

# Isolation of Two Cytotoxic Diterpenes from the Fern *Pteris multifida*

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From aerial parts of the fern *Pteris multifida* Poir. (Polypodiaceae) two diterpenes, *ent*-kaurane-2 $\beta$ ,16 $\alpha$ -diol and *ent*-kaur-16-ene-2 $\beta$ ,15 $\alpha$ -diol, were isolated by repeated column chromatography using silica gel and silica gel impregnated with silver nitrate. The structures were confirmed by spectroscopic methods. Both compounds showed a moderate cytotoxicity to Ehrlich ascites tumour cells.

## Introduction

*Pteris multifida* Poir. (Polypodiaceae) is a fern native to eastern and southern regions of China. Aerial and subterranean parts of the plant have been used in traditional Chinese medicine as a heat-clearing and detoxicating agent. The herb is still used in Chinese folk herbal medicine and known as *Feng Wei Cao*. Cytotoxic properties are ascribed to this fern, but it is yet unknown which constituents are responsible for the effect (Zhou and Wang, 1986; Qian, 1987; Huang, 1993). Phytochemical investigations that have been carried out with this species so far, revealed the presence of flavonoids, phenols and sterols (Zhou and Wang, 1986; Qian, 1987; Huang, 1993; Okuno *et al.*, 1994), essential oil (Okuno *et al.*, 1993), sesquiterpenes and diterpenes (Murakami *et al.*, 1985). This study reports on the isolation of two known *ent*-kaurane diterpenes, which were subsequently tested for cytotoxicity to Ehrlich ascites tumour cells using the microculture tetrazolium (MTT) assay.

## Materials and Methods

### Plant material

Dried aerial parts of *Pteris multifida* Poir. (Polypodiaceae) were purchased from the Chinese Medical Center, Utrecht, The Netherlands. The plant material was imported from China. A voucher specimen is deposited at the Department of Pharmaceutical Biology, University of Groningen, The Netherlands.

### Extraction and isolation

Ground (1 mm) plant material, 500 g, was percolated with subsequently 4.5 l hexane and 3.8 l MeOH, yielding 2.9 and 5.7 g crude extract, respectively. The MeOH extract was first submitted to column chromatography for prepurification (column length 12 cm, i.d. 8.5 cm), using silica gel, 63–200  $\mu$ m, 70–230 mesh ASTM (Merck) and gradient elution with CHCl<sub>3</sub>-Me<sub>2</sub>CO (from 100% CHCl<sub>3</sub> to 100% Me<sub>2</sub>CO, 300 ml fractions). Fractions containing **1** and **2** (TLC, GC) were pooled and chromatographed on silica gel 30–60  $\mu$ m (Baker) (column length 95 cm, i.d. 2.4 cm) successively with CHCl<sub>3</sub> (500 ml), CHCl<sub>3</sub>-Me<sub>2</sub>CO (99:1, 500 ml), CHCl<sub>3</sub>-Me<sub>2</sub>CO (98:2, 500 ml) and Me<sub>2</sub>CO (500 ml). Fractions containing **1** and **2** were pooled, the solvent evaporated and chromato-

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graphed on silica gel-10% AgNO<sub>3</sub> (column length 45 cm, i.d. 2 cm) successively with CHCl<sub>3</sub> (500 ml), CHCl<sub>3</sub>-Me<sub>2</sub>CO (99:1, 500 ml), CHCl<sub>3</sub>-Me<sub>2</sub>CO (98:2, 500 ml), CHCl<sub>3</sub>-Me<sub>2</sub>CO (96:4, 500 ml) and Me<sub>2</sub>CO (500 ml). Compounds **1** and **2** were now completely separated and fractions containing either **1** or **2** were pooled and the solvent was evaporated. To remove traces of AgNO<sub>3</sub> the compounds were extracted with MeOH, leaving the AgNO<sub>3</sub> behind, and purified by recrystallisation in Et<sub>2</sub>O, yielding 247 mg **1** and 109 mg **2**.

#### Quantitative determination of **1** and **2**

In order to find out which solvent was optimal for the extraction of **1** and **2**, their contents were determined in various extracts of *P. multifida* using GC under the following conditions: WCOT fused-silica CP-Sil 5 CB column, 10 m × 0.32 mm i.d., film thickness 0.12 µm (Chrompack, Middelburg, The Netherlands), oven temperature programme 175–325°C at 3°/min, injector temperature 250°C, inlet pressure 5 psi, detector (FID) temperature 300°C, carrier gas N<sub>2</sub>, linear gas velocity 32 cm/s, split ratio 1:56, injected volume 1.0 µl. Calibration curves were made with a solution of **1** and **2** in MeOH; 0.25–2.0 mg/ml. A linear relationship ( $r = 0.9999$ ) between peak area and concentration was found in this range. Plant material, 5.0 g, was extracted with 20 ml MeOH, EtOH 70%(v/v), Me<sub>2</sub>CO, CH<sub>2</sub>Cl<sub>2</sub> or hexane. After filtration the volume was adjusted to 20.0 ml with the solvent. This solution was used for GC analysis. Determinations were done in duplicate.

#### Ent-kaurane-2β,16α-diol (**1**)

Amorphous white powder, m.p. (uncorr.) 254.5–256.7°C;  $[\alpha]_D^{20}$  -46° (MeOH;  $c$  1.00); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 208.4 nm (0.87); IR (KBr)  $\nu_{\max}$  (cm<sup>-1</sup>): 3265 (–OH), 2975, 2942, 2863, 2847, 1458, 1443, 1370, 1215, 1164, 1125, 1065, 1042, 930, 876, 670; <sup>1</sup>H-NMR (CDCl<sub>3</sub>,  $\delta$  in ppm): 0.68 (dd, <sup>2</sup> $J_{AB}$  = 11.96 Hz, <sup>3</sup> $J$  = 11.54 Hz, 1H, H-1<sub>ax</sub>), 0.83 (dd, <sup>3</sup> $J$  = 11.99 Hz, <sup>3</sup> $J$  = 1.28 Hz, 1H, H-5), 0.91 (s, 3H, H-19), 0.96 (s, 3H, H-18), 1.08 (m, 1H, H-9), 1.12 (dd, <sup>2</sup> $J_{AB}$  = 12.01 Hz, <sup>3</sup> $J$  = 12.41 Hz, 1H, H-3<sub>ax</sub>), 1.14 (s, 3H, H-20), 1.37 (s, 3H, H-17), 1.40 (ddd, <sup>2</sup> $J_{AB}$  = 11.81 Hz, <sup>3</sup> $J$  = 11.99 Hz, <sup>3</sup> $J$  = 1.71 Hz, 1H, H-6<sub>ax</sub>), 1.53 (ddd, <sup>2</sup> $J_{AB}$  = 12.82 Hz, <sup>3</sup> $J$  = 11.98 Hz, <sup>3</sup> $J$  = 3.42 Hz, 1H, H-7<sub>ax</sub>), 1.58 (s, 2H, H-15), 1.63 (d, <sup>2</sup> $J_{AB}$  =

11.54 Hz, 1H, H-14S), 1.63 (m, 1H, H-13), 1.64 (m, 1H, H-11<sub>eq</sub>), 1.64 (m, <sup>2</sup> $J_{AB}$  = 11.81 Hz, 1H, H-6<sub>eq</sub>), 1.65 (m, 1H, H-11<sub>ax</sub>), 1.65 (m, <sup>2</sup> $J_{AB}$  = 12.82 Hz, 1H, H-7<sub>eq</sub>), 1.65 (m, 1H, H-13), 1.75 (ddd, <sup>2</sup> $J_{AB}$  = 12.01 Hz, <sup>3</sup> $J$  = 4.27 Hz, <sup>4</sup> $J$  = 1.71 Hz, 1H, H-3<sub>eq</sub>), 1.87 (m, <sup>3</sup> $J$  = 2.56 Hz, 1H, H-12), 1.93 (m, <sup>2</sup> $J_{AB}$  = 11.54 Hz, 1H, H-14R), 2.20 (ddd, <sup>2</sup> $J_{AB}$  = 11.96 Hz, <sup>3</sup> $J$  = 3.85 Hz, <sup>4</sup> $J$  = 1.71 Hz, 1H, H-1<sub>eq</sub>), 3.91 (m, 1H, H-2), 4.90 (s, br, 2H, OH); <sup>13</sup>C-NMR (CDCl<sub>3</sub>,  $\delta$  in ppm): 19.23 (CH<sub>2</sub>, C-11), 19.45 (CH<sub>3</sub>, C-20), 21.25 (CH<sub>2</sub>, C-6), 22.80 (CH<sub>3</sub>, C-19), 24.46 (CH<sub>3</sub>, C-17), 27.91 (CH<sub>2</sub>, C-13), 34.22 (CH<sub>3</sub>, C-18), 35.68 (C, C-10), 38.62 (CH<sub>2</sub>, C-14), 42.15 (C, C-4), 43.09 (CH<sub>2</sub>, C-7), 46.35 (C, C-8), 48.44 (CH, C-12), 50.43 (CH<sub>2</sub>, C-1), 51.99 (CH<sub>2</sub>, C-3), 57.14 (CH, C-5), 58.36 (CH, C-9), 58.77 (CH<sub>2</sub>, C-15), 65.53 (CH, C-2), 79.76 (C, C-16); HRMS:  $m/z$  306.256 [M<sup>+</sup>], EI-MS ( $m/z$ ) (rel. int.) 306 [M<sup>+</sup>] (1), 288 (8), 270 (8), 255 (19), 227 (19), 187 (19), 160 (17), 147 (39), 134 (61), 121 (72), 105 (57), 94 (100), 78 (35), 69 (21), 55 (38), 43 (62).

#### Ent-kaur-16-ene-2β,15α-diol (**2**)

Amorphous white powder, m.p. (uncorr.) 219.5–220.8°C;  $[\alpha]_D^{20}$  -84° (CHCl<sub>3</sub>;  $c$  1.00); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 214.4 (2.37); IR (KBr)  $\nu_{\max}$  (cm<sup>-1</sup>): 3383 (–OH), 2983, 2926, 2864, 1458, 1386, 1368, 1187, 1031, 1001, 896, 829, 701; <sup>1</sup>H-NMR (CD<sub>3</sub>OD,  $\delta$  in ppm): 0.70 (dd, <sup>2</sup> $J_{AB}$  = 11.65 Hz, <sup>3</sup> $J$  = 11.00 Hz, 1H, H-1<sub>ax</sub>), 0.86 (dd, <sup>3</sup> $J$  = 11.00 Hz, <sup>3</sup> $J$  = 0.91 Hz, 1H, H-5), 0.93 (s, 3H, H-19), 0.99 (s, 3H, H-18), 1.12 (m, 1H, H-9), 1.13 (dd, <sup>2</sup> $J_{AB}$  = 10.05 Hz, <sup>3</sup> $J$  = 11.31 Hz, 1H, H-3<sub>ax</sub>), 1.15 (s, 3H, H-20), 1.36 (ddd, <sup>2</sup> $J_{AB}$  = 12.25 Hz, <sup>3</sup> $J$  = 2.5 Hz, <sup>3</sup> $J$  = 1.5 Hz, 1H, H-6<sub>ax</sub>), 1.42 (m, H-14S), 1.52 (m, H-11<sub>eq</sub>), 1.52 (m, H-7<sub>ax</sub>), 1.54 (m, H-13), 1.72 (m, H-7<sub>eq</sub>), 1.72 (m, H-6), 1.73 (m, H-11<sub>ax</sub>), 1.74 (m, H-13), 1.87 (m, H-3<sub>eq</sub>), 1.95 (m, <sup>2</sup> $J_{AB}$  = 11.02 Hz, 1H, H-14R), 2.21 (ddd, <sup>2</sup> $J_{AB}$  = 11.65 Hz, <sup>3</sup> $J$  = 11.31 Hz, <sup>4</sup> $J$  = 1.5 Hz, 1H, H-1<sub>eq</sub>), 2.75 (m, 1H, H-12), 3.78 (m, 1H, H-15), 3.92 (m, 1H, H-2), 4.96 (dd, <sup>2</sup> $J_{AB}$  = 150 Hz, 1H, H-17), 5.38 (dd, <sup>2</sup> $J_{AB}$  = 150 Hz, 1H, H-17); <sup>13</sup>C-NMR (CD<sub>3</sub>OD,  $\delta$  in ppm): 19.27 (CH<sub>2</sub>, C-7), 19.37 (CH<sub>3</sub>, C-20), 20.30 (CH<sub>2</sub>, C-6), 22.92 (CH<sub>3</sub>, C-19), 33.86 (CH<sub>2</sub>, C-13), 34.31 (CH<sub>3</sub>, C-18), 35.64 (C, C-10), 36.51 (CH<sub>2</sub>, C-11), 37.40 (CH<sub>2</sub>, C-14), 42.36 (C, C-4), 43.68 (CH, C-12), 48.15 (C, C-8), 50.42 (CH<sub>2</sub>, C-1), 51.83 (CH<sub>2</sub>, C-3), 55.90 (CH, C-9), 57.03 (CH, C-5), 65.48 (CH, C-2), 83.83 (CH, C-

15), 109.03 (CH<sub>2</sub>, C-17), 160.39 (C, C-16); HRMS: *m/z* 304.240 [M<sup>+</sup>]; EI-MS (*m/z*) (rel. int.) 304 [M<sup>+</sup>] (1), 286 (21), 271 (44), 268 (10), 253 (19), 228 (18), 203 (33), 188 (27), 173 (16), 145 (39), 121 (76), 107 (65), 91 (70), 80 (47), 67 (28), 55 (73), 41 (100). The NMR spectra of compound **2** appeared to be highly sensitive to the purity, concentration and solvents used. Due to strongly overlapping signal groups not all the coupling constants were resolved.

### Cell line

Ehrlich ascites tumour cells were grown in suspension culture in RPMI 1640 (Gibco, Paisley, UK). The culture medium was supplemented with 10% heatinactivated fetal calf serum (Gibco) plus 0.2 mg.ml<sup>-1</sup> streptomycin and 200 IU.ml<sup>-1</sup> penicillin G. The cell line was cultured routinely at 37°C in a shaking incubator. The doubling time was 11–13 h. For all experiments, exponentially growing cells were used, with a viability exceeding 95%, as determined by trypan blue exclusion.

### MTT assay

The microculture tetrazolium (MTT) assay is a cytotoxicity assay that is based on the metabolic reduction of a soluble tetrazolium salt (MTT), by mitochondrial enzyme activity of viable tumour cells, into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolution in DMSO (Carmichael *et al.*, 1987). Under the experimental conditions used, the enzyme activity and the amount of formazan formed were proportional to the amount of cells.

Concentrated stock solutions (200×) of the test compounds were made in DMSO (Merck, Darmstadt, FRG) and stored at -20°C. Cisplatin (*cis*-dichlorodiammine-platinum(II)), used as a reference cytostatic agent, was from Aldrich (Milwaukee, WI). It was dissolved in water immediately before use.

After harvesting, the cells were counted and diluted appropriately with culture medium. Of the cell suspensions, 50 µl containing 700 cells were pipetted into the wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark). Subsequently, 50 µl of a solution of the test compounds, obtained by diluting the stock solution with the appropriate quantity of growth medium, were added to each well. The concentration range tested was from 0–

100 µM. The small amount of DMSO present in the wells (maximal 0.5%) was proved not to affect the experiments. The cells were exposed to the test compounds for 72 h. The plates were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After adding a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, Mo), the amount of formazan formed was measured spectrophotometrically at 520 nm as described (Woerdenbag *et al.*, 1993; Middel *et al.*, 1995).

Cell growth was calculated using the formula: cell growth (%) = (absorbance of treated cells – absorbance of culture medium) / (absorbance of untreated cells – absorbance of culture medium) × 100. The IC<sub>50</sub> value (the concentration of a test compound causing 50% cell growth inhibition or cell kill) was used as a parameter for cytotoxicity. IC<sub>50</sub> values were calculated using the curve fitting programme 'Graphpad'.

### Results and Discussion

The hexane extract of aerial parts of *P. multifida* spontaneously yielded a small crystalline fraction. It consisted of two compounds that were recognized as diterpenes, based upon the fragmentation patterns of their mass spectra, obtained from GC-MS analysis. The MeOH extract, containing much higher concentrations of both compounds, was used for further isolation steps. Isolation was achieved by repeated column chromatography. First, silica gel was used twice for the purification of the extract. Complete separation was achieved by column chromatography using silica gel impregnated with AgNO<sub>3</sub>.

Spectral data were recorded and compared with the literature. The compounds were identified as the *ent*-kaurane diterpenes, *ent*-kaurane-2β,16α-diol (**1**) and *ent*-kaur-16-ene-2β,15α-diol (**2**) (Fig. 1). Both diterpenes had been isolated earlier

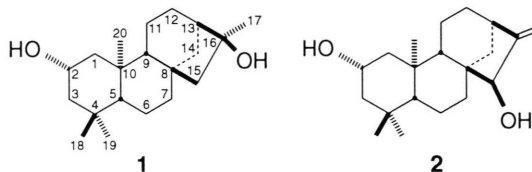


Fig. 1. Chemical structures of the diterpenes isolated from *Pteris multifida*. Compound **1**: *ent*-kaurane-2β,16α-diol; compound **2**: *ent*-kaur-16-ene-2β,15α-diol.

by Japanese researchers (Chen and Murakami, 1971; Murakami *et al.*, 1985), but the spectral data published for both compounds are rather limited. Therefore, we have included a complete list in the Materials and Methods section. The structures of **1** and **2**, were further confirmed with a COSY and NOESY spectrum. *Ent*-kaurane diterpenes form the largest group of diterpenes in ferns and 2- $\beta$ -hydroxy *ent*-kauranes are characteristic for the genus *Pteris* (Murakami and Tanaka, 1988).

With yields of 0.049% for compound **1** and 0.022% for compound **2**, we substantially isolated more from *P. multifida* than the previously reported 0.0008% and 0.0014% for **1** and **2**, respectively (Murakami *et al.*, 1985). MeOH was the most efficient extraction solvent for **1** and **2** from the plant material. A MeOH extract contained 0.077 and 0.066% on a dry weight basis, of **1** and **2**, respectively. For comparison: the contents of **1** and **2** in an extract prepared in the same way with EtOH 70% (v/v) were 0.061 and 0.055%, with acetone 0.063 and 0.054, with CH<sub>2</sub>Cl<sub>2</sub> 0.064 and 0.055%, and with hexane 0.023 and 0.023%, respectively.

Compounds **1** and **2** showed moderate cytotoxicity to Ehrlich ascites tumour cells after continuous incubation in the microculture tetrazolium (MTT) assay, as can be seen from Fig. 2. Progressive cell growth inhibition was found for both

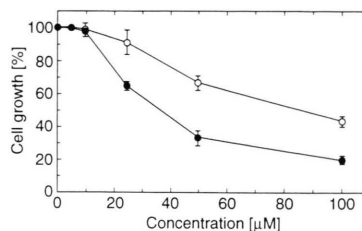


Fig. 2. The effect of *ent*-kaurane-2 $\beta$ ,16 $\alpha$ -diol (compound **1**; closed circles) and *entkaur*-16-ene-2 $\beta$ ,15 $\alpha$ -diol (compound **2**; open circles) on the growth of Ehrlich ascites tumour cells as measured by the microculture tetrazolium (MTT) assay. Cells were incubated for 3 days with different concentrations of the diterpenes. For each point the mean value ( $n=3$ )  $\pm$  sd (vertical bar) is given.

compounds at concentration from 10  $\mu$ M, but compound **1** was more active than compound **2**. At 25  $\mu$ M, only **1** displayed significant cytotoxicity. The IC<sub>50</sub> values of **1** and **2** were 37 and 83  $\mu$ M (11 and 25  $\mu$ g/ml), respectively. At 100  $\mu$ M, the highest concentration tested, 81% cell growth inhibition was found for compound **1** 58% for compound **2**. For comparison: the highly active reference cytostatic agent cisplatin had an IC<sub>50</sub> of 0.5  $\mu$ M in the applied test system.

In conclusion, the two diterpenes found in *P. multifida* may partly be responsible for the cytotoxic properties of the plant, as claimed in the Chinese literature.

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