Dimeric Cyanobacterial Cyclopent-4-ene-1,3-dione as Selective Inhibitor of Gram-positive Bacteria Growth: Bio-production Approach and Preparative Isolation by HPCCC

José Cheel[a], Kateřina Bogdanová[b], Svetlana Ignatova[c], Ian Garrard[c], Peter Hewitson[c], Milan Kolář[b], Jiří Kopecký[a], Pavel Hrouzek[a,*], Jan Vacek[d,*]

[a] Department of Phototrophic Microorganisms-ALGATECH, Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovicky mlyn, Trebon 379 81, Czech Republic

[b] Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic

[c] Advanced Bioprocessing Centre, Institute of Environment, Health & Societies, Brunel University London, Kingston Lane, Uxbridge, Middlesex UB8 3PH, United Kingdom

[d] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic

*corresponding authors:

P. Hrouzek (bio-production and isolation procedures) hrouzekp@gmail.com

J. Vacek (chemical analysis and biological testing) jan.vacek@upol.cz

Abstract

The need for new antimicrobial agents is greater than ever because of the emergence of multidrug resistance in common pathogens and incidence of new infections. Cyclopent-4-ene-1,3-diones (CPDs) have been reported as a new class of compounds with promising antimicrobial and antifungal properties. Herein we report the selective antibiotic properties of nostotrebin 6, a phenolic CPD produced biotechnologically by the culture of cyanobacterium *Nostoc* sp. str. Lukešová 27/97. High performance countercurrent chromatography (HPCCC) combined with gel permeation chromatography (GPC) was used for the isolation of nostotrebin 6 with a relatively high 0.53±0.1% yield (calculated from dried biomass) and final purity higher than 96 %. Nostotrebin 6 was tested for its antimicrobial and antifungal activities by using standard micro-dilution method, and the results were expressed as minimal inhibitory concentrations (MICs). Nostotrebin 6 unequivocally inhibited the growth of Gram-positive reference (*Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 4223 and Staphylococcus aureus CCM 3953) and multidrug-resistant (*Staphylococcus haemolyticus* A/16568, *Staphylococcus aureus* MRSA 4591 and *Enterococcus faecium* VanA 419/ana) strains. Its strongest effect was exerted against the Gram-positive bacteria with MICs ranging between 6.25 and 15.6 μg/mL. There was no effect on Gram-negative strains tested and yeasts. Our results suggest that nostotrebin 6 could serve as basic nucleus for further design of novel antibiotic agents and demonstrate that the bio-production approach based on HPCCC/GPC isolation endpoint is an efficient methodology for obtaining nostotrebin 6 in multi-gram scale. Furthermore, the presented isolation method can be easily up-scaled to process kilograms of the cyanobacterial biomass.

Keywords: phenolic cyclopentenedione, natural antibiotic, cyanobacteria, *Nostoc* sp., biomass production, high performance countercurrent chromatography

1. Introduction

Cyclopent-4-ene-1,3-diones (cyclopentenediones, CPDs) represent a relatively newly defined group of compounds derived from the natural constituents of plants, fungi and bacteria [1]. This group includes more than 100 chemical individuals, most of them prepared synthetically. The synthesis of novel CPDs is often devoted to modification of natural CPDs resulting in structure-activity relationship (SAR) studies and subsequent discoveries of novel bioactives with applicability in chemical technologies, biotechnology and biomedicine fields. Among well studied CPD representatives are lucidone and linderone with anti-inflammatory activities [2-5], cytostatic compound TX-1123 [6], involuton constituent of fungi *Paxillus involutus* [7-9], madindolines [10-13] and nostotrebin 6 [14-16] attributed to enzyme inhibitory effects. The novel synthetic approaches are primarily focused on total synthesis of above-mentioned CPDs and also on strategies for development of structurally simplified CPDs with biological, especially antibacterial and antifungal activities. This was exampled in the case of antibacterial coruscanones and their analogues [17-20]. For CPDs, with complex chemical structure, *e.g.* nostotrebin 6, the biotechnological production is preferred for preparation of a suitable amount of the pure compound for biological testing or consequent synthetic modifications or technological applications.

Nostotrebin 6, *i.e.* 2,2'-bis[4,5-bis(4-hydroxybenzyl)-2-(4-hydroxyphenyl)cyclopent-4-ene-1,3-dione], is a secondary phenolic metabolite produced by cyanobacterium *Nostoc* sp. strain Lukešová 27/97 [16], see Fig. 1. Nostotrebin 6 is an effective inhibitor of acetylcholinesterase and butyrylcholinesterase [16], and its cytotoxic and pro-apoptotic properties have been previously reported [15]. Nostotrebin 6 has been obtained from the cyanobacterial biomass by using conventional separation methods, including solid-phase extraction and two HPLC steps [16]. However, this multi-step procedure involved time and solvent consuming operations plus a risk of a loss of compound due to the inevitable

adsorption effects. High performance countercurrent chromatography (HPCCC) has been recently used, at a semi-preparative scale, for isolating nostotrebin 6 from *Nostoc* sp. [21].

HPCCC is a liquid-liquid partition chromatographic technique, which uses a liquid stationary phase. Consequently, the method eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive loss of sample, deactivation and contamination. HPCCC is cost effective, produces high sample recoveries and permits the direct introduction of crude extracts into the column without additional sample pre-treatement [22]. In addition, there is potential for reduced environmental impact of the purification as the amount of solvents used is low in comparison to classical column chromatography. This technique has been successfully applied to the separation of a number of drugs, toxins and natural products [23-26]. Concurrently, the use of HPCCC in combination with gel permeation chromatography (GPC) on Sephadex LH-20 has been advantageously used for the isolation and purification of structurally diverse bioactive compounds from complex matrices derived from plants and cyanobacteria [27-29]. HPCCC is considered orthogonal to GPC [23], because the retention of the solutes in both techniques is caused by different mechanisms. This favorable difference renders them complementary chromatographic techniques, which enables a high level of purification when they are used in combination [30].

In the present report, nostotrebin 6 was produced *via* cultivation of *Nostoc* sp. and isolated and purified by HPCCC combined with GPC, and tested for its antimicrobial activity against Gram-positive and Gram-negative strains, including multidrug-resistant Gram-positive bacteria and yeasts.

2. Experimental

2.1. Chemicals

All organic solvents used for HPCCC were of HPLC grade and purchased from Fisher Chemicals (Loughborough, UK). Ammonia was of reagent grade from Fisher Chemicals (Loughborough, UK). HPCCC water was purified from a Purite Select Fusion pure water system (Thame, UK). Organic solvents used for extraction and HPLC analyses were obtained from Analytika (Prague, Czech Republic). Solutions were prepared using reverse-osmosis deionized water (Aqua Osmotic, Tišnov, Czech Republic).

2.2. Cultivation of Nostoc *sp.*

Cyanobacterial strain *Nostoc* sp. Lukešová 27/97 (Fig. 1A) was obtained from the culture collection of soil algae and cyanobacteria of the Institute of Soil Biology of the Academy of Sciences of the Czech Republic. Maintenance and precultivation of uni-cyanobacterial strain was carried out on solid Allen & Arnon medium [31] in test tubes. Preparation of the inoculum for large scale cultivation was performed by strain cultivation in a 250 mL glass column and subsequent transfer into a 15.0 L photobioreactor containing liquid Allen & Arnon medium, using a semi-batch system, at the constant temperature of 25 ± 0.5 °C, with continuous illumination (351 μmol $m^{-1} \cdot s^{-1}$). The medium was stirred using a flow of mixed air and CO_2 (98:2; *v*/*v*). At the end of exponential growth phase the inoculum (10 L) was transferred into a 100 L bioreactor and cultivated at temperature 25 ± 2 °C, 300 μmol $m^{-1} \cdot s^{-1}$ and stirred by air enriched with CO_2 (98:2; *v*/*v*). The batch culture was cultivated for 14 days and the culture was harvested at the end of the exponential phase as assessed by nitrate depletion using an ion exchange chromatography system DIONEX ISC-90. It is important to note that there is only limited knowledge on the chemical composition of the strain Lukešová 27/97, which is a typical member of the genus *Nostoc* as characterized by vast

exopolysaccharide production, especially at the end of the exponential growth phase. In order to prevent cell lysis and subsequent loss of compound during the stationary phase, the time of harvest was set to the end of exponential phase as previously reported [16]. The biomass was harvested by sedimentation, subsequent centrifugation (Sorvall, Thermo Scientific) and finally freeze-dried prior to extraction.

2.3. Preparation of the Crude Extract

Freeze-dried biomass of *Nostoc* sp. Lukešová 27/97 strain was homogenized with sea-sand and then extracted three times with pure methanol (500 mL per 30 g of biomass). The resulting methanolic extracts were combined and evaporated to total dryness under reduced pressure at 40 °C affording the dried material (4 g in total). The dried extract was dissolved in cold (−20 °C) acetone and stored overnight to precipitate polar lipids [21]. The suspension obtained was separated from the precipitate by centrifugation (1350 × *g*, 10 min), and the precipitation was twice repeated. The supernatant was evaporated until dry and finally stored in a refrigerator for the subsequent HPCCC separation of nostotrebin 6. A representative amount of the extract (500 mg) was subjected to HPCCC separation (see below).

2.4. Separation and Purification Method

2.4.1. High Performance Countercurrent Chromatography (HPCCC)

The separation of nostotrebin 6 by HPCCC was performed on a Midi preparative instrument (Dynamic Extractions Ltd., Slough, UK), consisting of a coil of 912 mL and 4.0 mm bore polyfluoroalkoxy (PFA) tubing. The revolution speed was adjusted with a controller to 1,200 rpm. Fractions were collected with a Gilson FC202 fraction collector (Villiers-le-Bel, France). A preparative Knauer K-1800 HPLC pump (Berlin, Germany) was used to fill the HPCCC column with the stationary phase and pump the mobile phase. The effluent was continuously

monitored by a Knauer K-2501 spectrophotometer operating at 280 nm. Knauer EuroChrom software was used to record the chromatogram. The solvent system was composed of *n*-hexane–ethyl acetate–methanol–water (4:5:4:5, *v*/*v*/*v*/*v*). The phase system was made up classically by vigorously shaking in a separating funnel and allowing the phases to equilibrate overnight. The lower phase was separated and basified using NH_3 (1% NH_3 in lower phase, pH 8.7) to be used as a mobile phase. The sample solution was prepared by dissolving 500 mg of extract in 18 mL of equal volume of lower phase and upper phase of the solvent system selected for the separation. The resulting solution was filtered through a 0.45 µm PTFE membrane before use. The HPCCC column was initially filled with the upper phase (stationary phase). The apparatus was then rotated at 1,200 rpm, and the lower phase (mobile phase) was pumped into the column at a flow rate of 10 mL/min (reversed phase mode). After the mobile phase front emerged and hydrodynamic equilibrium was established, 18 mL of sample solution containing the crude extract (500 mg) was injected through the sample injection valve. The temperature of the apparatus was set at 30 °C. Fractions of 20 mL each were collected automatically for subsequent HPLC analysis. The retention of the stationary phase was calculated according to a previously reported method [21].

2.4.2. Gel permeation chromatography

The nostotrebin 6 fraction obtained by HPCCC was further cleaned-up by gel permeation chromatography (GPC) on Sephadex LH-20 (column length 31 cm, internal diameter 4 cm) with 50% methanol as eluent at a flow rate of 0.5 mL/min. Thirty-five fractions of 5 mL each were collected automatically and then evaporated under reduced pressure for subsequent off-line HPLC-ESI-HRMS analysis.

2.5. HPLC-ESI-HRMS

The analysis of the crude extract and the fractions obtained by HPCCC and GPC was performed on a A Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped with a diode array detector (DAD) and high resolution mass spectrometry with electrospray ionization source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker, Billerica, MA, USA). The samples were subjected to a reversed phase column (Phenomenex Kinetex C_{18} column, 150 × 4.6 mm, 2.6 μm) at 30 °C. The mobile phase consisted of a combination of A (0.05% formic acid in acetonitrile, *v*/*v*) and B (0.05% formic acid in water, *v*/*v*). The gradient was as follows: 0–1 min, 85% B; 1–20 min, 85%–0% B; 20–25 min, 0% B; 25–30 min, 0%–70% B, at a flow rate of 0.6 mL/min. The operating parameters of the mass spectrometer were as follows: the spray needle voltage was set at 3.5 kV, nitrogen was used both as nebulizing gas (2 bar) and drying gas (8 L/min), and the drying temperature was 200 °C. The scanning range was 50–2,000 *m*/*z* and the scanning rate 1 Hz operating in the positive ion mode. The DAD was set at 280 nm to record the peaks, and the UV–Vis spectra were recorded from 200 to 650 nm. The chemical identity of the isolated compound was confirmed by comparing with the authentic standard of 99 % purity [16]. HPLC-ESI-HRMS was also used for the analysis of nostotrebin 6 uptake in bacterial samples. For this purpose, the previously reported experimental protocol was used [15].

2.6. Antimicrobial activity assay

2.6.1. Bacterial strains

Bacterial suspensions were prepared as follows. Bacterial strains were inoculated into blood agar and incubated for 24 hours at 35 °C. Four to five well-isolated colonies were dissolved in 2 mL of Mueller Hinton broth (Himedia) and incubated for 2 hours at 35 °C. After that the broth was diluted in 10 mL of distilled water and used for testing of antimicrobial activity. In this study, the screening for the antimicrobial activity of nostotrebin 6 was performed against

Gram-positive (*Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 4223 and *Staphylococcus aureus* CCM 3953) and Gram-negative (*Escherichia coli* CCM 3954 and *Pseudomonas aeruginosa* CCM 3955) reference bacterial strains from the Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University Brno. Multidrug-resistant Gram-positive strains (fluoroquinolone-resistant *Staphylococcus haemolyticus* A/16568, methicillin-resistant *Staphylococcus aureus* MRSA 4591 and vancomycin-resistant *Enterococcus faecium* VanA 419/ana) and yeasts (*Candida albicans* 978, *Candida tropicalis* 5 and *Candida parapsilosis* 6) from the culture collection of Department of Microbiology (Faculty of Medicine and Dentistry, Palacky University Olomouc) were also tested. All tested microorganisms were stored in cryotubes (ITEST plus, Czech Republic) at -80 °C.

2.6.2. MIC assessment

The antimicrobial efficacy of nostotrebin 6 was determined using the micro-dilution method, and the results were expressed as minimal inhibitory concentrations (MICs). Samples were diluted exponentially and tested in microtiter plates. Brain Heart Infusion broth (Himedia), Mueller Hinton broth (Himedia) and glucose-peptone broth (Oxoid) were used as cultivation medium for Gram-positive, Gram-negative bacteria and yeasts, respectively. Plates were inoculated into a standard quantity of microbe (10^6 CFU/mL). MIC was subtracted after 24 and 48 hours for bacteria and yeast, respectively.

2.6.3. Growth curves assessment

This procedure was performed only with Gram-positive bacteria strains as they were significantly inhibited by nostotrebin 6. Stock solution of the compound was prepared in DMSO (c=50 mg/mL) and diluted in liquid cultivating medium to a concentration of 0.125 mg/mL and then diluted exponentially in 96-well microtiter plate. The last row of wells was

used as a positive control of bacterial growth. The plate was subsequently inoculated with bacterial suspensions, covered with foil to stop evaporation and moved to a spectrophotometer with built-in incubator. Optical density (at 630 nm) was measured every hour over 24 hours and the measured values were used to construct growth curves.

2.6. Statistical evaluation

Three independent experiments were performed in three replicates for each sample if not stated otherwise. Data were expressed as mean ± standard deviation (S.D.), which was lower than 5 % and 7 % for growth curves assessment and cell uptake LC-MS analysis, respectively. MIC data are of non-continuous nature, based on three independent experiments measured in doublets, expressed as average value. All data and error bars were evaluated and plotted using Microsoft Office Professional Edition (ver. 11, 2003).

3. Results and Discussion

Nostotrebin 6 was bio-produced using *Nostoc* sp. Lukešová 27/97 strain, Fig. 1A. This cyanobacterial strain was previously selected based on high production efficiency of nostotrebin 6 [16]. After 14 days pre-cultivation step (Fig. 1B) a 100 L photobioreactor was used for production of appropriate, *i.e.* large-scale multi-gram, amount of the *Nostoc* sp. biomass (Fig. 1C). Consequently, cyanobacterial biomass was harvested, homogenized and extracted (Fig. 1D). Due to the emulsification properties hampering the HPCCC separation, polar lipids in the biomass extract had to be removed by precipitation in cold acetone. The resulting delipidated extract was directly used for isolation and purification of nostotrebin 6 (Fig. 1E-F).

Recently, nostotrebin 6 has been separated from cultivated soil cyanobacteria in a two-step HPCCC operation [21]. This semi preparative HPCCC method combined isocratic and

gradient elution using a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (4:5:4:5, *v*/*v*/*v*/*v*), with the basic lower phase (1% NH_3 in lower phase, pH 8.7) employed as mobile phase [21]. In the present study, nostotrebin 6 was obtained from crude extract (Fig. 2A) by a preparative HPCCC method followed by gel permeation chromatography (GPC). The stationary phase retention accounted for the 80.82 % of the HPCCC column capacity. Nostotrebin 6 was identified in the HPCCC fraction eluted at a retention time (t_R) between 35 and 42 min (Fig. 1E) at a purity of 45 % (Fig. 2B). This fraction was subjected to gel permeation chromatography (GPC) on Sephadex LH-20 (Fig. 1F) to improve further its purity. As a result, nostotrebin 6 (20 mg) was obtained at purity 96.5±0.2% as determined by HPLC-ESI-HRMS. Its identity was confirmed using by the presence of the protonated molecule at *m/z* 799 and main fragment ions at *m/z* 399.1, 371.1, 343,1 and 307.0 as reported previously [16] (Fig. 2C).

Using the described bio-production approach, ~50 g of *Nostoc* sp. strain biomass was prepared during 14 days cultivation period in a 100 L bioreactor, with very reproducible results. The gain of the crude extract was 16.6±3.0 % with respect to dry biomass. Further purification of nostotrebin 6 resulted in a yield of 4 % and 0.53±0.1% on the basis of crude extract and dried biomass, respectively. The purity evaluation was based on the HPLC-ESI-HRMS methodology described in sec. 2.5. The procedure could be used for the easy-to-make isolation of multigram amounts of target molecule, which is crucial for its consequent biological testing, synthetic modifications and technological lab-scale applications. However, the real advantage of the approach described is the possibility of immediate and relatively easy transfer of this technology to a larger scale.

Preliminary data indicated that nostotrebin 6 is a bioactive substance affecting enzyme functions and cell proliferation [15, 16], which was also reported for many other CPDs [1]. Given that CPD structures were previously described as effective antibacterial agents [17-20],

the present study was focused on evaluation of nostotrebin 6 antibacterial action. The testing was based on a screening panel containing Gram-positive reference strains (*Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 4223 and *Staphylococcus aureus* CCM 3953), multidrug-resistant bacterial strains (*Staphylococcus haemolyticus* A/16568, *Staphylococcus aureus* MRSA 4591 and *Enterococcus faecium* VanA 419/ana), Gram-negative reference strains (*Escherichia coli* CCM 3954 and *Pseudomonas aeruginosa* CCM 3955) and yeasts (*Candida albicans* 978, *Candida tropicalis* 5, *Candida parapsilosis* 6). The antimicrobial and antifungal activities were tested using a standard micro-dilution method, and the results were expressed as minimal inhibitory concentrations (MICs). There was no effect on the growth of Gram-negative strains and yeasts in the concentration range from 1.95 µg/mL to 1 mg/mL. Conversely, the nostotrebin 6 MIC values against Gram-positive bacteria varied from 6.25 to 15.6 µg/mL (Fig. 3A). The dynamic growth curve of *Staphylococcus aureus* MRSA 4591 in the presence of different nostotrebin 6 concentrations (Fig. 3B), shows unequivocally that nostotrebin 6 inhibits bacterial growth in a dose-dependent manner, exerting its effect even at the lowest concentration used in the test (1.95 µg/mL).

Finally, we monitored nostotrebin 6 uptake into bacterial cells (*Enterococcus faecalis* CCM 4224 and *Staphylococcus aureus* CCM 3953) by HPLC-ESI-HRMS [15]. When increasing the concentration of nostotrebin 6, its accumulation can be observed in bacterial biomass (Fig. 3C). At a concentration around 4 µM (*i.e.* 3 µg/mL) the bacterial cells are saturated by nostotrebin 6, which corresponds well with strong inhibition of bacterial growth (Fig. 3A).

The recognition of a molecular mechanism of nostotrebin 6 antibiotic activity is important for further research. However, our results indicate that nostotrebin 6 could be synthesized by cyanobacteria as a protective factor against biotic stress. The reason for this hypothetical statement is based on the fact that the cyanobacteria producing nostotrebin 6 are

Gram-negative type bacteria, but nostotrebin 6 is a growth inhibitor only against Gram-positive ones. Thus, there is minimal self-toxicity for Gram-negative cyanobacteria producing nostotrebin 6, and simultaneously there is an antibiotic action against bacteria which are not cyanobacteria. For hypothesis confirmation, the results presented in this report have to be extended for consequent finding in the field of nostotrebin 6 biosynthesis and investigations of its antibiotic action at a molecular level.

4. Conclusion

A large-scale bio-production method aligned with the combined use of HPCCC and GPC enabled the isolation of nostotrebin 6 at over 96% purity. Nostotrebin 6 was shown to possess antibacterial activities against selected standard reference and multi-drug resistance strains of Gram-positive bacteria. As the nostotrebin 6 MICs values against multi-drug resistance strains varied from 6.25 to 15.6 µg/mL, this compound could serve as a basic nucleus for further design and synthesis of simplified CPD structures or for direct application as an antimicrobial product. The selectivity of nostotrebin 6 against Gram-positive bacteria supports its potential applicability, especially in biotechnological field, *e.g.* applications requiring Gram-positive bacteria elimination in presence of Gram-negative ones. The results presented here extend our knowledge in the field of antibiotic action of newly identified phenolic compounds [32, 33] and more specifically of CPDs [1] from cyanobacteria.

References:

[1] Z. Sevcikova, M. Pour, D. Novak, J. Ulrichova, J. Vacek, Chemical properties and biological activities of cyclopentenediones: A review, Mini-Rev. Med. Chem., 14 (2014) 322-331.

[2] Y. Cui, J. Wu, S.C. Jung, G.O. Kim, R. Kyeong Ko, H.J. Lee, E.S. Yoo, H.K. Kang, K. Suk, S.Y. Eun, Neuroprotective effect of methyl lucidone against microglia-mediated neurotoxicity, Eur. J. Pharmacol., 690 (2012) 4-12.

[3] K.J.S. Kumar, H.W. Hsieh, S.-Y. Wang, Anti-inflammatory effect of lucidone in mice via inhibition of NF-kappa-B/MAP kinase pathway, Int. Immunopharmacol., 10 (2010) 385-392.

[4] K.J.S. Kumar, H.L. Yang, Y.C. Tsai, P.C. Hung, S.H. Chang, H.W. Lo, P.C. Shen, S.C. Chen, H.M. Wang, S.Y. Wang, C.W. Chou, Y.C. Hseu, Lucidone protects human skin keratinocytes against free radical-induced oxidative damage and inflammation through the up-regulation of HO-1/Nrf2 antioxidant genes and down-regulation of NF-kB signaling pathway, Food Chem. Toxicol., 59 (2013) 55-66.

[5] K.J.S. Kumar, J.C. Yang, F.H. Chu, S.T. Chang, S.Y. Wang, Lucidone, a Novel Melanin Inhibitor from the Fruit of *Lindera erythrocarpa* Makino, Phytother. Res., 24 (2010) 1158-1165.

[6] H. Hori, H. Nagasawa, M. Ishibashi, Y. Uto, A. Hirata, K. Saijo, K. Ohkura, K.L. Kirk, Y. Uehara, TX-1123: An antitumor 2-hydroxyarylidene-4-cyclopentene-1,3-dione as a protein tyrosine kinase inhibitor having low mitochondrial toxicity, Bioorg. Med. Chem., 10 (2002) 3257-3265.

[7] R. Antkowiak, W.Z. Antkowiak, I. Banczyk, L. Mikolajczyk, A new phenolic metabolite, involutone, isolated from the mushroom *Paxillus involutus*, Can. J. Chem.-Rev. Can. Chim., 81 (2003) 118-124.

[8] R. Feling, K. Polborn, W. Steglich, J. Muhlbacher, G. Bringmann, The absolute configuration of the mushroom metabolites involutin and chamonixin, Tetrahedron, 57 (2001) 7857-7863.

[9] L. Mikolajczyk, W.Z. Antkowiak, Structure studies of the metabolites of *Paxillus involutus*, Heterocycles, 79 (2009) 423-426.

[10] M. Hayashi, Y.P. Kim, S. Takamatsu, A. Enomoto, M. Shinose, Y. Takahashi, H. Tanaka, K. Komiyama, S. Omura, Madindoline, a novel inhibitor of IL-6 activity from *Streptomyces* sp K93-0711. 1. Taxonomy, fermentation, isolation and biological activities, J. Antibiot., 49 (1996) 1091-1095.

[11] S. Hosokawa, S. Kobayashi, Total synthesis of madindoline A, J. Synth. Org. Chem. Jpn., 59 (2001) 1103-1108.

[12] S. Hosokawa, K. Sekiguchi, M. Enemoto, S. Kobayashi, Novel stereoselective construction of a quaternary carbon: application to synthesis of the cyclopentenedione moiety of madindolines, Tetrahedron Lett., 41 (2000) 6429-6433.

[13] S. Takamatsu, Y.P. Kim, A. Enomoto, M. Hayashi, H. Tanaka, K. Komiyama, S. Omura, Madindolines, novel inhibitors of IL-6 activity from Streptomyces sp. K93-0711. II. Physico-chemical properties and structural elucidation, J. Antibiot., 50 (1997) 1069-1072.

[14] J. Hrbac, P. Jakubec, V. Halouzka, P. Matejka, M. Pour, J. Kopecky, J. Vacek, The Permselective Layer Prepared onto Carbon and Gold Surfaces by Electropolymerization of Phenolic Cyclopentenedione-Nostotrebin 6, Electrochem. Commun., 38 (2014) 53-56.

[15] J. Vacek, J. Hrbac, J. Kopecky, J. Vostalova, Cytotoxicity and Pro-Apoptotic Activity of 2,2'-Bis[4,5-bis(4-hydroxybenzyl)-2-(4-hydroxyphenyl)cyclopent-4-en-1,3-dione], a Phenolic Cyclopentenedione Isolated from the Cyanobacterium Strain Nostoc sp. str. Lukesova 27/97, Molecules, 16 (2011) 4254-4263.

[16] P. Zelik, A. Lukesova, J. Cejka, M. Budesinsky, V. Havlicek, A. Cegan, J. Kopecky, Nostotrebin 6, a bis(cyclopentenedione) with cholinesterase inhibitory activity isolated from Nostoc sp str. Lukesova 27/97, J. Enzym. Inhib. Med. Chem., 25 (2010) 414-420.

[17] K.S. Babu, X.C. Li, M.R. Jacob, Q.F. Zhang, S.I. Khan, D. Ferreira, A.M. Clark, Synthesis, antifungal activity, and structure-activity relationships of coruscanone A analogues, J Med Chem, 49 (2006) 7877-7886.

[18] M.J. Riveira, B.L. Tekwani, G.R. Labadie, M.P. Mischne, Synthesis and biological activity profile of novel 2-cinnamylidene-1,3- diones related to coruscanone A: Promising new antileishmanial agents, MedChemComm, 3 (2012) 1294-1298.

[19] O.P. Shestak, V.L. Novikov, Synthesis of coruscanones A and B, metabolites of *Piper coruscans*, and related compounds, Russ. Chem. Bull., 59 (2010) 81-90.

[20] L. Tichotova, E. Matousova, M. Spulak, J. Kunes, I. Votruba, V. Buchta, M. Pour, Synthesis and biological activity of desmethoxy analogues of coruscanone A, Bioorg. Med. Chem. Lett., 21 (2011) 6062-6066.

[21] J. Cheel, P. Kucerova, I. Garrard, S. Ignatova, P. Hrouzek, J. Kopecky, Two-step separation of nostotrebin 6 from cultivated soil cyanobacterium (Nostoc sp.) by high performance countercurrent chromatography, Molecules, 19 (2014) 8773-8787.

[22] Y. Ito, Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography, J. Chromatogr. A, 1065 (2005) 145-168.

[23] J.B. Friesen, J.B. McAlpine, S.N. Chen, G.F. Pauli, Countercurrent Separation of Natural Products: An Update, J. Nat. Prod., 78 (2015) 1765-1796.

[24] R. Hu, Y. Pan, Recent trends in counter-current chromatography, TrAC Trends Anal. Chem., 40 (2012) 15-27.

[25] D. Jia, X. Chen, X. Ding, Z. Zhu, Y. Chai, C. Zhang, Preparative chromatography and its applications in drug research, Pharm. Care Res., 15 (2015) 161-165.

[26] K. Skalicka-Wozniak, I. Garrard, A comprehensive classification of solvent systems used for natural product purifications in countercurrent and centrifugal partition chromatography, Nat. Prod. Rep., 32 (2015) 1556-1561.

[27] J. Cheel, M. Minceva, P. Urajova, R. Aslam, P. Hrouzek, J. Kopecky, Separation of aeruginosin-865 from cultivated soil cyanobacterium (Nostoc sp.) by centrifugal partition chromatography combined with gel permeation chromatography, Nat. Prod. Commun., 10 (2015) 1719-1722.

[28] T. Shan, S. Lu, C. Luo, R. Luo, Y. Mou, M. Wang, Y. Peng, L. Zhou, Preparative separation of spirobisnaphthalenes from endophytic fungus Berkleasmium sp. Dzf12 by high-speed counter-current chromatography, Molecules, 18 (2013) 12896-12908.

[29] C. Yang, D. Li, X. Wan, Combination of HSCCC and Sephadex LH-20 methods. An approach to isolation and purification of the main individual theaflavins from black tea, J. Chromatogr. B, 861 (2008) 140-144.

[30] E.L. Ghisalberti, Detection and Isolation of Bioactive Natural Compounds, p. 11-65, In: Bioactive Natural Products: Detection, Isolation, and Structural Determination, S. M. Colegate and R. J. Molyneux (eds.) 2nd edition, CRC press, Taylor & Francis Group, (2008).

[31] M.B. Allen, D.I. Arnon, Studies on nitrogen-fiixing blue-green algae .1. Growth and nitrogen fixation by Anabaena-cylindrica Lemm, Plant Physiol., 30 (1955) 366-372.

[32] L. Onofrejova, J. Vasickova, B. Klejdus, P. Stratil, L. Misurcova, S. Kracmar, J. Kopecka, J. Vacek, Bioactive phenols in algae: The application of pressurized-liquid and solid-phase extraction techniques, J. Pharm. Biomed. Anal., 51 (2010) 464-470.

[33] J. Vacek, M. Snoblova, B. Klejdus, A short introduction to algal and cyanobacterial constituents - The occurrence of phenolic metabolites, Cs. Slov. Farm., 58 (2009) 103-108.

Figures:

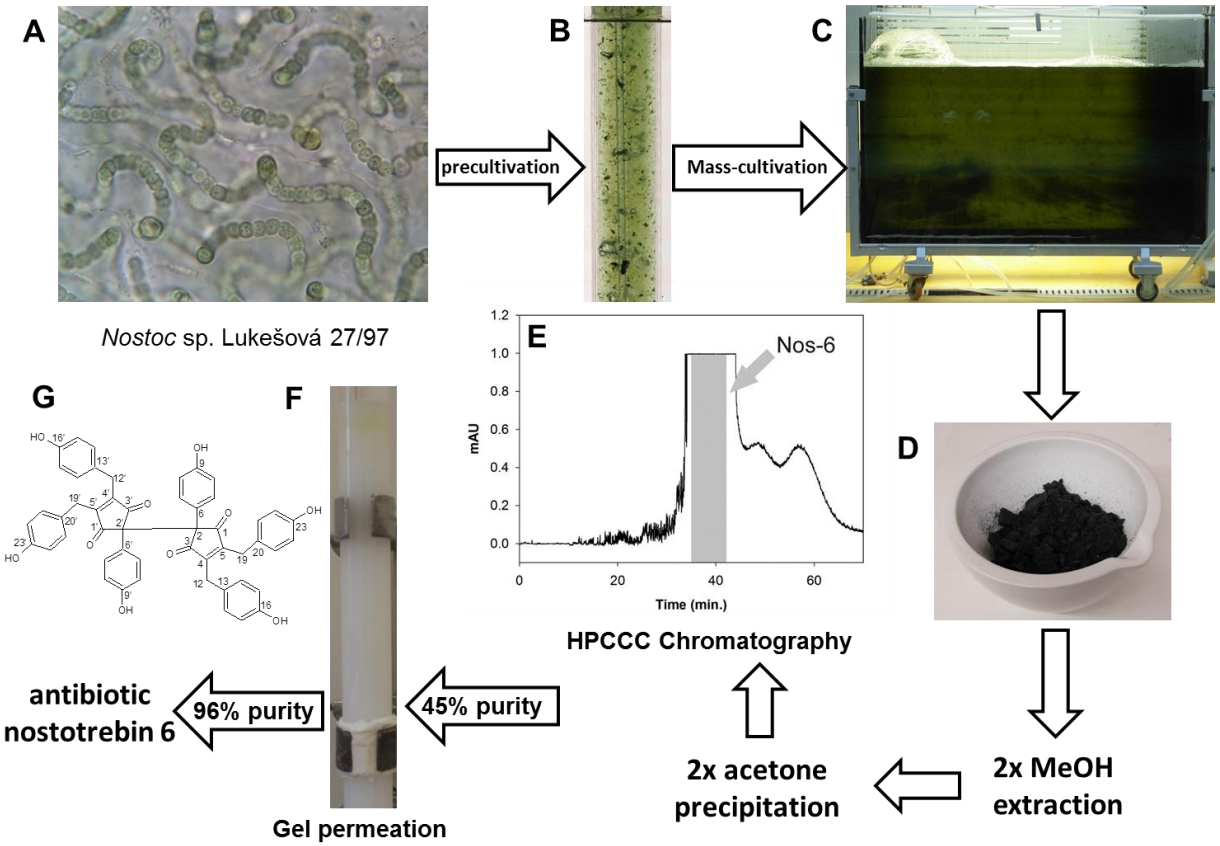

Fig. 1. General procedure for *Nostoc* sp. Lukešová 27/97 biomass production and purification by HPCCC and GPC method. For detail description of panels (**A-F)** see the main text. Nostotrebin 6 (Nos-6) chemical structure **(G)**.

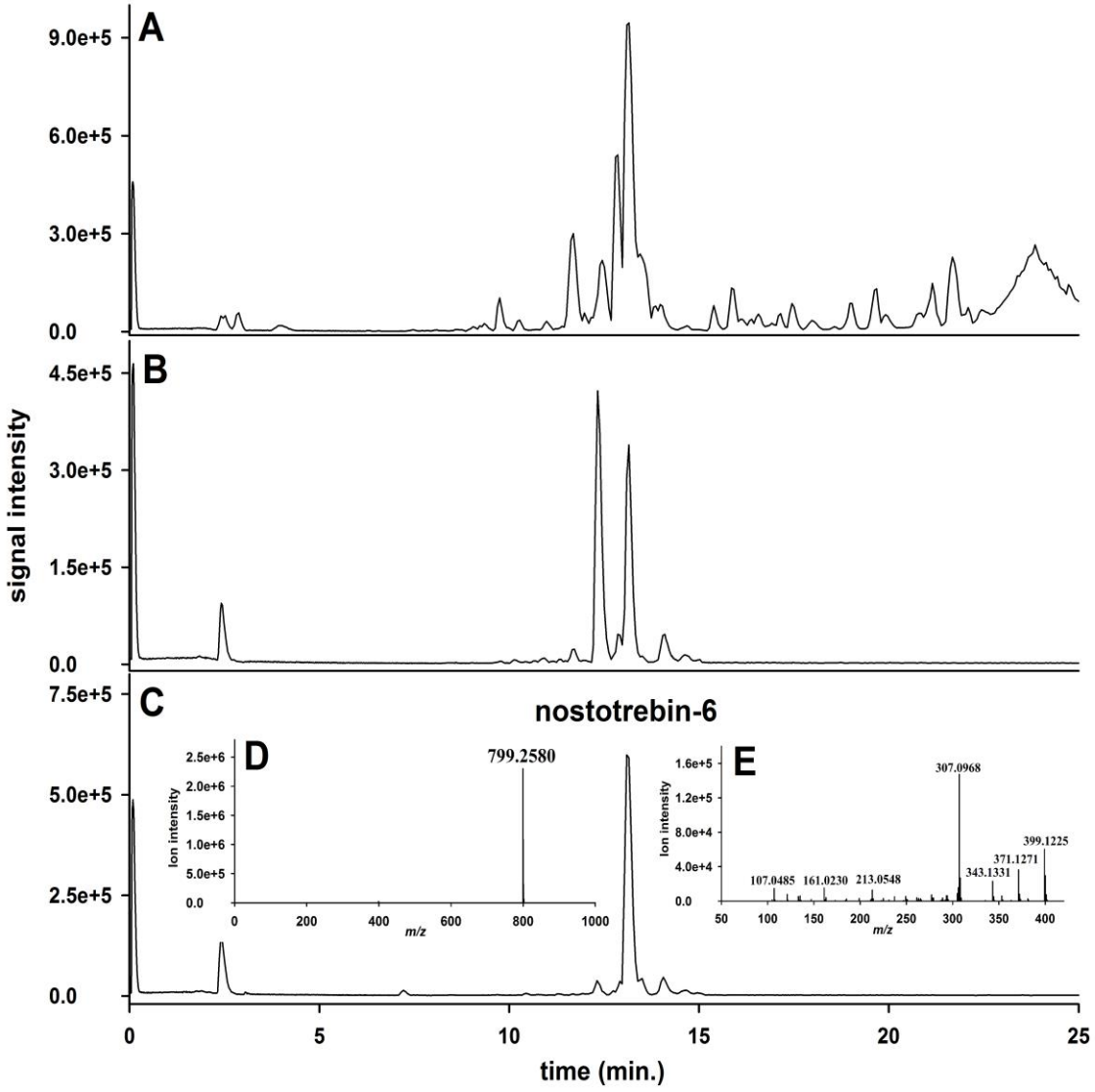

Fig. 2. Total ion HPLC-ESI-HRMS chromatograms of (**A**) *Nostoc* sp. Lukešová 27/97 crude extract, (**B**) nostotrebin 6 fraction obtained by HPCCC separation with retention time between 35 and 42 min, and (**C**) nostotrebin 6 fraction obtained by HPCCC separation followed by gel permeation chromatography on Sephadex LH-20. (**D**) Positive-ion mass spectrum of nostotrebin 6 showing the protonated molecule and (**E**) corresponding MS^2 spectrum.

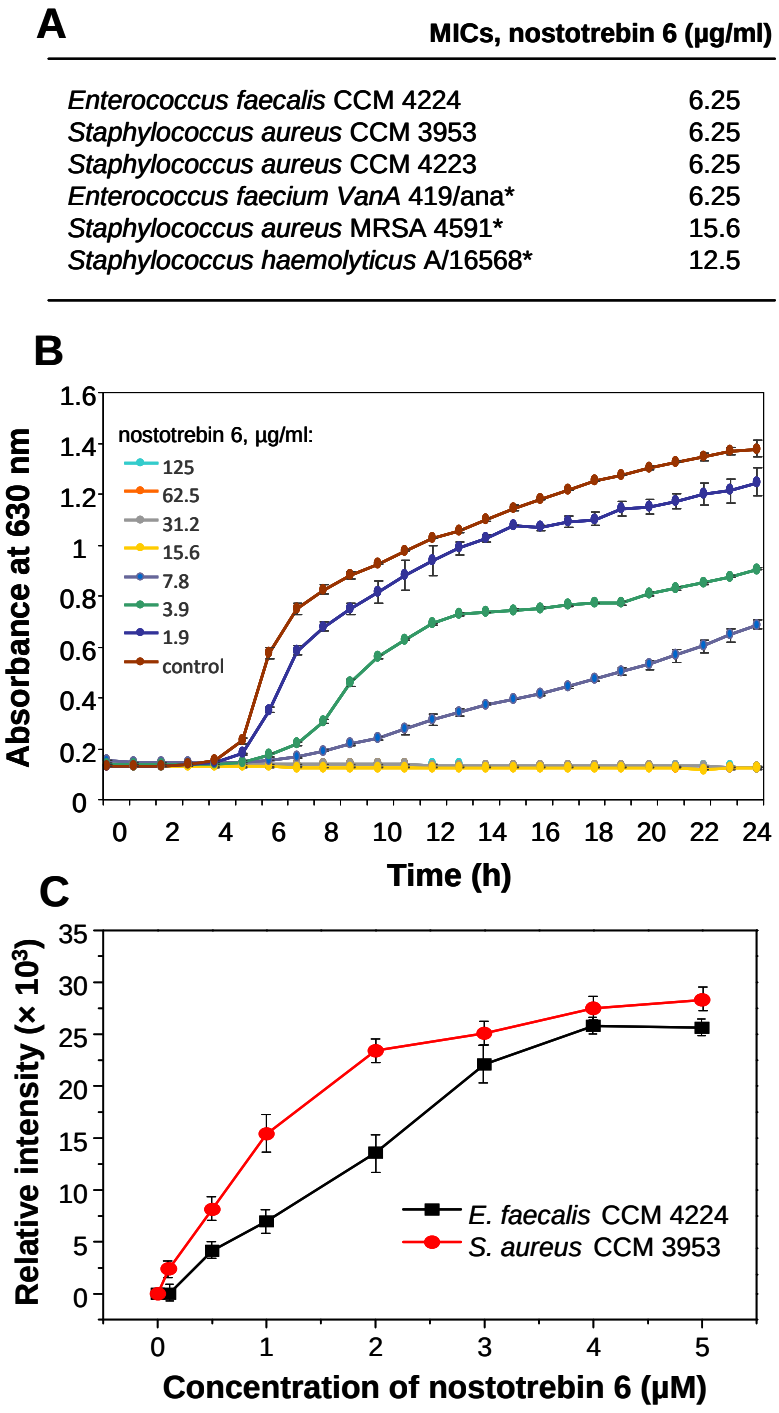

Fig. 3. (A) Minimal inhibition concentrations (MICs) of nostotrebin 6 for selected Gram-positive bacteria, (*) multi-resistant strains. MICs equal minimal bactericidal concentrations. **(B)** Growth curves for *Staphylococcus aureus* MRSA 4591 incubated with various concentrations of nostotrebin 6. **(C)** Nostotrebin 6 bacterial cell uptake, incubation period was 4 h. Data are means ±SD of three independent experiments measured in triplicates. Error bars smaller than the plotted symbols are not visible.