CHAPTER III

EXPERIMENTAL

Source of Plant Material

The stems of *Strychnos nitida* G.Don (Loganiaceae) were collected from Sakae-raj, Nakorn-Ratchasima province, Thailand in July,1993. The plant material was authenticated by comparison with the herbarium specimen at Royal Forest Department, Bangkok, Thailand.

A voucher specimen of the plant material has been deposited in the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

General Techniques

1. Chromatography

1.1 Thin Layer Chromatography (TLC)

Technique : one way ascending

Adsorbent : silica gel 60 G (No.7731, Merck) and silica gel 60 HF_{254} (No.7739, Merck) in ratio 2:1 30 g. in 60 ml. distilled water.

Plate sizes : 5 x 20 cm., 10 x 20 cm. or 20 x 20 cm.

Layer thickness : 0.25 mm.

Activation : air-dried for 15 min. and then warm in hot air oven at 110° C for 1 hour

Solvent system : various solvent systems depending on the material.

Distance : 15 cm.

Laboratory temperature: 28°-35° C

Detection : 1) UV light (254 and 366 nm.)

2) Ferric chloride-perchloric acid

The reagent was made by mixing 1 ml. of 0.5 M ferric chloride solution with 100 ml. of 35% aqueous perchloric acid solution.

The reagent gave a variety of colors depending on the nature of the substitution pattern in the aromatic part of the *N*-acylindoline nucleus and also on the different type of alkaloid skeletons. The color would either develope immediately after spraying or only after heating the chromatographic plate at 90° C for 5 to 30 minutes.

3) Dragendorff's Reagent

This reagent was used as a general alkaloidal detecting reagent which characterized the alkaloids by giving orange color. The stock solution consisted of bismuth oxynitrate 1.7 g., glacial acetic acid 20 ml., distilled water 80 ml. and 5% aqueous potassium iodide 100 ml.

The working solution was made by mixing 10 ml. of the stock solution with 20 ml. of glacial acetic acid and 70 ml. of distilled water.

1.2 Column Chromatography

1.2.1 Conventional Column Chromatography (CC)

Adsorbent : silica gel 60 G (No.9385 E. Merck).
Packing method : wet packing.
Solvent : various solvent systems depending on the material.

1.2.2 Flash Chromatography

Adsorbent : silica gel 60 (No.1511 E. Merck).
Packing method : wet packing.
Solvent : various solvent systems depending on the materials.

1.2.3 Medium Pressure Liquid Chromatography (MPLC)

Adsorbent : silica gel 60 (No.1511 E. Merck).
Packing method : wet packing.
Solvent : various solvent systems depending on the material.

1.2.4 Gel Chromatography

Adsorbent : Sephadex LH-20
Packing method : wet packing.
Solvent : chloroform: methanol (2:1)

2. Physical Constant

2.1 Melting Point

Melting points were determined on a Fisher-John Melting Point Apparatus (uncorrected) .

2.2 Optical rotation

Optical rotation was measured on a Perkin Elmer 341 polarimeter.

3. Spectroscopy

3.1 Ultraviolet (UV) Absorption Spectroscopy

Ultraviolet absorption spectra were obtained with a Hitachi U3400 spectrophotometer.

3.2 Infrared (IR) Absorption Spectroscopy

Infrared absorption spectra of SN-1 and SN-2 were obtained with a Nicolet Magna IR spectrophotometer 750 . The spectra of the rest of the compounds were measured on a Perkin Elmer FT-IR 1760X spectrophotometer. The material was examined as dry film on KBr cell. The absorption bands were reported in wave number (cm^{-1}).

3.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H NMR and ^{13}C NMR spectra were obtained with a JEOL JNM-A 500 (alpha series) 500 MHz spectrometer. Deuterochloroform ($CDCl_3$) and Deuteromethanol (CD_3OD) were used as operating solvents for compounds SN-1, SN-2, SN-3, SN-4, SN-8 and compounds SN-5, SN-6, SN-7 respectively. Chemical shifts were reported in ppm sclae using TMS as an internal standard. The operating parameters were adjusted to required experiments of ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC, HMBC ($^{n}J_{C-H}$= 8 Hz) and NOESY.

3.4 Mass Spectroscopy (MS)

The EIMS were determined by direct inlet system operating at 70 eV. A Fison VG Autospec mass spectrophotometer was used for SN-3 and a Fison AG Trio 2000 quadrupole mass spectrophotometer for the rest of the compounds.

Extraction and Isolation

1. Extraction

The dried coarsely powdered stems (35 kg) were exhaustively extracted by maceration for 24 hours with 95% ethanol and by percolation with the same solvent. After the percolate was concentrated, the residue (2.5 kg) was partitioned between hexane and methanol. The combined hexane extract was evaporated to dryness under reduced pressure to yield crude hexane extract (290 g). The combined methanol extract was dried by the same process to give crude methanol extract (2.1 kg).

The crude methanol extract was divided into 2 portions. One portion (400 g) was submitted to direct separation by chromatography. The other (1.7 kg) was further partitioned between 10% sulfuric acid and chloroform. The combined acid extract was basified with conc. ammonium hydroxide and extracted with chloroform. The combined chloroform extract was washed with a small volume of water, dried over anhydrous sodium sulfate and then evaporated to dryness under reduced pressure to yield the crude alkaloid mixture (70 g).

2. Isolation

Isolation of compounds from the crude alkaloid mixture

The crude alkaloid mixture was divided into 2 parts. Each part (35 g) was chromatographed on a column of silica gel (500 g, 9.2 x 13 cm.) by elution with a mixture of hexane-ethyl acetate-diethylamine (5:4:1) and then methanol. Fractions of 50 ml. were collected and examined by TLC (hexane-ethyl acetate-methanol (5:4:1); Dragendorff's and $FeCl_3/HClO_4$ reagents). The fractions of similar chromatographic patterns were combined together as shown in the table:

Portion	Fraction	Eluent	Weight (g)
A-1	1-22	|	0.6
A-2	23-42	hexane:ethyl acetate:diethylamine	1.4
A-3	43-70	5:4:1	15.4
A-4	71-112	|	21.8
A-5	113-130	methanol	28.2

(Similar results were obtained from both part of the crude alkaloid mixture . Corresponding portions were combined together).

The portion A-2, A-3 and A-4 were suggested by TLC to contain several alkaloids which gave a variety of colors (*eg.* pink, red, orange, grey, *etc*) with $FeCl_3/HClO_4$ reagent. Major alkaloids indicated by two intense-colored spots, a larger orange and a smaller red ones, were present in the portion A-4. This portion (21.8 g)was submitted to separation by MPLC using a column of silica gel (250 g, 5.3 x 27 cm.) with a mixture of hexane:ethyl acetate:diethylamine (7:2:1) as an eluent. The fractions of 30 ml. were collected and examined by TLC (hexane: ethylacetate : diethylamine (7:2:1), $FeCl_3/HClO_4$ reagent). Combined fraction 22-38, the chromatogram of which showed a large orange spot of the main component, was evaporated to dryness under reduced pressure and dissolved in a small amount of chloroform and ethyl acetate to yield colorless needle crystals, designated as SN-1 (1.5 g).

Combined fraction 39-51, which gave one major red spot along with other minor ones, was re-fractionated by MPLC on a silica gel column (80 g, 2.5 x 27 cm.) with the eluent as used in the former separation. Eluates containing a major component which gave a red color with $FeCl_3/HClO_4$ reagent were combined together. Crystallization from a mixture of chloroform and ethyl acetate afforded colorless prismatic crystals, designated as SN-2 (55 mg).

The portion A-3 (15.4 g) which contained several minor alkaloids was further investigated. The portion was separated by MPLC using a column of silica gel (250 g, 5.3 x 27 cm.) with a mixture of hexane-ethyl acetate-methanol (5:4:1) as an eluent. 30 ml. of eluates were collected; the fractions were combined together on the basis of TLC detection (hexane-ethyl acetate-methanol (5:4:1); $FeCl_3/HClO_4$ reagent).

The chromatogram of combined fraction 15-22 showed two interesting grey spots together with others of different colors. This fraction was fractionated by MPLC on a silica gel column (80 g, 2.5 x 27 cm.) with a mixture of hexane:ethyl acetate:diethylamine (7:2:1). Two compounds which gave the grey spots with $FeCl_3/HClO_4$ reagent were eluted separately. Each of the fractions containing these two compounds was further subJected to separation by Flash chromatography.

The fraction showing the grey spot with lower hR_f value was chromatographed on a silica gel column (20 g, 2 x 13 cm.) with a mixture of hexane-ethyl acetate-methanol (14:4:1). Fractions of 20 ml. were collected and monitored by TLC (hexane-ethyl acetate-methanol (5:4:1), $FeCl_3/HClO_4$ reagent). The same process of separation was repeated 4 times to give a pure compound, obtained in a white amorphous form, coded as SN-3 (7.0 mg).

The fraction containing the other interesting compound was submitted to fractionation over a column of silica gel (20 g, 2 x 13 cm.) using a mixture of hexane-ethyl acetate-diethylamine (14:4:1) as an eluent. The fractionation was repeated over four columns by using two different systems of eluents: a mixture of hexane-ethyl acetate-diethylamine (14:4:1) for the first two columns and that of hexane-ethyl acetate-methanol (14:4:1) for the others. From such fractionations a compound designated as SN-4 (8.0 mg) was isolated and purified, obtained as a white amorphous substance.

The portion A-2 (1.4 g) was also investigated. However, no pure compounds were obtained because of very small amounts of each compound together with its decomposition during the process of separation.

Isolation of compounds from the crude methanol extract

The crude methanol extract (400 g) was mixed with sufficient quantity of Kieselguhr and eluted with hexane until the eluates were diluted and clear. The elution was repeated by changing the eluent from hexane to chloroform, ethyl acetate and methanol respectively. Eluates from the same eluent were collected together. Four fractions from the eluents were detected by TLC (hexane-chloroform-methanol 2:3:1, $FeCl_3/HClO_4$ reagent). Ethyl acetate fraction (76 g) which gave some interesting spots was further investigated. Ethyl acetate fraction was evaporated to dryness and divided into 4 parts. Each part (19 g) was roughly fractionated by elution through a column of Sephadex LH-20 (85 g, 2.0 x 69 cm.) with the mixture of $CHCl_3$:MeOH (2:1). Fractions of 50 ml. were collected and examined by TLC (hexane-chloroform-methanol 2:3:1, $FeCl_3/HClO_4$ reagent). Those of similar chromatographic pattern were combined together as shown in the table below:

Portion	Fraction	Weight (g)
B-1	1-5	23.6
B-2	6-13	26.0
B-3	14-17	18.4
B-4	18-19	2.5
B-5	20-26	5.3

Portions B-2 (26.0 g) and B-4 (2.5 g), each of which contained one component which developed an intensed blue color with $FeCl_3/HClO_4$ reagent after heating about 5 minutes, were further investigated . The latter portion was separated by MPLC on a silica gel column (40g, 2.5 x 15 cm.) with chloroform-methanol mixtures of increasing polarity. Fractions of 30 ml. were collected and detected by TLC ($CHCl_3$-MeOH (9:1), $FeCl_3/HClO_4$ reagent). The combined fraction which showed the intensed-blue colored spot was rechromatographed by MPLC using a silica gel column (83 g, 2.5 x27 cm.) with a mixture of hexane -ethyl acetate-methanol (10:5:1) as an eluent. Fractions of 20 ml were collected and examined by TLC (hexane-ethyl acetate-methanol (6:3:1), $FeCl_3/HClO_4$ reagent). The eluates containing the compound of interest were combined together. Crystallisation from methanol afforded 8.0 mg of a white amorphous substance, designated as SN-5.

The portion B-2 was chromatographed on a column of silica gel (500 g, 9.2 x 14 cm.) by elution with a mixture of hexane-chloroform-methanol (2:3:1). After the 200 ml., fractions of 50 ml. were collected and combined together on the basis of TLC (hexane-chloroform-methanol 2:3:1, $FeCl_3/HClO_4$ reagent). Combined fraction 15-35 gave two major spots; one of higher hR_f value was red-violet in color and the other was the afroementioned blue one. The latter spot was also showed as only one major spot in the chromatogram of combined fraction 36-52.

The combined fraction 15-35 was further separated by flash chromatography using a column of silica gel (80 g, 2.5 x 24.5 cm.) with a mixture of toluene-ethyl acetate-methanol (4:1:1) as an eluent. Two major components of the fraction were eluted separately. The eluates showing the intensed blue spot were gathered into the combined fraction 36-52 of the former separation. This portion was submitted to fractionation by flash chromatography on a column of silica gel (80 g, 2.5 x 24.5 cm.) eluted with a mixture of chloroform-ethyl acetate-methanol (8:1:1), followed by

gel chromatography using Sephadex LH-20 as the stationary phase (15 g, 1.5 x 27 cm.) with chloroform-methanol (2:1) as an eluent. From these fractionations, the compound designated as SN-6 (700 mg), was obtained in a white amorphous form.

The eluates of the combined fraction 15-35 which contained the component giving a red-violet color with $FeCl_3/HClO_4$ reagent was evaporated to dryness under reduced pressure, dissolved in a small amount of chloroform-methanol mixture to yield colorless prismatic crystals designated as SN-7 (900 mg).

Isolation of compounds from the crude hexane extract.

The crude hexane extract (290 g) was dissolved in hexane to allow the precipitation. The precipitate and the filtrate were collected separately. The precipitate was further purified by crystallisation in a mixture of hexane and chloroform to yield colorless prismatic crystals, designated as SN-8 (10.6 g) which gave a red color with Lieberman-Burchard's reagent. The investigation of the filtrate was also carried out, indicating the presence of some long chain fatty acids as the major components.

Characterization of isolated compounds

1. SN-1

Melting point : 168-170° C

$[\alpha]_D$: +22.7° (c = 0.50 in MeOH)

UV λ_{max} (MeOH) nm : 209, 253, 270, 290

IR ν_{max} (KBr) cm^{-1} : 3400, 2950, 1640, 1595, 1500, 1400

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)

: 1.62 (m), 1.72 (d J=7.1 Hz), 1.93 (m), 2.31 (s), 2.41 (s), 2.79 (m), 2.93 (m), 3.04 (br s), 3.06 (br s), 3.18 (m), 3.25 2.80 (dd: J=10, 8 Hz), 3.4 (m), 3.73 (br s), 3.76 (br s), 2.81 3.80 (br s), 3.83 (br s), 3.88 (br s), 4.13 (d: J=7.5 2.82 Hz), 4.62 (d: J=7.9 Hz), 5.44 (q), 7.06 (m), 7.22 2.83 (m), 8.11 (d: J=8.1 Hz)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)

: 13.3, 13.4, 19.0, 19.2, 23.2, 23.9, 29.1, 29.8, 40.8, 41.1,

41.6, 42.6, 50.5, 51.8, 52, 55.2, 57.6, 58.1, 61.0, 61.5, 64.2, 64.5, 114.3, 117.2, 120.8, 121.8, 122, 122.1, 124.0, 124.4, 127.9, 128.3, 135.5, 136.3, 136.6, 137.8, 142.1, 142.9, 168.8

EIMS : *m/z* (% relative intensity)
338 (M^+ 79.23), 321 (13.85), 307, (13.06), 293(20.00), 185 (8.09), 166(100.00), 144 (39.23), 130(22.31).

2. SN-2

Melting point : 117-118 ° C

$[\alpha]_D$: +13.4° (*c* = 0.15 in MeOH)

UV λ_{max} (MeOH) nm : 223, 249, 291

IR ν_{max} (KBr) cm^{-1} : 3400, 2930, 1650, 1610

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)
: 1.60 (m), 1.70 (d: *J*=6.1 Hz), 1.92 (m), 2.31(s), 2.41 (s), 2.79 (m), 2.92 (m), 3.03 (br s), 3.81 (s), 4.12 (d: *J*=7.6 Hz), 4.59 (d: *J*=7.9 Hz), 5.44 (q), 6.61 (dd: *J*=8.2, 2.1 Hz), 6.67 (d: *J*=1.5 Hz), 6.95 (d: *J*=8.2 Hz), 7.02 (d: *J*=8.2 Hz), 7.80 (d: *J*=2.14 Hz)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)
: 13.4, 19.0, 19.1, 23.3, 24, 29.2, 30.0, 40.8, 41.1, 41.5, 42.6, 49.9, 51.2, 51.7, 51.9, 55.1, 55.6, 57.5, 58.1, 61.2, 61.6, 64.7, 65.1, 102.7, 103.5, 107.6, 110.3, 121.1, 121.9, 122.2, 122.4, 127.3, 129.9, 136.3, 136.6, 143.1, 144.0, 159.8, 160.0, 168.7,169.0

EIMS : *m/z* (% relative intensity)
368 (M^+ 47.69), 323(11.54), 215(11.54), 174(34.62), 166(21.54), 160(2.8).

3. SN-3

Melting point : 247-249 ° C

$[\alpha]_D$: +17.8° (*c* = 0.03 in MeOH)

UV λ_{max} (MeOH) nm : 226, 285, 295

IR ν_{max} (KBr) cm^{-1} : 2930, 1453, 1383

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)

: 1.59 (3H, d: *J*=6.7 Hz), 1.69 (1H, dt: *J*=12.5, ~3 Hz), 1.81 (1H, q: *J*=6-7Hz), 1.98 (1H, td: *J*=11-12, 2.1Hz), 2.64 (1H, d: *J*=15.3 Hz), 2.75 (1H, m), 2.78 (1H, t: *J*=5-6 Hz), 3.05 (1H, dd: *J*=15.3, 5.2 Hz), 3.50 (4H, m), 4.11 (1H, d: *J*=10 Hz), 5.29 (1H, q: *J*=6.7 Hz), 7.08 (1H, td: *J*=7.3-7.5), 7.13 (1H, td: J=7.3-7.4, 1.2 Hz), 7.30 (1H, d: *J*=7.4 Hz), 7.44 (1H, d: *J*=7.5 Hz), 8.18 (1H, br s)

^{13}C-NMR (125MHz in $CDCl_3$, δ in ppm)

: 12.7, 26.9, 27.6, 34.4, 44.2, 50.4, 54.4, 55.8, 65.0, 104.6, 110.9, 116.8, 118.1, 119.4, 121.4, 127.7, 135.4, 136.4, 137.9

EIMS : *m/z* (% relative intensity)

294 (M^+ 92.17), 293 (30.80), 263 (28.56), 169 (100.00), 168 (58.89)

4. SN-4

Melting point : 146-148 ° C

$[\alpha]_D$: +5.4° (*c* = 3.10 in MeOH)

UV λ_{max} (MeOH) nm : 232, 252,310

IR ν_{max} (KBr) cm^{-1} : 2910, 2830, 1735, 1640, 1115, 1080

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)

: 1.57 (3H, d: *J*=6.1 Hz), 1.67 (1H, m), 1.88 (1H, d: *J*=13.3 Hz), 2.02 (1H, dd: *J*=13.3, 3.4 Hz), 2.26 (1H, d: *J*=14.3 Hz), 2.61 (2H, br s), 2.75 (1H, br s), 2.97 (1H, d: *J*=16.4 Hz), 3.35 (2H, m), 4.13 (1H, d: *J*=16.4 Hz), 5.29 (1H, q: *J*=6.1 Hz), 6.89 (2H, br s), 7.08 (1H, m), 7.22 (1H, d: *J*=8.2 Hz), 9.00 (1H, br s)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)

: 12.7, 25.6, 29.9, 40.4, 42.8, 48.4, 57.3, 64.5, 82.1, 106.0,

110.7, 116.5, 118.6, 119.4, 121.7, 127.1, 134.7, 136.3, 138.2

EIMS : *m/z* (% relative intensity)
310 (M^+ 4.98), 184(10.75), 185(8.53), 138(9.25),

5. SN-5

Melting point : 190 - 193 ° C

$[\alpha]_D$: 0° (c 0.80, MeOH)

UV λ_{max} (MeOH) nm : 280

IR ν_{max} (KBr) cm^{-1} : 3440, 2940, 1610, 1445

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)
: 1.62 (1H, m), 1.96 (1H, m), 2.56 (1H, dd : *J*=15.1, 11.3 Hz), 2.69 (1H, dd : *J*=15.1, 4.9 Hz), 3.37 (1H, s), 3.48 (3H), 3.58 (1H, dd : *J*=10.7, 5.2 Hz), 3.73 (3H, s), 3.85 (3H, s), 4.30 (1H, d : *J*=5.5 Hz), 6.37 (2H, s), 6.58 (1H, s)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)
: 33.6, 40.9, 42.3, 48.9, 56.6, 56.8, 60.2, 66.8, 106.9, 107.8, 126.3, 130.2, 134.5, 138.9, 139.3, 147.7, 148.7, 149.0

EIMS : *m/z* (% relative intensity)
420 (m^+ 27.12), 402 (7.34), 371 (6.21), 210 (2.05), 205 (3.23), 197 (3.67)

6. SN-6

Melting point : 102-105 ° C

UV λ_{max} (MeOH) nm : 236

IR ν_{max} (KBr) cm^{-1} : 3400, 1610

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)
: 1.70 (m), 2.10 (m), 2.66 (m), 3.76 (s), 3.86, 4.13 (d: *J*=7.6), 4.23 (d : *J*=6.4 Hz), 4.28 (d : *J*=7.6 Hz), 4.42 (d :

J=6.1 Hz), 6.41 (s), 6.42 (s), 6.57 (br s)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)

: 33.8, 40.6, 41.2, 42.7, 43.2, 46.6, 46.7, 56.6, 56.9, 60.1, 60.2, 62.7, 62.8, 66.2, 71.5, 71.6, 72.0, 75.0, 75.1, 7.9, 78.1, 78.2, 104.2, 104.8, 106.9, 107.1, 107.8, 126.2, 126.4, 130.2, 134.5, 138.9, 139.3, 139.4, 147.5, 148.6, 148.9

EIMS : *m/z* (% relative intensity)
178(0.92), 154(5.54), 139(3.69)

7. SN-7

Melting point : 220-222 ° C

$[\alpha]_D$: -83.2° (*c* = 0.60 in MeOH)

UV λ_{max} (MeOH) nm : 238

IR ν_{max} (KBr) cm^{-1} : 3400, 1695, 1640, 1080, 1460

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)

: 1.09 (1H, d : *J*=7.0 Hz), 1.62 (1H, m), 1.87 (1H, m), 2.03 (1H, td : *J*=9.2, 4.4 Hz), 2.23 (1H, qd : *J*=13.2, 7.9, 1.4 Hz), 3.11 (1H, qd : *J*~8 Hz), 3.20 (1H, dd : *J*=9.0, 7.9 Hz), 3.30 (2H, m), 3.37 (1H, t: *J*=9.0 Hz), 3.67 (1H, dd : *J*=11.9, Hz), 3.69 (3H, s), 3.89 (1H, dd : *J*=11.9, 1.8 Hz), 4.4 (td : *J*=4.9, 1.4 Hz), 4.65 (d : *J*=7.9 Hz), 5.27 (d : *J*=4.4 Hz), 7.38 (d : *J*=1.2 Hz)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)

: 13.4, 32.1, 42.1, 42.7, 46.5, 62.7, 71.6, 74.7, 75.0, 78.0, 78.3, 97.7, 100.0, 114.0, 152.1, 169.5

EIMS : *m/z* (% relative intensity)
228(12.64), 210(15.53), 182(32.95), 179(46.74), 150(19.23), 139(29.89)

8. SN-8

IR ν_{max} (KBr) cm^{-1} : 3500 - 3200, 3000-2800, 1640, 1470, 1390, 1090.

^{1}H-NMR (500 MHz in $CDCl_3$, δ in ppm)

: 0.68-1.01, 3.52 (m), 5.35 (m)

^{13}C-NMR (125 MHz in $CDCl_3$, δ in ppm)

: 12.0, 19.0, 19.4, 19.8, 21.0, 23.0, 24.3, 26.1, 28.3, 29.1, 31.7, 32.0 (2C), 33.9, 36.2, 36.4, 37.2, 39.8, 42.3 , 45.9, 50.1, 56.0, 56.8, 72.0, 122.0, 140.5

EIMS : *m/z* (% relative intensity)

414(10.08), 381(5.04), 329(7.75), 273(8.14), 255 (25.58), 231(10.85), 213(26.74), 173(13.12), 147(29.84).

Bioactivity Determination

1 Brine Shrimp Lethality (BSL assay)

The bioassay was modified from the method of Maeyer *et al* [191]

1.1 Hatching the brine shrimp

The eggs of brine shrimp, *Artemia salina* Leach, (Sigma Union Inc., USA were hatched in a shallow rectangular box filled with artificial sea water which was prepared from a commercial salt mixture and double distilled water. The box was divided into 2 compartments by a perforated divider. The eggs were sprinkled into one compartment which was darkened. The other compartment was illuminated by a tungsten lamp. After 48 hours, the mature nauplii, which moved to the lighted side, were collected, having been separated from their shells by the divider.

1.2 Sample preparation

Tested compounds : SN-1, SN-2, SN-3, SN-4, SN-5, SN-6, SN-7.

The compound was dissolved in a proper solvent and diluted to the appropriate concentration. This stock solution was transferred to 3 vials in sufficient volumes, calculated for providing 3 final concentrations of 100, 10 and μg/ml respectively. The solution in each vial was allowed to dry in vacuum for overnight and 5 ml of artificial sea water were added. The dissolution was allowed with assistance of sonication. The determinations for each concentration were made in triplicate, using the solvent as controls.

1.3 Bioassay

Fifteen brine shrimps were transferred to each vial and the test solution was adjusted to 3 ml with artificial sea water immediately. The vials were maintained under illumination. After 24 hours, the number of dead brine shrimps in the test sample of each concentration was recorded. The results were reported in term of LD_{50} value ($BSLD_{50}$).

2 Antimicrobial activity

The activity was determined by means of the agar diffusion method. The assay was modified from the method of Cleeland and Grunberg [192] .

2.1 Microorganisms and media

The following microorganism strains were used in the test .

- gram positive bacteria - *Bacillus subtilis* ATCC6633
  - *Sarcina lutea* ATCC9341
  - *Staphylococcuc aureus* ATCC6538P
- gram negative bacteria - *Escherichia coli* ATCC25922
  - *Klebsiella pneumoniae* ATCC10031
  - *Pseudomonas aeruginosa* ATCC9721
  - *Salmonella typhimurium* ATCC14028
- yeast - *Candida albicans* ATCC10230
  - *Saccharomyces cerevisiae*ATCC9763

The bacteria were cultured overnight at 37° C in Muller Hinton (MH) broth and the yeasts were cultured for 48 hours at 37° C on Sabouraud Dextrose agar (SDA) slants. The media were sterilized prior to use by autoclaving at 121° C for 15 minutes.

2.2 Sample preparation

Tested compounds : SN-1, SN-2, SN-6 and SN-7.

Each of the compounds was dissolved in DMSO to give the stock solution for providing test solutions of various concentrations (2, 4 and 8 μg/ml for SN-1, SN-2 and SN-7; 2 and 4 μg/ml for SN-6).

2.3 Bioassay

The assay was performed under aseptic conditions. All glassware were sterilized prior to use with hot air oven at 180° C for 1 hour. The cultures of test organisms were diluted with saline solution. The turbidity of each suspension was adjusted to be equal to that of McFarland suspension no.0.5 (0.5 N $BaCl_2$ in 0.36 N H_2SO_4 w/v, equivalent to 10^8 cells/ml). The suspension was mixed with the melted agar medium (45-50° C) in the ratio 1:99 (the suspension : the medium). The mixture was poured into 10 cm. petri dishes (20 ml/dish) and allowed to be harden. In these test plates,

4 holes, 1 cm diameter, were made, with which 50 mcl of test solution were filled. All determinations were made in duplicate. Tetracycline HCl and tolnaftate were used as positive controls for the bacteria and the yeasts respectively. DMSO was used as a negative control.

3. Antiviral activity

The antiviral activity of the compounds was determined by using a simplified plaque-reduction assay [193] and acyclovir was used as a positive control.

3.1 Cell lines

Vero cells (African green monkey kidney cells) were used for viral infection.

3.2 Viruses

HSV type 1 (HSV-1) KOS strain and type 2 (HSV-2) Baylor 186 strain were employed in the experiment.

3.3 Sample preparation

Tested compounds: SN-1, SN-2, SN-4, SN-5, SN-6 and SN-7.

The tested compounds were dissolved in 10% DMSO in water at an appropriate concentration and diluted 5-fold with the same solvent. The samples at various concentrations were submitted to the test for cytotoxic activity [193] and a maximum non-cytotoxic concentration of each compound was recorded. The solution of the compound in 2.5% DMSO in complete medium prepared at the recorded concentration was used as the sample for antiviral bioassay.

3.4 Bioassay

Twenty-five microliters of the sample solution was mixed with 25µl of HSV-1 or HSV-2 suspension in complete medium containing 30 PFU per 25µl in 96 well microtiter plate. (Each sample was done in triplicate). After the incubation at 37° C for 1 hour, 50 µl of Vero cell suspension in complete medium, 6 x 10^{-5} cells/ml, was added to each well. After 3 hour-incubation at 37° C in CO_2-humidified incubation, the overlay medium containing the same concentration of each sample was added 100 µl/well. The plate was incubated for 24-48 hours at 37°C. The cells were observed and plaques were counted under inverted microscope. The cells were also fixed and stained with 4%

formalinized saline and 1% crystal violet in 0.25% formalin and the plaques were counted for confirmation. The positive control was done in the same manner using acyclovir.

4 Cardioactivity

The experiment with isolated auricles of the rat was employed for the determination of this activity. The effects of the compounds on the rate and the amplitude of the beats were observed through the results obtained from the kymograph [194].

4.1 Isolated auricles

A rat was killed by a blow on the head and cutting the throat, the chest was opened and the heart removed as quickly as possible and placed in Ringer-Locke solution at room temperature. All other tissue was cut away until nothing was left except the auricles. The left and right auricles were cut off separately. Each auricle was tied with two threads at the tip of each side. The preparation was mounted in Ringer-Locke solution at 30° C, through which a brisk stream of pure oxygen is blown. One thread was attached to a fixed pin in the bath and the other to a light spring-loaded lever writing on smoke drum, or to a force-displacement transducer connected to an amplifier and recorder. The volume of the organ bath was 20 ml.

4.2 Sample preparation

Tested compounds: SN-1, SN-2, SN-6 and SN-7.

The tested solution of the compound in absolute ethanol was prepared at concentration of 1×10^{-2} M.

4.3 Procedure

Twenty microliters of the test solution was pipetted into the organ bath containing each auricle. The final concentration for each compound was 1×10^{-5} M. Rate and amplitude of the beats were recorded .