1988

Search for Allelochemicals in Rice (Oryza Sativa L.) and Structure Determination of External Flavonoids of Calamintha Ashei.

Hidelisa Padua Hernandez
Louisiana State University and Agricultural & Mechanical College

Order Number 8917823

Search for allelochemicals in rice (*Oryza sativa* L.) and structure determination of external flavonoids of *Calamintha ashei*

Hernandez, Hidelisa Padua, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1988

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

SEARCH FOR ALLELOCHEMICALS IN RICE (*ORYZA SATIVA* L.) AND STRUCTURE DETERMINATION OF EXTERNAL FLAVONOIDS OF *CALAMINTHA ASHEI*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

Hidelisa Padua Hernandez
B.S., 1977; M.S., 1983
University of the Philippines
at Los Baños

December, 1988

for

Joe

Tatay and **Nanay**

ACKNOWLEDGEMENTS

I would like to thank the following people who made the task of completing this work easier:

Dr. Nikolaus H. Fischer for his support, guidance, patience and for imparting a lot of wisdom and good sense; Mrs. Helga Fischer for taking care of us;

Dr. C. Michael Smith for his help and guidance in conducting the biological part of this work; Dr. Ben H. Lye and Maureen Riley for their help in planting the rice and in conducting the assays;

Dr. Francisco Macias for introducing me to flavonoid chemistry;

Dr. Thomas C.-Y. Hsieh and coworkers for their assistance and for allowing me to use the laboratory facilities for the analysis of rice volatiles;

Linda Gauthier for an excellent typing job;

All my friends and housemates for providing friendship, company and much-needed humor that made this task a very pleasant experience;

My family for their moral support and prayers, and most of all to my husband, Joe, whose unselfishness, respect and trust sustained and inspired me all the time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The first part of this work was conducted to search for allelochemicals from the rice varieties PI346833 (moderately resistant) and Mars (susceptible) that may be responsible for resistance to fall armyworm, *Spodoptera frugiperda* (J. E. Smith) feeding. Mature tillers and seedlings were extracted with petroleum ether (PE), dichloromethane (DCM), acetone, methanol and water. The search for biological activity towards fall armyworm (FAW) was directed by growth inhibition bioassay. Significant antibiotic activity to FAW was observed with the PE and DCM crude extracts. Bioassay of the fractions of the PE and DCM extracts indicated that the fractions from PI346833 seedlings adversely affected the development of FAW, mainly by blocking pupation. Purification of one of the active fractions (PE-4) led to the isolation of the sitosterols β-sitosterol, stigmasterol and campesterol and, the triterpene isoarborinol. Dose-response bioassays with authentic stigmasterol and a sitosterol mixture adversely affected the development of FAW but did not show a positive correlation between dose and antibiosis.

Volatile compounds of the rice seedlings were collected by dynamic headspace sampling and trapping on Tenax TA. Thermal desorption and cryogenic focusing were used to introduce the volatile compounds to the column for analysis by gas chromatography-mass spectrometry. Among the compounds detected, twenty-eight compounds

were identified. Mars contained numerous and greater quantities of volatile compounds compared to PI346833. Twelve compounds were found only in Mars and two were found only in PI346833.

The second part of this research dealt with the isolation and identification of the major external flavonoids from leaves of *Calamintha ashei* (Lamiaceae). A new flavone, 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**1**), a new natural aglycone, 5,6,4'-trihydroxy-7,3'-dimethoxyflavone (**2**), 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone (**3**) and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (**4**) were identified by spectroscopic methods.

The NMR method INAPT (Insensitive Nuclei Assigned by Polarization Transfer) was used and demonstrated as a new general method of solving problems in flavonoid structure determination. INAPT was found to be a sensitive and selective method of distinguishing among 5,6,7-, 5,7,8-substituted flavonol/flavone and 5,6,7,8-substituted flavone.

PART I

SEARCH FOR ALLELOCHEMICALS IN RICE (*ORYZA SATIVA* L.)

GENERAL INTRODUCTION

The breeding of plants resistant to disease and insect pests is a very effective way of minimizing pest management problems. To accomplish this, knowledge of both allelochemical and agronomic factors are important for the identification of resistant varieties in plant breeding programs. The roles of plant allelochemicals in plant-insect interaction have received considerable attention during the past years (Hedin 1986). Allelochemicals are phytochemicals that affect the behavior, growth, health or physiology of insects (Whittaker and Feeny 1971). For this reason, identifying allelochemicals and understanding their roles in the mechanisms of insect-plant interactions are important in the development of resistant plants and in the management of insect pests.

Allelochemicals can have negative (allomones) or positive (kairomones) effects on insects (Whittaker and Feeny 1971). As a consequence, allelochemicals can act as repellants/attractants, feeding deterrents/stimulants, or oviposition deterrents/stimulants. Most of the allelochemicals shown to confer resistance or susceptibility to plants belong to the secondary plant metabolites (Hedin 1986). Both volatile and nonvolatile compounds play a significant role as allelochemicals.

In recent years, scores of allelochemicals have been isolated, structurally identified, and bioassayed for biological activity against insects. Hexanol, hexenols and hexenal isolated from potato leaves have been observed to attract the Colorado potato beetle *Leptinotarsa decemlineata* (Visser 1983). Sinigrin, a glucosinolate in crucifers, is a feeding deterrent to *Papilio polyxenes* but a feeding stimulant to cabbageworm *Pieres rapae* (Renwick 1983). Gossypol in cotton increases the resistance of this important crop to several pests (Seigler 1983). Many other plants have been investigated for their allelochemical content.

Rice (*Oryza sativa* L.) is a very important world food crop. It is the staple food in Asia. One of the major factors affecting rice production is pest management. Rice is infested by more than 60 diseases and attacked by 100 insect species, rats, birds and weeds. To solve these problems, the development of rice varieties resistant to pests has been given emphasis (Chaudhary et al. 1984).

Several studies have been conducted on the isolation and testing of allelochemicals in rice. Oryzanone (para-methylacetophenone), isolated from an insect-susceptible rice variety, is an attractant of adults and larvae of the striped stem borer, *Chilo suppressalis* Walker (Munakata et al. 1959). In the presence of high concentrations of benzoic acid and salicylic acid, the growth of stemborer larvae is inhibited (Ishii et al. 1962). On the other hand, salicylic acid is a probing and oviposition stimulant of the brown planthopper, *Nilaparvata lugens* Stal (Sekido and Sogawa 1976). Other probing stimulants of the brown planthopper include the

flavonoids orizatin and homoinetin (Sogawa 1976) and eight C-glycosyl flavones (Kim et al. 1985, Besson et al. 1985) which were also isolated from rice. Other studies on rice resistance to brown planthopper indicated that oxalic acid and maleic acid (Yoshihara et al. 1980) and β-sitosterol (Shigematsu et al. 1982) act as sucking inhibitors.

More recent research on volatiles from rice have been reported to affect the behavior of rice insect pests. Obata et al. (1983) isolated and identified 27 compounds from the susceptible rice 'Nihonbare'. Among the compounds identified, a mixture of carbonyl compounds and isocyanurate were found to be potent attractants to the brown planthopper. However, minor unidentified volatile compounds may be more potent attractants. Steam distillates from resistant and susceptible rice varieties have been tested for their effects on various rice pests. The steam distillate from a resistant rice variety was noted to change the behavior of the green leafhopper, *Nephotettix virescens* from phloem feeding to xylem drinking (Khan and Saxena 1985). Steam distillates also have significant effects on the behavior and physiology of striped stemborer larvae and brown planthoppers (Saxena 1986). However, no compounds from these steam distillates were identified.

Studies on the allelochemicals responsible for the resistance of rice to insects can be difficult. Chemical factors in the rice plant vary both in quantity and quality depending on the variety, cultural conditions and growth stages of the plant (Ishii et al. 1962). Moreover, allelochemicals are often present in very low

concentrations and activity is usually observed at a level much higher than those normally found in the plant. Ishii et al. (1962) demonstrated that benzoic acid and salicylic acid are highly active at more than 3% dry weight but the natural level of these acids in the plant is just 0.065% and 0.013%, respectively. Yoshihara et al. (1979) also found that crude extracts from the leaf sheaths of both resistant and susceptible rice varieties were equally inhibitory to sucking by the brown planthopper. It seems that the differences in the profile of allelochemicals between rice varieties are very subtle.

CHAPTER 1

ALLELOCHEMICALS FROM ORGANIC EXTRACTS OF RICE VARIETIES VARYING IN SUSCEPTIBILITY TO DEFOLIATION BY THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J. E. SMITH)

INTRODUCTION

The resistance of rice to fall armyworm, *Spodoptera frugiperda* (J. E. Smith) was identified in several varieties and its mode of resistance determined (Pantoja et al. 1986). In this work, plant introductions (PI) 346833 and PI160842 were found to have moderate resistance to fall armyworm (FAW). PI160842 was less preferred and had low defoliation. PI346833 also had low defoliation, but was highly preferred. 'Mars', a commonly cultivated variety that is highly preferred and highly defoliated, was used as a susceptible check.

The low defoliation and high preference for PI346833 indicate that its mode of resistance is antibiosis. On the other hand, the resistance of PI160842 was concluded to be due to non-preference (Pantoja et al. 1986).

Resistance by antibiosis is shown by the effect of allelochemicals on the growth and development of the insect pest. Antibiosis is manifested by reduced larval weight, reduced pupal weight, mortality, reduced fecundity and prolonged development (Seigler 1983).

This study was conducted to evaluate PI346833 and PI160842 for the allelochemical(s) responsible for resistance to FAW. Mars was

used as the susceptible check. Growth inhibition bioassays were conducted on the crude extracts and their fractions to monitor the isolation of the possible active allelochemical factor(s).

RESULTS AND DISCUSSION

Bioassays

Throughout the discussion of the bioassay results, the petroleum ether (PE) fractions will be referred to as PE-1, PE-2, etc., and the dichloromethane (DCM) fractions as DCM-1, DCM-2, etc.

Bioassays of crude rice extracts from mature rice foliage

Evaluation of the bioassay data (Table 1.1) showed that the PE, DCM and MeOH extracts had pronounced antibiotic activity. The acetone extract did not have an adverse effect on the development and survival of FAW.

Almost all of the larvae fed the PE and DCM extract diets were unable to pupate, resulting in very high mortality (90-100%), although larvae in these diets exhibited normal development. The weights of larvae fed these diets did not differ from those fed the control diet.

As the larvae fed extract diets grew older, they grew larger than larvae fed the control diet. Most of the larvae reached the prepupal stage. However, the larvae began to die on the eleventh and twelfth days of the experiment, the normal date of pupation. Some larvae reached an advanced prepupal stage and survived until the twenty-second day, but they were unable to pupate.

TABLE 1.1. Development and Survival of Fall Armyworm, *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Foliage Extracts from Mature Rice PI160842, PI346833, and Mars.

Plant Introduction and Extract	Mean Weight (mg) 7-Day Larvae**	Mean Weight (mg) Pupae*	Mean Life (days) Larvae**	Mean Life (days) Pupae*	Mortality %
PI160842					
Pet Ether	295a***	147a	12a	-----	100.0
DCM	285a	243bc	12a	7.3ab	90.0
Acetone	321a	247c	12a	7.5ab	10.0
MeOH	74b	249c	15b	7.6ab	19.4
PI346833					
Pet Ether	286a	266c	12a	7.3ab	90.9
DCM	302a	----	---	-----	100.0
Acetone	313a	245c	12a	7.6ab	16.7
MeOH	97b	259c	14b	8.0ab	23.3
'Mars'					
Pet Ether	259a	231c	12a	7.5ab	93.3
DCM	265a	220b	12a	8.0ab	93.3
Acetone	318a	249c	12a	7.4a	10.0
MeOH	96b	251c	14b	8.3b	29.0
Control	279a	255c	12a	7.7ab	6.7

*
*α = 0.01.
**α = 0.05.
Means in each column not followed by the same letter differ significantly as determined by Duncan's multiple range test.

The few larvae that pupated (1-4 larvae/treatment), successfully emerged as adults. One abnormal pupa was observed in each of the diets with PE and DCM extracts from PI160842. These abnormal pupae did not reach the adult stage.

The methanol extract from all varieties also showed a definite antibiotic effect (Table 1.1). Larvae had lower larval weights (26-35% of the control) and longer lives compared to the control and the other treatments. When larvae reached the pupal stage their development became normal, suggesting that the MeOH extract contains a growth inhibitor that the FAW can overcome. This was manifested by the low mortality rate.

The results of the particular bioassay indicate that the crude PE and DCM extracts have allelochemicals that are responsible for the high mortality of FAW. However, this activity was observed in both resistant and susceptible rice varieties. For this reason, the water extracts were tested.

Table 1.2 summarizes the bioassay results from the water extracts. The water extracts of PI346833, PI160842 and Mars reduced FAW larval weight and extended pupal life. However, the mortality observed was not as high as that with the organic extracts.

Since there were no differences observed with the antibiotic effects of the crude extracts from both the resistant and susceptible rice varieties, the extract dosage was reduced for subsequent assays of the extract fractions. Moreover, only PI346833 was used for the succeeding studies.

TABLE 1.2. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Water Extracts from Mature Foliage of Rice PI160842, PI346833 and Mars.

Rice	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
PI160842	144.2 ± 13.5b	225.3 ± 19.0a	13.9 ± 0.5a	9.6 ± 0.7a	16.7	16.7	33.4
PI346833	159.0 ± 23.0b	216.8 ± 25.5a	13.8 ± 0.5a	9.4 ± 0.8a	6.7	3.0	9.7
Mars	157.5 ± 21.5b	220.2 ± 25.1a	13.5 ± 0.5b	9.3 ± 2.2a	6.9	20.7	27.6
Control	257.1 ± 41.8a	199.4 ± 15.4b	12.9 ± 0.4c	8.2 ± 1.0b	6.7	6.7	13.4

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

TABLE 1.3. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Petroleum Ether (PE) Extract from Mature Rice PI346833.

Fraction	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
PE-1	135.4 ± 21.8b	221.5 ± 25.0b	14.2 ± 0.5d	9.0 ± 0.7bc	20.0	3.3	23.3
PE-2	139.3 ± 20.7b	228.3 ± 17.2ab	14.2 ± 0.5d	8.7 ± 1.0c	0	10.3	10.3
PE-3	149.7 ± 23.4a	226.4 ± 25.6ab	13.8 ± 0.6e	8.6 ± 0.9c	0	3.3	3.3
PE-4	68.2 ± 17.9c	229.1 ± 24.5ab	15.4 ± 0.8ab	9.3 ± 0.9ab	0	0	0
PE-5	71.1 ± 20.1c	239.6 ± 17.8a	15.3 ± 0.5b	9.7 ± 0.9a	16.7	6.7	23.7
PE-6	131.0 ± 22.4b	229.9 ± 31.7ab	14.5 ± 0.6c	9.3 ± 1.0ab	3.3	13.3	16.6
Control	55.5 ± 13.9d	233.0 ± 19.0ab	15.6 ± 0.6a	9.5 ± 0.9ab	10.0	6.7	16.7

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

Bioassays of Fractions from the Mature Foliage Extracts

Table 1.3 summarizes the data on growth inhibition by the PE fractions from PI346833. The PE fractions showed no adverse effects on the growth and development of FAW. Compared to the control, the PE extract fractions promoted higher larval weights, and the pupal weights were statistically the same. There was a significant increase in pupal life of those FAW reared with fractions PE-1, PE-4, PE-5, and PE-6, but the mortality rate was low.

Like the PE fractions, the DCM fractions from mature PI346833 tillers had little activity (Table 1.4). All of the fractions caused significantly higher larval weights. Only fractions DCM-5 and DCM-6 caused a significant reduction in pupal weight but the mortality rates of larvae fed these fractions were lower than those fed the control diet.

Compared to the assay results on the PE and DCM extract fractions from mature PI346833 foliage, the fractions from mature Mars tillers caused more adverse effects on the growth and survival of FAW (Table 1.5).

Mars fractions PE-5 caused the highest mortality rate (76.7%). The FAW larvae grown with this fraction also exhibited significantly lower weights. Likewise, larval life and pupal life were significantly higher than those of the control and the other treatment diets. Next to PE-5, PE-1 and PE-4 also caused high mortality rate (43.3% for each). The larval weights, pupal weights and larval life were statistically the same for PE-1 and PE-4 as the control.

TABLE 1.4. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Dichloromethane (DCM) Extract from Mature Foliage of Rice PI346833.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
DCM-1	92.0 ± 23.0b	225.9 ± 17.7bc	14.8 ± 0.6bc	8.9 ± 0.9bc	0	3.3	3.3
DCM-2	132.5 ± 19.1a	221.2 ± 19.1bcd	14.3 ± 0.5c	8.7 ± 0.8c	13.3	3.3	16.6
DCM-3	98.5 ± 21.3b	227.0 ± 21.2bc	14.8 ± 0.5bc	8.8 ± 0.9c	6.9	3.4	10.3
DCM-4	102.9 ± 25.0b	242.9 ± 19.2a	14.8 ± 0.5bc	9.2 ± 0.8abc	6.7	3.3	10.0
DCM-5	96.0 ± 27.2b	212.3 ± 31.0d	15.1 ± 2.1b	9.4 ± 1.2ab	6.7	6.7	13.4
DCM-6	101.2 ± 26.1b	219.8 ± 24.4cd	15.0 ± 0.7b	8.9 ± 1.2bc	0	3.4	3.4
Control	55.5 ± 13.9c	233.0 ± 19.0ab	15.6 ± 0.6a	9.5 ± 0.9a	10.0	6.7	16.7

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

TABLE 1.5. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Petroleum Ether (PE) Extract from Mature Foliage of Rice Variety Mars.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
PE-1	101.7 ± 19.0ab	225.6 ± 27.0a	14.4 ± 0.5d	9.2 ± 0.6ab	23.3	20.0	43.3
PE-2	67.6 ± 20.8c	225.1 ± 20.6a	15.1 ± 0.6b	8.8 ± 0.6bc	13.3	26.7	40.0
PE-3	107.1 ± 37.6a	224.3 ± 22.3a	14.4 ± 0.8d	8.8 ± 0.7bc	13.3	0	13.3
PE-4	89.3 ± 20.6b	225.2 ± 17.7a	14.5 ± 0.7cd	9.4 ± 0.6a	33.3	10.0	43.3
PE-5	32.2 ± 15.7d	222.0 ± 18.3a	16.2 ± 1.2a	9.2 ± 1.0ab	73.3	3.3	76.6
PE-6	70.2 ± 27.9c	224.9 ± 21.6a	14.9 ± 0.7bc	8.7 ± 0.5c	3.3	13.3	16.6
Control	88.7 ± 29.2b	229.8 ± 20.5a	14.6 ± 0.7cd	8.7 ± 0.8c	10.0	13.3	23.3

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

Among the DCM fractions from mature Mars foliage, fraction DCM-3 caused the highest mortality (56.7%), followed by DCM-6 (46.7%) and DCM-1 (43.3%) (Table 1.6). Larvae fed with DCM-3 amended diet had weights significantly higher than those fed the control diet, and larval life was shorter than on the control diet. The pupal life of larvae fed DCM-3 diet was not different from larvae fed control diet. The majority of the mortality on the DCM-3 diet occured in the larval and prepupal stages. The physiological effect of DCM-1 and DCM-6 differ from that of DCM-3. Larvae fed both DCM-1 and DCM-6 had greatly reduced weights (about one-third weights of larvae fed the control diet). The larval life in these fraction diets was also significantly longer than larvae fed the control diet. Pupal weights and pupal life were similar to that on control diet. Mortality in larvae fed both fractions was mainly due to failure of pupae to emerge (5 in DCM-1, 8 in DCM-6). Both fraction diets also had four larvae that failed to pupate.

PE and DCM extracts of mature rice foliage of Mars caused greater adverse physiological effects on FAW larvae than similar extracts of PI346833. This is surprising since Mars is a FAW susceptible variety. It is not unusual though for a susceptible variety to show some adverse effects on an insect pest. As Reese (1983) points out, even a susceptible plant contains some defensive factors against pests. Likewise, Yoshihara et al. (1979) also found that crude extracts from both resistant and susceptible varieties contain sucking inhibitors of brown planthopper.

TABLE 1.6. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Dichloromethane (DCM) Extract from Mature Foliage of the Rice Variety Mars.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
DCM-1	30.6 ± 10.2e	228.9 ± 29.1a	16.2 ± 0.8a	8.9 ± 0.8abc	30.0	13.3	43.3
DCM-2	59.0 ± 22.7c	226.0 ± 21.9ab	15.2 ± 0.8b	8.7 ± 0.6bc	6.7	6.7	13.4
DCM-3	118.9 ± 28.7a	214.3 ± 21.5b	14.0 ± 0.8d	9.2 ± 0.6ab	40.0	16.7	56.7
DCM-4	59.3 ± 23.0c	234.3 ± 21.5a	15.4 ± 0.7b	8.9 ± 1.0abc	13.3	13.3	26.6
DCM-5	44.3 ± 21.6d	228.4 ± 24.1a	15.9 ± 1.1a	8.6 ± 0.8c	23.3	13.3	36.6
DCM-6	28.4 ± 11.2e	229.5 ± 20.5a	16.2 ± 1.1a	9.3 ± 1.0a	36.7	10.0	46.7
Control	88.7 ± 29.2b	229.8 ± 20.5a	14.6 ± 0.7c	8.7 ± 0.8bc	10.0	13.3	23.3

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

Bioassays of Fractions from Seedling Extracts

Rice seedling extracts were also bioassayed, since the antibiotic effect of organic extracts from the mature foliage of PI346833 were less than that of Mars and because resistance to FAW in PI346833 was identified in the seedling stage (Pantoja et al. 1986).

Among the PE fractions from PI346833 seedlings, PE-6 and PE-4 caused the highest mortality (83% and 73%, respectively), followed by PE-3 and PE-5 (47% and 50%, respectively) (Table 1.7). Growth of FAW larvae fed PE-6 diet was most adversely affected at the prepupal stages, as indicated by reduction in larval weight and the significant increase in larval life. In the PE-3, PE-4, and PE-5 amended diets, larval and pupal weights and development times were similar to or better than those larvae fed control diet, but mortality was high in the pupal stage.

From the DCM extract, four fractions were detrimental to the survival of FAW (Table 1.8). Larvae fed diets amended with DCM-6 suffered high mortality (90%), greatly reduced larval weights, and greatly prolonged larval life. DCM-1 also caused considerable FAW mortality (70%), reduced larval weights, and significantly longer larval life. The pupal weight of larvae fed DCM-1 was higher (α = 0.05) than larvae fed control diet, but 42% of the pupae did not emerge. This suggests that greater pupal weight is not a good index of pupal viability.

TABLE 1.7. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Petroleum Ether (PE) Extract from Rice PI346833 Seedlings.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
PE-1	213.7 ± 52.8ab	204.3 ± 19.8ab	12.5 ± 0.8bc	8.4 ± 0.6a	3.3	3.3	6.6
PE-2	223.3 ± 69.5ab	212.8 ± 22.5ab	12.4 ± 0.6bc	8.7 ± 0.6a	20.0	13.3	33.3
PE-3	234.1 ± 47.0a	211.3 ± 31.8ab	12.4 ± 0.5bc	8.8 ± 0.6a	33.3	13.3	46.6
PE-4	174.1 ± 53.0cd	209.8 ± 28.4ab	13.1 ± 1.1a	8.8 ± 0.9a	56.7	16.7	73.4
PE-5	165.1 ± 46.3de	198.9 ± 26.5b	12.8 ± 0.4ab	8.6 ± 0.6a	40.0	10.0	50.0
PE-6	143.1 ± 52.9e	221.2 ± 15.3a	13.1 ± 0.5a	8.9 ± 0.9a	63.3	20.0	83.3
Control	197.8 ± 58.6bc	208.1 ± 21.5ab	12.1 ± 1.1c	8.6 ± 0.9a	6.7	16.7	23.4

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

TABLE 1.8. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Dichloromethane (DCM) Extract from Rice PI346833 Seedlings.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
DCM-1	169.2 ± 46.6cd	230.5 ± 18.5a	12.9 ± 0.3bc	8.3 ± 0.7ab	53.3	16.7	70.0
DCM-2	174.1 ± 53.0bc	208.3 ± 21.9c	12.6 ± 0.7c	8.6 ± 0.6ab	6.7	30.0	36.7
DCM-3	204.4 ± 63.3a	223.3 ± 20.9ab	12.6 ± 0.9c	8.9 ± 1.3a	26.7	26.7	53.4
DCM-4	146.2 ± 40.6d	222.2 ± 23.6abc	13.1 ± 0.5b	8.3 ± 0.8ab	13.3	3.3	16.6
DCM-5	188.2 ± 40.5abc	218.0 ± 27.3abc	12.7 ± 0.6bc	8.2 ± 0.6b	6.7	16.7	23.4
DCM-6	83.1 ± 46.5e	210.1 ± 18.2bc	15.2 ± 1.0a	8.6 ± 1.0ab	70.0	20.0	90.0
Control	197.8 ± 58.6ab	208.1 ± 21.5c	12.1 ± 1.1d	8.6 ± 0.9ab	6.7	16.7	23.3

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

The bioassays with the PE and DCM fractions from Mars seedlings indicated less biological activity in these extract fractions than in similar fractions from PI346833 extracts (Table 1.9). The highest mortality observed among the PE fractions was 33.4% (PE-6) which was mainly due to adult mortality. Note that with PE-6, the larval weight, larval life and pupal life are adversely affected, but this did not cause high mortality. In all the treatments with PE fractions, more than half of the mortality rates were due to abnormal and/or short-lived adults. In the assays with DCM fractions (Table 1.10), only DCM-3 had an adverse effect on FAW biology, including a 43% mortality rate.

The overall activity of the fractions from mature PI346833 and Mars tillers was lower than the activity observed with the fractions from the seedlings. These results indicated that qualitative or quantitative allelochemical content of seedling and mature foliage is different. Also, the higher biological activity of some PE and DCM fractions from PI346833 seedlings suggests that PI346833 contains allelochemicals that contribute to its resistance to defoliation by FAW.

The adverse effects of the active PE and DCM fractions from both PI346833 and Mars are consistent with the effects observed with the crude extracts. The same effects such as failure to pupate and death of pupa were the major causes of mortality. It was observed that larval death occurred at the prepupal stage when the larva has burrowed into the diet. At this stage, the larval size has been greatly reduced. In some cases tanning of the dorsal part of the

TABLE 1.9. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Petroleum Ether (PE) Extract from Rice Mars Seedlings.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
PE-1	47.7 ± 14.0b	221.7 ± 21.1ab	16.1 ± 0.7c	10.4 ± 1.0a	0	0	0
PE-2	45.5 ± 12.1b	225.1 ± 17.6ab	16.1 ± 0.7c	10.8 ± 0.9a	3.3	20.0	23.3
PE-3	48.0 ± 15.6b	220.3 ± 20.3b	16.3 ± 1.0bc	10.4 ± 1.0a	10.0	13.3	23.3
PE-4	36.1 ± 6.1c	222.4 ± 22.0ab	17.9 ± 0.8a	10.3 ± 0.6a	10.0	16.7	26.7
PE-5	52.5 ± 11.2ab	226.6 ± 20.9ab	16.7 ± 0.8b	10.7 ± 0.8a	0	0	0
PE-6	48.2 ± 13.5b	228.8 ± 24.2ab	16.3 ± 1.0bc	10.4 ± 0.8a	6.7	26.7	33.4
Control	55.5 ± 13.9a	233.0 ± 19.0a	15.6 ± 0.6d	9.5 ± 0.9b	10.0	6.7	16.7

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

TABLE 1.10. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Fractions of Dichloromethane (DCM) Extract from Rice Mars Seedlings.

Fractions	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
DCM-1	46.1 ± 7.9c	223.6 ± 18.7bc	16.2 ± 0.8b	10.5 ± 1.2ab	6.7	6.7	13.4
DCM-2	56.9 ± 16.2b	224.0 ± 19.2bc	16.0 ± 0.6bc	10.2 ± 1.0b	0	3.3	3.3
DCM-3	26.0 ± 10.0d	218.3 ± 12.0c	18.0 ± 0.8a	11.0 ± 1.1a	16.7	26.7	43.4
DCM-4	51.6 ± 14.6bc	224.1 ± 16.8bc	16.3 ± 0.9b	10.5 ± 0.9ab	0	0	0
DCM-5	71.1 ± 20.0a	240.4 ± 29.0a	15.4 ± 0.6d	10.3 ± 0.9b	6.7	3.3	10.0
DCM-6	26.8 ± 9.1d	226.7 ± 19.0bc	18.0 ± 0.8a	10.8 ± 1.0ab	3.3	3.3	6.6
Control	55.5 ± 13.9b	233.0 ± 19.0ab	15.6 ± 0.6cd	9.5 ± 0.9c	10.0	6.7	16.7

Means followed by the same letter are not significantly different at $\alpha = 0.05$ in Duncan's multiple range test.

head was observed. Soft and deformed pupae were also observed. The larval weight, pupal weight, larval life and pupal life were also affected but adverse effects on these biological parameters did not always lead to high mortality. This was demonstrated by most of the PE fractions from Mars seedlings.

The extract dosage also affected the physiological activity on FAW larvae. When the amount of the crude extract supplement in the diet was twice the amount that occurred in the rice foliage, mortality was observed at the prepupal stage. However, when the amount of the extract supplement was similar to that found in the plant, mortality was observed at both the prepupal and the pupal stage. Thus, these allelochemicals block pupation at higher dosage but at lower dosage, it also causes pupal mortality.

Based on the observed biological activity in the bioassays, it is possible that these active compound(s) specifically affects the endocrine mediated stages in insect development. An anti-juvenile hormone called precocene II has been reported by Mathai and Nair to cause precocious metamorphosis in armyworm (Bowers, 1985). This same compound has been found by Cupp et al. (1977) to inhibit pupation in the yellow fever mosquito, *Aedes aegypti*. How the allelochemicals from rice compare with the activity of the precocene II has to be verified.

Dose Response Bioassays of Sitosterols

The fractions from PI346833 seedlings (Table 1.7) that showed the highest activity were PE-6 (83.3%) and DCM-6 (90.0%), followed by

PE-4 (73.4%). The isolation and purification of compounds from PE-6 and DCM-6 proved to be very difficult due to its gummy consistency and high polarity. Thus, purification of PE-4 was performed, leading to the isolation of a mixture of the phytosterols: β-sitosterol, stigmasterol and campesterol and the triterpene isoarborinol. To verify the activity of these sterols, a dose response bioassay was performed on the authentic standards of stigmasterol and the mixture of β-sitosterol, stigmasterol and campesterol.

Some stigmasterol amended diets caused higher FAW mortality than the control diet, however mortality among all stigmasterol doses ranged from 17-60% (Table 1.11). A similar range of effects was noted among diets amended with different concentrations of the B-sitosterol mixture (23-70%) (Table 1.12). As observed previously with the rice foliage extracts, the adverse effects were noted at or after the prepupal stage.

Although the treatments in the dose-response assay showed higher mortality compared to the control, regression analysis showed no effect of sterol dose on the FAW growth parameters measured. The data indicate that the B-sitosterol mixture is slightly more toxic than stigmasterol alone. This may be due to the higher activity of pure B-sitosterol or pure campesterol or the mixture.

These phytosterols are needed by insects for normal growth and development, and specifically for the biosynthesis of ecdysteroids. At higher dosage, it is possible that the FAW larvae either sequester or detoxify excess sterols ingested in the diet. FAW larvae detoxify these sterols using its mixed function oxidases (MFO) in the presence

TABLE 1.11. Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Stigmasterol.

Stigmasterol ppm	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
125	289.2 ± 56.8	200.0 ± 19.8	12.1 ± 0.5	8.0 ± 0.7	16.7	23.3	40.0
250	227.5 ± 48.8	223.4 ± 23.8	12.4 ± 0.7	8.1 ± 0.8	30.0	3.3	33.3
500	239.2 ± 82.1	211.6 ± 27.0	12.8 ± 0.7	8.3 ± 0.8	10.0	20.0	30.0
1000	221.1 ± 49.1	221.4 ± 19.2	12.3 ± 0.6	8.2 ± 0.8	26.7	26.7	53.4
1500	164.7 ± 47.7	223.6 ± 23.7	13.0 ± 0.7	8.6 ± 0.8	40.0	20.0	60.0
2000	171.3 ± 31.5	217.1 ± 20.6	12.8 ± 0.6	8.1 ± 0.7	6.7	10.0	16.7
4000	285.8 ± 68.6	228.0 ± 20.4	12.3 ± 0.5	8.5 ± 0.8	36.7	16.7	53.4
Control	147.0 ± 30.7	224.5 ± 20.2	13.6 ± 0.7	8.8 ± 1.2	20.0	13.3	33.3

TABLE 1.12. **Development and Survival of Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) Reared on Diet Supplemented with Sitosterol Mixture.**

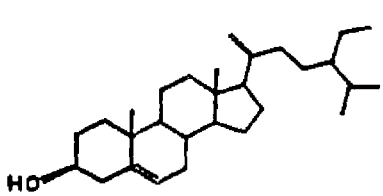
β-sitosterol ppm	Mean Weight (mg)		Mean Life (Day)		% Mortality		
	8 Day Larvae	Pupae	Larvae	Pupae	Larvae/Pupae	Adult	Total
125	211.3 ± 53.5	225.5 ± 26.6	12.8 ± 0.6	8.8 ± 0.4	46.7	16.7	63.3
250	120.2 ± 27.9	213.2 ± 13.5	14.0 ± 0.5	8.8 ± 0.7	26.7	16.7	43.3
500	211.4 ± 42.2	226.1 ± 24.8	12.6 ± 0.6	8.1 ± 0.9	6.7	23.3	30.0
1000	186.2 ± 40.2	224.9 ± 22.6	13.0 ± 0.5	8.6 ± 0.8	23.3	13.3	36.7
1500	205.9 ± 41.0	229.7 ± 29.5	12.9 ± 0.8	8.5 ± 1.0	56.7	0	56.7
2000	142.4 ± 28.7	219.6 ± 19.5	13.8 ± 0.7	8.7 ± 0.6	56.7	13.3	70.0
4000	114.8 ± 32.2	199.3 ± 32.7	14.5 ± 1.5	8.1 ± 0.9	10.0	13.3	23.3
Control	147.0 ± 30.7	224.5 ± 20.2	13.6 ± 0.7	8.8 ± 1.2	20.0	13.3	33.3

of more potent MFO inducers (Yu 1987). If these sterols are used by insects for the biosynthesis of ecdysteroids, it is possible that the physiological balance may be disturbed by producing too much ecdysteroid, especially if no detoxification mechanism is used.

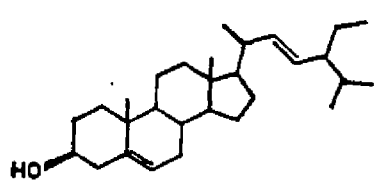
Isolation and Identification of Sitosterols

Among the active fractions, PE-4 was analyzed further for its constituents. PE-4 is composed mainly of triterpenoid compounds as indicated by the ^{1}H NMR of this fraction (Figure 1.1).

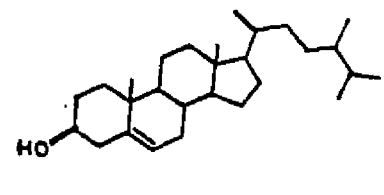
The main components of PE-4 were found to be the sitosterols. The MS data on this mixture showed molecular ions at 414, 412 and 400 which correspond to β-sitosterol, stigmasterol and campesterol, respectively. The identification of the three components were performed by the silylation of the mixture and the subsequent analysis of the silyl derivatives by GC-MS. Comparison of the NMR and GC-MS data of the rice sterols with the data from the authentic sample confirmed their identity.



β-Sitosterol



Stigmasterol



Campesterol

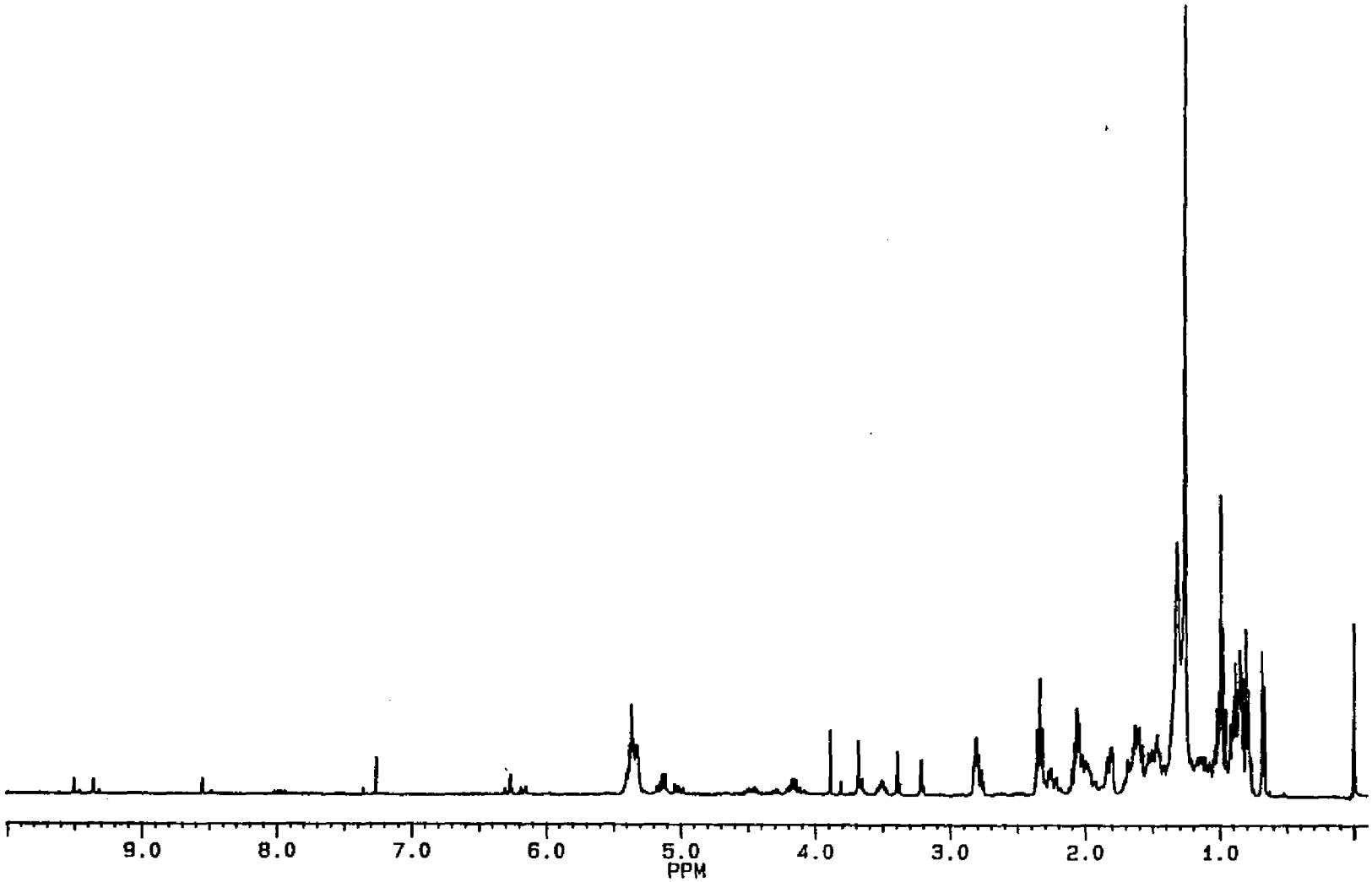


Figure 1.1. ^{1}H NMR spectrum of fraction PE-4 from PI346833 seedling.

Isolation and Identification of Isoarborinol

Another component isolated from the PE-4 fractions was a white solid which appeared to be less polar than the sitosterols. The ^{1}H NMR data indicated the presence of six methyl groups bonded to quaternary carbons and two methyl groups bonded to a tertiary carbon. These two methyl groups appeared as doublets at δ0.82 and δ0.89 and had the same coupling constant (J = 6.9 Hz) indicating the presence of an isopropyl group. A doublet at δ5.23 (J = 6.1 Hz) indicated the presence of an olefinic proton while the doublet of a doublet at δ3.21 (J = 4.1, 11.5 Hz) suggested the presence of a proton next to an alcohol group. The complex multiplet signals at the δ1.2 - 2.1 area which integrates for twenty-four protons indicated the presence of methylene and methine protons. These NMR data indicated that the molecule is a triterpene.

The ^{13}C NMR data (Figure 1.2 and Table 1.13) allowed for the determination of the number of carbons in the molecule. Moreover, DEPT (Distortionless Enhancement by Polarization Transfer) experiments allowed for the determination of the carbon multiplicities in the molecule (Derome 1987). The carbon doublet at δ114.3 and the carbon singlet at δ148.8 confirmed the presence of a trisubstituted alkene moiety. Likewise, the doublet at δ78.9 suggested the presence of a secondary alcohol.

The mass spectral data of this compound was very similar to the fragmentation pattern of the triterpene isoarborinol (Budzikiwiecz et al. 1963, Obafemi et al. 1979). Comparison of the melting point (Vorbruggen et al., 1963) and the ^{1}H NMR data (Nes et al. 1984) further confirmed the identity of the compound isolated.

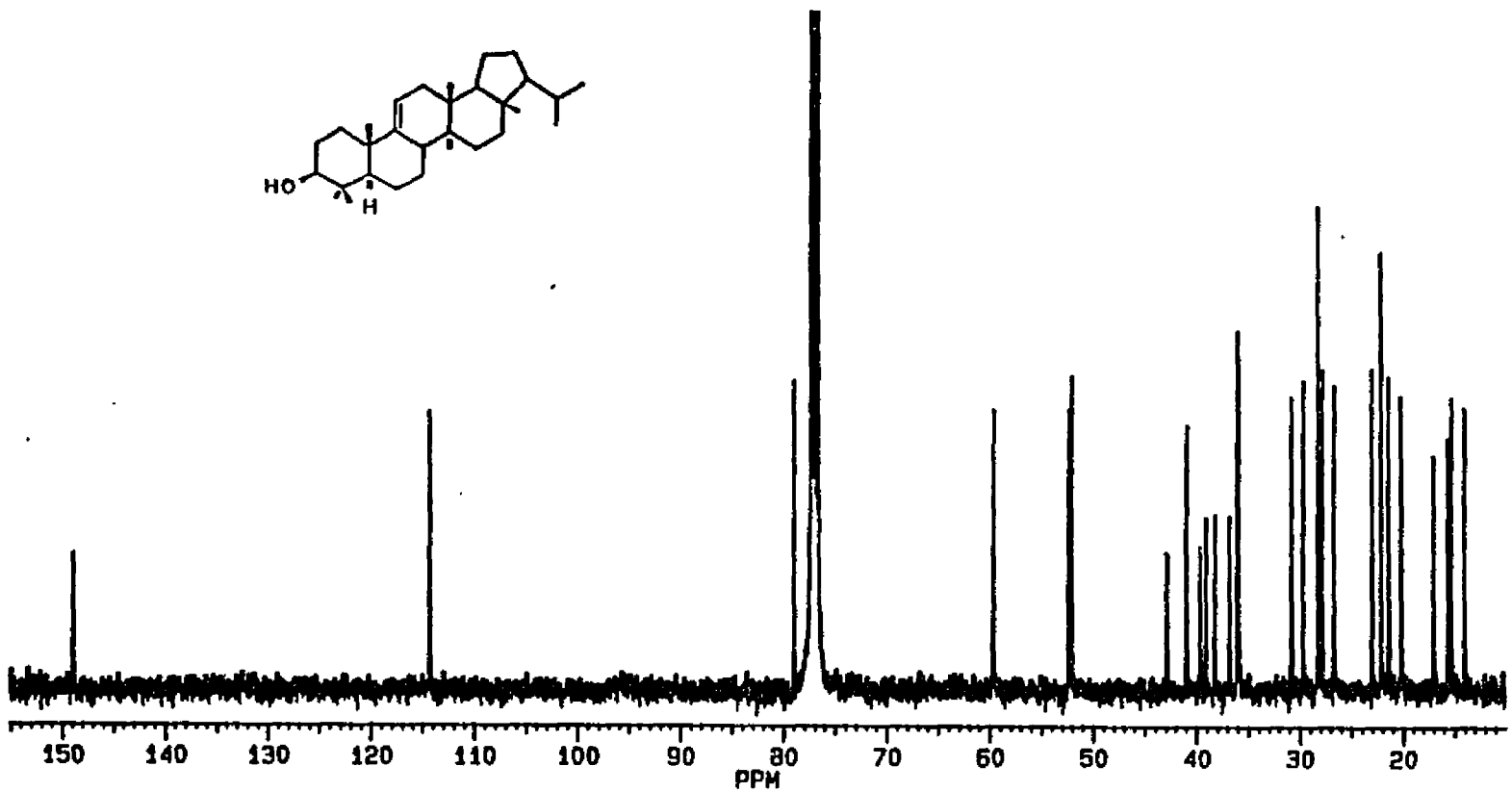


Figure 1.2. ^{13}C NMR Spectrum of isoarborinol.

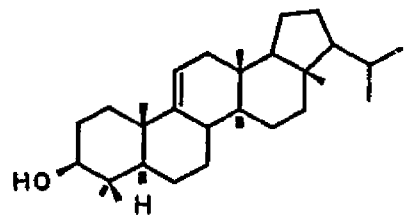
TABLE 1.13. ^{13}C NMR Spectral Data[a] of Isoarborinol.[b]

Carbon	δ, ppm	Carbon	δ, ppm
1	35.9 t	16	36.0 t
2	27.8 t	17	42.8 s
3	78.9 d	18	52.1 d
4	39.0 s	19	20.2 t
5	52.3 d	20	28.2 t
6	21.4 t	21	59.6 d
7	28.2 t	22	30.7 d
8	41.0 d	23	26.7 q
9	148.8 s	24	15.6 q
10	39.6 s	25	22.1 q
11	114.3 d	26	17.0 q
12	36.0 t	27	15.3 q
13	36.8 s	28	14.0 q
14	38.2 s	29	22.1 q
15	29.6 t	30	23.0 q

[a]Solvent: $CDCl_3$, TMS as internal standard. Carbon multiplicity was determined by DEPT NMR experiments.

[b]Assignment based on ^{13}C assignments of cylindrin (Blunt and Munro 1980).

from rice plants as isoarborinol. The assignment of the carbon signals was performed by comparison with the ^{13}C assignment of cylindrin, the methyl ether of isoarborinol (Blunt and Munro 1980). Isoarborinol has been isolated from the root and leaf callus tissues of rice (Yanagawa et al. 1972).



Isoarborinol

CONCLUSIONS

It is apparent that the PE and DCM extracts from rice foliage contain allelochemicals that cause adverse effects on the development of FAW and high mortality rates at the prepupal and pupal stages of growth. The higher activity of the PE and DCM extracts from PI346833 seedlings indicates that these extracts contain allelochemicals that may be responsible for moderate resistance to FAW defoliation. However, it is difficult to determine the specific varietal allelochemical factor that confers resistance to PI346833 because of the similar chemical profile of both PI346833 and Mars. To gain a better understanding of the chemical profile of these two rice varieties, the study of volatile allelochemicals emitted by rice to its environment will be important.

It is clear that the sitosterols and stigmasterol can cause deleterious effects on the growth of FAW. However, the growth inhibition bioassay results did not show a positive correlation between dose and toxicity.

EXPERIMENTAL

Plant Material

Rice plants were grown in the Louisiana State University greenhouse on Perkins Road, Baton Rouge, Louisiana. The plants were maintained in a flooded state. The mature tillers were harvested after eleven weeks at the prebooting stage while the seedlings were harvested at the four-leaf stage (Yoshida, 1981). The tillers were cut above ground, washed with water to remove soil, dried in a drying chamber at 60°C for 5 hours and ground in a Wiley mill.

Extraction

Ground rice samples were soaked in petroleum ether (110 g/l), filtered and the residue washed with fresh solvent twice. The extraction was repeated twice. The combined filtrate was concentrated in a rotary evaporator under vacuum at 30°C until a thick syrup was obtained. The plant residue was dried after extraction and then extracted in the same manner with DCM, acetone and methanol (MeOH).

Eighty grams of rice residue was soaked in ca 550 ml water and filtered. The water extract volume was adjusted to yield 283.3 ml to substitute the water part in the pinto bean diet (80 g dry wt.). The bioassay was carried out as described below for crude extracts except that no α-cellulose was used.

Fractionation of Crude Extracts

A crude PE extract from 40 g dry rice tiller was fractionated by vacuum liquid chromatography (Coll and Bowden 1986) on a 2.7 cm ht x 4 cm. o.d. column of TLC silica gel (25 g silica gel/g extract). The extract was introduced to the column by pre-adsorption. Fractionation of the PE crude extract was carried out with the solvents: hexane, hexane-benzene (1:1), benzene, DCM, ethy ether and MeOH. For the DCM crude extract the solvents used were: hexane, benzene, DCM, DCM-ethyl ether (1:1), and MeOH. In each case, ca. 10 ml solvent per 0.1 g extract were used, except for the elution with DCM, where up to 15 ml per 0.1 g extract was used. Six fractions (PE-1 to PE-6; DCM-1 to DCM-6) were collected and used for the bioassay experiments.

Bioassays of Crude Extracts

Extracts from 80 g dry mature rice foliage were bioassayed by dissolving the PE, DCM, acetone and MeOH extracts in DCM, acetone, water-acetone, and water, respectively. The solutions were added to 5.4 g α- cellulose, dried in a rotary evaporator (MacMillian et al. 1969) and then by a vacuum pump. Water was then added to make a thick slurry which was added to ca 250 ml of warm liquid pinto bean diet (Smith and Fischer 1983). Control diet was prepared by adding 5.4 g of α- cel treated with either DCM or acetone instead of the α-cellulose + extract mixture.

The diets were dispensed into polystyrene diet cups (30/treatment) and allowed to cool at room temperature for 4 hours. One neonate FAW larva (1 hr old) was introduced to each cup and the cups were capped with paper lids. All treatments were arranged randomly in trays and kept in a humid chamber at 28°C and 14:10 hr light:dark photoperiod. FAW growth and development were monitored by measuring its larval weight, pupal weight, larval life, pupal life and mortality. The mortality data were recorded as mortality in the larval and pupal stage and mortality due to emergence of abnormal and/or short-lived adults (less than one day). The data were subjected to a one-way analysis of variance and the means were separated by Duncan's multiple range test using Kramer's adjustment for unequal numbers of observations.

Bioassays of Extract Fractions

Fractions of the crude extracts from 40 g dry rice foliage were dissolved in DCM, dried under a gentle stream of nitrogen gas and then dried further using a vacuum pump. Pinto bean diet containing 40 g dry wt were prepared with the extract mixture and dispensed into 31 diet cups. Egg masses of FAW were stapled on the paper cups and allowed to hatch in one cup of treatment or control diet. Meanwhile, the other diets were kept in the refrigerator. The larvae were transferred to each diet cup [warmed to room temperature (RT)] four days after hatching. The same conditions were used as in the bioassay of the crude extracts.

Dose Response Bioassays of Sitosterols

Pinto bean diets containing 40 g dry ingredients were added with 5, 10, 20, 40, 60, 80, and 160 mg of authentic stigmasterol to formulate diets with sterol concentrations of 125, 250, 500, 1000, 1500, 2000, and 4000 ppm, respectively. The same amounts of the sitosterol mixture (β-sitosterol, stigmasterol and campesterol) were also used in a different experiment. α- Cellulose was not used. The same procedure for bioassay was used as described in the bioassays of extract fractions.

Isolation of Sitosterols and Isoarborinol

Fraction PE-4 (414 mg) was treated with activated carbon and filtered yielding a clear yellow solution. Vacuum liquid chromatography (VLC) of the solution using hexane and increasing polarity of hexane-DCM afforded 34 10 ml fractions. Preparative TLC of fractions 19-22 on a silica gel plate with 2% MeOH in DCM gave two spots corresponding to sitosterols (27.5 mg, Rf 0.54) and isoarborinol (10.2 mg, Rf 0.81).

Sitosterols. Colorless crystals; EIMS *m/z*: 414, 412, 400. The sterol mixture (0.4 mg) was placed in a screw cap vial with a teflon septum. Two hundred μl of Tri-Sil/TBT reagent (Pierce Chemical Co.) was introduced into the vial using a Hamilton syringe according to the method of Chambaz and Horning (1967). The mixture was allowed to stand at RT for at least 24 hr.

One μl of the silylated mixture was chromatographed using a polydiphenyldimethylsiloxane column (Alltech RSL-200) in a Hewlett Packard 5895 GC-MS instrument. GC conditions were: carrier gas He; initial temperature, 220°C (11 min); programming rate, 5°C/min; final temperature, 280°C (3 min); total run time 26 min. The GC retention time (min) and molecular ions were: campesterol 18.6, 400 mu: stigmasterol 19.3, 412 mu; β- sitosterol 20.5, 414 mu. An authentic sample of sitosterol mixture (Aldrich Co.) was analyzed in the same manner.

Isoarborinol. $C_{30}H_{50}O$, colorless solid; mp (uncorrected) 291-292°C [lit. (Vorbruggen et al. 1963) 295-296°C]; ^1H NMR (400 MHz, $CHCl_3$-d_1, TMS), δ in ppm: 0.76 (s, 28-CH_3), 0.77 (s, 25-CH_3), 0.81 (s, 24-CH_3), 0.82 (s, 23-CH_3), 0.98 (s, 27-CH_3), 1.03 (s, 26-CH_3), 0.82 (d, $J_{22,29}$ = 6.9 Hz, 29-CH_3), 0.89 (d, $J_{22,30}$ = 6.9 Hz, 30-CH_3), 3.21 (dd, $J_{2,3}$ = 4.1, 11.5, H-3), 5.23 (d, $J_{11,12}$ = 6.1 Hz, H-11), 1.2 - 2.1 (complex signals, 24H); ^{13}C NMR (100 MHz, $CHCl_3$-d_1) in Table 1.13 and Figure 1.2; EIMS, *m/z* (rel. int.): 426 (37, $M^{+\cdot}$ $C_{30}H_{50}O$), 411 (90, $M^{+\cdot}$-CH_3), 393 (21, $M^{+\cdot}$-CH_3-H_2O), 273 (15, $M^{+\cdot}$-$C_{10}H_{21}$), 259 (50, $M^{+\cdot}$-$C_{12}H_{23}$), 241 (26, 259-H_2O).

REFERENCES

1. Besson, E., Dellamonica, G., Chopin, J., Markham, K., Kim, M., Koh, H., and Fukami, H. (1985) Phytochemistry **24**:1061-1064.

2. Blunt, J. W. and Munro, M. H. G. (1980) Org. Magn. Res. **13**:26-27.

3. Bowers, W. S. (1985) Phytochemical disruption of insect development and behavior. In: Bioregulators of Pest Control (Hedin, P. A., ed.). ACS Symp. Ser. No. **276**:225-236. ACS, Washington, D. C.

4. Budzikiewicz, H., Wilson, J. M., and Djerassi, C. (1963) J. Am. Chem. Soc. **85**:3688-3699.

5. Chambaz, E. M. and Horning, E. C. (1967) Anal. Let. **1**:201-211.

6. Chaudhary, R. C., Khush, G. S., and Heinrichs, E. A. (1984) Insect. Sci. Applic. **5**:447-463.

7. Coll, J. C. and Bowden, B. F. (1986) J. Nat. Prod. **49**:934-936.

8. Cupp, E. W., Lok, J. B., and Bowers, W. S. (1977) Ent. Exp. Appl. **22**:23-28.

9. Derome, A. E. (1987) Modern NMR Techniques for Chemistry Research. Pergamon Press, Oxford, England.

10. Hedin, P. A. (1986) Developing research trends in the chemistry of plant resistance to pests. In: Natural Resistance of Plants to Pests. ACS Symp. Ser. No. **296**:2-14. ACS, Washington, D. C.

11. Ishii, S., Hirano, C., Iwata, Y., Nakasawa, M., and Miyagawa, H. (1962) Jap. J. Appl. Ent. Zool. **6**:281-288.

12. Khan, Z. R. and Saxena, R. C. (1985) J. Econ. Entom. **78**:562-566.

13. Kim, M., Koh, H., and Fukami, H. (1985) J. Chem. Ecol. **11**:441-452.

14. MacMillian, W. W., Bowman, M. C., Burton, Starks, K. J., and Wiseman, B. R. (1969) J. Econ. Entom. **62**:708-710.

15. Munakata, K., Saito, T. O. S., and Ishii, S. (1959) Bull. Agric. Chem. Soc. Jap. 23:64-65.

16. Nes, W. D., Heupel, R. C., Benson, M., Stafford, A. E., and Heddon, W. F. (1984) J. Chem. Soc. Chem. Commun., 223-225.

17. Obafemi, C. A., Ogunkoya, L., Quartey, J. A. K., and Waight, E. S. (1979) Phytochemistry 18:496-497.

18. Obata, T., Koh, H. S., Kim, M., and Fukami, H. (1983) Appl. Ent. Zool. 18:161-169.

19. Pantoja, A., Smith, C. M., and Robinson, J. F. (1986) J. Econ. Entomol. 79:1319-1323.

20. Reese, J. C. (1983) Nutrient-allelochemical interactions in host plant resistance. In: Plant Resistance to Insects (Hedin, P. A., ed.). ACS Symp. Ser. No. 208:231-244. ACS, Washington, D. C.

21. Renwick, J. A. A. (1983) Nonpreference mechanism: plant characteristics influencing insect behavior. In: Plant Resistance to Insects (Hedin, P. A., ed.). ACS Symp. Ser. No. 208:199-214. ACS, Washington, D. C.

22. Saxena, R. C. (1986) Biochemical bases of insect resistance in rice varieties. In: Natural Resistance of Plants to Pests (Green, M. B. and Hedin, P. A., eds.). ACS Symp. Ser. No. 296:142-159. ACS, Washington, D. C.

23. Seigler, D. S. (1983) Role of lipids in plant resistance to insects. In: Plant Resistance to Insects (Hedin, P. A., ed.). ACS Symp. Ser. No. 208:303-328. ACS, Washington, D. C.

24. Sekido, S. and Sogawa, K. (1976) Appl. Ent. Zool. 11:75-81.

25. Shigematsu, Y., Murofushi, N., Ito, K., Kaneda, C., Kawabe, S., and Takahashi, N. (1982) Agric. Biol. Chem. 46:2877-2879.

26. Smith, C. M. and Fischer, N. H. (1983) Ent. Exp. Appl. 33:343-345.

27. Sogawa, K. (1976) Appl. Ent. Zool. 11:160-164.

28. Visser, J. H. (1983) Differential sensory perceptions of plant compounds by insects. In: Plant Resistance to Insects (Hedin, P. A., ed.). ACS Symp. Ser. No. 208:215-230. ACS, Washington, D. C.

29. Vorbruggen, H., Pakrashi, S. C., and Djerassi, C. (1963) Annal. der Chem. **666**:57-76.

30. Whittaker, R. H. and Feeny, P. P. (1971) Science **171**:757-770.

31. Yanagawa, H., Kato, T., and Kitahara, Y. (1972) Phytochemistry **11**:1893-1897.

32. Yoshida, S. (1981) Fundamentals of Rice Crop Science. Int. Rice Res. Inst., Los Banos, Laguna, Philippines. 269 pp.

33. Yoshihara, T., Sogawa, K., Pathak, M. D., Juliano, B. O., and Sakamura, S. (1979) Ent. Exp. and Appl. **26**:314-322.

34. Yoshihara, T., Sogawa, K., Pathak, M. D., Juliano, B. O., and Sakamura, S. (1980) Ent. Exp. Appl. **27**:149-155.

35. Yu, S. J. (1987) J. Chem. Ecol. **13**:423-436.

CHAPTER 2

DYNAMIC HEADSPACE VOLATILES FROM THE FOLIAGE OF RICE CULTIVARS VARYING IN RESISTANCE TO THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J. E. SMITH)

INTRODUCTION

Volatile constituents of plants play a vital role in plant-insect interactions. Since volatiles are emitted by plants to their surroundings, insects can perceive their presence through sensory cells located on olfactory sensilla (1). For this reason, volatiles can act as insect attractants or repellants. Secondary plant metabolites also play a role in antibiosis, thus affecting the behavior and physiology of insects (2). To understand the function of volatiles in plant-insect interaction, it is vital to know the profile of volatiles in plants and identify the structures of the profile components.

Little is known about the volatile components of rice plants. Saxena et al. isolated volatile mixtures from rice plants by steam distillation that affected the behavior of the brown planthopper (*Nilaparvata lugens* Stal) (3), striped stemborer (*Chilo suppressalis*), and green leafhopper (*Nephotettix virescens* Distant) (2). However, the components of the mixture were not identified.

Obata et al. (4) identified twenty-seven compounds from the steam distillate of the susceptible rice variety Nihonbare. Among the compounds identified, a mixture of isocyanurate and carbonyl compounds were found to attract brown planthopper.

A major disadvantage of obtaining volatiles by steam distillation is the possible production of artifacts due to heating and the introduction of contaminants from the solvents and drying agents. Steam distillation also requires a large amount of sample. Moreover, grinding or maceration of leaves can lead to the formation of enzyme-catalyzed oxidation products (6).

Recently, dynamic headspace sampling (DHS) and concentration has been used in studying volatile compounds in environmental water and various food samples (7). This procedure can be used to trap volatiles without laborious sample preparation and does not require extensive sample heating thus, minimizing the formation of artifacts. DHS has been used for collecting volatiles from leaves of wheat (6), potato (1), and strawberry (8). In all instances, the trapped volatiles were eluted with a suitable solvent for gas chromatography-mass spectrometry (GC-MS) analysis.

To avoid the use of elution solvent, thermal desorption and cryogenic focusing has been most recently used to introduce the volatiles to the GC column for GC or GC-MS analysis (9).

Rice variety PI (plant introduction) 346833 is moderately resistant and 'Mars' susceptible to feeding by fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) larvae (10). In this study, these two rice varieties were analyzed for their volatile profiles and composition by DHS-GC-MS method. The major volatile components were identified by GC and mass spectral comparison with standards.

RESULTS AND DISCUSSION

The dynamic headspace volatiles from seedling foliage of the rice varieties 'Mars' and PI346833 were purged and trapped in a column of Tenax TA using the set-up shown in Figure 2.1. The trapped volatiles were desorbed in a Tekmar sample concentrator and analyzed by gas chromatography and mass spectrometry (GC-MS). The total ion chromatograms (TIC) (Figure 2.2) showed that the two varieties had remarkable qualitative and quantitative differences in their volatile profiles. The susceptible variety 'Mars' contained a greater number of headspace volatiles than the moderately resistant PI346833. It is also apparent that 'Mars' produced larger amounts of volatiles. The expanded TICs of the volatile compounds are shown in Figure 2.3 and Figure 2.4, respectively.

Table 2.1 lists the 28 compounds identified from both rice varieties. Their molecular structures are shown in Figure 2.5. Among those identified, fourteen compounds were present in both varieties, twelve compounds were present only in 'Mars' and two were found only in PI346833. The volatiles were composed of 3 hydrocarbons, 1 monoterpene, 4 aldehydes, 2 ketones, 3 enones, 2 dienones, 3 alcohols, 4 enals and 6 dienals. Sixteen compounds were identified by comparing their mass spectra and retention indices with those of standard compounds. Figure 2.6 shows an example of the mass spectrum of one of the volatile constituents in comparison with authentic E,E-2,4-heptadienal (peak 19). The other volatiles were tentatively identified by computer matching their mass spectra with those stored in the NBS/NIH/EPA/MSDC Data Base stored in the HP MSD

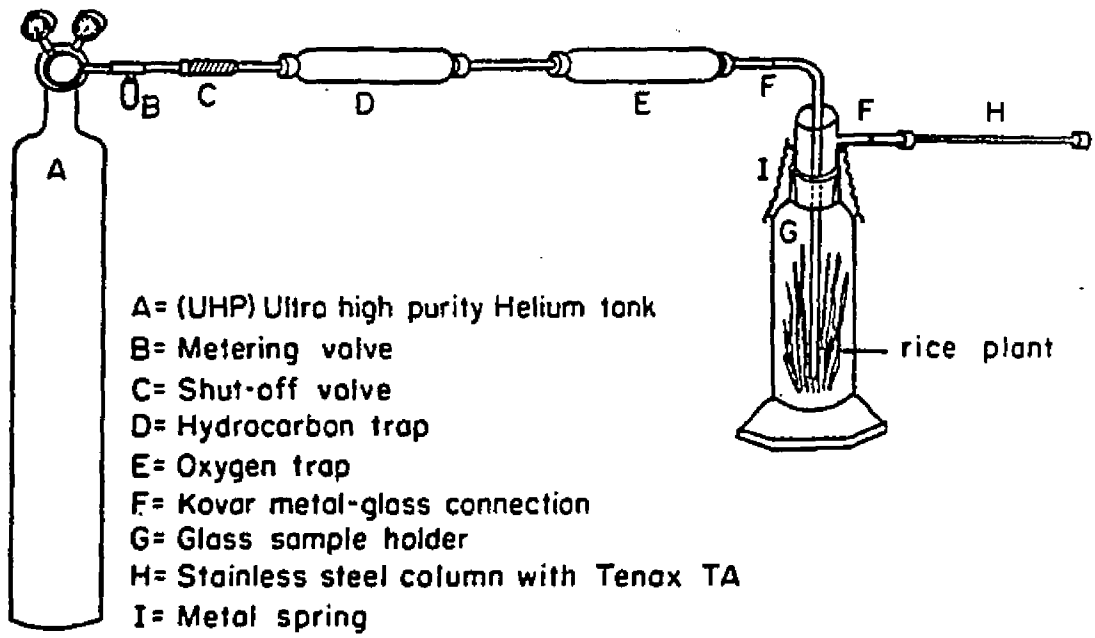


Figure 2.1. Set-up for the collection of volatiles from rice foliage by dynamic headspace sampling.

ChemStation. An example of the mass spectrum of a rice volatile compound, 6-methyl-5-hepten-2-one (peak 12) with the library mass spectrum is shown in Figure 2.7.

Based on the percent peak area of each component, the most abundant compounds in both rice varieties are hexanal, E,E-2,4-heptadienal, Z,Z-2,4-heptadienal and E-2-hexenal. Together, these four compounds comprise more than 50% of the total volatile components in each variety. Hexanal and E-2-hexenal have been known to be components of the "green leaf odor" of plants (1). These compounds commonly represent C6 aldehydes, alcohols and acetates which are produced by the oxidative degradation of unsaturated leaf

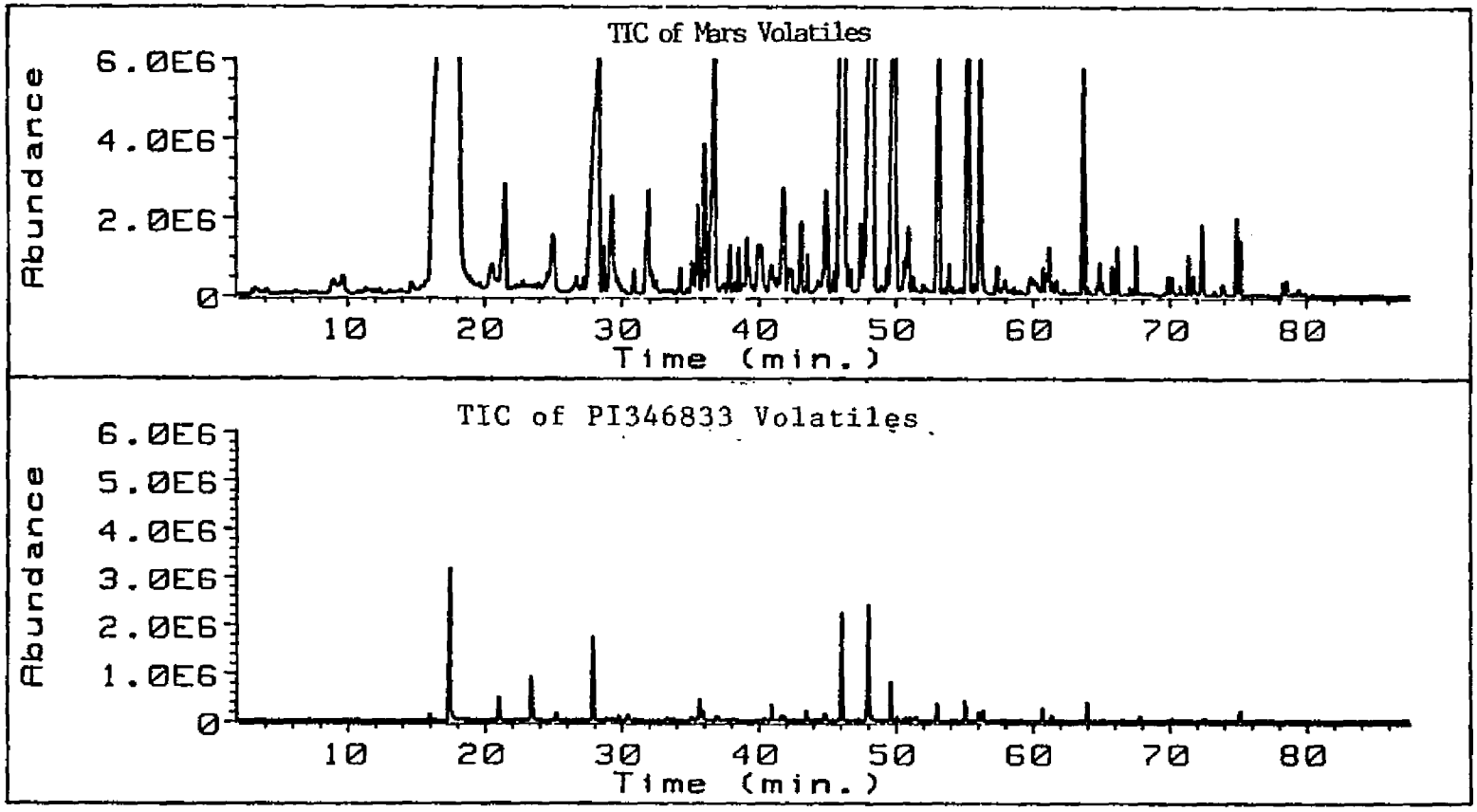


Figure 2.2. Total ion chromatograms of dynamic headspace volatile compounds from rice PI346833 (bottom) and 'Mars' (top).

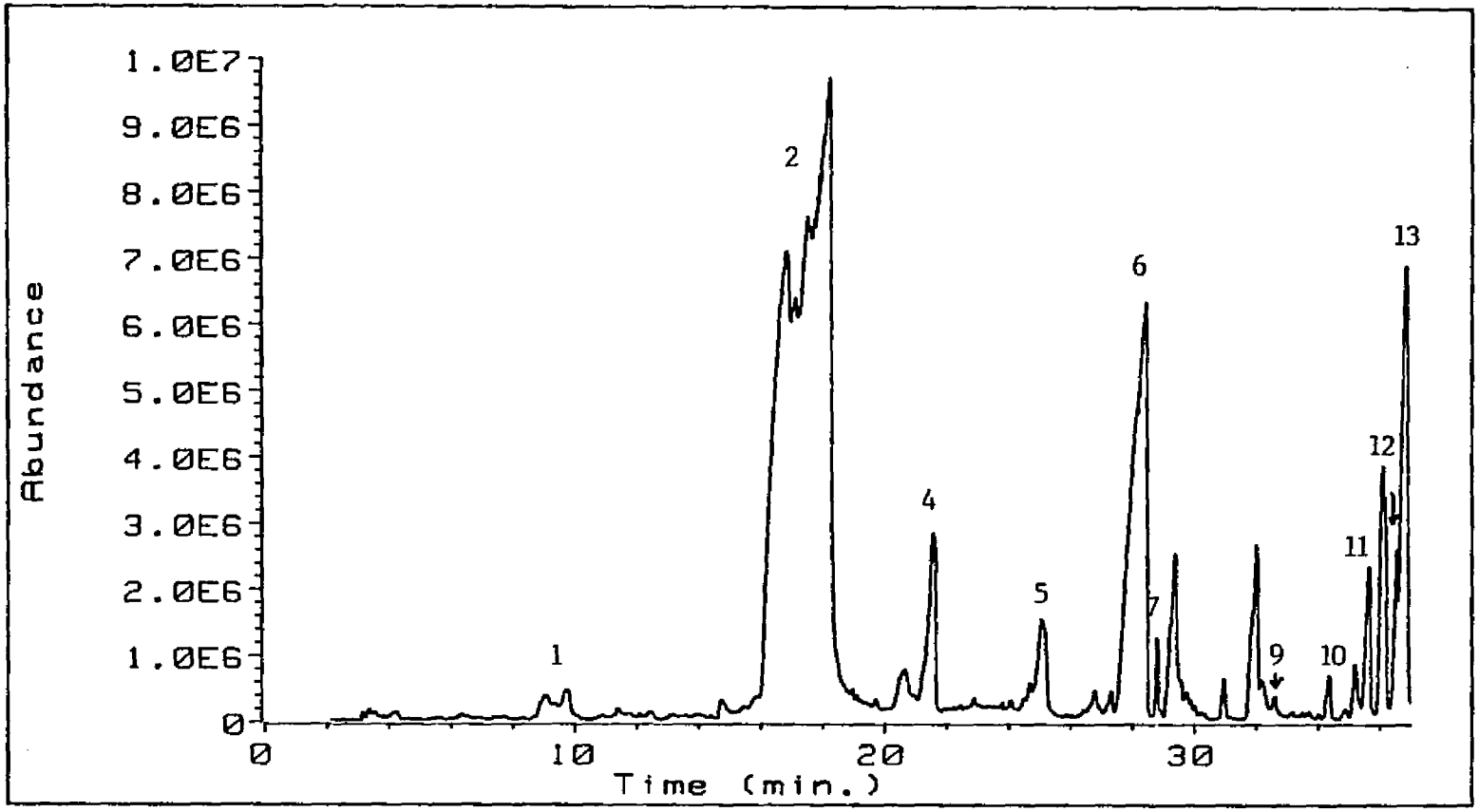


Figure 2.3. Total ion chromatogram (TIC) of dynamic headspace volatile compounds from rice variety 'Mars' seedlings. The peak number corresponds to the peak number in Table 2.1 and structure number in Figure 2.5.

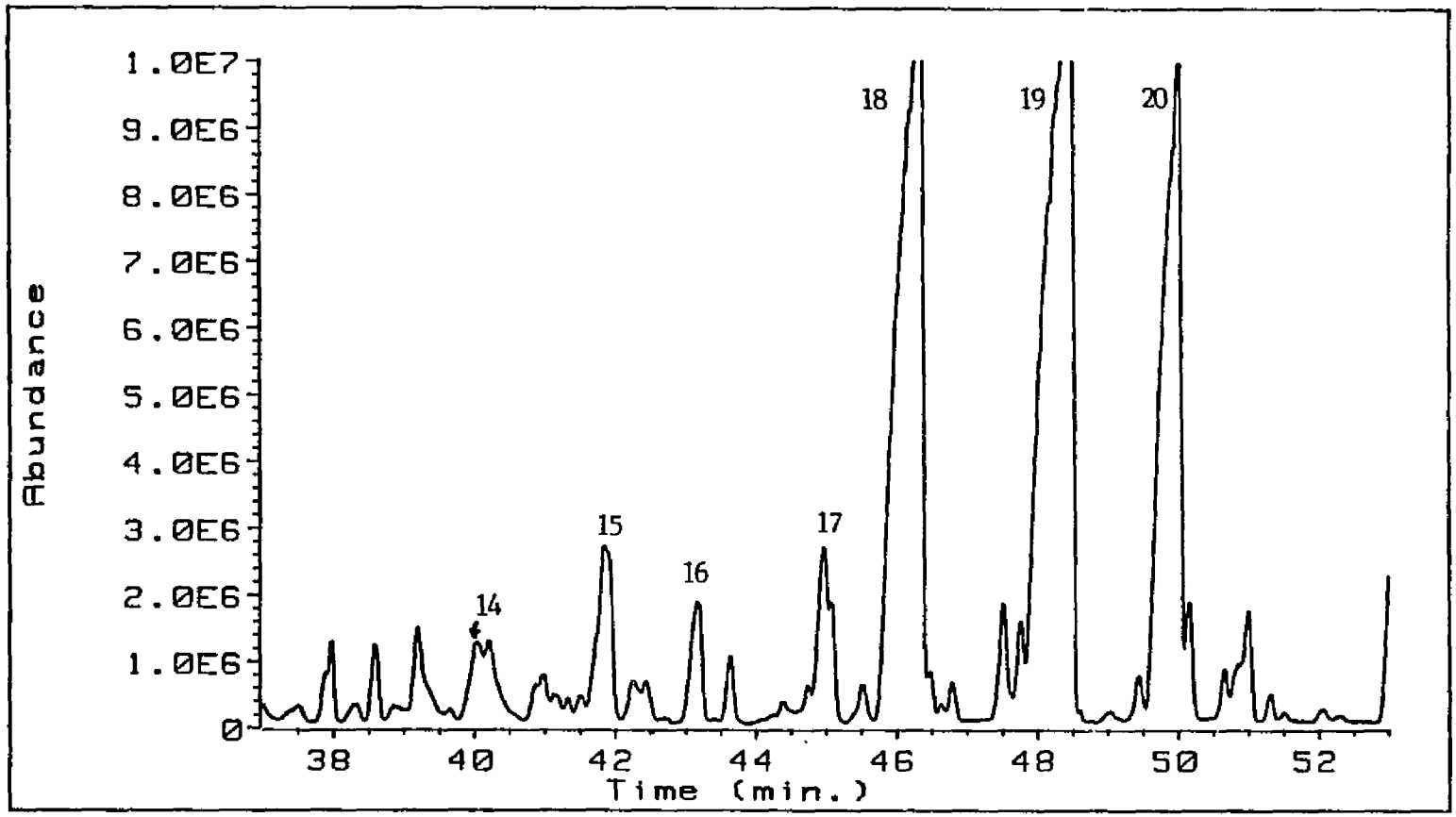


Figure 2.3. TIC of volatile compounds from 'Mars' (continued).

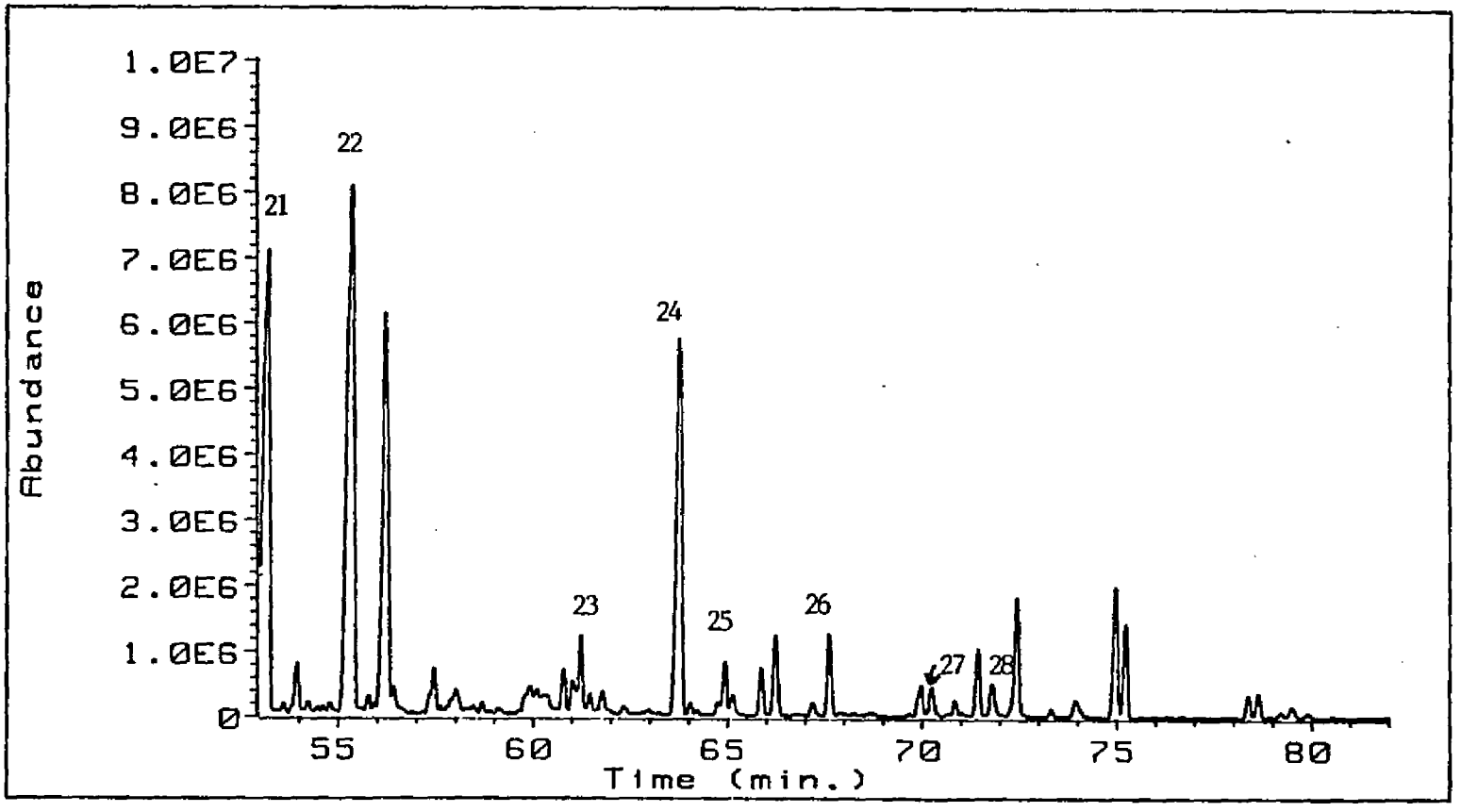


Figure 2.3. TIC of volatile compounds from 'Mars' (continued).

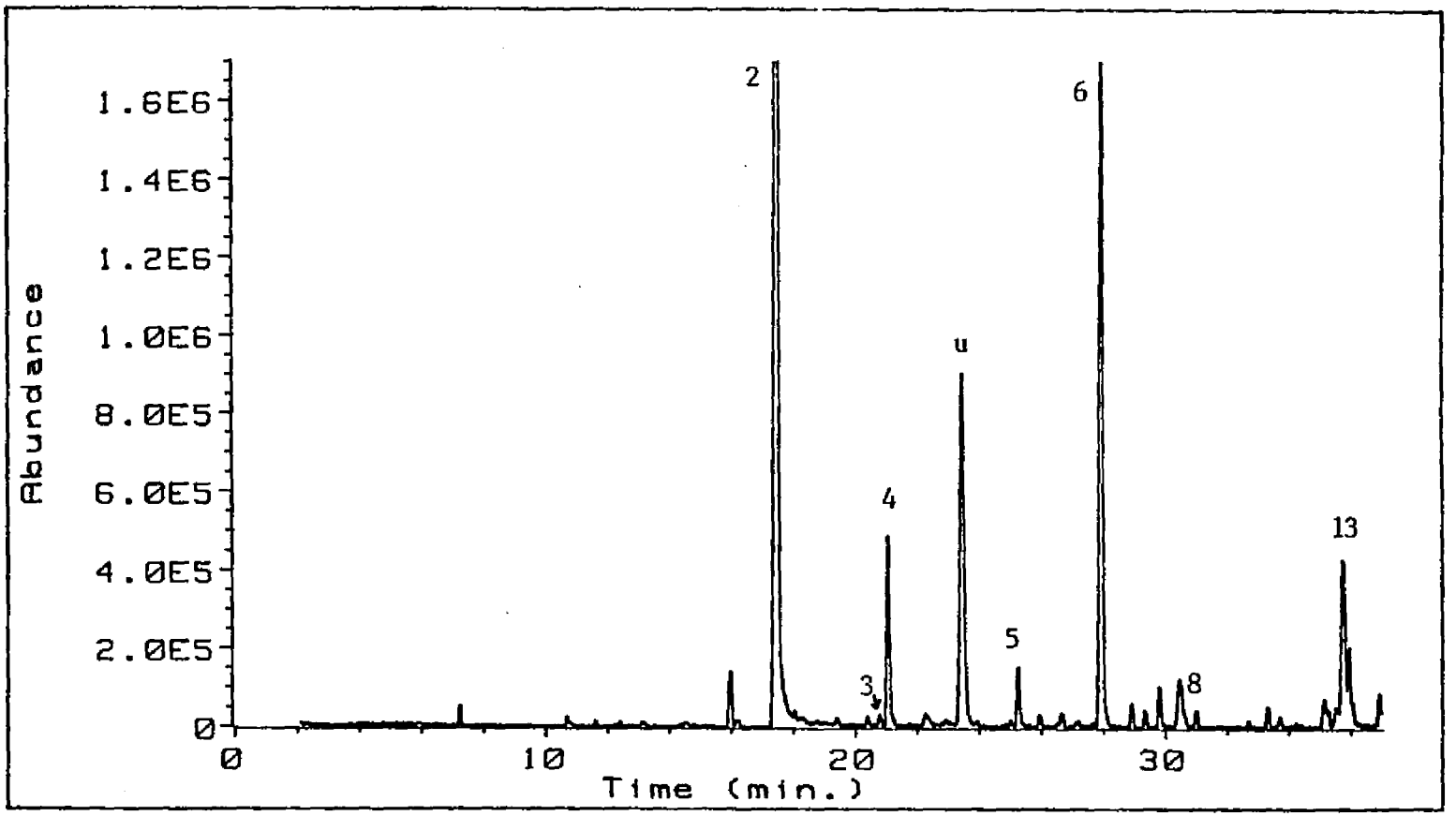


Figure 2.4. Total ion chromatogram (TIC) of dynamic headspace volatile compounds from rice PI346833 seedlings. The peak number corresponds to the peak number in Table 2.1 and structure number in Figure 2.5.

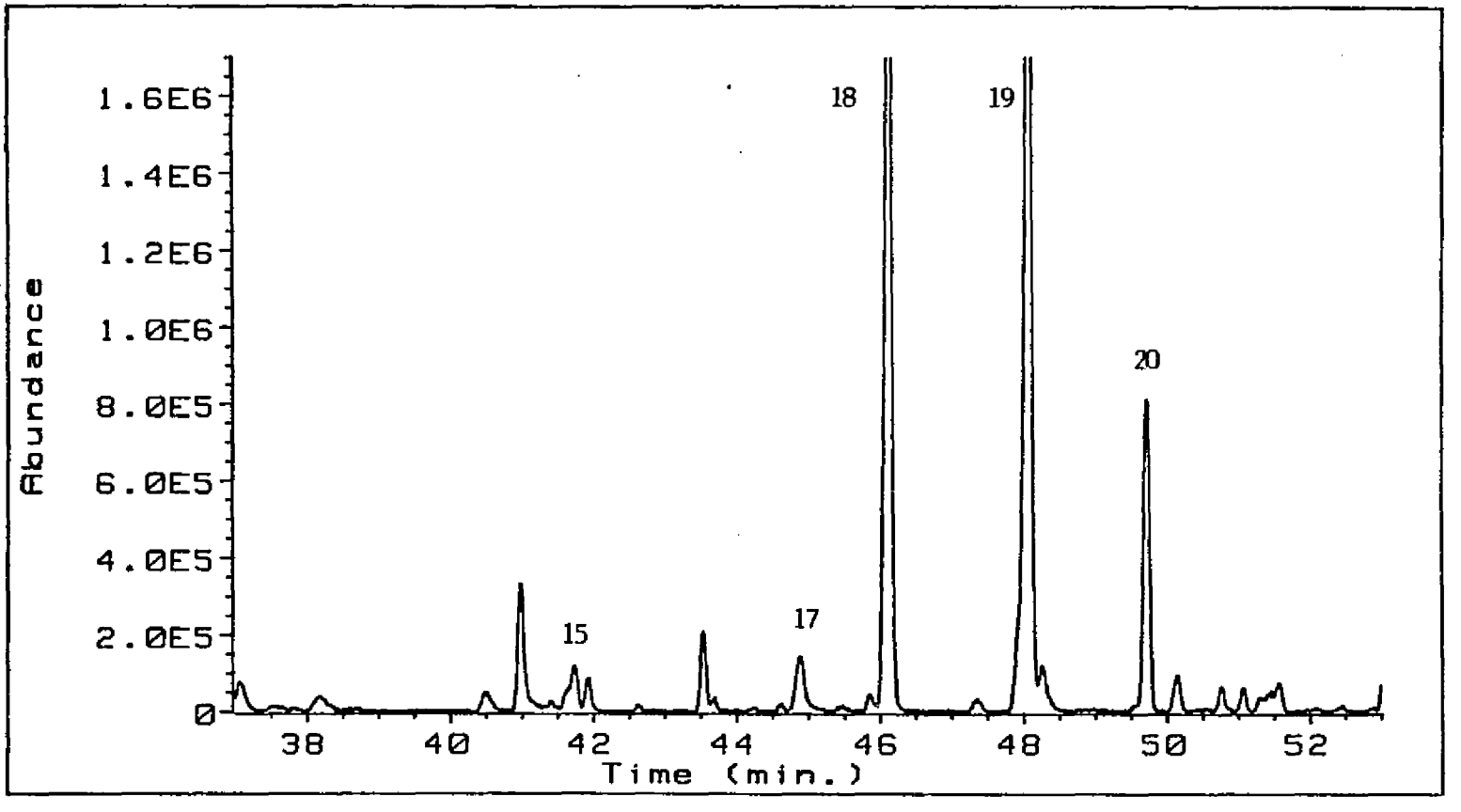


Figure 2.4. TIC of volatile compounds from PI346833 (continued).

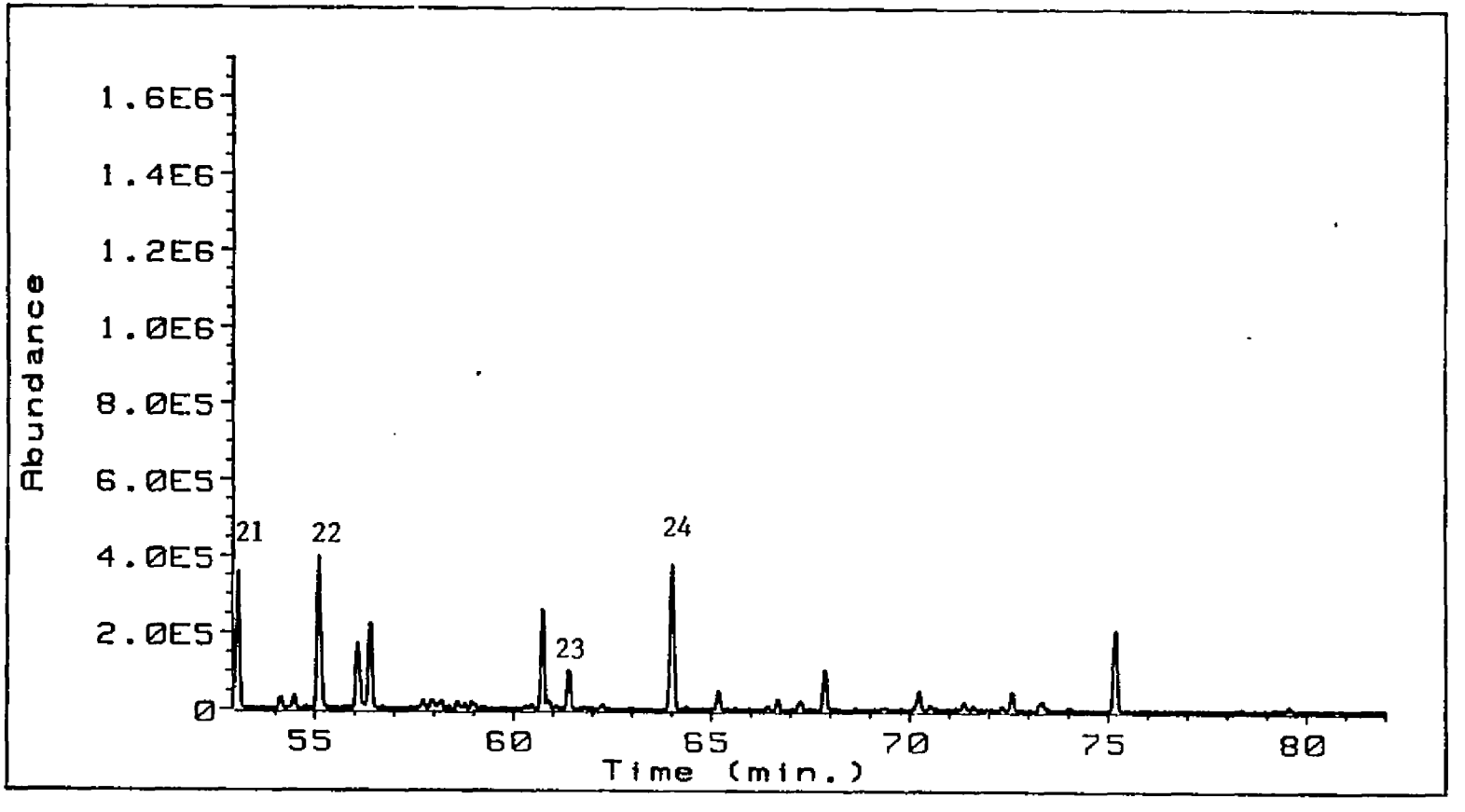


Figure 2.4. TIC of volatile compounds from PI346833 (continued).

Table 2.1. Dynamic Headspace Volatile Compounds Identified from Mars and PI346833 Rice Seedlings.

Peak No.[a]	Compound	Mars		PI346833		Standard
		RI[b]	% Peak Area	RI	% Peak Area	RI
1	Pentanal	966	0.30	------		982
2	Hexanal	1085	27.70	1094	20.91	1085
3	3-Penten-2-one	------		1137	0.15	1128
4	3-Methyl-2-butenal	1146	1.56	1140	2.31	----
5	Heptanal	1190	1.31	1192	0.75	1189
6	E-2-Hexenal	1231	6.33	1228	9.54	1222
7	6-Methyl-2-heptanone	1240	0.21	------		----
8	3,5-Octadiene	------		1270	0.20	----
9	Limonene	1292	0.16	------		1200
10	5-Nonen-2-One	1314	0.20	------		----
11	2-Heptenal	1331	0.76	------		1329
12	6-Methyl-5-hepten-2-one	1343	0.66	------		----
13	2-Penten-1-ol	1347	2.84	1332	4.54	1318
14	Nonanal	1387	0.51	------		1398
15	2,4-Hexadienal	1412	1.33	1410	0.84	----
16	E-2-Octenal	1431	0.68	------		1435
17	7-Octen-4-ol	1457	1.27	1456	1.06	----
18	Z,Z-2,4-Heptadienal	1477	8.10	1474	11.72	----
19	E,E-2,4-Heptadienal	1508	9.69	1502	14.19	1500
20	Z,Z-3,5-Octadien-2-one	1531	6.06	1527	3.79	----
21	E,E-3,5-Octadien-2-one	1580	2.72	1578	1.62	----
22	3-Methyl-4-heptanone	1614	3.12	1610	1.93	----
23	Z,Z-2,4-Nonadienal	1708	0.41	1711	0.44	1710
24	Naphthalene	1751	1.69	1755	1.92	1746
25	Z,Z-2,4-Decadienal	1770	0.34	------		1778
26	E,E-2,4-Decadienal	1816	0.26	------		1827
27	1H-Indene-1-ethylidene	1863	0.13	------		----
28	Benzyl Alcohol	1890	0.16	------		1891

[a]Peak number corresponds to the peak numbers on the TICs in Figures 2.3 and 2.4 and structure numbers in Figure 2.5.

[b]RI = retention index.

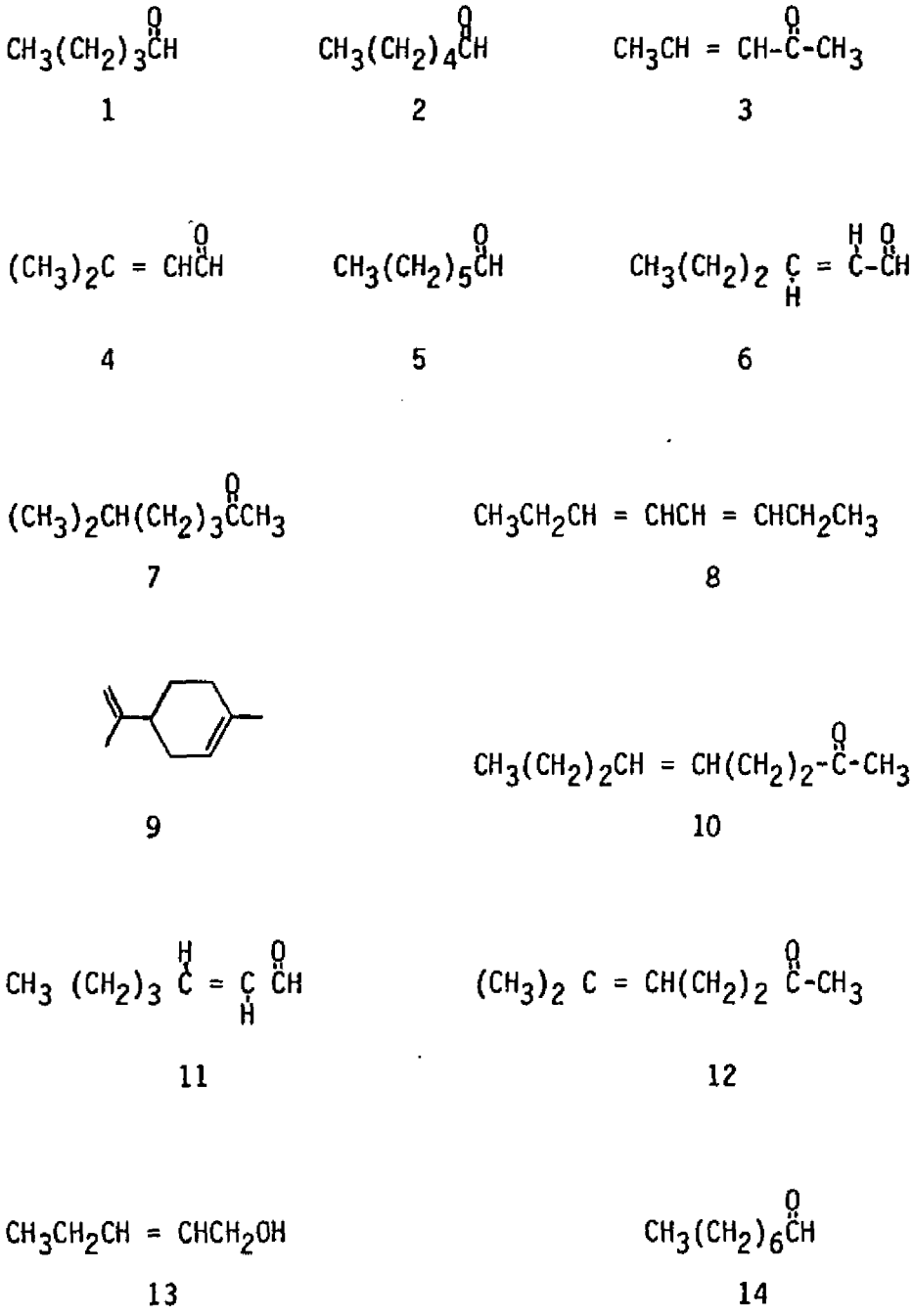
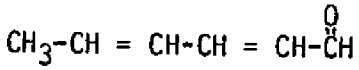
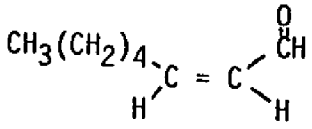


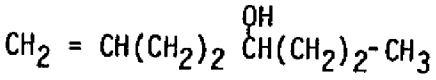
Figure 2.5. Structures of dynamic headspace volatile compounds from 'Mars' and PI346833 rice seedlings.



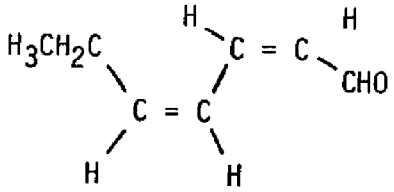
15



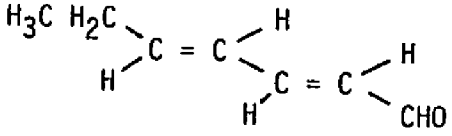
16



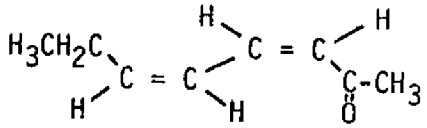
17



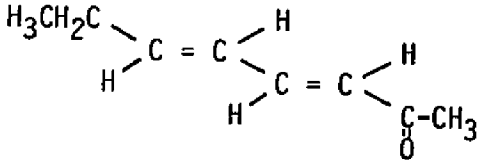
18



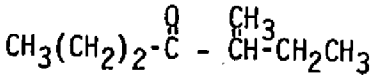
19



20

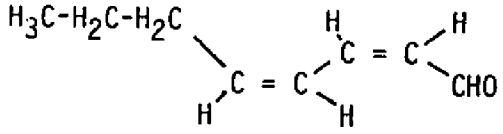


21

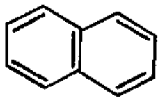


22

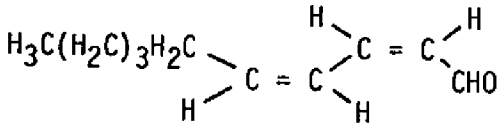
Figure 2.5. (continued)



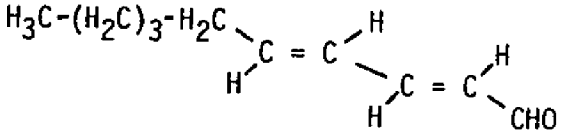
23



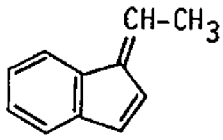
24



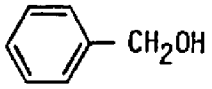
25



26



27



28

Figure 2.5. (continued)

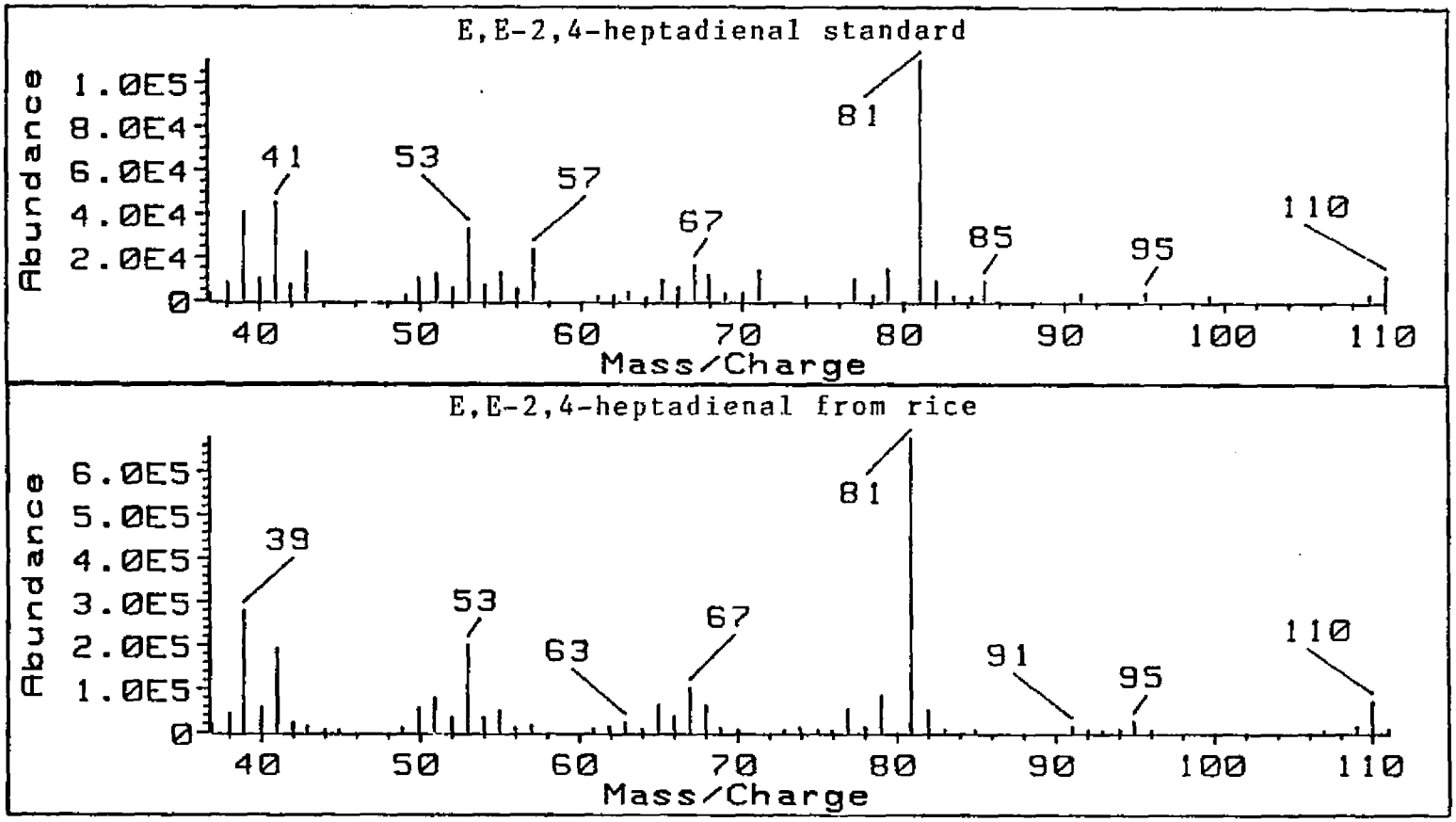


Figure 2.6. Bottom). Mass spectrum of E, E-2,4- heptadienal from rice (peak 19) and top). Mass spectrum of the E,E-2,4- heptadienal standard.

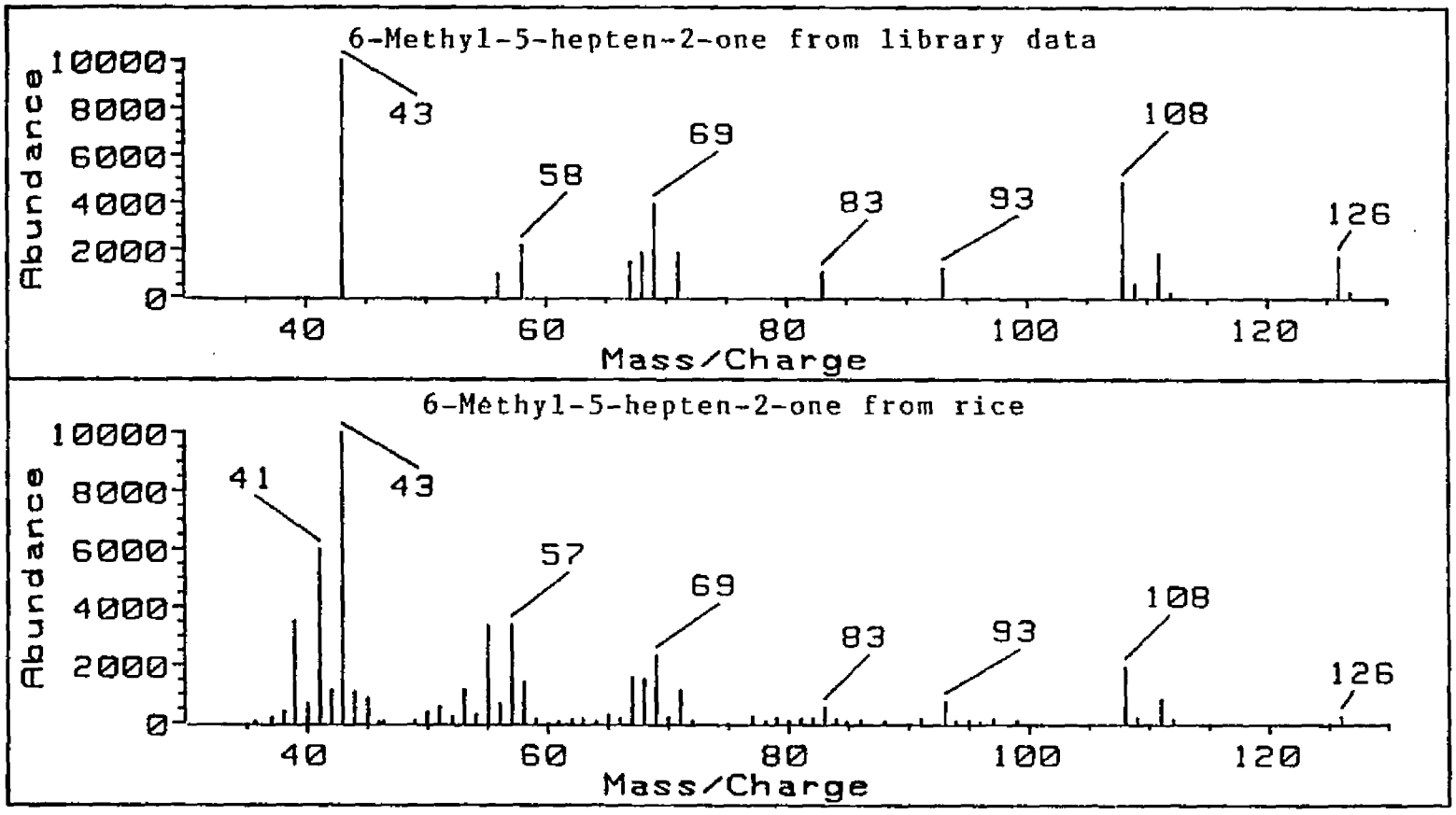


Figure 2.7. Bottom). Mass spectrum of 6-methyl-5-hepten-2-one from rice (peak 12) and top). Mass spectrum of 6-methyl-5-hepten-2-one from the library.

fatty acids such as linoleic and linolenic acids (1). Most of the other volatiles identified are also products of the oxidative degradation of unsaturated fatty acids (12).

Visser (1) studied the response of different insects toward C6 aldehydes, enols and acetate isolated from potato leaves. It was found that different insects responded differently to each compound. It is, therefore, possible that each insect could be attracted or repelled by a plant depending on the composition and relative concentration of each volatile component.

Some of the compounds identified from the rice seedlings have been found as volatile components of other cereal plants. E-2-Hexenal, nonanal, limonene (6) as well as hexanal, heptanal, E-2-heptenal and E-2-octenal (5) were detected in wheat leaves. Volatiles from whole oat leaves were reported to contain heptanal, nonanal, E,E-2,4-decadienal, limonene and 6-methyl-5-hepten-2-one (13). Likewise, benzyl alcohol has been found in barley leaves (14).

It is interesting to note that in comparison with PI346833, the rice variety 'Mars' produces a larger number of volatile compounds as well as in larger amounts. A quantitative comparison of the peak areas of the common volatiles produced by the two rice varieties in a single chromatogram showed that Mars produced from 17 to 46 times more volatiles than PI346833. The amount of heptanal was 46 times greater and E-2-hexenal was 17 times greater in 'Mars' than in PI346833.

Mars has been found to be highly susceptible to FAW based on its high defoliation rate and FAW's high feeding preference for this

rice variety (10). It is possible that 'Mars' produces large amounts of attractants which makes it a preferred host for this insect pest. Since there are 16 compounds (12 identified and 4 unknown) present in 'Mars' that were not detected in PI346833, it is possible that a specific constituent and/or a combination of several compounds may act as attractants and/or feeding stimulants of 'Mars' towards certain insect species. Conversely, 3-penten-2-one and 3,5-octadiene, which were not detected in 'Mars' but were present in very small amounts in PI346833, are potential candidates for laboratory tests on their effect on the behavior of FAW as feeding deterrents or repellants.

Speculation on the possible functions of these volatile leaf components that may make PI346833 more resistant to FAW than 'Mars' will require more detailed experimental verification.

Nonanal was found in 'Mars' but not in PI346833. This compound was found to be the chief volatile component in live wheat stem rust spores (*Puccinia graminis* var. *tritici*) (15). Nonanal is also a highly active germination stimulant of wheat stem rust, wheat leaf rust (*P. recondita*), oat crown rust (*P. recondita* F. sp. *avenae*) and common corn rust (*P. sorghi*) (16).

Another interesting study could involve structure-activity relationships of related volatiles such as 6-methyl-5-hepten-2-one and its saturated form 6-methyl-2-heptanone. 6-Methyl-5-hepten-2-one has been isolated as a minor component of the uredospores of wheat stem rust (15). This compound stimulated the germination of uredospores of wheat stem rust, oat crown rust (*P. coronata* F. sp.

avenae) (16). 6-Methyl-5-hepten-2-one is also the alarm pheromone of the *Iridomyrmex* species of ants (17). It will be interesting to know the role of these compounds in rice-insect interaction.

EXPERIMENTAL

Plant Material.

Rice varieties Mars and PI346833 were grown in the Louisiana State University Greenhouse on Perkins Road, Baton Rouge, Louisiana. Seeds were germinated on September 19, 1987 and planted on September 22. The plants were maintained in a flooded state. The aerial parts were harvested on October 13, 1987 at the 4-leaf stage (11), rinsed with water to remove soil, placed in a plastic bag, frozen, and then kept at -5°C. Plant samples were analyzed for volatiles on April 19-20, 1988.

Collection of Volatiles.

Whole aerial parts (45.3 g) of frozen rice seedlings were placed in a glass container (23 cm ht x 5.5 cm i.d.) fitted with a Kovar metal-glass connection. The volatiles were collected by passing ultra high purity (UHP) helium gas (99.999%, Linde Division, Union Carbide Corporation, Danbury, CT) through an oxygen trap, a hydrocarbon trap and then through the sample at a flow rate of 86 ml/min for 16 hr at room temperature (25°C). The volatiles were trapped in a 30.48 cm x 0.32 cm o.d. stainless steel column packed with Tenax TA (2,6-diphenyl-p-phenylene oxide polymer, 0.24 g, 60-80

mesh, Chrompack, Raritan, NJ). After the collection of volatiles, the trap was purged with UHP He to remove any water until a constant trap weight was obtained (9). The set-up used for this part is illustrated in Figure 2.1.

Gas Chromatography-Mass Spectrometry.

The volatiles were desorbed from the Tenax trap by a Tekmar model 4000 Dynamic Headspace Concentrator at 185°C for 15 min into a 60 m length x 0.25 mm i.d. x 0.25 μm film thickness Supelcowax 10 column (Supelco, Inc., Bellefonte, PA). Ethanol/dry ice mixture was used for the cryogenic focusing of the sample. The carrier gas head pressure was 30 psi and the injector split vent was closed during desorption to facilitate the transfer of volatiles from the trap to the column.

Gas chromatography was carried out on a Hewlett Packard 5792 gas chromatograph under the following conditions: carrier gas head pressure, 15 psi; carrier gas linear velocity, 25 cm/sec; carrier gas flow rate, 0.76 ml/min. The column temperature was programmed as follows: initial temperature, 40°C for 5 min, programming rate, 2°C/min; final temperature 175°C for 15 min; total run time, 87.5 min.

The chromatographic components were analyzed on an HP5970B mass selective detector. Electron ionization was carried out at 70 eV and electron multiplier voltage was set at 1800 V. Solvent delay was set at 2 min.

Calculation of Retention Indices.

The determination of the retention indices of the volatile compounds was performed by adding 200 ng of each normal alkanes (C9-C19) to 45.3 g of Mars seedlings. The same procedure for collection of volatiles and subsequent GC-MS analysis was followed, as described above. The retention index of each compound was calculated according to van den Dool and Kratz (18).

GC-MS of Standard Compounds.

Five µl of each standard and each C8-C22 n-alkanes were dissolved in 5 ml hexane. One µl of the standard mixture was injected at a 27:1 split ratio. The solvent delay was set at 6 min with the other chromatographic and MS conditions the same as above. The mass spectra and retention indices of the standards were used to identify the volatiles obtained from the rice samples.

When authentic standards were not available, tentative identification of some compounds were based on computer matching of the unknown mass spectra with the reference mass spectra of the NBS/NIH/EPA/MSDC Data Base Copyright 1984, 1986 installed on the HP MSD ChemStation.

Per Cent Peak Area.

The per cent peak area of each chromatographic peak was calculated by electronic integration with an HP59970C MS ChemStation Program. The baseline threshold was set at 16 and the peak width at 0.40 min.

REFERENCES

1. Visser, J. H. (1983) Differential sensory perceptions of plant compounds by insects. In: Plant Resistance to Insects. (Hedin, P. A., ed.). ACS Symp. Series No. 208: 215-230. Washington, D. C.

2. Saxena, R. C. (1986) Biochemical bases of insect resistance in rice varieties. In: Natural Resistance of Plants to Pests. (Green, M. B. and Hedin, P. A., eds.). ACS Symp. Series No. 296: 142-159. ACS, Washington, D. C.

3. Saxena, R. C. and Okech, S. H. (1985) J. Chem. Ecol. 11:1601-1606.

4. Obata, T., Koh, H. S., Kim, M., and Fukami, H. (1983) Appl. Ent. Zool. 18:161-169.

5. Hamilton-Kemp, T. R. and Andersen, R. A. (1984) Phytochemistry. 23:1176-1177.

6. Buttery, R. G., Xu, C. and Ling, L. C. (1985) J. Agric. Food Chem. 33:115-117.

7. Badings, H. T., de Jong, C., Dooper, R. P. M., and de Nijs, R. M. C. (1984) Rapid analysis of volatile compounds in food products by purge-and-cold trapping/capillary gas chromatography. In: Progress in Flavour Research 1984. Proceedings of the 14th Weurman Flavor Research Symposium, Bourdan, France, May 9-11, 1984. (Adda, J., ed.). Elsevier Science Publishing Co., Inc., N. Y.

8. Hamilton-Kemp, T. R., Andersen, R. A., Rodriguez, J. G., Laughrin, J. H. and Patterson, C. G. (1988) J. Chem. Ecol. 14:789-796.

9. Williams, S. S. and Hsieh, T. C.-Y. (1988) J. Agric. Food Chem. (submitted manuscript).

10. Pantoja, A., Smith, C. M. and Robinson, J. F. (1986) J. Econ. Entomol. 79:1314-1323.

11. Yoshida, S. (1981) Fundamentals of Rice Crop Science. Int. Rice Res. Inst., Los Banos, Laguna, Philippines. 269 pp.

12. Frankel, E. N. (1982) Prog. Lipid Res. 22:1-33.

13. Buttery, R. G., Ling, L. C., Wellso, S. G. (1982) J. Agric. Food Chem. 30:791-792.

14. Juneja, P. J., Gholson, R. K., Burton, R. L. and Starks, K. J. (1972) Ann. Entomol. Soc. Am. 65:961-964.

15. Rines, H. W., French, R. C. and Daasch, L. W. (1974) J. Agric. Food Chem. 22:96-100.

16. French, R. C., Gale, A. W., Graham, C. L. and Rines, H. W. (1975) J. Agric. Food Chem. 23:4-8.

17. Crewe, R. M. and Blum, M. S. (1971) Ann. Entomol. Soc. Am. 64:1007-1010.

18. Van Den Dool, H. and Kratz, P. D. (1963) J. Chromatog. 2:463-471.

PART II

STRUCTURE DETERMINATION OF EXTERNAL FLAVONOIDS OF *CALAMINTHA ASHEI*

CHAPTER 3

MAJOR EXTERNAL FLAVONOIDS FROM LEAVES OF *CALAMINTHA ASHEI*

INTRODUCTION

Calamintha ashei (Lamiaceae) is a member of the Florida scrub community which has been shown to have inhibitory effects on the growth of sandhill plants (1). Therefore, a search for the chemical constituents of *C. ashei* with possible allelopathic activities was performed. It was observed that *C. ashei* is very rich in monoterpenes (2), triterpenes and flavonoids (3). Moreover, it was found that menthofuran-type monoterpenes exhibit strong germination inhibitory activities on blue stem (*Schizachyrium scoparium*), a native grass of the sandhill community (4).

In continuation of the phytochemical analysis of *C. ashei*, this study was conducted to isolate and identify its major external flavonoid constituents. A new flavone, 5,6,4'-trihydroxy-7,8,3'-trimethoxy flavone (1) was identified by spectroscopic methods and single-crystal X-ray crystallography. The aglycone 2 (5,6,4'-trihydroxy-7,3'-dimethoxyflavone) was first obtained by synthesis (5) and by hydrolysis of its natural glycoside (6), but this is the first report of it's natural occurrence. The structures of the known flavonoids 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone (3) (7) and desmethoxynobiletin (4) (8) were established by comparison with reported spectral data.

RESULTS AND DISCUSSION

The crude dichloromethane extracts of fresh *C. ashei* leaves were chromatographed on silica gel by vacuum liquid chromatography (9). The more polar fractions contained flavonoids **1-4.**

The ^{1}H NMR spectrum of compound **1** (Figure 3.1) indicated the presence of three methoxyl groups and a hydrogen-bonded hydroxyl at C-5. The multiplicity and coupling constants of the aromatic protons at the δ 6.9 - δ 7.6 region suggested a 3',4'-substitution pattern in ring B. The doublet at δ 7.06 with an ortho coupling constant of J = 8.7 Hz was assigned to H-5' and the doublet at δ 7.42 (J = 1.8 Hz) to H-2'. The latter signal is coupled with H-6', which appears as a doublet of a doublet at δ 7.55 and is coupled to H-5' (J = 8.7 Hz) and H-2' (J = 1.8 Hz).

The presence of two hydroxyl groups and a hydrogen-bonded C-5 hydroxyl (δ 12.44) was confirmed by the ^{1}H NMR spectrum of compound **1a** (Figure 3.2). Acetylation of **1** resulted in the appearance of three acetate signals and the disappearance of the C-5-OH signal as shown in Figure 3.3. The ^{1}H NMR data are summarized in Table 3.1.

The position of the hydroxyl groups was determined by UV spectroscopy in MeOH and with the use of chemical shift reagents (10). UV spectral data are summarized in Table 3.2. The bathochromic shift of band I by 56 nm with an increase in the absorption in NaOMe signified a C-4'-OH. The band I bathochromic shift by 28 nm upon $AlCl_3$/HCl treatment suggested either a 6-hydroxylated/methoxylated 3-methoxyflavonol or a 6,7,8-substituted flavone (11).

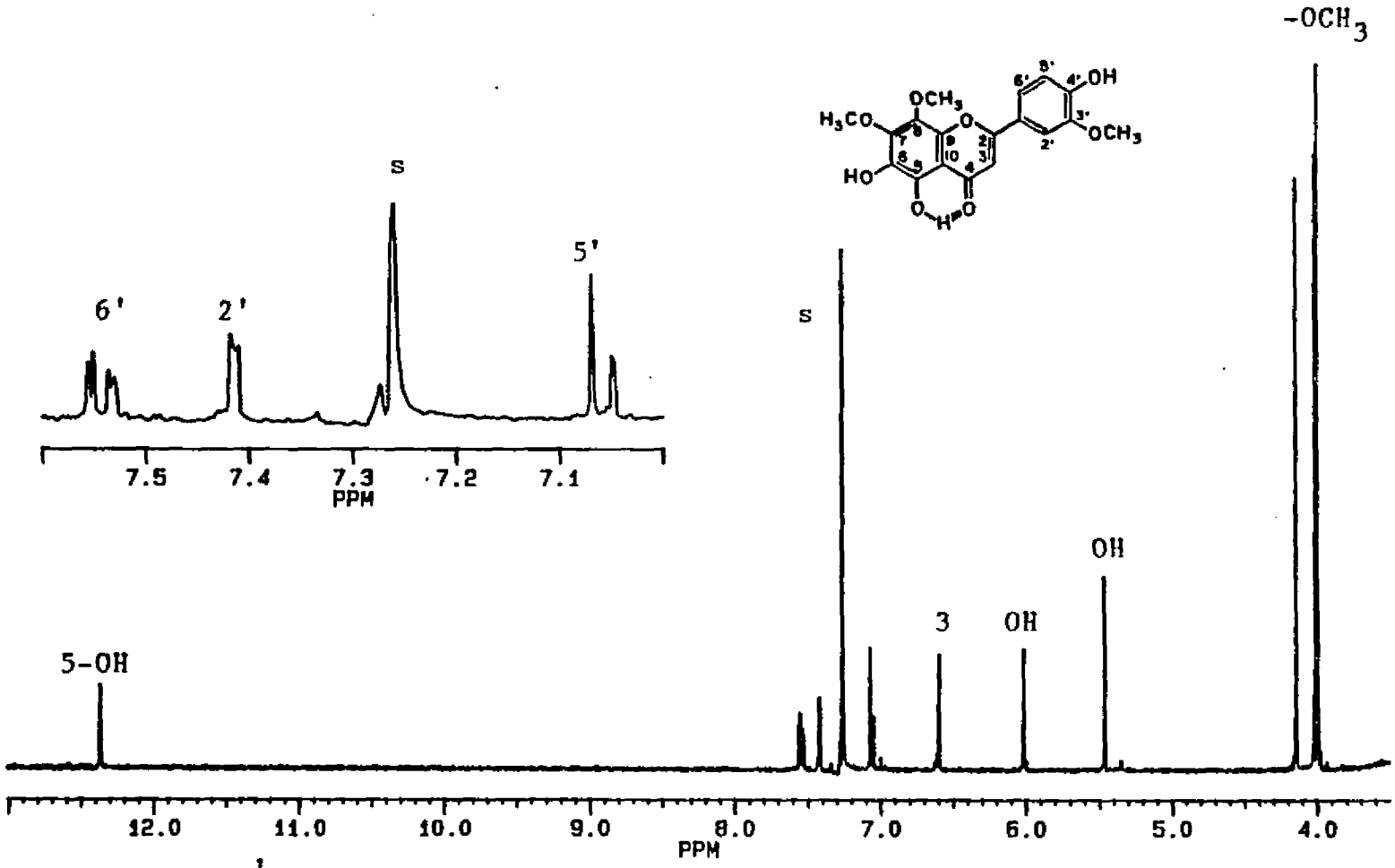


Figure 3.1. ^{1}H NMR spectrum of compound 1 in $CHCl_3$-d_1.

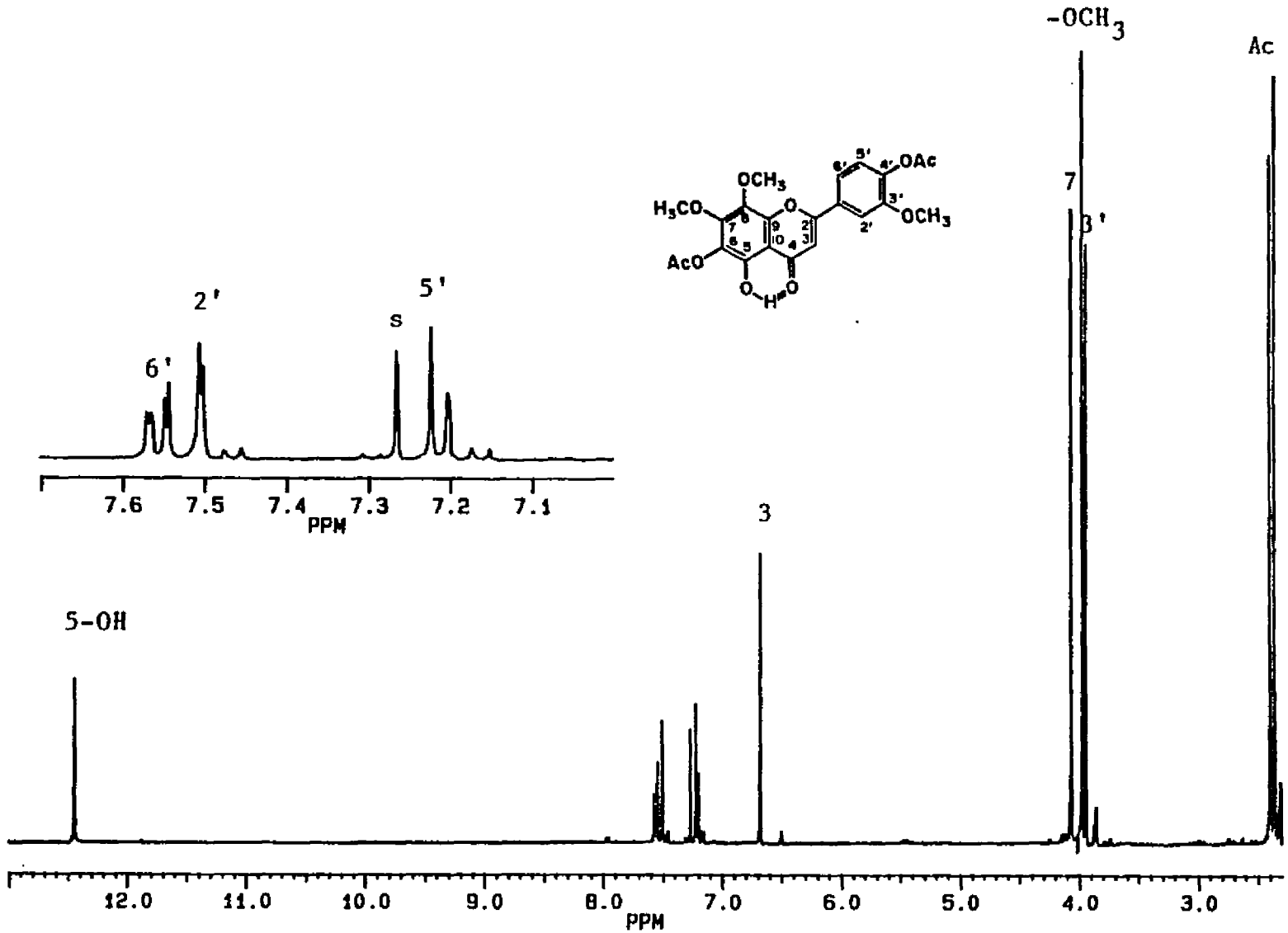


Figure 3.2. ^{1}H NMR spectrum of compound 1a in $CHCl_3$-d_1.

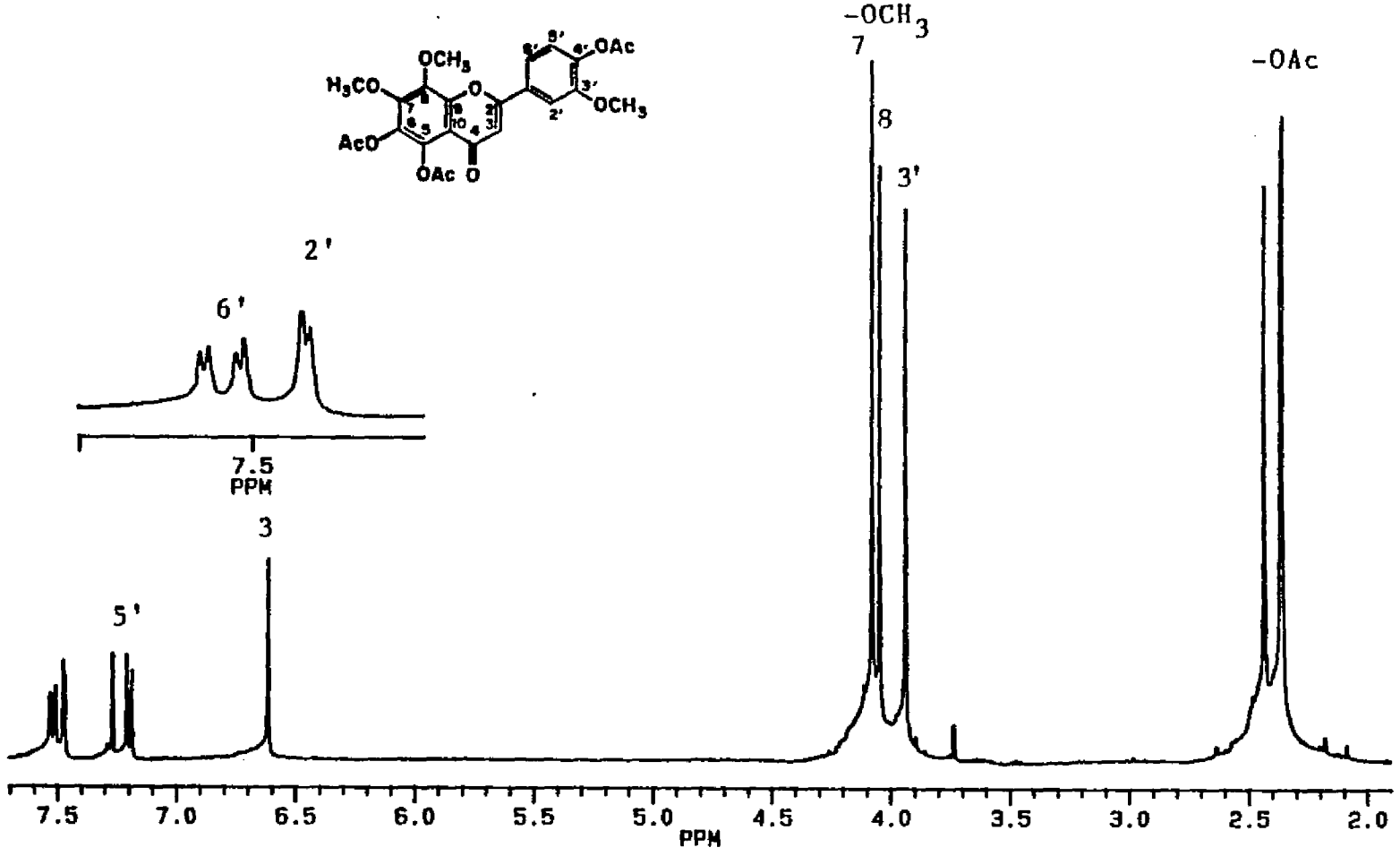


Figure 3.3. ^{1}H NMR spectrum of compound **1b** in $CHCl_3$-d_1.

Table 3.1. 400 MHz ^{1}H.NMR Spectral Data[a] of Compounds **1**, **1a**, and **1b**.

	δ,ppm (*J* in Hz)		
Proton	**1**	**1a**	**1b**
H-3	6.60 s	6.68 s	6.60 s
H-2'	7.42 d (1.8)	7.50 d (1.8)	7.45 d (1.8)
H-5'	7.06 d (8.7)	7.21 d (8.3)	7.16 d (8.2)
H-6'	7.55 dd (8.7, 1.8)	7.56 dd (8.3,1.8)	7.47 dd (8.2, 1.8)
5-OH	12.37 s	12.44 s	-----
7-OCH_3	4.15 s	4.07 s	4.06 s
8-OCH_3	4.01 s	3.98 s	4.02 s
3'-OCH_3	4.00 s	3.95 s	3.88 s
-OAc	---	2.36, 2.40 s	2.32, 2.35, 2.42 s

[a]Solvent: $CHCl_3$-d_1; internal standard: TMS.

Table 3.2. UV Spectral Data of Flavonoids from *Calamintha ashei*

Compound	λ max, nm				
	MeOH	+NaOMe	+$AlCl_3$	+$AlCl_3$/HCl	+NaOAc
1	290	326	306	304	290
	344	400	380	372	338
2	284	264	298	296	286
	342	400	376	372	344
3	254,280	266	288	264,288	276
	344	412	372	366	350
4	252,282	252,284	264,290	262,296	248,282
	340	338	366	360	340

The assignment of the singlet at δ 6.96 in compound **1** can not be derived from ^{1}H NMR spectra nor by UV methods (11). Since the NMR signal could be due to either H-8 or H-3, the proton's position was established by long-range ^{1}H-^{13}C correlation NMR experiment on flavonoids **1** and **1b**. This methodology correlates carbons which are separated by 2 bonds or 3 bonds from the coupled protons (12). A short range ^{1}H-^{13}C correlation spectrum was also obtained to check on residual short range ^{1}H-^{13}C couplings that may show up in the long-range spectrum. Figure 3.4 shows the contour plot of the short range ^{1}H-^{13}C 2D spectrum of compound **1**.

Figure 3.5 represents the contour plot of the long-range ^{1}H-^{13}C 2D NMR spectrum of **1b**. The proton singlet at δ 6.60 is coupled to two quaternary carbons at δ 113.0 and δ 160.9. Based on chemical shift considerations, these carbons are C-10 and C-2, respectively (13), suggesting that the proton is attached to C-3. The long-range coupling of C-2 with H-2' and H-6' also corroborates this assignment.

The ^{1}H-^{13}C 2D NMR data were used in the assignment of all carbons of compound **1** (Figure 3.7) and compound **1b** (Figure 3.8). Moreover, it allowed for the assignment of the carbon signals of compound **1a** (Figure 3.9). The long-range coupling of the C-5 hydroxyl proton (5-OH) in compound **1** verified that C-6 is hydroxlated (Figure 3.6). It will be noted that the C-5-hydroxyl proton correlates to three quaternary carbons which were assigned to C-10, C-5, and C-6. Since C-6 does not couple to a methoxyl signal, it must have a hydroxyl substituent. The ^{13}C NMR data are summarized in Table 3.3.

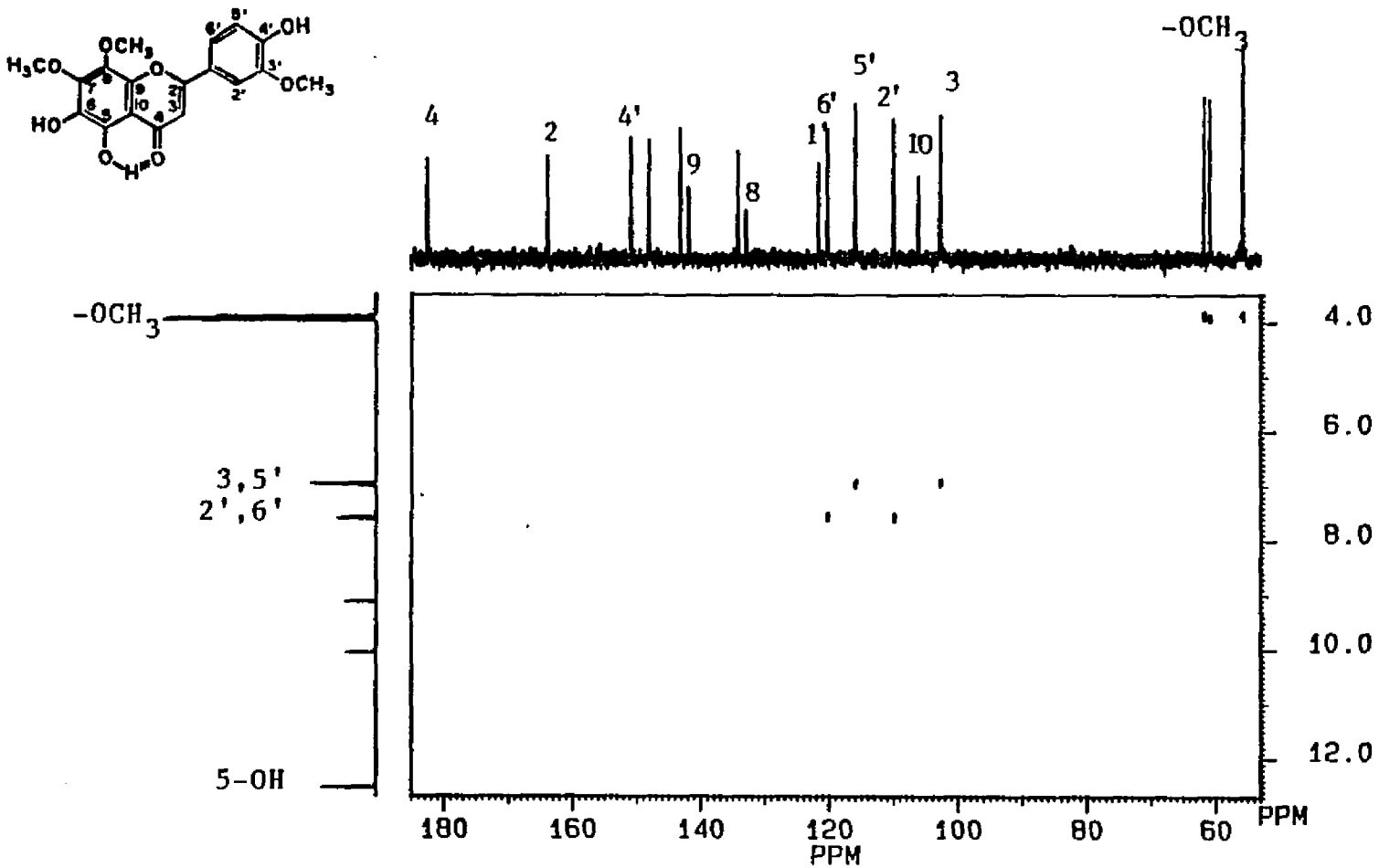


Figure 3.4. Contour plot of ^{1}H-^{13}C short range correlated NMR of compound 1 in DMSO-d_6.

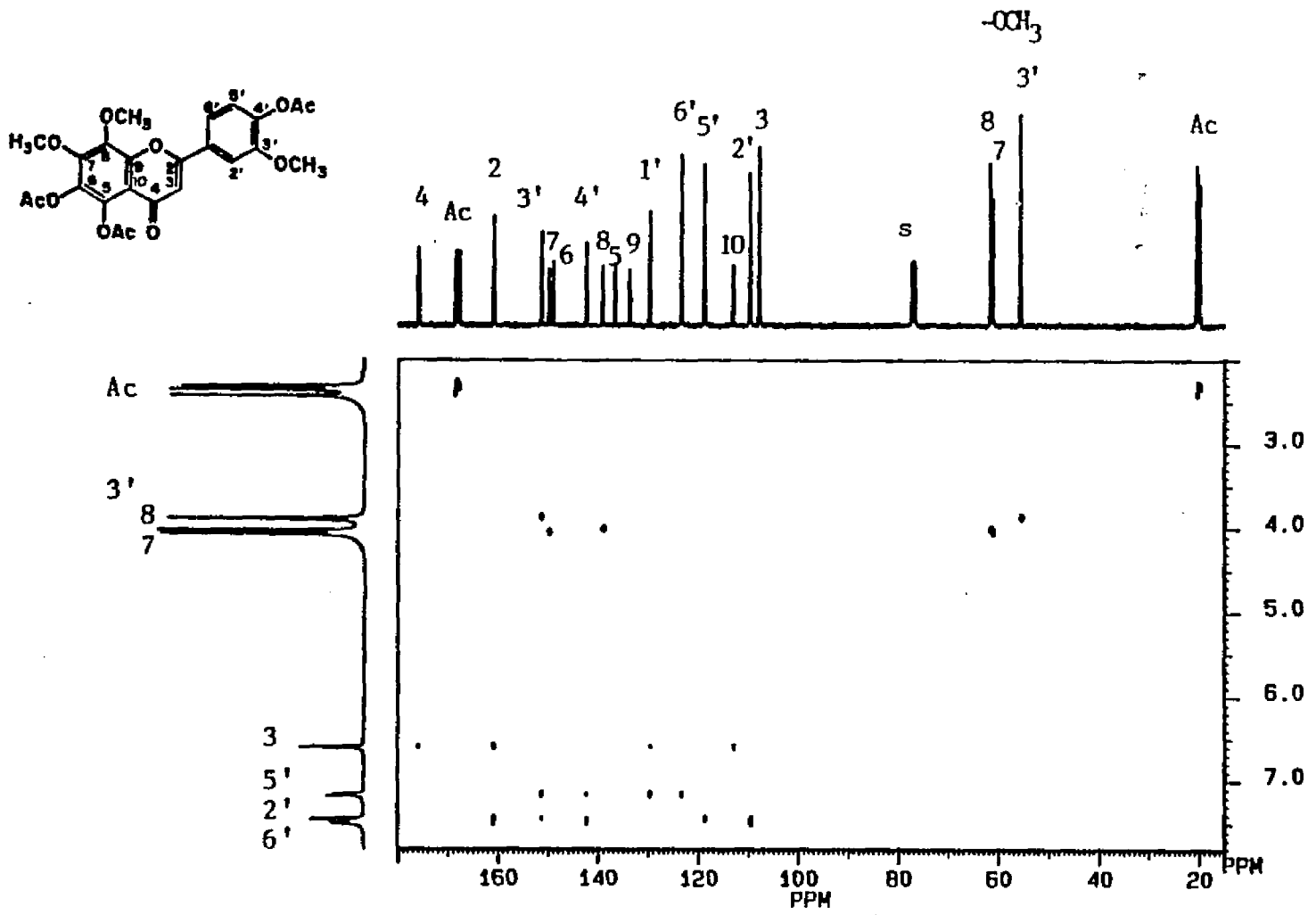


Figure 3.5. Contour plot of ^{1}H-^{13}C long range shift correlated NMR of compound 1b in $CHCl_3$-d_1.

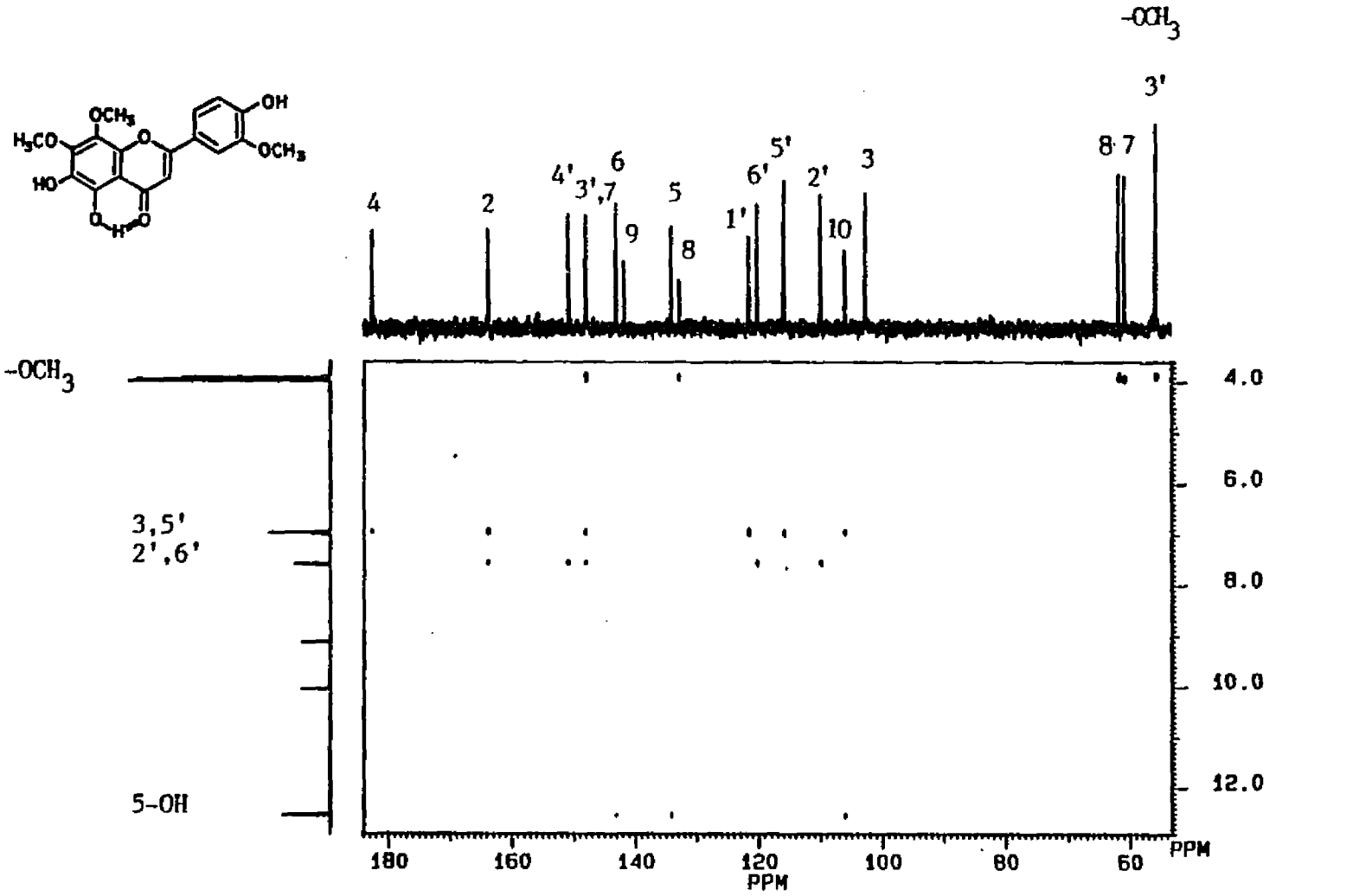


Figure 3.6. Contour plot of ^{1}H-^{13}C long range shift correlated NMR of compound 1 in DMSO-d_6.

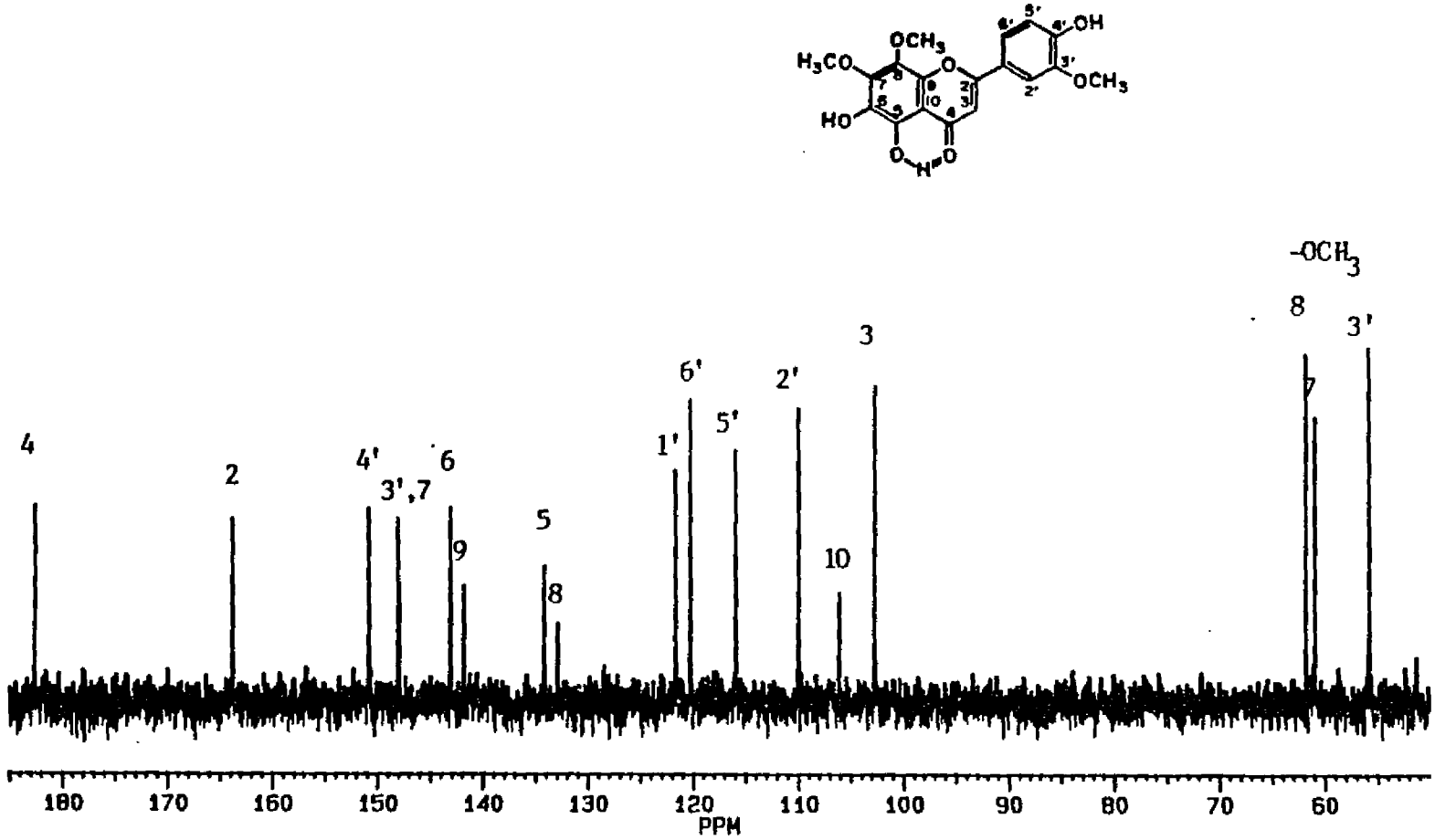


Figure 3.7. ^{13}C NMR spectrum of compound 1 in DMSO-d_6.

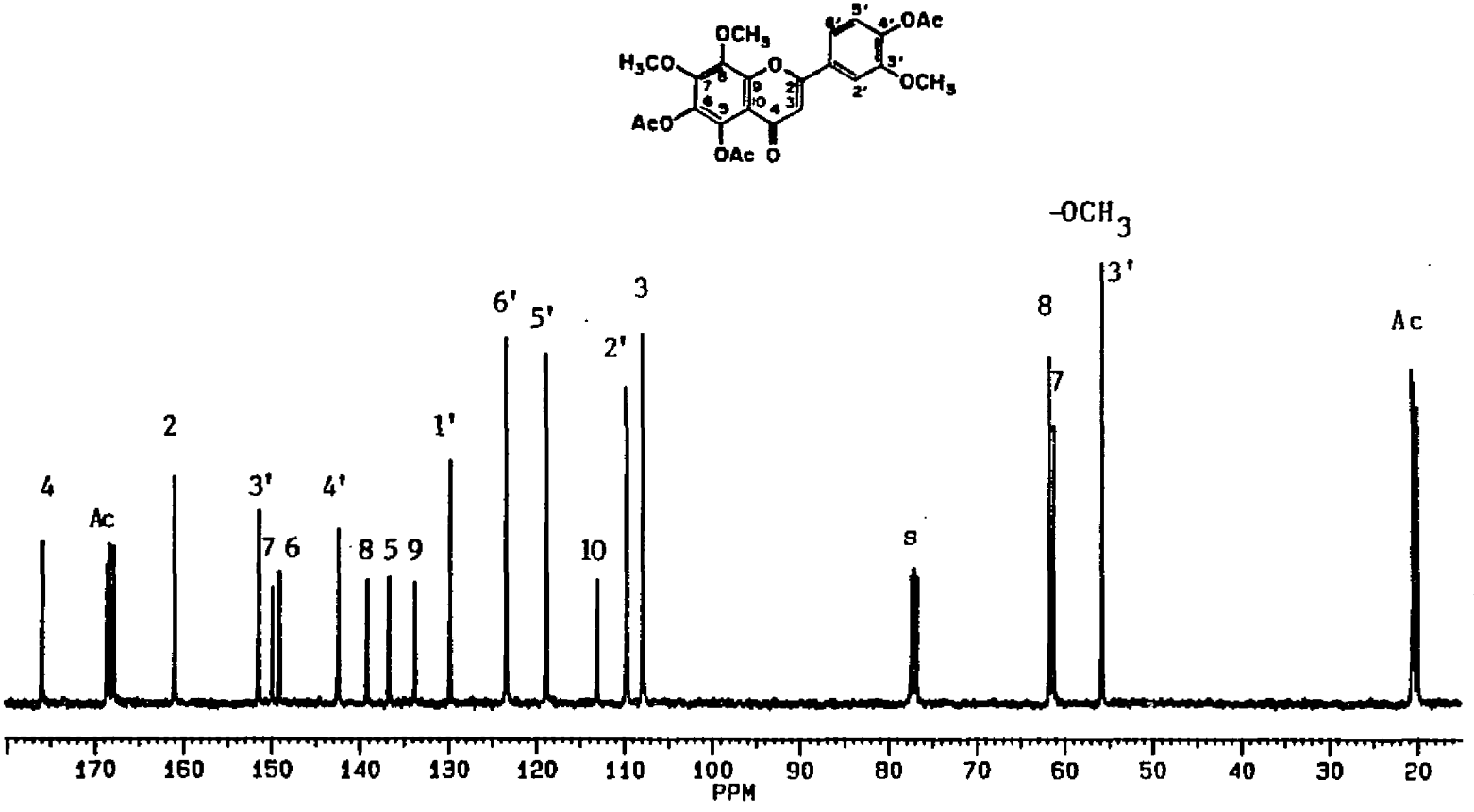


Figure 3.8. ^{13}C NMR spectrum of compound 1b in $CHCl_3$-d_1.

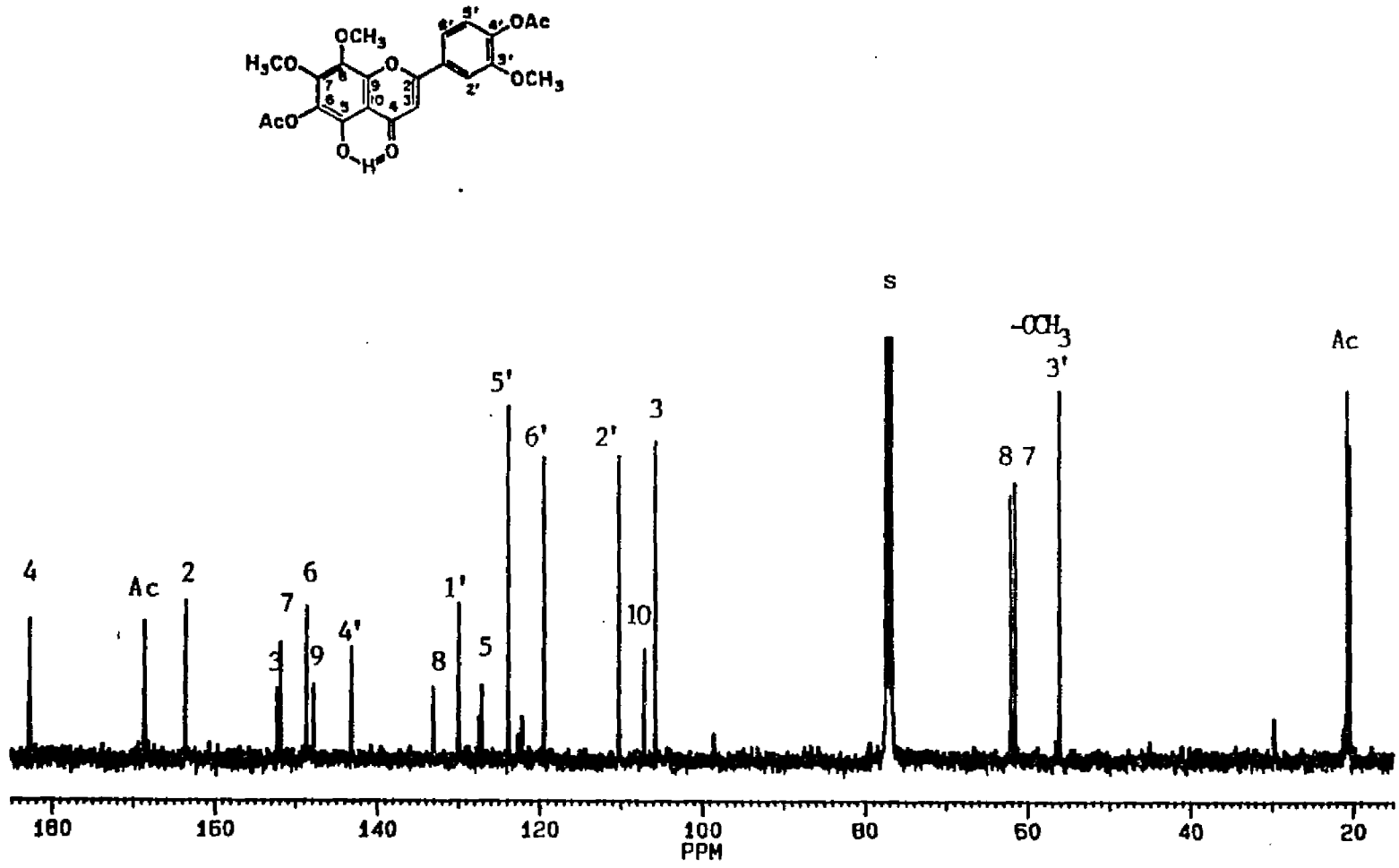


Figure 3.9. ^{13}C NMR spectrum of compound **1a** in $CHCl_3$-d_1.

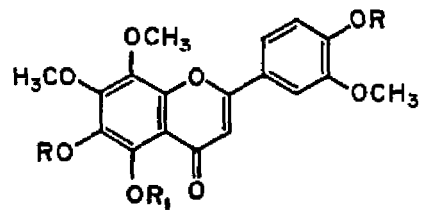
Table 3.3. 100 MHz ^{13}C NMR Spectral Data of Compounds **1**, **1a** and **1b**.

Carbon	δ,ppm 1[a]	1a[b]	1b[b]
C-2	163.8 s	163.5 s	160.9 s
C-3	102.7 d	105.7 d	107.8 d
C-4	182.6 s	182.8 s	175.9 s
C-5	134.1 s	127.0 s	136.6 s
C-6	143.1 s	148.6 s	149.0 s
C-7	132.9 s	152.2 s	149.8 s
C-8	147.9 s	133.0 s	139.1 s
C-9	141.8 s	147.8 s	133.7 s
C-10	106.1 s	107.1 s	113.0 s
C-1'	121.6 s	129.9 s	129.7 s
C-2'	110.0 d	110.2 d	109.6 d
C-3'	148.0 s	151.8 s	151.4 s
C-4'	150.8 s	143.1 s	142.3 s
C-5'	115.9 d	119.4 d	118.7 d
C-6'	120.2 d	123.8 d	123.3 d
7-OMe	60.9 q	61.5 q	61.2 q
8-OMe	61.8 q	62.0 q	61.6 q
3'-OMe	55.8 q	56.1 q	55.7 q
Ac(Me)	---	20.4 q	19.9 q
	---	20.6 q	20.3 q
	---	---	20.5 q
Ac(C=O)	---	168.5 s	167.8 s
	---	168.6 s	168.2 s
	---	---	168.5 s

[a] DMSO-d_6 as solvent and as internal standard, δ 39.5.
[b] $CHCl_3$-d_1 as solvent and as internal standard, δ 77.0.

The mass spectrum of compound **1** showed a molecular ion peak at *m/z* 360. The base peak at *m/z* 345 (M^+-CH_3) suggests a methoxyl group at C-8 of a flavone (14). A peak due to the loss of CH_3CO (43 mu) from the parent ion was not observed. This suggested that flavone **1** does not represent a 3-methoxylated flavonol (15). The mass spectra of **1a** and **1b** confirmed the presence of two and three acetate groups, respectively. It also agrees with the fragmentation pattern of the flavone. The mass spectral data of **1**, **1a**, and **1b** are summarized in Tables 3.4, 3.5, and 3.6.

On the basis of the spectroscopic data presented above, compound **1** was found to be 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone.



1: R = R_1 = H

1a: R = -$COCH_3$, R_1 = H

1b: R = R_1 = -$COCH_3$

The structure of compound **1b** was confirmed by single crystal X-ray crystallography.[a] The compound was found to exist in two conformations as shown in Figure 3.10.

---

[a]X-ray work was done by Dr. Frank R. Fronczek, Department of Chemistry, Louisiana State University.

Table 3.4. Mass Spectral Data of Compound 1.

m/z (%)	Fragmentation
361 (14)	$M^{+\cdot}$ + 1
360 (47)	$M^{+\cdot}$ $C_{18}H_{16}O_8$
346 (32)	$M^{+\cdot}$ - CH_3 + $H^{\cdot}$
345 (100)	$M^{+\cdot}$ - CH_3
330 (10)	$M^{+\cdot}$ - 2 CH_3
327 (16)	$M^{+\cdot}$ - CH_3 - H_2O
197 (17)	$A_1^{+\cdot}$ - CH_3
169 (11)	$A_1^{+\cdot}$ - CO
151 (4)	$A_1^{+\cdot}$ - CH_3 - H_2O - CO

Table 3.5. Mass Spectral of Compound 1a.

m/z (%)	Fragmentation
444 (7)	$M^{+\cdot}$ $C_{22}H_{20}O_{10}$
402 (67)	$M^{+\cdot}$ - CH_2CO
387 (18)	$M^{+\cdot}$ - CH_2CO - CH_3
360 (18)	$M^{+\cdot}$ - 3 CH_2CO
345 (100)	360 - CH_3
327 (10)	360 - CH_3 - H_2O

Table 3.6. Mass Spectral Data of Compound **1b**.

m/z (%)	Fragmentation
486 (1)	$M^{+\cdot}$ $C_{24}H_{22}O_{12}$
444 (18)	$M^{+\cdot}$ - CH_2CO
402 (100)	$M^{+\cdot}$ - 2 CH_2CO
387 (18)	$M^{+\cdot}$ - 2 CH_2CO - H_2O
372 (16)	$M^{+\cdot}$ - 2 CH_2CO - H_2O - CH_3
360 (18)	$M^{+\cdot}$ - 3 CH_2CO
359 (20)	360 - H
345 (57)	360 - CH_3
327 (8)	360 - CH_3 - H_2O
197 (9)	A_1^+ - CH_3

Table 3.7. 400 MHz ^1H NMR Spectral Data of Compounds **2** and **2a**.

	δ, ppm (*J* in Hz)	
	2[a]	**2a**[b]
H-3	6.70 s	6.56 s
H-8	6.85 s	6.94 s
H-2'	7.64 d (1.9)	7.37 d (1.9)
H-5'	7.01 d (8.5)	7.17 d (8.2)
H-6'	7.61 dd (8.5, 1.9)	7.46 dd (8.2, 1.9)
5-OH	12.71 s	----
3'-OCH_3	3.99 s	3.93 s
7-OCH_3	4.00 s	3.96 s
OAc	----	2.348 s
	----	2.351 s
	----	2.44 s

[a]Solvent: acetone-d_6 at 308°K; TMS as internal standard.
[b]Solvent: $CHCl_3$-d_1; TMS as internal standard.

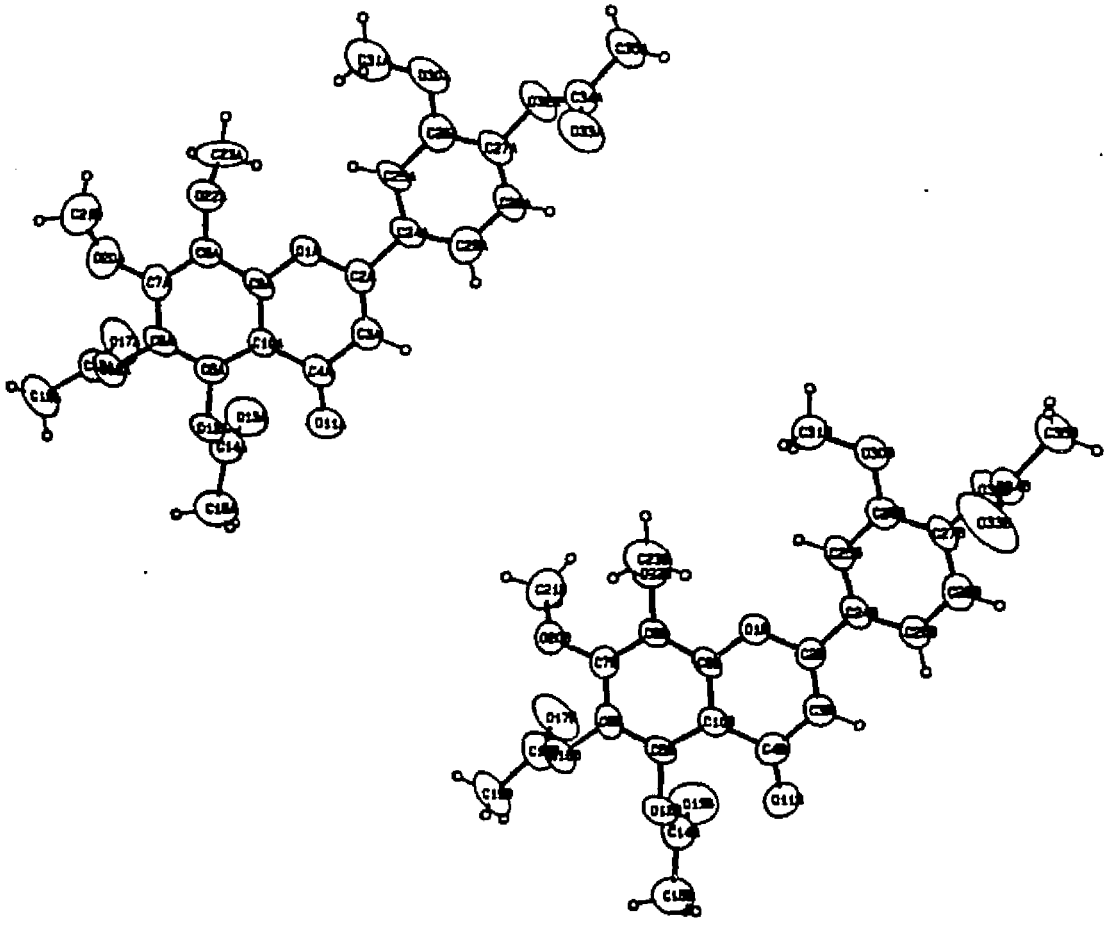


Figure 3.10. Two Conformations of Compound **1** Determined by X-ray Crystallography.

Compound **2** was the most polar flavonoid isolated from the DCM extract of *C. ashei*. Its UV spectral data (Table 3.2) is very similar to that of compound 1, indicating hydroxyl groups at C-4' and C-5, no hydroxyl group at C-7 (10) and a substituted C-6 (11).

The ^{1}H NMR data of compound 2 (Figure 3.11) and its acetate (2a) (Figure 3.12) indicated a 3',4'-substitution pattern in ring B, a proton at C-3 and the presence of two methoxyl groups. The proton signal at δ 12.71 also indicated a hydrogen-bonded C-5 hydroxyl group. Since the UV data suggested a hydroxyl group at C-4' but not at C-7, then the methoxyl groups must be at C-3' and C-7 and C-6 must be hydroxylated. The ^{1}H NMR data are summarized in Table 3.7.

The mass spectral data of compound 2 (Table 3.8) showed a molecular ion base peak. This is consistent with a flavone structure which lacks a methoxyl group at C-8 (15). The mass spectrum of compound 2a confirmed the presence of three acetate groups (Table 3.9).

The ^{13}C NMR spectrum of 2 (Figure 3.13) and 2a (Figure 3.14) confirmed the presence of two methoxyl groups, five aromatic protons and three hydroxyl groups. The complete assignment of the ^{13}C signals of compound 2 was derived with the aid of INAPT experiments, which will be discussed later, and by comparison with the ^{13}C NMR spectral data of compound 1 and 2a. The ^{13}C NMR data are summarized in Table 3.10.

The unambiguous assignments of the ^{13}C signals of compound 2a were carried out by short-range and long-range ^{1}H-^{13}C 2D NMR experiments. The contour plot of the short-range 2D experiment (Figure 3.15) shows the carbons directly bonded to a proton. On the other hand, the long-range 2D experiment (Figure 3.16) correlates carbons that are separated by two or three bonds from the protons. The proton singlet at δ 6.56 (H-3) is coupled to two quaternary

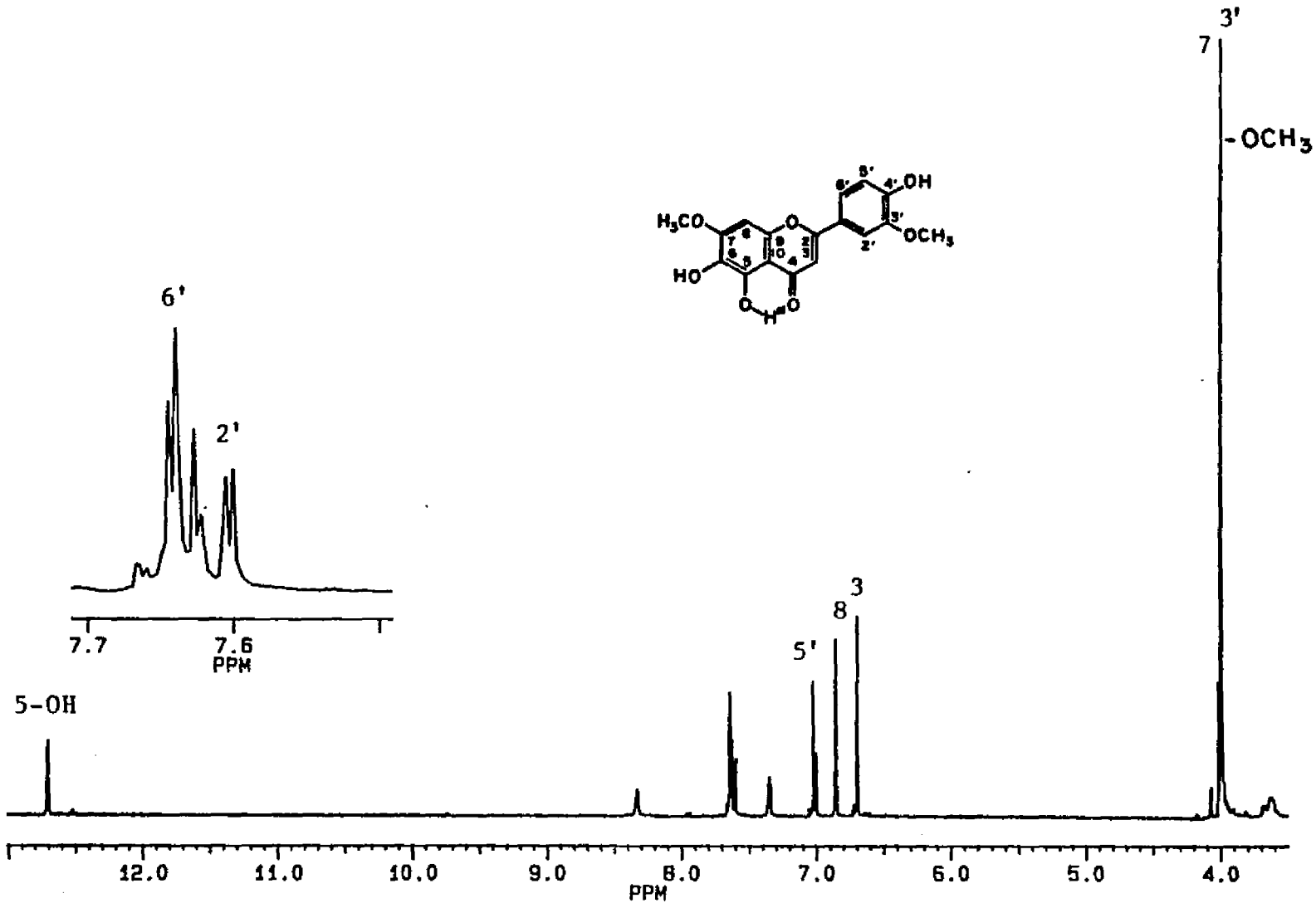


Figure 3.11. ^{1}H NMR spectrum of compound 2 in $(CD_3)_2CO$ at 35°C.

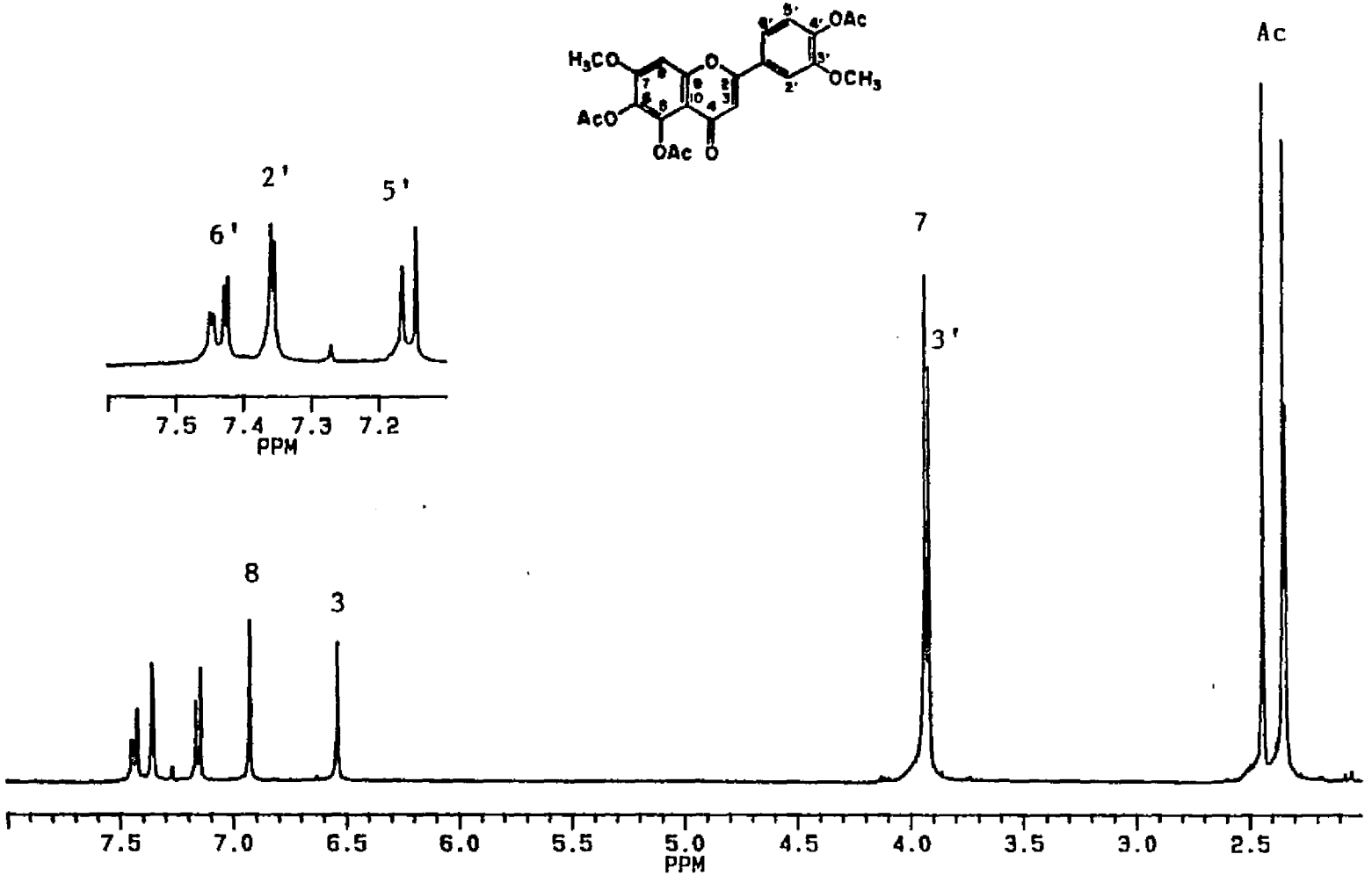


Figure 3.12. ^{1}H NMR spectrum of compound 2a in $CHCl_3$-d_1.

Table 3.8. Mass Spectral Data of Compound 2.

m/z (%)	Fragmentation
331 (26)	$M^{+\cdot}$ + H
330 (100)	$M^{+\cdot}$, $C_{17}H_{14}O_7$
329 (14)	$M^{+\cdot}$ - H
312 (38)	$M^{+\cdot}$ - H_2O
300 (6)	$M^{+\cdot}$ - 2 CH_3
284 (46)	$M^{+\cdot}$ - H_2O - CO
269 (5)	$M^{+\cdot}$ - H_2O - CO - CH_3
149 (14)	$B^{+\cdot}$ + H
148 (9)	$B^{+\cdot}$

Table 3.9. Mass Spectral Data of Compound 2a.

m/z (%)	Fragmentation
456 (2)	$M^{+\cdot}$, $C_{23}H_{20}O_{10}$
414 (11)	$M^{+\cdot}$ - CH_2CO
372 (100)	$M^{+\cdot}$ - 2 CH_2CO
330 (33)	$M^{+\cdot}$ - 3 CH_2CO
329 (17)	330 - H
312 (31)	330 - H_2O
284 (12)	330 - H_2O - CO
183 (5)	A_1^+ + H

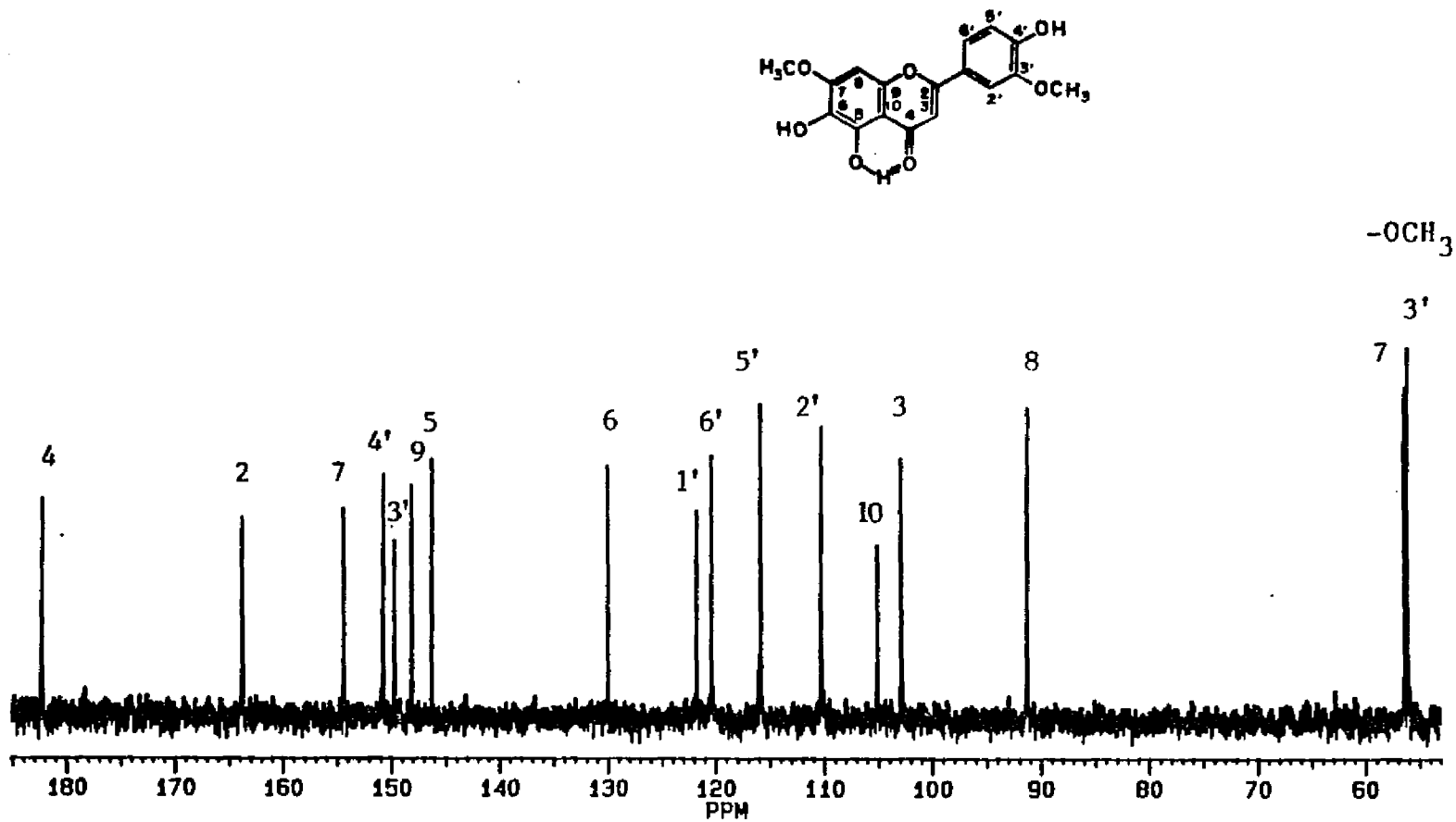


Figure 3.13. ^{13}C NMR spectrum of compound 2 in DMSO-d_6.

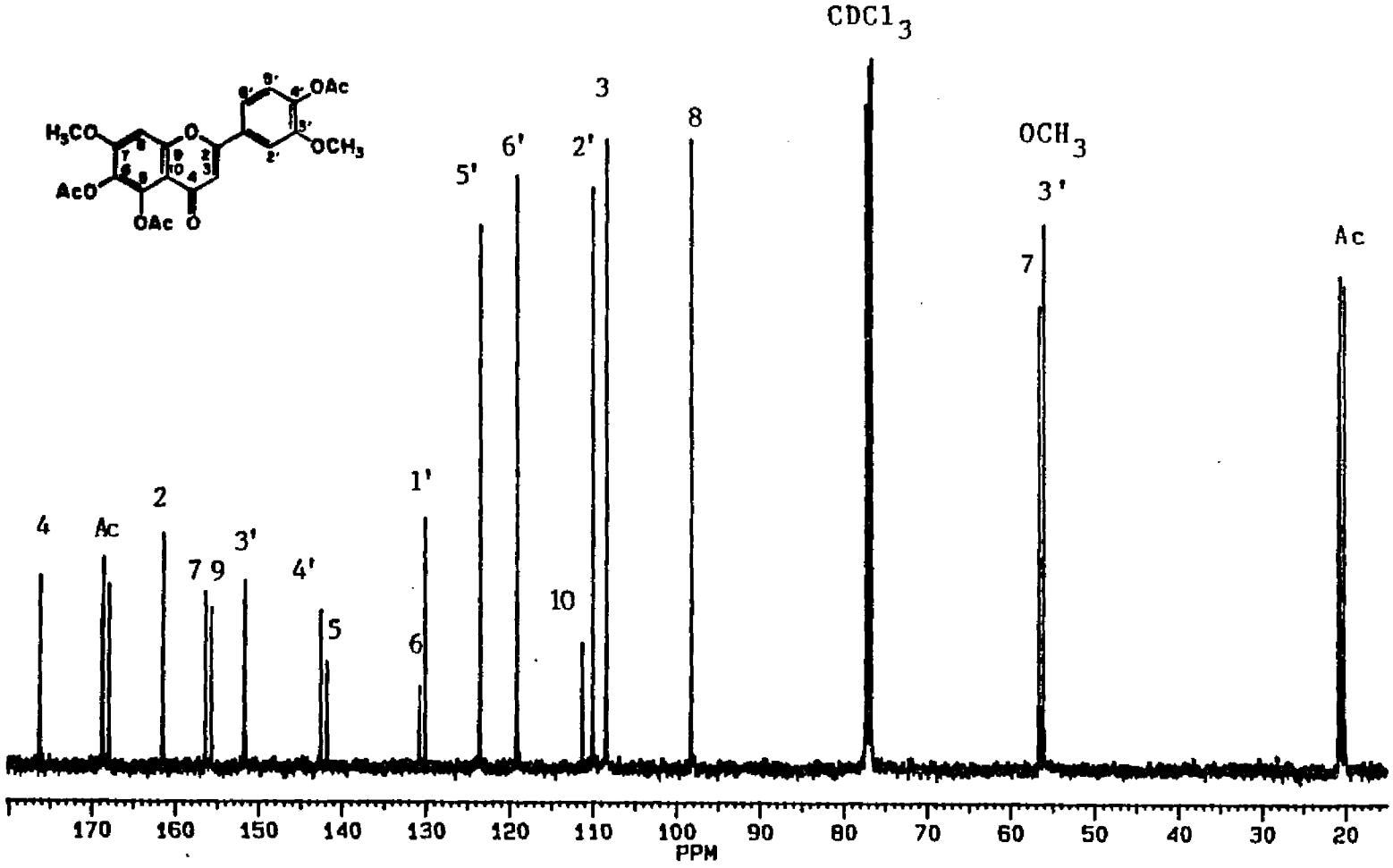


Figure 3.14. ^{13}C NMR spectrum of compound 2a in $CHCl_3$-d_1.

Table 3.10. 100 MHz ^{13}C NMR Spectral Data of Compounds 2 and 2a.

Carbon	δ, ppm	
	2[a]	2a[b]
C-2	163.7 s	161.3 s
C-3	102.8 d	108.3 d
C-4	182.2 s	176.1 s
C-5	146.2 s	141.7 s
C-6	130.0 s	130.6 s
C-7	154.3 s	156.2 s
C-8	91.2 d	98.2 d
C-9	148.0 s	155.6 s
C-10	105.0 s	111.2 s
C-1'	121.7 s	130.0 s
C-2'	110.2 d	109.9 d
C-3'	149.6 s	151.5 s
C-4'	150.7 s	142.5 s
C-5'	115.8 d	123.4 d
C-6'	120.3 d	119.0 d
7-OMe	56.3 q	56.6 q
3'-OMe	56.0 q	56.1 q
Ac(Me)	---	20.1 q
	---	20.6 q
	---	20.8 q
Ac(C=O)	---	167.8 s
	---	168.5 s
	---	168.6 s

[a] DMSO-d_6 as solvent and internal standard, δ 39.5.
[b] $CHCl_3$-d_1 as solvent and internal standard, δ 77.0.

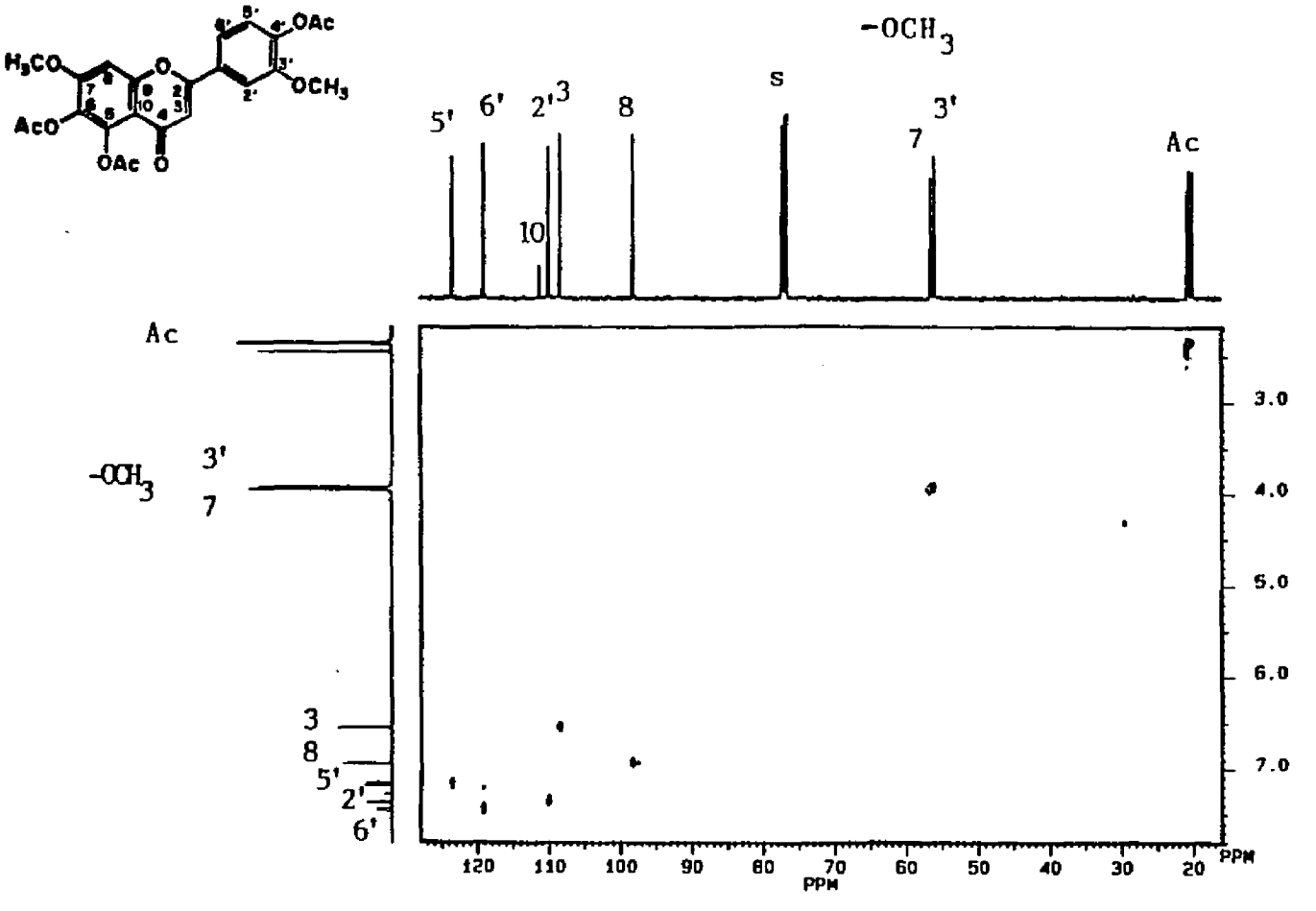


Figure 3.15. Contour plot of the ^{1}H-^{13}C short-range shift correlated NMR of compound 2a.

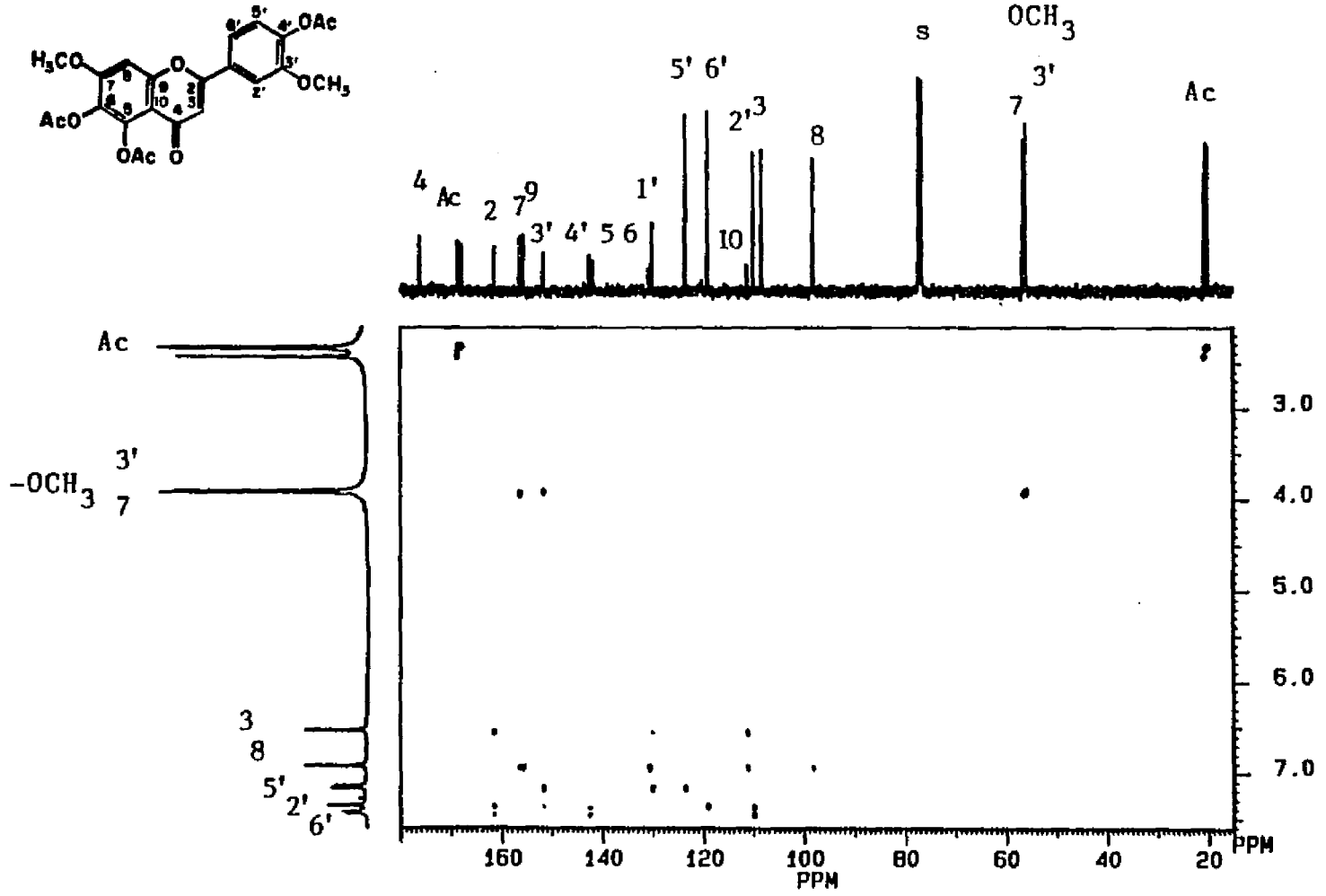
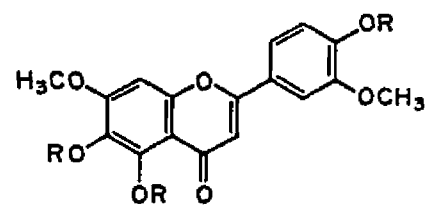


Figure 3.16. Contour plot of ^{1}H-^{13}C long-range shift correlated NMR of compound 2a.

carbons at δ 161.3 (C-2) and δ 111.2 (C-10). The proton at δ 6.94 (H-8) is coupled to four quaternary carbons at δ 111.2 (C,10), δ 130.6 (C-6) δ 155.6 (C-9) and δ 156.2 (C-7) and a short-range coupling to C-8 is observed. The C-7 signal is also correlated to one methoxyl signal. The other proton and carbon signals were analyzed in the same manner. Thus, the data confirm the structure of compound **2** to be 5,6,4'-trihydroxy-7,3'-dimethoxyflavone. This flavone has been previously synthesized (5) and was isolated from *Citharexylum subserratum* as its 6-glucoside (6).



2: R = H

2a: R = $COCH_3$

Compound **3** was isolated from the less polar chromatographic flavonoid fractions. The UV spectral data (Table 3.2) indicated the hydroxylation pattern of compound **3**. The band I bathochromic shift by 68 nm upon addition of NaOMe indicated a C-4'-OH (10). Treatment of compound **3** with $AlCl_3$/HCl caused a band I bathochromic shift by 22 nm which suggested a 6-substituted 3-methoxylated flavonol or a 6,7,8-substituted flavone (11). Addition of NaOAc did not cause a significant UV absorption shift which indicated the absence of C-7-OH. The ^1H NMR spectrum (Figure 3.17, Table 3.11) supported the

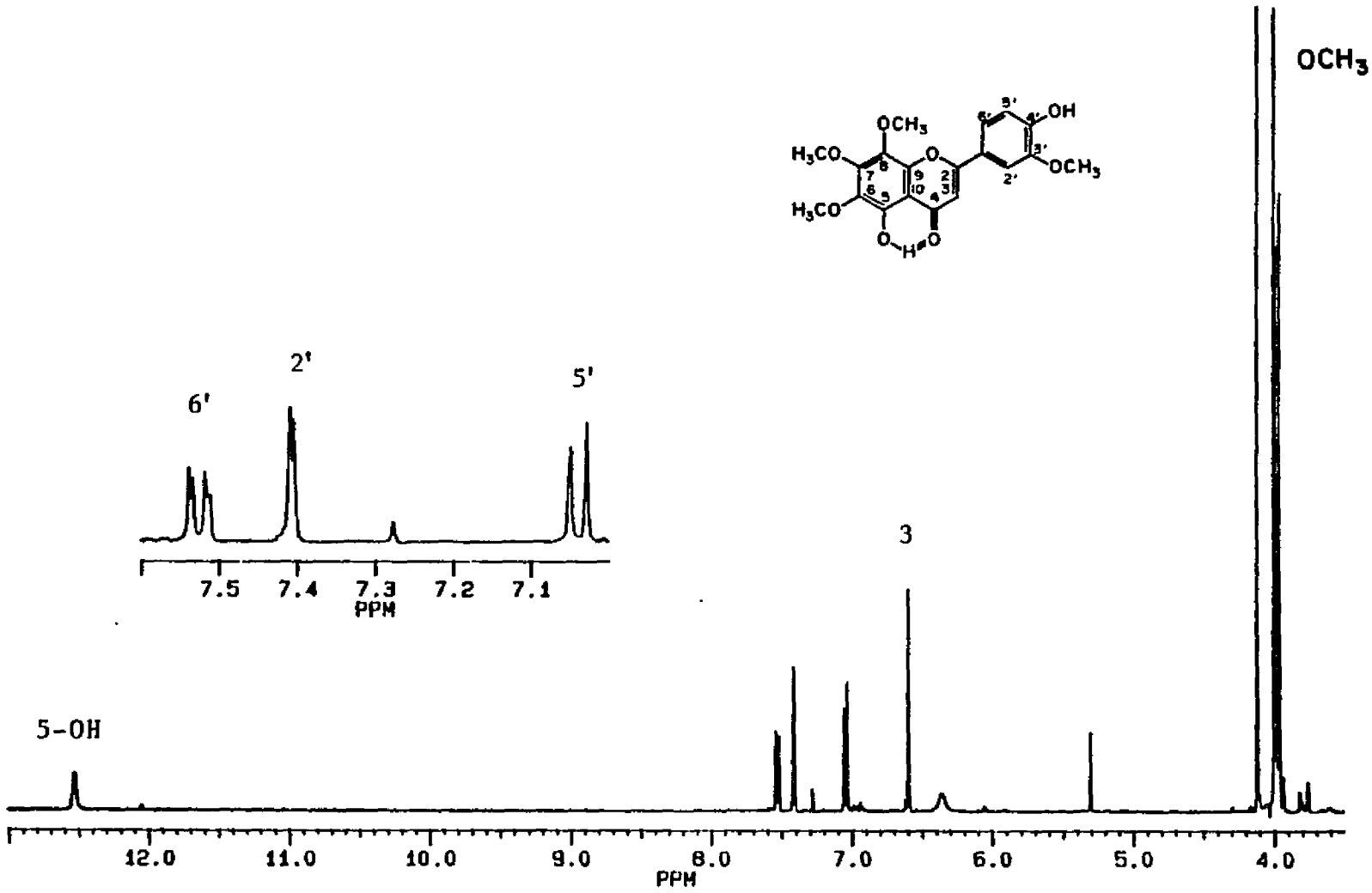


Figure 3.17. ^{1}H NMR spectrum of compound 3 in $CHCl_3$-d_1.

Table 3.11. 400 MHz ^{1}H NMR Spectral Data of Compounds **3** and **4**.

Proton	δ,ppm (J in Hz)	
	3[a]	**4**[a]
H-3	6.59 s	6.62 s
H-2'	7.40 d (1.7)	7.43 d (1.6)
H-5'	7.04 d (8.3)	7.00 d (8.6)
H-6'	7.53 dd (8.3, 1.7)	7.59 dd (8.6, 1.6)
5-OH	12.53 s	12.52 s
6-OCH_3	3.96 s	3.96 s
7-OCH_3	3.98 s	3.97 s
8-OCH_3	4.11 s	4.11 s
3'-OCH_3	3.99 s	3.98 s[c]

[a]$CHCl_3$-d_1 as solvent and TMS as internal standard.

[c]Proton signal integrated for 2 CH_3 which are assigned to 3'- and 4'-OCH_3.

presence of the hydrogen-bonded hydroxyl group at C-5, four methoxyl groups and a singlet at δ 6.59 due to H-3. As in the previously discussed flavones from *C. ashei*, a 3',4'-substitution pattern in ring B was observed. The ^{13}C NMR spectrum (Figure 3.18) of compound 3 confirmed the presence of four methoxyl groups and four methine protons. The complete assignment of the carbon signals were carried out by INAPT experiments (discussed in the next chapter) and by chemical shift considerations (Table 3.12).

The mass spectrum of compound 3 (Table 3.13) showed a base peak at *m/z* 359 $[M-15]^+$ which is indicative of a flavone with a C-8 methoxyl group (14,19). A retro-Diels-Alder fragmentation of compound 3 was suggested by peaks at *m/z* 211 and 183 (15) as shown in Figure 3.19 (15).

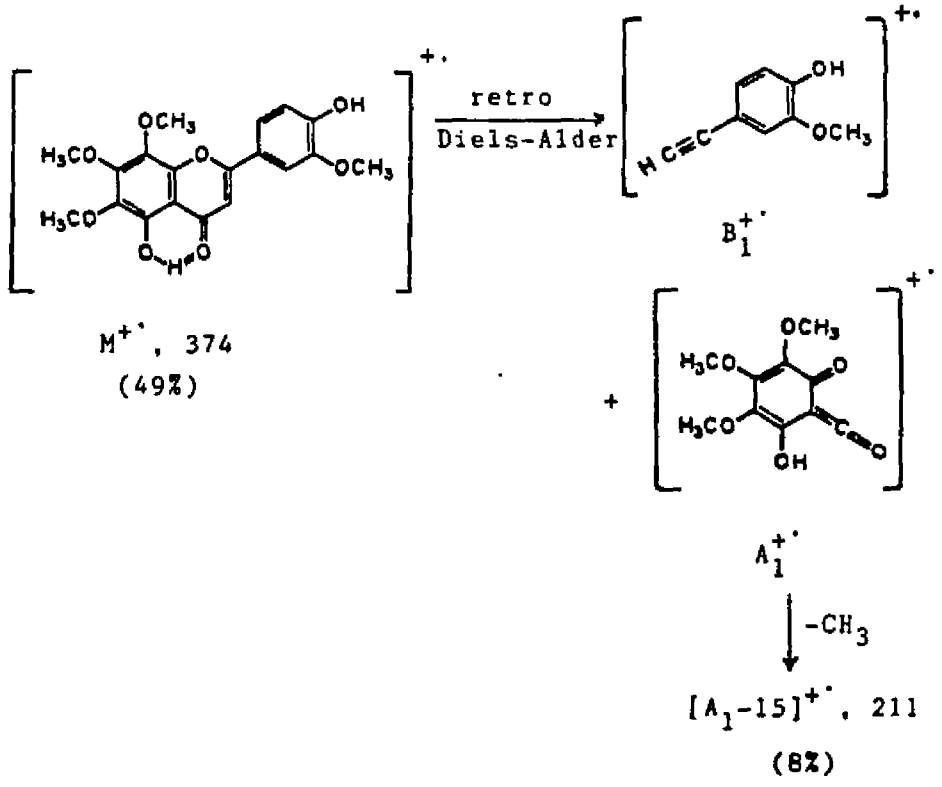


Figure 3.19. The retro-Diels-Alder fragmentation of compound 3.

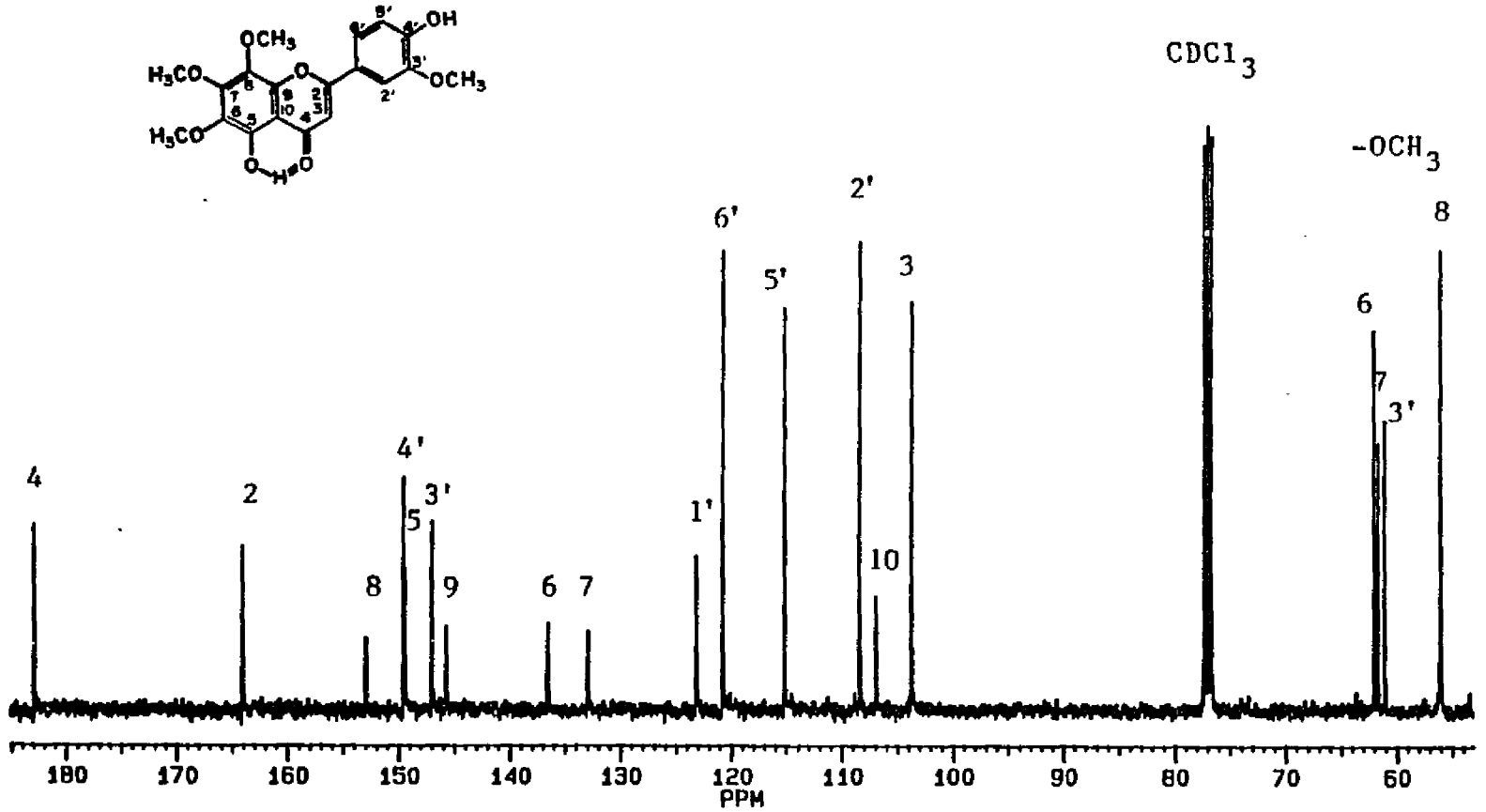


Figure 3.18. ^{13}C NMR spectrum of compound 3 in $CHCl_3$-d_1.

Table 3.12. 100 MHz ^{13}C NMR spectral data[a] of compounds 3 and 4.

Carbon	δ,ppm	
	3	4
C-2	164.0 s	164.0 s
C-3	103.7 d	104.0 d
C-4	182.9 s	183.0 s
C-5	149.4 s	145.8 s
C-6	136.5 s	136.6 s
C-7	132.9 s	133.0 s
C-8	152.9 s	153.0 s
C-9	145.7 s	149.4 s
C-10	106.9 s	107.0 s
C-1'	123.1 s	123.7 s
C-2'	108.4 d	108.9 d
C-3'	147.0 s	149.6 s
C-4'	149.6 s	152.5 s
C-5'	115.1 d	111.3 d
C-6'	120.7 d	120.2 d
6-OCH_3	62.0 q	62.0 q
7-OCH_3	61.6 q	61.7 q
8-OCH_3	56.0 q	56.0 q
3'-OCH_3	61.1 q	61.1 q
4'-OCH_3	----	56.1 q

[a] $CHCl_3$-d_1 as solvent and as internal standard, δ 77.0.

Table 3.13. Mass Spectral Data of Compound 3.

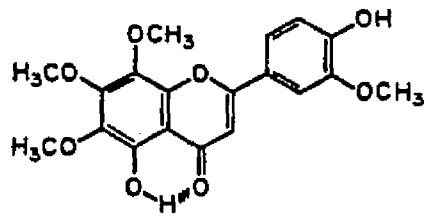
m/z (%)	Fragmentation
374 (49)	$M^{+\cdot}$ $C_{19}H_{18}O_8$
359 (100)	$M^{+\cdot}$ - CH_3
344 (9)	$M^{+\cdot}$ - 2 CH_3
211 (8)	A_1^+ - CH_3
183 (8)	A_1^+ - CH_3 - CO

Table 3.14. Mass Spectral Data of Compound 4.

m/z (%)	Fragmentation
388 (53)	$M^{+\cdot}$ $C_{20}H_{20}O_8$
373 (100)	$M^{+\cdot}$ - CH_3
211 (7)	A_1^+ - CH_3
183 (7)	A_1^+ - CH_3 - CO

The above spectroscopic data established the structure of flavone **3** as 5,4'-dihydroxy- 6,7,8,3'-tetramethoxyflavone.

The UV and ^{1}H NMR spectral data of compound **3** are in good agreement with data reported for the synthetic (16,17) and natural flavone (18). However, the experimental melting point (152-154°) is considerably lower than the reported values [164-165° (17), 163.5-165° (18)].

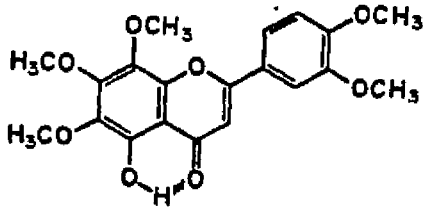


Compound 3

The ^{1}H NMR spectrum of compound **4** (Figure 3.20, Table 3.11) indicated the presence of five methoxyl groups, a hydrogen bonded hydroxyl group at C-5 and a 3',4'-substitution pattern at ring B. The proton singlet at δ 6.62 was assigned to H-3. As in compound **3**, the mass spectrum of compound **4** (Table 3.14) showed a base peak at *m/z* 373 $[M-CH_3]^+$ and a retro-Diels-Alder fragmentation pattern. The identical mass of the retro-Diels-Alder fragments from compounds **3** and **4** indicated that both flavones have the same ring A structure. The UV data (Table 3.2) of compound **4**, upon treatment with $AlCl_3$/HCl, suggested a C-6-substituted flavone. The absence of UV absorption shifts with NaOMe and NaOAc indicated that there are no hydroxyl groups at C-4' and C-7, respectively (10).

The ^{13}C NMR chemical shifts were assigned by correlation with those of compound **3**. Note that with a methoxylated C-4', both C-4' and C-3' were shifted downfield while C-5' had about 4 ppm upfield shift (Figure 3.21, Table 3.12).

On the basis of its spectral data, compound **4** was found to be 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone or 5-desmethoxynobiletin (8). Compound **4** melted at 116-118° which is about 30° below that reported (mp 147-148°) by Tatum and Berry (8). While the UV data of desmethoxynobiletin in 95% EtOH agrees with data of compound **4** in MeOH, comparison of other spectroscopic data could not confirm the identity of the newly isolated compound **4** and the previously isolated 5-desmethoxynobiletin (8).



Compound **4**

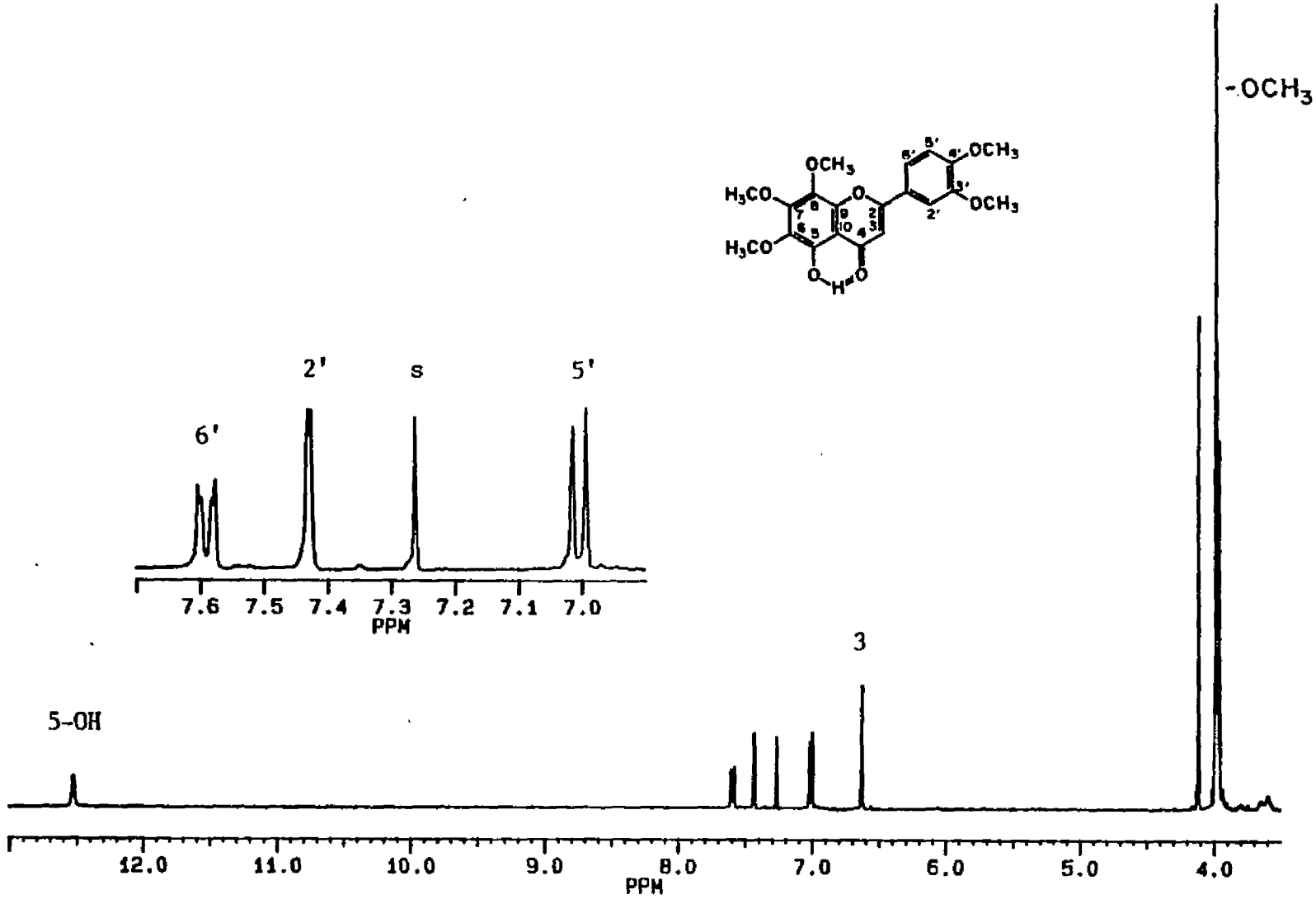


Figure 3.20. ^{1}H NMR spectrum of compound 4 in $CHCl_3$-d_1.

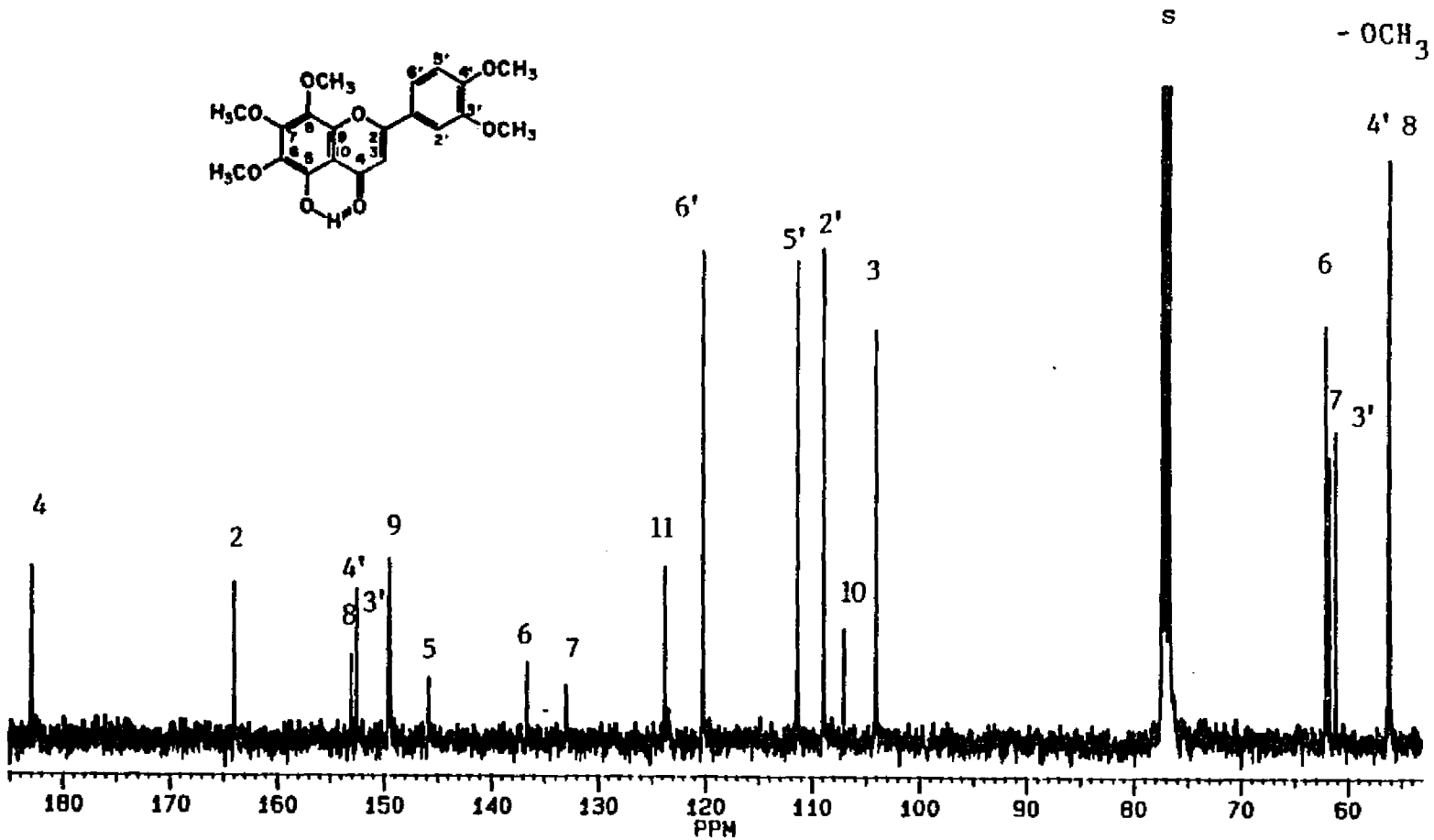


Figure 3.21. ^{13}C NMR spectrum of compound 4 in $CHCl_3$-d_1.

EXPERIMENTAL

Plant material. *Calamintha ashei* was collected by Dr. Don Richardson of the University of South Florida, Tampa, Florida in Polk County at Sunray, Florida in June, 1987. Voucher is deposited at the University of South Florida Herbarium at Tampa, sheet No. 137245.

General techniques. Vacuum liquid chromatography (VLC) was done on Macherey-Nagel silica N-HR using the method of Coll and Bowden (9). The sample was introduced to the column by pre-adsorption. Glass columns with medium pore sintered glass were used. Preparative TLC used 1 mm silica gel G 254 plates, CC silica gel (60-200 mesh).

Mps are uncorrected, obtained with Arthur Hoover capillary melting point apparatus.

UV spectral data were recorded on an HP8451A diode array spectrophotometer.

MS data were obtained by direct probe electron impact at 70 eV in an HP5985A GC-MS-DS instrument equipped with 2100 MX series computer.

All NMR spectral data were recorded in a Bruker AM-400 instrument using a $^{1}H/^{13}C$ tuned dual probe.

Extraction of plant material. Fresh leaves (1.0 kg) of *Calamintha ashei* were extracted with 12 L of water. The residue was dried and then extracted sequentially with hexane (36-60°C), DCM (2X) and MeOH (12 L each). The first DCM extract was concentrated under vacuum at 30°C until a slurry was obtained. Yellow-brown solids (5.4

g of air dried material) were obtained after decanting. The liquid portion was further concentrated giving 28.1 g of a thick brown syrup. The second DCM extract yielded, after concentration, 24.7 g of brown syrup. Total DCM extract: 58.2 g (5.8% of fresh leaf weight).

Isolation of compounds 1 and 2. VLC of the solid material (103.8 mg) from the first DCM extract was performed on a 2 x 4 cm column (hxdia) using toluene, toluene-ethyl acetate (EtOAc) (5% to 80% EtOAc) and EtOAc. Twenty-one 10 ml fractions were collected. Fractions 4-10 contained triterpenes (3) while fractions 11-21 provided flavonoids. Preparative TLC of the flavonoid mixture with toluene-EtOAc (7:3) afforded 14 mg of compound **1** (Rf 0.21) and 6 mg of compound **2** (Rf. 0.11).

5,6,4'-trihydroxy-7,8-3'-trimethoxyflavone (1). $C_{18}H_{16}O_8$; yellow crystals; mp 222-224°C; UV data in Table 3.2; ^1H NMR (400 MHz, $CDCl_3$) in Table 3.1 and Figure 3.1; ^{13}C NMR (100 MHz, DMSO-d_6) in Table 3.3 and Figure 3.7; EIMS *m/z* (rel. int.): 361 (14, M^++1), 360 (47, M^+, $C_{18}H_{16}O_8$), 345 (100, M^+-CH_3), 330 (10, M-2 CH_3), 327 (16, M-CH_3-H_2O), 197 (17, A_1^+-CH_3), 169 (11, A_1^+-H_2O).

5,6-4'-trihydroxy-7,3'-dimethoxyflavone (2). $C_{17}H_{14}O_7$; yellow solid; UV data in Table 3.2; ^1H NMR [400 MHz, $(CD_3)_2CO$, 35°C] in Table 3.7 and Figure 3.11; ^{13}C NMR (100 MHz, DMSO-d_6) in Table 3.10 and Figure 3.13. EIMS *m/z* (rel. int.): 331 (26,M^++1), 330 (100, M^+, $C_{17}H_{14}O_7$), 329 (14, M^+-H), 312 (38, M^+-H_2O), 300 (6, M^+-2CH_3), 284 (46, M^+-H_2O-CO), 269 (5, M^+-CH_3-H_2O-CO), 149 (14, B_1^+ +1), 148 (9, B_1^+).

Acetylation of a mixture of compounds 1 and 2. The crude solid (ca 1.5 g) from the first DCM extract was dissolved in 1.5 ml of dry pyridine and treated with 13.5 g acetic anhydride for 24 hrs at room temperature. Repeated addition of benzene and concn under vacuum was carried out until pyridine was removed, providing a thick dark brown residue. VLC of the reaction mixture on a 5 x 5 cm (hxdia) column with hexane and hexane-EtOAc yielded 15 10 ml fractions. Preparative TLC of fraction 6 with hexane-EtOAc (1:1) yielded compound **1a** (Rf 0.67). Fractions 9, 12, and 13 contained a yellow residue which gave colorless crystals after repeated washings with 75% EtOAc/hexane. Fraction 9 provided 155.8 mg of compound **1b** and fractions 12 and 13 yielded 226.5 mg compound **2a**.

Slow crystallization of fraction 10 (hexane-EtOAc, 1:1) produced white needle-like crystals of compound **1b** which were submitted for single crystal X-ray crystallography.

5-hydroxy-7,8,3'-trimethoxy-6,4'-diacetoxyflavone (**1a**). $C_{22}H_{20}O_{10}$; yellow crystals; mp 167-170°C. ^{1}H NMR (400 MHz, $CDCl_3$) data in Table 3.1, Figure 3.2. ^{13}C NMR (100 MHz, $CDCl_3$) data in Table 3.3 and Figure 3.9. EIMS *m/z* (rel. int.): 444 (7, M^+, $C_{22}H_{20}O_{10}$), 402 (67, M^+-CH_2CO), 387 (18, M^+-CH_2CO-CH_3), 360 (18, M^+-3 CH_2CO), 345 (100, 360-CH_3), 327 (10, 360-CH_3-H_2O).

7,8,3'-trimethoxy-5,6,4'-triacetoxyflavone (**1b**). $C_{24}H_{22}O_{12}$; colorless needle-like crystals; mp 190-191°C. ^{1}H NMR (400 MHz, $CDCl_3$) data in Table 3.1 and Figure 3.3. ^{13}C NMR (100 MHz, $CDCl_3$) in Table 3.3 and Figure 3.8. EIMS *m/z* (rel. int.): 486 (1, M^+, $C_{24}H_{22}O_{12}$), 444 (18, M^+-CH_2CO), 402 (100, M^+-$2CH_2CO$), 387 (18,

M^+-$2CH_2CO$-H_2O), 372 (6, M^+-$3CH_2CO$), 359 (20, 360-H), 345 (57, 360-CH_3), 327 (8, 360-CH_3-H_2O), 197 (9, A_1^+-CH_3).

7,3'-dimethoxy-5,6,4'-triacetoxyflavone (2a) (5,6). $C_{23}H_{20}O_{10}$; needle-like crystals; mp 213-214°C [lit. (6): 217-219°C]. ^1H NMR (400 MHz, $CDCl_3$) data in Table 3.7 and Figure 3.12. ^{13}C NMR (100 MHz, $CDCl_3$) data in Table 3.10 and Figure 3.14. EIMS *m/z* (rel. int.): 456 (2,M^+,$C_{23}H_{20}O_{10}$), 414 (11, M^+ - $2CH_2CO$), 372 (100, M^+ - $2CH_2CO$), 330 (33, M^+-$3CH_2CO$), 329 (17, 330-H), 312 (31, 330-H_2O), 284 (12, 330-H_2O-CO), 183 (5, A_1^+ +H).

Isolation of compounds 1, 3, and 4. The second DCM extract (12.9 g) was chromatographed by VLC on a 4.5 x 5 cm (hxdia) column using hexane and hexane-EtOAc with twenty-two 100 ml fractions being collected. Fractions 1-11 gave chlorophyll and triterpenes (3) while fractions 12-22 contained flavonoids. Fractions 18 and 19 represented a yellow fluffy solid which upon washing with hexane and DCM yielded 159.4 mg of compound **1**. Fractions 12-15 (1.3 g) were chromatographed on a silica gel column using hexane, hexane-DCM and DCM-MeOH, 20 and 30 ml fractions being collected. Fractions 148-153 (27.4 mg) were purified by prep. TLC using 2% MeOH in DCM (2x) to give compounds **3** (38.8 mg, Rf 0.43) and **4** (7.1 mg, Rf 0.57).

5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone (3). $C_{19}H_{18}O_8$; yellow crystals; mp 152-154°C [lit. (18) 162-164°C]. UV data in Table 3.2; ^1H NMR data in Table 3.11 and Figure 3.17; ^{13}C NMR data in Table 3.12 and Figure 3.18; EIMS *m/z* (rel. int.): 374 (49, M^+, $C_{19}H_{18}O_8$), 359 (100, M^+-CH_3), 344 (9, M-$2CH_3$), 211 (8, A_1^+-CH_3), 183 (8, A_1^+-CH_3-CO). The UV and ^1H NMR data are in good agreement with

data reported for the synthetic flavone (16) and the natural product (18).

5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (4) (8). $C_{20}H_{20}O_8$; yellow crystals; mp 116-118°C [lit. (8): 147-148°C]; UV data in Table 3.2; ^{1}H NMR data in Table 3.11 and Figure 3.20; ^{13}C NMR data in Table 3.12 and Figure 3.21; EIMS *m/z* (rel. int.): 388 (53, M^+, $C_{20}H_{20}O_8$), 373 (100, M^+-CH_3), 211 (7, $A_1^+-CH_3$), 183 (7, $A_1^+-CH_3-CO$).

^{1}H-^{13}C Chemical shift correlation by 2D NMR experiments (12). These experiments were performed using the following pulse sequence:

^{1}H: D0-90°-D0- D0-D3-90° -BB;

^{13}C: D1- -180°- -90°-D4-FID.

The data were acquired using 256 experiments (NE = 256) each with a block size of 4K and a recycle delay (D1) of 2 sec. Delay D0 was set to 3 μsec. The length of delays D3 and D4 were calculated with the observed one-bond ($^1J_{CH}$ = 140 Hz) and three-bond ($^3J_{CH}$ = 7 Hz) coupling constants. The short-range 2D NMR experiments used D3 = 0.0036 sec, D4 = 0.0018 sec while the long-range 2D NMR experiments, D3 = 0.071 sec, D4 = 0.036 sec.

The data were processed using sinebell multiplication in both dimensions (SSB1 = SSB2 = 0) and Gaussian multiplication in the second dimension (WDW2 = G; LB2 = 2.0) before Fourier transformation.

X-ray data of compound 1b. A crystal of dimensions 0.10 x 0.13 x 0.45 mm was used for data collection on an Enraf-Nonius CAD4 diffractometer equipped with Mo radiation (λ = 0.71073 Å) and a graphite monochromator. Crystal data are: $C_{22}H_{22}O_{11}$, M_r = 486.4,

a triclinic space group P1, a = 10.790 (2), b = 11.042 (2), C = 21.096 (3) Å, β = 98.23 (1) Å, V = 2291.7 (13) Å, Z = 4, dc = 1.410 g cm^{-3}, μ (Mo) = 1.06 cm^{-1}, T = 23°C. One quadrant of data having 1° < θ < 25° was measured and yielded 5970 unique data of which 3159 were used in the refinement.

Two independent molecules were observed. Convergence was achieved with R = 0.073, R_w = 0.052 for 632 variables.

REFERENCES

1. Richardson, D. R. (1985) Ph.D. Dissertation, University of South Florida, Tampa.
2. Macias, F. A., Fronczek, F. R. and Fischer, N. H. (1988). (submitted for publication).
3. Tanrisever, N. (1986) Ph.D. Dissertation, Louisiana State University, Baton Rouge, LA.
4. Tanrisever, N., Fischer, N. H., and Williamson, G. B. (1988) Phytochemistry **27**, 000 (manuscript No. 7677).
5. Fukui, K., Nakayama, M., and Matsui, T. (1969) J. Chem. Soc. Japan **90**, 1270.
6. Mathuram, S., Purushothaman, K. K., Sarada, A. and Connolly, J. D. (1976) Phytochemistry **15**, 838.
7. Rodriguez, B. (1977) Phytochemistry **16**, 800.
8. Tatum, J. H. and Berry, R. E. (1972) Phytochemistry **11**, 2283.
9. Coll, J. C. and Bowden, B. F. (1986) J. Nat. Prod. **49**, 934.

10. Markham, K. R. and Mabry, T. J. (1975) in The Flavonoids. (Harborne, J. B., Mabry, T. J. and Mabry, H. eds.), p. 45. Academic Press, New York.

11. Mears, J. A. and Mabry, T. J. (1972) Phytochemistry 11, 411.

12. Bax, A. and Morris, G. (1981) J. Magn. Res., 42, 501.

13. Markham, K. R. and Chari, V. M. (1982) in The Flavonoids: Advances in Research. (Harborne, J. B. and Mabry, T. J., eds.), p. 19. Chapman and Hall, London.

14. Barberan, F. A. T., Ferreres, F., and Tomas, F. and Guirado, A. (1986) Phytochemistry 25, 923.

15. Mabry, T. J. and Markham, K. R. (1975) In: The Flavonoids (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.), p. 78. Academic Press, New York.

16. Krishnamurti, M., Seshadri, T. R. and Sharma, N. D. (1970) Ind. J. Chem. 8, 575.

17. Wagner, H., Maurer, G., Horhammer, L., and Farkas, L. (1971) Chem. Ber. 104, 3357.

18. Rodriquez, B. (1977) Phytochemistry 16, 800.

19. Goudard, M., Faure-Bonvin,J., Lebreton, P., and Chopin, J. (1978) Phytochemistry 17, 145.

CHAPTER 4

THE USE OF INAPT NMR TECHNIQUE IN THE UNAMBIGUOUS STRUCTURE DETERMINATION OF FLAVONOIDS

INTRODUCTION

One of the major problems in the structure determination of flavonoids is the lack of a general method that can distinguish between a 5,6,7- and a 5,7,8-substituted compound. Likewise, it is difficult to distinguish a 5,6,7,8-substituted flavone from the above-mentioned types. In the past UV (1) and MS methods (2,3) have been applied to differentiate between certain types of flavonoid structures but these methods are limited in scope. Another problem encountered in structural studies of flavonoids is related to unambiguous assignments of ^{13}C NMR chemical shifts because of the unpredictable effect of the hydroxyl and/or methoxyl substituents on carbon chemical shifts, especially in the A- ring of a flavonoid.

Van Loo and coworkers (4) used selective decoupling of carbon signals as well as 2D ^{1}H-^{13}C correlation experiments to verify the assignments on the ^{1}H and ^{13}C chemical shifts of apigenin.

This study describes the use of Insensitive Nuclei Assigned by Polarization Transfer (INAPT) to be used as a complementary method in solving structural problems pertaining to flavonoids. This method was introduced by Bax (5) as a modified version of the refocused Insensitive Nuclei Enhanced by Polarization Transfer (INEPT)

experiment. It is a one-dimensional method for determining two- and three-bond connectivities and has been used for the unambiguous assignment of ^{13}C chemical signals of polycyclic aromatic hydrocarbons (6).

It is known that the hydrogen-bonded C-5-hydroxyl hydrogen of flavonoids exhibits long-range couplings to C-6, C-10 (7) and C-5 (4). This common C-5-hydroxyl substituent present in most flavonoids was very useful in the use of INAPT as an aid in structure determination. Figure 4.1 illustrates how INAPT experiments can be used to distinguish between different types of C-5-hydroxylated flavones and flavonols with one unsubstituted carbon in ring A. In principle, it should be applicable irregardless of the kind of substituent at the other carbons.

For flavones and flavonols with H-6 or H-8, polarization of the C-5-OH alone should be sufficient. As shown in Figure 4.1a, compounds bearing a hydrogen at C-6 would transfer to quaternary carbons C-5 and C-10 and to one tertiary carbon (C-6). On the other hand, molecules with H-8 and any non-hydrogen substituent at C-6 would transfer to three quaternary carbons (C-5, C-10, and C-6).

To determine whether a proton singlet is due to an H-8 or an H-3, two INAPT experiments should be performed. The expected INAPT results are illustrated in Figure 4.1b. In a 5,6,7-substituted flavonol, polarization of H-8 would transfer to quaternary carbons C-6, C-10, C-7, and C-9 while polarization of C-5-OH would transfer to quaternary carbons C-5, C-6, and C-10. In the 5,6,7-substituted flavonol, both INAPT spectra would show polarization transfers to C-6

1a

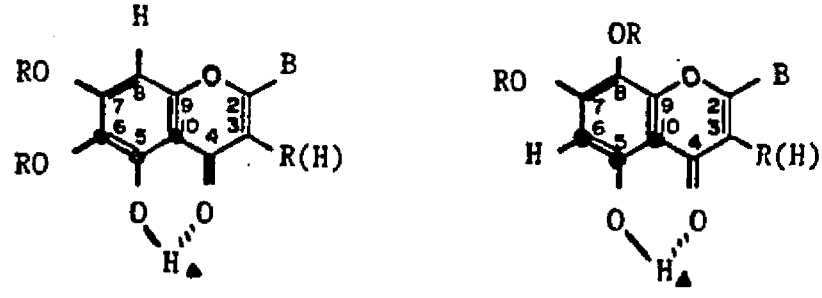


Figure 4.1a. Expected INAPT results from flavones/flavonols with H-6 or H-8.

1b

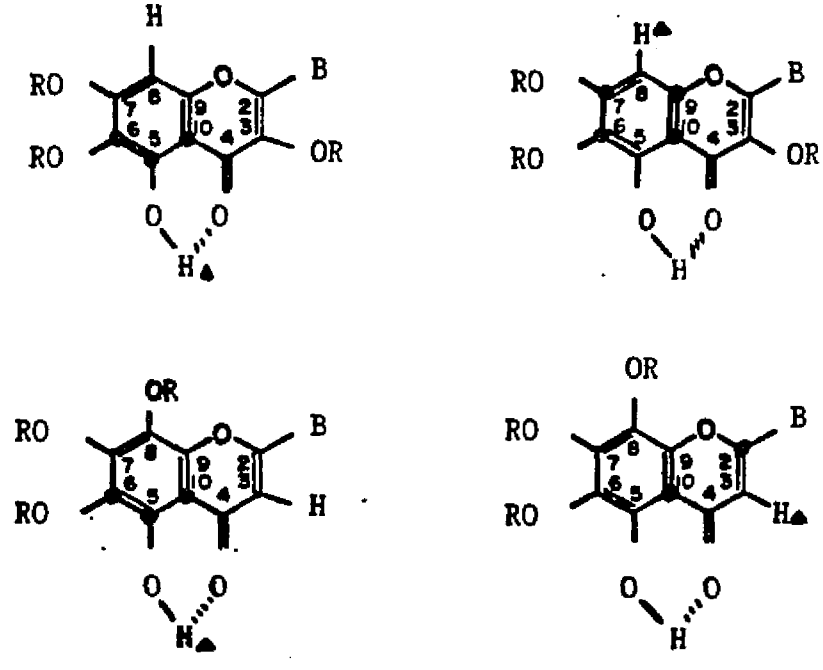


Figure 4.1b. Expected INAPT results from flavonol with H-8 and a flavon with fully substituted ring A. The triangle (▲) denotes the polarized proton; the dot (•) denotes the carbons that would be affected in the INAPT spectrum.

and C-10. For a 5,6,7,8-substituted flavone, polarization of H-3 would transfer to C-2 and C-10 while polarization of C-5-OH would transfer to C-5, C-6, and C-10. In this flavone system both INAPT spectra would show polarization transfers to C-10 only. Since the chemical shifts of C-10 and C-2 are very distinct, assignments could be performed unambiguously.

RESULTS AND DISCUSSION

The compounds that were used to demonstrate the utility of INAPT in structure elucidation studies were first analyzed by common spectroscopic methods like UV, MS, ^{1}H and ^{13}C NMR. These spectroscopic data were presented in Chapter 3. Whenever possible, the 2D ^{1}H-^{13}C correlation NMR data were obtained to verify the INAPT experiment results.

Compounds 1, 1a, and 1b.

Analysis of the spectroscopic data of compound 1 suggested that compound 1 has the following structural features: three methoxyl groups, a 3',4'-substitution pattern in ring B, three hydroxyl groups, and a proton singlet at δ 6.96 (Figure 4.2a). It was clear that two of the methoxyl groups are attached to C-7 and C-3' while two hydroxyl groups are at C-5 and C-4'. However, it was difficult to ascertain the position of the remaining hydroxyl, methoxy and the assignment of the proton singlet at δ 6.96 which could be due to an H-6, H-8, or H-3. The UV data, after treatment with $AlCl_3$/HCl, suggested either a flavone or a C-3-methoxylated

flavonol with a substituent at C-6 (1). However, the mass spectrum showed a base peak at m/z $[M-CH_3]^+$ which suggested that compound **1** could be either a C-8- or a C-6-methoxylated flavonol or flavone (2,3). The INAPT method was used to resolve these ambiguities.

Figure 4.2 shows the NMR spectra of compound **1**. Polarization of the distinct hydrogen-bonded C-5-hydroxyl proton at δ12.52 transferred to three quaternary carbons: C-10 (δ106.0), C-5 (δ134.1), and C-6 (δ143.1) (Figure 4.2c). This finding eliminated the possibility of a structure with a proton at C-6. When the proton singlet at δ6.96 was polarized, the INAPT spectrum showed four quaternary carbons (Figure 4.2d). These four carbons are due to the polarization of both H-3 and H-5'. It will be noted that since the resonance of H-3 is very close to H-5' polarization of this proton signal is not selective. This was confirmed by the identical INAPT spectrum generated by polarization of H-5'. Nevertheless, the carbon chemical shifts shown in Figure 4.2d indicated that the carbons can be assigned to C-10, C-2, C-3', and C-1'. This provided evidence that the proton singlet at δ6.96 is due to H-3 suggesting that compound **1** is a flavone.

The INAPT experimental results performed on compound **1b** were more conclusive because of its well-resolved proton chemical shifts in $CDCl_3$. Figure 4.3b-d shows the various INAPT spectra from the polarization selected protons. Polarization of H-3 showed resonances for C-2 and C-10 (Figure 4.3b) while H-5' transferred to C-1' and C-3' and a small two-bond transfer to C-4' (Figure 4.3c). Since the H-3 and H-5' chemical shifts of compound **1b** are well resolved, the

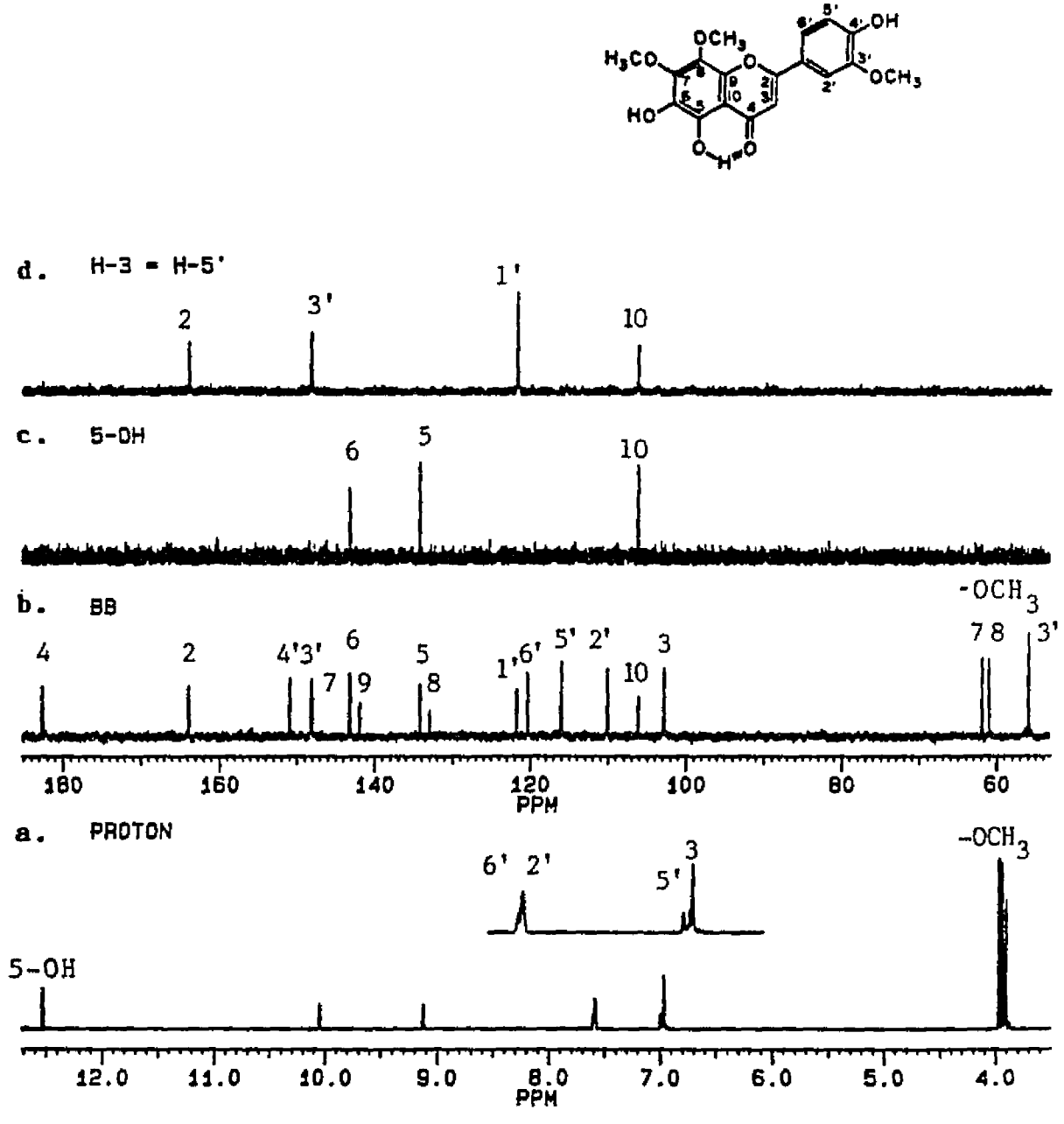
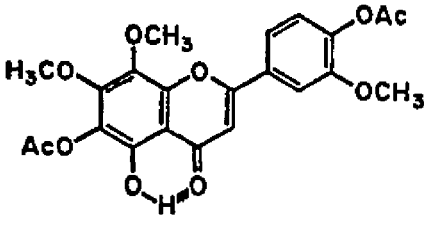


Figure 4.2. ^{1}H NMR, broad band ^{13}C NMR and INAPT spectra of compound 1.

polarization transfers were more selective. Polarization of H-6' and H-2' again produced the same INAPT spectra, showing C-2, C-3' and C-4' (Figure 4.3d).

When the acetate derivative of a polyhydroxy flavone/flavonol with a free C-5-hydroxyl group is studied, more INAPT informations can be obtained at a shorter time and the proton signals are often better resolved.

Compound **1a** is the diacetate derivative of compound **1** with a C-5-hydroxyl moiety. The ^{1}H NMR spectrum of



1a

compound **1a** is shown in Figure 4.4a; the ^{13}C NMR spectrum is given in Figure 4.4b.

The INAPT spectra of **1a** (Figure 4.4c-f) agree with those obtained from **1** (Figure 4.2) and **1b** (Figure 4.3). Polarization of the C-5 hydroxyl proton transferred to C-10, C-5 and C-6 (Figure 4.4c) while polarization of H-3 transferred to C-2 and C-10 (Figure 4.4d). As observed previously with compounds **1** and **1b**, the data on **1a** verified that C-6 is a quaternary carbon. Furthermore, C-6 must have an acetate substituent because polarization of the methoxyl groups (Figures 4.4e-f) did not exhibit any polarization transfer to C-6.

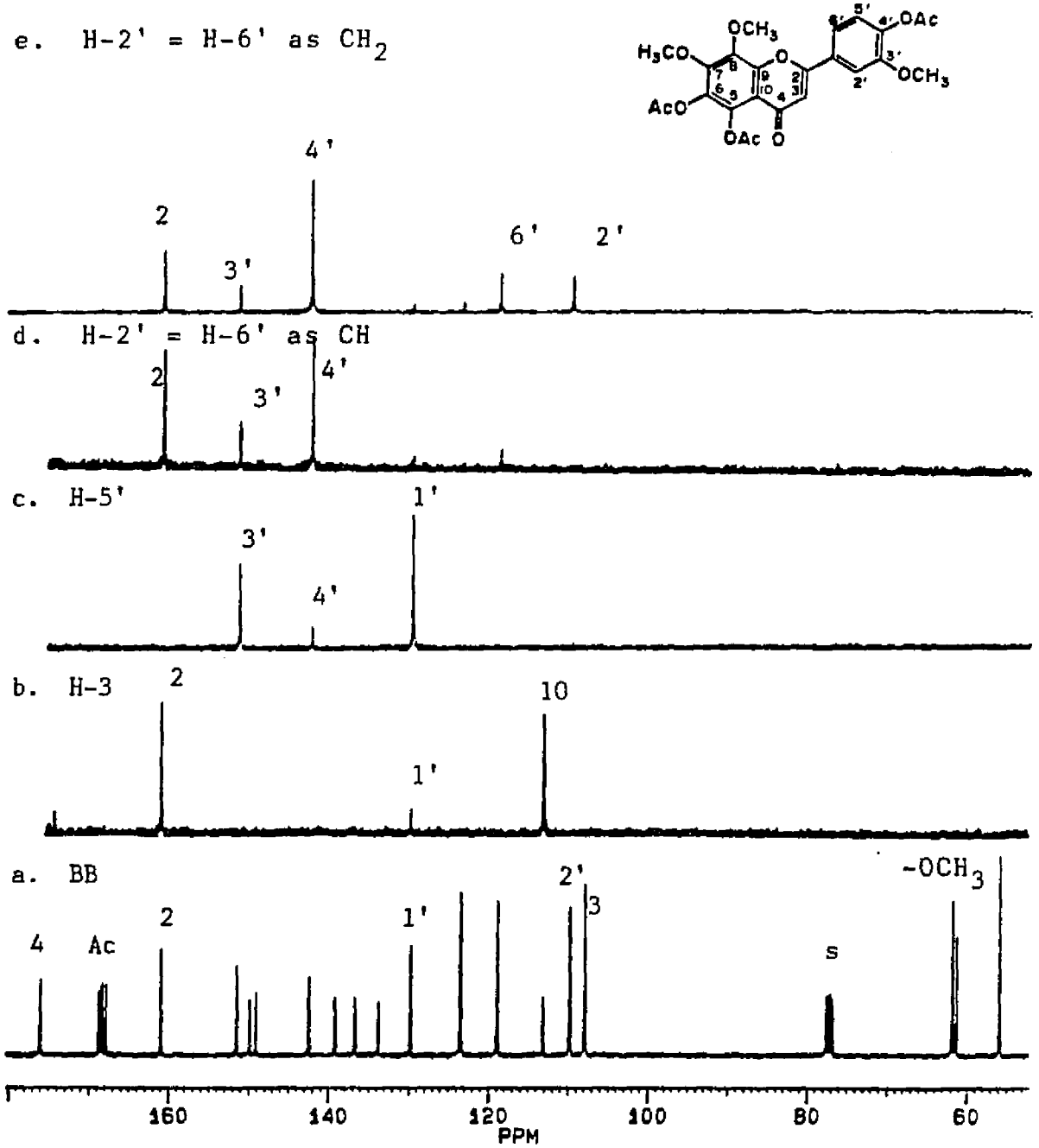


Figure 4.3. Broad band ^{13}C NMR and INAPT spectra of compound **1b**.

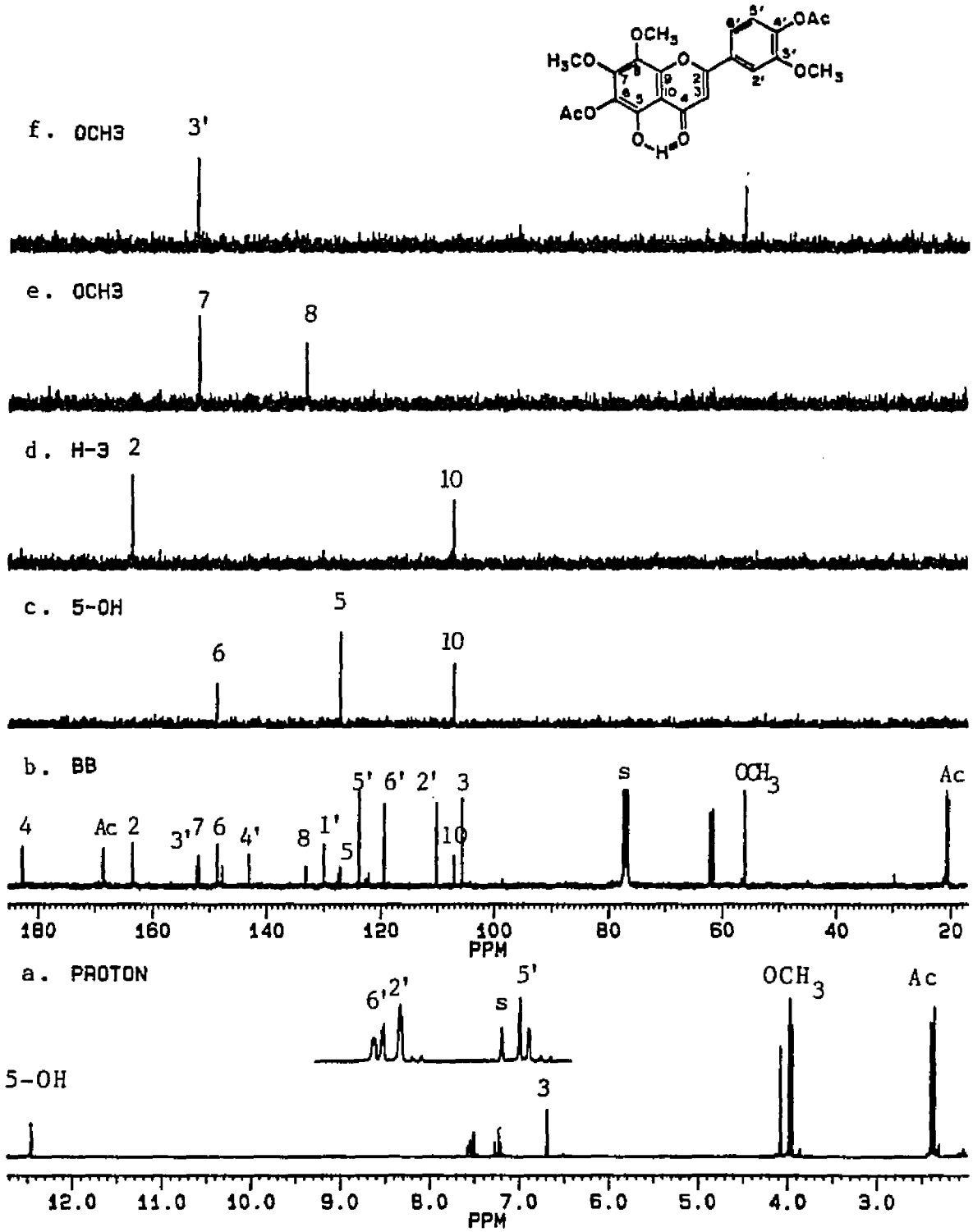


Figure 4.4. ^{1}H NMR, broad band ^{13}C NMR and INAPT spectra of compound **1a**.

The INAPT spectra of the remaining protons of **1a** were obtained for verification purposes. It was observed that for non-coupled protons with very close chemical shifts, polarization transfers are observed for the carbons coupled to both selected and non-selected protons. This is illustrated by the transfer from C-7- and C-8-methoxy methyls which were performed at different frequencies but gave similar spectra (Figure 4.4e). The difference between the chemical shift of the C-7- OCH_3 and the C-8- OCH_3 is 12 Hz for compound **1a**. For the C-3'- OCH_3 only the polarization transfer to C-3' was observed (Figure 4.4f). The chemical shift difference between C-3'-OCH_3 and the nearest proton signal is 37 Hz. Thus it seems that a 37 Hz frequency difference is sufficient to avoid spurious proton polarization transfers.

Compounds 2 and 2a

Only the C-5-hydroxyl proton in compound **2** produced a selective INAPT spectrum. As shown in Figure 4.5b, compound **2** also has a non-hydrogen substituent at C-6. An attempt to detect polarization transfers to the carbons two or three bonds away from H-3 led to polarization transfers from H-3, H-8, and H-5' since these protons are either overlapping or have very close resonances. However, the INAPT spectrum (Figure 4.5c) helped in the assignment of the carbon signals for C-5, C-6, C-7, and C-9. Since the signal at δ129.9 is affected in both INAPT spectra, it was assigned to C-6 while the signal at δ146.2 must be due to C-5.

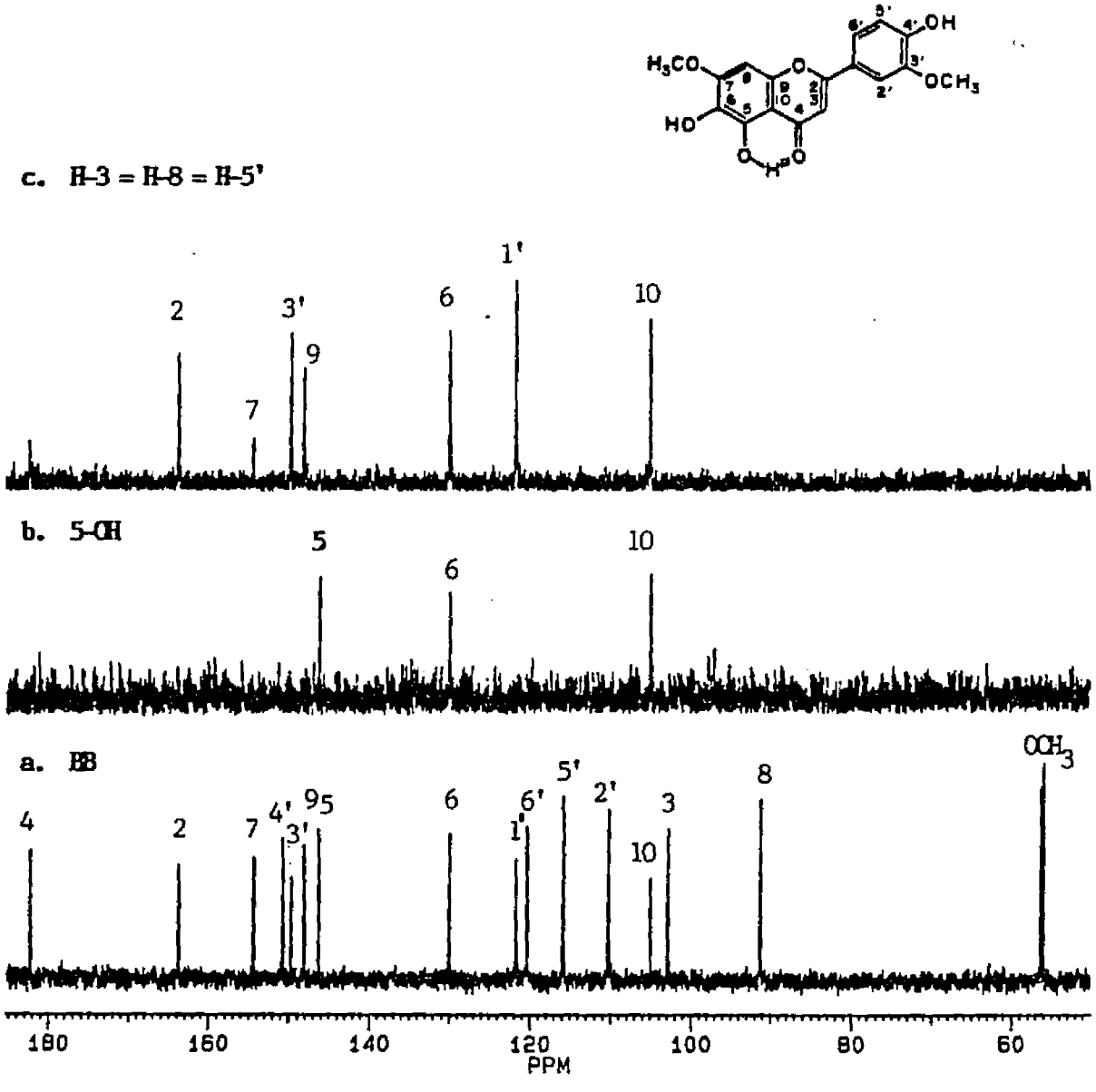


Figure 4.5. Broad band ^{13}C NMR and INAPT spectra of compound 2.

The INAPT spectra of the triacetate **2a** show more selective polarization transfers. In Figure 4.6d and 4.6e, H-2' and H-6' were polarized as methine protons. Polarization transfers from the methoxyl groups were nonselective due to the very similar resonances of these protons (Figure 4.6f). By comparing the INAPT spectrum of H-8 (Figure 4.6f) with those of the methoxyl groups, it was concluded that C-6 is acetylated. The results of the INAPT experiments correlate well with the short range (Figure 3.15) and long range (Figure 3.16) ^{1}H-^{13}C 2D NMR spectra.

Compound 3

The assignment of the carbon signals of compound **3** was based on relative chemical shifts and the INAPT results (Figure 4.7). Here, H-2' and H-6' were well resolved. Both, H-2' and H-6' were polarized as methylene protons thus giving a more sensitive transfer (Figure 4.8).

Artemetin (5)

Artemetin (**5**), (5-hydroxy-3,6,7,3',4'-pentamethoxyflavonol), a known flavonol, was also used as a model compound for the INAPT experiments. Its ^{1}H NMR spectrum (Figure 4.9) shows the presence of five methoxyl groups, a hydrogen bonded hydroxyl, and a 3',4'-substitution pattern in ring B. Its long range ^{1}H-^{13}C NMR correlation spectra (Figure 4.10) shows the one-bond, two-bond and three-bond proton carbon connectivities in this molecule.

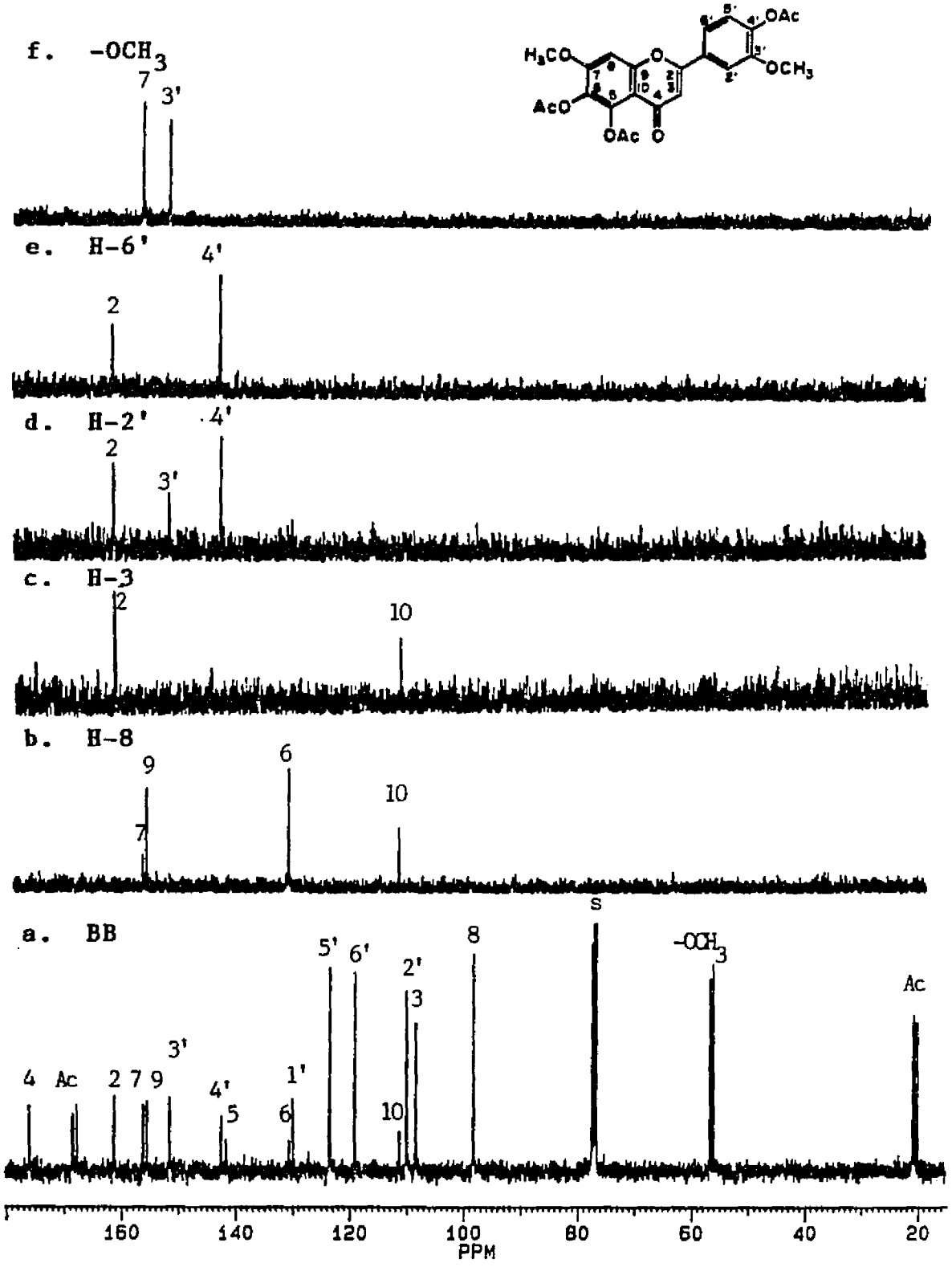


Figure 4.6. Broad band ^{13}C NMR and INAPT spectra of compound 2a.

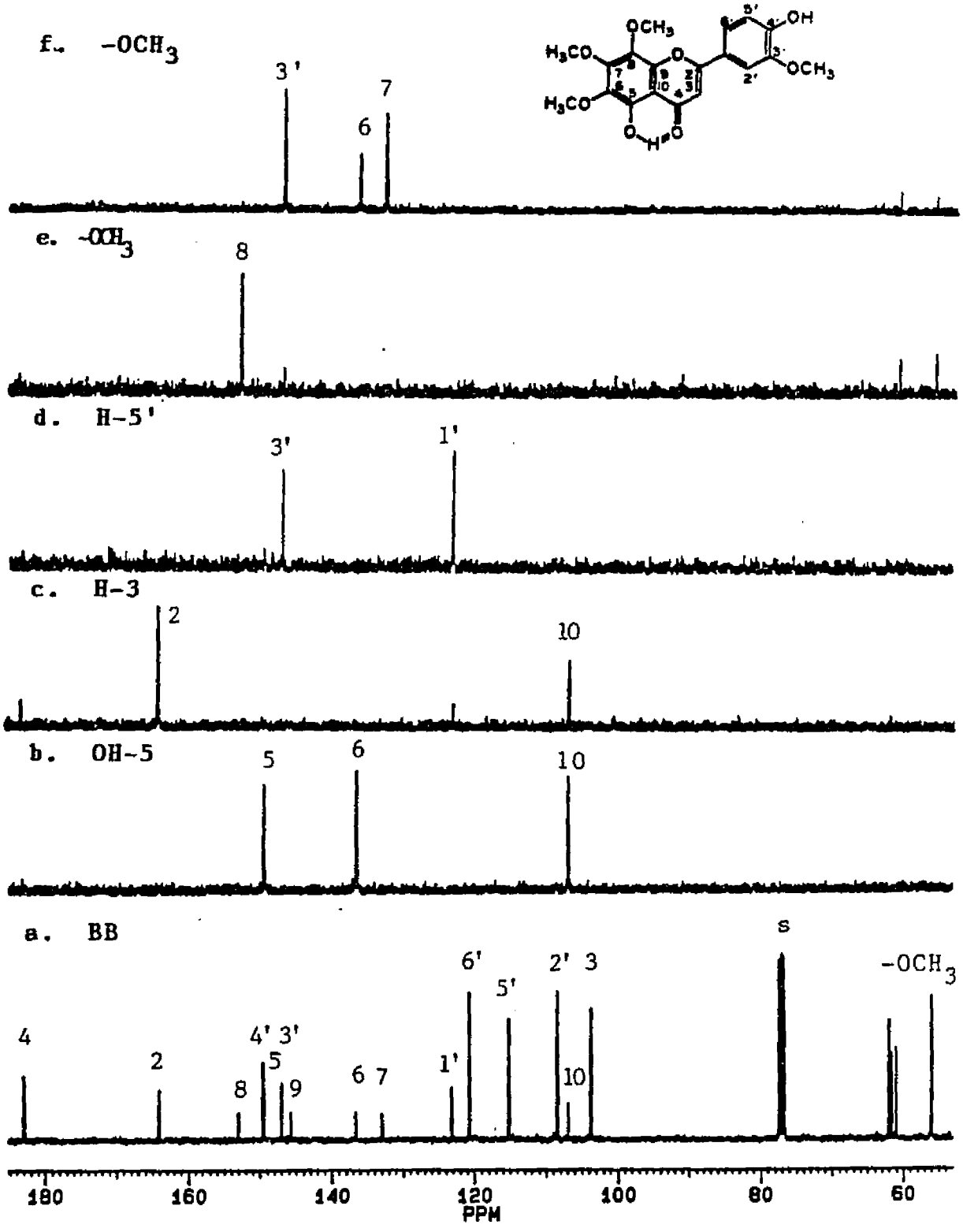


Figure 4.7. Broad band ^{13}C NMR and INAPT spectra of compound 3.

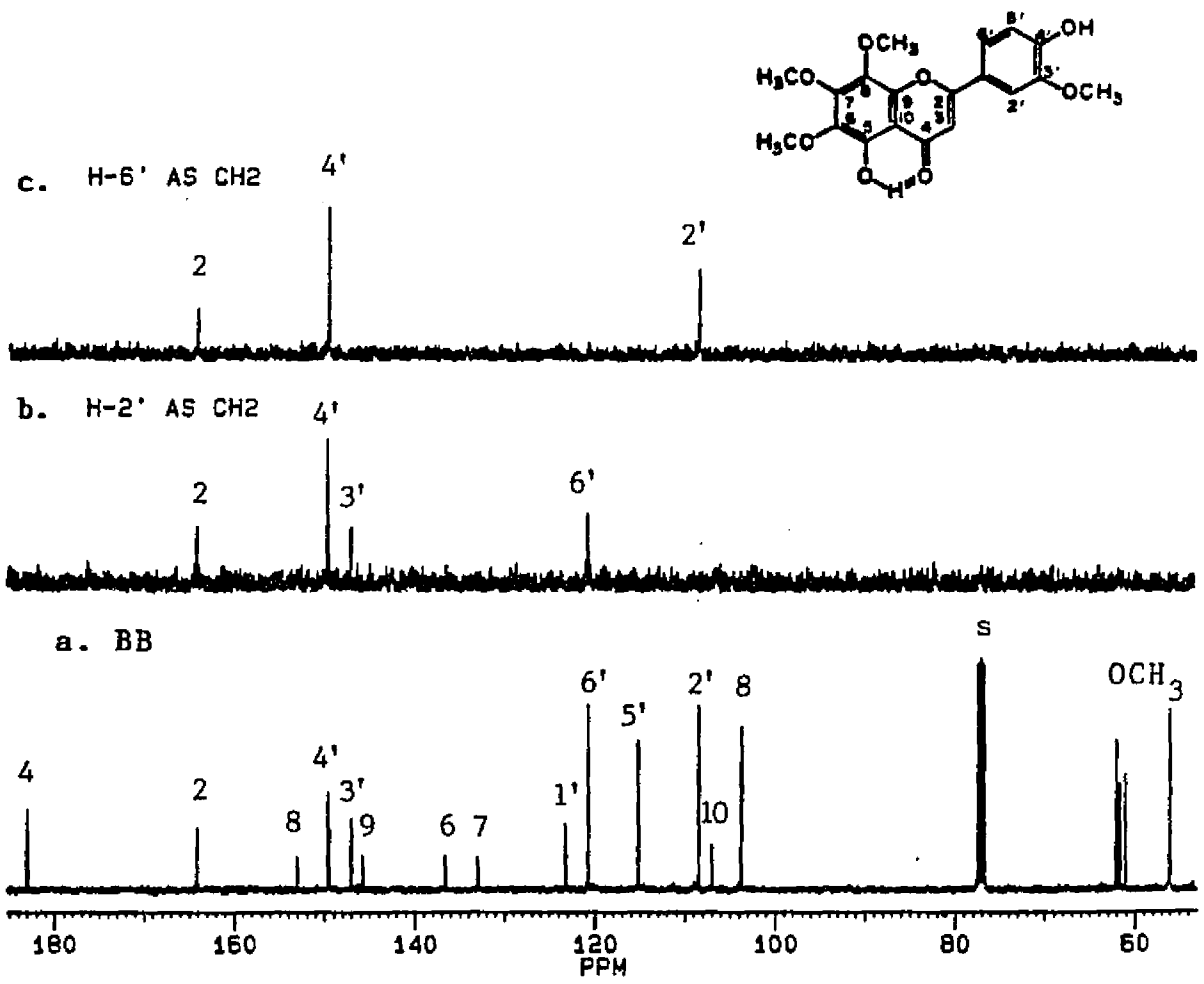


Figure 4.8. Broad band ^{13}C NMR and INAPT spectra of compound 3. Protons H-2' and H-6' are polarized as methylene protons.

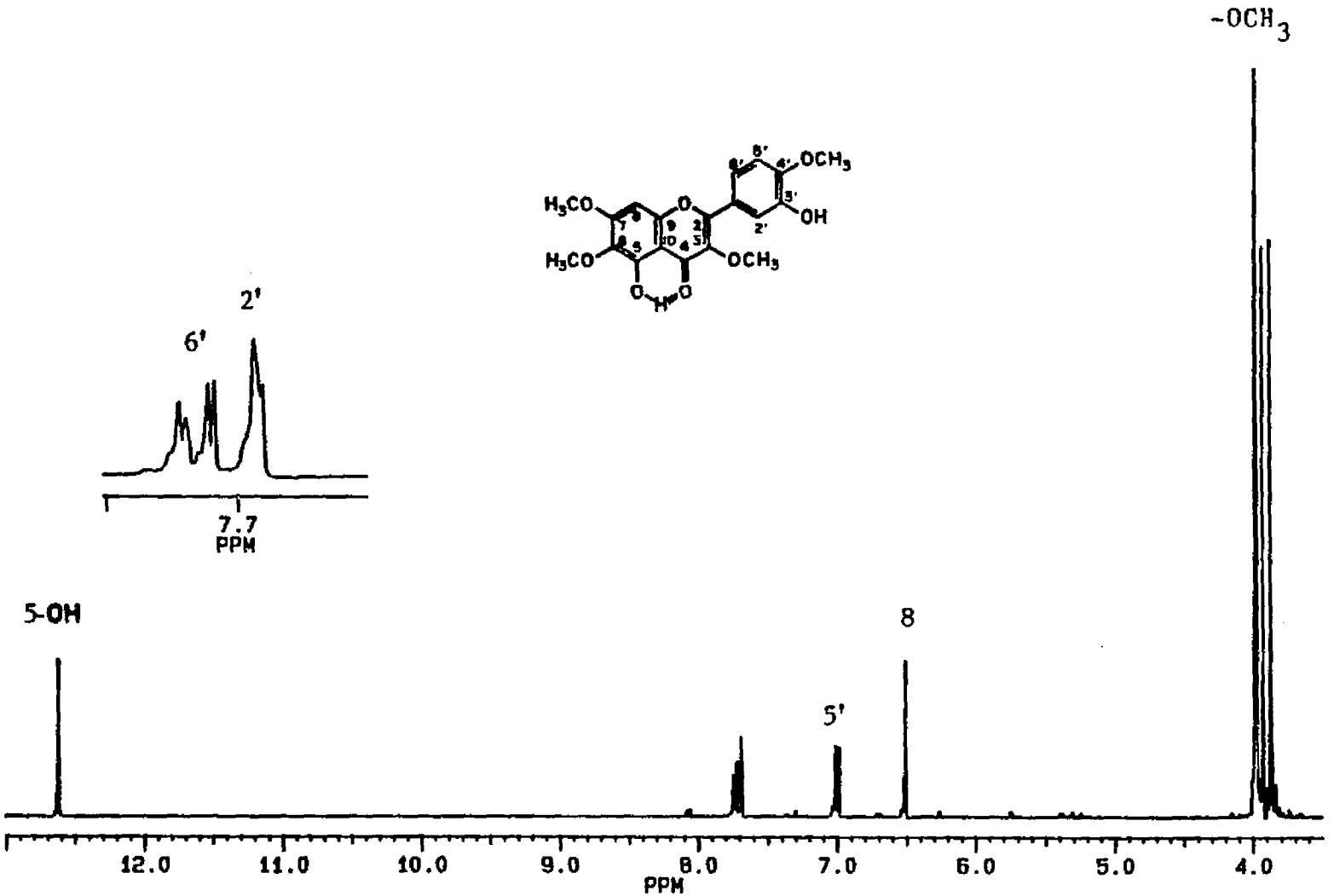


Figure 4.9. ^{1}H NMR spectrum of artemetin (5).

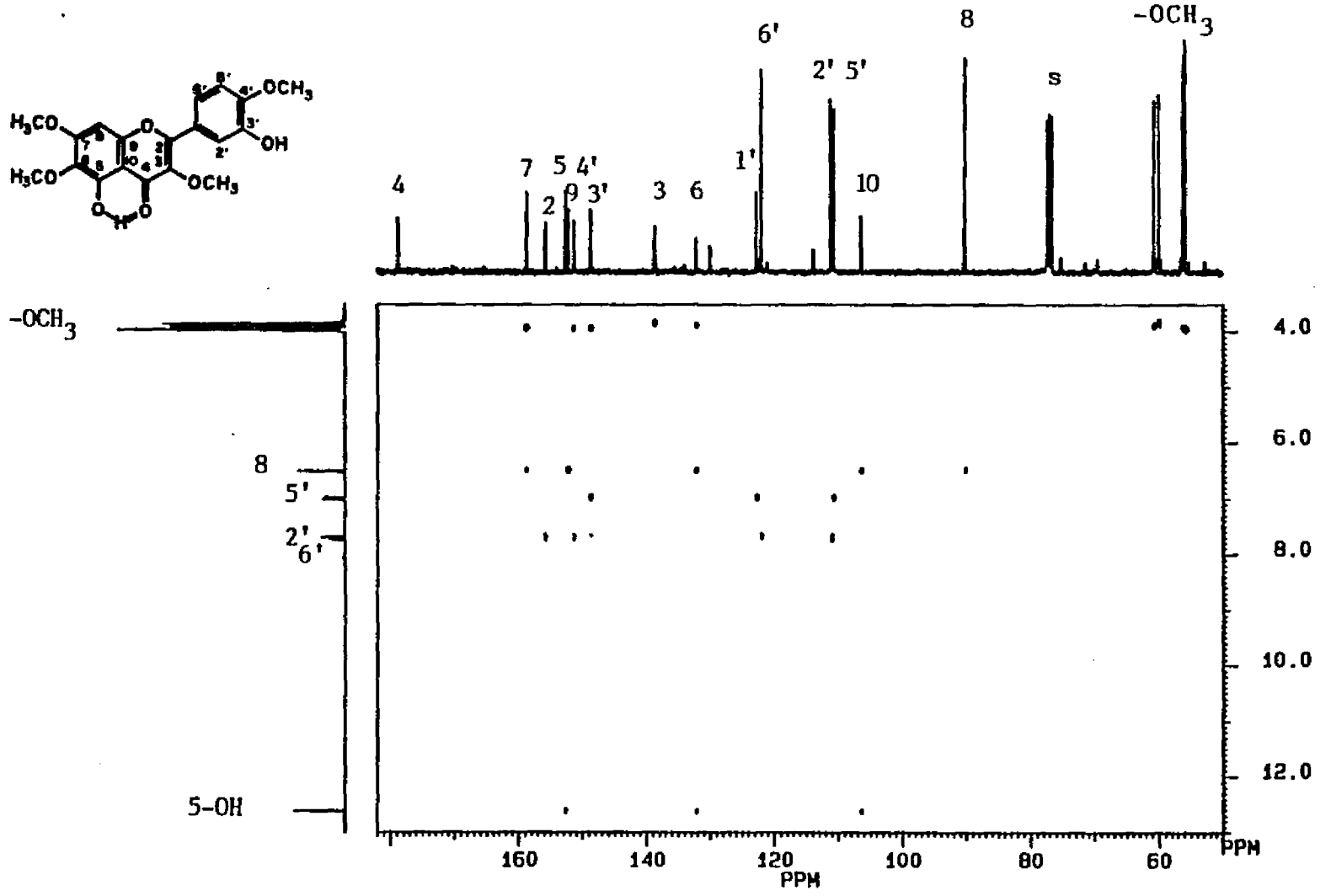
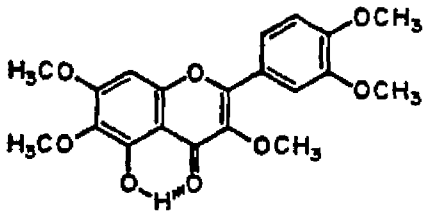


Figure 4.10. Contour plot of long range ^{1}H-^{13}C correlation NMR of artemetin (5).



Artemetin (5)

The INAPT spectra of artemetin (Figure 4.11) correlate well with the ^{1}H-^{13}C 2D spectra. Polarization of the C-5 hydroxyl proton transferred to C-5, C-6, and C-10 (Figure 4.11b). The singlet due to H-8 had a polarization transfer to C-6, C-7, C-9, and C-10 (Figure 4.11c). Both C-6 and C-7 must bear methoxyl groups since both carbon signals appear upon the polarization of the methoxyl protons (Figure 4.11 e-f). C-9 correlates only with H-8.

Comparison of the INAPT spectra of artemetin (5) and 5,6,4'-trihydroxy-7,8,3'-trimethoxy-flavone (1) shows that this method is a convenient method of differentiating a flavone from a flavonol. This is illustrated in Figure 4.1a. An H-8 would transfer to the nearby four carbons while an H-3 tranfers only to C-10 and C-2. Thus, polarization of the C-5-hydroxyl proton and the proton singlet would confirm the structure of a flavonoid.

INAPT Studies Involving Coupled Protons

The INAPT spectra for the strongly coupled proton H-5' have been shown to be both selective and sensitive. It always shows

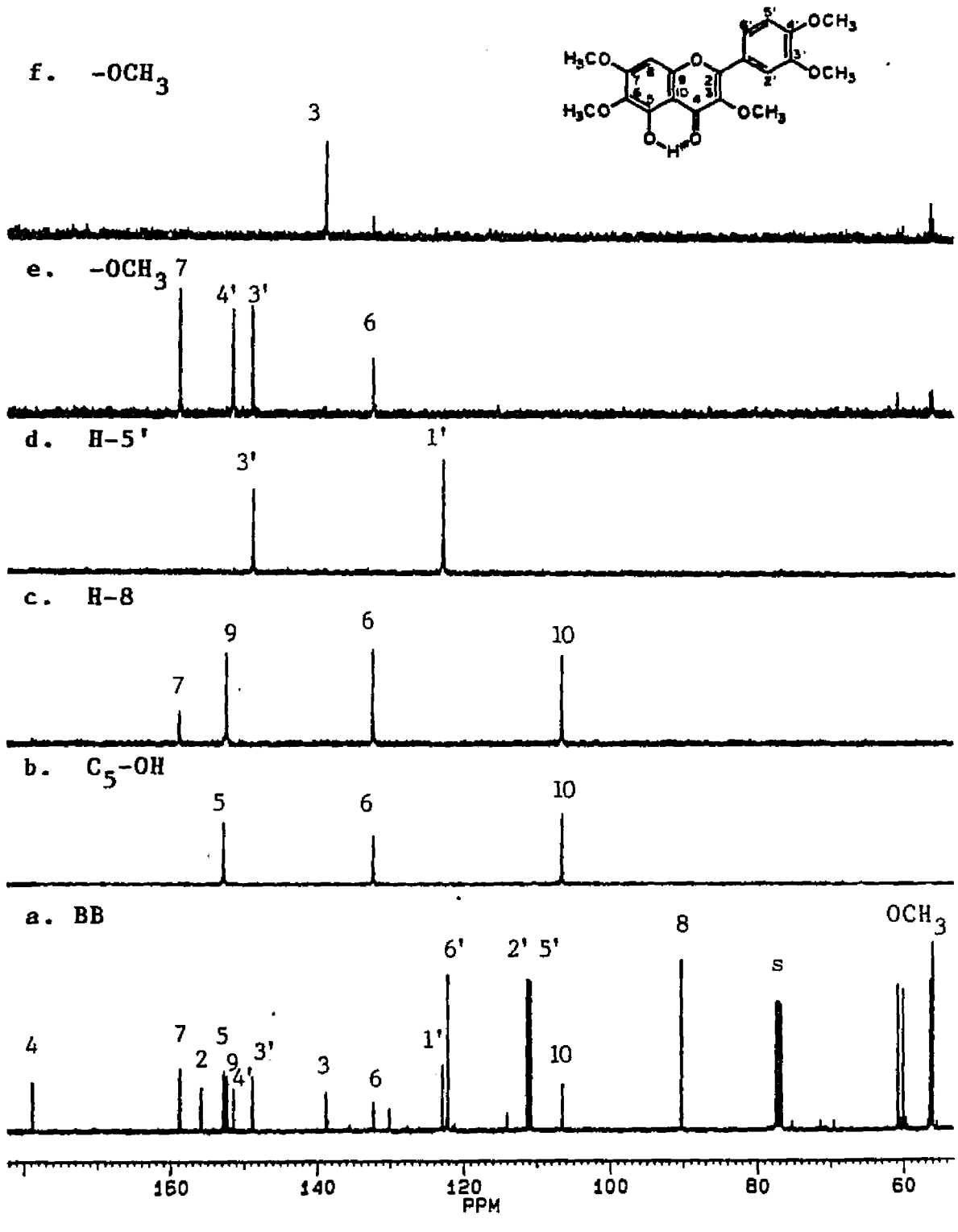


Figure 4.11. Broad band ^{13}C NMR and INAPT spectra of artemetin (5).

transfer to C-1', C-3' and sometimes to C-4'. However, when H-2' and H-6' are polarized using a D3 value of a methine [D3 (CH) = 26.0 msec] similar INAPT spectra are obtained even if the difference in the chemical shift is 31 Hz (see Figure 4.6 d-e). Using compounds **1b**, **5**, and **3**, it will be illustrated that both, D3 and ~ 30 Hz proton resonance difference, affects the INAPT selectivity of H-2' and H-6' in flavonoids.

Figure 4.12.b-c show the H-2' and H-6' INAPT spectra of compound **5** using D3 for a methine. Both protons exhibit polarization transfers to C-2 and C-4', accompanied by a small peak due to C-3'. When D3 for a methylene proton was used (Figure 4.12d), H-2' transfers to C-2, C-4', C-3', and C-2' were observed. The low selectivity of the polarization transfer might be due to the proton chemical shift difference which is about 20 Hz.

To learn if an increase in the difference of proton resonance would improve the selectivity of polarization transfer, the resonance of polarization for both, H-2' and H-6', were moved by 5-10 Hz. The INAPT spectra are shown in Figures 4.12 e-f. The best results were achieved when the proton resonance difference was about 31 Hz. Figure 4.12e shows that H-2' transferred to C-2, C-4', C-6', and C-3'. Figure 4.12f shows the transfer from H-6' to C-2, C-4', and C-2'. The chemical shifts of C-2' and C-6' agree with the 2D ^{1}H-^{13}C correlation spectra and its environment. C-2' has o- and m-OCH_3 and thus resonates upfield from C-6' that has m- and p-OCH_3 groups. Also, only H-2' shows a transfer to C-3' since it is the only proton in ring B with a two-bond C-H coupling to C-3'.

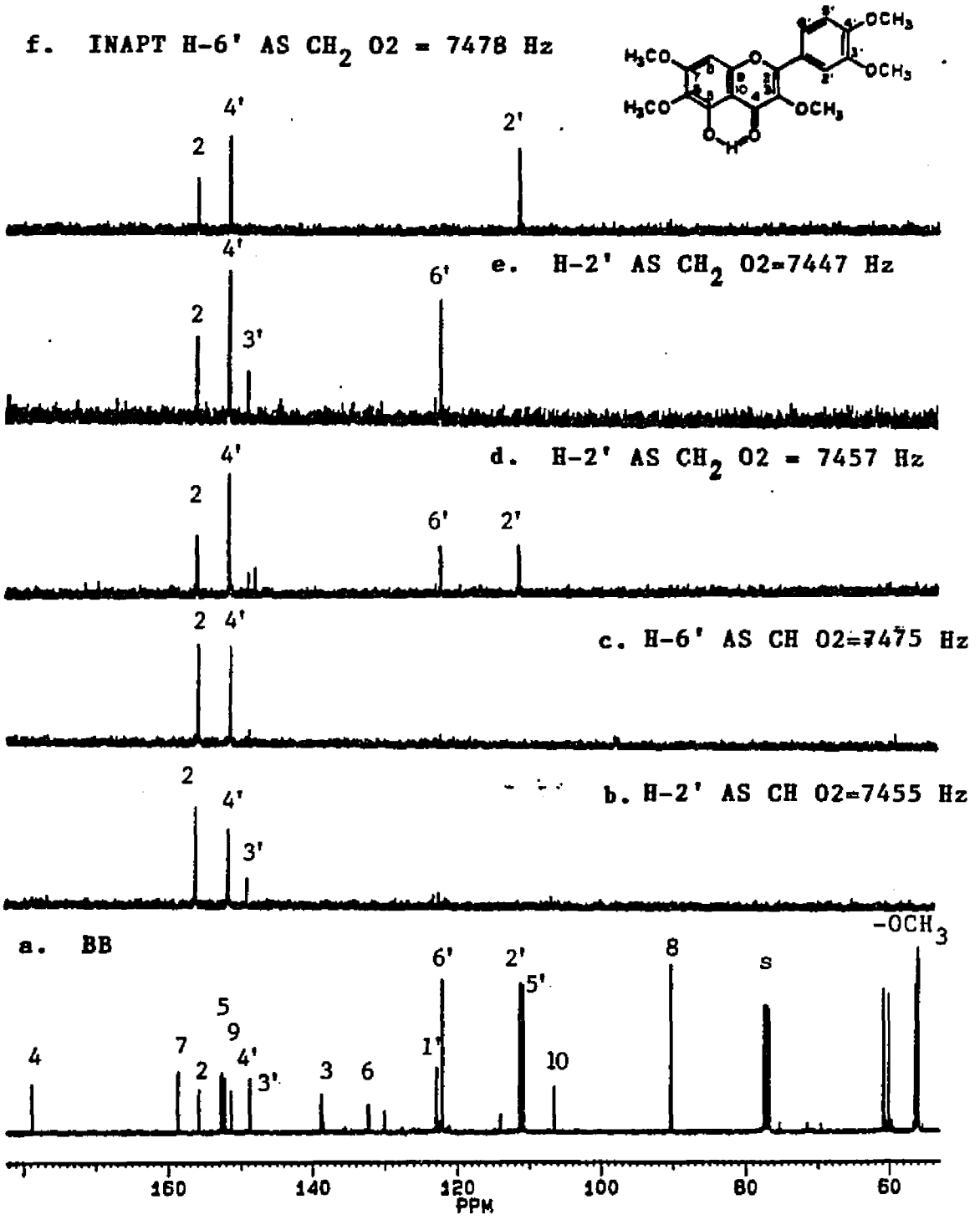


Figure 4.12. INAPT spectra of artemetin (5) Effect of D3 on selectivity and sensitivity of polarization transfer.

The INAPT spectra of H-2' and H-6' of compound **3** were also obtained using D3 for methylene protons. The resonance frequency difference in compound **3** was about 49 Hz so the resonance for polarization was not changed. Polarization of H-2' transferred to C-2, C-4', C-6' and C-3' (Figure 4.8b), whereas H-6' transferred to C-2, C-4', and C-2' (Figure 4.8c). Thus, for compound **3** polarization transfers from H-2' to H-6' were both selective and sensitive.

The transfer from H-2' and H-6' from compound **1b** also showed identical INAPT spectra when these protons used D3 value for a methine (Figure 4.3d). When a methylene D3 value was used, sensitivity increased thus showing transfers to C-2, C-4', C-3', as well as to C-2' and C-6' (Figure 4.3e). With compound **1b**, the proton resonance for polarization has to be changed drastically to achieve a 30 Hz resonance difference. Consequently, selectivity cannot be achieved in this case.

Thus, for strongly coupled protons like H-2' and H-6' that have three-bond H-C connectivity to each other, INAPT selectivity and sensitivity can be improved by treating the methine protons as methylene protons and by slightly changing the proton resonance values to increase its difference to about 30 Hz. These observations agree with the findings of Bax (5).

INAPT is a very powerful tool for resolving problems in the determination of flavonoid structure. It is experimentally simple and sensitive. With the availability of powerful NMR facilities, the experiment can be carried out with small amounts of material and in a reasonable amount of time. In cases where polarization of the

C-5-hydroxyl proton is sufficient, the process can be fast. Moreover, selectivity and sensitivity is high with C-5-hydroxyls because its resonance near δ12 is well separated from other flavonoid protons. When polarization experiments of other protons need to be performed and the proton signals are overlapping, acetylation and use of $CDCl_3$ as a solvent may be necessary to obtain a well resolved spectrum and consequently increase the selectivity of polarization transfers. Formation of acetates with a free C-5 hydroxyl group makes the task even easier.

INAPT is also useful for the assignment of proton and carbon chemical shifts. Although other types of flavonoids were not used in this study, the potential of this method as a complement to other spectroscopic methods is obvious.

EXPERIMENTAL

The NMR spectra were acquired at 25° on a Bruker AM400 spectrometer using a 5 mm dual tuned $^{1}H/^{13}C$ probe with observation frequencies of 400.13 and 100.62 MHz, respectively. All compounds were dissolved in DMSO-d_6 all compounds were dissolved in $CHCl_3$-d_1 except for compounds 1 and 2 which were dissolved in DMSO-d_6.

^{1}H-^{13}C Shift Correlated NMR

Experimental conditions and parameters are described in Chapter 3.

INAPT Experiments

The INAPT experiments were performed using the following pulse sequence (5,6):

^{1}H: D1-90°-D2-180°-D2-90°-D3-180°-D3-BB

^{13}C: -180°- -90° -180° -FID

The proton pulse widths were obtained by using dichloroacetic acid with $^{1r}J_{CH}$ = 2.4 Hz. Proton relaxation delay (D1) was set to 1.5 sec. Length of delays D2 and D3 were optimized for three-bond coupling constant $^{3}J_{CH} \cong$ 7-8 Hz. The delay D2 was set to 20.75 msec for all protons while D3 was set to 26.0 msec, 10.38 msec, and 7.25 msec for methine, methylene and methyl protons, respectively.

The INAPT spectra presented were processed with LB = 2 and with magnitude calculation after Fourier transformation to obtain positive signals.

REFERENCES

1. Mears, J. A. and Mabry, T. J. (1972) Phytochemistry **11**: 411-412.

2. Barberan, F. A. T., Ferreres, F., Tomas, F., and Guirado, A. (1986) Phytochemistry **25**: 923-925.

3. Goudard, M., Favre-Bonvin, J., Lebreton, P., and Chopin, J. (1978) Phytochemistry **17**: 145-146.

4. Van Loo, P., de Bruyn, A., and Budesinsky, M. (1986) Magn. Res. Chem. **24**: 879.

5. Bax, A. (1984) J. Magn. Res. **57**: 314-318.

6. Bax, A., Ferreti, J. A., Nashed, N., and Jerina, D. M. (1985) J. Org. Chem. 50: 3029-3034.

7. Wehrli, F. (1975) J. Chem. Soc. Chem. Comm. 663.

8. Wollenweber, E. (1982) In: The Flavonoids: Advances in Research. (Harborne, J. B. and Mabry, T. J., eds.) Chapman and Hall, London, p. 189-253.

VITA

Hidelisa Padua Hernandez was born on December 19, 1955 in Nabua, Camarines Sur, Philippines. She is the second child of Engracio R. Padua and Maria Palencia Padua. She attended the University of the Philippines at Los Baños (UPLB) where she obtained a Bachelor of Science in Agricultural Chemistry in May 1977 and a Master of Science in Agricultural Chemistry in April 1983. She has been a member of the teaching staff of the Department of Chemistry, UPLB since June 1977.

On July 3, 1982, she married Jose E. Hernandez.

In August of 1983, she obtained a graduate assistantship at the Department of Chemistry, Louisiana State University where she worked under the supervision of Dr. Nikolaus H. Fischer and Dr. C. Michael Smith. She is currently a candidate for the degree of Doctor of Philosophy in Chemistry.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Hidelisa P. Hernandez

Major Field: Chemistry

Title of Dissertation: Search for Allelochemicals in Rice (Oryza Sativa) and Structure Determination of External Flavonoids of Calamintha Ashei

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

June 13, 1988