

CHAPTER IV

RESULTS AND DISCUSSION

By means of several chromatographic techniques, two compounds, C-1 and C-2 were isolated from crude hexane extract of the stem bark of *Croton roxburghii* N.P. Balakr.

Spectroscopic data (UV, IR, MS, and NMR) were used to determine the chemical structure of the two compounds. The structures were confirmed by comparative analysis using previous reports as references.

Structure Determination of the isolated compounds

1. Structure determination of compound C-1

Compound C- 1 was obtained as colourless prism crystal (2.03 g) with a melting point of 147-148°C. The FT-IR spectrum (Figure 12) displayed bands indicating a conjugated carbonyl group (1655 cm^{-1}).

Table 6. The IR absorption band assignments of compound C-1

Wave number(cm^{-1})	Tentative assignments
3025, 3075	alkene C-H stretching
2860-2960	alkane C-H stretching
1655	C=O stretching
1615, 994, 913, 825	C=C stretching & bending

The ^1H -NMR spectrum (Figure 14) of compound C-1 showed five methyl group at δ_{H} 1.05 (3H, s; H-20), 1.08 (3H, s; H-19), 1.16 (3H, s; H-18), 1.32 (3H, s; H-16), 1.38 (3H, s; H-17) and five olefinic protons at 4.94 (1H, dd; H-15a), 5.15 (1H, dd; H-15b), 5.86 (1H, d; H-2), 5.89 (1H, dd; H-14), 7.10 (1H, d; H-1).

The ^{13}C -NMR spectrum (Figure 15) of compound C-1 showed twenty carbon resonances, four of which are olefinic carbons (δ_{C} 110.7, 125.8, 147.4, 157.6) and one of ketocarbonyl carbon (δ_{C} 205.1) was also observed.

In DEPT experiment (Figure16), three sp^2 methine carbon signals (δ_{C} 125.8, 147.4 and 157.6) together with two saturated methine carbons (δ_{C} 49.9 and 53.2) and five methylene carbon signals at δ_{C} 15.5, 20.2, 35.4, 44.6 and 110.7 were shown. The downfield δ_{C} 110.7 signal was assigned as a terminal methylene carbon. Five methyl signals resonated at δ_{C} 18.6, 21.9, 25.6, 27.6, 28.6. The two most downfield methyl signals their proximity to an oxygen atom in the molecule. According to the ^{13}C -NMR and experiment DEPT , it could be concluded that there were five quaternary carbons (δ_{C} 39.4, 42.3, 73.7, 75.0 and 205.1) in this structure.

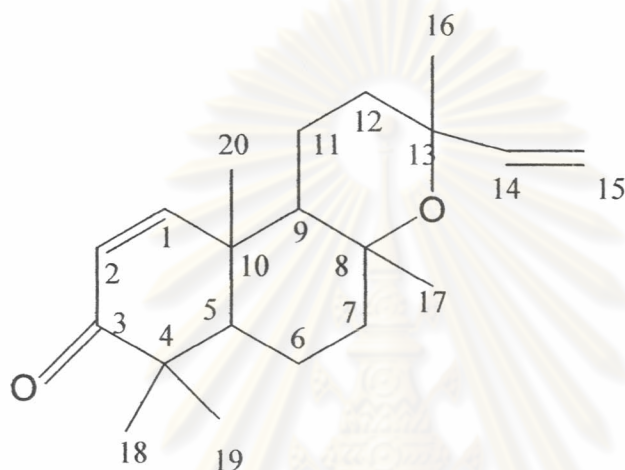
In the EI-MS spectrum (Figure 13), compound C-1 gave a molecular ion peak $[\text{M}]^+$ at m/z 302. The base peak at m/z 287 indicated the loss of a CH_3 group from the molecule.

The molecular formula of compound C-1 was assigned as $\text{C}_{20}\text{H}_{30}\text{O}_2$ based on elemental analysis, ^1H and ^{13}C NMR spectra (Table 7) and EIMS. The IR stretching indicated conjugated carbonyl group at 1655 cm^{-1} , olefinic groups at $1615, 994, 913, 825\text{ cm}^{-1}$. The ^{13}C NMR spectrum and DEPT experiments

revealed the presence of 20 non-equivalent carbons, of which 15 are sp^3 (five methyl, four methylene, two methine and four quaternary carbons) and four sp^2 (one methylene, three methine carbons) hybridized carbons, together with a carbonyl carbon [δ_c 205.1(s)], two double bond groups [δ_c 125.8 (d), 157.6 (d), 110.7 (t), 147.4 (d)] and two oxygenated carbons [δ_c 73.7 (s), 75.0 (s)]. The molecular formula $C_{20}H_{30}O_2$ of compound C-1 defined a degree of unsaturation of six; therefore, compound C-1 must consist of three rings in addition to the two double bonds and one carbonyl group. Several 2D-NMR techniques were then used to assist in the interpretation of the structure of this compound. All of the proton-proton spin systems were traced by using data from a COSY experiment (Figure 17). Heteronuclear correlation experiments, HMQC (Figure 18) and HMBC (Figure 19) allowed unambiguous assignment of all 1H -NMR and ^{13}C -NMR resonances in compound C-1.

From HMBC spectrum, methyl groups at 1.16 ppm (H-18) and 1.08 ppm (H-19) correlated with a conjugated carbonyl at 205.1 ppm. Therefore, the carbonyl group must be C-3 and the olefinic double bond was between C-1 and C-2. The methyl group at 1.05 ppm (H-20) correlated with olefinic carbon at 157.6 ppm. Therefore, this carbon must be C-1 and a carbon at 125.8 ppm must be C-2 with confirmed by the COSY spectrum that showed correlated between H-1 and H-2. The HMBC spectrum showed methyl group at 1.32 ppm (H-16) correlated with olefinic carbons at 147.4 ppm (C-14) and 110.7 ppm (C-15) with confirmed by the COSY spectrum that showed correlated between H-14 and H-15 together. Moreover, the methyl group at 1.32 ppm (H-16) was correlated with

a quaternary carbon at 73.7 ppm (C-13) that carbon connected to an oxygen atom and the methyl group at 1.38 ppm (H-17) was correlated with a quaternary carbon at 75.0 ppm (C-8) together. Thus the cyclic ether must form between C-13 and C-8. Therefore, the structure of compound C-1 should be as the following



From a comparison of the ^{13}C NMR data of compound C-1 with that of ribenone (Fraga, B. M. *et al.*, 1999) (Table 8). It was deduced that C-1 possesses olefinic carbon signals at δ_{C} 157.6 ppm and 125.8 ppm instead of methylene carbon signals at δ_{C} 38.1 ppm and 33.7 ppm at position C-1 and C-2 in ribenone, namely, 8, 13-epoxylabda-1,4-diene-3-one. This was verified by ^1H - ^{13}C chemical shift correlation spectroscopy (COSY) NMR. The relative stereochemistry of compound C-1 was established by X-ray crystallography (Figure 6). The circular dichroism (CD) spectrum of compound C-1 showed a positive cotton effect at 342.6 nm and negative at 260.6 nm (Figure 11), analogous to that of ribenone. Therefore, the absolute stereostructure of compound C-1 was determined to be as (5*S*, 8*S*, 9*S*, 10*R*, 13*S*)- 8, 13-epoxylabda-1,4-diene-3-one (Figure 5).

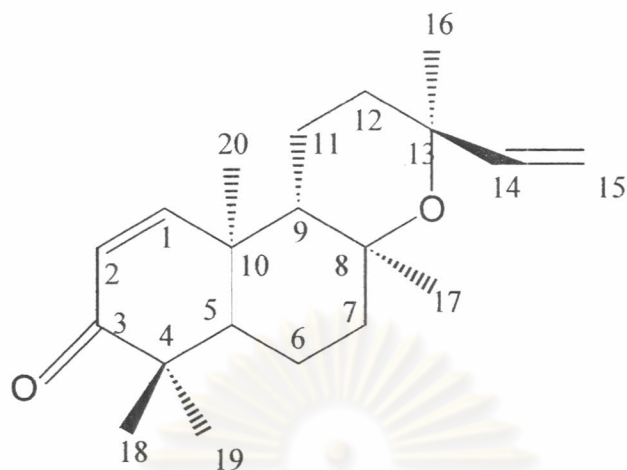


Figure 5: Structure of compound C-1

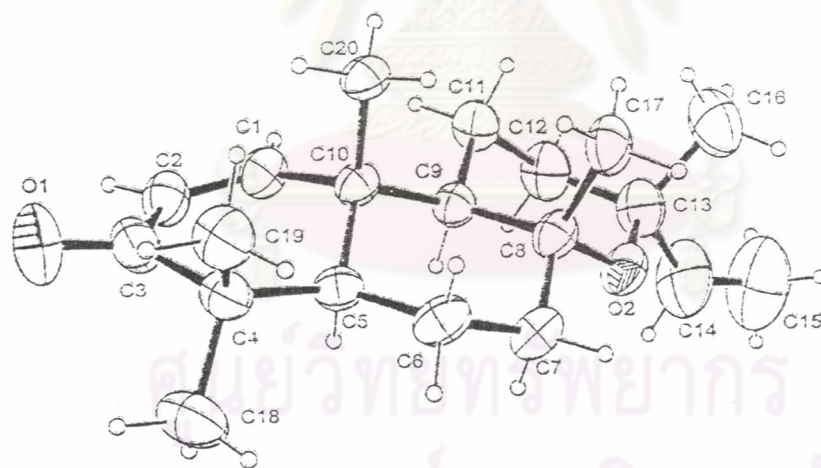


Figure 6: ORTEP structure of compound C-1

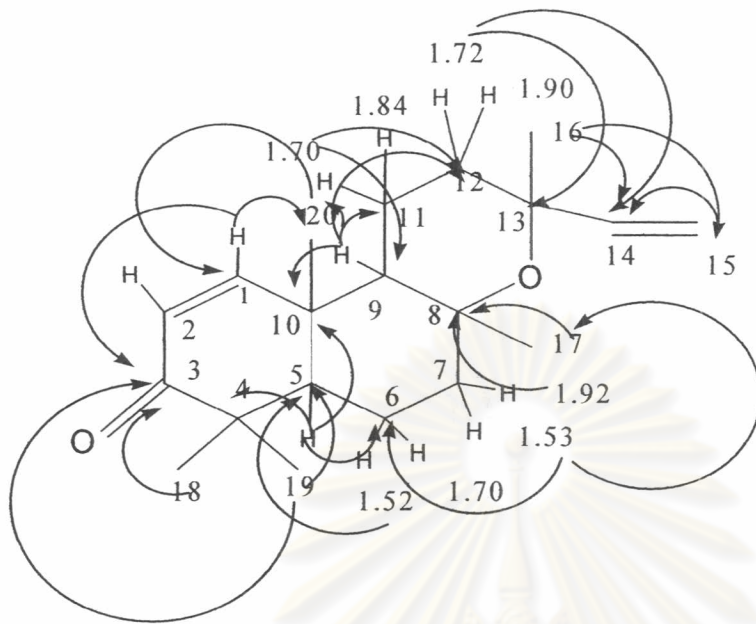


Figure 7: Long-range correlation from HMBC spectrum of compound C-1

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Table 7 $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and HMBC spectral data of compound C-1

Position	δ_{C} (ppm)	δ_{H} (ppm) J (Hz)	HMBC
1	157.6	7.10 d ($J=10.0$)	C-3, C-6, C-20
2	125.8	5.86 d ($J=10.0$)	-
3	205.1	-	-
4	42.3	-	-
5	53.2	1.78 m	C-4, C-6, C-7, C-9, C-10, C-20
6	20.2	1.52 m	C-4, C-5, C-8, C-17
		1.70 m	C-5, C-8
7	44.6	1.53 m	C-6, C-17
		1.92 m	C-5, C-8, C-9
8	75.0	-	-
9	49.9	1.62 m	C-8, C-10, C-11, C-12, C-17, C-20
10	39.4	-	-
11	15.5	1.70 m	C-9, C-12, C-13
		1.84 m	C-8, C-16
12	35.4	1.72 m	C-9, C-11, C-13, C-14, C-16
		1.90 m	C-9
13	73.7	-	-
14	147.4	5.89 dd ($J=16.0, 10.0$)	-
15	110.7	4.94 dd ($J=10.0, 2.0$)	-
		5.15 dd ($J=18.0, 2.0$)	C-14
16	28.6	1.32 s	C-12, C-13, C-14, C-15,
17	25.6	1.38 s	C-8, C-9
18	27.6	1.16 s	C-3, C-5, C-19
19	21.3	1.08 s	C-3, C-5, C-18
20	18.6	1.05 s	C-1, C-9, C-10

Table 8 ^{13}C NMR data of compound C-1 and ribenone

Position	δ_{C} Compound C-1 (ppm)	δ_{C} Ribenone (ppm)
1	157.6	38.1
2	125.8	33.7
3	205.1	217.4
4	42.3	47.2
5	53.2	54.5
6	20.2	20.7
7	44.6	42.0
8	75.0	75.5
9	49.9	57.5
10	39.4	36.3
11	15.5	16.3
12	35.4	34.7
13	73.7	73.6
14	147.4	147.2
15	110.7	109.7
16	28.6	32.5
17	25.6	23.2
18	27.6	26.6
19	21.3	20.8
20	18.6	15.4

2. Structure determination of compound C-2

Compound C-2 was obtained as long prism crystal (1.02g) with a melting point of 141-142 °C. The FT-IR spectrum of compound C-2 (Figure 22) displayed bands indicating a hydroxyl group at 3521 cm⁻¹ and a conjugated carbonyl group at 1660 cm⁻¹.

Table 9 The IR absorption band assignments of compound C-2

Wave number(cm ⁻¹)	Tentative assignments
3521	O-H stretching
3086	alkene C-H stretching
2925	alkane C-H stretching
1660	C=O stretching
1463, 992, 915, 828	C=C stretching & bending
1156, 1082	C-O-C stretching

The ¹H-NMR spectrum (Figure 24) of compound C-2 showed five methyl group at δ_{H} 1.04 (3H, s; H-20), 1.09 (3H, s; H-19), 1.16 (3H, s; H-18), 1.38 (3H, s; H-17), 1.39 (3H, s; H-16) and five olefinic protons at 5.26 (1H, dd; H-15a), 5.44 (1H, dd; H-15b), 5.81 (1H, dd; H-14), 5.88 (1H, d; H-2), 7.13 (1H, d; H-1).

The ¹³C-NMR spectrum (Figure 25) of compound C-2 showed twenty carbon resonances, four of which are olefinic carbons (δ_{C} 125.9, 142.5, 115.8, 157.3) and one ketocarbonyl carbon (δ_{C} 205.0) was also observed.

In DEPT experiment (Figure 26), three sp^2 methine carbon signals (δ_C 125.9, 142.5 and 157.3) were shown, together with three saturated methine carbons (δ_C 43.5, 53.2 and 70.0) and showed four methylene carbon signals at 20.1, 22.9, 41.5 and 115.8. The downfield δ_C 115.8 signal implied that methylene carbon should be olefinic carbon. Five methyl signals were shown at δ_C 18.9, 21.2, 25.3, 27.4, 27.6. According to the ^{13}C -NMR, DEPT data, it was concluded that there were five quaternary carbons (δ_C 38.7, 44.6, 75.3, 76.7 and 205.0) in this structure.

In the EIMS spectrum (Figure 23), compound C- 2 gave a molecular ion peak $[\text{M}]^+$ at m/z 318. Its mass spectrum exhibited a peak at m/z 300 corresponding to $[\text{M}^+ - \text{H}_2\text{O}]$.

Several 2D-NMR techniques were then used to assist the interpretation of the structure of this compound. All of the proton-proton spin systems were traced by using data from a COSY experiment (Figure 27). Heteronuclear correlation experiments, HMQC (Figure 28), HMBC (Figure 29) and NOESY (Figure 30) allowed unambiguous assignment of all ^1H -NMR and ^{13}C -NMR resonances in compound C-2.

The molecular formula of compound C-2 was assigned as $\text{C}_{20}\text{H}_{30}\text{O}_3$ base on elemental analysis, ^1H and ^{13}C NMR spectra and EIMS that defined a degree of unsaturation of six. The IR spectrum of compound C-2 showed a hydroxyl (3521 cm^{-1}), conjugated ketocarbonyl (1660 cm^{-1}), olefinic ($1463, 992, 915, 828\text{ cm}^{-1}$) and ether ($1156, 1082\text{ cm}^{-1}$) groups.

The ^1H and ^{13}C NMR spectra of compound C-2 were similar to those of compound C-1 (Table 11), except at methine proton on the carbon bearing a hydroxyl group of H-12 [δ_{H} 3.80 (q, $J = 2.5$ Hz)] and at oxygenated carbon of C-12 [δ_{C} 70.0 (s)]. From the HMBC spectrum, the methyl group at 1.39 ppm (H-16) correlated with an oxygen bearing carbon at 70.0 ppm. Therefore the hydroxyl group must connect to C-12. The compound C-2 was assigned as 8,13-epoxy-12-hydroxylabda-1,14-diene-3-one. These observation showed that the relative stereochemistry of compound C-2 was the same as compound C-1. The circular dichroism (CD) spectrum of compound C-2 showed a positive cotton effect at 342.6 nm and negative at 260.8 nm (Figure 21), analogous to that compound C-1 was determined to be (5*S*, 8*S*, 9*S*, 10*R*, 12*S*, 13)- 8, 13-epoxy-12-hydroxylabda-1, 14-diene-3-one (Figure 8).

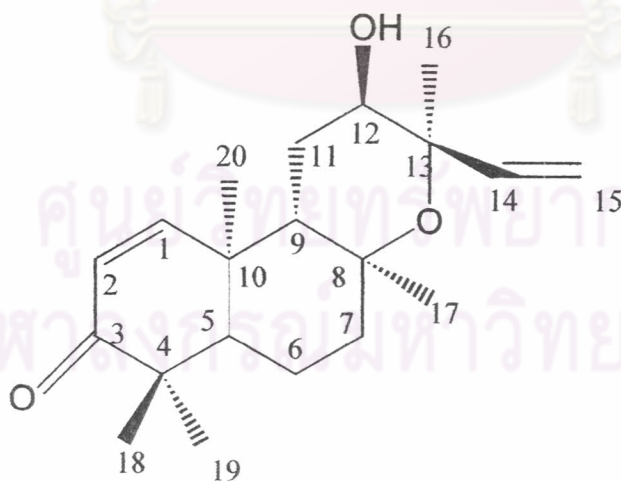


Figure 8: Structure of compound C-2

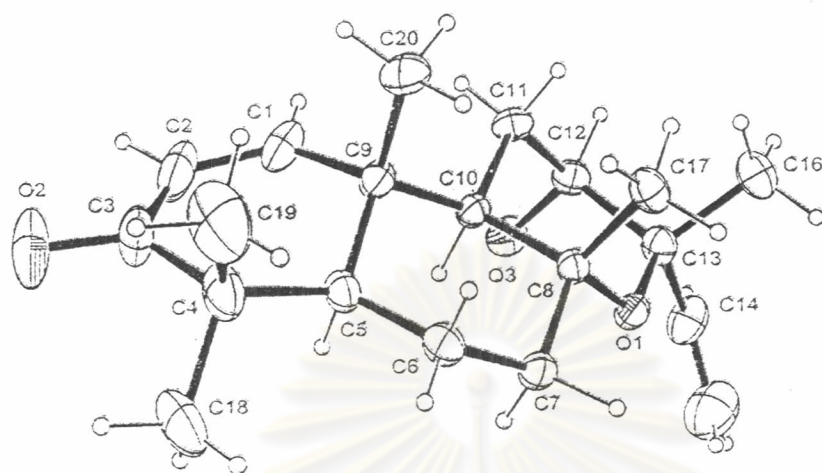


Figure 9: ORTEP structure of compound C-2

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Table 10. $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and HMBC spectral data of compound C-2

Position	δ_{C} (ppm)	δ_{H} (ppm) J (Hz)	HMBC
1	157.3	7.13 d ($J = 10.0$)	C-5, C-6, C-9, C-10
2	125.9	5.88 d ($J = 10.0$)	C-4, C-10
3	205.0	-	-
4	44.6	-	-
5	53.2	1.85 dd ($J = 10.0, 5.0$)	C-4, C-6, C-7, C-9, C-10, C-11, C-18, C-19, C-20
6	20.1	1.52 m	C-5, C-17, C-18
		1.72 dd ($J = 13.0, 3.0$)	C-7, C-10
7	41.5	1.58 m	C-5, C-6, C-10, C-13, C-17
		1.92 m	C-8, C-9
8	76.7	-	-
9	43.5	2.09 dd ($J = 10.0, 6.0$)	C-5, C-10, C-11, C-12, C-13, C-17, C-20
10	38.7	-	-
11	22.9	1.98 m,	-
		1.94 m	-
12	70.0	3.80 q ($J = 2.5$)	C-9
13	75.3	-	-
14	142.5	5.81 dd ($J = 18.0, 12.0$)	C-8, C-16
15	115.8	5.26 dd ($J = 12.0, 2.5$)	C-8, C-16
		5.44 dd ($J = 18.0, 2.5$)	C-14
16	27.4	1.39 s	C-12, C-13, C-14
17	25.3	1.38 s	C-16
18	27.6	1.16 s	C-4, C-5, C-19
19	21.2	1.09 s	C-18
20	18.9	1.04 s	C-1, C-5, C-9, C-10

Table 11. The ^1H and ^{13}C NMR spectra of compound C-1 and compound C-2.

Position	δ_{C} (ppm)		δ_{H} (ppm)	
	Compound C-1	Compound C-2	Compound C-1	Compound C-2
1	157.6	157.3	7.10	7.13
2	125.8	125.9	5.86	5.88
3	205.1	205.0	-	-
4	42.3	44.6	-	-
5	53.2	53.2	1.78	1.85
6	20.2	20.1	1.52	1.52
			1.70	1.72
7	44.6	41.5	1.53	1.58
			1.92	1.92
8	75.0	76.7	-	-
9	49.9	43.5	1.62	2.09
10	39.4	38.7	-	-
11	15.5	22.9	1.70	1.98
			1.84	1.94
12	35.4	70.0	1.72	3.80
			1.90	
13	73.7	75.3	-	-
14	147.4	142.5	5.89	5.81
15	110.7	115.8	4.94	5.26
			5.15	5.44
16	28.6	27.4	1.32	1.39
17	25.6	25.3	1.38	1.38
18	27.6	27.6	1.16	1.16
19	21.3	21.2	1.08	1.09
20	18.6	18.9	1.05	1.04

3. Results of Biological activity

The *in vitro* activity of some compounds (10 μ g/ml) from *Croton roxburghii*. against 5 cell lines, for example, KATO-3 (gastric cancer), SW 620 (colon cancer), BT 474 (breast cancer), HEP-G2 (hepatoma) and CHAGO (lung cancer) are reported in Table 12.

Table 12. Cytotoxicity data of the diterpenes from *Croton roxburghii*

Compounds (10 μ g/ml)	% Survival				
	KATO-3	SW620	BT474	HEP-G2	CHAGO
[1]	35	45	67	38	73
[2]	54	74	103	59	76
Hexane extract	19	6	38	13	29
EtOAc extract	17	8	31	13	8
Acetone extract	16	8	31	13	19

The results from table 12 showed that, compound C-1 and compound C-2 exhibited weak cytotoxic activity against all cancer cell lines. Therefore, hydroxyl group at 12 position of compound C-2 was not significantly to increase cytotoxic activity. Crude extracts (hexane extract, EtOAc extract and acetone extract) of this plant, showed more cytotoxic activity than isolated compounds (compound C-1 and compound C-2). Therefore, crude extracts should be have other isolated compounds that show higher cytotoxic activity than compound C-1 and compound C-2.