CHAPTER 3

Experimental

3.1. General experimental procedures

3.1.1. Chemicals and reagents

All organic solvents were analytical reagent grade: petroleum spirit 35-60ºC was obtained from J.T. Baker (USA). Hexane, ethyl acetate, butanol and acetone were purchased from RCI Labscan (Bangkok, Thailand), ethanol and methanol were obtained from Carlo Erba (France) and dichloromethane was purchased from Fisher Scientific (UK). Sulfuric acid (H_2SO_4), hydrochloric acid (HCl), acetic acid (AcOH) and ammonia solution were purchased from Carlo Erba (France). Folin-Ciocalteu's reagent, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$), sodium hydroxide (NaOH), potassium hydroxide (KOH) and potassium iodide (KI) were purchased from Merck (Darmstadt, Germany). ABTS (2,2'-Azino-bis(3-ethylbenzothaizoline-6-sulfonic acid)) and gallic acid were purchased from Sigma (St. Louis, USA). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), 3,5-dinitrobenzoic acid and cerium(IV) sulfate ($Ce(SO_4)_2$) were obtained from Aldrich (Milwaukee, USA). Potassium persulfate was obtained from UNILAB (AU). DPPH (2,2'-diphenyl-1-picrylhydrazyl), ascorbic acid and lead acetate were obtained from Fluka (Buchs, Switzerland). Quercetin was purchased from Acros organics (New Jersey, USA). Ammonium molybdate tetrahydrate ($(NH_4)_6MoO_{24}.4H_2O$) and bismuth subnitrate were obtained from Sigma-Aldrich (St. Louis, USA).

3.1.2. Chromatography

3.1.2.1. Column Chromatography

Column chromatographic purification of compounds was carried out by flash column chromatography on Merck GF 254 silica gel (70-230 mesh) or RSiL C18 HL chromatography silica gel, diameter 0.015-0.035

mm from Chemie Uetikon (Uetikon, Switzerland). All columns were packed by the slurry method. Most columns were developed using gradient elution of the solvent mixtures which are described in the experimental section.

3.1.2.2. Preparative Thin Layer Chromatography

PTLC was performed with aluminium backed Merck F254 sorbent silica gel. The desired compounds were observed under ultraviolet light at 254 and 365 nm.

3.1.2.3. Thin Layer Chromatography

TLC was performed with aluminium-backed Merck F254 sorbent silica gel. TLC plates were visualized by ultraviolet light or treatment with ceric ammonium molybdate solution (CAM), followed by development with a 1400 Watt heat gun.

3.1.3. Preparation of spraying reagents (89-92)

3.1.3.1. Preparation of Ceric Ammonium Molybdate solution (CAM)

One liter of ceric ammonium molybdate solution (CAM) contained water (900 mL), concentrated H_2SO_4 (100 mL), $(NH_4)_6MoO_{24}$ (50 g) and $Ce(SO_4)_2$ (10 g).

3.1.3.2. Preparation of Dragendorff's reagent

Solution **A**: 0.85 g of bismuth subnitrate in 10 mL of acetic acid and adjusted the volume to 50 mL with distilled water.

Solution **B**: 8 g of KI in 20 mL of distilled water

Dragendorff's reagent was prepared by mixing 1 mL of solution **A** and 1 mL of solution **B** in 20 mL of distilled water and 4 mL of acetic acid. The resulting solution should be kept well and freshly prepared every 1-2 weeks.

3.1.3.3. Preparation of Kedde's reagent

Solution **A**: 3% of 3,5-dinitrobenzoic acid in EtOH

Solution **B**: 2M NaOH

Kedde's reagent was prepared by mixing 5 mL of solution **A** and 5 mL of solution **B**.

3.1.3.4. Preparation of KOH reagent

A solution of 5 % KOH was prepared in EtOH.

3.1.3.5. Preparation of ferric chloride reagent

A solution of 5% $FeCl_3$ was prepared in EtOH.

3.1.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

3.1.4.1. ^{1}H-NMR spectra

All ^{1}H-NMR spectra were measured in $CDCl_3$ (with TMS (δ 0.00) as an internal standard) at 500 MHz using a Varian VNMR PS54-500 or a Varian INOVA-500 magnetic resonance spectrometer. The resonances were assigned as follows: chemical shift in ppm (mulplicity, coupling constants (*J*) in Hz, number of protons, assigned protons). The following abbreviations were used to explain the multiplicities: s (singlet), d (doublet), t (triplet), m (multiplet) and br (broad).

3.1.4.2. ^{13}C-NMR spectra

All ^{13}C-NMR spectra were obtained at 125 MHz using deuterated solvents, $CDCl_3$ ($CDCl_3$ reference at δ 77.16) and used the same NMR spectrophotometers as for the ^{1}H-NMR spectra. The resonances were assigned as follows: Chemical shift in ppm (assigned carbon).

3.2. Source and authentication of the plant materials

Three species of Thai medicinal plants were selected for this study.

The fresh leaves of *Graptophyllum pictum* (L.) Griff. were collected from the Medicinal Plants Garden, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand, in July and December, 2008 and July, 2011 (for extraction) and May, 2009 (for oil isolation).

The fresh leaves of *Solanum spirale* Roxb. were collected from Chiang Kham District, Phayao Province, Thailand, in July, 2009.

The fresh leaves of *Gynura divaricata* (L.) DC. were collected from the Medicinal Plants Garden, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand, in November, 2008 and August, 2011 (for extraction) and July, 2009 (for oil isolation).

All plant materials were identified by Dr. J. F. Maxwell, a botanist of the Herbarium of the Department of Biology, Faculty of Science, Chiang Mai University. All voucher specimens were deposited at the Herbarium of the Department of Biology. The voucher specimen numbers of *G. pictum*, *S. spirale* and *G. divaricata* are N. Jiangseubchatveera 1, S. Keawsa-ard 01 and N. Jiangseubchatveera 2, respectively.

3.3. Extraction of the essential oils

The fresh leaves of *G. pictum* ([**OP1]**; 5.06 kg) and the fresh leaves of *G. divaricata* (**[OP3**]; 1.83 kg) were homogenized and hydrodistilled in a modified Clevenger-type apparatus for 6 h. The distillate was stored at 4 °C until further analysis.

3.4. Analysis of the essential oils

The Gas chromatography (GC-FID) analysis of the essential oils **[OP1]** and **[OP3]** was performed on a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan). Separation was achieved using H_2 carrier gas (1.5 mL/min @ 40 ºC) in a constant total flow mode using a fused silica capillary column: Rxi-5MS (Nonpolar 5% diphenyl 95% dimethylpolysiloxane phase) 30 m x 0.25 mm i.d., 0.25 µm film thickness (Restek, Bellefonte, PA, USA). Injector and detector temperatures were 260 °C and 300 °C, respectively, with an oven temperature programme starting at 40 °C and increasing at 6 °C/min to 290 °C. Programmed-temperature Kovàts retention indices (RI) for each constituent were obtained by GC-FID analysis of an aliquot of the essential oil spiked with an *n*-alkane mixture containing each homologue from *n*-C_7 to *n*-C_{30}.

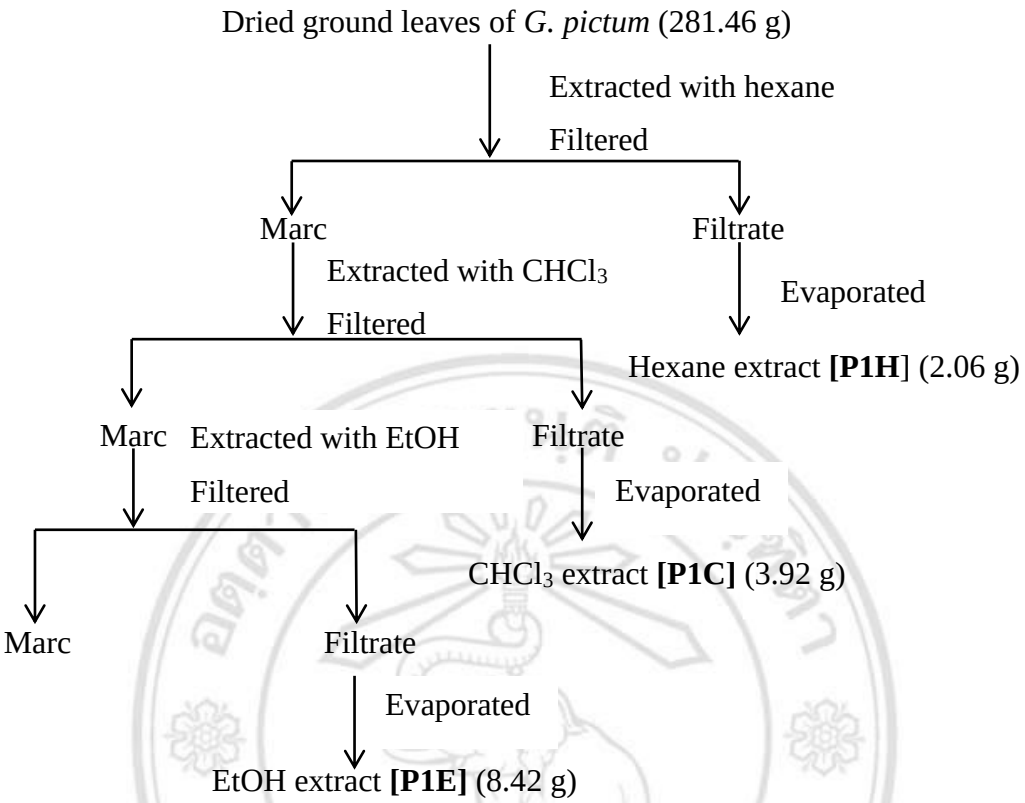
The Gas chromatography-Mass spectrometry (GC-MS) analysis was performed in the electron impact (EI) mode at 70 eV using a Shimadzu QP5050A GC-MS system

(Shimadzu). The column and GC-MS chromatographic conditions were as above for the GC-FID analysis with He used as the carrier gas. All determinations were performed in triplicate. Identification of the essential oil constituents was performed by comparison of their mass spectra with the NIST and NISTREP mass spectra libraries of the GC-MS data system and from a comparison of their RI with those in the literature (93-102).

3.5. Extraction of crude extracts

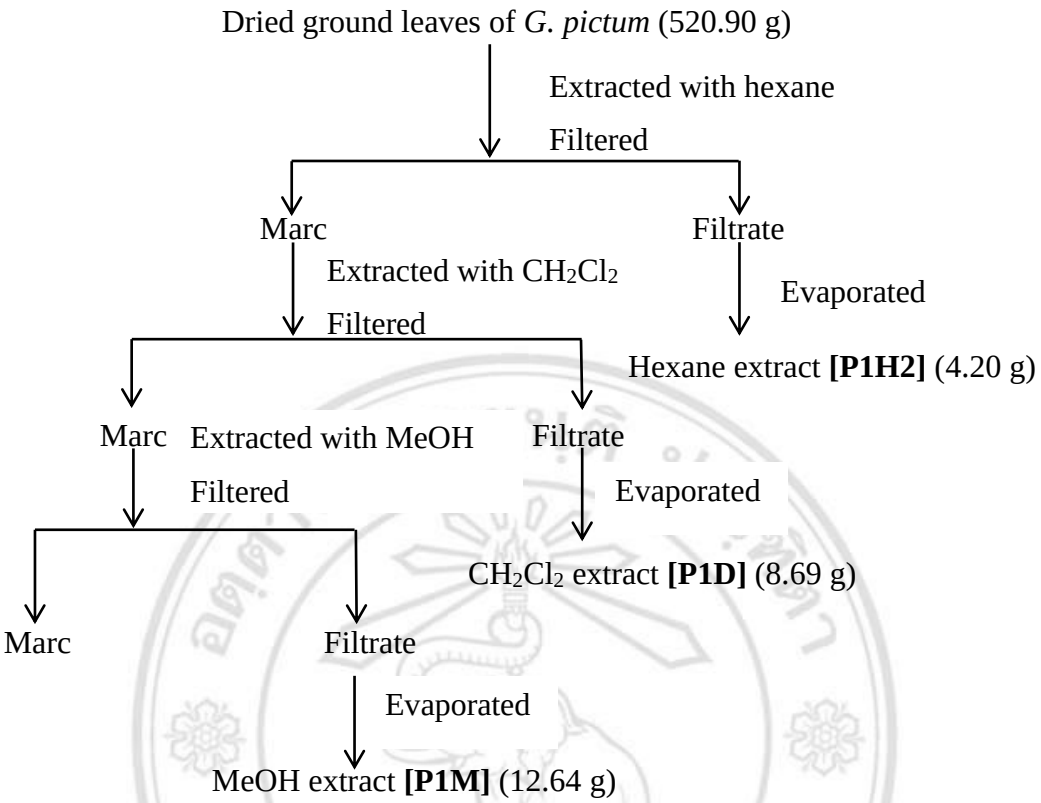
3.5.1. Maceration of the leaves of *G. pictum* [P1]

The leaves of *G. pictum* (July 2008) were washed with distilled water and dried in a hot air oven at 40 ºC for 24 h. Then the dried leaves (281.46 g) were ground and extracted sequentially with 1.2 L of hexane, chloroform ($CHCl_3$) and 95% ethanol (EtOH) at room temperature for 3 days. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the crude extracts as follows: the crude hexane extract (2.06 g), the crude $CHCl_3$ extract (3.92 g) and the crude EtOH extract (8.42 g). All samples were stored at 4 ºC until further analysis. The extraction sequence is shown in Scheme 3.1.



Scheme 3.1 Maceration of *G. pictum* leaves

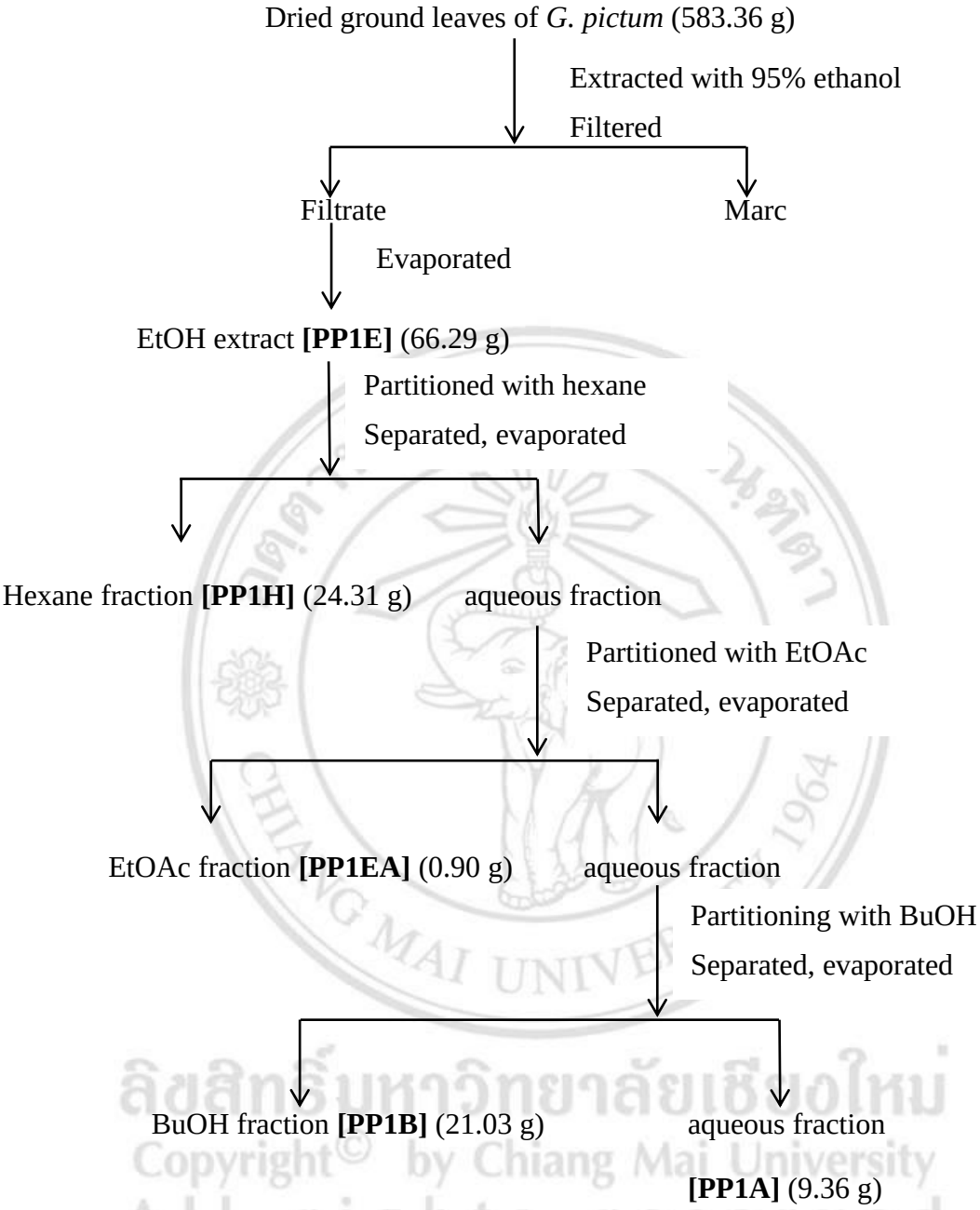
The leaves of *G. pictum* (December 2008) were washed with distilled water and dried in a hot air oven at 40ºC for 24 h. Then the dried leaves (520.90 g) were ground and extracted sequentially with 2 liters of hexane, dichloromethane (CH_2Cl_2) and methanol (MeOH) at room temperature for 3 days. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the crude extracts as follows: the crude hexane extract (4.20 g), the crude CH_2Cl_2 extract (8.69 g) and the crude MeOH extract (12.64 g). All samples were stored at 4ºC until further analysis. The extraction sequence is shown in Scheme 3.2.



Scheme 3.2. Maceration of *G. pictum* leaves

3.5.2. Liquid-liquid extraction of the leaves of *G. pictum* [PP1]

The leaves of *G. pictum* (July 2011) were washed with distilled water and dried in a hot air oven at 40°C for 24 h. Then the dried leaves (583.36 g) were ground and soaked in 95% ethanol (EtOH) (6.0 L) for one week at room temperature with shaking day by day. The extract was filtered and evaporated to dryness under pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the ethanolic extract (66.29 g). Then this extract was partitioned with hexane, ethyl acetate (EtOAc), *n*-butanol (BuOH) (6 x 500 mL of each solvent), respectively. Each fraction and the residue (aqueous fraction) were concentrated to obtain the fractions as follows: the hexane fraction (24.31 g), the EtOAc fraction (0.90 g), the BuOH fraction (21.03 g) and the aqueous fraction (9.36 g). All fractions were kept at 4 °C for further analysis. The extraction sequence is shown in Scheme 3.3.

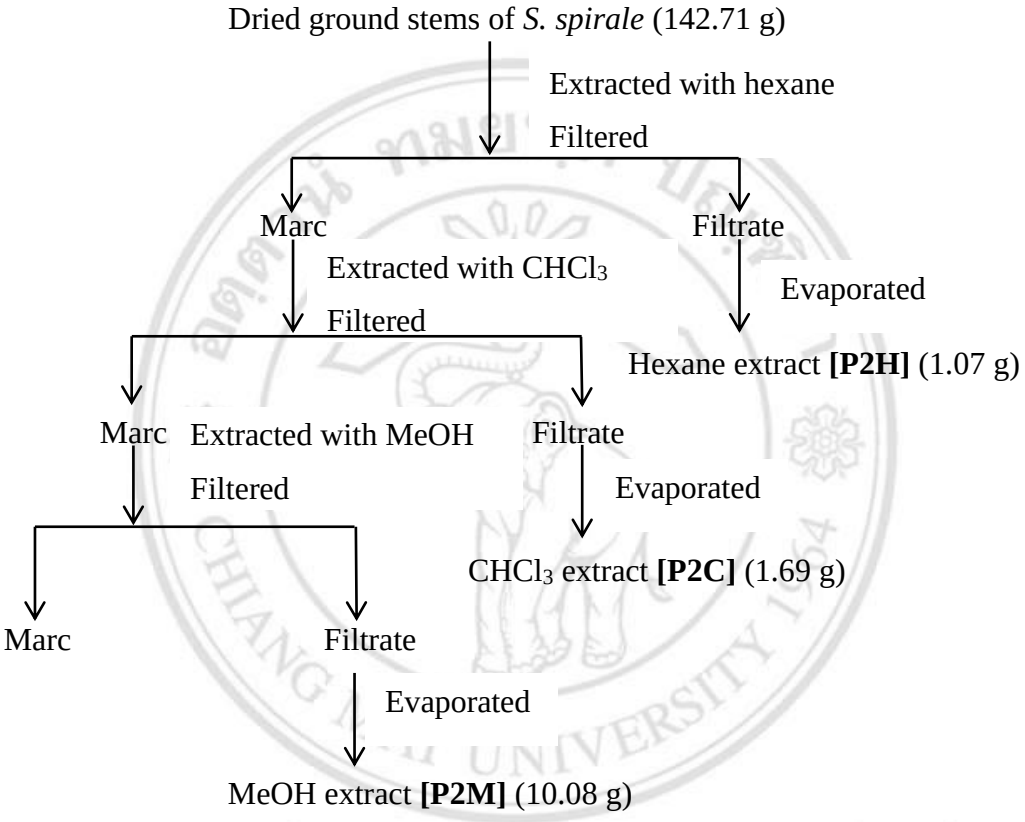


Scheme 3.3. Liquid-liquid extraction of *G. pictum* leaves

3.5.3. Maceration of the stems of *S. spirale* [P2]

The stems of *S. spirale* were washed with distilled water and dried in a hot air oven at 40 ºC for 24 h. Then the dried stems (142.71 g) were ground and extracted sequentially with 3 L of hexane, $CHCl_3$ and MeOH at room temperature for a week. Each extract was filtered and evaporated to dryness under reduced

pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the crude extracts as follows: the crude hexane extract (1.07 g), the crude $CHCl_3$ extract (1.69 g) and the crude MeOH extract (10.08 g). All samples were stored at 4 ºC until further analysis. The extraction sequence is shown in Scheme 3.4.

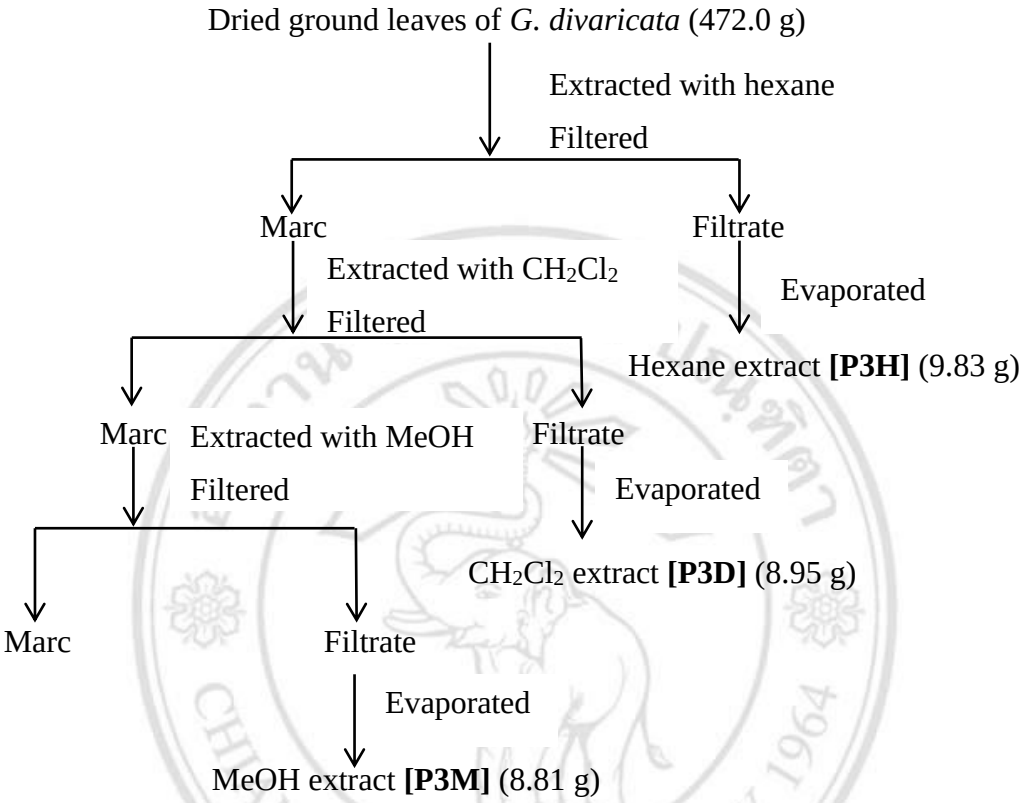


Scheme 3.4. Maceration of *S.spirale* stems

3.5.4. Maceration of the leaves of *G. divaricata* [P3]

The leaves of *G. divaricata* (November 2008) were washed with distilled water and dried in a hot air oven at 40 ºC for 24 h. Then the dried leaves (472.0 g) were ground and extracted sequentially with 2 L of hexane, CH_2Cl_2 and MeOH at room temperature for 3 days. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the crude extracts as follows: the crude hexane extract (9.83 g), the crude CH_2Cl_2 extract (8.95 g) and

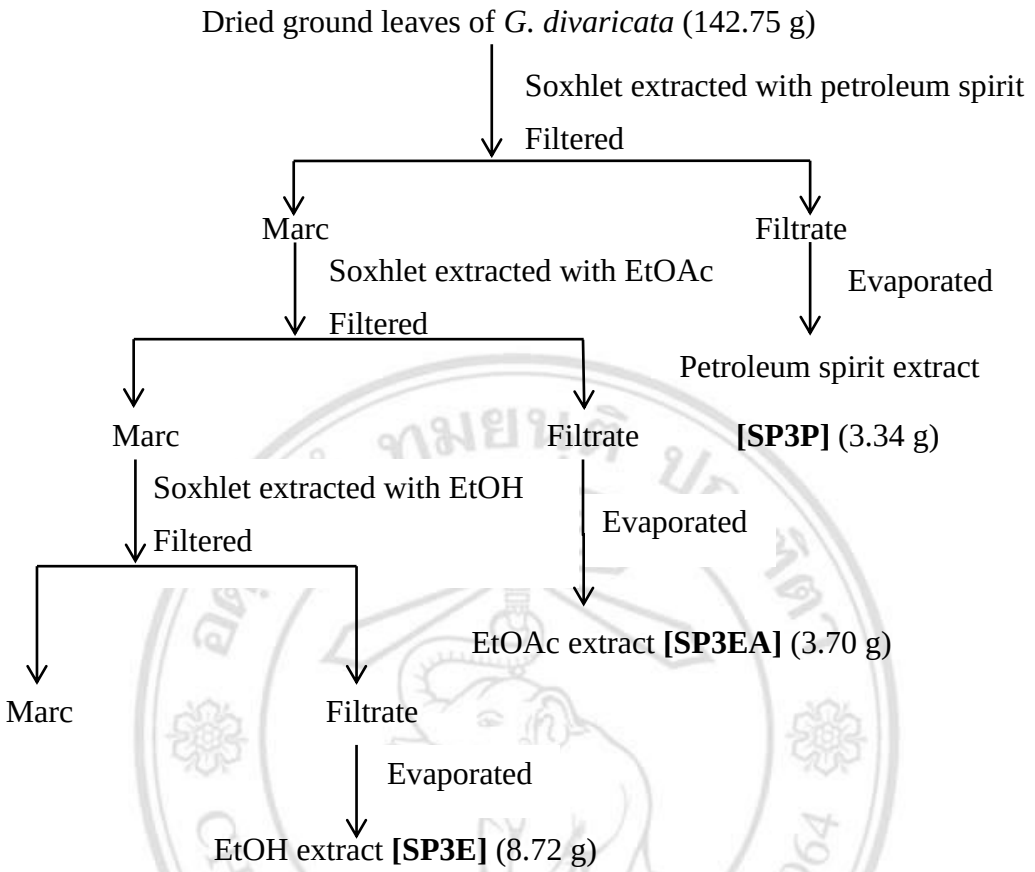
the crude MeOH extract (8.81 g). All samples were stored at 4ºC until further analysis. The extraction sequence is shown in Scheme 3.5.



Scheme 3.5. Maceration of *G. divaricata* leaves

3.5.5. Soxhlet extraction of the *G. divaricata* [SP3]

The leaves of *G. divaricata* (August, 2011) were washed with distilled water and dried in a hot air oven at 40 ºC for 24 h. Then the dried leaves (142.75 g) were ground and sequentially extracted in a Soxhlet extractor using 3 liters of petroleum spirit, ethyl acetate (EtOAc) and 95% Ethanol (EtOH). The extraction time for each solvent was 9 hours. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator (EYELA, rotary vacuum evaporator, Tokyo Rikakikai Co., Ltd., Japan) to obtain the crude extracts as follows: the crude petroleum spirit extract (3.34 g), the crude EtOAc extract (3.70 g) and the crude EtOH extract (8.72 g). All samples were stored at 4ºC until further analysis. The extraction sequence is shown in Scheme 3.6.



Scheme 3.6. Soxhlet extraction of *G. divaricata* leaves

3.6. Determination of Cytotoxic Activity

The cytotoxicities of the essential oils of *G. pictum* **[OP1]** and *G. divaricata* **[OP3]**, the crude extracts and the fractions of *G. pictum* (**[P1]** and **[PP1]**), and the crude extracts of *G. divaricata* (**[P3]** and **[SP3]**) were determined against the KB (epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF-7 (breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (small cell lung carcinoma, ATCC CRL-5804) cancerous human-cell lines using the Resazurin microplate assay (REMA), using the method described by O'Brien *et al*. (103). The extracts were first diluted to 50 µg/mL in 0.5% DMSO and then subjected to a doubling series of dilutions. Cells at a logarithmic growth phase were harvested and diluted to $7x10^4$ cells/mL for KB and $9x10^4$ cells/mL for MCF-7 and NCI-H187 in fresh medium. Successively, 5 µL of each sample solution and 45 µL of cell suspension were added to 384-well plates, incubated at 37 ºC in 5% CO_2 in an incubator. After the incubation period (3 days for KB and MCF-7, and 5 days

for NCI-H187), 12.5 µL of resazurin solution (62.5 µg/mL) was added to each well, and the plates were then incubated at 37 ºC for 4 h. The fluorescence was measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. The percentage inhibition of cell growth (%) was calculated using the following equation:

$$[1– (FU_T/FU_C)] \times 100$$

Where FU_T and FU_C were the mean fluorescent units from treated and untreated conditions, respectively. The inhibition of cell growth by 50% (IC_{50}) values were derived from the dose response curves using the SOFTMax Pro software (Molecular Devices). Triplicate determinations were performed. Ellipticine, doxorubicin and tamoxifen were used as positive controls and 0.5% DMSO was used as a negative control.

3.7. Determination of Cytotoxicity Assay against Vero cell

The cytotoxicities of the essential oils of *G. pictum* **[OP1]** and *G. divaricata* **[OP3]**, the crude extracts and the fractions of *G. pictum* (**[P1]** and **[PP1]**), and the crude extracts of *G. divaricata* (**[P3]** and **[SP3]**) were determined against Vero cells (African green monkey kidney, ATCC CCL-81) using the Green Fluorescent Protein (GFP) detection method (104). The assay was carried out by adding 45 µL of cell suspension at $3.3x10^4$ cells/mL to each well of 384-well plates containing 5 µL of test compounds, which previously diluted in 0.5% DMSO, and then incubating for 4 days in an incubator at 37°C with 5% CO_2. The fluorescence was measured using a SpectraMax M5 microplate reader in the bottom-reading mode at the excitation and emission wavelengths of 485 and 535 nm. Triplicate determinations were performed. The percentage of cytotoxicity and the IC_{50} value of each sample were calculated. Ellipticine was used as a positive control and 0.5% DMSO was used as a negative control.

3.8. Antimycobacterial activity

Antimycobacterial activities of the essential oils of *G. pictum* **[OP1]** and *G. divaricata* **[OP3]** and the CH_2Cl_2 and the MeOH crude extracts of *G. pictum* (**[P1D]** and **[P1M]**) and *G. divaricata* (**[P3D]** and **[P3M]**) were determined against *Mycobacterium*

tuberculosis $H_{37}Ra$ with the Green Fluorescent Protein (GFP)-based fluorescent detection (105-106). $H_{37}Ra$ *gfp* was cultivated on 7H10 agar containing 30 μg/mL kanamycin at 37 ºC for 4 weeks or until the growth was observed. Starter cultures were prepared by fully looping 2-3 single colony into 7H9 broth supplemented with 0.2% v/v glycerol, 0.1% w/v casitone, 0.05% v/v Tween 80, 10% v/v Middlebrook OADC enrichment solution (BD Biosciences, USA) and 30 μg/mL of kanamycin. The mixture was then incubated at 37 ºC in a 200 rpm shaker incubator until the optical density (OD) at 550 nm was between 0.5 and 1. For batch cultivation, the starter cultures were transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37 ºC in a 200 rpm shaker incubator until the OD550 nm was approximately 0.5 to 1. The cells were pelleted, washed and suspended in PBS buffer, and then sonicated 8 times for 15 seconds each. The sonicated samples were then aliquoted and frozen at –80 ºC for up to 2 to 3 months prior to use. Titer stocks were determined by the colony forming unit (cfu) assay and the seeding density for the anti-TB assay was optimized by serial dilutions. The dilution that grew at logarithmic phase on day 7 was used as an optimal bacterial seeding density. For assay in 384-well formats, the seeding was approximately $2x10^4$ to $1x10^4$ cfu/mL/well. Each well contained 5μL of test samples serially diluted in 5% dimethyl sulfoxide, followed by 45 μL of cell suspension prepared as described above. Plates were incubated at 37 ºC for 7 days. The fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices) in the bottom-reading mode at the excitation and emission wavelengths of 485 nm and 535 nm. Triplicate determinations were performed. The percentage inhibition of cell growth (%) was calculated using the following equation:

$$[1- (FU_T/FU_C)] \times 100$$

Where FU_T and FU_C were the mean fluorescent units from treated and untreated conditions, respectively. The lowest drug concentration that inhibited cell growth by 90% was reported as the Minimum Inhibitory Concentration (MIC). Rifampicin, streptomycin, isoniazid and ofloxacin were used as positive controls, and 0.5% DMSO was used as a negative control.

3.9. Anti-herpes simplex virus type-1 activity

Anti-herpes simplex virus type-1 activities of the essential oils of *G. pictum* **[OP1]**, the crude extracts and the fractions of *G. pictum* (**[P1]** and **[PP1]**), and the crude extracts of *G. divaricata* (**[P3]** and **[SP3]**) were determined against HSV-1 (ATCC VR260) with Green fluorescent protein (GFP) detection (104). All compounds were tested in the range of non-cytotoxic concentrations to the host cells, before testing the anti-viral assay. Ten µL of sample solutions, which were dissolved in 10% DMSO, were added to 96-well plates followed by 190 µL of infected host cells (GFP-expressing Vero cell suspension at $1x10^5$ cells/mL was premixed with $5x10^5$ PFU/mL of HSV-1 inoculums). The plates were incubated in a 37 °C humidified incubator with 5% CO_2 for 4 days. The fluorescence signals were measured on day zero and day 4 using SpextraMax M5 multi-detection microplate reader (Molecular Devices, USA) in the bottom-reading mode with excitation and emission wavelengths of 485 and 535 nm. The percentage of viral inhibition (%) was calculated using the following equation:

$$\% \text{ viral inhibition} = \{1-[(FU_C-FU_{VT}) / (FU_C-FU_{VC})]\} \times 100$$

Where FU_C, FU_{VT} and FU_{VC} were average fluorescence units of non-infected cells, virus-infected cells treated with drug, and untreated, respectively. The IC_{50} values were derived from dose-response curves by the SOFTMax Pro software (Molecular devices). Acyclovir was used as a positive control and 0.5% DMSO was used as a negative control.

3.10. Antimicrobial Activity

The antibacterial activities of the essential oils of *G. pictum* **[OP1]** and *G. divaricata* **[OP3]** and the fractions of *G. pictum* **[PP1]** were determined using the disc diffusion assay (107) against three bacteria (*Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922), which were obtained from the Central Diagnostic Laboratory, Faculty of Medicine, CMU). Each bacterial suspension which has been adjusted to 0.5 McFarland was uniformly spread using a cotton swab on a nutrient agar Petri dish. Five sterile paper discs (5 mm filter paper disc, Whatmann no. 1) were placed on the surface of each agar plate and

were impregnated with 10 µL of the diluted concentration of 9.40, 7.05, 4.70, 3.525, 2.35, 1.7625 and 1.175 mg/mL **[OP1]**, 100.0, 70.0, 50.0, 30.0, 10.0, 5.0 and 2.5 mg/mL **[OP3]**, 25.0, 12.5, 6.25, 3.125 and 1.5625 mg/mL **[PP1H],** 18.0, 9.0, 4.5, 2.25 and 1.125 mg/mL **[PP1EA],** 24.0, 12.0, 6.0, 3.0 and 1.5 mg/mL **[PP1B]** and 18.4, 9.2, 4.6, 2.3 and 1.15 mg/mL **[PP1A]**. Plates were incubated for 24 h at 36±0.1 °C under appropriate cultivation conditions. Antibacterial activity as MIC was determined as the lowest concentration of the sample which inhibits the growth of bacteria. A disc impregnated with ethanol served as a negative control and discs with vancomycin 30 µg, amikacin 30 µg (Oxoid, UK) and norfloxacin 5 µg (Sigma-Aldrich, US) served as positive controls. Tests were performed in triplicate.

The antimicrobial activities of the crude extracts of *G. pictum* **[P1]** and *G. divaricata* **[P3]** were determined using the agar diffusion assay (108). The samples were tested against the three aforementioned bacterial strains *and Aspergillus flavus*, *Candida albican* and *Trichophyton mentagrophyte*. All microorganisms were obtained from the Central Diagnostic Laboratory, CMU. The microorganisms were maintained in agar conservation at room temperature. The cell suspensions were adjusted to 0.5 McFarland standard turbidity (10^{7-8} CFU/mL) for bacteria and 1.0 McFarland standard turbidity (10^{15} CFU/mL) for fungi. Every cell suspension was spread over plates containing Nutrient Agar (NA) for bacteria and Potato Dextrose Agar (PDA) for fungi. In each of these plates, 4 wells (9 mm) were cut out using sterilized cork borer. The extracts were diluted with ethanol and screened for antibacterial activity using concentrations of 20 mg/mL. One hundred microtiter sample solution was carefully transferred to the wells and the same volume of ethanol was used as a negative control. The samples were incubated at 37 ºC for 24 h for bacterial pathogens and 3 days for fungal pathogens. After incubation, the diameter of the inhibition zone was measured. A positive control was also assayed to check the sensitivity of the tested organisms using gentamicin (75 µg/mL) and ketoconazole (250 µg/mL). Experiments were performed in triplicate.

3.11. Antioxidant activity

3.11.1. ABTS radical cation scavenging assay

The antioxidant activities of the essential oils of *G. pictum* **[OP1]** and *G. divaricata* **[OP3]** were investigated using the ABTS radical cation scavenging assay, which was carried out according to the procedure described by Re *et al*. (109) with some modifications. ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting 5 mL of ABTS stock solution (7 mM) with 2.5 mL of potassium persulfate (2.4 mM) in darkness for 16 h at room temperature. Prior to assay, the freshly prepared $ABTS^{\bullet+}$ solution was diluted with deionized water to obtain the absorbance at 734 nm of 0.700 ± 0.020 determined using a Jasco 7800 UV-Visible spectrophotometer (USA). The essential oils were prepared in EtOH; **[OP1]** (10.6 mg/mL) and **[OP3]** (50 mg/mL). For the assay, 20 µL of the essential oil was mixed with 2.0 mL of diluted $ABTS^{\bullet+}$ solution and incubated at room temperature for 5 min. The decrease in absorbance at 734 nm was measured. Trolox and ascorbic acid (concentration range; 0.13-0.63 mg/mL of trolox and 0.09-0.44 mg/mL of ascorbic acid solutions) were used as the standards. Triplicate determinations were performed. The appropriate solvent blank was run in each assay. The percentage inhibition of free radical by $ABTS^{\bullet+}$ (I%) was calculated using the following equation:

$$[(A_{blank}-A_{sample})/A_{blank}] \times 100$$

Where A_{blank} and A_{sample} are the absorbance of the control reaction (containing all reagents except the test compound) and the absorbance of the test compound. The standard curves were plotted between the percentage inhibitions (I%) and concentrations of trolox and ascorbic acid. The ABTS of samples were determined from standard curves and expressed as milligram of trolox equivalent antioxidant capacity (TEAC) per gram of the extract and milligram of ascorbic acid equivalent capacity (VCEC) per gram of the extract, respectively.

The antioxidant activity of the fractions of *G. pictum* **[PP1]** was measured using the ABTS radical scavenging assay using the above method. The

freshly prepared ABTS•$^{+}$ solution was diluted with deionized water to obtain the absorbance at 734 nm of 0.700 ± 0.050 determined using a spectrophotometer (Shimadzu UV-2450, Japan). Each fraction was prepared in EtOH; **[PP1H]** (25.0 mg/mL), **[PP1EA]** (9.0 mg/mL), **[PP1B]** (6.0 mg/mL) and **[PP1A]** (4.0 mg/mL). Trolox and ascorbic acid were used as reference standards in the concentration range 0.01-0.5 mg/mL.

3.11.2. DPPH radical scavenging assay

The free radical scavenging activity of the crude extracts of *G. pictum* **[P1]** and *G. divaricata* **[P3]** was determined according to the DPPH method (110-111) with some modifications. All crude extracts were prepared in the concentration of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 10.0 mg/mL except the hexane extract of *G. pictum* **[P1H]** (15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 and 50.0 mg/mL) using EtOH as a solvent, Twenty microliters of each sample was added to 180 µL of DPPH in EtOH (0.0066 % w/v or 10 µM) in a 96-well microtiter plate. After incubation for 30 min in the dark, the absorbance of each well was measured at 520 nm spectrophotometrically (spectrophotometer: Multimode detector, Beckman Coulter DTX 880, USA). The DPPH solution was used as a negative control. Trolox and ascorbic acid were used as reference standards in the concentration range 0.01-0.3 mg/mL. Triplicate determinations were performed. The percentage of DPPH scavenging activity was using the following equation:

$$[(A_c-A_s)/ A_c] \times 100$$

Where A_c is the absorbance of the control and A_s is the absorbance of the sample. The IC_{50} values denote the concentration of the sample which is required to scavenge 50% of DPPH free radicals.

The antioxidant activity of the fractions of *G. pictum* **[PP1]** was determined using the same DPPH method which was described above. All fractions were prepared in EtOH at various concentrations; **[PP1H]** (1.0, 3.0, 5.0, 7.0, 10.0, 15.0, 20.0 and 25.0 mg/mL), **[PP1EA]** (0.09, 0.18, 0.36, 0.54, 0.72, 0.90, 1.80 and 2.70 mg/mL), **[PP1B]** (0.6, 1.2, 1.8, 2.4, 3.0, 3.6, 4.2 and 6.0

mg/mL) and **[PP1A]** (3.68, 5.52, 7.36, 9.20, 11.04, 12.88, 14.72 and 18.40 mg/mL).

3.12. Total phenolic contents determination

Total phenolic content of all fractions of *G. pictum* **[PP1]** was determined using the Folin-Ciocalteu assay (112). Different concentrations of the fractions in MeOH were prepared; **[PP1H]** (1.2 mg/mL), **[PP1EA]** (0.5 mg/mL), **[PP1B]** (0.9 mg/mL) and **[PP1A]** (4.1 mg/mL). Gallic acid (0.01 - 0.07 mg/mL) was prepared in MeOH and used as a standard. Sample (300 µL) was introduced into a test tube followed by 1.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 1.2 mL of 7.5% w/v Na_2CO_3. The mixture was allowed to stand in the dark for 30 min at room temperature. The absorbance at 765 nm was measured by a spectrophotometer (Shimadzu UV-2450, Japan). Triplicate determinations were performed. The results were expressed as milligram gallic acid equivalents (mg GAE) per gram of the extract by comparison with the standard curve of gallic acid.

3.13. Total flavonoid content determination

The total flavonoid content of the fractions of *G. pictum* **[PP1]** was determined according to the method of Ramamoorthy *et.al* with some modification (113). Quercetin (0.001 - 0.07 mg/mL) was prepared in MeOH for standard solutions. One milliliter of 2% $AlCl_3$ in MeOH was mixed with 1 mL of sample (1 mg/mL). The mixture was incubated for 10 min at room temperature. The absorbance was measured using a spectrophotometer (Shimadzu UV-2450, Japan) at 415 nm against a blank sample consisting of 1 mL of sample solution with 1 mL of MeOH without $AlCl_3$. Triplicate determinations were performed. The calibration line was plotted between the absorbance and the concentration of quercetin. The content of flavonoids was expressed in terms of quercetin equivalent (mg QE) per gram of the extract.

3.14. Preliminary phytochemical screening of *G. pictum*

The preliminary phytochemical screening for the secondary metabolites in different fractions was performed using standard procedures (90-92).

3.14.1. Flavonoids

(a) Shinoda's test

One milliliter of 80% ethanolic extract was added a small piece of magnesium followed by 8-9 drops of concentrated hydrochloric acid. The formation of a pink, reddish or brown color indicated the presence of flavonoids.

3.14.2. Steroids

(a) Salkowski reaction

To 1 mL of $CHCl_3$ extract was slowly added a few drops of sulfuric acid by the sides of a 5-mL test tube. The formation of a red color indicated the presence of steroids.

3.14.3. Tannins

A few drops of 5% w/v ferric chloride solution was added to 1-2 mL of the 80% ethanolic extract. A greenish color indicated the presence of gallotannins, while a brown color indicated the presence of pseudotannins.

3.14.4. Resins

One milliliter of ethanolic extract was dissolved in acetone and the solution was poured into distilled water. A turbidity indicated the presence of resins.

3.14.5. Coumarins

(a) Dry sample (0.2 g) was transferred into a 5-mL test tube. The test tube was sealed with 20% NaOH coated filtered paper, then heated on a water bath at 70 °C for 5 min. The filtered paper was observed under long wavelength UV at 365 nm. A greenish blue spot indicated the presence of volatile coumarins.

(b) To the same test tube, 0.5-1.0 mL of 35% EtOH was added and heated on a water bath for 15 min. The clear solution was dropped onto a 20% NaOH coated filtered paper. A greenish blue spot under UV-365 nm indicated the presence of non-volatile coumarins.

3.14.6. Saponins

One gram of dry sample was extracted with 5 mL of distilled water, heated on a water bath for 5 min and filtered. The extract was divided into 2 test tubes, (a) The test tube was shaken vigorously. (b) One milliliter of 10% H_2SO_4 was added and heated on a water bath for 5 min, cooled and then shaken vigorously. The formation of a honeycomb like froth which stayed for 10 min, indicated the presence of saponins.

3.14.7. Anthraquinones

Dry sample (0.5 g) was extracted with 5 mL of methanol, heated on a water bath for 10-15 min, filtered and concentrated to 1-2 mL. The extract (20 µL) was applied to a TLC plate (3.3x10 cm) and using EtOAc: MeOH: H_2O (80:10:8 v/v) as the mobile phase. The chromatogram was sprayed with KOH reagent and viewed under long wavelength UV (365 nm). A yellow/ orange/ red spot indicated the presence of anthraquinones.

3.14.8. Cardiac glycosides

Dry sample (0.5 g) was extracted with 5 mL of 50% MeOH and 10% lead acetate, heated on a water bath for 10-15 min, filtered and then extracted with 2.5 mL of $CHCl_3$ (2-3 times) and concentrated to 1-2 mL. The extract (20 µL) was applied to a TLC plate (3.3x10 cm) and using EtOAc: MeOH: H_2O 80:10:8 as a mobile phase. After spraying the chromatogram with Kedde's reagent, a pink/ yellow/ violet spot indicated the presence of glycosides and digoxin.

3.14.9. Phenolics

Dry sample (1 g) was extracted with 10 mL of dichloromethane, heated on a water bath, filtered and concentrated to 1 mL. The extract (25 µL) was applied to a TLC plate (3.3x10 cm) and using CH_2Cl_2: EtOAc: MeOH 90:5:5 as the mobile phase. The chromatogram was sprayed with $FeCl_3$ reagent. A blackish blue/ blackish green spot indicated the presence of phenolics.

3.14.10. Sugars

Dry sample (1 g) was extracted with 10 mL of distilled water, heated on a water bath for 10-15 min, filtered and concentrated to 1-2 mL. The extract (10 µL) was applied to a TLC plate (3.3x10 cm) and using MeOH: H_2O 60:40 as the mobile phase. The chromatogram was sprayed with 10% H_2SO_4 reagent and heated at 100 °C for 5-10 min. A blackish gray/ blackish blue spot indicated the presence of sugars.

3.14.11. Alkaloids

Dry sample (1 g) was extracted with 80% EtOH, heated on a water bath for 30-60 min, then 1 mL of 28% ammonia solution and 1 mL of $CHCl_3$ were added, shaken and the $CHCl_3$ layer was separated and then concentrated to 1-2 mL. The extract (40 µL) was applied to a TLC plate (3.3x10 cm) and using EtOAc: hexane 60:40 as the mobile phase. The chromatogram was sprayed with Dragendorff's reagent. Orange spot on a yellow background indicated the presence of alkaloids.

3.15. Analysis of the hexane fraction of *G. pictum* [PP2H] and the $CHCl_3$ extract of *S. spirale* [P2C]

Due to the anticancer activity of the hexane fraction of *G. pictum* **[PP2H]** and the moderately anti-herpes simplex virus type-1 activity of the $CHCl_3$ extract of *S. spirale* which was report by Keawsa-ard (114), the fractions **PP2H** and **P2C** were selected for GC-MS analysis.

The Gas chromatography-Mass spectrometry (GC-MS) analysis was performed on an Agilent HP 6890 (Agilent, CA, USA) coupled with a Hewlett-Packard 5973 mass selective detector fitted with a fused silica capillary column: AT-5MS (Altech 15807) 30 m x 0.25 mm i.d., 0.25 µm film thickness (Alltech Associates, Inc., Deerfield, IL, USA). Helium (He) was used as the carrier gas at a flow rate of 1.0 mL/min. Injector and detector temperatures were 260 °C and 325 °C, respectively, with an oven temperature programme starting at 40 °C and increasing at 6 °C/min to 290 °C. Significant quadrupole MS operating parameters: interface temperature 280 °C; mass

quadrupole temperature 150 °C; mass source temperature 230 °C; electron impact ionization (EI) at 70 eV with scan mass range of 29-550 *m/*z.

Programmed-temperature Kovàts retention indices (RI) for each constituent were obtained by GC analysis of an aliquot of the sample spiked with an *n*-alkane mixture containing each homologue from *n*-C_7 to *n*-C_{30}. The identification of the each constituent was performed by comparison of their mass spectra with the Wiley7n.l and W8N05ST.L mass spectra libraries of the GC-MS data system and from a comparison of their RI with those in the literature (55,115-122).

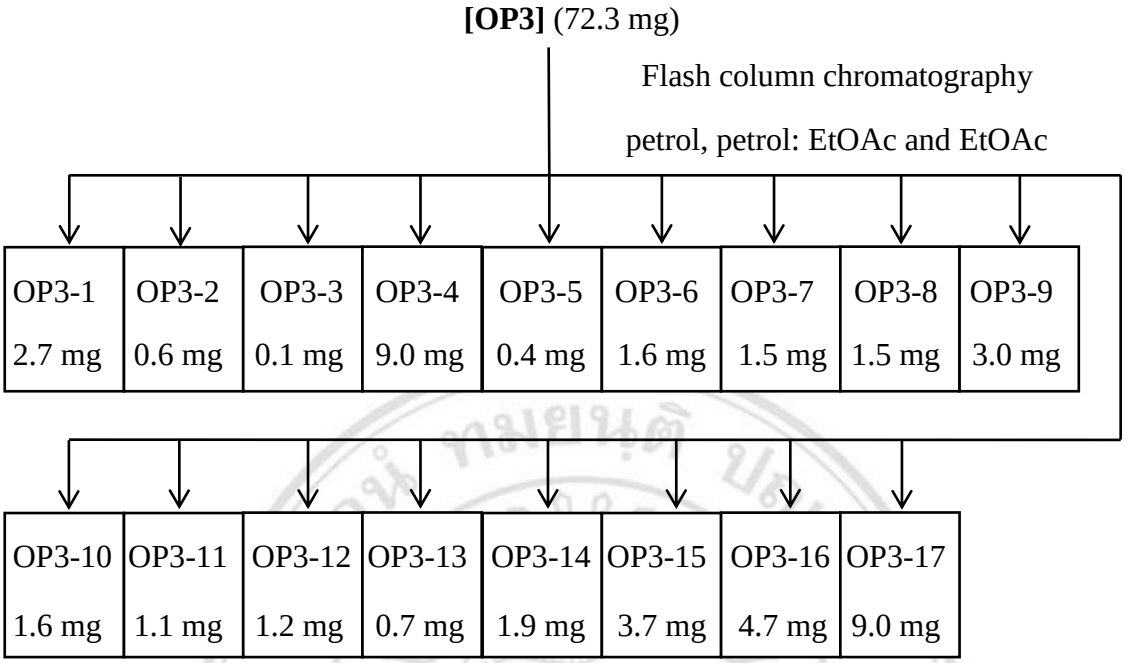
3.16. Isolation and purification of the essential oil of *G. divaricata* [OP3]

The essential oil of the leaves of *G. divaricata* **[OP3]** showed moderate activity against KB (oral cavity cancer), MCF-7 (breast cancer) and NCI-H187 (small cell lung cancer) cell lines with IC_{50} values of 5.79, 47.44, and 17.65 µg/mL, respectively and also had a MIC 50 µg/mL against *Mycobacterium tuberculosis* H_{37}Ra. Therefore, this oil was selected for further purification. The essential oil (72.3 mg) was subjected to normal phase flash column chromatography over silica gel (diameter 1 cm, height 14 cm), using a gradient of petroleum spirit (petrol) and ethyl acetate (EtOAc) as eluent. One hundred and twenty fractions were collected (10 mL/fraction). The constituents of each fraction were examined on silica gel TLC plates with petrol /EtOAc (9:1) as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation and flushing with dry nitrogen gas. Groups of fractions are shown in Table 3.1.

Table 3.1 Groups of fractions and eluent conditions of the isolation and purification of **[OP3]**

Groups	Fractions	Eluent (petrol:EtOAc)
OP3-1	1-10	100:0
	11-20	99:1
	21-24	98:2
OP3-2	25-26	98:2
OP3-3	27-28	98:2
OP3-4	29-44	98:2
OP3-5	45	98:2
OP3-6	46-52	98:2
OP3-7	53-58	98:2
OP3-8	59-60	98:2
	61-67	97:3
OP3-9	68-70	97:3
	71-80	95:5
	81-84	90:10
OP3-10	85-90	90:10
OP3-11	91-93	80:20
OP3-12	94-95	80:20
OP3-13	96-97	80:20
OP3-14	98-100	80:20
	101	50:50
OP3-15	102-103	50:50
OP3-16	104-106	50:50
OP3-17	107-110	50:50
	111-120	0:100

All groups were identified using NMR spectroscopy. Further **[OP3-4]** and **[OP3-6]** showed clean ^{1}H-NMR spectra which were selected for further identification using GC-MS and their cytotoxic activities were determined using KB, MCF-7 and NCI-H187 cell lines. Their antimycobacterial activities were also tested. A summary of the isolation and purification procedure of **[OP3]** is shown in Scheme 3.7.



Scheme 3.7. A summary of the isolation and purification procedure of the essential oil of the leaves of *G. divaricata* **[OP3]**

3.17. Isolation and purification of the fractions of *G. pictum* [PP1]

The hexane fraction **[PP1H]**, the EtOAc fraction **[PP1EA]** and the aqueous fraction **[PP1A]** from the liquid-liquid extraction of the leaves of *G. pictum* **[PP1]** showed anticancer activity against MCF-7 (breast cancer cell line) with the IC_{50} values of 38.66, 26.01 and 20.41 μg/mL. Therefore, these fractions were selected for further isolation and purification.

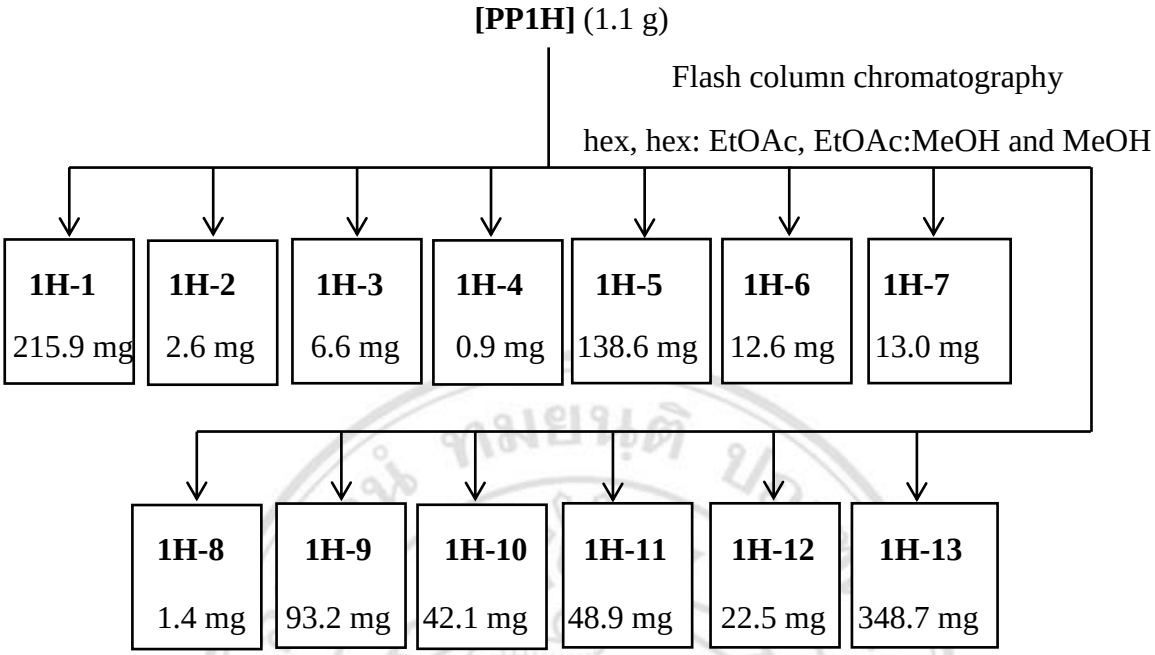
3.17.1. Isolation and purification of the hexane fraction of *G. pictum* [PP1H]

The hexane fraction of *G. pictum* (**[PP1H]**, 1.1 g) was subjected to normal phase flash column chromatography over silica gel (diameter 3 cm, height 15 cm), using a gradient of hexane, EtOAc and MeOH (each, 50 mL) as eluent. The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (4:1) as the eluent. Eighteen fractions were collected (50 mL/fraction). The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.2.

Table 3.2 Groups of fractions and eluent conditions of the isolation and purification of **[PP1H]**

Groups	Fractions	Eluent
		hexane:EtOAc
1H-1	1	100:0
1H-2	2	100:0
1H-3	3	90:10
1H-4	4	90:10
1H-5	5	90:10
1H-6	6	80:20
1H-7	7	80:20
1H-8	8	60:40
1H-9	9	50:50
1H-10	10	40:60
1H-11	11-12	20:80
1H-12	13-14	0:100
		EtOAc: MeOH
	15	50:50
1H-13	16-17	50:50
	18	0:100

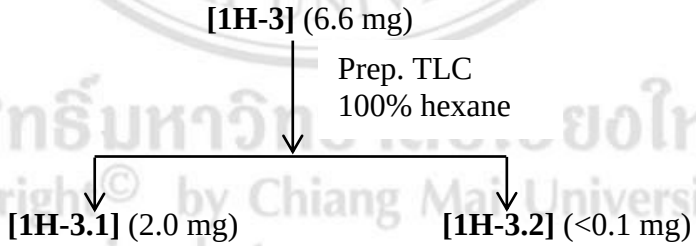
Fraction **[1H-3]** showed one major compound, while fraction **[1H-9]** exhibited crystals mixed with a green paste. Therefore, fractions **[1H-3]** and **[1H-9]** were selected for further purification. A summary of the isolation and purification procedure for this study of **[PP1H]** is shown in Scheme 3.8.



Scheme 3.8. A summary of the isolation and purification procedure of the hexane fraction of *G. pictum* **[PP1H]** of the leaves of *G. pictum* **[PP1]**

3.17.1.1. Isolation and purification of [1H-3]

The fraction **1H-3** (6.6 mg) was purified using preparative TLC on aluminium backed Merck F254 sorbent silica gel to yield **1H-3.1** and **1H-3.2**. This fractionation process is shown in Scheme 3.9.



Scheme 3.9. The isolation and purification process of **[1H-3]**

The ^{1}H-NMR spectrum of **[1H-3.1]** detected as a long chain fatty acid. The GC-MS was used to identify and confirm this compound.

3.17.1.2. Isolation and purification of [1H-9]

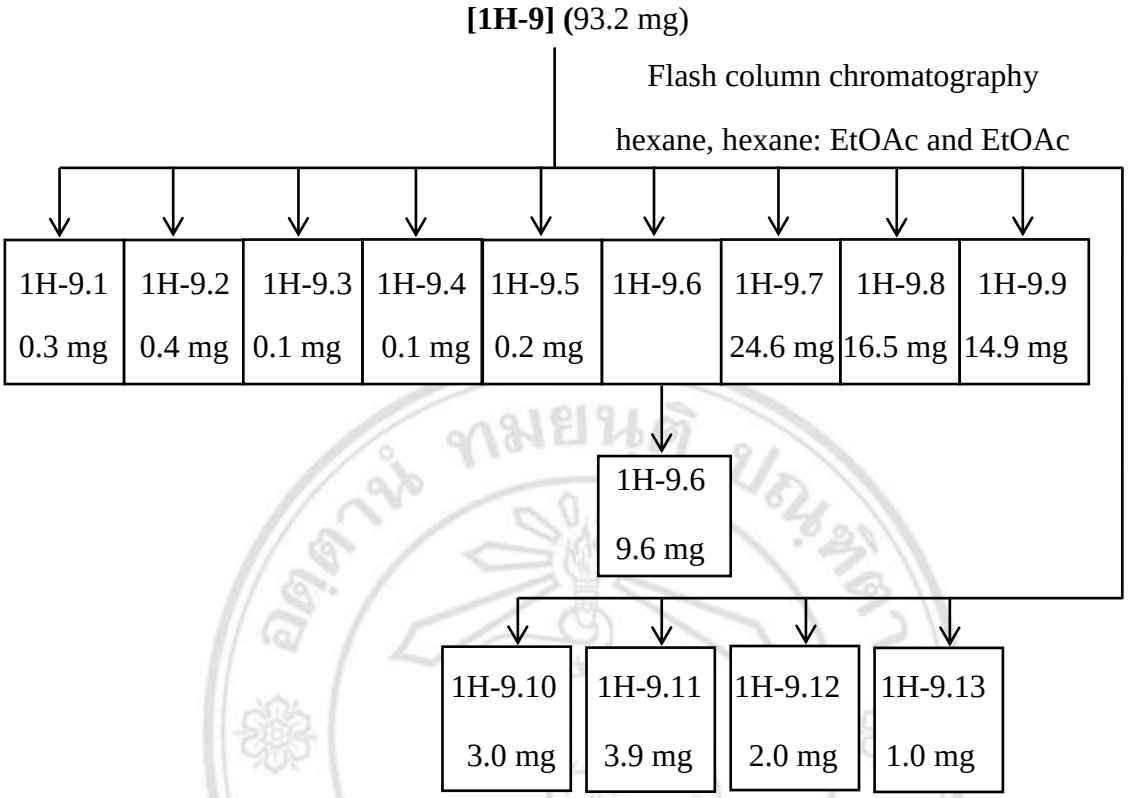
The fraction **[1H-9]** (93.2 mg) was subjected to normal phase flash column chromatography over silica gel (diameter 2 cm, height 18 cm),

using a gradient of hexane and EtOAc (each, 50 mL) as eluent. The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (3:2) as the eluent. Fifteen fractions were collected (50 mL/fraction). The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.3.

Table 3.3 Groups of fractions and eluent conditions of the isolation and purification of the fraction **[1H-9]**

Groups	Fractions	Eluent
		hexane:EtOAc
1H-9.1	1-2	100:0
	3	90:10
1H-9.2	4	90:10
1H-9.3	5	90:10
1H-9.4	6	90:10
1H-9.5	7	90:10
1H-9.6	8	80:20
1H-9.7	9	80:20
1H-9.8	10	80:20
1H-9.9	11	80:20
1H-9.10	12	80:20
1H-9.11	13	60:40
1H-9.12	14	50:50
1H-9.13	15	0:100

Fraction **[1H-9.6]** showed green crystals. Recrystallization of **[1H-9.6]** from hexane afforded white crystalline needles which were further identified using NMR spectroscopy. A summary of the isolation and purification procedure of **[1H-9]** is shown in Scheme 3.10.



Scheme 3.10. A summary of the isolation and purification procedure of the fraction **[1H-9]**

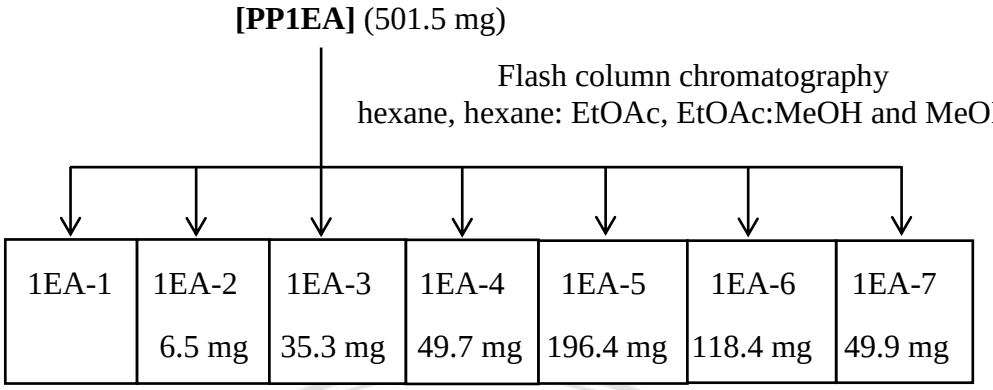
3.17.2. Isolation and purification of the EtOAc fraction of *G. pictum* [PP1EA]

The EtOAc fraction of *G. pictum* (**[PP1EA]**, 501.5 mg) was subjected to normal phase flash column chromatography over silica gel (diameter 2 cm, height 12 cm), using a gradient of hexane, EtOAc and MeOH as eluent. Fifty-four fractions were collected (20 mL/fraction). The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (1:4) as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.4.

Table 3.4 Groups of fractions and eluent conditions of the isolation and purification of **[PP1EA]**

Groups	Fractions	Eluent
		hexane:EtOAc
1EA-1	1-4	100:0
	5-6	80:20
1EA -2	7	80:20
	8-9	70:30
1EA-3	10	70:30
	11-13	60:40
	14-16	50:50
	17-18	40:60
1EA-4	19	40:60
	20-22	30:70
	23-25	20:80
	26-28	10:90
	29-30	0:100
1EA-5	31	0:100
		EtOAc:MeOH
	32-34	90:10
	35-37	80:20
1EA-6	38-40	70:30
	41-42	60:40
1EA-7	43	60:40
	44-46	50:50
	47-49	30:70
	50-54	0:100

The TLC analysis of fraction **[1EA-1]** indicated no compounds. Fractions **[1EA-3]** and **[1EA-4]** showed the same major compound. Therefore, they were selected for further purification. A summary of the isolation and purification procedure of **[PP1EA]** is shown in Scheme 3.11.



Scheme 3.11. A summary of the isolation and purification procedure of the fraction **[PP1EA]**

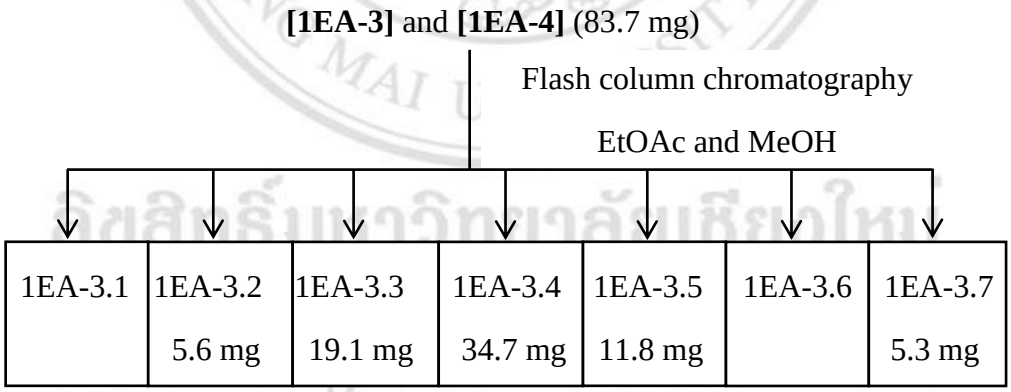
3.17.2.1. Isolation and purification of [1EA-3] and [1EA-4]

The combined fractions **[1EA-3]** and **[1EA-4]** (83.7 mg) were subjected to normal phase flash column chromatography over silica gel (diameter 2 cm, height 10 cm), using an isocratic elution of EtOAc (100 mL) followed by MeOH (30 mL). The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (1:4) as the eluent. Seventeen fractions were collected (10 mL/fraction). The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.5.

Table 3.5 Groups of fractions and eluent conditions of the isolation and purification of **[1EA-3]** and **[1EA-4]**

Groups	Fractions	Eluent
		EtOAc
1EA-3.1	1	100%
1EA-3.2	2	100%
1EA-3.3	3	100%
1EA-3.4	4-6	100%
1EA-3.5	7-13	100%
		MeOH
1EA-3.6	14-15	100%
1EA-3.7	16-17	100%

TLC analysis of fractions [**1EA-3.1]** and **[1EA-3.6]** showed no compounds. Fractions **[1EA-3.3], [1EA-3.4]** and **[1EA-3.5]** showed the same major compound. Therefore, they were selected for further purification using column chromatography. A summary of the isolation and purification procedure of **[1EA-3]** and **[1EA-4]** is shown in Scheme 3.12.



Scheme 3.12. A summary of the isolation and purification procedure of the fractions **[1EA-3]** and **[1EA-4]**

3.17.2.1.1. Isolation and purification of [1EA-3.3], **[1EA-3.4**] **and [1EA-3.5**]

The combined fractions **[1EA-3.3**], **[1EA-3.4**] and **[1EA-3.5**] (65.6 mg) were subjected to normal phase flash column

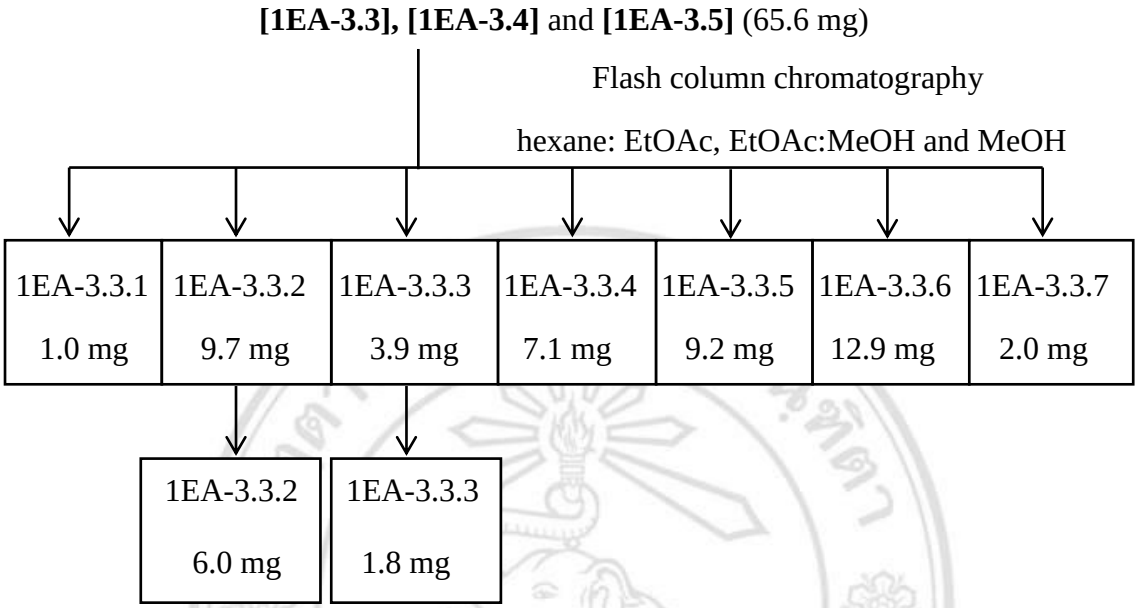
chromatography over silica gel (diameter 2 cm, height 20 cm), using a gradient of hexane, EtOAc and MeOH as eluent. Thirty-eight fractions were collected (10 mL/fraction). The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (1:1) as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.6.

Table 3.6 Groups of fractions and eluent conditions of the isolation and purification of **[1EA-3.3], [1EA-3.4]** and **[1EA-3.5]**

Groups	Fractions	Eluent
		hexane:EtOAc
1EA-3.3.1	1-2	60:40
1EA -3.3.2	3-6	50:50
	7	40:60
1EA-3.3.3	8-9	40:60
1EA-3.3.4	10	40:60
	11-12	20:80
1EA-3.3.5	13-14	20:80
	15-17	0:100
1EA-3.3.6	18	0:100
		EtOAc:MeOH
	19-25	95:5
	26-27	90:10
1EA-3.3.7	28-29	90:10
	30-33	80:20
	34-38	0:100

Fractions **[1EA-3.3.2]** and **[1EA-3.3.3]** showed green crystals. Therefore, they were recrystallized from hexane to afford yellow crystalline needles which were further identified using NMR

spectroscopy. A summary of the isolation and purification procedure of **[1EA-3.3], [1EA-3.4]** and **[1EA-3.5]** is shown in Scheme 3.13.



Scheme 3.13. A summary of the isolation and purification procedure of the fractions **[1EA-3.3], [1EA-3.4]** and **[1EA-3.5]**

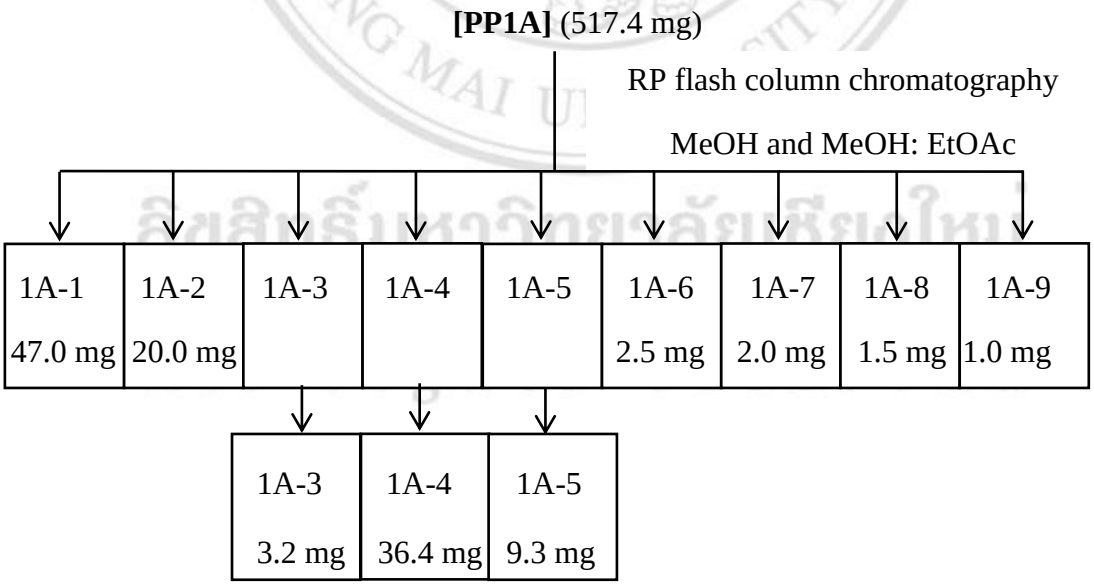
3.17.3. Isolation and purification of the aqueous fraction of *G. pictum* [PP1A]

The aqueous fraction of *G. pictum* (**[PP1A]**, 517.4 mg) was subjected to reverse phase flash column chromatography over RP-C18 silica gel (diameter 2 cm, height 10 cm), using a gradient of MeOH and EtOAc as eluent. Nine fractions were collected. The solvent was removed by evaporation. Groups of fractions are shown in Table 3.7.

Table 3.7 Groups of fractions and eluent conditions of the isolation and purification of **[PP1A]**

Groups	Fractions	Eluent
		MeOH
1A-1	1	100%
1A-2	2	100%
1A-3	3	100%
		MeOH:EtOAc
1A-4	4	80:20
1A-5	5	80:20
1A-6	6	80:20
1A-7	7	80:20
1A-8	8	50:50
1A-9	9	20:80

Fraction **[1A-3]**, **[1A-4]** and **[1A-5]** showed brown crystals. They were recrystallized from MeOH to afford yellow crystalline needles. A summary of the isolation and purification procedure of **[PP1A]** is shown in Scheme 3.14.



Scheme 3.14. A summary of the isolation and purification procedure of the fraction **[PP1A]**

3.18. Isolation and purification of the crude extract of *S. spirale* [P2]

Keawsa-ard (26) reported that the $CHCl_3$ extract of the leaves of *S. spirale* showed moderately anti-herpes simplex virus type-1 (HSV-1) activity (% viral inhibition > 35-50%). Therefore, the crude $CHCl_3$ extract **[P2C]** was selected for further isolation and purification.

3.18.1. Isolation and purification of the crude $CHCl_3$ extract of *S. spirale* [P2C]

The crude $CHCl_3$ extract of *S. spirale* **[P2C]** (1.7 g) was subjected to normal phase flash column chromatography over silica gel (diameter 3 cm, height 15 cm), using a gradient elution, hexane, EtOAc and MeOH were used as eluent. Seventy-nine fractions were collected (30 mL/fraction). The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (1:4) as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. The fractions that showed the similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.8.

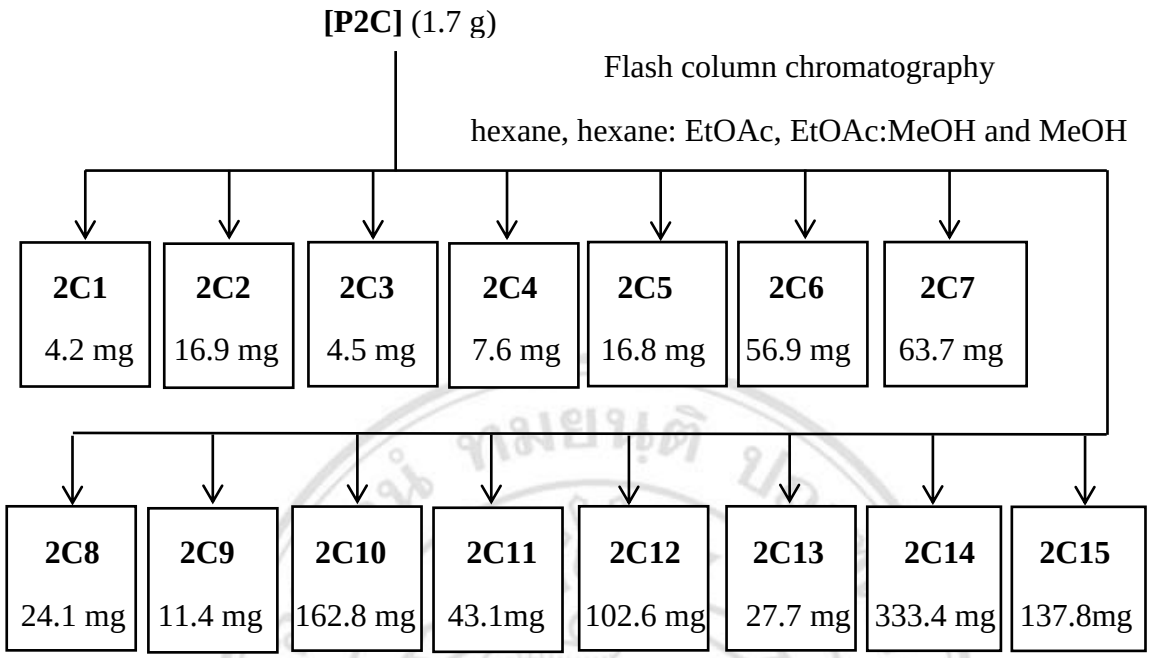
Table 3.8 Groups of fractions and eluent conditions of the isolation and purification of **[P2C]**

Groups	Fractions	Eluent
		hexane:EtOAc
2C1	1-5	100:0
	6-8	99:1
	9-10	98:2
2C2	11-12	98:2
	13	97:3
2C3	14-15	97:3
	16	96:4
2C4	17-19	96:4
	20-21	93:7

Table 3.8 (continued)

Groups	Fractions	Eluent
		hexane:EtOAc
2C5	22	93:7
	23-26	90:10
	27-28	80:20
2C6	29	80:20
	30-32	70:30
2C7	33	70:30
	34-36	60:40
2C8	37-38	50:50
2C9	39-40	50:50
	41	40:60
2C10	42-43	40:60
	44-47	30:70
	48	20:80
2C11	49-50	20:80
	51-52	10:90
2C12	53	10:90
	54-57	0:100
		EtOAc:MeOH
	58-60	90:10
2C13	61-63	80:20
2C14	64	80:20
	65-69	50:50
2C15	70-73	50:50
	74-79	0:100

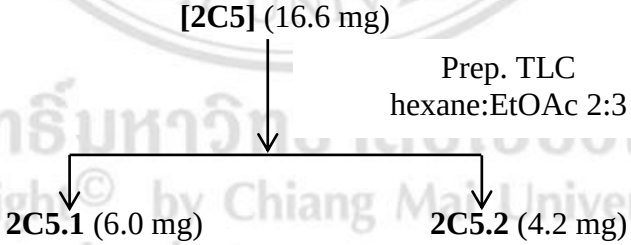
Fractions **[2C5]**, **[2C6]** and **[2C8]** were further purified using preparative TLC. Moreover, after visualization, a blue spot was observed under long wavelength UV (365 nm) for the fraction **[2C10]**. Therefore, it was selected for further purification using flash column chromatography. A summary of the isolation and purification procedure of **[P2C]** is shown in Scheme 3.15.



Scheme 3.15. A summary of the isolation and purification procedure of the fraction **[P2C]**

3.18.1.1. Isolation and purification of [2C5]

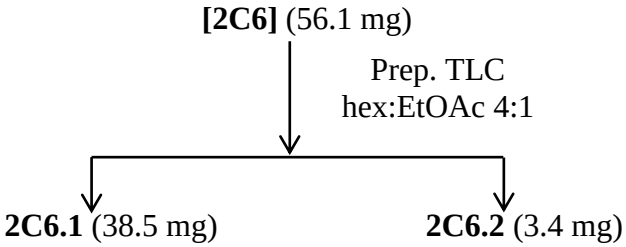
The fraction **[2C5]** (16.6 mg) was purified using preparative TLC on aluminium backed Merck F254 sorbent silica gel to yield **2C5.1** and **2C5.2**. The fractionation process of **[2C5]** is shown in Scheme 3.16.



Scheme 3.16. A summary of the isolation and purification procedure of the fraction **[2C5]**

3.18.1.2. Isolation and purification of [2C6]

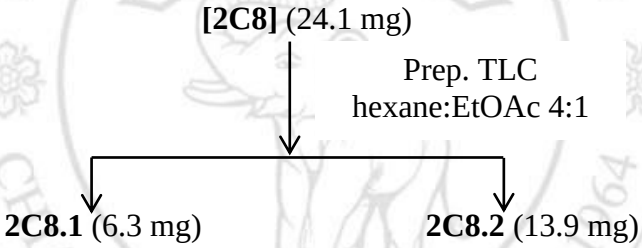
The fraction **[2C6]** (56.1 mg) was purified using preparative TLC on aluminium backed Merck F254 sorbent silica gel to yield **2C6.1** and **2C6.2**. The fractionation process of **[2C6]** is shown in Scheme 3.17.



Scheme 3.17. A summary of the isolation and purification procedure of the fraction **[2C6]**

3.18.1.3. Isolation and purification of [2C8]

The fraction **[2C8]** (24.1 mg) was purified using preparative TLC on aluminium backed Merck F254 sorbent silica gel to yield **2C8.1** and **2C8.2**. The fractionation process of **[2C8]** is shown in Scheme 3.18.



Scheme 3.18. A summary of the isolation and purification procedure of the fraction **[2C8]**

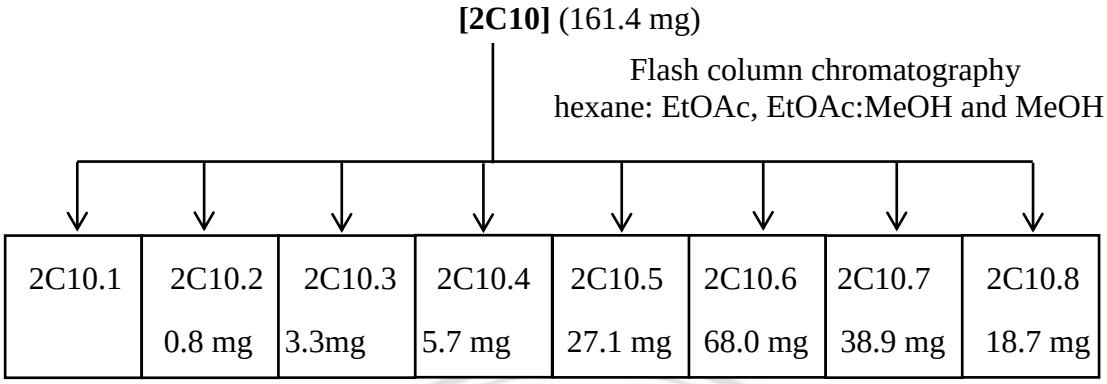
3.18.1.4. Isolation and purification of [2C10]

The fraction **[2C10]** (161.4 mg) was subjected to normal phase flash column chromatography over silica gel (diameter 2 cm, height 12 cm), using a gradient elution of hexane, EtOAc and MeOH (each, 50 mL). The constituents of each fraction were examined on silica gel TLC plates with EtOAc as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation and flushing with dry nitrogen gas. Groups of fractions are shown in Table 3.9.

Table 3.9 Groups of fractions and eluent conditions of the isolation and purification of **[2C10]**

Groups	Fractions	Eluent
		hex:EtOAc
2C10.1	1	60:40
2C10.2	2	60:40
2C10.3	3-4	60:40
	5	50:50
2C10.4	6	50:50
2C10.5	7-8	50:50
	9	40:60
2C10.6	10-12	40:60
	13	30:70
2C10.7	14-16	30:70
	17-19	20:80
2C10.8	20	20:80
	21-24	10:90
	25-28	0:100
		EtOAc:MeOH
	29-32	90:10
	33-36	80:20
	37-42	0:100

After TLC analysis, no compounds were observed in fraction **[2C10.1]**. Fractions **[2C10.6]** and **[2C10.7]** showed the same major compound. Therefore, they were selected for further purification using column chromatography. A summary of the isolation and purification procedure of **[2C10]** is shown in Scheme 3.19.



Scheme 3.19. A summary of the isolation and purification procedure of the fraction **[2C10]**

3.18.1.4.1. Isolation and purification of [2C10.6] and [2C10.7]

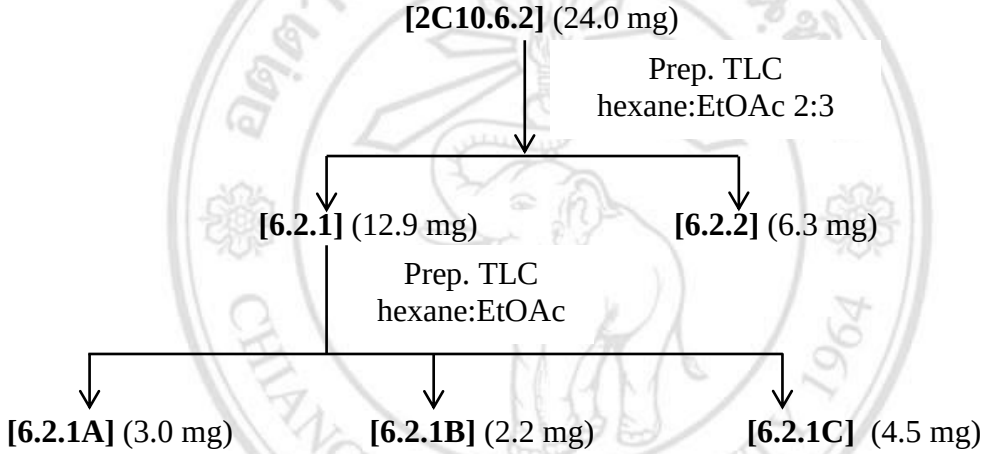
The combined fractions **[2C10.6]** and **[2C10.7]** (106.9 mg) were subjected to normal phase flash column chromatography over silica gel (diameter 2 cm, height 12 cm), using an isocratic elution of hexane:EtOAc 40:60 (100 mL). Twenty-two fractions were collected. The constituents of each fraction were examined on silica gel TLC plates with hexane/EtOAc (2:3) as the eluent. The TLC plates were stained with CAM and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined together and the solvent was removed by evaporation. Groups of fractions are shown in Table 3.10.

Table 3.10 Groups of fractions and eluent conditions of the isolation and purification of **[2C10.6]** and **[2C10.7]**

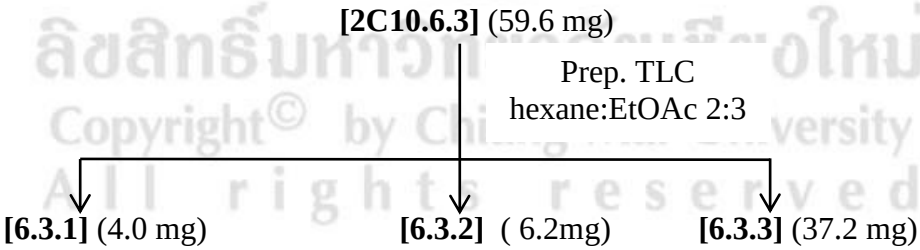
Groups	Fractions	Eluent
		hexane:EtOAc
2C10.6.1	1-6	40:60
2C10.6.2	7-9	40:60
2C10.6.3	10-17	40:60
2C10.6.4	18-22	40:60

After TLC analysis, no compounds were observed in fractions **[2C10.6.1]** and **[2C10.6.4]**. Fractions **[2C10.6.2]** and **[2C10.6.3]**

showed the same major compound. Therefore, they were selected for further purification using preparative TLC on aluminium backed Merck F254 sorbent silica gel. Fraction **[2C10.6.2]** was separated into 2 fractions (**[6.2.1]** and **[6.2.2]**). Fraction **[6.2.1]** was further purified using preparative TLC to obtain 3 fractions (**[6.2.1A]**, **[6.2.1B]** and **[6.2.1C]**). The fractionation process of **[2C10.6.2]** is shown in Scheme 3.20, while fraction **[2C10.6.3]** was separated to yield 3 fractions (**[6.3.1]**, [**6.3.2]** and **[6.3.3]**). The fractionation process of **[2C10.6.3]** is shown in Scheme 3.21.



Scheme 3.20. A summary of the isolation and purification procedure of the fraction **[2C10.6.2]**



Scheme 3.21. A summary of the isolation and purification procedure of the fraction **[2C10.6.3]**

Fractions **[6.2.1C]** and **[6.3.2]** were identified using NMR spectroscopy and their antibacterial activities were also tested.

CHAPTER 3

Experimental

3.1 General Experimental

3.1.1 General reaction condition

All reagents were used as received from commercial sources without further purification. Solvents were purchased as Analytical Reagents (AR) grade. Petroleum spirit (Chem-Supply, Australia) refers to the hydrocarbon fraction of bp 40-60 °C. Tetrahydrofuran (Sigma, USA) was stored over KOH pellets (Univar, Australia) until needed, then distilled over sodium wire under nitrogen, using benzophenone (Sigma, USA) as indicator. Anhydrous CH_2Cl_2 and MeOH were purchased from Aldrich (USA). Progress of reactions was monitored by thin-layer chromatographic (TLC) analysis. Reactions were stirred using Teflon-coated magnetic stirring bars.

3.1.2 Nuclear Magnetic Resonance Spectroscopy

3.1.2.1 ^{1}H-NMR spectra

All ^{1}H-NMR spectra were measured in $CDCl_3$ (with TMS (δ 0.00) as an internal standard) or D_2O (with H_2O (δ 4.80) as internal standard) at 500 MHz using a Varian VNMR PS54-500 or a Varian INOVA-500 magnetic resonance spectrometer. The resonances were assigned as follows: chemical shift in ppm (mulplicity, coupling constants (*J*) in Hz, number of protons, assigned protons). The following abbreviations were used to explain the multiplicities: s (singlet), d (doublet), t (triplet), q (quatet), m (multiplet) and br (broad).

3.1.2.2 ^{13}C-NMR spectra

All ^{13}C-NMR spectra were obtained at 125 MHz using deuterated solvents, $CDCl_3$ ($CDCl_3$ reference at δ 77.16) or D_2O (with MeOH (δ 49.51) as internal standard) and used the same NMR spectrophotometers as for the ^{1}H-NMR spectra. The resonances were assigned as follows: Chemical shift in ppm (assigned carbon). ^{13}C-NMR assignments were made from APT and/or gHSQC experiments.

3.1.3 Chromatography

3.1.3.1 Column Chromatography

Column chromatographic purification of products was carried out by flash column chromatography on Merck GF 254 silica gel (70-230 mesh) which was packed by the slurry method. Small scale separations (< 500.0 mg) were performed using 15 mm diameter columns, and the large scale separations (> 1.0 g) were performed using 40 mm diameter columns with suitable solvent systems. Most columns were developed using gradient elution of the solvent mixtures which are described in the experimental section.

3.1.3.2 Preparative Thin Layer Chromatography

PTLC was performed with aluminium backed Merck $_{F254}$ sorbent silica gel. The desired compounds were observed under ultraviolet light at 254 and 365 nm.

3.1.3.3 Thin Layer Chromatography

TLC was performed with aluminium-backed Merck $_{F254}$ sorbent silica gel. TLC plates were visualized by ultraviolet light or treatment with aqueous solution of ammonium molybdate and cerium(IV) sulphate, followed by development with a 1400 Watt heat gun. One liter of the molybdate dip contained water (900 mL), concentrated H_2SO_4 (100 mL), $(NH_4)_6MoO_{24}$ (50 g) and $Ce(SO_4)_2$ (10 g).

3.1.3.4 Basic Ion-Exchange Chromatography

Basic ion-exchange chromatography was performed using a Pasteur pipette with Amberlyst A-26(OH) resin. Before the purification, the resins were mixed with 14% NH_3/H_2O and rested in the column for 15 min. After that, the resins were washed with distilled water until neutral pH. The compounds were applied to the column and eluted with water.

3.1.4 Polarimetry

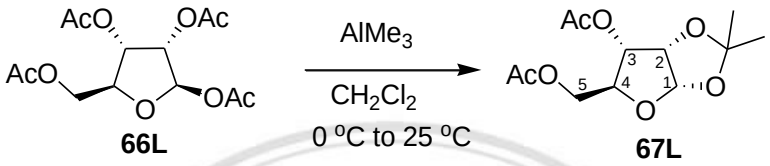
Polarimetry was carried out using a JASCO P-2000 Digital Polarimeter and the measurements were made at the sodium D-line with a 1 dm pathlength cell. Concentrations (*c*) are given in gram per 100 mL. The specific rotations are reported as: specific rotation [10^{-1}.deg.cm^3.g^{-1}] (concentration, solvent). The values were calculated from the average of ten measurements.

3.1.5 Mass spectrometry

Low-resolution mass spectra were obtained on a Waters LCZ single quadropole (ESI). High-resolution mass spectra (HRMS) were recorded on a Waters QTOF (ESI), a Waters Xevo (ESI) or a Waters Xevo (ASAP).

3.2 Method for the synthesis of the β-L-ribofuranose derivative [65L]

3.2.1. ((3a*S*,5*S*,6*S*,6a*S*)-6-Acetoxy-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxol-5-yl)methyl acetate [67L] (242)



To a solution of the β-L-ribofuranose 1,2,3,5-tetraacetate [**66L**] (5.00 g, 15.71 mmol) in anhydrous CH_2Cl_2 (126.0 mL) at 0 °C under an atmosphere of N_2 was added trimethylaluminium (2.0 M in hexane, 15.7 mL, 31.42 mmol) dropwise via a dropping funnel. The reaction mixture was allowed to slowly warm at rt and was stirred for 15 h. The reaction mixture was re-cooled to 0 °C and quenched by the dropwise addition of a saturated sodium potassium tartrate (Rochelle's salt) solution (126 mL). CH_2Cl_2 (126 mL) was added and the mixture was stirred for 2 h. The organic layer was separated and the aqueous layer was extracted once with CH_2Cl_2 (252 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to afford the crude product mixture. Purification by flash column chromatography (increasing polarity from 85:15 to 75:25 petrol/EtOAc as eluent) afforded the title compound (3.39 g, 79%) as a clear viscous oil. R_f 0.50 (1:1 petrol/EtOAc).

$[\alpha]_D^{25}$ –121.3 (*c* 1.8, $CHCl_3$), lit. (243) (enantiomer) $[\alpha]_D^{22}$ +120.5 (*c* 2.8, $CHCl_3$).

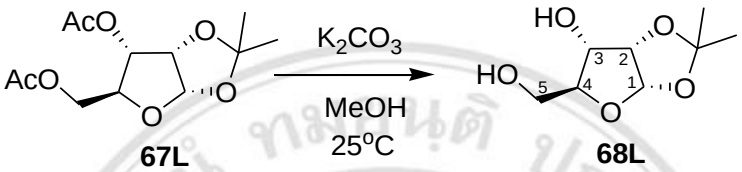
MS (ESI +ve) *m/z* 297.07 ($M+Na^+$), 100%.

IR (cm^{-1}): 2990, 2942, 1740, 1436, 1372, 1215, 1168, 1119, 1077, 1017, 870.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.83 (d, *J* = 3.5 Hz, 1H, H-1), 4.83 (brs, 1H, H-2), 4.68 (dd, *J* = 9.0, 5.0 Hz, 1H, H-3), 4.37 (d, *J* = 12.0 Hz, 1H, H-5a), 4.33-4.30 (m, 1H, H-4), 4.15 (dd, *J* = 12.5, 5.0 Hz, 1H, H-5b), 2.14 (s, 3H, Ac), 2.09 (s, 3H, Ac), 1.57 (s, 3H, CH_3), 1.35 (s, 3H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (C=O), 170.3 (C=O), 113.3 ($C(CH_3)_2$), 104.3 (C-1), 77.2 (C-3), 75.5 (C-2), 72.3 (C-4), 62.5 (C-5), 26.7 (2 CH_3), 20.8 (CH_3), 20.7 (CH_3).

3.2.2. (3a*S*,5*S*,6*S*,6a*S*)-5-(Hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxol-6-ol [68L] (244)



67L **68L**

To a solution of **67L** (3.36 g, 12.25 mmol) in dry MeOH (60 mL) was added anhydrous K_2CO_3 (5.08 g, 36.75 mmol) at rt. The reaction mixture was stirred for 2 h at rt and quenched with H_2O (12 mL). The precipitates were filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (60 mL), dried with $MgSO_4$ (25 g), filtered from inorganic salts and evaporated to afford the title compound as a white solid (1.98 g, 85%).

$[\alpha]_D^{25}$ –43.3 (*c* 1.2, $CHCl_3$), lit. (245) $[\alpha]_D^{25}$ –31.50 (*c* 0.62, $CHCl_3$).

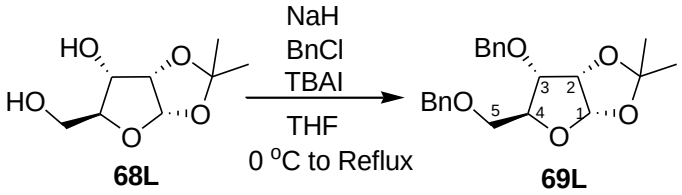
MS (ESI +ve) *m/z* 213.07 ($M+Na^+$), 100%.

IR (cm^{-1}): 3236, 2956, 2922, 2887, 1377, 1214, 1115, 1014, 874.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.82 (d, *J* = 4.0 Hz, 1H, H-1), 4.59 (t, *J* = 4.5 Hz, 1H, H-2), 4.00 (dd, *J* = 9.0, 5.5 Hz, 1H, H-3), 3.94 (dd, *J* = 12.5, 2.5 Hz, 1H, H-5a), 3.85 (ddd, *J* = 9.0, 3.0, 2.5 Hz, 1H, H-4), 3.75 (dd, *J* = 12.5, 3.5 Hz, 1H, H-5b), 2.67 (brs, 1H, OH), 2.34 (brs, 1H, OH), 1.58 (s, 3H, CH_3), 1.38 (s, 3H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ 112.9 ($C(CH_3)_2$), 104.1 (C-1), 80.7 (C-2), 78.9 (C-4), 71.0 (C-3), 60.9 (C-5), 26.7 (CH_3), 26.6 (CH_3).

3.2.3. (3a*S*,5*S*,6*S*,6a*S*)-6-(Benzyloxy)-5-(benzyloxymethyl)-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxole [69L] (246)



68L **69L**

To the suspension of sodium hydride (60% dispension in mineral oil, 1.44 g, 35.98 mmol of NaH) in anhydrous THF (36 mL) was slowly added a solution of **68L** (1.96 g, 10.28 mmol) in anhydrous THF (15 mL) at 0 °C under an atmosphere of N_2. The mixture was then heated under reflux for 5 min. After re-cooling to rt, benzyl chloride (3.3 mL, 3.64 g, 28.78 mmol) and TBAI (532 mg, 1.44 mmol) were added and the mixture was heated to reflux for further 30 min after which time TLC control confirmed full consumption of the starting material. The reaction mixture was then quenched by the addition of water (18 mL) under cooling in an ice bath and subsequently extracted with Et_2O (3×18 mL). The combined ethereal extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (increasing polarity from 100:0 to 80:20 petrol/EtOAc as eluent) to afford the title compound (2.72 g, 72%) as a pale yellow viscous oil. R_f 0.32 (1:1 petrol/Et_2O).

$[\alpha]_D^{25}$ –84.3 (*c* 2.0, $CHCl_3$), lit. (247) (enantiomer) $[\alpha]_D^{20}$ +84.5 (*c* 0.65, $CHCl_3$).

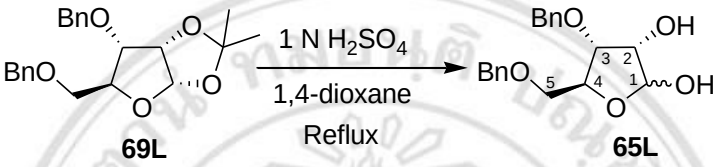
MS (ESI +ve) *m/z* 393.36 ($M+Na^+$), 100%.

IR (cm^{-1}): 2987, 2935, 2865, 1453, 1372, 1310, 1247, 1214, 1167, 1128, 1097, 1020, 873, 736, 697.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33-7.27 (m, 10H, Ar), 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 4.72 (AB_q, J_{AB} = 12.0 Hz, 1H, OC*H*HPh), 4.56 (AB_q, J_{AB} = 12.0 Hz, 1H, OC*H*HPh), 4.55 (t, *J* = 9.5 Hz, 1H, H-2), 4.54 (AB_q, J_{AB} = 12.0 Hz, 1H, OC*H*HPh), 4.49 (AB_q, J_{AB} = 12.0 Hz, 1H, OC*H*HPh), 4.18 (m, 1H, H-4), 3.85 (dd, *J* = 9.0, 4.5 Hz, 1H, H-3), 3.76 (dd, *J* = 11.0, 1.5 Hz, 1H, H-5a), 3.57 (dd, *J* = 11.0, 4.0 Hz, 1H, H-5b), 1.59 (s, 3H, CH_3), 1.35 (s, 3H, CH_3).

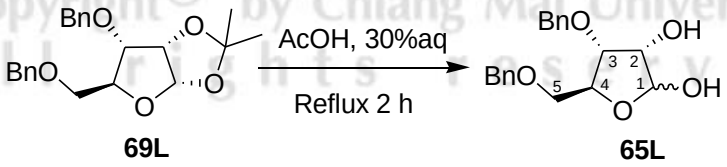
^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2 (C), 137.8 (C), 128.5 (CH), 128.4 (CH), 128.1 (2CH), 127.8 (CH), 127.7 (CH), 113.0 $(CH_3)_2$), 104.2 (C-1), 78.1 (C-2), 77.5 (C-3), 77.3 (C-4), 73.6 (2C*H*HPh), 72.3 (2C*H*HPh), 68.1 (C-5), 26.9 (CH_3), 26.7 (CH_3).

3.2.4. (3*S*,4*R*,5*S*)-4-(Benzyloxy)-5-(benzyloxymethyl)tetrahydrofuran-2,3-diol [65L]



69L **65L**

Procedure A: To solution of **69L** (1.65 g, 4.45 mmol) in 1,4-dioxane (22 mL was added 1 N H_2SO_4 (3 mL) at rt. The reaction mixture was heated under reflux (100 °C) and stirred for 3 h. The reaction was monitored by TLC analysis every hour. The mixture was then cooled to rt and neutralized by addition of 1 M $NaHCO_3$ solution (3.1 mL). The organic solvent was evaporated *in vacuo* and reduced to approximately 1/10 of the original volume. This residue was diluted with CH_2Cl_2 (9 mL) and 1 M citric acid (4 mL). The aqueous phase was separated and extracted with CH_2Cl_2 (5x9 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* (248). The residue was purified by flash column chromatography (increasing polarity from 50:50 to 0:100 petrol/Et_2O as eluent) to afford the desired compound [**65L**] (138.5 mg, 9%) as a white solid. R_f 0.45 (1:1 petrol/Et_2O).



69L **65L**

Procedure B: A solution of **69L** (2.71 g, 7.32 mmol) in 30% aqueous acetic acid (55 mL) was stirred under reflux conditions (~112 °C) for 2 h, the reaction mixture was then cooled in an ice bath over 1 h and precipitation of the product was observed. The reaction mixture was diluted with CH_2Cl_2 (55 mL) then quenched with sat. $NaHCO_3$ (2x55 mL) (249). The CH_2Cl_2 solution was dried

over $MgSO_4$, filtered and concentrated *in vacuo* to afford the product as a white solid (2.28 g, 94%), α:β 1:1.

$[\alpha]_D^{25}$ –52.4 (*c* 1.0, $CHCl_3$), lit. (250) (enantiomer) $[\alpha]_D^{25}$ +27.0 (*c* 1.0, $CHCl_3$).

MS (ESI +ve) *m/z* 353.26 ($M+Na^+$), 100%.

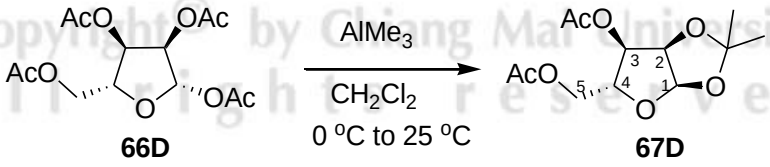
IR (cm^{-1}): 3406, 3320, 2946, 2870, 1454, 1136, 1097, 1082, 1051, 1011, 940, 755, 738, 697.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.27 (m, 10H, Ar), 5.26 (dd, *J* = 9.5, 4.5 Hz, 1H, H-1α), 5.23 (d, *J* = 6.5 Hz, 1H, H-1β), 4.61-4.46 (m, 4H, OC*H*HPh), 4.27-4.24 (m, 2H, H-3β, H4α), 4.20-4.19 (m, 1H, H4β), 4.13 (dd, *J* = 8.0, 6.0 Hz, 1H, H-2α), 4.03 (t, *J* = 3.5 Hz, 1H, H-2β), 3.96 (dd, *J* = 5.5, 4.0 Hz, 1H, H-3α), 3.63 (dd, *J* = 10.5, 3.0 Hz, 1H, H-5β), 3.50 (dd, *J* = 10.5, 4.0 Hz, 1H, H-5β), 3.48-3.44 (m, 2H, H-5α, H-5α).

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.0 (C), 137.3 (C), 137.1 (C), 137.1 (C), 102.6 (C-1β), 97.2 (C-1α), 81.1 (C-4β), 80.4 (C-4α), 78.3 (C-3β), 78.1 (C-3α), 74.7 (C-2β), 73.7 (2C*H*HPh), 73.7 (2C*H*HPh), 73.2 (2C*H*HPh), 73.1 (2C*H*HPh), 70.8 (C-2α), 69.9 (C-5β), 69.8 (C-5α).

3.3 Method for the synthesis of the β-D-ribofuranose derivative [65D]

3.3.1. ((3a*R*,5*R*,6*R*,6a*R*)-6-Acetoxy-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxol-5-yl)methyl acetate [67D] (242)



66D **67D**

To a solution of β-D-ribofuranose 1,2,3,5-tetraacetate [**66D**] (2.52 g, 7.91 mmol) in anhydrous CH_2Cl_2 (63 mL) at 0 °C under an atmosphere of N_2 was added trimethylaluminium (2.0 M in hexane, 7.9 mL, 15.82 mmol) dropwise *via* a dropping funnel. The reaction mixture was allowed to slowly warm to rt and stirred for 15 h. The reaction mixture was re-cooled to 0 °C and quenched by the

dropwise addition of a saturated sodium potassium tartrate (Rochelle's salt) solution (63 mL). CH_2Cl_2 (63 mL) was added and the mixture was stirred for 2 h. The organic layer was separated and the aqueous layer was extracted once with CH_2Cl_2 (126 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to afford the crude product mixture. Purification by flash column chromatography (increasing polarity from 85:15 to 75:25 petrol/EtOAc as eluent) afforded the title compound (1.74 g, 80%) as a clear viscous oil. R_f 0.50 (1:1 petrol/EtOAc).

$[\alpha]_D^{25}$ +125.9 (*c* 1.1, $CHCl_3$), lit. (243) $[\alpha]_D^{22}$ +120.5 (*c* 2.8, $CHCl_3$).

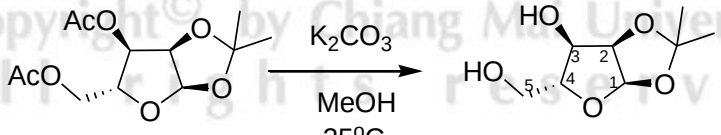
MS (ESI +ve) *m/z* 297.13 ($M+Na^+$), 100%.

IR (cm^{-1}): 2990, 2942, 1740, 1436, 1372, 1215, 1168, 1119, 1077, 1017, 870.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.84 (d, *J* = 3.5 Hz, 1H, H-1), 4.83 (t, *J* = 4.5 Hz, 1H, H-2), 4.68 (dd, *J* = 9.5, 5.0 Hz, 1H, H-3), 4.37 (dd, *J* = 12.5, 2.5 Hz, 1H, H-5a), 4.33-4.30 (m, 1H, H-4), 4.15 (dd, *J* = 13.0, 5.0 Hz, 1H, H-5b), 2.14 (s, 3H, Ac), 2.10 (s, 3H, Ac), 1.57 (s, 3H, CH_3), 1.35 (s, 3H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (C=O), 170.3 (C=O), 113.3 ($C(CH_3)_2$), 104.3 (C-1), 77.2 (C-3), 75.5 (C-2), 72.4 (C-4), 62.5 (C-5), 26.7 ($2CH_3$), 20.9 (CH_3), 20.8 (CH_3).

3.3.2. (3a*R*,5*R*,6*R*,6a*R*)-5-(Hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxol-6-ol [68D] (244)



67D **68D**

To a solution of **67D** (1.74 g, 6.34 mmol) in dry MeOH (32 mL) was added anhydrous K_2CO_3 (2.63 g, 19.01 mmol) at rt. The reaction mixture was stirred for 2 h at rt and quenched with H_2O (6.3 mL). The precipitates were filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (32

mL), dried with $MgSO_4$ (12.68 g), filtered from inorganic salts and evaporated to afford the title compound as a white solid (1.13 g, 94%).

$[\alpha]_D^{25}$ +44.2 (*c* 1.3, $CHCl_3$), lit. (245) (enantiomer) $[\alpha]_D^{25}$ –31.50 (*c* 0.62, $CHCl_3$).

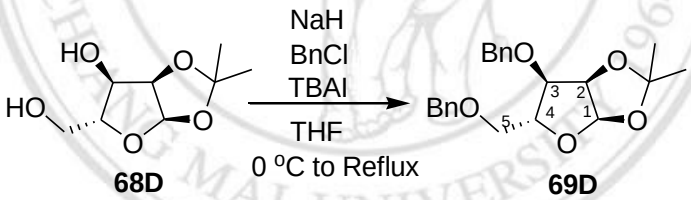
MS (ESI +ve) *m/z* 213.10 ($M+Na^+$), 100%.

IR (cm^{-1}): 3217, 2956, 2921, 2887, 1371, 1240, 1214, 1115, 1017.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.81 (d, *J* = 4.0 Hz, 1H, H-1), 4.58 (t, *J* = 4.0 Hz, 1H, H-2), 4.00 (dd, *J* = 9.0, 5.0 Hz, 1H, H-3), 3.93 (dd, *J* = 12.5, 2.5 Hz, 1H, H-5a), 3.85 (ddd, *J* = 9.0, 3.0, 2.5 Hz, 1H, H-4), 3.74 (dd, *J* = 12.5, 3.5 Hz, 1H, H-5b), 2.65 (brs, 2H, 2OH), 1.57 (s, 3H, CH_3), 1.38 (s, 3H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ 112.9 ($C(CH_3)_2$), 104.1 (C-1), 80.7 (C-2), 78.4 (C-4), 71.0 (C-3), 60.9 (C-5), 26.6 ($2CH_3$).

3.3.3. (3a*R*,5*R*,6*R*,6a*R*)-6-(Benzyloxy)-5-(benzyloxymethyl)-2,2-dimethyltetrahydrofuro[3,2-*d*][1,3]dioxole [69D] (246)



68D **69D**

To the suspension of sodium hydride (60% dispension in mineral oil, 751.3 mg, 18.79 mmol of NaH) in anhydrous THF (19 mL) was slowly added a solution of **68D** (1.02 g, 5.37 mmol) in anhydrous THF (8 mL) at 0 °C under an atmosphere of N_2. The mixture was then heated under reflux for 5 min. After re-cooling to rt, benzyl chloride (1.7 mL, 1.90 g, 15.03 mmol) and TBAI (277 mg, 0.75 mmol) were added and the mixture was heated under reflux for further 30 min after which time TLC control confirmed full consumption of the starting material. The reaction mixture was then quenched by the addition of water (9.4 mL) under cooling in an ice bath and subsequently extracted with Et_2O (3×9.4 mL). The combined ethereal extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography

(increasing polarity from 100:0 to 80:20 petrol/EtOAc as eluent) to afford the title compound (1.68 g, 84%) as a pale yellow viscous oil. R_f 0.32 (1:1 petrol/Et_2O).

$[\alpha]_D^{25}$ +88.5 (*c* 1.5, $CHCl_3$), lit. (247) $[\alpha]_D^{20}$ +84.5 (*c* 0.65, $CHCl_3$).

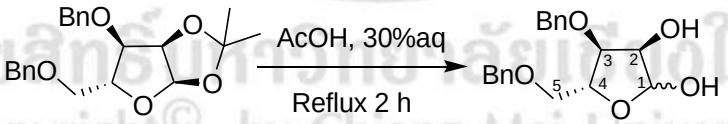
MS (ESI +ve) *m/z* 393.40 ($M+Na^+$), 100%.

IR (cm^{-1}): 2988, 2865, 1497, 1453, 1372, 1310, 1247, 1214, 1167, 1126, 1097, 1020, 873, 736, 697.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33-7.27 (m, 10H, Ar), 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 4.73 (AB_q, J_{AB} = 12.5 Hz, 1H, OC*H*HPh), 4.56 (AB_q, J_{AB} = 11.5 Hz, 1H, OC*H*HPh), 4.55 (t, *J* = 9.5 Hz, 1H, H-2), 4.54 (AB_q, J_{AB} = 11.5 Hz, 1H, OC*H*HPh), 4.49 (AB_q, J_{AB} = 12.0 Hz, 1H, OC*H*HPh), 4.18 (m, 1H, H-4), 3.86 (dd, *J* = 9.0, 4.5 Hz, 1H, H-3), 3.76 (dd, *J* = 11.5, 1.5 Hz, 1H, H-5a), 3.57 (dd, *J* = 11.0, 3.5 Hz, 1H, H-5b), 1.59 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2 (C), 137.8 (C), 128.5 (CH), 128.4 (CH), 128.1 (2CH), 127.8 (CH), 127.7 (CH), 113.0 (CH_3)$_2$), 104.2 (C-1), 78.1 (C-2), 77.5 (C-3), 77.3 (C-4), 73.6 (2C*H*HPh), 72.4 (2C*H*HPh), 68.1 (C-5), 26.9 (CH_3), 26.7 (CH_3).

3.3.4. (3*R*,4*S*,5*R*)-4-(Benzyloxy)-5-(benzyloxymethyl) tetrahydrofuran-2,3-diol [65D] (249)



69D **65D**

A solution of **69D** (1.41 g, 3.79 mmol) in 30% aqueous acetic acid (28 mL) was stirred under reflux conditions (~112 °C) for 2 h, the reaction mixture was cooled in an ice bath over 1 h and precipitation of the product was observed. The reaction mixture was diluted with CH_2Cl_2 (28 mL) then quenched with sat. $NaHCO_3$ (2x28 mL). The CH_2Cl_2 solution was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the product as a white solid (1.24 g, 99%), α:β = 1:3.

[α]$_D^{25}$ +56.9 (*c* 1.0, $CHCl_3$), lit. (250) [α]$_D^{25}$ +27.0 (*c* 1.0, $CHCl_3$).

MS (ESI +ve) *m/z* 353.25 ($M+Na^+$), 100%.

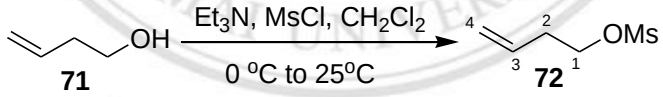
IR (cm^{-1}): 3408, 3318, 2946, 2871, 1454, 1372, 1136, 1097, 1051, 1011, 940, 755, 739, 697.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 10H, Ar), 5.26 (dd, *J* = 9.0, 4.0 Hz, 1H, H-1α), 5.23 (d, *J* = 6.5 Hz, 1H, H-1β), 4.61-4.46 (m, 4H, OC*H*HPh), 4.27-4.24 (m, 2H, H-3β, H4α), 4.20-4.18 (m, 1H, H4β), 4.13 (dd, *J* = 7.5, 5.5 Hz, 1H, H-2α), 4.03 (t, *J* = 4.5 Hz, 1H, H-2β), 3.96 (dd, *J* = 5.5, 3.5 Hz, 1H, H-3α), 3.63 (dd, *J* = 10.5, 3.0 Hz, 1H, H-5β), 3.50 (dd, *J* = 10.5, 4.5 Hz, 1H, H-5β), 3.48-3.44 (m, 2H, H-5α, H-5α).

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.0 (C), 137.3 (C), 137.1 (C), 137.0 (C), 102.6 (C-1β), 97.2 (C-1α), 81.1 (C-4β), 80.4 (C-4α), 78.3 (C-3β), 78.1 (C-3α), 74.7 (C-2β), 73.7 (2C*H*HPh), 73.7 (2C*H*HPh), 73.2 (2C*H*HPh), 73.1 (2C*H*HPh), 70.8 (C-2α), 69.9 (C-5β), 69.8 (C-5α).

3.4 Method for the synthesis of 3-buten-1-amine [70] (251)

3.4.1. But-3-enyl methanesulfonate [72]

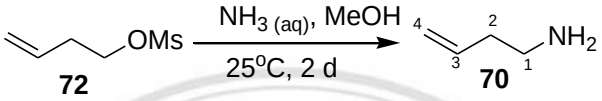


71 **72**

To a solution of 3-buten-1-ol [**71**] (120 µL, 1.39 mmol) and triethylamine (290 µL, 2.09 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 ºC was added *via* syringe methanesulfonyl chloride (160 µL, 2.09 mmol) under an atmosphere of N_2. The reaction mixture was allowed to slowly warm at rt and stirred for 18 h. The mixture was diluted with CH_2Cl_2 (2 mL) then quenched with sat. $NaHCO_3$ (2x2 mL) and 2 mL brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the product [**72**] as a yellow oil (208.3 mg, 100%). R_f 0.57 (3:2 petrol/EtOAc).

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 4.73 (AB_q, J_{AB} = 12.5 Hz, 1H, OC*H*HPh), 3.86 (dd, *J* = 9.0, 4.5 Hz, 1H, H-3), 1.59 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

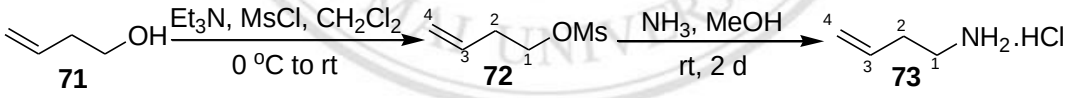
3.4.2. 3-Buten-1-amine [70]



To a solution of **72** (208.3 mg) in MeOH (3 mL) was added 28% aqueous ammonia solution (5 mL). The reaction mixture was stirred at the rt for 2 days. The mixture was diluted with water (3 mL) and extracted with CH_2Cl_2 (3x5 mL). The combined organic extracts were dried ($MgSO_4$), filtered and carefully concentrated *in vacuo* to give the volatile amine [**70**] as a yellow oil (7.9 mg, 8%).

^{1}H NMR (500 MHz, $CDCl_3$) δ) δ 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 4.73 (AB_q, J_{AB} = 12.5 Hz, 1H, OC*H*HPh), 3.86 (dd, *J* = 9.0, 4.5 Hz, 1H, H-3), 1.59 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

3.5 Method for the synthesis of 3-butenylamine.hydrochloride [73] (251)



To a solution of **71** (590 µL, 6.93 mmol) and triethylamine (800 µL, 10.4 mmol) in anhydrous CH_2Cl_2 (10 mL) at 0 ºC was added *via* syringe methanesulfonyl chloride (1.45 mL, 10.4 mmol) under an atmosphere of N_2. The reaction mixture was allowed to slowly warm at rt and stirred for 15 h. The mixture was diluted with CH_2Cl_2 (10 mL) then quenched with sat. $NaHCO_3$ (2x10 mL) and 10 mL brine. The organic phase was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the product [**72**] as a yellow oil (1041 mg, 100%). R_f 0.57 (3:2 petrol/EtOAc).

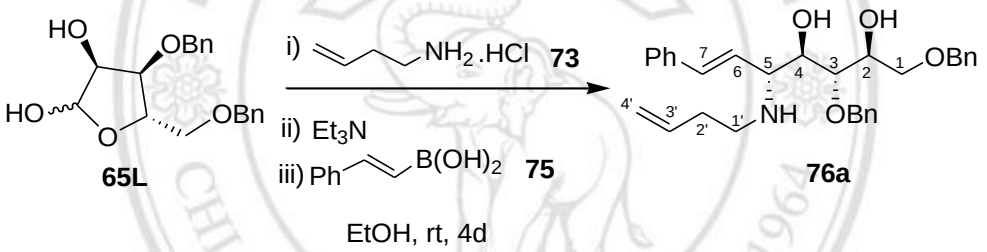
To a solution of **72** (1041mg) in MeOH (15mL) was added 28% aqueous ammonia solution (23 mL). The reaction mixture was stirred at rt for 2 days. The mixture was diluted with water (15 mL) and extracted with Et_2O (3x23 mL). The

combined organic extracts were dried ($MgSO_4$), filtered and then 2M HCl in Et_2O (5 mL) was added. The aqueous phase was separated and concentrated *in vacuo* to give the product [**73**] as a white solid (104.5 mg, 21%).

^{1}H NMR (500 MHz, D_2O) δ 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 5.75 (d, *J* = 3.5 Hz, 1H, H-1), 4.73 (AB_q, J_{AB} = 12.5 Hz, 1H, OC*H*HPh), 3.86 (dd, *J* = 9.0, 4.5 Hz, 1H, H-3), 1.59 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

3.6 Method for the synthesis of 10-*nor*-steviamine [6] and its triacetate derivative [74a]

3.6.1. ((2*S*,3*R*,4*R*,*E*)-1,3-Bis(benzyloxy)-5-(but-3-enylamino)-7-phenylhept-6-ene-2,4-diol [76a]



To a solution of **65L** (30.4 mg, 0.09 mmol) in absolute EtOH (0.75 mL) was added 3-butenylamine·hydrochloride [**73**] (9.7 mg, 0.09 mmol) followed by Et_3N (0.013 mL, 0.09 mmol) and *trans*-2-phenylvinylboronic acid [**75**] (13.3 mg, 0.09 mmol, commercial sample from Aldrich). The mixture was stirred at rt for 4 days, followed by evaporation of all volatiles *in vacuo*. The residue was dissolved in CH_2Cl_2 (4 mL) and washed with sat. aq. $NaHCO_3$ (2×4 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford a brown foam (252). Purification by flash column chromatography (increasing polarity from 0:100 to 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (26.3 mg, 59%) as a brown foam. R_f 0.45 (10:90 MeOH/CH_2Cl_2).

$[\alpha]_D^{25}$ +51.5 (*c* 1.3, $CHCl_3$).

MS (ESI +ve) *m/z* 488.5 ($M+H^+$), 100%.

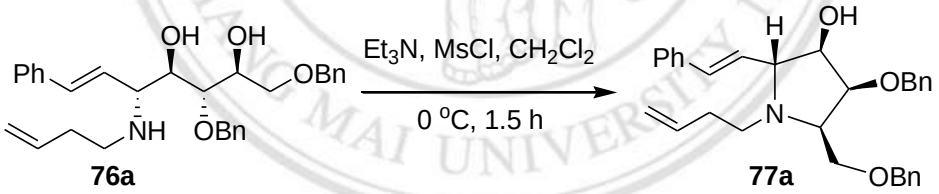
HRMS (ESI +ve) calculated for $C_{31}H_{38}NO_4$ ($M+H^+$) 488.2801, found 488.2784.

IR (cm^{-1}): 3376, 3029, 1452, 1088, 1072, 1028.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39-7.19 (m, 15H, Ar), 6.51 (d, *J* = 16.0 Hz, 1H, H-7), 6.23 (dd, *J* = 16.0, 9.0, 1H, H-6), 5.77-5.67 (m, 1H, H-3′), 5.07 (d, *J* = 18.0 Hz, 1H, H-4′a), 5.03 (d, *J* = 10.5 Hz, 1H, H-4′b), 4.64, 4.56 (ABq, J_{AB} = 12.5 Hz, 2H, C*H*HPh), 4.55, 4.47 (ABq, J_{AB} = 11.5 Hz, 2H, C*H*HPh), 4.04-4.00 (m[a], 2H, H-2 and H-4), 3.75-3.68 (m[a], 3H, H-1 and H-5), 3.65 (dd, *J* = 7.0, 4.5 Hz, 1H, H-3), 2.76-2.71 (m, 1H, H-1′a), 2.60-2.55 (m, 1H, H-1′b), 2.26 (q, *J* = 7.0 Hz, 2H, H-2′) [[a] indicates the overlapping of signals]. (Appendix B Figure 1, page 271)

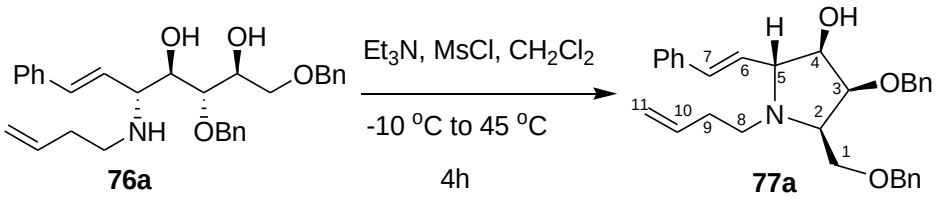
^{13}C NMR (125 MHz, $CDCl_3$) δ 138.5 (C), 138.0 (C), 136.4 (C), 135.7 (C-3′), 134.6 (C-7), 128.8 (2CH), 128.5 (4CH), 128.1 (3CH), 128.0 (2CH), 127.9 (CH), 127.7 (CH), 126.8 (2CH), 126.8 (C-6), 117.1 (C-4′), 79.7 (C-3), 73.8 (CH_2(Ph)), 73.5 (C-4), 72.9 (CH_2(Ph)), 71.7 (C-1), 68.8 (C-2), 62.8 (C-5), 46.0 (C-1′), 33.8 (C-2′). (Appendix B Figure 2, page 271)

3.6.2. (2*R*,3*R*,4*S*,5*R*)-4-(Benzyloxy)-5-(benzyloxymethyl)-1-(but-3-enyl)-2-styrylpyrrolidin-3-ol [77a]



76a **77a**

Procedure A: To a solution of **76a** (16.9 mg, 0.035 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C was added *via* syringe triethylamine (48 µL, 0.035 mmol) and 0.13 M solution of methanesulfonyl chloride in CH_2Cl_2 (0.27 mL, 0.035 mmol $MeSO_2Cl$). The reaction mixture was stirred at 0 °C for 1.5 h and quenched with sat. $NaHCO_3$. The organic phase was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford a brown oil (253). Purification by flash column chromatography (increasing polarity from 100:0 to 0:100 CH_2Cl_2/MeOH as eluent) afforded the title compound (6.5 mg, 40%) as a yellow oil. R_f 0.86 (1:9 MeOH/ CH_2Cl_2).



76a **77a**

Procedure B: To a solution of **76a** (80.0 mg, 0.164 mmol) in anhydrous CH_2Cl_2 (9 mL) at 0 °C was added Et_3N (0.023 mL, 0.164 mmol) under an atmosphere of N_2. The mixture was then cooled to –10 °C followed by the addition of a 0.13 M solution of methanesulfonyl chloride in anhydrous CH_2Cl_2 (1.51 mL, 0.197 mmol $MeSO_2Cl$). After complete addition, the reaction mixture was gradually warmed to 45 °C over 3 h and stirred for further 30 min under gentle reflux. The solution was subsequently concentrated *in vacuo* to afford a brown oil (254). Purification by flash column chromatography (increasing polarity from 10:90 to 20:80 EtOAc/petrol and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (63.9 mg, 83%) as a yellow oil. R_f 0.52 (3:7 EtOAc/petrol).

$[\alpha]_D^{25}$ –18.2 (*c* 0.7, $CHCl_3$).

MS (ESI +ve) *m/z* 470.4 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{31}H_{36}NO_3$ ($M+H^+$) 470.2695, found 470.2674.

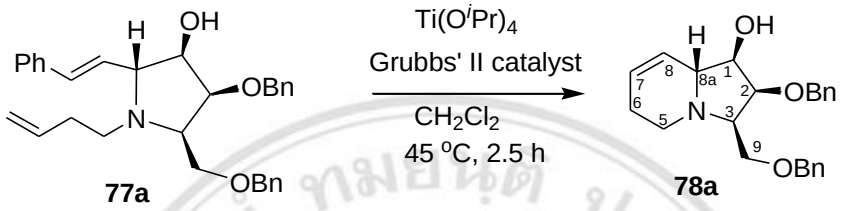
IR (cm^{-1}): 3405, 3061, 3028, 2863, 1640, 1599, 1495, 1452, 1363, 1099, 1055.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.20 (m, 15H, Ar), 6.56 (d, *J* = 16.0 Hz, 1H, H-7), 6.03 (dd, *J* = 16.0, 9.0 Hz, 1H, H-6), 5.79-5.72 (m, 1H, H-10), 4.99 (d, *J* = 17.0 Hz, 1H, H-11), 4.95 (d, *J* = 10.0 Hz, 1H, H-11′), 4.76, 4.51 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.60, 4.57 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.18 (t, *J* = 7.0 Hz, 1H, H-3), 4.01 (brs, 1H, H-4), 3.69-3.61 (m[a], 3H, H-1 and H-5), 3.43 (d, *J* = 8.0 Hz, 1H, H-2), 2.78-2.66 (m, 2H, H-8), 2.25-2.14 (m, 2H, H-9) [[a] indicates the overlapping of signals]. (Appendix B Figure 3, page 272)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 137.6 (C), 137.1 (C), 136.9 (C-10), 131.8 (C-7), 130.3 (C-6), 128.7 (2CH), 128.6 (2CH), 128.5 (2CH), 128.0 (2CH), 127.9 (CH), 127.8 (3CH), 127.6 (CH), 126.5 (2CH), 115.6 (C-11), 77.6 (C-3),

74.7 (C-4), 73.8 (CH_2(Ph)), 73.4 (C-5), 71.8 (CH_2(Ph)), 66.4 (C-1), 61.4 (C-2), 47.9 (C-8), 33.1 (C-9). (Appendix B Figure 4, page 272)

3.6.3. (1*R*,2*S*,3*R*,8a*R*)-2-(Benzyloxy)-3-(benzyloxymethyl)-1,2,3,5,6,8a-hexahydroindolizin-1-ol [78a]



To a solution of **77a** (63.9 mg, 0.136 mmol) in anhydrous CH_2Cl_2 (4.8 mL) under a N_2 atmosphere was added *via* syringe a solution of Ti(O^i-Pr)$_4$ (0.008 mL, 0.0272 mmol) in anhydrous CH_2Cl_2 (1.6 mL). The above solution was stirred at rt for 0.5 h, then Grubbs II catalyst (13.84 mg, 0.0163 mmol) was added. The reaction mixture was heated under reflux at 45 °C for 2.5 h, when TLC analysis showed complete consumption of **77a**. The reaction mixture was then diluted with CH_2Cl_2 (17 mL) and washed with sat. aq. $NaHCO_3$ (11 mL). The aqueous layer was further extracted with CH_2Cl_2 (17 mL). The organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to afford a dark brown oil (255). Purification by flash column chromatography (increasing polarity from 20:80 to 10:90 petrol/EtOAc and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (30.9 mg, 62%) as a brown oil. R_f 0.28 (1:4 petrol: EtOAc).

$[\alpha]_D^{25}$ +108.9 (*c* 0.2, $CHCl_3$).

MS (ESI +ve) *m/z* 366.3 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{23}H_{28}NO_3$ ($M+H^+$) 366.2069, found 366.2053.

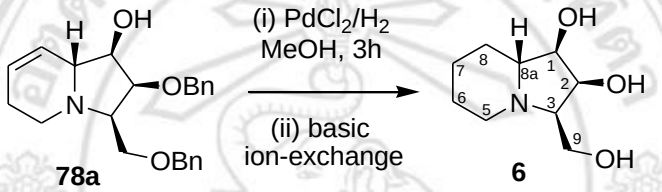
IR (cm^{-1}): 3259, 2922, 2854, 2364, 1731, 1631, 1452, 1362, 1143, 1084, 1025.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 10H, Ar), 5.84-5.82 (m, 1H, H-7), 5.62 (d, *J* = 9.0 Hz, 1H, H-8), 4.68, 4.54 (AB_q, J_{AB} = 11.5 Hz, 2H, CH*H*Ph), 4.62, 4.53(AB_q, J_{AB} = 11.5 Hz, 2H, CH*H*Ph), 4.15 (dd, *J* = 7.5, 4.0 Hz, 1H, H-2), 3.96 (brs, 1H, H-1), 3.77 (brs, 1H, H-8a), 3.63 (dd, *J* = 9.5, 4.5 Hz, 1H, H-9), 3.55 (dd,

J = 9.5, 3.0 Hz, 1H, H-9′), 3.29-3.27 (m, 1H, H-3), 3.04-3.00 (m, 2H, H-5), 2.24-2.22 (m, 1H, H-6), 1.77 (d, *J* = 17.5 Hz, 1H, H-6′) [[a] indicates the overlapping of signals]. (Appendix B Figure 5, page 273)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2 (C), 137.9 (C), 128.5 (2CH), 128.4 (2CH), 128.4 (C-8), 128.1 (2CH), 127.9 (2CH), 127.8 (CH), 127.7 (CH), 127.2 (C-7), 78.6 (C-2), 73.9 (CH_2(Ph)), 73.4 (C-1), 72.6 (CH_2(Ph)), 68.8 (C-9), 64.2 (C-8a), 60.9 (C-3), 44.6 (C-5), 19.3 (C-6). (Appendix B Figure 6, page 273)

3.6.4. (1*R*,2*S*,3*R*,8a*R*)-3-(Hydroxymethyl)octahydroindolizine-1,2-diol (10-*nor*-steviamine) [6]



To a solution of **78a** (28.6 mg, 0.078 mmol) in MeOH (1.7 mL) was added $PdCl_2$ (20.8 mg, 0.117 mmol). The mixture was stirred at rt under an atmosphere of H_2 (balloon) for 3 h. The mixture was filtered through a pad of Celite and the solids were washed with MeOH. The combined filtrates were evaporated *in vacuo* and the residue was dissolved in water (1.5 mL) and applied to a column of Amberlyst A-26 (OH^-) resin (3 cm) (256). Elution with water followed by evaporation *in vacuo* afforded the title compound (14.6 mg, 100%) as a brown oil.

$[\alpha]_D^{25}$ –7.7 (*c* 0.6, H_2O), $[\alpha]_D^{25}$ -11.4 (*c* 0.6, MeOH), lit. (194) $[\alpha]_D^{22}$ –8.7 (*c* 1.2, H_2O).

MS (ESI +ve) *m/z* 188.2 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_9H_{18}NO_3$ ($M+H^+$) 188.1287, found 188.1288.

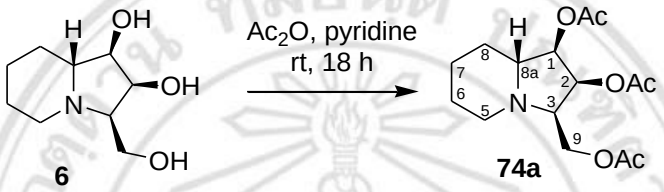
IR (cm^{-1}): 3324, 2929, 1636, 1596, 1445, 1141, 1105, 1083, 1049, 1007.

^{1}H NMR (500 MHz, D_2O) δ 4.40 (t, *J* = 6.5 Hz, 1H, H-2), 3.87 (dd, *J* = 12.0, 5.5 Hz, 1H, H-9), 3.82 (t, *J* = 6.0 Hz, 1H, H-1), 3.81 (dd, *J* = 12.0, 5.0 Hz, 1H, H-9′), 3.30 (dd, *J* = 12.5, 5.5 Hz, 1H, H-3), 2.96-2.93 (m, 1H, H-5α), 2.76-2.74 (m, 1H, H-8a), 2.72-2.67 (m, 1H, H-5β), 1.87-1.84 (m, 1H, H-8), 1.80-1.76 (m, 1H, H-7),

1.62-1.58 (m, 1H, H-6), 1.54-1.46 (m, 1H, H-6′), 1.41-1.32 (m, 1H, H-7′), 1.29-1.21 (m, 1H, H-8′). (Appendix B Figure 7, page 274)

^{13}C NMR (125 MHz, D_2O) δ 74.5 (C-1), 70.1 (C-2), 64.0 (C-3), 63.8 (C-8a), 58.5 (C-9), 47.3 (C-5), 27.2 (C-8), 23.1 (C-6), 22.7 (C-7). (Appendix B Figure 8, page 274)

3.6.5. (1*R*,2*S*,3*R*,8a*R*)-3-(Acetoxymethyl)octahydroindolizine-1,2-diyldiacetate [74a]



To a solution of **6** (3.0 mg, 0.016 mmol) in dry pyridine (0.055 mL, 0.68 mmol) was added Ac_2O (0.060 mL, 0.64 mmol). The mixture was stirred at rt for 18 h followed by the evaporation of all volatiles (257). The oily residue was purified by flash column chromatography (increasing polarity from 100:0 to 0:100 petrol/EtOAc and 20:80 MeOH/CH_2Cl_2 as eluent) to afford the title compound (4.3 mg, 86%) as a yellow oil. R_f 0.38 (1:1 petrol: EtOAc).

$[\alpha]_D^{25}$ +9.4 (*c* 0.2, $CHCl_3$).

MS (ESI +ve) *m/z* 314.3 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{15}H_{24}NO_6$ ($M+H^+$) 314.1604, found 314.1595.

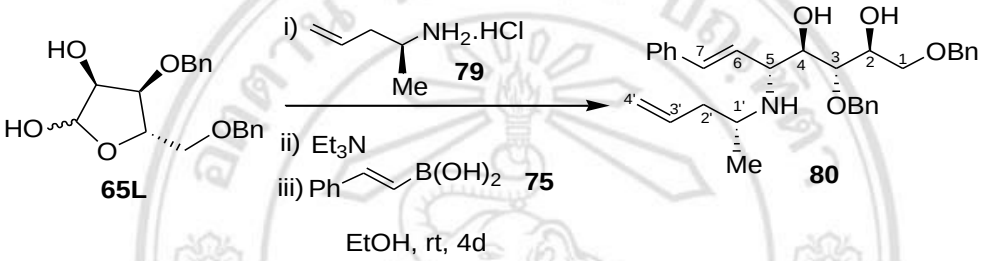
IR (cm^{-1}): 2935, 2855, 1738, 1440, 1369, 1220, 1148, 1131, 1091, 1038.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.44 (t, *J* = 6.5 Hz, 1H, H-2), 4.98 (dd, *J* = 6.5, 4.0 Hz, 1H, H-1), 4.26 (dd, *J* = 11.5, 6.5 Hz, 1H, H-9), 4.09 (dd, *J* = 11.5, 5.5 Hz, 1H, H-9′), 3.59 (dd, *J* = 13.0, 6.0 Hz, 1H, H-3), 3.04 (d, *J* = 12.5 Hz, 1H, H-5α), 2.93 (dt, *J* = 11.5, 4.0 Hz, 1H, H-8a), 2.75-2.69 (m, 1H, H-5β), 2.07 (s, 3H, Ac), 2.05 (s, 6H, 2Ac), 1.82-1.75 (m[a], 2H, H-6, H8), 1.49-1.42 (m[a], 2H, H-7), 1.36-1.26 (m, 1H, H-6′), 1.23-1.15 (m, 1H, H-8′) [[a] indicates the overlapping of signals]. (Appendix B Figure 11, page 277)

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (C=O), 170.4 (C=O), 169.9 (C=O), 75.3 (C-1), 70.2 (C-2), 62.8 (C-8a), 61.7 (C-9), 59.6 (C-3), 47.7 (C-5), 28.3 (C-8), 23.9 (C-6), 23.1 (C-7), 21.1 (CH_3), 20.9 (CH_3), 20.7 (CH_3). (Appendix B Figure 12, page 277)

3.7 Method for the synthesis of (–)-steviamine [1]

3.7.1. (2*S*,3*R*,4*R*,*E*)-1,3-Bis(benzyloxy)-5-((*R*)-pent-4-en-2-ylamino)-7-phenylhept-6-ene-2,4-diol [80]



To a solution of **65L** (1.00 g, 3.03 mmol) in absolute ethanol (25 mL) was added (*R*)-pent-4-en-2-amine·hydrochloride [**79**] (368 mg, 3.03 mmol, a commercial sample from NetChem, Inc. USA, >95% ee, $[\alpha]_D^{25}$ +4.0 (*c* 1.0, EtOH)) followed by Et_3N (0.42 mL, 3.03 mmol) and *trans*-2-phenylvinylboronic acid [**75**] (448 mg, 3.03 mmol). The mixture was stirred at rt for 4 days, followed by evaporation of all volatiles *in vacuo*. The residue was dissolved in CH_2Cl_2 (10 mL) and washed with sat. aq. $NaHCO_3$ (2×10 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford a brown foam (252). Purification by flash column chromatography (increasing polarity from 0:100 to 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (1.16 g, 77%) as a yellow foam. R_f 0.48 (10:90 MeOH/CH_2Cl_2).

$[\alpha]_D^{25}$ +47.9 (*c* 2.0, $CHCl_3$).

MS (ESI +ve) *m/z* 502.4 ($M+H^+$), 100%.

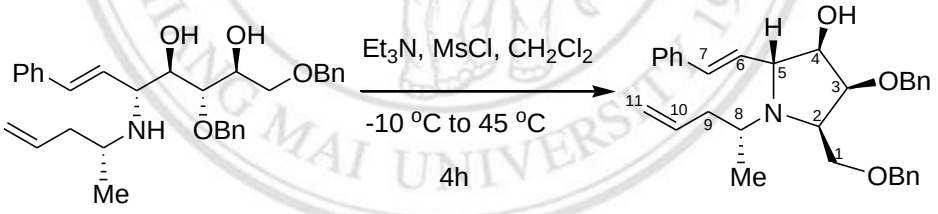
HRMS (ESI +ve) calculated for $C_{32}H_{40}NO_4$ ($M+H^+$) 502.2957, found 502.2938.

IR (cm^{-1}): 3289, 3072, 1452, 1072, 1028.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41-7.20 (m, 15H, Ar), 6.47 (d, *J* = 16.0 Hz, 1H, H-7), 6.16 (dd, *J* = 16.0, 9.0 Hz, 1H, H-6), 5.75-5.67 (m, 1H, H-3′), 5.05 (d, *J* = 16.0 Hz, 1H, H-4′a), 5.04 (d, *J* = 12.0 Hz, 1H, H-4′b), 4.63, 4.54 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.56, 4.48(AB_q, J_{AB} = 11.5 Hz, 2H, C*H*HPh), 4.06-4.03 (m, 1H, H-2), 3.95 (t, *J* = 4.5 Hz, 1H, H-4), 3.78 (dd, *J* = 9.0, 5.0 Hz, 1H, H-5), 3.75-3.66 (m^a, 3H, H-1, H-3), 2.88-2.76 (m, 1H, H-1′), 2.22-2.10 (m^a, 2H, H-2′), 1.04 (d, *J* = 6.5 Hz, 3H, CH_3) [^{a} indicating the overlapping of signals]. (Appendix B Figure 14, page 279)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 138.2 (C), 136.5 (C), 135.1 (C-3′), 133.9 (C-7), 128.7 (2CH), 128.5 (4CH), 128.0 (5CH), 127.9 (CH), 127.7 (CH), 126.5 (2CH), 127.5 (C-6), 117.9 (C-4′), 80.0 (C-3), 74.0 (C-4), 73.7 (CH_2(Ph)), 73.0 (CH_2(Ph)), 71.7 (C-1), 69.1 (C-2), 59.6 (C-5), 49.2 (C-1′), 42.1 (C-2′), 19.2 (CH_3). (Appendix B Figure 15, page 279)

3.7.2. (2*R*,3*R*,4*S*,5*R*)-4-(Benzyloxy)-5-(benzyloxymethyl)-1-((*R*)-pent-4-en-2-yl)-2-styrylpyrrolidin-3-ol [81]



80 **81**

To a solution of **80** (1.02 g, 2.02 mmol) in anhydrous CH_2Cl_2 (6 mL) at 0 °C was added Et_3N (1 mL, 7.08 mmol) under an atmosphere of N_2. The mixture was then cooled to –10 °C followed by the addition of a 0.13 M solution of methanesulfonyl chloride in anhydrous CH_2Cl_2 (17 mL, 2.18 mmol $MeSO_2Cl$). After complete addition, the reaction mixture was gradually warmed to 45 °C over 3 h and stirred for further 30 min with heating under a gentle reflux. The solution was subsequently concentrated *in vacuo* to afford a brown oil (254). Purification by flash column chromatography (increasing polarity from 10:90 to 20:80 EtOAc/petrol and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (642.2 mg, 66%) as a yellow oil. R_f 0.62 (3:7 EtOAc:Petrol).

[α]$_D^{25}$ –29.4 (*c* 1.0, $CHCl_3$).

MS (ESI +ve) *m/z* 484.3 ($M+H^+$), 100%.

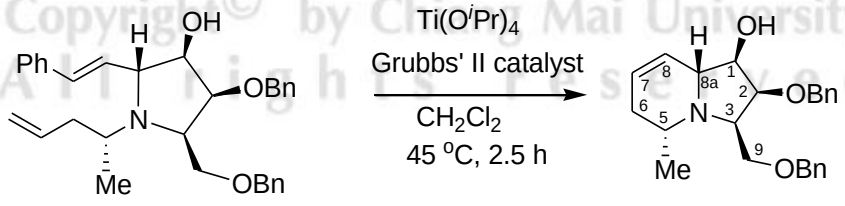
HRMS (ESI +ve) calculated for $C_{32}H_{38}NO_3$ ($M+H^+$) 484.2852, found 484.2832.

IR (cm^{-1}): 3393, 3062, 3027, 2906, 2869, 1496, 1452, 1365, 1178, 1138, 1116, 1098, 1055, 1026.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.19 (m, 15H, Ar), 6.52 (d, *J* = 16.0 Hz, 1H, H-7), 5.97 (dd, *J* = 16.0, 9.0 Hz, 1H, H-6), 5.84-5.76 (m, 1H, H-10), 5.03 (d, *J* = 17.0 Hz, 1H, H-11), 4.97 (d, *J* = 10.0 Hz, 1H, H-11′), 4.76, 4.48 (AB_q, J_{AB} = 12.0 Hz, 2H,C*H*HPh), 4.62 (d, *J* = 10.5 Hz, 1H, OH), 4.59, 4.55 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.15 (t, *J* = 5.5 Hz, 1H, H-3), 3.97 (dd, *J* = 10.5, 5.5 Hz, 1H, H-4), 3.88 (d, *J* = 9.0 Hz, 1H, H-5), 3.62-3.56 (m[a], 3H, H-1 and H-2), 3.08-3.02 (m, 1H, H-8), 2.40-2.34 (m, 1H, H-9), 2.17-2.11 (m, 1H, H-9′), 1.03 (d, *J* = 6.5 Hz, 1H, CH_3) [[a] indicates the overlapping of signals]. (Appendix B Figure 16, page 280)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.5 (C), 137.6 (C), 137.3 (C-10), 137.2 (C), 132.5 (C-6), 130.5 (C-7), 128.7 (2CH), 128.5 (2CH), 128.4 (2CH), 127.9 (3CH), 127.7 (3CH), 127.4 (CH), 126.4 (2CH) 115.7 (C-11), 77.9 (C-3), 74.9 (C-4), 73.8 (CH_2(Ph)), 71.4 (CH_2(Ph)), 70.0 (C-5), 68.0 (C-1), 58.9 (C-2), 51.4 (C-8), 39.6 (C-9), 17.9 (CH_3). (Appendix B Figure 17, page 280)

3.7.3. ((1*R*,2*S*,3*R*,5*R*,8a*R*)-2-(Benzyloxy)-3-(benzyloxymethyl)-5-methyl-1,2,3,5,6,8a-hexahydroindolizin-1-ol [82]



81 **82**

To a solution of **81** (490.7 mg, 1.01 mmol) in anhydrous CH_2Cl_2 (36 mL) under a N_2 atmosphere was added *via* syringe a solution of Ti(O^i-Pr)$_4$ (0.06 mL, 0.203 mmol) in anhydrous CH_2Cl_2 (11 mL). The above solution was stirred at rt for 0.5 h, then Grubbs II catalyst (155.3 mg, 0.183 mmol) was added. The reaction

mixture was heated at reflux at 45 °C for 2.5 h, when TLC analysis showed complete consumption of **81**. The reaction mixture was then diluted with CH_2Cl_2 (125 mL) and washed with sat. aq. $NaHCO_3$ (87 mL). The aqueous layer was further extracted with CH_2Cl_2 (125 mL). The organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to afford a dark brown oil as a crude product (255). Purification by flash column chromatography (increasing polarity from 50:50 to 0:100 petrol/EtOAc as eluent) afforded the title compound (294.2 mg, 76%) as a brown oil. R_f 0.28 (1:4 Petrol/EtOAc).

$[\alpha]_D^{25}$ +38.6 (*c* 1.4, $CHCl_3$).

MS (ESI +ve) *m/z* 380.2 ($M+H^+$), 100%.

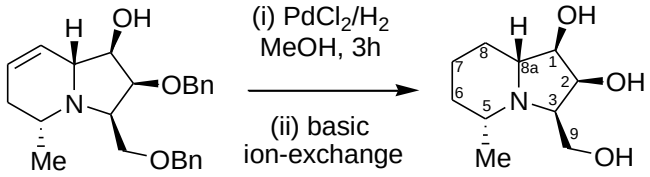
HRMS (ESI +ve) calculated for $C_{24}H_{30}NO_3$ ($M+H^+$) 380.2226, found 380.2214.

IR (cm^{-1}): 3382, 3015, 2928, 2874, 2316, 1496, 1451, 1152, 1055.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34-7.21 (m, 10H, Ar), 5.79-5.76 (m, 1H, H-7), 5.53 (d, *J* = 10.5 Hz, 1H, H-8), 4.93 (d, *J* = 9.5 Hz, 1H, OH), 4.71, 4.45 (AB_q, J_{AB} = 11.5 Hz, 2H, CH*H*Ph), 4.69, 4.54 (AB_q, J_{AB} = 12.0 Hz, 2H, CH*H*Ph), 4.12 (dd, *J* = 9.5, 4.5 Hz, 1H, H-2), 3.97 (dd, *J* = 9.5, 4.5 Hz, 1H, H-1), 3.91 (brs, 1H, H-8a), 3.58 (d, *J* = 9.0 Hz, 1H, H-9), 3.41 (dd, *J* = 9.5, 3.0 Hz, 1H, H-9′), 3.34 (brd, *J* = 9.5 Hz, 1H, H-3), 3.27-3.20 (m, 1H, H-5), 2.04-1.98 (m, 1H, -6), 1.77-1.72 (m, 1H, H-6′), 1.17 (d, *J* = 7.0 Hz, 3H, CH_3). (Appendix B Figure 18, page 281)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 137.5 (C), 129.1 (C-8), 128.4 (2CH), 128.3 (2CH), 128.2 (2CH), 127.8 (2CH), 127.6 (2CH), 127.7 (C-7), 78.2 (C-2), 74.0 (CH_2(Ph)), 71.5 (CH_2(Ph)), 71.5 (C-1), 70.6 (C-9), 66.7 (C-8a), 55.7 (C-3), 50.6 (C-5), 26.1 (C-6), 21.3 (CH_3). (Appendix B Figure 19, page 281)

3.7.4. (1*R*,2*S*,3*R*,5*R*,8a*R*)-3-(Hydroxymethyl)-5-methyloctahydroindolizine-1,2-diol ((–)-steviamine) [1]



82 **1**

To a solution of **82** (183.5 mg, 0.484 mmol) in MeOH (10 mL) was added $PdCl_2$ (171.5 mg, 0.967 mmol). The mixture was stirred at rt under an atmosphere of H_2 (balloon) for 3 h. The mixture was filtered through a celite pad and the solids were washed with MeOH. The combined filtrates were evaporated *in vacuo* and the residue was dissolved in water (10 mL) and applied to a column of Amberlyst A-26 (OH^-) resin (3 cm) (256). Elution with water followed by evaporation *in vacuo* afforded the title compound (98.0 mg, 100%) as a brown oil.

$[\alpha]_D^{25}$ –23.8 (*c* 1.0, MeOH), lit. (189) $[\alpha]_D^{25}$ –22.0 (*c* 1.0, MeOH).

MS (ESI +ve) *m/z* 202.0 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{10}H_{20}NO_3$ ($M+H^+$) 202.1443, found 202.1465.

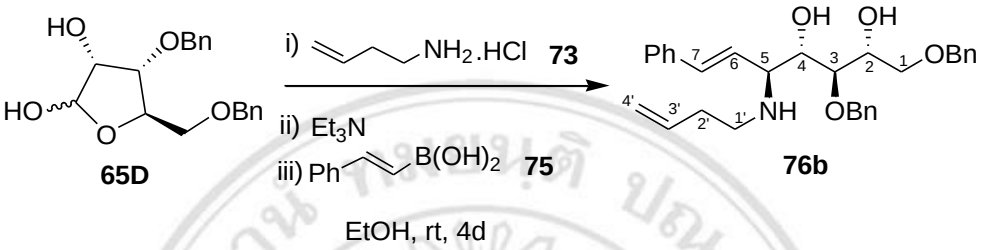
IR (cm^{-1}): 3329, 2929, 2855, 1631, 1441, 1379, 1315, 1214, 1137, 1097, 1079, 1036, 1006.

^{1}H NMR (500 MHz, D_2O) δ 4.33 (t, *J* = 7.5 Hz, 1H, H-2), 3.96 (dd, *J* = 12.5, 5.5 Hz, 1H, H-9), 3.91 (dd, *J* = 12.0, 3.5 Hz, 1H, H-9′), 3.80 (t, *J* = 7.0 Hz, 1H, H-1), 3.52 (dd, *J* = 9.5, 6.5 Hz, 1H, H-3), 2.85-2.82 (m, 1H, H-5), 2.67-2.64 (m, 1H, H-8a), 2.00 (brd, *J* = 12.5 Hz, 1H, H-8), 1.81(brd, *J* = 13.0 Hz, 1H, H-7), 1.74 (brd, *J* = 13.0 Hz, 1H, H-6), 1.42-1.34 (m, 1H, H-7′), 1.21-1.12 (m^a, 5H, H-6′, H-8′ and CH_3) [^{a} indicates the overlapping of signals]. (Appendix B Figure 20, page 282)

^{13}C NMR (125 MHz, D_2O) δ 74.1 (C-1), 69.3 (C-2), 66.9 (C-8a), 61.5 (C-3), 56.7 (C-9), 52.8 (C-5), 33.6 (C-6), 29.5 (C-8), 23.9 (C-7), 19.4 (CH_3). (Appendix B Figure 21, page 282)

3.8 Method for the synthesis of 10-*nor*-*ent*-steviamine [83] and its triacetate derivative [74b]

3.8.1. (2*R*,3*S*,4*S*,*E*)-1,3-Bis(benzyloxy)-5-(but-3-enylamino)-7-phenylhept-6-ene-2,4-diol [76b]



To a solution of **65D** (200.0 mg, 0.605 mmol) in absolute ethanol (5 mL) was added 3-butenylamine·hydrochloride [**73**] (65.1 mg, 0.605 mmol) followed by Et_3N (0.084 mL, 0.605 mmol) and *trans*-2-phenylvinyl boronic acid [**75**] (89.6 mg, 0.605 mmol). The mixture was stirred at rt for 4 d, followed by evaporation of all volatiles *in vacuo*. The residue was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. $NaHCO_3$ (2×5 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford a brown foam (252). Purification by flash column chromatography (increasing polarity from 0:100 to 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (167.9 mg, 57%) as a brown foam. R_f 0.48 (10:90 MeOH/CH_2Cl_2).

$[\alpha]_D^{25}$ –58.7 (*c* 1.0, $CHCl_3$).

MS (ESI +ve) *m/z* 488.6 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{31}H_{38}NO_4$ ($M+H^+$) 488.2801, found 488.2798.

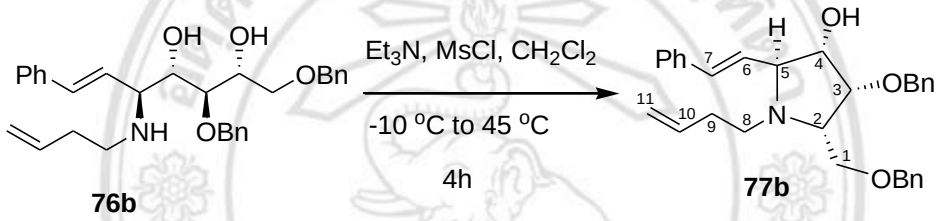
IR (cm^{-1}): 3381, 3063, 3028, 2906, 2858, 1496, 1452, 1089, 1073, 1028.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38-7.18 (m, 15H, Ar), 6.48 (d, *J* = 16.0 Hz, 1H, H-7), 6.21 (dd, *J* = 16.0, 9.0, 1H, H-6), 5.76-5.68 (m, 1H, H-3′), 5.05 (d, *J* = 18.0 Hz, 1H, H-4′a), 5.02 (d, *J* = 10.5 Hz, 1H, H-4′b), 4.62, 4.54 (ABq, J_{AB} = 12.5 Hz, 2H, C*H*HPh), 4.53, 4.45 (ABq, J_{AB} = 11.0 Hz, 2H, C*H*HPh), 4.07-4.04 (m, 1H, H-2), 3.98-3.96 (t, *J* = 4.5 Hz, 1H, H-4), 3.76-3.69 (m^a, 2H, H-1), 3.65-3.63 (m^a, 2H,

H-3 and H-5), 2.72-2.67 (m, 1H, H-1′a), 2.56-2.51 (m, 1H, H-1′b), 2.24-2.20 (m, 2H, H-2′) [[a] indicates the overlapping of signals].

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 138.0 (C), 136.4 (C), 135.8 (C-3′), 134.1 (C-7), 128.6 (2CH), 128.4 (2CH), 128.3 (2CH), 128.0 (2CH), 127.9 (3CH), 127.8 (CH), 127.5 (CH), 126.6 (2CH), 127.1 (C-6), 116.8 (C-4′), 79.6 (C-3), 73.6 (CH_2(Ph)), 73.6 (C-4), 72.8 (CH_2(Ph)), 71.6 (C-1), 68.7 (C-2), 62.7 (C-5), 45.9 (C-1′), 33.8 (C-2′).

3.8.2. (2*S*,3*S*,4*R*,5*S*)-4-(Benzyloxy)-5-(benzyloxymethyl)-1-(but-3-enyl)-2-styrylpyrrolidin-3-ol [77b]



76b **77b**

To a solution of **76b** (162.9 mg, 0.334 mmol) in anhydrous CH_2Cl_2 (1.5 mL) at 0 °C was added Et_3N (0.16 mL, 1.169 mmol) under an atmosphere of N_2. The mixture was then cooled to –10 °C followed by addition of a 0.13 M solution of methanesulfonyl chloride in anhydrous CH_2Cl_2 (2.8 mL, 0.359 mmol $MeSO_4Cl$). After complete addition, the reaction mixture was gradually warmed to 45 °C over 3 h and stirred for further 30 min under gentle reflux. The solution was subsequently concentrated *in vacuo* to afford a brown oil (254). Purification by flash column chromatography (increasing polarity from 10:90 to 20:80 EtOAc/petrol and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (90.5 mg, 58%) as a yellow oil. R_f 0.52 (3:7 EtOAc/petrol).

$[\alpha]_D^{25}$ +35.4 (*c* 0.8, $CHCl_3$).

MS (ESI +ve) *m/z* 470.1 ($M+H^+$), 100%.

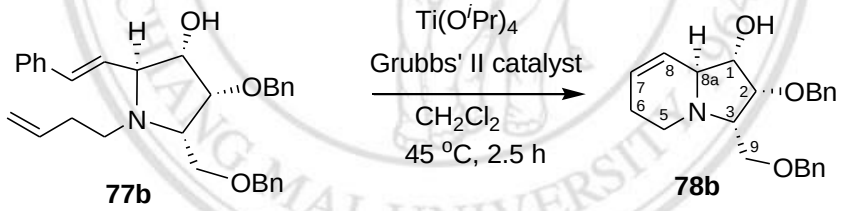
HRMS (ESI +ve) calculated for $C_{31}H_{36}NO_3$ ($M+H^+$) 470.2695, found 470.2709.

IR (cm^{-1}): 3405, 3061, 3028, 2863, 1640, 1599, 1495, 1452, 1363, 1099, 1055.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.19 (m, 15H, Ar), 6.55 (d, *J* = 16.5 Hz, 1H, H-7), 6.03 (dd, *J* = 15.5, 9.0 Hz, 1H, H-6), 5.80-5.72 (m, 1H, H-10), 4.99 (d, *J* = 17.0 Hz, 1H, H-11), 4.95 (d, *J* = 10.0 Hz, 1H, H-11′), 4.75, 4.51 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.59, 4.56 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.17 (t, *J* = 6.5 Hz, 1H, H-3), 4.02-4.00 (m, 1H, H-4), 3.68-3.61 (m[a], 3H, H-1 and H-5), 3.42 (d, *J* = 8.0 Hz, 1H,H-2), 2.78-2.65 (m, 2H, H-8), 2.27-2.14 (m, 2H, H-9) [[a] indicates the overlapping of signals].

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.3 (C), 137.6 (C), 137.0 (C), 136.9 (C-10), 131.7 (C-7), 130.4 (C-6), 128.6 (2CH), 128.5 (2CH), 128.4 (2CH), 127.9 (3CH), 127.7 (3CH), 127.5 (CH), 126.4 (2CH), 115.5 (C-11), 77.6 (C-3), 74.7 (C-4), 73.8 (CH_2(Ph)), 73.4 (C-5), 71.7 (CH_2(Ph)), 66.4 (C-1), 61.3 (C-2), 47.9 (C-8), 33.0 (C-9).

3.8.3. (1*S*,2*R*,3*S*,8a*S*)-2-(Benzyloxy)-3-(benzyloxymethyl)-1,2,3,5,6,8a-hexahydroindolizin-1-ol [78b]



To a solution of **77b** (85.5 mg, 0.182 mmol) in anhydrous CH_2Cl_2 (6.4 mL) under a N_2 atmosphere was added *via* syringe a solution of Ti(O^i-Pr)$_4$ (0.0107 mL, 0.036 mmol) in anhydrous CH_2Cl_2 (2.1 mL). The above solution was stirred at rt for 0.5 h, then Grubbs II catalyst (27.8 mg, 0.033 mmol) was added. The reaction mixture was heated under reflux at 45 °C for 2.5 h, when TLC analysis showed complete consumption of **77b.** The reaction mixture was then diluted with CH_2Cl_2 (23 mL) and washed with sat. aq. $NaHCO_3$ (15 mL). The aqueous layer was further extracted with CH_2Cl_2 (23 mL). The organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to afford a dark brown oil (255). Purification by flash column chromatography (increasing polarity from 50:50 to 0:100 petrol/EtOAc and 100% CH_2Cl_2 as eluent) afforded the title compound (41.8 mg, 63%) as a brown oil. R_f 0.28 (1:4 petrol/EtOAc).

[α]$_D^{25}$ –114.5 (*c* 0.2, $CHCl_3$).

MS (ESI +ve) *m/z* 366.1 ($M+H^+$), 100%.

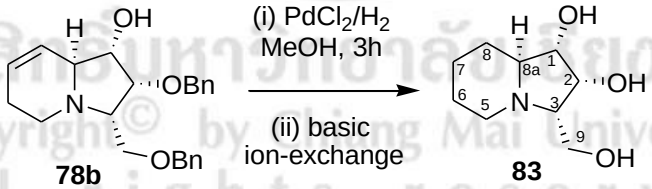
HRMS (ESI +ve) calculated for $C_{23}H_{28}NO_3$ ($M+H^+$) 366.2069, found 366.2065.

IR (cm^{-1}): 3317, 3062, 3029, 2922, 2862, 2359, 2314, 1634, 1453, 1363, 1207, 1141, 1082, 1055, 1026.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34-7.24 (m, 10H, Ar), 5.84-5.81 (m, 1H, H-7), 5.61 (d, *J* = 9.5 Hz, 1H, H-8), 4.68, 4.52 (AB_q, J_{AB} = 12.0 Hz, 2H, CH*H*Ph), 4.62, 4.54 (AB_q, J_{AB} = 12.0 Hz, 2H, CH*H*Ph), 4.14 (dd, *J* = 7.5, 4.0 Hz, 1H, H-2), 3.96 (brs, 1H, H-1), 3.76 (brs, 1H, H-8a), 3.62 (dd, *J* = 10.0, 5.0 Hz, 1H, H-9), 3.55 (dd, *J* = 9.5, 3.0 Hz, 1H, H-9′), 3.29-3.26 (m, 1H, H-3), 3.06-2.97 (m, 2H, H-5), 2.26-2.20 (m, 1H, H-6), 1.76 (d, *J* = 17.5 Hz, 1H, H-6) [[a] indicates the overlapping of signals].

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2 (C), 137.8 (C), 128.4 (2CH), 128.3 (2CH), 128.0 (CH), 128.0 (C-8),127.8 (4CH), 127.7 (CH), 127.1 (C-7), 78.5 (C-2), 73.8 (CH_2(Ph)), 73.3 (C-1), 72.6 (CH_2(Ph)), 68.7 (C-9), 64.1 (C-8a), 60.9 (C-3), 44.5 (C-5), 19.2 (C-6).

3.8.4. (1*S*,2*R*,3*S*,8a*S*)-3-(Hydroxymethyl)octahydroindolizine-1,2-diol (10-*nor*-*ent*-steviamine)[83]



To a solution of **78b** (37.3 mg, 0.102 mmol) in MeOH (2.2 mL) was added $PdCl_2$ (36.2 mg, 0.204 mmol). The mixture was stirred at rt under an atmosphere of H_2 (balloon) for 3 h. The mixture was filtered through a pad of Celite and the solids were washed with MeOH. The combined filtrates were evaporated *in vacuo* and the residue was dissolved in water (3 mL) and applied to a column of Amberlyst A-26 ($OH^–$) resin (3 cm) (256). Elution with water followed by evaporation *in vacuo* afforded the title compound (19.2 mg, 100%) as a brown oil.

$[\alpha]_D^{25}$ +70.9 (c 1.0, H_2O), $[\alpha]_D^{25}$+23.8 (*c* 0.6, MeOH).

MS (ESI +ve) *m/z* 188.1($M+H^+$, 100%).

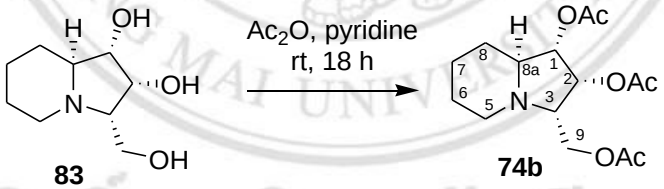
HRMS (ESI +ve) calculated for $C_9H_{18}NO_3$ ($M+H^+$) 188.1287, found 188.1250.

IR (cm^{-1}): 3299, 2931, 2856, 1634, 1595, 1446, 1339, 1139, 1106, 1086, 1049, 1034.

^{1}H NMR (500 MHz, D_2O) δ 4.40 (t, *J* = 6.5 Hz, 1H,H-2), 3.88 (dd, , *J* = 12.0, 5.5 Hz, 1H H-9), 3.83 (t, *J* = 6.5 Hz, 1H, H-1), 3.81 (dd, *J* = 12.5, 5.0 Hz, 1H, H-9′), 3.30 (dd, *J* = 12.0, 5.0 Hz, 1H, H-3), 2.98-2.94 (m, 1H, H-5α), 2.80-2.76 (m, 1H, H-8a), 2.74-2.69 (m, 1H, H-5β), 1.88-1.84 (m, 1H, H-8), 1.80-1.76 (m, 1H, H-7), 1.62-1.58 (m, 1H, H-6), 1.55-1.46 (m, 1H, H-6′), 1.41-1.32 (m, 1H, H-7′), 1.30-1.22 (m, 1H, H-8′).

^{13}C NMR (125 MHz, D_2O) δ 74.4 (C-1), 70.4 (C-2), 64.4 (C-3), 64.1 (C-8a), 58.8 (C-9), 47.7 (C-5), 27.5 (C-8), 23.3 (C-6), 22.9 (C-7).

3.8.5. (1*S*,2*R*,3*S*,8a*S*)-3-(Acetoxymethyl)octahydroindolizine-1,2-diyl diacetate [74b]



To a solution of **83** (5.0 mg, 0.027 mmol) in dry pyridine (0.11 mL, 1.36 mmol) was added Ac_2O (0.12 mL, 1.27 mmol). The mixture was stirred at rt for 18 h followed by evaporation of all volatiles (257). The oily residue was purified by flash column chromatography (increasing polarity from 100:0 to 0:100 petrol/EtOAc and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (5.3 mg, 63%) as a yellow oil. R_f 0.25 (1:1 petrol: EtOAc).

$[\alpha]_D^{25}$ –9.2 (*c* 0.2, $CHCl_3$).

MS and HRMS (ESI +ve) were the same as those for compound **74a** (enantiomer).

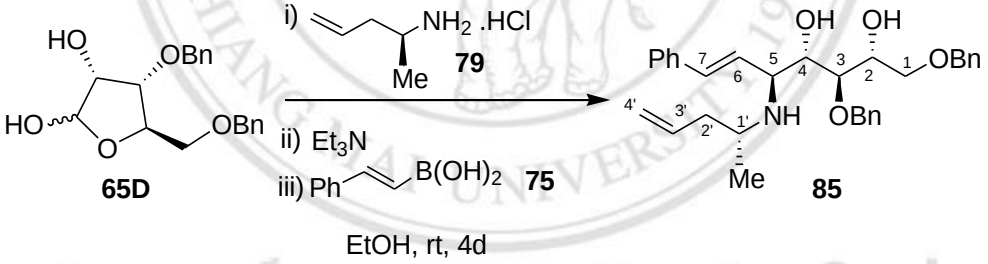
IR (cm^{-1}): 2936, 1738, 1440, 1369, 1220, 1148, 1131, 1091, 1038.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.45 (t, *J* = 7.0 Hz, 1H, H-2), 4.98 (dd, *J* = 6.5, 4.0 Hz, 1H, H-1), 4.26 (dd, *J* = 11.5, 6.0 Hz, 1H, H-9), 4.09 (dd, *J* = 11.5, 6.0 Hz, 1H, H-9′), 3.59 (dd, *J* = 13.0, 6.0 Hz, 1H, H-3), 3.04 (d, *J* = 12.5 Hz, 1H, H-5α), 2.94 (dt, *J* = 11.5, 3.5 Hz, 1H, H-8a), 2.75-2.70 (m, 1H, H-5β), 2.08 (s, 3H, Ac), 2.05 (s, 6H, 2Ac), 1.82-1.75 (m[a], 2H, H-6, H8), 1.49-1.42 (m[a], 2H, H-7), 1.36-1.25 (m, 1H, H-6′), 1.23-1.15 (m, 1H, H-8′). [[a] indicates the overlapping of signals].

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9 (C=O), 170.4 (C=O), 170.0 (C=O), 75.3 (C-1), 70.2 (C-2), 62.7 (C-8a), 61.7 (C-9), 59.5 (C-3), 47.7 (C-5), 28.2 (C-8), 23.8 (C-6), 23.1 (C-7), 21.1 (CH_3), 21.0 (CH_3), 20.7 (CH_3).

3.9 Method for the synthesis of 5-*epi*-*ent*-steviamine [7] and its triacetate derivative [84]

3.9.1. (2*R*,3*S*,4*S*,*E*)-1,3-Bis(benzyloxy)-5-((*R*)-pent-4-en-2-ylamino)-7-phenylhept-6-ene-2,4-diol [85]



To a solution of **65D** (200.0 mg, 0.605 mmol) in absolute ethanol (5 mL) was added (*R*)-pent-4-en-2-amine·hydrochloride [**79**] (73.6 mg, 0.605 mmol) followed by Et_3N (0.084 mL, 0.605 mmol) and *trans*-2-phenylvinylboronic acid [**75**] (89.6 mg, 0.605 mmol). The mixture was stirred at rt for 4 d, followed by the evaporation of all volatiles *in vacuo*. The residue was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. $NaHCO_3$ (2×5 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford a brown foam (252). Purification by flash column chromatography (increasing polarity from 0:100 to 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (248 mg, 82%) as a brown oil. R_f 0.45 (10:90 MeOH/CH_2Cl_2).

$[\alpha]_D^{25}$ –35.7 (*c* 0.8, $CHCl_3$).

MS (ESI +ve) *m/z* 502.3 ($M+H^+$), 100%.

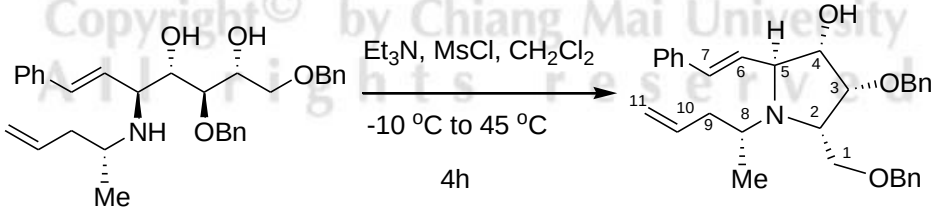
HRMS (ESI +ve) calculated for $C_{32}H_{40}NO_4$ ($M+H^+$) 502.2957, found 502.2941.

IR (cm^{-1}): 3366, 3062, 3029, 2925, 2863, 1641, 1599, 1495, 1452, 1373, 1092, 1072.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39-7.16 (m, 15H, Ar), 6.48 (d, *J* = 16.0 Hz, 1H, H-7), 6.24 (dd, *J* = 16.0, 9.0 Hz, 1H, H-6), 5.78-5.69 (m, 1H, H-3′), 5.08 (d, *J* = 11.5 Hz, 1H, H-4′a), 5.07 (d, *J* = 14.5 Hz, 1H, H-4b′), 4.63, 4.55 (AB_q, J_{AB} = 12.5 Hz, 2H, C*H*HPh), 4.52, 4.45 (AB_q, J_{AB} = 11.0 Hz, 2H, C*H*HPh), 4.05-4.02 (m, 1H, H-2), 3.95 (t, *J* = 4.0 Hz, 1H, H-4), 3.82 (dd, *J* = 8.5, 4.0 Hz, 1H, H-5), 3.76-3.69 (m[a], 2H, H-1), 3.64 (dd, *J* = 7.5, 4.5 Hz, 1H, H-3), 2.82-2.78 (m, 1H, H-1′), 2.25-2.20 (m, 1H, H-2′a), 2.12-2.07 (m, 1H, H-2′b), 1.06 (d, *J* = 6.5 Hz, 3H, CH_3) [[a] indicates the overlapping of signals]. (Appendix B Figure 22, page 283)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 138.0 (C), 136.5 (C), 134.6 (C-3′), 133.8 (C-7), 128.7 (2CH), 128.5 (2CH), 128.4 (2CH), 128.0 (5CH), 127.9 (CH), 127.5 (CH), 126.7 (2CH), 126.5 (2CH)127.6 (C-6), 118.0 (C-4′), 79.5 (C-3), 73.8 (C-4), 73.7 (CH_2(Ph)), 72.8 (CH_2(Ph)), 71.6 (C-1) 68.6 (C-2), 60.2 (C-5), 49.4 (C-1′), 39.5 (C-2′), 21.2 (CH_3). (Appendix B Figure 23, page 283)

3.9.2. (2*S*,3*S*,4*R*,5*S*)-4-(Benzyloxy)-5-(benzyloxymethyl)-1-((*R*)-pent-4-en-2-yl)-2-styrylpyrrolidin-3-ol [86]



85 **86**

To a solution of **85** (241.2 mg, 0.481 mmol) in anhydrous CH_2Cl_2 (1.6 mL) at 0 °C was added Et_3N (0.23 mL, 1.68 mmol) under an atmosphere of N_2. The mixture was then cooled to –10 °C followed by addition of a 0.13 M solution of methanesulfonyl chloride in anhydrous CH_2Cl_2 (4.10 mL, 0.529 mmol $MeSO_2Cl$).

After complete addition, the reaction mixture was gradually warmed to 45 °C over 3 h and stirred for further 30 min under gentle reflux. The solution was subsequently concentrated *in vacuo* to afford a brown oil as a crude product (254). Purification by flash column chromatography (increasing polarity from 10:90 to 20:80 EtOAc/petrol and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (161 mg, 69%) as a brown oil. R_f 0.62 (3:7 EtOAc/petrol).

$\mathbf{[\alpha]_D^{25}}$ +20.0 (*c* 0.5 $CHCl_3$).

MS (ESI +ve) *m/z* 484.3 ($M+H^+$, 100%).

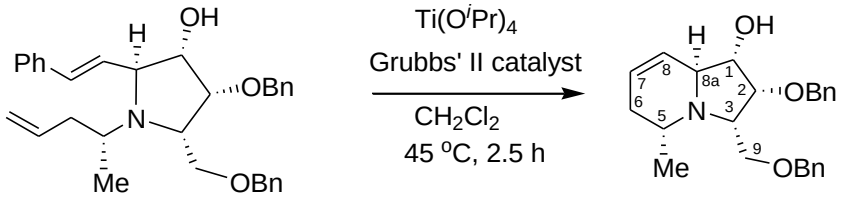
HRMS (ESI +ve) calculated for $C_{32}H_{38}NO_3$ ($M+H^+$) 484.2852, found 484.2837.

IR (cm^{-1}): 3384, 3062, 3028, 2931, 2869, 1725, 1640, 1495, 1452, 1174, 1140, 1087, 1054, 1026.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37-7.20 (m, 15H, Ar), 6.51 (d, *J* = 16.5 Hz, 1H, H-7), 5.98 (dd, *J* = 16.0, 9.0 Hz, 1H, H-6), 5.81-5.72 (m, 1H, H-10), 4.97 (d, *J* = 15.5 Hz, 1H, H-11), 4.95 (d, *J* = 11.5 Hz, 1H, H-11′), 4.77, 4.49 (AB_q, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.71 (d, *J* = 10.0 Hz, 1H, OH), 4.61, 4.57 (AB_q, J_{AB} = 11.5 Hz, 2H, C*H*HPh), 4.19 (dd, *J* = 8.0, 5.5 Hz, 1H, H-3), 3.99 (dd, *J* = 8.0, 6.5 Hz, 1H, H-4), 3.91 (d, *J* = 9.0 Hz, 1H, H-5), 3.62-3.54 (m^a, 3H, H-1 and H-2), 3.06-2.99 (m, 1H, H-8), 2.43-2.38 (m, 1H, H-9), 1.98-1.92 (m, 1H, H-9′), 1.12 (d, *J* = 7.0 Hz, 1H, CH_3) [^{a} indicates the overlapping of signals]. (Appendix B Figure 24, page 284)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.5 (C), 137.5 (C), 137.3 (C-10), 137.3 (C), 132.5 (C-6), 130.6 (C-7), 128.7 (2CH), 128.6 (2CH), 128.5 (2CH), 128.0 (2CH), 127.9 (CH), 127.7 (3CH), 127.5 (CH), 126.4 (2CH), 116.0 (C-11), 77.8 (C-3), 74.9 (C-4), 73.8 (CH_2(Ph)), 71.5 (CH_2(Ph)), 70.5 (C-5), 68.1 (C-1), 59.8 (C-2), 52.1 (C-8), 40.4 (C-9), 18.5 (CH_3). (Appendix B Figure 25, page 284)

3.9.3. (1*S*,2*R*,3*S*,5*R*,8a*S*)-2-(Benzyloxy)-3-(benzyloxymethyl)-5-methyl-1,2,3,5,6,8a-hexahydroindolizin-1-ol [87]



86 **87**

To a solution of **86** (62.5 mg, 0.129 mmol) in anhydrous CH_2Cl_2 (4.4 mL) under a N_2 atmosphere was added *via* syringe a solution of Ti(O^i-Pr)$_4$ (0.0076 mL, 0.026 mmol) in anhydrous CH_2Cl_2 (1.5 mL). The above solution was stirred at rt for 0.5 h, then Grubbs II catalyst (19.8 mg, 0.023 mmol) was added. The reaction mixture was heated under reflux at 45 °C for 2.5 h, when TLC analysis showed complete consumption of **86**. The reaction mixture was then diluted with CH_2Cl_2 (16 mL) and washed with sat. aq. $NaHCO_3$ (11 mL). The aqueous layer was further extracted with CH_2Cl_2 (16 mL). The organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to afford a dark brown oil (255). Purification by flash column chromatography (increasing polarity from 20:80 to 10:90 petrol/EtOAc as eluent) afforded the title compound (23.8 mg, 83%) as a brown oil. R_f 0.28 (1:4 EtOAc/petrol).

[α]$_D^{25}$ –7.7 (*c* 0.9, $CHCl_3$).

MS (ESI +ve) *m/z* 380.2 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{24}H_{30}NO_3$ ($M+H^+$) 380.2226, found 380.2227.

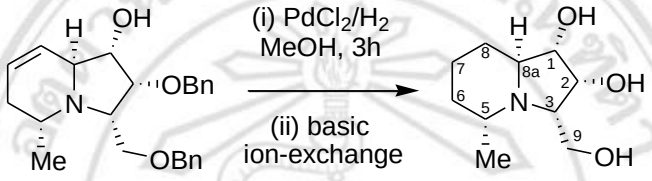
IR (cm^{-1}): 3376, 3030, 2925, 2869, 2358, 1636, 1453, 1375, 1265, 1207, 1087, 1060, 1027.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 10H, Ar), 5.73-5.70 (m, 1H, H-7), 5.60 (dd, *J* = 10.0, 1.0 Hz, 1H, H-8), 4.68, 4.57 (ABq, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.68, 4.60 (ABq, J_{AB} = 12.0 Hz, 2H, C*H*HPh), 4.13 (dd, *J* = 8.0, 4.0 Hz, 1H, H-2), 3.96 (brs, 1H, H-1), 3.71 (brs, 1H H-8a), 3.63 (dd, *J* = 9.5, 5.0 Hz, 1H, H-9), 3.55 (brd, *J* = 8.5 Hz, 1H, H-9′), 3.25-3.21 (m ,1H, H-3), 3.04 (brs, 1H, H-

5), 2.37-2.32 (m, 1H, H-6), 1.63 (m, 1H, H-6′), 1.18 (d, *J* = 7.0 Hz, 3H, CH_3). (Appendix B Figure 26, page 285)

^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4 (C), 138.2 (C), 128.5 (2CH), 128.4 (2CH), 127.9 (2CH), 127.8 (3CH), 127.7 (CH), 127.7 (C-8), 125.3 (C-7), 78.4 (C-2), 73.8 (CH_2(Ph)), 73.2 (C-1), 72.6 (CH_2(Ph)), 69.3 (C-9), 62.3 (C-3), 60.6 (C-8a), 48.8 (C-5), 25.6 (C-6), 20.0 (CH_3). (Appendix B Figure 27, page 285)

3.9.4. (1*S*,2*R*,3*S*,5*R*,8a*S*)-3-(Hydroxymethyl)-5-methyloctahydroindolizine-1,2-diol (5-*epi*-*ent*-steviamine) [7]



87 **7**

To a solution of **87** (37.0 mg, 0.098 mmol) in MeOH (2 mL) was added $PdCl_2$ (34.6 mg, 0.195 mmol). The mixture was stirred at rt under an atmosphere of H_2 (balloon) for 3 h. The mixture was filtered through a pad of Celite and the solids were washed with MeOH. The combined filtrates were evaporated *in vacuo* and the residue was dissolved in water (1.5 mL) and applied to a column of Amberlyst A-26 (OH^-) resin (3 cm) (256). Elution with water followed by evaporation *in vacuo* afforded the title compound (19.6 mg, 100.0%) as a brown oil.

$[\alpha]_D^{25}$ –4.6 (*c* 1.0, MeOH), lit. (193) $[\alpha]_D^{20}$ –1.2 (*c* 1.0, MeOH).

MS (ESI +ve) *m/z* 202.1 ($M+H^+$), 100%.

HRMS (ESI +ve) calculated for $C_{10}H_{20}NO_3$ ($M+H^+$) 202.1443, found 202.1450.

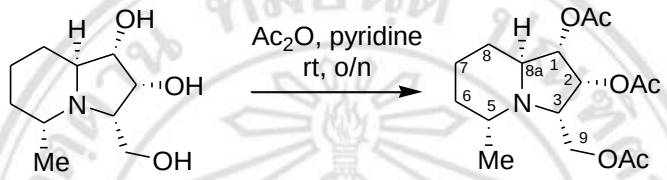
IR (cm^{-1}): 3352, 2931, 2863, 1629, 1596, 1455, 1381, 1339, 1121, 1096, 1059, 1028.

^{1}H NMR (500 MHz, D_2O) δ 4.37 (t, *J* = 6.0 Hz, 1H, H-2), 4.09 (dd, *J* = 10.5, 5.5 Hz, 1H, H-1), 3.77 (dd, *J* = 11.0, 9.5 Hz, 1H, H-9), 3.60 (dd, *J* = 11.0, 5.5 Hz, 1H, H-9′), 3.29-3.25 (m, 1H, H-3), 3.00-2.97 (m,1H, H-8a), 2.55-2.51 (m, 1H, H-5), 1.77-1.75 (m, 1H, H-8), 1.71-1.63 (m[a], 2H, H-6, H-8′), 1.56-1.52 (m, 1H, H-7),

1.45-1.37 (m, 1H, H-7′), 1.16-1.08 (m, 1H, H-6′), 1.02 (d, *J* = 6.0 Hz, 3H, CH_3) [[a] indicates the overlapping of signals]. (Appendix B Figure 28, page 286)

^{13}C NMR (125 MHz, D_2O) δ 70.6 (C-1), 69.8 (C-2), 66.2 (C-3), 60.4 (C-9), 58.5 (C-8a), 55.1 (C-5), 32.0 (C-6), 22.7 (C-8), 20.4 (CH_3), 18.1 (C-7). (Appendix B Figure 29, page 286)

3.9.5. (1*S*,2*R*,3*S*,5*R*,8a*S*)-3-(Acetoxymethyl)-5-methyloctahydroindolizine-1,2-diyldiacetate [84]



7 **84**

To a solution of **7** (5.0 mg, 0.025 mmol) in dry pyridine (0.11 mL, 1.36 mmol) was added Ac_2O (0.12 mL, 1.27 mmol). The mixture was stirred at rt for 18 h followed by evaporation of all volatiles (257). The oily residue was purified by flash column chromatography (increasing polarity from 100:0 to 0:100 petrol/EtOAc and 20:80 MeOH/CH_2Cl_2 as eluent) afforded the title compound (4.1 mg, 50%) as a clear oil. R_f 0.4 (1:1 petrol: EtOAc).

$[\alpha]_D^{25}$ –11.2 (*c* 0.2, $CHCl_3$).

HRMS (ESI +ve) calculated for $C_{16}H_{26}NO_6$ ($M+H^+$) 328.1800, found 328.1760.

IR (cm^{-1}): 2966, 2933, 2865, 1737, 1455, 1439, 1370, 1219, 1164, 1102, 1087, 1073, 1062, 1036.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.58 (dd, *J* = 7.5, 5.5 Hz, 1H, H-2), 5.08 (t, *J* = 6.0 Hz, 1H, H-1), 4.13-4.07 (m, 2H, H-9), 3.76 (dd, *J* = 14.0, 7.0 Hz, 1H, H-3), 3.33 (dd, *J* = 12.0, 7.0 Hz, 1H, H-8a), 2.78-2.74 (m, 1H, H-5), 2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.72-1.56 (m[a], 3H, H-6, H-7, H-8), 1.51-1.45 (m, 1H, H-7′), 1.42-1.37 (m, 1H, H-8′), 1.26-1.20 (m, 1H, H-6′), 1.11(d, *J* = 6.5 Hz, 1H, CH_3) [[a] indicates the overlapping of signals]. (Appendix B Figure 30, page 287)

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9 (C=O), 170.5 (C=O), 170.0 (C=O), 73.3 (C-1), 70.4 (C-2), 63.7 (C-9), 61.1 (C-3), 57.5 (C-8a), 52.5 (C-5), 29.8 (C-6), 25.0 (C-8), 21.0 (CH_3), 21.0 (CH_3), 20.7 (CH_3), 18.6 (C-7). (Appendix B Figure 31, page 287)

3.10 The procedure of the glycosidase inhibition assay (258)

Four synthetic compounds [**1**, **6**, **7** and **83**] were tested for glycosidase inhibition activity by Prof. Robert J. Nash at Phytoquest Limited, IBERS, Aberystwyth University, Aberystwyth, UK using commercially available enzymes and *para*-nitrophenyl substrates from Sigma, with the exception of beta-mannosidase which came from Megazyme. The enzymes were assayed at 27 ºC in 0.1 M citric acid / 0.2 M disodium hydrogen phosphate buffers at the optimum pH for the enzyme. The incubation mixture consisted of 10 µL enzyme solution, 10 µL of 1 mg/mL aqueous solution of the extract and 50 µL of the appropriate 5 mM *para*-nitrophenyl substrate made up in buffer at the optimum pH for the enzyme. The reactions were stopped by addition of 70 µL 0.4 M glycine (pH 10.4) during the exponential phase of the reaction, which had been determined at the beginning using uninhibited assays in which water replaced inhibitor. Final absorbances were read at 405 nm using a Versamax microplate reader (Molecular Devices). The assays were carried out in triplicate, and the values given are means of the three replicates per assay.