CHAPTER IV

RESULT AND DISCUSSION

The 10 kg of dried ground entire plant of *Bidens biternata* Merr. & Sherff was marcerated with methanol. The methanol extract was then partitioned with the process that showed in section 3 chapter 3. BB-1 and BB-2 were separated from chloroform crude extract. Compound BB-3 and BB-4 were separated from methanol extract. The structure elucidations of the isolated compounds were discussed as follow.

1. Structure Elucidation of BB-1

BB-1 was obtained as white amorphous compound from F-007 (Table 3)by chromatographic techniques using silica gel column (gradient system of hexane-chloroform-methanol) to yield 200 mg (2 x 10^{-3}%based on dry weight of *B.biternata*).

The infrared absorption spectrum of BB-1 (Figure 5) exhibited O-H stretching at 3400-3200 cm^{-1}, C-O stretching at 1,062 cm^{-1}, C-H stretching of methyl and methylene groups at 2,918, 2,849 cm^{-1}, C-H bending of methyl and methylene groups at 1,473, 1,464 cm^{-1} and C-H rocking of methylene groups ($(CH_2)_n$, n>4) at 720 cm^{-1}

The ^{1}H-NMR spectrum of BB-1 (Figure 6) showed the signal of proton attaching to oxygenated carbon at δ 3.62 ppm (t, J = 6 Hz) which coupled to methylene protons with the coupling constant of 6 Hz. The signals at δ 1.35 and 1.23 ppm were the signals of methylene protons. The signal at δ 0.85 ppm (t, J = 6 Hz) is the signal of terminal methyl group that coupled to methylene protons.

The IR and ^{1}H-NMR data suggested that BB-1 was long chain alcohol. The EIMS showed the different spectra (Figure 7). It indicated that BB-1 was the mixture of at least 2 compounds . Both spectra showed characteristic fragmentation pattern of long chain compounds by the clusters of peaks, and the corresponding peaks of each cluster were 14 (CH_2) mass units apart because the loss of CH_2=CH_2 (Silverstein *et al.*, 1991). The example were *m/e*$^-$ 139, 125, 111 (139 - CH_2=CH_2), 97 (125 - CH_2=CH_2), 83 (111 - CH_2=CH_2) and 69 (97 - CH_2=CH_2).

The gas chromatography was used to identified this mixture of long chain alcohols by comparing with the authentic long chain alcohols (C_{14},C_{16},C_{18},C_{20},C_{22} and C_{30}). The retention times of compound BB-1 were 11.67, 19.20, 25.50 and 32.57 minute. From the linear graph of log retention times and number of carbons of autentic long chain alcohols (figure 4) indicated that BB-1 was the mixture of C_{26}-C_{30} long chain alcohols as follow, the relative amounts were calculated from area under peaks of GC chromatogram.

$C_{26}H_{53}OH$ (hexacosanol)	4.8%
$C_{28}H_{57}OH$ (octacosanol)	68.7%
$C_{29}H_{59}OH$ (nonacosanol)	2.9%
$C_{30}H_{61}OH$ (triacotanol)	23.6%

2. Structure Elucidation of BB-2

BB-2 was obtained as white needles from F-008 (Table 3) using a silica gel column (gradient system of hexane-chloroform-methanol) and was recrystallized in hexane to yeild 70 mg (7×10^{-4}% based on dry weight of *B.biternata*). It gived green color to Libermann-Burchard's test. Thus, it tended to be the steroidal compound.

The IR spectrum of BB-2 (Figure 10) suggested that functional groups of BB-2 were hydroxy group, methyl group, methylene group and alkyl group.

Table 7 The IR spectrum's assignment of BB-2

range of absorption (cm^{-1})	intensity	assignment
3500-3200	medium	OH stretching of R-OH
2960-2860	high	CH stretching of CH_3, CH_2
1640	weak	C=C stretching of alkene
1460	medium	C-H bending of CH_3 (asymmetric), CH_2
1380,1370	medium	C-H bending of CH_3 (symmetric)
1060	medium	C-O stretching
970-960	weak	out-of-plane C-H bending of trans-disubstituted alkene
840,800	weak	out-of-plane C-H bending of trisubstituted alkene

The ^{1}H-NMR spectrum of BB-2 (Figure 11) showed the signals at δ 0.65-1.01 ppm which were the signals of methyl proton that substituted at C-18, C-19 and at side chain of the steroidal compounds. The signals at δ 1.1-2.3 ppm were the signals of methylene and methine proton of steroids. The signal at δ 3.51 ppm (m) was the signal of proton at C-3. The olefinic signals at δ 5.14 ppm (dd, J = 15.1, 8.0 Hz) and δ 4.98 ppm (dd, J = 15.1, 8.0 Hz) are *trans*-disubstituted vinyl protons (H-22 and H-23). And the olefinic signal at δ 5.33 (br d J=6.2 Hz) could be assigned as H-6 which was trisubstituted vinyl proton.

The ^{13}C NMR spectrum of BB-2 (Figure 12) showed the signals that were close to the signals from β-sitosterol and stigmasterol (Table 8).

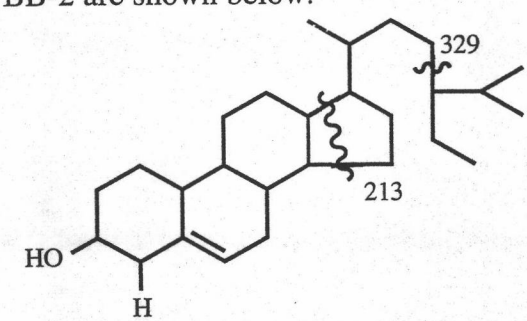
Table 8 The ^{13}C NMR chemical shifts of β-sitosterol, stigmasterol and BB-2

Carbon	Chemical Shift (ppm)		
	β-sitosterol	stigmasterol	BB_2
1	37.1	37.4	37.25
2	31.8	31.7	31.65
3	71.9	71.8	71.78
4	42.4	42.4	42.3
5	140.9	140.0	140.76
6	121.8	121.7	121.69
7	32.0	31.9	31.87, 31.9
8	32.0	31.9	31.87, 31.9
9	50.3	50.3	50.14, 50.16
10	36.6	36.6	36.51
11	21.1	21.1	21.21, 21.07
12	39.9	39.8	39.78, 39.68
13	42.4	42.4	42.3, 42.21
14	56.8	57.0	56.77, 56.86
15	24.3	24.4	24.29, 24.36
16	28.2	28.9	28.23, 28.90
17	56.2	56.0	56.07, 55.96
18	11.9	12.2	11.97, 12.23
19	19.4	19.4	19.38

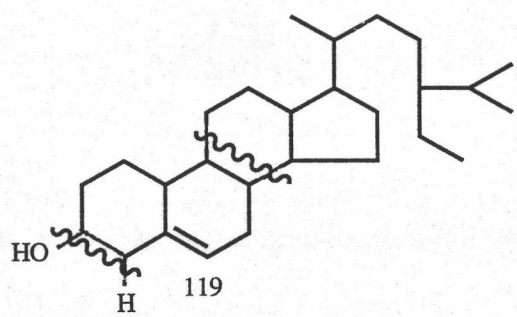
Table 8 (continued)

Carbon	Chemical Shift (ppm)		
	β-sitosterol	stigmasterol	BB_2
20	36.2	40.5	36.14, 40.47
21	19.1	21.1	19.03, 21.07
22	34.0	138.4	33.95, 138.30
23	29.3	129.4	129.28, 29.67
24	50.3	51.3	51.23
25	26.2	31.9	26.09, 29.69
26	18.8	19.0	18.77, 18.97
27	19.8	21.1	19.8, 21.07
28	23.1	25.4	23.07, 25.4
29	11.9	12.0	11.85, 12.03

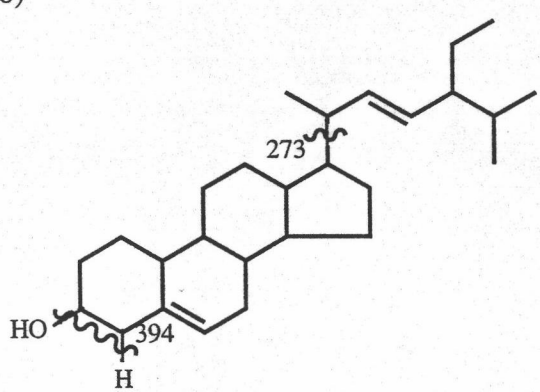
MS spectrum of BB-2 showed the characteristic fragmentation peak of C_{29} steroid. The molecular ion peak observed at *m/z* 414 indicated that compound BB-2 was consist of β-sitosterol. The peak at *m/z* 412 did not agreed with β-sitosterol. It tended to be the molecular ion peak of stigmasterol. The proposed fragmentation of BB-2 are shown below.



(18)

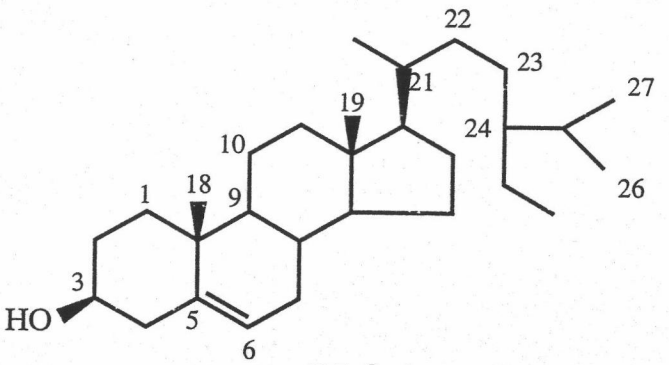


(19)

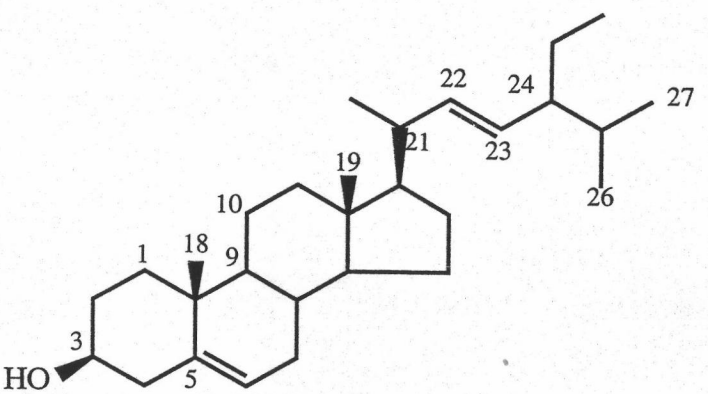


(20)

From the spectroscopic data, BB-2 was proposed to be the mixture of steroids.The identification of this compound was done by means of gas chromatography. By comparing the retention times with the authentic steroids, BB-2 was identified as the mixture of 25.8% β-sitosterol (21) and 74.2% stigmasterol (22).



(21) β-sitosterol



(22) stigmasterol

3. Stucture Elucidation of BB-3

Compound BB-3 was obtained as golden-yellow needle crystals from F-028 (Table 6) by repeated chromatographic techniques using sephadex LH-20 with 10% methanol in chloroform and 5% methanol in chloroform as eluents to yield 8 mg (8 x 10^{-5} % based on dry weight of *B.biternata*).

The APCI spectrum of compound BB-3 (figure 32) exhibited the $[M+H]^+$ at m/z 533(22%) and established the proposed molecular formula of $C_{25}H_{24}O_{13}$. The UV adsorption bands at λ max 250 nm (log ε 4.03) and 408 nm (log ε 4.39) (Figure 16) showed the characteristic of an aurone chromophore. The bathochromic shift (50 nm) of the band II in the UV spectrum with the presence of $AlCl_3$ (Figure 17) was effected

from the acid-labile $AlCl_3$ complex with B ring *ortho*-dihydroxy groups which disappeared when added hydrochloric acid (Markham,1982).

The ^1H NMR spectrum (Figure 18) showed signal of 19 protons, of which six were aromatic or olefinic and thirteen were aliphatic. The singlets at δ 2.01 and 2.03 ppm with the intensity of three protons indicated that there were two acetyl groups. The signal at δ 5.69 ppm (d, J = 7.9 Hz) (Figure 20) showed the characteristic coupling constant of the anomeric proton H-1" of a β-glucose. The six signals of protons attached to oxygenated carbon at δ 4.1-5.52 ppm could be assigned as signals of glucose moiety's protons. The proton H-2" at δ 4.12 ppm (dd, J = 8.6, 8.0 Hz), H-3" at δ 4.37 ppm (dd, J = 9.1, 9.2 Hz), H-4" at δ 5.52 ppm (t, J = 9.8 Hz) showed the large coupling constants indicated *trans* relationship with their adjacent protons. The sugar proton assignment was confirmed by $^1H^1H$ COSY (Figure 22) that showed correlation between the 2-3 bonds neighbouring protons.The acetyl groups were supposed to be located at C-4" and C-6" because the typical downfields of C-4" methine proton and C-6" methylene protons were observed.

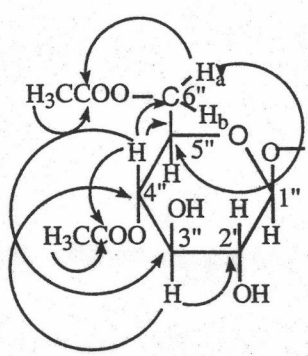
The expansion of ^1H NMR spectrum (7.1-8.2 ppm) (Figure 19) showed signals indicative of the presence of 2,5,6-related aromatic protons, *ortho*-related protons, and one olefinic proton. By comparison of the recorded data with literature values, 1H- signal at δ 7.6 ppm (dd, J = 8.2, 2 Hz) could be assigned as the signal of H-6' proton of ring B that *ortho*-coupled with H-5' (δ 7.13 ppm, d, J = 8.2 Hz) and *meta*-coupled with H-2'(δ 8.09 ppm, d, J = 2 Hz). The singlet at δ 7.18 ppm was assigned as the signal of exocyclic olefinic proton (H-10). The two isolated *ortho*-related proton at δ 7.46 ppm (d, J = 8.3 Hz) and 7.36 ppm (d, J = 8.3 Hz) were H-4 and H-5 of ring A respectively. The assignment of H-4 and H-5 will be discussed later. The relationship of aromatic protons were confirmed by ^1H-^1H COSY(Figure 23).

The ^{13}C NMR spectrum of BB-3 (Figure 24) indicated twentyfive carbon signals. The two acetyl functional groups were confirmed by the presences of carbonyl ester carbons at δ 170.34 and 170.55 ppm and methyl carbons at δ 20.68 and 20.81 ppm. The signal at δ 103.2 ppm was the signal of anomeric carbon C-1". The five signals at δ 60-75 ppm were the signals of sp^3 oxygenated carbons of glucose moiety. The assignment of glucose carbons was achieved by ^{13}C-^1H COSY experiment. From the expansion spectrum of ^{13}C-^1H COSY (Figure 26) the signals at δ 74.56, 75.30,

71.56, 73.09 and 63.04 ppm could be assigned as the signal of C-2", C-3", C-4", C-5" and C-6", respectively since they showed correlations to their proton signals.

The fifteen sp^2-carbons were the carbons of aurone moiety. The ketone carbonyl carbon at δ 184 ppm was unambiguously assigned as C-3 from its typical chemical shift . The methine sp^2-carbon at δ 113.60, 113.84, 114.40, 116.95, 119.35 and 125.62 ppm was assigned by the 1-bond C-H correlation from ^{13}C-^{1}H COSY (figure 26) to be C-5, C-10, C-4, C-5', C-2' and C-6' respectively. The remaining six oxygenated quarternary sp^2-carbon (δ 135-160 ppm) and two non-oxygenated quarternary carbon (δ 119.14 and 124.67 ppm) were assigned by the aid of ^{1}H-detected multiple bond heteronuclear multiple quantum coherent (HMBC) experiment.

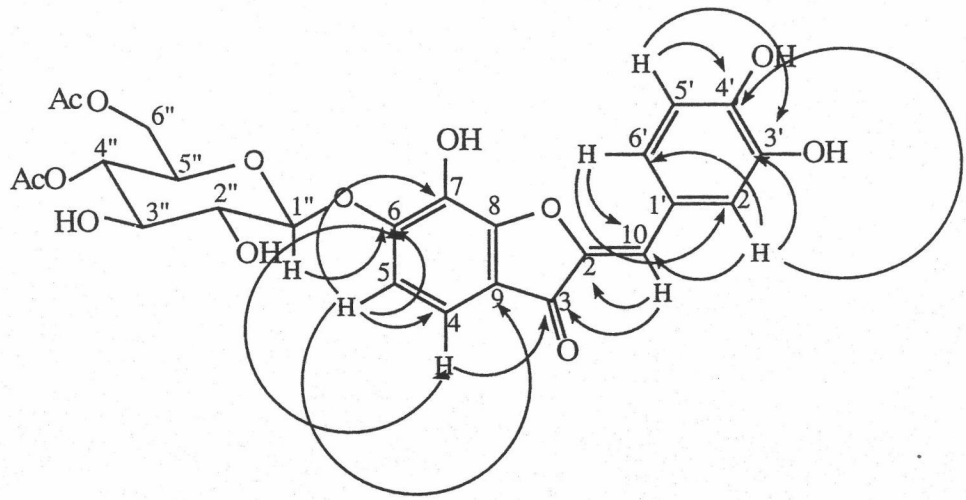
From the HMBC operated at J = 5 Hz spectrum (Figure 27) exhibited the C-H long range (2-3 bonds) correlation of BB-3. The correlations of carbonyl ester carbons of acetyl groups at 170.34 and 170.55 ppm to H-4" and H-6" of sugar moiety respectively confirmed that the acetyl groups attached to 4" and 6"hydroxyl position of glucose. The C-H correlations from HMBC spectrum (Figure 28) of sugar moiety (23) are shown below.



(23)

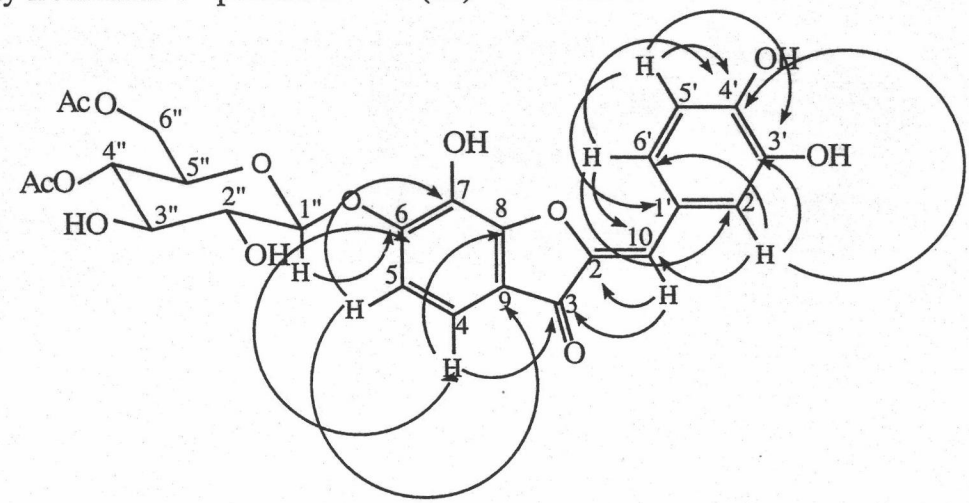
The carbon signals of the aurone moiety were unequivocally assigned by the HMBC operated at J = 5 Hz and 8 Hz. The expansion of HMBC operated at 5 Hz spectrum (Figure 29) exhibited the correlation of methine aromatic proton at δ 7.46 ppm (d, J = 8.3 Hz) to the ketone carbon C-3, thus this methine proton was assigned as H-4 proton that coupled to H-5 proton at δ 7.13 ppm (d, J = 8.3 Hz). H-4 and H-5 protons also showed the correlation to oxygenated quarternary carbon at 153.06 ppm thus this carbon was assigned as C-6 carbon. The oxygenated quarternary carbon at δ 135 ppm could be assigned as C-7 from its correlation to H-5. This C-7 assignment was supported by the fact that the chemical shift of this carbon was relatively upfield

from any oxygenated carbon because of mesomeric effect of electron donating group at C-6 and C-8. The oxygenated quarternary sp^2 carbon at δ 147.49 ppm was assigned as C-2 since there was the correlation between this carbon and H-10. The C-H long-range correlations from HMBC operated at 5 Hz of aurone moiety (24) are shown below.



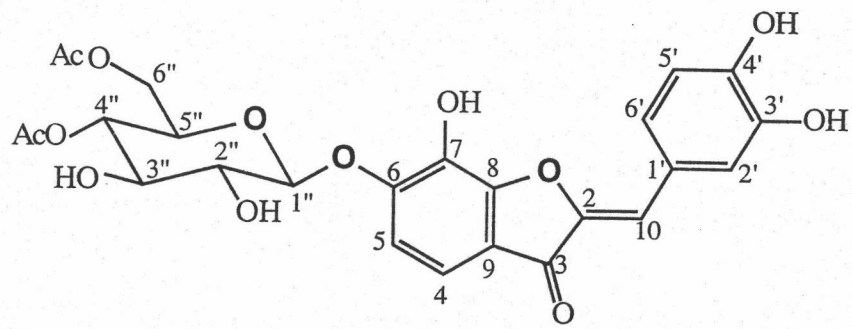
(24)

By the HMBC operated at 8 Hz spectrum (Figure 31), the two oxygenated carbons, C-3' and C-4' could be assigned from the fact that C-4' (δ 149.62 ppm) showed the correlation to H-6'. The C-3' was the carbon signal at δ 146.83 ppm, thus the rest oxygenated quarternary sp^2 carbon (δ 156.22 ppm) was assigned as C-8 which correlated only H-4 proton. The assignments of C-1' and C-9 unoxygenated carbon were clear from the correlation between H-5' to C-1' (δ 124.67 ppm) and H-5 to C-9 (δ 119.14 ppm). The relationship of carbons and neighbouring proton of aurone moiety from HMBC operated at 8 Hz (25) are shown below.



(25)

Both HMBC at 5 Hz and 8 Hz spectra showed the correlation of 1" β-anomeric proton of glucose to C-6. It indicated that sugar moiety attached to the 6-hydroxyl position of aurone. Consequently, the structure of compound BB-3 is, then, identified as *(Z)* -6-O-(4,6-O-diacetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone which is a new member of aurones. The proposed configuration is *Z*-isomer because in the *E*-isomer protons H-2' and H-6' are deshielded by the carbonyl group and have a chemical shift of δ 8.15 (Brady et al, 1973).This geometric isomer is confirmed by the chemical shift of C-10 (113.84 ppm).According to Pelter and Ward (1979), the exocyclic olefinic carbon atom of *E*-isomer locates at more greater down field than this (about 120 ppm). The structure of BB-3 is shown below.



(Z) -6-O-(4,6-O-diacetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone

(26)

The summary of carbon and proton assignments including correlations between protons and carbons by HMBC spectrum are shown in Table 9.

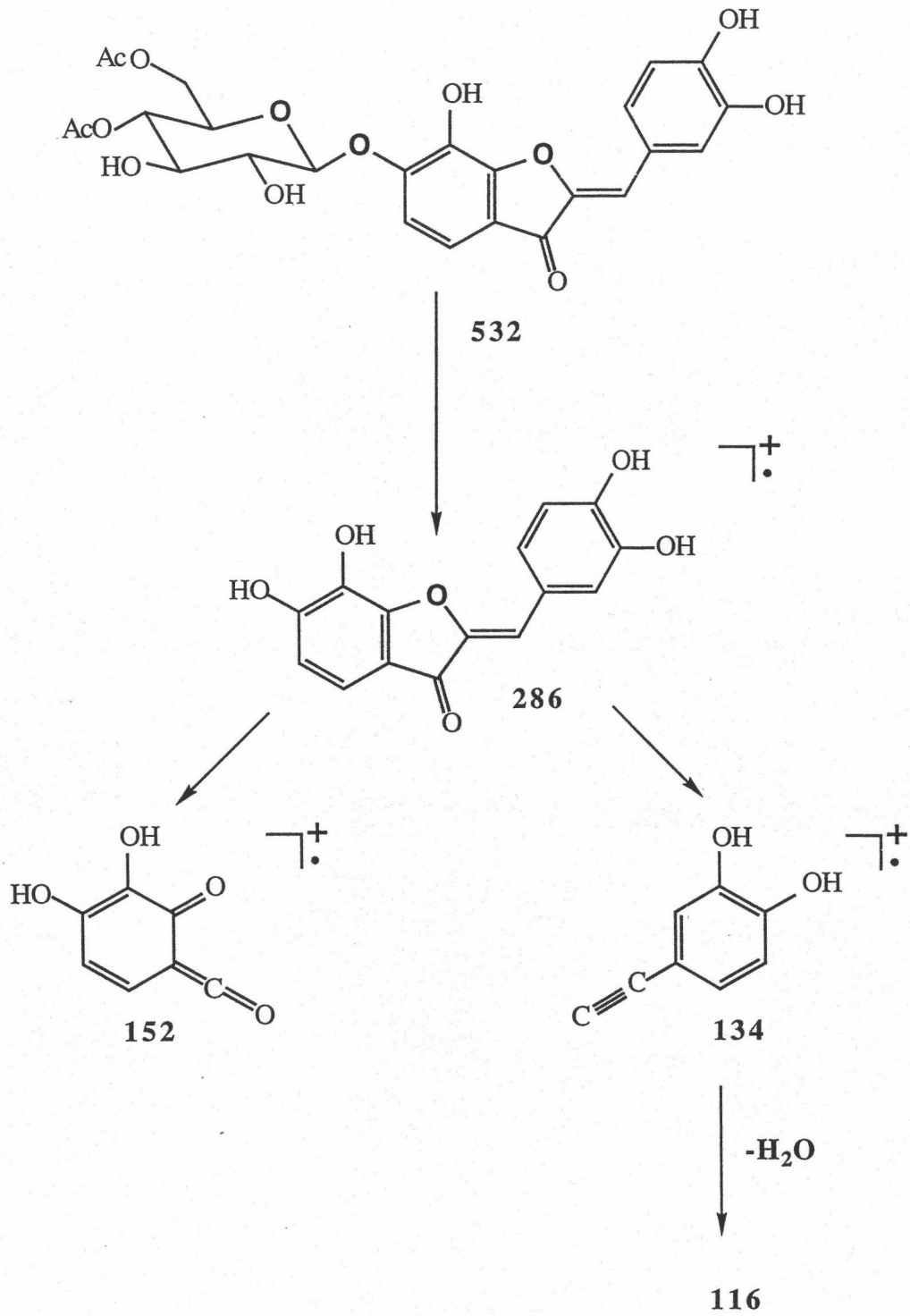
Table 9 Carbon and proton assignments of BB-3 and long-range correlations between protons and carbons in HMBC spectra

Positiond	δC(ppm)	δ H(ppm) (multiplicity, *J* Hz)	long-range correlation from H to C in HMBC spectrum (*J* = 5 Hz)	long-range correlation from H to C in HMBC spectum (*J* = 8 Hz)
2	147.49			
3	184			
4	114.40	7.46(d,8.6)	C-3, C-6	C-3, C-6, C-8
5	113.60	7.36(d,8.6)	C-4, C-6, C-7, C-9	C-7, C-9
6	153.06			

Table 9 (Continued)

Positiond	δC(ppm)	δ H(ppm) (multiplicity, *J* Hz)	long-range correlation from H to C in HMBC spectrum (*J* = 5 Hz)	long-range correlation from H to C in HMBC spectum (*J* = 8 Hz)
7	135.54			
8	156.22			
9	119.14			
10	113.84	7.18(s)	C-6',C-2, C-3	C-6', C-2, C-3
1'	124.67			
2'	119.35	8.09(d,2)	C-3', C-4', C-6', C-10	C-3', C-4', C-6', C-10
3'	146.83			
4'	149.62			
5'	116.95	7.13(d,8.2)	C-3', C-4'	C-1', C-3', C-4'
6'	125.62	7.6(dd,8.2,2)	C-2', C-10	C-2', C-4', C-10
1"	103.2	5.69(d,7.93)	C-6	C-6
2"	74.56	4.12(dd,8.8, 8.2)	C-3"	C-1", C-3"
3"	75.30	4.37 (dd,9.1,9.2)	C-2", C-4"	C- 2", C-4"
4"	71.56	5.52(t,9.8)	C-3", C-5", C-6", 4"-O-carbonyl	C-3", C-5", C-6", 4"-O-carbonyl
5"	73.09	4.24(m)		
6"	63.04	a)4.46 (dd,12.2,5.6) b)4.42 (dd,12.2,2.4)	C-5", 6"-O-carbonyl	6"-O-carbonyl
4"-O carbonyl	170.34			
6"-O carbonyl	170.55			
CH_3 of acetyl group	20.68,20.81	2.01(s),2.03(s)	C-carbonyl of acetyl group	C-carbonyl of acetyl group

This proposed structures of compound BB-3 was finally supported by mass fragmentation (Scheme 4) from EIMS (Figure 33). The loss of sugar moiety caused the fragment at *m/z* 286 (2%). The retro Diels-Alder cleavage of the aurone provided the fragment at *m/z* 152 (3%) and 134 (2%). The fragment of 116 (8%) was caused by the loss of H_2O of fragment at *m/z* 134.



Scheme 4 proposed mass fragmentations of compound BB-3

4. Stucture Elucidation of BB-4

BB-4 was obtained as orange compound from F-028(Table 6) by gel filtration chromatographic techniques using three columns of sephadex LH 20 with eluants of 50%methanol in chloroform, methanol and 5% water in methanol respectively. It yielded 23 mg (2.3 x 10^{-4}% based on dry weight of *B.biternata*).

The APCI spectrum of compound BB-4 exhibited the $[M-H]^+$ at *m/z* 489 and established the proposed molecular formula of $C_{23}O_{12}H_{22}$. The UV absorption bands at a λ max 244 (log ε 3.99) and 409 (log ε 4.26) exhibited the characteristic of an aurone chromophore.The 43 nm bathochromic shift on UV spectrum in the presences of $AlCl_3$ showed the existence of free *ortho*-hydroxyl group in B-ring. The IR spectrum also exhibited the band at ν 3400(broad), 1649 cm^{-1} indicated the presences of hydroxyl and α,β unsaturated ketone groups.

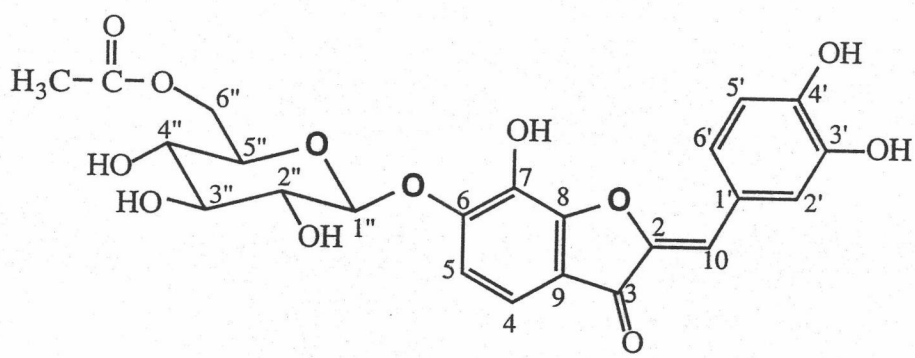
BB-4 could be assigned as a known aurone glycoside, *(Z)* -6-O-(6-acetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone by analyses of its ^1H and ^{13}C NMR spectra. The ^1H NMR spectrum of BB-4 (Figure 34) showed the signals of 6 olefinic or aromatic protons, 7 oxygenated aliphatic protons and 1 acetyl group. The ^{13}C NMR spectrum (Figure 25) suggested the presences of 1 carbonyl carbon, 1 carbonyl ester carbon, 14 sp^2 carbons, 6 oxygenated sp^3 carbons and 1 methyl carbon.

Spectral data of BB-4 was closely related to that of BB-3 except for the absence of one acetyl group at 4" hydroxyl position of glucose informed by the fact that signal of H-4" appeared at higher field than that of BB-3. The acetyl group was substitued at 6" hydroxyl position of glucose because a typical downfield of H-6". The signal at δ 5.15 ppm (d, J = 7.4 Hz) showed the characteristic coupling constant of the anomeric proton H-1" of a β-glucose. The signal of C-1" was located at δ 103.18 ppm in ^{13}C-NMR spectrum.

The 3',4',6,7 tetrahydroxyaurone moiety of BB-4 could be observed by the presence of 2,5,6-related aromatic protons, *ortho*-related protons and one olefinic proton. The signal of H-6' of ring B (δ 7.38 ppm) showed the *ortho* coupling and *meta* coupling to H-5' (d 6.98 ppm) and H-2' (d 7.67 ppm) with coupling constants of 8 and 1.8 Hz respectively. The signal at δ 7.30 ppm coupled with the signal at δ 7.16 ppm with coupling constant 8.5 Hz that could be assigned as the signal of H-4 and H-5 of rong B respectively. The signal of H-5 located at further upfield than that of H-4

because of resonance effect of electron donating group at C-6. The singlet at d 6.81 ppm could be assigned as an exocyclic olefinic proton H-10.

The assignments of proton and carbon were confirmed by comparison of the recorded data with literature value. Thus, BB-4 was determined as *(Z)*-6-O-(6-acetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone, which has been isolated previously from *B. pilosa* Linn. by Sashida *et al* (1991). The structure of BB-4 is shown below. The proton and carbon assignments of BB-4 are summerized in Table 10.

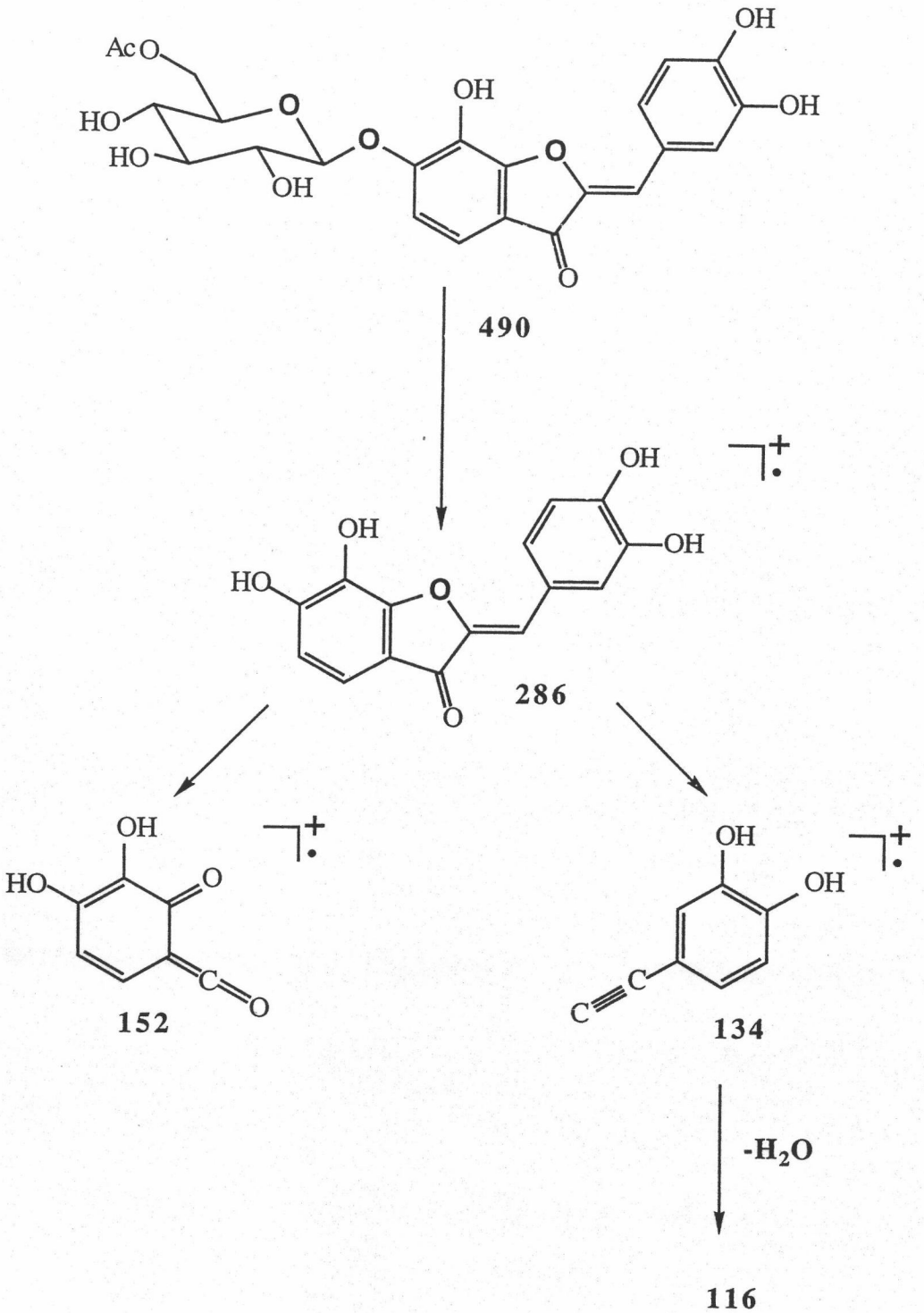


(Z) -6-O-(6-acetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone (27)

Table 10 Proton and carbon assignments of BB-4

Position	δC (ppm)	δH (ppm) (multiplicity, *J* Hz)
2	147.39	
3	185.07	
4	115.53	7.30 (d, 8.6)
5	113.61	7.16 (d, 8.3)
6	153.54	
7	134.64	
8	156.21	
9	119.44	
10	115.32	6.81 (s)
1'	125.36	
2'	119.23	7.67 (d, 1.8)
3'	147.02	
4'	149.54	
5'	116.89	6.93 (d, 8)
6'	126.65	7.38 (dd, 8, 1.8)
1"	103.18	5.15 (d, 7.4)
2"	74.81	3.73-3.75
3"	77.58	(overlapping)
4"	71.55	3.58 (dd, 8.9, 9.1)
5"	75.80	3.85 (ddd, 9.6, 6.7, 2.1)
6"	64.65	4.56 (dd, 11.9, 1.9)
		4.38 (dd, 11.9, 6.4)
Carbonyl ester	172.17	
Methyl	20.80	

This structure was confirmed by the analyses of mass fragmentations (Scheme 5) from EIMS (Figure 42). The fragment of an aglycone was observed at *m/z* 286. The other fragments were provided by the retro Diels-Alder cleavage like those of BB-3 that were discussed before.



Scheme 5 Proposed mass fragmentations of compound BB-4