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CHAPTER 5

SPECTROSCOPIC ANALYSIS OF ISOLATED COMPOUND

The active compounds, 10 mg each of CF2A and CF2C, were dissolved in deuterated chloroform ($CDCl_3$) and sent for NMR analysis with a 300 MHz Varian NMR machine (Oxford instruments). Both compounds gave similar NMR spectra, however CF2C showed higher purity than CF2A.

The active compound isolated as CF1b together with CF2C was sent for NMR and mass spectroscopic analysis. The NMR and mass spectra of both CF1b and CF2C showed that they are similar compounds but isolated through different procedures. CF1b was however, the purest.

Table 16 ^{1}H-NMR (300MHz) and ^{13}C- NMR (75MHz) spectra data for isolated compound (CF1b). Data obtained in $CDCl_3$

Carbon Position	Chemical shift (δ, ppm)	
	^{1}H	^{13}C
1	-	133.4
2	6.40	105.1
3	-	146.8
4	-	132.2
5	-	146.8
6	6.40	105.1
1'	-	121.5
2'	-	142.1
3'	-	132.7
4'	6.54	145.2
5'	-	102.3
6'	6.35	120.1
1a	3.84	36.5
1'a	3.84	32.1
3, 5 OMe	2.82	56.2
4' OMe	2.82	56.1

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The ^{1}H-NMR spectrum of CF1b, showed signals for three aromatic protons at 6.5 ppm (1H, d, J= 8.4Hz), 6.40 ppm (2H, s) and 6.36 ppm (1H, d, J=8.4Hz), a signal at 3.84 ppm integrating for three methoxyl groups and a complex multiplet at 2.82 ppm [Spectrum 1]. The complex multiplet arises from an ethane bridge, which is typical of bibenzylic, compounds (Majumder et al, 1999; Katerere, 2001).

Mass spectroscopy confirmed the bibenzylic nature of CF1b. It gave a molecular ion at m/z 320 (41%) corresponding to $C_{17}H_{20}O_6$ and two major fragments at m/z 153 $C_8H_9O_3$ (100%, base peak) and 167 $C_9H_{11}O_3$ (89.9%). The fragments are tropylium derivatives of the phenolic ring which is typical of bibenzyls (Letcher et al., 1972; Katerere, 2001)[Fig. 53]. This suggested that one ring contained two methoxyl groups and one hydroxyl group and the other one methoxyl group and two hydroxyl groups. The exact positions of the hydroxyl and methoxyl groups around the two aromatic rings were ascertained from the chemical shift and the splitting patterns of signals of the aromatic protons. 2D NMR was also attempted but gave equivocal results.

The isolated active compound was divided into aromatic rings arbitrarily labeled as 'A' and 'B' [Fig. 52]. Mass spectroscopy of CF1b gave fragment of m/z 167 ($C_9H_{11}O_3$) representing aromatic ring A. It has two methoxyl and one-hydroxyl functions.

For ring A, ^{1}H-NMR showed a singlet at 6.40 ppm, which correspond to two protons. These are *meta*-coupled and magnetically equivalent and also imply that ring A is probably symmetrical. Therefore, the protons were placed at position 2 and 6. Positions 3, 5, 6 would have to be oxygenated as seen from the mass spectroscopic fragment m/z 167 ($C_9H_{11}O_3$).

The other fragment of m/z 153 ($C_8H_9O_3$) from mass spectroscopy of CF1b represents the aromatic ring B. The proton at 6.54 ppm is *ortho*- coupled to that at 6.35 ppm. This is implied from the coupling constant J=8.4. The protons were placed at 5' and 6' leaving the other positions to be taken up by two hydroxyl and a methoxyl group.

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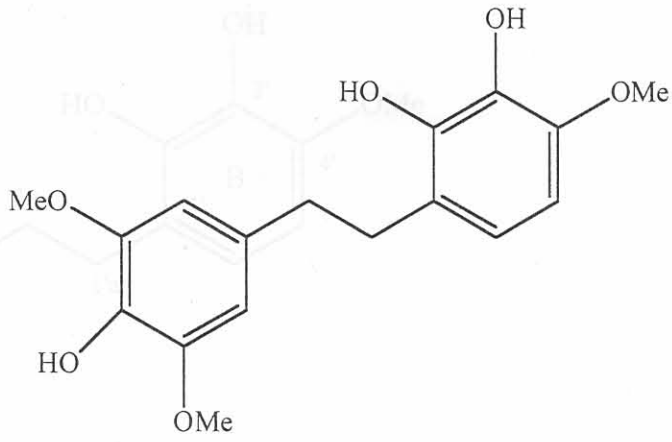

Fig. 50 Proposed structure for isolated active compound.

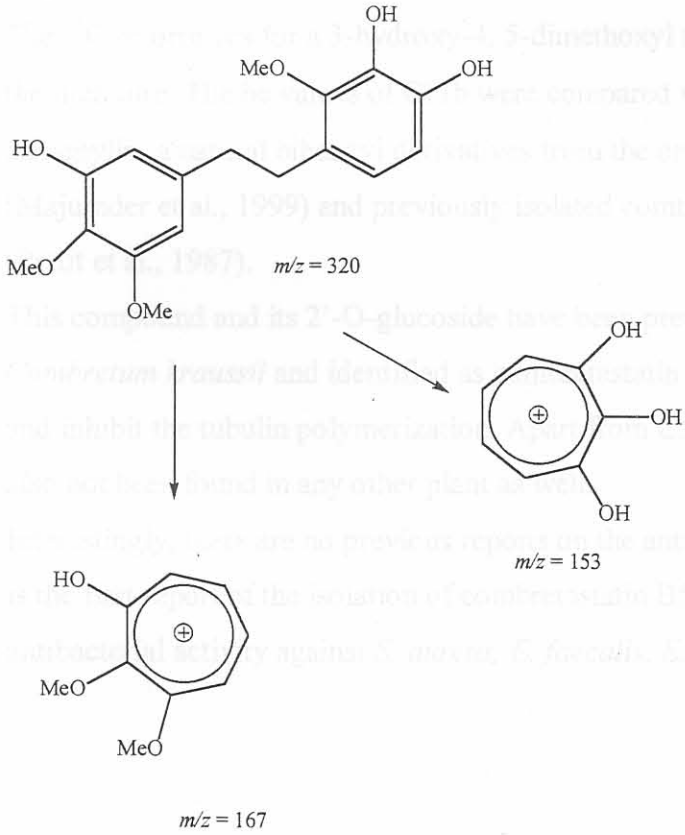

Fig. 51 The isolated active compound and its fragmentation into two tropylium ions.

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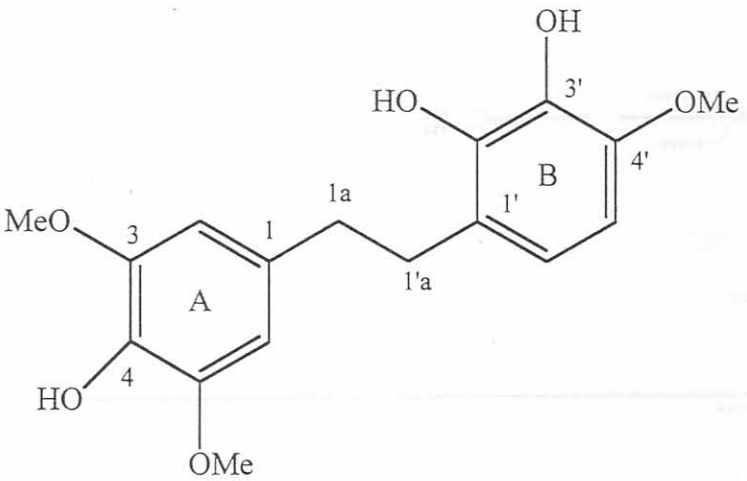

Fig. 52 Isolated active compound with its two aromatic rings labeled as 'A' and 'B'

The compound isolated was proposed to be 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl [Fig. 50].

The ^{13}C resonances for a 3-hydroxy-4, 5-dimethoxyl ring were compared experimental data in the literature. The δc values of CFIb were compared with structurally related carbon atoms of amoenylin, a natural bibenzyl derivatives from the orchid *Dendrobium amoenumm* (Majumder et al., 1999) and previously isolated combretastatins A-1 and B-I from *C. caffrum* (Pettit et al., 1987).

This compound and its 2'-O-glucoside have been previously isolated from seeds of *Combretum kraussii* and identified as combretastatin B5. They showed *in vitro* cytotoxicity and inhibit the tubulin polymerization. Apart from C. *krausii*, this compound has apparently also not been found in any other plant as well.

Interestingly, there are no previous reports on the antibacterial activity of this compound. This is the first report of the isolation of combretastatin B5 from *C. woodii* as well as its antibacterial activity against *S. aureus, E. faecalis*, *E. coli*, and *Ps. aeruginosa*.

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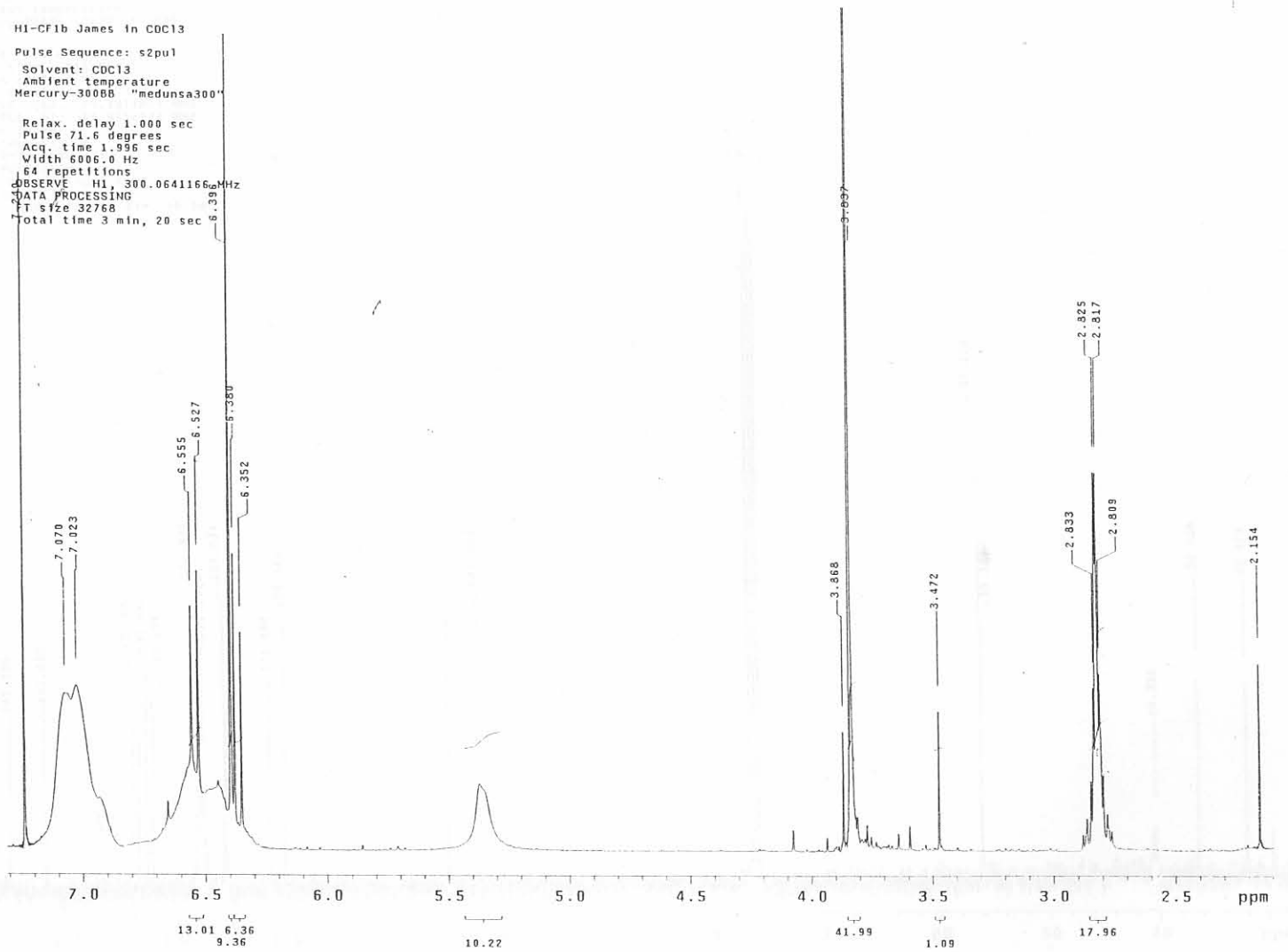
Spectrum 1 ^{1}H-NMR Spectroscopy of CF1b



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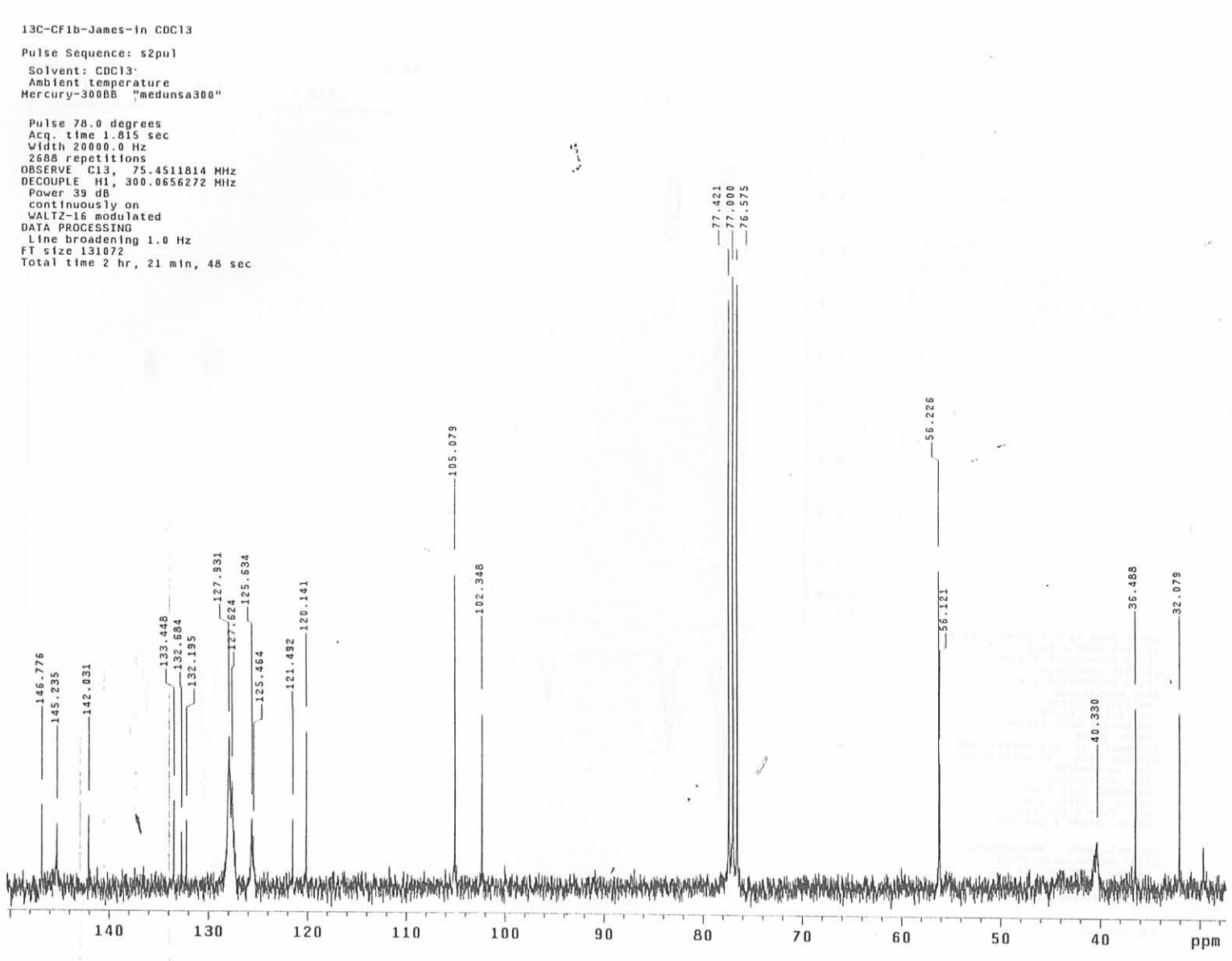
Spectrum 2 ^{13}C-NMR Spectroscopy of CF1b



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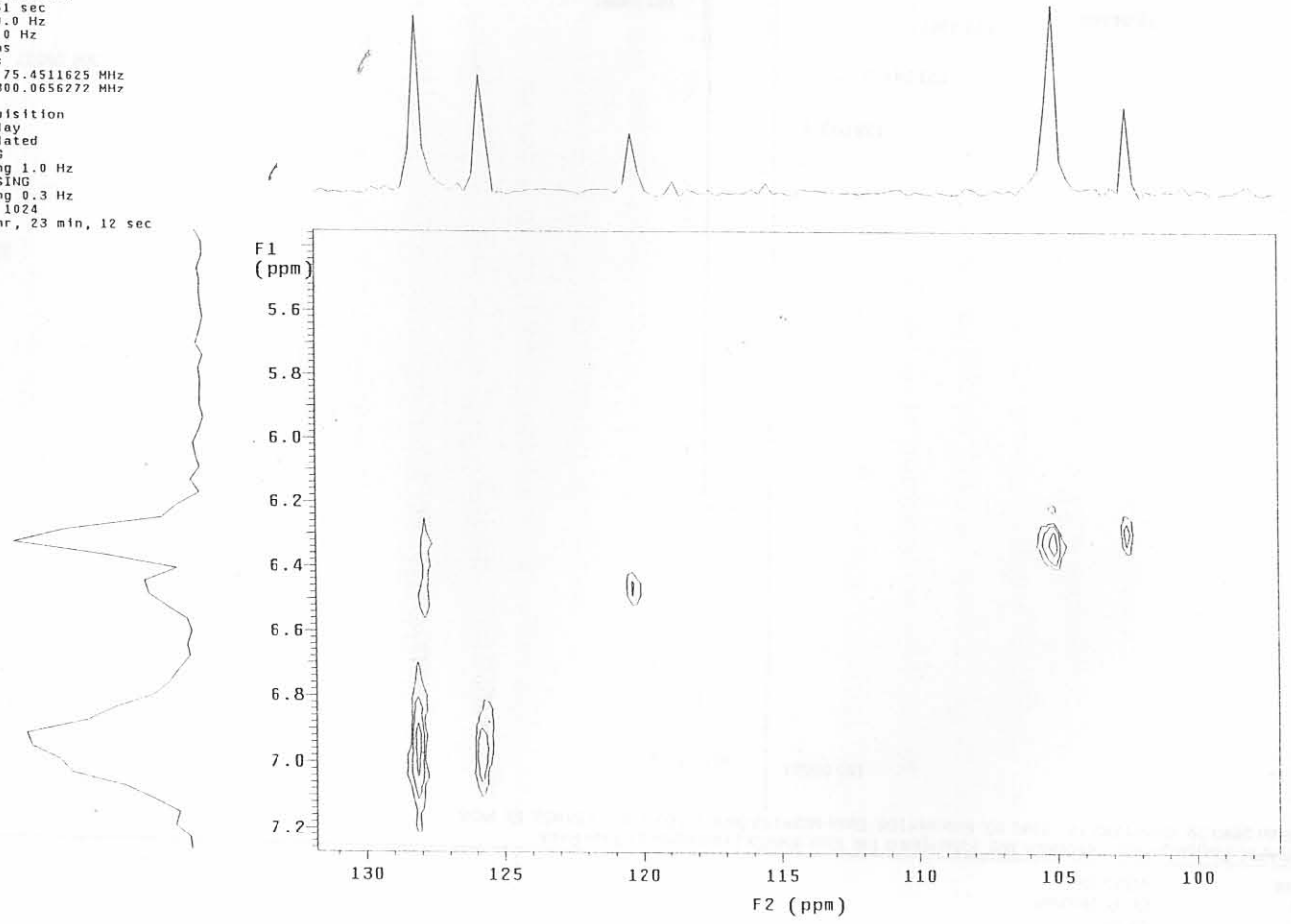
13C OBSERVE

Pulse Sequence: hetcor
Solvent: CDCl3
Ambient temperature
File: Hetcor-CF1b-James
Mercury-300BB "medunsa300"

Relax. delay 1.000 sec
Acq. time 0.051 sec
Width 20000.0 Hz
2D Width 6006.0 Hz
128 repetitions
256 increments
OBSERVE C13, 75.4511625 MHz
DECOUPLE H1, 300.0656272 MHz
Power 39 dB
on during acquisition
off during delay
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
F1 DATA PROCESSING
Line broadening 0.3 Hz
FT size 2048 x 1024
Total time 10 hr, 23 min, 12 sec



Spectrum 3 2D NMR Spectroscopy of CF1b

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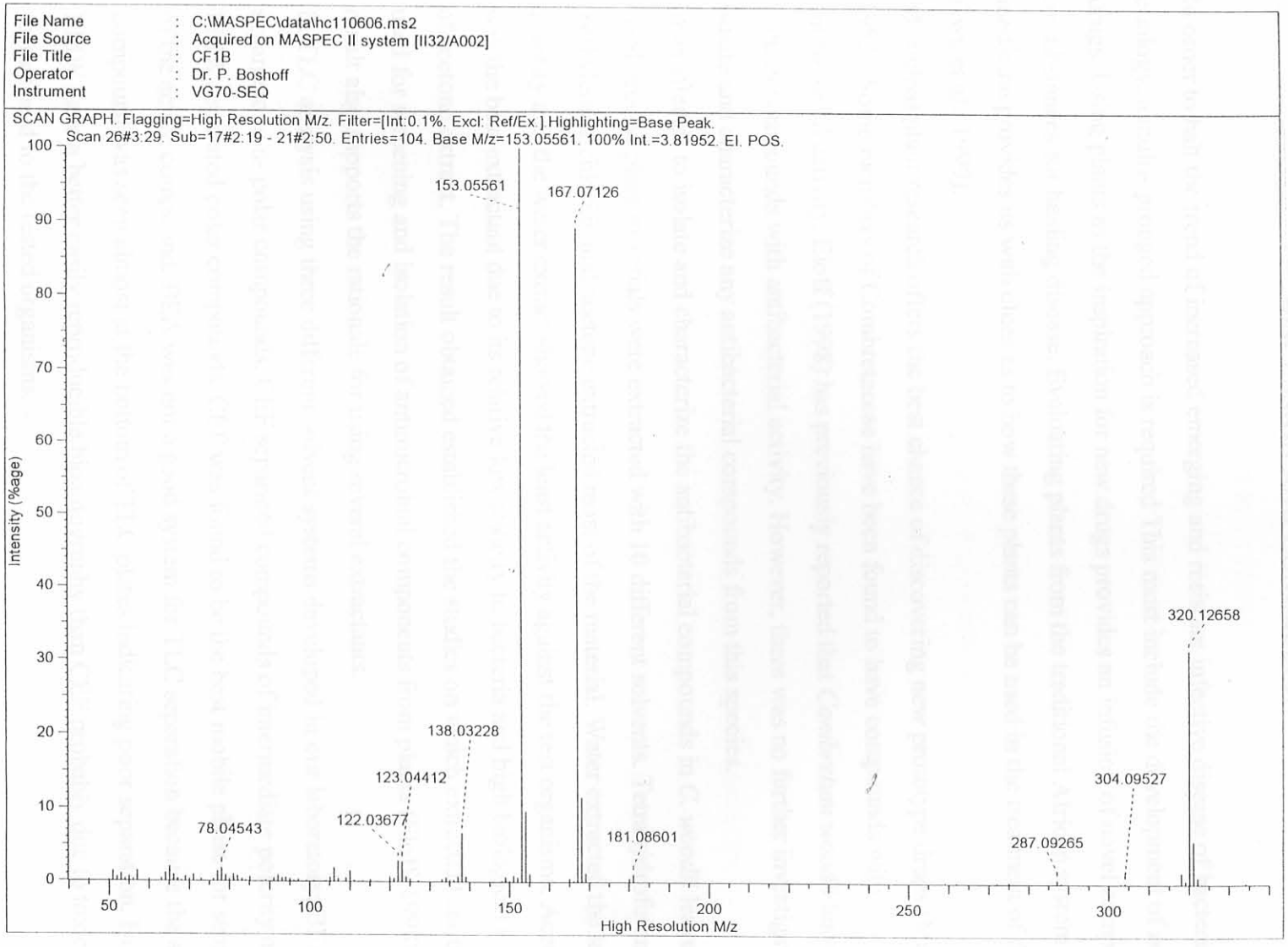
Spectrum 4 Mass spectrometry of CF1b



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DISCUSSION AND CONCLUSION

In order to halt the trend of increased emerging and resistant infective disease of bacterial etiology, a multi- pronged approach is required This must include the development of new drugs. Using plants as the inspiration for new drugs provides an infusion of novel compounds or substances for healing disease. Evaluating plants from the traditional African system of medicine provides us with clues as to how these plants can be used in the treatment of disease (Iwu et al., 1999).

Medicinal plant research offers the best chance of discovering new prototype drugs (Malone, 1983). Some members of Combretaceae have been found to have compounds with antibacterial activity. Eloff (1998) has previously reported that *Combretum woodii* leaves contain compounds with antibacterial activity. However, there was no further investigation to isolate and characterize any antibacterial compounds from this species.

In an attempt to isolate and characterize the antibacterial compounds in *C. woodii* leaves, the dried ground plant materials were extracted with 10 different solvents. Tetrahydrofuran and methylene dichloride and acetone extracted most of the material. Water extracted the least quantity and the water extract showed the least activity against the test organisms. Acetone was the best extractant due to its relative low toxicity to bacteria and high biological activity of acetone extract. The result obtained established the studies on which extractant should be used for screening and isolation of antimicrobial components from plants (Eloff, 1998). This result also supports the rationale for using several extractants.

In TLC analysis using three different solvent systems developed in our laboratory, BEA separated non- polar compounds, CEF separated compounds of intermediate polarity and EMW separated polar compounds. CEF was found to be the best mobile phase for separation of the active compound. BEA was not a good system for TLC separation because the active compound was seen almost at the bottom of TLC plates indicating poor separation. However, EMW gave a better easily reproducible bioautography than CEF probably due to toxicity of formic acid to the tested organisms.

Both vanillin- sulphuric acid and *p*-anisaldehyde-sulphuric acid spraying reagents were initially used. However, more compounds were visible with vanillin than with anisaldehyde spray reagent. Vanillin-sulphuric acid spray reagent was therefore routinely used; it appears to be a good spray reagent for detection of the active compound in *C. woodii* leaves. However, not all the compounds that were seen under on UV light were revealed by it, but they were not the compounds with antibacterial activity.

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After solvent- solvent fractionation, most of the activity resided in chloroform and hexane fractions. Bioassay-guided isolations resulted in the identification of the responsible antibacterial compounds in the leaves of this plant. There were many antibacterial compounds shown by bioautography. However, attempts were directed towards isolating one major compound, with R_f value of 0.74 in EMW solvent systems, present in all the fractions (except water fraction) obtained. Column chromatography of the chloroform fraction led to the successful isolation of this compound. This isolated compound exhibited *in vitro* antibacterial activity against all the four test organisms. Both Gram-positive organisms and Gram-negative organisms were sensitive to the compound. *S. aureus* was the most sensitive of all the four tested with an MIC value of 16 µg/ml followed by *E. faecalis* (125 µg/ml) and *Ps. aeruginosa* (125 µg/ml) and *E. coli* (250 µg/ml). Activities on three of the bacteria tested were higher than the standard antibiotics compared.

NMR and mass spectra of the isolated active compound led to its structural elucidation. This antibacterial compound was found to be a bibenzylic compound, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxyl bibenzyl. This compound is also a stilbene. The stilbenes are phytoalexins, which are antimicrobial compounds which accumulates in response to a pathogen (Kuc, 1990) Therefore, the possible role of the bibenzyl in *C. woodii* is to protect the plant against any invading microorganism. This same compound also named as combretastatin B5 previously isolated from seeds of *C. krausii* has been found to be antimitotic (Pellizzoni et. al, 1992). There are no previous reports of its antibacterial activity. Based on the MIC values of this compound, further investigation is necessary for treating ailments caused by bacteria, mostly *S. aureus*, *Ps. aeruginosa* and *E. faecalis*. Infections caused by the *S. aureus* and *Ps. aeruginosa* organisms are amongst the most difficult to treat with conventional antibiotics (Salie et al, 1996).

With the presence of antibacterial compounds, first time isolated in leaves, this plant or isolated constituent, which is accessible, could be an inexpensive additional means for treating bacterial infections

Generally, *C. woodii* leaves contain many antibacterial compounds of which only one was isolated and characterized. The results obtained with *C. woodii* indicated that not only the stability of the biologically active component, but also probably the high concentration of the desired component enhances the applicability of all the techniques used. This, in fact, suggested that *C. woodii* has a high biological activity, since it is impossible to detect antibacterial activity if the quantity of material is limited.

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Because this plant contains many compounds, further work needs to be carried out on isolation and characterization of other antibacterial compounds.

These results validate the ethnobotanical use of many *Combretum* species for bacteria infections.

Furthermore, it is recommended for future work that the spectrum of antimicrobial activity of the active compound isolated be determined by testing its activity against many bacteria and fungi.