CHAPTER IV

RESULTS AND DISCUSSION

1. Isolation of C3, BB21-C and BB28-C

Chemical constituents of the ethyl acetate extract from the culture broth of the endophyte, *Phomopsis* sp. AANN8 were investigated by antileukemic-guided fractionation. Initially, the crude extract showed cytotoxicity toward humam acute monocytic leukemia (THP-1) cell lines at EC_{50} 4.69 mg/ml. Afterward the white precipitate BC was obtained from the crude extract and was further purified by a Sephadex LH-20 column and crystallization to provide compound C3. The residue fraction BB1 from the crude extract was fractionated by a silica gel column to yield 5 subfractions (BB2-BB6). Among the five subfractions obtained, BB4 exhibited the most potent antileukemic activity. Therefore, subfraction BB4 was selected for the next separation step by a Sephadex LH-20 column to provide 7 subfractions (BB7-BB13). Only subfractions BB8-BB10 having suitable amounts for further separation steps were choosen for cytotoxicity testing. The result showed that subfraction BB10 displayed higher potent antileukemic activity than the others and was selected for subsequent isolation by a silica gel column and a C-18 reversed phase HPLC column to give compound BB21-C. Finally, a silica gel column and a C-18 reversed phase HPLC column were employed to isolate compound BB28-C from subfraction BB9 displaying an interesting TLC pattern. The structure determination of all three isolates (C3, BB21-C and BB28-C) was achieved through spectroscopic techniques, including

MS and NMR, and further confirmed by comparison with literatures. The isolated compounds were examined for their antileukemic activity against THP-1 cell line.

2. Structure Determination of C3

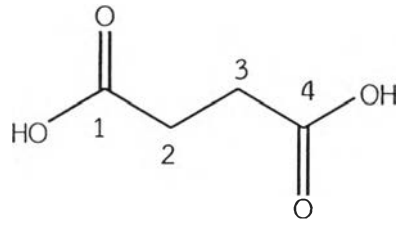
Compound C3 was obtained as colorless needles (783.7 mg, 1.96 %w/v yield of the broth). According to its FABMS (**Figure 15**) showing a pseudomolecular ion peak at *m/z* 117 [M-H], the molecular formula was suggested as $C_4H_6O_4$ with two degrees of unsaturation. The IR spectrum (**Figure 14**) showed absorption bands at 3041 (very broad) and 1694 cm^{-1}, indicating the presence of the carboxylic functional group.

Based on the NMR and MS data, this compound could be a symmetry molecule. The ^{1}H (**Table 4, Figure 16**) and ^{13}C (**Table 4, Figure 17**) NMR spectra respectively showed a singlet at δ_H 2.58 (H_2-2 and H_2-3) and a singlet at δ_c 28.4 (C-2 and C-3), corresponding to two methylene groups. In addition, a carbonyl signal at δ_c 174.8 (C-1 and C-4) was readily assigned to two carboxylic carbonyl carbons.

Based on the above spectral evidence and by comparison of its NMR data with those previously published (Choi *et al.*, 2006) as shown in **Table 4**, compound C3 was identified as a dicarboxylic acid, succinic acid **[46]**.

Succinic acid (1,4-butanedioic acid) is an intermediate of the Kreb's cycle and one of a fermentative byproduct. Several microorganisms, mostly bacteria such as *Actinobacillus succinogenes* (Guettler *et al.*, 1999), *Anaerobiospirillum succiniciproducens* (Lee *et al.*, 2001) and recombinant *Escherichia coli* (Lin *et al.*, 2005) as well as fungi, *Aspergillus fumigatus* (Ling *et al.*, 1978), *Penicillium simplicissimum* (Gallmetzer *et al.*, 2002) and *Saccharomyces cerevisiae* (Magnuson

and Lasure 2004), were screened and studied for succinic acid natural and non-natural production. However, this is the first report of succinic acid produced from the fungus *Phomopsis*. Amount of succinic acid produced in high yield from the fungus *Phomopsis* AANN8 was close to amount from the natural bacteria but less than recombinant *E. coli* about 8 times (Beauprez *et al.*, 2010). Succinic acid has been wildly used in food industrial as a flavoring agent or preservative and also in pharmaceutical industrial as a surfactant, chelating agent, pH modifier and excipient in effervescent tablets (Zeikus *et al.*, 1999). Due to the large quantity of succinic acid utilization, this fungus may be applied for commercial natural succinic acid production for industrials. Furthermore, a succinic acid derivative, 2-hexylidene-3-methyl-succinic acid, was reported to exhibit potent cytotoxic activity against K562 cell line by apoptosis with EC_{50} 4.81 μg/ml (Hazalin *et al.*, 2012).



Succinic acid **[46]**

Table 4. ^{1}H- and ^{13}C-NMR spectral data of C3 in CD_3OD.

Position	C3		Succinic acid*	
	δ_C	δ_H, *mult.*	δ_C	δ_H, *mult.*
1, 4	174.8	-	179.9	-
2, 3	28.4	2.58, 4H, *s*	31.8	2.54, 4H, *s*

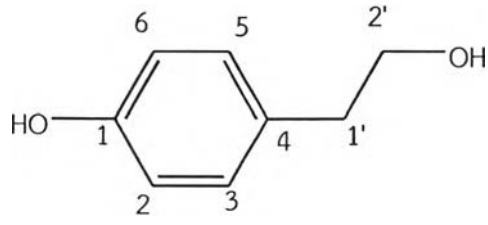
*(Choi *et al.*, 2006)

3. Structure Determination of BB21-C

BB21-C was obtained as colorless needles (9.5 mg, 0.02 %w/v yield of the broth) and appeared as a red spot on a silica gel TLC plate upon spraying with anisaldehyde-sulfuric acid reagent (Rf = 0.5, mobile phase = hexane : EtOAc 4:6). The HR-EIMS (**Figure 20**) showed a molecular ion peak at *m/z* 138.0680, corresponding to its molecular formula of $C_8H_{10}O_2$ (calc *m/z* 138.0681) with four degrees of unsaturation. The IR spectrum (**Figure 19**) displayed a broad absorption of hydroxyl stretching at 3151 cm^{-1}. Furthermore, the IR spectrum exhibited absorption bands at 3392, 1598, 1512 and 818 cm^{-1}, revealing the presence of a 1,4-disubstituted aromatic ring.

The ^{1}H-NMR spectrum (**Table 5, Figure 21**) showed two doublets of four aromatic protons at δ_H 7.03 (H-3 and H-5) and 6.72 (H-2 and H-6) with an *ortho* coupling constant of 8.5 Hz indicating a 1,4-disubstituted phenyl ring and a pair of triplets of two coupled methylenes at δ_H 3.66 (H_2-2′) and 2.69 (H_2-1′). The ^{13}C NMR (**Table 5, Figure 22**) and DEPT135 (**Figure 23**) spectra confirmed the 1,4-disubstituted phenyl moiety by a pair of two-carbon signals of aromatic methine carbons at δ_C 130.6 (C-3 and C-5) and 115.8 (C-2 and C-6), one oxygenated quaternary aromatic carbon at δ_C 156.5 (C-1) and one quaternary aromatic carbon at δ_C 131.6 (C-4) as well as the ethylene moiety by two methylene carbons at δ_C 64.2 (C-2′) and 39.4 (C-1′). Through comparison of NMR data of BB21-C with previous reports (Park *et al.*, 2011) (**Table 5**) led to the identification of this compound as tyrosol **[47]**.

Tyrosol (4-hydroxyphenethyl alcohol) was reported as a well-known phenolic compound presented in plants, such as *Acorus gramineus* (Park *et al.*, 2011) and *Olea europaea* (Briante *et al.*, 2002) as well as fungi, such as *Neofusicoccum parvum* (Evidente *et al.*, 2010) and *Glomerella cingulata* (Guimarães *et al.*, 2008). However, this compound was also isolated from the endophytic fungus *Phomopsis oblonga* in the outer bark of *Ulmus glabra* (Claydon *et al.*, 1985). In addition, tyrosol was reported to possess various biological activities such as anti-inflammatory activity by inhibiting 5-lipoxygenase enzyme with an EC_{50} 0.5 mM (de la Puerta *et al.*, 1999) and antioxidant activity with an EC_{50} 1 mM (de la Puerta *et al.*, 2001). Moreover, tyrosol also exhibited a dose-dependent neuroprotective effect against the sensory motor dysfunction that peaked at 64.9 % in rats treated with 30 mg/kg of tyrosol (Bu *et al.*, 2007).



Tyrosol **[47]**

Table 5. ^{1}H- and ^{13}C-NMR spectral data of BB21-C in acetone-d_6.

Position	BB21-C		Tyrosol*	
	δ_C	δ_H, *mult.* (*J* in Hz)	δ_C	δ_H, *mult.* (*J* in Hz)
1	156.5	-	155.6	-
2, 6	115.8	6.72, 2H, *d* (8.5)	114.9	6.70, 2H, *dd* (8.0, 2.1)
3, 5	130.6	7.03, 2H, *d* (8.5)	129.7	7.02, 2H, *dd* (8.0, 2.1)
4	131.6	-	130.7	-
1'	39.4	2.69, 2H, *t* (7.2)	38.2	2.71, 2H, *t* (7.0)
2'	64.2	3.66, 2H, *t* (7.2)	63.4	3.67, 2H, *t* (7.3)
1-OH	-	8.15, 1H, *br*	-	-
2'-OH	-	3.69, 1H, *br*	-	-

*(Park *et al.*, 2011)

4. Structure Determination of BB28-C

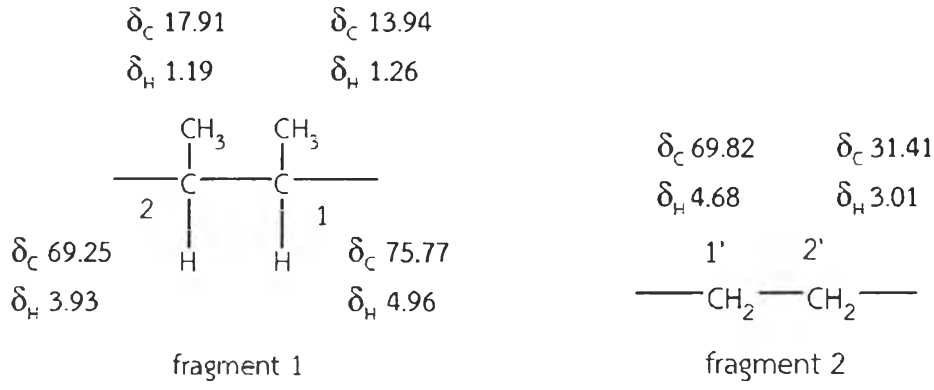
BB28-C was obtained as a colorless amorphous powder (6.9 mg, 0.02 %w/v yield of the broth) and appeared as a blue spot on a silica gel TLC plate upon spraying with anisaldehyde-sulfuric acid reagent (Rf = 0.5, mobile phase = hexane : EtOAc 3:7). The IR spectrum (**Figure 24**) exhibited a broad strong absorption of hydroxyl stretching at 3435 cm^{-1} and an absorption band at 1733 cm^{-1}, indicating the presence of the carbonyl functional group.

The ^{1}H-NMR spectrum (**Table 6**, **Figures 26** and **27**) displayed two sets of the similar coupled protons with the ratio of 3:2, suggesting the mixture of two closely related compounds. The proton signals of the major component were two coupled methylene protons at δ_H 4.68 (2H, t, *J*=6.0 Hz) and 3.01 (2H, t, *J*=6.0 Hz), two methine protons at δ_H 4.96 (1H, dq, *J*=6.3, 3.3 Hz) and 3.93 (1H, dq, *J*=6.3, 3.3 Hz), and two methyl protons at δ_H 1.26 (3H, d, *J*=6.3 Hz) and 1.19 (3H, d, *J*=6.3 Hz). The proton signals of the minor component also appeared as two coupled methylene protons at δ_H 4.69 (2H, t, *J*=6.0 Hz) and 3.02 (2H, t, *J*=6.0 Hz), two methine protons at δ_H 4.86 (1H, pentet, *J*=6.3 Hz) and 3.79 (1H, pentet, *J*=6.3 Hz), and two methyl protons at δ_H 1.27 (3H, d, *J*=6.3 Hz) and 1.21 (3H, d, *J*=6.3 Hz).

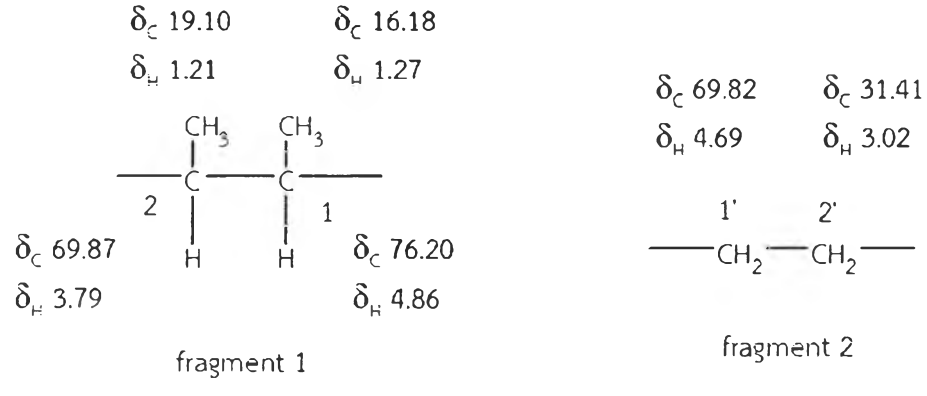
The ^{13}C-NMR (**Table 6**, **Figure 28**) and DEPT135 (**Figure 29**) spectra also confirmed the mixture of two inseparable compounds with the ratio 3:2. The carbon signals of the major component were two methylene carbons at δ_C 69.82 and 31.41, two methine carbons at δ_C 75.77 and 69.25, two methyl carbons at δ_C 17.91 and 13.94, and a carbonyl carbon at δ_C169.01. The carbon signals of the minor component also appeared as two methylene carbons at δ_C 69.82 and 31.41, two

methine carbons at δ_C 76.20 and 69.87, two methyl carbons at δ_C 19.10 and 16.18, and a carbonyl carbon at δ_C 169.10.

The connectivity of protons and carbons were readily assigned based on analyses of the coupling constants, ^1H-^1H COSY (**Figure 30**) and HSQC (**Figure 31**) correlations to propose two similar partial structures (fragments 1 and 2) for both compounds as shown in **Figure 12**. The HMBC spectrum (**Figure 32**) exhibited correlations of H-1 (δ_H 4.96), H-1′ (δ_H 4.68) and H-2′ (δ_H 3.01) to the carbonyl carbon (δ_C 169.01), establishing the proximity of fragments 1 and 2 to the carbonyl of the major compound. In the same manner, the HMBC spectrum (**Figure 32**) also confirmed the proximity of fragments 1 and 2 to the carbonyl of the minor compound by the similar correlations of H-1 (δ_H 4.86), H-1′ (δ_H 4.69) and H-2′ (δ_H 3.02) to the carbonyl carbon (δ_C 169.10).



Major compound



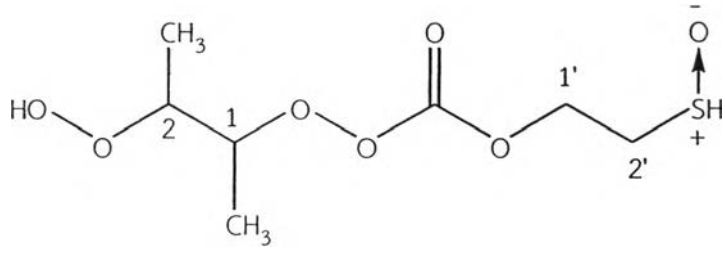
Minor compound

Figure 12. Partial structures of major and minor components in the mixture BB28-C.

The observe information provided the formula of $C_7H_{12}O$ fragment according for 112 mass unit. Since the EIMS (**Figure 26**) of the compound showed the molecular ion peak at *m/z* 242, the remaining 130 mass unit might be 5 possible fragments, including H_2S_4 , $H_2S_3O_2$, $H_2S_2O_4$, H_2SO_6 and H_2O_8. However, the possible molecular formula for BB28-C was proposed as $C_7H_{14}O_7S$ by the following evidence.

The more downfield chemical shifts of the methines at C-1 (δ_H 4.96, δ_C 75.77) and C-2 (δ_H 3.93, δ_C 69.25) and the methylene at C-1′ (δ_H 4.68, δ_C 69.82) for the major compound and the methines at C-1 (δ_H 4.86, δ_C 76.20) and C-2 (δ_H 3.769, δ_C 69.87) and the methylene at C-1′ (δ_H 4.69, δ_C 69.82) for the minor compound were suggestive of their positions adjacent to oxygen atoms. Consequently, the downfield shifts of the two methine carbons at C-1 and C-2 were compared with the carbons of the peroxide derivatives (Klochkov *et al.,* 1987) to propose the connectivity of the methine carbons with the peroxide groups. In addition, the strong HMBC correlations of the methylene protons at C-1′ to the carbonyl carbon suggested their connectivity through an oxygen. Finally, the less downfield shifts of the methylene carbons at C-2′ (δ_C 31.41) were compared with those of the thiol and the sulfoxide compounds (Freeman and Angeletakis 1983, Suwanborirux *et al.,* 2002) to propose the connectivity of this methylene carbon to a sulfoxide functional group.

Thus, the structure of BB28-C was proposed as a mixture of two diastereomers namely *O*-1-(2-hydroperoxy-1,2-dimethylethyl)-*O*-1′-(mercaptoethyl-*S*-oxide)-peroxocarbonate **[48]**. Each compound had two chiral carbons at C-2 (δ_C 69.25 and 69.87) and C-1 (δ_C 76.20 and 75.77).



[48]

Table 6. ^{1}H- and ^{13}C-NMR spectral data of BB28-C in $CDCl_3$.

Position	Major		Position	Minor	
	δ_C	δ_H, *mult.* (*J* in Hz)		δ_C	δ_H, *mult.* (*J* in Hz)
1	75.77	4.96, *dq* (1H, 6.3, 3.3)	1	76.20	4.86, *pentet* (1H, 6.3)
2	69.25	3.93, *dq* (1H, 6.3, 3.3)	2	69.87	3.79, *pentet* (1H, 6.3)
1-CH_3	13.94	1.26, *d* (3H, 6.3)	1-CH_3	16.18	1.27, *d* (3H, 6.3)
2-CH_3	17.91	1.19, *d* (3H, 6.3)	2-CH_3	19.10	1.21, *d* (3H, 6.3)
1′	69.82	4.68, *t* (2H, 6.0)	1′	69.82	4.69, *t* (2H, 6.0)
2′	31.41	3.01, *t* (2H, 6.0)	2′	31.41	3.02, *t* (2H, 6.0)
CO	169.01	-	CO	169.10	-

5. *In Vitro* Antileukemic Activity

Antileukemic activity of fractions BB1-BB6, BB8-BB10, together with isolated compounds, succinic acid (C3), tyrosol (BB21-C), and *O*-1-(2-hydroperoxy-1,2-dimethylethyl)-*O*-1′-(mercaptoethyl-*S*-oxide)-peroxocarbonate (BB28-C) against THP-1 cell line were reported as cytotoxicity percentage against THP-1 cell line and EC_{50} values in **Table 7**. Ellipticine was used as the positive control. The results showed that fraction BB1 exhibited more potent antileukemic activity than ellipticine. After fraction BB1 was fractionated by a silica gel column, the obtained fraction BB4 presented higher antileukemic activity than fraction BB1. Unexpectedly, the antileukemic activity of fractions BB7-BB13 separated from fraction BB4 dramatically decreased. This result suggested that there might be complexing conditions in fraction BB4 influencing its antileukemic activity, for example some co-factors required for the cytotoxicity were disappeared during the separation or several compounds would be synergistically involved in such a bioactivity. The isolated compounds, succinic acid and tyrosol, which were isolated from the active fraction, showed no cytotoxicity against THP-1 cell line. However, *O*-1-(2-hydroperoxy-1,2-dimethylethyl)-*O*-1′-(mercaptoethyl-*S*-oxide)-peroxocarbonate which isolated from the inactive fraction exhibited weak antileukemic activity with EC_{50} 131 μM.

Table 7. Cytotoxicity percentages against THP-1 cell line of fractions, C3, BB21-C, and BB28-C.

Fraction	%cytotoxicity (at 20 µg/ml)	EC_{50} (µg/ml)	EC_{50} (µM)
Ellipticine	nt	19.5	79
BB1	59.80	17.1	nt
BB2	16.13	nt	nt
BB3	-12.26	nt	nt
BB4	89.20	0.7	nt
BB5	31.15	nt	nt
BB6	15.25	nt	nt
BB8	1.97	nt	nt
BB9	0.53	nt	nt
BB10	52.98	nt	nt
C3	2.13	nt	nt
BB21-C	-0.93	nt	nt
BB28-C	35.57	31.6	131

nt = not tested