

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

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## Abstract

*Notopterygium* roots (Qiang Huo) have been used in traditional Chinese medicine for treating colds, inflammatory diseases like rheumatoid arthritis, and as an analgesic. The anti-inflammatory activity of the roots of *Notopterygium incisum* has been evaluated by testing the inhibitory activity on nitric oxide production by inducible nitric oxide synthase. The apparent authenticity of the sample was checked by DNA sequence comparison. Using activity-guided isolation, different compounds were isolated and structurally characterized by means of NMR and mass spectrometry. Eight polyacetylenes could be identified and were tested on their inhibitory activity on nitric oxide production in RAW 264.7 mouse macrophages using the Griess assay. Different 3-hydroxy allyl polyacetylenes exhibited significant activity (IC<sub>50</sub>: 8-acetoxyfaltarinol, 20.1 μM; faltarindiol, 9.2 μM; 9-epoxyfaltarindiol, 8.8 μM; and crithmumdiol, 23.6 μM).

## Key words

*Notopterygium incisum* · Apiaceae · polyacetylenes · nitric oxide · iNOS · Griess assay · DNA sequencing

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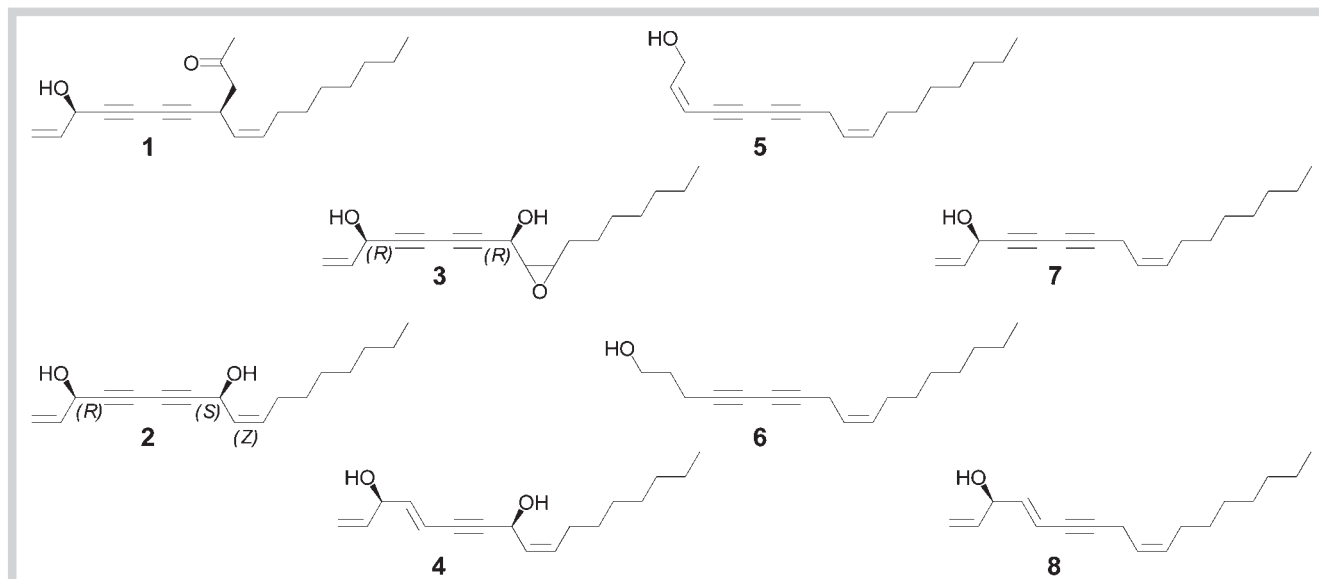
The dried rhizomes and roots of both *Notopterygium incisum* C. C. Ting ex H. T. Chang and *N. forbesii* Boiss (Apiaceae or Umbelliferae) are the source of *Rhizoma et Radix Notopterygii* (Qiang Huo) listed in the Chinese Pharmacopeia [1]. With a history of more than two thousand years of usage, these two Chinese herbs were not differentiated from *Angelica pubescens* (Apiaceae) until about one thousand years ago [2]. As a traditional Chinese medicine (TCM), it is widely used for treating colds, inflammatory diseases like rheumatoid arthritis, and as an analgesic [3]. Pharmacological research revealed that one of the major coumarins, notopteronol, has analgesic and anti-inflammatory activity [4]. We previously reported phenethyl ferulate and faltarindiol from the *n*-hexane extracts as having inhibitory effects on 5-lipoxygenase and cyclooxygenase [5]. Phenethyl ferulate was later reported as being able to attenuate proinflammatory responses to lipopolysaccharide in RAW 264.7 macrophages, while faltarindiol was found to induce immunosuppressive effects *in vitro* by inhibiting

dendritic cell maturation [6, 7]. An aqueous extract was found to inhibit picryl chloride-induced contact sensitivity through downregulating matrix metalloproteinase activities [3].

Nitric oxide (NO) released by the inducible NO synthase (iNOS) plays a vital role in host defense as it possesses cytotoxic effects on bacterial, virus, and tumor cells. On the other hand, in high concentrations, it can lead to tissue damage. Therefore, it plays an important role in inflammatory processes and in the pathophysiology of many diseases. Hence, inhibition of NO production in biological systems turned out to be an interesting target in the field of anti-inflammatory drug research [8, 9]. We now report on the activity-guided isolation elucidating the compounds with inhibitory effects on NO production by iNOS.

In our previous screening of medicinal plants that have been traditionally used in China to treat inflammatory diseases, dichloromethane and methanol extracts of 79 Chinese medicinal herbs were investigated for their inhibitory activity on NO production using the murine macrophage cell line RAW 264.7. Cytotoxic activity of all extracts was checked by determining the cell viability in two different cell lines at each tested concentration. All cytotoxic extracts were excluded from further investigations. *Radix Notopterygii* (Qiang Huo) turned out as a promising hit in this screening, since bioassay results revealed that the DCM extract showed significant inhibitory activity on NO production at 10 μg/mL (18% of control). Unambiguous identification of plant material used in scientific research is an important first step, but it is sometimes difficult to assess by morphological and phytochemical analysis, especially in cases where the plant material has been processed. Therefore, DNA-based methods for identification have become a valuable supplementation to authenticate plant material [10, 11]. Genomic DNA was extracted from dried roots and rhizomes of the sample and leaf fragments of the herbarium specimen using a modified CTAB protocol [12]. Internal transcribed spacer (ITS) of herbarium specimens of *Notopterygium* spp. was sequenced to improve the reliability of a DNA-based identification approach. 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 were obtained from the herbarium specimen (GenBank accession numbers JF694084 to JF694088) using MEGA4 [13]. By comparison of ITS with published and newly sequenced references, the sample used in this investigation could be identified unambiguously as *N. incisum*. Further details can be found as Supporting Information.

The dichloromethane extract of *N. incisum* roots and rhizomes, which has shown inhibitory effects on NO production in RAW 264.7 macrophages, was subjected to activity-guided isolation, with successive chromatographic methods like silica gel fractionation, SPE (RP-18)/ODS medium pressure column fractionation, prep/semiprep HPLC, etc. Eight compounds were obtained and through comparing their NMR data with the literature, their structures (with relative configuration) were identified as 8-acetoxyfaltarinol (**1**) [14], faltarindiol (**2**) [15, 16], 9-epoxyfaltarindiol (**3**) [15, 17–19], crithmumdiol (**4**) [20, 21], (2*Z*,9*Z*)-heptadecadiene-4,6-diyn-1-ol (**5**) [22], (9*Z*)-heptadecene-4,6-diyn-1-ol (**6**) [23], faltarinol (panaxynol) (**7**) [24–26], and 4,5-dihydropanaxynol (**8**) [27, 28] (● Fig. 1). Polyacetylene derivatives **3**, **4**, **6**, and **8** were isolated from *N. incisum* for the first time. The purity of the isolated compounds was over 95% for **1–4**, **7**, and **8** and above 90% for **5** and **6**, determined by HPLC profile and <sup>1</sup>H-NMR analysis. Additionally, all isolated compounds were subjected to



**Fig. 1** Polyacetylenes from *Notopterygium incisum*.

$\Delta\delta = \delta_{S\text{-ester}} - \delta_{R\text{-ester}}$ (Hz)						Carbinol configuration		
H-1E	H-1Z	H-2	H-9	H-10	H-11	H-12	C-3	C-8
48	42	60	-60	-30	-12	-6	R	S

**Table 1** Stereochemical analysis of faltarindiol with (S)- and (R)- $\alpha$ -methoxy- $\alpha$ -trifluoromethylphenylacetate esters.

GC-MS analysis in order to determine the molecular weight and confirm their structures, especially the alkyl chain length. Detailed information about the isolation and structure elucidation are given as Supporting Information.

The absolute configuration of compounds **2**, **3**, **4**, **7**, and **8** were determined by comparing their optical rotation with references [16, 17, 20, 24, 28]. To further determine the absolute configuration of faltarindiol (**2**) from *Notopterygium*, (S)- and (R)- $\alpha$ -methoxy- $\alpha$ -trifluoromethylphenylacetate (MTPA Mosher), esters were synthesized using a reported method [29, 30]. Through comparing chemical shifts of two sets of protons in the nearby respective acylated chiral centers (see **Table 1**), its absolute configuration was determined as 3R, 8S. This is consistent with that of faltarindiol reported from *Daucus carota*, another species of the Umbelliferae family [25] (**Fig. 1**).

The isolated compounds were assayed for inhibition of NO production by iNOS in RAW 264.7 macrophages. Some of the polyacetylenes exhibited remarkable activities with  $IC_{50}$  values of 10 to 30  $\mu$ M (**Table 2**).

Compounds **1–8** possess the same chain length of C 17 but differ in their number of double or triple bonds and substitution pattern at positions 1, 3, and 8. Comparing the activity of **2** and **4**, which possess the same substitution but differ in the number of triple bonds, it is obvious that the triple bond at position 4 is important for activity, indicating that the linear arrangement enables a better interaction of the hydroxyl moieties. The results also revealed that 1-hydroxy-polyacetylenes (like **5** and **6**) showed no relevant activity. In contrast, 3-hydroxy allyl polyacetylenes (like **2** and **3**) show significant inhibitory potential on NO production, moderated by a substitution at position 8 (like **1**). The findings of the importance of the 4-hydroxyl-allyl moiety are in

**Table 2** Inhibitory activity of the polyacetylenes from *Notopterygium incisum* on NO production of iNOS. Activity is referred to nitrite accumulation of cells treated with lipopolysaccharides/interferon  $\gamma$ /DMSO (0.1%).  $IC_{50}$  determinations were performed in at least six concentrations, each in at least three independent experiments, each time in duplicate.  $IC_{50}$  values were calculated with the SigmaPlot program package employing the 4-parameter logistic regression model. The data shown are means  $\pm$  SD.

Compound	$IC_{50} \pm SD$ ( $\mu$ M)
8-Acetoxyfaltarindiol ( <b>1</b> )	20.1 $\pm$ 3.7
Faltarindiol ( <b>2</b> )	9.2 $\pm$ 4.6
9-Epoxyfaltarindiol ( <b>3</b> )	8.7 $\pm$ 2.5
Crithmundiol ( <b>4</b> )	23.6 $\pm$ 4.9
(2Z,9Z)-heptadecadiene-4,6-diyne-1-ol ( <b>5</b> )	> 100
(9Z)-heptadecene-4,6-diyne-1-ol ( <b>6</b> )	> 100
Faltarindiol (panaxynol) ( <b>7</b> )	25.3 $\pm$ 1.8
4,5-Dihydropanaxynol ( <b>8</b> )	62.5 $\pm$ 1.3

good agreement with previous studies of faltarindiol [31]. That study proved a reduction of IKK and JAK, giving rise to nuclear blockage of NF- $\kappa$ B and Stat1 resulting in a reduced induction of iNOS. Therefore, we conclude that polyacetylenes must contribute to the anti-inflammatory activity of *N. incisum* through the inhibition of NO production.

## Materials and Methods

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**Cell culture and nitric oxide measurements:** Cells were stimulated with lipopolysaccharides (LPS; Sigma) and interferon  $\gamma$  (IFN $\gamma$ ; Roche) for induction of iNOS gene expression. The effects on NO

production were determined by photometrical quantification of nitrite accumulation in cell culture supernatants using the Griess assay (Griess reagent from Sigma) compared with a sodium nitrite standard curve after 16 hours of incubation with the respective sample as described by Baer et al. [32], with slight modifications [33]. Activity is referred to nitrite accumulation of cells treated with LPS/IFN- $\gamma$ /DMSO (final concentration of 0.1% DMSO served as the solvent control). L-NMMA (N<sup>l</sup>-monomethyl-L-arginine; purity  $\geq$  99%; Alexis) is a known, relatively nonselective inhibitor of all NOS isoforms. It is the archetypal NOS inhibitor to which other inhibitors are most often compared. Therefore, it has been used as a positive control in the described assay. Further information about the bioassay and statistics are given as Supporting Information.

### Supporting information

Detailed information about plant material and its DNA-based confirmation of identity, extraction, isolation, and structure elucidation of the polyacetylenes by means of multidimensional NMR spectroscopy and mass spectrometry (GC-MS), as well as the preparation of the (S)- and (R)-MTPA esters of faltarindiol can be found as Supporting Information.

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### Conflict of Interest

The authors declare no conflict of interest.

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