3,5-Nonadiyne Isolated from the Rhizome of *Cachrys ferulacea* Inhibits Endogenous Nitric Oxide Release by Rat Peritoneal Macrophages

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3,5-Nonadiyne, *in vitro*, selectively inhibits endogenous nitric oxide release (IC_{50}=6.7±0.8 μM) by rat peritoneal macrophages at doses that do not inhibit T cell proliferation. 3,5-Nonadiyne was isolated from root essential oil of *Cachrys ferulacea* (L.) CALESTANI, synonym *Prangos ferulacea* (L.) LINDLEY, obtained by hydrodistillation and spectrometricaly identified unambiguously.

Key words 3,5-nonadiyne; *Cachrys ferulacea*; *Prangos ferulacea*; endogenous nitric oxide; inhibition

Cachrys ferulacea (L.) CALESTANI, synonym *Prangos ferulacea* (L.) LINDLEY Family Apiaceae, is a plant widely distributed in the Mediterranean region,[1] as well as in the Caucasus, in Turkey, Iraq, Iran, and India. In Serbia, this taxon is critically endangered.[2] Plant leaves are used in the Caucasus as salad and for therapy of some digestive disorders. *Cachrys* species have been used in Turkish folk medicine as a tonic and antiflatulent, for treatment of intestinal worms and wounds, and to stop external bleeding.[3] Roots of *Cachrys* species are used in Turkey as an aphrodisiac.[3] Manunta,[4] suggested that *C. ferulacea* is botanically identical to the plant on coins of the old Greek colony Cyrene (Kyrene), nowadays Shahhat in Libya, north Africa, which is well known under name Silphion or Latinized Silphium, indicating antique interest. The plant is mainly used as fodder on arid pastures and transfers a pleasant smell to milk and derivatives. In Serbia and surrounding areas, preparations from the plant root are used as herbal folk medicine for curing intestinal wounds and especially hemorrhoids.

The plant seeds were analyzed as a possible source of industrial oil and found to contain high amounts of petroselinic acid.[5] Numerous furocoumarins were isolated from the plant.[6] Essential oil was analyzed several times by Russian,[7] Turkish,[8] Iranian,[9] and Italian,[10] research groups, but no acetylenic compounds were mentioned. More so, Bohlmann *et al.*[11] reported the absence of acetylenic compounds in the plant, based on UV spectra of the extract, which cannot detect simple diyne chromophore.

Nitric oxide (NO) is a gas produced by nitric oxide synthase (NOS), an enzyme that has three isoforms and acts as regulator of numerous and complex functions of various vital processes in cells and organs. The best known is regulation of vasodilatation and inflammation. Rat macrophages are potent producers of NO whether unstimulated or following stimulation with lipopolysaccharide (LPS) and thus are suitable for *in vitro* studies on NO release. Mitogen lectin-stimulated rat T lymphocyte proliferation is often employed for evaluating the immunomodulatory activity of various substances. It is comparable to the human lymphocyte proliferation test and is used for immunotoxic studies.

Results and Discussion

Hydrodistillation of essential oil from the roots gave a yield of 0.4% and the oil was immediately analyzed by GC and GC/MS. The main component (85% by GC-FID) had retention between that of γ-terpinene, reported as one of main components,[10] and terpinolene on an SE-54-type column. Due to the fact that both compounds were identified by the AMDIS program,[12] but at a trace level, we isolated the compound using preparative GC, and recorded >99.5% GC purity. Spectral data of isolated compound are in agreement with data for 3,5-nonadiyne from the roots of *Selinum tenuifolium*.[13] This is the second report on the occurrence of 3,5-nonadiyne in nature. Our suggestion is that, up to now, this substance has not been reported as a constituent of the *C. ferulacea* essential oil, due to the lack of its spectrum in mass spectral databases and thus it can be missed in analysis.

The biologically relevant activity of 3,5-nonadiyne was evaluated by measuring its effect on macrophage NO production and T cell proliferation. Concentration-dependent inhibition of endogenous NO production revealed an inhibitory concentration (IC_{50}) of 6.7±0.8 μM (Fig. 1). For comparison, amino guanidine (AG), a well known NOS inhibitor, was employed and showed an value IC_{50} of 236±13 μM similar to the reported value,[14] of 255 μM. LPS stimulated production of NO was inhibited slightly in 1000 fold higher concentration.

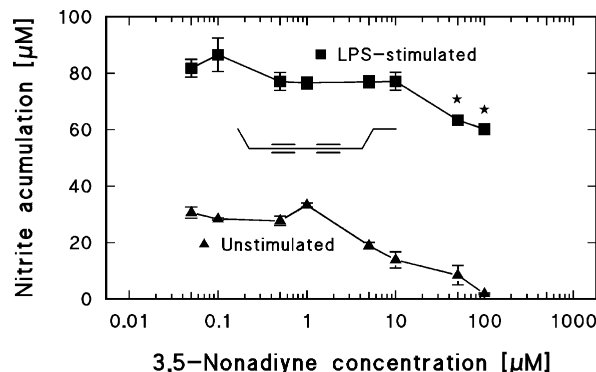

Fig. 1. Accumulation of Nitrites by Unstimulated (Triangles) and LPS (100 ng/ml) Stimulated (Solid Squares) Rat Peritoneal Macrophages in the Presence of Various Concentrations of 3,5-Nonadiyne (10^{-2}—10^{2} μM)

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Elimination of basal emission of NO by 3,5-nonadiyne does not interfere with LPS-stimulated NO production in macrophages. This rather unusual behavior may be explained by the highly lipophilic nature of the compound. As such, the probability of interaction with water-soluble iNOS is very low.

Mitogen lectin-stimulated rat T lymphocyte proliferation was employed for evaluating the immunotoxic activity of 3,5-Nonadiyne. The compound did not inhibit rat T lymphocyte proliferation, indicating low toxicity.

Experimental

General Procedures NMR, Varian Gemini 2000 ^{1}H-NMR (200 MHz, $CDCl_3$, TMS); ^{13}C-NMR (50 MHz, $CDCl_3$, TMS); IR, Perkin Elmer FTIR 1725X, neat liquid; MS, Finnigan-MAT 8230 BE geometry, resolution 1000, EI-CI source at 200 °C. EI 70 eV 0.5 mA; CI, 1 mtorr of isobutane 150 eV 0.2 mA. A GC/MS Finnigan Ion Trap Detector ITD-705 was used with a Varian 3400 GC equipped with a split/splitless injector (1 : 20) operated at 266 °C. Column, Supelco PTE-5 30 m, 0.25 mm i.d., 0.25 mm film, inserted directly in the ion trap *via* a transfer line at 240 °C. Carrier gas, hydrogen, 1 ml/min, measured at 210 °C. Ion manifold and exit nozzle temperatures of 240 °C were used. Column temperature was linearly programmed from 60 °C to 285 °C at 4.3 °C/min. Scan range was 39—333 Da, 1 scan/s (5 microscans were averaged). Tuning parameters were: 10, 50, 100, 100; B 9000; AGC on, background mass 33. Version 3 of ITDS software was used. GC-FID analysis was done on a Varian 3400 GC equipped with split/splitless injector (1 : 99) operated at 266 °C. Column J&W Scientific DB-5 30 m, 0.25 mm i.d., 0.25 μm film. Carrier gas, hydrogen, 1 ml/min, measured at 210 °C. Column temperature was linearly programmed from 60 °C to 290 °C at 4.3 °C/min. Detector temperature was 310 °C. Preparative GC was done on a Varian 920 GC with TC detector at 200 °C. A quartz insert in the injector operated at 200 °C. Glass column, 2 m, 6 mm i.d., with 8% OV-101 or 12% Carbowax-20M on Chromosorb G-hp 80—100 mesh. Carrier gas, hydrogen, 80 ml/min, measured at 111 °C. Isothermal operation at 111 °C.

A library search and mass spectral deconvolution and extraction were performed using AMDIS[12] (Automated Mass Spectral Deconvolution and Identification System) software version 2.0β or v2.1, using RI calibration data analysis parameters with "strong" level and 7% penalty for compounds without a retention index. The search was performed against our own library, which includes 4951 spectra.

Isolation Procedure Dried, powdered root (100 g) with 233 g of distilled water was subjected to distillation in a Clavenger apparatus and 0.4 ml (0.33 g) of oil was separated from water. The oil was subjected to preparative GC and 200 mg of substance was isolated.

3,5-Nonadiyne (**1**): Colorless oil, ^{13}C-NMR (50 MHz, $CDCl_3$, TMS) δ 78.33 (C-6); δ 77.26 (C-3); δ 65.3 C-5 (C-4); δ 64.6 C-4 (C-5); δ 21.8 CH_2-2 (C-7); δ 21.0 CH_2-7 (C-2); δ 13.3 (2×CH_3-1 & 9); δ 12.8 CH_2-8; ^{1}H-NMR δ 0.987 (t, 3H, J=7.3 Hz, CH_3-9); δ 1.152 (t, 3H, J=7.4 Hz, CH_3-1); δ 1.546 (sx, 2H, J=>7.3 Hz, CH_2-8); δ 2.227 (tt, 2H, $J1$=7.3 Hz, $J2$=1.1 Hz, CH_2-7); δ 2.257 (tq, 2H, $J1$=>7.4 Hz, $J2$=1.1 Hz, CH_2-2); IR 2160, 2250 cm^{-1} (ν C≡C); UV λ_{max} (CH_3CN) nm (ε) 194 2.57; 211 0.109; 226 0.1748; 239 0.1745; 253 0.116; MS: (m/z %) 120 73 (M^+); 119 17; 117 10; 115 8; 105 76; 103 50; 91 46; 79 100; 78 6; 77 5; CI-MS: (m/z %) 121 40 $(M+H)^+$; 177 100 $(M+\text{isobutene}+H)^+$; 241 37 $(2M+H)^+$; RI_{pte5}=1076 (retention index on Supelco PTE-5 fused silica capillary column). Identification was done using MS, CIMS, IR, UV, ^{1}H-NMR, ^{13}C-NMR, DEPT, 2D-NMR HH-COSY, and HETCOR. Copies of the original spectra are obtainable from the author for correspondence.

Plant Material Whole plant samples including roots were collected in August 2000 in Crna Gora (Montenegro) near Kolašin, locality "Bljuštuvni do" at an altitude of 1808 m and voucher sample BEOU-13521 was deposited at herbarium of Belgrade University Botanical Garden. Identification of plant material was done by Prof. Vladimir Stevanović and Asst. Prof. Dmitar Lakušić.

Macrophage Preparation and Culture Three-month-old male inbred Albino Oxford (AO) rats (Breeding Facility of the Institute for Biological Research, Belgrade) were used in experiments in adherence with the NIH Guidelines for the Use of Experimental Animals, with the permission of the Ethical Committee of the Institute. Macrophages were obtained by peritoneal lavage with culture medium RPMI-1640 (Gibco, BRL) supplemented with 5% (v/v) fetal calf serum and 5 U/ml heparin. Cell were seeded in wells of 96-well microtiter plates at 105 cells/well and maintained in an incubator (Hereaus, Germany) in a humidified atmosphere at 37 °C, 5% CO_2, for 48 h. LPS (100 ng/ml) was added for LPS-stimulated experiments. 3,5-Nonadiyne and AG were dissolved as stock solution in 20% dimethyl sulfoxide (DMSO) and diluted further with RPMI-1640 culture medium. The highest concentration of DMSO in culture was 0.017%. Control tests were carried out with the respective concentrations of DMSO instead of sample solution. The level of nitrites in the incubation medium was zero within experimental error.

T Cell Proliferation Assay Thymic T lymphocytes (thymocytes) were isolated under sterile conditions and resuspended in culture medium RPMI-1640 supplemented with 5% (v/v) fetal calf serum. Cells (5×10^5/well) were cultured for 72 h (37 °C, humidified atmosphere with 5% CO_2) in 96-well microplates in the presence of concanavalin A (2.5 mg/ml culture) and various concentrations (10^{-8}—10^{-4} M) of substance. The level of DNA synthesis was evaluated *via* incorporation of ^{3}H-thymidine present during the last 6—8 h of culture. Results are expressed as counts per minute.

Cell Viability Determination A quantitative colorimetric assay in which MTT (three-4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazolium bromide) is metabolically reduced to the colored end product formazan, was employed to estimate cell survival of macrophages and lymphocytes. Briefly, 10 ml of MTT solution (5 mg/ml) was added to each well after 48 h (for macrophages) or 72 h (for lymphocytes) of culture and cultures were incubated for an additional 3 h at 37 °C in an atmosphere of 5% CO_2/95% humidified air. Formazan produced by cells was dissolved by overnight incubation in SDS-Cl (10% SDS in 0.01 N HCl) and absorbance was measured at dual wavelength of 570/650 nm with an ELISA 96-well plate reader.

Griess Assay for Nitrite Determination Cells were seeded in 96-well plates (10^5/200 μl) and cultured for 2 d without or with LPS (100 ng/ml). Nitrite concentrations were determined in the Griess reaction. In brief, 50 μl of cell culture supernatant was removed and combined with 50 μl of a mixture of 1% sulfanilmide in 5% H_3PO_4 and 0.1% naphtyl-ethylenediamine dihydrochloride in H_2O in a 96-well microtiter plate. Absorbance was measuerd at 570 nm (reference wavelength 650 nm) with an ELISA microplate reader (Behring, Germany). The level of nitrites in incubation medium was zero within experimental error. Nitrite concentrations in macrophage culture supernatants were determined *via* the sodium nitrite standard curve.

Results are expressed as mean±S.E.M. for independent quadruplicate cultures of macrophages or T lymphocytes. Statistical significance was determined by the Student's t-test and p values less than 0.05 were considered significant. All statistical calculations were done with program "FigP" from FigP Software Corporation, Durham, NC, U.S.A.

Acknowledgments The authors are grateful to Daniel Vincek from Montenegro Botanical Garden for help in collecting samples, and to the Ministry for Science, Technology and Development of Serbia (projects No. 1755 and 1568) for financial support.

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