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► To cite this version:

Josette Linda Toussi Matchi, Diderot Tchamo NOUNGOUÉ, Isabelle Kuhn, Jérôme Boissier, Jean Claude Tchouankeu, et al.. Manniindole, an indole derivative from the roots of Anonidium mannii and combined antischistosomal and enzymatic activities. *Natural Product Research*, 2021, 35 (24), pp.5665-5673. 10.1080/14786419.2020.1824227 . hal-02996085

HAL Id: hal-02996085

<https://hal.science/hal-02996085>

Submitted on 2 Apr 2021

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Manniindole, an indole derivative from the roots of *Anonidium mannii* and combined antischistosomal and enzymatic activities

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ABSTRACT

A new alkaloid, manniindole 1, together with four known compounds: aristolactam AII 2, aristolactam BII 3, piperolactam D 4 and polycarpol 5 were isolated from the crude extract EtOH:H₂O (8:2) of the roots of *Anonidium mannii* by chromatographic separation. The structure elucidation was performed on the basis of a spectroscopic analysis (IR, HRESI MS, 1D and 2D NMR) as well as a comparison of their spectral data with those reported in the literature. For the first time, the crude extract and those isolated compounds were evaluated for their antischistosomal activity against *Schistosoma mansoni* and for cytotoxicity activity against Huh7 and A549 cells. Furthermore, they were also tested in vitro on the recently characterized *Schistosoma mansoni* NAD⁺ catabolizing enzyme (SmNACE) for their impact on this enzyme which is localized on the outer surface of the adult parasite. Compound 2 displayed quite good worm killing capability, while 4 showed significant inhibition of SmNACE

KEYWORDS

Anonidium mannii ; Annonaceae; phytochemical and biological investigation ; indole alkaloid ; antischistosomal activity ; enzymatic activity (SmNACE) ; cytotoxicity

1. Introduction

The genus *Anonidium*, belonging to the Annonaceae family, comprises 7 tropical African tree and shrub species (Pellegrin 1947), but this genus is not well studied. *Anonidium mannii* (Oliv.) Engl. & Diels, is known to be located to Central Africa [Cameroon (Bankomo, Makenene, Ndikinemeki, Kribi), Gabon and Congo], and has been used in the respective countries of origin for the treatment of various ailments including female infertility (Abondo et al. 1991), treatment of abscess (Taffou et al. 2017), open wounds, hemorrhoids, intestinal spasms, diarrhea (Musuyu Muganza et al. 2012), arthritis, rheumatism, dysentery, paralysis, epilepsy, convulsion, stomach trouble (Erhenhi and Obadoni 2015), cancer (Kuede et al. 2013) malaria (Betti 2004) and Schistosomiasis (Messi 1999). Otherwise, Schistosomiasis, also known as bilharzia, is a disease caused by parasitic worms infecting people worldwide. This neglected tropical disease (OMS 2010; Zhang et al. 2010) comes second after malaria as the most devastating parasitic disease in terms of impact. It is estimated that 600 million people are at risk of infection, 200 million people are infected, and at least 200 thousand deaths per year are associated with the disease (OMS 2014). The parasites that cause schistosomiasis live in certain types of freshwater snails, from which their infectious form, known as cercariae, can emerge into the water and infect humans through skin contact. In most human infections, acute schistosomiasis is caused by *Schistosoma mansoni*, *S. haematobium*, or *S. japonicum*, and is characterized by fever, headache, myalgia, and respiratory symptoms, and occasionally by eosinophilia and painful hepato-and/or splenomegaly. No vaccine is currently available and the antiparasitic drug mostly used to treat schistosomiasis is Biltricide, which active ingredient (Praziquantel) belongs to the alkaloid family (OMS 2020; Doenhoff et al. 2009). However, it is only effective against adult worms and it has showed many side effects. More worrying, because the question of the reduction of the efficacy of praziquantel molecule was raised recently, it is thus urgent to find new, safe antischistosomal drugs and new therapeutic targets (Fallon and Doenhoff 1994). SmNACE is an ecto-enzyme recently discovered which has a favorable topology because it is one of the rare identified and characterized target on the tegument of adult schistosomes responsible for serious clinic troubles. In fact, SmNACE is extremely interesting due to its accessibility of drugs and thus a potential target for conception of future therapeutic agents (Goodrich et al. 2005; Kuhn et al. 2010; Jacques et al. 2015).

The Annonaceae family is known to contain acetogenins, alkaloids, essential oils, terpenoids, and some flavonoids (Leboeuf 1982). Previous phytochemical investigation on the specie *Anonidium mannii* revealed the presence of alkaloids, phenols, polyphenols, saponins, tannins and steroids (Djeussi et al. 2013; Ngangoue et al. 2020). Some prenylated indoles and bisindole alkaloids have been identified from the stem bark of *A. mannii* (Achenbach and Renner 1985) and some have been reported to have the following biological activities: antibacterial, immunosuppressive and radical scavenging (Southon and Buckingham 1989). As a part of our ongoing project on the structurally and biologically interesting secondary metabolites from *Anonidium mannii*, and based on previous reports on the antiparasitic activity of this species, the aim of this work was to carry out a phytochemical and biological investigation on the roots of the plant collected from Cameroon.

2. Results and discussion

Herein report the isolation and characterization by the spectroscopies means, of the novel indole derivative 1, together with four known compounds identified as, aristolactam A II 2 (Sun et al. 1987), aristolactam B II 3 (Akasu et al. 1974), piperolactam D 4 (Desai et al. 1990) and polycarpol 5 (Hammoni re et al. 1976) respectively (Figure 1); and also their cytotoxicity, enzymatic and antischistosomal activities evaluation

Compound 1 was isolated as a yellow-orange amorphous powder, which gave a positive Dragendorff test characteristic of alkaloid. Its molecular formula $C_{18}H_{23}N_3O$, could be deduced

from its positive high resolution electrospray ionization mass spectrum (HRESI-MS) which showed protonated molecule $[M + H]^+$ ion peak at m/z 298. 1924 (calc. 298. 1919), indicating nine degrees of unsaturation. Its infrared spectrum displayed maxima at around $3\ 480\text{ cm}^{-1}$ which could be due by a vibration of the N–H group for a indole skeleton (Benesova et al. 1969), and we can also observe absorptions attributable to the N-carbamoylpyrrolidine at (cm^{-1}) 3270 and 3100 (NH₂); 1651 (amid carbonyl, N₂C=O); 1519, 1494 (N–CO–N) (D'Ambrosio et al. 1986).

The ¹³C NMR spectrum (Table S1) displayed 18 carbon signals which were sorted by DEPT into two methyls, four sp³ methylenes, six methines (three aromatics, two olefinics and one sp³) five sp² quaternary carbons and one carbonyl at δ_c 157.2 characteristic of N-carbamoyl group.

The ¹H NMR spectrum of **1** (Table S1) showed in the low field region, two broad singlets at δ_H 10.80 and 7.03 which might confirm the existence in **1** of an indoyl N-H and olefinic (H-2) protons respectively (Benesova et al. 1969), and furthermore it exhibited also three coupled aromatic protons each other at δ_H 7.34 (1H, bd, $J=7.8$ Hz, H-4), δ_H 6.90 (1H, (1H, t, $J=7.5$ Hz, H-5) and δ_H 6.85 (1H, bd, $J=7.0$ Hz, H-6), this assume that the single aromatic cycle of **1** was trisubstituted. In addition, this spectrum showed the characteristic resonances of a 3,3-dimethylallyl moiety [δ_H 1.71 (6H, s, H-4'' and H-5''); 3.50 (2H, d, $J=7.4$ Hz, H-1'') and 5.43 (1H, m, H-2''). After all, we can also observe aliphatic protons at δ_H [1.86 (2H, m, H-3''); 1.89 (1H, m, H-4a), 2.17 (1H, m, H-4b); 3.43 (1H, m, H-2a); 3.51 (1H, m, H-2b) and 5.11 (1H, bd, $J=7.2$ Hz; H-5'')], together with amide protons (CONH₂) at δ_H 5.45 (2H, s,); According to its molecular formula, all these data corroborate the presence in the compound **1** of a N-carbamoylpyrrolidine group. Combined analysis of these data above indicated that compound **1** had a framework of an indole type alkaloid which was substituted by prenyl and N-carbamoylpyrrolidine groups respectively. In its ¹H-¹H COSY spectrum, coupling was observed between olefinic proton (H-2) and N-H proton of the pyrrole ring of the indole moiety and this assume that C-3 was substituted, and associate with the HMQC, that spectrum revealed connectivity of pyrrolidine ring (C-2', C-3', C-4' and C-5' resp.) and prenyl group (C-1'', C-2'' and C-4''/5'' resp.). Key HMBC correlations between aliphatic protons (H-2'' and H-1'') of the 3,3-dimethylallyl moiety and aromatic carbons (C-7 and C-6; C-7a resp.) of the indole skeleton allowed us to fix the prenyl group on C-7, and interaction between H-5' and C-2 enabled us to attach N-carbamoylpyrrolidine on carbon C-3. This was confirmed by its NOESY correlations observed between H-5' and H-2 and (CONH₂) protons respectively (Figure S2). The compound **1** was showed optical activity, due to its chiral carbon (C-5) which deflected the polarized light to the left (levorotatory) based on the polarimetry performed. Hence, its structure was identified as (-)-3-(5'-N-carbamoylpyrrolidine)-7-(3''-methyl-2''-butenyl) indole and has been given the trivial name manniindole.

In the meantime, new compound **1**, and compounds **2**, **3** and **4** were subjected to antischistosomal, enzymatic and/or cytotoxicity activities, the results were quite encouraging.

The human lung cancer cells (A549) and the human hepatocarcinoma cells (Huh7) were chosen for evaluating the cytotoxicity of compound **1** due to the fact that the lung and liver are the focal points of pathogenic insult and subsequent pathological damage in schistosomiasis (De Oliveira et al. 2013). At the concentration C=100 μM , the percentage of cell viability is about 15% (Huh7 cells) and 80% (A549 cells). This showed that the compound **1** was toxic at this concentration for Huh7 cells line and no toxic for A549 cells line (Figure S3).

The new compound **1** and the compounds **2** and **3** were evaluated in vitro for their activity against adult *Schistosoma mansoni*. Only compound **2** displayed quite good worm killing capability after 6 h at the concentrations of 100 μM , Praziquantel was taken as positive control (Table S2).

The compounds **1**, **2**, **3** and **4** were also tested for their ability to inhibit the catalytic activity of recombinant *SmNACE in vitro* using 1,N⁶-etheno NAD⁺ as substrate. This enzyme was chosen due to its expression on the outer surface of the *Schistosoma mansoni* adult worm tegument. In these sense, it could be an interesting pharmacological target for antischistosomal therapy (Goodrich et al., 2005). The IC₅₀ values were determined fluorometrically using 1,N⁶-etheno NAD⁺ as substrate. Only the compound **4** showed a significant inhibition with a IC₅₀ value about 10–20 μM compared to the reference *SmNACE* inhibitor (Table S3).

3. Experimental

3.1. General methods

IR spectrum was obtained using a Thermo Electron (Nicolet 380) FT-IR spectrometer. Column chromatography was carried out using silica gel (Merck 60–120, 70–230 and 230–400 mesh). Rotating power was recorded on a P-2000 Jasco polarimeter. Thin layer chromatography was performed on percolated 0.5mm thick Merck Si gel 60 F254 aluminium sheets. Separated compounds were visualized under UV light and by spraying with H₂SO₄–EtOH (1:9, v/v) followed by mild heating for about 2–3 min. The mass spectra were recorded on an Agilent MS instrument (Agilent Technologies 6520, Accurate mass Q-ToF). NMR spectra were recorded on a Bruker Avance DRX-500 instrument operating at 500 MHz (¹H) and 125 MHz (¹³C), using a deuterated dimethylsulfoxide (DMSO-*d*₆) as solvent. Chemical shifts (δ) were quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS) and the coupling constants (*J*) are given in Hz. Different mixtures of n-hexane, EtOAc, CH₂Cl₂ and MeOH were used as eluting solvents. They were distilled prior to use.

3.2. Plant material

Roots of *Anonidium mannii* were collected in 2016 at Mount Kalla (Latitude 3° 30' North, Longitude 11° 13' East), from Bankomo locality, Mefou et Akono Sub-division, Center Region of Cameroon and identified by M. Victor NANA, a botanist of National Herbarium, Yaounde, Cameroon; where a voucher specimen is deposited under the voucher number 45582HNC.

3.3. Extraction and isolation

The air-dried and powdered roots (3.4 kg) of *A. mannii* was macerated in 25 L of a mixture of EtOH-H₂O (8:2) for 72 h at room temperature. After filtration and solvent evaporation, a residue of 130.41 g was obtained. A part of the crude extract (100 g) was subjected to flash column chromatography (FCC) on silica gel employing a step gradient of hexane-ethyl acetate and ethyl acetate-methanol to afford ten fractions Fr1-Fr10 based on TLC monitoring.

Fraction Fr6 (2.51 g) was purified employing a step gradient of hexane (1:0)-ethyl acetate (7:3) to yield polycarpol **5** (15.0mg). A part of the fraction Fr9 (3.24 g), eluted with a gradient dichloromethane (1:0)–methanol (9.5:0.5) lead to aristolactam AII **2** (8mg). Fraction Fr10 (9.1 g) was purified by flash chromatography (MPLC) over silica gel with a gradient ethyl acetate (1:0)-methanol (8.5:1.5) and subfractions [30–55] (250mg) were purified by semi-preparative HPLC using a gradient (5–95% CH₃CN, in 60min) to give piperolactam D **4** (11.2mg), aristolactam BII **3** (12mg) and manniindole (**1**) (6mg). For ¹H and ¹³C NMR data, see Table S2.

3.4. Bioassay

3.4.1. Cytotoxicity activity

Cytotoxicity was tested against the Huh7 and A549 cells using a MTS assay. Huh7 cells (Human Hepato carcinoma cells) are cultured in DMEM medium 1 g/L glucose (SIGMA), 2µM Glutamine and 10% FCS (fetal calf serum, SIGMA). The A549 cells (Human Lung Tumor cells) are cultured in HamF12 medium, 2 µM Glutamine and 10% FCS. 24 hours before the toxicity experiment, the cells are seeded in a 96-well plate (Nunc Edge 2.0, ThermoScientific) at the rate of 4000 cells/well (Huh7); 6000 cells/well (A 549) in 100 µL and cultured in an incubator at 37°C. with 5% CO₂. The extracts to be tested are diluted in medium with serum and added at a rate of 20 µL/well to obtain the desired final concentration. All tests were done in triplicate. 48 hours after the addition of the extracts, 20 µL of MTS solution (Cell Titer 96 R Aqueous One Cell Proliferation Assay Solution, Promega) are added to each well. After 1 hour of incubation at 37°C., the ODs are read at 490nm and 700nm (Safas, Monaco).

The OD or absorbance of the extracts to be assayed (490 nm) are analyzed after deducting the OD at 700 nm, the OD of the plastic, the OD of the cells alone.

Praziquantel and DMEM medium were used as positive and negative controls, respectively.

3.4.2. Determination of activity against adult *Schistosoma mansoni* (in vitro)

The host-parasite system used was an albino variety of *Biomphalaria glabrata* from Brazil and a strain of *Schistosoma mansoni* from Puerto Rico (NMRI strain). Female hamsters (*Mesocricetus auratus*; Janvier Labs; Le genest-Saint-Isle, France) were percutaneously exposed to 400 cercariae following standard procedures previously published (Dumont et al. 2007). Forty days' post-exposition adult *S. mansoni* were recovered from the hepatic portal system and mesenteric veins by hepatic perfusion technique (Boissier et al. 2003). Living worms were immediately deposited in RPMI 1640 culture medium (supplemented with 5% inactivated foetal calf serum (iFCS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO₂. Ten to twelve worms with equilibrated sex ratio were deposited in each well of 24 well plates. For the determination of activity against adult flukes all compounds were initially tested at a concentration of 100 µg/mL, using DMSO stock solutions (conc 10 µg/mL; final concentration of DMSO: 0.2) diluted in supplemented RPMI 1640 medium with a final volume of 2mL per well. Wells with RPMI and DMSO in medium served as negative controls. PZQ served as positive control, and concentrations of 10, 50, and 100 µg/mL were used to evaluate the schistosome's mortality. Each test was performed in duplicate or triplicate. Parasites were subsequently observed for body contractility and movement each hour for 6 hours. Parasites showing no body contractions during a 30-s observation were considered dead [no worm started to move again after 30 s without motor activity (Boissier et al. 2009)].

3.4.3. Enzymatic activity (in vitro)

*Sm*NACE activity was determined by a fluorometric assay using 1, N⁶-etheno NAD⁺ (ε-NAD⁺, Sigma) as substrate. This assay consists in measuring the appearance of the reaction product ε-ADP-ribose by the increase of fluorescence at $\lambda_{em} = 410\text{nm}$ ($\lambda_{exc} = 310\text{nm}$) at 37°C in 10mM potassium phosphate buffer, pH 7.4, containing 0.05% (w/v) emulphogen (1 mL final volume) in a quartz tank.

In the spectrofluorimeter (*Shimadzu RP-5301 PC*), the buffer was added to the tank at 37°C and with stirring. The substrate was then added; its final concentration being 20 µM. After that, inhibitors were introduced. Before introducing the enzyme, the tracing was started in order to see the basic fluorescence F_0 . Once the enzyme has been added, the kinetics were exploited at the end of the measurement time (2 minutes) using a non-linear regression program

(GraphPad, Prism) for the determination of catalytic activity. All the inhibitors were initially tested at a concentration of 100mM diluted in DMSO (less than 2% added). In case that we observed fluorescence or quenching, the concentrations were decreased. So, concentrations of 10 μ M, 100 μ M, 1 mM, 10mM and 100mM were used. Each test was performed twice.

The approximate IC₅₀ values were determined based on concentrations giving approximately 50% inhibition.

4. Conclusion

In conclusion, phytochemical and biological investigation which was carried out on the roots of *Anonidium mannii* led to the isolation of five major compounds among which one, **1**, was newly described. Though there are well known in the Annonaceae family, this is the first time that lactams were found in *A. mannii*. Those isolated compounds were submitted for the first time to the combined biologic tests on the schistosomes (parasitological and enzymatic tests). Two compounds, respectively **2** and **4**, were potentially having good activity on adult *Schistosoma mansoni* in vitro and good inhibition of a new member of the ADP-ribosyl cyclase family of enzymes (*SmNACE*) in the Platyhelminthes trematodes, *Schistosoma mansoni* and *Schistosoma japonicum*.

Acknowledgments

The authors are thankful to AUF for providing a fellowship to J L T M.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethic statement

The laboratory where the experiments were done has permission A66040 from «Ministère de l'Enseignement supérieur de la Recherche et de l'Innovation (France)» for animal experimentation. Experimenters possess the certificate for animal experimentation (authorization 007083, decree 87-848 and 2012201-0008). Housing, breeding and animal care followed the national and European ethical requirements.

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Abstract figure

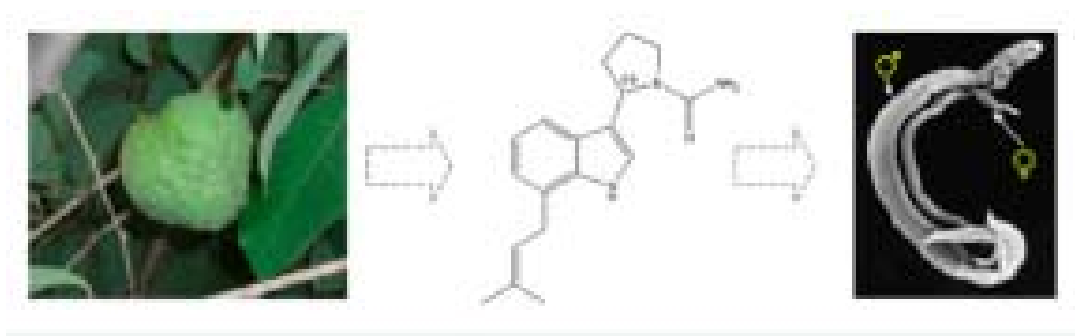
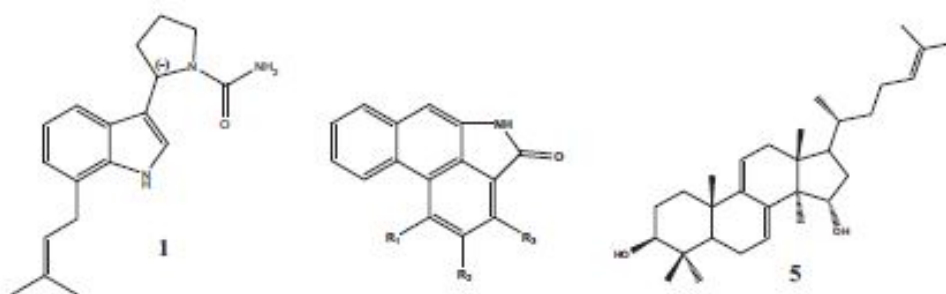


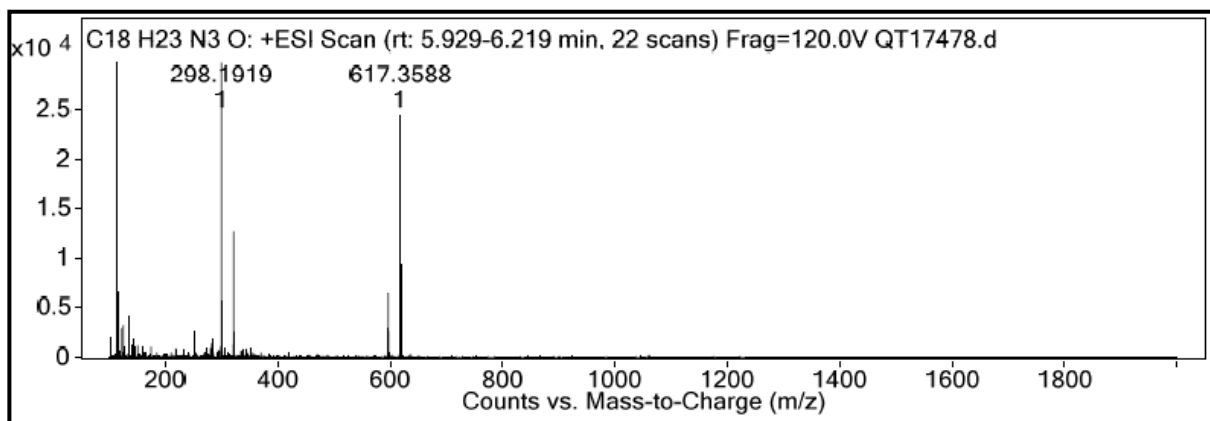
Figure 1. Chemical structures of compounds 1–5.



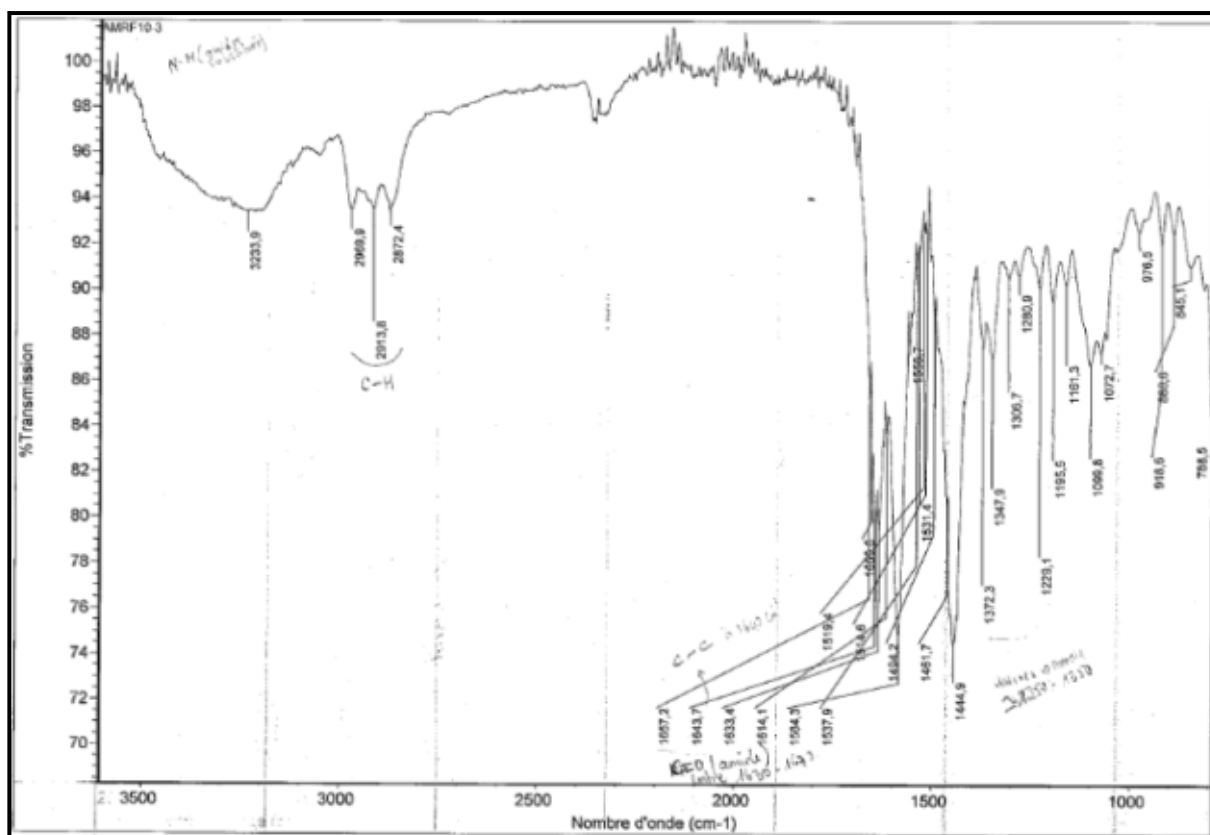
2 $R_1 = \text{OCH}_3$; $R_2 = \text{OH}$; $R_3 = \text{H}$

3 $R_1 = \text{OCH}_3$; $R_2 = \text{OCH}_3$; $R_3 = \text{H}$

4 $R_1 = \text{OCH}_3$; $R_2 = \text{OCH}_3$; $R_3 = \text{OH}$



1. HRESI-MS of Compound 1



2. IR Spectrum of Compound 1

3. ALPHA-D of Compound 1

[Data Information]

Creation Date 28/06/2019 15:57

[Measurement Information]

[Comment]

Sample name

Comment

User

Instrument Name Oberon
 Model Name P-2000
 Serial No. a072861232
 Polarizer Dichrom
 Faraday Cell Flint Glass

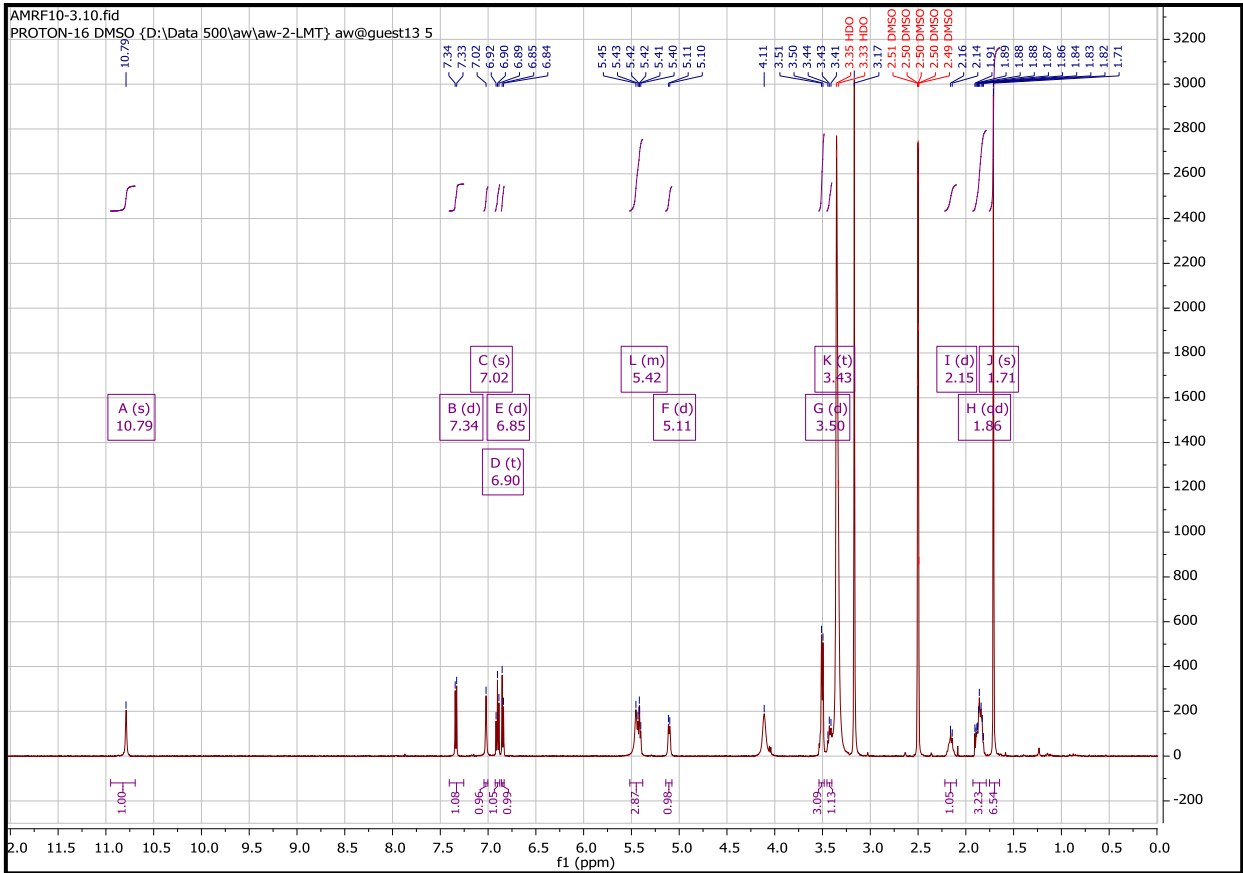
Division
 Company Faculté de pharmacie

Accessory PTC-203
 Accessory S/N A031561234
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 Sensor Holder
 Monitor Sensor
 Holder
 Start Mode Keep target temperature +/-0.10 C while 5 seconds
 Light Source Na
 Monitor wavelength 589
 nm
 D.I.T.
 31 sec No. of
 cycle 5
 Cycle interval 10
 sec Temp. Monitor
 Holder Temp. Corr.
 Factor None
 Aperture(S)
 8.0mm Aperture(L)
 Auto
 Mode
 Specific O.R. Path
 Length 10 mm
 Concentration 0.4
 w/v%
 Water content of sample
 0 % Factor 1

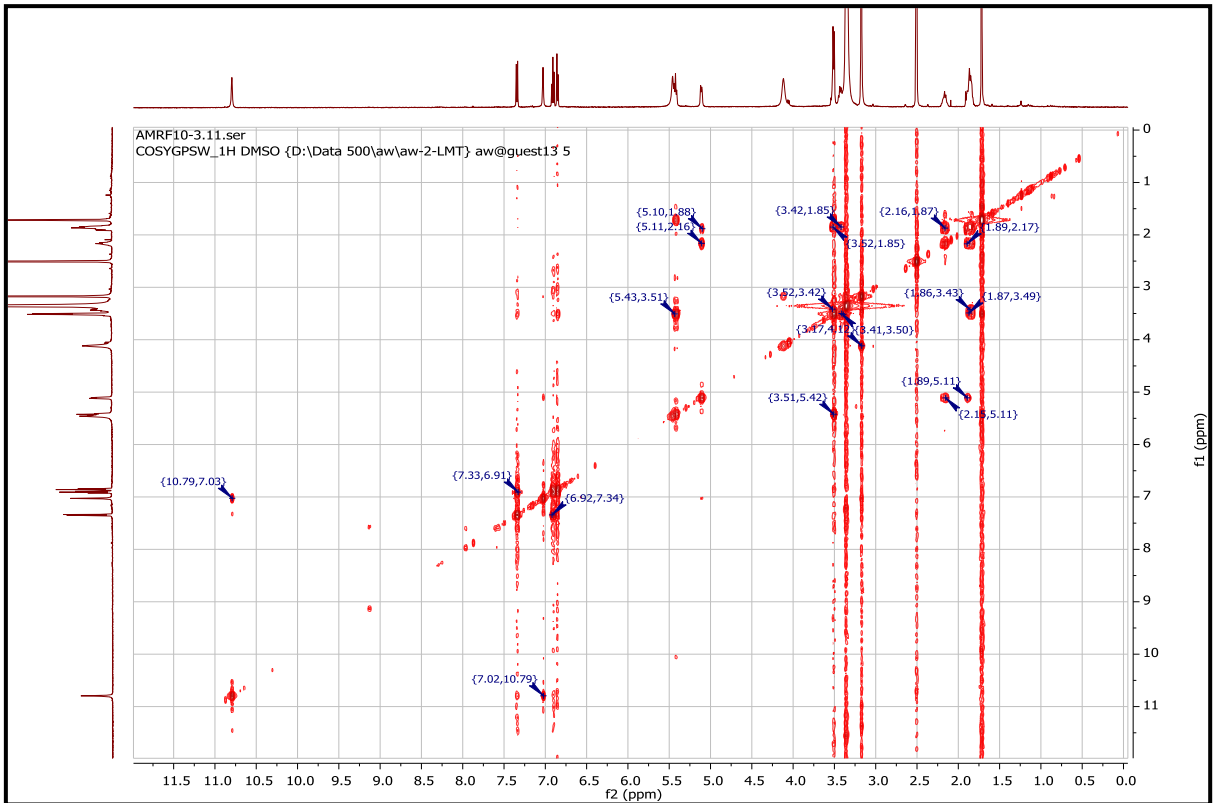
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3	3-2	28/06/2019 15:55	244	25.00	-0.0146
4	3-3	28/06/2019 15:56	243	25.02	-0.0152
5	3-4	28/06/2019 15:56	243	24.97	-0.0153
6	3-5	28/06/2019 15:57	244	25.02	-0.0154

No.	Specific O.R.	Path Length[mm]	Concentration[w/v%]	Water content[%]	Factor	S.D.	C.V.
1	-37.5000	10	0.4000	0	1	1.0458	2.7889
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3	-36.5000						
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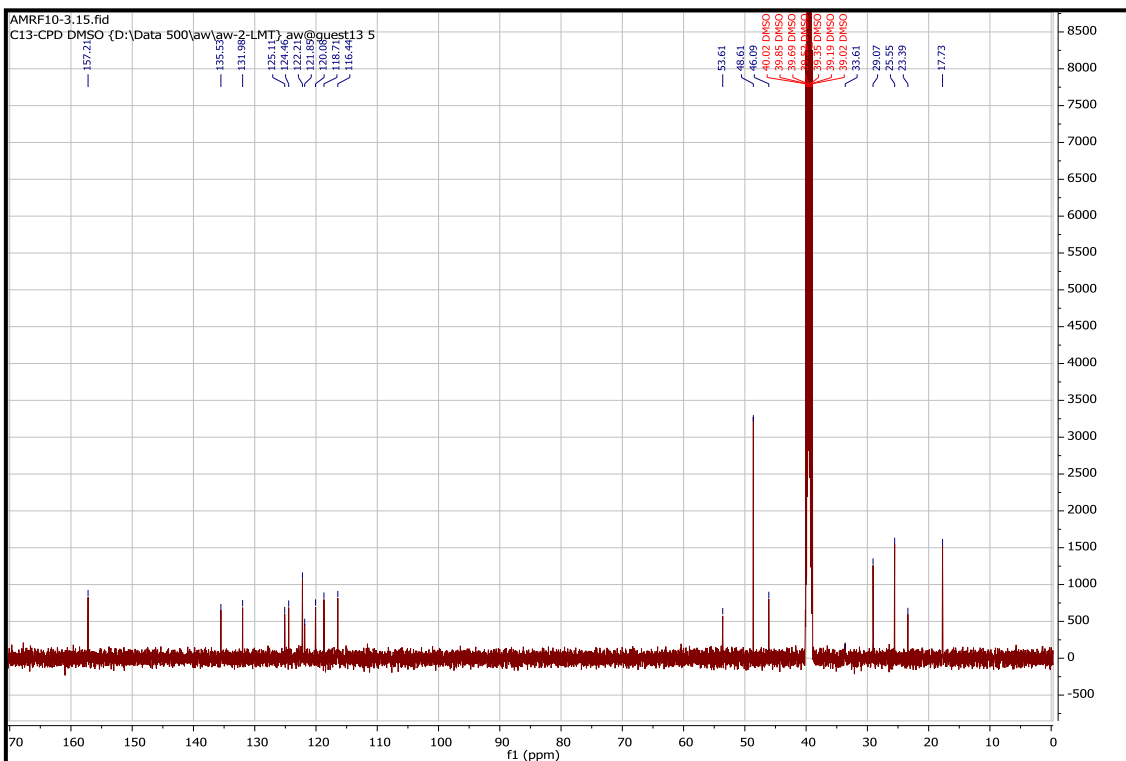
No.	Comment
1	
2	
3	
4	
5	



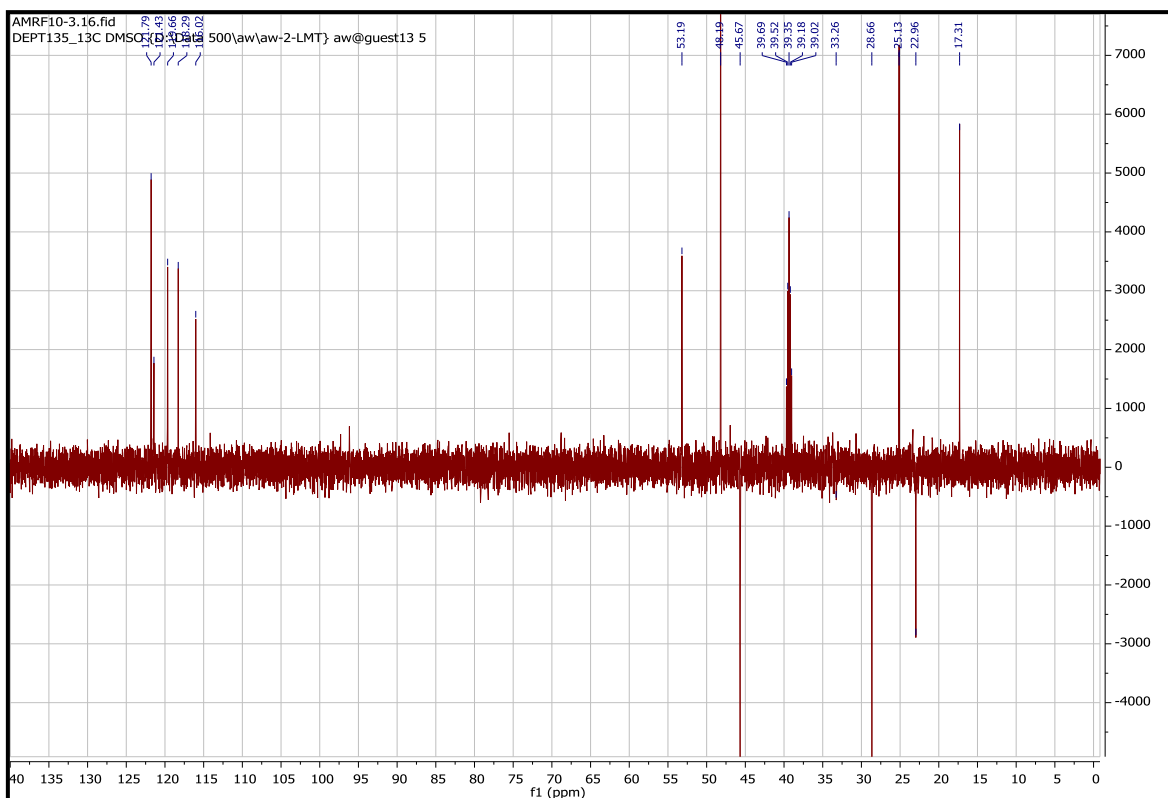
4. ¹H NMR (500 MHz, DMSO-d₆) spectrum of Compound 1



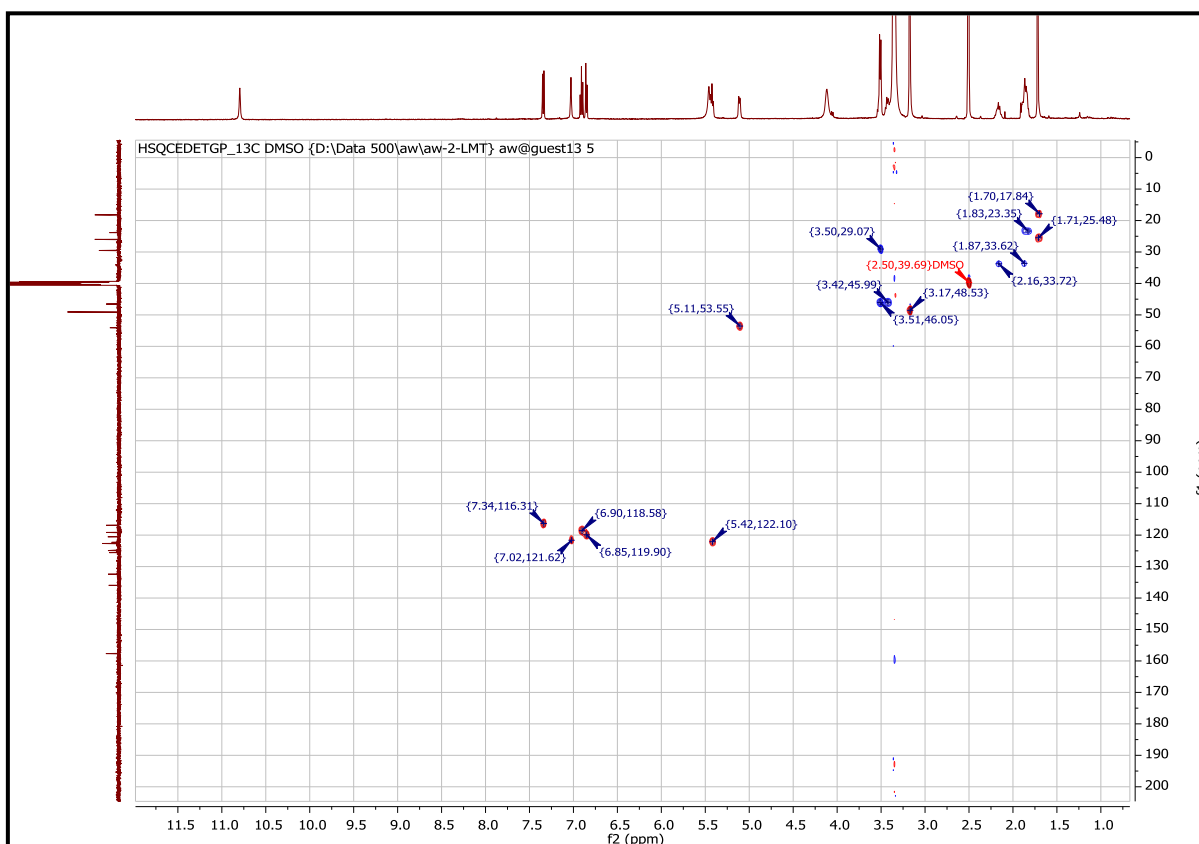
5. ^1H - ^1H COSY (500 MHz, DMSO- d_6) Spectrum of Compound 1



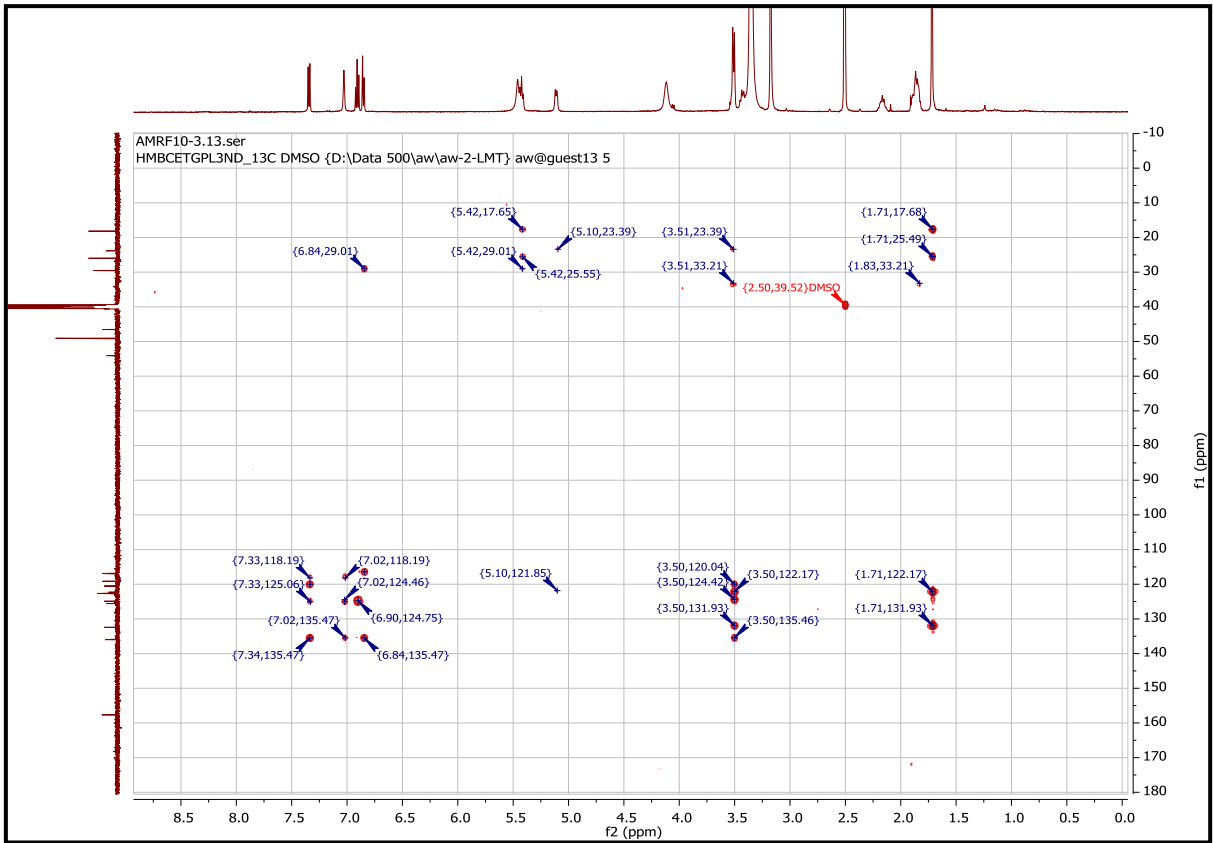
6. ^{13}C NMR (125 MHz, DMSO- d_6) spectrum of Compound 1



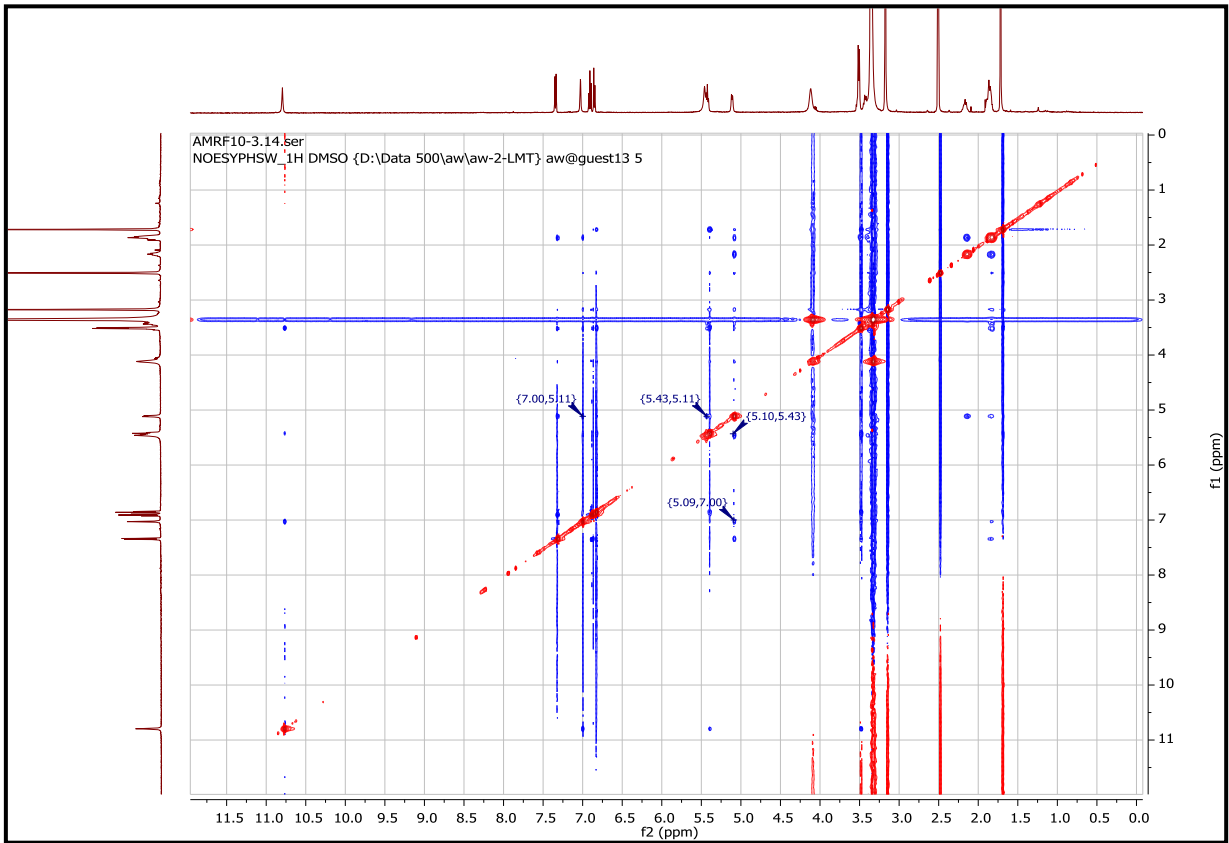
7. DEPT 135 Spectrum of Compound 1



8. HSQC Spectrum of Compound 1



9. HMBC Spectrum of Compound 1



NOESY Spectrum of Compound 1

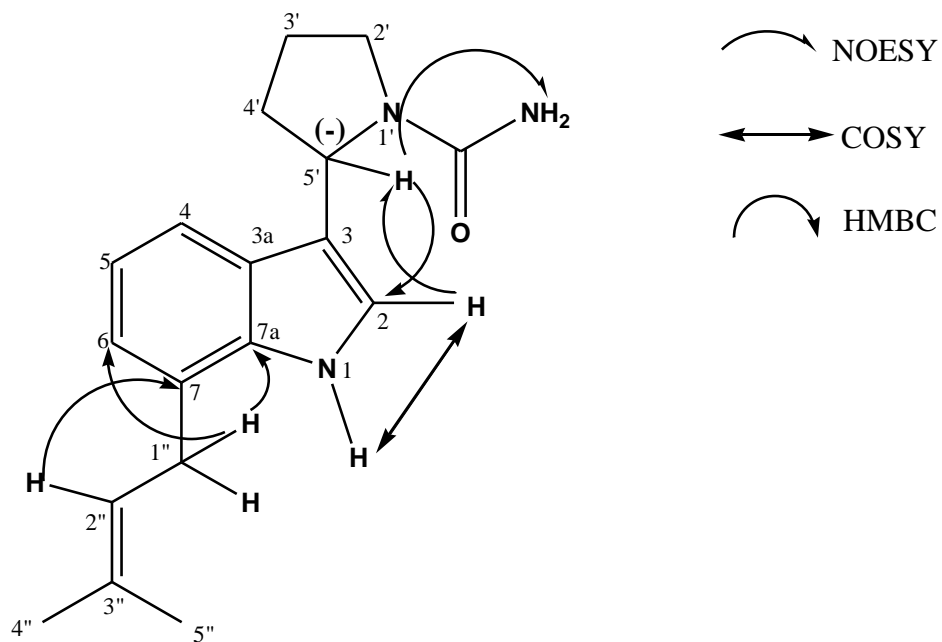


Figure S2. Key HMBC, COSY and NOESY Correlations between indole moiety and the two substituents of **1**.

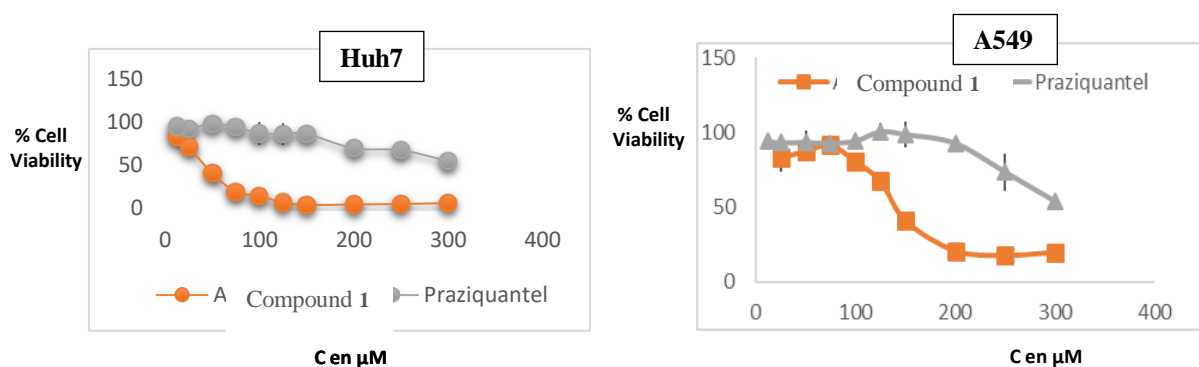


Figure S3. The cell viability of Compound **1** and Praziquantel assayed by MTT.

Table S1. ^{13}C (125 MHz) and ^1H NMR (DMSO- d_6 , 500 MHz) [J (Hz), δ (ppm)] data for compound (**1**).

Attributions	1	
	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (nH, mult., J (Hz))
1	-	10.79 (1H, s)
2	121.8	7.02 (1H, s)
3	117.8	-
3a	125.1	-
4	116.4	7.34 (1H, d, $J = 7.5$)
5	118.7	6.90 (1H, t, $J = 7.5$)
6	119.7	6.85 (1H, d, $J = 7.5$)
7	124.5	-
7a	135.5	-
N-CO-NH ₂	157.2	5.45 (2H, s)
2'	46.1	3.43 (1H, m) 3.51 (1H, m)
3'	23.4	1.86 (2H, m)
4'	33.7	1.89 (1H, m) 2.17 (1H, dd, $J = 13.4 ; 6$)
5'	53.6	5.11 (1H, bd, $J = 7.2$)
1''	29.1	3.50 (2H, d, $J = 7.4$)
2''	122.2	5.43 (1H, dd, $J = 16.1 ; 8.7$)
3''	131.9	-
4''	25.6	1.71 (3H, s)
5''	17.7	1.71 (3H, s)

Assignments were based on HSQC, HMBC, COSY and NOESY experiments.

Table S2. Effects of the extract and the isolated compounds on adult *Schistosoma mansoni* in *vitro*.

Extract/Compound (No.)	Concentration (μM)	Mobile worm after 6 hours of incubation (%)
Roots extract	100	44.4
	50	100
	10	Not Done
1 Manniindole	100*	70
	50*	95
	10*	100
2 Aristolactam AII	100	9.1
	50	66.7
	10	71
3 Aristolactam BII	100	62.5
	50	77.3
	10	94.4
Praziquantel	100	0
	50	7
	10	Not Done
Control (RPMI)	-	100

*Experiments were performed twice. The others were performed three times. For each experiment, 10-12 worms were used with equilibrated sex-ratio.

Table S3. Approximate IC₅₀ values for the inhibition of *SmNACE* by compounds.

The roots extract was active on *SmNACE* with inhibition rate of 76.13%. at C=100 µg/mL being a mixture of compounds, its IC₅₀ could not be determined.

Compound (No.)	IC₅₀ (µM)
1	> 100
2	> 100
3	> 100
4	10-20
Cyanidin*	2.3
Delphinidin*	6.0

*Cyanidin and Delphinidin are the natural products reference *SmNACE* inhibitors (Kuhn et al. 2010).