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Nicrophorusamides A and B, Antibacterial Chlorinated Cyclic Peptides from a Gut Bacterium of the Carrion Beetle *Nicrophorus concolor*

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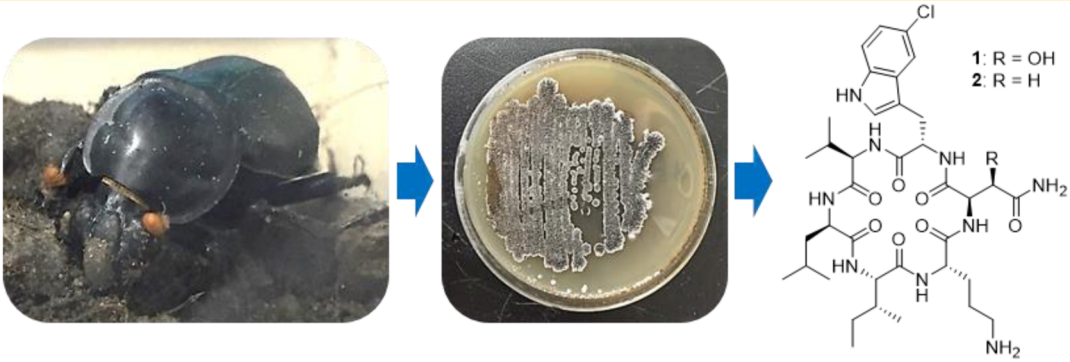
Supporting Information



ABSTRACT: Nicrophorusamides A and B (**1** and **2**) were discovered from a rare actinomycete, *Microbacterium* sp., which was isolated from the gut of the carrion beetle *Nicrophorus concolor*. The structures of the nicrophorusamides were established as new chlorinated cyclic hexapeptides bearing uncommon amino acid units mainly based on 1D and 2D NMR spectroscopic analysis. The absolute configurations of the amino acid residues 5-chloro-L-tryptophan, D-*threo*-*β*-hydroxyasparagine/D-asparagine, L-ornithine, L-*allo*-isoleucine, D-leucine, and D-valine were determined using Marfey's method and chemical derivatization with 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate followed by LC/MS analysis. Nicrophorusamide A (**1**) showed antibacterial activity against several Gram-positive bacteria.

Investigating the secondary metabolites produced by bacteria associated with insect hosts has become a promising method of discovering novel bioactive small molecules.[1] Carrion beetles (Coleoptera, Silphidae) are ecologically interesting because they utilize vertebrate carrion to rear their offspring.[2] Carrion beetles are, thus, exposed to carrion-borne bacteria, which may be pathogenic to them during development.[3] A recent analysis of the gut microbiome of six carrion beetle species belonging to the genus *Nicrophorus* indicated that these carrion-feeding beetles harbor gut microbial communities that are distinctively different from those of herbivorous, xylophagous, humivorous, omnivorous, and predatory beetles.[2] Although that study proposed hypothetical roles of carrion beetles' gut symbionts, such as carcass degradation, detoxification, and defense,[2] the mechanisms by which carrion beetles defend themselves against entomopathogenic bacteria originating from carrion have not yet been clearly elucidated. Previous chemical studies of symbionts in beetle-type insects (order: Coleoptera) led to the discovery of structurally novel bioactive compounds, including antimicrobial compounds. For example, an antifungal polyene peroxide was isolated from a symbiotic *Streptomyces* sp. in the southern pine beetle *Dendroctonus frontalis*.[4] Additionally, bacterial strains associated with the dung beetle *Copris tripartitus* produced a dichlorinated indanone that inhibits histone demethylase[5] and the highly modified cyclic peptides coprisamides A and B.[6] In this context, we assumed that the gut

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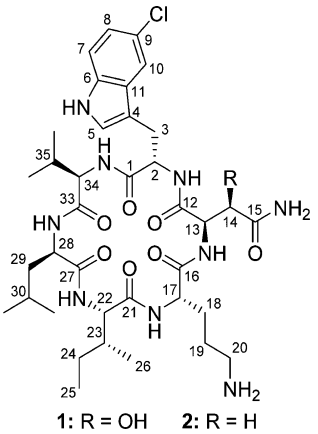
symbiotic bacteria of the carrion beetle *Nicrophorus concolor* may be a potential source of antimicrobial compounds. Therefore, we extracted the intestinal parts from an *N. concolor* specimen and isolated the bacterial strains, targeting chemically prolific actinobacteria. The isolated actinobacterial strains were cultivated and subjected to chemical analysis. During our chemical analysis, a rare actinomycete strain (UTG9) belonging to the genus *Microbacterium* was found to produce a series of previously unreported compounds based on ultraviolet (UV) spectroscopic data and low-resolution electrospray ionization mass spectrometry (LRESIMS) data with a characteristic isotopic pattern corresponding to chlorination. This initial chemical evaluation prompted a large-scale cultivation (72 L) and deeper chemical investigation by chromatographic purification and spectroscopic analysis of the two major compounds: nicrophorusamides A and B (**1** and **2**). Here, we report the structures and antibacterial activity of nicrophorusamides A and B.



RESULTS AND DISCUSSION

Nicrophorusamide A (**1**) was purified as a white powder, and the molecular formula was deduced as $C_{37}H_{56}ClN_9O_8$, which has an unsaturation number of 14, based on high-resolution fast atom bombardment MS (HRFABMS) data. The ^{1}H and HSQC NMR spectra of **1** identified 11 exchangeable protons (δ_H 11.03, 8.43, 7.98, 7.87, 7.81, 7.66 (2H), 7.62, 7.45, 7.31, and 7.28) and four aromatic protons (δ_H 7.57, 7.32, 7.15, and 7.05) in the downfield region below δ_H 7.0. One more heteroatom-bound proton was detected at δ_H 5.85, and six α-amino proton resonances were observed at δ_H 4.7−3.5 (δ_H 4.62, 4.60, 4.24, 4.23, 4.01, and 3.75). This initial analysis indicated that nicrophorusamide A is likely a peptide-derived compound with an aromatic ring structure. In addition, the ^{1}H NMR and HSQC data revealed the existence of a methine proton (δ_H 4.47) directly bonded to an oxygen-bearing carbon (δ_C 70.8). Further analysis of the ^{1}H NMR and multiplicity-edited HSQC spectra of **1** identified six aliphatic methylene and three methine protons between 3.11 and 1.16 ppm and six methyl groups (δ_H 0.87, 0.86, 0.85, 0.83, 0.72, and 0.69).

The ^{13}C NMR data of **1** (in DMSO-d_6 at 125 MHz) revealed seven carbonyl carbon signals (δ_C 173.2, 172.1, 171.7, 171.1, 170.6, 170.4, and 169.4), eight aromatic carbon resonances (δ_C 134.5, 128.4, 125.4, 122.9, 120.7, 117.5, 112.7, and 109.8), one oxygenated carbon (δ_C 70.8), and six amino acid α-carbon peaks (δ_C 60.4, 56.5, 55.8, 53.5, 53.1, and 51.5), reflecting the characteristic features of a peptide-class compound. The ^{13}C NMR data of nicrophorusamide A (**1**) also displayed 15 aliphatic carbon resonances at δ_C 39.6−11.3, including six methyl carbons (δ_C 22.7, 21.8, 18.8, 18.5, 14.5, and 11.3). All the one-bond ^{1}H−^{13}C correlations were established by analyzing the ^{1}H, ^{13}C, and HSQC NMR spectral data together.

Because nicrophorusamide A (**1**) was revealed to be a peptide with several amino acid units, individual amino acid moieties were elucidated by interpreting COSY, TOCSY, and HMBC NMR data. A 2-NH (δ_H 7.98)/H-2 (δ_H 4.62) COSY correlation connected the nitrogen to the C-2 α-carbon (δ_C 53.5). The COSY correlations from H-2 to H_2-3 (δ_H 3.11 and 2.89) showed connectivity between the α-carbon and β-carbon of the amino acid unit. The HMBC correlations from H_2-3 to C-4 (δ_C 109.8), C-5 (δ_C 125.4), and C-11 (δ_C 128.4) revealed that this amino acid has an aromatic ring structure in its side chain group.

The aromatic ring was constructed by analyzing COSY and HMBC NMR data. A ^{1}H−^{1}H coupling between H-7 (δ_H 7.32 [d, J = 8.5 Hz]) and H-8 (δ_H 7.05 [dd, J = 8.5, 2.0 Hz]) established the C-7−C-8 connectivity. The $^3J_{H7H8}$ value (8.5 Hz) indicated that this spin system belongs to a six-membered aromatic ring. This six-membered aromatic ring was assigned based on three-bond HMBC correlations from H-7 to C-9 and C-11, from H-8 to C-6 and C-10, and from H-10 to C-6 and C-8. The other aromatic proton, H-5 (δ_H 7.15), correlated with 5-NH (δ_H 11.03) in the COSY NMR data, allowing for C-5-N connectivity. The HMBC correlations from H-5 to C-4, C-6 (δ_C 134.5), and C-11 and from 5-NH to C-4, C-6, and C-11 indicated an indole ring structure, thereby identifying a tryptophan moiety (Figure 1a). An array of COSY correlations between 13-NH (δ_H 7.62), H-13 (δ_H 4.60), H-14 (δ_H 4.47), and 14-OH (δ_H 5.85) showed 13-NH-C-13-C-14-OH connectivity. This spin system was also confirmed by their TOCSY correlations. The two-bond H-13/C-12 (δ_C 169.4) ^{1}H−^{13}C coupling along with HMBC correlations from 15-NH_2b (δ_H 7.28) and H-14 to C-15 (δ_C 173.2) indicated β-hydroxyasparagine (Figure 1b). An ornithine unit was assigned based on the ^{1}H−^{1}H couplings of 17-NH (δ_H 8.43), H-17 (δ_H 4.01), H_2-18 (δ_H 1.84 and 1.64), H_2-19 (δ_H 1.50), H_2-20 (δ_H 2.79), and 20-NH_2 (δ_H 7.66) in the COSY and TOCSY NMR data, along with an H-17/C-16 (δ_C 170.6) HMBC correlation (Figure 1c).

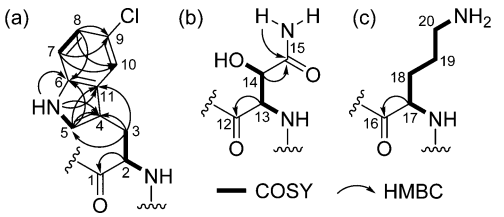


Figure 1. Structure determination of the uncommon amino acid units in **1** based on COSY and HMBC correlations: (a) 5-chlorotryptophan, (b) β-hydroxyasparagine, and (c) ornithine.

Other amino acid units, including valine, leucine, and isoleucine, were identified by further analyzing COSY, TOCSY, HSQC, and HMBC NMR data. Based on these results, nicrophorusamide A is a peptide with six amino acid units. After elucidating the six amino acids, the chlorine atom was assigned at the carbon C-9 in the tryptophan unit based on the molecular formula and the ^{13}C chemical shift, corresponding to 5-chlorotryptophan (Figure 1a).

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The discrete structures of 5-chlorotryptophan, β-hydroxyasparagine, ornithine, valine, leucine, and isoleucine bearing seven carbonyl carbons, four double bonds, and two rings explain 13 out of 14 double-bond equivalents. Therefore, nicrophorusamide A (**1**) was deduced to possess an additional ring. The last ring was constructed by connecting these six amino acid partial structures based on HMBC and ROESY correlations (Figure 2). The HMBC correlations from 2-NH of 5-chlorotryptophan

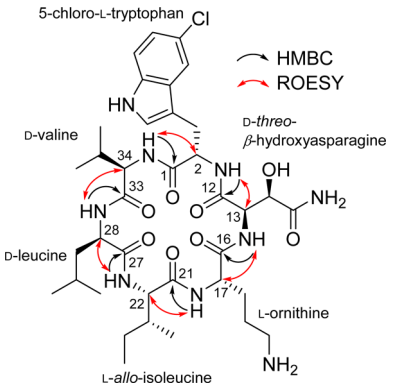

Figure 2. Identification of the amino acid sequence in **1** based on HMBC and ROESY NMR spectra.

to C-12 of β-hydroxyasparagine showed a linkage between the 5-chlorotryptophan and β-hydroxyasparagine units. The HMBC correlation of 13-NH to C-16 revealed the connectivity between β-hydroxyasparagine and ornithine. Isoleucine was located next to ornithine by the $^{1}H-^{13}C$ long-range correlation from 17-NH of ornithine to the amide carbon C-21 (δ_C 171.7) of isoleucine. The HMBC signal between 22-NH (δ_H 7.87), the amide proton of isoleucine, and C-27 (δ_C 172.1), the carbonyl carbon of leucine, indicated that leucine can have connectivity with isoleucine. The valine unit was placed adjacent to leucine by long-range $^{1}H-^{13}C$ couplings from 28-NH (δ_H 7.45) to C-33 (δ_C 170.4). A macrocyclic ring was closed by an HMBC correlation from 34-NH (δ_H 7.81) to C-1, completing the cyclic hexapeptide structure of nicrophorusamide A (**1**). ROESY correlations between amide NH's and α-protons further confirmed the amino acid sequence of **1** identified based on the HMBC correlations (Figure 2).

The absolute configurations at the α-carbons of the six amino acid units were determined by applying the advanced Marfey's method with the L- and D-forms of 1-fluoro-2,4-dinitrophenyl-5-alanine amide (FDAA).[7] Nicrophorusamide A (**1**) was hydrolyzed to obtain free amino acids, which were derivatized with L- and D-FDAA and analyzed by LC/MS. Comparing the LC/MS analysis results revealed 5-chloro-L-tryptophan, D-β-hydroxyasparagine, L-ornithine, L-isoleucine, D-leucine, and D-valine (Table S1, Figures S17 and S18).

An additional chiral center at C-14 of D-β-hydroxyasparagine had to be subjected to further stereochemical analysis. As β-hydroxyasparagine is converted to β-hydroxyaspartic acid during acid hydrolysis, samples of both *threo*-β-hydroxyaspartic acid and *erythro*-β-hydroxyaspartic acid (see the Supporting Information) were prepared and compared to the product from **1** using LC/MS; the latter was shown to be D-*threo*-β-hydroxyaspartic acid, thus determining a 14*R* configuration. To elucidate the absolute configuration at C-23 of the L-isoleucine unit, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) derivatization was used.[8] Derivatizing authentic L-isoleucine and L-*allo*-isoleucine with GITC and subsequently comparing their retention times revealed that isoleucine in **1** is L-*allo*-isoleucine (Figure S20).

Nicrophorusamide B (**2**) was isolated as a white powder, the molecular formula of which was determined to be $C_{37}H_{56}ClN_9O_7$ by HRFABMS and 1D ^{1}H and ^{13}C NMR spectral data (Table 1). Based on its molecular formula, nicrophorusamide B (**2**) has one fewer oxygen atom than **1**. The NMR and UV spectra of **2** displayed high degrees of similarity to those of **1**, indicating that this compound is analogous to nicrophorusamide A (**1**). Comparison of 1D and 2D NMR spectroscopic data with those of **1** revealed that D-*threo*-β-hydroxyasparagine was replaced with D-asparagine, whereas the other amino acids were identical. The absolute configurations of the amino acid units in **2** were deduced to be identical to those in **1** based on their identical circular dichroism (CD) data (Figure S16) and the common biosynthetic origin.

Other cyclic hexapeptides sharing similar amino acid units to the nicrophorusamides have been reported. For example, desotamides A–D, discovered from deep-sea-derived *Streptomyces scopuliridis*, contain L-tryptophan, glycine, L-asparagine, L-*allo*-isoleucine/L-valine, D-leucine, and L-leucine.[9] Their biosynthetic gene cluster was also recently reported along with a new analogue produced by heterologous expression.[10] However, the desotamides and nicrophorusamide A share only L-*allo*-isoleucine and D-leucine both in different positions in their hexapeptide sequences. Another series of cyclic hexapeptides, wollamides A and B, which were isolated from an Australian soil *Streptomyces*, are more similar to the desotamides rather than the nicrophorusamides; these antimycobacterial compounds contain D-ornithine in place of the glycine found in the destoamides.[11] The most closely related cyclic hexapeptide to nicrophorusamides (**1** and **2**) is longicatenamycin A, an antibacterial compound previously discovered from *Streptomyces diastaticus* strain S-520.[12] Longicatenamycin A is a cyclic hexapeptide containing 5-chlorotryptohan and ornithine as found in **1** and **2**, but with glycine, homohomoleucine, valine, and β-hydroxyglutamic acid replacing the valine, leucine, isoleucine, and β-hydroxyasparagine of the nicrophorusamide A. The absolute configuration of the amino acids in **1** is opposite that of the amino acids in longicatenamycin A, of which the structure was confirmed by total synthesis.[12b] Nicrophorusamide A (**1**) has the sequence L-D-D-L-L-D, whereas longicatenamycin A possesses the sequence D-NA-L-D-D-L.

The biological activities of the nicrophorusamides were evaluated against pathogenic bacterial strains (Table 2). Nicrophorusamide A (**1**) showed antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, and *Salmonella enterica* ATCC 14028 with minimum inhibitory concentration (MIC) values of 8–16 μg/mL. Nicrophorusamide B (**2**), which bears D-asparagine instead of D-*threo*-β-hydroxyasparagine, displayed 8 times lower activity against these bacteria than **1**, implying that D-*threo*-β-hydroxyasparagine could play a significant role in determining bioactivity. The structural importance of the hydroxy group in nicrophorusamide A (**1**) can be rationalized by the conformational difference between nicrophorusamides A and B. Careful analysis of the ROESY NMR spectra and density functional theory (DFT) modeling for the energy minimization of nicrophorusamide A (**1**) clearly reveals a ROESY correlation between the hydroxy proton (14-OH) of D-*threo*-β-hydroxyasparagine and the terminal amine

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Table 1. ^{1}H and ^{13}C NMR Data for Nicrophorusamide A and B (1 and 2) in DMSO-d_6

	1[a]				2[b]			
position	δ_C,	type	δ_H,	mult (J in Hz)	δ_C,	type	δ_H,	mult (J in Hz)
1	171.1,	C			170.8,	C		
2	53.5,	CH	4.62,	ddd (7.5, 7.5, 7.5)	53.4,	CH	4.51,	ddd (7.5, 7.5, 7.5)
2-NH			7.98,	d (7.5)			7.80,	d (7.5)
3a	26.7,	CH_2	3.11,	dd (14.5, 7.5)	26.5,	CH_2	3.18,	dd (14.5, 7.5)
3b			2.89,	dd (14.5, 7.5)			2.88,	dd (14.5, 7.5)
4	109.8,	C			109.8,	C		
5	125.4,	CH	7.15,	d (2.0)	125.7,	CH	7.18,	d (2.0)
5-NH			11.03,	d (2.0)			11.06,	d (2.0)
6	134.5,	C			134.5,	C		
7	112.7,	CH	7.32,	d (8.5)	112.8,	CH	7.32,	d (8.5)
8	120.7,	CH	7.05,	dd (8.5, 2.0)	120.7,	CH	7.04,	dd (8.5, 2.0)
9	122.9,	C			123.0,	C		
10	117.5,	CH	7.57,	d (2.0)	117.5,	CH	7.54,	d (2.0)
11	128.4,	C			128.4,	C		
12	169.4,	C			170.6,	C		
13	55.8,	CH	4.60,	dd (8.5, 2.5)	49.3,	CH	4.55,	dd (8.0, 6.0)
13-NH			7.62,	d (8.5)			7.95,	d (8.0)
14	70.8,	CH	4.47,	dd (6.0, 2.5)	36.0,	CH_2	2.56,	d (6.0)
14-OH			5.85,	d (6.0)				
15	173.2,	C			171.8,	C		
15-NH_2a			7.31,	d (2.0)				
15-NH_2b			7.28,	d (2.0)				
16	170.6,	C			170.5,	C		
17	53.1,	CH	4.01,	ddd (7.5, 7.5, 6.0)	52.3,	CH	4.19,	ddd (7.5, 7.5, 6.0)
17-NH			8.43,	d (6.0)			7.81,	d (7.5)
18a	26.8,	CH_2	1.84,	m	28.0,	CH_2	1.85,	m
18b			1.64,	m			1.60,	m
19	23.7,	CH_2	1.50,	m	23.8,	CH_2	1.52,	m
20	38.4,	CH_2	2.79,	br s	38.5,	CH_2	2.79,	m
20-NH_2			7.66,	br s			7.76,	t (7.0)
21	171.7,	C			171.2,	C		
22	56.5,	CH	4.24,	dd (7.5, 6.0)	57.1,	CH	4.11,	dd (7.5, 7.0)
22-NH			7.87,	d (7.5)			8.05,	d (7.0)
23	36.3,	CH	1.89,	m	35.9,	CH	1.92,	m
24a	25.6,	CH_2	1.35,	m	25.7,	CH_2	1.29,	m
24b			1.16,	m			1.19,	m
25	11.3,	CH_3	0.86,	d (7.5)	11.5,	CH_3	0.84,	d (7.0)
26	14.5,	CH_3	0.85,	d (7.5)	14.6,	CH_3	0.85,	d (7.0)
27	172.1,	C			172.8,	C		
28	51.5,	CH	4.23,	dd (7.5, 7.5)	51.4,	CH	4.43,	dd (7.0, 6.5)
28-NH			7.45,	d (7.5)			7.61,	d (7.0)
29	39.6,	CH_2	1.57,	dd (7.5, 7.5)	41.0,	CH_2	1.50,	m
30	24.3,	CH	1.47,	m	24.2,	CH	1.49,	m
31	22.7,	CH_3	0.87,	d (6.5)	22.5,	CH_3	0.90,	d (6.0)
32	21.8,	CH_3	0.83,	d (6.5)	22.6,	CH_3	0.85,	d (7.0)
33	170.4,	C			170.4,	C		
34	60.4,	CH	3.75,	dd (7.0, 7.0)	59.8,	CH	3.84,	dd (8.0, 7.5)
34-NH			7.81,	d (7.0)			7.92,	d (8.0)
35	29.1,	CH	1.84,	m	29.8,	CH	1.90,	m
36	18.5,	CH_3	0.72,	d (6.5)	19.0,	CH_3	0.70,	d (6.5)
37	18.8,	CH_3	0.69,	d (6.5)	18.1,	CH_3	0.64,	d (6.5)

[a] ^{1}H 600 MHz, ^{13}C 125 MHz. [b] ^{1}H 500 MHz, ^{13}C 125 MHz.

protons (20-NH_2) of L-ornithine, which enables an additional ring to form that is closed by a 14-O−20-NH hydrogen bond (Figure 3a). In contrast, nicrophorusamide B (**2**), which lacks the hydroxy group at C-14, is unable to make a rigid conformation with the corresponding hydrogen bond (Figure 3b).

Nicrophorusamides A and B did not inhibit the pathogenic fungi *Candida albicans* ATCC 10231, *Aspergillus fumigatus* HIC 6094, *Trichophyton rubrum* NBRC 9185, or *T. mentagrophytes* IFM 40996. In cytotoxicity tests against various human cancer cell lines, such as A-549, HCT-116, SNU-638, SK-HEP-1,

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Table 2. Inhibitory Activities of Nicrophorusamides A and B (1 and 2) against Bacterial Strains (MIC values in μg/mL)

strain	1	2	ampicillin
S. aureus	8	64	0.13
E. faecalis	16	128	0.5
E. faecium	16	128	0.25
S. enterica	16	not active	0.13
E. coli	not active	not active	8

MDA-MB-231, and K-562, nicrophorusamides A and B exhibited no significant cytotoxicity.

The rare actinomycete genus *Microbacterium* has not been the subject of many chemical investigations, with most such studies focusing on simple sugars.[13] The only peptide-derived metabolites isolated from this genus are cytotoxic microbacterins A and B from deep-sea-inhabiting *M. sediminis*.[14] The nicrophorusamides constitute the second example of peptide-derived metabolites from the rare actinomycete genus *Microbacterium*, reflecting the untapped chemical potential of this relatively unstudied actinomycete.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained at 25 °C using a JASCO P-2000 polarimeter with a 1 cm cell. UV spectra were obtained using a PerkinElmer LAMBDA 25 UV/vis spectrophotometer with a 1 cm cuvette. CD spectra were recorded at 25 °C using an Applied Photophysics Chirascan-Plus circular dichroism spectrometer and a 1 mm CD cell. Infrared (IR) spectral data were acquired by a JASCO FT/IR-4200 FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR experiments were conducted with Bruker Avance 500 and 600 MHz spectrometers. LC/MS data and LRESIMS data were obtained by an Agilent Technologies 1200 series high-performance LC (HPLC) coupled with an Agilent Technologies 6130 quadrupole MS. HRFABMS data were acquired on a JEOL JMS-700 high-resolution MS. The UV/vis spectrophotometer, NMR spectrometer, and HRFABMS were located at the National Center for Interuniversity Research Facilities (NCIRF) in Seoul National University.

Bacterial Isolation. Two identical carrion beetles were collected using pitfall traps from Maebong Mountain, Seoul, Republic of Korea, in July 2015. The carrion beetles were identified as *N. concolor* by morphological classification. The carrion beetle samples were soaked in 70% aqueous ethanol (EtOH/aq) for 1 min and rinsed with sterilized distilled water to eliminate residual EtOH. The guts of the carrion beetles were expelled with a sterilized surgical blade and diluted with 40 mL of sterilized water to extract the bacterial strains from the intestines. For bacterial isolation from the guts of the carrion beetles, the bacterial suspension was spread on various isolation agar media (actinomycetes isolation agar medium, YPM agar medium, YPG agar medium, A1 agar medium, K agar medium, Czapek-Dox agar medium, ISP1 agar medium, ISP4 agar medium, starch-casein agar medium, chitin-based agar medium, and R2A agar medium), and the plates were cultivated at 25 °C for more than 14 days. The actinobacterial strain UTG9, which produces nicrophorusamides A and B (**1** and **2**), was separated on starch-casein agar medium (10 g of soluble starch, 1 g of casein, 0.5 g of K_2HPO_4, 18 g of agar, and 100 mg of cycloheximide per 1 L of sterilized water). The actinomycete strain was identified as *Microbacterium* sp. (97% identity with *Microbacterium paraoxydans*) by analyzing its 16S rDNA sequence (GenBank accession number: MF000987).

Cultivation and Extraction. To obtain nicrophorusamides A and B (**1** and **2**) produced by the *Microbacterium* sp. UTG9 strain, a spore of the bacterial strain was transferred and inoculated into a 500 mL flask containing 125 mL of YPM liquid medium (2 g of yeast extract, 2 g of peptone, and 4 g of mannitol per 1 L of sterilized water) and cultivated on a rotary shaker at 200 rpm and 27 °C. After 2 days of fermentation, 10 mL of the liquid culture was moved to a 2.8 L large flask with 1 L of YPM liquid medium for scale-up and incubated on a rotary shaker at 160 rpm and 27 °C for 6 days. The entire culture (72 L) was extracted with an equivalent volume of ethyl acetate (EtOAc) in a separation funnel. The EtOAc layer was segregated from the water layer and dried by a rotary evaporator *in vacuo* to acquire 10 g of the crude extract.

Purification of Nicrophorusamides A and B (1 and 2). The entire extraction was absorbed on Celite and loaded onto 20 g of C_{18} resin for fractionation. Then, 200 mL aliquots of 20%, 40%, 60%, 80%, and 100% aqueous methanol (MeOH/aq) were collected, and a portion of each fraction was analyzed by LC/MS under a gradient solvent system (flow rate: 0.7 mL/min; UV detection: 210, 230, 254, 280, and 360 nm; 10% to 100% aqueous acetonitrile [CH_3CN/H_2O] with 0.1% formic acid over 20 min) with a Phenomenex column (Luna, 100 × 4.6 mm, C_{18}, 5 μm). Analyzing the LC/MS data confirmed that nicrophorusamides A and B (**1** and **2**) were present in the 80% and 100% MeOH/aq fractions. To acquire the nicrophorusamides, the fractions were purified by semipreparative reversed-phase HPLC with a YMC column (250 × 10 mm, C_{18}, 5 μm) under gradient solvent conditions (flow rate: 2 mL/min; UV detection: 280 nm; 35% to 62% CH_3CN/H_2O with 0.2% trifluoroacetic acid over 50 min). Nicrophorusamides A (**1**, 12 mg) and B (**2**) eluted at 28.0 and 28.5 min, respectively. Nicrophorusamide B (**2**) was purified again with another gradient solvent system (flow rate: 2 mL/min; UV detection: 280 nm; 30% to 45% CH_3CN/H_2O with 0.2% trifluoro-

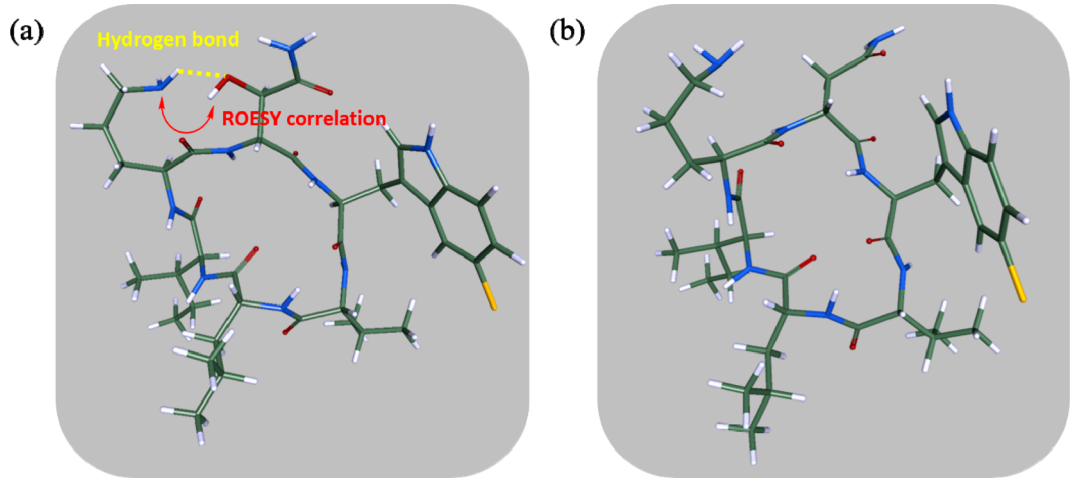

Figure 3. Energy-minimized conformations of nicrophorusamides A and B: (a) nicrophorusamide A (**1**), (b) nicrophorusamide B (**2**).

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acetic acid over 50 min) for refinement. Finally, pure nicrophorusamide B (**2**, 4 mg) was obtained at 31.0 min after injection.

*Nicrophorusamide A (**1**):* white powder; $[\alpha]_D^{25}$ −18 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.85), 290 (3.02) nm; IR (neat) ν_{max} 3693, 3262, 2923, 1679, 1633, 1540, 1206, 1140 cm^{-1}; for ^1H and ^{13}C NMR spectral data, Table 1; HRFABMS $[M + H]^+$ *m*/*z* 790.4022 (calcd for $C_{37}H_{57}ClN_9O_8$, 790.4019).

*Nicrophorusamide B (**2**):* white powder; $[\alpha]_D^{25}$ −20 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.79), 290 (2.97) nm; IR (neat) ν_{max} 3413, 2926, 1682, 1634, 1543, 1207, 1138 cm^{-1}; for ^1H and ^{13}C NMR spectral data, Table 1; HRFABMS $[M + H]^+$ *m*/*z* 774.4066 (calcd for $C_{37}H_{57}ClN_9O_7$, 774.4069).

Determination of the Absolute Configurations at the α-Carbons of the Amino Acid Units in Nicrophorusamide A (1). One milligram of **1** was dissolved in 1 mL of 6 N HCl and heated at 115 °C for 1 h. The heated suspension was cooled in a 0 °C ice bath for 5 min, and the HCl was vaporized *in vacuo*. To eliminate residual HCl in the vial, 1 mL of water was added to the vial and then evaporated under low pressure three times. Subsequently, the hydrolysate mixture was lyophilized for 24 h and divided into two vials. Each hydrolysate sample was dissolved in 200 μL of 1 N $NaHCO_3$. Then, 100 μL of 10 mg/mL L-FDAA in acetone was added to one of the two vials, and an equivalent proportion of D-FDAA in acetone was added to the other vial. The two reaction vials were heated at 80 °C for 3 min to accelerate the chemical derivatization. Then, 100 μL of 2 N HCl was added to neutralize both vials, and the two reaction mixtures were diluted with 300 μL of 50% CH_3CN/H_2O solution. Ten microliters of each reaction mixture was injected into the LC/MS under a gradient solvent system (flow rate: 0.7 mL/min; UV detection: 360 nm; 10% to 60% CH_3CN/H_2O with 0.1% formic acid over 40 min) with a Phenomenex column (Luna, 100 × 4.6 mm, C_{18}, 5 μm) (Table S1). The retention times of the FDAA derivatives of the free amino acids were observed via LC/MS analysis.

Determination of the Absolute Configuration at the β-Carbon of D-β-Hydroxyasparagine in Nicrophorusamide A (1). Of the four diastereomers of β-hydroxyaspartic acid, only L-*threo*-β-hydroxyaspartic acid [(2*S*,3*R*)-2-amino-3-hydroxysuccinic acid, CAS No. 7298-99-9, Tocris] is commercially available. L-*erythro*-β-Hydroxyaspartic acid [(2*S*,3*S*)-2-amino-3-hydroxysuccinic acid] was synthesized via several reaction steps as described in the Supporting Information. These two synthetic amino acids were derivatized with L-FDAA and injected into LC/MS under a gradient solvent system (flow rate: 0.7 mL/min; UV detection: 360 nm; 10% to 30% CH_3CN/H_2O in water solution with 0.1% formic acid over 40 min) to compare retention times. The retention times of the L-FDAA derivatives of L-*threo*-β-hydroxyaspartic acid (2*S*,3*S*-L) and L-*erythro*-β-hydroxyaspartic acid (2*S*,3*R*-L) were 19.3 and 26.8 min, respectively. To elucidate the absolute configuration at the β-carbon of D-β-hydroxyasparagine in **1**, these L-FDAA derivatives were co-injected with the D-FDAA derivative of D-β-hydroxyaspartic acid in the hydrolysate of **1**. The LC/MS results revealed that the D-FDAA derivative of D-β-hydroxyaspartic acid from the hydrolysate of **1** showed the identical retention time with the L-FDAA derivative of authentic L-*threo*-β-hydroxyaspartic acid (Figure S19). This result demonstrated that the D-FDAA derivative of D-β-hydroxyaspartic acid (2*R*-2-amino-3-hydroxysuccinic acid) from the hydrolysate of **1** is an enantiomer of the L-FDAA derivative of authentic L-*threo*-β-hydroxyaspartic acid, thus possessing a 3*R* configuration. Therefore, D-β-hydroxyasparagine in nicrophorusamide A (**1**) was elucidated as D-*threo*-β-hydroxyasparagine.

Determination of the Absolute Configuration of the β-Carbon of L-Isoleucine in Nicrophorusamide A (1). The hydrolysate of **1** was obtained by the same procedures described above and dissolved in 1 mL of water. For the chemical derivatization of the L-isoleucine residue in **1**, 100 μL of triethylamine and 100 μL of 1% GITC solution in acetone were added to the reaction vial. The reaction was processed at room temperature (25 °C) for 15 min and quenched with 100 μL of 5% acetic acid solution. Then, 20 μL of the reaction mixture was analyzed by HPLC under a gradient solvent system (flow rate: 0.3 mL/min; UV detection: 254 nm; 35% to 50% CH_3CN in water with 0.1% formic acid over 80 min) with a Phenomenex column (Gemini, 250 × 4.6 mm, C_{18}, 5 μm). The GITC derivative eluted at 60.1 min. GITC derivatives of authentic L-isoleucine (CAS No. 73-32-5, Sigma-Aldrich) and L-*allo*-isoleucine (CAS No. 1509-34-8, Sigma) were prepared via the same procedure and injected into the HPLC using the same analysis conditions. The GITC derivatives of authentic L-isoleucine and L-*allo*-isoleucine eluted at 60.7 and 60.0 min, respectively. Finally, the amino acid residue in **1** was identified as L-*allo*-isoleucine.

Molecular Modeling of Nicrophorusamides A and B (1 and 2). To identify the conformational difference between nicrophorusamides A and B, computational DFT calculations were performed. The initial structural energy minimizations of **1** and **2** were established by using Avogadro 1.2.0 with the UFF force field.[15,16] An array of energy optimizations were processed via Tmolex 4.3.1 with the DFT settings (B3-LYP functional/M3 grid size) and geometry optimization options (energy 10^{-6} hartree, gradient norm $|dE/dxyz| = 10^{-3}$ hartree/bohr).[17]

Antibacterial Activity Assay. The antibacterial activities of nicrophorusamides A and B (**1** and **2**) were determined by evaluating their MIC values; ampicillin was used as a reference compound. Three species of Gram-positive bacteria (*S. aureus* ATCC 25923, *E. faecalis* ATCC 19433, and *E. faecium* ATCC 19434) and two species of Gram-negative bacteria (*S. enterica* ATCC 14028 and *E. coli* ATCC 25922) were used in this study. The bacteria were grown overnight in Mueller Hinton broth (MHB) at 37 °C, harvested by centrifugation, and washed twice with sterile distilled water. Each compound was dissolved in DMSO and diluted with MBH to prepare serial 2-fold dilutions of 128 to 0.03 μg/mL. The final DMSO concentration was maintained at 1% by adding DMSO to the medium. In each well of a 96-well plate, 190 μL of MBH containing **1**, **2**, and ampicillin was mixed with 10 μL of broth containing approximately 10^7 colony-forming units (cfu)/mL of the test bacteria (final: 5×10^5 cfu/mL). The plates were incubated for 24 h at 37 °C, and the MIC value was defined as the lowest concentration of the test compound that inhibited bacterial growth.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00506.

1D and 2D NMR spectra, CD spectra, HRMS data, energy-minimized coordinates for **1** and **2**, synthesis of L-β-hydroxyaspartic acid, detailed procedure for determination of absolute configurations of amino acid units (PDF)

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Notes

The authors declare no competing financial interest.

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