# CHAPTER III

# DISCUSSION

## 3.1 Structural elucidation of Mixture I

Mixture I was a white powder with a melting point of 72-73 °C. Its IR spectrum (Figure 3) showed absorption bands of carboxylic acid consisting of the band of hydroxyl group at 3500-2400 $cm^{-1}$ and carbonyl group at 1715 $cm^{-1}$. The C-H stretching vibration peaks of aliphatic C-H were presented at 2980-2860 $cm^{-1}$. The IR spectroscopic data suggested that Mixture I might be a mixture of long-chain aliphatic acid.

**Table 3.1** The IR absorption band assignments of Mixture I

| Frequency ($cm^{-1}$) | Band type | Assigments |
|---|---|---|
| 3500-2400 | weak, broad | O-H stretching vibration of -COOH |
| 2980,2860 | strong | C-H stretching vibration of $-CH_2-$,$-CH_3$ |
| 1715 | stong | C=O stretching vibration of -COOH |
| 1470, 1390 | medium | C-H bending of $-CH_2-$,$-CH_3$ |
| 1310 | medium | C-O stretching vibration |
| 720 | weak | $CH_2$ rocking in $C-(CH_2)_n-C$ |

To study the chemical constituents of Mixture I , this mixture was converted to the correspondting methyl ester by reacting with diazomethane [19] and analyzed by GC-MS . The GC-MS chromatogram was shown in Figure 4 and the mass spectrum of each peak was compared with authentic spectra through library search (NIST database). The experimental results are tabulated in Table 3.2 .

**Table 3.2** Number of carbon in methyl ester derivative of Mixture I

| Compound | Molecular formular | Retention time | *m/z* |
|---|---|---|---|
| Docosanoic acid, methyl ester | $C_{23}H_{46}O_2$ | 23.15 | 354 |
| Tricosanoic acid, methyl ester | $C_{24}H_{48}O_2$ | 24.36 | 368 |
| Tretracosanoic acid, methyl ester | $C_{25}H_{50}O_2$ | 25.52 | 382 |
| Pentracosanoic acid, methyl ester | $C_{26}H_{52}O_2$ | 26.51 | 396 |
| Hexacosanoic acid, methyl ester | $C_{27}H_{54}O_2$ | 27.49 | 410 |
| Heptacosanoic acid, methyl ester | $C_{28}H_{56}O_2$ | 28.39 | 424 |
| Octacosanoic acid, methyl ester | $C_{29}H_{58}O_2$ | 29.25 | 438 |

From GC-MS analysis, Mixture I was identified as a mixture of long chain carboxylic acids and their structures were shown in Table 3.3.

**Table 3.3** The mixture of long chain carboxylic acids in Mixture I

| Compound | Molecular formula | Structural formula |
|---|---|---|
| Docosanoic acid | $C_{22}H_{44}O_2$ | $CH_3$-$(CH_2)_{19}$-$CH_2$-COOH |
| Tricosanoic acid | $C_{23}H_{46}O_2$ | $CH_3$-$(CH_2)_{20}$-$CH_2$-COOH |
| Tetracosanoic acid | $C_{24}H_{48}O_2$ | $CH_3$-$(CH_2)_{21}$-$CH_2$-COOH |
| Pentracosanoic acid | $C_{25}H_{50}O_2$ | $CH_3$-$(CH_2)_{22}$-$CH_2$-COOH |
| Hexacosanoic acid | $C_{26}H_{52}O_2$ | $CH_3$-$(CH_2)_{23}$-$CH_2$-COOH |
| Heptacosanoic acid | $C_{27}H_{54}O_2$ | $CH_3$-$(CH_2)_{24}$-$CH_2$-COOH |
| Octacosanoic acid | $C_{28}H_{56}O_2$ | $CH_3$-$(CH_2)_{25}$-$CH_2$-COOH |

### 3.2 Structural elucidation of Compound II

Compound II was bright white needle-like crystals and the melting point was 136-137 °C . The color test of this compound suggested the presence of a steroidal skeleton whereby the compound gave a green color (positive test) with Liebermann-Burchard's reagent.

The IR spectrum of Compound II (Figure 12) indicated important absorption band of hydroxyl group at 3640-3080 $cm^{-1}$ and absorption band of unsaturated C=C at 1650 $cm^{-1}$. The principle IR absorption bands can be assigned as shown in Table 3.4

**Table 3.4** The IR absorption band assignments of Compound II

| Frequency($cm^{-1}$) | Band type | Assignments |
|---|---|---|
| 3690-3080 | strong, broad | O-H stretching vibration of R-OH |
| 2940,2860 | strong | C-H stretching vibration of $-CH_3$,$-CH_2-$ |
| 1640 | weak | C=C stretching vibration |
| 1460,1380 | medium | C-H bending vibration of $-CH_2-$,$-CH_3$ |
| 1060 | weak | C-O stretching vibration |
| 800 | weak | C-H out of plane bending vibration of $R_1R_2C=CHR_3$ |

The $^{1}$H-NMR spectrum ( Figure 13 ) showed signals of protons at 0.66-2.26 ppm corresponding to the signals of methyl , methylene and methine protons . The chemical shift at 3.47 ppm indicated the proton attached to a carbon bearing a hydroxyl group (C<u>H</u>-OH) and the signal at 5.32 ppm indicated the persence of olefinic proton (-C<u>H</u>=C-) .

$^{13}$C-NMR spectrum ( Figure 14 ) showed 25 signals in the region of 11.84-56.75 ppm which indicated $sp^3$ $CH_3$, $CH_2$ and CH. The olefinic carbon signals were evidenced at 121.72 ppm (<u>C</u>H=C) and 140.74 ppm (CH=<u>C</u>).

The color test, IR spectrum and NMR spectra suggested that compound II could be a steroidal compound having a hydroxyl group. GLC tecnique was then used to identify compound II by comparison the chromatogram of compound II with that of the standard mixture of steroids : campesterol, stigmaterol, β-sitosterol ( Figure 15 ). The retention times of the standard steroid were 21.52, 22.57 and 25.66 min respectively. The retention time of compound II was 25.76 min which revealed that compound II was β-sitosterol. EI mass spectra ( Figure 16 ) revealed a molecular ion peak at $M^+$= 414 which also corresponded to the molecular formula $C_{29}H_{26}O$ of β-sitosterol.

Comparison of the $^{13}$C-NMR spectrum of compound II and β-sitosterol [20] was also carried out to further confirm the structure as presented in Table 3.4 .

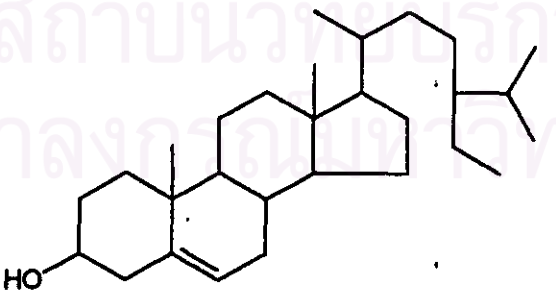
**Table 3.5** $^{13}$C-NMR spectrum of Compound II compared with β-sitosterol

| Position | chemical shift (ppm) | |
|---|---|---|
| | β-sitosterol | compound II |
| 1 | 37.4 | 37.2 |
| 2 | 31.8 | 31.6 |
| 3 | 71.9 | 71.8 |
| 4 | 42.4 | 42.3 |
| 5 | 140.9 | 140.7 |
| 6 | 121.8 | 121.7 |
| 7 | 32.0 | 31.9 |
| 8 | 32.0 | 31.9 |
| 9 | 50.3 | 50.1 |
| 10 | 36.6 | 36.5 |
| 11 | 21.1 | 21.1 |
| 12 | 39.9 | 39.8 |
| 13 | 42.4 | 42.3 |
| 14 | 56.8 | 56.8 |
| 15 | 24.3 | 24.3 |
| 16 | 28.2 | 28.2 |
| 17 | 56.2 | 56.0 |
| 18 | 11.9 | 12.0 |
| 19 | 19.4 | 19.4 |

**Table 3.5** (continued)

| Position | Chemical shift (ppm) | |
|---|---|---|
| | β-sitosterol | Compound II |
| 20 | 36.2 | 36.1 |
| 21 | 19.1 | 19.0 |
| 22 | 34.0 | 33.9 |
| 23 | 29.3 | 29.1 |
| 24 | 50.3 | 50.1 |
| 25 | 26.2 | 26.0 |
| 26 | 18.8 | 18.8 |
| 27 | 19.8 | 19.8 |
| 28 | 23.1 | 23.0 |
| 29 | 11.9 | 11.8 |

These results suggested that Compound II was β-sitosterol.




β-sitosterol.

### 3.3 *Structural elucidation of Compound III*

Compound III was a white amorphous solid , m.p. 255-257 °C (dec.) having an $R_f$ value 0.43 ( $SiO_2$ : 10% methanol in dichloromethane ). The color test of Compound III with Liebermann-Burchard's reagent gave a positive test (green color) which suggested the presence of a steroidal skelaton.

The IR spectrum (Figure 17) showed a broad absorption band of hydroxyl group (O-H) at 3400$cm^{-1}$, C-O stretching vibration of a glycosidic linkage at 1020-1075 $cm^{-1}$. and an anomeric axial C-H deformation of β-glycoside at 890 $cm^{-1}$. The IR absorption band is assigned as shown in Table 3.6

**Table 3.6** The IR absorption band assignments of Compound III

| Frequency | Band type | Assignments |
|---|---|---|
| 3400 | broad, strong | O-H stretching vibration of R-OH |
| 2940, 2860 | strong | C-H stretching vibration of $-CH_2-$,$-CH_3$ |
| 1650 | weak | C=C stretching vibration |
| 1465, 1380 | medium | C-H bending of $-CH_2-$,$-CH_3$ |
| 1020 | broad, strong | C-O stretching vibration |
| 800 | weak | C-H out of plane bending vibration of $R_1R_2C=CHR_3$ |

IR spectrum and color test indicated that Compound III might be steroid glycoside .

The EI mass spectrum (Figure 18) did not reveal the molecular ion ($M^+$) , due to the extensive fragmentation of the molecule. The spectrum exhibited the dominant fragmentation ion peaks at *m/z* 414 and others minor fragments at 396, 381, 329, 303, 273, 255, 213, 145, 107, 81 and 55 which was similar to the fragmentation pattern of β-sitosterol .

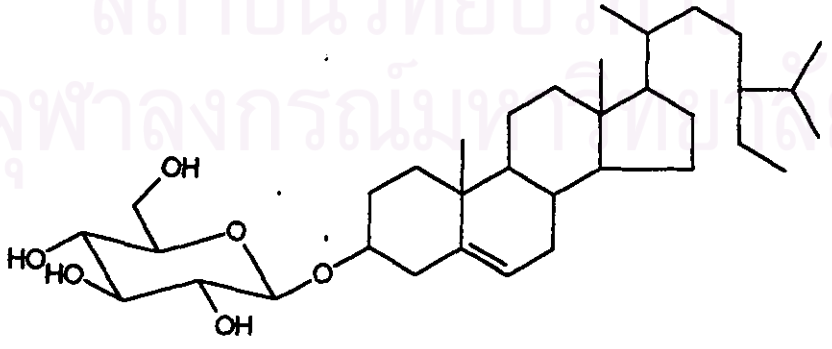
The $^1$H-NMR spectrum ( Figure 19 ) showed signals of proton at 0.66-2.50 ppm which corresponding to methyl, methylene and methine protons of the steroid. The chemical shift at 5.32 ppm indicated olefinic proton ( *CH*=C) . In addition, the signals at 2.94 - 3.69 ppm was observed and these were assigned to the proton of the sugar moiety and a doublet at 4.25 ppm (1H, d, J = 7.6 Hz) which was the anomeric proton of β-D-glucose .

The $^{13}$C-NMR spectrum ( Figure 20 ) showed two signals of olefinic carbons at 140.6 (CH=C) and 121.0 ppm (CH=C), as well as the signals of $sp^3$ carbon at 11.65-56.73 ppm which were similar to the carbons signals of β-sitosterol. Furthermore, the signals at 100.9, 76.9, 76.7, 73.6, 70.4 and 61.4 ppm corresponding to the signals of β-D-glucose were also observed [21]. The $^{13}$C-NMR spectrum of Compound III was compared with that of β-sitosterol-3-*O*-β-D-glucopyranoside and they were closely resembled ( Table 3.7 ).

**Table 3.7** $^{13}$C-NMR spectrum of Compound III compared with β-sitosterol-3-*O*-β-D-glucopyranoside (only the sugar part shown)

| position | chemical shift (ppm) | |
|---|---|---|
| | β-sitosterol-3-*O*-β-D-glucopyranoside (sugar part) | Compound III |
| G1 | 100.7 | 100.9 |
| G2 | 73.4 | 73.6 |
| G3 | 76.9 | 76.9 |
| G4 | 70.1 | 70.4 |
| G5 | 76.7 | 76.7 |
| G6 | 61.1 | 61.4 |

All these results suggested that Compound III was β-sitosterol-3-*O*- β-D-glucopyranoside .




β-sitosterol-3-*O*-β-D-glucopyranoside .

### 3.4 Structural elucidation of Compound IV

Compound IV was a dark green solid with a melting point of 145-147 °C. It gave a red fluorescence when irradiated with long-wave UV light which indicated a rigid chromophoric structure. Its principal IR absorption peaks ( Figure 21 ) showed the presence of a broad absorption band of N-H stretching vibration of amine at 3445 $cm^{-1}$ , the C=O stretching band of ester and conjugated ketone at 1740, 1730 and 1700 $cm^{-1}$ , respectively and C=C aromatic band around 1550 $cm^{-1}$ ( Table 3.8 ).

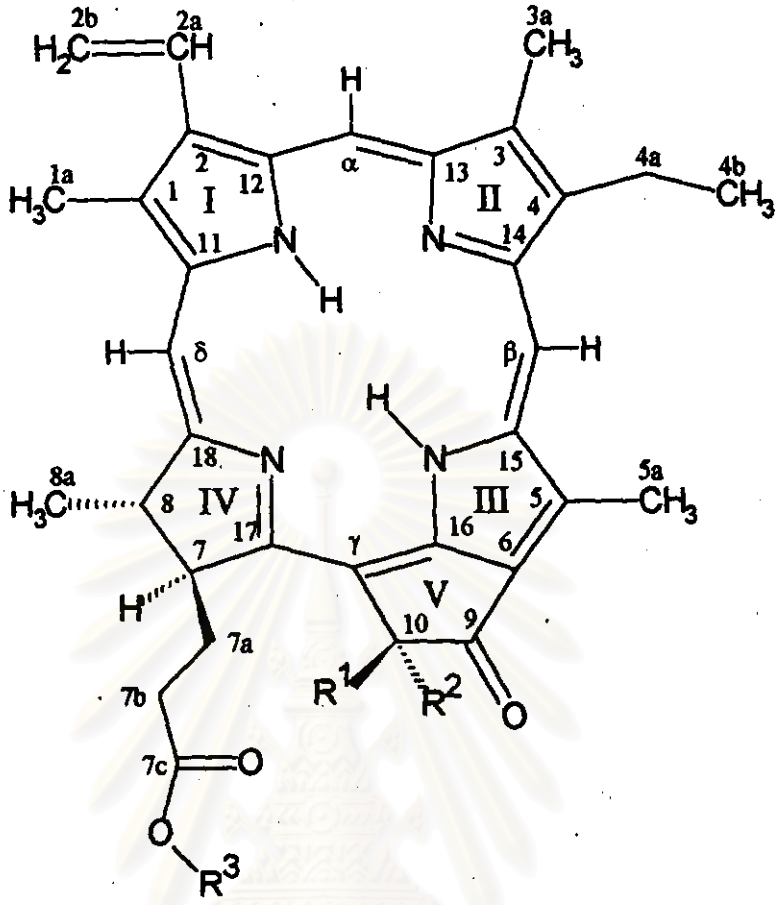
**Table 3.8** The IR absorption band assignments of Compound IV

| Frequency ($cm^{-1}$) | Band type | Assignments |
|---|---|---|
| 3445 | broad, medium | N-H stretching vibration of amine |
| 3110 | weak | C-H stretching vibration of aromatic |
| 2980,2930,2850 | strong | C-H stretching vibration of aliphatic |
| 1740,1730 | strong | C=O stretching vibration of ester |
| 1700 | strong | C=O stretching vibration of conjugated ketone |
| 1640 | medium | C=C stretching and C=N stretching vibration |
| 1155,1460 | medium | C=C stretching vibration of aromatic |
| 1500 | medium | N-H bending of 2° amine |
| 1300 | medium | C-N stretching vibration |
| 1200,1160 | strong | C-O stretching vibration |

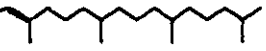
The $^{1}$H-NMR spectrum of Compound IV (Figure 22) showed signals in an unusually down field region at δ 9.46 (1H, s), 9.32 (1H,s) and 8.54 (1H,s), three signals of vinylic protons at δ 7.94 (1H,dd, $J$ = 11.6,18.0 Hz ; C<u>H</u>=C<u>H</u>$_2$), 6.15 (1H,d, $J$ = 0.9,11.6 Hz ; CH=C<u>H</u>$_2$), and 6.26 (1H,d, $J$ = 0.9,18.0 Hz ; CH=C<u>H</u>$_2$), three methine protons at δ 6.24 (1H, s), 4.20 and 4.45 (each 1H, m), two methoxy groups at δ 3.87 and 3.66 (each 3H, s), three methylene protons at δ 3.62 (2H, m), 2.23, 2.31, 2.50 and 2.62 (each 1H, m), five methyl protons at δ 3.17, 3.37 and 3.56 (each 3H,s), 1.80 (3H,d,$J$ = 7.6 Hz) and 1.66 (3H,t,$J$ = 7.6 Hz) and two broad signals at δ 0.50 and -1.66 ppm (each 1H,s) .

The $^{13}$C , DEPT 135 and DEPT 90 spectra (Figure 23-24) revealed that Compound IV had 17 methyl carbons, 4 methylene carbons, 7 methine carbons and 18 quaternary carbons and also consisted of one ethylene group, two methoxy groups and three carbonyl groups .

Its fluorescent properties suggested that Compound IV might have a rigid chromophore, probably having a porphyrin structure . Examination of $^{13}$C and $^{1}$H-NMR data of Compound IV further support the hypothesis. $^{13}$C-NMR spectrum of Compound IV was found to correspond to pheophytin a (a) and a' (b) reported in the literature [22] (Table 3.9) except that the phytol ester side chain disappeared in Compound IV and was replaced by a methyl ester.




(a) pheophytin a : $R^1 = H$, $R^2 = CO_2CH_3$, $R^3 = -CH_2$ 

(b) pheophytin a' : $R^1 = CO_2CH_3$, $R^2 = H$, $R^3 = -CH_2$ 

(c) methyl pheophorbide a : : $R^1 = H$, $R^2 = CO_2CH_3$, $R^3 = CH_3$

(d) methyl 10-epipheophorbide a : $R^1 = CO_2CH_3$, $R^2 = H$, $R^3 = CH_3$

**Table 3.9** $^{13}C$ chemical shifts of Compound IV compared with the literature

| Position | Chemical shifts (ppm) | | |
|---|---|---|---|
| | Pheophytin a | Pheophytin a' | Compound VI |
| 1 | 132.1 | 132.2 | 131.8 |
| 2 | 136.4 | 136.3 | 136.2 |
| 3 | 136.0 | 136.1 | 136.1 |
| 4 | 145.2 | 145.2 | 145.2 |
| 5 | 128.9 | 128.8 | 129.0 |
| 6 | 130.1 | 130.0 | 129.1 |
| 7 | 52.1 | 52.8 | 51.1 |
| 8 | 50.8 | 51.6 | 50.1 |
| 9 | 189.2 | 189.3 | 189.6 |
| 10 | 66.4 | 66.4 | 64.7 |
| 11 | 142.2 | 142.2 | 142.1 |
| 12 | 136.5 | 136.5 | 136.5 |
| 13 | 155.6 | 155.6 | 155.6 |
| 14 | 151.4 | 151.3 | 150.9 |
| 15 | 138.5 | 138.3 | 137.9 |
| 16 | 149.9 | 150.5 | 151.0 |
| 17 | 162.1 | 161.9 | 161.2 |
| 18 | 172.8 | 173.1 | 173.3 |

**Table 3.9** (continued)

| Position | Chemical shifts (ppm) | | |
|---|---|---|---|
| | Pheophytin a | Pheophytin a' | Compound IV |
| α | 97.5 | 97.5 | 97.5 |
| β | 104.4 | 104.4 | 104.4 |
| γ | 106.8 | 107.3 | 105.2 |
| δ | 93.9 | 93.9 | 93.1 |
| 1a | 11.9 | 11.9 | 12.1 |
| 2a | 129.7 | 129.7 | 129.1 |
| 2b | 122.1 | 122.1 | 122.7 |
| 3a | 10.8 | 10.7 | 11.1 |
| 4a | 19.5 | 19.5 | 19.4 |
| 4b | 17.4 | 17.4 | 17.4 |
| 5a | 11.7 | 11.6 | 12.0 |
| 7a | 31.7 | 31.2 | 29.9 |
| 7b | 30.2 | 29.8 | 31.0 |
| 7c | 172.9 | 172.9 | 172.2 |
| 8a | 23.3 | 23.5 | 23.1 |
| 10a | 169.9 | 171.1 | 169.5 |
| 10b | 52.5 | 52.4 | 52.8 |

Two dimensional NMR tecniques, namely HMQC, HMBC , COSY and NOESY were used to futher confirm the structure of Compound IV .

One bond C-H correlation (HMQC) spectra ( Figure 25 ) were used to assign protons and carbons directly attached to each other and the long range C-H coupling information was obtained by HMBC experiments ( Figure 26 ) . The data were as presented in Table 3.10

**Table 3.10** One bond and mutiple bond C-H correlation of Compound IV

| $^{13}C$ (ppm) | One bond correlation with proton at $\delta_H$ | mutiple bond correlation with proton at $\delta_H$ |
|---|---|---|
| 189.6 | - | 6.24 |
| 173.3 | - | 3.56, 2.50, 2.23 |
| 172.2 | - | 4.45, 1.80 |
| 169.6 | - | 6.25, 3.87 |
| 161.2 | - | - |
| 155.6 | - | 3.17 |
| 151.0 | - | 3.62 |
| 149.7 | - | 6.24 |
| 145.2 | - | 9.46, 3.62, 3.17, 1.66 |

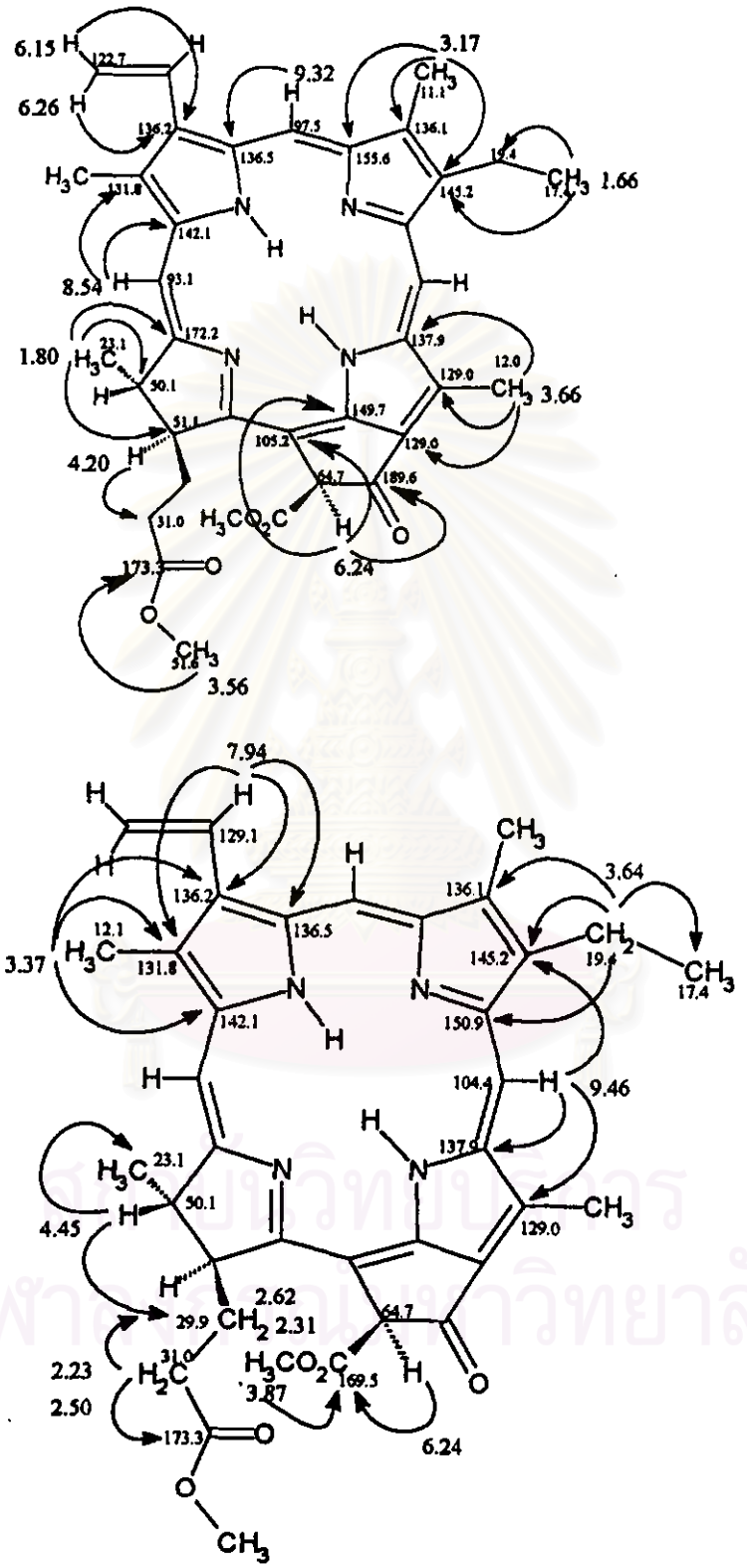
**Table 3.10** (continued)

| $^{13}C$ (ppm) | One bond correlation with proton at $\delta_H$ | One bond correlation with proton at $\delta_H$ |
|---|---|---|
| 142.1 | - | 8.54, 3.37 |
| 137.9 | - | 9.46, 3.66 |
| 136.5 | - | 9.32, 7.94 |
| 136.2 | - | 7.94, 6.15, 6.26, 3.37 |
| 136.1 | - | 3.62, 3.17 |
| 131.8 | - | 8.54, 7.94, 3.37 |
| 129.0 | - | 9.46, 3.66 |
| 128.9 | 7.94 | - |
| 128.9 | - | 3.66 |
| 122.8 | 6.15, 6.26 | - |
| 105.2 | - | 6.24 |
| 104.4 | 9.46 | - |
| 97.5 | 9.32 | - |
| 93.1 | 8.54 | - |
| 64.7 | - | 6.24 |
| 52.8 | 3.87 | - |
| 51.7 | 3.56 | - |
| 51.1 | 4.20 | 1.80 |

**Table 3.10** (continued)

| $^{13}C$ (ppm) | One bond correlation with proton at $\delta_H$ | One bond correlation with proton at $\delta_H$ |
|---|---|---|
| 50.1 | 4.45 | 1.80 |
| 31.0 | 2.50, 2.23 | 4.20, 3.56 |
| 29.9 | 2.26, 2.31 | 4.45, 2.50 |
| 23.1 | 1.80 | 4.45 |
| 19.4 | 3.62 | 1.66 |
| 17.4 | 1.66 | 3.62 |
| 12.1 | 3.37 | - |
| 12.1 | 3.66 | - |
| 11.2 | 3.17 | - |

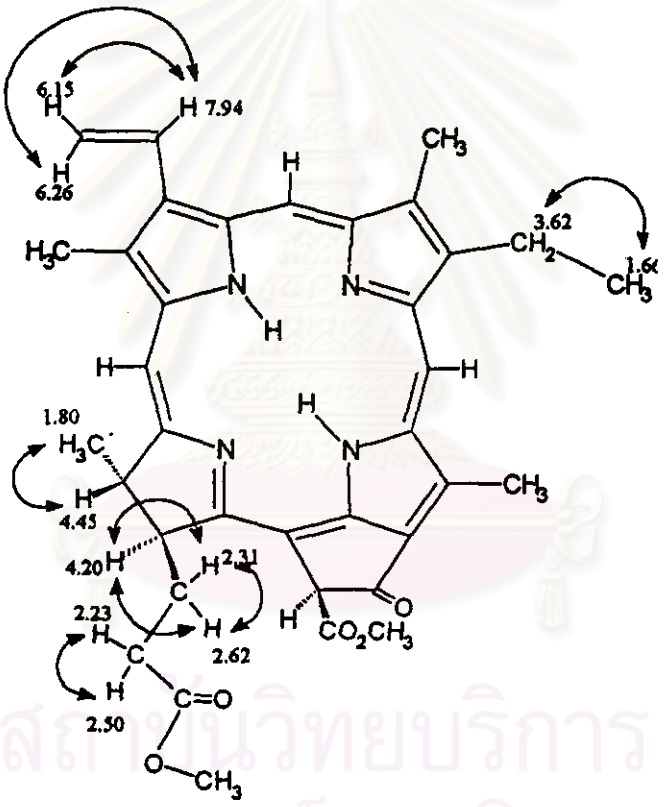
The possible structure of Compound IV was thus confirmed by these data .




**Most significant correlation obsered in HMBC of Compound IV**

COSY spectrum revealed (Figure 27) that the vinylic proton at 7.94 ppm coupling to the vinylic proton at 6.15 and 6.26 ppm , the methine proton at 4.45 ppm coupling to the methyl proton at 1.80 ppm , the methine proton at 4.20 ppm coupling to methylene proton at 2.31 and 2.62 ppm and the methylene proton at 3.62 ppm coupling to the methyl proton at 1.66 ppm .

This can be schematically summarised below



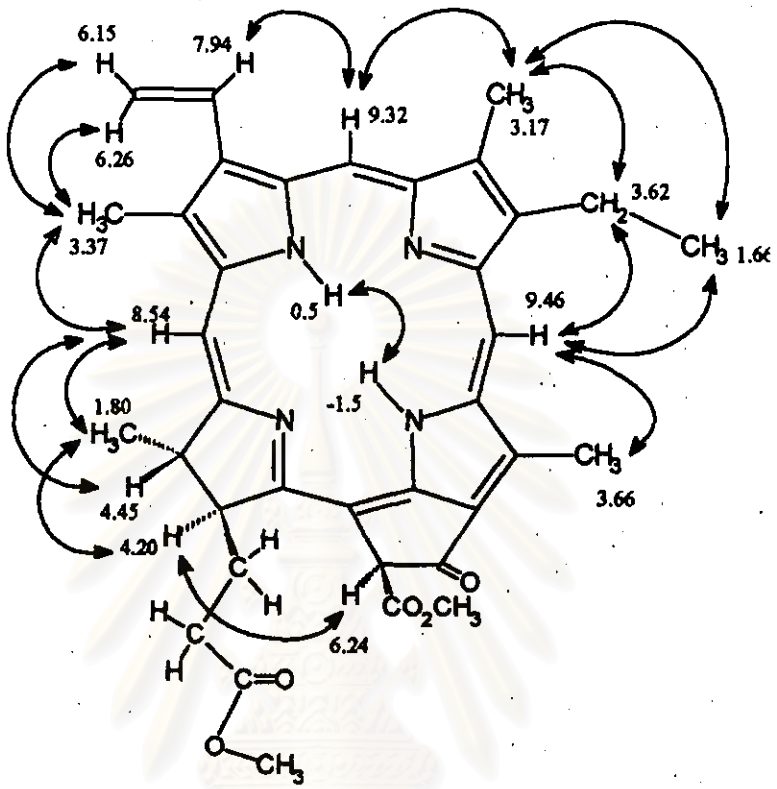

**Most significant H-H correlation observed in COSY spectra of Compound IV**

NOESY experiments further revealed correlations between protons closing to each other in space ( Table 3.11 and Figure 28) . This also agreed with the proposed structure .

**Table 3.11** H-H correlation in space (NOESY) of Compound IV

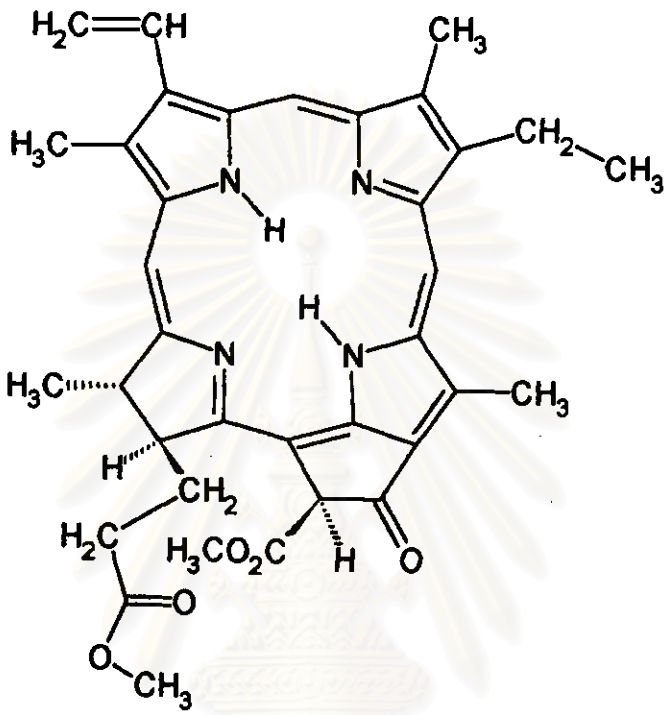
| Proton (ppm) | Correlated protons (ppm) |
|---|---|
| 9.46 | 1.66, 3.66, 3.62 |
| 9.32 | 3.17, 7.94 |
| 8.54 | 1.80, 3.37, 4.45 |
| 7.94 | 9.32, 6.15, 6.26 |
| 6.24 | 4.20, 2.62 |
| 6.15, 6.26 | 7.94, 3.37 |
| 4.45 | 8.54, 2.50, 4.20 |
| 4.20 | 6.24, 2.62, 1.80 |
| 3.87 | - |
| 3.66 | 9.45 |
| 3.62 | 3.17, 1.66, 9.45 |
| 3.56 | - |
| 3.37 | 8.54 |
| 3.17 | 9.32, 1.66 |
| 2.62 | 2.31 |
| 2.50 | 2.23 |
| 2.31 | 2.62 |
| 2.23 | 2.50 |
| 1.80 | 8.54 |
| 1.66 | 9.45 |
| 0.52 | -1.66 |

The NOESY data confirmed the possible structure of Compound IV as follow




The structure had two possible isomers corresponding to pheophytin a and a' ( (c) and (d) ) which could be differentiated again by NOESY data. $^{1}H$-$^{1}H$ NOESY position of two form was not different except the methine protons at 4.20 and 6.24 ppm correlated to each other which indicated that they were on the same side of the ring.

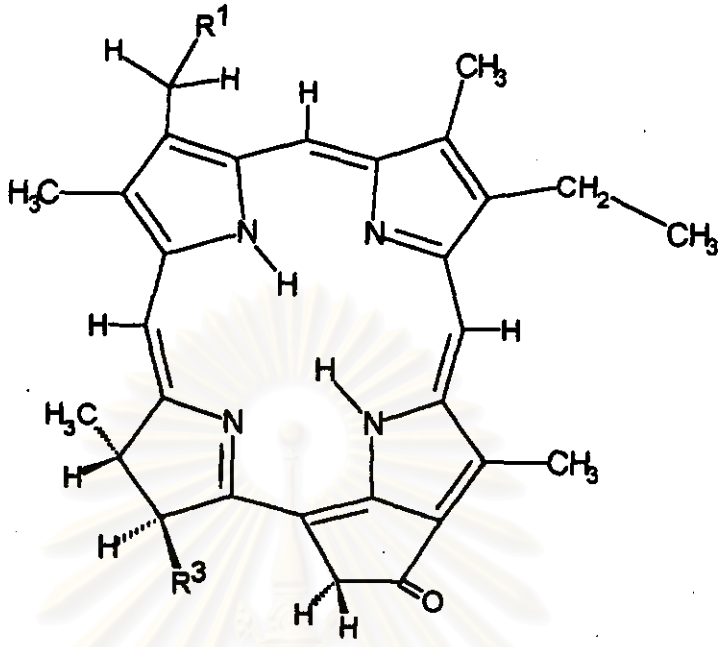
The spectrum data and literature comparison are consistent with Compound IV being methyl 10-epipheophorbide-a (d) which was previously discovered in fresh spinach leaves [23] .



methyl 10-epipheophorbide-a

Literature surveys on biological activity of this type of molecule revealed that a series of long wavelength absorbing photosensitizers related to pheophorbide-a, pyropheophorbide (e) and bacteriopheophorbide-a are useful in cancer diagnosis and therapy, expecially in photodynamic therapy [24,25] .

The pyropheophorbide derivatives are accumulated in tumor tissue to a higher degree than surrounding normal tissue , when injected into a host. When the pyropheophorbides are exposed to a particular wavelength of light, they become cytotoxic and destroy the tumor or diseased tissue without causing reversible normal tissue damage [26] .




(e) pyropheophorbide : $R^1 = CH_2OR_2$

$R^2$ = primary or secondary alkyl containing 1 to 20 carbons

$R^3 = CO_2R_4$

$R^4$ = H or alkyl containing 1 to 20 carbons

### 3.5 Structural elucidation of Compound V

Compound V was a yellow amorphous solid with melting point of 264-266 °C. Its IR spectrum (Figure 29) indicated absorption band of hydroxyl group at 3500-3200 $cm^{-1}$ and absorption of C=O stretching vibration of conjugated ketone at 1660 $cm^{-1}$ . The C=C stretching vibration of aromatic at 1618, 1500 $cm^{-1}$ and other IR absorption bands were shown in Table 3.12 .

**Table 3.12** The IR absorption band assignments of Compound V

| Frequency | Band type | Assignments |
|---|---|---|
| 3432 | strong , broad | O-H stretching vibration of R-OH |
| 3030 | weak | C-H stretching vibration of aromatic |
| 1656 | medium | C=O stretching vibration |
| 1616, 1571, 1508 | medium | C=C stretching vibration of aromatic |
| 1178 | medium | C-O stretching vibration and O-H bending |

The $^{1}$H-NMR spectrum ( Figure 30 ) [27] showed a characteristic pattern of para-substituted aromatic system at δ 8.11 (d, $J$ = 9.0 Hz) , 7.02 (d, $J$ = 9.0 Hz) ppm ($A_2B_2$ system), meta aromatic protons at δ6.24(d, $J$= 2.0 Hz), 6.51(d,$J$= 2.0Hz) ppm and hydroxyl protons at δ 9.13 and 9.15 ppm .

The $^{13}$C-NMR spectrum (Figure 31) showed 13 signals . DEPT 135 and 90 NMR spectrum (Figure 32) revealed four aromatic methine carbons at 130.4 (2C), 116.2 (2C), 99.1 and 94.4 ppm .

The remaining signals were due to quaternary carbons resonating at 104.1, 123.2, 136.6, 146.9, 157.7, 160.1, 162.2, 164.9 and 176.5 ppm . The most down field signal at 176.5 ppm was due to a carbonyl group, probably a conjugated one.

The EI mass spectrum ( Figure 33 ) showed molecular ion peak ($M^+$) at 286 and the other major fragmentation at *m/z* 257, 229, 213 , 153, 121, 93 and 69 respectively. The fragmentation pattern of Compound V very closely matched the fragmentation pattern of kaempferol from library search (NIST database). The molecular ion peak ($M^+$) at *m/z* 286 was consistent with a molecular weight of $C_{15}H_{10}O_5$ . Comparison of $^{13}$C-NMR spectra Compound V and kaemferol was carried out in order to confirm the structure [28] . The results were shown in Table 3.13 .

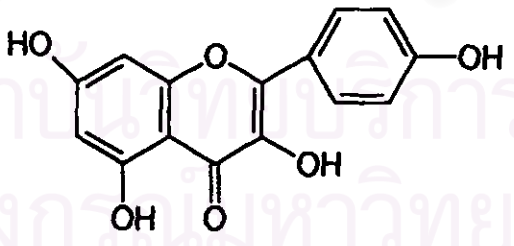
**Table 3.13** $^{13}$C-NMR spectal data of Compound V compared with kaempferol

| Position | Chemical shift ( ppm) | |
|---|---|---|
| | Kaempferol | Compound V |
| C-2 | 146.8 | 146.9 |
| C-3 | 135.6 | 136.6 |
| C-4 | 175.9 | 176.5 |
| C-5 | 156.2 | 157.7 |
| C-6 | 98.2 | 99.1 |
| C-7 | 163.9 | 164.9 |
| C-8 | 93.5 | 94.4 |

**Table 3.13** (continued)

| Position | Chemical shift (ppm) | |
|---|---|---|
| | Kaempferol | Compound V |
| C-9 | 160.7 | 162.2 |
| C-10 | 103.1 | 104.1 |
| C-1' | 121.7 | 123.2 |
| C-2' | 129.5 | 130.4 |
| C-3' | 115.4 | 116.2 |
| C-4' | 159.2 | 160.1 |
| C-5' | 115.4 | 116.2 |
| C-6' | 129.5 | 130.4 |

All of these spectroscopic data and literature comparison suggested that Compound V was kaempferol ( 3,4',5,7-tatrahydroxy flavone) .




Kaempferol

Kaempferol was reported to possess anti-enzyme cyclic AMP-dependent protein kinase of mice's liver ($IC_{50}$ = 1 μM) and anti-enzyme myosin light chain kinase of chicken ($IC_{50}$ = 4 μM) activities [29,30] . More recently , the anti-mutagenic effects of kaempferol at concentration 300 μg/plate on the mutagenicity of $AFB_1$ in *Samonella typhimurium* TA 100 showed inhibition 70% [31] . Moreover, the kaempferol was discovered to possess other activities such as cancer-preventive, cholertic, diuretic, mutagenic, natriuretic [32] .

### 3.6 Structural elucidation of Compound VI

Compound VI was white needle-like crytals with melting point at 237-238 °C (dec). Its IR spectrum ( Figure 34 ) revealed broad absorption band of hydroxyl group at 3496-2538 $cm^{-1}$, C=O stretching vibration of conjugated C=O of carboxylic acid at 1651$cm^{-1}$ and C=C stretching vibration of aromatic at 1615, 1542 $cm^{-1}$. The IR absorption band assignments of Compound VI were shown in Table 3.14 .

**Table 3.14** IR absorption band assignments of Compound VI

| Frequency ($cm^{-1}$) | Band type | Assignments |
|---|---|---|
| 3496-2538 | strong, broad | O-H stretching vibration of R-OH and COOH |
| 3013 | strong | C-H stretching vibration of aromatic |
| 1651 | strong | C=O stretching vibration |
| 1613, 1542 | strong | C=C stretching vibration of aromatic |
| 1320 | medium | C-O stretching vibration of COOH |
| 1221 | strong | C-O stretching vibration of R-OH |

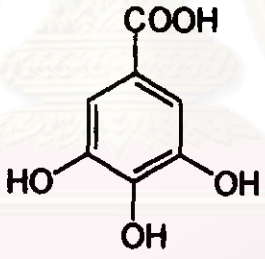
The $^{1}$H-NMR spectrum (Figure 35) showed only one signal of aromatic protons at δ 7.1 ppm and hydroxyl proton at δ 8.25 ppm .

The $^{13}$C-NMR spectrum (Figure 36) revealed five signals of carbon at 167.90, 145.93, 138.63, 121.92 and 110.03 ppm . DEPT 135 and 90 (Figure 37) showed one signal of methine $sp^2$ carbons (=CH) at 110.03 ppm which indicated that they are symmetric methine carbon of an aromatic ring.

The others were quternary carbons at 167.90, 145.93 (2C), 138.63 and 121.92 ppm . The most down field signal at 167.90 ppm was assigned to a conjugated carbonyl group .

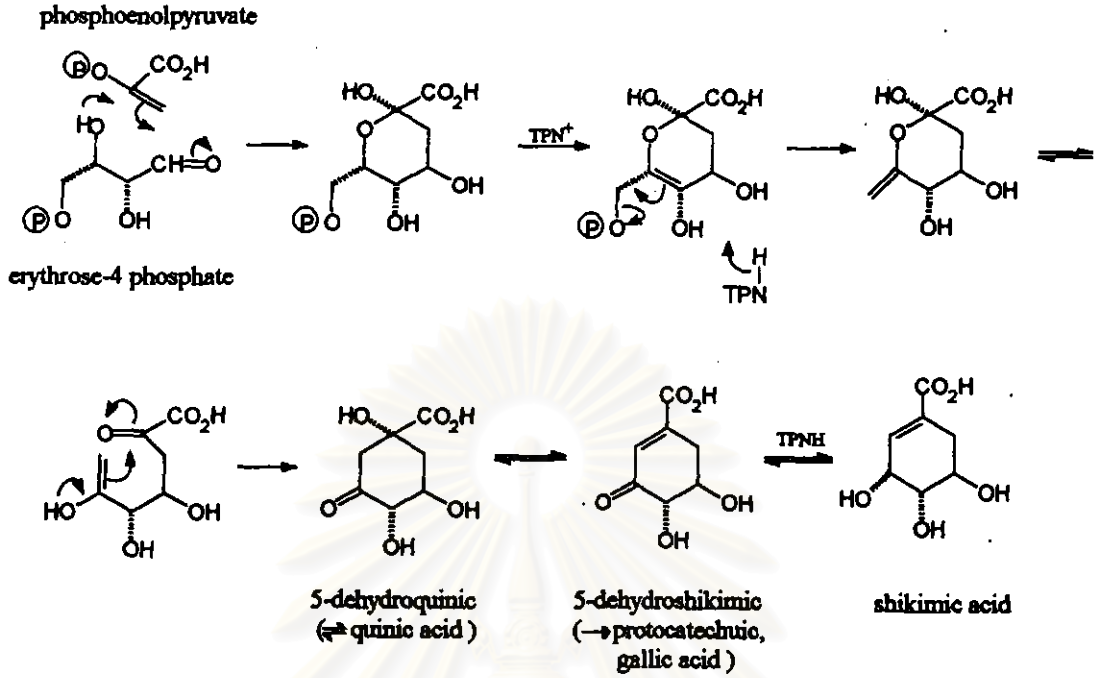
The EI mass spectrum ( Figure 38 ) indicated moleclar peak ($M^+$) at *m/e* 170 and other fragments at 153, 126, 108, 97 , 80 and 52 . From comparison of the fragmentation pattern with library compounds (NIST database) , the fragmentation pattern of Compound VI was found to be similar to 3,4,5-trihydroxybenzoic acid which had a molecular weight of 170 corresponding to a molecular formula of $C_7H_6O_5$.

The above results indicated that Compound VI was 3,4,5-trihydroxybenzoic acid (gallic acid).




gallic acid

Shikimic acid was found in root and stem of this plant since it is reasonable to propose that both compounds derive from the same biological pathway as shown in Scheme 3.1 [33] .




**Scheme 3.1** Biological pathway of gallic acid and shikimic acid

Gallic acid was formerly used as astringent and styptic, and has been used as intestinal astringent [34] .

### 3.7 Structural elucidation of Compound VII

Compound VII was obtained as white needle like crytal with melting point at 239-240°C. Its IR spectrum ( Figure 39 ) indicated the presence of hydroxyl group (O-H stretching vibration peak at 3650-3069 $cm^{-1}$ and C-O stretching vibration peak at 1095 $cm^{-1}$ ). The IR absorption bands were assigned as shown in Table 3.15 .

**Table 3.15** IR absorption bands assignments of Compound VII

| Frequency ($cm^{-1}$) | Band type | Assignments |
|---|---|---|
| 3450 | strong , broad | O-H stretching vibration of R-OH |
| 2966 | medium | C-H stretching vibration of aliphatic |
| 1135 | medium | C-O stretching vibration |

The $^{1}$H-NMR spectrum of Compound VII ( Figure 40 ) showed three signals of hydroxylic protons at δ4.27- 4.61 ppm and three signals of C-H group at δ3.27-3.63 ppm .

The $^{13}$C-NMR spectrum ( Figure 41 ) showed signals of carbon at δ73.10, 72.15, 70.68 ppm which revealed that they were attached to hydroxyl groups. The DEPT 135 and 90 experiments ( Figure 42 ) indicated the three peaks belong to three $sp^3$ methine carbons .

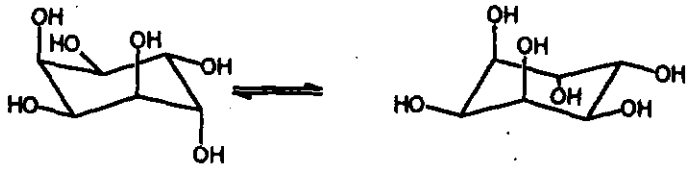
The EI mass spectrum ( Figure 43 ) showed extensive fragmentation and the molecular ion was not observed. However, the fragmentation pattern in the mass spectrum of Compound VII was found to be similar to fragmentation patterns of inositols ($C_6H_{12}O_6$) from library search NIST database and from literature[35].

There are nine possible sterioisomeric inositols appeared in the literature [36,37]. The presence of only three $^{13}C$ signals matches only with *allo*- and *chiro*-inositol as presented in Table 3.16 .

**Table 3.16** $^{13}C$-NMR spectra of the inositols compared with compound VII

| sterioisomeric inositols | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| *scyllo*-Inositol (135/246) | 74.7 | 74.7 | 74.7 | 74.7 | 74.7 | 74.7 |
| *myo*-Inositol (1235/46) | 72.1 | 73.2 | 72.1 | 73.4 | 75.3 | 73.4 |
| *epi*-Inositol (12345/6) | 72.6 | 72.6 | 76.5 | 72.6 | 72.6 | 71.2 |
| *neo*-Inositol 123/456) | 70.3 | 72.6 | 70.3 | 70.3 | 72.6 | 70.3 |
| *muco*-Inositol (1245/36) | 73.2 | 73.2 | 71.0 | 73.2 | 73.2 | 71.0 |
| *chiro*-Inositol (125/346) | 72.6 | 71.5 | 73.8 | 73.8 | 71.5 | 72.6 |
| *allo*-Inositol[(a)] (1234/56) | 72.7 | 71.1 | 71.1 | 72.7 | 70.5 | 70.5 |
| *allo*-Inositol[(b)] (1a) | 75.3 | 66.6 | 75.6 | 70.4 | 68.0 | 72.8 |
| *cis*-Inositol[(c)] (123456/0) | 68.9 | 74.5 | 68.9 | 74.5 | 68.9 | 74.5 |
| Compound VII | 72.2 | 70.7 | 73.1 | 73.1 | 70.7 | 72.2 |

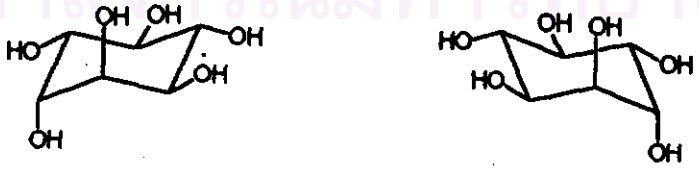
[(a)] At 95 °C , [(b)] At -20 °C , [(c)] At 2 °C



(1a) (1b)

The $^{13}C$ NMR spectra of Compound VII was compared well with both *allo-* and *chiro*-inositol . However, there was evidence that interconversion of the two chair from (1a and 1b) of allo-inositol was slow at room temperature and thus the spectrum of allo-inositol was indistinct, indicating in complex averaging ; the temperature had to be incresed to 95 °C in order to obtain a spectrum of three sharp lines . At -20 °C , the spectrum of separate chair forms was obtained as six sharp lines . On the other hand, the spectrum of *chiro*-inositol exhibited three lines at ambient temperature which is in accord with Compound VII . Furthermore , the melting point of Compond VII (239-240 °C) apporached to chiro-inositol (246 °C) .

From all these results, the Compound VII appeared to be *chiro*-inositol . however, the exact stereochemistry (D or L) could not be determined as there was not enough material to measure the optical rotation .



D-*chiro*-inositol L-*chiro*-inositol

D-*chiro*-inositol was found in various plants as its monomethyl ether, pinitol, which was a constituent of the exudate of *Pinus lambertiance* Dougl. and *Ceratonia siliqua* Linn. Moreover, it has been discovered in the heartwood (*Sequoia sempervirens*) and in two varieties of the loco weed , *Astragalus earlei* and *Oxytropis lamberlii* . The L-*chiro*-inositol also occurs as a monomethyl ether which was presented in Euphorbiaceae and Compositae [38] .

Literature surveys indicated that the chiro-inositol is an essential element for the synthesis of an insulin-directed mediator apparently responsible for the activation of pyruvate dehydrogenase-phosphatase. Disease conditions commonly associated with insulin-resistance, such as hypertention, lactic acidosis, obesity, coronary artery disease and the like, are treated by administration of sufficient *chiro*-inositol to meet normal metabolic levels [39] .

### 3.8 Structural elucidation of Compound VIII

Compound VIII was white crystals with melting point above 300 °C. It was soluble in water and insoluble in organic solvents which indicated that the Compound VIII was inorganic salt. When the compound reacted with sodium cobaltinitrite ($Na_3[Co(NO_2)_6]$) a yellow precipitate of potassium cobaltinitrite, insoluble in dilute acetic acid, as shown in the equation below [40].

$$Na_3[Co(NO_2)_6]_{(aq)} + 3K^+_{(aq)} \longrightarrow K_3[Co(NO_2)_6]_{(s)} + 3Na^+_{(aq)}$$

Purple-red color from flame test further confirm that the Compound VIII had $K^+$ as its cation. Reaction of Compound VIII with silver nitrate solution ($AgNO_3$) gave white precipitate of silver chloride (AgCl) which was soluble in dilute ammonia solution and precipitate back on addition of dilute nitric acid. These indicated that Compound VIII had $Cl^-$ as its anion as shown in the equation below.

$$Ag^+_{(aq)} + Cl^-_{(aq)} \longrightarrow AgCl_{(s)}$$

$$AgCl_{(s)} + 2NH_{3\,(aq)} \longrightarrow [Ag(NH_3)_2]^+_{(aq)} + Cl^-_{(aq)}$$

$$[Ag(NH_3)_2]^+_{(aq)} + Cl^-_{(aq)} + 2H^+_{(aq)} \longrightarrow AgCl_{(s)} + 2NH_4^+{}_{(aq)}$$

From chemical analysis, the Compound VIII was KCl .