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Antifungal Activity of Verticillin D Isolated from *Clonostachys rosea* EC28 against *Alternaria burnsii* and *Sclerotium rolfsii*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Alternaria leaf spot and white crown rot caused respectively by *Alternaria* sp and *Sclerotium rolfsii* are responsible for significant economic losses in tomato cultivation in Côte d'Ivoire. Chemical control of these diseases has its limits due to the harmful effects of synthetic fungicides on the environment and the health of farmers and populations. As a result, biological control through the use of biocontrol agents represents one of the alternative solutions. *Clonostaschys rosea* EC 28, a biocontrol agent is an endophyte fungus isolated from *Ceiba pentandra,* whose objective of this study was to show the antifungal properties of verticillin D produced by this fungus against *A. burnsii* and *S. rolfsii. C. rosea* EC 28 was cultured on rice medium and extracted with ethyl acetate. Open silica gel column chromatography of this crude extract led to the purification of two secondary metabolites that were identified by mass spectrometry and NMR as verticillin D and ergosterol. The activity *in vitro* antigerminative of *A. burnsii* spores and against growth mycelial growth of *S. rolfsii* of these molecules have been determined. Verticillin D inhibited *A. burnsii* spore germination and growth mycelial growth of *S. rolfsii with* respective MICs of 0.25 mg / mL and 5mg/ mL. Ergosterol n

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/ A present none activity antifungal. These results provide a database for *the* use of *Clonostachys rosea* EC 28 as a potential biocontrol agent for two major tomato diseases in Ivory Coast.

Keywords: Alternaria burnsii; antifungal; biocontrol agent; Clonostachys rosea EC 28; Côte d'Ivoire; Sclerotium rolfsii; verticillin D.

1. INTRODUCTION

In Côte d'Ivoire, the tomato is the most widely grown vegetable in rural, urban, and periurban areas. Its cultivation is an income-generating activity for populations, especially women [1]. However, this culture is subject to many abiotic and biotic constraints that limit yields causing financial losses. Among the biotic constraints, the attacks of certain pathogens responsible for various diseases make it difficult to grow tomatoes in Côte d'Ivoire [2]. Among these diseases, Alternaria is the most harmful causing a significant drop in tomato production and a deterioration in its quality [2]. The necrotrophic species of the genus *Alternaria* responsible for this disease can lead to yield losses of around 90% [1,3,4]. In addition to Alternaria, white crown rot caused by *Sclerotium rolfsii* causes enormous damage to tomato crops in Côte d'Ivoire. In production areas, this disease is responsible for crop losses estimated at 41 %. In addition, the fight against *S. rolfsii* remains difficult because of the way it is preserved in the soil, in the form of sclerotia [5]. To control plant diseases in general and tomatoes in particular, farmers commonly use synthetic fungicides [6]. However, their high cost and the harmful effects of their use on the environment and the health of the population contrast with their effectiveness. Therefore, alternatives to synthetic fungicides are strongly desired in tomato cultivation. The use of biocontrol agents could constitute one of these alternatives. These biocontrol agents, which are microorganisms, have the ability to inhibit the pathogen by different mechanisms of action, either by parasitism, or by induction of host plant defenses, or by secretion of antibiotic compounds [7]. In the latter case, biocontrol agents like *Trichoderma* sp species are known to produce various and varied antifungal molecules involved in their mechanism of antagonism against plant pathogens [8]. Like *Trichoderma* sp, *Clonostachys rosea* (Sch.) Schroers & Samuels is a biocontrol agent for several plant pathogens like *Verticillium dahliae* [9], *Sclerotinia sclerotiorum* [10] *Fusarium culmorum* [11] and *Botrytis cinerea* [12]. Studies have shown that the production of secondary metabolites belonging to the family of peptaibiotics is part of one of the biological control mechanisms of *C. rosea* against *S. sclerotiorum* [13]. The objective of this study was to show the antifungal properties of verticillin D produced by *C. rosea* EC28, an endophyte fungus of *Ceiba pentandra*, against *A. burnsii* and *S. rolfsii.*

2. MATERIALS AND METHODS

2.1 Fungal Strains

The strains of *Alternaria burnsii* and *Sclerotium rolfsii* were provided respectively by the Biochemical Pharmacodynamics Pedagogical and Research Unit and the Plant Physiology Pedagogical and Research Unit of the Félix Houphouët Boigny University in Côte d'Ivoire. These strains were isolated from diseased tomato plants. They were cultured on PDA medium. *Clonostachys rosea* EC28 was isolated from the stem bark of *Ceiba pentandra*, a medicinal plant [14].

2.2 Secondary Metabolites of *C. rosea* EC28

A volume of 2 mL of a suspension of EC 28 spores was distributed over rice medium (40 g of rice and 40 mL of distilled water supplemented with 5% glucose, 5 g/l of peptone, and extract of yeast) and incubated at 28°C for 21 days. At the end of the 21 days of incubation, the cultures of EC 28 were extracted with ethyl acetate. The organic phases were combined, dried ($MgSO_4$), and evaporated under vacuum at 35°C. The crude extract obtained (2.5 g) was fractionated by chromatography on an open silica gel column to give 4 fractions numbered from F1 to F4. According to the analysis of the thin layer chromatography profiles, the F2 and F3 fractions were further purified. Fraction F2 (627 mg) was chromatographed on silica gel with an elution system (C_6H_{12} /EtOAc, 70:30) to give compound 1 (325 mg). Fraction F3 (254 mg) was also subjected to column chromatography on silica gel with an elution system (C_6H_{12} /EtOAc, 60:40 to 40:60), to give a compound 2 (117mg).1D and

2D NMR spectra were recorded in DMSO-d6 with Bruker 400 spectrometers using TMS as an internal standard. The chemical shifts were given in δ (ppm). HR-ESI-MS and fragmentation MS spectra were obtained with an Applied Biosystem QSTAR Pulsar I mass spectrometer.

2.3 Antifungal Properties of Secondary Metabolites *C. rosea* EC28

2.3.1 Antigermination activity of secondary metabolites against *A. burnsii*

The antifungal activity of the secondary metabolites of *C. rosea* EC28 was carried out against the spores of *A. burnsii* according to the method described by Fawole et al. [15] with some modifications. The strain of *A. burnsii* was cultured on PDA medium for 7 days. The spore suspension was prepared from this culture in sterilized distilled water. The spore suspension was adjusted to 10^4 spores/mL with a Malassez cell. A volume of 50 µL of this suspension was distributed in the then of a 96-well plate containing 100 µL of Potato Dextrose Broth (PDB). The secondary metabolite solubilized in DMSO (50 µl) was added to the wells to give concentrations of 1 mg/ml; 0.5mg/ml; 0.25mg/ml; 0.1mg/ml; 0.06mg/ml and 0.03mg/ml. The control was incubated with DMSO alone. The antifungal Clotrimazole at 5 mg/ml served as a positive control. All experiments were done in triplicate. Plates were incubated on orbital shaker (200 rpm) at 28°C. The germination rate was recorded at 24, 48, and 72 h under a phase contrast microscope (*Leica)*. A minimum inhibitory concentration (MIC) was defined as a concentration for which no germination was observed. The well showing no germination when observed under a microscope was cultured on PDA medium for verification.

2.3.2 Antifungal activity of secondary metabolites against mycelial growth of *S. rolfsii*

Sclerotium rolfsii was cultured on PDA agar and incubated at 28°C for 10 days. A concentration range of each compound was achieved by the half dilution method, ranging from 20 to 5 mg/mL homogenized in sterile distilled water. Then, 200 µl of each concentration were spread in Petri dishes 90 mm in diameter containing the PDA medium. A pellet of mycelium about 6 mm in diameter from *S. rolfsii* 10 days old was placed in the center of the Petri dish. The negative control was prepared with water. Clotrimazole (reference) prepared at 10 mg/ml was used as a positive control. The tests were carried out in triplicate. The Petri dishes were incubated in an oven at 28°C. until the negative control box was filled. The measurements of mycelial growth inhibition diameters were carried out on the 10th day. For each concentration, the percentage of growth inhibition I (%) was expressed by the reduction in the diameter of the fungal colony compared to the control according to the following equation [16]:

$$I\ (\%) = (1- C_n / C_o) \times 100$$

- $\mathbf{C_n}$ is the average diameter of mycelium growth in the presence of verticillin D.
- $\mathbf{C_o}$ is the average diameter of mycelium growth in the absence of verticillin D.

2.4 Statistical Analysis

The statistical analysis was carried out by one-way ANOVA analysis of variance followed by Tukey's test for the comparison between the concentrations of Verticillin D and Clotrimazole on the one hand and between the tests. All the results obtained were expressed as mean ± standard deviation. Probability values less than ($P<0.05$) were considered statistically significant. All results were analyzed using *GraphPad Prism 5 statistical analysis software.*

3. RESULTS

3.1 Identification of Secondary Metabolites

Fraction F2 gave compound **1** as white crystals of mass 325 mg. analyzes of the proton ^{1}H NMR 1D spectrum and the carbon ^{13}C spectrum (DEPT-Q) as well as the 2D NMR showed that compound **1** at ESI $[M-H]^-$ *m/z* 395.3297 (calc for $C_{28}\ H_{44}O - H^+$: 395.330) corresponds to Ergosterol (Fig. 1). Fraction F3 led to compound **2** as a white powder of mass 117 mg. Analysis of the mass spectrum of compound **2** made it possible to demonstrate a high-resolution molecular ion peak ESI $[M+H]^+$ *m/z* 757.123 (calc. for $C_{32}\ H_{32}\ N_6\ O_8\ S_4 + H^+$: 757.124). This analysis, combined with the 1D (proton ^{1}H and DEPT-Q) and 2D NMR data, made it possible to determine the chemical structure of the compound in accordance with the data in the literature. It was verticillin D (Fig. 2) [17].

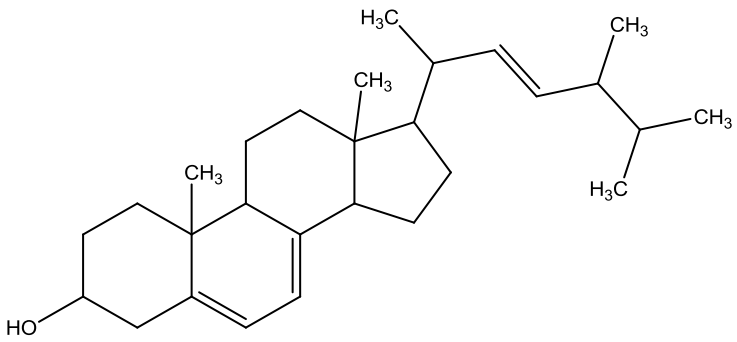

Fig. 1. Structure of Ergosterol

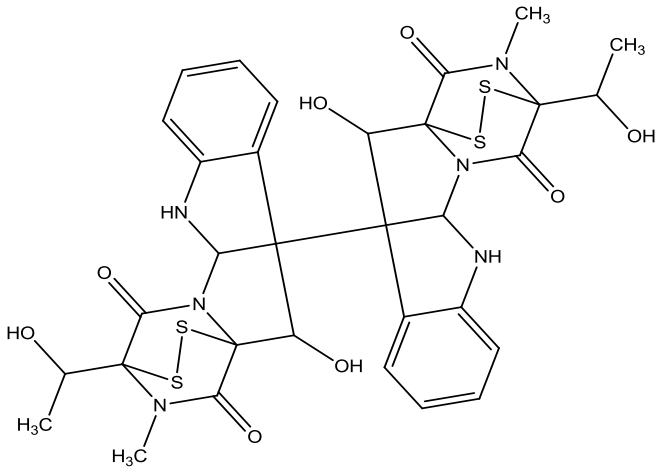

Fig. 2. Structure of Verticillin D

3.2 Activity of Secondary Metabolites against *A. burnsii*

Ergosterol and verticillin D have been tested against spore germination of *A. burnsii.* Experiments were conducted in a 96-well plate by incubating *A. burnsii spores* in PDB medium with varying concentrations of purified secondary metabolites. After 24 hours, ergosterol had no anti-germination activity regardless of the concentration tested, unlike verticillin D, which inhibited spore germination up to a concentration (MIC) of 0.25 mg/ml (Table 1). Clotrimazole (reference antifungal) completely inhibited the germination of *A. burnsii* spores at a concentration of 1.25 mg/ml. Growth was observed in the negative control wells.

3.3 Antifungal activity of secondary metabolites against mycelial growth of *S. rolfsii*

Inhibition of mycelial growth of *S. rolfsii* is a function of verticillin D concentrations (Table 2). Indeed, inhibition rates of 99.66 ±0.57%; 90±1%, and 59.33±0.57% were observed respectively for the concentrations of 20; 10 and 5 mg/ml on the 10th^{day} of incubation compared to the negative control (Fig. 3). The statistical analyzes of the results represented by the histograms show a significant difference ($P<0.01$) on the one hand between the concentrations of verticillin D and on the other hand between the concentrations of Verticillin D and the reference (Clotrimazole) (Fig. 4). Ergosterol showed no activity against the two phytopathogens tested.

Table 1. Inhibition of *A. burnsii* spore germination by verticillin D

Verticillin D Concentrations mg/ml)	Inhibitory effect
1	+
0.5	+
0.25	+
0.1	-
0.06	-
0.03	-

+ spore inhibition; - no spore inhibition

Table 2. Rate of inhibition of mycelial growth of *S. rolfsii*

Verticillin D Concentrations (mg/ml)	Inhibition rate (%)
20	99.66 ±0.57% **a**
10	90±1% **b**
5	59.33±0.57% **c**
Clotrimazole	56±1 %

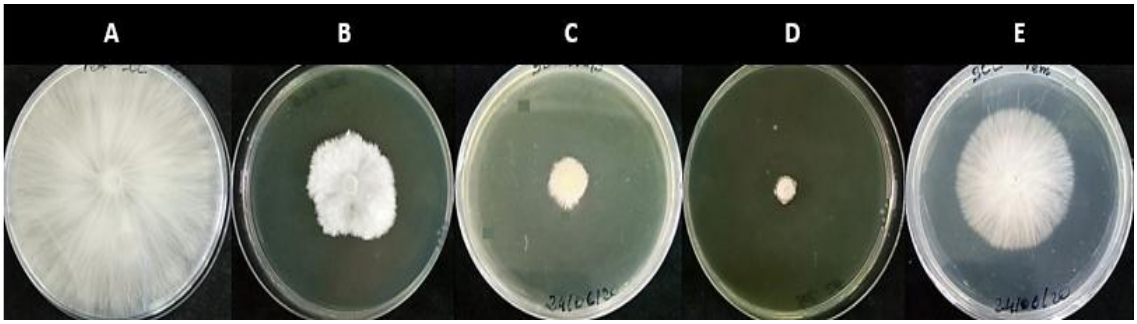

Fig. 3. Inhibition of mycelial growth of *S. rolfsii* after 10 days of incubation by different concentration range of verticillin D

(A): water control (B): 5 mg/mL (C): 10 mg/mL (D): 20 mg/mL (E): positive control Clotrimazole at 10 mg/mL

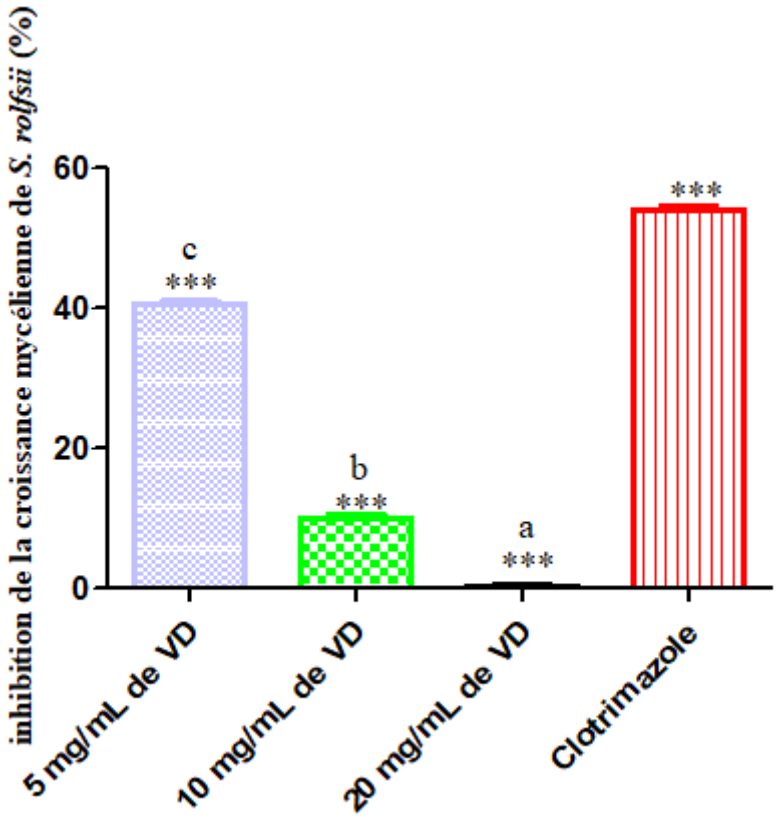

Fig. 4. Histograms of *S. rolfsii mycelial growth inhibition* as a function of verticillin D concentrations

****: very highly significant difference between the different concentrations of verticillin D on the one hand and between the concentrations of verticillin D and Clotrimazole on the other hand (P< 0.001)*

4. DISCUSSION

Clonostachys rosea (same species *Gliocladium roseum*) has a wide geographical distribution. He lives in forests, bushes, meadows, wastelands, and deserts this fungus is widely distributed in the arctic, temperate, and subtropical regions and tropical regions of the world [18,19]. He has a life of common saprophytism in the soil of the world [20]. However, it can also be isolated from plants as an endophytic fungus. In this study, this fungus, coded EC28, was isolated from *Ceiba pentandra,* a medicinal plant from Côte d'Ivoire. *C. rosea* is a biological control agent highly resistant to many phytopathogenic fungi, such as the spore formation of *Botrytis cinerea* [21], *Fusarium wilt* of wheatears [22]. Just like biological control agents. *C. rosea* has the ability to inhibit the pathogen by different mechanisms of action including parasitism, competition, induction of host plant defenses, or secretion of antibiotic compounds [7]. Antibiosis is the mechanism of action widely used by fungal biocontrol agents. The species of the genus Trichoderma and Clonostachys are the most studied thanks to their significant production of bioactive secondary metabolites of various natures. In particular polyketides, alkaloids, terpenoids, peptaiboles and epidithiodioxopiperrazines, the most important of which are viridines and gliovirins, glisoprenins and gliotoxins. They are toxic at concentrations that depend on the compound and the target [23,24]. The metabolites secreted by these biocontrol agents act by antibiosis operating at several levels such as the alteration or inhibition of the germination of the spores, the growth and/or the sporulation of the pathogen, the distortion of the hyphae of the pathogen, the modification of the appearance of colonies and production of specific forms such as pseudo-parenchyma or by inhibiting their development [25,26]. This study, which is part of the latter case, aimed to determine the inhibitory activity of the compounds produced by *C. rosea* EC 28 against *Alternaria burnsii* and *Sclerotium rolfsii* two major pathogens of tomato cultivation.

Purification by silica gel chromatography of the crude *C. rosea extract* led to the identification of two secondary metabolites identified as ergosterol and verticillin D. The antifungal activity achieved with ergosterol did not show no effect on the pathogens tested. Because this compound is devoid of antifungal activity. Being the main sterol of fungal membranes, it is essential for the growth of fungi. However, this compound becomes active when combined with other compounds [27]. The biological tests carried out with verticillin D have made it possible to demonstrate the antigerminative activity of verticillin D against *A. burnsii spores* and the inhibitory power of this molecule against the mycelial growth of *S. rolfsii*. Verticillin D was first described by Joshi et al., [17] and produced from *the* fungus *Gliocladium catenulatum*. This compound belongs to epidithiodioxopiperrazines. It is a dimeric molecule that contains in its chemical structure two disulphide bridges. Members of this class of molecules exhibit various biological activities including cytotoxic, antifungal, and antibiotic.This activity could be explained by the presence of two di-disulfide ridges contained in the chemical structure of this molecule. De Clercq et al., and Rightsel et al., [28,29] reported that the antiviral activity of gliotoxin, an epepidithiodioxopiperazine as due to the presence of the disulfide bridge contained in its chemical structure. Moreover, the removal of sulfur atoms or the addition of the reducing agent dithiothreitol completely suppresses this activity [30,31]. Moreover, the gliovirin produced by *Trichoderma* sp was able to inhibit *Phytophthora species* [8]. The antigerminative and inhibitory capacity of the mycelium of the 2 pathogenic agents constitute a real advantage for *C. rosea* EC 28 as a potential biological control agent.

5. CONCLUSION

The objective of this work was to determine the antifungal effect of verticillin D, a compound isolated from *Clonostachys rosea* EC 28 from *Ceiba pentandra*. The chemical study of *C. rosea* subjected to fractionation on a chromatographic column in silica gel led to the identification of ergosterol and verticillin D. The antifungal tests carried out *in vitro* with these two compounds showed that only the verticillin D possesses an antifungal effect against the phytopathogens tested namely *A. burnsii* and *S. rolfsii.* Verticillin D could be used as an antifungal in the field of agriculture.

SUPPLEMENTARY MATERIALS

Supplementary materials available in this link: https://journaljamb.com/index.php/JAMB/libraryFiles/downloadPublic/10.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Display Report

Analysis Info

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Comment			

Acquisition Parameter

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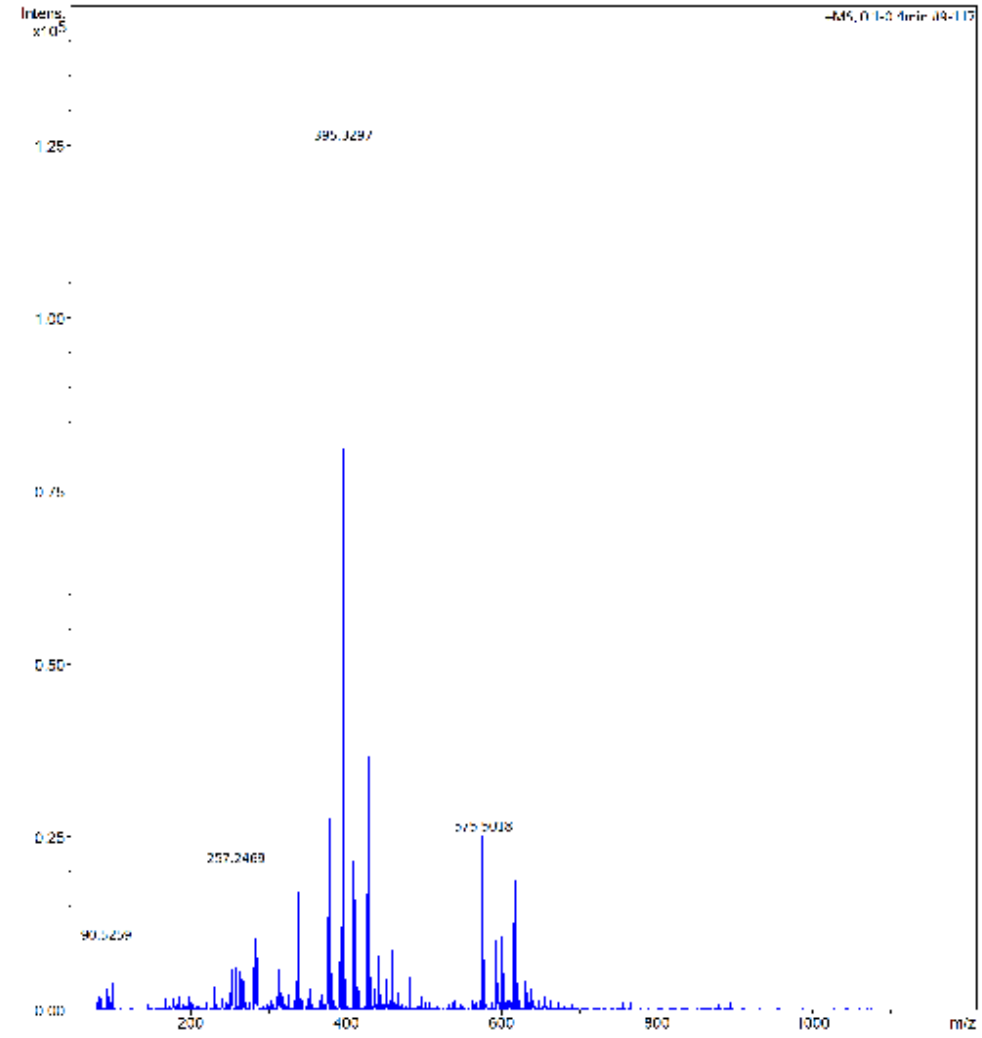

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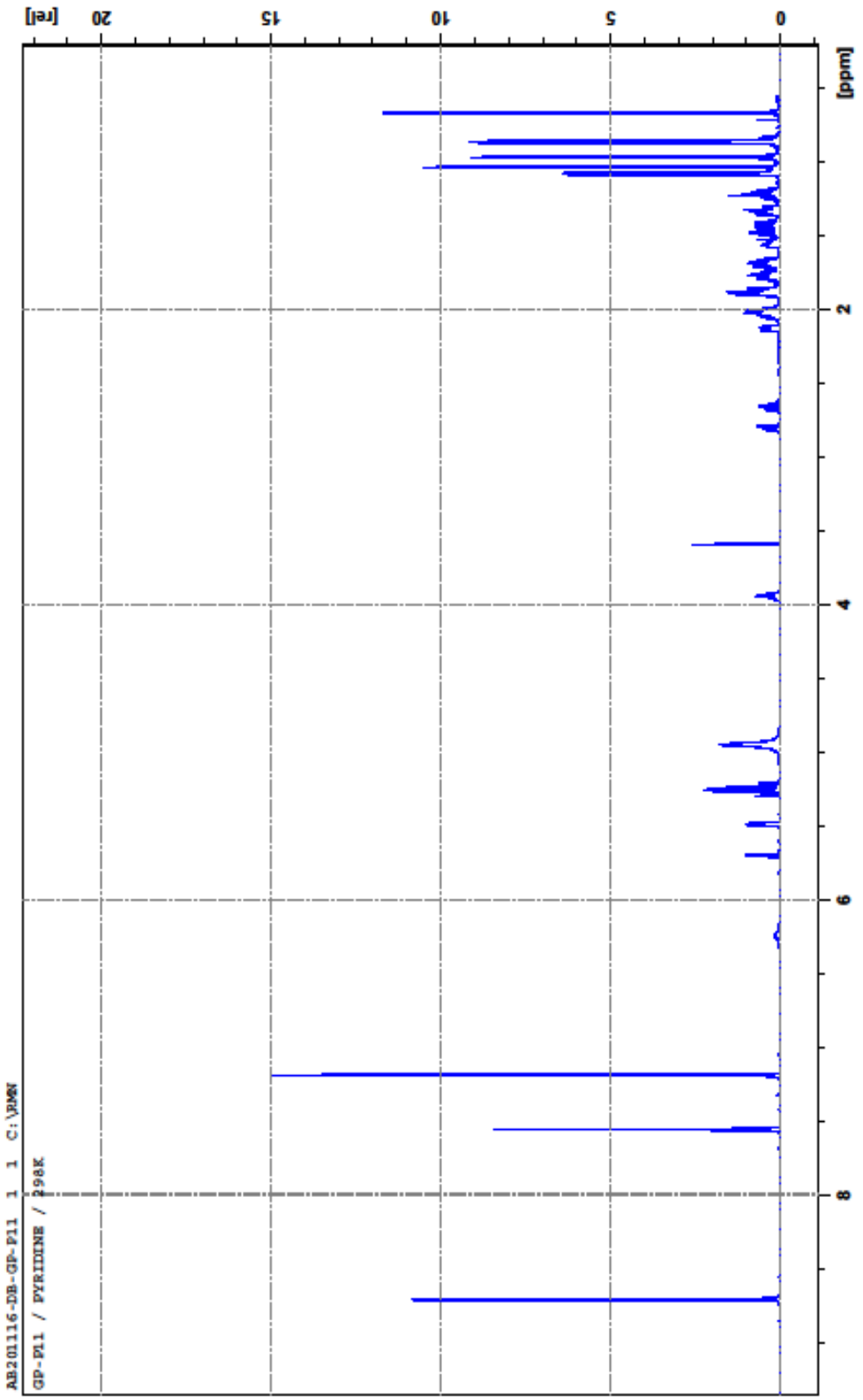
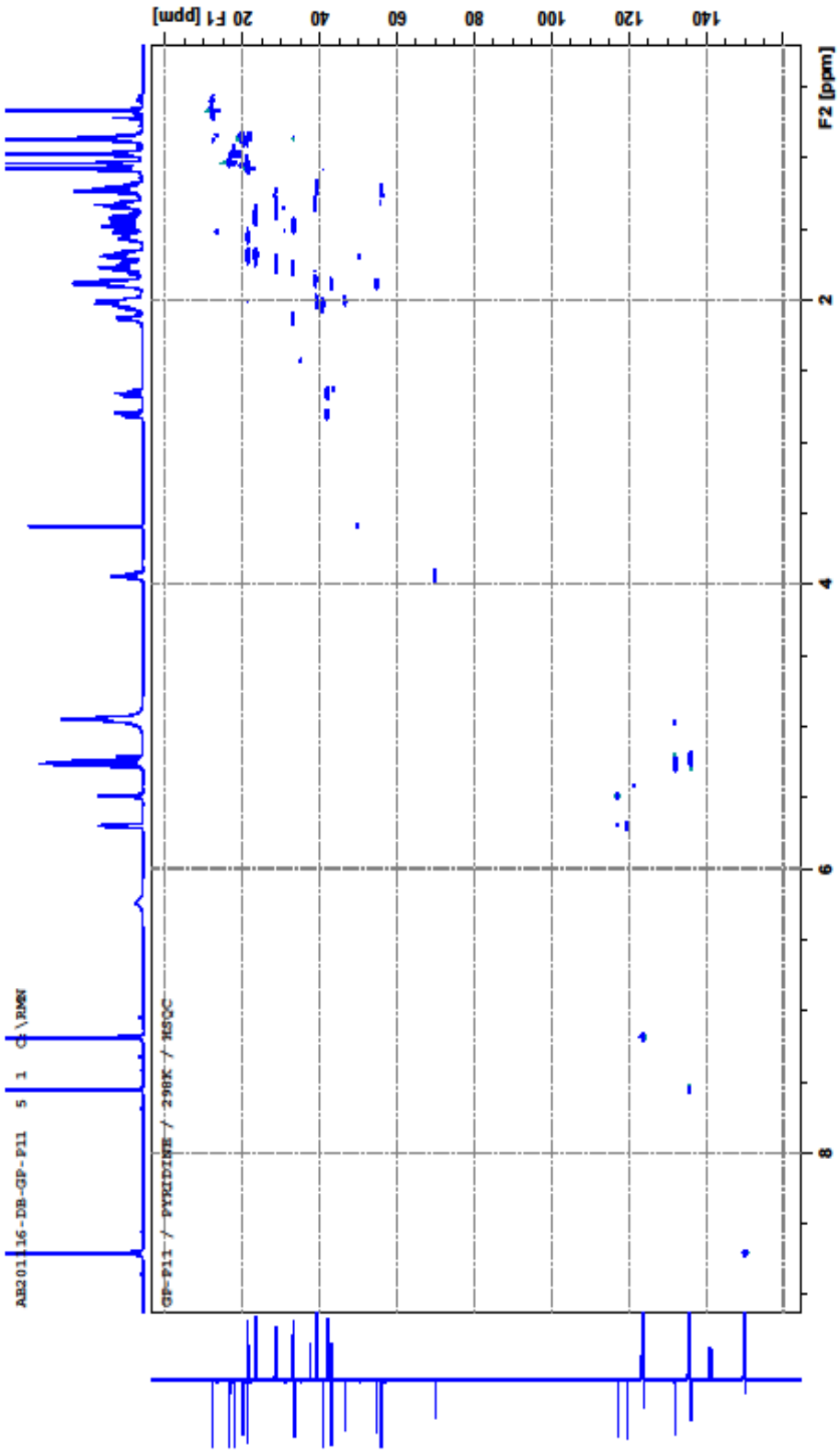
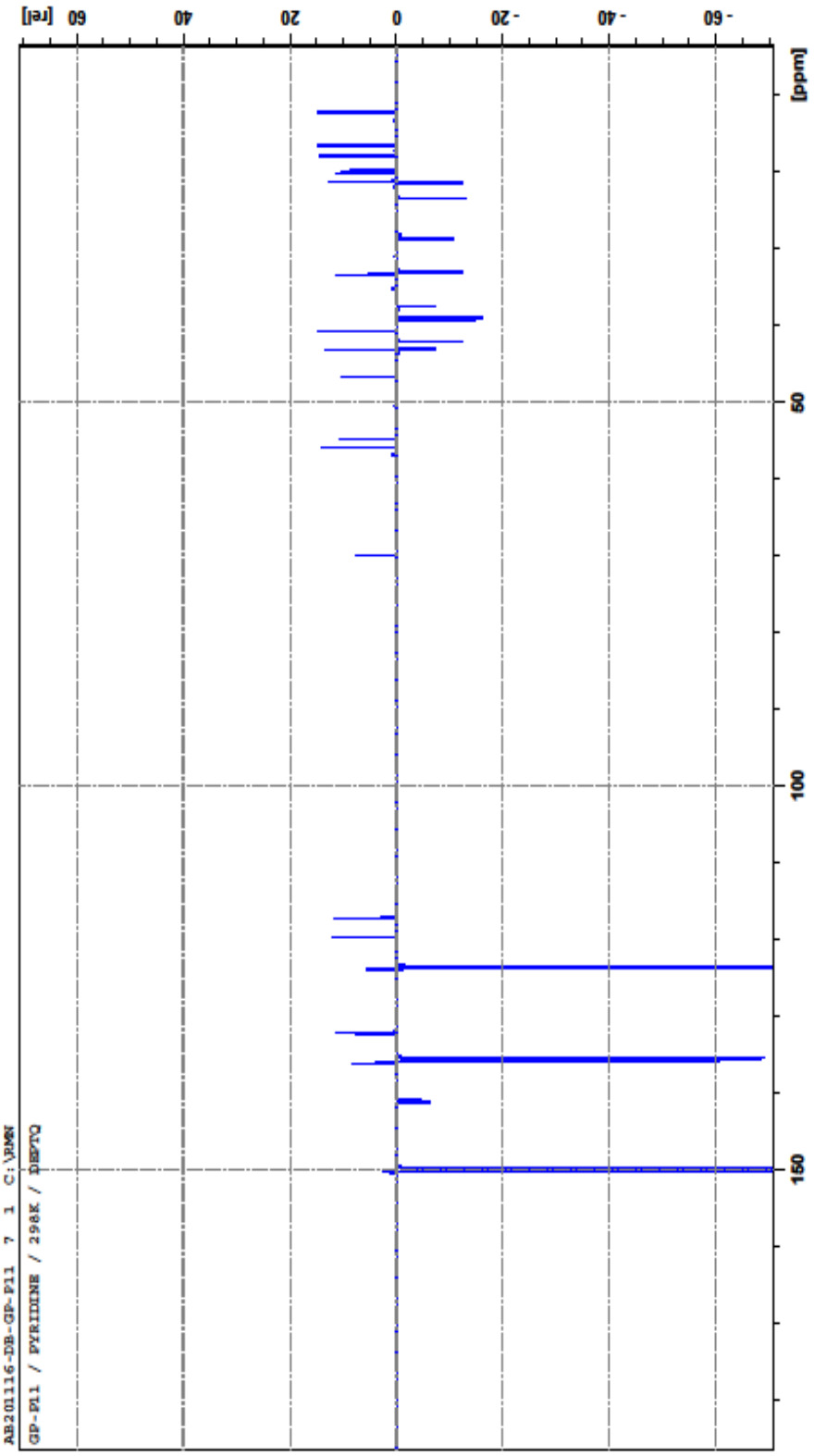
Appendix 1. Mass spectrum of compound C1



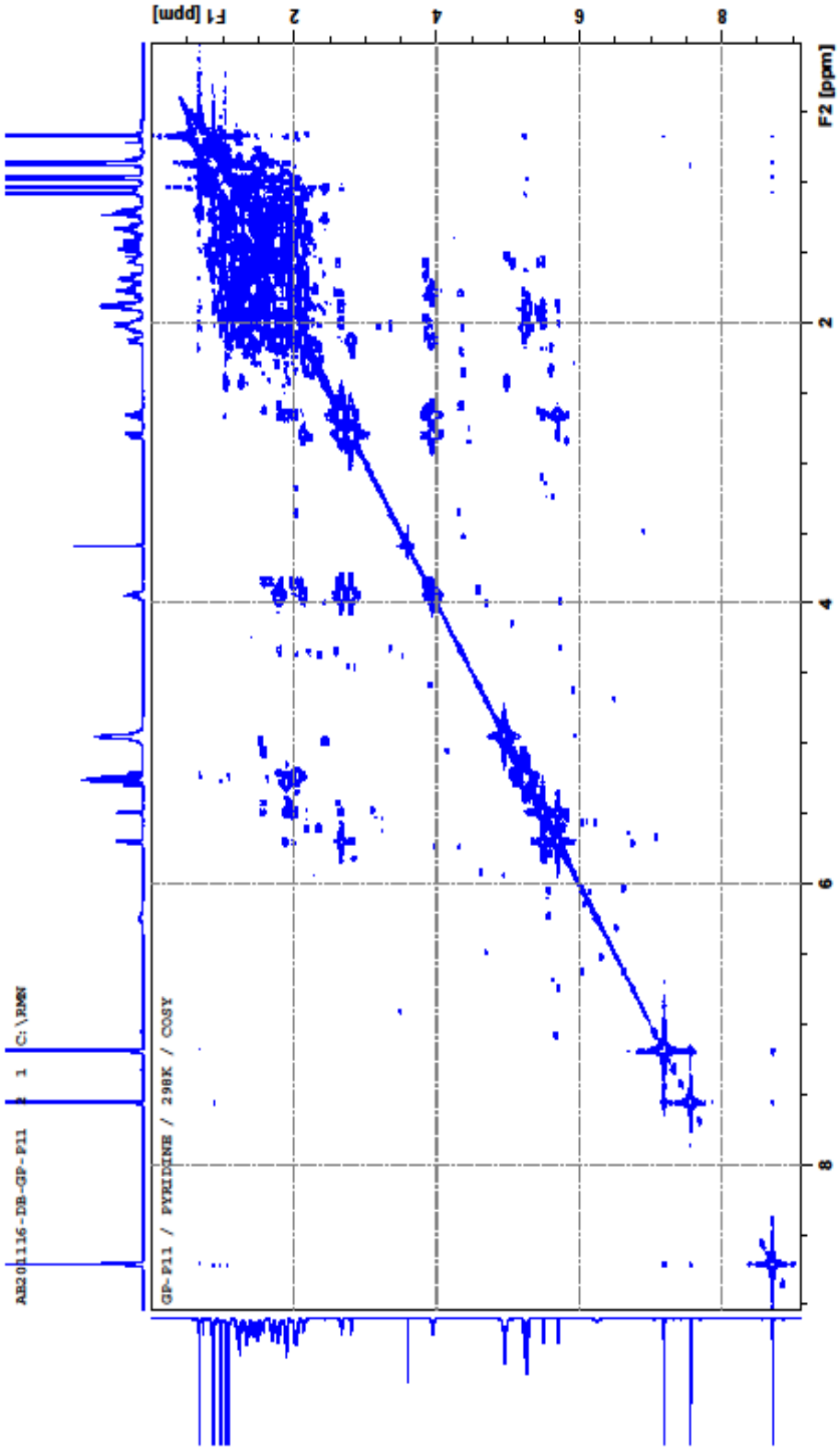
Appendix 2. Proton spectrum of compound C1



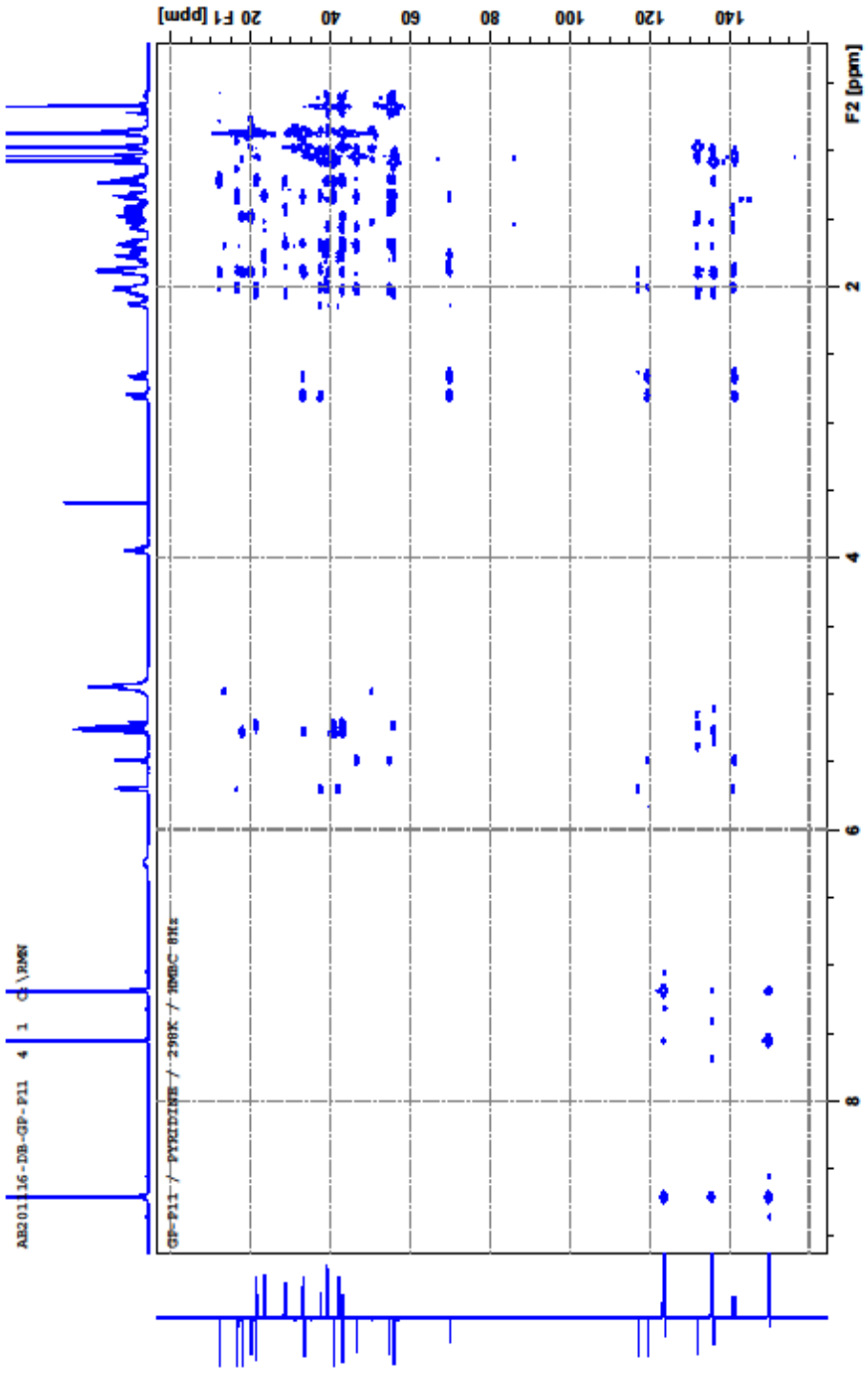
Appendix 3. HSQC spectrum of the NMR of compound C1



Appendix 4. NMR DEPT spectrum of compound C1



Appendix 5. COZY NMR spectrum of compound C1



Appendix 6. HMBC NMR spectrum of compound C1

Peer-review history:
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