CHAPTER II
EXPERIMENTAL

2. Experimental

2.1 Materials

The calf thymus DNA (ct-DNA) was purchased from Sigma Chemicals and the hexamer $d(CGTACG)_2$ was purchased from Bio Service Unit Thailand. They were used without further purification. Deionized double distilled water and analytical grade reagents were used throughout. All other reagents were obtained form Sigma. Etoposide (positive control drug for topoisomerase II study) and plasmid PBR 322 DNA were purchased from Sigma. Topoisomerase II were purchased from Amersham (UK).

2.2 Extraction of 6-deoxyclitoriacetal from the dried roots of *Stemona collinsae* Craib.

The ground dried roots of *Stemona collinsae* Craib. were extracted with CH_2Cl_2. The CH_2Cl_2 phase was concentrated to obtain the dark brown crude extract and the CH_2Cl_2 crude extract was separated on silica gel column using hexane: ethyl acetate: dichloromethane = 2 : 1 : 2 (v/v) as eluent. The fractions showing similar spots were combined and then concentrated to dryness. The MeOH was used to crystallize 6-deoxyclitoriacetal from the other rotenoid compounds. The obtained compounds were characterized by spectroscopic method [9].

2.3 Cytotoxic activity of the obtained rotenoid compounds

The obtained rotenoid compounds were studied in cytotoxicity against various types of human carcinoma, such as KB (Human mouth carcinoma), BC (Breast cancer), NCL-H187 (Human small lung cancer) [31].

2.4 Drug-DNA binding studies

The calf thymus DNA (ct-DNA) and hexamer d(CGTACG)$_2$ were used in the spectral measurements and NMR spectroscopic studies to observe the binding ability of rotenoid compounds with DNA, respectively. The DNA concentration was determined by absorbance measurements at 260 nm with the molar extinction coefficient 6600 $M^{-1}cm^{-1}$ [32]. The purity of the DNA was checked by monitoring the value of *A260/280* [33]. The ratio was in excess of 1.80 for all samples used in the experiments so that the contents of residual proteins should be small.

Aqueous stock solution of rotenoid compounds were prepared by wetting these compounds with DMSO (1:10 v/v) followed by addition of deionized double distilled water. Working stock solutions of 6-deoxyclitoriacetal were prepared by dilution of an aqueous stock solution with phosphate buffer, pH 7.0.

2.4.1 Spectral measurements

2.4.1.1 UV-titration experiments

The UV titration experiment was performed on a Varian - CARY 50 spectrophotometer using 10 mm light-path at 25 ± 0.2 °C. Aliquots of a concentration of calf thymus-DNA were added to a cell containing a constant amount of a 6-deoxylcitoriacetal solution (3.25 x 10^{-5} M) and thoroughly mixed to obtain different DNA to compound ratios. After the absorbance is stabilized (10-15 min) the absorbance was read against a blank (10 mM sodium phosphate pH 7.0) [34].

2.4.1.2 DNA melting studies

T_m experiments were performed on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.) equipped with a thermal melt system. The sample for T_m measurement was prepared by mixing calculated amounts of stock oligonucleotide and rotenoid solutions together to give final concentration of nucleotides and sodium phosphate buffer (pH 7.0) and the final volumes were adjusted to 3.0 mL by addition of deionized water. The samples were transferred to a 10 mm quartz cell with a Teflon stopper and equilibrated at the starting temperature

for 10 min. The A_{260} was recorded in steps heating from 20-90 °C, cooling 90-20 °C and reheating 20-90 °C with a temperature ramp of 1 °C/min. The temperature recorded was the actual temperature measured by a built-in temperature probe. Only the result taken from the last heating cycle was used and was normalized by dividing the absorbance at each temperature by the initial absorbance T_m was obtained from derivative plot after smoothing using KaliedaGraph 3.6 (Synergy Software) and analysis of the data was performed on a PC compatible computer using Microsoft Excel XP [27, 28].

2.4.1.3 Circular dichroism spectroscopy (CD)

CD experimets were performed on a J700 spectropolarimeter. To a solution containing the DNA (0.12 mM) and 10 mM sodium phosphate buffer pH 7.0 (2.5 mL) was added aliquot of a concentrated stock solution of rotenoids (concentration = 1 mM). The sample was prepared by mixing calculated amounts of stock oligonucleotide and rotenoid solutions together in a 10 mm quartz cell and the final volumes were adjusted to 2.5 mL by addition of deionized water containing an appropriate amount of sodium phosphate buffer pH 7.0 to give the appropriate concentration of each component as described in the text. The spectra were measured at 25 °C from 200 to 400 nm and averaged 3 times then subtracted from a spectrum of 10 mM sodium phosphate buffer pH 7.0 under the same condition. The induced CD is defined as the CD of the rotenoids-DNA mixture minus the CD of DNA alone at the same wavelength and is expressed as ellipticity in millidegrees (mdeg) [35-37].

2.4.1.4 NMR studies

For the studies of compound-DNA complexes, a stock solution 10 mM of rotenoid compounds were prepared by dissolved in DMSO. The stock solution 60 µl was added to the NMR tube. To the NMR tube, the solution of $d(CGTACG)_2$ 4 mM in phosphate buffer solution (50 mM sodium phosphate, pH 7.0, 150 mM NaCl in 99.8 % D_2O) were added to give the desired mole ratio of 1/2 and $d(CGTACG)_2$. Then the mixtures of 1/2 and $d(CGTACG)_2$ were subjected to NMR analysis. 1D NMR spectra were recorded on a Varian Mercury+ 400 NMR Spectroscopy. 2D

NMR spectra were recorded on a JNM-A 500 spectroscopy. Phase sensitive NOESY spectra were recorded as 512 t_1 block during the recycle delay of 1.0 sec. The chemical shifts were referenced to the signal of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) with resonance at δ_H 0.00. The 1D data were processed with the MESTREC software.

The solutions of $d(CGTACG)_2$ in phosphate buffer solution (50 mM sodium phosphate, pH 7.0, 150 mM NaCl in 99.8 % D_2O) were titrated with rotenoid compounds by increasing the mole ratio of compounds at 0.5, 1, 2, 3 equivalent, respectively. 1D NMR spectra were recorded on a Varian Mercury+ 400 NMR Spectrometer. 2D NMR spectra were recorded on a JNM – A 500 spectroscopy. Phase sensitive NOESY spectra were recorded as 512 t_1 block during the recycle delay of 1.0 sec. The chemical shifts were referenced to the 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), sodium salt as the internal standard. The 1D data were processed with the MESTREC software [38, 39].

2.5 Synthesis of 6-deoxyclitoriacetal analogues

Materials

All organic solvents were reagent grade. All of the chemical reagents were purchased from Aldrich Chem. Co.

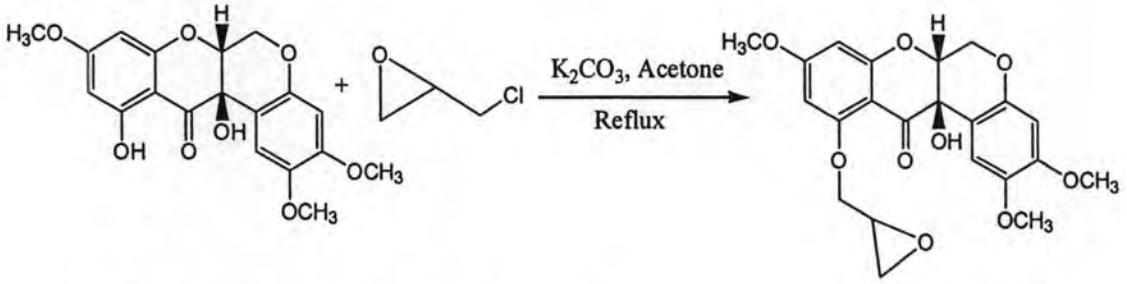
Three groups of 6-deoxyclitoriacetal analogues were synthesized. The first group that is 6-deoxyclitoriacetal added the epoxy ring before opening it with amino acid (compound A, A1-A5). The second group that is 6-deoxyclitoriacetal added the tosylate group before adding the pyrimidine base (B, B1-B3). The last group that is 6-deoxyclitoriacetal condensed with carbonic acid (C-G).

2.5.1 The 6-deoxyclitoriacetal – amino acid analogues

In order to study the effect of functional group of amino acid and the length of side chain of amino acid to the cytotoxicity, five amino acids were chosen to synthesize with 6-deoxyclitoriacetal. They composed of glycine, alanine, isoleucine, cystein and arginine. The 6-deoxyclitoriacetal was added with epoxide ring (**A**) before opening it with each amino acids (**A1-A5**).

2.5.1.1 Synthesis of 12a-hydroxy-2,3,9-trimethoxy-11-oxiranylmethoxy-6a,12a-dihydro-6*H*-chromeno[3,4-*b*]chromen-12-one. (A)

To the reaction mixture of 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and K_2CO_3 (180 mg, 1.3 mmol) in anhydrous acetone (15 mL) was refluxed for 3 h. under nitrogen condition and then added epichlorohydrin (0.10 mL, 13.4 mmol). The reaction mixture was refluxed overnight and solvent was removed under reduced pressure. The residue was extracted with CH_2Cl_2 and the organic layer was washed respectively with water and brine. After evaporation of solvent, the residue was purified by silica gel column chromatography (eluent: hexane: dichloromethane: ethyl acetate 2: 2: 1) to afford a yellow solid (scheme 1) [40].



6-deoxyclitoriacetal epichlorohydrin compound **A**

Scheme 1 Synthesis of 12a-hydroxy-2,3,9-trimethoxy-11-oxiranylmethoxy-6a,12a-dihydro-6*H*-chromeno[3,4-*b*]chromen-12-one. (**A**)

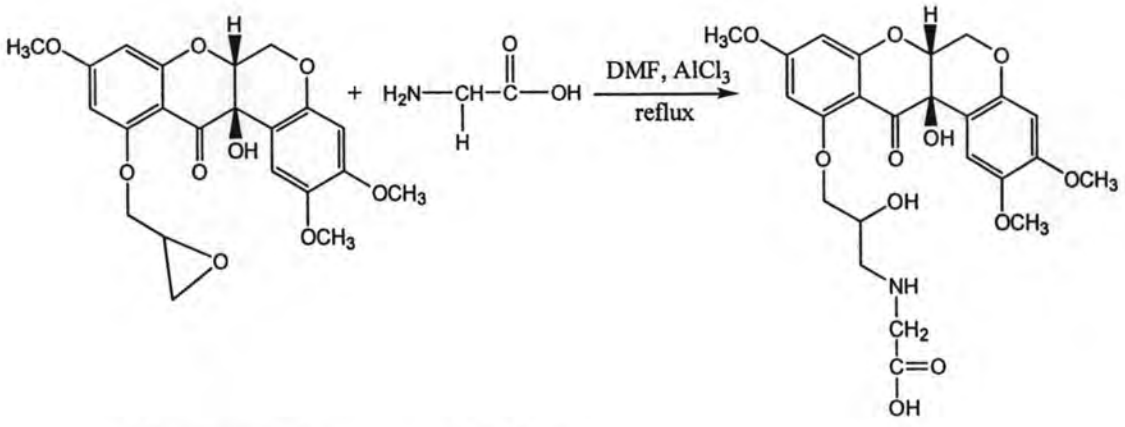
The ring opening of compound A with amino acids

Reactions of the epoxide with amino acids were reported [41,42]. Five amino acids (glycine, alanine, isoleucine, cystein, arginine) were used in condensation of compound **A** in the presence of dimethylformamide (DMF) as a solvent and anhydrous aluminium chloride as a catalyst.

2.5.1.2 Synthesis [2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-acetic acid (A1)

Equimolar amounts of compound **A** and glycine (0.14 mmol) were heated under reflux in DMF (5 cm^3) in the presence of catalytic amounts of $AlCl_3$ for 3 h.

The reaction mixture was poured into water and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 2) [43].

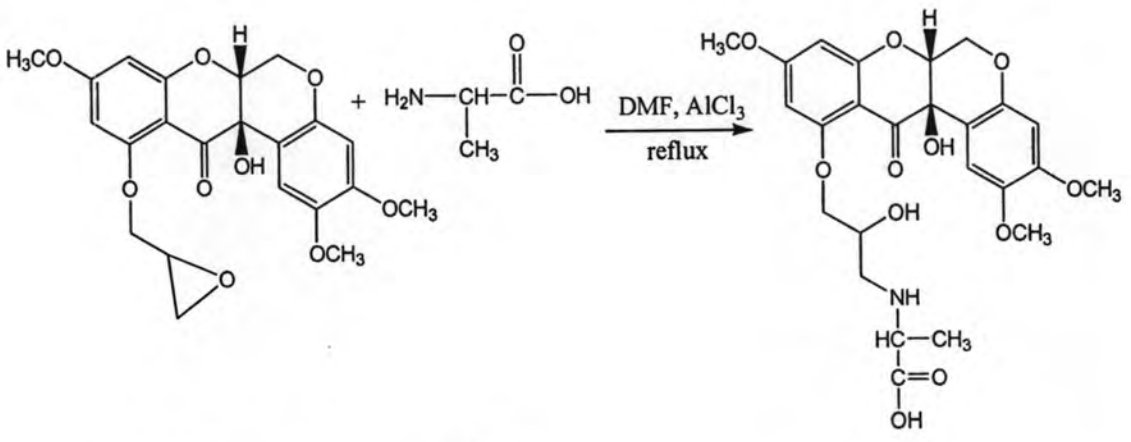


Compound **A** glycine **A1**

Scheme 2 Synthesis of [2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-acetic acid **(A1)**

2.5.1.3 Synthesis 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-propionic acid (A2)

Equimolar amounts of compound **A** and alanine (0.14 mmol) were heated under reflux in DMF (5 cm^3) in the presence of catalytic amounts of $AlCl_3$ for 3 h. The reaction mixture was poured into water and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 3) [43].

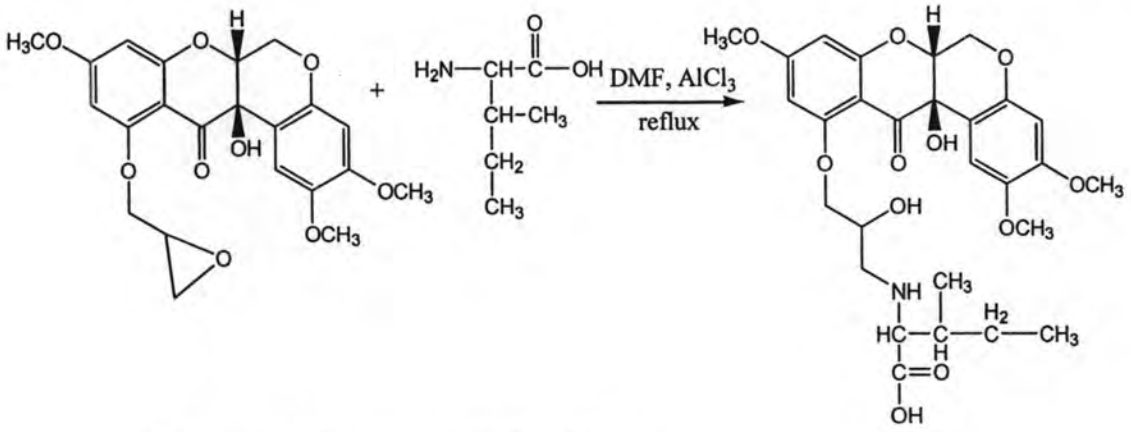


Compound **A** alanine **A2**

Scheme 3 Synthesis of 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-propionic acid (**A2**)

2.5.1.4 Synthesis 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-3-methyl-pentanoic acid (A3)

Equimolar amounts of compound **A** and isoleucine (0.14 mmol) were heated under reflux in DMF (5 cm^3) in the presence of catalytic amounts of $AlCl_3$ for 3 h. The reaction mixture was poured into water and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 4) [43].

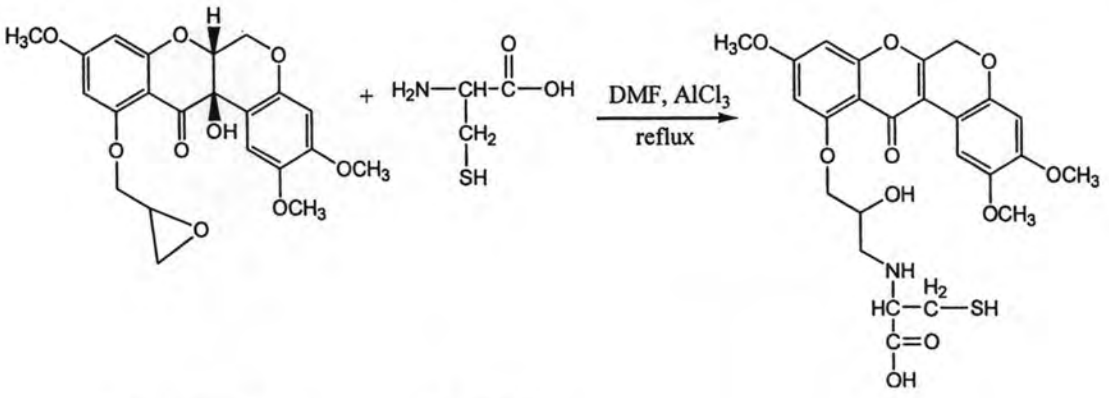


Compound **A** isoleucine **A3**

Scheme 4 Synthesis of 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-3-methyl-pentanoic acid (**A3**)

2.5.1.5 Synthesis 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-3-mercapto-propionic acid (A4)

Equimolar amounts of compound **A** and cystein (0.14 mmol) were heated under reflux in DMF (5 cm^3) in the presence of catalytic amounts of $AlCl_3$ for 3 h. The reaction mixture was poured into water and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 5) [43].

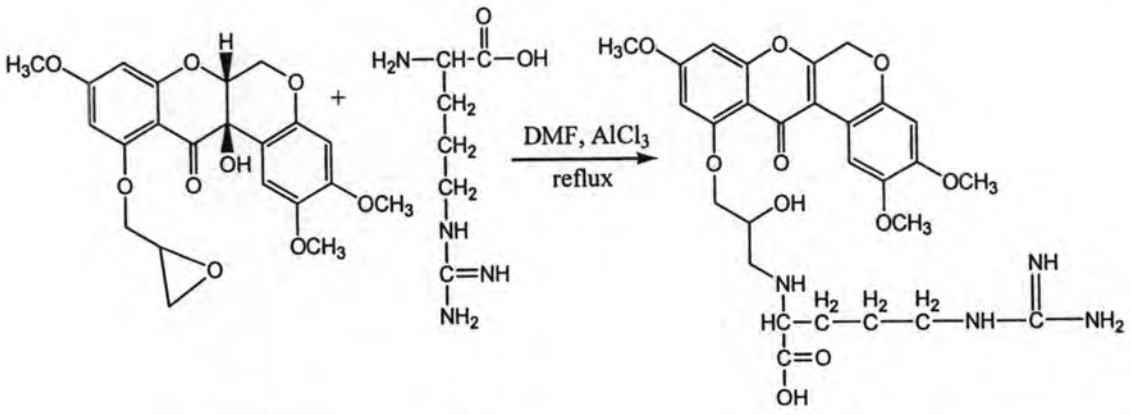


Compound **A** cystein **A4**

Scheme 5 Synthesis of 2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-3-mercapto-propionic acid (**A4**)

2.5.1.6 Synthesis 5-guanidino-2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-pentanoic acid (A5)

Equimolar amounts of compound **A** and arginine (0.14 mmol) were heated under reflux in DMF (5 cm^3) in the presence of catalytic amounts of $AlCl_3$ for 3 h. The reaction mixture was poured into water and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 6) [43].



Compound **A** arginine **A5**

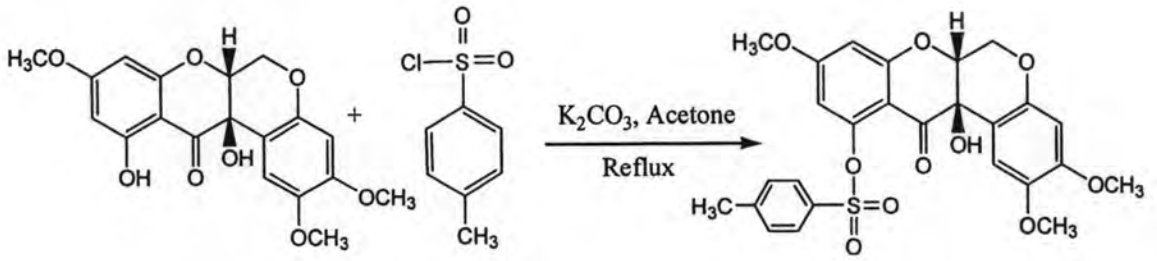
Scheme 6 Synthesis of 5-guanidino-2-[2-hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yloxy)-propylamino]-pentanoic acid (**A5**)

2.5.2 The 6-deoxyclitoriacetal – pyrimidine base analogues

In order to study the effect of functional groups on pyrimidine base to the cytotoxicity, three pyrimidine bases were chosen to synthesize with 6-deoxyclitoriacetal. They composed of cytosine, thymine and uracil, respectively. The 6-deoxyclitoriacetal was added with tosylate group (**B**) before removed it with each pyrimidine base (**B1-B3**).

2.5.2.1 Synthesis of toluene-4-sulfonic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydrochromeno[3,4-*b*]chromen-11-yl ester (B)

To the reaction mixture of 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and K_2CO_3 (92 mg, 0.67 mmol) in anhydrous acetone (15 mL) was refluxed for 2 hr. and then added toluene-4-sulfonyl chloride (51 mg, 0.27 mmol). The reaction mixture was refluxed for 3 hours and solvent was removed under reduced pressure. After evaporation of solvent, the residue was purified by silica gel column chromatography (eluent: hexane: dichloromethane: ethyl acetate 2: 2: 1, v/v) to afford yellow solid (scheme 7) [44].

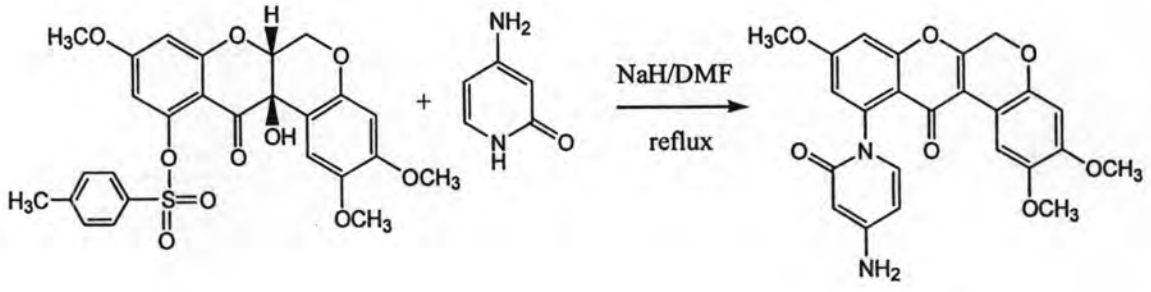


6-deoxyclitoriacetal toluene-4-sulfonyl chloride **B**

Scheme 7 Synthesis of toluene-4-sulfonic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydrochromeno[3,4-*b*]chromen-11-yl ester (**B**)

2.5.2.2 Synthesis 4-amino-1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-1*H*-pyridine-2-one (B1)

To the reaction mixture of the cytosine and NaH in DMF (15 mL) was refluxed for 3 hours and then added the compound **B** in DMF. The reaction mixture was poured into ice bath and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 8) [45].

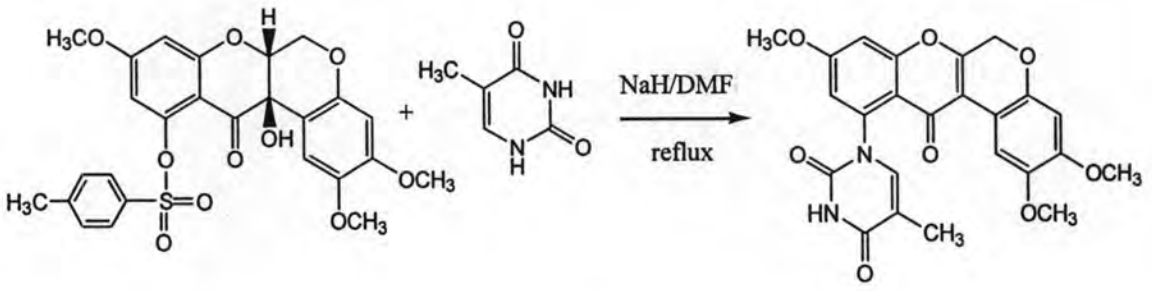


Compound **B** cytosine **B1**

Scheme 8 Synthesis of 4-amino-1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-1*H*-pyridine-2-one (**B1**)

2.5.2.3 Synthesis 1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-5-methyl-1*H*-pyrimidine-2,4-dione (B2)

To the reaction mixture of the thymine and NaH in DMF (15 mL) was refluxed for 3 hours and then added the compound **B** in DMF. The reaction mixture was poured into ice bath and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 9) [45].

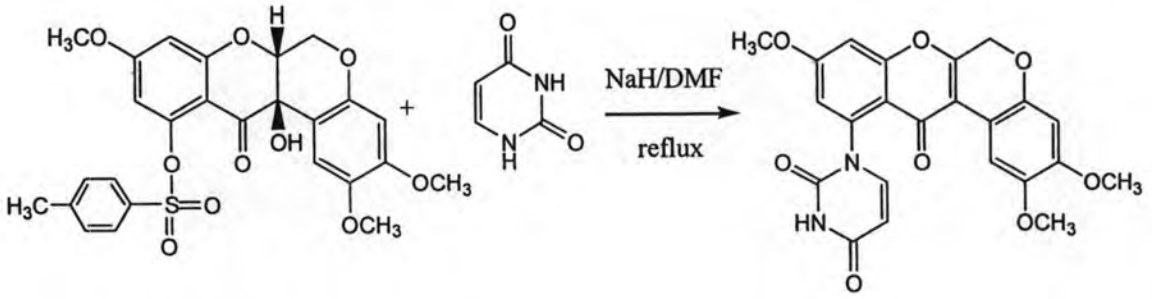


Compound **B** thymine **B2**

Scheme 9 Synthesis of 1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-5-methyl-1*H*-pyrimidine-2,4-dione (**B2**)

2.5.2.4 Synthesis 1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-1*H*-pyridine-2,4-dione (B3)

To the reaction mixture of the uracil and NaH in DMF (15 mL) was refluxed for 3 hours and then added the compound **B** in DMF. The reaction mixture was poured into ice bath and extracted with dichloromethane. The combined extract was dried over $MgSO_4$ and the solution was filtered. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (1:2:2, v/v) (scheme 10) [45].



Compound **B** uracil **B3**

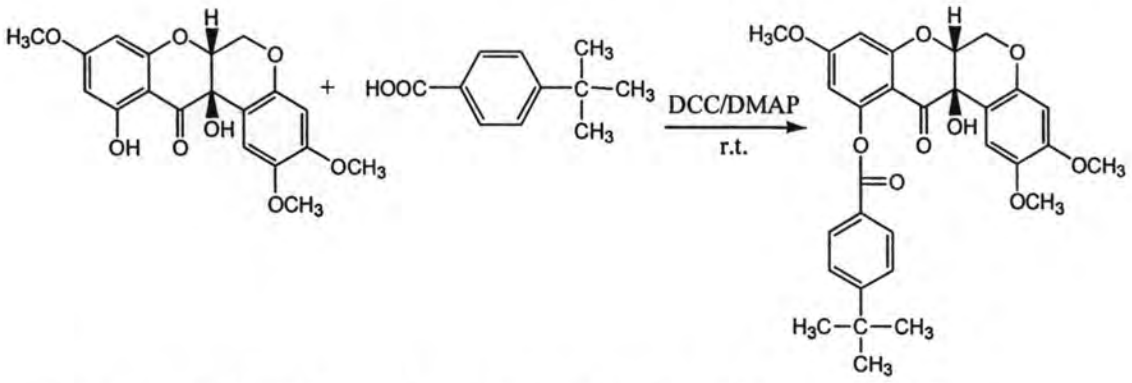
Scheme 10 Synthesis of 1-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl)-1*H*-pyridine-2,4-dione (**B3**)

2.5.3 The 6-deoxyclitoriacetal – aromatic carboxylic analogues

In order to study the effect of functional group on aromatic carboxylic compound to the cytotoxicity, five aromatic carboxylic compounds were chosen to synthesize with 6-deoxyclitoriacetal. They composed of 4-*tert*-butylbenzoic acid, benzoic acid, 4-chlorobenzoic acid, 4-hydroxybenzoic acid and 4-aminobenzoic acid. Each compound was referred to **C-G**.

2.5.3.1 Synthesis 4-*tert*-butyl-benzoic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl ester (C)

The 4-*tert*-butylcarboxylic acid (0.13 mmol) was added to a solution of 1,3-dicyclohexylcarbodiimide (DCC) (30.9 mg, 0.15 mmol), 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and N,N-dimethylaminopymidine (DMAP) (3.25 mg) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature. After completion of the reaction, the reaction mixture was filtered and the filtrate was shaken with 100 mL of 2N HCl and separated. The organic phase was washed with water and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (2:1:2, v/v) (scheme 11) [46, 47].

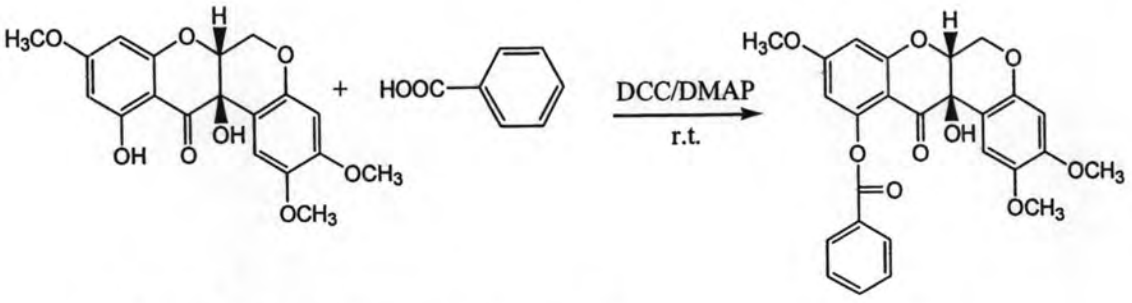


6-deoxyclitoriacetal 4-*tert*-butylcarboxylic acid **C**

Scheme 11 Synthesis of 4-*tert*-butyl-benzoic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl ester (**C**)

2.5.3.2 Synthesis benzoic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl ester (D)

The benzoic acid (0.13 mmol) was added to a solution of 1,3-dicyclohexylcarbodiimide (DCC) (30.9 mg, 0.15 mmol), 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and N,N-dimethylaminopymidine (DMAP) (3.25 mg) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature. After completion of the reaction, the reaction mixture was filtered and the filtrate was shaken with 100 mL of 2N HCl and separated. The organic phase was washed with water and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (2:1:2, v/v) (scheme 12) [46, 47].

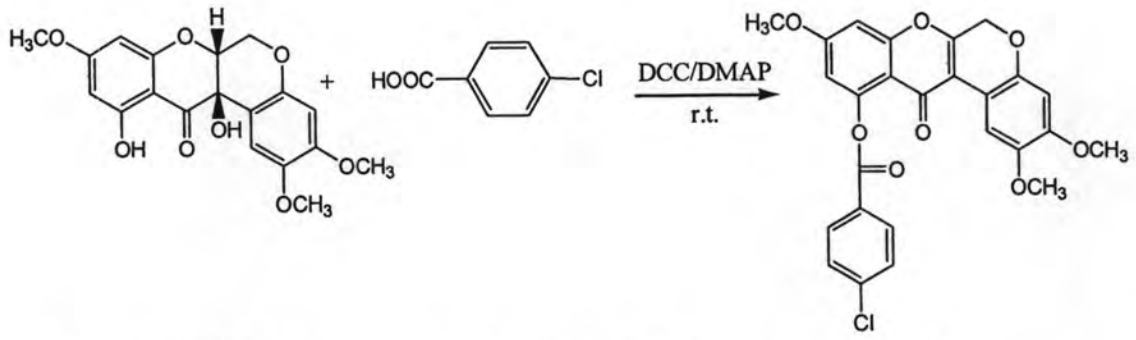


6-deoxyclitoriacetal benzoic acid **D**

Scheme 12 Synthesis of benzoic acid 12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-*b*]chromen-11-yl ester **(D)**

2.5.3.3 Synthesis 4-chloro-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester (E)

The chlorobenzoic acid (0.13 mmol) was added to a solution of 1,3-dicyclohexylcarbodiimide (DCC) (30.9 mg, 0.15 mmol), 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and N,N-dimethylaminopymidine (DMAP) (3.25 mg) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature. After completion of the reaction, the reaction mixture was filtered and the filtrate was shaken with 100 mL of 2N HCl and separated. The organic phase was washed with water and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (2:1:2, v/v) (scheme 13) [46, 47].

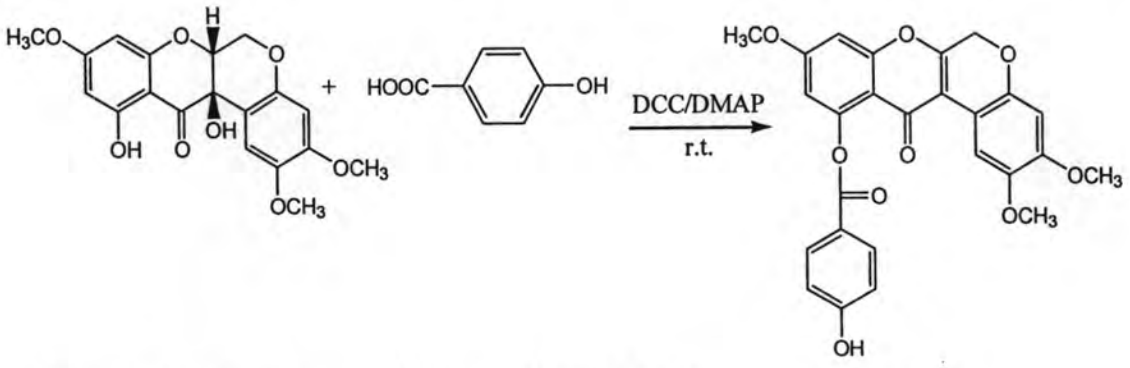


6-deoxyclitoriacetal chlorobenzoic acid **E**

Scheme 13 Synthesis of 4-chloro-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester **(E)**

2.5.3.4 Synthesis 4-hydroxy-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester (F)

The 4-hydroxybenzoic acid (0.13 mmol) was added to a solution of 1,3-dicyclohexylcarbodiimide (DCC) (30.9 mg, 0.15 mmol), 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and N,N-dimethylaminopymidine (DMAP) (3.25 mg) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature. After completion of the reaction, the reaction mixture was filtered and the filtrate was shaken with 100 mL of 2N HCl and separated. The organic phase was washed with water and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (2:1:2, v/v) (scheme 14) [46, 47].

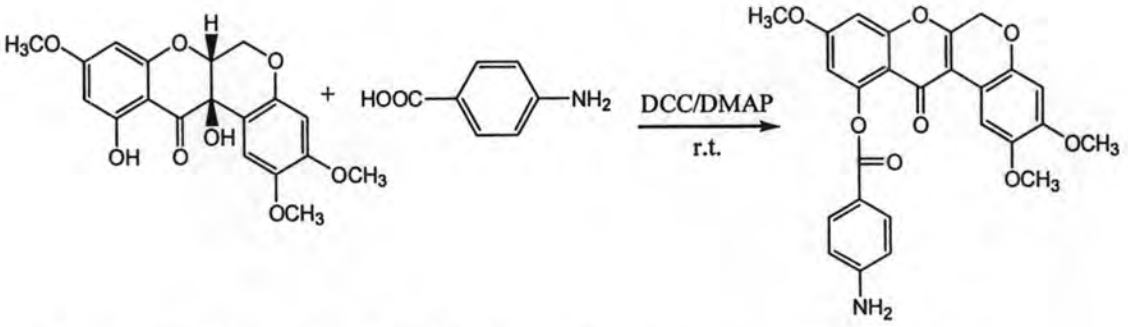


6-deoxyclitoriacetal 4-hydroxybenzoic acid **F**

Scheme 14 Synthesis of 4-hydroxy-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester **(F)**

2.5.3.5 Synthesis 4-amino-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester (G)

The 4-aminobenzoic acid (0.13 mmol) was added to a solution of 1,3-dicyclohexylcarbodiimide (DCC) (30.9 mg, 0.15 mmol), 6-deoxyclitoriacetal (50 mg, 0.13 mmol) and N,N-dimethylaminopymidine (DMAP) (3.25 mg) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature. After completion of the reaction, the reaction mixture was filtered and the filtrate was shaken with 100 mL of 2N HCl and separated. The organic phase was washed with water and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography eluting with hexane: ethyl acetate: dichloromethane (2:1:2, v/v) (scheme 15) [46, 47].



6-deoxyclitoriacetal 4-aminobenzoic acid **G**

Scheme 15 Synthesis of 4-amino-benzoic acid 2,3,9-trimethoxy-12-oxo-6,12-dihydro-chromeno[3,4-*b*]chromen-11-yl ester **(G)**

2.6 Cytotoxic activity of the 6-deoxyclitoriacetal analogues

The obtained compounds were studied in cytotoxicity against various types of human carcinoma, such as KB (Human mouth carcinoma), BC (Breast cancer), NCL-H187 (Human small lung cancer) [31].

2.7 Assay of topoisomerase II activity

Topoisomerase II is an important nuclear enzyme controlling DNA topology through catalysis of a breakage of double-stranded DNA, allowing for the passage of double-stranded DNA followed by a resealing of the DNA. Relaxation of DNA supercoils by topoisomerase II is considered crucial to its role in DNA replication and in transcription. To further elucidate the mechanism of action of 6-deoxyclitoriacetal, stemonal and 6-deoxyclitoriacetal analogues for cytotoxicity, the relaxation of supercoiled plasmid pBR322 DNA was evaluated. Topoisomerase II relaxation assay was conducted using human topoisomerase II (Amersham). Etoposide are selective topoisomerase II inhibitors was used as positive control.

Rotenoid compounds were screened for the topoisomerase II inhibition function. The activity of the compounds on the relaxation of DNA topoisomerase IIα was determined by measuring the conversion of supercoiled PBR 322 plasmid DNA to its relaxed form. Topoisomerase II relaxation assay was conducted by using human topoisomerase II with etoposide as a positive control.

Rotenoid compounds and etoposide were prepared as stock solutions (10, 25 mM, respectively) in DMSO and stored at -20 °C. The reaction mixture contained 10

mM Tris-HCl (pH 7.9), 175 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5 % glycerol, 1 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.2 μg pBR322 plasmid DNA, 0.3 U DNA topoisomerase IIα, and test compounds in a final volume of 50 μL. The reactions were incubated for 30 min at 37 °C and terminated by the addition of 3 μL of solution containing 0.77 % sodium dodecyl sulfate, and 77 mM EDTA. Samples were mixed with 2 μL of solution containing 30 % sucrose, 0.5 % bromophenol blue and 0.5 % xylene cyanol, and subjectded to electrophoresis on a 1 % agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris-borate-EDTA. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and quantities by an image analyzer and Syngene software [48-49].