University of Leicester
(Nene College)

The Minor Theaflavins of Black Tea

A thesis submitted in fulfilment of the requirements of a
Master of Philosophy Degree
by

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Completed at Unilever Research, Sharnbrook, Bedfordshire,
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A fool who persists in his folly eventually becomes wise.
[Anon].

Perhaps.

Abstract

This thesis describes the isolation, characterisation and synthesis of three novel tea pigments.

This isolation was achieved by the solvent extraction of a Malawian black tea (Ruo Mimosa) followed by the chromatography of the ethyl acetate-soluble portion on Sephadex LH-20 (ethanol elution), which produced three fractions containing orange pigments. Further separation of these fractions under various chromatographic conditions yielded ten orange pigments (**1**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**). These compounds have been structurally characterised by a number of spectroscopic techniques including 1D and 2D NMR. Theaflavate B (**1**) has been shown to be formed from (-)-epicatechin-3-*O*-gallate (ECG) and (-)-epicatechin (EC). This is the first compound to be isolated from black tea which contains a benzotropolone moiety formed from a galloyl ester group of a flavan-3-ol gallate. In addition, the theaflavin isomers, isotheaflavin-3'-*O*-monogallate (**3**) and neotheaflavin-3-*O*-monogallate (**4**) have also been characterised. Compound **5** was identified as isotheaflavin, compound **6** as epitheaflagallin-3-*O*-monogallate, compound **10** as theaflavic acid and compound **11** as epitheaflavic acid. Compounds **7** - **9** were found to be theaflavin, theaflavin-3-*O*-monogallate and theaflavin-3'-*O*-monogallate.

This thesis also describes the investigation of the formation of theaflavins and related compounds under chemical oxidation conditions. The results clearly showed the importance of size (galloylation of flavan-3-ol at the 3 position on the C-ring) and configuration (2,3-*cis* vs 2,3-*trans*) of the flavan-3-ol precursors in determining the yields of these compounds. An attempt was made using molecular modelling techniques, to further our understanding of the relative yields of theaflavins obtained in chemical oxidation studies of flavan-3-ols.

Acknowledgements

Many have suffered during the course of my studies but a few deserve special mention for their help and guidance.

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Mr F. Trussell because without his permission many moons ago, none of this would have been possible.

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Abbreviations

i) Compounds

C	(+)-catechin
GC	(+)-gallocatechin
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-*O*-gallate
EGCG	(-)-epigallocatechin-3-*O*-gallate
Tf	Theaflavin
Tf-3-mg	Theaflavin-3-*O*-monogallate
Tf-3'-mg	Theaflavin-3'-*O*-monogallate
Tfdg	Theaflavin-3,3'-*O*-digallate
ITf	Isotheaflavin
ITf-3'-mg	Isotheaflavin-3'-*O*-monogallate
NTf	Neotheaflavin
NTf-3-O-mg	Neotheaflavin-3-*O*-monogallate
Tfate A	Theaflavate A
Tfate B	Theaflavate B
Tfate C	Theaflavate C
ETfgg	Epitheaflagallin-3-*O*-gallate
TR	Thearubigin
Pur. Acid	Purpurogallin carboxylic acid
GA	Gallic acid
G	Galloyl ester group
PPO	Polyphenol oxidase
GAQ	Gallic acid quinone

ii) Techniques

HPLC	High performance liquid chromatography
NMR	Nuclear Magnetic Resonance
COSY	Correlation spectroscopy
NOE	Nuclear Overhauser Enhancement effect
NOESY	Nuclear Overhauser Enhancement effect spectroscopy
ROE	Rotating Overhauser Enhancement effect
ROESY	Rotating-frame Overhauser Enhancement effect spectroscopy
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum connectivity
MS	Mass spectrometry
UV/Vis	Ultra Violet and Visible spectroscopy

iii) General Terms

CTG	Consultative Tea Group, Croydon
1D	One dimensional
2D	Two dimensional
Lin.	Linear
Isocrat.	Isocratic
EDTA	Ethylene diamine tetraacetic acid disodium salt
SX	Sephadex LH-20

Chapter 1. Introduction.

Plant polyphenols (vegetable tannins) have been defined as water-soluble phenolic compounds with molecular weights between 500 and 3,000 Daltons, and in addition to giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins[1]. The biological importance of polyphenolic compounds in plants is not completely understood, but they are thought to be functional in a number of ways. Their roles may include part of the plant's defence mechanism providing protection against fungal[2] and insect attack[3]. In addition, the polyphenolic anthocyanins provide some of the vivid pigments which colour the flowers of many plants and help to attract nectar-collecting birds and insects which facilitate cross pollination prior to fertilisation[4].

Phenolics also contribute to the autumnal colour changes of leaves; the process initiated by the disappearance of the green chlorophylls is continued by the enzymic oxidation of colourless flavan-3-ols to a range of coloured polymeric compounds. Enzymic oxidation of polyphenols (principally chlorogenic acids) is also responsible for the browning associated with the bruising of many varieties of fruit. As a result of these enzymic oxidations, the polymeric pigments become water soluble and readily dissolve in hot water.

The tea plant, *Camellia sinensis*, is unique in the plant world in the quantity and diversity of polyphenolic compounds present in its leaves, whilst the processed black tea leaf contains a vast range of pigmented polyphenolic compounds. Aqueous infusions of fresh green tea leaves have been consumed for around 5,000 years in China, whilst black tea entered Europe in the early 17th century.

1.1 Composition of the Fresh Green Leaf

The principal phenolic constituents of the fresh green tea leaf are flavan-3-ols, flavones, flavonols and their glycosides; these are shown in Figure 1.1. The exact composition of these polyphenols in the leaf can vary with a number of factors including climatic and agronomic conditions[5], plant and clonal variety. The accepted ring classification system for flavonoid compounds is also shown (Figure 1.1).

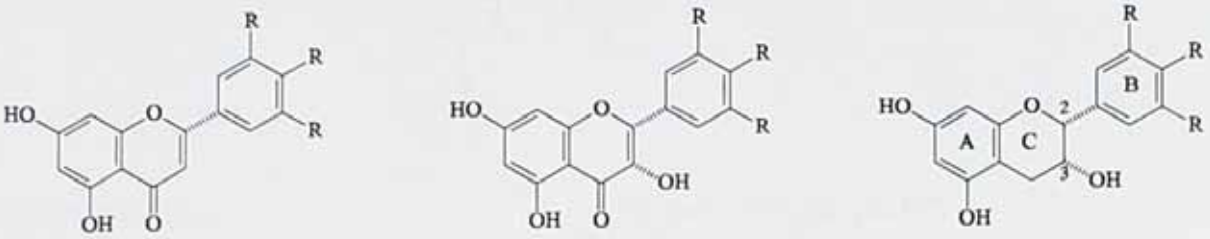
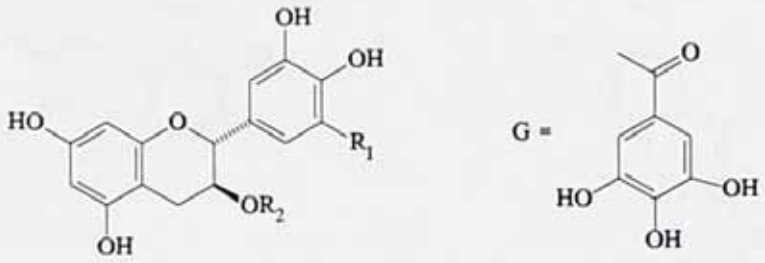


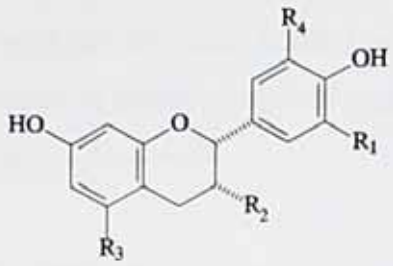
Figure 1.1 Flavonoids of the fresh green tea leaf

The flavan-3-ols predominate and are found in seven main forms, differing on the basis of their stereochemistry and presence of galloyl ester groups. The structures of these compounds are shown in Figure 1.2 and 1.3. A number of other minor flavan-3-ol derivatives has been isolated from fresh green tea leaves and these are included in Figure 1.3.



(+)-catechin (C)
R_1 = R_2 = H
(+)-catechin-3-*O*-gallate (CG)
R_1 = H; R_2 = galloyl ester (G).
(+)-gallocatechin (GC)
R_1 = OH; R_2 = H

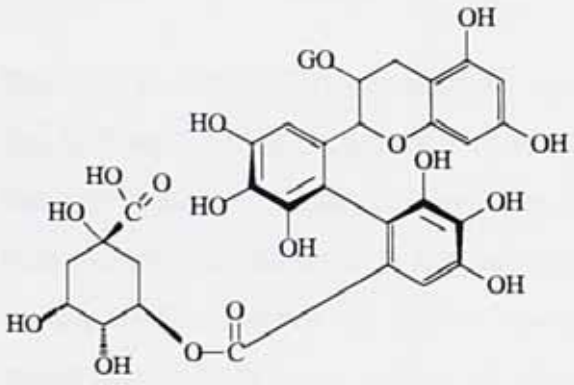
Figure 1.2 Catechin series of flavan-3-ols isolated from fresh green tea leaves.

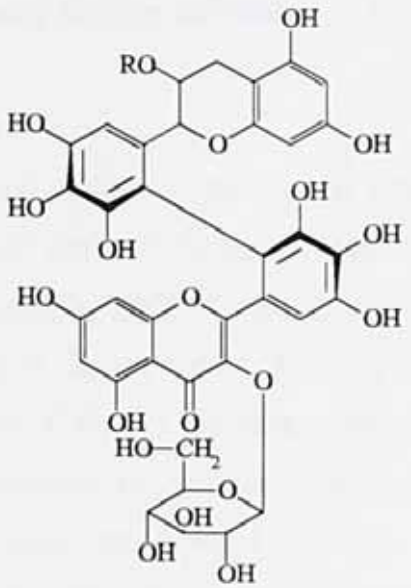


(-)-Epicatechin (EC)
R_1= H; R_2= R_3= R_4= OH
(-)-Epicatechin-3-*O*-gallate (ECG)
R_1= H; R_2= galloyl ester; R_3= R_4= OH
(-)-Epicatechin-3,5-*O*-digallate[6]
R_1 = H; R_2 = R_3 = galloyl ester; R_4= OH
(-)-Epicatechin-3-*O*-,(3 methyl)-gallate[7]
R_1= H; R_2= (3-methyl)-galloyl ester;
R_3= R_4= OH;

(-)-Epigallocatechin (EGC)
R_1= R_2= R_3= R_4= OH
(-)-Epigallocatechin-3-*O*-gallate (EGCG)
R_1= OH; R_2= galloyl ester; R_3= R_4= OH
(-)-Epigallocatechin-3,5-*O*-digallate[6]
R_1 = OH; R_2= R_3= galloyl ester; R_4= OH
(-)-Epigallocatechin-3-*O*-,(3-methyl)-gallate[7]
R_1= R_3= R_4= OH;
R_2 = (-3-methyl)-galloyl ester.

Figure 1.3 Epi-catechin series of flavan-3-ols isolated from fresh green tea leaves.





Theogallinin[9]
G = galloyl ester.

Theaflavonin[9]
R = galloyl ester.
Desgalloyl Theaflavonin[9]
R = H.

Figure 1.4 Trace novel dimers isolated from fresh green tea leaves.

Proanthocyanidins (condensed tannins) have also been reported in fresh green tea leaves[9]. These are mainly dimeric flavan-3-ols with acid labile C-4-C-8 or C-4-C-6 interflavanoid linkages. More recently, a number of novel groups of compounds has been isolated and these are summarized in Figure 1.4.

The flavonols, quercetin, kaempferol and myricetin, which are common in fresh tea leaves, are predominantly present as their 3-glycosides. Investigation of fresh green tea leaves has resulted in the identification of 14 flavonol-3-glycosides. This number is due to the incorporating various sugar moieties (e.g. glucose, fructose, rhamnose and galactose) in mono-, di- or tri- glycosides[10].

1.2 Black Tea

The methods and processes used in black tea manufacturing have been reviewed extensively[8]. Flavan-3-ols in fresh green leaf are oxidised during fermentation to yield the many polyphenolic pigments characteristic of black tea. The vast majority of these pigments remain uncharacterised due to their heterogeneity and the difficulties in their separation and purification.

Bradfield and Penny[11] fractionated an aqueous tea infusion with ethyl acetate and found that 50% of the total polyphenols of black tea liquor were removed by this process. Roberts[12] built on this advance by using paper chromatography (PC) to separate further both the ethyl acetate-soluble and aqueous soluble solids of the tea liquor. The ethyl acetate fraction yielded two yellow/orange spots (labelled X and Y), an orange/brown streak (labelled SI) and a number of other minor substances on PC analysis. The aqueous fraction yielded an identical orange/brown streak to SI which was labelled SIa. The aqueous fraction also yielded a dark brown spot with a Rf value of zero (labelled SII), some flavonol glycosides and three colourless products through their visualisation with iron (III) chloride spray reagent.

Further studies by Roberts[13] revealed that eight of these compounds were the result of reactions of, or between, EGC and EGCG. In addition, the three colourless compounds have been shown to be members of the theasinensin series of compounds[9] (Table 1.3 and Figure 1.5). These are formed via a single C-C bond between the B-rings of two flavan-3-ol molecules. Recent reports also indicated that a number of other minor components are formed in a similar manner.

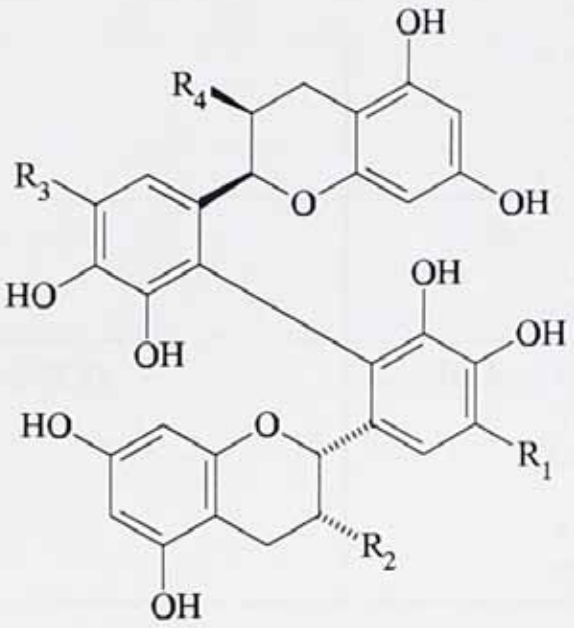


Figure 1.5 General theasinensin structure.

Table 1.1 Theasinensins of black tea[9].

Precursors	Products
EGCG + EGCG	R_1=R_3= OH, R_2= R_4 = Galloyl ester. Theasinensin A (Configuration R) Theasinensin D (Configuration S)
EGC + EGCG	R_1= R_2= R_3= OH, R_4 = Galloyl ester. Theasinensin B (Configuration R) Theasinensin E (Configuration S)
EGC + EGC	R_1= R_2= R_3= R_4= OH Theasinensin C (Configuration R)
ECG + EGCG	R_1= H, R_2 =R_4=Galloyl ester, R_3= OH. Theasinensin F (Configuration R) Theasinensin G (Configuration S)

The yellow/orange compound, X, was named theaflavin. The second, Y, was later named theaflavin gallate when it was found to yield theaflavin and gallic acid upon treatment with gallate esterase (tannase) isolated from *Aspergillus niger*[14].

The orange/brown pigments labelled SI, SIa and SII were named thearubigins.

1.2.1. Enzymes involved in Black Tea Production

Roberts and Wood[15] demonstrated that polyphenol oxidase (PPO) was the key enzyme responsible for the oxidation of the flavan-3-ol B-rings (Figure 1.2) to their respective *o*-quinones and that the trihydroxy-flavan-3-ols (EGC, EGCG and GC) are consumed in preference to the dihydroxy-analogues (EC, C, ECG), reflecting their lower redox potentials[16]. It has also been shown that PPO plays a part in the degallation and

epimerisation of the flavan-3-ol gallates present in the fresh green tea leaves during fermentation[17]. PPO was fully characterised by Gregory and Bendall[18]; the enzyme was shown to be a copper-containing protein (0.32 % (w/w)) with a molecular weight of 144,000 ± 16,000 Daltons.

1.2.2 Polyphenols of Black Tea

i) Theaflavins

The theaflavin fraction of black tea has been reported to make important contributions to the visual and organoleptic properties of its aqueous infusions[19]. The theaflavins are also considered to play an important role in the formation of tea cream[20].

Roberts and Myers[21] isolated crystalline theaflavin, but were unsuccessful in their attempts to isolate crystalline theaflavin gallate. This has since been shown to be due to the fact that it was a mixture of two monogalloyl esters of theaflavin and a digalloyl ester of theaflavin[22].

Model studies on the oxidation of flavan-3-ols by both enzymic and chemical means showed that theaflavins were formed from various pairs of these compounds[23, 24, 25]. Takino proposed a structure of theaflavin[24] (Figure 1.6) and a mechanism for its formation which was based on the tentative mechanisms for the formation of purpurogallin[26] (Figure 1.8).

This structure was confirmed by a series of NMR studies[22, 27, 28], which also showed that the relative configurations of the flavan-3-ol precursors were unchanged during theaflavin formation. Theaflavin and its galloyl esters were all observed to have 2,3-*cis*-2',3'-*cis*- relative configurations[22, 27]. Further work resulted in the characterisation of two novel isomers of theaflavin, isotheaflavin with a 2,3-*trans*-2',3'-*cis*- relative configuration[22, 27] and neotheaflavin with a 2,3-*cis*-2',3'-*trans*- relative configuration[22]. Six theaflavins have been isolated from black tea, and a further three are feasible based on the flavan-3-ols normally found in fresh green tea leaves (Table 1.2 and Figures 1.6 and 1.8).

† Tea cream is the term given to the precipitate which forms during cooling due to the interaction of caffeine and tea polyphenols in an aqueous tea infusion.

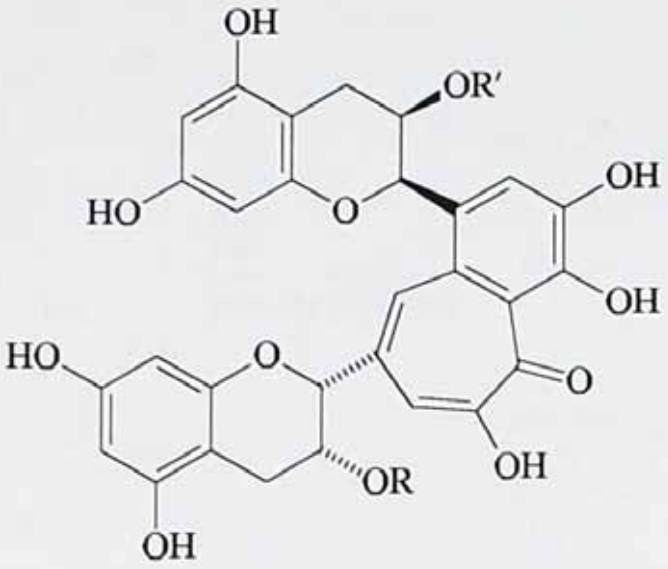
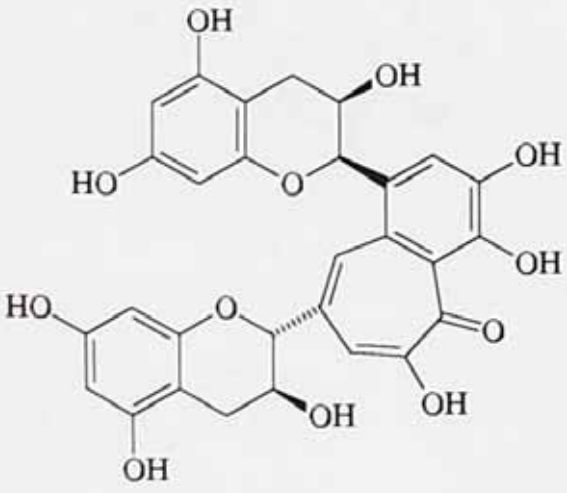
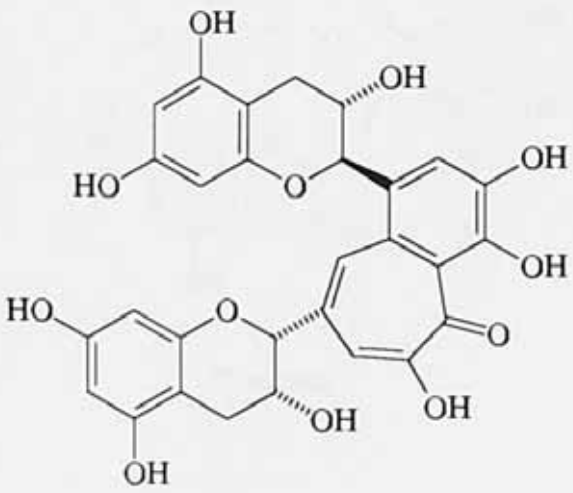


Figure 1.6 The structures of the major theaflavins of black tea[22, 27, 30]

Theaflavin:	R = R' = H.
Theaflavin-3-*O*-monogallate	R = G; R' = H.
Theaflavin-3'-*O*-monogallate	R = H; R' = G.
Theaflavin-3,3'-*O*-digallate	R = G; R' = G.



Isotheaflavin



Neotheaflavin

Figure 1.7 The known minor theaflavins of black tea[22, 27, 30].

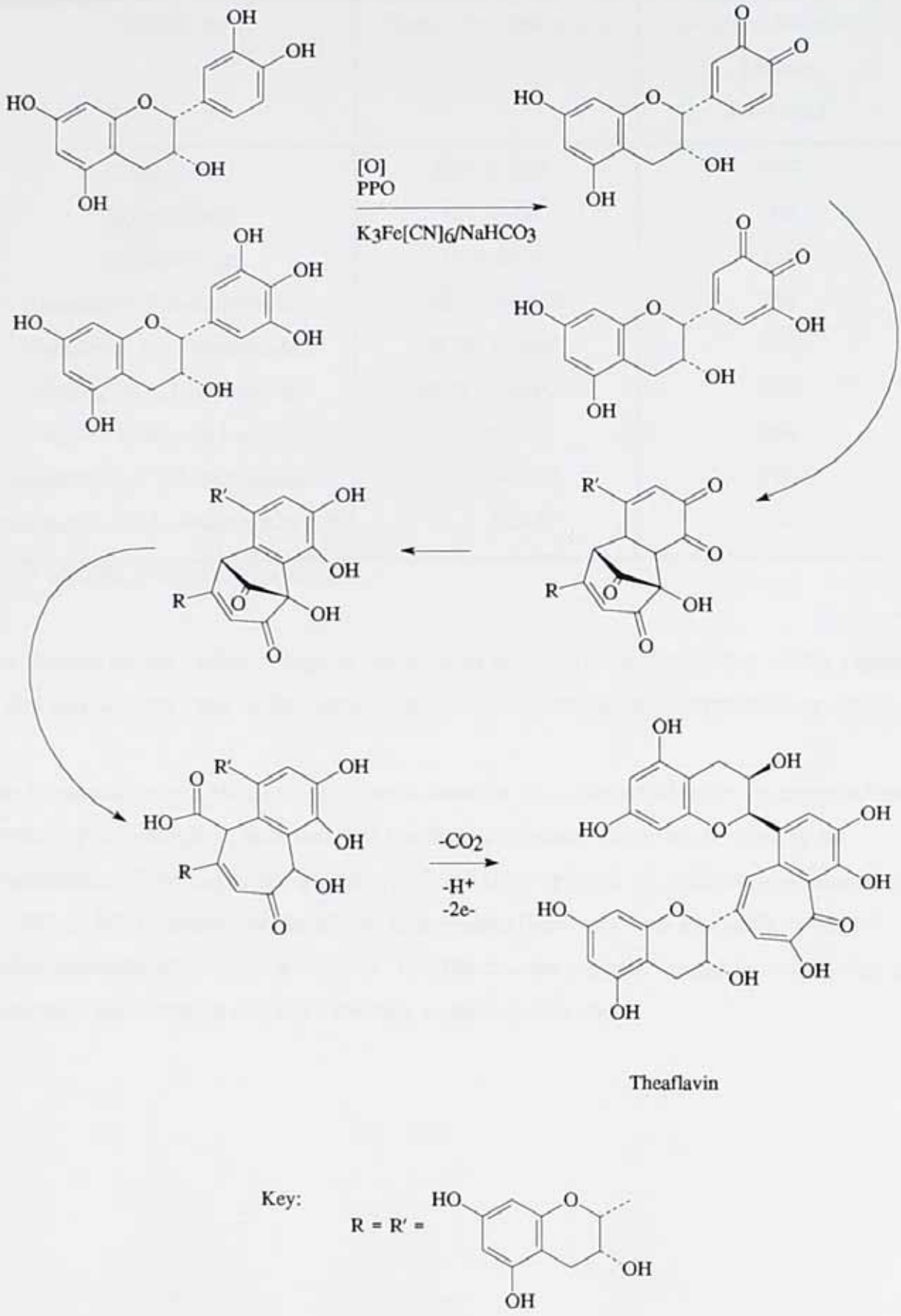


Figure 1.8 Proposed mechanism for Theaflavin formation[24].

Table 1.2 Synthesis of the known theaflavins of black tea[22, 27, 30] (Figures 1.6 and 7).

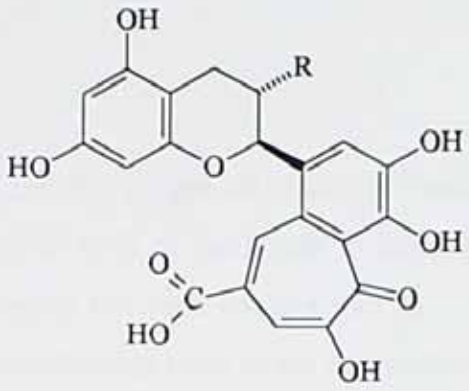
Theaflavin	Flavan-3-ol Precursors	Relative Molecular Masses / Daltons
Theaflavin	EC + EGC	564
Isotheaflavin	EC + GC	564
Neotheaflavin	C + EGC	564
Theaflavin-3-*O*-monogallate	EC + EGCG	716
Theaflavin-3'-*O*-monogallate	ECG + EGC	716
Theaflavin-3,3'-*O*-digallate	ECG + EGCG	868
Fourth Theaflavin Isomer *	C + GC	564
Isotheaflavin-3'-*O*-monogallate *	ECG + GC	716
Neotheaflavin-3-*O*-monogallate *	C + EGCG	716

* Not isolated from black tea to date.

The theaflavins are yellow/orange in colour with absorption maxima in the visible region at 380 and 460 nm[30] due to the electron delocalisation around the benzotropolone moiety.

The fermentation process also yields small amounts of a number of other benzotropolone containing compounds. These included the theaflavic acids which are formed by the condensation of the *o*-quinone of gallic acid and the *o*-quinone of a dihydroxy-flavan-3-ol (C, EC or ECG), producing theaflavic acid, epitheaflavic acid and epitheaflavic acid-3-gallate respectively[22, 29, 30] (Figure 1.9). Theaflavic acids are also orange in colour with an absorption maximum at 400 nm extending to around 500 nm.

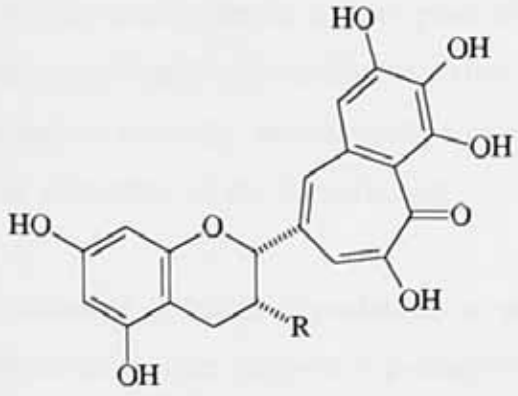
Epitheaflavic Acid. R = OH
Epitheaflavic Acid-3-gallate. R = Galloyl ester.



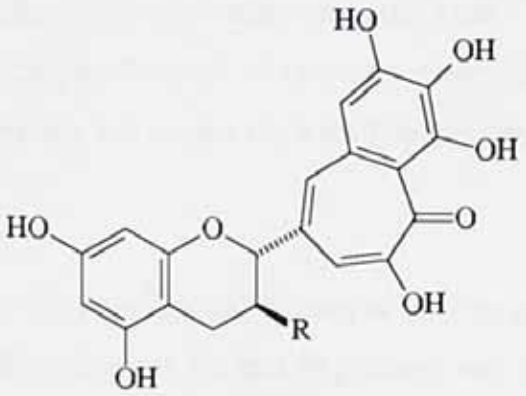
Theaflavic Acid. R = OH

Figure 1.9 The theaflavic acids of black tea[22, 29, 30].

A third group of red pigments related to theaflavins have recently been reported. The theaflagallins[9] (Figure 1.10), as they have been designated, were shown to be the condensation products of the quinones of a trihydroxy-flavan-3-ol and gallic acid resulting the formation of 7,8,9 trihydroxy substituted benzotropolone system, rather than the 8,9-dihydroxy benzotropolone system of theaflavin. Three of these compounds [theaflagallin, epitheaflagallin and epitheaflagallin-3-*O*-monogallate] have been isolated from a black tea liquor.



Epitheaflagallin. R = OH.
Epitheaflagallin-3-gallate. R = Galloyl ester.



Theaflagallin. R = OH.

Figure 1.10 The theaflagallins of black tea[9].

The theaflavin fraction of black tea has been estimated to account for 3-6 % (w/w) of the total soluble solids[19].

ii) Thearubigins

The thearubigins constitute between 10 and 20% of the dry weight of black tea[19] and, due to their hot water solubility, represent approximately 30-60% of the solids in liquors after infusion[31]. In over forty years of research, little progress has been made either in structurally characterising the thearubigins or in understanding their exact contribution to black tea quality.

A wide range of separation techniques has been applied to various thearubigin fractions, including:

Liquid-liquid partition chromatography[12]
Paper and Thin-Layer Chromatography[12]
Multi-stage liquid-liquid partition chromatography[32]
Reverse phase HPLC[33]
Normal phase HPLC[34]
Counter-current chromatography[35]
Column chromatography[36, 37]
Ultrafiltration[38]

All of these techniques have been generally ineffective in providing any structural information about the thearubigins. However, despite this lack of concrete evidence, the observations made by various groups of workers has led to two theories being proposed for the structures of the thearubigins.

The results of chemical degradations of various TR fractions led Brown *et al.*[32] to propose that thearubigins are polymeric proanthocyanidins. Support for this hypothesis was given by proposing a mechanism for the formation of interflavanoid bond at the 4 and 8 positions (Figure 1.11)[32].

Subsequent work by Cattell and Nursten[37] led to a totally different view of the thearubigins. These workers proposed that thearubigins are pentameric flavan-3-ols, flavan-3-ol gallates and benzotropolone moieties, linked by both hydrolysable and nonhydrolysable interflavonoid links[37].

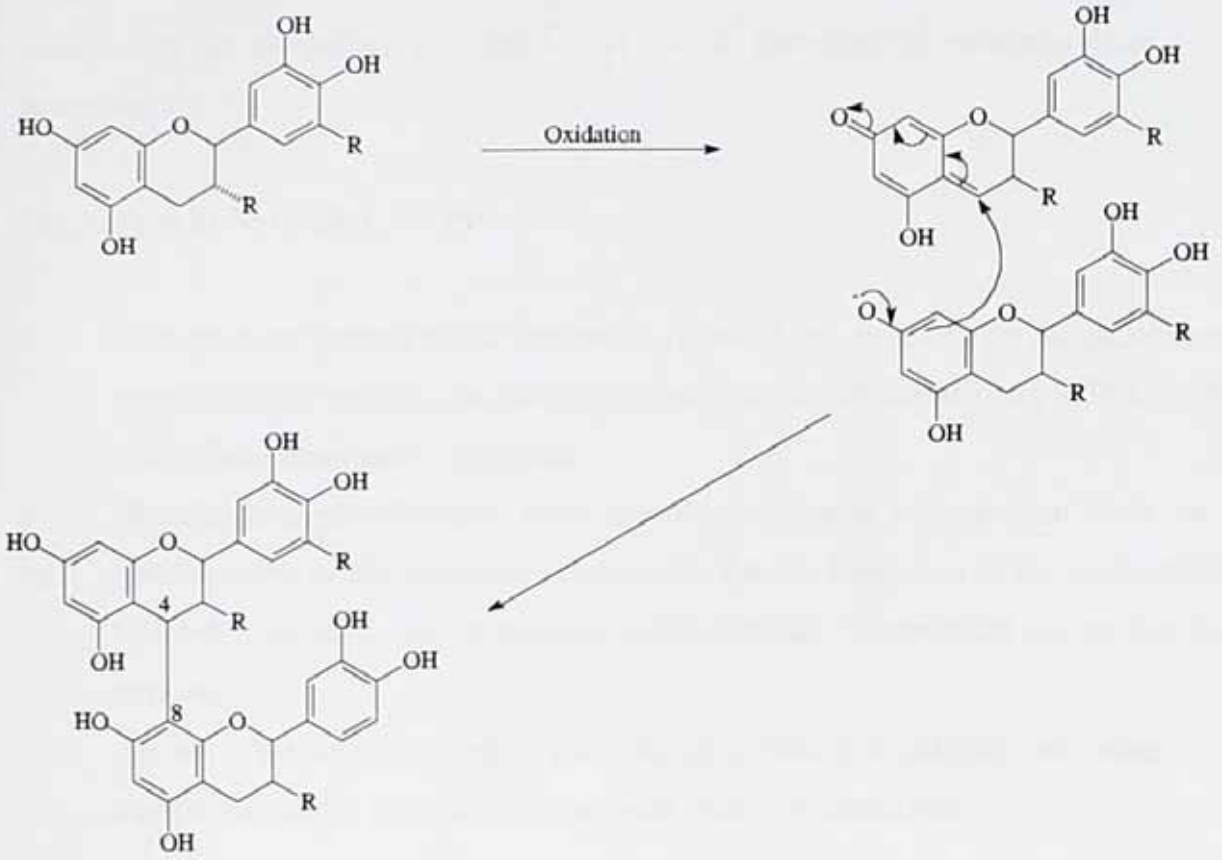


Figure 1.11 Mechanism for the formation of a 4-8 interflavanoid link (Brown *et al.* [32]).

1.5 Aims of Current Study

The general aims of this study are to isolate and characterise minor polyphenol components of black tea which have not been studied previously.

Specifically, the project aims to investigate the ethyl acetate fraction for the presence of the previously unidentified theaflavin-type compounds whose presence in black tea can be predicted by the mechanism of Takino *et al.* for the formation of benzotropolone structures[24].

The study will be divided into four parts:

i) A large-scale extraction and separation of black tea, followed by the isolation and purification of specific theaflavin-type compounds, as identified by HPLC analysis with photo-diode array detection.

ii) The structural elucidation of those specific compounds isolated from black tea.

iii) Confirmation of the precursors responsible for the formation of the compounds isolated from black tea, by model oxidative studies via chemical and/or enzymic conversion.

iv) An investigation of the factors affecting the yields of theaflavins and related compounds under standardised chemical oxidation conditions.

Chapter 2. Isolation and Characterisation of Novel Theaflavins.

2.1 Introduction

i) Isolation and Separation

The first polyphenolic compounds identified from plant sources were isolated by classical crystallization techniques. The application of such a method requires two criteria to be met:

1) the compound must readily crystallize, and
2) the sample should have a high content of this component.

This technique, used in the early part of this century, yielded a number of polyphenols contained in tea including (-)-epicatechin, (+)-catechin, gallic acid and ellagic acid. Roberts *et al.*[19, 21] used this technique in combination with paper chromatography to effect the initial isolation of theaflavin from black tea.

More recent applications of solvent extraction and fractionation, in combination with classical open column chromatography using the increasing variety of stationary phases available, has led to the isolation of a very large number of natural products. The most significant improvements in isolation and purification have been achieved by the utilisation of high performance liquid chromatography (HPLC). Hoefler and Coggon[39] published one of the first papers on the application of HPLC analysis to tea components. They showed that, under appropriate conditions, it was possible to separate the major catechins and also some of the major theaflavins. This, and subsequent research on tea analysis, have encountered difficulties in separating theaflavin-3'-*O*-monogallate from theaflavin-3,3'-*O*-digallate and the theaflavin monogallates from each other. Advances in the design of the materials used for stationary phase have allowed the routine analytical separation[20] and preparation of all four major theaflavins in milligram to gram amounts[40].

The four major theaflavins are considered to constitute only 2% of the dry weight of black tea (3 - 6% (w/w) of the soluble solids) [19]. However, the theaflavin content and the ratio of non-galloyl to galloyl ester theaflavins have been shown to vary from clone to clone and from country to country[40].

This indicates that the minor theaflavin related compounds will be present at even lower levels (0.1% or less of dry weight). Thus, simple extraction and HPLC separation will be inappropriate in this case. Consideration must therefore be given to designing an extraction and separation method which enhances the minor theaflavin content by removing the catechins, caffeine, chlorophylls, flavonol glycosides and the thearubigins, the last interfering with the HPLC separation process by binding irreversibly to the active sites on reverse phase columns.

ii) Structural Elucidation

The structural elucidation of polyphenols by one-dimensional NMR can be difficult due to the carbon-rich nature of these compounds. This leads to a large number of quaternary carbons and a lack of information about the proton-proton connectivities, which makes linking the various fragments of the molecule together difficult.

A number of two-dimensional techniques have been developed which allow information to be gathered about proton-proton and proton-carbon correlations within molecules such as polyphenols (Table 2.1 and the examples below).

Table 2.1 NMR techniques for polyphenol structural elucidation.

	Experiment	Information
^{1}H-^{1}H Correlations	Correlation Spectroscopy COSY	^{1}H-^{1}H coupling partners
	Nuclear Overhauser Enhancement Effect Spectroscopy NOESY (Figure 2.1)	^{1}H-^{1}H through-space interactions (+ve & -ve enhancements dependent on molecular size)
	Rotating-frame Overhauser Enhancement Effect Spectrscopy ROESY (Figure 2.2)	^{1}H-^{1}H through-space interactions (+ve enhancement independent of molecular size)
^{1}H-^{13}C Correlations	Heteronuclear Multiple Quantum Connectivity HMQC[41]	Direct attachment of proton to carbons
	Heteronuclear Multiple Bond Correlation HMBC[42] (see Figure 2.3)	^{1}H-^{13}C interactions over two or three bonds

Nuclear Overhauser Enhancement effect (NOE) difference spectroscopy (Figure 2.1) has been used successfully to differentiate between the 4α and 4β protons which are present in the aliphatic C-ring of all flavan-3-ols[43] and their derivatives such as the theaflavins[44], theaflavic acids and theaflagallins. An NOE is observed for the 4β proton when H-2 is irradiated, while no similar effect is seen for the 4α proton. Thus this technique is also reported to show the pseudo-axial or equatorial positioning of these protons on the C-ring[43, 44].

Rotating-frame Overhauser Enhancement effect (ROE) spectroscopy (Figure 2.2) can also be useful in polyphenol studies. For example, Davis *et al.*[44] reported its use to identify the galloyl ester signals in theaflavin-3,3'-*O*-digallate, as these were unresolved by 1D techniques and the HMBC experiment. The ROE spectrum showed clearly spatial interactions between benzotropolone proton H-g and galloyl ester proton H-G2', whilst benzotropolone proton H-c interacted with the galloyl ester proton H-G2, so identifying both groups of signals.

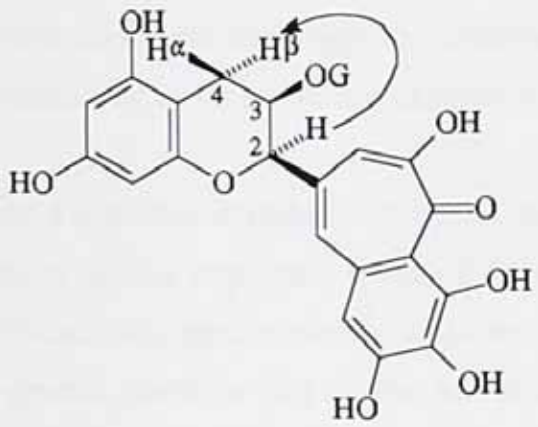


Figure 2.1 Example of NOE results from Epitheaflagallin-3-gallate. Showing NOE interaction of H-2 and H-4β.

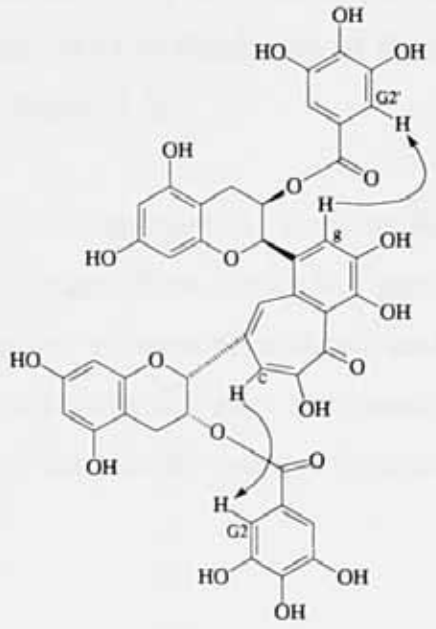


Figure 2.2 An Example of ROE in theaflavin-3,3'-digallate

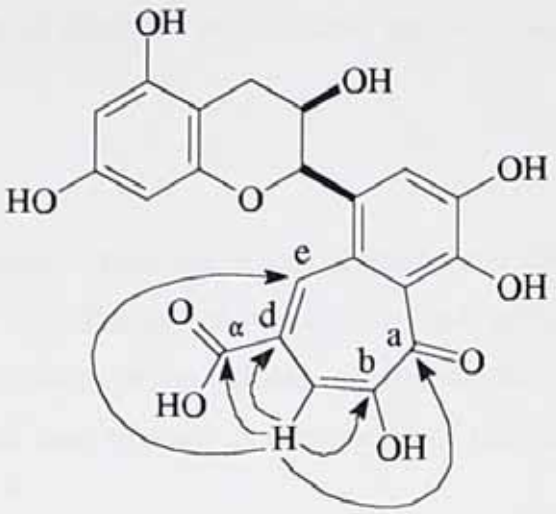


Figure 2.3 Example of HMBC results from epitheaflavic acid.

The Heteronuclear Multiple Bond Correlation (HMBC)[42] experiment is a very powerful technique, allowing the determination of long-range (two- and three-bond) ^{1}H-^{13}C connectivities. This is of particular importance in the study of theaflavins and other related benzotropolone derivatives. In these cases, the connectivities of the benzotropolone protons allows the unambiguous determination of the points of attachment of flavan units or carboxylic acids to the benzotropolone ring (see Figure 2.3).

Once a structural hypothesis of a novel theaflavin-type component has been established, support for this hypothesis is sought through their synthesis from flavan-3-ol precursors predicted from the mechanism of Takino *et al.*[24] and the subsequent oxidation studies[22, 45]. In general, there are two distinct sets of oxidation conditions that have been used in the study of fermentation processes and understanding of the accompanying polyphenol oxidation reactions.

iii) Synthesis

a) Chemical Oxidation

Chemical oxidation of polyphenols, principally using potassium hexacyanoferrate (III) in cold sodium hydrogen carbonate solution, has been studied[46] and utilised extensively.

This oxidant has been used to investigate and establish the nature of the precursors used in the formation of a number of black tea polyphenols, including theaflavins[22], theaflagallins[9] and theasinensins[9].

b) Enzymic Oxidations

A number of enzyme systems, which use these polyphenols as their substrates, have been used in a range of conditions. The enzymes studied include peroxidase, mushroom tyrosinase and crude preparations of tea oxidase (polyphenol oxidase)[45]. These enzyme preparations have also been used to study the formation of theaflavins[21, 24, 47], theaflavic acids[17] and thearubigins[48, 49].

Enzymic and chemical oxidations generally give mixtures of products and further purification is required[22, 24, 48]. In this study, chemical oxidation of specified flavan-3-ol pairs will be used to confirm the structures proposed for any new compounds isolated.

2.3 Experimental

2.3.1 Extraction and Isolation

The extraction and isolation procedures are summarized in Figure 2.4. All solvents were supplied by Sherman Chemicals, Sandy, Bedfordshire. All chemicals were supplied by Sigma Chemicals, Poole, Dorset.

Black tea (Ruo Mimosa, Malawi, supplied by Consultative Tea Group) (3.0 kg in 1.0 kg batches) was extracted twice with methanol (3.0 dm^3, A.R. grade) with stirring (30 minutes). The spent leaf was discarded and the methanol extracts combined and concentrated to a known volume (500 cm^3). An equal volume of distilled water was added and the residual methanol removed. The aqueous extract was heated (333 K) and extracted with chloroform (2 x 1 dm^3) and then with ethyl acetate (8 x 1 dm^3). The chloroform extracts, containing caffeine and chlorophylls, were discarded. The ethyl acetate washings were combined and concentrated to dryness and the residual red/purple solid (35 g) retained.

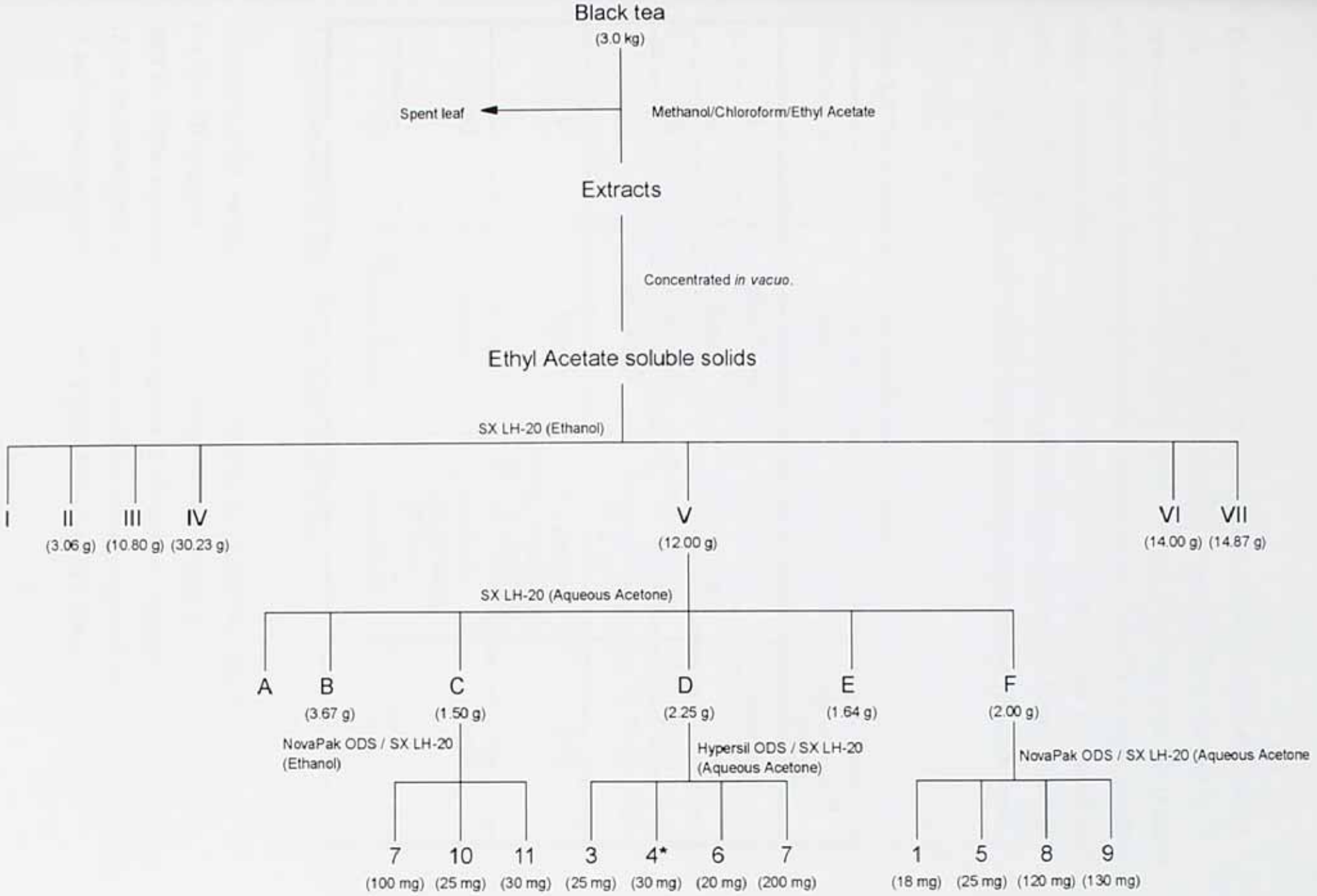


Figure 2.4 Summary of experimental procedure.

* indicates sample was isolated and characterised as a mixture of compounds.

The ethyl acetate soluble solids (100 g) were applied to a column of Sephadex LH-20 (26 cm x 12 cm), pre-swollen in ethanol. Elution with ethanol (63 dm^3) followed by aqueous acetone (28% v/v, 10 dm^3) afforded seven fractions (see Table 2.2).The progress of the separation was followed by thin layer chromatography (T.L.C.) on cellulose plates (Merck Aluminium-backed Cellulose-F) developed in a mixture of butan-2-ol: acetic acid: water [14:1:5]. The T.L.C. plates were visualised by spraying with a freshly prepared aqueous mixture of iron (III) chloride and potassium hexacyanoferrate (III) (1% each, 1:1).

Table 2.2 The separation of ethyl acetate soluble extract.

Fraction Numbers	Colour	T.L.C. Identification	Comments and Yield / g
I	Yellow/Green	Chlorophyll	Discarded
II	Purple	Unidentified	3.06
III	Orange/red	Mixed Catechins	10.78
IV	Bright red	Theaflavin rich Rf 0.35	30.28
V	Red	Theaflavin Monogallates Rich Rf 0.46 and 0.56	12.00
VI	Orange	Theaflavin Digallate Rich Rf 0.62	14.00
VII	Yellow/brown	Unidentified*	14.87

* Assumed to be ethyl acetate-soluble thearubigins.

Fractions I to III consisted largely of chlorophylls, anthocyanidins and flavan-3-ols. Fraction VII consisted of an undefined red/brown material and so all of these fractions were not further examined. Chromatography of Fraction IV on Sephadex LH-20 (26 cm x 12 cm i.d.) eluted with distilled water containing increasing proportions acetone (28% v/v, 25 dm^3 followed by 35% v/v 10 dm^3) yielded 6 fractions (see Table 2.3)

Table 2.3 Separation of the theaflavin fraction.

Fraction	Colour	T.L.C. Identification	Comments and Yield / g
A	Colourless	-	Discarded
B	Yellow	Mixed Catechins	3.67
C	Orange	ECG, Tf and Theaflavic Acid	4.57
D	Orange/red	Theaflavin mixture Major Comp. Rf 0.35	3.14
E	Orange/red	"Pure" theaflavin Rf 0.35	1.64
F	Orange/red	Theaflavin Monogallate mixture Rf 0.46 (Tf-3-mg) Rf 0.56 (Tf-3'-mg)	4.56

Fractions B and E were found to contain mainly a mixture of catechins and theaflavin by HPLC analysis (analytical conditions see Table 2.4) and were not examined further. Separation of Fraction C (1.5 g) by preparative HPLC (Preparative HPLC conditions Table 2.4) yielded orange pigments **7**, **10** and **11**. These were further purified on Sephadex LH-20 (ethanol) to yield pure samples of **7:** 100 mg, **10:** 25 mg and **11:** 30 mg. Chromatography (Preparative HPLC conditions Table 2.4) of Fraction D (2.25 g) yielded a mixture of orange/red pigments **3**, **4**, **6** and **7**, which were separated by chromatography over Hypersil C_{18} (semi-prep HPLC conditions Table 2.4) and Sephadex LH-20 (water, aqueous acetone (50% v/v)) to yield the following samples **3:** 25 mg; **4:** 30 mg (as a mixture); **6:** 20 mg and **7:** 200 mg. Fraction F (2.0g) was separated on Waters NovaPak C_{18} (Preparative HPLC conditions Table 2.4) and yielded samples of orange/red pigments **1**, **5**, **8** and **9**. These were further purified on Sephadex LH-20 (water, aqueous acetone (50% v/v)) to yield the following pure samples (**1:** 18 mg; **5:** 25 mg; **8:** 120 mg; **9**: 130 mg).

Table 2.4 Chromatography conditions

	Preparative HPLC Conditions	Semi-preparative HPLC Conditions	Analytical Conditions
System	Waters Delta-Prep 4000 with Fraction Collector.	Waters Delta-Prep 4000 with Fraction collector.	Perkin-Elmer with Shimadzu Photo-diode array detector.
Column	NovaPak C_{18} (30 cm x 4.7 cm) Waters	Hypersil C_{18} (30 cm x 1 cm) Fisons	Nucleosil C_{18} (15 cm x 0.47 cm) Phenomenex
Col. Temp.	293 K	293 K	303 K
Solvent: A	CH_3CN	CH_3CN	CH_3COOH:CH_3CN [2:98]
B	H_2O: CH_3COOH: CH_3CN [79.5:0.5:20]	H_2O: CH_3COOH: CH_3CN [81.5:0.5:18]	CH_3COOH: H_2O [2:98]
Elution Profile	1) 100% B: 50 min. (isocrat) 2) 50% B: (step) 3) 50% B: 5 min. (isocrat) 4) 100% B (step) 5) 100% B 10 min. (isocrat)	1) 100% B: 50 min. (isocrat) 2) 50% B: (step) 3) 50% B: 5 min. (isocrat) 4) 100% B (step) 5) 100% B 10 min. (isocrat)	1) 8% - 40% A 55 min (lin) 2) 40% A 5 min (isocrat) 3) 40% - 8% A 1 min (lin) 4) 8% A 15 min (isocrat)
Detection	280 nm (UV)	280 nm (UV)	260 nm to 500 nm (UV/Vis)
Flow Rates	75 cm^3/min	4.75 cm^3/min	1.00 cm^3/min
Sample mass per injection	100 mg	20 mg	-
Inj Volume	4 cm^3	200 mm^3	20 mm^3

2.3.2 Characterisation Techniques:

2.3.2.1 HPLC and UV/Visible Analysis

Samples **1** to **11** (5 mg) were individually dissolved in aqueous 'stabiliser' solution (250 ppm EDTA, 250 ppm ascorbic acid and 20% acetonitrile v/v) (5 cm^3) and analysed under the analytical HPLC conditions described in Table 2.4 above.

2.3.2.2 Mass Spectrometry

Mass spectra were obtained on a VG Biotech Quattro triple quadrupole mass spectrometer. Electrospray conditions were as follows:

Nitrogen bath gas flow	300 dm^3/hr
Nebulising gas	15 dm^3/hr.
Source temperature	333 K.
Capillary potential	3.5 kV
HV lens	0.20 kV.

Negative ion mode acquiring continuum data, m/z 100-1000, with cone voltage set alternately at 30 V and 70 V.

Detector	650 V.

2.3.2.3 NMR spectroscopy

NMR spectra were measured on a Bruker AMX400 spectrometer operating at a probe temperature of 303 K or 293 K using either a dual $^1H/^{13}C$ 5 mm probe or a multinuclear 5 mm inverse probe as appropriate. Sample concentrations were typically *ca.* 2-3 mg per 0.5 cm^3. Heteronuclear Multiple Quantum Connectivites (HMQC) and Heteronuclear Multiple Bond Correlations (HMBC) experiments were acquired with the parameters described previously[44].

2.3.3 Chemical Synthesis

2.3.3.1 Materials and Methods

(+)-Gallocatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate were prepared as described previously[43]. (-)-Epicatechin, (+)-catechin were used as supplied by Sigma Chemicals, Poole, Dorset. Sodium hydrogen carbonate and anhydrous magnesium sulfate was used as supplied by Sherman Chemicals, Sandy, Beds. Hydrochloric acid was supplied by Merck (BDH) Chemicals.

Synthesis of Compounds **1, 2, 4, 6, 10, 11**

An ice-cooled solution of sodium hydrogen carbonate (200 mg) and potassium hexacyanoferrate (III) (300 mg) in distilled water (10 cm^3) was added to an ice-cooled solution of specified flavan-3-ols (Table 2.5) in distilled water (10 cm^3). The reaction mixture was allowed to stand, with ice cooling and no stirring for 15 minutes. The solution was acidified (hydrochloric acid (1 mol dm^{-3}) to pH 2.5) and extracted with ethyl acetate (3 x 20 cm^3). The organic extracts were combined, washed (distilled water, 2 x 20 cm^3) and dried (magnesium sulfate). The dried solution was filtered (Whatman 541) and evaporated to dryness under reduced pressure.

Table 2.5 Precursors and predicted products of the synthetic preparation of the natural dimers (Compounds **1, 2, 4, 6, 10, 11**) .

Compound Number	Precusors		Predicted Products (Yields)	
			Major	Minor
1	EC (120 mg)	ECG (180 mg)	Tfate B (30 mg)	Tfate A
2	C (120 mg)	ECG (180 mg)	Tfate C (35 mg)	Tfate A
4	C (120 mg)	EGCG (190 mg)	NTf-3-mg (20 mg)	
6	GA (70 mg)	EGCG (190 mg)	ETfgg (25 mg)	
10	C (120 mg)	GA (70 mg)	TFA (35 mg)	PA (25 mg)
11	EC (120 mg)	GA (70 mg)	ETFA (40 mg)	PA (25 mg)

Compounds **1**, **2**, **4**, **6** and **11** were isolated by semi-preparative HPLC under the conditions described in Table 2.4. Compound **10** was isolated on Sephadex LH-20 with ethanol elution.

Synthesis of Compounds **3**, **5**, **7**, **8** and **9**.

These compounds were synthesized on a small scale as described in Chapter 3, Section 3.2

2.4 Data Interpretation

The HPLC and spectroscopic data are shown in Tables 2.6 to 2.10 (page 40-49) and Figure 2.13 (page 39). The ^{1}H and ^{13}C NMR spectra are shown in Appendix 1 in compound number order (page 78-103).

2.4.1 Theaflavates

Theaflavate B (Compound **1**)

The UV/Visible spectrum of theaflavate B is characteristic of a carboxy-substituted hydroxy-benzotropolone compound[22], such as epitheaflavic acid (absorbance maxima at 284 and 406 nm) and theaflavate A[50] (absorbance maxima at 282 and 398 nm). The molecular mass of this molecule is 700 Daltons, i.e. an empirical formula of $C_{36}H_{28}O_{15}$. The difference in molecular masses between theaflavate A (852 Daltons) and theaflavate B is 152 Daltons which is equivalent to a single galloyl ester group. These data indicate that theaflavate B has been formed from the coupling of (-)-epicatechin-3-O-gallate (442 Daltons) and (-)-epicatechin (290 Daltons) with the loss of carbon dioxide (32 Da) i.e. 442 + 290 -32 = 700.

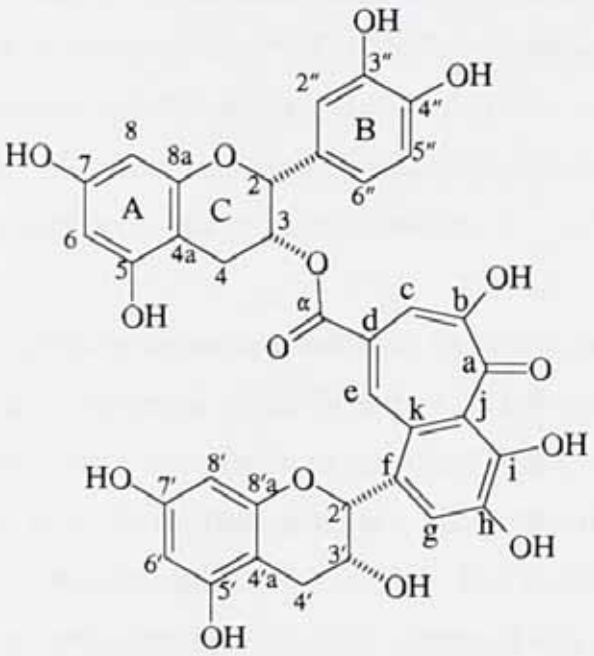


Figure 2.5 Structure of theaflavate B labelled for NMR data.

Analysis of the ^{1}H and ^{13}C-{^{1}H} NMR spectra leads to the conclusion that the oxidation condensation takes place between the B-ring of the EC molecule and the galloyl ester group of the ECG. These data is summarised in Tables 2.7 - 2.10 and the spectra are shown in Appendix 1. The structure and numbering scheme for the proposed product is shown in Figure 2.5.

The ^{1}H NMR spectrum between (7.5 ppm to 8.5 ppm) contains resonances H-e, H-g and H-c which are characteristic of the benzotropolone protons. The three signals in the region 6.4 to 7.1 ppm (H-2", H-5" and H-6"), have chemical shifts, integrals and a coupling pattern which are indicative of the presence of a single 1,3,4 substituted aromatic system, similar to the B-ring proton signals observed in (-)-epicatechin. There are no resonances observed which are consistent with the presence of a galloyl ester group. The signals which occur in the expected regions of the ^{1}H and ^{13}C spectra are too weak to be due to the main species and are produced by the trace impurities present in this sample. This strongly supports the proposal that the galloyl ester group and the second B-ring of the precursor molecules are used in the formation of the interflavonoid linkage.

The two pairs of doublets (J = 2.3 Hz i.e. *meta* coupling) in the region 5.9 to 6.3 ppm (H-6, H-6', H-8 and H-8') have the chemical shifts and coupling constants expected for the A-ring protons in the proposed structure. The aliphatic resonances, H-4β, H-4α, H-3 and H-2 and their counterparts (H-4β', H-4α', H-3' and H-2'), are also similar to those observed in theaflavin-3,3'-digallate[44] and theaflavinate A[50]. These are consistent with the patterns expected for the C-rings in the proposed structure.

The use of 2D ^{13}C-^{1}H correlation techniques confirms the above conclusions and allows an unambiguous and complete assignment of the ^{1}H and ^{13}C NMR spectra (Table 2.8). The resonance for C-a is immediately identifiable on chemical shift grounds and this gives a long-range correlation to H-c. Correlations from H-c allow identification of C-b (which is hydroxylated based on its chemical shift) and C-e. Both H-c and H-e are observed to correlate to C-d and to an ester carbonyl C-α. This carbonyl also correlates to H-3 confirming that the seven membered ring of the benzotropolone moiety is linked to one aliphatic ring by an ester group with the point of attachment being C-d.

Proton H-e also correlates to C-f and C-j, which can be provisionally assigned to the structure at this point by comparison of their chemical shifts to those of the analogous carbons in theaflavin-3,3'-*O*-digallate. In addition, H-g correlates to C-f and also to C-k, C-h and C-i, but not to C-j. This confirms the assignment of H-g as a benzotropolone proton and also confirms the assignment of C-j. The assignments of C-f and C-k are determined by the relative intensities of their correlations to H-g in the HMBC spectrum, as described previously[53]. The chemical shifts of C-i and C-h confirm the presence of a hydroxy substituent; the assignment of these carbons is also determined by the relative intensities of their correlations to H-g. The proton H-2' is observed to correlate to C-f, C-g and C-k which demonstrates that the point of attachment of the second aliphatic ring is at C-f. The chemical shifts of C-a to C-j are all entirely consistent with the structure (Figure 2.5) suggested by their comparison to those obtained for theaflavin and its gallate esters.

The structure of the remainder of the molecule can be similarly determined from the data given in Table 2.7. Correlations from H-3 to C-1", C-2" and C-6" give the point of attachment of the 1,3,4-substituted aromatic B-ring. The stereochemistry of the molecule at C-2 and C-3 (and likewise at C-2'and C-3') can be deduced from the proton spectrum. The measurable coupling constants around the aliphatic rings being those expected from a *cis* 2,3 geometry (epicatechin-like) rather than a *trans* 2,3 geometry (catechin-like) in each ring[51]. The coupling constants, $J_{2,3}$ and $J_{2',3'}$, are both small (1.2 Hz), indicating that a pseudo axial-axial arrangement of these protons in their respective rings is very unlikely. Assignment of the H-4α and H-4β resonances (and their primed counterparts) follows the work of Porter *et al.*[51]. These workers noted that $J_{3,4\alpha}$ is always smaller than $J_{3,4\beta}$ in epicatechin regardless of the conformation of the ring and thus it is reasonable to assume that H-4β and H-4'β are the resonances with the larger coupling to H-3 and H-3' respectively. Further evidence for this proton assignment is found by comparison of the proton NMR spectrum of this molecule to that of theaflavate A[50] or theaflavin digallate[52] where ROESY spectra have unequivocally demonstrated that H-4β resonates at higher frequency than H-4α (and similarly for the primed counterparts).

Comparison of the H-2 and H-3 resonances to those of epicatechin[43] and the H-2' and H-3' resonances to those of theaflavin[44], reveals very strong similarities are in both cases. and supports these assignment.

Further confirmation of the proposed structure was obtained when a compound with identical chromatographic and spectroscopic properties was isolated from the chemical oxidation products of the oxidation (-)-epicatechin and (-)-epicatechin-3-*O*-gallate.

The spectroscopic and chemical oxidation findings indicate that theaflavate B has *cis* relative configurations at its chiral centres[52]. The literature shows that fresh green tea leaves contain (-)-epicatechin-3-*O*-gallate and (-)-epicatechin, as used in the chemical synthesis, rather than their enantiomers[11, 52, 53]. In this case, the absolute configurations of both of these has been reported to be 2 R and 3 R[52, 53]. Therefore, the evidence leads to the proposal that theaflavate B would have a 2 R, 3 R, 2' R, and 3' R absolute configuration. Further studies are required to support this proposal.

Theaflavate C (Compound **2**)

The UV/Visible spectrum of theaflavate C is very similar to those observed for theaflavates A and B, which is characteristic of a carboxy-substituted hydroxy-benzotropolone compound[22]. The molecular mass of this molecule is 700 Daltons. This suggests theaflavate C has an empirical formula of $C_{36}H_{28}O_{15}$ which is identical to that of theaflavate B. It is also consistent with theaflavate C being formed from the oxidative condensation of (-)-epicatechin-3-*O*-gallate (442 Daltons) and (+)-catechin (290 Daltons) as was expected based on the precursors used in this synthesis, i.e. (442+290-32 = 700). Examination of the ^{1}H and ^{13}C-{^{1}H} NMR spectra confirm these conclusions and is supported the interpretation of theaflavate B (compound **1**). These data are summarised in Tables 2.7 (^{13}C-{^{1}H}), 2.8 (HMBC), 2.9 (^{1}H) and 2.10 (coupling constants), while spectra are shown in Appendix 1. The proposed structure for theaflavate C is shown in Figure 2.6.

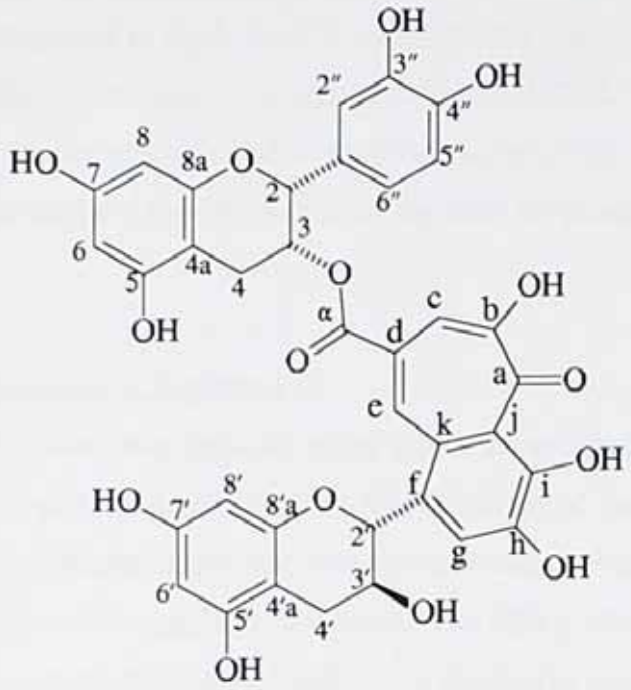


Figure 2.6 Structure of theaflavate C labelled for NMR data.

The ^{1}H spectrum contains resonances in the range (7.5-8.5 ppm) whose chemical shifts are characteristic of benzotropolone protons. The group of resonances in region of 6.7 to 7.1 ppm confirm the presence of a single 1,3,4 substituted aromatic system as found in (+)-catechin. The two pairs of doublets (J = 2.3 Hz, *i.e. meta* coupling) in the region 5.9 to 6.2 ppm (H-6, H-6', H-8 and H-8') of the ^{1}H NMR spectrum have the chemical shifts and coupling constants expected for the A-ring protons in the proposed structure. The aliphatic resonances are characteristic of those expected from protons in flavan-3-ol C-rings.

The coupling constants for the aliphatic protons H-2, H-3 and H-2', H-3' indicate that theaflavate C has the following relative configurations; one flavanol group has a *cis*-2,3 geometry (epicatechin-like; $J_{2,3} < 1$ Hz) and the other flavanol unit has a *trans*-2,3 geometry (catechin-like) as characterized by a large (*ca.* 9 Hz) $J_{2',3'}$ coupling constant[51] as would be expected in the light of the precursor flavan-3-ols used in this synthesis.

The spectroscopic findings confirm that theaflavate C has *cis* and *trans* relative configurations at its chiral centres. The synthesis used (-)-epicatechin-3-*O*-gallate and (+)-catechin, rather than their enantiomers. In this case, the absolute configurations of these compounds have been reported to be 2 R, 3 R and 2 R, 3 S, respectively[52, 53]. Therefore, the evidence indicates that theaflavate C would have a 2 R, 3 R, 2' R, and 3' S absolute configuration. Further studies are required to confirm the presence of this compound in black tea extracts and to support the absolute configuration proposed here.

2.4.2 Theaflavins

Isotheaflavin-3'-*O*-monogallate (Compound **3**)

Isotheaflavin-3'-*O*-monogallate was isolated as the major component of a mixture (2:1 with epitheaflagallin-3-*O*-monogallate). The UV-Vis spectrum of the sample was characteristic of a theaflavin-type compound and the molecular mass of this compound was 716 Da which is typical of a monogalloyl theaflavin. The HPLC data was consistent with the hypothesis that this compound was an isomer of a theaflavin monogallate.

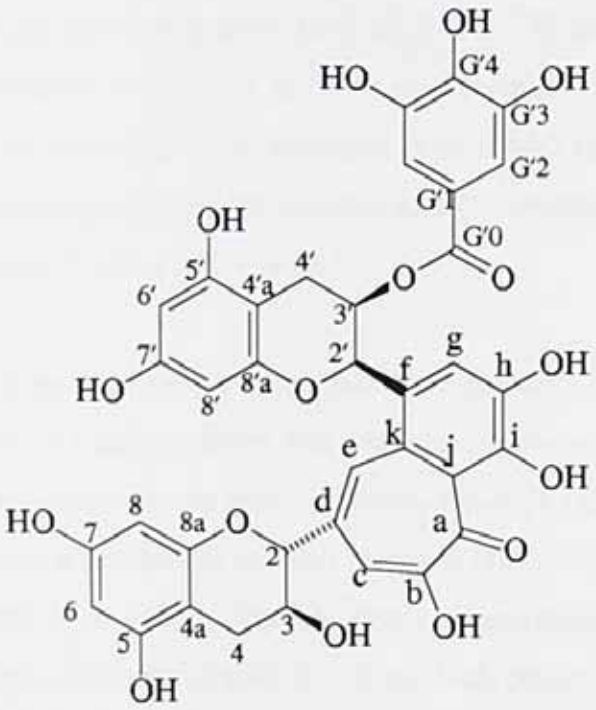


Figure 2.7 Structure of Isotheaflavin-3'-O-monogallate.

The NMR data for compound **3** is summarised in Tables 2.7a (^{13}C-{^{1}H}), 2.8 (HMBC), 2.9a (^{1}H) and 2.10 (coupling constants) The spectra are shown in Appendix 1.

The ^{1}H NMR spectrum of this molecule confirms that it is a theaflavin containing a single galloyl ester group (indicated by a singlet resonance at 7.0 ppm which integrates to be two proton equivalents). The proton signals in the region 2.5 to 6.0 ppm are characteristic of the aliphatic C-ring protons (H-2, H-3, H-4α, H-4β and their primed counterparts). The coupling constants which are observed in these signals confirm that one flavanol group has a *cis*-2,3 relative configuration (c.f. epicatechin; $J_{2',3'} < 1$ Hz) whilst the other has a *trans*-2,3 geometry (c.f. catechin; $J_{2,3}$ 8.6 Hz). Further confirmation of this finding is found by the comparison of the aliphatic signals of this molecule with those seen in the NMR spectra of theaflavin and its gallates (Table 2.7 and 2.7a). This shows that H-2, H-3 and H-2', H,3' signals of isotheaflavin-3'-*O*-monogallate very closely match those of theaflavin-3'-*O*-monogallate. The use of 2D ^{13}C-^{1}H correlation techniques (Table 2.8) supports these conclusions. Correlation from H-2' ($J_{2',3'}$ - 0.8 Hz) to C-g and C-f showing that the epicatechin-like flavanol unit is linked to the six membered ring of the benzotropolone moiety, whilst H-2 correlates to C-c, C-e and C-d indicating the catechin-like flavanol unit is linked to the seven membered ring. Finally, the position of the galloyl ester group is confirmed by correlation from H-3' to C-G'0. These assignments are consistent with those described by Davis *et al.*[44] for theaflavin and its galloyl esters. Therefore, the structure of isotheaflavin-3'-*O*-gallate is as shown in Figure 2.8, suggesting that it is formed from coupling of (+)-gallocatechin and (-)-epicatechin-3-*O*-gallate based on the work of Takino *et al.*[24] and Collier *et al.*[22].

A large scale synthesis of isotheaflavin-3'-*O*-gallate from the catechin precursors was not possible since insufficient (+)-gallocatechin was available. However, a small scale chemical oxidation of (+)-gallocatechin and (-)-epicatechin-3-*O*-gallate produced a reaction mixture containing a compound with an identical HPLC retention time to that observed for isotheaflavin-3'-*O*-gallate. The absolute configuration of this molecule was derived from the recorded configurations of the flavan-3-ols precursors present in tea (ECG- 2 R, 3 R and GC- 2R, 3 S)[52, 53] and is proposed to be 2 R, 3 S and 2' R, 3'R.

Neotheaflavin-3-*O*-monogallate (Compound **4**)

The UV-Visible spectrum of the sample was characteristic of a theaflavin-type compound and the mass spectrum showed that the molecular mass of the main component was 716 Daltons which is typical of a monogalloyl theaflavin. The HPLC data was consistent with the hypothesis that this compound was an isomer of a theaflavin monogallate. All analysis techniques suggested that the sample was a mixture of compounds.

The high levels of impurities made the 1D spectra of this compound more difficult to interpret (Tables 2.7a and 2.9a; Spectra in Appendix 1). More informative data was obtained by the application of 2D ^{1}H-^{13}C techniques (Table 2.8). The ^{1}H NMR spectrum indicated the presence of benzotropolone resonances (H-c, H-e and H-g), a galloyl ester group (H-G2, singlet at 6.99 ppm), A-ring signals (H-6, H-8 (unresolved signals) and H-6',H-8') and the C-ring resonances (Table 2.9a).

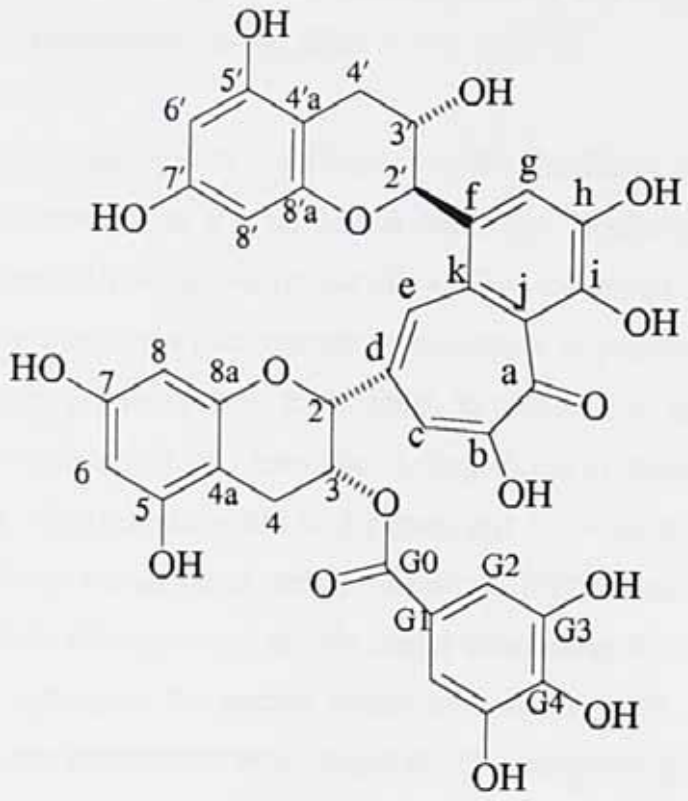


Figure 2.8 Structure of Neotheaflavin-3-*O*-monogallate

The observation of these signals supports the assumption that the major component in this mixture is a theaflavin monogallate. Furthermore, the coupling constants (Table 2.10) around the C-rings of this molecule shows one has a *trans*-2,3 geometry (c.f. catechin $J_{2',3'}$ 8.2 Hz), whilst the other has a *cis*-2,3 relative configuration (c.f. epicatechin $J_{2,3}$ <2 Hz). Correlations from H-2' ($J_{2',3'}$ 8.2 Hz) to C-g and C-f show it is attached to the six membered ring of the benzotropolone moiety. H-2 ($J_{2,3}$ < 2 Hz) correlates to C-c, C-d and C-d confirming its position on the seven membered ring. The positioning of the galloyl ester group cannot be achieved directly from the HMBC spectrum as no correlations are observed to the galloyl ester carbon C-G0. The findings of previous studies on the flavan-3-ols of green tea[43], have shown that the chemical shift of H-3 is i 1.5 ppm downfield when a galloyl ester group is also attached at this position. A similar observation was made for the theaflavin series[44] (Table 2.7a). This strongly suggests that this is the 3-*O*-gallate as H-3 appears at 5.69 ppm whilst H-3' occurs at 4.25 ppm. This conclusion is further supported by the comparison of the ^{1}H and ^{13}C data obtained for this compound with those of theaflavin and its gallates. Clearly the closest match is achieved with theaflavin-3-*O*-monogallate (Table 2.7a)

These data suggest that compound **4** is an isomer of the theaflavin monogallates. However, the HPLC data demonstrates that it is neither of the major theaflavin monogallates nor isotheaflavin-3'-*O*-monogallate. Based on the flavan-3-ol composition of the fresh green leaf [8], only one other theaflavin monogallate stereoisomer is possibly present, neotheaflavin-3-*O*-monogallate (Figure 2.8), which is predicted to be formed from (-)-epigallocatechin-3-*O*-gallate and (+)-catechin. A large scale synthesis of neotheaflavin-3-*O*-monogallate from (-)-epigallocatechin-3-*O*-gallate and (+)-catechin yielded a reaction mixture which contained a compound with an identical HPLC retention time and UV/Visible spectrum to that observed for the major component of the mixture isolated from black tea. The spiking of the natural isolate with the synthetic product supported the assumption that the two compounds were identical. This supports the assignment of the structure shown in Figure 2.8. The absolute configurations of this molecule are derived from the recorded configurations of the flavan-3-ols precursors present in tea (C- 2 R, 3 S and EGCG- 2R, 3 R)[52, 53] and are proposed to be 2 R, 3 R and 2' R, 3' S.

Isotheaflavin (Compound **5**)

The UV-Vis spectrum of this sample was characteristic of a theaflavin and an electrospray mass spectrum indicated the presence of a theaflavin-like molecule (MW = 564 Daltons). However, the retention time of this compound confirmed that this was an isomer of theaflavin. The assignments of the ^{1}H and ^{13}C spectra of compound **5** follow from the assignments made for isotheaflavin-3'-*O*-monogallate and the major theaflavins described by Davis *et al.*[44]. Characteristic signals are observed for the benzotropolone, A-ring and C-ring protons. The spectra for this compound are shown in Appendix 1.

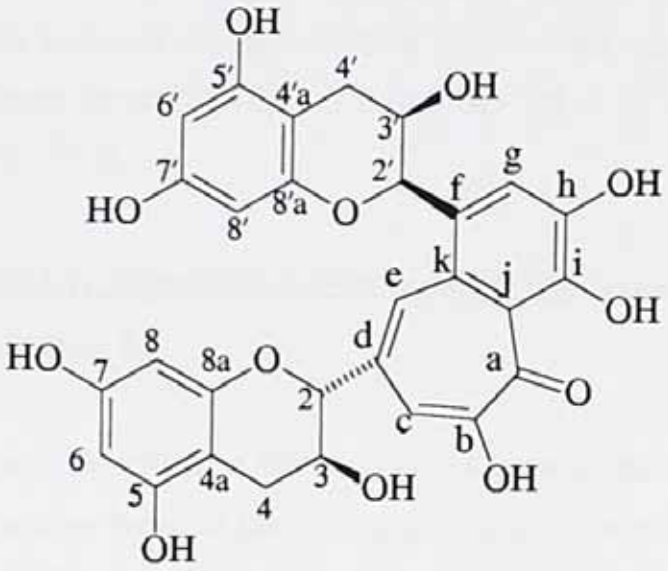


Figure 2.9 Structure of Isotheaflavin

The coupling constants of the C-ring protons are in agreement with those observed in isotheaflavin-3'-*O*-monogallate indicating that these compounds share the same configurations on these rings. One flavanol group has a *cis*-2,3 geometry (epicatechin-like; $J_{2',3'} < 1$ Hz) and the other flavanol unit has a *trans*-2,3 geometry (catechin-like) as characterized by a large (*ca.* 9 Hz) $J_{2,3}$ coupling constant[51] (see Table 2.10 for all recorded coupling constants).

Comparison of the positions of the aliphatic resonances of this molecule to those observed in the NMR spectra of theaflavin and its mono- and di-galloyl esters allowed the structure to be deduced as that shown in Figure 2.9, the chemical shifts of H-2, H-3 and their dashed counterparts most closely match those of theaflavin, (see Table 2.9a ^{1}H data and Table 2.7a ^{13}C). This suggests compound **5**, isotheaflavin, is formed from the coupling of (+)-gallocatechin and (-)-epicatechin based on these data and the proposed mechanism for theaflavin formation[22] and the previous report of this compound isolation by Collier *et al*[22].

The chemical synthesis of isotheaflavin from (+)-gallocatechin and (-)-epicatechin produced a reaction mixture containing a compound with an identical HPLC retention time and UV/Visible spectrum. This supports the proposed structure (Figure 2.9). The absolute configurations in this molecule are derived from the recorded configurations of the flavan-3-ols precursors present in tea (EC- 2 R, 3 R and GC- 2R, 3 S)[39, 40] and are proposed to be 2 R, 3 S and 2' R, 3' R.

Theaflavin (Compound **7**), Theaflavin-3-*O*-monogallate (Compound **8**) and Theaflavin-3'-*O*-monogallate (Compound **9**)

These compounds were identified on the basis of comparison their chromatographic and physical properties against those of known standards and those recorded in the literature [22, 44]. The NMR data for these compounds are summarised in Tables 2.7a (^{13}C-{^{1}H}), 2.9a (^{1}H) and 2.10 (coupling constants). (The spectra are shown in Appendix 1).

2.4.3 Theaflagallins and Theaflavic Acids:

Compound **6** (Epitheaflagallin-3-*O*-monogallate)

The UV-Vis spectrum of compound **6** (absorbance maxima (λ_{max}) at 284, 304, 373 and 430 nm) is not similar to that of either theaflavates (λ_{max} at 284 and 406 nm) or the theaflavins (λ_{max} at 280, 380 and 460 nm). The molecular mass of compound **6** is 552 Daltons, corresponding to an empirical formula of $C_{27}H_{20}O_{13}$.

Examination of the ^{1}H and ^{13}C-{^{1}H} NMR spectra (Appendix 1) confirms these conclusions. These data are shown in Tables 2.7a (^{13}C-{^{1}H}), 2.7 (HMBC), 2.9a (^{1}H) and 2.10 (coupling constants).

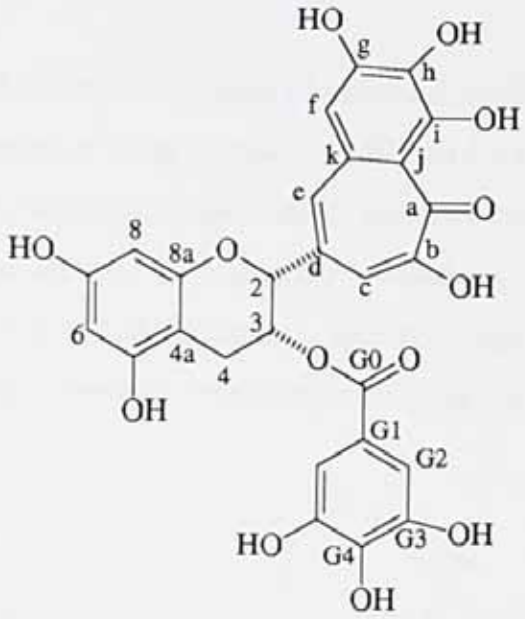


Figure 2.10 Structure of Epitheaflagallin-3-*O*-gallate.

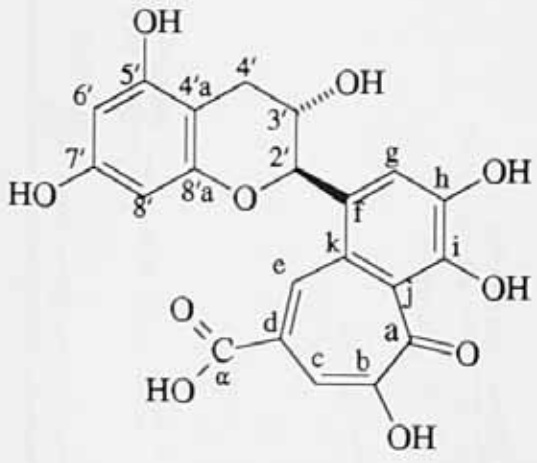
The ^{1}H spectrum contains resonances in the range (7.02-7.69 ppm) whose chemical shifts are characteristic of benzotropolone protons. A galloyl ester is also identifiable from the signal at 7.01 ppm which integrates to two proton equivalents and the ^{13}C spectrum also contains an ester carbonyl (C-G0) signal at 165.89 ppm. The remaining resonances can be assigned to the A and C-ring protons.

The use of 2D techniques confirms these assignments and shows that the benzotropolone ring is attached to the C-ring at the 2 position (correlation C-G0 to H-3), supporting the proposed structure (Figure 2.10) and suggesting that precursors of this compound are (-)-epigallocatechin-3-*O*-gallate and gallic acid, as proposed by Nishioka *et al*[9].

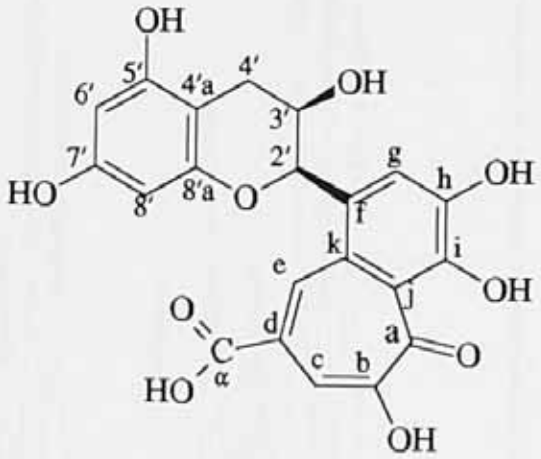
This proposed structure was further supported by the isolation of a compound with identical physical, spectroscopic and chromatographic properties, from the reaction mixture produced by the chemical oxidation of (-)-epigallocatechin-3-*O*-gallate and gallic acid. The configuration of this compound follows from that proposed for (-)-epigallocatechin-3-*O*-gallate (2 R, 3 R)[52, 53] and so would also be 2 R, 3 R.

Compound **10** (Theaflavic Acid) and Compound **11** (Epitheaflavic Acid)

The UV-visible spectra, HPLC data and results of chemical synthesis studies were all consistent with the identification of these compounds **10** and **11** as theaflavic acids. The NMR data (Tables 2.7a, 2.9a) confirmed the identification of compound **11** as epitheaflavic acid (Spectra are shown in Appendix 1), based on its coupling constants around the aliphatic C-ring (Table 2.9). Chemical synthesis supported compound **10** as being theaflavic acid. All data were also consistent with those reported in the literature[22].



10



11

Figure 2.11 Structures of theaflavic acid (**10**) and epitheaflavic acid (**11**)

Compound **12** (Purpurogallin Carboxylic Acid)

Compound **12** , isolated from those chemical oxidations involving gallic acid, was identified as a benzotropolone ring system with a carboxylic acid group attached at carbon C-d. The NMR data is consistent with that previously reported by Nishioka *et al.*[9]. (Spectra are shown in Appendix 1, whilst the data are summarised in Tables 2.7a (^{13}C-{^{1}H}), 2.9a (^{1}H) and 2.10 (coupling constants)).

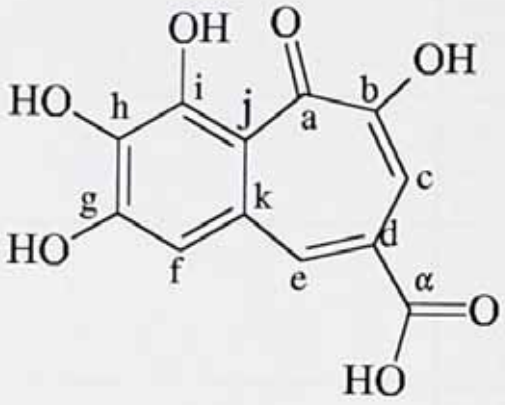


Figure 2.12 Structure of Purpurogallin-carboxylic acid

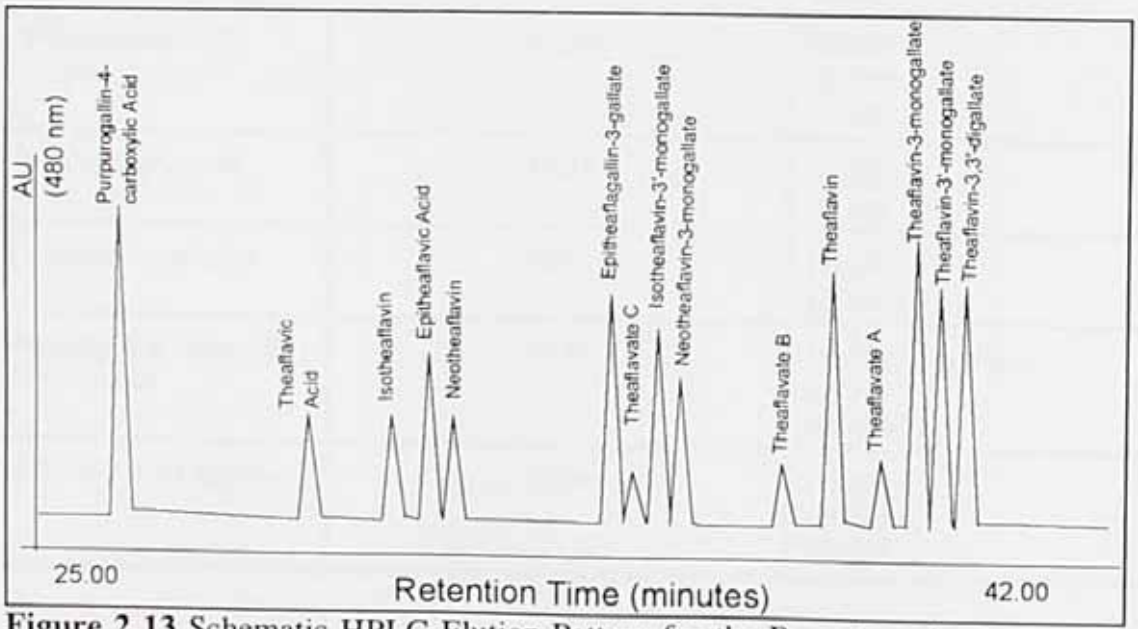


Figure 2.13 Schematic HPLC Elution Pattern for the Benzotropolone Derivatives of Black Tea.

Table 2.6 HPLC and UV/Vis Spectroscopy Data

	Retention Time /minutes	UV-Visible Spectrum Data (λ_{max} / nm) Solvent: Water-CH_3CN-CH_3COOH
1 Theaflavate B	38.99	(1) 284 (2) 406
1a Theaflavate A	41.43	(1) 282 (2) 398
2 Theaflavate C	36.39	(1) 284 (2) 400
3 Isotheaflavin-3'-*O*-monogallate	36.32	(1) 284 (2) 375 (3) 455
4 Neotheaflavin-3-*O*-monogallate	36.71	(1) 277 (2) 373 (3) 451
5 Isotheaflavin	32.28	(1) 273 (2) 375 (3) 455
6 Epitheaflagallin-3-*O*-gallate	35.62	(1) 284 (2) 304 (3) 373 (4) 430
7 Theaflavin	39.52	(1) 273 (2) 375 (3) 455
8 Theaflavin-3-*O*-monogallate	41.43	(1) 273 (2) 375 (3) 455
9 Theaflavin-3'-*O*-monogallate	42.54	(1) 273 (2) 375 (3) 455
10 Theaflavic Acid	30.38	(1) 282 (2) 396
11 Epitheaflavic Acid	33.34	(1) 282 (2) 400
12 Purpurogallin carboxylic acid	25.76	(1) 292 (Shoulder) (2) 311 (3) 400
Theaflavin-3,3'-*O*-digallate	42.99	(1) 273 (2) 375 (3) 455

Table 2.7 13C-{^{1}H} NMR data for Compounds **1**, **1a**, **2** and Theaflavin-3,3'-*O*-digallate.

	1 / ppm	**1a**[50] / ppm	**2** / ppm	Tfdg / ppm
α	166.78	167.72	166.63	-
a	185.99	186.79	186.05	185.12
b	154.44	155.24	154.27	155.02
c	115.76	115.61	116.03	117.81
d	124.21	124.72	124.04	134.94
e	132.02	131.38	134.45	126.64
k	126.39	126.51	128.98	128.44
f	134.77	133.36	135.11	130.34
g	123.76	122.81	122.74	123.15
h	148.97	149.40	149.31	146.51
i	151.52	151.95	152.05	151.12
j	121.92	122.57	122.01	121.16
2	77.43	77.82	77.50	80.53
3	71.65	71.95	71.59	69.69
4	26.37	26.53	26.38	26.72
4a	98.81	99.29	98.79	98.82
5	157.40	157.74	157.42	157.97[cd]
6	96.90	97.06	96.76	96.97[c]
7	157.95	157.99	157.82	157.64[cd]
8	96.27	96.34	96.15	95.90[c]
8a	156.77	156.91	156.82	156.44[c]
2'	76.67	75.52	79.63	75.10
3'	66.02	68.74	69.30	68.02
4'	29.81	27.17	30.10	27.06
4'a	100.15	99.92	101.15	99.36
5'	157.78	157.92	157.06	157.97[cd]
6'	96.90	97.23	97.00	96.97[c]
7'	157.62	157.99	157.92	157.54[cd]
8'	96.04	96.23	95.94	96.04[c]
8'a	157.04	157.01	156.79	157.07[c]
G0	-	167.13	-	166.04
G1	-	120.85	-	121.37
G2	-	110.12	-	109.99
G3	-	146.30	-	145.98
G4	-	139.91	-	139.01
G'0	-	-	-	166.04
G'1	-	-	-	121.24
G'2	-	-	-	109.99
G'3	-	-	-	145.98
G'4	-	-	-	139.01
1"	130.95	131.02	131.09	-
2"	113.97	114.00	114.27	-
3"	145.82	146.25	145.74	-
4"	145.48	145.88	145.49	-
5"	115.86	116.27	115.96	-
6"	118.72	118.85	118.82	-

[a] Measured at 300 K in acetone-d_6 unless otherwise stated and referenced relative to internal TMS or the solvent peak = 29.83 ppm.

[c] Assignment follows from HMBC connectivities and unequivocal data from analogous compounds very strongly suggesting that δC-6 > δC-8.

[d] Assignment interchangeable.

Table 2.7a ^{13}C-{^{1}H} NMR data for Compounds 3 to 12 including Purpurogallin for reference.

	3 / ppm	**4** / ppm	**5** / ppm	**6** / ppm	**7** / ppm	**8** / ppm	**9** / ppm	**11** / ppm	**12** / ppm	Pur / ppm
α	-	-	-	-	-	-	-	168.18	167.88	-
a	185.39	185.22	182.74	185.31	184.90	185.03	185.00	186.03	183.76	183.26
b	155.44	154.60	154.59	155.21	154.72	154.99	154.90	154.48	154.25	155.70
c	117.88	117.79	116.65	118.04	199.24	117.61	119.44	116.10	114.89	116.98
d	135.19	133.94	134.58	134.63	135.20	133.80	135.88	124.73	125.52	124.73
e	128.08	128.40	133.60	128.57	127.17	126.36	126.81	132.40	139.39	135.61
k	128.43	129.66	134.08	128.62	128.83	128.50	128.73	126.86	132.23	134.96
f	130.28	132.15	111.98	131.77	131.77	131.61	130.32	134.55	114.65	111.37
g	123.31	122.90	152.14	124.11	123.90	124.00	123.16	123.60	151.88	152.06
h	146.44	146.76	135.32	146.40	146.15	146.42	146.22	148.65	137.41	135.37
i	151.13	151.11	152.46	150.82	150.16	150.83	150.92	151.01	153.26	155.56
j	121.68	121.84	115.70	121.72	121.68	121.55	121.69	122.23	116.13	115.94
G0	-	166.43	-	-	-	166.02	-	-	-	-
G1	-	121.08	-	-	-	121.31	-	-	-	-
G2	-	109.98	-	-	-	109.88	-	-	-	-
G3	-	145.99	-	-	-	145.90	-	-	-	-
G4	-	139.18	-	-	-	139.04	-	-	-	-
G'0	165.74	-	-	-	-	-	165.69	-	-	-
G'1	121.38	-	-	-	-	-	121.46	-	-	-
G'2	109.93	-	-	-	-	-	109.90	-	-	-
G'3	145.99	-	-	-	-	-	145.96	-	-	-
G'4	138.96	-	-	-	-	-	138.90	-	-	-

Table 2.7a Continued.

	3 / ppm	**4** / ppm	**5** / ppm	**6** / ppm	**7** / ppm	**8** / ppm	**9** / ppm	**11** / ppm	**12** / ppm	Pur / ppm
2	85.97	80.29	85.99	79.86	81.37	79.70	81.54	-	-	-
3	68.24	70.00	68.28	68.87	66.42	69.21	66.48	-	-	-
4	29.87	26.70	29.82	26.84	29.49	26.62	29.55	-	-	-
4a	100.79	98.49	100.76	98.82	99.47	98.79	99.55	-	-	-
5	157.23[c]	157.92[cd]	157.21	157.59	157.83[d]	158.02[cd]	157.93[d]	-	-	-
6	96.70[c]	96.89[c]	96.66	96.98	96.60	97.03[c]	96.66	-	-	-
7	158.03[c]	157.84[cd]	157.98	157.99	157.72[d]	157.75[cd]	157.76[d]	-	-	-
8	95.54[c]	95.62[c]	95.53	95.90	95.70	95.80[c]	95.74	-	-	-
8a	156.44[c]	156.23[c]	156.41	156.50	156.66	156.24[c]	156.73	-	-	-
2'	75.30	79.19	76.66	-	76.84	76.68	75.48	76.89	-	-
3'	67.85	68.96	65.46	-	65.25	65.56	67.94	65.86	-	-
4'	26.95	29.17	29.67	-	29.72	29.76	27.01	29.92	-	-
4'a	99.28	100.49	99.95	-	100.04	99.77	99.37	99.62	-	-
5'	157.61[c]	157.53[d]	157.79	-	157.72[d]	157.86[cd]	157.76[d]	157.77	-	-
6'	97.02[c]	96.66	96.66	-	96.60	96.74[c]	96.99	96.60	-	-
7'	157.93[c]	157.34[d]	157.66	-	157.63[d]	157.55[cd]	157.65[d]	157.68	-	-
8'	96.02[c]	95.34	95.94	-	95.90	95.98[c]	96.02	95.94	-	-
8'a	156.97[c]	156.60	157.16	-	157.17	157.09[c]	157.03	157.09	-	-

[a] Measured at 300 K in acetone-d_6 unless otherwise stated and referenced relative to internal TMS or the solvent peak = 29.83.
[b] Measured at 303 K
[c] Assignment follows from HMBC connectivities and unequivocal data from analogous compounds very strongly suggesting that δC-6 > δC-8.
[d] Assignment interchangeable.

Table 2.8 Long-range proton-carbon correlations of compounds **1**, **3**, **4**, **6** and **11** measured using the HMBC experiment[a].

	1	**3**	**4**	**6**	**11**
c	α, a, b, d, e	2, a, b, d, e	2, a, b, d, e	2, a, b, d, e	α, a, b, d, e
e	α, c, d, f, j	2, c, d, f, j	2, c, d, f, j	a, e, g, h, j	b_w, c, d, f, i_w , j, k_w
g	2', f_w, h_w, i_s, k_s	2', f_w, h_w, i_s, k_s	2', h_w, i_s, k_s	-	α, 2', f, h, i, k
2	3, 1", 2", 6"	3, 4, c, d, e	3, c, d, e	3, c, d, e	-
3	α, 4a	4a	4a	4a, G0	-
4α	2, 3, 4a, 5, 8a	2, 3, 4a, 5, 8a	(2, 3, 4a, 5, 8a)	2, 3, 4a, 5, 8a	-
4β	4a, 5, 8a	3, 4a, 5, 8a	4a, 5, 8a	4a, 5, 8	-
6	4a, 5, 7, 8	4a, 5, (7), 8	(4a, 5, 7, 8)	4a, 5, 7, 8	-
8	4a, 6, 7, 8a	4a, 6, 7, 8a	(4a, 6, 7, 8a)	4a, 6, 7, 8a	-
2'	f, g	3', f, g, k	3', 4', 8'a, f, g	-	3', 4', 8'a, f, g, k
3'	4a'	4'a, G'0	n.o.	-	4a'
4α'	2', 3', 4'a, 5, 8'a	2', 3', 4'a,8'a	(2', 3', 4'a, 5', 8'a)	-	2', 3', 4'a, 5', 8'a
4β'	4'a, 5', 8'a	4'a, 5', 8'a	4'a, 5', 8'a	-	2', 3', 4'a, 5', 8'a
6'	4'a, 5', 7', 8'	4'a, 5', 7', 8'	4'a, 8', (5', 7')	-	4'a, 5', 7', 8'
8'	4'a, 6', 7', 8'a	4'a, 6', 7', 8'a	4'a, 6', 8'a, (7')	-	4'a, 5', 7', 8'
G2	-	-	-	-	-
G2'	-	G'1, G'2, G'3, G'4, G'0	-	-	-

Table 2.8 Long-range proton-carbon correlations of compounds **1**, **3**, **4**, **6** and **11** measured using the HMBC experiment[a]. (continued)

	1	**3**	**4**[b]	**6**	**11**
2"	2, 3", 4", 6"	-	-	-	-
5"	1", 3", 4"	-	-	-	-
6"	2, 2", 4"	-	-	-	-
OH-5	-	-	-	4a, 5, 6	-
OH-7	-	-	-	6, 7, 8	-
OH-G3	-	-	-	G2, G3, G4	-
OH-G4	-	-	-	G3, G4	-
OH-b	-	-	-	a, b, c, d	-
OH-g	-	-	-	f, g, h	-
OH-h	-	-	-	g, h, i	-
OH-i	h, i, j	n.m.[d]	n.m.[d]	h, i, j	-

[a] Measured at 303 K in acetone-d_6 plus D_2O. Only those correlations with sufficient signal-to-noise to be fully reliable are listed. Numbers in parentheses represent correlations that cannot be unambiguously assigned due to signal overlap. Where assignments were made on the basis of the intensity of the correlation the following notation is used: Subscript s = Strong; Subscript w = Weak. If no correlation from H-2 to C-8a (or H-2' to C-8'a) was noted then ambiguities in A-ring assignments due to the inability to distinguish H-6 from H-8, etc. were resolved by ^{13}C shifts (δC-6 > δC-8).

[b] Measured in acteone-d_6 plus D_2O.

[c] Not observed.

[d] Not measured.

Table 2.9 {^{1}H} NMR data for Compounds **1**, **1a**, **2** and Theaflavin-3,3'-*O*-digallate.

	1 / ppm	**1a**[50h] / ppm	**2** / ppm	Tfdg / ppm
c	7.69	7.67	7.70	7.76
e	8.46	8.36	8.46	7.97
g	8.04	7.82	8.04	8.04
2	5.17	5.11	5.17	5.46
3	5.69	5.70	5.64	5.74
4α	3.04	3.04	2.99	2.96
4β	3.17	3.11	3.10	3.07
6	6.09	5.98	6.07	6.11[e]
8	6.21	6.15	6.05	6.14[e]
2'	5.65	5.65	5.46	6.10
3'	4.22	5.53	4.18	5.79
4'α	2.93	2.91	3.04	2.99
4'β	3.27[d]	3.32	2.76[d]	3.28
6'	6.17[e]	6.10	6.15[e]	6.13[e]
8'	6.09[e]	6.11	5.97[e]	6.15[e]
G2	-	-	-	7.02
G'2	-	6.79	-	6.98
2"	7.01	6.84	7.04	-
5"	6.97	6.53	6.95	-
6"	6.65	6.88	6.73	-
OH-i	14.77[f]	n.m.[g]	14.97[f]	14.87[f]

[a] Measured at 300 K in acetone-d_6 plus D_2O and referenced relative to internal TMS or the solvent peak at 2.087 ppm unless otherwise stated.
[b] Measured at 303 K.
[c] The following assignments were obtained from additional spectra measured at 263 K in acetone-d_6.
[d] Distinguish from 4α (or 4'α as appropriate) by coupling chemical shift .
[e] Assignment follows from HMQC *and* unequivocal data from analogous compounds showing δC-6 > δC-8.
[f] Measured in acetone-d_6 and referenced relative to internal TMS or the solvent peak = 2.052 ppm.
[g] Not measured.
[h] Measured at 303 K in methanol-d_4 solvent and quoted in ppm relative to solvent peak at 3.304 ppm.

Table 2.9a {^{1}H} NMR data for Compounds 3 to 12 including Purpurogallin for reference.

	3 / ppm	**4**[b] / ppm	**5**[b] / ppm	**6**[c] / ppm	**7** / ppm	**8** / ppm	**9**[b] / ppm	**11** / ppm	**12** / ppm	Pur / ppm
c	7.42	7.67	7.40	7.52	7.58	7.66	7.59	7.90	7.77	7.14
d	-	-	-	-	-	-	-	-	-	6.81
e	7.83	8.26	7.79	7.69	7.99	8.02	8.02	8.78	8.33	7.40
f	-	-	-	7.03	-	-	-	-	7.23	7.02
g	8.07	7.70	8.05	-	8.05	8.06	8.07	8.09	-	-
2	4.80	5.32	4.71	5.21	5.03	5.36	5.09	-	-	-
3	4.04	5.69	4.03	5.69	4.41	5.76	4.42	-	-	-
4α	3.10	2.96	3.06	3.00	2.85	3.00	2.87	-	-	-
4β	2.57[d]	3.05[d]	2.56	3.13	2.94[d]	3.10[d]	2.95[d]	-	-	-
6	6.08[e]	6.08	6.06	6.11	6.07	6.11[e]	6.07	-	-	-
8	5.96[e]	6.08	5.95	6.12	6.00	6.16[e]	6.00	-	-	-
2'	6.02	5.67	5.72	-	5.74	5.81	6.00	5.73	-	-
3'	5.74	4.25	4.40	-	4.49	4.56	5.77	4.46	-	-
4'α	2.96	2.97	2.84	-	2.81	2.92	2.95	3.06[d]	-	-
4'β	3.17[d]	2.70[d]	3.00	-	3.00[d]	3.11[d]	3.15[d]	2.93	-	-
6'	6.09[e]	6.11	6.07	-	6.09	6.12[e]	6.11	6.11	-	-
8'	6.13[e]	5.98	6.03	-	6.04	6.07[e]	6.14	6.05	-	-

Table 2.9a Continued.

	3 / ppm	**4** / ppm	**5** / ppm	**6** / ppm	**7** / ppm	**8** / ppm	**9** / ppm	**11** / ppm	**12** / ppm	Pur / ppm
G2	-	6.99	-	7.01	-	6.94	-	-	-	-
G'2	6.97	-	-	-	-	-	6.96	-	-	-
OH-5	-	-	-	8.77[c]	-	-	-	-	-	-
OH-7	-	-	-	8.56[c]	-	-	-	-	-	-
OH-G3	-	-	-	8.58[c]	-	-	-	-	-	-
OH-G4	-	-	-	8.34[c]	-	-	-	-	-	-
OH-b	-	-	-	8.90[c]	-	-	-	-	-	-
OH-g	-	-	-	9.58[c]	-	-	-	-	-	-
OH-h	-	-	-	8.85[c]	-	-	-	-	-	-
OH-i	14.92[f]	n.m.[g]	14.94[f]	15.03[f]	14.98[f]	14.86[f]	14.96[f]	14.82[f]	14.89[f]	15.04[f]

[a] Measured at 300 K in acetone-d_6 plus D_2O and reference relative to internal TMS or the solvent peak at 2.087 ppm unless otherwise stated.
[b] Measured at 303 K.
[c] The following assignments were obtained from additional spectra measured at 263 K in acetone-d_6.
[d] Distinguish from 4α (or 4'α as appropriate) by coupling chemical shift .
[e] Assignment follows from HMQC and unequivocal data from analogous compounds showing δC-6 > δC-8.
[f] Measured in acetone-d_6 and referenced relative to internal TMS or the solvent peak = 2.052.
[g] Not measured.
* Measured at 303 K in methanol-d_4 solvent and quoted in ppm relative to solvent peak at 3.304 ppm.

Table 2.10 ^{1}H NMR coupling constant* data for compounds **1** to **9**, **11** and theaflavin-3,3'-O-digallate[a].

	1 / Hz	**2**[b] / Hz	**3** / Hz	**4**[b] / Hz	**5**[b] / Hz	**6**[b] / Hz	**7** / Hz	**8** / Hz	**9**[b] / Hz	**11** / Hz	Tfdg / Hz
J (4α, 4β)	17.7	17.8	15.8	17.2	15.9	17.5	16.8	17.5	16.8	-	17.4
J (3, 4β)	4.6	4.6	9.6	4.5	9.6	4.5	4.3	4.6	4.1	-	4.4
J (3, 4α)	2.2	2.3	5.6	n.m.	5.8	1.9	2.4	2.2	2.7	-	2.2
J (2, 4α)	n.r.	n.r.	n.r.	n.m.	n.r.	0.4	n.r.	0.8	0.6	-	n.r.
J (2, 3)	1.2	1.2	8.6	≤ 2	8.6	1.3	1.3	1.3	1.3	-	1.3
J (6, 8)	2.3	2.3	2.3	n.r.[c]	2.3	2.3	2.3	2.3	2.3	-	2.3
J (4'α, 4β')	17.1	16.0	17.7	16.1	16.8	-	16.9	17.0	17.5	16.8	17.6
J (3, 4'β)	4.6	9.3	4.6	8.8	4.4	-	4.6	4.4	4.6	4.5	4.6
J (3', 4'α)	1.8	5.7	1.7	5.4	2.2	-	2.1	1.9	1.9	2.2	1.8
J (2', 4'α)	n.r.	n.r.	n.r.	n.r.	n.r.	-	0.6	0.6	n.r.	0.7	n.r.
J (2', 3')	n.r.	8.6	0.8	8.2	1.2	-	0.9	0.8	0.8	0.8	0.7
J(6', 8')	2.3	2.3	2.3	2.4	2.3	-	2.3	2.3	2.3	2.3	2.3
J (2, 2")	0.6	n.r.	-	-	-	-	-	-	-	-	-
J (2, 6")	0.7	0.6	-	-	-	-	-	-	-	-	-
J (2", 6")	2.1	2.1	-	-	-	-	-	-	-	-	-
J (5", 6")	8.2	8.2	-	-	-	-	-	-	-	-	-
J (2, e)	-	-	n.r.	n.r.	n.r.	0.8	n.r.	n.r.	n.r.	-	n.r.
J (e, f)	-	-	-	-	-	0.8	-	-	-	-	-
J (c, e)	1.3	1.4	1.2	1.1	1.2	1.6	1.3	1.3	1.2	1.2	1.2

All values in Hertz (Hz).

[a] Measured at 300 K in acetone-d_6 plus D_2O and typically accurate to ± 0.2 Hz.

[b] Measured at 303 K.

[c] H-6 and H-8 are coincident.

n.m = not measured.

n.r. = not resolved.

2.5 Discussion

The isolation of a new product theaflavate B from black tea extracts is reported here. It is the first compound to have been characterised which has been formed through the oxidative coupling of two flavan-3-ols via the galloyl ester group. Previously, the galloyl ester group had been assumed to be relatively chemically inactive during the fermentation of black tea.

2.5.1 Mechanism of Formation of Theaflavates:

There are a number of important factors which must be considered when postulating a possible mechanism for the formation of the theaflavates.

Firstly, enzyme specificity studies which have been carried out on polyphenol oxidase, the enzyme responsible for initiating the fermentation of black tea, have concluded that it does not readily use gallic acid as a substrate[17]. However, gallic acid has been identified as a precursor of a number of fermentation products including the theaflavic acids[17, 22, 29] and the theaflagallins[9]. This suggests that the oxidation of gallic acid during tea fermentation is more likely to take place via an indirect oxidation pathway.

Secondly, the presence of the ester linkage in the flavan-3-ol gallate esters would serve to deactivate the aromatic ring to electrophilic attack by withdrawing electrons from the ring system.

Therefore, it seems possible that theaflavates follow a formation mechanism similar to that of the theaflavic acids reported by Berkowitz *et al.*[17] and theaflagallins[9]. These workers[17] suggested that gallic acid and flavan-3-ol quinones undergo a chemical redox reaction producing a gallic acid quinone (GAQ) and a flavan-3-ol. The GAQ is then incorporated into the fermentation reaction pathway which yields theaflavic acids and theaflagallins.

The proposed mechanism for theaflavate formation is shown below (Figure 2.14)

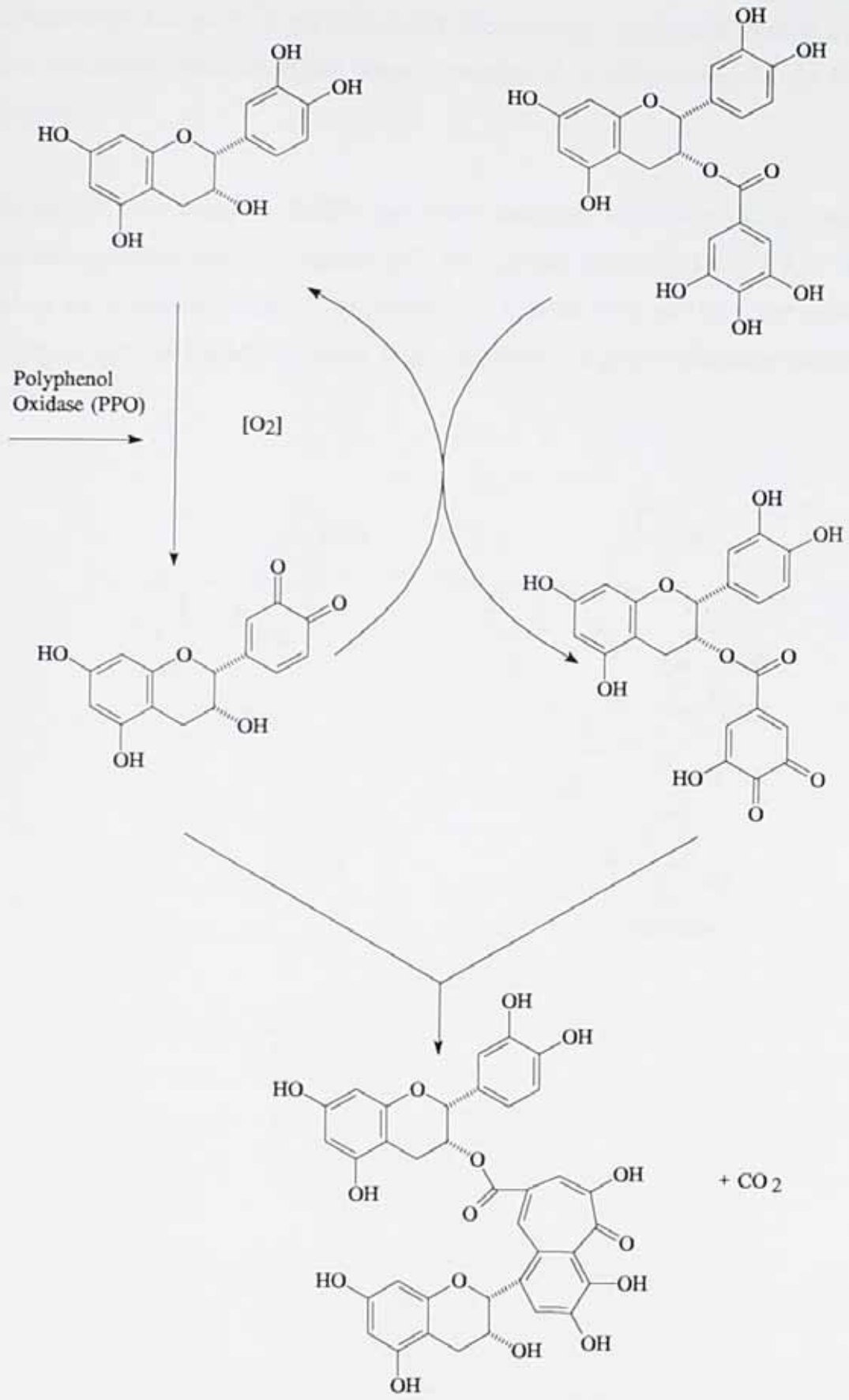


Figure 2.14 Mechanism of galloyl ester oxidation in theaflavate formation. Adapted from Berkowitz *et al.*[17]

2.5.2 Theaflavates and their Possible Involvement in Thearubigin Production:

The identification in this work of the trihydroxy-benzene ring of the galloyl ester group as a reactive site for non-enzymic oxidation opens a number of possible routes for the formation of thearubigins:

a) The galloyl ester rings of EGCG and ECG molecules which are incorporated into proanthocyanidins (eg. procyanidin B-2,3'-*O*-gallate, procyanidin B-2 3, 3'-di-*O*-gallate and procyanidin B-4 3'-*O*-gallate) can now be proposed as a site at which oxidation may take place resulting in the formation of polymeric materials (Figure 2.15).

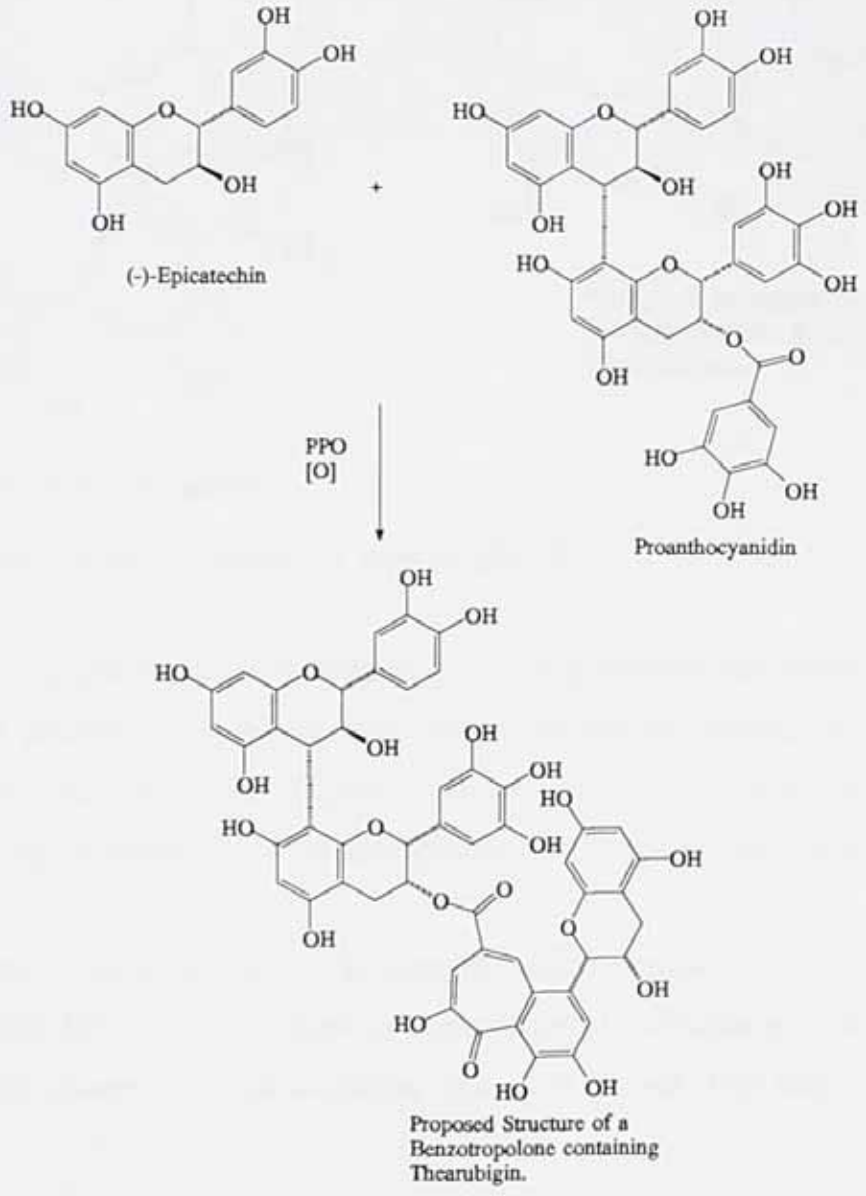


Figure 2.15 Route for the production of thearubigins 1

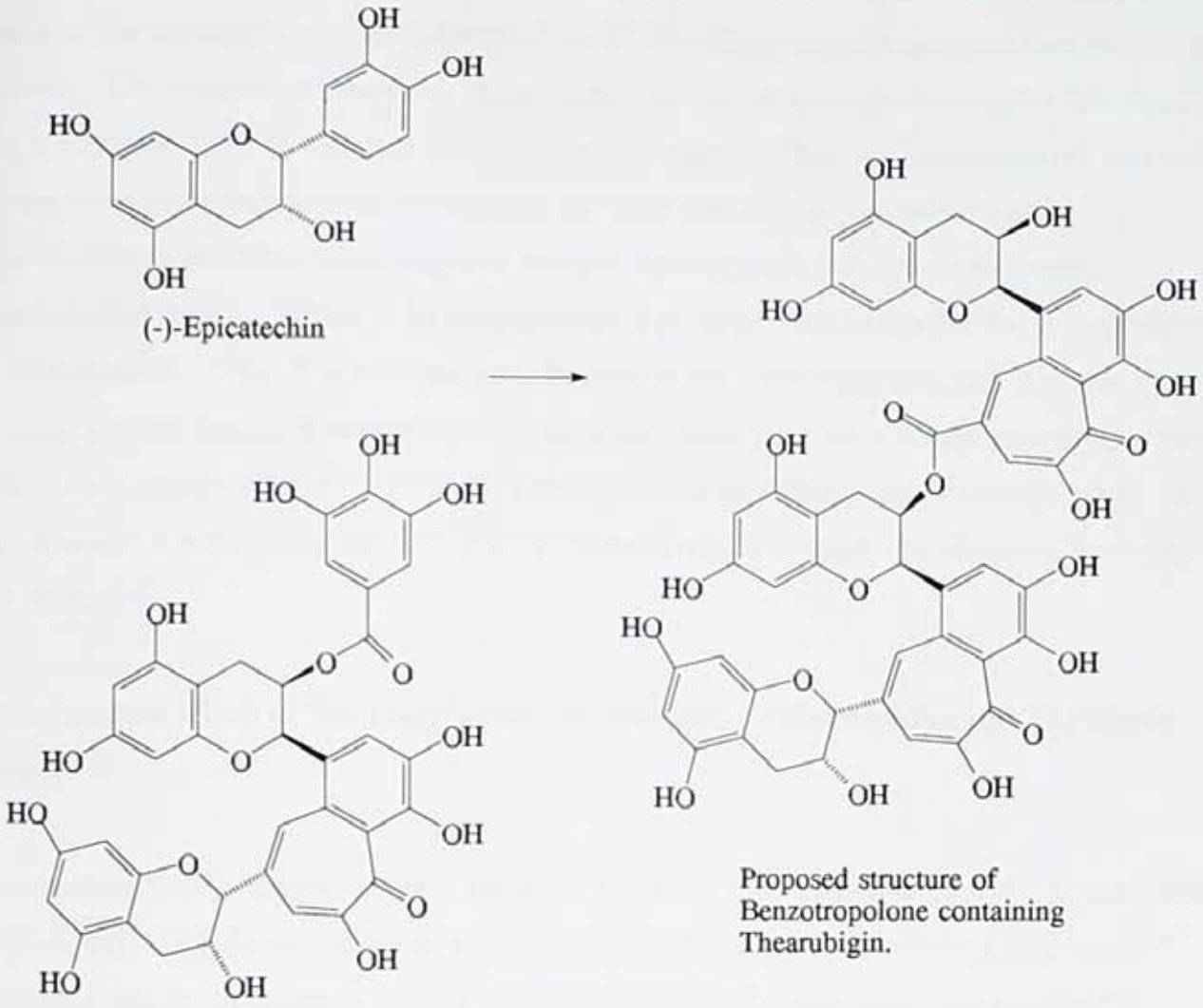


Figure 2.16 Route for the production of thearubigins 2.

b) Likewise, the galloyl esters present in mono- and digalloyl-theaflavins may also provide a mechanism by which these compounds are polymerised by the formation of theaflavate-type compounds (Figure 2.16) i.e. monomeric flavan-3-ols and theaflavins linked by the formation of a benzotropolone system using galloyl ester groups.

The efficacy of these routes is likely to be low, but their existence is now more probable than had previously been believed. Both of these potential mechanisms of thearubigin formation produce compounds with molecular masses of around 1000 Daltons.

However, it is possible that further flavan-3-ols could be incorporated into the trimer by means of the formation of C-4-C-8 or C-2"-C-2" interflavanoid linkages and benzotropolone moieties. The resultant tetramers or larger molecules would have structural characteristics that are similar to those reported by Cattell and Nursten[37]. These workers reported that ethyl acetate soluble thearubigins are pentameric flavan-3-ols, flavan-3-ol gallates and benzotropolone moieties joined together by both hydrolysable and non-hydrolysable interflavanoid bonds. Figure 2.14 demonstrates a possible mechanism for the incorporation of hydrolysable C-4-C-8 bonds into thearubigins, it has been suggested that this type of bond can also formed during fermentation[32]. In addition, incorporation of theasinensins with free galloyl ester groups (Table 1.1) via the formation of a theaflavate-type structure would allow the presence of non-hydrolysable C-2"-C-2" interflavanoid linkages, as observed by Cattell and Nursten[37].

2.5.3 Configurations of the Theaflavins, Theaflavates, Theaflagallins and Theaflavic Acids

The absolute configurations of these black tea pigments remain unclear and the measurements taken in this study do not shed any further light on this subject. However, Collier *et al.*[22] concluded that on the basis of the ^1H coupling constants ($J_{2,3}$ and $J_{2',3'}$) "the relative 2,3 configurations and flavan conformations (half chair or sofa[55]) of the precursor flavan-3-ol are retained in the derived natural and model theaflavins". Similarly, Nishioka[9] used this argument to propose the absolute configuration of theaflagallin based on its production from (+)-gallocatechin. Based on the work of Collier *et al.*[22], it is possible to propose the following absolute configurations for the compounds isolated in this study (Table 2.11).

Table 2.11 Configuration of the flavan-3-ols, theaflavins, theaflavic acids and theaflavates

	Established Relative Configuration		Proposed or Established (*) Absolute Configuration			
	2, 3	2', 3'	2	3	2'	3'
(-)-EC (*), (-)-EGC (*), (-)-EGCG (*), (-)-ECG (*)	*cis*		R	R		
(+)-Catechin	*trans*		R	S		
Theaflavin	*cis*	*cis*	R	R	R	R
Theaflavin-3-*O*-monogallate	*cis*	*cis*	R	R	R	R
Theaflavin-3'-*O*-monogallate	*cis*	*cis*	R	R	R	R
Theaflavin-3,3'-digallate	*cis*	*cis*	R	R	R	R
Epitheaflavic Acid		*cis*			R	R
Theaflavic Acid		*trans*			R	S
Epitheaflagallin-3-*O*-gallate	*cis*		R	R		
Theaflavate A	*cis*	*cis*	R	R	R	R
Theaflavate B	*cis*	*cis*	R	R	R	R
Theaflavate C	*cis*	*trans*	R	R	R	S
Isotheaflavin	*trans*	*cis*	R	S	R	R
Isotheaflavin-3'-*O*-monogallate	*trans*	*cis*	R	S	R	R
Neotheaflavin-3-*O*-monogallate	*cis*	*trans*	R	R	R	S

Adapted from Collier *et al.*[22].

These absolute configurations are proposed on the basis of the known absolute configurations of the flavan-3-ol precursors [52, 53].

In this study, it has been shown that tea leaf flavan-3-ol gallates, especially (-)-epicatechin-3-*O*-gallate, can be enzymically transformed by oxidative coupling into theaflavate B and potentially into a series of other related theaflavate derivatives. In addition, it has also been shown that isotheaflavin-3'-*O*-monogallate and neotheaflavin-3-*O*-monogallate are formed during traditional enzymic black tea production.

2.6 Conclusions

1 Polyphenols in enzymically fermented black tea, have been shown to include a novel class of compound which has been designated the theaflavates. Specifically, theaflavate B has been isolated and characterised.

2 Two minor theaflavins have been isolated and characterised, namely isotheaflavin-3'-*O*-monogallate and neotheaflavin-3-*O*-monogallate.

3 An NMR database has been established for the dimers of black tea.

4 A number of known tea polyphenols have also been isolated and characterised.

Chapter 3

Studies on the Formation of Theaflavins and Related Compounds via Chemical Oxidation.

3.1 Introduction:

In the previous chapter, reference to the use of chemical oxidation has been made to confirm the identification of the catechin precursors of novel theaflavins, and to show the possible existence of other theaflavate compounds in black tea. A number of studies have investigated the formation of theaflavins and theaflavic acids in both chemical[55] and enzymic[56] oxidation systems. However, the lack of authentic reference standards does not permit quantitative data to be gathered from these studies. In the light of the work detailed in the previous chapters, it was of great interest to carry out a quantitative study addressing the following questions;

1) How do the changes in the configuration and esterification of the flavan-3-ol precursors affect the yields of the theaflavin and theaflavate classes of compounds?

2) Do these changes cause these chemical oxidation systems to yield varying levels of thearubigin material?

The composition of black tea suggests that the epicatechin series are the most favourable substrates for the formation of theaflavins. This may be a result of the initial composition of the fresh green leaf, which is rich in the epicatechin compounds and low in catechin compounds, or because of the 2,3-*cis* configuration of the epicatechins.

In chemical oxidation model systems, it is possible to compare the reactivity of flavan-3-ol pairs which can be varied in terms of their molecular size (presence of galloyl ester groups) and/or stereo-structure. The effect of these factors can be assessed on product yield, reaction efficiency (residual precursor concentration) and the extent to which oxidation to thearubigin compounds occurs.

While these recent studies were only qualitative in nature[55, 56], they did convey some valuable information. The data suggested that stereo-structure and molecular size (the presence of galloyl ester groups) were important in determining the yield of benzotropolone derivatives in these systems. However, they did not indicate why these factors may be important. To assess their importance, it is necessary to understand the mechanism of formation of theaflavins and theaflavates. A number of mechanisms have been put forward[24, 57] (Figure 1.8). However, none of them have been experimentally proven and it was not part of this study to investigate these reaction mechanisms.

3.2 Methods and Materials

3.2.1 Materials:

Refer to Chapter 2, Section 2.3.1.

3.2.2 Oxidation conditions[22]

An ice-cooled solution of sodium hydrogen carbonate (50 mg) and potassium hexacyanoferrate (III) (75 mg) in distilled water (2.5 cm^3) was added to an ice-cooled solution of the specified flavan-3-ol precursors (see Table 3.1) in distilled water (5 cm^3). The reaction mixture was allowed to stand, with ice cooling and no stirring for 15 minutes. The solution was acidified (hydrochloric acid (1 mol dm^{-3}) to pH 2.5) and extracted with ethyl acetate (3 x 10 cm^3). The organic extracts were combined, washed (distilled water, 2 x 10 cm^3) and dried (magnesium sulfate). The dried solution was filtered (Whatman 541) and evaporated to dryness under reduced pressure.

Table 3.1 Precursors and predicted products of the synthetic studies.

Flavan-3-ol Precursors		Predicted Major Product
Dihydroxyl	Trihydroxyl	
EC: 13.75 mg	EGC: 14.74 mg	Theaflavin
EC: 13.53 mg	EGCG: 22.62 mg	Theaflavin-3-monogallate
ECG: 22.42 mg	EGC:14.92 mg	Theaflavin-3'-monogallate
ECG: 21.85 mg	EGCG: 22.00 mg	Theaflavin-3,3'-digallate
EC: 13.98 mg	GC: 15.67 mg	Isotheaflavin
ECG: 22.75 mg	GC: 16.52 mg	Isotheaflavin-3'-monogallate
C: 14.14 mg	EGC: 18.21 mg	Neotheaflavin
C: 11.43 mg	EGCG: 17.85 mg	Neotheaflavin-3-monogallate
ECG: 35.47 mg	-	Theaflavate A
EC: 12.69 mg	ECG: 25.11 mg	Theaflavate B
C: 13.62 mg	ECG: 18.90 mg	Theaflavate C

3.3 Quantitation of Synthetic Products:

3.3.1 Sample Preparation

3.3.1.1 Theaflavins.

Samples of each standard (approximately 1.5 mg) were dissolved in stabiliser solution (2% acetic acid in 20% acetonitrile with 250 ppm ascorbic acid and EDTA) with sonication to give exactly a 2 mg/cm^3 stock solution. An aliquot of each stock solution (0.1 cm^3) was diluted ten-fold with stabiliser solution and dispensed into vials to produce mixed standards.

3.3.1.2 Flavan-3-ols and Gallic Acid

A sample (9.5 mg) of each of the compounds shown below (Table 3.2) was dissolved in an exact amount of stabiliser solution such that the final concentration of the stock solution was 1 mg/cm^3. The stock solutions were then placed into a water bath at 313 K for 20 minutes with intermittent shaking to ensure complete dissolution. Aliquots (see Table 3.2) of each of the stock solutions were mixed to produce a combined working standard.

Table 3.2 Composition of mixed flavan-3-ol standard.

Gallic Acid	250 μl
(+)-Gallocatechin	100 μl
(-)-Epigallocatechin	1000 μl
(+)-Catechin	500 μl
Caffeine	500 μl
(-)-Epicatechin	500 μl
(-)-Epigallocatechin-3-*O*-gallate	500 μl
(-)-Epicatechin-3-*O*-gallate	500 μl

3.3.1.3 Synthetic Standards

A sample (~5 mg) of each of the compounds, shown in Table 3.3, was dissolved in stabiliser solution (5 cm^3).

Table 3.3 Synthetic standards

Isotheaflavin
Isotheaflavin-3'-*O*-monogallate
Neotheaflavin
Neotheaflavin-3-*O*-monogallate
Theaflavate A
Theaflavate B
Theaflavate C

3.3.1.4 Calibration

A range of volumes (5 μl to 90 μl) of each of the standards described above were analysed using the HPLC conditions described in Chapter 2, Section 2.3.1, Table 2.5 (under analytical conditions).

3.3.2 Sample Preparation

Samples of the dried reaction mixtures were dissolved in stabiliser solution, to give solutions in the concentration range 2 to 6 mg/cm^3.

3.3.3 HPLC Analysis Conditions

The HPLC analysis was carried out according to the conditions described in Chapter 2, Section 2.3.1, Table 2.4 (under analytical conditions).

3.4 Results and Discussion:

3.4.1 Reaction Mixture Analysis.

As described previously in Section 3.3, each reaction mixture was analyzed for residual flavan-3-ols (C, EC, ECG, EGC, GC, EGCG and gallic acid) content and all known theaflavin/theaflavate-derived products. The results of the qualitative analysis are summarized in Table 3.4, while the quantitative results for the major products of each reaction are summarized in Table 3.5. Based on these results, it is possible to estimate the extent to which each of these catechin pairs favours the formation of thearubigin-type (TR-type) compounds compared with the formation of predicted benzotropolone derivatives. The estimate for each of the catechin pairs after chemical oxidation (incubation time 15 minutes) is shown in Table 3.6.

Tables 3.5 and 3.6 show that the flavan-3-ols with a trihydroxyl B-ring (GC, EGC, EGCG) are utilised more efficiently in chemical oxidations than their dihydroxyl counterparts. This observation is in agreement with that made by other workers[15, 45]. Indeed, the reported redox potentials for the flavan-3-ols show that EGC, GC and EGCG have the lowest potentials and so are most efficiently oxidised. The dihydroxyl flavan-3-ols (EC, C and ECG) have higher redox potentials and so their quinones readily undergo redox reactions with trihydroxyl flavan-3-ols (EGC, GC and EGCG). The stereostructure and size of the catechin precursors (eg. galloyl ester derivatives) clearly play an important role in defining how each reaction mixture behaves in terms of the yield of the desired theaflavin/theaflavate compound, and the extent to which they produce thearubigins.

Table 3.4 Qualitative analysis of reaction mixtures

Reaction Mixture	Tf	Tf3mg	Tf3'mg	Tfdg	ITf	ITf3'mg	NTf	NTf3mg	TFA	ETFA	Tfate A	Tfate B	Tfate C	Pur. Acid
EC+EGC	✓✓													
EC+EGCG		✓✓												
ECG+EGC			✓✓								✓			
ECG+EGCG				✓✓							✓			
EC+GC					✓✓									
ECG+GC					✓	✓✓					✓			
C+EGC							✓✓							
C+EGCG								✓✓						
C+GA									✓✓					✓
EC+GA										✓✓				✓
ECG											✓✓			
EC+ECG											✓	✓✓		
C+ECG											✓		✓✓	

Key:

✓✓	Major product.
✓	Minor identified product.
	Product not present in reaction mixture.
Pur. Acid	Purpurogallin-4-carboxylic Acid.

Table 3.5 Quantitative analysis of reaction mixtures.

Amount of Residual Catechins (μmol)		Amount of Benzotropolone Product (μmol/ mmol flavan-3ol precursor)
EC - 24.7 EGC - N/D	Theaflavin	382
EC - 40.3 EGCG - 8.58	Theaflavin-3-*O*-monogallate	79
ECG - 26.9 EGC - N/D	Theaflavin-3'-*O*-monogallate	76
ECG - 28.2 EGCG - 1.57	Theaflavin-3,3'-*O*-digallate	40
EC - 12.5 GC - N/D	Isotheaflavin	110
ECG - 12.3 GC - N/D	Isotheaflavin-3'-*O*-monogallate	44
C - 32.1 EGC - N/D	Neotheaflavin	146
C - 24.4 EGCG - N/D	Neotheaflavin-3-*O*-monogallate	71
ECG - 30.0	Theaflavate A	42
EC - 27.8 ECG - 6.0	Theaflavate B	91
C - 15.0 ECG - 3.0	Theaflavate C	64

Table 3.6 Comparison of theaflavins, theaflavates and thearubigins production.

Reaction Mixture and Major Product	Thearubigin Material (% w/w)		Benzotropolone Product (% w/w)	Ratio (TR:TF)
	Ethyl Ethanoate Soluble	Water Soluble		
Theaflavin EC + EGC	30	24	27	2:1
Theaflavin-3-*O*-monogallate EC + EGCG	33	16	7	7:1
Theaflavin-3'-*O*-monogallate ECG + EGC	19	42	7	9:1
Theaflavin-3,3'-*O*-digallate ECG + EGCG	46	19	4.1	16:1
Isotheaflavin EC + GC	38	34	13	6:1
Isotheaflavin-3'-*O*-monogallate ECG + GC	43	32	5	16:1
Neotheaflavin C + EGC	29	30	12	5:1
Neotheaflavin-3-*O*-monogallate C + EGCG	40	29	7	11:1
Theaflavate A ECG + ECG	34	20	8	7:1
Theaflavate B EC + ECG	22	25	6	8:1
Theaflavate C C + ECG	40	29	6	11:1

3.4.2 Effect of Esterification of Flavan-3-ol Precursors on the Yield of Theaflavins, Theaflavates and Thearubigins:

To assess the effects of esterification of the flavan-3-ol precursors on the yields of theaflavins produced by chemical oxidation, it is necessary to examine the yields for formation of the four major theaflavins which show the following trend:

$$\text{Theaflavin} > \text{Tf-3-mg} \approx \text{Tf-3'-mg} > \text{Tf dg}$$

This trend clearly shows that galloylation of the either C-ring hydroxyl group reduces the yield of the desired theaflavin product while the yield of TR-type compounds, increases. There appears to be a number of possible reasons for this:

1) Galloylation of a galloyl ester group sterically hinders formation of one of the reaction intermediates, by partially blocking the approach of other epicatechin quinones. This steric effect is further investigated in Section 3.4.3;

2) Galloylated products may be less stable than their free hydroxyl counterparts and hence may be readily converted into more stable non-theaflavin species.

3.4.3 Effect of Initial Flavan-3-ol Quinone Conformation and Esterification on Theaflavin/Theaflavate Formation:

The preferred conformations of the initial flavan-3-ol *ortho*-quinones are difficult to deduce from NMR or by other means. However, using molecular modelling techniques on a Silicon Graphics Indigo Work-station using Biosym Insight II Version 9.50 Molecular Simulation Software, it is possible to produce a model of the most favourable conformation of the catechin quinones. From these models, it has been possible to estimate the extent to which these conformations will hinder the approach of other catechin quinones and hence discourage the formation of theaflavins. To enable this investigation to be carried out a number of assumptions has been made to simplify the system and its interpretation.

1) Reaction is initiated by the B-rings of the catechin quinones attaining either a parallel or perpendicular arrangement.
2) Reaction takes place when both reactants are in their minimum energy conformations.
3) The reactants align themselves to minimise the interaction of their A and C rings.
4) Solvent effects on the conformation of the catechin quinones are negligible.

Catechin Quinone:

The most favourable conformation for catechin quinone is shown in Appendix 2.1 (Figure 3.1). This model suggests that the B-ring is held at angle of around 108° to the plane of the A and C rings, and with the B-ring parallel to the plane of these flavan backbone (A and C rings). This positioning of the B-ring suggests that one face is sterically protected by the A and C rings against attack. This implies that for catechin quinone to take part in theaflavin formation the majority of reactions will be initiated from the sterically unprotected face of the B-ring.

Epicatechin Quinone:

The most favourable conformation for epicatechin quinone is shown in Appendix 2.1 (Figure 3.2). This model suggests that the B-ring is held at 135° to the plane of the A and C rings. In epicatechin quinone the B-ring is closer to the plane of the A and C rings, than was suggested for catechin quinone, and so any possible interactions and steric

hinderance are minimized. The distortion of the non-aromatic C-ring results in the *cis*-hydroxyl group at the 3 position also being held in a position where it appears not to hinder sterically the approach of other catechin quinones. In comparison to catechin quinone, the extent of the steric hinderance expected for epicatechin quinone is significantly less because its structure holds the B-ring in a position which allows reaction from either face without significant steric interference.

Gallocatechin Quinone:

The most favourable conformation for gallocatechin quinone is shown in Appendix 2.1 (Figure 3.3). This model suggests that the B-ring is held at a very similar angle to that of the other catechin quinones, at 109°. Here, the model predicts that the *trans*-3-hydroxyl group is sterically important. The conformation of the C-ring causes this group to marginally block one face of the B-ring. This situation is different from that of catechin quinone where it is the A and C rings which protect the B-ring from attack on one face. However, the model suggests that gallocatechin quinone should be reactive due to its conformational mobility allowing the reaction to be initiated from either side of the B-ring.

Epigallocatechin Quinone:

The most favourable conformation for epigallocatechin quinone is shown in Appendix 2.1 (Figure 3.4). This model suggests that the B-ring is held at a very similar angle to that of catechin quinones, at 113°. The conformation of EGC is similar to that of GC in that distortion of the C-ring minimises the steric effects of the 3-hydroxyl group. This results in a structure which holds the B-ring in a position which allows reaction from either side of this ring without significant steric interference.

Epicatechin Gallate Quinone:

The most favourable conformation for epicatechin gallate quinone is shown in Appendix 2.1 (Figure 3.5). This model suggests that the B-ring is held at a very similar angle to that of the other epicatechin quinones, at 114°. The galloyl ester appears to be partially blocking this ring. The addition of the galloyl ester in place of the hydroxyl group significantly increases the amount of steric hinderance other catechin quinones will experience when approaching the B-ring from beneath. The model predicts a structure

which holds the B-ring in a position which will predominantly only allow the initiation of reaction from one side of this ring due to significant steric interference.

Epicatechin Galloyl Ester Quinone:

The most favourable conformation for epicatechin galloyl ester quinone is shown in Appendix 2.1 (Figure 3.6). This model suggests that the galloyl ester is positioned at approximately 90 degrees to the A, B and C-rings. The addition of the -COO- linkage also serves to reduce the steric effects of these other parts of the molecule, by increasing the distance between the galloyl ring and the remainder of the molecule. Therefore the model predicts a structure which is open and would allow reaction to occur at either face of the galloyl ring.

Epigallocatechin Gallate Quinone:

The most favourable conformation for epigallocatechin gallate quinone is shown in Appendix 2.1 (Figure 3.7). This model suggests that the B-ring is held at a very similar angle to that of the other epicatechin quinones, at 113°. It can seen that the B-ring is available for further reaction from above. From below, the B-ring and the galloyl ester ring are predicted to be staggered, although not to the same extent as in ECG quinone, so only marginally reducing this blocking effect. Despite this staggering, galloylation of the 3-hydroxyl group significantly increases the amount of steric hinderance other catechin quinones will experience when approaching the B-ring in this direction. Therefore the model predicts a structure which holds the B-ring in a position which will only allow the initiation of reaction from one side of this ring due to significant steric interference.

Table 3.7a Summary of flavan-3-ol quinone conformation studies

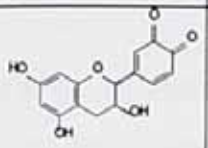
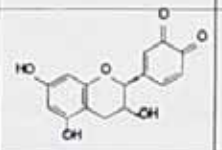
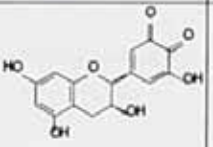
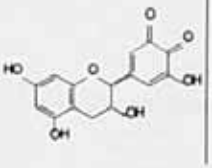
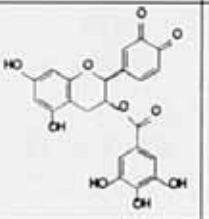
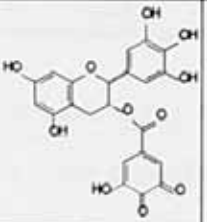
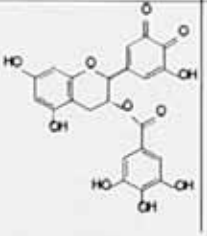
	Structure	*Predicted Potential Directions of B-ring Attack*
Catechin Quinone		Above
Epicatechin Quinone		Above and Below
Gallocatechin Quinone		Above and Below
Epigallocatechin Quinone		Above

Table 3.7b Summary of flavan-3-ol quinone conformation studies.

	Structure	Predicted Potential Directions of B-ring Attack
Epicatechin Gallate Quinone		Above
Epicatechin Galloyl Ester Quinone		Above and Below
Epigallocatechin Gallate Quinone		Above

The preceding discussions are summarized in Tables 3.7a and b. From these models, the following trend is suggested in terms of the steric hinderance of the B-ring:

Lowest: Highest:

EC ≈ EGC ≈ GC < Galloyl ester quinone of ECG < Catechin < ECG < EGCG

Based on this trend, it is possible to predict the order of the expected yields of the theaflavins, their isomers and the theaflavates. These predictions are shown in Table 3.8:

Table 3.8 Comparison of predicted order of the yields of theaflavins, their isomers and theaflavates with their experimental yields.

Product	Reactants	Predicted Yield Based on Steric Interactions of Reactants†	Experimental Yield (μmol/mmol of flavan-3-ol precursor)
Theaflavin	EC + EGC	5	382
Theaflavin-3-O-monogallate	EC + EGCG	2	79
Theaflavin-3'-O-monogallate	ECG + EGC	3	76
Theaflavin-3,3'-O-digallate	ECG + EGCG	1	40
Isotheaflavin	EC + GC	5	110
Isotheaflavin-3'-O-monogallate	ECG + GC	3	44
Neotheaflavin	C + EGC	4	146
Neotheaflavin-3-O-monogallate	C + EGCG	1	71
Theaflavate A	ECG + ECG	2	42
Theaflavate B	EC + ECG	4	91
Theaflavate C	C + ECG	3	64

† 5 = High Yield (Both molecules have open, unhindered B-rings).

1 = Low Yield (Both molecules have closed and significantly hindered B-rings).

The data, shown in Table 3.8, indicates considerable agreement between the predicted yields of these compounds and those observed in the experimental study. However, the predicted levels of yield appear to be only qualitative indicators of the observed yields and do not reflect the absolute levels of the products produced. This qualitative explanation is in line with the assumptions which were made at the start of this work. This is also in-line the predictions which can be made on the basis of the redox potentials of the flavan-3-ols[15]. It confirms that the initial conformations of the reactants are important in theaflavin formation.

3.4.4 Effect of Stereo-structure on the Yield of Theaflavin, Theaflavate and Thearubigins:

Comparison of the yields of theaflavin with those of iso- and neo-theaflavin:

$$\text{Theaflavin} > \text{Neotheaflavin} \approx \text{Isotheaflavin}$$

clearly demonstrates the pronounced effect that the change in the stereo-structure of the catechin precursors has.

The theaflavins with 2,3 *cis* and 2',3' *cis* configuration is clearly favoured over the other possible configurations (2,3-*trans* or 2',3'-*trans*). The *cis* configuration also reduces the amount of TR-type compounds produced. Isotheaflavin (EC + GC) with a 2,3-*trans* stereochemistry and neotheaflavin (C + EGC) with a 2',3'-*trans* one are present at comparable levels. The relative yields of theaflavin-3-monogallate and neotheaflavin-3-monogallate and theaflavin-3'-monogallate and isotheaflavin-3'-monogallate are as follows:

$$\text{Tf-3-mg} \approx \text{NTf-3-mg} \approx \text{Tf-3'-mg} > \text{ITf-3'-mg}$$

3.5 Conclusions from Chemical Oxidation Experiments:

1) The molecular size and configuration of the flavan-3-ol precursors are important in determining the yield of theaflavin/theaflavate compounds.
2) Galloylation of the 3 position on the C-ring favours the production of TR compounds.
3) Changing the configuration of flavan-3-ols precursors (from *cis* to *trans*) reduces the yield of the benzotropolone-derived product.
4) Changing the configuration of the flavan-3-ol precursors (from *cis* to *trans*) increases the production of TR compounds.
5) From the yields of the theaflavin/theaflavate derivatives obtained, the catechin series is more sterically hindered than the epicatechin series of flavan-3-ol precursors.
6) Galloylation of the 3-hydroxyl groups significantly increases the steric hinderance of these compounds relative to their non-galloyl counterparts, and reduces the yields of theflavins/theaflavates.
7) Molecular modelling is a potentially useful tool in the study of the mechanisms of theaflavin formation.

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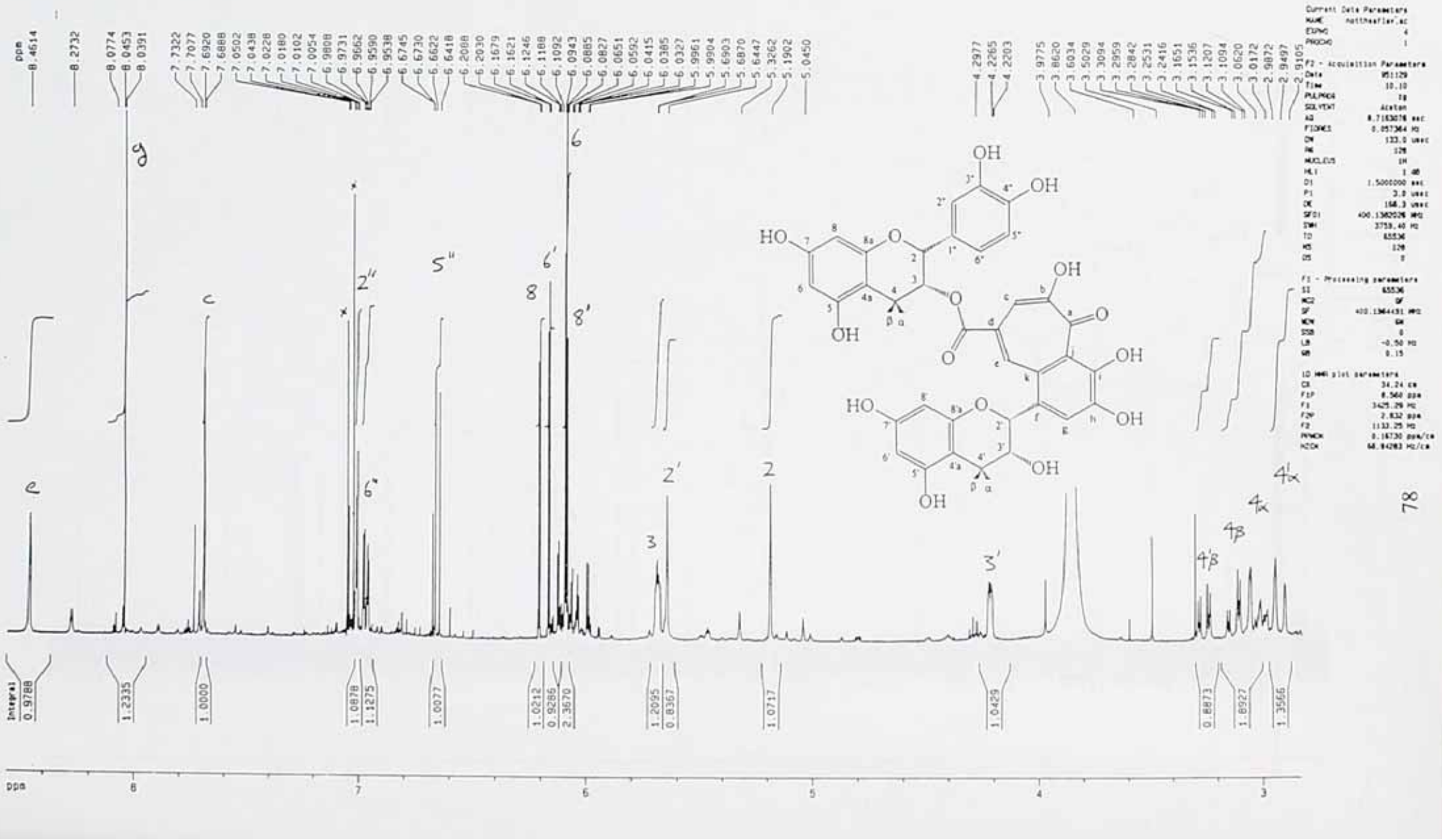
Appendix 1:

NMR Spectra:

Figure 1H Theaflavate B in d_6 acetone plus D_2O.
^1H NMR Spectrum at 303 K.



78

Figure 1C Theaflavate B in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 303 K.

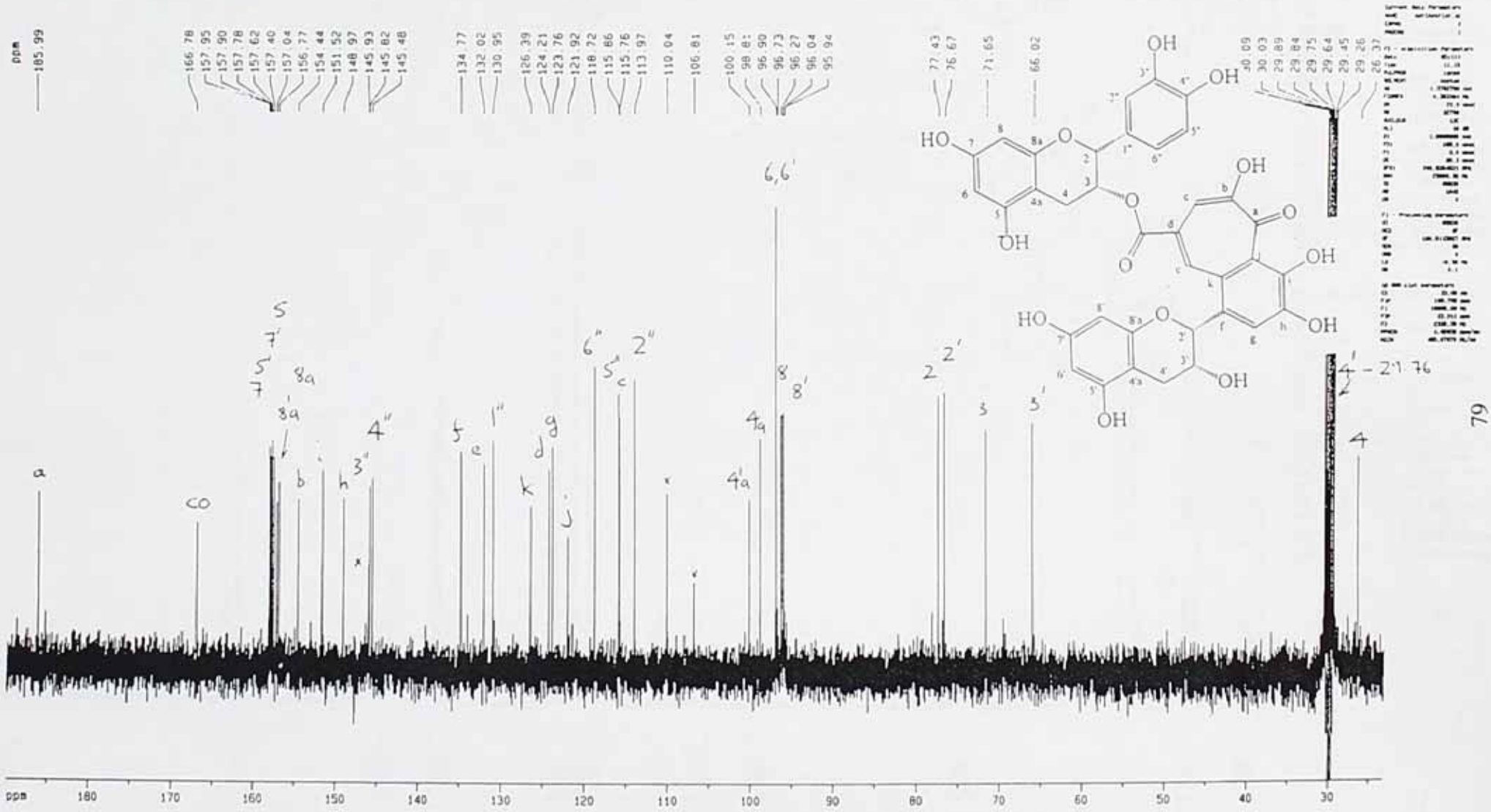
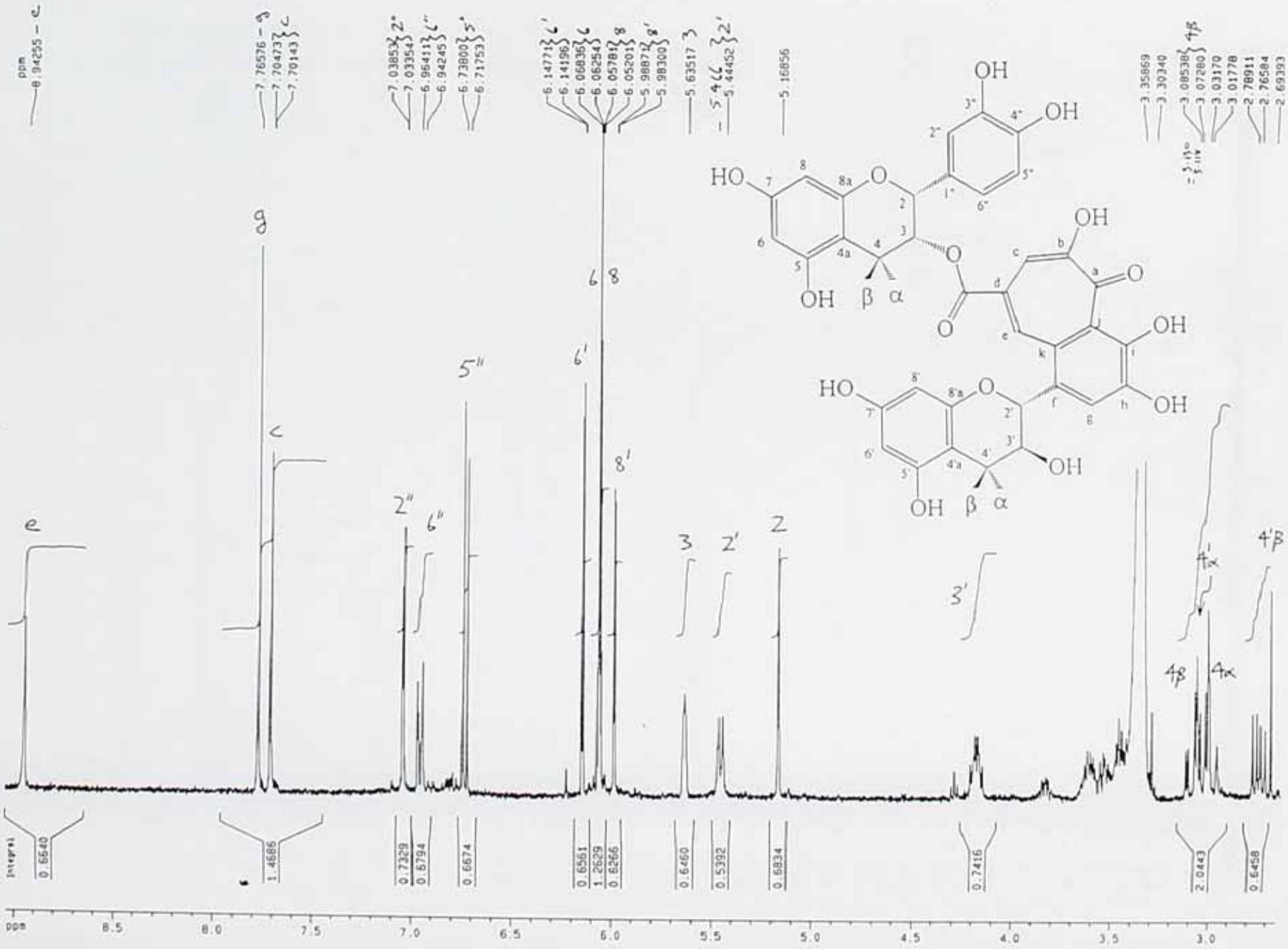


Figure 2H Theaflavate C in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.



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NAME	110496
EXPNO	8
PROCNO	1

F2 - Acquisition Parameters

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Time	9.10
INSTRUM	AMX400T
PROBHD	5 mm Dual 13
PULPROG	zg
TD	65536
SOLVENT	Aceton
NS	227
DS	0
SWH	4672.897 Hz
FIDRES	0.071303 Hz
AQ	7.0124021 sec
RG	4096
DW	107.000 usec
DE	133.75 usec
TE	300.0 K
D1	1.00000000 sec
P1	5.00 usec
DE	133.75 usec
SFO1	400.1387764 MHz
NUCLEUS	1H

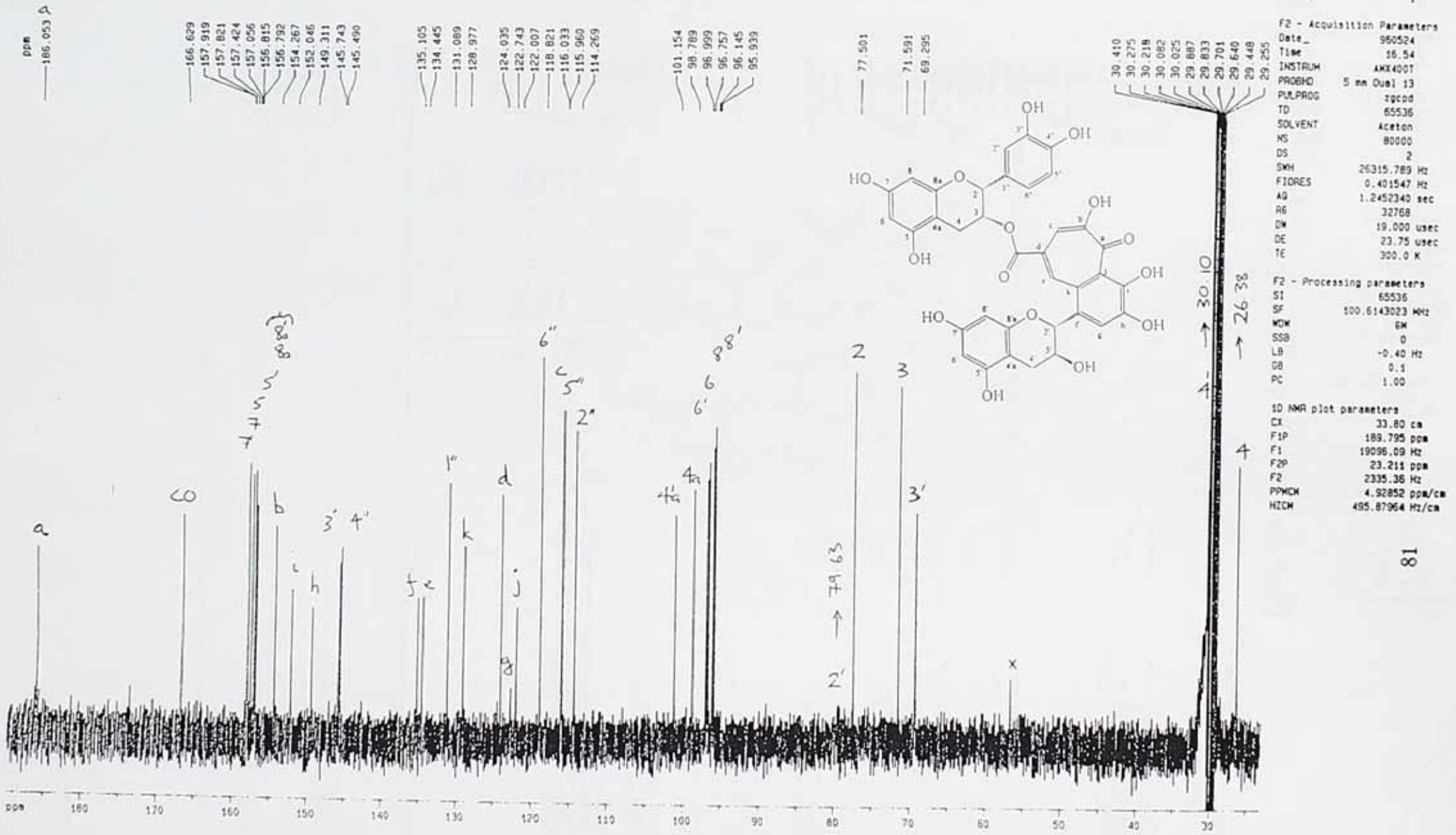
F2 - Processing parameters

SI	65536
SF	400.1364594 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.1
PC	10.00

1D NMR plot parameters

CX	32.00 cm
F1P	9.039 ppm
F1	3615.78 Hz
F2P	2.645 ppm
F2	1058.51 Hz
PPMCM	0.19980 ppm/cm
HZCM	79.94582 Hz/cm

Figure 2C Theaflavate C in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 300 K.



Current Data Parameters

NAME	110496
EXPNO	7
PROCNO	1

F2 - Acquisition Parameters

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Time	16.54
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PROBHD	5 mm Dual 13
PULPROG	zgcpd
TD	65536
SOLVENT	Aceton
NS	80000
DS	2
SWH	26315.789 Hz
FIDRES	0.401547 Hz
AQ	1.2452340 sec
RG	32768
DW	19.000 usec
DE	23.75 usec
TE	300.0 K

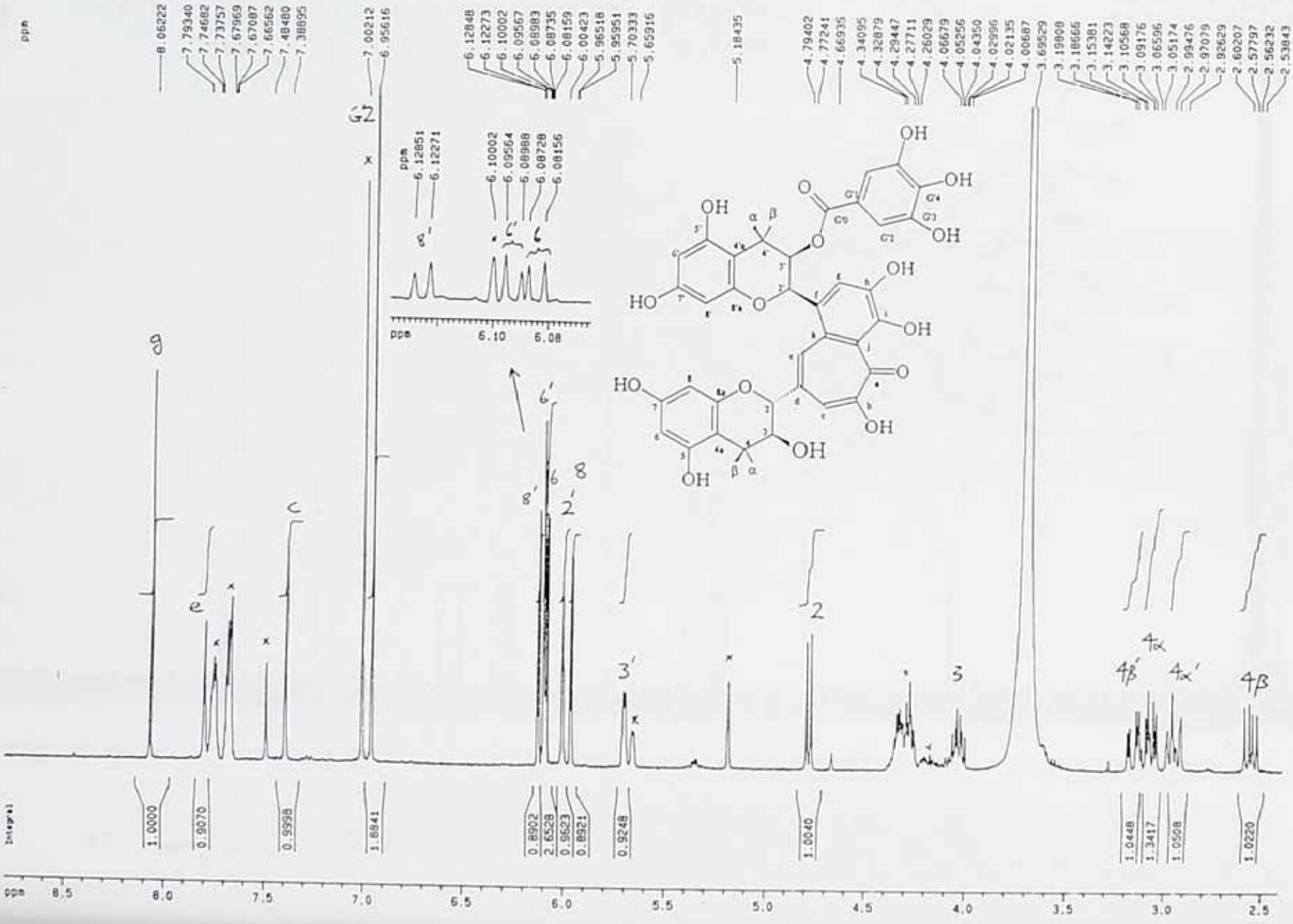
F2 - Processing parameters

SI	65536
SF	100.6143023 MHz
WDW	EM
SSB	0
LB	-0.40 Hz
GB	0.1
PC	1.00

1D NMR plot parameters

CX	33.80 cm
F1P	189.795 ppm
F1	19096.09 Hz
F2P	23.211 ppm
F2	2335.36 Hz
PPMCM	4.92852 ppm/cm
HZCM	495.87964 Hz/cm

Figure 3H Isotheaflavin 3'-O-gallate in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.

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EXPNO                   2
PROCNO                  1

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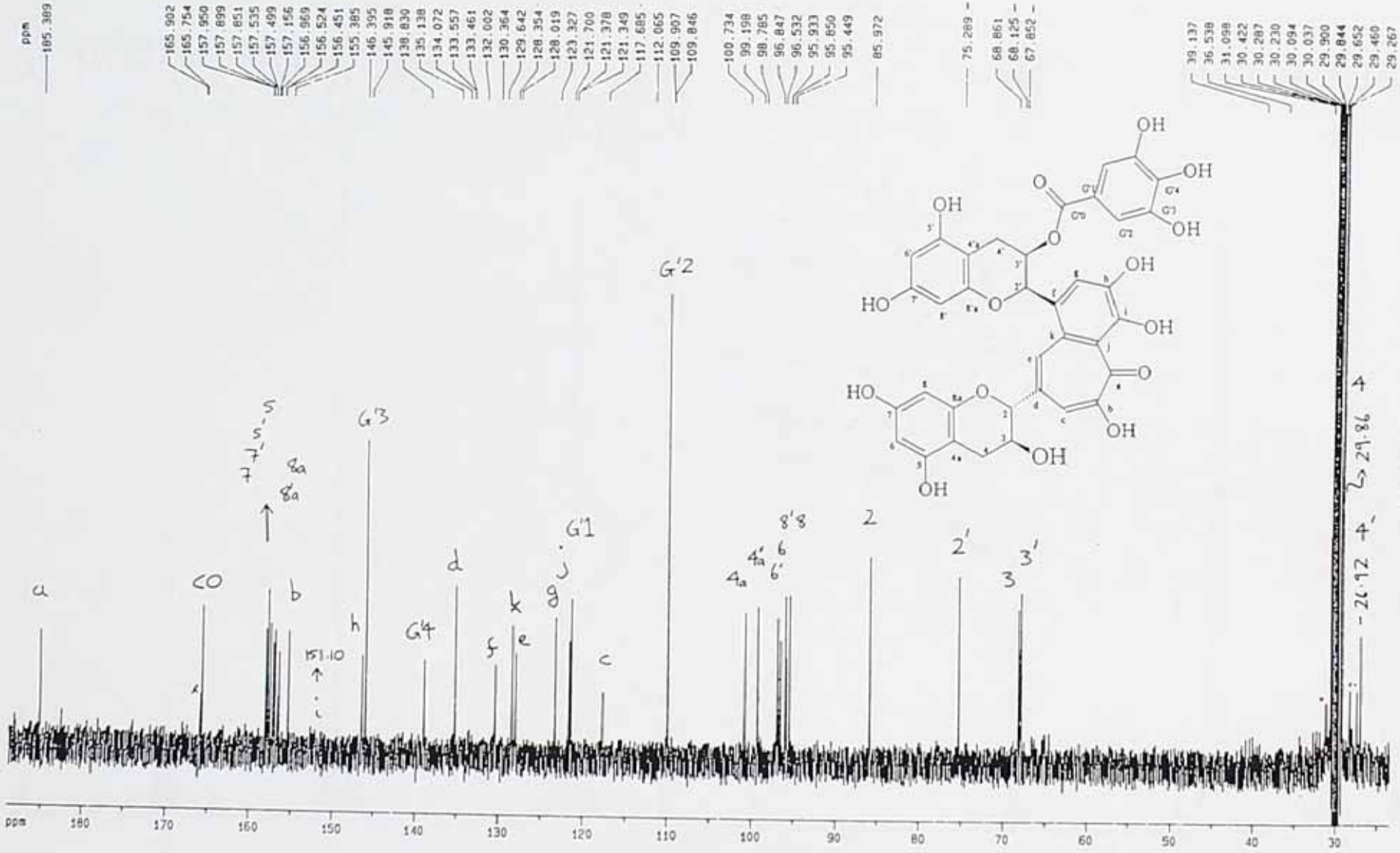
PULPROG                 zg
TD                  65536
SOLVENT           Aceton
NS                     286
DS                       0
SWH           7245.377 Hz
FIDRES        0.110571 Hz
AQ            4.5220342 sec
RG                     512
DW              69.000 usec
DE              86.25 usec
TE                 360.0 K
D1            1.00000000 sec
P1              3.00 usec
DE              86.25 usec
SFO1          400.1400639 MHz
NUCLEUS               1H

F2 - Processing parameters
SI                  65536
SF            400.1364581 MHz
WDW                    EM
SSB                     0
LB                -0.30 Hz
GB                    0.1
PC                  10.00

1D NMR plot parameters
CX                31.94 cm
F1P               8.792 ppm
F1                3518.10 Hz
F2P               2.410 ppm
F2                964.38 Hz
PPMCM          0.19980 ppm/cm
HZCM           79.94727 Hz/cm
```

Figure 3C Isotheaflavin 3'-O-gallate in d_6 acetone.
^{13}C-{^{1}H} NMR Spectrum at 300 K.



Current Data Parameters

NAME	ITFMG
EXPNO	3
PROCNO	1

F2 - Acquisition Parameters

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PULPROG	zgpgd
TD	65536
SOLVENT	Aceton
NS	8176
DS	0
SWH	26315.789 Hz
FIDRES	0.401547 Hz
AQ	1.2452340 sec
RG	32768
DW	19.000 usec
DE	23.75 usec
TE	300.0 K

F2 - Processing parameters

SI	65536
SF	100.5143023 MHz
WDW	EM
SSB	0
LB	-0.70 Hz
GB	0.25
PC	1.00

1D NMR plot parameters

CX	33.60 cm
F1P	189.095 ppm
F1	19025.66 Hz
F2P	23.511 ppm
F2	2365.54 Hz
PPMCM	4.92852 ppm/cm
HZCM	495.87967 Hz/cm

N.B. impurity peaks "edited out"

Figure 4H Neotheaflavin-3-O-gallate in d_6 acetone plus D_2O
^1H NMR Spectrum at 303 K

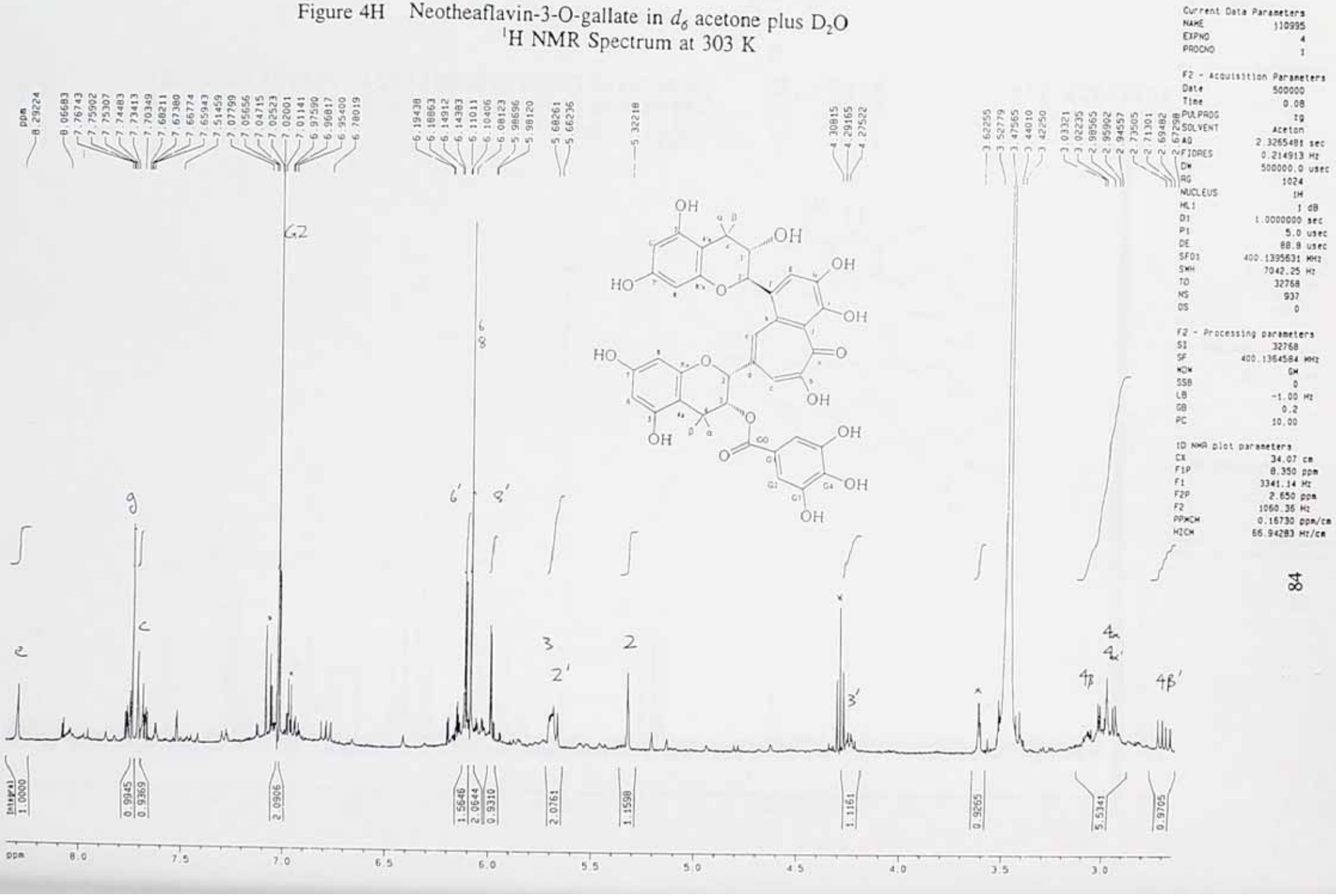
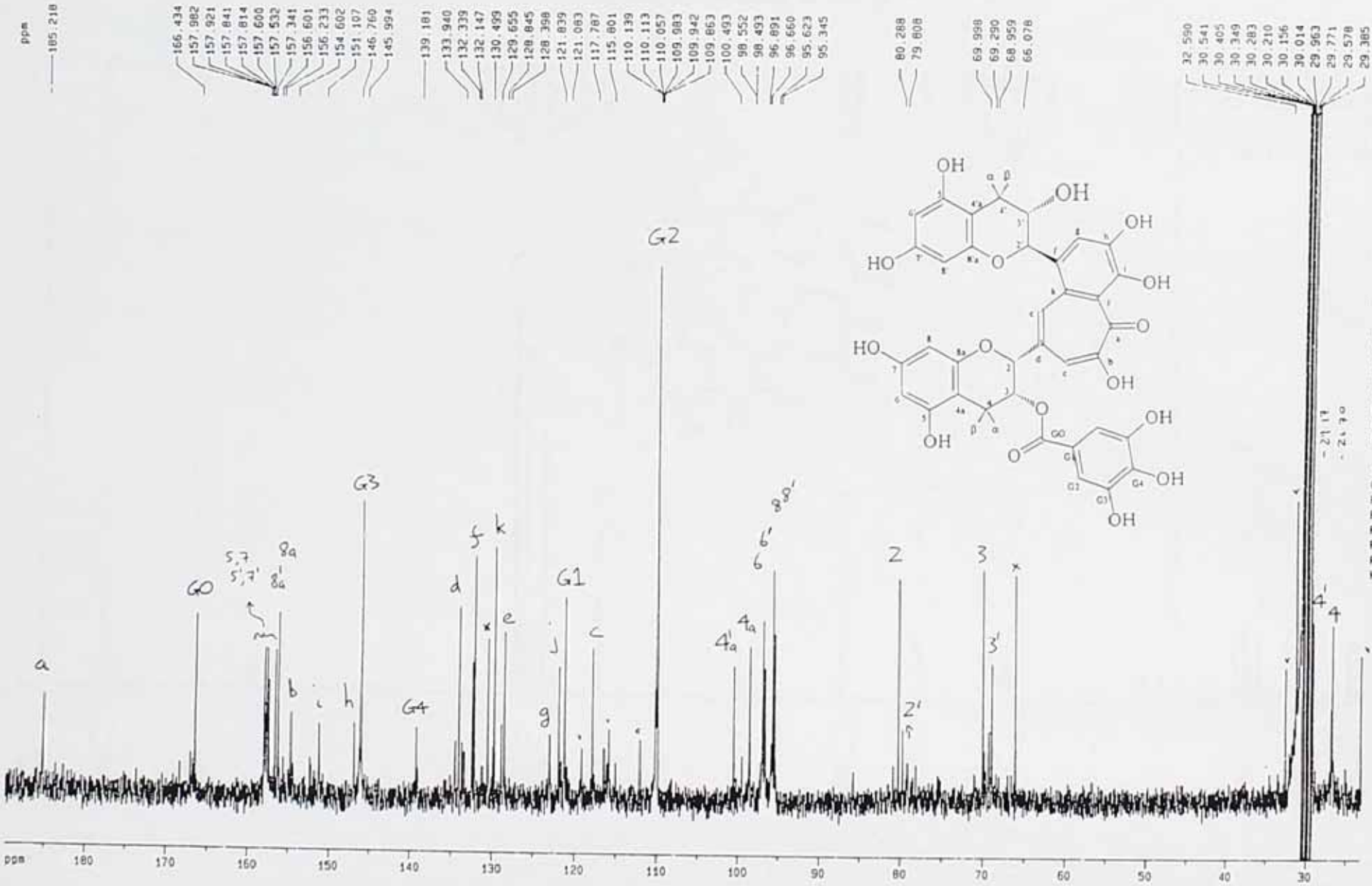


Figure 4C Neotheaflavin-3-O-gallate in d_6 acetone plus D_2O
^{1}13C-{^{1}H} NMR Spectrum at 303 K



x = imp

Current Data Parameters
NAME 110995
EXPNO 14
PROCNO 1

F2 - Acquisition Parameters
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Time 0.08
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SOLVENT Aceton
AQ 1.2452040 sec
FIDRES 0.401547 Hz
DW 500000.0 usec
RG 32768
NUCLEUS 13C
HL1 18 dB
D1 1.5000000 sec
P31 105.0 usec
P1 3.0 usec
DE 23.8 usec
SFO1 100.6247401 MHz
SWH 26315.79 Hz
TD 65536
NS 80000
DS 0

F2 - Processing parameters
SI 65536
SF 100.6143023 MHz
WDW GM
SSB 0
LB -1.00 Hz
GB 0.1
PC 0.10

1D NMR plot parameters
CX 33.80 cm
F1P 189.795 ppm
F1 19096.09 Hz
F2P 23.211 ppm
F2 2335.36 Hz
PPMCM 4.92852 ppm/cm
HZCM 495.87964 Hz/cm

Figure 5H Isotheaflavin in d_6 acetone plus D_2O.
^{1}H NMR Spectrum at 303 K.

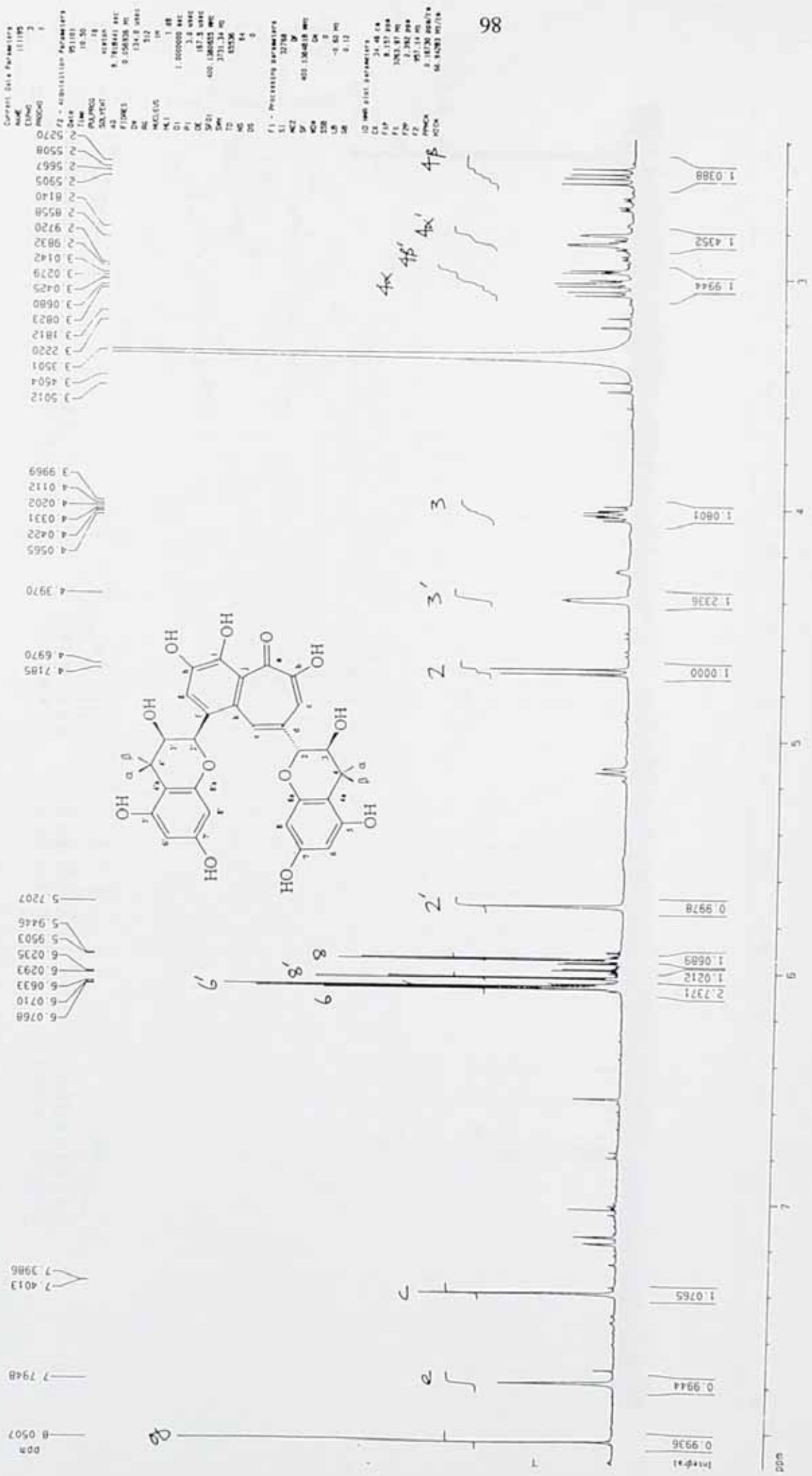
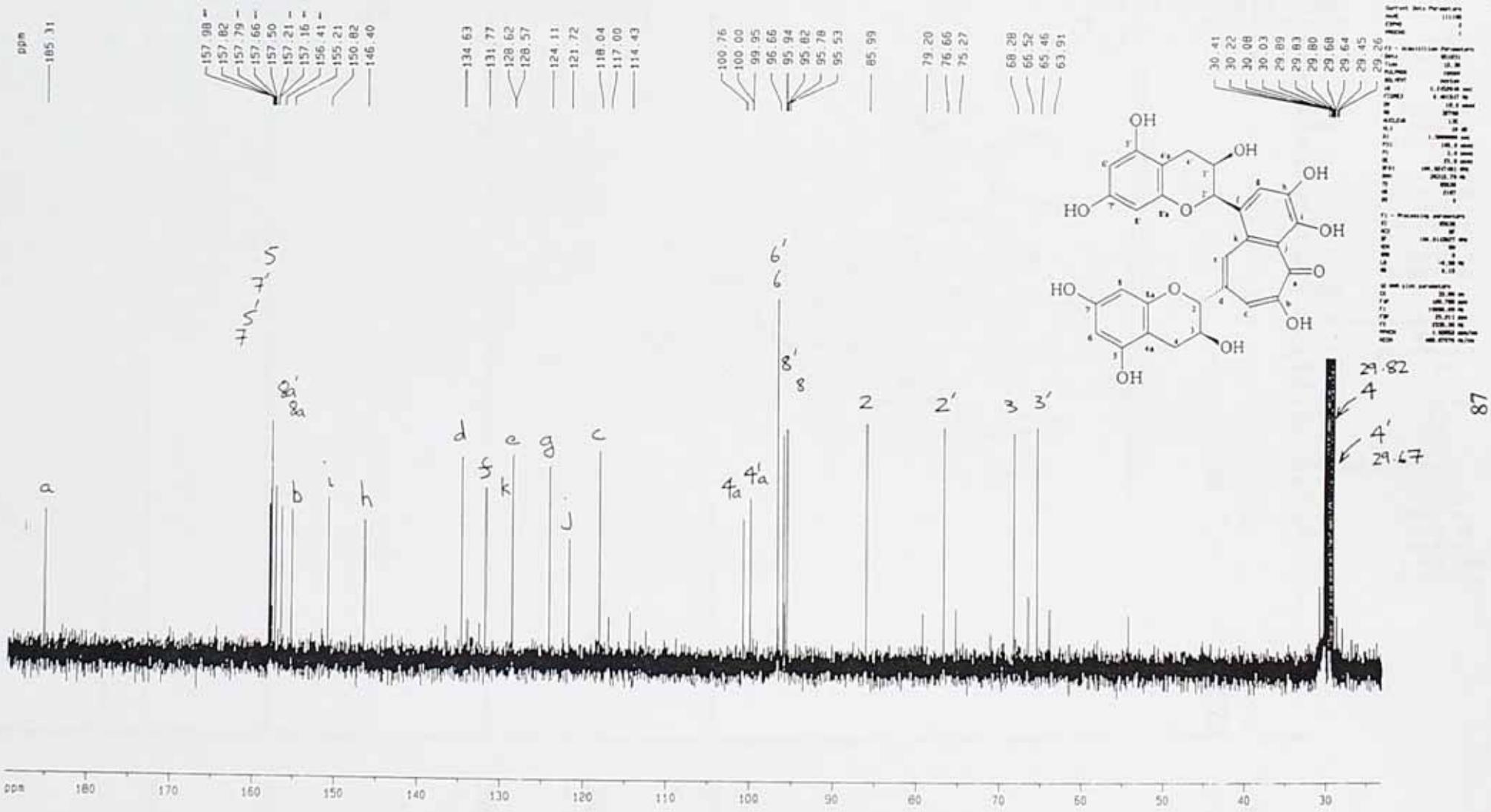
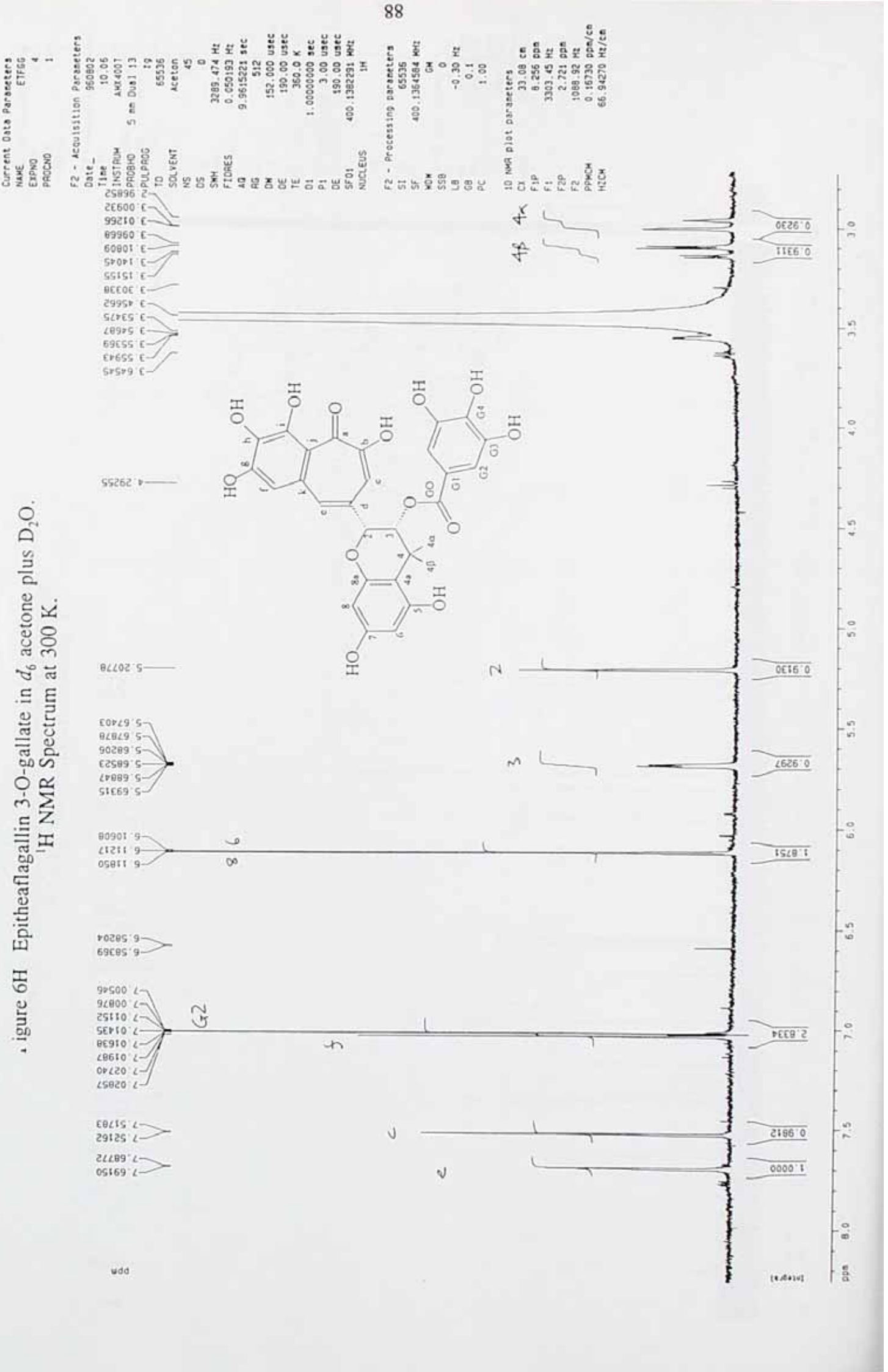


Figure 5C Isotheaflavin in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 303 K.





igure 6H Epitheaflagallin 3-O-gallate in d_6 acetone plus D_2O.
^{1}H NMR Spectrum at 300 K.

Figure 6C Epitheaflagallin 3-O-gallate in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 300 K.

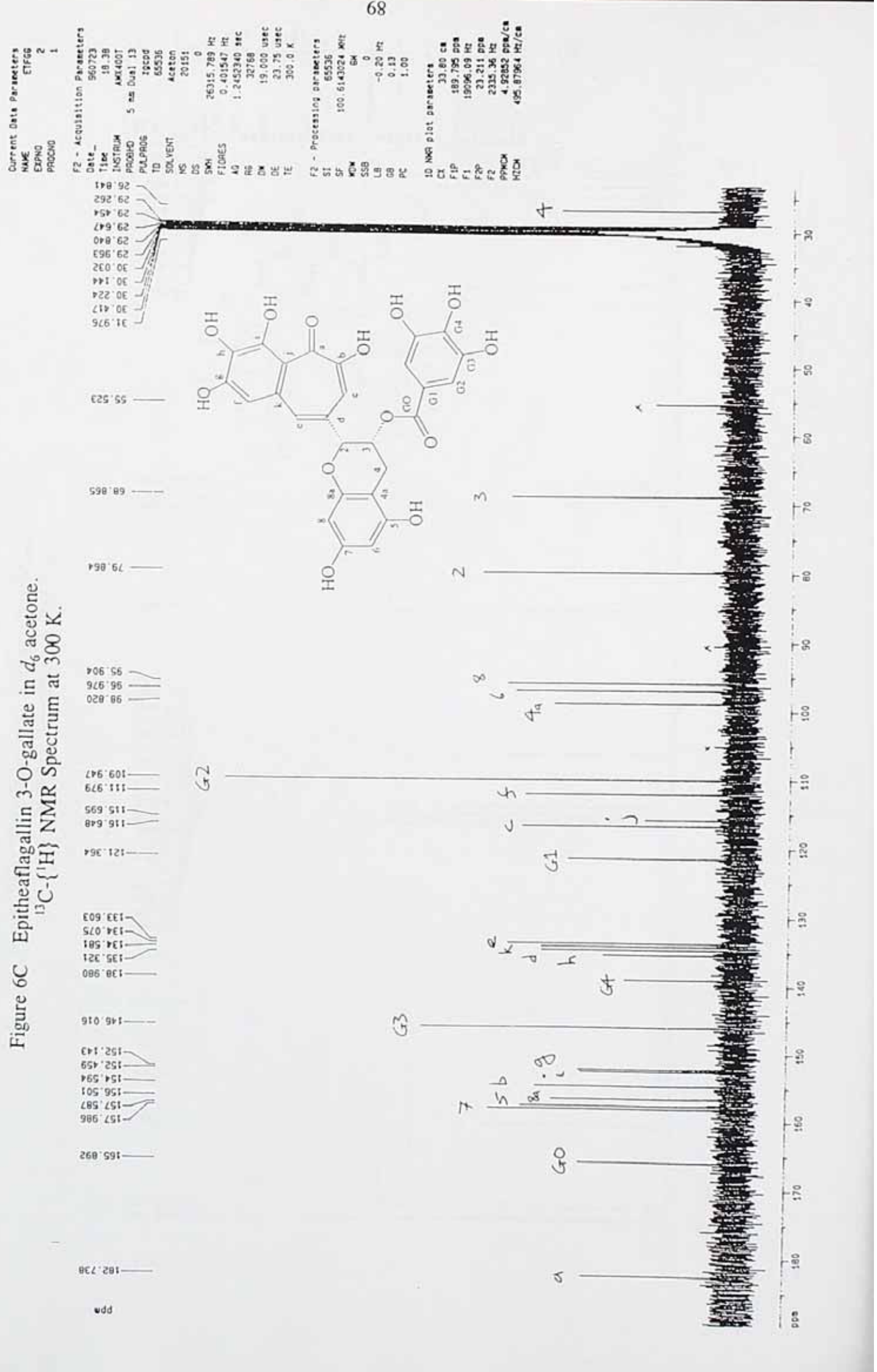


Figure 7H Theaflavin in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.

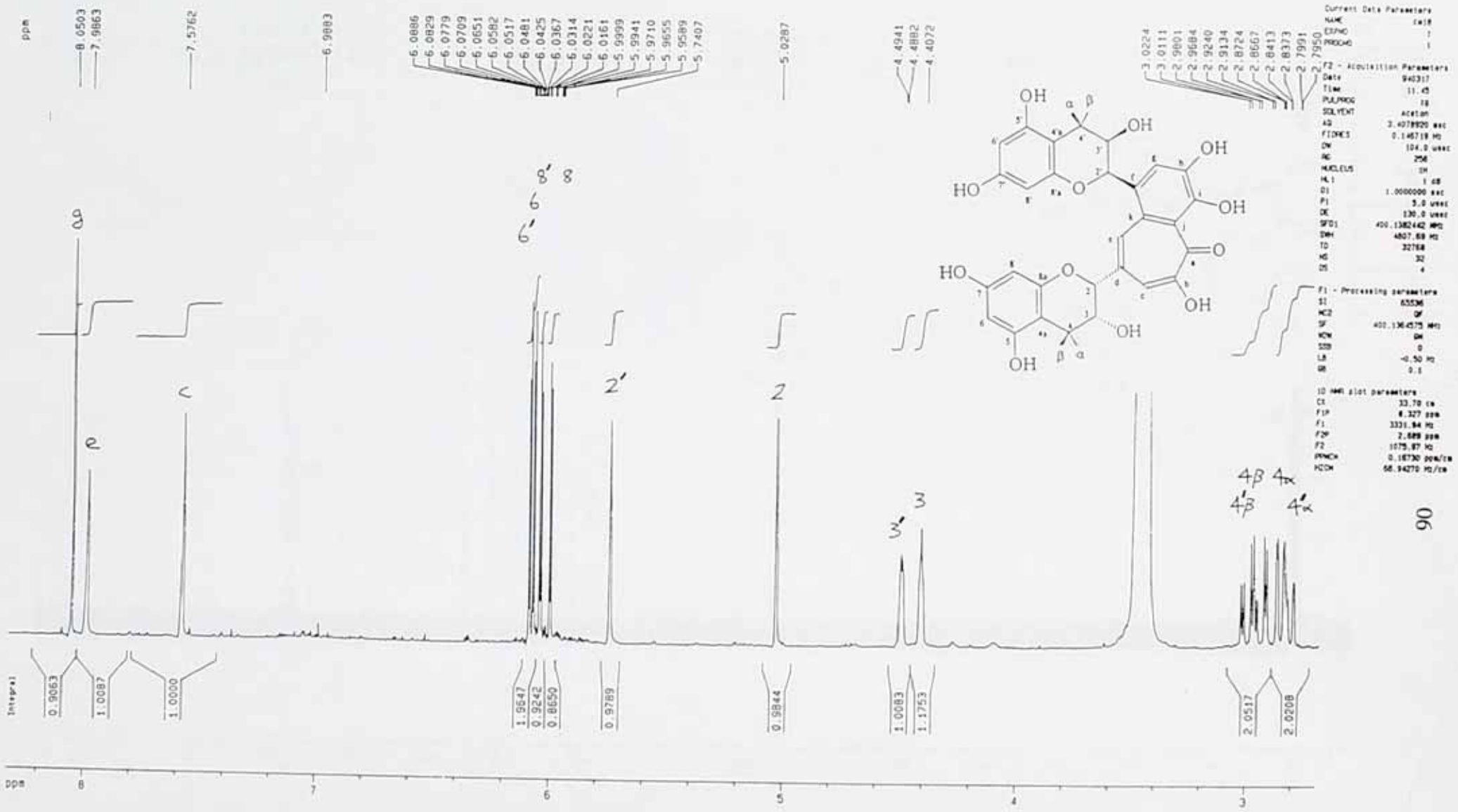


Figure 7C Theaflavin in d_6 acetone.
^{13}C-{^{1}H} NMR Spectrum at 300 K.

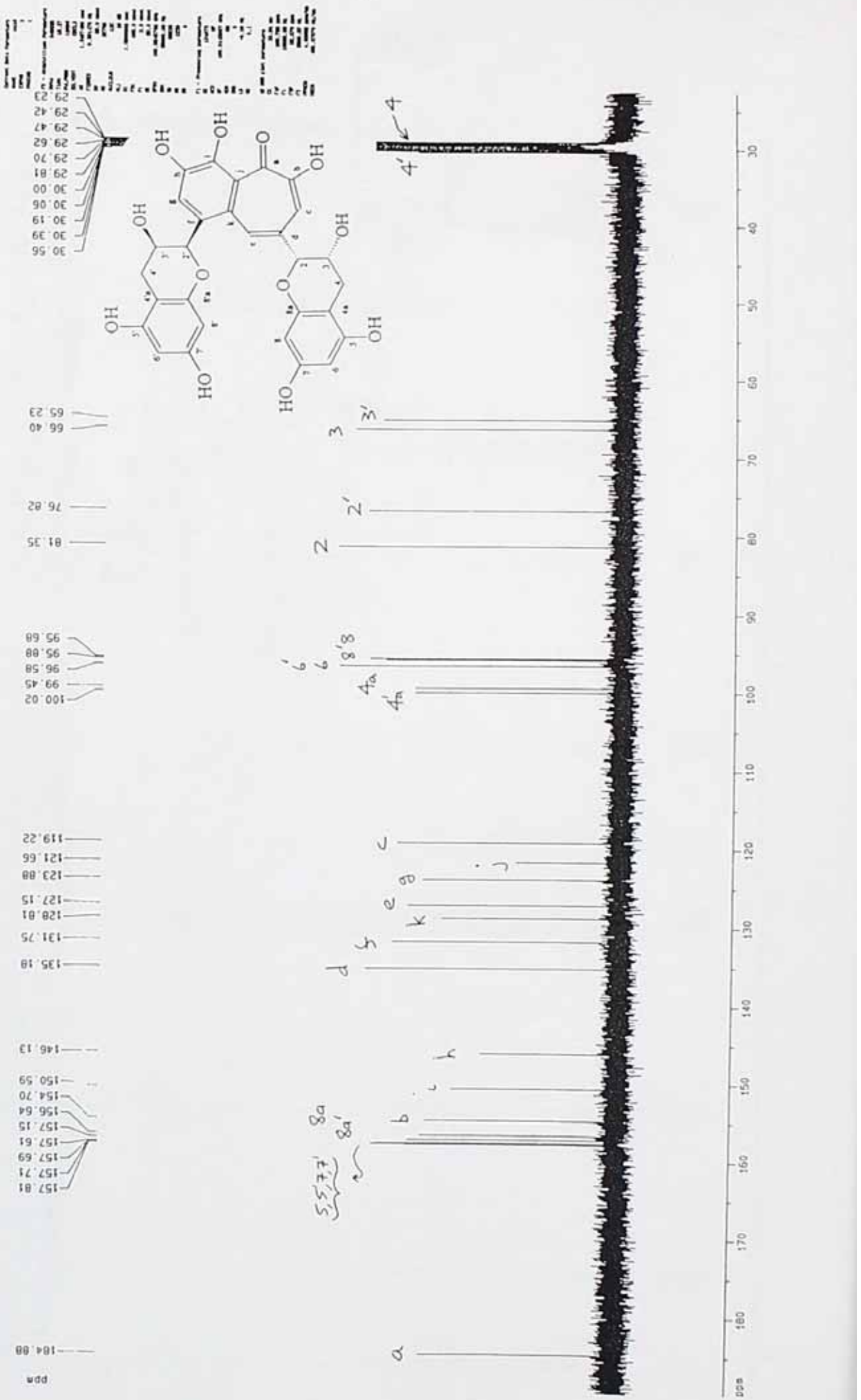


Figure 8H Theaflavin 3-O-gallate in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.

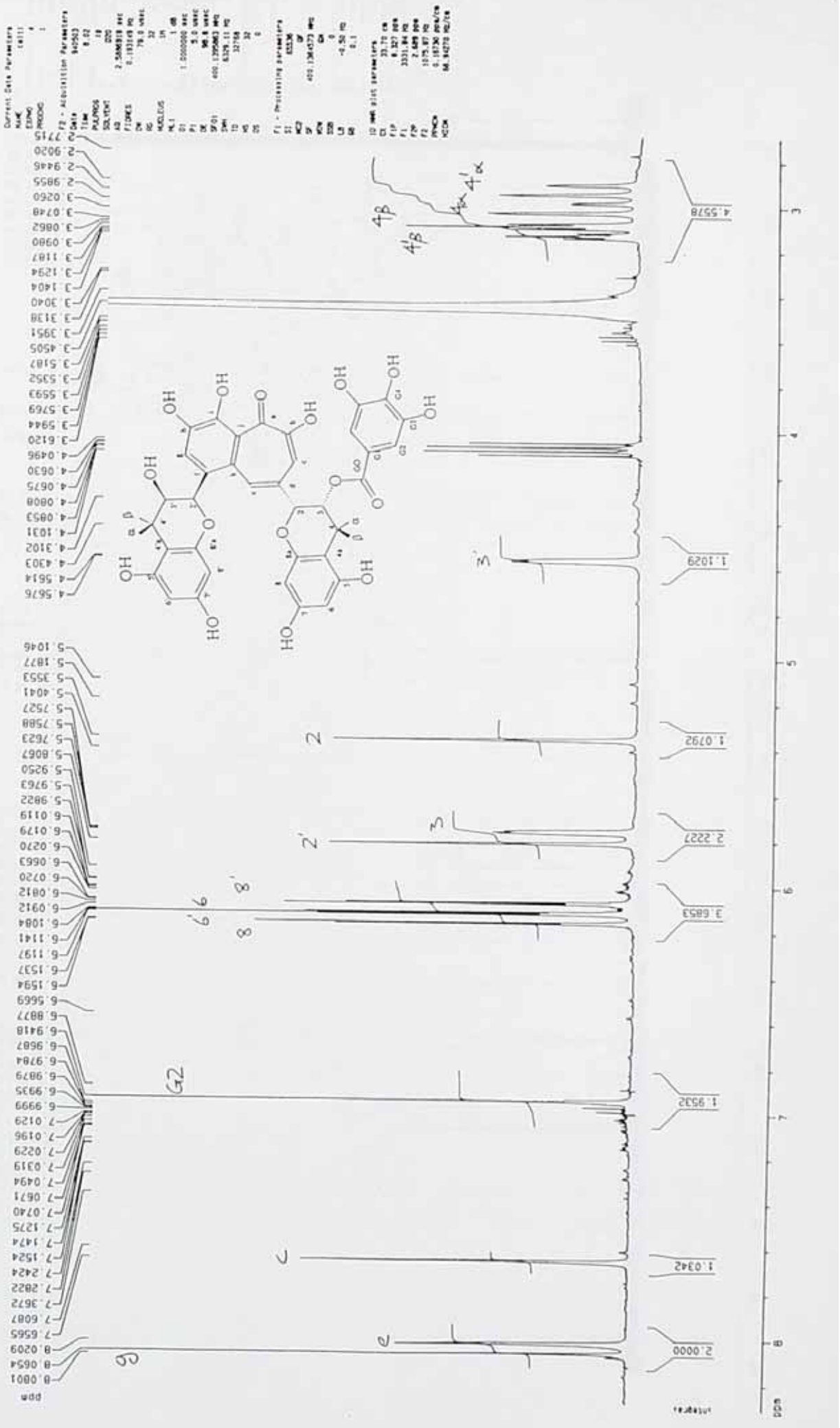


Figure 8C Theaflavin 3-O-gallate in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 300 K.

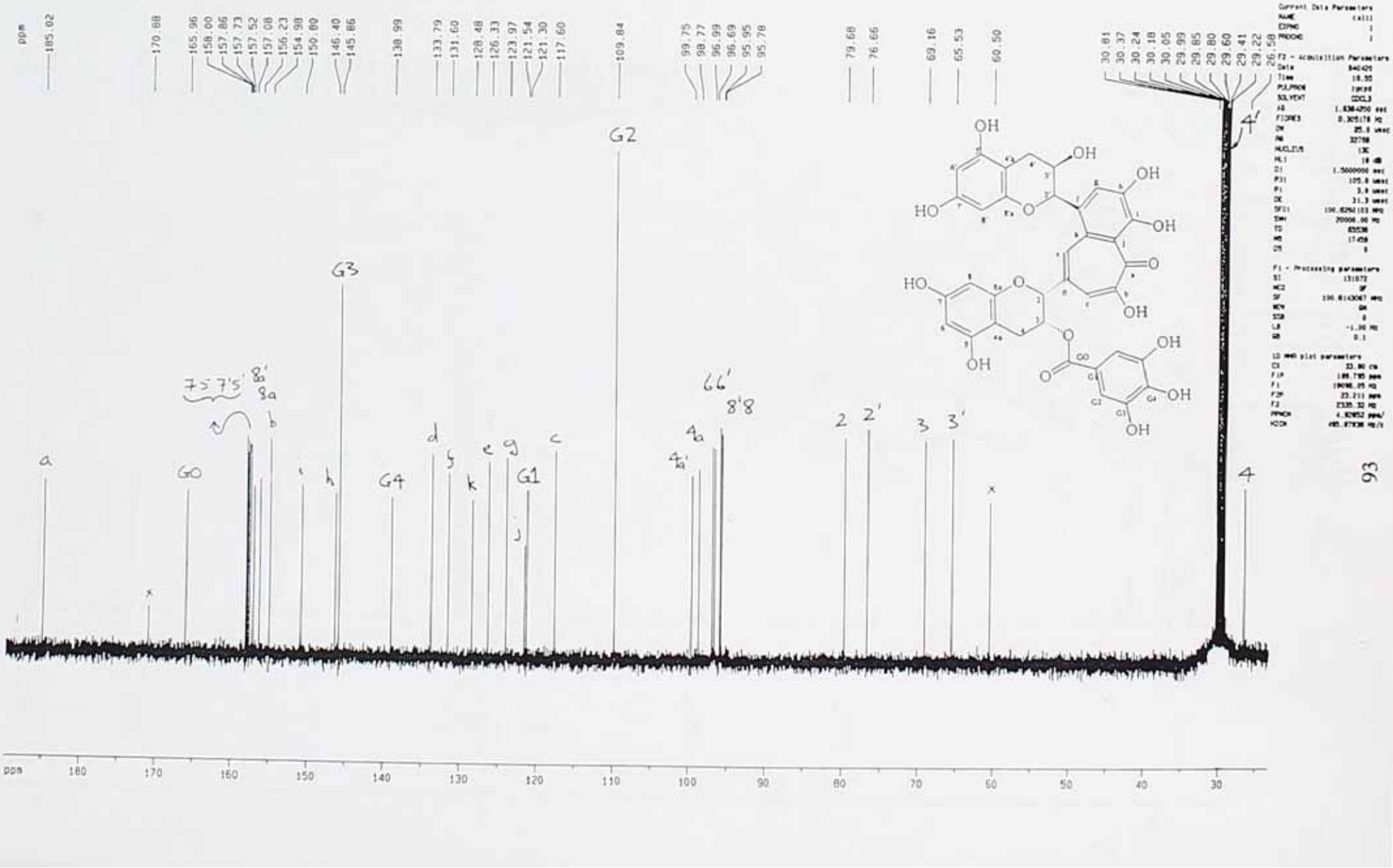


Figure 9H Theaflavin 3'-O-gallate in d_6 acetone plus D_2O.
^1H NMR Spectrum at 303 K.

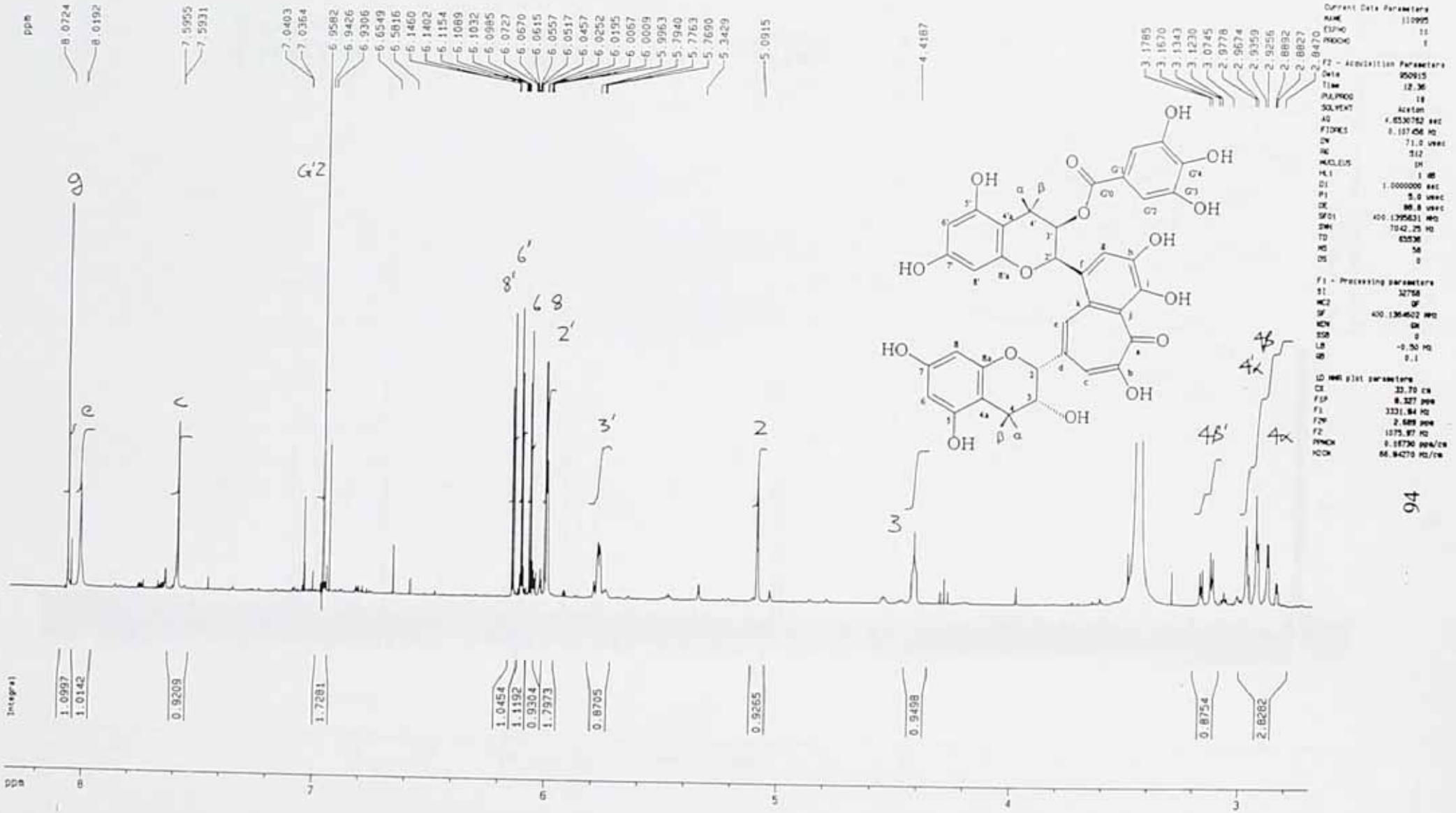


Figure 9C Theaflavin 3'-O-gallate in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 303 K.

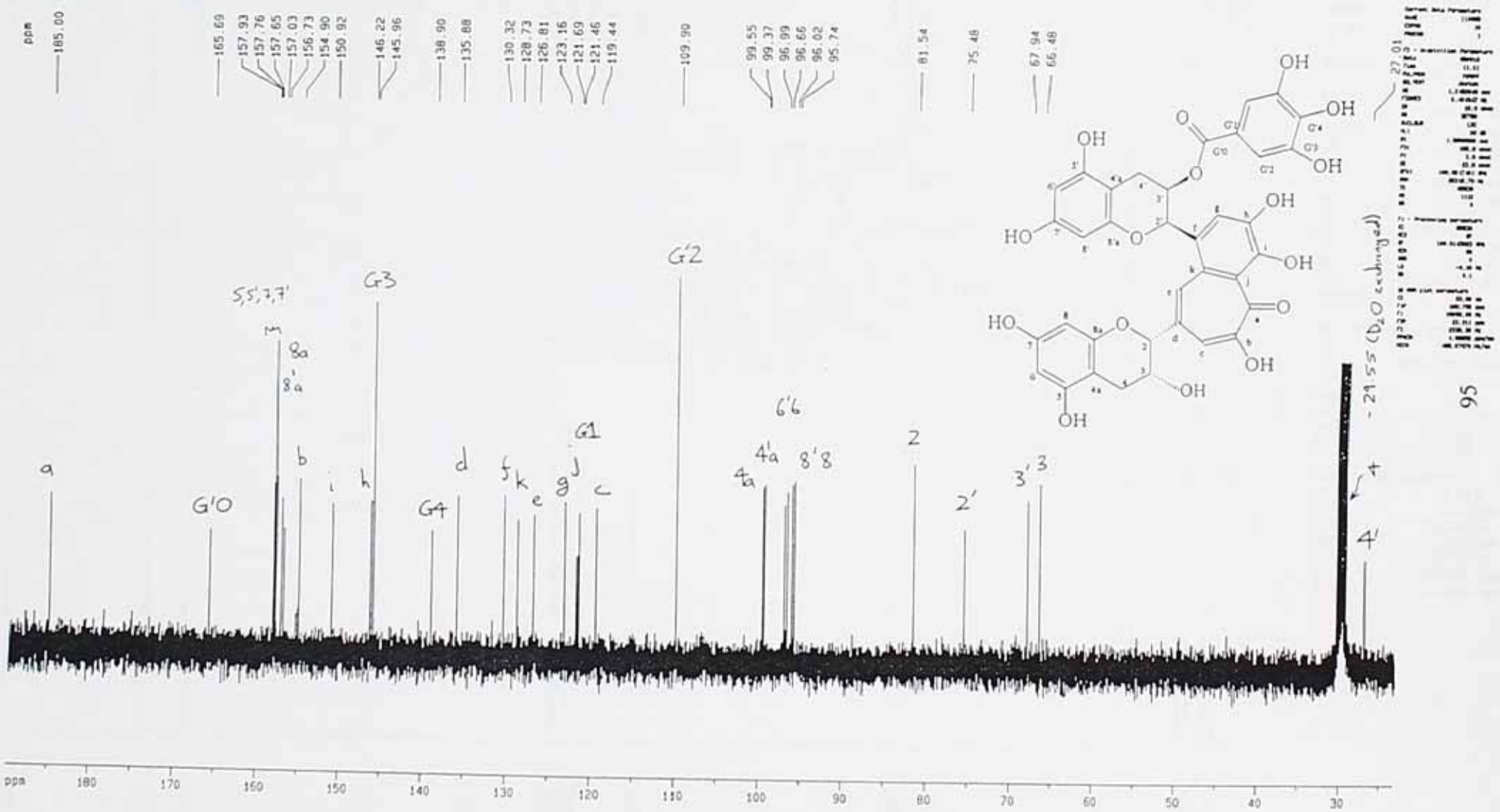
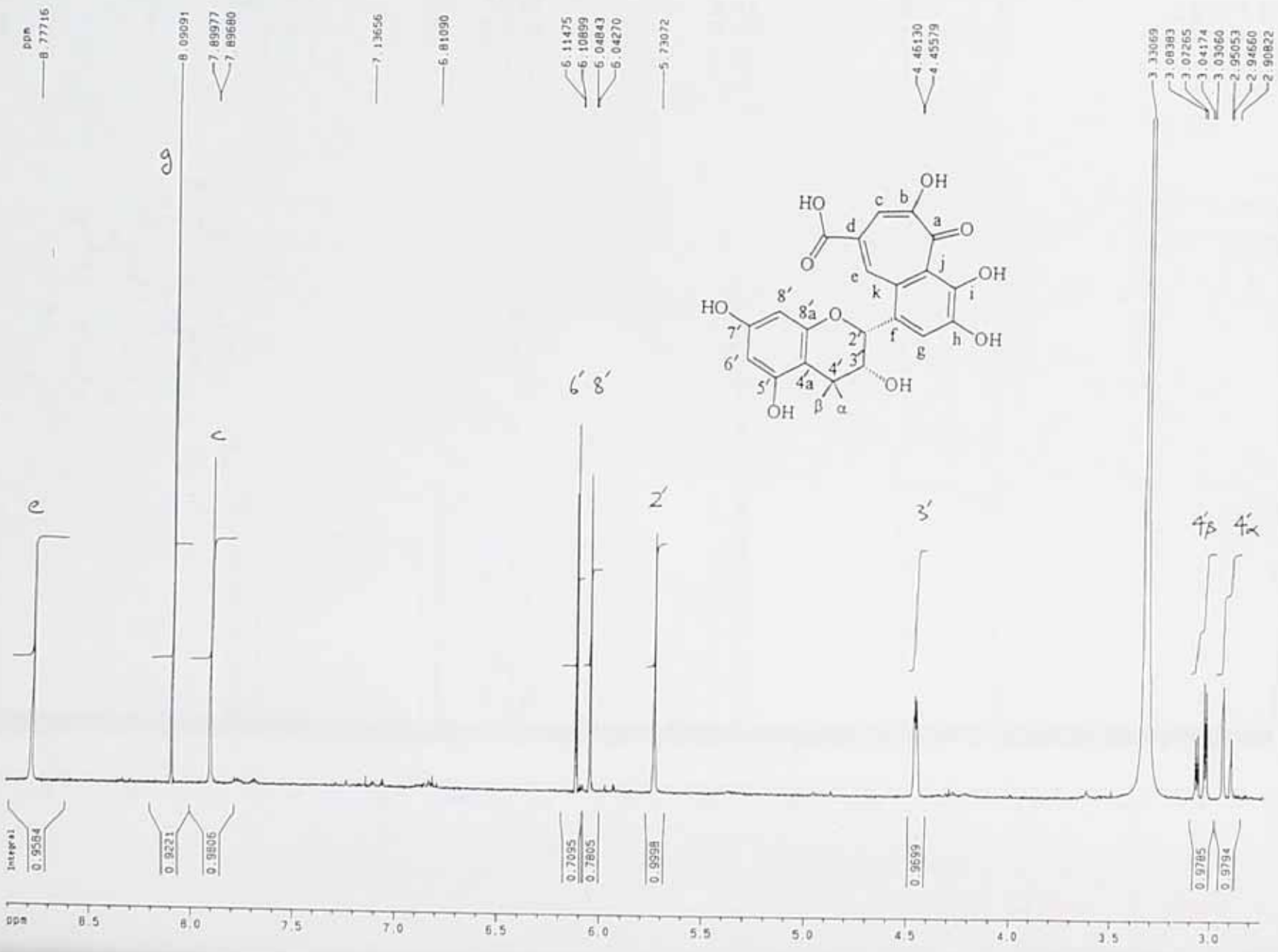


Figure 10H Epitheaflavic Acid in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.

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EXPNO                   6
PROCNO                  1

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Time                11.27
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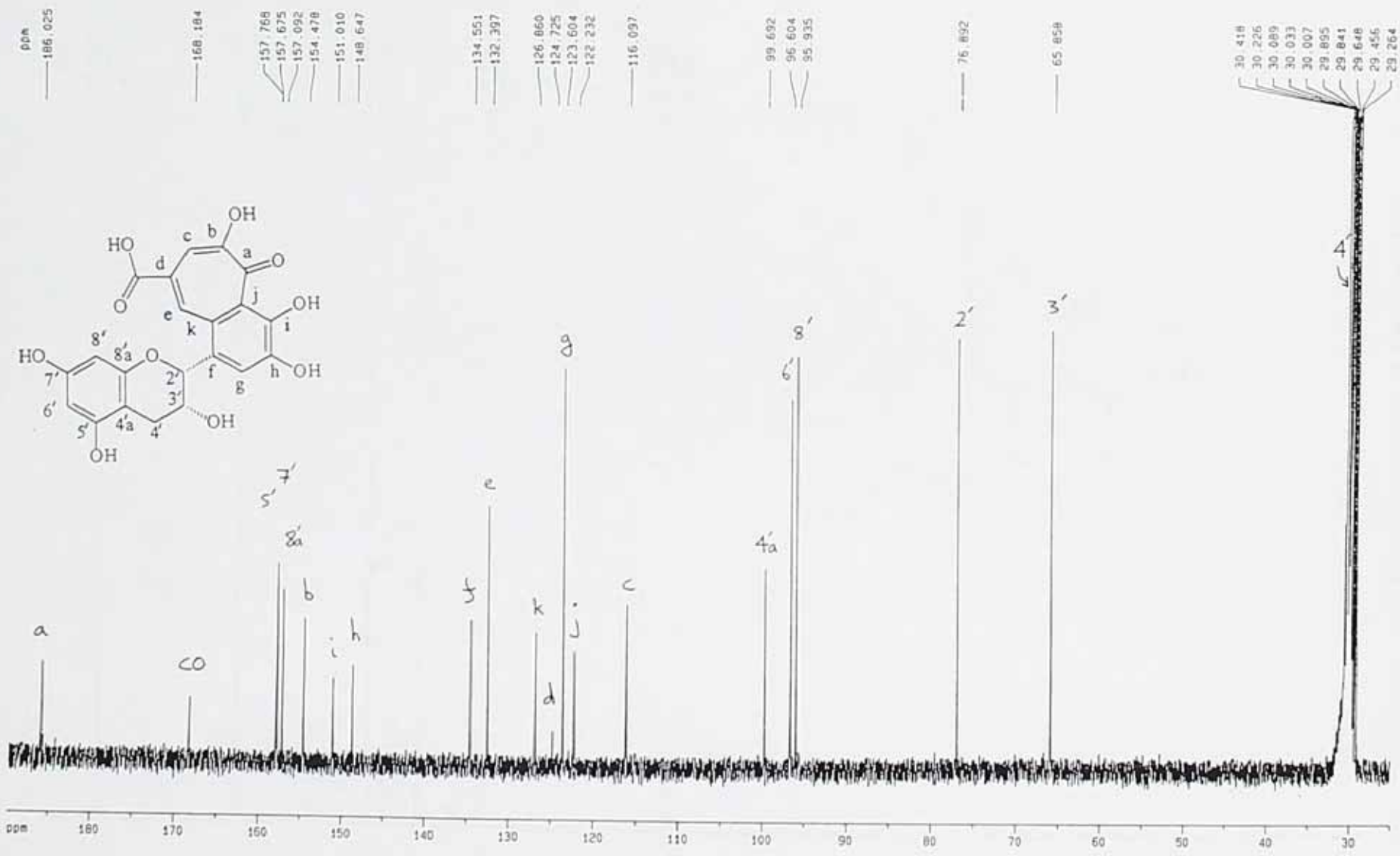
PROBHD   5 mm Dual 13 C/H
PULPROG               zg
TD                 32768
SOLVENT           Aceton
NS                   128
DS                     0
SWH           3875.969 Hz
FIDRES        0.118285 Hz
AQ            4.2271218 sec
RG                   512
DW             129.000 usec
DE             161.25 usec
TE                 360.0 K
D1            1.00000000 sec
P1               5.00 usec
DE             161.25 usec
SFO1          400.1383793 MHz
NUCLEUS               1H

F2 - Processing parameters
SI                 32768
SF            400.1364575 MHz
WDW                   EM
SSB                    0
LB               -0.50 Hz
GB                   0.2
PC                  3.00

1D NMR plot parameters
CX                30.84 cm
F1P               8.907 ppm
F1              3563.83 Hz
F2P               2.745 ppm
F2              1098.21 Hz
PPMCM         0.19980 ppm/cm
HZCM          79.94582 Hz/cm
```

Figure 10C Epitheaflavic Acid in d_6 acetone.
^{13}C-{^{1}H} NMR Spectrum at 300 K.



Current Data Parameters

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EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

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PROBHD	5 mm Dual 13
PULPROG	zgcpd
TD	65536
SOLVENT	Aceton
NS	80000
DS	0
SWH	26315.789 Hz
FIDRES	0.401547 Hz
AQ	1.2452340 sec
RG	32768
DW	19.000 usec
DE	23.75 usec
TE	300.0 K

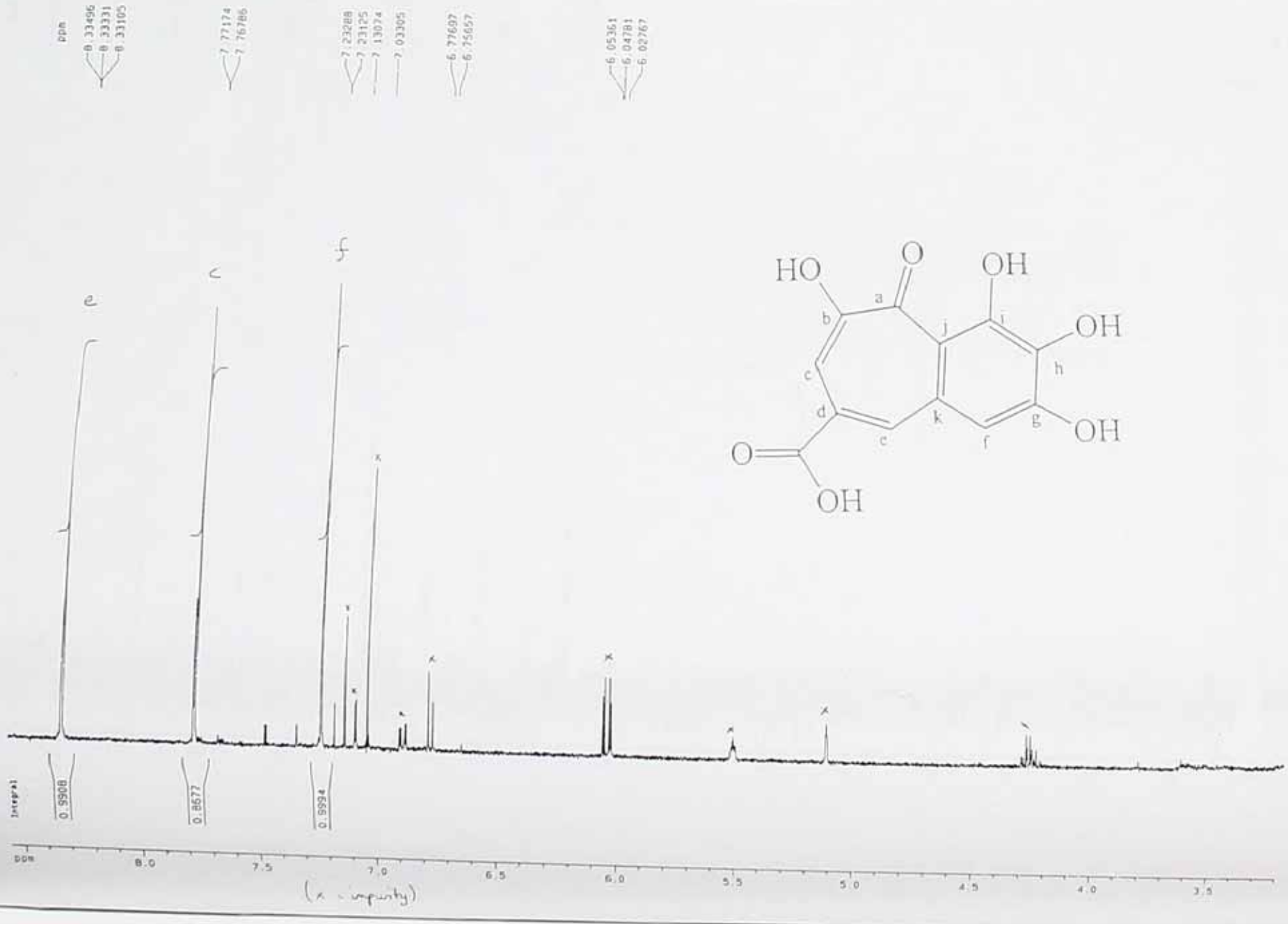
F2 - Processing parameters

SI	65536
SF	100.6143015 MHz
WDW	GM
SSB	0
LB	-0.20 Hz
GB	0.1
PC	1.00

1D NMR plot parameters

CX	33.44 cm
F1P	189.795 ppm
F1	19096.09 Hz
F2P	25.000 ppm
F2	2515.36 Hz
PPMCM	4.92652 ppm/cm
HZCM	495.87973 Hz/cm

Figure 12H Purpurogallin-4-carboxylic acid in d_6 acetone plus D_2O
^1H NMR Spectrum at 300K

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PROCNO                     1

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PULPROG                  zg
TD                    65536
SOLVENT           ACETONE-D6
NS                       128
DS                         4
SWH                8064.516 Hz
FIDRES             0.123055 Hz
AQ                 4.0632820 sec
RG                       512
DW                  62.000 usec
DE                   88.57 usec
TE                   300.0 K
D1                0.10000000 sec
P1                    6.00 usec
DE                   88.57 usec
SFO1              400.1388558 MHz
NUCLEUS                  1H

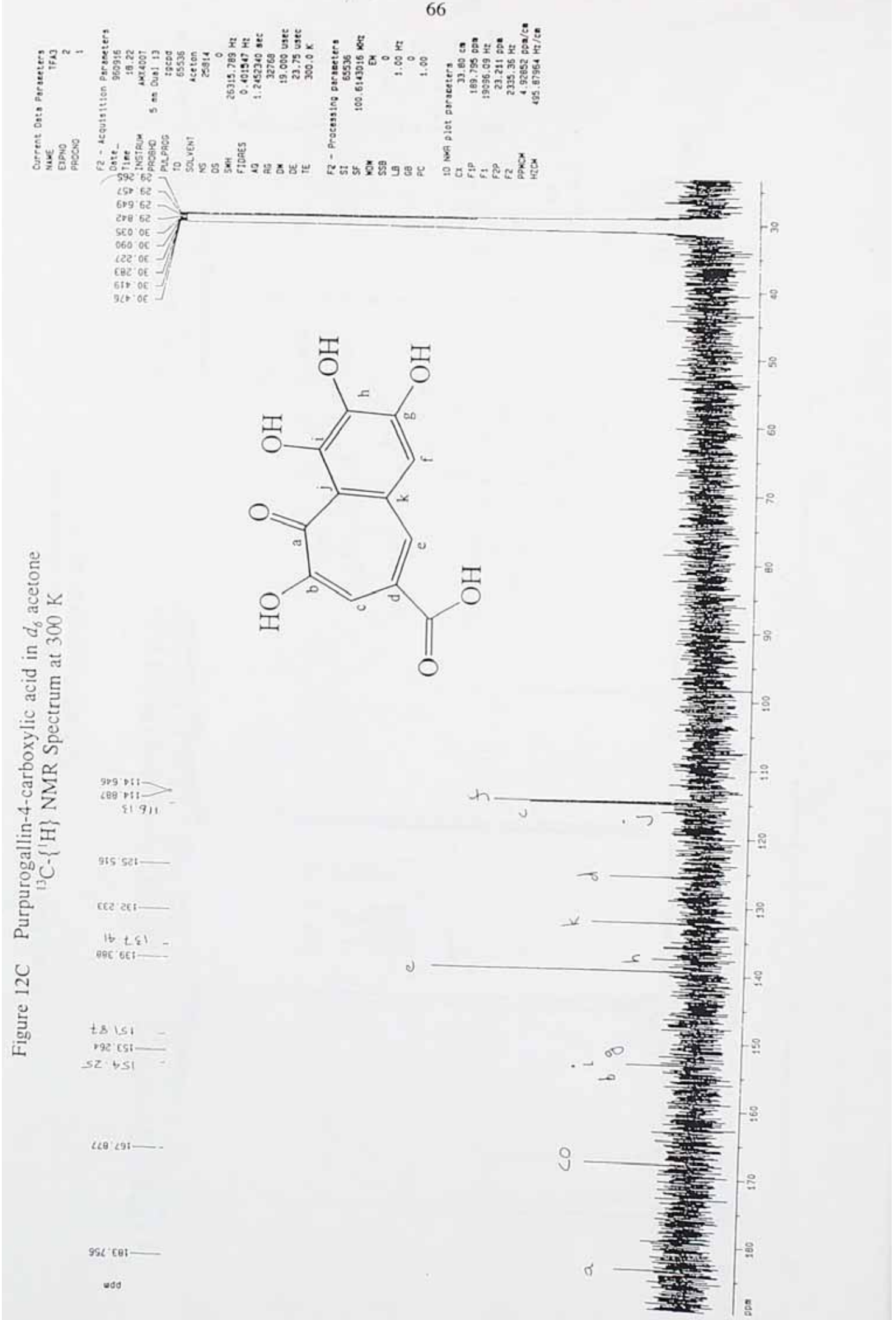
F1 - Acquisition parameters
NDO                        2
TD                       512
SFO1              100.6221 MHz
FIDRES            10.494602 Hz
SW                 53.400 ppm

F2 - Processing parameters
SI                     65536
SF                400.1364663 MHz
WDW                       GM
SSB                        0
LB                   -0.30 Hz
GB                     0.15
PC                    10.00

F1 - Processing parameters
SI                       512
MC2                     TPPI
SF                100.6139748 MHz
WDW                       no
SSB                        2
LB                   -11.49 Hz
GB                     0.437

1D NMR plot parameters
CX                   32.04 cm
F1P                 8.560 ppm
F1                 3425.17 Hz
F2P                 3.200 ppm
F2                 1280.44 Hz
PPMCM            0.16730 ppm/cm
HZCM             66.94283 Hz/cm
```

Figure 12C Purpurogallin-4-carboxylic acid in d_6 acetone
^{13}C-{^1H} NMR Spectrum at 300 K



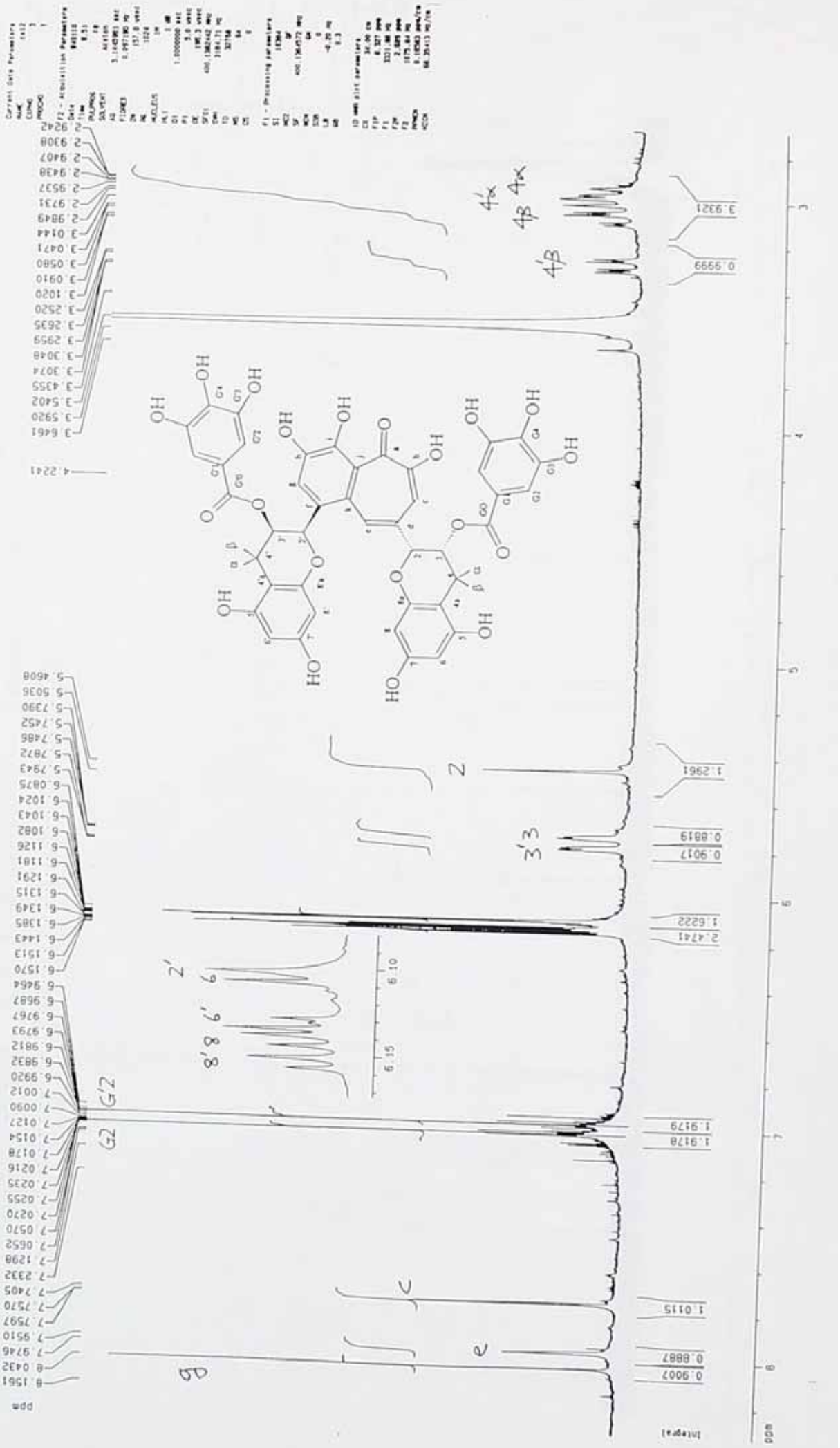


Figure Ref1 Theaflavin 3,3'-di-O-gallate in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.

Figure Ref2 Theaflavin 3,3'-di-O-gallate in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 300 K.

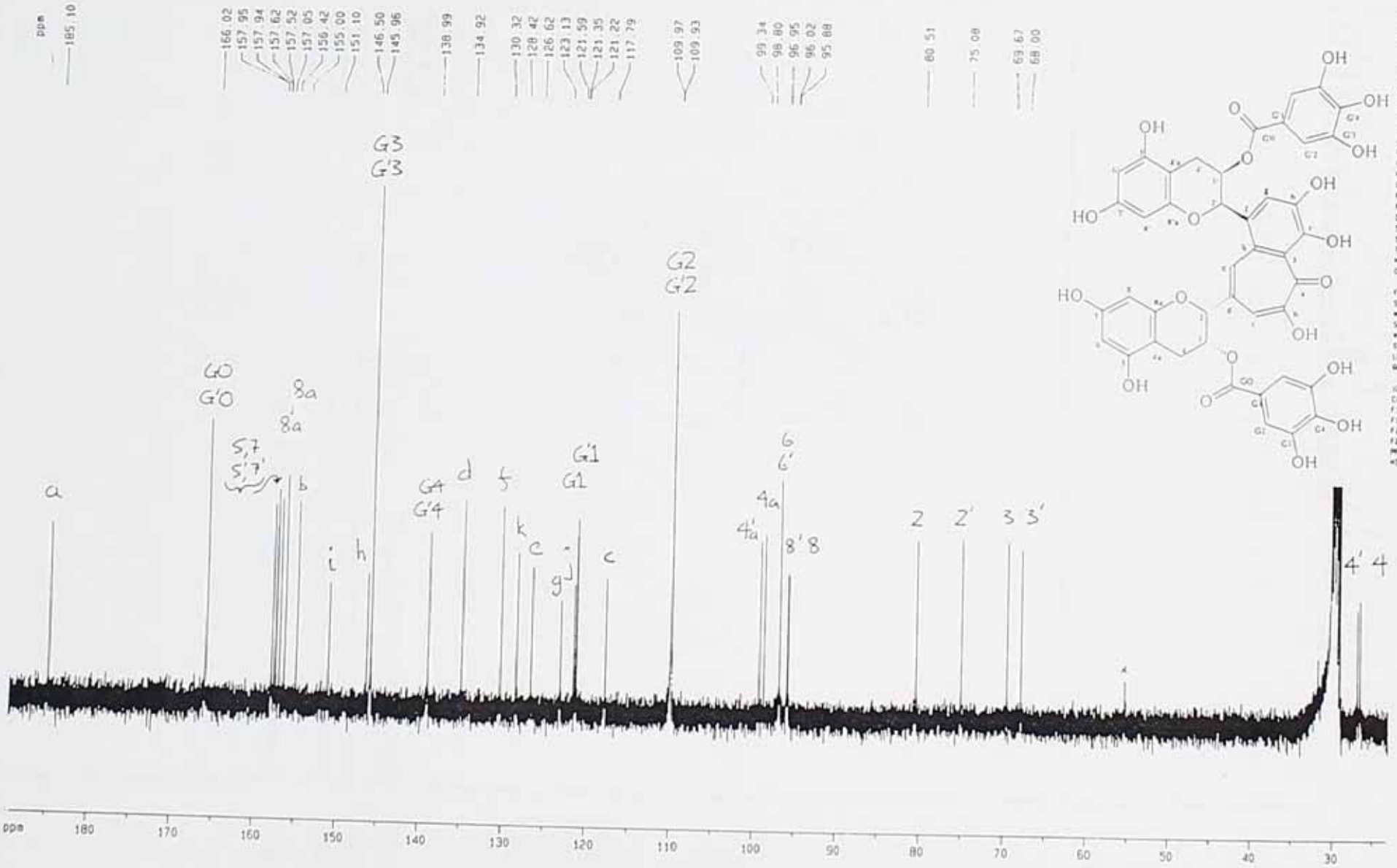
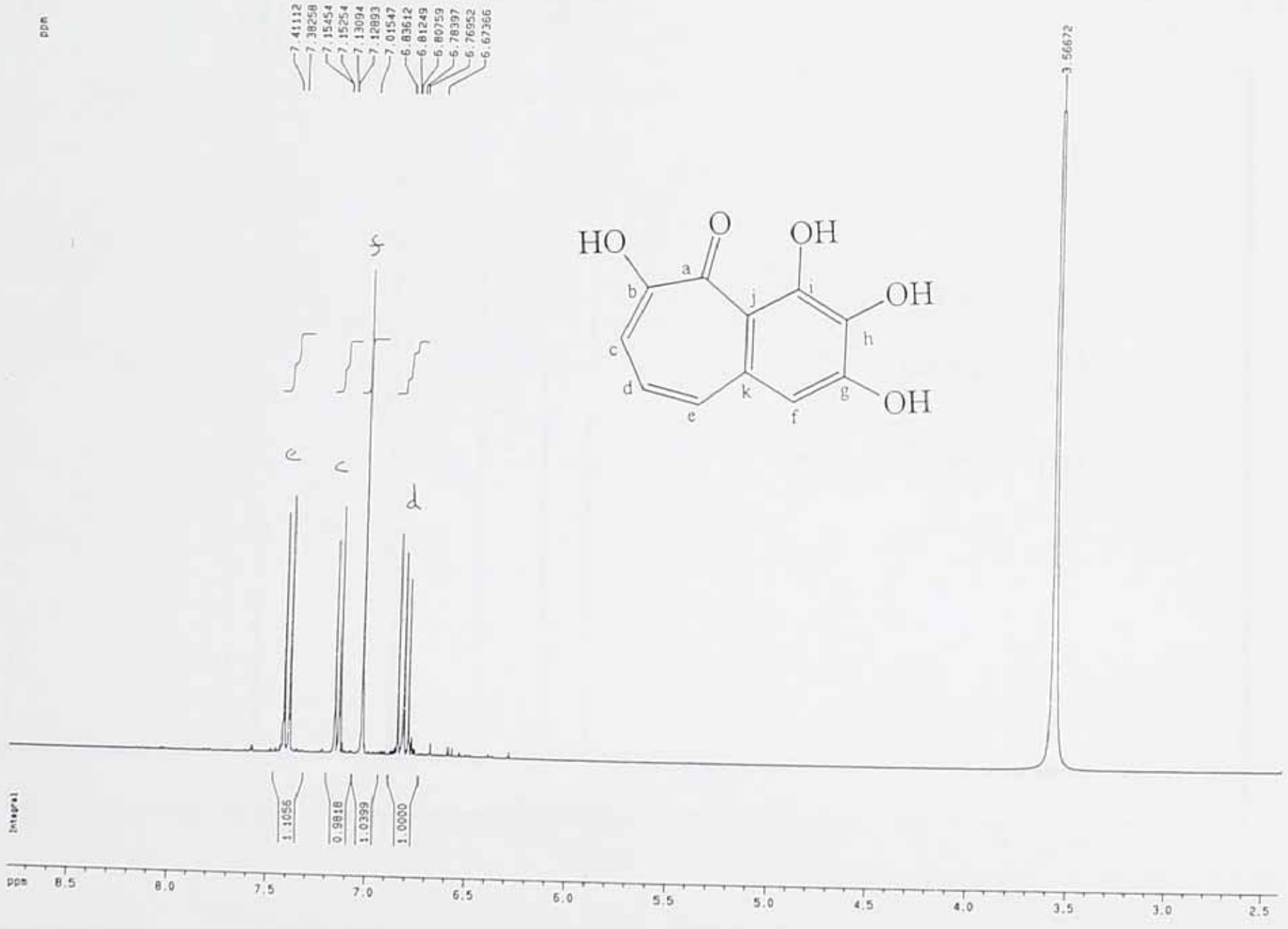


Figure Ref3 Purpurogallin in d_6 acetone plus D_2O.
^1H NMR Spectrum at 300 K.



Current Data Parameters

NAME	PUR
EXPNO	4
PROCNO	1

F2 - Acquisition Parameters

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Time	8.56
INSTRUM	AMX400T
PROBHD	5 mm Dual 13
PULPROG	zg
TD	65536
SOLVENT	Aceton
NS	32
DS	0
SWH	7575.758 Hz
FIDRES	0.115597 Hz
AQ	4.3254261 sec
RG	256
DW	66.000 usec
DE	82.50 usec
TE	360.0 K
D1	1.00000000 sec
P1	5.00 usec
DE	82.50 usec
SF01	400.1396955 MHz
NUCLEUS	1H

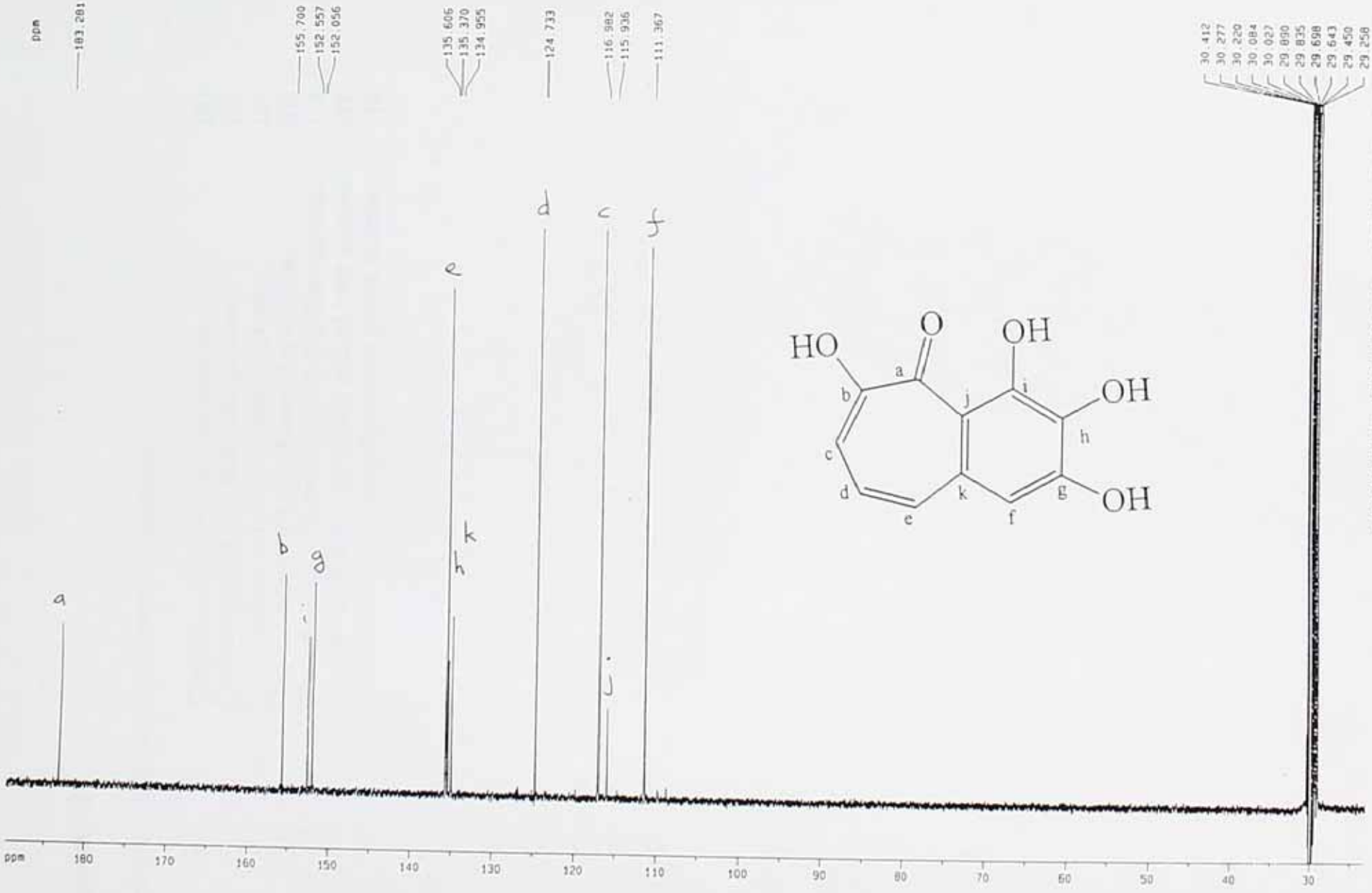
F2 - Processing parameters

SI	65536
SF	400.1364562 MHz
WDW	GM
SSB	0
LB	-0.30 Hz
GB	0.15
PC	20.00

1D NMR plot parameters

CX	32.00 cm
F1P	8.792 ppm
F1	3518.10 Hz
F2P	2.410 ppm
F2	964.38 Hz
PPMCM	0.19944 ppm/cm
HZCM	79.80363 Hz/cm

Figure Ref4 Purpurogallin in d_6 acetone.
^{13}C-{^1H} NMR Spectrum at 300 K.



Current Data Parameters

NAME	PUR
EXPNO	3
PROCNO	1

F2 - Acquisition Parameters

Date_	961024
Time	18.57
INSTRUM	AMX4001
PROBHD	5 mm Dual 13
PULPROG	lgcpd
TD	65536
SOLVENT	Aceton
NS	14598
DS	0
SWH	26315.789 Hz
FIDRES	0.401547 Hz
AQ	1.2452340 sec
RG	32768
DW	19.000 usec
DE	23.75 usec
TE	300.0 K

F2 - Processing parameters

SI	65536
SF	100.6143036 MHz
WDW	GM
SSB	0
LB	-0.40 Hz
GB	0.1
PC	1.00

1D NMR plot parameters

CX	33.80 cm
F1P	189.795 ppm
F1	19096.09 Hz
F2P	23.211 ppm
F2	2335.36 Hz
PPMCM	4.92852 ppm/cm
HZCM	495.87964 Hz/cm

Appendix 2:

Predicted configuration of flavan-3-ol quinones.

Figure 3.1 Catechin Quinone

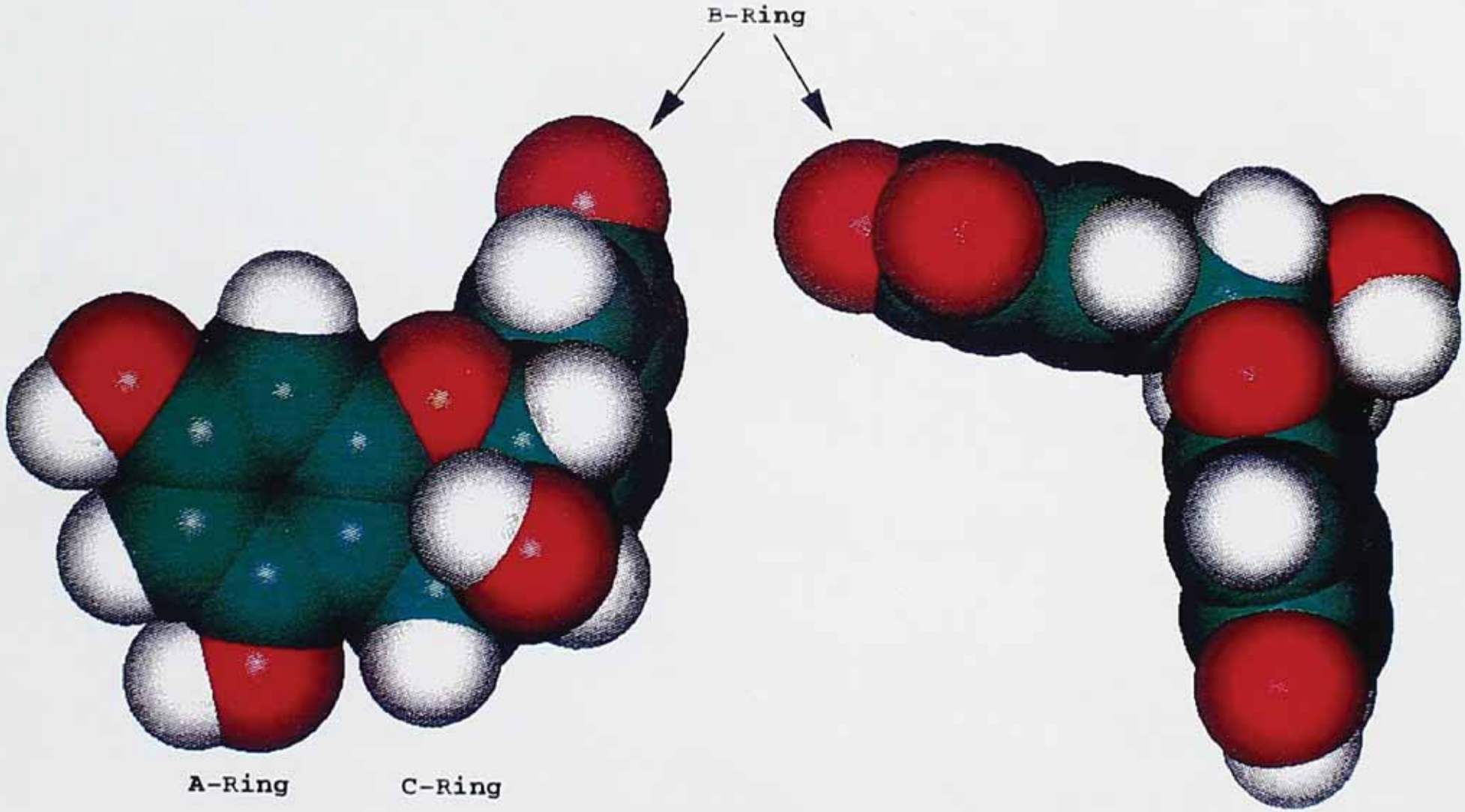


Figure 3.2 Epicatechin Quinone

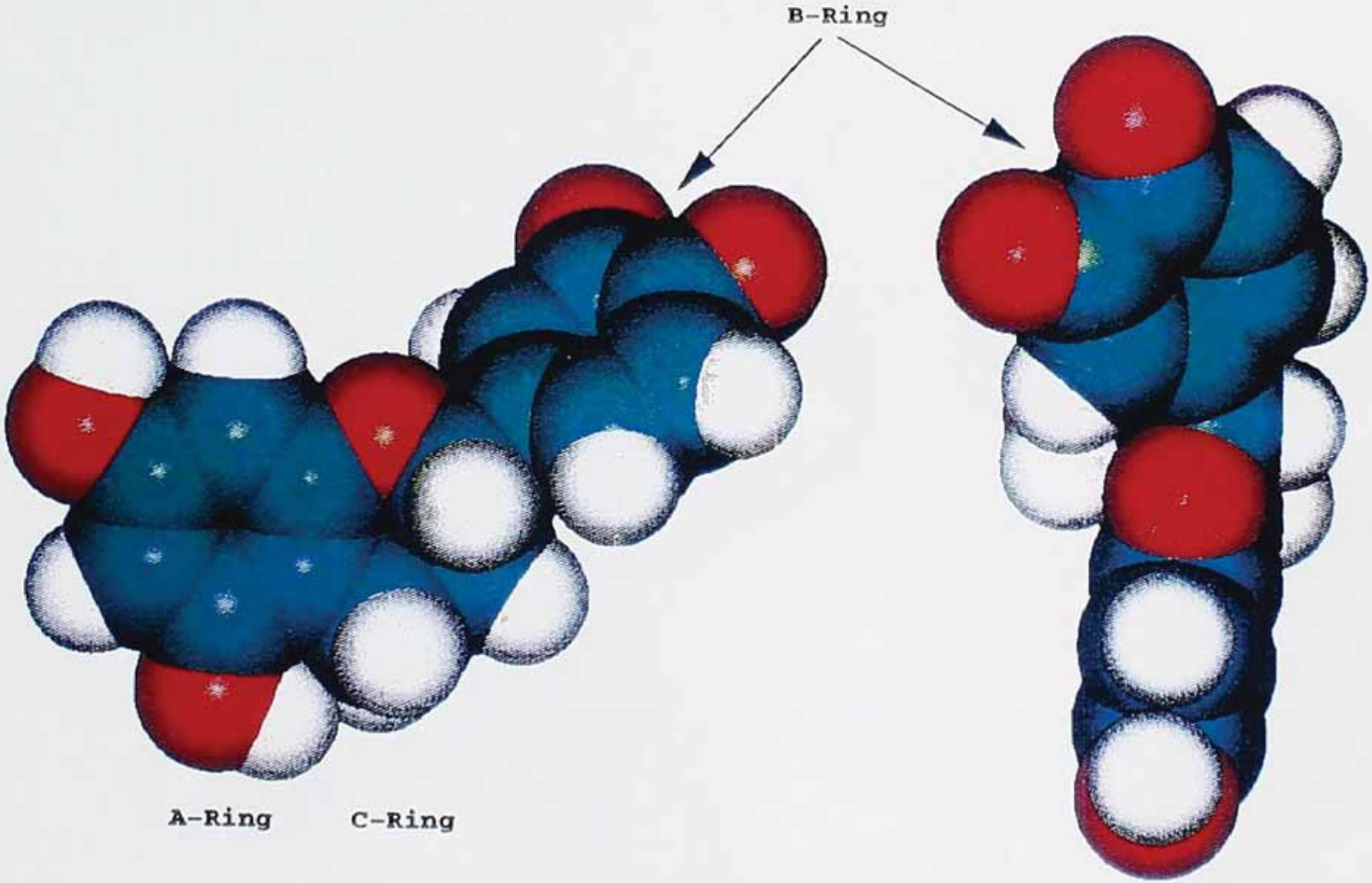


Figure 3.3 Gallocatechin Quinone

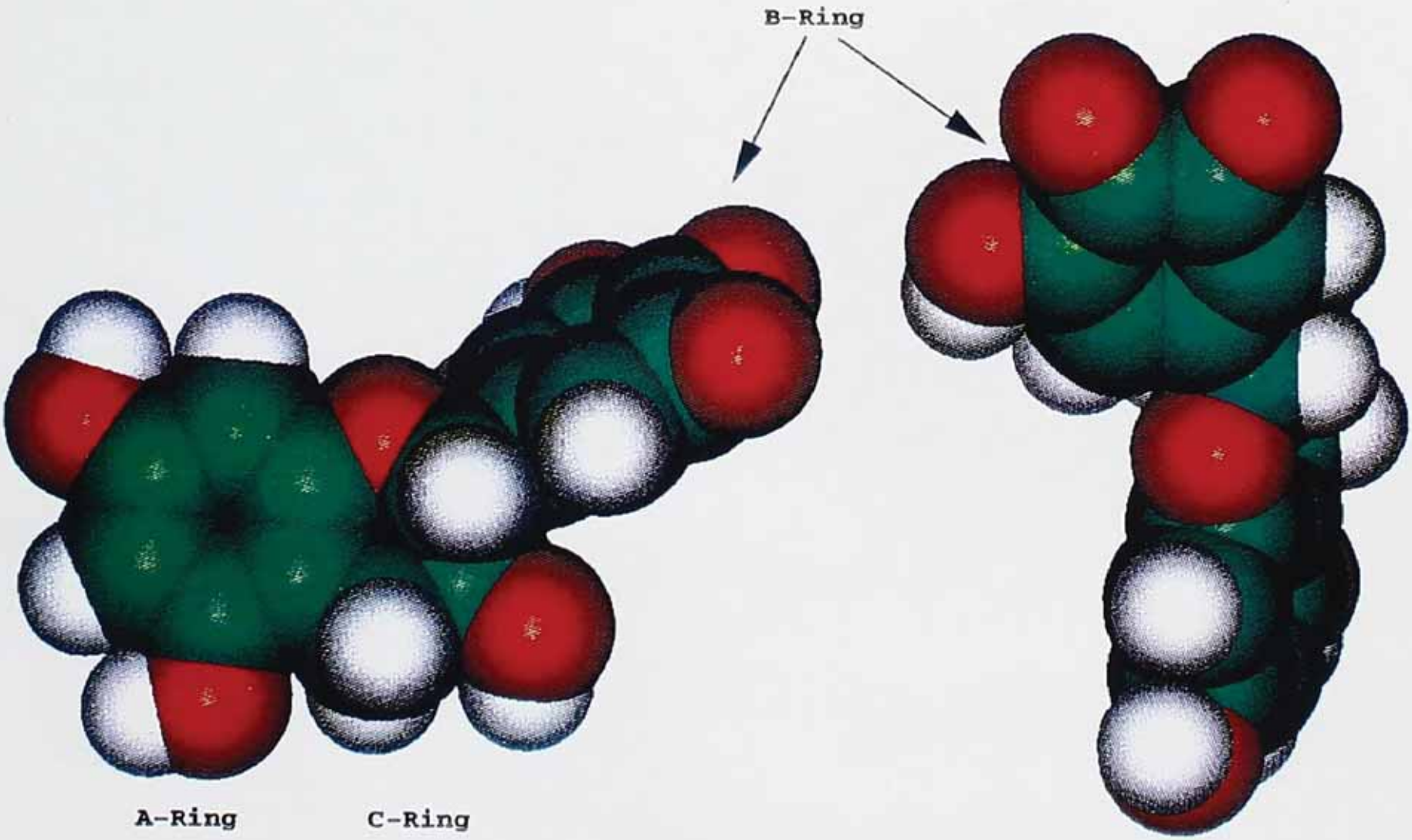


Figure 3.4 Epigallocatechin Quinone

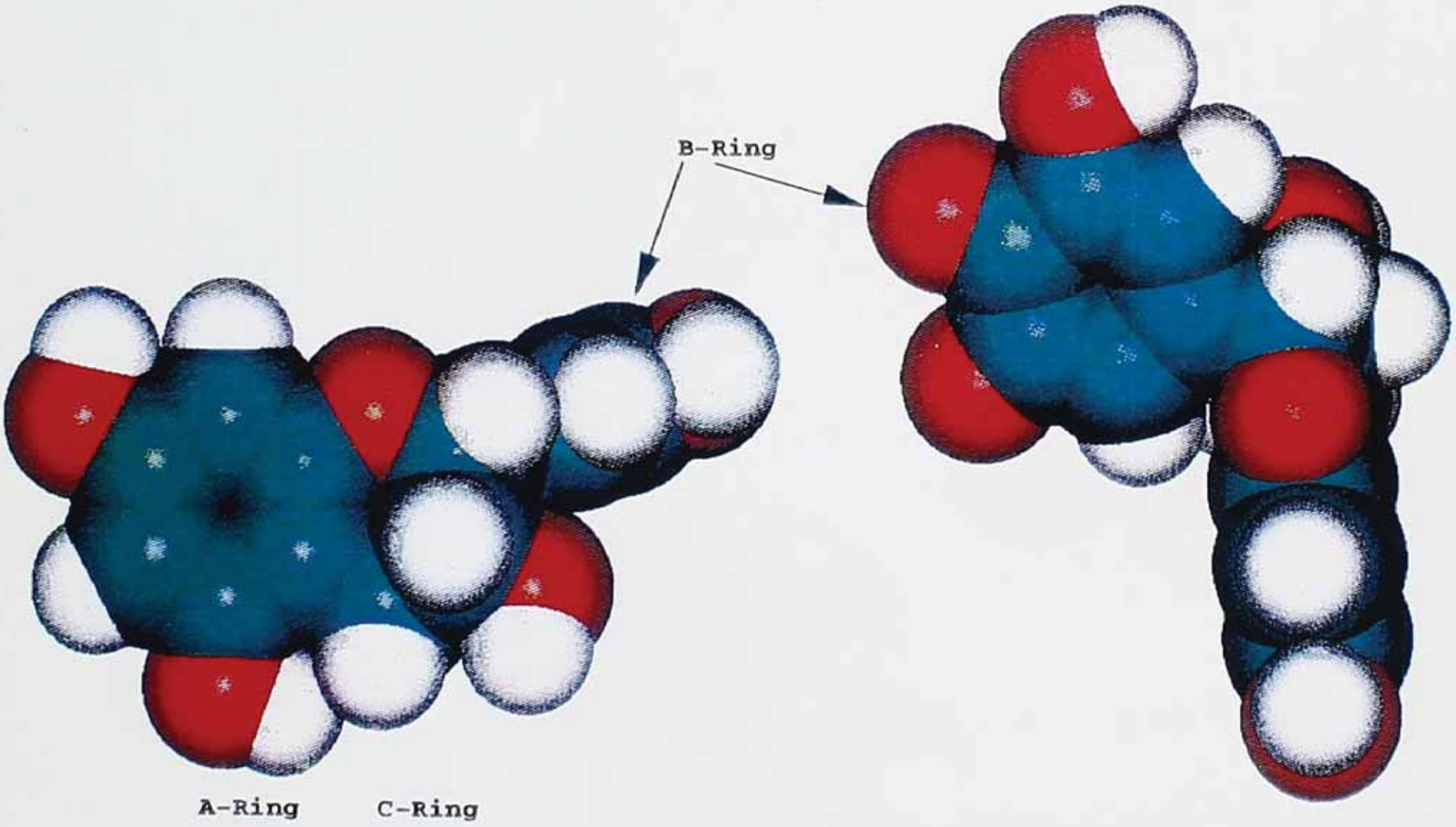


Figure 3.5 Epicatechin Gallate Quinone

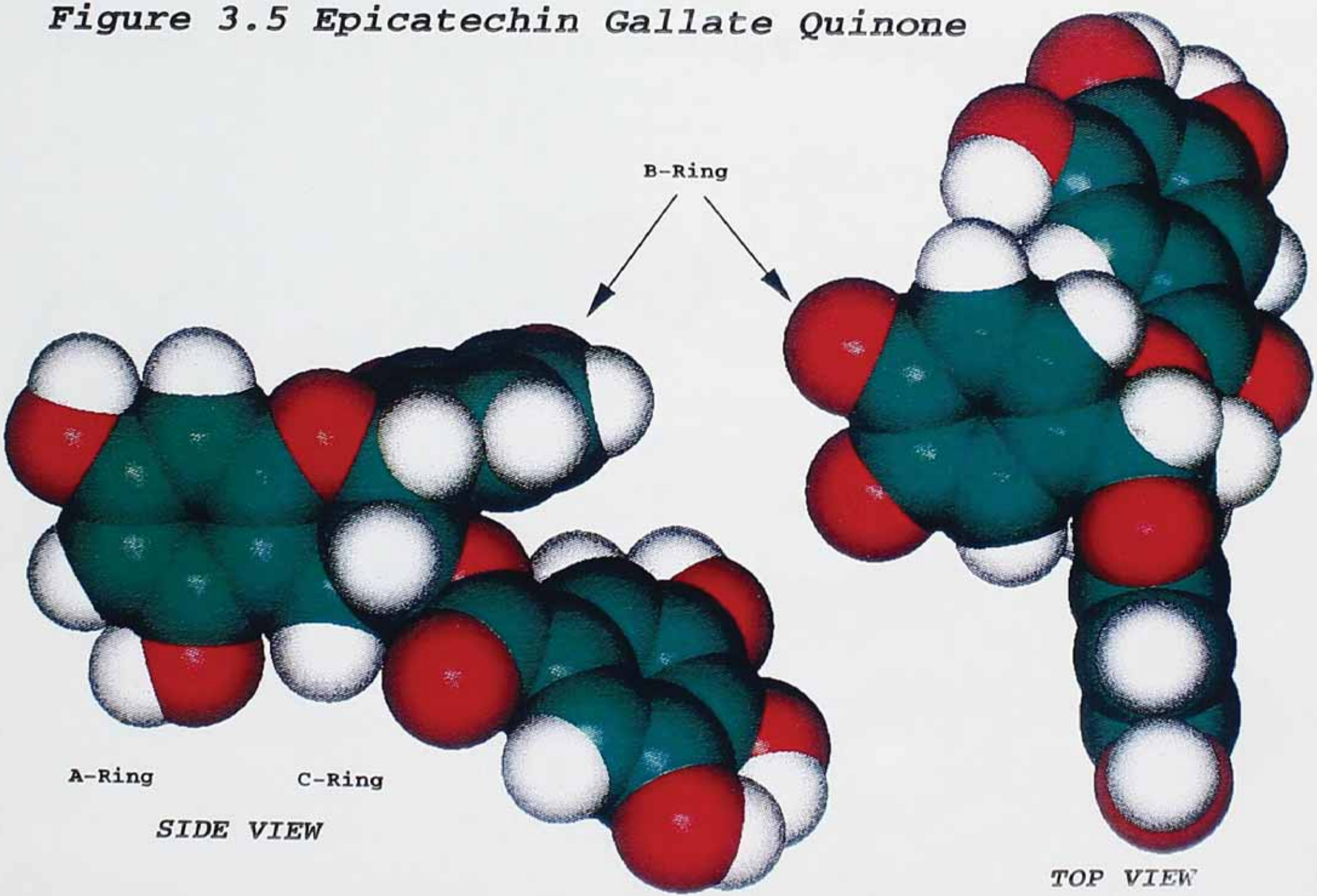


Figure 3.6: Epicatechin Galloyl Ester Quinone

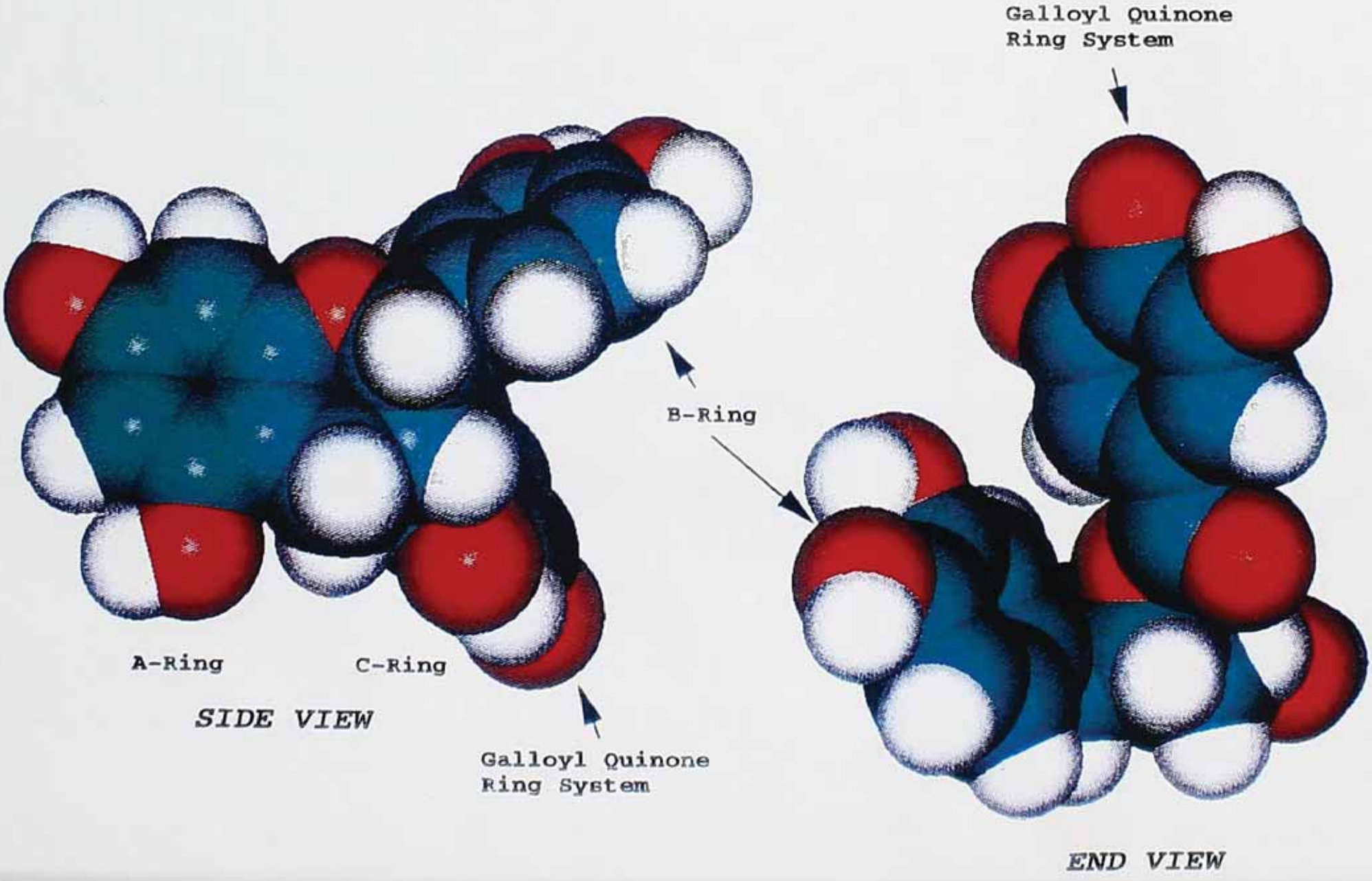


Figure 3.7: Epigallocatechin Gallate Quinone