Lignans, Phenylpropanoids and Polyacetylenes from *Chaerophyllum aureum* L. (Apiaceae)

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Sub-aerial parts of *Chaerophyllum aureum* L. yielded two polyacetylenes, falcarinol (**1**), falcarindiol (**2**), three lignans, namely nemerosin (**3**), deoxypodorhizone (**4**), deoxypodo-phyllotoxin (**5**), two phenylpropanoids, 1′-hydroxymyristicin (**6**) and its angeloyl ester (**7**). Compounds **6** and **7** were isolated for the first time from plant material and their structures were elucidated by means of extensive 1- and 2-dimensional NMR spectroscopy and high resolution mass spectrometry. In bioautographic tests on TLC plates the dichloromethane extract showed a significant antimicrobial activity. Falcarindiol was identified as the main active principle whereas the phenylpropanoids and lignans showed no activity.

Key words: 1′-Hydroxymyristicin, 1′-Angeloyloxymyristicin, Antimicrobial Activity

Introduction

Chaerophyllum aureum L. (Apiaceae, tribe Scandiceae) is a perennial herb growing in the mountainous to sub-alpine regions of Europe. The aromatic smell of this plant is similar to that of carrots. *Ch. aureum* has considerable nutritional values and is used as pasture herb by cattle (Hegi, 1975). Due to morphological similarities and identical habitats it is easily confused with the related *Anthriscus sylvestris* (L.) Hoffm. (Adler *et al.*, 1994). Characteristics of *Ch. aureum* are the red speckled stem and the non-beaked fruit bearing styles which are arranged in a right angle. Furthermore, the bracts are missing and the bracteoles (5 to 10) are continuously pointed and have a membranous and ciliated margin. The Latin (*aureum*) and German species name (*Gold*-Kälberkropf) are derived from the fact that the colour of the fruit turns to gold as they ripen.

There is a number of phytochemical and pharmacological investigations of *A. sylvestris* (Ikeda *et al.*, 1998a, b, and literature cited therein). In contrast, there are no phytochemical reports about *Ch. aureum*, except for the flavonoid glycosides isolated from its leaves (Gonnet, 1985, 1986). This paper deals with the isolation and structure elucidation of apolar compounds from *Ch. aureum* and their potential antimicrobial activity.

Results and Discussion

Chromatographic separation of the dichloromethane extract resulted in the isolation of **1–7** (Fig. 1). Compounds **1** and **2** were identified by NMR experiments as the polyacetylenes falcarinol and falcarindiol, the latter representing the main constituent of the crude extract. Their proton and carbon assignments are in accordance with published reports (Gafner *et al.*, 1989; Zheng *et al.*, 1999). Structures of the isolated lignans, nemerosin (**3**), (–)-deoxypodorhizone (**4**), and deoxypodophyllotoxin (**5**, also known as anthricin), were elucidated by comparing the results of the NMR experiments with literature data (Ikeda *et al.*, 1998a, b).

HR Mass spectrometric analysis of **6** indicated a molecular formula of $C_{11}H_{12}O_4$. ^{1}H NMR data showed two aromatic protons at $\delta_{H\text{-}2}$ 6.57 and $\delta_{H\text{-}6}$ 6.56, a sharp singlet integrating for two protons ($\delta_{H\text{-}OCH2O}$ 5.96), further signals at $\delta_{H\text{-}2'}$ 6.00 (ddd, J = 17.5, 10.5, 6.5), at $\delta_{H\text{-}3'}$ 5.35 (dt, J = 17.5, 1.5), 5.19 (dt, J = 10.5, 1.5), and at $\delta_{H\text{-}1'}$ 5.10 (d, J = 6.5) integrating for one proton, each. The ^{13}C NMR

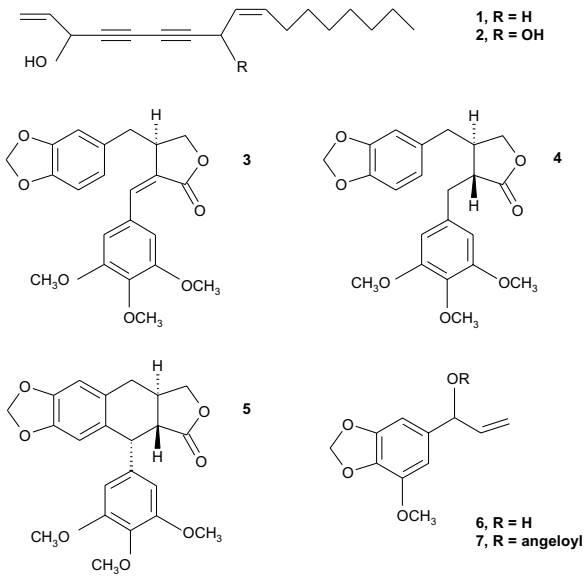

Fig. 1. Structures of two polyacetylenes, three lignans, and two phenylpropanoids from *Chaerophyllum aureum* (**1**–**7**). Falcarinol (**1**), falcarindiol (**2**), nemerosin (**3**), deoxypodorhizone (**4**), deoxypodophyllotoxin (**5**), 1′-hydroxymyristicin (**6**) and 1′-angeloyloxymyristicin (**7**).

spectrum showed six aromatic carbon signals, corresponding to four quarternary carbons ($\delta_{C\text{-}1}$ 137.4, $\delta_{C\text{-}3}$ 143.6, $\delta_{C\text{-}4}$ 134.7 and $\delta_{C\text{-}5}$ 148.9) and two methins ($\delta_{C\text{-}2}$ 105.8 and $\delta_{C\text{-}6}$ 100.6). Additionally, there were signals for two further methins ($\delta_{C\text{-}1'}$ 75.2, $\delta_{C\text{-}2'}$ 140.0), two methylene carbons ($\delta_{C\text{-}OCH2O}$ 101.5, $\delta_{C\text{-}3'}$ 115.2) and one methoxy group ($\delta_{C\text{-}CH3}$ 56.6). The methylenedioxy group ($\delta_{C\text{-}OCH2O}$ 101.5) is linked to the aromatic carbons C-4 and C-5 which was established by HMBC cross peaks between the methylene group signal at $\delta_{H\text{-}OCH2O}$ 5.96 and the quarternary carbon signals at $\delta_{C\text{-}4}$ 134.7 and $\delta_{C\text{-}5}$ 148.9. Localisation of the methoxy group was established by the HMBC cross peak of the methyl protons to the aromatic carbon at $\delta_{C\text{-}3}$ 143.6. The remaining quarternary aromatic carbon C-1 was substituted with an allyl group. Its downfield shifted carbons C-2′ and C-3′ were assigned to a vinyl group. The two terminal methylene protons ($\delta_{H\text{-}3'}$ 5.35, dt, J = 17.5, 1.5 and 5.19, dt, J = 10.5, 1.5) coupled with the proton H-2′ ($\delta_{H\text{-}2'}$ 6.00, ddd, J = 17.5, 10.5, 6.5) which in turn showed an HMBC cross peak to the oxygen bearing methin C-1′ at 75.2. In the FTIR spectrum a distinct broad valence vibration between 3600 to 3200 cm^{-1} clearly indicated the presence of a hydroxyl group in the molecule. The connection of the aromatic ring and allyl moiety was also established by HMBC spectroscopy (cross peaks between H-1′ and the aromatic methins C-2 and C-6). Thus, compound **6** is the phenylpropanoid 1′-hydroxymyristicin, 1-(7-methoxy-benzo [1,3]dioxol-5-yl)-prop-2-en-1-ol.

The HR mass spectrum of compound **7** indicated a molecular formula of $C_{16}H_{18}O_5$. NMR spectra were very similar to those of compound **6**. The main difference between the spectra of **6** and **7** was the pronounced downfield shift of the proton signal assignable to H-1′ of **7** ($\delta_{H\text{-}1'}$ 6.23 instead of 5.10) indicating esterification in that position. Furthermore, the ^1H NMR spectrum showed three additional signals at $\delta_{H\text{-}3''}$ 6.10 (qq, J = 7.5, 1.5), $\delta_{H\text{-}4''}$ 1.99 (dq, J = 7.5, 1.5) and $\delta_{H\text{-}2''Me}$ 1.93 (dq, J = 1.5, 1.5), integrating for one, three and three protons, respectively. The ^{13}C NMR spectrum was also very similar, but additional signals for two methyl carbons ($\delta_{C\text{-}2''Me}$ 20.6 and $\delta_{C\text{-}4''}$ 15.8), one methin ($\delta_{C\text{-}3''}$ 138.5) and two quarternary carbons ($\delta_{C\text{-}2''}$ 127.8 and $\delta_{C\text{-}1'}$ 166.8) were detectable. Taking into account results from 2D NMR experiments and literature data (Zidorn and Perry, 2001; Stuppner *et al.*, 2002) this partial structure was assigned to a angeloyl moiety. The downfield shifts of the methine proton H-1′ and carbon C-1′, together with an HMBC correlation between position 1′ and the carboxylic carbon C-1″ identified compound **7** as the 1′-angeloyl ester of compound **6**. These findings were confirmed by FTIR spectroscopy: In contrast to compound **6**, the hydroxyl valence vibration was missing in the spectrum of compound **7**, whereas a strong absorption band at 1718 cm^{-1} was detectable. The position of this carbonyl stretching vibration is indicative of an α, β-unsaturated ester. ^1H and ^{13}C NMR data of compounds **6** and **7** are given in Table I. All signal assignments were verified by 2D NMR experiments (HSQC, HMBC). Important HMBC cross peaks for compound **7** are shown in Fig. 2.

Up to now 1′-hydroxymyristicin (**6**) has never been observed as plant constituent, although related phenylpropenes, especially myristicin, widely occur in a number of members of the Apiaceae family (Harborne, 1971). However, compound **6** was found as a metabolite of myristicin formed in

Table I. ^{1}H and ^{13}C NMR data of compound **6** and **7***.

Position	**6** ^{1}H NMR	**6** ^{13}C NMR	**7** ^{1}H NMR	**7** ^{13}C NMR
1		137.4		138.0
2	6.57 1H δ (1.5)	105.8	6.56 1H ≥ (1.5)	107.1
3		143.6		143.5
4		134.7		135.0
5		148.9		148.9
6	6.56 1H ≥ (1.5)	100.6	6.57 1H ≥ (1.5)	101.4
O-CH_3	3.90 3H s	56.6	3.90 3H s	56.6
O-CH_2-O	5.96 2H s	101.5	5.96 2H s	101.5
1′	5.10 1H br ≥ (6.5)	75.2	6.23 1H dt (5.5, 1.5)	75.7
2′	6.00 1H ddd (17.5, 10.5, 6.5)	140.0	6.00 1H ddd (17.0, 10.5, 5.5)	136.5
3′	5.35 1H dt (17.5, 1.5)	115.2	5.31 1H dt (17.0, 1.5)	116.5
	5.19 1H dt (10.5, 1.5)		5.23 1H dt (10.5, 1.5)	116.5
Angeloyl moiety				
1″				166.8
2″				127.8
2″-Me			1.93 3H dq (1.5, 1.5)	20.6
3″			6.10 1H qq (7.5, 1.5)	138.5
4″			1.99 3H dq (7.5, 1.5)	15.8

* Measured in $CDCl_3$ at 500 and 125 MHz, respectively; referenced to solvent signals at 77.0 ppm and solvent residual signals at δ_H = 7.26 ppm, respectively.

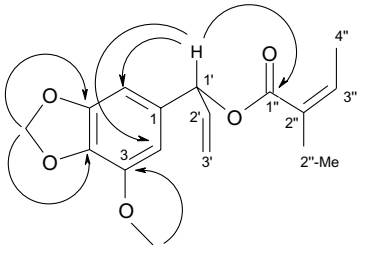

Fig. 2. Selected HMBC correlations for compound **7**.

the liver of mice (Hattori *et al.*, 1993) and rats (Lee *et al.*, 1998). 1′-Angeloyloxymyristicin (**7**), represents a new natural compound.

The crude dichloromethane extract from the sub-aerial parts of *Ch. aureum* was tested for antimicrobial activity in an agar diffusion assay, and showed activities against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes* strains. For monitoring the active principles the dichloromethane extract and the isolated compounds were tested in a TLC bioautographic assay. Falcarindiol (**2**), the main compound of the crude extract, exhibited activity against all of the selected strains (*Escherichia coli* NCTC 9001, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* Δ68), with the exception of *Escherichia coli* ATCC 0120. Interestingly in our assay, falcarinol (**1**) exhibited activity only against *Streptococcus pyogenes* Δ68. The antimicrobial activity of **1** and **2** is well known [Matsuura *et al.*, 1996 and literature cited herein; Villegas *et al.*, 1988; Lutomski *et al.*, 1992]. Therefore, no quantitative evaluation of their antimicrobial activity was performed.

Experimental

General experimental procedures

Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The FTIR spectra were recorded on a Bruker IFS 25, FTIR spectrometer in transmission mode within the range of 4000 to 600 cm^{-1}. Samples were applied to a ZnSe disk (2 mm thickness). The mass spectra (HR-FAB) were recorded on a Finnigan MAT 95S mass spectrometer. NMR spectra were either recorded in $CDCl_3$ on a Bruker Avance 300 or a Varian Unity*plus* 500 spectrometer. Column chromatography (CC) was performed using silicagel 60 (Merck, 0.040–0.063 mm, 230–400 mesh) and Sephadex LH-20. Semi preparative reversed phase HPLC was performed on a Dionex P 580 pump with a LichroCART® (250 × 10 mm) column with LiChrospher® 100 RP-18 material (particle size 10 μm). TLC was performed with silica gel 60 F_{254} plates (0.25 mm, Merck).

Plant material

Ch. aureum was collected in July 2001 between Igls and Patsch near Innsbruck, Tyrol, Austria (altitude 980 m) and identified according to Adler *et al.* (1994). Voucher specimens (JR-20010707A-1) have been deposited in the herbarium of the Department of Pharmacognosy, Institute of Pharmacy, University of Innsbruck, Austria.

Extraction and isolation

Fresh underground parts (2.0 kg) were lyophilized, crushed to coarse powder (450 g) and extracted three times with 1.5 l CH_2Cl_2 for one day. The crude extract (18.5 g) was suspended in CH_2Cl_2 and roughly fractionated by vacuum liquid CC (2.5 × 50 cm) using a step-gradient of PE-CH_2Cl_2-MeOH (PE; PE-CH_2Cl_2 95:5, 90:10, 75:25, 50:50, 25:75; CH_2Cl_2; CH_2Cl_2-MeOH 90:10, 75:25, 50:50 (v/v); MeOH; 500 ml each) to give eleven fractions (A 1–11). Fraction A 6 (1.2 g) was further separated by silica gel (50 g) flash CC (1.8 × 35 cm) with a step-gradient of CH_2Cl_2-EtOAc-MeOH (CH_2Cl_2; CH_2Cl_2-EtOAc 99:1, 95:5, 90:10, 80:20, 50:50, 25:75; EtOAc; EtOAc-MeOH 50:50; MeOH; 250 ml each) to give ten fractions (B 1–10). B 4 (585 mg; between 400 and 550 ml) was further purified by Sephadex CC (2 × 100 cm; CH_2Cl_2-$(CH_3)_2CO$, 85:15; 2.3 ml/min) to yield pure compound **6** (10.7 mg; t_R of **6** between 504 and 560 ml). B 5 (298 mg; between 575 and 700 ml) was separated by Sephadex CC (2 × 100 cm; CH_2Cl_2-$(CH_3)_2CO$, 85:15; 2.3 ml/min) to give a fraction enriched with **3–5** (137 mg; t_R of 3–5: between 168 and 196 ml). Isolation of these compounds was achieved by semi-preparative HPLC using a mixture of H_2O-MeCN (75:25 to 55:45 (v/v) within 10 min; then isocratic run 55:45; 3 ml/min) to yield **3** (6.4 mg; t_R between 128 and 138 ml), **4** (7.2 mg; t_R between 114 and 123 ml), and **5** (12.8 mg; t_R between 93 and 111 ml). B 6 (33 mg; between 725 and 825 ml) was purified by Sephadex CC (2 × 100 cm; CH_2Cl_2-$(CH_3)_2CO$, 85:15; 2.7 ml/min) to yield pure compound **2** (16.8 mg; t_R of **2**: between 456 and 656 ml).

Fraction A 4 (1.3 g) was re-chromatographed by silica gel (70 g) flash CC (1.8 × 55 cm) with a step-gradient of PE-CH_2Cl_2 (PE; PE-CH_2Cl_2 80:20, 60:40, 50:50, 40:60, 30:70, 15:85; CH_2Cl_2; 250 ml each) to give thirteen fractions (C 1–13). Fraction C 4 (255 mg; between 775 and 1025 ml) enriched with **1** and **7** was subjected to Sephadex CC (1.8 × 55 cm; CH_2Cl_2-$(CH_3)_2CO$, 85:15; 2.3 ml/min) to yield pure compounds **7** (8.1 mg; t_R of **7**: between 84 and 91 ml) and **1** (8.3 mg; t_R of **1**: between 112 and 133 ml). Detection of eluates by TLC (silica gel), CH_2Cl_2/EtOAc (7:1), was performed with methanolic vanillin – sulfuric acid reagent. R_f **1**: 0.84, **2**: 0.35, **3**: 0.62, **4**: 0.57, **5**: 0.51, **6**: 0.48, **7**: 0.87.

Spectroscopic data

1′-Hydroxymyristicin (**6**): Pale yellow oil; $[\alpha]_D^{20}$ + 18.3° ($CHCl_3$, c 4.7); UV (H_2O/MeCN): λ_{max} = 211 nm, 244 nm, 275 nm; FTIR ν_{max}: 3400 cm^{-1} (br), 2914 cm^{-1}, 2896 cm^{-1}, 1636 cm^{-1}, 1510 cm^{-1}, 1457 cm^{-1}, 1432 cm^{-1}, 1319 cm^{-1}, 1195 cm^{-1}, 929 cm^{-1}, 840 cm^{-1}, 751 cm^{-1}; HR-FAB-MS *m/z* 208.0738 $[M]^+$ (calc. for $C_{11}H_{12}O_4$, 208.0736).

1′-Angeloyloxymyristicin (**7**): Whitish crystals; $[\alpha]_D^{20}$ – 19.0° ($CHCl_3$, c 4.0); UV (H_2O/MeCN): λ_{max} = 213 nm, 246 nm, 274 nm, 284 nm.

FTIR ν_{max}: 2955 cm^{-1}, 2917 cm^{-1}, 2849 cm^{-1}, 1718 cm^{-1}, 1636 cm^{-1}, 1473 cm^{-1},1462 cm^{-1}, 1432 cm^{-1}, 1229 cm^{-1}, 1136 cm^{-1}, 1044 cm^{-1}, 731 cm^{-1}, 719 cm^{-1}; HR-FAB-MS *m/z* 290.11541 $[M]^+$ (calc. for $C_{16}H_{18}O_5$, 290.11488).

Test organisms

Bacteria were purchased from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and the National Collection of Type Cultures (NCTC). *Staphylococcus aureus* B9 and *Streptococcus pyogenes* Δ68 were in-house reference strains.

Bioassays

For testing Müller-Hinton agar (Oxoid, UK) inoculated with suspensions of respective test organisms, was used. Wells for the test solutions were punched into the agar using pipette tips. The assay was performed in triplicates with 265 μg (10 μl, c 25.6 mg/ml DMSO) of the crude extract, ampicillin (2 mg/ml dissolved in water) was used as control antibiotic and pure DMSO as vehicle control added into each well. Then, the Petri dish was incubated at 37 °C for 20 h. Extract wells showing growth inhibition zones were visually evaluated as active.

Bioautographic TLC assay

The bioautographic assay was performed according to Wedge and Nagle (2000). TLC was carried out with about 200 μg of crude extract (20 μl, c 10 mg/ml dichloromethane) and about 10 μg (10 μl, c 1 mg/ml dichloromethane) of pure compounds, respectively, on silica gel 60 F_{254} plates (0.25 mm, Merck) in duplicates. Plates were devel-

oped using mixtures of dichloromethane and ethylacetate (7:1 v/v). One plate was detected under UV_{254} and UV_{366} light, and sprayed with methanolic vanillin-sulfuric acid and heated for detection of substance spots. The second plate was overlaid with nutrition medium (Müller Hinton Agar, Oxoid, UK) which was inoculated with suspensions of the respective test organisms and then incubated for 16–20 h at 37 °C in humidified atmosphere. After incubation bacterial growth was detected by spraying 0.9% NaCl solution containing 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Roth, Germany) and re-incubation for 20 min. Dehydrogenase activity of living bacteria converted the tetrazolium salt into violet-colored formazan. Spots with antimicrobial activity remained as clear zones and were compared with the reference plate.

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