COMPONENTS FROM SOUTH AFRICAN APPLE CIDER: STRUCTURE AND SYNTHESIS.

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By

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I dedicate this thesis to the loving memory of my father

Dawid Benjamin Fourie

26/03/1945 - 06/12/2001

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Key words: Apple, apple cider, polyphenols, flavonoids, dihydrochalcones, glycosides, chalcone glycoside synthesis, antioxidants, phloridzin, high-resolution NMR.

Apple cider, fermented from assorted apple cultivars, contains a variety of polyphenols including flavonoids. Polyphenolic compounds are critical in the design of juice products. These compounds play an important role in taste, flavor and coloration of juices and their products.

The cider under investigation is artificially sweetened after fermentation, using sugar cane by-products. In our investigation both commercially available sweetened, and partially processed unsweetened products were investigated. Extraction with ethyl acetate followed by chromatographic separation (column Sephadex and preparative thin-layer) afforded flavonols, dihydrochalcones, flavan-3-ols, dihydroflavonols and one C_6C_3-type phenol. The structures of these compounds were characterized, mainly through high-resolution (300 MHz) Nuclear Magnetic Resonance spectroscopy (including NOESY, COSY, DEPT and ^{13}C NMR experiments). All the compounds isolated from the cider are known to occur in the *Malus* genus (apples). One compound, taxifolin (dihydroflavonol), have not been previously isolated from apple cider.

Characteristically, the phloretin glycoside, phloridzin, were isolated along with the diglycoside, 2',4',6',4-tetrahydroxy dihydrochalcone-2'-*O*-β-D-(6"-β-D-xylopyranosyl)-β-D-glucopyranoside. Quercetin and two analogue glycosides, quercetrin and 3',4',5,7-tetrahydroxy flavonol-3-β-D-arabinopyranosyl were also isolated.

Synthesis of phloridzin, which could serve as a model reaction for synthesis of dihydrochalcones with more complex glycosides, was attempted. A glycoside was attached to an appropriate acetophenone and used in a base-catalyzed aldol condensation with a benzaldehyde to yield the precursor chalcone to dihydrochalcones. In a second procedure, the chalcone was synthesized first, using the same aldol-type

reaction, followed by attachment of the glycoside. Although the synthesis of chalcones by an aldol condensation is a common high-yielding procedure, difficulty with the condensation, due to the attachment of the glycosyl unit to the acetophenone was anticipated and encountered. Synthesis of chalcone glycosides was accomplished, but no appropriate protection and deprotection protocols could be established.

Flavonoids constitute an important part of polyphenols in apple juice, and their ability to influence a wide variety of biological functions has been asserted. Antioxidants have recently come under much investigation and the obvious advantages to human health, makes the understanding of these compounds in apple juice and its products (e.g. cider) important. Vitamins E and C, flavonoids and other polyphenols act as primary antioxidants, having the ability to quench superoxides, hydroxy and peroxy radicals. The results of this investigation clearly indicate the presence of compounds with potential antioxidant properties in the cider.

LITERATURE SURVEY

CHAPTER 1

POLYPHENOLS FROM APPLES, APPLE JUICE AND APPLE CIDER

1.1 Overview

The study and understanding of the polyphenolic composition and the factors that affect these phenolic compounds are critical in the design of juice products. These compounds play an important role in taste, flavor, and coloration of juices and their products. Flavonoids constitute an important part of polyphenols in apple juice, and their ability to influence a wide variety of biological functions have been asserted.[1]

The biological role of these polyphenols remains to be elucidated, but there is growing evidence that an increase in dietary levels of these substances may be of long-term benefit to human health. The value of antioxidants to human health has recently come under much investigation and the obvious advantages that have already been shown, makes the understanding of these compounds in apple juice and its products (e.g. cider) important.[2] Considering that the average American dietary intake of these polyphenols have been estimated at 1g or more per day, it is clear that these substances could play an important role in human nutrition.[3]

Apple juices are made from a wide variety of apple species which all belong to the *Malus* genus. Although there is a variety of species (25 in the *Malus* genus),[4] the polyphenolic constituents in apple leaf and bark seldom vary. There is, however, a marked difference in the concentrations of these polyphenols in the fruit of the different species.[5]

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[1] McClure, J.W., *The Flavonoids,* Ed. Harborne, J.B., Mabry T.J and Mabry H., Chapman & Hall, London, **1975**, pp. 970

[2] Harborne, J.B., Middleton, E., Kandaswami, C., *The Flavonoids: Advances in research since1986*, Ed. Harborne, J.B., Chapman & Hall, London, **1994**, pp. 619-645

[3] Kuhnau, J., The Flavonoids. A class of semi-essential food components: their role in human nutrition *World Rev. Nutr. Diet* **1976**, *24*, pp. 1673-1681

[4] Rehder, A., *Manual of Cultivated Trees and Shrubs*, 2nd ed. rev., The McMillan Co., New York. **1940**, pp. 996.

[5] Williams, A. H., *Chem. Ind. (London)* **1958**, pp. 1200

This makes the selection of different apples for different products, like apple juice, apple cider, dessert apples, culinary apples, *etc.* important in the light of some of the mentioned attributes of polyphenols.

1.2 The Phenolics of Ciders: Bitterness and Astringency

The association between bitterness and astringency and the phenolic fractions of cider has long been recognized.[6] The most important compounds in this regard have been identified as procyanidins based on epicatechin (oligomeric flavonoids), which are found in high levels (2-3 g litre^{-1}) in bittersweet apples. The characterization of some of these procyanidins suggests the presence of a range of procyanidin oligomers in cider, up to at least a seven-fold degree of polymerization. Lower members of the series have been readily isolated by counter-current distribution and column chromatography, and identified.[7,8]

"Bitterness" and "astringency" are sometimes regarded as synonymous even in the cider industry, a confusion that probably arises because both sensations are always present in bittersweet fruit such as cider apples. Bitterness can be defined as a penetratingly unpleasant sensation perceived mostly at the back and sides of the tongue, with the vegetable alkaloids such as caffeine and quinine, giving some of the purest and well-known sensations of bitterness. Astringency can however be defined as a dry, puckering sensation in the mouth, which tends to affect the whole of the tongue, displayed at its best in certain unripe fruit such as sloes, quinces and perry pears.[9]

Rossi and Singleton[10] found that in 'leucoanthocyanin' extracts derived from grapes, that the low molecular weight materials were predominantly bitter and the high molecular weight materials astringent. Lea and Arnold[11] made similar observations and concluded

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[6] Knight, T.A., *A Treatise on the Culture of Apple and Pear and the Manufacture of Cider and Perry.* H. Proctor, Ludlow, pp. 1801
[7] Lea, A.G.H., Timberlake, C.F., *J. Sci. Fd Agric.* **1974**, *25*, pp. 1537
[8] Lea, A.G.H., *J. Sci. Fd Agric.* **1978**, *29*, pp. 471
[9] Lea, A.G.H., Arnold, G.M., *J. Sci. Fd Agric.* **1978**, *29*, pp. 478
[10] Rossi, J.A., Singleton, V.L., *Am. J. Enol. Vitic.* **1966**, *17*, pp. 240
[11] Lea, A.G.H., Arnold, G.M., *J. Sci. Fd Agric.* **1978**, *29*, pp. 482

that the balance of bitterness and astringency in ciders is determined by the balance between oligomeric (1-5 units) and polymeric (6-10 units) procyanidins respectively.

At molecular level, the phenomenon of astringency is universally believed to result from non-specific and irreversible hydrogen bonding between *o*-diphenolic groups and proteins in the mouth. The larger the procyanidin, the greater its capacity for hydrogen bonding and the more astringent it will seem.[12,13] Bitterness is generally regarded as an interaction between polar molecules and the lipid portion of the taste papillae membrane. The relative lipid solubility of the bitter materials is thus critical for this sensation.[14] Only the oligomeric procyanidins would be sufficiently small to be fat-soluble, pass into the lipid membrane, and interact with receptors. Similar considerations can also explain the effect of ethanol on the taste of cider: it will increase the solubility of oligomeric procyanidins by co-solubility and simultaneously reduce hydrogen bonding, thus increasing bitterness, and reducing astringency.[15]

1.3 The Phenolics of Ciders: Effect of Processing conditions

The nature and concentrations of procyanidins are the main distinguishing factor between bittersweet apples. Maintaining or even increasing these concentrations during processing has received much interest, and attention has been given to the methods of extraction.

Traditional methods involve crushing and milling the fruit to a pulp, which is built up in alternate layers, and subsequently pressed by a vertical pack press.[16] Although the total extraction of sugar can be very high, the recovery of procyanidins is incomplete, and it has been shown that solvent extractions of the pomace (crushed apples) contain useful quantities of phenolic material.[17] Considerable losses occur at the point of milling by

[12] Joslyn, M.A., Goldstein, J.L., *Adv. Fd Res.* **1964**, *13*, pp. 179
[13] Bate-Smith, E.C., *Phytochemistry.* **1973**, *12*, pp. 907
[14] Koyama, N., Kurihara, K., *Biochim. biophys. Acta* **1972**, *288*, pp. 22
[15] Lea, A.G.H., Arnold, G.M., *J. Sci. Fd Agric.* **1978**, *29*, pp. 482
[16] Beech, F.W., *J. Inst. Brew.* **1972**, *78*, pp. 477
[17] Burroughs, L.F., *Rep. Long Ashton Res. Stn for* **1971**, pp. 168

oxidation and precipitation of polyphenols on the pomace.[18,19] Pack press operations are a batch-process and are labor intensive. This merits the use of continuous systems of extraction such as the hot water diffusion system, used for sugar beet and fruit juice extraction. This system consists of a hollow steel drum through which apple slices are passed using an Archimedean screw. Hot water passes slowly through the drum in the opposite direction and soluble solids diffuse into the liquor. Although sugar concentrations may be lower than pack-press methods, the total extraction efficiency may be high. Because of the use of concentrators, the dilution effect of this method is not perceived as a distinct disadvantage.[20]

Pectolytic enzymes have been employed to improve the pressing characteristics of the pulped fruit and to increase the yield. Although similar enzyme preparations have been used to cleave phenolic acid esters for analytical purposes,[21] the use of pectolytic enzymes have no detrimental effect on polyphenols in apple juice, as long as oxidation is prevented by prior use of SO_2. The total juice yield is markedly improved, but the tannin concentrations seem to be unchanged. There also seems to be no evidence to suggest the hydrolysis of phenolic acids or phloretin glycosides.

Temperature plays an equally important role in juice extraction. In an experiment by Lea and Timberlake (1978)[22] using Dabinett apples, the temperature dependence of extraction efficiency was clearly shown. A mean gain of 28% for total phenolics under warm sulfiting conditions was obtained and even a larger gain of 50% for the organoleptically important oligomeric and polymeric procyanidins under warmer conditions. The reason for this is that procyanidins are poorly soluble in water and have a low rate of diffusion at low temperatures.[23]

[18] Lea, A.G.H., Timberlake, C. F. *J. Sci. Fd Agric.* **1974**, *25*, pp. 1537
[19] Johnson, G., Donnelly, B., Johnson, D.K., *Fd. Technol.* **1969**, *23*, pp. 1312
[20] Lüthi, H.R., Glunk, U., *Fluss. Obst.* **1974**, *41*, pp. 498
[21] Mosel, H.D., Herrmann, K., *J. Sci. Fd Agric.* **1974**, *25*, pp. 251
[22] Lea, A.G.H., Timberlake, C. F. *J. Sci. Fd Agric.* **1978**, *29*, pp. 484-492

1.4 Oxidation of Polyphenols by Polyphenoloxidase: An important reaction in juice production

The enzymatic oxidation of fruit polyphenols is a result of the presence of the enzyme polyphenoloxidase. This oxidation takes place when damaged fruit is exposed to molecular oxygen, as in the case of milled apples.[24] With *o*-diphenols as substrate, the so-called "catecholase" activity of the enzyme results in the primary formation of *o*-quinones with an accepted stoichiometry of 0.5 mol of oxygen consumed per mol of phenol degraded and *o*-quinone formed.[25] Depending on the phenol, stability of the *o*-quinone formed varies considerably. These quinones undergo subsequent reactions leading to dark-colored pigments, which vary from one phenol to another. Variable amounts of phenol can also be consumed during non-enzymatic oxidation of *o*-quinones.[26,27]

Sulfiting agents have been the conventional chemicals to inhibit enzymatic-browning reactions in fruits and vegetables. However, there have been concerns over the possible harmful effects of sulfiting agents to sensitive consumers, especially asthmatics.[28] The search for sulfite substitutes has led to several alternatives.[29] Agents controlling enzymatic browning include ascorbic acid,[30] it's derivatives and proteases, specifically ficin,[31] to inactivate polyphenoloxidase. Wide varieties of sulfite substitutes have however lacked the versatility of sulfiting agents to control both enzymatic and non-enzymatic browning.

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[23]Lea, A.G.H., Timberlake, C. F. *J. Sci. Fd Agric.* **1978**, *29*, pp. 484-492
[24] Vamos-Vigyazo, L., Polyphenoloxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, pp. 49-127
[25] Mayer, A.M. Polyphenoloxidase in plants. Recent progress. *Phytochemistry* **1987**, *26*, pp. 11-20
[26] Lee, C.Y., Jaworski, A.W., Phenolics and browning potential of white grapes grown in New York. *Am. J. Enol. Vitic.* **1988**, *39*, pp.337-340
[27]Rouet-Mayer, M.A., Roles of *o*-quinones and their polymers in the enzymatic browning of apples. *Phytochemistry* **1990**, *29*, pp. 435-440
[28] Taylor, S.L., Higley, N.L., Bush, R.K., Adv. Food Res. **1986**, *30*, pp. 1
[29] Sapers, G.M., Hicks, K.B., Phillips, J.G., Garzarella, L., Pondish, D.L., Matulaitis, R.M., McCormack, T.J., Sondey, S.M., Seib, P.A., El-Atawy, Y.S., J. Food Sci. **1989**, *52*, pp. 997
[30] Sapers, G.M., Miller, R.L., Douglas, J.R. Hicks, K.B., J. Food Sci. **1991**, *56*, pp. 419
[31] Labuza, T.P., Cereal Food World, **1989**, *34*(4), pp. 353

Resorcinol derivatives have been employed and patented as anti-browning agents.[32,33] In the family of resorcinol derivatives, 4-hexylresorcinol has been effective in preventing shrimp blackspot due to the action of polyphenoloxidase.[34] It has a long history of use in pharmaceuticals and exhibits no systemic toxicity.[35] It has also been shown that in combination with ascorbic acid, it is an effective anti-browning agent that compares favorably with sulfiting agents.

There are primarily two ways of controlling browning chemically. One is the reduction of the quinones produced, and the other is by inhibition of the enzyme. Although both ascorbic acid-2-phosphate and 4-hexylresorcinol are effective in preventing enzymatic browning, the inhibitory mechanisms are different. Ascorbic acid-2-phosphate reduces quinones generated by polyphenoloxidase and thus retards browning,[36] whereas 4-hexylresorcinol is a specific inhibitor of polyphenoloxidase.[37]

Ascorbic acid-2-phosphate, sodium sulfite and 4-hexylresorcinol have been tested on apple slices, as anti-browning agents at different temperatures. At storage temperatures (35 ^{0}C) non-enzymatic browning predominated because of the temperature dependence of enzymes and only sodium sulfite was more effective. At temperatures above 45 ^{0}C, browning occurred regardless of anti-browning treatments.[38] Sodium sulfite, contrary to 4-hexylresorcinol is also effective against non-enzymatic browning, and explains why it was effective in the experiment. Sodium sulfite forms hydroxysulphonate complexes that exhibit much lower browning potentials than the precursor intermediates, which dehydrate to α,β-unsaturated dicarbonyls and eventually form brown pigments or melanoidins.[39]

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[32] McEviley A.J., Iyengar, R., Gross, A., Competition and methods for inhibiting browning in food using rescorcinol derivatives. U.S. patent 5,059,438, October 22, **1991.**
[33] McEviley A.J., Iyengar, R., Otwell, W.S., Food Technol. **1991,** *45*(9), pp. 80
[34] McEviley A.J., Iyengar, R., Otwell, W.S., Food Technol. **1991,** *45*(9), pp. 80
[35] Frankos, V.H., Schmidt, D.F., Haws, L.C., McEviley A.J., Iyengar, R., Miller, S.A., Munro, I.C., Clydesdale, F.M., Forbes, A.L., Sauer, R.M., Reg. Toxic. Pharmacol. **1991,** *14,* pp. 202
[36] Sapers, G.M., Hicks, K.B., Phillips, J.G., Garzarella, L., Pondish, D.L., Matulaitis, R.M., McCormack, T.J., Sondey, S.M., Seib, P.A., El-Atawy, Y.S., J. Food Sci. **1989,** *52,* pp. 997
[37] McEviley A.J., Iyengar, R., Otwell, W.S., Food Technol. **1991,** *45*(9), pp. 80
[38] Monsalve-Gonzales, A., Barbosa-Canovas, G.V., Cavalieri, R.P., McEvily, A.J., Iyengar, R., J. Food Sci. **1993,** *58*(4), pp. 797-800
[39] Wedzicha, B.L., Int. J. Food Sci. Technol. **1987,** *22,* pp. 433

The onset of browning correlates with the depletion, oxidation, or chemical transformation of the anti-browning agents.[40] Depletion of ascorbic acid and derivatives occurs due to oxidation by quinones, which are enzymatic products of polyphenoloxidase.[41] Hydrogen sulfite, the predominant form of sodium sulfite at the pH of apple juice, is lost by irreversible binding, formation of sulfate or as gaseous SO_2.[42] Ascorbic acid in combination with 4-hexylresorcinol is an effective anti-browning agent that compares favourably with sodium sulfite at 25 ^{0}C.[43]

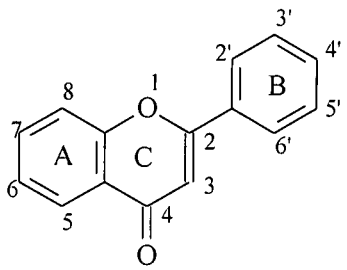
[40] Bolin, H.R., Boyle, H.R., Food. Prod. Dev. **1972,** *7,* pp. 84
[41] Labuza, T.P., Saltmarch, M., In *Water Activity: Influence on Food Qualit,* L.B. Rockland and G.F. Stewart. (Ed.), Academic Press, New York. **1981,** pp. 855
[42] Wedzicha, B.L., Int. J. Food Sci. Technol. **1987,** *22,* pp. 433
[43] Monsalve-Gonzales, A., Barbosa-Canovas, G.V., Cavalieri, R.P., McEvily, A.J., Iyengar, R., J. Food Sci. **1993,** *58*(4), pp. 800

CHAPTER 2

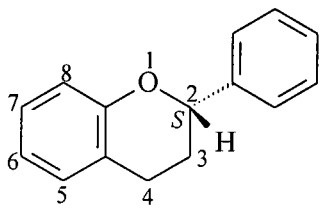
FLAVONOIDS: AN OVERVIEW OF STRUCTURE, GLYCOSIDES, OCCURRENCE AND BIOSYNTHESIS

2.1 Structure

The term flavonoid was first applied by Geisman and Hinreiner (1952)[44] to embrace all those compounds whose structure is based on that of flavone (2-phenyl-chromone) **(1).** Occasionally the term is misspelt as flavanoid, which may be a better term to use since the parent compound of the group is actually flavan (2-phenylchroman) **(2)**, in which the heterocyclic ring is fully reduced.



2-phenylchromone (Flavone) **(1)**
(numbering shown)

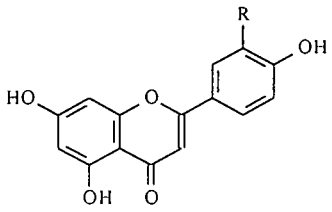


2-phenylchroman (Flavan) **(2)**
Note the 2*S* form is shown (phenyl group above plane of heterocyclic ring)

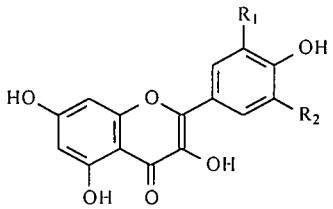
Fig. 2.1 The basic structure of flavonoids

Flavone consists of two benzene rings (A and B) joined together by a three-carbon link that form the C-ring (γ-pyrone ring). The various classes of true flavonoids differ only by the state of oxidation of this C_3 link. There is a limitation to the number of structures found in nature, which vary in their oxidation states from flavan-3-ols to flavonols and anthocyanins. Also included in the flavonoids are the flavanones, flavanonols (dihydroflavonols) and the flavan-3,4-diols.

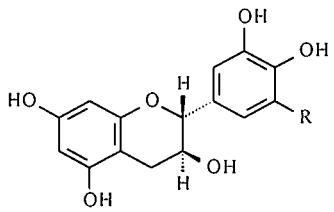
[44] Geisman, T.A., Hinreiner, E., *Bot. Rev.* **1952,** *18,* p. 77



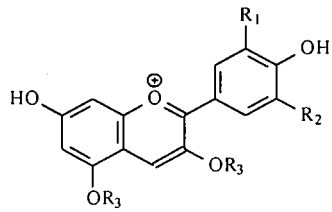
Flavones
R = H apigenin **(3)**
R = OH luteolin **(4)**



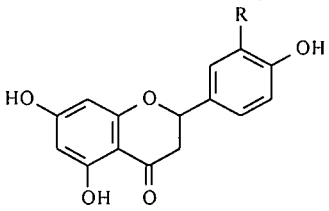
Flavonols (3-hydroxyflavones)
R_1R_2 = H kaempferol **(9)**
R_1 = OH, R_2 = H quercetin **(10)**
R_1R_2 = OH myricetin **(11)**



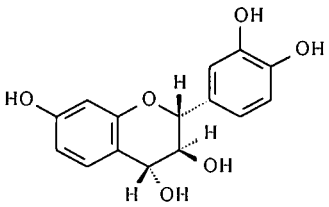
Flavan-3-ols
(2*R*, 3*S* as shown)
R = H catechin **(14)**
R = OH gallocatechin **(15)**
(2*R*, 3*R*: opposite configuration at C-3)
R = H epicatechin **(16)**
R = OH epigallocatechin **(17)**



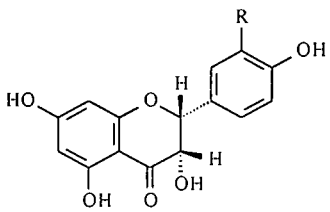
Anthocyanidins (R_3 = H)
R_1R_2 = H pelargonidin **(5)**
R_1 = OH, R_2 = H cyanidin **(6)**
R_1R_2 = OH delphinidin **(7)**
Anthocyanins (R_3 = glycosyl) **(8)**



Flavanones
R = H naringenin **(12)**
R = OH eriodictyol **(13)**



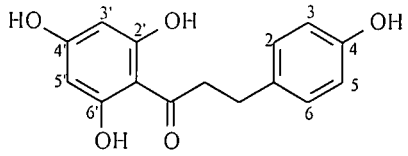
Flavan-3,4-diols
Mollisacacidin (2*R*, 3*S*, 4*R*) **(18)**
Leucofisitinidin has opposite configuration
(2*S*, 3*R*, 4*S*) **(19)**



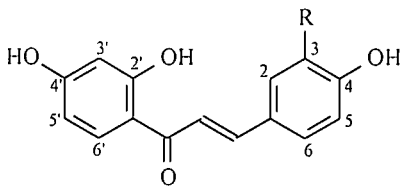
Dihydroflavanols (flavanones)
Note the steriochemistry at C-2 and C-3 (2*R*, 3*S*)
is the same as (-)-epicatechin **(16)**
R = H dihydrokaempferol **(20)**
R = OH dihydroquercetin (taxifolin) **(21)**

Fig. 2.2 Structure of classes flavonoids

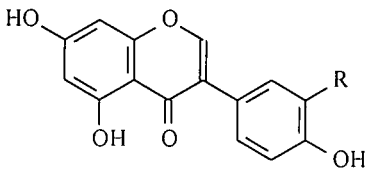
There are also five classes of compounds that do not essentially possess the basic 2-phenylchroman skeleton, but are so closely related both chemically and biosynthetically to the true flavonoid types, that they are included in the flavonoid group. These are the chalcones (Gr. *Chalcos,* copper), dihydrochalcones, isoflavones, neoflavones and aurones (L. *aurum*, gold).



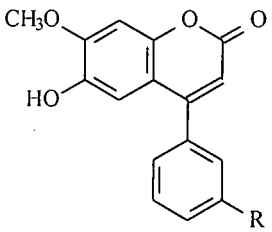
Dihydrochalcones (note numbering)
phloretin **(22)**



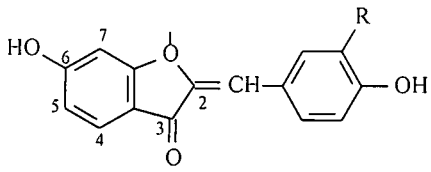
Chalcones (note numbering)
R = H isoliquiritigenin **(23)**
R = OH butein **(24)**



Isoflavones (numbering same as flavones)
R = H genistein **(25)**
R = OH orobol **(26)**



Neoflavones (dalbergins or 4-phenylcoumarins)
R = H dalbergin **(27)**
R = OH stevenin **(28)**



Aurones (note numbering)
R = H hispidol **(29)**
R = OH sulphuretin **(30)**

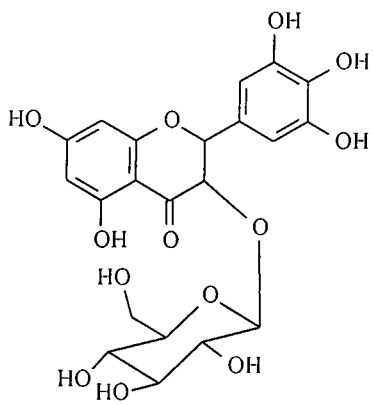
Fig. 2.3 Structures of some of the minor flavonoids

The individual compounds within each class are distinguished mainly by the number and position of hydroxy, methoxy and other groups substituted on the two benzene rings (A and B). These groups have generally restricted patterns of substitution, reflecting the different biosynthetic origins of the two aromatic nuclei. In the A-ring, the majority of hydroxy groups are substituted at both C-5 and C-7, or only at C-7, and generally are

unmethylated. One, two or three hydroxy groups or methoxy groups on the other hand generally substitute the B-ring. The first, which is seldom methylated, is substituted *para* [C-4'(B)] to the point of attachment of this ring to the rest of the molecule, with the second and third groups *ortho* to it at C-3'(B) and C-5'(B), with the latter two groups often being methylated. The hydroxylation of the A-ring reflects its origin from malonate or acetate precursors, whilst the hydroxylation pattern of the B-ring resembles that found in the commonly occurring cinnamic acids, and reflects their common biosynthetic origin from shikimic acid and its congeners (see section 2.4).

2.2 Glycosides

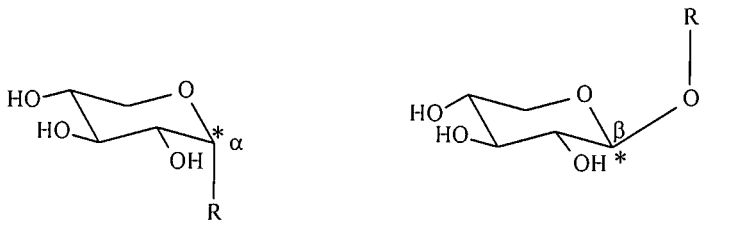
Flavonoids often exist as glycosides in plants, except in non-living woody tissue. One or more hydroxy groups are joined by a hemiacetal link to C-1 of a sugar. The sugar-free compound is called an aglycone, and although their presence has often been reported in non-woody tissue, it is probable that in most cases they are formed as artifacts during extraction, since most living tissue contains glycosidases, which work even in the presence of high concentrations of organic solvents. Glycosidation is responsible for *in vitro* solubility of the otherwise generally water-insoluble aglycones. It also improves stability, especially for the anthocyanidins and more highly hydroxylated compounds. A good example is quercetin **(10)** and myricetin **(11)**, which are susceptible to oxidation catalyzed by phenolase, but the corresponding 3-O-glycosides **(31)** are stable.[45]



Myricetin-3-O-β-D-glucopyranosyl **(31)**

[45] Roberts E.A.H., *Nature, Lond.* **1960**, *185*, pp. 536

Sugars that have been found in flavonoid glycosides include simple hexoses and pentoses (monosides), di- and trisaccharides (biosides and triosides). These sugars, when connected to the aglycone *via* oxygen at C-1 (anomeric carbon) or another carbon, are referred to as an O-glycoside **(33)**. A sugar connected directly through a carbon atom is called a C-glycoside **(32)**. The nature of the bond is almost invariably such that the link is β. D-glucose, occurring either alone or as part of a disaccharide, is the most common sugar in glycosides.



α-D-xylopyranosyl **(32)** (C-glycoside)

β–D-xylopyranosyl **(33)** (O-glycoside)

R = Aglycone (flavonoid)

Fig. 2.4 Xylopyranosyl glycosides (note the configuration at the anomeric* carbon).

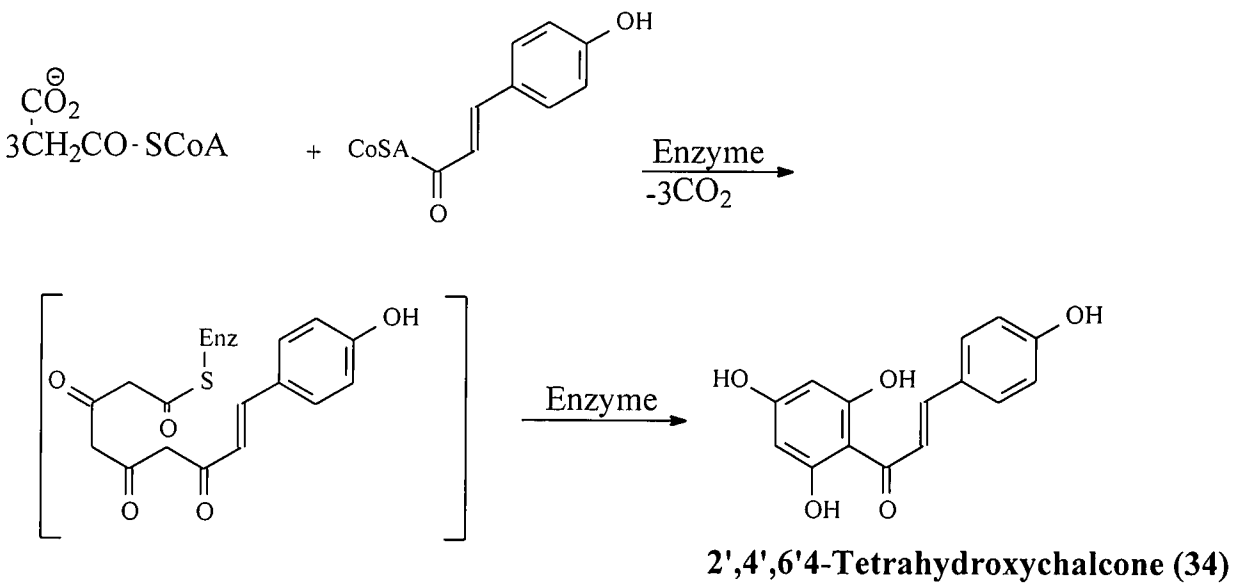
2.3 Occurrence

Flavonoid compounds are widely distributed in higher plants[46,47,48] with glycosides of quercetin occurring in 62% of leaves of dicotyledons examined by Bate-Smith (1962).[49] They have been isolated from all the different parts of plants, with variations in the type of compounds found in different anatomical tissues of any one plant.[50]

[46] Harborne, J.B., *Comparative Biochemistry of the Flavonoids*, Academic press, London and New York. **1967**
[47] Harborne, J.B., *Phytochemical Methods*, Chapman and Hall, London. **1973**
[48] Harborne, J.B., Williams, C., *The Flavonoids* (J.B Harborne *et al.*, eds). Chapman and Hall, London. **1975**
[49] Bate-Smith, E.C., *J. Linn. Soc. (Bot)* **1962**, *58*, pp. 95
[50] Griffiths, L.A., *Biochem. J.* **1958**, 70, pp. 120

2.4 Biosynthesis

The basic $C_6C_3C_6$ skeleton of flavonoids have long been postulated to arise from the condensation of a C_6C_3 unit with three acetate units (via malonyl-CoA).[51,52] The C_6C_3 unit is a cinnamic acid giving rise to a C_{15} flavonoid prototype **(Fig. 2.5)**.

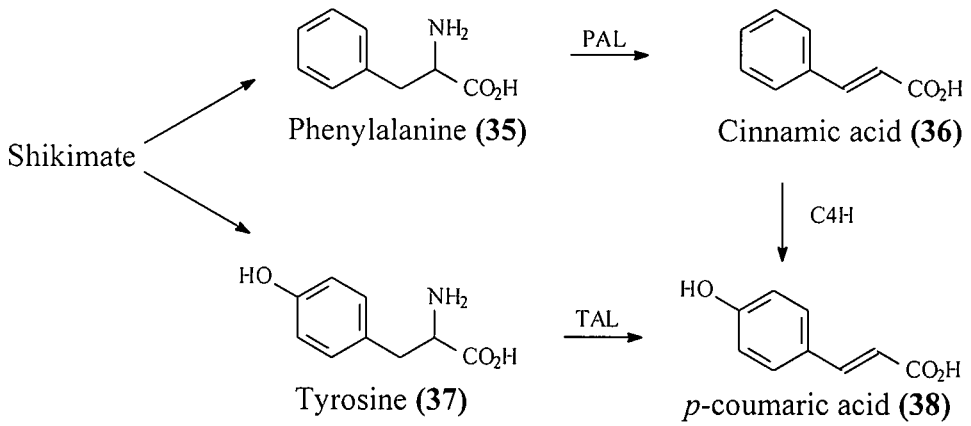


2',4',6'4-Tetrahydroxychalcone (34)

Fig. 2.5 The origin of the $C_6C_3C_6$ skeleton of flavonoid compounds.

Phenylalanine **(35)** is the immediate biogenetic precursor to cinnamic acid **(36)**, the enzyme responsible for the conversion being phenylalanine ammonialyase (PAL) **(Fig.2.6)**. This aromatic amino acid serves as the branching point from primary metabolism to this major area of secondary plant metabolism. With few exceptions, flavonoid compounds possesesses oxygen at C-4' of the B-ring. This strongly suggests that *p*-coumaric acid **(38)** instead of cinnamic acid **(36)** itself is the direct phenylpropanoid intermediate for a great majority of flavonoid compounds. The enzyme catalyzing the formation of *p*-coumaric acid from cinnamic acid is cinnamic acid 4-hydroxylase (C4H). The biosynthesis of *p*-coumaric acid is also possible via tyrosine **(37)**, but this route seems to be of significance in plants of the Gramineae family only.[53]

[51] Birch, A.J., Donovan, F.W., *Aust. J. Chem.* **1953**, *6*, pp. 360-368
[52] Grisebach, H., *Planta med.* **1962**, *10*, pp. 385-397
[53] Wong, E., Chemistry and Biochemistry of Plant Pigments, Second ed. Vol. 1, Ed. T.W. Goodwin, Academic Press, London, New York, San Francisco, **1976**, pp. 467



Ezymes: PAL = phenylalanine ammonialyase
TAL = tyrosine ammonialyase
C4H = cinnamic acid 4-hydroxylase

Fig. 2.6 Possible routes to *p*-coumaric acid

2.4.1 Phenylalanine ammonialyase (PAL)

Phenylalanine ammonialyase is the key enzyme in phenylpropanoid (C_6C_3) metabolism and was first report by Koukol and Conn (1961).[54] The enzyme has a wide distribution[55] and has been isolated from various plant sources and from certain fungi.[56] The reaction catalyzed by PAL is the antiperiplanar deamination of phenylalanine to yield *trans*-cinnamic acid.[57] **(Fig. 2.7)**

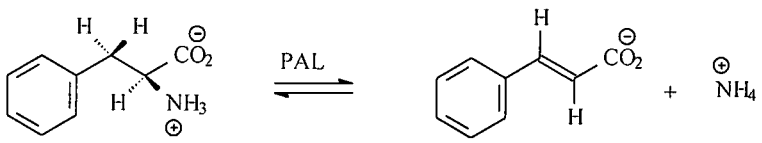


Fig. 2.7 Antiperiplanar deamination of L-phenylalanine

Reversibility of the reaction *in vitro* has been demonstrated,[58] but the biosynthetic significance of this remains obscure. Preparations of the enzyme from the grass family[59]

[54] Koukol, J., Conn, E.E., *J. Biol. Chem.* **1961**, *236*, pp. 2692-2698
[55] Towers, G.H.N., Subba Rao, P.V., *Recent Adv. Phytochem.* **1972**, *4*, pp. 1-43
[56] Camm, E.L., Towers, G.H.N., *Phytochemistry.* **1973**, *12*, pp. 961-973
[57] Hanson, K.R., Havir, E.A., *Recent Adv. Phytochem.* **1972**, *4*, pp. 45-85
[58] Subba Rao, P.V., Moore, K., Towers, G.H.N., *Can. J. Biochem.* **1967**, *45*, pp. 1863-1872
[59] Neish, A.C., *Phytochemistry.* **1961**, *1*, pp. 1-24

and some yeasts[60] also deaminates L-tyrosine to *trans-p*-coumaric acid, although always to a lesser extent. PAL is inhibited by cinnamic acids and benzoic acid (end product or negative feedback inhibition) and by some flavonoid compounds such as kaempferol and quercetin.[61]

2.4.2 Cinnamic acid 4-hydroxylase

Cinnamic acid 4-hydroxylase (C4H) is the second key enzyme in the phenylpropanoid/flavonoid biosynthetic pathway. It catalyses the *para*-hydroxylation of cinnamic acid **(Fig. 2.8)** and was first isolated from spinach by Nair and Vining (1965).[62] C4H has been isolated from pea seedlings by Russel and Conn (1967) and many of its properties have been studied. C4H from pea seedlings is a mixed function oxidase that requires molecular oxygen and NADPH for activity. 2-Mercaptoethanol is required for optimal activity but does not serve as an external reductant for the hydroxylation, but is needed to maintain the structural integrity of the enzyme. C4H is specific for cinnamic acid, and has a high affinity for it (K_m = 1.7 x 10^{-5}). It does not hydroxylate *p*-coumaric acid or phenylalanine and low concentrations of *p*-coumaric acid inhibits it. This high degree of inhibition indicates that regulation of C4H activity is an important control point in phenolic biosynthesis.[63]

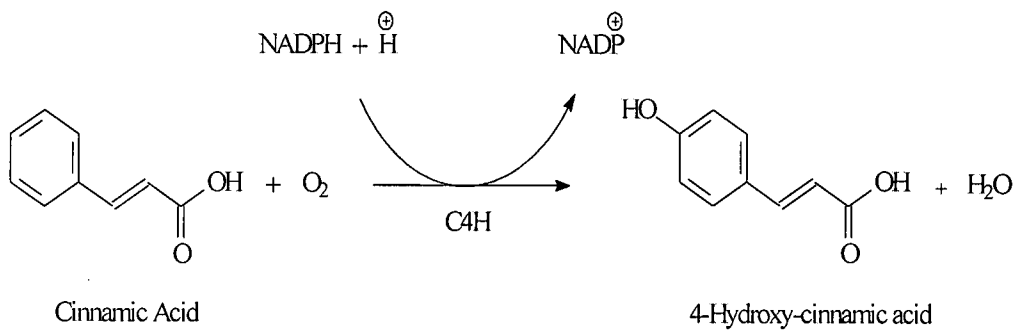


Fig. 2.8 4-Hydroxylation of cinnamic acid

[60] Camm, E.L., Towers, G.H.N., *Phytochemistry.* **1973**, *12*, pp. 961-973
[61] Attridge, T.H., Stewart, G.R., Smith, H., *FEBS Lett.* **1971**, *17*, pp. 84-86
[62] Nair,P.M., Vining, L.C., *Phytochemistry.* **1965**, *4*, pp. 161-168
[63] Russell, D.W., *J. Biol. Chem.* **1971**, *246*, pp. *3870-3878*

2.4.3 Activation of hydroxycinnamic acids

Hydroxycinnamic acids have to be activated for further condensation, reduction, or transfer reactions. There are two primary possibilities of activation, i.e. formation of a coenzyme-A ester (CoA) or a 1-*O*-glucose ester **(Fig. 2.9)**. Both esters can be used by relevant transferases to give new esters that may serve as substrates in a secondary set of transfer reactions. Coenzyme-A esters are the exclusive substrates for chalcone synthase (CHS), the first committed enzyme of flavonoid biosynthesis and for some acyltransferases involved in the modification of the carbohydrate moiety of anthocyanins. Strong evidence exists that 4-coumaroyl-CoA is preferably used as substrate by chalcone synthase, while the anthocyanin-related acyl transferases even accept the highly substituted sinapoyl-CoA. [64] Coenzyme-A ligase enzymes are responsible for the esterification of 4-coumaric acid and other isoforms. It also accepts hydroxycinnamic acids with more complex substitution patterns. These enzymes have long been known from various plant species. The ligase reaction strictly requires ATP and Mg^{2+} as cofactors. The reaction proceeds via an acyl-AMP (adenosine 5'-mono-phosphate) intermediate, characterizing the enzyme as a synthetase.[65] Formation of glucose esters has frequently been demonstrated with enzymes from various plant sources, using UDP-glucose as glucose donor.[66]

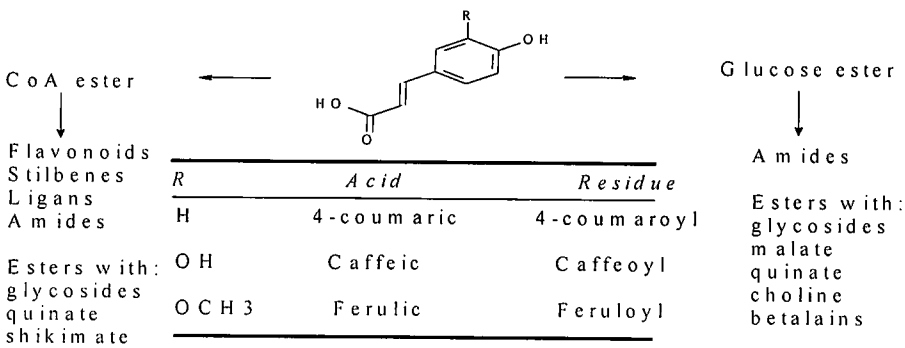


Fig. 2.9 This scheme illustrates the central position of activated hydroxycinnamic acids in flavonoid biosynthesis.

[64] Heller, W., Forkman, G., *The Flavanoids: Advances in research Since 1980, (ed. J.B. Harborne)* Chapmann and Hall, London, **1988**, pp. 399-425
[65] Heller, W., Forkman, G., *The Flavanoids: Advances in research Since 1980, (ed. J.B. Harborne)* Chapmann and Hall, London, **1988**, pp. 399-425
[66] Strack, D., Mock, H.P., *Methods in Plant Biochemistry,* vol. 9, (eds. P.M. Dey, J.B. Harborne), Academic Press, London. **1992**

2.4.4 Modification of 4-coumaric acid

There are two different pathways of 4-coumaric acid modifications: modification at the free acid level or modification of conjugates like CoA, shikimate and quinate esters. 4-Coumaric acid has frequently been described to be 3-hydroxylated to caffeic acid by phenolase type enzymes.[67,68] Methylation of the 3-hydroxy function by methyltransferases (COMs) using S-adenosyl-L-methionine as methyl donor is a well-known reaction. The enzymes are often produced through environmental stress, and have therefore been extensively studied. [69] Hydroxylation of 4-coumaric acid conjugate was first observed by Kamsteeg *et al.* (1981),[70] who described hydroxylation of 4-coumaroyl-CoA by a phenolase-like enzyme. A similar reaction was later studied in *Daucus carota* cell suspension cultures (Kneusel *et al.,* 1989).[71] No further modification on the CoA ester level have been described so far, but similar reactions with a number of 4-coumaric acid conjugates have been detected (Kühnl *et al.*, 1987).[72]

2.4.5 Chalcone synthase (CHS)

CHS provides the basic C_{15} chalcone intermediate from which all other flavonoids are derived. CHS catalyses the condensation of three molecules malonyl-CoA with 4-coumaroyl-CoA. Besides 4-coumaroyl-CoA, the main substrate of CHS, enzymes from some plant species additionally accept caffeoyl-CoA, and even feruloyl-CoA as substrates.[73] The central function of CHS, and the fact that no cofactors are required for the condensation reaction, identifies CHS as a typical key enzyme. Strong inhibition of 4CHS from *Avena* and *Secale* was observed with apigenin and leutolin. Flavanones and

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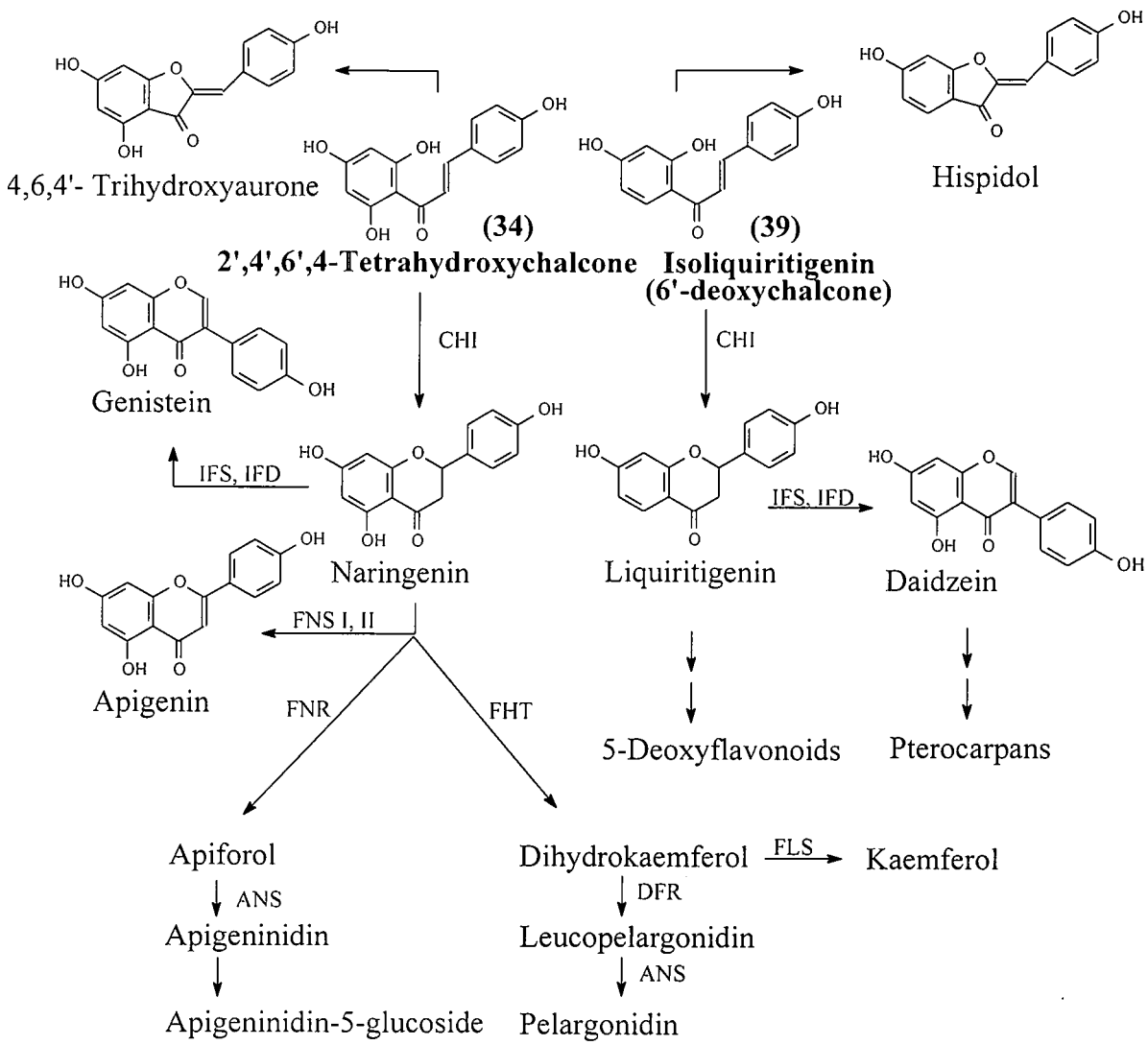
[67] Heller, W., Forkman, G., *The Flavanoids: Advances in research Since 1980, (ed. J.B. Harborne)* Chapmann and Hall, London, **1988**, pp. 399-425
[68] Gross, G.G., *Biosynthesis and Biodegradation of Wood Components* (ed. T. Higuchi), Academic Press, London, **1985**, pp. 229-266
[69] Gowri, G., Bugos, R.C., Campbell, W.H., Maxwell, C.A., Dixon, R.A., *Plant Physiol.* **1991**, *97,* pp.7
[70] Kamsteeg, J., van Brederode, J., Verschuren, P.M., van Nigtevecht, G., *Z.Pflanzenphysiol.* **1981**, *102,* pp.435
[71] Kneusel, R.E., Matern, U., Nicolay, K., *Arch. Biochem. Biophys.* **1989**, *269,* pp. 455
[72] Kühnl, T., Koch, U., Heller, W., Wellmann, E., *Arch. Biochem. Biophys.* **1987**, *258,* pp. 226
[73] Heller, W., Forkman, G., *The Flavanoids: Advances in research Since 1980, (ed. J.B. Harborne)* Chapmann and Hall, London, **1988**, pp. 399-425

coenzyme A showed similar inhibition effects. This inhibition may be indicative of regulation through a feedback mechanism.[74]

2.4.6 Biosynthesis of classes of flavonoids

2',4',6',4-Tetrahydroxychalcone **(34)** is the central branch point for most flavonoids. It is important to note that an important analogue of the tetrahydroxy chalcone exists as 2',4',4-trihydroxychalcone **(39)**. This is a 6'-deoxychalcone which is an intermediate for the important group of 5-deoxyflavonoids, that includes several major phytoalexins. The individual synthesis of each class of flavonoid is discussed by Heller and Forkmann (1994) who gives a concise discussion of the biosynthesis of different classes of flavonoids and all relevant enzymes, in "*The Flavonoids,* Advances in research since 1986" [75]. (see also **Fig. 2.10**)

[74] Harker, C.L., Ellis, T.H.E., Coen, E.S., *Plant Cell,* **1990**, *2,* pp.185
[75] Heller, W., Forkman, G., *The Flavanoids: Advances in research Since 1986*, (ed. J.B. Harborne) Chapmann and Hall, London, **1994**, pp. 499-535



Enzyme	Acronym
Chalcone isomerase	**CHI**
2-Hydroxyisoflanvanone synthase	**IFS**
2-Hydroxyisoflavanone dehydratase	**IFD**
Flavone synthase I	**FNS I**
Flavone synthase II	**FNS II**
Flavanone 4-reductase	**FNR**
Flavanone 3-hydroxylase	**FHT**
Flavonol synthase	**FLS**
Dihydroflavonol 4-reductase	**DFR**
Anthocyanidin synthase	**ANS**

Fig. 2.10 Scheme to illustrate the pathway to major classes of flavonoids. The relevant enzymes involved in each transformation are also shown. The central position of 2',4',6,'4-Terahydroxychalcone **(34)** and its 6'-deoxychalcone **(39)** can be seen.

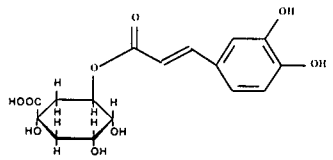
CHAPTER 3

PHENOLIC CONSTITUENTS OF APPLES

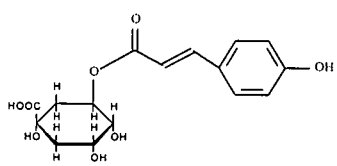
3.1 Introduction

There are six classes of polyphenols in apples **(Fig. 3.1)**[76]. The anthocyanins and flavonol glycosides are mainly found in the skin and may also be present in the juice. The phenolic acids are chlorogenic acid **(40)** and *p*-coumaroylquinic acid **(41)**, which belongs to the cinnamate family.

1. Phenolic acids

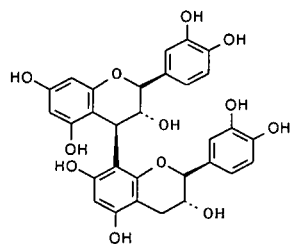


Chlorogenic acid **(40)**
(5-caffeoyl quinic acid)



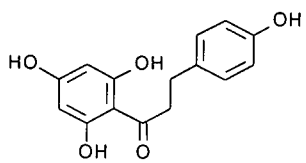
p-coumaroyl quinic acid **(41)**

4. Procyanidins

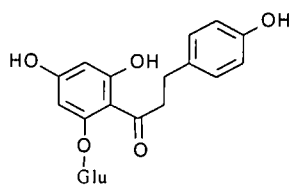


4-β-8-linked epicatechin **(44)**

2. Dihydrochalcones

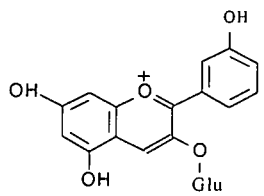


Phloretin **(22)**



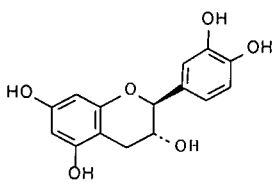
Phloridzin **(42)**
(Phloretin glycoside)

5. Anthocyanins

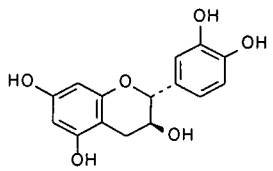


Cyanidin-3-glucoside **(43)**

3. Catechins

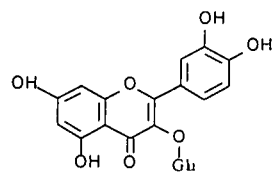


epicatechin **(14)**



catechin **(16)**

6. Flavonol glycosides



quercetin-3-glucoside **(45)**

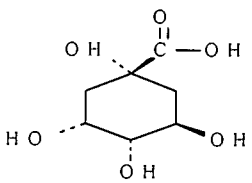
Fig. 3.1 Six classes of polyphenols found in apples

[76] Lea, A.G.H., Timberlake, C.F., The phenolics of ciders. *J. Sci. Food Agric.* **1974**, *25*, pp. 1537-1545

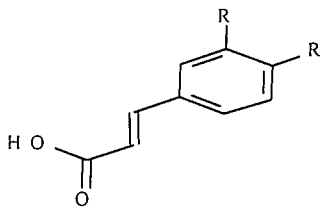
The dihydrochalcones are phloretin glucoside (phloridzin) **(42)** and xyloglucoside. The main flavan-3-ol is epicatechin **(16)**, and the procyanidins are the 4-β-8-linked epicatechin **(44)** series with some mixed catechin /epicatechin. The constitution of apple juice and apple cider differs from that of apples. The compounds found in apple juice are directly related to the process that is used to extract the juice. If for instance the apples were peeled before pulping, one would not expect to find anthocyanins in the juice.

3.2 Phenolic acids

Chlorogenic acid **(40)** and *p*-coumaroylquinic acid **(41)** belongs to the cinnamate family of acids, which is principally derived from cinnamic acid **(48)**. Chlorogenic acid was first isolated from coffee beans by Gorter (1907).[77] Chlorogenic acid is a derivative of quinic acid, and is hydrolyzed by tannase[78] (enzyme) into equimolar amounts of quinic acid **(46)** and caffeic acid **(47)**.



Quinic acid **(46)**



R = OH Caffeic acid **(47)**
R = H Cinnamic acid **(48)**

Chlorogenic acid is wide-spread in its occurrence, and is found in the fruit, leaves and other tissue of dicotyledonous plants.[79,80] The first synthesis of chlorogenic acid was carried out by Panizzi *et al.* (1955).[81] Williams (1958)[82] first isolated 3-*O*-*p*-coumaroylquinic acid from cider apples, but it also occurs in tea, tobacco, and pears.[83,84] 3-*O*-*p*-Coumaroylquinic acid was first synthesized by Haslam' *et al.* (1961).[85] Chlorogenic acid is oxidized by polyphenol oxidase, and contributes to browning of apple

[77] Gorter, *Annalen*, **1907,** *358,* pp. 328
[78] Freudenberg, *Ber.* **1920,** *53,* pp. 232
[79] Bate-Smith, *Chem. and Ind.* **1954,** pp. 1454
[80] Hermann, *Pharmazie,* **1958,** *11,* pp. 433
[81] Panizzi, Scarpati, Oriente, *Experientia,* **1955,** *11,* pp. 383
[82] Williams, *Chem. and Ind.* **1958,** pp. 1200
[83] Cartwright, Flood, Roberts, Williams, *Chem. and Ind.* **1955,** pp. 1062
[84] Griffiths, *Biochem. J.* **1958,** *70,* pp. 120
[85] Haslam, E., *et. al, J. Chem. Soc.* **1961**, pp. 5153

juices. 3-*O*-*p*-Coumaroylquinic acids, on the other hand are not oxidized, since they contain no *o*-phenolic groups. Other phenolic acids that have been reported to occur in apples include caffeic, ferulic, cinnamic, benzoic, malic and citric acid. [86]

3.3 Dihydrochalcones

The occurrence of dihydrochalcone glycosides (phloretin glucoside and phloretin xyloglucoside) in apple juice has been known for a long time (Johnson *et al.*, 1968).[87] In fact, these substances are characteristic of apples since they have not been detected in any other fruit (Herrmann, 1990),[88] and therefore their analysis is useful in food authenticity studies. Dihydrochalcones are also important since they oxidize easily,[89] and their oxidations contribute to apple juice browning.[90]

The glycosides of dihydrochalcones represent an interesting group of compounds from a chemi-taxonomic point of view,[91] as they are of a limited distribution but widespread enough to have some taxonomic significance.

The parent compound of the two mentioned dihydrochalcones, 2',4',6',4-tetrahydroxydihydrochalcone-2'-*O*-β-D-glucopyranoside (phloridzin) **(42)** and 2',4',6',4-tetrahydroxy dihydrochalcone-2'-*O*-β-D-(6''-β-D-xylopyranosyl)-β-D-glucopyranoside, is phloretin **(22)**, and the best known of the group is phloridzin. Phloridzin was first isolated by De Koninck (1835a).[92] He reported that it was found in the bark of apple, cherry, and plum, when, in fact it was only isolated from the root bark of apple (De Koninck

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[86] Miller, N.J., Diplock, A.T., Rice-Evans, C.A., *J. Agric. Food Chem.* **1955,** *43,* pp. 1794-1801

[87] Johnson, G., Donnelly, B.J., Johnson. D.K., The chemical nature and precursors of clarified apple juice sediment. *J. Food Sci.*, **1968**, *33,* pp. 254-257

[88] Herrmann, K., Occurrence and contents of falvonoids in fruit. Part II. Flavonol glycosides, anthocyanins and dihydrochalcones. *Ewerbsobstbau,* **1990,** *32,* pp. 32-38

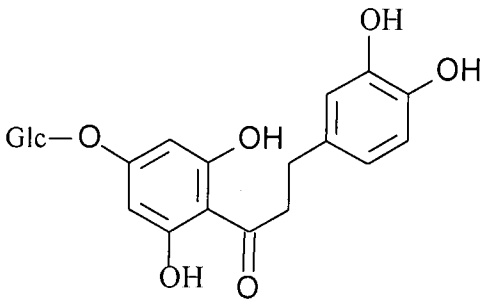
[89] Dziedzic, S.Z., Hudson, B.J.F., Barbers G., Polyhydroxychalcones as antioxidants for lard. *J. Agric. Food Chem.*, **1985,** *33,* pp. 244-246

[90] Oszmianski, J., Lee, C.Y., Enzymatic oxidation of phloretin glucoside in a model system. *J. Agric, Food Chem.*, **1991,** *39,* pp. 1050-1052

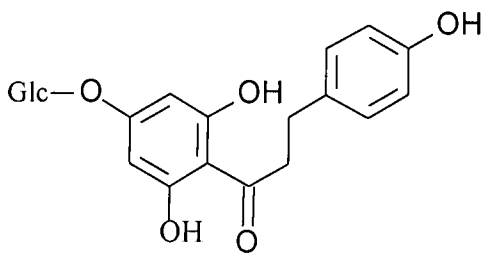
[91] Williams, A.H., "Comparative Phytochemistry" (T. Swain, ed.), Academic Press, New York, **1966,** pp. 297-307

[92] De Koninck, L., *Justus Liebigs Ann. Chem.* **1835,** *15,* pp. 75-77

1835b).[93] According to Williams (1966),[94] various authors noted the non-occurrence of phloridzin in pears. Williams states that extensive studies carried out by him have definitely shown that phloridzin is confined to apples, and does not occur in any of the fruit trees mentioned, nor in any of their relatives among the Rosaceae family. Rehder (1940)[95] lists 25 species in the genus *Malus*. Leaf extracts of these species were found to contain phloridzin in the majority of instances, but in some species the phloridzin was either accompanied or replaced by another glucoside *e.g.*, sieboldin **(49)**, which was found to possesesses the β-D-glucopyranoside in the 4'-position of the dihydrochalcone with an additional phenolic group in the C-3 position. It is found in most but not all of the species in the series Sieboldianae, *M. floribunda, M. zumi, M. sieboldii,* and *M. sargenti.* Sieboldin has not been found outside the *Sieboldianae* series or their hybrids except in one variety of *M. prunifolia* Rinki, which could be a hybrid with *Sieboldianae* species in its pedigree.



Sieboldin **(49)**
Glc = Glucose



Trilobatin **(50)**
Glc = Glucose

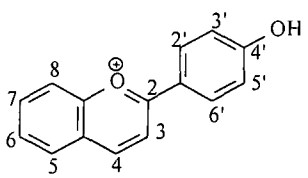
M. trilobata contains the glucoside trilobatin **(50)**, which is isometic with phloridzin. It has the glucose in the 4'-position of the dihydrochalcone parent compound. *M. trilobata* seems to contain no phloridzin; the Glycosidation occurs only in the 4'-position. Three other glycosides of phloretin occur in *Malus* species. These are all polyglycosides of the parent compound, and they occur in the common apple tree together with phloridzin but in much smaller amounts. Phloretin 2'–xyloglycoside occurs mainly in very young leaves. A similar compound with arabinose attached to the phloridzin glucose is known and a

[93] De Koninck, L., *Justus Liebigs Ann. Chem.* **1835,** *15,* pp. 258-263
[94] Williams, A.H., "Comparative Phytochemistry" (T. Swain, ed.), Academic Press, New York, **1966,** pp. 297-307

compound with three molecules attached to phloretin has been identified. These additional glycosides have been studied only in *M. pumila.*

3.4 Anthocyanins

The pigments responsible for the color of apple skin, and flowers, is chlorophyll, carotenoids located in plastids, and the phenolic pigments located in the vacuole. The flavonols and proanthocyanins do not contribute significantly to overall coloration but are important in enhancing anthocyanin coloration through copigmentation. Pelargonidin **(52)**, cyanidin **(53)** and delphinidin **(54)** (anthocyanins), produce scarlet, crimson and blue-mauve shades, respectively [absorbance maxima (λ max) of 520, 535 and 546 nm in 0.001 % HCL in methanol].[96]



Flavylium cation **(53)**

	Substitution pattern					
Anthocyanidin	3	5	6	7	3'	5'
Common basic structure						
Pelargonidin (Pg)*(**54**)	OH	OH	H	OH	H	H
Cyanidin (Cy)*(**55**)	OH	OH	H	OH	OH	H
Delphinidin (Dp)*(**56**)	OH	OH	H	OH	OH	OH

* Abbreviation

Anthocyanins are glycosides and acyl glycosides of anthocyanidins. The primary structures of anthocyanins are based on 2-phenylbenzopyrylium (flavylium cation) **(51),** but other secondary structures exist in aqueous acidic solutions **(Fig. 3.2).**

[95] Rehder, A., "Manual of Cultivated Trees and Shrubs." 2nd ed. rev., The McMillan Co., New York. **1940,** pp. 996

[96] Osawa, Y., Anthocyanins as food colours. P. Markakis ed. Academic Press, New York, **1982,** pp. 41-65

This is a mixture of the quinonoidal base(s) and the carbinol pseudobase.[97] There are also four possible stabilization mechanisms possible that lead to "tertiary structures." These mechanisms include self-association, inter- and intra-molecular copigmentation and metal complex copigmentation.[98] Copigmentation results in an increase in absorbance and a shift in λ_{max} to longer wavelengths (bathochromic shift). Thus, copigmentation results in "bluing" of red shades. The mechanism of copigmentation is detailed by Mazza and Brouillard (1990).[99] Copigmentation is probably also the most efficient protection mechanism, avoiding nucleophilic attack of the quinonoidal structures by water in the vacuole.[100]

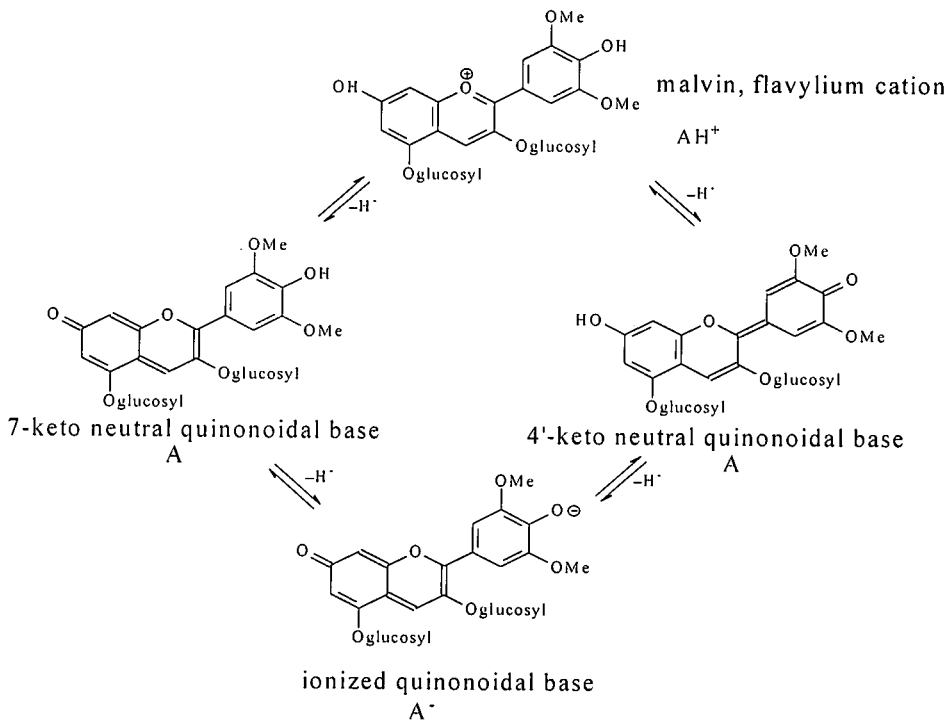
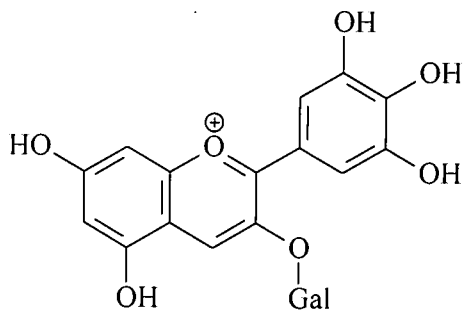


Fig. 3.2 Fast acid-base equilibria between the flavyllium cation AH^+, the neutral quinonoidal base A and the ionized quinonoidal base A^-.

[97] Brouillard, R., Anthocyanins as food colours. P. Markakis ed. Academic Press, New York, **1982,** pp. 1-40
[98] Brouillard, R., The *in vivo* expression of anthocyanin colour in plants. Phytochemistry **1983,** *22,* pp. 1311-1323
[99] Mazza, G., Brouillard, R., The mechanism of copigmentation of anthocyanins in aqueous solutions. Phytochemistry, **1990,** *29,* pp. 1097-1102
[100] Strack, D., Wray, V., *The Flavonoids* (J.B Harborne *et al.,* eds). Chapman and Hall, London. **1994,** pp. 6-7

Anthocyanins occur as 3-mono-, -bio and -triosides as well as 3,5-diglycosides. 3,7-Diglycosides also exist but are rare. The sugars generally associated with anthocyanidins are glucose, galactose, rhamnose, arabinose, and xylose. Apple skin contains mainly cyanidin-3-galactoside **(55)**[101] and high concentrations of flavonols (quercetin glycosides) and proanthocyanidins.[102] Cyanidin 3-glucoside, cyanidin 3-arabinoside and cyanidin 3-xyloside have all been found in the fruits of apple trees.[103]

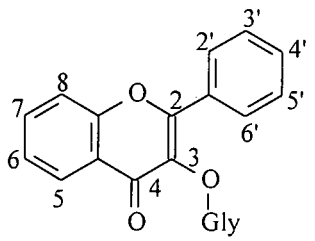


Cyanidin 3-galactose **(55)**

[101] Sun, B.H., Francis, F.J., Apple anthocyanins: Identification of cyanidin 7-arabinoside. *J. Food Sci.* **1967,** *32,* pp. 647-648
[102] McRae, K.B., Lidster, P.D., De Marco, A.C., Dick, A.J., Comparison of the polyphenol profiles of apple fruit cultivars by correspondence analysis. *J. Sci. Food Agric,* **1990,** *50,* pp. 329-342
[103] Mazza, G., Velioglu, Y.S., *Food Chem.* **1992,** *43,* pp. 113

3.5 Flavonol glycosides

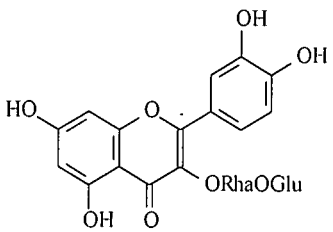
The structure of flavonols is based on 2-phenylchromanone **(1)** that is hydroxylated at the 3-position of the C-ring. Most flavonols have sugars attached at no more than two hydroxy groups (usually 3-,7-,3,7-) of the flavonoid nucleus **(Fig. 3.3).** Some triglycosides have been reported [rhamnetin-3-galactoside-3',4'-bisglucoside (Barberà et. al,. 1986)] but are rare.



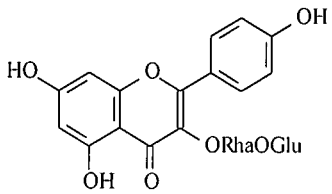
Basic structure of flavonol glycosides
Hyroxylation generally at the 5-,7-, and 4' positions.
Gly = glycoside

Fig. 3.3 The basic structure of flavonol glycosides.

The major flavonol glycosides in apples are those with quercetin as the aglycone. Quercetin glycosides in apple juices are glycosilated at the 3-position of the aglycone, via oxygen. Six monosides have been reported as glycosides from the skin of some cultivars (Granny Smith and Splendour) using reverse phase HPLC. These include galactose, glucose, rhamnose, xylose, arabinopyranose and arabinofuranose. One bioside(O-α-L-Rhamnosyl-(1→6)-glucose) **(56)** have also been reported.[104] This compound is also known as rutin, and a kaempferol analogue **(57)** have been identified in apricots (Simòn et. al.1992).[105] Rutin is an excellent copigment, and plays an important physiological role in plants, insects and mammals.



Quercetin 3-rutinoside **(56)**
(Rutin)

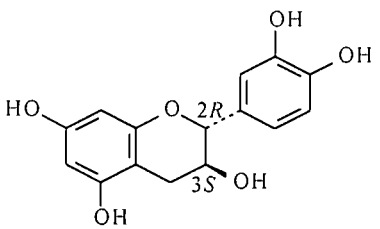


Kaempherol 3-rutinoside **(57)**

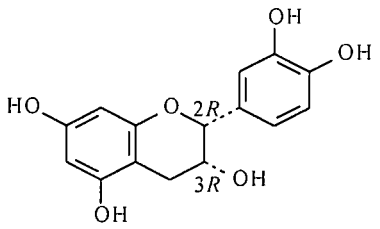
[104] Lancaster, J.E., Lister, C.E., Sutton, K.H., *J. Sci Food Agric.* **1994,** pp.155-161
[105] Simòn, B.F., de, Pèrez-Ilzarbe, J., Hernàndez, T., Gòmez-Cordovèz, C., Estrella, I., *J. Agric. Food Chem.***1992,** *42,* pp. 1531-1535

3.6 Flavans and Proanthocyanidins

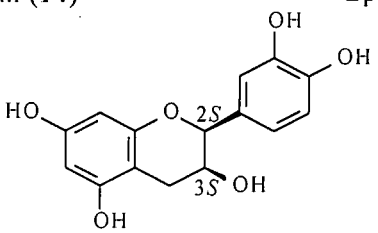
The structure of flavans is based on 2-phenylchroman with hydroxylation primarily at the 5-,7-,3' and 4' positions. Flavan 3-ols normally exist with a configurations of 2*R*,3*S*. Flavan-3-ols with configurations of 2*R*, 3*R* are prefixed with 'epi' and those with a 2*S* configuration distinguished by the enantio (*ent*) prefix.



Catechin **(14)**

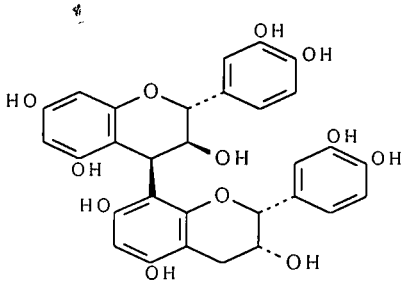


Epicatechin **(16)**



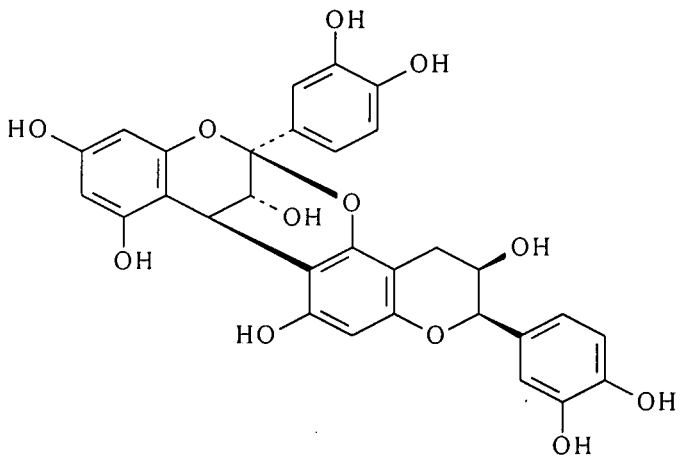
ent-epicatechin **(58)**

These compounds (flavans and flavan 3-ols) serve as building blocks (monomers) for the formation of proanthocyanidins. They are linked directly to each other through the flavonoid backbone or through an oxygen bridge, as is often the case with double interflavonoid bonds. The configuration of the interflavonoid bond is indicated either as α or β, in accordance with IUPAC (1979) nomenclature. The bond and its direction are indicated in parenthesis, and describe the two carbon atoms on the two monomers involved.



Catechin-(4α → 8)- epicatechin **(59)**

Dimers and trimers occur most frequently, but tetramers[106] and pentamers[107] have been isolated. The linkage between monomers is diverse, with (β4→6) and (β4→8) bonds being most abundant. Proanthocyanidins with double interflavonoid bonds **(60)** also exist and are common. The nomenclature for flavans and proanthocyanidins was first suggested by Hemmingway *et al.* (1982)[108] and is outlined by Porter (1994).[109] According to this system proanthocyanidins are named according to the monomers they consist of e.g., procyanidins consist of catechin monomers and its isomers like epicatechin. These individual proanthocyanidins are also named using a system, by which dimers are described by the letter B, trimers by C etc. and the individual configurations by numbers.



Epicatechin-(2β→ 5, 4β→6)-epicatechin **(60)**

Proanthocyanidins consisting of catechin and epicatechin (procyanidins) have been isolated from apples and apple products such as ciders. Procyanidins up to trimeric levels of polymerization were isolated from ciders by Lea and Timberlake (1974)[110], thus Procyanidin dimers B1, B2, (Vallés *et. al.* 1994)[111] and B3, B4, B5, have been characterized, together with trimer C1 and some tetramers.[112]

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[106] Hwang, T., Kashiwada, Y., Nonaka, G., Nishioka, I., *Phytochemistry,* **1990,** *29,* pp. 279.
[107] Foo, L.Y., Karchesy, J.J., *Phytochemistry,* **1991,** *30,* pp. 667
[108] Hemmingway, R.W., Foo, L.Y., Porter, L.J., *J. Chem. Soc., Perkin Trans.I,* **1982,** pp. 1209
[109] Porter, L.J., *The Flavanoids: Advances in research Since 1986, (ed. J.B. Harborne)* Chapmann and Hall, London, **1994**, pp. 23-25
[110] Lea, A.G.H., Timberlake, C. F. *J. Sci. Fd Agric.* **1974**, *25*, pp. 1537-1545
[111] Vallés, B.S., Victorero, J.S., Alonso, J.J.M., Gomis, D.B., *J. Agric. Food Chem.* **1994,** *42,* pp. 2732-2736
[112] Pèrez-Ilzarbe, J., Martínez, V., Hernàndez, T., Estrella, I., *J .Liq. Chromatogr.* **1992,** *15,* pp. 637-646

CHAPTER 4

PHYSIOLOGICAL PROPERTIES OF POLYPHENOLS FOUND IN APPLES AND ITS PRODUCTS

4.1 Antioxidants in apples

Flavonoids are the most abundant polyphenols found in apples and apple juices, and play an important physiological role in mammals. Phenolic acids, the other class of polyphenols in apples, posses antibacterial and antifungal properties[113] and some are antioxidants.[114]The effect that free radicals, such as superoxide[115] ions, singlet oxygen and lipid peroxy-radicals have on human health have been well documented.[116,117] These radicals are formed during the normal metabolism of oxygen, but the human body is protected against these species by an enzyme, superoxide dismutase (SOD). This enzyme quenches excess superoxides, but modern life styles that include smoking, alcohol abuse, excess fat intake and exposure to high levels of radiation, increase these free radicals, and make this enzyme alone insufficient. Vitamins (E,C), flavonoids and other polyphenols act as primary antioxidants, having the ability to quench superoxides, hydroxy- and peroxy-radicals.[118]

4.2 Antioxidants

To evaluate the total antioxidant activity (TAA) of a compound, a system was devised by Miller *et al.* (1993)[119] that compare the antioxidant activity of any compound with a water-soluble Vitamin E analogue. This method estimates the relative ability of the

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[113] Martindale: The Extra Pharmacopoeia, Ainley Wade ed. The Pharmaceutical Press, London **1977,** pp.1278- 1279
[114] Miller, N.J., Diplock, A.T., Rice-Evans, C.A., *J. Agric. Food Chem.* **1955,** *43,* pp. 1794-1801
[115] Robak, J., Gryglewski, R.J., *Biochem. Pharmacol.* **1988,** *37,* pp. 837-841
[116] Thomas, M.J., *Crit. Rev. Food Sci. Nutr.* **1990,** *29,* pp. 273
[117] Halliwell, B., Murcia, M.A., Chirico, S., Auroma, O.O., *Crit. Rev. Food Sci. Nutr.* **1995,** *7,* pp. 35
[118] Torel, J., Cillard, J., Cillard, P., *Phytochemistry.* **1986,** *25,* pp. 383
[119] Miller, N.J., Rice-Evans, C.A., Davies, M.J., Gopinathan, V., Milner, A., *Clin. Sci.* **1993,** *84,* pp. 407-4

antioxidant substance to scavenge the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\cdot+}$) in the aqueous phase, as compared to standard amounts of the synthetic antioxidant, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the water-soluble vitamin E analogue. This allows the measurement of antioxidant activity as apposed to concentration, and the antioxidant activity of mixtures. This ability to measure the TAA of mixtures allows us the ability to distinguish between additive and synergistic effects, if the molar concentration of the contributing antioxidant is known. In an experiment by Miller *et al.* (1995), where two apple juices were evaluated, chlorogenic acid was found to be the biggest contributor to the TAA value of the juice that was not supplemented with vitamin C. In the second juice, where vitamin C was added (6% juice fortified with 300mg/L of vitamin C), vitamin C activity was the major contributor to the TAA. The antioxidant with the highest concentration in the unsupplemented juice was found to be chlorogenic acid **(40)**(257μmol/L). The Trolox Equivalent Antioxidant Capacity (TEAC) was also measured for compounds found in apple juice. This measures the millimolar concentration of Trolox solution having the antioxidant capacity equivalent to a 1,0 mM solution of the substance under investigation. By this method, quercetin **(10)** (TEAC = 4.72) proved to be the most powerful antioxidant potentially present in the apple juices. Epicatechin **(45)** (TEAC = 2.50) and phloridzin **(42)** (TEAC = 2.38) was approximately half as potent, but still much more than vitamin C (TEAC = 0.99).

Substance	TEAC
ascorbic acid	0.99
chlorogenic acid	1.24
quercetin	4.72
rutin(quercetin-3-rutinoside)	2.42
phloridzin	2.38
epicatechin	2.50
catechin	2.40
cyanidin	4.42
citric acid	0.00
benzoic acib	0.00

Table 4.1 Trolox Equivalent Antioxidant Capacity (TEAC) values of apple juice constituents.

Chlorogenic acid proved to be a more active antioxidant than ascorbic acid, and the most significant antioxidant present (due to its relatively high concentration) in the apple juice that was not supplemented by ascorbic acid. The relative contribution of each antioxidant, based on its concentration in the juice could now be determined. This is done by multiplying the TEAC value of each substance with its molar concentration in the juice, and expressing it as a percentage of the total TAA. Chlorogenic acid contributed 38.7% of the TAA of the unsupplemented juice, thus, representing the major single antioxidant. Phloridzin and phloretin xyloglucoside contributed 11.7% of the TAA and ascorbic acid only 1.0% of the TAA of the unsupplemented juice. In the supplemented juice, it constituted 94.1% of the TAA.

	% Contribution to the TAA	
Substance	**Type I juice**	**Type II juice**
ascorbic acid	94.1	1.0
chlorogenic acid	1.0	31.9
p-coumarouylquinic acid	0.2	6.8
phloretin glucosides*	0.5	11.2
epicatechin		0.5
remaining activity (unmeasured substances or synergistic interactions)	4.2	48.6

Type I juice supplemented with 300 mg/L ascorbic acid

* Phloridzin + phloretin xyloglucoside

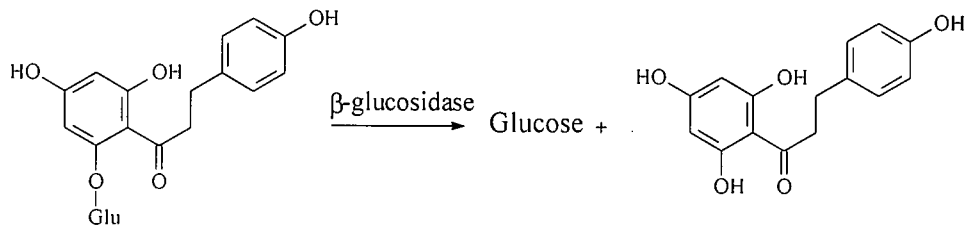
Table 4.2 Relative Contribution of Antioxidant Substances to the TAA of apple Juices (Based on TEAC x Concentration for Each substance as a Percentage of the TAA).

4.3 Physiology of flavonoids occurring in apples

Phloridzin **(42)** has been studied as a promoter of urinary glucose excretion. By preventing renal tubular glucose reabsorbtion and promoting excretion of glucose, by inhibiting the Na^+-glucose cotransporter (SGLT), blood glucose levels can be controlled.[120] Based on this action of phloridzin, it has been used as a blood glucose-lowering reagent to verify the glucose toxicity theory. This procedure entails the

[120] Alvarado, F., Crane, R.K., *Biochim. Biophys. Acta.* **1962,** *56,* pp. 170-172

normalizing of a diabetic condition in animals, by controlling of the blood glucose level of the diabetic animal to a normal level. This is achieved with subcutaneous injections of phloridzin, for a long period, without using insulin.[121] Phloridzin is hydrolyzed to glucose and phloretin **(22)** by β-glucosidase in the intestine[122] **(Fig. 4.1)**. Phloretin inhibits facilitated glucose transporters (GLUT) and is toxic to the kidneys.[123] The ability to inhibit GLUT, and the toxicity, was determined to be linked to the 4'-hydroxy group of the B ring.[124] Because of these findings, 4'-deoxy analogues of phloridzin have been synthesized and tested, with promising results.[125]



Phloridzin **(42)** Phloretin **(22)**

Fig. 4.1 Enzymatic hydrolysis of phloridzin.

The roots of *Malus* contain large quantities of phloridzin, which have a marked retarding effect on the growth of roots, when applied in dilute solutions. Apple trees planted in old orchards show symptoms of disease, known as 'soil sickness.' In studies done by Börner (1959),[126] he found phloretin, phloroglucinol, *p*-hydroxyhydrocinnamic acid, and *p*-hydroxybenzoic acid, as breakdown products (fungus) in the soil, believed to be responsible for the disease symptoms. Phloridzin also acts as a feeding stimulant, and deterrent, for several monophagous and oligophagous insects, especially those of the order Homoptera.[127]Quercetin, **(10)** and its glycosides, influences a profoundly wide range of physiological functions in plants and insects, but even more so in mammalian

[121] Dimitrikoudis D., Vranic, M., Klip, A., *J. Am. Soc. Nephrology.* **1992,** *3,* pp. 1087-1091
[122] Malathi, P., Crane, R.K., *Biochim. Biophys. Acta.* **1969,** *173,* pp. 245-256
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[124] Hase, J., Kobayashi, K., Kobayashi, R., *Chem. Pharm. Bull.* **1973,** *21,* pp. 1076-1079
[125] Tsujihara, K., Mitsuya, H., Saito, K., Inamasu, M., Arakawa, K., Oku, K., Matsumoto, M., *Chem. Pharm. Bull.* **1996,** *44*(6), pp. 1174-1180
[126] Börner, H., *Naturwissenchaften,* **1958,** *45,* pp. 138-139
[127] Klingauf, F., *Z. Ang. Entomol.***1971,** *68,* pp. 41

and human physiology. Quercetin influences enzyme systems, immune systems, smooth muscle, antiviral and lipid per oxidation, and has cancer-related properties.[128] Of interest is the effect quercetin and rutin **(58)** have on lipid peroxidation and oxyradical production. Oxidative degradation of polyunsaturated fatty acids, have been implicated in several pathological conditions, including aging, hepatotoxicity, haemolysis, cancer, arteriosclerosis, tumor promotion, inflammation and metal toxicity.[129,130] Iron ion-dependent lipid peroxidation is inhibited by rutin and quercetin, presumably due to metal chelation.[131] Ascorbic acid-induced non-enzymatic lipid peroxidation is also inhibited. The inhibition of the formation of hydroxy- and lipid-peroxy radicals was suggested.[132] Catechin **(14)** has been reported to inhibit lipid peroxidation in rats, as well as haloalkane-induced hepatotoxicity.[133] Selected flavonoids can exert protective effects against cell damage created by lipid peroxidation. This is due in part to their antioxidative properties. Flavonoids could prove to be promising therapeutic agents for protection against free-radical-mediated cell injury.

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[131] Afanase'ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., Potapovich, A.I., *Biochem. Pharmacol.* **1989,** *38,* pp. 1763
[132] Afanase'ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., Potapovich, A.I., *Biochem. Pharmacol.* **1989,** *38,* pp. 1763
[132] Kappus, H., Koster-Albrecht, D., Remmer, H., *Toxicology.* **1979,** *2,* pp. 321

[133] Kappus, H., Koster-Albrecht, D., Remmer, H., *Toxicology.* **1979,** *2,* pp. 321

DISCUSSION

CHAPTER 5

PHENOLIC COMPOUNDS FROM A SOUTH AFRICAN APPLE CIDER

5.1 Introduction

Considering the potential health benefits associated with compounds found in apples, the analyses of ciders and other juice products of apples have become very important. The aim of this study was to elucidate the structure of phenols, and in particular polyphenols, found in a South African apple cider. The cider under investigation is artificially sweetened after fermentation, using sugar cane by-products. In our investigation both commercially available sweetened, and partially processed unsweetened products, were investigated. No comparison was made between the two, due to a lack of sufficient phenolic material isolated from the sweetened cider.

The identification of cider compounds, by Nuclear Magnetic Resonance (NMR), which requires separation and purification, have hitherto generally been disregarded in favor of faster techniques such as High Performance Liquid Chromatography (HPLC). Unfortunately it has limited the identification to known compounds and restricts characterization of compounds such as proanthocyanidins, where interflavonyl bonds and configurations vary considerably.

5.2 Results and Discussion

After removal of excess gas by stirring, the ethyl-acetate extract of the commercial cider yielded mixtures of phenolic compounds after column chromatography fractionation on Sephadex LH-20 in ethanol. Due to the complexity of the mixtures, these fractions were derivatised by means of acetylation to obtain the pure compounds. Invariably this derivatisation leads to losses, due to the nature of the reactions and subsequent

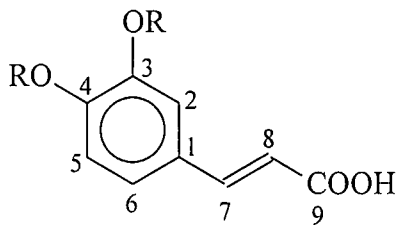
purifications. The amount of material obtained restricted the use of hydrolysis reactions to identify aglycones separated from their glycosides, but the nature of the flavonoid-glycosides which have been isolated, were established unambiguously, mainly by the application of high-resolution NMR.

The fractions obtained from the cider yielded one phenolic acid (caffeic acid), dihydrochalcone glycosides, flavonols (two as glycosides), flavan-3-ols and one dihydroflavanone. Not all the anticipated polyphenols were isolated from the cider, most noticeably chlorogenic acid, *p*-coumarouylquinic acid and flavonoid polymers. Although many interesting compounds were detected by ^{1}H NMR, not all could be unambiguously identified due to a lack of material. The dihydroflavonol 2,3-*trans*-3',4',3,5,7-pentahydroxy flavonol have not been previously isolated from apple cider.

CHAPTER 6

$C_6.C_1$ –TYPE PHENOL

Fraction 8 of the unsweetened cider together with fraction 6 of the sweetened cider yielded the biosynthetic precursor[134] caffeic acid **(61),** and is identified by comparison of the ^1H NMR spectrum of its *O*-acetyl derivative, with authentic spectra in the literature.[135] The ^1H NMR spectrum [plate 1 (Ac-D_6-296K)] of the free phenol displays an aromatic ABX spin system and two doublets, one of which is deshielded. The doublets are assigned to 8-H (δ 7.50, d, 15.0 Hz) and 7-H (δ 6.24, d, 15.0 Hz) of the aliphatic chain. The chemical shift downfield of one of the doublets (8-H) is attributed to the presence of the adjacent carboxylic functionality. The acetate derivative **(62)** displays the same resonances, but the presence of two acetoxy groups is evident [plate 2 (Ac-D_6 – 296K)].



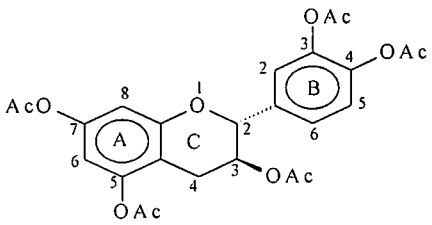
R = H **(61)**
R = -$COCH_3$ **(62)**

[134] See **Fig. 2.9** on page 16.
[135] Kamara, B. I.; *Structure and synthesis of polyphenols from honeybush tea (Cyclopia intermadia) and the potential of flavonoids as active oxygen scavengers.*; Ph.D-thesis; UOVS; **1999**; pp. 39

CHAPTER 7

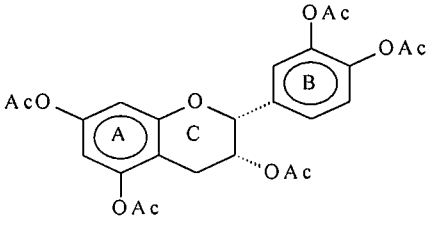
FLAVAN-3-OLS

Fraction 16 of the unsweetened cider yielded the two diastereoisomers catechin **(63)** and epicatechin **(64).** These two compounds were identified by comparison of their ^{1}H NMR spectral data with those of *O*-acetyl derivatives in the literature.[136] The ^{1}H NMR spectrum [plate 3 ($CDCl_3$-296K)] of **(63)** shows an ABX spin-system [δ 7.27, dd, 2.0 Hz, 8.0 Hz, 6'-H(B), δ 7.21, d, 8.0 Hz, 5'-H(B), δ 7.18, d, 2.0 Hz, 2'-H(B)], two *meta*-doublets [δ 6.68, d, 2.0 Hz, 8-H(A), δ 6.61, d, 2.0 Hz, 6-H(A)], characteristic 2-H(C) [δ 5.16, d, 6.0 Hz] and 3-H(C) [δ 5.27, m] resonances and the presence of one $-CH_2-$ [δ 2.88, dd, 5.0 Hz, 17.0 Hz, 4-H(C), δ 2.68, dd, 6.0 Hz, 17.0 Hz, 4-H(C)] resonating as two doublets of doublets. The conformation of the C-ring that makes the two 4-H(C) chemically unequivalent, is evident in the different coupling constants between 3-H(C) and the two $-CH_2-$ protons. The unequivalence of the two $-CH_2-$ protons is a result of the non-planar nature of the C-ring. The ^{1}H NMR spectrum [plate 4 ($CDCl_3$-296K)] of compound **(64)** displays the same resonances, which includes an ABX spin-system [δ 7.29, dd, 2.0 Hz, 8.0 Hz, 6'-H(B), δ 7.22, d, 8.0 Hz, 5'-H(B), δ 7.37, d, 2.0 Hz, 2'-H(B)], two *meta*-doublets [δ 6.69, d, 2.0 Hz, 8-H (B), δ 6.58, d, 2.0 Hz, 6-H (B)] and the C-ring protons [δ 3.10, dd, 5.0 Hz, 18.0 Hz, 4-H(C), δ 2.90, dd, 2.0 Hz, 18.0 Hz, 4-H(C), δ 5.13, s, 2-H(C), δ 5.40, m, 3-H(C)].



Catechin (2*R*, 3*S*)

(63)



Epicatechin (2*R*, 3*R*)

(64)

[136] Kamara, B. I.; *Structure and synthesis of polyphenols from honeybush tea (Cyclopia intermadia) and the potential of flavonoids as active oxygen scavengers.*; Ph.D-thesis; UOVS; **1999**

CHAPTER 8

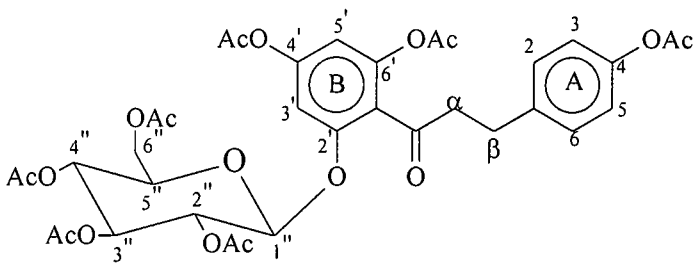
DIHYDROCHALCONES

8.1 Dihydrochalcones

Following acetylation, fraction 11 of the ethyl-acetate extract of the unsweetened cider and fraction 3 of the sweetened cider yielded two dihydrochalcone glycosides[137] **(65)** and **(66)** as acetate derivatives. The ^{1}H NMR spectra display multiplets (δ 3.0 to δ 3.2 ppm) integrating for four protons, indicating the presence of ethylene (-CH_2-) protons, and the absence of C-ring protons, characteristic of dihydrochalcones.

8.2 *O*-Glycosilated dihydrochalcones

The ^{1}H NMR spectrum of compound **(65)** [plate 5 ($CDCl_3$-296K)] displays two aromatic *meta*-doublets, an aromatic AA'BB' spin system, two -CH_2- resonances and resonances typical of a monomeric glycoside. The absence of C-ring protons is a characteristic of chalcones **(23)**, aurones **(29)** and also flavonols **(9)**, but these possibilities are ruled out by a COSY experiment on compound **(65)** [plate 5a 1 ($CDCl_3$-296K)], which clearly shows coupling between one of the ethylene protons (δ 2.94, m, β-H) and the 2,6-H (A) [(δ 7.28, d, 8.0 Hz,)] and the aromatic AA'BB' spin system of the A-ring. This is confirmed by a NOESY experiment [plate 5b 1 ($CDCl_3$-296K)], which shows NOE association between the ethylene β-H and the 2,6-H (A) of the A-ring.

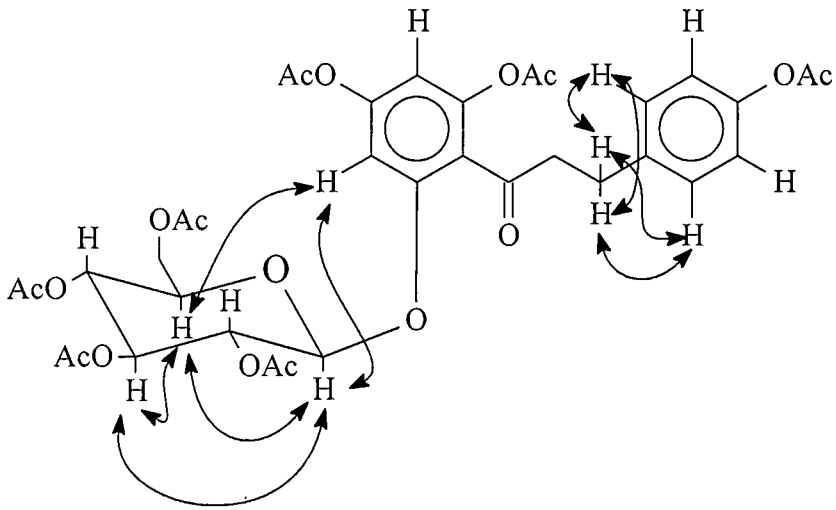


(65)

[137] Johnson, G., Donnelly, B.J., Johnson. D.K., The chemical nature and precursors of clarified apple juice sediment. *J. Food Sci.*, **1968**, *33*, pp. 254-257

The presence of three -CH_2- groups in the DEPT spectrum [plate 5d ($CDCl_3$-296K)], one of which can be attributed to the sugar moiety, confirms the linkage between the A and B-ring to be fully reduced. COSY [plate 5a 2 ($CDCl_3$-296K)] couplings between α-H and β-H are evident, as well as NOE association, which confirms their positions on adjacent carbons.

NOE associations [plate 5b 3 ($CDCl_3$-296K) between 1"-H (δ 5.03, d, 8.0 Hz) and 5"-H (δ 3.90, m) of the glycosyl unit and the 3'-H(B) [δ 6.81, d, 2.0 Hz] of the B-ring is observed. Considering this evidence in conjunction with the fact that no NOE couplings are observed between the 1"-H and 5"-H and 5'-H(B) of the B-ring, the point of attachment, *via* an ether bridge, of the glycosyl unit to the flavonoid backbone is determined to be at C-2 of the B-ring.

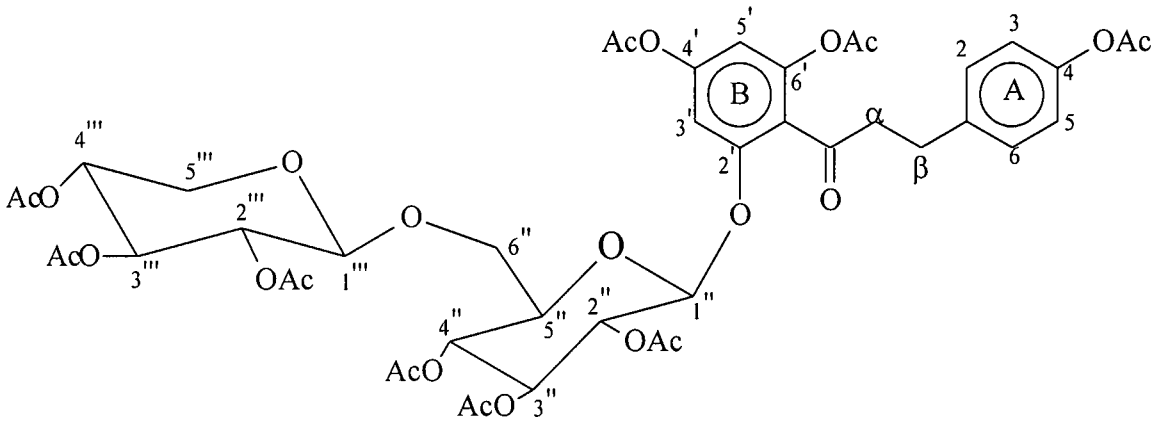


(65 a) Observed NOE associations

The glycosidic protons are allocated to the glycosyl unit according to the strong consecutive couplings established in the COSY spectrum [plate 5a 2 ($CDCl_3$-296K)]: 1"-H (δ 5.03, d, 8.0 Hz); 2"-H (δ 5.27, overlapping 3"'-H); 3"-H (δ 5.27, overlapping 2"'-H); 4"-H (δ 5.14, dd, 9.0 Hz, 9.0 Hz); 5"-H (δ 3.90, m); 6"-H (δ 4.19, dd, 2.0 Hz, 12.0 Hz); 6"-H (δ 4.28, dd, 5.0 Hz, 12.0 Hz) for the glucopyranosyl unit. The β-D-configuration of the glucopyranosyl unit is evident from the strong NOE association [plate 5b 2 ($CDCl_3$-296K)] between the 1"-H (δ 5.03, 8.0 Hz, s) and the di-axial 3"-H and 5"-H of the glucoside and the large coupling constant observed for the anomeric proton.

Seven acetoxy groups (δ 2.0 – 2.32 ppm), two of which are deshielded, are observed in the ^{1}H NMR spectrum. A ^{13}C NMR spectrum of compound **(65)** [plate 22 ($CDCl_3$-296K)] together with a HMQC spectrum [plate 5c ($CDCl_3$-296K)], which allows allocation of some of the carbons in the ^{13}C NMR spectrum, confirms the proposed structure to be the acetate derivative of 2',4',6',4-tetrahydroxydihydrochalcone-2'-*O*-β-D-glucopyranoside **(phloridzin).**

The ^{1}H NMR spectrum of compound **(66)** [plate 6 (Ac-D_6-296K)] displays the same aromatic region as **(65)**. The same -CH_2- resonances (δ 3.10, m, α-H, δ 2.92 m, β-H) are observed together with a more complex glycosyl unit. Nine acetoxy resonances occur in the ^{1}H NMR spectrum [plate 6 2 ($CDCl_3$-296K)]. The AA'BB' spin system [δ 7.33, d, 8.0 Hz, 2,6-H(A), δ7.04, d, 8.0 Hz, 3,5-H(A)] and the two *meta*-doublets [δ 7.02, d, 2.0 Hz, 3'-H(B), δ 6.81, d, 2.0 Hz, 5'-H(B)] are allocated to the A- and B-rings respectively.

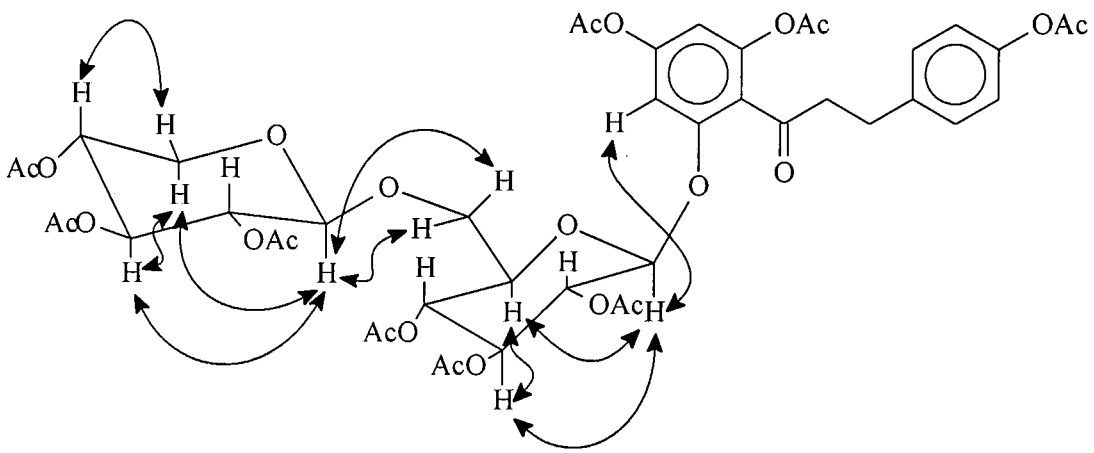


(66)

The point of attachment of the glycosyl unit through oxygen, is indicated by clear NOE association [plate 6b (Ac-D_6-296K)] between the 1"-H (δ 5.48, d, 8.0 Hz) and 5"-H (δ 4.21, m) protons of the glycosyl unit and the 3'-H(B) [δ 7.02, d, 2.0 Hz] of the B-ring. The ^{1}H NMR spectrum of compound **(66)** and strong consecutive couplings established in the COSY spectrum [plate 6a (Ac-D_6-296K)], are used to assign the glycosidic protons to the glucosyl and xylopyranosyl units, respectively. Hence the following allocation of

resonances for the glucosyl unit: 1"-H (δ 5.48, d, 8.0 Hz); 2"-H (δ 5.18, dd, 8.0 Hz, 10.0 Hz); 3"-H (δ 5.38, dd, 10.0 Hz, 9.0 Hz); 4"-H (δ 5.06, dd, 9.0 Hz, 10.0 Hz); 5"-H (δ 4.21, m); 6"-H (δ 3.70, dd, 6.0 Hz, 11.0 Hz); 6"-H (δ 3.95, dd, 11.0 Hz, 2.0 Hz). The β-D-configuration of the anomeric proton is evident from the NOE association [plate 6b (Ac-D_6-296K)] between the 1"-H and the di-axial 3"-H and 5"-H of the glucoside and the large coupling constant of the anomeric proton (J = 8.0 Hz).

The allocation of the xylopyranosyl protons are as follows: 1"'-H (δ 4.66, d, 7.0 Hz); 2"'-H (δ 4.88, dd, 7.0 Hz, 9.0 Hz); 3"'-H (δ 5.16, dd, 9.0 Hz, 9.0 Hz); 4"'-H (δ 4.90, m); 5"'-H (δ 4.08, dd, 5.0 Hz, 11.0 Hz); 5"'-H (δ 3.48, dd, 11.0 Hz, 9.0 Hz). Clear NOE association [plate 6b (Ac-D_6-296K)] between 1"'-H and the di-axial 3"'-H and one 5"'-H confirms the β-D-configuration at the anomeric position of the xylopyranoside. The position of the glycosidic linkage is established by NOE association between the 6"-H of the glucoside and the anomeric 1"'-H of the xylopyranoside. Compound **(66)** is thus identified as the acetate derivative of 2',4',6',4-tetrahydroxy dihydrochalcone-2'-*O*-β-D-(6"-β-D-xylopyranosyl)-glucopyranoside.



(66 a) Observed NOE association

CHAPTER 9

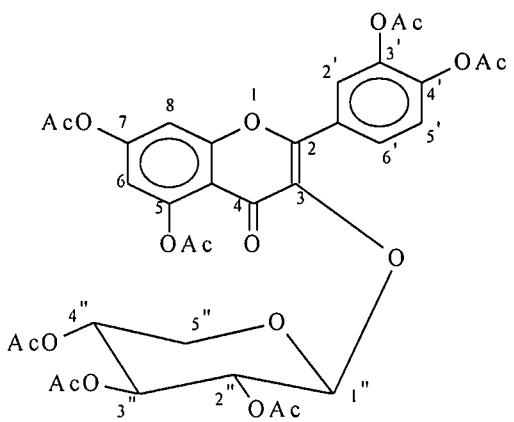
FLAVONOLS

9.1 Introduction

Following acetylation, fractions 16 and 14 of the ethyl acetate extract of the unsweetened cider afforded two flavonol glycosides and one aglycone from fraction 7 of the sweetened cider. All three display the same aromatic resonances that consist of an aromatic ABX spin-system and two *meta*-doublets. No heterocyclic C-ring protons are present in the ^{1}H NMR spectra of compounds **(67), (68)** and **(69),** a characteristic of flavonols possessing an enolic functionality at the C-ring.

9.2 *O*-Glycosylated flavonols

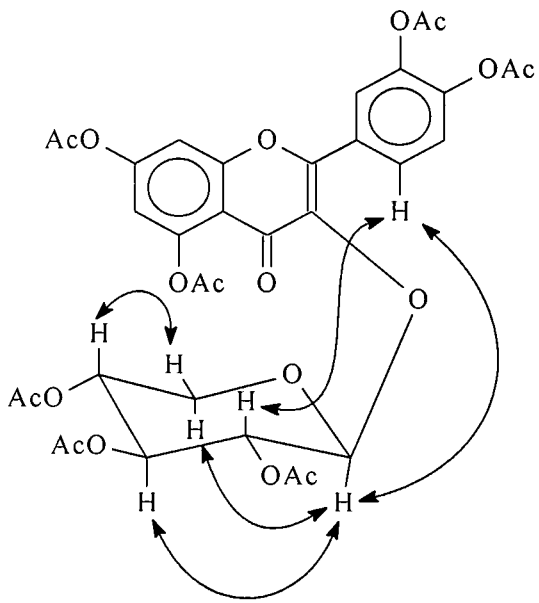
The ^{1}H NMR spectrum of compound **(67)** displays an aromatic ABX spin system, two *meta*-doublets, glycosyl resonances, four aromatic- and three aliphatic acetoxy groups. In the ^{1}H NMR spectrum [plate 7 ($CDCl_3$-296K)] the 3',4'-*O*-di-acetyl substituted B-ring displays an aromatic ABX spin system [δ 7.93, d, 2.0 Hz, 2'-H(B)[overlapping 6'-H(B)], δ 7.95, dd, 2.0 Hz, 9.0 Hz, 6'-H(B) and δ 7.36, d, 9.0 Hz, 5'-H(B)]. The 6'-H(B) and 2'-H(B) is clearly deshielded due to the conjugation with the C-ring. The *meta*-doublets [δ 7.32, d, 2.0 Hz, 6-H(A) and δ 6.85, d, 2.0 Hz, 8-H(A)] are assigned to the 5,7-*O*-di-acetyl substituted A-ring.



(67)

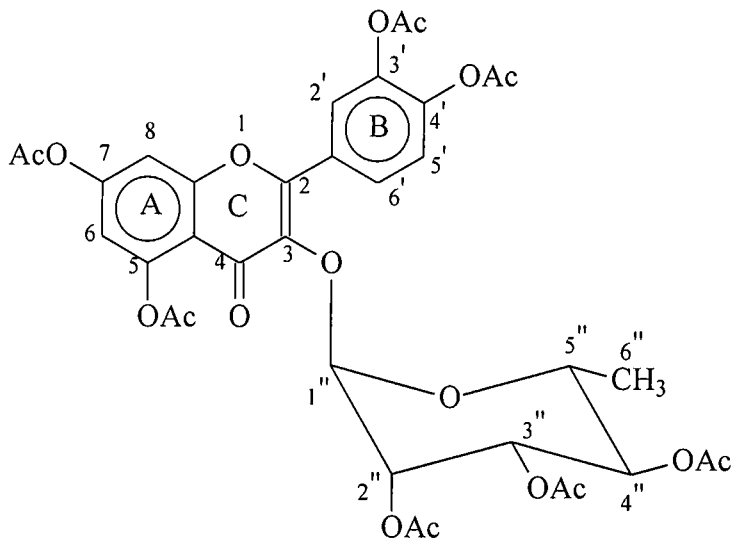
The position of coupling *via* an ether bridge, is determined to be at C-3 of the C-ring, based on NOE coupling [plate 7b 1 ($CDCl_3$-296K)] between 1"-H and 2"-H of the glycosyl unit and the 2'-H(B) and 6'-H(B) of the B-ring. Consecutive couplings displayed by the COSY spectrum [plate 7a ($CDCl_3$-296K)] together with the ^1H NMR spectrum are used to assign the glycosidic protons. They are assigned as follows: 1"-H (δ 5.62, d, 6.0 Hz); 2"-H (δ 5.20, dd, 6.0 Hz, 9.0 Hz); 3"-H (δ 5.26, dd 9.0 Hz, 7.5 Hz); 4"-H (δ 4.94, m); 5"-H (δ 3.25, dd, 12.0 Hz, 8.0 Hz); 5"-H (δ 3.88, dd, 12.0 Hz, 5.0 Hz).

The β- configuration of the sugar at the anomeric position is deduced from NOE coupling [plate 7b 2 ($CDCl_3$-296K)] between 1"-H, 3"-H and 5"-H (δ 3.25, dd, 12.0 Hz, 8.0 Hz). NOE coupling between 2"-H, 4"-H, and 5"-H (δ 3.88, dd, 12.0 Hz, 5.0 Hz) is observed and in conjunction with a clear trans-diaxial arrangement between 2"-H and 3"-H (J = 9.0 Hz) confirms this to be β-D-xylopyranose unit. Four deshielded aromatic- (δ 2.47, δ 2.38, δ 2.36 and δ 2.35) and three aliphatic acetoxy groups (δ 2.02, δ 2.08 and δ 2.14) are observed in the ^1H NMR spectrum. Compound **(67)** is identified as the acetate derivative of 3',4',5,7-tetrahydroxy flavonol-3-β-D-xylopyranosyl.



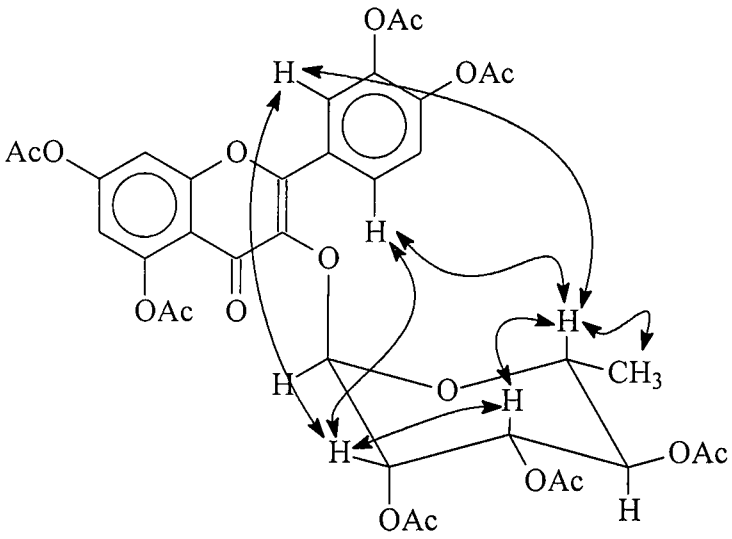
(67 a) Observed NOE association

The ^{1}H NMR spectrum [plate 8 (C_6D_6 – 296K)] of compound **(68)** displays the same aromatic resonances as compound **(67)**, which includes an aromatic ABX system and two *meta*-doublets. The glycosyl resonances in the ^{1}H NMR spectrum are distinctly different from that of compound **(67)** owing to the presence of a $-CH_3-$ resonance (δ 1.28). The 2'-H(B) and 6'-H(B) are deshielded, as with compound **(67),** as a result of the conjugated nature of the C-ring. The ABX system is again attributed to the 3',4'-*O*-di-acetyl substituted B-ring and the *meta*-doublets to the 5,7-*O*-di-acetyl substituted A-ring.



(68)

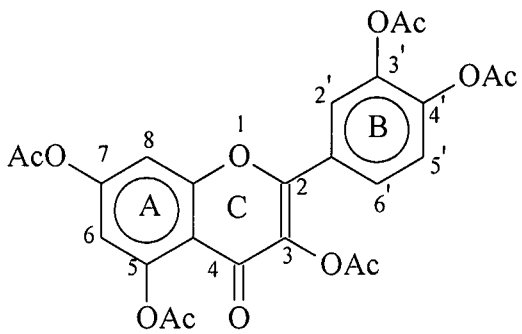
The coupling of the glycosyl unit at C-3 of the C-ring is determined by NOE association [plate 8b 1 (C_6D_6 – 296K)] between 2"-H, 3"-H and 5"-H and the ABX system of the B-ring. The ^{1}H NMR and COSY spectrum [plate 8b 2 (C_6D_6 – 296K)] is used to assign the glycosyl protons as follows: 1"-H (δ 6.35, d, 2.0 Hz); 2"-H (δ 6.32, dd, 2.0 Hz, 4.50 Hz); 3"-H (δ 5.84, dd 4.50 Hz, 10.0 Hz); 4"-H (δ 5.63, dd, 10.0 Hz, 10.0 Hz); 5"-H (δ 3.78, dd, 10.0 Hz, 6.0 Hz); 6"-H ($-CH_3-$) (δ 1.28, d, 6.0 Hz). The α-configuration at the anomeric proton is evident from the absence of NOE association between the anomeric proton, 3"-H, and 5"-H and also the small coupling constant (J = 2.0 Hz) between the anomeric proton and 2"-H. Thus compound **(68)** is identified as 3,3',4',5,7-Pentahydroxyflavone-3-*O*-α-L-rhamnopyranosyl **(quercetrin).**



(70 a) Observed NOE association

9.3 Flavonols

The ^{1}H NMR spectrum of compound **(69)** [plate 9 ($CDCl_3$-296K)] displays the same aromatic resonances as **(67)** and **(68),** but contains no glycosyl resonances. The 2"-H and 6"-H are clearly deshielded, as with **(67)** and **(68),** because of the conjugated C-ring. The same 3',4'-*O*-di-acetyl and 5,7-*O*-di-acetyl substitution pattern is observed in the ^{1}H NMR spectrum, resulting in an ABX spin system [δ 7.74, dd, 2.0 Hz, 8.5 Hz, 6'-H(B), δ 7.70, d, 2.0 Hz, 2'-H(B), δ 7.37, d, 8.5 Hz, 5'-H(B)] and two *meta*-doublets [δ 7.35, d, 2.0 Hz, 6-H(A), δ 6.90, d, 2.0 Hz, 8-H(A)] Compound **(69)** is identified as the acetate derivative of 3',4',3,5,7-pentahydroxy flavonol (**quercetin**).



(69)

Chapter 10

DIHYDROFLAVONOLS

(3-HYDROXYFLAVANONES)

10.1 Introduction

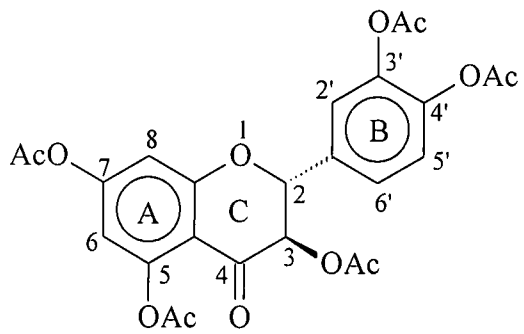
Compound **(70)** **(taxifolin)** was isolated from fraction 7 of the sweetened cider. This compound, a dihydroflavonol, is closely related to flavanones, but is hydroxylated at C-3 of the C-ring, making this position chiral. The ^{1}H NMR spectrum of compound **(70)** clearly shows the presence of only two C-ring protons, distinguishing it from a flavanone. The presence of two chiral carbons (C-2 and C-3) makes four diastereoisomers possible, but the (2*R*, 3*R*) stereochemistry is much more frequently encountered.

10.2 Dihydroflavonols

The ^{1}H NMR spectrum [plate 10 (Ac-D_6-296K)] of compound **(70)** displays an aromatic ABX spin system with 2'-H(B) and 6'-H(B) deshielded, and two *meta*-doublets. The deshielding is not as articulated as with compounds **(67)**, **(68)** and **(69)**, because of the unconjugated nature of the C-ring. The ABX spin system is attributed to the 3',4'-*O*-di-acetyl substitution pattern of the B-ring. Hence the following allocation of the B-ring protons: [δ 7.61, dd, 2.0 Hz, 8.0 Hz, 6'-H(B), δ 7.52, d, 2.0 Hz, 2'-H(B), δ 7.37, d, 8.0 Hz, 5'-H(B)]. The two *meta*-doublets are attributed to the 5,7-*O*-di-acetyl substitution pattern of the A-ring and are allocated as follows: [δ 6.88, d, 2.0 Hz, 6-H(A), δ 6.72, d 2.0 Hz, 8-H(A)]. Five acetoxy groups are present in the ^{1}H NMR spectrum.

The large coupling constant (10.0 Hz) between 2-H and 3-H gives a good indication of the dihedral angle and subsequently the stereochemistry of these two positions. In a (2*S*, 3*R*) or (2*R*, 3*S*) configuration the large coupling constant can be explained only if the conformation between 2-H and 3-H is fully eclipsed and the C-ring completely flat. Unfavorable steric interaction between the synperiplanar B-ring and acetoxy group, in

conjugation with the expected ring-strain associated with this conformation, makes this conformation highly unlikely. The (2*R*, 3*R*) and (2*S*, 3*S*) configuration with the 2-H and 3-H in an antiperiplanar conformation, would explain the large coupling constant. This puts the B-ring and acetoxy group in a synclinical orientation and relieves the ring-strain. The CD-spectrum of compound **(70)** [plate 19] displays the same Cotton effects associated with 2,3-*trans*-dihydroflavonols and confirms an (2*R*, 3*R*) absolute configuration for compound **(70).**[138] Compound **(70)** is thus identified as the *O*-acetyl derivative of 2,3-*trans*-3',4',3,5,7-pentahydroxy flavonol.



(70)

[138] Fourie, T.G., du Preez, I.C., Roux, D.G., *Phytochemistry*, **1972,** *11*, pp. 1763-1770

Chapter 11

SYNTHESIS OF DIHYDROCHALCONE-GLYCOSIDES

11.1 Introduction.

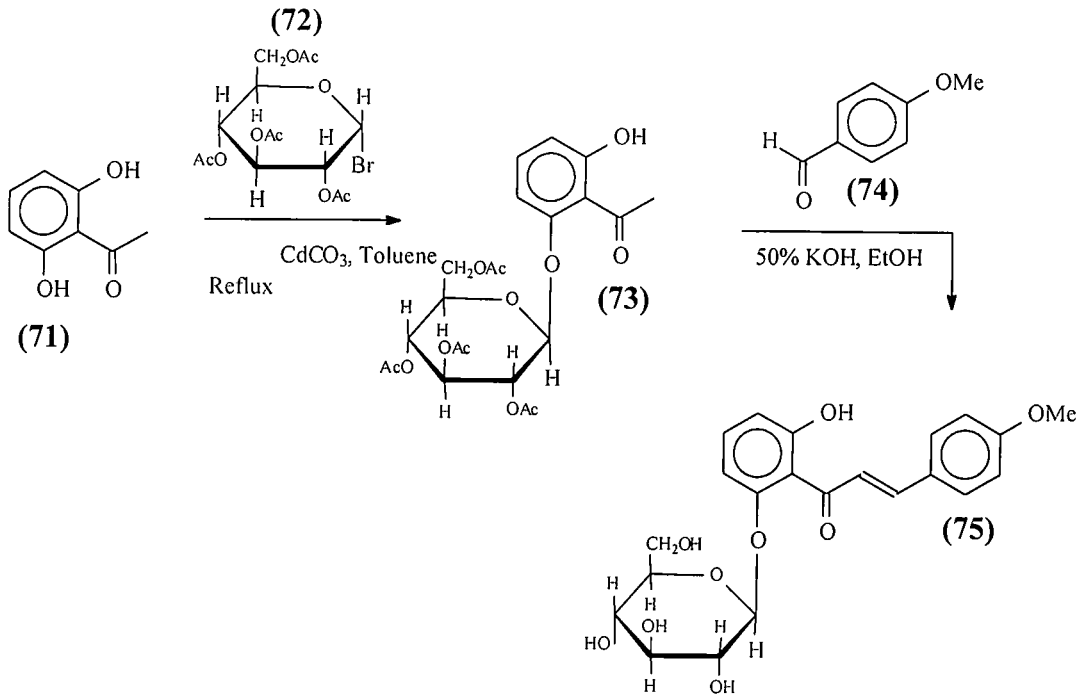
Synthesis of phloridzin **(65),** which could serve as a model reaction for synthesis of dihydrochalcones with more complex glycosides, was attempted by two routes. In the first procedure, the glycoside was attached to an appropriate acetophenone and used in a base-catalyzed aldol condensation with a benzaldehyde, with successive reduction of the chalcone to a dihydrochalcone. In the second procedure, the chalcone was synthesized first, using the same aldol-type reaction, followed by attachment of the glycoside. Although the synthesis of chalcones by an aldol condensation is a common high-yielding procedure, difficulty with the condensation, due to the attachment of the glycosyl unit to the acetophenone was anticipated and encountered.

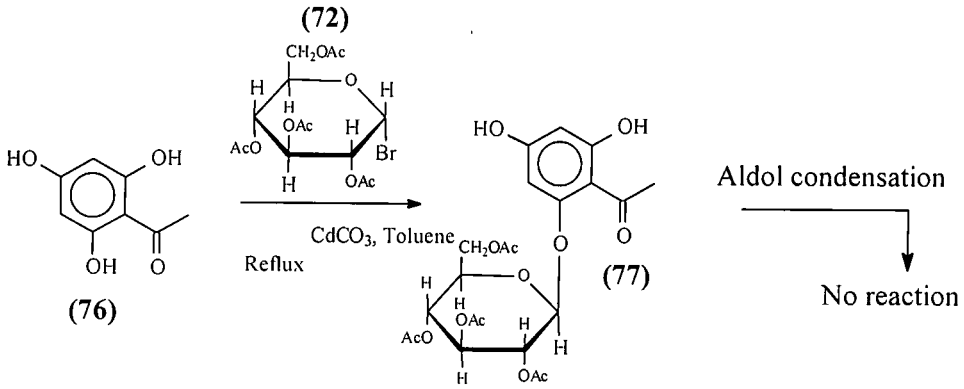
11.2 Chalcone synthesis *via* glycosylated acetophenone.

The procedure outlined by Dick[139] and previously employed by Tsujihara,[140] was used to synthesize 2',4'-dihydroxy-4-methoxydihydrochalcone, as a model reaction. Thus, **(71)** and **(72)** (synthesized from glucose)[141] (2 eq mol) were reacted with $CdCO_3$ (4 eq mol) as a base, in refluxing toluene with removal of generated water to afford the desired ether **(73)** in 65% yield. According to Winget's procedure, **(73)** was condensed with the aldehyde **(74)** (1.2 eq mol) in a mixture of 50% aqueous KOH solution and ethanol at room temperature to provide the chalcone **(75)** as yellow needles (70% yield). The same procedure was employed using 2,4,6-trihydroxy acetophenone **(76)** for the synthesis of phloridzin. Thus, **(72)** and **(76)** (2 eq mol) were reacted with $CdCO_3$ (4 eq mol) as a base

[139] Dick, W.E Jr., *Carbohydr. Res.* **1979,** *70,* pp. 313-318
[140] Tsujihara, K., Mitsuya, H., Saito, K., Inamasu, M., Arakawa, K., Oku, K., Matsumoto, M., *Chem. Pharm. Bull.* **1996,** *44*(6), pp. 1174-1180
[141] Redemann, C.E., Niemann, C., *Organic Synthesis,* vol. 3, pp. 11-14

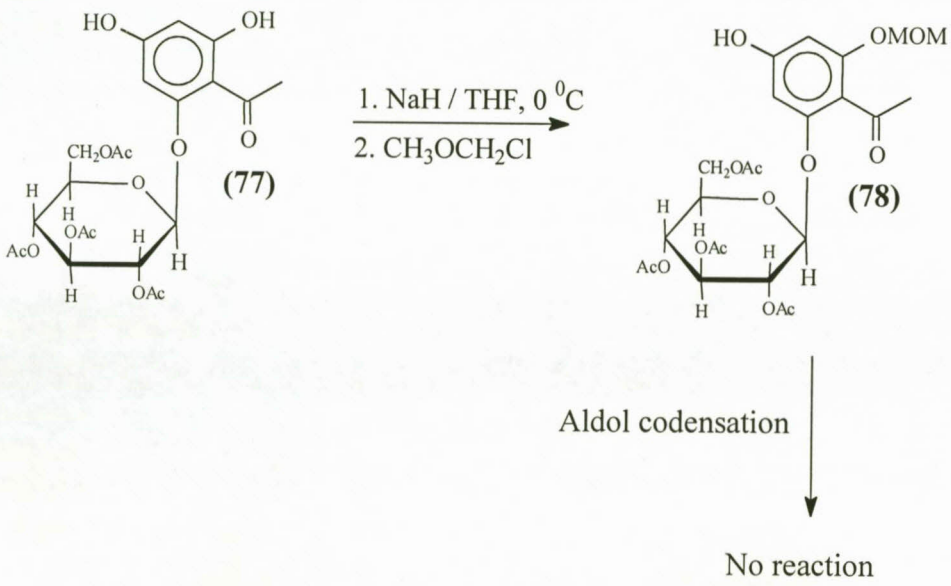
in refluxing toluene with removal of generated water to afford the desired acetophenone (77) (65% yield). The latter was condensed with the aldehyde (74) (1.2 eq mol) in a mixture of 50% aqueous KOH solution and ethanol at room temperature, but the reaction mixture formed two immiscible layers and no reaction took place.





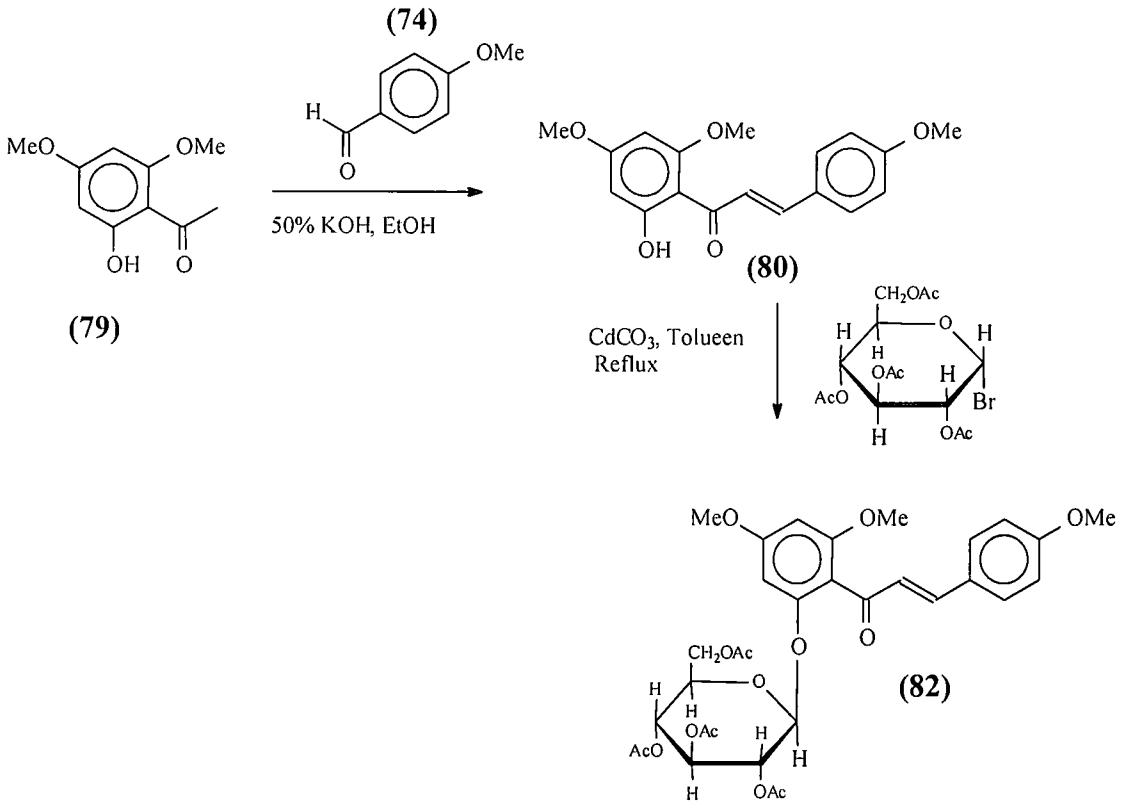
Synthesis of chalcones *via* aldol condensation with unprotected reactants is known to be problematic due to the lack of solubility of the free-phenols in the aqueous reaction conditions. The presence of the glycosyl moiety in the case of the initial model reaction increases solubility, but seems inadequate in the case of the trihydroxyacetophenone. Thus, protection of acetophenone (77), by dissolving the ketone in a mixture of dry THF

and NaH (4 eq mol) and adding chloromethyl methyl ether (2.4 eq mol), yielded the partially protected compound **(78).** Methoxymethyl-protected hydroxy groups are commonly deprotected by catalytic hydrogenation, thus providing an ideal protective group in this protocol where reduction of the double bond is required in the last step of the synthesis. Although methylation as protection is an option, deprotection was considered problematic, considering the stable nature of methoxy groups and resultant hard deprotection protocol, which would probably lead to the loss of the glycosyl unit. Compound **(78)** was subjected to the same protocol as **(73)** and **(76),** but no observable reaction took place.



11.3 Synthesis of chalcone-glycosides by direct glycosyl attachment

Compound **(80)** (2'-hydroxy-4,4',6'-trimethoxychalcone) was synthesized by an aldol condensation between 6-hydroxy-2,4-dimethoxyacetophenone **(79)** and 4-methoxy-benzaldehyde **(74).** Compound **(80)** was subjected to the same procedure as **(76),** yielding the chalcone glucoside **(82).**



Synthesis of chalcones, and subsequently dihydrochalcones, using the two protocols described above, is a viable option but protection of starting materials, solubility and hydroxylation patterns do however restrict the general application of these methods. Modification of reaction conditions, phase-transfer catalysts and other glycosylation methods need to be explored further to make this approach universally applicable.

EXPERIMENTAL

CHAPTER 12

STANDARD EXPERIMENTAL TECHNIQUES

Unless specified to the contrary, the following experimental techniques were used during this project.

12.1 Chromatographic techniques

12.1.1 Paper Chromatography

Two-dimensional paper chromatography was performed using Whatman nr. 1 paper (28.5 x 46 cm). The two eluent systems used were water-saturated butan-2-ol in the first direction and aqueous acetic acid (2%,v/v) in the second direction. The chromatograms were air-dried and developed with benzidine.

12.1.2 Column Chromatography (CC)

Sephadex LH-20 was used as the stationary phase in CC separations with pure ethanol as the eluent. The column dimensions were as follows: 12 cm x 165 cm. An ISCO automatic fraction collector was used to collect the various fractions of approximately 15 ml at 1ml/min.

12.1.3 Thin-layer Chromatography

Qualitative thin layer chromatography (TLC) was performed using Merck pre-coated aluminum sheets (silica gel $PF_{254,}$ 0.25 mm). The plates were developed using an H_2SO_4-HCHO mixture (40:1,v/v) and subsequent heating with an open flame. **Preparative scale thin-layer chromatography** (PLC) was performed using glass plates (20x20 cm) pre-coated with Kieselgel PF_{254} (1 mm). The plates (loaded with 10-15 mg material per plate) were developed in an appropriate eluent, and dried in a stream of air. A UV lamp (254 nm) was used to distinguish between the different bands, which were subsequently scraped off the plates.

The various compounds were eluted from the stationary phase (Kieselgel) with acetone. The latter was removed by a rotary evaporator under reduced pressure at *ca.* 40^0C. Pre-coated (0.25 mm) Merck PLC plates (silica gel 60 PF_{254}) was used to separate material less than 3 mg.

The following abbreviations were used to describe the solvent systems used in the separations using TLC and PLC.

A= Acetone
B= Benzene
C= Chloroform
H= Hexane
M= Methanol

12.2 Spraying reagents

12.2.1 Formaldehyde-sulphuric acid[142]

All TLC plates were sprayed using a 2% (v/v) solution of formaldehyde (40%) in concentrated sulphuric acid. The plates were heated for maximum coloration.

12.2.2 Benzidine spraying reagent[143]

All paper chromatograms were sprayed using benzidine reagent, which was prepared by mixing the benzidine solution (benzidine (5 g) in HCl (14 ml) made up to 1 liter with H_2O) with $NaNO_2$ (10 % w/v) in a ratio of 3:2.

[142] Saayman, H.M., Roux, D.G., *Biochem J.*, **1965**, *96*, pp. 36
[143] Linstedt, G., *Acta. Chem. Scand.*, **1960**, *4*, pp. 65

12.3 Chemical methods

12.3.1 Acetylation[144]

The phenolic material was dissolved in the minimum volume of pyridine. Acetic anhydride (2x the volume of pyridine) was added. The reaction was left for 12-18 hours at 30-40 ^{0} C followed by addition of crushed ice and subsequent precipitation of the acetylated material. After the ice melted, the material was collected through vacuum filtration and washed with cold water.

12.4 Spectroscopic methods

12.4.1 Nuclear Magnetic Resonance Spectrometry (NMR)

NMR-spectrometry was performed on a 300MHz Bruker DRX 300 spectrometer at 296K (23°C) with tetramethylsilane as the internal standard. The solvents used were deuteriochloroform ($CDCl_3$), deuterioacetone [$(CD_3)_2CO$] or deuteriobenzene (C_6D_6) as indicated. Chemical shifts are reported in parts per million (ppm) on the δ-scale and coupling constants are given in Hz. The following abbreviations are used:

s	singlet
d	doublet
dd	doublet of doublets
m	multiplet
br	broadened
t	triplet

[144] Kametani, T., Kano, S., *J. Pharm. Soc. Japan.* **1962,** *82*, pp. 1059

12.4.2 Circular dichroïsm (CD)

CD spectra were recorded on a Jasco J-710 spectropolarimeter with methanol as solvent.

12.5 Freeze-drying

Phenolic material were freeze-dried using a Virtis Freezemobil 12SL at 40 millitorr.

CHAPTER 13

ISOLATION OF PHENOLIC COMPOUNDS FROM APPLE CIDER

13.1 Extraction of the cider.

Excess gas (CO_2) was removed from the sweetened and unsweetened cider, by stirring the ciders (700 ml) in an Erlenmeyer flask (1L), for 6 h. Each batch was divided into three portions and extracted with ethylacetate (3x). After drying ($MgSO_4$) the ethylacetate was evaporated on a rotavapor and all fractions combined, yielding a dark-brown oil from the sweetened cider ($\mathbf{O_s}$) (7.6 g from 6.0 L extracted) and the unsweetened cider (**O**) (22.1 g from 32.64 L extracted).

13.2 Separation of phenolic material from the unsweetened cider

The phenolic material from the unsweetened cider (22 g) was separated on a Sephadex LH-20/EtOH column (5 x 160 cm) with a flow rate of 1.5 ml/min, collecting 32 min fractions. Following TLC on the collected volumes, the following combinations were made:

Tubes:	Fraction:	Yield:
51-59	O1	0.290 g
62-70	O2	3.385 g
74-82	O3	2.087 g
84-90	O4	3.365 g
94-108	O5	0.080 g
112-116	O6	0.075 g
118-122	O7	0.158 g
126-134	O8	0.125 g

136-140	O9	0.036 g
142-144	O10	0.038 g
146-164	O11	0.070 g
166-172	O12	0.055 g
176-186	O13	0.194 g
188-208	O14	0.040 g
210-212	O15	0.015 g
252-260	O16	0.220 g
260-290	O17	0.180 g
292-350	O18	0.160 g

13.3 Isolation of compounds from fraction O8

Fraction O8 yielded compound **(61)** as a *white crystalline* solid (125 mg). Acetylation of fraction O8, followed by PLC purification (B:A 9:1 v/v) yielded compound **(62)** as a white crystalline solid. (12.3 mg R_f 0.47)

^{1}H NMR plate 1 **(61)**

^{1}H NMR plate 2 **(62)**

Table 1

13.4 Isolation of compounds from fraction O11

Acetylation of fraction O11 (200 mg), followed by PLC purification [B:A:M 7:2:1 (v/v)], gave two bands, O11.1 (5.0 mg, R_f 0.77), O11.2 (6.1 mg, R_f 0.49).

13.4.1 2',4',6',4-tetrahydroxydihydrochalcone-2'-O-β-D-glucopyranosyl (65)[145]

Fraction O11.1 yielded compound **(65)**, without further purification, as a *pale-yellow oil* (5.0 mg, R_f 0.77).

[145] Tomàs-Barberàn, F.A., Garcìa-Viguera, C., Nieto, J.L., Ferreres, F., Tomàs-Lorente,F., *Food Chemistry*, **1993**, *46*, pp. 33-36

^{1}H NMR	plate 5
COSY	plate 5a
NOESY	plate 5b
HMQC	plate 5c
DEPT	plate 5d
Table 3	

13.4.2 2',4',6',4-tetrahydroxy dihydrochalcone-2'-*O*-β-D-(6''-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (66)[146]

Fraction O11.2 yielded compound **(66)**, without further purification, as a *white amorphous solid* (6.1 mg, R_f 0.49).

^{1}H NMR	plate 6
COSY	plate 6a
NOESY	plate 6b
Table 3	

13.5 Isolation of compounds from fraction O14

Acetylation of fraction O14 (20 mg), followed by PLC purification [B:A 9:1 x 3 (v/v)], gave one band, O14.1 (12.2 mg, R_f 0.58).

13.5.1 3,3',4',5,7-Pentahydroxyflavone-3-*O*-α-L-rhamnopyranosyl (68)[147]

Fraction O14.1 yielded compound **(68)**, without further purification, as a *pale-brown amorphous* solid (12.2 mg, R_f 0.58).

^{1}H NMR	plate 8

[146] Tomàs-Barberàn, F.A., Garcìa-Viguera, C., Nieto, J.L., Ferreres, F., Tomàs-Lorente,F., *Food Chemistry*, **1993,** *46*, pp. 33-36

[147] Vallés, B.S., Victorero, J.S., Alonso, J.J.M., Gomis, D.B., *J. Agric. Food Chem.* **1994,** *42,* pp. 2732-2736

NOESY plate 8b

Table 4

13.6 Isolation of compounds from O16

Acetylation of fraction O16 (50 mg), followed by PLC purification [B:A 9:1 x 3 (v/v)], gave three bands, O16.1 (14.8 mg, R_f 0.61), O16.2 (16.3 mg, R_f 0.59) and O16.3 (6.2 mg, R_f 0.47).

13.6.1 (2*R*, 3*S*) 3',4',5,7-Tetrahydroxyflavan-3-ol (63)[148]

PLC purification of fraction O16.1 [B:A 98:2 x 3 (v/v)], yielded compound **(63)** as a *pale-brown amorphous* solid (9.0 mg, R_f 0.75).

^{1}H NMR plate 3

Table 2

13.6.2 (2*R*, 3*R*) 3',4',5,7-Tetrahydroxyflavan-3-ol (64)[149]

PLC purification of fraction O16.2 [B:A 98:2 x 3 (v/v)], yielded compound **(64)** as a *pale-brown amorphous* solid (15 mg, R_f 0.65).

^{1}H NMR plate 4

Table 2

13.6.3 3,3',4',5,7-Tetrahydroxyflavonol-3-*O*-β-D-xylopyranosyl (67)[150]

PLC purification of fraction O16.3 [B:A 98:2 x 3 (v/v)], yielded compound **(67)** as a *white amorphous* solid. (5.1 mg, R_f 0.51)

[148] Lea, A.G.H., Timberlake, C.F., *J. Sci. Fd Agric.* **1974**, *25*, pp. 1537
[149] Lea, A.G.H., Timberlake, C.F., *J. Sci. Fd Agric.* **1974**, *25*, pp. 1537
[150] Simòn, B.F., de, Pèrez-Ilzarbe, J., Hernàndez, T., Gòmez-Cordovèz, C., Estrella, I., *J. Agric. Food Chem.* **1992**, *42*, pp. 1533

^{1}H NMR	plate 7
COSY	plate 7a
NOESY	plate 7b
Table 4	

Fractions O1-7, O9-10, O12-13, O15, O17-18 did not contain compounds of interest pertaining to this investigation.

13.7 Separation of fraction O_s

The phenolic material from the sweetened cider (7.6 g) was separated on Sephadex LH-20/EtOH (5 x 160 cm) with a flow rate of 1.5 ml/min, collecting 32 min fractions. Following TLC on the collected volumes, the following combinations were made:

Tubes:	Fraction:	Yield:
75-82	O_s1	0.356 g
84-88	O_s2	0.153 g
90-96	O_s3	0.195 g
98-100	O_s4	0.015 g
102-104	O_s5	0.030 g
106-110	O_s6	0.055 g
112-116	O_s7	0.120 g

13.8 Isolation of compounds from O_s3

Acetylation of fraction O_s3 (110 mg), followed by PLC purification [B:A 8:2 (v/v)], gave one band, $O_s3.1$ (11.9 mg, R_f 0.72).

13.8.1 2',4',6',4-Tetrahydroxydihydrochalcone-2'-O-β-D-glucopyranosyl (65)

Fraction $O_s3.1$ yielded compound **(65)**, without further purification as a *pale-yellow oil.* (11.9 mg, R_f 0.72)

^{1}H NMR	plate 5
COSY	plate 5a
NOESY	plate 5b
DEPT	plate 5c
Table 3	

13.9 Isolation of compounds from O_s6

Acetylation of fraction O_s6 (35 mg), followed by PLC purification [B:A 9:1 (v/v)], gave two bands, $O_s6.2$ (7.2 mg, R_f 0.26) and $O_s6.3$ (5.5 mg, R_f 0.22).

13.9.1 3',4',5,7-Tetrahydroxyflavone-3-*O*-rhamnoside (68)[151]

Fraction $O_s6.2$ yielded compound **(68)**, without further purification, as a *white amorphous* solid (7.2 mg, R_f 0.26).

^{1}H NMR	plate 8
NOESY	plate 8b
Table 4	

13.9.2 3-(3,4-Diacetoxyphenyl)-2-propenoic acid (62)[152]

Fraction $O_s6.3$ yielded compound **(62)** (5.5 mg, R_f 0.22), without further purification, as a *white crystalline* solid.

^{1}H NMR	plate 2
Table 1	

[151] Simòn, B.F., de, Pèrez-Ilzarbe, J., Hernàndez, T., Gòmez-Cordovèz, C., Estrella, I., *J. Agric. Food Chem.* **1992,** *42,* pp. 1533

[152] Simòn, B.F., de, Pèrez-Ilzarbe, J., Hernàndez, T., Gòmez-Cordovèz, C., Estrella, I., *J. Agric. Food Chem.* **1992,** *42,* pp. 1532

13.10 Isolation of compounds from O_s7

Acetylation of fraction O_s7 (70 mg), followed by PLC purification [B:A 8:2 (v/v)], gave two bands, $O_s7.1$ (10.8 mg, R_f 0.61) and $O_s7.2$ (2.2 mg, R_f 0.52).

13.10.1 3,3',4',5,7-Pentahydroxyflavonol (69)[153]

Fraction $O_s7.2$ yielded compound **(69)** (10.8 mg, R_f 0.61), without further purification, as a *white amorphous* solid.

^{1}H NMR plate 9

Table 4

13.10.2 3,3',4',5,7-Pentahydroxyflavanone (70)

Fraction $O_s7.1$ yielded compound **(70)** (10.8 mg, R_f 0.61), without further purification, as *white amorphous* solid.

^{1}H NMR plate 10

Table 5

Fractions O_s1, O_s2, O_s4 and O_s5 did not contain compounds of interest pertaining to this investigation.

[153] Vallés, B.S., Victorero, J.S., Alonso, J.J.M., Gomis, D.B., *J. Agric. Food Chem.* **1994,** *42,* pp. 2732-2736

CHAPTER 14

SYNTHESIS OF CHALCONE-GLYCOSIDES

14.1 2,6-Dihydroxyacetophenone-2-*O*-(2',3',4',6'-tetraacetyl-β-D-glucopyranoside) (73)

A mixture of 2,6-dihydroxyacetophenone **(71)** (5.0 g, 0.033 mol) and $CdCO_3$ (22.7 g, 0.066 mol) in toluene was refluxed (2h) with removal of the generated water, in a Dean-Stark apparatus. Acetobromoglucose **(72)** (27.0 g, 0.066 mol) was added and the mixture was heated at reflux (18 h). The hot mixture was filtered through a Celite plug, and the solid washed with hot $CHCl_3$. The filtrate was triturated (MeOH) to provide a pale-yellow solid, followed by flash CC [B:A (9 : 1)], to afford **(73)** (R_f 0.67) as colorless needles (10.9 g, 70 %). ^{1}H NMR ($CDCl_3$): δ 7.36 (*t,* 8.0 Hz, 4-H), δ 6.72 (*dd,* 8.0 Hz, 1.0 Hz, 3-H), δ 6.49 (*dd,* 8.0 Hz, 1.0 Hz, 5-H), δ 5.35 (*m,* 1'-H, 2'-H, 3'-H overlapping), δ 5.21 (*dd,* 10.0 Hz, 10.0 Hz, 4'-H), δ 4.31 (*dd,* 5.0 Hz, 12.0 Hz, 6'-H), δ 4.16 (*dd,* 2.0 Hz, 12.0 Hz, 6'-H), δ 3.90 (*m,* 5'-H), δ 2.50 (*s,* $-OCH_3$) *δ* 2.05-δ 2.09 (4 x *s,* OAc).

14.2 2,4,6-Trihydroxyacetophenone-2-*O*-(2',3',4',6'-tetraacetyl-β-D-glucopyranoside) (77)

A mixture of 2,4,6-trihydroxyacetophenone (1.2 g, 0.006 mol) and $CdCO_3$ (4 g, 0.012 mol) in toluene was refluxed (2h) with removal of the generated water, in a Dean-Stark apparatus. Acetobromoglucose **(72)** (5.4 g, 0.012 mol) was added and the mixture was heated at reflux (18 h). The hot mixture was filtered through a Celite plug, and the solid washed with hot $CHCl_3$. The filtrate was triturated (MeOH) to provide a yellow solid, followed by flash CC with [B:A (8 : 1)], to afford **(77)** (R_f 0.45) as a white crystalline solid (256 mg, 63 %). ^{1}H NMR ($CDCl_3$): δ 6.08 (*d,* 2.0 Hz, 3-H), δ 6.04 (*d,* 2.0 Hz, 5-H), δ 5.35 (*m,* 1'H, 2'-H, 3'-H overlapping), δ 5.17 (*dd,* 10.0 Hz, 10.0 Hz, 4'-H), δ 4.27 (*dd,* 5.0 Hz, 12.0 Hz, 6'-H), δ 4.17 (*dd,* 2.0 Hz, 12.0 Hz, 6'-H), δ 3.93 (*m,* 5'-H), δ 2.50 (*s,* $-OCH_3$) δ 2.03-δ 2.09 (4 x *s,* OAc).

14.3 4,6-Di-*O*-acetyl-2-hydroxyacetophenone-2-*O*-(2',3',4',6'-tetraacetyl-β-D-glucopyranoside) (83)

Acetylation of **(77)** yielded the per-*O*-acetyl derivative **(83)**: ^{1}H NMR ($CDCl_3$): δ 6.82 (*d,* 2.0 Hz , 3-H), δ 6.71 (*d,* 2.0 Hz, 5-H), δ 5.27 (*m,* 1',2',3'-H overlapping), δ 5.15 (*dd,* 10.0 Hz, 10.0 Hz, 4'-H), δ 5.05 (*d,* 8.0 Hz, 1'-H), δ 4.23 (*dd,* 5.0 Hz, 12.0 Hz, 6'-H), δ 4.19 (*dd,* 2.0 Hz, 12.0 Hz, 6'-H), δ 3.85 (*m,* 5'-H), δ 2.45 (*s,* -OCH_3) δ 2.05-δ 2.09 (*6* x *s,* OAc).

14.4 2,4-Dihydroxy-6-methoxymethylacetophenone-2-*O*-(2,'3,'4,'6'-tetraacetyl-β-D-glucopyranoside) (78)

Acetophenone **(77)** (0.3g) was dissolved in a mixture of dry THF and NaH (0.1 g) and the mixture stirred (20 min., 0°C). Chloro-dimethylether (0.120 g) was added (drop-wise) and the reaction stirred (2h), followed by filtration of the reaction mixture and the evaporation of the solvent yielding pale-yellow oil. Following TLC purification [B:A (9:1)] the reaction mixture yielded **(78)** (R_f 0.59), as a white amorphous solid (0.131 g, 57 %). ^{1}H NMR ($CDCl_3$): δ 6.36 (*d,* 2.0 Hz , 3-H), δ 6.17 (*d,* 2.0 Hz, 5-H), δ 5.30 (*m,* 1',2',3',4'-H overlapping), δ 5.20 (*s,*-OCH_2-), δ 4.29 (*dd,* 5.0 Hz, 12.0 Hz, 6'-H), δ 4.20 (*dd,* 2.0 Hz, 12.0 Hz, 6'-H), δ 3.93 (*m,* 5'-H), δ 3.49 (*s,* -OCH_3), δ 2.56 (*s,* -CH_3) δ 2.04-δ 2.11 (4 x *s,* OAc).

14.5 General procedure for the preparation of chalcones

50% (m/v) *aq.* KOH (2.5 ml) was mixed with a solution of the appropriate acetophenone (0.5 g) in EtOH (10 ml), stirred at room temperature for 30 min and an excess of 2-hydroxy-4,6-methylenedioxydenzaldehyde **(74)** (0.5 g) in EtOH (5ml) added drop wise. After depletion of the acetophenone (18-24 h), H_2O (10 ml) was added, the mixture acidified with 10 % (v/v) H_2SO_4 and extracted with EtOAc (4 x 20 ml). Drying of the extract (Na_2SO_4) followed by evaporation of the solvent and flash CC afforded the pure chalcone.

14.6 2,6-Dihydroxy-4-methoxychalcone-2-*O*-β-D-glucopyranoside (75)

Flash CC of the reaction product with [B:A (9:1)] gave the chalcone **(75)** as a yellow amorphous solid (0.295 g, 71 %). The product **(75)** was subsequently acetylated to yield **(75a)**: (identified as per-*O*-acetyl derivative) ^{1}H NMR ($CDCl_3$): δ 7.50 (*d,* 9.0 Hz, 2,6-H), δ 7.39 (*t,* 6.0 Hz, 4'-H), δ 7.21 (*d,* 10.0 Hz, α-H), δ 7.10 (*dd,* 1.0 Hz, 6.0 Hz, 3'-H), δ 6.95 (*dd,* 1.0 Hz, 6.0 Hz, 5'-H), δ 6.90 (*d,* 9.0 Hz, 3,5-H), δ 6.75 (*d,* 10.0 Hz, β-H), δ 5.15 (*m,* 1''-H, 2''-H, 3''-H, 4''-H overlapping), δ 4.30 (*dd,* 5.0 Hz, 12.0 Hz, 6''-H), δ 4.18 (*dd,* 2.0 Hz, 12.0 Hz, 6''-H), δ 3.89 (*m,* 5''-H overlapping -OCH_3), δ 3.87 (*s,* -OCH_3 overlapping 5'-H), 2.0 – 2.2 (*s,* OAc)

14.7 6-Hydroxy-2,4,4'-trimethoxychalcone (80)

Flash CC of the reaction product with [B:A:M (6:3:1)] afforded the chalcone **(80)** as a yellow amorphous solid (0.156 g, 54 %). ^{1}H NMR ($CDCl_3$): δ 7.82 (*s,* α,β-H), δ 7.58 (*d,* 9.0 Hz, 2,6-H), δ 6.95 (*d,* 9.0 Hz, 3,5-H), δ 6.13 (*d,* 2.0 Hz, 3'-H), δ 5.80 (*d,* 2.0 Hz, 5'-H), δ 3.84-3.95 (3 x *s,* -OCH_3)

14.8 2-Hydroxy-4,4',6'-trimethoxychalcone-2-*O*-β-D-glucopyranoside (82)

A mixture of 2,6-dihydroxy-4-methoxychalcone-2-*O*-β-D-glucopyranoside **(75)** (0.1 g, 0.30 mmol) and $CdCO_3$ (0.2 g, 1.162 mmol) in toluene was refluxed (2h) with removal of the generated water, in a Dean-Stark apparatus. Acetobromoglucose **(72)** (0.18 g, 0.44 mmol) was added and the mixture was heated at reflux (18 h). The hot mixture was filtered through a Celite plug and the solid washed with hot $CHCl_3$. The filtrate was triturated (MeOH) to provide a pale-yellow solid, followed by flash CC with [B:A (8 : 1)], to afford **(82)** (R_f 0.71) as a yellow crystalline solid (0.07 g, 35 %) ^{1}H NMR ($CDCl_3$): δ 7.19 (*d,* 15.0 Hz, α-H), δ 7.07 (*d,* 8.0 Hz, 2,6-H), δ 6.85 (*d,* 8.0 Hz, 3,5-H), δ 6.75 (*d,* 15.0 Hz, β-H), δ 6.40 (*d,* 2.0 Hz, 3'-H), δ 6.27 (*d,* 8.0 Hz, 5'-H), δ 5.33, δ 5.05, δ 4.21 (3 x *m,* overlapping sugar protons), δ 4.22 (*m,* 6''-H), δ 4.22 (*m,* 6''-H), δ 3.90 (*m,* 5''-H) δ 3.75 – δ 3.92 (4 x *s,* -OCH_3), δ 1.96 - δ 2.18 (4 x *s,* OAc).

APPENDIX A

Protons	(61) Ac-D_6 - 296K	(62) $CDCl_3$ - 296K
2-H	7.15 (*d*, 2.0 Hz)	7.41(*d*, 2.0 Hz)
5-H	6.85 (*d*, 8.0 Hz)	7.45 (*d*, 8.0 Hz)
6-H	6.98 (*dd*, 2.0Hz, 8.0 Hz)	7.26 (*dd*, 2.0Hz, 8.0 Hz)
7-H	6.24 (*d*, 15.0 Hz)	6.41(*d*, 15.0 Hz)
8-H	7.50 (*d*, 15.0 Hz)	7.73 (*d*, 15.0 Hz)
OAc		2.33

Table 1: ^{1}H NMR peaks of C_6C_3-type phenol and it's *O* -acetyl derivative, **(61)** and **(62)** from apple cider at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	(63) $CDCl_3$ - 296K	(64) $CDCl_3$ - 296K
2-H(B)	7.18 (*d*, 2.0 Hz)	7.37 (*d*, 2.0 Hz)
5-H(B)	7.21 (*d*, 8.0 Hz)	7.22 (*d*, 8.0 Hz)
6-H(B)	7.27 (*dd*, 2.0Hz, 8.0 Hz)	7.29 (*dd*, 2.0Hz, 8.0 Hz)
6-H(A)	6.61 (*d*, 15.0 Hz)	6.58 (*d*, 15.0 Hz)
8-H(A)	6.68 (*d*, 15.0 Hz)	6.69 (*d*, 15.0 Hz)
3-H(C)	5.27 (*m*)	5.40 (*m*)
2-H(C)	5.16 (*d*, 6.0 Hz)	5.13 (*s*)
4-H(C)	2.68 (*dd*, 6.0Hz, 18.0 Hz)	2.90 (*dd*, 2.0Hz, 18.0 Hz)
4-H(C)	2.88 (*dd*, 5.0Hz, 18.0 Hz)	3.00 (*dd*, 5.0Hz, 18.0 Hz)
OAc	2.02 - 2.31	1.94 - 2.33

Table 2: ^{1}H NMR peaks of flavan-3-ols derivatives, **(63)** and **(64)** from apple cider at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	(65) $CDCl_3$ - 296K	(66) Ac-D_6 - 296K
3,5-H(A)	7.01 (*d*, 8.0 Hz)	7.03 (*d*, 8.0 Hz)
2,6-H(A)	7.27 (*d*, 8.0 Hz)	7.33 (*d*, 8.0 Hz)
3-H(B)	6.81 (*d*, 2.0 Hz)	7.02 (*d*, 2.0 Hz)
5-H(B)	6.72 (*d*, 2.0 Hz)	6.81 (*d*, 2.0 Hz)
α-H	3.10 (*m*)	3.10 (*m*)
β-H	2.95 (*m*)	2.91 (*m*)
1"-H	5.03 (*d*, 8.0 Hz)	5.48 (*d*, 8.0 Hz)
2"-H	5.27 (*m*, overlapping 3"'-H)	5.19 (*m*, overlapping 3"'-H)
3"-H	5.27 (*m*, overlapping 2"'-H)	5.16 (*m*, overlapping 2"'-H)
4"-H	5.15 (*dd*, 9.0 Hz, 9.0 Hz)	5.05 (*dd*, 9.0 Hz, 9.0 Hz)
5"-H	3.90 (*m*)	4.21 (*m*)
6"-H	4.19 (*dd*, 2.0 Hz, 12.0 Hz)	3.70 (*dd*, 6.0 Hz, 12.0 Hz)
6"-H	4.28 (*dd*, 5.0 Hz, 12.0 Hz)	3.95 (*dd*, 2.0 Hz, 12.0 Hz)
1"'-H		4.66 (*d*, 7.0 Hz)
2"'-H		4.88 (*dd*, 7.0 Hz, 9.0 Hz)
3"'-H		5.16 (*dd*, 9.0 Hz, 9.0 Hz)
4"'-H		4.90 (*m*)
5"'-H		3.48 (*dd*, 9.0 Hz, 11.0 Hz)
5"'-H		4.08 (*dd*, 11.0 Hz, 5.0 Hz)
OAc		2.02 - 2.32

Table 3: ^{1}H NMR peaks of dihydrochalcone-glycoside derivatives, **(65)** and **(66)** from apple cider at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	**(67)** $CDCl_3$ - 296K	**(68)** C_6D_6 - 296K	**(69)** $CDCl_3$ - 296K
2-H(B)	7.93 [*d*, 2.0 Hz, overlapping 6-H(B)]	7.74 (*d*, 2.0 Hz)	7.70 (*d*, 2.0 Hz)
6-H(B)	7.95 (*dd*, 2.0 Hz, 9.0 Hz)	7.55 (*dd*, 2.0 Hz, 9.0 Hz)	7.74 (*dd*, 2.0 Hz, 8.5 Hz)
5-H(B)	7.36 (*d*, 9.0 Hz)	7.29 (*d*, 9.0 Hz)	7.37 (*d*, 8.5 Hz)
8-H(A)	6.85 (*d*, 2.0 Hz)	6.75 (*d*, 2.0 Hz)	6.90 (*d*, 2.0 Hz)
6-H(A)	7.32 (*d*, 2.0 Hz)	6.98 (*d*, 2.0 Hz)	7.35 (*d*, 2.0 Hz)
1"-H	5.62 (*d*, 6.0 Hz)	6.35 (*d*, 2.0 Hz)	
2"-H	5.20 (*dd*, 6.0Hz, 9.0 Hz)	6.32 (*dd*, 2.0Hz, 4.50 Hz)	
3"-H	5.26 (*dd*, 9.0 Hz, 7.5 Hz)	5.84 (*dd*, 4.50 Hz, 10.0 Hz)	
4"-H	4.94 (*m*)	5.63 (*dd*, 10.0 Hz, 10.0 Hz)	
5"-H	3.25 (*dd*, 8.0 Hz, 12.0 Hz)	3.78 (*dd*, 10.0 Hz, 6.0 Hz)	
5"-H	3.88 (*dd*, 12.0 Hz, 5.0 Hz)	3.88 (*dd*, 12.0 Hz, 5.0 Hz)	
6"-H (CH_3)		1.28 (*d*, 6.0 Hz)	
OAc (arom.)	2.36 - 2.46		
OAc (aliph.)	2.02 - 2.15		
OAc		1.61 - 2.31	2.35 - 2.47

Table 4: ^{1}H NMR peaks of flavonol-glycoside derivatives, **(67)** and **(68)** and one flavonol, **(69)** from apple cider at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	(70) Ac-D_6- 296K
2-H(B)	7.52 (*d*, 2.0 Hz)
6-H(B)	7.61 (*dd*, 2.0 Hz, 8.0 Hz)
5-H(B)	7.37 (*d*, 8.0 Hz)
8-H(A)	6.72 (*d*, 2.0 Hz)
6-H(A)	6.88 (*d*, 2.0 Hz)
2-H(C)	5.71 (*d*, 10.0 Hz)
3-H(C)	5.87 (*d*, 10.0 Hz)
OAc	2.03 - 2.10

Table 5: ^{1}H NMR peaks of dihydroflavonol derivative, **(70)** from apple at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	**(73)** $CDCl_3$ - 296K	(77) CDCl3 - 296K	**(78)** CDCl3 - 296K
3-H	6.72 (*dd*, 8.0 Hz, 1.0 Hz)	6.08 (*d*, 2.0 Hz)	6.36 (*d*, 2.0 Hz)
4-H	7.36 (*t*, 8.0 Hz)	OH	OH
5-H	6.49 (*dd*, 8.0 Hz, 1.0 Hz)	6.04 (*d*, 2.0 Hz)	6.17 (*d*, 2.0 Hz)
1"-H	5.30 - 5.42 (overlapping 2',3'-H)	5.29 - 5.40 (overlapping 2',3'-H)	5.15 - 5.40 (overlapping 2',3',4'-H)
2"-H	5.30 - 5.42 (overlapping 1',3'-H)	5.29 - 5.40 (overlapping 1',3'-H)	5.15 - 5.40 (overlapping 1',3',4'-H)
3"-H	5.30 - 5.42 (overlapping 1',2'-H)	5.29 - 5.40 (overlapping 1',2'-H)	5.15 - 5.40 (overlapping 1',2',4'-H)
4"-H	5.21 (*dd*, 10.0 Hz, 10.0 Hz)	5.17 (*dd*, 10.0 Hz, 10.0 Hz)	5.15 - 5.40 (overlapping 1',2',3'-H)
5"-H	3.90 (*m*)	3.93 (*m*)	3.93 (*m*)
6"-H	4.31 (*dd*, 5.0 Hz, 12.0 Hz)	4.27 (*dd*, 5.0 Hz, 12.0 Hz)	4.29 (*dd*, 5.0 Hz, 12.0 Hz)
6"'-H	4.16 (*dd*, 12.0 Hz, 2.0 Hz)	4.17 (*dd*, 12.0 Hz, 2.0 Hz)	4.20 (*dd*, 12.0 Hz, 2.0 Hz)
~OCH_2~			5.20 (*s*)
~OCH_3			3.49 (*s*)
CH_3	2.50 (s)	2.50 (s)	2.56 (*s*)
OAc	2.05 - 2.09	2.03 - 2.09	2.04 - 2.11

Table 6: ^{1}H NMR peaks of **(73), (77)** and **(78)** from the synthesis of chalcone-glycosides at 300 MHz. Splitting patterns and *J* (Hz) values are given in parenthesis.

Protons	**(75)** $CDCl_3$ - 296K	**(80)** $CDCl_3$ - 296K	**(82)** $CDCl_3$ - 296K
3,5-H(A)	6.90 (*d*, 9.0 Hz)	6.95 (*d*, 9.0 Hz)	6.85 (*d*, 8.0 Hz)
2,6-H(A)	7.50 (*d*, 9.0 Hz)	7.58 (*d*, 8.0 Hz)	7.07 (*d*, 8.0 Hz)
3-H(B)	7.10 (*dd*, 1.0 Hz, 6.0 Hz)	6.13 (*d*, 2.0 Hz)	6.40 (*d*, 2.0 Hz)
4-H(B)	7.39 (*t*, 6.0 Hz)	OH	OH
5-H(B)	6.95 (*dd*, 1.0 Hz, 6.0 Hz)	5.80 (*d*, 2.0 Hz)	6.27 (*d*, 2.0 Hz)
α-H	7.21 (*d*,10.0 Hz)	7.82 (*s*)	7.19 (*d*,15.0 Hz)
β-H	6.75 (*d*,10.0 Hz)	7.82 (*s*)	6.75 (*d*, 15.0 Hz)
1"-H	5.15 (*m*, overlapping 2",3",4"-H)		5.10 (*m*, overlapping 2",3",4"-H)
2"-H	5.15 (*m*, overlapping 1",3",4"-H)		5.10 (*m*, overlapping 1",3",4"-H)
3"-H	5.15 (*m*, overlapping 1",2",4"-H)		5.10 (*m*, overlapping 1",2",4"-H)
4"-H	5.15 (*m*, overlapping 1",2",3"-H)		5.10 (*m*, overlapping 1",2",3"-H)
5"-H	3.89 (*m*)		3.90 (*m*)
6"-H	4.18 (*dd*, 2.0 Hz, 12.0 Hz)		4.22 (*m*)
6"'-H	4.30 (*dd*, 5.0 Hz, 12.0 Hz)		4.22 (*m*)
~OCH_3~	3.87 (*s*)	3.84 - 3.95 (s)	3.75 - 3.92 (*s*)
OAc	2.0 - 2.2 (*s*)		1.96 - 2.18

Table 7: ^{1}H NMR peaks of chalcone-glycosides **(75)**, **(80)** and **(82)** at 300 MHz. Splitting patterns and *J*(Hz) values are given in parenthesis.

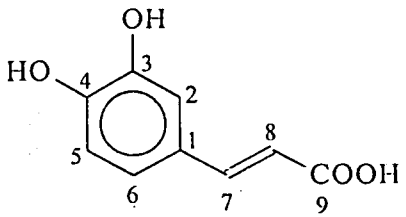
Carbon	(65) $CDCl_3$ - 296K
C1	139.0
C2	148.0 - 154.0
C3	107.0
C4	148.0 - 154.0
C5	112.0
C6	148.0 - 154.0
C7	201.0
C8	46.5
C9	29.0
C10	130.0
C11	121.5
C12	148.0 - 154.0
C13	122.0
C14	130.0
C15	99.5
C16	71.0
C17	73.0
C18	68.5
C19	73.0
C20	62.5
$\mathbf{C}DCl_3$	77.0
$O\mathbf{C}OCH_3$	170.0
$OCO\mathbf{C}H_3$	21.5

Table 8: ^{13}C NMR peaks of dihydrochalcone-glycoside derivative, **(65)** from apple cider

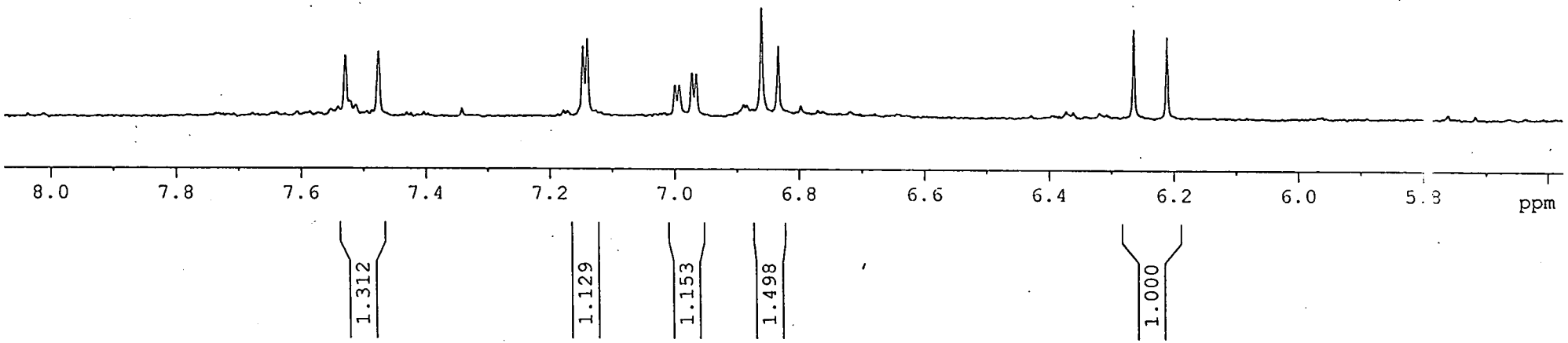
APPENDIX B

[Plate 1 (Ac-D6 - 296K)]

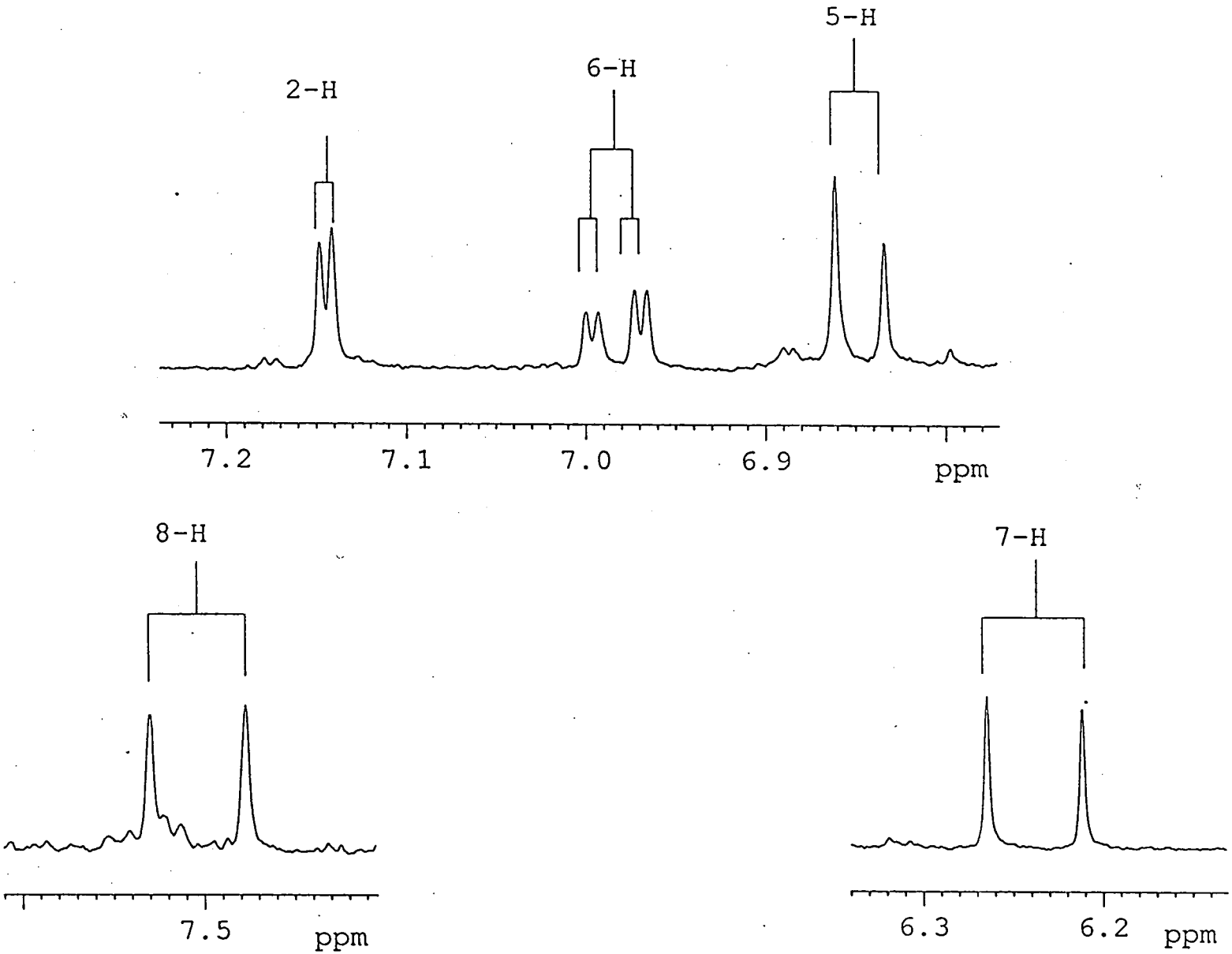
^{1}HNMR



(61)

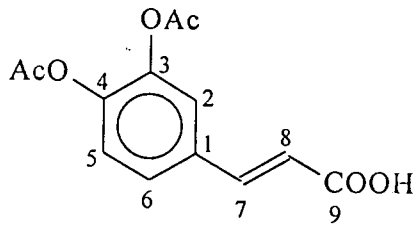


[Plate 1 1 (Ac-D6 - 296K)]

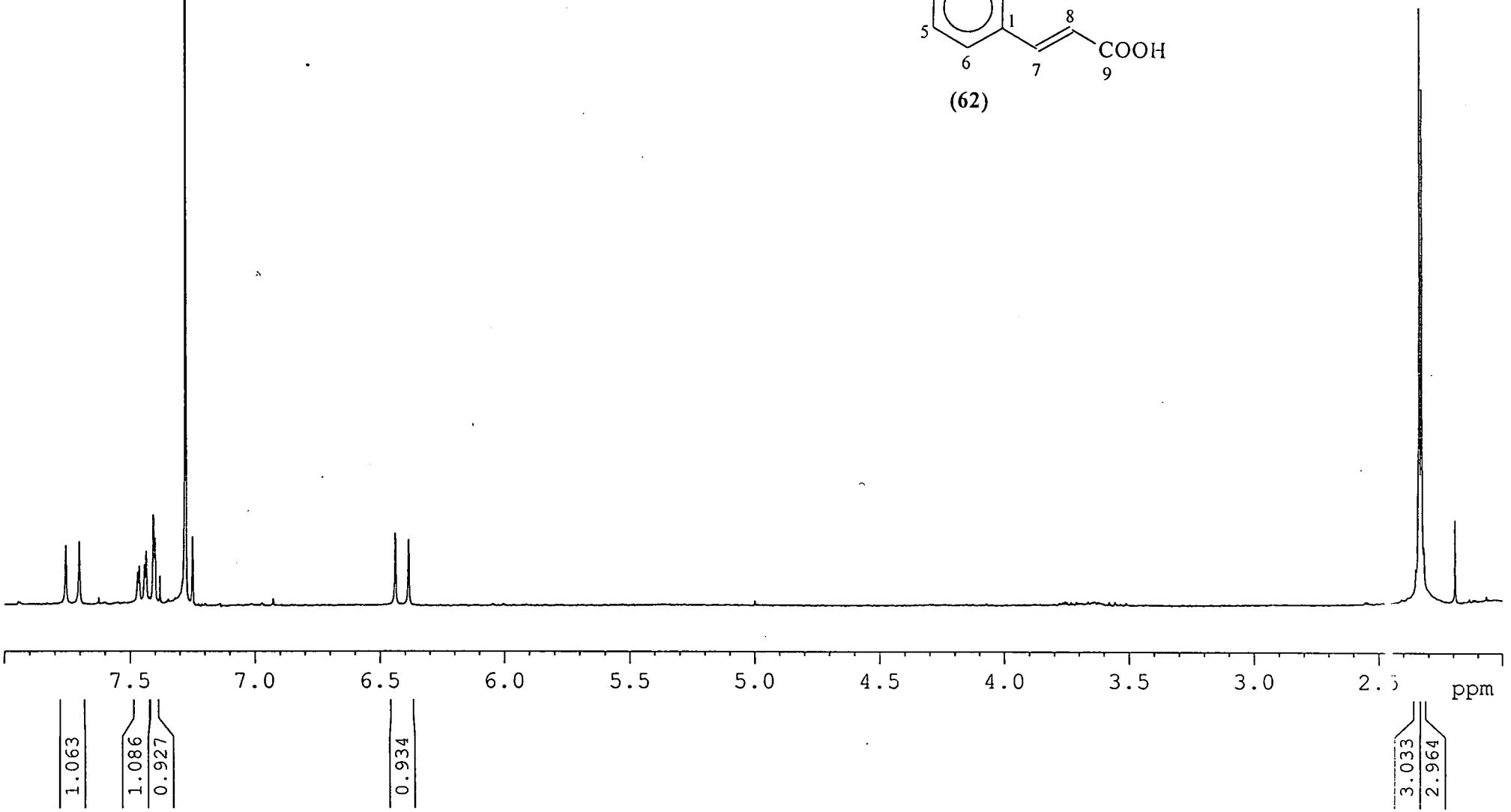


[Plate 2 (CDCl3 - 296K)]

^{1}H NMR



(62)



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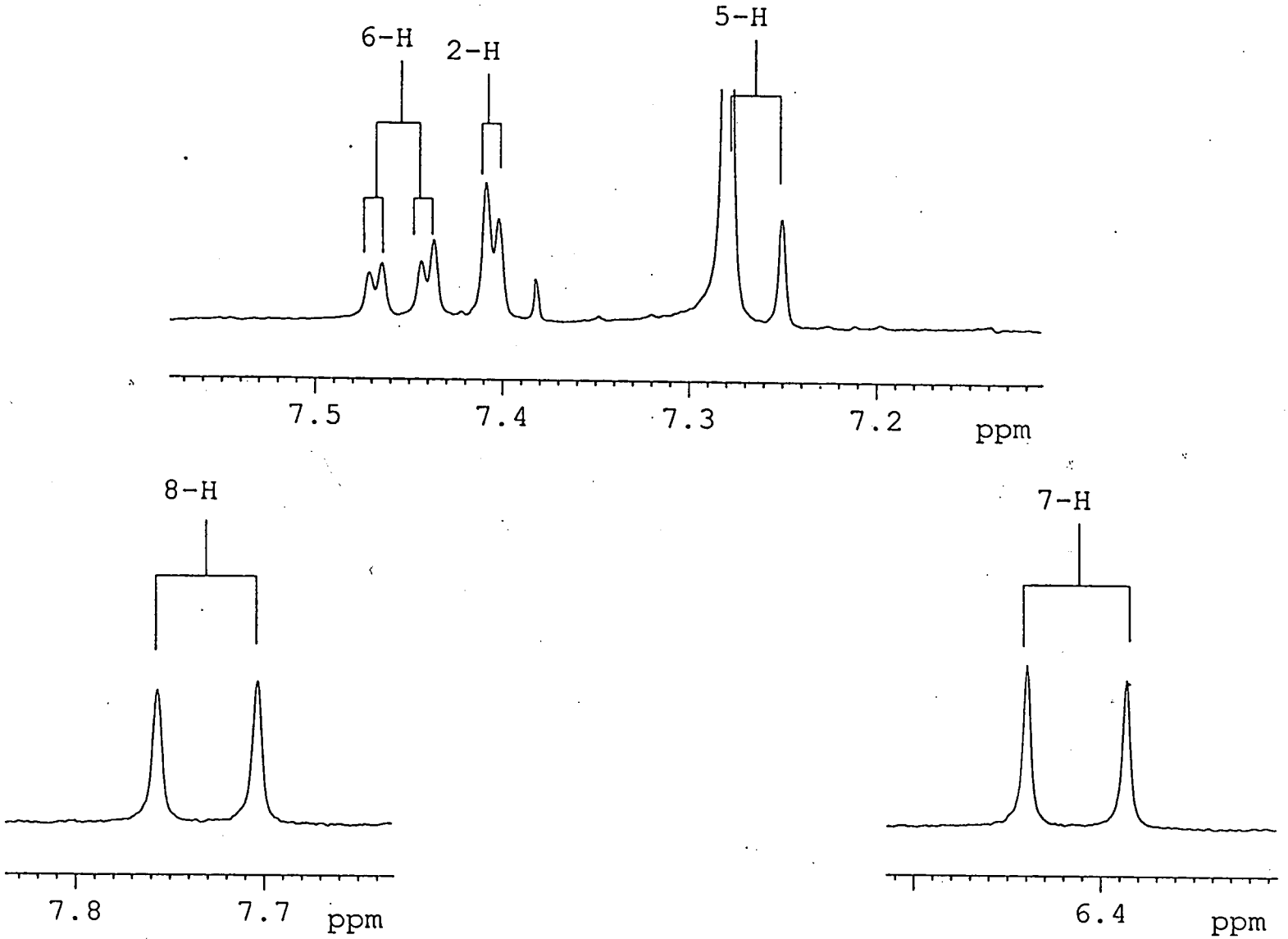
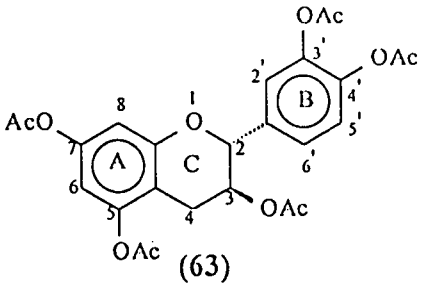


[Plate 3 (CDCl3 - 296K)]

^{1}H NMR



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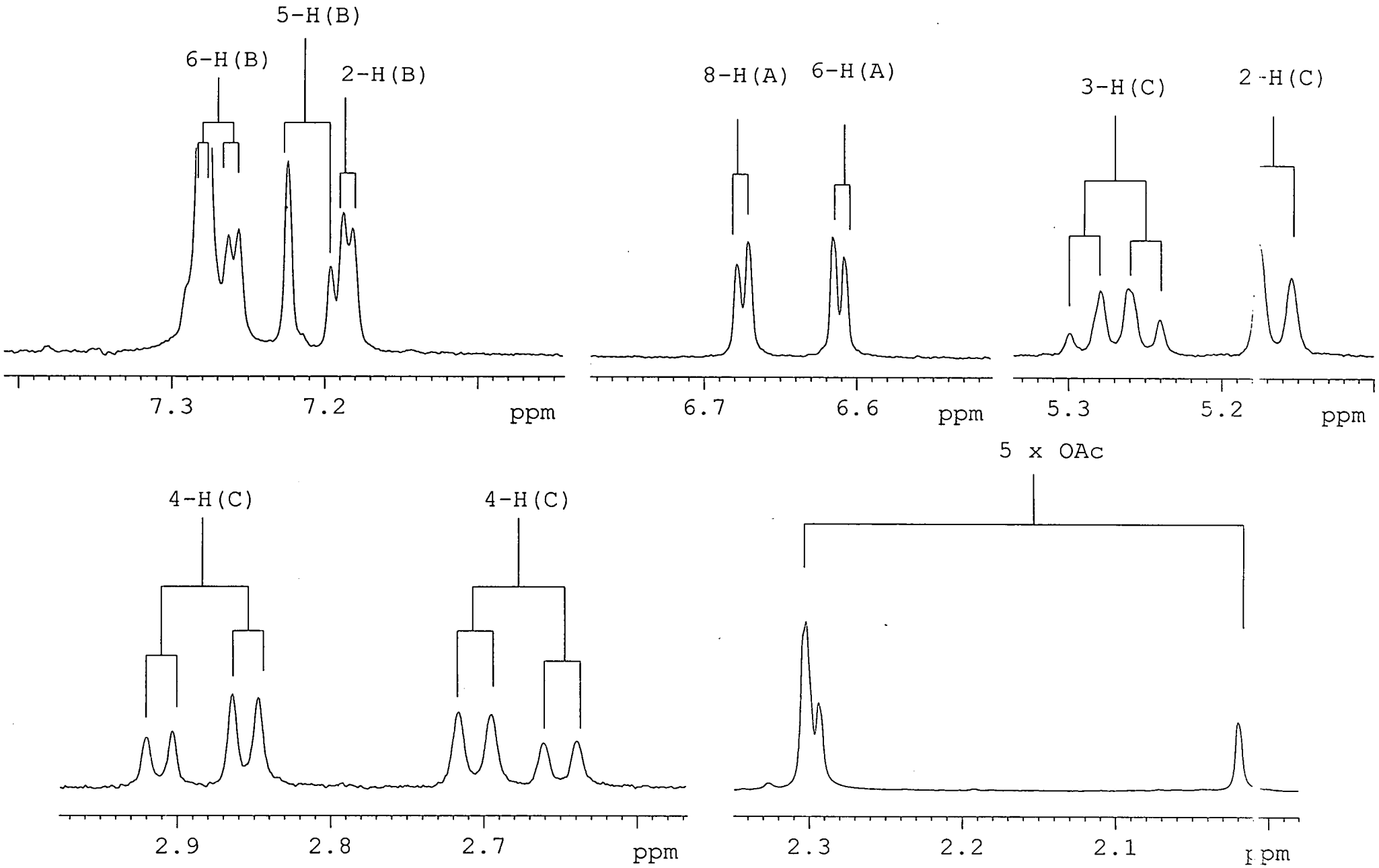
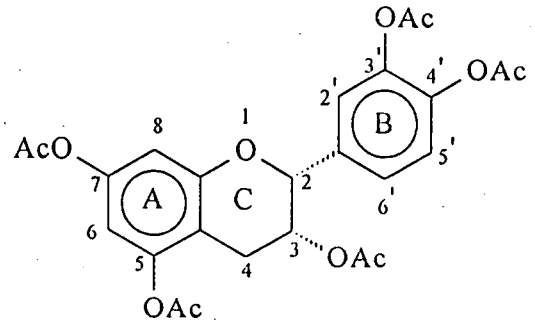
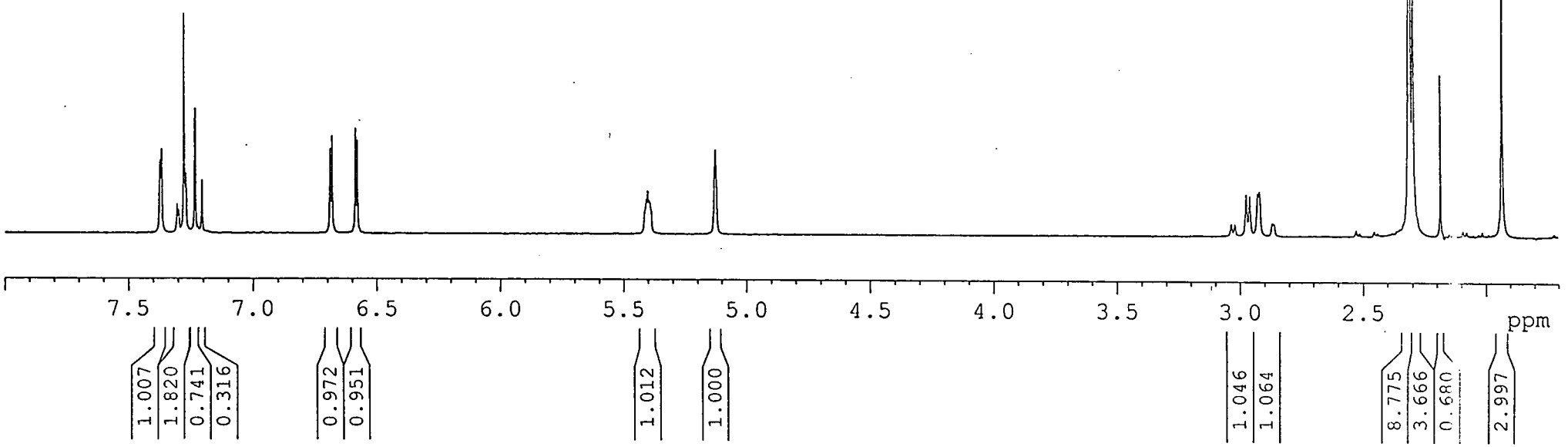


[Plate 4 (CDCl3 - 296K)]

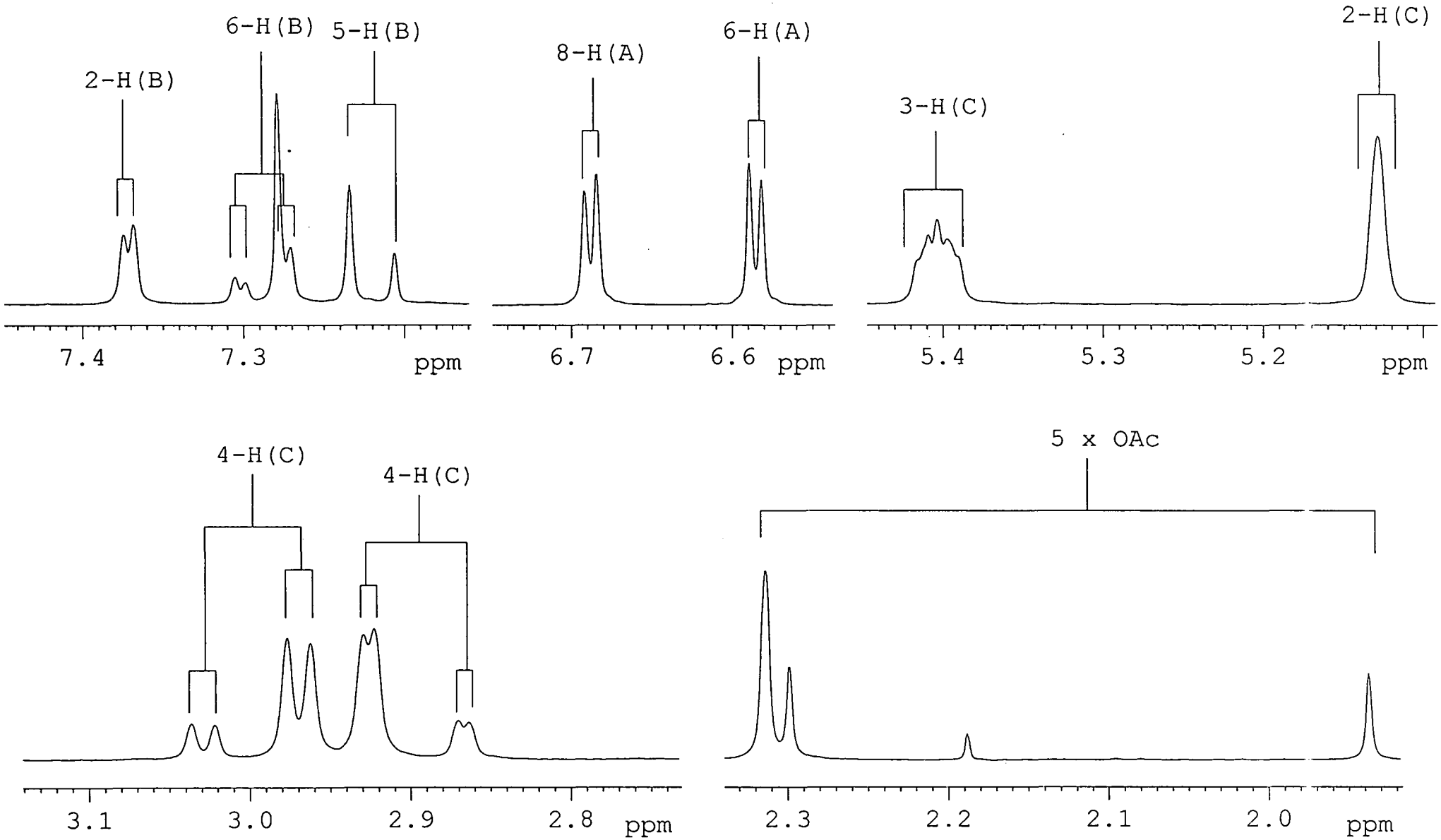
^{1}H NMR



(64)

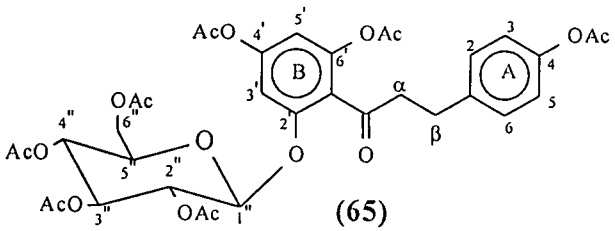


[Plate 4 1 (CDCl3 - 296K)]



[Plate 5 (CDCl3 - 296K)]

^{1}H NMR



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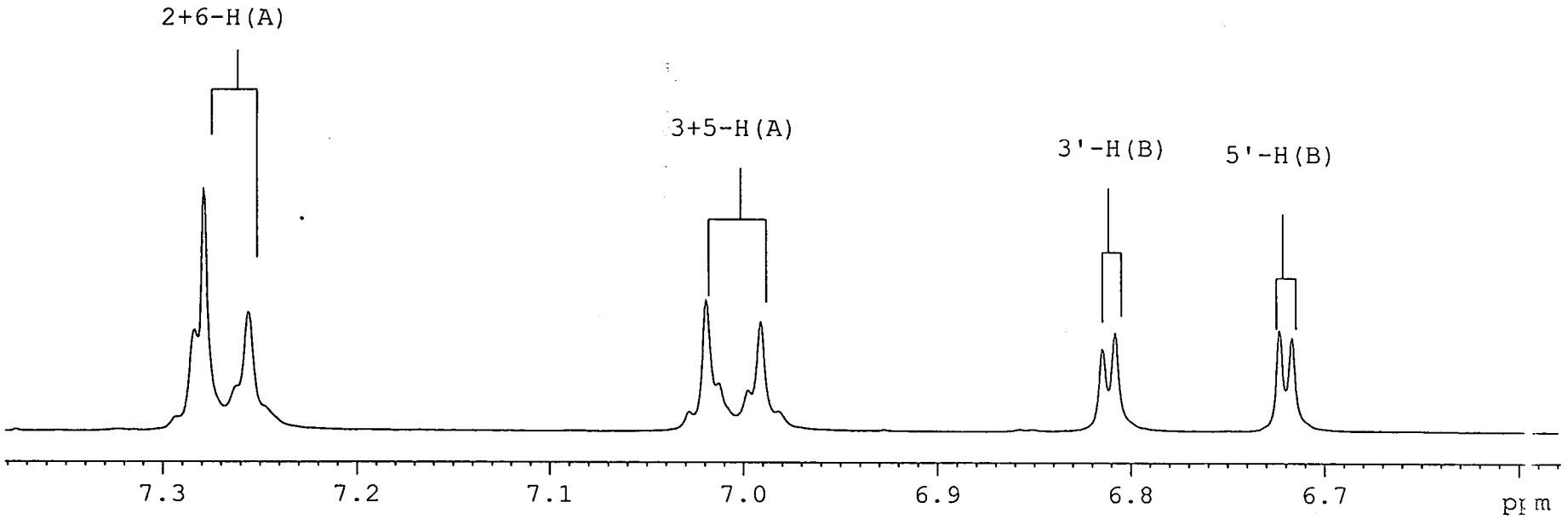
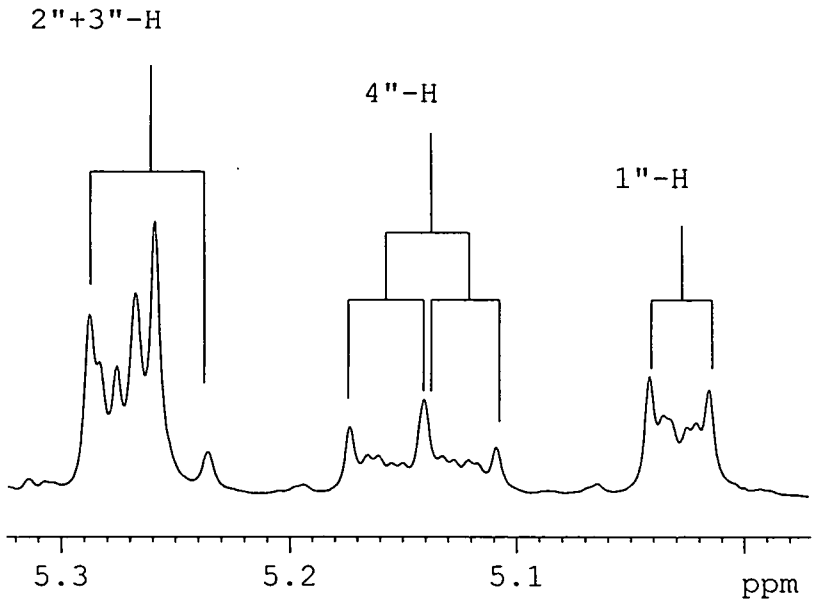
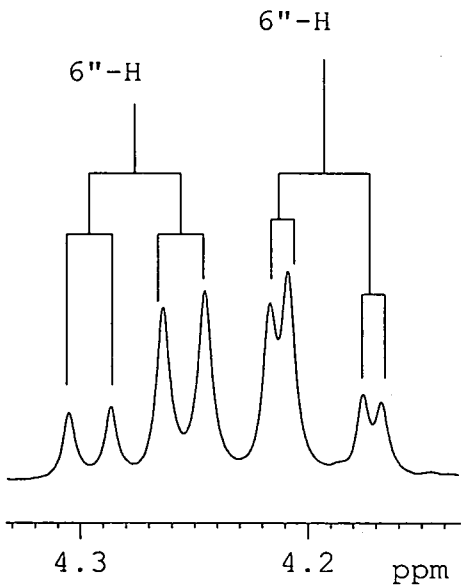
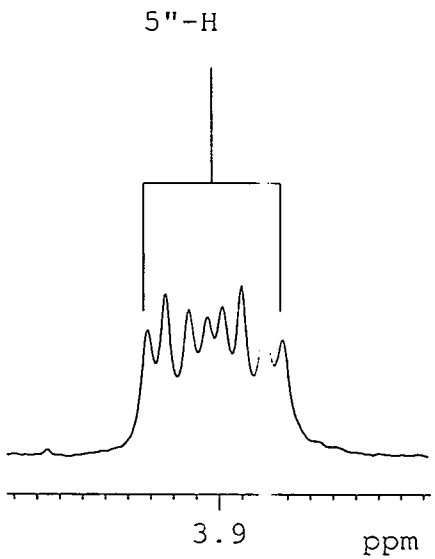
[Plate 5 1 (CDCl3 - 296K)]

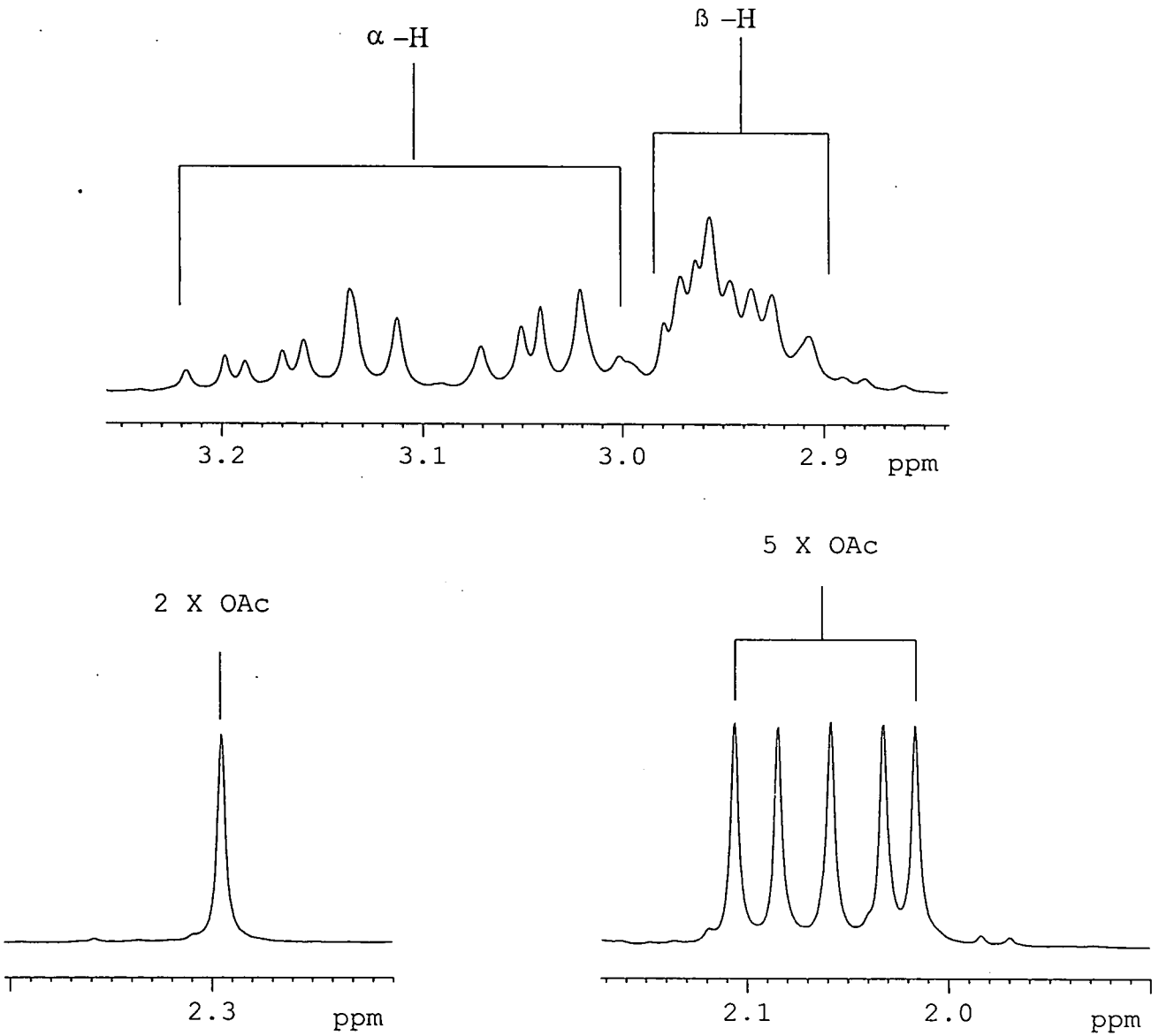




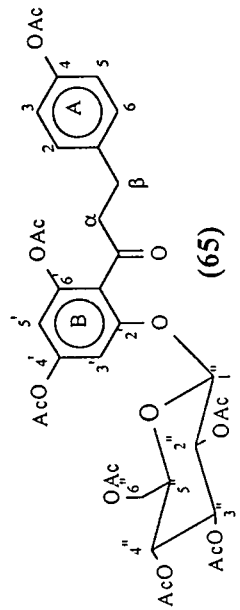




[Plate 5 2 (CDCl3 -296K)]



[Plate 5a 1 (CDCL3 -296K)]
COSY



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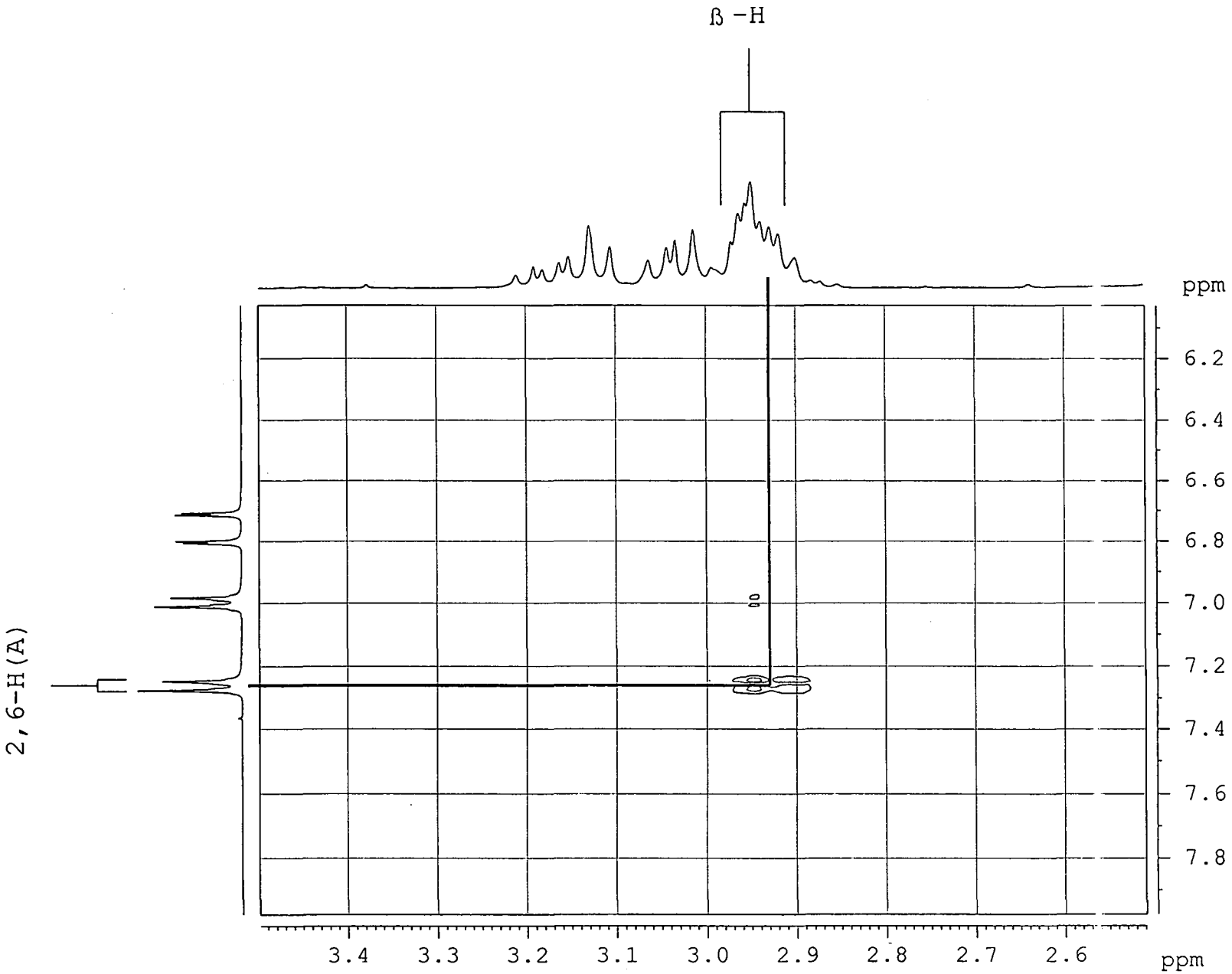

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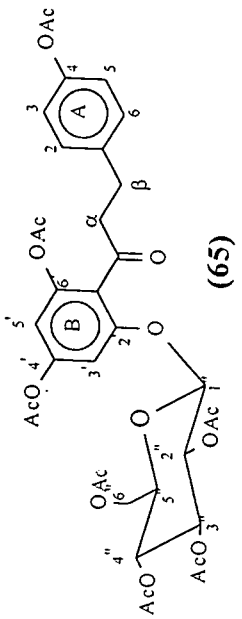

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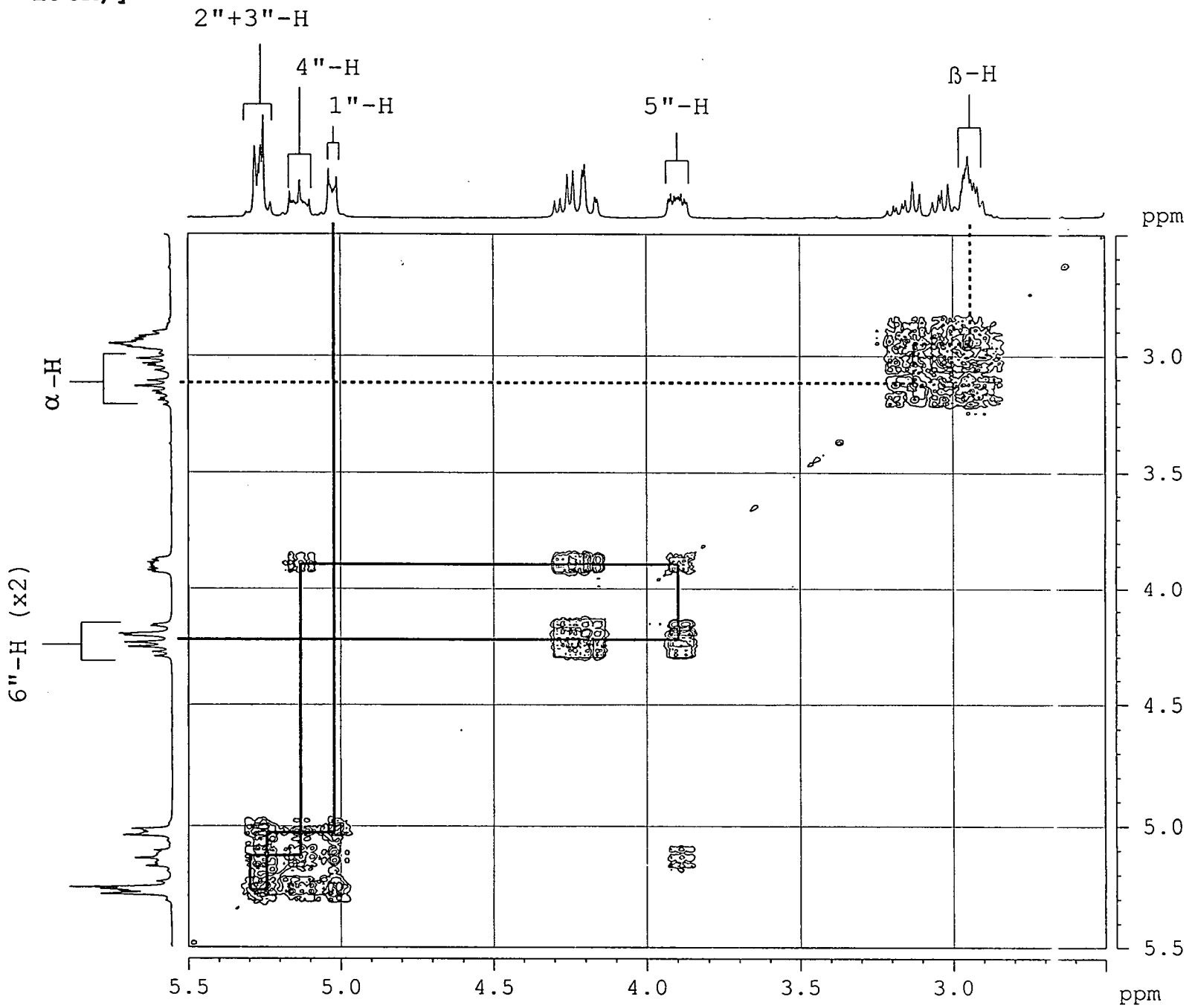

[Plate 5b 1 (CDCl3 - 296K)]
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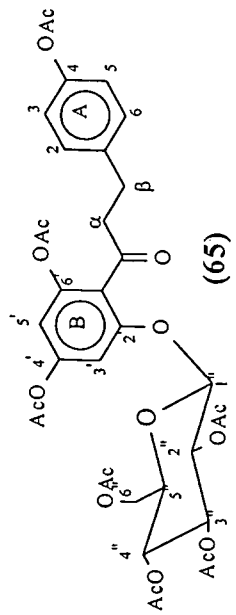

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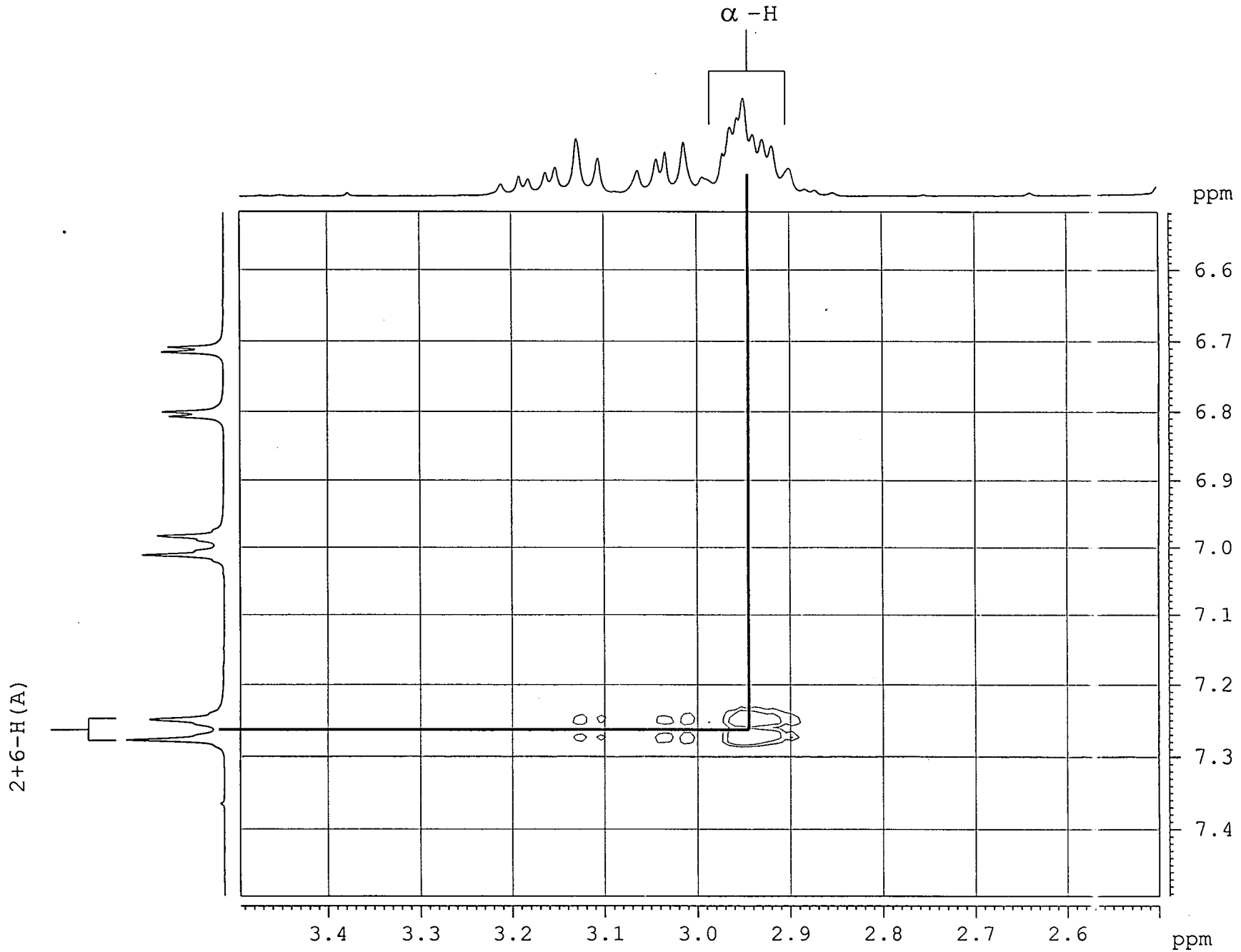
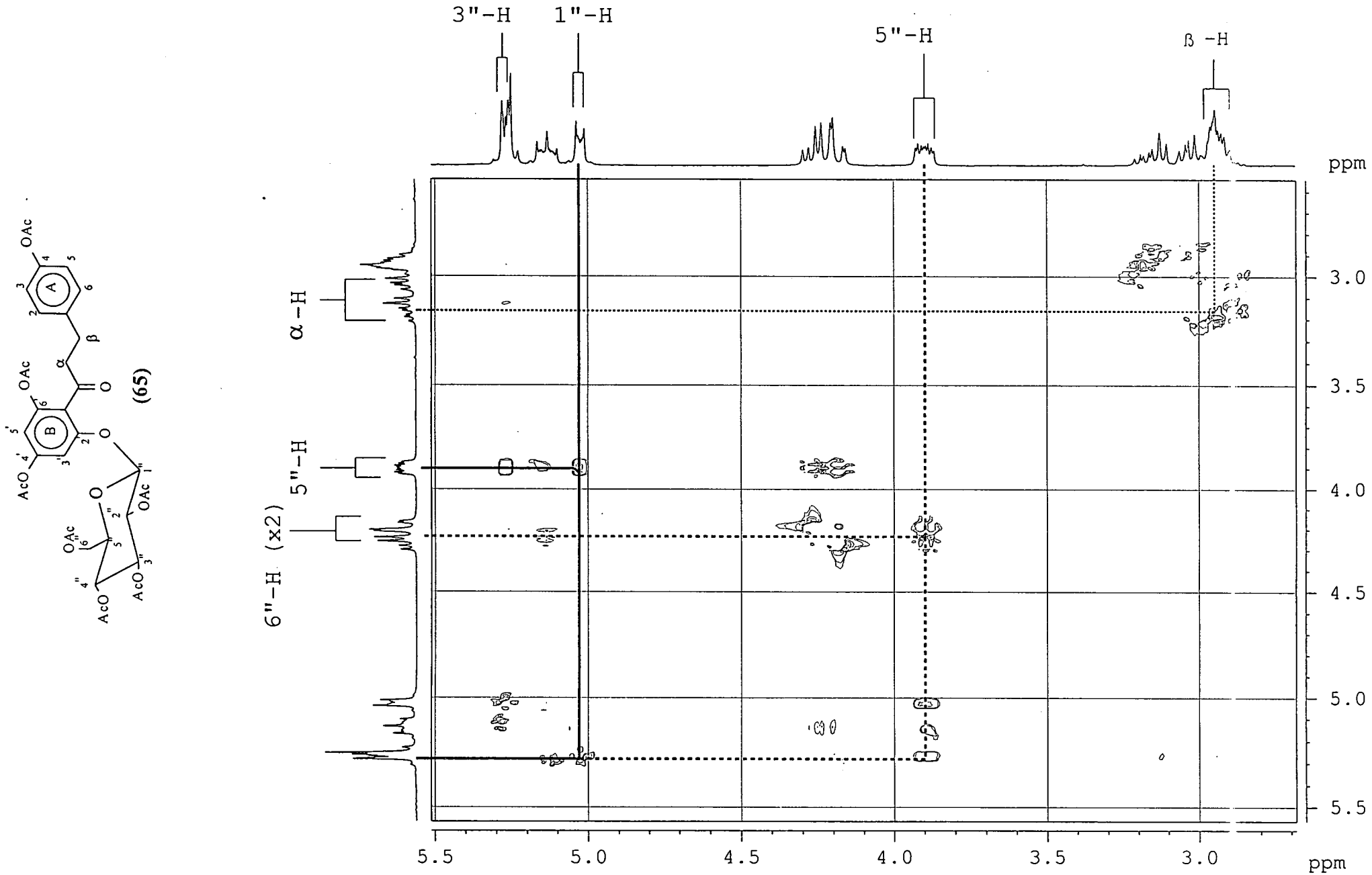
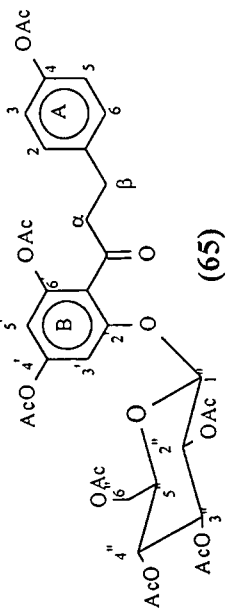


[Plate 5b 2 (CDCl3 - 296K)]
NOESY



[Plate 5b 3 (CDCl3 - 296K)]
NOESY



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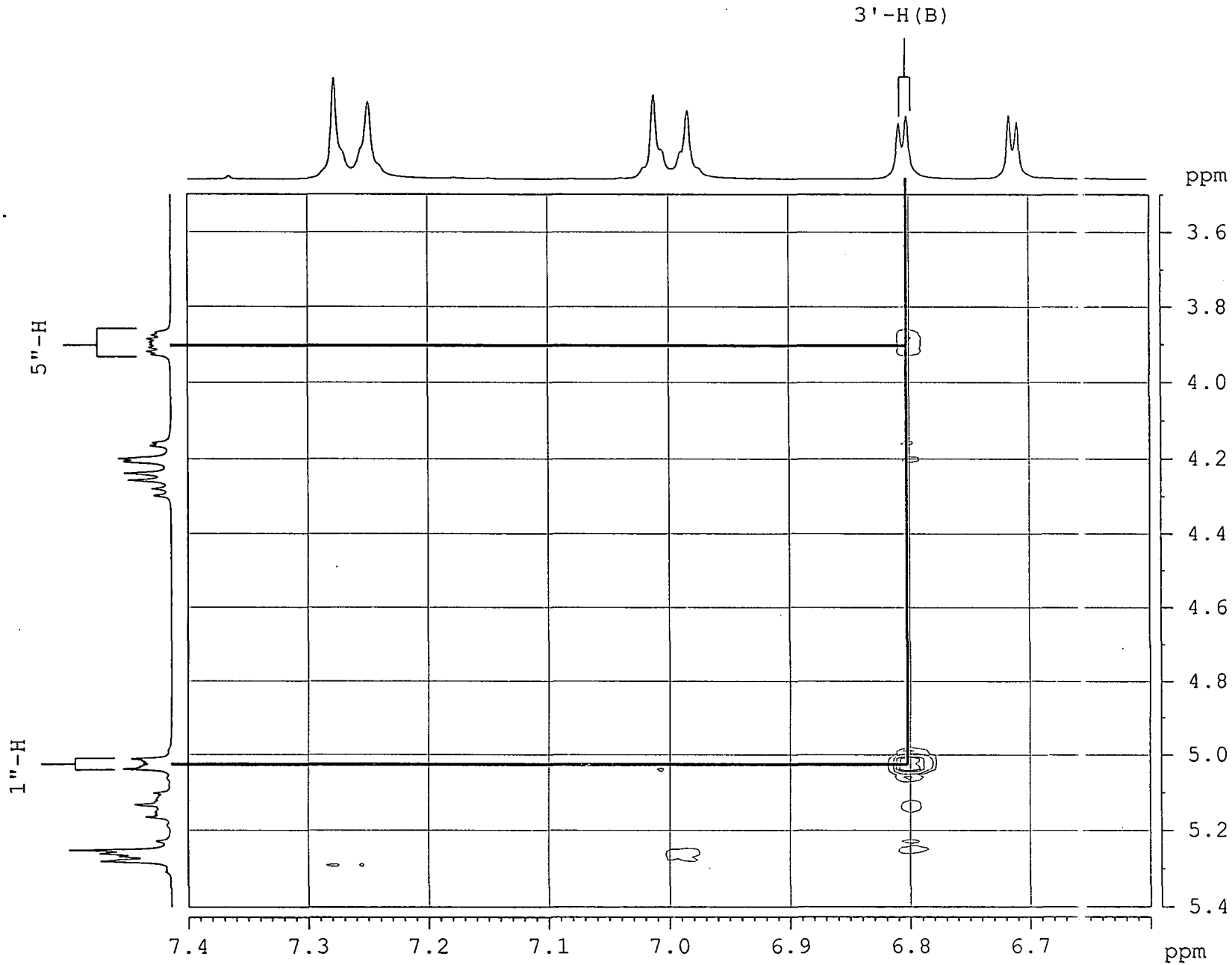
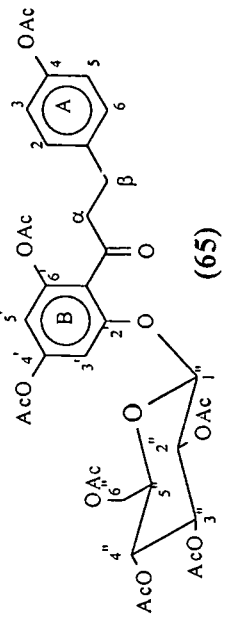
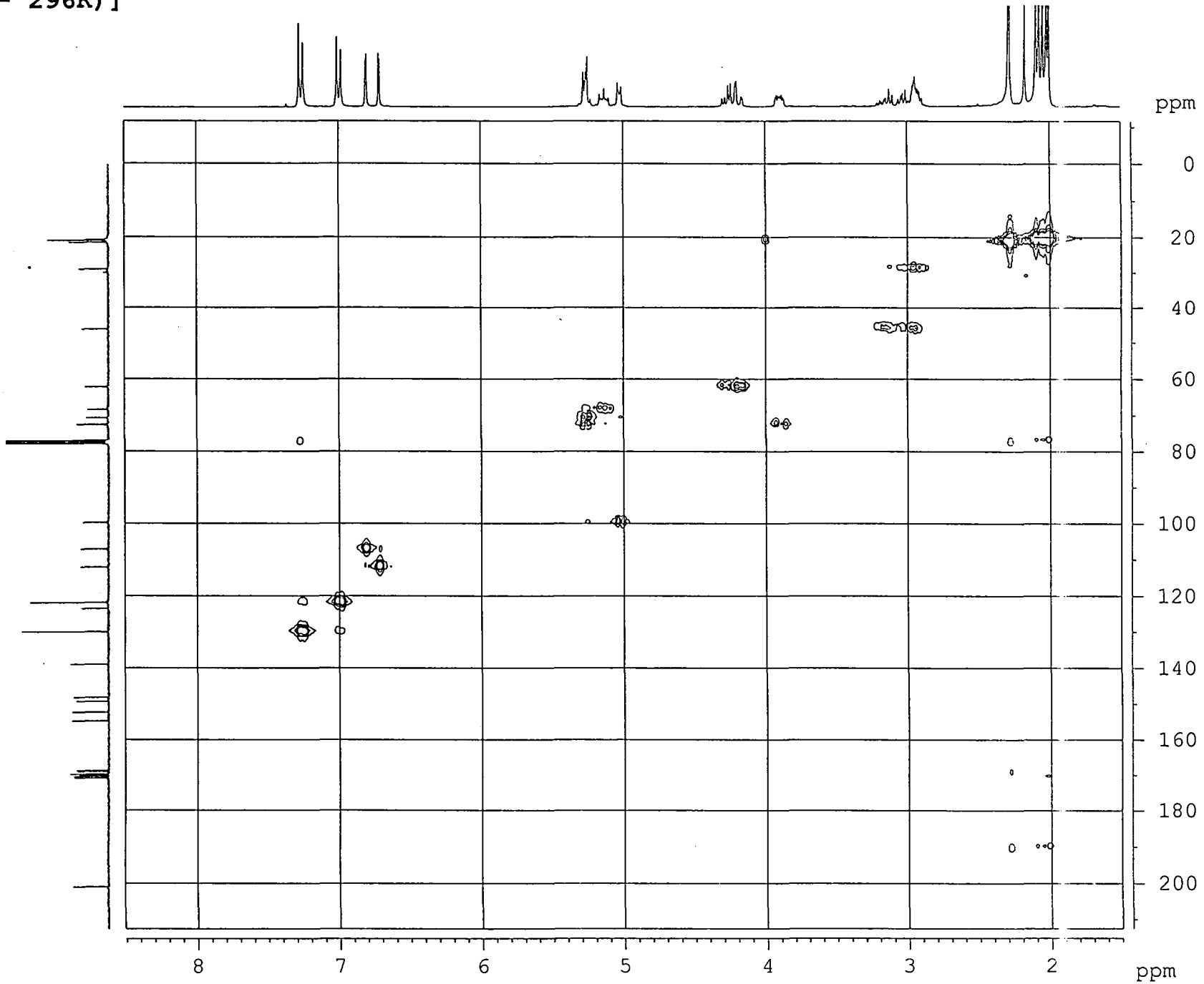


[Plate 5c (CDCl3 - 296K)]
HMQC





[Plate 5d (CDCl3 - 296K)]
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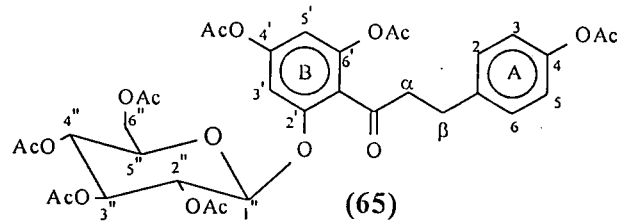

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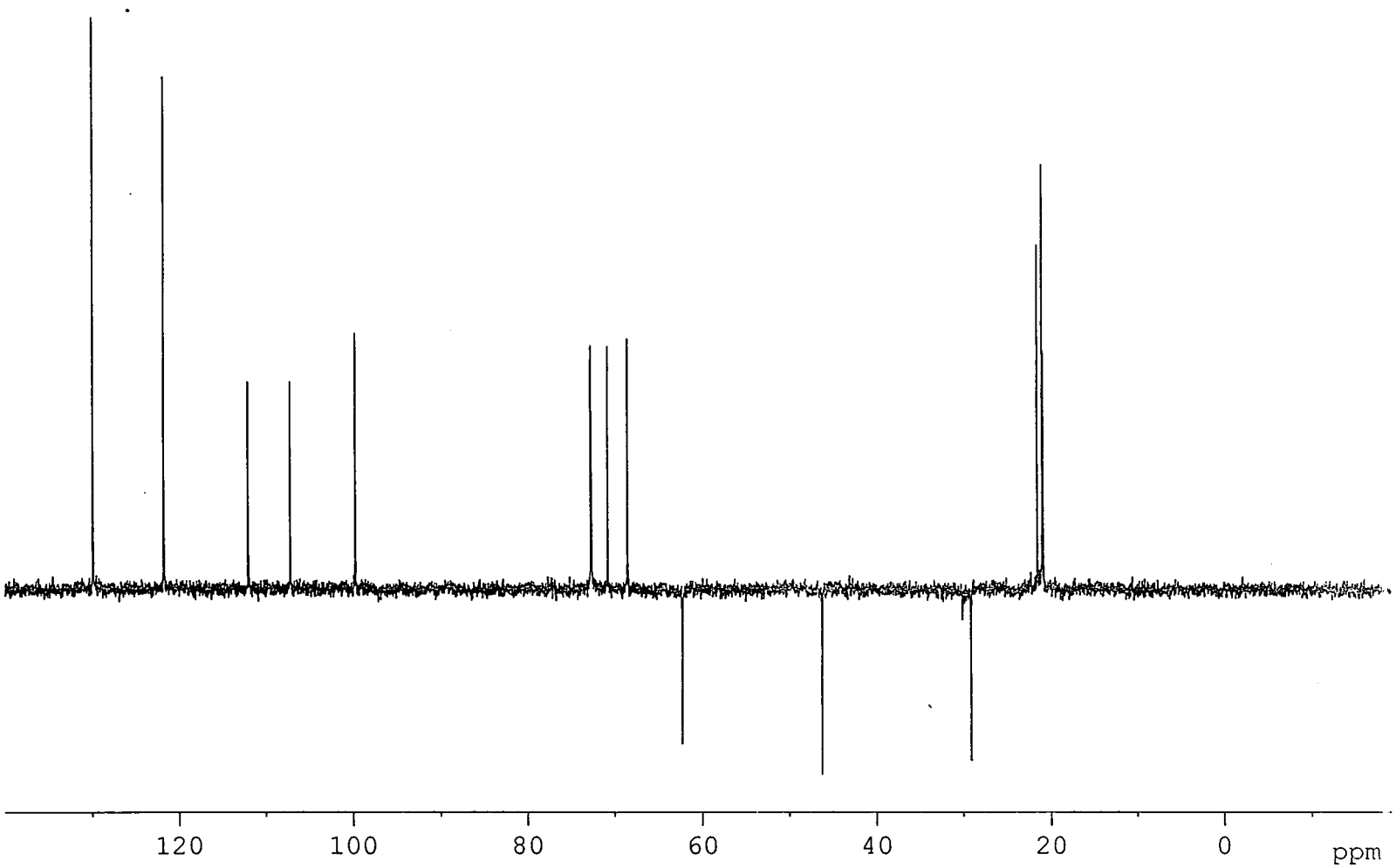
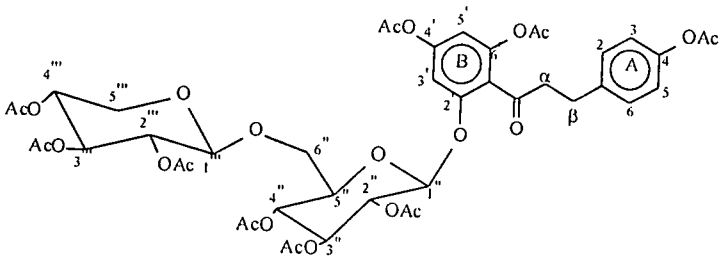


[Plate 6 (Ac-D6 - 296K)]

^{1}H NMR



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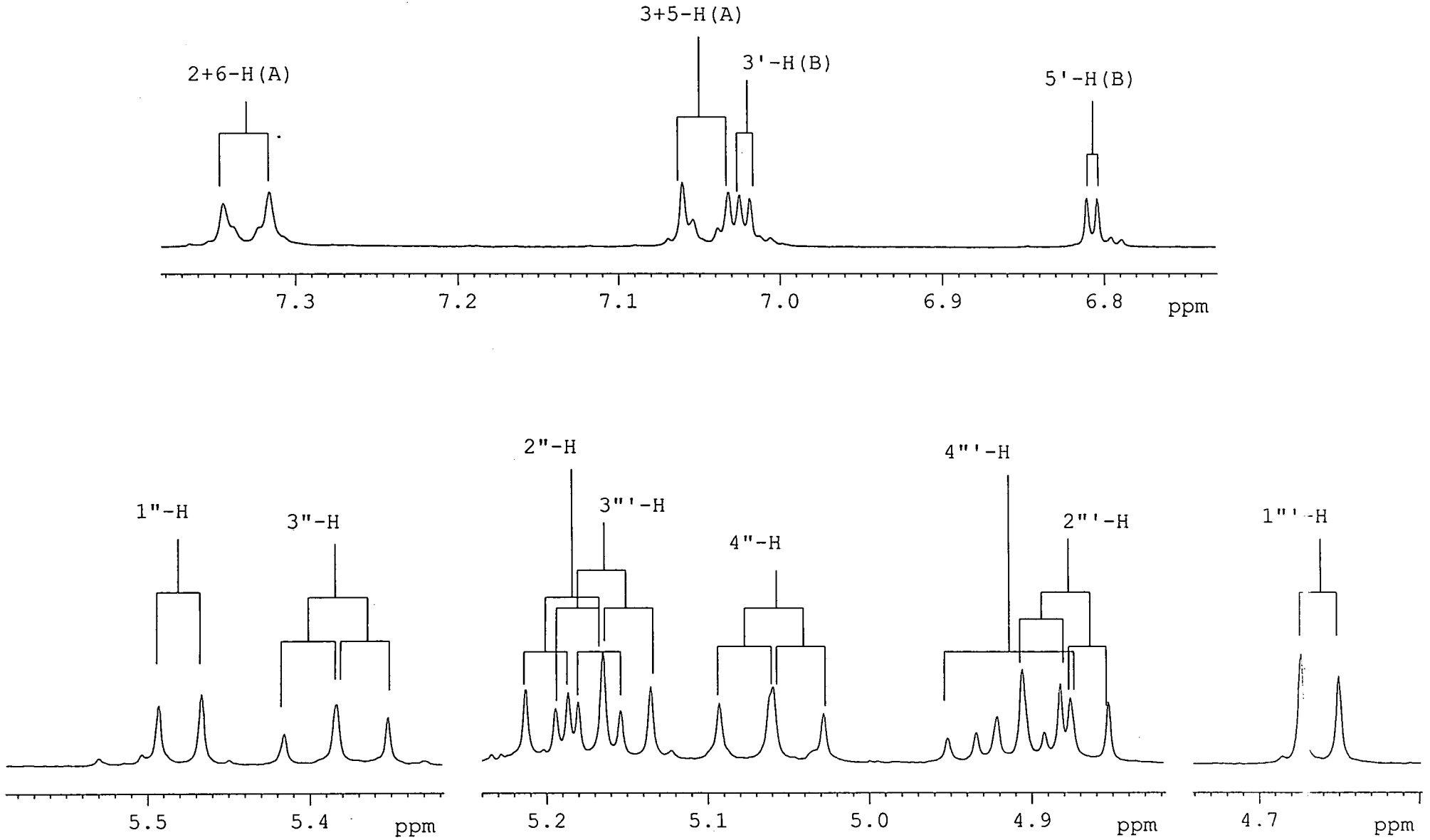
[Plate 6 1 (Ac-D6 - 296K)]



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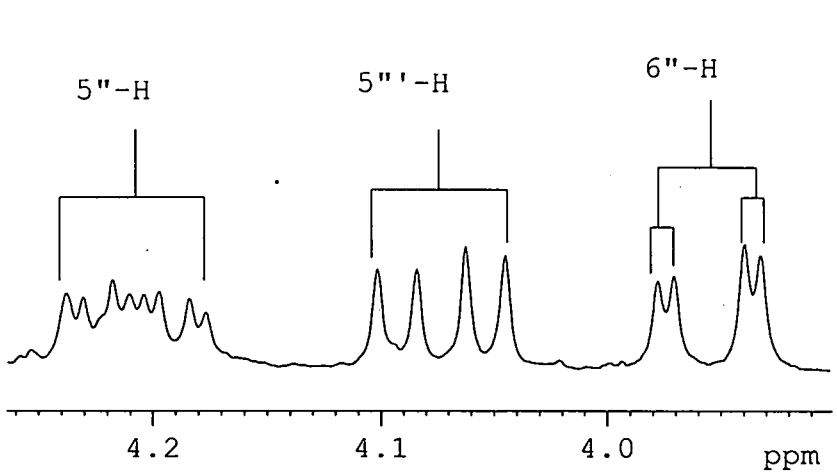

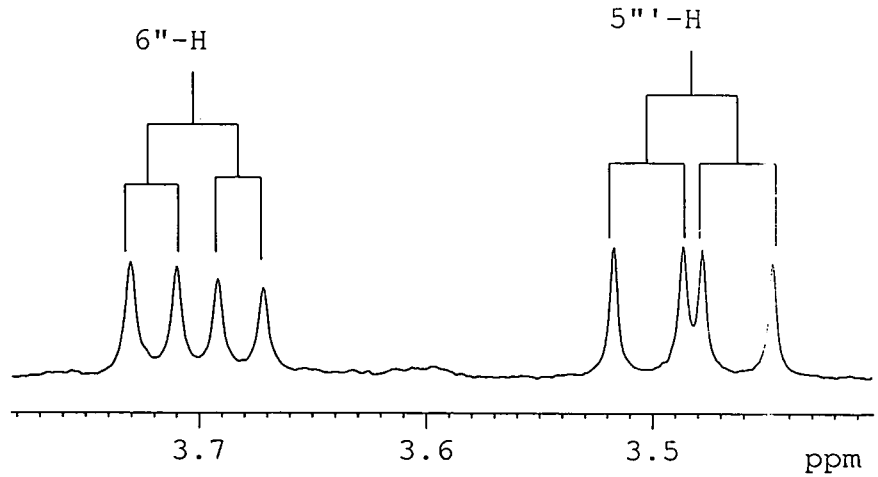

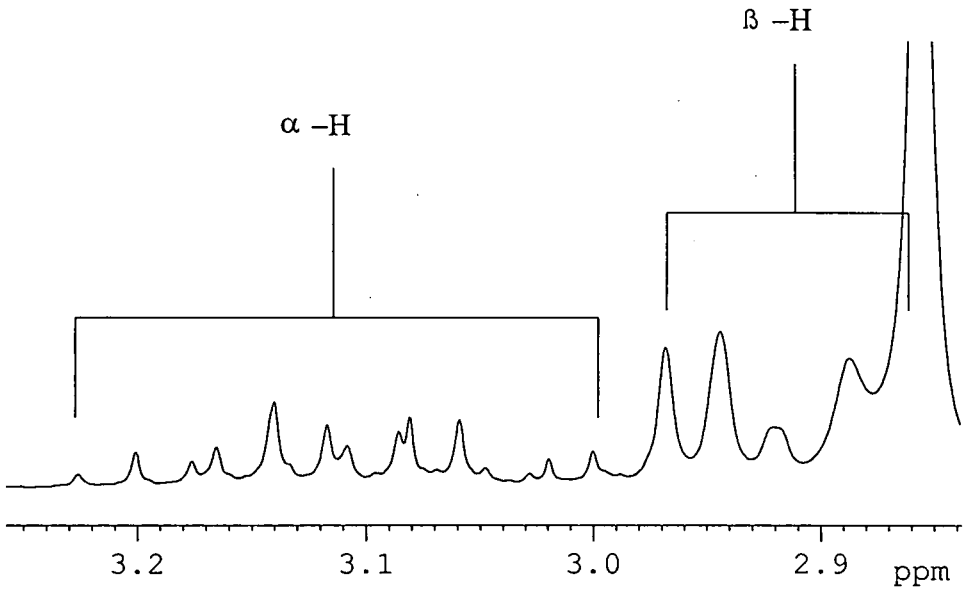

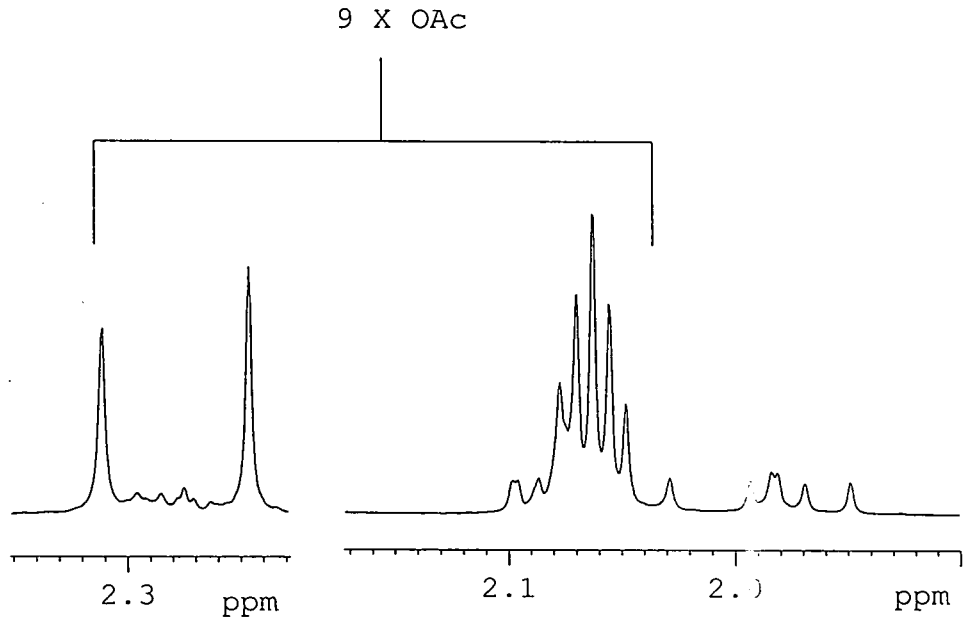

[Plate 6a (CDCl3 - 296K)]
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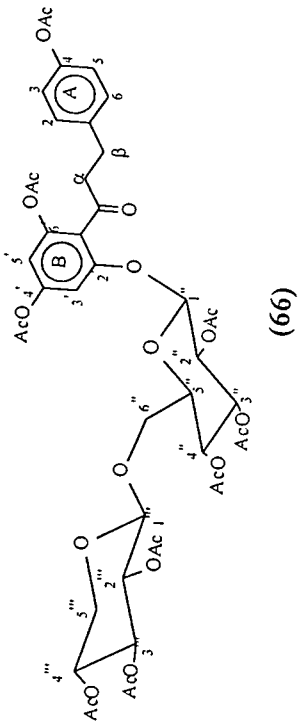

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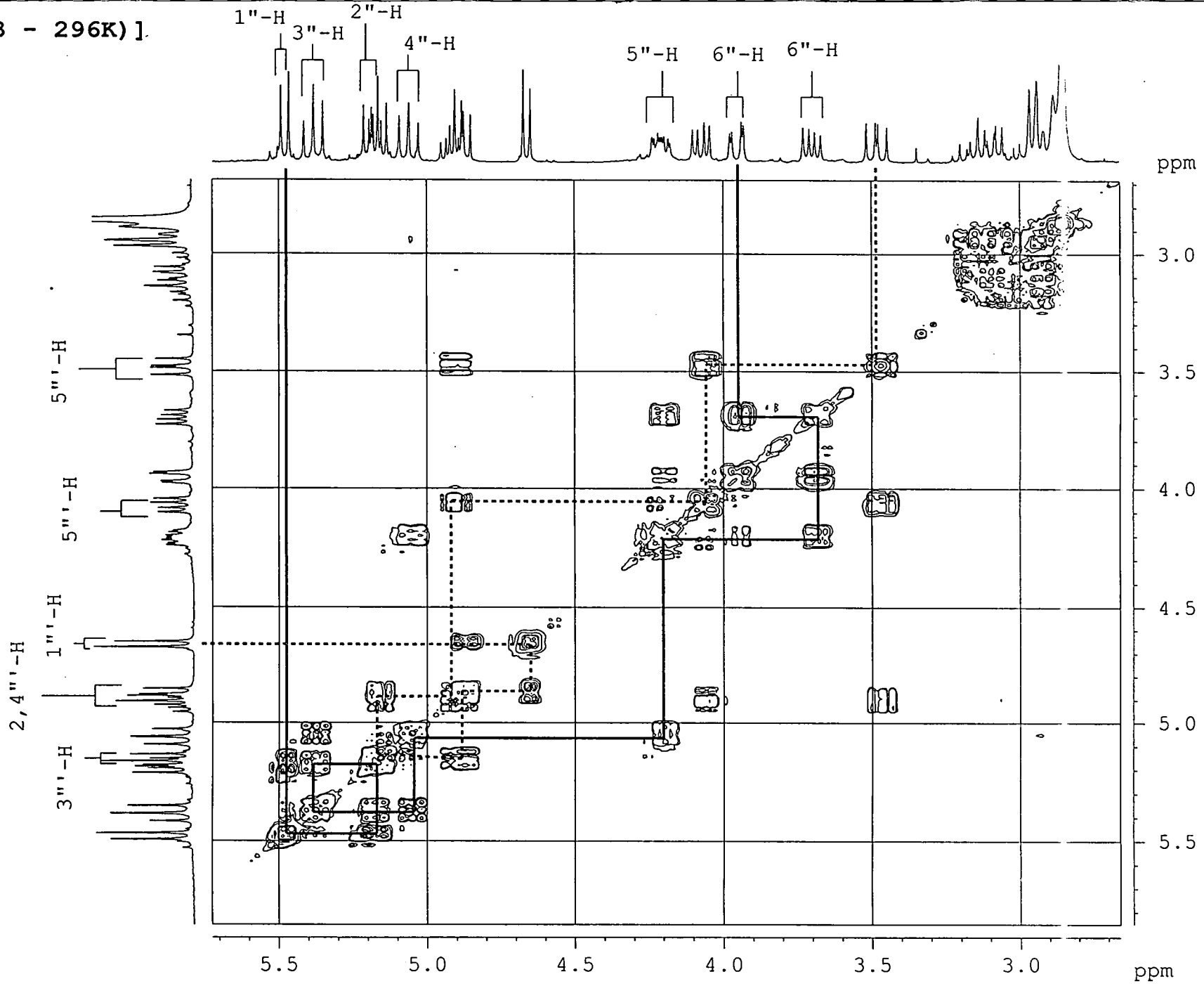

[Plate 6b 1 (Ac-D6 - 296K)]
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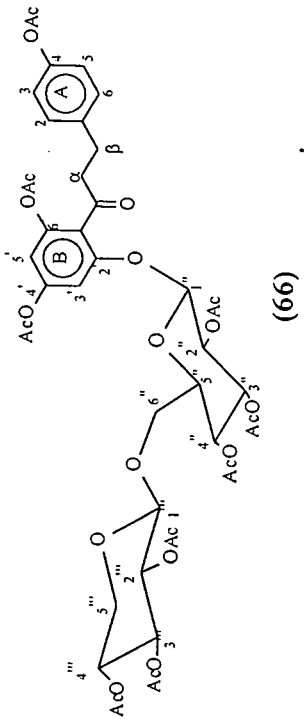

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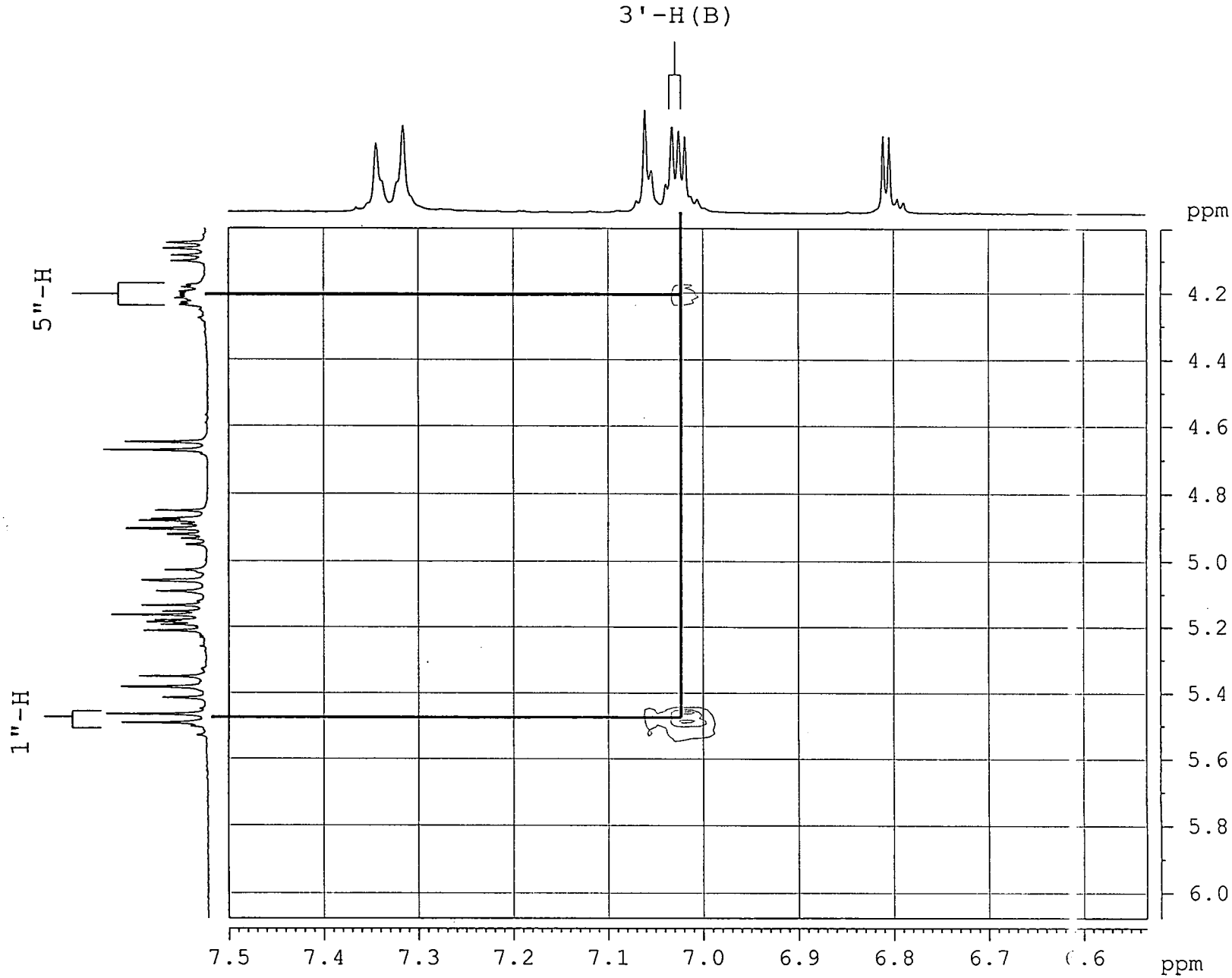

[Plate 6b 2 (Ac-D6 - 296K)]
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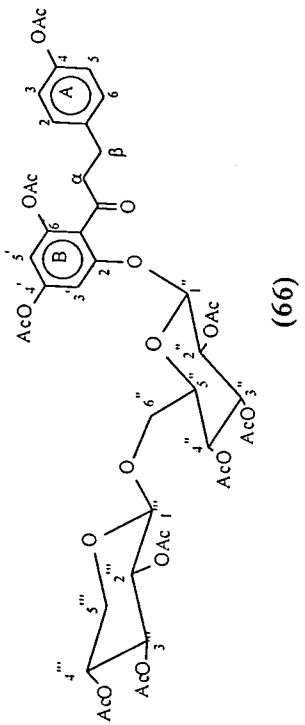

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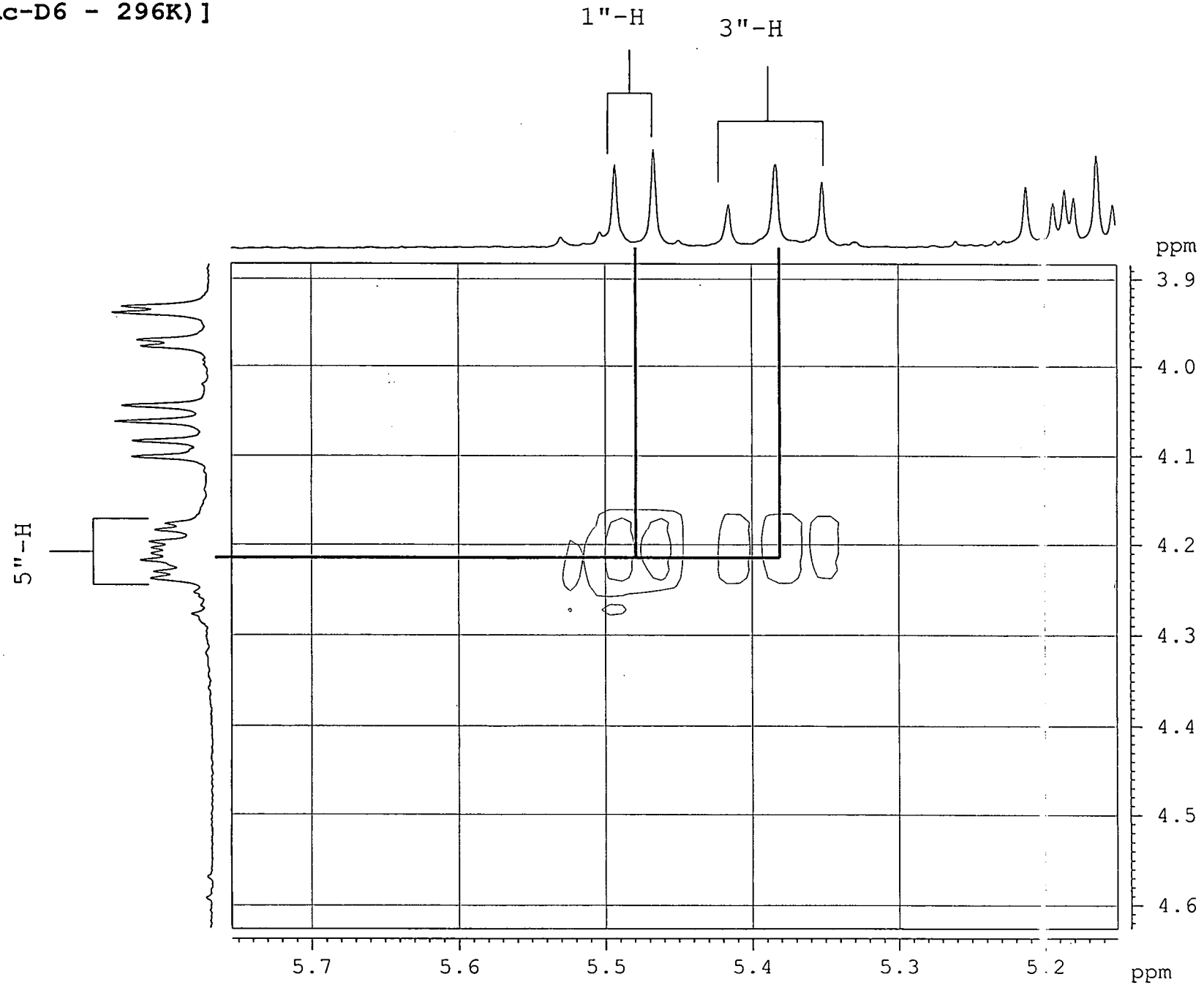

[Plate 6b 3 (Ac-D6 - 296K)]
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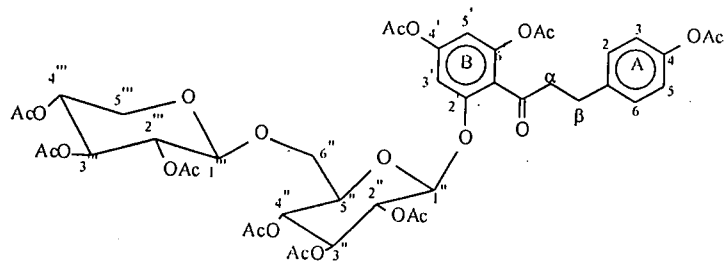

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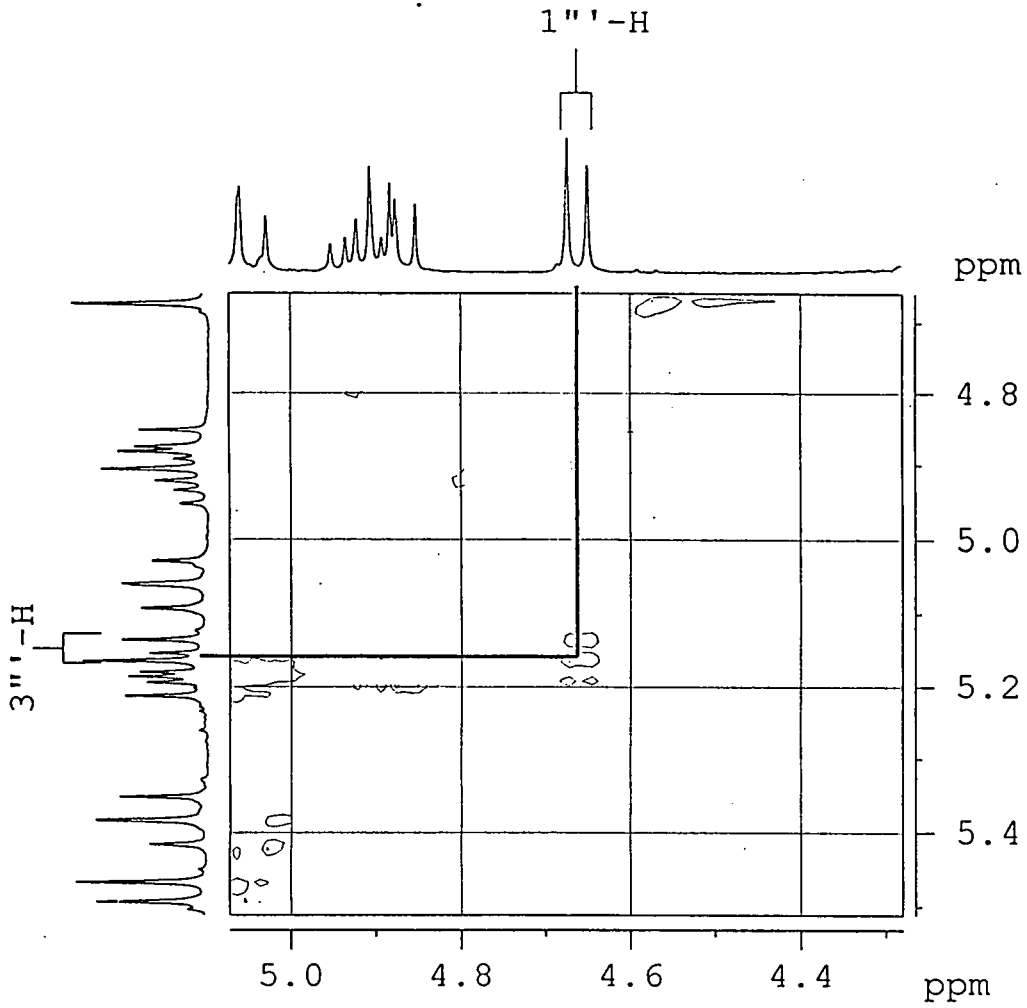
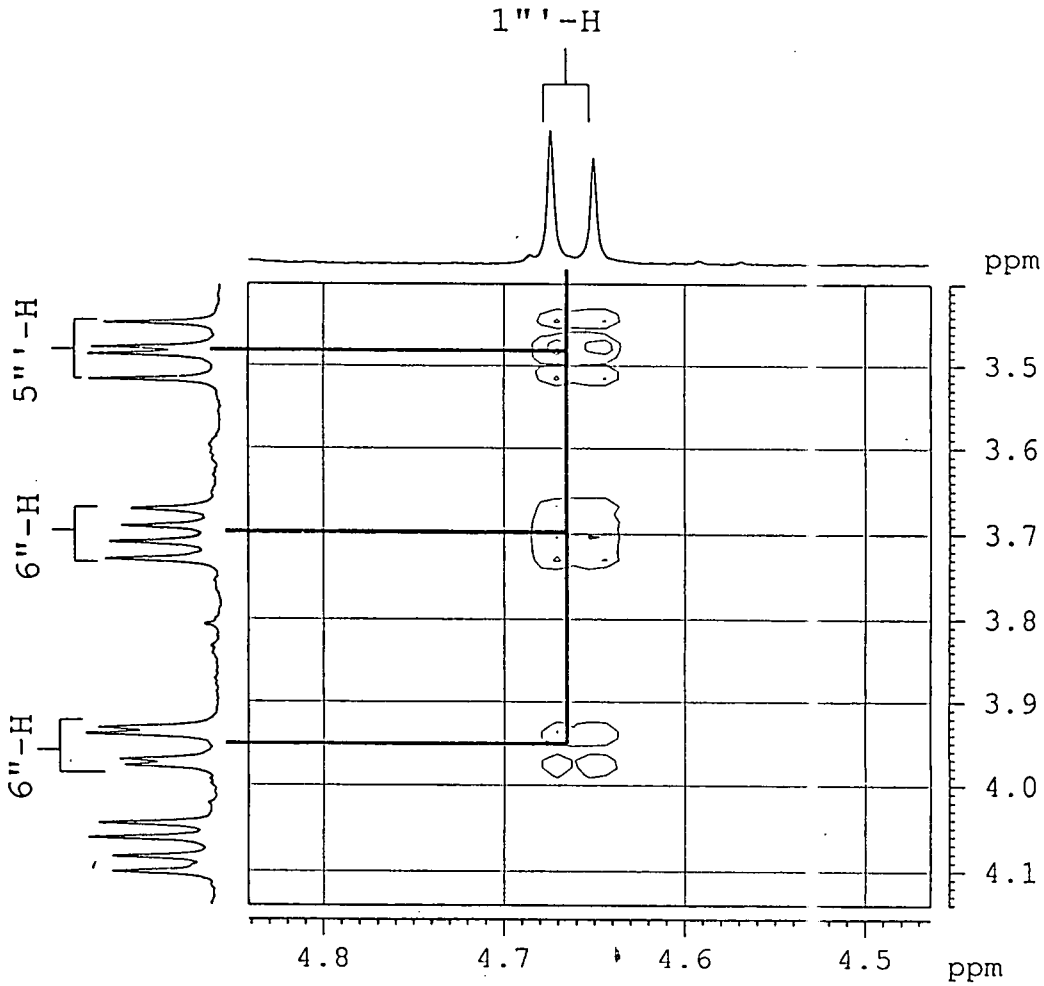
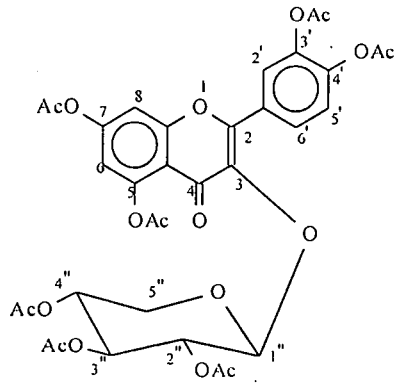




[Plate 7 (CDCl3 - 296K)]

^{1}H NMR



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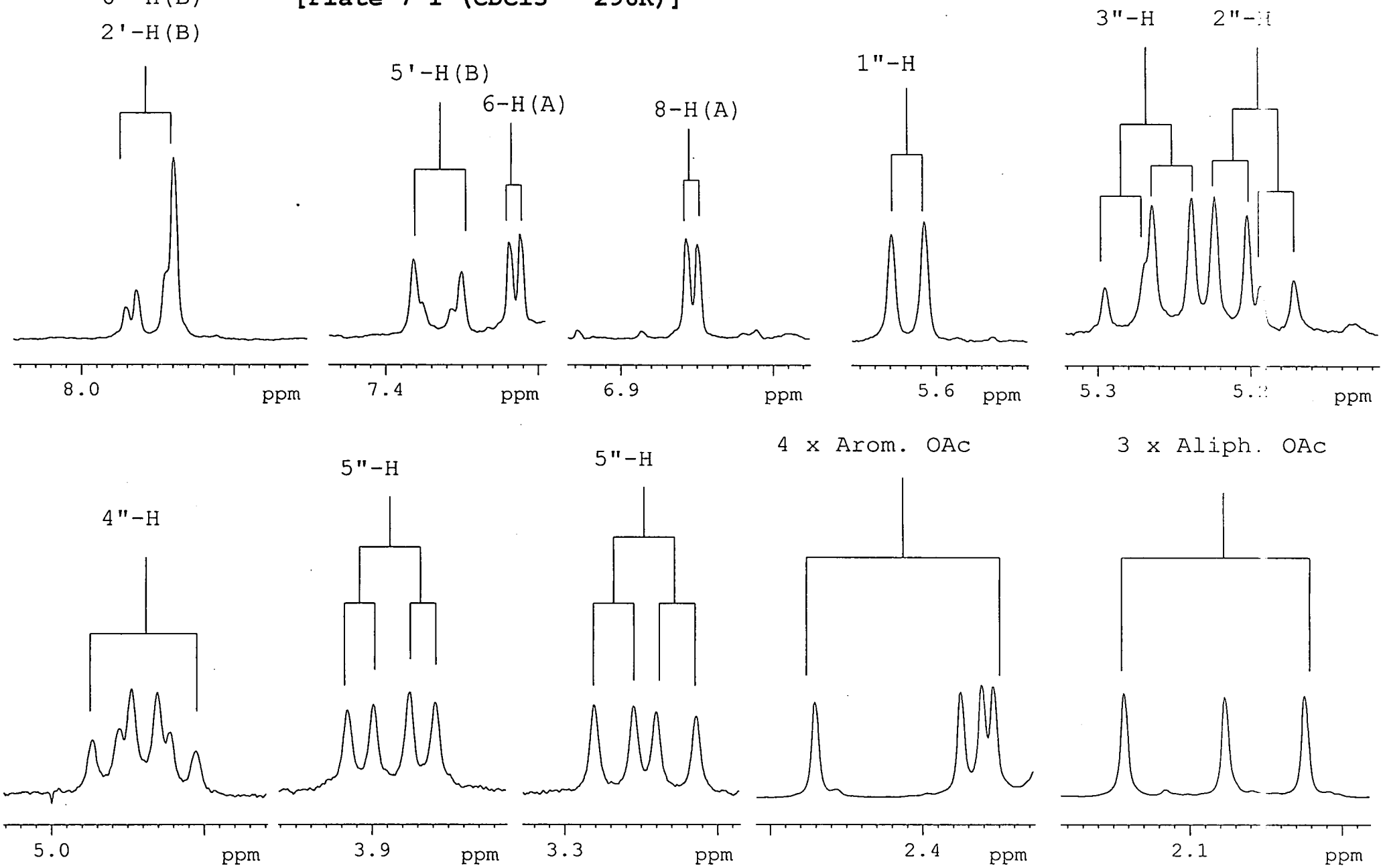
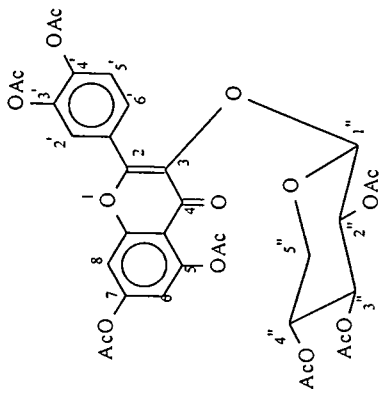
[Plate 7 1 (CDCl3 - 296K)]



[Plate 7a 1 (CDCl3 - 296K)]
COSY



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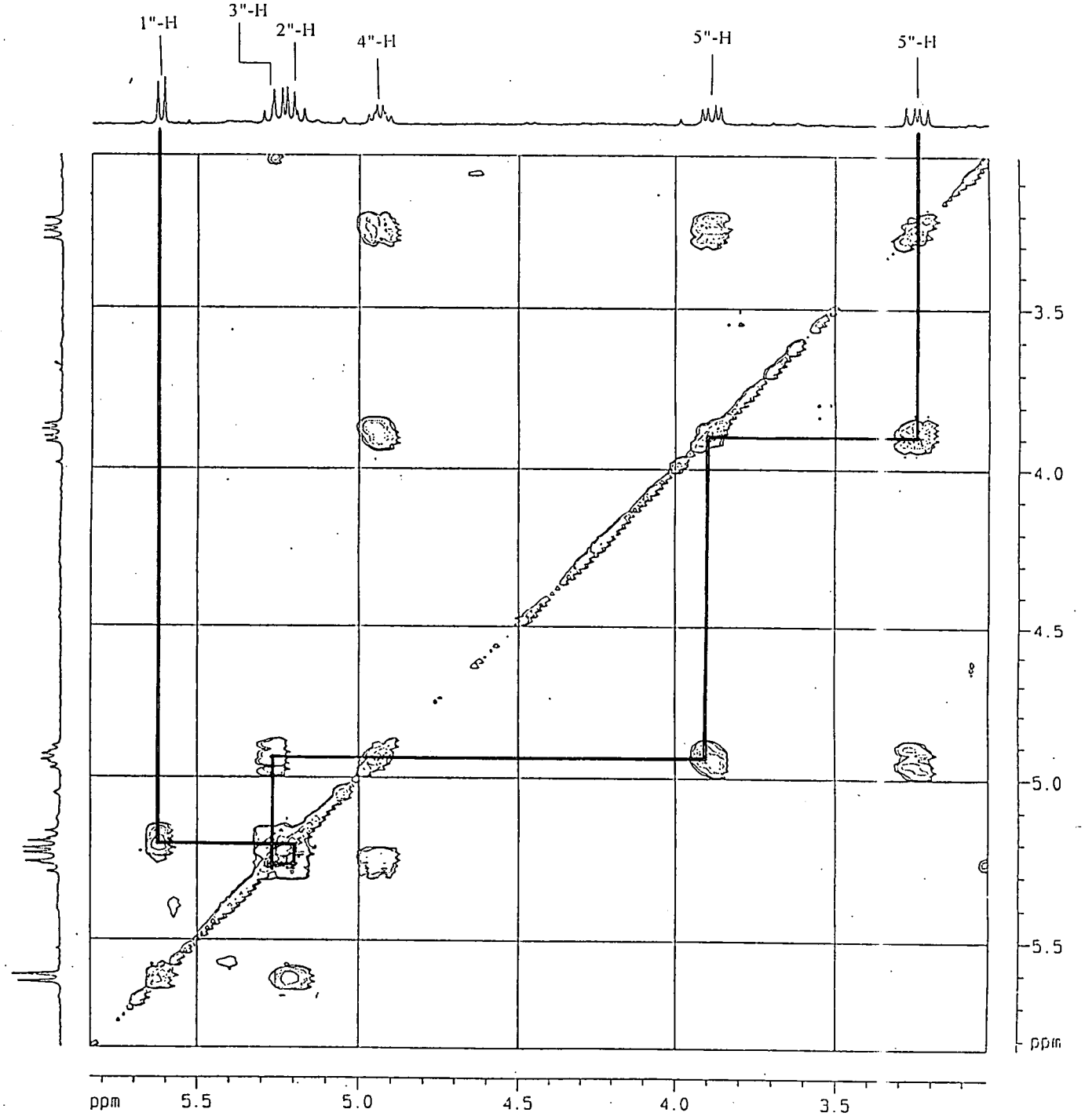

[Plate 7b 1 (CDCl3 - 296K)]
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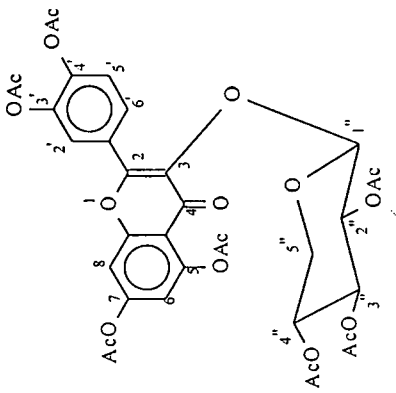

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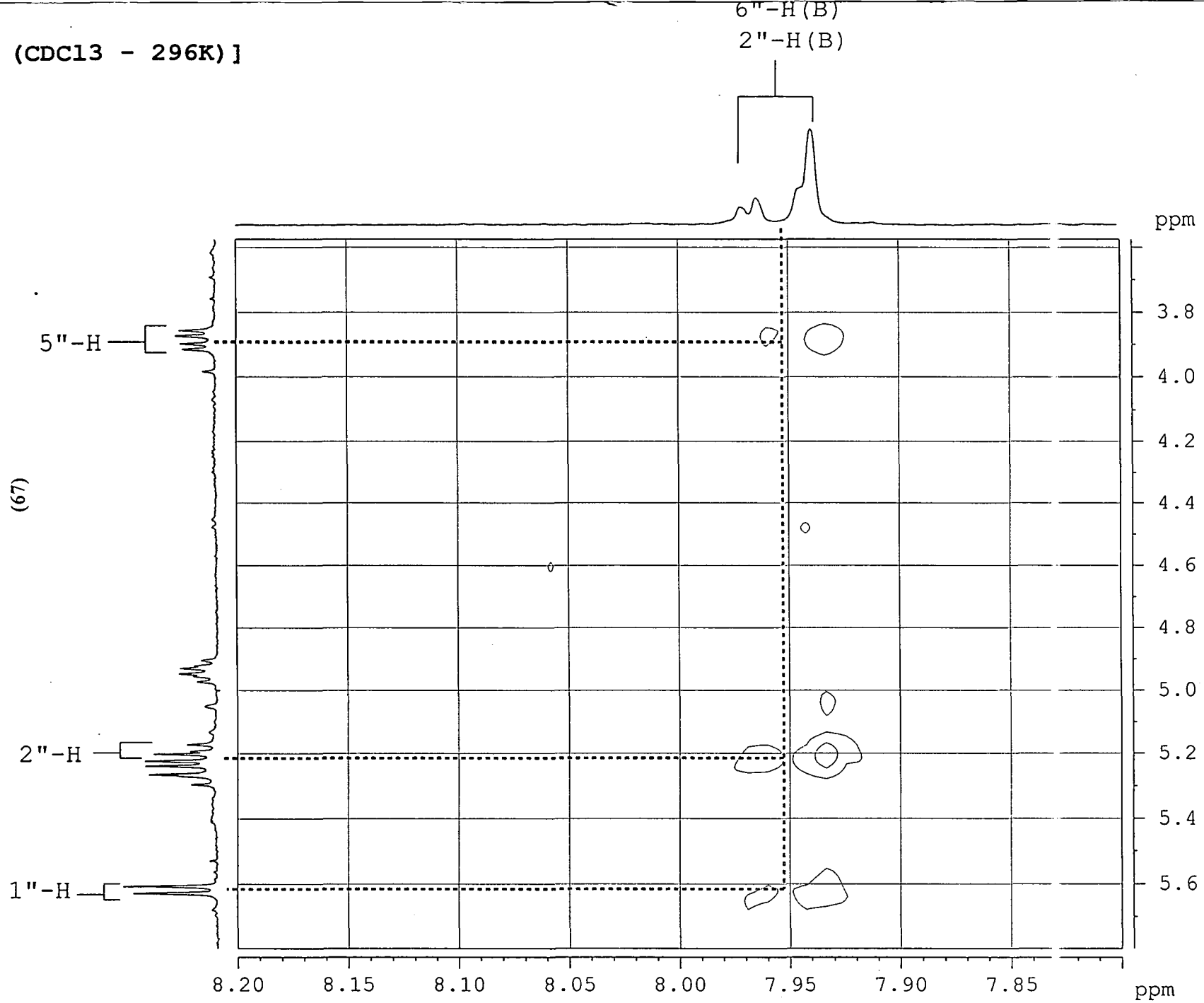

[Plate 7b 2 (CDCl3 - 296K)]
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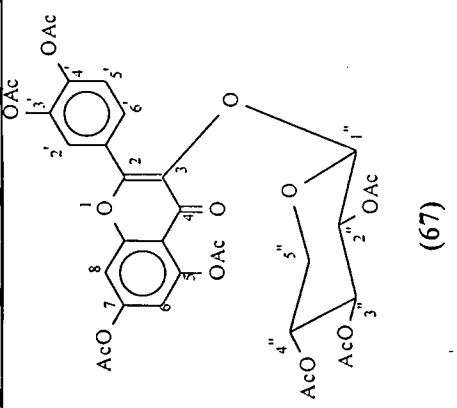

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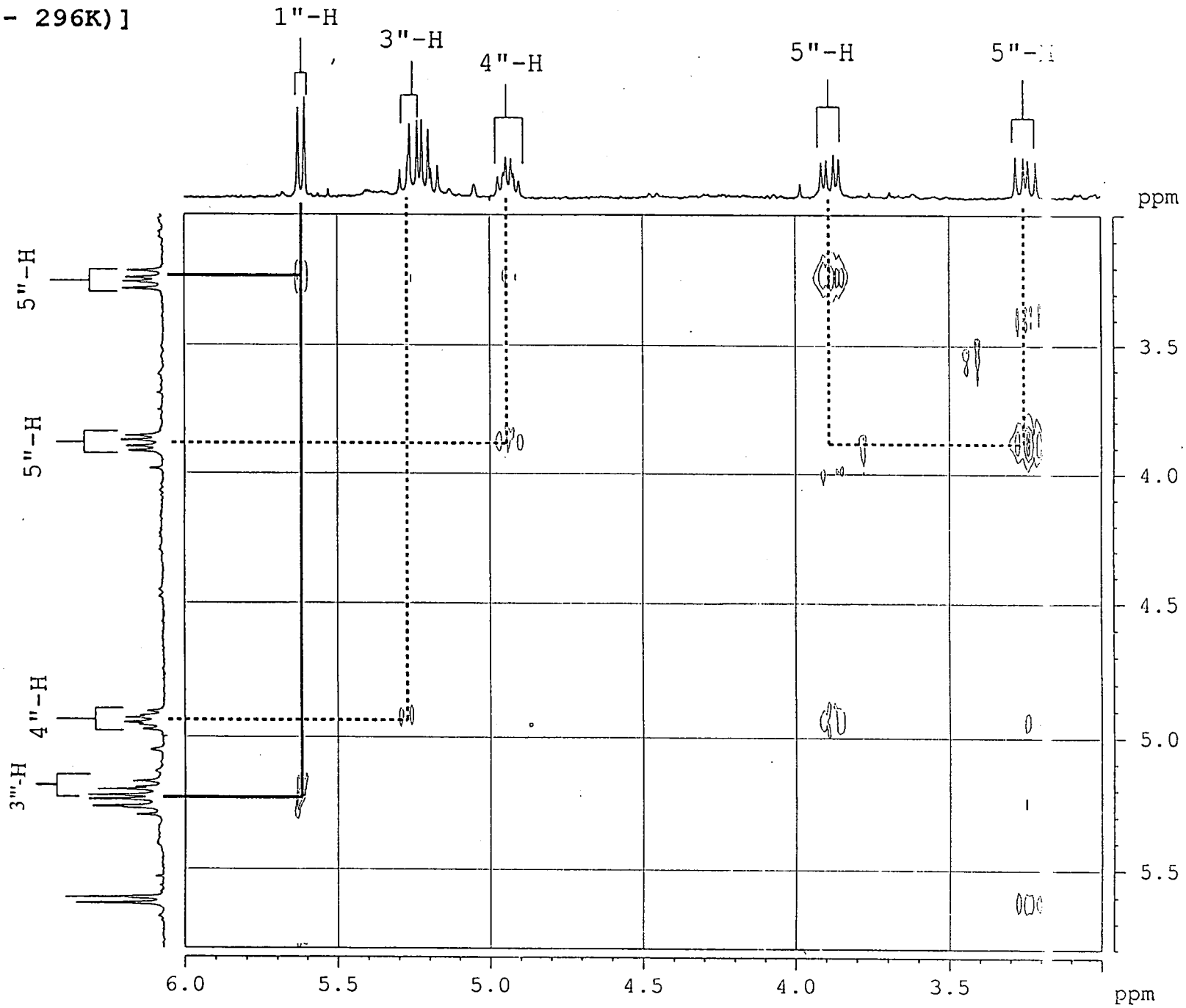
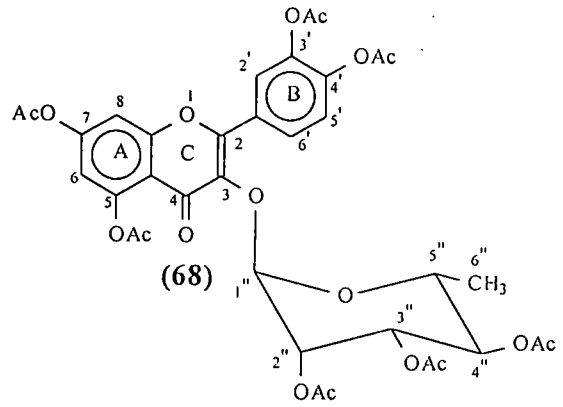


[Plate 8 (C6D6 - 298K)]

^{1}H NMR



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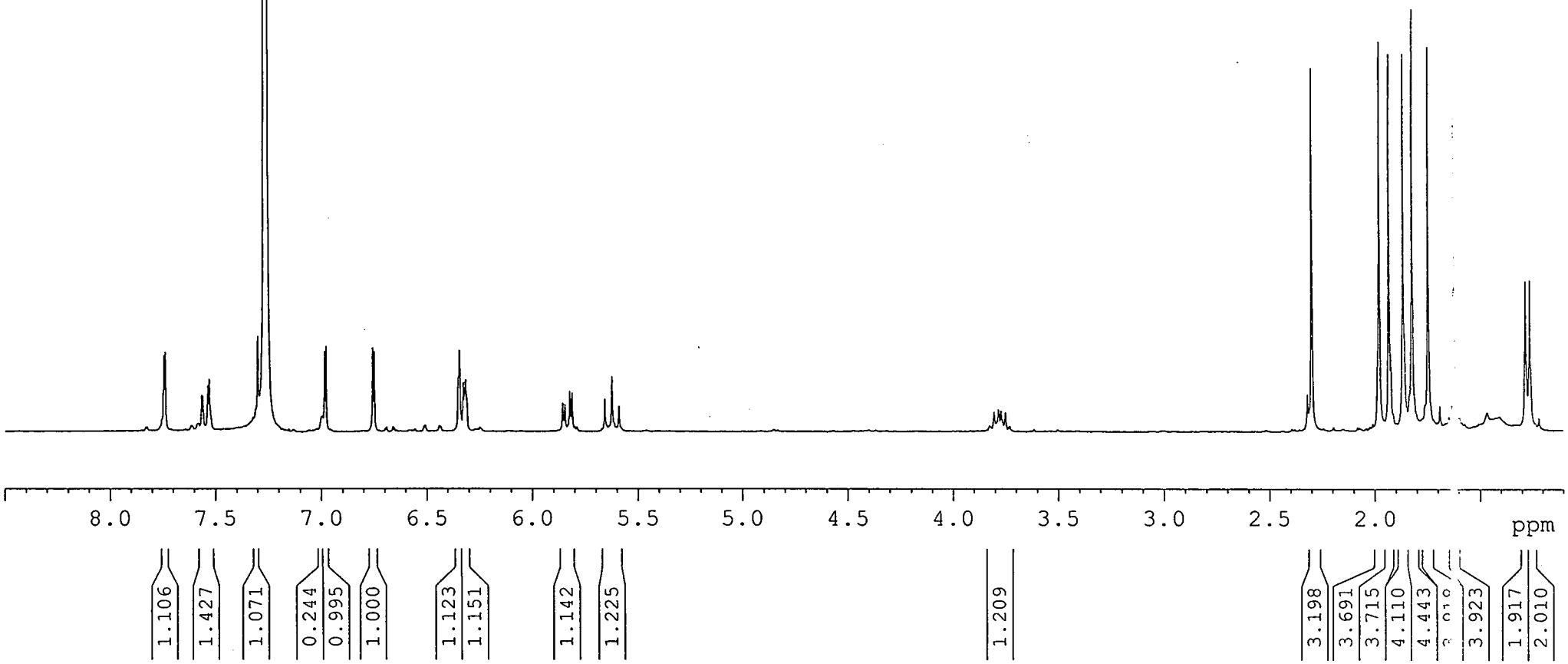
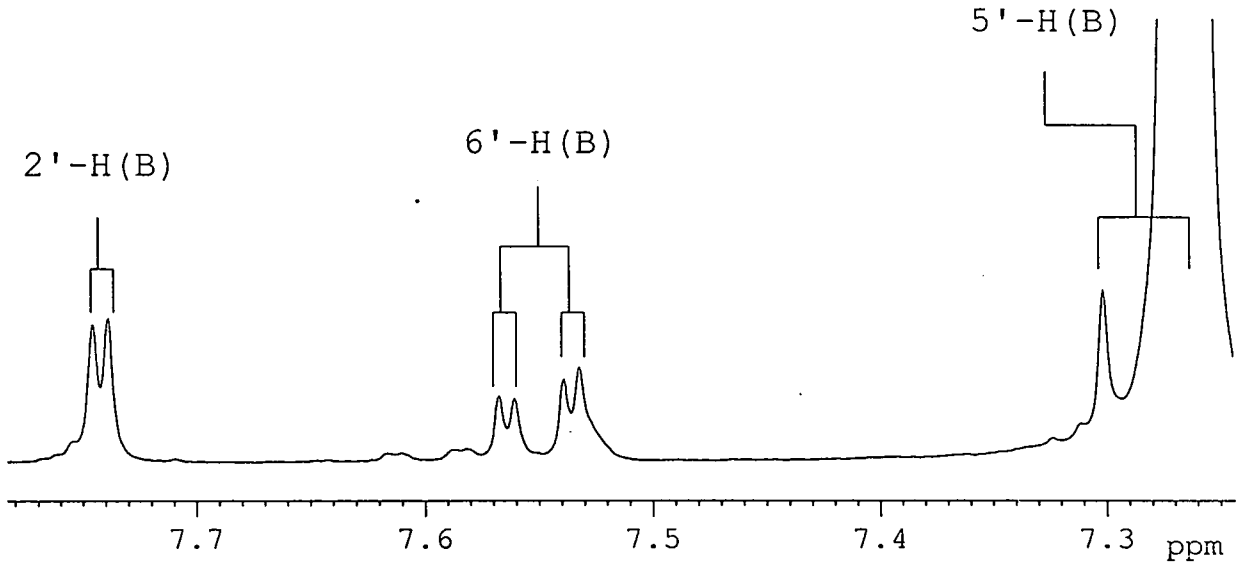
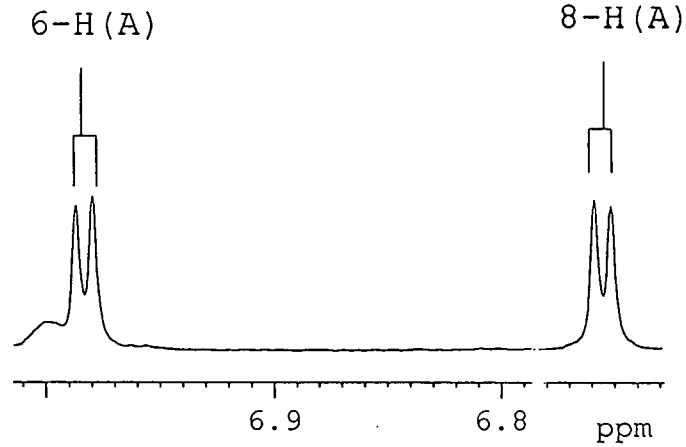
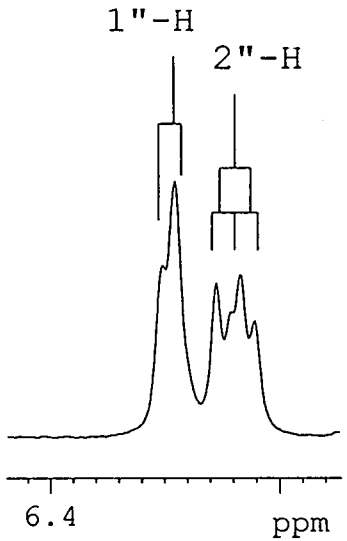
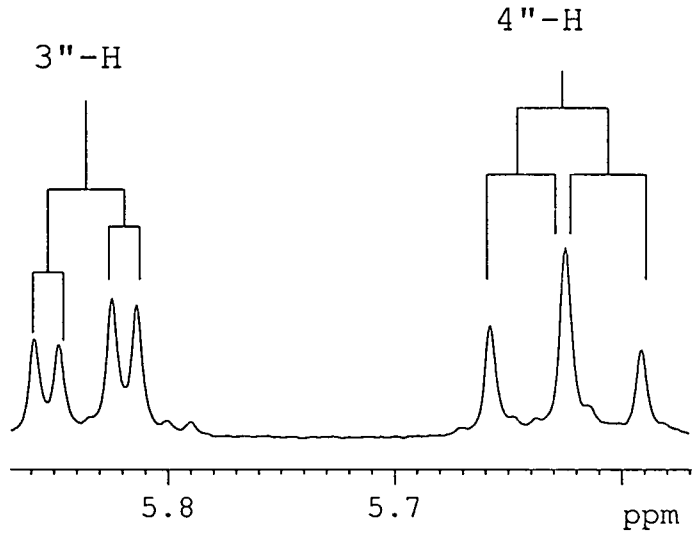


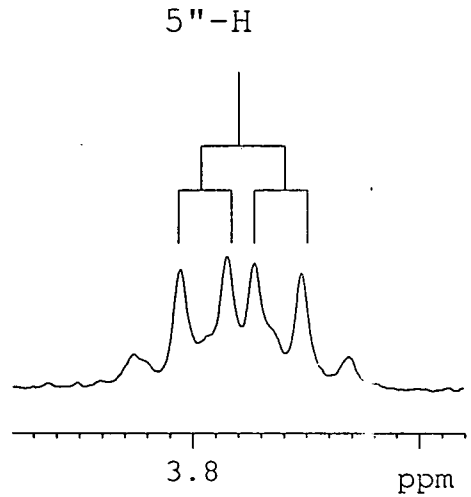
[Plate 8 1 (C6D6 - 296K)]



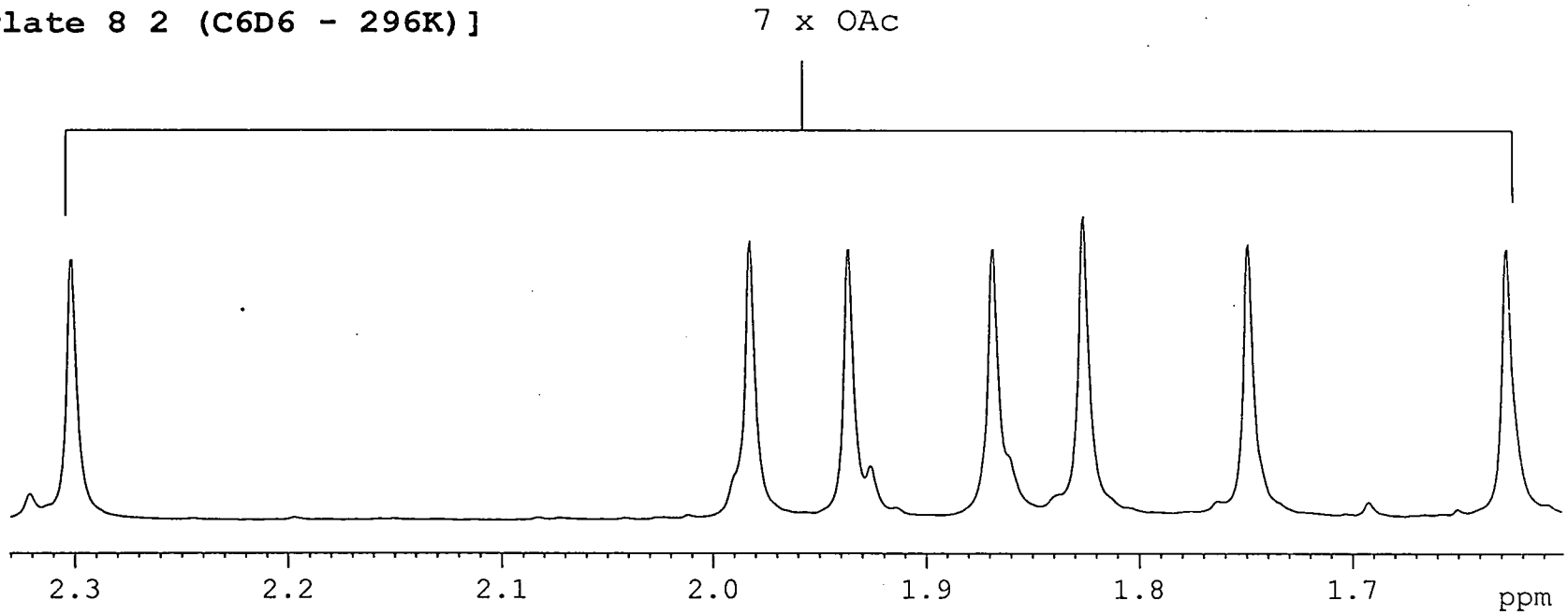


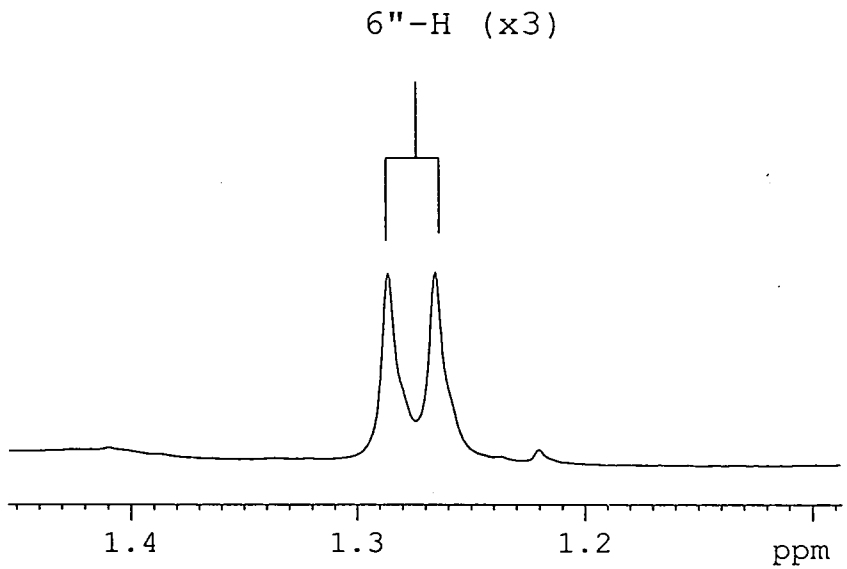




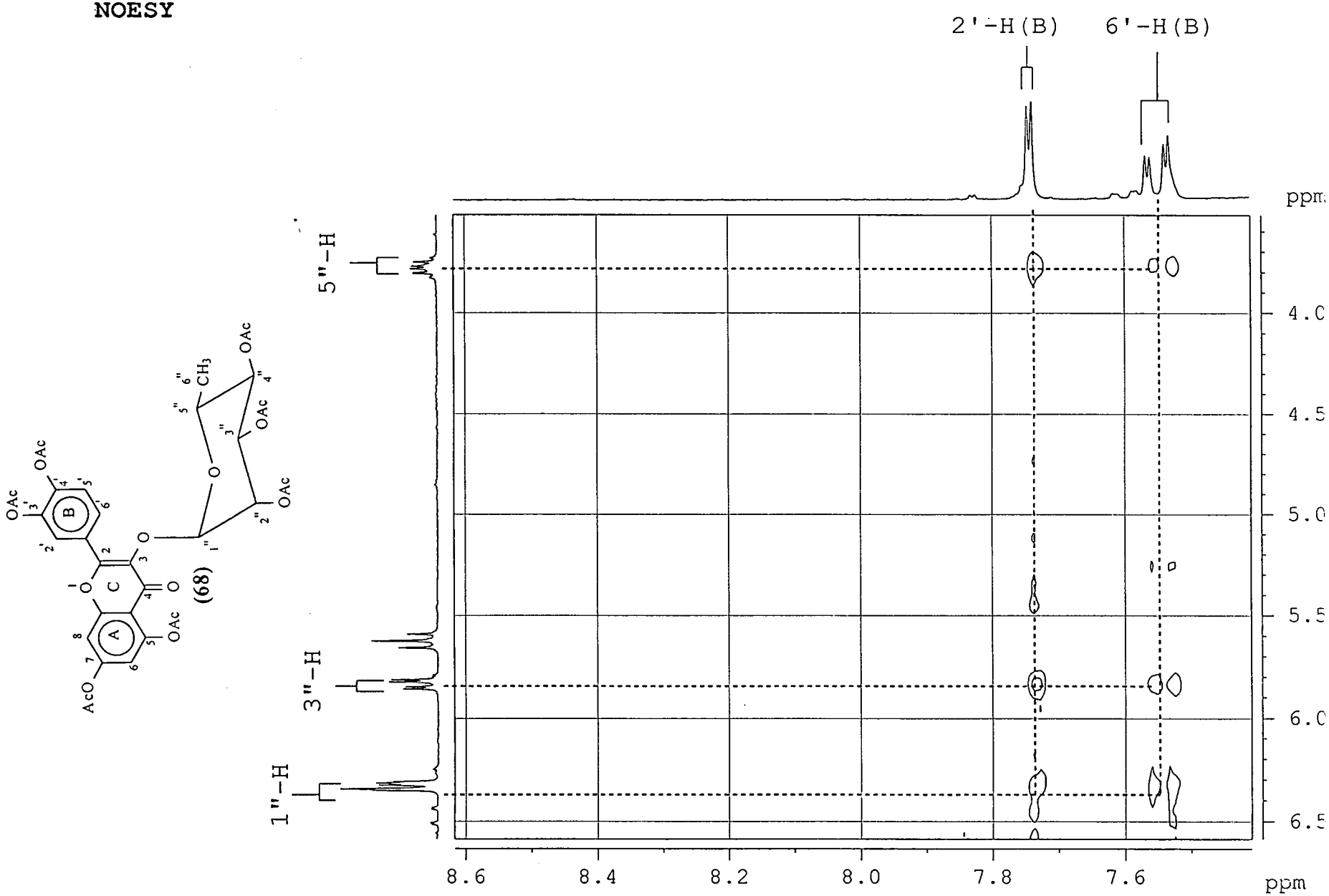


[Plate 8 2 (C6D6 - 296K)]

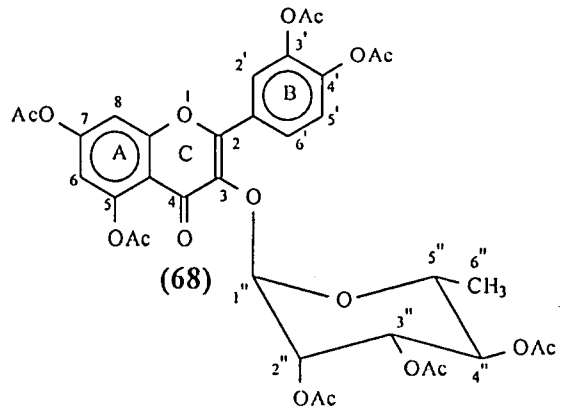




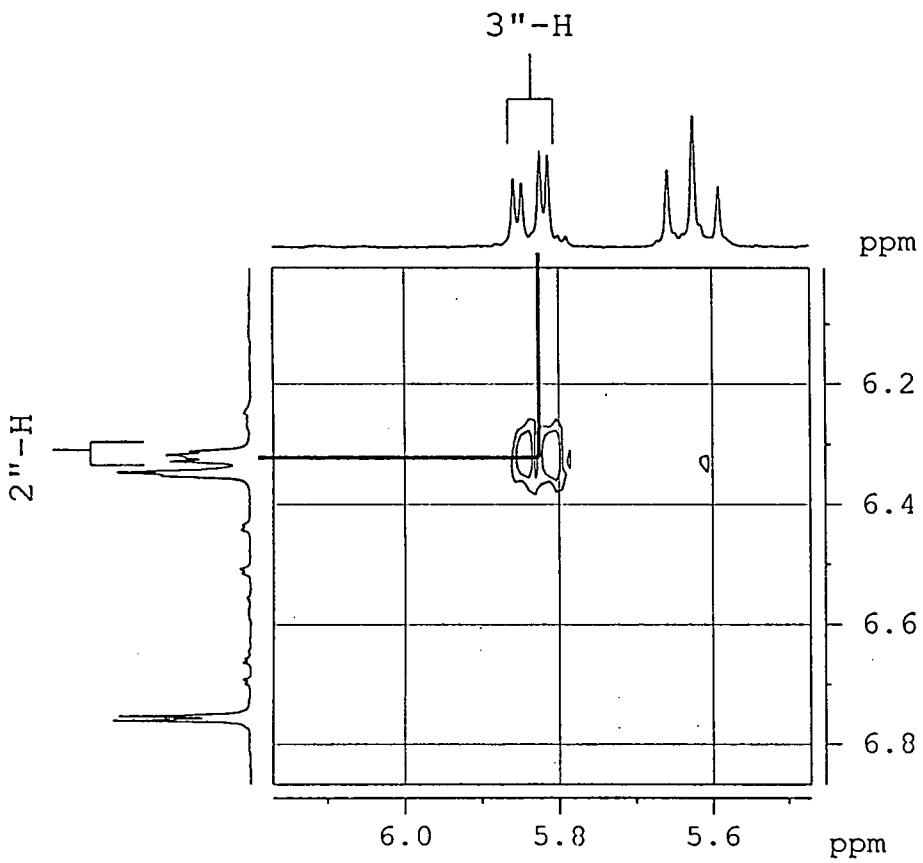
[Plate 8b 1 (C6D6 - 296K)]
NOESY

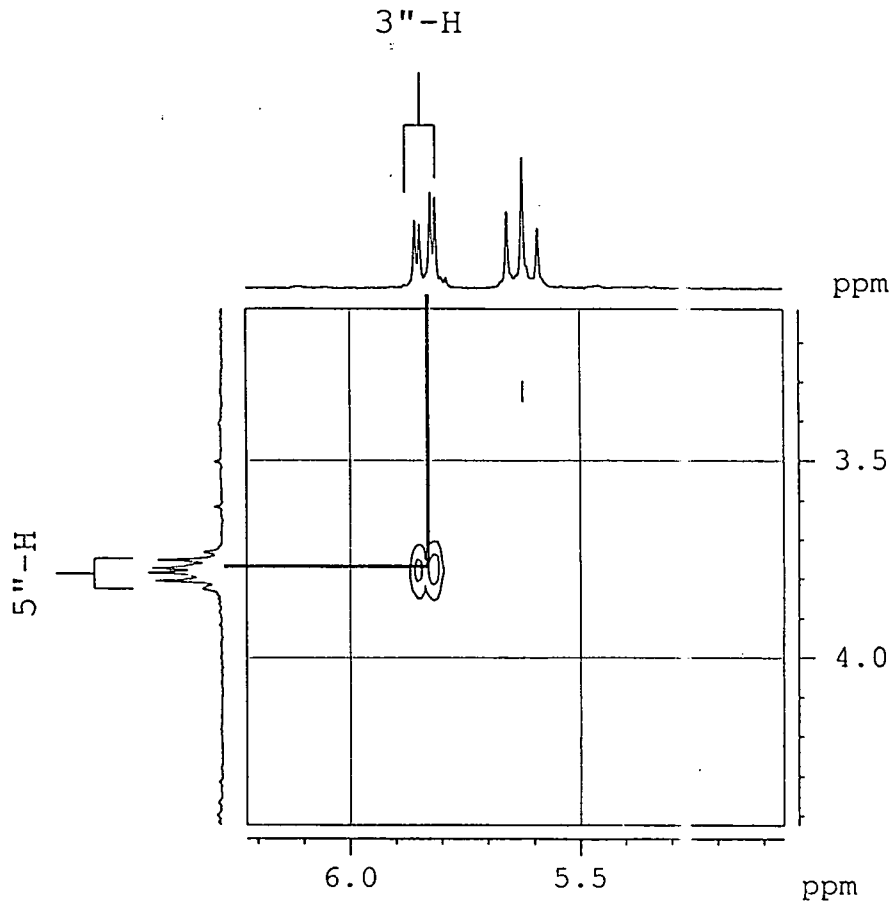


[Plate 8b 2 (C6D6 - 296K)]
NOESY



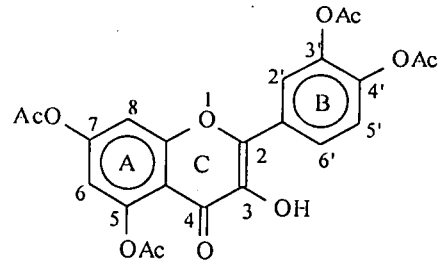
(68)





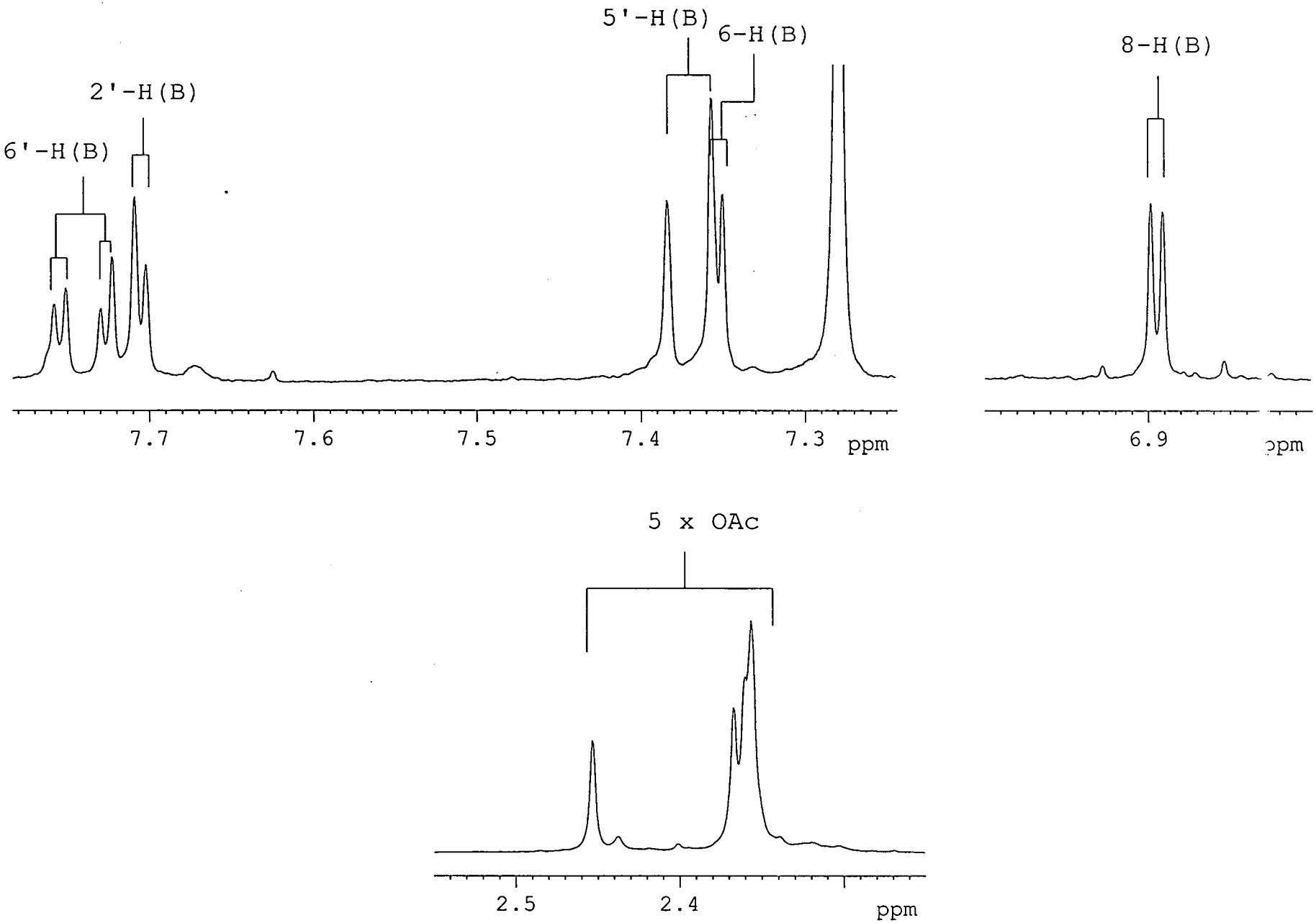
[Plate 9 (CDCl3 - 296K)]

^{1}H NMR



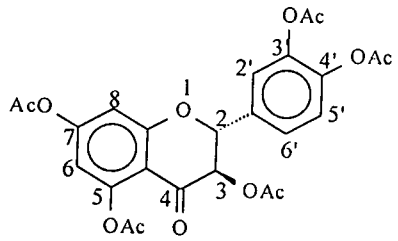
(69)

[Plate 9 1 (CDCl3 - 296K)]



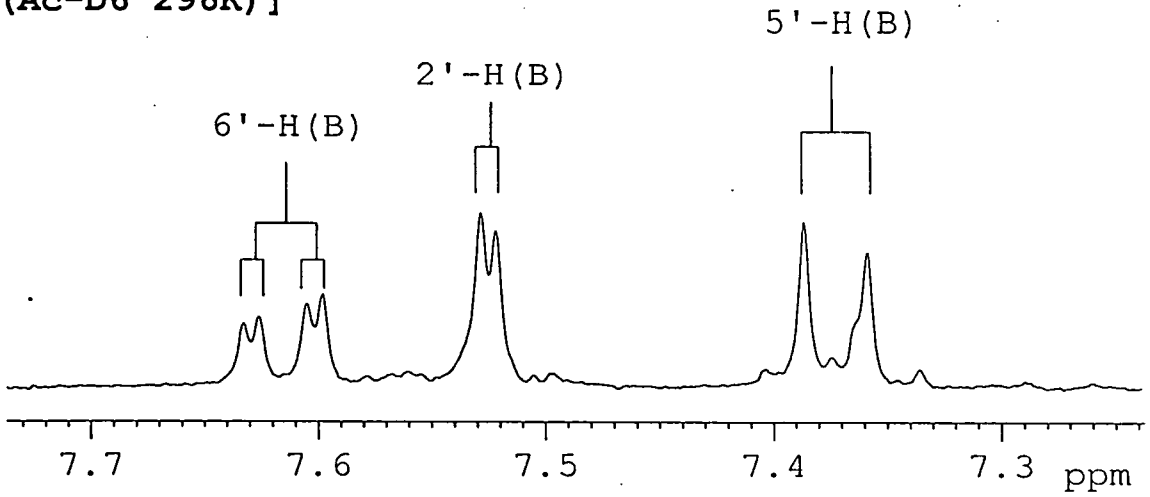
[Plate 10 (Ac-D6 - 296K)]

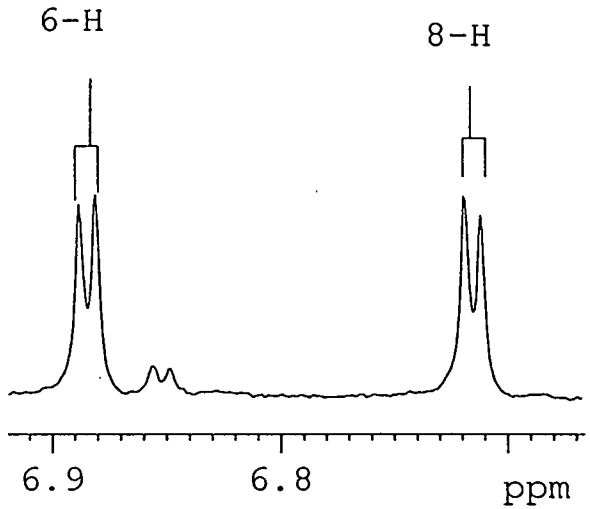
^{1}H NMR

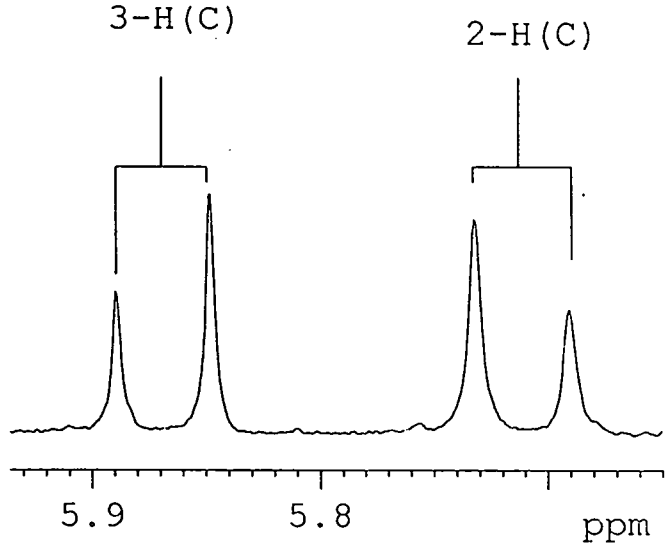


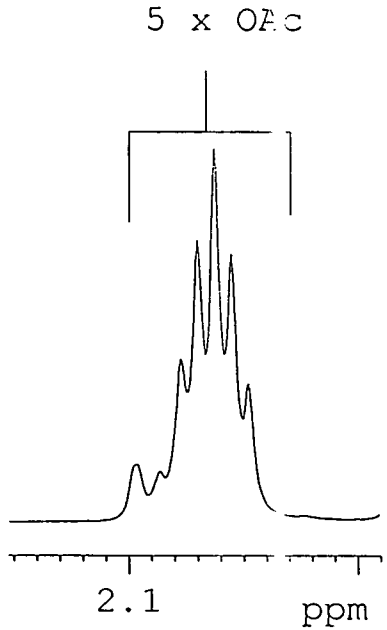
(70)

[Plate 10 1 (Ac-D6 296K)]

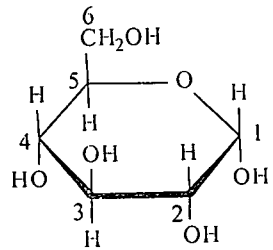






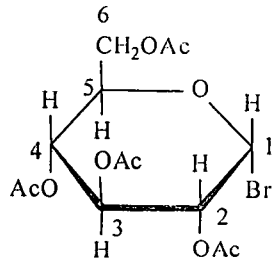


[Plate 11 (CDCl3 - 296K)]



(83)

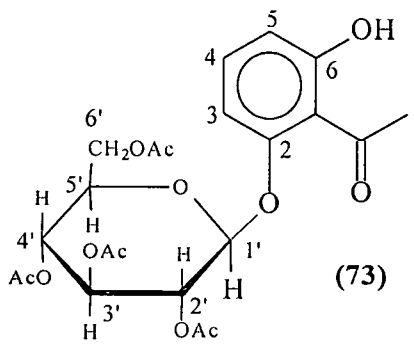
[Plate 12 (CDCl3 - 296K)]



(72)

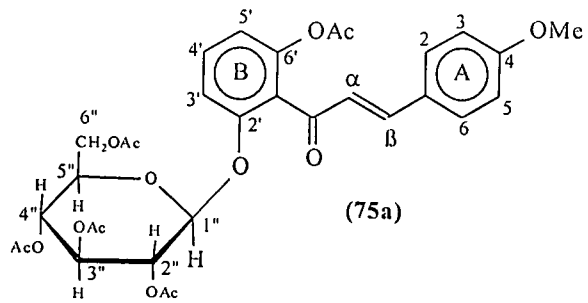
[Plate 13 (CDCl3 - 296K)]

^{1}H NMR



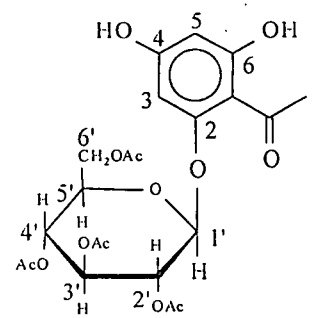
(73)

[Plate 14 (CDCl3 - 296K)]



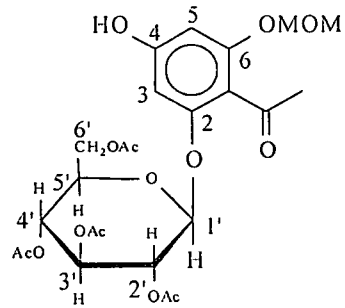
(75a)

[Plate 15 (CDCl3 - 296K)]

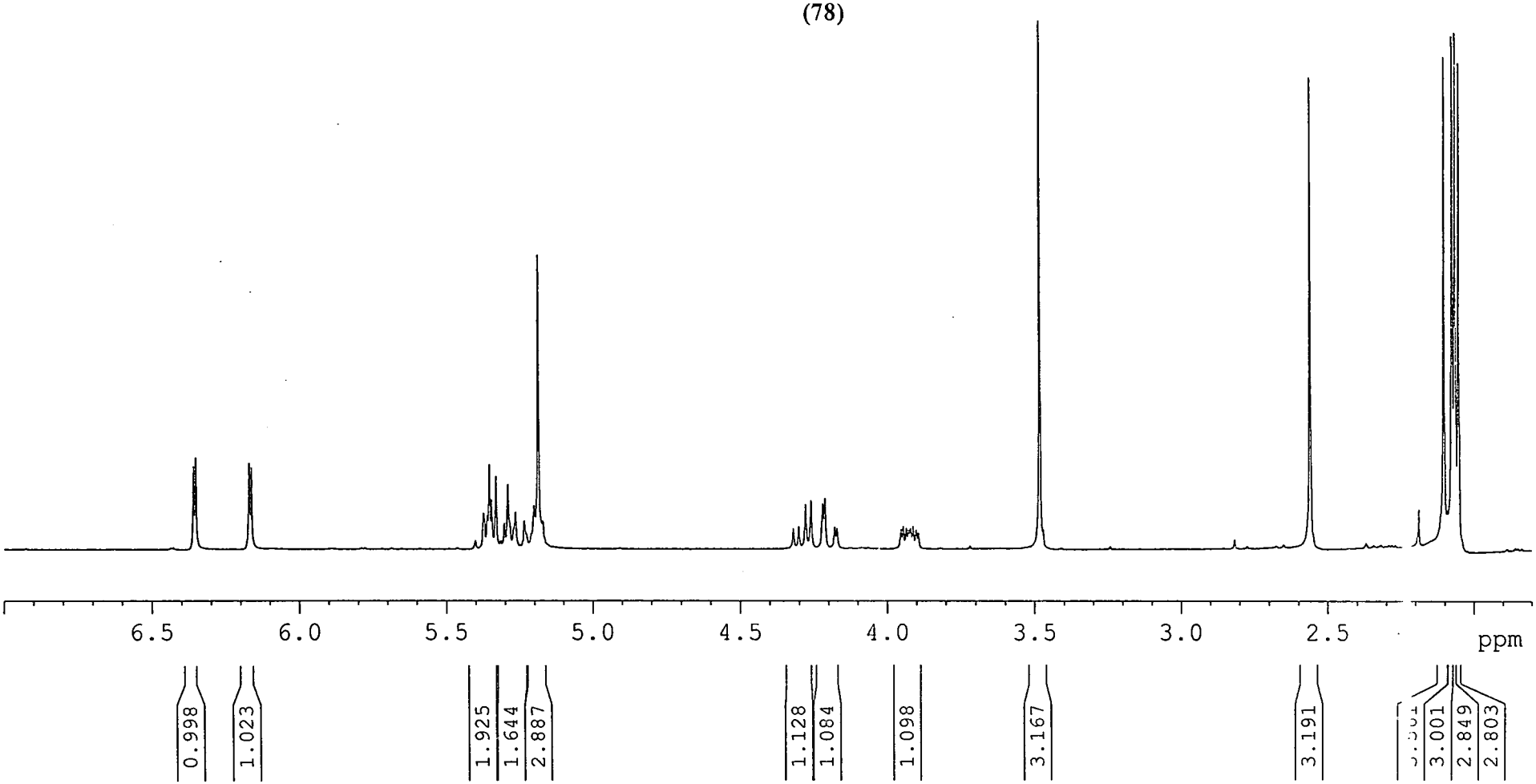


(77)

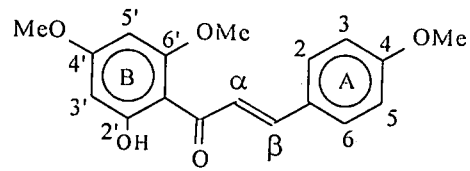
[Plate 16 (CDCl3 - 296K)]



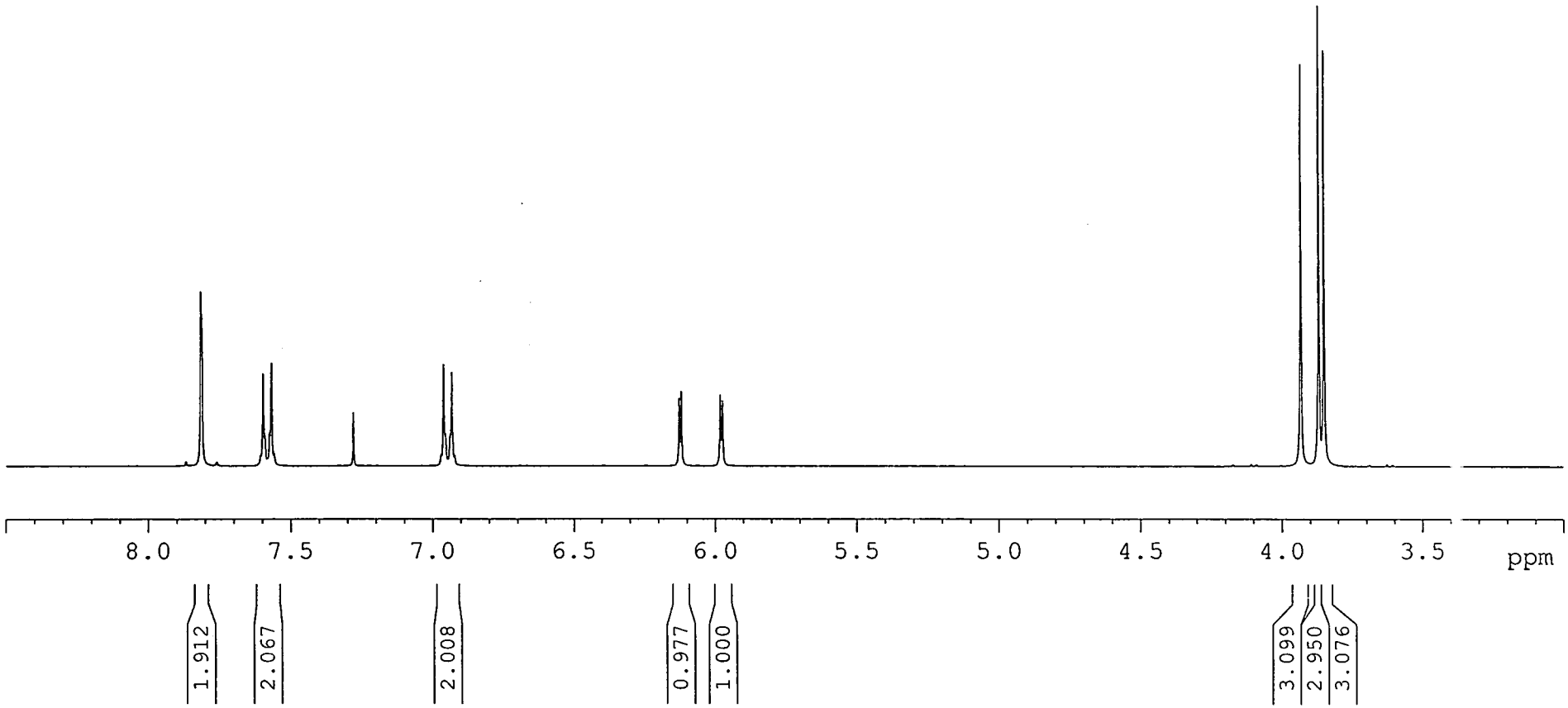
(78)



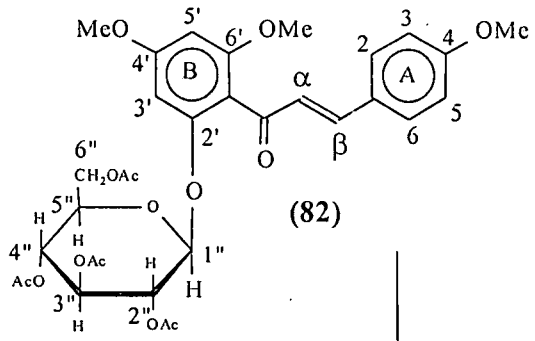
[Plate 17 (CDCl3 - 296K)]

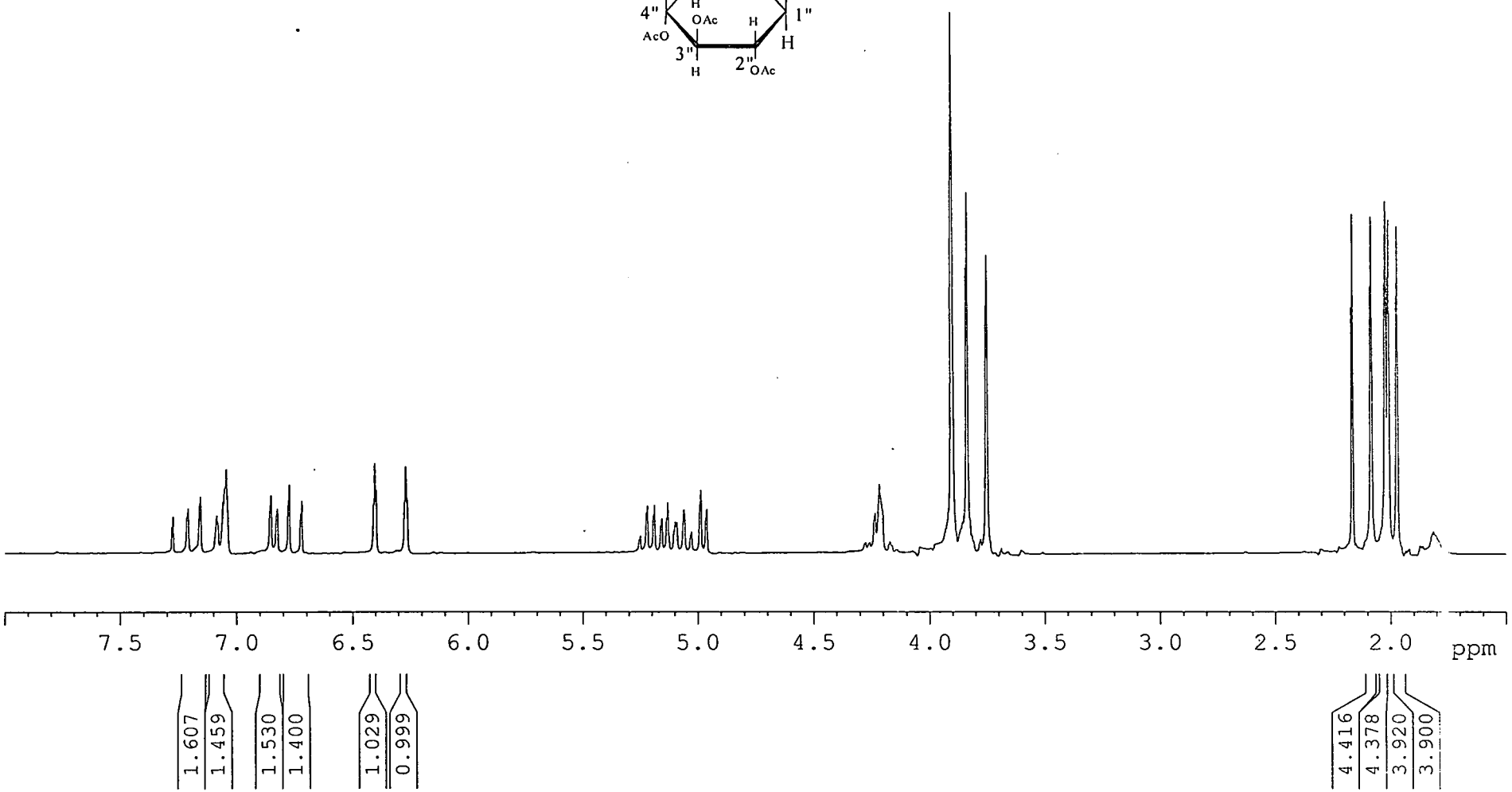


(80)

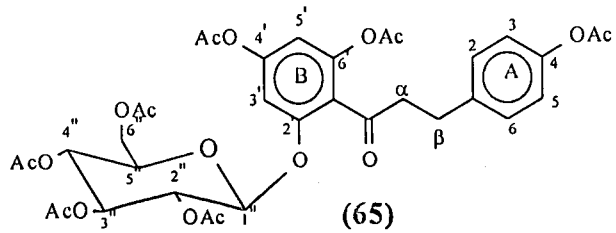


[Plate 18 (CDCl3 - 296K)]





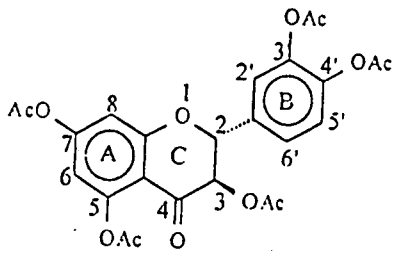
[Plate 22 (CDCl3 - 296K)]
^{13}C NMR



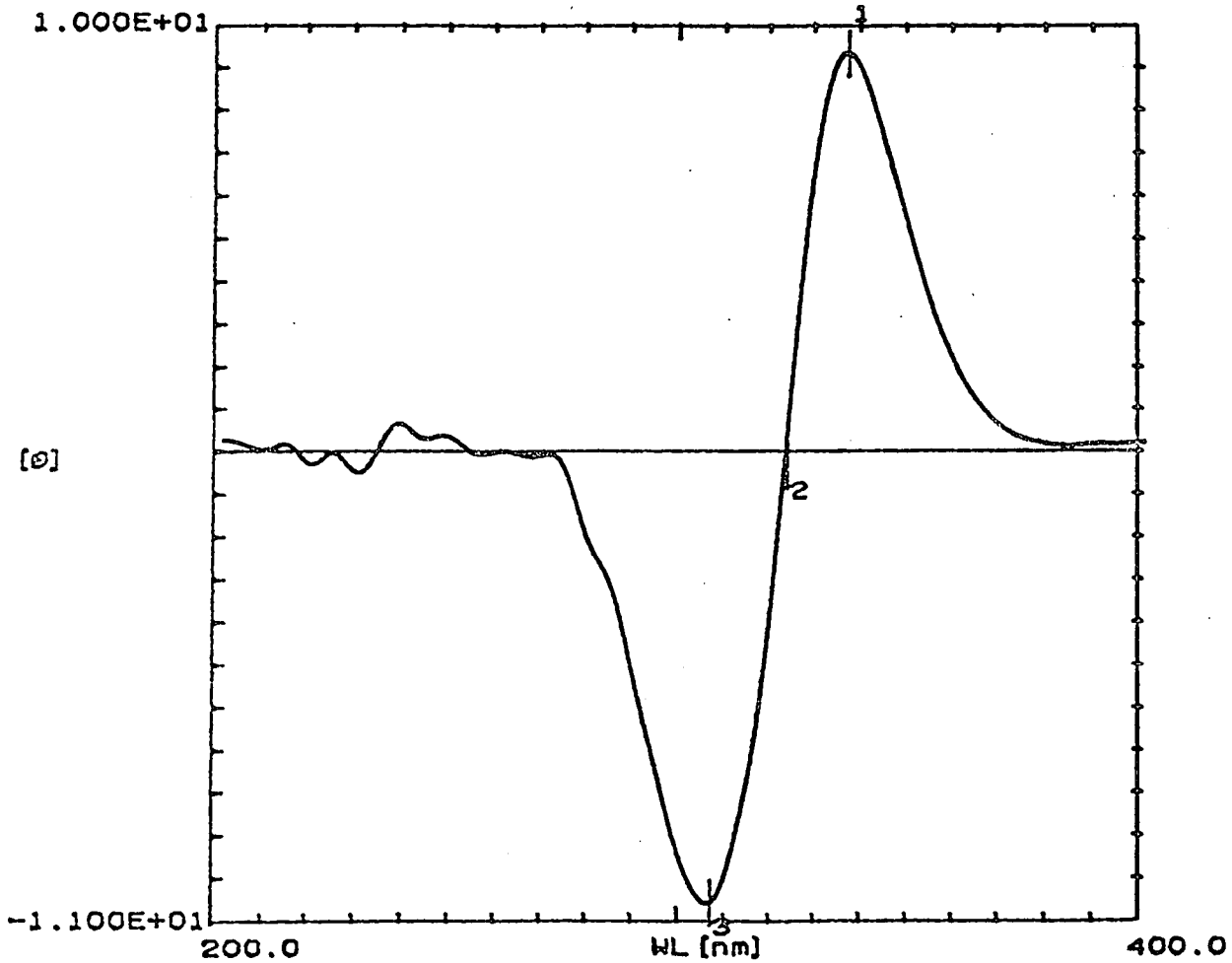
(65)

APPENDIX C

[Plate 19 (1.02mg/10ml in MeOH)]
CD Spectrum

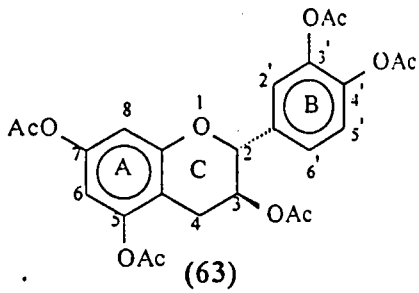


(70)

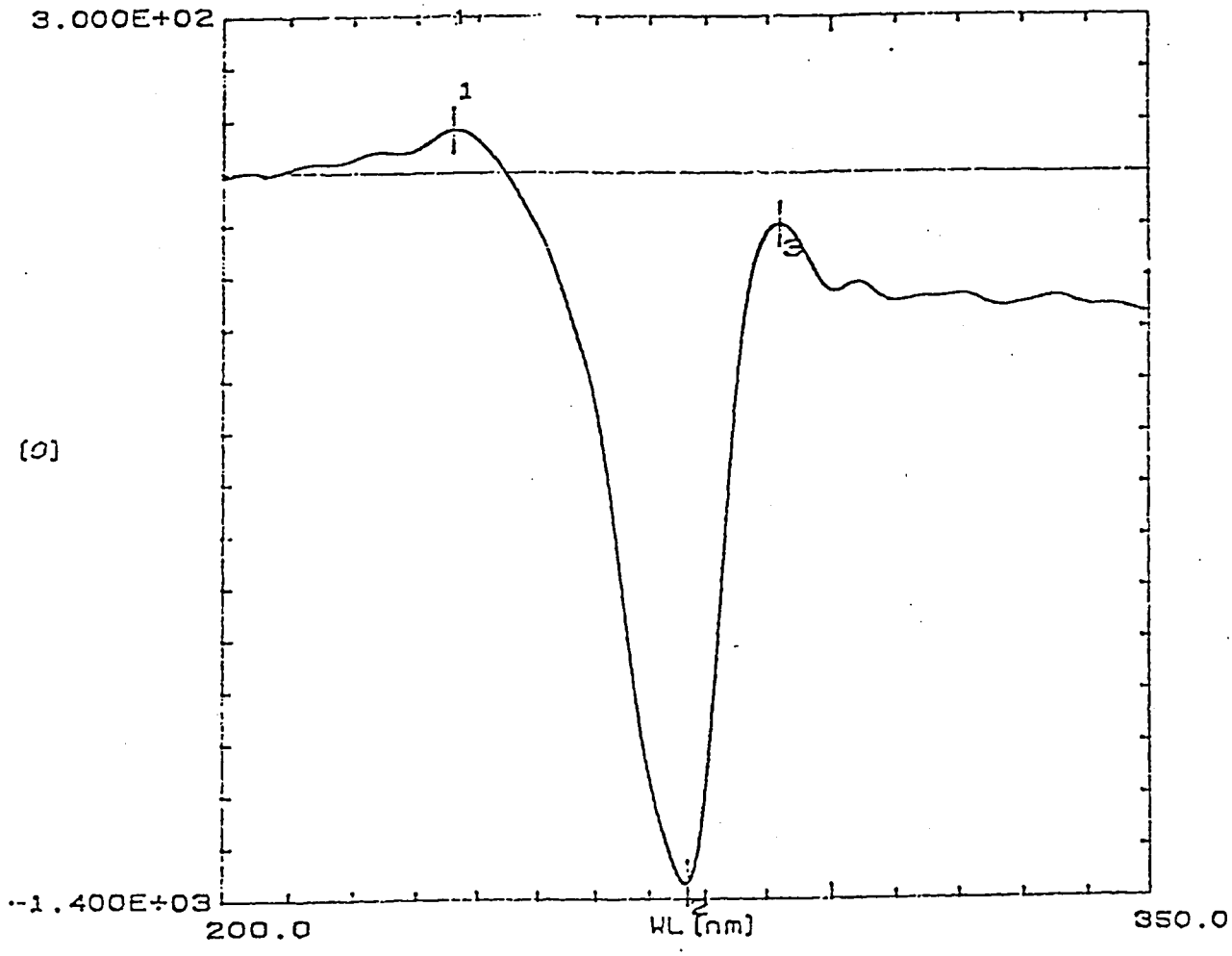


No	Wavelength	Value
1	335.60 nm	9.267E+00
2	322.10 nm	-3.950E-01
3	[illegible]	[illegible]

[Plate 20 (1.01mg/10ml in MeOH)]
CD Spectrum

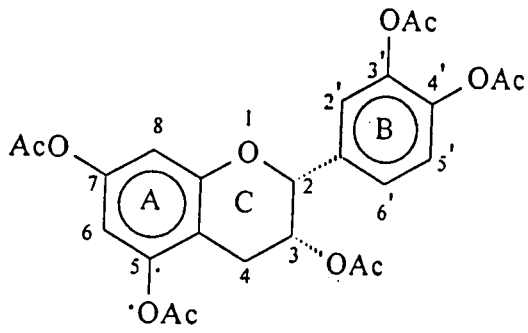


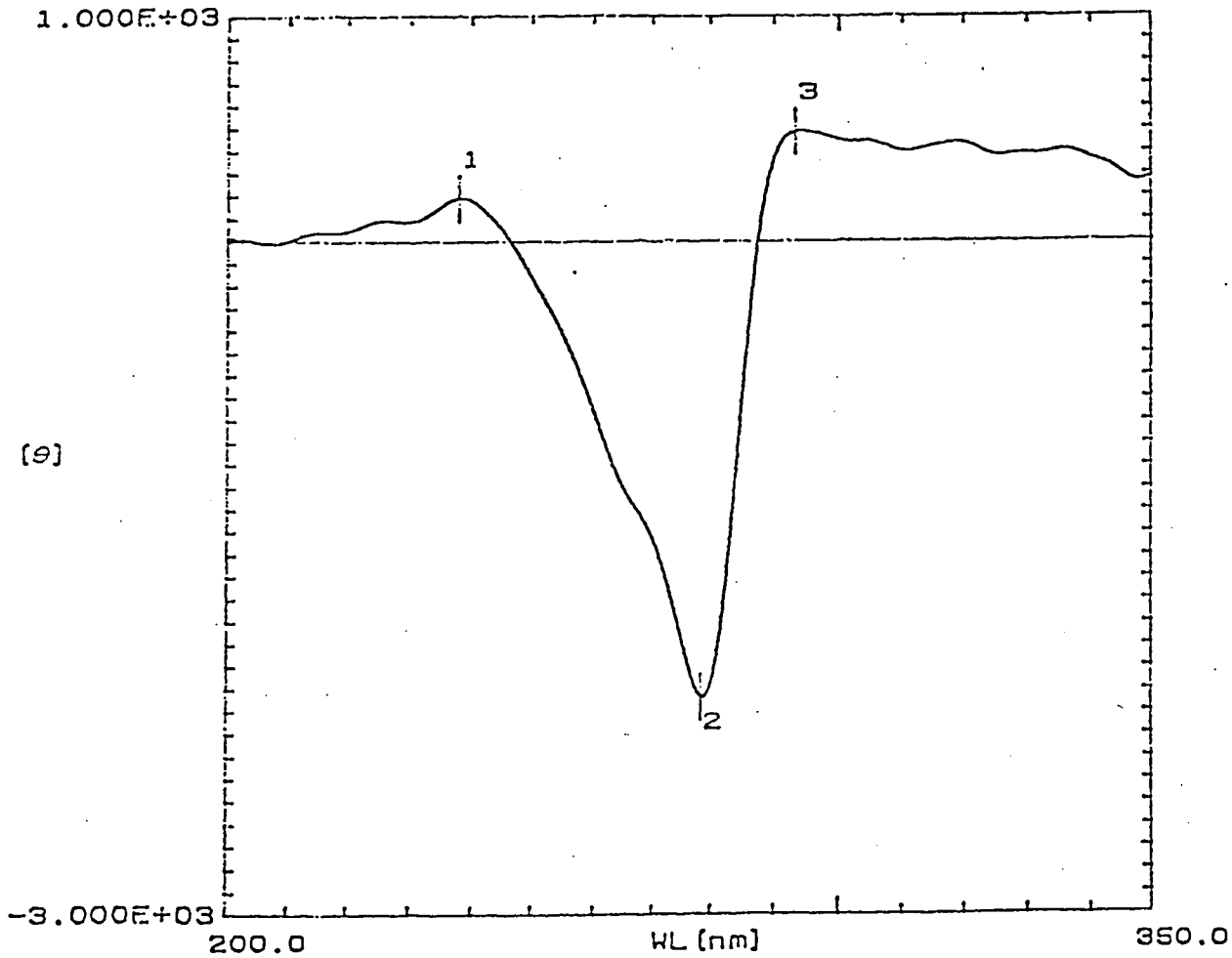
(63)



No.	Wavelength	Value
1	235.00 nm	2.214E+02
2	277.00 nm	-1.367E+03
3	292.00 nm	-9.890E+01

[Plate 21 (0.99mg/10ml in MeOH)]
CD Spectrum





No.	Wavelength	Value
1	237.90 nm	1.947E+02
2	278.40 nm	-2.033E+03
3	293.40 nm	4.870E+02

OPSOMMING

Sleutel woorde: Appels, applebier, polifenole, flavanoïede, dihidrochalkone, glikosiede, chalkooon glikosied sintese, antioksidante, phloridzin, hoë-resolusie KMR.

Appelbier, gefermenteer van 'n verskeidenheid appel cultivars, bevat 'n omvattende groep polifenole, insluitend flavanoïede. Polifenoliese verbindings is krities in die produksie van versapte produkte. Hierdie verbindings speel 'n belangrike rol in smaak, geur en die kleur van sappe en hul produkte.

Die appelbier wat ondersoek is, word sinteties versoet na fermentering m.b.v. suikerriet by-produkte. In ons ondersoek is beide kommersiële beskikbare en gedeeltelik verwerkte produkte ondersoek. Ekstraksie met etielasetaat gevolg deur chromatografiese skeiding (kolom Sephadex en dun-laag) het flavonole, dihidrochalkone, flavan-3-ols, dihidroflavonols en een C_6C_3-tipe fenol opgelewer. Die struktuur van hierdie verbindings is gekarakteriseer hoofsaaklik m.b.v. hoë-resolusie (300 MHz) Kern Magnetiese Resonans spektrometrie (insluitend NOESY, COSY, DEPT en ^{13}C KMR eksperimente). Al die verbindings geïsoleer uit die bier kom in die *Malus* genus (appels) voor. Een verbinding, taxifolin, is nie van te vore uit appel bier geïsoleer nie.

Die phloretin glikosied, phloridzin, is kenmerkend tesame met die diglikosied, 2',4',6',4-tetrahidroksie-dihidrochalkoon-2'-*O*-β-D-(6"-β-D-xylopiranosiel)-β-D-glikopiranosied geïsoleer uit die appelbier. Quercetin en twee analoë glikosiede, quercetrin en 3',4',5,7-tetrahidroksie flavonol-3-β-D-arabinopiranosiel is ook geïsoleer.

Sintese van phloridzin, wat as 'n model reaksie vir die sintese van dihidrochalkone met meer komplekse glikosiede kan dien, is probeer. 'n Glukosied is verbind aan 'n toepaslike asetofenoon en gebruik in 'n basis-gekataliseerde aldol kondensasie met 'n benzaldehied, wat die chalkoon voorloper vir dihidrochalkone gelewer het. In die tweede prosedure is die chalkoon eerste gesintetiseer d.m.v. dieselfde aldol-tipe reaksie, gevolg deur reaksie met die glukosied.

Alhoewel die sintese van chalkone d.m.v. aldol kondensasies 'n algemene hoë-opbrengs reaksie is, is probleme met die kondensasie geantisipeer en ook ondervind. Sintese van chalcone glikosiede is wel vermag, maar geen aanvaarbare beskerming en ontskerming protokol kon vasgestel word nie.

Flavanoïede is 'n belangrike deel van polifenole in appelsap en die vermoë van hierdie verbinding om 'n verskeidenheid biologiese funksies te beïnvloed is al vasgestel. Antioksidante is onlangs meer intensief ondersoek en die klaarblyklike voordele wat hierdie verbindings vir die menslike gesondheid inhou, maak 'n begrip van hierdie verbindings in appelsap en produkte (bv. appelbier) belangrik. Vitamiene E en C, flavanoïede en ander polifenole dien as primêre antioksidante, wat superoksiede, hidroksie- en peroksie-radikale opneem. Die resultate van hierdie ondersoek wys dat verbindings met potensiële antioksidant eienskappe wel in appelbier voorkom.