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Application of HPCCC Combined with Polymeric Resins and HPLC for the Separation of Cyclic Lipopeptides Muscotoxins A–C and Their Antimicrobial Activity

José Cheel ^{1*}, Jan Hájek ¹, Marek Kuzma², Iva Smýkalová³, Eliška Ondráčková³, Petra Urajová ¹, Dai Long Vu ¹, Karine Faure ⁴, Jiří Kopecký ¹ and Pavel Hrouzek ¹

¹ Laboratory of Algal Biotechnology-Centre ALGATECH, Institute of Microbiology of the Czech Academy of Sciences, Opatovický mlýn, Novohradská 237, 379 81, Třeboň, Czech Republic; jcheel@alga.cz (J.C.); hajek@alga.cz (J.H); urajova@alga.cz (P.U.); longvu1182@gmail.com (D.L.V.); kopecky@alga.cz (J.K.); hrouzek@alga.cz (P.H.)

² Laboratory of Molecular Structure Characterization, Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic; kuzma@biomed.cas.cz

³ Plant Biotechnology Department, AGRITEC Plant Research Ltd., Zemědělská 2520/16, 787 01 Šumperk, Czech Republic; smykalova@agritec.cz; ondrackova@agritec.cz

⁴ University of Lyon, CNRS, Université Claude Bernard Lyon 1, Ens de Lyon, Institut des Sciences Analytiques, UMR 5280, 5 rue de la Doua, 69100, Villeurbanne, France; karine.faure@isa-lyon.fr

* Correspondence: jcheel@alga.cz; jcheel@email.cz; Tel.: +420-384-340-465

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Abstract: Muscotoxins are cyclic lipopeptides of cyanobacterial origin which share similarities in the chemical structure with lipopeptide therapeutic agents. Therefore, the development of an efficient isolation method is relevant to speed up their pharmacological and biotechnological evaluation. After enriching a crude extract of producing cyanobacterial strain *Desmonostoc muscorum* CCALA 125 by consecutive adsorption on Amberlite XAD-16 and XAD-7 resins, a two-step high performance countercurrent chromatography (HPCCC) method was applied to separate four muscotoxins variants from the resin-treated extract. In the HPCCC first step, the two-phase solvent system composed of *n*-hexane, ethyl acetate, ethanol, and water (1:5:1:5, v/v/v/v) was used to obtain the compounds **2**, **3** and a **1**-enriched fraction from the resin-treated extract. In the HPCCC second step, the two-phase solvent system composed of *n*-hexane, ethyl acetate, ethanol, water and acetic acid (1:5:1:5:1 v/v/v/v/v) was used to separate the compound **1** from the **1**-enriched fraction. In both HPCCC steps, the lower phases of the corresponding two-phase solvent systems were used as mobile phases at a flow rate of 1 mL/min. The rotational speed and temperature of the HPCCC column were 1200 rpm and 28°C, respectively. Compounds **1** (3.5 mg, 98% purity), **2** (130 mg, 98% purity) and **3** (8 mg, 95% purity) were obtained from HPCCC, and were further re-purified by HPLC affording the compounds **1**, **2** and **3** with purity over 98%. The chemical identity of the isolated compounds **1-3** were assigned to muscotoxins C, A, B, respectively, on the basis of their MS and NMR spectral data. Muscotoxin C was identified as a novel chemical variant occurring in *D. muscorum*. The developed HPCCC method proved to be an efficient chromatographic tool for obtaining cyclic lipopeptides from cyanobacterial biomass. These structurally closely related muscotoxin variants exhibited different antifungal activity against plant pathogenic fungus

Sclerotinia sclerotiorum. Interestingly, a change of proline for γ -methyl-proline in the vicinity of the fatty acid moiety led to the improvement of the antifungal activity.

Keywords: Cyanobacteria; cyclic lipopeptides; muscotoxins; countercurrent chromatography; HPCCC; CPC; CCC; anti-fungal activity; *Sclerotinia*; γ -methyl-Proline.

1. Introduction

Cyanobacteria are gram-negative photosynthetic microorganisms that have attracted increasing attention as source of potential therapeutically useful compounds [1]. One of these groups is represented by cyclic lipopeptides (CLPs), which have been found to comprise up to 14 amino acid residues. CLPs are biosynthesized by multifunctional protein complexes called polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) [2]. In these structures, a modified fatty acid tail is linked to the peptidic macrocycle leading to the formation of CLPs. The pharmacological potential of CLPs is represented by their antifungal [3], antibiotic [4], cytotoxic [5] and antiproliferative activities [6, 7]. CLPs have been approved for clinical use as in the case of cyclosporine A and daptomycin. The first one is currently used in medicine as an immunosuppressant agent [8], whereas the later one is the first CLP that was approved to be used as antibiotic agent in humans over the past decade [9]. Pharmacodynamically, CLPs offers advantages as compared to the open forms because a cyclized peptide is less flexible and therefore more pre-organized, thus exerting a better affinity to their biological target due to the reduced entropy needed for target ligand interaction [10,11]. In addition, CLPs are less sensitive to proteolytic degradation thereby favoring their stability and bioavailability [11].

Muscotoxins are cyanobacterial CLPs comprising 11 amino acid units and a β -amino fatty acid [12]. Our group reported the separation and identification of cyclic undeca-lipopeptides muscotoxin A and B, as a mixture, from the soil cyanobacterium *Desmonostoc muscorum* strain CCALA125 (synonymous to Lukešová 1986/14 and NIVA-CYA 817). The structure of these compounds was determined by MS and NMR analysis, and Marfey's reagent was used for chiral amino acid analysis [12]. Muscotoxins A and B were observed to differ in the substitution of proline by methylproline, respectively. So far, the separation of muscotoxins from cyanobacterial biomass has only been performed by the application of multi-step procedures including the use of liquid-liquid partitioning and chromatographic methods of solid support such as solid-phase extraction on cartridges and HPLC [12]. However, these methods did not exert enough resolution capacity for separating muscotoxin A from its congener B, and were not throughput competitive to isolate minor muscotoxins from cyanobacterial biomass. Muscotoxins possess a chemical structure that is similar to those of approved therapeutic agents; therefore, they may serve as a chemical template for developing new antibiotic and antifungal agents. Moreover, they could be interesting targets for addressing combinatorial biosynthesis strategies aimed at enhancing their potential pharmacological efficacy or mitigating toxicity issues. Hence, the development of an efficient and high-throughput separation method for obtaining these compounds from cyanobacterial biomass is warranted to enable their extensive *in vitro* and *in vivo* studies and to efficiently support drug discovery strategies focused on CLPs of medical importance.

High performance countercurrent chromatography (HPCCC) is a solid support-free chromatographic technique that uses two immiscible solvent phases. The solvent phase that is used as stationary phase is retained in the column by a centrifugal force field, while the other solvent phase is used as the mobile phase [13]. The principle of the separation using HPCCC is based on the difference in partitioning of each target compound between the two immiscible phases. The liquid nature of the stationary phase renders HPCCC with many advantages over traditional solid liquid chromatography techniques. Those advantages have been recently reviewed, and encompass the absence of irreversible adsorption of target molecules; high sample loading capacity and recovery;

low risk of sample denaturation; and low solvent consumption [14]. The successful application of HPCCC for isolating a variety of bioactive components from higher plants [15, 16, 17, 18, 19] and cyanobacteria [20,21] has been extensively reported. However, the HPCCC chromatographic capacity toward CLPs has been a little explored. In the present study we inform the separation of muscotoxins from cyanobacteria by HPCCC combined with polymeric resin treatment and HPLC. The isolated compounds were tested for their antifungal activity against the plant pathogenic fungus *Sclerotinia sclerotiorum*.

2. Results and Discussion

2.1. HPLC–ESI–HRMS analysis of the crude extract

The chemical identity of the target compounds in the crude extract was determined by comparing their MS and MS/MS spectra with the literature data [12]. The molecular ion peaks corresponding to the target compounds are shown in the base peak chromatogram (Figure 1a). The compound **2**, the major CLP in the extract, was assigned to muscotoxin A. Compounds **3** and **1**, which were present at trace amounts in the crude extract, were identified as muscotoxin B and a muscotoxin congener, respectively. In order to achieve the isolation of the minor target compounds, the enrichment of the crude extract was necessary prior their isolation.

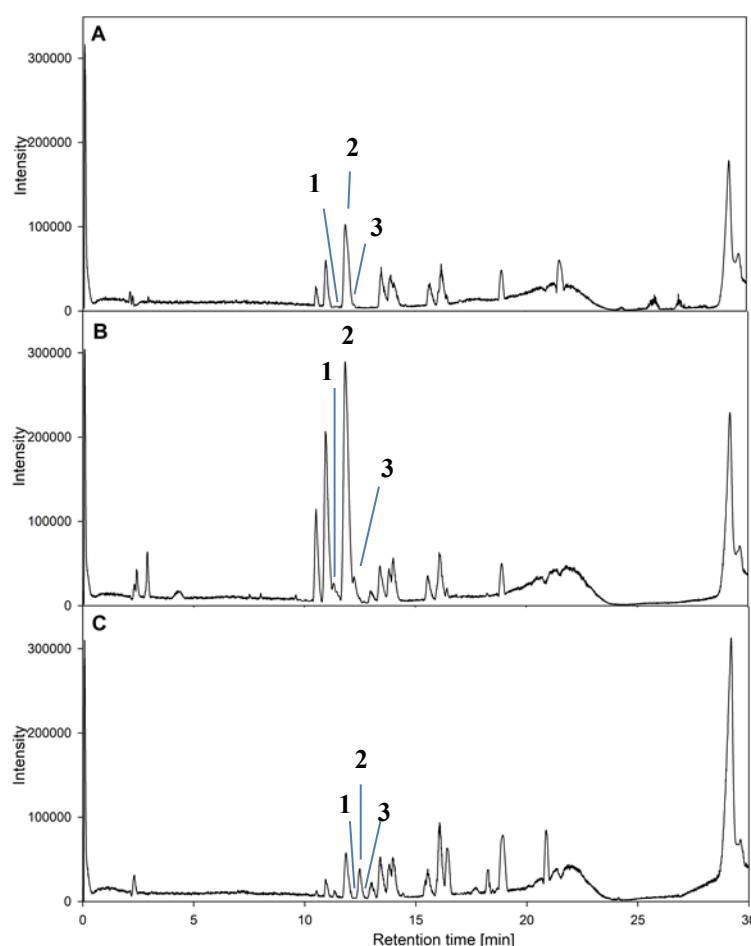


Figure 1. High-performance liquid chromatography (HPLC)–electrospray ionization high resolution mass spectrometry (ESI–HRMS) chromatogram (base peak chromatogram) (a) of the crude extract from *D. muscorum* CCALA125. Base peak chromatograms of the muscotoxins-enriched extract obtained by consecutive treatment of the crude extract from *D. muscorum* CCALA125 on Amberlite XAD-16 (b) and Amberlite XAD-7 (c) adsorption resins.

117

118 2.2. Enrichment of the crude extract

119 The *D. muscorum* CCALA 125 crude extract was enriched in the target compounds by
 120 consecutive adsorption on two kinds of non-ionic polymeric resins made of a hydrophobic styrene-
 121 divinylbenzene matrix (Amberlite XAD-16 resin) and a moderately polar acrylic matrix (Amberlite
 122 XAD-7 resin). The application of these non-ionic polymeric resins either using a single type of resin
 123 [22-25] or combining different resins [25-26] has shown a great efficiency in the purification of cyclic
 124 peptides from cyanobacterial and bacterial extracts. In the current study, some 6200 mg of
 125 *Desmonostoc muscorum* crude extract were processed by non-ionic polymeric resins affording 700 mg
 126 of enriched extract from Amberlite XAD-16 resin treatment and 300 mg of enriched extract from
 127 Amberlite XAD-7 resin treatment. As observed in the chromatograms (Figure 1b,c), Amberlite XAD-
 128 16 and Amberlite XAD-7 enriched extracts contained the target compounds and exhibited
 129 qualitatively similar profiles. The higher peak purities of the target compounds were observed in the
 130 Amberlite XAD-16 enriched extract (Figure 1b). The peak purity values reflected the presence of other
 131 contaminants in the sample, but did not account for the amount of compounds recovered by the
 132 resins. Thus, to determine possible losses of target compounds during the prepurification by the
 133 resins, the recovery (%) was calculated by the use of an external standard HPLC–HRMS method.
 134 Seven experimental points were used to create calibration curves. The regression equation for
 135 muscotoxins A–C (0.3–100 µg/mL with 10 µL injection) were $y = 645395x - 10108$ ($R^2 = 0.9979$),
 136 $y = 469079x - 933572$ ($R^2 = 0.9993$), and $y = 389999x - 1E+06$ ($R^2 = 0.9978$) respectively, where y is the
 137 peak area of the target compound and x is the concentration (µg/mL) of the target compound. The
 138 Amberlite XAD-16 resin treatment showed the highest contribution to the total recovery of
 139 muscotoxins (51-59%). Owing to the chromatographic profiles of both the Amberlite XAD-16 and
 140 Amberlite XAD-7 enriched extracts were similar, thus they were pooled affording 1000 mg of
 141 enriched extract, which was subsequently used for the HPCCC separation of the target compounds.
 142 The overall recovery values of the target compounds using the pooled enriched extracts ranged
 143 between 65 % and 93 %, as shown in Table 1. One gram of enriched extract in the three target
 144 compounds was obtained after passing the crude extract (6.138 g) on resins, which demonstrated the
 145 enriching effect of the operation.

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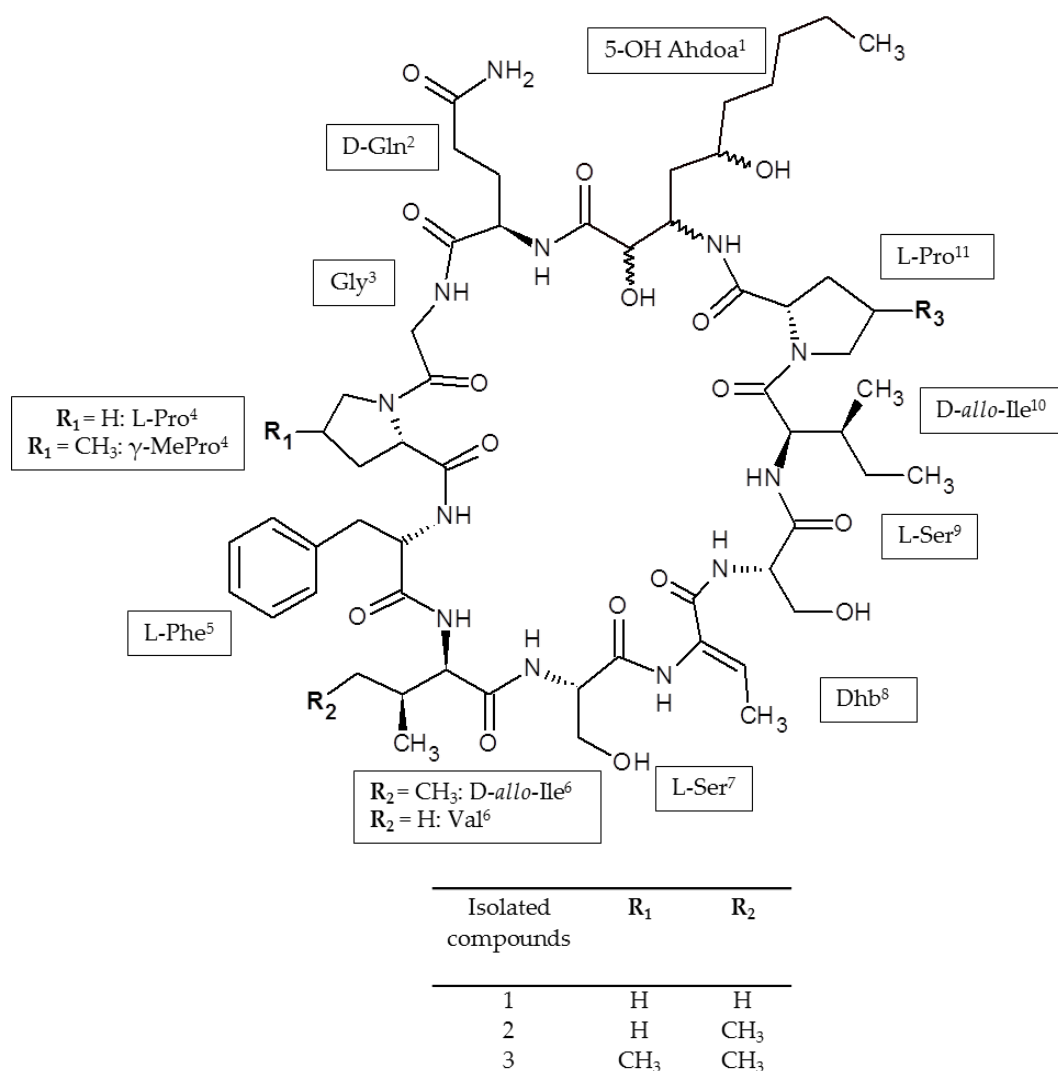


Figure 2. Chemical structures of muscotoxins isolated from the soil *D. muscorum* CCALA 125. Ahdoa¹ (3-amino-2-hydroxydecanoic acid), D-Gln² (D-glutamine), Gly³ (glycine), L-Pro^{4,11} (L-proline), γ-MePro⁴ (γ-methylproline), L-Phe⁵ (L-phenylalanine), D-allo-Ile^{6,10} (D-alloisoleucine), Val⁶ (valine), L-Ser^{7,9} (L-serine), Dhb⁸ (2,3-didehydrobutyric acid).

2.3. Optimization of the HPCCC conditions

When working with HPCCC, the searching for a suitable two-phase solvent system is the first step which has been informed to represent the major part of the work involved in the HPCCC separation [13]. A suitable two-phase solvent system is the one which provides a partition coefficient (K) of the target compounds within the range $0.5 \leq K \leq 2.5$, and exhibit a short settling time (less than 30 s). Additionally, for achieving a good resolution between two closely eluting peaks, the separation factor (α) between the two compounds ($\alpha = K_2/K_1$, $K_2 > K_1$) should be greater than 1.5 [13, 27]. It is well described that compounds with smaller K values are eluted closer to the solvent front exhibiting a lower resolution, whereas a larger K value gives better resolution, but wider peaks and more dilute peak fractions because of a later elution time [13]. Moreover, a short settling time of a two-phase solvent system is correlated with a high amount of stationary phase retained inside the HPCCC column [13,27], which favors the chromatographic resolution. In the present study, different two-phase solvent systems (Table 2) were examined for their capacity to provide suitable K values of the target compounds. The K value is frequently estimated by using HPLC with UV detection, which is calculated by deviding the peak area of the target compound in the appear phase by that of the peak

area of the same target compound in the lower phase. However, given that UV chromatogram provided insufficient peak areas for minor muscotoxins, thus HPLC–ESI–HRMS was used to determine the K values. By using this approach, the peak area of the target molecular ions were selectively retrieved from the base peak chromatogram. As shown in the Table 2, solvent system 10 (*n*-hexane, ethyl acetate, ethanol, and water in a ratio of 1:5:1:5) provided suitable K values and a good separation factor (α) for compounds **2** and **3**, as well as a short settling time. However, the solvent system 10 gave a small K value ($K \leq 0.5$) for compound **1**, and therefore this compound would be eluted near the solvent front and close to other contaminants, resulting in poor peak resolution. It was concluded that to have a suitable K value, the solubility of compound **1** should be favored in the upper phase. The improvement of the K value of compound **1** could be achieved by finding out a new solvent system or by modifying the composition of the already selected solvent system. In the present study, the system 10 was acidified by adding acetic acid leading to the system 11 (*n*-hexane, ethyl acetate, ethanol, water and acetic acid in a ratio of 1:5:1:5:1. This modification favored the solubility of compound **1** in the upper phase of the solvent system 11 giving rise an appropriate partition coefficient ($K = 1.09$), a short settling time and a suitable density difference. Overall, the results indicated that the separation of compounds **1–3** would demand the application of a two-steps HPCCC method. The solvent system 10 was found to be selective for compounds **2** and **3**, whereas solvent system 11 was selective for compound **1**. In HPCCC, it is very well established that the chromatographic resolution is improved when the volume of the stationary phase retained in the column is increased [28]. Flow rate, rotational speed and sample loading are operational parameters that affect the retention of the stationary phase. By reducing the flow-rate and increasing the revolution speed, the retention of the stationary phase is improved. In this study, the flow rate for the first-step and second-step HPCCC operations was 1 mL/min and the revolution speed was set at 1200 rpm. Moreover, it was observed that a good resolution between compounds **2** and **3** was achieved when injecting 100 mg of enriched extract.

Table 1. Recovery of muscotoxins from *D. muscorum* CCALA 125 after enrichment by polymeric resins.

Compounds	Recovery (%)		Total recovery (%)
	XAD-16 resin-purified extract	XAD-7 resin-purified extract	
1	50.52	14.21	64.73
2	58.86	33.91	92.77
3	52.16	19.57	71.73

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198
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Table 2. The partition coefficient (K) and separation factor (α) values of three muscotoxins from *D. muscorum* CCALA 125 in different two-phase solvent systems and the settling times.

Solvent Systems	Composition	Relative proportions of solvents (v/v/v/v)	Phase volume ratio (UP/LP)	Settling time (s)	Density difference (LP-UP, g mL ⁻¹)	Partition coefficient (K) of muscotoxins		
						1	2	3
1	<i>n</i> -Hex-EtOH-H ₂ O	10:5:5	1.22	7	0.272	0.00	0.03	0.01
2	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	9:1:5:5	1.00	10	0.226	0.00	0.03	0.01
3	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	8:2:5:5	1.00	11	0.197	0.00	0.01	0.00
4	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	7:3:5:5	0.85	14	0.229	0.01	0.06	0.02
5	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	6:4:5:5	0.82	18	0.197	0.01	0.09	0.04
6	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	5:5:5:5	0.82	33	0.183	0.00	0.01	0.01
7	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	4:5:4:5	1.00	33	0.133	0.02	0.14	0.05
8	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	3:5:3:5	1.00	21	0.152	0.08	0.32	0.27
9	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	2:5:2:5	1.12	18	0.139	0.40	0.97	1.22
10	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O	1:5:1:5	1.00	13	0.112	0.36	0.94	1.41
11	<i>n</i> -Hex-EtOAc-EtOH-H ₂ O-AcOH	1:5:1:5:1	0.97	24	0.119	1.09	1.97	2.45

200 n-Hex: n-hexane. EtOAc: ethyl acetate. EtOH: ethanol. AcOH: acetic acid

2.4. HPCCC separation of the target compounds

Using the previously described optimized conditions, some 100 mg of enriched extract from *D. muscorum* was subjected to HPCCC using the lower phase of the solvent system 10 as mobile phase at a flow rate of 1 mL/min. This operation was repeated 10 times in order to process 1 g of enriched extract. The retention of the stationary phase during the HPCCC separation was 72 %. A representative HPCCC chromatogram is shown in Figure 3a. Three peaks fractions that were eluted within the retention time range from 60 to 80 min, from 110 to 135 min, and from 150 to 170 min corresponded to the compound **1**-containing fraction (**Fr.1**), compound **2** and compound **3**, respectively. They were collected and concentrated under reduced pressure at 38 °C yielding 20 mg of **Fr.1**, 130 mg of **2** (90 % purity) and 8 mg of **3** (90 % purity). The **Fr.1** was further subjected to HPCCC using the solvent system 11 with its lower phase as the mobile phase at a flow rate of 1 mL/min. The target compound **1** was eluted within the retention time range from 172 to 188 min (Figure 3b), which was collected and concentrated at reduced pressure, yielding 3.5 mg of compound **1** (90 % purity). The retention of the stationary phase at the end of the HPCCC separation was 70 %. A representative HPCCC chromatogram is shown in Figure 3b. The HPLC chromatograms of the three target fractions obtained by HPCCC are shown in Figure 4a. In the present study, the isolation of compound **1** is reported for the first time. The separation of these kinds of CLPs with closely related chemical structures demonstrates the powerful selectivity of HPCCC.

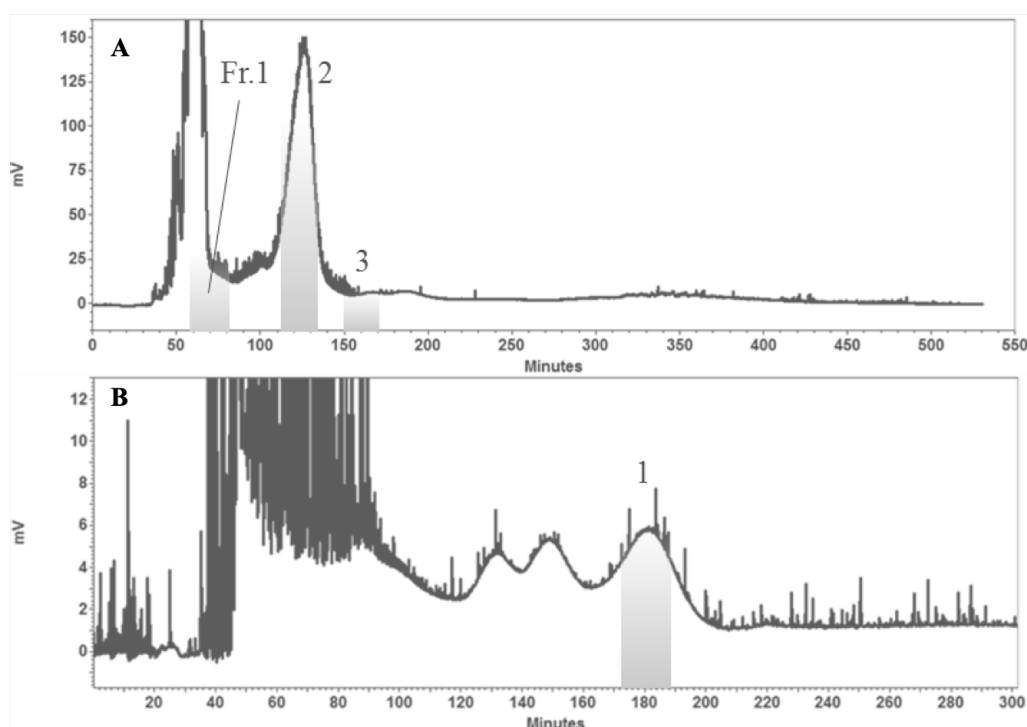


Figure 3. HPCCC chromatograms of enriched extract (a) and compound 1-containing fraction (Fr.1) (b) from *D. muscorum* CICALA 125. For the HPCCC first-step (a): two-phase solvent system, *n*-hexane, ethyl acetate, ethanol, and water (1:5:1:5, v/v/v/v); material to process, 1000 mg of enriched extract; loading per injection, 100 mg in 3 mL lower phase; runs required: 10. For the HPCCC second-step (b): Two-phase solvent system, *n*-hexane, ethyl acetate, ethanol, water and acetic acid (1:5:1:5:1, v/v/v/v); material to process: 20 mg of Fr.1; loading per injection, 20 mg in 3 mL lower phase; runs required: 1. Mobile phase: lower phase. Flow rate, 1 mL/min; rotational speed, 1200 rpm; temperature, 28 °C.

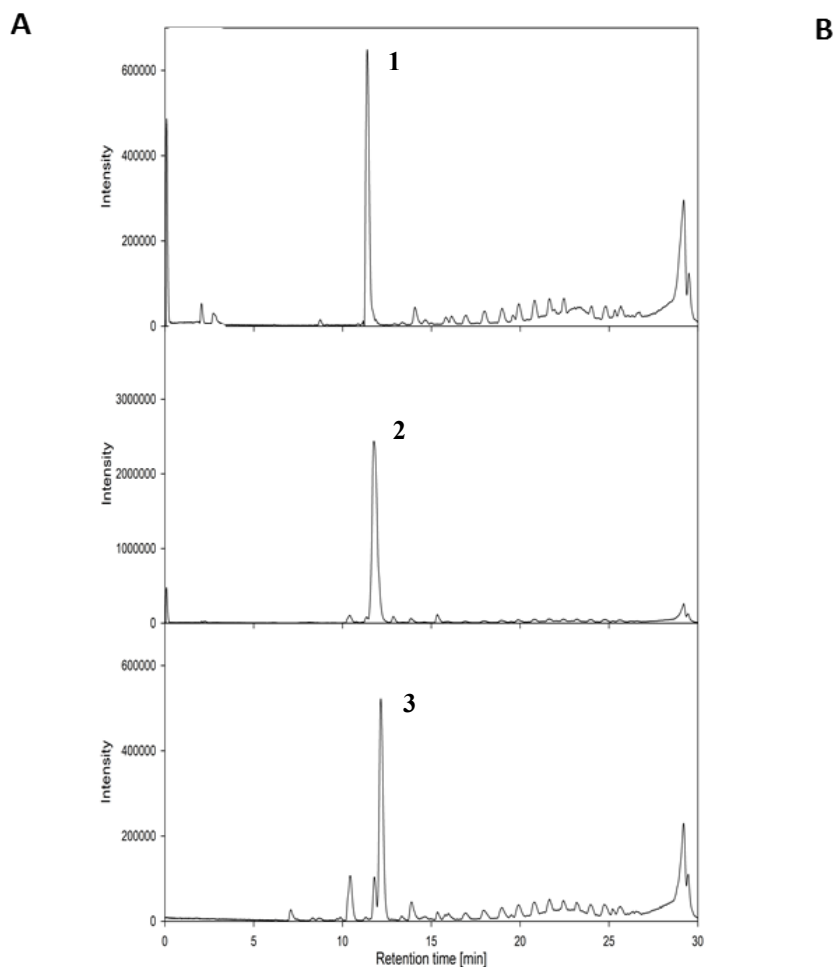


Figure 4. The HPLC–ESI–HRMS chromatograms of muscotoxins separated by HPCCC (a). The HPLC-ESI-HRMS chromatograms of muscotoxins obtained by HPCCC and followed by HPLC purification (b).

2.5. HPLC purification of the compounds obtained from HPCCC

The combined use of HPCCC and HPLC has been successfully applied to the isolation of a number of natural products including CLPs [26]. This chromatographic complementarity is due to HPCCC is considered orthogonal to HPLC, which is explained by the fact that the mechanisms underlying the separation of solutes in both techniques are different. In the present study, the HPCCC peak fractions corresponding to compounds **1** (3.5 mg), **2** (130 mg) and **3** (8 mg) were further cleaned up by HPLC to increase their purity. The HPLC–ESI–HRMS chromatograms of the compounds purified by HPLC are shown in Figure 4b. The purified compounds **1** (0.95 mg, ...% purity), **2** (53.4 mg, ... % purity) and **3** (0.8 mg, % purity) were obtained by HPLC.

2.6. Identification of the target compounds

As can be seen in Table 3, the fragmentation of molecular ions at m/z 1211.67 (**2**) and m/z 1225.68 (**3**) with a collision energy of 60 eV generated fragment ions that were in excellent agreement with those of the characteristic amino acid sequence of muscotoxins A and B [12], respectively. It was observed that muscotoxins A and B differ from each other only in a $-\text{CH}_3$ group (methyl group), as

determined by the substitution of L-proline for γ -methylproline [12]. The fragmentation of molecular ion at m/z 1197.65 (**1**) led to fragment ions [X^1 , Gln^2 , Gly^3 , Pro^4 , Phe^5 , Val^6 , Ser^7 , Dhb^8 , Ser^9 , Ile^{10} , Pro^{11}] (Figure S1), which were consistent with those formed by the successive cleavage of amino acids typically found in muscotoxin A, but differing from each other only in the substitution of Ile^6 for Val^6 in compound **1**. The mass fragmentation spectra of compounds **1-3** are shown in supplementary materials (Figure S1). The structure of **1** was determined by NMR data as shown in supplementary materials (Figure S2).

2.7. *In vitro* antifungal activity against plant pathogenic fungus *Sclerotinia sclerotiorum*.

The three isolated compounds were tested for their antifungal activity against the common plant pathogen *S. sclerotiorum*. The lipopeptides such as iturin and fengycin are important antifungal agents [29]. However, the antifungal potency of the cyanobacterial lipopeptide muscotoxin A is entirely unknown. Our isolation protocol enabled testing antifungal properties not only for the major variant produced by the cyanobacterium *D. muscorum* CCALA 125 but also for the variants occurring in this strain in low quantities. Surprisingly, although the chemical structures of the isolated muscotoxin variants differed only in one amino acid position, we recognized interesting shift of their bioactive effect. Muscotoxin C showed no inhibition even at the highest concentration (Figure 5). Muscotoxin A exhibited inhibition at doses 250 μ g (inhibition of $32 \pm 7\%$) and 100 μ g (inhibition of $22 \pm 2\%$). Finally, slight but significant increase of bioactivity was found for muscotoxin B, which manifested higher inhibition values at 250 μ g and 100 μ g compared to muscotoxin A ($43 \pm 8\%$ and $30 \pm 3\%$, respectively) and the minor inhibition affect was recognized also for the 50 μ g dose ($5 \pm 0.1\%$). Despite the observed antifungal activities of muscotoxin variants are rather moderate; the obtained data about the shift in antifungal activity via exchange of one amino acid residue can provide important information for future development of antifungal agents. Apparently such a minor change as the addition of one methyl group of the peptide core in the vicinity of the fatty acid moiety can improve the antifungal properties of muscotoxin and related compounds. This fact is demonstrated in the case of muscotoxin A and muscotoxin B variants containing proline versus γ -methyl-proline. Although there is a clear evidence showing that the fatty acid part of the molecule is responsible for the antifungal effect in various types of lipopeptides [30], the importance of the peptide core for the bioactivity has been shown in some lipopeptide groups as well [30]. In the case of iturins, the different antifungal activity was attributed to changes in peptide core folding which is necessary for manifesting the activity and the change in one amino acid position can compromise the compound bioactivity. This can be also the case of the isolated muscotoxin variants. The present study shows that the presence of a methyl group in muscotoxins has a key role as a structural pre-requisite for augmenting the antifungal activity of compounds **1-3** against plant pathogenic fungus *S. sclerotiorum*.

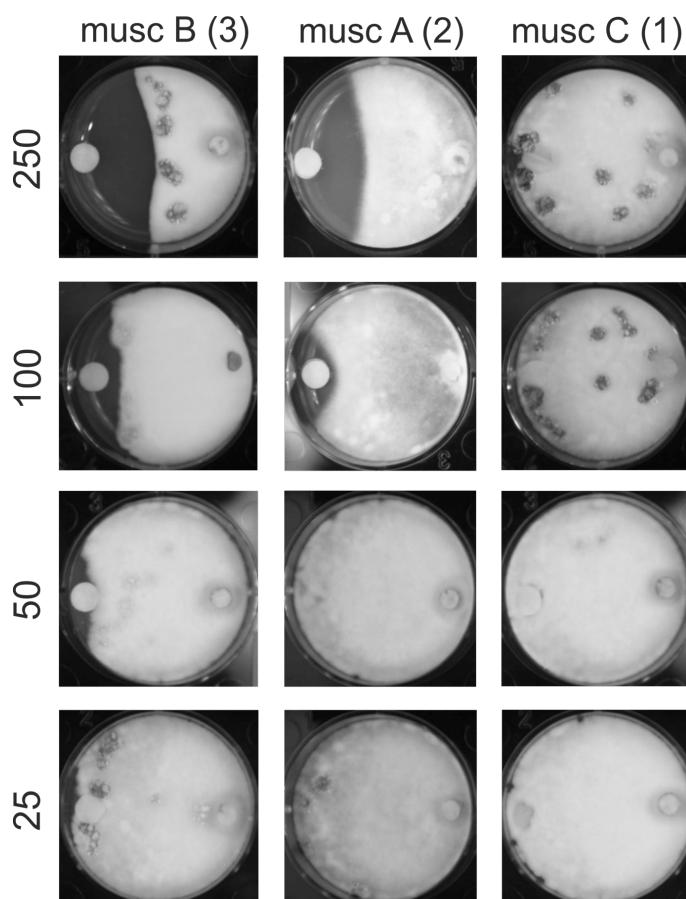


Figure 5. *In vitro* antifungal bioassay against phytopathogenic fungi *S. sclerotiorum*. Increasing antifungal effect was recorded for muscotoxin C, A and B variants, respectively. Numbers in the left part of the graph denote the dose of compound in μg applied on the paper target to the left side of the Petri dish, on the right side of the Petri dish mycelial targets were placed.

Table 3. HPLC-ESI-HRMS measurements of muscotoxins isolated from soil cyanobacterium *Desmonostoc muscorum*

Peaks	Molecular ion [M+H] ⁺ (<i>m/z</i>)	Formula	Fragment ions <i>m/z</i> (ESI HR MS ² spectra) 60 eV	Compounds
1	1197.65	C ₅₇ H ₈₉ N ₁₂ O ₁₆	1098.5947 [M+H-Val]; 1011.5569 [M+H-Val-Ser]; 928.5164 [M+H-Val-Ser-Dhb]; 841.4867 [M+H-Val-Ser-Dhb-Ser]; 728.4033[M+H-Val-Ser-Dhb-Ser-Ile]; 484.276 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro]; 427.2565 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro-Gly]; 302.1512 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro-Gly-Gln]	Muscotoxin C
2	1211.67	C ₅₈ H ₉₁ N ₁₂ O ₁₆	1098.586 [M+H-Ile]; 1011.5527 [M+H-Ile-Ser]; 928.5158 [M+H-Ile-Ser-Dhb]; 841.4829 [M+H-Ile-Ser-Dhb-Ser]; 728.4005 [M+H-Ile-Ser-Dhb-Ser-Ile]; 484.2757 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro]; 427.2547 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro-Gly]; 299.1971 [M+H-Val-Ser-Dhb-Ser-Ile-Phe-Pro-Gly-Gln]	Muscotoxin A [12]
3	1225.69	C ₅₉ H ₉₃ N ₁₂ O ₁₆	1112.6098 [M+H-Ile]; 1025.5732 [M+H-Ile-Ser]; 942.5349 [M+H-Ile-Ser-Dhb]; 855.5052 [M+H-Ile-Ser-Dhb-Ser]; 742.4175 [M+H-Ile-Ser-Dhb-Ser-Ile]; 498.2859 [M+H-Ile-Ser-Dhb-Ser-Ile-Phe-γ-MePro]; 441.2701 [M+H-Ile-Ser-Dhb-Ser-Ile-Phe-γ-MePro-Gly]; 313.1772 [M+H-Ile-Ser-Dhb-Ser-Ile-Phe-γ-MePro-Gly-Gln]	Muscotoxin B [12]

Gln (Glutamine), Gly (glycine), Pro (Proline), γ-MePro (γ-methyl-proline), Phe (Phenylalanine), Ile (Isoleucine), Val (Valine), Ser (Serine), Dhb (2,3-didehydrobutyric acid), Ahdoa (3-amino-2-hydroxydecanoic acid).

322

323 4. Materials and Methods

324 *Chemicals and reagents*

325

326 All organic solvents used in HPCCC separation works were of HPLC grade and purchased from
327 VWR (Leuven, Belgium) and Analytika (Prague, Czech Republic). Methanol used for extraction was
328 obtained from Analytika (Prague, Czech Republic). Acetonitrile and water for HPLC-HRMS analyses
329 were obtained either from Sigma-Aldrich (Germany) or Merck (New Jersey, USA) and were of LC-
330 MS grade purity. Deionized water was obtained using reverse-osmosis (Ultrapur, Watrex, Prague,
331 Czech Republic). Non-ionic polymeric adsorbents (Amberlite XAD16 and Amberlite XAD-7) were
332 from Sigma Aldrich (St. Louis MO, USA).

333

334 *Culture growth conditions and biomass extraction*

335

336 The filamentous cyanobacterium *D. muscorum* CCALA 125 was grown in a glass cuvette (150 L)
337 on A-D Anabaena medium by bubbling CO₂-enriched air (2%) at 28°C for 10 days [12]. Cells were
338 harvested by centrifugation (1500 g, 15 min), frozen at -40 °C, and lyophilized. Freeze-dried biomass
339 was disintegrated with sea sand and extracted with methanol (1L). The extraction was repeated three
340 times on the same biomass. The resulting suspension (3L) was centrifuged (5,000 rpm, 10 min) and
341 the supernatant was subjected to a rotary evaporator under reduced pressure at 40°C for removing
342 methanol. Some 6.200 g of dried crude extract were obtained, which were stored at 2°C for
343 subsequent enrichment in the target compounds.

344

345 *Enrichment of crude extract*

346

347 Two different kinds of non-ionic polymeric resins (XAD-16 and XAD-7 Amberlite resins) were
348 consecutively used for enriching the dried crude extract. Some 6.200 g of dried crude extract were
349 suspended in 500 mL of water, and loaded into an Amberlite XAD-16 resin column (22 cm × 5.5 cm,
350 0.4 Kg resin). The column was thoroughly rinsed with water (1 L) and the aqueous solution (1.5 L)
351 eluted from XAD-16 resin column containing non-adsorbed components was further passed through
352 the XAD-7 resin column (22 cm × 5.5 cm, 0.4 Kg resin). To release the adsorbed target compounds
353 from the resins, methanol was used as eluent (1.5 L, each column). The resulting methanol eluates
354 from XAD-16 and XAD-7 resins column were separately subjected to a rotary evaporator under
355 reduced pressure at 40°C for removing methanol, and subsequently analyzed by HPLC-ESI-HRMS
356 for determining the peak purity of the target compounds and their recovery (%). The isolated target
357 compounds were used as reference compounds for quantification purposes. The recovery values of
358 the target compounds were estimated using the following equation:

359

360
$$\text{Recovery (\%)} = \left(\frac{P2 \times W2}{P1 \times W1} \right) \times 100$$

361

362 where P1 is the concentration (mg mL⁻¹) of the target compound in the crude extract before
363 passing through resins; W1 the amount of the crude extract before passing through resins; P2 the
364 concentration (mg/mL) of compounds in the extract desorbed from resins, and W2 is the amount of
365 extract desorbed from resins.

366

367 *HPCCC separation*

368

369 *HPCCC Apparatus*

370

The separation of the target compounds from the enriched extract was performed on a HPCCC apparatus (Model Spectrum, Dynamic Extractions Ltd., Slough, UK) using a semi-preparative column with a total volume of 134 mL (PTFE bore tubing = 3.2 mm). The β -value range varied from 0.52 at internal to 0.86 at the external terminal ($\beta = r / R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation of the column was controlled by a speed regulator installed into the HPCCC apparatus. A H50/H150 Smart Water Chiller (LabTech Srl, Sorisole Bergamo, Italy) was used for adjusting the temperature of separation work. The stationary and mobile phases were pumped with a Q-Grad pump (LabAlliance, State College, PA, USA). The effluent from the column was continuously monitored with a Sapphire UV-VIS spectrophotometer (ECOM spol. s.r.o., Prague, Czech Republic) operating at 240 nm. The chromatographic run was processed using an EZChrom SI software platform (Agilent Technologies, Pleasanton, CA, USA).

Selection of the two-phase solvent system

Different biphasic solvent systems composed of *n*-hexane, ethyl acetate, ethanol, water and acetic acid [13] were prepared (Table 1) and tested for their capacity to provide a proper partition coefficient (K) of the target compounds as well as a short settling time and an adequate density difference. The partition coefficient (Table 2) was determined by adding 2 mg of extract into a test tube containing 2 mL of two-phase solvent system (1 mL per each phase). The test tube was shaken vigorously and then left to equilibrate the two phases for 20 min. Equal volumes of each phase were then analyzed by HPLC-ESI-HRMS to obtain the partition coefficients (K), which were calculated by dividing the peak area of the target compound in the upper phase by the peak area of compound in the lower phase. The settling time of each biphasic solvent system was calculated as stated in a previous report [27]. The density difference between the upper and lower phases of each biphasic solvent system was estimated by weighting 1 mL of each phase with a micro balance.

Preparation of the two-phase solvent system and sample solution

The best biphasic solvent system selected from the previous step was prepared at a large scale for separating the target compounds by HPCCC. The components of the selected system were added into a separating funnel and shaken vigorously and then were left to equilibrate for 20 min. The upper and lower were separated and degassed by sonication shortly before their use. The sample solution was prepared by dissolving the enriched extract in 3 mL of the lower phase of the selected solvent system.

HPCCC separation procedure

A two-step HPCCC method was used for the separation of the target compounds from the enriched extract. In the HPCCC first step, the solvent system composed of *n*-Hex–EtOAc–EtOH–H₂O (1:5:1:5, v/v/v/v) was used in reverse phase for obtaining compounds **2-3** and the **1**-containing fraction. In the HPCCC second step, the solvent system composed of *n*-Hex–EtOAc–EtOH–H₂O–AcOH, 1:5:1:5:1 was used in reverse phase for obtaining the compound **1**. The elution mode in reverse phase implies that the lower phase of the solvent system is used as the mobile phase and the upper phase as the stationary phase. At the beginning, the HPCCC column was filled with the upper phase (stationary phase) and it was rotated at 1200 rpm. After that, the lower phase (mobile phase) was pumped through the column at a flow rate of 1 mL/min. When reaching the hydrodynamic equilibrium, which is the steady stage when a clear mobile phase is eluted from the column, the sample solution was injected through the injection valve. The temperature of the apparatus was set at 28°C. The fractions collected from HPCCC were analyzed offline by HPLC-ESI-HRMS. The retention of the stationary phase (S_f) during the HPCCC run was calculated as follows:

$$Sf (\%) = \frac{V_s}{V_c} \times 100$$

where V_c is the known column volume and V_s is the volume of the stationary phase in the column.

Subsequent purification by using preparative HPLC

The HPCCC peak fractions corresponding to the target compounds were re-purified on a Reprosil 100 C18 column (250×4mm 5μm) using an Agilent 1100 HPLC system coupled with a diode array detector (DAD) with a mobile phase composed of acetonitrile (A) and water (B) using the following gradient: 0–2 min, 70% B; 2–6 min, 70%–60% B; 6–15 min, 60%–30% B; 15–16 min, 30%–0% B; 16–20 min, 0%–0% B; 20–21 min, 0%–70% B. The mobile phase was pumped at a flow rate of 2 mL min⁻¹ and the column temperature was set at 28°C. The DAD detector was set at 240 nm to record the peaks, and the UV–Vis spectra were recorded from 200 to 700 nm.

HPLC-ESI-HRMS analysis of extracts and HPCCC fractions

The analysis of extracts and HPCCC fractions was performed using an Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) coupled with a diode array detector (DAD) and high resolution mass spectrometer with electrospray ionization source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker, Billerica, MA, USA). The separations were performed on a reversed phase column (Phenomenex Kinetex C18 column, 150 × 4.6 mm, 2.6 μm) held at constant temperature of 30 °C. The chromatographic run was performed using as mobile phase the combination of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) using the following gradient: 0–1 min, 85% A; 1–20 min, 85%–0% A; 20–25 min, 0% A; 25–30 min, 0%–85% A, which was pumped at a constant flow rate of 0.6 mL min⁻¹. The source parameters were as follows: the spray needle voltage was set at 3.8 kV, nitrogen was used both as the nebulizing gas (3 bar) and the drying gas (12 L/min) and the temperature was 210 °C. Nitrogen was used as collision gas to induce fragmentation of molecular ions. The collision energy was set to 60 eV for the determination of cyclic oligopeptide part. The scanning range was 50–2,600 m/z operating in the positive ion mode. The DAD detector was set at 240 nm to record the peaks, and the UV–Vis spectra were recorded from 200 to 700 nm.

Structural identification of the isolated target compounds

The chemical identity of the target compounds was determined by ESI-HRMS and NMR analysis in comparison with literature data [12]. The MS data compounds **1–3** are presented in Table 3, and their corresponding mass fragmentation spectra are shown in supplementary materials (Figure S1). The structure of compound **1** was determined by NMR data as presented in supplementary materials (Figure S2). NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with TCI CryoProbe (600.23 MHz for ¹H, 150.93 MHz for ¹³C, Bruker Biospin GmbH, Rheinstetten, Germany) in CD₃OD, 303.2 K. The residual solvent signals were used as an internal standard (δ_H 3.330 ppm and δ_C 49.05 ppm). ¹H NMR, ¹³C NMR, COSY, TOCSY, ¹H-¹³C HSQC, ¹H-¹³C HMBC, ¹H-¹³C HSQC-TOCSY and *J*-resolved spectra were measured using the standard manufacturer's software. The ¹H NMR spectrum was zero filled to 2-fold data points and multiplied by a window function (two parameter double-exponential Lorentz-Gauss function) before Fourier transformation to improve the resolution. The ¹³C NMR spectrum was zero filled to 2-fold data points. Subsequently, the line broadening (1 Hz) was used to improve signal-to-noise ratio. Protons were assigned by COSY, TOCSY, and HSQC-TOCSY and assignment was transferred to carbons by HSQC. The

chemical shifts are given on the δ scale (ppm) and coupling constants are given in Hz. The digital resolution allowed us to present the proton and carbon chemical shifts to three or two decimal places. The proton chemical shift readouts from HSQC are reported to two decimal places. The operating conditions of the ESI-HRMS NMR instruments are indicated in the previous section.

In vitro antifungal disc bioassay and evaluation of antifungal activity

The isolated compounds were dissolved in MeOH at different concentrations and further transferred (20 μ L) on layer filter paper targets (size 5 mm). For the minimal inhibitory concentration, the following doses were applied: 250, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μ g. The organic solvent was evaporated from the targets, and then each one was placed on Czapek-Dox medium in Petri dish (on average 35 mm) at the border of dishes. The mycelial target of the plant pathogenic fungus *S. sclerotiorum* (0.5 cm in diameter) was inoculated on the opposite sites of dish. The fungicide RANCONA 15 ME (active substance – ipconazole, Crompton Registration Ltd.) and methanol were used as positive and negative control, respectively. Each experiment was performed in triplicates. The growth of fungal mycelium after 4 days in the presence of the studied compounds was evaluated using digital image analysis. The macro-optic system, consisting of a digital camera DUS-1 (5Mpx) and objective lens type Cosmocar (Nikon Instruments, CS-Optoteam Ltd., Prague, Czech Republic) were used for acquisition of images of the Petri dishes. The images (TIFF format) were converted by thresholds and the area of binary objects, corresponding to size of grown mycelium, were measured and evaluated by software NIS-ELEMENTS AR ver. 3.2 (LIM - Laboratory Imaging Ltd. Prague, Czech Republic) and by a method published previously [31]. The inhibition of the mycelium growth (inhibition zone in %) was calculated as the difference between the measurements (in px) of the total area of fungal mycelium (negative controls) and of the area that is covered with mycelium (treated dishes).

5. Conclusions

Three CLPs (muscotoxins A-C) were isolated and purified from the filamentous cyanobacterium *Desmonostoc muscorum* by the combined use of adsorption on polymeric resins, HPLC and HPLC methods. As these three chromatographic methods are recognized to be orthogonal against each other; therefore, their combined use rendered the separation of closely chemically related compounds. Muscotoxin C was isolated for the first time and its chemical structure was established by MS and NMR spectral data. The described isolation method is an efficient approach for obtaining CPLs from cyanobacterial biomass and represents a methodological reference than might be scaled up for obtaining these compounds at higher amounts. The isolated compounds were tested for their antifungal activity against the common plant pathogen *S. sclerotiorum* showing how a methyl group, so often visualized as chemically inert, is able to influence the biological properties of CLPs.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: mass fragmentation spectra of compounds 1-4. Figure S2: NMR data of isolated compound 1.

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