SUPPLEMENTARY MATERIAL

Curvulin and spirostaphylotrichins R and U from extracts produced by two endophytic *Bipolaris* sp. associated to aquatic macrophytes with antileishmanial activity

Tiago Tognolli de Almeida[1,2]; Marcos Alessandro dos Santos Ribeiro[1,3]; Julio Cesar Polonio[1]; Francielle Pelegrin Garcia[4]; Celso Vataru Nakamura[4]; Eduardo Cesar Meurer[5]; Maria Helena Sarragiotto3; Débora Cristina Baldoqui[3]; João Lúcio Azevedo[6] and João Alencar Pamphile[1*]

[1] *Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, PR, Brazil*
[2] *Centro de Energia Nuclear na Agricultura (CENA), Universidade de São Paulo (USP), Piracicaba, SP, Brazil*
[3] *Departamento de Química, Universidade Estadual de Maringá, Maringá, PR, Brazil*
[4] *Programa de Pós-graduação em Ciências Biológicas, Laboratório de Inovação Tecnológica no Desenvolvimento de Fármacos e Cosméticos, Universidade Estadual de Maringá, Maringá, PR, Brazil*
[5] *Fenn Mass Spectrometry Laboratory, Universidade Federal do Paraná, Jandaia do Sul, PR, Brazil*
[6] *Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, SP, Brazil*

**Corresponding author:*
João Alencar Pamphile
Department of Biotechnology, Genetics and Cell Biology, State University of Maringá, Av. Colombo, 5790, Jardim Universitário, 87020-900, Maringá, Paraná, Brazil
Tel: +44-3011-4342
e-mail: prof.pamphile@gmail.com

Abstract: In the present study biological activity and chemical composition of two crude extracts of endophytic fungal strains of *Bipolaris* genera isolated from two species of aquatic macrophytes: *Eichhornia azurea* (Kunth) and *E. crassipes* (Mart.) were investigated. The nuclear magnetic resonance and mass spectrometry data provided the identification of three main compounds: curvulin (**1**), spirostaphylotrichin R (**2**) and U (**3**). The fragmentation mechanism of the precursor ions towards collision induced dissociation (CID) tandem mass spectrometry experiment (MS/MS) is also proposed. Furthermore, biological screening of the crude extracts displayed antileishmanial activity with IC_{50} values ranging from 70 to 84.2 $\mu g.mL^{-1}$.

Keywords: *Eichhornia azurea*; *Eichhornia crassipes*; endophytes; MS/MS fragmentation mechanism;

Experimental

Chemical characterization

^{1}H and ^{13}C-NMR spectra were recorded on a Varian Mercury Plus spectrometer operating at 300 MHz and 75.5 MHz, respectively, using $CDCl_3$ as solvent, and tetramethylsilane (TMS) as internal reference. TLC was performed on normal phase pre-coated silica gel 60 G or 60 GF254 (Merck) plates. Visualization of the compounds on TLC was accomplished by UV irradiation at 254 and 366 nm, and/or by spraying with a H_2SO_4/MeOH (1:1) or H_2SO_4/anisaldehyde/ acetic acid (1:0.5:50 mL) solution followed by heating at 100 °C.

The ^{1}H NMR spectrum from crude extracts showed high similarity, as seen in Figure S1. In addition, due to the high concentration of **1**, **2** and **3** in the crude extracts, were not necessary purification steps to identify these compounds using NMR and MS data. For this reason, only the spectra obtained for the extract of the fungus *Bipolaris* sp. AZ26 are shown in the Figures S2 to S20. The ^{1}H NMR spectrum of ethyl 2-acetyl-3,5-dihydroxyphenylacetate (curvulin) (**1**) showed signals for aromatic hydrogens at δ_H 6.32 and 6.34 (each 1H, br s) relative to H-8 and H-6, a methylene group attached to a carbonyl at δ_H 3.80 (2H, s), an acetyl group at δ_H 2.55 (3H, s), and an carboethoxy group at δ_H 1.23 (3H, t) and 4.13 (2H, q).

The ^{13}C NMR spectrum displayed two carbonyl carbons at δ_C 203.6 and 171.6 of ester and acetyl groups, respectively, two phenolic carbons at δ_C 161.8 and 164.8, a methylene group at δ_C 41.7, and an ethoxy group at δ_C 14.3 and 61.6.

The chemical shift values for hydrogens and carbons were in accordance with the literature data of **1** (Varma et al. 2006).

Compounds spirostaphylotrichin R (**2**) and U (**3**) displayed very similar resonances in the ^{1}H and ^{13}C NMR spectra. The difference observed between them were the signals in the ^{13}C NMR spectrum at δ_C 23.6 (C-11), 68.7 (C-4) and 86.6 (C-3) of spirostaphylotrichin R and at δ_C 18.7 (C-11), 73.1(C-4) and 90.5 (C-3) of spirostaphylotrichin U. Comparison of the obtained spectroscopic data with those reported confirmed the chemical structure of the compounds **2** and **3** as spirostaphylotrichin R and U, respectively (Abraham et al. 1995).

Mass spectra analysis of the EtOAc extract were performed at low resolution with electrospray ionization (ESI-MS) on a MICROMASS® Quattro MicroTM API. The mass spectra were recorded with ESI in the positive mode. The parameters were as follows: voltage of the employed capillar: 2.00 kV, cone voltage: 20 V, source temperature: 100 °C, desolvation temperature: 250 °C, desolvation gas flow rate: 400 L h^{-1}, cone gas flow rate: 100 L h^{-1}, scanning range: from 50 to 500 amu. These parameters were optimized in preliminary experiments to get the highest abundance of the targeted molecular-related ions. N_2 was used as both dry gas and nebulizer gas. As mentioned in the main text, the mass spectrum of EtOAc extract showed a mixture of three compounds with *m/z* 239.0 and *m/z* 298.0, attributed to curvilin (**1**) and spirostaphylotricin R (**2**) and U (**3**), respectively. The collision induced dissociation (CID) tandem mass spectrometry (MS/MS) using positive mode electrospray ionization of the $[M+H]^+$ precursor ion of the compounds are demonstrated in the Figures S21 and S22.

Curvulin (1): The brown solid (20 mg), **^{1}H-NMR** (300 MHz, $CDCl_3$): δ 1.23 (2H, t, *J* = 7.2 Hz, (C1)OCH_2CH_3), 2.55 (3H, s, (C-2')-$COCH_3$), 3.80 (2H, s, 2-*H*), 4.13 (2H, q, *J* = 7.2 Hz, (C1)OCH_2CH_3), 6.32 (1H, sbr, 6'-*H*), 6.34 (1H, sbr, 4'-*H*); **^{13}C-NMR** (75.5 MHz, $CDCl_3$): δ 14.3 ((C1)OCH_2CH_3), 32.1 ((C-2')-$COCH_3$), 41.7 (C-2), 61.3 ((C1)OCH_2CH_3), 103.1 (C-4'), 113.1 (C-6'), 116.1 (C-2'), 137.4 (C-1'), 161.8 (C-3'), 164.8 (C-5'), 171.6 (C-1), 203.6 ((C-2')-$COCH_3$); **ESI-MS** (positive) *m/z* 239.0 $[M+H]^+$.

Spirostaphylotrichin R (2): The brown solid (30 mg), **^{1}H-NMR** (300 MHz, $CDCl_3$): δ 1.03 (3H, s, 14-*H*), 1.61 (3H, s, 11-*H*), 2.19 (2H, m, 13-*H*), 3.96 (3H, s, 15-*H*), 4.07 (1H, s, 4-*H*), 4.75 (2H, s, 6-*H*), 5.90 (1H, d, *J* = 9.0 Hz, 8-*H*), 6.14, (1H, t, *J* = 7.5 Hz, 12-*H*), 7.05 (1H, d, *J* = 9.0 Hz, 9-*H*); **^{13}C-NMR** (75.5 MHz, $CDCl_3$): δ 13.2 (C-14), 23.6 (C-11), 23.7 (C-13), 56.8 (C-5), 64.8 (C-15), 68.7 (C-5), 73.4 (C-6), 86.8 (C-3), 120.8 (C-8), 127.7 (C-10), 150.7 (C-12), 153.0 (C-9), 167.7 (C-1), 196.1 (C-7); **ESI-MS** (positive) *m/z* 298.0 $[M+H]^+$.

Spirostaphylotrichin U (3): The brown solid (30 mg), **^{1}H-NMR** (300 MHz, $CDCl_3$): δ 1.01 (3H, s, 14-*H*), 1.55 (3H, s, 11-*H*), 2.19 and 2.25 (2H, m, 13-*H*), 3.84 (1H, s, 4-*H*), 3.97 (3H, d, *J* = 3.0 Hz, 15-*H*), 4.74 (2H, s, 6-*H*), 5.87 (1H, d, *J* = 9.0 Hz, 8-*H*), 6.27 (1H, s, 12-*H*), 7.01 (1H, d, *J* = 9.0 Hz, 9-*H*); **^{13}C-NMR** (75.5 MHz, $CDCl_3$): δ 13.5 (C-14), 18.8 (C-11), 23.4

(C-13), 56.9 (C-5), 64.6 (C-15), 73.3 (C-4), 73.5 (C-6), 90.8 (C-3), 120 (C-8), 128.9 (C-10), 153.4 (C-9), 153.7 (C-12), 167.5 (C-1), 197.6 (C-7); **ESI-MS** (positive) *m/z* 298.0 $[M+H]^+$.

Figure S1. Comparison of ^{1}H Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract of *Bipolaris* sp. AZ26 and *Bipolaris* sp. C36.

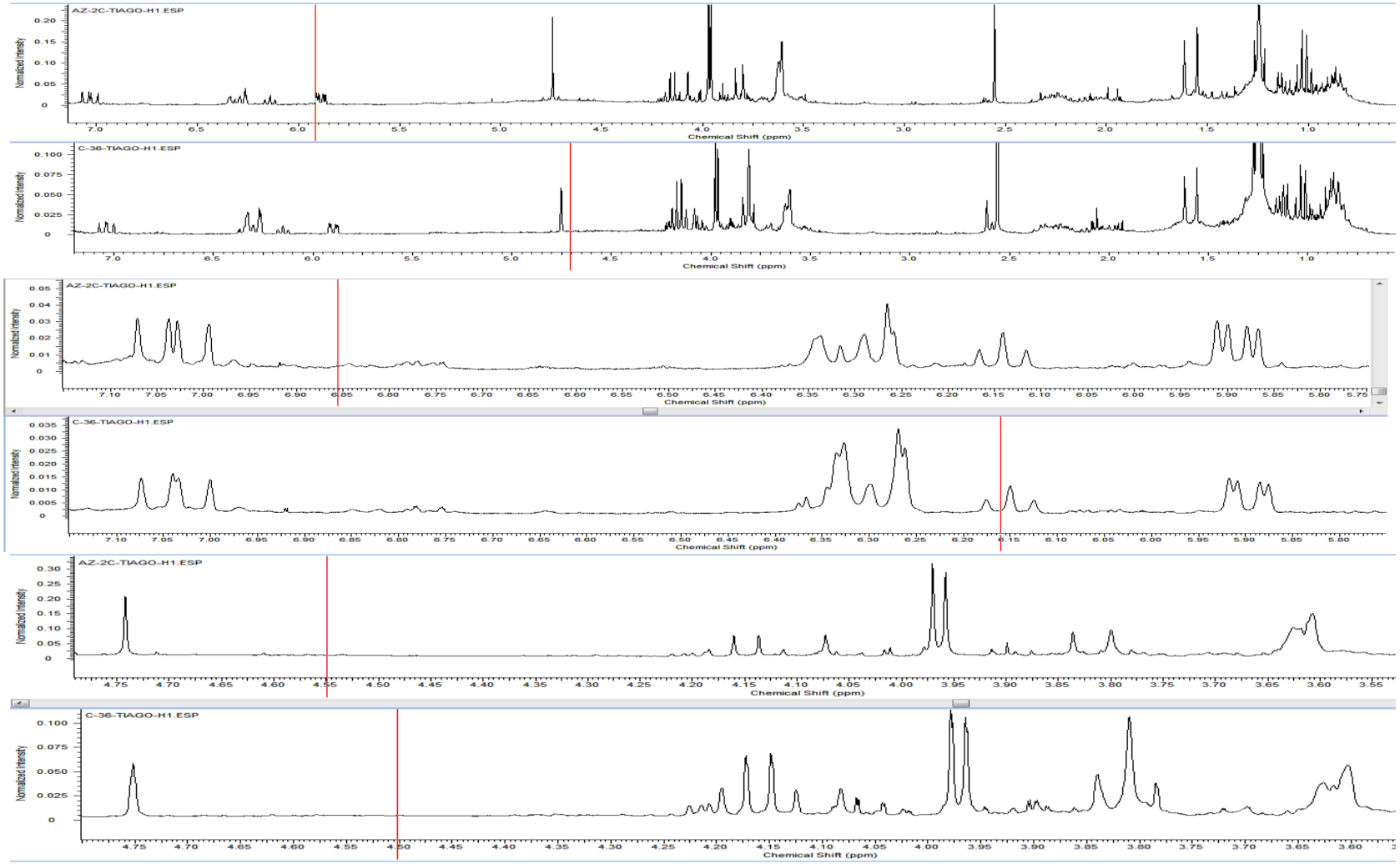


Figure S2. ^{1}H Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

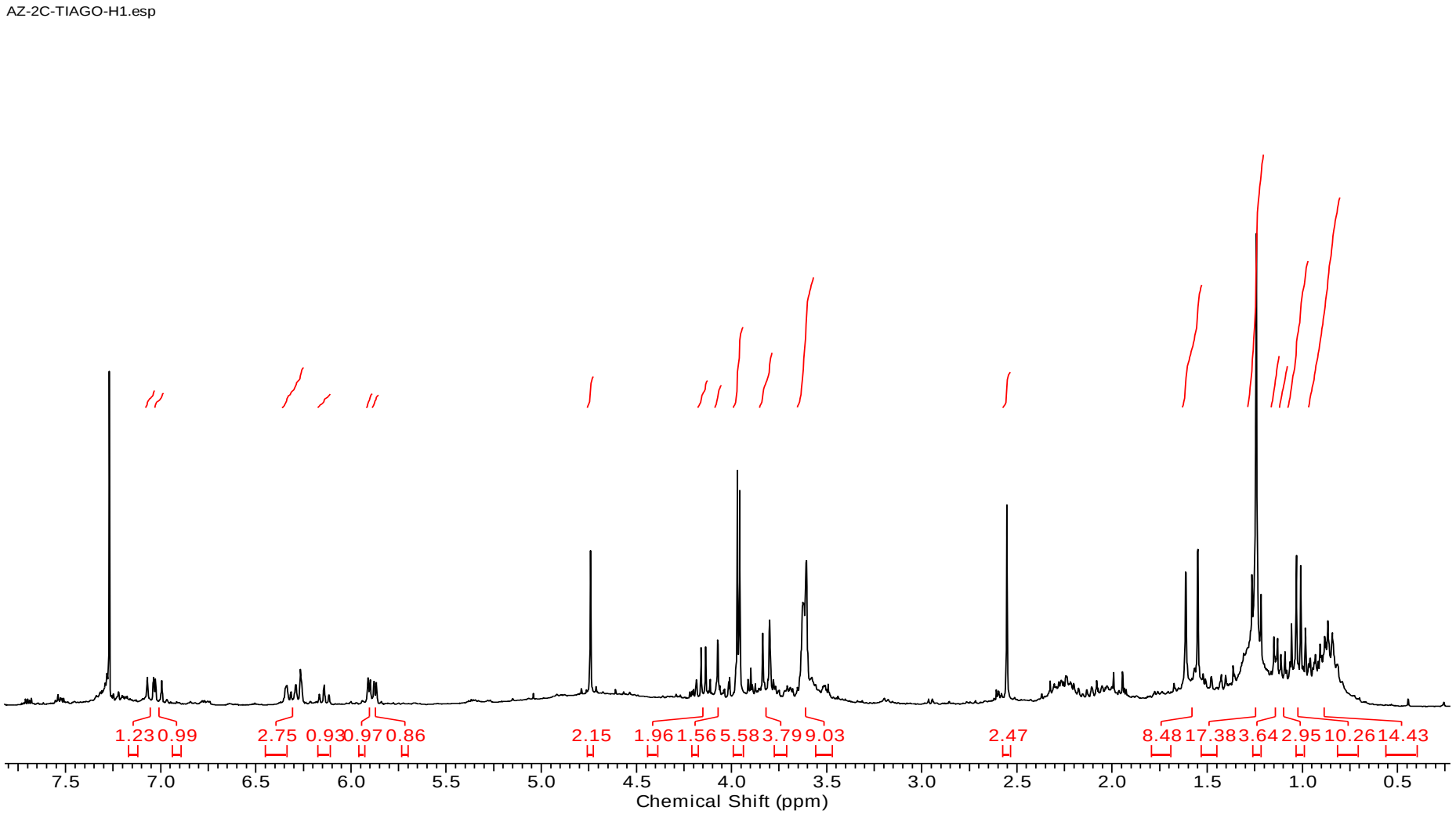


Figure S3. ^{1}H Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

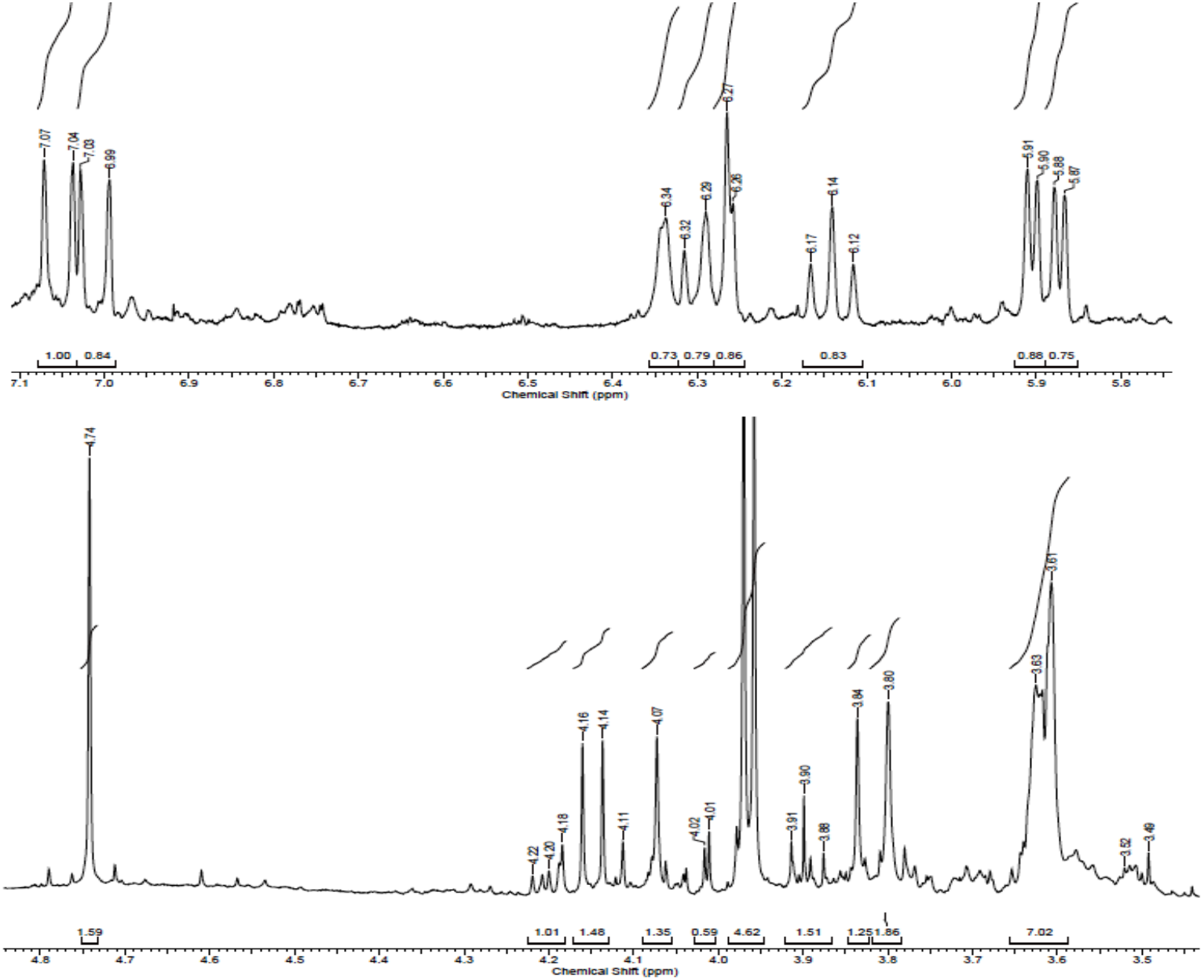


Figure S4. ^{1}H Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

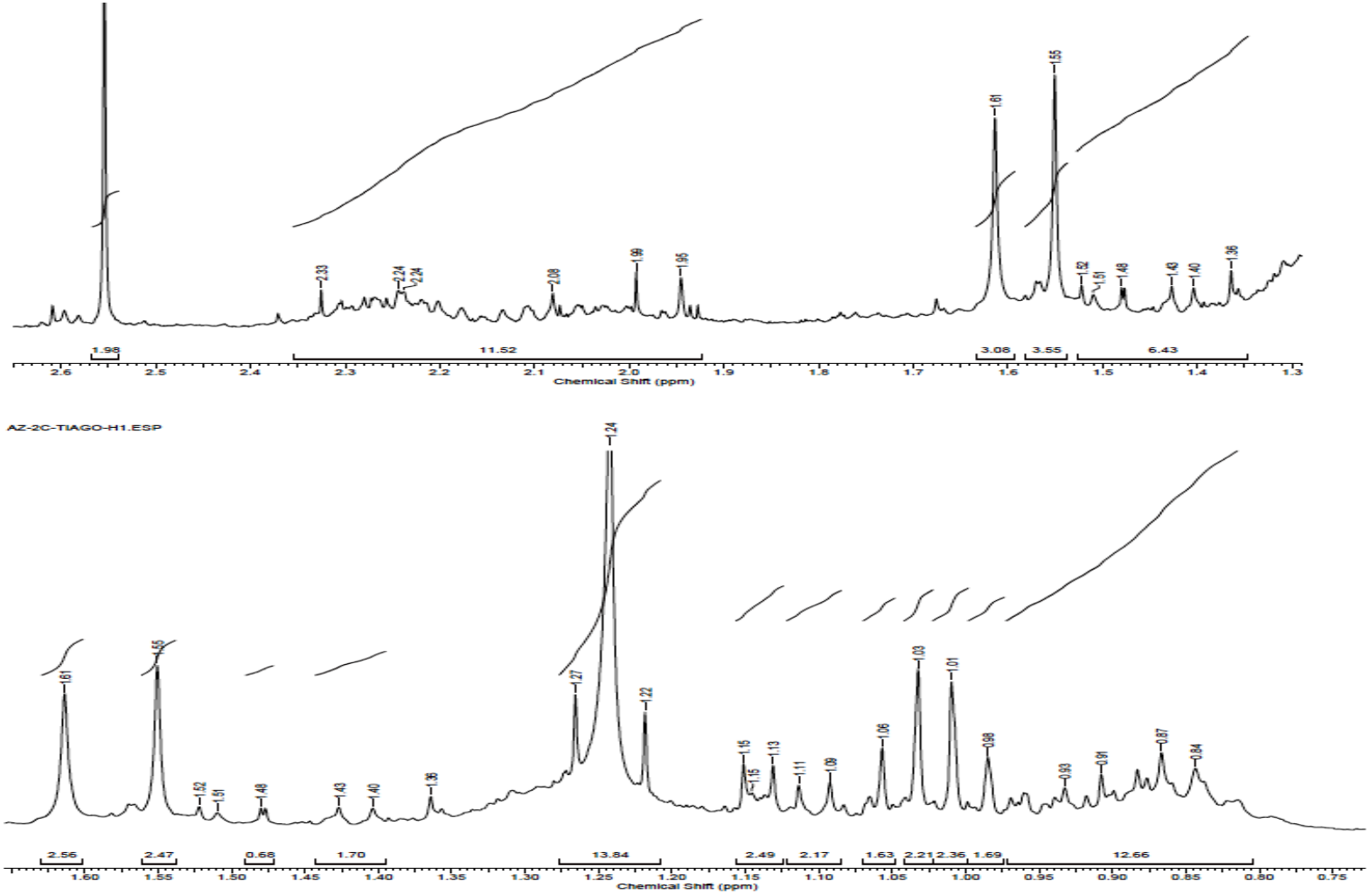


Figure S5. COSY spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

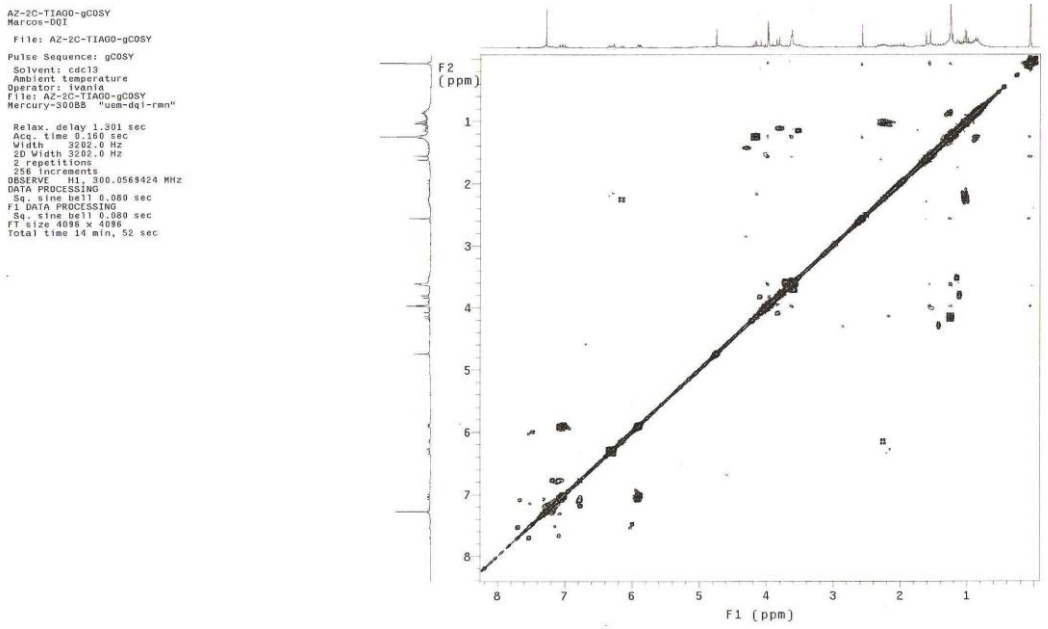


Figure S6. COSY spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

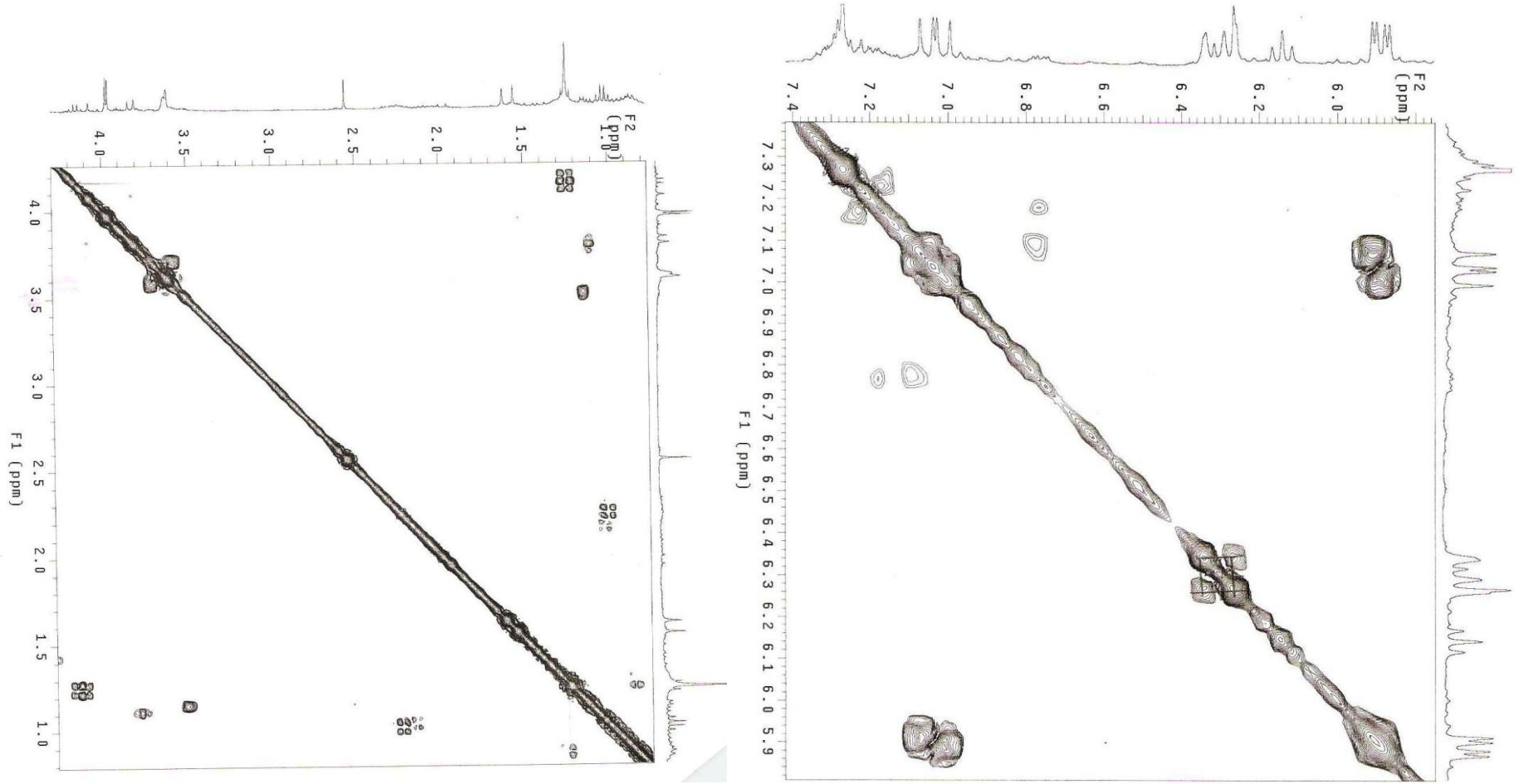


Figure S7. ^{13}C Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

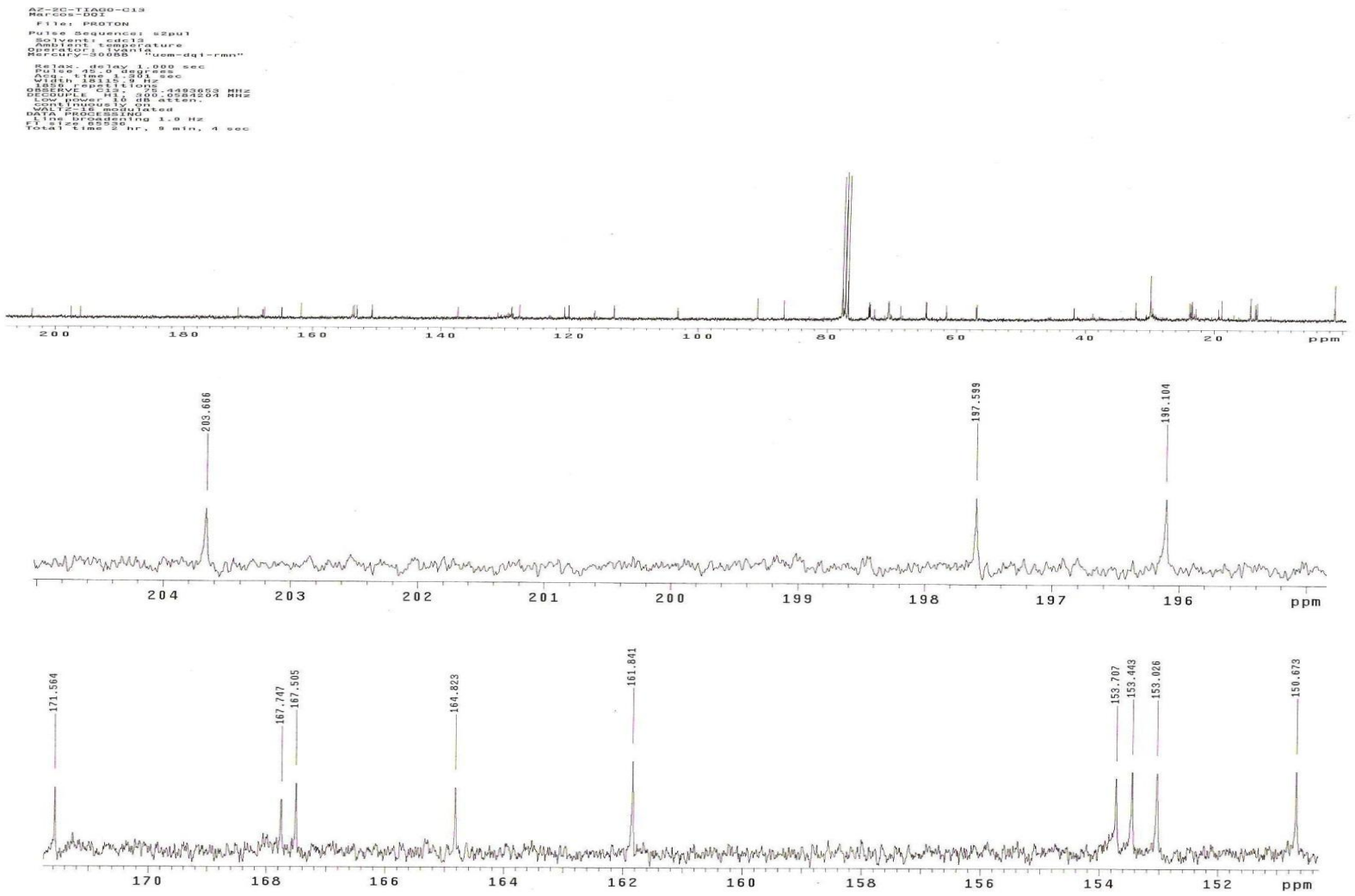


Figura S8. ^{13}C Nuclear magnetic resonance spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

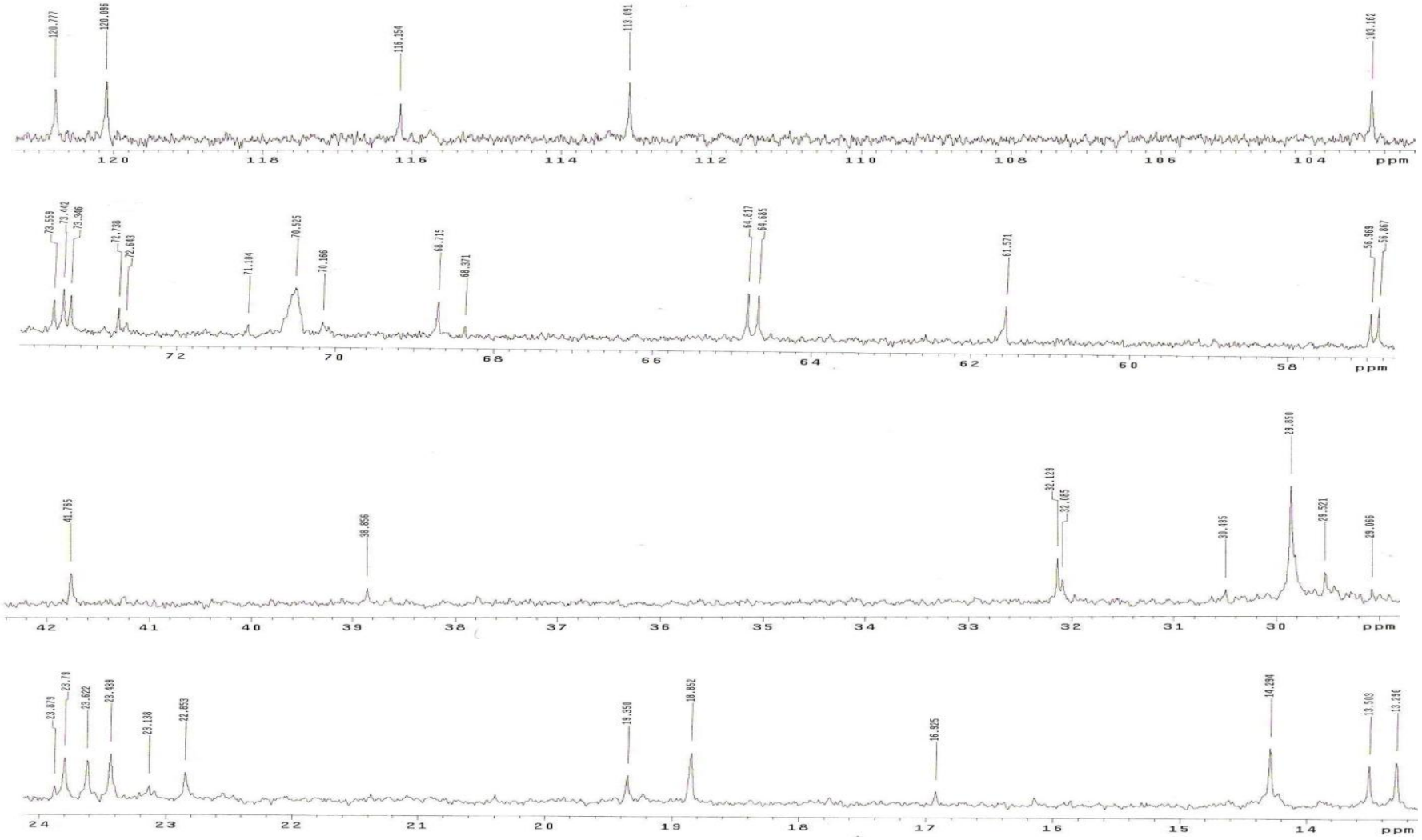


Figure S9. DEPT spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

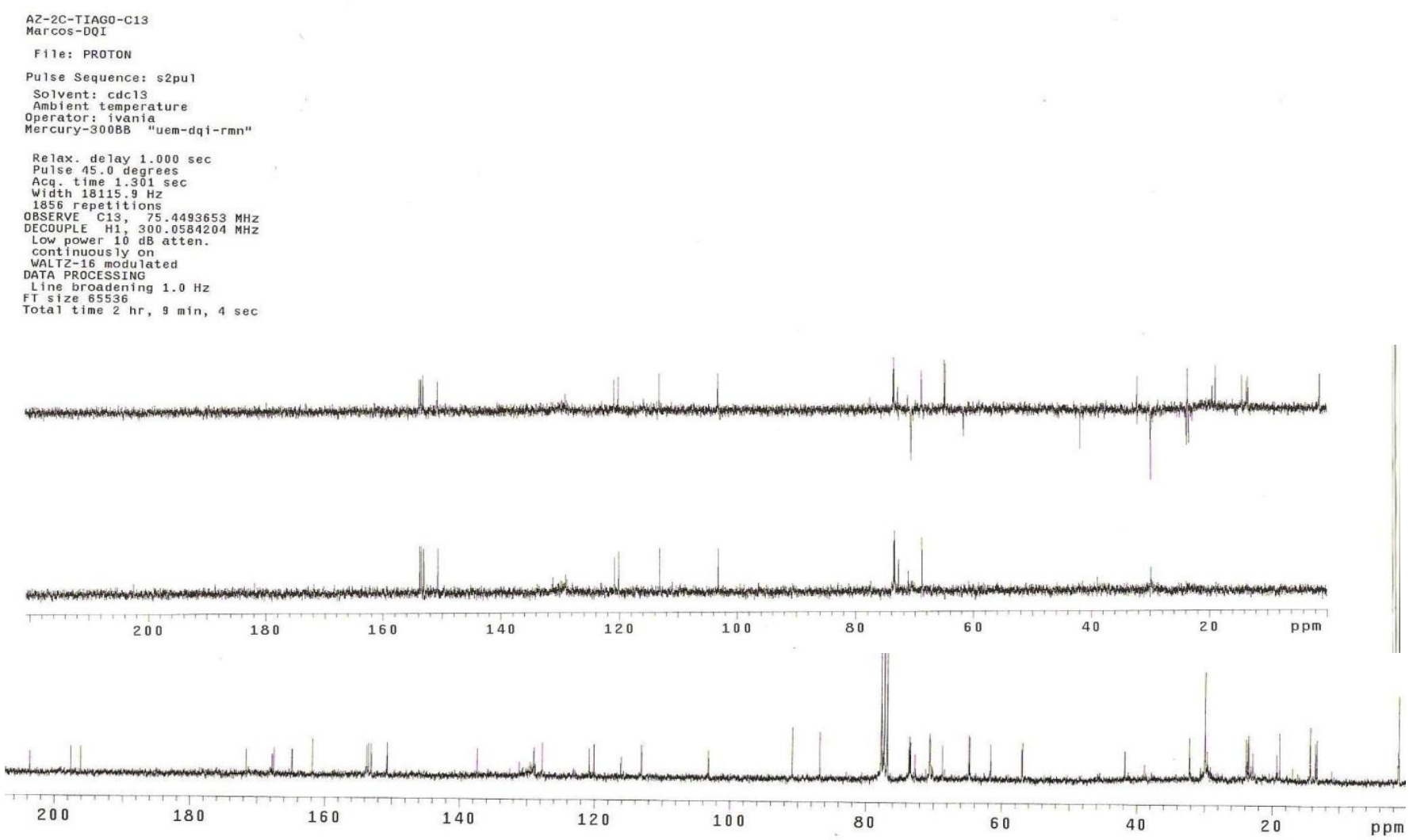


Figure S10. HSQC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

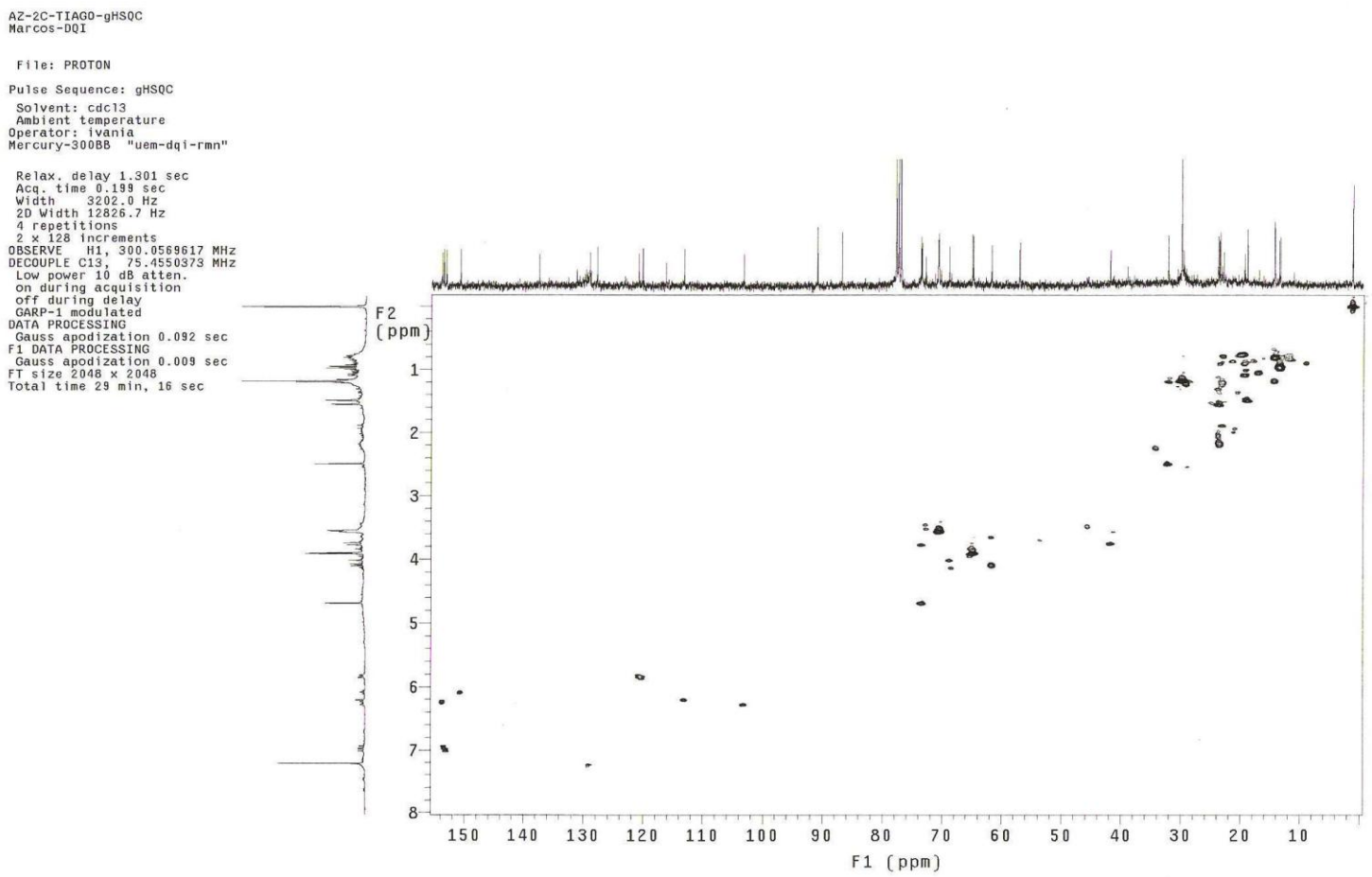


Figure S11. HSQC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

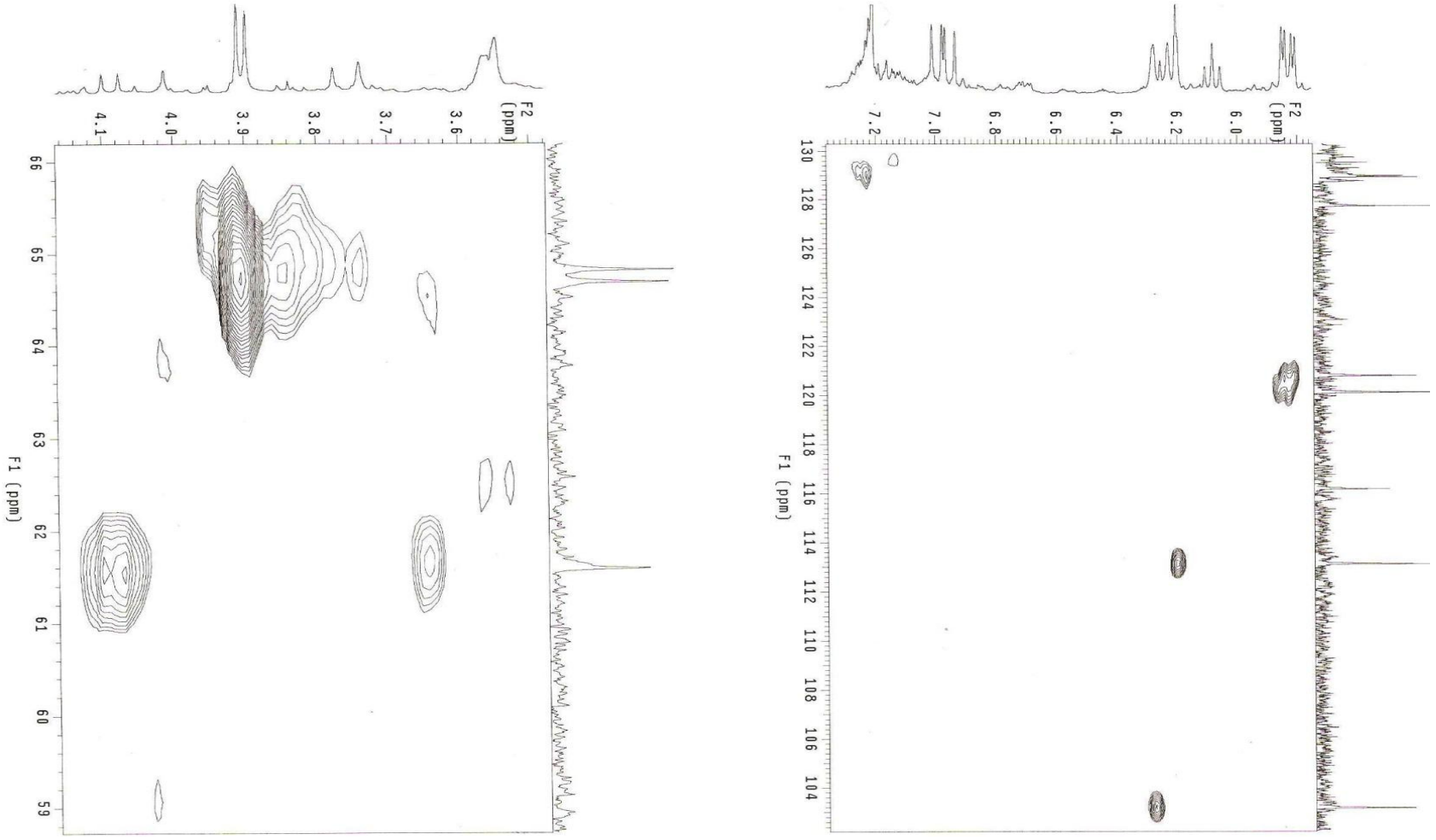


Figure S12. HSQC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

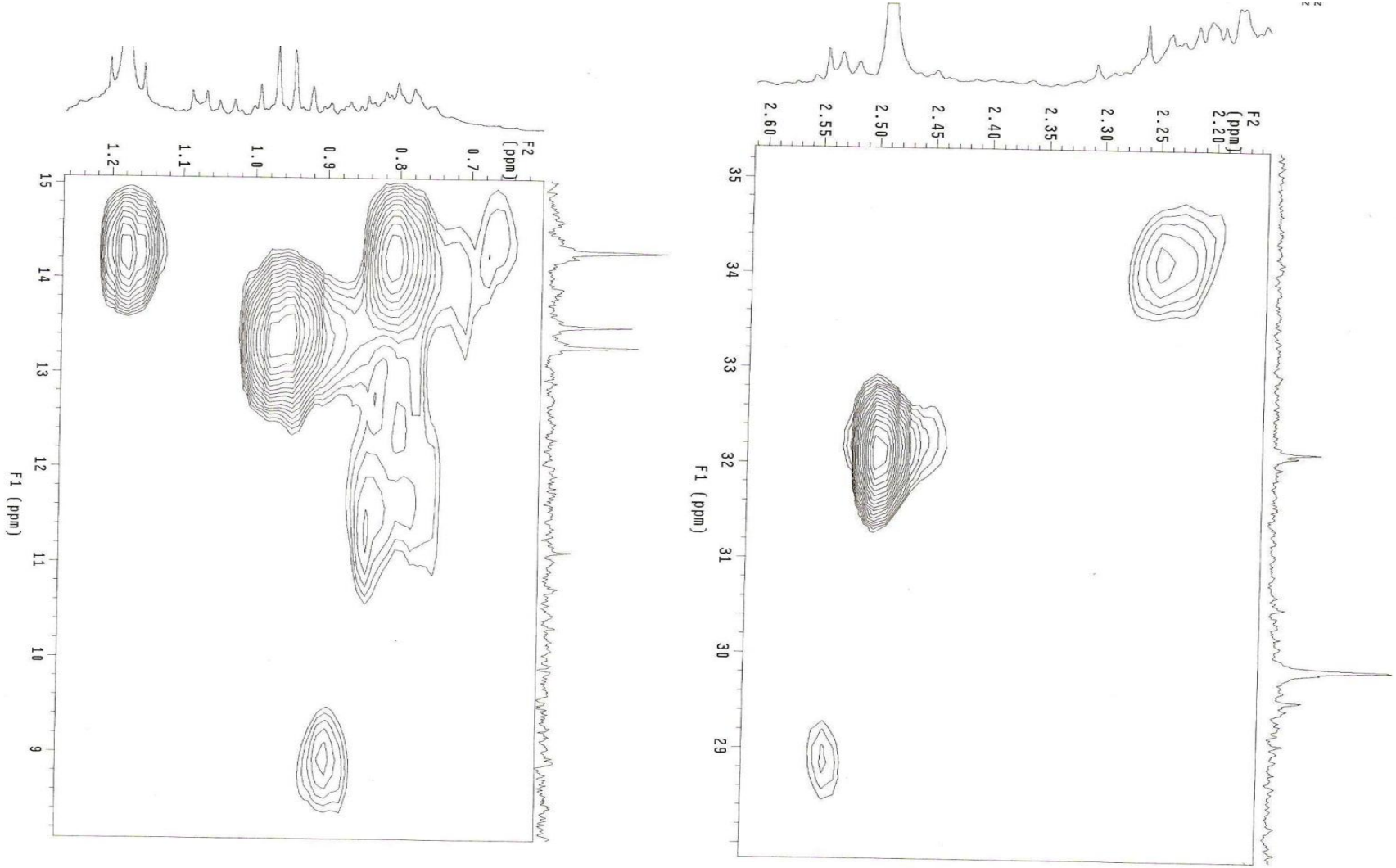


Figure S13: HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

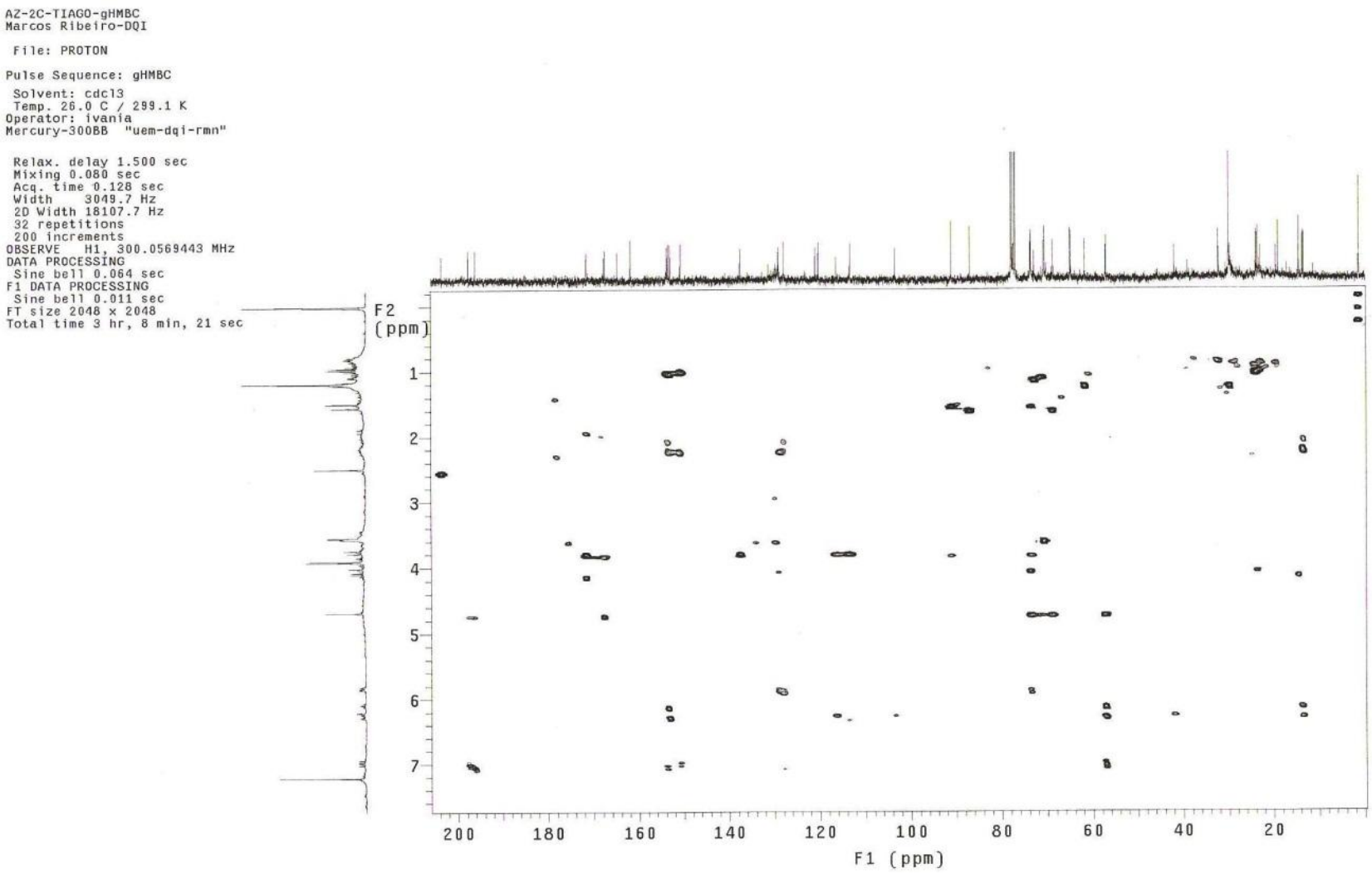


Figure S14. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

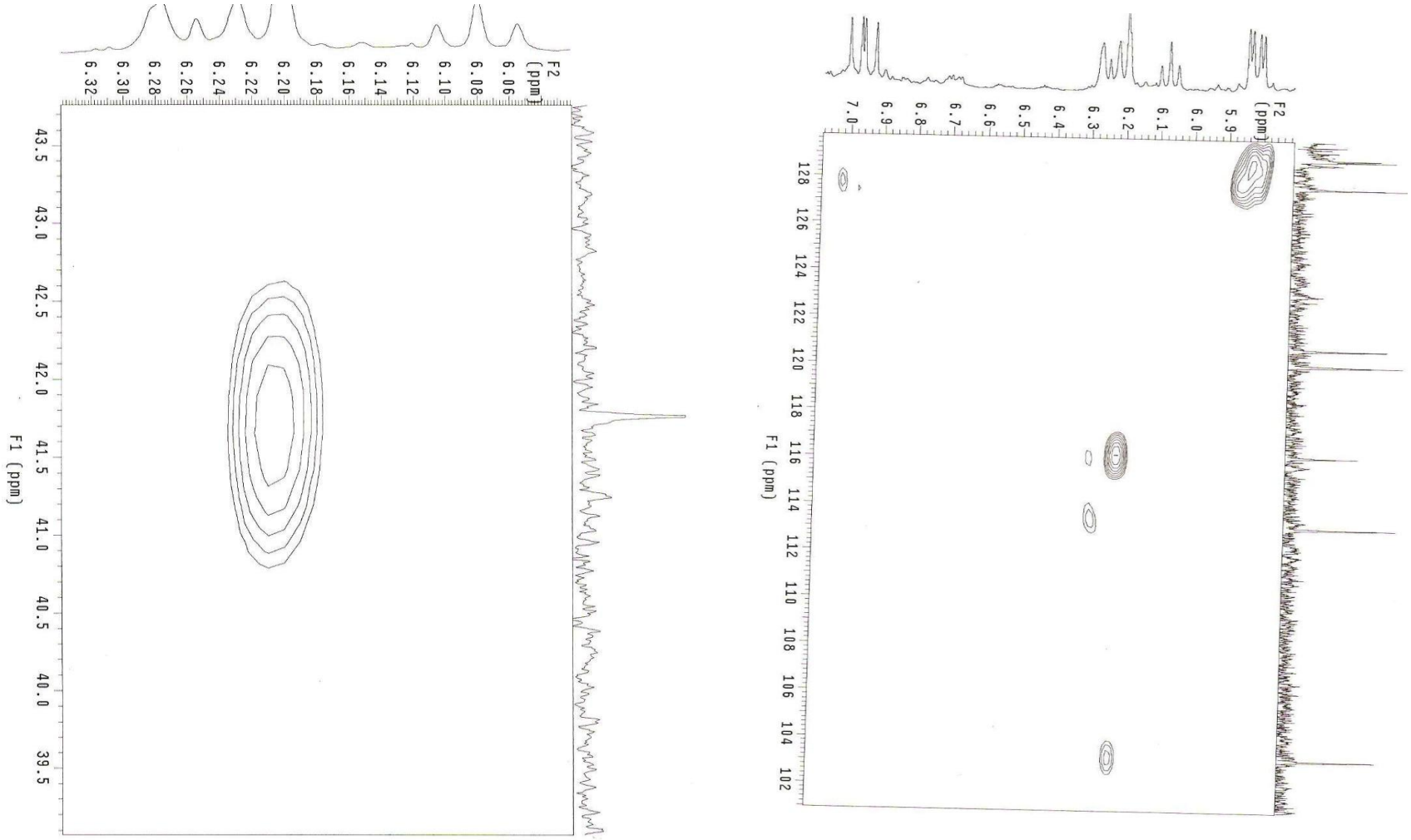


Figure S15. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

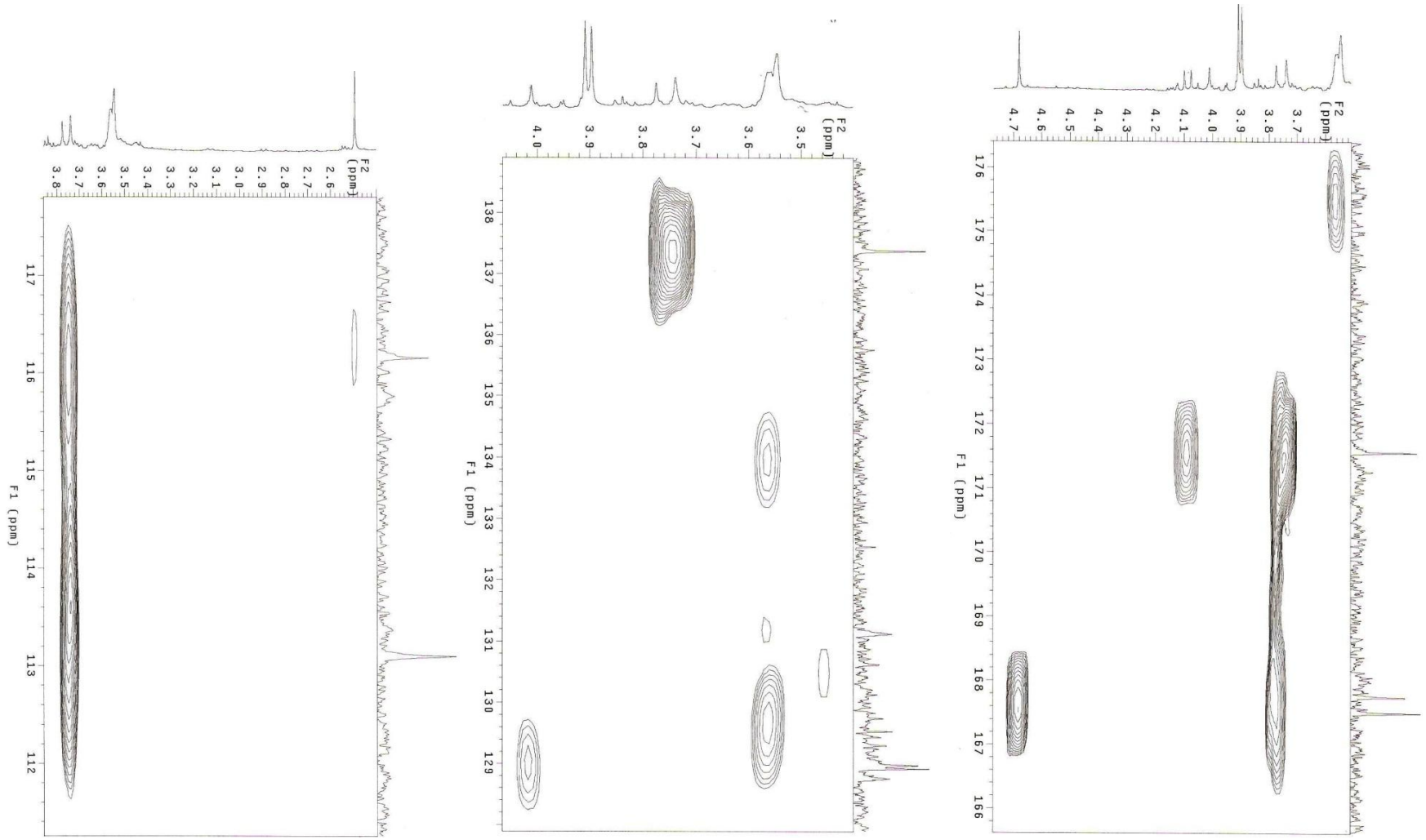


Figure S16. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

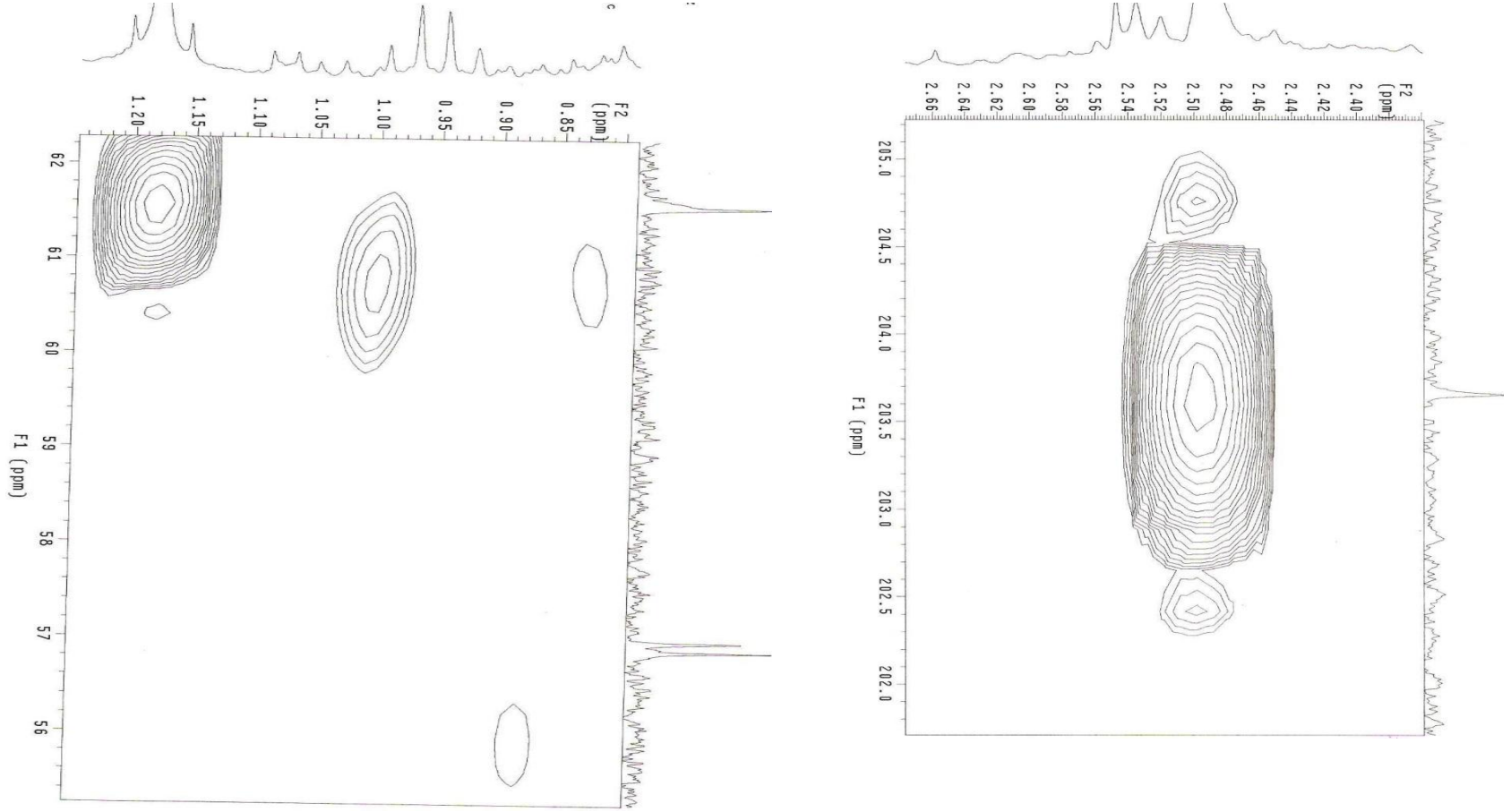


Figure S17. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

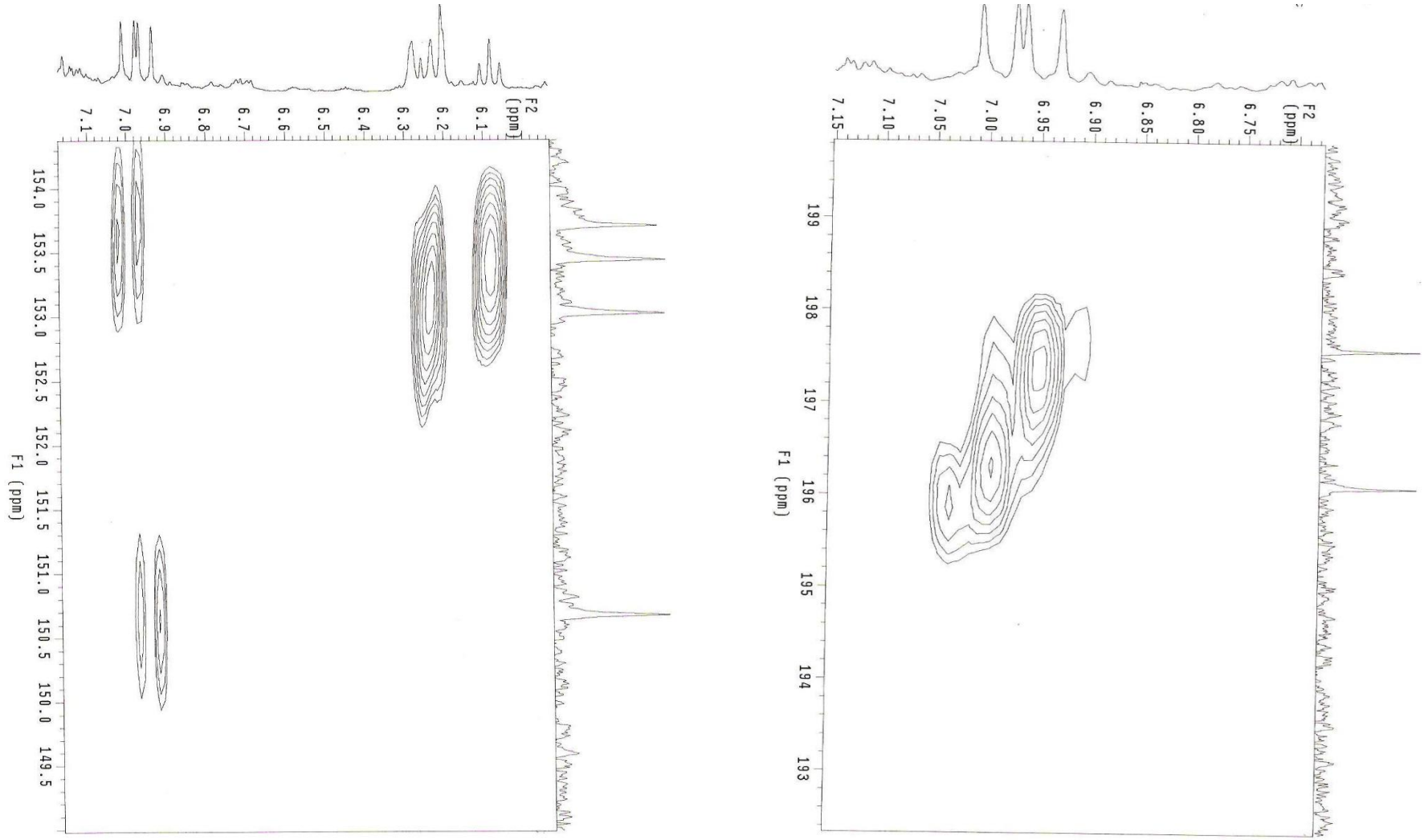


Figure S18. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

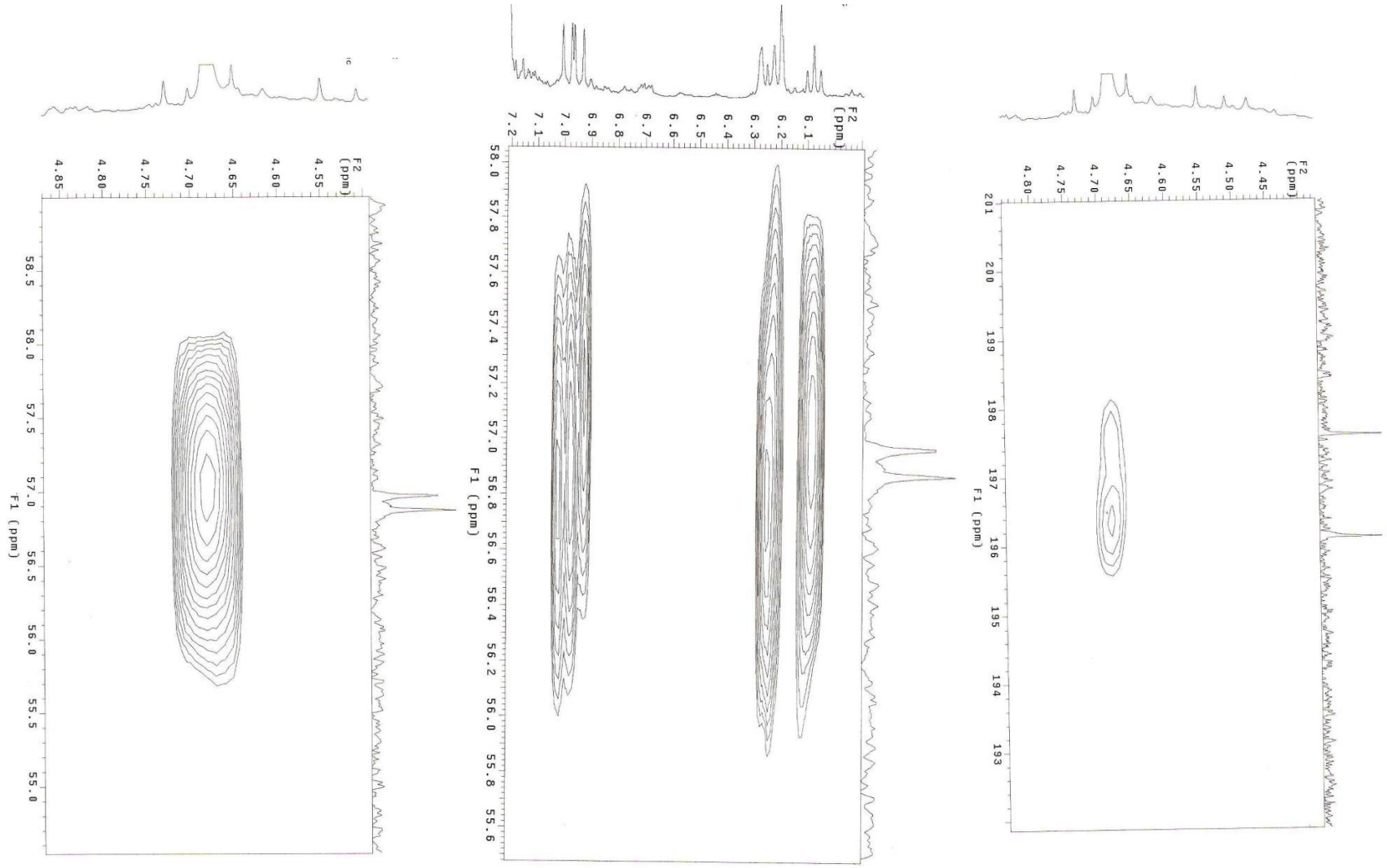


Figure S19. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

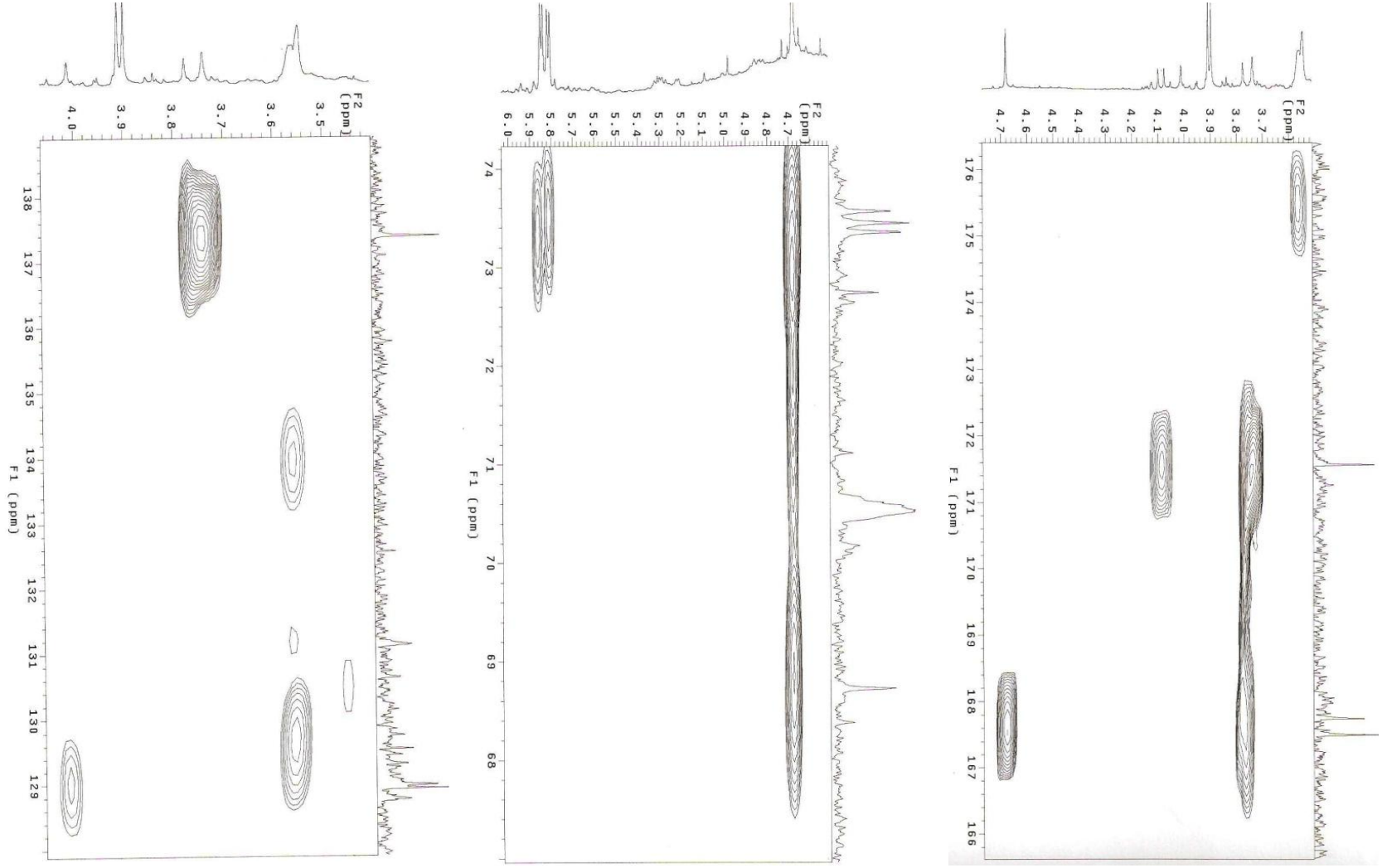


Figure S20. HMBC spectra ($CDCl_3$; 300 MHz) of EtOAc extract AZ-26.

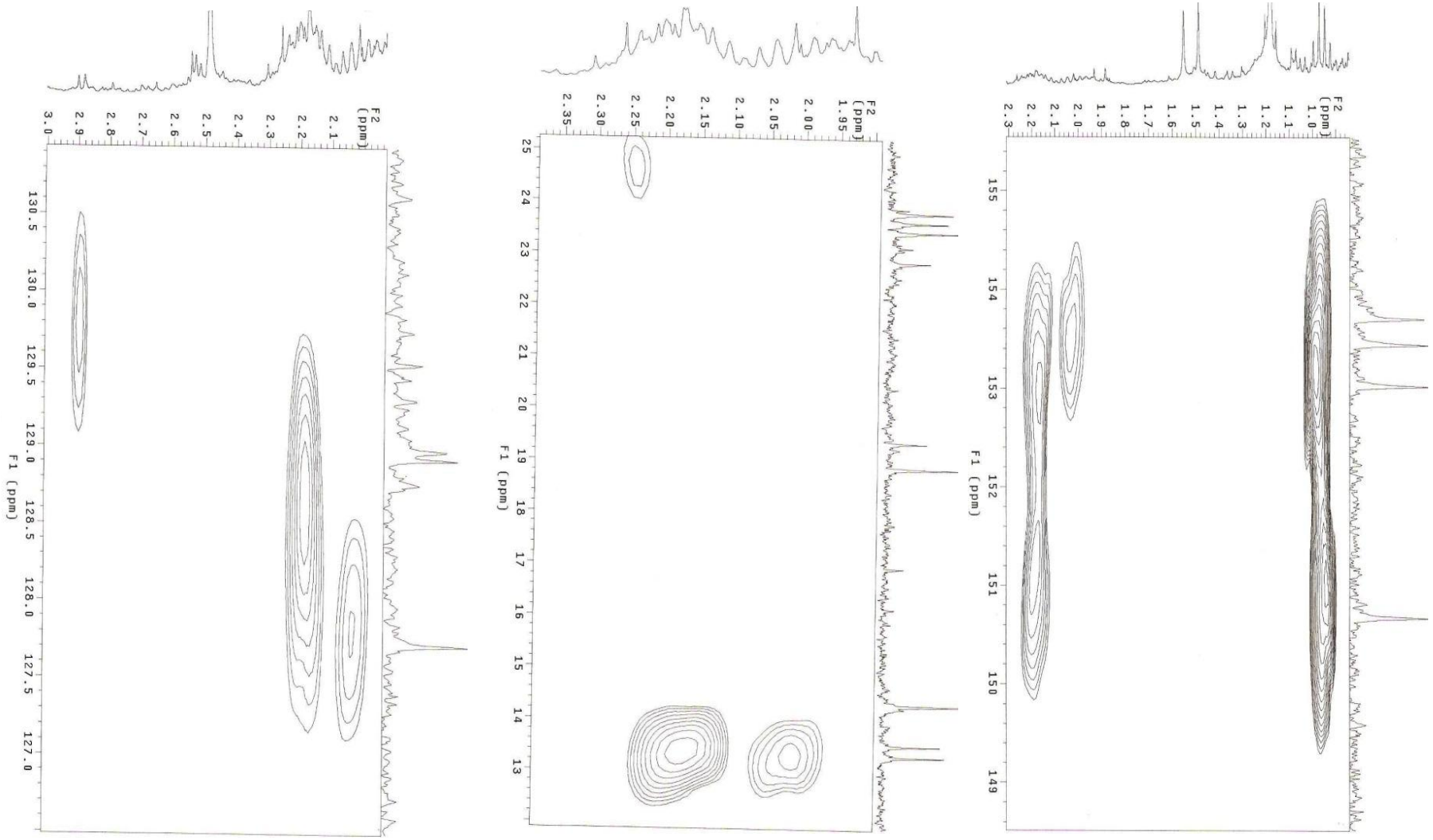


Figure S21. Collision induced dissociation (CID) tandem mass spectrometry (MS/MS) using positive mode electrospray ionization of the $[M+H]^+$ precursor ion from curvulin with *m/z* 239.

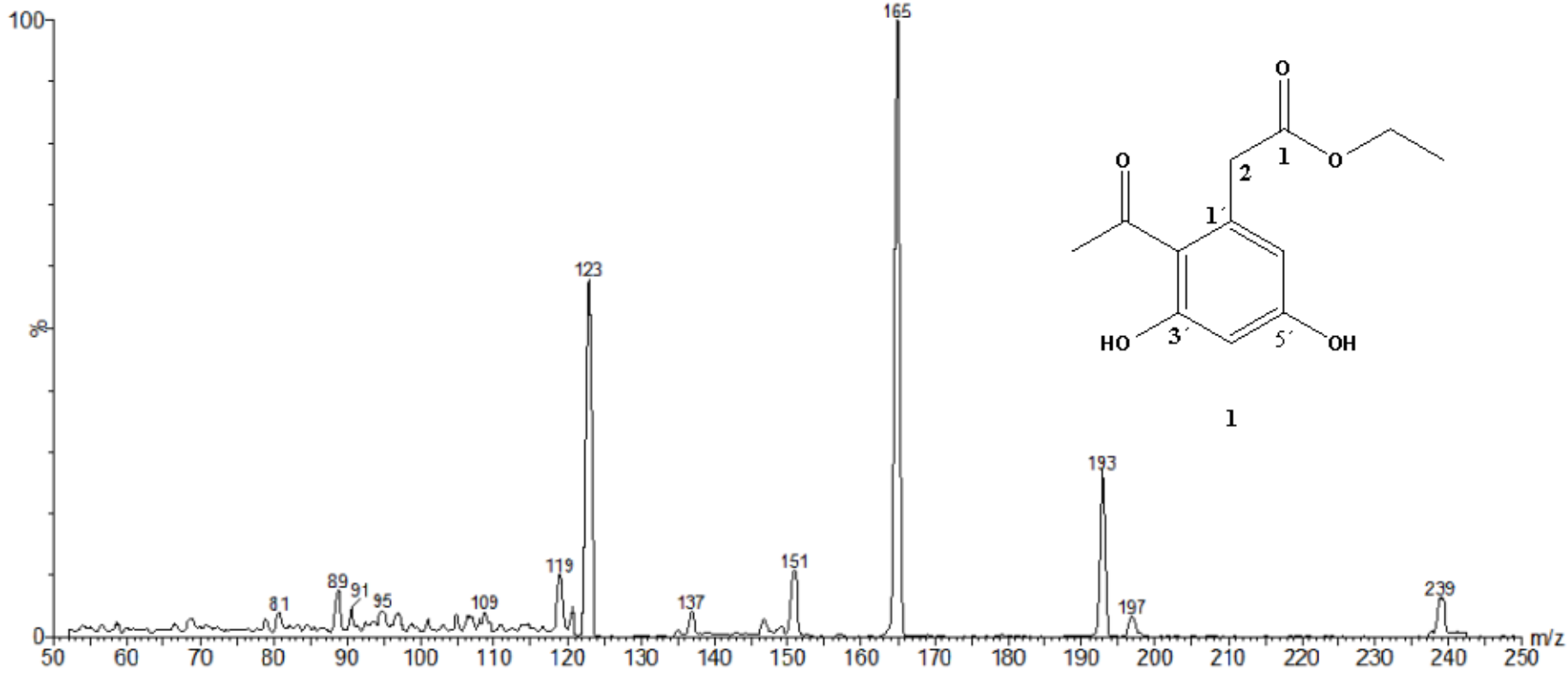
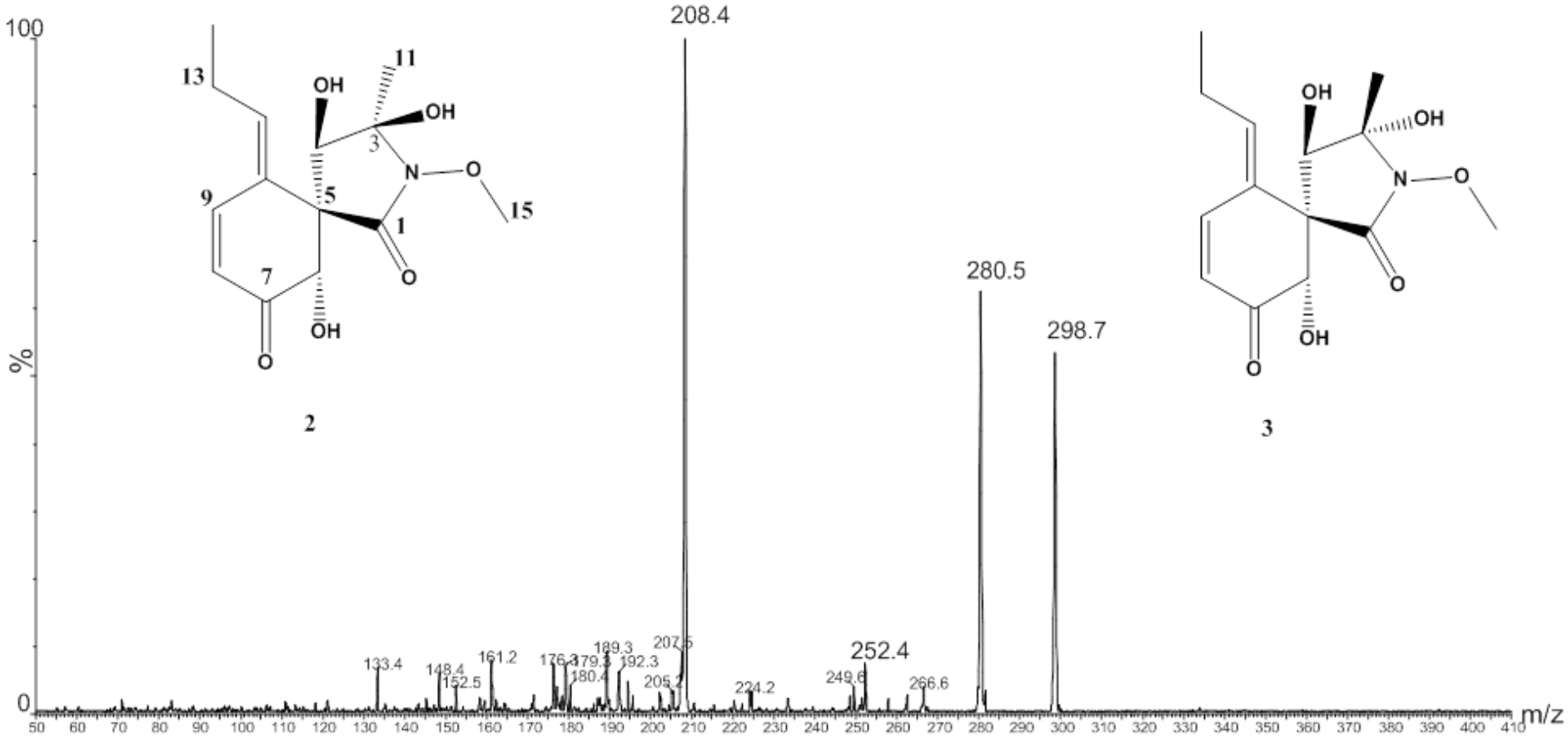


Figure S22. Collision induced dissociation (CID) tandem mass spectrometry (MS/MS) using positive mode electrospray ionization of the $[M+H]^+$ precursor ion from a mixture of spirostaphylotricin R and U with *m/z* 298.



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