

CHAPTER 5

Experimental

5.1 Plant Material

Vernonia staeheleinoides plant material was collected in April 1999 from Perseverance Farm in the Magaliesburg region of Gauteng. The collection was undertaken by Errol Nienaber, a plant collector contracted by the CSIR. Voucher specimen (EN00323) containing flowers and aerial parts of the plant was identified and retained at the National Herbarium (Pretoria).

The initial sample of *Oncosiphon piluliferum* plant material was also collected by Errol Nienaber in October 1999 from Dwarsvlei Farm near Middelburg in the Eastern Cape. The recollection of *O. piluliferum* plant material from the original collection site was conducted by Jean Meyer of the South African National Botanical Institute (SANBI) in November 2002. Voucher specimen (EN00579) containing flowers and aerial parts of the plant was identified and retained at the National Herbarium.

5.2. Extract Preparation

Plant material was dried in an oven at 30 – 60 °C. Dried material was then ground to a coarse powder using a hammer mill. Powdered plant material was sequentially extracted with cold dichloromethane, dichloromethane/methanol (1:1) and purified water.

For each extraction procedure the plant material was steeped in sufficient solvent for 4 - 5 h at room temperature, with occasional stirring. The solvent was subsequently drained and the plant material was air-dried before extraction with the next solvent. Organic extracts were concentrated by rotary vacuum evaporation below 45 °C and then further dried in vacuo at ambient temperature for 24 h. The aqueous extract of *V. staeheleinoides* was concentrated by freeze-drying. All extracts were stored at -20 °C and the yields of the extracts were recorded in terms of starting plant material.

5.3 *In Vitro* Antiplasmodial Activity

The *in vitro* antiplasmodial assays were conducted by the Pharmacology Department at the University of Cape Town. The chloroquine sensitive (D10) and chloroquine-resistant (K1) strains of *Plasmodium falciparum* were continuously cultured according to the methods described by Trager and Jensen¹. The parasites were maintained at a 5% haematocrit with RPMI 1640 (Biowhittaker) medium supplemented with Albumax II (lipid rich bovine serum albumin) (GibcoBRL) (25 g/L), hypoxanthine (44 mg/L), HEPES (N-[2-Hydroxyethyl]-piperazine-N'-[2-Ethansulphonic acid]) (Sigma-Aldrich) (6 g/L), sodium hydrogen carbonate (Sigma-Aldrich) (2.1 g/L) and gentamycin (Sigma-Aldrich) (50 mg/L). The cultures were incubated at 37 °C in an atmosphere of 93% N₂, 4% CO₂ and 3% O₂.

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity.² This enzymatic assay differentiates between pLDH and host LDH activity by using 3-acetylpyridine adenine dinucleotide (APAD). The pLDH uses APAD as a coenzyme in the conversion of pyruvate to lactate and reduces it to APADH. The formation of APADH can be measured by the subsequent reduction of a yellow nitroblue tetrazolium (NBT) salt to a blue formazan product, the absorbance of which can be monitored on a microplate reader.

The *in vitro* assays were performed as described by Clarkson *et al.*³ Microtitration techniques were used to measure the activity of a large number of samples over a wide range of concentrations. The microtitre plates (Greiner) consisted of 96 wells arranged in a matrix of eight rows (A to H) and 12 columns (1 to 12). Rows A to H in column 1 contained unparasitised RBC (blank), column 2 served as a parasite control (parasitised RBC in the trophozoite stage, adjusted to a 2% parasitaemia and 2% haematocrit, and no drug) and columns 3 to 12 contained parasites and varying concentrations of the drug. A solution of chloroquine diphosphate (Sigma) in Millipore water served as a positive control in all experiments. The initial concentration of chloroquine was 1000 ng/ml. All tests were performed in duplicate

¹ W. Trager and J.B. Jensen, *Science*, 1976, **193**, 673.

² M.T. Makler J.M. Ries, J.A. Williams, J.E. Bancroft, R.C. Piper, B.L. Gibbins and D.J. Hinrichs, *Am. J. Trop. Med. Hyg.*, 1993, **48**, 739.

³ C. Clarkson, W.E. Campbell and P. Smith, *Planta Med.*, 2003, **8**, 720.

and no attempt was made to determine 50% inhibitory concentration (IC₅₀) values in excess of 100 µg/ml.

Crude plant extracts received from the CSIR were stored at -20 °C prior to testing and stock solutions were made up a day before the experiment and stored at -20 °C. Crude extracts were first dissolved in methanol or DMSO, depending on their solubility, sonicated for 10 minutes and then diluted in Millipore water to give a 2 mg/ml solution. This was further diluted in RPMI 1640 medium to give 200 µg/ml stock solutions. The highest concentration of solvent that the parasites were exposed to was 0.5%, which was shown to have no measurable effect on parasite viability.⁴ Extracts were tested in nine serial twofold dilutions (final concentration range: 100 -0.2 µg/ml) in the 96-well microtitre plates. Fractions and pure compounds were dissolved in 10% methanol and were further diluted in complete medium on the day of the experiment. The starting concentration for a full dose-response was 100 µg/ml, which was diluted 2-fold in complete medium to give ten concentrations, with the lowest concentration being 0.195 µg/ml. The microtitre test plates were placed in a desiccator cabinet, flushed with a gas mixture consisting of 93% N₂, 4% CO₂ and 3% O₂, sealed and incubated at 37 °C for 48 h.

The pLDH activity was measured using a 1.96 mM NBT (Sigma) and 0.24 mM phenazine ethosulphate (PES) (Sigma) solution in Millipore water, and the Malstat reagent containing triton (1ml/L), APAD (0.33 g/L) and TRIS buffer (3.3 g/L) in Millipore water. Malstat reagent (100 µl) and NBT/PES (25µl) solution were added to all the wells of another 96-well microtitre plate. The test plate was removed from the desiccator after the 48 h incubation period and the parasites were re-suspended in each well and then transferred (15 µl) with a multi-channel dispenser to the corresponding wells in the plate containing the Malstat and NBT/PES solution. This plate was placed in a 7520 Microplate Reader (Cambridge Technology), blanked on the wells in column 1 and the absorbance of the blue formazan salt was measured at λ 620 nm. Since the amount of formazan produced is proportional to parasite viability, the percentage parasite survival in each well was calculated using the formula:

⁴ C. Clarkson V.J. Maharaj, N.R. Crouch, O.M. Grace, P. Pillay, M.G. Matsabisa, N. Bhagwandhin, P.J. Smith, P.I. Folb., *J. Ethnopharmacol.*, 2004, **92**, 177.

$$\% \text{ Parasite Viability} = \frac{A_{\lambda 620} \text{ test well (PRBC + drug)}}{A_{\lambda 620} \text{ parasite control well (PRBC + no drug)}} \times 100$$

Dose response curves were constructed using non-linear dose-response curve fitting analyses with GraphPad Prism v.4.00 software. The concentration of the drug that inhibits 50% of the parasites (IC₅₀ values) was established from the dose response curves using GraphPad Prism.

5.4 Bioassay-guided Fractionation, Targeted Purification and Selected Derivatisations of Active Compounds

The fractionation process involved column and thin layer chromatographic techniques. Different sized columns, ranging from 1.5 – 6 cm in diameter, were used depending on the amount of sample and the purification stage. Silica gel column chromatography was conducted using Silica gel 60 (0.063 - 0.2 mm) and flash silica gel chromatography was carried out using 35 - 75 micron flash silica gel (Merck Art).

Thin layer chromatography was carried out on 0.20 mm pre-coated (SIL-25 UV₂₅₄) glass-backed plates. The plates were first viewed under UV, developed using a vanillin : concentrated H₂SO₄ (1 g : 100 ml) spray reagent and then heated.

5.4.1 Bioassay-guided Fractionation of P01009A

(i) Fractionation of P01009A

The crude dichloromethane extract (1.5 g) was fractionated on a silica gel column (87 x 2.5 cm) using a gradient eluent of increasing polarity (ethyl acetate-hexane 1:9 → ethyl acetate-hexane 3:2). The column was stripped with ethyl acetate. A total of 6 pooled fractions (1A - 1F) were generated. These fractions were bioassayed against *P. falciparum* D10.

(ii) Further Purification of Fraction 1D

Fraction 1D (70 mg) was chromatographed on a silica gel column (50 x 1.5 cm). Silica gel was eluted with dichloromethane. Polarity was gradually increased by addition of methanol in 1% increments to methanol-dichloromethane (1:9). Column was stripped with methanol. Eight fractions were generated (2A - 2F) and bioassay

results revealed that two of these showed improved antiplasmodial activity, *ie.* 2C (2.6 mg) and 2D (1.3 mg).

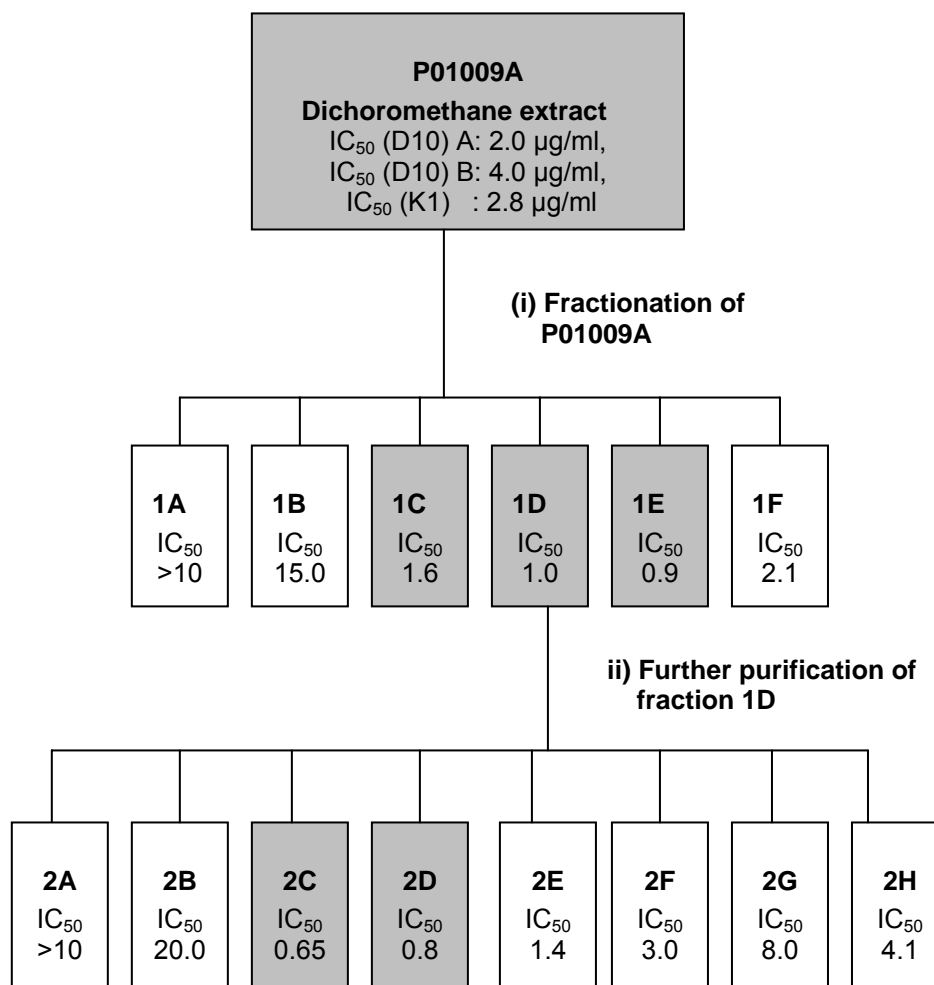


Figure 5.1 Fractionation of dichloromethane extract of *V. staeheleinoides*. IC₅₀ values are in µg/ml

5.4.2 Bioassay-guided Fractionation of P01009B

(i) Fractionation of P01009B

The crude 1:1 dichloromethane/methanol extract (2 g) was subjected to silica gel column chromatography (92 x 2.5 cm) using a gradient eluent of increasing polarity (ethyl acetate-hexane 1:9→ ethyl acetate-hexane 3:2). Column was stripped with ethyl acetate. A total of 7 pooled fractions (3A - 3G) were generated. These fractions were bio-assayed against *P. falciparum* D10.

(ii) Purification of Fraction 3E

Fraction 3E (107 mg) was subjected to flash silica gel chromatography. Flash silica gel (15 g) was eluted with ethyl acetate-hexane (1:4). Ten pooled fractions were generated (4A - 4J). These fractions were bio-assayed against *P. falciparum* D10.

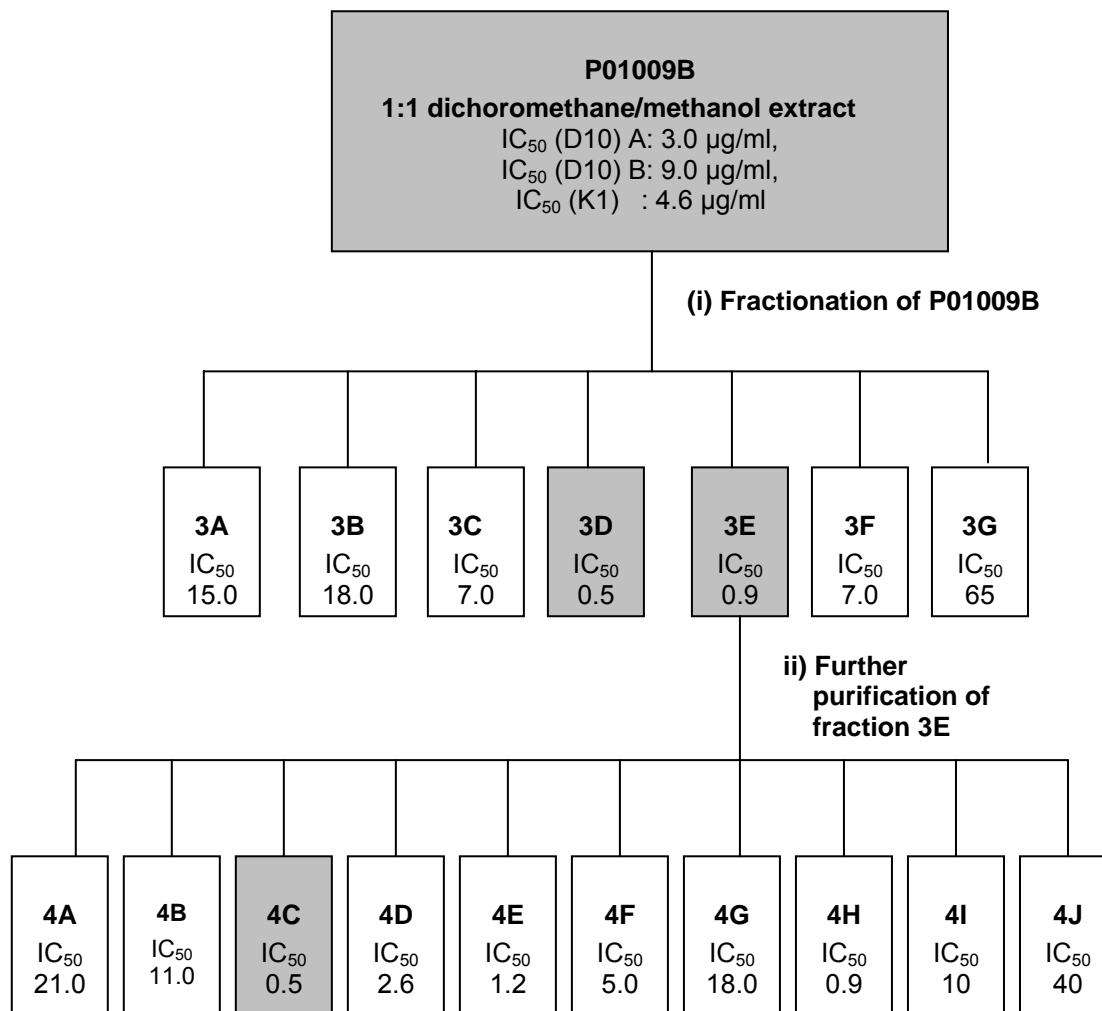


Figure 5.2 Fractionation of the dichloromethane/methanol (1:1) extract of *V. staeheleinoides*. IC₅₀ values are in µg/ml

5.4.3 Targeted Purification of Active Compounds from P01009A**(i) Liquid-liquid Partitioning of P01009A**

Crude extract (2 g) was dissolved in methanol-water (9:1) (200 ml), and extracted with hexane (3 x 100 ml) (Figure 5.3). The combined hexane layers were evaporated under reduced pressure to yield 5A, the hexane-soluble fraction (0.4 g). The methanol from the methanol/water layer was evaporated off under reduced pressure. An additional 30 ml of water was added to the remaining water layer,

which was subsequently extracted with dichloromethane (3 x 100 ml). The combined dichloromethane layers were evaporated under reduced pressure to yield 5B, the dichloromethane-soluble fraction (1.4 g). The water layer was freeze-dried to yield 5C, the aqueous fraction (0.1 g).

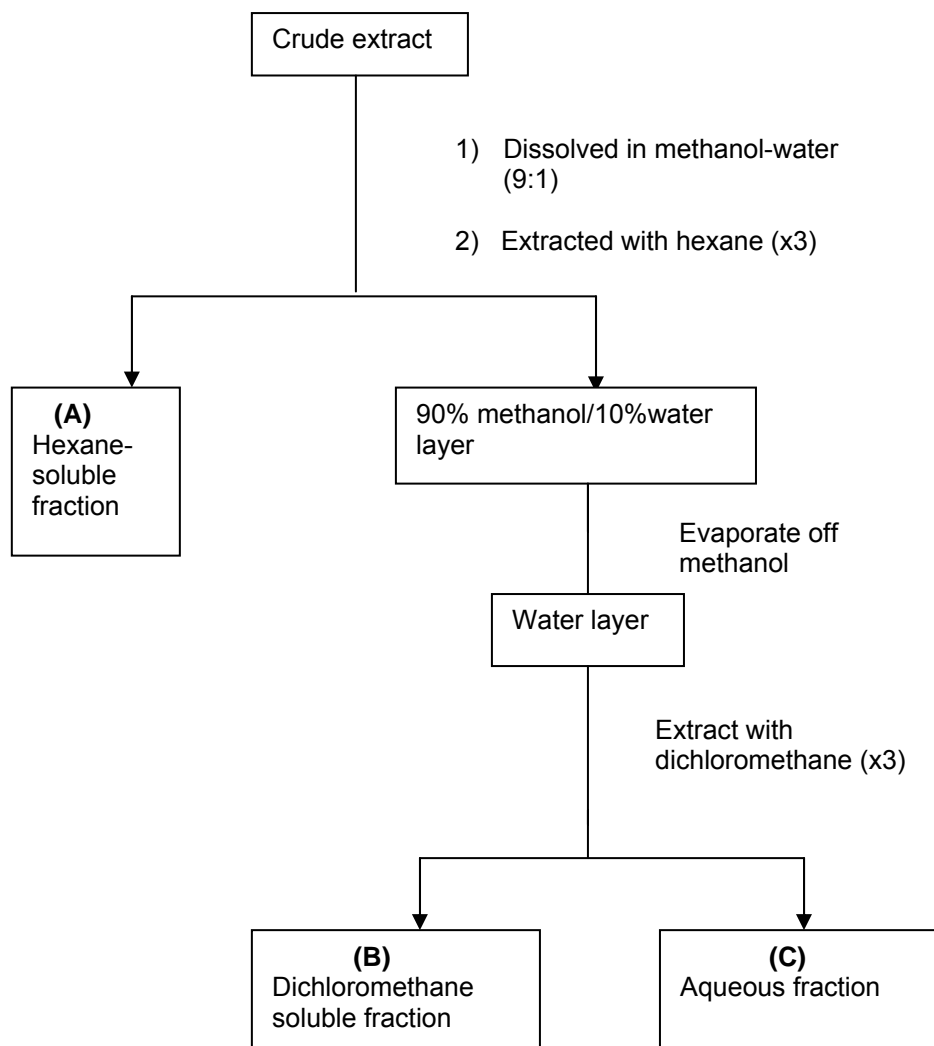


Figure 5.3 Liquid/liquid partitioning method

(ii) Flash Silica Gel Chromatography of the Dichloromethane-soluble Fraction (A)

The dichloromethane-soluble fraction from liquid/liquid partitioning of P01009A was subjected to flash silica gel chromatography using ethyl acetate-hexane-dichloromethane (1:5.75:5.75) to yield 4 pooled fractions (6A - 6D). Target compounds were concentrated in fractions 6A and 6C.

(iii) Purification of Compound (50)

Target compound **(50)** was concentrated in fraction 6A; easily discernable by TLC analysis (dark orange colour when sprayed with vanillin). Fraction 6A was purified by flash silica gel chromatography with acetone-hexane-dichloromethane (7:46.5:46.5). All fractions containing **(50)** were pooled and further purified by flash silica gel chromatography with acetone-hexane-dichloromethane (6:47:47), to yield semi-purified **(50)**. Further purification by flash silica gel chromatography using acetone-hexane-dichloromethane (3:48.5:48.5) yielded compound **(50)** as a colourless gum (7.7 mg); R_f 0.29 (acetone:hexane:dichloromethane 1:4.5:4.5); $[\alpha]_D -77.4$ (c 0.31, CHCl_3)

^1H and ^{13}C NMR data : Listed in Table 3.3 (Chapter 3)

EI-MS : m/z 316 $[\text{M} - \text{C}_3\text{H}_5\text{COOH}]^+$. Exact mass: Calculated for $\text{C}_{17}\text{H}_{16}\text{O}_6$, 316.0947; Found, 316.0868

(iv) Purification of Compound (51)

Target compound **(51)** was concentrated in fraction 6C; easily discernable by TLC analysis (dark brown colour when sprayed with vanillin). Flash silica gel chromatography of fraction 5C with acetone-hexane-dichloromethane(1:4.5:4.5) yielded **(51)** in a semi-purified state. Further purification by flash silica gel chromatography, using acetone-hexane-dichloromethane (7:46.5:46.5), yielded a colourless gum **(51)** (11.2 mg); R_f 0.23 (acetone:hexane:dichloromethane 1:4.5:4.5); $[\alpha]_D -209.8$ (c 0.51, CHCl_3)

^1H and ^{13}C NMR data : Listed in Table 3.4 (Chapter 3)

EI-MS : m/z 316 $[\text{M} - \text{C}_7\text{H}_{10}\text{O}_4]^+$. Exact mass: Calculated for $\text{C}_{17}\text{H}_{16}\text{O}_6$, 316.0947; Found, 316.0885

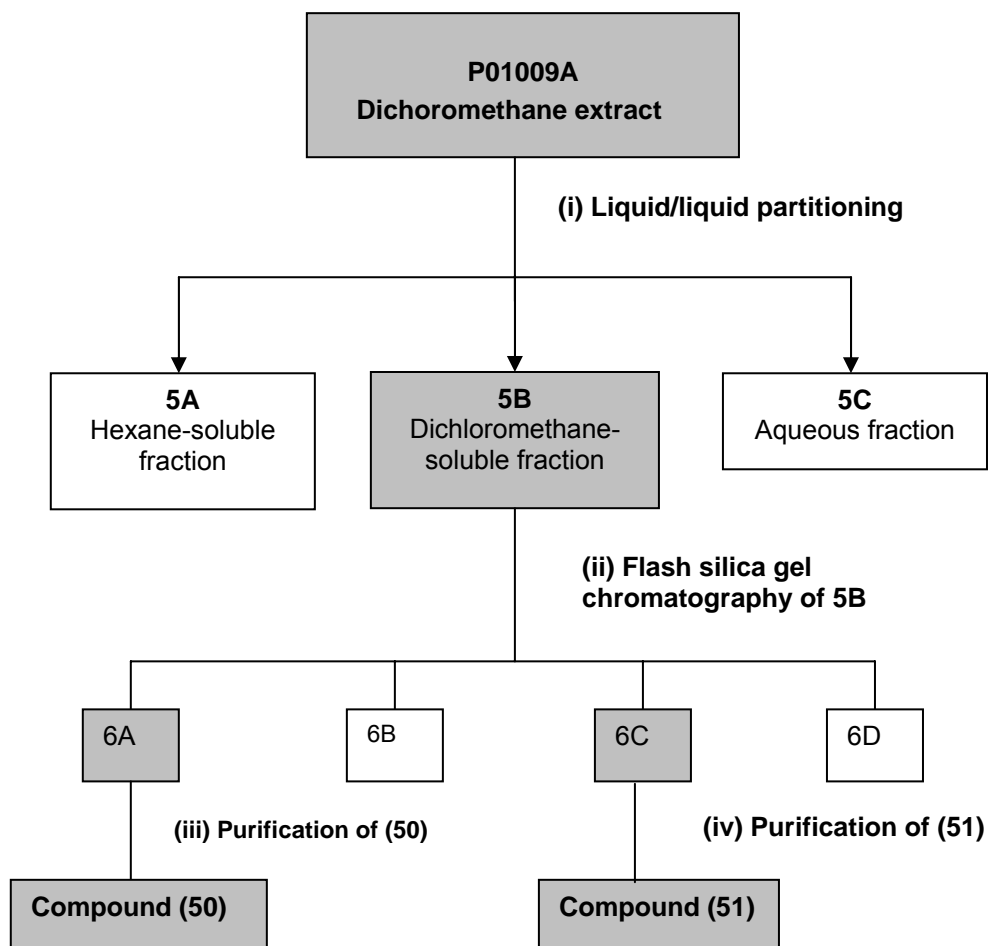


Figure 5.4 Targeted purification of compounds **(50)** and **(51)** from P01009A

5.4.4 Bioassay-guided Fractionation of P01609A

(i) Primary Fractionation of P01609A

The crude dichloromethane extract (15 g) was subjected to silica gel column chromatography using a gradient eluent of increasing polarity (ethyl acetate-hexane (1:20)→ ethyl acetate-hexane (3:2)→100% ethyl acetate) to yield a total of 32 pooled fractions. These fractions were bio-assayed against *P. falciparum* D10. Solubility problems were encountered with the first twelve fractions and the bio-assay results of these were subsequently disregarded. The remaining 20 fractions were assigned as 7A - 7T.

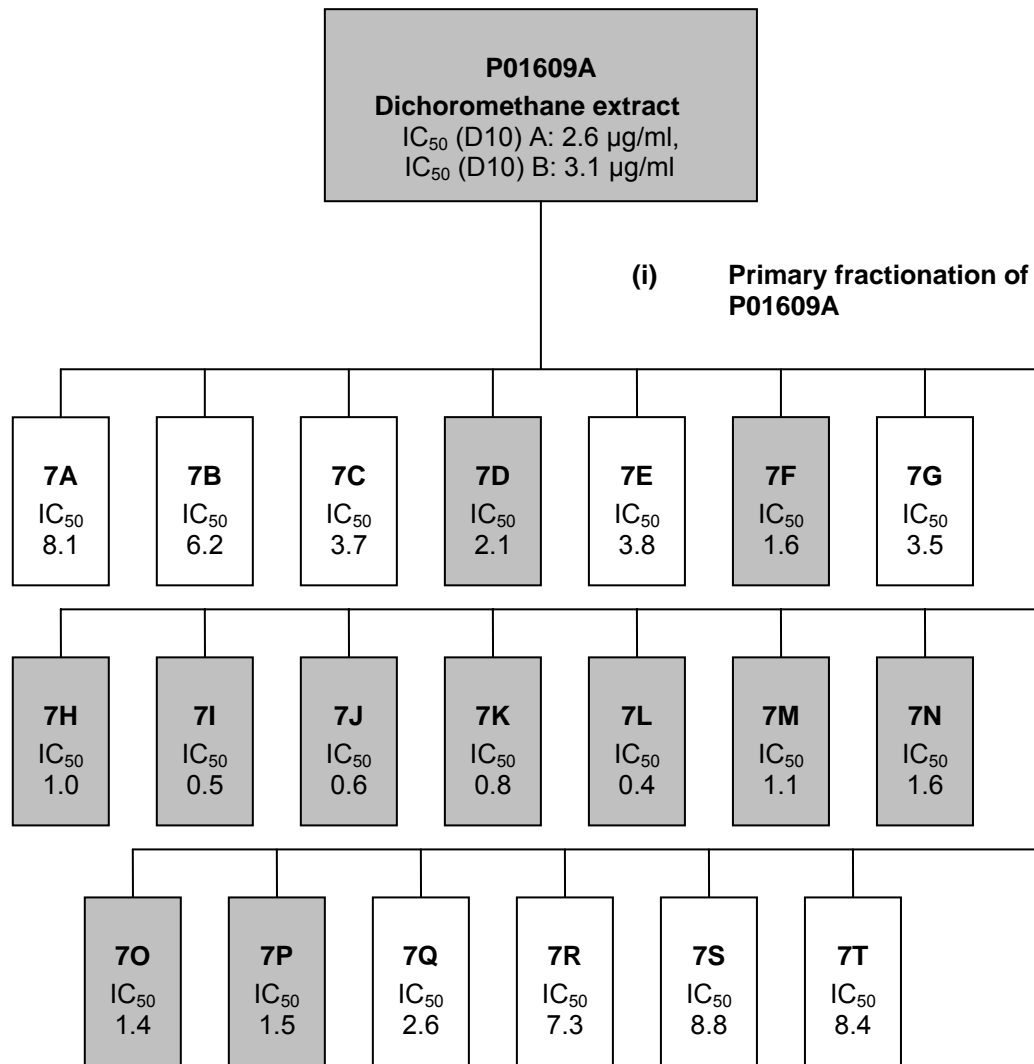


Figure 5.5 Summary of IC₅₀ values of the primary fractions generated from silica gel column chromatography of P01609A. IC₅₀ values are in µg/ml

(ii) Further Fractionation of 7I

Fraction 7I (840 mg) was chromatographed on a silica gel column (2.5 x 40 cm). The silica gel was eluted with acetone-hexane-dichloromethane (1:2:2). A total of 8 pooled fractions (8A - 8H) were generated and these were bio-assayed against *P. falciparum* D10 (Figure 5.6).

(iii) Further Fractionation of 8D

Fraction 8D (200 mg) was subjected to flash silica gel chromatography, using 13 g of flash silica gel and acetone-dichloromethane (1:99). Two major fractions were generated (9A + 9B), with 9B being a semi-pure compound which was easily discernible on TLC by its dark pink colour when sprayed with vanillin.

(iv) Further Purification of 9B

Approximately 45 mg of 9B was subjected to successive flash silica gel purifications; first using ethyl acetate-hexane-dichloromethane (1:4.5:4.5) and then decreasing the polarity of the eluent slightly to ethyl acetate-hexane-dichloromethane (1:11.5:11.5). This yielded 16 mg of compound (**59**), which decomposed during NMR analysis.

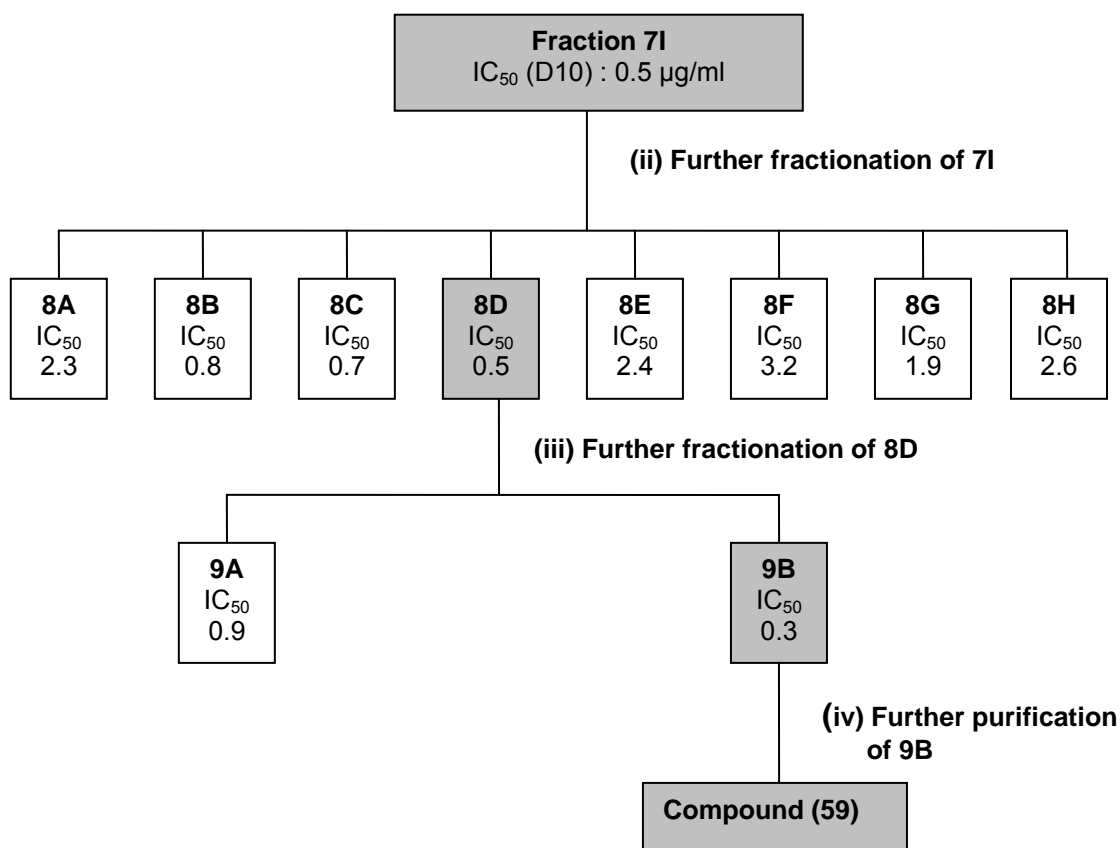


Figure 5.6 Further purification of fraction 7I. IC₅₀ values are in µg/ml

(v) Further Fractionation of 7M

Fraction 7M (450 mg) was chromatographed on a silica gel column (Figure 5.7). Silica gel was eluted with acetone-hexane-dichloromethane (1:1.5:1.5). Four pooled fractions were generated (10A - 10D). Major fraction 10C showed crystalline properties.

(vi) Crystallisation of 10C

Fraction 10C (132 mg) was first decolourised with charcoal and then crystallised from ethyl acetate/hexane. The crystals were filtered and submitted for assaying.

(vii) Further Purification of 11A

Successive flash silica gel purifications of 11A (80 mg), first using methanol-dichloromethane (1:49) and then methanol-dichloromethane (1:99), yielded fraction 12A (18 mg) and colourless crystals of **(60)** (12 mg); mp. 278 – 280 °C {Lit.⁵ : 280 °C}; R_f 0.27 (methanol-dichloromethane 1:24); $[\alpha]_D +2.0$ (c 0.49, MeOH)

¹H and ¹³C NMR data : Listed in Table 4.6 (Chapter 4)

EI-MS : m/z 264 $[M]^+$. Exact mass: Calculated for C₁₅H₂₀O₄, 264.1362;

Found, 264.1347

Crystallographic data : *Appendix (A)*

(viii) Further Purification of Fraction 7O

Successive purifications of 7O (335 mg) by silica gel column chromatography (Figure 5.8), first using acetone-hexane-dichloromethane (6:7:7), and then ethyl acetate-hexane (2:3) yielded colourless crystals of **(63)** (20mg); mp. 159 – 161 °C {Lit.⁶: 158-159 °C}; R_f 0.35 (acetone:hexane:dichloromethane 3:3.5:3.5); $[\alpha]_D +24.0$ (c 0.50, MeOH) {Lit.⁶: $[\alpha]_D^{23} +34.8$ (c 1.84, MeOH)}

¹H and ¹³C NMR data : Listed in Table 4.10 (Chapter 4)

EI-MS : m/z 246 $[M - H_2O]^+$. Exact mass: Calculated for C₁₅H₁₈O₃, 246.1256;

Found, 246.1219

Crystallographic data: *Appendix (D)*

⁵ M.L Cardona, I. Fernández, B. Garcia and J. R. Pedro, *J. Nat. Prod.*, 1990, **53**, 1042

⁶ A.I Yunusov, N.D. Abdullaev, S.Z. Kasymov and G.P. Sidyakin., *Khim. Prir. Soedin.*, 1976, **2**, 263.

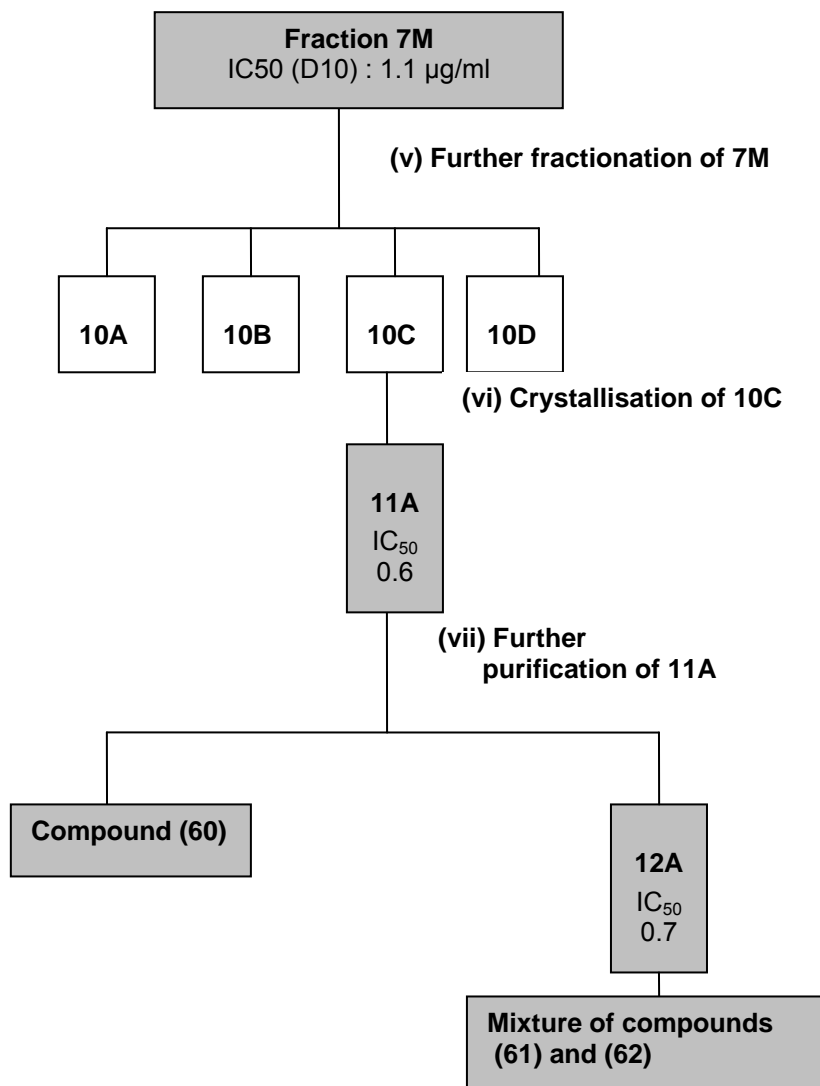


Figure 5.7 Further purification of fraction 7M. IC₅₀ values are in µg/ml

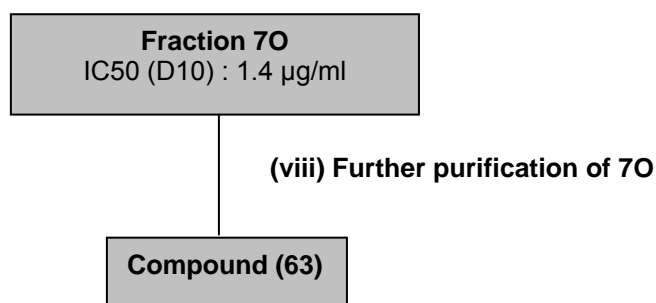


Figure 5.8 Further purification of fraction 7O. IC₅₀ values are in µg/ml

5.4.5 Targeted Purification and Selected Derivatisations of Active Compounds from P01069A

(ix) Liquid-liquid Partitioning of P01609A

57 g of the crude extract was subjected to liquid/liquid partitioning as illustrated in Figure 5.3. The combined chloromethane layers were evaporated under reduced pressure to yield 13B, the dichloromethane-soluble fraction (25 g).

(x) Fractionation of 13B

24 g of 13B was chromatographed on a silica gel column (7 x 57 cm). The silica gel was eluted with acetone-hexane-dichloromethane (1:2:2). A total of 8 pooled fractions were generated (14A – 14H).

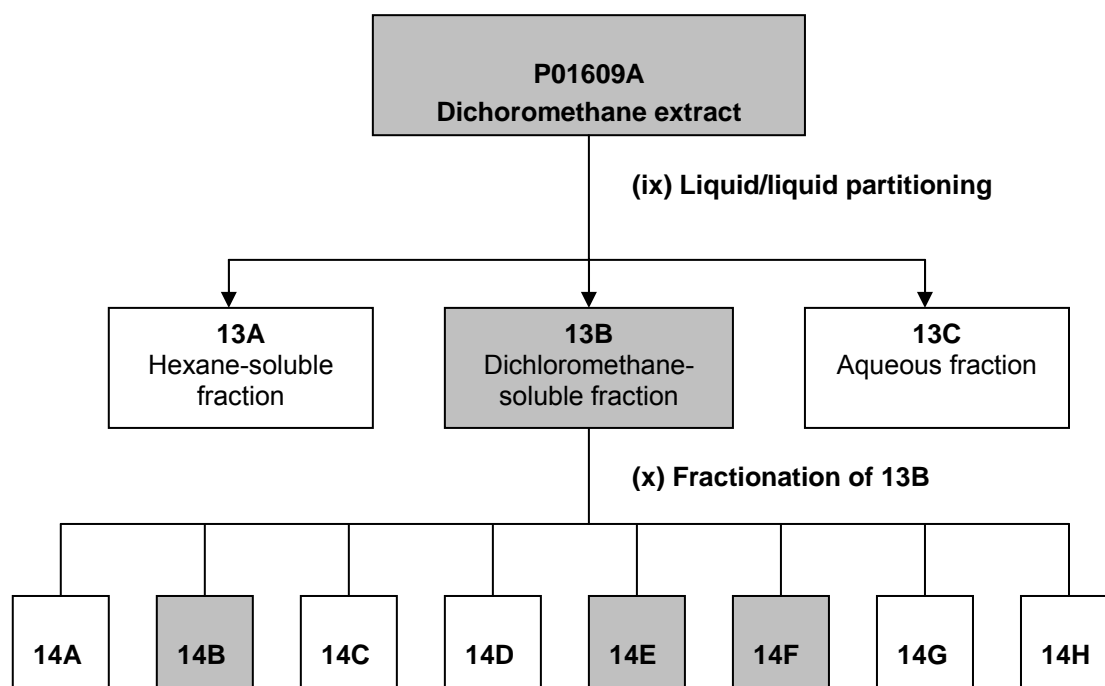


Figure 5.9 Fractionation of PO1609A.

(xi) Targeted Isolation of Compound (59)

8.7 g of 14B was subjected to a series of successive flash silica gel purifications, first using ethyl acetate-hexane-dichloromethane (1:24.5:24.5), then ethyl acetate-hexane-dichloromethane (2:9:9) followed by ethyl acetate-hexane-dichloromethane (4:23:23). Compound (59) was concentrated in sub-fraction 14B5 (98 mg) (Figure 5.10). 25 mg of 14B5 was purified on a preparative silica

plate to yield the colourless gum **(59)** (18 mg); R_f 0.26 (ethyl acetate-hexane 2:3); $[\alpha]_D$ and MS data not obtained due to decomposition of compound.

^1H NMR data : listed in Table 4.4 (Chapter 4)

(xii) Acetylation of Compound (59)

Acetic anhydride (0.5 ml, 5 mmol) was added to 14B5 (30 mg, 0.11 mmol) in 3 ml of anhydrous pyridine. The mixture was stirred at room temperature for 15 h. Chloroform (50 ml) was added to the reaction and the organic layer was washed with saturated citric acid (4 x 100 ml), saturated sodium hydrogen carbonate solution (100 ml) and water (100 ml). The chloroform layer was dried over calcium chloride and evaporated to dryness under reduced pressure. Flash silica gel chromatography of the crude product using acetone-hexane-dichloromethane (1:9.5:9.5) yielded the yellow gum **(64)** (16mg, 48%); R_f 0.41 (acetone-hexane-dichloromethane 1:4.5:4.5); $[\alpha]_D$ +41.2 (c 0.34, CHCl_3)

^1H NMR and ^{13}C NMR data : Listed in Table 4.5 (Chapter 4)

EI-MS : m/z 264 $[\text{M} - (\text{CH}_2=\text{C}=\text{O})]^+$ Exact mass: Calculated for $\text{C}_{15}\text{H}_{20}\text{O}_4$
264.1362; Found, 264.1313

(xiii) Benzoylation of Compound (59)

28mg (0.106 mmol) of 14B5 was dissolved in 5 ml of chloroform. *p*-nitrobenzoylchloride (20 mg, 0.107 mmol) and DMAP (20.9 mg, 0.171 mmol) was added and the reaction mixture was stirred at room temperature. The reaction was monitored on TLC and after 24 h only a minor trace of product was observed and the starting material was still concentrated. The reaction mixture was heated (60 °C) under reflux conditions but no improvement in the product yield was observed. Excess *p*-nitrobenzoylchloride and DMAP were added and a marked improvement in the product yield was subsequently observed. The reaction was quenched once a large quantity of salt began to precipitate out of solution and no more improvement in product yield was observed. Chloroform (50 ml) was added to the reaction mixture and the salt was filtered off. The chloroform solution was washed with 0.1 M HCl (100 ml) and water (100 ml), dried over calcium chloride. The crude

product (48 mg) was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (1:19.5:19.5) to yield the white crystals **(65)** (6 mg, 14%).

^1H NMR data: Listed in Table 4.4 (Chapter 4)

Various solvents, combinations of solvents and other crystallisation techniques were attempted to produce crystals of **(65)** suitable for X-ray crystallography. Compound eventually showed evidence of decomposition and turned yellowish so no additional physical data was obtained on **(65)**.

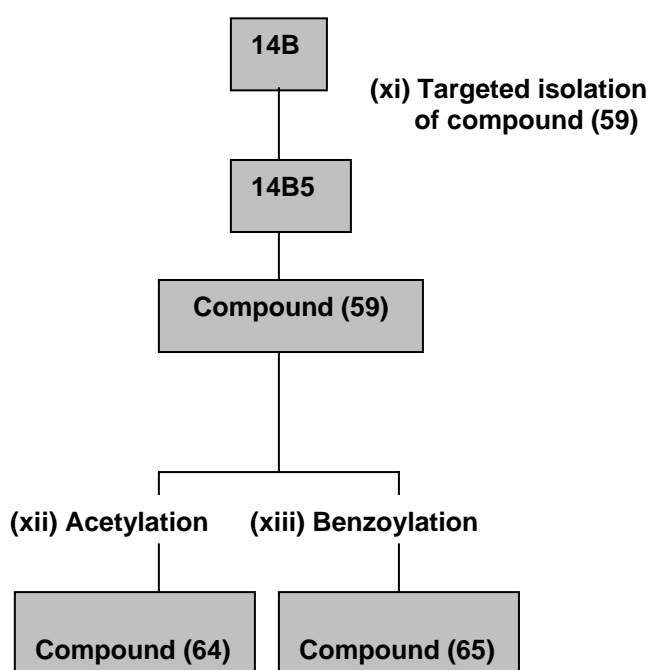


Figure 5.10 Isolation and derivatisation of **(59)**.

(xiv) Targeted isolation of compounds (60), (61) and (62)

520 mg of fraction 14E was subjected to flash silica gel chromatography using acetone:hexane:dichloromethane (1:2:2), to yield 4 sub-fractions (14E1 – 14E4). Sub-fraction 14E2 crystallised on standing to yield coarse white crystals (80 mg), which were subjected to flash silica gel chromatography using methanol-dichloromethane (1:49) to yield 42 mg of compound **(60)** (recrystallised from ethyl acetate) and white crystals of **(61)** (6 mg); mp. 234 - 236 °C; R_f 0.21 (methanol-dichloromethane 1:24); $[\alpha]_D -38.5$ (c 0.39, MeOH).

^1H NMR and ^{13}C NMR data : Listed in Table 4.7 (Chapter 4)

EI-MS : m/z 264 $[\text{M}]^+$ Exact mass: Calculated for $\text{C}_{15}\text{H}_{20}\text{O}_4$, 264.1362; Found, 264.1261

Sub-fractions 14E3 and 14 E4 were combined and crystallised from ethyl acetate/hexane to yield white needle-like crystals of compound **(62)** (104 mg); mp. 158 – 160 °C {Lit. ⁷ :160 - 161 °C}; R_f 0.26 (methanol-dichloromethane 1:24); $[\alpha]_D$ –54.0 (c 0.50, MeOH) {Lit. : $[\alpha]_D^{25}$ –32.0 (c 1.0, MeOH)}

^1H NMR and ^{13}C NMR data : Listed in Table 4.7 (Chapter 4)

EI-MS : m/z 264 $[\text{M}]^+$ Exact mass: Calculated for $\text{C}_{15}\text{H}_{20}\text{O}_4$, 264.1362; Found, 264.1293

40 mg of **(62)** was recrystallized from acetone in a hexane-saturated atmosphere to yield dense cubic crystals, suitable for X-ray analysis.

Crystallographic data: *Appendix (B)*

(xv) Acetylation of Compound (62)

Acetic anhydride (0.1 ml, 0.96 mmol) was added to compound **(62)** (30 mg, 0.12 mmol) in 0.5 ml of anhydrous pyridine. The mixture was stirred at room temperature for 24 h. Chloroform (50 ml) was added to the reaction and the organic layer was washed with saturated citric acid (3 x 100 ml), saturated sodium hydrogen carbonate solution (100 ml) and water (100 ml). The chloroform layer was dried over calcium chloride and evaporated to dryness under reduced pressure. The crude product was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (1:4.5:4.5) to yield white needle-like crystals of **(66)** (19 mg, 50%) mp. 203 – 205 °C; R_f 0.33 (methanol:dichloromethane 1:24). No additional physical data was obtained due to

⁷ A.I. Yunusov, G.P. Sidyakin and A.M. Nigmatullaev, *Khim. Prir. Soedin.*, 1979, **1**, 101.

poor recovery of product from attempts at recrystallisation to produce better quality crystals for X-ray analysis.

^1H NMR data : Listed in Table 4.9 (Chapter 4)

Crystallographic data : *Appendix (C)*

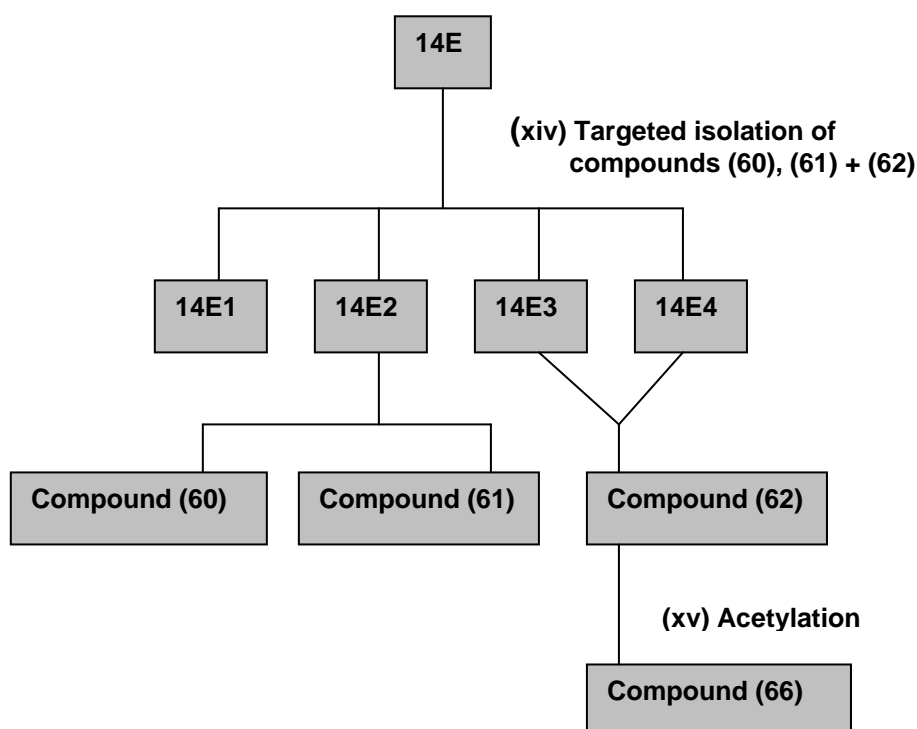


Figure 5.11 Isolation and derivatisation of (60),(61) and (62).

(xvi) Targeted Isolation of Compound (63)

730 mg of 14F was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (6:7:7). 170 mg of (63) was subsequently recovered (Figure 5.12). Crystals were formed at the bottom of test tubes during chromatography and these were recovered for X-ray analysis.

(xvii) (R)-Mosher Esterification

Oxalyl chloride (0.065 ml, 0.75 mmol) was added to a solution of *R*(+)- α -methoxy- α -trifluoromethylphenyl acetic acid (98.5 : 1.5) (58.9 mg, 0.25 mmol) and DMF (19 μl , 0.25 mmol) in anhydrous hexane (6 ml). The mixture was stirred at room temperature for 1 h. The solution was then filtered through a small cotton wool

plug to filter off the white DMFCl precipitate formed. The filtrate was evaporated under reduced pressure to yield (S)-MTPA-Cl (59.0 mg, 0.23 mmol).

A solution of compound **(63)** (33.2 mg, 0.13 mmol) in dichloromethane (4 ml), triethylamine (0.3 ml, 0.25 mmol) and a spatula tip of DMAP was added to a solution of the acid chloride in dichloromethane. The reaction was quenched after 48 h with water and the organic layer was washed with 0.1 M HCl and saturated NaHCO₃ solution. The organic solution was dried over Na₂SO₄, filtered and evaporated. The crude product (57 mg) was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (1:4.5:4.5). 20.1 mg of the diester **(67)**; R_f 0.85 (acetone-hexane-dichloromethane 3:8.5:8.5), 16.1 mg of one monoester **(68)**; R_f 0.55 (acetone-hexane-dichloromethane 3:8.5:8.5), and 5.1 mg of the other monoester **(69)**; R_f 0.31 (acetone-hexane-dichloromethane 3:8.5:8.5) were recovered. Relative $\Delta\delta$ values were determined from ¹H and COSY NMR data. Products showed signs of deterioration and thus no additional physical data were obtained on these.

(xviii) (S)-Mosher Esterification

Oxalyl chloride (0.065 ml, 0.75 mmol) was added to a solution of of S(-)- α -methoxy- α -trifluoromethylphenyl acetic acid (98.5 : 1.5) (58.3 mg, 0.25 mmol) and DMF (19 μ l, 0.25 mmol) in anhydrous hexane (6 ml). The mixture was stirred at room temperature for 1 h. The solution was then filtered through a small cotton wool plug to filter off the white DMFCl precipitate formed. The filtrate was evaporated under reduced pressure to yield 52.2 mg (0.20 mmol) of (R)-MTPA-Cl.

A solution of **(63)** (33.4 mg, 0.13 mmol) in dichloromethane (4 ml), triethylamine (0.3 ml, 0.25 mmol) and a spatula tip of DMAP was added to a solution of the acid chloride in dichloromethane. The reaction was quenched after 48 h with water and the organic layer was washed with 0.1 M HCl and saturated NaHCO₃ solution. The organic solution was dried over Na₂SO₄, filtered and evaporated. The crude product (52 mg) was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (1:4.5:4.5). 9.8 mg of the diester **(70)**; R_f 0.57 (acetone-hexane-dichloromethane 1:4.5:4.5), 22.4 mg of one monoester **(71)**; R_f 0.19 (acetone-hexane-dichloromethane 1:4.5:4.5), and 1.7 mg of the other monoester

(72); R_f 0.10 (acetone-hexane-dichloromethane 1:4.5:4.5), were recovered. Relative $\Delta\delta$ values were determined from ^1H and COSY NMR data. Products showed signs of deterioration and thus no additional physical data were obtained on these.

(ixx) NaBH_4 Reduction of Compound (63)

15 mg (0.06 mmol) of (63) was dissolved in anhydrous methanol (10 ml). The solution was cooled to 0 °C, and NaBH_4 (78 mg, 2 mmol) was added. The mixture was stirred at 0 °C for 10 min, after which 20% acetic acid (10 ml) was added. The solution was diluted further with water (20 ml) and extracted twice with chloroform (50 ml). The crude product was subjected to flash silica gel chromatography using acetone-hexane-dichloromethane (1:8.5:8.5) which yielded a pale yellow gum, (73) (5 mg, 34%); R_f 0.25 (acetone-hexane-dichloromethane 1:4.4:4.5). No additional physical data could be obtained on the compound due to its instability.

^1H NMR data : listed in Table 4.12

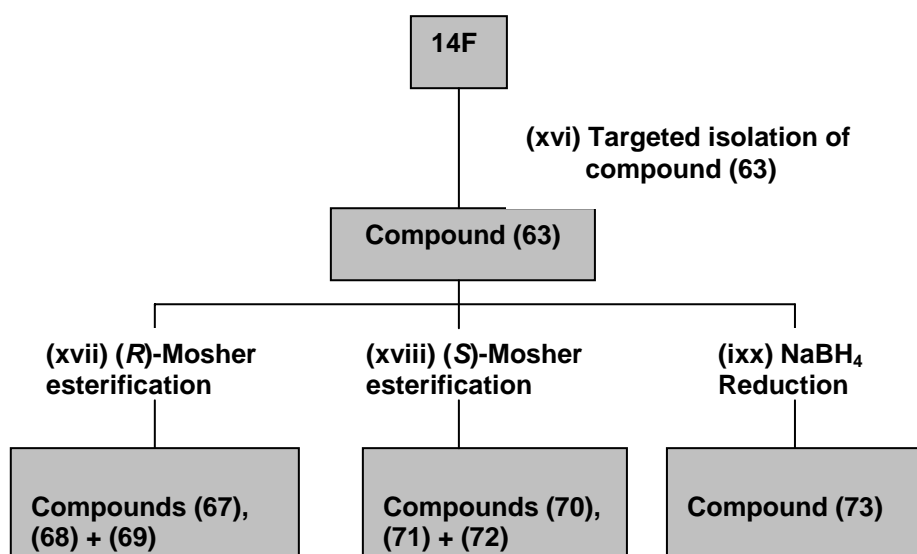


Figure 5.12 Isolation and derivatisation of (63).

5.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy was carried out on a Varian 400 MHz Unity spectrometer by Sue Rhodes and Madelyn Bekker of the Analytical Function at Bio/Chemtek (now Biosciences), CSIR. All the spectra were recorded at room temperature in either deuteriated acetone, deuteriated chloroform, or deuteriated benzene. The chemical shifts were all recorded in ppm relative to TMS.

5.6 Mass Spectrometry

High resolution mass spectra were recorded on a VG 70SEQ HRMS instrument, in the positive EI mode with a resolution of 7500 at 8kV. The mass spectrometry was performed by Dr. T van der Merwe at the University of the Witwatersrand.

5.7 X-ray Crystallography

X-ray crystallography experiments were conducted by Dr Dave Liles at the University of Pretoria on a Bruker (Siemens) P4 4-circle diffractometer fitted with a Bruker SMART 1000 CCD area detector using Mo-K α X-radiation and a graphite-crystal monochromator. Data reduction was conducted using Bruker SAINT software (2001) and structure solution and refinement was accomplished using Bruker SHELXTL (2001).

5.8 Optical Rotations

Optical rotations were measured in chloroform or methanol at room temperature on a Perkin-Elmer 241 polarimeter at 589 nm (Na D-line) using a 1 dm cell.

5.9 Melting Point Determinations

Melting points were determined using a Reichert hot stage apparatus and are uncorrected.

5.10 *In Vitro* Cytotoxicity Assay

Compounds were tested for *in vitro* cytotoxicity against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay.⁸ This colorimetric assay is based on the ability of viable cells to metabolise a yellow water-soluble tetrazolium salt into a water-insoluble purple formazan product. The amount of formazan produced can be measured spectrophotometrically and is proportional to the metabolic activity and number of cells in the test plate. This assay was conducted by the UCT Pharmacology Department. The CHO cells were cultured in Dulbecos Modified Eagles Medium (DMEM) : Hams F-12 medium (1:1) supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamycin (0.04 µg/ml). The medium reagents were obtained from Highveld Biological, South Africa.

Samples were dissolved in methanol:water (1:9). Stock solutions (2 mg/ml) were prepared and were stored at -20 °C until use. The highest concentration of methanol to which the cells were exposed had no measurable effect on the cell viability. Emetine was used as the positive control in all cases. The initial concentration of emetine was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/ml. The same dilution technique was applied to all test samples with an initial concentration of 100 µg/ml to give 5 concentrations, with the lowest concentration being 0.01 µg/ml.

In the initial stage of the experiments, the cells were adjusted to a concentration of 10^5 / ml and 100 µl of this cell suspension were seeded in all wells except in column 1 (blank) in a 96 well culture plate (Costar). The plates were incubated at 37 °C for 24 h in a humidified 5% CO₂-air atmosphere. After the incubation period, the medium was carefully aspirated out of the wells and 100 µl of the different test substances (drug solutions) were added in quadruplicate to columns 3 through to 9. A further 100 µl of culture medium was then added to all of the wells containing cells and drugs (columns 3 to 9), and 200 µl of medium was dispensed to the wells in column 1 (blank) and column 2 (cells and no drug). The microplate was then incubated at 37 °C for 48 h.

After the 48 h incubation period, 25 µl of sterile MTT (5 mg/ml in PBS) was added to each well and incubation was continued for 4 h at 37 °C. The plates were then

⁸ T. Mosmann, *Journal of Immunological Methods*, 1983, **65**, 55.

centrifuged at 2050 rpm for 10 min and the supernatant was carefully aspirated from the wells, ensuring that the formazan crystals were not disturbed. The formazan crystals were dissolved in DMSO (100 μ l) and the plate was gently shaken for 5 min on a microtitre plate shaker. The plate was blanked on the wells in column 1 and the absorbance of the crystals was measured at λ 540 nm on a Microtitre Plate Reader (Cambridge Technologies). The cell viability was calculated in each well using the formula :

$$\% \text{ Cell Viability} = \frac{A_{\lambda 540} \text{ test well (cells + drug)}}{A_{\lambda 540} \text{ cell control well (cells + no drug)}} \times 100$$

The concentration of drug that inhibits 50% of the cells (IC_{50} values) for these samples were obtained from dose-response curves, using a non-linear dose-response curve fitting analyses via GraphPad Prism v.2.01 software.