




A New Alkaloid from Marine-Derived Actinomycete *Actinoalloteichus cyanogriseus* G631

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Abstract: A new alkaloid, isocyanogranamide (**1**) together with six known compounds, including cyanogranamide (**2**), marinacarboline F (**3**), marinacarboline H (**4**), caerulomycinamide (**5**), cerulomyconitrile (**6**), and 5'-deoxyuridine (**7**) were isolated from the agar culture of the marine-derived actinomycete *Actinoalloteichus cyanogriseus* G631. Compounds **1-7** were evaluated for antimicrobial activity against Gram (+) bacteria, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Bacillus cereus*, Gram(-) bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and the yeast, *Candida albicans*. All compounds significantly exhibited antibacterial effects on three tested Gram-(+) bacteria and the yeast *C. albicans* with the MIC values ranging from 64 to 256 µg/mL. Compounds **4**, **5**, and **7** significantly inhibited *B. cereus* bacteria with MIC values of 16, 128, and 128 µg/mL, respectively.

Keywords: *Actinoalloteichus cyanogriseus*; marine actinomycetes; isocyanogranamide; alkaloid; antibacterial activity; antifungal activity. © 2023 ACG Publications. All rights reserved.

1. Introduction

Actinoalloteichus genus belongs to the family Pseudonocardaceae [1-3]. *Actinoalloteichus* species are also known as a producer of different types of secondary metabolites, including polycyclic tetramate macrolactams [4], polyene macrolactams [5], pyridine alkaloids [6, 7], and cyclopentenone

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derivatives [8]. As part of our project on investigating natural products that possess antimicrobial effects from marine-derived actinomycetes, the crude extract of *Actinoalloteichus cyanogriseus* G631 fermentation (isolated from the soft coral sample (*Sarcophyton infundibuliforme* Tixier-Durivault, 1958)) was found to exhibit antimicrobial effects against three Gram-(+) bacteria (*E. faecalis*, *S. aureus*, and *B. cereus*), one Gram-(−) bacteria (*S. enterica*), and one yeast (*C. albicans*) with MIC values of 64, 2, 16, 256, and 2 µg/mL respectively. Thus, chemical components of *A. cyanogriseus* G631 fermentation have been evaluated. Herein, we report the isolation, structural elucidation, and antimicrobial activity of one new and six known alkaloid compounds from the fermentation of the actinomycete strain *A. cyanogriseus* G631.

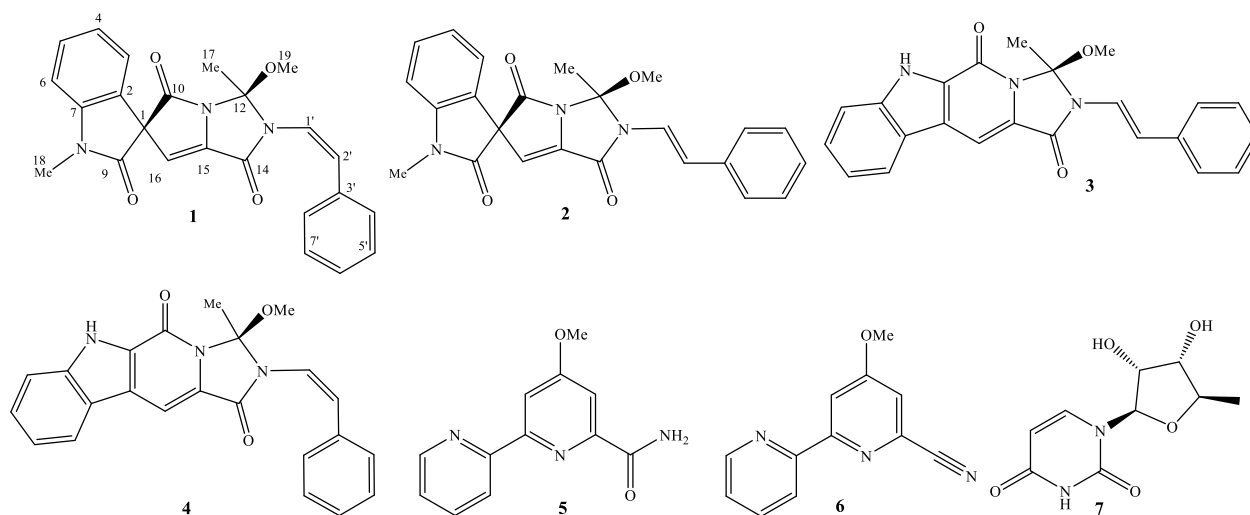


Figure 1. Chemical structures of compounds 1-7

2. Materials and Methods

2.1. General Experimental Procedures and Fermentation

Detailed information of experimental procedure were given in supporting information file of the article.

2.2. Isolation and Taxonomy Identification

Actinoalloteichus cyanogriseus G631 strain was isolated from a soft coral (*Sarcophyton infundibuliforme* Tixier-Durivault, 1958), collected at a depth of 8 meters at Phu Quy, Binh Thuan, Vietnam in April 2021. The soft coral was identified by Prof. Do Cong Thung, Institute of Marine Environment and Resources, VAST. *A. cyanogriseus* G631 were identified according to its morphological characteristics and 16S rRNA gene sequences (GenBank accession number OM190414).

2.3. Extraction and Isolation

The agar culture of *A. cyanogriseus* G631 (30 L) was minced and then extracted with ethyl acetate (EtOAc) (4 times, each 5 L) using a sonicator. The EtOAc layer was concentrated to give an EtOAc extract (EG631, 5.6 g). The EG631 extract was chromatographed on a silica gel chromatography column (CC) eluting with gradient solvents of *n*-hexane:CH₂Cl₂ (2:1, 1:1, 1:0, v/v) and then CH₂Cl₂:MeOH (40:1, 20:1, 10:1, 5:1, v/v) to afford seven fractions (F1-F7). F5 fraction (508 mg) was subjected to a Sephadex LH-20 column chromatography eluting with MeOH to give five fractions (F5.1-F5.7). F5.2 fraction was chromatographed on a silica gel CC using eluent solvent of *n*-

hexane:acetone (5:1, v/v) to yield **6** (3.5 mg). The F5.3 fraction was purified on an HPLC (J'sphere H-80 column, 250 mm length \times 20 mm ID, a flow rate of 3 mL/min eluting with 90 % MeOH in water) to yield **1** (2.0 mg, t_R = 32 min) and **2** (15 mg, t_R = 27 min). The F5.5 fraction was separated on an HPLC eluting with 90 % MeOH in water to yield **3** (3.0 mg, t_R = 31 min) and **4** (2.5 mg, t_R = 36 min). F6 fraction was subjected to a Sephadex LH-20 CC eluting with MeOH and then followed by a silica gel CC (CH₂Cl₂: acetone, 10:1, v/v) to yield compound **5** (4.0 mg). Compound **7** (4.5 mg) was obtained from the F7 fraction by purifying on a silica gel CC eluting with CH₂Cl₂:MeOH (5:1, v/v).

Isocyanogranamide (1): Yellow oil, $[\alpha]_D^{25}$ -59.3 (c 0.2, CH₂Cl₂); CD: λ_{\max} ($\Delta\epsilon$, rel) 221 (-0.58), 238 (+0.55), 259 (-1.00), 278 (+0.21), and 309 (-0.27) nm; HR-ESI-MS m/z : 416.1610 [M+H]⁺ (calcd. for [C₂₄H₂₂N₃O₄]⁺, 416.1605); ¹H-NMR and ¹³C-NMR, see Table 1.

2.4. Antimicrobial Assays

Antimicrobial activity studies were carried out in accordance with the method given in the literature. In order to avoid unnecessary repetition in the main text, the details are given in the supporting information file of this article in accordance with the relevant literature [9-11].

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as a yellow oil. The HR-ESI-MS of **1** showed a pseudo-molecular ion peak [M+H]⁺ at m/z 416.1610, confirming the molecular formula of C₂₄H₂₁N₃O₄. The ¹H-NMR spectrum of **1** showed proton signals of one mono-substituted benzene ring at δ_H 7.28 (1H, d, J = 7.2 Hz, H-6'), 7.32 (2H, d, J = 7.2 Hz, H-4'/H-8'), and 7.37 (2H, t, J = 7.2 Hz, H-5'/H-7'), one *ortho*-disubstituted benzene ring at δ_H 6.93 (1H, d, J = 7.8 Hz, H-6), 7.11 (1H, dd, J = 7.2, 7.8 Hz, H-4), 7.13 (1H, d, J = 7.8 Hz, H-3), and 7.40 (1H, dd, J = 7.2, 7.8 Hz, H-5), two olefinic protons at δ_H 5.86 (1H, d, J = 9.6 Hz, H-1') and 6.65 (1H, d, J = 9.6 Hz, H-2'), suggested the presence of a double bond, three methyl groups at δ_H 1.95, 3.27, and 3.31 (each 3H, s). The ¹³C-NMR and HSQC spectra of **1** exhibited signals of 24 carbons, including three carbonyls at δ_C 154.3, 167.5, and 170.0, six-non-protonated carbons at δ_C 70.2, 98.6, 124.4, 135.0, 140.2, and 144.9, twelve olefinic methines at δ_C 106.4, 109.2, 115.9, 123.5, 124.3, 128.3, 128.4 \times 2, 128.5 \times 2, 130.3, and 130.8, and three methyl carbons at δ_C 23.8,

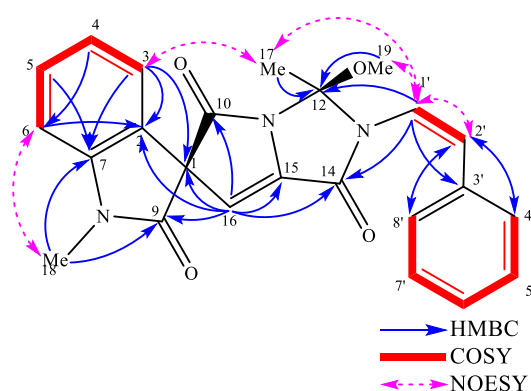


Figure 2. The key HMBC, COSY, and NOE correlations of **1**

27.1, and 50.2 (Table 1). Analysis of ¹H- and ¹³C-NMR data of **1** indicated its structure was similar to that of cyanogranamide (**2**), a compound that has been isolated from the same species, *Actinoalloteichus cyanogriseus* [12]. The HMBC correlations between H-16 (δ_H 5.84) and C-1 (δ_C 70.2)/C-2 (δ_C 124.4)/C-10 (δ_C 167.5)/C-14 (δ_C 154.3)/C-15 (δ_C 140.2) and between *N*-methyl protons (δ_H 3.27) and C-7 (δ_C 144.9)/C-9 (δ_C 170.0) proved the presence of spiro rings at C-1 and carbonyl groups at C-9

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and C-10 (Figure 2). The *ortho*-disubstituted benzene ring at C-2/C-7 was confirmed by HMBC correlations between H-3 (δ_{H} 7.14) and C-1 (δ_{C} 70.2)/C-2 (δ_{C} 124.4)/C-7 (δ_{C} 144.9), between H-6 (δ_{H} 6.93) and C-2 (δ_{C} 124.4)/C-7 (δ_{C} 144.9) as well as the COSY correlations of H-3 (δ_{H} 7.14)/H-4 (δ_{H} 7.11)/H-5 (δ_{H} 7.40)/H-6 (δ_{H} 6.93). The presence of phenyl ethenyl was confirmed by HMBC correlations between H-2' (δ_{H} 6.65) and C-1' (δ_{C} 115.9)/C-3' (δ_{C} 135.0)/C-4' (8') (δ_{C} 128.4) and COSY correlations of H-4' (δ_{H} 7.32)/H-5' (δ_{H} 7.37)/H-6' (δ_{H} 7.28)/H-7' (δ_{H} 7.37)/H-8' (δ_{H} 7.32) and H-1' (δ_{H} 5.86)/H-2' (δ_{H} 6.65). In addition, the coupling constant between H-1' and H-2', $J = 9.6$ Hz and also NOESY correlations between H-1' (δ_{H} 5.86) and H-2' (δ_{H} 6.65) suggested the geometry of the double bond of C-1' and C-2' is *Z* [11]. The position of this group at *N-13* of tetrahydroimidazole was confirmed by HMBC correlations from H-1' (δ_{H} 5.86) and C-12 (δ_{C} 98.6)/C-14 (δ_{C} 154.3). Both methyl and methoxy groups at C-12 was proved by HMBC correlations from H-17 (δ_{H} 1.95) and methoxy proton (δ_{C} 3.31) to C-12 (δ_{C} 98.6). Compound **1** has two stereogenic centers, C-1 and C-12. Thus, the experimental ECD has been recorded and shown the negative Cotton effects (CEs) 221, 259 and 309 nm and positive CEs at 238 and 278 nm (Figure 3), similar to that of cyanogramide (**2**) [13], suggesting the absolute configurations of two stereogenic centers at C-1 and C-12 to be *R* and *S*, respectively. Consequently, new structure of compound **1** was elucidated as spirocyclic pyrrolo[1,2-*c*]imidazole, named isocyanogramide.

Table 1. NMR Data for Compounds **1** and **2**, and reference compound

C	1		2		$\delta_{\text{C}}^{\text{b}}$
	$\delta_{\text{C}}^{\text{a}}$	$\delta_{\text{H}}^{\text{a}}$ mult. (<i>J</i> in Hz)	$\delta_{\text{C}}^{\text{a}}$	$\delta_{\text{H}}^{\text{a}}$ mult. (<i>J</i> in Hz)	
1	70.2	-	70.1	-	69.8
2	124.4	-	124.4	-	124.6
3	124.3	7.14 (1H, d, <i>J</i> = 7.8 Hz)	124.2	7.10 (1H, d, <i>J</i> = 7.5 Hz)	124.5
4	123.5	7.11 (1H, dd, <i>J</i> = 7.2, 7.8 Hz)	123.5	7.11(1H)*	123.1
5	130.3	7.40 (1H, dd, <i>J</i> = 7.2, 7.8 Hz)	130.3	7.40 (1H, dd, <i>J</i> = 7.5, 7.5 Hz)	130.1
6	109.2	6.93 (1H, d, <i>J</i> = 7.8 Hz)	109.2	6.93 (1H, d, <i>J</i> = 7.5 Hz)	109.6
7	144.9	-	144.9	-	144.8
9	170.0	-	169.8	-	169.5
10	167.5	-	167.4	-	167.4
12	98.6	-	99.1	-	98.7
14	154.3	-	154.6	-	154.6
15	140.2	-	139.3	-	138.6
16	106.4	5.84 (1H, s)	106.4	5.90 (1H, s)	107.2
17	23.8	1.95 (3H, s)	23.0	2.10 (3H, s)	21.8
18	27.1	3.27 (3H, s)	27.2	3.27 (3H, s)	26.9
19	50.2	3.31 (3H, s)	50.4	3.36 (3H, s)	49.4
1'	115.9	5.86 (1H, d, <i>J</i> = 9.6 Hz)	118.5	7.22 (1H, d, <i>J</i> = 15.0 Hz)	118.9
2'	130.8	6.65 (1H, d, <i>J</i> = 9.6 Hz)	118.7	6.96 (1H, d, <i>J</i> = 15.0 Hz)	118.0
3'	135.0	-	135.5	-	135.5
4', 8'	128.4	7.32 (2H, d, <i>J</i> = 7.2 Hz)	126.1	7.42 (1H, d, <i>J</i> = 7.5 Hz)	126.1
5', 7'	128.5	7.37 (2H, t, <i>J</i> = 7.2 Hz)	128.8	7.35 (1H, t, <i>J</i> = 7.5 Hz)	128.8
6'	128.3	7.28 (1H, t, <i>J</i> = 7.2 Hz)	127.8	7.27 (1H, t, <i>J</i> = 7.5 Hz)	127.6

^a)recorded in CDCl₃, ^b) δ_{C} of cyanogramide recorded in DMSO-d₆ [10], *overlapped signals.

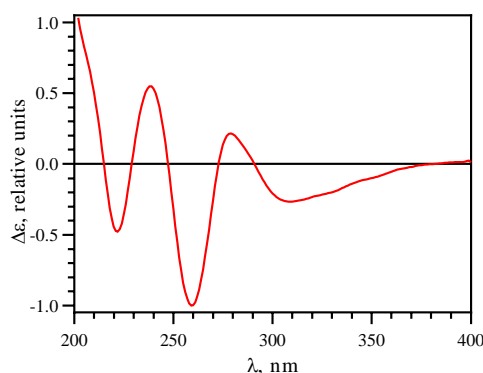


Figure 3. Experimental CD spectrum 1

The known compounds were determined to be cyanogranamide (**2**) [12], marinacarboline F (**3**) [14], marinacarboline H (**4**) [14], caerulomycinamide (**5**) [6], cerulomyconitrile (**6**) [6], and 5'-deoxyuridine (**7**) [14]. Their chemical structures were elucidated by their spectroscopic data and comparing with those reported NMR data in the literature data.

3.2 Antibacterial and Antifungal Activities

All compounds were evaluated for their antibacterial and antifungal activities against Gram-(+) and Gram(-) bacteria and yeast, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus cereus*, and *Candida albicans* (Table 2). Streptomycin and cycloheximide were used as positive controls. All compounds exhibited significant antibacterial effects on Gram-(+) bacteria, *E. coli*, *S. aureus* and the yeast *C. albicans* with MIC values ranging from 64 to 256 $\mu\text{g/mL}$. Compounds **4**, **5**, and **7** significantly inhibited *B. cereus* bacteria with MIC values of 16, 128, and 128 $\mu\text{g/mL}$, respectively. In addition, compounds **2** and **4** also showed potent inhibitory activity against the Gram(-) bacteria *P. aeruginosa* with a MIC value of 64 $\mu\text{g/mL}$. This is the first report of the antimicrobial activity of isolated compounds. These compounds may be potential antibacterial sources against Gram-(+) bacteria.

Table 2. Antibacterial and antifungal activities of compounds 1-7

Compounds	MIC ($\mu\text{g/mL}$)						
	Gram-(+)			Gram(-)			Yeast
	<i>E. faecalis</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>C. albicans</i>
1	128	64	-	-	-	-	128
2	128	128	-	32	64	-	32
3	64	128	-	256	-	-	64
4	64	128	16	-	64	-	128
5	128	64	128	-	-	-	32
6	128	128	-	-	-	-	128
7	64	256	128	-	-	-	64
Streptomycin	256	256	128	32	256	128	
Cyclohexamide							32

(-) compound did not exhibit antimicrobial and antifungal activities (MIC >256 $\mu\text{g/mL}$)

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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