CHAPTER III

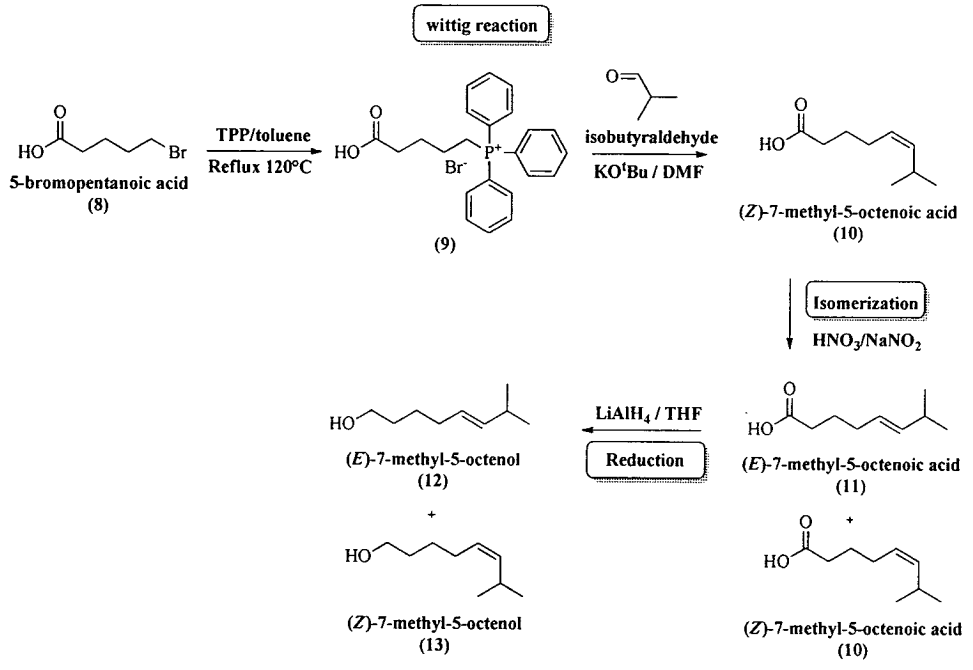
RESEARCH METHODOLOGY

Investigation of capsinoid analogues **(4-7)** was divided into three parts as following: (I) Design and syntheses of capsinoid analogues **(4-7)**, (II) Study of their stability in polar protic solvent comparing with capsiate and (III) Evaluation of cytotoxicity *via* MTT assays on Caco-2 cell line.

Overview synthesis of capsinoid analogues (4-7)

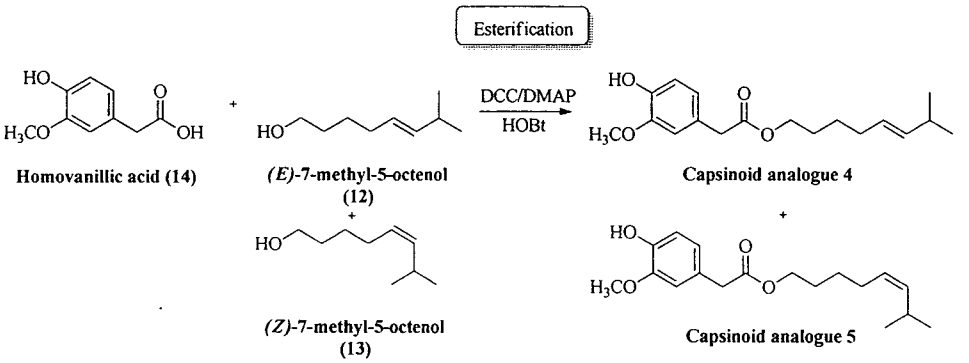
Synthesis of capsinoid analogues **(4-7)** were categorized into three parts, (I) Synthesis of *(E)*-7-methyl-5-octenol (**12**), (II) Synthesis of capsinoid analogue **4** and **5** and (III) Synthesis of capsinoid analogue **6** and **7** *via* esterification between homovanillic acid (**14**) with unsaturated alcohol **17** and saturated alcohol **18**

1. Synthesis of (*E*/Z)-7-methyl-5-octenol (12-13)



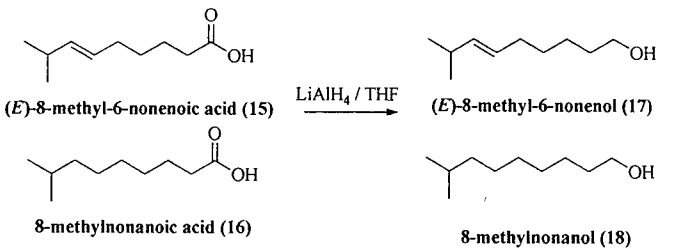
Scheme 5 Synthesis diagram of *(E/Z)*-7-methyl-5-octenol (12-13)

2. Synthesis of capsinoid analogue 4 and 5



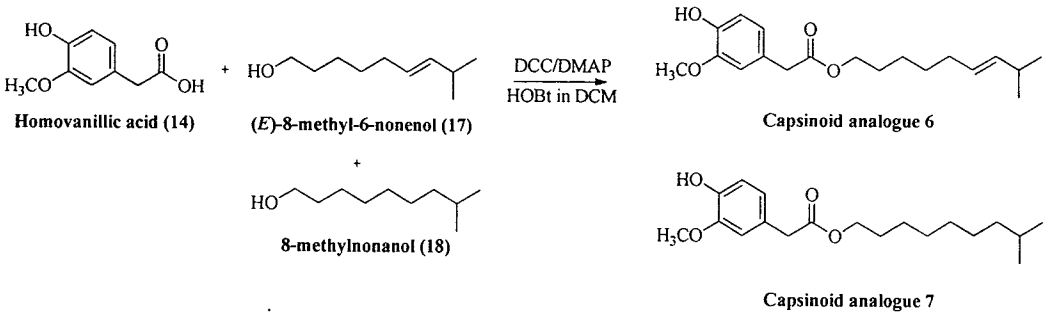
Scheme 6 Synthesis diagram of capsinoid analogue 4 and 5

3. Synthesis of mixture lipophilic chain carbon, *(E)*-8-methyl-6-nonenol (17) and 8-methylnonanol (18)



Scheme 7 Synthesis of *(E)*-8-methyl-6-nonenol (17) and 8-methylnonanol (18) *via* reduction

4. Synthesis of capsinoid analogue 6 and 7



Scheme 8 Synthesis diagram of capsinoid analogue 6 and 7

Stability test of capsinoid analogues (4-7) in polar protic solvent

The stability test of capsinoid analogues (**4-7**) were studied in a mixture solvent between CH_3OH:H_2O with ratio of 20:80 in presence of 0.025 M CH_3COOH. The stability test was investigated in the term of percent remaining of capsinoid derivatives comparing with capsiate *via* HPLC technique.

Investigation of Cell Viability on Caco-2 cell *via* cytotoxicity MTT assays

Evaluation of toxicity on Caco-2 cell by using cytotoxicity MTT assays for observing the effective dose of capsinoid analogues (**4-7**) was a preliminary study to obtain a toxicity information before further study biological activities in the future. (Figure 16)

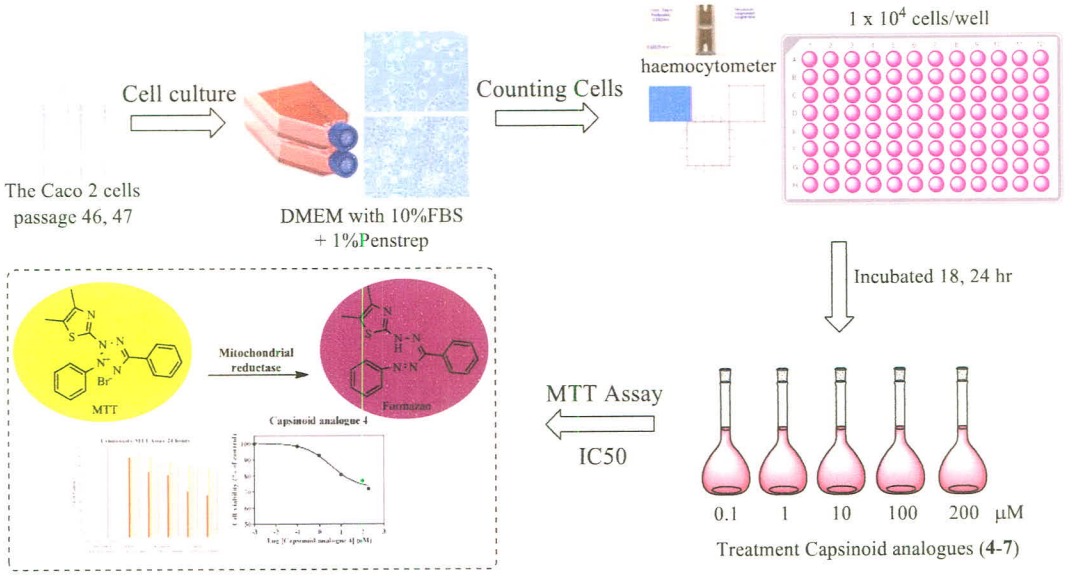


Figure 16 cytotoxicity MTT assays procedure

General Procedure

1. Equipment

Unless otherwise stated, all glasswares were oven dried. The weights of all chemical substances were determined on sartorius electrical balance. Evaporation of solvents was operated on Büchi Rotavapor R-114 with a water aspirator model B-480 or a Refco Vacubrand pump. Column chromatography was performed on silica gel having 60-200 µm for column chromatography. Visualization of TLC plates was accomplished using either UV light (254 nm), *p*-anisaldehyde or phosphomolybdic acid. The progresses of reactions were monitored by thin layer chromatography (TLC) (Merck D.C. silica gel 60 F254 0.2 mm-pre-coated aluminium plates).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker NMR spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in appropriate deuterated solvents. Chemical shifts (δ) were reported in part per million (ppm) relative to either tetramethylsilane (TMS) or the residual protonated solvent signal as a reference.

The capsiate and capsinoid analogues were submitted to quantitative analysis for stability testing using reverse phase HPLC on an Agilent 1100 series controller system equipped with gradient pump and Agilent 1100 series photodiode

array detector. A Vertisep^{TM} C_{18} HPLC column 5 μm, particle size 4.6 x 250 mm was used for the stability test. Peak monitoring and data processing were performed on the base Empower software. The fractions were manually collected and assisted by real-time HPLC chromatogram monitoring. Mass spectra were measured in positive ion mode with an Agilent 6540 Q-TOF mass spectrometer.

2. Materials

All commercially available chemicals were purchased from Fluka Co., Ltd, Merck Co., Ltd, Acros Co., Ltd, Aldrich Co., Ltd, Chempep Co., Ltd and Lab Scan Co., Ltd, Thai can biotech Co., Ltd. and all chemicals were used without further purification unless otherwise stated. Commercial grade solvents for column chromatography were distilled prior to use. Solvent for reactions were AR grade and were used without purification unless otherwise stated. *N,N*-Dimethylformamide (DMF), tetrahydrofuran (THF) and dichloromethane (DCM) were dried over 3 Å molecular sieves under N_2 atmosphere. HPLC grade methanol and glacial acetic acid for HPLC experiments, obtained from Lab Scan, were filtered through 0.45 μm nylon membrane before use. DI water was obtained from ultrapure water system with ELGA (England). For capsinoid analogues synthesis, anhydrous DMF, THF and dichloromethane (DCM) ($H_2O \leq 0.01$ % dried over activated 3 Å molecular sieves) using as solvent was purchased from Merck. Reducing and Coupling agents were obtained from commercial source and used without purification.

Chemicals synthesis grade (IUPAC name) 99% 5-Bromovaleric acid, 99% Isobutylraldehyde, 98% Potassium tert-butoxide, 99% Triphenyl phosphine, 99% 4-(dimethylamino)pyridine (DMAP), 99% *N,N'*-Dicyclohexylcarbodiimide (DCC), 99% 1-hydroxybenzotriazole (HOBt), 95% Lithium aluminium hydride ($LiAlH_4$), 65% Nitric acid, 37% Hydrochloric acid, 99% Sodium nitrite, 98% Acetic anhydride, 99% Sodium sulfate anhydrous (Na_2SO_4).

Chemicals for cells culture and cytotoxicity MTT assay, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (JRH Biosciences, Lexena, KS), Penicillin Streptomycin (Pen Strep), phosphate buffered saline (PBS) at pH 7.4, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

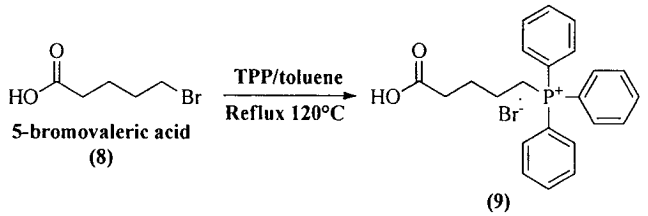
Deuterated solvent for NMR characterization, Chloroform-*d* ($CDCl_3$), Dimethyl sulfoxide (DMSO-d_6) and Deuterium oxide (D_2O) were purchased from Aldrich.

Synthesis of capsinoid analogues (4-7)

1. Synthesis of capsinoid analogue 4 and 5

Synthesis of (*E/Z*)-7-methyl-5-octenol (12 and 13)

1.1 Synthesis of (4-carboxybutyl) triphenylphosphonium Bromide (9)

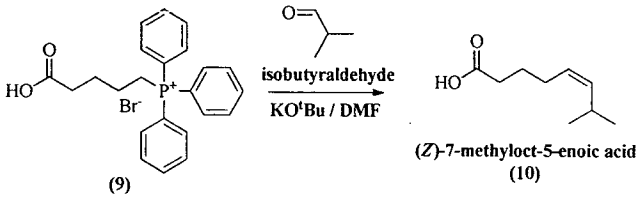


Scheme 9 Synthesis of (4-carboxybutyl)triphenylphosphonium bromide (9)

A mixture of 5-bromovaleric acid (**8**) (1.00 g, 5.52 mmol) and triphenylphosphine (1.74 g, 6.63 mmol) was dissolved in toluene 5.00 mL and then refluxed at 120 °C for 24 hours. After that, it was cooled to room temperature and the toluene was removed in *vacuo*. The cooled glassy mixture was triturated with CH_2Cl_2 and diethyl ether in ratio 1:1, then the precipitates were obtained and recrystallized in cooled diethyl ether, affording (4-carboxybutyl)triphenylphosphonium bromide (**9**) as a white powder in 98% yields (2.41 g).

^{1}H NMR (D_2O) 400 MHz δ: 7.94 – 7.76 (*m*, 15H, Ar), 3.40 – 3.32 (*m*, 2H), 2.43 (*t*, J = 6.80 Hz, 2H), 1.89 – 1.74 (*m*, 4H).

1.2 Synthesis of *(Z)*-7-methyl-5-octenoic acid (10)

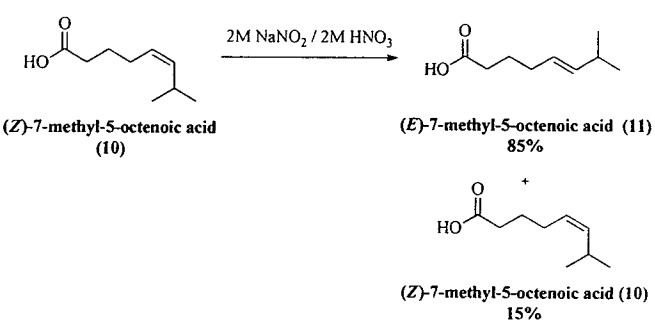


Scheme 10 Synthesis of *(Z)*-7-methyl-5-octenoic acid (10) *via* Wittig reaction

Wittig reaction was initiated by preparing sublimation of Potassium tert-butoxide (KO^tBu) at 220 °C about 20 minutes before use. The mixture of (4-carboxybutyl)triphenylphosphonium bromide (**9**) (1.50 g, 3.38 mmol) and isobutyral- dehyde (0.62 mL, 6.77 mmol) was dissolved in anh. DMF and then added to suspension of KO^tBu (1.52 g, 13.53 mmol) in anh. DMF at 0 °C (in ice-salt bath) by dropwise stirred for 15 minutes. After that, the reaction mixture was removed from ice-salt bath and allowed to stir at room temperature for 24 hours. Precipitated triphenylphosphine oxide was removed by suction filtration and acidified with 1M HCl. The product was extracted with diethyl ether (3 x 20 mL) and brine solution (3 x 20 mL), dried over anh. Na_2SO_4. The crude product was evaporated *in vacuo* to give colorless oil of acid and purified by column chromatography (silica gel, EtOAc:Hexane/30:70 v/v) to give (*Z*)-7-methyl-5-octenoic acid (**10**) in 68% yields (0.36 g). R_f = 0.43 (EtOAc:Hexane/30:70 v/v).

^1H NMR ($CDCl_3$) 400 MHz δ: 5.29 – 5.12 (*m*, 2H), 2.64 – 2.47 (*m*, 1H), 2.36 (*t*, *J* = 8.0 Hz, 2H), 2.18 – 2.01 (*m*, 2H), 1.69 (*q*, *J* = 7.5 Hz, 2H), 0.97 – 0.92 (*m*, 6H).

1.3 Synthesis of *(E)*-7-methyl-5-octenoic acid (11)



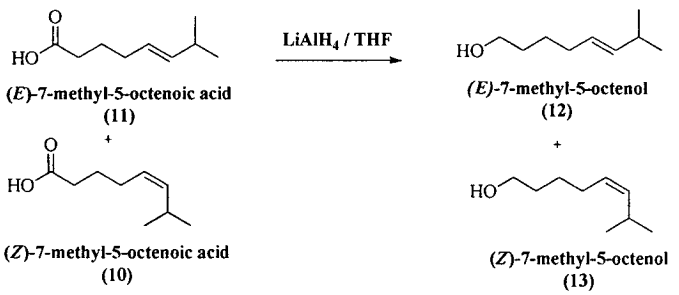
Scheme 11 Synthesis of (*E*)-7-methyl-5-octenoic acid (11)

The isomerization of (*Z*)-7-methyl-5-octenoic acid (**10**) to (*E*)-7-methyl-5-octenoic acid (**11**) can be successfully prepared by the mixture of 2M $NaNO_2$ and 2M HNO_3 (1 : 3) was rapidly added to (*Z*)-7-methyl-5-octenoic acid (0.36 g, 2.30 mmol) at 0 °C stirred for 15 minutes then heated up to 70 °C for 1.5 hour. After that, the reaction was quenched by adding cooled diethyl ether and stirred at 0 °C for 20 minutes and the mixture was extracted with diethyl ether (3 x 20 mL) and brine solution (3 x 20 mL) and the extract was dried over anhydrous Na_2SO_4. Diethyl ether was removed by evaporated *in vacuo* to give the mixture between 85% of (*E*)-7-methyl-5-octenoic acid (**11**) and 15% of (*Z*)-7-methyl-5-octenoic acid (**10**) with 95% yields (0.35 g), R_f = 0.43 (EtOAc:Hexane/30:70 v/v).

^{1}H NMR ($CDCl_3$) 400 MHz δ: 5.42 – 5.20 (*m*, 1H), 2.36 (*t*, 2H, *J* = 3.2 Hz), 2.34 – 2.25 (*m*, 1H), 2.01 – 1.97 (*m*, 1H), 1.67 – 1.61 (*m*, 2H), 1.44 – 1.38 (*m*, 2H), 0.95 (*d*, *J* = 6.8 Hz, 6H),

FT-IR (KBr) cm^{-1}: 3,362 (O-H stretching), 1,658 (C=O stretching)

1.4 Synthesis of *(E/Z)*-7-methyl-5-octenol (12-13)



Scheme 12 Synthesis of *(E/Z)*-7-methyl-5-octenol (12-13) *via* reduction

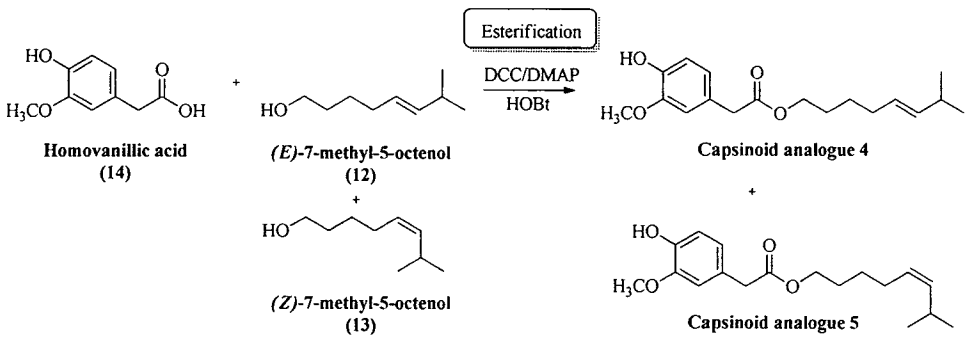
(E/Z)-7-methyl-5-octenol (**12-13**), To a one-necked 25 mL round bottomed flask equipped with a Teflon-coated magnetic stir bar is added lithium aluminum hydride ($LiAlH_4$) (0.25 g, 6.50 mmol). The flask was fitted with a septum and nitrogen inlet needle. The solution was cooled in dry-ice acetone bath under an atmosphere of nitrogen gas. *(Z/E)*-7-methyloct-5-enoic acid (**10-11**) (0.35 g, 2.24 mmol) was dissolved in anh.THF (5 mL) and added over the course of 15

minutes by dropwise into $LiAlH_4$. The reaction mixture was allowed to warm slowly to room temperature overnight with stirring (20 h). When the reaction was complete as determined by TLC, the crude reaction mixture was poured slowly into ethyl acetate (10 mL) in a 250 mL Erlenmeyer flask equipped with a magnetic stir bar and cooled in an ice bath. The excess $LiAlH_4$ is quenched by the slow addition of water (10 mL) to the stirred mixture over 30 minutes. Additional distilled water is added (10 mL) and the mixture was stirred until appeared two distinct layers form (about~30 minutes). The mixture was transferred to a 150 mL separatory funnel and the layers separated. The aqueous layer was extracted with ethyl acetate (3 x 20 mL). The combined organic layers were transferred to a 150 mL separatory funnel, washed with brine (3 x 20 mL) and dried for 30 minutes over anh. Na_2SO_4. After filtration, the solvent was removed by rotary evaporation in *vacuo* to give a colorless oil in 98% yields of *(E)*-7-methyl-5-octenol (**12**) and *(Z)*-7-methyl-5-octenol (**13**) (0.31 g.), R_f = 0.53 (EtOAc:Hexane/30:70 v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.44 – 5.27 (*m*, 2H), 3.67 – 3.62 (*m*, 2H), 2.36 – 2.19 (*m*, 1H), 2.01 (*dd*, J_1 = 12.4, J_2 = 6.3, Hz, 2H), 1.61 – 1.52 (*m*, 2H), 1.46 – 1.38 (*m*, 2H), 0.95 (*t*, *J* = 7.0 Hz, 6H).

FT-IR (KBr) cm^{-1}: 3,361 (O-H stretching)

2. Synthesis of capsinoid analogue 4 and 5



Scheme 13 Synthesis of capsinoid analogue 4 and 5 *via* esterification

The mixture of homovanillic acid (**14**) (0.48 g, 2.62 mmol), 1-hydroxybenzotriazole (HOBt) (0.59 g, 4.36 mmol), (*E/Z*)-7-methyl-5-octenol (0.31 g,

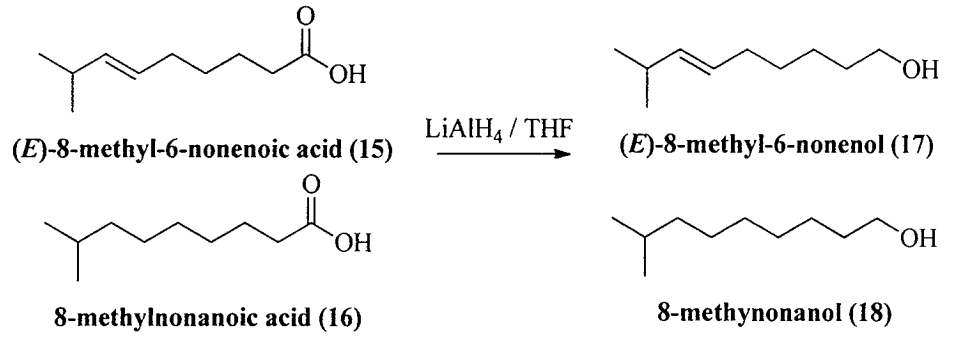
2.18 mmol) (**12-13**) and 4-dimethylaminopyridine (4-DMAP) (0.53 g, 4.36 mmol) was dissolved in dried dichloromethane (5.00 mL) and cooled in water-ice bath under nitrogen inlet needle. *N,N'*-dicyclohexylcarbodiimide (DCC) (0.89 g, 4.36 mmol) was dissolved and added in dried DCM (2.00 mL) dropwise into the mixture about 20 minutes at 0 °C under an atmosphere of nitrogen gas and stirred over period of 24 hours. After that, the precipitate of *N,N'*-dicyclohexylurea (DCU) was filtered by suction filtration and washed with cooled dichloromethane. The mixture was extracted with diethyl ether (3 x 20 mL), and brine solution (3 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated in *vacuo* to yield a crude product as colorless oil. Finally, crude product was purified by column chromatography (EtOAc:Hexane/30:70 v/v) to give mixture of capsinoid analogue **4** and **5** as colorless oil in 62% yields (0.41 g), R_f = 0.54 (30:70 EtOAc:Hexane). Mixture of capsinoid analogue **4** and **5** was purified by using HPLC to separate *(E/Z)*-isomer mixture with condition column, VertiSep^{TM} UPS C18, 4.6 x 250 mm, 5 μm, mobile phase, CH_3OH:H_2O/80:20 v/v with 0.025 M CH_3COOH, flow rate 0.5 mL/min, detection UV 280 nm.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.85 (*d*, *J* = 8.0 Hz, 1H), 6.81 (*d*, *J* = 2.0 Hz, 1H), 6.76 (*dd*, *J* = 8.1, 2.1 Hz, 1H), 5.41 – 5.24 (*m*, 2H), 4.10 – 4.05 (*m*, 2H), 3.87 (*s*, 3H), 3.53 (*s*, 2H), 2.22 (*dq*, *J* = 13.4, 6.7 Hz, 1H), 2.01 – 1.93 (*m*, 2H), 1.61 (*ddd*, *J* = 18.7, 10.0, 4.9 Hz, 2H), 1.44 – 1.32 (*m*, 2H), 0.97 – 0.93 (*m*, 6H).

FT-IR (KBr) cm^{-1}: 3,447 (O-H stretching), 1,735 (C=O stretching), 1,035 (C-O stretching).

3. Synthesis of capsinoid analogue 6 and 7

3.1 Synthesis of mixture Fatty chain alcohol, *(E)*-8-methyl-6-nonenol (17) and 8-methylnonanol (18)



Scheme 14 Synthesis of *(E)*-8-methyl-6-nonenol (17) and 8-methylnonanol (18) *via* reduction

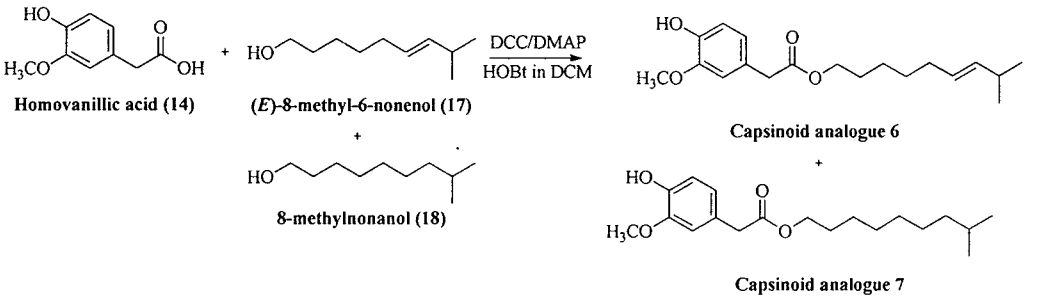
(E)-8-methyl-6-nonenol (**17**) and 8-methylnonanol (**18**), To a one-necked 25 mL round bottomed flask equipped with a Teflon-coated magnetic stir bar was added lithium aluminum hydride (0.32 g, 8.53 mmol). The flask was fitted with a septum and nitrogen inlet needle. The solution was cooled in a dry-ice acetone bath under an atmosphere of nitrogen gas. Then the mixture of *(E)*-8-methyl-6-nonenoic acid (**15**) and 8-methylnonanoic acid (**16**) (0.50 g, 2.94 mmol) was dissolved in anh. THF (5 mL). After that the mixture solution was added over the course of 15 minutes by dropwise. The reaction mixture was allowed to warm slowly to room temperature overnight with stirring (20 h). When the reaction was complete as determined by TLC, the crude reaction mixture was poured slowly into ethyl acetate (10 mL) in a 250 mL Erlenmeyer flask equipped with a magnetic stir bar cooled in an ice bath. The excess $LiAlH_4$ is quenched by the slow addition of water (10 mL) to the stirred mixture over 30 minutes. Additional distilled water was added (10 mL) and the mixture was stirred until displayed two distinct layers form (about~30 minutes). The mixture was transferred to a 250 mL separatory funnel and the layers separated. The aqueous layer was extracted with ethyl acetate (3 x 20 mL). The combined organic layers was transferred to a 250 mL separatory funnel, washed with brine (3 x 20 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed by rotary evaporation in *vacuo* to give a colorless oil in 95% yields the mixture of *(E)*-8-methyl-6-nonenol (**17**) and 8-methylnonanol (**18**) (0.44 g.).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42 – 5.27 (*m*, 2H), 3.68 – 3.58 (*m*, 4H), 2.28 – 2.16 (*m*, 1H), 2.02 – 1.93 (*m*, 2H), 1.79 – 1.63 (*m*, 1H), 1.61 – 1.44 (*m*,

4H), 1.40 – 1.29 (*m*, 2H), 1.29 – 1.20 (*m*, 2H), 1.18 – 1.08 (*m*, 1H), 0.95 (*d*, *J* = 6.7 Hz, 6H), 0.90 – 0.80 (*m*, 6H).

FT-IR (KBr) cm^{-1}: 3,369 (O-H stretching).

3.2 Synthesis of capsinoid analogue 6 and 7



Scheme 15 Synthesis of capsinoid analogue 6 and 7 *via* esterification

The mixture of homovanillic acid (**14**) (0.62 g, 3.38 mmol), 1-hydroxybenzotriazole (HOBt) (0.76 g, 5.64 mmol), the mixture of *(E)*-8-methyl-6-nonenol (**17**) and 8-methylnonanol (**18**) (0.44 g, 2.82 mmol) and 4-dimethylaminopyridine (4-DMAP) (0.69 g, 5.64 mmol) were dissolved in dried dichloromethane (5.00 mL) and stirred in water-ice bath under atmosphere of nitrogen gas. Then *N,N'*-Dicyclohexylcarbodiimide (DCC) (1.16 g, 5.64 mmol) was dissolved in dried DCM (2.00 mL) dropwise into the mixture about 20 minutes at 0 °C under N_2 inlet needle and continuous stirred over period of 24 hours. After that, the precipitate of *N,N'*-dicyclohexylurea (DCU) was filtered by suction filtration and washed with cooled dichloromethane. The mixture was extracted with diethyl ether (3 x 20 mL), and brine solution (3 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated in *vacuo* to yield a crude product as colorless oil. Finally, crude product was purified by column chromatography (EtOAc:Hexane/30:70 v/v) to give the mixture of capsinoid analogues **6** and **7** as colorless oil in 77% yields (0.56 g), R_f = 0.52 (EtOAc:Hexane/30:70 v/v). The mixture of capsinoid analogue **6** and **7** was purified by using HPLC with condition column, VertiSep^{TM} UPS C18, 4.6 x 250 mm, 5 μm, mobile phase, CH_3OH:H_2O/80:20 v/v with 0.025 CH_3COOH, flow rate 0.5 mL/min, detection UV 280 nm.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 – 6.80 (*m*, 6H), 5.44 – 5.21 (*m*, 2H), 4.12 – 3.99 (*m*, 4H), 3.92 – 3.88 (*m*, 6H), 3.76 (*s*, 2H), 3.57 (*s*, 2H), 2.30 – 2.12 (*m*, 1H), 2.03 – 1.86 (*m*, 2H), 1.72 – 1.55 (*m*, 4H), 1.53 – 1.43 (*m*, 1H), 1.40 – 1.18 (*m*, 12H), 1.18 – 1.07 (*m*, 2H), 0.99 – 0.91 (*m*, 6H), 0.90 – 0.80 (*m*, 6H).

FT-IR (KBr) cm^{-1}: 3,447 (O-H stretching), 1,735 (C=O stretching), 1,035 (C-O stretching).

Stability test of capsinoid analogues (4-7) in polar protic solvent

Aliquot of capsinoid analogues (**4-7**) (1 mM approximately) was dissolved in polar protic solvent (CH_3OH:H_2O/80:20 v/v with 0.025 M CH_3COOH). Immediately after the preparation, the sample was kept at 25 °C. An aliquot of the sample (20 μl) was taken at adequate time to analyze by HPLC every 6 hour for 24 hours. HPLC was carried out under the following conditions: column, VertiSep^{TM} UPS C18, 4.6 x 250 mm, 5 μm, mobile phase, CH_3OH:H_2O/80:20 v/v with 0.025 M CH_3COOH, flow rate 0.5 mL/min, detection UV 280 nm. Peak area of (*E*/Z)-isomer of capsinoid analogues were used to calculate in term of remaining percentage of capsinoid analogues (**4-7**) with the period of time.

Investigation of Cell Viability on Caco-2 cell *via* cytotoxicity MTT assays

Cell culture: Human colon epithelial cells, Caco-2 cells line (ATCC® HTB-37™), were kindly provided by Associate Professor Dr.Anchalee Srichamroen, department of Agro-industry, Faculty of Agriculture, Natural Resources and Environment, Naresuan University. They were used between passages 46 and 47, and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences, Lexena, KS), 1% Penicillin Streptomycin (Pen Strep) and washed with phosphate buffered saline (PBS) at pH 7.4 solution in a humidified atmosphere (5% CO_2, 95% air, 37°C). The Caco-2 cells in cryo tube were removed from liquid nitrogen and warmed in a water bath at 37°C (an appropriate temperature for the cell line) by submerging only the lower half of the cryo tube. The cells were allowed to thaw until a small amount of ice remains in the vial (usually 1-2 minutes). The cryo tube was transferred to safety cabinet, a T75 flask was prepared that contained 10 mL DMEM supplemented with 10% (v/v) fetal bovine serum, and

1% Penicillin Streptomycin (Pen Strep). After that, cells were slowly pipetted into DMEM T75 flask and were incubated overnight. The Caco-2 cells were grown under standard conditions until 70–80% confluency and sub-cultured as necessary. Subculture cells were done by trypsinizing cells to T75 flasks.

First, DMEM media was removed from T75 flask and then the Caco-2 cells were washed with PBS pH 7.4 and PBS was removed. After that 3 mL of 0.25% trypsin/EDTA were added for releasing cells from T75 flask and the cells were incubated (5% CO_2, 95% air, 37°C) for 5 minutes. Then, 5 mL of DMEM were added to neutralize trypsin and this was centrifuged at 2500 rpm for 5 minutes. The solution was removed and new medium solution 3 mL were added to re-suspend the cells. After that, counting cells was performed by using a haemocytometer. About 1 mL of cell suspension was taken out using a serological pipette and placed in an eppendorf tube. 100 µL of cells solution were mixed with 100 µl trypan blue and this was re-suspended gently again. The mixture was carefully filled in haemocytometer chamber by gently resting the end of the Gilson tip at the edge of the chambers focus on the grid lines of the haemocytometer using the 10X objective of the microscope and only live cells that look healthy (unstained by trypan blue) were counted. 1 mL of cells was splitted into 3 T75 flasks and the culture medium was added 10 mL. DMEM medium was changed every second day. The cells were left to differentiate for 3-5 days. The Caco-2 cells passages 48 were used for all of the experiments. The cells were seeded in 96-well Transwell® plates at a seeding density of 1×10^4 cells/cm^2 100 µL/well.

Cytotoxicity MTT assays: The 96-well Transwell® plates with a seeding density of 1×10^4 cells/cm^2 were incubated overnight then DMEM was removed from each 96-well. The cells were treated with capsinoid analogues (**4-7**), capsaicin was used as negative control, capsiate was used as positive control, 1%PEG+DMEM and DMEM were used as a vehicle. The variation of dose was in the range of 0.1, 1, 10, 100, and 200 µM, respectively. 96-well plates were treated and incubated at 18 and 24 hours after that added MTT solution (5mg/mL) in PBS, 20 µL/well and incubated for 2 hours. After 2 hours, the supernatant was removed and then 150 µL DMSO were added to dissolve the formazan and this was centrifuged for 10 minutes. The absorbance was read by using spectrophotometer microplate reader at 595 nm.

IC_{50} values investigation: To observe the effects of different concentrations of capsinoid analogues (**4-7**) on Caco-2 cell viability, 96-well Transwell® plates with a seeding density of 1×10^4 cells/cm^2 were incubated overnight then DMEM was removed from each 96-well. The cells were treated with capsinoid analogues (**4-7**), 1%PEG+DMEM and DMEM was used as a control. The variation of dose was in the range of 0.1, 1, 10, 50, 100, 150 and 200 μM, respectively. 96-well plates were treated and incubated at 24 hours after that MTT solution (5mg/mL) in PBS was added at 20 μL/well and incubated for 2 hours. After 2 hours, the supernatant was removed and then 150 μL DMSO were added to dissolve the formazan. The 96-well plate was centrifuged for 10 minutes and the absorbance was read by using spectrophotometer microplate reader at 595 nm. The IC_{50} value was calculated *via* GraphPad Prism software version 6 plot between %cell viability of control and Log of concentration of each capsinoid analogues (**4-7**).