CHAPTER IV

RESULTS AND DISCUSSION

The preliminary screening for bioactivity of the dichloromethane extract from the Thai sponge, *Biemna fortis* (Topsent), presented significant cytotoxicity against P-388, A-549 and HT-29 tumor cell lines (ED_{50}= 2.5 μg/ml). The initial fractionation of the extract was guided by chemical constituents detected by anisaldehyde sulfuric acid reagent and UV light at 254 and 365 nm on silica gel TLC to yield 3 steroid compounds (K057, K068, and K084). The cytotoxicity was used to guede the isolation of bioactive compounds from the interesting cytotoxic fractions. Finally, ^{1}H-NMR spectroscopy was very helpful to predict and monitor further isolation to give mixture of bioactive compounds F201.

THE ISOLATION AND STRUCTURE ELUCIDATION OF BIOACTIVE COMPOUNDS (F201)

The preliminary bioactivity screenings by means of brine shrimp lethality test, antimicrobial activity test, sea urchin egg lethality test, cytotoxicity test against P-388, A-549, HT-29 tumor cell lines were used to examine bioactivity of the dichloromethane extract (F042), the hexane extract (F044), the methanol extract (F045) and the butanol extract (F046). Only cytotoxicity against tumor cell lines were found in F042, F045 and F046 as listed in Table 9. F042 also inhibited cell division of fertilized sea urchin eggs, this method was not chosen to monitor the isolation because of the difficulty in collecting sea urchins.

Table 9. Cytotoxicity of the fractions from *Biemna fortis* (Topsent)

Fraction No.	Cytotoxicity (IC_{50}; μg/ml)		
	P-388	A-549	HT-29
F042*	2.5	2.5	2.5
F044	5	5	5
F045*	0.5	0.5	0.5
F046	2	2	2
F047*	0.2	0.2	0.2
F048	2	2	2
F149	5	5	5
F050	>20	>20	>20
F051*	0.2	0.2	0.2
F052	2	2	2
F053	2.5	2.5	2.5
F054	2	2	2
F055	20	>20	>20
F056	5	5	5
F057	2.5	2.5	2.5
F058	5	5	5
F059	10	10	10
F060	>20	>20	>20
F139	2	2	2
F140	2	0.5	0.5
F141	2.5	2.5	2.5
F142	2.5	2.5	2.5
F149	10	2	2
F150*	0.1	0.1	0.1
F151	1	0.25	0.25
F167	>10	>10	>10
F168	2	2	2
F169*	0.25	0.25	0.25
F170	1	1	1
F171	5	5	5
F183	>20	>20	>20
F184	5	5	5
F185	2.5	2.5	2.5
F186	2.5	2.5	2.5
F187*	0.5	0.1	0.1
F188	20	20	20
F190*	0.2	0.02	0.02

-The asterisk (*) represents interesting bioactive value.

Table 10. Antiviral (HIV-I) activity of the fractions from *Biemna fortis* (Topsent)

Sample No.	Antiviral Activity		
	Concentration (ml)	CEM Cells (% cytotoxicity)	Antiviral (% inhibition)
F150	1.6	100	0
	1	0	0
	0.5	0	0
	0.25	0	33
	0.16	0	0
	0.125	0	0
	0.016	0	42
F169	18	98	
	1.8	3	30
	0.18	10	0
	0.018	1	28

The cytotoxicity against tumor cell lines of the dichloromethane extract (F042) showed IC_{50} of 2.5 μg/ml and further distributed to the methanol extract (F045, IC_{50} 0.5 μg/ml). Sephadex LH-20 column chromatography using hexane, chloroform and methanol as mobile phases were applied to isolate bioactive constituents from the methanol extract to yield 4 fractions, F047 - F050. It was found that the bioactivity was presented in the earlier fractions. This investigation indicated the large molecules property of the bioactive components.

Two portions of F048 were separately fractionated by means of both SiO_2 (F055-F060) and sephadex LH20 column chromatographies (F051-F054). The fractions (F055-F060) from SiO_2 column did not shown any interesting cytotoxicity while the fractions from sephadex LH-20 (F051-F054) did as listed in Table 9. Thus, F051 which showed the highest cytotoxicity was then considered for further isolation by using sephadex LH20 column. Similarly, the cytotoxicity was further distributed to F169 by this isolation pathway. During the isolations of F169, the isolations were directed by ^{1}H-NMR instead of cytotoxicity. This technique was very useful because the spectral data could show interesting besides the ratio of constituents. In addition,

this non destructive NMR technique is very effective for analysing small amount of samples.

It was found that ^{1}H-NMR spectrum of F169 (Figure 2) showed interesting and distinguish peaks as discussed below:

1) at δ 7.46, δ 7.65 ppm and at δ 7.18, δ 7.22 ppm were proposed to be protons of aromatic compounds.

2) at δ 5.05 to 5.4 ppm indicated proton of sp^2 carbon.

3) at δ 4.1, δ 4.2 and δ 4.3 ppm with a splitting pattern showed protons of carbons that connecting to the heteroatom.

4) at δ 3.62 ppm also indicated protons of carbons which connected to the heteroatom.

5) the large group of protons of sp^3 carbons.

We wished to isolate bioactive compounds by following interesting peaks in 1) - 4). Fortunately, the cytotoxicity of F169 exhibited the most interesting value (IC_{50} = 0.25 μg/ml) among F167 - F171 group and antiviral HIV-I activity as shown in Table 10. F169 was then isolated to yield F183 - F188. It was considered that the expected interesting peaks at δ 7.46, 7.65 ppm were also presented in F183 and F184 (Figure 3, 4). However, these fractions had no interesting cytotoxicity. It was then intended to analyze the other fractions by comparison the ^{1}H-NMR pattern of each fraction with its cytotoxicity. It was found that the more increasing cytotoxicity, the more intensity of peaks at δ 7.18 and δ 7.22 ppm in contrast to the δ 7.46 and δ 7.65 ppm. Furthermore, F187 which exhibited the most cytotoxicity showed not only the intense peaks at δ 7.18 and δ 7.22 ppm of aromatic protons but also δ 6.8 ppm, δ 4.12 (t), δ 3.82 (t) and δ 3.45 - 3.75 ppm (broad and large peaks) as shown in Figure 5. Up to this step, each fraction had a small amount left, therefore, the further isolation and detection should be done carefully. F187 was further purified by several sephadex LH-20 columns with different polarity of the solvent mixtures to concentrate the bioactive

compounds. Finally, F209 was obtained by RP-2 TLC using 20% H_2O in MeOH as the developing solvent. In order to increase the amount of the bioactive components, the remaining F045 was fractionated using the similar process as described above.

It was found that F150 from the first column chromatography of F045 showed not only cytotoxicity, but also significantly antiviral activity (HIV-I) as shown in Table 10. F150 was repeatedly purified by sephadex LH-20 columns and RP-2 TLC to eliminate some impurities and yield F201. F201 was obtained as pale yellow syrupy mass from F190 by RP-2 TLC with 20% H_2O in MeOH to yield 3.4 mg (2.19×10^{-6} % based on wet weight of sponge). The ^{1}H-NMR of F201 (Figure 9) showed similar pattern to that of F209 (Figure 6), but cleaner. The mass spectral data indicated F201 a mixture of more than one compound which should be isomerism. In the last step, HPLC technique of reversed phase RP-8, RP-18 and phenylpropanolamine with various ratio of eluents were all applied to F201 to obtain A004 (Figure 15). However, these tecniques could not isolate isomerism compounds.

The chromatogram of FAB MS (Figure 10) indicated that F201 contained at least 3 main components. The first component had retention time at 3.0 min with scan number 70 to 79, the second component had retention time at 4.4 min with scan number 103 to 115, and the third component had retention time at 6.1 min with scan number 141 to 171. The FAB MS of each components (Figure 11 - 13) exhibited the highest *m/e* peak at 658.3 (4%), 613 (2%) and 656.3 (10%), respectively. Because F201 was a mixture of similar components and also had small amount, the structure elucidation could not be completed.

The 500 MHz ^{1}H-NMR spectrum of F201 (Figure 9) showed signals of either aromatic or olifinic protons at δ 7.1 - 7.25 ppm, the signals of oxymethylene protons between δ 3.6 - 4.2 ppm and up field signals below d 1.8 ppm.

The ^{13}C-NMR (Figure 16) and DEPT 135° (Figure 17) spectra exhibited some carbon signals, sp^2 quaternary carbons of 6 signals resonated at δ 163.2, 159.6, 156.4, 156.3, 142.9, 142.3 ppm. sp^2 methine carbon of 12 signals resonated at δ 127.6, 127.5, 127.4, 127.0, 126.9, 126.7, 114.0, 113.9, 113.88, 113.8, 113.6 ppm. A large number of oxygenated methylene carbons resonated at δ 72.6, 70.8, 70.6, 70.5, 70.3, 69.8, 67.4, 67.3, 67.2, 63.5, 61.7 ppm. Particularly, the broad large signal resonated at δ 70.6 and 70.5 ppm indicating a large number of methylene carbons.

In HMQC experiment (Figure 21, 22) which provided information on correlation between directly bonded of proton and carbon, the group of protons (c) at δ 6.82 - 6.85 ppm showed correlation with olefinic carbon (C) at δ 113.9 ppm. Moreover, triplet protons (d) at δ 4.1 ppm correlated with methylene carbon (D) at δ 67.2 ppm and triplet protons (e) at δ 3.8 ppm correlated with methylene carbon (E) at δ 69.8 ppm. A large peaks (f, g, h, j) at δ 3.6 ppm correlated with a large methylene carbon (F, G, H, J) at δ 70.5 ppm.

DQF long range experiment (Figure 18, 19) and HOHAHA experiment (Figure 20) indicated correlation of cross peak between two triplet of (d) δ 4.1 ppm and (e) δ 3.8 ppm. It was suggested the presence of -O-CH_2-CH_2-O- fragment in the molecule. The peak at low field (d) δ 4.1 ppm was proposed to connect to aromatic or olifinic group therefore these protons resonated at down field. This was confirmed by calculated chemical shift to have approximate value of 4 ppm and aromatic carbon, connected to heteroatom, were also calculated to have approximate value of 159 ppm. Apart form the large methylene protons which had the center at δ 3.65 ppm should represent the repeat units -[-O-CH_2-CH_2-O-]-$_n$. The presence of this unit was confirmed by 44 amu. mass difference of some fragment ions in FAB MS spectrum.

According to ^{1}H-NMR, ^{13}C-NMR, 2D-NMR and FAB MS experiments exhibited interesting peaks in a range of carbinol methylene groups. The structure of F201 was proposed to have symmetric carbons and protons of polyether.

The aromatic or olefinic part which contained protons (a) at δ 7.23 ppm, (b) δ 7.16 ppm and (c) δ 6.82 - 6.85 ppm were exhibited correlation to each others in decoupling experimets (Figure 23, 24). Moreover, the member of this part should be suggested to be *para* - benzene substituted fragment based on signal at 801 cm^{-1} in IR specturm (Figure 25). The UV spectral data (Figure 26) also displayed λmax at 276 nm.to indicated characteristic of aromatic which connected to auxochromic groups to show longer wavelength than regular aromatic (λmax 255 nm).

The proposed partial structures were proposed by analysis from a combination of all physical methods' data in this work. However, these data were insufficient for complete analysis include the limit of small amount of sample F201. The discussion was wished to benefit for further investigation of these compounds which had interesting cytotoxicity. They were also interested in being in doubt of their symmetry and structure in term of large molecule.

STRUCTURE ELUCIDATION OF K057

The present work had led to the isolation of a crystalline steroid K057 which was identified as 24 methylcholesta-7,22-diene-3,5,6-triol or ergosta-7,22-diene-3,5,6-triol or 3β,5α,6β,22*E*,24*R*- form of Cerevisterol (Hill *et al.*,1991). Information of this compound such as IR, UV, MS, ^{13}C NMR (in pyridine-d_6) and some proton assignments (^{1}H NMR in chloroform-d) had already been published. Although the compound had been studied, this work was assigned for the further study to complete carbon and proton assignments by means of ^{13}C NMR, ^{1}H NMR and 2D-NMR such as HMBC, HSQC, HOHAHA, NOESY, PDQFH and DEPT 135° in 30% methanol-d_4 in chloroform-d. The compared ^{13}C-NMR and ^{1}H-NMR data in term of chemical shifts between published data and data in present work were shown in Table 17.

Compound K057 was obtained in a form of white needle crystal from the methanol extract (F045), as shown in Scheme I, by using sephadex LH-20 column with chloroform as eluent (2 times) and finally using successive recrystallization technique in methanol to yield 20.9 mg (1.3×10^{-5} % based on wet weight of the sponge). The way to confirm this structure required a combination of several methods ; IR, UV, acetylation reaction, EIMS, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR and decoupling experiments. Details of each method were discussed as follows:

The IR spectrum of K057 (Figure 27) was used to confirm functional groups, such as hydroxyl groups, cyclic alcohols and sp^2 carbon in molecule. The ranges of absorption to identify each group were shown in Table 11.

Table 11. The IR spectrum assignment of K057

Range of absorption (cm^{-1})	Intensity	Assignment
3442	broad	hydrogen bonded OH stretch
2990, 2850	high	$-CH_3$, $-CH_2$ stretch
1052	medium	cyclic alcohol C-O stretch
990	medium	=CH out of-plane bending

The UV spectrum (Figure 28) showed absorption bands at λ_{max} 252 nm (ε_{max} 2211.4).

An acetylated derivative of K057 was prepared to confirm the presence of hydroxyl groups in the structure. Complete reaction was detected, at 11 hrs after added acetic anhydried, by TLC technique with 10% MeOH in $CHCl_3$. It was found that the acetylated compound exhibited Rf value of 0.8 instead of the initial sample value of 0.1. Both spots were positive to anisaldehyde in sulfuric acid reagent as violet color spots. Preparative RP-2 TLC technique was then applied to isolate the acetylated compound from the remaining pyridine. The pure acetylated compound of K057 was analyzed by ^{1}H-NMR technique. The ^{1}H-NMR spectrum of the acetylated K057 compound (Figure 29) exhibited peaks of two methyl groups of the acetyl functional group at δ 2.1 and 2.05 ppm. This suggested the occurrence of two secondary hydroxyl groups.

The EIMS spectrum of K057 (Figure 30) did not show the molecular ion peak, instead the peak of *m/e* 412 (2%) indicating the loss of H_2O from the molecule (M^+ - H_2O). Furthermore, 28 carbon peaks as shown in ^{13}C-NMR spectrum confirmed this compound to be a steroid compound in which signals in the region for 4 sp^2 carbons, 21 sp^3 carbons and 3 methoxy carbons were shown Thus, it established the tentative molecular formula of $C_{28}H_{46}O_3$.

The compound K057 was dissolved in 30% CD_3OD in $CDCl_3$ using TMS as internal standard for NMR measurement. The ^{1}H-NMR spectrum of K057 showed characteristic peaks of steroid or triterpenoid compound. The spectrum showed 43 signals for 3 olefinic protons, 2 carbinol methine protons, 20 methine and methylene protons, and 18 methyl protons. The analysis of its ^{1}H-NMR spectra will be discussed in the following paragraphs.

Total number of protons in this molecule was 46 protons. Here, the ^{1}H NMR spectrum (Figure 31) showed signals for only 43 protons. Other disappeared three protons should be hydroxyl protons which were exchanged in $CDCl_3$ solvent. Within 43 protons, there were three signals of olefinic protons of K057. One of them, the lowest field signal, was H-7 as a ddd signal at δ 5.31 ppm (J = 2.0, 2.2, 5.2 Hz). The coupling constant of 5.2 Hz was the value of coupling with adjacent proton. The two smaller coupling constants (2.0, 2.2 Hz) could be long-range couplings via π-bond with H-9 and H-14 (See expansion ^{1}H-NMR spectrum Figure 32, 33). The remaining two dd olefinic protons were the signals of H-22 and H-23 at δ 5.23 ppm (J = 7.4, 15.3 Hz) and δ 5.17 ppm (J = 7.4, 15.3 Hz), respectively. The large coupling constant of 15.3 Hz indicated that these olefinic protons were *trans* conformation.

One of two carbinol methine protons at δ 3.9 ppm (tt, J = 5.1, 11.2 Hz) was assigned as 3-OCH which was the results of axial-axial coupling with large coupling constant of H_{ax}-3 to H_{ax}-2, H_{ax}-4 and axial-equatorial coupling with smaller coupling constant of H_{ax}-3 to H_{eq}-2 and H_{eq}-4 (Figure 32). The other carbinol methine proton at δ 3.57 ppm (ddd, J = 2, 2, 5.2 Hz) was assigned as 6-OCH which was coupled to the adjacent olefinic proton at 7 position with 5.2 Hz coupling constant. Two smaller coupling constants could be the homoallylic coupling of H-7 and H-9, H-14. Signals for twenty methine and methylene protons showing in the range of δ 2.07 - δ 1.30 ppm were too crowded (overlap). In this case, 2D-NMR and decoupling experiments were used to analyze each proton, however, protons at δ 2.07, 1.67 and 1.86 ppm could be analyzed without applying the experiment although they were not separated signals. Signal at δ 2.07 ppm (H_{ax}-4) showing dd splitting pattern (J = 11.1, 13.3 Hz) indicated vicinal coupling with H-3 in term of axial-axial position with J = 11.1 Hz and geminal coupling with H_{eq}-4 with J = 13.3 Hz. Signal at δ 1.67 ppm (H_{eq}-4) also showed coupling patternas ddd which indicated geminal coupling (J = 13.3 Hz), axial-equatorial coupling with H_{ax}-3 (J = 5 Hz) and W-shape (4 bonds) coupling with H_{eq}-2 (J = 2.5 Hz). Proton signal at δ 1.86 ppm (H-24) with dq (J = 7.7 Hz) pattern was

received from vicinal coupling with H-23 to be doublet form and vicinal coupling with CH_3-28 to be quartet form.

For methyl groups analysis, two singlets of methyl protons which attached with quaternary carbons at δ 1.06 ppm and δ 0.61 ppm were respectively assigned as CH_3-19 and CH_3-18. Four remaining CH_3-21, CH_3-28, CH_3-26 and CH_3-27 were dd signals at δ 1.03, 0.92, 0.82 and 0.85 ppm, respectively with J = 6.7 Hz (Figure 32). This assignment of methyl groups by NMR did not allow discrimination between CH_3-26 and CH_3-27 and also could not be made from HMBC spectra, although it could assign each carbon of C-26 and C-27 with their methyl proton signals by HMBC spectra.

^{13}C-NMR spectra (Figure 34) were recorded with a JEOL A 500 spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University under the following condition: frequency 33.89 MHz, acquisition time 0.4833 sec, flip angle ca. 90° internal pulse lock to D in $CDCl_3$ (solvent) , temperature 28.9°C, concentration ca. 40 mg/ml. For off resonance decoupling, protons were irradiated at δ_H ca. 6.25 ppm ($\gamma H_2/2\pi$ ca.1800 or 3800 Hz).

The types of carbons and protons were achieved by the analysis of the phase sensitive ^{1}H-detected heteronuclear single quantum coherent (HSQC) (Figure 35, 36) which provided information on correlation's between directly bonded ^{1}H and ^{13}C. This experimental measurement directly observed ^{1}H signal, thus it could be done in a shorter time compared with ^{13}C-^{1}H COSY, in which ^{13}C was observed. DEPT 135° (Distortionless Nuclei Enhanced by Polarization Transfer) (Figure 37) experiment indicated -CH, -CH_3 as the up signals and -CH_2 as the down signal, but quaternary carbons were absent, as listed in Table 12.

Table 12. Assignments of protonated carbons based on the correlation of protons and carbons in the HSQC and DEPT 135° spectra

Position	δ C (ppm)	δ H (ppm)	Type of Carbon
1	32.95	1.6 H_{eq} (m) 1.52 H_{ax} (m)	$-CH_2$
2	30.6	1.83 H_{eq} (m) 1.43 H_{ax}(m)	$-CH_2$
3	67.45	3.9 (tt, J = 5.1, 11.2 Hz)	-OCH
4	39.1	2.07 H_{ax} (dd, J = 11.1, 13.3 Hz) 1.67 H_{eq} (ddd, J = 2.5, 5, 13.3 Hz)	$-CH_2$
5	76.12	-	-OC-
6	73.31	3.57 (ddd, J = 2, 2, 5.2 Hz)	-OCH
7	117.69	5.31 (ddd, J = 2,2.2,5.2 Hz)	=CH
8	143.52	-	=C-
9	43.35	1.99 (m)	-CH
10	37.2	-	-C-
11	22.14	1.57 (m)	$-CH_2$
12	39.48	1.34 (m)	$-CH_2$
13	43.83	-	-C-
14	56.12	1.92 (m)	-CH
15	23.08	1.58 H_{α} (m) 1.46 H_{β} (m)	$-CH_2$
16	28.12	1.75 H_{α} (m) 1.31 H_{β} (m)	$-CH_2$
17	65.20	1.3 (m)	-CH
18	12.35	0.61 (s)	$-CH_3$
19	18.41	1.06 (s)	$-CH_3$
20	40.59	2.05 (m)	-CH
21	21.21	1.03 (d, J = 6.7 Hz)	$-CH_3$
22	135.69	5.17 (dd, J = 7.6, 15.3 Hz)	=CH
23	132.27	5.23 (dd, J = 7.4, 15.3 Hz)	=CH
24	43.02	1.86 (dq, J = 7, 7 Hz)	-CH
25	33.26	1.48 (m)	-CH
26	20.02	0.85 (d, J = 6.7 Hz)	$-CH_3$
27	19.71	0.82 (d, J = 6.7 Hz)	$-CH_3$
28	17.69	0.92 (d, J = 7 Hz)	$-CH_2$

Table 13. Chemical shifts comparison of carbons and protons between K057 (30% CD_3OD in $CDCl_3$) and Cerevisterol (pyridine)

Position	δ ^{13}C-NMR (ppm)		δ ^{1}H-NMR (ppm)	
	K057	Cerevisterol	K057	Cerevisterol
1	32.95	32.6	1.6 H_{eq} (m) 1.52 H_{ax} (m)	-
2	30.6	33.9*	1.83 H_{eq} (m) 1.43 H_{ax}(m)	-
3	67.45	67.6	3.9 (tt, J = 5.1, 11.2 Hz)	4.08 (m)
4	39.1	41.9*	2.07 H_{ax}(dd, J = 11.1, 13.3 Hz) 1.67 H_{eq}(ddd, J = 2.5, 5, 13.3 Hz)	2.14 H_{ax}(dd, J = 12.8, 12.8Hz) 1.78 H_{eq}(dd, J = 4.9, 12.8 Hz)
5	76.12	76. 2	-	-
6	73.31	74.3	3.57 (ddd, J = 2, 2, 5.2 Hz)	3.63 (bs)
7	117.69	120.5*	5.31 (ddd, J = 2, 2.2, 5.2 Hz)	5.35 (bd, J = 4.9 Hz)
8	143.52	141.6*	-	-
9	43.35	43.8or43.2	1.99 (m)	-
10	37.2	38.1	-	-
11	22.14	22.5	1.57 (m)	-
12	39.48	40.0	1.34 (m)	-
13	43.83	43.8	-	-
14	56.12	55.3	1.92 (m)	-
15	23.08	23.5	1.58 H_{α} (m) 1.46 H_{β} (m)	-
16	28.12	28.5	1.75 H_{α} (m) 1.31 H_{β} (m)	- -
17	56.20	56.2	1.3 (m)	-
18	12.35	12.6	0.61 (s)	0.59 (s)
19	18.41	18.8	1.06 (s)	1.08 (s)
20	40.59	40.8	2.05 (m)	-
21	21.21	21.5	1.03 (d, J = 6.7 Hz)	1.04 (d, J = 6.9 Hz)
22	135.69	136.2	5.17 (dd, J = 7.6, 15.3 Hz)	5.15 (dd, J = 7.9, 14.8 Hz)
23	132.27	132.2	5.23 (dd, J = 7.4, 15.3 Hz)	5.20 (dd, J = 6.9, 14.8 Hz)
24	43.02	43.8 or 43.2	1.86 (dq, J = 7, 7 Hz)	-
25	33.26	33.4	1.48 (m)	-

Table 13. Chemical shifts comparison of carbons and protons between K057 (30% CD_3OD in $CDCl_3$) and Cerevisterol (pyridine), (continued)

Position	δ ^{13}C-NMR (ppm)		δ ^{1}H-NMR (ppm)	
	K057	Cerevisterol	K057	Cerevisterol
26	20.02	20.2 or 19.9	0.85 (d, J = 6.7 Hz)	0.85 or 0.82 (d, J = 6.9 Hz)
27	19.71	20.2 or 19.9	0.82 (d, J = 6.7 Hz)	0.85 or 0.82 (d, J = 6.9 Hz)
28	17.69	17.9	0.92 (d, J = 7 Hz)	0.91 (d, J = 6.9 Hz)

The partial assignment of structure was assigned by the analysis of three methods as follows :

-PDQFH (Phase Sensitive Double Quantum Filter ^{1}H-^{1}H COSY) (Figure 38, 39) provides the correlations between protons and protons through 1-bond with clearer and easier spectrum than ^{1}H-^{1}H COSY. In some case, the coupling constant can be observed from its positive and negative phase cross peak. Since all correlation peaks due to *J* scalar couplings are obtained as positive and negative pure absorption signals, analysis is not complicated when correlation peaks closely exist to diagonal peaks.

-HMBC (Heteronuclear multiple bond coherence spectra (Figure 40 - 41) provides the correlation between protons and carbons through long-range coupling It also be observed for quaternary carbons' assignment as well as the information from their chemical shifts as shown in Table 14.

-Decoupling experiment irradiated well separated proton signals. The coupled protons were influenced to show simpler pattern signals.

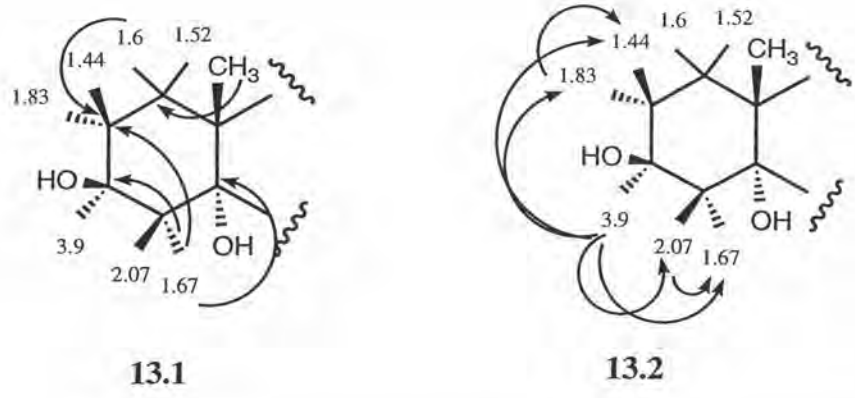
The carbinol methine proton 3-OCH at δ 3.9 ppm showed cross peak with protons at δ 2.07, δ 1.67, δ 1.83, δ 1.44 ppm and the cross peaks could be observed as larger for δ 1.44 and δ 2.07 ppm (H_{ax}-2 and H_{ax}-4, respectively). Thus, the smaller cross peaks of 2 protons at δ 1.83 ppm (H_{eq}-2) and δ 1.67 ppm (H_{eq}-4) were equatorial - axial conformation with 3-OCH. It was also shown further correlation of carbon at δ 30.6 ppm (C-2) with protons at δ 1.6 ppm (H-1) in the HMBC spectrum. As the result of these correlations, the partial structure of ring A could be achieved as shown in Figure IV.1). In case of confirming H_{eq}-2 position at δ 1.83 ppm, proton signal at δ 1.83 ppm was irradiated to change signals of H-3, H_2-1, H_{ax}-2 and H_{eq}-4 at δ 3.9, 1.52-1.6, 1.44 and 1.67 ppm, respectively, in decoupling experiment (Figure 47, 48).

Other partial structures achieved from data of PDQFH. Olefinic proton of H-7 (δ 5.31 ppm) showed very small cross peak to methine proton, H-14, (δ 1.92 ppm). It could be confirmed by HMBC in which C-7 (δ 117.69 ppm) correlated with H-6 (δ 3.57 ppm) and H-9 (δ 1.99 ppm) C-6 (δ 73.31 ppm) also showed correlation with H-4 (δ 1.67 ppm). The quaternary carbons as a junction between ring A and B could be analyzed by using HMBC spectrum. With this spectrum, C-5 (δ 76.12 ppm) showed correlation with H-7, H-19, H-6 and H-4 (δ 5.31, δ 1.06, δ 3.57, δ 1.67 ppm, respectively). Meanwhile, H-19, H_{eq}-4, H-9 and H-6 (δ 1.06, δ 1.67, δ 1.99, δ 3.57, respectively) were correlated by C-10 (δ 37.20 ppm). The quaternary carbon C-8 (δ 143.52 ppm), junction of ring B and C, correlated with H-6, H-9 and H-14 (δ 3.57, δ 1.99, δ 1.92, respectively). This achieved partial structure was shown in Figure IV.2.

In ring C and D of steroid nucleus, C-9 (δ 43.35 ppm) showed correlation with H-7 (δ 5.31 ppm), H-19 (δ 1.06 ppm), H-14 (δ 1.92 ppm) and H-12 (δ 1.34 ppm) in HMBC spectrum. δ 1.57 ppm (H-11) showed cross peak in PDQFH spectrum to H-9 (δ 1.99 ppm) and H-12 (δ 1.34 ppm). C-13, the junction of ring C and D, correlated with H_3-18 (δ 0.61 ppm) and H-16 (δ 1.31 ppm). For C-17 in ring D, which attached with side chain, it correlated to H-20 (δ 2.05 ppm), H_3-21 (δ 1.03 ppm) and H_3-18 (δ

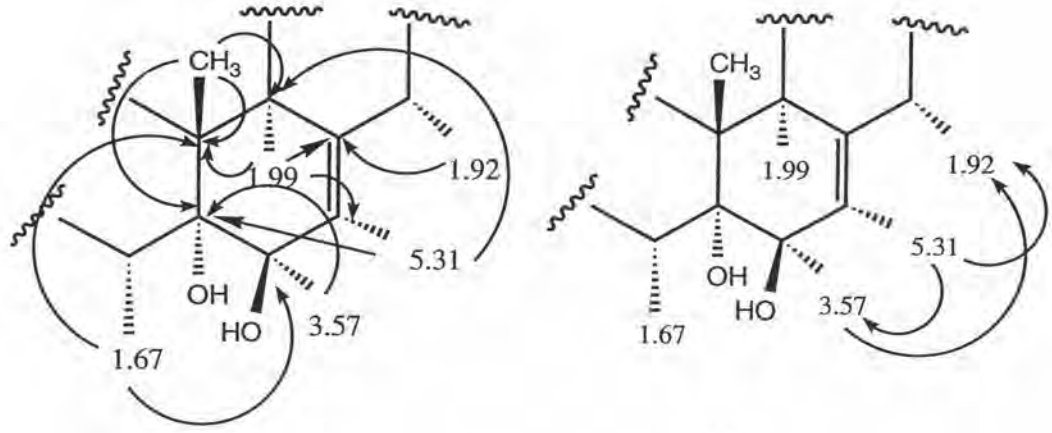
0.61 ppm). The information of decoupling experiment could confirm some proton assignments in ring D. Irradiation of the proton at δ 1.99 ppm (H-9). Protons at δ 1.57 ppm (H_2-11) changed to simpler pattern and long-range effect via π-bond where H-7 (δ 5.31 ppm) was influenced from ddd pattern to dd pattern (Figure 49). The protons at δ 1.57 ppm (H_2-11) was further irradiated to influence protons at δ 1.99 ppm (H-9) and δ 1.34 ppm (H_2-12) (Figure 50). In another side of this ring, irradiation at δ 1.92 ppm (H-14) exhibited influencing to δ 1.46, 1.58 ppm (H_2-15) and also H-7 (δ 5.31 ppm) to form dd pattern and so did irradiation at δ 1.99 ppm (Figure 51). Proton at δ 1.75 ppm was suggested to be assigned as H_α-16 from its correlation with H-17 (δ 1.31 ppm) and also influenced to protons at δ 1.46 ppm (H_α-15) (Figure 52)

Expansion of PDQFH spectrum exhibited the connection of H-20 (δ 2.05 ppm) to H_3-21 and to H-22 at δ 1.03 and δ 5.23 ppm, respectively. The H-23 (δ 5.17 ppm) correlated with H-24 (δ 1.86 ppm) which further correlated with H_3-28. This partial side chain path was confirmed and added by HMBC spectrum in which C-20 (δ 40.59 ppm) respectively correlated with H_3-21 and H-22 (δ 1.03, δ 5.17 ppm). C-23 further showed correlation with H_3-28, H-24 and H-25 at δ 0.92, δ 1.86 and δ 1.48 ppm, respectively, which confirmed that the methyl group attached to C-24 of side chain. In the terminal of side chain, C-25 showed correlation to 26 and 27 methyl protons at δ 0.82, δ 0.85 ppm. This side chain was also confirmed by decoupling experiments that proton at δ 1.48 ppm (H-25), δ 5.17 ppm (H-23) and δ 0.92 ppm (H_3-28). They were influenced by irradiate at δ 1.86 ppm (H-24) (Figure 53). This terminal of side chain was also confirmed by irradiation at H-27 (δ 0.85 ppm) (Figure 54), the proton H-25 signal was changed. The partial structure of this side chain was shown in Figure IV.3. All of these fragments were linked together to provide a structure as shown in Figure IV.4.



13.1 **13.2**

Figure IV.1 Partial structure of ring A in compound K057



14.1 **14.2**

Figure IV.2 Partial structure of ring B in compound K057

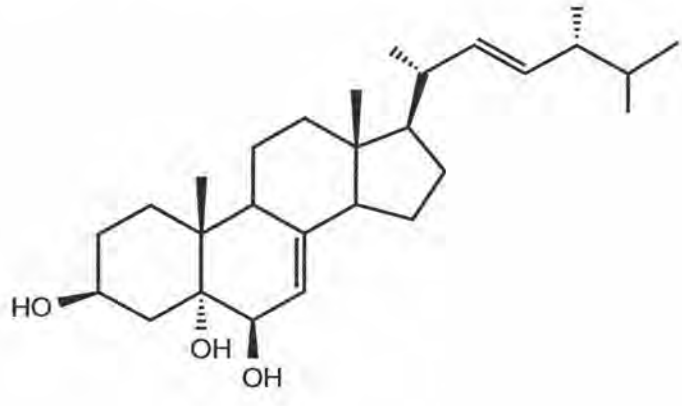
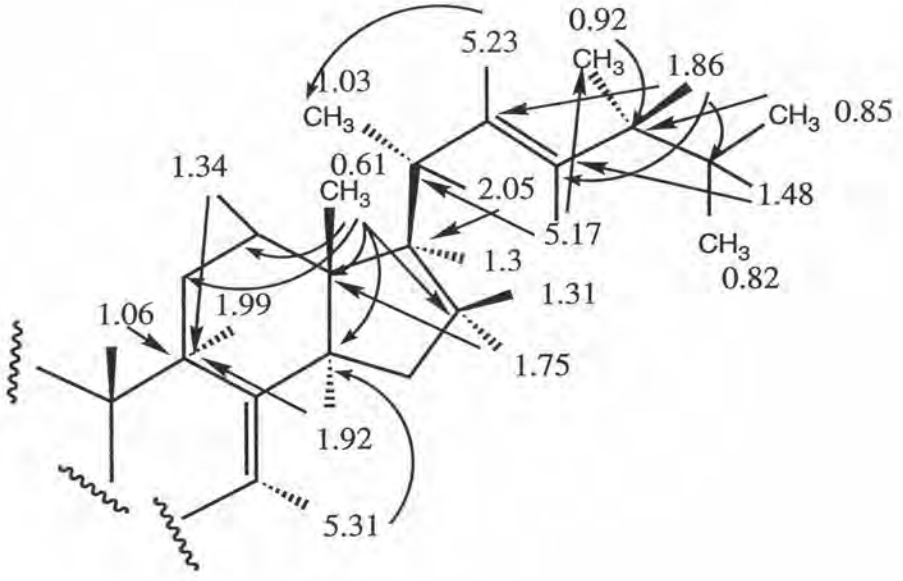
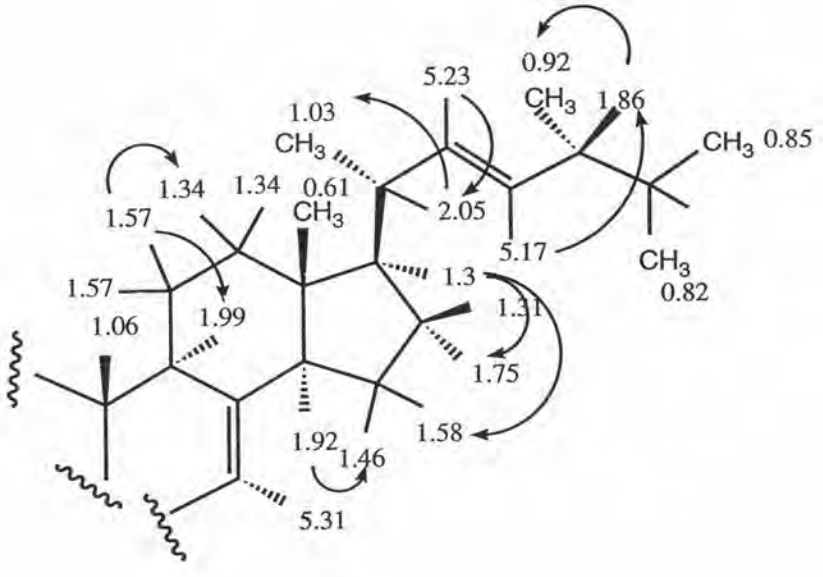


Figure IV.4 Structure of compound K057



15.1



15.2

Figure IV.3 Partial structure of ring C, D and side chain in compound K057

Table 14. Assignments of protons and carbons based on correlation between protons-protons in PDQFH and protons-carbon in HMBC.

Position	δ C (ppm)	δ H (ppm)	PDQFH	HMBC
1	32.95	1.6 H_{eq} 1.52 H_{ax}	-	H-19
2	30.6	1.83 H_{eq} 1.43 H_{ax}	H_{ax}-2	H-1, H_{eq}-4, H_{ax}-4
3	67.45	3.9	H_{eq}-2, H_{ax}-2, H_{eq}-4, H_{ax}-4	H_{eq}-4, H_{ax}-4
4	39.1	2.07 H_{ax} 1.67 H_{eq}	H_{eq}-4	-
5	76.12	-	-	H_{eq}-4, H-6, H-7, H-19
6	73.31	3.57	H-14	H_{eq}-4
7	117.69	5.31	H-6, H-14	H-6, H-9
8	143.52	-	-	H-6, H-9, H-14
9	43.35	1.99	H-11	H-1, H-7, H-19, H-12, H-14
10	37.2	-	-	H_{eq}-4, H-9, H-14, H-19
11	22.14	1.57	H-12	H-9, H-18, H-19
12	39.48	1.34	-	H-18, H-16
13	43.83	-	-	H-16, H-18
14	56.12	1.92	H-15	H-7, H-18
15	23.08	1.58 H_{α} 1.46 H_{β}	-	-
16	28.12	1.75 H_{α} 1.31 H_{β}	H-17, H-15	H-17, H-18
17	65.20	1.3	-	H-18, H-20, H-21
18	12.35	0.61	-	H-12, H-14, H-17
19	18.41	1.06	-	H-9
20	40.59	2.05	H-21	H-21, H-23
21	21.21	1.03	-	H-22
22	135.69	5.17	H-20	H-21, H-23, H-24
23	132.27	5.23	H-24	H-24, H-25
24	43.02	1.84	H-28	H-22, H-26, H-27, H-28
25	33.26	1.48	-	H-24, H-26, H-27, H-28
26	20.02	0.85	-	H-24
27	19.71	0.82	-	H-24
28	17.69	0.92	-	H-23, H-21

The proton-proton correlations, which exhibited via space to other protons depending on protoned carbon and further correlated due to homonuclear spin coupling until reaching quaternary carbon or heteroatom, were shown in HOHAHA (Homonuclear Hartman Halm) spectrum (Figure 55, 56). The HOHAHA (J = 8 Hz) experiment could confirm partial structure of this compound. The data of HOHAHA experiment was shown in Table 15.

The proposed fragmentation of K057, Figure IV.5, was one of formations to confirm the structure. This mass fragmentation's analysis exhibited the losing two H_2O fragment of m/e 394 (10%) and the cleavage of the side chain, later, provided the fragments of m/e 269 (7%). The losing three H_2O fragment was also observed at m/e 376 (38%). Subsequently, this fragment cleavaged in 3 types as follows :

1) Cleavage of the side chain provided the fragment of m/e 251 (100%)

2) Cleavage of the ring D leading to cleavage of C14 - C15 bond showed peak at m/e 224 (14%) and also showed peak of losing C19 methyl group at m/e 209 (32%)

3) Apart from the cleavage of the ring D, electrons rearrangement were occurred to cleavage C8 - C9 bond and lose C19-methyl group resulting in providing fragment of m/e 361 (6%)

The relative stereochemistry of this compound was determined by the coupling constants in ^{1}H-NMR and the NOESY spectrum (Figure 57 - 59) which was presented in terms of the nuclear overhauser effect (NOE) to show chemical exchange.

The stereochemistry of H-3 in axial position was analysed depended on coupling constants that indicated axial - axial and axial - equatorial coupling as mentioned before. The relative stereochemistry of steroid nucleus was analyzed from the data of NOESY experiment and from proposed relative stereochemistry. The NOESY spectrum showed the correlations between H-3, which was established to be

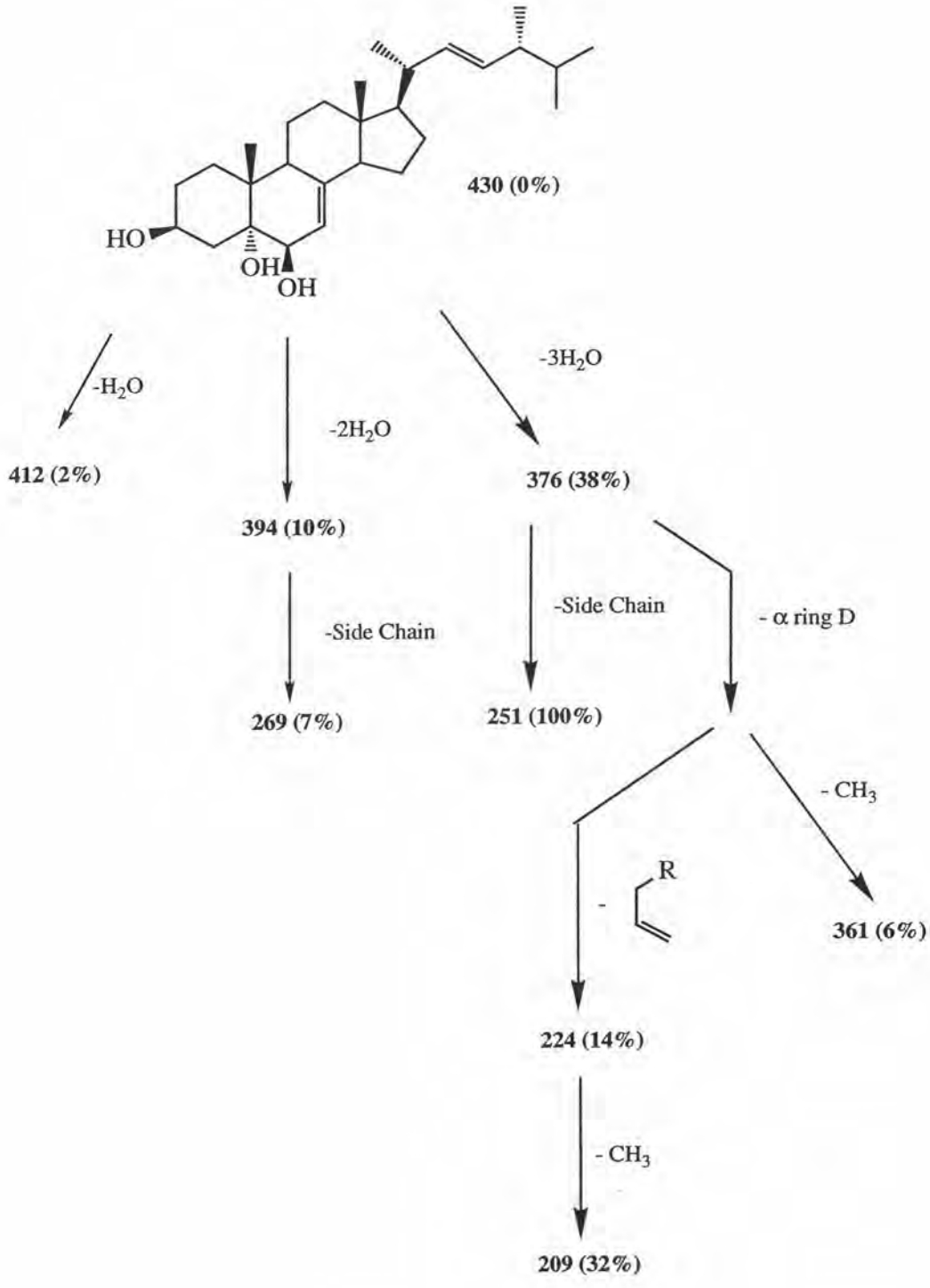


Figure IV.5 Proposed mass fragmentation of compound K057

axial position in ring A, and H_{eq}-2, H_{eq}-4. H_{eq}-2 correlated with H-1 (δ 1.52 ppm) and this correlation indicated the position of H-1 (δ 1.52 ppm) to be axial position. On the other hand H_{ax}-2 and H_{ax}-4, effects to H-19 ring A, were proposed to have chair form conformation. H_{eq}-4 showed correlation with H-6 to indicate that the hydroxyl group at C-6 was in axial position and it could confirm H-6 did not show cross peak to CH_3-19 (β-position). Moreover, CH_3-18 showed relative stereochemistry to CH_3-19 in which was pointed out that CH_3-18 was in the β-position. Because protons attached to C-15, C-16 and C-17 which liked those of cyclopentane were eclipsed, they were then assigned in term of α,β–orientation. Proton at δ 1.58 ppm was proposed to be α-orientation (H_α-15) when it showed cross peak with H-14 which was anti configulation to CH_3-18 . This assignment could be confirmed by a relationship between CH_3-18 and H_β-15 (δ 1.46 ppm). In the same way, CH_3-18 which was in the β-position correlated with H_β-16 (δ 1.31 ppm) and H_α-16 (δ 1.75 ppm) correlated with H-17. The relative stereochemistry of steroid nucleus was shown in Figure IV.6.

The stereochemistry of the unsaturated side chain (Figure IV.7) comprised *trans*-conformation of C22-C23 and R-configuration of C24. The R-configuration of C24 were assigned by comparison in term of ^{13}C-NMR chemical shifts with the known configuration C-24 of the marine sterols, (22*E*, 24*R*)-24-methylcholesta-5,22-dien-3β-ol (I) and (22*E*, 24*S*)-24-methylcholesta-5,22-dien-3β-ol (II) as shown in Table 16. The 24*S* and 24*R* diastereoisomers influenced other side chain carbons to have different chemical shifts. The ^{13}C-NMR chemcal shifts of K057 tended to exhibit nearly value with 24*R* diastereomer.

This relative stereochemistry of K057 compound Figure IV.10 was described as having three six - membered rings of chair form and a five - membered ring in the *trans*, anti, *trans*, anti, *trans* configuration (Fieser, 1959). Ring fusions 5,10-, 8,9-, and 13,14- were all *trans*-configuration and bonds 10,9- and 8,14-, each of which connects two rings, were in the anti arrangement

Table 15. Correlations between protons-protons in HOHAHA and Decoupling-experiments

Position	δ H (ppm)	HOHAHA	Decoupling - experiment	NOESY
2	1.83 (H_{eq})	H_{ax}-2, H_{eq}-4	H-1, H_{ax}-2, H-3, H_{eq}-4	H_{ax}-2
	1.43 (H_{ax})	-	-	H_3-19
3	3.9	-	-	H_{eq}-2, H_{eq}-4
4	2.07 (H_{ax})	H-1, $H_{eq,ax}$-2, H_{eq}-4	-	H_{eq}-4, H_3-19
6	3.57	H-14	-	H_{eq}-4
7	5.31	H-9, H-14	-	2H-15, H-6
9	1.99	H-11, H-12	H-7, H-11	-
11	1.57	H-12	H-9, H-12	H_3-18, H_3-19
14	1.92	H-15, H_α-16	H-7, H-15	H-17, $H_{\alpha-15}$
15	1.58 (H_α)	H_β-16, H-21	H_α-16	-
	1.46(H_β)	-	-	H_3-18
16	1.75 (H_α)	H-11, H-15, H_β-16, H-21	H-15, H_α-16, H-17	H-17
	1.31 (H_β)	H-21	-	H_3-18
19	1.06	-	-	H_3-18
20	2.05	H-21	-	H_3-18, H_2-12
22	5.23	H-21, H-24	-	H_β-16, H-24
23	5.17	H-20, H-28	-	H_3-28, H_3-21
24	1.86	H-26, H-28	H-23, H-25, H-28	H_3-27
27	0.85	-	H-25	-
28	0.92	H-26	-	H_3-26

Table 16. ^{13}C-NMR chemical shifts comparison between K057 and C24 methyl epimer of sterols

Position	Chemical Shift (ppm)		
	I	II	K057
16	28.86	28.58	28.12
20	40.33	40.24	40.59
22	136.05	135.83	135.69
23	131.84	131.76	132.27
24	43.12	42.90	43.02
25	33.28	33.16	33.26
26	19.69	20,02	20.02
27	20.19	19.69	19.71
28	18.08	17.68	17.69

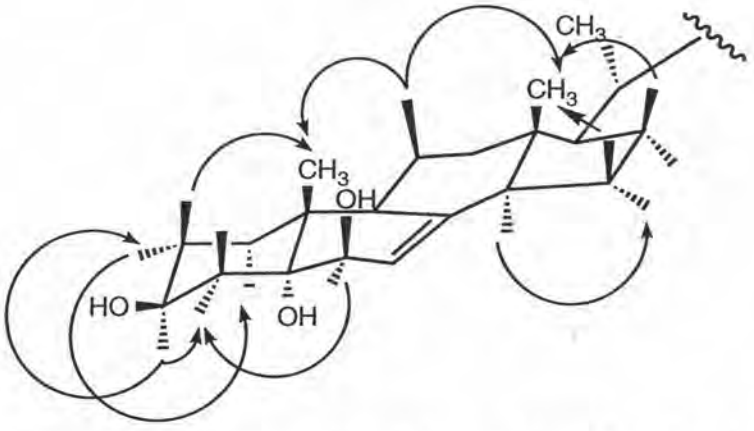


Figure IV.6 Partial stereochemistry of steroid nucleus in compound K057

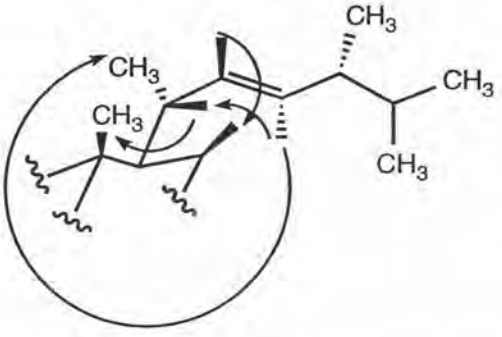


Figure IV.7 Partial stereochemistry of side chain in compound K057

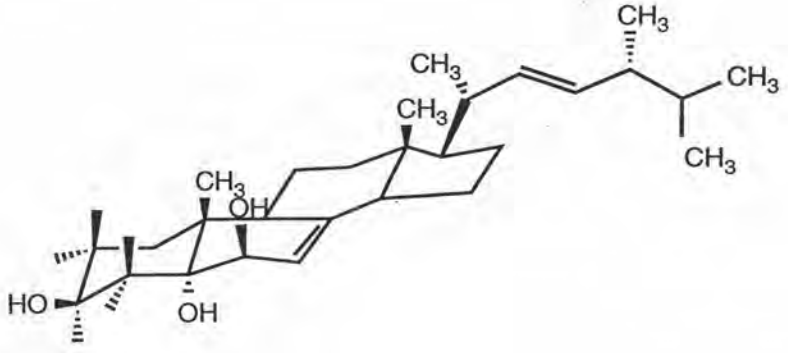


Figure IV.8 Stereochemistry of compound K057

STRUCTURE ELUCIDATION OF K068

Compound K068 was other steroid compound that was isolated from fraction F048 in Scheme I by repeated silica gel column chromatographies with gradient chloroform and methanol (3 times) crystallized in methanol and finally, preparative TLC technique in 5% ethyl acetate in chloroform was used. Both crystallization (6.8 mg) and preparative TLC technique (9.9 mg) gave 16.70 mg of this steroid (0.01 % based on wet weight of *Biemna fortis* (Topsent)).

Compound K068 was crystallized as white needle crystal. It was soluble in chloroform but slightly soluble in methanol. The compound had less polarity than K057. According to the spectroscopic data, this steroid K068 was identified as (22*E*)-ergosta-5,22-dien-3β-ol or (22*E*,24R)-24-methylcholesta-5,22-dien-3β-ol (brassicasterol) by comparison with the published data (Wright *et al.*, 1978). Which reported its carbon assignment and presentation of unique methyl group patterns for each C-24 epimer of 220 MHz ^{1}H-NMR spectra, (Rubinstein *et al.*, 1976 ; William *et al.*, 1976)

The structure of compound K068 was presented comparative carbon assignment by comparison with ^{13}C-NMR spectra of Brassicaserol. Whereas its proton assignment was proposed by mustering both 1D and 2D-NMR data in this work.

The IR. spectrum of K057 (Figure 60) was assigned in Table 17.

Table 17. The IR spectrum's assignment of K068

Range of absorption (cm^{-1})	Intensity	Assignment
3450	broad	hydrogen bonded OH stretch
2990, 2890	high	$-CH_3$, $-CH_2$ stretch
1052	medium	cyclic alcohol C-O stretch
980	small	=CH out of-plane bending

The UV spectrum (Figure 61) showed absorption band at λ_{max} 241 nm (ε_{max} 394.9). The EIMS spectrum of K068 (Figure 62) showed fragments of structure that could be analyzed to propose tentative molecular formula. The spectrum exhibited molecular ion peak m/e 398 (13%). The fragmentation of molecule was analyzed to cleavage in 3 pathways as follow (Figure IV.9)

1) Cleavage C13-C17 bond in ring D, subsequently rearrangement of proton at C8 to attach C17 and then C14-C15 bond cleavage to give a fragment ion at m/e 231 (7%)

2) Cleavage of side chain to provide the fragment of m/e 273 (10%)

3) Loss of H_2O to provide peak of m/e 380 (12%) and further cleavage togive 3 fragment ions at *m/e* 255, 365 and 213, as follows:

3.1) Cleavage of side chain to show peak of m/e 255 (36%)

3. 2) Cleavage of CH_3-19 to provide peak of m/e 365 (5%)

3. 3) Cleavage of ring D at C13 - C17 and subsequent rearrangement of protons, cleavage C15 - C16 and cleavage CH_3-19 to give peak of m/e 213 (16%)

^{1}H-NMR spectra were recorded on a JEOL A 500 MHz (Figure 63). Characteristic peaks of steroid were exhibited and it was not only that the unique six methyl groups pattern were proposed to be steroid with 28 carbons, the unique two sp^2 protons of 22, 23 unsaturated side chain, one sp^2 proton in steroid nucleus and one carbinol methine proton with tt splitting pattern of C-3 were also presented in ^{1}H-NMR spectra. Some of remained 22 sp^3 proton signals were too overlap to assign, so 2D-NMR must be used to solve the assignment.

In order to discuss the correlation in term of coupling constant values, the expansion ^{1}H-NMR spectra were observed (Figure 64, 65). The up-fields chemical shifts of unique six methyl groups exhibited signals at δ 0.91 ppm (d, J = 6.7 Hz), δ 0.83 ppm (d, J = 6.7 Hz), δ 0.82 ppm (d, J = 6.7 Hz), δ 1.01 ppm (d, J = 6.41 Hz),

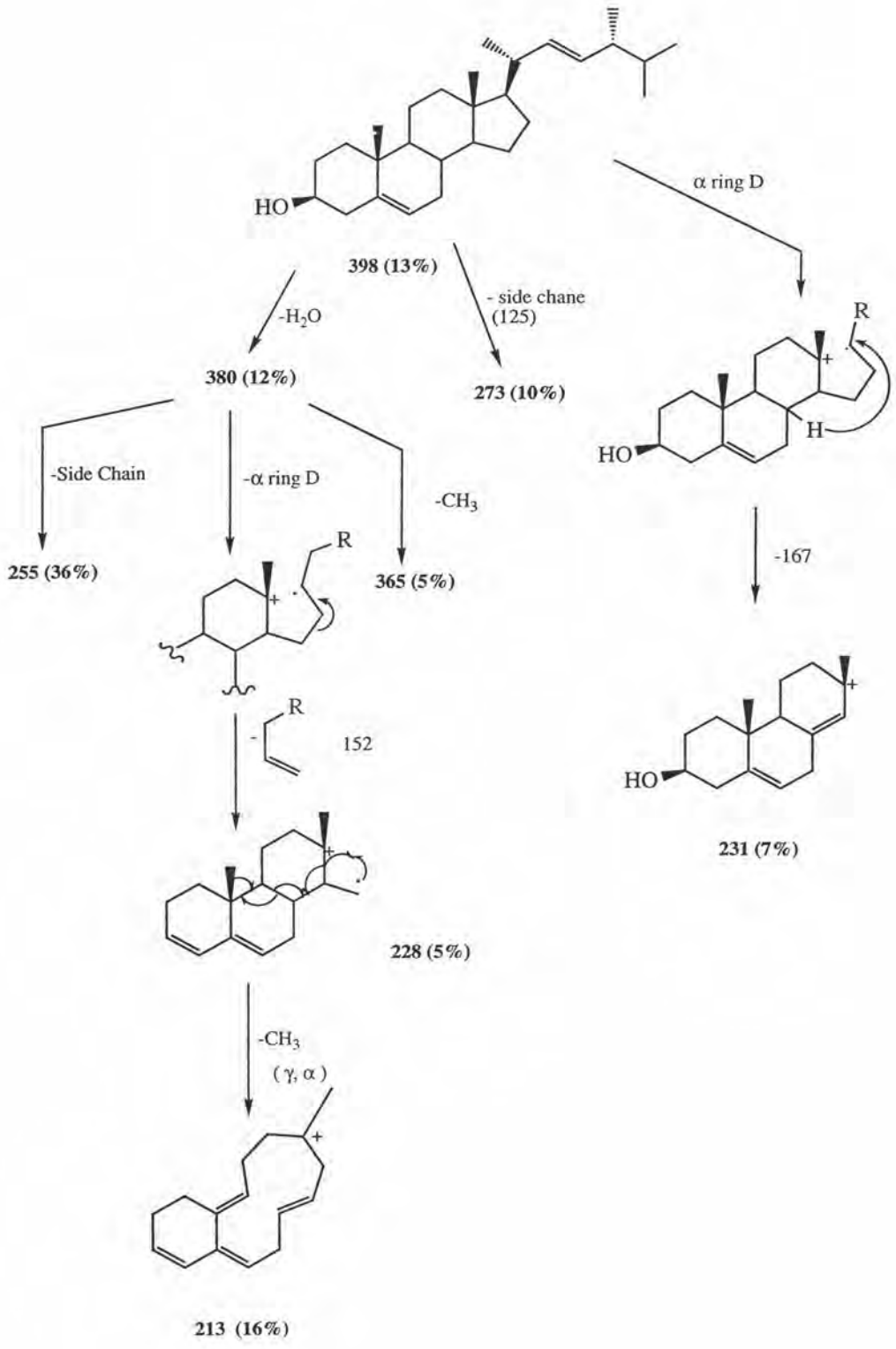


Figure IV.9 Proposed mass fragmentation of compound K068

δ 1.00 ppm (s) and δ 0.69 ppm (s) which were assigned as CH_3-28, CH_3-27, CH_3-26, CH_3-21, CH_3-19 and CH_3-18, respectively. It was considerable that methyl groups which attached with methine carbon must exhibit adjacent methine proton with coupling constant value ca. 7 Hz. In the down field chemical shift of 22, 23 unsaturated side chain, respectively showed two sp^2 proton signals at δ 5.21 ppm (dd, J = 15.3, 7.0 Hz) and δ 5.16 ppm (dd, J = 15.3, 7.1 Hz). The large coupling constant of 15.6 Hz indicated *trans*-orientation of these two olefinic protons and the smaller coupling constant of ca. 7 Hz indicated vicinal coupling with adjacent the methine proton. The remaining sp^2 proton at δ 5.35 ppm (dt, J = 5.2, 1.8 Hz) was assigned as H-6 which locals in ring B of steroid nucleus. The carbinol methine proton signal at δ 3.52 ppm (tt, J = 11.3, 4.8 Hz) showed large coupling constant (11.3 Hz) for axial-axial coupling and the smaller one (4.8 Hz) for axial-equatorial coupling. In the other words, dihedral angle of protons between two closed carbons could be discussed in term of the relation between coupling constant value and dihedral angle that coupling constant value ca. 11 Hz and ca. 9 Hz were effected by 0° or 180° and 60° of dihedral angle, respectively. At the chemical shift range of methylene and methine protons, proton H_{eq}-4 (δ 2.29 ppm) could be analyzed as ddd splitting pattern (J = 2.1, 4.8, 13.3 Hz). The large couplling constant value of 13.3 Hz was geminal coupling with H_{ax}-4 (δ 2.23 ppm), coupling constant value 4.8 Hz for vicinal coupling with H-3 and J = 2.14 Hz for W-shape coupling with H_{eq}-2. It had transfer spin information between H_{eq}-4 and H_{eq}-2 that they was arranged in W-pattern in structure. Similary, H_{ax}-4 (δ 2.23 ppm showed ddd splitting pattern (J = 2.4, 5, 11.3, 13.1 Hz). J = 11.3 Hz indicated axial-axial conformation based on H-3, J = 2.4 Hz which was also for W-shape coupling with H_{ax}-2 and J = 5 Hz was suggested to be coupling via π-bond to H-6 which was firmly supported by correlation in ^1H-^1H COSY.

^{13}C-NMR spectra were recorded with a JEOL A 500 spectrometer at the Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University under the following condition; frequency 33.89 MHz, acquisition time 0.4833 sec, pulse width 4.75 μsec, flip angle ca. 90° internal pulse lock to Din $CDCl_3$

(solvent), temperature 29.9 ^{o}C, concentration ca. 1.4 mg/ml. For off resonance decoupling, protons were irradiated at δ_H ca. 6.25 ppm ($\gamma H_2/2\pi$ ca.1800 or 3800 Hz).

The ^{13}C-NMR spectra of K068 (Figure 66) showed 28 carbon signals that were closed to the signals from Brassicasterol. Compound K068 prefered values for carbon assignment by comparison with brassicasterol (Which obtains from a Varian XL-100/ 15 Fourier transform spectrometer), as listed in Table 18.

Table 18. The ^{13}C-NMR chemical shifts of brassicasterol and K068 in $CDCl_3$

Position	Chemical Shift (ppm)	
	Brassicasterol	K068
1	37.36	37.27
2	31.69	31.69
3	71.76	71.81
4	42.30	42.25
5	140.79	140.78
6	121.60	121.69
7	31.97	31.92
8	31.97	31.92
9	50.27	50.20
10	36.58	36.53
11	20.13	21.08
12	39.77	39.70
13	42.35	42.33
14	56.96	56.86
15	24.39	24.29
16	28.58	28.51
17	56.06	56.05
18	12.09	12.08
19	19.42	19.40
20	40.24	40.13
21	21.06	20.96
22	135.83	135.83
23	131.76	131.75
24	42.90	42.81
25	33.16	33.10
26	20.02	19.95
27	19.69	19.63
28	17.68	17.61

The remaining 22 protons were exhibited ambiguous signals in the range of 1.1 to 2.3 ppm. In this case, the CHSHF spectrum which was used with carbon-13 decoupling during acquisition and presents cross peaks to indicate the relation between carbons and their protons (Figure 67 - 69). The CHSHF data were obtained by using the condition that frequency of 25.06 MHz for carbon-13 and 4.33 MHz for proton, 32 scans, 4 dummy, acquisition time of 0.0204 sec, pulse delay of 2 sec, digital resolutions of 8.32 Hz per point in the column, temperature of 28.3 ^{o}C, 19.00 μsec of 90^{o} pulse width and bottom threshold of 1.0 . This data showed distinguish correlation of protons resonating at δ 5.36, 3.52 and 0.69 ppm to C-6, C-3 and C-18, respectively. The expansion spectrum of CHSHF in the same condition at changing bottom threshold to be 0.7, presented the correlation of proton signals at δ 2.29 ppm and 2.23 ppm to C-4. Proton signals at δ 1.99, 1.96 and 1.56 ppm were correlated with C-7 and C-8, proton signals at δ 1.01, 1.0, 0.91 and 0.83 ppm showed correlation to C-21, C-19, C-28 and C-26(27), respectively.

Whatever, it was not enough data for proton assignments although the lower values of bottom threshold were changed to reduce T_1- noise interruption.

We suggested that the correlation of proton through adjacent proton and further long range proton must be very useful informations for proton assignments. So, proton correlated spectroscopy (^{1}H-^{1}H COSY) was made by JEOL A 500 MHz under the condition of 4.55 MHz frequency, 32 scans, 4 dummy, 0.1124 sec of acquisition time, 0.8870 sec pulse delay, digital resolutions of 8.89 Hz per point in the column. Temperature of 28.5 ^{o}C, 90^{o} pulse with of 10 μsec and bottom threshold of 1.5 (Figure 70 - 71). A sine-square window function was applied before Fourier Transformation.

At the lowest field signal, H-6 at δ 5.35 ppm showed cross peak to H_2-7 at δ 1.96, and 1.99 ppm and also showed small cross peak to H_{ax}-4 at δ 2.23 ppm which was achieved from the correlation through π bond. Subsequently, H_2-7 signals

showed correlation to H-8 at 1.56 ppm. In addition, H_{ax}-4 had W-shape (4 bonds) coupling to H_{ax}-2 (δ 1.50 ppm) by showing small cross peak. This H_{ax}-2 also respectively correlated to H_{eq}-2 and H_2-1 at δ 1.81, 1.83 and 1.86 ppm. The unique carbinol methine proton (H-3) signal of steroid compound showed correlation to H_{ax}-2 (δ 1.50 ppm) and H_2-4 (δ 2.29 and 2.23 ppm). On the other hand, the correlation of H-3 to H_{eq}-2 (δ 1.81 ppm), H_{eq}-4 (δ 2.29 ppm) and H_{ax}-4 (δ 2.23 ppm) were shown in ^1H-^1H COSY which was operated bottom threshold at 2.0 (Figure 70, 71). The indicated conformation of ring A was firmly supported by coupling constant value of H-3. The larger coupling constant value of 11.3 Hz was coupling with H_{ax}-4 and the smaller one (4.8 Hz) was coupling with H_{eq}-4. Proton H_{ax}-4 further appeared the resonance with small coupling constant of 2.44 Hz for W-shape coupling.

For unsaturated side chain, one olefinic proton H-22 (δ 5.16 ppm) showed correlation to H-20 (δ 2.02 ppm) and correlation between H-20 and H_3-21 (δ 1.01 ppm) was also shown. Another olefinic proton H-23 (δ 5.21 ppm) correlated to H-24 (δ 1.85 ppm). H-24 had vicinal coupling to H-28 and H-25 at δ 0.91 and 1.45 ppm, respectively. H-25 also showed correlation to H_3-26 (δ 0.82 ppm) and H_3-27 (δ 0.83 ppm). The partial structure of ring D and side chain refered to this spectroscopic data as shown in Figure IV.11.

Apart from ^1H-^1H COSY based on vicinal coupling, proton assignment of compound K068 was also accomplished by mean of the HOHAHA experiment (proton-proton correlations which exhibited via space to others protons at conditions of 64 scans, acquisition time of 0.1124 sec, pulse delay of 1.3878 sec, the digital resolution of 7.89 Hz per point in the columns 90° pulse width of 10 μsec., bottom threshold of 0.3 as shown in Figure 72, 73. The remained protons were assigned.

The HOHAHA spectra confirmly showed correlations between protons in ring A (Figure IV.12). Protons in ring B assignment (Figure IV.13) were also confirmed by

the long-range correlations of H-6 to H-8. Similarly, the correlation of protons in side chain also exhibited to assign protons of side chain as shown in Figure IV.14.

Steroid structure of K068 was confirmed the carbon and proton assignments by using a long-range reverse detected heteronuclear multiple bonds coherence (HMBC) experiment which optimized for 4 Hz coupling (Figure 74 - 76). The proton at δ 1.0 ppm (H_3-19) coupled to four carbons resonating at δ 140.78, 50.18, 37.27 and 36.53 ppm of C-5, C-9, C-1 and C-10, respectively. Proton at δ 0.69 ppm (H_3-18) related to four neighbor carbons at C-12, C-13, C-14 and C-17 of δ 39.70, 42.34, 56.86 and 56.04, respectively as shown in Figure IV.15.

For side chain analysis, the other protons of methyl groups of CH_3-21, CH_3-26, CH_3-28 similarly showed correlations to adjacent and the next carbons such as CH_3-21 (δ 1.01 ppm) correlated to C-20 and to C-22, C-27. Protons of CH_3-26 showed cross peak to C-25 and to C-24. Protons of CH_3-28 correlated to C-24 and to C-25, C-23. The up-field part of H-22 and H-23 at δ 5.16 and 5.12 ppm only showed cross peak to adjacent of their sp^2 carbon. H-23 correlated to C-24 and H-22 correlates to C-20. Although HMBC experiment only analyzed the up field of methylene protons and the down field of methyl protons. These correlation data could support the assignment of compound K068 as shown in Figure IV.14.

The correlations of proton-proton which were appeared in 2D-NMR experiment were listed in Table 19 as shown below ;

Table 19. Proton assignments of K068 by CHSHF, proton- proton correlations of ^{1}H-^{1}H COSY and long range correlation in HOHAHA experiment

position	δ H (ppm)	^{1}H-^{1}H COSY	HOHAHA
1	1.86 m	H_{ax}-2	-
	1.83 m	H_{ax}-2	H_{ax}-2
2	1.81 m (H_{eq})	H_{ax}-2	-
	1.50 m (H_{ax})	-	-
3	3.52 tt, J = 4.8, 11.3 Hz	H_{ax}-2, H_{ax}-4, H_{eq}-4 H_{eq}-2*	H_{ax}-2, H_2-1, H_{eq}-4
4	2.29 ddd, J = 2.1, 4.8, 13.3 Hz (H_{eq})	-	H_{ax}-2, H_2-1
	2.23 dddd, J = 2.4,5,11.4,13.1 Hz(H_{ax})	H_{ax}-2**	H_{ax}-2, H_2-1
6	5.35 dt, J = 1.8,5.2 Hz	H_2-7, H_{ax}-4**	H_2-7, H-8
7	1.99 m (H_a)	H-8	
	1.96 m (H_b)	H-8	
8	1.56 m	-	-
16	1.69 tt, J= 4, 9.5 Hz		H-21
18	0.69 s	-	-
19	1.00 s	-	-
20	2.03 dtq, J = 7.1, 9.2 Hz	H-21	H-21
21	1.01 d, J = 6.4 Hz	-	-
22	5.16 dd, J = 7.6, 15.3 Hz	H-20**	H-20, H-21#, H-28#
23	5.21 dd, J = 7.0,15.3 Hz	H-24**	H-24, H-28#
24	1.85 q, J = 6.4 Hz	H-28, H-25	H-25, H-27#, H-28#
25	1.45 m	H-26, H-27	H-27#
26	0.82 d, J = 6.7 Hz	-	-
27	0.83 d, J = 6.7 Hz	-	-
28	0.91 d, J = 6.7 Hz	-	-

Asterisk (*) represented the data of ^{1}H-^{1}H COSY experiment which was optimized with bottom threshold 8.0 and 2.0.

Asterisk (**) represented the data of ^{1}H-^{1}H COSY experiment which was optimized with bottom threshold 1.0.

datch (#) represented the data of HOHAHA experiment which was operated at bottom threshold 0.3062.

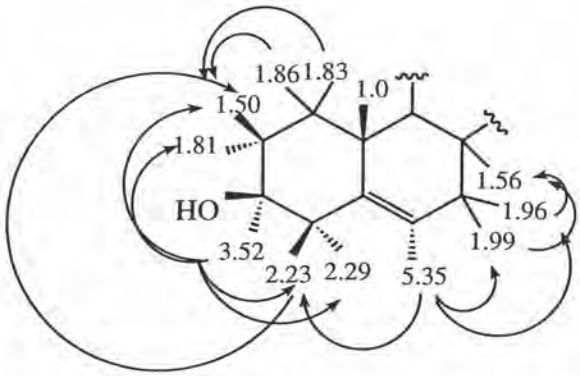


Figure IV.10 Partial structure of K068 assignment from ^{1}H-^{1}H COSY

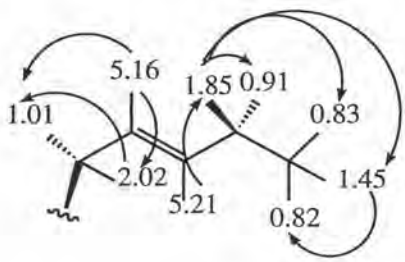


Figure IV.11 Side chain assignment from ^{1}H-^{1}H COSY

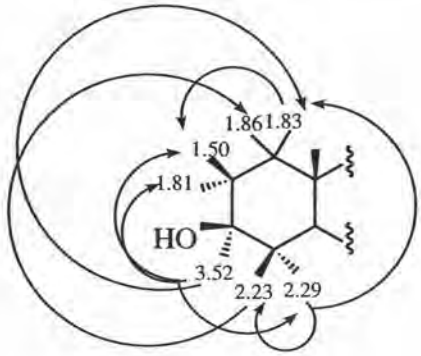


Figure IV.12 The protons correlation in ring A
of K068 from HOHAHA experiment

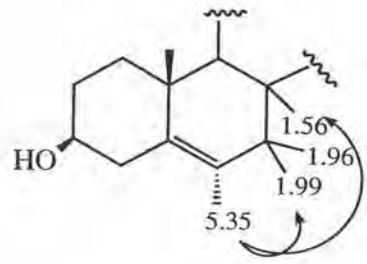


Figure IV.13 The protons correlation in ring B
of K068 from HOHAHA experiment

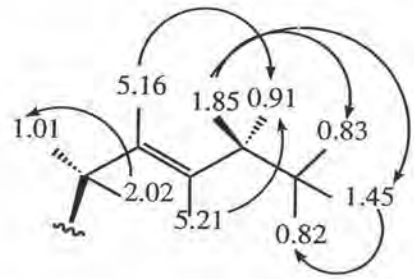


Figure IV.14 The protons correlation in and side chain
of K068 from HOHAHA experiment

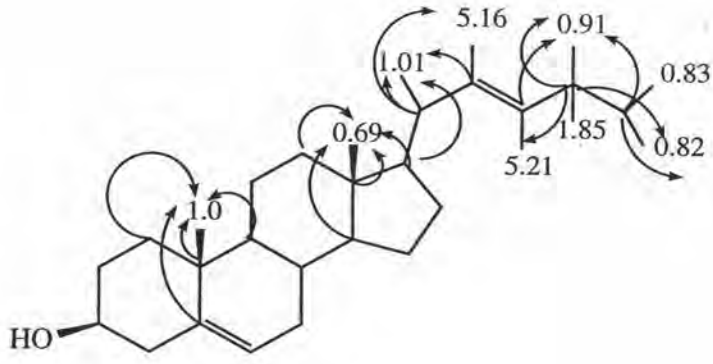


Figure IV.15 The correlation between carbon and proton from HMBC spectra

STRUCTURE ELUCIDATION OF K084

Compound K084 was the third steroid compounds that was purified from fraction F057 as shown in Scheme I. It was received by processes of repeated silica gel column chromatogrophies with gradient chloroform and methanol and followed by crystallization in methanol. On the other band, ^{1}H-NMR of this crystal was still showed mixture of steroids. HPLC technique was successively purified K084 with 2% MeOH in $CHCl_3$ as solvent system of eluent, retention time = 11.49 min, flow rate = 1.0 ml/min, silica gel column 4 x 125 mm.(Figure 77). Finally, K084 was yielded 1.6 mg or 1.03 x 10^{-6} % based on wet weight of *Biemna fortis* (Topsent).

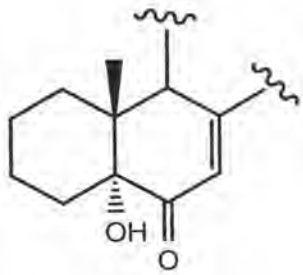
Compound K084 was received in term of white needle crystal. It was extremely solubled in chloroform but slightly solubled in methanol. It showed Rf value of 0.4 on TLC pattern (Figure 78) in 5% MeOH in $CHCl_3$ solvent system. The Rf value of 4 located between Rf value of K068 (0.94) and K057 (0.08). Compound K084 showed brown color to positive anisaldehyde test and to positive Iodine-vapor test. Positive UV light detector at 254 nm, K084 also showed quenching spot.

The IR spectrum of K084 (Figure 79) was assigned in Table 20.

Table 20. The IR spectrum's assignment of K084

Range of absorption (cm^{-1})	Intensity	Assignment
3450, 3200	broad	hydrogen bonded OH stretch
2990, 2850	high	$-CH_3$, $-CH_2$ stretch
1675	high	C=O stretch of unsat. ketones
1627	weak	C=C stretch
990, 890	medium	=CH out of-plane bending

The absorption at 1675 cm^{-1} was importance to indicate unsaturated ketones group in molecule. Furthermore, UV spectrum (Figure 80) confirmly showed absorption bands at λ_{max} 256 nm (ε_{max} = 527.6) and λ_{max} 323 nm (ε_{max} = 41.2) which were the characteristic of unsaturated ketone which connected to hydroxyl groups' chromophore (Scott *et al.*, 1964) as shown in fragment of Δ^7-6-one **(1)**.



(1)

The EIMS spectrum of K084 (Figure 81) exhibited the molecular ion peak at *m/e* 428 (1.29%). The losing H_2O, both one and two molecules, fragments also showed in the EIMS spectrum at *m/e* 410 (9.29%) and *m/e* 392 (16.14%), respectively to indicate two hydroxyl groups in this steroid compound. Both losing H_2O, one and two molecules, fragments were further analyzed the mass fragmentation in 2 types of cleavage as follows :

1) Cleavage of side chain to provide the fragment of *m/e* 285 (4.04%) and *m/e* 267 (5.95%), respectively.

2) Cleavage of ring D at the C13 - C17 bond and further cleavage the C14 - C15 bond to respectively exhibit peaks at *m/e* 258 (2.92%) and *m/e* 240 (3.82%). After that the losing C19 methyl group's fragments at *m/e* 243 (3.91%) and *m/e* 225 (5.24%) were also respectively shown in the spectrum.

This steroid K084 was established the tentative molecular formula of $C_{28}H_{44}O_3$. The proposed fragmentation of K084 was shown in the Figure IV.16.

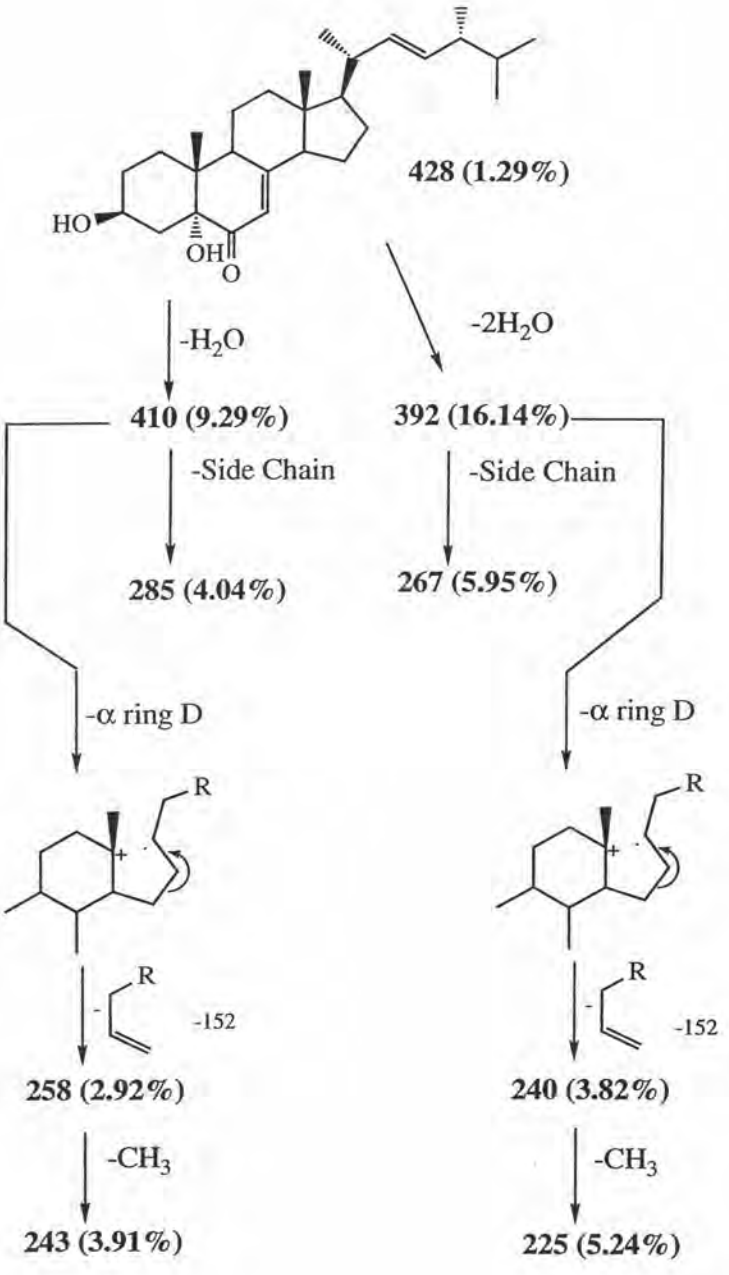


Figure IV.16 Proposed mass fragmentation of compound K084

The 500 MHz ^{1}H-NMR spectra (Figure 82 - 85) of K084 exhibited characteristic peaks of steroid and also showed the unique six methyl groups pattern of C28-steroid, the unique two sp^2 protons of 22, 23 unsaturated side chain , one sp^2 proton in steroid nucleus and one carbinol methine proton with ddt splitting pattern of H-3. The other 21 sp^3 proton signals were assigned by using 2D-NMR spectral data.

The 125 MHz ^{13}C-NMR spectra (Figure 86) of K084 compound showed 28 carbons signals which were listed in Table 21.

The carbon 28 atoms in molecule were analyzed. Carbonyl carbon resonated at the most down field signal (δ 198.1 ppm), which belonged to carbonyl ketone carbon.. The notice one signal (δ 165.1 ppm) of four sp^2 carbons resonated more sown field than regular sp^2 signal region. This signal was β-quaternary carbon of unsaturated ketone's signal and it was withdrawed electron to resonance at down field region. Two carbinol carbons resonated at δ 77.8, 67.4 ppm. The remained 21 sp^3 carbons resonated at up field region.

The assignment of carbons was also up on HMQC experiment (Figure 87 - 89), ^{1}H-^{1}H COSY (Figure 90, 91) and K057 comparison because it was suggested that K084 compound had carbonyl carbon at C6 instead of carbinol carbon at C6 in K057 compound. This was only one point of structure that different from K057. However, unsaturated ketone group in this structure resulted in slightly different of chemical shift of carbons and protons in steroid nucleus. The carbons, protons assignment from analysis of MS, UV, IR, 1D and 2D-NMR were listed in Table 21. The correlation of carbon - proton and proton - proton were analyzed and also listed in Table 21. Meanwhile, the conditions of 2D-NMR were listed in Table 22.

Table 21. Carbons and protons assignment

Position	Carbon (δ, ppm)	Proton (δ, ppm)	HMBC	^{1}H-^{1}H COSY
1	30.3	1.62 (1a) 1.42 (1b)	C13 -	Hb-1 -
2	30.2	1.89 (2_{eq}) 1.80 (2_{ax})	- C10	Hb-1, Ha-1 Hb-1, Ha-1
3	67.4	4.01, tdd, J = 5.5, 11.6, 15.0 Hz.	-	H_{ax}-4, H_{eq}-4, H_{ax}-2, H_{eq}-2
4	38.8	2.11 (4_{eq}) 1.45 (4_{ax})	C1, C10, C3, C5	H_{ax}-4, H_{eq}-2
5	77.8	-	-	-
6	198.1	-	-	-
7	119.7	5.64, dd, J = 2.1, 2.1 Hz.	C9, C14, C5	H-14, H-9
8	165.1	-	-	-
9	43.9	2.50, ddd, J =2.1,7.1,11.9Hz.	C19, C11, C8	Hb-11, Hb-12
10	40.4	-	-	-
11	21.9	1.78 (11a) 1.60 (11b)	- C12, C10	Hb-11 -
12	36.5	2.09 (12a) 1.73 (12b)	C13 -	Hb-12, Ha-11 -
13	44.7	-	-	-
14	55.7	2.13	C18, C13, C8, C7	-
15	22.4	-	-	-
16	27.8	1.8 (16a) 1.37 (16b)	-	Hb-16
17	56.0	1.36	C18	-
18	12.6	0.59, s	C17, C14	-
19	16.4	0.93, s	C1, C10, C9, C5	-
20	40.2	2.02, q, J = 8.24 Hz.	-	H-17, H-21
21	21.1	1.01, d J = 6.41 Hz.	C20, C17	-
22	134.9	5.14, dd, J = 8.24, 15.26 Hz.	C21, C20, C24, C23	H-20
23	132.5	5.21, dd, J = 7.63, 15.26 Hz.	C28, C20, C24	H-24
24	42.8	1.86	C28, C26, C27, C25	H-28

Table 21. Carbons and protons assignment (continued)

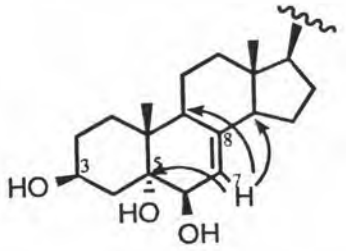
Position	Carbon (δ, ppm)	Proton (δ, ppm)	HMBC	^{1}H-^{1}H COSY
25	33.1	1.48	C26, C14	H-26, H-27
26	19.9	0.82, d, J = 6.7 Hz.	C25, C24	-
27	19.5	0.82, d, J = 6.7 Hz.	C25, C24	-
28	17.5	0.9, d J = 7.0 Hz.	C25, C24	-

Table 22. The conditions of NMR experiments

Condition	^{13}C-NMR	^{1}H-^{1}H COSY	HMQC	HMBC
Frequency (Hz)	33898.31	4231.91	4027.39	4027.39
Scans	12000	32	128	128
Acquisition time (sec)	0.4833	0.1210	0.2543	0.2543
Pulse delay (sec)	2.5	1.50	1.5	2.0
Pulse width 1 (μsec)	4.75	6.50	23.50	6.5
PI_1	-	100.0	100.00	62.50
Point	16384	512	1.0 K	1.0 K
Temperature (^{o}C)	28.7	28.0	27.7	29.2
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$
Threshold bottom	-	0.2	0.075, 0.04, 0.05	0.05

The proton assignment and analysis were done by using 2D-NMR experiments and splitting pattern of proton signals. However, in term of splitting pattern analysis could not analyzed most of proton signals because of the overlapping of 21 protons signals in range of chemical shift of 1.3 - 2.2 ppm. In the down field region, H-7 (δ 5.63 ppm) showed dd (J = 2.14, 2.14 Hz.) of 4 bond coupling with H-9 and H-14. Proton H-9 showed ddd signal coupling with H-7 (J = 2.14 Hz.), axial - axial proton coupling with H-11 (J = 11.9 Hz.) and axial - pseudoaxial with H-14 (J = 7.1 Hz.). This part of structure **(2)** could be confirmed by the correlations between proton-

proton in ^{1}H-^{1}H COSY spectrum. Proton H-7 showed correlation to H-9 and H-9 further exhibited correlation to 2H-11. A part from carbon - proton correlation in HMBC spectrum, H-7 showed correlation to C9, C14 and C5 (Figure 92 - 94).



(2)

The position of H-14 was confirmed by the correlation to C12, C13, C8 and C7 in HMBC spectrum. Proton H-9 shows correlation to C19, C11 and C8. Protons H_3-19 correlated to C1, C10, C9 and C5. These data also confirmed partial structure **(2)**. It was only ring B part in steroid K084 that was different from structure of steroid K057.

A part from the relative stereochem of unsaturated side chain part, the 500 MHz ^{1}H-NMR spectra showed H-23 and H-22 signal of dd splitting pattern. The large coupling constance (15.26 ppm) indicated *trans* - coupling meanwhile the small coupling constance (7.63 or 8.24 ppm) indicated vicinal coupling. The other part of this side chain were received from the comparison of ^{13}C-NMR chemical shifts with C24 methyl epimer of sterols, (22*E*,24*R*)-24-methylcholesta-5,22-dien-3β-ol (I) and (22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-ol (II) as shown in Table 23. The comparison indicated the conformation of side chain to be (22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-ol.

Table 23. ^{13}C-NMR chemical shifts comparisons between K084 and C24 methyl epimer of sterols

Position	Chemical Shift (ppm)		
	I	II	K084
16	28.86	28.58	27.8
20	40.33	40.24	40.2
22	136.05	135.83	134.9
23	131.84	131.76	132.5
24	43.12	42.90	43.8
25	33.28	33.16	33.1
26	19.69	20.02	19.9
27	20.19	19.69	19.5
28	18.08	17.68	17.5