CHAPTER 2

EXPERIMENTAL

2.1 General

Unless stated otherwise, solvents for general purposes were commercial grade and were re-distilled prior to use. All preparative HPLC solvents were analytical grade and were filtered through membrane filter 0.45 μm and degassed by ultrasonic sonicator prior to use. Analytical TLC was performed on Merck® pre-coated siliga gel 60 F_{254} plates (layer thickness 0.20 mm). Visualization was accomplished by observation under UV light (254 nm) and by staining with phosphomolybdic acid (10% solution in ethanol) followed by heating. The size-exclusion chromatography was conducted on a column of Sephadex® LH-20, which was allowed to saturate with eluting solvents as indicated for an overnight prior to use. Flash column chromatography was carried out using Merck® siliga gel 60 (particle size 0.04-0.06 mm, 230-400 mesh ASTM), according to the procedure described by Still, Kahn and Mitra (1978).

HPLC was performed on Waters® multisolvent delivery system (model 600E) connected to Waters® tunable absorbance detector (model 486). This was equiped with a Rheodyne® injector port (model 7125). Reverse phase HPLC was performed using Thermo Hypersil® BDS C_{18} (5 μ, 250×4.6 mm) or Hamilton®

PRP-1 semi-preparative (10 μ, 305×7.0 mm) C_{18} polymer-based columns. The normal phase HPLC column used was Econosil® semi-preparative (10 μ, 250×7.0 mm) column.

NMR spectra were recorded on a FTNMR, Varian Unity® Inova 500 spectrometer (500 MHz for proton and 125 MHz for carbon-13). The chemical shifts were reported on the δ-scale relative to the solvent signals. The operating NMR solvents used were benzene-d_6 (7.15 ppm of residual C_6HD_5 for ^{1}H NMR and 128.0 ppm for ^{13}C NMR) and chloroform-*d* (7.24 ppm of residual $CHCl_3$ for ^{1}H NMR and 77.0 ppm for ^{13}C NMR). Signal multiplicities were indicated by s, d, t, br, and m; denoting singlet, doublet, triplet, broad, and multiplet, respectively. IR spectra were recorded on a Jasco® IR-810 infrared spectrometer. UV spectra were obtained from a Spectronic® Genesys 5 spectrophotometers. Mass spectra were obtained from a Micromass® LCT mass spectrometer or HP 5890 GC series 2 plus-HP 5972 mass selective detector. Optical rotation and CD spectra were recorded in methanol on a Jasco® J-810 spectropolarimeter, using sodium D-line wavelength at 589 nm.

2.2 Sponge material

The sponge (Figure 3) was collected at the depth of 18-20 meters from Koh-Tao, Surat Thani, Thailand in April 2001, and in April 2002. It was identified as *Brachiaster* sp. (Order Astrophorida, Family Pachastrellidae) by Mr.Somchai Busaravich of Phuket Marine Biological Center. The sponge is lumpy- and

khaki internally. The outer texture is prickly, with tiny unpenetrating spines covering over the surface. When touched, the specimen appears very tough, incompressible, and resistible to be cut. The sponge voucher specimen (AP 01-008-03) was preserved in 70% ethanolic solution and was deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The remaining specimens were preserved at –20 °C until the time of extraction.

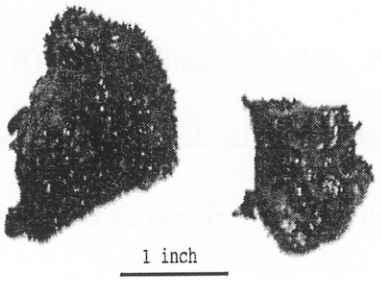


Figure 3 The Thai sponge, *Brachiaster* sp.

2.3 Bioactivity determination

2.3.1 Antituberculosis activity

The antituberculosis activity was assessed against *Mycobacterium tuberculosis* $H_{37}Ra$ using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The activity determination was kindly supported by Dr.Prasat Kittakoop of the National Center for Genetic Engineering and Biotechnology.

Initial sample dilutions were prepared in either DMSO or distilled deionized water, and subsequent two-fold dilutions, starting from 200 μg/mL, were performed in 0.1 mL of 7H9GC (no Tween 80) in the microplate. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC, and 0.1 mL was added to each well. Subsequent determination of bacterial titers yielded 2.5×10^6 CFU/mL in plate wells for $H_{37}Ra$. Frozen inocula were initially diluted 1:20 in BACTEC 12 B medium followed by a 1:50 dilution in 7H9GC. Addition of 1/10 mL to wells resulted in final bacterial titer of 5×10^4 CFU/mL. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37° C. Starting at day 4 of incubation, 20 μL of 10% Alamar blue solution and 12.5 μL of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink. If the B wells became pink within 24 h, reagent was added to the entire plate. If the wells remained blue, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C and resulted colors were recorded at 24 h post-reagent addition. For the positive control, isoniazid and kanamycin sulfate were used as standard drugs. The MICs of both agents in the test system were 0.040-0.090 and 2.0-5.0 μg/mL, respectively.

Visual MICs were defined as the lowest concentration of samples that prevented a color change.

2.3.2 Cytotoxic activity

The determination was kindly supported by Assist. Prof. Supreeya Yuenyongsawad of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The cell lines utilized as the target cells in this test were MCF-7 (breast adenocarcinoma), HeLa (human cervical cancer), HT-29 (colon cancer) and KB (human oral cancer). The sulphorhodamine B (SRB) assay was used in this study to assess growth inhibition. The following protocol is modified from that originally described by Skehan *et al.* (1990).

For the assay, monolayered culture of each cell line in a 96-well microtiter plate (2×10^3 cells/well) was treated with a serial dilution (at least five concentrations) of each sample in suitable culture medium. All the plates were incubated according to the reported condition for seven days, at the midway of which time the medium was refreshed once (exposure time of 72 h). On the seventh day of culture period, ice-cold 40% trichloroacetic acid (TCA) was added to each well. The plates were washed five times with water. The TCA-fixed cells were stained for 30 min with 0.4% SRB in 1% acetic acid. The plates were washed five times with 1% acetic acid and allowed to dry overnight. Once dried, bound dye was solubilized with 10 mM Tris base for 20 min on a gyratory shaker. Survival percentage was measured via the intensity of the resulted purplish-pink color at 492 nm (Power Wave X plate reader). The IC_{50} values were calculated

from the dose-response curves obtained by plotting the survival percentage against the concentrations of tested samples.

2.4 Isolation and purification

The freeze-dried sponge (158.60 g) from the first collection was crushed and macerated exhaustively (5×1.5 L) in methanol to yield a crude extract, which was then subjected to a solvent partitioning scheme using the procedure modified from Kupchan and Tsou *et al.* (1973). Hexane-, CH_2Cl_2- and *n*-BuOH-soluble materials were obtained (1.11 g, 0.70%; 1.64 g, 1.03%; 1.32 g, 0.83%, respectively). The hexane and CH_2Cl_2 fractions, which exhibited antituberculosis activity against *Mycobacterium tuberculosis* $H_{37}Ra$ with MIC of 6.25 μg/mL, were chosen for the isolation of the bioactive compounds (Scheme 1).

The CH_2Cl_2 fraction was fractionated by the chromatographic technique using a column of Sephadex LH-20 with methanol as eluent. The eluates were collected approximately 20 mL per fraction. Fractional pool, monitored by TLC technique (10% MeOH in CH_2Cl_2 as developing system) and comfirmed by antituberculosis assay, led to two major active fractions (MICs 6.25 and 3.125 μg/mL). These active fractions were further purified separately over a SiO_2 column (3% EtOAc in CH_2Cl_2), then repeatedly re-crystallized to yield compound **18** as white needles (99 mg, 6.0% of CH_2Cl_2 fraction), which was later identified as heteronemin.

The hexane fraction, which was also active, was chromatographed over a SiO_2 column (5% EtOAc in CH_2Cl_2). Fraction combination, achieved as stated above yield two active fractions (MIC 0.39 μg/mL). Each was separately further purified using reverese phase HPLC (5 μ, 250×4.6 mm) with isocratic 87% aqueous CH_3CN (flow rate 1 mL/min, UV detector, 220 nm). Two white non-crystallized compounds, **41** (10 mg, 0.9% of hexane fraction) and **42** (2 mg, 0.2% of hexane fraction), eluted at retention times 18.0 and 14.8 min, respectively, were collected. They were identified as heteronemin acetate and 12-epi-19-deoxyscalarin, respectively.

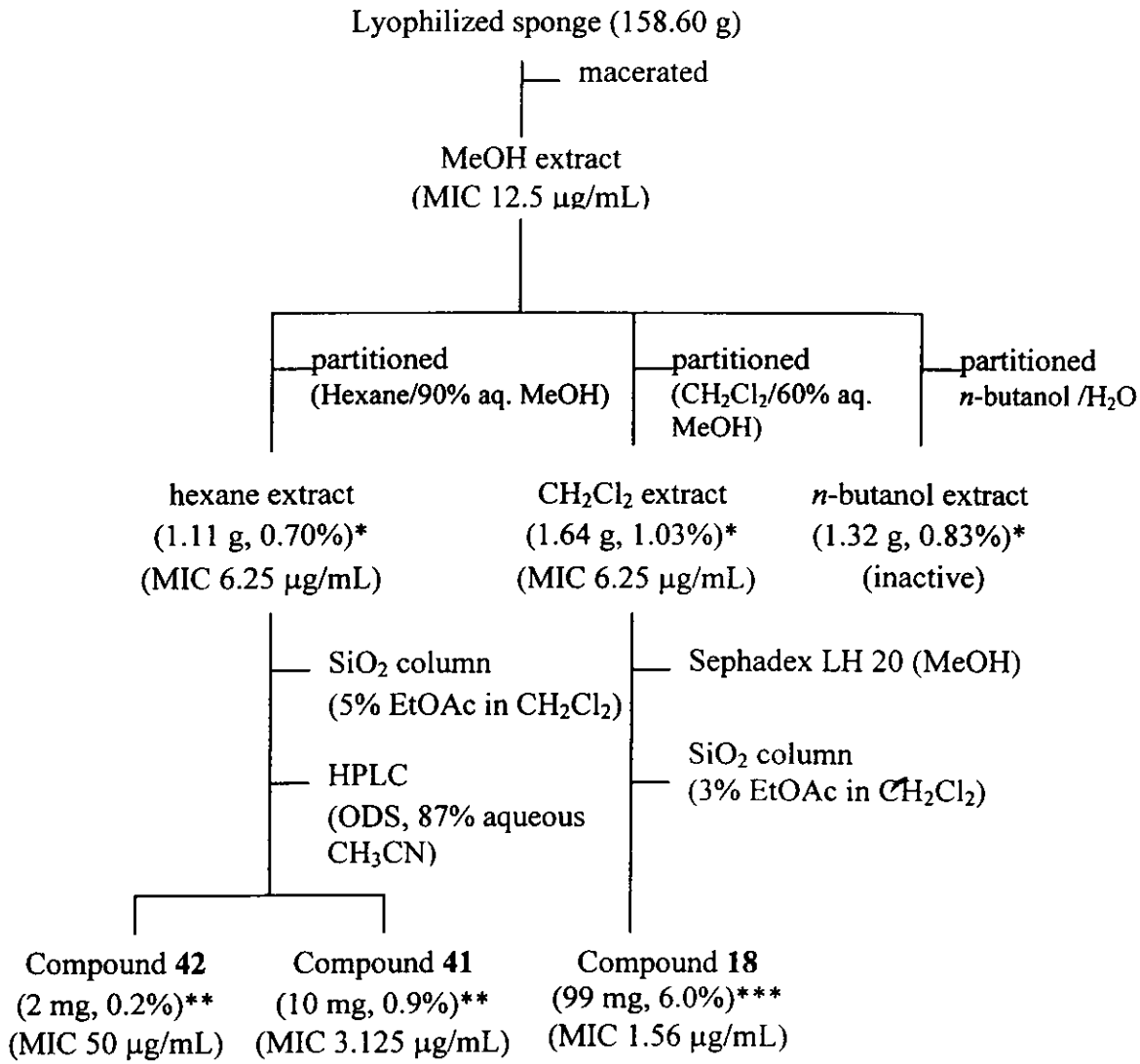
The freeze-dried sponge (212.33 g) from the second expedition was crushed and consecutively extracted with hexane (3×2 L), CH_2Cl_2 (3×2 L) and MeOH (3×2 L) (13.49 g, 6.35%; 1.36 g, 0.64%; 26.90 g, 12.67%, respectively). The large hexane fraction, which also exhibited antituberculosis activity (MIC of 3.125 μg/mL), was chosen for the further isolation of the bioactive compounds (Scheme 2).

An aliquot of the hexane fraction (4.30 g) was isolated by the flash chromatographic technique over a column of SiO_2 (20:5:75 of EtOAc:acetone: hexane). Fractions with similar chromatographic pattern were combined to yield four main fractions. The first fraction was re-crystallized with CH_2Cl_2-methanol mixture (1:3) to afford compound **18** (390 mg, 9.1% of hexane fraction), which was identical to that obtained from the previous expedition, was identified as heteronemin.

The second fraction was further fractionated using semi-preparative normal phase HPLC (10 μ, 250×7.0 mm) with isocratic 5% isopropanol in hexane as mobile phase (flow rate 2 mL/min; UV detector, 220 nm) to afford compound **43** (2 mg, 0.05% of hexane fraction, T_R 17.5 min) as white needles. It was identified as 12-deacetyl-12-epi-19-deoxyscalarin. The residue from this fractionation step was combined and further purified using reverese phase HPLC (5 μ, 250×4.6 mm) with isocratic 75% aqueous CH_3CN as mobile phase (flow rate 1 mL/min; UV detector, 220 nm). Two viscous colorless liquids, compounds **44** and **45** (4 and 7 mg; 0.1 and 0.2% of hexane extract, respectively) were obtained at 24.5 and 28.3 min. They were identified as (*E*) and (*Z*)-neomanoalide diacetates, respectively.

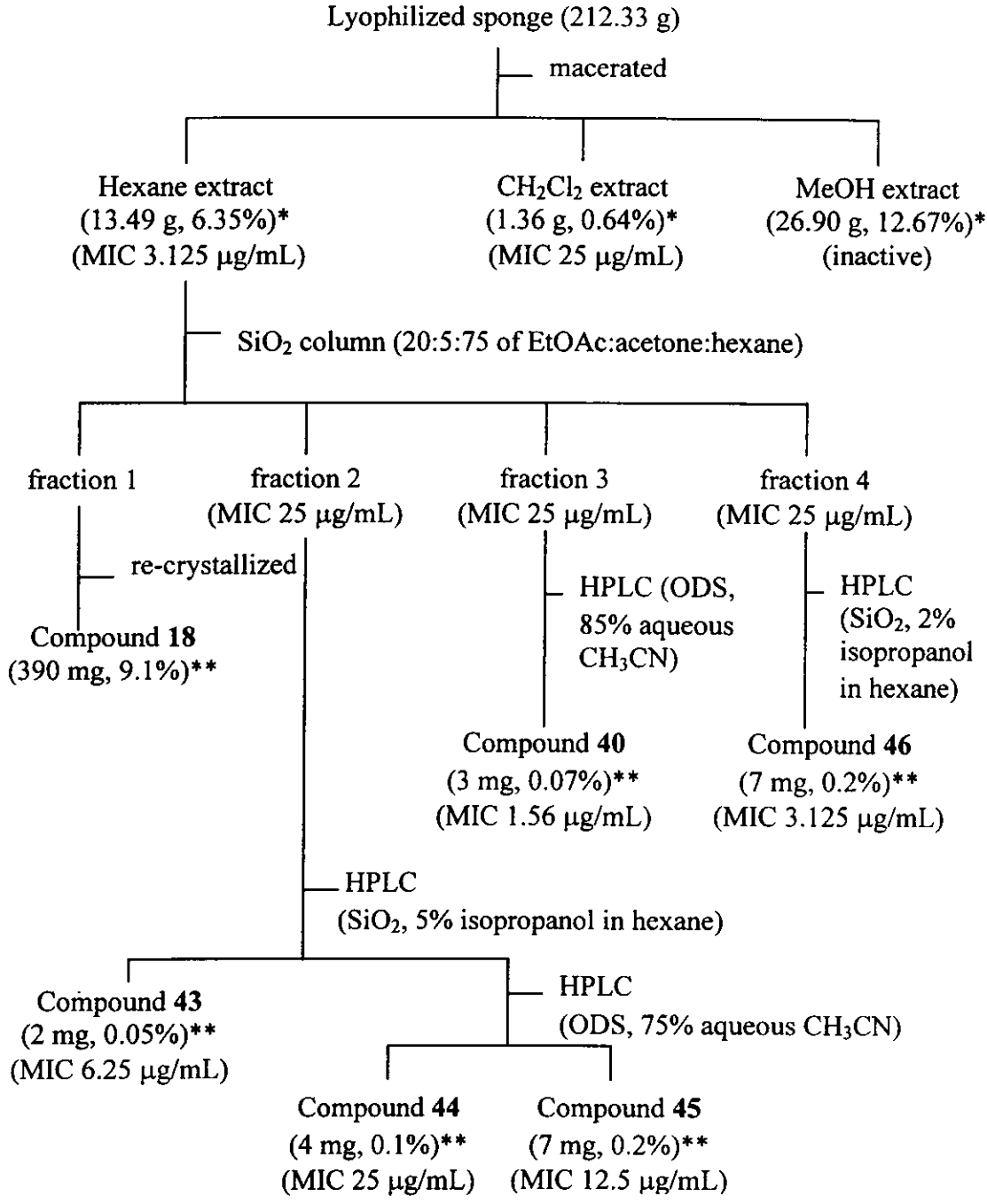
The third fraction was further purified using reverese phase HPLC (5 μ, 250×4.6 mm) with isocratic 85% aqueous CH_3CN as mobile phase (flow rate 1 mL/min; UV detector, 220 nm). A white non-crystallized compound, compound **40** (3 mg, 0.07% of hexane fraction), was obtained from the eluate at 27.1 min. It was identified as 12-deacetoxy-scalarin acetate. Along with **40**, the presence of **41** and **42** was also observed via TLC detection. The two compounds, however, were not further isolated in this step.

The last fraction was further isolated using semi-preparative normal phase HPLC (10 μ, 250×7.0 mm) with isocratic 2% isopropanol in hexane as mobile phase (flow rate 2 mL/min; UV detector, 220 nm) to give compound **46** (7 mg, 0.2% of hexane fraction, T_R 21.5 min) as white needles (re-crystallized from 1:5 of isopropanol:hexane mixture). It was identified as manoalide-25-acetate.



* % of dried weight of sponge
** isolated yield, % of hexane extract
***isolated yield, % of CH_2Cl_2 extract

Scheme 1 Extraction and isolation scheme of the Thai sponge, *Brachiaster* sp. (April 2001 expedition)



* % of dried weight of sponge
** isolated yield, % of an aliquot of hexane extract

Scheme 2 Extraction and isolation scheme of the Thai sponge, *Brachiaster* sp. (April 2002 expedition)

2.5 Physical properties of isolated compounds

12-Deacetoxy-scalarin acetate (40): white amorphous solid; $[\alpha]_D^{25}$ –24.3° (c=0.014, MeOH); IR (thin film) ν_{max} 1770, 1730 cm^{-1}; UV (MeOH) λ_{max} (log ε) 227 (3.78) nm; CD (c=1.58×10^{-4} M, MeOH) θ (nm) 0 (272), +2177 (251), 0 (239); ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 4; ESIMS *m/z* (relative intensity) 429 ($[M+H]^+$, 29), 407 (29), 369 (100), 358 (29), 336 (29); HREIMS *m/z* 428.2910 [$C_{27}H_{40}O_4$ requires 428.2927].

Heteronemin (18): white needles (CH_2Cl_2:methanol = 1:3); $[\alpha]_D^{20}$ –71.4° (c=0.055, $CHCl_3$); IR (thin film) ν_{max} 3500, 1740, 1235 cm^{-1}; UV (MeOH) λ_{max} (log ε) 229 (2.34) nm; ^1H and ^{13}C NMR (500 MHz for ^1H, $CDCl_3$) see Table 5; ESIMS *m/z* (relative intensity) 511 ($[M+Na]^+$, 23), 429 (13), 369 (100), 351 (54), 191 (13), 148 (8).

Heteronemin acetate (41): white amorphous solid; $[\alpha]_D^{20}$ –48.6° (c=0.075, $CHCl_3$); IR (thin film) ν_{max} 1740, 1235 cm^{-1}; UV (MeOH) λ_{max} (log ε) 241 (2.52) nm; ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 5; ESIMS *m/z* (relative intensity) 553 ($[M+Na]^+$, 26), 411 (18), 351 (100), 233 (18), 221 (52), 163 (26).

12-Epi-19-deoxyscalarin (42): white amorphous solid; $[\alpha]_D^{25}$ –33.0° (c=0.004, MeOH); IR (thin film) ν_{max} 1765, 1735, 1240 cm^{-1}; UV (MeOH) λ_{max} (log ε) 223 (3.77) nm; ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 6; ESIMS *m/z* (relative intensity) 429 ($[M+H]^+$, 3), 369 (100), 352 (3), 149 (2).

12-Deacetyl-12-epi-19-deoxyscalarin (43): white needles (CH_3CN); $[\alpha]_D^{25}$ –61.4° (c=0.004, MeOH); IR (thin film) ν_{max} 3450, 1745 cm^{-1}; UV (MeOH) λ_{max} (log ε) 224 (3.72) nm; ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 6; ESIMS *m/z* (relative intensity) 387 ($[M+H]^+$, 100).

***E*-Neomanoalide diacetate (44):** vicous colorless liquid; $[\alpha]_D^{25}$ –32.9° (c=0.023, MeOH); IR (thin film) ν_{max} 1755, 1225 cm^{-1}; UV (MeOH) λ_{max} (log ε) 224 (3.72) nm; CD (c=4.73×10^{-4} M, MeOH) θ (nm) –5504 (218), –6299 (200); ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 7; ESIMS *m/z* (relative intensity) 509 ($[M+Na]^+$, 100), 172 (24); HRESIMS *m/z* 509.2898 [$C_{29}H_{42}O_6Na$ requires 509.2868].

***Z*-Neomanoalide diacetate (45):** vicous colorless liquid; $[\alpha]_D^{25}$ –23.3° (c=0.015, MeOH); IR (thin film) ν_{max} 1755, 1225 cm^{-1}; UV (MeOH) λ_{max} (log ε) 224 (3.72) nm; CD (c=3.04×10^{-4} M, MeOH) θ (nm) 0 (271), –10485 (214), –9760 (204); ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 7; ESIMS *m/z* (relative intensity) 509 ($[M+Na]^+$, 100), 172 (24); HRESIMS *m/z* 509.2794 [$C_{29}H_{42}O_6Na$ requires 509.2868].

Manoalide-25-acetate (46): white needles (*i*-PrOH:C_6H_{14} = 1:5); $[\alpha]_D^{25}$ +25.0° (c=0.004, MeOH); IR (thin film) ν_{max} 3430, 1790, 1770, 1235 cm^{-1}; UV (MeOH) λ_{max} (log ε) 224 (3.81) nm; CD (c=9.60×10^{-5} M, MeOH) θ (nm) 0 (219), –20000 (200); ^1H and ^{13}C NMR (500 MHz for ^1H, C_6D_6) see Table 6; ESIMS *m/z* (relative intensity) 481 ($[M+Na]^+$, 100).