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Carbamidocyclophanes F and G with Anti-*Mycobacterium tuberculosis* Activity from the Cultured Freshwater Cyanobacterium *Nostoc* sp.

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Abstract

Two new (**1** and **2**) and three known (**3–5**) carbamidocyclophanes were isolated from a cultured freshwater cyanobacterium *Nostoc* sp. (UIC 10274) obtained from a sample collected at Des Plaines, Illinois. Their planar structures and stereoconfigurations were determined by extensive spectroscopic analysis including 1D/2D NMR experiments, HRESIMS as well as CD spectroscopy. Carbamidocyclophane F (**1**) showed potent anti-*Mycobacterium tuberculosis* activity in the microplate Alamar blue assay and low-oxygen-recovery assay with MIC values of 0.8 and 5.4 μ M, respectively. Carbamidocyclophane F (**1**) also displayed antimicrobial activities against the gram positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis* with MIC values of 0.1 and 0.2 μ M, respectively. Carbamidocyclophane F (**1**) and Carbamidocyclophane G (**2**) both showed antiproliferative activity against MDA-MB-435 and HT-29 human cancer cell lines with IC₅₀ values in the range from 0.5 to 0.7 μ M.

Keywords

Cyanobacteria; *Nostoc* sp.; [7.7]paracyclophane; Anti- *Mycobacterium tuberculosis* Activity

Cyanobacteria are prolific producers of structurally diverse and bioactive natural products.^{1,2} Several metabolites containing a [7.7]paracyclophane skeleton have been discovered from cyanobacteria. Nostocyclophanes A–D and cylindrocyclophane A, were

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ASSOCIATED CONTENT

Supplementary data (Morphological and phylogenetic characterization of *Nostoc* sp. UIC 10274; Experimental detail; ¹H NMR, DEPTQ, COSY, HSQC and HMBC spectra of **1**; ¹H NMR, COSY, HSQC and HMBC spectra of **2**; CD spectra of **1** and **2**, LC-MS detection of **1** and **2** in crude extract) associated with this article can be found in the online version at <http://dx.doi.org/xx.xxxx>

isolated from *Nostoc linckia* (Roth) Bornet (UTEX B1932) and *Cylindrospermum licheniforme* Kutzing (ATCC 29204), respectively.^{3,4} These compounds displayed moderate cytotoxicity against KB and LoVo tumor cell lines. Subsequent these initial reports, cylindrocyclophanes B–F, carbamidocyclophanes A–E, cylindrocyclophanes A₁–A₄, C₁–C₄, F₄ and merocyclophanes A–B were discovered in various cyanobacterial strains. These cyclophanes exhibited wide range of biological activities including antimicrobial, antiproliferative and proteasome inhibitory activities.^{5–8} Isotope feeding experiments revealed a unique polyketide biosynthetic pathway in the formation of [7.7]paracyclophane skeleton.⁹ In the recent communication by Nakamura et al, the biosynthesis of cylindrocyclophane has been analyzed in detail.^{10,11}

The extract from the cultured freshwater *Nostoc* sp. (UIC 10274) was found to be active against *M. tuberculosis* in microplate Alamar blue assay (MABA).^{12,13} Bioassay-guided fractionation revealed two new (**1** and **2**) and three known (**3**–**5**) carbamidocyclophanes. We evaluated all five [7.7]paracyclophanes for anti-*M. tuberculosis* activity in MABA and low-oxygen-recovery (LORA) assays.¹⁴ Herein we describe the isolation, structure elucidation, and biological evaluation of these novel [7.7]paracyclophanes.

Nostoc sp. (UIC 10274) was obtained from a field sample collected in Des Plaines, IL. The extract showed significant inhibitory activity against *M. tuberculosis* in the microplate Alamar blue assay and was subjected to a bioassay guided fractionation scheme, which included a Diaion™ vacuum liquid chromatography (VLC) step. Diaion™ fractions eluting with 40–70% iPrOH displayed significant activity. Chemical dereplication using ESI-TOF-MS and ¹H NMR spectroscopy revealed the presence of five chlorinated [7.7]paracyclophanes, including two potentially new compounds. The final isolation of **1**–**5** was achieved by reversed-phase HPLC to obtain carbamidocyclophanes F (**1**, 2.3 mg, 0.09% of dry biomass), G (**2**, 1.2 mg, 0.05% of dry biomass), and three previously known analogues, carbamidocyclophanes A (**3**, 8.86 mg, 0.35% of dry biomass), B (**4**, 2.7 mg, 0.11% of dry biomass) and C (**5**, 1.3 mg, 0.05% of dry biomass).⁶

Carbamidocyclophane F (**1**)¹⁵ was obtained as white amorphous powder. Negative mode HRESIMS analysis indicated a tetrachlorinated molecule with a molecular formula of C₃₇H₅₃Cl₄NO₇ (m/z 764.2476 [M – H][–]). The ¹H NMR spectrum of **1** (Table 1) contained a group of aromatic protons (δ_H 6.08–6.25), a downfield triplet indicating the presence of dichlorinated methyl (δ_H 5.82), a benzylic proton multiplet (δ_H 3.20), 14 alkyl protons (δ_H 0.63–2.20), and two methyl doublets (δ_H 1.00 and 1.06). Together this pattern resembled those of carbamidocyclophane A (**3**)⁶ and cylindrocyclophane A₄⁷ and indicated that **1** possessed a tetrachlorinated [7.7]paracyclophane skeleton. Both carbamidocyclophane A (**3**) and cylindrocyclophane A₄ possess a C₂ axis of symmetry, and thus produce half of the NMR signals expected. However, **1** exhibited two sets of resonances. The large chemical shift difference of the oxymethine doublets (δ_H 3.75, 4.81) strongly indicated asymmetrical substituent pattern at C-1 and C-14. The doubling of several carbon signals (Table 1) as compared to carbamidocyclophane A (**3**) and cylindrocyclophane A₄ also corroborated the asymmetric structure of **1**. Analysis of the carbon and proton chemical shifts at C-1, C-2, C-3, C-23, C-24, C-25, C-35 in **1** indicated the presence of hydroxyl group at C-1 position, as observed in cylindrocyclophane A₄. Comparison of chemical shifts at C-14, C-15, C-16,

C-10, C-11, C-12, C-36 to carbamidocyclophane A (**3**) in combination with elemental composition indicated the presence of a carbamate group at C-14. HMBC correlation between H-14 (δ_{H} 4.81) and carbamate carboxyl (δ_{C} 159.8) confirmed the attachment of carbamate moiety to C-14 (Figure 1). Complete carbon and proton chemical shift assignments were achieved by analysis of ^1H , DEPTQ, COSY, TOCSY, HSQC, HMBC spectra and confirmed the structure of **1**.

Carbamidocyclophane G (**2**)¹⁶ was obtained as white, amorphous powder. The HRESIMS data (m/z 806.2616 [$\text{M} - \text{H}$]⁻) established the molecular formula to be $\text{C}_{39}\text{H}_{54}\text{Cl}_4\text{NO}_8$. The isotopic distribution pattern was consistent with that of **1**, indicating **2** also contained a tetrachlorinated moiety. The ^1H NMR spectrum of **2** closely resembled that of **1**, except for the change in chemical shift of H-1 from δ_{H} 3.75 to 4.99, and the presence of a methyl singlet at δ_{H} 1.99, both indicating the presence of an acetate group attached to C-1. HMBC correlations from both acetate methyl proton and H-1 to carbonyl carbon supported the attachment of an acetate moiety to C-1. The presence of acetate group is consistent with the molecular formula of **2**, which bears two more carbons than that of **1**. Structure determination and chemical shift assignments were completed by detailed study of ^1H , COSY, HSQC, HMBC spectra and confirmed the structure of **2**.

The stereoconfigurations of **1** and **2** were determined by a combination of coupling constant analysis and CD spectra comparison. The relative configurations of **1** at C-1, C-2 and C-14, C-15 were both determined to be “anti” based on the large coupling constants observed ($^3J_{\text{H-1,H-2}} = 9.7$ Hz and $^3J_{\text{H-14,H-15}} = 10.3$ Hz). The absolute configurations were established by comparison of the CD spectrum of **1** to that of nostocyclophanes A–D, cylindrocyclophane A₄ and merocyclophanes A and B.^{4,7,8} The negative Cotton effects at 215 nm ($\epsilon -3.72$) and 284 nm ($\epsilon -2.85$) were similar to those observed for nostocyclophanes A–D, cylindrocyclophane A₄ and merocyclophanes A and B, suggesting the same absolute configuration. Therefore, we submit that the absolute configurations at C-1 and C-14 are “R”, while C-7 and C-20 are “S”. The coupling constants for **2** ($^3J_{\text{H-1,H-2}} = ^3J_{\text{H-14,H-15}} = 10.3$ Hz) indicated the same relative configuration at C-1, C-2, C-14, C-15, as found in **1**. The CD spectrum with negative Cotton effects at 217 nm ($\epsilon -4.27$) and 284 nm ($\epsilon -4.57$) were similar to the values observed for **1** and we submit that **2** has the same absolute configurations at C-1, C-7, C-14 and C-20 as **1**. Both **1** and **2** could be detected by LC-MS analysis of the crude extract of *Nostoc* sp. (UIC 10274), indicating that they are not artifacts from isolation (S14, Supporting Information).

Taxonomic identification of the strain UIC 10274 was performed based on traditional morphological analysis and phylogenetic analysis of a partial 16S rRNA gene sequence (GenBank Accession No. **JX188019**). The phylogenetic distances between [7.7]paracyclophane-producing strains (S15, Supporting Information) raises the possibility of horizontal gene transfer. However, further genetic and biosynthetic studies would be needed to validate this hypothesis.^{7,8}

Both **1** and **2** contain the [7.7]paracyclophane ring with branched methyls at C-2/15, and are chlorinated at C-11/24, as previously found in carbamidocyclophanes A–E (Supporting Information).⁶ But unlike carbamidocyclophanes A–E, which have carbamate groups at both

C-1/14, **1** and **2** have only one carbamate moiety at C-14. Comparing to other known [7.7]paracyclophanes, **1** and **2** have the same carbon skeleton as cylindrocyclophanes, and only differ by substituent groups at C-1/14, indicating **1** and **2** to have very similar biosynthetic pathway as cylindrocyclophanes.^{10,11} However, the differences in [7.7]paracyclophane carbon skeleton between **1**, **2** and nostocyclophanes and merocyclophanes suggested different carbon elongation pathways.¹⁰

Compounds **1**–**5** were evaluated for their anti-*M. tuberculosis* activity in the microplate Alamar blue assay (MABA). This assay assesses the inhibitory effect against the rapidly growing *M. tuberculosis*.^{12,13} In the treatment of tuberculosis, the physiological state of nonreplicating persistence (NRP) accounts for antimicrobial tolerance.¹⁷ The low-oxygen-recovery assay (LORA) has been established to represent the NRP phenotype of *M. tuberculosis*.¹⁴ Carbamidocyclophane F (**1**) exhibited potent activity in MABA with MIC of 0.8 μ M, and moderate activity in LORA (MIC= 5.4 μ M) (Table 2). However, carbamidocyclophane G (**2**) showed more than two fold less potent activity in MABA (MIC= 1.8 μ M), and no activity in LORA at 10 μ M. The structures of **1** and **2** are almost identical except that the hydroxyl group at C-1 in **1** was replaced by acetate moiety in **2**. This indicates that the hydroxyl group plays a significant role for anti-*M. tuberculosis* activity in both rapidly growing and NRP phenotypes. A comparison of MABA and LORA activities between **1** and **3** indicated that the substitution of hydroxyl group (C-1) in **1** with carbamate moiety also deprives **1** of its anti-*M. tuberculosis* activity. The five isolated carbamidocyclophanes were also evaluated for their antimicrobial activity against *Mycobacterium smegmatis*, *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumonia* and *Candida albicans* (Table 3). Neither **1** nor **2** were active against *M. smegmatis*, a fast growing and non-pathogenic model species in the genus *Mycobacterium*. This indicates **1** and **2** selectively inhibit the growth of *M. tuberculosis* within *Mycobacterium* genus. None of isolates were active against gram negative bacteria *A. baumannii*, *E. coli* and *P. aeruginosa*, whereas all exhibited inhibitory activity against gram positive bacteria *S. aureus* and *E. faecalis* (MIC values 0.1–1.1 μ M). Evaluation of cytotoxic activities against human cancer cell lines MDA-MB-435 (melanoma) and HT-29 (colon) revealed that compound **1** exhibited antiproliferative activity with IC₅₀ of 0.7 μ M in both cell lines, while **2** displayed slightly higher potency with IC₅₀ of 0.5 μ M in both cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Carbamidocyclophane F (**1**): white, amorphous powder; $[\alpha]_D^{22}$ 0 (c 0.2, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 217 (4.32), 276 (3.38) nm; CD (c 0.01, MeOH) $\lambda_{\max}$ (log ϵ) 215 (–3.72), 238 (–2.42), 262 (–2.13), 284 (–2.85) nm; IR (neat) $\nu_{\max}$ 3356 (br), 2932, 2857, 1704, 1619, 1594, 1432, 1375, 1335, 1021, 987, 834, 747 cm^{-1} ; ^1H and ^{13}C NMR see Table 1; HR-ESI-TOF-MS (–) m/z 764.2476 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{37}\text{H}_{52}\text{Cl}_4\text{NO}_7$, 764.2468).
- Carbamidocyclophane G (**2**): white, amorphous powder; $[\alpha]_D^{22}$ +3.4 (c 0.06, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 219 (4.40), 276 (3.50) nm; CD (c 0.0025, MeOH) $\lambda_{\max}$ (log ϵ) 217 (–4.27), 234 (–2.86), 263 (–2.98), 284 (–4.57) nm; IR (neat) $\nu_{\max}$ 3390 (br), 2933, 2858, 1707, 1621, 1594, 1433, 1374, 1338, 1257, 1019, 831, 748, 659 cm^{-1} ; ^1H and ^{13}C NMR see Table 1; HR-ESI-TOF-MS (–) m/z 806.2616 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{39}\text{H}_{54}\text{Cl}_4\text{NO}_8$, 806.2574).
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Highlights

- Two new carbamidocyclophanes were isolated from a cultured freshwater cyanobacterium *Nostoc* sp. (UIC 10274).
- Carbamidocyclophane F showed potent anti-*Mycobacterium tuberculosis* activity in MABA and LORA assays.
- Both carbamidocyclophane F and G exhibited antiproliferative activity against human melanoma and colon cancer cell lines.

Figure 1.
Key 2D correlations for structure determination of **1** and **2**.

	R ₁	R ₂	R ₃	R ₄
Carbamidocyclophane F (1)	CHCl ₂	CHCl ₂	OH	OCONH ₂
Carbamidocyclophane G (2)	CHCl ₂	CHCl ₂	OCOCH ₃	OCONH ₂
Carbamidocyclophane A (3)	CHCl ₂	CHCl ₂	OCONH ₂	OCONH ₂
Carbamidocyclophane B (4)	CHCl ₂	CH ₂ Cl	OCONH ₂	OCONH ₂
Carbamidocyclophane C (5)	CHCl ₂	CH ₃	OCONH ₂	OCONH ₂

Table 1

NMR Spectroscopic Data of Carbamidocyclophanes F and G (1–2) in MeOH-*d*₄

Carbamidocyclophane F (1)					Carbamidocyclophane G (2)		
position	δ_C^a , mult.	δ_H^b , mult. (J in Hz)	COSY	HMBC	position	δ_C^c , mult.	δ_H^d , mult. (J in Hz)
1	81.7, CH	3.75, d (9.7)	2	2, 23, 24, 25, 35	1	83.3, CH	4.99, d (10.3)
2	42.0, CH	1.55, m	1, 35, 3		2	40.1, CH	1.77, m
3	35.2, CH ₂	0.63, m	2, 4	1, 2, 4, 35	3/16	34.5, CH ₂	0.71, m
4/17	29.7 ^d , CH ₂	0.74, m	3/16, 5/18	3/16, 5/18	4/17	29.6 ^e , CH ₂	0.79, m
	29.8 ^d , CH ₂	1.44, m					0.85, m
5/18	30.5 ^d , CH ₂	0.72, m	4/17, 6/19	4/17, 6/19	5/18	30.5 ^e , CH ₂	1.45, m
	30.6 ^d , CH ₂	0.95, m					0.73, m
6/19	35.3 ^d , CH ₂	1.33, m	5/18, 7/20	5/18, 7/20	6/19	35.3 ^e , CH ₂	0.96, m
	35.4 ^d , CH ₂	2.06, m					1.33, m
7/20	36.3 ^d , CH	3.20, m	27/31, 6/19	5/18, 6/19, 8/21, 9/22, 13/26, 27/31, 28/32	7/20	36.4 ^e , CH	2.06, m
	36.4 ^d , CH						3.20, m
8	117.4, C				8	117.1, C	
9	158.8, C				9	158.6, C	
10	105.2, CH	6.21, s		8, 9, 11, 12, 14	10	105.3, CH	6.21, s
11	140.1, C				11	140.0, C	
12	109.4, CH	6.13, s		8, 10, 13, 14	12	109.4, CH	6.13, s
13	157.0, C				13	156.2, C	
14	83.5, CH	4.81, d (10.3)	15	10, 11, 12, 15, 36, OCON	14	83.5, CH	4.81, d (10.3)
15	40.4, CH	1.73, m	14, 36, 16	14	15	40.4, CH	1.73, m
16	34.5, CH ₂	0.71, m	15, 17	14, 15, 17, 36			
		0.79, m					
21	116.7, C				21	117.1, C	
22	158.9, C				22	159.2, C	

Carbamidocyclophane F (1)				Carbamidocyclophane G (2)		
position	δ_C^a , mult.	δ_H^b , mult. (J in Hz)	COSY	HMBC	position	δ_C^c , mult. δ_H^b , mult. (J in Hz)
23	105.0, CH	6.25, s		1, 21, 22, 24, 25	23	105.1, CH
24	144.2, C				24	139.2, C
25	108.8, CH	6.08, s		1, 21, 23, 26	25	109.6, CH
26	157.1, C				26	154.0, C
27/31	33.7 <i>d</i> , CH ₂	1.51, m	7/20, 28/32	7/20, 8/21, 28/32, 29/33	27/31	33.7 <i>e</i> , CH ₂
	33.8 <i>d</i> , CH ₂	2.04, m				2.02, m
28/32	25.7 <i>d</i> , CH ₂	1.36, m	27/31, 29/33	27/31, 29/33, 30/34	28/32	25.8 <i>e</i> , CH ₂
	25.8 <i>d</i> , CH ₂					1.38, m
29/33	45.0 <i>d</i> , CH ₂	2.06, m	28/32, 30/34	27/31, 28/32, 30/34	29/33	45.1 <i>e</i> , CH ₂
	45.1 <i>d</i> , CH ₂	2.20, m				2.18, m
30/34	75.3 <i>d</i> , CH	5.82, t (6.2)	29/33	28/32, 29/33	30/34	75.3 <i>e</i> , CH
	75.4 <i>d</i> , CH	5.83, t (6.2)				5.83, t (6.2)
35	17.0, CH ₃	1.06, d (6.5)	2	1, 2, 3	35	16.6, CH ₃
36	16.6, CH ₃	1.00, d (6.5)	15	14, 15, 16	36	16.6, CH ₃
OCON	159.8, C				OCONH2	159.7, C
H2					OAc	172.1, C
					OAc	21.2, CH ₃
						1.99, s

^a DEPTQ experiment recorded at 226 MHz.

^b Recorded at 600 MHz.

^c Determined indirectly using HSQC and HMBC.

^d Carbon chemical shifts interchangeable in **1**.

^e Since carbon chemical shifts of **2** were determined indirectly using HSQC and HMBC on a 600 MHz instrument, carbon chemical shifts differ less than 0.5 ppm could not be resolved.

Table 2Anti-*Mycobacteria tuberculosis* Activity of Isolated Carbamidocyclophanes (1–5)

compound	R ₁	R ₂	R ₃	R ₄	MABA MIC (μM)	LORA MIC (μM)
1	CHCl ₂	CHCl ₂	OH	OCONH ₂	0.8	5.4
2	CHCl ₂	CHCl ₂	OCOCH ₃	OCONH ₂	1.8	>10
3	CHCl ₂	CHCl ₂	OCONH ₂	OCONH ₂	>10	>10
4	CHCl ₂	CH ₂ Cl	OCONH ₂	OCONH ₂	>10	>10
5	CHCl ₂	CH ₃	OCONH ₂	OCONH ₂	>10	>10

Table 3Evaluation of Antimicrobial Selectivity of Isolated Carbamidocyclophanes (**1–5**)

MIC (μ M)	compound				
	1	2	3	4	5
<i>M. smegmatis</i>	>10	>10	>10	>10	>10
<i>A. baumannii</i>	>10	>10	>10	>10	>10
<i>E. coli</i>	>10	>10	>10	>10	>10
<i>P. aeruginosa</i>	>10	>10	>10	>10	>10
<i>S. aureus</i>	0.1	0.7	0.2	0.4	0.4
<i>E. faecalis</i>	0.2	1.1	0.2	0.2	0.4
<i>S. pneumoniae</i>	>10	>10	5.6	8.2	>10
<i>C. albicans</i>	2.9	>10	5.5	1.3	>10