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Abstract

Puwainahycins, a recently described group of *β*-amino fatty acid cyclic lipopeptides, are secondary metabolites of cyanobacterial origin possessing interesting biological activities. Therefore, the development of an efficient method for their isolation from natural sources is necessary. Following the consecutive adsorption of the crude extract on Amberlite XAD-16 and XAD-7 resins, high performance countercurrent chromatography (HPCCC) was applied to separate seven puwainaphycin variants from soil cyanobacterium (*Cylindrospermum alatosporum* CCALA 988). The resin-enriched extract was first fractionated by HPCCC into fraction I and II using the system *n*-hexane–ethyl acetate–ethanol–water (1:5:1:5, v/v/v/v) at a flow rate of 2 mL min^{-1} and rotational speed of 1,400 rpm. The fraction I was subjected to HPCCC, using the system ethyl acetate–ethanol–water (5:1:5, v/v/v/v), affording the compounds **1** and **2**. The fraction II was subjected to HPCCC, using the system *n*-hexane–EtOAc–ethyl acetate–water (1:5:1:5, v/v/v/v), affording the compounds **3**-**7**. In both cases, the lower phases were employed as mobile phases at a flow rate of 1 mL min^{-1} with a rotational speed of 1,400 rpm and temperature of 28 °C. The **1**, **2** and **5** target fractions obtained by HPCCC were re-purified by semi-preparative HPLC. As a result, compounds **1-7,** with purities of 95%, 95%, 99%, 99%, 95%, 99% and 90%; respectively, were isolated with a combination of HPCCC and HPLC. The chemical identity of the isolated puwainaphycins (**1**-**7**) was confirmed using ESI-HRMS and NMR analyses. Three new puwainaphycin variants (**1**, **2** and **5**) with hydroxy- or chloro- substitution on their fatty acid side chains are reported for the first time. As such, this study supplies a new approach for the isolation of puwainaphycins from cyanobacterial biomass.

Keywords cyclic lipopeptides, puwainaphycins, cyanobacteria, high performance countercurrent chromatography,

Introduction

Cyanobacteria (also known as blue-green algae) are being recognized as a promising source of novel pharmaceutical lead compounds and secondary metabolites of a large structural diversity [1, 2]. A hardly explored group of cyanobacterial secondary metabolites are cyclic lipopeptides (CLPs). These compounds are composed of a modified fatty acid tail linked to a short oligopeptide which forms a peptidic macrocycle. The cyclic part of these structures has been found to comprise up to 14 amino acid residues, which are often represented by non-standard amino acids. The presence of unusual amino acids is explained by the fact that CLPs are biosynthesized by non-ribosomal pathways [3] analogously to many other cyanobacterial peptides [4] [5]. CLPs have been reported to exhibit antifungal [6], antibiotic [7], cytotoxic [8] and antiproliferative activities [5, 9]. Cyclosporine A, a widely used immunosuppressant agent, is plausibly the CLP of major relevance in medicine [10]. Daptomycin represents the first new class of antibiotic CLP that was approved for clinical use in the past decade [4]. Although daptomycin was first reported to be toxic, its administration scheme was optimized giving rise to the amelioration of the adverse effects [11].

Puwainaphycins are CLP of cyanobacterial origin, which are composed of ten amino acid units and a *β*-amino fatty acid. The *β*-amino fatty acid is incorporated into the cycle by two peptide bonds formed from a carboxyl group and a *β*-amino group [3]. Puwainaphycins A−E have been isolated from the soil cyanobacterium *Anabaena* sp. [12, 13] and their structures were determined by ESI-HRMS, and 1D and 2D NMR, and the stereo configurations were assigned by advanced Marfey's analysis after acid hydrolysis. Our group reported the obtaining, identification and structural elucidation of puwainaphycin F and G as a mixture [8] from cyanobacterial strain *Cylidrospermum alatosporum* CCALA 988 (C24/1989). These two compounds were observed to differ in the substitution of asparagine by glutamine. The isolation of a structural variant of puwainaphycin F from the same cyanobacterial strain, differing in length of fatty acid chain and its substitution, was also reported by our group [3]. More recently, a number of minor puwainaphycin variants have been detected in *C. alatosporum* CCALA 988 by HPLC-HRMS/MS [14], most of which have been neither isolated nor biologically assessed due to their occurrence in the cynobacterial biomass at tiny concentrations. So far, puwainaphycins have only been obtained

from the biomass of cyanobacteria by using conventional separation methods, including solid-phase extraction, a column of C-18 silica and HPLC [8,12,13]. However, these methods were neither sufficiently resolutive for puwainaphycins F and G nor preparative enough for obtaining minor puwainaphycin variants present in cyanobacteria. Puwainaphycins share common chemical features with approved therapeutic agents and may lead to the development of a novel class of antifungal and antibiotic agents. Moreover, they offer opportunities for designing combinatorial biosynthesis strategies to improve their potential therapeutic efficacy or to solve potential toxicity aspects**.** Therefore, the development and application of a high-throughput chromatographic method for the separation of puwainaphycins from complex matrices is pivotal in order to enable extensive *in vitro* and *in vivo* studies targeted to drug discovery.

High performance countercurrent chromatography (HPCCC) is a liquid-liquid chromatographic technique that uses a support-free liquid stationary phase. Two immiscible liquid phases are used for the separation of target compounds. One of the two liquid phases (the stationary phase) is retained in the column by centrifugal force, while the other (the mobile phase) is pumped through the column [15]. In HPCCC, the chromatographic separation is based on the different partition coefficients of each target compound in these two immiscible phases. Given that HPCCC uses a liquid stationary phase without solid support, consequently the method offers many advantages over traditional solid liquid chromatography, such as the absence of irreversible adsorption of target molecules; high loading capacity; total recovery of the injected sample; low risk of sample denaturation; and low solvent consumption [16]. This chromatographic method is considered a cost-effective, high-throughput and scalable technology for the extraction of bioactive substances from natural sources. HPCCC has been successfully applied to the isolation and purification of a wide variety of bioactive components from higher plants [17–21] and cyanobacteria [22, 23]. So far, there is no report on the separation of cyclic lipopeptides from cyanobacteria biomass by using HPCCC. Herein, we report the separation of seven puwainaphycins from cyanobacteria by HPCCC combined with microporous resin treatment and HPLC.

Materials and methods

Chemicals and reagents

All organic solvents used in the experiments were of HPLC grade and purchased from Scharlab S.L. (Barcelona, Spain) and Analytika (Prague, Czech Republic). Organic solvents used for extraction and HPLC analyses were obtained from Analytika (Prague, Czech Republic). Acetonitrile and water for HPLC-HRMS analyses were obtained either from Sigma-Aldrich (Germany) or Merck (New Jersey, USA) and were of LC-MS grade purity. Solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, Czech Republic). Polymeric resins (Amberlite XAD16 and XAD-7) were purchased from Sigma Aldrich (St. Louis MO, USA).

Culture growth conditions

The cyanobacterial strain *Cylindrospermum alatosporum* CCALA 988 (C24/89) was grown in 200 mL tubes on liquid Alen−Arnold medium [41] at 30°C, constant irradiation of 150 μE and mixing with 2% CO_2-enriched air for 10−14 days. The resulting biomass suspension was transferred to 15 L photobioreactor for larger scale cultivation under the same conditions. Cells were harvested by centrifugation (1500 g, 15 min), frozen at −40 °C, and lyophilized.

Biomass extraction

Freeze-dried biomass of cultivated *Cylindrospermum alatosporum* C24/89 (ca. 32 g) was homogenized with sea-sand and then extracted with methanol (800 mL). The extraction was repeated with methanol (800 mL), three times. The obtained suspension was separated from the solid material by centrifugation (5,000 rpm, 10 min). The extraction procedure was repeated 3 times with a same amount of methanol. The resulting methanolic extracts were combined, and concentrated under reduced pressure at 40°C, yielding 5.543 g of green dried extract, which were stored in the refrigerator for its subsequent enrichment in the target compounds.

Enrichment of crude extract

The dried crude extract was consecutively enriched on non-ionic polymeric resins (XAD-16 and XAD-7 Amberlite resins). Some 5.543 g of crude extract were dissolved in 500 mL of water, and passed through an Amberlite XAD-16 resin column (22 cm × 5.5 cm, 0.4 Kg resin). The column was then washed with water (1 L). The resulting aqueous solution (1.5 L), which contained non-adsorbed components on XAD-16 resin, was re-loaded into a XAD-7 resin column (22 cm × 5.5 cm, 0.4 Kg resin). Finally, the adsorbed materials were desorbed from XAD-16 and XAD-7 resins with methanol (1.5 L, each column). The resulting methanolic eluates were separately evaporated to dryness under reduced pressure at 35°C, and subsequently analyzed by HPLC-ESI-HRMS. The peak purity of the target compounds in the resins-treated extracts was estimated by HPLC-HRMS and their recovery (%) after treatment by resins was calculated by external-standard HPLC-HRMS method using the isolated target compounds as reference compounds. Recoveries of puwainaphycins were calculated as follows:

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

where P1 is the concentration of the target compound in crude extract before absorption on resins; W1 the amount of crude extract before absorption on resins; P2 the concentration of compound in the extract after absorption on resins, and W2 is the amount of extract after absorption on resins.

HPCCC separation

HPCCC Apparatus

The separation of target compounds from enriched extract was performed by high performance countercurrent chromatography (HPCCC), which consisted of a semi-preparative apparatus of Model Spectrum (Dynamic Extractions Ltd., Slough, UK). The equipment uses two columns connected in series (PTFE bore tubing = 3.2 mm, total volume = 134 mL). The β-value range varied from 0.52 at internal to 0.86 at the external terminal (β = 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 rotational speed was controlled by a speed regulator installed into the HPCCC chassis. The experimental temperature was adjusted by a H50/H150 Smart Water Chiller (LabTech Srl, Sorisole Bergamo, Italy. A Q-Grad pump (LabAlliance, State College, PA, USA) was used both to fill the columns with the stationary phase and elute the mobile phase. The effluent was continuously monitored by a Sapphire UV-VIS spectrometer (ECOM spol. s.r.o., Prague, Czech Republic) operating at 240 nm. The EZChrom SI software platform (Agilent Technologies, Pleasanton, CA, USA) was used to record the HPCCC chromatograms.

Selection of the two-phase solvent system

Different solvent systems composed of *n*-hexane, ethyl acetate, *n*-butanol, ethanol, water and acetic acid were prepared in accordance with a pre-defined list of solvent systems reported in literature [24], with some modifications (Table 1). The partition coefficient (*K*) of target compounds in each of the selected biphasic systems (Table 2) was determined as follows: 2 mg of extract was dissolved in 1 mL of each phase of the pre-equilibrated two-phase solvent system. The samples were shaken vigorously and allowed to stand for 30 min. The two phases were separated and analyzed by HPLC-ESI-HRMS. The *K* values of target compounds were calculated as the ratio of the peak areas of the target molecular ions obtained from the extracted ion chromatogram. K = AU/AL, where AU is the peak area of the selected ion in the upper phase, and AL, is the peak area of the selected ion in the lower phase. The settling time of each solvent system was determined according to previously reported method [25]. The density difference between the upper and lower phases was also measured by weighing l mL of each phase with a micro balance.

Preparation of the two-phase solvent system and sample solution

The selected solvent systems for HPCCC separation of target compounds were prepared by vigorous mixing of the corresponding portions of solvents. The phases were left to equilibrate overnight and separated using a separating funnel. Shortly before their use, the phases were

degassed by sonication. The sample solution was prepared by dissolving the extract in 6 mL (for HPCCC fractionation) or 3 mL (for HPCCC separation) of the lower phase of the selected solvent system. The resulting sample solution was filtered through a 0.45 μm membrane before use.

HPCCC separation procedure

The enriched extract was first fractionated by HPCCC. The resulting fractions were further subjected to HPCCC for the separation of target compounds. For the HPCCC fractionation, the solvent system composed of *n*-Hex–EtOAc–EtOH–H_2O (1:5:1:5, v/v/v/v) was used in reverse phase. For HPCCC separations, the two solvent systems composed of EtOAc–EtOH–H_2O (5:1:5, v/v/v/v) and *n*-Hex–EtOAc–EtOH–H_2O (1:5:1:5, v/v/v/v) were used in reverse phase. When working in reverse phase, the lower phase of the solvent system is used as the mobile phase and the upper phase as the stationary phase The HPCCC column was initially filled with the upper phase (stationary phase). The HPCCC column was rotated at 1,400 rpm, and the lower phase (mobile phase) was pumped into the column at 2 mL min^{-1} and 1 mL min^{-1} for the HPCCC fractionation and HPCCC separation operations, respectively. After the mobile phase front emerged and hydrodynamic equilibrium was reached, the sample solution was injected through the sample injection valve. The effluent from the outlet was continuously monitored at 240 nm. The temperature of the apparatus was set at 28°C. Fractions were collected manually according to the chromatographic profile and then analyzed by HPLC-ESI-HRMS. The retention of the stationary phase (S*f*) was estimated both after the hydrodynamic equilibrium was reached and at the end of the HPCCC separation run. The S*f* value (%) was calculated as follows:

$$Sf\ (\%) = \frac{\mathrm{Vs}}{\mathrm{Vc}} \times 100$$

where Vc is the known column volume and Vs is the volume of the stationary phase in the column.

Subsequent purification by using preparative HPLC

The target peak fractions obtained with HPCCC were further purified using an Agilent 1100 HPLC system equipped with a diode array detector (DAD). The **1** and **5** HPCCC peak fractions were purified through a Reprosil 100 C18 column (250×4mm 5µm) held at constant temperature of 28°C, and 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, which was pumped at a constant flow rate of 2 ml/min. The **2** HPCCC peak fraction was purified through a Reprosil 100 Phenyl column (250×8mm 5µm), held at constant temperature of 28°C. For compound 2, the mobile phase was composed of acetonitrile (A) and water (B) using the following gradient: 0–2 min, 70% B; 2–6 min, 70%–55% B; 6–13 min, 55%–45% B; 13–15 min, 45%–0% B; 15–16 min, 0%–0% B; 16–18 min, 0%–70% B; 18–19 min, 70%–70% B, which was pumped at a constant flow rate of 2 mL min^{-1}. For compound 3, 6 and 7 the mobile phase was composed of methanol (A) and water (B) using the following gradient: 0–2 min, 50%–30% B; 2–15 min, 30%–10% B; 15–16 min, 10%–0% B; 16–20 min, 0%–0% B; 20–20.1 min, 0%–50% B, which was pumped at a constant flow rate of 2.4 mL min^{-1}.

HPLC-ESI-HRMS analysis of extracts and fractions obtained by HPCCC and prep-HPLC

A Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped 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) was used for the analysis of the crude extract and fractions. 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 mobile phase was made up from 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 operating parameters of the mass spectrometer were as follows: the spray needle voltage was set at 3.8 kV, nitrogen was used both as nebulizing gas (3 bar) and drying gas (12 L min^{-1}), and the drying temperature was 210°C. For fragmentation of selected molecular ions, nitrogen was used as collision gas. The collision energy was set to 60-70 eV for the determination of cyclic oligopeptide part and to 100 eV for determination of β-amino fatty acid part. The scanning

range was 50–2,600 m/z and the scanning rate 2 Hz 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. Identification of target compounds was performed by comparing their MS data (molecular and fragment ions) with those previously reported in literature [14].

Chemical identification of isolated target compounds

The isolated target compounds were structurally identified using ESI-HRMS and NMR experiments. The NMR spectra were recorded on a Bruker Avance III 700 MHz spectrometer equipped with TCI CryoProbe (700.13 MHz for 1H, 176.05 MHz for 13C, Bruker Biospin GmbH, Rheinstetten, Germany) in DMSO-d_6 303.2 K. The residual solvent signals were used as an internal standard (δ_H 2.499 ppm and δ_C C 39.46 ppm). ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, *J*-resolved, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC-TOCSY, band selective ^{1}H-^{13}C HMBC, ROESY, NOESY, and 1D-TOCSY spectra were measured using the standard manufacturer's software. The ^{1}H 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 ^{13}C NMR spectrum was zero filled to 2-fold data points. Subsequently, the line broadening (1 Hz) was used to improve signal-to-noise ratio. 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. MS data for each compound are shown in Table 3. Complete NMR data of the compounds are presented in Supplementary Data.

Results and discussion

HPLC-ESI-HRMS analysis of crude extract

The identities of seven target compounds in the crude extract were confirmed on the basis of their MS spectra after comparison with data reported in literature [3, 8, 14]. The molecular ion peaks corresponding to the seven target puwainaphycins are shown in the BPC chromatogram (Fig. 1a). Signals of molecular ion peaks were selectively retrieved from the BPC chromatogram and used

for detection purposes. As depicted in the Figure 1b most of the target compounds were observed to be poorly UV absorbing compounds. Compounds **3** and **6** were eluted closely with minor target compounds **4** and **7**, respectively. Compounds **1**, **2** and **5**, are three minor puwainaphycin variants whose *β*-amino fatty acid side chain have not been fully structurally elucidated by ESI-HRMS [14]. Accordingly, the enrichment of the crude extract in the seven target compounds and their separation by HPCCC were applied.

Enrichment of crude extract

To enrich the crude extract in the seven target compounds, two kinds of polymeric resins were used. Appropriate resin selection is crucial to assure an efficient recovery and purification of target compounds from complex matrices. XAD-2 polymeric resin has been used for the purification of puwainaphycin C from cyanobacterial extracts [13]. XAD-16 resin has been used for enriching bacterial extracts in cyclic peptides by removing water-soluble impurities [26-28]. The consecutive adsorption on XAD-2 and XAD-7 resins has been successfully applied for enriching a bacterial extract in cyclic peptides [28]. XAD-2 Amberlite resin is chemically similar to XAD-16 Amberlite resin, as both resins are made of styrene-divinylbenzene; however, XAD-16 resin is considered to be more efficient due to its higher surface area. In the current study, *C. alatosporum* crude extract was enriched by consecutive adsorption on XAD-16 and XAD-7 polymeric resins. As a result, 757 mg and 190 mg of enriched extracts were obtained from XAD-16 and XAD-7 resins, respectively. As observed in the chromatograms (Fig. 1c, d), XAD-16 and XAD-7 enriched extracts contained the seven target compounds and exhibited qualitatively similar profiles. The peak purities of compounds **1**-**7** in the XAD-16 enriched extract were higher than those found in the crude extract (Table 1). Conversely, no increase of the target peak purity in the XAD-7 enriched extract was observed due to the presence of an increased relative abundance of contaminants eluting after 15 min. Peak purity values reflected the presence of other contaminants in the sample, but did not account for the amount compounds recovered by resins. Thus, to determine possible losses of target compounds during the pre-purification by resins, the recovery (%) was calculated by using an external standard HPLC-HRMS method. Seven experimental points were employed for establishing calibration curves. The regression lines for compounds **1**, **2**, **3**, **4**, **5**, **6** and **7** were y= 903.89x + 4677.3 (R^2 = 0.9998), y =709,79x + 4305.403 (R^2 = 0.9961), y = 3793.3x+8162.2 (R^2 =

0.9997), y = 4603.6x-17111.0 (R2 = 0.9996), y = 903,89x + 4677,3 (R^2 = 0.9998), y = 1930,1x +44729.0 (R^2 = 0.8954) and y = 4555,2x +5387,7 (R^2 = 0.9900), respectively, where y was the peak area of analyte, and x was the concentration of analyte (μg/mL). We found out total recoveries between 35 and 77 % of the target compounds. Recovery data are presented in Table 1. The XAD-16 resin was found to significantly contribute to the total recovery of puwainaphycins. Given that both XAD-16 and XAD-7 enriched extracts exhibited similar chromatographic profiles, they were pooled affording 900 mg of enriched extract, which was subsequently used for the HPCCC separation of the target compounds. Some 900 g of resin-treated extract containing the seven target compounds were obtained after treatment of 5.5 g of crude extract, which demonstrated the enriching effect of the operation.

Optimization of HPCCC conditions

In order to perform a successful HPCCC separation, the searching for a suitable two-phase solvent system is the first and crucial step, which is estimated to represent about 90% of the whole HPCCC separation work [15]. An ideal two-phase solvent system for HPCCC separation should exhibit a short settling time (< 30 s) and provide a partition coefficient (K) of the target compounds within the range $0.5 \leq K \leq 2.5$. Additionally, the separation factor (α) between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be greater than 1.5 [15, 25]. Compounds with smaller K values elutes closer to the solvent front with lower resolution while a larger K value gives better resolution, but broader peaks and more dilute peak fractions due to a longer elution time [15]. In HPCCC, it has been demonstrated that the peak resolution is improved when the volume of the stationary phase retained in the HPCCC column is increased [29]. Moreover, the settling time of a two-phase solvent system is inversely correlated with the amount of stationary phase retained inside the HPCCC column [15, 25].

In the present study, a series of two-phase solvent systems (Table 2) were examined to optimize the K values of the target compounds. Habitually, HPLC-UV is used to measure K values by comparing the area under the compound peak in each phase. However, some impurities may co-elute with the target compounds leading to an imprecise measure of peak areas. Additionally, the quantity of some analytes might be insufficient to obtain well resolved UV peak. Therefore, in

order to obtain an accurate partition coefficient of both major and minor puwainaphycin variants, HPLC-ESI-HRMS was used for determining the *K* values. In this way, the molecular ions of the target compounds were selectively retrieved from the BPC chromatogram. As shown in the Table 2, the system 10 (*n*-Hex–EtOAc–EtOH–H_2O in ratio 1:5:1:5) provided suitable *K* values and good separation factors (α) for compounds **3**-**7**, as well as a short settling time. However, it gave small *K* values ($K \leq 0.5$) for the compounds **1** and **2**, therefore they would elute close to the solvent front and with a lower resolution. It was concluded that for obtaining proper *K* values systems, the solubility of compounds **1** and **2** should be favoured to the upper phase. Thus, solvent systems of increased polarity (systems 12-16, Table 2) were tested. Systems 12-16 provided too high *K* values for compounds **1** and **2**, because the solubility of the target compounds was extremely favoured to the upper phase (organic phase). Thus, another strategy for optimizing the *K* values of compounds **1** and **2** consisted in increasing the polarity of the system 10 by removing *n*-hexane from its global composition. Thus, the system 10 was converted to the system 17 (EtOAc-EtOH-H_2O 5:1:5 v/v/v). As shown in the Table 2, this modification favored the solubility of compounds **1** and **2** in the upper phase of the system 17 in such a way that appropriate *K* values, a good separation factor ($\alpha = 2.77$) and a short settling time were obtained. Unlike system 10, the density difference between the two immiscible phases composing the system 17 was slightly lower, but sufficiently convenient to maintain stationary phase inside the column. Overall, the results indicated that two groups of compounds of differing polarity, i.e. one group (fraction I) composed of compounds **1** and **2** and the other one (fraction II) composed of compounds from **3** to **7** were present in the enriched extract. The system 10 was found to be selective for compounds **1** and **2**, while system 17 was selective for compounds **3**-**7**. A single solvent system would not be enough to cover the separation of the seven target compounds. Therefore, a previous fractionation of the enriched extract was necessary, followed by the separation of the target compounds from fractions I and II in independent operations. Since the system 10 was observed to efficiently divide the extract into fractions I and II, it was thus selected for fractionation purposes.

It has been well established that a high retention of the stationary phase affords a good peak resolution in HPCCC [29]. Thus, given that the rotational speed and flow rate can affect the retention of the stationary phase; therefore, these parameters were optimized. It is worth mentioning that in HPCCC a high rotational speed favours the retention of the stationary phase,

but a high flow rate is detrimental to the retention of the stationary phase. Conversely, a low flow rate can produce good separations, but leads to long elution times. In the present study, different rotational speeds (1200-1500 rpm) and flow rates (1-3 mL min^{-1}), at reverse phase, were examined to optimize separation conditions. For fractionation purposes, where the system 10 was selected, the results showed that when the flow rate and the rotation speed were 2 mL min^{-1} and 1400 rpm; respectively, the S*f* value was 68.66 %. Thus, a fast and efficient fractionation of the enriched extract could be achieved under these conditions, where the separation of enriched extract into fraction I and II is only required. On the other hand, for the purpose of separation of target compounds, suitable S*f* values with the systems 10 (S*f*=76.12%) and 17 (S*f*=74.63%) were reached at a flow rate of 1 mL min^{-1} and with a rotational speed of 1400 rpm. Therefore, under these conditions good separations of compounds **1**-**7** could be achieved.

The sample loading is another important parameter in HPCCC. A large loading of sample is expected to favor the throughput of the HSCCC operation, but it can also cause a loss of the stationary phase from the column by affecting the physical properties of the solvent systems. Therefore, this parameter was optimized to enable an efficient HPCCC fractionation. In the present study, it was observed that when the sample loading is increased from 60 to 400 mg of enriched extract (dissolved in 6 mL of the system 10, with 3 mL of each phase), the stationary phase retention decreases from 44% to 25 %. A good fractionation of the enriched extract into fractions I (compounds **1** and **2**) and II (compounds **3**-**7**) was achieved when loading a mass of 60 mg (Fig. 2b) and 300 mg (Fig. 2c) of enriched extract. It was also observed that the loss of stationary phase resulted that compounds with *K* values < 1 eluted at increasing retention times, whereas compounds with *K* values > 1 eluted at decreasing retention times. This phenomenon was more marked when loading 400 mg of enriched extract causing the overlapping of chromatographic elution of target compounds from fraction I and II (Fig. 2a). Therefore, a sample loading of 300 mg of enriched extract was selected for fractionation purposes. As far as the sample loading for separation purposes was concerned, loadings of 180 mg of fraction I and 60 mg of fraction II were used for an efficient separation of target compounds.

HPCCC separation of target compounds

Under the aforementioned optimized conditions, some 300 mg of enriched extract were fractionated by HPCCC using the system 10 in reverse phase mode at a flow rate of 2 mL min^{-1}. This procedure was repeated for three times to process 900 mg of enriched extract. The retention of the stationary phase at the end of the HPCCC separation was 31%. A representative HPCCC chromatogram is shown in Figure 2b. Fractions I and II eluting within the retention time range from 46 to 52 min and from 55 to 160 min; respectively, were collected and concentrated under reduced pressure at 38°C. As a result, 180 mg of fraction I containing the target compounds **1** and **2**, and 300 mg of fraction II containing the target compounds **3**-**7** were yielded.

Fraction I (180 mg) was further subjected to HPCCC in reverse phase mode using the system 17. The target compounds **1** and **2** eluted within retention time ranges from 110 to120 min and from 210 to 230 min, respectively (Fig. 3a). The target peak were collected and concentrated at reduced pressure yielding 10 mg of **1** (72.2 % purity) and 5 mg of **2** (80 % purity). The retention of the stationary phase at the end of the HPCCC separation was 74 %. Fraction II was subjected to HPCCC in reverse phase mode using the system 10. The target peak fractions **3**, **4**, **5**, **6** and **7** eluted within the retention time range 100-120 min, 127-143 min, 170-185 min, 225-275 min and 300-386 min, respectively (Fig. 3b). They were concentrated at reduced pressure yielding 75 mg of **3** (99 % purity), 21 mg of **4** (99 % purity), 6 mg of **5** (86 % purity), 34 mg of **6** (99 % purity) and 12 mg of **7** (90 % purity). The HPLC chromatograms of the seven target fractions obtained by HPCCC are shown in Figure 4a. The retention of the stationary phase at the end of the HPCCC separation was 76 %. In the present study, the isolation of compounds **1**, **2**, **4**, **5**, **6** and **7** was reported for the first time. The compounds **3** and **4** as well as **6** and **7**, only differ from each other in a - CH_2 - group (methylene group), as determined by the substitution of asparagine by glutamine [14]; therefore, the powerful selectivity of HPCCC for separating compounds of closely related structures is demonstrated.

HPLC purification of fractions obtained from HPCCC

HPCCC is considered orthogonal to HPLC, which means that the retention of the solutes in both techniques is caused by different mechanisms. This advantageous difference renders HPCCC and HPLC into two complementary chromatographic techniques. In the present study, **1**, **2** and **5** peak

fractions separated by HPCCC were further cleaned up by HPLC to increase their purity. The HPLC-ESI-HRMS chromatograms of the purified compounds by HPLC are shown in Figure 4b. Compounds **1** (1.57 mg, 95% purity), **2** (0.65 mg, 95 % purity) and **5** (1.41 mg, 95 % purity) were obtained from HPLC.

Identification of target compounds

The chemical identity of the isolated compounds was confirmed by comparing their MS/MS spectra obtained at fragmentation energy of 35 eV (Table 3) with those previously reported in literature [3, 8, 14]. Compounds **1**, **2**, **3**, **5** and **7** were identified as Puwainaphycin F variants, while compounds **4** and **7** were identified as Puwainaphycin G variants. Puwainaphycin F and G differ in the substitution of asparagine by glutamine, as previously reported [8]. Variants derived from these two compounds differ in the lengths and substitution of the *β*-amino fatty acid side chain [14]. As can be seen in the Table 3, the fragmentation of molecular ions using collision energy of 50 eV generated fragment ions that were consistent with those of a characteristic amino-acid sequence of the peptidic macrocycle of Puwainaphycin F/G variants [3, 8, 14], whereas fragmentations of molecular ions using collision energy of 100 eV generated diagnostic *β*-amino fatty immonium ions. Hence, compounds **3**, **4**, **6** and **7** were fully characterized on the basis of their MS data as 4-methyl-Ahdoa-Puw F, 4-methyl-Ahdoa-Puw G, 4-methyl-Ahtea-Puw F and 4-methyl-Ahtea-Puw G, respectively. However, since MS experiments were insufficient for determining the position of both the hydroxyl-substituent in the *β*-amino fatty acid side chain of compound **1** and chloro-substituent in the *β*-amino fatty acid side chains of compounds **2** and **5**, this difficulty was overcome by combining the NMR assignments with the MS data. Consequently, NMR data (supplementary material) allowed to determine the partial structure of the *β*-amino-fatty acid residue, namely for **1** it is C(=O)-CH(OH)-CH(NH)-$CH(CH_3)$-CH_2-$(CH_2)_X$-CH_2-CH_2-CH(OH)-CH_2-CH_3, for **2** it is C(=O)-CH(OH)-CH(NH)-$CH(CH_3)$-CH_2-(CH_2)x-CH_2-CH(Cl)-CH_3 and for **5** it is C(=O)-CH(OH)-CH(NH)-$CH(CH_3)$-CH_2-$(CH_2)_X$-CH_2-CH_2-CH(Cl)-CH_2-CH_3. Given that the length of aliphatic part was confirmed from MS spectra obtained by the fragmentation in 100 eV, as described earlier [14], thus we were able to assign compound **1** as 12-hydroxy-4-methyl-Ahtea Puw F and compound **5** as 12-chloro-4-methyl-Ahtea Puw F. The spectra of compound **2** showed a dehydrated 4-methyl-Ahdoa immonium ion and thus its structure was

assigned to 11-chloro-4-methyl-Ahdoa Puw F (supplementary material). The structures of the seven target compounds are shown in Figure 5.

Conclusions

Seven cyclic lipopeptides puwainaphycins were isolated and purified from *Cylindrospermum alatosporum* by a combination of resins treatment, HPCCC and HPLC methods. The complementary and orthogonality of these three methods enabled the separation of closely chemically related compounds. Three new puwainaphycin variants (**1**, **2** and **5**), bearing hydroxy- and chloro- substitution on the fatty acid side chain, were fully characterized and their structures were established by MS and NMR spectral data. The described method represents a good strategy to systematically purify puwainaphycins from cyanobacterial biomass and a good reference to scale up the obtaining of higher amounts of these cyclic lipopeptides.

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Conflict of interest: The authors declare that they have no conflict of interest.

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Fig. 1 HPLC-HRESI-MS chromatogram (base peak chromatogram - BPC) (**a**) and HPLC-DAD chromatogram (detection wavelength, 240 nm) (**b**) of the crude extract from *C. alatosporum*. Base peak chromatograms (BPC) of the cyclopeptide-enriched extract (CEE) obtained by consecutive treatment of the crude extract from *C. alatosporum* on XAD-16 (**c**) and XAD-7 (**d**) adsorption resins.

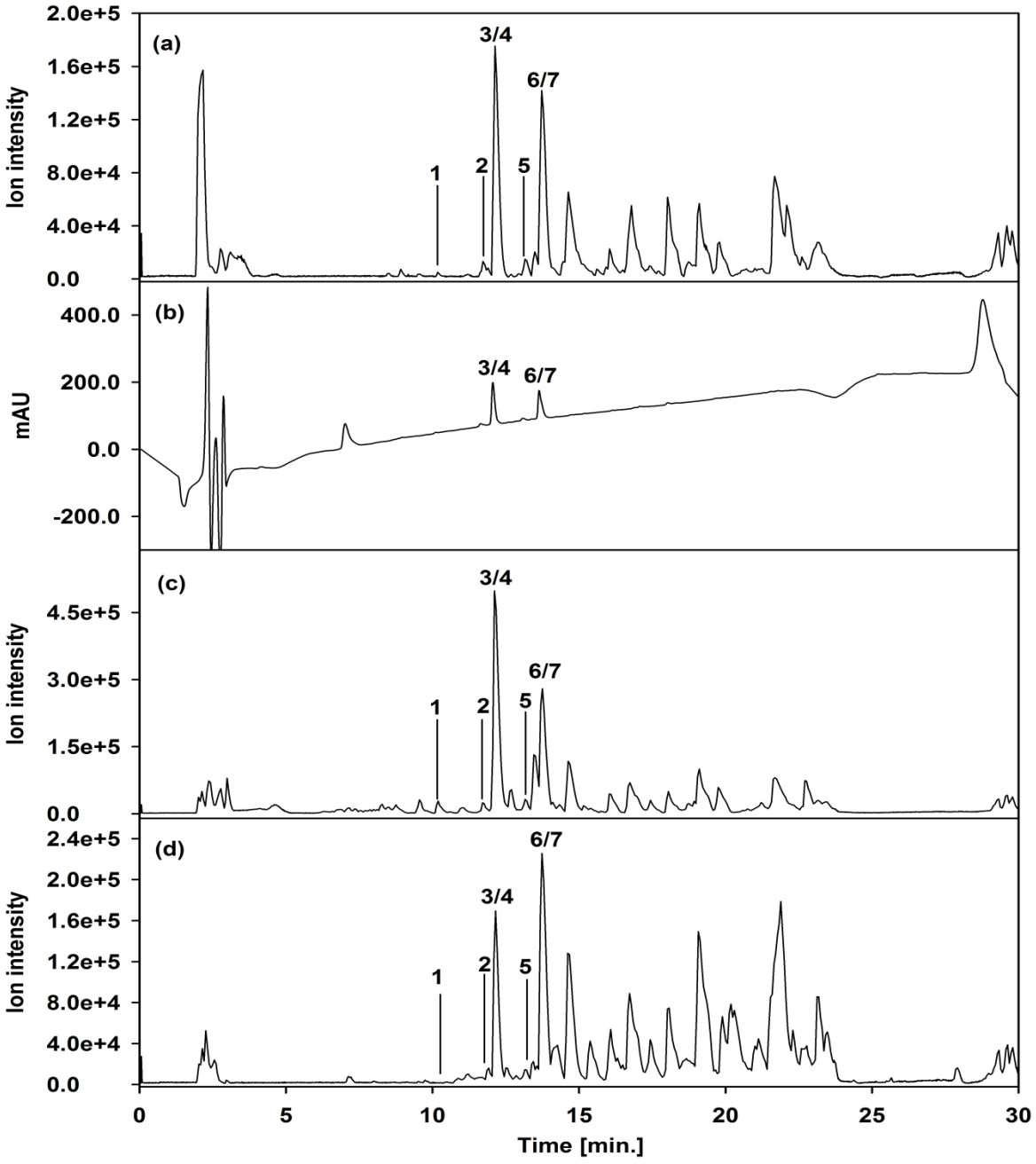


Fig. 2 Effect of sample loading on HPCCC fractionation of enriched extract of *Cylidrospermum alatosporum* CCALA 988 (C24/1989). Two-phase solvent system: *n*-hexane–ethyl acetate–ethanol–water (1:5:1:5, v/v/v). Stationary phase, upper phase; mobile phase, lower phase; rotation speed, 1400 rpm; injection volume, 6 ml; detection wavelength, 240 nm; flow rate, 2 ml/min; separation temperature, 28 °C. Sample loading of 400 mg (**a**), 300 mg (**b**), and 60 mg (**c**) of enriched extract. Fr. I: Fraction I (compounds 1 and 2). Fr. II: fraction II (compounds 3-7).

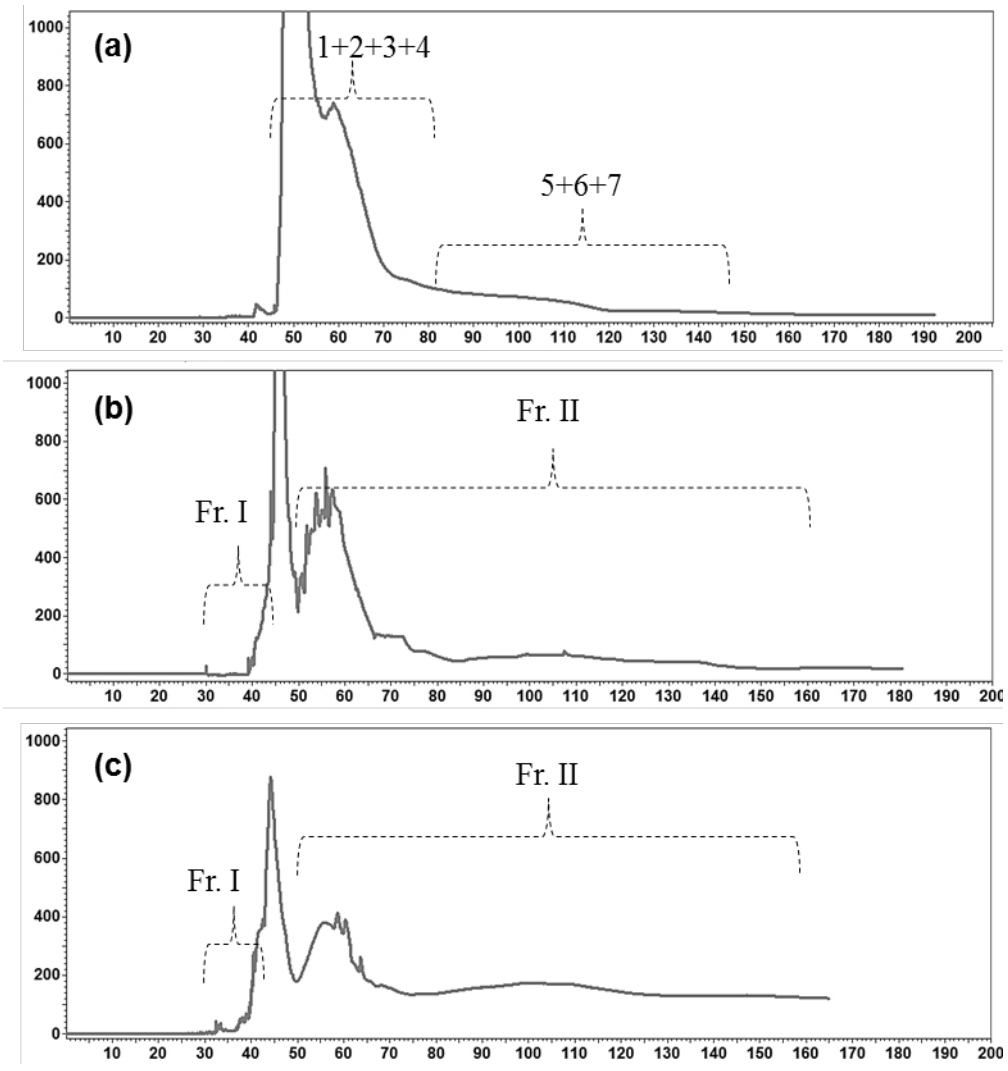


Fig. 3 HPCCC chromatograms of fraction I (**a**) and fraction II (**b**) from enriched extract of *Cylidrospermum alatosporum* CCALA 988 (C24/1989). For fraction I: two-phase solvent system, ethyl acetate–ethanol–water (5:1:5, v/v/v/v); crude to process: 180 mg fraction I; loading per injection, 180 mg in 3 mL lower phase; runs required, 1. For fraction II: Two-phase solvent system, *n*-hexane–ethyl acetate–ethanol–water (1:5:1:5, v/v/v); crude to process: 300 mg of fraction II; loading per injection, 60 mg in 3 mL lower phase; runs required: 5. Mobile phase, lower phase; flow rate, 1 mL/min; rotational speed, 1400 rpm, temperature, 28 °C.

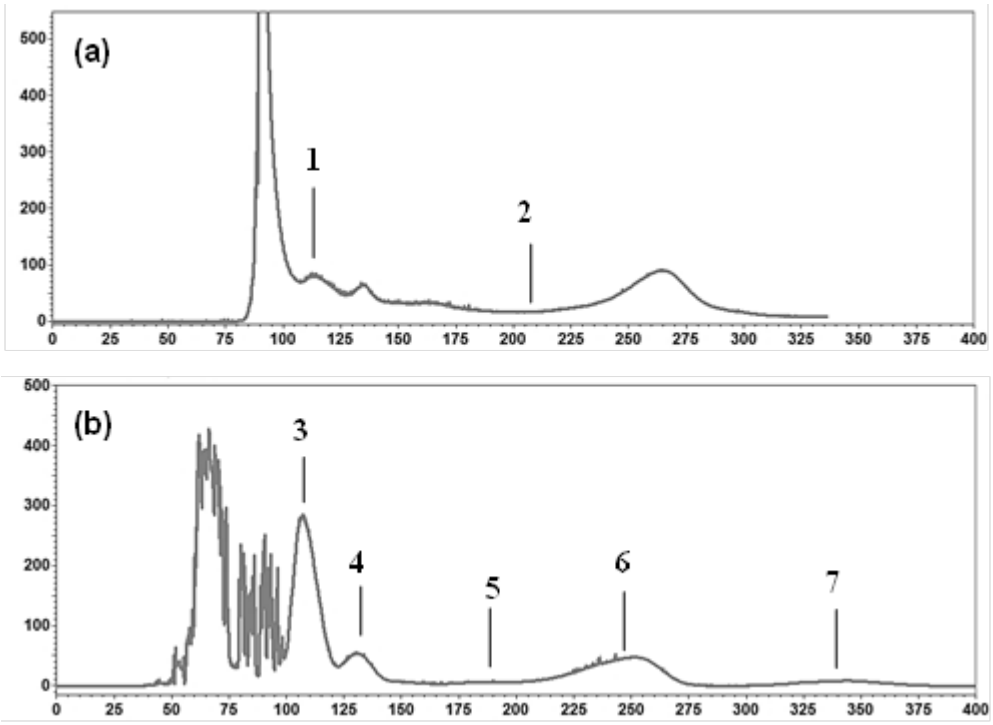


Fig. 4 The HPLC-ESI-HRMS chromatograms of compounds **1**-**7** separated by HPCCC (**a**). The HPLC-ESI-HRMS chromatograms of compounds **1**, **2** and **5** obtained by HPCCC and followed by HPLC purification (**b**).

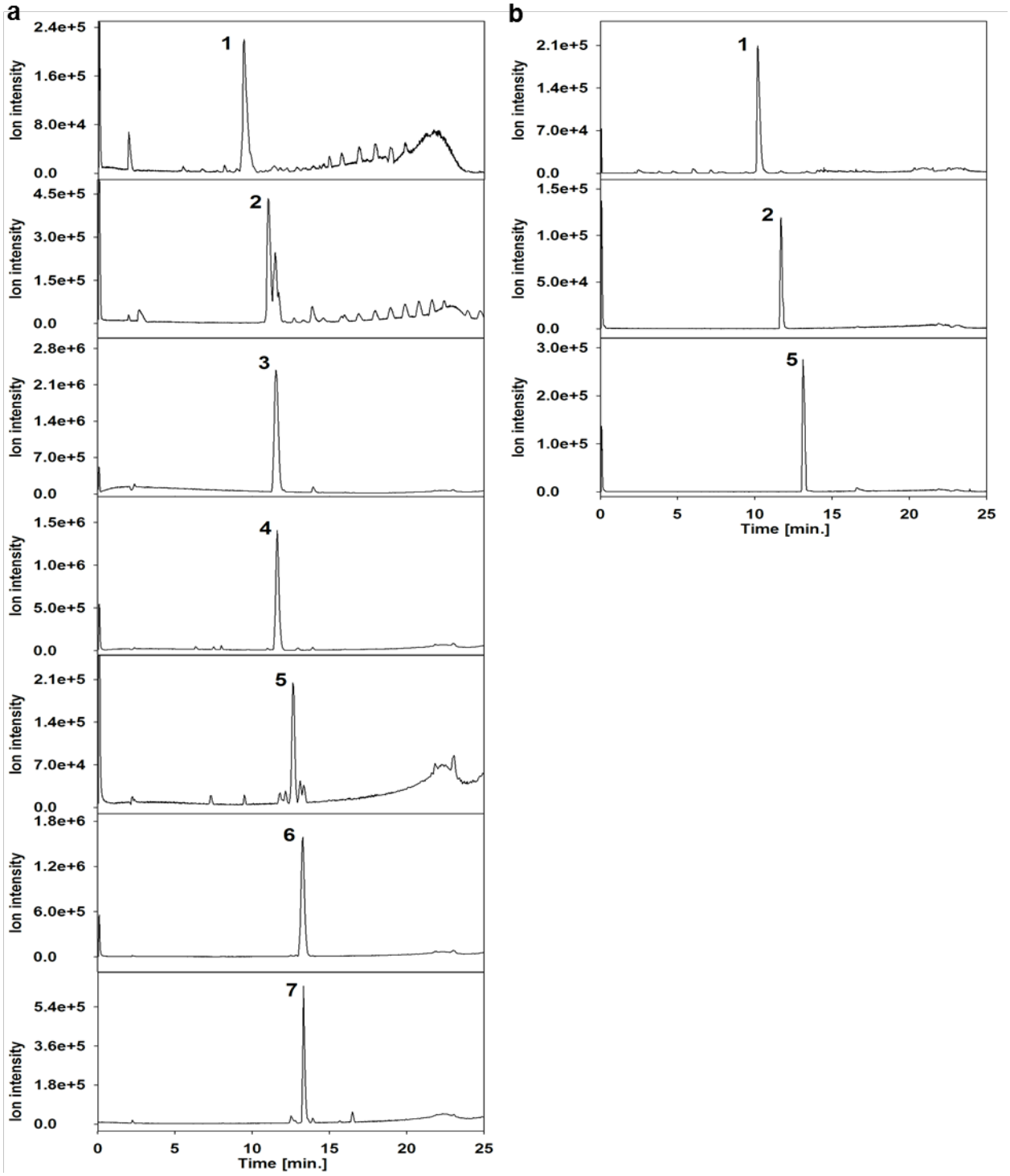
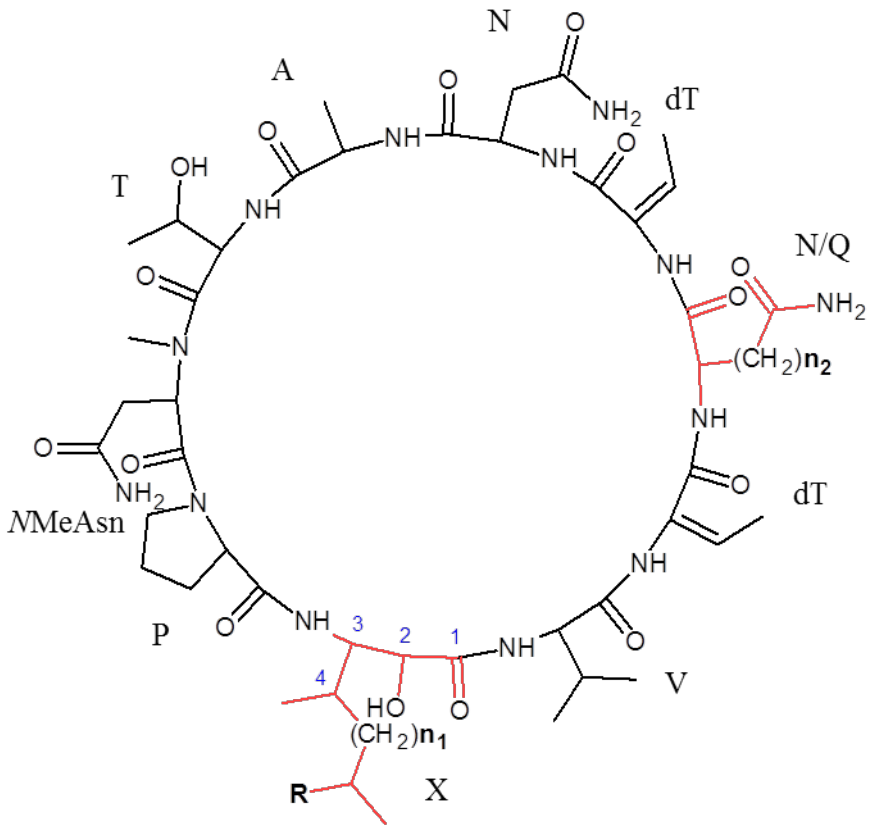


Fig. 5 Chemical structures of puwainaphycins isolated from soil cyanobacterium *Cylidrospermum alatosporum* CCALA 988 (C24/1989). P: L-proline. X: *β*-amino fatty acid. V: L-valine. dT: L-dehydrothreonine. T: L-threonine. Q: L-glutamine. A: L/D-alanine. N: L-asparagine. NMeAsn: L-*N*-methylasparagine.



Compounds	**R**	n_1	n_2
1	OH	8	1
2	Cl	6	1
3	H	6	1
4	H	6	2
5	Cl	8	1
6	H	8	1
7	H	8	2

Table 1 Recovery of the seven puwainaphycins from *Cylidrospermum alatosporum* CCALA 988 (C24/1989) after enrichment by polymeric resins.

Compounds	Recovery (%)		Total recovery (%)
	XAD-16 resin-purified extract	XAD-7 resin-purified extract	
1	54.62	1.71	56.77
2	46.66	0.00	47.06
3	73.36	3.38	77.47
4	66.58	3.64	71.08
5	31.66	3.27	35.62
6	64.41	9.03	74.54
7	61.46	9.43	72.21

682 **Table 2**. The partition coefficient (K_D) and separation factor (α) values of puwainaphycins in different
683 two-phase solvent systems

684 and the settling times

685

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)	Partition coefficient (K_D) of cyclopeptides						
						1	**2**	**3**	**4**	**5**	**6**	**7**
1	*n*-Hex–EtOAc–EtOH–H_2O	10:5:5	1.22	7	0.272	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	*n*-Hex–EtOAc–EtOH–H_2O	9:1:5:5	1.00	10	0.226	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	*n*-Hex–EtOAc–EtOH–H_2O	8:2:5:5	1.00	11	0.197	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	*n*-Hex–EtOAc–EtOH–H_2O	7:3:5:5	0.85	14	0.229	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	*n*-Hex–EtOAc–EtOH–H_2O	6:4:5:5	0.82	18	0.197	0.00	0.00	0.00	0.00	0.00	0.02	0.00
6	*n*-Hex–EtOAc–EtOH–H_2O	5:5:5:5	0.82	33	0.183	0.00	0.00	0.01	0.00	0.00	0.01	0.02
7	*n*-Hex–EtOAc–EtOH–H_2O	4:5:4:5	1.00	33	0.133	0.00	0.00	0.04	0.00	0.03	0.07	0.09
8	*n*-Hex–EtOAc–EtOH–H_2O	3:5:3:5	1.00	21	0.152	0.01	0.06	0.21	0.23	0.20	0.36	0.43
9	*n*-Hex–EtOAc–EtOH–H_2O	2:5:2:5	1.12	18	0.139	0.03	0.20	0.49	0.64	0.77	1.17	1.52
10	*n*-Hex–EtOAc–EtOH–H_2O	1:5:1:5	1.00	13	0.112	0.03	0.30	0.60	1.00	1.50	2.20	3.20
11	EtOAc–H_2O	5:5	1.00	17	0.082	0.00	0.60	0.70	1.30	3.70	5.50	9.10
12	EtOAc–*n*-BuOH–H_2O	4:1:5	0.96	26	0.102	3.80	>10	>10	>10	>10	>10	>10
13	EtOAc–*n*-BuOH–H_2O	3:2:5	1.08	35	0.120	>10	>10	>10	>10	>10	>10	>10
14	EtOAc–*n*-BuOH–H_2O	2:3:5	1.08	25	0.124	>10	>10	>10	>10	>10	>10	>10
15	EtOAc–*n*-BuOH–H_2O	1:4:5	1.22	45	0.167	>10	>10	>10	>10	>10	>10	>10
16	*n*-BuOH–H_2O	5:5	1.27	75	0.192	>10	>10	>10	>10	>10	>10	>10
17	EtOAc–EtOH–H_2O	5:1:5	0.83	15	0.080	0.65	1.80	2.34	1.77	6.67	7.45	1.07
18	EtOAc–EtOH–H_2O	5:2:5	0.94	23	0.056	0.72	1.08	1.41	3.46	2.56	2.68	10.7

Separation factor (α)						
System 17	System 10					
$\alpha_{1/2}$	$\alpha_{3/4}$	$\alpha_{4/5}$	$\alpha_{5/6}$	$\alpha_{6/7}$	$\alpha_{3/7}$
2.77	1.73	1.50	1.46	1.45	5.33

686 *n*-Hex: *n*-hexane. EtOAc: ethyl acetate. EtOH: ethanol. *n*-BuOH: *n*-butanol. AcOH: acetic acid.

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692 **Table 3**. HPLC-ESI-HRMS measurements of seven puwainaphycin variants isolated from soil
693 cyanobacterium *Cylindrospermum alatosporum* CCALA 988 (C24/89)

694

	Molecular ion $[M+H]^+$ (*m/z*)	Formula	Fragment ions m/z (HRESI-MS^2 spectra)		Compounds
			50 eV	100 eV	
1	1162.6478	$C_{53}H_{87}N_{13}O_{16}$	1144.6361; 1016.5791; 933.5364; 862.5043; 748.4626; 665.4232; 551.3809	196.2065; 214.2152	12-hydroxy-4-methyl-Ahtea-Puw-F
2	1152.5815	$C_{51}H_{82}ClN_{13}O_{15}$	1134.5705; 1006.5101; 923.4765; 852.4389; 738.3947; 655.3569; 541.3144	168.1745, 204.1525	11-chloro-4-methyl-Ahdoa-Puw-F
3	1118.6194	$C_{51}H_{83}N_{13}O_{15}$	1100.6117; 972.5537; 889.5163; 818.477; 704.435; 621.3983; 507.3548	170.1907	4-methyl-Ahdoa-Puw-F
4	1132.6352	$C_{52}H_{85}N_{13}O_{15}$	1114.6237; 986.5585; 903.5297; 832.4903; 718.4506; 635.4137; 507.3541	170.1900	4-methyl-Ahdoa-Puw-G
5	1180.6146	$C_{53}H_{86}ClN_{13}O_{15}$	1162.6024; 1034.5435; 951.5062; 880.4724; 766.4278; 683.3898; 569.3466	196.2058; 232.1841	12-chloro-4-methyl-Ahtea-Puw-F
6	1146.6527	$C_{53}H_{87}N_{13}O_{15}$	1128.6408; 1000.5826; 917.545; 846.5071; 732.4664; 649.4288; 535.3856	198.2220	4-methyl-Ahtea-Puw-F (Puwainaphycin F)
7	1160.6701	$C_{54}H_{89}N_{13}O_{15}$	1142.6539; 1014.596; 931.5623; 860.5242; 746.4813; 663.4426; 535.3863	198.2220	4-methyl-Ahtea-Puw-G (Puwainaphycin G)

695 Ahtea: 3-amino-2-hydroxy tetradecanoic acid. Ahdoa: 3-amino-2-hydroxy dodecanoic acid.

696