CHAPTER III

EXPERIMENTAL

1.Source of plant material

The root of *Siphonodon celastrineus* Griff. 610.0 g was collected from Wangthong District , Pitsanulok Province, in September 2001. It was identified by Dr. Thawatchai Santisuk , and by comparison with the herbarium specimen , collector number C. Niyomdham No. 1962 at the Royal Forest Department , Bangkok , Thailand.

2. Phytochemical techniques

2.1) Chromatographic techniques

2.1.1) Thin layer chromatography (TLC)

Technique : One way ascending

Adsorbent : Silica gel $60F_{254}$ (E. Merck) precoated plate

Layer thickness : 0.2 mm

Solvent system : Various solvent systems depending on materials.

Distance : 6 cm

Temperature : Laboratory temperature (30 - 35 $^{\circ}$C)

Detection :1.Ultraviolet light (254 and 365 nm)

2. 10% Sulfuric acid in ethanol and heated at 105 $^{\circ}$C for 10 minutes

2.1.2) Column chromatography

2.1.2.1) Conventional column chromatography (cc)

Column sizes : Glass column of 3/4-4 inches in diameter were used depending on the quantity of sample to be separated.

Adsorbent : Silica gel 60 (No. 1.09385 , E. Merck , particle size 0.040 - 0.063 mm)

Packing method : Wet packing

Solvent system : Various solvent systems depending on materials.

2.1.2.2) Gel filtration chromatography

Column size : Glass column *1* inche in diameter

Adsorbent : Sephadex$^{@}$ LH20 (Pharmacia Biotech)

Packing method : Wet packing

Solvent system : Various solvent systems depending on materials

2.2) Melting point

Melting points were determined on a Yanakimoto micro melting point apparatus (Molecular Structure and Biological Function Laboratory, Faculty of Pharmaceutical Sciences, Chiba University, Japan)

2.3) Optical Rotation

Optical rotations were measured on a Perkin Elmer 341 polarimeter (Pharmaceutical Research Instrument Center , Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4) Spectroscopy

2.4.1) Ultraviolet (UV) absorption spectra

UV spectra were obtained on a Shimadzu UV-160A UV / VIS spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.2) Infrared (IR) spectra

IR spectra were recorded on a Perkin-Elmer Spectrum 2000 FT - IR spectrophotometer (Pharmaceutical Research Instrument Center , Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were prepared as KBr pellets.

2.4.3) Mass spectra (MS)

The FAB - MS were measured on a JEOL HX –110 A mass spectrometer (Research Instrument Center, Chiba University, Japan) , whereas the EI – MS

determined by Fisons AG Trio 2000 quadrupole Mass Spectrometer (Department of Chemistry, Faculty of Sciences, Chulalongkorn University) , using direct inlet system operating at 70 eV.

2.4.4) Proton and carbon nuclear magnetic resonance (^{1}H - and ^{13}C - NMR) spectra .

The ^{1}H - and ^{13}C - NMR spectra were obtained on a JEOL ECP 600 , (600 MHz) NMR Spectrometer and 500 MHz JEOL JNM - A500 (Alpha series) NMR Spectrometer (Faculty of Pharmaceutical Sciences, Chiba University, Japan). Deuterochloroform ($CDCl_3$) containing 0.03 % v / v tetramethylsilane (TMS) was used as the operating solvent. The chemical shifts were reported in ppm scale using the chemical shift of TMS at 0.00 ppm as the reference signal. The operating parameters were adjusted to those required for experiments of ^{1}H – NMR , ^{13}C – NMR , DEPT , ^{1}H -^{1}H COSY, HMQC and HMBC ($^{n}J_{C\text{-}H} = 8$ Hz)

2.5) Solvents

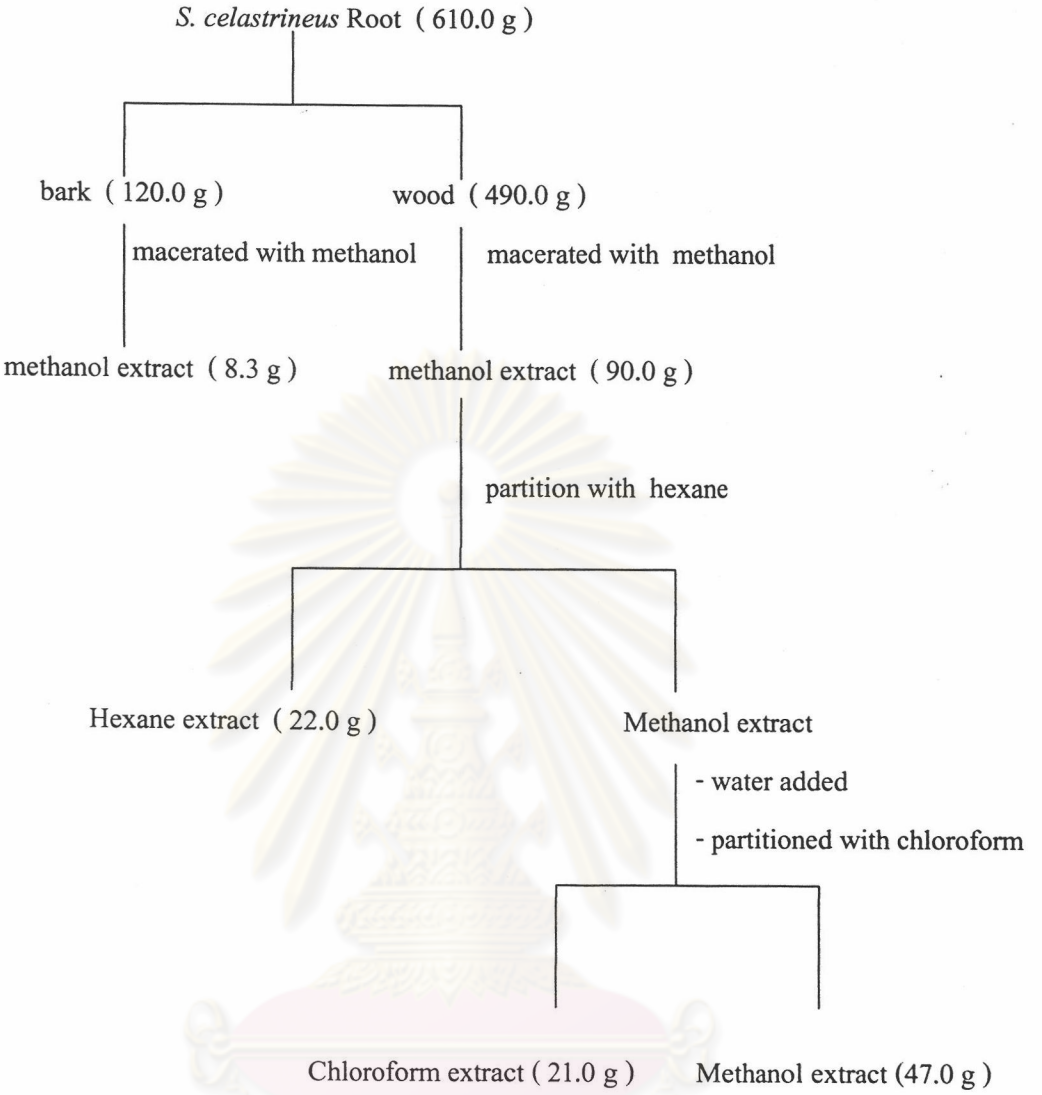
Solvents of commercial grade were redistilled before used.

3. Extraction of the root of *Siphonodon celastrineus* Griff.

The extraction of chemical constituents from the root of *Siphonodon celastrineus* are summarized in Scheme 2. The process was as follows:

The root bark was separated from the root of *S. celastrineus* .The air - dried and ground root bark (120.0 g) was extracted successively by maceration with methanol (7 days , for 4 times). The methanol extract was filtered and the filtrate evaporated under reduced pressure to give 8.3 g of dark-red gummy residue.

The air - dried ground root wood (490.0 g) was similarly extracted by maceration with methanol (7 days , 4 times). The dark brown methanol extract (90.0 g) was then partitioned with hexane and chloroform, respectively. The hexane , chloroform , and methanol layer were evaporated to yield 22.0 g , 21.0 g , and 47.0 g of extract , respectively.



Scheme 2. Extraction scheme for the root of *Siphonodon celastrineus* Griff.

4. Isolation of chemical constituents of *S. celastrineus* root

The isolation of chemical constituents of the root of *S. celastrineus* is summarized in Scheme 3 , 4 and 5. The process was as follows:

The methanol extract of the root bark of *S. celastrineus* 8.3 g was subjected to silica gel column chromatography, eluted with hexane - ethyl acetate (from 9 : 1 to 1 : 9) to give 180 fractions of 30 ml each. The eluents were used in the order as shown below :

Hexane – ethyl acetate 9 : 1 for fractions 1 - 20

Hexane – ethyl acetate 8 : 2 for fractions 21 - 40

Hexane – ethyl acetate 7 : 3 for fractions 41 - 60

Hexane – ethyl acetate 6 : 4 for fractions 61 - 80

Hexane – ethyl acetate 1 : 1 for fractions 81 - 100

Hexane – ethyl acetate 4 : 6 for fractions 101 -120

Hexane – ethyl acetate 3 : 7 for fractions 121 -140

Hexane – ethyl acetate 2 : 8 for fractions 141 - 160

Hexane – ethyl acetate 1 : 9 for fractions 161 -180

The fractions were combined according to their TLC pattern using hexane - ethyl acetate (9 : 1) as the developing system to give 7 fractions , A001 (fractions 1- 10) , A002 (fractions 11) , A003 (fractions 12 - 37) , A004 (fractions 38 - 49) , A005 (fractions 50 - 69) , A006 (fractions 70 – 143) and A007 (fractions 144 - 180)

The hexane extract from the root wood of *S. celastrineus* (22.0 g) was subjected to silica gel column chromatography, eluted with hexane / ethyl acetate (from 9 : 1 to 1 : 9) to give 180 fractions of 30 ml each. The fractions were then combined according to their TLC pattern using hexane - ethyl acetate (9 : 1) as the developing system to give 5 fractions , B001 (fraction 1 – 35) , B002 (fractions 36 - 103) , B004 (fractions 104 – 120) and B005 (fractions 121 - 180). The eluents were used in the order as shown below :

Hexane – ethyl acetate 9 : 1 for fractions 1 - 20

Hexane – ethyl acetate 8 : 2 for fractions 21 - 40

Hexane – ethyl acetate 7 : 3 for fractions 41 - 60

Hexane – ethyl acetate 6 : 4 for fractions 61 - 80

Hexane – ethyl acetate 1 : 1 for fractions 81 - 100

Hexane – ethyl acetate 4 : 6 for fractions 101 -120

Hexane – ethyl acetate 3 : 7 for fractions 121 -140

Hexane – ethyl acetate 2 : 8 for fractions 141 - 160

Hexane – ethyl acetate 1 : 9 for fractions 161 –180

Purification of compound SC1

Fraction A002 (190 mg) was recrystallized in chloroform at room temperature to give compound SC1 (110 mg) as colorless needles.

Purification of compound SC2

A004 (250 mg) was recrystallized in chloroform at room temperature to give compound SC2 (140 mg) as colorless needles.

The same compound was also isolated from fraction B002 (2.1 g) through similar recrystallization in chloroform to give 1.0 g of pure compounds.

Isolation and purification of compound SC3

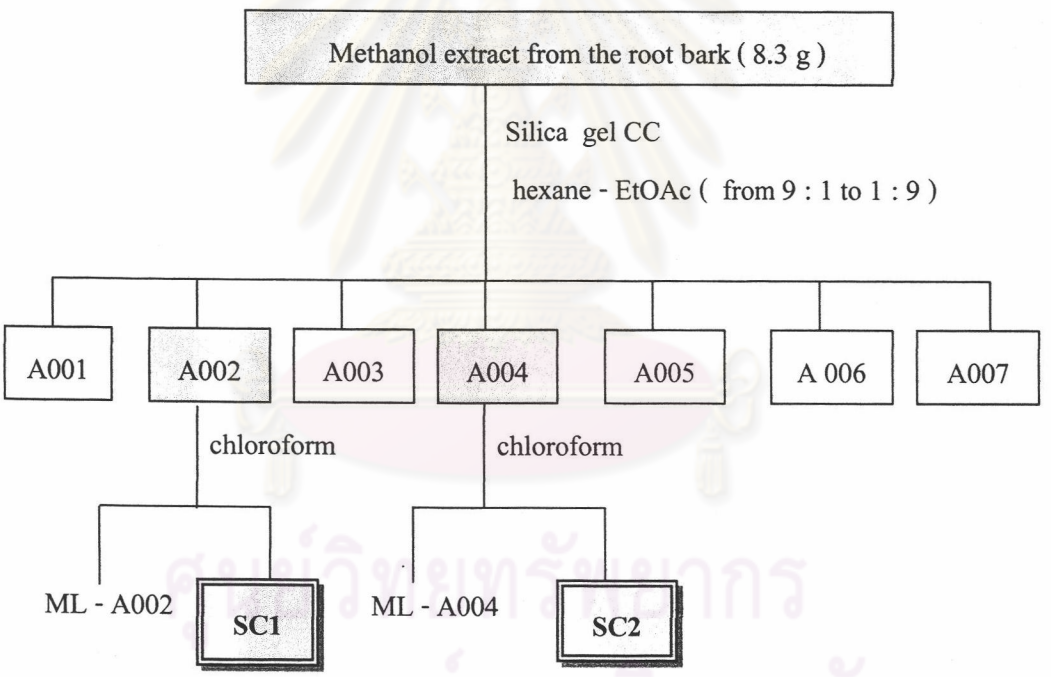
Fraction A006 (1.12 g) was subjected to silica gel column chromatography, eluted with hexane – chloroform – acetone (8 : 1 : 1) to give 50 fractions of each 20 ml. The fractions were then combined according to their TLC pattern using hexane – chloroform – acetone (7 :2 :1) as the developing system to give 5 fractions , A008 (fractions 1- 6) , A009 (fractions 7 - 10) , A010 (fractions 11 – 30) and A011 (fractions 31 – 50).

Fraction A009 (320 mg) was subjected to column chromatography, eluted with hexane - acetone (9:1) to give 40 fractions of each 10 ml. The fractions were then combined according to their TLC pattern using hexane – chloroform (7 : 3) as the developing system to give 4 fractions, A012 (fractions 1 - 8) , A013 (fractions 9 –16) , A014 (fractions 17 – 30) and A015 (fractions 31 – 40)

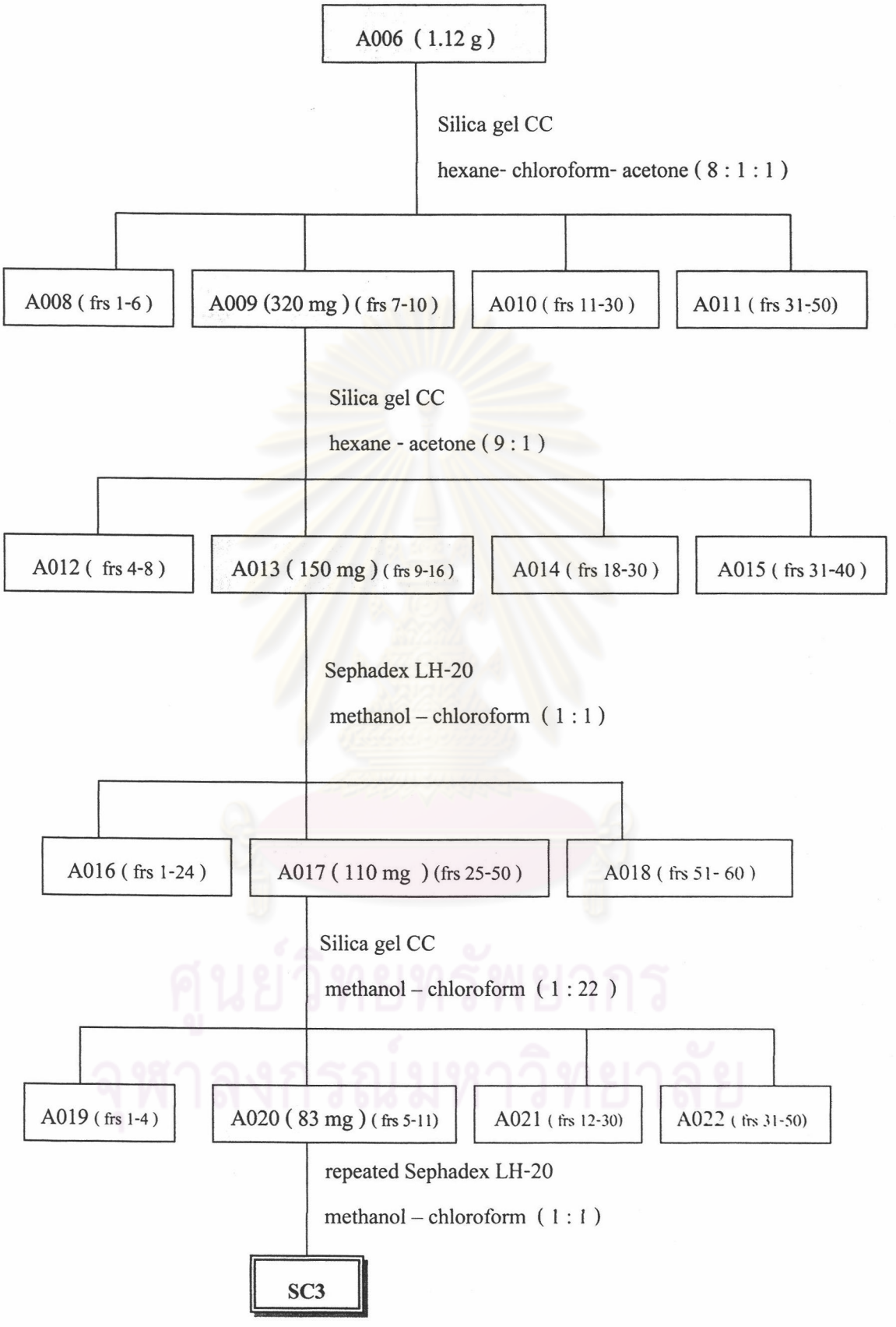
A013 (150 mg) was subjected to gel filtration column chromatography using a solvent system of methanol -chloroform (1 : 1) to give 60 fractions of 1 ml each. The fractions were then combined according to their TLC pattern using methanol – chloroform (1 : 9) as the developing system to give 3 fractions , A016 (fractions 1 - 24) , A017 (fractions 25 – 50) , and A018 (fractions 51 – 60)

A017 (110 mg) was subjected to column chromatography, eluted with methanol – chloroform (1 : 22) to give 50 fractions of 1 ml each. The fractions were then combined according to their TLC pattern using methanol – chloroform (1 : 22) as the developing system to give 4 fractions, A019 (fractions 1-4) , A020 (fractions 5 –11) , A021 (fractions 12 – 30) , and A022 (fraction 31 – 50).

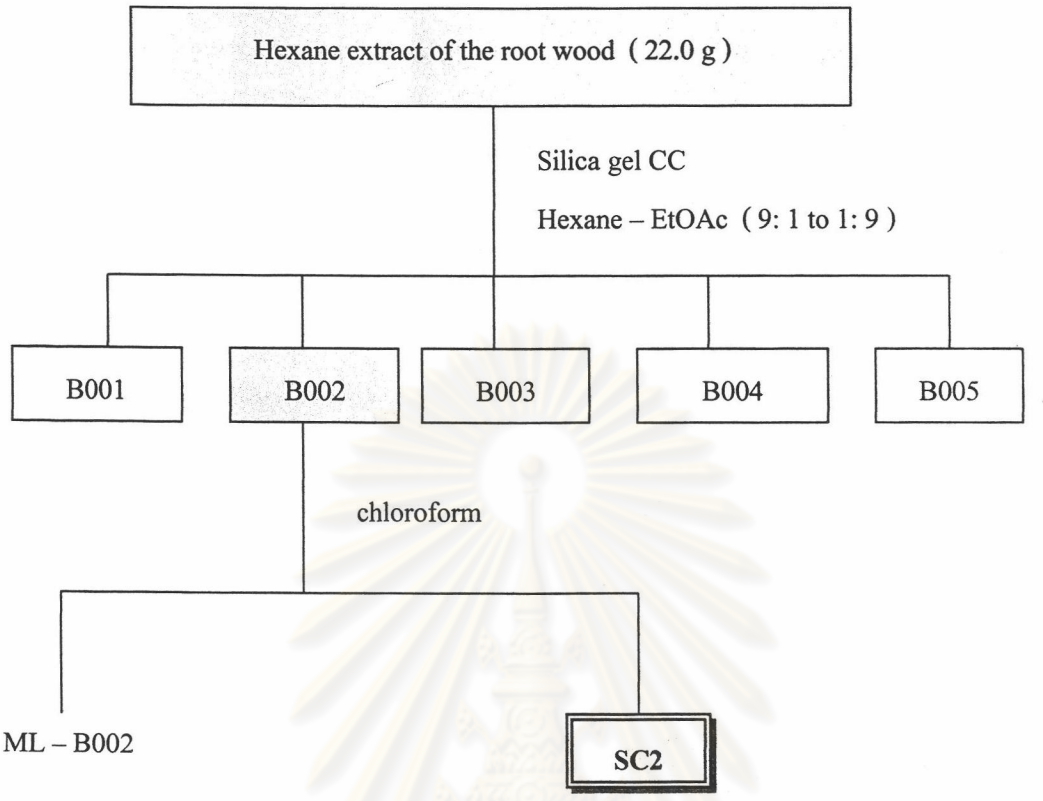
A020 (83 mg) was subjected to repeated Sephadex LH - 20 gel filtration column chromatography using a solvent system of methanol - chloroform (1 : 1) to give compound SC3 (11 mg) as red crystals.



Scheme 3. Isolation of pure compounds SC1and SC2 from the root bark of *S. celastrineus.*



Scheme 4. Isolation of compound SC3 from the root bark of *S. celastrineus*.



Scheme 5. Isolation of compound SC2 from the root wood of *S. celastrineus*.

5. Characterization of the isolated compounds

Compound SC1

Colorless needles (chloroform).

Melting point : 310 ^{0}C .

Soluble in methanol ,ethyl acetate, and acetone.

$[\alpha]_D$ +11^{0}(c = 0.048 , $CHCl_3$).

UV λ_{max} (MeOH) nm (log ε) :229 (4.40) (Figure 6, page 85)

IR ν_{max}, (KBr), cm^{-1}: 3468 , 2929 , 1716 , 1638 , 1278 , 1117 , 1028 , 710 (Figure 7 , page 86)

FAB - MS , m / z (rel. int.) :

643 [M + Na]$^{+}$(7), 621 [M+H]$^{+}$(3), 603 (10), 543 (1), 514 (1), 498 (5), 411(3), 208 (10), 121(15), 105(100), 77(25) ,43(20) (Figure 8 , page 87)

^{1}H - NMR (600 MHz , $CDCl_3$):

8.07 (2 H, *d*, *J* = 7.4 Hz) , 7.57 (1 H, *t* , *J* = 7.4 Hz) , 7.45 (2 H , *t* , *J* = 7.4 Hz), 6.44 (1 H, *d*, *J* = 12.6 Hz) , 4.48 (1 H , *dd* , *J* = 11.8 , 4.6 Hz), 3.50 (1 H , br *s*), 2.16 (1 H, *m*) , 2.14 (1 H , *m*), 2.06 (1 H , *d* , *J* = 12.6 Hz), 2.02 (3 H , *s*) , 1.98 (1 H , *m*) , 1.88 (1 H , *m*), 1.63 (2 H , *m*), 1.58 (1 H , *m*) , 1.56 (1 H , *m*), 1.52 (1 H , *m*) , 1.51 (1 H , *m*) , 1.49 (3 H , *s*) , 1.43 (1 H , *m*), 1.41 (1 H , *m*), 1.34 (1 H , *m*) , 1.23 (3 H , *s*) , 1.22 (1 H , *m*), 1.21 (1 H , *m*) , 1.20 (2 H , *m*) , 1.13 (1 H , *m*) , 1.12 (1 H , *m*) , 1.04 (3 H , *s*) , 1.03 (3 H , *s*) , 0.90 (3 H , *s*) , 0.88 (1 H , *m*) , 0.87 (3 H , *s*) , 0.86 (3 H , *s*) , 0.84 (3 H , *s*) (Figure 9, pages 88 - 90)

^{13}C - NMR (150 MHz, $CDCl_3$) :

202.2 (*s*) , 171.2 (*s*) , 165.6 (*s*) , 133.2 (*d*) , 133.2 (*s*) , 130.1 (2 C) (*d*) , 128.6 (2C) (*d*) , 83.1 (*s*) , 80.2 (*d*) , 75.1 (*d*) , 55.2 (*d*) , 54.2 (*d*) , 49.1 (*d*) , 44.8 (*s*) , 44.1 (*s*) , 40.3 (*t*) , 39.3 (*s*) , 39.1 (*t*) , 38.4 (*s*) , 38.3 (*t*) , 34.5 (*t*) , 34.0 (*t*) , 33.5 (*s*) , 32.3 (*q*) , 31.5 (*q*) , 31.4 (*s*) , 30.0 (*t*) , 28.2 (*q*) , 24.5 (*q*) , 23.9 (*t*) , 22.9 (*t*) , 21.4 (*q*) , 20.9 (*q*) , 18.9 (*q*) , 17.7 (*t*) , 16.4 (2 C) (*q*) (Figure 10 , pages 91 – 92)

Compound SC2

Colorless needles (chloroform).

Melting point : 142 – 145 ° C . Soluble in methanol , ethyl acetate , and acetone.

UV λ_{max} (MeOH) nm (log ε) : 207 (2.92) , 281 (1.81)
(Figure 16 , page 102)

IR ν_{max} , (KBr), cm^{-1}: 3500 , 3000 , 1640 , 1470 , 1090 (Figure 17 , page 103)

EI – MS , m / z (rel. int) :

414 [M]$^{+}$(100) , 396 (40) , 381 (20) , 354 (7) , 329 (42) , 303 (45) , 273 (23) , 255 (25) , 213 (30) , 159 (33) , 145 (50) , 133 (40) , 119 (48) , 107 (65) , 95 (70) , 91(60) , 81(65) , 69 (45) , 55 (65)
(Figure 18 , page 104)

^{1}H - NMR (500 MHz , $CDCl_3$) :

5.35 (1 H , *m*) , 3.52 (1 H , *m*), 1.00 (3 H , *s*) , 0.92 (3 H , *d* , *J* = 6.5 Hz) , 0.86 (3 H , *t* , *J* = 7.2 Hz) , 0.85 (3 H , *d* , *J* = 6.8 Hz) , 0.82 (3 H , *d* , *J* = 6.8 Hz) , 0.70 Hz (3 H , *s*) (Figure19 , pages 105 – 106)

^{13}C - NMR (125 MHz, $CDCl_3$):

140.8 , 121.7 , 71.8 , 56.8 , 56.0 , 50.1 , 45.8 , 42.3 (2 C) , 39.8 , 37.2 , 36.5 , 36.1 , 33.9 , 31.9 (2 C) , 31.7 , 29.1 , 28.2 , 26.1 , 24.3 , 23.1 , 21.1 , 19.8 , 19.4 , 19.0 , 18.8 , 12.0 , 11.9
(Figure 20 , pages 107 - 108)

Compound SC3

Orange - red crystal (chloroform).

Melting point 219 – 220 °C.

Soluble in methanol, ethyl acetate, and acetone.

$[\alpha]_D$ -188^0 (c = 0.058, MeOH).

UV λ_{max} (MeOH) nm (log ε) : 255 (2.34) , 426 (2.52)(Figure 21, page 109)

IR ν_{max}, (KBr), cm^{-1}: 3414 , 2929 , 1727 , 1615 , 1438 , 1384 , 616 (Figure 22, page 110)

FAB – MS , m / z (rel . int.) :

487 [M + Na]$^+$(20) , 465 [M + H]$^+$(30) , 464 [M]$^+$(10) , 263 (10) , 202 (35), 201(100) (Figure 23, page 111)

^{1}H - NMR (500 MHz , $CDCl_3$) :

7.01 (1 H , *dd* , J = 7.0 , 1.5 Hz) , 6.96 (1 H , br *s*) , 6.53 (1 H , *d*, J = 1.5 Hz) , 6.34 (1 H , *d* , J = 7.0 Hz) , 3.55 (3 H , *s*) , 2.45 (1 H , *d* , J = 15.6 Hz), 2.20 (3 H , *s*) , 2.15 (1 H, *dd*, J = 11.6 , 4.6 Hz) , 2.14 (1 H , *m*) , 2.05 (1 H , *t d*, J = 14.3 , 10.1 Hz) , 1.87 (1 H , *t d*, J = 14.0 , 6.1 Hz) , 1.82 (1 H, *dd*, J = 12.8 , 5.5 Hz) , 1.78 (1 H , *m*) , 1.70 (1 H , *d* , J = 15.6 Hz) , 1.65 (1 H , *m*) , 1.57 (1 H , *m*) , 1.55 (1 H , *m*) , 1.53 (1 H , *m*) , 1.50 (1 H , *m*), 1.45 (3 H , *s*) , 1.38 (1 H, *t d*, J = 14.0 , 4.6 Hz) , 1.26 (3 H , *s*) , 1.17 (3 H , *s*) , 1.10 (3 H , *s*) , 0.99 (1 H , *dt* , J = 14.3 , 4.5 Hz) , 0.53 (3 H , *s*)

(Figure 24 , pages 112 – 114)

^{13}C - NMR (125 MHz , $CDCl_3$) :

178.7 (*s*) , 178.3 (*s*) , 170.0 (*s*) , 164.8 (*s*) , 146.0 (*s*) , 134.0 (*d*) , 127.4 (*s*) , 119.6 (*d*) , 118.1 (*d*) , 117.1 (*s*) , 51.6 (*q*) , 45.0 (*s*) , 44.3 (*d*) , 42.9 (*s*) , 40.4 (*s*) , 39.4 (*s*) , 38.3 (*q*) , 36.4 (*t*) , 34.8 (*t*) , 33.6 (*t*) , 32.7 (*q*) , 31.6 (*q*) , 30.9 (*t*) , 30.5 (*s*) , 29.9 (*t*) , 29.6 (*t*) , 28.6 (*t*) , 21.6 (*q*) , 18.3 (*q*) , 10.2 (*q*)

(Figure 25 , pages 115 – 116)