NOTE

Identification of Ostruthin from *Peucedanum ostruthium* Rhizomes as an Inhibitor of Vascular Smooth Muscle Cell Proliferation

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Supporting Information

**ABSTRACT:** Inhibition of vascular smooth muscle cell (VSMC) proliferation is of substantial interest in combating cardiovascular disease. A dichloromethane extract from the rhizomes of *Peucedanum ostruthium*, a traditionally used Austrian medicinal plant with anti-inflammatory properties, was examined for a putative antiproliferative activity in rat aortic VSMC. This extract inhibited serum (10%)-induced VSMC proliferation concentration dependently. Further identification and biological testing of its major constituents revealed that the coumarin ostruthin (7) is the major antiproliferative substance. In summary, a new bioactivity of *P. ostruthium* rhizomes is described, and 7 has been identified as the responsible compound.

Coronary artery disease induced by atherosclerosis is one of the major causes of death in the Western world. One major pathological change associated with atherosclerosis is thickening of the arterial wall and progressive narrowing of the arterial lumen, leading to insufficient blood flow. Proliferation of vascular smooth muscle cells (VSMC) in the intima layer of the arterial wall is one of the major components of the narrowing process, besides ongoing inflammatory processes. There are already several pharmaceuticals used to combat pathological VSMC proliferation, but their action is not optimal due to long-term side effects. Therefore, there is still a need to identify new active compounds.

Natural products represent an excellent resource for the identification of new lead structures. Austria and its adjacent regions have a rich history in traditional folk medicine. In order to conserve this ethnopharmacological knowledge, the VOLKS MED database was created. Since inflammation is associated with very diverse pathological conditions, including atherosclerosis, plants used traditionally to treat inflammatory conditions were retrieved from the VOLKS MED database. In an ongoing screening program, extracts of these plants were tested for their ability to inhibit VSMC proliferation. This bioactivity screening procedure revealed an activity of the dichloromethane (CH$_2$Cl$_2$) extract from *Peucedanum ostruthium* (L.) WDJ Koch (Apiaceae) rhizomes. *Peucedanum* species, especially the rhizomes of *P. ostruthium*, have a long history in Austrian traditional medicine for the treatment of inflammatory diseases. Furthermore, an anti-inflammatory action of a *P. ostruthium* root extract has been demonstrated in vivo on oral administration to rats with carrageenan-induced rat paw edema.

To test whether the CH$_2$Cl$_2$ extract of *P. ostruthium* rhizomes concentration dependently inhibits serum (10%)-induced proliferation of rat aortic VSMC, this extract was applied at concentrations of 3 to 30 μg/mL (Figure 1), and resazurin conversion after 48 h was measured. Serum-stimulated VSMC were taken as positive control and serum-starved cells as negative control. Data quantification revealed that the CH$_2$Cl$_2$ extract of...
P. ostruthium rhizomes inhibits the proliferation of VSMC with an IC₅₀ value of 24 ± 14 μg/mL.

It has been reported previously that coumarins are major bioactive constituents of extracts from P. ostruthium rhizomes. Furthermore, in a parallel study, we have performed a comprehensive qualitative and quantitative analysis of the main coumarins in extracts from P. ostruthium rhizomes. High-performance liquid chromatography—diode-array detection—mass spectrometry (HPLC-DAD-MS) profiling of the CH₂Cl₂ extract investigated in this study (Figure 2) led to the identification of seven coumarins as major constituents (1–7). The total coumarin content in this CH₂Cl₂ extract was determined to be 78%, whereas the content per gram dry weight of plant material was 6%. Ostruthin (7) was quantified as the main coumarin (41% of the total coumarin content), followed by oxypeucedanin (2, 18%), ostruthol (3, 13%), imperatorin (4, 12%), isoimperatorin (6, 9%), oxypeucedanin hydrate (1, 6%), and osthole (5, 1%).

After the identification of the seven major coumarin constituents of the CH₂Cl₂ extract of P. ostruthium rhizomes, their individual antiproliferative activities in serum-stimulated VSMC were investigated. Compounds 1, 2, and 4–6 were obtained from commercial sources. Ostruthol (3) and ostruthin (7) were isolated by preparative HPLC, the identity confirmed by mass spectrometry and 1D and 2D NMR spectroscopy, and their purity was shown to be >98% by HPLC-DAD, HPLC-ELSD, and NMR. Compounds 1–7 were all applied at 30 μM, but only 7 exhibited significant antiproliferative activity (Figure 3). Since 7 was the main compound in the extract (Figure 2), as well as the most active among the seven major constituents (Figure 3), it is concluded that 7 is the major active compound in the CH₂Cl₂ extract of P. ostruthium rhizomes inhibiting VSMC proliferation. Furthermore, 7 constitutes around 32% of the total extract. The IC₅₀ value determined for the CH₂Cl₂ extract, i.e., 24 μg/mL, corresponds to about 26 μM of 7. Since 7 inhibited VSMC proliferation with an IC₅₀ of 11 ± 2 μM, its activity can entirely explain the inhibitory properties of the plant crude extract.

The resazurin assay method used is based on the metabolic conversion of resazurin to the fluorescent resoruin, which correlates to the cell number. To validate the potential of 7 to inhibit VSMC proliferation, the rate of de novo DNA synthesis via BrdU incorporation was measured (Figure 4). As expected, ostruthin concentration dependently inhibited the serum-induced DNA synthesis, with an IC₅₀ of 17 ± 6 μM. Furthermore, the IC₅₀ value at which the CH₂Cl₂ extract inhibited DNA synthesis was 18 ± 4 μg/mL. Since 18 μg/mL extract solution corresponds to 19 μM ostruthin, the DNA synthesis inhibitory properties of the plant crude extract can also be entirely explained by that single chemical constituent. No significant differences in the cell viability assessed by trypan blue exclusion test was detected in the presence of ostruthin in the used concentration range, with a cell viability of 98 ± 2% and 92 ± 6% for the untreated and ostruthin (30 μM)-treated cells, respectively (mean ± SD, n = 4, p > 0.05, Student’s t test).

An antiproliferative action of 7 in VSMC has not been reported before. However, one study examined an antiproliferative action (IC₅₀ of 13.6 ± 1.8 μM) of the structurally similar osthole (5) isolated from Angelica pubescens in the smooth muscle cell line A10. In contrast, when applied at 30 μM we did not observe any significant inhibitory action by osthole in our model system utilizing primary rat aorta VSMC (Figure 3). Since the structurally similar linear furanocoumarins (Figure 3) also do not have an effect on the proliferation of VSMC, it was concluded...
that the geranyl group at the C-6 position may be necessary for the inhibitory effect. To validate this specific effect of the geranyl group, the inhibitory potential for several further coumarins and furanocoumarins was tested, namely, auraptene, bergamottin, bergapten, bergaptol, heraclenin, isobergapten, isopimpinellin, scopoletin, and umbelliferone (Figures S1 and S2, Supporting Information), which all share some structural similarity to 7, but lack the C-6 geranyl group. Indeed, none of these compounds significantly inhibited VSMC proliferation at a concentration of 30 μM, underlining the specific action of 7, among the compounds tested.

### EXPERIMENTAL SECTION

**Reagents.** Cell culture reagents including Dulbecco’s modified Eagle’s medium (DMEM) without phenol red containing 4.5 g/L glucose, glutamine, calf serum, penicillin, and streptomycin were obtained from Lonza Group Ltd. (Basel, Switzerland). Trypsin was purchased from Invitrogen (Carlsbad, CA), and DMSO was from Fluka (Buchs, Switzerland). Acetonitrile, dichloromethane, ethyl acetate, hexane, and methanol (MeOH) were HPLC grade and purchased from VWR (Vienna, Austria). Heracleum (Carlsbad, CA), and DMSO was from Fluka (Buchs, Switzerland). The identification of the main coumarins in extracts from P. ostruthium rhizomes was recently described elsewhere. Briefly, analytical HPLC separation was performed on an Acclaim C18 reversed-phase column from Dionex (2.1 × 150 mm, 3 μm) at 38 °C and a flow rate of 0.5 mL/min with a water (A) and acetonitrile (B) gradient containing 0.01% acetic acid. The gradient was 25–37% B in 6 min, 37–45% B in 8 min, 45–65% B in 10 min, 65–95% B in 1 min, and isocratic at 95% B for 5 min. Tentative identification of the main coumarins was facilitated by HPLC-DAD-MS in an automated data-dependent acquisition mode. These analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). The identity of 1, 2, and 4–6 was confirmed by comparison of their retention times and UV spectra with authentic references. The main coumarins were quantified by a validated HPLC-DAD method at a detection wavelength of 310 nm with imipramine as external standard. A Shimadzu Prominence HPLC system was used for these analyses.

**Resazurin Conversion Assay.** The assay is based on the metabolic conversion of resazurin to fluorescent resorufin, which correlates with cell number. Cells were seeded in transparent 96-well plates (1 × 10^4 cells/well), serum-starved for 24 h, pretreated with the indicated concentrations of the respective samples or vehicle for 30 min, and subsequently stimulated for 48 h with calf serum (10%). Then, cells were washed with PBS and incubated in DMEM containing 10 μg/mL resazurin for 2 h. Samples were measured by monitoring the increase

![Figure 4. Ostruthin (7) inhibits serum-stimulated proliferation of VSMC in a concentration-dependent manner. Starved serum VSMC were pretreated with the indicated concentrations of 7 or DMSO (0.1%) for 30 min, BrdU (10 μM) was added, and cells were stimulated with 10% serum for 20 h. Incorporation of BrdU was measured at the end of the stimulation period. All values were normalized to the signal obtained from the unstimulated, vehicle (0.1% DMSO)-treated cells and therefore represent the fold activation of proliferation above the basal level (SEM, n = 3, *p < 0.05; ANOVA/Tukey).](image-url)
in fluorescence at a wavelength of 580 nm using an excitation wavelength of 535 nm in a 96-well plate reader (Tecan GENios Pro; Tecan Group Ltd., Männedorf, Switzerland).

**5-Bromo-2′-deoxyuridine Incorporation.** As a measurement of DNA synthesis, 5-bromo-2′-deoxyuridine (BrdU) incorporation was determined using a cell proliferation kit (Roche Diagnostics) as previously described. Briefly, VSMC were seeded into black (transparent flat bottom) 96-well plates (1 × 10^4 cells/well), serum-starved for 24 h, pretreated with test samples or vehicle (0.1% final concentration of DMSO in all samples) for 30 min, and subsequently stimulated with calf serum (10%). After 30 min, BrdU (10 μM) was added, and the cells were cultivated for another 20 h. Afterward, cells were fixed and stained with an enzyme-coupled anti-BrdU antibody according to the manufacturer’s instructions. Relative light units were determined using a 96-well plate reader (Tecan GENios Pro; Tecan Group Ltd., Männedorf, Switzerland).

**Trypan Blue Exclusion Test.** The trypan blue viability test is based on the principle that living cells possess intact cell membranes that are not permeable to the trypan blue dye, whereas dead cells have disrupted membrane integrity and in the presence of the dye are colored blue. To assess the cell viability, VSMC were seeded in six-well plates (1 × 10^5 cells/well), serum-starved for 24 h, pretreated with ostruthin for 30 min, and subsequently stimulated with calf serum (10%). The viability of the cells was assessed 24 h later by trypan blue exclusion utilizing an automated cell viability analyzer (ViCell, Beckman Coulter, Brea, CA).

**Statistical Analysis.** Data were evaluated as means ± standard deviation from at least three independent experiments. IC_{50} values were determined by nonlinear regression using Data Analysis Toolbox software (MDL Information Systems Inc., Nashville, TN). Statistical significance was determined by ANOVA using Tukey post hoc test, and the results with p < 0.05 were considered significant.

### ASSOCIATED CONTENT

**Supporting Information.** Results regarding the antiproliferative activity of additional structurally related coumarins and furanocoumarins are available free of charge via the Internet at http://pubs.acs.org.

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