CHAPTER IV

RESULT AND DISCUSSION

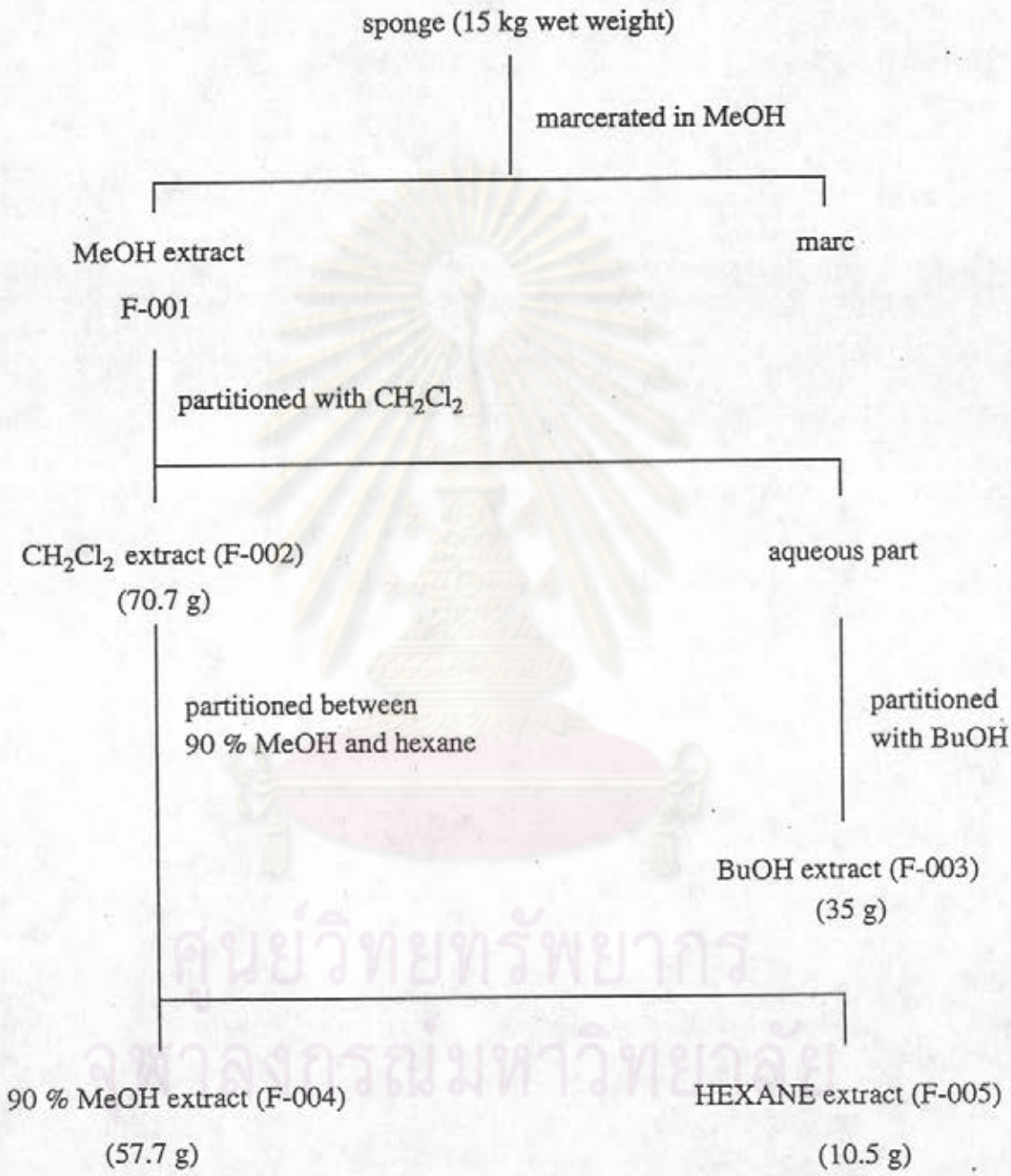
The preliminary study on the bioactivity screening of the dichloromethane extract from the sponge, *Reniera* sp., revealed several significant bioactivities, such as antimicrobial, ichthyotoxic, and cytotoxic activities. The sponge, then, was selected for study on the isolation and structure elucidation of bioactive constituents. The structure-activity relationship of some isolated compounds will be also discussed.

1. Isolation of the Bioactive Constituents

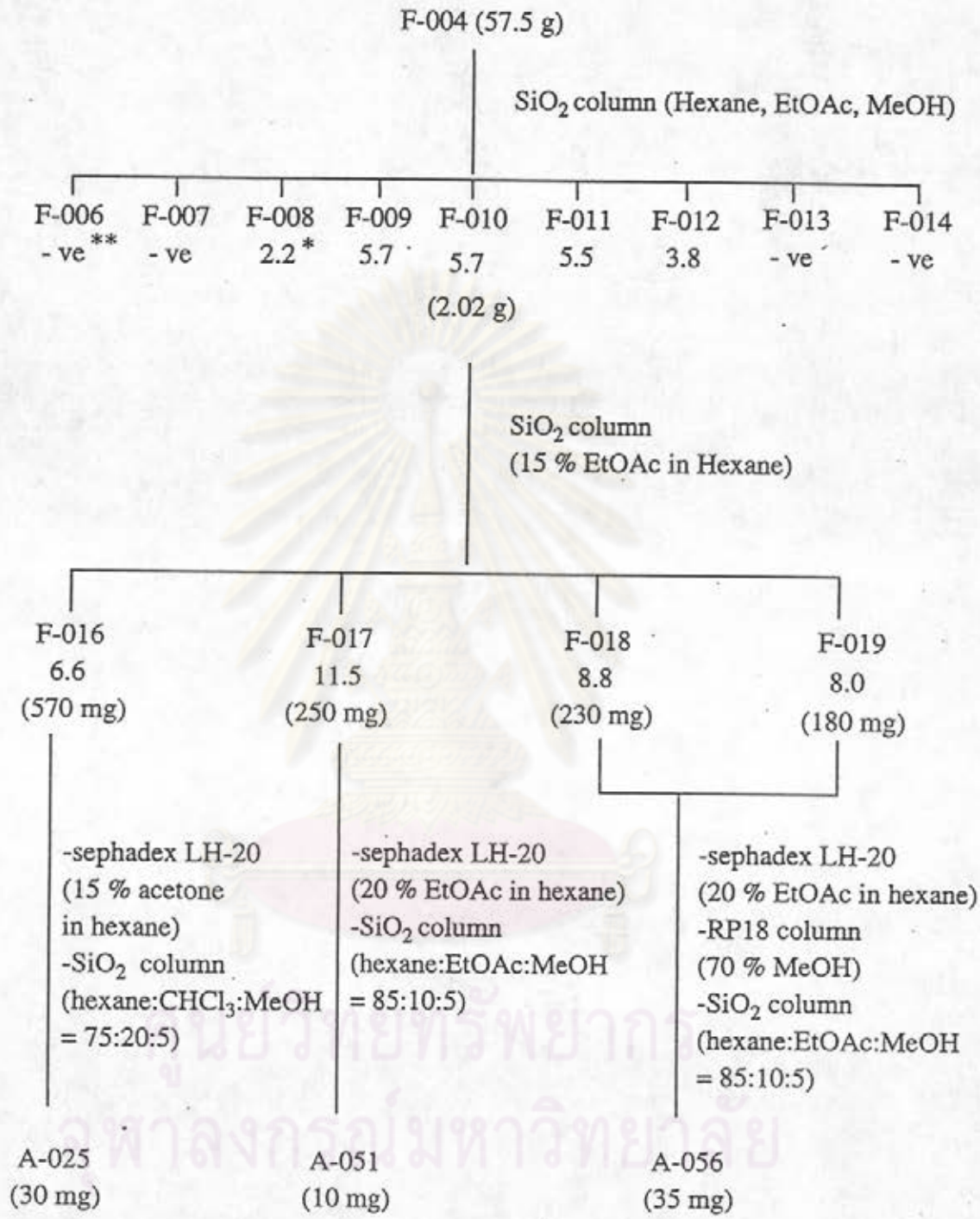
Further investigation on the large scale collection of the sponge, *Reniera* sp. (15 kg, wet weight), guided by antimicrobial assay (against *S. aureus*, concentration 0.1 mg/disc) led to the isolation of 6 isoquinoline quinone alkaloids. After the repeated marceration of the sponge in methanol and fractionation by solvent partition processes (Scheme 1), the active methanolic extract (F-004, 57.5 g) was further investigated by successive chromatographic techniques to yield 9 fractions, F-006 - F-014. The antimicrobial assay revealed that the antimicrobial activity mainly distributed from F-009 to F-012.

Fraction F-010 (2.02 g), which was the most active fraction, was subjected to further fractionation by repeated column chromatographies (Scheme 2). It yielded 3 antimicrobial compounds, A-025 (30 mg), A-051 (10 mg), and A-056 (35 mg), which were later identified as the known isoquinoline quinones, renierone [**17**], 1,6-dimethyl-7-methoxy-5,8-dihydroisoquinoline-5,8-dione [**21**], and *N*-formyl-1,2-dihydrorenierone [**19**], respectively.

Fraction F-012 (6.62 g), which exhibited less activity, was also investigated. Using the successive column chromatographic techniques (Scheme 3), This fraction provided 3 compounds, A-073 (5 mg), A-082 (35 mg), and A-129 (30 mg). They were



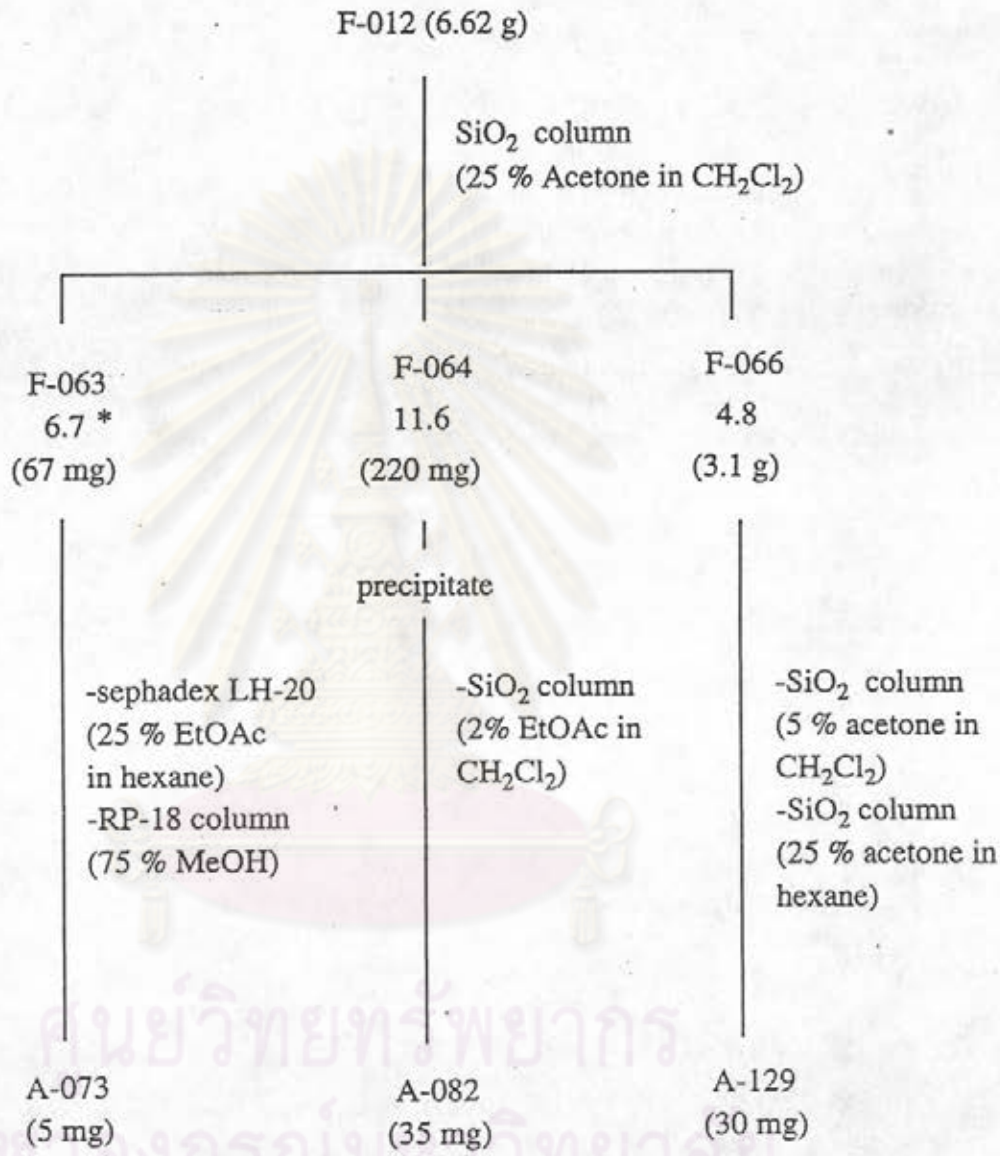
Scheme 1 Extraction scheme of the sponge, *Reniera* sp.



note ; * The diameter of the inhibition zone (mm) against *S. aureus* at the concentration of 0.1 mg/disc

** The negative result in the antimicrobial assay

Scheme 2 Isolation scheme of the bioactive constituents from the sponge, *Reniera* sp., from fractions F-004 and F-010



note ; * The diameter of the inhibition zone (mm) against *S. aureus* at the concentration of 0.1 mg/disc

Scheme 3 Isolation scheme of the bioactive constituents from the sponge, *Reniera* sp, from fraction F-012

later identified as *N*-(1"*E*-buten-3"-onyl)-1,2-dihydrorenierone [**47**], mimosamycin [**16**], and renierine B [**55**], respectively.

2. The Structure Elucidation of the Isolated Compounds

2.1 Compound A-025

Compound A-025 was obtained as yellow needles from F-016 by successive chromatographic techniques using sephadex LH-20 (20 % acetone in hexane) and silica gel column (hexane:chloroform:methanol = 70:20:5) to yield 30 mg (0.04 % of F-002). It shows moderate antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* (concentration 0.1 mg/disc, diameter of inhibition zone 6.5 and 6.9 mm, respectively).

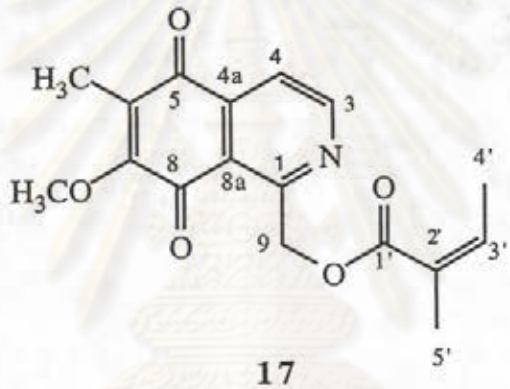
The ei mass spectrum of A-025 (Figure 16,page 90) exhibits the molecular ion peak at *m/z* 315 (48.5 %), and establishes the tentative molecular formula of $C_{17}H_{17}NO_5$. The uv spectrum (Figure 17, page 91) shows the absorption bands at λ_{max} 244 (ε 26365) and 353 nm (ε 7560) which are the characteristic of the conjugated quinone part. The ir spectrum (Figure 18, page 92) confirms the presence of the quinone carbonyls at ν 1680 cm^{-1} and also suggests the ester carbonyl at ν 1732 cm^{-1}.

A-025 can be assigned as a known isoquinoline quinone, renierone [**17**], by the analysis of its ^1H and ^{13}C nmr spectra. The ^1H nmr spectrum of A-025 (Figure 19, page 93) shows the signals of 3 olefinic protons, 2 benzylic methylene protons, 1 methoxy, and 3 methyl groups. The ^{13}C nmr spectrum (Figure 20, page 94) suggests the presences of 3 carbonyl carbons, 9 sp^2 carbons, 2 oxygenated sp^3 carbons, and 3 methyl carbons.

The angelate ester part is observed by the couplings of the olefinic proton, H-3', at δ 6.05 ppm (qq) to the methyl protons, H_3-4' and H_3-5', at δ 1.99 (dq) and 1.96 ppm (quintet) with the coupling constants of 7.1 and 1.2 Hz, respectively. This part is confirmed by the signals in ^{13}C nmr spectrum at δ 137.9, 128.0, 20.9, and 15.9 ppm which are respectively assigned as C-3', C-2', C-5', and C-4', together with the ester carbonyl carbon, C-1', at δ 167.4 ppm. The angelate ester part connects to a benzylic carbon, C-9, which is observed at δ 65.2 ppm in ^{13}C nmr spectrum, corresponding to the methylene protons, H_2-9, at δ 5.70 ppm (s) in ^1H nmr spectrum.

The signals of the isoquinoline part of A-025 can be found at δ 8.87 (d, J = 4.9 Hz) and 7.73 ppm (d, J = 4.9 Hz) which are assigned as H-3 and H-4. These two protons are substituted at C-3 (δ 153.9 ppm) and C-4 (δ 118.3 ppm), respectively. The remaining quarternary carbons provide the signals at δ 184.7, 181.8, 158.4, 157.1, 138.9, 130.1, and 122.1 ppm which are assigned as C-5, C-8, C-7, C-1, C-4a, C-6, and C-8a, respectively.

The assignments of protons and carbons of compound A-025 are confirmed by comparison with the data of renierone [**17**] previously reported by McIntyre *et al* (1979) and Kitahara *et al* (1985). The assignments are summarized in Tables 12 and 13 (pages 59 and 60). The structure of renierone [**17**] is shown below.



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This structure is confirmed by the analysis of the mass fragmentation (Figure 9). The α-cleavage of the ester carbonyl provides the fragments of *m/z* 232 (14.4 %) and 83 (73.4 %) and the ether bond cleavage also gives the fragment at *m/z* 216 (24.1 %).

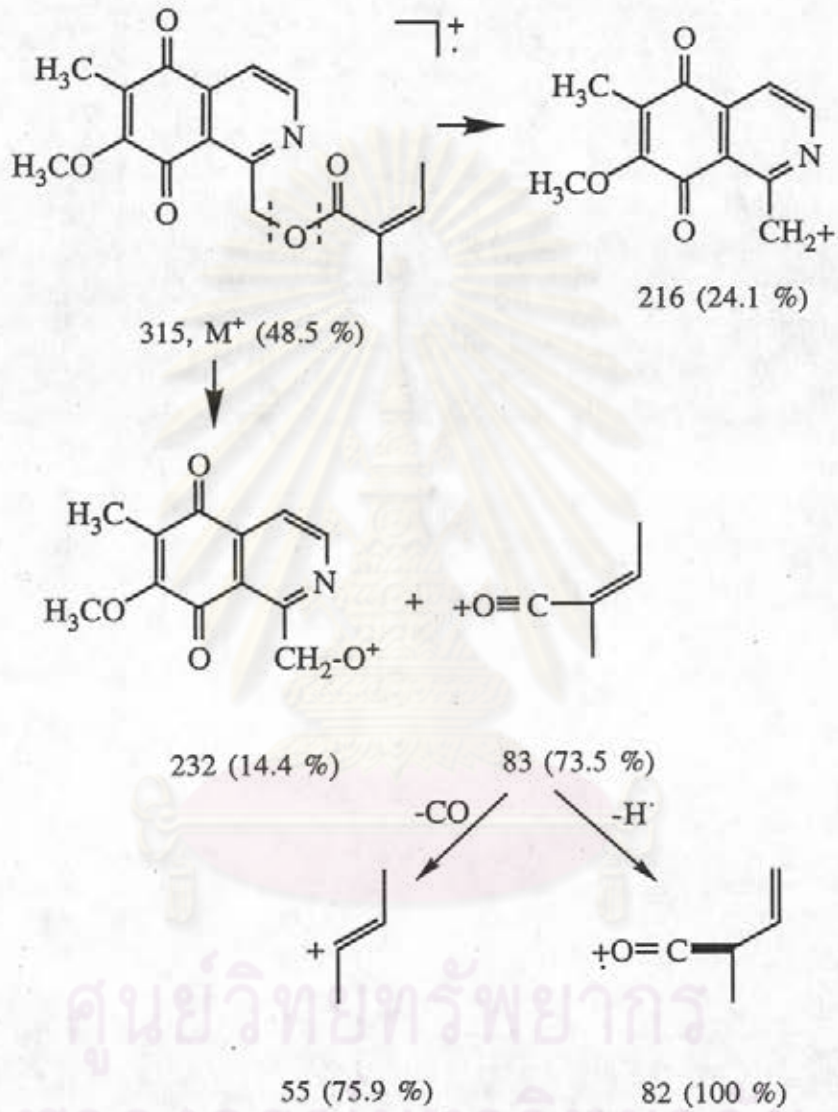


Figure 9 Proposed mass fragmentation of compound A-025

2.2 Compound A-051

Compound A-051 was obtained as yellow needles from F-017 by chromatographic techniques using sephadex LH-20 (20 % ethyl acetate in hexane) and silica gel column (hexane:ethyl acetate:methanol = 85:10:5). It yields 10 mg (0.014 % of F-002). This compound shows antimicrobial activity against *S. aureus*, *B. subtilis*, and *C. albicans* (concentration 0.1 mg/disc, diameter of inhibition zone 14.0, 9.7, and 2.6 mm, respectively).

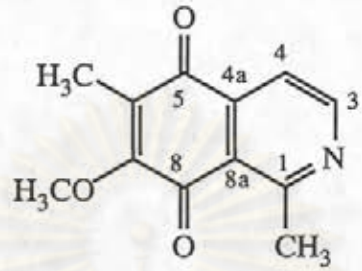
The eims spectrum of A-051 (Figure 21, page 95) exhibits the stable molecular ion peak at *m/z* 217 (100 %) and establishes the tentative molecular formula of $C_{12}H_{11}NO_3$. The uv absorptions at λ_{max} 251 (ε 14190) and 324 nm (ε 5600) (Figure 22, page 96) suggest the conjugated quinone part which is confirmed by the ir absorption band at ν 1680 cm^{-1} (Figure 23, page 97).

Compound A-051 is assigned as a known isoquinoline quinone, 1,6-dimethyl-7-methoxy-5,8-dihydroisoquinoline-5,8-dione [**21**], by the analysis of the ^1H and ^{13}C nmr spectra. The ^1H nmr spectrum (Figure 24, page 98) provides the signals of 2 olefinic protons, 1 methoxy, and 2 methyl groups. The ^{13}C nmr spectrum (Figure 25, page 99) shows the signals of 2 carbonyl carbons, 7 sp^2 carbons, 1 methoxy carbon, and 2 methyl carbons.

The signals of the olefinic protons in ^1H nmr spectrum at δ 8.79 (d, J = 4.9 Hz) and 7.74 ppm (d, J = 4.9 Hz) are assigned as H-3 and H-4, respectively. The remaining signals are the signals of 7-OCH_3 (δ 4.12 ppm, s), 1-CH_3 (δ 2.94 ppm, s), and 6-CH_3 (δ 2.03 ppm, s).

The ^{13}C nmr spectrum supports this structure. The protonated sp^2 carbon signals at δ 153.4 and 117.1 ppm are assigned as C-3 and C-4, respectively, and the protonated sp^3 carbons at δ 60.8, 25.5, and 8.9 ppm are assigned as 7-OCH_3, 1-CH_3, and 6-CH_3. The quarternary signals are the signals of C-5 (δ 184.3 ppm), C-8 (δ 181.7 ppm), C-1 (δ 160.2 ppm), C-7 (δ 158.1 ppm), C-4a (δ 138.7 ppm), C-6 (δ 129.6 ppm), and C-8a (δ 122.8 ppm).

The assignments of carbons and protons of A-051 are supported by comparison with the reports of Frincke and Faulkner (1982) and Kitahara *et al.*(1985). The chemical shifts of protons and carbons are summarized in Tables 12 and 13 (pages 59 and 60), respectively. The chemical structure of compound A-051 is shown below.



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2.3 Compound A-056

Compound A-056 was obtained as a deep-red non-crystallized solid from F-018 and F-019. Both fractions were fractionated by repeated chromatographic techniques using sephadex LH-20 (20 % ethyl acetate in hexane), RP-18 column (60 % methanol in water), and silica gel column (20 % acetone in hexane). It yields 35 mg (0.05 % of F-002). This compound is antimicrobial active against *S. aureus* and *B. subtilis* (concentration 0.1 mg/disc, diameter of inhibition zone 11.4 and 8.2 mm, respectively).

The eims spectrum (Figure 26, page 100) shows the molecular ion peak at *m/z* 345 (3.4 %) and establishes the proposed molecular formula of $C_{18}H_{19}NO_6$. The uv absorption at λ_{max} 242 (ε 15145), 270 (ε 16835), and 351 nm (ε 6485) (Figure 27, page 101) suggests the conjugated quinone part, and can be comfirmed by the ir absorption at ν 1670 cm^{-1} (Figure 28, page 102). The ester carbonyl absorption at ν 1720 cm^{-1} is also observed.

Compound A-056 is assigned as a known isoquinoline quinone, *N*-formyl-1,2-dihydrorenierone **[19]**. The ^1H nmr (Figure 29, page 103) and ^{13}C nmr spectra of this compound (Figure 30, page 105) suggest the mixture of two inseparable isomers in the ratio of 2:1. The signals of each isomer in ^1H nmr spectrum consist of 1 formyl proton, 3 olefinic protons, 2 methylene protons coupling to 1 methine proton, 1 methoxy, and 3 methyl groups. The ^{13}C nmr spectrum suggests the presences of 4

carbonyl carbons, 8 sp^2 carbons, 3 sp^3 carbons connecting to the heteroatom, and 3 methyl carbons.

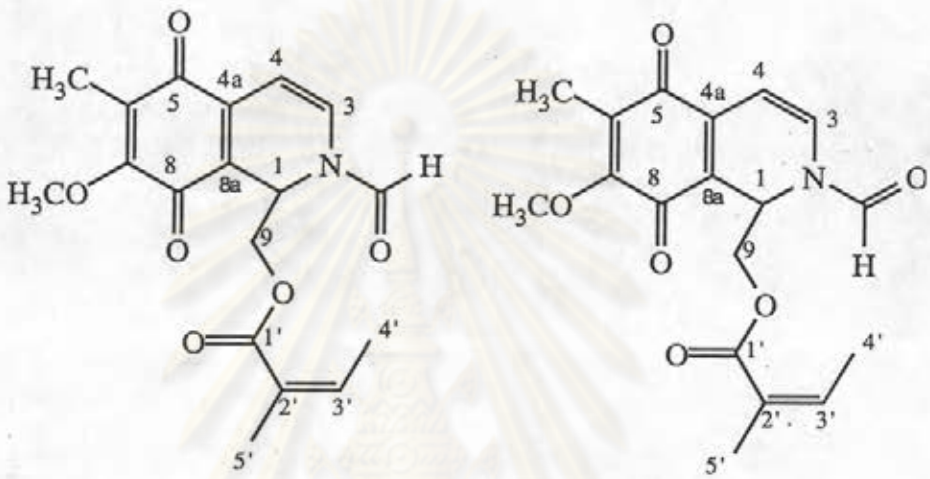
The signals of angelate ester part of the major isomer are found in the ^1H nmr spectrum at δ 6.01 ppm (qq) coupling to 2 methyl groups at δ 1.86 ppm (dq) and 1.73 ppm (quintet) with the coupling constants of 7.3 and 1.4 Hz, respectively. They are respectively assigned as H-3', H3-4', and H3-5'. The signals of this part in ^{13}C nmr spectrum can be assigned as follows; C-1' (δ 167.2 ppm), C-2' (δ 126.9 ppm), C-3' (δ 139.6 ppm), C-4' (δ 15.6 ppm), and C-5' (δ 20.5 ppm).

The signals in ^1H nmr spectrum of A-056 at δ 6.89 (d, J = 7.5 Hz) and 5.98 ppm (d, J = 7.5 Hz) are assigned as H-3 and H-4. The signal of H-1 at δ 5.92 ppm (dd) couples to 2 methylene protons , H-9a and H-9b, at δ 4.15 (dd) and 4.31 ppm (dd) (J = 11.9 Hz) with the coupling constants of 3.0 and 4.6 Hz, respectively. The remaining signals at δ 8.39 (s), 4.02 (s), and 1.90 ppm (s) are continuously assigned as N-CHO, 7-OCH_3, and 6-CH_3.

The ^{13}C nmr spectrum of compound A-056 confirms the proposed structure. Four carbonyl carbons at δ 184.7, 180.1, 167.2, and 162.0 ppm are assigned as C-5, C-8, C-1', and N-CHO, respectively. The protonated sp^2 carbons, C-3 is at δ 133.1 ppm, and C-4 at δ 100.8 ppm. The quarternary signals of carbons in the isoquinoline part are C-4a (δ 135.4 ppm), C-5 (δ 184.7 ppm), C-6 (δ 127.0 ppm), C-7 (δ 156.2 ppm), C-8 (δ 180.1 ppm), and C-8a (δ 123.9 ppm). The remaining signals in the upfield region are the signals of C-9 (δ 63.0 ppm) and C-1 (δ 47.3 ppm).

The structure elucidation and assignments of protons and carbons in the minor isomer can be carried out in the same manner. The proton and carbon assignments of A-056 are confirmed by those previously given by Frincke and Faulkner (1982) and Kitahara *et al.* (1985), and summarized in Tables 12 and 13 (pages 59 and 60), respectively. The restricted rotation of the *N*-formyl group causes the geometrical difference of the two isomers. The structures of the two rotamers, as shown below, were proposed by Frincke and Faulkner based on the report of Lewin and Frucht (1975). This suggestion is confirmed by the analysis of NOESY spectrum (Figure 32,page 106). Two cross peaks between the formyl proton and H-3 of the major and between the formyl

proton and H-1 of the minor indicate the proximity of each pair of these protons. Moreover, the careful observation of a very small coupling ($J < 0.5$ Hz) of the signal of H-4 of the minor isomer also supports its proposed conformation (Figure 30, page 104). This coupling is caused by the long-range coupling via a zigzag path between H-4 and the formyl proton of the minor. The conformation of the major will not provide a coupling in this way.



19 A (major) **19 B** (minor)

The proposed structure of A-056 is finally confirmed by the analysis of the mass fragmentation (Figure 10). The loss of formaldehyde causes the fragment at *m/z* 315 (9.7 %) which can further loss the angelate ester part to provide the fragment of *m/z* 232 (81.2 %). The loss of the angelate side chain followed by loss of carbonmonoxide causes the base peak at *m/z* 204 . The ether bond cleavage also provides the fragment at *m/z* 245 (4.03%).

2.4 Compound A-082

Compound A-082 was obtained as yellow crystals by purifying the precipitates from F-064 using a silica gel column (2 % ethyl acetate in dichloromethane) to yield 35 mg (0.05 % of F-002). This compound exhibits antimicrobial activity against *S. aureus*, *B. subtilis*, and *C. albicans* (concentration 0.1 mg/disc, diameter of inhibition zone 12.6, 6.7 and 6.6 mm, respectively).

The stable molecular ion peak at *m/z* 233 (100 %) in the eims spectrum of A-082 (Figure 33, page 107) establishes the proposed molecular formula of $C_{12}H_{11}NO_4$.

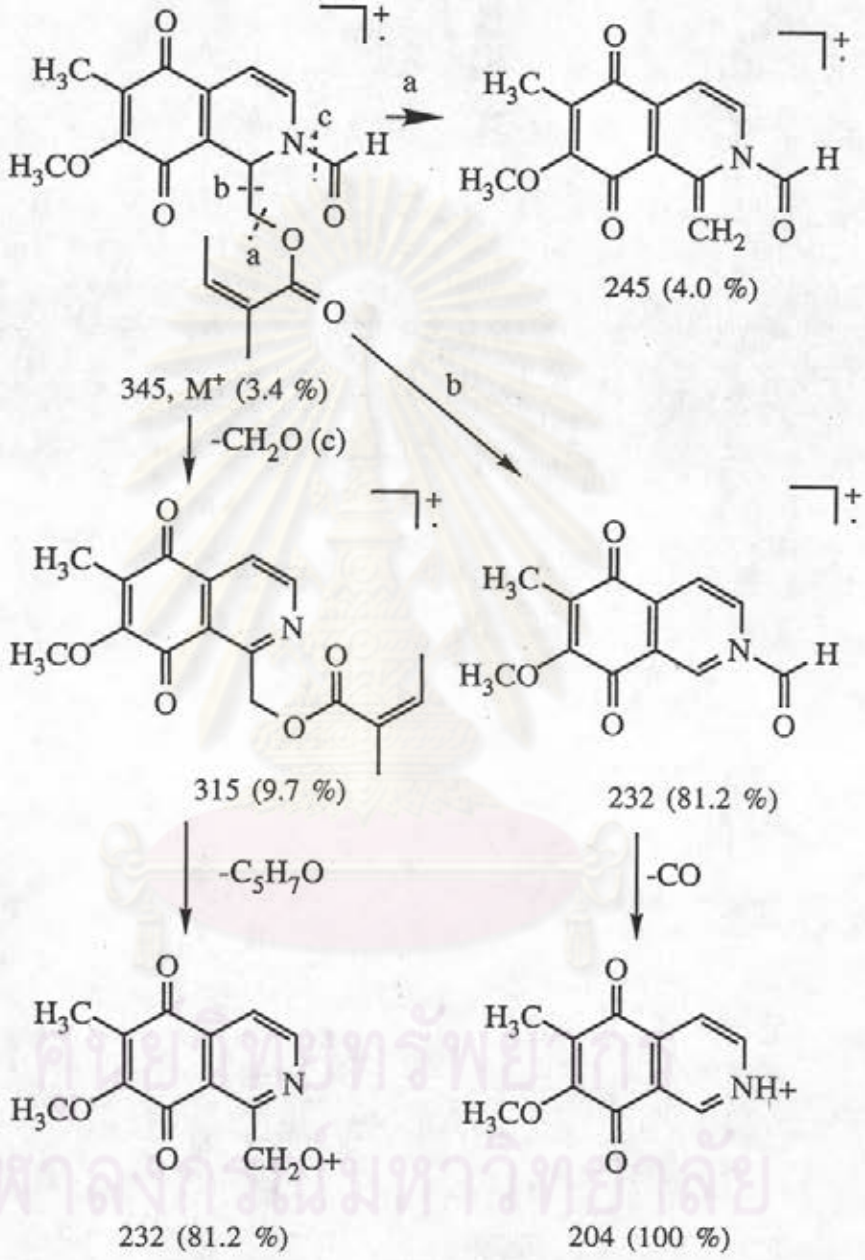


Figure 10 Proposed fragmentation of compound A-056

The uv absorption bands at λ_{max} 244 (ε 8925), 323 (ε 10810), and 399 nm (ε 3122) (Figure 34, page 108) suggests the conjugated quinone moiety. The ir absorption at ν 1695 cm^{-1} also supports the presence of the quinone carbonyl (Figure 35, page 109).

Compound A-082 is assigned as a known isoquinoline quinone, mimosamycin [**16**], by the analysis of the nmr spectra. The ^{1}H nmr spectrum (Figure 36, page 110) shows the signals of 2 olefinic protons, 1 methoxy, 1 N-methyl, and 1 methyl groups. The ^{13}C nmr spectrum (Figure 37, page 111) shows the signals of 3 carbonyl carbons, 5 sp^2 carbons, and 3 sp^3 carbons.

Two singlet olefinic protons in ^{1}H nmr spectrum of A-082 at δ 8.24 and 7.08 ppm are respectively assigned as H-1 and H-4. Three remaining singlet signals are the signals of 7-OCH_3 (δ 4.14 ppm), N-CH_3 (δ 3.64 ppm), and 6-CH_3 (δ 2.04 ppm). The carbonyl carbon signals in ^{13}C nmr spectrum at δ 183.5, 177.3, and 162.8 ppm are assigned as C-5, C-8, and C-3. The quarternary sp^2 carbons are C-1 (δ 142.1 ppm), C-4a (δ 138.9 ppm), C-4 (δ 116.7 ppm), C-6 (δ 113.2 ppm), C-7 (δ 159.5 ppm), and C-8a (δ 111.3 ppm). The methyl signals at δ 61.3, 29.7, and 9.6 ppm are assigned as 7-OCH_3, N-CH_3, and 6-CH_3, respectively.

The proton and carbon assignments of A-082 are confirmed by comparison with the data reported by Fukumi *et al.*(1978). The assignments are summarized in Tables 12 and 13 (pages 59 and 60).

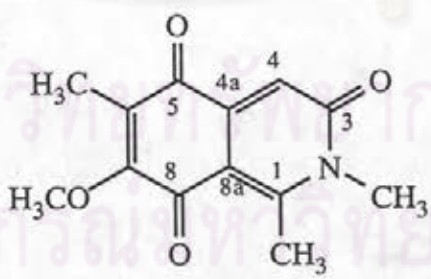


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Table 12 Proton assignments of compounds A-025, A-051, A-056, and A-082

	δH (ppm)				
proton	A-025	A-051	A-056A	A-056B	A-082
1	-	-	5.92,dd (3.0,4.6)	5.32,dd (3.5,9.3)	8.24,s
3	8.87,d (4.9)	8.79,d (4.9)	6.89,d (7.5)	7.39,d (7.5)	-
4	7.73,d (4.9)	7.74,d (4.9)	5.98,d (7.5)	6.18,d (7.5)	7.08,s
9-Ha	5.70,s	-	4.15,dd (11.9,3.0)	3.85,dd (12.4,3.5)	-
9-Hb	-		4.31,dd (11.9,4.6)	4.18,dd (12.4,9.3)	
3'	6.05,qq (7.1,1.2)	-	6.01,qq (7.3,1.4)	6.10,qq (7.2,1.4)	-
4'	1.99, dq (7.1,1.2)	-	1.86,dq (7.3,1.4)	1.94,dq (7.2,1.4)	-
5"	1.96,quintet (1.2)	-	1.73,quintet (1.4)	1.89,quintet (1.4)	-
1-CH_3	-	2.94,s	-	-	-
6-CH_3	2.05,s	2.03,s	1.90,s	1.92,s	2.04,s
7-OCH_3	4.15,s	4.12,s	4.02,s	4.01,s	4.14,s
N-CH_3	-	-	-	-	3.64,s
N-CHO	-	-	8.39,s	8.18,s	-

note The numbers in the parenthesis are the coupling constants in Hz

Table 13 Carbon assignments of compounds A-025, A-051, A-056, and A-082

	δC (ppm)				
carbon	A-025	A-051	A-056A	A-056B	A-082
1	157.1	160.2	47.3	49.7	142.1
3	153.9	153.4	133.1	129.3	162.8
4	118.3	117.1	100.8	102.8	116.7
4a	138.9	138.7	135.4	136.1	138.9
5	184.7	184.3	184.7	184.6	183.5
6	130.1	129.6	127.0	127.9	133.2
7	158.4	158.1	156.2	155.9	159.5
8	181.8	181.7	180.1	180.1	177.3
8a	122.1	122.8	123.9	123.1	111.3
9	65.2	-	63.0	63.0	-
1'	167.4	-	167.2	166.6	-
2'	128.0	-	126.9	126.4	-
3'	137.9	-	139.6	140.7	-
4'	15.9	-	15.6	15.8	-
5'	20.9	-	20.5	20.4	-
1-CH_3	-	25.5	-	-	-
6-CH_3	9.2	8.9	8.5	8.6	9.6
7-OCH_3	61.1	60.8	61.0	60.8	61.3
N-CH_3	-	-	-	-	29.7
N-CHO	-	-	162.0	161.2	-

2.5 Compound A-073

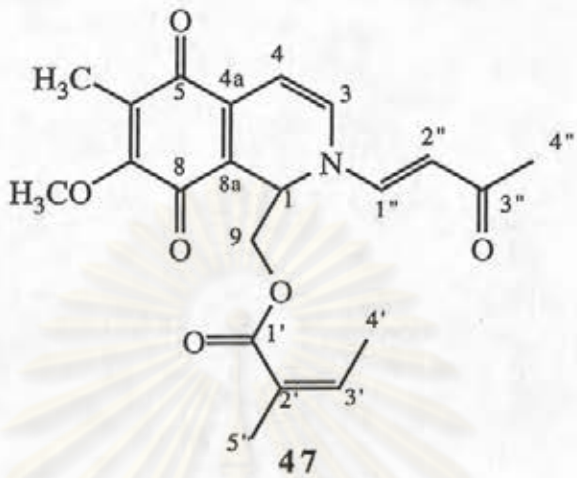
Compound A-073 was obtained as a deep-blue non-crystallized solid from fraction F-063 by chromatographic techniques using sephadex LH-20 (25 % acetone in hexane) and RP-18 column (75 % methanol in water) to yield 5 mg (0.007 % of F-002). It shows antimicrobial activity against *S. aureus* and *B. subtilis* at the concentration of 0.1 mg/disc (diameter of inhibition zone, 8.0 and 9.8 mm, respectively).

The ei mass spectrum of compound A-073 (Figure 38, page 112) exhibits the molecular ion peak at *m/z* 385 (2.9 %) and establishes the proposed molecular formula of $C_{21}H_{23}NO_6$. The uv absorption bands at λ_{max} 243 (ε 18425), 310 (ε 23000), and 580 nm (ε 6125) (Figure 39, page 113) indicate the presence of the quinone moiety with the highly conjugated system, and the ir absorption band at ν 1720 cm^{-1} (Figure 40, page 114) also confirms the presence of quinone structural unit.

The ^1H nmr spectrum of compound A-073 (Figure 41, page 115) is very similar to that of compound A-056. The olefinic signal at δ 6.06 ppm (qq, J = 7.3, 1.3 Hz) and 2 methyl signals at δ 1.93 (dq, J = 7.3, 1.3 Hz) and 1.77 ppm (quintet, J = 1.3 Hz) indicate the presence of the angelate ester moiety. The methine signal at δ 5.47 ppm (br.dd, J = 6.1, 3.9 Hz) can be assigned as H-1 which couples with 2 signals of the methylene protons (δ 4.52 ppm, dd, J = 11.5, 6.1 Hz, and δ 4.05 ppm, dd, J = 11.5, 3.9 Hz) on the oxygenated carbon, C-9. Two olefinic signals at δ 6.76 (dd, J = 7.3, 1.0 Hz) and 5.96 ppm (d, J = 7.3 Hz) have been already assigned as H-3 and H-4 of the isoquinoline part, respectively. The smaller coupling constant (1.0 Hz) at the H-3 signal can be discussed as the long-range coupling via a zigzag path with H-1. This coupling also causes the broadening in the signal of H-1. These nmr evidences establish a 1,2-dihydroisoquinoline quinone nucleus which is common to compound A-056, *N*-formyl-1,2-dihydrorenierone **[19]**.

The additional signals are 2 olefinic protons (δ 7.39 and 5.89 ppm, both d, J = 13.7 Hz) and 1 methyl group (δ 2.20 ppm, s). The chemical shifts of both olefinic protons suggest the presence of an α,β-unsaturated ketone system, and the coupling constant indicates their *trans*-relationship. The methyl chemical shift suggests its attachment to a carbonyl functional group. The above information supports the replacement of the formyl group in compound A-056 by the butenonyl group at the nitrogen atom.

Compound A-073, finally, can be identified as a new member of the isoquinoline quinones, named as *N*-(1"*E*-buten-3"-onyl)-1,2-dihydrorenierone [**47**].



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The complete carbon assignments are achieved by the analysis of the ^{1}H-detected heteronuclear multiple quantum coherent (HMQC) and ^{1}H-detected multiple bond heteronuclear multiple quantum coherent (HMBC) spectra (Figures 44 and 45, pages 118 and 119), which provide the correlations between protons and carbons through 1-bond and long-range coupling, respectively. The protonated carbons are continually assigned based on the correlation between protons and carbons in the HMQC spectrum as followed; H-1 (δ 5.47 ppm) - C-1 (δ 51.3 ppm), H-3 (δ 6.76 ppm) - C-3 (δ 137.8 ppm), H-4 (δ 5.96 ppm) - C-4 (δ 99.7 ppm), H-9a (δ 4.05 ppm) and H-9b (δ 4.32 ppm) - C-9 (δ 61.1 ppm), H-3' (δ 6.06 ppm) - C-3' (δ 140.3 ppm), H3-4' (δ 1.93 ppm) - C-4' (δ 15.8 ppm), H3-5' (δ 1.77 ppm) - C-5' (δ 20.6 ppm), 6-CH_3 (δ 1.93 ppm) - 6-$\underline{C}H_3$ (δ 8.6 ppm), 7-OCH_3 (δ 4.03 ppm) - 7-O$\underline{C}H_3$ (δ 60.8 ppm), H-1" (δ 7.39 ppm) - C-1" (δ 145.6 ppm), H-2" (δ 5.89 ppm) - C-2" (δ 105.0 ppm), and H3-4" (δ 2.20 ppm) - C-4" (δ 29.3 ppm).

The quarternary carbons are assigned depended on the long-range coupling observed from the HMBC spectrum, as well as the information from their chemical shifts. Four signals at the lowest field are of the carbonyl functional groups. The signal at δ 196.0 ppm is assigned as C-3" and confirmed by the long-range coupling with the signals of H-1" (δ 7.39 ppm, d), H-2" (δ 5.89 ppm, d), and H3-4" (δ 2.20 ppm, s). The next two signals are the signals of carbonyl carbons on quinone part, C-5 and C-8, respectively. The signal at δ 185.1 ppm shows the long-range correlation with H-4 (δ 5.96 ppm, d) and 6-CH_3 (δ 1.93 ppm, s) and is assigned as C-5, while the signal at δ 180.3

ppm correlates with H-1 (δ 5.47 ppm, br.dd) and H-4 (δ 5.96 ppm, d) and is assigned as C-8. The signal at δ 167.2 ppm is assigned as C-1' because of its correlations with H-9a (δ 4.05 ppm, dd), H-9b (δ 4.32 ppm, dd), and H3-5' (δ 1.77 ppm, quintet).

The signals in the olefinic region belong to C-4', C-6, C-7, C-8a, and C-2'. The signal at δ 156.3 ppm shows long-range correlations with 6-CH_3 (δ 1.93 ppm, s) and 7-OCH_3 (δ 4.03 ppm, s) while the signal at δ 127.3 ppm correlates with 6-CH_3, only. Therefore, these two signals are assigned as C-7 and C-6, respectively. The signal of C-4a at δ 135.9 ppm shows long-range couplings with H-1 (δ 5.47 ppm, br.dd) and H-3 (δ 6.76 ppm, dd) while the signal of C-8a at δ 120.0 ppm couples with these two protons and also with H-4 (δ 5.96 ppm, d), H-9a (δ 4.05 ppm, dd), and H-9b (δ 4.32 ppm, dd). The last signal at δ 125.8 ppm is the signal of C-2' whish is confirmed by the long-range correlation with H3-5' (δ 1.77 ppm, quintet). The chemical structure of compound A-073 with the long-range correlations observed from the HMBC spectrum is shown in Figure 11.

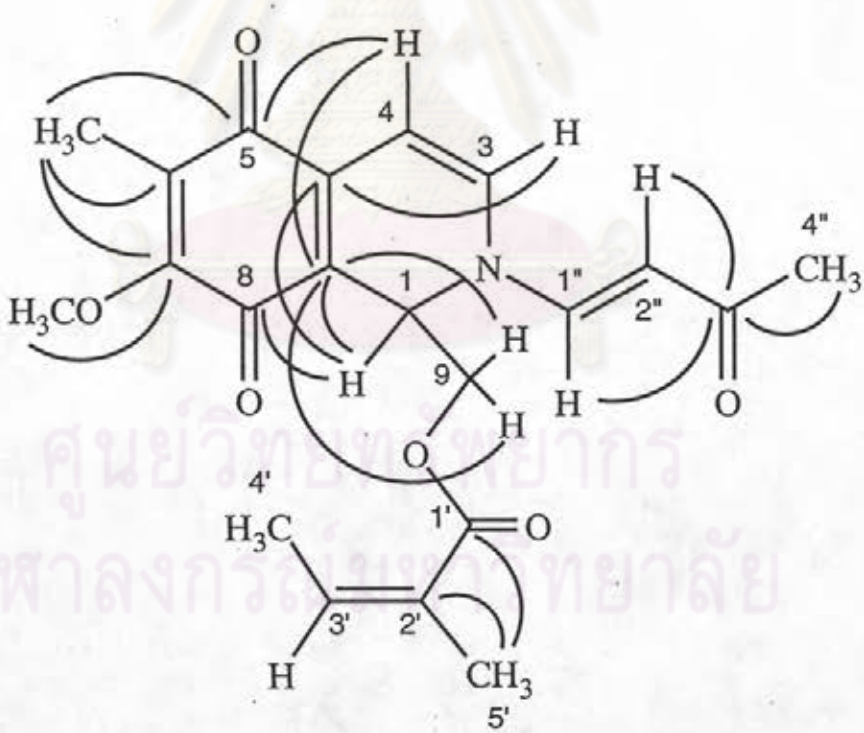


Figure 11 The long-range correlations between carbons and protons of compound A-073 observed from HMBC spectrum

The unambiguous assignments of carbons and protons with the long-range correlations between carbons and protons are summarized in Table 14 (page 65).

The proposed structure of compound A-073 is finally supported by the analysis of mass fragmentations from eims (Figure 12). The fragmentations of the angelate ester part can be observed like those found in compound A-056. The additional fragmentations are proposed to be on the butenonyl side chain. The α cleavage of the keto group (c) provides the fragments of *m/z* 43 (29.2 %). The loss of butenone (b) provides the fragment of *m/z* 315 (0.9 %), and the odd-electron shift (a) also causes the fragment of m/z 232 (100 %).

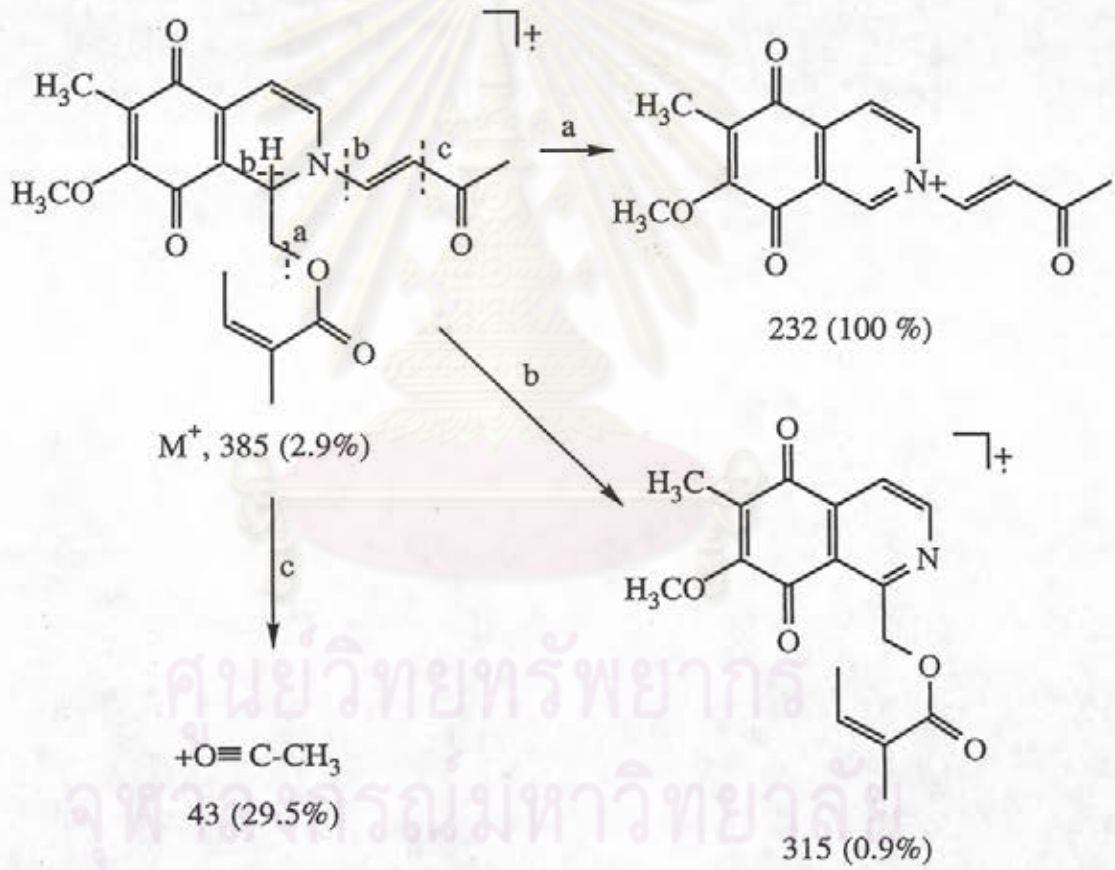


Figure 12 Proposed mass fragmentations of compound A-073

Table 14 Carbon and proton assignments of compound A-073 and long-range correlation between carbons and protons.

position	δC (ppm)	δH (ppm)	long-range correlation from C on H observed in HMBC spectrum
1	51.3	5.47, br.dd (3.9,6.1)	H-9a, H-9b, H-3, H-4"
3	137.8	6.76, dd (7.3, 1.0)	H-1, H-4, H-4"
4	99.7	5.96, d, (7.3)	H-3
4a	135.9	-	H-1, H-3
5	185.1	-	H-6-CH_3, H-4
6	127.3	-	6-CH_3
7	156.3	-	6-CH_3, 7-OCH_3
8	180.3	-	H-1, H-4
8a	120.0	-	H-9a, H-9b, H-1, H-3,H-4
9	61.1	Ha; 4.05, dd (11.5, 3.9) Hb; 4.32, dd (11.5, 6.1)	H-1
1'	167.2	-	H-9a, H-9b, H-5'
2'	126.8	-	H-5'
3'	140.3	6.06, qq (7.3, 1.3)	H-4', H-5'
4'	15.8	1.93, dq (7.3, 1.3)	H-3'
5'	20.6	1.77, quintet (1.3)	H-3'
6-CH_3	8.6	1.93, s	-
7-OCH_3	60.8	4.03, s	-
1"	145.6	7.39 (d, 13.7)	H-1, H-3, H-3"
2"	105.0	5.89, d (13.7)	H-1"
3"	196.0	-	H-1", H-3", H-4"
4"	29.3	2.20, s	H-3"

note : The numbers in the parenthesis are the coupling constants in Hz.

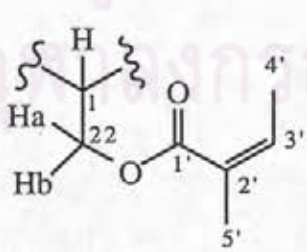
2.6 Compound A-129

Compound A-129 was crystallized as greenish-yellow crystals from the mixture of chloroform and hexane (1:1). It was obtained from fraction F-066 by repeated silica gel column chromatographies using 5 % acetone in dichloromethane and 25 % acetone in hexane as the eluants. It yields 30 mg (0.05 % of F-002). This compound is not active against all microorganisms used in this work at the concentration of 0.1 mg/disc.

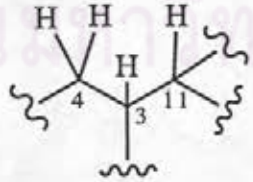
The ei mass spectrum of compound A-129 (Figure 46, page 120) shows a molecular ion peak at *m/z* 622 (0.9 %) and establishes the tentative molecular formula of $C_{33}H_{38}N_2O_{10}$ suggesting the unsaturating degree of 15. The uv absorption band (Figure 47, page 121) at λ_{max} 271 nm (ε 25400) indicates the quinone moiety which is confirmed by the ir absorption band at ν 1623 cm^{-1}. The ir spectrum (Figure 48, page 122) also exhibits the bands at ν 3289 (broad), 1712, and 1655 cm^{-1} indicating the presences of hydroxyl, ester carbonyl, and carbonyl connecting to an aromatic ring functional groups, respectively.

The assignments of protons of compound A-129 are achieved by the analysis of the chemical shifts and coupling constants in 1-D ^1H nmr spectrum (Figure 49, page 123), and the correlations observed in 2-D nmr (^1H,^1H COSY; Figure 50, page 124) spectrum. Its 1-D ^1H nmr spectrum shows the typical signals of angelate ester part at δ 5.82 (qq, J = 7.2, 1.4 Hz), 1.71 (dq, J = 7.2, 1.4 Hz), and 1.31 ppm (quintet, J = 1.4 Hz).

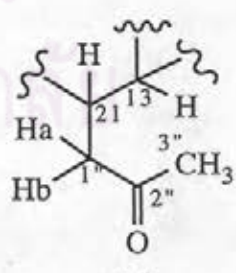
The aliphatic region of the spectrum provides three structural parts [**48** - **50**] as followed:



48



49



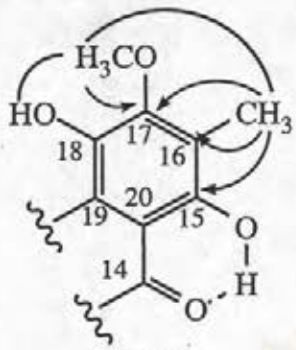
50

Fragment **48** is established from a signal of H-1 at δ 3.83 ppm (ddd, J = 4.8, 3.6, 1.8 Hz) coupling to the signals of the methylene protons, H-22a (δ 4.13 ppm, dd, J = 13.0, 4.8 Hz) and H-22b (δ 3.82 ppm, dd, J = 13.0, 3.6 Hz). The chemical shifts of H-22a and H-22b suggest the connectivity to the oxygenated carbon of the angelate ester part.

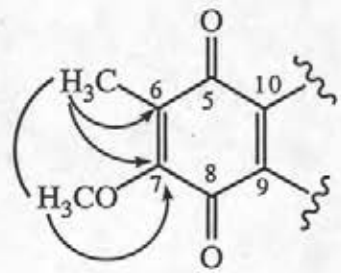
Fragment **49** contains the connectivity of H-3 (δ 3.14 pm, dt, J = 10.9, 2.8 Hz), H-4α (δ 2.99 ppm, dd, J = 17.0, 2.8 Hz), H-4β (δ 1.46 ppm, ddd, J = 17.0, 10.9, 1.8 Hz), and H-11 (δ 4.20 ppm, dd, J = 2.7, 0.8 Hz). The methylene protons (H-4α and H-4β, J = 17.0 Hz) couple to H-3 with the coupling constants of 2.8 and 10.9 Hz, respectively and H-3 couples to H-11 with the coupling constant of 2.8 Hz.

Fragment **50** exhibits the signals of H-13 (δ 3.09 ppm, dd, J = 1.1, 0.8 Hz), H-21 (δ 3.81 ppm, ddd, J = 9.3, 2.1, 1.1 Hz), H-1"a (δ 3.44 ppm, dd, J = 17.2, 9.3 Hz), and H-1"b (δ 2.51 ppm, dd, J = 17.2, 2.1 Hz). The signal of H-13 couples to H-21 with the coupling constant of 1.1 Hz, and H-21 couples to the methylene protons (H-1"a and H-1"b, J = 17.2 Hz) with the coupling constants of 9.3 and 2.1 Hz, respectively. The chemical shifts of H-1"a and H-1"b indicate the attachment to the carbonyl carbon of a methyl keto functional group. The presence of a methyl keto functional group is based on a methyl proton signal at δ 2.21 ppm (s) in the ^{1}H nmr spectrum and a carbonyl carbon at δ 207.2 ppm and a methyl carbon at δ 30.7 ppm in the ^{13}C nmr spectrum.

Fragments **51** and **52** are proposed by the analyses of the chemical shifts in 1-D ^{13}C nmr spectrum (Figure 51, page 125) and the C-H correlations observed in the HMBC spectrum (Figure 52, page 126) as shown below.



51



52

→ C-H long-range correlation

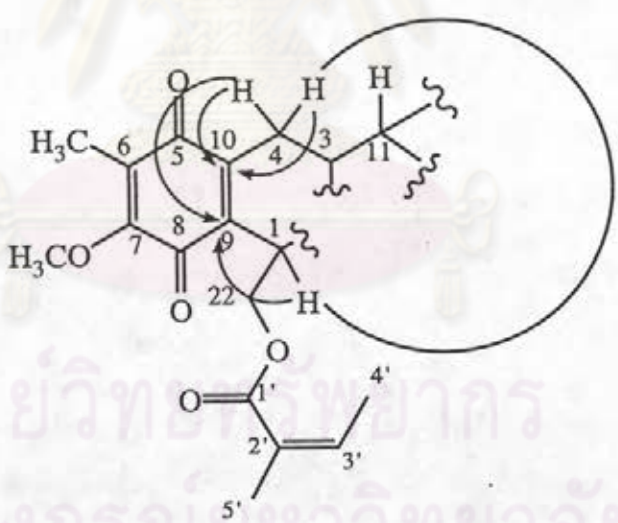
—— nOe observed between two protons

Fragment **51** is established as a hydroquinone part by analyses of three oxygenated aromatic carbons (δ 154.2, 152.1, and 138.9 ppm), three quarternary aromatic carbons (δ 117.9, 117.8, and 112.6 ppm), one carbonyl carbon (δ 203.4 ppm), one methoxy carbon (δ 60.7 ppm), and one methyl carbon (δ 8.7 ppm). The placements of the methyl and the methoxy group on aromatic carbons, C-16 (δ 117.8 ppm) and C-17 (δ 152.1 ppm), are assigned by the C-H correlations in the HMBC spectrum (Figure 52). The methyl protons (δ 2.13 pm) correlates with C-16 and C-17 while the methoxy protons (δ 3.82 ppm) correlates with C-17, only. The oxygenated aromatic carbon at δ 154.2 ppm is assigned as C-15 by its correlation with the methyl protons. The proximity of the exchangeable hydroxyl proton at δ 5.41 ppm to the methoxy group obtained by the NOESY spectrum (Figure 55, page 129) indicates the placement of this hydroxyl group on C-18 (δ 138.9 ppm). C-15 (δ 154.2 ppm), therefore, is substituted by another hydroxyl group (δ 11.82 ppm, s). The down field chemical shift of this proton (δ 11.82 ppm) caused by the hydrogen bond formation suggests the peri-relationship of this hydroxyl group to the carbonyl signal at δ 203.4 ppm. This carbonyl carbon is assigned as C-14.

Fragment **52** consists of the signals of two carbonyl carbons (δ 185.9 and 180.9 ppm), four sp^2 carbons (δ 156.1, 141.5, 137.0, and 127.7 ppm), one methoxy carbon (δ 60.7 ppm), and one methyl carbon (δ 8.5 ppm). The chemical shifts of two carbonyl carbons at δ 185.9 and 180.9 ppm suggest the presence of a quinone structural unit and are able to be assigned as C-5 and C-8, respectively. Compared with the spectral data of other known isoquinoline quinones, the sp^2 carbon signals at δ 156.1, 141.5, 137.0, and 127.7 ppm are continuously assigned as C-7, C-9, C-10, and C-6. From the

HMBC spectrum of compound A-129 (Figure 52), the methyl protons at δ 1.87 ppm show the long-range correlations with C-6 and C-7 while the methoxy protons at δ 3.97 ppm correlate with C-7, only. These evidences suggest the placements of the methyl group on C-6 and the methoxy group on C-7. The nOe observed between these two groups supports their proximity.

All of these fragments are linked together based on the analyses of H-H long-range couplings and C-H long-range correlations. Fragment **53** is proposed by the connection of fragments **48**, **49**, and **52**. The HMBC spectrum of compound A-129 (Figure 52) exhibits the C-H long-range correlations of C-9 (δ 141.5 ppm) and H-1 (δ 3.83 ppm, ddd), H-4α (δ 2.99 ppm, dd), and H-22b (δ 3.82 ppm, dd). The C-H long-range correlations of C-10 (δ 137.0 ppm) and H-4α, H-4β (δ 1.46 ppm, ddd) are also observed. These evidences suggest the successive connectivity of C-1, C-4 and C-9, C-10, respectively. This connectivity is supported by the long-range homoallylic coupling of H-1 and H-4β through the double bond between C-9 and C-10 with the coupling constant of 1.8 Hz.



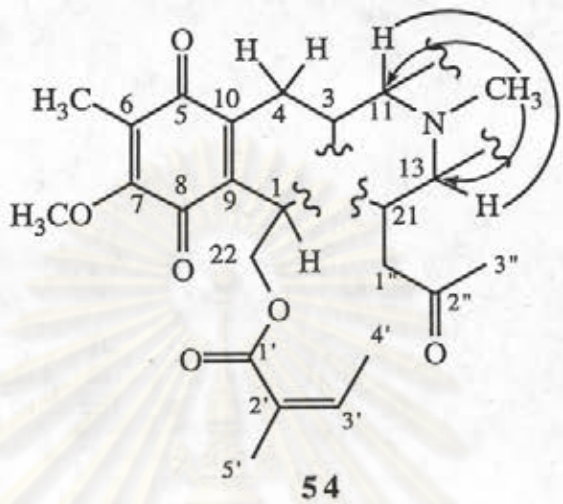
53

→ C-H long-range correlation

—— H-H long-range coupling

The linking of fragments **50** and **53** is executed by insertion of a heteroatom between C-11 and C-13 to provide a long-range coupling via a zigzag path between H-11 (δ 4.20 ppm, dd) and H-13 (δ 3.09 ppm, dd) with the coupling constant of

0.8 Hz. The heteroatom inserted is a nitrogen atom which is adjacent to a methyl group (δ 2.31 ppm, s). This suggestion is confirmed by the C-H long-range correlations from C-11 and C-13 to N-CH3.



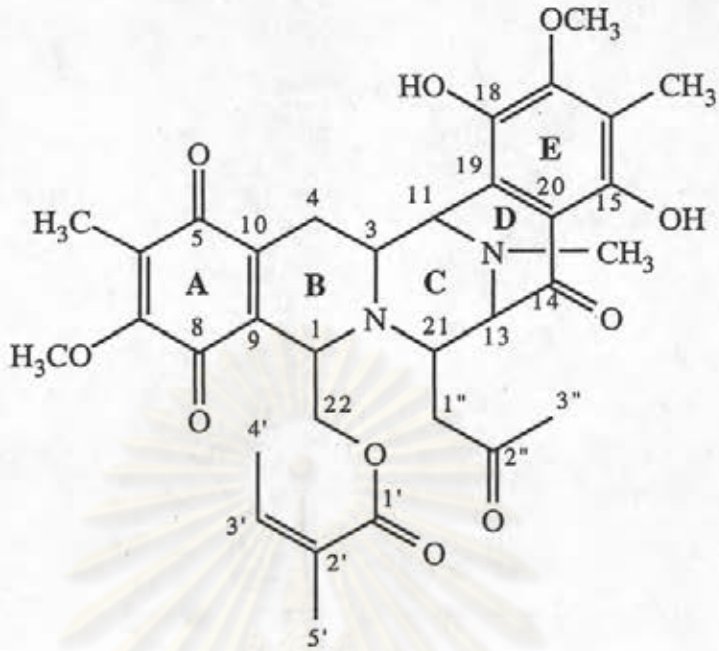
54

→ C-H long-range correlation

—— H-H long-range coupling

Units **51** and **54** are placed between C-19 and C-11 depended on the down field chemical shift of H-11 at δ 4.20 ppm. This chemical shift is the characteristic of the proton on the heteroatom carbon connected to an aromatic ring. The carbonyl C-14 of fragment **51** must be connected to C-13 of fragment **54** to form a 6-membered ring.

After their linking, there is only one nitrogen atom left. This nitrogen, therefore, has to be inserted among C-1, C-3, and C-21. The planar structure of compound A-129 are proposed as shown.



55

This compound is a new member of the saframycin-type isoquinoline quinones possessing an alkyl group substituted at position 21. It is named as renierine B [**55**]. Its structure is finally supported by the comparison with other known compounds in the same group which exhibit the common spectral pattern (Lown, Joshua, and Chen, 1981; Frincke and Faulkner, 1982; He and Faulkner, 1989).

The assignments for the protonated carbons are achieved by the analysis of the $^{13}C,^{1}H$ COSY spectrum (Figure 53-54, pages 127-128). The correlations between each pair of proton and carbon are as followed; H-1 (δ 3.83 ppm) - C-1 (δ 53.4 ppm), H-3 (δ 3.14 ppm) - C-3 (δ 51.5 ppm), H-4α (δ 2.99 ppm) and H-4β (δ 1.46 ppm) - C-4 (δ 24.1 ppm), H-11 (δ 4.20 ppm) - C-11 (δ 57.2 ppm), H-13 (δ 3.09 ppm) - C-13 (δ 67.6 ppm), H-21 (δ 3.81 ppm) - C-21 (δ 55.9 ppm), H-22a (δ 4.13 ppm) and H-22b (δ 3.82 ppm) - C-22 (δ 64.3 ppm), H-3' (δ 5.82 ppm) - C-3' (δ 139.1 ppm), H-4' (δ 1.71 ppm) - C-4' (δ 15.3 ppm), H-5' (δ 1.31 ppm) - C-5' (δ 19.8 ppm), H-1"a (δ 3.44 ppm) and H-1"b (δ 2.51 ppm) - C-1" (δ 37.5 ppm), H-3" (δ 2.21 ppm) - C-3" (δ 30.7 ppm), 6-CH_3 (δ 1.87 ppm) - 6-$\underline{C}H_3$ (δ 8.5 ppm), 7-OCH_3 (δ 3.97 ppm) - 7-$O\underline{C}H_3$ (δ 60.9 ppm), 16-CH_3 (δ 2.13 ppm) - 16-$\underline{C}H_3$ (δ 8.7 ppm), 17-OCH_3 (δ 3.82 ppm) - 17-$O\underline{C}H_3$ (δ 60.7 ppm), and N-CH_3 (δ 2.31 ppm) - N-$\underline{C}H_3$ (δ 42.6 ppm).

The assignments for all protons and carbons as well as the long-range correlations observed in the HMBC spectrum are summarized in Table 15 (page 73).

The relative stereochemistry of this compound is determined by analysis of the coupling constants and analysis of nOe observed from the NOESY spectrum (Figure 55, page 129). Compared with the known compounds, the chemical shift of H-4β at δ 1.46 ppm moves to the high field region because of the ring current effect from the aromatic E ring, and indicates the β-orientation of this proton. The homoallylic coupling via π-bond of this proton with H-1 (J = 1.8 Hz) indicates the α-orientation of H-1. In this position, the allylic angles (the angle between the plane of the double bond and the substituted proton) of H-4β and H-1 are close to 90° and provide the maximum homoallylic coupling constant. If these angles are close to 0° or 180°, there will be no coupling found (Jackman and Sternhell, 1969).

The NOESY spectrum shows the correlations between the protons as followed; H-4α - H-11, H-3 - H-11, H-11 - N-CH_3, N-CH_3 - H-13, and H-1 and H-1"b, and indicates that all of these protons are in the α-orientation. In the other hand, the nOe observed between H-21 and H-22a suggests the β–orientations of H-21 and C-22. All nOe's are summarized in Table 16. The proposed conformation of compound A-129 is shown in Figure 13.

Table 15 Carbon and proton assignments of compound A-129, nOe observed between protons, and long-range correlations between carbons and protons

position	δC (ppm)	δH (ppm)	nOe observed between protons in NOESY spectrum	long-range correlations from C to H in HMBC spectrum
1	53.4	3.83, ddd (4.8,3.6,1.8)**	H-1"b	-
3	51.5	3.14, dt (10.9,2.8)	H-11	H-4β
4	24.1	α; 2.99,dd (17.0,2.8)	H-11,H-4β	-
		β; 1.46, ddd (17.0,10.9,1.8)	H-4α	-
5	185.9	-	-	-
6	127.7	-	-	6-CH_3
7	156.1	-	-	6-CH_3,7-OCH_3
8	180.9	-	-	-
9	141.5	-	-	H-4α,H-1,H-22b
10	137.0	-	-	H-4α,H-4β
11	57.2	4.20, dd (2.7,0.8)	H-3,H-4α, N-CH_3	N-CH_3,H-4α
13	67.6	3.09, dd (1.1,0.8)	N-CH_3,H-21	N-CH_3
14	203.4	-	-	-
15	154.2	-	-	16-CH_3
16	117.8	-	-	16-CH_3
17	152.1	-	-	16-CH_3,17-OCH_3
18	138.9	-	-	-
19	117.9*	-	-	-
20	112.6*	-	-	-
21	55.9	3.81, ddd (9.3,2.1,1.1)**	H-13,H-22a	-
22	64.3	Ha; 4.13, dd (13.0,4.8)	H-21,H-22b	-
		Hb; 3.82,dd (13.0,3.6)**	H-22a	

Table 15 (cont.)

position	δC (ppm)	δH (ppm)	nOe observed between protons in NOESY spectrum	long-range correlations from C to H in HMBC spectrum
1'	167.0	-	-	H-5'
2'	126.8	-	-	H-4',H-5'
3'	139.1	5.82, qq (7.2,1.4)	H-4',H-5'	H-4',H-5'
4'	15.3	1.71, dq (7.2,1.4)	H-3'	-
5'	19.8	1.31, quintet (1.4)	H-3'	-
1"	37.5	Ha; 3.44, dd (17.2,9.3)	H-1"b	-
		Hb; 2.51, dd (17.2,2.1)	H-1,H-1"a,H-3"	
2"	207.2	-	-	-
3"	30.7	2.21, s	H-1"b	-
6-CH_3	8.5	1.87, s	7-OCH_3	-
7-OCH_3	60.7	3.97, s	6-CH_3	-
16-CH_3	8.7	2.13, s	17-OCH_3	-
17-OCH_3	60.9	3.82, s	16-CH_3, 18-OH	-
N-CH_3	42.6	2.31, s	H-11,H-13	-
15-OH	-	11.82, s	-	-
18-OH	-	5.42, s	17-OCH_3	-

note ; The numbers in the parenthesis are the coupling constants in Hz.

* The chemical shifts are interchangeable.

** The splitting pattern and the coupling constants of these signals are estmated from other coupled signals.

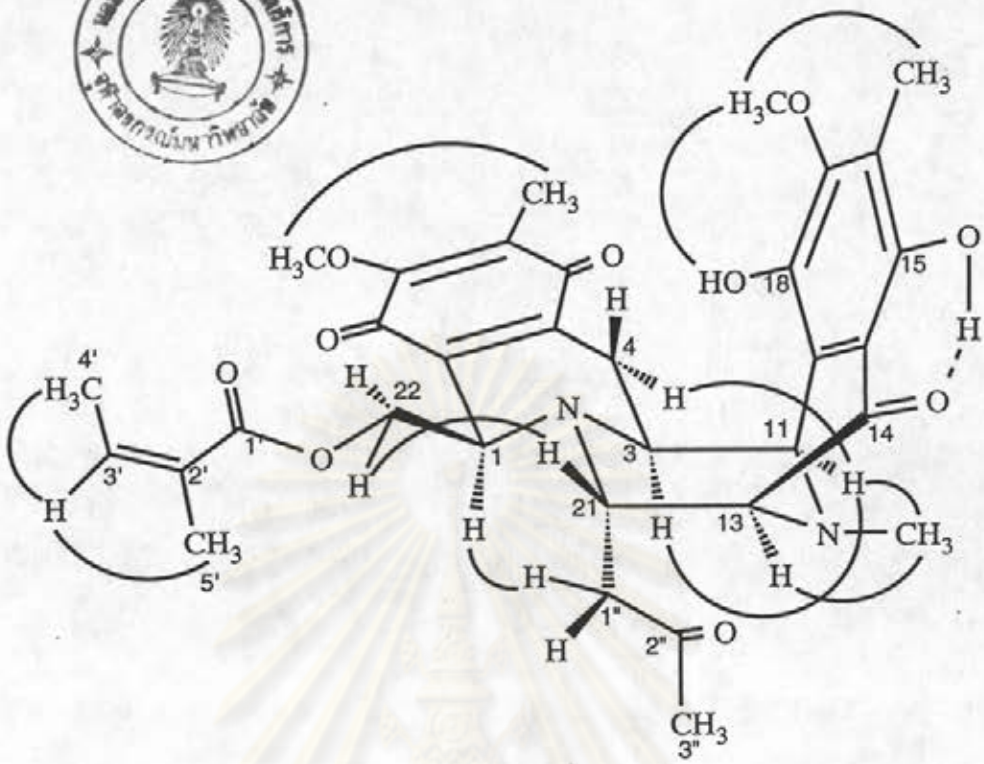


Figure 13 The conformation of compound A-129

The structure proposed from the analyses of nmr spectra is supported by the eims fragmentation (Figure 14). The main fragmentations of this compound are proposed to be occurred by the C ring cleavage to give the fragments at *m/z* 388 (14.3 %) and 236 (100 %) This fragmentation pattern can be found in the fragmentations of the ecteinascidins, the tetrahydroisoquinolines from a tunicate, *Ecteinascidia turbinata*, which perform the common skeleton to the dimeric isoquinoline quinones (Rinehart *et al*, 1990, b). Other fragments found are caused by the cleavages of the angelate ester part like those discussed for compounds A-025, A-056, and A-073.

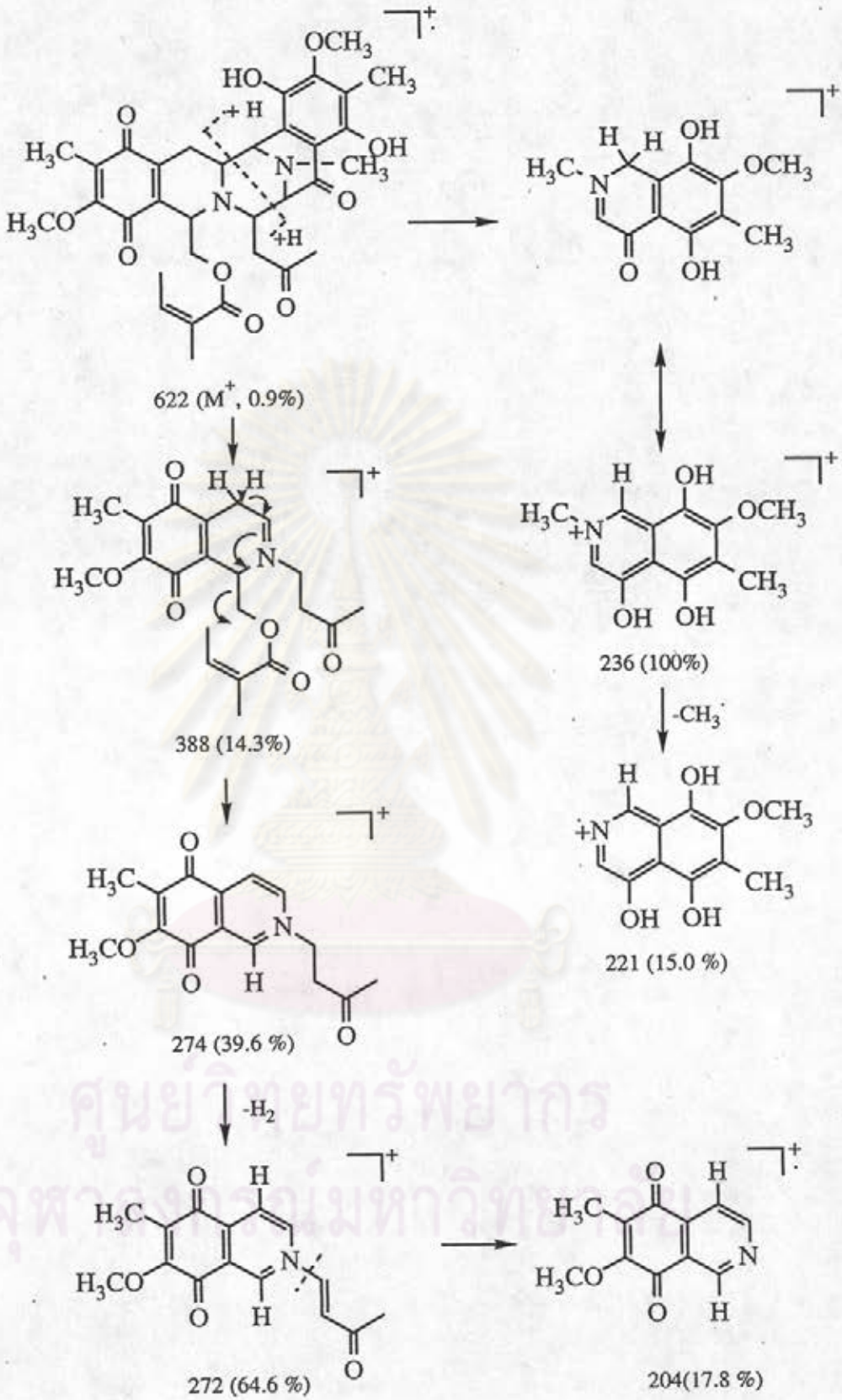


Figure 14 Proposed mass fragmentations of compound A-129

3. Bioactivity

All isolated compounds were determined their antimicrobial activity against 5 microbes, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, using disc method at the concentration of 0.1 mg/disc. The results are shown in Table 16.

Table 16 Antimicrobial activity of isolated compounds

compounds	diameter of inhibition zone (mm)				
	S, aureus	*B. subtilis*	*E. coli*	*Ps. aeruginosa*	*C. albicans*
A-025	6.5	6.9	- ve	- ve	- ve
A-051	14.0	9.7	-ve	-ve	2.6
A-056	11.4	8.2	- ve	- ve	- ve
A-073	8.0	5.8	-ve	-ve	-ve
A-082	12.6	6.7	-ve	-ve	6.6
A-129	- ve	- ve	-ve	-ve	- ve

Among these compounds, the monomeric isoquinoline quinones, compounds A-025, A-051, A-056, A-073, and A-082, are active against gram-positive bacteria. The most active compounds are compounds A-051 and A-082 which also exhibit the activity against *C. albicans*. The compounds A-056 and A-073 are moderately active and compound A-025 are the least active compound in this group.

While the isolated monomeric compounds shows antimicrobial activity, compound A-129, a dimeric one, is not active against all microbes used in this work at the concentration of 0.1 mg/disc. The decreasing activity of this compound may be caused by the alkyl substitution at position 21.

As reported by Lown *et al.* (1982), the cyano residue at position 21 of saframycin A is the active site of this compound. It was suggested that the cyano group was eliminated to generate an active immonium ion which could bind with the nucleophiles like DNA. Davidson (1991) also proposed that the amide linkage between the nitrogen (N-2) and C-21 increased the stability but decreased the activity of renieramycin G.

The decreasing of activity of compound A-129 should be explained in the same way. The substitution by the propyryl side chain, a bad leaving group, prevents the formation of the immonium ion. This compound, therefore, cannot react with the nucleophile, and the activity will be reduced.

Because of the 90 % methanol extract of this sponge also showed the toxicity against tumor cell lines and ichthyotoxicity, these activities will be determined in all isolated compounds, too. The determination will reveal other interesting activities and guide to the study in the structure-activity relationship of the isolated compounds and other isoquinoline quinones.

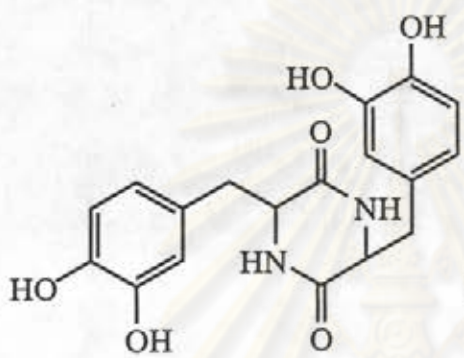
4. Possible Sources and Origins of Isoquinoline Quinones from the *Reniera*

There are 2 major sources of naturally occurring isoquinoline quinones, certain species of the *Streptomyces* for the mimocin and saframycin series, and the sponge, *Reniera* sp., for the renierone and renieramycin series. There are also other minor sources for the compounds in this class, but only a few compounds were found.

Isoquinoline quinones from both major sources have something in common. They have the same skeleton with some of the same substituents resided on the same positions, such as the methyl and methoxy group on positions 6 and 7, respectively. This phenomenon indicates the closeness between the producers of isoquinoline quinones from both sources. This similarity suggests the possibility of the actinomycetes source of the *Reniera* isoquinoline quinones (Frincke and Faulkner, 1982). The actinomycetes may be the symbiont or epibiont of the sponge. On the other hand, the microorganisms may be fed by the sponge and their metabolites are accumulated in the sponge's body.

Although there has been no study on the biogenesis of the isoquinoline quinones, their biogenesis can be deduced to be in the same route as those of

ecteinascidins, the tetrahydroisoquinolines which have the common skeleton to the saframycins. As proposed by Rinehart *et al.* (1990 b), the tetrahydroisoquinoline nucleus of ecteinascidins is arisen from the condensation of carbonyl groups of dopa or dopamine equivalents, perhaps involving the intermediate diketopiperazine **[56]**. The B ring of this system is closed by the condensation with a serine-derived glycoaldehyde to from structure **57**. Oxidation and methylation would complete this main structure.



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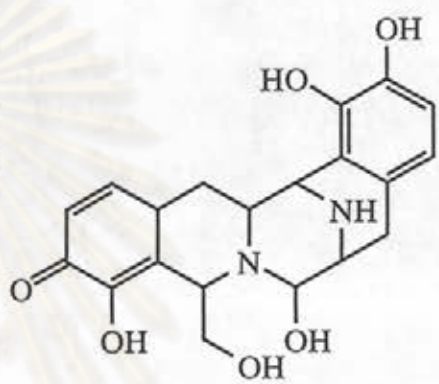

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It was found that some dimeric isoquinoline quinones are unstable. Renieramycin E **[40]** and F **[41]** can degrade very quickly and provide renierone **[17]** and mimosamycin **[16]** as their decomposition products. It was suggested that the monomeric isoquinoline quinones previously isolated were the degradation products from the oxidative cleavages of the dimeric isoquinoline quinones (He and Faulkner, 1989). This suggestion, however, has to be proved.