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Carbamidocyclophanes A−E, Chlorinated Paracyclophanes with Cytotoxic and Antibiotic Activity from the Vietnamese Cyanobacterium *Nostoc* sp.

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Five new paracyclophanes, carbamidocyclophanes A−E (**1**−**5**), characterized by carbamido side chains at a symmetric [7.7]paracyclophane ring, have been isolated from the biomass of the Vietnamese *Nostoc* sp. CAVN 10. Structure elucidation by spectroscopic methods showed that **1**−**5** vary in the substitution pattern of the chlorinated butyl side chains. The compounds exhibited cytotoxic activity against MCF-7 (breast cancer cell line) and Fl cells (human amniotic epithelial cell line) and moderate antibacterial activity against the Gram-positive bacterium *Staphylococcus aureus*.

In the search for novel bioactive metabolites for pharmaceutical use, cyanobacteria have been identified as one of the most promising sources of highly complex natural products, which show a high diversity in their chemical structures and biological activities. Mainly lipopeptides but also alkaloids, fatty and amino acids, amides, esters, and lactones exhibiting cytotoxic, antibiotic, and enzyme-inhibiting activity have been isolated from marine cyanobacteria.[1] Metabolites belonging to the group of polyketides have been isolated less frequently from cyanobacteria.[2]

In the 1990s the first natural paracyclophanes, nostocyclophanes and cylindrocyclophanes, originating from the polyketide pathway were isolated from *Nostoc linckia* Roth (Bornet) and *Cylindrospermum licheniforme* Kützing, respectively. These cyclophanes exhibited cytotoxic effects against KB and LoVo cell lines.[3−5] Nostocyclyne, a triple bond containing paracyclophane isolated from a natural bloom of *Nostoc* sp., inhibited the growth of several Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* and showed a weak inhibition of photosynthesis in green algae.[2] Here we report five novel paracyclophanes isolated from laboratory cultures of a *Nostoc* strain collected in Northern Vietnam.

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Results and Discussion

Cyanobacterial strains isolated from various locations in Northern Vietnam were screened for antibiotic activity against a variety of bacteria and one yeast. The methanol extract prepared from the biomass of *Nostoc* sp., strain CAVN 10, showed inhibiting activity against Gram-positive bacteria such as *Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 6538 as well as against the yeast *Candida maltosa* SBUG 700. Further, the growth of coagulase-negative methicillin-resistant *Staphylococcus aureus* strains 535 and 847 was inhibited by the extract. Bioassay-guided separation of the extract on silica gel followed by preparative RP-HPLC resulted in five novel paracyclophanes, carbamidocyclophanes A−E (**1**−**5**).

Carbamidocyclophane A (**1**) was the last peak in the RP-HPLC of a group of compounds with similar UV spectra. Compound **1** provided molecular ion clusters during HPLC-ESIMS showing a conspicuous isotope distribution of *m*/*z* 824, 826, and 828 $[M + NH_4]^+$ in the positive ESIMS mode. In the negative mode a corresponding pattern of *m*/*z* 805, 807, and 809 $[M - H]^-$ was determined. Both clusters indicated a nominal molecular mass of 806 Da. High-resolution ESITOFMS of **1** showed a matching sodium cluster $[M + Na]^+$ with *m*/*z* 829.257 (74%), 831.254 (100%), 833.253 (53%), and 835.253 (16%), from which an elemental composition of $C_{38}H_{54}N_2O_8NaCl_4$ was calculated [829.253; (71:100:55:15%)].

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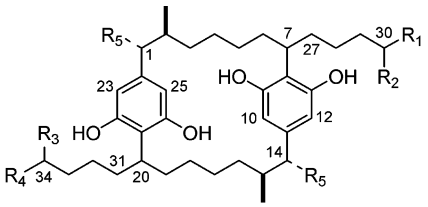

		R_1	R_2	R_3	R_4	R_5
Carbamidocyclophane A	(**1**)	Cl	Cl	Cl	Cl	$OCONH_2$
Carbamidocyclophane B	(**2**)	Cl	Cl	Cl	H	$OCONH_2$
Carbamidocyclophane C	(**3**)	Cl	Cl	H	H	$OCONH_2$
Carbamidocyclophane D	(**4**)	Cl	H	H	H	$OCONH_2$
Carbamidocyclophane E	(**5**)	H	H	H	H	$OCONH_2$
Cylindrocyclophane A	(**6**)	H	H	H	H	OH
Cylindrocyclophane D	(**7**)	H	H	H	H	OAc

The elemental composition was corroborated by the NMR data of **1** in DMSO-d_6 (Table 1). Since the ^{13}C NMR spectrum showed only 19 carbon signals, a symmetrical compound was expected. According to the ^{13}C DEPT NMR spectrum, **1** consisted of five quaternary, six methine, seven methylene, and one methyl carbon, each in duplicate. All signals in the ^1H NMR spectrum were correlated to their respective carbon signals in the $^1H,^{13}C$ HMQC NMR spectrum unambiguously, leaving just a sharp OH singlet at δ 8.82 and a broad NH signal at δ 6.36 of two and four protons each. Together, the correlated signals accounted for the $C_{38}H_{54}$ part of the elemental composition.

From the $^1H,^1H$ COSY NMR spectrum at 600 MHz the structure from C-1 to C-30 could be assembled, which had unassigned bonds at C-1, C-7, and C-30 (Figure 1). Six of the remaining carbon signals showed the presence of a *para*-disubstituted dihydroxybenzene unit: phenolic carbon signals at δ 155 and 157, aromatic methine signals at δ 103 and 107, and quaternary carbon signals at δ 115 and 138 in the ^{13}C NMR spectrum. The signal of C-8 at δ 115 showed the only $^1H,^{13}C$ HMBC correlation with the aromatic OH proton signal besides two equal and stronger correlations with the aromatic protons (H-10 and H-12). Further, the signal of C-8 had correlation signals with H-7 and the methylene protons at C-6 and C-27 (Figure 1). Thus the structure of the dihydroxybenzene unit and its connection to C-7 in the aliphatic part of **1** was shown. The aromatic methine groups C-10 and C-12 had correlations with each other and equally strong with H-14, which is identical to H-1 and belongs to the second, identical half of the molecule. In addition, the C-1/14 had strong HMBC correlations with both aromatic pairs of methines (H-10/23, H-12/25).

The $^1H,^{13}C$ HMBC correlation signal between the oxymethines H-1/14 and the carbonyl signal C-35/37 at δ 156.3 indicated the ester function, which was required to provide the acylation shift of their ^1H NMR signal at δ 4.72. Though no correlation signal with the broad N−H signal was observed, the NH_2 groups had to be connected to C-35/37 in order to account for their ^{13}C chemical shift of δ 156.3, which is characteristic for carbamate groups. Their presence was also shown in the DCI mass spectrum by the double loss of 61 Da from the molecular ion cluster, which corresponds to the stepwise elimination of two carbamic acid residues.

Similarly, all four chlorine atoms were necessary as substituents in pairs at the methine 30/34 residues to account for their NMR shifts (δ_H 6.21 and δ_C 74.96). A literature search revealed the cylindrocyclophanes A (**6**) and D (**7**), metabolites of the terrestrial cyanobacterium *Cylindrospermum licheniforme*, as the next structural relatives of **1**. However, **6** and **7** lack the chlorine atoms and the carbamoyl groups. Carbamidocyclophane A (**1**) has the same relative configuration, because the vicinal coupling constant $J_{1,2} = J_{14,15}$ was determined as 10.6 Hz, as observed with cylindrocyclophane D (**6**), which bears acetate residues at C-1/14.

The preferred orientation of the aromatic ring was derived using a $^1H,^1H$ ROESY NMR spectrum, where correlation signals between the aromatic methine H-10/23 ($\delta_{H/C}$ 6.10/103.7) and H-2/15 and another correlation between H-12/25 ($\delta_{H/C}$ 6.04/107.9) and H-1/14 indicated syn relationships, respectively (Figure 1). In contrast, Moore et al.[6] showed an image of a X-ray structure with NMR designation of the signal at δ 102.9 opposite that of the methine C-12/25, while the methine signal at δ 107 was assigned to C-10/23. They were depicted as syn to H-1/14 and to H-2/15, respectively.

Carbamidocyclophane B (**2**) had a similar UV spectrum but showed a sodium ion cluster of *m*/*z* 795.297 in the high-resolution ESITOFMS with an isotope distribution of 96:100:39:6 for *m*/*z* 795:797:799:801 corresponding to the elemental composition of $C_{38}H_{55}N_2O_8NaCl_3$ (calculated 795.291; 93:100:39:6). The mass spectrum had an equally abundant ion cluster of a fragment at *m*/*z* 651, corresponding to loss of two carbamic acid residues and sodium. From these data, carbamidocyclophane B (**2**) was expected to be a mono-des-chloro variant of **1**.

Comparison of the ^{13}C NMR data (Table 1) showed all signals of **1** and some further carbon signals. Thus, at least one-half of **2** was identical to that of **1**. The loss of one chlorine substituent from the side chain of the second half was indicated by a new chloromethylene carbon signal at δ 45.8 in the ^{13}C NMR spectrum and triplet of two corresponding protons at δ 3.38 in the ^1H NMR spectrum. This group was connected to three further methylene groups of the modified side chain by correlation signals in the $^1H,^1H$ COSY and $^1H,^{13}C$ HMBC NMR spectra. Only a few ^{13}C NMR signals of the cyclophane ring in **2** were doubled.

Carbamidocyclophane C (**3**) was recognized as a carbamidocyclophane from its similar UV spectrum. The nominal molecular mass *m*/*z* 738 was indicated by a molecular ion cluster at *m*/*z* 737/739/741 Da (∼100:67:15) in the negative ESIMS and a ion cluster with ammonia $[M + NH_4]^+$ at *m*/*z* 756/758/760 (∼100:66:18) in the positive mode ESIMS. The isotope pattern was in good agreement with the presence of two chlorine atoms in the elemental composition $C_{38}H_{56}N_2O_8Cl_2$ [*m*/*z* 738.341 (100:75:17)] of **3**.

Again the complete set of NMR signals known for one-half of **1** was identified in the spectrum (Table 1) of **3**. Additionally, a new triplet of a methyl group at δ_H 0.86 was observed, which was connected to three further methylene groups of the side chain by a set of correlation signals in the $^1H,^1H$ COSY and $^1H,^{13}C$ HMBC NMR spectra. These findings indicated that the second half of the molecule contained a pure aliphatic side chain. It was characterized as an *n*-butyl group by ^{13}C shifts of δ 14.5 for the methyl group and δ 23.9, 31.6, and 34.8 for the methylene carbons.

Carbamidocyclophane D (**4**) gave an intense $[M + NH_4]^+$ ion cluster at *m*/*z* 722 in the positive mode ESIMS and a $[M - H]^-$ ion at *m*/*z* 703 in the negative mode ESIMS, which indicated a nominal molecular mass of 704 Da. The loss of 34 Da compared to **3** and the isotope distribution 100:41:57:15 for *m*/*z* 722, 723, 724, 725 (calcd 100:43:43:15) showed the presence of one chlorine atom in the elemental composition $C_{38}H_{57}N_2O_8Cl_1$. The structure of **4** was derived by comparison of the NMR spectra (Table 1). The signals of an *n*-butyl side chain residue as observed in carbamidocyclophane C (**3**) were present for one-half as well as the signals of a monochlorobutyl side chain for the second half, which was previously seen in carbamidocyclophane B (**2**).

Finally, traces of carbamidocyclophane E (**5**) were isolated. **5** has the elemental composition of $C_{38}H_{58}N_2O_8$ (670.419 Da) according to the $[M + NH_4]^+$ ion cluster at *m*/*z* 688 in the positive mode ESIMS and a $[M - H]^-$ ion at *m*/*z* 669 in the negative mode ESIMS, both lacking any chlorine isotope peaks.

In spite of the low amount, the quality of the NMR spectra was good, because in this symmetrical carbamidocyclophane all signal intensities were duplicated. The structure of **5** with two *n*-butyl

Table 1. NMR Data of Carbamidocyclophanes A−E (**1**−**5**) in Methanol-d_4[e]

1[a]					**1**				**2**					**3**					**4**					**5**				
C	δ_H	M	δ_C[b]	m	δ_H	m	δ_C[c]	m	C	δ_H	m	δ_C	m	C	δ_H	m	δ_C	m	C	δ_H	m	δ_C	m	C	δ_H	m	δ_C	m
1/14	4.72	d	80.23	d	4.87	m[d]	83.49	d	1/14	4.85	d	83.51	d	1/14	4.85	d	83.58	d	1/14	4.85	d	83.65	d	1/14	4.85	d	83.59	d
2/15	1.58	m	38.64	d	1.77	m	40.39	d	2/15	1.76	m	40.38	d	2/15	1.76	m	40.38	d	2/15	1.76	m	40.45	d	2/15	1.76	m	40.39	d
3/16	0.68	m	33.15	t	0.83	m	34.51	t	3/16	0.82	m	34.51	t	3/16	0.83	m	34.52	t	3/16	0.83	m	34.58	t	3/16	0.83	m	34.54	t
	0.58	m			0.74	m				0.76	m				0.74	m				0.75	m				0.74	m		
4/17	1.38	m	28.00	t	1.49	m	29.59	t	4/17	1.28	m	29.57	t	4/17	1.48	m	29.60	t	4/17	1.48	m	29.68	t	4/17	1.48	m	29.66	t
	0.74	m			0.88	m				0.88	m				0.87	m	29.55	t		0.87	m				0.87	m		
5/18	0.89	m	29.37	t	1.00	m	30.44	t	5/18	1.00	m	30.43	t	5/18	0.98	m	30.49	t	5/18	0.99	m	30.56	t	5/18	0.99	m	30.53	t
	0.56	m			0.76	m				0.77	m	30.40	t		0.76	m	30.40	t		0.75	m	30.50	t		0.76	m		
6/19	1.92	m	33.41	t	2.09	m	36.25	t	6/19	2.09	m	35.25	t	6/19	2.08	m	35.25	t	6/19	2.07	m	35.32	t	6/19	2.06	ddt	35.27	t
	1.28	m			1.37	m				1.37	m				1.37	m				1.36	m				1.36	m		
7/20	3.09	m	34.60	d	3.24	m	36.36	d	7	3.24	m	36.37	d	7	3.23	m	36.37	d	7	3.22	m	36.61	d	7/20	3.20	m	36.81	d
									20			36.55	d	20	3.21	m	36.80	d	20	3.20	m	36.87	d					
8/21			115.22	s			117.37	s	8			117.44	s	8			117.45		8			117.92	s	8/21			118.38	s
									21			117.87	s	21			118.44		21			118.50	s					
9/22			157.06	s			158.78	s	9/22			158.75	s	9/22			158.74		9/22			157.02	s	9/22			158.73	s
																						156.99	s					
10/23	6.10	s	103.76	d	6.27	m	105.27	d	10/23	6.24	m	105.33	d	10/23	6.26	m	105.34	d	10/23	6.26	m	105.40	d	10/23	6.26	m	105.31	d
11/24			138.58	S			140.09	s	11			140.10	s	11			140.11		11/24			140.02	s	11/24			139.74	s
									24			139.96	s	24			139.74					139.80	s					
12/25	6.04	s	107.81	D	6.14	m	109.38	d	12/25	6.17	m	109.43	d	12/25	6.14	m	109.45	d	12/25	6.14	m	109.51	d	12/25	6.13	m	109.43	d
13/26			155.32	S			157.02	s	13/26			157.01	s	13/26			159.79		13/26			158.80	s	13/26			156.93	s
27/31	1.88	m	32.16	t	2.06	m	33.69	t	27	2.05	m	33.71	t	27	2.08	m	33.72	t	27	2.02	m	34.27	t	27/31	1.95	ddt	34.82	t
	1.50	m			1.56	m				1.58	m				1.56	m				1.54	m				1.53	ddt		
28/32	1.28	m	24.27	t	1.42	m	25.81	t	28	1.43	m	25.79	t	28	1.42	m	25.79	t	28	1.34	m	26.63	t	28/32	1.21	m	31.64	t
																				1.28	m							
29/33	2.13	m	43.20	t	2.23	ddt	45.07	t	29	2.23	m	45.08	t	29	2.23	m	45.08	t	29	1.78	m	34.18	t	29/33	1.11	m	23.94	t
	2.05	m			2.10	m				2.10	m				2.10	m				1.70	m				1.27	m		
30/34	6.21	t	74.96	d	5.86	t	75.29	d	30	5.86	t	75.28	d	30	5.86	t	75.28	d	30	3.84	t	45.88	t	30/34	0.86	t	14.48	q
									31	2.02	m	34.20	t	31	1.95	ddt	34.82	t	31	1.95	m	34.89	t					
															1.53	m				1.54	m							
NH_2	6.36	s							32	1.32	m	26.57	t	32	1.21	m	31.64	t	32	1.21	m	31.70	t					
OH	8.82	s													1.11	m				1.11	m							
									33	1.73	m	34.12	t	33	1.28	m	23.92	t	33	1.28	m	23.99	t					
									34	3.38	t	45.82	t	34	0.86	t	14.46	q	34	0.85	t	14.52	q					
35/37			156.41	s			159.82	s	35/37			159.79	s	35/37			159.84	-	35/37			159.91	s	35/37			159.87	s
																						159.88	s					
36/38	0.91	d	15.88	q	1.04	d	16.55	q	36/38	1.04	d	16.54	q	36/38	1.04	d	16.54	q	36/38	1.04	d	16.61	q	36/38	1.04	d	16.55	q
J: 1/14-H 10.6, 30/34-H 6.0, 36/38-H 6.4 Hz					*J*:1/14-H*d*,30/34-H 6.2, 36/38-H 6.4 Hz				*J*: 1/14-H 10.2,30-H 6.2, 34-H 7.2, 36/38-H 6.4 Hz					*J*: 1/14-H 10.2, 30-H 6.2, 27-Ha 14.6, 9.4, 5.8, 31-Ha 13.2, 5.3, 9.4, 34H 7.2, 36/38-H 6.4 Hz					*J*: 1/14-H 10.2, 30-H 7.0, 34-H 7.2, 36/38H 6.4 Hz					*J*: 1/14-H 10.2, 6/19-Ha 3.8, 12.1, 12, 27/31-Ha, 4.9, 13.4, 9.4, 27/31-Hb, 10, 13.4,5.6, 30/34-H, 7.2, 36/38-H 6.4 Hz				

[a] In DMSO-d_6. [b] ^{13}C 150 MHz. [c] ^{13}C 75 MHz. [d]Under OH signal. [e] ^{13}C NMR shifts are reported with two decimal places in order to discriminate between narrow signals although the last digit may vary slightly between different measurements.

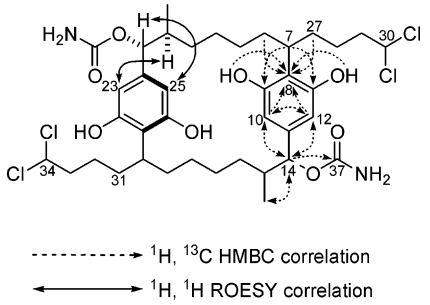

Figure 1. Crucial NMR long-range correlations of carbamidocyclophane A (**1**) in DMSO-d_6.

side chains was elucidated by comparison of the NMR data with those of the corresponding aliphatic parts of **3** and **4** (Table 1). Carbamidocyclophane E (**5**) is closely related to cylindrocyclophane A (**6**), which bears hydroxy groups instead of the carbamido groups, and also to cylindrocyclophane D (**7**), the corresponding diacetyl ester.[3,5]

Because the coupling constant $J_{1,2} = J_{14,15}$ was determined as 10.6 Hz, carbamidocyclophane A (**1**) was assigned the same relative configuration as observed for cylindrocyclophane D (**7**) with acetate residues at the C-1/14 instead of the carbamido groups. From biosynthetic considerations, both [7.7]cyclophane types should have an identical absolute configuration. Since the absolute configuration of cylindrocyclophane A (**6**) has been determined by Mosher's method,[5] a comparison between cylindrocyclophane D (**7**), the corresponding 1,14-diacetate, and the most similar structure of the carbamidocyclophanes was done to provide the absolute configuration. As expected for an acylated cylindrocyclophane, carbamidocyclophane E (**5**) gave a positive specific optical rotation ($[\alpha]^{22}_D$ +5.7).

All carbamidocyclophanes contain the [7.7]paracyclophane ring previously found in the cylindrocyclophanes from *Cylindrospermum licheniforme*,[3] but they differ in their characteristic carbamido substituents in the 1,14-positions. Additionally, compounds A−D (**1**−**4**) are variably chlorinated at the end of their butyl side chains, where the number of chlorine atoms decreases by one in each case. Only the low abundant metabolite carbamidocyclophane E (**5**) does not contain chlorine in its completely symmetrical molecule.

The three main components A−C (**1**−**3**) obtained with yields of 0.2−0.1% presented MIC values of 0.1 mmol (**1**), 0.04 mmol (**2**), and 0.06 mmol (**3**) against *Staphylococcus aureus* ATCC 6538, revealing a moderate antibacterial activity. Estimation of cytotoxic activities with Fl cells (human amniotic epithelial cell line) resulted in IC_{50} values of 3.3 μmol (**1**), 4.2 μmol (**2**), and 5.1 μmol (**3**), respectively. The IC_{50} values of 0.86, 1.8, and 2.2 μmol, determined with MCF7 cells (breast adenocarcinoma cell line), indicated that the carbamidocyclophanes possess stronger cytotoxic activity against these tumor cells than against nontransformed Fl cells.

Experimental Section

General Experimental Procedures. UV spectra were recorded in MeOH (Uvasol, Merck, Germany) with a UV-2102 PC UV−vis scanning spectrophotometer (Shimadzu). One-dimensional (^{1}H, ^{13}C, DEPT-135) and two-dimensional (COSY, HMQC, HMBC) NMR spectra were recorded at 300 K on a Bruker DMX600 NMR spectrometer (^{1}H NMR: 600 MHz; ^{13}C NMR: 150 MHz) locked to the major resonance of the solvent. Chemical shifts are shown in δ values (ppm) with the solvent signal as an internal standard. HPLC-ESIMS spectra were obtained with a PE Sciex API 2000 MS/MS spectrometer attached to an Agilent HPLC Series 1100 instrument system. Positive ion high-resolution electrospray mass spectra (HRESITOF) were recorded on a quadrupole time-of-flight QTOF-2 mass spectrometer (Micromass, UK) equipped with a nanospray ion source, and a voltage of approximately 1000 V was applied. The resulting ions were separated by the orthogonal TOF mass analyzer, and the isotopic composition of the sample was determined in the accurate mass mode using reserpine ($[M + H]^+ = 609.2811$ Da) as an internal reference compound.

Column chromatography was carried out on silica gel (Si 60, 0.015−0.04 mm, Merck, Germany). Fractions were monitored by TLC (Si 60 GF 254 nm, Merck, Germany) with ethyl acetate−acetone−MeOH (50:50:2) as mobile phase. Detection was done under UV light at 254 nm or by spraying with anisaldehyde/sulfuric acid reagent and heating. Analytical and preparative HPLC were performed on a component system (Kontron Instruments, Italy), consisting of pumps 422 and 422 S, auto sampler 360, and diode array detector (DAD 440). A Synergi POLAR-RP column (250 × 4.6 mm, 4 μm, 80 Å Phenomenex) and a gradient of deionized water (Clear UV plus SG, Water Preparation and Recycling GmbH, Germany) and MeOH (gradient grade, ROTH, Germany) from 50% aqueous MeOH to 100% MeOH in 30 min were used for analytical HPLC with a flow rate of 1.0 mL min^{-1}. Preparative HPLC was performed on an equivalent POLAR-RP column (250 × 10.0 mm) with a flow rate of 2 mL min^{-1}. HPLC runs were recorded using the Geminyx HPLC data system 1.91 SST version 1.6. All chemicals were used as received, and solvents were distilled prior to use except for HPLC.

Culture Conditions. The filamentous *Nostoc* sp. strain CAVN 10 was isolated from a soil sample collected in Northern Vietnam and established as a laboratory culture by Dr. Nhi V. Tran (Institute for Biotechnology, Hanoi). The strain is maintained in the culture collection of the Institute of Pharmacy, EMAU Greifswald, as a stock culture. The cyanobacterium was cultured in a glass column containing 30 L of BG 11 medium.[7] After 30 days the biomass was harvested by centrifugation and filtration. The yield of lyophilized biomass was 0.6 g L^{-1}.

Extraction and Isolation. Freeze-dried cells (5 g) were extracted three times with 250 mL of *n*-hexane followed by three portions of 250 mL of MeOH under stirring for 1 h, respectively. Residue and supernatant were separated by centrifugation at 4500 rpm at 4 °C for 20 min. The methanolic supernatants were pooled and evaporated to provide a crude extract of about 1 g. A portion of 500 mg of extract was separated on silica gel (column 4 × 60 cm) using a stepwise gradient of toluene−ethyl acetate−MeOH (92:5:3, 600 mL), toluene−ethyl acetate−MeOH (75:25:20, 600 mL), ethyl acetate−acetone−MeOH (50:50:2, 600 mL), and MeOH (600 mL) at 1 mL min^{-1} to obtain 11 fractions. After evaporation of the solvents 500 μg of each fraction was tested for antibiotic activity. Fraction F2 [eluted with toluene−ethyl acetate−MeOH (75:25:20), yield 50 mg of a white powder] exhibited the strongest activity. Further separation of F2 was done by preparative RP-HPLC. Pure compounds were obtained using a solvent gradient starting with 10% aqueous MeOH increasing to 75% MeOH−H_2O in 5 min followed by 75 to 85% MeOH−H_2O in 30 min and 85−100% MeOH−H_2O for 3 min. The separation was monitored by UV absorption (226 nm), and pure carbamidocyclophanes E (**5**), D (**4**), C (**3**), B (**2**), and A (**1**) eluted at retention times of 20.9, 21.9, 22.8, 23.7, and 24.6 min, respectively. After evaporation the yields of the pure, amorphous compounds were 10 (**1**), 8 (**2**), 5 (**3**), 1 (**4**), and 0.5 mg (**5**), respectively.

Carbamidocyclophane A (1): $C_{38}H_{54}Cl_4N_2O_8$, M = 808.65; $[\alpha]^{22}_D$ −1.2 (*c* 0.4, methanol); UV (MeOH) λ_{max} (log ϵ) 208 (4.817), 228 (sh 4.27), 276 (3.510), 283 (3.530) nm; IR (KBr) ν_{max} 3500, 3404, 2933, 2858, 1706, 1620, 1593, 1433, 1391, 1336, 1050, 1017 cm^{-1}; NMR data in DMSO-d_6 and in methanol-d_4, see Table 1; ESI (+) *m*/*z* 824, 826, 828 (73:100:53%) (calcd 78:100:48%) for $C_{38}H_{54}N_2O_8Cl_4$ as $[M + NH_4]^+$; ESI (−) *m*/*z* 805, 807, 809 $[M - H]^-$; HRESITOF (+) *m*/*z* 829.2553, calcd 829.2532 for $C_{38}H_{54}N_2O_8Cl_4$ as $[M + Na]^+$.

Carbamidocyclophane B (2): $C_{38}H_{55}Cl_3N_2O_8$, M = 774.21; $[\alpha]^{22}_D$ −0.3 (*c* 0.34, methanol); UV (MeOH) λ_{max} (log ϵ) 209 (4.873), 228 (sh), 276 (3.446), 282 (3.461) nm; IR (KBr) ν_{max} 3502, 3397, 2933, 2857, 1706, 1620, 1594, 1433, 1391, 1336, 1050, 1018 cm^{-1}; NMR data in methanol-d_4, see Table 1; HRESITOF (+) *m*/*z* 795:797:798 = 100:101:40% (calcd 100:105:40%); *m*/*z* 795.2945 calcd 772.2922 for $C_{38}H_{55}N_2O_8Cl_3$ as $[M + Na]^+$.

Carbamidocyclophane C (3): $C_{38}H_{56}Cl_2N_2O_8$, M = 739.77; $[\alpha]^{22}_D$ +8.5 (*c* 0.56, methanol); UV (MeOH) λ_{max} (log ϵ) 208 (4.894), 229 (sh), 276 (3.603), 282 (3.613) nm; IR (KBr) ν_{max} 3500, 3399, 2931, 2857, 1706, 1620, 1593, 1433, 1384, 1336, 1050, 1019 cm^{-1}; NMR data in methanol-d_4, see Table 1; ESI (+) *m*/*z* 756, 758, 760 (100:77:18%), calcd 100:75:17% for $C_{38}H_{56}N_2O_8Cl_2$ as $[M + NH_4]^+$;

ESI (−) *m*/*z* 737, 739, 741 $[M - H]^-$; HRESITOF (+) *m*/*z* 761.3316, calcd 761.3311 for $C_{38}H_{56}N_2O_8Cl_2$ as $[M + Na]^+$.

Carbamidocyclophane D (4): $C_{38}H_{57}ClN_2O_8$, M = 705.32; $[\alpha]^{22}_D$ +5.6 (*c* 0.26, methanol); UV (MeOH) λ_{max} (log ϵ) 210 (4.803), 229 (sh), 276 (3.464), 282 (3.482) nm; NMR data in methanol-d_4, see Table 1; ESI (+) *m*/*z* 722, 723, 724, 725 (100:41:57:15%), calcd 100:43:43:15% for $C_{38}H_{57}N_2O_8Cl$ as $[M + NH_4]^+$; ESI (−) *m*/*z* 703.704 $[M - H]^-$; HRESITOF (+) *m*/*z* 727.3697, calcd 727.3701 for $C_{38}H_{57}N_2O_8Cl$ as $[M + Na]^+$.

Carbamidocyclophane E (5): $C_{38}H_{58}N_2O_8$, M = 670.88; $[\alpha]^{22}_D$ +5.7 (*c* 0.12, methanol); UV (MeOH) λ_{max} (log ϵ) 208 (4.916), 229 (sh), 276 (3.596), 282 (3.585) nm; NMR data in methanol-d_4, see Table 1; ESI (+) *m*/*z* 688 (100:43:10%) for $C_{38}H_{58}N_2O_8$ as $[M + NH_4]^+$; ESI (−) *m*/*z* 669 $[M - H]^-$; ESITOF (+) *m*/*z* 693.4077, calcd 693.4091 for $C_{38}H_{58}N_2O_8$ as $[M + Na]^+$.

Antibiotic Assay. Crude extracts (2 mg/6 mm paper disc) and fractions (0.5 mg/6 mm paper disc) were tested in an agar plate diffusion assay[8] against the ATCC strains *Bacillus subtilis* 6051, *Staphylococcus aureus* 6538, and *Escherichia coli* 11229, *Pseudomonas aeruginosa* 27853 and the yeast *Candida maltosa* SBUG 700. Diameters of the inhibition zones were measured over the whole zone including the paper disc. Determination of MIC (minimal inhibition concentration) of carbamidocyclophanes **1**−**5** was done with *Staphylococcus aureus* ATCC 6538 according to the methods of the European Pharmacopoeia.[9]

Cytotoxicity Assay. The cytotoxicity of **1**−**5** was measured by a modified cellular test according to Bracht et al.[10] with MCF-7 cells, a breast adenocarcinoma cell line obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany), and Fl cells, a human amniotic epithelial cell line (ATCC, CCL 62, RIE 81, USA, evaluated by the ZBV, Riems, Germany, 2005). Cells were maintained in Isocove's modified Dulbecco's medium (IMDM Invitrogen) with 10% FBS (Biochrome AG, Germany), and 15 000 cells were seeded in 150 μL of IMDM per well of a 96-well microtiter plate. After 48 h at 37 °C in a humidified 5% carbon dioxide atmosphere the test substances were added. The substances were solved in 10 μL of MeOH (MeOH gradient grade Roth) per 1 mL of medium, and 50 μL of this mixture was added per well, resulting in final concentrations between 15 and 0.0002 μg mL^{-1}. After incubation for 48 h the cells were stained with Crystal Violet: the culture medium was removed, and the cells were rinsed with 100 μL of PBS per well and fixed with 2.5% glutaraldehyde in buffer for 30 min. The fixing solution was discarded; the cells were washed with double distilled water and stained with 0.02% Crystal Violet for 30 min. After washing and drying the cell-bound dye was dissolved with 70% EtOH under shaking for 4 h and the optical density was measured at λ = 550 nm with an Anthos ht II plate reader (Anthos, Austria). Inhibition of cell growth was calculated as decrease of optical density of the cells incubated with the test substances in comparison to the control (cells with medium), and the IC_{50} values were calculated.[10]

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