



Chlorinated *bis*-indole alkaloids from deep-sea derived *Streptomyces* sp. SCSIO 11791 with antibacterial and cytotoxic activities

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Abstract

Two new chlorinated *bis*-indole alkaloids, dionemycin (**1**) and 6-OMe-7',7''-dichlorochromopyrrolic acid (**2**), along with seven known analogs **3–9**, were isolated from the deep-sea derived *Streptomyces* sp. SCSIO 11791. Their structures were elucidated by extensive HRESIMS, and 1D and 2D NMR data analysis. In vitro antibacterial and cytotoxic assays revealed that, compound **1**, shows anti-staphylococcal activity with an MIC range of 1–2 µg/mL against six clinic strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from human and pig. Additionally, compound **1** displayed cytotoxic activity against human cancer cell lines NCI-H460, MDA-MB-231, HCT-116, HepG2, and noncancerous MCF10A with an IC₅₀ range of 3.1–11.2 µM. Analysis of the structure-activity relationship reveals that the chlorine atom at C-6'' could be pivotal for conferring their bioactivities, thus providing hints on chemical modifications on *bis*-indole alkaloid scaffold in drug design.

Introduction

bis-Indole natural products are structurally defined by two monomeric indole alkaloids that contribute to their diverse biological activities. Compounds in the *bis*-indole family are

Dedicated to Professor William Fenical in recognition of his contributions to marine derived secondary metabolites.

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ubiquitously distributed in plants [1] and microorganisms [2]. Since the early 1960s, this class of compounds has been successfully utilized in combination therapies for cancer [3, 4]. The first anti-cancer *bis*-indole alkaloids being introduced into clinical use were vinblastine and vincristine. Thereafter, subsequent progress has inspired enduring interest in the discovery of new *bis*-indole alkaloids from natural sources [5, 6]. Indolocarbazoles are a type of *bis*-indoles that possess inhibitory activities against protein kinases or DNA topoisomerases, as exemplified by K-252a to d [7], rebeccamycin [8], and staurosporine [9]. Moreover, compounds similarly classified in this class such as UCN-01 and BMY-27557 had been explored in clinical trials for cancer chemotherapy [10, 11]. Marine-derived *bis*-indole compounds typically contain halogen atoms in their structures. Such halogenated *bis*-indole alkaloids generally display potent cytotoxic and/or antibacterial activities, and are thus considered as promising anti-cancer or antibacterial leads [12]. The marine-derived brominated *bis*-indole alkaloids BrBIN is a potential inducer apoptosis in human cancer cells [13, 14]. A series of marine-derived chlorinated *bis*-indoles was shown to inhibit methicillin-resistant *Staphylococcus aureus* (MRSA) pyruvate kinase significantly, with their halogenated indole ring being implicated as a critical pharmacophore [15].

Marine-derived *Actinomycetes* have been valued as an important reservoir of drug resources of interest to the treatment of various cancers and multi-drug resistant

bacteria in clinic [16]. Previously, the cytotoxic properties of the chlorinated bisindole alkaloids spiroindimicins A–D [17] and indimicins A–E [18] were reported from a marine-derived *Actinomycetes*, *Streptomyces* sp. SCSIO 03032. As part of our effort to identify bioactive natural products, the deep-sea derived strain *Streptomyces* sp. SCSIO 11791 was subjected to intensive screening. Chemical investigation on this strain led to identification of nine chlorinated *bis*-indole alkaloids (**1–9**), including two new compounds dionemycin (**1**) and 6-OMe-7',7''-dichlorochromopyrrolic acid (**2**) (Fig. 1). Herein, we describe the isolation, structure elucidation, cytotoxic and antibacterial activities of these compounds, and the discussion of their structure-activity relationship (SAR).

Results and discussion

Isolation and structure elucidation

The strain SCSIO 11791 was isolated from a sediment sample collected from the South China Sea. It was fermented scale-up to 21 L. The subsequent extraction, extensive isolation by silica gel chromatography, medium pressure liquid chromatography (MPLC) and HPLC purification led to the isolation of compounds **1–9**. Compound **3**, as a symmetric structure, was identified as an inhibitor of protein kinase C in a German patent [19]. Compounds **4–6**, **8**, and **9** were identified as lynamincins A (**4**), B (**5**) and D (**6**) [20], dichlorochromopyrrolic acid (**8**) [21], and spiroindimicin B (**9**) [17], respectively. Compound **7** was reported in a PCT patent [22] (Fig. 1).

Compound **1** was isolated as an orange amorphous powder. Its molecular formula ($C_{20}H_{10}Cl_3N_3O_2$) was determined upon analysis of the HRESIMS peak at m/z 429.9912 $[M + H]^+$, requiring 16 degrees of unsaturation. The isotope pattern was consistent with the presence of

three chlorines. 1H NMR spectra showed seven aromatic hydrogens, while ^{13}C NMR spectra showed twenty carbon signals from δ_C 107.0 to δ_C 174.5 (Table 1). Detailed analysis on 2D NMR spectra confirmed the presence of twenty aromatic carbon signals, including seven methine carbons and thirteen non-hydrogen bearing carbons. HMBC correlations from H-2' (δ_H 7.84, s) to C-3', C-3a', and C-7a', from H-4' to C-5', C-6', C-7a', and C-3', from H-6' to C-5', C-4', and C-7a', and from H-7' to C-3a' and C-5' determined the presence of the indole moiety (A). HMBC correlations from H-4', H-6', and H-7' to the carbon C-5', as well as the coupling constants of H-4' (d, $J = 2.0$ Hz), H-6' (dd, $J = 8.5, 2.0$ Hz), and H-7' (d, $J = 8.5$ Hz) in 1H NMR data revealed the presence of a 5'-substituted indole. Similarly, another indole moiety (B) was elucidated according to the HMBC correlations. However, the ABX system (H-4', 6', and 7') in moiety-A was replaced by two aromatic singlet signals at δ_H 6.81 (H-4'') and 7.53 (H-7''), indicating that H-6'' was replaced by one chlorine atom in moiety-B. In addition, HMBC correlations from H-2' to C-3 and the carbonyl at C-2, and from H-2'' to C-4 and the carbonyl at C-5 were observed. Thus, a 3,4-*bis*-indole-pyrrole-2,5-dione moiety was established. Accordingly, the two remaining chlorine atoms were placed at C-5' and C-5'' in indole rings. Therefore, the structure of compound **1** was proposed as that in Fig. 2, and named dionemycin.

Compound **2** was purified as a pale-yellow amorphous powder. Its molecular formula ($C_{23}H_{15}Cl_2N_3O_4$) was acquired by the HRESIMS peak at m/z 468.0502 $[M + H]^+$, which contained 17 unsaturated degrees, and the isotope pattern confirmed the presence of two chlorines. 1D and 2D NMR spectra revealed two carbonyl carbons (C-6, 8), twelve aromatic non-hydrogen bearing carbons, eight aromatic methines, and one methoxy (-OCH₃(C-7)) groups. Detailed comparison showed that 1H and ^{13}C NMR data were similar to those *bis*-indole compounds **6** [20] and 7',7''-dichlorochromopyrrolic acid (CCA) [23] (Table S1). Coupling

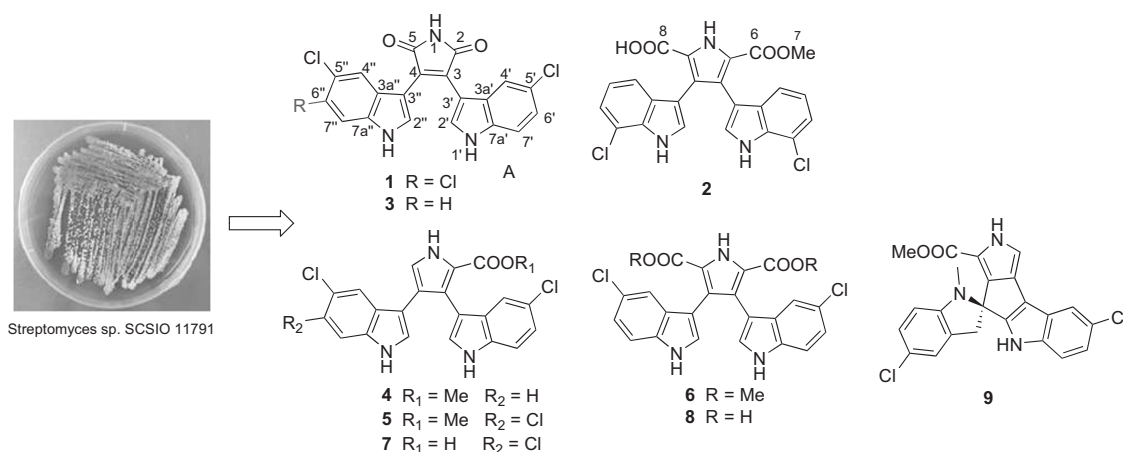
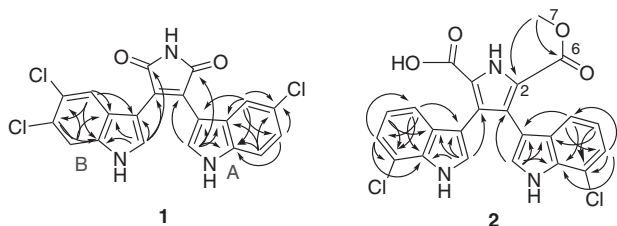


Fig. 1 Structures of chlorinated *bis*-indole alkaloids **1–9** from *Streptomyces* sp. SCSIO 11791

Table 1 ^1H (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of compounds **1** and **2** in CD_3OD

Pos.	Compound 1		Compound 2	
	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)
2	174.5		123.5	
3	129.7		125.8	
4	128.6		126.2	
5	174.5		125.2	
6			162.8	
7			51.9	3.71, s
8			164.0	
2'	131.4	7.84, s	127.7	6.98, s
3'	107.0		109.4	
3a'	126.5		130.1	
4'	121.6	6.67, d (2.0)	120.2	7.04, d (8.0)
5'	128.0		113.1	7.20, t (8.0)
6'	123.4	6.98, dd (8.5, 2.0)	121.9	6.94, dd (8.0, 2.0)
7'	113.8	7.35, d (8.5)	125.5	
7a'	136.2		135.7	
2''	132.1	7.85, s	127.7	6.96, s
3''	107.2		109.4	
3a''	124.4		130.1	
4''	123.4	6.81, s	120.1	7.04, d (8.0)
5''	126.7		113.1	7.19, t (8.0)
6''	126.9		122.0	6.92, dd (8.0, 2.0)
7''	114.0	7.53, s	125.5	
7a''	136.6		135.7	

**Fig. 2** Selected HMBC (arrows) correlations of compounds **1** and **2**

constants of H-4'/H-4'' (dd, $J = 8.0$ Hz), H-5'/H-5'' (t, $J = 8.0$ Hz), and H-6'/H-6'' (dd, $J = 8.0, 2.0$ Hz) in ^1H NMR data indicated the presence of two 1,2,3-trisubstituted benzene rings in **2**. The position of the methoxy group at C-6 (-OCH₃ (C-7), δ_{H} 3.71, 3 H, s; δ_{C} 51.9) was determined by HMBC correlation of H₃-7 to C-6. Moreover, HMBC correlations (Fig. 2), especially with respect to H-4'/4'' to C-3'/3'', H-5'/5'' to C-3a'/3a'', C-7'/7'' to C-4'/4'' and C-7a'/7a'' confirmed that the two chlorine atoms were attached to C-7' and C-7'' in the two indole rings, respectively. The carboxy group was placed at C-5 as the only leaving substitution position. Therefore, the structure of compound **2** was finally elucidated as 6-OMe-7',7''-dichlorochromopyrrolic acid.

Table 2 Cytotoxicities of five compounds **1**, **2**, **5**, **7**, **9** against a panel of human cancer and noncancerous cell lines (IC₅₀ value, μM)

	1	2	5	7	9	EPI ^a
MDA-MB-435	3.9	19.4	6.9	13.8	3.0	0.3
MDA-MB-231	11.2	>25.0	28.6	30.4	12.2	0.9
NCI-H460	3.6	>25.0	12.2	21.9	7.0	0.02
HCT-116	4.3	13.1	8.7	8.8	2.2	0.4
HepG2	8.2	18.5	11.6	27.7	6.7	0.1
MCF10A	3.1	2.9	1.9	3.1	3.4	0.1

^aEPI (epirubicin) was used as a positive control

Cytotoxic activities

Compounds **1–9** were tested for cytotoxicity against MDA-MB-435 human breast cancer cells across a concentration gradient of up to 50 μM . Compounds **1**, **2**, **5**, **7**, **9** showed moderate cytotoxicity with IC₅₀ values of 3.9, 19.4, 6.9, 13.8 and 3.0 μM , respectively. These five compounds were further evaluated against MDA-MB-231 human breast adenocarcinoma cells, NCI-H460 human non-small-cell lung cancer cells, HCT-116 colon cancer cells, HepG2 liver cancer cells, and the non-cancerous MCF10A human breast epithelial cells. Our results indicated that these five compounds exhibited moderate cytotoxicity against the above-mentioned cells with IC₅₀ values ranging from 2.2 to 30.4 μM (Table 2). Compounds **1**, **5** and **7** possessing a chlorine atom at C-6'' produced greater cytotoxicity than compounds **3**, **4**, **6**, **8**, (IC₅₀ > 50 μM against MDA-MB-435), which could be potentially useful in rational drug design.

Antibacterial activities

Preliminary screens of compounds **1–9** for antibacterial activities were conducted by using the disk diffusion method against Gram-positive bacteria including *Micrococcus luteus* ML01, *Staphylococcus aureus* ATCC 29213, and a panel of MRSA isolated from human patients (MRSA 991, MRSA 1862, MRSA 669 A and MRSA A2) and pig (MRSA GDQ6P012P and MRSA GDE4P037P), and Gram-negative bacteria including *Acinetobacter baumannii* ATCC 19606, *Vibrio coralliilyticus* ATCC BAA-450 and *Vibrio alginolyticus* XSBZ14. Our results show that **1–9** could inhibit the growth of Gram-positive bacteria, but not Gram-negative bacteria at 10 μg per filter paper. Subsequently, compounds **1–6** were further validated for their MIC values by using the broth microdilution method for SAR. Our results (Table 3) suggest that all the tested compounds, except compound **2**, demonstrated antibacterial activities against *Micrococcus luteus* ML01 and *Staphylococcus aureus* ATCC 29213 with MIC values ranging between 0.2 and 4 $\mu\text{g}/\text{mL}$. Also, these compounds showed antibacterial activities against MRSA strains with MIC values of 1–16 $\mu\text{g}/\text{mL}$. We found that

Table 3 Antibacterial activities (MIC, $\mu\text{g}/\text{mL}$) of six selected compounds

	1	2	3	4	5	6	Kan ^a	Van ^a	Clinical source
<i>Micrococcus luteus</i> ML01 Ju1	0.5	16	0.5	0.25	0.25	2	1	0.5	
<i>Staphylococcus aureus</i> ATCC 29213	1	3	2	1	0.5	4	2	0.5	
MRSA 991	2	64	4	4	1	16	128	2	From human
MRSA 1862	2	32	4	2	1	8	64	1	
MRSA 669A	2	32	4	2	2	8	8	1	
MRSA A2	2	32	2	2	1	8	>128	1	
MRSA GDQ6P012P	2	128	4	4	2	16	128	1	From pig
MRSA GDE4P037P	2	64	4	4	1	16	>128	8	

^aKan: kanamycin; Van: vancomycin

compounds **1** and **5**, which contained a chlorine atom at C-6'', showed more potent antibacterial activities than compounds **3** and **4**, supporting the conclusion that the chlorine atom at C-6'' is functionally significant.

Conclusion

Chemical investigation on deep-sea derived *Streptomyces* sp. SCSIO 11791 led to the identification of nine chlorinated bis-indole alkaloids **1–9**, including two new compounds **1** and **2**. These chlorinated bis-indole alkaloids showed cytotoxic activities and antibacterial activities in bioactive tests. Notably, compound **1** showed moderate cytotoxicity against a panel of human cancer cell lines with an IC₅₀ range of 3.1–11.2 μM . In addition, **1** showed significant antibacterial activity against Gram-positive pathogenic bacteria, including clinical MRSA strains with MIC values of 0.5–2 $\mu\text{g}/\text{mL}$. SAR analysis of these compounds indicates that the chlorine atom at C-6'' is a key functional atom. This finding may prove information to SAR studies carrying the purpose of developing novel cytotoxic or antibacterial drugs.

Materials and methods

General experimental procedures

NMR spectra were collected with an Avance 500 spectrometer (Bruker) at 500 MHz for the ¹H nucleus and 125 MHz for the ¹³C nucleus. HRESIMS data were operated by using a MaXis quadrupole-time-of-flight mass spectrometer (Bruker). CC (column chromatography) was performed on 100–200 mesh silica gel (Yantai Jiangyou Silica Gel Development Co., Ltd.). RP-MPLC was carried out using CHEETAHTM MP200 (Agela Technologies) and Claricep Flash S Silica columns. RP-HPLC analyses were carried out using a Hitachi HPLC with YMC-Pack ODS-A column (250 × 10 mm, 5 μm).

Strains and fermentation

Strains

The strain SCSIO 11791 was isolated from a sediment sample collected from the South China Sea at a depth of 1,765 m using HRA medium after incubation at 28 °C for 4 weeks [24]. It was preserved at the RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences. According to the morphological characteristics and phylogenetic analysis, it was identified as previously described [24]. Its 16S rRNA gene sequence was deposited in GenBank with accession no. MK764932, which demonstrated the highest similarity value to *Streptomyces specialis* GW41-1564^T (97.70%).

Fermentation

The strain was inoculated using a modified ISP-2 agar plate (glucose 0.4%, yeast extract 0.4%, malt extract 1%, crude sea salt 3%, pH 7.4) for 5 days at 28 °C. The growth mycelia and spores were transferred into 250-mL Erlenmeyer flasks containing 50 mL modified ISP-4 medium (0.5% soluble starch, 0.05% yeast extract, 0.1% peptone, 0.1% K₂HPO₄, 0.1% MgSO₄·7H₂O, 0.2% (NH₄)₂SO₄, 0.4% NaCl, 3% crude sea salt, 0.2% CaCO₃, pH 7.4) and incubated for 36 h at 28 °C on rotary shakers (200 rpm) as seed cultures. Then, each flask of seed culture was transferred as an inoculum into a 1-L flask containing 200 mL modified ISP-4 medium and incubated for 9 days at 28 °C on rotary shakers (200 rpm). In total, 21 L liquid culture was obtained.

Extraction and isolation

The culture was harvested and centrifuged at 3600 rpm for 10 min to yield the supernatant and mycelia cake, which was extracted with butanone and acetone, respectively [25]. The extracted residues were combined for purification after HPLC analyses.

The combined extract was first subjected to silica gel CC (column A) by gradient elution with CHCl₃/MeOH (100/0, 98/2, 96/4, 94/6, 92/8, 90/10, 80/20, 50/50, v/v, 200 mL for each gradient) to obtain fractions A1–A8. The fractions A1 and A2 were combined and isolated with silica gel CC (column B) eluting with gradient ratios of petroleum ether/EtOAc (100/0, 90/10, 85/15, 80/20, 75/25, 72/28, 70/30, 60/40, v/v, 200 mL for each gradient) to afford fractions B1–B8. The fractions B4–B6 were combined and further subjected to MPLC purification using an ODS column (column C), eluting with a linear gradient of MeCN/H₂O from 25/75 to 80/20 for 60 min, and then holding at 100% MeCN for 30 min at a flow rate of 10 mL/min to get fractions C1–C11. Fraction C4 was purified by a semi-preparative HPLC system, eluting with CH₃CN/H₂O from 40/60 to 100/0 over 20 min and then holding at 100% MeCN for 5 min at a flow rate of 2.5 mL/min to yield compounds **5** (*t*_R 23.3 min, 103.6 mg) and **6** (*t*_R 21.2 min, 28.4 mg). Fraction C6 was subjected to MPLC purification by using an ODS column eluting by MeCN/H₂O mixture from 40/60 to 80/20 over 60 min to yield compound **9** (*t*_R 45.8 min, 21.0 mg). Fractions A4–A8 were combined and isolated by MPLC using an ODS column (column E), eluting with a linear gradient of MeCN/H₂O from 10/90 to 100/0 over 90 min, and then holding at 100% MeCN for 30 min at a flow rate of 10 mL/min to get fractions E1–E11. Fraction E6 was repeatedly eluted with a linear gradient of MeCN/H₂O/HAc from 10/90/0.1 to 60/40/0.1 over 60 min by ODS MPLC to obtain compounds **4** (*t*_R 35.6 min, 21.6 mg) and **8** (*t*_R 49.3 min, 3.4 mg). Fraction E7 was eluted with CHCl₃/MeOH (v/v, 50 mL for each gradient, increasing 0.5% MeOH) from 100/0 to 96/4 to get compounds **1** (3.6 mg) and **7** (12.0 mg) at 99/1 and 98.5/1.5, respectively. Fraction E8 was purified by semi-preparative HPLC with CH₃CN/H₂O from 35/60 to 100/0 over 20 min at a flow rate of 2.5 mL/min to give compounds **3** (*t*_R 16.4 min 5.2 mg) and **2** (*t*_R 17.8 min 16.7 mg).

dionemycin (**1**): orange amorphous powder; UV (MeOH) λ_{\max} (log ϵ) 442 (3.72), 357 (3.67), 287 (3.99), 217 (4.55) nm; IR (ATR) ν_{\max} 1701, 1526 cm⁻¹; see Table 1 for ¹H and ¹³C NMR spectroscopic data; (+)-HRESIMS *m/z* 429.9912 [M + H]⁺ (calcd for C₂₀H₁₁Cl₃N₃O₂, 429.9917).

6-OMe-7',7''-dichlorochromopyrrolic acid (**2**): pale-yellow amorphous powder; UV (MeOH) λ_{\max} (log ϵ) 261 (4.4), 230 (4.73) nm; IR (ATR) ν_{\max} 3418, 1694, 1458, 1238 cm⁻¹; see Table 1 for ¹H and ¹³C NMR spectroscopic data; (+)-HRESIMS *m/z* 468.0502 [M + H]⁺ (calcd for C₂₃H₁₆Cl₂N₃O₄, 468.0528).

Assays for cytotoxicity and antibacterial activities

Cytotoxic activities were evaluated by a previously reported MTT method [26]. MDA-MB-435 human breast cancer cells, MDA-MB-231 human breast adenocarcinoma cells,

NCI-H460 human non-small-cell lung cancer cells, HCT-116 human colon cancer cells, HepG2 human liver cancer cells, and the non-cancerous MCF10A human breast epithelial cells were used.

The antibacterial activities were evaluated by using filter paper agar plates and broth microdilution-based antimicrobial susceptibility tests for aerobic bacteria [27]. Gram-positive bacteria including *Micrococcus luteus* ML01, *Staphylococcus aureus* ATCC 29213, and a panel of clinical MRSA strains isolated from human and pig, as well as Gram-negative bacteria including *Acinetobacter baumannii* ATCC 19606, *Vibrio corallilyticus* ATCC BAA-450, and *Vibrio alginolyticus* XSBZ14 were used. Minimal inhibitory concentrations (MIC values) of antimicrobial agents that maximally inhibited cell growth in microdilution wells were determined by visual inspection and absorption measurements.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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