

THE ABSOLUTE STEREOCHEMISTRY AND CYTOTOXICITY OF THE ASCIDIAN-DERIVED METABOLITE, LONGITHORONE J

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The absolute stereochemistry of longithorone J (**1**) from the ascidian *Aplidium longithorax* has been determined using the advanced Mosher method. Based on biosynthetic reasoning and chiroptical data comparison the absolute stereochemistry for longithorone K (**2**) was also assigned. Longithorone J was tested for cytotoxicity against the cell lines SHSY5Y, HEK293T and A549. Compound **1** showed minimal cytotoxicity towards the SHSY5Y and HEK293T cell lines.

Keywords: Ascidian; *Aplidium longithorax*.; Natural product; Longithorone J; Longithorone K; Absolute stereochemistry; Mosher method; Cytotoxicity

INTRODUCTION

Longithorones A-K are a unique class of macrocyclic C₂₁ or C₄₂ prenylated quinone derivatives, which have all been isolated from ascidian sources. Longithorones A–I were isolated from *Aplidium longithorax* collected in Palau as part of a search for new antitumour agents from marine sources [1,2], while longithorones J (**1**) and K (**2**) were purified during chemical investigations of an Australian *A. longithorax* [3] (Figure 1). To date the only biological activity reported for these marine metabolites pertains to longithorone A (**3**), which was shown to display cytotoxicity against P388 murine leukemia cells with an IC₅₀ of ~10 µg/mL [1]. In the first reported isolation of longithorones J and K it was noted that both metabolites were unstable in CDCl₃ [3]. Although the gross structures and relative stereochemistry of both **1** and **2** were elucidated, their instability prevented further stereochemical analyses or biological testing. Recently we have re-isolated longithorone J (**1**, 0.7 mg) from a fraction resulting from the initial chemical investigations of *A. longithorax*. This paper reports the absolute stereochemistry of longithorone J using the advanced Mosher method and the cytotoxicity of **1** against the cell lines SHSY5Y, HEK293T and A549. The absolute stereochemistry of longithorone K (**2**) is also reported.

RESULTS AND DISCUSSION

A fraction obtained during the initial chemical investigations of *A. longithorax* was subjected to the previously published chromatographic protocol used for the isolation of **1** and **2** [3]. This yielded a pure compound (**1**, 0.7 mg) which was spectroscopically identical to the previously published data for longithorone J [3].

Prior to synthesizing the MTPA esters of compound **1**, molecular modeling studies using Macromodel[®] were performed in order to determine whether the secondary alcohol at C-17 was located in a sterically crowded environment. It has been reported that sterically hindered alcohols can produce erroneous assignments of absolute stereochemistry when using the advanced Mosher method [4,5]. Monte-Carlo conformational searching found 181 unique conformations for **1** with the global minimum energy conformation ($E = 71.01$ kcal/mol) occurring 40 times. Analysis of all 181 conformers revealed that 17-OH was unhindered. Low energy conformer 2 ($E = 71.66$ kcal/mol) (Figure 2) appeared to best fit the previously reported experimental NMR data [3]. The ^1H - ^1H coupling constants calculated from this conformer between H-16 and H-21 α /H-21 β were almost identical to the observed coupling constants in the initial structure elucidation studies [$J_{\text{H16, H21}\alpha}$ 4.8 Hz (obsd) [3], 4.6 Hz (calcd); $J_{\text{H16, H21}\beta}$ 2.4 Hz (obsd) [3], 2.0 Hz (calcd)]. Further analysis of conformer 2 also allowed the distance between H-17 and H-

21 α to be calculated as 2.60 Å. This distance was in agreement with the observed strong ROESY correlation between these H-17 and H-21 α in **1**. [3].

Since 17-OH of **1** had been determined to be sterically unhindered the advanced Mosher method [6] was employed. Compound **1** was esterified with the appropriate MTPA-Cl in anhydrous pyridine at room temperature. The (*S*)-MTPA ester (**4**) and (*R*)-MTPA (**5**) were purified by DIOL SPE followed by DIOL semi-preparative HPLC. Compounds **4** and **5** were characterised using ¹H and gCOSY NMR analysis. All proton signals for both compounds could be assigned, and diagnostic ¹H NMR chemical shift differences ($\delta\Delta = \delta_S - \delta_R$) were calculated. Analysis of the $\delta\Delta$ values (Figure 3) revealed that the absolute stereochemistry of C-17 was *R*. Since the relative stereochemistry of **1** had been previously determined the absolute stereochemistry of C-16 was *R*.

Longithorones J (**1**) and K (**2**) differ structurally only on account of their C-17 oxidation state. It is postulated that in nature the selective reduction of **2** would produce **1** or the oxidation of **1** would afford **2**. Based on this biosynthetic reasoning and by comparison of the chiroptical data for **1** and **2** [For **1**: $[\alpha]_D +170$ (*c* 0.085, CH₂Cl₂), CD (CH₃OH) 209 ($\Delta\epsilon$ -12.6), 245 ($\Delta\epsilon$ +19.5), 339 nm ($\Delta\epsilon$ -1.8); For **2**: $[\alpha]_D +61$ (*c* 0.046, CH₂Cl₂), CD (CH₃OH) 209 ($\Delta\epsilon$ +1.9), 251 ($\Delta\epsilon$ +1.7),

376 nm ($\Delta\epsilon$ -0.4)][3] we predict *R* absolute stereochemistry at C-16 for longithorone K.

Longithorone J (**1**) was tested for cytotoxicity against the cell lines SHSY5Y (human neuroblastoma), HEK293T (SV40 T antigen transformed human embryonal kidney cells), and A549 (human non-small cell lung carcinoma) using the colourimetric sulphorhodamine B assay [7]. Compound **1** showed no cytotoxicity in the A549 assay when tested at 2 and 20 $\mu\text{g/mL}$, however **1** displayed minimal activity at 20 $\mu\text{g/mL}$ in the SHSY5Y and HEK293T assays with cell death of 28% and 16%, respectively. Due to the low cytotoxic activity of **1** and the scarcity of this natural product no further biological evaluations were undertaken.

EXPERIMENTAL

General

NMR spectra were recorded at 30 °C on a Varian 600 MHz Unity INOVA at 599.926 MHz for ^1H . The ^1H chemical shifts were referenced to the solvent peak for CDCl_3 at δ 7.26. Waters 600 pump equipped with a Waters 996 PDA detector and Waters 717 autosampler were used for HPLC. An Alltech DIOL cartridge

(200 mg, 30-40 μ m, 60 Å) was used for SPE work. A YMC DIOL column 5 μ m 120 Å (10-mm D \times 150-mm L) was used for HPLC semi-preparative work. All solvents were Merck Omnisolv grade and the H₂O was Millipore Milli-Q PF filtered. Anhydrous pyridine (Sigma-Aldrich) and (*S*)- and (*R*)-MTPA-Cl (Fluka) were used in the preparation of the Mosher esters, paying particular attention that the (*S*)-MTPA-Cl gives the (*R*)-MTPA ester and *vice versa*.

Animal Material

Longithorone J (**1**) was re-isolated from *A. longithorax* [3] that was collected by scuba diving (-24 m) off Gannet Cay, at the Swains Reefs, Queensland, Australia. Voucher specimen QMG305411 has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation

The re-isolation of longithorone J (**1**, 0.7 mg) was performed using the identical chromatographic protocol to that used for the initial isolation of **1** and **2** [3].

Preparation of MTPA Esters for Longithorone J (1**)**

(*S*)- or (*R*)-MTPA-Cl (29 μ L, 160 μ mol) was added to the starting alcohol (**1**, 0.25 mg, 0.8 μ mol) in anhydrous pyridine (100 μ L), and the resulting mixture was allowed to stand at room temperature for 4 h. CH₂Cl₂ (2 mL) was added and the resulting solution was chromatographed over a DIOL packed SPE cartridge (200 mg) using 100% CH₂Cl₂ (2 mL) as the eluent. The resulting solvent was concentrated under N₂ and chromatographed on a DIOL HPLC column using isocratic conditions of 15% EtOAc/85% hexanes for 5 min at 4 mL/min. The two HPLC separations yielded the pure (*S*)-MTPA ester of longithorone J (**4**, 0.2 mg, 46% yield) and the (*R*)-MTPA ester of longithorone J (**5**, 0.2 mg, 46% yield).

(*S*)-MTPA ester of longithorone J (**4**). Stable yellow gum; ¹H-NMR (600 MHz, CDCl₃): δ 1.55 (3H, s, H-14), 1.57 (3H, s, H-15), 1.68 (1H, m, H-8b), 1.68 (3H, s, H-13), 1.72 (1H, m, H-9b), 1.83 (1H, m, H-8a), 1.83 (1H, m, H-12b), 1.83 (1H, m, H-9a), 1.91 (1H, ddd, J = 12.6, 12.6, 4.0 Hz, H-4b), 2.04 (1H, m, H-5a), 2.15 (1H, m, H-5b), 2.15 (1H, m, H-4a), 2.35 (1H, dd, J = 17.4, 4.2 Hz, H-21 α), 2.39 (1H, dd, J = 14.4, 9.6 Hz, H-1a), 2.45 (1H, dd, J = 13.2, 13.2 Hz, H-12a), 2.54 (1H, dd, J = 17.4, 4.5 Hz, H-21 β), 2.71 (1H, m, H-16), 3.35 (1H, dd, J = 14.4, 9.6 Hz, H-1b), 3.57 (3H, s, MTPA-OCH₃), 4.89 (1H, dd, J = 7.2, 6.6 Hz, H-6), 4.91 (1H, m, H-2), 5.37 (1H, dd, J = 7.2, 6.6 Hz, H-10), 6.14 (1H, br d, J = 5.4 Hz, H-17), 6.34 (1H, s, H-18), 7.44 (3H, m, MTPA-ArH), 7.57 (2H, m, MTPA-ArH).

(R)-MTPA ester of longithorone J (**5**). Stable yellow gum; ¹H-NMR (600 MHz, CDCl₃): δ 1.56 (3H, s, H-14), 1.48 (3H, s, H-15), 1.68 (1H, m, H-8b), 1.70 (3H, s, H-13), 1.72 (1H, m, H-9b), 1.83 (1H, m, H-8a), 1.60 (1H, m, H-12b), 1.83 (1H, m, H-9a), 1.91 (1H, ddd, *J* = 12.6, 12.6, 4.0 Hz, H-4b), 2.04 (1H, m, H-5a), 2.15 (1H, m, H-5b), 2.15 (1H, m, H-4a), 2.33 (1H, dd, *J* = 17.4, 4.9 Hz, H-21 α), 2.41 (1H, dd, *J* = 13.8, 4.5 Hz, H-1a), 2.38 (1H, dd, *J* = 13.2, 13.2 Hz, H-12a), 2.52 (1H, dd, *J* = 17.4, 1.8 Hz, H-21 β), 2.69 (1H, m, H-16), 3.37 (1H, dd, *J* = 13.8, 10.2 Hz, H-1b), 3.63 (3H, s, MTPA-OCH₃), 4.89 (1H, dd, *J* = 7.2, 7.2 Hz, H-6), 4.92 (1H, m, H-2), 5.33 (1H, dd, *J* = 7.8, 7.2 Hz, H-10), 6.13 (1H, br d, *J* = 5.4 Hz, H-17), 6.44 (1H, s, H-18), 7.44 (3H, m, MTPA-ArH), 7.58 (2H, m, MTPA-ArH).

Molecular Modeling Studies

Molecular modeling studies were performed using Macromodel[®] version 6.0 on a Silicon graphics workstation. Monte-Carlo conformational searching was employed using a MM2 force-field and TNCG algorithm. 10000 structures were generated with 181 unique conformations found; all 181 conformations minimised with good convergence.

Cells and Culture Conditions

The SHSY5Y, HEK293T and A549 cell lines were obtained from the American Type Culture Collection. All cells were maintained in Dulbeccos modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin 50 units/mL and streptomycin (50 µg/mL). Cultures were incubated at 37 °C in a humidified 5% CO₂ atmosphere in T-25 cm² tissue culture flasks.

Cell Proliferation Assay

SHSY5Y, HEK293T and A549 cells were seeded in 96-well culture plates at 4000 cells/well. After 24 h, longithorone J (**1**) was added to the cells and following 72 h of drug treatment cell viability was determined by measuring the amount of sulforhodamine B bound to viable-cell protein [7]. Assay results were read using a Molecular Devices Spectra Max 250 plate reader at 520 nm. Longithorone J was tested in triplicate at 2 and 20 µg/mL and was solubilized in 100% DMSO with a final DMSO concentration of 0.5% in each well. All three cell lines responded typically when treated with the laboratory standards, lissoclinotoxins E and F [8]. For example, the IC₅₀ of lissoclinotoxin F towards the HEK293T cells was ~2 µg/mL.

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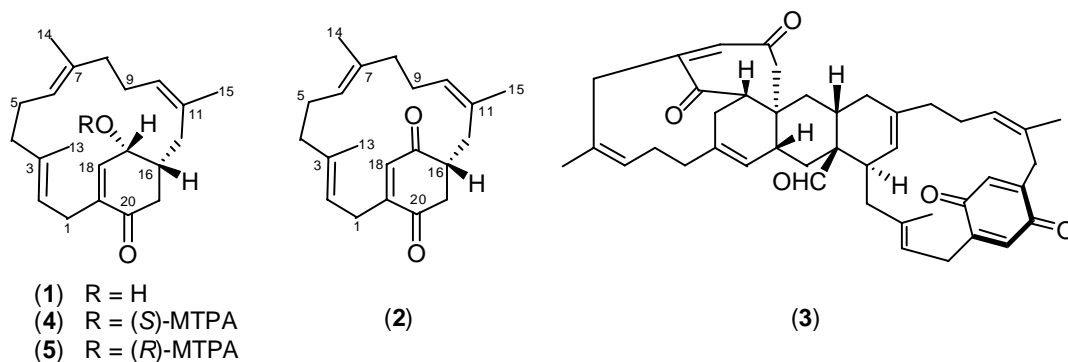


Figure 1. Chemical structures for compounds 1-5.

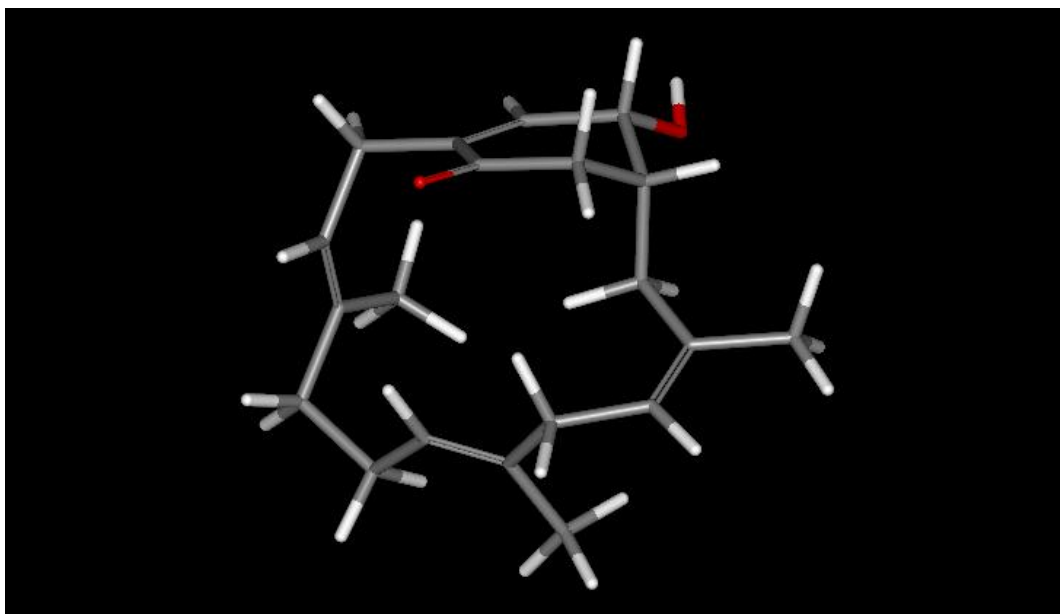


Figure 2. Low energy conformer of longithorone J (**1**) showing unhindered 17-OH substituent.

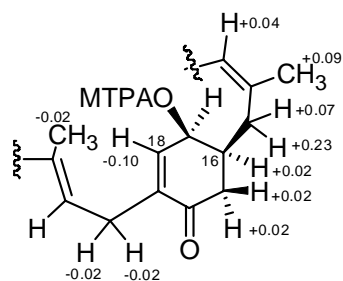


Figure 3. Diagnostic ^1H NMR $\delta\Delta$ values (ppm) from Mosher method analysis of **4** and **5**.