

# Hapalindoles from the Cyanobacterium *Hapalosiphon* sp. Inhibit T Cell Proliferation

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## ABSTRACT

Novel immunomodulating agents are currently sought after for the treatment of autoimmune diseases and cancers. In this context, a screening campaign of a collection of 575 cyanobacteria extracts for immunomodulatory effects has been conducted. The screening resulted in several active extracts. Here we report the results of subsequent studies on an extract from the cyanobacterium *Hapalosiphon* sp. CBT1235. We identified 5 hapalindoles as the compounds responsible for the observed immunomodulatory effect. These indole alkaloids are produced by several strains of the cyanobacterial family Hapalosiphonaceae. They are known for their anti-infective, cytotoxic, and other bioactivities. Modulation of the activity of human immune cells has not yet been described. The immunomodulatory activity of the hapalindoles was characterized *in vitro* using flow cytometry-based measurements of T cell proliferation after carboxyfluorescein diacetate succinimidyl ester staining, and apoptosis and necrosis induction after annexin V/propidium iodide staining. The most potent compound, hapalindole A, reduced T cell proliferation with an IC<sub>50</sub> of 1.56 μM, while relevant levels of apoptosis were measurable only at 10-fold higher concentrations. Hapalindole A-formamide and hapalindole J-formamide, isolated for the first time from a natural source, had much lower activity than the nonformylated derivatives while, at the same time, being less selective for antiproliferative over apoptotic effects.

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## ABBREVIATIONS

CFSE	carboxyfluorescein diacetate succinimidyl ester
CPT	camptothecin
CsA	cyclosporine A
PBMC	peripheral blood mononuclear cells

## Introduction

Cyanobacteria are an intriguing source for structurally diverse and biologically active natural products. Especially the genera *Microcystis*, *Nostoc*, and *Lyngbya* or *Moorea* are chemically well-characterized [1–7]. Several strains of the family Hapalosiphonaceae produce indole alkaloids [8]. The major classes of these indole alkaloids include hapalindoles, ambiguines, fischerindoles, and welwitindolinones, of which more than 80 variants have been described in the literature [9–24]. They share several common motifs: an indole or oxindole core, a cyclohexane fused to that core, an isonitrile or isothiocyanate functional group, a chlorine substituent, or an additional ring [25]. The largest group of these alkaloids are the hapalindoles. So far 30 different hapalindoles have been reported [9, 10, 15, 18, 21, 26]. The first hapalindoles, hapalindoles A and B, were discovered in 1984 by Moore et al. from *Hapalosiphon fontinalis* [9]. Since then, diverse hapalindoles have been isolated from various strains of the genera *Hapalosiphon*, *Fischerella*, and *Westiellopsis* [9, 17, 21, 26].

Hapalindoles have a broad spectrum of biological activities, e.g., activity against bacteria [17, 19, 21, 27], fungi [19, 27], and algae [27]. Studies by Doan et al. suggested inhibition of RNA polymerase and consequently the disturbance of the protein biosynthesis as a possible mode of action for the antibacterial activity [27, 28]. Furthermore, cytotoxic activity against normal mammalian and cancer cell lines has been reported for various hapalindoles [21, 27]. However, no mode of action for this activity has been described yet. Finally, they have been shown to be toxic to insects [18, 29] and vertebrates [23]. The insecticidal activity could be explained by sodium channel modulating activity of the indole alkaloids [30]. Interestingly, inhibition of sodium channels did not lead to any cytotoxicity in neuroblastoma cell lines. Thus, compared to insects, a different mode of action must underlie the cytotoxicity on mammalian cells. Although various bioactivities can be attributed to hapalindoles, modulation of the activity of human immune cells has not yet been described. Immunomodulatory drugs are used as modifiers of the immune system to either enhance the immune response against infectious diseases, tumors, and immunodeficiency, or to suppress the immune reaction in organ transplants or to treat autoimmune responses. Screening of a cyanobacteria extract collection (575 extracts) derived from strains from all cyanobacteria orders for inhibition of T cell proliferation resulted in 35 extracts with an activity at 1  $\mu\text{g}/\text{mL}$  or lower. One of the active extracts was derived from a *Hapalosiphon* sp. strain. Bioassay-guided fractionation of the extract led to the isolation of 3 known hapalindoles, hapalindole A (1) [9], D (2), and M (3) [10], as well as 2 formamide-bearing hapalindoles that are reported here for the first time from a natural

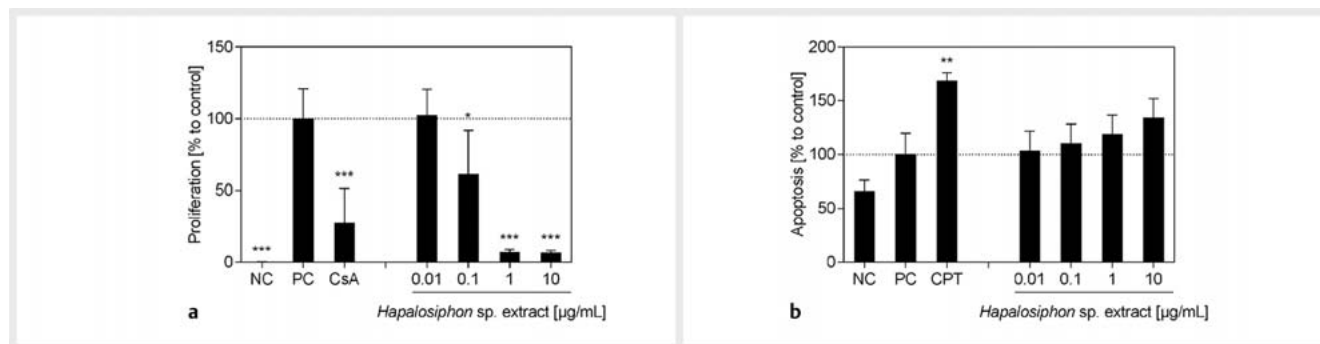
source, namely hapalindole A-formamide (4) [31] and hapalindole J-formamide (5) [32]. Here, we report the isolation, structure elucidation, and the investigation of the immunomodulatory properties of the hapalindole derivatives isolated from *Hapalosiphon* sp. CBT1235.

## Results and Discussion

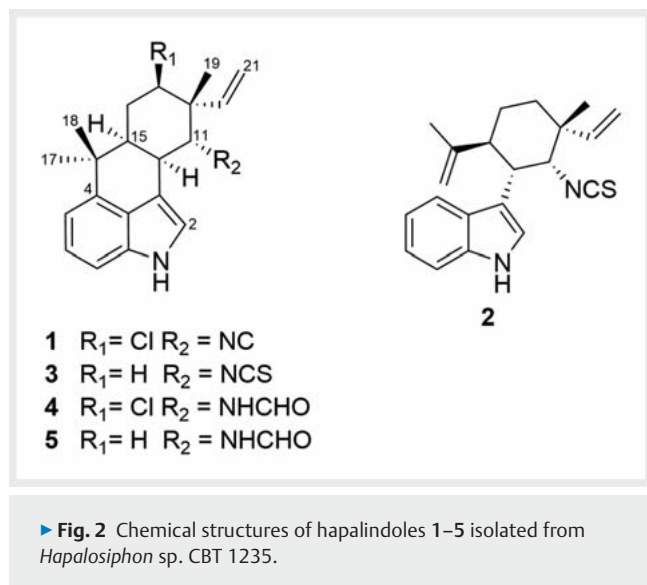
Our initial screening of a cyanobacteria extract collection for immunomodulating activity on human immune cells resulted in 35 extracts with an activity at a concentration of 1  $\mu\text{g}/\text{mL}$  or lower. Nine out of the 11 most potent extracts were derived from cyanobacteria of the genus *Nostoc*. Dereplication of the natural products in these *Nostoc* strain extracts showed the presence of the well-known cytotoxic compound cryptophycin-1 in most of these strains [33–36], so they were excluded from further investigation. Chromatographic evaluation of the remaining extracts by HPLC-DAD/MS showed that few of them featured prominent peaks. In the HPLC-DAD chromatogram of an *Hapalosiphon* sp. extract, several prominent peaks were observed; thus, this strain was selected for follow-up work. T cell proliferation was inhibited by this extract with an  $\text{IC}_{50}$  of 0.11  $\mu\text{g}/\text{mL}$  (► Fig. 1 a). At the same time, a trend to an increased amount of apoptotic cells at an extract concentration of 10  $\mu\text{g}/\text{mL}$  could be observed (statistically non-significant, ► Fig. 1 b). No induction of necrosis has been observed (data not shown). Microfractionation of the extract and subsequent bioassays showed that indeed the major compounds observable in the chromatogram were responsible for the bioactivity and led to the significant retardation of T cell proliferation (Fig. 1S a–c in the Supporting Information).

Five compounds present in the active micro-fraction were isolated from the extract by semi-preparative HPLC (1–5).  $^1\text{H}$  NMR spectra of the pure compounds displayed typical signals of hapalindoles. The molecular formulas were deduced from HRESIMS data. The structures of 1–5 were elucidated based on 1D and 2D NMR data. NMR and MS spectral data of compounds 1–3 matched the published data for hapalindole A (1), hapalindole D (2), and hapalindole M (3), respectively (structures of all isolated compounds, see ► Fig. 2). NOESY experiments as well as the determination of specific rotation values confirmed the absolute configuration of compounds 1–3 as originally described in detail by Moore et al. [10]. Hapalindole A (1) was the main compound isolated from *Hapalosiphon* sp. CBT1235.

Compounds 4 and 5 were hapalindole derivatives not yet described as natural products. Compound 4 was isolated as a brown oil. HRESIMS showed an  $[\text{M} + \text{H}]^+$  ion with  $m/z$  357.1723, corresponding to the molecular formula  $\text{C}_{21}\text{H}_{25}\text{N}_2\text{OCl}$  (calcd. 357.1728,  $\Delta$  1.54 ppm). The  $^1\text{H}$ -spectrum of 4 was almost identical with the respective spectrum of 1 but with 2 additional peaks in the low-field region (8.04 ppm and 8.57 ppm) and a deshielded H-11 (4.84 ppm) compared to 1 (4.37 ppm), suggesting C-11 to be substituted with a formamide moiety (► Table 1). Key HMBC and COSY correlations confirmed the planar structure (► Fig. 3). Evaluation of the NOESY spectrum showed the relative configuration of 4 to be the same as hapalindole A with the formamide group being attached axially to C-11 (strong correlations of the N-formamide proton with H-10, H-13 and H-15) [9]. Comparing the specific rotation



► **Fig. 1** Effects of *Hapalosiphon* sp. CBT1235 extract on T cell proliferation and apoptosis induction. Primary human lymphocytes were cultured in the presence of medium (NC) or were stimulated with anti-human CD3 and anti-human CD28 mAb (PC; 100 ng/mL). Activated T cells were further incubated with cyclosporine A (CsA; 5 µg/mL), camptothecin (CPT; 30 µg/mL), or different concentrations of the *Hapalosiphon* sp. CBT1235 extract. Cell division analysis was carried out using CFSE staining and flow cytometry. Levels of apoptosis were determined using flow-cytometric analysis of annexin V-stained cells. **a** Effects of *Hapalosiphon* sp. CBT1235 extract on lymphocyte proliferation. **b** Induction of apoptosis by *Hapalosiphon* sp. CBT1235 extract. Data of 4 (**a**) or 3 (**b**) independent experiments are presented as mean ± SD in relation to stimulated T cells (PC = 100%). Asterisks indicate significant differences from PC controls (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



values with already published data confirmed our assignment of the absolute configuration of 4 [37]. Therefore, compound 4 was identified as the new natural product hapalindole A-formamide.

The main difference with 5 compared to 1 and 4 was the absence of the chlorine. Compound 5 was also isolated as a brown oil. HRESIMS showed an  $[M+H]^+$  ion with  $m/z$  323.2114, corresponding to the molecular formula  $C_{21}H_{26}N_2O$  (calcd. 323.2118,  $\Delta$  1.08 ppm). Evaluation of the NMR spectra confirmed the presence of a formamide group at C-11 of the hapalindole backbone, as well (► **Fig. 3** and **Table 1**). The absolute configuration of 5 matches the one of hapalindole J, the nonchlorinated variant of hapalindole A (1), and has been confirmed by comparing the NOESY correlations and the specific rotation values with those originally published by Moore et al. [10]. Additional NOESY correlations for the axial formamide could be observed (► **Fig. 4**). Com-

pound 5 was thus confirmed to be the new natural product hapalindole J-formamide.

Coupling constants between H-22 (NH) and H-23 were found to be 1.37 Hz and 1.45 Hz for hapalindole A-formamide (4) and J-formamide (5), respectively, indicating a *cis* conformation of the amide bond in both compounds [31].

Hapalindole formamides have been described as intermediates during the total synthesis of hapalindoles, as derivatization products with formic acid, and they can also form during storage in *d*-chloroform [13, 31, 32, 38]. Here, we report for the first time the isolation of 2 hapalindole formamides as natural products from *Hapalosiphon* sp. CBT1235. 4 and 5 could readily be detected by HPLC-MS in a fresh *Hapalosiphon* sp. CBT1235 extract that has not been in contact with formic acid or other acids (**Fig. 2S**, Supporting Information), ruling out the possibility that the isolated formamides are processing or isolation artifacts.

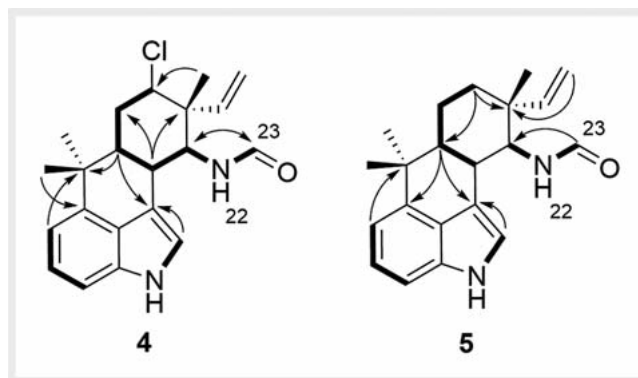
All isolated hapalindoles showed significant effects in the T cell proliferation assay (► **Fig. 5**). Hapalindole A (1) displayed the highest antiproliferative activity with an  $IC_{50}$  of 1.56 µM. Hapalindoles D (2) and M (3) showed a weaker activity and suppressed proliferation with an  $IC_{50}$  value of 27.15 µM for hapalindole D. Hapalindoles A-formamide (4) and J-formamide (5) again showed lower activity (► **Fig. 5**). Except for hapalindole J-formamide, all hapalindoles induced T cell apoptosis at highest concentrations (► **Fig. 6**).

Although relevant amounts of T cell apoptosis were detected, retardation of T cell proliferation was measurable at up to 10-fold lower concentrations. The most potent compound, hapalindole A, was effective at a concentration of 3.0 µM without showing cytotoxic effects. Therefore, potentially a therapeutic range for an application as an anti-inflammatory remedy is given. The isonitrile functional group seems to be crucial for the antiproliferative bioactivity of the hapalindoles, as the formamide derivatives possess a weaker activity. This is in agreement with previous findings, where a reduced antifungal and antibacterial activity of the hapalindole formamides compared to their isonitrile and isothiocyanate counterparts has been reported [31]. Our results, therefore,

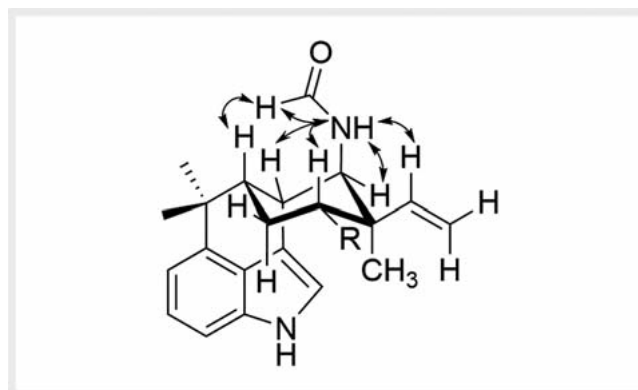
► **Table 1**  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments for **4** and **5** (600 MHz for  $^1\text{H}$ , 150 MHz for  $^{13}\text{C}$ ,  $\text{DMSO-d}_6$ ).  $^{13}\text{C}$  chemical shifts for **5** were extracted from the 2D spectra.

Position	4		5	
	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
1	10.81, s	indole N	10.69, s	indole N
2	7.24, t (1.8)	120.5	7.13, m	119.7
3		110.6		111.8
4		137.1		138.0
5	6.81, d (7.0)	112.3	6.79, br d (7.02)	111.9
6	7.02, t (7.6)	121.9	6.99, t (7.4)	121.5
7	7.14, d (7.9)	108.7	7.10, d (8.0)	108.1
8		133.3		133.4
9		124.0		124.4
10	3.29	36.7	3.30	36.3
11	4.84, m	54.6	4.59, s	51.6
12		44.6		39.1
13 ax	4.61, dd (12.4, 4.0)	65.3	1.74, td (13.2, 3.5)	30.2
13 eq			1.27, m	
14 ax	1.26, m	31.3	0.85, m	19.3
14 eq	1.96, td (13.0, 3.4)		1.60, br d (10.5)	
15	2.11, br td (13.1, 3.8)	44.4	1.87, m	43.4
16		37.4		37.3
17	1.46, s	24.3	1.43, s	24.5
18	1.04, s	31.9	1.06, s	31.5
19	0.85, s	19.6	0.74, s	26.4
20	5.82, dd (17.1, 11.0)	144.2	5.87, m	147.7
21	5.08, m	114.0	4.87, m	109.9
22	8.57, br d (9.9)	N	8.23, br d (9.16)	N
23	8.04, d (1.37)	159.7	8.00, br d (1.45)	159.6

strengthen the key role of this functional group in regard to the bioactivity of members of the hapalindole family. Our work shows that antiproliferative effects on human T cells are more pronounced than toxicity on human T cells, adding this activity to the wide range of reported bioactivities of the hapalindoles. The mode of action in human immune cells remains to be investigated. Moreover, concerning the fact that hapalindoles have been described to be neurotoxic metabolites [30], detailed studies which further discriminate toxicity and immunomodulatory effects need to be carried out in order to estimate whether or not a development as immunomodulatory drugs would be possible.



► **Fig. 3**  $^1\text{H}$ - $^1\text{H}$  COSY (bold connections) and selected HMBC correlations (arrows) of **4** and **5**.



► **Fig. 4** Selected NOESY correlations (arrows) of compounds **4** ( $\text{R} = \text{Cl}$ ) and **5** ( $\text{R} = \text{H}$ ).

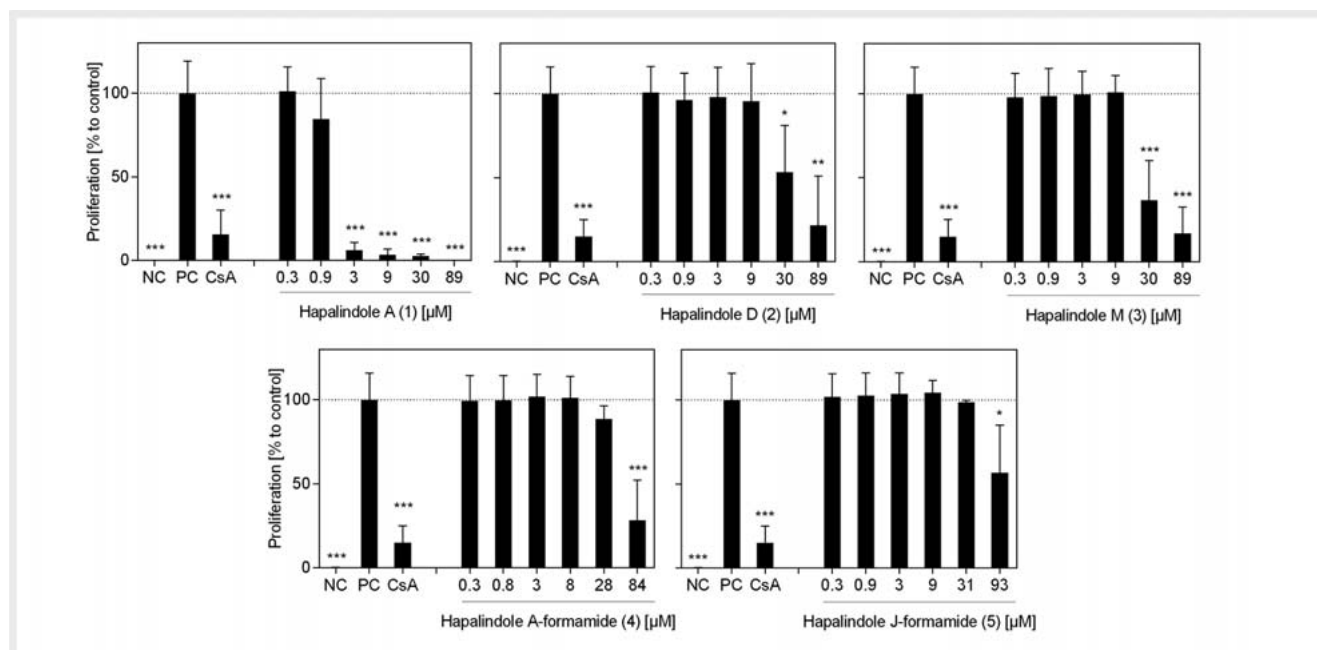
## Materials and Methods

### General experimental procedures

HRESIMS data were obtained using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 HPLC system (Thermo Fisher Scientific). Semi-preparative HPLC was conducted on an UltiMate 3000 HPLC system (Thermo Fisher Scientific). NMR spectra were either recorded at 600 MHz ( $^1\text{H}$  frequency) on a Bruker AV-III spectrometer using a cryogenically cooled 5 mm TCI-triple resonance probe equipped with 1-axis self-shielded gradients at 300 K or at 400 MHz ( $^1\text{H}$  frequency) on an Agilent DD2 spectrometer. Spectra were referenced indirectly. If 1D spectra were not separately recorded,  $^{13}\text{C}$  chemical shifts were extracted from the 2D spectra (compounds 1–3, and 5).

### Cyanobacterial material

*Hapalosiphon* sp. CBT1235 was taxonomically identified as *Hapalosiphon* sp. on the basis of its morphology. The strain has kindly been provided by Algenol Biotech Inc. (USA) as ABCC 804 and is deposited in the culture collection of the Cyano Biotech GmbH



► **Fig. 5** Effects of different hapalindoles on T cell proliferation. Primary human lymphocytes were cultured in the presence of medium (NC) or were stimulated with anti-human CD3 and anti-human CD28 mAb (PC; 100 ng/mL). Activated T cells were further incubated with cyclosporine A (CsA; 5  $\mu$ g/mL) or different concentrations of 1–5. Cell division analysis was carried out using CFSE staining and flow cytometry. Data of 3 independent experiments are presented as mean  $\pm$  SD in relation to stimulated T cells (PC = 100%). Asterisks indicate significant differences from PC controls (\* $p$  < 0.01, \*\*\* $p$  < 0.001).

(Germany) under the accession number CBT 1235. The strain was cultivated in BG11 medium [39] at 28 °C under continuous light (60–80  $\mu$ mol  $m^{-2} \cdot s^{-1}$ ) in 20 L scale photobioreactors and harvested semi-continuously over a period of several weeks.

### Extraction, bioassay-guided fractionation, and isolation of compounds 1–5

Cyanobacterial cells were harvested and lyophilized. Seven grams dry biomass were suspended in 100 mL 50% methanol in water (v/v), treated with an ultrasonication rod (Bandelin), and extracted on a shaker for 30 min at room temperature. After centrifugation (20 min, 10 800 g), the biomass was extracted using 100 mL 80% methanol (v/v). The solutions were combined and dried under reduced pressure, yielding 0.4 g of biomass extract. For micro-fractionation, 4 mg of extract were suspended in 0.1 mL acetonitrile and separated into 23 fractions by HPLC using a  $C_{18}$  column (250  $\times$  4.6 mm, 5  $\mu$ m, 100  $\text{Å}$ , Luna, Phenomenex) and 5–100% acetonitrile-water as the mobile phase at 1 mL/min in 23 min. All fractions were tested for inhibitory activity on T cell proliferation.

For preparative isolation of the active compounds, the remaining extract was dissolved in acetonitrile and fractionated by semi-preparative HPLC using a phenyl-hexyl column (250  $\times$  10 mm, 5  $\mu$ m, 100  $\text{Å}$ , Luna, Phenomenex) and 60–80% acetonitrile-water as the mobile phase at 4.7 mL/min in 25 min to afford 11 fractions. Fraction 1 ( $t_R$  6.8 min) was further purified by an additional round of semi-preparative HPLC using a phenyl-hexyl column (250  $\times$  100 mm, 5  $\mu$ m, 100  $\text{Å}$ , Luna, Phenomenex) and 40–47%

acetonitrile-water as the mobile phase at 9.5 mL/min in 20 min to afford hapalindole A-formamide (**4**, 5.0 mg,  $t_R$  16.5 min) and hapalindole J-formamide (**5**, 2.0 mg,  $t_R$  15.1 min). Fraction 6 ( $t_R$  15.2 min) was further purified by semi-preparative HPLC using a pentafluorophenyl column (250  $\times$  100 mm, 5  $\mu$ m, 100  $\text{Å}$ , Luna, Phenomenex) and 45–63% acetonitrile-water as mobile phase at 9.5 mL/min in 30 min yielding hapalindole A (**1**, 16.1 mg,  $t_R$  18.4 min). No further purification was needed for fraction 8 ( $t_R$  19.2 min) and fraction 10 ( $t_R$  22.4 min), which corresponded to hapalindole D (**2**, 5.3 mg) and hapalindole M (**3**, 6.4 mg), respectively.

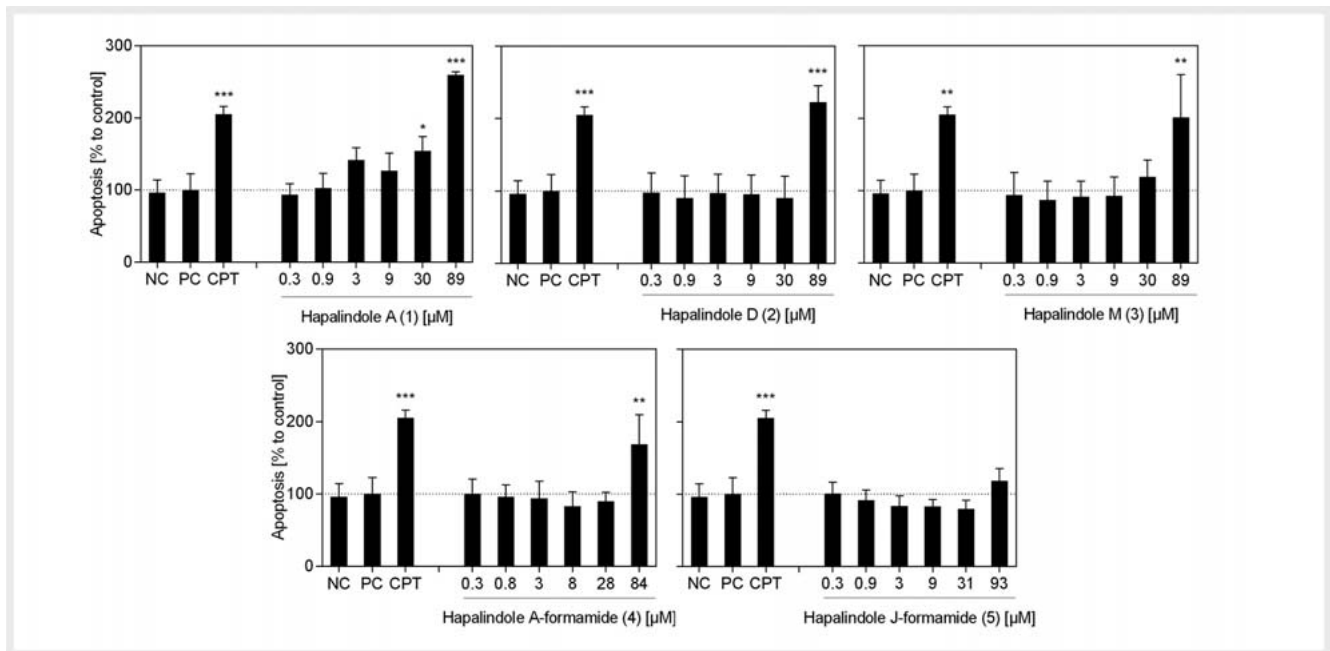
**Hapalindole A (1):**  $[\alpha]_D^{23} - 64.2^\circ$  ( $CH_2Cl_2$ , c 1.2); HRESIMS (positive ion mode):  $m/z$  339.1616  $[M + H]^+$ ; NMR spectra, see **Fig. 3S**, **4S**.

**Hapalindole D (2):**  $[\alpha]_D^{23} + 45.2^\circ$  ( $CH_2Cl_2$ , c 0.31); HRESIMS (positive ion mode):  $m/z$  337.1727  $[M + H]^+$ ; NMR spectra, see **Fig. 5S**, **6S**.

**Hapalindole M (3):**  $[\alpha]_D^{23} - 6.7^\circ$  ( $CH_2Cl_2$ , c 0.45); HRESIMS (positive ion mode):  $m/z$  337.1727  $[M + H]^+$ ; NMR spectra, see **Fig. 7S**, **8S**.

**Hapalindole A-formamide (4):** brown oil;  $[\alpha]_D^{23} - 56.2^\circ$  ( $CH_2Cl_2$ , c 0.45); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ): 223 (4.38), 282 (3.60), 292 (3.52) nm; HRESIMS (positive ion mode):  $m/z$  357.1723  $[M + H]^+$  (calcd. for  $C_{21}H_{25}N_2OCl$ , 357.1728); NMR data, see ► **Table 1**; NMR spectra **Fig. 9S–14S**.

**Hapalindole J-formamide (5):** brown oil;  $[\alpha]_D^{23} + 12.2^\circ$  ( $CHCl_3$ , c 0.9); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ): 224 (4.24), 282 (3.50), 292 (3.41) nm; HRESIMS (positive ion mode):  $m/z$  323.2114  $[M + H]^+$  (calcd.



► **Fig. 6** Levels of T cell apoptosis after treatment with different hapalindoles. Primary human lymphocytes were cultured in the presence of medium (NC) or were stimulated with anti-human CD3 and anti-human CD28 mAb (PC; 100 ng/mL). Activated T cells were further incubated with camptothecin (CPT; 30 μg/mL) or different concentrations of 1–5. Levels of apoptosis were determined using flow-cytometric analysis of annexin V-stained cells. Data of 3 independent experiments are presented as mean ± SD in relation to stimulated T cells (PC = 100%). Asterisks indicate significant differences from PC controls (\*\*p < 0.01, \*\*\*p < 0.001).

for C<sub>21</sub>H<sub>26</sub>N<sub>2</sub>O, 323.2118); NMR data, see ► **Table 1**; NMR spectra **Fig. 155–195**.

### Ethics statement

Written informed consent was obtained from patients prior to blood donation for research purposes. All experiments conducted on human material were approved by the ethics committee of the University Freiburg (55/14; February 11th, 2014).

### Preparation and cultivation of human immunocompetent cells

Human PBMC were isolated from the blood of adult donors obtained from the Blood Transfusion Centre (University Medical Center Freiburg). Venous blood was centrifuged on a LymphoPrep gradient (density: 1.077 g/cm<sup>3</sup>, 20 min, 500 g, 20 °C; Progen). Afterwards, cells were washed twice with medium and cell viability and concentration was determined using the trypan blue exclusion test. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen) at 37 °C in a humidified incubator with a 5% CO<sub>2</sub>/95% air atmosphere. PBMC were additionally stimulated with anti-human CD3 (clone OKT3) and anti-human CD28 (clone 28.6) mAb (100 ng/mL; both from eBioscience). Incubation was carried out as indicated in the figure captions in the presence of medium alone, camptothecin (CPT; 30 μg/mL; Tocris), cyclosporine A (CsA, 5 μg/mL, Sandimmun 50 mg/mL, Novartis), or different

concentrations of the *Hapalosiphon* sp. CBT1235 extract (0.01, 0.1, 1, 10 μg/mL), fractions (semi-quantitative using dilutions 1:250, 1:500, 1:1000, 1:2000) or various hapalindoles (0.1, 0.3, 1, 3, 10, 30 μg/mL).

### Cell division tracking using CFSE

PBMC were harvested, washed twice in cold PBS (Invitrogen) and resuspended in PBS at a concentration of 5 × 10<sup>6</sup> cells/mL. CFSE (5 mM; Sigma) was diluted 1/1000 and incubated for 10 min at 37 °C. The staining reaction was stopped by washing twice with complete medium. PBMC were activated as described above and cultured with the *Hapalosiphon* sp. CBT1235 extract, fractions, hapalindoles, or DMSO as a solvent control for 72 h. Cell division progress was analyzed from 3 independent experiments with a BD FACSCalibur flow cytometer using BD CellQuest Pro Software.

### Determination of apoptosis and necrosis using annexin V and propidium iodide staining

Cells were cultured as described, and levels of apoptosis and necrosis were determined using annexin V-FITC apoptosis detection kit (eBioscience) according to the manufacturer's instructions. After annexin V and propidium iodide staining, cells were analyzed by flow cytometry. CPT and Triton-X 100 (0.5%; Sigma-Aldrich) were used as positive controls for apoptosis and necrosis, respectively.

## Data analysis

For statistical analysis, data were processed with Microsoft Excel and SPSS software (IBM, Version 22.0). Data were adjusted in relation to untreated control cells (= 100% ± SD) and values are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* pairwise comparisons. P values <0.05 were considered as statistically significant (\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001). IC<sub>50</sub> and EC<sub>50</sub> values were determined using GraphPad Prism 6.

## Supporting information

<sup>1</sup>H, <sup>13</sup>C, HSQC, HMBC and NOESY spectra, MS data as well as a chromatogram of the micro-fractionation and the results of the inhibition of T cell proliferation by different *Hapalosiphon* sp. CBT1235 fractions are available as Supporting Information.

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## Conflict of Interest

HE is CSO and co-owner of Cyano Biotech GmbH, DE is CEO and co-owner of Cyano Biotech GmbH. The company does not have any financial interest in the research presented here. The other authors declare no conflicts of interest. The funding sponsors had no role in the design, writing and publishing strategy of the study, nor in collection, analysis or interpretation of the data.

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