SUPPLEMENTARY MATERIAL

Secondary metabolites from *Triclisia gilletii* (De Wild) Staner (Menispermaceae) with antimycobacterial activity against *Mycobacterium tuberculosis*

Eric Robert Tiam[a], Dominique Serge Ngono Bikobo[a,e*], Auguste Abouem A Zintchem[a,b], Norbert Mbabi Nyemeck II[a,c], Esther Del Florence Moni Ndedi[d], Patrick Hervé Betote Diboué[d], Maximilienne Ascension Nyegue[d], Alex de Théodore Atchadé[a], Dieudonné Emmanuel Pegnyemb[a], Christian G. Bochet[e] and Ulrich Koert[c].

[a] *Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, P.O Box 812, Yaoundé, Cameroon;* [b] *Department of Chemistry, Higher Training College, University of Yaoundé I, P.O Box 47, Yaoundé, Cameroon;* [c] *Philipps-Universität Marburg, Faculty of Chemistry, Hans-Meerwein-Strasse, D-35032 Marburg, Germany;* [d] *Department of Microbiology, Faculty of Science, University of Yaoundé I, P.O Box 812, Yaoundé, Cameroon;* [e] *Department Chemie, Universität Fribourg, CH du Musée 9, 1700 Fribourg, Switzerland*

[*]Corresponding author: Tel: +237 675 05 54 68; Fax: +237 242 22 18 73 E-mail: ngono_serge@yahoo.fr (D. S. Ngono Bikobo)

Abstract

Triclisinone (**2**), a new ochnaflavone derivative, was isolated from the aerial parts of *Triclisia gilletii*, along with known drypemolundein B (**1**) and eight other known compounds. The chemical shifts of drypemolundein B (**1**) have been partially revised based on reinterpretation of NMR spectroscopic data. The eight other secondary metabolites are composed of: (+)-nonacosan-10-ol (**3**); stigmasterol (**4**), 3-*O*-β-D-glucopyranosylsitosterol (**5**), 3-*O*-β-D-glucopyranosylstigmasterol (**6**); oleanic acid (**7**); myricetin (**8**), quercetin (**9**) and 3-methoxyquercetin (**10**). Their structures were elucidated using IR, MS, NMR 1D and 2D, ^{1}H and ^{13}C and comparison with literature data. Furthermore, compounds **1**, **2**, **5, 6, 8, 9** and the crude extract were tested against *Mycobacterium tuberculosis*. Compounds **1, 2, 8** and **9** displayed moderate to very good activity against resistant strain (codified AC 45) of *M. tuberculosis* with minimum inhibitory concentrations (MICs) ranged from 3.90 to 62.5 µg/mL.

Keywords: *Triclisia gelletii*; Menispermaceae; ochnaflavone derivative; NMR chemical shift analysis; antimycobacterial activity.

Experimental section (Annex)

General procedures

Melting points were uncorrected and were measured on a Mettler Toledo instrument. IR spectra were recorded on an Alpha FT-IR Spectrometer from Bruker, while 1D and 2D NMR spectra were obtained on a Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C spectra) spectrometer (Bruker, Rheinstetten, Germany) with chemical shifts reported in δ (ppm) using TMS (δ_H) as an internal standard. The HR-ESI-MS were obtained on LTQ-FT instrument (Thermo Scientific). LC-MS were measured with Shimadzu LC-MS system using a L^{-}column 2 ODS (I.D. 2.1 × 100 mm, Chemical Evaluation and Research Institute, Japan), at a flowrate of 0.2 mL min^{-1}, a detection wavelength of 350 and 300 nm, and FMW (HCOOH/MeCN/H_2O = 1:12:87) as eluent, ESI^{+} 4.5 kV, ESI^{-} 3.5 kV, 250°C. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Silica gel 60 (230–400 mesh E. Merck, Darmstadt, Germany) and Sephadex LH-20 were employed for column chromatography, the solvent mixing systems for elution were mainly CH_2Cl_2/MeOH for the phytochemical study with increasing polarity and pure MeOH, while precoated aluminium sheets silica gel 60 F_{254} were used for TLC.

Extraction and isolation

Dried and powered leaves of *T. gilletii* (109 g) were extracted for 48 h with MeOH (3 x 1L) at room temperature. After filtration and evaporation of the solvent, the crude MeOH extract (12 g) was subjected to column chromatography CC (SiO_2, eluting with a gradient solvent system (CH_2Cl_2/MeOH) giving four main fractions: I (2.4 g), II (1.6 g), III (3.8 g), IV (4.2 g). Fraction I (2.4 g) was submitted to a silica gel column using solvent system CH_2Cl_2/MeOH (60/1) to give three sub-fractions (Ia, Ib and Ic). Sub-fraction Ib (0.42 g) was subjected to a silica gel column (SiO_2) using solvent system CH_2Cl_2/MeOH (60/1-40/1) in gradient elution to obtain compound **3** (6 mg). Sub-fraction Ic (0.38 g) was purified using silica gel column with gradient CH_2Cl_2/MeOH (40/1-30/1) to give compound **4** (9 mg). Fraction II was chromatographed as described above to afford three sub-fractions (IIa, IIb and IIc). Sub-fraction IIa (0.28 g) was fractionated by silica gel column in gradient elution of CH_2Cl_2/MeOH (40/1-20/1) to obtain compounds **5** (16 mg) and **6** (7 mg). Compound **1** (13 mg) was isolated from the sub-fraction IIb (0.49 g) using silica gel column with solvent system CH_2Cl_2/MeOH (40/1-30/1). Sub-fraction IIc (0.83 g) was fractionated and purified using silica gel column and eluted with solvent system CH_2Cl_2/MeOH (40/1-20/1) to afford **7** (2.8 mg). Fraction III was submitted to a silica gel column as above eluted with solvent

system CH_2Cl_2/MeOH (20/1 to 5/1) to give five sub-fractions (IIIa, IIIb, IIIc, IIId and IIIe). Sub-fraction IIIc (0.74 g) was subjected to sephadex LH-20 column, eluted with MeOH to afford compounds **8** (11 mg) and **9** (4 mg). Sub-fraction IIId (0.42 g) was also purified via sephadex LH-20 to obtain compound **10** (4.8 mg). Fraction IV was subjected to a silica gel as described above and eluted with solvent system CH_2Cl_2/MeOH (20/1 to 5/1) to give four sub-fractions (IVa, IVb, IVc and IVd). Sub-fraction IVc (0.89 g) was successively chromatographed using a silica gel column in gradient elution of CH_2Cl_2/MeOH (20/1-1/1) to obtain compound **2** (68 mg).

Antitubercular activity

For the present study, the mycobacteria (*M. tuberculosis*) used was a clinical isolated strain resistant to Isoniazid codifies as AC 45 (this strain was obtained from Sangmelima district's Hospital in South Region of Cameroon). The genetical profile of the resistance has been carried out at Laboratory for Tuberculosis Research (Biotechnology Centre, University of Yaoundé I) through Line probe Assay method. The mycobacteria strain has been cultured at 37 ^oC for two week in Middlebrook 7H9 (Himedia, India) supplemented with 0.05% (v/v), 2% glycerol and 10% OADC (oleic acid-albumin-dextrose-catalase of Liofilchem s.r.l, Italia). The optical density of 0.45 to 0.55 was measured using spectrophotometer at 550 nm to obtain a suspension of 1.5 $x10^8$ UFC/mL (Collins et al. 1997). The activity of all phytochemicals (extract and pure compounds) against the aforementioned *M. tuberculosis* strains was tested using the microplate Alamar Blue assay as described previously by Collins et al. 1997 and Jiménez-Arellanes et al. 2003; 2007. In a 96 well microplates, all wells received 100 µL of supplemented Middlebrook 7H9 broth, then working metabolites solutions (100 µL) were poured into the first well of each row, from which two-fold dilution series were made through the microplate column. The test inoculum (100 µL) was added to all testing wells, as well as to the drug-free control wells. The final concentration of DMSO in wells was 7% v/v. The final concentrations tested ranged from 250 to 0. 244 µg/mL for pure compounds and 5000 to 4,882 µg/mL for extracts. Rifampicin was used as standard drug. Each concentration was assayed in triplicate. Each microplate was sealed with parafilm paper and incubated for 14 days at 37^oC. After that, 40 µL of Alamar blue solution was added to two columns of each triplicate in other to show mycobacterial growth and the plate were re-incubated at 37^oC for 24 h. After one day of incubation, the Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of phytochemicals that inhibited the bacterial growth (prevents a color change from blue to pink) after incubation time (CLSI 2011). The Minimal Bactericidal

Concentration (MBC) determination, 50 µL of each wells which concentration were ≥ MIC was sub-cultured in 150 µL of Mbk 7H9 medium and incubated at 37 ° C. for 10 days, then mycobacterial growth was carried out by addition of 40 µL of alamar blue. MBC was defined as the lowest concentration of extract at which no visible growth of the germ was observed (CLSI 2011).

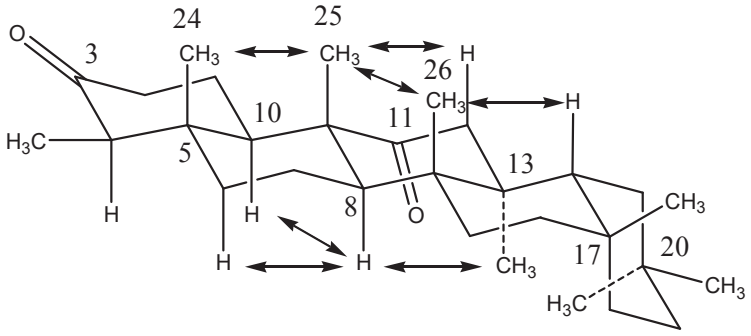
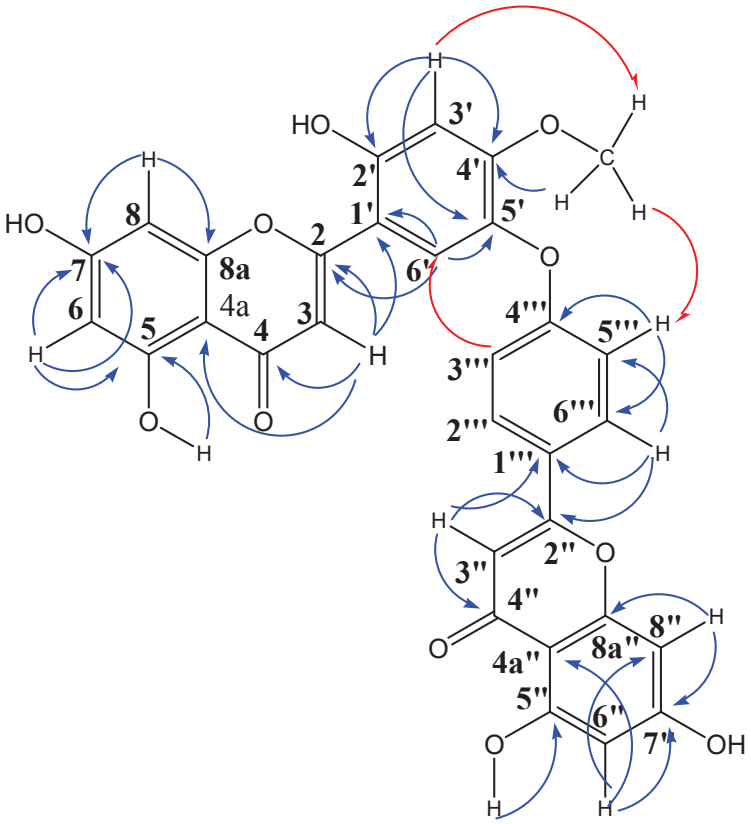


Figure S1. Some characteristic NOESY correlations of compound **1**



Isoetin unit (I)

Apigenin unit (II)

Figure S2. selected HMBC (→) and NOESY (—→) correlations of compound **2**

Table S1: ^{1}H and ^{13}C-NMR spectroscopic data[a] of compound **1** (500 and 125 MHz in $CDCl_3$) δ in ppm and drypemolundein B. (300 and 75 MHz in $CDCl_3$)

	Compound **1**		drypemolundein B	
No.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	22.4 t	1.99; 1.71 (m)	22.2 t	-
2	41.5 t	2.21 (-[b])	40.8 t	-
3	207.8 s		212.1 s	
4	57.9 d	2.12 (m)	57.9 d	-
5	41.9 s		42.2 s	
6	37.6 t	1.70 (m); 1.30 (-[b])	41.0 t	-
7	18.3 t	1.56 (m)	18.4 t	-
8	52.8 d	1.41 (m) ([c])	53.0 d	2.38 (m)
9	59.6 s ([c])		55.5 s	
10	59.6 d ([c])	1.40 (-[b])	53.1 d	-
11	213.8 s		214.2 s	
12	41.6 t	2.23; 2.15 (m)	51.2 t	2.62; 2.01 (m, 11.4)
13	38.3 s ([c])	-	44.0 s	-
14	39.5 s ([c])	-	43.8 s	-
15	31.2 t	1.30; 1.00 (m)	31.6 t	-
16	36.1 t	0.99 (-[b])	36.1 t	-
17	29.9 s	-	29.6 s	-
18	42.1 d	1.60 (-[b])	36.4 d	-
19	38.4 t	1.80; 1.31 (m)	35.4 t	-
20	28.4 s	-	28.3 s	-
21	32.8 t	0.99 (-[b])	33.0 t	-
22	39.3 t	-	38.9 t	-
23	6.8 q	0.88 (d, 6.4)	6.8 q	0.86 (d, 3.1)
24	14.8 q	0.77 (s)	14.5 q	0.73 (s)
25	18.1 q	0.87 (s)	18.1 q	0.90 (s)
26	20.3 q	1.01 (s)	19.0 q	0.88 (s)
27	18.6 q	1.02 (s)	19.8 q	1.39 (s) [(d)]
28	31.3 q	1.18 (s)	31.8 q	0.91 (s)
29	35.0 q ([c])	0.96 (s)	31.7 q	1.04 (s) [(d)]
30	31.6 q ([c])	1.00 (s)	34.2 q	0.91 (s)

[a)] Assignments were confirmed by DEPT-135, HSQC, HMBC, COSY and NOESY experiments. [b)] Overlapping signals. [c)] The assignments are different from those in Wandji et al. (2000). [d)] Mismatched chemical shift values beared by the same carbon atom (CH_3-29).

Table S2: ^{1}H and ^{13}C-NMR spectroscopic data[a] of compound **2** (500 and 125 MHz in DMSO-d_6) δ in ppm

Position	δ_C	δ_H (*J* in Hz)
1	/	/
2	164.4 s	
3	108.0 d	7.14 (s)
4	182.2 s	
4a	104.7 s	
5	161.6 s	
6	98.9 d	6.16 (d, 2.0)
7	164.5 s	
8	94.4 d	6.48 (d, 2.0)
8a	160.6 s	
1′	109.5 s	
2′	155.0 s	
3′	102.1 d	6.82 (s)
4′	157.3 s	
5′	133.4 s	
6′	122.3 d	7.79 (s)
1″	/	/
2″	164.4 s	
3″	103.3 d	6.87 (s)
4″	182.1 s	
4a″	103.5 s	
5″	161.5 s	
6″	99.0 d	6.33 (d, 2.0)
7″	164.6 s	
8″	93.9 d	6.53 (d, 2.0)
8a″	160.6 s	
1‴	122.6 s	
2‴	129.0 d	7.96 (d, 8.6)
3‴	116.2 d	6.90 (d, 8.6)
4‴	157.6 s	
5‴	116.2 d	6.90 (d, 8.6)
6‴	129.0 d	7.96 (d, 8.6)
4′-OCH_3	56.2 q	3.78 (s)

[a] Assignments were confirmed by DEPT-135, HSQC, HMBC, COSY and NOESY experiments.

Table S3. MIC and MBC values of the methanol extract and the isolated compounds against *Mycobacterium tuberculosis* (AC 45)

Plant species/Compounds	MIC[a] (µg/mL)	MBC[b] (µg/mL)	MBC/MIC
T. gilletii	1250	5000	4
1	3.90	15.62	4
2	62.5	125	2
5	62.5	125	2
6	62.5	250	4
8	7.81	31.25	4
9	62.5	125	2
RMP	0.97	7.81	8

[a]Minimum Inhibitory Concentration
[b]Minimum Bactericidal Concentration
RMP = Rifampicin

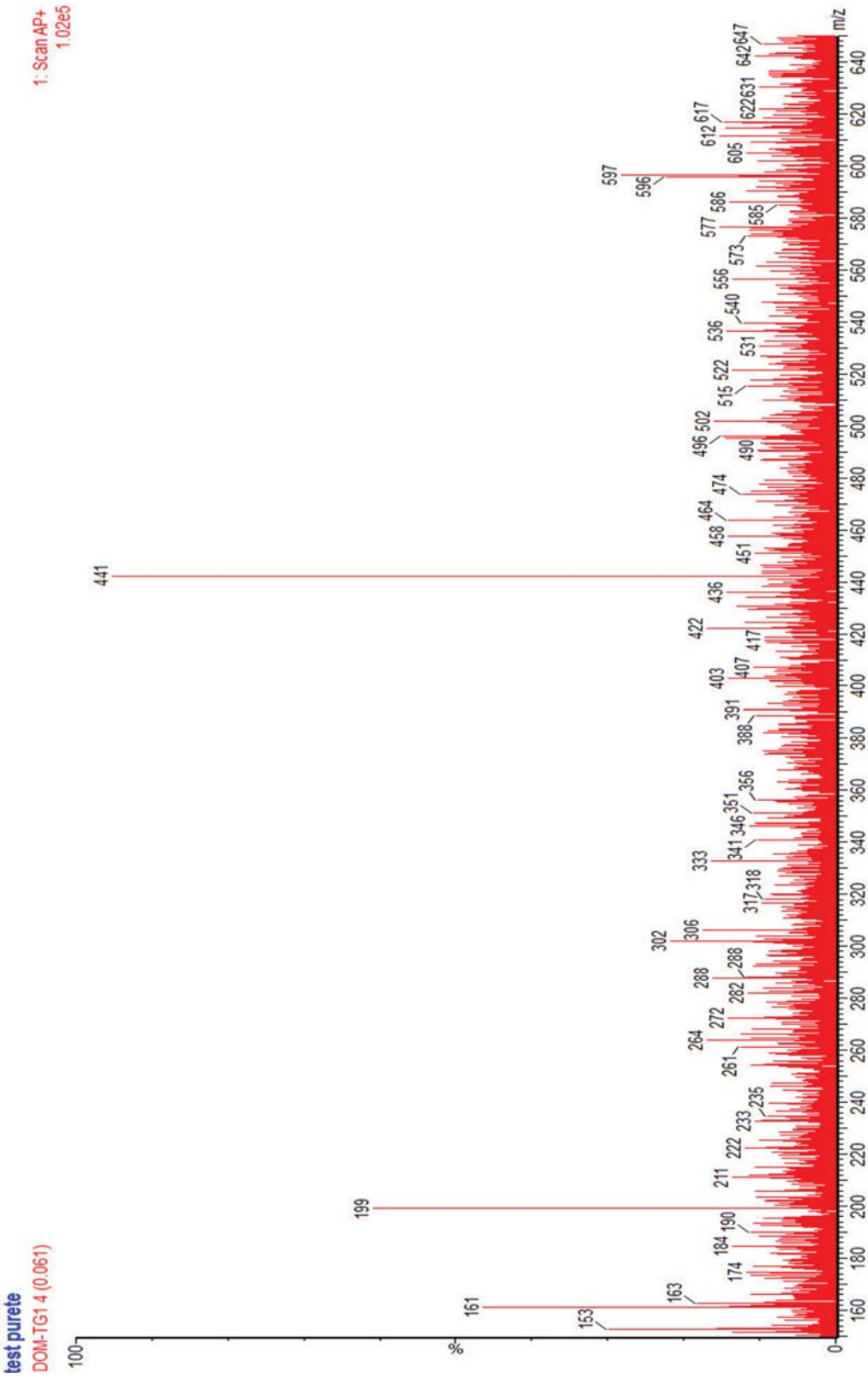


Figure S3. LC-MS spectrum of compound **1**

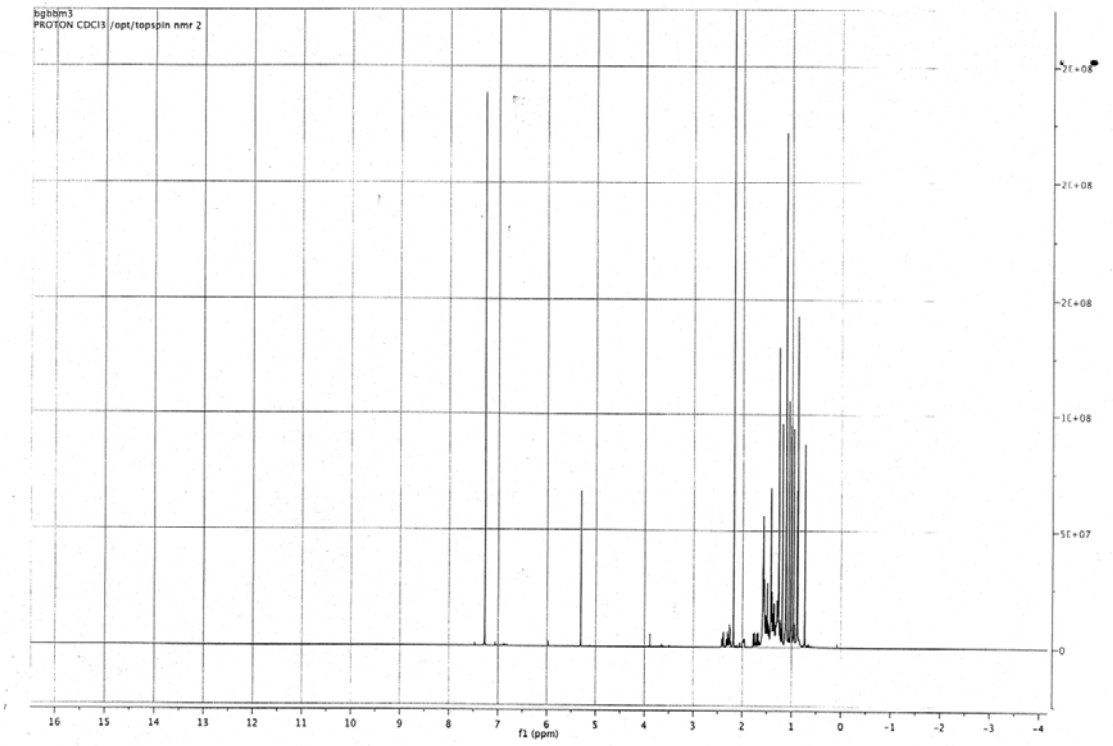


Figure S4. ^{1}H NMR spectrum of compound **1**

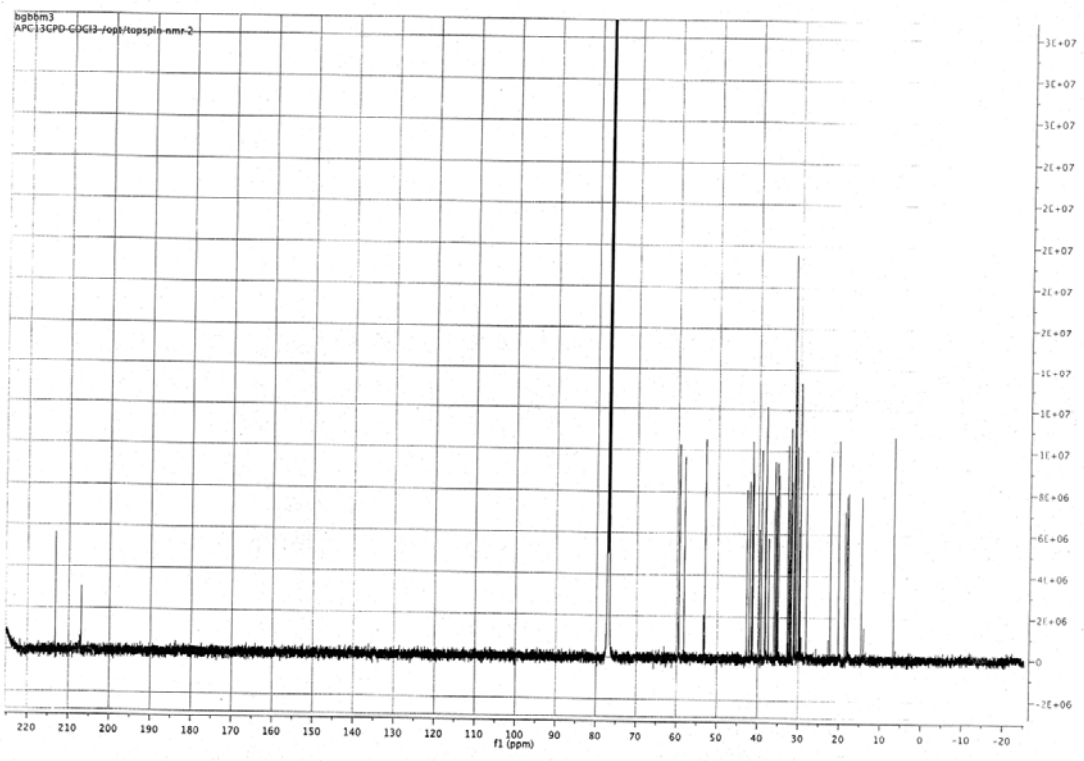


Figure S5. ^{13}C NMR spectrum of compound **1**

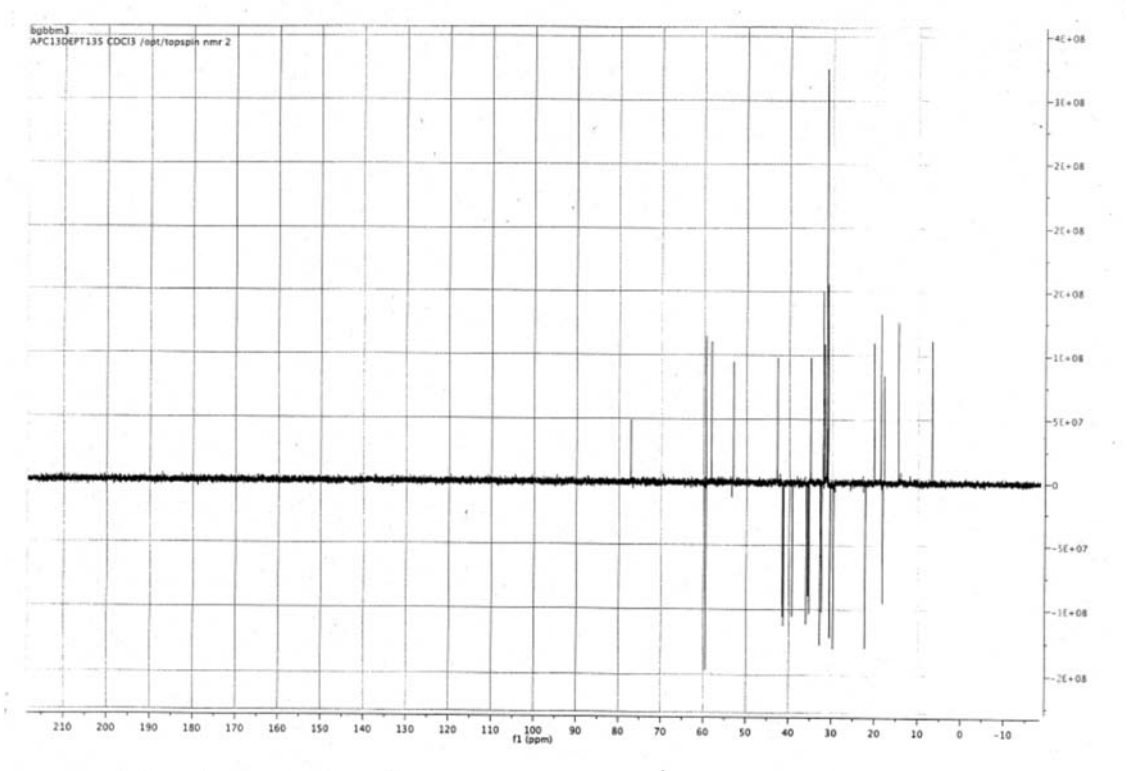


Figure S6. DEPT 135 spectrum of compound **1**

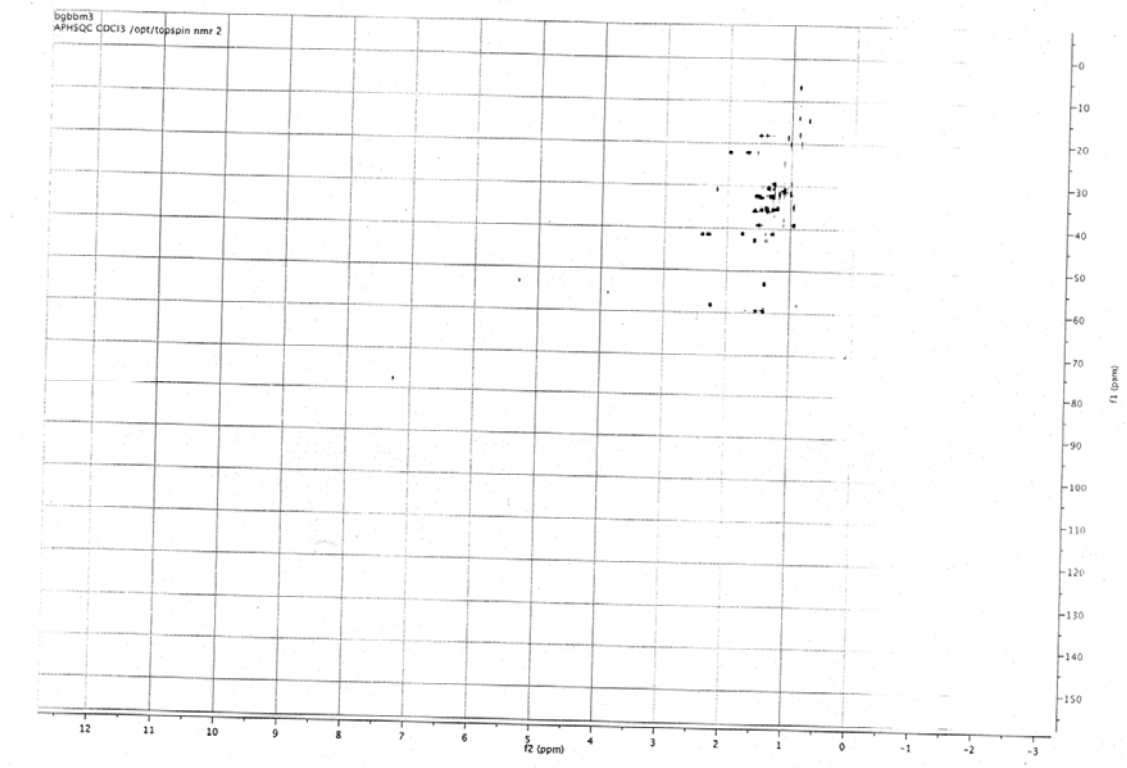


Figure S7. HSQC spectrum of compound **1**

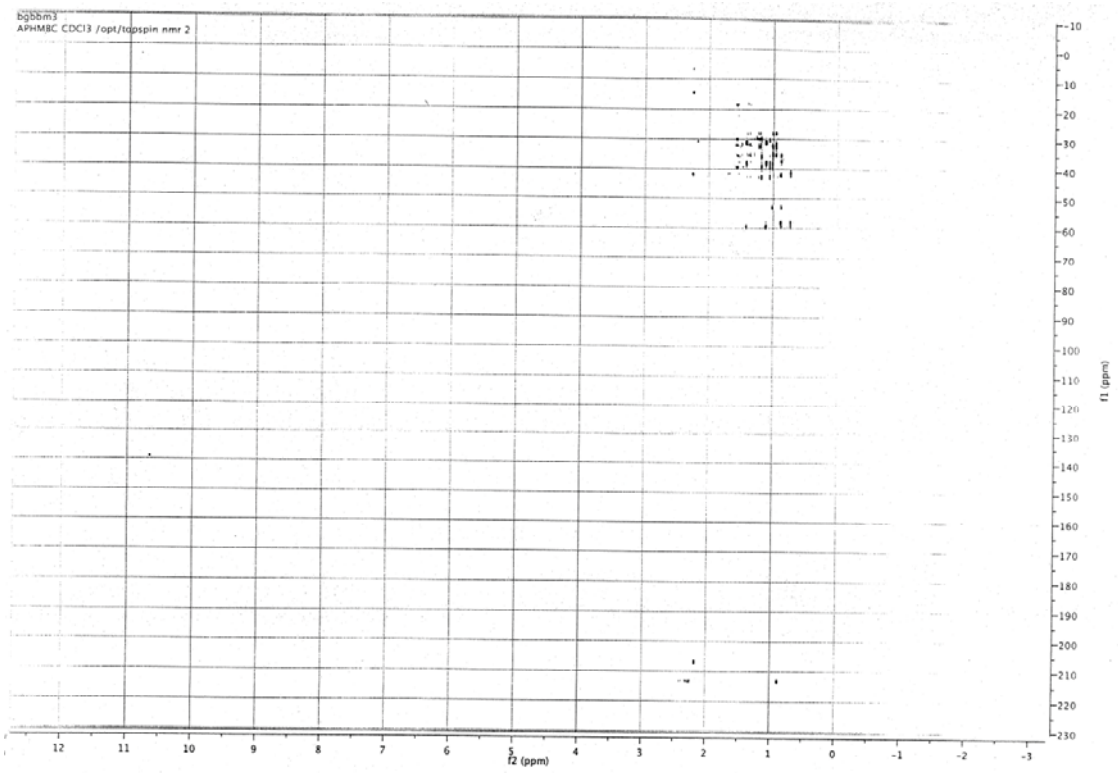


Figure S8. HMBC spectrum of compound **1**

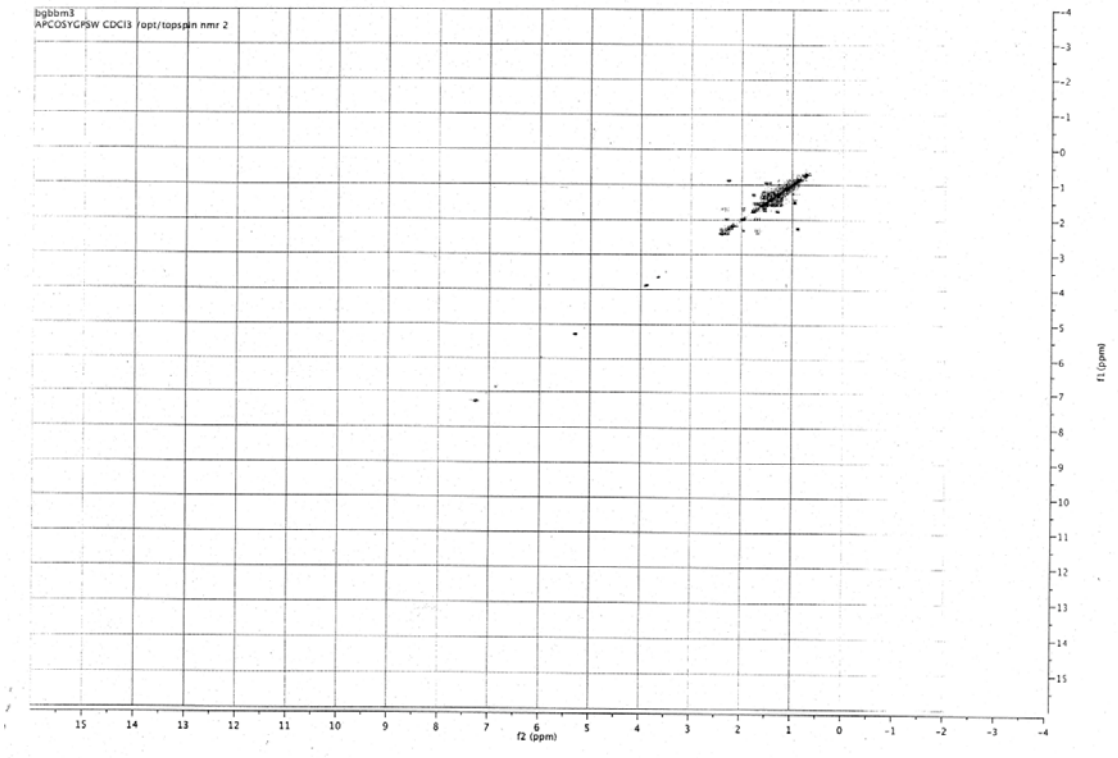


Figure S9. ^{1}H-^{1}H COSY spectrum of compound **1**

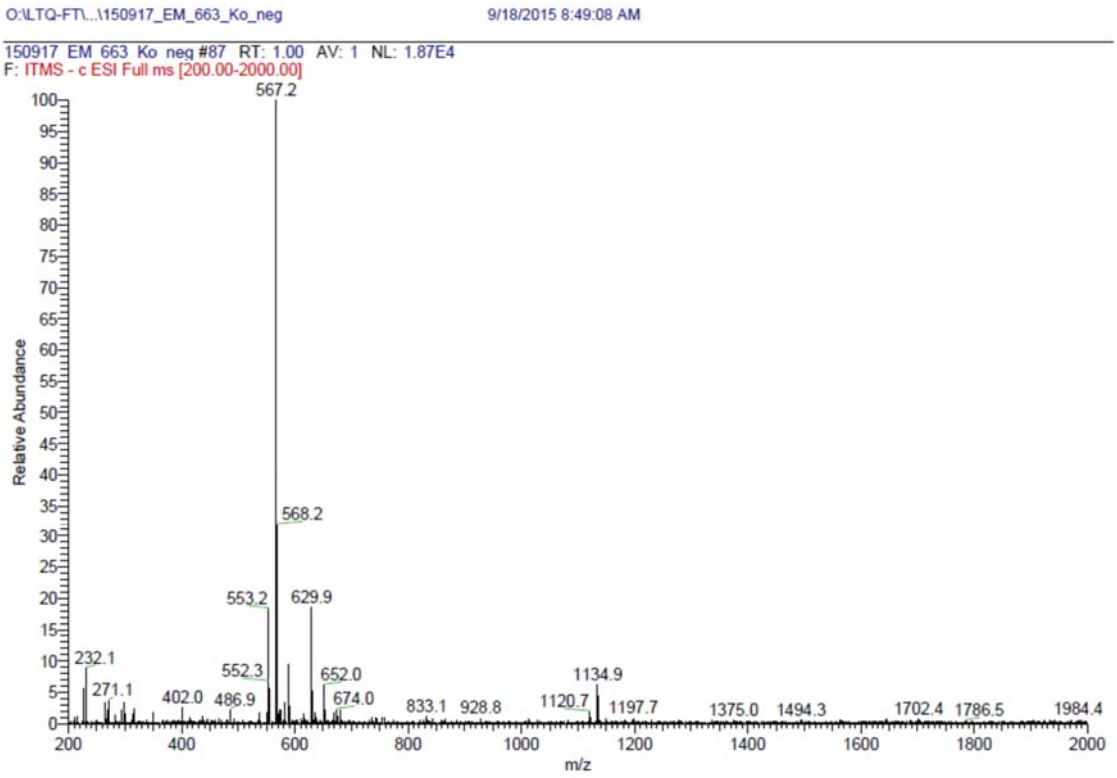


Figure S10. ESI-MS spectrum of compound **2**

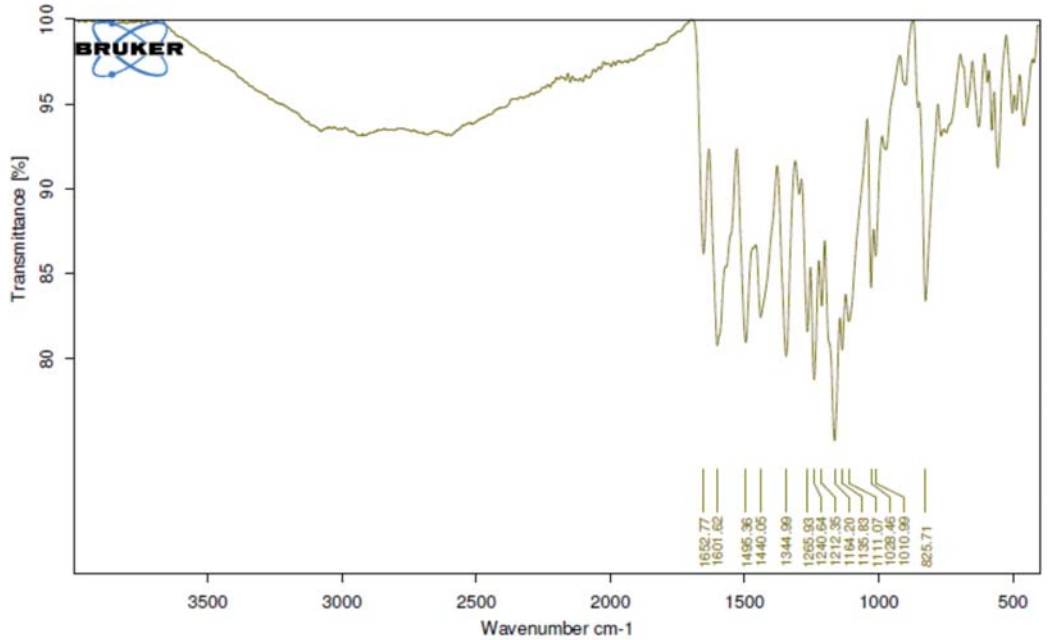


Figure S11. IR spectrum of compound **2**

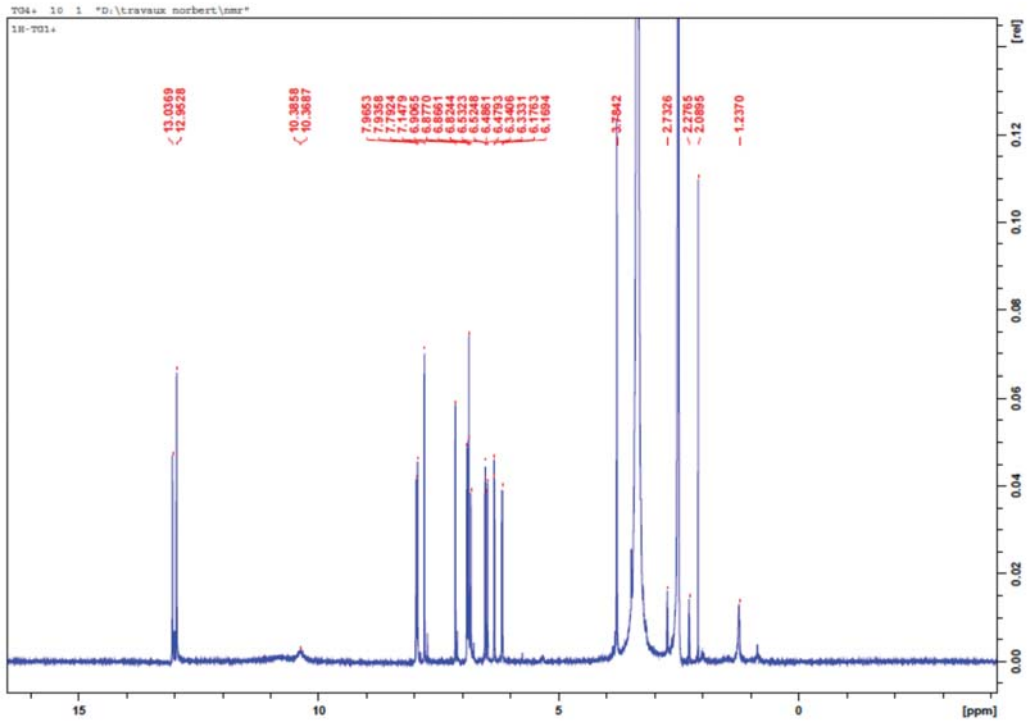


Figure S12. ^{1}H NMR of compound **2**

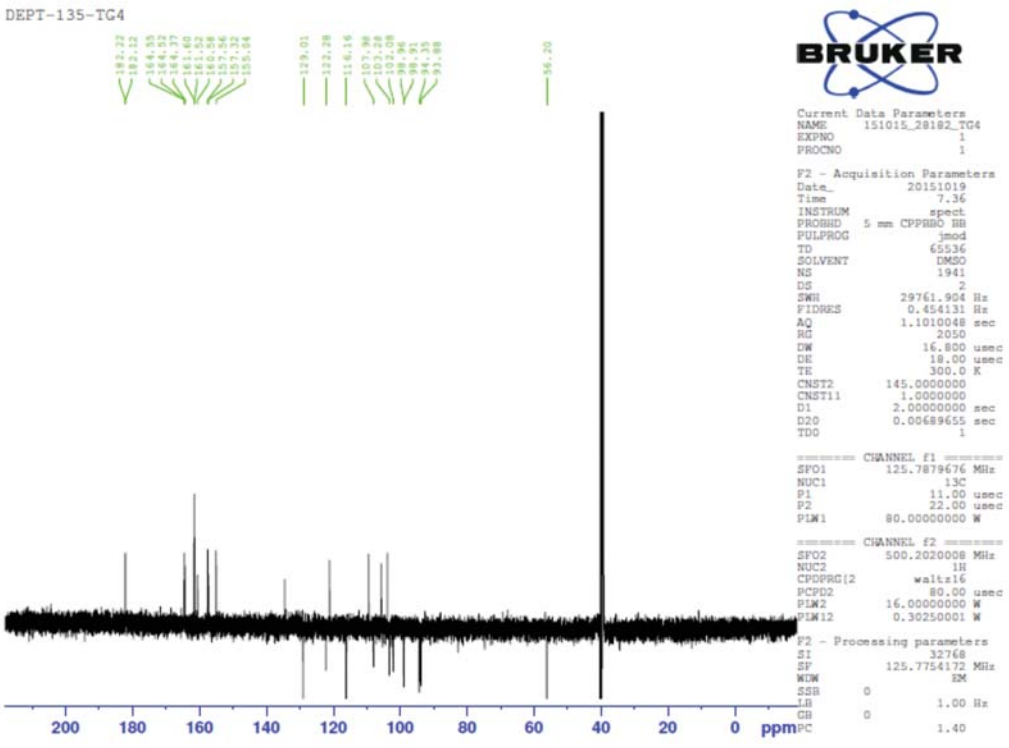


Figure S13. DEPT 135 of compound **2**

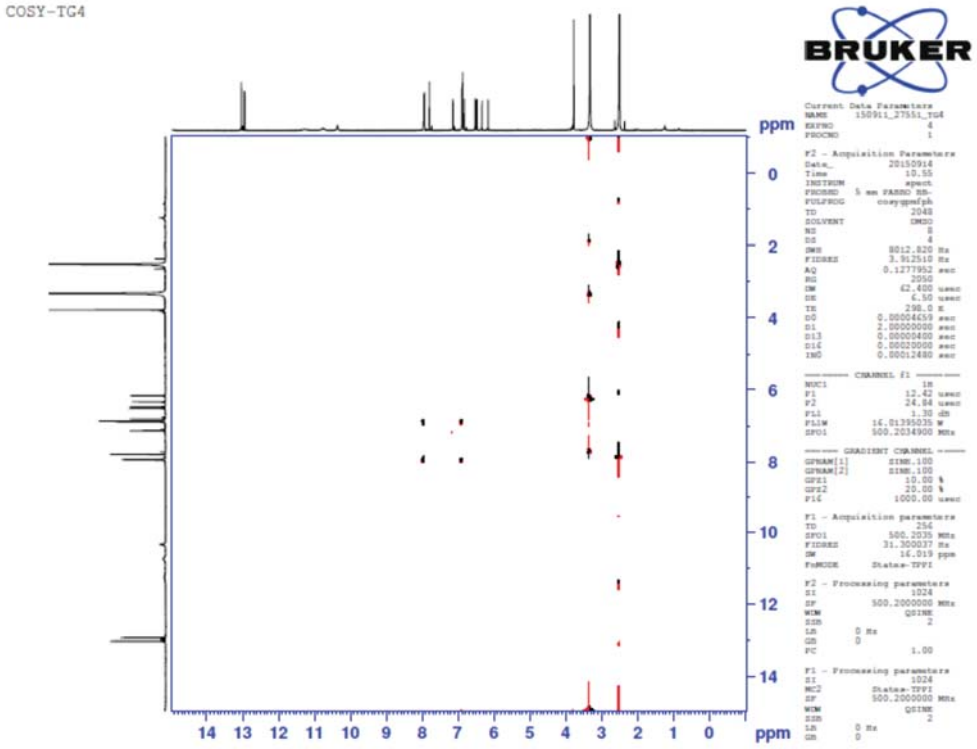


Figure S14. ^{1}H-^{1}H spectrum of compound **2**

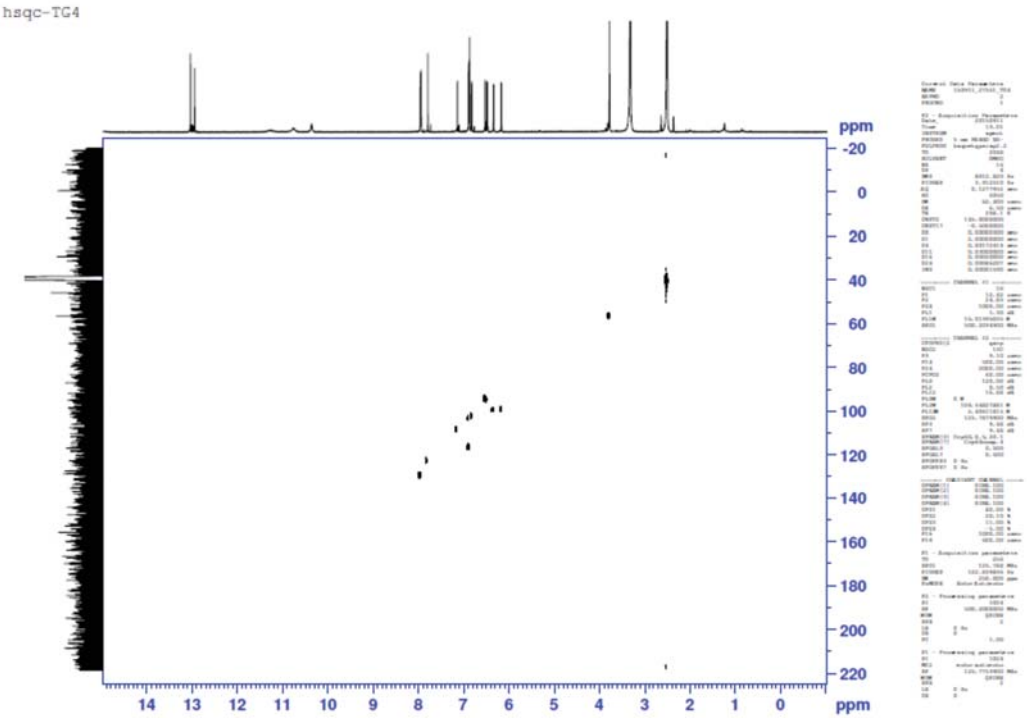


Figure S15. HSQC spectrum of compound **2**

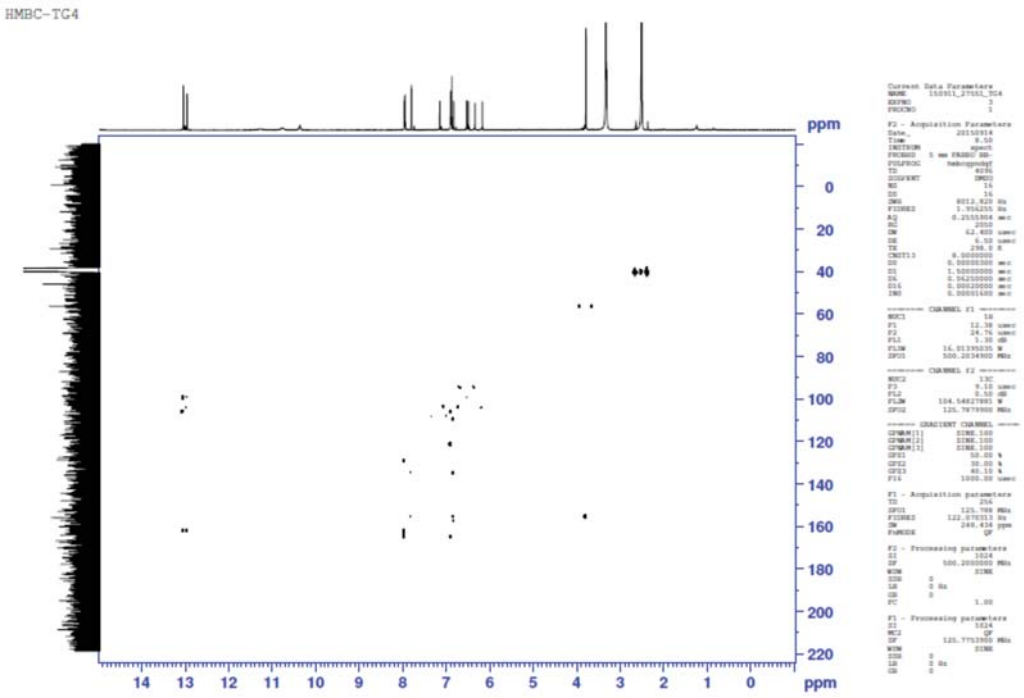


Figure S16. HMBC spectrum of compound **2**

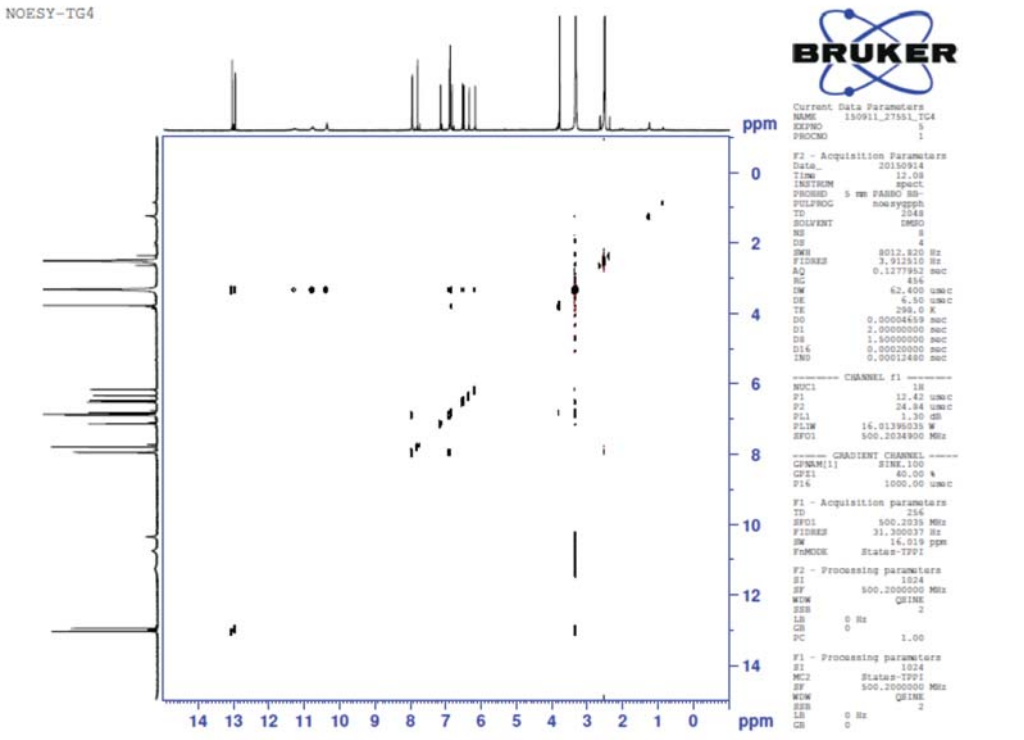


Figure S17. NOESY spectrum of compound **2**

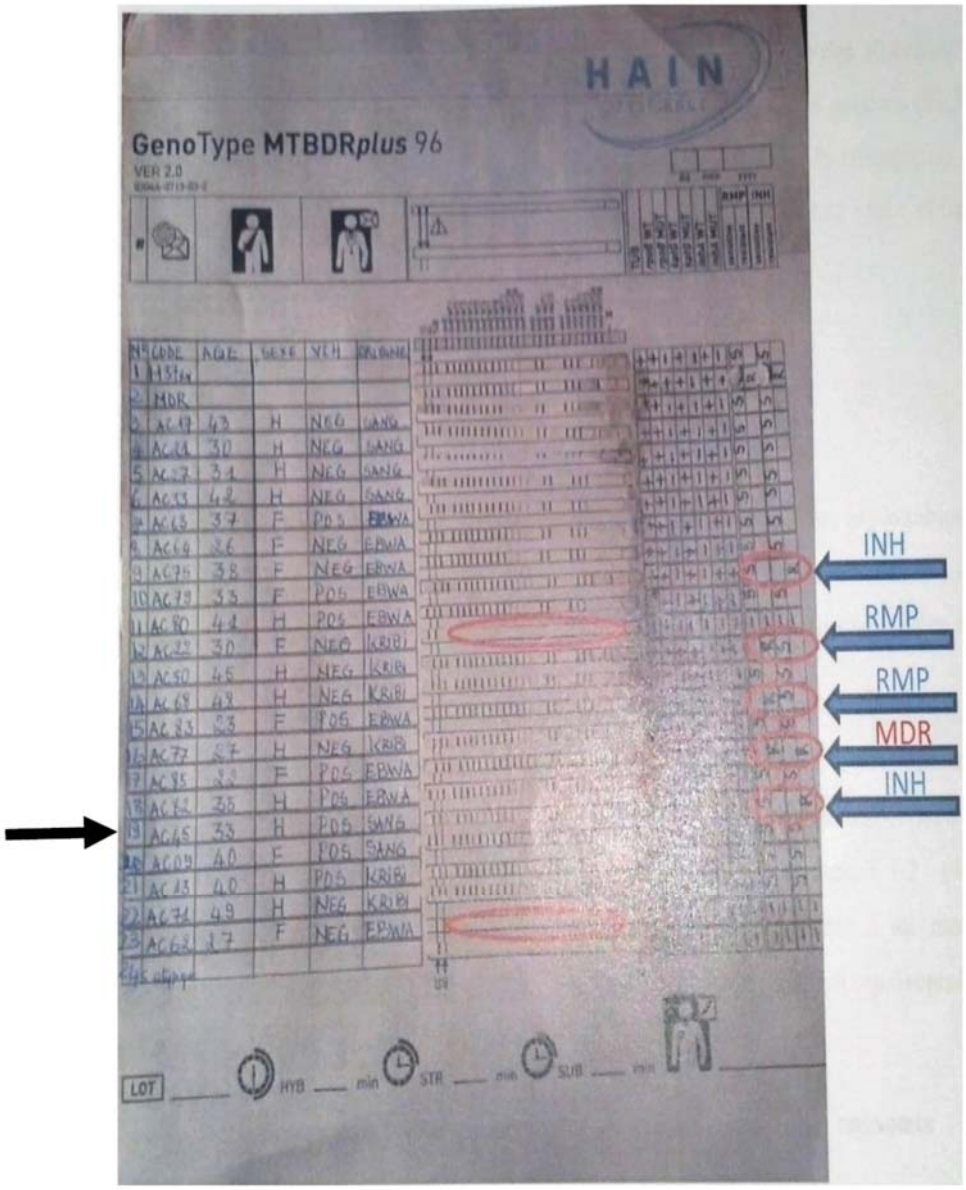


Figure S18. Genotype profile of *M. tuberculosis* codifies AC 45.

References

Clinical and Laboratory Standards Institute. 2011. Susceptibility Testing *of Mycobacteria, Nocardiae,* and Other Aerobic Actinomycetes-Second Edition*:* Approved Standard M24-A2 Wayne, PA, USACLSI.

Collins L, Franzblau SG. 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother. 41: 1004–1009.

Jiménez-Alleranes A, Meckes M, Ramirez R, Torres J, Herrera-Luna J. 2003. Activity against multidrug-resistant *Mycobacterium tuberculosis* in Mexican plants used to treat respiratory diseases. Phytother Res. 17: 903-908.

Jiménez-Alleranes A, Meckes M, Torres J, Herrera-Luna J. 2007. Antimycobacterial triterpenoids from *Lantana hispida* (Verbenaceae). J Ethnopharmacol. 111: 202-205.