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

ROHAN A. DAVIS, a ANTHONY R. CARROLL, a DIANNE WATTERS, b and RONALD J. QUINN a,*

a Natural Product Discovery, Eskitis Institute, Griffith University, Brisbane, QLD 4111, Australia
b Cell Biology, Eskitis Institute, Griffith University, Brisbane, QLD 4111, Australia

* Corresponding author. Tel.: 61 7 3735 6006. Fax: 61 7 3735 6001. E-mail: r.quinn@griffith.edu.au
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 C$_{21}$ or C$_{42}$ 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 IC$_{50}$ of ~10 μg/mL [1]. In the first reported isolation of longithorones J and K it was noted that both metabolites were unstable in CDCl$_3$ [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 \(^1\)H-\(^1\)H coupling constants calculated from this conformer between H-16 and H-21α/H-21β were almost identical to the observed coupling constants in the initial structure elucidation studies [\(J_{H16, H21\alpha}\) 4.8 Hz (obsd) [3], 4.6 Hz (calcd); \(J_{H16, H21\beta}\) 2.4 Hz (obsd) [3], 2.0 Hz (calcd)]. Further analysis of conformer 2 also allowed the distance between H-17 and H-
21α to be calculated as 2.60 Å. This distance was in agreement with the observed strong ROESY correlation between these H-17 and H-21α 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 $^1$H and gCOSY NMR analysis. All proton signals for both compounds could be assigned, and diagnostic $^1$H 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, CH$_2$Cl$_2$), CD (CH$_3$OH) 209 ($\Delta\varepsilon$ -12.6), 245 ($\Delta\varepsilon$ +19.5), 339 nm ($\Delta\varepsilon$ -1.8); For 2: $[\alpha]_D$ +61 (c 0.046, CH$_2$Cl$_2$), CD (CH$_3$OH) 209 ($\Delta\varepsilon$ +1.9), 251 ($\Delta\varepsilon$ +1.7),
376 nm ($\Delta\varepsilon$ -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 $\mu$g/mL, however 1 displayed minimal activity at 20 $\mu$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 $^1$H. The $^1$H chemical shifts were referenced to the solvent peak for CDCl$_3$ at $\delta$ 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, 30-40µm, 60 Å) was used for SPE work. A YMC DIOL column 5 µm
120 Å (10-mm D × 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 μL, 160 μmol) was added to the starting alcohol (1, 0.25 mg, 0.8 μmol) in anhydrous pyridine (100 μL), 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-21α), 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-21β), 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; $^1$H-NMR (600 MHz, CDCl$_3$): $\delta$ 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$\alpha$), 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$\beta$), 2.69 (1H, m, H-16), 3.37 (1H, dd, $J$ = 13.8, 10.2 Hz, H-1b), 3.63 (3H, s, MTPA-OCH$_3$), 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 Dulbecco’s 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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REFERENCES AND NOTES


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 $^1$H NMR $\delta$Δ values (ppm) from Mosher method analysis of 4 and 5.