 (s)	56.1
OH	12.27 (s)	

[a]Spectra were recorded at 300 MHz for ^1H NMR and 75 MHz for ^{13}C NMR. [b]Multiplicity and coupling constant *J* in Hz are in parenthesis.

*Isosulochrin (**2**)*

Yellowish amorphous solid; mp 180-185 °C (MeOH); lit.[17] 182-189 °C; $C_{17}H_{16}O_7$; ^1H NMR (300 MHz, $CDCl_3$) δ 10.2 (1H, br s, OH), 6.89 (1H, d, *J* 2.1 Hz, H-4), 6.54 (1H, d, *J* 2.1 Hz, H-6), 6.13 (2H, s, H-5', H-3'), 3.52, 3.64 (each 3H, s, CH_3O), 2.15 (3H, s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.5 (CO), 167.3 ($COOCH_3$), 161.0 (C-5), 160.2 (C-6', C-2`), 156.2 (C-1), 147.9 (C-4'), 131.2 (C-3), 122.4 (C-2), 109.8 (C-1'), 108.5 (C-5', C-3'), 107.1 (C-4), 105.5 (C-6), 55.4 (CH_3O), 52.2 (CH_3O), 21.8 (CH_3).

*Chloroisosulochrin (**3**)*

Yellowish amorphous solid; mp 166-169 °C (MeOH); lit.[17] 170-173 °C. $C_{17}H_{15}O_6Cl$, ESIMS *m/z* 367.2 $[M-H]^-$, ^1H NMR (300 MHz; CD_3OD) δ 7.15 (1H, s, H-4); 6.13 (2H, s, H-5', H-3'); 3.68, 3.92 (each 3H, s, CH_3O), 2.19 (3H, s, CH_3); ^{13}C NMR (75 MHz; CD_3OD) δ 200.6 (CO), 167.5 ($COOCH_3$), 163.3 (C-6', C-2'), 156.5 (C-5), 151.8 (C-1), 149.5 (C-4'), 129.5 (C-3), 127.2 (C-6), 115.7 (C-2), 110.5 (C-1'), 108.8 (C-5', C-3'), 104.8 (C-4), 55.4 (CH_3O), 52.2 (CH_3O), 21.8 (CH_3).

Antimicrobial bioassay

The antimicrobial properties of compounds **1**, **2** and **3** were evaluated by the disc diffusion method.[19,20] The study was carried out with the following microorganisms species: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosas* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans.*

The compounds **1**, **2** and **3** were weighed under aseptic conditions in sterile flasks and dissolved with DMSO to obtain 5 mg mL^{-1} solutions. These solutions were impregnated on sterile paper discs of 6 mm diameter (20 µL *per* disc) and the discs were let to dry overnight to remove any residual solvent. The solvent control (DMSO) did not show any antimicrobial activity.

Seeded agar plates were prepared and inoculated with 0.1 mL of inoculum. Discs were then placed on the seeded agar plates. The zones of growth inhibition around the discs were measured after 24 h of incubation at 37 °C. All determinations were made in triplicate.

Results and Discussion

The MeOH extract obtained from the cultivation of *Pestalotiopsis guepinii* afforded after chromatographic separation, three anthraquinones derivatives (Figure 1).

The compounds **2** and **3** in conformity with literature refer to isosulochrin and chloroisosulochrin.[17] The molecular formula of compound **1** was established as $C_{17}H_{14}O_6$ (11 degrees of insaturation) on the basis of HRESIMS (*m/z* 337.0691 $[M+Na]^+$; Δ −0.3 mmu] analysis and the NMR data (Table 1). The UV spectrum showed absorption maxima at 255 and 313 nm. The IR spectrum showed characteristic absorption bands of a hydroxy group (3000 cm^{-1}), carbonyl of a lactone (1712 cm^{-1}), carbonyl

of a α, β-unsaturated ketone (1653 cm^{-1}) and characteristic absorption bands of aromatic ring (1618-1600 cm^{-1}). The ^{13}C NMR spectrum of **1** showed 17 signals attributed to: two carbonyls carbons (δ 158.0 and δ 179.7 ppm), eight non-hydrogenated carbons, four aromatics CH carbons, one aromatic methyl and two methoxyl groups (Table 1), identified by DEPT. The ^{1}H NMR spectrum of compound **1** (Table 1) showed a singlet at δ 12.27 ppm from a hydroxyl group H-bonded to a carbonyl, two doublets attributed to *meta*-coupled aromatic hydrogens, H-5 (δ 6.88 ppm) and H-7 (δ 6.86 ppm) and two double doublets at δ 6.60 (*J* 1.5 and 0.6 Hz) and δ 6.68 (*J* 1.5 and 0.6 *Hz*) from H-2 and H-4, respectively. The COSY spectrum confirmed the coupling between one hydrogen from the methyl group (δ 2.41 ppm) and the aromatic hydrogens (H-2 and H-4). The signals at δ 3.93 and 4.01 ppm are characteristic of OCH_3 groups. A nOe difference experiment with irradiation at δ 6.60 ppm (H-2) resulted in the enhancement of the signal of the CH_3 and also of phenolic OH and confirmed the position of the hydroxyl group. The confirmation of the position of the other substituents on the rings was obtained from HMBC data shown in Figure 2.

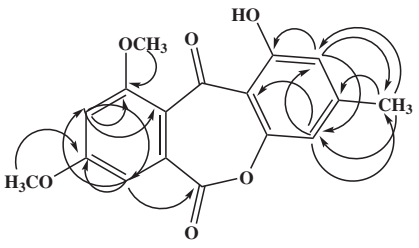

Figure 2. Key HMBC (H → C) correlations of compound **1**.

The antimicrobial activity of guepinone (**1**), isosulochrin (**2**) and cloroisosulochrin (**3**) were evaluated in the presence of *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*. Guepinone (**1**) and isosulochrin (**2**) were completely inactive against the microorganisms tested and cloroisosulochrin (**3**) was toxic only to *Staphylococcus aureus* (13 mm inhibition zone).

Supplementary Information

Supplementary information data (Figure S1-S33) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgements

We thank CAPES/PROCAD for financial support and a fellowship (M. N. O.), to CNPq and Fundação de Amparo a Pesquisa do Estado do Pará (FAPESPA) for financial support.

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Submitted: November 26, 2009
Published online: February 8, 2011

FAPESP has sponsored the publication of this article.

J. Braz. Chem. Soc., Vol. 22, No. 5, S1-S17, 2011.
Printed in Brazil - ©2011 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Supplementary Information

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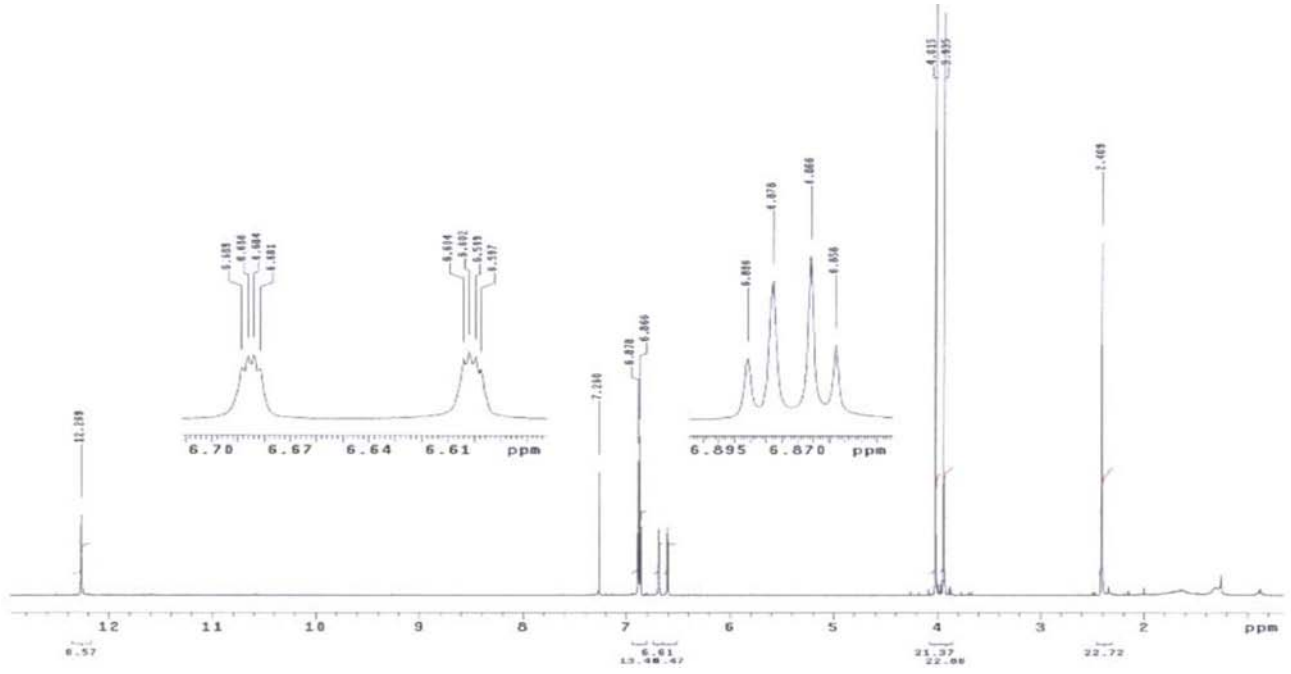

Figure S1. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of compound **1.**

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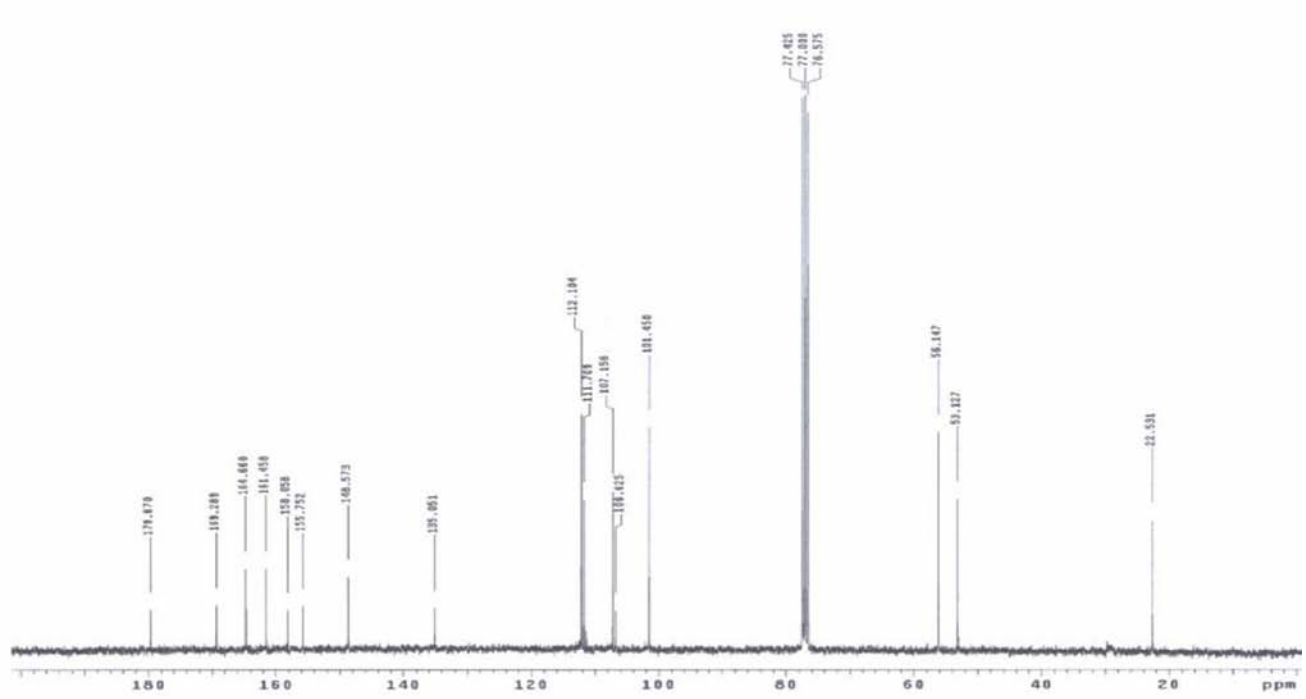

Figure S2. ^{13}C NMR spectrum (75 MHz, $CDCl_3$) of compound **1**.

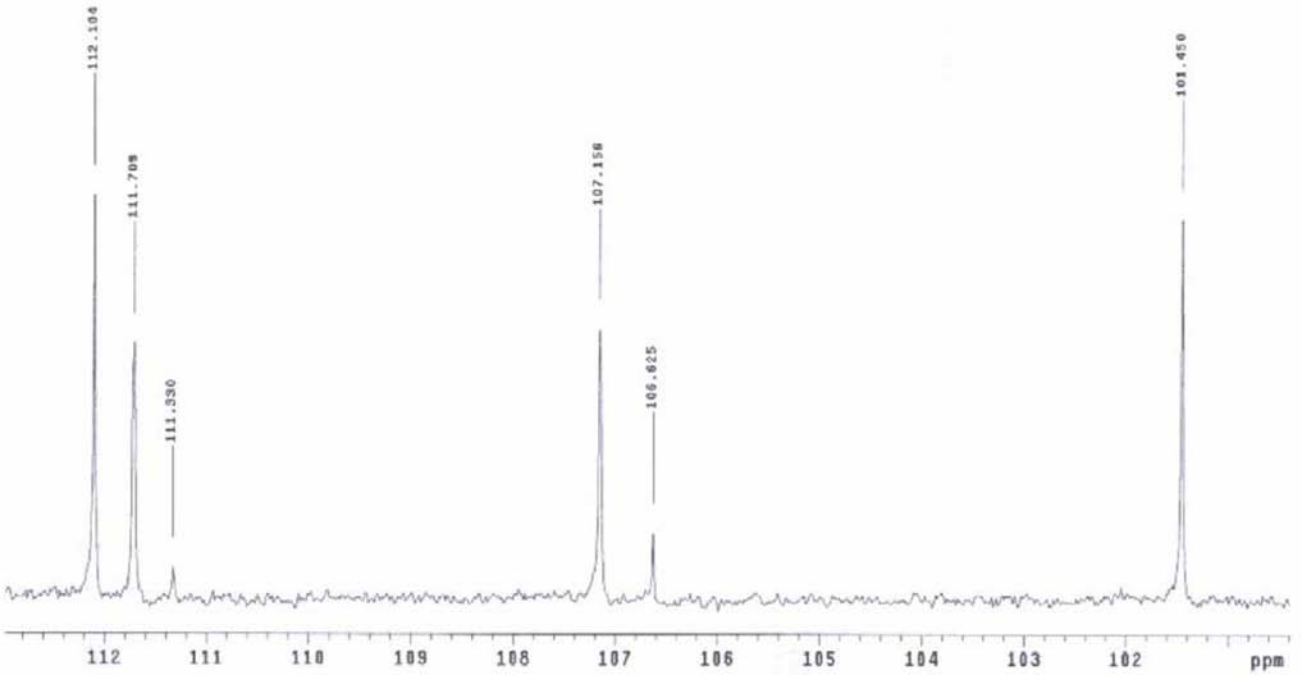

Figure S3. ^{13}C NMR spectrum (75 MHz, $CDCl_3$) of compound **1** (expansion).

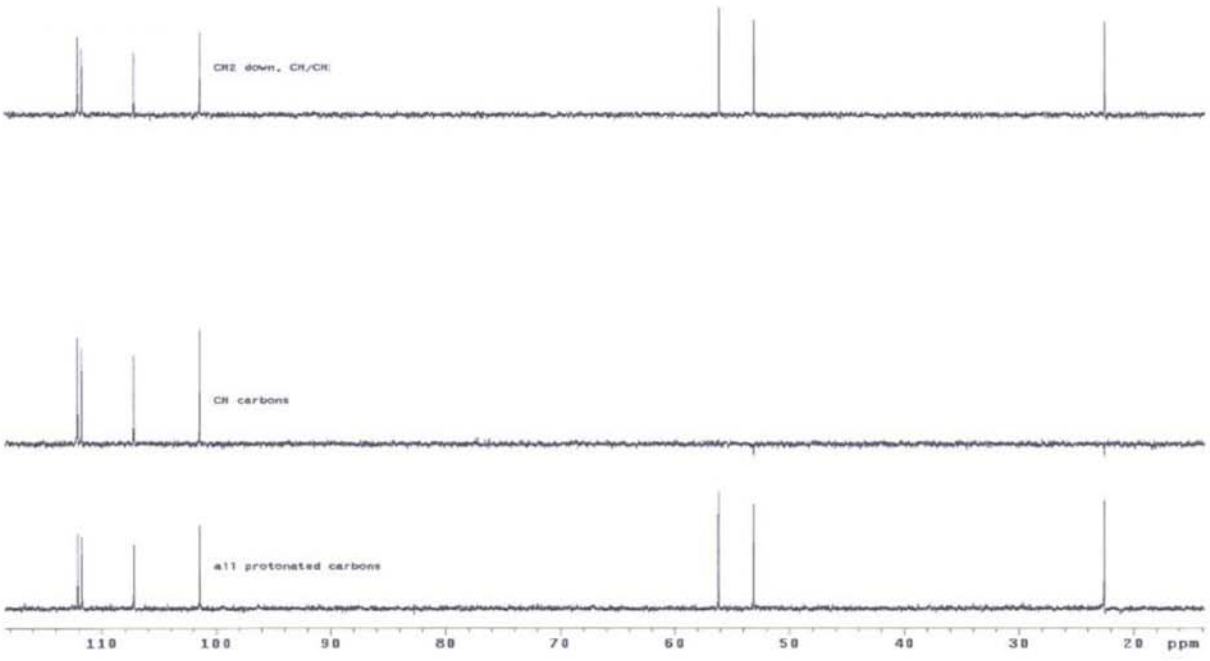

Figure S4. DEPT spectrum (75 MHz, $CDCl_3$) of the compound **1**.

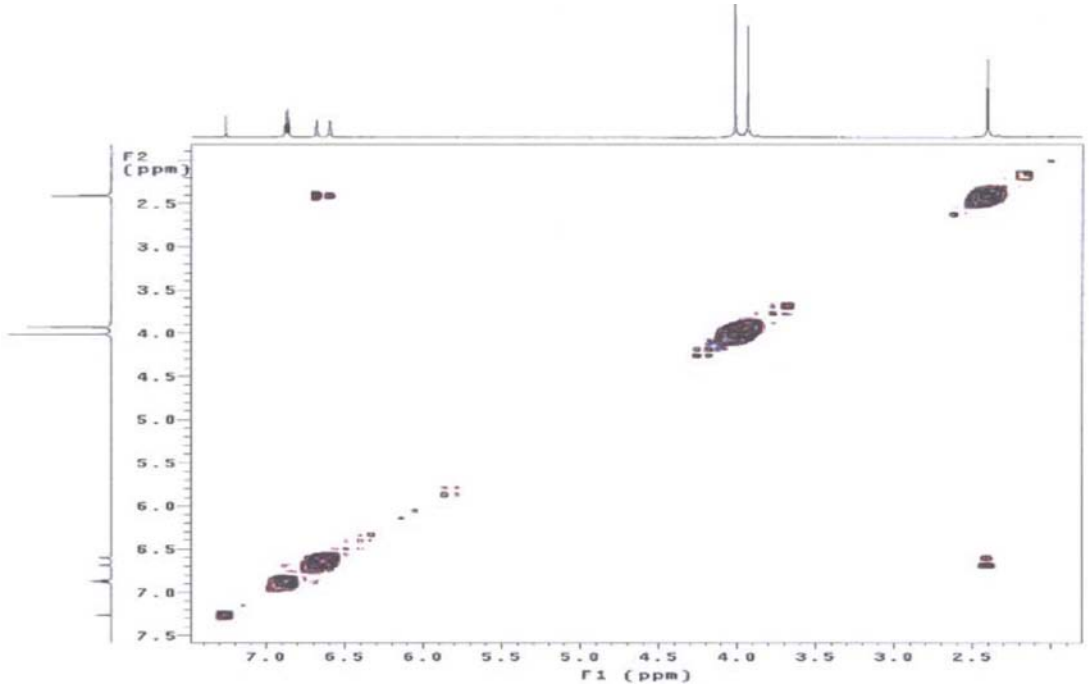

Figure S5. COSY spectrum (75 MHz, $CDCl_3$) of compound **1.**

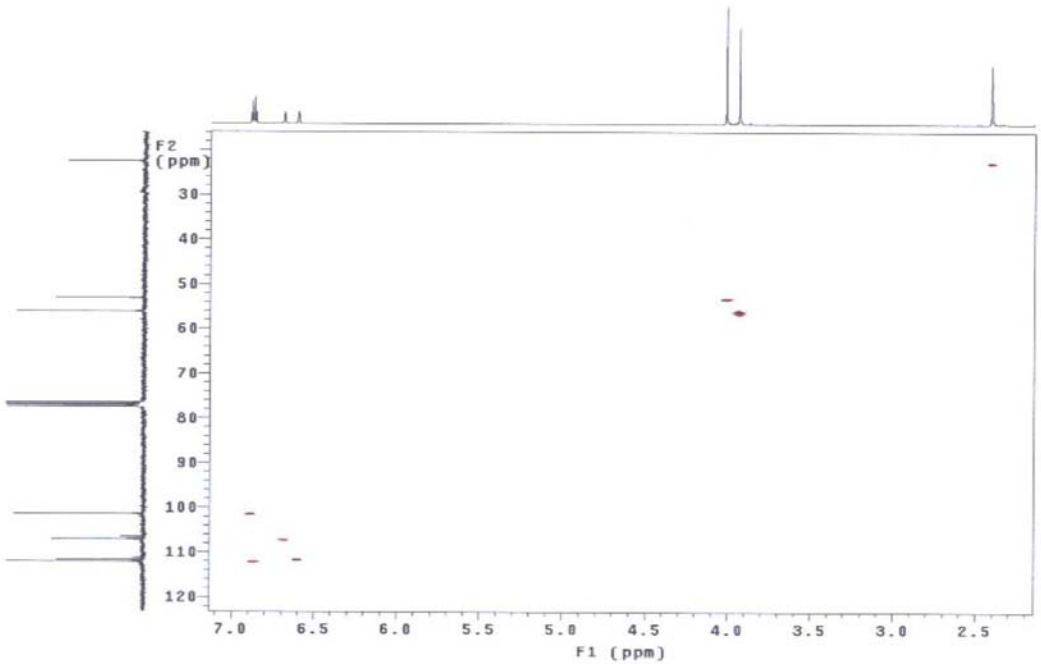

Figure S6. HETCOR spectrum ($CDCl_3$) of compound **1**.

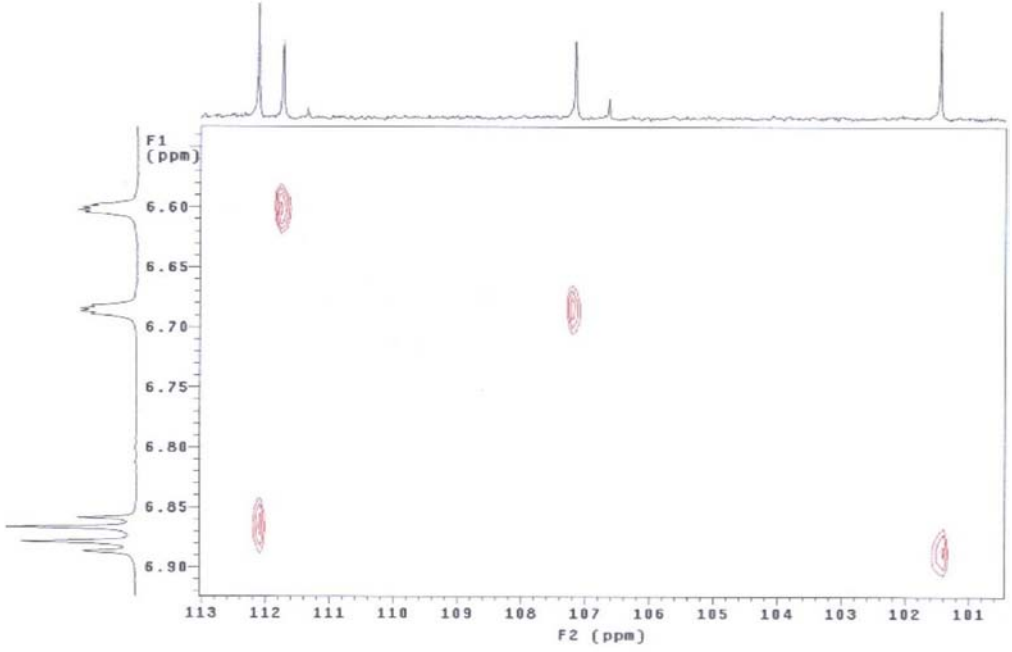

Figure S7. HETCOR spectrum ($CDCl_3$) of compound **1** (expansion).

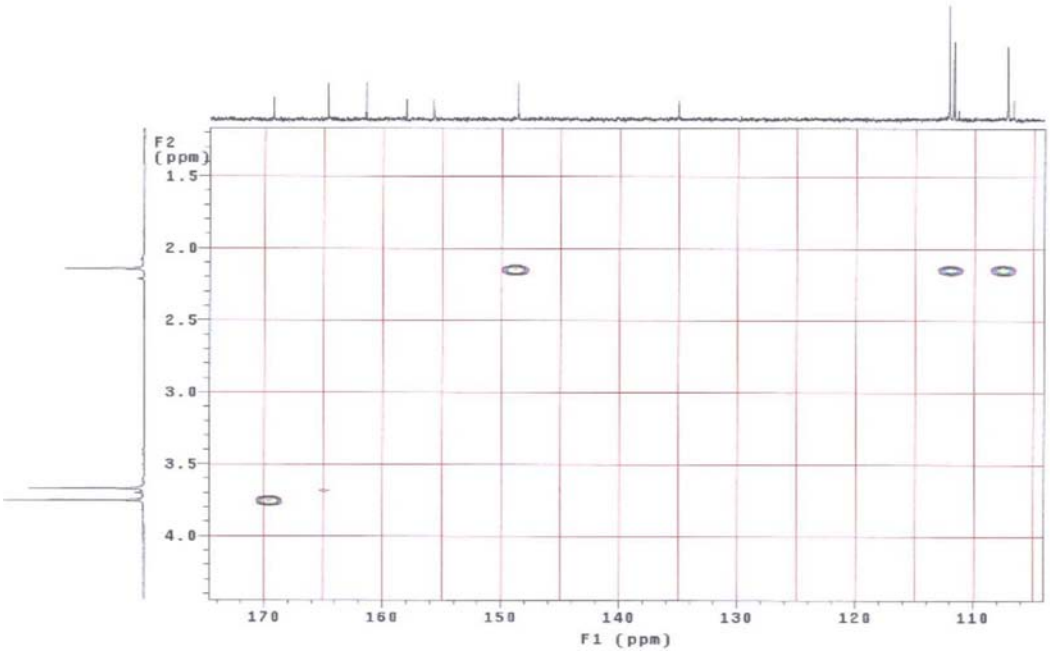

Figure S8. HMBC spectrum of compound **1** (expansion).

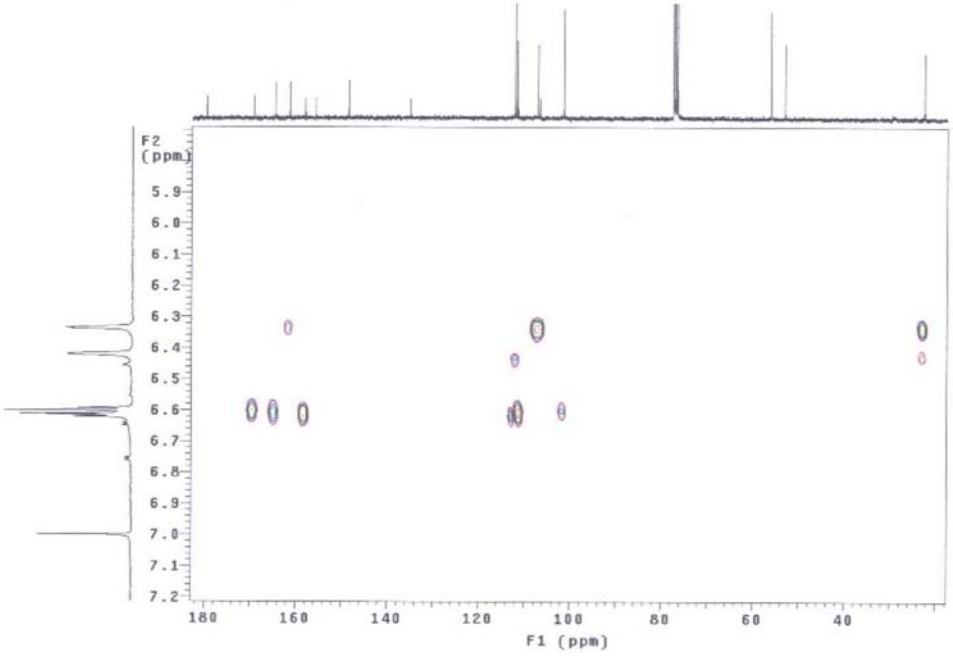

Figure S9. HMBC spectrum ($CDCl_3$) of compound **1** (expansion).

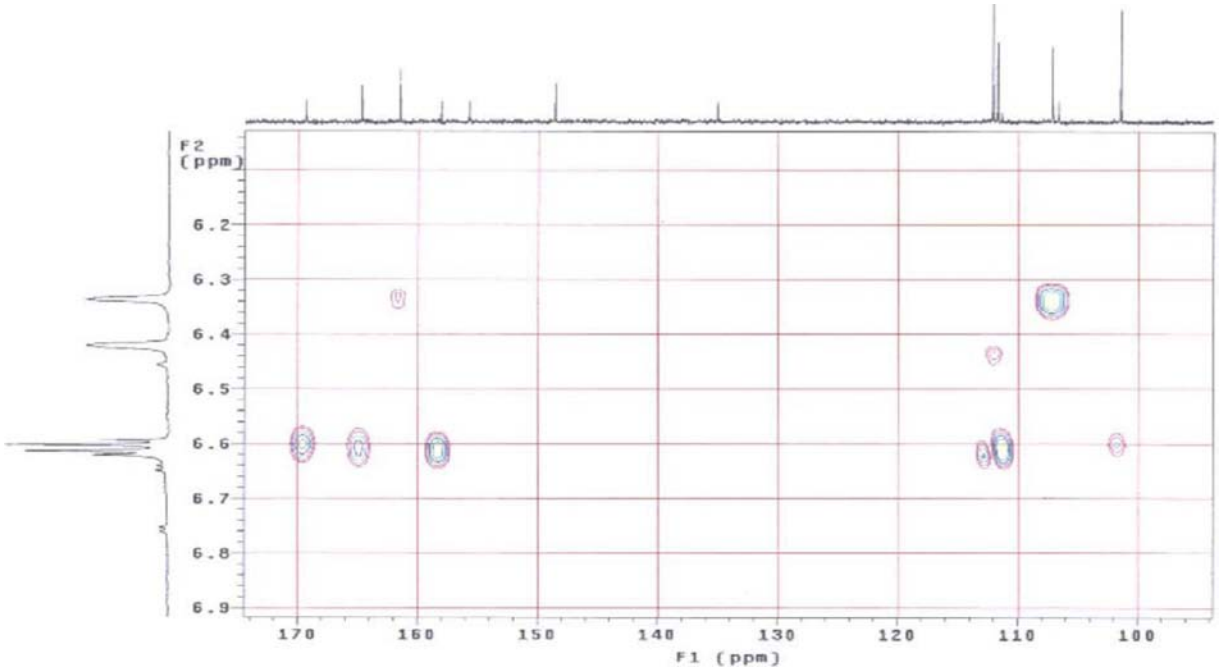

Figure S10. HMBC spectrum ($CDCl_3$) of compound **1** (expansion).

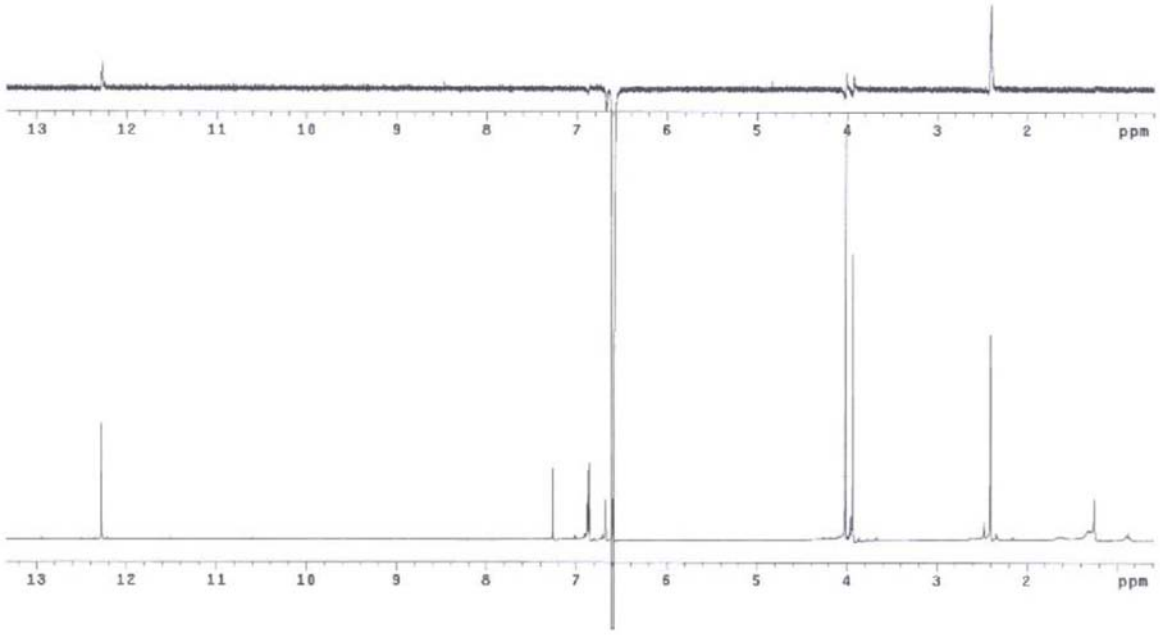

Figure S11. NOEDIF spectrum (irradiation at 6.60 ppm) of compound **1**.

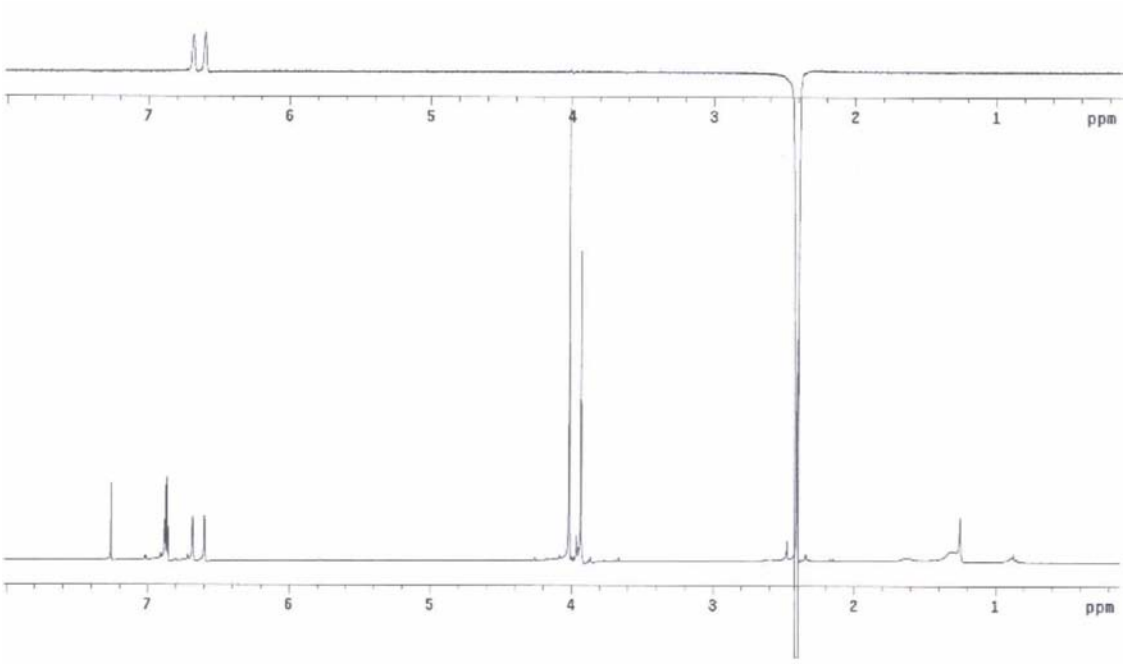

Figure S12. NOEDIF spectrum (irradiation at 2.41 ppm) of compound **1**.

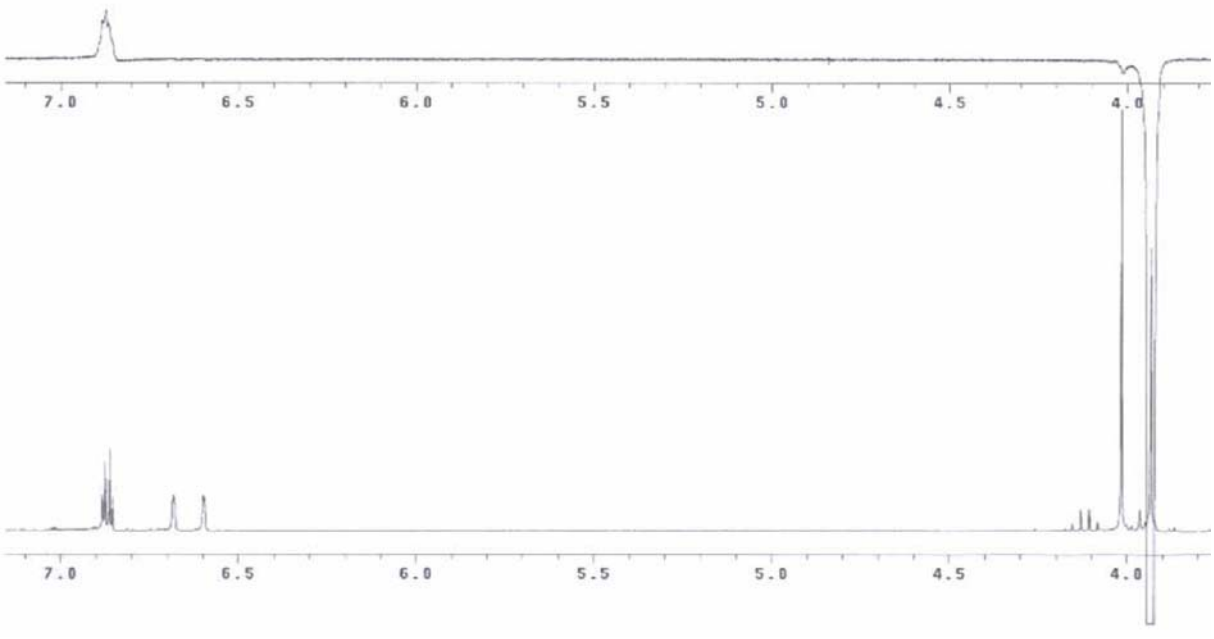

Figure S13. NOEDIF spectrum (irradiation at 3.93 ppm) of compound **1**.

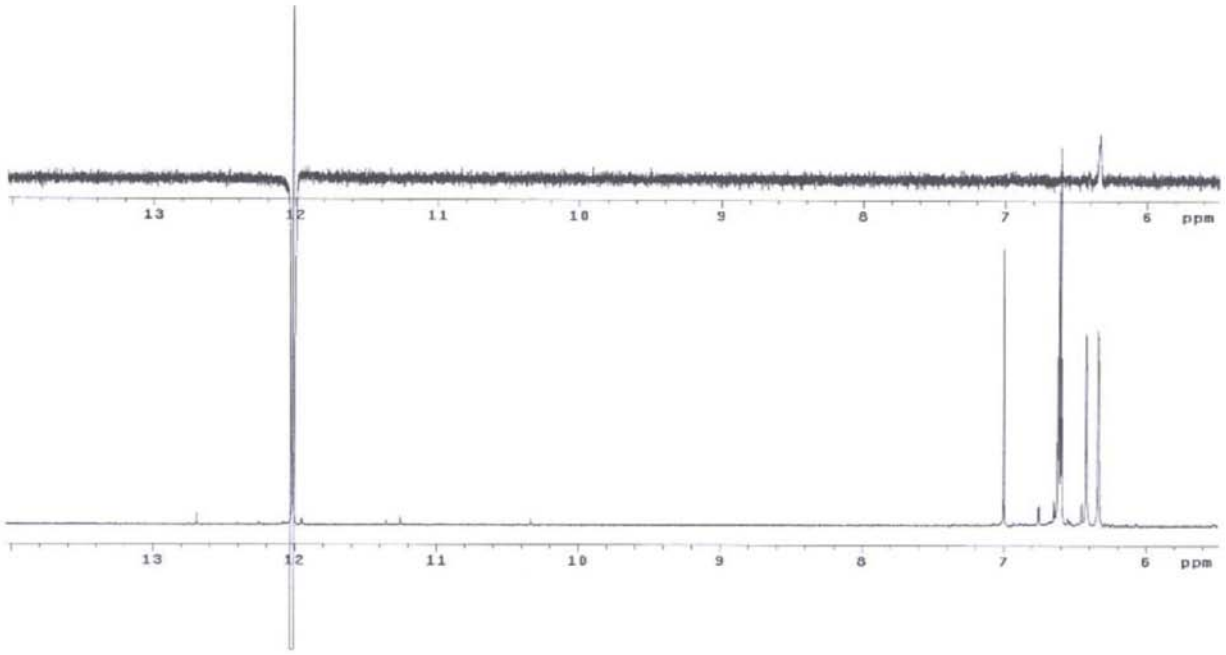

Figure S14. NOEDIF spectrum (irradiation at 12.0 ppm) of compound **1**.

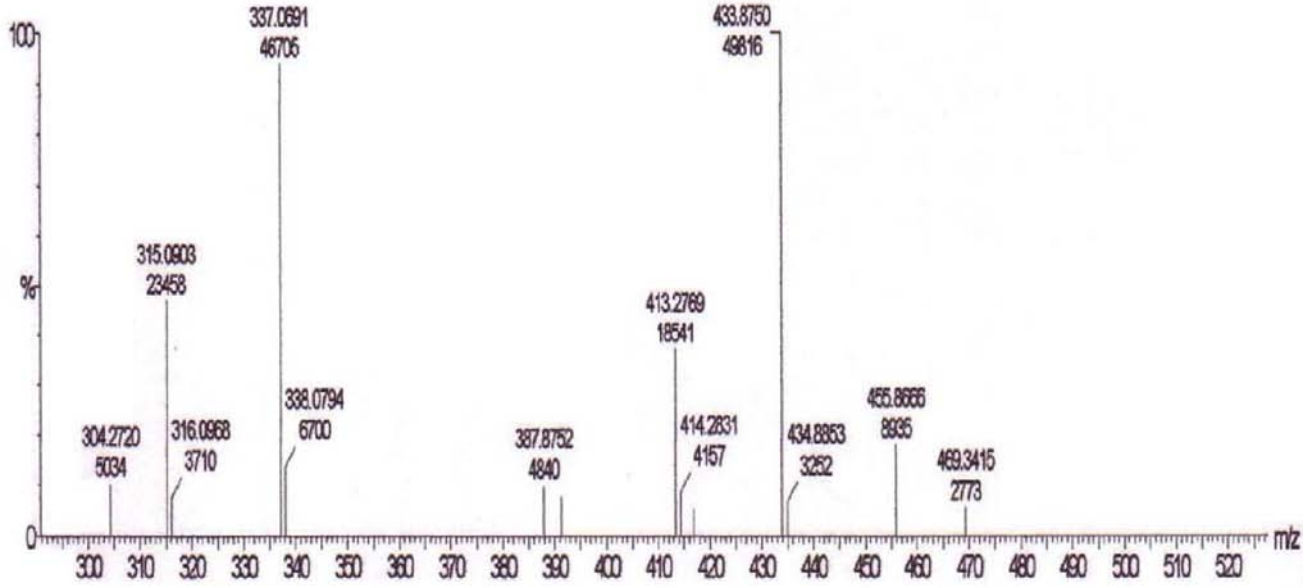

Figure S15. High resolution mass spectrum of compound **1**.

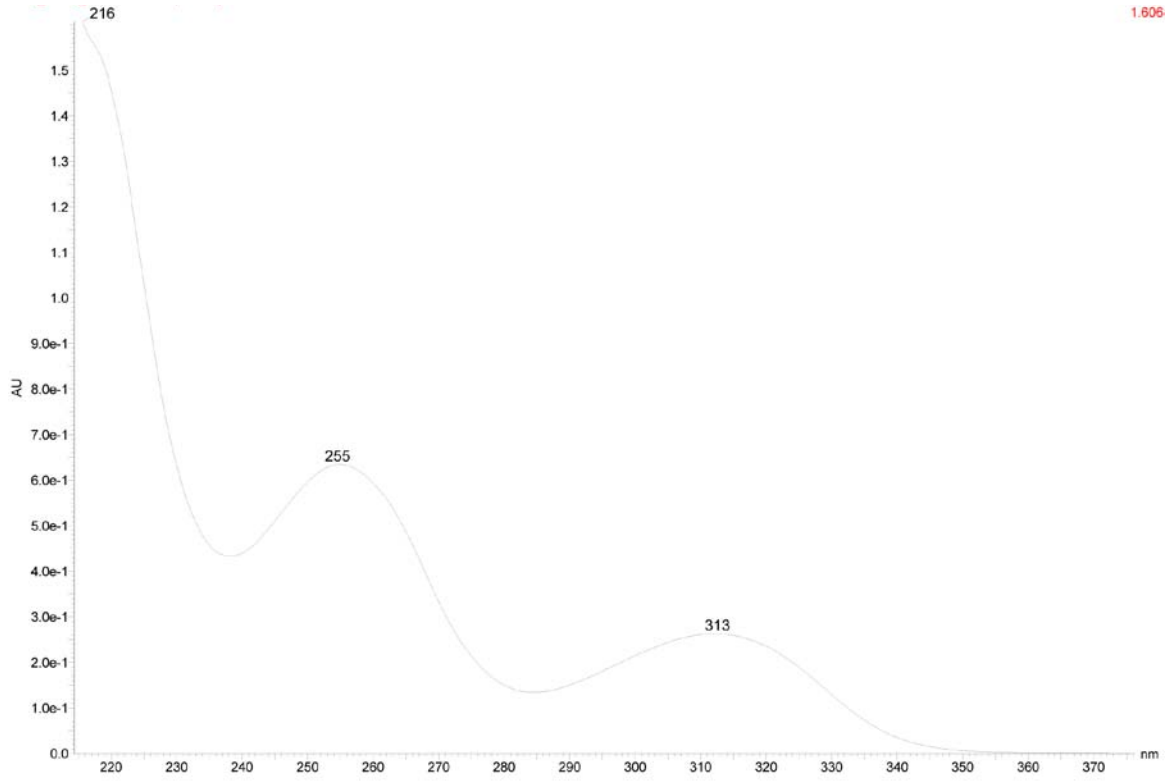

Figure S16. UV spectrum (MeOH) of compound **1**.

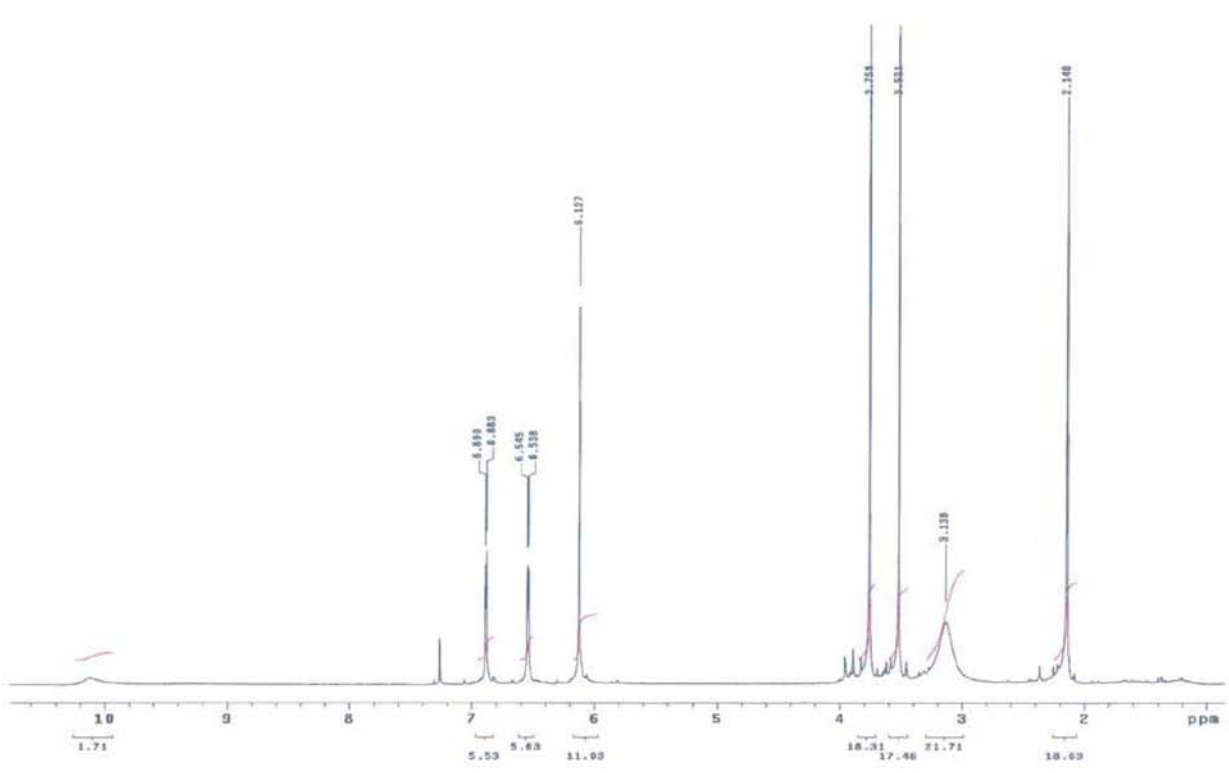

Figure S17. ^{1}H NMR spectrum (300 MHz, $CDCl_3$/drops CD_3OD) of compound **2.**

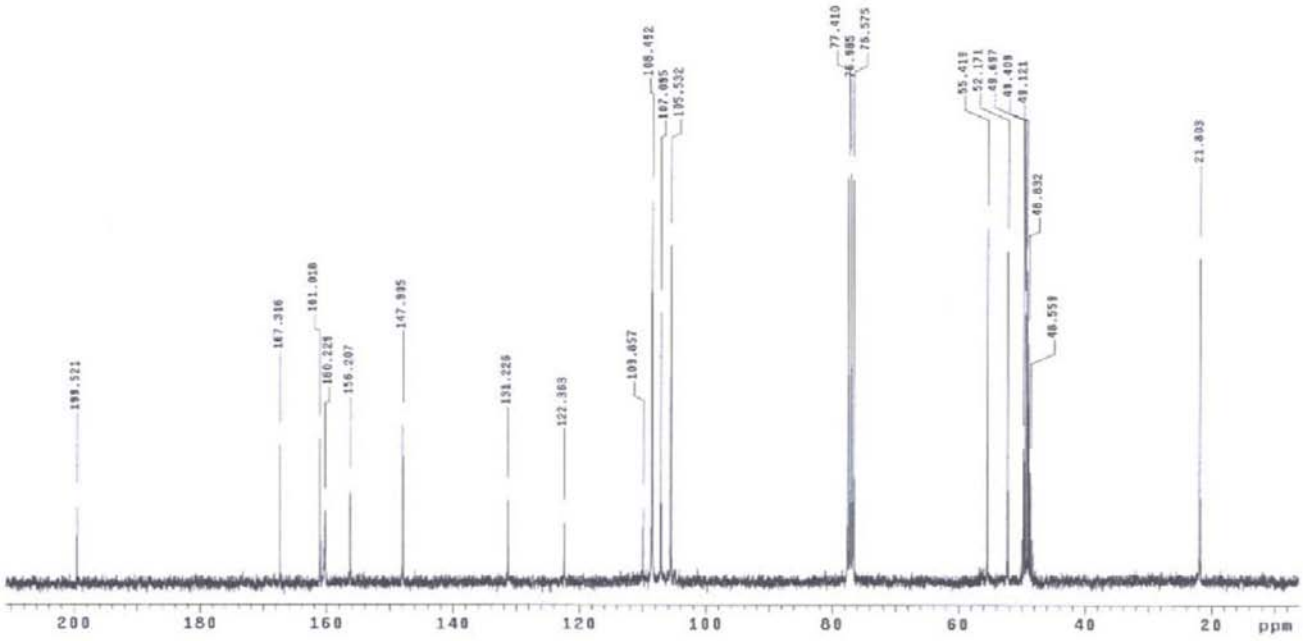

Figure S18. ^{13}C NMR spectrum (75 MHz, $CDCl_3$/drops CD_3OD) of compound **2**.

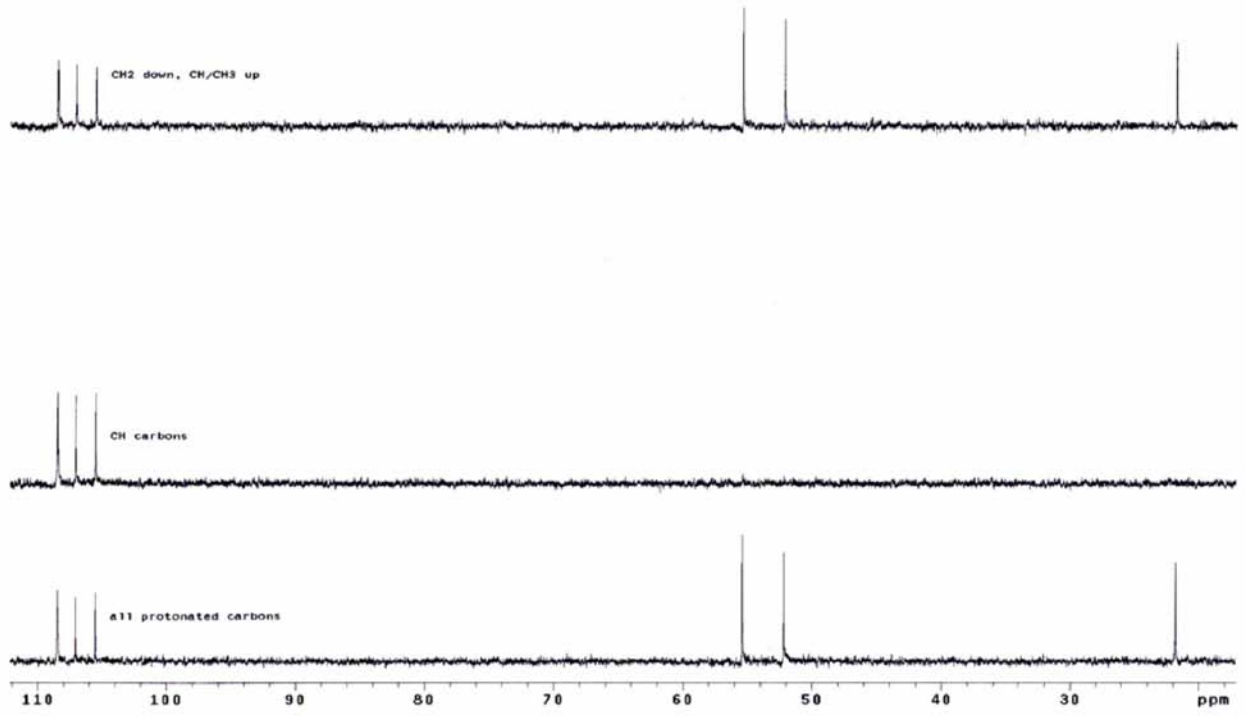

Figure S19. DEPT spectrum (75 MHz, $CDCl_3$/drops CD_3OD) of compound **2**.

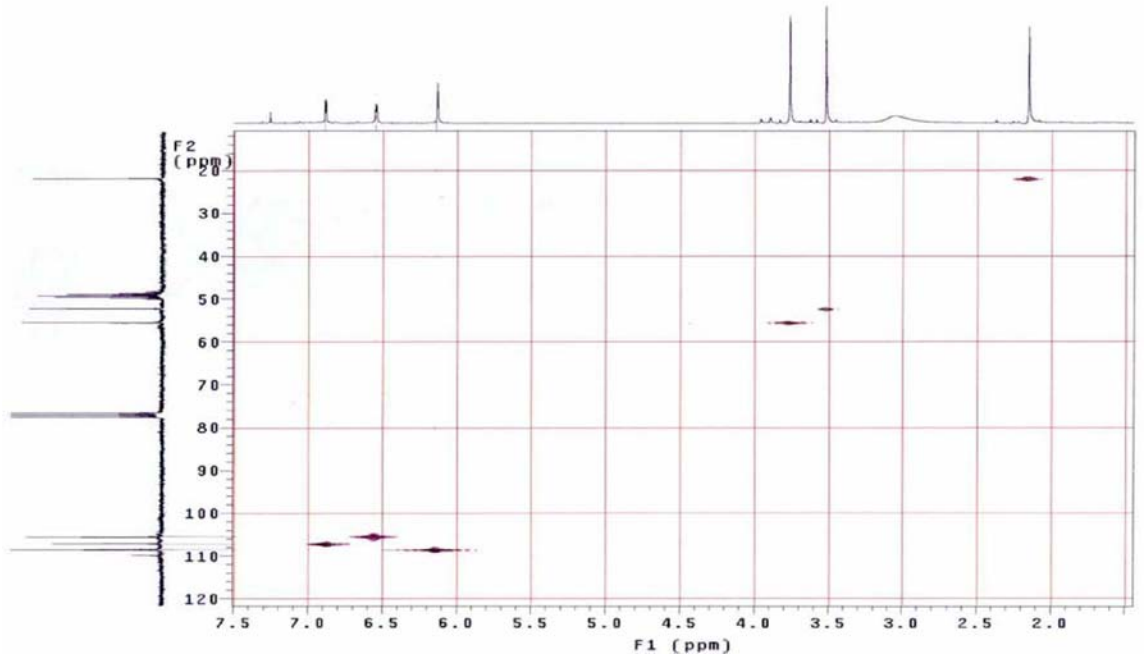

Figure S20. HETCOR spectrum ($CDCl_3$/drops CD_3OD) of compound **2**.

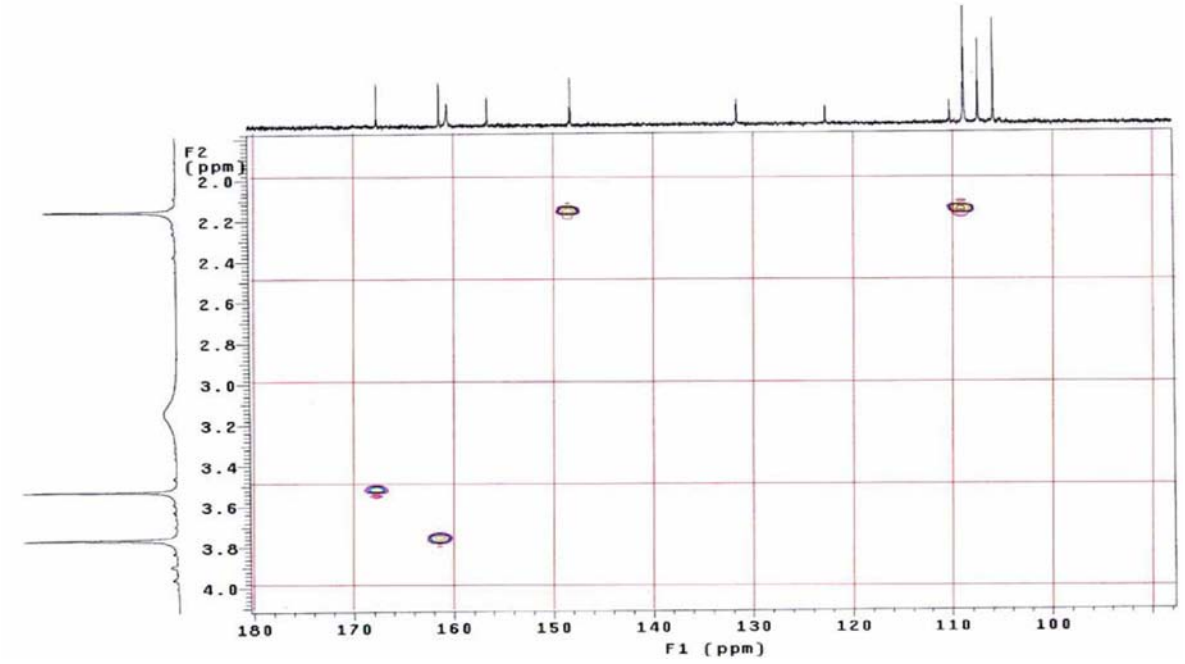

Figure S21. HMBC spectrum ($CDCl_3$/drops CD_3OD) of compound **2** (expansion).

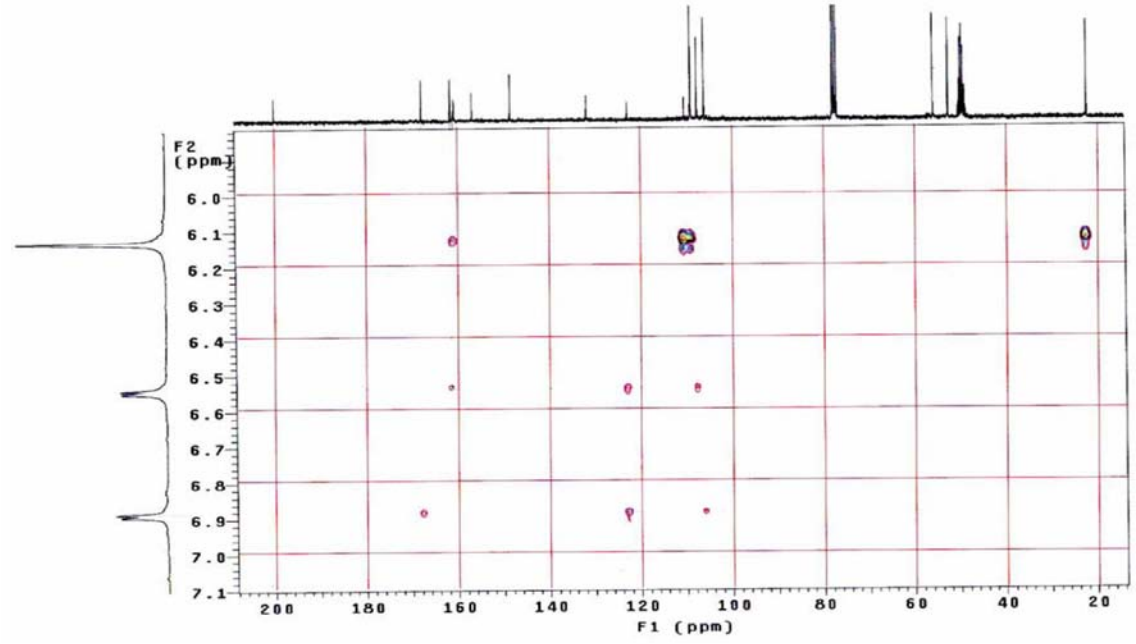

Figure S22. HMBC spectrum ($CDCl_3$/drops CD_3OD) of compound **2** (expansion).

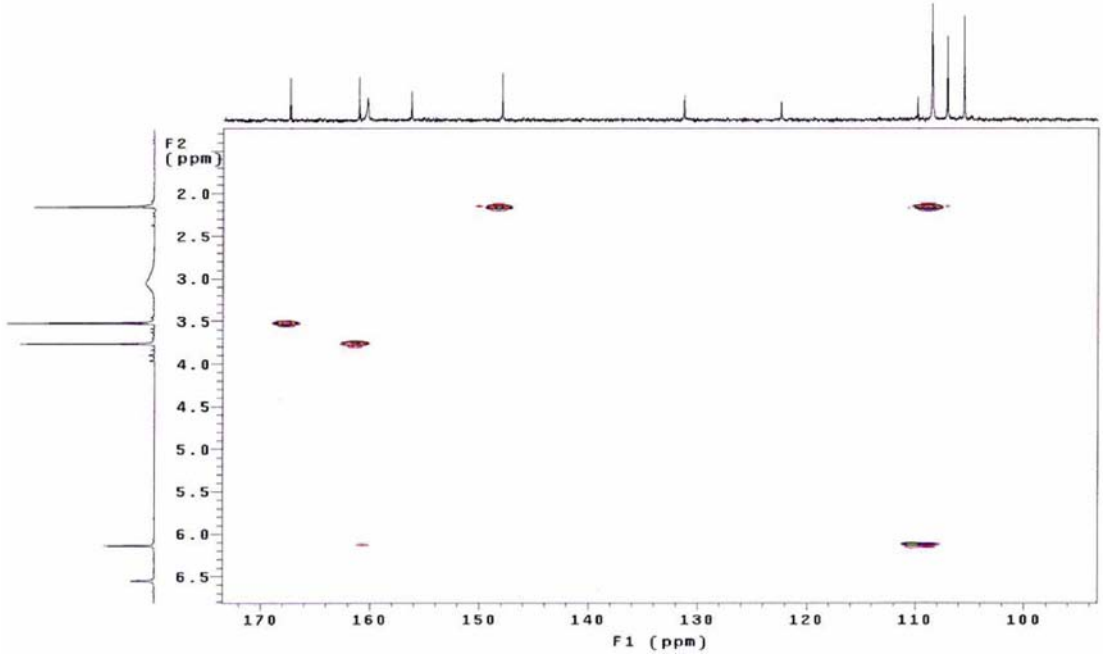

Figure S23. HMBC spectrum ($CDCl_3$/drops CD_3OD) of compound **2** (expansion).

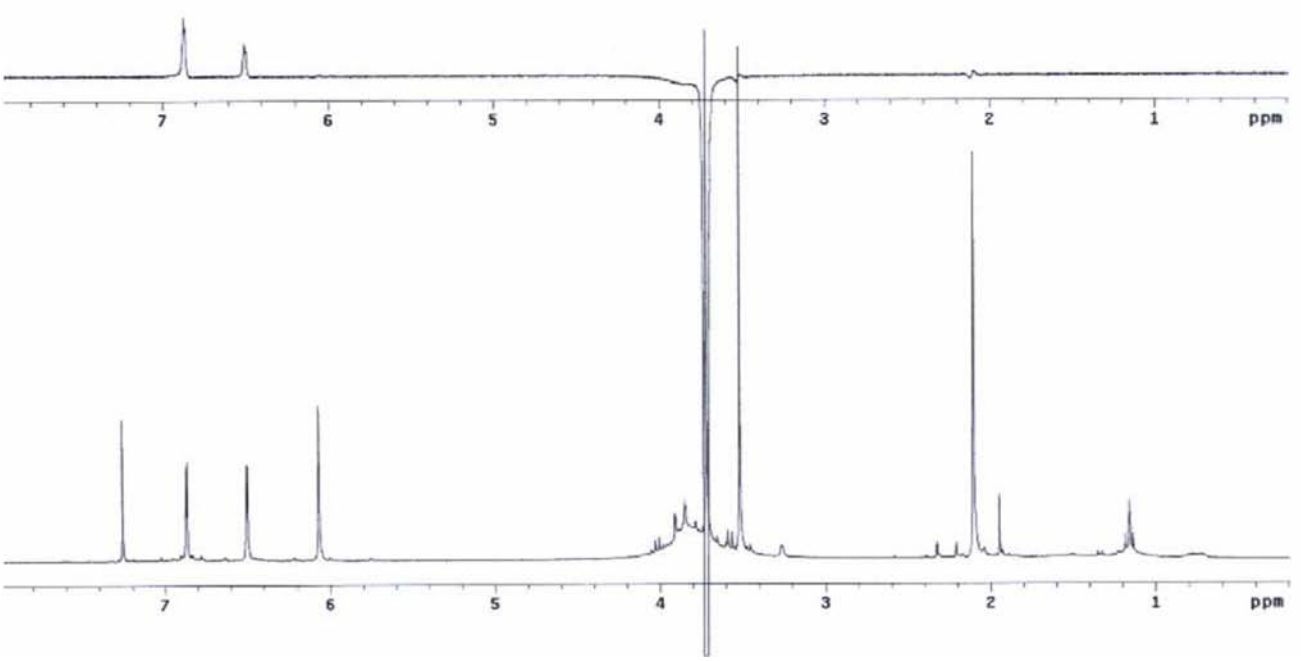

Figure S24. NOEDIF spectrum (irradiation at 3.76 ppm) of compound **2**.

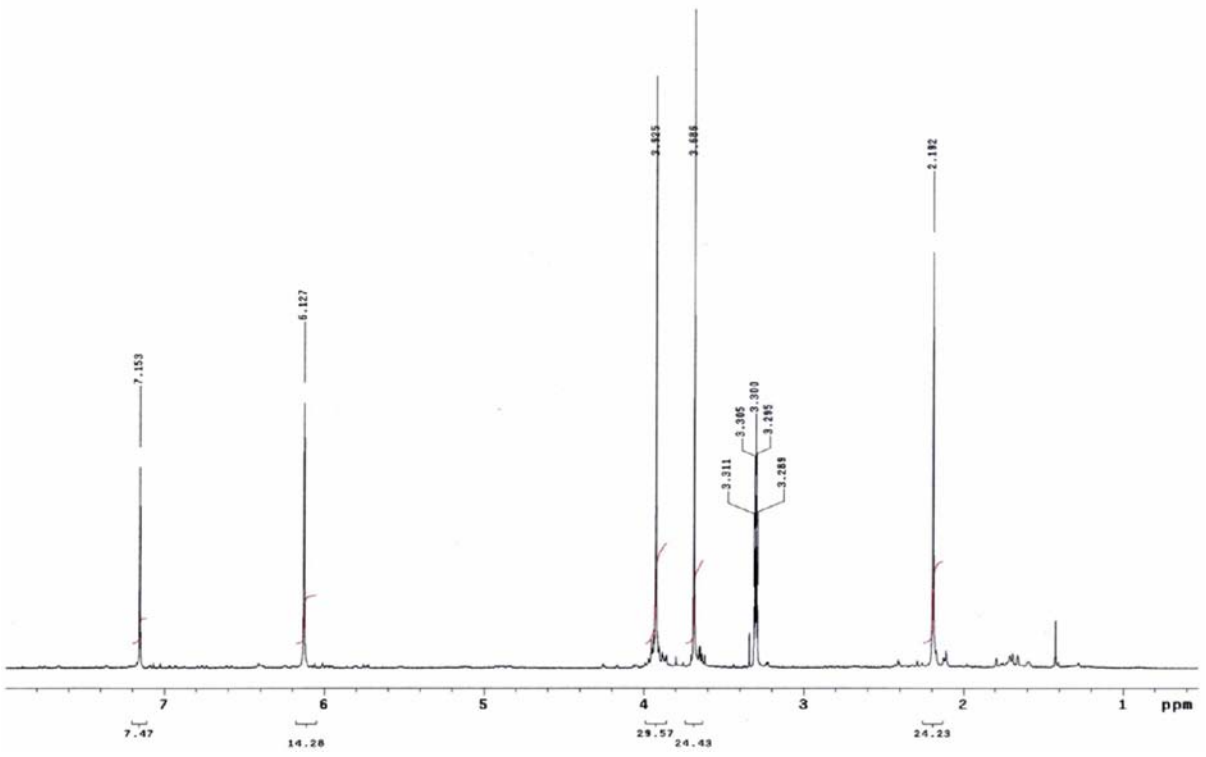

Figure S25. ^{1}H NMR spectrum (300 MHz, CD_3OD) of compound **3.**

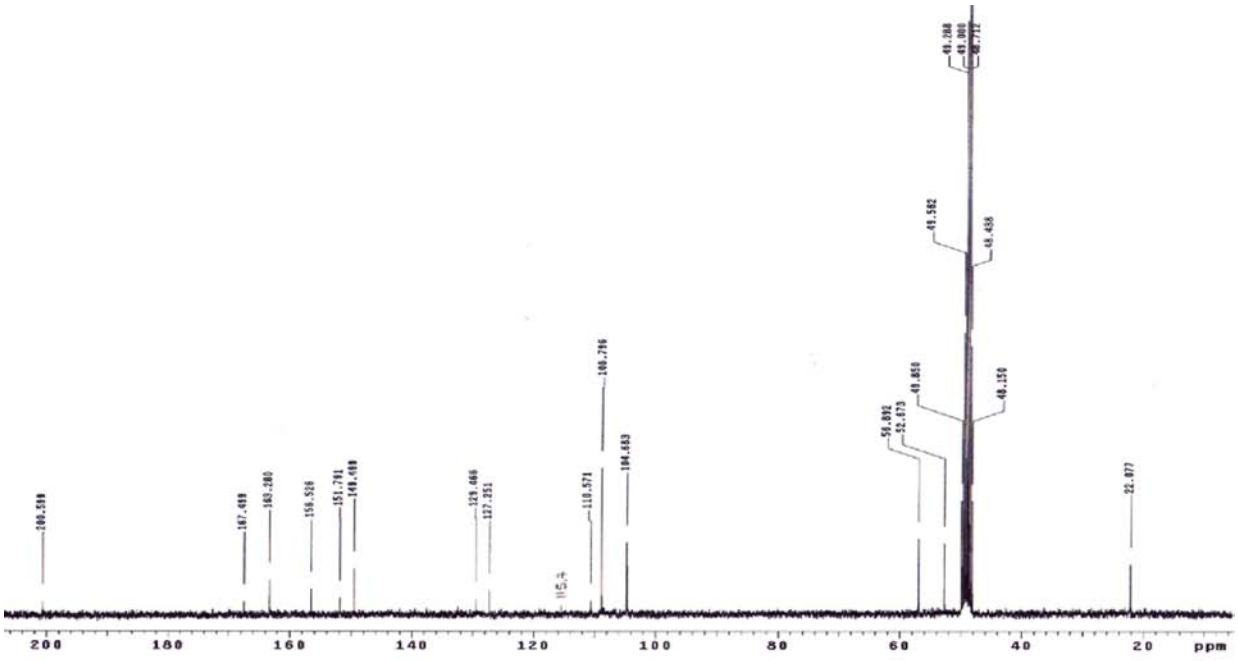

Figure S26. ^{13}C NMR spectrum (75 MHz, CD_3OD) of compound **3**.

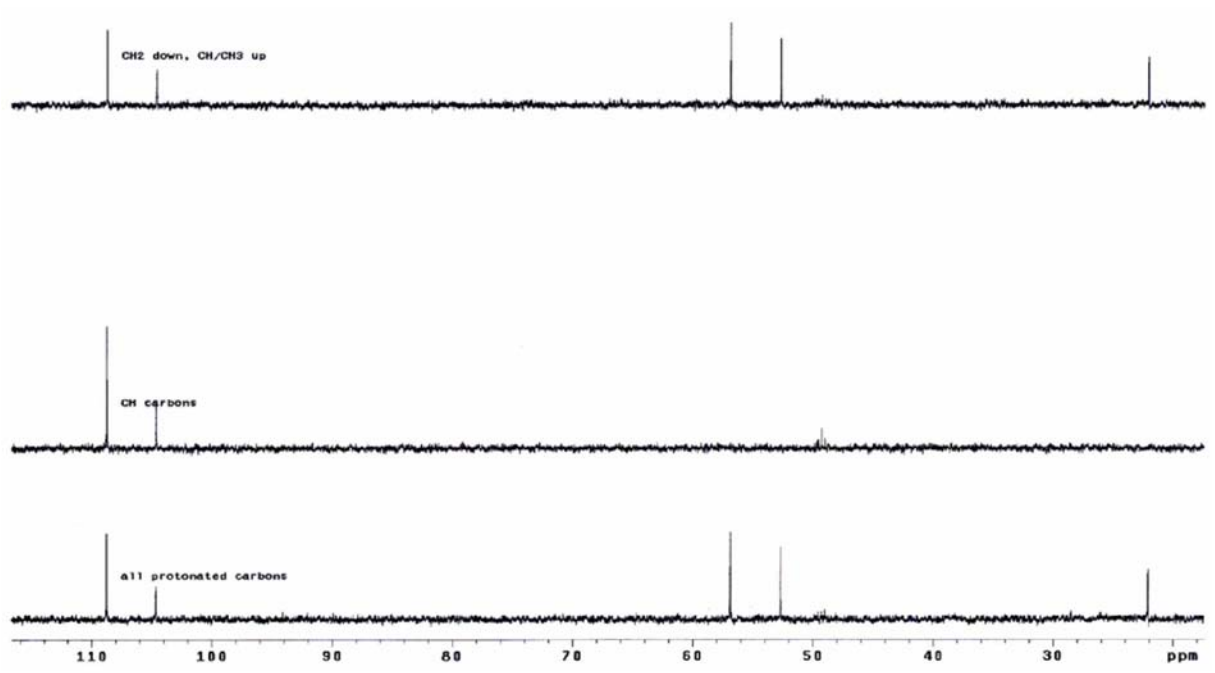

Figure S27. DEPT spectrum (75 MHz, CD_3OD) of compound **3**.

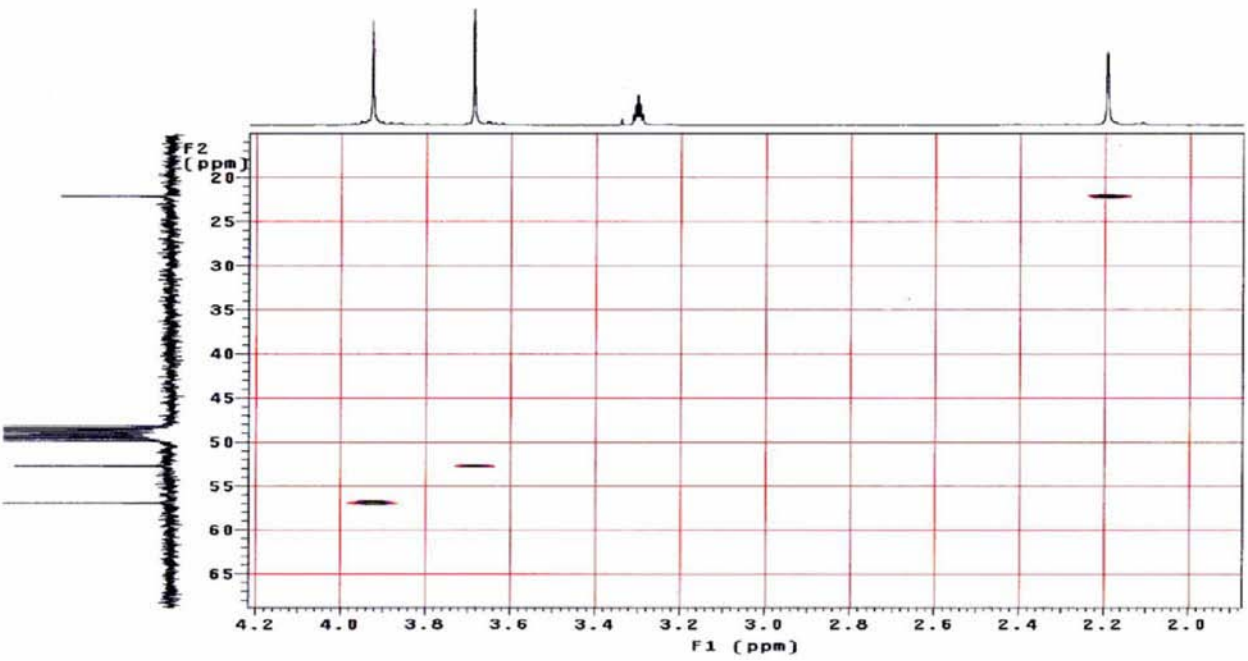

Figure S28. HETCOR spectrum (CD_3OD) of compound **3** (expansion).

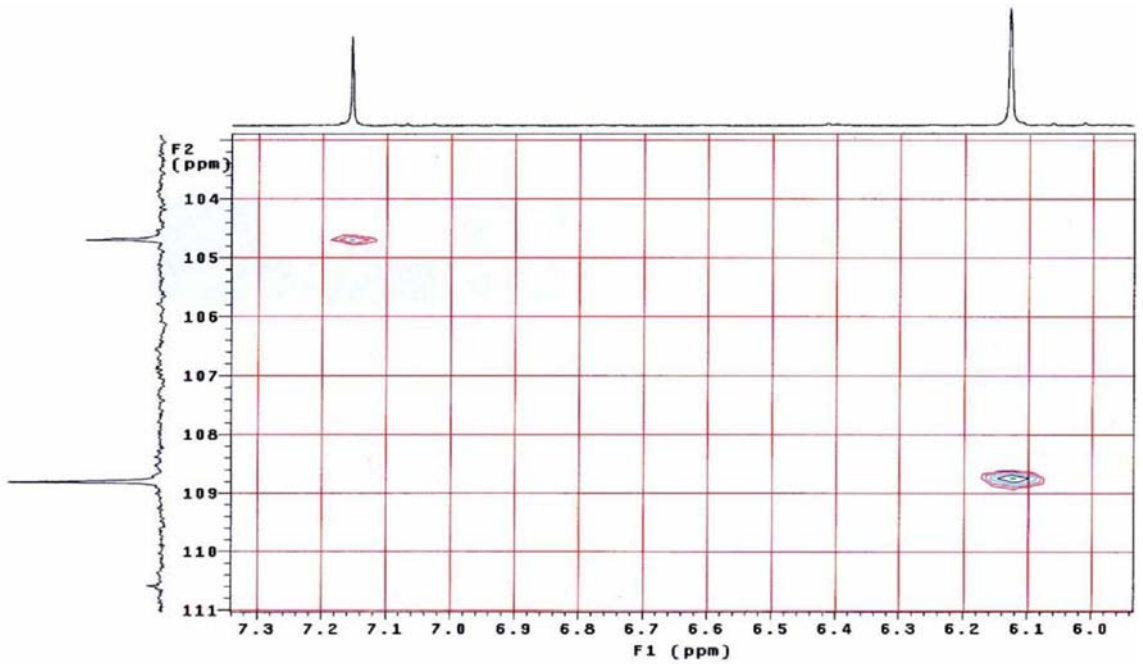

Figure S29. HETCOR spectrum (CD_3OD) of compound **3** (expansion).

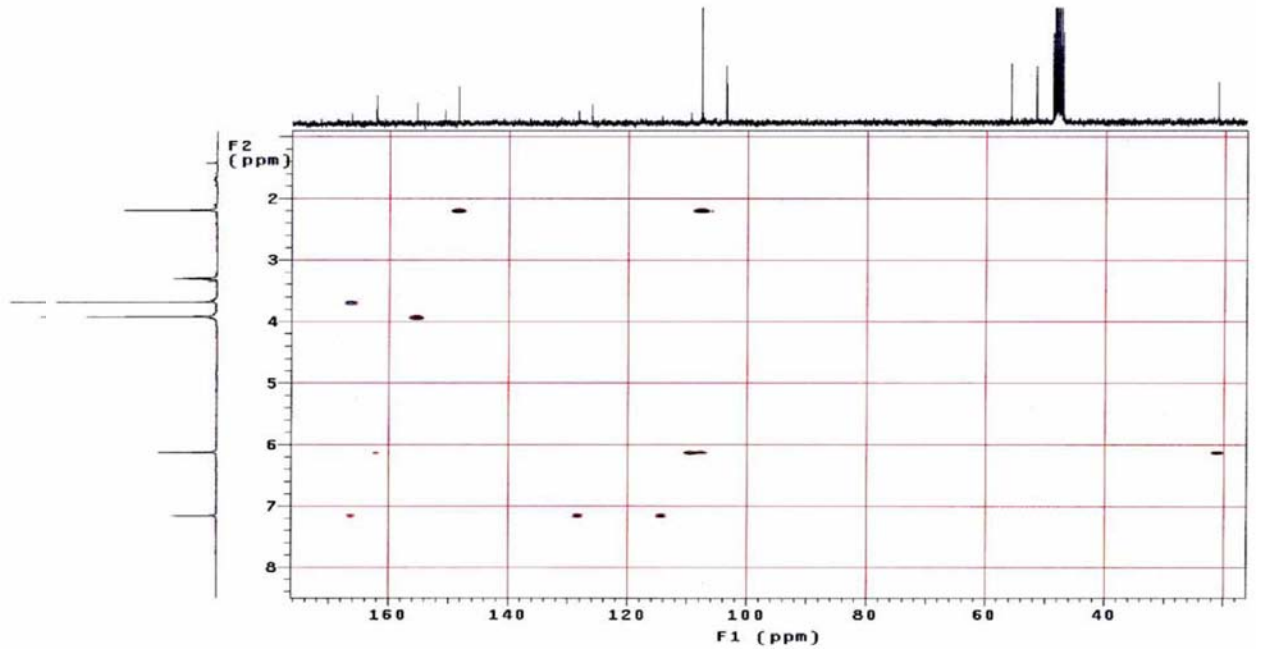

Figure S30. HMBC spectrum (CD_3OD) of compound **3**.

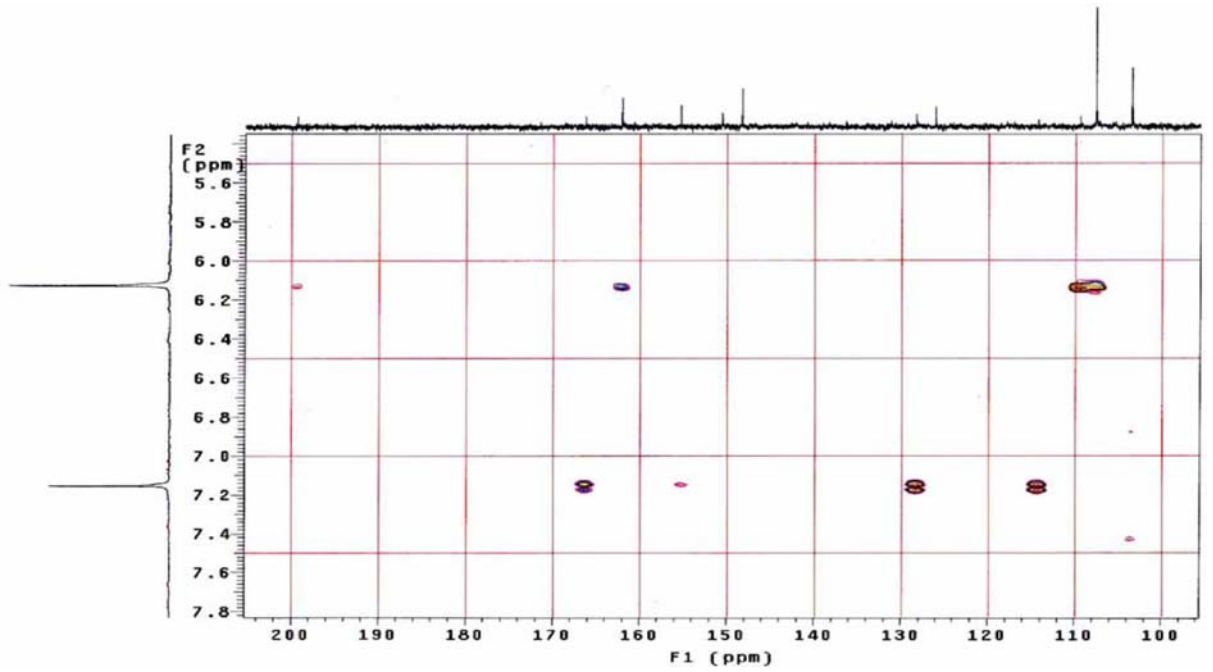

Figure S31. HMBC spectrum (CD_3OD) of compound **3** (expansion).

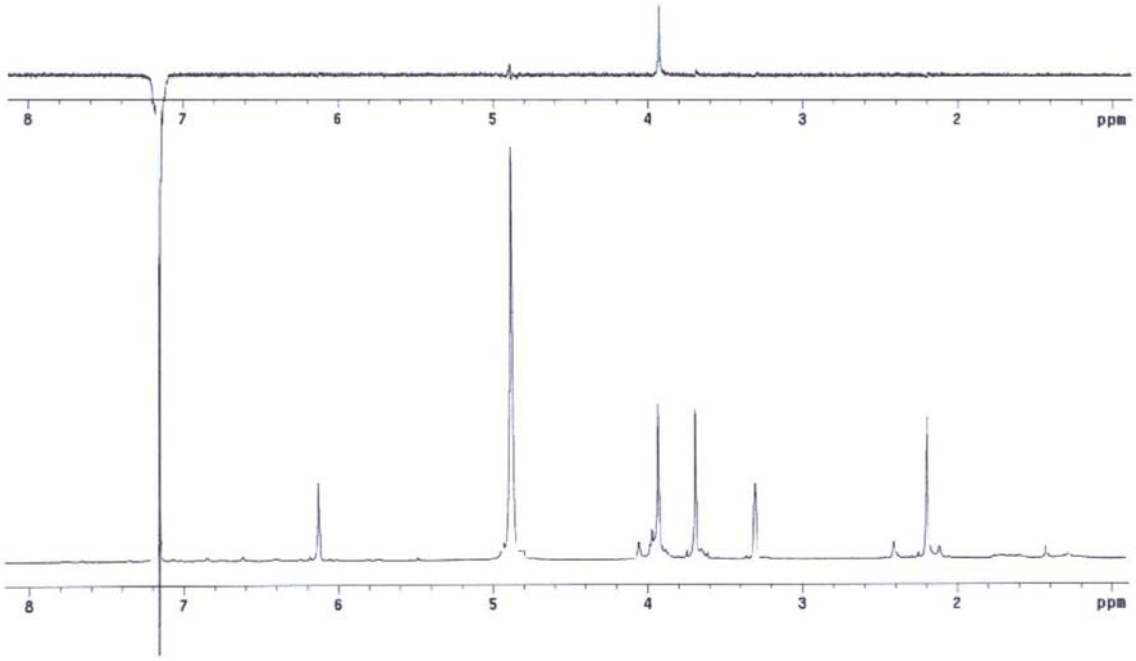

Figure S32. NOEDIF spectrum (irradiation at 7.15 ppm) of compound **3**.

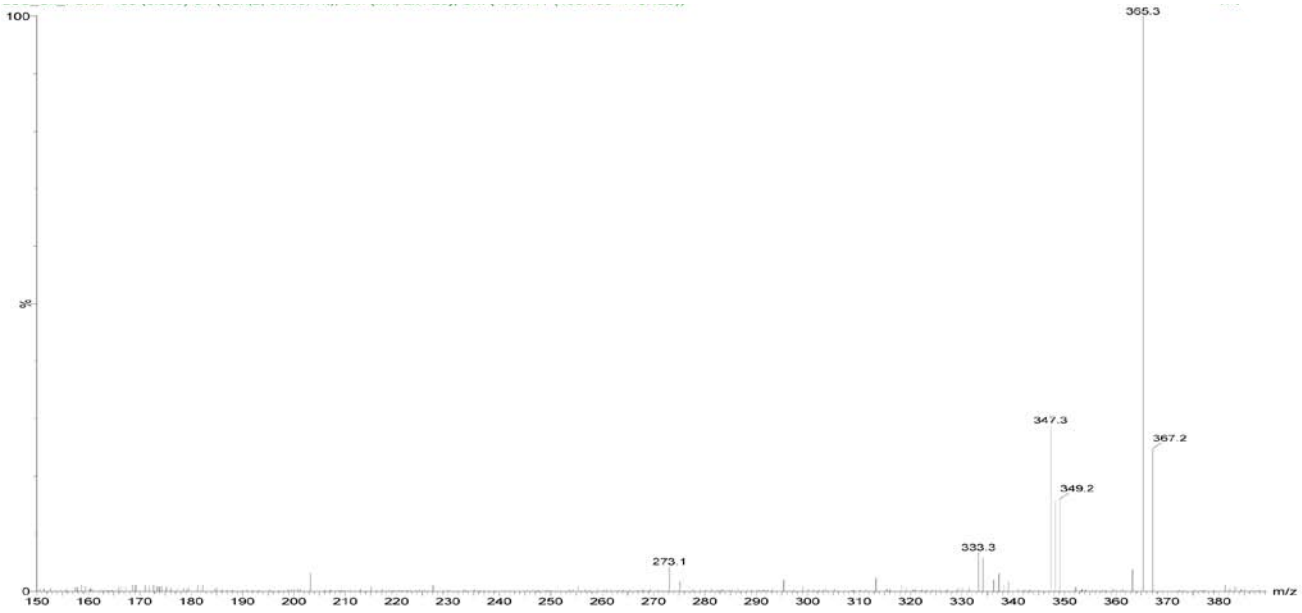

Figure S33. Low resolution mass spectrum of compound **3**.