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1 **Separation of cyclic lipopeptides puwainaphycins from cyanobacteria by HPCCC**
2 **combined with polymeric resins and HPLC**

3
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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,

63 **Introduction**

64

65 Cyanobacteria (also known as blue-green algae) are being recognized as a promising source of
66 novel pharmaceutical lead compounds and secondary metabolites of a large structural diversity [1,
67 2]. A hardly explored group of cyanobacterial secondary metabolites are cyclic lipopeptides
68 (CLPs). These compounds are composed of a modified fatty acid tail linked to a short oligopeptide
69 which forms a peptidic macrocycle. The cyclic part of these structures has been found to comprise
70 up to 14 amino acid residues, which are often represented by non-standard amino acids. The
71 presence of unusual amino acids is explained by the fact that CLPs are biosynthesized by non-
72 ribosomal pathways [3] analogously to many other cyanobacterial peptides [4] [5]. CLPs have
73 been reported to exhibit antifungal [6], antibiotic [7], cytotoxic [8] and antiproliferative activities
74 [5, 9]. Cyclosporine A, a widely used immunosuppressant agent, is plausibly the CLP of major
75 relevance in medicine [10]. Daptomycin represents the first new class of antibiotic CLP that was
76 approved for clinical use in the past decade [4]. Although daptomycin was first reported to be
77 toxic, its administration scheme was optimized giving rise to the amelioration of the adverse effects
78 [11].

79

80 Puwainaphycins are CLP of cyanobacterial origin, which are composed of ten amino acid units
81 and a β -amino fatty acid. The β -amino fatty acid is incorporated into the cycle by two peptide
82 bonds formed from a carboxyl group and a β -amino group [3]. Puwainaphycins A–E have been
83 isolated from the soil cyanobacterium *Anabaena* sp. [12, 13] and their structures were determined
84 by ESI-HRMS, and 1D and 2D NMR, and the stereo configurations were assigned by advanced
85 Marfey's analysis after acid hydrolysis. Our group reported the obtaining, identification and
86 structural elucidation of puwainaphycin F and G as a mixture [8] from cyanobacterial strain
87 *Cylindrospermum alatosporum* CCALA 988 (C24/1989). These two compounds were observed to
88 differ in the substitution of asparagine by glutamine. The isolation of a structural variant of
89 puwainaphycin F from the same cyanobacterial strain, differing in length of fatty acid chain and
90 its substitution, was also reported by our group [3]. More recently, a number of minor
91 puwainaphycin variants have been detected in *C. alatosporum* CCALA 988 by HPLC-HRMS/MS
92 [14], most of which have been neither isolated nor biologically assessed due to their occurrence in
93 the cyanobacterial biomass at tiny concentrations. So far, puwainaphycins have only been obtained

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

104
105 High performance countercurrent chromatography (HPCCC) is a liquid-liquid chromatographic
106 technique that uses a support-free liquid stationary phase. Two immiscible liquid phases are used
107 for the separation of target compounds. One of the two liquid phases (the stationary phase) is
108 retained in the column by centrifugal force, while the other (the mobile phase) is pumped through
109 the column [15]. In HPCCC, the chromatographic separation is based on the different partition
110 coefficients of each target compound in these two immiscible phases. Given that HPCCC uses a
111 liquid stationary phase without solid support, consequently the method offers many advantages
112 over traditional solid liquid chromatography, such as the absence of irreversible adsorption of
113 target molecules; high loading capacity; total recovery of the injected sample; low risk of sample
114 denaturation; and low solvent consumption [16]. This chromatographic method is considered a
115 cost-effective, high-throughput and scalable technology for the extraction of bioactive substances
116 from natural sources. HPCCC has been successfully applied to the isolation and purification of a
117 wide variety of bioactive components from higher plants [17–21] and cyanobacteria [22, 23]. So
118 far, there is no report on the separation of cyclic lipopeptides from cyanobacteria biomass by using
119 HPCCC. Herein, we report the separation of seven puwainaphycins from cyanobacteria by
120 HPCCC combined with microporous resin treatment and HPLC.

121

122

123 **Materials and methods**

124

125 Chemicals and reagents

126

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

135

136 Culture growth conditions

137

138 The cyanobacterial strain *Cylindrospermum alatosporum* CCALA 988 (C24/89) was grown in 200
139 mL tubes on liquid Alen–Arnold medium [41] at 30°C, constant irradiation of 150 μE and mixing
140 with 2% CO₂-enriched air for 10–14 days. The resulting biomass suspension was transferred to 15
141 L photobioreactor for larger scale cultivation under the same conditions. Cells were harvested by
142 centrifugation (1500 g, 15 min), frozen at –40 °C, and lyophilized.

143

144 Biomass extraction

145

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

153

154 Enrichment of crude extract

155

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

168

$$169 \quad \text{Recovery (\%)} = \left(\frac{P2 \times W2}{P1 \times W1} \right) \times 100$$

170

171

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

175

176 HPCCC separation

177

178 *HPCCC Apparatus*

179

180 The separation of target compounds from enriched extract was performed by high performance
181 countercurrent chromatography (HPCCC), which consisted of a semi-preparative apparatus of
182 Model Spectrum (Dynamic Extractions Ltd., Slough, UK). The equipment uses two columns
183 connected in series (PTFE bore tubing = 3.2 mm, total volume = 134 mL). The β -value range
184 varied from 0.52 at internal to 0.86 at the external terminal ($\beta = r / R$, where r is the distance from
185 the coil to the holder shaft and R is the revolution radius or the distance between the holder axis

186 and central axis of the centrifuge). The rotational speed was controlled by a speed regulator
187 installed into the HPCCC chassis. The experimental temperature was adjusted by a H50/H150
188 Smart Water Chiller (LabTech Srl, Sorisole Bergamo, Italy. A Q-Grad pump (LabAlliance, State
189 College, PA, USA) was used both to fill the columns with the stationary phase and elute the mobile
190 phase. The effluent was continuously monitored by a Sapphire UV-VIS spectrometer (ECOM spol.
191 s.r.o., Prague, Czech Republic) operating at 240 nm. The EZChrom SI software platform (Agilent
192 Technologies, Pleasanton, CA, USA) was used to record the HPCCC chromatograms.

193

194 *Selection of the two-phase solvent system*

195

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

208

209

210

211

212 *Preparation of the two-phase solvent system and sample solution*

213

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

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

220

221 *HPCCC separation procedure*

222

223 The enriched extract was first fractionated by HPCCC. The resulting fractions were further
224 subjected to HPCCC for the separation of target compounds. For the HPCCC fractionation, the
225 solvent system composed of *n*-Hex–EtOAc–EtOH–H₂O (1:5:1:5, v/v/v/v) was used in reverse
226 phase. For HPCCC separations, the two solvent systems composed of EtOAc–EtOH–H₂O (5:1:5,
227 v/v/v/v) and *n*-Hex–EtOAc–EtOH–H₂O (1:5:1:5, v/v/v/v) were used in reverse phase. When
228 working in reverse phase, the lower phase of the solvent system is used as the mobile phase and
229 the upper phase as the stationary phase. The HPCCC column was initially filled with the upper
230 phase (stationary phase). The HPCCC column was rotated at 1,400 rpm, and the lower phase
231 (mobile phase) was pumped into the column at 2 mL min⁻¹ and 1 mL min⁻¹ for the HPCCC
232 fractionation and HPCCC separation operations, respectively. After the mobile phase front
233 emerged and hydrodynamic equilibrium was reached, the sample solution was injected through
234 the sample injection valve. The effluent from the outlet was continuously monitored at 240 nm.
235 The temperature of the apparatus was set at 28°C. Fractions were collected manually according to
236 the chromatographic profile and then analyzed by HPLC-ESI-HRMS. The retention of the
237 stationary phase (*Sf*) was estimated both after the hydrodynamic equilibrium was reached and at
238 the end of the HPCCC separation run. The *Sf* value (%) was calculated as follows:

239

$$240 \quad Sf (\%) = \frac{V_s}{V_c} \times 100$$

241

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

243

244 Subsequent purification by using preparative HPLC

245

246 The target peak fractions obtained with HPCCC were further purified using an Agilent 1100 HPLC
247 system equipped with a diode array detector (DAD). The **1** and **5** HPCCC peak fractions were
248 purified through a Reprisil 100 C18 column (250×4mm 5µm) held at constant temperature of
249 28°C, and with a mobile phase composed of acetonitrile (A) and water (B) using the following
250 gradient: 0–2 min, 70% B; 2–6 min, 70%–60% B; 6–15 min, 60%–30% B; 15–16 min, 30%–0%
251 B; 16–20 min, 0%–0% B; 20–21 min, 0%–70% B, which was pumped at a constant flow rate of 2
252 ml/min. The **2** HPCCC peak fraction was purified through a Reprisil 100 Phenyl column
253 (250×8mm 5µm), held at constant temperature of 28°C. For compound 2, the mobile phase was
254 composed of acetonitrile (A) and water (B) using the following gradient: 0–2 min, 70% B; 2–6
255 min, 70%–55% B; 6–13 min, 55%–45% B; 13–15 min, 45%–0% B; 15–16 min, 0%–0% B; 16–
256 18 min, 0%–70% B; 18–19 min, 70%–70% B, which was pumped at a constant flow rate of 2 mL
257 min⁻¹. For compound 3, 6 and 7 the mobile phase was composed of methanol (A) and water (B)
258 using the following gradient: 0–2 min, 50%–30% B; 2–15 min, 30%–10% B; 15–16 min, 10%–
259 0% B; 16–20 min, 0%–0% B; 20–20.1 min, 0%–50% B, which was pumped at a constant flow
260 rate of 2.4 mL min⁻¹.

261

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

263

264 A Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped with
265 a diode array detector (DAD) and high resolution mass spectrometer with electrospray ionization
266 source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker, Billerica, MA, USA) was used for
267 the analysis of the crude extract and fractions. The separations were performed on a reversed phase
268 column (Phenomenex Kinetex C18 column, 150 × 4.6 mm, 2.6 µm) held at constant temperature
269 of 30 °C. The mobile phase was made up from the combination of 0.1 % formic acid in water (A)
270 and 0.1 % formic acid in acetonitrile (B) using the following gradient: 0–1 min, 85% A; 1–20 min,
271 85%–0% A; 20–25 min, 0% A; 25–30 min, 0%–85% A, which was pumped at a constant flow rate
272 of 0.6 mL min⁻¹. The operating parameters of the mass spectrometer were as follows: the spray
273 needle voltage was set at 3.8 kV, nitrogen was used both as nebulizing gas (3 bar) and drying gas
274 (12 L min⁻¹), and the drying temperature was 210°C. For fragmentation of selected molecular ions,
275 nitrogen was used as collision gas. The collision energy was set to 60-70 eV for the determination
276 of cyclic oligopeptide part and to 100 eV for determination of β-amino fatty acid part. The scanning

277 range was 50–2,600 m/z and the scanning rate 2 Hz operating in the positive ion mode. The DAD
278 detector was set at 240 nm to record the peaks, and the UV–Vis spectra were recorded from 200
279 to 700 nm. Identification of target compounds was performed by comparing their MS data
280 (molecular and fragment ions) with those previously reported in literature [14].

281

282 Chemical identification of isolated target compounds

283

284 The isolated target compounds were structurally identified using ESI-HRMS and NMR
285 experiments. The NMR spectra were recorded on a Bruker Avance III 700 MHz spectrometer
286 equipped with TCI CryoProbe (700.13 MHz for ^1H , 176.05 MHz for ^{13}C , Bruker Biospin GmbH,
287 Rheinstetten, Germany) in DMSO- d_6 303.2 K. The residual solvent signals were used as an internal
288 standard (δ_{H} 2.499 ppm and δ_{C} C 39.46 ppm). ^1H NMR, ^{13}C NMR, COSY, TOCSY, J -resolved,
289 ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC, ^1H - ^{13}C HSQC-TOCSY, band selective ^1H - ^{13}C HMBC, ROESY,
290 NOESY, and 1D-TOCSY spectra were measured using the standard manufacturer's software. The
291 ^1H NMR spectrum was zero filled to 2-fold data points and multiplied by a window function (two
292 parameter double-exponential Lorentz-Gauss function) before Fourier transformation to improve
293 the resolution. The ^{13}C NMR spectrum was zero filled to 2-fold data points. Subsequently, the line
294 broadening (1 Hz) was used to improve signal-to-noise ratio. The chemical shifts are given on the
295 δ scale (ppm) and coupling constants are given in Hz. The digital resolution allowed us to present
296 the proton and carbon chemical shifts to three or two decimal places. The proton chemical shift
297 readouts from HSQC are reported to two decimal places. MS data for each compound are shown
298 in Table 3. Complete NMR data of the compounds are presented in Supplementary Data.

299

300 **Results and discussion**

301

302 HPLC-ESI-HRMS analysis of crude extract

303

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

308 for detection purposes. As depicted in the Figure 1b most of the target compounds were observed
309 to be poorly UV absorbing compounds. Compounds **3** and **6** were eluted closely with minor target
310 compounds **4** and **7**, respectively. Compounds **1**, **2** and **5**, are three minor puwainaphycin variants
311 whose β -amino fatty acid side chain have not been fully structurally elucidated by ESI-HRMS
312 [14]. Accordingly, the enrichment of the crude extract in the seven target compounds and their
313 separation by HPLC were applied.

314

315 Enrichment of crude extract

316

317 To enrich the crude extract in the seven target compounds, two kinds of polymeric resins were
318 used. Appropriate resin selection is crucial to assure an efficient recovery and purification of target
319 compounds from complex matrices. XAD-2 polymeric resin has been used for the purification of
320 puwainaphycin C from cyanobacterial extracts [13]. XAD-16 resin has been used for enriching
321 bacterial extracts in cyclic peptides by removing water-soluble impurities [26-28]. The consecutive
322 adsorption on XAD-2 and XAD-7 resins has been successfully applied for enriching a bacterial
323 extract in cyclic peptides [28]. XAD-2 Amberlite resin is chemically similar to XAD-16 Amberlite
324 resin, as both resins are made of styrene-divinylbenzene; however, XAD-16 resin is considered to
325 be more efficient due to its higher surface area. In the current study, *C. alatosporum* crude extract
326 was enriched by consecutive adsorption on XAD-16 and XAD-7 polymeric resins. As a result, 757
327 mg and 190 mg of enriched extracts were obtained from XAD-16 and XAD-7 resins, respectively.
328 As observed in the chromatograms (Fig. 1c, d), XAD-16 and XAD-7 enriched extracts contained
329 the seven target compounds and exhibited qualitatively similar profiles. The peak purities of
330 compounds **1-7** in the XAD-16 enriched extract were higher than those found in the crude extract
331 (Table 1). Conversely, no increase of the target peak purity in the XAD-7 enriched extract was
332 observed due to the presence of an increased relative abundance of contaminants eluting after 15
333 min. Peak purity values reflected the presence of other contaminants in the sample, but did not
334 account for the amount compounds recovered by resins. Thus, to determine possible losses of
335 target compounds during the pre-purification by resins, the recovery (%) was calculated by using
336 an external standard HPLC-HRMS method. Seven experimental points were employed for
337 establishing calibration curves. The regression lines for compounds **1**, **2**, **3**, **4**, **5**, **6** and **7** were $y =$
338 $903.89x + 4677.3$ ($R^2 = 0.9998$), $y = 709.79x + 4305.403$ ($R^2 = 0.9961$), $y = 3793.3x + 8162.2$ ($R^2 =$

339 0.9997), $y = 4603.6x - 17111.0$ ($R^2 = 0.9996$), $y = 903,89x + 4677,3$ ($R^2 = 0.9998$), $y = 1930,1x$
340 $+44729.0$ ($R^2 = 0.8954$) and $y = 4555,2x + 5387,7$ ($R^2 = 0.9900$), respectively, where y was the
341 peak area of analyte, and x was the concentration of analyte ($\mu\text{g/mL}$). We found out total recoveries
342 between 35 and 77 % of the target compounds. Recovery data are presented in Table 1. The XAD-
343 16 resin was found to significantly contribute to the total recovery of puwainaphycins. Given that
344 both XAD-16 and XAD-7 enriched extracts exhibited similar chromatographic profiles, they were
345 pooled affording 900 mg of enriched extract, which was subsequently used for the HPCCC
346 separation of the target compounds. Some 900 g of resin-treated extract containing the seven target
347 compounds were obtained after treatment of 5.5 g of crude extract, which demonstrated the
348 enriching effect of the operation.

349

350 Optimization of HPCCC conditions

351

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

364

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

370 order to obtain an accurate partition coefficient of both major and minor puwainaphycin variants,
371 HPLC-ESI-HRMS was used for determining the K values. In this way, the molecular ions of the
372 target compounds were selectively retrieved from the BPC chromatogram. As shown in the Table
373 2, the system 10 (*n*-Hex–EtOAc–EtOH–H₂O in ratio 1:5:1:5) provided suitable K values and good
374 separation factors (α) for compounds **3-7**, as well as a short settling time. However, it gave small
375 K values ($K \leq 0.5$) for the compounds **1** and **2**, therefore they would elute close to the solvent front
376 and with a lower resolution. It was concluded that for obtaining proper K values systems, the
377 solubility of compounds **1** and **2** should be favoured to the upper phase. Thus, solvent systems of
378 increased polarity (systems 12-16, Table 2) were tested. Systems 12-16 provided too high K values
379 for compounds **1** and **2**, because the solubility of the target compounds was extremely favoured to
380 the upper phase (organic phase). Thus, another strategy for optimizing the K values of compounds
381 **1** and **2** consisted in increasing the polarity of the system 10 by removing *n*-hexane from its global
382 composition. Thus, the system 10 was converted to the system 17 (EtOAc–EtOH–H₂O 5:1:5 v/v/v).
383 As shown in the Table 2, this modification favored the solubility of compounds **1** and **2** in the
384 upper phase of the system 17 in such a way that appropriate K values, a good separation factor (α
385 = 2.77) and a short settling time were obtained. Unlike system 10, the density difference between
386 the two immiscible phases composing the system 17 was slightly lower, but sufficiently convenient
387 to maintain stationary phase inside the column. Overall, the results indicated that two groups of
388 compounds of differing polarity, i.e. one group (fraction I) composed of compounds **1** and **2** and
389 the other one (fraction II) composed of compounds from **3** to **7** were present in the enriched extract.
390 The system 10 was found to be selective for compounds **1** and **2**, while system 17 was selective
391 for compounds **3-7**. A single solvent system would not be enough to cover the separation of the
392 seven target compounds. Therefore, a previous fractionation of the enriched extract was necessary,
393 followed by the separation of the target compounds from fractions I and II in independent
394 operations. Since the system 10 was observed to efficiently divide the extract into fractions I and
395 II, it was thus selected for fractionation purposes.

396

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

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

412
413 The sample loading is another important parameter in HPCCC. A large loading of sample is
414 expected to favor the throughput of the HSCCC operation, but it can also cause a loss of the
415 stationary phase from the column by affecting the physical properties of the solvent systems.
416 Therefore, this parameter was optimized to enable an efficient HPCCC fractionation. In the present
417 study, it was observed that when the sample loading is increased from 60 to 400 mg of enriched
418 extract (dissolved in 6 mL of the system 10, with 3 mL of each phase), the stationary phase
419 retention decreases from 44% to 25 %. A good fractionation of the enriched extract into fractions
420 I (compounds **1** and **2**) and II (compounds **3-7**) was achieved when loading a mass of 60 mg (Fig.
421 2b) and 300 mg (Fig. 2c) of enriched extract. It was also observed that the loss of stationary phase
422 resulted that compounds with *K* values < 1 eluted at increasing retention times, whereas
423 compounds with *K* values > 1 eluted at decreasing retention times. This phenomenon was more
424 marked when loading 400 mg of enriched extract causing the overlapping of chromatographic
425 elution of target compounds from fraction I and II (Fig. 2a). Therefore, a sample loading of 300
426 mg of enriched extract was selected for fractionation purposes. As far as the sample loading for
427 separation purposes was concerned, loadings of 180 mg of fraction I and 60 mg of fraction II were
428 used for an efficient separation of target compounds.

429
430 HPCCC separation of target compounds

431

432 Under the aforementioned optimized conditions, some 300 mg of enriched extract were
433 fractionated by HPCCC using the system 10 in reverse phase mode at a flow rate of 2 mL min⁻¹.
434 This procedure was repeated for three times to process 900 mg of enriched extract. The retention
435 of the stationary phase at the end of the HPCCC separation was 31%. A representative HPCCC
436 chromatogram is shown in Figure 2b. Fractions I and II eluting within the retention time range
437 from 46 to 52 min and from 55 to 160 min; respectively, were collected and concentrated under
438 reduced pressure at 38°C. As a result, 180 mg of fraction I containing the target compounds **1** and
439 **2**, and 300 mg of fraction II containing the target compounds **3-7** were yielded.

440
441 Fraction I (180 mg) was further subjected to HPCCC in reverse phase mode using the system 17.
442 The target compounds **1** and **2** eluted within retention time ranges from 110 to 120 min and from
443 210 to 230 min, respectively (Fig. 3a). The target peak were collected and concentrated at reduced
444 pressure yielding 10 mg of **1** (72.2 % purity) and 5 mg of **2** (80 % purity). The retention of the
445 stationary phase at the end of the HPCCC separation was 74 %. Fraction II was subjected to
446 HPCCC in reverse phase mode using the system 10. The target peak fractions **3**, **4**, **5**, **6** and **7** eluted
447 within the retention time range 100-120 min, 127-143 min, 170-185 min, 225-275 min and 300-
448 386 min, respectively (Fig. 3b). They were concentrated at reduced pressure yielding 75 mg of **3**
449 (99 % purity), 21 mg of **4** (99 % purity), 6 mg of **5** (86 % purity), 34 mg of **6** (99 % purity) and 12
450 mg of **7** (90 % purity). The HPLC chromatograms of the seven target fractions obtained by HPCCC
451 are shown in Figure 4a. The retention of the stationary phase at the end of the HPCCC separation
452 was 76 %. In the present study, the isolation of compounds **1**, **2**, **4**, **5**, **6** and **7** was reported for the
453 first time. The compounds **3** and **4** as well as **6** and **7**, only differ from each other in a -CH₂- group
454 (methylene group), as determined by the substitution of asparagine by glutamine [14]; therefore,
455 the powerful selectivity of HPCCC for separating compounds of closely related structures is
456 demonstrated.

457
458 HPLC purification of fractions obtained from HPCCC

459
460 HPCCC is considered orthogonal to HPLC, which means that the retention of the solutes in both
461 techniques is caused by different mechanisms. This advantageous difference renders HPCCC and
462 HPLC into two complementary chromatographic techniques. In the present study, **1**, **2** and **5** peak

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

467

468 Identification of target compounds

469

470 The chemical identity of the isolated compounds was confirmed by comparing their MS/MS
471 spectra obtained at fragmentation energy of 35 eV (Table 3) with those previously reported in
472 literature [3, 8, 14]. Compounds **1**, **2**, **3**, **5** and **7** were identified as Puwainaphycin F variants, while
473 compounds **4** and **6** were identified as Puwainaphycin G variants. Puwainaphycin F and G differ
474 in the substitution of asparagine by glutamine, as previously reported [8]. Variants derived from
475 these two compounds differ in the lengths and substitution of the β -amino fatty acid side chain
476 [14]. As can be seen in the Table 3, the fragmentation of molecular ions using collision energy of
477 50 eV generated fragment ions that were consistent with those of a characteristic amino-acid
478 sequence of the peptidic macrocycle of Puwainaphycin F/G variants [3, 8, 14], whereas
479 fragmentations of molecular ions using collision energy of 100 eV generated diagnostic β -amino
480 fatty immonium ions. Hence, compounds **3**, **4**, **6** and **7** were fully characterized on the basis of
481 their MS data as 4-methyl-Ahdoa-Puw F, 4-methyl-Ahdoa-Puw G, 4-methyl-Ahtea-Puw F and 4-
482 methyl-Ahtea-Puw G, respectively. However, since MS experiments were insufficient for
483 determining the position of both the hydroxyl-substituent in the β -amino fatty acid side chain of
484 compound **1** and chloro-substituent in the β -amino fatty acid side chains of compounds **2** and **5**,
485 this difficulty was overcome by combining the NMR assignments with the MS data. Consequently,
486 NMR data (supplementary material) allowed to determine the partial structure of the β -amino-fatty
487 acid residue, namely for **1** it is C(=O)-CH(OH)-CH(NH)-CH(CH₃)-CH₂-(CH₂)_x-CH₂-CH₂-
488 CH(OH)-CH₂-CH₃, for **2** it is C(=O)-CH(OH)-CH(NH)-CH(CH₃)-CH₂-(CH₂)_x-CH₂-CH(Cl)-CH₃
489 and for **5** it is C(=O)-CH(OH)-CH(NH)-CH(CH₃)-CH₂-(CH₂)_x-CH₂-CH₂-CH(Cl)-CH₂-CH₃.
490 Given that the length of aliphatic part was confirmed from MS spectra obtained by the
491 fragmentation in 100 eV, as described earlier [14], thus we were able to assign compound **1** as 12-
492 hydroxy-4-methyl-Ahtea Puw F and compound **5** as 12-chloro-4-methyl-Ahtea Puw F. The spectra
493 of compound **2** showed a dehydrated 4-methyl-Ahdoa immonium ion and thus its structure was

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

496

497 **Conclusions**

498

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

507

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512

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

514

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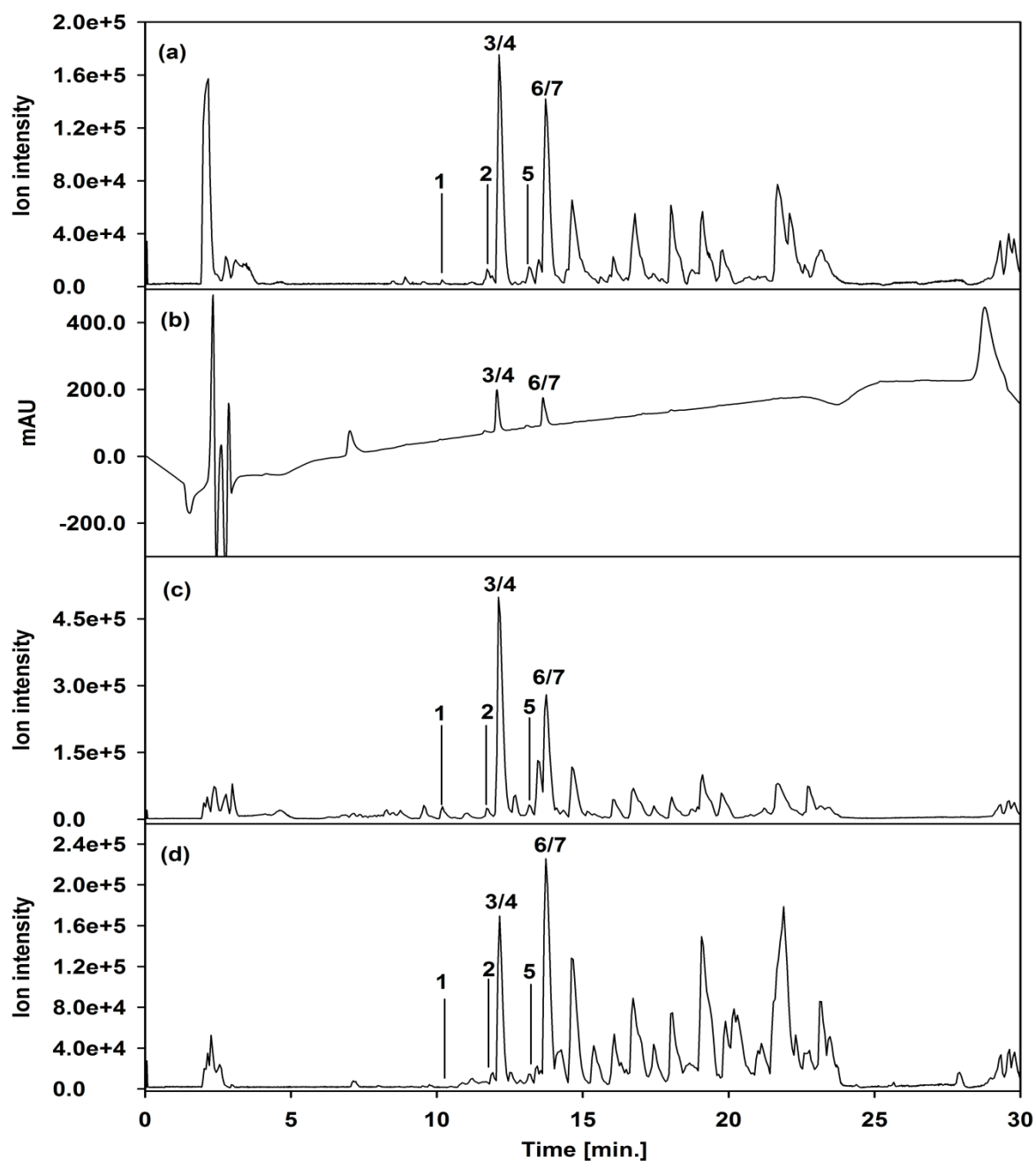
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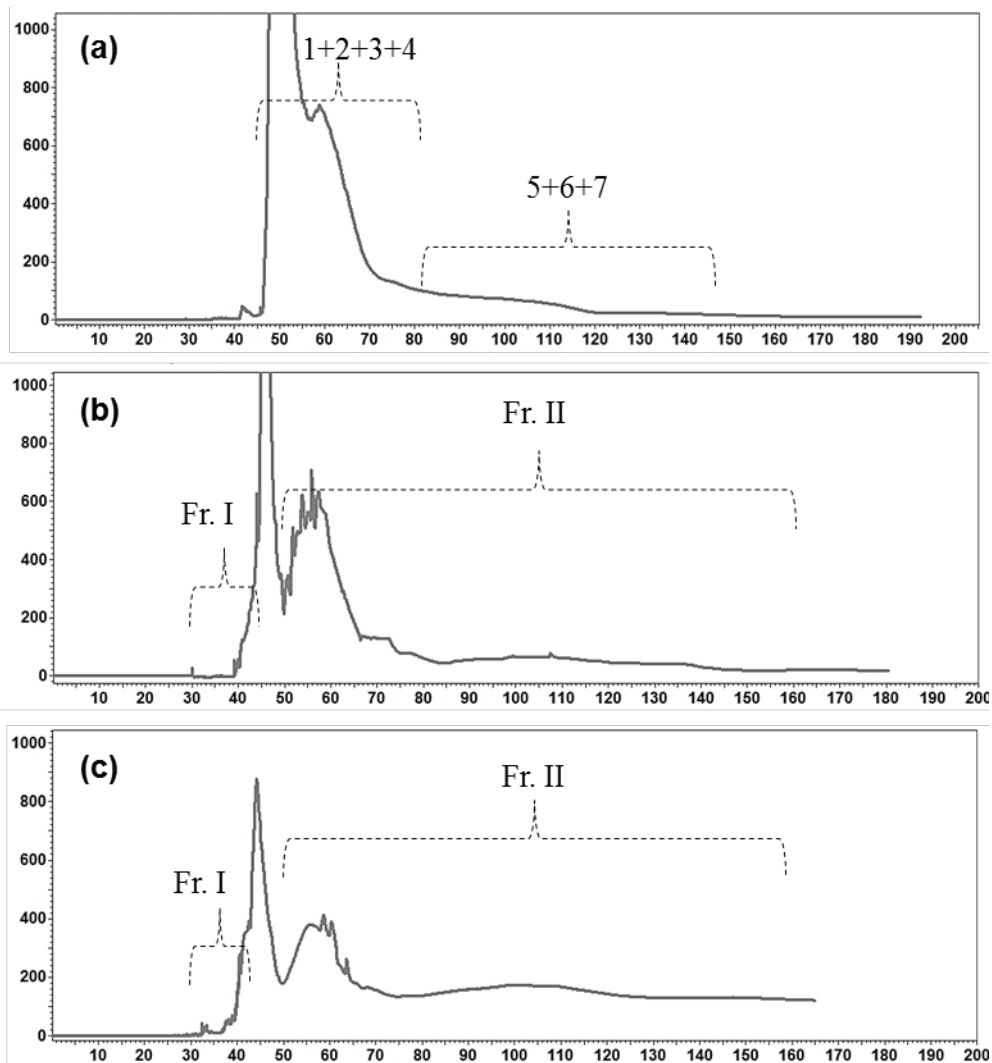
632 **Fig. 1** HPLC-HRESI-MS chromatogram (base peak chromatogram - BPC) (a) and HPLC-DAD
633 chromatogram (detection wavelength, 240 nm) (b) of the crude extract from *C. alatosporum*. Base
634 peak chromatograms (BPC) of the cyclopeptide-enriched extract (CEE) obtained by consecutive
635 treatment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



637

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

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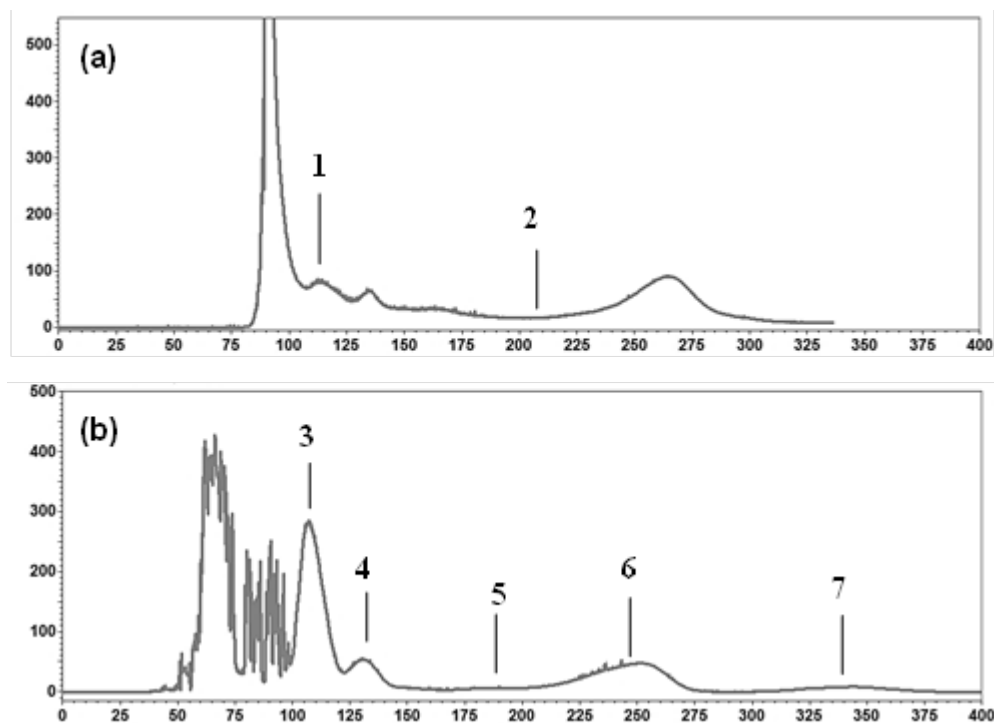
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648 **Fig. 3** HPLC chromatograms of fraction I (a) and fraction II (b) from enriched extract of
649 *Cylindrospermum alatosporum* CCALA 988 (C24/1989). For fraction I: two-phase solvent system,
650 ethyl acetate–ethanol–water (5:1:5, v/v/v); crude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

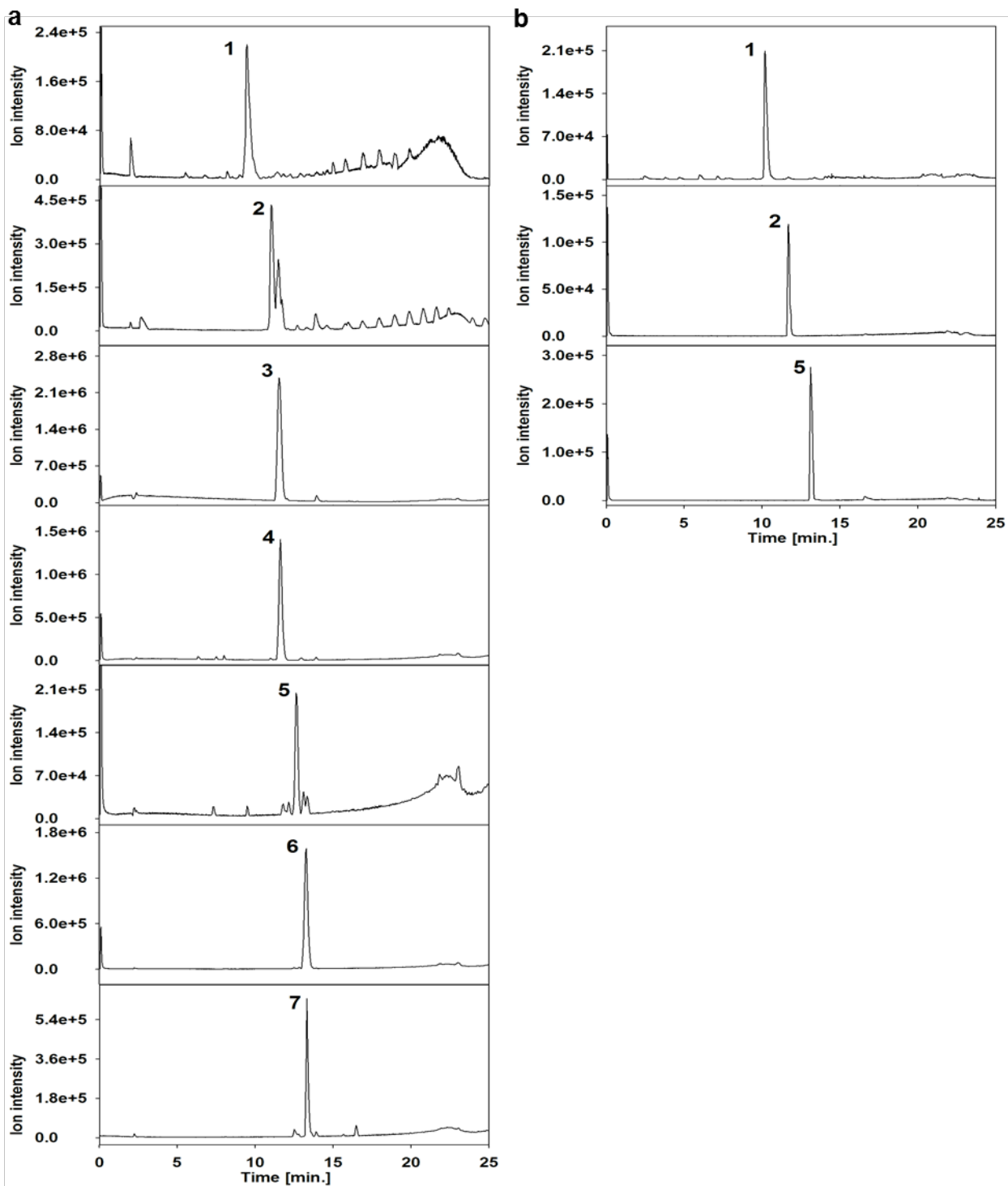
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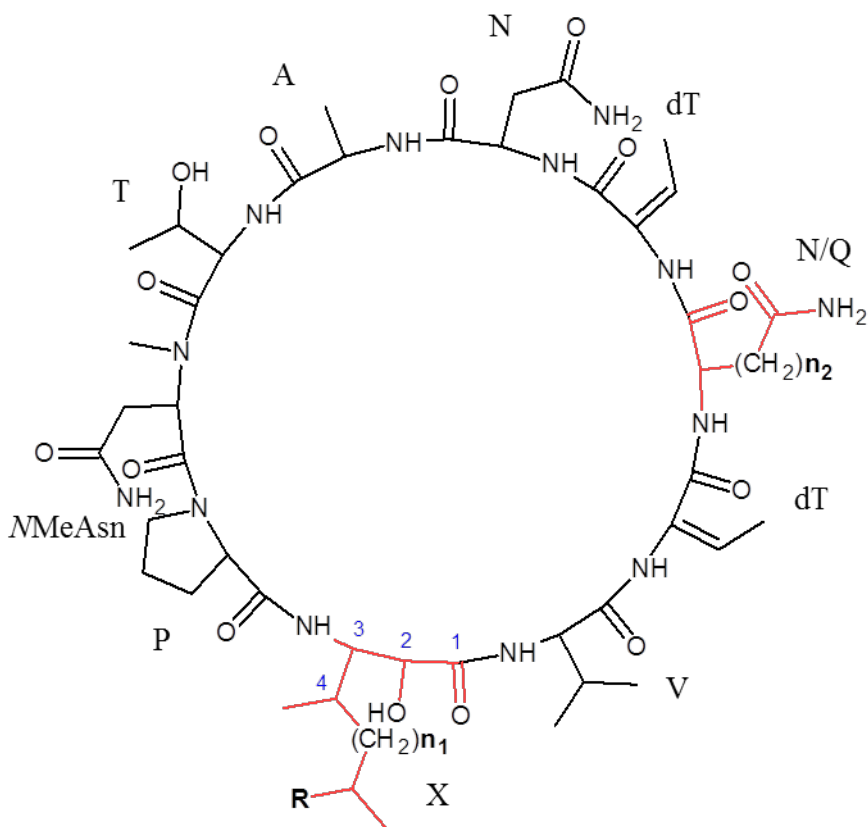
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658 **Fig. 4** The HPLC-ESI-HRMS chromatograms of compounds **1-7** separated by HPCCC (a). The
659 HPLC-ESI-HRMS chromatograms of compounds **1, 2** and **5** obtained by HPCCC and followed by
660 HPLC purification (b).



661

662 **Fig. 5** Chemical structures of puwainaphycins isolated from soil cyanobacterium *Cylindrospermum*
 663 *alatosporum* CCALA 988 (C24/1989). P: L-proline. X: β -amino fatty acid. V: L-valine. dT: L-
 664 dehydrothreonine. T: L-threonine. Q: L-glutamine. A: L/D-alanine. N: L-asparagine. NMeAsn: L-
 665 *N*-methylasparagine.
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| Compounds | R | n₁ | n₂ |
|-----------|----------|----------------------|----------------------|
| 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 |

674 **Table 1** Recovery of the seven puwainaphycins from *Cylindrospermum alatosporum* CCALA 988
675 (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 |

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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 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 10:5:5 | 1.22 | 7 | 0.272 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 9:1:5:5 | 1.00 | 10 | 0.226 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 8:2:5:5 | 1.00 | 11 | 0.197 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 4 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 7:3:5:5 | 0.85 | 14 | 0.229 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 5 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 6:4:5:5 | 0.82 | 18 | 0.197 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 |
| 6 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 5:5:5:5 | 0.82 | 33 | 0.183 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.01 | 0.02 |
| 7 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 4:5:4:5 | 1.00 | 33 | 0.133 | 0.00 | 0.00 | 0.04 | 0.00 | 0.03 | 0.07 | 0.09 |
| 8 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 3:5:3:5 | 1.00 | 21 | 0.152 | 0.01 | 0.06 | 0.21 | 0.23 | 0.20 | 0.36 | 0.43 |
| 9 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 2:5:2:5 | 1.12 | 18 | 0.139 | 0.03 | 0.20 | 0.49 | 0.64 | 0.77 | 1.17 | 1.52 |
| 10 | <i>n</i> -Hex-EtOAc-EtOH-H ₂ O | 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 ₂ O | 5:5 | 1.00 | 17 | 0.082 | 0.00 | 0.60 | 0.70 | 1.30 | 3.70 | 5.50 | 9.10 |
| 12 | EtOAc- <i>n</i> -BuOH-H ₂ O | 4:1:5 | 0.96 | 26 | 0.102 | 3.80 | >10 | >10 | >10 | >10 | >10 | >10 |
| 13 | EtOAc- <i>n</i> -BuOH-H ₂ O | 3:2:5 | 1.08 | 35 | 0.120 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| 14 | EtOAc- <i>n</i> -BuOH-H ₂ O | 2:3:5 | 1.08 | 25 | 0.124 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| 15 | EtOAc- <i>n</i> -BuOH-H ₂ O | 1:4:5 | 1.22 | 45 | 0.167 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| 16 | <i>n</i> -BuOH-H ₂ O | 5:5 | 1.27 | 75 | 0.192 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| 17 | EtOAc-EtOH-H ₂ O | 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 ₂ O | 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)

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| | 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.

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