

SUPPLEMENTARY MATERIAL

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

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Abstract

Triclisinone (**2**), a new ochnaflavone derivative, was isolated from the aerial parts of *Triclisia gillettii*, 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, ¹H and ¹³C 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 gillettii*; 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 ^1H 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 ($\text{HCOOH/MeCN/H}_2\text{O} = 1:12:87$) 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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ for the phytochemical study with increasing polarity and pure MeOH, while precoated aluminium sheets silica gel 60 F₂₅₄ were used for TLC.

Extraction and isolation

Dried and powered leaves of *T. gillettii* (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 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$) 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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (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 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (40/1-30/1). Sub-fraction IIc (0.83 g) was fractionated and purified using silica gel column and eluted with solvent system $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (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 CH₂Cl₂/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 CH₂Cl₂/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 CH₂Cl₂/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 °C 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 x10⁸ 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°C. After that, 40 µL of Alamar blue solution was added to two columns of each triplicate in order to show mycobacterial growth and the plate were re-incubated at 37°C 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

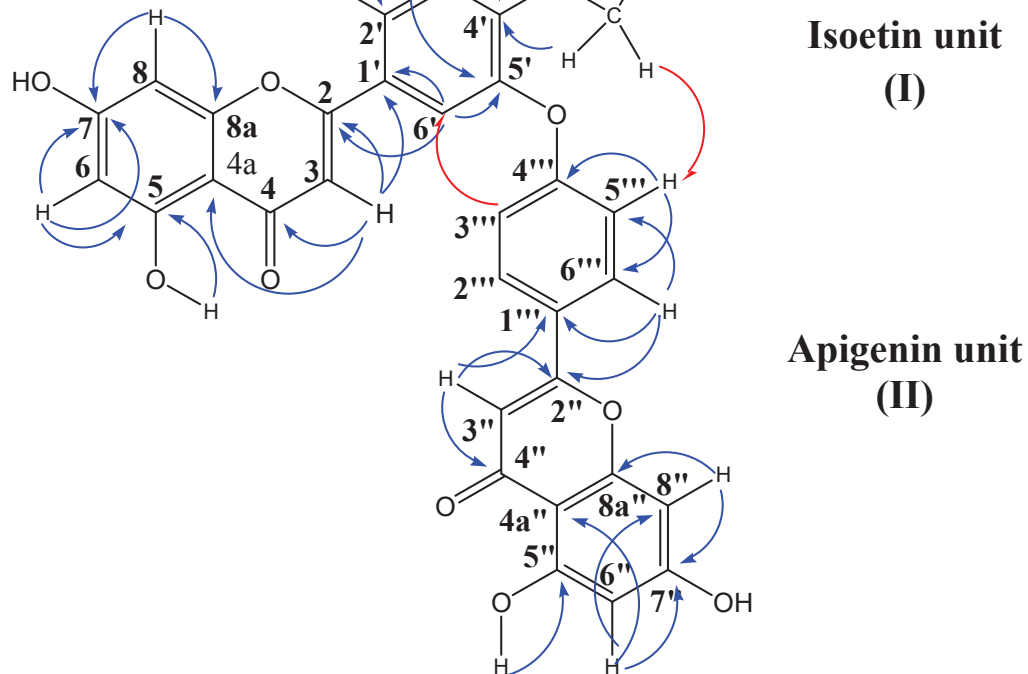


Table S1: ^1H and ^{13}C -NMR spectroscopic data^a of compound **1** (500 and 125 MHz in CDCl_3)
 δ in ppm and drypamolundein B. (300 and 75 MHz in CDCl_3)

Compound 1			drypamolundein 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: ^1H 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
<i>T. gillettii</i>	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

^aMinimum Inhibitory Concentration

^bMinimum Bactericidal Concentration

RMP = Rifampicin

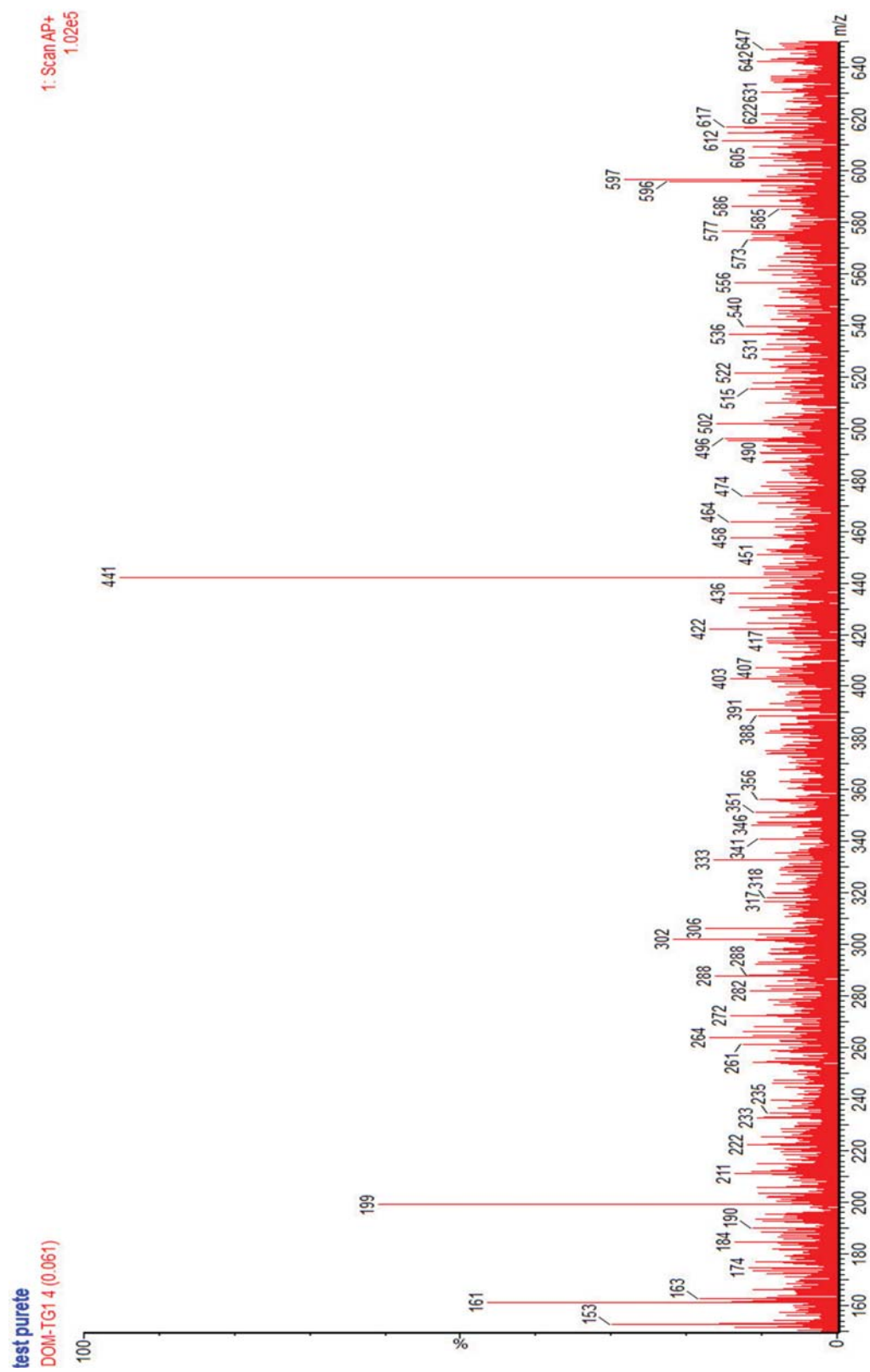


Figure S3. LC-MS spectrum of compound 1

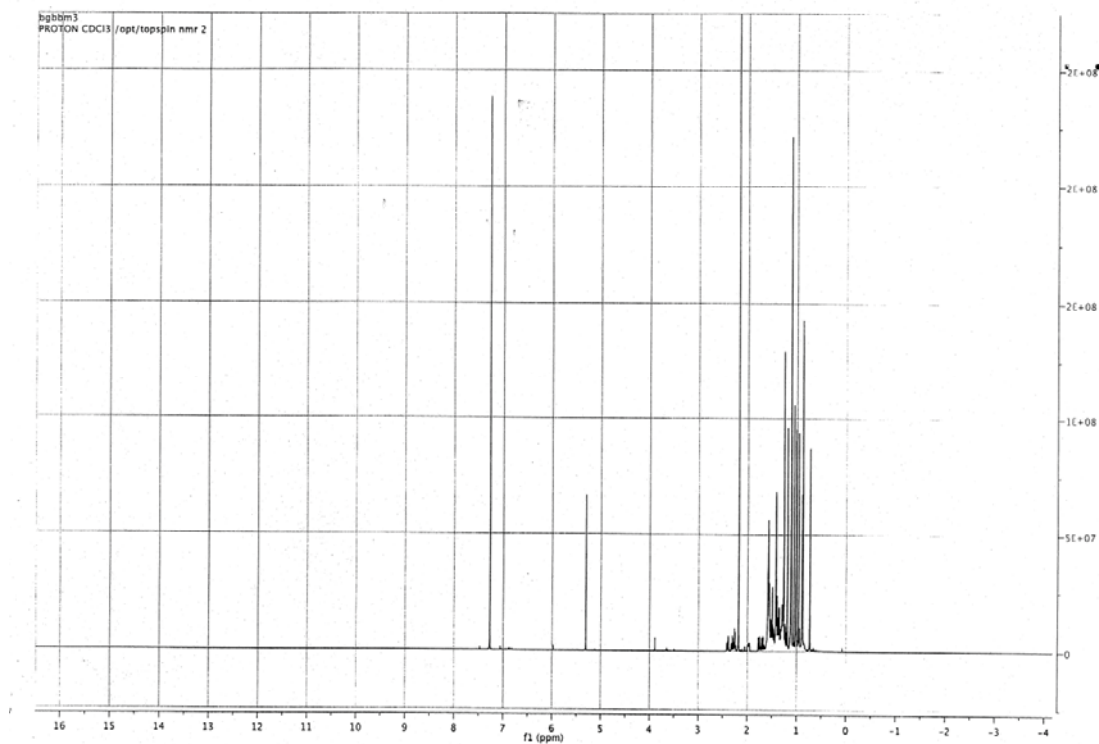


Figure S4. ^1H NMR spectrum of compound 1

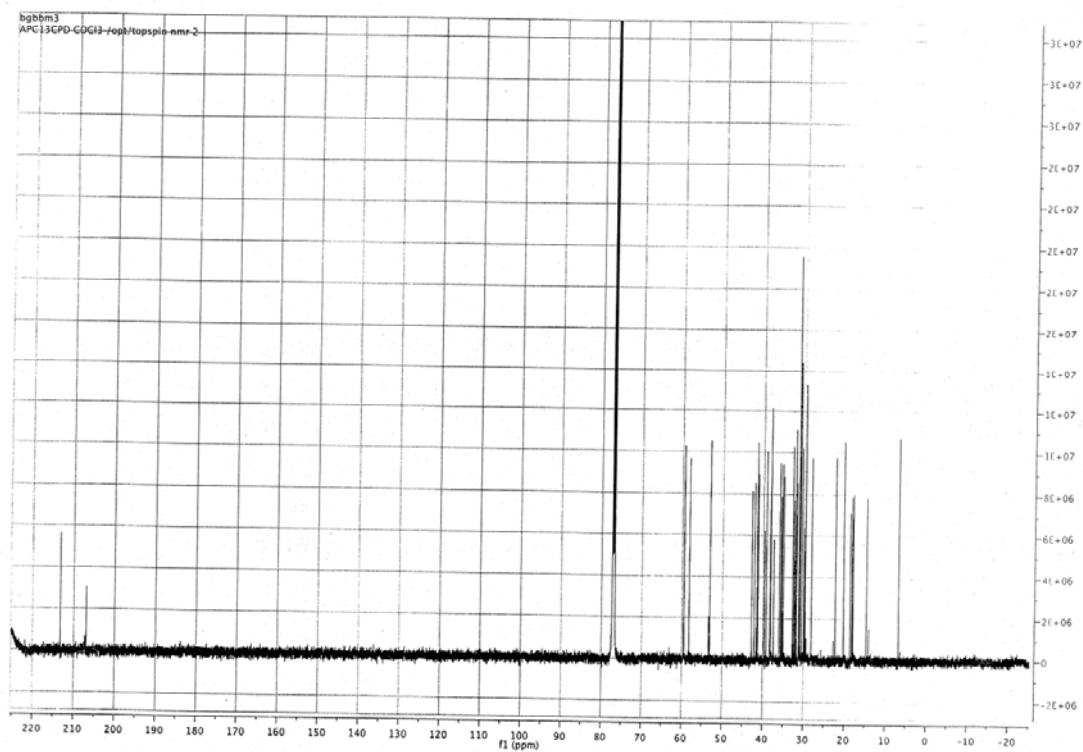


Figure S5. ^{13}C NMR spectrum of compound 1

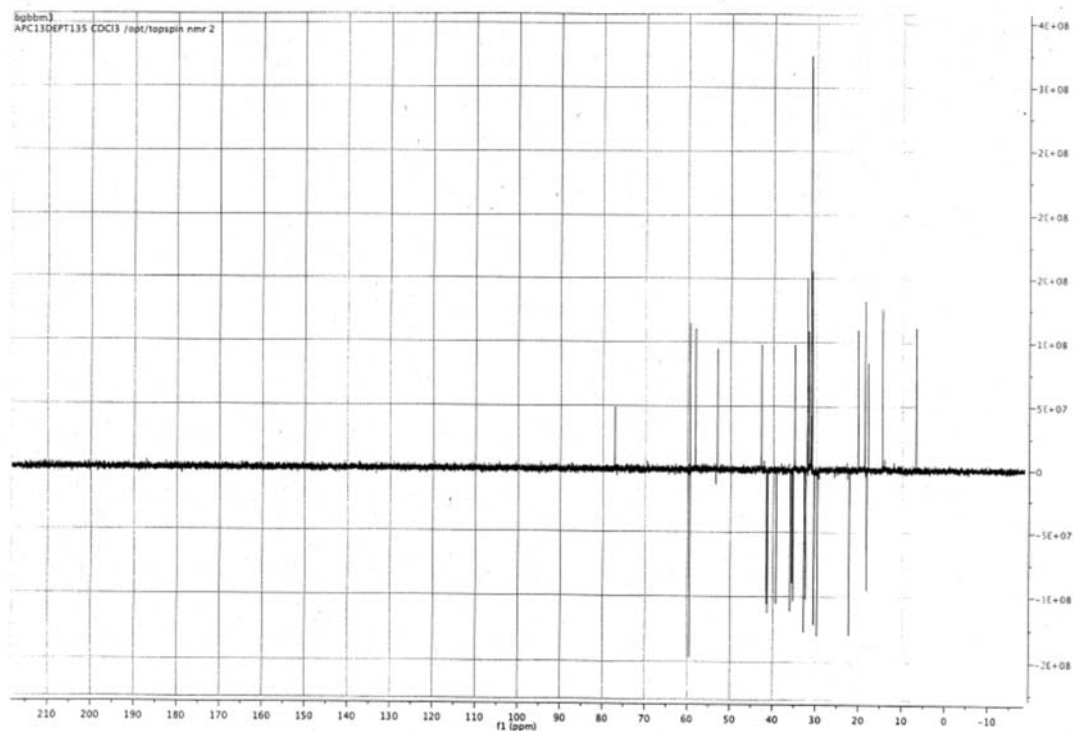


Figure S6. DEPT 135 spectrum of compound **1**

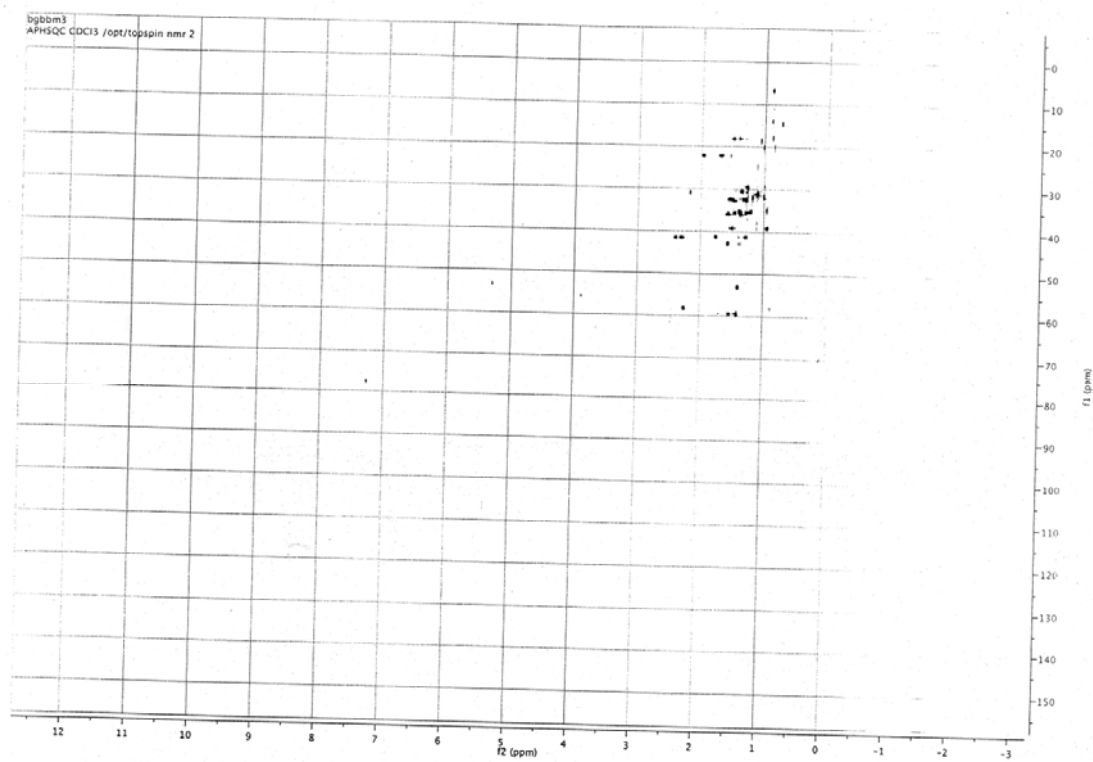


Figure S7. HSQC spectrum of compound **1**

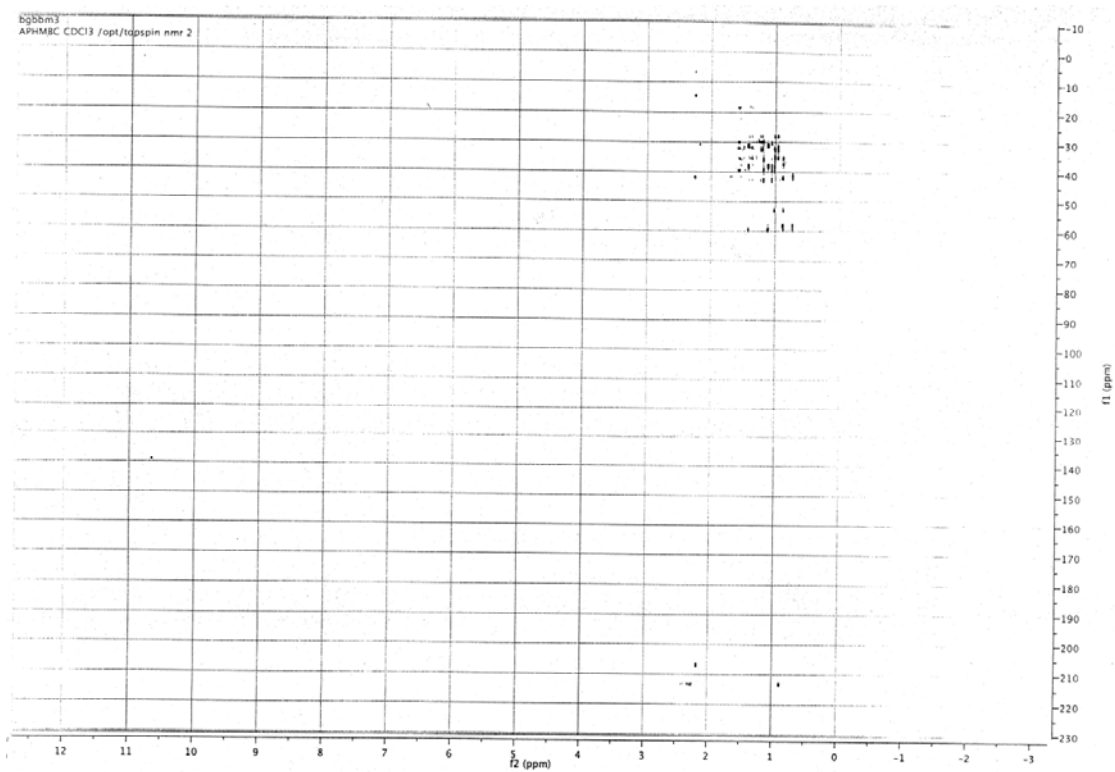


Figure S8. HMBC spectrum of compound **1**

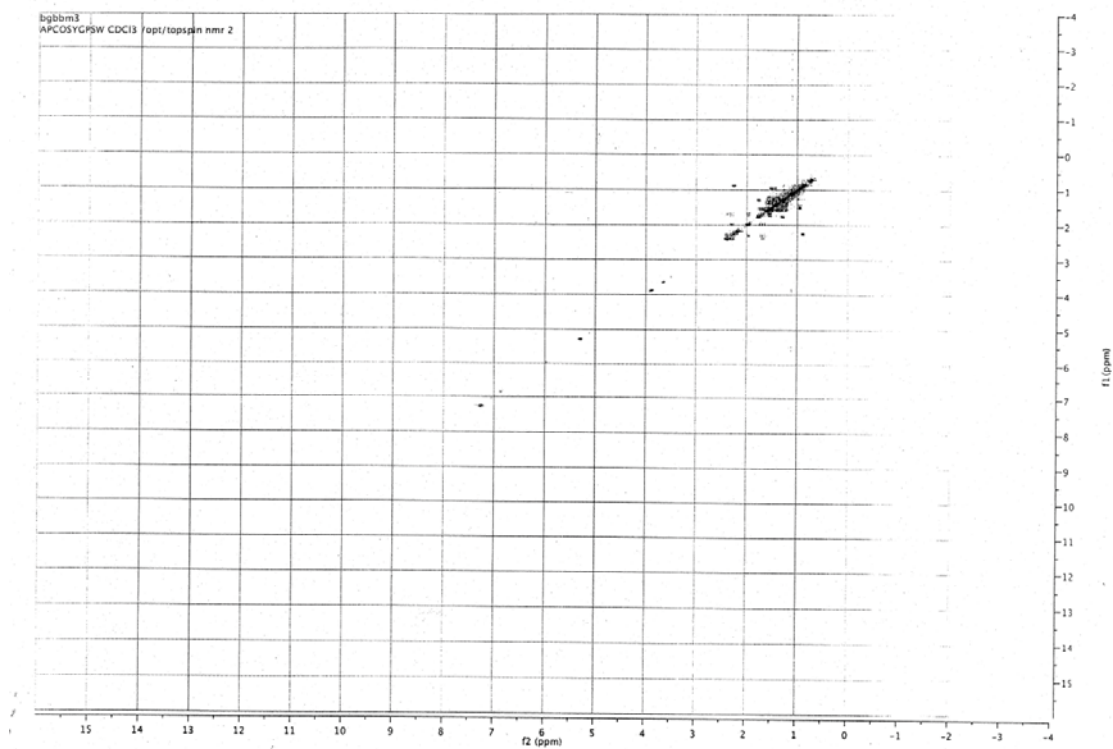


Figure S9. ^1H - ^1H COSY spectrum of compound **1**

150917 EM 663 Ko neg #87 RT: 1.00 AV: 1 NL: 1.87E4

F: ITMS - c ESI Full ms [200.00-2000.00]

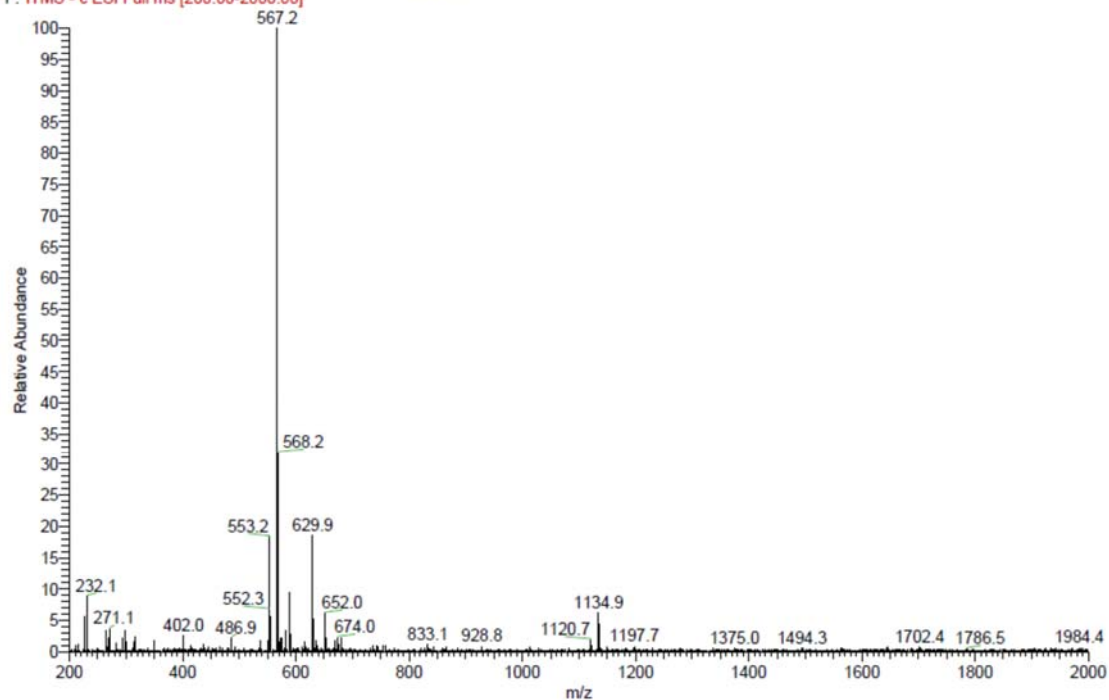


Figure S10. ESI-MS spectrum of compound 2

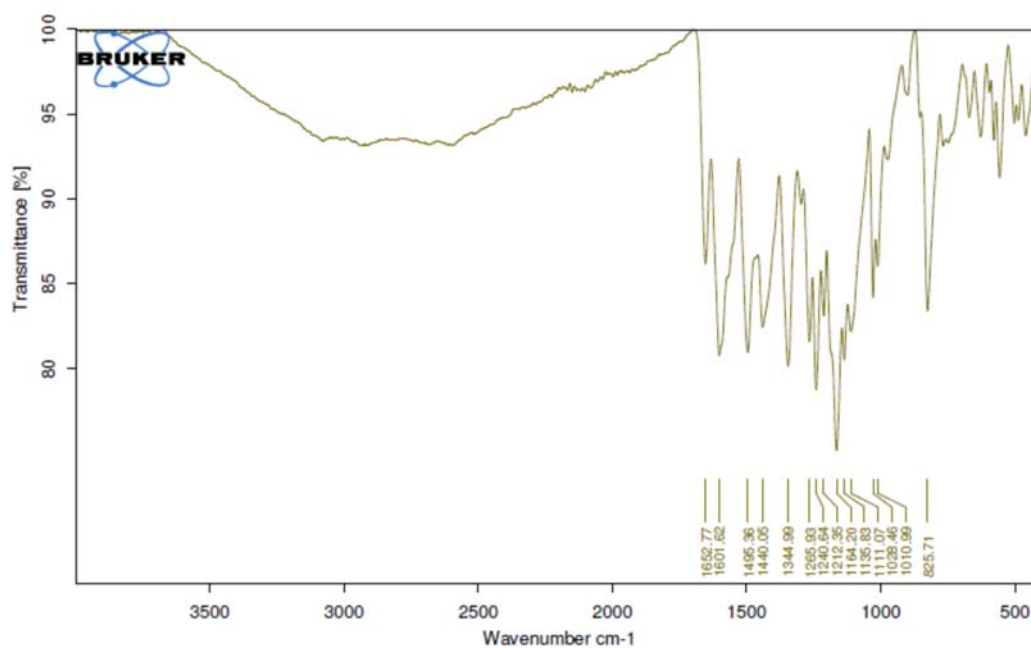


Figure S11. IR spectrum of compound 2

COSY-TG4

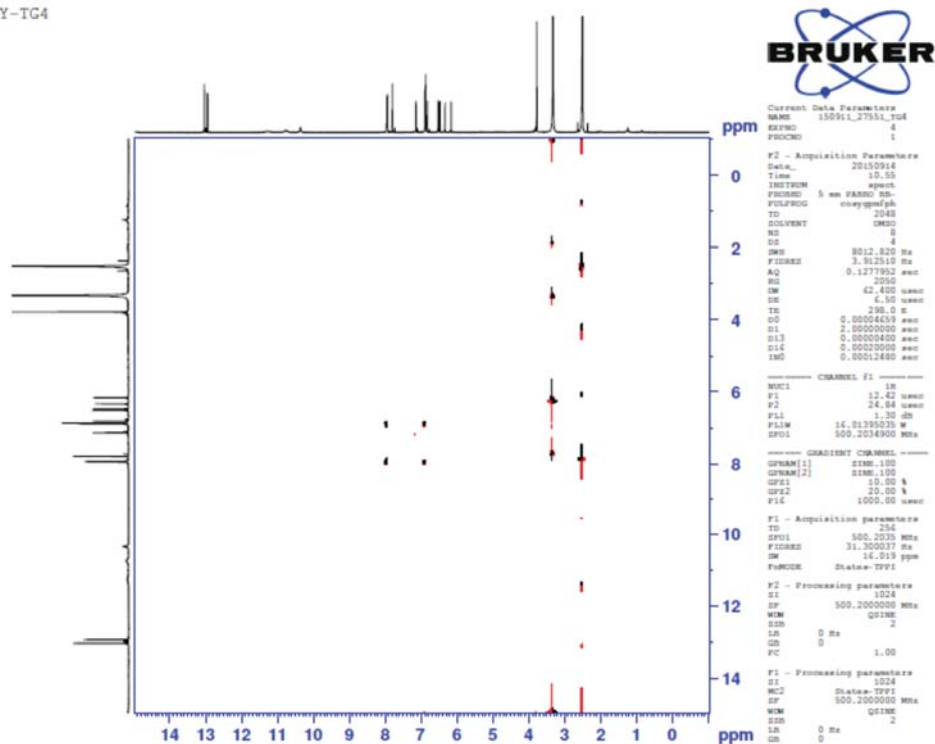


Figure S14. ^1H - ^1H spectrum of compound 2

hsqc-TG4

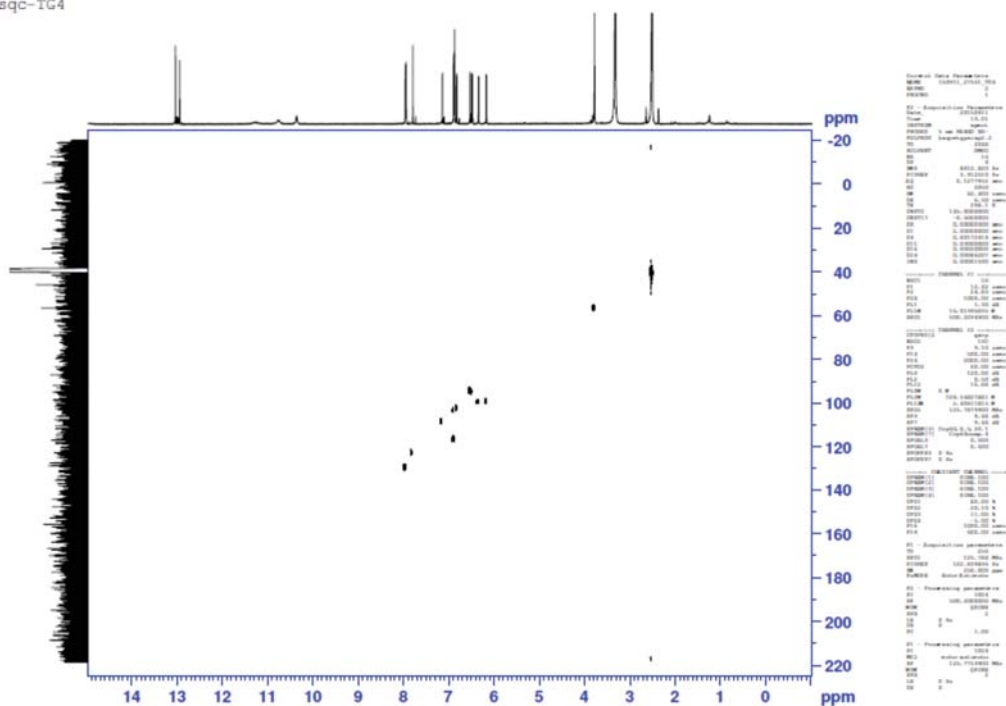


Figure S15. HSQC spectrum of compound 2

HMBC-TG4

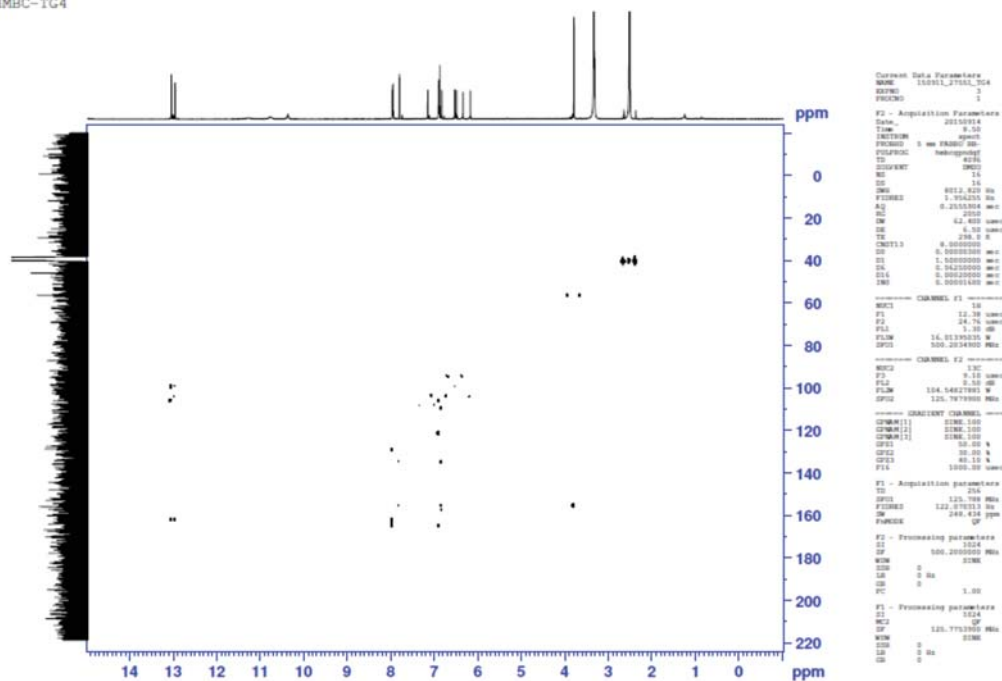


Figure S16. HMBC spectrum of compound 2

NOESY-TG4

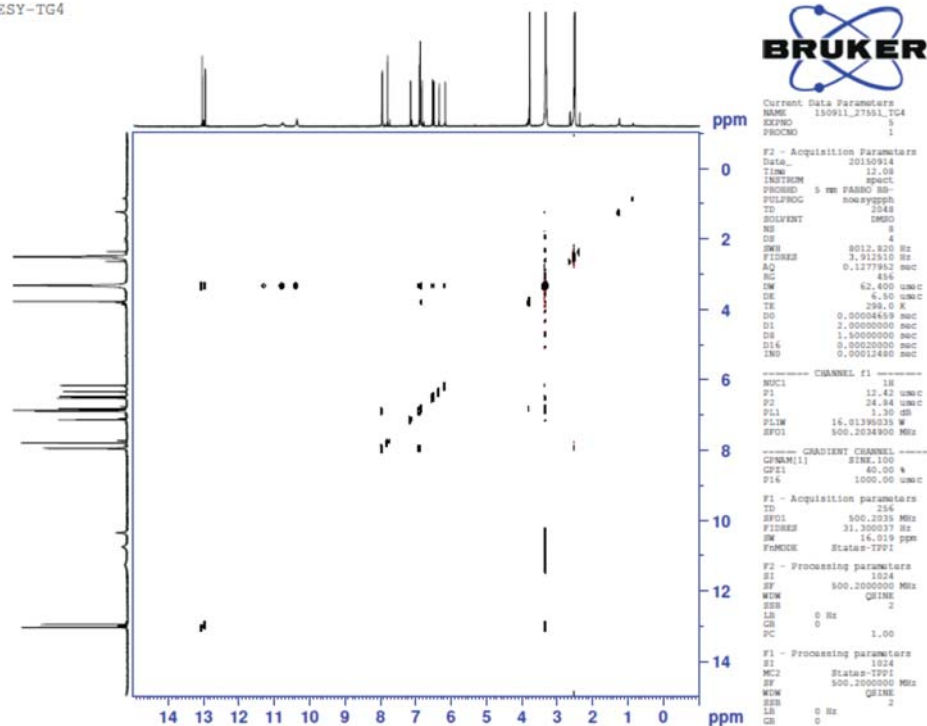


Figure S17. NOESY spectrum of compound 2

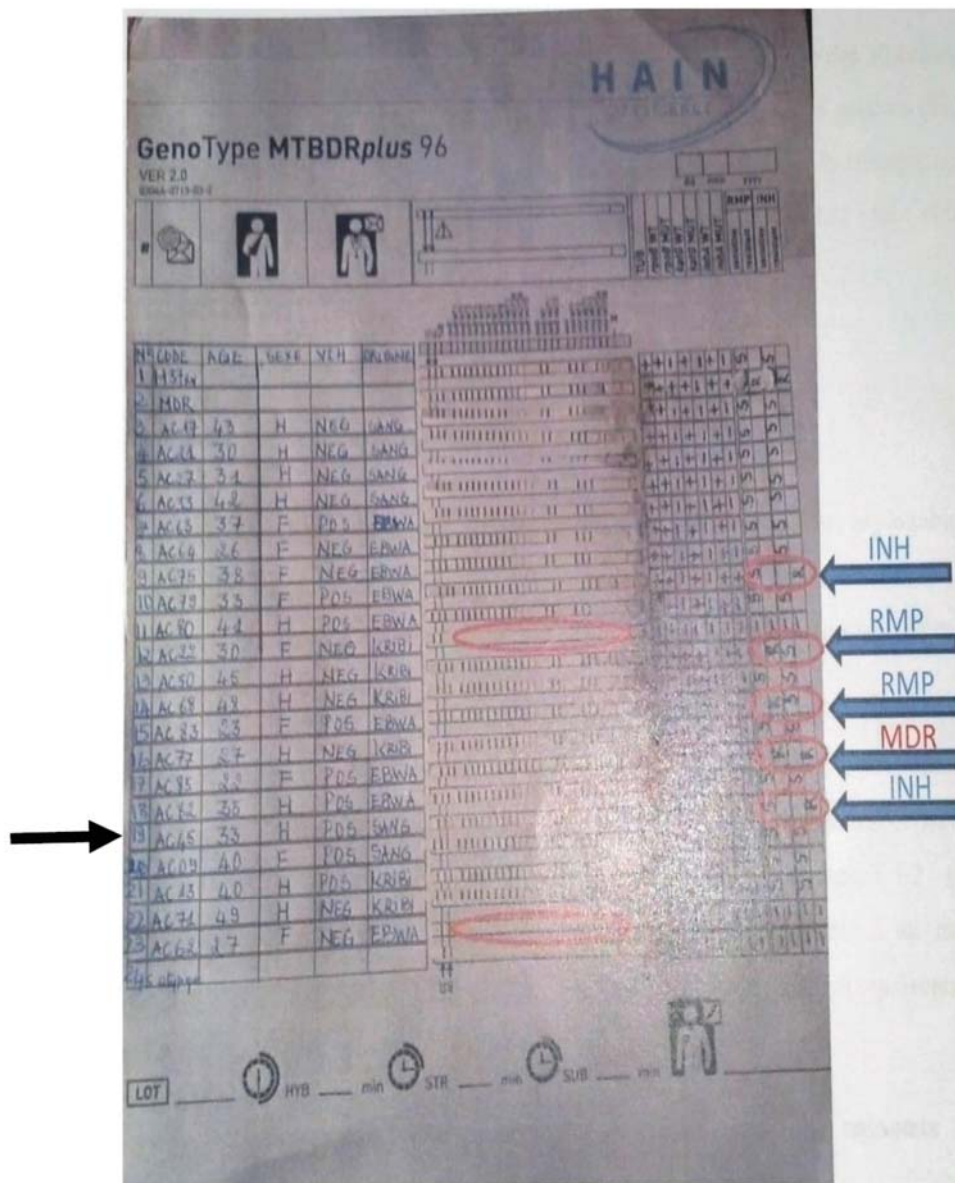


Figure S18. Genotype profile of *M. tuberculosis* codifies AC 45.

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