# CHAPTER III

# EXPERIMENTAL

## Source and Authentication of the Sponge

The colonies of a sponge, investigated in this work were found at the depth of 10-15 m. in the vicinity of Phi Phi Island, Krabi, Thailand, and were identified by Dr. John N. A. Hooper of Queensland Museum, Australia as *Phakellia cavernosa* (*Acanthella cavernosa* Dendy). The voucher specimens of the sponge were preserved in 70% ethanolic solution and kept at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The remaining samples were frozen at -20 °C prior to extraction.

## General Techniques

1. Chromatography

   1.1 Analytical Thin Layer Chromatography

   Adsorbent : Silica gel 60 F-254 precoated plate ( E. Merck)

   Layer thickness : 250 μm

   Technique : One way, ascending

   Distance : 5 cm

   Temperature : Room temperature 25-30 °C

   Detection : 1) Visual detection under daylight

   2) Ultraviolet light at the wavelengths of 254 and 365 nm

   3) Spraying with anisaldehyde-sulphuric acid solution and heating at 100-110 °C for a few minutes

1.2 Column Chromatography

1.2.1 Conventional column chromatography

Column size : The glass columns 1.5-5.0 cm in diameter were used depending on the quantity of sample to be seperated.

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing method : Wet packing

Solvent : Various solvent systems depending on materials

Sample loading : The sample extract was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates : Fractions were examined by TLC under ultraviolet light at the wavelengthsof 254 and 365 nm and by exposing to anisaldehyde-sulphuric acid solution, respectively.

1.2.2 Gel filtration chromatography

Column size : The glass column 2.2 cm in diameter

Adsorbent : Sephadex LH-20

Packing method : The adsorbent was suspended in the eluent and left to swell for 24 hours (before using), then poured into the column and allowed to settle.

Sample loading : The sample extract was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates : Fractions were examined in the same manner as described in section 1.2.1.

2. Spectroscopy

2.1 Ultraviolet (UV) Absorption Spectra

The ultraviolet absorption spectra were obtained on a Milton Roy Sprotronic 3000 Ray Spectrometer. Methanol was employed as the solvent for all compounds.

2.2 Infrared (IR) Absorption Spectra

The spectra were obtained on a Shimadzu IR-440 infrared spectrophotometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3 Mass Spectra (MS)

The atmosphere pressure c mass spectra (APCI-MS) were obtained on a Finnigan 700 equipped with Finnigan Electroscopy Source, operating at -31 eV CID voltage with capilary temperature of 230$^{0}$.

2.4 Proton and Carbon-13 Nuclear Magnetic Resonance ($^{1}H$ and $^{13}C$ NMR) spectra

The nmr spectra were obtained on a JEOL JMN-A500 (Alpha series) 500 MHz NMR Spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University) and a Bruker Avance DPX-300 300 MHz NMR Spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3. Optical Rotations

Optical Rotations of the isolated compounds were determined with a Perkin-Elmer Model 241 Polarimeter (Faculty of Pharmaceutical Sciences, Chulalongkorn University), using a one-decimeter micro cell and spectral grade solvent. Concentrations are expressed as g per 100 ml.

4. Solvent

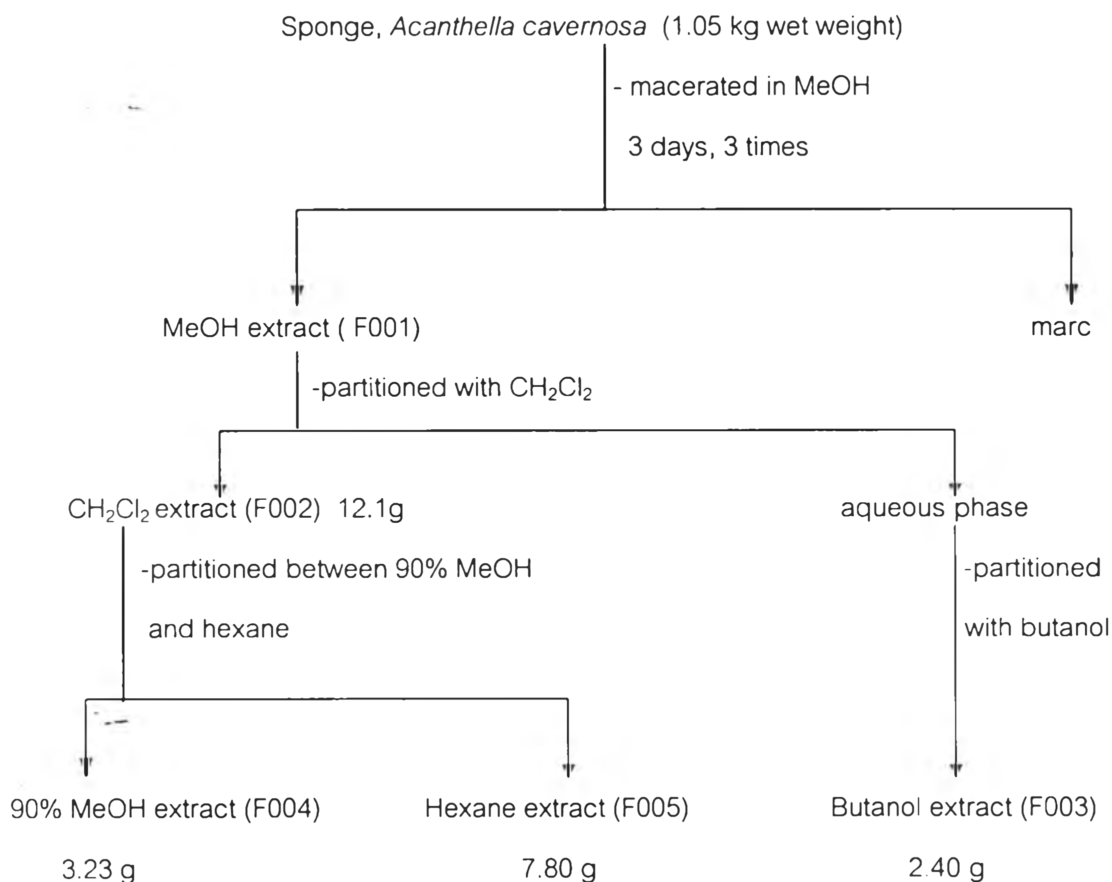
Throughout this work, all organic solvents used in this work, excluding the deuterated solvents for NMR spectra, were commercial grade and had to be redistilled prior to use.

Extraction

The sponges (1.05 kg wet weight) were chopped into small pieces. They were repeatedly macerated for three times in methanol (3 liters and 3 days each ) and then filtered. The filtrate of each maceration was combined and concentrated to give a final volume of approximately 1 liter, called F001, which was then partitioned with 1 liter of dichloromethane. The dichloromethane extract (F002) was evaporated to dryness under reduced pressure to yield 12.1 g of brown mass

(1.15% of sponge wet weight). A little amount of this fraction (about 30 mg) was reserved as a reference sample. The aqueous phase was subsequently partitioned with butanol to yield 2.4 g of butanol extract (F003) (0.23% of sponge wet weight ).

Fraction F002 was dissolved in 400 ml of 90% methanol solution and partitioned with hexane (400 ml). Both aqueous methanol and hexane extracts were evaporated to dryness under reduced pressure to yield 3.23 and 7.80 g (0.31% and 0.67% of sponge wet weight ) of fractions F004 and F005, respectively. A little amount of these three fractions was also reserved as reference.




**Scheme 1.** Extraction scheme of *Acanthella cavernosa*

Isolation

The hexane extract (F005, 7.8 g) was subjected to silica gel column chromatography. The extract was dissolved in a small volume of chloroform and applied to the top of a column (4.5 x20cm) already packed with a slurry of silica gel (150 g) in hexane-acetone (9:1). This same solvent mixture was employed as eluting solvent and the collected fractions (30 ml each) were monitored by TLC, with hexane-acetone (4:1) as the developing solvent system. Seventy-three fractions were collected and combined according to their TLC profiles into seven major fractions (F006-F012) as shown in Table 3.

Table 3. Combined fractions from the hexane extract, F005

| Code | Number of eluates | Weight of combined fraction (g) |
|---|---|---|
| F006 | 1 - 8 | 1.38 |
| F007 | 9 - 12 | 0.13 |
| F008 | 13 -16 | 0.17 |
| F009 | 17 - 29 | 4.30 |
| F010 | 30 - 51 | 0.90 |
| F011 | 52 - 63 | 0.38 |
| F012 | 64 - 73 | 0.20 |

1. Isolation of chemical constituents of fraction F008

Fraction F008 was separated by column chromatography using a column of sephadex LH-20 (2.2x100 cm) with chloroform-methanol (1:1) as the eluent. The fraction (0.17 g) was dissolved in a small volume of chloroform-methanol (1:1) and loaded on the top of the column. This same solvent mixture was employed as eluting solvent. The fractional volumn was about 30 ml. All eluates were collected and combined by using TLC examination,with hexane-acetone (10:1) as the developing solvent system. Fractionation of F008 gave four fractions (F013-F016) as summarized in Table 4.

Table 4. Combined fractions from F008

| Code | Number of eluates | Weight of combined fraction (g) |
|---|---|---|
| F013 | 1 - 12 | 59.4 |
| F014 | 13 - 19 | 28.7 |
| F015 | 20 - 21 | 6.3 |
| F016 | 22 -40 | 72.1 |

1.1 Isolation of compound K020

Fraction F016 was subjected to a column chromatography. The fraction(72 mg) was dissolved in a small volume of toluene-ethyl acetate (20:1) and applied to the top of a column (4.5 x10 cm) already packed with a slurry of silica gel (75 g) in identical solvent system. The same mixture was employed as eluting solvent and the collected fractions were examined by TLC, with toluene-ethyl acetate (15:1) as the developing system. The fractional volume was approximately 25 ml. Thirty-nine fractions were collected. The fractions were combined according to their TLC profiles into five fractions (F017-F021) as shown in Table 5.

Table 5. Combined fractions from F016

| Code | Number of eluates | Weight of combined fraction (mg) |
|---|---|---|
| F017 | 1-10 | 18.9 |
| F018 | 11-18 | 12.2 |
| F019 | 19-22 | 7.8 |
| F020 | 23-33 | 16.7 |
| F021 | 34-39 | 12.5 |

Fraction F020 (16.7 mg) appeared as a blue spot on TLC plate after being sprayed with anisaldehyde-sulfuric acid solution and heated for 5 minutes. Recrystallization of the fraction in methanol gave the compound K020 as a white powder (16.2 mg, 0.0015% of wet weight ).

1.2 Isolation of compound K023

F013, another fraction obtained from F008, displayed several blue spots on TLC plate upon detection with anisaldehyde-sulfuric acid solution and, thus, was further investigated. The sample (59.4 mg) was dissolved in a small portion of toluene-ethyl acetate (40:1) and loaded onto the top of a silica gel column (20 g, 2.3x10 cm). Elution was performed utilizing the same solvent system. Each 15 ml fraction was collected and compared by TLC, using toluene-ethyl acetate (20:1) as developing solvent system. Twenty-five fractions were combined into three major fractions (F022-F024) as shown in Table 6.

Table 6. Combined fractions from F013

| Code | Number of eluates | Weight of combined fraction (mg) |
|---|---|---|
| F022 | 1-6 | 13.7 |
| F023 | 7-11 | 18.4 |
| F024 | 12-25 | 26.1 |

After recrystallization of F023, 18 mg (0.0017% of wet weight ) of white needle crystal were recieved and given the code K023. NMR technique, both 1D and 2D were applied to identify the chemical structure of K023. Compared with the known compounds, isolated from *Acanthella cavernosa*, this compound was identified as Kalihinol Y(39) (Chang, *et al.*,1987).

2. Isolation of chemical constituents of fraction F009

Fraction F009 was fractionated by column chromatography using a column of silica gel (75g, 4.5x10 cm) with hexane-acetone (19:1) as packing solvent. The sample (4.3 g) was dissolved in a small volumn of hexane-acetone (19:1) and loaded on the top of the column. Elution was performed utilizing the same solvent system. Each 25 ml fraction was collected and combined according to TLC patterns. All eluates were combined as summarized in Table 7.

Table 7. Combined fractions from F009

| Code | Number of eluates | Weight of combined fraction (g) |
|---|---|---|
| F025 | 1-10 | 0.96 |
| F026 | 11-30 | 1.83 |
| F027 | 31-40 | 1.27 |

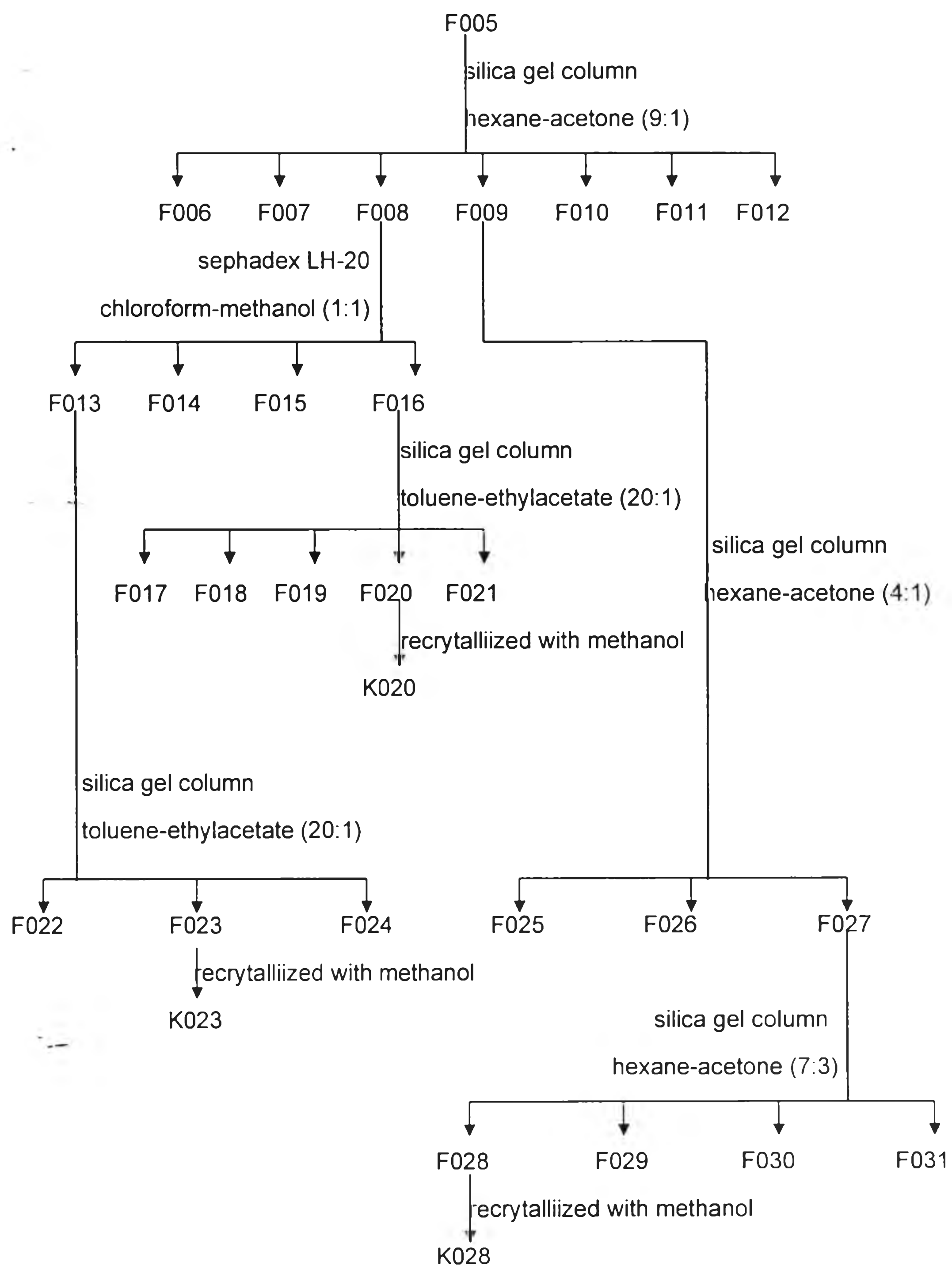
1.1 Isolation of compound K028

Fraction F027 (1.27 g), also obtained from fractionation of F009, was selected for further isolation by using a silica gel column (50 g, 2.3x25 cm) with hexane-acetone (4:1) as the eluent. The volume of each collected fraction was approximately 25 ml. Forty fractions were collected and combined according to their TLC patterns to give four major fractions (F028-F031) as shown in Table 8.

Table 8. Combined fractions from F027

| Code | Number of eluates | Weight of combined fraction (g) |
|---|---|---|
| F028 | 1-7 | 0.08 |
| F029 | 8-13 | 0.17 |
| F030 | 14-25 | 0.40 |
| F031 | 26-40 | 0.53 |

When developed with hexane-acetone (7:3), fraction F028 (0.08g) displayed a blue spot on TLC plate after being sprayed with anisaldehyde-sulfuric acid solution and heated for 5 minutes. After recrystallization of F028 with methanol, 25 mg of a white powder was obtained. NMR experiments indicated that the powder was a mixture of two compounds, of which the major one, named K028, could be identified as will be later discussed. The isolations of these compounds can be summarized as shown in scheme 2.




Scheme 2. Isolation scheme of compounds from the hexane extract

Characterization of the isolated compounds

1. Compound K020

White amorphous powder (MeOH). Soluble in chloroform, ethyl acetate, acetone and methanol.

$[\alpha]_D^{20}$ = -30° (c=0.05, MeOH)

UV $\lambda_{max}$, (MeOH), nm, (logε) : 244 (3.11) (Figure 6)

IR $\nu_{max}$, (film), $cm^{-1}$ : 3553, 3464, 3414, 2093 and 1683 (Figure 5)

APCI-MS *m/z* (% rel. int.) : 443 (100), 425 (52), 407.5 (16) and 348 (7) (Figure 4)

$^1$H NMR (δ ppm, 500 MHz, $CDCl_3$): 8.12 (1H, d, *J* = 11.0 Hz), 6.55 (1H, br t, J = 11.0 Hz), 4.26 (1H, d, *J* = 10.1 Hz), 3.65 (1H, dd, *J* = 12.0, 4.0 Hz), 2.41 (1H, dt, *J* = 11.0, 2.0 Hz), 2.09 (1H, ddd, *J* = 13.4, 3.6, Hz ), 1.99 (1H, ddd, *J* = 13.7, 7.6, 3.7 Hz), 1.86 (1H, ddd, *J* = 13.4, 3.7, 3.1 Hz), 1.76 (1H, m), 1.38 (3H, s), 1.36 (3H, s), 1.34 (3H, s), 1.30 (3H, s) and 1.19 (3H, s) (Figures 11-12)

$^{13}$C NMR (δ ppm, 125 MHz, $CDCl_3$): 166.7 (d), 132.0 (s), 79.1 (s), 77.2 (s), 71.0 (s), 64.4 (d), 63.7 (s), 59.3 (d), 45.9 (d), 43.0 (d), 38.8 (t), 38.0 (t), 36.3 (d), 33.3 (t), 31.4 (q), 28.8 (q), 27.7 (q), 27.6 (t), 23.5 (q), 22.4 (t), 22.4 (t) and 19.7 (q) (Figures 7-8)

2. Compound K028

White amorphous powder (MeOH). Soluble in chloroform, ethyl acetate, acetone and methanol.

$[\alpha]_D^{20}$ = -24° (c=0.05, MeOH)

UV $\lambda_{max}$, (MeOH), nm, (logε) : 245 (3.23) (Figure 23)

IR $\nu_{max}$, (film), $cm^{-1}$ : 3396 and 2102 (Figure 22)

APCI-MS *m/z* (% rel. int.) : 457 (56), 439 (16), 421 (99), 398 (100) and 304 (7) (Figure 21)

$^1H$ NMR (δ ppm, 500 MHz, $CDCl_3$) : 4.58 (1H, br s, $W_{1/2}$=8 Hz), 3.75 (1H, dd, $J$ = 12.5, 4.5 Hz), 2.18 (1H, m), 2.10 (1H, dd, $J$ = 12.5, 3.7 Hz), 2.01 (1H, dd, $J$ = 13.4, 3.7 Hz), 1.95 (1H, dt, $J$ = 13.7, 3.4 Hz), 1.84 (1H, d, $J$ = 4.6 Hz), 1.78 (1H, m), 1.62 (1H, m), 1.51 (1 H, dd, $J$ = 13.4, 3.4 Hz), 1.46 (1H, dd, $J$ = 11.3, 3.4 Hz), 1.44 (1H, m), 1.41 (3H, s), 1.40 (3 H, s), 1.36 (3H, s), 1.35 (3H, s) and 1.25 (3H, s) (Figures 27-28)

$^{13}C$ NMR (δ ppm, 125 MHz, $CDCl_3$) : 132.1 (s), 77.2 (s), 75.9 (s), 70.1 (s), 64.3 (s), 64.3 (d), 63.7 (d), 48.6 (d), 43.3 (d), 39.3 (t), 38.0 (t), 36.4 (d), 32.4 (t), 30.6 (q), 28.7 (q), 27.5 (q), 27.4 (t), 22.8 (q), 22.2 (t), 21.8 (t) and 19.1 (q) (Figures 24-25 )

3. Compound K023

White needle crystal (acetone). Soluble in chloroform, ethyl acetate, acetone and methanol.

IR $\nu_{max}$, (film) $cm^{-1}$ : 3353, 2107 and 759 (Figure 38)

APCI-MS *m/z* (% rel. int.) : 366 (34), 348 (14), 339 (67) and 330 (33) (Figure 37)

$^1H$ NMR (δ ppm, 300 MHz, $CDCl_3$) : 4.69 (1H, br s, $W_{1/2}$ = 6.2 Hz), 4.59 (1H, br s, $W_{1/2}$ = 6.2 Hz), 4.48 (1H, br s, $W_{1/2}$ = 7.8 Hz), 3.75 (1H, dd, $J$ = 11.8, 4.8 Hz), 1.40 (3H, s), 1.35 (3H, s), 1.33 (3H, s) and 1.13 (3H, s) (Figure 40)

$^{13}C$ NMR (δ ppm, 125 MHz, $CDCl_3$) : 105.9 (s), 105.3 (t), 77.1 (s), 75.8 (s), 70.8 (s), 64.5 (d), 64.1 (d), 49.7 (d), 42.1 (d), 38.4 (t), 37.9 (d), 35.8 (t), 32.7 (t), 30.8 (q), 29.0 (q), 28.9 (t), 27.7 (t), 24.2 (t), 22.9 (q) and 19.3 (q) (Figure 39)