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Full Length Research Paper

3β-hydroxyllup-20(29)-en-28-oic acid: Structural elucidation, *Candidosis* and mold inhibition evaluation

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3β-hydroxyllup-20(29)-en-28-oic acid (Betulinic acid) a Pentacyclic lupane triterpene known for anti-HIV activity and cytotoxic activity against various malignant versus nonmalignant cancer cell lines, was isolated from *malaleuca bracteata*. The structure was elucidated on the basis of spectroscopy analysis, including 2D-NMR correlation spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum correlation (HSQC) experiments. The inhibitory zone (mm) ranges from 20 ± 0.02 to 30 ± 0.01 against the test organisms. *Trichophyton tonsurans* was the most sensitive organism (30 ± 0.01) which was observed to be greater than the standard drugs Fluoconazole (14 ± 0.90), Nystatin (17 ± 0.03) and Fulcin (23 ± 0.50) used as positive control. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) results indicated that a concentration of 0.625 and 2.5 mg/ml inhibited and completely kill *Candida guielemondi, Candida stelletoidea, Candida parapsilosis, Candida pseudotropicalis, T. tonsurans* and *Microsporum canis* except *T. tonsurans* which was kill at 1.25 mg/ml. While the rest of the organisms were inhibited and kill by a concentration of 1.25 and 2.5 mg/ml respectively, the results shows that the compound have great potential as antifungal drug.

Key words: Betulinic acid, pentacyclic triterpene, antifungal, nuclear magnetic resonance spectroscopy.

INTRODUCTION

The pentacyclic triterpenes have been widely investigated for pharmacological activities. Pentacyclic triterpenoids are the dominant constituents of the triterpenoids class and are all based on a 30-carbon skeleton comprising five, six-membered rings and one five-membered ring. These compounds occur commonly and are mostly concentrated in fruits, vegetables, leaves, stem bark and several medicinal plants (Nagaraj et al., 2000). They are devoid of any prominent toxicity. These naturally occurring triterpenoids include the lupane, ursane, oleanane, lanostane, dammarane and some miscellaneous scaffolds (Dang et al., 2009). *Melaleuca bracteata* commonly called revolution gold belong to the Myrtaceae family. The genus *Melaleuca* is entirely Australian and contains more than 100 species of trees and shrubs which are mostly cultivated for their ornamental characters. The leaves and the volatile oils are used in enthnomedicine of West Africa as a remedy for numerous diseases (Oliver, 1960; Irvine, 1961; Howes, 1974; Goldstein et al., 1990; Belousova and Denisova, 1992). *M. bracteata* var. revolution gold and *M. bracteata* var. revolution green are hybrids of *M. bracteata* and are indigenous in South Africa. The essential oil obtained from the fresh leaves and twigs are recognized for their antiseptic, germicidal and insecticidal properties (Cribb and Cribb, 1981; Yatagai, 1997). Examination of the leaves of *M. bracteata* has lead to the isolation of Betulinic acid. The inhibitory activities of Betulinic acid against tumor promotion was identified as a melanoma-specific cytotoxicity mediated by the induction of apoptosis (Pisha et al., 1995). Chemical modification of Betulinic acid at the C-3 position results in a compound that inhibit the HIV-1 maturation as oppose to entry, 3-O-(3',3'-dimethylsuccinyl) betulinic acid (Bevirimat) which shows extremely potent anti-HIV

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activity with a reported EC_{50} value of less than 0.00035 μM and a therapeutic index value greater than 20,000 which is currently under clinical trial. Unmodified Betulinic acid was found to be inactive or exhibited weak activity against HIV-1 replication (Kashiwada et al., 2004). *Candida* infections are very common throughout the world. Superficial infection may affect the skin, nails, mucous membranes of the mouth and vagina. Mucosal infections are the commonest form of superficial *Candidosis* while oral *Candidosis* occurs most frequently in infancy and old age, or in severely immune-compromised patients, including those with acquired immune deficiency syndrome (AIDS). Some individual antibiotics therapy may results in a painful *Candida* infection of the tongue, chronic oral *Candidosis* may also occur with extensive leucoplakia and infection of the angles of the mouth (*angular cheilitis*). Vaginal *Candidosis* is common especially during pregnancy; it is usually accompanied by itching, soreness and a non-homogenous white discharge (Med. Micro, 1992). In this paper we investigate the *Candidosis* and mold inhibition properties of the compound JH30.

MATERIALS AND METHODS

Plant material

Leaves of *M. bracteata* var. revolution gold were collected from trees growing on the Westville Campus, University of KwaZulu-Natal in March, 2009, identified and authenticated by Mr. Teddy Govender of Ethekwini Parks and Gardens Nursery, Burnwood Road, Durban, South Africa.

Extraction and isolation

Dried leaves of *M. bracteata* var. revolution gold were exhaustively extracted with dichloromethane at room temperature by maceration. After evaporation of the solvent from the extract, a dark green solid mass was obtained. This mass was washed with n-hexane twice to remove oily materials leaving behind a light green solid. A portion of the residue was subjected to chromatographic separation on silica gel (60 - 120 mesh) column (20 x 5.5 cm). Elution with hexane: ethyl acetate (8:2 →7:3) afforded cream solid which was re-crystallized from methanol to give a white crystalline material in a yield of 1.07%.

GENERAL EXPERIMENTAL PROCEEDURE

The melting point (uncorrected) was determined on a Stuart Scientific SMP1 apparatus.

The nuclear magnetic resonance (NMR) spectra (1D and 2D) were recorded on Bruker-Advance 400 MHz FT-NMR Spectrometer, using the residual solvent ($CDCl_3$) peaks as internal standards. The EIMS (LC-MS) was performed on Agilent Technologies 1200 Series Binary SL. The Infra-red spectroscopy was conducted on Perkin Elmer Spectrum 100 FTIR Spectrometer.

BIOLOGICAL STUDIES

Test organism

The clinical pathogenic yeast (*Candida guielemondi, Candida tropicalis, Candida pseudotropicalis, Candida stelletoidea, Candida albicans, Candida krusei,* and *Candida parapsilosis*) and molds (*Trichophyton tonsurans* and *Microsporum canis*) used in the study were obtained from the Department of Medical Microbiology Ahmadu Bello University teaching hospital (ABUTH) Shika Zaria. All the isolates were purified and maintained in slants of Sabouraud dextrose agar (SDA).

Determination of antifungal activity

The antifungal activity was carried out by utilizing the well-in-plate diffusion technique as reported by Karou et al. (2006). Pure culture of the organisms was inoculated on to Sabauroud Dextrose Agar (SDA) incubated for 24 h at 30°C. About 5 discrete colonies were aseptically transferred with a sterile wire loop into a tube containing sterile normal saline (0.85% NaCl) and were adjusted to a turbidity of 0.5 MacFarland standards. The suspension was used to streak on the surface of SDA plates with a sterile swap. A sterile 6 mm diameter cork borer was used to make holes into the set agar in the Petri dishes containing the fungi culture. The wells were filled with 2.5 mg mL^{-1} concentrations of the compounds. Standard antifungal drugs Fulconazole (2.5 $mgmL^{-1}$), Nystatin (2.5 $mgmL^{-1}$) and Fulcin (2.5 $mgmL^{-1}$) were used as positive control. The plates were incubated for 1 - 7 days at 30°C. All the tests were performed in triplicate and the antifungal activities were expressed as mean diameter of inhibition zones (mm) produced by the compound and the standard drugs (Table 2).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was carried out using the micro broth dilution technique in accordance with National Committee for Clinical Laboratory Standard (2006). Serial dilution of the concentration of the compounds and drug that showed activity was prepared using test tubes containing 9 mL of double strength broth. The tests tubes were inoculated with the suspension of the standardized inocular and incubated at 30°C for 7 days. MIC was recorded as the lowest concentration of the compound and drug showing no visible growth of the broth (Table 3).

Determination of minimum fungicidal concentration (MFC)

The minimum fungicidal concentration was determined by aseptically inoculating aliquots of culture from MIC tubes that showed no growth, on sterile nutrient agar plates and incubated at 30°C for 7 days. The MFC was recorded as the lowest concentration of the compound and drug showing no colony growth (Tables 4 and 5).

RESULTS AND DISCUSSION

The compound JH30 was assigned the molecular formula $C_{30}H_{48}O_3$ by electron impact mass spectroscopy (EIMS), LC-MS (Appendix 8) shows a molecular ion at m/z 457.1 $[M^+ +H]$ which is in agreement with the formula mass 456.7 $gmol^{-1}$. The Infra-Red (IR □$_{max,}$ cm^{-1}) Spectra indicate the presence of hydroxyl and carbonyl groups (3300 cm^{-1} and 1685 cm^{-1}) confirming the presence of carboxylic acid functional group (C-28, Table 1). The signal between 3000 and 2850 cm^{-1} is attributed to C-H stretching vibration from alkane (CH_3, CH_2), the

Table 1. ^{13}C–NMR (400 MHz δ, $CDCl_3$) data.

Carbon position	^{13}C δ(ppm)	CH_n	HSQC (ppm)
1	38.66	CH_2	
2	27.26	CH_2	0.79, 0.76
3	78.42	CH	2.79
4	38.76	C	-
5	55.26	CH	0.30
6	18.20	CH_2	1.02, 0.89
7	34.25	CH_2	0.99
8	40.62	C	-
9	50.42	CH	0.88
10	37.06	C	-
11	20.78	CH_2	1.31
12	25.43	CH_2	0.58
13	38.10	CH	
14	42.32	C	-
15	30.56	CH_2	1.56, 0.98
16	32.22	CH_2	1.01
17	55.99	C	-
18	46.80	CH	1.18
19	49.09	CH	2.65
20	150.76	C	-
21	29.61	CH_2	1.89, 1.86
22	37.06	CH_2	1.89
23	27.67	CH_3	1.56
24	15.42	CH_3	1.20
25	15.94	CH_3	1.14
26	16.04	CH_3	0.56
27	14.58	CH_3	0.37
28	178.43	COOH	-
29	109.27	CH_2	4.35, 4.21
30	19.27	CH_3	1.31

absorption at 1452 cm^{-1} characterize the bending vibration of CH_2 methylene groups. The band at 1379 cm^{-1} corresponds to the bending vibration of methyl groups CH_3. ^{13}C–NMR spectrum (Table 1 and Appendix 2) reveals the presence of 30 carbon signals, which were assigned by DEPT experiments (Appendix 3) as five methine CH, eleven methylene CH_2, six methyl CH_3 and seven quaternary carbons. The two sp^2 carbons observed at δ_C 150.76 and δ_C 109.27 in the ^{13}C–NMR spectrum confirmed the $\Delta^{20,29}$ functionality of a lupene skeleton. The ^1H-NMR spectrum (Table 1 and Appendix 1) showed five methyl groups [δ_H 1.56, 1.20, 1.14, 0.56, 0.37, (each 3H, s)] and isopropenyl group [δ_H 1.31 (3H, s), 4.35 (1H, s) and 4.21 (1H, s)]. Spectra data (IR and NMR) with the application of correlation spectroscopy (COSY) relates protons on adjacent carbon while nuclear overhauser enhancement spectroscopy (NOESY) relates protons in space. However, heteronuclear single quantum correlation (HSQC) this shows the relationship between ^1H-^{13}C (J_{CH}) one bond away, and heteronuclear multiple bond correlation (HMBC) shows the relationship between proton-carbon two to three bonds away ($^2J_{CH}$ and $^3J_{CH}$) were used to assign the structure 3β-hydroxyllup-20(29)-en-28-oic acid (Appendices 4, 5, 6, 7 and 9). The melting point was recorded as 264 - 266°C.

The result of the well-in-plate agar diffusion method (Table 2) shows a marked inhibitory zone (mm) as compared to the standard drugs used as positive control. *T. tonsurans,* a mold, was the most sensitive organism on the compound JH30 with a inhibitory zone of 30 ± 0.01 which was observed to be greater than the control for the same organism; Fluoconazole (14 ± 0.9), Nystatin (17 ± 0.03) and Fulcin 23 ± 0.5). The smallest inhibitory zones for JH30 was recorded against C. stelletoidea (20 ± 0.02) this was lower than the result observed for Fluoconazole (30 ± 0.33) but higher than the other standards, which were resistant to the organism. The MIC and MFC results (Tables 3, 4 and 5) indicated that a concentration of

Table 2. Sensitivity test of JH30 (Zone of inhibition in mm).

Test organism	JH30	FZ	NY	FL
C. guielemondi	25 ± 0.01	20 ± 0.20	R	R
C. stelletoidea	20 ± 0.02	30 ± 0.33	R	R
C. albicans	27 ± 0.03	20 ± 0.50	17 ± 025	R
C. Krusei	22 ± 0.01	21 ± 0.05	14 ± 0.05	R
C. parapsilosis	24 ± 0.03	24 ± 1.50	20 ± 0.25	17 ± 0.33
C. tropicalis	28 ± 0.05	21 ± 0.80	R	R
C. pseudotropicalis.	28 ± 0.10	21 ± 0.035	R	R
T. tonsurans	30 ± 0.01	14 ± 0.90	17 ± 0.90	23 ± 0.50
M. canis	20 ± 0.08	R	R	24 ± 0.05

FZ = *Fluoconazole*, NY = *nystatin*, FL = Fulcin (FZ, NY, FL = positive control), R = resistant.

Table 3. Minimum inhibition concentration.

Test organism	2.500	1.250	0.625	0.3125	0.1563
C. guielemondi	-	-	**	+	++
C. stelletoidea	-	-	**	+	++
C. albicans	-	**	+	++	+++
C. Krusei	-	**	+	++	+++
C. parapsilosis	-	-	**	+	++
C. tropicalis	-	-	**	+	++
C. pseudotropicalis.	-	-	**	+	++
T. tonsurans	-	-	**	+	++
M. canis	-	**	+	++	+++

- = no growth, ** = MIC, + = slight growth, ++ = dense growth, +++ = very dense growth.

Table 4. Minimum fungicidal concentration.

Test organism	2.5000	1.2500	0.625	0.3125	0.1563
C. guielemondi	*	+	++	ND	ND
C. stelletoidea	*	+	++	ND	ND
C. albicans	*	+	++	ND	ND
C. Krusei	*	+	++	ND	ND
C. parapsilosis	*	+	++	ND	ND
C. tropicalis	*	+	++	ND	ND
C. pseudotropicalis.	*	+	++	ND	ND
T. tonsurans	-	*	+	ND	ND
M. canis	*	+	++	ND	ND

- = no growth, * = MFC + = slight growth, ++ = dense growth, ND = not determined.

625 and 2.50 mg/ mL inhibited and completely killed *C. guielemondi, C. stelletoidea, C. parapsilosis, C. tropicalis* and *C. pseudotropicalis,* respectively. The only exceptions are *C. albicans, C. krusei*, *M. canis* (MIC = 1.25 mg/mL; MFC = 2.50 mg/mL) and *T. tonsurans* (MIC = 0.625 mg/mL, MFC = 1.25 mg/mL). When the results were compared with the MIC and MFC of the standard Fluoconzole (Table 5) only *C. stelletoidea* (MIC = 0.625 mg/mL, MFC = 1.25 mg/mL) and *C. parapsilosis* (MIC = 1.25 mg/mL, MFC = 1.25 mg/mL), compare with those of the compound JH30, the rest of the organisms showed0. MIC (2.50 mg/mL) and MFC (2.50 mg/mL) which were lower. The result of the studies indicates that the compound JH30 has great potential as antifungal agent

Table 5. MIC and MFC results of fluoconazole.

Test organism	Concentration (mg/mL)	
	MIC	MFC
C. guielemondi	2.50	2.50
C. stelletoidea	0.63	1.25
C.albicans	2.50	2.50
C. Krusei	2.50	2.50
C. parapsilosis.	1.25	1.25
C. tropicalis	2.50	2.50
C. pseudotropicalis.	2.50	1.25
T. tonsurans	2.50	2.50

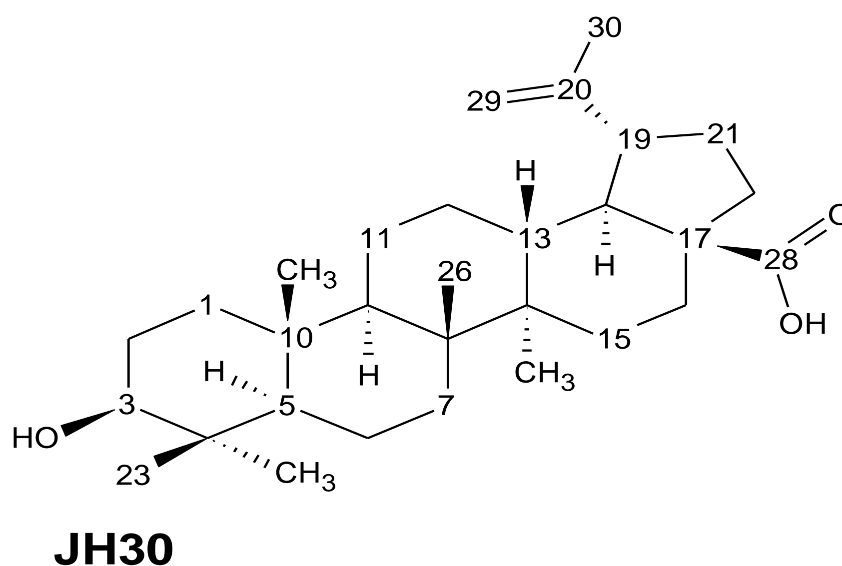

Figure 1. 3β-hydroxyllup-20(29)-en-28-oic (Betulinic acid).

apart from its other numerous applications (Figure 1).

Conclusion

The results of the investigation shows that bioactive Pentacyclic triterpene from plants have the ability not only to inhibit fungi and mold, but to completely kill them. This has listed Betulinic acid a Pentacyclic triterpene, as one of the compounds with potential that could be used in the treatment of infection caused by *Candida* species and molds, among its many other applications.

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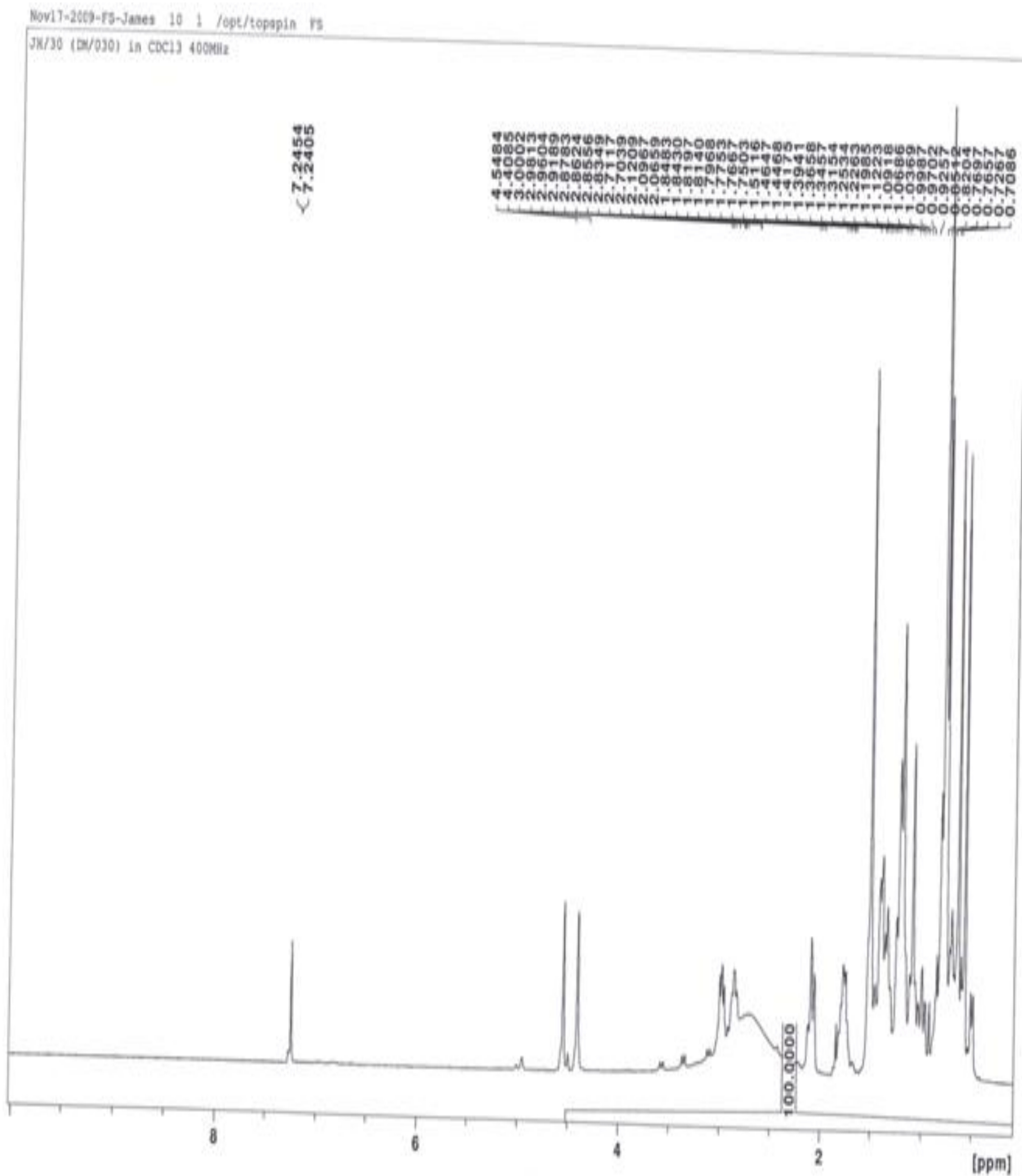
APPENDICES



Appendix 1. ^{1}H-NMR.

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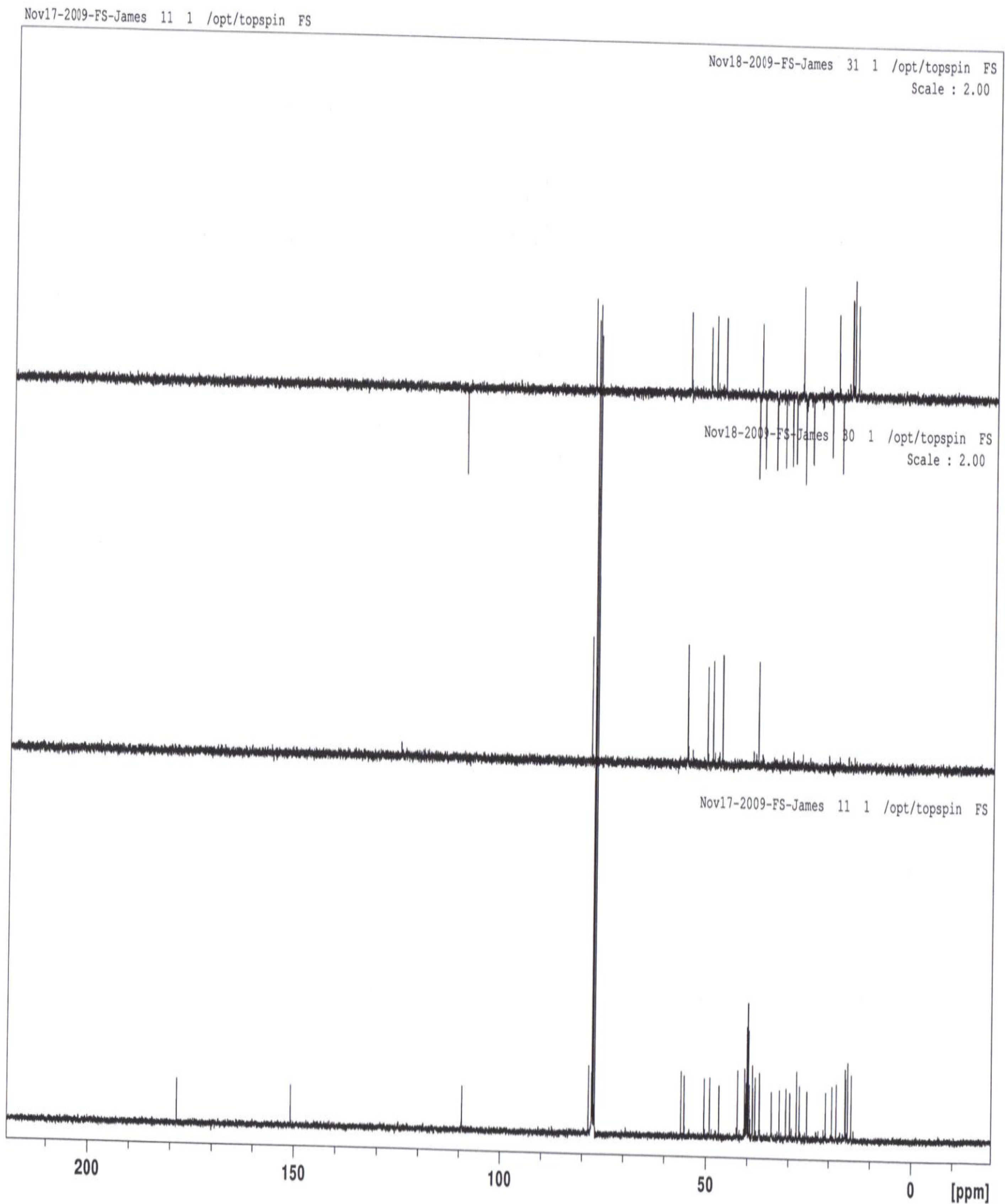
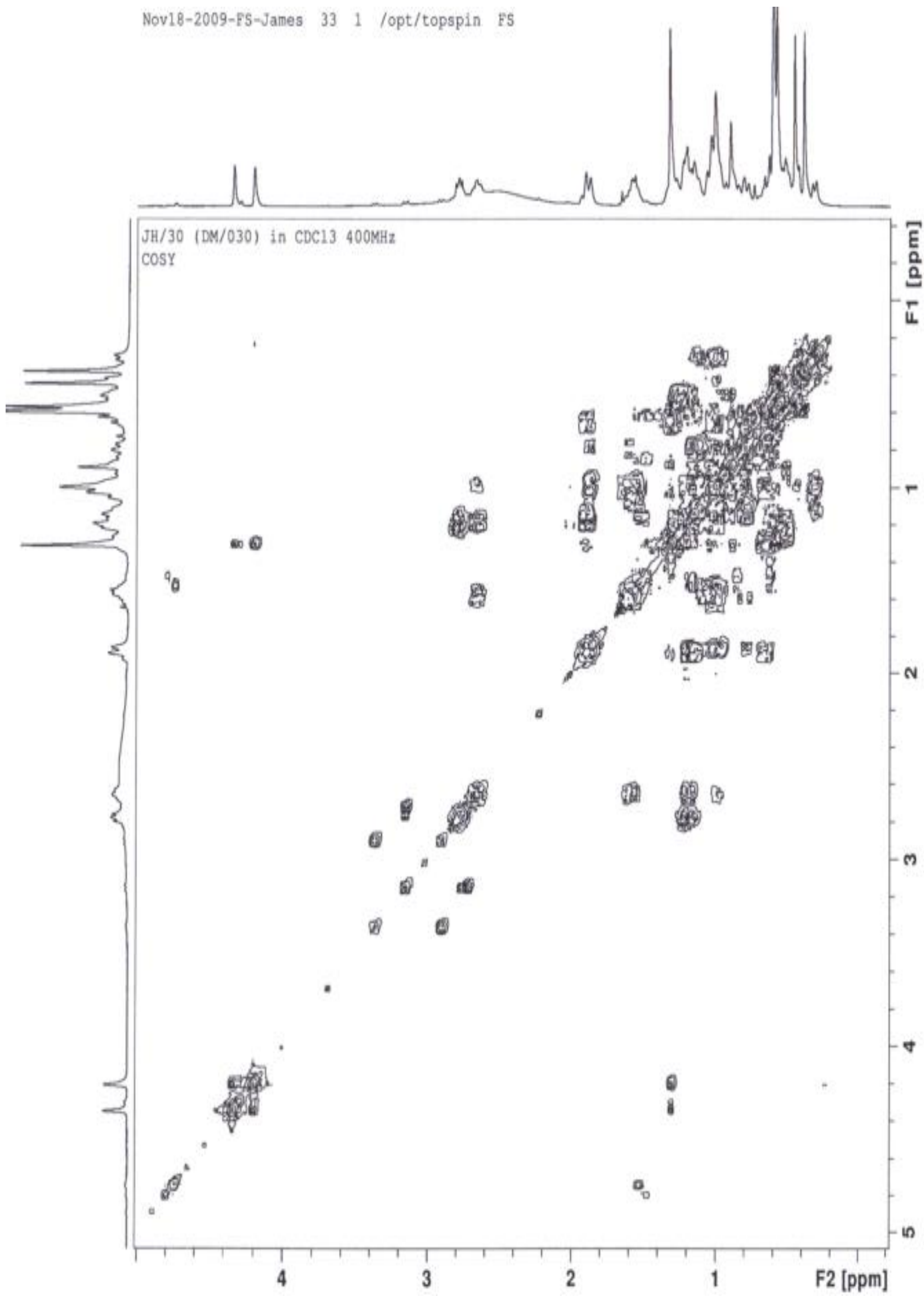
JH/30 (DH/030) in CDCl3 400MHz

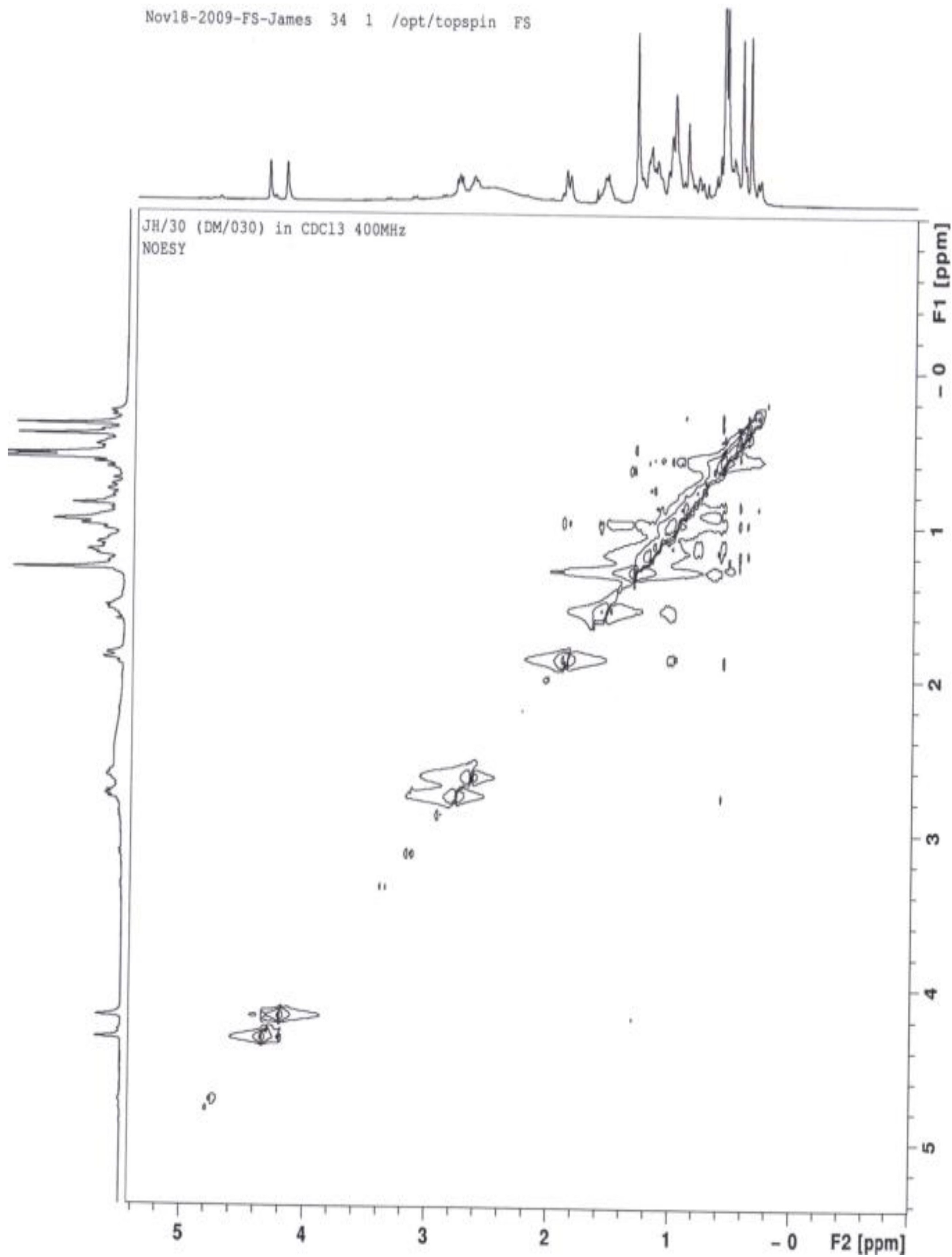
Appendix 2. ^{13}C-NMR.



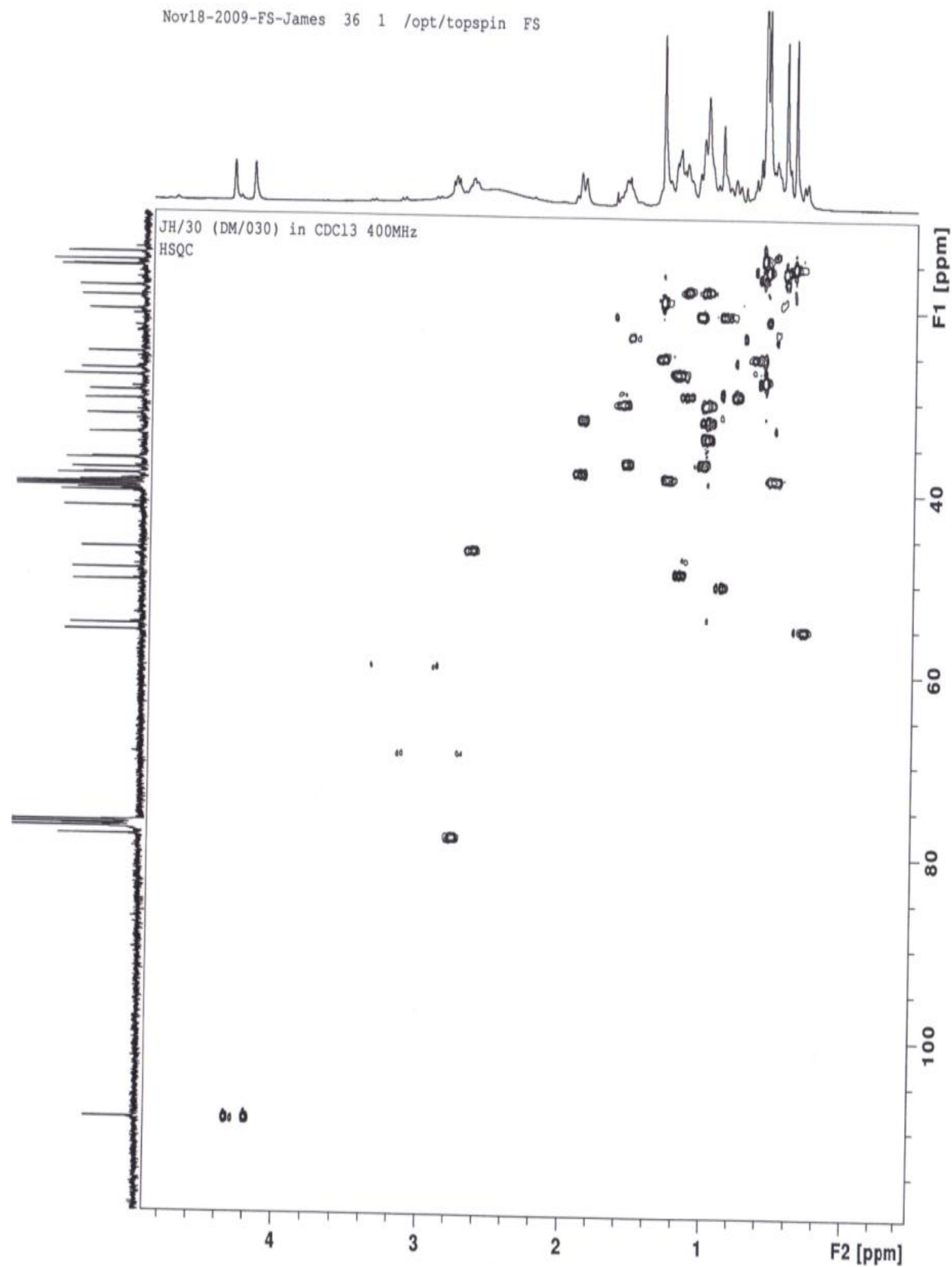
Appendix 3. DEPT experiment.



Appendix 4. COSY experiment.



Appendix 5. NOESY experiment.



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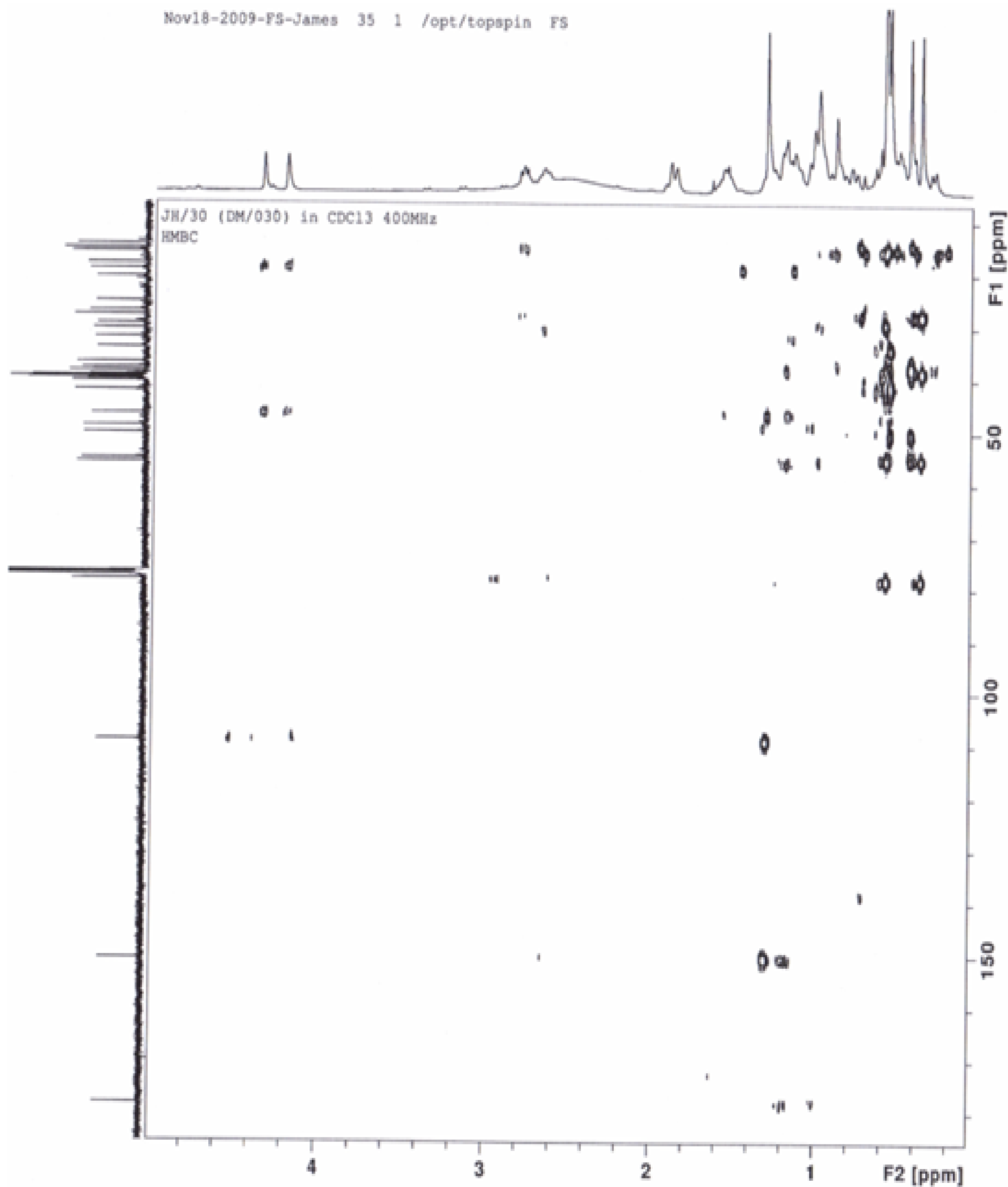

Appendix 7. HMBC experiment.

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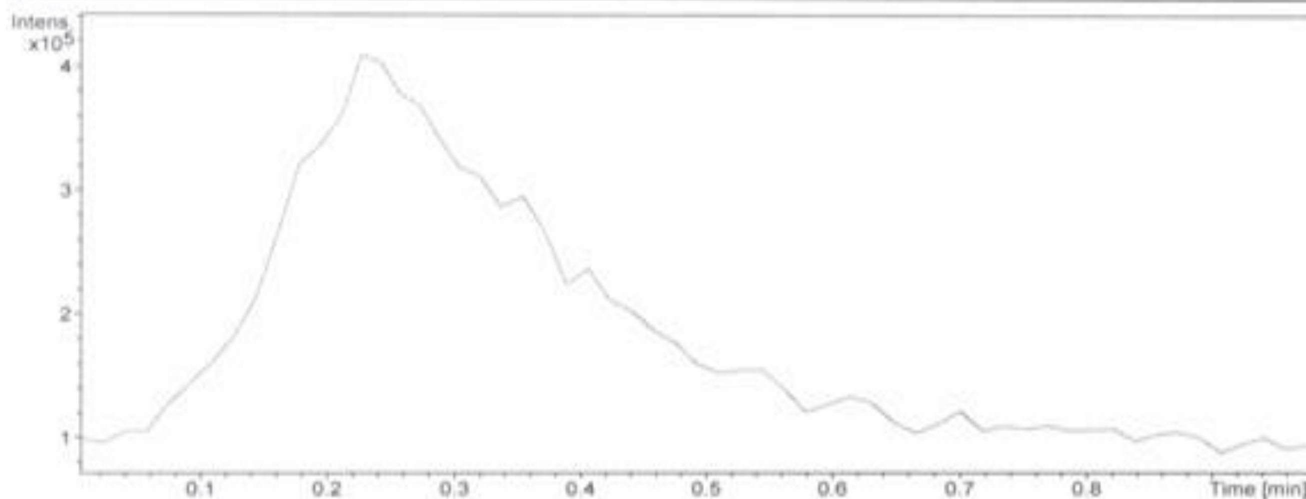
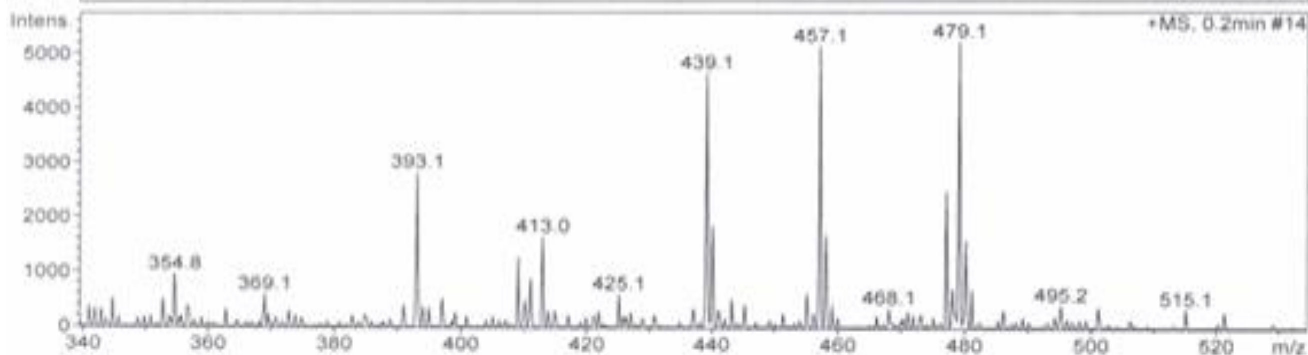
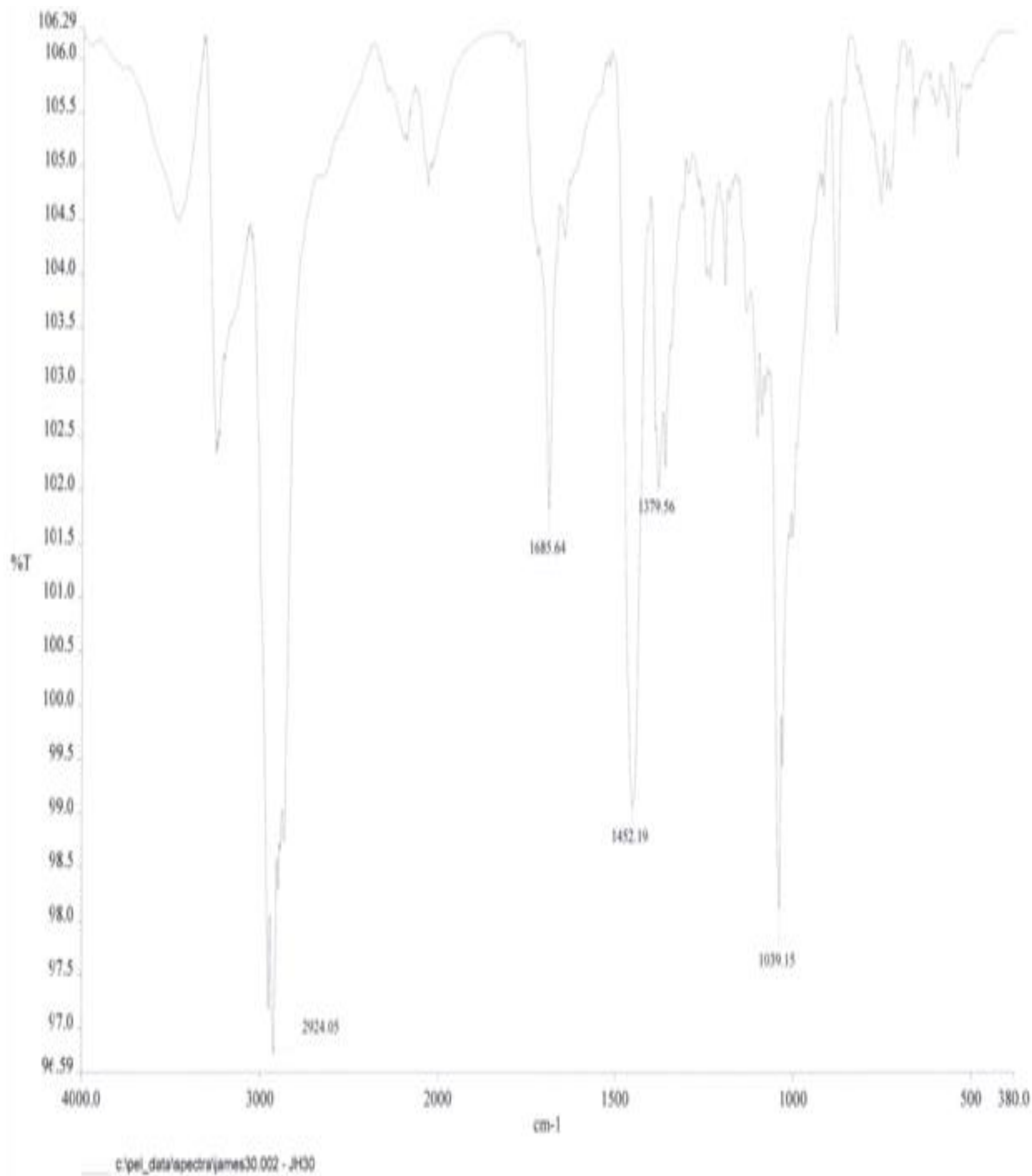




MSD Trap Report v5.2 (A4-Opt1) Page 1 of 1 Agilent Technologies

Appendix 8. LC-MS spectra.



Appendix 9. IR-Spectra.