CHAPTER III

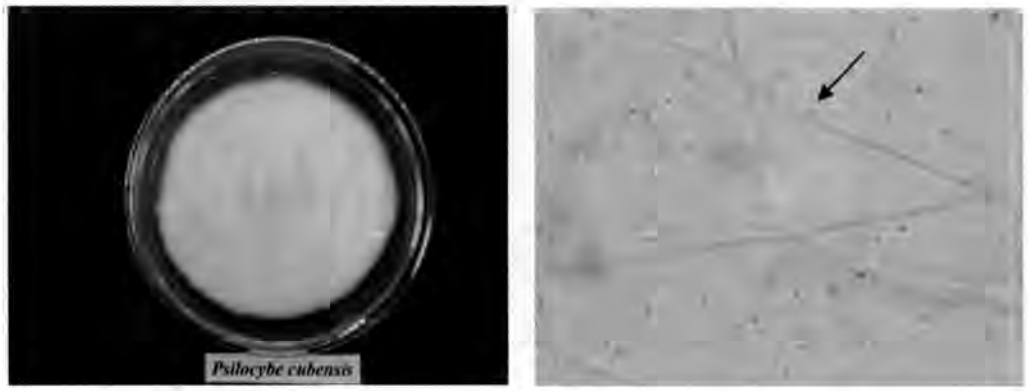
EXPERIMENTS

3.1 Isolation of *ent*-kaur-16-en-19-oic-acid

Ent-kaur-16-en-19-oic-acid or *ent*-kaurenoic acid obtained from the plant *Croton oblongifolius* Roxb. which was collected from Aumphoe Kui buri, Prachuap khiri khan Province, Thailand. The stem bark of *C. oblongifolius* was milled and soaked with hexane three times. The extract was evaporated under reduced pressure by rotary evaporator to remove the solvent. After the evaporation, a viscous dark-yellow oil was obtained. The dark-yellow oil hexane crude was separated by quick column chromatography using silica gel. *Ent*-kaurenoic acid was obtained from quick column chromatography which was eluted with 5% ethyl acetate in hexane. The fraction was purified by crystallization using ethyl acetate and hexane, resulting in a white crystalline solid. The structure of *ent*-kaurenoic acid was confirmed by spectroscopic technique (Figure C1 and C2), the chemical structure of *ent*-kaurenoic acid was shown in Figure 2.

3.2 Microorganism and culture condition

Psilocybe cubensis (Figure 16) was obtained from the Department of Microbiology, Chulalongkorn University, Thailand. The fungus was grown in a Petri dish containing PDA (Potato Dextrose Agar) (appendix A1) and stock cultures were prepared by inoculating the pieces of 10-days mycelia on PDA in sterilized water and kept at room temperature. The fungus was grown fresh before used.



A B

Figure 16. The mycelium of *Psilocybe cubensis*

A) *P. cubensis* on potato dextrose agar; **B**) Camp connection of *P. cubensis*

3.3 Biotransformation of *ent*-kaurenoic acid by *Psilocybe cubensis*

A piece of mycelium from stock cultures growing on PDA was inoculated on to a Petri dish of PDA and incubated at room temperature for 10 days. Pieces of mycelium were inoculated into 100 ml. of Czapex peptone medium (Appendix A2) in 250 Erlenmeyer flasks and were allowed to grow in a rotary shaker (150 rpm.) at room temperature for 5 days. After 5 days of growth, the substrate, dissolved in ethanol, was evenly distributed into each flask to a final concentration of 0.2 g/l. The incubations were made under the same conditions for 2 days. In the second experiment incubation was maintained for 9 days.

Culture controls consisted of fermentation blanks in which the fungus was grown without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate without the fungus and incubated under the same condition.

The product formation was monitored every two days by taking out 1 ml of the culture medium and extracted 3 times with equal volumes of ethyl acetate. The solvent was removed from the sample to obtain the ethyl acetate crude. The sample was applied onto the TLC plate using $CHCl_3$: CH_3OH (4:1) as solvent system and visualized by vanillin solution.

3.4 Product extraction and purification

After the end of incubation, the cultures were filtrated. The broth was extracted three times with equal volumes of EtOAc and the mycelium was washed with the same solvent. The extracts were combined and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure in a rotary evaporator to obtain the viscose brown oil. The metabolites were separated by silica gel column chromatographic procedures. The column was packed with 30 % ethyl acetate in hexane and eluted with mixture of Hexane-EtOAc and EtOAc-CH_3OH by gradient in a stepwise fashion. The similar fractions were combined and the solvent was removed under reduced pressure using a rotary evaporator.

The culture from 9-days incubation was extracted and purified using the same procedure as described above.

Elution of EtOAc crude from 2-day and 9-day culture experiments with 30 % EtOAc in hexane obtained Compound **1**, white solid powder, for 20.0 % and 19.8 % respectively. Compound **1** was identified as *ent*-kaurenoic acid using TLC and H^1, C^{13} NMR.

3.4.1 Purification and properties of compound 2

From the 2 days incubation culture extract, compound **2** (21.1 %), white solid powder, was eluted with 60% EtOAc. From the 9 day incubation culture extract, compound **2** (11.5 %) was obtained using the same conditions as the 2 days experiment. Compound **2** is soluble in methanol.

Rf: 0.57 [$CHCl_3$: CH_3OH (4:1)] ;

mp.: 261-263 °C ;

$[\alpha]^{20}_D$: -65° (c 0.1, CH_3OH) ;

FT-IR (KBr) ν_{max} cm^{-1}: 3678-3068 (br), 2943 (s), 1696 (s), 868 (m) (Figure C5, Table 4);

^1H NMR spectrum (CD_3OD, 400 MHz) (Fig.), δ: 0.86 (1H, dt, 1-H), 1.01 (3H, s, 20-H), 1.04 (1H, m, H-9), 1.05 (1H, m, 3-H), 1.10 (1H, dd, J = 6, 8.4), 5-H), 1.21 (3H, s, 18-H), 1.42 (1H, d, J = 14.8, 15-H), 1.43 (1H, br, 2-H), 1.49 (1H, m, 7-H), 1.52 (1H, m, 12-H), 1.54-1.67 (2H, m, 12-H), 1.56 (1H, d, J = 14, 15-H), 1.64 (1H, m, 12-H), 1.65 (1H, m, 7-H), 1.68 (1H, m, 14-H), 1.81-1.90 (2H, m, 6-H), 1.88

(1H, m, 1-H), 1.94 (1H, br, 2-H), 1.95 (1H, d, *J* = 12, 14-H), 2.05 (1H, brs, 13-H), 2.15 (1H, br, 3-H), 3.63 (1H, d, *J* = 12.2, 17-H), 3.74 (1H, d, *J* = 11.6, 17-H) ppm, (Figure C6, Table 5) ;

^{13}C NMR (CD_3OD, 400 MHz,) δ:14.8 (C-20), 18.2 (C-11), 18.9 (C-2), 21.9 (C-6), 25.9 (C-12), 28.1 (C-18), 36.8 (C-14), 37.8 (C-3), 39.4 (C-10), 40.5 (C-1), 41.9 (C-7), 43.2 (C-4), 44.4 (C-8), 44.8 (C-13), 52.3 (C-15), 55.9 (C-9), 56.6 (C-5), 65.4 (C-17), 81.5 (C-16), 180.2 (C-19) (Figure C5, Table 5) ;

LC-MS (rel.int.), *m/z*: 359 $(M+Na)^+$ (Figure C4)

3.4.2 Purification and properties of compound 3

Following experimentation as described above, the crude extract from the 9-day culture was eluted with mixture of Hexane-EtOAc and EtOAc-CH_3OH. Compound **3** (8.3 %), colorless crystal, was eluted at 100% EtOAc. Compound **3** is soluble in methanol.

Rf: 0.41 [$CHCl_3$: CH_3OH (4:1)] ;

mp.: 253-255 °C ;

$[\alpha]^{20}_D$: -60° (c 0.16, CH_3OH) ;

FT-IR (KBr) ν_{max} cm^{-1}: 3667-3052 (br), 2932 (s), 1679 (s), 862 (m) (Figure C11, Table 8) ;

^{1}H NMR (CD_3OD, 400 MHz) δ: 0.89 (1H, m,1-H), 0.96 (3H, s, 20-H), 1.05 (1H, dt, 3-H), 1.12 (1H, d, *J* = 8.8, 5-H), 1.18 (1H, dd, *J* = 2.4, 11.6, 9-H), 1.22 (3H, s, 18-H), 1.45 (1H, d, *J* = 14.4,15-H), 1.46 (1H, m, 2-H), 1.52 (1H, dd, *J* = 4, 12.8, 7-H), 1.57 (1H, d, *J* = 14.4, H-15), 1.60 (1H, m, 11-H), 1.70 (1H, m, 1-H), 1.70 (1H, m, 14-H), 1.82 (1H, m, 6-H), 1.85 (1H, m, 6-H), 1.88 (1H, m, 14-H), 1.91 (1H, m, 1-H), 1.92 (1H, m, 11-H), 1.93 (1H, m, 1-H), 2.16 (1H, br, 2-H), 2.21 (1H, dd, *J* = 4,12.8, 13-H), 3.64 (1H, d, J = 12.0, 17-H), 3.89 (1H, d, *J* = 12.0, 17-H), 3.91 (1H, br, 12-H) (Figure C14, Table 9) ;

^{13}C NMR (CD_3OD, 400 MHz) δ: 15 (C-20), 18.9 (C-2), 21.8 (C-6), 27.9 (C-11), 28.1 (C-6), 36.1 (C-14), 37.7 (C-3), 38.9 (C-10), 40.5 (C-1), 40.8 (C-7), 43.3 (C-3), 44.1 (C-8), 52.0 (C-13), 52.5 (C-15), 56.4 (C-4), 67.1 (C-17), 70.1 (C-12), 81.4 (C-16), 180.2 (C-19) (Figure C15, Table 9);

HRESIMS m/z: 375.2149 (calcd for $C_{20}H_{32}O_5Na$, 375.2147) (Figure C12 and C13)

Compound **3** was crystallized with methanol and water resulting in white solid crystals.

3.4.3 Purification and properties of compound 4

Compound **4** (2.8 %), white solid powder, was isolated from the 9-days culture material eluted with and 5% methanol in ethyl acetate. Compound **4** is soluble in methanol.

Rf: 0.31 [$CHCl_3$: CH_3OH (4:1)] ;

mp.: 238-240 °C ;

$[\alpha]^{20}_D$: -67° (c 0.165, CH_3OH) ;

FT-IR (KBr) ν_{max} cm^{-1}: 3657-3042 (br), 2941 (s), 1680 (s), 865 (m) (Figure C20, Table 16) ;

^1H NMR spectrum (CD_3OD, 400 MHz) δ: 0.87 (3H, s, 20-H), 1.04 (1H, dt, *J* = 4, 13.6, 3-H), 1.10 (1H, m, 5-H), 1.09 (1H, m, 1-H), 1.18 (1H, m, 9-H), 1.18 (3H, s, 18-H), 1.20 (1H, d, *J* = 14.4, 15-H), 1.42 (1H, dr, 2-H),1.47 (1H, m, 7-H), 1.64 (1H, m, 7-H), 1.68 (1H, m, 14-H), 1.73 (1H, m, 12-H), 1.76 (1H, m, 14-H), 1.80 (2H, m, 6-H), 1.90 (1H, m, 2-H), 1.91 (1H, m, 1-H), 1.93 (1H, m, 1-H), 1.98 (1H, m, 12-H), 2.03 (1H, br, 13-H), 2.09 (1H, d, *J* = 14.4, 15-H), 2.11 (1H, br, 3-H), 3.70 (1H, d, *J* = 11.2, 17-H), 3.85 (1H, d, *J* = 6.8, H11), 4.12 (1H, d, *J* = 11.2, 17-H) (Figure C23, Table 17) ;

^{13}C NMR (CD_3OD, 400 MHz) δ: 14.4 (C-20), 18.8 (C-2), 21.7 (C-6), 28.1 (C-18), 35.6 (C-12), 35.9 (C-14), 37.7 (C-3), 38.3 (C-10), 39.6 (C-1), 41.9 (C-7), 42.8 (C-8), 43.3 (C-4), 44.5 (C-13), 50.3 (C-15), 56.5 (C-5), 64.8 (C-11), 64.9 (C-9), 65.9 (C-17), 81.8 (C-16), 180.2 (C-19) (Figure C24, Table 17) ;

HRESIMS m/z: 375.2154 (calcd for $C_{20}H_{32}O_5Na$, 375.2147) (Figure C21 and C22)

3.5 Epoxidation of compound 1

Compound **1** (200 mg, 0.66 mmol) was epoxidised with m-CPBA ($C_7H_5ClO_3$, 120 mg, 0.69 mmol, 1:1 eq) in 6 ml of dichloromethane and the reaction was

performed in a 50 ml round-bottom flask. The reaction mixture was stirred for 5 hours. After 5 hours, the reaction was stopped with water and extracted with EtOAc. The mixture was separated by column chromatography on silica gel and eluted with 10% EtOAc in hexane. The similar fractions were combined and the solvent was removed by rotary evaporation under reduced pressure. Compound **1a** was obtained (40.8 mg) and further purified by re-crystallization with EtOAc and hexane. The structure of compound **1a** was confirmed by NMR spectroscopy. The NMR spectra of compound **1a** were compared with the literature previously described (Sirimongkhon, 2000). The ^{1}H of Compound **1a** is shown in Figure C29.

3.6 Biotransformation of compound 1a by *P. cubensis*

Compound **1a** in ethanol was added at the concentration of 0.2 g/l to the culture using the same procedure as in 3.3 accept the medium was adjusted to pH 5.5. The cultures were allowed to grow in a rotary shaker (150 rpm.) at room temperature for 2 days. After 2 day of incubation, the cultures were extracted with EtOAc and the solvent was removed by rotary evaporation under reduce pressure. The product was determined by TLC using $CHCl_3$: CH_3OH (4:1) as solvent system and visualized by vanillin solution.

3.7 Biotransformation of compound 2 by *P. cubensis*

The biotransformation of compound **2** was performed as described in 3.6 but the incubation time was continued for 9 days.

3.8 Biological evaluation

3.8.1 Antimicrobial activity assay

The microorganisms which were used in antimicrobial activity assay were *Bacillus cereus*, *Staphylococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922 and *Pseudomonas aeruginosa* ATTC 27853. Pure bacterial colonies were inoculated into 5 ml of Mueller-Hinton broth and incubated at 37°C overnight. The turbidity of the bacterial suspension was accomplished by adjusting with Mueller-Hinton broth to equal the turbidity of 0.5 McFarland turbidity standard.

The inoculation procedure was performed on Mueller-Hinton plate. A sterile cotton swab is dipped into the suspension, rotated several times, and pressed

firmly on the inside wall of the tube above the fluid level to removed the excess inoculum from the swab. The swab was then streaked over the entire surface of an agar plate three times with the plate rotated by approximately 60°. The final swab is made around the agar rim. The plate was then left for 3-5 min to allow the excess moisture to be absorbed before the disks were applied. Each disc was prepared by impregnated with 10 µl of 250 µg of the test compound in DMSO. The discs were placed on Mueller-Hinton agar plates seeded with the test cultures and incubated at 37 °C for 16-24 h. After the incubation period, the zones of inhibition were examined.

Controls were carried out using DMSO which was used to dissolve the compounds. Streptomycin and Chloramphenical were used as positive controls for all microorganisms.

3.8.2 Cytotoxicity test

3.8.2.1 Cytotoxicity test on human tumor cell lines

Each compound was tested for cytotoxic activity towards 5 cell lines which contain HEP-G2 (hepatoma), SW620 (colon), Chago (lung), KATO-3 (gastric) and BT474 (breast) *in vitro*. The method was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.

3.8.2.3 Antiproliferation on human erythromyeloblastoid leukemia cell line (K-562)

Each compound was tested against K-562 cells (human promyelocytic leukemia cells). The method was performed by MTS assay. MTS assays were performed according to the manufacturer's instruction described in CellTiter 96® Non-Radioactive Cell Prolifearion Assay Technical Bulletin #TB112 (Promega Corp.; Madison, WI) with only minor modifications (Chuchawankul *et al.*, 2006). Briefly, K562 at 5,000 cell/well were seeded into 96-well plates (Corning Costar; Corning, NY) and exposed to DMSO (0.5%, as the vehicle) or Compound **1**, **2**, **3**, and **4** at 100 ug/ml for 72 h. After treatment, MTS tetrazolium compound (Owen's reagent) 20 µl was added directly to 100 µl culture wells and incubated for 4 h in a 5% CO_2 humidified incubator at 37 °C. The MTS tetrazolium compound was bioreduced by metabolically active cells into a colored formazan product that was

soluble in tissue culture medium. In this assay, the quantity of formazan product formed is directly proportional to the number of viable cells in the cultures. The colored formazan is correlative with the metabolic state of the cells and cell viability (Wiepz et al., 2006). Absorbance was recorded at 490 nm by an ELISA reader (Anthos, model zenyth 340).

Cell lines and culture conditions

K562 was a gift from Professor Dr. Apiwat Mutirangura (Faculty of Medicine, Chulalongkorn University, Thailand). Cells were maintained in RPMI-1640 (GIBCO, Invitrogen, NY) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc.; Logan, UT), 50 units/ml of penicillin, 50 µg/ml of streptomycin and anti-mycotic (GIBCO, Invitrogen, NY) in a humidified atmosphere at 37 °C with 5% CO_2. Cell numbers and cell viability were determined by hemocytometer counting and viability was assessed by trypan blue dye exclusion method (Talwar, 1974).

3.9 Enzyme study

3.9.1 Preparation of cell-free extract

The organism was grown as described previously in 3.4. After cultivation for the indicated time, the mycelia were harvested by vacuum filtration on Whatman No. 1 filter paper and rinsed extensively with 50 mM sodium phosphate buffer pH 7.4. The mycelium was stored frozen at -80 °C until used. All subsequent operations were carried out at 0-4 °C. After washed with sodium phosphate buffer, the mycelium was resuspended in a small volume of 0.1 M Tris-HCl, pH 7.6 and, then, disrupted by homogenizer. The broken mycelium was resuspended in 0.1 M Buffer A (Appendix B2) with a ratio of 1 g cell (wet wt) per 2 ml. buffer. After addition of protease inhibitor ,1 ml of cocktail solution for 100 ml cell-free extract from 20 g cell (wet wt), the suspension was passed through a chilled French pressure cell at 2.5 ton/1" for three times. The suspension was centrifuged at 10,000 x *g* for 30 min at 4 °C. The post mitochondrial (S_{10}) supernatant was further centrifuged at 100,000 x *g* for 60 min at 4 °C to obtain the microsomal (P_{100}) and cytosolic (S_{100}) fraction. The

microsomal fraction (100,000 x *g* pellet) was resuspended in buffer B (Appendix B3). Both fractions, microsomal and cytosolic fraction, were assayed for enzyme activity.

3.9.2 Enzyme activity test

3.9.2.1 Enzyme assay

The oxidation of *ent*-kaurenoic acid using cell-free extract preparations was assayed in the reaction mixture containing 0.15 mM *ent*-kaurenoic acid, 4 μM FAD, and 200 μg protein sample. Each mixture was brought to 800 μl with buffer A. The reaction was initiated by the addition of NADPH to a final concentration of 0.15 mM. After mixing of the contents, change of absorbance at 340 nm was monitored up to 60 min at 30 °C using a spectrophotometer. An extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used to calculate the enzyme activity. One unit of enzyme activity was determined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per min. The activity is calculated as the following equation.

Calculations:

$\Delta A_{340nm} = \varepsilon bc;$

ΔA_{340nm} = A340 (Time 2)-A340(Time 1)/T2-T1

ε = Molar extinction coefficient ($M^{-1}cm^{-1}$)

b = cuvette cell length (cm)

c = absorptivity constant

Molar extinction coefficient of NADPH at 340 nm = 0.00622

Specific activity = Units/μg protein

3.9.2.2 Assay of *ent*-kaurenoic acid activity in subcellular fraction

The *ent*-kaurenoic acid activity was determined by the following method. The incubation mixture contained 2 mg protein of the active fraction, 0.15 mM *ent*-kaurenoic acid, 4 μM FAD, 0.15 mM NADPH, and 0.1 M Tris-HCl, pH 7.6 in a total volume of 2 ml. The reaction was initiated by adding of NADPH and the mixture was incubated aerobically for 60 min at 30 °C. The reaction was terminated by the addition of 2 ml of methanol and the mixture was extracted with 4 ml of chloroform. The organic layer was concentrated and subjected to TLC

using $CHCl_3$: CH_3OH (4:1) as a solvent system and visualized by vanillin solution. Control experiments were performed by 1) heating the sample in boiling water for 10 min before use and, 2) incubating the reaction without substrate.

3.9.3 Constitutive and *ent*-kaurenoic acid-inducible oxygenase activities

The cultures were prepared as described in 3.3 but divided into two experiments. *Ent*-kaurenoic acid was added in one the experiment and another was performed in the absence of *ent*-kaurenoic acid. The cultures were incubated under the same conditions for two days. The presence of constitutive hydroxylating activities or the ability of the mycelia to induce this activity in the presence of *ent*-kaurenoic acid, the activity of cell-free extract was determined using the same procedure as described in 3.9.2.

3.9.4. Localization of the enzyme

The activity was tested as described above on each of the samples derived from 3.9.1, except for the pellet from the 10,000 x *g* centrifugation.

3.9.5 Protein determination

Protein content was determined by the Bradford test (Bollag, Rozycki and Edelstein, 1996), using bovine serum albumin as the standard.

3.9.6 Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was carried out in a separating gel containing 12.5 % (w/v) acrylamide, 0.5 % (w/v) stacking gel. Tris-glycine buffer, pH 8.3 containing 0.15 % (w/v) SDS was used as the electrode buffer. Samples to be analyzed were treated with the sample buffer and boiled at least 5 min before being applied to the gel. The electrophoresis was performed at a constant current of 20 mA per slab gel at room temperature on an electrophoresis unit from cathode towards anode. The low molecular weight calibration kit was used as standard molecular weight marker proteins; Phosphorylase b (97 kDa), Albumin (66 kDa), Carbonic anhydrase (45 kDa), Trypsin inhibitor (20.1 kDa), and α-Lactalbumin (14.4 kDa). After electrophoresis, proteins were stained with Coomassie brilliant blue.

3.9.7 MALDI-MS and protein identification

The peptides from SDS-PAGE of active fraction were selected for identification using a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI/Tof MS). Peptide mass fingerprinting database was searched via the MASCOT program (http://www.matrixscience.com) and scored with Mowse score.

3.10 Instruments and equipments

Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, HSQC, HMBC and NOESY) were obtained on a Varian, Mercury 400 using methanol-d_4 (CD_3OD).

X-ray Diffractrometer

Results for the X-ray diffractrometer were obtained on SIENEN AMART diffractrometer at the Department of Physics, Faculty of Science, Thammasart University.

Fourier Transform-Infrared spectrophotometer (FT-IR)

The IR spectra of all samples were recorded as KBr pellets using Nicolet Impect 410 Spectrophotometer.

Mass spectrometer (MS)

Low resolution mass spectra were obtained with a Agilent 1100 HPLC-system connected to Agilent 1100 mass spectrometer with electrospray ionization in positive mode (ESP+).

Height resolution mass spectra were obtained with Mass Spectrometer LCT, Micromass UK Limited.

Specific Optical Rotation

The optical rotation values were taken on a Perkin-Elmer 341 polarimeter at 20°C.

Melting point Apparatus

Melting points were obtained on a Fisher-John Melting apparatus.

Rotary Evaporator

The Buchi rotary evaporator was used for the removal of volatile solvents.

Autoclave

Sterilization was performed by using the autoclave from Isuzu Seisakusho Co., Ltd.

Incubator

Microorganisma were incubated in the Gallenkamp incubator.

Incubator Shaker

The cultures were incubated and shaken by New Brunswick Scientific Incubator Shaker.

Centrifuge

The refrigerator centrifugation was performed by Bacman Avanti Tm J-25 centrifuge.

Beckman L-80 Ultracentrifuge was used for high-speed centrifugation.

UV spectrometer

The Perkin Elmer UV/VIS spectrometer was used for measurement of NADPH consumption.

Electrophoresis

The electrophoresis was performed using Electrophoresis unit: Model Mini-Protean II cell from Bio-Rad Applied Bio system company, USA.

3.11 Chemicals

3.11.1 Solvents

The solvents for column chromatography and extraction such as chloroform, ethyl acetate, hexane and methanol had used in this research were of commercial grade and were purified prior to use by distillation.

The solvents used for TLC and crystallization were of analytical grade. Chloroform and methanol are from Merck.

The NMR solvent, Methanol D, is from Merck.

3.11.2 Other chemicals

Chemical	Company
TLC aluminum sheet, Art. 5554 silica gel 60 F_{254} (1.05554.0001)	Merck
Silica gel 60 No. 109385.1000 (230-400 mesh ATMS)	Merck
Acetic acid	BDH
Ammonium sulfate $(NH_4)_2SO_4$	BDH
Dimethyl sulfoxide (DMSO)	Merck
Dithiothreital (DTT)	Oxford Glyco System
Ethylenediaminetetraacetate (EDTA)	BDH
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	BDH
Flavin adenine dinucleotide (FAD) disodium salt	Sigma
Glucose	BDH
Glycerol	BDH
Hydrochloric acid (HCL)	BDH
High Molecular Weight Standard Mixture for Gel Electrophoresis	Sigma
Magnesium sulfate ($MgSO_4$)	BDH
Mueller-Hinton broth	Oxiod
Nicotinamine Adinine dinucleotide Phosphate (NADPH) tetrasodium salt	Sigma
Polypeptone	Oxoid
Potassium dihydrogen phosphate (KH_2PO_4)	BDH

Chemical	Company
Potassium Chloride (KCl)	BDH
di-Potassium hydrogen phosphate (K_2HPO_4)	BDH
Protease Inhibitor Cocktail	Sigma
di-Sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)	BDH
Sodium chloride (NaCl)	BDH
Standard molecular weights marker proteins	GE Healthcare Bio
Sucrose	BDH
Sulfuric acid (H_2SO_4)	Merck
2-hydroxymethyl-2-methyl-1-1,3-propanediol (Tris)	BDH
Vanillin	Fluka