CHAPTER II

EXPERIMENTAL

2.1 Instruments and Equipments

Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 F_{254}) and column chromatography was performed in silica gel (Merck Kieselgel 60 G) (Merck Kgaa, Darmstadt, Germany). Melting points were determined with a Stuart Scientific Melting Point SMP 1 (Bibby Sterilin, Ltd., UK.). Broad band UVA (320-400 nm) was generated by F24T12/BL/ HO (PUVA) lamp (National Biological Corporation, Twinsburg, Ohio, USA) and broad band UVB (280-320 nm) was generated by FSX24T12/UVB/HO lamp (National Biological Corporation, Twinsburg, Ohio, USA). UV Irradiance was measured by using UVA-400C and UVB-500C power meter (National Biological Corporation, Twinsburg, Ohio, USA).

The FT-IR spectra were recorded on a Nicolet Fourier Transform Infrared spectrophotometer: Impact 410 (Nicolet Instruments Technologies, Inc. WI, USA). Solid samples were incorporated into a pallet of potassium bromide. The ^{1}H-NMR spectrum was obtained in deuterated chloroform (CDCl$_3$) or deuterated dimethylsulfoxide (DMSO-d_6) using ACF 200 spectrometer which operated at 400.00 MHz for ^{1}H nuclei (Varian Company, USA). Molecular weight was determined by gel permeation chromatography (GPC): Waters 600E Multisolvent Delivery System (Waters, MA, USA) using Waters styragel HR columns. Ultraviolet absorption spectra were obtained with the aid of HP 8453 UV-Vis spectrophotometer (Agilent Technologies, CA, USA). The UV absorbance was recorded using a sample in the 1 cm quartz cell. Freez-drying was performed with Freezone 6 Liter Benchtop model 77520 (Lab Conco, Kansas City, Missouri, USA). Membranes used for dialysis experiments were seamless cellulose tubing, with MWCO 12,400 Dalton, size 76/49 mm (Sigma-Aldrich, Steinheim, Germany). Mass spectra were acquired using Mass Spectrometer: Waters Micromass Quattro micro API (Waters, MA USA).

2.2 Chemicals

Solvents used in syntheses and spectroscopic techniques were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Solvents used in column chromatography were purified from commercial grade solvents prior to use by distillation. Curcumin or 1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxy-benzotriazole (HOBt) and iodomethane were purchased from Acros Organics (New Jersey, USA).

2-Ethylhexyl-4-methoxycinnamate (EHMC) was obtained from Merck Co. Ltd. (Bangkok, Thailand). Succinic acid was purchased from Carlo Erba Reagent (Milan, Italy). Potassium carbonate was purchased from Fluka Chemical Company (Buchs, Switzerland).

## 2.3 Synthesis of 1,7-bis-(3,4-dimethoxyphenyl)-4-methyl-1,6-heptadiene-3,5-dione (trimethylcurcumin, TMC)

1,7-Bis[4-hydroxy-3-methoxy phenyl]-1,6-heptadiene-3,5-dione
(curcumin)

1,7-Bis-(3,4-dimethoxyphenyl)-4-methyl-1,6-heptadiene-3,5-dione
(trimethylcurcumin, TMC)
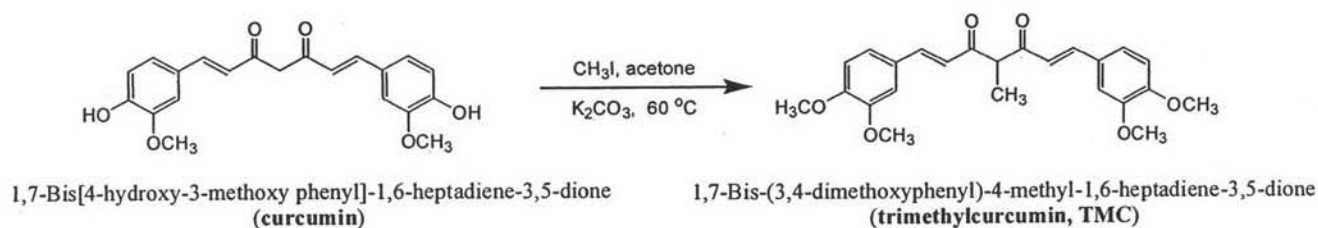
Scheme 2.1 Synthesis of trimethylcurcumin (**TMC**)

Into a two-necked, round-bottomed flask containing 20 mL, acetone was placed 0.5228 g (1.42×10^{-3} mol) of curcumin and 4 mL (6.42×10^{-2} mol) of CH_3I and 0.5821 g (3.62×10^{-3} mol) of K_2CO_3 under N_2 atmosphere. The mixture was kept stirring for 48 h under N_2 atmosphere at 60 °C. After that, K_2CO_3 was filtered out and washed by acetone.

Curcumin: yellow solid, m.p. 177 °C, R_f 0.56 (75% EtOAc/Hex), IR (KBr, cm^{-1}) : 3432, 1629, 1598, 1511, 1276 and 1153; UV-Vis, λ_{max} 431 nm; ε = 52,092 $mol^{-1}Lcm^{-1}$; ^{1}H-NMR (CDCl$_3$) δ (ppm): 7.63 (d, *J*=15.8 Hz, 1H, Ar-**CH**=), 7.16 (d, *J*=8.6 Hz, 1H, Ar-**H**), 7.09 (s, 1H, Ar-**H**), 6.97 (d, *J*=8.3 Hz, 1H, Ar-**H**), 6.52 (d, *J*=15.7 Hz, 1H, CO-**CH**=), 5.90 (s, 1H, CO-**CH**=COH), 5.84 (s, 1H, CO-**CH₂**-CO), 3.99 (s, 3H, O**CH₃**).

1,7-Bis-(3,4-dimethoxyphenyl)-4-methyl-1,6-heptadiene-3,5-dione
(**trimethylcurcumin, TMC**): orange oil (yield: 63%), R_f 0.57 (75% EtOAc/Hex), IR (KBr, cm^{-1}): 2900, 1760, 1629, 1588, 1516, 1260 and 1127; UV-Vis, λ_{max} 345 nm; ε = 62,955 $mol^{-1}Lcm^{-1}$; ^{1}H-NMR (CDCl$_3$) δ (ppm): 7.63 (d, *J*=15.5 Hz, 1H, Ar-**CH**=), 7.05 (s, 1H, Ar-**H**), 6.99 (s, 1H, Ar-**H**), 6.82 (d, *J*=7.9 Hz, 1H, CH$_3$O-Ar-**H**), 6.65 (d, *J*=15.4 Hz, 1H, CO-**CH**=), 3.90 (s, 3H, O**CH₃**) and 1.44 (s, 3H, CO-CH(**CH₃**)-CO).

2.4 Synthesis of bis(4-((1E,6E)-7-(2-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate (curcumin oligomer)

Bis(4-((1E,6E)-7-(2-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate
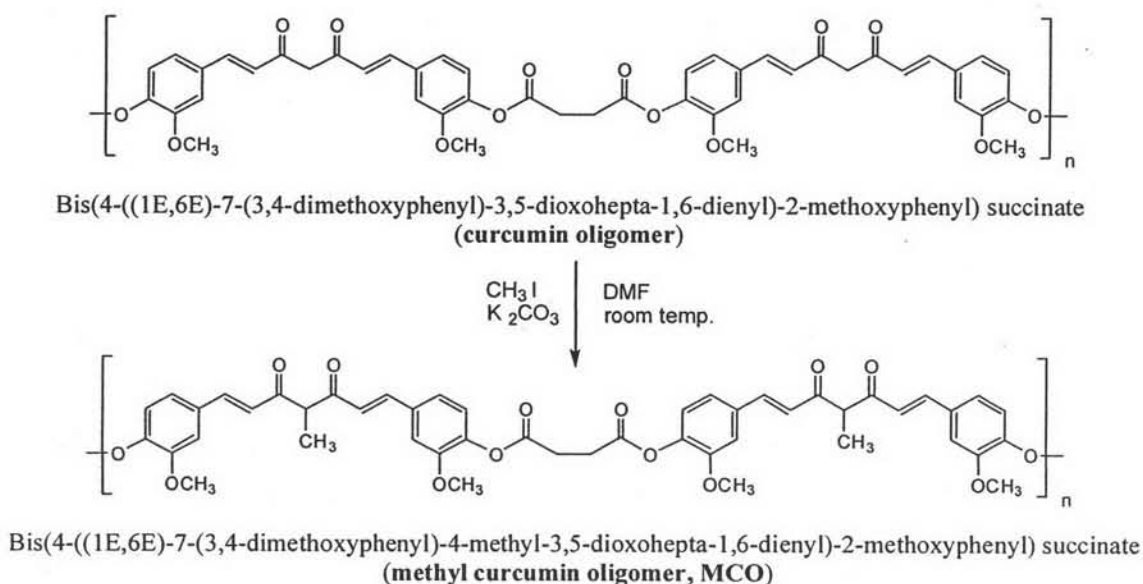(**curcumin oligomer**)
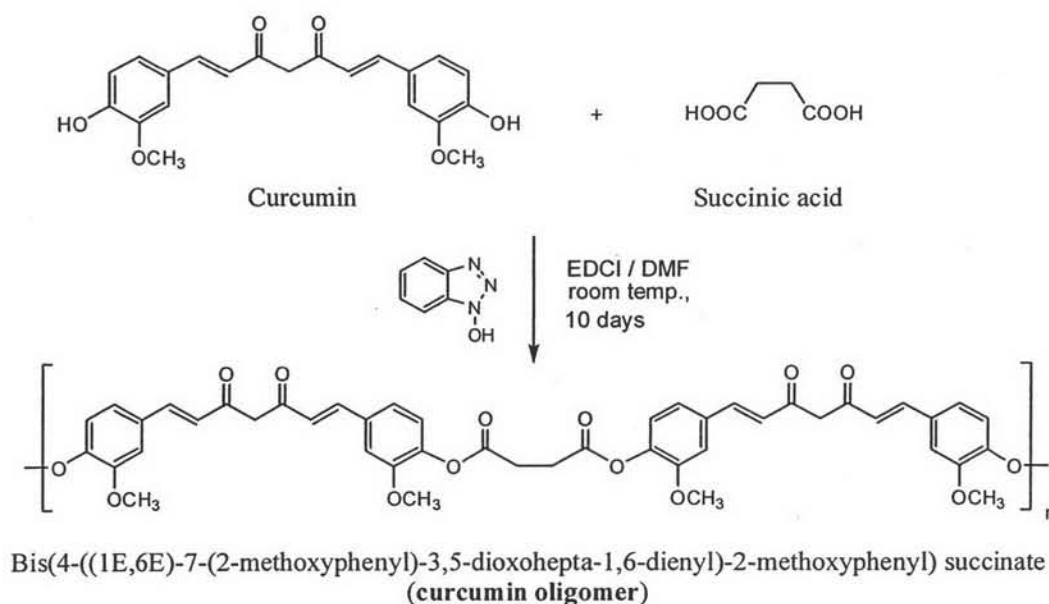
Scheme 2.2 Synthesis of curcumin oligomer

Into a two-necked, round-bottomed flask containing 50 mL dry DMF was placed 0.7368 g (0.0020 mol) of curcumin, 0.2362 g (0.0020 mol) of succinic acid, 1.1600 g (0.0060 mol) of EDCI and 0.8160 g (0.0060 mol) of HOBt. The reaction mixture was stirred at room temperature for 10 days. Progress of the reaction was checked by TLC using 75:25 ethylacetate:hexane as mobile phase. The disappearing of curcumin spot and the appearance of new spot indicated curcumin oligomer. The reaction was quenched by adding 1000 ml of water. The reaction mixture was then transferred to a dialysis bag and dialyzed against water; the dialyzed product was then dried by freeze drying. The product was analyzed by ^{1}H-NMR, IR, UV-Vis spectroscopy and gel permeation chromatography (GPC).

Bis(4-((1E,6E)-7-(2-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxy phenyl) succinate (**curcumin oligomer**): orange solid (yield: 29%), m.p. 126-129 °C, R_f 0.50 (75% EtOAc/Hex), IR (KBr, cm^{-1}): 3432, 1757, 1629, 1588, 1511, 1280 and 1127; UV-Vis, λ_{max} 431 nm; ^{1}H-NMR (CDCl$_3$) δ (ppm): 7.52 (d, J=15.4 Hz, 1H, Ar-

CH=), 7.06 (d, J=7.1 Hz, 1H, Ar-**H**), 6.99 (s, 1H, Ar-**H**), 6.87 (d, J=7.9 Hz, 1H, Ar-**H**), 6.41 (d, J=15.4 Hz, 1H, CO-C**H**=), 5.80 (broad, 1H, CO-C**H**=COH), 5.74 (s, 1H, CO-C**H$_2$**-CO), 3.89 (s, 3H, OC**H$_3$**) and 3.00 (t, 2H, CO-C**H$_2$**-CH$_2$-CO).

2.5 Synthesis of bis(4-((1E,6E)-7-(3,4-dimethoxyphenyl)-4-methyl-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate (MCO)

Bis(4-((1E,6E)-7-(3,4-dimethoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate
(**curcumin oligomer**)

CH$_3$I   DMF
K$_2$CO$_3$ room temp.

Bis(4-((1E,6E)-7-(3,4-dimethoxyphenyl)-4-methyl-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate
(**methyl curcumin oligomer, MCO**)

Scheme 2.3 Synthesis of mixture **MCO**

Into a two-necked, round-bottomed flask containing 30 mL, dry DMF was placed 0.0217 g (8.64 x 10^{-6} mol equivalent of OH) of curcumin oligomer and 1 mL (0.0161 mol) of CH$_3$I and 4.0 g (0.0289 mol) of K$_2$CO$_3$ under N$_2$ atmosphere. The mixture was kept stirring for 44 h (still under N$_2$ atmosphere) at room temperature. After that, K$_2$CO$_3$ was filtered out and the reaction mixture was then diluted with 20 mL CH$_2$Cl$_2$ and wash with water 3 times until the pH of the water was around 7. Product was dehydrated with anhydrous Na$_2$SO$_4$. Solvent was then evaporated under reduced pressure. The product was analyzed by ^{1}H-NMR, IR, UV-Vis spectroscopy and GPC.

Bis(4-((1E,6E)-7-(3,4-dimethoxyphenyl)-4-methyl-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenyl) succinate (**MCO**): dark orange solid (yield: 86%), m.p. 93-95 ^{o}C,

R_f 0.46 (75% EtOAc/Hex), IR (CH$_2$Cl$_2$, cm^{-1}) : 2924, 2837, 1705, 1670, 1588, 1516 and 1260; UV-Vis, λ_{max} 351 nm; ε = 59,058 mol^{-1}Lcm^{-1}; ^{1}H-NMR (CDCl$_3$) δ (ppm): 7.62 (d, J=15.5 Hz, 1H, Ar-CH=), 7.06 (d, J=8.9 Hz, 1H, Ar-**H**), 6.93 (s, 1H, Ar-**H**), 6.77 (d, J=7.9 Hz, 1H, Ar-**H**), 6.57 (d, J=15.6 Hz, 1H, CO-CH=), 3.83 (s, 3H, OCH$_3$), 3.42 (s, 1H, CO-CH(CH$_3$)-CO), 2.85 (d, 4H, CO-(CH$_2$)$_2$-CO), and 1.40 (d, 3H, CO-CH(CH$_3$)-CO).

2.6 Purification the curcumin oligomer by dialysis method

Dialysis cellulose membrane was soaked for 10 minutes in water prior to use. The membrane tube was sealed at one end and 400 mL of the solution was poured into the membrane. The top end of the membrane was then sealed by allowing an additional dead space approximately ¼ of the volume taken up by the sample. The bag was then placed into a beaker containing 4000 mL of water. Dialysis was performed for 4 days with 10 changes of water.

2.7 General procedure for molar absorptivity measurements

Tested compounds were dissolved in hexane or butanol to the concentration of 1 mol/L. The resulting stock solution was then diluted to selected concentrations using corresponding solvents. The UV absorbance of each final dilution was recorded by scanning wavelengths between 200 and 800 nm. The molar absorptivity (ε) at the wavelength of maximum absorbance (λ_{max}) was calculated using Beer's law:

$$A \quad = \quad \varepsilon bc$$

where A is absorbance

b is the cell path length (1 cm)

c is the concentration of the absorbing species in mol per liter

2.8 Franz cell absorption test [54-56]

In vitro permeation studies were conducted with vertical Franz diffusion cells (Figure 2.1) with a 13 mL capacity receptor compartment and 2.27 cm^2 diffusion areas. The abdominal skin of 2 week olds baby mouse (*Mus Musculus* Linn.) was removed by surgical procedure. The skin samples were stored at room temperature, -20 °C (freezer) and -80 °C. Skin was cut into two suitable small pieces and carefully mounted onto the receiver compartment of the diffusion cells with the stratum corneum facing in the direction of the donor compartment. When the donor compartment was fastened to the receptor compartment with a clamp, the skin acted as a seal between the two half-cells. The receptor medium consisted of isotonic phosphate buffered saline pH 7.4 (PBS-buffer) with 1% w/v Tween 20. The buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.44 g Na_2HPO_4 and 10 mL Tween 20 in 1 litre distilled water. This medium was maintained at 37 °C and constantly stirring with magnetic bar. The sunscreen solution (200 µL) was added into the donor compartment of the cell. At five time intervals; 0, 1, 2, 4 and 24 h, the 3.4 mL of receptor volume was withdrawn and replaced with fresh receptor medium. Care was given to avoid any air bubble in the receptor fluid. The concentration of the UV absorbers in each withdrawn receptor volume was then determined by UV-Vis spectrophotometer. The tests were done at least in duplicate. Since skins from different mice gave different penetration rates, each sample was compared to the penetration rate of 2-Ethylhexyl-4-methoxycinnamate (EHMC) using skin from the same mouse.
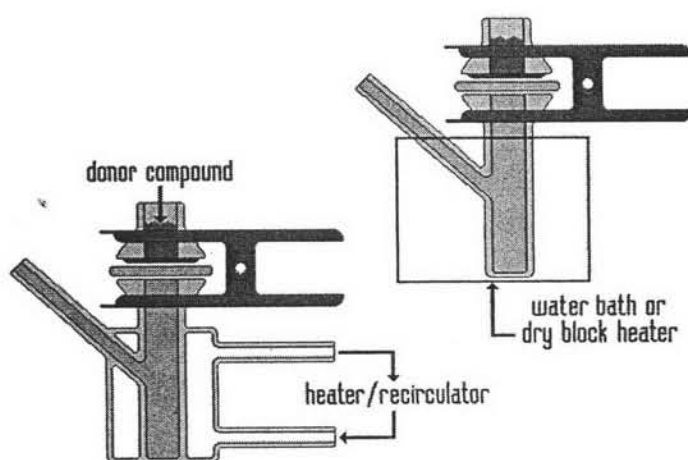
Figure 2.1 Franz-type glass diffusion cells [54].

2.9 General procedure for photostability test

The photostability tests were performed in dichloromethane. Stock solution of each compound was prepared in a 50 mL volumetric flask. The resulting 10 ppm solution was divided into two parts. One part was kept away from light (covered with foil) at room temperature (dark sample) while another part was irradiated by artificial UV lamp (irradiated sample) at 3.0 mW/cm^2 UVA and 0.19 mW/cm^2 UVB. Then UV absorption profile of each sample was analyzed on UV-Vis spectrometer. The absorbance of irradiated sample at various irradiant times was compared to samples that were kept in dark.

The calculation of percent relative absorbance of each irradiated sample is given by:

$$\text{Percent of relative absorbance} = \left(\frac{\text{Absorbance of irradiated sample at time X}}{\text{Absorbance of dark sample (starting time)}} \right) \times 100$$

Percent relative absorbance of 10 ppm **MCO** compared with 10 ppm of **TMC**, EHMC and curcumin, respectively.