Isolation and Identification of a Novel Chlorophenol from a Cell Suspension Culture of *Helichrysum aureonitens*

Seyed Mahdi Ziaratnia,[a,b,#] Kiyoshi Ohyama,[c,#] Ahmed Abdel-Fattah Hussein,[a,1)] Toshiya Muranaka,[c,d] Namrita Lall,[a] Karl Josef Kunert,[a,b] and Jacobus Johannes Marion Meyer*[,a]

[a] *Department of Plant Science, University of Pretoria;* [b] *Forestry and Agricultural Biotechnology Institute, University of Pretoria; Pretoria 0002, South Africa:* [c] *RIKEN Plant Science Centre; Yokohama, Kanagawa 230–0045, Japan: and* [d] *Kihara Institute for Biological Research, Yokohama City University; Yokohama, Kanagawa 244–0813, Japan.*
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A novel chlorophenol, 4-chloro-2-(hepta-1,3,5-triyn-1-yl)-phenol (1), was isolated as the major phenolic compound from the cells of *Helichrysum aureonitens* suspension cultures. Compound 1 has been proposed to be an intermediate in the acetylene biosynthetic pathway of other acetylenic compounds in *Helichrysum* spp. The ethanol extract of cell suspension cultures and compound 1 were evaluated for their cytotoxicity against monkey kidney Vero (Vero cells) and human prostate epithelial carcinoma (DU145) cell lines, also, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Mycobacterium tuberculosis* H37Rv were determined as well.

Key words *Helichrysum aureonitens*; polyacetylene; cell suspension; chlorophenol; anticancer

The genus *Helichrysum* (Asteraceae) is represented by approximately 600 species in Africa, of which 244 are growing in South Africa.[2)] *Helichrysum* species are often used to treat respiratory conditions and tuberculosis.[3)] Several *Helichrysum* species such as *H. odoratissimum*, *H. melanacme* and *H. caespititium* have been reported to have activity against *Mycobacterium tuberculosis*.[4—6)] The medicinal properties of this genus are mainly attributed to the presence of flavonoids, but the genus is rich in several other interesting compounds (coumarins, phenolic acids, antioxidant micronutrients, *e.g.* Cu, Mn, Zn)[7)] with proven medicinal characteristics.[8)] Polyacetylene compounds are present in many plants including the *Helichrysum* genus and display important biological activities.[9,10)] *H. aureonitens* Sch. Bip is a hairy perennial herb which grows mostly in the Kwazulu-Natal province of South Africa[11)] and is widely used in folk medicine.[12)] Previously 3,5,7-trihydroxyflavone (galangin) with antiviral, antifungal and antibacterial activity was isolated from *H. aureonitens*.[11,13)]

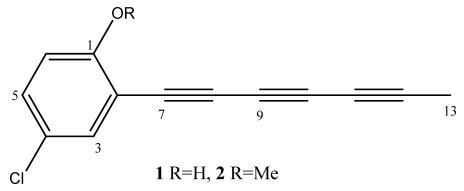
In our preliminary experiments with the cell suspension cultures of *H. aureonitens* to optimize the conditions for the production of phenolic compounds *e.g.* galangin, it was found that compound **1** existed as major metabolite. In this article we report the isolation and identification of **1** from a cell suspension culture of *H. aureonitens* and its significance in the biosynthesis of other polyacetylenic compounds in the *Helichrysum* genus. The results of antitubercolusis activity and toxicity of a crude extract and the chloronated phenol isolated from *H. aureonitens* cell suspension cultures are also discussed.

Results and Discussion

Compound **1** was isolated as a semi-solid from the non-polar part of the ethanolic extract of *H. aureonitens* cell suspension cultures. HR-ESI-MS (negative mode) gave 213.0181 $(M-1)^+$ corresponding to a molecular formula of $C_{13}H_7OCl$. ^{1}H-NMR showed four signals at 7.33 (d, 2.7 Hz), 7.23 (dd, 8.7, 2.7 Hz), and 6.87 (d, 8.7 Hz), in addition to a methyl single signal at 2.06. The ^{13}C-NMR showed 13 carbons, six of them were aromatic 157.5, 132.2, 131.6, 125.2, 116.6, 109.2, six carbons of acetylenic bonds at 82.1, 80.1, 70.1, 68.1, 64.6, 57.8 and a signal of a methyl carbon at 4.7. HMBC cross-peak showed correlations of H-3/C-1, C-4, C-5, C-7, H-5/C-1, C-2, C-3, C-4, C-6, H-6/C-1, C-2, C-4, C-5, C-7. The foregoing data indicated the compound **1** to be 4-chloro-2-(hepta-1,3,5-triyn-1-yl)-phenol. The other data including 2D-NMR, HMQC and COSY also supported the structure of **1** (Fig. 1). Compound **1** has not been isolated previously.

The methyl ether derivative (**2**) of compound **1** was isolated from the roots of *H. coriaceum*[14)] and the other two unusual chlorophenol acetylenes, helitenuin and helitenuone from *H. tenuifolium*, which are related to **1**.[15)] It is proposed that fatty acids are the precursors in the biosynthetic pathway of the isolated acetylenes.[16,17)] Based on that, **1** is proposed to be an intermediate in the acetylene biosynthetic pathway and the substrate for both helitenuin and helitenuone.

Compound **1** was also detected in the aerial parts of intact plants and tissue cultured plants of *H. aureonitens* by LC-MS analysis, but at very low concentrations (data not shown). This indicates the higher induction of the polyacetylene biosynthesis enzyme under the cell suspension conditions. This response of the cells in the culture could have been driven by the exogenously applied plant growth regulators through upregulating the involved genes in **1** production biosynthetic pathway. Bohlmann *et al.*[14,15)] proposed the



1 R=H, **2** R=Me

Fig. 1. 4-Chloro-2-(hepta-1,3,5-triyn-1-yl)-phenol (**1**), and Its Methyl Ether Derivative (**2**)

* To whom correspondence should be addressed. e-mail: marion.meyer@up.ac.za
# These authors contributed equally to this work.

presence of **1** in the roots of *H. coriaceum* and *H. tenuifolium* (which normally grow under darkness) and in our experiment **1** was produced in the dark grown cells of *H. aureonitens* suspension cultures. It might therefore be that dark conditions are necessary for the induction of **1**, but more experimentation is needed to confirm this. This result was confirmed in another experiment in which **1** could not be detected after 72 h exposure of cell cultures to UV light (data not shown).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanol extracts of cell suspension cultures against *M. tuberculosis* H37Rv was found to be 1000 μg/ml and 2000 μg/ml respectively. The antimycobacterial activity of the crude extract of *H. aureonitens* cell suspension cultures was found to be rather weak as compared to the other ethanol extract of the aerial parts of the plants whereas MIC observed against H37Rv was found to be 200 μg/ml (unpublished data) and **1** did not show antituberculosis activity at the highest concentration (200 μg/ml) tested. The MIC for the positive control "isoniazid" was found to be 0.2 μg/ml.

The anticancer activity of acetylenic compounds has already been reported.[10] Polyacetylenes found in ginseng[18] and other medicinal plants[19] have been reported to exhibit anticancer activity. There are also some reports on the anticancer activities of *Helichrysum* genus. The extracts from *H. pallassi*, *H. armenium*, *H. plicatum* have been reported to have a considerable inhibition on DNA topoisomerase I, which represent a major class of anticancer drugs.[20] The ethanol extract of cell suspension cultures and **1** were evaluated *in vitro* for their cytotoxicity against the monkey kidney Vero (Vero cells) and human prostate epithelial carcinoma (DU145) cell lines. **1** was about 8 times more toxic (IC_{50} value, 1.51 μg/ml) than the crude ethanol extract (12.11 μg/ml) on a Vero cell line. The crude extract and **1** showed similar toxicity on a prostate cancer cell line with IC_{50} values of 3.52 and 2.14 μg/ml respectively. This indicates that other toxic compounds could be present in the extract. These results also showed that the anticancer activity of the crude extract and compound **1** was found to be significant and comparable with that of the positive control tested in the present study. The IC_{50} values of the positive control "Zearalenone" were found to be 1.63 and 0.53 μg/ml against Vero and DU145 cell lines respectively. Compound **1** warrants further investigation for its potential as an anticancer drug.

Experimental

Establishment of Callus and Cell Suspension Cultures Callus tissues were induced from young leaves of *H. aureonitens* plants. The leaves were surface sterilized (70% ethanol for 30 s, 0.5% sodium hypochlorite for 30 min) and inoculated onto the culture medium with the inner surface in contact with the medium. These explants grew on Murashige and Skoog (MS) medium[21] supplemented with 3% sucrose (w/v), 5.37 μM naphtaleneacetic acid (NAA), 0.85 μM 6-benzyladenine (BA) on 0.30% agar; the pH was adjusted to 5.70 prior to autoclaving for 20 min at 1.05 kg/cm^2 and 121 °C. The cultures were incubated in a growth chamber at 25 °C in the darkness. After 4—6 weeks, the callus tissues formed, mainly from the surface of leaves and were then subcultured to obtain friable callus. Cell suspension cultures were established from selected *H. aureonitens* CALLI. Callus pieces (0.1 g) were inoculated into 100 ml flasks containing 20 ml MS medium supplemented with 3% sucrose, and similar concentrations of NAA and BA to the callus induction experiment, and incubated in the dark at 25 °C on a rotary shaker at 120 rpm. Cell cultures were passed through a stainless steel sieve (1 mm^2) every 2 weeks for 6 months in order to obtain a fine cell suspension culture. For the cell suspension subculturing, 0.20 g of cells (fresh mass) were inoculated into 20 ml of the above-mentioned liquid medium in 100 ml flasks.

Extraction and Isolation of 1 Cells were filtrated from the medium and around 100 g fresh cells were used for extraction. The extraction was carried out with 95% EtOH followed by Sephadex LH-20 column chromatography, using ethanol as eluent. The fractions that contained the impure **1**, were further purified on another Sephadex column under the same conditions to yield 20 mg pure compound.

4-Chloro-2-(hepta-1,3,5-triyn-1-yl)-phenol amorphous powder, IR (KBr disk) 3310, 2120 cm^{-1}, ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.33 (1H, d, J=2.7 Hz, H-3), 7.23 (1H, dd, J=8.7, 2.7 Hz, H-5), 6.87 (1H, d, J=8.7 Hz, H-6), 2.06 (Me, s, H-13); ^{13}C-NMR (100 MHz) δ: 157.5 (C-1), 132.2 (C-3), 131.6 (C-5), 125.2 (C-4), 116.6 (C-6), 109.2 (C-2), 82.1, 80.1, 70.1, 68.1 (C-7), 64.6, 57.8, 4.7 (C-13), C-8, 9, 10, 11, and 12 could not be assigned; HR-ESI-MS (negative-ion mode) m/z: 213.0181 (M-1) (Calcd for $C_{13}H_7OCl$, 214.0186).

Bioassays. Antituberculosis Activity The radiometric respiratory technique using the BACTEC system (Becton Dickinson Diagnostic Instrument, Sparks, MD, U.S.A.) was used for susceptibility testing of *M. tuberculosis* H37Rv (ATCC 27264).[22,23]

Cytotoxicity Assay Microtitre well plates with Vero and DU145 cells were used for toxicity analysis of the ethanolic crude extract and compound **1**. The positive drug control, Zearalenone was tested at final concentrations of 7.52—0.26 μg/ml.[24]

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