CHAPTER III

RESEARCH METHODOLOGY

Materials

Krebs' solution(mM): (NaCl 122; KCl 5; [N-(2-Hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid)] HEPES 10; KH_2PO_4 0.5; NaH_2PO_4 0.5; $MgCl_2$ 1; glucose 11 and $CaCl_2$ 1.8; adjusted to pH 7.3 with 1N NaOH), acetylcholine(ACh), phenylephine(PE), CV Buffer (100 mM Tris-HCl, pH 7.5; 100 mM imidazole; 15 mM $MgCl_2$; 1.0 mg/ml BSA and 2.5 mg/ml snake venom), CH Buffer (100 mMTris-HCl, pH 7.5 100 mM imidazole, 15 mM $MgCl_2$, 1.0 mg/ml BSA and 0.5 mg/ml histone were obtained from Sigma (St. Louis, MO, USA). Pentobarbital sodium solution (Nembutal®) was obtained from Ceva Animal Health (Bangkok, Thailand). QAE resin(QAE SephadexTM A-25) was purchased from GE Healthcare(Sweden). The PDE5 was prepared from transfected cells expressing human PDE5A and PDE6 was prepared from chicken retinas.

Preparations of compounds

The natural compounds, curcumin (1), demethoxycurcumin (2) and bisdemethoxycurcumin (3), were obtained from the rhizomes of *C longa* as described previously. Compound 4 was synthesized from compound 1 by the literature method [77]. Aldol condensation of substituted cinnamones (10a and 10b) and substituted cinnamaldehydes (11a, 11b and 11c) under base-catalyzed condition gave the trienones 5-7 (Figure 8). For compounds 8 and 9, the acid group-containing analogues of 7 were synthesized by coupling of the alkyne analogues 12 and 13 with the azide (2-azidoacetic acid) 14 via click chemistry approach as shown in Figure 9 to produce 8 and 9. All the tested compounds 4-9 were characterized by spectroscopic methods and the results are consistent with their structures. The samples were kept at -20 °C until use.

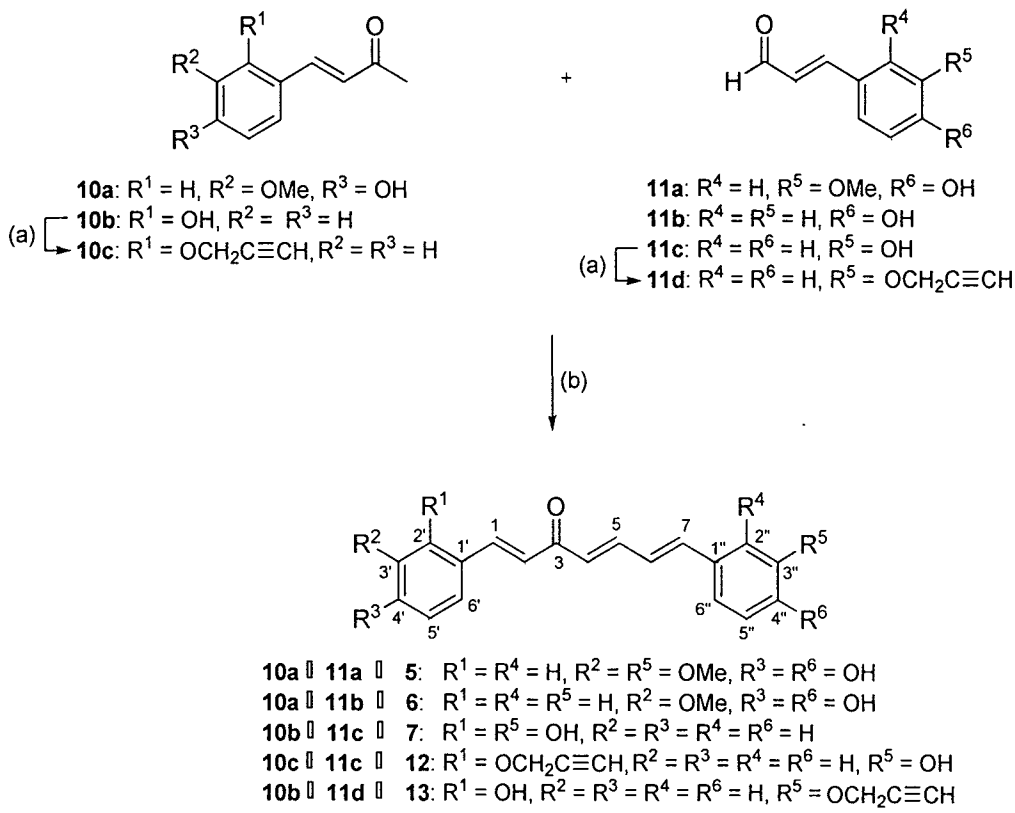

Figure 8 Preparation of compounds 5–7. Reagents and conditions: (a) propargyl bromide, K_2CO_3, acetone, room temperature; (b) 20% aq. NaOH, EtOH, 0°C– room temperature

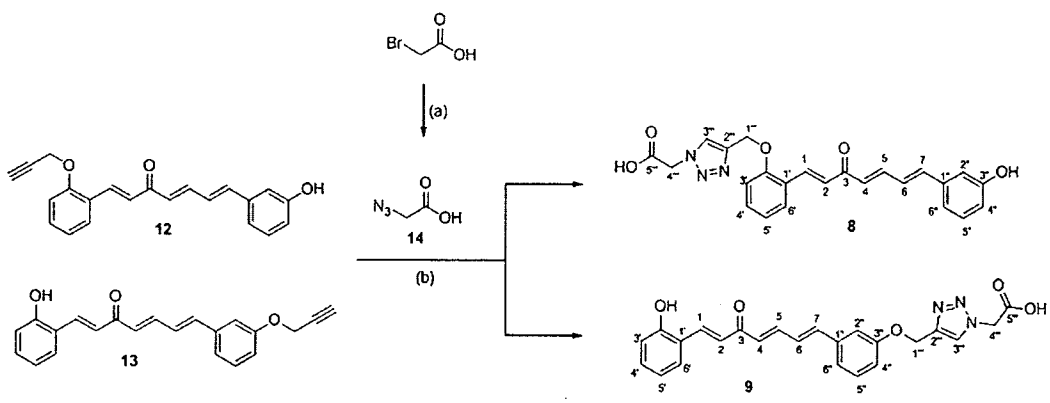

Figure 9 Preparation of compounds 8 and 9. Reagents and conditions: (a) NaN_3, H_2O, room temp, overnight; (b) $CuSO_4$, sodium ascorbate, THF:H_2O (9:1)

Determination of PDE5 inhibitory activity

All samples were dissolved in DMSO and diluted with water such that the final concentration of DMSO in sample solution was 5%. PDE5 was extracted from PDE5A transfected cells expressing human PDE5A. The PDE5 assay was conducted using the two-step radioactive procedure which has been modified from Sonnenburg*et al.*, 1998[111]. 20 µl of the following reagents were added to 96 well plate: CV buffer, EGTA, PDE5 solution and test samples or control (5% DMSO in buffer). The reaction was started by adding 20 µl of 5 µM [^{3}H]cGMP (~50,000 cpm) to the reaction mixture and incubated at 30°C for 40 min. Then, 100 µl of 50% QAE resin in water was added to the wells for purified product. The plate was shaken for 10 min and left for the resin to sediment for 20 min. The supernatants (100 µl) were transferred to new microplate wells containing 100 µl of 50% QAE resin. The plate was again shaken for 10 min and left to settle for another 20 min. Then, 100 µl supernatant was mixed and shaken with 200µl of Microscint® 20 for 2 h. The radioactivity was counted by TopCount NXT (PerkinElmer, USA) for 1 well/min. The PDE5 activity in the study was standardized to have a hydrolysis activity of 20-25% of the total substrate counts. The calculation of hydrolysis is shown in equation (1). The PDE5 inhibitory activity was calculated from equation (2)

$$\%\,\text{hydrolysis}_{sample} = \left[\frac{(CPM_{sample} - CPM_{background})}{(CPM_{totalcount1} - CPM_{background})}\right] x\,100 \text{--------(1)}$$

where CPMsample is the radioactive count rate of the assay with enzyme and CPMbackground is the count rate without enzyme. CPMtotal count is the count rate of 20 µl of substrate plus 100 ml of buffer 1.

$$\%\text{PDE5 inhibition} = \left[1 - \left(\frac{\%\text{hydrolysis}_{sample}}{\%\text{hydrolysis}_{control}}\right)\right] x\,100 \text{ ----------(2)}$$

where $\%\text{hydrolysis}_{sample}$ and $\%\text{hydrolysis}_{control}$ were the enzyme activities of the sample and solvent (1% DMSO) used in the assay, respectively. The IC_{50} values were determined using the test samples at >80% PDE5 inhibition.

Determination of PDE6 inhibitory activity

PDE6 activity was conducted using the procedure previously reported [112] which has been modified from Huang, et al. [113]. 25 ml of the followings reagents were added to tube: CH buffer, EGTA, PDE6 solution and test samples or control (5% DMSO in buffer). The reaction was started by adding 25 µl of 5 µM [3H]cGMP and incubated at 30°C for 10 min. Then, the reaction was stopped by placing the tube in boiling water for 1 min and cooled for 5 min. The second step of reaction, 25 µl of 2.5 mg/ml snake venom was added to the reaction, incubated at 30 °C for 5 min. After that, 250 µl of 20 mM Tris-HCl, pH 6.8 (buffer I) was added. The reaction was transferred to a QAE resin column and eluted 4 times with 500 µl of buffer I. The eluent was mixed with a scintillant cocktail and the radioactivity was measured using a β-counter. The %hydrolysis of PDE6 was similarly calculated as for PDE5.

Animals

Male Wistar rats (200-250 g) were obtained from the National Laboratory Animal Center, Mahidol University, Nakhornpathom, Thailand. The study was approved by the Animal Ethics Committee, Naresuan University, Phitsanulok, Thailand (NU-AE540416). Animals were housed under standard environmental conditions at 25±2°C, 12 h-light and dark cycle, fed with standard rodent diet and tap water in the Center For Animal Research, Naresuan University, Thailand.

Tissue preparation and vasorelaxation protocol

Rats were anesthetized by pentobarbital (65 mg/kg, intraperitoneal injection) and the lungs and aorta were isolated. Intra-pulmonary artery was removed from lung and soaked in Krebs' solution to wash off the surrounding loose connective tissue. The vessel was cut into rings of 2-3 mm in length and mounted in organ chambers via a pair of intraluminal wires. The chambers contained Krebs' solution at 37°C and bubbled with air. The rings were incubated for 45-60 min at an optimum tension of 1 g during which the solution was replaced every 15 min. The wires were connected to force transducers to measure isometric tension via a MacLab A/D converter (Chart V5, A.D. Instruments, Castle Hill, Australia) (Figure 10), stored and displayed on

personal computer. The arterial contraction and relaxation was tested by sequential application of 10 μM phenylephrine (PE) and 10 μM acetylcholine (ACh). Only the vessels showing 80-100% relaxation to ACh were considered as endothelium-intact, while in some experiments the endothelium was predenuded mechanically, and relaxations of <20% were considered as successfully denuded. After washing for 45-60 min, vessels were precontracted by adding 10 μM PE again. When stable contractions were obtained, the samples (containing compounds **1-9**) at concentrations of 0.1-100 μM were cumulatively added (Figure 11). The samples were dissolved in DMSO and then diluted with water to obtain the final concentrations of 300, 100, 30, 10, 3, 1, 0.1 μM in 2 ml organ bath (final solutions contained <0.1% DMSO). Sildenafil was diluted in the same fashion to final concentrations of 100, 10, 1, 0.1, 0.03, 0.1, 0.003, 0.01, 0.0003 and 0.0001 μM in 2 ml organ bath. Authors conducting these measurements were blinded with respect to the compound identities. All the compounds were coded so that the person conducting the following experiments had no knowledge of the compound structures or their PDE5 inhibitory potency.

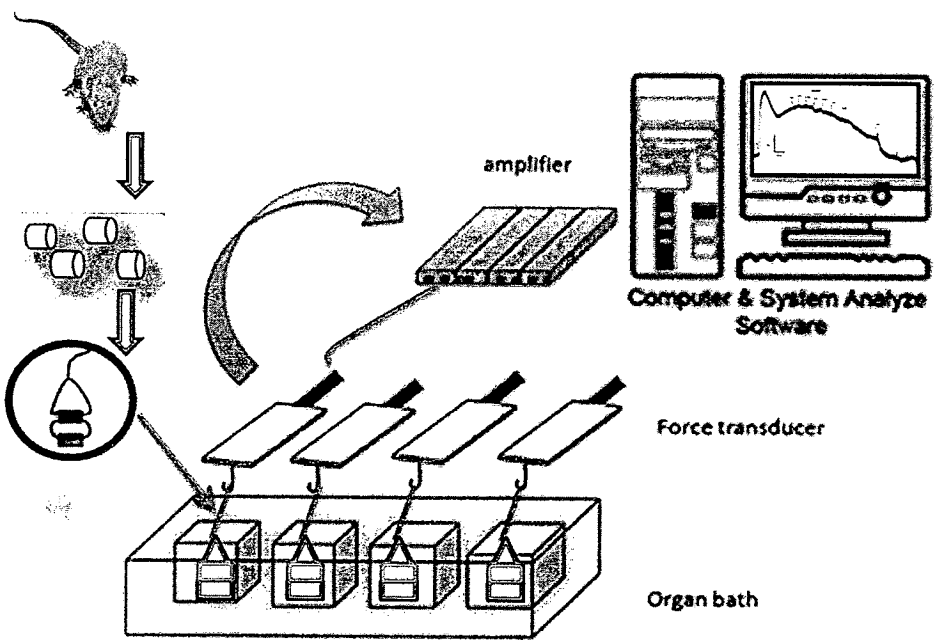

Figure 10 Mac Lab setting for evaluation of vasorelaxation effect

Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M). Statistical analysis was conducted using *t-test* : unpaired and *One-way* ANOVA, followed by *Tukey's* post hoc test. p-values of <0.05 were considered significant.