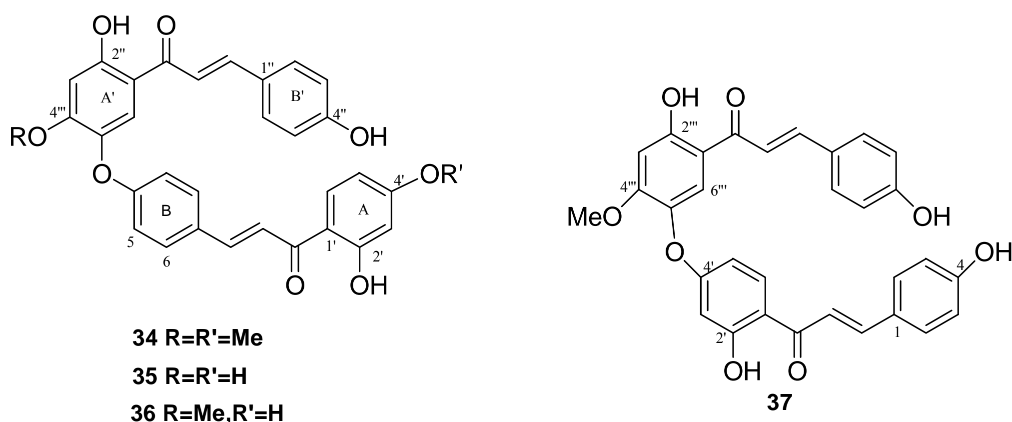
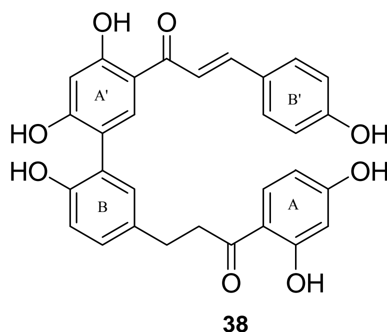
In a recent report, five new bichalcones namely: rhuschalcone I (**34**), rhuschalcone II (**35**), rhuschalcone III (**36**), rhuschalcone IV (**37**), and, rhuschalcone V (**38**) have been recorded. All of their structures were determined by spectroscopic and chemical methods. Upon testing, they exhibited selective cytotoxicity against some colon tumor cells.





2.4.2 Biological activity

Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) suggest that many animals, including humans, ingest significant quantities in their diet.

Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory (Yamamoto *et al*., 2004), anti-microbial (Cushnie *et al*., 2005) and anti-cancer activity. Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancer and cardiovascular diseases. The beneficial effects of fruits, vegetables, and tea or even red wine have been attributed to flavonoid compounds rather than to known nutrients and vitamins (Félicien, 2008).

2.4.3 Health benefits besides antioxidant properties

In recent times, research conducted on Free Radical Biology and Medicine indicates that inside the human body, flavonoids themselves are of little or no direct antioxidant value (Lotito, 2006). Body conditions prove to be unlike controlled test tube conditions, and the flavonoids are poorly absorbed (less than 5%), with most of what is absorbed being quickly metabolized and excreted. The huge increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused directly by the flavonoids themselves, but most likely is due to increased uric acid levels. An interesting quotation from David, (2007) reads as follows: - "we can now follow the activity of flavonoids in the body, and one thing that is clear is that the body sees them as foreign compounds and is trying to get rid of them."

The process of gearing up the body to get rid of unwanted compounds induces the formation of the so-called Phase II metabolic enzymes that also help eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids can induce mechanisms that help kill cancer cells and inhibit tumor invasion (Ladislaus, *et al*., 2003).

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Cancer researchers have found that human subjects who ate foods containing certain flavonoids seemed to be protected from developing lung cancer (David, 2007). Some of the flavonoids that have appeared to be the most protective include quercetin (**27**) and kaempferol (**28**). Researchers have indicated that only small amounts of flavonoids are necessary to see these medical benefits. Taking large dietary supplements provides no extra benefit and may pose some risks.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents, equipment and materials

3.1.1 Reagents

Sulphuric acid, acetic acid, *p*-anisaldehyde, vanillin were bought from Kobian chemicals, Nairobi Kenya; Solvents: Dichloromethane, *n*-hexane, ethyl acetate, chloroform, petroleum ether, acetone, toluene and methanol bought from Kobian chemicals, Nairobi Kenya. Analytical grade or double distilled solvents were bought from Fluka AG Switzerland. Deuteriated solvent MeOH used for spectroscopic analysis was bought from Sigma-Aldrich South Africa.

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3.1.2 Equipment

3.1.2.1 Solvent evaporation

Solvent evaporation was performed on a Buchi Rotavapor RE 111 with a water bath at 40^0C and 14 mbar.

3.1.2.2 Chromatography

3.1.2.2.1 Thin layer chromatography (TLC)

Analytical thin layer chromatography was performed on both aluminium and plastic pre-coated plates of silica gel 60 F_{254} with a 0.2 mm layer thickness. Visualization of the TLC spots was achieved under UV light at 254nm or 366nm and by spraying with the *p*-anisaldehyde reagent prepared by mixing 0.5ml anisaldehyde mixed with 10ml glacial acetic acid, followed by 85ml of chilled methanol and 5ml of 98% sulphuric acid (Randerath *et al.*, 1968).

Preparative thin layer chromatography (PTLC) was performed using normal phase silica gel 60 F_{254} pre-coated on glass plates (20 x 20), with varying thickness (0.25, 0.5, 1.0, or 2.0 mm). Detection was done under UV light at 256 or 366 nm.

3.1.2.2.2 Column chromatography (CC)

Glass columns (20-25 mm diameter) wet packed with silica gel 60 (0.040-0.063mm) (230-400 Mesh ASTM, Merck) were used for column chromatography. Size-exclusion chromatography was carried out using Sephadex® LH-20 (Pharmacia), eluting with either MeOH or MeOH-$CHCl_3$.

3.1.3 Spectroscopy

3.1.3.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were measured on Varian-600 NMR machine based at the Department of Chemistry at the University of the Stellenbosch, South Africa and a Bruker-300NMR at the University of Botswana. Deuteriated methanol (CD_3OD) was used as the solvent. Chemical shifts were recorded in δ (ppm).

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3.2 Plant material

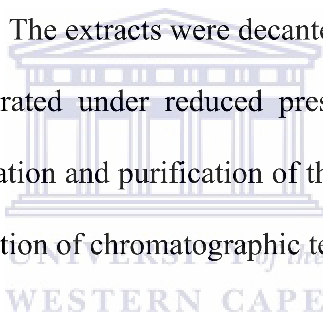
3.2.1 Plant samples collection and identification

Plant material was collected in Kenya, near the Thika river within Gatanga division in June 2008. The three plant parts namely; root bark, stem bark and leaves were dried for a period of three months, stored under controlled conditions in order to minimize undue chemical changes. The material was ground into a powder prior to extraction.

The plant was taxonomically identified by Mr. Simon Mathenge of the Department of Botany, University of Nairobi, Kenya. The voucher specimen (HM 2008/01) was deposited at the Department of Botany Herbarium, University of Nairobi, Kenya.

3.2.2 Extraction

The powdered material was extracted sequentially with solvents of increasing polarity starting with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and finally methanol (MeOH). Each extraction involved maceration for a period of about 48 hours, with three repetitions for each solvent except for methanol which was done twice. Occasional swirling was done to ensure thorough extraction. The extracts were decanted and filtered using Whatman filter paper. The extracts were concentrated under reduced pressure using a rotary evaporator at temperatures less than50 ^oC. Separation and purification of the plant constituents was carried out using one or the other of a combination of chromatographic techniques.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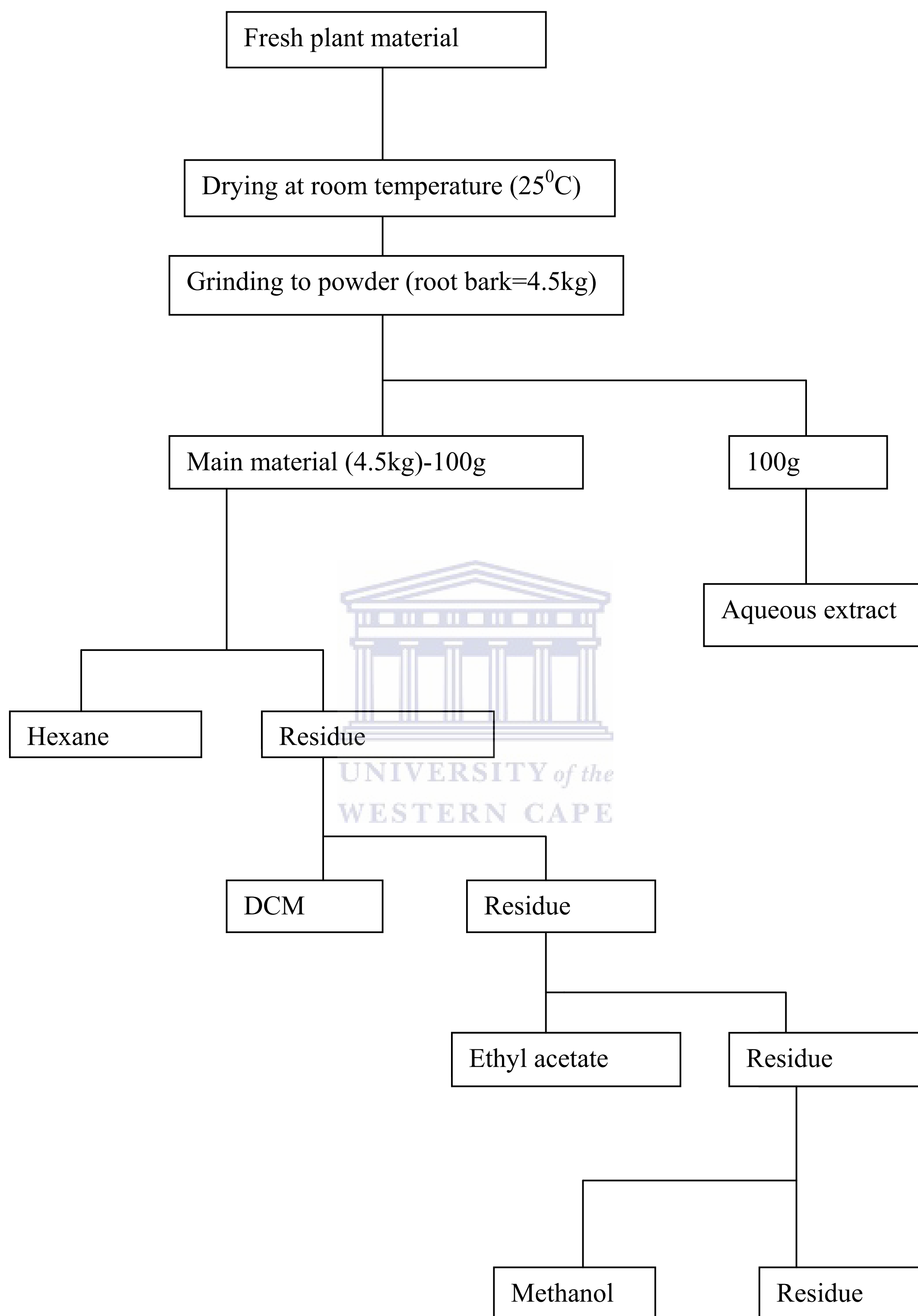

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Scheme 2. A flow chart for the preparation of the plant extracts

3.3 Antibacterial and anti-fungal screening tests

Bioassay of the crude extracts against test organisms was carried out using the agar diffusion technique. Micro-organisms used in these tests were obtained from the National Public Health Laboratories (NPHL). These included local and standard clinical isolates. The micro-organisms used included Gram positive bacteria, *Staphylococcus aureas* (ATCC 25923), *Escherichia coli* (ATCC 25922) Gram negative bacteria, *Pseudomonas aureginosa* (clinical isolate). The fungi used *Cryptococcus neoformans* (clinical isolate), *Trichophyton mentagrophytes* (clinical isolate), *Penicillium spp* (environmental isolate) and *Candida albicans*. These test strains of bacteria were kept refrigerated at 4^oC in Muller-Hutton agar slants during the storage period. They were then tested biochemically for purity before use (Elgayyar *et al.,* 2000). A colony of each bacterial strain was put in some agar slants and incubated for 18 hours at normal body temperature.

Sterile Petri-dishes containing about 20ml of sterile medium of yeast, malt extracts and glucose agar for fungi and nutrient broth agar for bacteria were inoculated with an 18 hour culture of the appropriate test organism. After 6 hours, a diluted sub-culture was prepared with a similar broth. The plant extract was then dissolved in dimethylsulphoxide (DMSO) and 20µl were loaded onto a 6mm filter paper. A standard antibiotic, chloramphenicol 2mg and fluconazole 2mg for the fungi were used as controls. The various Petri-dishes were then inoculated with 0.1ml of the diluted bacteria or fungi culture directly from the 18 hour broth. The inoculations were then spread using a sterile glass rod to avoid contamination and allowed to stand for 2 – 3 minutes in a refrigerator which was set at 4 ^oC. Discs loaded with the plant extracts were then placed onto the seeded plates. All the Petri-dishes were then incubated at 37 ^oC for 24 hours for the bacterial pathogens and 3 days for the fungal pathogens.

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After that period of incubation, antibacterial or antifungal activity was assessed by measuring the diameter of all the zones of inhibition which were measured in millimeters, taking triplicate values of each by measuring in different directions and then calculating the average, using a standard laboratory ruler (Elgayyar *et al.,* 2000).

3.4 Isolation of the pure compounds

3.4.1 Fractionation of the root hexane extract

The hexane extract (10.6g) was adsorbed on silica gel and fractionated on a column by gravity elution using the following solvent systems; 500ml of 100% hexane, then 250ml volumes of mixtures with DCM at the following ratios (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100%DCM. This was further followed by 250 ml volumes of DCM-EtOAc mixtures using similar ratios until 100% EtOAc. Finally small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH (98.5:2.5), (95:5), (92.5:7.5), (90:10), (95:15). Fractions collected were analyzed by TLC using hexane-EtOAc (7:3). Fractions with similar Rf values were pooled together. The combined fraction from vials 26-29 (400mg) was loaded onto a sephadex column for further separation. DCM-MeOH (50:50) 500ml was used as eluent leading to the isolation of compound **41** as a clear liquid (171mg).

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3.4.2 Fractionation of the EtOAc extract of the root

The EtOAc extract (69.1g) was adsorbed on silica gel and fractionated on a column by gravity elution using the following solvent system; 250ml of 100% hexane, then 250ml volumes of mixtures with DCM at the following ratios (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100%DCM. This was further followed by 500 ml volumes of DCM-EtOAc mixtures using similar ratios until 100% EtOAc. Finally, small portions of MeOH were introduced at 5% intervals in the mixture with EtOAc of up to 50% MeOH; (95:5), (90:10), (85:15), (80:20), (75:25), (70:30), (65:35), (60:40), (50:50).

Fractions collected were subjected to TLC analysis using DCM-MeOH (9.5:0.5) ratio. Pooling of fractions with similar TLC profiles was done. The combined fraction from vials 39-51 (800mg) upon drying was chromatographed on Sephadex LH-20 using the DCM-MeOH (50:50) ratio. Fractions collected were analyzed by TLC in DCM-MeOH (9:1). Preparative TLC using DCM-MeOH (9:1) gave compounds E2 (10.5mg) and **40** (14.9mg). Re-crystallization and subjecting other portions to sephadex column chromatography using DCM-MeOH (50:50), and further analysis of the fractions by TLC using DCM-MeOH (8:2) gave a yellow powder (321mg). This was further purified by preparative TLC using DCM-MeOH (8:2) ratio. Compounds E3 (11.2mg) and **39** (13.5mg) were obtained as yellow products. Pooling of combined fractions from vials 72-78 (400mg) and subjecting the collection to sephadex column chromatography using DCM-MeOH (7:3) as eluent; compounds E5 (12.9mg), a crystalline yellow powder and E6 (9.9mg), deep yellow crystals, were obtained.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Crude plant extracts

The finely ground *R. natalensis* leaves, stem and root barks were available as shown in table 3. Only the root bark and stem bark materials that were subjected to sequential extraction with the solvents hexane, DCM, EtOAc and finally MeOH.

Table 3. Initial weights of the plant collection.

Part of the plant	Weight in Kgs
Root bark	4.5
Stem bark	4.0
Leaves	2.0

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Some of the crude concentrated extracts deposited crystals on standing while others remained in the form of a paste. The yields obtained are presented in table 4. About 1g of each of these crude extracts was set aside for use in bioassays.

Table 4. Summary data for the crude yields.

Part of plant extracted	Hexane	DCM	Ethyl acetate	Methanol
Root bark	10.6g	13.9g	69.1g	85.2g
Stem bark	9.6g	16.6g	32.5g	59.6g

4.2 Bioassays

4.2.1 Bioassay of the crude extracts

Bioassay of the crude extracts against selected test organisms was carried out as described previously. The data obtained was used to determine MIC breakpoints. The antibacterial and antifungal activities of the extracts in terms of diameters of inhibition zones were reported as in Table 5

Table 5. Inhibition zones of micro-organisms.

EXTRACTS/ CONC 0.5mg/ml	**TEST MICROORGANISMS/INHIBITION ZONE IN MM**						
	Bacteria			**Fungi**			
	Sau	*Eco*	*Pseudo*	*Penici*	*TM*	*Can*	*Crypto*
1	7	6	9	7	6	6	0
2	18(0.5)	6	9	6	6	6	7
3	15(0.03)	7	15(0.03)	7	8	8	7
4	17(0.015)	6	14(0.015)	10(0.5)	10(0.5)	7	7
5	6	7	15(0.03)	11(0.5)	7	7	6
6	14(0.25)	6	10(0.5)	8	6	6	6
7	7	7	13(0.25)	8	8	7	7
8	6	6	12	6	6	7	6
9	20	18	17	-	-	-	-
10	-	-	-	18	19	15	16

Each value is the mean of three replications ± standard deviation in bracket measured in 6mm disc diameter.

KEY:

1. Root bark DCM
2. Root bark hexane
3. Stem bark EtOAC
4. Stem bark MeOH
5. Root bark EtOAC
6. Stem bark DCM
7. Root bark MeOH
8. Stem bark Hexane
9. Chloramphenicol standard
10. Fluconazole standard

Test Micro-organisms

- Sau-*Staphylococcus aureus* ATCC 25923
- E. coli-*Escherichia coli* ATCC 25922
- Pseudo- *Pseudomonas aureginosa* clinical isolate
- Can- *Candida albicans* ATCC 90028
- Crypto-*Cryptococcus neoformans* clinical isolate
- Penici-*Penicillium spp* environmental isolate
- TM-*Trichophyton mentagrophytes* clinical isolate

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The method of testing the efficacy of the extracts was done by introducing a sample into the middle of a bacteria-laden or fungi Petri-dish. Standards were also used in order to compare the results. A clear zone indicated bactericidal activity. The larger the diameter of the zone of inhibition, the higher the efficacy of that extract. The average data was determined and compared to the positive controls used. The inhibition zones against microbial growth are presented in Table 5. The three bacterial test organisms used showed a wide range of activity; *P. aureginosa* (gram negative bacterium) was the most sensitive to all the extracts. *S. aureus* (gram positive bacterium) that plays a considerable role in skin infections was highly sensitive to the root hexane extract with an inhibition zone of 18mm. It showed low sensitivity to the stem hexane extract, an indicator that the roots had greater potency. *E. coli* showed intermediate resistance to all the extracts in comparison to the standard chloramphenicol. Values greater than 10mm could be considered as reflecting moderate activity. However the differences in susceptibility between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacteria cell wall outer membrane appears to act as a barrier to many substances including antibiotics (Tortora *et al*., 2001).

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The choice of the fungus *C. albicans* was made due to the serious systemic infections, it causes including the opportunistic infections that are common with HIV positive patients. These various crude extracts demonstrated limited antifungal activity. The activity on fungi was much lower than that on bacteria. There was no activity recorded at all for the root DCM extract against *C. neoformans*. Moderate activities of the extracts against the fungi were recorded for *Penicillium ssp* at 11mm (0.5) and 10mm (0.5) as well as *T. mentagrophytes* at 10mm (0.5) which were clinical isolates. *C albicans* and *C. neoformans* displayed intermediate resistance.

However, the antifungal activity was far from comparable (as determined by the zones of inhibition) to that of fluconazole used as the standard. The observed biological activities could be due to the flavonoids present in the plant under study, as these compound types have been known to posses antibacterial and antifungal activity. Traditionally this plant is used in the treatment of oral candidiasis and athlete's foot. The results show that the methanol and ethyl acetate extracts have a greater potential as a source of antibacterial or antifungal agents of natural origin. Preliminary phytochemical studies have shown that the aerial and root parts of the genus *Rhus* contain biflavonoids responsible for these antimicrobial activities.

Table 6. Antimicrobial activity of isolated compounds

Name of extracts	Wt	Volume of DMSO used	Amount /disc	Antibacterial assay			Antifungal Assay		
				Sau	Eco	Pseudo	Can	TM	Mg
HM/RN/H/1(**41**)	5mg	400µl	20µl	9	0	0	0	0	0
HM/RN/E/2a(**40**)	5mg	400µl	20µl	17	0	0	0	0	0
HM/RN/E/4 (**39**)	5mg	400µl	20µl	21	0	0	0	0	0
Chloramphenicol	2mg	400µl	20µl	20	18	17	-	-	-
Fluconazole	2mg	400µl	20µl	-	-	-	15	12	14

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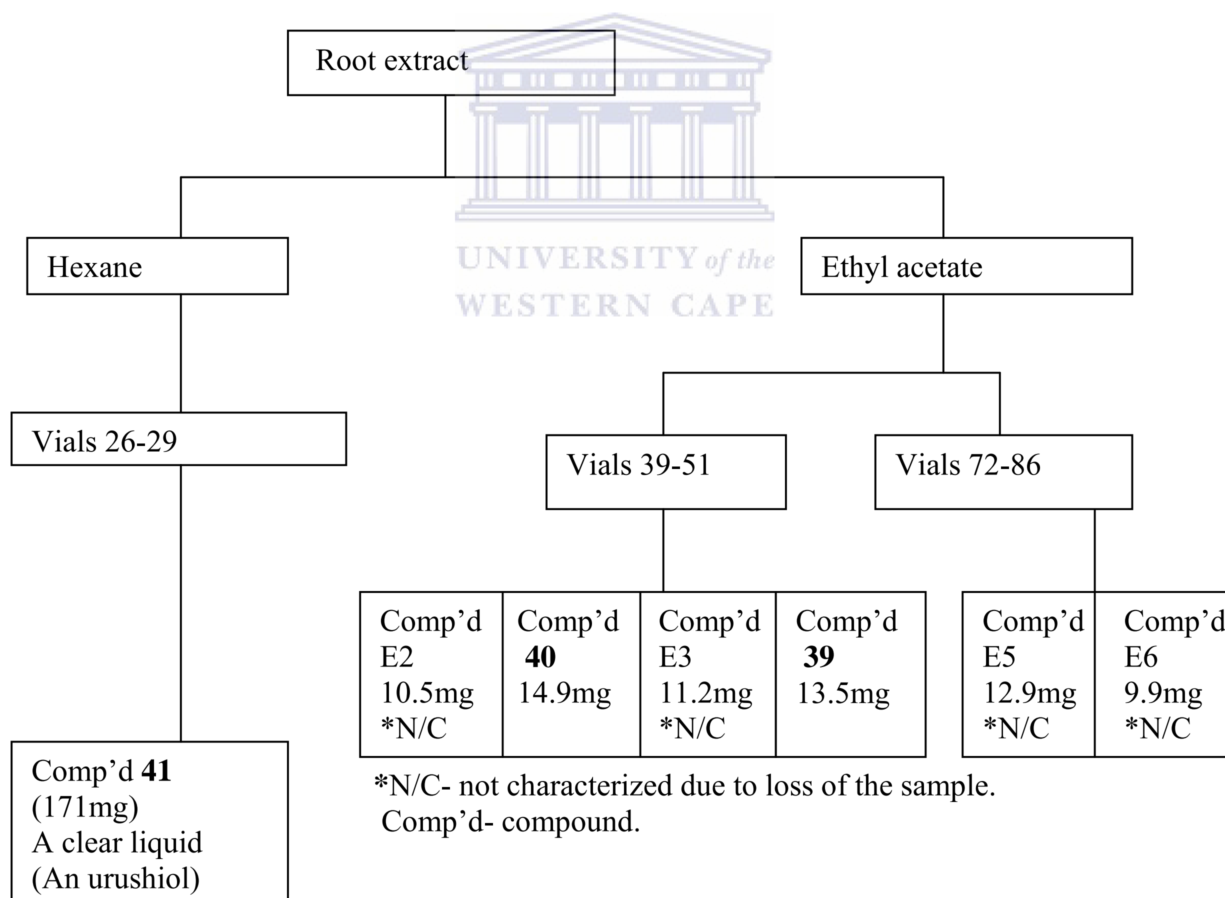
KEY:

1. Sau-*Staphylococcus aureus* ATCC 25923
2. Eco-*Escherichia coli* ATCC 25922
3. Pseudo-*Pseudomonas aureginosa* clinical isolate
4. Can-*Candida albicans* ATCC90028
5. Tm-*Trichophyton mentagrophytes* clinical isolate
6. Mg-*Microsporum gyseum* clinical isolate

The results in table 6 showed that the isolated compounds expressed some level of activity against the bacterium *S.aureus* in comparison to the chloramphenicol. Compound **39** showed a more pronounced zone of inhibition (21mm) against *S. aureus*. The compounds did not register any activity with *E. coli* and *P. aureginosa*. No activity on the fungi was recorded for the *C. albicans*, *T. mentagrophytes* or *M. gyseum* as compared to fluconazole standard. Comparing these results with those of the crude extracts may suggest that the potency of individual compounds is compromised by their isolation or that they were not used at sufficient concentrations. On the other hand, it is possible that the more potent compounds evaded the isolation process.



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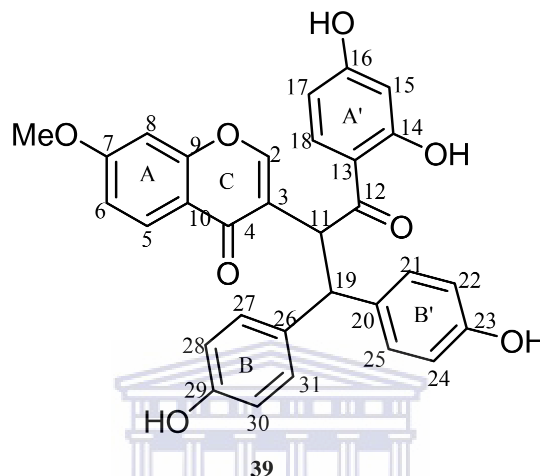
Scheme 3. A flow diagram for the compounds extracted.

4.3. 3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromen-4-one (39)

Compound (**39**), was obtained as an amorphous yellow powder, revealing a pseudo molecular ion $[M+H]^+$ at m/z 524, corresponding to the molecular formula $C_{31}H_{24}O_8$.



39

The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) (Table 7, Appendix 1a-k). The ^{1}H NMR spectrum assigned with the aid of the COSY experiment enabled the distinction of two 1, 4-disubstituted aromatic rings that were identical (B, B') and two 1, 2, 4-trisubstituted (A, A') aromatic rings. Long range interaction of two *meta*-protons H-6 (δ 6.44, *dd, J*= 2.1, 2.1Hz) and H-8 (δ 6.28, *d*, *J*= 2.1Hz) as well as H-15 (δ 6.69, *d*, *J*=2.1Hz) and H-17 (δ 6.83, *dd*, *J*=2.1, 2.1Hz) were confirmed by COSY. A typical singlet for proton H- 2 of the isoflavone moiety was observed at δ 8.20, (*s*) and an AB system with a bridge between H-11 (δ 6.02, *d*, *J*=12Hz) and H-19 (δ 4.67, *d*, *J*= 12.0 Hz) were detected. There was long range coupling between the methine proton at H-19 (δ 4.67, C- δ 54.5) and H-21/H-25 (δ 7.15, C-129.9) of the two aromatic identical rings B and B' which are interchangeable, hence the rings are related to that methine group by ^3J (**figure 1a**). The upfield part of the ^{1}H NMR spectrum showed one methoxy resonance at δ3.76.

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The location of this methoxy substituent in the A- ring was deduced on the basis of NOESY enhancement between proton H-6 (*δ* 6.4, *dd*, *J*=2.4, 2.4Hz) and H-8 (*δ* 6.28, *d*, *J*=2.4Hz). The methoxy group showed through-space interaction with two protons only, and these were meta-coupled to each other (**figure 1b**). Proton H-6 (*δ* 6.44, *dd*, *J*=2.4, 2.4Hz) showed a NOESY relationship with the proton H-5 (*δ* 8.21, *d*, *J*=3.9Hz). Another important NOESY was observed between H-2 (*δ* 8.20, *s*) and H-11 (*δ* 6.02, *d*, *J*= 12.0Hz) then H-19 (*δ* 4.67, *d*, *J*= 12Hz) with H-21/25 (*δ* 7.15, *d*, *J*= 9.0Hz). Proton H-21/25 (*δ* 7.15, *d*, *J*= 9.0Hz) with H22/24 (*δ* 6.57, *d*, *J*= 8.4Hz). Aromatic protons were observed at; *δ* 8.21 (*d*, *J*=3.9Hz, H-5), *δ* 6.44 (*dd*, *J*=2.4, 2.4Hz, H-6) and *δ* 6.28 (*d*, *J*=2.4Hz, H-8). The 2, 4-dihydroxyphenyl signals in ring A' were observed at *δ* 6.69 (*d*, *J*=2.1Hz, H-15), *δ* 6.83 (*dd*, *J*=2.1, 2.1Hz, H-17), and *δ* 7.87 (*d*, *J*=8.7 Hz, H-18). The protons H-11 and H-19 were found to be in a trans-conformation due to their *J* value of 12.0 Hz (Ghogomu *et al* 1987). The assignment of H-11 was confirmed from HMBC's with C-2 (*δ* 157.5), C-3 (*δ* 122.6), C-4(*δ* 177.2), C-12 (*δ* 205.4), and C-13(*δ* 135.9). Similarly H-19 was confirmed from HMBC's with C-11(*δ* 45.1), C-12(*δ* 205.4), and C-21/25 (*δ* 129.9).

^{13}C NMR spectrum showed the typical methine signals for C-11, C-19 at *δ* 45.1 and *δ* 54.5 respectively and this was confirmed by HMQC. There were two carbonyl signals at *δ*205.4 and *δ* 177.0. Other carbons bearing oxygen atoms appeared between *δ* 156.8 to *δ* 168.9. H-11 (*δ* 6.02, *d, J*= 12Hz) showed connectivity to the A, C isoflavone ring moiety. The B, B' identical rings were bound together to C-19 (*δ* 54.5). The carbonyl group at *δ* 205.4 was linked to ring A' and the methine at C-11. The ^{13}Cspectrum displayed signals for 31 carbons which were edited by DEPT into 1 methyl, 17 methines and 13 non-protonated carbons consisting of some two carbonyls and 9 oxygenated carbons. Detailed analysis of the HMBC and HMQC data enabled the complete assignment of the proton and carbon signals leading to the new structure **39**.

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Table7. ^{1}H NMR and ^{13}C NMR data of compound 39 in CD_3OD at 300MHz

Position	^{1}H NMR (*δ* multiplicity, *J*)	^{13}C NMR (*δ*)	COSY	HMQC	HMBC
2	8.20 (*s*)	157.5		8.20	
3		122.6			
4		177.2			
5	8.21 (*d*, *J*= 3.9)	134.1	H-6	8.21	C-4, C-7, C-9
6	6.44 (*dd* , *J*= 2.1, 2.1)	108.6	H-5,8	6.44	C-8, C-10
7		166.9			
8	6.28 (*d*, *J*= 2.1)	101.9	H-6	6.28	C-6, C-7, C-10
9		159.5			
10		115.2			
11	6.02 (*d*, *J*= 12.0)	45.1	H-19	6.02	C-2, C-3, C-4, C-12, C-13
12		205.4			
13		135.9			
14		168.2			
15	6.69 (*d*, *J*=2.1)	103.4	H-17	6.69	C-14, C-16, C-17
16		164.9			
17	6.83 (*dd*, *J*=2.1,2.1)	116.7	H-15,18	6.83	C-13, C-15
18	7.87 (*d*, *J*=8.7)	128.3	H-17	7.87	C-14, C-16
19	4.67(*d*, *J*= 12)	54.5	H-11,21	4.67	C-11, C-12, C-21/C-25
20		134.9			
21	7.15 (*d*, *J*= 9.0)	129.9	H-22	7.15	C-19, C-23, C-25
22	6.57 (*d*, *J*= 8.4)	116.2	H-21	6.57	C-20, C-23
23		156.8			
24	6.57 (*d*, *J*= 8.4)	116.3			
25	7.15 (*d*, *J*= 9.0)	129.9			
26		134.9			
27	7.15 (*d*, *J*= 9.	130.6			
28	6.57 (*d*, *J*=8.4)	116.2			
29		156.9			
30	6.57 (*d*, *J*=8.4)	116.3			
31	7.15 (*d*, *J*= 9.0)	130.6			
-OCH_3	3.76 *s*	56.2		3.76	

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B and B' values may be reversed

Figure 1a. Important HMBC's (full arrow) and COSY (half arrow) correlations for compound **39**

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Figure 1b. Important NOESY correlations for compound **39**

Such types of biflavonoids are relatively rare in nature, and 3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromen-4-one (Rhuschromone) is, to our knowledge, the first hydroxyphenyl chromen-4-one to be reported from *Rhus natalensis* and possibly within the entire *Rhus* genus. The other phenoxychromone to be isolated was chamaechromone (**42**) from the roots of *Stellera chamaejasme L* (Thymelaeaceae) (Masatake *et al*., 1984). Subsequently, R. Ghogomu *et al*., (1987) isolated Lophirone A (**43**), a phenoxychromone from the stem bark of *Lophira lanceolata* (Ochnaceae) and Calodenone (**44**) (Messanga, et al., 1992) isolated from the stem bark of *Ochna calodendron* (Ochnaceae). The reported biological activity of the latter did not include any significant antibacterial activity. On the contrary Rhuschromone was found to possess a relatively high activity as an antimicrobial zone of inhibition (21mm). It was speculated that the activity observed may be associated with the unique positioning of the 7-OCH_3 group in ring A. From a biogenetic point of view, Rhuschromone differs from known biflavonoids in having involved an aryl shift of ring B from one flavone unit to the next resulting to an isoflavone. The term 'isobiflavonoid' would best suit this class of compounds.

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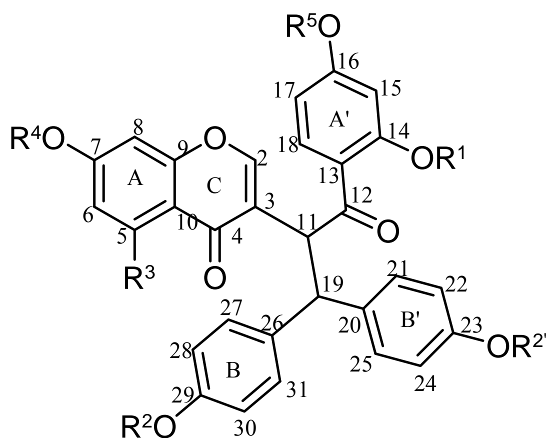
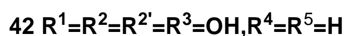
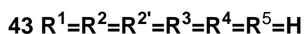
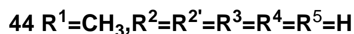


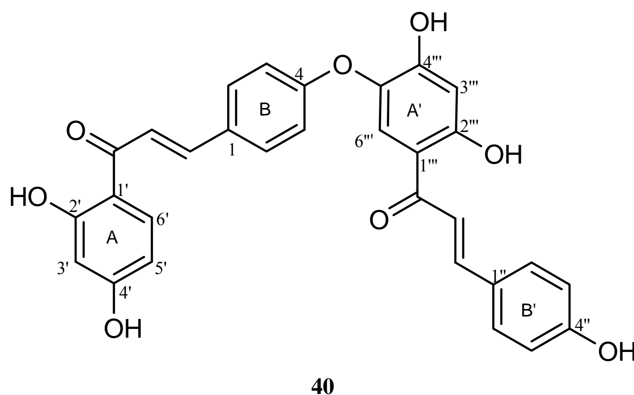






4.4. 2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (40)

Compound **40**, was obtained as a yellow solid. MS indicated an ion at m/z 510.1212 (M+, calculated 510.1314) consistent with a molecular formula of $C_{30}H_{22}O_8$.



40

The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) (Table 8, Appendix 2a-i). The ^{1}H NMR spectrum (300MHz, CD_3OD-d_6) showed signals of a three ABC proton spin system observed at H-3'(δ 6.31, *d, J*= 2.4 Hz) that had a long range meta-coupling with signal at H-5' (δ 6.42, *dd, J*=9.0,2.4Hz), and H-6' (δ 8.01,*d*, *J*=9.0 Hz) characteristic of a 1,2,4-trisubstituted benzene unit. There were two singlet signals integrating for one proton each observed at H-6''' (δ 7.94, *s*) and H-3''' (δ 6.61, *s*). Two pairs of the AA'BB'-type signals, where one pair was at H-2/6 (δ 7.75, *d, J*=8.41Hz) and H-3/5 (δ 6.96, *d, J*=8.8 Hz) and the other pair at H-2''/6'' (δ 7.67, *d, J*=8.4Hz) and H-3''/5'' (δ 6.87, *d, J*=8.7Hz), were observed for the two *para*-substituted phenolic moieties. The given data strongly suggested compound **40** to be a bichalcone. Signals of *trans*-alkene protons of the bichalcone system were not clearly observed due to weak resolution of some peaks. Multiplets at H-α (δ 7.57 *m*) and H-α' (δ 7.68 *m*) for hydrogen protons connected to α and α' carbons (δ_{CH} 118.3) could only be tentatively assigned.

The ^{13}C NMR spectrum of compound **40** displayed signals for 30 carbon atoms which were edited by DEPT into 17 methines and 13 non-protonated carbons amongst which were two carbonyls and seven oxygenated carbons. The HMBC spectrum showed the chemical shift of a carbonyl group whose carbon signal had ^{3}J interactions with the H-6''' singlet at (δ 7.94 *s*) and the H-β' signal at (δ 7.85 *d*, J=15.3 Hz) and this was assigned to with the upfield C=O' signal at δ_C 192.4. Likewise, the second H-β signal at (δ 7.83 *d*, J=15.3Hz) was correlated to the other C=O resonance at δ_C 192.0 (**figure 2a**). The presence of the two C=O at δ 192.0 and δ 192.4, allowed the proposal of the bichalcone structure (Table 8). NOESY irradiation gave some important information in establishing the inter-chalcone linkage in that the signals belonging to H-5' and H-6' of ring A of one of these chalcone moieties had no corresponding correlation with the signals for H-3''' and H-6''' of ring A' of the other. The linkage was determined to be at C-4 (δ 158.4) and C-5''' (δ 127.3) forming the C-4-*O*-5'''connection. The ^{13}C (DEPT), COSY and HMBC spectra results were in agreement with those of a chalcone reported on, to a great extent in the literature namely, Rhuschalcone II (Ladislaus *et al*., 2003) from the root bark of *Rhus pyroides*. The accompanying NMR data facilitated the proposal of a similar bichalcone as a constituent of the root bark of *Rhus natalensis*. The tentative structure of the chalcone was therefore deduced to be the 2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (**40**)

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Table 8. ^{1}H NMR and ^{13}C NMR data of compound 40 in CD_3OD at 300MHz

Position	^{1}H NMR (δ multiplicity, J)	^{13}C NMR (δ)	COSY	HMQC	HMBC
1		129.9			
2	7.75 (*d*, *J*= 8.4)	128.8	H-3	7.75	C-β, C-3/5, C-4
3	6.96 (*d*, *J*= 8.7)	115.6	H-2	6.96	C-1, C-2/6, C-4
4		158.4			
5	6.96 (*d*, *J*= 8.7)	115.6	H-6	6.96	C-1, C-2/6, C-4
6	7.75 (*d*, *J*= 8.4)	130.6	H-5	7.75	C-β, C-3/5, C-4
1’		165.2			
2’		113.9			
3’	6.31 (*d*, *J*= 2.4)	102.4	H-5’	6.31	C-1’, C-2’, C-5’
4’		166.2			
5’	6.42 (*dd*, *J*= 9.0,2.4)	108.1	H-3’, H-6’	6.42	C-1’, C-3’
6’	8.01 (*d*, *J*= 9.0)	132.1	H-5’	8.01	C=O, C-2’, C-4’,
1’’		129.1			
2’’	7.67 (*d*, *J*= 8.4)	130.2	H-3’’	7.67	C-β’, C-3’’/5’’, C-4’’
3’’	6.87 (*d*, *J*= 8.7)	116.7	H-2’’	6.87	C-1’’, C-2’’/6’’
4’’		160.3			
5’’	6.87 (*d*, *J*= 8.7)	115.3	H-6’’	6.87	C-1’’, C-2’’/6’’
6’’	7.67 (*d*, *J*= 8.4)	128.0	H-5’’	7.67	C-β’, C-3’’/5’’C-4’’
1’’’		113.3			
2’’’		160.4			
3’’’	6.61 (*s*)	107.8		6.61	C-1’’’, C-5’’’
4’’’		157.9			
5’’’		127.3			
6’’’	7.94 (*s*)	126.4		7.94	C-2’’’, C-4’’’, C=O
α	7.57 (*m*)	118.3	H-β	7.57	C-1, C-β
β	7.83 (*d*, *J*=15.3)	143.6	H-α	7.83	C-1, C-α, C2/6, C=O
α’	7.68 (*m*)	118.3	H-β’	7.68	C-1’’, C-β’
β’	7.85 (*d*, *J*=15.3)	144.8	H-α’	7.85	C-α’, C=O’, C-2’’/6’’
C=O		192.0			
C=O’		192.4			

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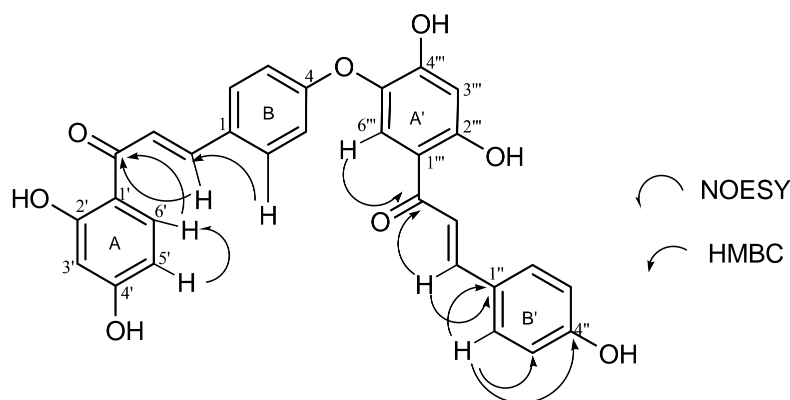

Figure 2a.Important HMBC correlations (full arrow) and NOESY (half arrow) for compound **40**

This is another biflavonoid that is of a relatively rare bichalcone class in nature, and is being reported for the first time as isolated from *Rhus natalensis*. On the part of the biological activity, 2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (**40**), exhibited significant antibacterial activity (Table 6). It showed relative activity as an antimicrobial, zone of inhibition (17mm) though much lower than the standard chloramphenicol on *Staphylococcus aureus*. Reported biological activity of rhuschalcone II was carried out for *in vitro* primary cytotoxic screening using human tumor cell lines (Ladislaus, *et al*., 2003). Varying degrees of cytotoxicity activity were recorded.

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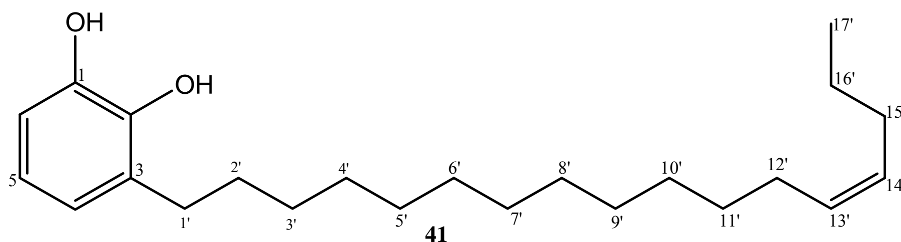
4.5. 3-((Z)-heptadec-13-enyl) benzene-1, 2-diol (41)

Compound **41,** was obtained as a clear liquid, revealing a pseudo molecular ion $[M+H]^+$ at m/z 347.2945, corresponding to the molecular formula $C_{23}H_{38}O_2$ (calculated 347.2945).



The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY and HMBC) (Table 9, Appendix 3a-h). The ^{1}H and ^{13}C NMR showed the typical pattern of a 5-alkenyl substituted catechol with a linear 17-carbon side chain containing one double bond at δ 5.33 (H-13'/H-14'), δ130.8 and 130.2 (C-13'/ C-14'), 14 methylene groups between δ_C 23.7 and δ_C 36.9 and one methyl group resonating at H-17' (δ 0.89 *t*) and C-17' (δ 14.4) (Valcic *et al.*, 2002; Jin *et al.*, 2006). The existence of a phenol ring originating from catechol could be identified with the signals between 6ppm and 7 ppm. Six aromatic carbons at C-1 (δ 158.3), C-2 (δ 145.6), C-3 (δ 130.9), C-4 (δ 116.2), C-5 δ 120 and C-6 (δ 113.5) as well as three proton signals at H-4 (δ 6.63, *d*, *J*=7.2), H-5 (δ 7.05 *t*) and H-6 (δ 6.57, *d, J*=7.8) indicating a catechol moiety in compound **41**. δ_H (600MHz, CD_3OD), 0.89 (3H, *t*, Me), 1.29 (18H, $(CH_2)_9$), 2.03 (4H, *m*, $2CH_2CH=$), 2.52 (2H, *t*, CH_2Ar), 5.33 (2H, *dd*, CH=CH, *J*=5.4,9.6Hz), 6.63 (*d, J*=7.2, H-4), 6.57 (*d*, *J*=7.8, H-6) and 7.05 (*t*, H-5).

The first four positions 1'-4' of the side chain were assigned by ^{1}H, ^{13}C (HMBC)-long range correlations, COSY and NOESY (**Figure 3a** and 3**b**).There was long range coupling between the proton H-14' (δ5.33) and H-12' (δ2.03).

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The ^{13}C peak selection offered that possibility of determining HSQC and the HMBC spectra for the crowded region of the methylene carbons of compound **41** (δ10-40ppm) with high resolution. HMBC correlations that are there between the protons of the terminal methyl group at δ0.89 and the carbons at δ_C23.7 and δ_C30.8 assisted in establishing the C-16' and C-15' positions respectively. There was a $^2J^1H$-^{13}C- connectivity between the latter carbon and the proton signal of H-14' at δ5.33 which shows a ^3J HMBC correlation with the allylic C-12' (δ 28.1) that assisted locate the double bond at C-13' (δ 130.8). This assignment was confirmed by HMBC correlation between both the olefinic resonance H-14' (δ5.33) and C-16'at δ23.7 and the latter with the methyl group H-17' (δ 0.89). Homo-nuclear decoupling experiments on the allylic protons as described in previous literature enabled establish the position of the double bond (Gonzales *et al*, 1996) and determination of the Z-configuration (J=9.6).

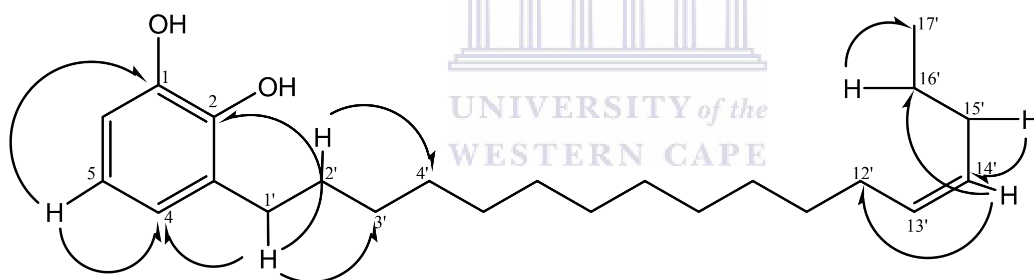

Figure 3a. Important HMBC

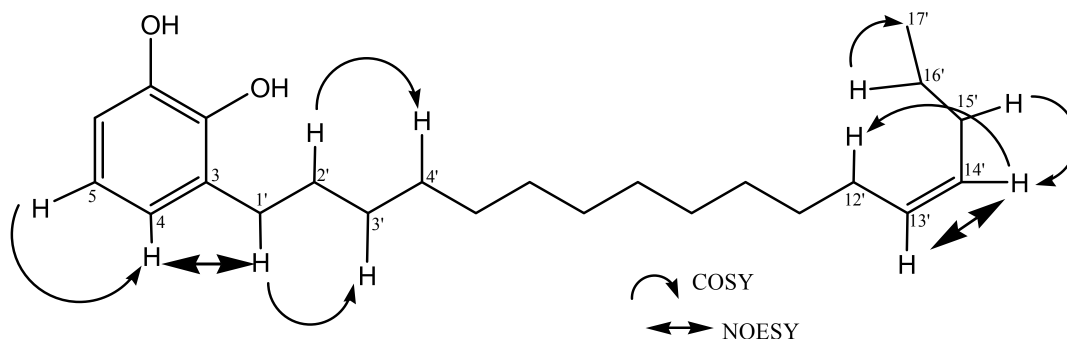

Figure 3b. Important COSY and NOESY (double arrow)

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Table 9. ^{1}H NMR and ^{13}C NMR data of compound 41 in CD_3OD at 600MHz

Position	^{1}H NMR δ, multiplicity, *J*	^{13}CNMR δ (ppm)	HSQC	HMBC
1		158.3		
2		145.6		C-4, C-6
3		130.9		
4	6.63 *d, J*=7.2	116.2	6.63	C-2, C-6
5	7.05 t	120.7	7.05	C-1, C-3
6	6.57 *d*, *J*=7.8	113.5	6.57	C-2, C-4
1’	2.52 *t*	36.9	2.52	C-2. C-3, C-4, C-2’
2’	1.58, *q*	33.0	1.58	C-1’, C-3
3’	1.29 *ov*	30.3		
4’	1.29 *ov*	30.4		
5’	1.29 *ov*	30.6		
6’	1.29 *ov*	30.7		
7’	1.29 *ov*	30.7		
8’	1.29 *ov*	30.7		
9’	1.29 *ov*	30.7		
10’	1.29 *ov*	30.7		
11’	1.29 *ov*	32.6		
12’	2.03 *m*	28.1		C-11’, C-13’, C-14
13’	5.33 *dd, J*=5.4, 9.6	130.8	5.33	C-12’, C-15
14’	5.33 *dd, J*=5.4, 9.6	130.2	5.33	C-12, C-15, C-16
15’	2.03 *m*	30.8		
16’	1.37 *ov*	23.7		
17’	0.89, *t*	14.4		C-15’, C-16’

Ov- overlapped by other signals

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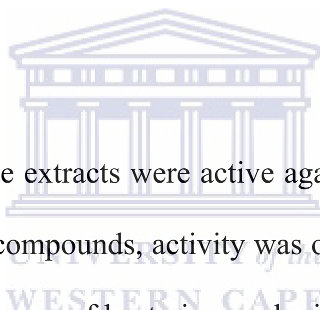
3-((Z)-heptadec-13-enyl) benzene-1, 2-diol (**41**) is a major constituent of the resulting monomeric urushiols extracted from the roots of *Rhus natalensis*. In addition to other compounds extracted from the plant, this catechol is the only one of its kind obtained from the hexane extract. Biological activity of the compound did not exhibit any major antibacterial activity. It was found to possess only a moderate to low activity as an antimicrobial zone of inhibition (9 mm) as compared to the standard chloramphenicol. Previous literature reports that meta-substituted catechol functionalities are present in a large number of natural compounds with beneficial effects on the human body. They have been used in the production of some anti-hypertensive pharmaceuticals (Kieboom *et al*, 2001). Compound **41** is one of such natural products.



In this study, a number of the crude extracts were active against both Gram-positive and Gram-negative bacteria, but for the pure compounds, activity was observed only against Gram-positive bacteria. The activity against both types of bacteria may be indicative of the presence of a broad spectrum of antibiotics or simply general metabolic toxins in these extracts. The antibacterial activity of biflavonoids against both Gram-positive and Gram-negative bacteria has been reported. Activity against bacteria like *S. aureus* was demonstrated mainly by compounds that contained hydroxyl groups in ring B. Biflavonoid compounds having two to three hydroxyl groups in rings A or B were more active against such Gram-positive bacteria. It has been documented (Grosvenor, *et al*., 1995; Martinez, *et al*., 1996; Chariandy, *et al*., 1999; Stickler and King, 1992) that *S. aureus* is one of the bacteria most susceptible to plant extracts. Other biflavonoids are known to be active against antibiotic-resistant bacteria.

Such bacteria are inhibited by those compounds carrying hydroxyl groups in ring B, for example quercetin (**27**), and kaempherol (**28**).

The results of the present investigation have clearly indicated that the antibacterial and antifungal activity vary with the different plant parts (Hoffman *et al*., 1993). This study is a preliminary evaluation of antimicrobial activity of *R. natalensis*. It indicates that the plant has the potential to generate novel metabolites. The crude extracts demonstrating anti-candidal activity could lead to the discovery of novel anti-candidal agents. The plant has demonstrated a broad spectrum of activity that may help discover new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases. These findings provide yet another example of the validity of making more advantageous structural modifications of seemingly rather thoroughly examined anti-infective drug in further researches. Compound **39** with the name Rhuschromone, is a new biflavonoid that is naturally isolated and with high antibacterial activity.

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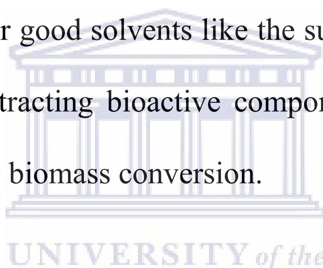
CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The research efforts on *Rhus* extracts indicate a promising potential for the plant family to provide renewable bio-products with the following desirable bioactivities: antimicrobial, antifungal, antimalarial, antiviral, antifibrogenic, antiinflammatory, antimutagenic, antioxidant, antithrombin, antitumorigenic, cytotoxic, hypoglycaemic, and leukopenic. More bioactive components can be extracted from the plant material using environmentally benign solvents like methanol, and water that allow for both food and industrial end-uses. A substantial opportunity exists to investigate the use of other good solvents like the sub-critical and super-critical liquids, ionic liquids among others for extracting bioactive components plus other phytochemicals in processing the residue for complete biomass conversion.



This study validates and documents, in a systematic way, the antimicrobial properties of the genus *Rhus* used for many years by many people of the world. It also provides valuable information for further phytochemical isolation and characterization studies of active compounds, necessary for the development of new drugs. At present the search for compounds active against antibiotic-resistant strains of bacteria is continuing among the flavonoids, compounds which are non-toxic or have low toxicity (Narayana, *et al*., 2001). However, as this overview demonstrates, the previous work had focused on only a few members of this large plant family. In addition, not all of the species studied to date have been fully characterized for potential bioactivities.

Thus, there remains a significant research gap spanning the range from lead chemical discovery through process development and optimization in order to better understand the full bioactive potential of the *Rhus* genus as part of the global green technology based bio-product and bio-process research programs.

5.2 Recommendations

- The results obtained from the parts of *Rhus natalensis* plant should be considered for further studies aimed at isolating and identifying more active compounds having discovered the antimicrobial activities observed in this research work.
- Strong activity against Gram-positive bacteria particularly by the compounds extracted should make this plant a target for further studies aimed at discovering new and more potent antimicrobial drugs which are effective.
- Further research could be carried out to screen for any immune boosting in the human body especially against viral attacks by drugs from this plant.
- *In vivo* activities should be initiated as soon as *in vitro* results have provided clear evidence for proof of activity.
- The dispensary details should be determined in order to provide the proper dosage for correct administration of such drugs.
- The Government should offer support to sustainable harvesting of these medicinal plants and put systems in place enabling the marketing of such drugs obtained from this kind of plants.
- Conservation measures of such plants, having proven antimicrobial activity, are very important. This is by conserving all the major forests from extinction.

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- Further studies should be extended to other related species of the plant from the different geographical regions to evaluate their activity against microbials.

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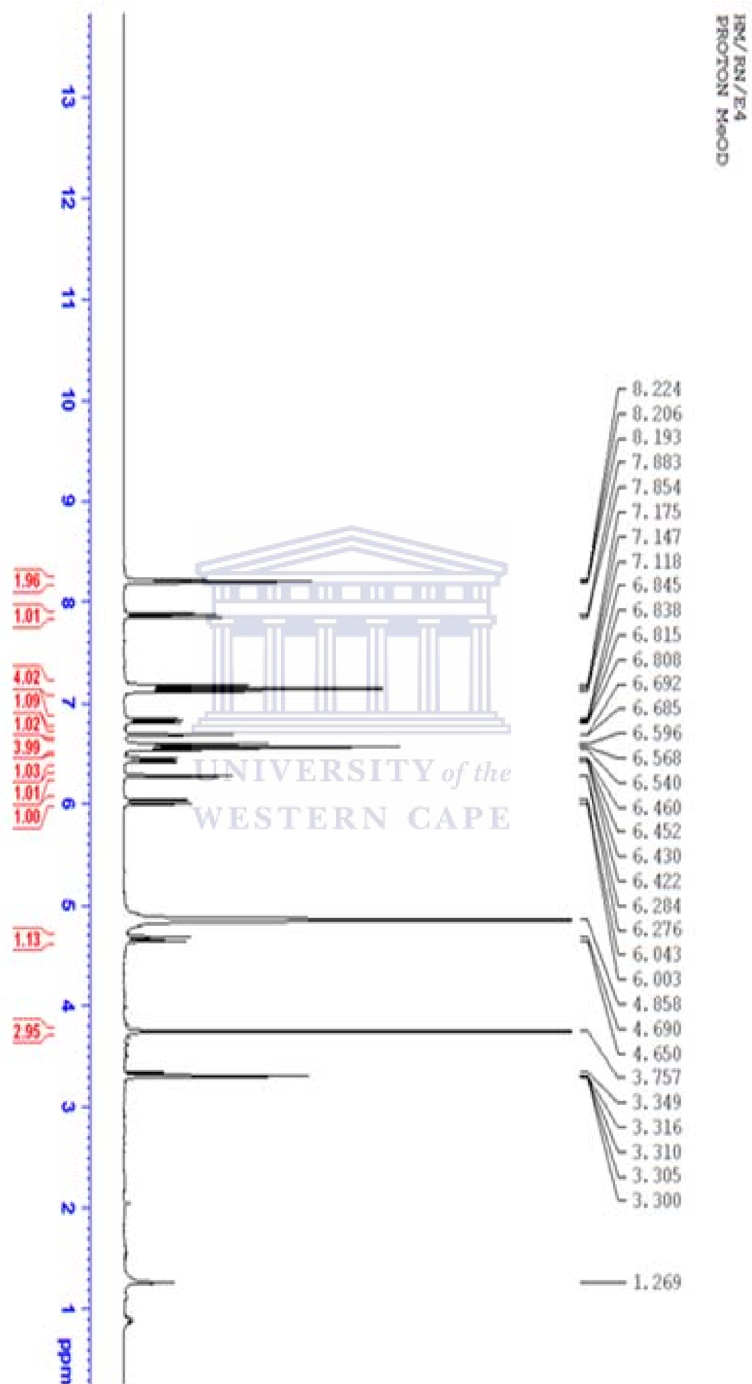
APPENDIX

Appendix 1a ^{1}H NMR of **39**



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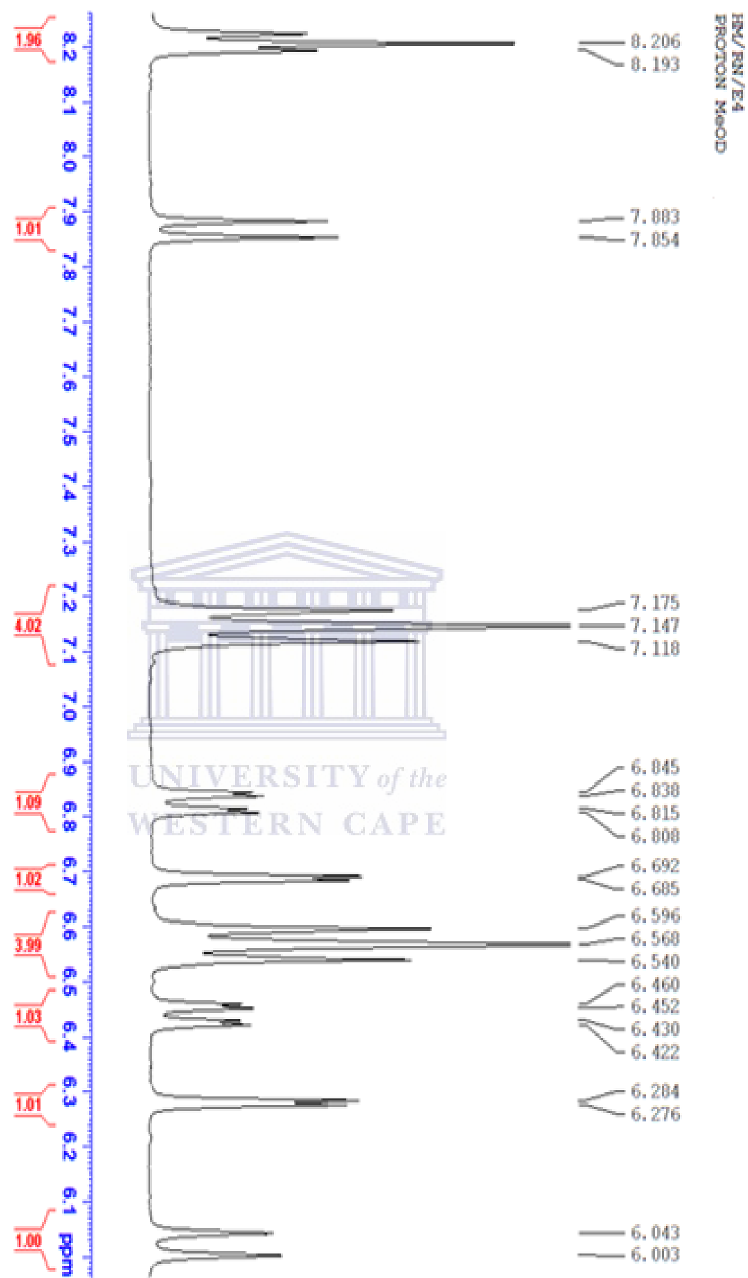
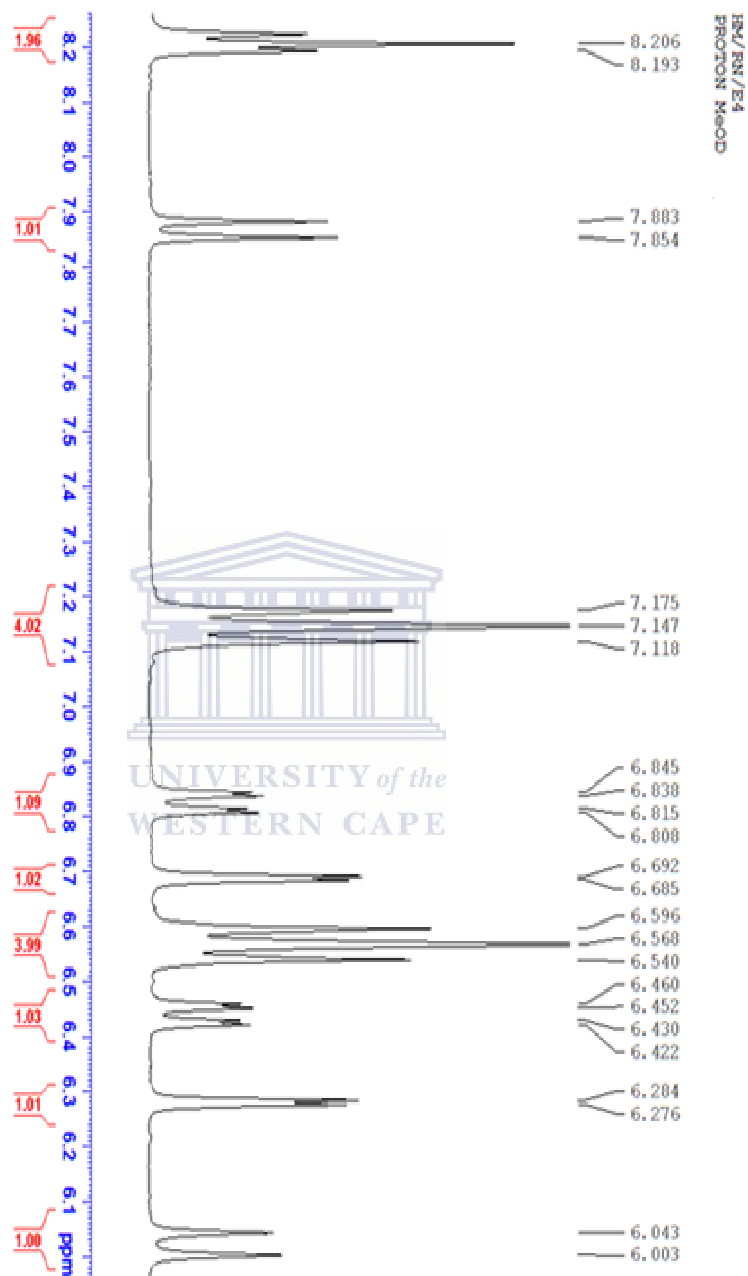
Appendix 1b ^{1}H NMR of **39**



Appendix 1c ^{1}H NMR of **39**



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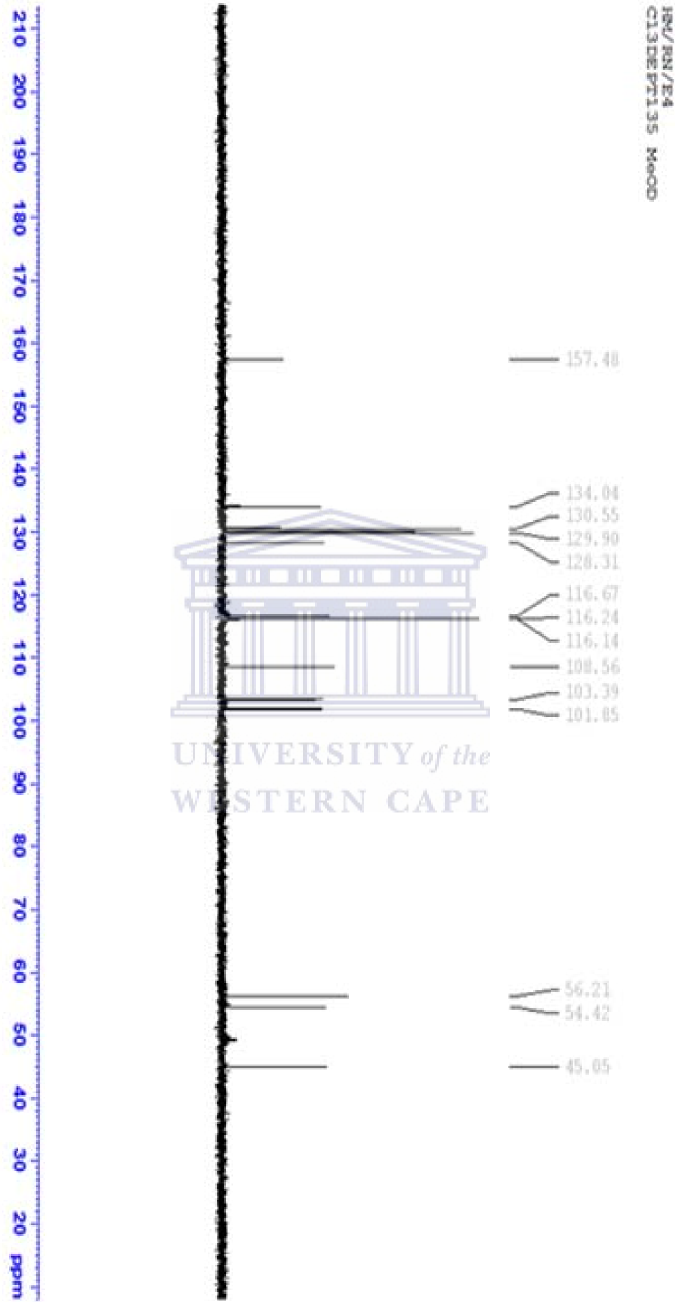
Appendix 1d DEPT 135 of **39**



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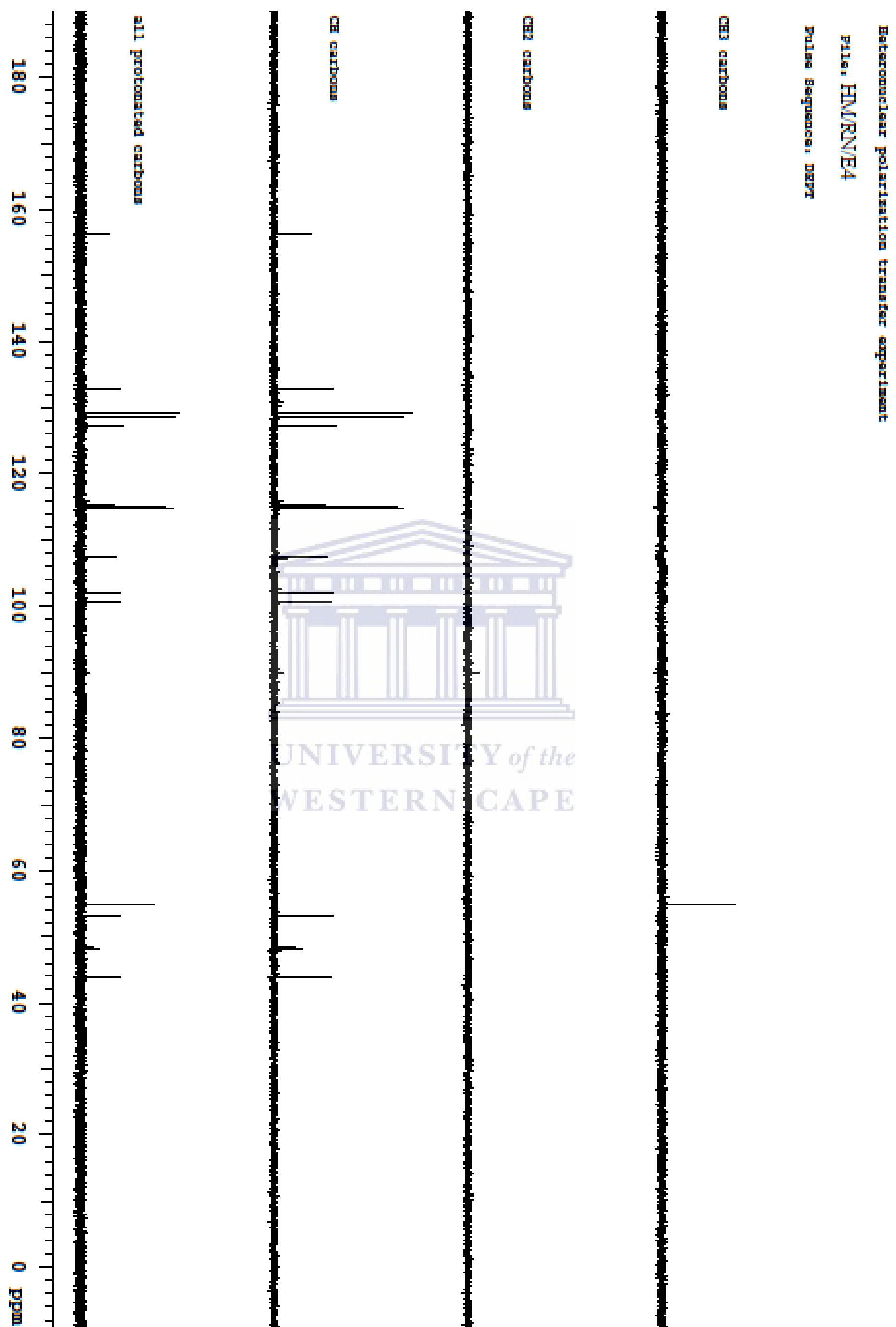
Appendix 1e DEPT of **39**



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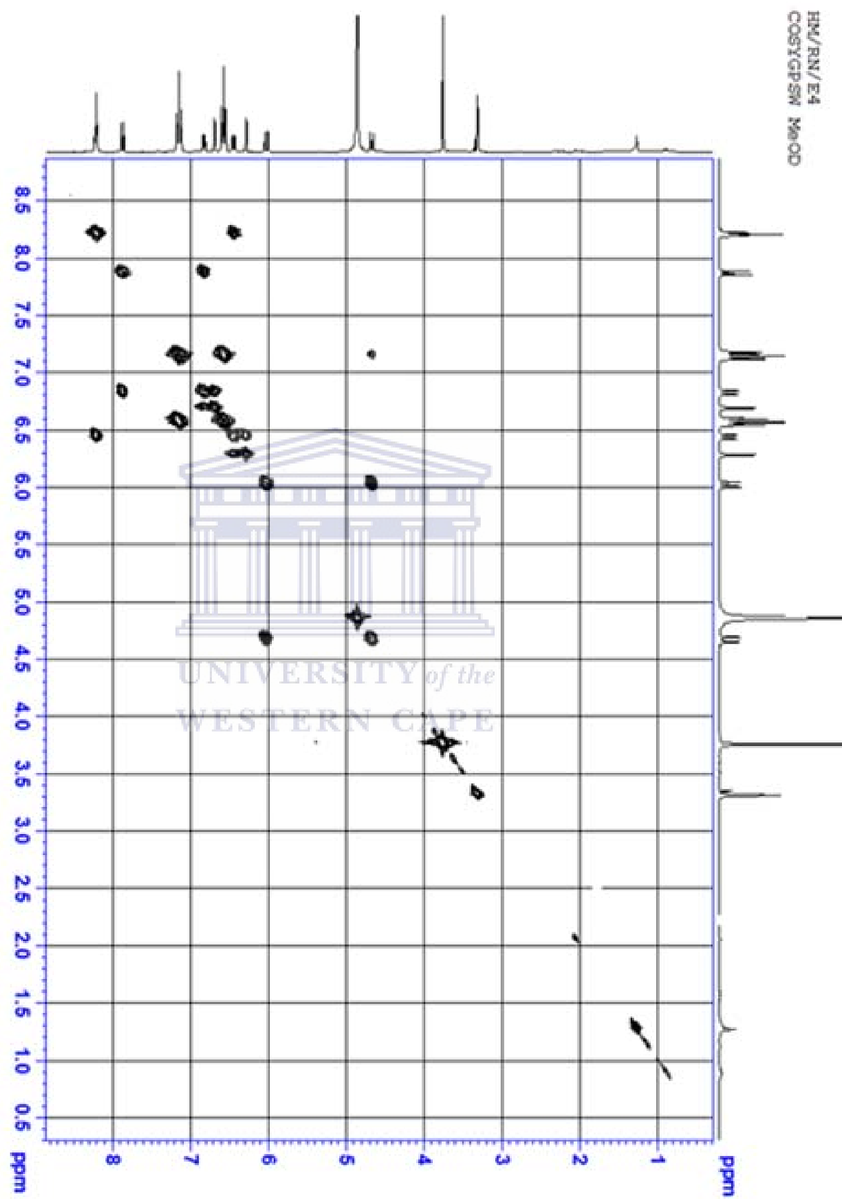
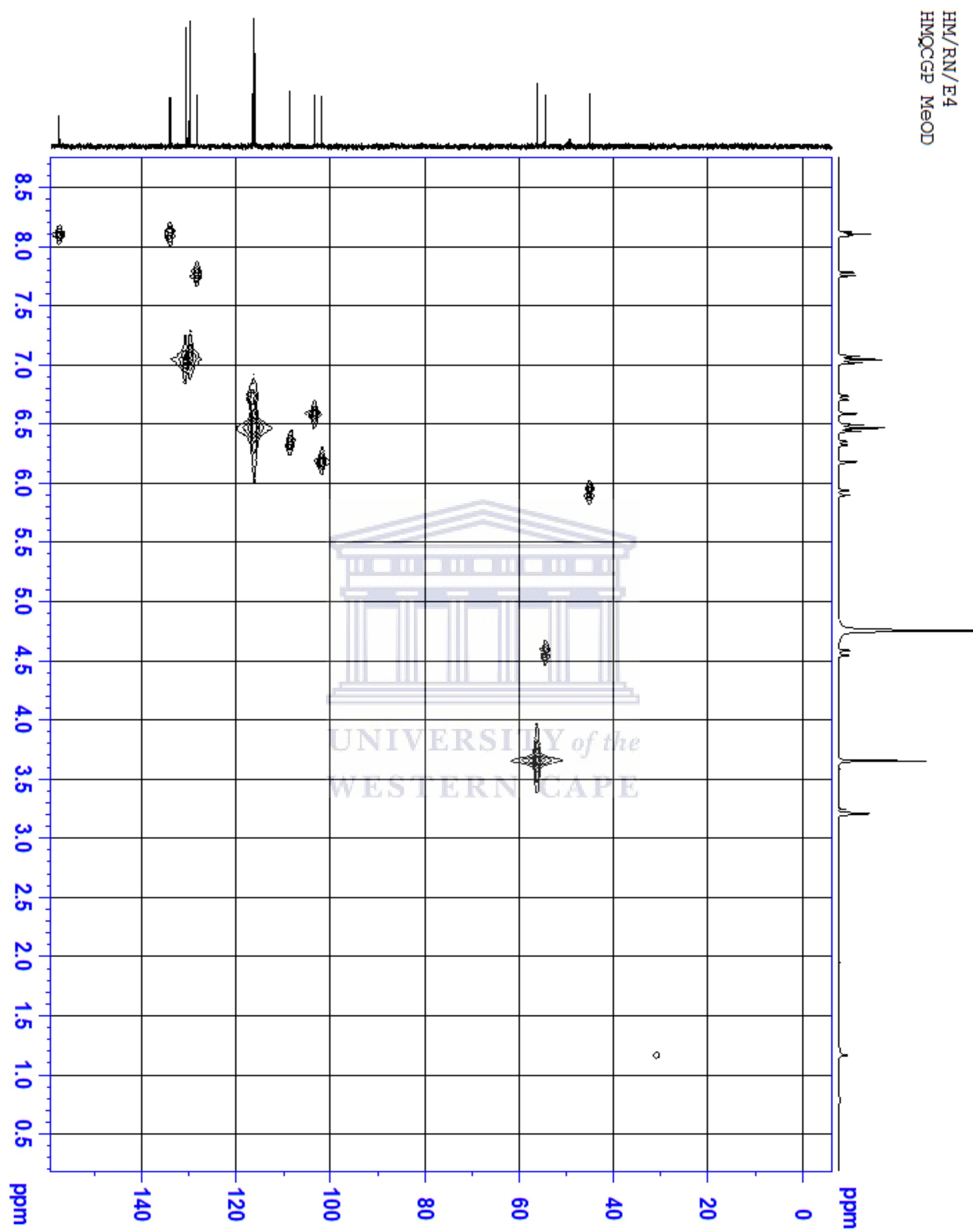
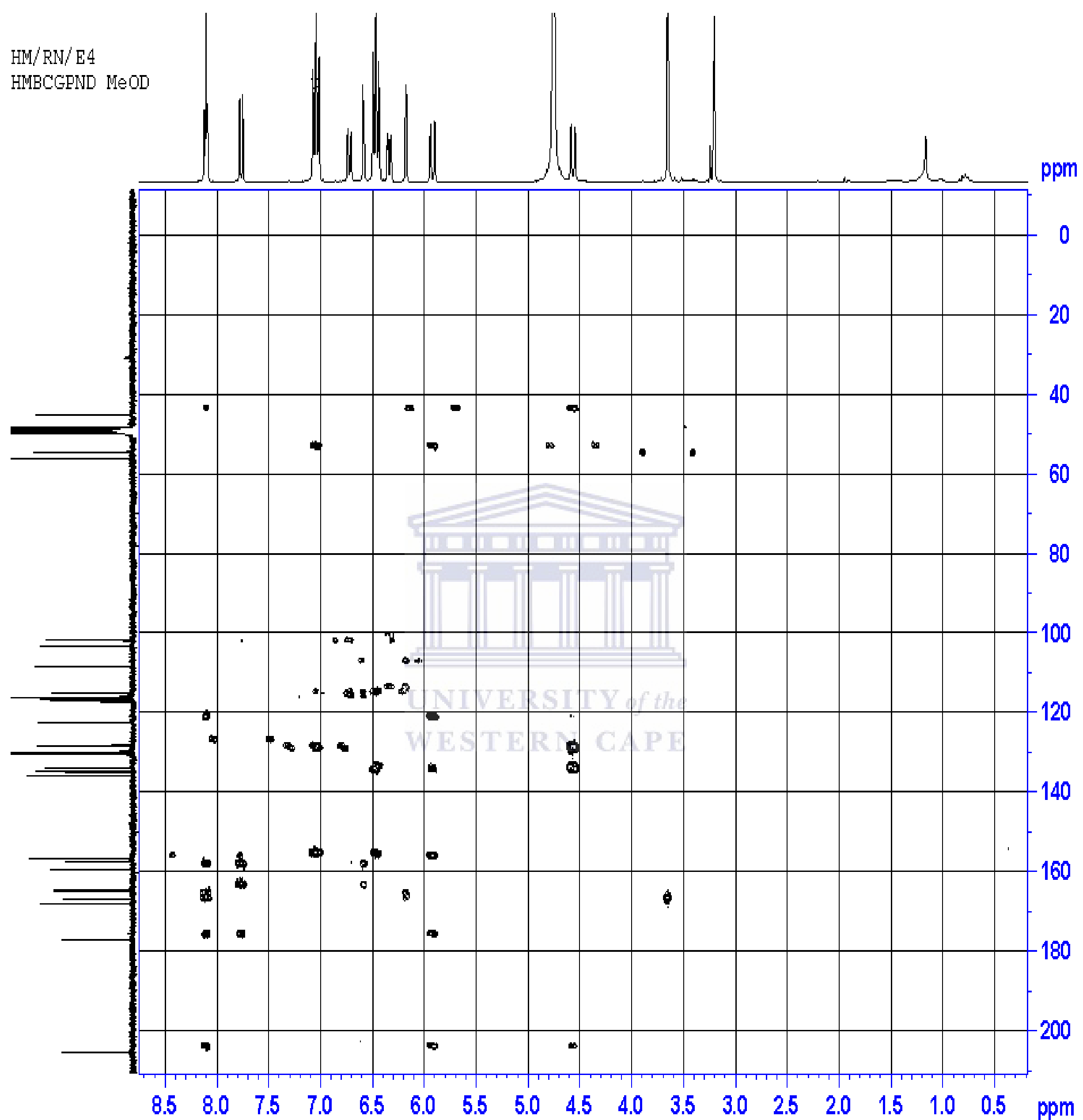
Appendix 1f COSY of **39**



Appendix 1g HMQC of **39**



Appendix 1h HMBC of **39**



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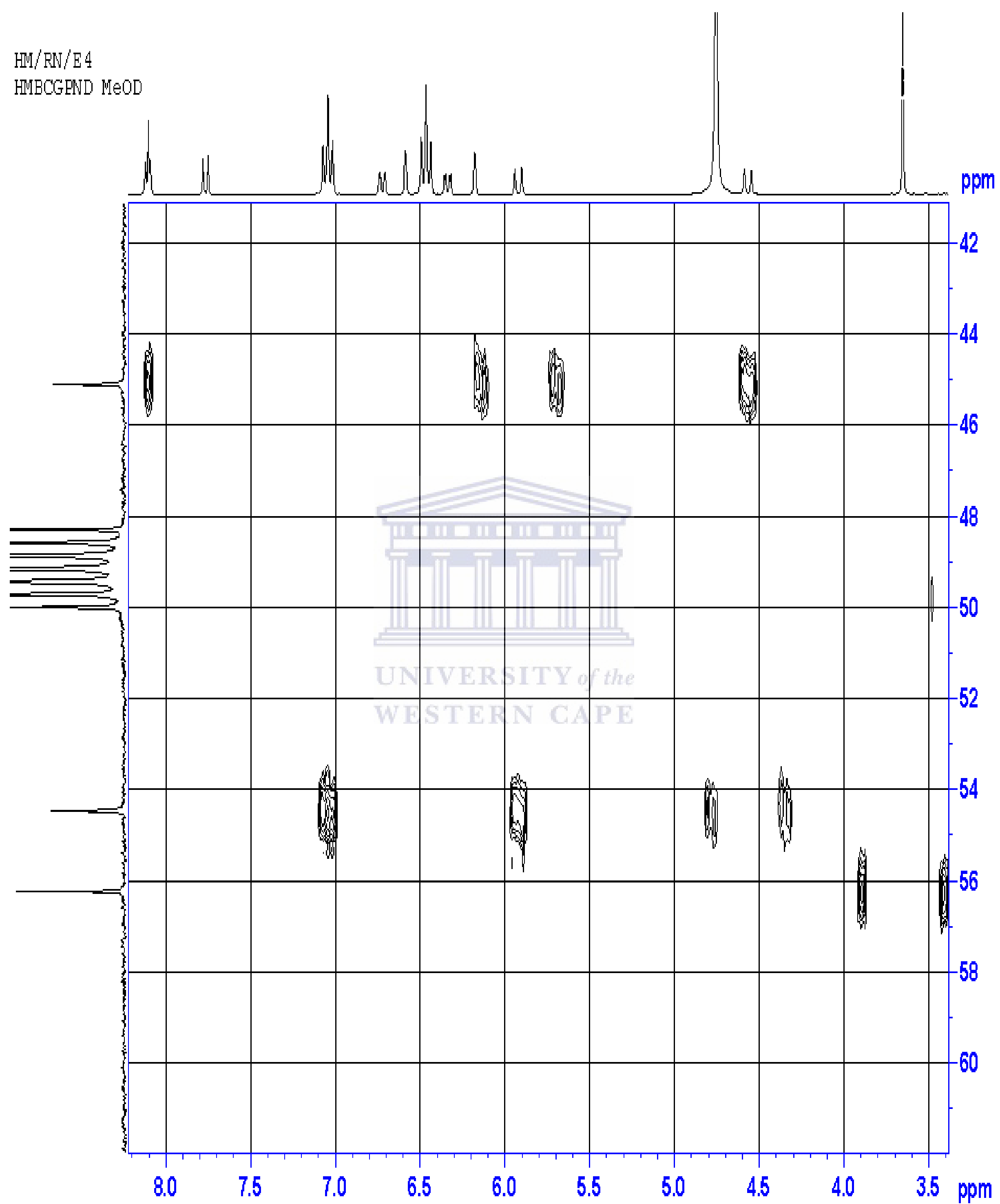
Appendix 1i HMBC of **39**



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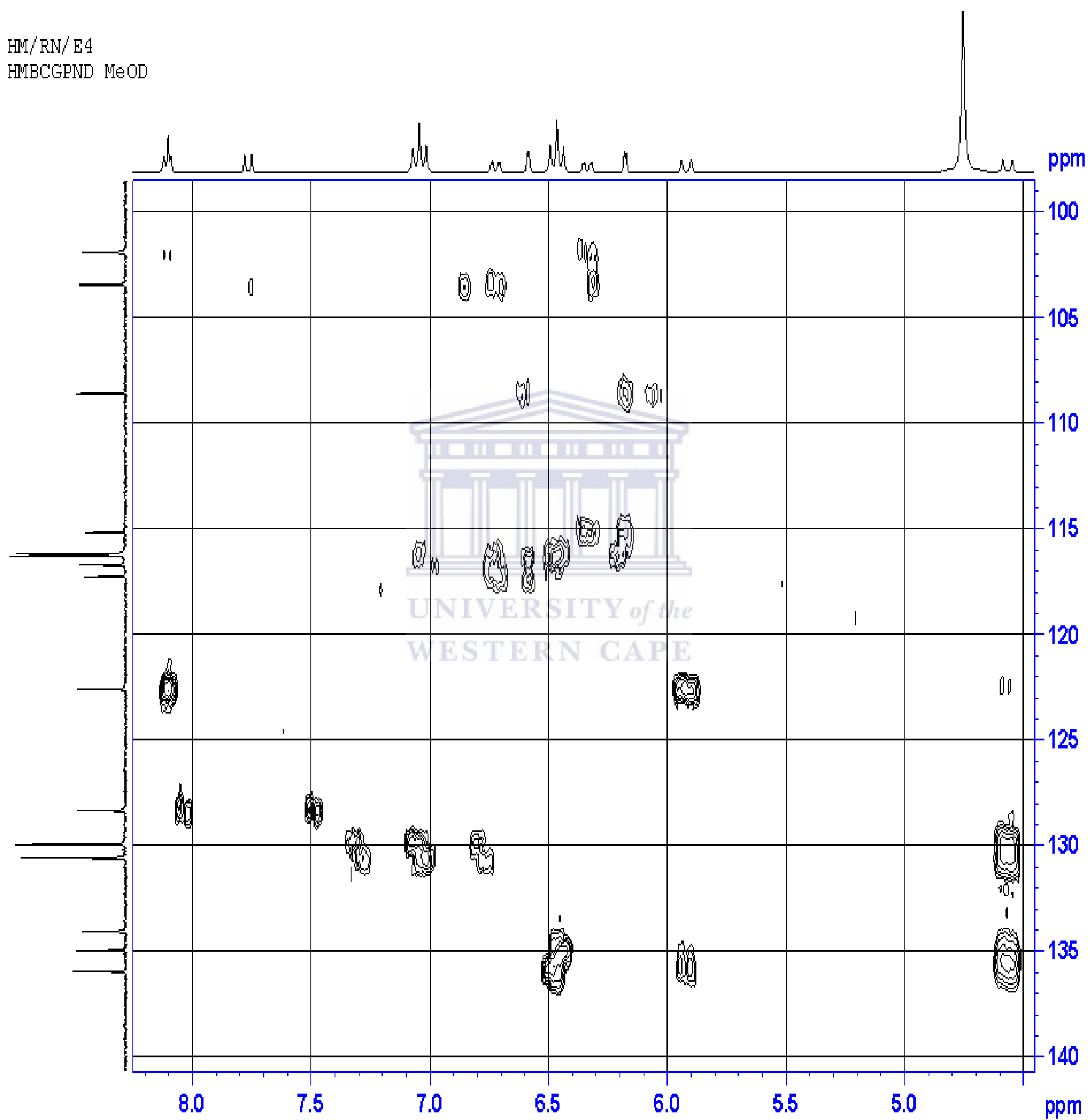
Appendix 1j HMBC of **39**



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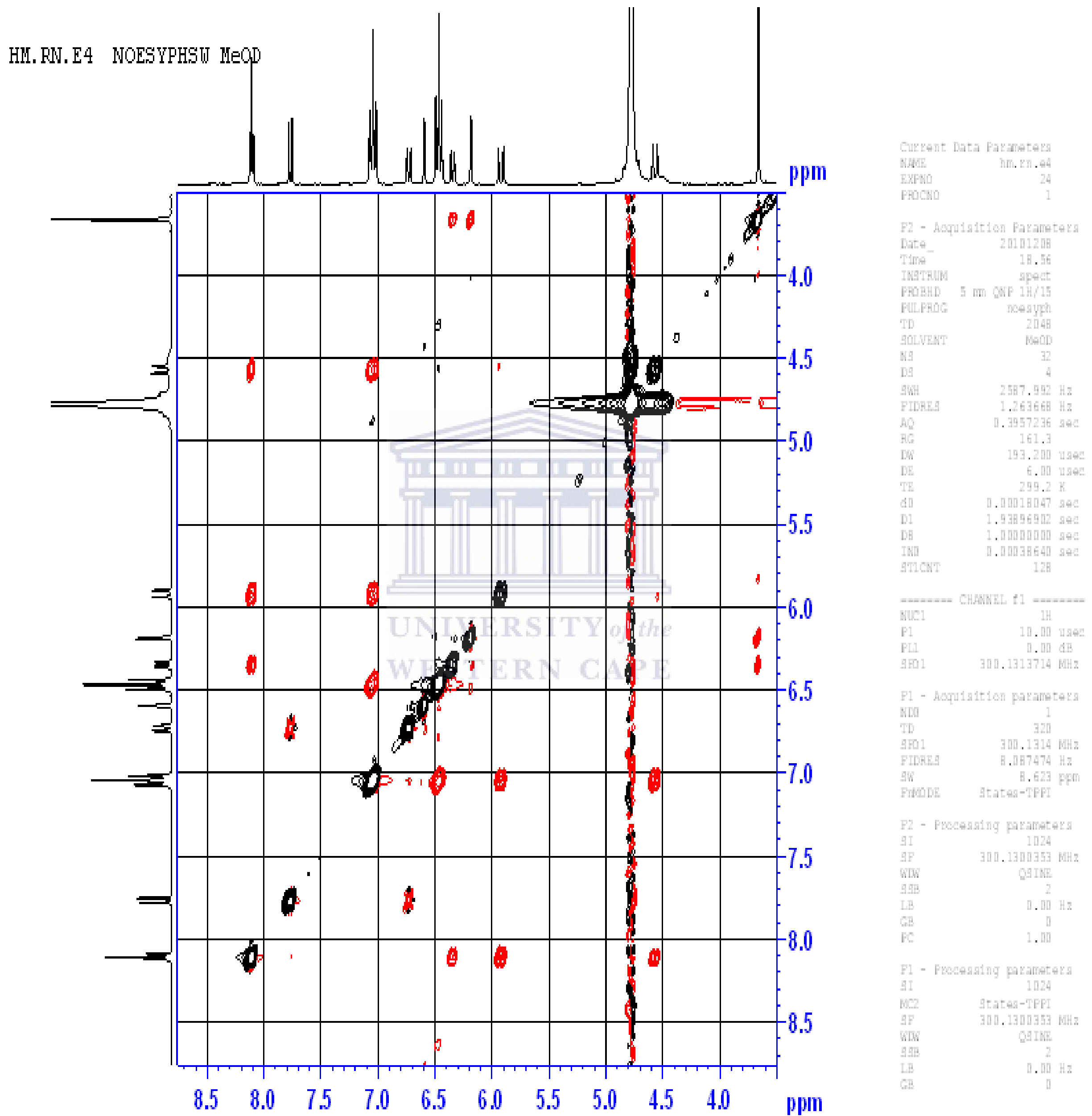
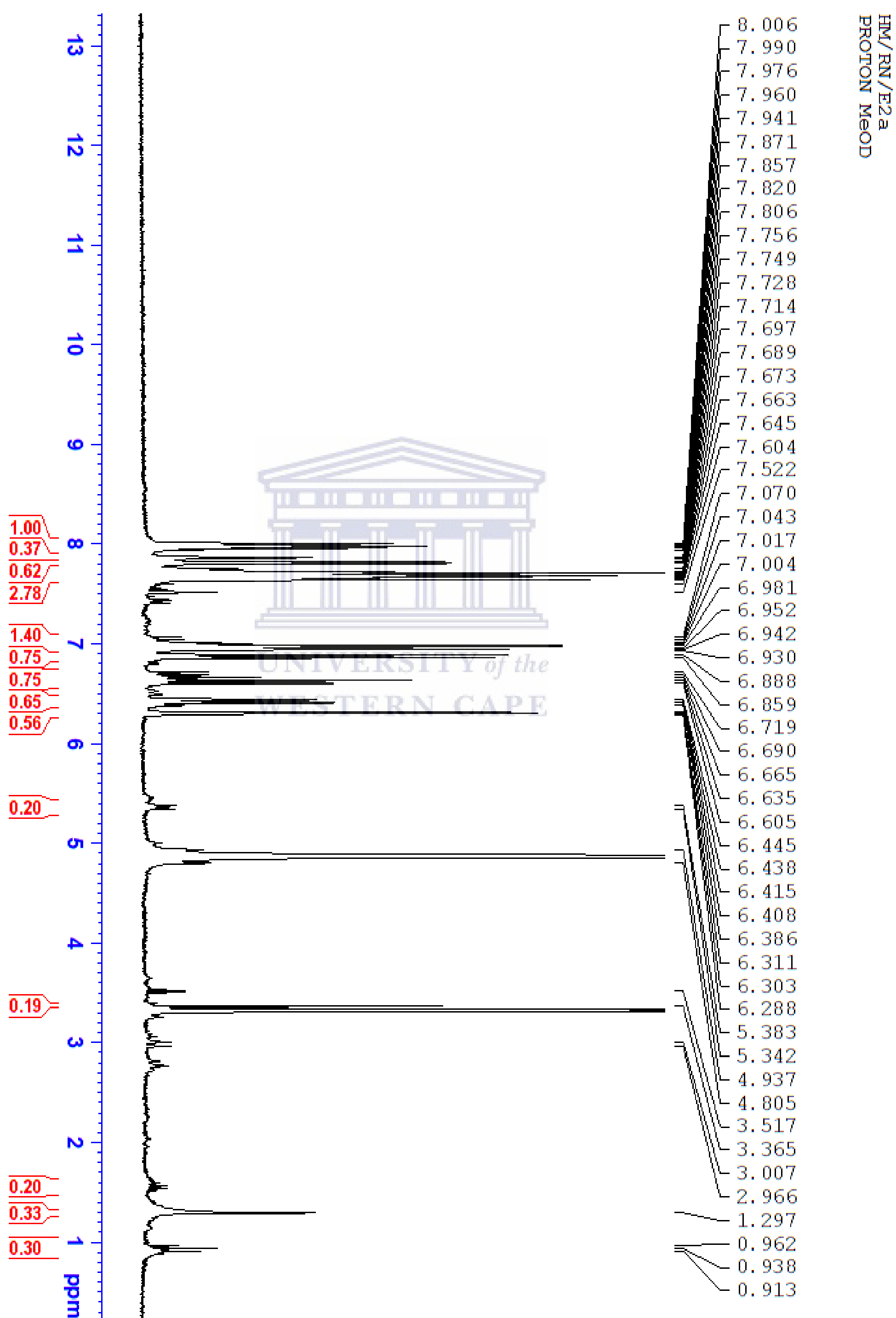
Appendix 1k NOESY of **39**



Appendix 2a ^{1}H NMR of **40**



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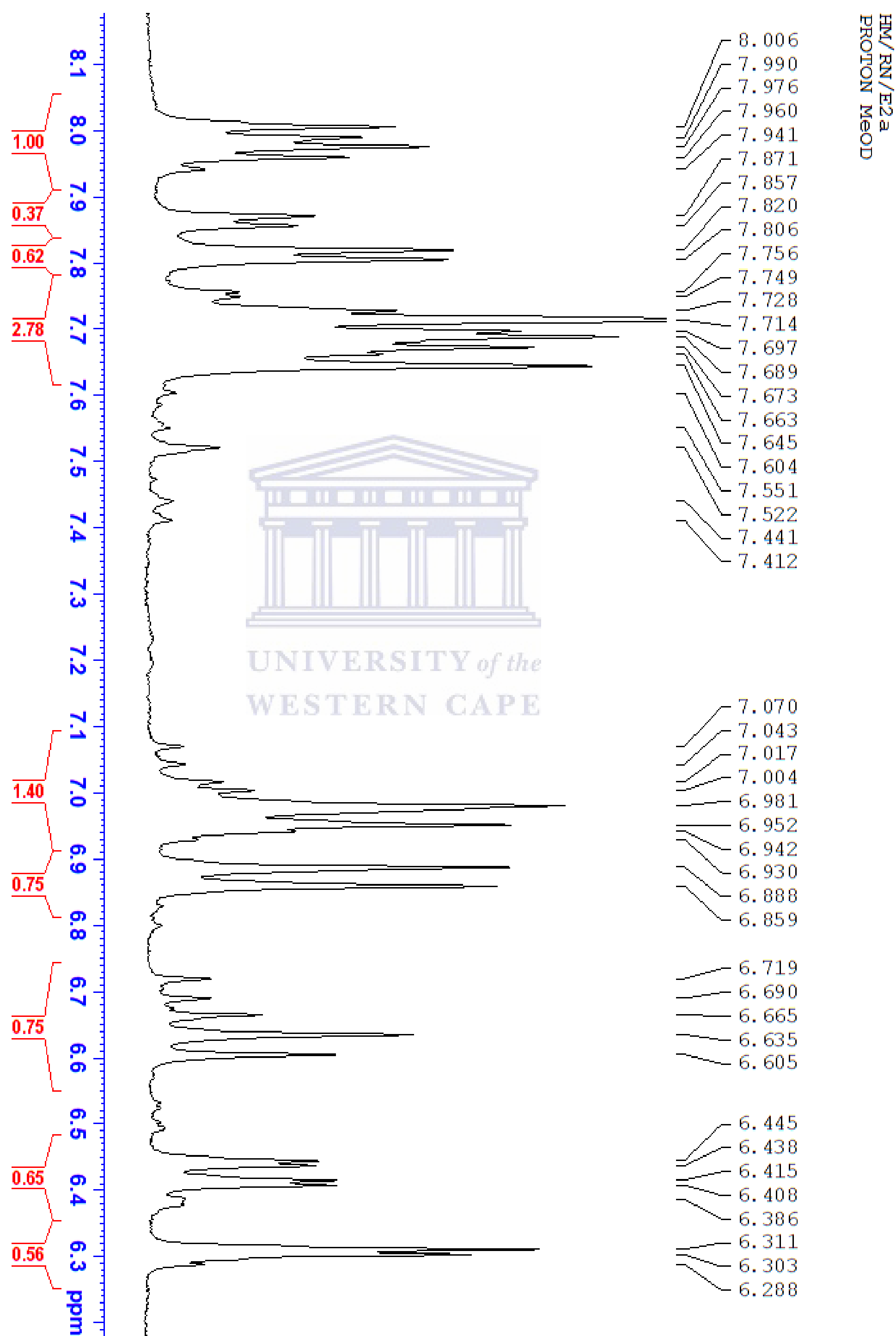

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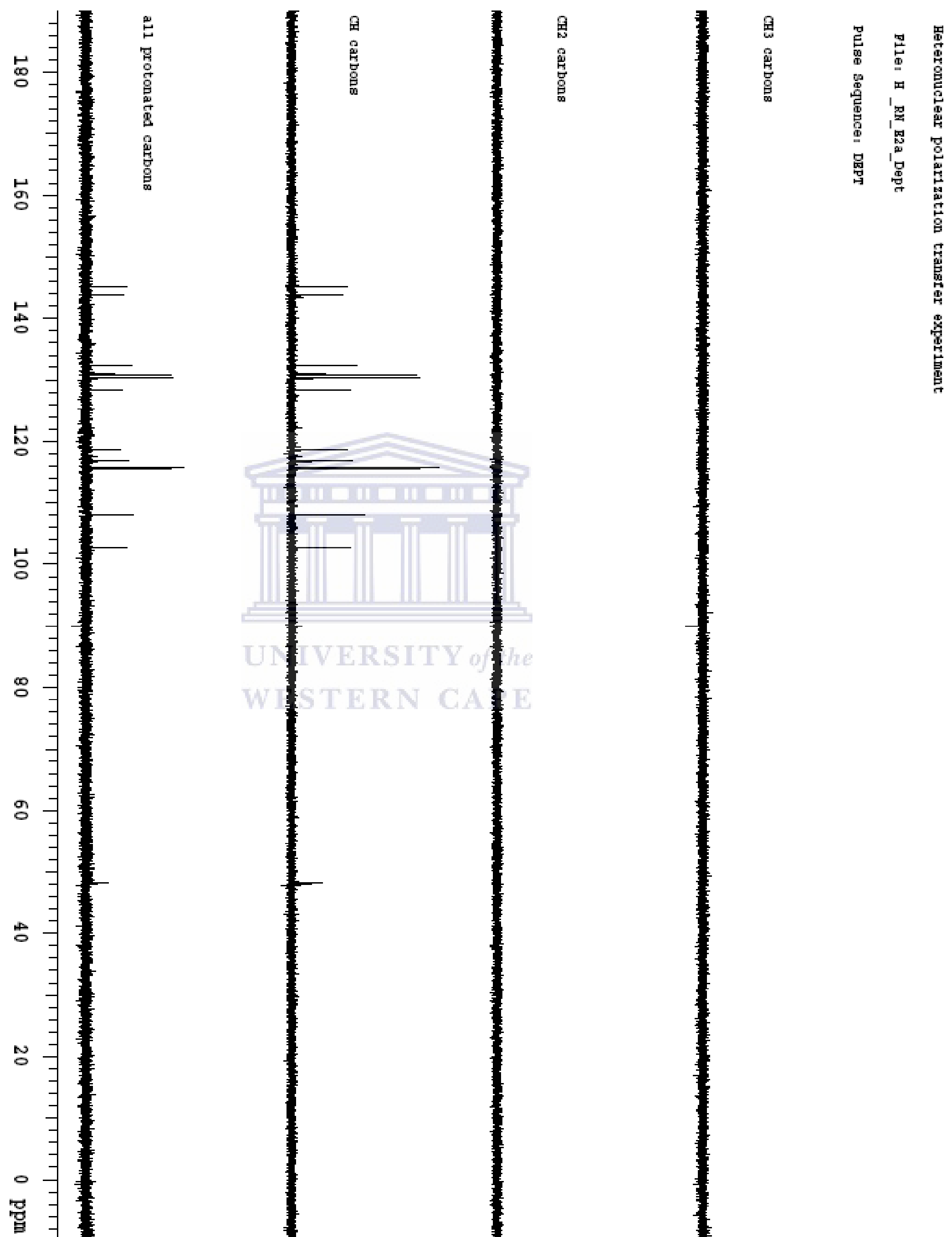
Appendix 2c DEPT of **40**



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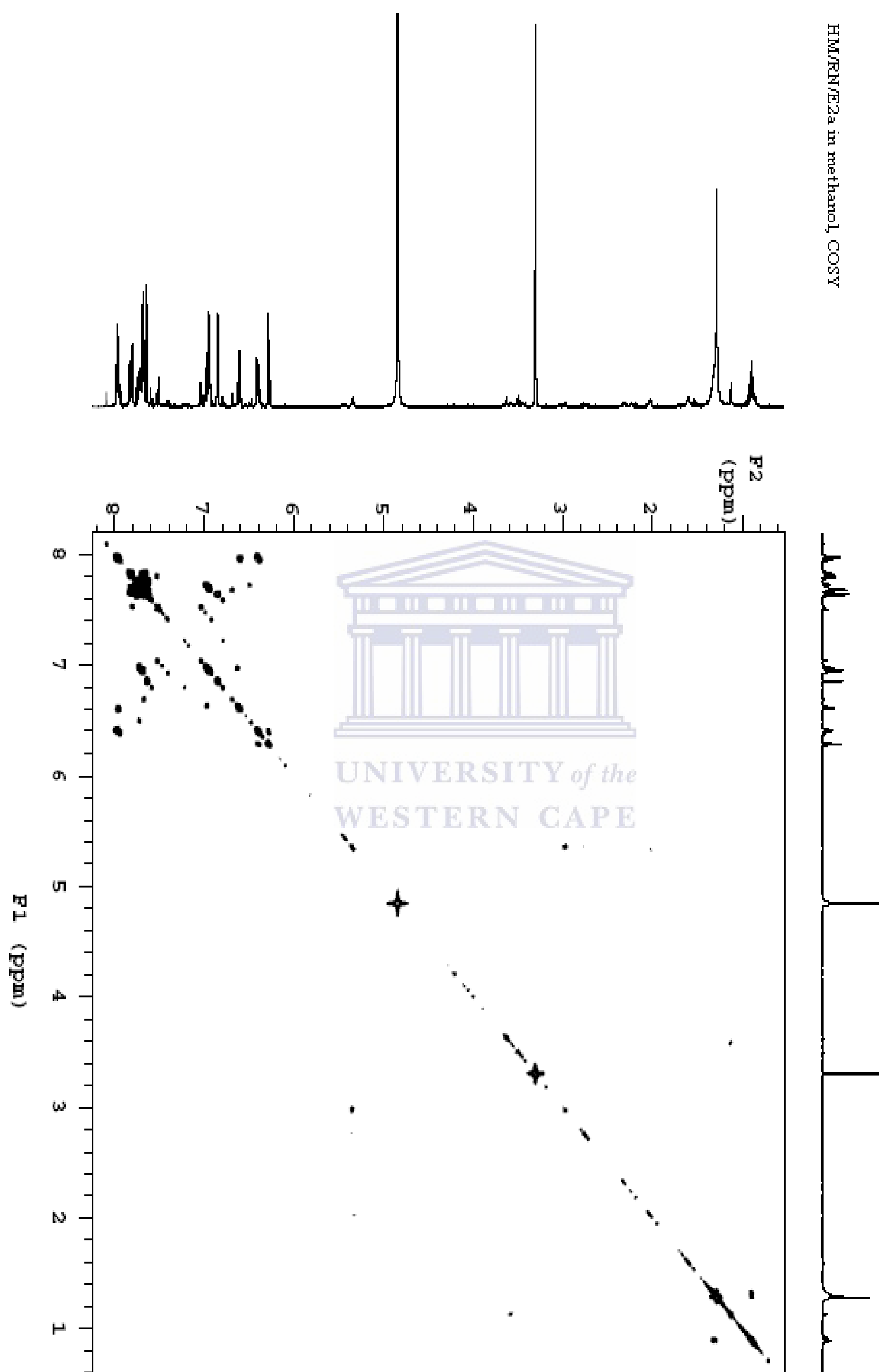
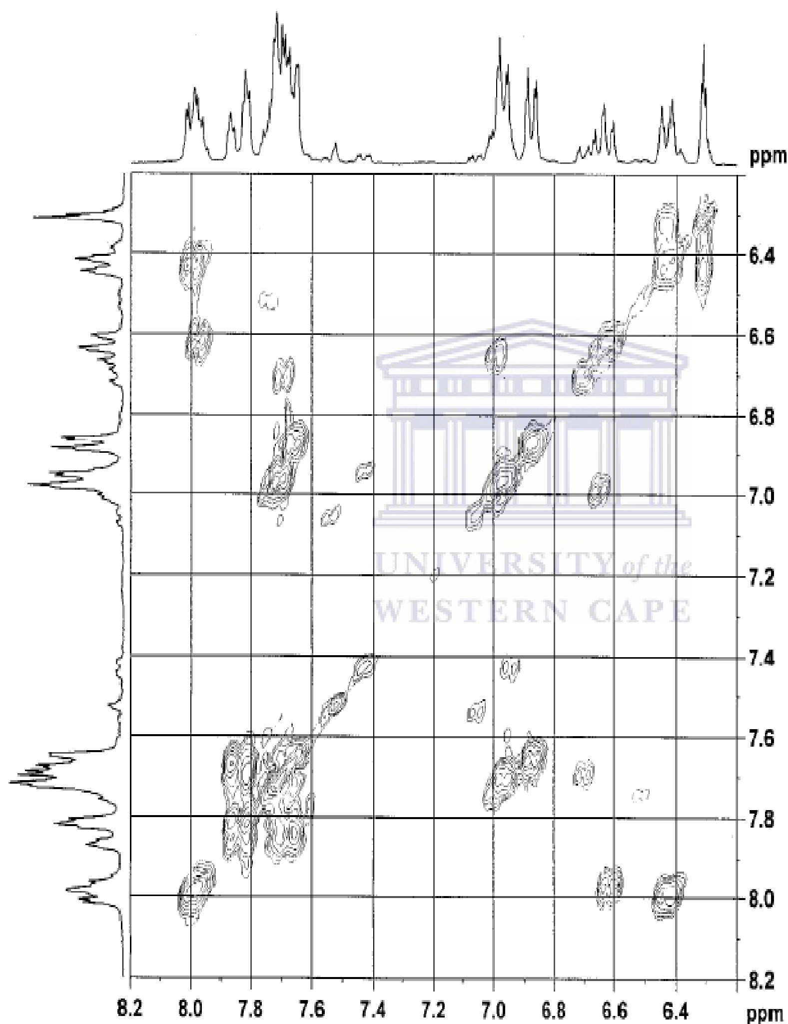
Appendix 2d ^{13}C in CD_3OD of **40**

Appendix 2e COSY of **40**



Appendix 2f COSY of **40**

hm-rn-e2a in methanol-d4, COSYGPSW



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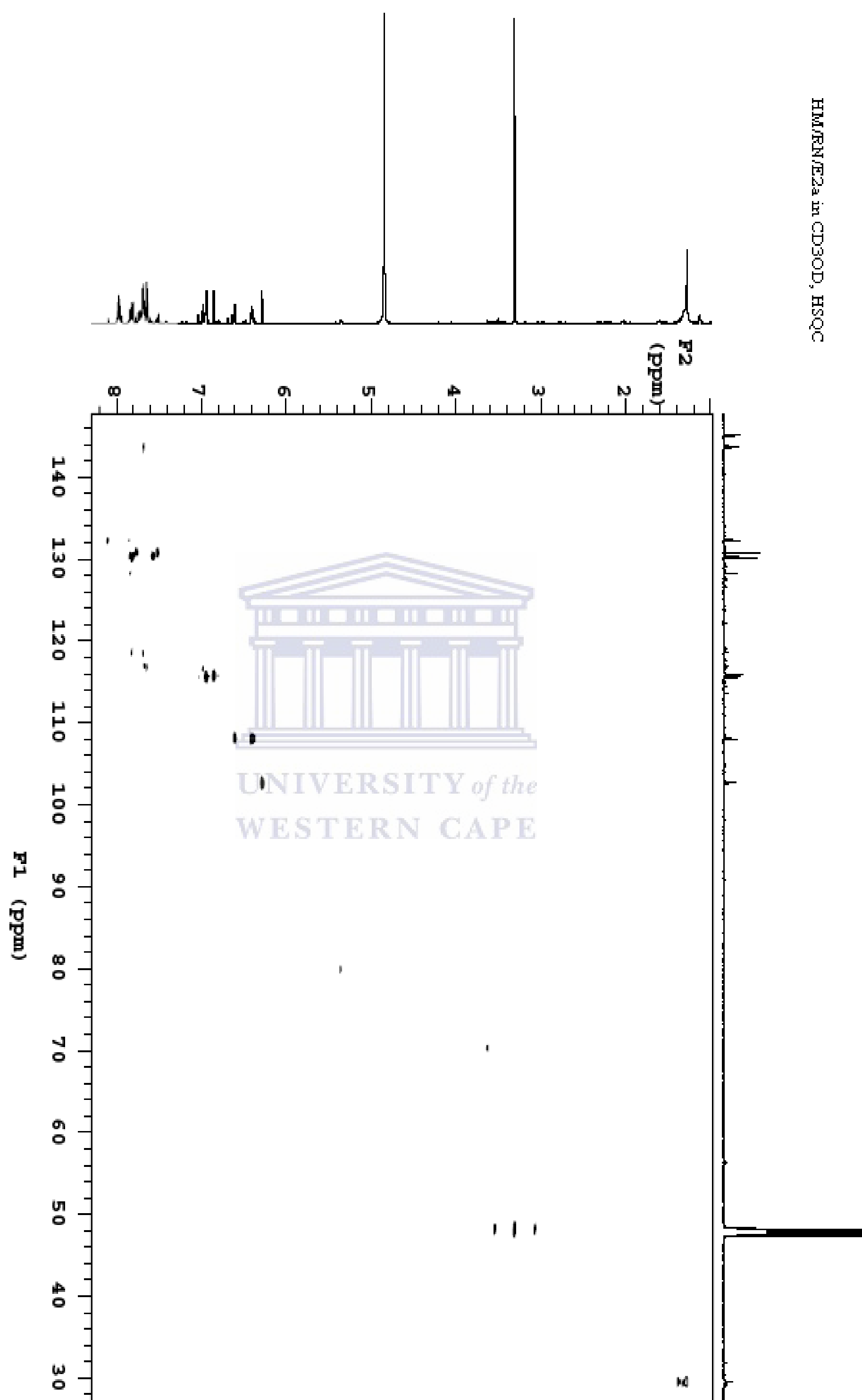
Appendix 2g HSQC of **40**



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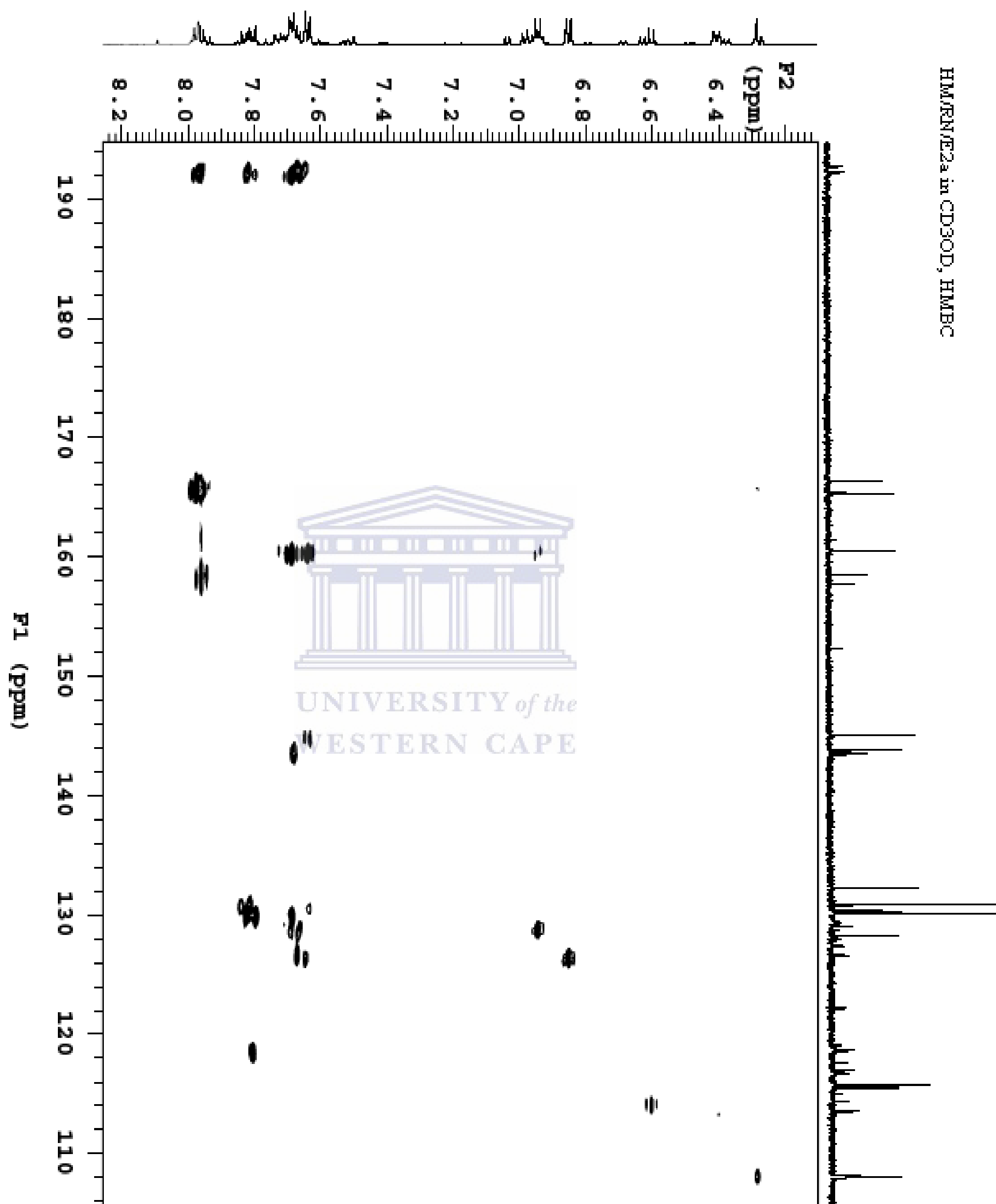
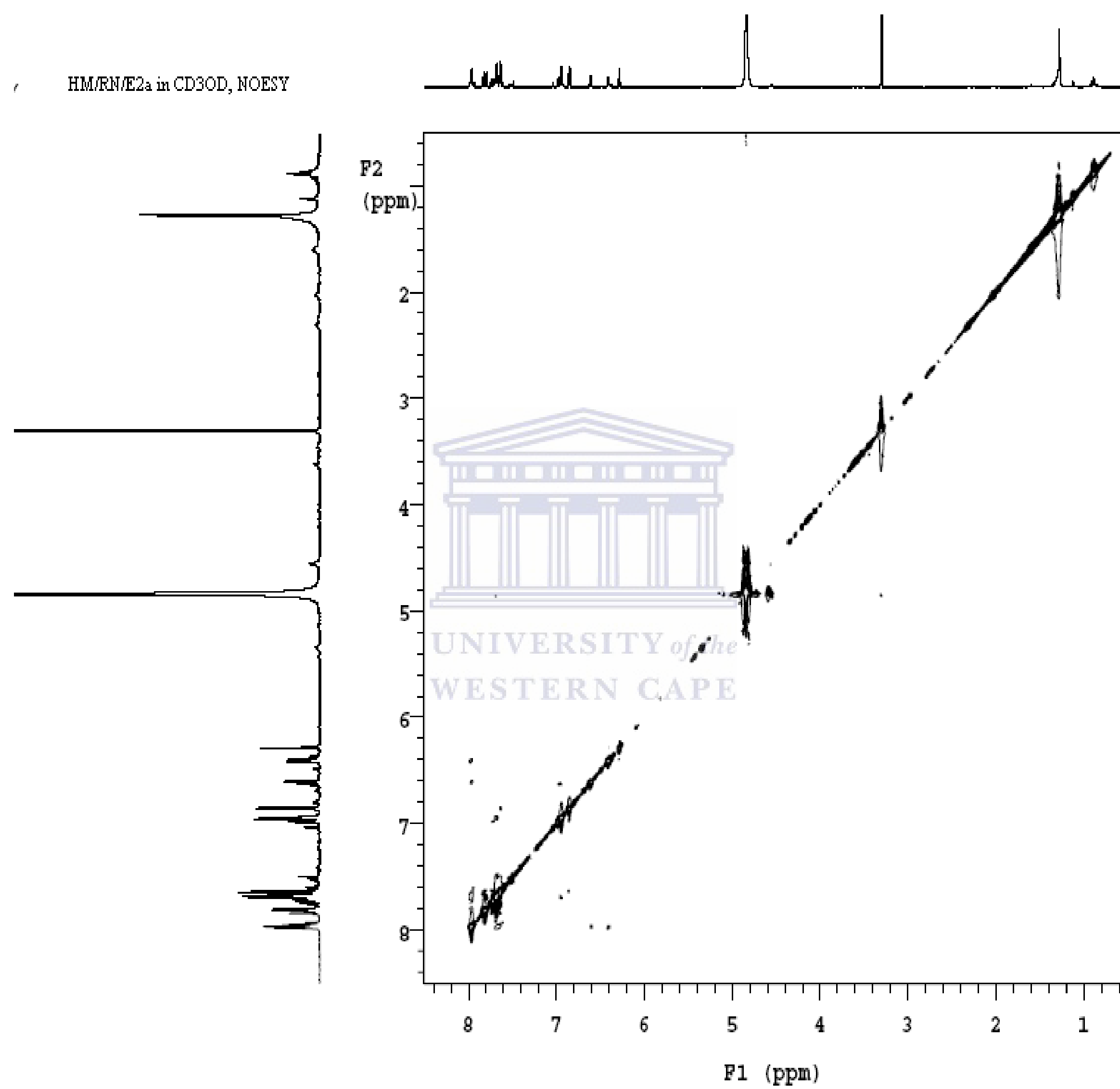
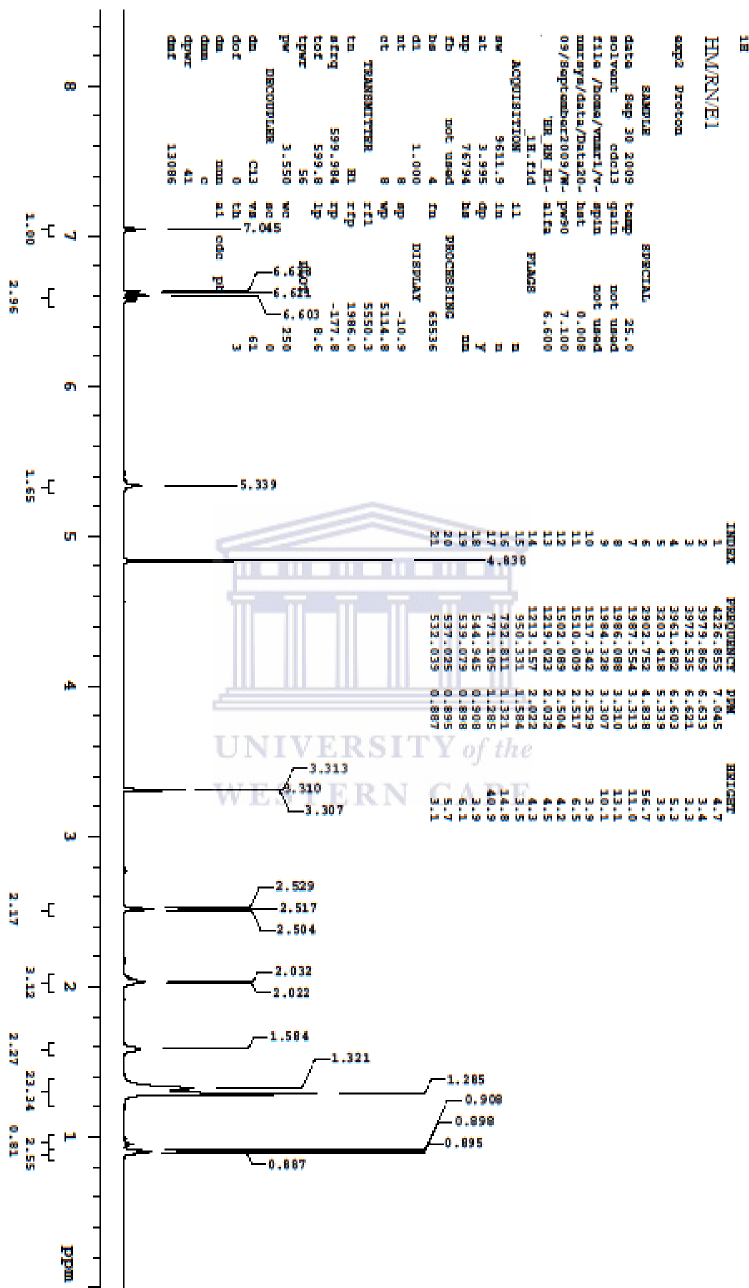


Appendix 2i NOESY of **40**



Appendix 3a ^{1}H NMR of **41**



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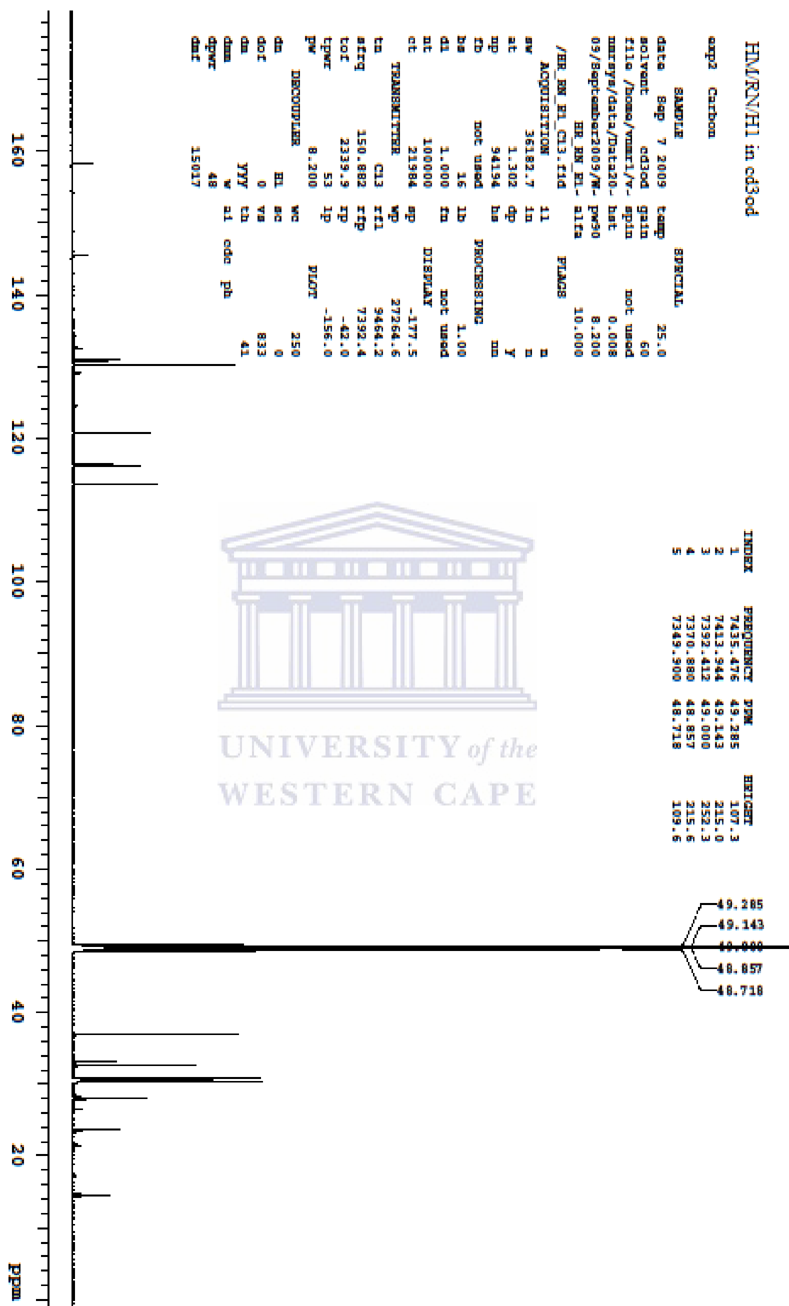
Appendix 3b ^{13}C NMR of **41**



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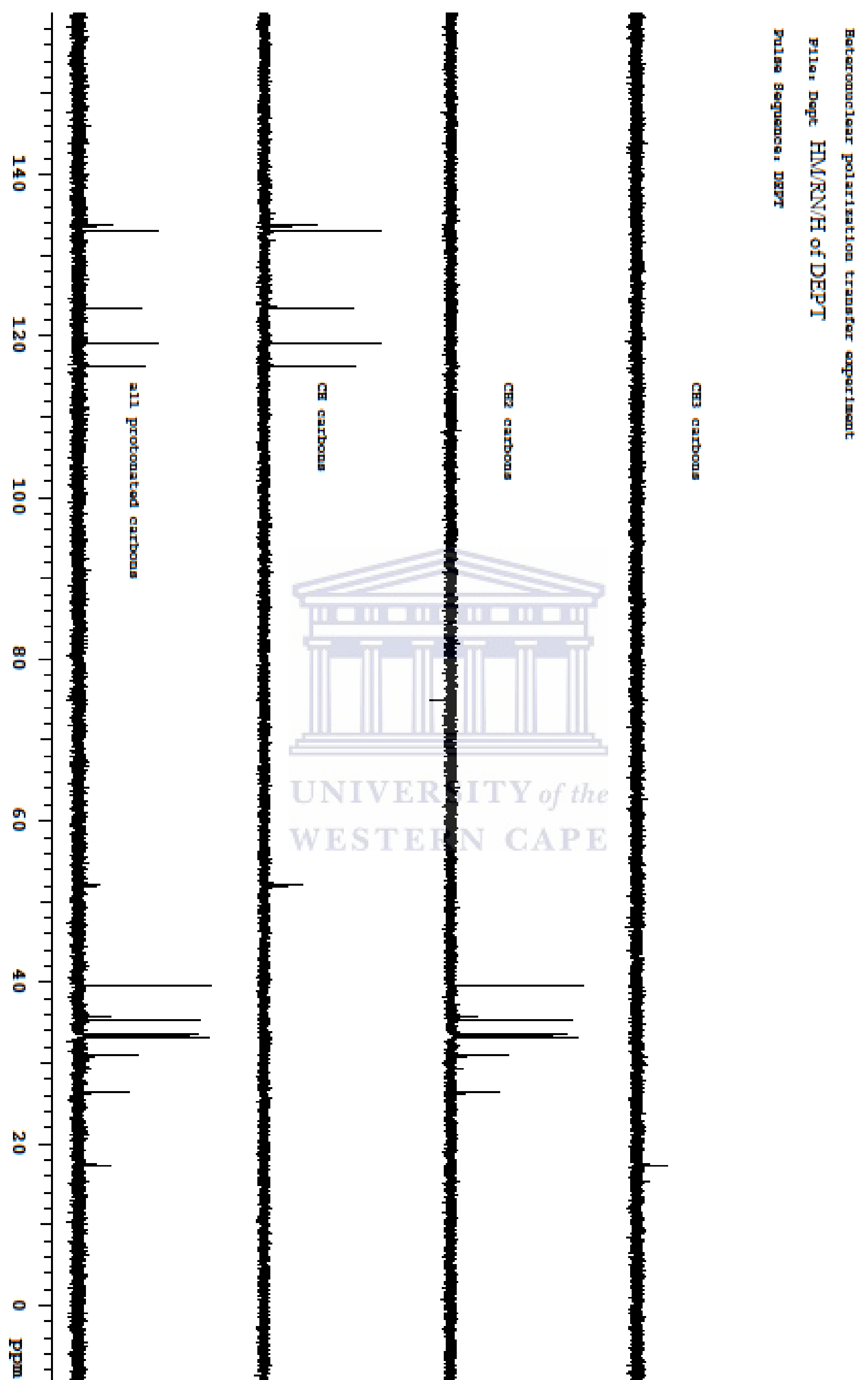
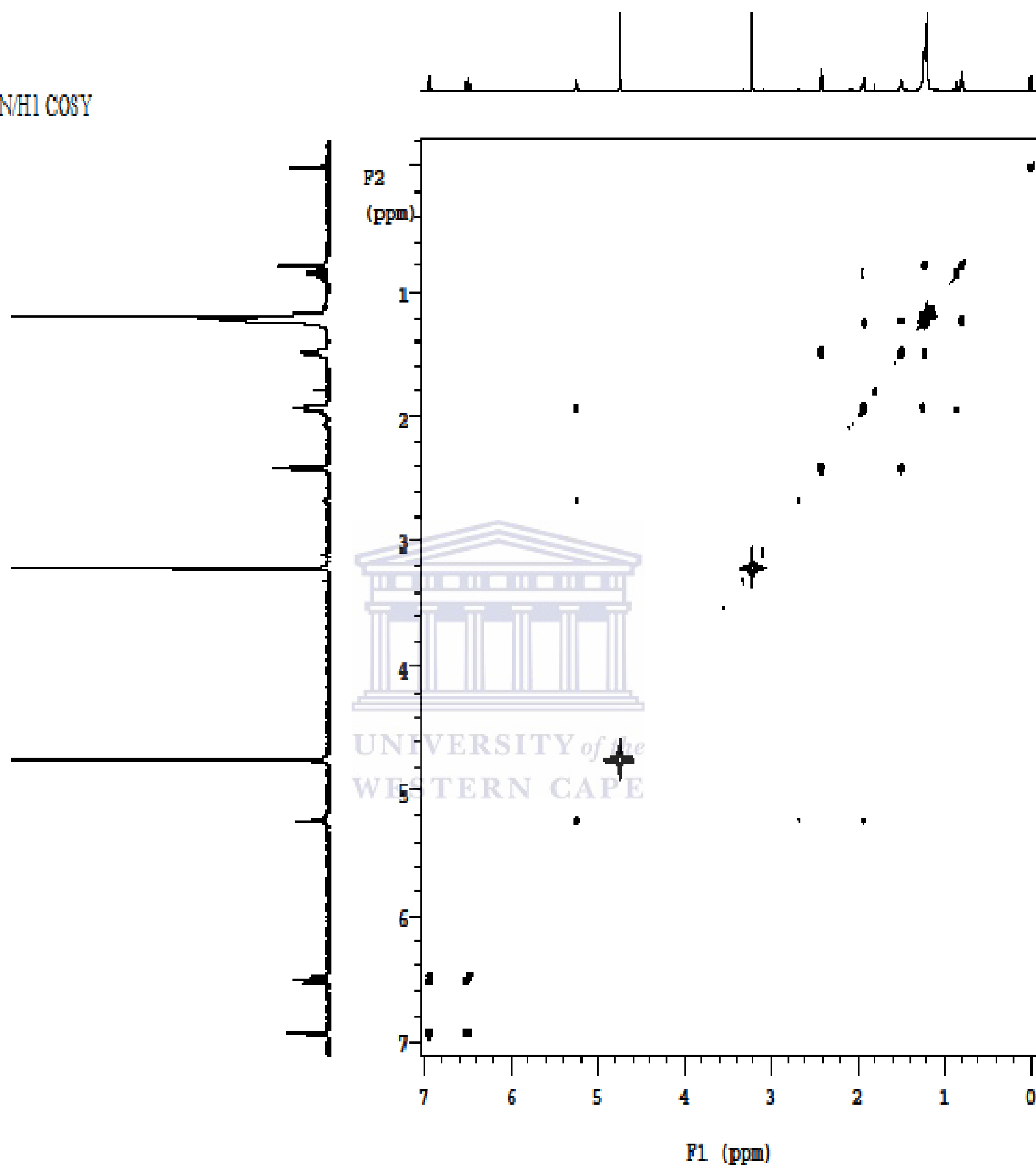
Appendix 3c DEPT NMR of **41**



Appendix 3d COSY NMR of **41**



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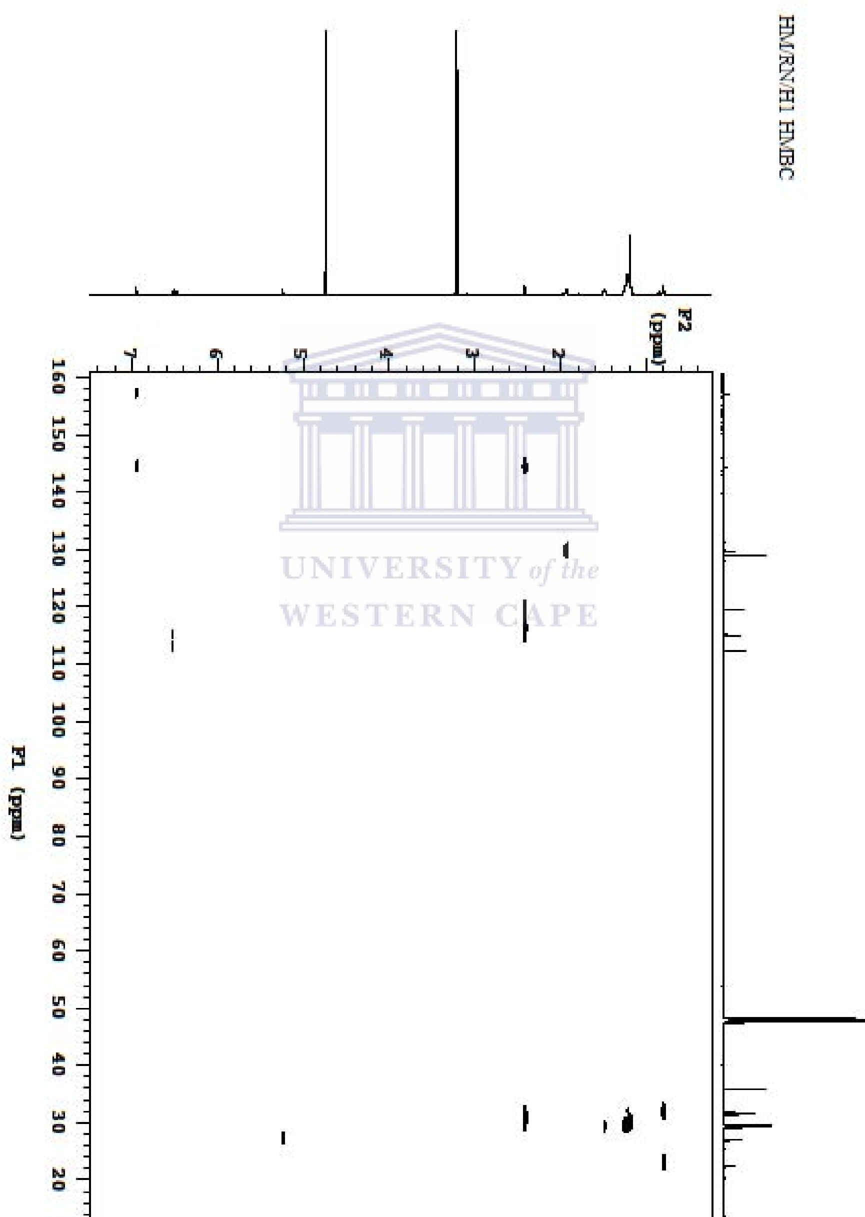
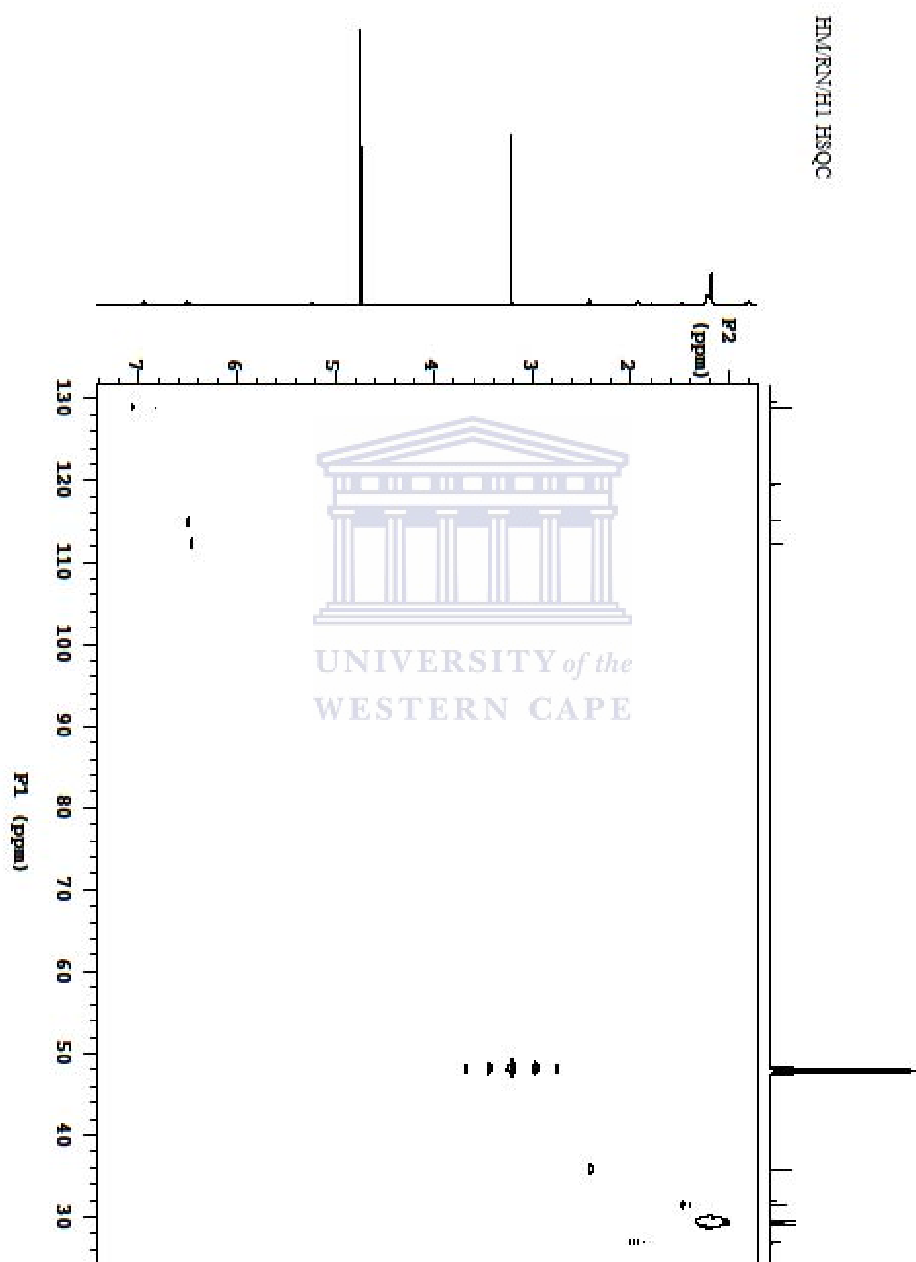
Appendix 3e HMBC NMR of **41**



Appendix 3f HSQC NMR of **41**



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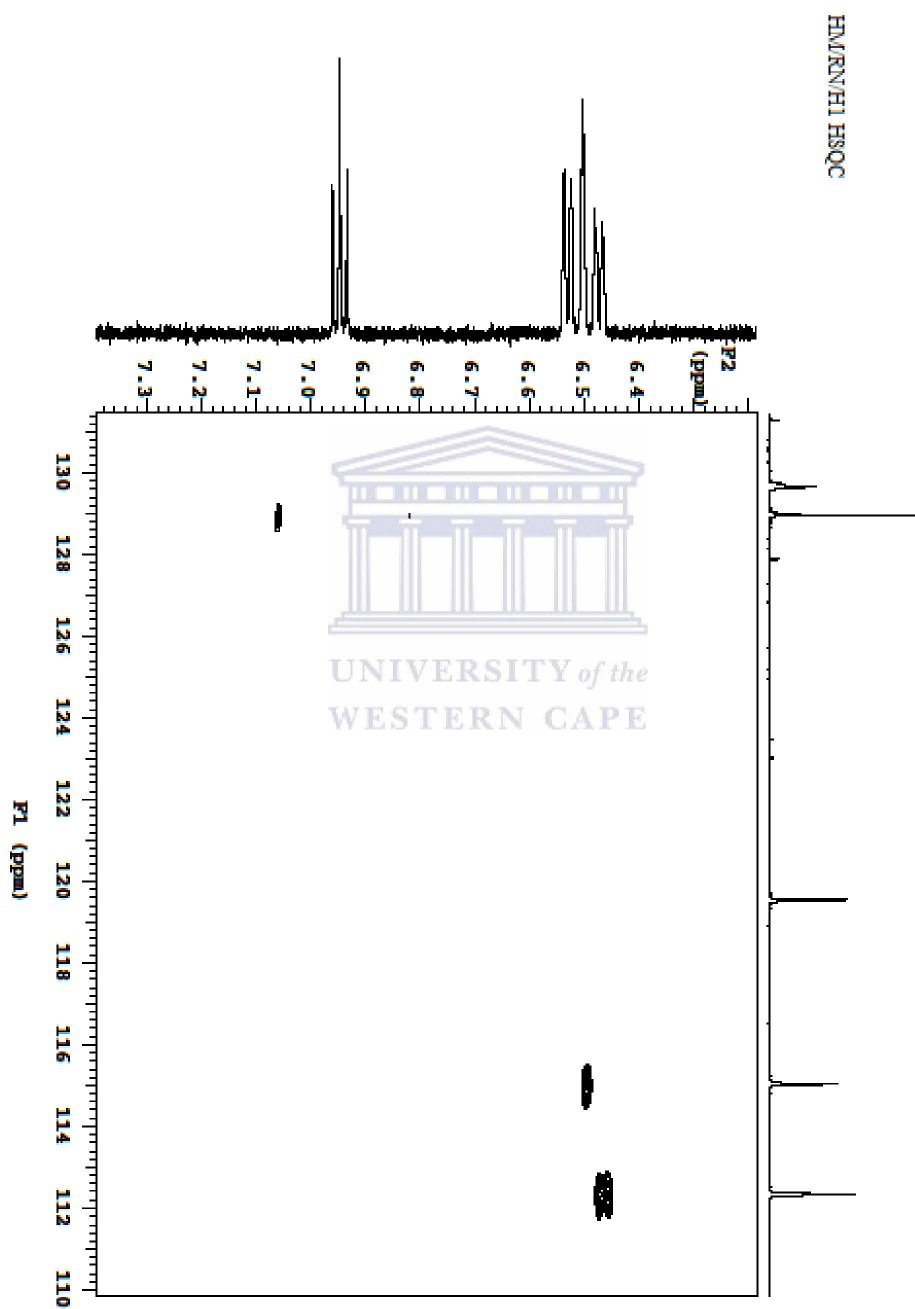
Appendix 3g HSQC NMR of **41**



Appendix 3h NOESY NMR of **41**