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Spiroindimicins A–D: New Bisindole Alkaloids from a Deep-Sea-Derived Actinomycete

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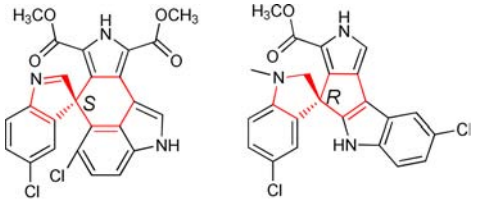
ABSTRACT



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A PCR-based screening approach led to the identification of a deep-sea-derived *Streptomyces* sp. SCSIO 03032 capable of producing new bisindole alkaloids spiroindimicins A–D (1–4). Structural elucidation of these compounds revealed the presence of unusual [5,6] or [5,5] spiro-ring containing skeletons. Spiroindimicins B–D (2–4) with a [5,5] spiro-ring exhibited moderate cytotoxicities against several cancer cell lines.

Bisindole alkaloids, including indolocarbazoles, comprise a large family of natural products with diverse biological activities, exemplified by the protein kinase inhibitor staurosporine and the topoisomerase inhibitor rebeccamycin, two anticancer drug leads.[1] Their excellent bioactivities inspired great interest in their structure diversification by chemical and biosynthetic methods. To date, more than 250 naturally occurring or chemo(bio)synthetic bisindole alkaloid family members have been described (Figure S1, Supporting Information).[1,2] Recently, cladoniamides and BE-54017 were discovered to have rearranged and degraded alkaloid skeletons, putatively originating from indolocarbazole precursors,[3] indicating that nature can continually contribute to new chemical entities of bisindole alkaloids. In the past decade, marine actinomycetes have been documented as a significant resource for producing novel secondary metabolites of

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high value for drug development,[4] and many new natural products with unique bioactivity were reported from marine-derived actinomycetes.[5] Given the recent discovery of new antibacterial, antimalarial, and antitumor natural products from our reservoir of marine-derived actinomycetes,[6] we were motivated to search for new bisindole alkaloids from the ocean.

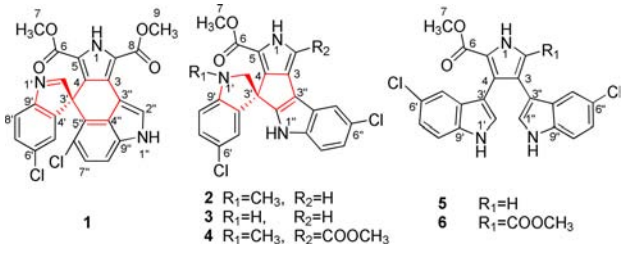

Figure 1. Chemical structures of compounds **1**–**6**.

To facilitate the discovery of potential bisindole alkaloid-producing marine actinomycetes, we adopted a PCR-based screening approach by using a pair of degenerate primers[3b] to target on conserved regions of tryptophan dimerization genes (*staD*/*rebD*/*atmD*/*inkD*/*vioB*, etc.), which are commonly found in the biosynthetic gene clusters for indolocarbazole natural products (staurosporine, rebeccamycin, AT-2433, k-252a)[7] as well as bisindole alkaloid violacein.[8] From 238 actinomycetes derived from the South China Sea and Indian Ocean, only two positive hits were identified (SCSIO 03032 and SH04). Bioinformatic analysis of the deduced products encoded by the cloned and sequenced PCR DNA fragments revealed their high similarities to RebD/StaD/AtmD/VioB (Figure S2, Supporting Information). The strain SH04 was identified as a *Salinispora arenicola* (GenBank accession no. JQ692175 for the 16S rRNA gene) and was found to be a 5′-hydroxystaurosporine producer (Table S1, Figure S3, Supporting Information),[9] while the strain SCSIO 03032, which was isolated from a sediment sample (E 87°59.7′, N 9°59.3′) at a depth of 3412 m from the Bay of Bengal in the Indian Ocean, was identified as a *Streptomyces* strain on the basis of its 16S rRNA gene sequence (GenBank accession no. JN798514) and was capable of producing four new spiro-bisindole alkaloids, spiroindimicins A–D (**1**–**4**, Figure 1). Their structures were elucidated by spectral analyses and X-ray diffraction studies (for **1** and **2**). Spiroindimicin A (**1**) possesses a [5,6] spiro-ring system, whereas spiroindimicins B–D (**2**–**4**) contain a distinct [5,5] spiro-ring system. To the best of our knowledge, spiroindimicins represent the first example of unusual spiro-ring containing bisindole alkaloids of bacteria origin, distinct from plant-derived vobtusine and its analogues,[10] and yeast-derived pityriarubins (Figure S1, Supporting Information).[11] Herein we presented details of the isolation, structure elucidation, biological activity, and proposed biogenesis of these new compounds.

An EtOAc extract prepared from the resin XAD-16 absorbent of the cultures of SCSIO 03032 in a seawater-based medium, was subjected to various chromatographic methods (Supporting Information) and led to the isolation of four new compounds, spiroindimicins A–D (Figure 1, **1**–**4**), and two known compounds, lynamicins A (**5**) and D (**6**) (Figure 1).[12] The ^{1}H and ^{13}C NMR spectra of **5** and **6** (Table S2, Figures S4 and S5, Supporting Information) were consistent with those previously reported.[12] Lynamicin D (**6**) was further confirmed by X-ray diffraction studies (Figure S5, Supporting Information).

Spiroindimicin A (**1**) was assigned a molecular formula of $C_{24}H_{15}Cl_2N_3O_3$ (18 degrees of unsaturation) through HRESIMS (*m*/*z* 478.0353, $[M - H]^-$, calcd 478.0361), whose isotope pattern was consistent with the presence of two chlorine atoms. The inspection of the ^{1}H, ^{13}C and HSQC NMR spectroscopic data of **1** (Table S3, Figure S6, Supporting Information) revealed the presence of two exchangeable protons, two methoxyl groups, 19 olefinic and/or aromatic carbons (seven of which are protonated), two ester carbonyl carbons, and one unprotonated sp^3 carbon. All of these ^{1}H and ^{13}C NMR resonances accounted for 12 degrees of unsaturation. The remaining

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6 degrees of unsaturation indicated that **1** is a hexacyclic compound.

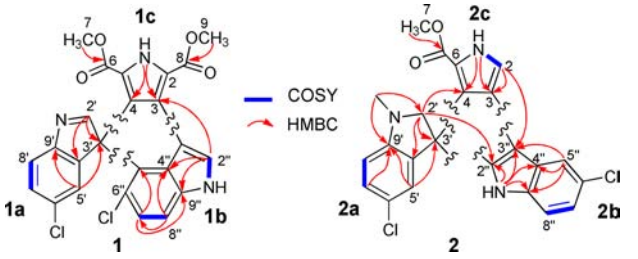

Figure 2. Selected key COSY and HMBC correlations for spiroindimicins A (**1**) and B (**2**).

The ^{1}H NMR and COSY correlation spectra of **1** revealed a characteristic ABX spin system of H-8′/H-7′/H-5′ (Table S3, Figure S6, Supporting Information), typical for a 1, 2, 4-trisubstituted benzene ring, which was supported by further HMBC correlations from H-5′ to C-7′/C-9′, H-7′ to C-5′/C-6′/C-9′ and from H-8′ to C-4′/C-6′/C-9′ (Figure 2). Downfield chemical shifts of a sp^2 methine (δ_H 8.04, δ_C 169.2, C-2′) implied that this methine carbon linked with a nitrogen atom to form an endo double bond. The HMBC correlations from the methine proton H-2′ to C-4′ and C-3′ suggested that the latter C=N was connected with the phenyl at C-4′ through a quaternary carbon C-3′. This was confirmed by the HMBC correlation from H-5′ to C-3′. The linkage of the nitrogen atom with the phenyl at C-9′ was deduced by the low field ^{13}C NMR chemical shift of C-9′ at δ_C 157.5. A chlorine was presumed to locate at the sp^2 quaternary carbon (δ_C 130.5, C-6′) according to the structures of **5** and **6**. Thus, the moiety **1a** was established (Figure 2). The presence of an indole ring (the moiety **1b**) was deduced from two proton spin systems by the ^{1}H−^{1}H COSY correlations of **1**, H-7″/H-8″ and H-2″/NH-1″. The former indicated the presence of a pair of *ortho*-coupled protons (J = 8.5 Hz) in a benzene ring. The latter suggested a "=CH−NH−" fragment. The moiety **1b** was further supported by HMBC correlations from H-7″ to C-5″/C-9″, H-8″ to C-4″/C-6″, NH-1″ to C-2″/C-3″/C-4″/C-9″, and H-2″ to C-4″/C-9″ (Figure 2). A chlorine was deduced to locate at the sp^2 quaternary carbon (C-6″) according to **5** and **6**, thus constructing the substructure of **1b**. The moiety **1c** was deduced by comparing NMR data of **1** and lynamycin D (**6**).[12] Two formyls were inferred by the HMBC correlations from methoxyl protons to the carbonyl carbons (H-7/C-6, H-9/C-8). The remaining atoms, C_4HN, were assigned to a tetrasubstituted pyrrole on the basis of key HMBC correlations from the exchangeable proton (δ_H 12.20, brs, NH-1) to C-3 and C-4, thus establishing a 2,5-diformyl-3,4-bisubstituted pyrrole moiety of **1c** (Figure 2).

The three moieties **1a**, **1b**, and **1c** accounted for pentacyclic rings, indicating that one more ring should be formed by connecting these three moieties to satisfy the deduced hexacyclic ring system for **1**. The linkage of C-3″ to C-3 between **1b** and **1c** was inferred from the key HMBC correlation from H-2″ to C-3. Additionally, **1** had two sp^2 quaternary carbons [δ_C 120.7 (s, C-5″), 120.1 (s, C-4)] and one sp^3 quaternary carbon [δ_C 61.2 (s, C-3′)]. Thus, a hexatomic ring was presumably formed by connecting C-3′ with C-4 and C-5″. This presumption was finally confirmed by X-ray crystallographic analysis of **1** (Figure 3). Locations of the two chlorine atoms at C-6′ and C-6″ were confirmed by the X-ray structure of **1**. Also, the presence of chlorine atoms in **1** allowed the unambiguous assignment of an *S* configuration at C-3′ on the basis of the refined Flack parameter value [x = 0.02 (5)].[13] Hence, spiroindimicin A (**1**) was determined to be a bisindole alkaloid with an unprecedented [5,6] spiro-ring skeleton (Figure 1).

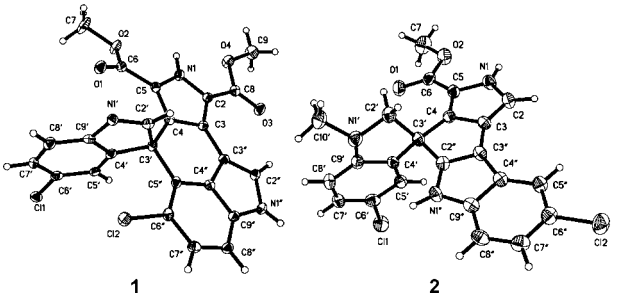

Figure 3. X-ray crystal structures of **1** and **2**.

Spiroindimicin B (**2**) was assigned a molecular formula of $C_{23}H_{17}Cl_2N_3O_2$ (16 degrees of unsaturation) by HRESIMS (m/z 436.0602, [M − H]$^-$, calcd 436.0620). The analysis of the ^{1}H, ^{13}C and HSQC NMR spectroscopic data of **2** (Tables S3, S4 and Figures S7, S8, Supporting Information) revealed two exchangeable protons, one *N*-methyl [δ_H 2.88 (1H, s)], one methoxyl [δ_H 3.61 (3H, s)], one methylene, 18 olefinic and/or aromatic carbons (seven of which are protonated), one unprotonated sp^3 carbon, and one ester carbonyl. The comparison of NMR data of **1** and **2** suggested that **2** shared partial structural features with **1**. Two ABX spin systems (H-7′H-8′/H-5′; H-7″/H-8″/H-5″), similar to that of **1a**, were observed in the ^{1}H NMR and COSY correlation spectra of **1**, indicating the presence of two 1, 2, 4-trisubstituted benzene rings in **2**, which were assigned to moieties of **2a** and **2b** and confirmed by key HMBC correlations (Figure 2). In addition, the HMBC correlations from *N*-methyl protons (H-10′) to C-2′ and C-9′ located the N-methyl group at N-1′ in **2a**. The moiety **2c** was given rise from the obvious HMBC correlations from H-2 to C-3/C-4/C-5, coupling with one proton spin system H-2/NH-1 observed from ^{1}H−^{1}H COSY correlations. The single ester group was located at C-5 by HMBC correlation from H-2 to C-3″. Different from the [5,6] spiro-ring system in **1**, **2** was found to have a distinct spiro-ring by connecting C-3′ with C-2″,

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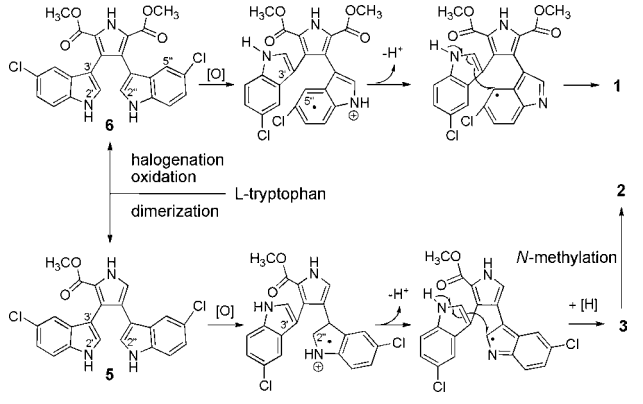
supported by key HMBC correlations from H-2′ to C-4 and H-2′ to C-2″ (Figure 2). Finally, the structure of **2**, which contained a [5,5] spiro-ring adopting an *R*-configuration at C-3′, was unambiguously confirmed by X-ray crystallographic analysis (Figure 3), on the basis of refined Flack parameter value [0.04(6)].[13]

The molecular formula of optically active spiroindimicin C (**3**), $C_{22}H_{15}Cl_2N_3O_2$, was derived from HRESIMS (*m*/*z* 422.0499 $[M - H]^-$, calcd 422.0463). The comparison of ^1H and ^{13}C NMR spectra of **3** (Table S3, Figure S9, Supporting Information) with those of **2** revealed that **3** differed from **2** only by losing the N-1′ methyl group (Figure 1). Spiroindimicin D (**4**) was assigned a molecular formula of $C_{25}H_{19}C_{l2}N_3O_4$ by HRESIMS (*m*/*z* 494.0661 $[M - H]^-$, calcd 494.0674). Comparing their ^1H and ^{13}C NMR spectroscopic data (Table S3, Figure S10, Supporting Information), **4** differed from **2** by having an additional methyl ester moiety. The sp^2 methine [δ_H 6.91 (d, J = 3.0 Hz, H-2); δ_C 110.3 (d, C-2)] in **2** was replaced by a sp^2 quaternary carbon [δ_C 111.5 (s, C-2)] in **4**, locating the additional methyl ester moiety at C-2.

The electronic circular dichroism (ECD) spectra derived from quantum-chemical calculations were shown to be useful for assigning the stereochemistry of natural products.[14] Comparison of the calculated ECD spectra with the experimental ones of **1**–**4** (Figure S11, Supporting Information), revealed good agreements of an *S*-absolute configuration at C-3′ in **1** and an *R*-absolute configuration at C-3′ in **2**–**4**.

Scheme 1. Proposed Biogenesis for Spiroindimicins A–C (**1**–**3**)



Spiroindimicins A–D (**1**–**4**) are new bisindole alkaloids with unprecedented skeletons, featuring [5,6] (**1**) or [5,5] (**2**–**4**) spiro rings. In staurosporine biosynthesis, the formation of the indolocarbazole planar skeleton was suggested by an aryl–aryl coupling of C-2′ and C-2″ of chromopyrrolic acid, catalyzed through a key indole cation radical intermediate.[15] By analogy to this radical mechanism, the [5,6] spiro-ring in spiroindimicin A (**1**) was proposed to be derived from lynamicin D (**6**) by an aryl–aryl coupling of C-3′ and C-5″, whereas the aryl–aryl coupling of C-3′ and C-2″ of lynamicin A (**5**) would lead to the [5,5] spiro-ring in spiroindimicin C (**3**), which was further modified to produce **2** (Scheme 1).

The *in vitro* cytotoxicities of compounds **1** to **6** were evaluated against 5 cancer cell lines including MCF-7, HepG2, B16, H460, and CCRF-CEM by MTT assays (Table S5, Supporting Information).[16] Spiroindimicin B (**2**) exhibited moderate cytotoxic activities against CCRF-CEM, B16 and H460 with IC_{50} values of 4, 5, and 12 μg/mL, respectively, which are comparable to those of 5′-hydroxy-staurosporine (Table S5, Supporting Information). Spiroindimicin C (**3**) inhibited the growth of HepG2 and H460 with IC_{50} values of 6 and 15 μg/mL. Spiroindimicin D (**4**) showed moderate inhibitory effects against HepG2, B16, and H460. In contrast, compounds **1**, **5**, and **6** showed no obvious inhibitory activities.

In summary, taking advantage of a PCR-based screening approach, we discovered a deep-sea-derived *Streptomyces* sp. SCSIO 03032 capable of producing four new bisindole alkaloids, spiroindimicins A–D (**1**–**4**), with two distinct, unprecedented spiro-containing skeletons from a reservoir of 238 marine-derived actinomycetes. The spiroindimicins B–D (**2**–**4**), featuring a [5,5] spiro-ring system, exhibited moderate antitumor activities. These new structures may present future opportunities for chemical, enzymatic, or genetic engineering approaches to generate new analogues.

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Supporting Information Available. Experimental details, tables of NMR assignments, structure characterization data, and cytotoxicity data for compounds **1**–**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.