CHAPTER III

EXPERIMENTAL

1. Animal material

1.1 Sample collection

The grayish black marine sponge (Figure 10) was collected at the dept of 5-10 meters from Sichang Island, Chonburi Province, Thailand, in February 2003. The sponge was identified as *Pachastrissa nux* by Dr. John N. A. Hooper of Queensland Museum, South Brisbane, Australia. Voucher specimens have been deposited at the Queensland Museum, South Brisbane, Australia (QM G320226) and at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (SC 602-03).

1.2 Identification and characterization of the *Pachastrissa nux* sponge

The sponge was identified and characterized by Dr. John N. A. Hooper from Queensland Museum, South Brisbane, Australia. The identification and characterization of this sponge were conducted as follows:

Scientific name: *Pachastrissa nux* (de Laubenfels, 1954) [QM species file number # 965]

Phylum	:	Porifera
Class	:	Demospongiae
Order	:	Astrophorida
Family	:	Calthropellidae
Known distribution	:	Thailand (Sichang, Gulf of Thailand), central west Pacific (Palau islands)
Habitat	:	On rock and dead coral, thick lumps spreading over substratum; shallow subtidal to about 10 m depth.

|		
Growth form	:	With small fistules growing from a massive partially burrowing base.
Color	:	Exterior greenish gray alive, fistules blackish; interior whitish brown with black margins; gray in ethanol.
Texture	:	Firm, brittle, harsh.
Oscules	:	Not visible in preserved material, but possibly on ends of small surface fistules.
Surface ornamentation	:	Membranous, opaque, slightly rough and corrugated.
Ectosomal skeleton	:	Dense layer of oxeas as upright brushes or sometimes paratangential to the surface.
Choanosomal skeleton	:	Vague reticulate to disorganized skeleton of oxeas and calthrops, with predominant silica over spongin ratio. Mesohyl collagen is dense and granular in appearance, especially due to the dense concentration of pigment cells.
Megascleres	:	Oxeas of two size classes 1500-1700 µm long, 25-40 µm wide, and 230-740 µm long, 12-19 µm wide. Calthrops small, with longer single axial ray and all rays equidistant: 50-100 µm at longest ray.
Microscleres	:	Absent.
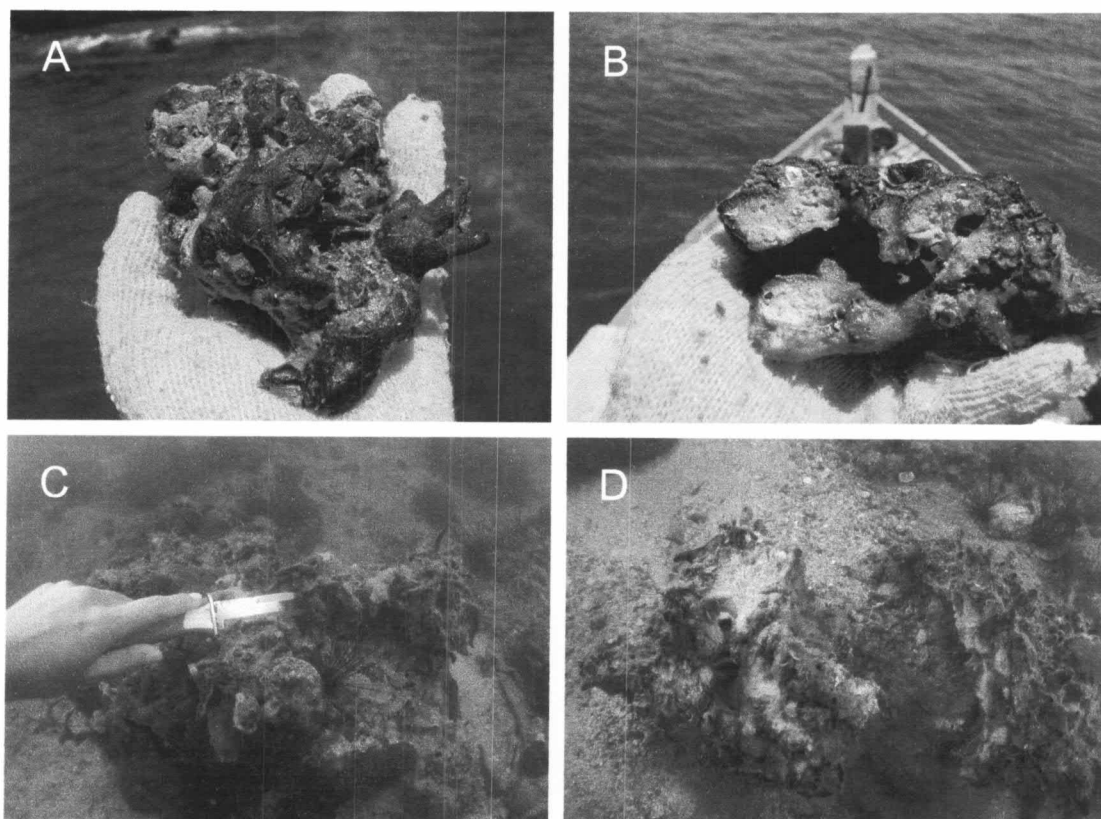
Figure 10 The *Pachastrissa nux* sponge: (A) the outer color on the surface, (B) the inner color on the surface, (C) the outer color under water, and (D) the inner color under water.

2. Chromatographic techniques

2.1 Thin-layer chromatography (TLC)

Technique	:	One dimension ascending
Adsorbent	:	1. Silica gel 60 F_{254} (Merck) coated plate
		2. C_{18}-Silica gel F_{254} (Analtech TLC Uniplates) coated plate
Layer thickness	:	250 µm
Distance	:	5-20 cm
Temperature	:	Room temperature (20-35 °C)
Detection	:	1. Visual detection under daylight.
		2. Ultraviolet light at wavelengths of 254 and 365 nm.

3. Spraying with anisaldehyde reagent and heated until colors developed.

4. Spraying with Dragendorff's reagent.

5. Spraying with fluorescamine solution.

6. Visual detection in iodine vapor.

2.2 Column chromatography

2.2.1 Vacuum liquid column chromatography

Adsorbent	:	Silica gel 60 (No. 7734), particle size 0.063-0.200 mm (70-230 mesh ASTM, Merck)
Packing method	:	The adsorbent was suspended in the eluant. The adsorbent slurry was poured into the sintered glass filter funnel and then allowed to settle by using vacuum. The height of silica gel is about 3-4 cm.
Sample loading	:	The sample was dissolved in a small volume of MeOH and dispersed with silica gel. After drying, the sample was placed gently on top of the column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in Section 2.1.

2.2.2 Flash column chromatography

Adsorbent	:	Silica gel 60 (No. 7734), particle size 0.063-0.200 mm (70-230 mesh ASTM, Merck)
Packing method	:	The adsorbent was suspended in the eluant. The adsorbent slurry was poured into the column and then allowed to settle.
Sample loading	:	The sample was dissolved in a small volume of the eluant and loaded on top of the column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in Section 2.1.

2.2.3 High performance liquid column chromatography (HPLC)

Column	:	Merck, LichroCART® 250 × 10 mm, LiChrospher 100 RP-18, 10 µm
Mobile phase	:	MeOH/H$_2$O (78:22)
Flow rate	:	3 ml/min
Sample preparation	:	The sample was dissolved in a small amount of MeOH and filtered through 0.45 µm filter paper before injection.
Injection volume	:	150 µL
Pump	:	constaMetric® 4100
Recorder	:	SpectroMonitor® 4100
Temperature	:	Room temperature (30-35 °C)

2.2.4 Gel filtration chromatography

Gel filter	:	Sephadex LH-20 (Pharmacia Biotech AB)
Packing method	:	Sephadex gel was suspended in the eluant and left standing overnight to swell prior to use. It was poured into the column and allowed to settle.
Sample loading	:	The sample was dissolved in a small volume of the eluant and loaded on top of the column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in Section 2.1.

3. Spectroscopy

3.1 Ultraviolet (UV) absorption spectroscopy

UV spectra were obtained on a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand) and a Shimadzu UV-Visible Spectrophotometer (GM's lab, Department of Physiology, University of Wisconsin-Madison, Wisconsin, USA).

3.2 Infrared (IR) absorption spectroscopy

IR spectra were obtained on a Perkin Elmer 2000 FT-IR spectrometer (film, Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand) and a Mattson Polaris FT-IR spectrometer (CH_2Cl_2, Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA).

3.3 Mass spectroscopy (MS)

Electrospray Ionization-Time of Flight (ESI-TOF) mass spectra were recorded on a Micromass LCT mass spectrometer [National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Science Park, Phathumthani, Thailand and Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA]. Electron impact ionization (EI) mass spectra were recorded on a Micromass Autospec (magnetic and electric sector analyzers, Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA).

3.4 Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopy

^{1}H and ^{13}C NMR, DEPT 90 and 135, HMQC, HMBC and ^{1}H-^{1}H COSY spectra were obtained on a Bruker AVANCE DPX-300 FT-NMR spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand) and a Bruker AC 300 spectrometer (Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA), operating at 300 MHz for protons and 75 MHz for carbons, and a Varian INOVA-500 spectrometer (Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA), operating at 500 MHz for protons and 125 MHz for carbons. The chemical shifts (ppm) of the residual deuterated solvents ($CDCl_3$ and DMSO-d_6) or internal standard (TMS) were used as reference. Proton detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 4 and 8 Hz) pulse sequences.

3.5 Optical rotation

Optical rotations were obtained on a Perkin-Elmer 341 polarimeter (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand) and a Perkin-Elmer 241 polarimeter (Department of Chemistry, University of Wisconsin-Madison, Wisconsin, USA) using a sodium lamp operating at 589 nm and at room temperature of about 23 °C.

3.6 Fluorescence spectroscopy

Fluorescence emission spectra and anisotropy were obtained on an Aminco-Bowman Series 2 Luminescence Spectrophotometer (GM's lab, Department of Physiology, University of Wisconsin-Madison, Wisconsin, USA). Fluorescence emission spectra were obtained by excitation and emission polarizers aligned at 0° and 54°, respectively, and corrected for the instrument response (Marriott et al., 1988). Fluorescence anisotropy was measured according to Heidecker et al. (1995). All spectra were recorded at room temperature.

4. Microscope

4.1 Confocal microscope

Confocal fluorescence images of living cells were obtained from a Bio-Rad MRC-1024 Laser Scanning Confocal Microscope (W. M. Keck Laboratory for Biological Imaging, University of Wisconsin-Madison, Wisconsin, USA).

5. Chemicals for synthesis

3-Aminopropyne (propargylamine)	Sigma-Aldrich
5(6)-Carboxyfluorescein diacetate N-succinimidyl ester	Fluka
5-(and-6)-Carboxytetrametylrhodamine, succinimidyl ester	Molecular Probes
Copper (I) iodide (CuI)	Sigma-Aldrich
Dapoxyl carboxylic acid, succinimidyl ester	Molecular Probes

Diisopropyl azadicarboxylate (DIAD)	Sigma-Aldrich
N-Ethyl-N'-[5-(N''-succinimidyloxycarbonyl)pentyl]-3, 3, 3', 3'-	
tetramethyl-2, 2'-indodicarbocyanine chloride (IC5-OSu)	Dojindo
N-(9H-fluoren-9-yl-methoxycarbonyloxy) succinimide (Fmoc-NHS)	Fluka
Piperidine	Sigma-Aldrich
Rhodol green carboxylic acid, succinimidyl ester (mixed isomer)	Molecular Probes
Sodium azide (NaN$_3$)	Sigma-Aldrich
Sulfuric acid, concentrated (H$_2$SO$_4$)	Sigma-Aldrich
Triethylamine (Et$_3$N)	Sigma-Aldrich
Triphenylphosphine (PPh$_3$)	Sigma-Aldrich

6. Solvents

The commercial grade organic solvents used in extraction and isolation were redistilled prior to use. The HPLC grade organic solvents were used for HPLC. The analytical grade organic solvents used in synthesis were dried prior to use. THF was dried over benzophenone and sodium, CH$_2$Cl$_2$ was passed through Al$_2$O$_3$ (activity I) and dried over CaH$_2$, and DMSO was opened immediately before use.

7. Biological activities

7.1 Antifungal activity

An antifungal activity of the isolated fractions and pure compounds was examined by the agar disc diffusion method (Lorian, 1980) against *Candida albicans* ATCC 10231. The yeast strain was cultivated on Sabouraud's dextrose agar (SDA, Difco®) slant at 30 °C for 24 hours. After that, the culture was suspended in sterile normal saline solution (NSS). Each 20 ml of molten culture media was poured into 9-cm diameter petri dish and allowed to gel. To prepare seed plates, a sterile cotton applicator swab was dipped into the suspension of the test organism, expressed excess liquid and streaked onto a sterile petri dish containing gelatinous culture media in all directions. Test samples were dissolved in suitable solvent and then applied on sterile

paper discs (6 mm diameter) or silica gel TLC aluminum sheets, and blank paper discs were impregnated with 20 µL of the solvent used. After the disc or TLC plates were dried, they were applied onto the seed plates and allowed to prediffuse at 4 °C for 2 hours. The plates and discs were placed in an incubator for 24 hours at 30 °C. The diameter and R$_f$ values of inhibition zones were measured. The fractions that exhibited antifungal activity were subsequently selected for further study.

7.2 Cytotoxic activity

Cytotoxic activity against human cervix carcinoma (HeLa) cells was tested. The HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®) supplemented with 10% v/v heat-activated fetal bovine serum (FBS, Gibco®), 1% v/v penicillin (10,000 unit/mL), and streptomycin (10 mg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Tested compound was dissolved in MeOH and diluted to 10, 100 and 1000 nM in culture medium immediately prior to use. The final concentration of MeOH in this experiment was 0.1% v/v and was nontoxic to the cells. Phase contrast images of control and drug-treated cells were recorded after 16 hours.

7.3 G-actin binding assay

7.3.1 Preparation of rabbit muscle acetone powder

Fresh rabbit back and leg muscle was put into ice and minced two times through prechilled meat grinder. Myosin was extracted from the minced muscle by stirring in 1 L of ice-cold Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, pH 6.5) for 10 minutes. After centrifugation at 3,000 × g for 10 minutes at 4 °C, the pellet was resuspended in 1 L cold 0.05 M $NaHCO_3$. The suspension was stirred at 4 °C for 5 minutes and filtered through 3-layer cheesecloth. The muscle residue was washed three times with 2 L of cold Milli-Q water and quickly filtered through cheesecloth. The residue was next extracted three times with 1 L of cold acetone. The clumps of residue were broken up by stirring during each extraction and then filtered through cheesecloth. The final residue was placed in a large tray and allowed to dry in a hood. The muscle acetone powder was stored at -20 °C.

7.3.2 Preparation of actin from muscle acetone powder

The rabbit muscle acetone powder was extracted at 4 °C by stirring with 20 mL of G-buffer [2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 1 mM dithiothreitol (DTT), pH 8] per gram of rabbit muscle acetone powder for 20 minutes. The mixture was filtered through three-layer of cheesecloth. The filtrate was then filter through 0.2 μM sterile-filter paper and polymerized by addition of 2 mM $MgCl_2$ and 100 mM KCl. The polymerization was performed at room temperature for 1 hour and KCl was added to 0.5 M. The solution was centrifuged at 100,000 × g for 1 hour. The pellet of F-actin was resuspended in G-buffer and dialyzed against G-buffer at 4 °C overnight. The dialyzed actin was centrifuged at 100,000 × g for 1 hour (Spudich and Watt, 1971). G-actin remained in the supernatant and the concentration of actin was determined by using the molar extinction coefficient for actin at 290 nm of 2.6 × 10^4 $M^{-1} \cdot cm^{-1}$ (Figure 36) (Gordon et al., 1976).

7.3.3 Preparation of prodan-G-actin

G-actin was labeled with the thiol probe, acrylodan, using a standard protocol (Marriott et al, 1988). G-actin was dialyzed in dialysis buffer (DB, 2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, pH 8) overnight and further treated with 2 molar excess of acrylodan in DMF. The reaction was left in the dark for 1 hour at room temperature and then dialyzed overnight at 4 °C against DB containing 1 mM DTT (G-buffer). The labeled G-actin was polymerized for 1 hour at room temperature by the addition of $MgCl_2$ and KCl to final concentrations of 2 mM and 100 mM, respectively, and centrifuged at 4 °C for 1 hour at 100,000 × g. The labeled actin pellet was resuspended in G-buffer and dialyzed against G-buffer as described above. Prodan labeled G-actin was recovered from the supernatant following a 100,000 × g spin at 4 °C for 1 hour. The labeling ratio of prodan/actin monomer was determined by using the molar extinction coefficient for actin at 290 nm of 2.6 × 10^4 $M^{-1} \cdot cm^{-1}$ (Gordon et al., 1976) and for prodan at 375 nm of 1.85 × 10^4 $M^{-1} \cdot cm^{-1}$ (Weber and Farris, 1979). The actin concentration for prodan-G-actin was calculated after correction for the absorbance of prodan at 290 nm (Figure 46); at this wavelength, the molar extinction coefficient is also 1.85 × 10^4 $M^{-1} \cdot cm^{-1}$.

Prodan-G-actin behaves as unlabeled G-actin in all functional assays tested (Marriott et al., 1988; Roy et al., 2001).

7.3.4 Prodan-G-actin binding experiment

The steady state substrate binding experiments of prodan-G-actin were performed at room temperature. Substrate binding was analyzed from the relative change of the fluorescence intensity of prodan-G-actin at varying substrate concentration. The freshly prepared solutions of test compounds in MeOH were added to the solution of prodan-G-actin in G-buffer. The concentration of prodan-G-actin was calculated as described in Section 7.3.3. The concentration of FDE-KabC was calculated by using the molar extinction coefficient for kabiramide C at 247 nm of 2.67×10^4 $M^{-1} \cdot cm^{-1}$. The fluorescence emission spectra were recorded with excitation at 385 nm and emission collected between 400 and 650 nm with a 4 nm bandwidth for both excitation and emission.

7.3.5 G-actin binding experiment

The steady state fluorescence measurements were performed at room temperature. In this experiment tested compounds, which are fluorescent derivatives of kabiramide C, were freshly prepared as a solution in G-buffer. The fluorescence emission spectra of fluorescent kabiramide C probes were measured by titrating with increasing amounts of unlabeled G-actin. The concentration of fluorescent kabiramides was calculated by using their molar extinction coefficient. The wavelengths of excitation and emission were varied for each fluorescent kabiramide C derivatives as follows.

Tetramethylrhodamine derivative of kabiramide C (TMR-KabC)

Excitation wavelength (nm)	:	546
Emission scan range (nm)	:	550-700
Spectral band width (ex, em; nm)	:	4, 4
Molar extinction coefficient (nm, $M^{-1} \cdot cm^{-1}$)	:	546, 9.5×10^4

Rhodol green derivative of kabiramide C (RG-KabC)

Excitation wavelength (nm)	:	499
Emission scan range (nm)	:	500-650
Spectral band width (ex, em; nm)	:	4, 4
Molar extinction coefficient (nm, $M^{-1} \cdot cm^{-1}$)	:	502, 7.3×10^4

IC5 derivative of kabiramide C (IC5-KabC)

Excitation wavelength (nm)	` :	630
Emission scan range (nm)	:	640-750
Spectral band width (ex, em; nm)	:	4, 4
Molar extinction coefficient (nm, $M^{-1} \cdot cm^{-1}$)	:	640, 15×10^4

Dapoxyl derivative of kabiramide C (DAP-KabC)

Excitation wavelength (nm)	:	385
Emission scan range (nm)	:	420-700
Spectral band width (ex, em; nm)	:	4, 4
Molar extinction coefficient (nm, $M^{-1} \cdot cm^{-1}$)	:	395, 2×10^4

7.3.6 Fluorescence resonance energy transfer (FRET) experiment (Valeur, 2002).

Resonance energy transfer is potentially possible whenever the emission spectrum of the donor fluorophore overlaps the absorption spectrum of the acceptor, donor excitation energy can be transferred non-radiatively to the acceptor. The net result is quenching of the donor fluorescence emission and an increase in the emission intensity of the acceptor. In this experiment, two fluorescent probes were used: one coupled to tested compounds, fluorescent kabiramides, while the other, prodan, coupled to G-actin. If the test compounds bound to G-actin and the emission spectrum of the donor, prodan-G actin, was overlapped with the absorption spectrum of the acceptor, fluorescent kabiramides, the FRET can be observed (Figures 11-12). In addition, the appropriate distance between the donor and acceptor molecules should be in the range of 10-100 Å. Analysis of the steady state fluorescence intensity ratio at

the center of prodan-G actin, and fluorescent kabiramides, as functions of increasing amounts of fluorescent kabiramides could provide the stoichiometric binding of fluorescent kabiramides to G-actin. This experiment was performed at room temperature. The solutions of the tested compounds in MeOH were added to the solution of prodan-G-actin in G-buffer. The fluorescence emission spectra were measured using the excitation wavelength at 385 nm and the emission was collected from 400 to 650 nm. The concentration of prodan-G-actin and tested compounds were calculated as described in Section 7.3.3 and 7.3.5, respectively.
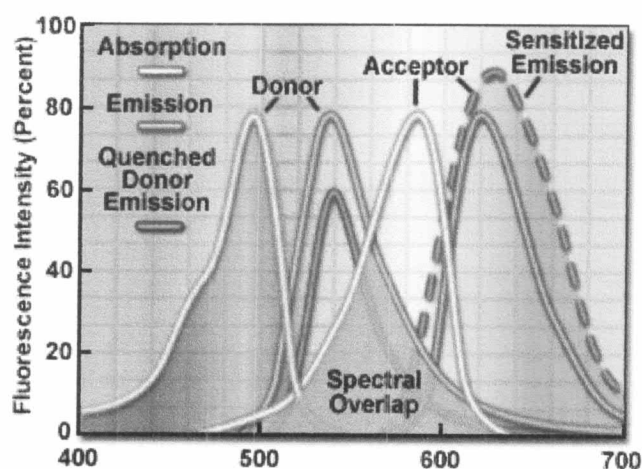

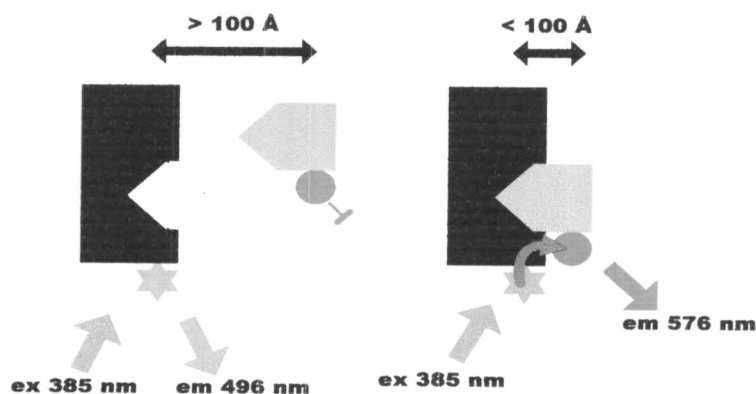Figure 11   FRET donor and acceptor spectral profiles (Olympus America Inc., 2005)

Figure 12   Schematic drawing of FRET between donor and acceptor.

7.3.7 Fluorescence Anisotropy (FA or *r*) experiment (Valeur, 2002).

Observation and measurement of fluorescence anisotropy is based on the photoselective excitation of fluorophores due to the transient alignment of the absorption dipole moment with an oriented electric field vector of illuminating photons (polarized light). Most of the fluorescence chromophores absorb light along a preferred direction depending on the electronic state. In contrast, the emission transition moment is the same whatever the excited state reached by the molecule upon excitation. When the chromophore was illuminated by linearly polarized incident light, the molecules whose absorption transition dipole moment are oriented in a direction parallel (or close) to the electric vector of polarized light are preferentially excited, this is called photoselective. Any change in direction of transition moment during the lifetime of excited state will cause the decrease of this anisotropy. A measurement of the relative angle between the excitation and emission transition dipole moments determines the degree of anisotropy (r), as given by the equations:

$$r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence emission measured in the plane parallel and perpendicular to the plane of excitation, respectively.

The determination of the emission requires the four fluorescence intensity measurements including I_{VV}, I_{VH}, I_{HV}, and I_{HH} (V: vertical; H: horizontal; Figure 13). The first subscript corresponds to the orientation of excitation polarizer and the second subscript corresponds to the orientation of emission polarizer. The G factor, $G = I_{HV}/I_{HH}$, is introduced into the equation as a correcting factor for the difference in sensitivity of the direction for vertically and horizontally polarized light. The equation then becomes

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

Fluorescence anisotropy is suited to the equilibrium binding studies, when free and bound components involve in the equilibrium have different rotational rates. In solution, small molecular weight fluorophores have a rotational correlation time

ranging between 50 and 100 picoseconds, which is significantly faster than the rate of emission, thus, the molecules are able to rotate numerous times before emission. As a result, the polarized emission is randomized so that the net anisotropy is zero. Binding of the fluorophore with a much larger macromolecule slows the rotational motion of the fluorescent probe that can be expected to higher anisotropy (Figure 14).
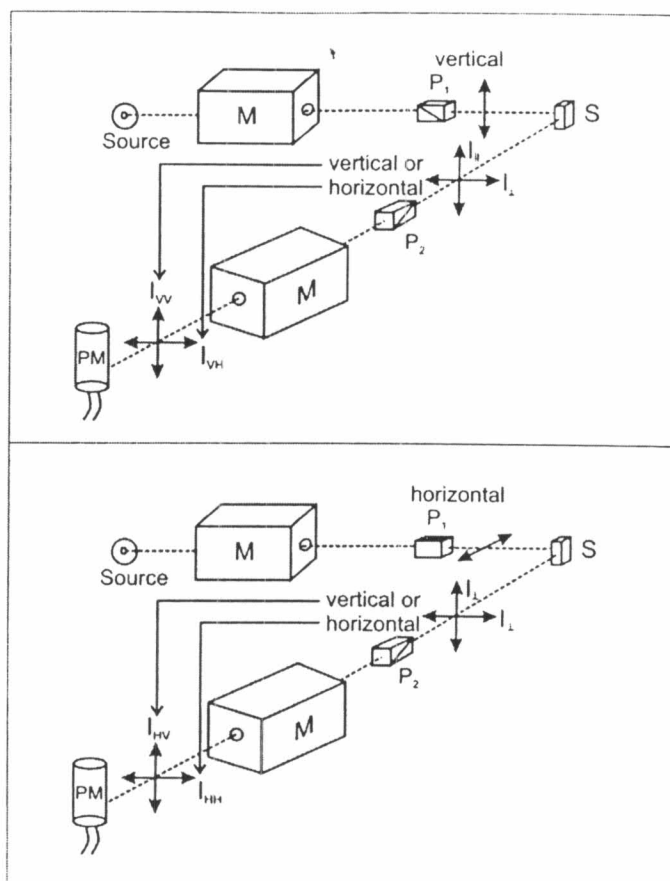

Figure 13 Diagram showing how the four intensity components are measured for determination of the steady state anisotropy. M: monochromator; S: sample; P_1, P_2: polarizers; and PM: photomultiplier tube (Valeur, 2002).
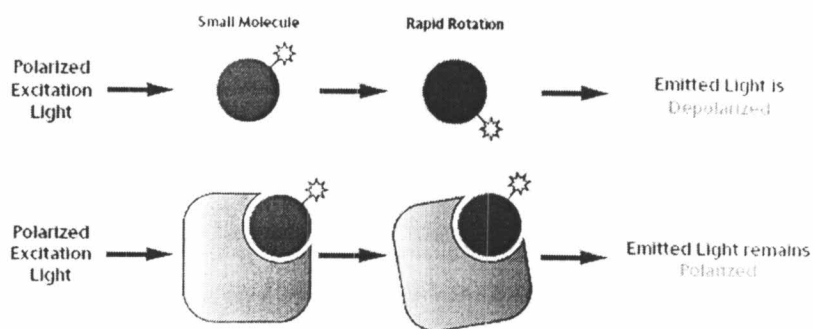
Figure 14 Schematic drawing of fluorescence anisotropy differences between small and large complexes (Invitrogen Corporation, 2004).

The tested compounds, fluorescent derivatives of kabiramide C, were freshly prepared as a solution in G-buffer. The fluorescence anisotropy of fluorescent kabiramide C probes was measured by titrating with increasing amounts of unlabeled G-actin. The concentration of G-actin and tested compounds were calculated as described in Section 7.3.2 and 7.3.5, respectively. The wavelengths of excitation and emission were varied for each fluorescent kabiramide C derivatives as follows.

Tetramethylrhodamine derivative of kabiramide C (TMR-KabC)

Excitation wavelength (nm)   :   546

Emission scan range (nm)   :   575-585

Spectral band width (ex, em; nm)   :   4, 4

Rhodol green derivative of kabiramide C (RG-KabC)

Excitation wavelength (nm)   :   499

Emission scan range (nm)   :   520-530

Spectral band width (ex, em; nm)   :   4, 4

IC5 derivative of kabiramide C (IC5-KabC)

Excitation wavelength (nm)   :   630

Emission scan range (nm)   :   655-665

Spectral band width (ex, em; nm)   :   4, 4

7.3.8 Iodide quenching (Valeur, 2002).

The fluorescence quenching experiments with the collisional quencher iodide were used to determine the environment of probes. The fluorescence was measured after aliquots of freshly prepared potassium iodide were added to the fluorescent kabiramide C probes solution in G-buffer either in the presence or in the absence of G-actin. The concentration of G-actin and tested compounds were calculated as described in Section 7.3.2 and 7.3.5, respectively. The iodide quenching data were fitted to the Stern-Volmer equation.
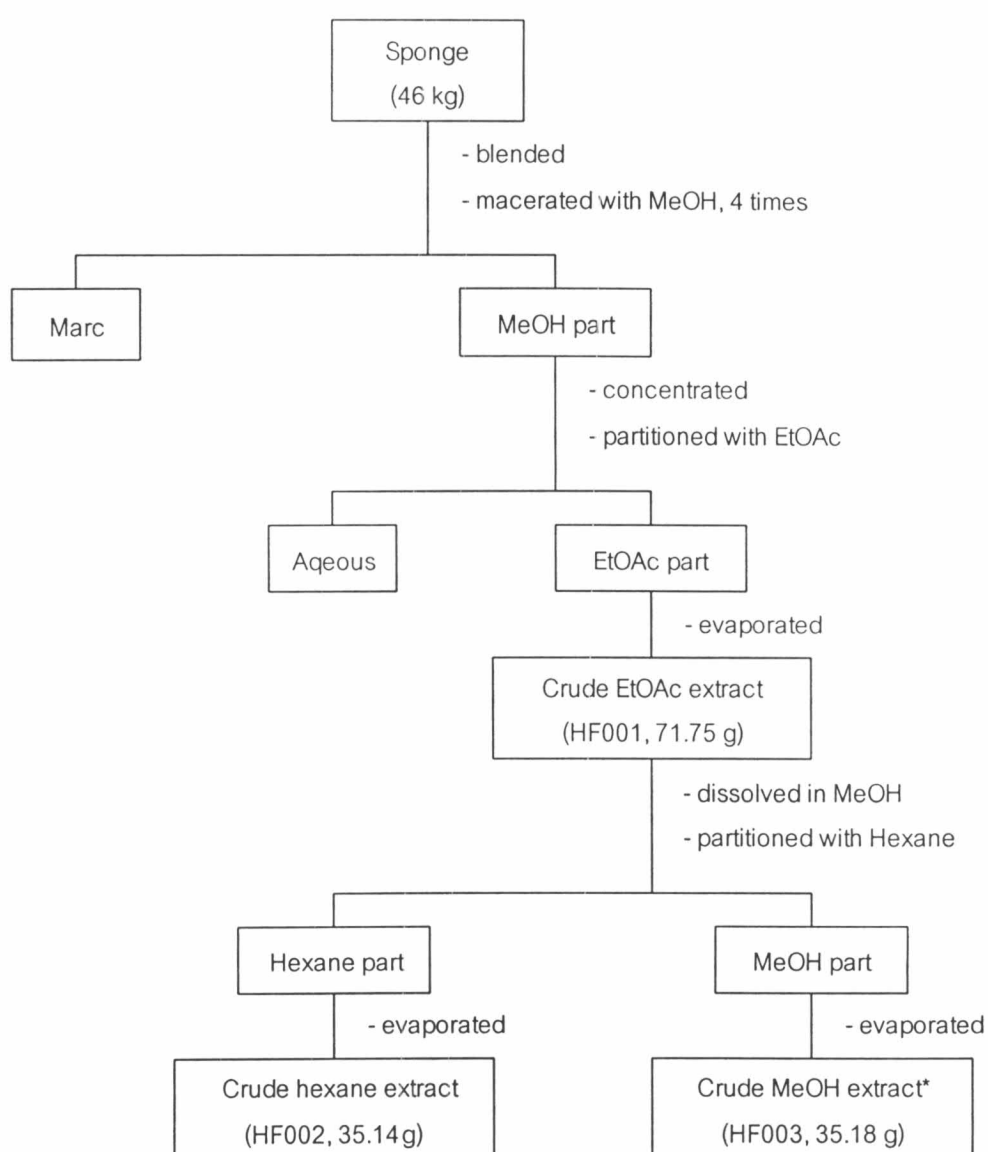
$$ I_0/I \quad = \quad 1 + K_{SV}[Q] $$

Where I_0 and I are the steady state fluorescence intensities in the absence and in the presence of quencher, respectively. K_{SV} is the Stern-Volmer constant and [Q] is the concentration of quencher. Generally, the ratio I_0/I is plotted against the quencher concentration (Stern-Volmer plot). If the variation is found to be linear, the slope gives the Stern-Volmor constant. Additionally, $K_{SV} = k_q \tau_0$, where k_q is rate constant for bimolecular process and τ_0 is excited-state lifetime. Then k_q can be calculated if the excited-state lifetime in the absence of quencher is known. For TMR-KabC, the excitation wavelength was 546 nm and the emission was measured from 550 to 700 nm. For RG-KabC, the excitation wavelength was 499 nm and the emission was measured from 500 to 650 nm.

8. Fluorescent images in living cells

The NIH 3T3 cells were cultured in DMEM supplemented with 10% v/v heat-activated FBS, 1% v/v penicillin (10,000 unit/mL), and streptomycin (10 mg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Each fluorescent probe was dissolved in MeOH and diluted to 100 nM in culture medium immediately prior to use. Cells were treated with medium containing 100 nM of each fluorescent probe for 10-30 minutes. After washing cells with fresh medium, fluorescent images were recorded by confocal microscope

9. Extraction and isolation

The sponge (46 kg, wet weight) was homogenized and extracted four times with MeOH. After filtration and concentration, the residue was partitioned three times with EtOAc. The EtOAc layers were combined and evaporated under reduced pressure at temperature not exceeding 50 °C to obtain the crude EtOAc extract (HF001, 71.75 g). The crude EtOAc extract was then dissolved in MeOH and partitioned with hexane. Both parts were evaporated to give 35.14 g of the hexane extract (HF002) and 35.18 g of the MeOH extract (HF003) (Scheme 1).

```
                        ┌─────────────┐
                        │   Sponge    │
                        │   (46 kg)   │
                        └─────────────┘
                               │  - blended
                               │  - macerated with MeOH, 4 times
             ┌─────────────────┴─────────────────┐
        ┌─────────┐                        ┌─────────────┐
        │  Marc   │                        │  MeOH part  │
        └─────────┘                        └─────────────┘
                                                 │  - concentrated
                                                 │  - partitioned with EtOAc
                           ┌─────────────────────┴───────┐
                      ┌──────────┐              ┌──────────────┐
                      │  Aqeous  │              │  EtOAc part  │
                      └──────────┘              └──────────────┘
                                                      │  - evaporated
                                          ┌──────────────────────┐
                                          │  Crude EtOAc extract  │
                                          │  (HF001, 71.75 g)     │
                                          └──────────────────────┘
                                                      │  - dissolved in MeOH
                                                      │  - partitioned with Hexane
                      ┌───────────────────────────────┴─────────────────────┐
                ┌─────────────┐                                      ┌─────────────┐
                │ Hexane part │                                      │  MeOH part  │
                └─────────────┘                                      └─────────────┘
                       │  - evaporated                                      │  - evaporated
          ┌──────────────────────┐                          ┌──────────────────────┐
          │ Crude hexane extract │                          │  Crude MeOH extract* │
          │  (HF002, 35.14 g)    │                          │  (HF003, 35.18 g)    │
          └──────────────────────┘                          └──────────────────────┘
```

*The fractions that contain active compounds.

Scheme 1 Extraction of the sponge *Pachastrissa nux.*

The MeOH extract exhibited antifungal activity against *Candida albicans* ATCC 10231 at the concentration of 1 mg/disc with the diameter of inhibition zone of 28 mm in agar disc diffusion method (Lorian, 1980). Additionally, the bioautograph showed that the active compounds appeared at R_f 0.4-0.5 (Silica gel TLC, CH_2Cl_2/MeOH, 10:1).
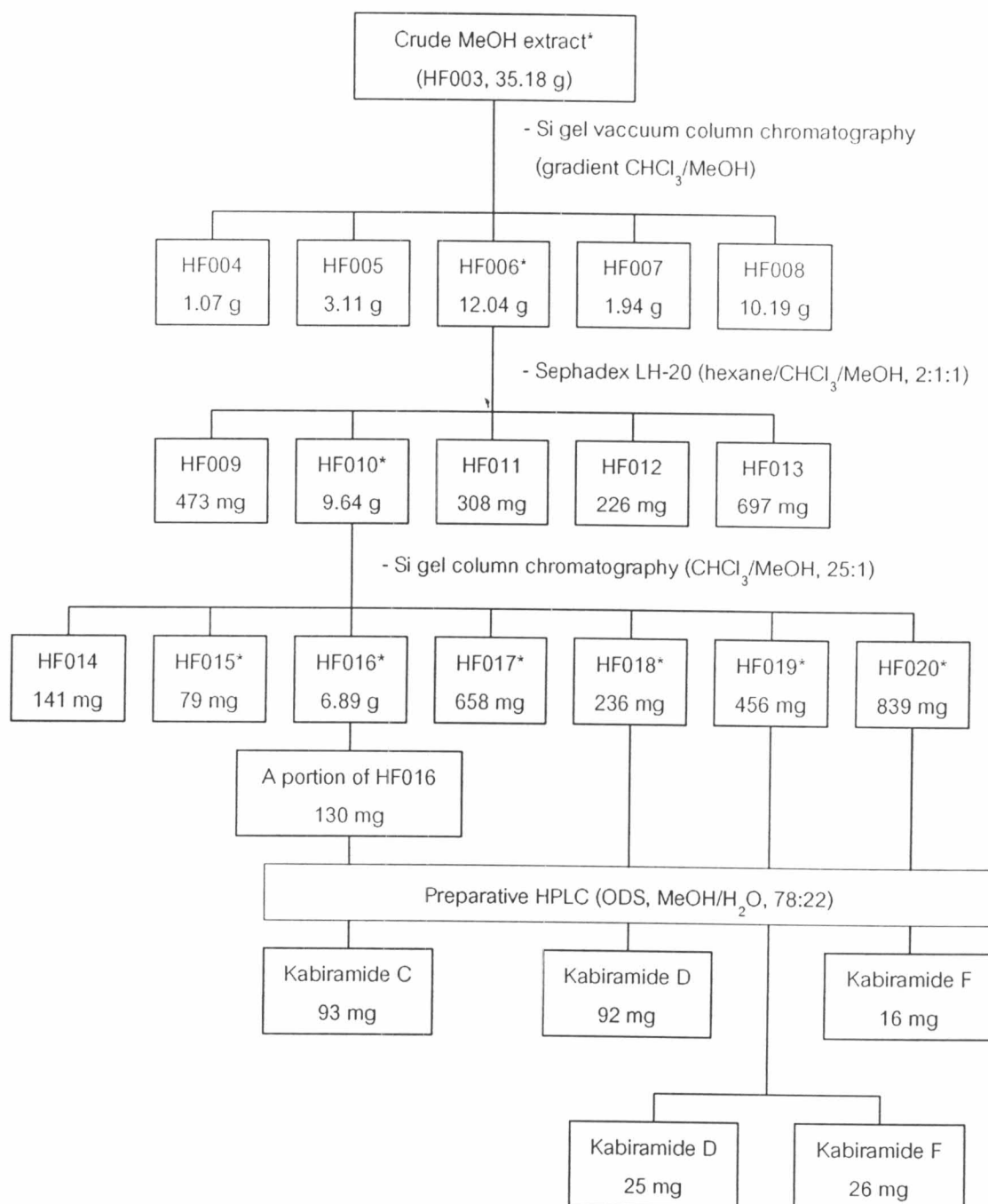
The MeOH extract (35.18 g) was fractionated by vacuum liquid column chromatography using a sintered glass filter column of silica gel (10.5 cm inner diameter and 4.5 cm long, silica gel 250 g). A step-gradient of $CHCl_3$ and MeOH was used to elute the column and 200-ml fractions were collected. Similar fractions guided by TLC technique were combined (section 2.1, Silica gel TLC, CH_2Cl_2/MeOH, 9:1) to give five pooled fractions (HF004-HF008). The TLC pattern showed that the active compounds were in the fraction HF006 (12.04 g), which was further purified by a Sephadex LH-20 gel filtration column (4.8 cm inner diameter and 40 cm long). The column was eluted with a mixture of hexane/$CHCl_3$/MeOH (2:1:1). The 15-ml fractions were collected and then combined by using TLC technique to obtain five pooled fractions (HF009-HF013). Fraction HF010 (9.60 g) was chromatographed on a silica gel column (4.8 cm inner diameter and 11 cm long), using an isocratic elution of $CHCl_3$/MeOH (25:1) to give seven fractions (HF014-HF020; Scheme 2).

A portion of fraction HF016 (130 mg) was purified by preparative HPLC (Merck LichroCART, 250 × 10 mm, Lichrosphere 100 RP18, 10 μM, 10 mg/injection) eluted with MeOH/H_2O (78:22) to give kabiramide C (93 mg).

Fraction HF018 (236 mg) was purified by preparative HPLC in the same condition as previously described to give kabiramide D (92 mg).

Fraction HF019 (456 mg) was purified by preparative HPLC using the same condition as previously described to give kabiramide D (25 mg) and the more polar compound, kabiramide F (26 mg).

Fraction HF020 (839 mg) was purified by preparative HPLC as previously described to give kabiramide F (16 mg).

Crude MeOH extract*
(HF003, 35.18 g)

- Si gel vaccuum column chromatography
(gradient CHCl$_3$/MeOH)

HF004	HF005	HF006*	HF007	HF008
1.07 g	3.11 g	12.04 g	1.94 g	10.19 g

- Sephadex LH-20 (hexane/CHCl$_3$/MeOH, 2:1:1)

HF009	HF010*	HF011	HF012	HF013
473 mg	9.64 g	308 mg	226 mg	697 mg

- Si gel column chromatography (CHCl$_3$/MeOH, 25:1)

HF014	HF015*	HF016*	HF017*	HF018*	HF019*	HF020*
141 mg	79 mg	6.89 g	658 mg	236 mg	456 mg	839 mg

A portion of HF016
130 mg

Preparative HPLC (ODS, MeOH/H$_2$O, 78:22)

Kabiramide C	Kabiramide D	Kabiramide F
93 mg	92 mg	16 mg

Kabiramide D	Kabiramide F
25 mg	26 mg

*The fractions that contain active kabiramide compounds.

Scheme 2 Fractionation of the crude MeOH extract obtained from the sponge *Pachastrissa nux.*

Physical and chemical properties of the isolated compounds

Kabiramide C

ESIMS	:	$[M+Na]^+$ m/z 964.4866 (calcd for $C_{48}H_{71}N_5O_{14}Na$, 964.4895); Figure 38
$[\alpha]^{23}_D$	:	+8.33° (c 0.047, in $CHCl_3$)
UV	:	λ_{max} nm (log ε), in MeOH; 247 (4.37); Figure 39
IR	:	ν_{max} cm^{-1}, film; 3455, 2929, 1716, 1653; Figure 40
^{1}H NMR	:	δ ppm, 300 MHz, in $CDCl_3$; Figure 41, Table 8
^{13}C NMR	:	δ ppm, 75 MHz, in $CDCl_3$; Figure 42, Table 8

Kabiramide D

ESIMS	:	$[M+Na]^+$ m/z 921.4836 (calcd for $C_{47}H_{70}N_4O_{13}Na$, 921.4837); Figure 48
$[\alpha]^{23}_D$	:	-13.51° (c 0.094, in MeOH)
UV	:	λ_{max} nm (ε), in MeOH; 245 (4.46); Figure 49
IR	:	ν_{max} cm^{-1}, film; 3443, 2964, 2934, 1728, 1712, 1691, 1656; Figure 50
^{1}H NMR	:	δ ppm, 300 MHz, in $CDCl_3$; Figure 51, Table 9
^{13}C NMR	:	δ ppm, 75 MHz, in $CDCl_3$; Figure 52, Table 9

Kabiramide F

ESIMS	:	$[M+Na]^+$ m/z 907.4679 (calcd for $C_{46}H_{68}N_4O_{13}Na$, 907.4681); Figure 58
$[\alpha]^{23}_D$	:	-14.99° (c 0.085, in MeOH)
UV	:	λ_{max} nm (ε), in MeOH; 245 (4.42); Figure 59
IR	:	ν_{max} cm^{-1}, film; 3419, 2961, 2927, 1713, 1693, 1655; Figure 60
^{1}H NMR	:	δ ppm, 300 MHz, in DMSO-d_6 and in $CDCl_3$; Figures 61 and 62, Table 10

¹³C NMR : δ ppm, 75 MHz, in DMSO-d_6 and in CDCl$_3$; Figures 63 and 64, Table 10

10. Synthesis and purification of 7-(4-aminomethyl-1H-1,2,3-triazol-1-yl)kabiramide C

Hydrazoic acid

A paste of sodium azide (260 mg, 4 mmol) in H$_2$O (260 µL) was stirred on ice bath, and benzene (1.6 mL) was added. Concentrated sulfuric acid (106 µL, 2 mmol) was carefully added dropwise to the reaction mixture. After stirring for 10 minutes, the organic layer containing hydrazoic acid (HN$_3$) was separated and dried over anhydrous MgSO$_4$.

7-Azidokabiramide C

To the mixture of kabiramide C (286 mg, 304 µmol) and triphenylphosphine (PPh$_3$; 158 mg, 602 µmol) in dry THF (3 mL) stirred on an ice bath under nitrogen atmosphere, the solution of hydrazoic acid in benzene (300 µL) was added. After stirring for 15 minutes, diisopropyl azadicarboxylate (DIAD; 117 µL, 604 µmol) was added dropwise over 1 minute to the reaction mixture. The reaction mixture was allowed to warm to room temperature and stirred for 4 hours. The reaction mixture was concentrated and purified by a Sephadex LH-20 column (hexane/CH$_2$Cl$_2$/MeOH, 2:1:1) and preparative silica gel TLC (CH$_2$Cl$_2$/MeOH, 20:1) to give 7-azidokabiramide C as a white amorphous solid (119 mg, 40.5%).

ESIMS : [M+Na]$^+$ m/z 989.4691 (calcd for C$_{48}$H$_{70}$N$_8$O$_{13}$Na, 989.4690); Figure 70

$[\alpha]^{23}_D$: -6.27° (c 0.047, in CHCl$_3$)

UV : λ_{max} nm (log ϵ), in MeOH; 247 (4.12); Figure 71

IR : ν_{max} cm^{-1}, CHCl$_3$; 3464, 3347, 3166, 3020-2935, 2104, 1720, 1657; Figure 72

¹H NMR : δ ppm, 300 MHz, in CDCl$_3$; Figure 73, Table 13

^{13}C NMR : δ ppm, 75 MHz, in CDCl$_3$; Figure 74, Table 14

3-(Fluoren-9-yl-methoxycarbonyl)aminopropyne

A suspension of N-(9H-fluoren-9-yl-methoxycarbonyloxy) succinimide (168 mg, 0.5 mmol) in dry THF was cooled with an ice bath, and 3-aminopropyne (propargylamine, 35 μL, 0.55 mmole) was added dropwise. The reaction mixture was stirred and allowed to warm to room temperature over 2 hours. The solvent was removed under reduced pressure to give a residue. The residue was dissolved in EtOAc and washed with water. The organic layer was dried over anhydrous MgSO$_4$ and concentrated. Recrytallisation from EtOAc gave 3-(fluoren-9-yl-methoxycarbonyl) aminopropyne as white needles (102 mg, 73.7%).

EIMS : [M]$^+$ m/z 277.1114 (calcd for C$_{18}$H$_{15}$NO$_2$, 277.1103); Figure 75

UV : λ$_{max}$ nm (log ε), in MeOH; 266 (4.24); Figure 76

IR : ν$_{max}$ cm^{-1}, CHCl$_3$; 3453, 3307, 3066-2954, 1725, 1510; Figure 77

^{1}H NMR : δ ppm, 300 MHz, in CDCl$_3$; Figure 78, Table 11

^{13}C NMR : δ ppm, 75 MHz, in CDCl$_3$; Figure 79, Table 11

7-[4-N-(9H-Fluoren-9-yl-methoxycarbonyl)aminomethyl-1,2,3-triazol-1-yl)kabiramide C

7-Azidokabiramide C (119 mg, 123 μmol) was dissolved in a small volume of MeOH followed by water (6 mL). To the suspension of 7-azidokabiramide C, 3-(fluoren-9-yl-methoxycarbonyl)aminopropyne (51 mg, 184 μmol), Et$_3$N (35 μL, 251 μmol), and Cu(I)I (3 mg, 16 μmol) were added. The reaction mixture was stirred at room temperature for 1 hour and then extracted with EtOAc (6 mL × 4). The combined extracts were washed with brine, dried over anhydrous MgSO$_4$, and concentrated to give a crude product which was purified by silica gel TLC (CH$_2$Cl$_2$/MeOH, 20:1) to give 7-[4-N-(9H-fluoren-9-yl-methoxycarbonyl)aminomethyl-1,2,3-triazol-1-yl)kabiramide C as a white amorphous solid (97 mg, 63.4%).

ESIMS	:	[M+Na]$^+$ m/z 1266.6033 (calcd for $C_{66}H_{85}N_9O_{15}Na$, 1266.6063); Figure 80
[α]$^{23}_D$	:	+3.06° (c 0.032, in $CHCl_3$)
UV	:	λ_{max} nm (log ε), in MeOH; 299 (3.82), 255 (4.49); Figure 81
IR	:	ν_{max} cm^{-1}, $CHCl_3$; 3453, 3348, 3072, 3006-2883, 1721, 1655, 1513; Figure 82
^{1}H NMR	:	δ ppm, 300 MHz, in $CDCl_3$; Figure 83, Table 13
^{13}C NMR	:	δ ppm, 75 MHz, in $CDCl_3$; Figure 84, Table 14

7-(4-Aminomethyl-1H-1,2,3-triazol-1-yl)kabiramide C (AMT-KabC)

To remove the Fmoc protecting group, 7-[4-N-(9H-fluoren-9-yl-methoxycarbonyl)aminomethyl-1,2,3-triazol-1-yl)kabiramide C (97 mg, 78 µmol) was treated with 20% v/v piperidine in dry CH_2Cl_2 (2 mL) for 20 minutes. Water (3 mL) was added and the reaction mixture was extracted with CH_2Cl_2 (3 mL × 4). The organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and concentrated to give a residue which was purified by silica gel TLC (CH_2Cl_2/MeOH, 20:3) to give AMT-KabC as a white amorphous solid (25 mg, 30.8%).

ESIMS	:	[M+H]$^+$ m/z 1022.5574 (calcd for $C_{51}H_{76}N_9O_{13}$, 1022.5562); Figure 85
[α]$^{23}_D$	:	-3.87° (c 0.012, in $CHCl_3$)
UV	:	λ_{max} nm (log ε), in MeOH; 246 (4.12); Figure 86
IR	:	ν_{max} cm^{-1}, $CHCl_3$; 3464, 3352, 3072-2884, 1722, 1655; Figure 87
^{1}H NMR	:	δ ppm, 500 MHz, in $CDCl_3$; Figure 88, Table 13
^{13}C NMR	:	δ ppm, 125 MHz, in $CDCl_3$; Figure 89, Table 14

11. Synthesis of fluorescent derivatives of kabiramide C

11.1 Synthesis of tetramethylrhodamine derivative of 7-(4-aminomethyl-1H-1,2,3-triazol-1-yl)kabiramide C (TMR-KabC)

To a solution of AMT-KabC (1 mg, 0.98 μmole) in dry CH_2Cl_2 (100 μL), a solution of 5-(and-6)-carboxytetrametylrhodamine succinimidyl ester (0.6 mg, 1.14 μmole) in DMSO (100 μL) was added. The reaction mixture was stirred at room temperature in the dark under nitrogen atmosphere. After 8 hours, the mixture was concentrated and isolated by silica gel TLC (CH_2Cl_2/MeOH, 20:3) and ODS TLC (MeOH/H_2O, 7:3) to give TMR-KabC.

ESIMS   : $[M+Na]^+$ m/z 1456.6838 (calcd for $C_{76}H_{95}N_{11}O_{17}Na$, 1456.6805); Figure 90

UV   : λ_{max} nm, in MeOH; 236, 251, 545; Figure 91

11.2 Synthesis of rhodol green derivative of 7-(4-aminomethyl-1H-1,2,3-triazol-1-yl) kabiramide C (RG-KabC)

To a solution of AMT-KabC (1 mg, 0.98 μmole) in dry CH_2Cl_2 (100 μL), a solution of rhodol green carboxylic acid succinimidyl ester (0.5 mg, 1.06 μmole) in DMSO (100 μL) was added. The mixture was stirred at room temperature in the dark under nitrogen atmosphere for 8 hours. The mixture was concentrated and purified by silica gel TLC (CH_2Cl_2/MeOH, 8:2) to give RG-KabC.

ESIMS   : $[M+Na]^+$ m/z 1401.6028 (calcd for $C_{72}H_{86}N_{10}O_{18}Na$, 1401.6019); Figure 92

UV   : λ_{max} nm, in MeOH; 245, 480, 502; Figure 93

11.3 Synthesis of IC5 derivative of 7-(4-aminomethyl-1H-1,2,3-triazol-1-yl) kabiramide C (IC5-KabC)

To a solution of AMT-KabC (1 mg, 0.98 μmole) in dry CH_2Cl_2 (100 μL), a solution of IC5-OSu (0.7 mg, 1.11 μmole) in DMSO (70 μL) was added and stirred at room temperature in the dark under nitrogen atmosphere. After being stirred for 8

hours, the mixture was concentrated and purified by preparative silica gel TLC (CH_2Cl_2/MeOH, 10:1) to give IC5-KabC.

ESIMS   :   [M+Na]$^+$ *m/z* 1501.8665 (calcd for $C_{84}H_{114}N_{11}O_{14}Na$, 1501.8619); Figure 94

UV   : λ_{max} nm, in MeOH; 245, 643; Figure 95

11.4 Synthesis of dapoxyl derivative of 7-(4-aminomethyl-1*H*-1,2,3-triazol-1-yl) kabiramide C (DAP- KabC)

To a solution of AMT-KabC (1 mg, 0.98 µmole) in dry CH_2Cl_2 (100 µL), a solution of dapoxyl carboxylic acid succinimidyl ester (0.5 mg, 1.23 µmole) in DMSO (100 µL) was added and stirred at room temperature in the dark under nitrogen atmosphere. After 8 hours, the mixture was concentrated and purified by silica gel TLC (CH_2Cl_2/MeOH, 10:1) to give DAP-KabC.

ESIMS   :   [M+Na]$^+$ *m/z* 1334.6503 (calcd for $C_{69}H_{89}N_{11}O_{15}Na$, 1334.6437); Figure 96

UV   : λ_{max} nm, in MeOH; 245, 370; Figure 97

11.5 Synthesis of fluorescein diester derivative of 7-(4-aminomethyl-1*H*-1,2,3-triazol-1-yl)kabiramide C (FDE-KabC)

To a solution of AMT-KabC (1 mg, 0.98 µmole) in dry CH_2Cl_2 (100 µL), a solution of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (0.8 mg, 1.44 µmole) in dry CH_2Cl_2 (100 µL) was added and stirred at room temperature in the dark under nitrogen atmosphere. After 8 hours, the mixture was purified by silica gel TLC (CH_2Cl_2/MeOH, 20:1) to give FDE- KabC.

ESIMS   :   [M+Na]$^+$ *m/z* 1486.6056 (calcd for $C_{76}H_{89}N_9O_{21}Na$, 1486.6071); Figure 98

UV   : λ_{max} nm, in MeOH; 245; Figure 99