CHAPTER III

EXPERIMENTAL

1. Sources and Authentication of Sponges

The colonies of a sponge, *Reniera* sp., were collected by snorkeling at the depth of 2-3 m from Si-Chang Island, Chonburi, Thailand, in July, 1992. The sponge voucher specimens were preserved in 70 % ethanolic solution and deposited at Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, The remaining samples were frozen at -20 ^{o}C until they were extracted.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Adsorbent ; The TLC plates for routine works were
(i) precoated TLC plates of silica gel 60 F-254 (E. Merck)
(ii) precoated TLC plates of silica gel C-18 F-254 (E. Merck).

Layer thickness ; 250 μm

Technique ; One way, ascending

Distance ; 5.5 mm

Temperature ; Laboratory temperature (30-35 ^{o}C)

Detection ; 1) Visual detection under day light
2) Visual detection under ultraviolet light at the wavelengths of 254 and 365 nm

2.2 Column Chromatography

2.2.1 Quick Column Chromatography

Adsorbent ; Silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing ; Adsorbent was wet-packed. The slurry of adsorbent in hexane was poured into the column. The solvent was sucked out, and the adsorbent was pressed tightly.

Addition of the sample extract ;

The extract was dissolved in a small volume of dichloromethane, triturated with sufficient quantity of kieselguhr, air-dried and then dried under the vacuum. The material was finely ground and put on the top of the column.

Examination of the eluates ;

Fractions were examined by TLC using visual detection under day light and ultraviolet light at wavelengths of 254 and 365 nm.

2.2.2 Flash Chromatography

Adsorbent ; Silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing ; Adsorbent was wet-packed. The slurry of adsorbent in the eluant was poured into the column and allowed to be settled tightly under the pressure of the nitrogen gas.

Addition of the sample extract ;

The extract was dissolved in a small volume of eluant and loaded on the top of the column.

Examination of eluates ;

Fractions were examined in the same manner as described in section 2.2.1.

2.2.3 Gel Filtration Chromatography

Adsorbent ; Sephadex LH-20

Packing ; Adsorbent was suspended in the eluant and left standing to swell prior to use for 24 hours. The adsorbent, then, was poured into the column and allowed to be settled tightly.

Addition of the sample extract ;

The extract was dissolved in a small volume of eluant and put on the top of the column.

Examination of eluates ;

Fractions were examined in the same manner as described in section 2.2.1.

2.2.4 Medium Pressure Liquid Chromatography (MPLC)

Adsorbent ; All columns used were pre-packed columns,
pre-packed column Lichroprep^{R} Si-60 (0.040-0.063 mm) for normal phase chromatography,
pre-packed column Lichroprep^{R} RP-18 (0.040-0.063 mm) for reverse phase chromatography.

Chromatographic pump ;
Buchi 681 chromatographic pump

Saturation time ; Column was saturated with eluant prior to add the sample extract for 30 minutes.

Addition of sample extract ;
The extract was dissolved in a small volume of eluant, filtered through Toyopak^{R} C-18 filter, and loaded on the top of the column.

Examination of eluates ;
Fractions were examined in the same manner as described in section 2.2.1.

2.3 Spectroscopy

2.3.1 Ultraviolet (uv) Absorption Spectra

The spectra were obtained on a Shimadsu double beam spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were dissolved and adjusted the concentrations using chloroform (analar grade, E. Merck) as solvent.

2.3.2 Infrared (ir) Absorption Spectra

The spectra were obtained on a Perkin-Elmer infrared spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University), or on a Shimadsu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University), using potassium bromide discs to determined the spectra.

2.3.3 Mass Spectra (ms)

The electron impact mass spectra (eims) were determined on a Jeol FX 3000 double focusing spectrometer which was operated at 70 ev with inlet

temperature of 150-240 ^{o}C (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C nmr) Spectra

The nmr spectra were obtained with a Bruker BZH-200 spectrometer (200 MHz for ^{1}H nmr and 50 MHz for ^{13}C nmr) (Department of Chemistry, Faculty of Sciences, Chulalongkorn University) or a Jeol JMN-GSX spectrometer (500 MHz for ^{1}H nmr and 125 MHz for ^{13}C nmr) (Faculty of Pharmaceutical Sciences, University of Tokyo) or GE NMR spectrometer (500 MHz for ^{1}H nmr and 125 MHz for ^{13}C nmr) (University of Hawaii).

The operating solvent for nmr spectra was deuterated chloroform ($CDCl_3$). The chemical shifts were reported in ppm scale using the chemical shift of chloroform at 7.24 ppm (^{1}H nmr) or 77 ppm (^{13}C nmr) as the reference signal.

2.4 Solvents

Throughout this work, all organic solvents were commercial grade and had to be redistillated prior to use, excluding the solvents for MPLC which were analar grade. The solvents for MPLC were also filtered through filter paper (Whatman^{R} no.1) before use.

3. Bioactivity Determination

3.1 Antimicrobial Activity

The determination of antimicrobial activity was executed by the disc method.

3.1.1 Microorganisms

The microorganisms used were kindly supplied by Assistant Professor Sathaporn Sirotamarat of Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. These microorganisms are

- *Staphylococcus aureus* ATCC 6538 P
- *Bacillus subtilis* ATCC 6633
- *Escherrichia coli* ATCC 25922

- *Pseudomonas aeruginosa*
- *Candida albicans*

All of these microorganisms were subcultured in suitable media prior to use in order to intensify their activities. The inoculation was carried out at 37 ^{o}C, 18-24 hours for bacteria or 48 hours for yeast.

3.1.2 Media

- Nutrient agar (NA)

Formula per liter of purified water

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g

Final pH 7.4

All ingredients were suspended in purified water 1 l and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to completely dissolve. The medium was sterilized by autoclaving at 121^{o}C for 15 minutes.

This medium was prepared for NA slants which were used in subculturing bacteria.

- Trypticase soy agar (TSA)

The medium used was BBL^{R} Trypticase^{R} Soy Agar (Becton-Dickinson Microbiology System).

Formula per liter of purified water

Pancreatic digest of casein	15.0 g
Papaic digest of casein	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

Final pH 7.3 $\pm$ 0.2

40 g of Powder was suspended in purified water 1 l and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to completely dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes.

This medium was prepared for inoculating the bacteria during the assay.

- Sabouraud dextrose agar (SDA)

The medium used was BBL^{R} Sabouraud Dextrose Agar (Becton-Dickinson Microbiology System).

Formula per liter of purified water	
Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Dextrose	40.0 g
Agar	15.0 g
Final pH	5.6 ± 0.2

65 g of Powder was suspended in purified water 1 l and thoroughly mixed. It was heated with frequent agitation and boiled for 1 minute to completely dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes.

This medium was prepared for SDA slants, which were used in subculturing yeast, and for yeast inoculation during the assay.

3.1.3 Sample Preparation

The test sample was dissolved in methanol and diluted to the concentration of 5 mg/ml. 0.02 ml of This solution was transfered to a 6.0 mm disc (Whatman^{R} AA disc for antibiotic assay) and allowed to dry. The final concentration was 0.10 mg/disc. The determination was made in duplicate.

3.1.4 Bioassay

The procedures in this bioassay were carried out by the aseptic technique. All glasswares and materials had to be sterilized before use with hot air oven at 180 °C for 1 hour.

Each microorganism was suspended in sterilized normal saline solution. The turbidity of the suspensions was adjusted to be equal to that of MacFarland suspension no.1 (equivalent to 300,000,000 cell/ml). Each microorganism suspension, then, was three-dimensionally spreaded on the surface of suitable medium plate.

The sample discs were put on the microbe-spreaded medium plates These plates, then, were incubated at 37 ^{o}C for 18-24 hours for bacteria, or for 48 hours for yeast. After inoculation, if the test sample was antimicrobial, the clear zone would appear. The diameter of inhibition zone was measured and reported in scale of millimeter.

3.2 Brine Shrimp Lethality Activity

The bioassay was applied from the method of Meyer *et al.* (1982).

3.2.1 Brine Shrimp

Brine shrimp's (*Artemia salina* Leach) eggs (Aquarium product^{R}) were hatched in a shallow rectangular dish filled with artificial sea water. The dish was divided into 2 sections by a plastic divider bearing a few of 2-mm holes. One section containing the shrimp's eggs was darken, while another one was illuminated by a tungsten lamp. After 48 hours, the nauplii of the shrimp, which had hatched and been allowed to be mature, would move to the lighted part, and the shells were separated by the divider.

3.2.2 Artificial Sea Water

The artificial sea water was prepared from the commercial salt mixture (Instant Ocean, Aquarium System). 38 g of This salt mixture was dissolved in 1 l of purified water. The solution, then, was filtered through filter paper (Whatman^{R} filter paper no.1).

3.2.3 Sample Preparation

The test sample was dissolved in methanol and diluted to proper concentrations. Sufficient volumes of this stock solution were transfered to 3 vials. Each vial was allowed to dry and added 5 ml of artificial sea water. Final concentrations were 0.1, 0.01, and 0.001 mg/ml, respectively. All determination were made in triplicate. The sonication was able to be obtained to help dissolving. Control vials were prepared by using only methanol and carried out in the same fashion as test sample.

3.2.4 Bioassay

Ten shrimps were transfered to each vial with a disposable pipette. The nauplii were able to be counted in the stem of the pipette against the lighted background. One drop of yeast suspension (3 mg of dry yeast in 5 ml of artificial sea water) was added into each vial as food for the nauplii. The vials, then, were maintained under illumination. After 24 hours, survivors were counted. The number of dead shrimps in test vials of each concentration, subtracted with the number of dead shrimps in control vials, was used to compute and reported in term of LD_{50} value.

3.3 Ichthyotoxic Activity

3.3.1 Fish

The guppy fish (*Poecilis reticulata* or *Lebistes reticulatus*) were used. The fish size was approximately 2 cm long.

3.3.2 Sample Preparation

The test sample was dissolved in ethanol and adjusted the concentration to be 10 mg/ml. 0.1 ml of Sample solution was transfered to a beaker containing 100 ml of tap water. Therefore, the final concentration of sample in water was 0.1 mg/100 ml, and the concentration of ethanol in this solution was not more than 0.1 ml/100 ml. The control solution was prepared by using only 0.1 ml of ethanol and followed the same process as the test solution.

3.3.3 Bioassay

Five guppies were transfered to each beaker. After 12 to 24 hours, dead fish in each beaker were counted and compared with the number of dead fish in control beaker. The ichthyotoxicity was given by number of dead animals at both times.

3.4 Cytotoxic Activity

The aims of the bioassay are concentrated on testing of the cytotoxicity of the test sample against human tumor cell lines and cultured tumor cells from the test animals. The determination was helpfully supported by Professor Tatsuo Higa of Department of Marine Sciences, University of the Ryukyus.

3.4.1 Target Cells

The cell lines utilized as the target cells in this test were

- P388 (a methylcholanthrene-induced lymphoid neoplasm in a DBA/2 mouse, a non-anchorage dependent cell line),
- A-549 (human non-small cell lung carcinoma),
- HT-29 (human colon adrenocarcinoma, moderately well differentiated).

The A-549 and HT-29 cell lines were the human tumor cell lines while the P388 was the induced tumor cell line in animals.

3.4.2 Bioassay

For P388 cell line, the cells were incubated in the presence of the test sample for 48 hours. Cell growth was determined by counting cell using the electronic counting device. The result was obtained by computing the ED_{50} of each test sample.

In human tumor assay, cells were plated overnight in 96-well microtiter plates. Serial dilutions of the test sample were added and cells were incubated for 4-6 days. The measurement of cell growth was performed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a formazan dye, which would be cleaved by active mitochondria to produce a blue color. Optical density which was directly proportional to cell number was measured by spectrometer (Mosmann, 1983).

4. The Extraction

The sponges (15 kg wet weight), were chopped into small pieces. They were repeatedly marcerated for five times in methanol (each, for 30 l, 3 days) and then filtered. The filtrate of each marceration was concentrated to remove methanol under reduced pressure at temperature not over 50 ^{o}C. Remaining volume of this fraction, called F-001, was approximately 3 l. The fraction F-001 was partitioned with dichloromethane (3 l). The dichloromethane extract (F-002) was separated and evaporated to dryness under reduced pressure to yield 70.7 g of brown mass (0.60 % based on wet weight of sponge). A little amount of this fraction (about 30 mg) was reserved for a reference sample and for bioactivity screening.

All of the aqueous phase from fraction F-001 was combined and partitioned with butanol. The butanol extract was separated and evaporated under reduced pressure. This extract (F-003) yielded 35 g (0.29 % based on wet weight of sponge).

Fraction F-002 was dissolved in methanol (900 ml) and added water to make a solution of 90 % methanol. It was partitioned with hexane (2 l). Both extracts were evaporated to dryness under reduced pressure to yield 57.7 and 10.5 g (0.38 and 0.07 % based on wet weight of sponge) and named as F-004 and F-005, respectively. A little amount of these three fractions was also reserved for references and for bioactivity screening.

5. The Bioassay-directed Isolation of Bioactive Constituents from the Sponge, *Reniera* sp.

In order to search for the fractions with interesting bioactivities, fractions F-002 - F-005 were determined for antimicrobial, brine shrimp lethality, ichthyotoxic, and cytotoxic activities. The results were shown in Tables 6 and 7.

Table 6 Antimicrobial activity of fraction F-002 - F-005

fractions	diameter of inhibition zone (mm)				
	S, aureus	*B. subtilis*	*E. coli*	*Ps. aeruginosa*	*C. albicans*
F-002	3.8	2.6	- ve	- ve	- ve
F-003	- ve	-ve	-ve	-ve	-ve
F-004	3.7	2.0	- ve	- ve	- ve
F-005	- ve	-ve	-ve	-ve	-ve

Table 7 Bioactivities of fraction F-002 and F-003

activities	F-002	F-003
- brine shrimp lethality (LD_{50} : µg/ml)	> 100	15
- Ichthyotoxicity (% dead animals)	100 (6 hrs)	- ve
- cytotoxicity (ED_{50} ; µg/ml)		
- P388	1	2.5
- A-549	1	2.5
- HT-29	1	2.5

The antimicrobial assay against *S. aureus* was chosen to monitor the isolation of the bioactive compounds. In each time of assay, the concentration of the test sample was 0.1 mg/disc. The comparison of the potency of each fraction was depended on the diameter of inhibition zone measured.

Fraction F-004 (57.5 g) was dissolved in a small volume of methanol and chloroform and triturated with kieselguhr (70 g). This mixture was dried under the vacuum. It, then, was fractionated by the quick column chromatographic technique using a scintered glass filter column of silica gel (700 g, 20 x 10 cm). The eluant were used in the order as shown below.

- hexane	1,000 ml	fractions # 1-6
- hexane : ethyl acetate (9:1)	1,000 ml	fractions # 7-11
- hexane : ethyl acetate (8:2)	2,000 ml	fractions # 12-21
- hexane : ethyl acetate (3:1)	2,000 ml	fractions # 22-31
- hexane : ethyl acetate (1:1)	2,000 ml	fractions # 32-41
- ethyl acetate	1,500 ml	fractions # 42-46
- ethyl acetate : methanol (1:1)	4,000 ml	fractions # 47-65

Methanol was used to wash the column until the eluates were diluted and clear comparing to the former ones.

The eluates were examined by TLC using 50 % ethyl acetate in hexane as developing solvent. The fractions giving similar chromatographic pattern were combined and designated. All combined fractions, then, were determined their antimicrobial activity against *S. aureus*. The results were shown in Table 8.

Table 8 The combined fractions from F-004

fractions	number of eluates	weight (g)	diameter of inhibition zone (mm)
F-006	1-10	2.03	- ve
F-007	11-16	0.20	- ve
F-008	17-20	0.25	2.2
F-009	21-25	0.67	5.7
F-010	26-34	2.02	5.7
F-011	35-41	1.40	5.5
F-012	42-46	6.62	3.8
F-013	47-65	15.00	- ve
F-014	MeOH eluted	3.82	- ve

5.1 The Isolation of Bioactive Constituents from Fraction F-010

Fraction F-010 was fractionated by the flash chromatographic technique, using a column of silica gel (160 g, 5 x 20 cm). The sample (2.02 g) was dissolved in 15 % ethyl acetate in hexane and loaded on the top of the column. The column was eluted with 15 % ethyl acetate in hexane (2,500 ml), and the fractional volume was about 50 ml. The eluates were examined by TLC using 15 % ethyl acetate in hexane as developing solvent, combined, and designated in the usual manner. All combined fractions were also monitored for antimicrobial activities. The results were shown in Table 9.

Table 9 The combined fractions from F-010

fractions	number of eluates	weight (mg)	diameter of inhibition zone (mm)
F-015	1-10	300	- ve
F-016	11-14	570	6.6
F-017	15-19	250	11.5
F-018	20-24	230	8.8
F-019	25-34	180	8.0
F-020	35-50	240	4.3

5.1.1 The Isolation for Compound A-025

The TLC chromatogram of fraction F-016 showed that there was one major yellow compound which became red quickly under ultraviolet light. This fraction (570 mg) was isolated by the chromatographic technique using a column of sephadex LH-20 (2 x 45 cm) with 20 % acetone in hexane as eluant. The eluates were collected based on the color bands (approximately 20 ml per fraction). The fractions performing as yellow solution were combined after examining with TLC. This combined fraction yielded 275 mg and was further purified using a column of silica gel (60 g, 2 x 35 cm) with a mixture of hexane:chloroform:methanol (75:20:5) as eluant. The eluates was also collected based on the color bands (approximately 15 ml per fraction).

One yellow compound was crystallized from fractions 3 - 8. It yielded 30 mg (0.04 % of F-002) and was named as A-025. This compound was identified as renierone [17].

5.1.2 The Isolation for Compound A-051

Examined by TLC, fraction F-017 contained one major yellow compound which became red quickly under ultraviolet light. This fraction was further purified by a two-step method.

F-017 (250 mg) was fractionated using a column of sephadex LH-20 (2 x 45 cm) with 20 % ethyl acetate in hexane as eluant. The fractions were collected based on the color bands (approximately 15 ml per fraction). The fractions of yellow solution was examined by TLC and combined to yield 50 mg. This combined fraction was further purified using a column of silica gel (40 g, 2 x 30 cm) with hexane:ethyl acetate:methanol (85:10:5) as eluant. The eluates were collected depended on the color bands (approximately 15 ml per fraction).

A yellow compound, from fractions 5 - 6 was recrystallized with methanol as yellow needles. It yielded 10 mg (0.014 % of F-002), was named as A-051, and was identified as 1,6-dimethyl-7-methoxy-5,8-dihydroisoquinoline-5,8-dione **[21]**.

5.1.3 The Isolation for Compound A-056

The TLC chromatograms of fractions F-018 and F-019 showed their similar constituents with one major red compound. The isolation for the red compound was carried out by a three-step process.

Each fraction (230 and 180 mg) was isolated using a column of sephadex LH-20 (2 x 45 cm) and eluted with 20 % ethyl acetate in hexane. 15 ml-Fractions were collected based on the color bands. The fractions of the red compound, after examined by TLC, were combined and isolated using the MPLC reversed phase column (Lichroprep^{R} RP-18 column, 2 x 30 cm). The MPLC pump was operated using 70 % methanol in water as an eluant (operating pressure 1-2 bar, flow rate 3-4 ml/min). The red band was separated out and examined to combine. The combined fraction was purified using a column of silica gel (50 g, 2 x 30 cm) with 20 % acetone in hexane as an eluant. The eluates were collected depended on the color bands.

The red compound was obtained as a deep-red non-crystallized solid. It yielded 35 mg (0.050 % of F-002), was named as A-056, and was identified as *N*-formyl-1,2-dihydrorenierone **[19]**.

5.2 The Isolation of Bioactive Constituents from Fraction F-012

Fraction F-012 (6.62 g) was equally divided into 2 portions, and further fractionated by flash chromatography using the column of silica gel (160 g, 5 x 20 cm) with 25 % acetone in dichloromethane (2,500 ml) as an eluant. 40 ml of Eluates were

collected based on the color band. The eluates were examined by TLC, and the fractions showing the same pattern were combined and designated. All of these newly combined fractions were determined their antimicrobial activity against *S. aureus* at the concentration of 0.1 mg/disc. The results were shown in Table 10.

Table 10 The combined fractions from F-012

fractions	number of eluates	weight (mg)	diameter of inhibition zone (mm)
F-058	1-4	8	3.2
F-059	5-9	18	8.4
F-060	10-13	15	4.1
F-061	14-17	17	9.0
F-062	18-23	25	7.4
F-063	24-28	67	6.7
F-064	29-35	220	11.6
F-065	36-45	280	2.6
F-066	46-60	3.1 g	4.8

5.2.1 The Isolation for Compound A-073

The fraction F-063 contained one major deep-blue compound. This fraction (60 mg) was isolated using a column of sephadex LH-20 (2 x 40 cm) with 25 % ethyl acetate in hexane as an eluant. The fractions were collected based on the color bands (approximately 15 ml per fraction). After examining by TLC, the fractions containing the blue compound were further purified using the MPLC reversed phase column (Lichroprep^{R} RP-18 column, 2 x 30 cm). The MPLC was operated using 75 % methanol as an eluant, pressure at 1-2 bar, flow rate at 2.5 ml/min. 10 ml-Fractions were collected and examined.

A deep-blue non-crystallized compound was obtained from eluates 2-4. It yielded 5 mg (0.007 % of F-002) and was named as A-073. This compound was identified as *N*-(1"*E*-buten-2"-onyl)-1,2-dihydrorenierone [**47**].

5.2.2 The Isolation for Compound A-082

Brown precipitate was obtained from the solution of F-064 in 25 % ethyl acetate in hexane. The precipitate (50 mg) contained one major yellow compound. It was purified using a column of silica gel (60 g, 3 x 30 cm) with 2 % ethyl acetate in dichloromethane as an eluant. Fractions were collected depended on the color bands (approximately 20 ml per fraction). The fractions of yellow solution were examined and combined to yield a yellow compound. This compound was recrystallized in chloroform as the yellow flake. It yielded 35 mg (0.050 % of F-002), was designated as A-082, and was identified as mimosamycin [**16**].

5.2.3 The Isolation for Compound A-129

The fraction F-066 (3.10 g) was divided into 2 equal portions, and each one was fractionated using a column of silica gel (160 g, 5 x 40 cm). The eluants were 5 % acetone in dichloromethane (2,500 ml), 15 % acetone in dichloromethane (1,500 ml), and 50 % methanol in dichloromethane (500 ml). 50 ml-Fractions were collected, and the eluates were examined and combined. All combined fractions were determined their antimicrobial activity against *S. aureus* at the concentration of 0.1 mg/disc. The results were shown in Table 11.

Table 11 The combined fractions from F-066

fractions	number of eluates	weight (mg)	diameter of inhibition zone (mm)
F-117	1-17	35	5.7
F-118	18-22	45	5.8
F-119	23-26	370	3.2
F-120	27-31	75	- ve
F-121	32-36	440	3.8
F-122	37-49	470	1.7
F-123	50-63	550	3.7
F-124	64-75	1.58 g	3.0

Fraction F-121 (440 mg), then, was further purified using a column of silica gel (70 mg, 3 x 30 cm) with 25 % acetone in hexane as an eluant. After partial evaporation, a yellow compound crystallized from fractions 18-23. This compound was combined and recrystallized in a mixture of chloroform and hexane to give a greenish-yellow compound (A-129). This compound was identified as a new isoquinoline quinone derivative and named as renierine B [**55**] (30 mg, 0.042 % of F-002).

6. Spectral Data of Isolated Compounds

6.1 Compound A-025

eims ; *m/z* (relative intensity) ; Figure 16, page 90
315 (48.5, M^+), 232 (14.4), 216 (24.1), 82 (100.0), 55 (79.5)

uv ; λ_{max} nm (ε), in chloroform; Figure 17, page 91
244 (26365), 323 (7560)

ir ; ν cm^{-1}, KBr disc; Figure 18, page 92
1732, 1681

^1H nmr ; δ ppm, 200 MHz, in chloroform-d ; Fugure 19, page 93
1.96 (3 H, quintet, J = 1.2 Hz), 1.99 (3 H, dq, J = 7.1, 1.2 Hz), 2.05 (3 H, s),

4.15 (3 H, s), 5.70 (2 H, s), 6.05 (1 H, qq, J = 7.1, 1.2 Hz), 7.73 (1 H, d, J = 4.9 Hz), 8.87 (1 H, d, J = 4.9 Hz)

^{13}C nmr ; δ ppm, 50 MHz, in chloroform-d ; Figure 20, page 94

9.2 (q), 15.9 (q), 20.9 (q), 61.1 (q), 65.2 (t), 118.3 (d), 122.1 (s), 128.0 (s), 130.1 (s), 137.9 (d), 138.9 (s), 153.8 (d), 157.1 (s), 167.4 (s), 181.8 (s), 184.4 (s)

6.2 Compounds A-051

eims ; *m/z* (relative intensity) ; Figure 21, page 95

217 (100.0, M^+)

uv ; λ_{max} nm (ε), in chloroform; Figure 22, page 96

252 (14190), 324 (5600)

ir ; ν cm^{-1}, KBr disc; Figure 23, page 97

1680

^{1}H nmr ; δ ppm, 200 MHz, in chloroform-d ; Fugure 24, page 98

2.03 (3 H, s), 2.94 (3 H, s), 4.12 (3 H, s), 7.74 (1 H, d, J = 4.9 Hz), 8.79 (1 H, d, J = 4.9 Hz)

^{13}C nmr ; δ ppm, 50 MHz, in chloroform-d ; Figure 25, page 99

8.9 (q), 25.5 (q), 60.8 (q), 117.1 (d), 122.8 (s), 129.6 (s), 138.7 (s), 153.4 (d), 158.1 (s), 160.2 (s), 181.7 (s), 184.3 (s)

6.3 Compound A-056

eims ; *m/z* (relative intensity) ; Figure 26, page 100

345 (3.4, M^+), 315 (9.7), 245 (4.0), 232 (81.2), 204 (100.0), 82 (50.2), 55 (51.1)

uv ; λ_{max} nm (ε), in chloroform; Figure 27, page 101

242 (15145), 270 (16835) , 351 (6485)

ir ; ν cm^{-1}, KBr disc; Figure 28, page 102

1720, 1670

^{1}H nmr ; δ ppm, 200 MHz, in chloroform-d ; Fugure 29, page 103

(A) 1.73 (3 H, quintet, J = 1.4 Hz), 1.86 (3 H, dq, J = 7.3, 1.4 Hz), 1.90 (3 H, s), 4.02 (3 H, s), 4.15 (1 H, dd, J = 11.9, 3.0 Hz), 4.32 (1 H, dd, J = 11.9, 4.6 Hz), 5.92 (1 H, dd, J = 4.6, 3.0 Hz), 5.98 (1 H, d, J = 7.5 Hz), 6.01 (1 H, qq, J = 7.3, 1.4 Hz), 6.89 (1 H, d, J = 7.5 Hz), 8.39 (1 H, s)

(B) 1.83 (3 H, quintet, J = 1.4 Hz), 1.92 (3 H, s), 1.96 (3 H, dq, J = 7.3, 1.4 Hz), 3.85 (1 H, dd, J = 12.4, 3.5 Hz),4.01 (3 H, s), 4.18 (1 H, dd, J = 12.4, 9.3 Hz), 5.32 (1 H, dd, J = 9.3, 3.5 Hz), 6.10 (1 H, qq, J = 7.3, 1.4 Hz), 6.18 (1 H, d, J = 7.7 Hz), 7.39 (1 H, d, J = 7.7 Hz), 8.18 (1 H, s)

^{13}C nmr ; δ ppm, 50 MHz, in chloroform-d ; Figure 31, page 105

(A) 8.5 (q), 15.6 (q), 20.5 (q), 47.3 (d), 61.0 (q), 63.0 (t), 100.8 (d), 123.9 (s), 126.9 (s), 127.0 (s), 133.1 (d), 135.4 (s), 139.6 (d), 156.2 (s), 162.0 (d), 167.2 (s), 180.1 (s), 184.7 (s)

(B) 8.6 (q), 15.8 (q), 20.4 (q), 49.7 (d), 60.8 (q), 63.0 (t), 102.8 (d), 123.1 (s), 126.4 (s), 127.9 (s), 129.3 (d), 136.1 (s), 140.7 (d), 155.6 (s), 161.2 (d), 166.6 (s), 180.1 (s), 184.6 (s)

6.4 Compounds A-073

eims ; *m/z* (relative intensity) ; Figure 38, page 112

385 (2.9, M^+), 355 (0.5), 342 (0.4), 315 (0.9), 285 (1.2), 272 (100.0), 83 (8.5), 55 (15.5), 43 (29.2)

uv ; λ_{max} nm (ε), in chloroform; Figure 39, page 113

243 (18425), 310 (23000) , 580 (6125)

ir ; ν cm^{-1}, KBr disc; Figure 40, page 114

1720, 1660

^{1}H nmr ; δ ppm, 500 MHz, in chloroform-d ; Fugure 41, page 115

1.77 (3 H, quintet, J = 1.4 Hz), 1.93 (3 H, s),1.93 (3 H, dq, J = 7.3, 1.4 Hz), 2.20 (3 H, s), 4.03 (3 H, s), 4.05 (1 H, dd, J = 11.5, 3.9 Hz), 4.32 (1 H, dd, J = 11.5, 6.1 Hz), 5.43 (1 H, br.dd, J = 6.1, 3.9 Hz), 5.89 (1 H, d, J = 13.7 Hz), 5.96 (1 H, d, J = 7.3 Hz), 6.06 (1 H, qq, J = 7.3, 1.4 Hz), 6.76 (1 H, dd, J = 7.3, 1.0 Hz), 7.39 (1 H, d, J = 13.7 Hz)

^{13}C nmr ; δ ppm, 125 MHz, in chloroform-d ; Figure 43, page 117

8.6 (q), 15.8 (q), 20.6 (q), 29.3 (q), 51.3 (d), 60.8 (q), 61.1 (t), 99.7 (d), 105.0 (d), 119.9 (s), 126.8 (s), 127.3 (s), 135.9 (s), 137.8 (d), 140.3 (d), 145.6 (d), 156.3 (s), 167.2 (s), 180.3 (s), 185.1 (s), 196.0 (s)

6.5 Compound A-082

eims ; *m/z* (relative intensity) ; Figure 33, page 107

233 (100.0, M^+)

uv ; λ_{max} nm (ε), in chloroform; Figure 34, page 108
244 (8925), 323 (10810), 399 (3120)

ir ; ν cm^{-1}, KBr disc; Figure 35, page 109
1695, 1645

^{1}H nmr ; δ ppm, 200 MHz, in chloroform-d ; Fugure 36, page 110
2.04 (3 H, s), 3.64 (3 H, s), 4.14 (3 H, s), 7.08 (1 H, s), 8.24 (1 H, s)

^{13}C nmr ; δ ppm, 50 MHz, in chloroform-d ; Figure 37, page 111
9.6 (q), 38.4 (q), 61.3 (q), 111.3 (s), 116.7 (d), 133.2 (s), 138.9 (s), 142.1 (d), 159.5 (s), 162.8 (s), 177.3 (s), 183.5 (s)

6.6 Compound A-129

eims ; *m/z* (relative intensity) ; Figure 46, page 120
622 (0.1, M^+), 567 (0.1), 565 (0.3), 524 (0.6), 522 (0.6), 509 (2.3), 388 (14.3), 272 (64.6), 236 (100.0), 100 (8.7), 83 (28.9), 55 (33.5)

uv ; λ_{max} nm (ε), in chloroform; Figure 47, page 121
271 (25400)

ir ; ν cm^{-1}, KBr disc; Figure 48, page 122
3289 (broad), 1712, 1655, 1623

^{1}H nmr ; δ ppm, 500 MHz, in chloroform-d ; Fugure 49, page 123
1.31 (3 H, quintet, *J* = 1.4 Hz), 1.46 (1 H, ddd, *J* = 17.0, 10.9, 1.8 Hz), 1.71 (3 H, dq, *J* = 7.2, 1.4 Hz), 1.87 (3 H, s), 2.13 (3 H, s), 2.21 (3 H, s), 2.31 (3H, s), 2.51 (1 H, dd, *J* = 17.2, 2.1 Hz), 2.99 (1 H, dd, *J* = 17.0, 2.8 Hz), 3.09 (1 H, dd, *J* = 1.1, 0.8 Hz), 3.14 (1 H, dt, *J* = 11.2, 2.8 Hz), 3.44 (1 H, dd, *J* = 17.2, 9.3 Hz), 3.81 (1 H, ddd, *J* = 9.3, 2.1, 0.8 Hz), 3.82 (1 H, dd, *J* = 13.0, 3.6 Hz), 3.82 (3 H, s), 3.83 (1 H, ddd, *J* = 4.8, 3.6, 1.8 Hz), 3.97 (3 H, s), 4.13 (1 H, dd, *J* = 13.0, 4.8 Hz), 4.20 (1 H, dd, *J* = 2.7, 0.8 Hz), 5.41 (1 H, s), 5.82 (1 H, qq, *J* = 7.2, 1.4 Hz), 11.82 (1 H, s)

^{13}C nmr ; δ ppm, 125 MHz, in chloroform-d ; Figure 51, page 125
8.5 (q), 8.7 (q), 15.3 (q), 19.8 (q), 24.1 (t), 30.8 (q), 37.5 (t), 42.6 (q), 51.5 (d), 53.4 (d), 55.9 (d), 57.2 (d), 60.7 (q), 60.9 (q), 64.3 (t), 67.6 (d), 112.3 (s), 117.8 (s), 117.9 (s), 126.8 (s), 127.7 (s), 137.0 (s), 138.9 (s), 137.1 (d), 141.5 (s), 152.6 (s), 154.2 (s), 156.1 (s), 167.0 (s), 180.9 (s), 185.9 (s), 203.4 (s), 207.2 (s)