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ORIGINAL ARTICLE

Anti-MRSA-acting carbamidocyclophanes H–L from the Vietnamese cyanobacterium *Nostoc* sp. CAVN2

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The methanol extract of the Vietnamese freshwater cyanobacterium *Nostoc* sp. CAVN2 exhibited cytotoxic effects against MCF-7 and 5637 cancer cell lines as well as against nontumorigenic FL and HaCaT cells and was active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae.* High-resolution mass spectrometric analysis indicated the presence of over 60 putative cyclophane-like compounds in an antimicrobially active methanol extract fraction. A paracyclophanes-focusing extraction and separation methodology led to the isolation of 5 new carbamidocyclophanes (1–5) and 11 known paracyclophanes (6–16). The structures and their stereochemical configurations were elucidated by a combination of spectrometric and spectroscopic methods including HRMS, 1D and 2D NMR analyses and detailed comparative CD analysis. The newly described monocarbamoylated [7.7]paracyclophanes (1, 2, 4 and 5) differ by a varying degree of chlorination in the side chains. Carbamidocyclophane J (3) is the very first reported carbamidocyclophane bearing a single halogenation in both butyl residues. Based on previous studies a detailed phylogenetic examination of cyclophane-producing cyanobacteria was carried out. The biological evaluation of 1–16 against various clinical pathogens highlighted a remarkable antimicrobial activity against MRSA with MICs of 0.1–1.0 μM, and indicated that the level of antibacterial activity is related to the presence of carbamoyl moieties.
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INTRODUCTION

Since the first synthesis of di-*p*-xylylene by Brown and Farthing[1] in 1949, and the first description of [m.n]paracyclophanes by Cram and Steinberg[2] in 1951, cyclophanes have become a widespread and well-known class of organic molecules in nearly all fields of chemistry.[3] Yet, it took almost another four decades until the first naturally occurring [7.7]paracyclophanes with cytotoxic effects against different cancer cell lines, nostocyclophane D and cylindrocyclophane A, have been isolated from the cyanobacterial strains *Nostoc linckia* (Roth) Bornet (UTEX B1932) and *Cylindrospermum licheniforme* (ATCC 29204), respectively.[4] Subsequently, numerous molecules with a varying substitution pattern of the slightly modified [7.7]paracyclophane skeleton have been isolated and reported from several terrestrial cyanobacteria belonging to the order Nostocales. Besides cylindrocyclophanes A−F,[5] A_1−A_4, C_1−C_4, F_4 and A_{B4},[6] nostocyclophanes A−D[7] and merocyclophanes A and B[8] with diverse biological effects, cytotoxically and antimicrobially active carbamidocyclophanes A−G[9,10] have been described from the cyanobacteria *Nostoc* spp. CAVN10 and UIC 10274. In comparison with the cylindrocyclophane/carbamidocyclophane carbon skeleton, the nonhalogenated merocyclophanes possess α-branched methyls at C-1/C-14, and lack in the presence of β-branched methyl groups at C-2/C-15, respectively. Nostocyclophanes contain neither α- nor β-branched methyls, but including exclusively chlorine atoms at C-3 and C-16. Furthermore, nostocyclophane A and B are glycosylated derivatives (see Figure 1a and Supplementary Information S0). The carbamidocyclophane subgroup is characterized by the presence of carbamoyl moieties attached to C-1/C-14 of the [7.7]paracyclophane scaffold. Both mono- and dicarbamoylated carbamidocyclophanes exhibited cytotoxic activity against several tumor cell lines and antimicrobial activity against Gram-positive bacteria; for example, *Mycobacterium tuberculosis*, *Entercoccus faecalis* and *Staphylococcus aureus*.[9,10] A cytotoxicity-guided evaluation of different extracts from various filamentous cyanobacteria revealed a new [7.7]paracyclophane-biosynthesizing strain, the Vietnamese freshwater cyanobacterium *Nostoc* sp. CAVN2. In this article, we describe an optimized extraction and separation procedure for the detection and isolation of closely related [7.7]paracyclophanes as well

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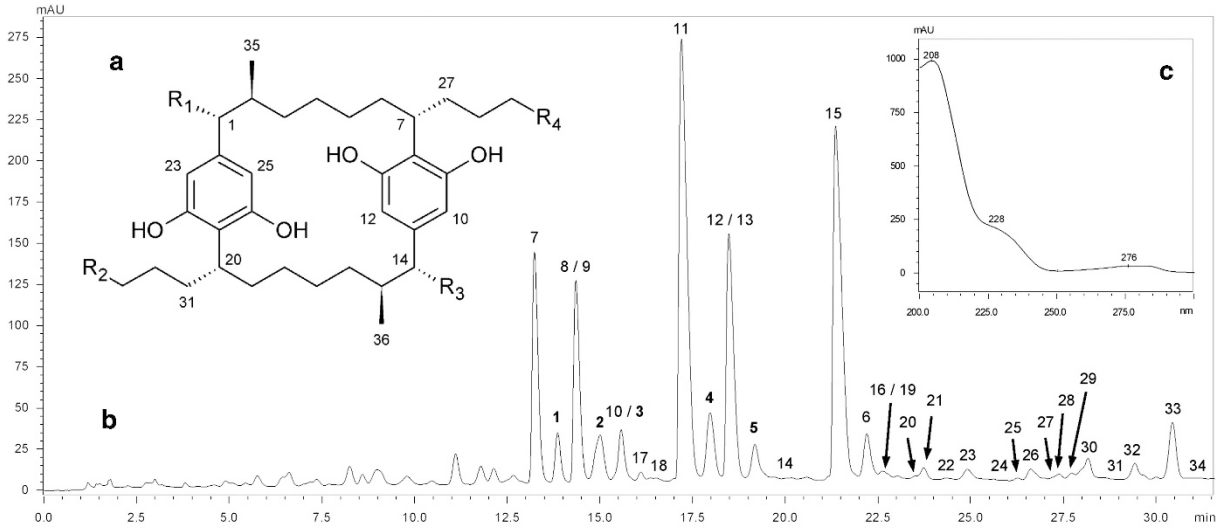


compound	cyclophane	monoisotopic mass *m/z* [M-H]$^-$ measured	monoisotopic mass *m/z* [M-H]$^-$ calculated	molecular formula	R_1	R_2	R_3	R_4	first described
7	carbamidocyclophane E	669.4124	669.4120	$C_{38}H_{58}N_2O_8$	$OCONH_2$	CH_3	$OCONH_2$	CH_3	Bui *et al.*, 2007 [9]
1	**carbamidocyclophane H**	**626.4070**	**626.4062**	**$C_{37}H_{57}NO_7$**	**$OCONH_2$**	**CH_3**	**OH**	**CH_3**	**this study**
8	cylindrocyclophane A	583,4005	583.4004	$C_{36}H_{56}O_6$	OH	CH_3	OH	CH_3	Moore *et al.*, 1990 [4]
9	carbamidocyclophane D	703.3741	703.3731	$C_{38}H_{57}ClN_2O_8$	$OCONH_2$	CH_3	$OCONH_2$	CH_2Cl	Bui *et al.*, 2007 [9]
2	**carbamidocyclophane I**	**660.3680**	**660.3673**	**$C_{37}H_{56}ClNO_7$**	**$OCONH_2$**	**CH_3**	**OH**	**CH_2Cl**	**this study**
10	cylindrocyclophane A_1	617.3624	617.3614	$C_{36}H_{55}ClO_6$	OH	CH_3	OH	CH_2Cl	Chlipala *et al.*, 2010 [6]
3	**carbamidocyclophane J**	**737.3339**	**737.3341**	**$C_{38}H_{56}Cl_2N_2O_8$**	**$OCONH_2$**	**CH_2Cl**	**$OCONH_2$**	**CH_2Cl**	**this study**
17	putative cyclophane [a], constitutional isomer of **4**	694.3289	694.3283	$C_{37}H_{55}Cl_2NO_7$	n.i. [b]	n.i.	n.i.	n.i.	this study
18	putative cyclophane [a], constitutional isomer of 12	651.3215	651.3225	$C_{36}H_{54}Cl_2O_6$	n.i.	n.i.	n.i.	n.i.	this study
11	carbamidocyclophane C	737.3356	737.3341	$C_{38}H_{56}Cl_2N_2O_8$	$OCONH_2$	CH_3	$OCONH_2$	$CHCl_2$	Bui *et al.*, 2007 [9]
4	**carbamidocyclophane K**	**694.3293**	**694.3283**	**$C_{37}H_{55}Cl_2NO_7$**	**$OCONH_2$**	**CH_3**	**OH**	**$CHCl_2$**	**this study**
12	cylindrocyclophane A_2	651.3211	651.3225	$C_{36}H_{54}Cl_2O_6$	OH	CH_3	OH	$CHCl_2$	Chlipala *et al.*, 2010 [6]
13	carbamidocyclophane B	771.2953	771.2951	$C_{38}H_{55}Cl_3N_2O_8$	$OCONH_2$	CH_2Cl	$OCONH_2$	$CHCl_2$	Bui *et al.*, 2007 [9]
5	**carbamidocyclophane L**	**728.2879**	**728.2893**	**$C_{37}H_{54}Cl_3NO_7$**	**$OCONH_2$**	**CH_2Cl**	**OH**	**$CHCl_2$**	**this study**
14	cylindrocyclophan A_3	685.2846	685.2835	$C_{36}H_{53}Cl_3O_6$	OH	CH_2Cl	OH	$CHCl_2$	Chlipala *et al.*, 2010 [6]
15	carbamidocyclophane A	805.2555	805.2562	$C_{38}H_{54}Cl_4N_2O_8$	$OCONH_2$	$CHCl_2$	$OCONH_2$	$CHCl_2$	Bui *et al.*, 2007 [9]
6	carbamidocyclophane F	762.2502	762.2503	$C_{37}H_{53}Cl_4NO_7$	$OCONH_2$	$CHCl_2$	OH	$CHCl_2$	Luo *et al.*, 2014 [10]
19	putative cyclophane [a]	610.4120	610.4113	$C_{37}H_{57}NO_6$	n.i.	n.i.	n.i.	n.i.	this study
16	cylindrocyclophane A_4	719.2456	719.2445	$C_{36}H_{52}Cl_4O_6$	OH	$CHCl_2$	OH	$CHCl_2$	Chlipala *et al.*, 2010 [6]
20	cylindrocyclophane C [a]	567.4056	567.4055	$C_{36}H_{56}O_5$	OH	CH_3	H	CH_3	Moore *et al.*, 1992 [5]
21	putative cyclophane [a]	644.3732	644.3723	$C_{37}H_{56}ClNO_6$	n.i.	n.i.	n.i.	n.i.	this study
22	cylindrocyclophan C_1 [a]	601.3655	601.3665	$C_{36}H_{55}ClO_5$	OH	CH_3	H	CH_2Cl	Chlipala *et al.*, 2010 [6]
23	putative cyclophane[a], constitutional isomer of 26	678.3314	678.3334	$C_{37}H_{55}Cl_2NO_6$	n.i.	n.i.	n.i.	n.i.	this study
24	putative cyclophane[a], constitutional isomer of 28	635.3267	635.3276	$C_{36}H_{54}Cl_2O_5$	n.i.	n.i.	n.i.	n.i.	this study
25	putative cyclophane[a], constitutional isomer of 27	726.3176	726.3181	$C_{37}H_{55}Cl_2NO_9$	n.i.	n.i.	n.i.	n.i.	this study
26	putative cyclophane[a], constitutional isomer of 23	678.3314	678.3334	$C_{37}H_{55}Cl_2NO_6$	n.i.	n.i.	n.i.	n.i.	this study
27	putative cyclophane[a], constitutional isomer of 25	726.3172	726.3181	$C_{37}H_{55}Cl_2NO_9$	n.i.	n.i.	n.i.	n.i.	this study
28	cylindrocyclophane C_2 [a]	635.3268	635.3276	$C_{36}H_{54}Cl_2O_5$	OH	CH_3	H	$CHCl_2$	Chlipala *et al.*, 2010 [6]
29	putative cyclophane [a]	712.2928	712.2944	$C_{37}H_{54}Cl_3NO_6$	n.i.	n.i.	n.i.	n.i.	this study
30	putative cyclophane [a]	773.3087	773.3108	$C_{38}H_{57}Cl_3N_2O_8$	n.i.	n.i.	n.i.	n.i.	this study
31	cylindrocyclophane C_3 [a]	669.2876	669.2886	$C_{36}H_{53}Cl_3O_5$	OH	CH_2Cl	H	$CHCl_2$	Chlipala *et al.*, 2010 [6]
32	cylindrocyclophane F [a]	551,4109	551.4106	$C_{36}H_{56}O_4$	H	CH_3	H	CH_3	Moore *et al.*, 1992 [5]
33	putative cyclophane [a]	746.2548	746.2554	$C_{37}H_{53}Cl_4NO_6$	n.i.	n.i.	n.i.	n.i.	this study
34	cylindrocyclophane C_4 [a]	703.2496	703.2496	$C_{36}H_{52}Cl_4O_5$	OH	$CHCl_2$	H	$CHCl_2$	Chlipala *et al.*, 2010 [6]

[a] putative identification, based on UV and HRMS data
[b] not identified so far

Figure 1 Structurally diverse paracyclophanes biosynthesized by *Nostoc* sp. CAVN2. (**a**) Carbamidoyclophane and cylindrocyclophane core structure and (**b**) HPLC-UV chromatogram ($\lambda = 228$ nm) of the cyclophane-rich solid-phase extraction (SPE) fraction. (**c**) UV reference spectrum of carbamidocyclophane A.[9] The table describes structures and structural proposals belonging to selected peaks of (**b**).

as the complete structure elucidation of novel carbamidocyclophanes with a partly hitherto unknown halogen atom distribution and the biological evaluation of all isolates.

RESULTS AND DISCUSSION

Cytotoxicity screening

Initially, 53 dried extracts from 14 cyanobacterial strains, belonging to the orders Nostocales and Oscillatoriales, were evaluated for cytotoxic activity against several cell lines (Supplementary Information S1–S3). A total of 30 extracts were inactive (IC_{50} $>500\,\mu g\,ml^{-1}$) and 21 exhibited only marginal cytotoxicity ($20\,\mu g\,ml^{-1} \leq IC_{50} \leq 500\,\mu g\,ml^{-1}$). Merely the ethyl acetate extract of the culture medium and the methanolic biomass extract from *Nostoc* sp. CAVN2 were found to have significant inhibitory activity against breast adenocarcinoma MCF-7 cells (IC_{50} $<13.5\,\mu g\,ml^{-1}$). In addition, the MeOH extract was active against the human urinary bladder carcinoma cell line 5637 ($IC_{50} = 6\,\mu g\,ml^{-1}$), but also exhibited moderate cytotoxicity against nontumorigenic FL ($IC_{50} = 11.6\,\mu g\,ml^{-1}$) and HaCaT ($IC_{50} = 14\,\mu g\,ml^{-1}$) cells. Furthermore, it was strongly active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* with an MIC of 0.8 and $3.2\,\mu g\,ml^{-1}$, respectively. No activity was observed against Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Therefore, 93 mg of the methanol extract from *Nostoc* sp. CAVN2 were subjected to bioactivity-guided fractionation utilizing solid-phase extraction (SPE) and *S. aureus* ATCC 6538 as indicator organism. An aliquot of the bioactive fraction, eluted with 80% MeOH in H_2O, was subjected to analytical HPLC-DAD-ESI-TOF-HRMS analysis. Using a pentafluorophenyl endcapped core-shell column, we were able to distinguish over 60 compounds with UV spectra comparable to that of carbamidocyclophane A (**15**).[9] Extensive HRMS data examination of each compound, including critical evaluation of its isotopic distribution pattern and predicted degree of double-bond equivalents, indicated the presence of already described compounds (carbamidocyclophanes A–F; cylindrocyclophanes A, A_1–A_4, C, C_1–C_4 and F) alongside unknown putative cyclophanes differing in the level of esterification and halogenation; that is, from nonhalogenated to trichlorinated molecules with only one carbamoyl moiety on C-1 or C-14 (see Figure 1). On the basis of obtained MS data, we assumed that the second carbamoyl group might be substituted by a hydroxyl group or just by a hydrogen atom. Furthermore, the LC-MS data also indicated several glycosylated cyclophanes in the retention time range from 4.5 to 11.5 min (data not shown) as it was reported for nostocyclophanes A and B.[7] In some cases, several peaks were detected at different retention times when analyzing distinct monoisotopic mass selected ion chromatograms. This might indicate the presence of different constitutional isomers, depending on the substituent distribution of the cyclophane core structure, confirming *Nostoc* sp. CAVN2 as a producer of a high diversity of cyclophanes.

Isolation procedure and structure elucidation

For isolation of cyclophanes, the methanol extract of the *Nostoc* sp. CAVN2 biomass was also fractionated via the SPE procedure. The cyclophane-containing fraction, eluting with 80% methanol in water, was collected. This sample was subjected to semi-preparative reversed-phase HPLC on a polar endcapped ether-linked phenyl phase to obtain five fractions: P1 to P5. Each of these contained paracyclophanes with an equal degree of halogenation, but the level of chlorination continuously increased from P1 to P5. The rich diversity of closely related cyclophane analogs in *Nostoc* sp. CAVN2 made this prepurification step necessary to achieve a proper separation, for example of the binary mixtures **8/9**, **10/3**, **12/13** and **16/19**, as the compounds differ only slightly in their physicochemical properties. Novel cyclophane derivatives **1–5** were isolated besides the previously described cyclophanes (**6–16**) by a second round of semi-preparative HPLC, this time using a pentafluorophenyl stationary phase (Figure 2).

Analytical HPLC-DAD-MS analysis of fraction P1 indicated the presence of six compounds with UV spectra comparable to carbamidocyclophane A (**15**) (Figure 1c) and in negative mode $[M–H]^-$ ions at *m/z* 933.3, 933.3, 646.4, 669.4, 626.4 and 583.4. The compounds occurred in a relative ratio (%) of 4.4:4.7:1.9:100:21.0:1.4 (Figure 2).

The final semi-preparative RP-HPLC separation of P1 yielded three white, amorphous substances successively eluting in the following

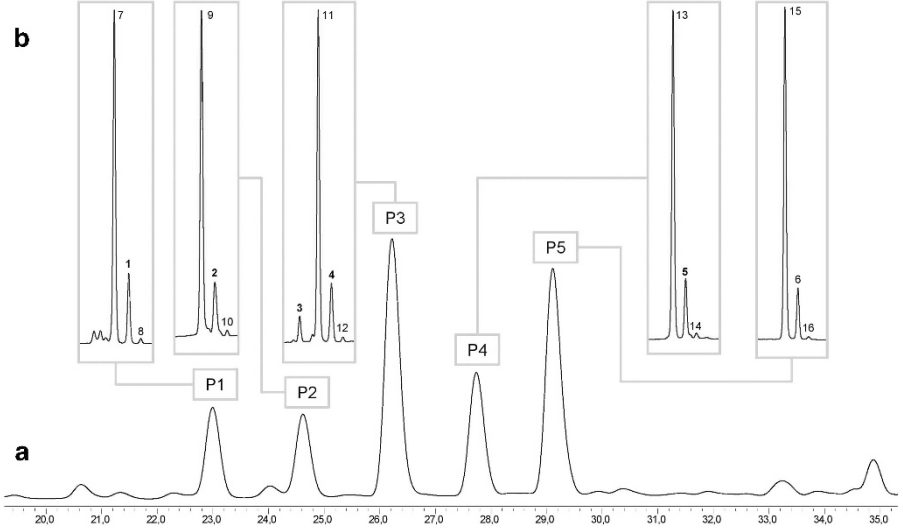
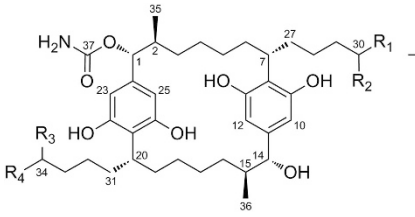


Figure 2 Separation and isolation procedure of novel and previously reported [7.7]paracyclophanes. (**a**) First semi-preparative HPLC of cyclophane-containing fraction utilizing an ether-linked phenyl phase. (**b**) Second semi-preparative HPLC of fractions P1 to P5 using a pentafluorophenyl stationary phase. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

order: **7** (4.3 mg, 0.18% of dry biomass) as the major compound, and **1** (1.0 mg, 0.04% of dry biomass) and **8** (0.2 mg, 0.01% of dry biomass) as minor metabolites. According to their spectroscopic data and by comparison with reported values, compound **7** was identified as the known nonhalogenated carbamidocyclophane E, and **8** as its bidescarbamoyl analog cylindroyclophane A.[4,5,9]

The negative-mode HR-ESI-TOF-MS analysis of **1** agreed with a molecular formula of $C_{37}H_{56}NO_4$ (found *m/z* 626.4070, calculated 626.4062 for $[M–H]^-$, Δ 1.28 p.p.m.). The isotope distribution indicated, in accordance with **7** and **8**, the absence of halogen atoms in **1**. The observed molecular mass of **1** is 43 Da lower than the molecular mass of **7**, and 43 Da higher compared with **8**, suggesting the presence of only one carbamoyl moiety in **1**.

As presented in Table 1, the ^{1}H-NMR data of **1** and the two known derivatives, carbamidocyclophane E (**7**)[9] and cylindrocyclophane A (**8**),[4,5] were very similar even though the spectra of **1** showed the double set of signals because of the loss of symmetry compared with **7** and **8** (Figure 1). The ^{1}H spectra included signals for aromatic protons (δ 6.0–6.5), methine proton signals at δ ~ 3.2 (H-7 and H-20), and in the range δ ~ 1.5–1.8 (H-2 and H-15), methyl groups (δ 0.7–1.2) and numerous methylene proton signals in the range δ 0.5 to δ 2.1. In addition, the ^{1}H NMR spectrum of **1** showed one oxymethine signal at δ 4.81, comparable to the signal for H-1/H-14 in **7**, and one oxymethine signal at δ 3.74, similar to that exhibited for the signal of H-1/H-14 in **8**, indicating an unsymmetrical substitution pattern for C-1 and C-14 as described for carbamidocyclophane F (**6**).[10] Analysis of homo- and heteronuclear 2D NMR data (COSY, TOCSY, HSQC and HMBC) revealed typical correlations for carbamidocyclophanes.[9,10] The 2D NMR signals and correlations corresponding to the core structure (C-1–C-26) matched perfectly with those described for **6**, whereas the substituents (C-27–C-30 and C-31–C-34) showed the typical signal pattern for alkyl chains and matched with the values described for **7** and **8**. HMBC correlation of H-1 (δ 4.81) to a quaternary carbon atom with a chemical shift value of δ 159.5 (C-37) corroborated the monocarbamoylation indicated by MS analysis. Taking into account all analytical data, compound **1** was identified as the nonhalogenated congener of **6** and was named carbamidocyclophane H (Figure 3).



#	R_1	R_2	R_3	R_4
6*	Cl	Cl	Cl	Cl
5	Cl	Cl	Cl	H
4	Cl	Cl	H	H
2	Cl	H	H	H
1	H	H	H	H

Figure 3 Monocarbamoylated carbamidocyclophanes **1**, **2**, **4**, **5** and **6***. *The structure was published by Luo *et al.*[10] during preparation of this article.

Table 1 NMR data of carbamidocyclophanes H–L (1–5) in MeOH-*d*$_4$

	1		***2***		***3***		***4***		***5***	
Position	δ_C, *mult.*	δ_H (*J in Hz*)	δ_C, *mult.*	δ_H (*J in Hz*)	δ_C, *mult.*	δ_H (*J in Hz*)	δ_C, *mult.*	δ_H (*J in Hz*)	δ_C, *mult.*	δ_H (*J in Hz*)
1	83.3, CH	4.81, *d* (10.2)	83.4, CH	4.80, *d* (10.3)	83.3, CH	4.81, *d* (10.1)	83.4, CH	4.81, *d* (10.2)	83.4, CH	4.81, *d* (10.3)
2	40.2, CH	1.73, *m*	40.3, CH	1.73, *m*	40.1, CH	1.73, *m*	40.2, CH	1.73, *m*	40.1, CH	1.73, *m*
3	34.2, CH_2	0.79, 0.70, *m*	34.3, CH_2	0.78, 0.69, *m*	34.1, CH_2	0.79, 0.72, *m*	34.4, CH_2	0.78, 0.72, *m*	34.3, CH_2	0.77, 0.72, *m*
4	29.6, CH_2	1.43, 0.83, *m*	29.6, CH_2	1.42, 0.82, *m*	29.4, CH_2	1.44, 0.83, *m*	29.6, CH_2	1.43, 0.83, *m*	29.6, CH_2	1.45, 0.83, *m*
5	30.4, CH_2	0.95, 0.71, *m*	30.4, CH_2	0.94, 0.71, *m*	30.2, CH_2	0.95, 0.73, *m*	30.4, CH_2	0.95, 0.72, *m*	30.3, CH_2	0.95, 0.71, *m*
6	35.1, CH_2	2.03, 1.32, *m*	35.2, CH_2	2.03, 1.31, *m*	35.1, CH_2	2.05, 1.33, *m*	35.1, CH_2	2.07, 1.33, *m*	35.2, CH_2	2.06, 1.32, *m*
7	36.7, CH	3.16, *m*	36.5, CH	3.19, *m*	36.3, CH	3.18, *m*	36.2, CH	3.20, *m*	36.2, CH	3.20, *m*
8	117.4, C	—	117.3, C	—	117.4, C	—	116.4, C	—	116.6, C	—
9	158.6, C	—	158.9, C	—	158.5, C	—	158.8, C	—	158.8, C	—
10	104.7, CH	6.24, *s*	105.0, CH	6.24, *s*	104.9, CH	6.20, *s*	104.6, CH	6.25, *s*	104.7, CH	6.25, *s*
11	143.5, C	—	144.0, C	—	139.5, C	—	144.1, C	—	144.0, C	—
12	108.5, CH	6.07, *s*	108.9, CH	6.07, *s*	109.0, CH	6.13, *s*	108.5, CH	6.08, *s*	108.7, CH	6.07, *s*
13	156.7, C	—	157.1, C	—	156.7, C	—	156.8, C	—	156.9, C	—
14	81.5, CH	3.74, *d* (9.6)	81.5, CH	3.74, *d* (9.7)	83.3, CH	4.81, *d* (10.1)	81.6, CH	3.75, *d* (9.5)	81.5, CH	3.75, *d* (9.7)
15	41.6, CH	1.55, *m*	41.8, CH	1.54, *m*	40.1, CH	1.73, *m*	41.8, CH	1.55, *m*	41.9, CH	1.54, *m*
16	34.9, CH_2	0.74, 0.63, *m*	35.0, CH_2	0.73, 0.64, *m*	34.1, CH_2	0.79, 0.72, *m*	35.0, CH_2	0.72, 0.63, *m*	34.9, CH_2	0.72, 0.63, *m*
17	29.6, CH_2	1.43, 0.83, *m*	29.6, CH_2	1.42, 0.82, *m*	29.4, CH_2	1.44, 0.84, *m*	29.6, CH_2	1.42, 0.83, *m*	29.6, CH_2	1.41, 0.82, *m*
18	30.4, CH_2	0.95, 0.71, *m*	30.4, CH_2	0.94, 0.71, *m*	30.2, CH_2	0.95, 0.73, *m*	30.4, CH_2	0.95, 0.72, *m*	30.3, CH_2	0.93, 0.71, *m*
19	35.1, CH_2	2.03, 1.32, *m*	35.2, CH_2	2.03, 1.31, *m*	35.1, CH_2	2.05, 1.33, *m*	35.1, CH_2	2.03, 1.32, *m*	35.2, CH_2	2.04, 1.32, *m*
20	36.7, CH	3.15, *m*	36.5, CH	3.16, *m*	36.3, CH	3.18, *m*	36.6, CH	3.16, *m*	36.2, CH	3.18, *m*
21	118.1, C	—	118.2, C	—	117.4, C	—	118.1, C	—	117.6, C	—
22	156.5, C	—	157.1, C	—	158.5, C	—	158.8, C	—	158.8, C	—
23	105.0, CH	6.20, *s*	105.3, CH	6.19, *s*	104.9, CH	6.20, *s*	104.9, CH	6.21, *s*	105.1, CH	6.20, *s*
24	139.5, C	—	139.9, C	—	139.5, C	—	139.5, C	—	139.7, C	—
25	109.0, CH	6.11, *s*	109.3, CH	6.12, *s*	109.0, CH	6.13, *s*	109.0, CH	6.13, *s*	109.2, CH	6.13, *s*
26	158.4, C	—	158.9, C	—	156.7, C	—	156.8, C	—	156.9, C	—
27	34.5, CH_2	1.93, 1.48, *m*	34.0, CH_2	2.00, 1.49, *m*	33.8, CH_2	1.98, 1.51, *m*	33.5, CH_2	2.05, 1.50, *m*	33.5, CH_2	2.03, 1.51, *m*
28	23.8, CH_2	1.28, 1.20, *m*	26.3, CH_2	1.29, 1.25, *m*	26.3, CH_2	1.30, 1.24, *m*	25.5, CH_2	1.37, *m*	25.6, CH_2	1.36, *m*
29	31.3, CH_2	1.16, 1.06, *m*	33.9, CH_2	1.73, 1.65, *m*	33.8, CH_2	1.74, 1.66, *m*	44.8, CH_2	2.19, 2.06, *m*	44.8, CH_2	2.19, 2.06, *m*
30	14.3, CH_3	0.81, *t* (7.4)	45.6, CH_2	3.44, *q* (7.3)	45.4, CH_2	3.44, *dt* (6.9, 0.9)	75.0, CH	5.82, *q* (5.7)	74.9, CH	5.83, *dt* (6.2, 3.9)
31	34.5, CH_2	1.93, 1.48, *m*	34.6, CH_2	1.92, 1.47, *m*	33.8, CH_2	1.98, 1.51, *m*	34.5, CH_2	1.93, 1.48, *m*	33.9, CH_2	1.99, 1.49, *m*
32	23.8, CH_2	1.28, 1.20, *m*	23.7, CH_2	1.29, 1.20, *m*	26.3, CH_2	1.24, 1.30, *m*	23.6, CH_2	1.28, *m*	26.3, CH_2	1.25, *m*
33	31.3, CH_2	1.16, 1.06, *m*	31.5, CH_2	1.16, 1.05, *m*	33.8, CH_2	1.74, 1.66, *m*	31.3, CH_2	1.16, 1.06, *m*	33.9, CH_2	1.73, 1.65, *m*
34	14.3, CH_3	0.81, *t* (7.4)	14.3, CH_3	0.81, *t* (7.4)	45.4, CH_2	3.44, *dt* (6.9, 0.9)	14.4, CH_3	0.82, *t* (7.4)	45.6, CH_2	3.43, *dt* (7.1, 7.5)
35	16.3, CH_3	1.01, *d* (6.4)	16.4, CH_3	1.01, *d* (6.4)	16.3, CH_3	1.00, *d* (6.4)	16.4, CH_3	1.00, *d* (6.1)	16.2, CH_3	1.00, *d* (6.4)
36	16.7, CH_3	1.06, *d* (6.4)	16.8, CH_3	1.06, *d* (6.4)	16.3, CH_3	1.00, *d* (6.4)	16.7, CH_3	1.06, *d* (6.2)	16.7, CH_3	1.06, *d* (6.4)
37	159.5, C	—	159.9, C	—	159.5, C	—	159.6, C	—	159.6, C	—
38	—	—	—	—	159.5, C	—	—	—	—	—

Fraction P2 contained five compounds that showed UV spectra comparable to the compounds in P1 and monoisotopic $[M–H]^-$ ions at *m/z* 703.4, 723.3, 660.4, 683.4 and 617.4, occurring in given order with a relative ratio in % to the most abundant substance of 100:<0.1:20.6:0.6:1.5 (Figure 2).

Compounds **9**, **2** and **10** were also obtained as white, amorphous powders after semi-preparative HPLC with a quantity of 3.4 mg (0.15% of dry biomass), 1.5 mg (0.06% of dry biomass) and 0.4 mg (0.02% of dry biomass). Comparing the analytical data of compounds **9** and **10** with data published by Bui *et al.*[9] and Chlipala *et al.*,[6] **9** was revealed as carbamidocyclophane D and **10** as cylindrocyclophane A_1. HR–ESI-TOF-MS of **2** in negative mode showed an isotopic pattern for a monochlorinated molecule and suggested the elemental composition of $C_{37}H_{55}ClNO_7$ (found *m/z* 660.3680, calculated 660.3673 for $[M–H]^-$, Δ 1.06 p.p.m.). Again, the observed mass difference of 43 Da for **2** compared with **9** and **10** indicated the presence of only one carbamoyl group within the molecule, which could be proven by NMR spectroscopic analysis. Like for carbamidocyclophane H (**1**), the NMR spectra of **2** showed one oxymethine signal with chemical shifts of δ_H 4.80/δ_C 83.4, correlating to a carbonyl C-atom at δ_C 159.9, and a second one with shifts of δ_H 3.74/δ_C 81.5. All other signals from 2D NMR analysis matched perfectly with those of **1**, with the only difference in one side chain (C-27–C-30), where signals were shifted downfield compared with those of **1**. As C-30 is represented by a signal corresponding to a methylene group with a chemical shift of δ_H 3.44/δ_C 45.6, monochlorination of C-30 was demonstrated.

The analytical HPLC-DAD-MS investigation of fraction P3 indicated the presence of six compounds with similar UV spectra as described above (Figure 1c) and $[M–H]^-$ ions at *m/z* 703.4, 737.3, 694.3, 737.3, 694.3 and 651.3 in a percentage ratio of 0.5:07.6:1.8:100:19.8:1.3 based on the integrated peak areas in the UV chromatogram at λ = 228 nm (Figure 2). For each substance, the observed isotope distribution was in good agreement with the presence of two chlorine atoms within the molecule. Because of the observed isotopic patterns and the detection of identical monoisotopic mass peaks at different retention times, the presence of constitutional isomers was assumed once again, and hence the combination of fractions containing compounds with the same *m/z* values was avoided.

The processing of P3 resulted in four pure compounds **3**, **11**, **4** and **12** as white, amorphous powders. Compound **3** was collected as the first peak of fraction P3 (0.7 mg, 0.05% of dry biomass), showing the same high-resolution monoisotopic mass ion *m/z* 737.3339 $[M–H]^-$ (calculated 737.3341 for $[M–H]^-$, Δ −0.27 p.p.m.) in negative mode and an equal isotopic pattern as the known compound carbamidocyclophane C (**11**). This resulted in the proposed elemental composition of $C_{38}H_{55}Cl_2N_2O_8$ that is identical to the determined one for **11**. However, differing retention times suggested **3** being a constitutional isomer of **11**. This assumption was confirmed by NMR analysis. The analysis of 1D and 2D NMR spectra led to a symmetrical structure, similar to carbamidocyclophane A (**15**), because of only 19 detected carbon signals compared with the MS analysis, suggesting 38 carbon atoms in the molecular formula. NMR signals and correlations corresponding to the core structure (C-1–C-26) matched those described for **15** and therefore demonstrating bicarbamoylation of **3**. Differences were detected for the chemical shift values of the side chains (C-27–C-30 and C-31–C-34). The monochlorination of chain end C-30, respectively C-34, in **3** was undoubtedly proven by the combination of HSQC and COSY/TOCSY experiments, showing a CH_2-group (C-30/C-34) with chemical shifts of δ_H 3.44, δ_C 45.4 attached to a C_3H_6 unit bound to C-7, respectively C-20 of the core structure (Table 1). These results corroborated the suggested structure from HRMS analysis, and thus compound **3** was named carbamidocyclophane J and is shown in Figure 4.

To the best of our knowledge, carbamidocyclophane J (**3**) is the first reported naturally occurring C-30/C-34-dihalogenated [7.7]paracyclophane. Having identified this new variant as a constitutional isomer of **11**, the LC-HRMS data analysis of the initial cyclophane-containing fraction (Figure 1) indicated further putative constitutional isomers of structurally confirmed, geminally dichlorinated cyclophanes. At least for the extracted ion chromatogram (EIC) for the $[M–H]^-$ ion of carbamidocyclophane K (**4**) as well as of cylindrocyclophane A_2 (**12**), several peaks were detected at different retention times, always showing a congruent isotopic pattern consistent with the structurally elucidated dichlorinated congeners (see Figure 1). Misinterpretations due to unwanted ESI fragmentations could be excluded by comparison of retention times in the EICs of relevant $[M–H]^-$ ions of fraction P3 (see Supplementary Information S4). Compound **12**, the latest eluting compound of this fraction with a yield of 0.2 mg (0.01% of dry biomass), and **11**, the major molecule in this fraction with a yield of 9.2 mg (0.60% of dry biomass), were identified by comparison with previously reported data as cylindrocyclophane A_2[6] and carbamidocyclophane C,[9] respectively. Compound **4** eluted between **11** and **12** and was obtained as white, amorphous solid in a yield of 1.4 mg (0.09% of dry biomass). The HR-ESI-TOF-MS analysis of **4** indicated the molecular formula of $C_{37}H_{54}Cl_2NO_4$ (found *m/z* 694.3293, calculated 694.3283 for $[M–H]^-$, Δ 1.44 p.p.m.) with an isotopic pattern consistent with the predicted degree of chlorination and differing from **11** and **12** by 43 Da, suggesting to also contain only one carbamoyl moiety. Evidence for the monocarbamoylation could be received via NMR spectroscopy. Similar to what could be shown for **1** and **2**, again for derivative **4** two different sets of chemical shifts for C-1 and C-14 are present (δ_H 4.81/δ_C 83.4 and δ_H 3.75/δ_C 81.6). Also in this case, the signal appearing more downfield shows a HMBC correlation with a carbonyl C-atom at δ_C 159.6, proving connectivity to the carbamoyl moiety. Dichlorination at C-30 could be shown by the chemical shift-pair δ_H 5.82/ δ_C 75.0 for a CH group attached to the alkylic side chain (C-27–C-29).

The analytical HPLC-DAD-MS data of fraction P4 uncovered a set of three main compounds with corresponding cyclophane-related UV spectra (Figure 1c) and $[M–H]^-$ ions at *m/z* 771.3, 728.3 and 685.3 that occurred in a ratio (%) of 100:18.8:1.4 (Figure 2). The compounds **13**, **5** and **14** were obtained in mentioned order as white, amorphous powders after isolation with yields of 7.8 mg (0.32% of dry biomass), 1.2 mg (0.05% of dry biomass), and 0.2 mg (0.01% of

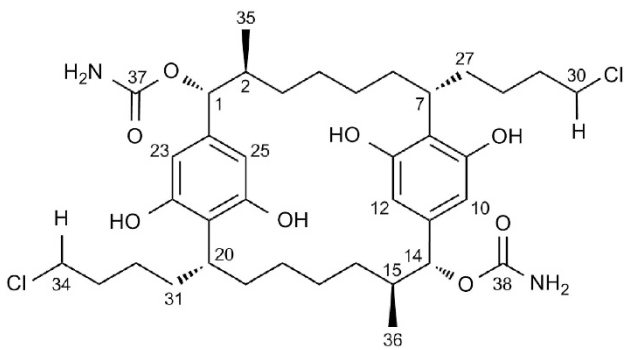


Figure 4 Carbamidocyclophane J (**3**) with a novel halogenation pattern of the paracyclophane core structure.

dry biomass). According to reported data by Bui *et al.*[9] and Chlipala *et al.*,[6] **13** was revealed as carbamidocyclophane B, and **14** as cylindrocyclophane A_3. HRMS data of **5** in negative mode showed the isotopic pattern of a trichlorinated molecule and agreed with the elemental composition $C_{37}H_{53}Cl_3NO_7$ (found *m/z* 728.2879, calculated 728.2893 for $[M–H]^-$, $\Delta -1.92$ p.p.m.). Once again, a mass difference of 43 Da indicated only one carbamoyl residue in compound **5** compared with **13** and **14**, and was in agreement with the investigations of the already described cyclophane clusters in P1 to P3. Similar to **1**, **2** and **4**, the monocarbamoylation could be proven by NMR spectroscopic analysis. Again, two different sets of signals corresponding to oxymethine groups are present in the spectra of **5**. One of them shows chemical shifts of δ_H 4.81/δ_C 83.4 and correlates (HMBC) to a carbonyl C-atom with a chemical shift of δ_C 159.6. The other oxymethine-related signal shows chemical shifts of δ_H 5.83/δ_C 74.9 that are typical for CHOH groups. Evidence for the chlorination pattern (dichlorination in C-30 and monochlorination in C-34) was received via analysis of chemical shifts for one methine signal (δ_H 5.83/δ_C 74.9) and one methylene signal (δ_H 3.43/δ_C 45.6) in combination with COSY and HMBC correlations.

Fraction P5 comprised three compounds with similar UV spectra and monoisotopic ions of *m/z* of 805.2, 762.3 and 719.2 $[M–H]^-$ in negative MS mode in a relative ratio to the most abundant derivative (calculated in %) of 100:15.4:0.9 (Figure 2). The final semi-preparative isolation yielded three white, amorphous solids eluted in following order: **15** (10.8 mg, 0.64% of dry biomass), **6** (1.6 mg, 0.09% of dry biomass) and **16** (0.1 mg, 0.01% of dry biomass). For each compound, the observed isotopic pattern indicated a tetrachlorinated molecule. Based on their recorded spectroscopic data, compounds **15** and **16** were identified as the previously reported carbamidocyclophane A[9] and cylindrocyclophane A_4,[6] respectively.

Recently, Luo *et al.*[10] described the isolation of carbamidocyclophane G besides the carbamidocyclophanes A–C (**15**, **13** and **11**) from the freshwater cyanobacterium *Nostoc* sp. (UIC 10274). The comparison of NMR and MS data with literature data identified **6** as carbamidocyclophane F, also produced by *Nostoc* sp. UIC 10274. However, in contrast to already published HRESIMS data, the monoisotopic mass of **6** (*m/z* 762.2502 for $[M–H]^-$) and the resultant isotopic distribution pattern were used to deduce the elemental composition of **6** and to confirm its degree of chlorination, as it was previously reported for tetrachlorinated paracyclophanes.[6,9] Taken together, NMR analysis revealed **1**, **2**, **4**, **5** and **6** as monocarbamoylated cyclophane structures with different degrees of chlorination in the substituents named carbamidocyclophane H (**1**), I (**2**), K (**4**), L (**5**) and F (**6**)[10] (Figure 3 and Table 1). The chlorination pattern was deciphered via analysis of 2D NMR data (TOCSY, COSY and HMBC). Key correlations are shown exemplary for **5** in Figure 5.

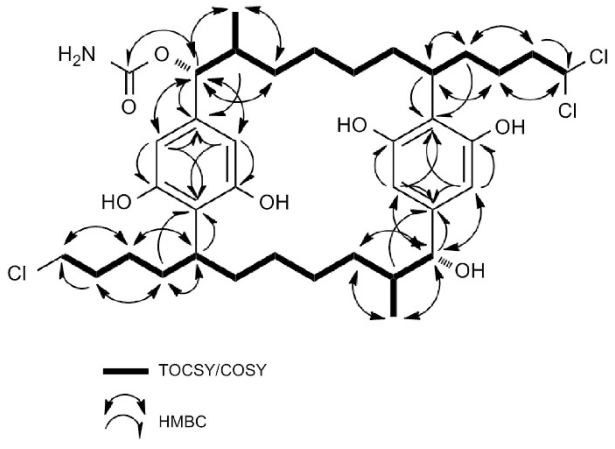


Figure 5 Selected TOCSY/COSY and HMBC correlations for carbamidocyclophane L (**5**).

Stereoconfigural analysis

The stereochemical configuration of isolated cyclophanes was established by careful analysis of NMR and CD spectroscopic data. As previously reported for the monocarbamoylated carbamidocyclophane F(**6**) as well as for the dicarbamoylated carbamidocyclophanes A–E (**15**, **13**, **11**, **9** and **7**), large ^3J coupling constants were determined for **1–5** in the range 10.2–10.3 Hz for H-1/H-2 and 9.5–10.1 Hz for H-14/H-15.[9,10] These data are congruent with an anti-conformation of H-1/H-2 and H-14/H-15 and a pseudoequatorial position of the methyl residues on C-2 and C-15, respectively.[4–6,9,10] The recorded CD spectra of **1**, **2**, **4** and **5** were comparable with recently described data of carbamidocyclophane F (**6**) as well as similar to previously reported cylindrocyclophanes A, A_4, C, F, nostocyclophanes A–D and merocyclophanes A and B, showing a negative Cotton effect at ~217–219 nm ($\Delta\varepsilon$ −0.55 to −1.21) with a negative shoulder extending from 220 to 230 nm and a weaker negative broad peak in the region between 265 and 280 nm with a second negative Cotton effect at ~278–281 nm ($\Delta\varepsilon$ −0.34 to −1.06).[5–8,10] In addition, we examined CD data for cylindrocyclophanes A_1–A_3 (**10**, **12** and **14**), as they were not described in the literature. Derivatives **10**, **12** and **14** showed comparable CD spectra with negative Cotton effects at ~217–218 nm ($\Delta\varepsilon$ −1.42 to −2.87) and at ~278–280 nm ($\Delta\varepsilon$ −0.34 to −0.52) to those of **1**, **2**, **4, 5** and **6**, underlining the results of the configurational analysis by Chlipala *et al.*[6] To corroborate our results, we measured the CD spectrum of cylindrocyclophane A (**8**) as a reference, because its absolute stereochemical configuration has been confirmed by Mosher's method.[5] Identical CD behavior of **1**, **2**, **4**, **5**, **6**, **10**, **12** and **14** compared with **8** suggests the same absolute stereochemical configurations. In contrast to this, carbamidocyclophane J (**3**), a dicarbamoylated paracyclophane, displayed a significantly different CD spectrum compared with the derivatives described above. A broad positive peak could be detected in the range from 220 to 240 nm with a positive Cotton effect at 233 nm ($\Delta\varepsilon$ 2.78) in addition to the familiar negative Cotton effect at 280 nm of $\Delta\varepsilon$ −0.94. These values are in good agreement with the data reported by Moore *et al.*[5] for cylindrocyclophane D, the 1,14-diacetylated cylindrocyclophane A, that is the most similar known structure to carbamidocyclophane J (**3**). Furthermore, CD data for dicarbamoylated carbamidocyclophanes A–E (**15**, **13**, **11**, **9** and **7**) were recorded and could confirm the previously suggested absolute configurations.[9] All five compounds showed comparable CD spectra with a positive Cotton effect at ~233–235 nm ($\Delta\varepsilon$ 1.88–2.31) and the negative Cotton effect at ~279–281 nm ($\Delta\varepsilon$ −0.74 to −1.03) to those of **3** and cylindrocyclophane D, which was also available as a reference standard during this study. The parallel biosynthesis of the configurationally determined cylindrocyclophane A and various carbamidocyclophane derivatives in *Nostoc* sp. CAVN2 supports the assumption that these paracyclophanes widely share the same biogenetically coded pathway. Therefore, an identical absolute configuration of all stereogenic carbon atoms is reasonable and promoted by all recorded data. As structurally typical for this class of paracyclophanes, we conclude that **1–16** do have the same absolute configuration at C-1, C-2, C-14 and C-15; namely, 1*R*, 2*S*, 14*R* and 15*S*. In addition, it is assumed that the compounds do not differ in their absolute configuration at C-7/C-20. However, only the stereo

descriptors have to be altered because of a priority change of the residues from *S* to *R* in case of any halogenation of C-30/C-34.

Biological evaluation of compounds 1–16

The initial screening results (see cytotoxicity screening) of the MeOH extract from CAVN2 are in good agreement with previously reported antimicrobial and cytotoxic data of [7.7]paracyclophane-containing extracts.[4–10] Bui *et al.*[9] reported an inhibition of MRSA strains 535 and 847 by the methanol extract obtained from the Vietnamese *Nostoc* sp. strain CAVN10 in addition to the cytotoxicity of the pure carbamidocyclophanes. Because of the increase of nosocomial and community-acquired infections with antibiotic-resistant staphylococci, especially of MRSA and vancomycin-resistant *S. aureus*, the search for novel pharmaceutical leads has become of crucial importance.[11–14] Having all these facts in mind, compounds **1**−**16** were examined for antibacterial activity against selected, clinically relevant pathogens.

As presented in Table 2, all tested isolates exhibited remarkable effects against *S. pneumoniae* (MICs of 0.25–2.10 μM) and even better results against MRSA (MICs of 0.10–1.02 μM). Furthermore, **1**−**16** displayed stronger antibacterial activity against both strains than commercially available antibiotics such as vancomycin (MIC 1.35 μM) or fusidic acid (MIC 3.77 μM) that were used as positive controls, and the pathogenic strains tested are not described to be intermediate or resistant to these antibiotics. No significant correlation between bioactivity and the degree of halogenation could be observed. However, slightly higher antibacterial activity was found for cyclophanes containing one or two carbamoyl moieties compared with noncarbamoylated derivatives with a 5- to 10-fold higher MIC of the latter. In accordance with the report of Luo *et al.*,[10] no activity against Gram-negative bacteria could be found up to a concentration of 50 μg ml^{-1}.

Based on the fact that initial colonizations of MRSA and *S. pneumoniae* usually affect the nose atrial, the throat or other areas of the skin, we have chosen immortal human keratinocytes, HaCaT cells, to evaluate the cytotoxicity of **1**−**16** by the CellTiter-Blue cell viability assay. Not surprisingly, as numerously reported by previous investigations, the IC_{50} values of **1**−**16** (2.8–11.5 μM) indicated a moderate cytotoxicity. The values are in the same range as those previously published for other naturally occurring paracyclophanes against various tumorigenic cell lines (0.5–5 μM) as well as nontransformed FL cells (IC_{50}s of 3.3–5.1 μM).[5,6,8–10] In summary, all compounds **1**−**16** possess stronger activity against Gram-positive pathogens, especially against MRSA, than cytotoxicity against HaCaT keratinocytes. However, some carbamoylated cyclophanes exhibited larger distances between determined *in vitro* concentrations for antibacterial activity and unwanted cytotoxic effects. Of these, carbamidocyclophane H (**1**) and D (**9**) are the most promising derivatives revealing MICs at least 50-fold lower than their corresponding IC_{50} values.

Taxonomic identification

The initial phenotypical characterization of the filamentous Vietnamese freshwater strain CAVN2 was conducted by microscopy

Table 2 Antibacterial and cytotoxic activity of isolated [7.7]paracyclophanes 1–16

	Antimicrobial testing					*Cytotoxic testing*
	Gram-positive [a]		*Gram-negative*			
Compound	*MRSA MIC (μM)*	*S. pneumoniae MIC (μM)*	*E. coli MIC (μM)*	*K. pneumoniae MIC (μM)*	*P. aeruginosa MIC (μM)*	*HaCaT* [b] *IC_{50} (μM)*
No carbamoyl moiety						
Cylindrocyclophane A (**8**)	0.45	0.97	NA[c]	NA	NA	5.0
Cylindrocyclophane A_1 (**10**)	1.02	2.10	NA	NA	NA	11.3
Cylindrocyclophane A_2 (**12**)	0.96	1.99	NA	NA	NA	11.5
Cylindrocyclophane A_3 (**14**)	0.45	0.92	NA	NA	NA	8.6
Cylindrocyclophane A_4 (**16**)	0.43	0.87	NA	NA	NA	9.3
One carbamoyl moiety						
Carbamidocyclophane H (**1**)	0.13	0.32	NA	NA	NA	7.6
Carbamidocyclophane I (**2**)	0.12	0.30	NA	NA	NA	5.8
Carbamidocyclophane K (**4**)	0.11	0.29	NA	NA	NA	3.8
Carbamidocyclophane L (**5**)	0.11	0.27	NA	NA	NA	4.6
Carbamidocyclophane F (**6**)	0.10	0.26	NA	NA	NA	4.7
Two carbamoyl moieties						
Carbamidocyclophane J (**3**)	0.11	0.27	NA	NA	NA	3.0
Carbamidocyclophane E (**7**)	0.12	0.30	NA	NA	NA	3.7
Carbamidocyclophane D (**9**)	0.11	0.28	NA	NA	NA	5.6
Carbamidocyclophane C (**11**)	0.11	0.27	NA	NA	NA	2.8
Carbamidocyclophane B (**13**)	0.10	0.26	NA	NA	NA	4.4
Carbamidocyclophane A (**15**)	0.10	0.25	NA	NA	NA	4.8

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; NA, not active.
[a]Positive control: vancomycin (MIC 1.35 μM) and fusidic acid (MIC 3.77 μM).
[b]Positive control: mitoxantrone (IC_{50} 3.9 μM), reference antibiotics: vancomycin and fusidic acid (IC_{50} > 100 μg ml^{-1}; that is, uncalculable in tested concentration range from 100 to 0.002 μg ml^{-1}).
[c]Not active in tested concentration range (50–0.01 μg ml^{-1}).

observation. Based on examined morphological features,[15–20] CAVN2 was supposed to be a member of the genus *Nostoc* just as the other two carbamidocyclophane-producing cyanobacteria, *Nostoc* sp. UIC 10274 and *Nostoc* sp. CAVN10.[9,10] For further taxonomic identification and molecular phylogenetic analysis, a 1.4-kb fragment of the 16S rRNA gene from CAVN2 was sequenced. A primary online

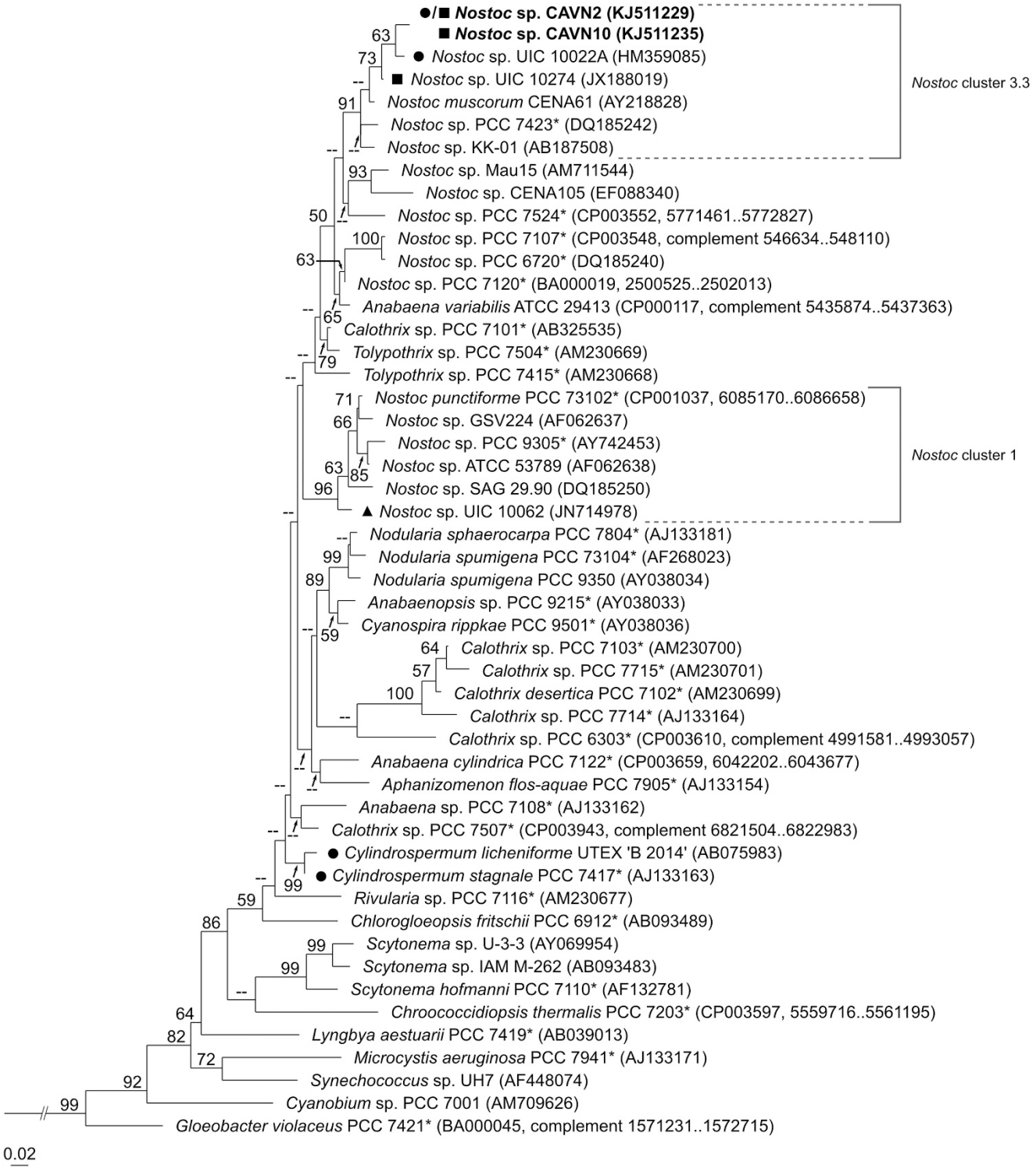


Figure 6 Phylogenetic tree based on a secondary structure alignment of cyanobacterial 16S rRNA gene sequences. The tree was inferred using a maximum likelihood method. Numbers given on the branches display bootstrap proportions as percentage of 1000 replicates for values ≥50%. The investigated cyanobacterial strains *Nostoc* sp. CAVN2 and *Nostoc* sp. CAVN10 are shown in bold. Filled circles (●), squares (■) and triangles (▲) denote cylindro-, carbamido- and merocyclophane producers, respectively. Reference strains according to Bergey's Manual of Systematic Bacteriology[19] are marked with an asterisk (*). The INSDC accession numbers are given in brackets. For entries that represent a whole genome, the genomic location of the considered sequence is provided additionally.

BLAST[21] search (http://blast.ncbi.nlm.nih.gov) and comparison of the partial CAVN2 16S rDNA nucleotide sequence with available GenBank sequence data revealed high homologies to various *Nostoc* and *Anabaena* strains with best hits for *Nostoc* sp. PCC 7423, KK-01 and CENA61 or *Anabaena variabilis* ATCC 29413. In order to infer the phylogenetic relationship of *Nostoc* sp. CAVN2 and these strains as well as previously reported paracyclophane-producing cyanobacteria, a phylogenetic tree was constructed on the basis of available 16S rDNA data using the maximum likelihood method (Figure 6). To make this report more comparable to previous studies, 16S rRNA gene sequences of at least 1 kb from Bergey's reference strains and other related or former investigated species were added to the sequence alignment. In addition, the so far unknown partial 16S rRNA gene sequence of the first reported carbamidocyclophane producer, *Nostoc* sp. CAVN10, was also investigated.

The resulting phylogenetic tree in Figure 6 revealed that *Nostoc* sp. CAVN2 is a member of the same monophyletic clade, including the cylindrocyclophane- and carbamidocyclophane-producing strains *Nostoc* spp. UIC 10022A and UIC 10274, previously reported and designated as *Nostoc* cluster 3.3 by Chlipala *et al.*[6] and Luo *et al.*[10] The assignment of the genus *Nostoc* to strain CAVN2 by the initial phenotypic characterization is in accordance with the presence of reference strain *Nostoc* sp. PCC 7423 in this group. Although CAVN2 shows a sequence homology of 98% to *Nostoc* sp. UIC 10274 as well as to *Nostoc* sp. UIC 10022A based on primary structure information, the phylogenetic tree based on a secondary structure alignment could elucidate that CAVN2 and UIC 10022A share a more recent common ancestor.

To our surprise, the 16S rDNA sequence data of CAVN2 and CAVN10 were completely identical. Based on the generally high conservation of the 16S rRNA, it is not an adequate phylogenetic marker gene when studying taxonomic relations among closely related species. Therefore, we examined several other molecular markers such as *het*R, *rbc*LX intergenic spacer, the phycocyanin intergenic spacer (PC-IGS) and the 16S-(tRNA^{Ile}-tRNA^{Ala})-23S rRNA internal transcribed spacer that are assumed to reveal more explanatory significance between strains at the intraspecific level. These markers all share either a unique distribution among filamentous cyanobacteria or at least a partial relatively high sequence variation.[22–26] As with the 16S rDNA sequence data, we could find no nucleotide differences between both strains in the aforementioned marker genes. Nevertheless, we recommend both strains CAVN2 and CAVN10 to be understood as independent and individual *Nostoc* sp. strains as they differ in the diversity of cyclophanes they are producing. This could undoubtedly be shown by a comparison of the chromatograms of carbamidocyclophanes A–E containing fraction F2 of CAVN10 and paracyclophanes **1–16** containing fraction F2$_{CAVN2}$ of CAVN2. When using the same cultivation, extraction and separation procedure as well as equal HPLC conditions described by Bui *et al.*,[9] only in F2$_{CAVN2}$ distinct double peaks could be detected, indicating the absence of mono- and nonesterified paracyclophanes in CAVN10 (see Supplementary Information S5). In addition, to exclude that **1–5** are only artifacts derived from dicarbamoylated cyclophanes during separation or isolation procedures, all compounds could be detected by LC-MS analysis of the crude extract from *Nostoc* sp. CAVN2 (see Supplementary Information S6).

METHODS

General experimental procedures

Optical rotations were determined on a P-2000 polarimeter (JASCO, Gross-Umstadt, Germany) at 20 °C and 589 nm. UV spectra were measured on a BioPhotometer plus (Eppendorf AG, Hamburg, Germany) in the wavelength range from 190 to 320 nm. CD spectra were recorded on a J-810 CD spectropolarimeter (JASCO), measuring the ellipticity θ in dependence of the wavelength from 200 to 300 nm at 20 °C and were analyzed with Spectra Manager Software (version 1.53.01; JASCO). Used concentrations are given in μmol l^{-1}. Cylindrocyclophane A and D were used as references. Attenuated total reflexion-IR (ATR-IR) spectra were recorded using a Nicolet IR 200 Fourier transform-IR spectrometer (Thermo Scientific, Bremen, Germany) at 22 °C. Raw data were processed with OMNIC Spectra Software (Thermo Scientific). NMR spectra were recorded in MeOH-d_4 on a 500 MHz *Avance III* (UltraShield) spectrometer (Bruker BioSpin, Rheinstetten, Germany) or on a 700 MHz *Avance III* (Ascend), each one equipped with a cryoplatform, at 298 K, if not specified differently. Chemical shift values δ of ^{1}H- and ^{13}C-NMR spectra are reported in p.p.m. relative to the residual solvent signal given as an internal standard.[27] Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad; corrected coupling constants are reported in Hz. HPLC-UV-MS analysis was conducted on a Shimadzu LC20A Prominence comprising a CBM-20A controller, a DGU-14A degasser, LC20A pumps, a SIL-AC HT auto sampler, a CTO-10-ASvp column oven, SPD-M20A Diode Array Detector (DAD) coupled to a LCMS-8030 triple quadrupole (QqQ) mass spectrometer (Shimadzu, Kyoto, Japan). High-resolution mass spectra were recorded on an ion trap-time of flight-MS (IT-TOF-MS, Shimadzu) equipped with an ESI source and attached to the above-mentioned HPLC set-up. Semi-preparative HPLC was performed on a Shimadzu HPLC system consisting of SCL-10Avp system controller, a LC-10ATvp liquid chromatograph, a FCV-M10Avp low pressure gradient unit, a SPD-M10Avp DAD (Shimadzu) and a JETSTREAM 2 PLUS column thermostat (Goebel Instrumentelle Analytik, Hallertau, Germany).

Cyanobacterial strains and culture conditions

The cyanobacterial strains investigated in this research included 13 freshwater and 1 brackish water strain belonging to the orders Nostocales and Oscillatoriales (Supplementary Information S1 and S2).[15–19] The freshwater cyanobacteria were originally isolated from samples of rice fields and shallows in Northern Vietnam (Thanh Hoa, Thai Binh, Nam Dinh and Hanoi) and established as unialgal laboratory cultures by Dr Nhi V Tran (Institute for Biotechnology, Hanoi, Vietnam). The strains were incorporated into the culture collection of the Institute of Pharmacy, University of Greifswald. The brackish water cyanobacterial strain was isolated from the Baltic Sea near the coast of Grabow (Ruegen island) by B Cuypers (University of Greifswald).

For the preparation of extracts for bioactivity screening, strains were cultured in 500 ml aliquots of BG11 medium[28] in 1.8 l Fernbach Flasks under continuous fluorescent light (8 μmol m^{-2} s^{-1}) and the temperature was maintained at 20 ± 1 °C. Only *Nostoc* sp. CAVN2 was cultivated in modified WC medium[29] (MBL medium) without the described vitamin mix, but buffered with 0.5 mM TES to a pH of 7.2, under otherwise identical conditions. After 6–8 weeks of growth, the cyanobacterial cells were harvested and separated from the medium by centrifugation. Biomasses were lyophilized and supernatants were evaporated to dryness and finally stored at −20 °C until use.

For isolation of paracyclophanes, *Nostoc* sp. CAVN2 was cultured in a glass column containing 35 l of MBL medium under continuous fluorescent light (20 μmol m^{-2} s^{-1}), the temperature was maintained at 22 ± 1 °C and pH was adjusted to 8.5 using CO_2 supplementation.[30] After 28 days, the cells were harvested by centrifugation, lyophilized and stored at −20 °C. The yield of freeze dried biomass was 286 mg l^{-1}.

The axenic cultures of *Nostoc* sp. CAVN2 and CAVN10 were achieved by a combined use of traditional microalgae isolation techniques.[31] Both strains were cultivated in BG11 medium at 28 ± 1 °C under continuous light (15 μmol m^{-2} s^{-1}) and aerated with 0.5% CO_2 in air. Then, 50 ml aliquots (log-phase) were harvested and centrifuged. Biomass pellets were washed with sterilized distilled water, centrifuged again and stored at −20 °C until use.

For comparison of [7.7]paracyclophane biodiversity between *Nostoc* sp. CAVN10 and *Nostoc* sp. CAVN2, culture, extraction and separation conditions reported by Bui *et al.*[9] were used.

Morphological characterization and identification

Phenotypic characterization of investigated cyanobacteria was based on literature data.[15–19]

Different parameters were used for the identification of the isolates; for example, shape (length and width) and relative size of vegetative and end cells, presence and distribution of heterocysts and akinetes, trichome polarity, level of branching and general morphological structure of the filaments. Microscopic examinations were carried out in culture media, mostly BG11 medium, using an inverted light microscope (Axioskop 2 *plus*, Carl Zeiss, Oberkochen, Germany) with coupled digital camera (AxioCam MRc camera, software Axiovision version 4).

DNA isolation, PCR amplification and sequencing

The DNA extraction procedure was performed according to Franche and Damerval,[32] modified as follows: biomass pellets of *Nostoc* sp. CAVN2 and CAVN10 were thawed and aliquots were resuspended in 1 ml TE buffer (50 mM EDTA, 50 mM Tris–HCl, pH 8). Cell wall breakage was performed by a Sonopuls UW 2200 ultrasound-homogenizer (BANDELIN Electronic, Berlin, Germany). The centrifuged pellet (20 000 *g*, 20 °C, 10 min) was combined with 300 µl STET buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA, 50 mM Tris–HCl, pH 8), 15 µl chloroform/isoamyl alcohol (Roti-C/I, Carl Roth, Karlsruhe Germany) and 35 µl Lytic Enzyme Solution (QIAGEN, Hilden, Germany), and the samples were incubated at 37 °C for 1 h. Then, 100 µl 10% SDS and 100 µl 5 M NaCl were added and samples were treated at 65 °C until cell lysis was completely achieved. After adding 200 µl of 1 M NaCl and incubation for another 15 min, the aqueous phase was rid of proteins by chloroform/isoamyl alcohol addition and genomic DNA was precipitated in a new 1.5 ml centrifuge tube with isopropyl alcohol at −20 °C. After incubation for 2 h, the centrifuged DNA pellet (20 000 *g*, 4 °C, 15 min) was washed with 500 µl EtOH and separated from the supernatant again. The briefly air-dried DNA was dissolved in a proper volume of TE buffer and finally subjected to RNA digest at 37 °C for 1 h by adding 1 µl RNAse (20 µg µl^{-1}, Sigma-Aldrich, Hamburg, Germany). The solution was heated to 65 °C for 10 min to inactivate the RNAse and then stored at −20 °C until use. PCR amplification was carried out with a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA) utilizing Opti *Taq* DNA polymerase (5 U µl^{-1}, Roboklon, Berlin, Germany) and listed oligonucleotides (see Table 3) according to the manufacturer's recommendations. PCR products were verified and separated by electrophoresis on 1.5% agarose gels in 1 × TBE buffer. Excised PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Hilden Germany) as described in the manual provided by the manufacturer. Gel-purified amplicons were sequenced by MWG Operon (Ebersberg, Germany) using primers in Table 3. The resulting sequence data were deposited with GenBank under accession numbers KJ511227–KJ511238 (Table 4).

Phylogenetic tree construction

Single sequencing reads were assembled in Geneious version 6.1.7 (available from http://www.geneious.com). The 16S rDNA sequences of *Nostoc* sp. CAVN2 and *Nostoc* sp. CAVN10 were automatically aligned according to the SILVA SSU Ref NR99 r115 database (available from http://www.arb-silva.de)[33] using the Silva INcremental Aligner (SINA) version 1.2.11.[34] SINA-aligned sequences and an additional sequence (AB075983) from the SILVA web release r117 database were imported into the ARB software package version 5.5.[35] The alignment of 52 sequences, also including previously used reference strains and available [7.7]paracyclophane-producing cyanobacterial strains,[10] was manually refined taking into account the secondary structure information of the rRNA. Phylogenetic reconstruction was performed with 51 sequences (CAVN2 and CAVN10 share 100% identity and were only considered once during reconstruction) using a maximum likelihood method. The final tree was calculated with RAxML version 8.0.14 (GTRGAMMA model)[36] and based on 638 distinct alignment patterns. The best tree out of 1000 independent inferences (Figure 6) is presented without outgroup sequences of *Bacillus subtilis* DSM 10 (AJ276351) and *E. coli* ATCC 25922 (DQ360844). The sequences of strains CAVN2 and CAVN10 are available from the INSDC (International Nucleotide Sequence Database Collaboration) databases; that is, DDBJ, EMBL and GenBank, under accession numbers KJ511229 and KJ511235 (Table 4).

Cytotoxicity assays

The cytotoxicity screening of crude extracts from investigated cyanobacteria against a breast adenocarcinoma cell line (MCF-7), human amniotic epithelial Fibroblast-Like (FL) cells and a human urinary bladder carcinoma cell line (5637) were performed by using either the crystal violet or the neutral red uptake assay as previously described.[9,37] The cell viability investigation for the cytotoxic evaluation of **1–16** was done by using the CellTiter-Blue assay (Promega, Mannheim, Germany) and HaCaT cells (human adult low calcium high temperature keratinocytes). HaCaT cells were obtained from German Cancer Research Center DKFZ (Heidelberg, Germany) and were cultured in calcium-free Gibco DMEM medium (Life Technologies, Vienna, Austria) with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). HaCaT cells were

Table 3 Used oligonucleotides as primers for PCR and sequencing

Primer	*Sequence (5′–3′)*	*Target locus*	*Position*	*Reference*
GM3F	AGAGTTTGATCMTGGC	16S rRNA gene	8–23[a]	Muyzer *et al.*[43]
GM4R	TACCTTGTTACGACTT		1492–1507[a]	
16S-SeqF[b]	TACAACCCAAGAGCCTTCC		ND[c]	This study
907FK[b,d]	AAACTCAAAKGAATTGACGG		ND	This study
ITS14	TGTACACACCGCCCGTC	16S–23S rRNA internal transcribed spacer (ITS)	1334–1350 (16S)[e]	Wilmotte *et al.*[44]
ITS18k[f]	CTCTGTGTGCCTAGGTATCC		26–45 (23S)[e]	
ITS-SeqR[b]	CACCATGGAAGCTGGCAACG		ND	This study
cpcB-F	CCKGGTGGTAAYGCTTACACCARCCG	Phycocyanin intergenic spacer (PC-IGS)	ND	This study
cpcA-R	TTGATGTRCTTSAGAGCTTCWAYRTACC			
hetR-F	AAGTGTGCMATWTACATGACHTATCTAGAGC	hetR gene	ND	This study
hetR-R	CRTAGAAGGGCATTCCCCAAGG			
rbcL-F	CGTAGCTTCCGGTGGTATCCAC	rbcL-rbcX-rbcS gene region	ND	This study
rbcS-R	GAAAGGGTTTCGTAACGACGCTC			

Abbreviation: ND, not determined.
[a]Position according to the *E. coli* gene numbering.[45]
[b]Primer only used for sequencing.
[c]Position not determined.
[d]Reverse complement of primer 907R.[46]
[e]Position in the corresponding genes of *Synechococcus* sp. PCC 6301.
[f]Partial sequence of primer 18 by Wilmotte *et al.*[44]

Table 4 Overview of accession numbers from resulting sequence data

Target locus	*Sequence accession number for strain*	
	CAVN2	CAVN10
Long 16S–23S rRNA internal transcribed spacer (ITS)[a]	KJ511227	KJ511233
Short 16S–23S rRNA ITS	KJ511228	KJ511234
16S rRNA gene	KJ511229	KJ511235
Phycocyanin intergenic spacer (PC-IGS)	KJ511230	KJ511236
hetR gene	KJ511231	KJ511237
rbcL-rbcX-rbcS gene region	KJ511232	KJ511238

[a]Contains $tRNA^{Ile}$ and $tRNA^{Ala}$ coding sequences.

plated at 3×10^4 cells per ml in 96-well plates (100 µl per well) for 18 h before stimulating them with different concentrations (serial dilution from 100 to 0.002 µg ml^{-1}) of crude extract or compounds, dissolved in DMSO, or left untreated (negative control) for 24 h. Subsequently, cells were incubated for 3 h with 10% (v/v) of CellTiter-Blue Reagent (Promega), and cell viability was assessed at an excitation wavelength of 530 nm and an emission wavelength of 590 nm in a multiplate reader (Infinite 200 PRO, Tecan, Gröding, Austria). Tests were performed twice in triplicates and values are reported as average of the determined IC_{50} values.

Determination of MIC

Tests of *Nostoc* sp. CAVN2 crude extract and isolated compounds were carried out according to the EUCAST criteria[38–40] in a 96-well dilution assay with compound concentrations, for crude extract or purified substances, between 50 and 0.01 µg ml^{-1} and incubated with bacteria solution in a concentration of 10^4 bacteria per ml. After 24 h, the MIC of a substance was determined. Tests were done in triplicates and reported values are shown as average of multiple MIC tests. All tested bacteria belong to the SeaLife Pharma MDR pathogen collection and have been isolated from infected patients. The screening included MRSA, *S. pneumoniae*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

Extraction, separation and isolation of [7.7]paracyclophanes

Initial screening for cytotoxic activity was performed with crude *n*-hexane, methanol and water extracts of biomasses and the ethyl acetate extracts of media from investigated cyanobacterial strains (Supplementary Information S3). For this purpose, 1 g dried biomass of each strain was successively extracted with *n*-hexane, followed by methanol and water (each three times for 1 h under stirring) to yield three crude extracts after removal of the solvents. The corresponding ethyl acetate extract was obtained by a threefold extraction of 3 l medium from the culture supernatant with a 1:1 mixture of EtOAc/H_2O over 24 h, followed by subsequent combination of the upper phases and evaporation to dryness. For analytically scaled structural investigations of *Nostoc* sp. CAVN2 biomass, portions of ~100 mg freeze dried cells (in total 400 mg) were extracted five times with 25 ml MeOH under stirring (750 r.p.m.) for 30 min. Cell wall breakage was provided by sea sand grinding and usage of ultrasonication. The methanolic supernatants were separated from the residues by centrifugation (3300 *g*, 10 min, 15 °C) and filtration through Whatman filter papers No. 1 (GE Healthcare, Buckinghamshire, UK), pooled and evaporated to yield altogether 93.3 mg of crude extract. This methanol extract was subjected to SPE utilizing a Strata C18-E 10 g/60 ml (55 µm, 70 Å) SPE cartridge (Phenomenex, Torrance, CA, USA) and a MeOH/H_2O step gradient of 0, 10, 25, 50, 80 and 100% (v/v) MeOH in water. Eluates were concentrated to dryness and 500 µg of each fraction were tested for antibacterial activity against *S. aureus* ATCC 6538 using previously described agar diffusion assay.[41,42] Bioactive fraction (37.5 mg), eluting with 80% MeOH, was subjected to HPLC-DAD/ESI-IT-TOF-HRMS. Separation of paracyclophanes was performed with a Kinetex PFP column (100 × 4.6 mm, 2.6 µm, 100 Å; Phenomenex) and a gradient of MeOH in deionized water with a flow rate of 0.6 ml min^{-1} from 60 to 77.5% MeOH in 35 min at 40 °C. Structural formulas and structure proposals were made on the basis of monoisotopic mass ions and corresponding isotopic distribution patterns from obtained high-resolution mass spectra of detected cyclophanes with ChemBioDraw Ultra software version 12.0 (CambridgeSoft, Cambridge, MA, USA) and the Formula Predictor tool provided by LabSolutions software version 3.60.361 (Shimadzu).

A 2.71 g biomass aliquot from the 35 l cultivation of *Nostoc* sp. CAVN2 (in total 10.02 g of lyophilized biomass) was successively extracted with methanol as already described for analytical investigations to yield 491 mg crude extract. This extract was also subjected to SPE under above-mentioned conditions to obtain 169.7 mg of cyclophane-containing fraction. A portion (156 mg) of cyclophane-rich fraction, eluted with 80% methanol, was separated by semi-preparative HPLC with a Synergi Polar RP column (250 × 10.0 mm, 4 µm, 80 Å; Phenomenex) and a binary gradient of MeOH in deionized water with a flow rate of 3.1 ml min^{-1} from 62 to 85% MeOH in 32 min at 25 °C. Multiple rounds of isolation yielded fractions P1 (9.4 mg), P2 (9.9 mg), P3 (24.1 mg), P4 (12.5 mg) and P5 (23.4 mg). Initial analytical HPLC-DAD-QqQ-MS analysis of P1 to P5 as well as the purity control of isolated compounds from P1 to P5 and the detection of **1**–**5** in the crude extract (Supplementary Information S6) was performed by using a Luna PFP(2) column (250 × 4.6 mm, 5 µm, 100 Å, Phenomenex) and a MeOH–H_2O gradient with a flow rate of 0.8 ml min^{-1} from 60 to 85% MeOH in 32 min at 25 °C. The relative abundance of detected compounds in P1 −P5 was calculated based on obtained areas at 226 nm in the UV chromatogram. The area of the major peak of every fraction was set to 100%. For final isolation of compounds **7**, **1** and **8**, fraction P1 (9.0 mg) was subjected to semi-preparative HPLC utilizing a Luna PFP(2) column (250 × 10.0 mm, 5 µm, 100 Å, Phenomenex) and the same conditions previously described for the separation of cyclophane-rich SPE fraction, using a flow rate of 3.5 ml min^{-1}. Several rounds of separation yielded 4.3 mg of **7**, 1 mg of **1** and 0.2 mg of **8** in described order. A portion (9.2 mg) of P2 yielded **9** (3.4 mg), **2** (1.5 mg) and **10** (0.4 mg) when utilizing the same HPLC column, but with a binary methanol water gradient from 63 to 86% MeOH in 32 min at 25 °C. Pure compounds **3** (0.7 mg), **11** (9.2 mg), **4** (1.4 mg) and **12** (0.2 mg) were obtained by separation of fraction P3 (14.9 mg) carried out with a MeOH–H_2O gradient from 59 to 82% MeOH in 32 min at 30 °C and a flow rate of 3.5 ml min^{-1}. The semi-preparative HPLC of P4 (12.4 mg) and P5 (15.9 mg) was performed as already described for P1. Compounds **13** (7.8 mg), **5** (1.2 mg), **14** (0.2 mg) were obtained from P4 and **15** (10.8 mg), **6** (1.6 mg), and **16** (0.1 mg) from fraction P5. The percentaged yields of obtained compounds **1**–**16** were calculated based on initially used 2.71 g lyophilized *Nostoc* sp. CAVN2 biomass. Because of the small amounts of **8**, **10**, **12**, **14** and **16**, additional rounds of mentioned extraction and separation procedures were performed. Only focusing on the isolation of these minor derivatives, finally, **8**, **10**, **12**, **14** and **16** yielded 0.5, 0.8, 0.4, 0.7 and 0.5 mg, respectively.

Carbamidocyclophane H (***1***). White, amorphous powder; $[\alpha]_D^{20} + 12.5$ (c = 0.8 g per 100 ml; MeOH); UV (c = 5 µM; MeOH) λ_{max} (log ε) 205 (5.250), 208 (5.225), 210 (5.164), 226 (4.580), 275 (3.903); CD (c = 30 µM; MeOH) λ_{max} (Δε) 211 (4.09), 218 (−0.92), 233 (0.24), 261 (−0.30), 278 (−0.64) nm; ATR-IR (film) $\tilde{\nu}_{max}$ 3384 (br), 2927, 2856, 1699, 1590, 1431, 1375, 1331, 1017, 834 cm^{-1}; for ^{1}H and ^{13}C NMR data see Table 1; HRMS (ESI): calcd for $C_{37}H_{56}NO_7$ $[M–H]^-$: 626.4062, found *m/z* 626.4070.

Carbamidocyclophane I (***2***). White, amorphous powder; $[\alpha]_D^{20}$ −6.7 (c = 0.9 g per 100 ml; MeOH), UV (c = 6 µM; MeOH) λ_{max} (log ε) 204 (5.181), 208 (5.054), 229 (4.426), 274 (3.824) nm; CD (c = 65 µM; MeOH) λ_{max} (Δε) 211 (1.72), 219 (−0.55), 229 (−0.07), 238 (0.66), 258 (0.22), 278 (−0.34) nm; ATR-IR (film) $\tilde{\nu}_{max}$ 3366 (br), 2927, 2854, 1699, 1591, 1431, 1375, 1017, 832, 650, 619 cm^{-1}; for ^{1}H and ^{13}C NMR data see Table 1; HRMS (ESI): calcd for $C_{37}H_{55}ClNO_7$ $[M–H]^-$: 660.3673, found *m/z* 660.3680.

Carbamidocyclphane J (***3***). White, amorphous powder; $[\alpha]_D^{20} + 2.0$ (c = 0.5 g per 100 ml; MeOH); UV (c = 3 µM; MeOH) λ_{max} (log ε) 205 (5.355), 207 (5.342), 210 (5.315), 229 (4.669), 275 (4.938) nm; CD (c = 30 µM; MeOH) λ_{max} (Δε) 211 (4.90), 224 (1.79), 233 (2.78), 251 (0.43), 280 (−0.94), 297 (0.09) nm;

ATR-IR (film) $\tilde{\nu}_{max}$ 3363 (br), 2929, 2854, 1699, 1590, 1432, 1374, 1334, 1040, 1018, 832, 648 cm^{-1}; for ^{1}H and ^{13}C NMR data see Table 1; HRMS (ESI): calcd for $C_{38}H_{55}Cl_2N_2O_8$ $[M-H]^-$: 737.3341, found *m/z* 737.3339.

Carbamidocyclophane K (***4***). White, amorphous powder; $[\alpha]_D^{20}$ −2.5 (c = 1.2 g per 100 ml; MeOH); UV (c = 4 μM; MeOH) λ_{max} (log ε) 205 (5.456), 208 (5.385), 211 (5.342), 228 (4.686), 275 (3.933) nm; CD (c = 82 μM; MeOH) λ_{max} (Δε) 211 (2.09), 219 (−1.21), 238 (0.47), 252 (−0.05), 279 (−1.06) nm; ATR-IR (film) $\tilde{\nu}_{max}$ 3370 (br), 2928, 2856, 1683, 1590, 1431, 1376, 1335, 1016, 833, 743, 653, 620 cm^{-1}; for ^{1}H and ^{13}C NMR data see Table 1; HRMS (ESI): calcd for $C_{37}H_{54}Cl_2NO_7$ $[M-H]^-$: 694.3283, found *m/z* 694.3293.

Carbamidocyclophane L (***5***). White, amorphous powder; $[\alpha]_D^{20}$ −6.0 (c = 1.0 g per 100 ml; MeOH); UV (c = 5 μM; MeOH) λ_{max} (log ε) 206 (5.301), 209 (5.246), 210 (5.220), 229 (4.602), 275 (3.903) nm; CD (c = 46 μM; MeOH) λ_{max} (Δε) 208 (2.00), 217 (−0.78), 228 (−0.44), 242 (0.70), 251 (0.11), 258 (0.42), 281 (−0.75) nm; ATR-IR (film) $\tilde{\nu}_{max}$ 3360 (br), 2927, 2855, 1678, 1586, 1430, 1393, 1375, 1336, 1012, 988, 829, 746, 648, 620 −cm^{-1}; for ^{1}H and ^{13}C NMR data see Table 1; HRMS (ESI): calcd for $C_{37}H_{53}Cl_3NO_7$ $[M-H]^-$: 728.2893, found *m/z* 728.2879.

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