Supporting Information

Polyacetylenes from Radix et Rhizoma Notopterygii Incisi with an Inhibitory Effect on Nitric Oxide Production *In Vitro*

Martina Blunder[1], Xin Liu[1], Olaf Kunert[2], Nora Anna Winkler[1], Andreas Schinkovitz[1], Corinna Schmiderer[3], Johannes Novak[3], Rudolf Bauer[1]

Affiliations

[1]Institute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Graz, Graz, Austria

[2]Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, University of Graz, Graz, Austria

[3]Institute of Applied Botany and Pharmacognosy, University of Veterinary Medicine, Vienna, Austria

Correspondence

Rudolf Bauer
Institute of Pharmaceutical Sciences
Department of Pharmacognosy
University of Graz
Universitaetsplatz 4
8010 Graz
Austria
Phone: +43 316 380 8700
Fax: +43 316 380 9860
rudolf.bauer@uni-graz.at; martina.blunder@uni-graz.at

Materials and Methods

Plant material and DNA-based confirmation of identity

The plant material of Radix et Rhizoma Notopterygii was purchased through Plantasia (batch number 770107, Austria, 2008). A voucher specimen (No. 650107) has been deposited at the Institute of Pharmaceutical Sciences – Department of Pharmacognosy (University of Graz, Austria).

To supplement the scarce number of published sequences of *Notopterygium* spp., leaf samples were obtained from RBGE herbarium specimen of *Notopterygium incisum* C.C. Ting ex H.T. Chang (syn. *Hansenia weberbaueriana* (Fedde ex H. Wolff) Pimenov & Kljuykov) (vouchers E00132748 and E00041974),), *Notopterygium franchetii* H. de Boissieu (syn. *Hansenia forbesii* (H. Boissieu) Pimenov & Kljuykov) (vouchers E00132729 and E00132739), and *Notopterygium forrestii* H. Wolff (syn. *Hansenia forrestii* (H. Wolff) Pimenov & Kljuykov) (voucher E00000110).

Genomic DNA was extracted from dried roots and rhizomes of the sample and leaf fragments of the herbarium specimen using a modified CTAB protocol.[1] The extraction mixture per sample consisted of 950 µL CTAB detergents, 0.95 µL β-mercaptoethanol, 4.1 µL proteinase K, 10 mg polyvinylpyrrolidone K30 (all reagents from Carl Roth, Karlsruhe, Germany), and 41 µL 10% sodium dodecyl sulphate (Merck, Vienna, Austria) per sample.

The internal transcribed spacer (ITS) region of nuclear DNA, including ITS1, ITS2 and the 5.8S gene, was amplified and sequenced with the primers ITS4 and ITS5 .[2] For a 15 µL PCR reaction, 1 µL of genomic DNA solution (1:50 dilution of the original DNA extract) was added to a master mix containing 1 × PCR buffer B, 2.5 mM $MgCl_2$, 133 µM dNTPs, 0.6 U HotFire Taq polymerase (all reagents from Solis BioDyne, Tartu, Estonia) and 0.6 µM forward and reverse primer (Invitrogen, Lofer, Austria). The PCR cycle profile included an initial denaturation step at 95 °C for 15 min, followed by 35 cycles at 95 °C for 45 s, at 55 °C for 45 s, and a final elongation step at 72 °C for 90 s. The PCR product was purified with the enzymes ExoI and SAP and was sequenced by an external company (Ibl, Gerasdorf, Austria).

The obtained sequence of the sample was aligned with published data (GenBank accession numbers EU236180.1, AY038209.1 and AY038208.1) and the new sequences obtained from the herbarium specimen (GenBank accession numbers JF694084 to JF694088) using MEGA4 [3].

Extraction and isolation

Isolation of compound 1-4:

2 kg of Rhizoma et Radix Notopterygii was ground and percolated with dichloromethane (DCM) (17.5 L). Altogether 250 g extract was obtained after evaporation of the solvent. 100 g of the crude extract were fractionated on a silica gel column (column diameter: 8,5 cm, h: 23.5 cm, Silicagel 60 (262 g) utilizing a hexane to EtOAc to MeOH gradient, with each collected subfraction having a volume of approx. 300-400 mL. Gradients and fractions were as follows:

A (Hex 120) A1-A8 (2400 mL), B (Hex 100/EtOAc 20) B1-B8 (2400 mL), C (Hex 80/EtOAc 40) C1-C3 (1200 mL), D (Hex 60/EtOAc 60) D1-D7 (2400 mL), E (Hex 40/EtOAc 80) E1-E7 (2400 mL), F (Hex 20/EtOAc 100) F1-F7 (2400 mL), G (EtOAc 120) G1-G7 (2400 mL), H (EtOAc 100/MeOH 20) H1-H7 (2400 mL), I (EtOAc 80/MeOH 40) I1 -I6 (2400 mL), J (EtOAc 60/MeOH 60) J1-J5 (2400 mL), K (MeOH 120) K1-7 (2400 mL).

All fractions were monitored using analytical TLC and HPLC and tested for their inhibition on NO production and most active ones showing an inhibition of more than 25% of control (at 10 µg/mL) were subjected to further isolation and purifications steps. Fractions C1, C2, D3 included main constituents of interest. After preliminary purification over RP-18 SPE, compounds 1-4 were isolated by semi-preparative HPLC on an RP-18 column (LiChrospher, 7 µm, 21x250 mm, Merck, Darmstadt, Germany) using an acetonitrile/water gradient (yields: 10.8 mg (**1**, isolated from fraction C1), 23 mg (**2**, isolated from fraction C2), 3.1 mg (**3**, isolated from fraction D3), 1.9 mg (**4**, isolated from fraction D3).

Isolation of compound 5-8:

Another 145 g of the DCM extract was partitioned twice between n-hexane and MeOH (1.5:1, v/v), to obtain hexane layer (39 g) and MeOH layer (104 g). MeOH layer was further partitioned twice between dichloromethane and 60% MeOH water solution (1:1, v/v) to obtain DCM layer (94 g) and aqueous MeOH layer (1.5 g).

The DCM layer was then fractionated by MCI CHP-20P resin, with the mobile phase being a gradient of MeOH-H_2O (40% to 100% MeOH), to afford 313 fractions. Fraction 1-10: 40 % MeOH (7 L); fraction 11-30: 50 % MeOH (14 L); fraction 31-96: 60 % MeOH (45 L); fraction 97-162: 65 % MeOH (45 L); fraction 163-213: 70 %

MeOH (30 L); fraction 214-230: 75 % MeOH (12 L); fraction 231-255: 80 % MeOH (18 L); fraction 256-312: 90 % MeOH (38 L); fraction 313: 100 % MeOH (5 L). To facilitate following bio-assay, 0.3% of each of these fractions were sampled and recombined according to their TLC profile to afford 10 pooled fractions, which was assayed together with the DCM layer. Pool 1: fraction 1-10; pool 2: fraction 11; pool 3: fraction 12; pool 4: fraction 13-28; Pool 5: fraction 29-49; pool 6: fraction 50-105; pool 7: fraction 106-166; pool 8: fraction 167-226; pool 9: fraction 227-312; pool 10: fraction 313.

Fractions 223 to 226 from MCI resin were combined (0.7 g), subjected to ODS C-18 (25-40 μm, 40×4.2 cm), eluted with MeOH/H_2O (85 %, v/v) solution to afford 13 sub-fractions (250 mL each). Sub-fraction 8 from ODS C-18 was further subjected to silica gel column, (15-40 μm, 28×3 cm), eluted with hexane/ethyl acetate (4.2:1, v/v), to afford series of sub-fractions (15 mL each), among which sub-fraction 13 afforded compound **5** (9.5 mg), sub-fractions 21-28 afforded compound **6** (11 mg).

Fractions 205 to 215 from MCI resin were combined (0.7 g), subjected to ODS C-18 (25-40 μm, 40×4.2 cm), eluted with MeOH/H_2O (85 %, v/v) solution to afford 12 sub-fractions (250 mL each). Sub-fractions 9 and 10 from ODS C-18 were combined, and subjected to silica gel column, (15-40 μm, 3×22 cm), eluted with hexane/ethyl acetate (8:1, v/v), to afford series of sub-fractions (15 mL each), among which sub-fraction 5 afforded compound **7** (10.5 mg). Sub-fractions 11 and 12 from ODS C-18 were combined and subjected to silica gel chromatography the same way, with sub-fraction 12 and 13 affording compound **8** (1.7 mg).

NMR experiments

^{1}H-, ^{13}C-, and 2D-NMR experiments (HSQC, HMBC, DQF-COSY) were performed on a Varian UnityInova spectrometer operating at a proton frequency of 600 MHz. Compounds were dissolved in $CDCl_3$ and spectra were recorded at 25 °C. TMS was used as internal standard. Experimental parameters were as published in Seebacher et al. [4]. Identification of the isolated compounds was accomplished by 2D-NMR and by comparison with literature data for 8-acetoxyfalcarinol (**1**) [5], falcarindiol (**2**), [6], 9-epoxyfalcarinol (**3**) [6-8], crithmumdiol (**4**) [9,10], (2*Z*,9*Z*)-heptadecadiene-4,6-diyn-1-ol (**5**) [11], (9*Z*)-heptadecene-4,6-diyn-1-ol (**6**) [12], falcarinol (panaxynol) (**7**) [13,14], and 4,5-dihydropanaxynol (**8**) [15].

Because the reported NMR data for compound **6** is limited, the full assignment of its ^{1}H and ^{13}C NMR are made:

^{1}H NMR (600 MHz, $CDCl_3$) d (ppm): 5.49 (1H, *m*, H-10), 5.37 (1H, *m*, H-9), 3.74 (2H, *t*, *J* = 9.2 Hz, H_2-1), 3.00 (2H, *d*, *J* = 10.4 Hz, H_2-8), 2.39 (2H, *t*, *J* = 10.4 Hz, H_2-3), 2.05 (2H, *m*, H_2-11), 1.77 (2H, *m*, H_2-2).
^{13}C NMR (150 MHz, $CDCl_3$) d (ppm): 132.84 (C-10), 122.63 (C-9), 76.97 (C-7), 75.89 (C-4), 65.92 (C-5), 65.04 (C-6), 61.59 (C-1), 31.98 (C-15), 31.13 (C-2), 29.42 (C-14), 29.34 (C-13), 29.31 (C-12), 27.32 (C-11), 22.80 (C-16), 17.74 (C-8), 15.90 (C-3), 14.24 (C-17).

Mass Spectrometry (GC-MS)

Additionally isolated compounds were subjected to GC-MS analysis in order to determine the molecular weight and confirm their structures, especially alkyl chain length. Mass spectra were recorded by an Agilent 7890A GC System connected to Agilent 5975C MSD operating at 70 eV, ion source temperature 230 °C and interface temperature 280 °C. A fused silica capillary column 5% phenylmethylsiloxane (HP-5MS 30 m x 250 µm x 0.25 µm, Agilent J & W, USA) was used. Injection volumes were 1µL and injector temperature was 240°C. The temperature program was as follows: 1 min at 100 °C, then to 300 °C at 8 °C/min, finally held at 300 °C for 20 min. The carrier gas was helium 5.6 at a flow rate 1.2 mL/min. Data acquisition was performed with Agilent GC/MSD ChemStation Version E.02.00 for the mass scan range 40–500u. Data are given as m/z.
Compound **1** 302.3 $[M]^+$; 285.2 $[M\text{-}H_2O]^+$, 241.1 $[M\text{-}OAc\text{-}H_2O]^+$,
Compound **2** 242.2 $[M\text{-}H_2O]^+$,
Compound **3** 276.1 $[M]^+$; 177.1; 135.1,
Compound **4** 262.1 $[M]^+$; 244.2 $[M - H_2O]^+$

Optical rotations

Optical rotations were measured with a Jasco P-2000 polarimeter.
Compound **1**: $[\alpha]_{20D}$: +152.9° (*c* 1.0, iPrOH)
Compound **2**: $[\alpha]_{20D}$: +247.0° (*c* 1.5, MeOH)
Compound **3**: $[\alpha]_{20D}$: +122.7° (*c* 0.02, MeOH)
Compound **4**: $[\alpha]_{20D}$: +51.3° (*c* 0.02, MeOH)

Compound **7**: $[\alpha]_{20D}$: -27.4° (*c* 0.1, MeOH)

Compound **8**: $[\alpha]_{20D}$: -19.4° (*c* 0.01, MeOH)

Preparation of the (*S*)- and (*R*)-MTPA ester of falcarindiol

2 mg falcarindiol and one granule of DMAP were dried together for 3 hours under vacuum with oil pump, and then dissolved in a mixture of 0.5 mL dichloromethane and 0.2 mL pyridine (both anhydrous). The system was added with 30 μL of *R*-(-)-α-methoxy-α- (trifluoromethyl) phenylacetyl chloride, and then stirred under nitrogen protection for 3 hours at room temperature, when TLC detection showed the reaction was complete. Afterwards the reaction mixture was dried under a nitrogen gas stream, and re-dissolved with small amount of dichloromethane, then introduced to a silica gel column (3 cm in length) made from glass pasteur pipette. Eluent of hexane-ethyl acetate (8:1 v/v, 4 mL) was subjected to further purification by semi-preparative RP-18 HPLC (10 × 250 mm), with the mobile phase being acetonitrile-water (95 % v/v, 3 ml/min). Retention time of target (*S*)- MTPA ester was 14.5 min.

(*R*)-MTPA ester was made with similar procedure from (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride.

Cell Culture and NO measurements

The bioassay for inhibition of NO production was carried out in a 24-well-plate format. RAW 264.7 mouse macrophages were cultivated at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (supplements: 10% (v/v) heat-inactivated foetal calf serum, 100 units/mL penicillin/streptomycin, 1.25 μg/mL amphotericin B). Cells (1×10^6 cells/mL) were seeded onto 24-well plates and left to rest for 24 hours. All reagents used for the cell culture were purchased from Invitrogen, if not stated otherwise. Lipopolysaccharides (LPS (Salmonella typhosa) from Sigma, 0.5 μg/mL) and interferon-gamma (IFNγ, mouse (mIFN) from Roche, 25 U/mL) were added into renewed culture medium (phenol red free DMEM) in presence or absence of the respective sample. After 16 hours of exposure, cell culture supernatants were centrifuged and NO release was quantified photometrically with a microplate reader as its stable product nitrite in the supernatants using the Griess assay and comparing with a sodium nitrite standard curve as described by Baer et al. [16] with slight modifications [17]. Aliquots of 100 μL were mixed with the same volume of Griess reagent (Sigma) and the absorbance at 535 nm was measured.

For the preliminary screening test, samples were dissolved in 10% DMSO in PBS to a final concentration of 100 µM. Samples showing moderate inhibitory activity were then subjected to further studies. Each experiment was performed at least eight times in independent experiments run in duplicate. Activity is referred to nitrite accumulation of cells treated with LPS/IFN-γ/DMSO (0.1%). The total solvent part never exceeded 0.1% and no effects were detected with solvent control only. Positive control for this test system was L-NMMA (purity ≥99%, Alexis) showing an IC_{50} value of 100 µM. Results are given as mean ±S.D.

Statistics

IC_{50} determinations were performed in at least six concentrations, each in at least three independent experiments, every time in duplicate. IC_{50} values were calculated with the SigmaPlot program package employing the 4-parameter logistic regression model.

References

1. Doyle J. DNA protocols for plants. In: Hewitt GM, Johnston AWB, Young JPW eds, Molecular Techniques in Taxonomy. First ed. Berlin: Springer Verlag; 1991:283-285
2. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ eds, PCR Protocols: A Guide to Methods and Applications. New York: Academic Press; 1990:315-322
3. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 2007;24:1596-1599
4. Seebacher W, Simic N, Weis R, Saf R, Kunert O. Complete assignments of ^{1}H and ^{13}C NMR resonances of oleanolic acid, 18-a-oleanolic acid, ursolic acid and their 11-oxo derivatives. Magn Reson Chem 2003;41:636-638
5. Dang NH, Zhang XF, Zheng MS, Son KH, Chang HW, Kim HP, Bae K, Kang SS. Inhibitory constituents against cyclooxygenases from *Aralia cordata* Thunb. Arch Pharmacal Res 2005;28:28-33
6. Eckenbach U, Lampman RL, Seigler DS, Ebinger J, Novak RJ. Mosquitocidal Activity of Acetylenic Compounds from *Cryptotaenia canadensis*. J Chem Ecol 1999;25:1885-1893
7. Deng S, Chen S-N, Yao P, Nikolic D, van Breemen RB, Bolton JL, Fong HHS, Farnsworth NR, Pauli GF. Serotonergic Activity-Guided Phytochemical Investigation of the Roots of *Angelica sinensis*. J Nat Prod 2006;69:536-541
8. Deng S, Wang Y, Inui T, Chen SN, Farnsworth NR, Cho S, Franzblau SG, Pauli GF. Anti-TB polyynes from the roots of *Angelica sinensis*. Phytotherapy Research 2008;22:878-882
9. Ruberto G, Vincenzo A. Crithmumdiol: A new C17-acetylene derivative from *Crithmum maritimum*. Planta Med 1999;65:681-682

10. Yamazoe S, Hasegawa K, Shigemori H. Growth inhibitory indole acetic acid polyacetylenic ester from Japanese ivy (*Hedera rhombea* Bean). Phytochemistry 2007;68:1706-1711
11. Huang H-Q, Zhang X, Shen Y-H, Su J, Liu X-H, Tian J-M, Lin S, Shan L, Zhang W-D. Polyacetylenes from *Bupleurum longiradiatum*. J Nat Prod 2009;72:2153-2157
12. Bohlmann F, Miethe R. Polyacetylenverbindungen, 196. Synthesen von Acetylenverbindungen aus *Oenanthe crocata* L. und *Pittosporum buchanani* Hook. Chem Ber 1971;104:1362-1374
13. Mayer SF, Steinreiber A, Orru RVA, Faber K. Chemoenzymatic Asymmetric Total Syntheses of Antitumor Agents (3R,9R,10R)- and (3S,9R,10R)-Panaxytriol and (R)- and (S)-Falcarinol from *Panax ginseng* Using an Enantioconvergent Enzyme-Triggered Cascade Reaction. The Journal of Organic Chemistry 2002;67:9115-9121
14. Kobayashi M, Mahmud T, Umezome T, Wang W, Murakami N, Kitagawa I. The absolute stereostructures of the polyacetylenic constituents of Ginseng Radix Rubra. Tetrahedron 1997;53:15691-15700
15. Hirakura K, Morita M, Nakajima K, Ikeya Y, Mitsuhashi H. Three acetylenic compounds from roots of *Panax ginseng*. Phytochemistry 1992;31:899-903
16. Baer HP, Schmidt K, Mayer B, Kukovetz WR. Pentamidine does not interfere with nitrite formation in activated RAW 264.7 macrophages but inhibits constitutive brain nitric oxide synthase. Life Sci 1995;57:1973-1980
17. Konkimalla VB, Blunder M, Bauer R, Efferth T. Inhibition of inducible nitric oxide synthase by bis(helenalinyl)glutarate in RAW264.7 macrophages. Biochem Pharmacol 2010;79:1573-1580