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Indoxamycins A−F. Cytotoxic Tricycklic Polypropionates from a Marine-Derived Actinomycete

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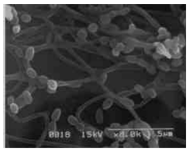
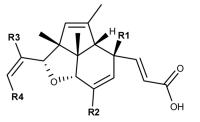
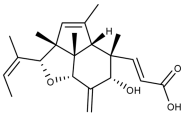




Indoxamycin A: R1 = CH_3, R2 = CH_3, R3 = CH_3, R4 = CH_3
Indoxamycin B: R1 = CH_2OH, R2 = CH_3, R3 = CH_3, R4 = CH_3
Indoxamycin C: R1 = CH_3, R2 = CH_2OH, R3 = CH_3, R4 = CH_3
Indoxamycin D: R1 = CH_3, R2 = CH_3, R3 = CH_2OH, R4 = CH_3
Indoxamycin E: R1 = CH_3, R2 = CH_3, R3 = CH_3, R4 = CH_2OH



Indoxamycin F

Six antitumor antibiotics of a new structure class, indoxamycins A−F (**1**−**6**), were isolated from a saline culture group of marine-derived actinomyces whose strains showed approximately 96% sequence homology of 16S rDNA with the family streptomycetaceae. The structures of these indoxamycins, which are unusual polyketides composed of six consecutive chiral centers, were assigned by combined spectral and chemical methods. In feeding experiments using a stable isotope label, indoxamycin A was assembled from propionate units initially forming the "aglycon" pentamethyl indeno furan. The discovery of these unprecedented compounds from marine-derived actinomycetes, a low gene homology genus, offers a significant opportunity for drug discovery.

Introduction

The actinomycetes are Gram-positive bacteria which for more than 50 years have provided a significant source of bioactive secondary metabolites. Since the discovery of actinomycin, cultured bacteria have been a prolific resource for drug discovery, with more than 13 000 bioactive metabolites described to date. More than 70% of these bioactive compounds have been isolated from cultured, primarily soil-derived actinomycetes.[1] In the late 1980s, however, the frequency of discovery of new drug candidates from terrestrial actinomycetes began to decrease, and the discovery of new chemical diversity is therefore essential. Research on the metabolites of marine plants and animals began in the early 1970s, and to date has yielded large numbers of novel compounds that possess potent biological activities.[2] However, bioactive compounds from marine plants and animals are often available only in small amounts, which make it difficult to supply for requirement. Recently, with the dramatic increase in the emergence of drug-resistant infectious diseases, there is an urgent need to identify and develop new antibiotics.[3] Unfortunately, current sources of chemical diversity do not seem to be generating new antibiotic drugs in response to this emerging challenge. New concepts are needed in order for new medicine candidate materials from natural products to be discovered.

Recently, Mincer et al. reported that actinomycetes adapted for life in the sea do indeed occur.[4] Terrestrial actinomycetes are valuable sources that produce various secondary metabolites with the ability to supply necessary amounts by large-scale cultivation. Therefore these actinomycetes isolated from marine sources were also expected to produce various secondary metabolites and supply for requirement. Fenical et al. have focused considerable attention on marine actinomycetes, which have been shown to produce many appealing novel metabolites with antitumor

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(1) Berdy, J. *J. Antibiot*. **2005**, *58*, 1–26.
(2) (a) Faulkner, J. D. *Nat. Prod. Rep*. **2000**, *17*, 1–6. (b) Blunt, J. W.; Copp, B. R.; Hu, W; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. *Nat. Prod. Rep*. **2008**, *25*, 35–94.
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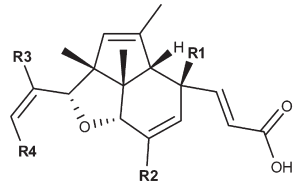
properties, similar to mangicols, sporolides, marinomycins, and marinopyrroles.[5]

In the present study, we examined the sequence homology of 16S rDNA to improve accuracy, and defined marine-derived actinomycetes with a sequence homology of 97% or less as candidate strains. Using various sampling tools, including methods that provide access to ocean sediments, and selection mediums, modified humic acid vitamin agar, we isolated multiple low-sequence homology actinomycetes from marine samples.

Actinomycete strain NPS-643, obtained from a marine sediment sample collected near Kochi Harbor, Japan, at a depth of 30 m, desired seawater for growth and shared 96.0% 16S rRNA gene sequence identity with its nearest neighbor (*Streptomyces cacaoi*). This level of similarity suggests that it may be a new *Streptomyces* species.[6] Strain NPS-643 was cultivated in a seawater-based medium and extracted with ethyl acetate. The ethyl acetate layer was separated by centrifugation and the organic solvent was removed under reduced pressure. The resulting organic extract demonstrated in vitro cytotoxicity against the HT-29 human colon adenocarcinoma cell line. Activity-guided fractionation using various chromatographic methods led to the isolation of one known compound (pironetin)[7] and six novel polyketides, indoxamycins A−F (**1**−**6**), which possess a novel tricyclic ring system and constitute a new class structure.



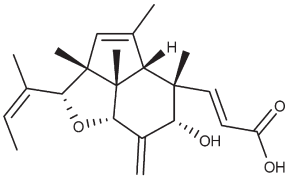
1: **R1** = CH_3, **R2** = CH_3, **R3** =CH_3, **R4** =CH_3

2: **R1** = CH_2OH, **R2** = CH_3, **R3** = CH_3, **R4** =CH_3

3: **R1** = CH_3, **R2** = CH_2OH, **R3** = CH_3, **R4** =CH_3

4: **R1** = CH_3, **R2** = CH_3, **R3** = CH_2OH, **R4** = CH_3

5: **R1** = CH_3, **R2** = CH_3, **R3** =CH_3, **R4** = CH_2OH



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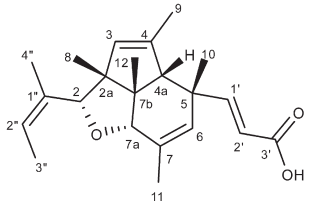
Indoxamycin numbering scheme:



Results and Discussion

Indoxamycin A (**1**) was obtained as a colorless oil with a molecular formula $C_{22}H_{30}O_3$ based on the interpretation of high-resolution electrospray-ionization time-of-flight mass spectrometry (HR-ESI-TOF-MS; obsd $[M+H]^+$ m/z 343.2270, calcd $[M+H]^+$ m/z 343.2275) and NMR spectral data. Additional evidence from MS data was the presence of one free hydroxyl functionality based on fragment ion peaks at 325 $[M + H - H_2O]^+$. This was corroborated by IR absorptions characteristic of conjugated carboxyl functionalities at 3423, 1694 cm^{-1}. The ^1H NMR spectrum of **1** in methanol-d_4 (Table 1) showed five olefinic protons, three allylic protons, and seven methyl groups.

TABLE 1. ^1H, ^{13}C, and HMBC NMR Spectral Data for Indoxamycin A (1) in Methanol-d_4

No.	δ_H,[a] mult (J [b])	δ_C,[c] H[d]	HMBC
1			
2	4.02, s	92.1, CH	2a, 3, 7a, 7b, 8, 2″, 4″
2a		61.2, C	
3	5.19, s	136.9, CH	2, 2a, 4a, 7b, 8, 9
4		139.6, C	
4a	2.27, s	65.9, CH	2a, 3, 4, 5, 6, 7a, 7b, 9, 10, 1′
5		42.3, C	
6	5.61, s	131.5, CH	4a, 5, 7a, 10, 11, 1′
7		136.7, C	
7a	4.10, s	85.1, CH	2, 2a, 4a, 7b, 12
7b		58.1, C	
8	0.84, s	17.7, CH_3	2, 3, 7b
9	1.66, s	19.0, CH_3	3, 4, 4a
10	1.25, s	30.2, CH_3	4a, 5, 6, 1′
11	1.81, s	20.7, CH_3	6, 7, 7a
12	1.19, s	26.6, CH_3	2a, 4a, 7a, 7b
1′	6.97, d (15.9)	153.7, CH	5, 6, 10, 3′
2′	5.83, d (15.9)	123.7, CH	5, 3′
3′		174.2, C	
1″		135.2, C	
2″	5.49, d (6.3)	120.3, CH	2, 3″, 4″
3″	1.64, d (6.3)	12.9, CH_3	1″, 2″
4″	1.59, s	14.4, CH_3	2, 1″, 2″

[a]400 MHz. [b]Coupling constants in Hz. [c]100 MHz. [d]Assignments by edited gs-HSQC experiments.

The ^{13}C NMR spectrum of **1** showed one carboxylic carbon, eight olefinic carbons, two oxygenated carbons, and eleven additional carbon signals between δ 12.9 and 65.9. Edited gs-HSQC experiment[8] indicated seven CH_3, eight CH, and seven quaternary carbons, and showed that $\alpha-\beta$ unsaturated carboxylic acid proton signals [δ 5.83 (1H, d, J=15.9 Hz, H-2′), 6.97 (1H, d, J=15.9 Hz, H-1′)] correlated with ^{13}C NMR signals at δ 123.7 and 153.7, and that HMBC experiments on their protons correlated with ^{13}C NMR signals at δ 174.2 (C-3′) and 42.3 (C-5). Additionally, the H-1′ signal correlated with resonances at δ 30.2 (C-10), 131.5 (C-6), and 65.9 (C-4a), and key HMBC correlations of the olefinic proton H-6 (δ 5.61) to ^{13}C NMR signals at δ 20.7 (C-11), 85.1 (C-7a), and C-4a were observed. Furthermore, the H-7a proton [δ 4.10 (1H, s)] correlated with ^{13}C NMR signals at δ 58.1 (C-7b), 26.6 (C-12), and C-4a. These correlations indicate trimethylcyclohexene substructure A (Figure 1).

Subsequently, substructure A was extended to pentamethylindenyl substructure B by HMBC correlations from H-9 [δ 1.66 (3H, s)] and H-3 [δ 5.19 (1H, s)] to C-4a, from H-3 to C-2a (δ 61.2), C-8 (δ 17.7), and C-7b, and from H-12 [δ 1.19 (3H, s)] and H-7a to C-2a. In addition, the correlation from H-3 and H-8 [δ 0.84 (3H, s)] to C-2 (δ 92.1) suggests that C-2a and C-2 are adjacent. An ether bond between C-2 and C-7a was indicated based on the interpretation of HMBC

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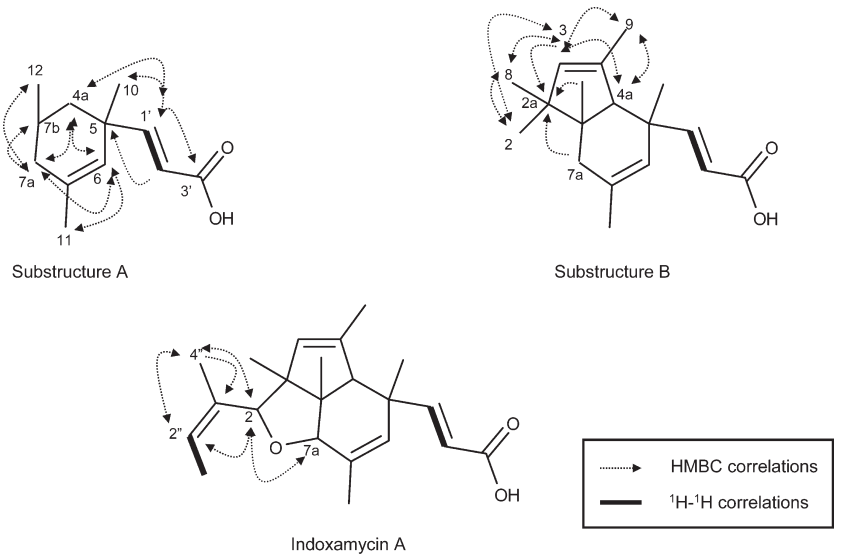

FIGURE 1. Key HMBC correlations of indoxamycin A (**1**).

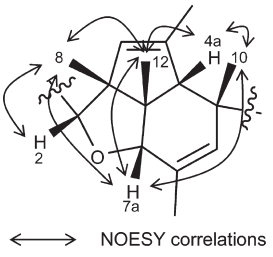

FIGURE 2. Key NOESY of indoxamycin A (**1**).

correlations from H-7a to C-2. Additionally, HMBC correlations from H-4″ [δ 1.59 (3H, s)] to C-2, C-1″ (δ 135.2) and C-2″ (δ 120.3) established attachment of an isobutene group (C1″- C4″) at C-2.

The relative configurations of the stereocenters of the tricyclic ring system in indoxamycin A were determined by interpretation of both 2D NOESY and 1D NOE experiments. Some key NOE correlations are illustrated in Figure 2.

The NOESY NMR experiment for **1** showed correlations between H-4a and H_3-10 and H_3-12, and between H-7a and H_3-10 and H_3-12. In addition, a 1D NOE experiment with **1** showed enhancement of H_3-12, H-2, and H-7a when H_3-8 was irradiated. These correlations allow the allylic proton (H-4a, H-7a, and H-2) and the methyl groups (H_3-10, H_3-12, and H_3-8) to be placed on the same face of the tricyclic ring system and the ether group to be placed on the opposite face, thus establishing the C-4a/C-7b, C-2a/C-7b, and C-7a/C-7b ring fusions as cis.

The absolute stereochemistry of indoxamycin A was determined by application of the modified Mosher ester NMR method,[9] using the tetrahydroxylated analogue of indoxamycin A. Indoxamycin A was first treated with trimethylsilydiazomethane[10] to give the corresponding methyl ester since Mosher esterification without the protection of carboxylic acid yielded multiple products. Asymmetric dihydroxylation[11] induced no reaction, thus the indoxamycin double bond seemed to exhibit low reactivity toward a sterically bulky chiral ligand. Treatment with OsO_4,[12] however, afforded tetraol (dr >11:1) with high stereoselectivity for steric hindrance of two methyl group (H_3-10, H_3-12; Scheme 1).

In this reaction, diol (dr = 6:1) was also obtained as a byproduct. These products and stereoisomers were separated readily by TLC purification. Treatment of tetraol in separate experiments, with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl acid (*R*-MTPA) and (*S*)-(+)-MTPA, yielded the *R*-Mosher ester **1a** and *S*-Mosher ester **1b**, respectively. Relative stereochemistry at C-6 was determined by the 2D NOESY experiments of **1b**. Correlation was observed between H-6 and H_3-11, and between H_3-11 and H-7a, suggesting that H-6 and H-7a are placed on the same face, with OMTPA-6 and H-7a on the opposite face (Figure 3). Analysis of ^{1}H NMR chemical shift differences ($\Delta\delta_{S-R}$) between **1a** and **1b** revealed that the absolute stereochemistry of C-6 is *S* (Figure 3). This result supports the assignment of the absolute stereochemistry at C-5 and C-7b as *S*, while C-2, C-2a, C-4a, and C-7a were assigned as *R*.

Indoxamycin B (**2**) was isolated as a colorless oil that analyzed for the molecular formula $C_{22}H_{30}O_4$ by HR-ESI-TOF-MS data (obsd $[M + H]^+$ at *m*/*z* 359.2227, $[M + H]^+$ *m*/*z* 359.2224), and by comprehensive analysis of NMR data (Tables 2 and 3). While the ^{1}H and ^{13}C NMR spectra for indoxamycin A and B appeared very similar, the difference between the two compounds was suggestive of the presence of an additional hydroxyl group in **2**. Also evident from the MS data was the presence of at least two free hydroxyl functionalities based on fragment ion peaks at 341 $[M + H - H_2O]^+$ and 323 $[M + H - 2H_2O]^+$. This was corroborated

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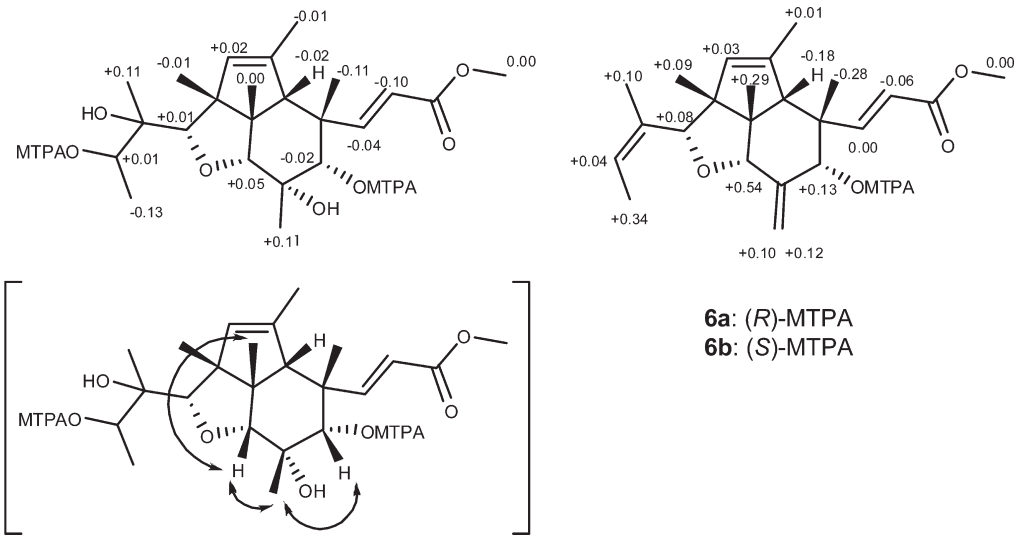

FIGURE 3. $\Delta\delta_{S-R}$ values for the Mosher esters.

SCHEME 1. Chemical Conversion for Mosher Ester Analysis

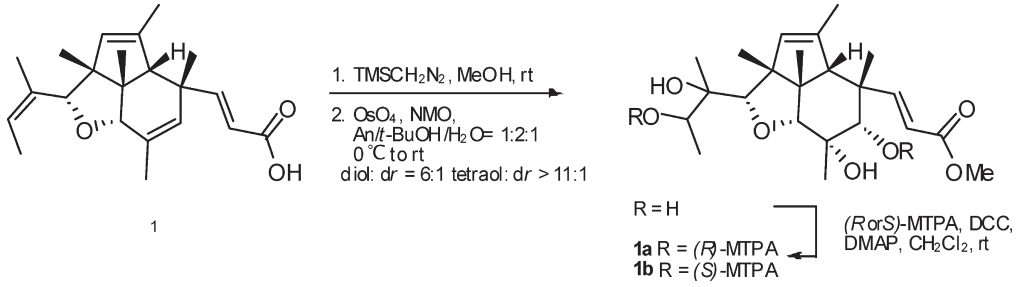

TABLE 2. ^{1}H NMR data for Indoxamycins B−F (2−6) in Methanol-d_4

	δ,[a] mult (J[b])				
no.	indoxamycin B (**2**)	indoxamycin C (**3**)	indoxamycin D (**4**)	indoxamycin E (**5**)	indoxamycin F (**6**)
1					
2	3.97, s	4.11, s	4.12, s	4.01, s	4.02, s
2a					
3	5.17, s	5.19, s	5.22, s	5.19, s	5.15, s
4					
4a	2.62, s	2.27, s	2.29, s	2.27, s	2.37, s
5					
6	5.68, s	5.68, s	5.66, s	5.64, s	4.51
7					
7a	4.07, s	4.26, s	4.22, s	4.10, s	4.14, s
7b					
8	0.82, s	0.85, s	0.79, s	0.86, s	0.82, s
9	1.69, s	1.70, s	1.64, s	1.67, s	1.65, s
10	3.37, 3.50 d (10.8)	1.29, s	1.22, s	1.23, s	1.28, s
11	1.83, s	4.13, 4.15, s	1.80, s	1.79, s	5.26, 5.06, s
12	1.19, s	1.17, s	1.22, s	1.18, s	1.18, s
1′	6.81, d (16.1)	6.87, d (16.0)	6.85, d (16.0)	6.85, d (16.0)	6.89, d (15.8)
2′	5.90, d (16.1)	5.85, d (16.0)	5.86, d (16.0)	5.85, d (16.0)	5.90, d (15.8)
3′					
1″					
2″	5.48, q (6.7)	5.49, q (6.2)	5.70, q (6.2)	5.65, t (6.2)	5.55, q (6.8)
3″	1.63, d (6.7)	1.63, d (6.2)	1.77, d (6.2)	4.17, d (6.2)	1.65, d (6.8)
4″	1.57, s	1.58, s	3.95, 4.20, d (11.9)	1.63, s	1.64, s

[a]400 MHz. [b]Coupling constants in Hz.

by IR absorptions characteristic of hydroxyl and conjugated carboxyl functionalities. The particularly different NMR signals between **1** and **2** were H-10, C-4a, C-5, C-6, and C-1′. The HMBC correlations observed for the H-10 geminal methylene protons (δ_H 3.50, 3.90) to ^{13}C NMR signals at δ 58.7 (C-4a), 61.2 (C-5), 128.1 (C-6), and 148.5 (C-1′)

TABLE 3. ^{13}C NMR Data for Indoxamycins B−F (2−6) in Methanol-d_4

	δ,[a] H[b]				
no.	indoxamycin B (**2**)	indoxamycin C (**3**)	indoxamycin D (**4**)	indoxamycin E (**5**)	indoxamycin F (**6**)
1					
2	91.2, CH	90.7, CH	83.6, CH	90.9, CH	90.3, CH
2a	61.2, C	59.8, C	59.7, C	61.1, C	62.0, C
3	136.4, CH	135.3, CH	135.2, CH	136.9, CH	135.4, CH
4	140.1, C	138.3, C	138.6, C	140.4, C	135.4, CH
4a	58.7, CH	65.0, CH	64.2, CH	65.7, CH	66.1, CH
5	61.2, C	40.5, C	40.6, C	42.1, C	44.2, C
6	128.1, CH	131.0, CH	130.1, CH	131.6, CH	80.3, CH
7	138.6, C	133.9, C	137.3, C	136.4, C	134.8, C
7a	84.4, CH	80.4, CH	87.7, CH	85.0, CH	85.7, CH
7b	57.8, C	56.8, C	57.4, C	58.1, C	57.5, C
8	17.2, CH_3	17.6, CH_3	18.7, CH_3	17.6, CH_3	17.6, CH_3
9	19.2, CH_3	20.1, CH_3	20.3, CH_3	19.2, CH_3	19.0, CH_3
10	70.1, CH_2	29.4, CH_3	29.4, CH_3	30.6, CH_3	29.1, CH_3
11	20.7, CH_3	63.1, CH_2	22.3, CH3	20.4, CH_3	120.0, CH_2
12	26.2, CH_3	25.1, CH_3	25.5, CH_3	27.8, CH_3	28.0, CH_3
1′	148.5, CH	149.5, CH	150.4, CH	151.7, CH	150.0, CH
2′	128.0, CH	128.5, CH	120.0, CH	125.7, CH	127.4, CH
3′	175.7, C	174.2, C	174.5, C	175.8, C	175.8, C
1″	138.6, C	138.2, C	135.5, C	136.3, C	134.8, C
2″	120.0, CH	118.7, CH	123.3, CH	125.2, CH	125.2, CH
3″	12.9, CH_3	12.9, CH_3	15.9, CH_3	59.1, CH_2	12.9, CH_3
4″	14.3, CH_3	15.7, CH_3	56.7, CH_2	14.7, CH_3	14.2, CH_3

[a]100 MHz. [b]Assignments by edited gs-HSQC experiments.

implied **2** contained a similar skeleton to **1**. These correlations and chemical shifts indicated that **2** was a C-10 hydroxymethyl analogue of **1**. The relative configurations of the stereocenters in **2** were assigned on the basis of correlations observed in the NOESY NMR spectrum, which showed that they were the same as those found in **1**. This result also suggests that the ring fusions were all cis. Indoxamycin B, with same chiral centers of **1**, showed the same sign, −49.2 (*c* 0.10, MeOH), as indoxamycin A.

Indoxamycin C (**3**), which was isolated as a colorless oil, had the molecular formula $C_{22}H_{30}O_4$ based on HR-ESI-TOF-MS data (obsd $[M + H]^+$ at *m*/*z* 359.2224) and combined NMR spectral data. The results of 2D NMR analysis established just a positional isomer of **2**, with NMR placing OH at C-11 in **3**. The key HMBC correlations were observed for the H-11 geminal protons (δ_H 4.15, 4.13) to ^{13}C NMR signals at δ 133.9 (C-7), 80.4 (C-7a), and 131.0 (C-6). The relative configurations of the stereocenters in **3** were also assigned C-4a/C-7b, C-2a/C-7b, and C-7a/C-7b ring fusions as cis. Additionally, the specific optical rotation of **3** showed −20.5 (*c* 0.08, MeOH).

Indoxamycin D (**4**) also analyzed for $C_{22}H_{30}O_4$ based on HR-ESI-MS data ($[M + H]^+$ at *m*/*z* 359.2224) and combined NMR and IR spectral data. The structure was also established as the C-4″ positional isomer of **2** by HMBC correlations from H-4″ geminal protons (δ_H 3.95, 4.20) to ^{13}C NMR signals at δ 135.5 (C-1″), 123.3 (C-2″), and 83.6 (C-2). The results of the 2D NOESY experiments also suggested that the C-4a/C-7b, C-2a/C-7b, and C-7a/C-7b ring fusions were cis. Additionally, the specific optical rotation of **4** shared the same sign, −36.9 (*c* 0.10, MeOH), with other indoxamycins.

Indoxamycin E (**5**) also analyzed for $C_{22}H_{30}O_4$ based on HR-ESI-MS data ($[M + H - H_2O]^+$ at *m*/*z* 341.2084, calcd 341.2038) and combined NMR spectral data. Indoxamycin E was also just a positional isomer of **2**. The key correlation was observed from H-3″ proton (δ_H 4.17) to H-2″ olefinic proton (δ_H 5.65) by ^{1}H−^{1}H COSY spectra and to ^{13}C NMR signals at δ 125.2 (C-2″) and 136.3 (C-1″) by HMBC spectra. These correlations indicated that **5** was a C-3″ positional isomer of **2**. The relative stereochemistry was also determined by 2D NOESY experiments, and the correlations of **5** were almost identical with those of the other indoxamycins. Thus, all of the ring fusions of **5** were also identified as cis. The specific optical rotation of **5** showed −30.6 (*c* 0.10, MeOH).

Indoxamycin F (**6**) was also obtained as a colorless oil that analyzed for the molecular formula $C_{22}H_{30}O_4$ based on HR-ESI-TOF-MS (obsd $[M + H]^+$ at *m*/*z* 359.2223). This molecular formula was supported by ^{1}H and ^{13}C NMR data. Additionally, MS data indicated the presence of at least two free hydroxyl functionalities based on fragment ion peaks at 341 $[M + H - H_2O]^+$ and 323 $[M + H - 2H_2O]^+$. This was corroborated by IR absorptions characteristic of hydroxyl and conjugated carboxyl functionalities at 3420, 1397, and 1647 cm^{-1}, respectively. The ^{13}C NMR data of indoxamycin F were analyzed by edited gs-HSQC spectra, which revealed the presence of one carbonyl, eight sp2 carbons (one methylene, four methines, and three quaternary carbons), four sp3 methines, three quaternary carbons, and six methyl groups. The NMR signal that was characteristic to indoxamicin F was the sp2 methylene signal (δ_H 5.26, 5.06; δ_C 120.0). The HMBC correlation of the sp2 methylene proton signals showed ^{13}C NMR signals at δ 85.7 (C-7a) and 80.3 (C-6). These correlations and chemical shifts indicated the attachment of the exomethylene group at C-11. Furthermore, the chemical sifts of C-7a and C-6 indicated the presence of an additional hydroxyl or ether group in respective carbons. Additionally, HMBC correlations from H-7a to C-2 suggested that C-7a and C-2 connected by ether bond. And then, the hydroxyl group was confirmed to exist at C-6 by estimated molecular formula, IR spectra (3420 cm^{-1}), and C-6 chemical shifts (δ_H 4.51; δ_C 80.3). The relative configurations of the stereocenters in indoxamycin F (**6**) were assigned on the basis of 2D NOESY experiments. The 2D NOESY

NMR experiment showed correlations from H-6 and H-4a to both H_3-10 and H_3-12, from H-2 to H_3-12 and H-8, and from H-7a to H_3-12. These correlations allowed the allylic proton (H-4a, H-7a, H-2, and H-6) and the methyl groups (H_3-10, H_3-12, and H_3-8) to be placed on the same face of the tricyclic ring system and the ether group and hydroxyl group to be placed on the opposite face, thus establishing the C-4a/C-7b, C-2a/C-7b, and C-7a/C-7b ring fusions as cis.

The absolute stereochemistry of indoxamycin F was also determined by the application of the modified Mosher ester NMR method as in the case of **1**. Indoxamycin F was converted to methyl ester, then esterified to give corresponding (*R*)- and (*S*)-MTPA ester **6a** and **6b**. According to the analysis of the Mosher ester, the absolute stereochemistry of indoxamycin F was determined at C-5, C-6, and C-7b as *S*, while C-2, C-2a, C-4a, and C-7a were assigned as *R* (Figure 3). Thus, the absolute stereochemistries of indoxamycins A and F were found to correspond completely.

The absolute stereochemistry of indoxamycins B−E (**2**−**5**), indoxamycin A hydroxymethyl analogue, was estimated by comparison of their optical rotations and CD spectra. Their optical rotations detected the same sign. Additionally, Their CD spectra also showed measurable correspondence to indoxamycin A (Figure S37, Supporting Information). These results of indoxamycins B−E, with a common optical complex of indoxamycin A, might signify that indoxamycins B−E have the same absolute stereochemistry as indoxamycin A.

Biological Activity of the Indoxamycins. Indoxamycins A−F (**1**−**6**) were tested for possible antiproliferative activity in the HT-29 tumor cell line, at five final assay concentrations of 0.03, 0.1, 0.3, 1, and 3 μM. Indoxamycins A (**1**) and F (**6**) caused significant growth inhibition (< 50% of growth) at concentrations between 0.3 and 3 μM relative to the vehicle-treated control (simple IC_{50} value: **1**, 0.59 μM; **6**, 0.31 μM). These inhibition activities achieved the same level as that of mitomycin (simple IC_{50} value: 0.66 μM). However, other indoxamycin hydroxymethyl analogues (**2**−**5**) did not cause any significant growth inhibition at any of the five tested assay concentrations. This result, that inhibition activity depends upon the presence of a lipophilic core, might be a clue to the solution of cytotoxic site.

Biosynthesis of the Indoxamycins. The biosynthetic origin of each carbon of **1** was investigated by ^{13}C NMR analyses of ^{13}C-enriched **1**, which were obtained from the cultured broth of *Streptomyces* strain NPS-643. Unfortunately, by using sodium [1, 2-^{13}C]-acetate and sodium [2-^{13}C]-acetate, ^{13}C labels were absorbed into other metabolic compounds (e.g., pironetin). Pironetin has been shown to derive from four acetate units, two propionate units, one butyrate unit, and one methyl unit of methionine.[13] Additional feeding experiments with sodium [1-^{13}C]-propionate indicated that the olefinic carbons (C-3, C-6, and C-1′) and allylic carbons (C-2, C-4a, and C-7) were derived from propionate with isotopic enrichment of each carbon. These results might indicate that indoxamycin "aglycon" pentamethyl indeno furan is constructed from six C_3 units (Figure 4).

Metabolites derived entirely from propionate are comparatively rare in actinomycetes. A notable example, the macrolide antibiotic erythromycins from *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*) is derived from seven C_3 units (polypropionates) that are composed of one unit of propionyl-CoA and six of methylmalonyl-CoA.[14] On the other hand, many other polypropionates have been found in marine invertebrates, specifically molluscs,[15] including the sacoglossan molluscs[16] and air breathing pulmonates. From this point of view, the biosynthetic pathway of indoxamycins is very interesting as a notable example of a second metabolite with C_3 units produced by an actinomycete. It is also interesting that their biosynthetic pathway might be acquired by the result of adjusting to the sea environment.

In summary, indoxamycins have a novel tricyclic skeleton that has yet to be defined in the secondary metabolites. These indoxamycin structures are unique for several reasons. First, while the compounds appear to be polyketides and therefore derived from acetate units, the existence of six consecutive chiral carbons is highly unusual, particularly with propionate biosynthetic subunits. The tricyclic structures of indoxamycins are completely unprecedented though many secondary metabolites from actinomycetes have been previously reported. The biological activities and biosynthetic pathways of these compounds are currently under investigation. To date, limited testing has shown that **1**−**6** have moderate cytotoxicity against human colon adenocarcinoma HT-29 cell line (simple IC_{50} value: **1**, 0.59 μM; **2**, > 3 μM; **3**, > 3 μM; **4**, > 3 μM; **5**, > 3 μM; **6**, 0.31 μM). The discovery of the indoxamycins provides additional evidence that taxonomically unique marine-derived actinomycetes have great potential as a source of secondary metabolites with novel structures. This result suggests that continued investigation of marine-derived actinomycetes, especially those cultured from sea sediments, represents a promising strategy for the discovery of structurally unique molecules.

Experimental Procedures

Isolation, Cultivation, and Extraction of the NPS-643 Strain. *Streptomyces* strain NPS-643 was isolated on modified humic acid vitamin (HV) agar (1.0 g of humic acid, 0.5 g of Na_2HPO_4, 1.71 g of KCl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate, and *p*-aminobenzoic acid, 0.25 mg of biotin, 50 mg of cycloheximide, 18 g of agar, 18 g of Daigo's artificial seawater sp, and 1 L of distilled water) from a marine sediment collected at a depth of 30 m near Kochi Harbor, Japan. The strain was cultured in 500 × 100 mL volumes of medium MKG (25 g of maltose, 15 g of soytone, 2 g of yeast extract, 4 g of $CaCO_3$, 18 g of Daigo's artificial seawater, 800 μL of 2 M NaOH, and 1 L of distilled water) under shaking at 200 rpm and at a temperature of 28 °C for 6 days. The pH was adjusted to 6.9 before autoclaving. At the end of the fermentation period, the culture solution was centrifuged (3000 rpm for 10 min) and the upper layer was

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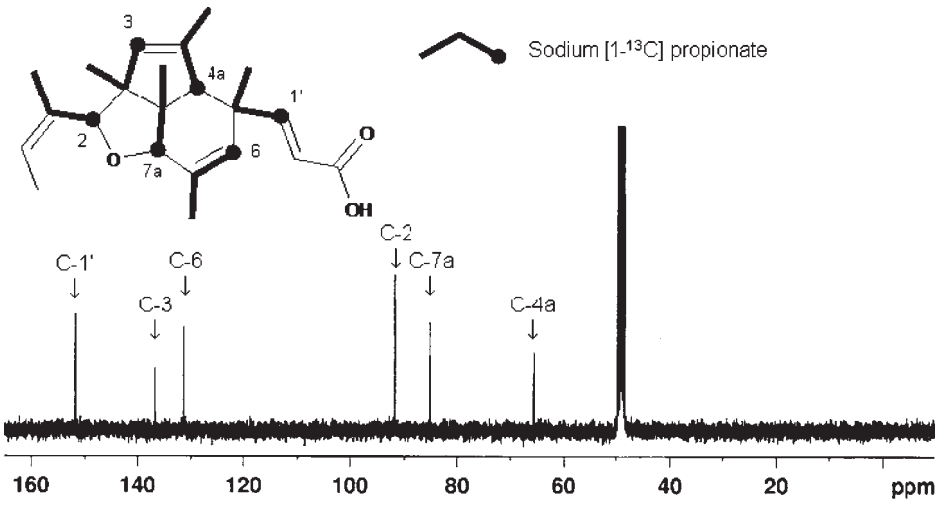

FIGURE 4. Sodium [1-^{13}C]-propionate labeling study of indoxamycin A (**1**).

partitioned with EtOAc, providing approximately 110 mg of dry extract per 1 L of culture after removal of the solvent.

Isolation and Purification of Indoxamycins A−F (1−6). The combined EtOAc extract from 500 × 100 mL fermentations (5.5 g) was subjected to C-18 reverse-phase column chromatography purification eluting with solvent mixtures of H_2O−MeOH (3:1), H_2O−MeOH (1:1), H_2O−MeOH (1:3), and 100% MeOH, successively. The H_2O−MeOH (1:3) eluting fraction (S643-3) showed moderately potent HT-29 cytotoxicity. These compounds were conspicuous on silica gel and C-18 TLC because of their characteristic pink color development when the plates were heated after maceration with a *p*-anisaldehyde reagent. Fraction S643-3 was refractionated by C-18 reverse-phase HPLC with MeOH−H_2O (9:1) to obtain pure indoxamycin A (**1**) and impure indoxamycins B−F (**2**−**6**) as major components (retention times: S643-3-A, 12.22−13.54 min; S643-3-BE, 8.82−10.39 min). Final purification of indoxamycins B−F (**2**−**6**) was achieved by preparative TLC with $CHCl_3$−MeOH (9:1) [R_f values: S643-3-B, 0.19; S643-3-C, 0.28; S-643-3-D, 0.30; S-643-3-E, 0.26; S-643-3-F, 0.34]. Typical recoveries of indoxamycins A−F from a 1 L culture were 21.8, 0.1, 0.1, 0.1, 0.2, and 0.4 mg, respectively.

Sodium [1-^{13}C]-Propionate Feeding Experiment and Purification of [^{13}C] Indoxamycin A. The NPS-643 strain was cultured in 100 mL volumes of medium (8.3 g of glucose, 15.0 g of soytone, 1.0 g of yeast extract, 1.0 g of sodium [1-^{13}C]-propionate, and 1 L of distilled water) under shaking at 200 rpm for 6 days. At the end of the fermentation period, the culture solution was centrifuged (3000 rpm for 10 min), the upper layer was partitioned with EtOAc, and combined EtOAc extracts were purified by preparative TLC with $CHCl_3$−MeOH (9:1). Thus, 0.9 mg of [^{13}C] indoxamycin A was obtained.

Indoxamycin A (1): colorless oil, $[\alpha]_D$ −5.1 (*c* 0.10, MeOH); IR (KBr) 3423, 2967, 2916, 2870, 1694, 1638, 1445, 1618, 1381, 1293, 1219, 1069, 1015 cm^{-1}; HR-ESI-TOF-MS obsd *m*/*z* 343.2270 $[M + H]^+$, $C_{22}H_{30}O_3$ + H requires 343.2275. See Table 1 for NMR data.

Indoxamycin B (2): colorless oil, $[\alpha]_D$ −49.2 (*c* 0.10, MeOH); IR (KBr) 3422, 2967, 2929, 2870, 1645, 1559, 1454, 1398, 1060 cm^{-1}; HR-ESI-TOF-MS obsd *m*/*z* 359.2227 $[M + H]^+$, $C_{22}H_{30}O_4$ + H requires 359.2224. See Tables 2 and 3 for NMR data.

Indoxamycin C (3): colorless oil, $[\alpha]_D$ −20.5 (*c* 0.08, MeOH); IR (KBr) 3421, 2968, 2923, 2869, 1637, 1560, 1458, 1394, 1314, 1087 cm^{-1}; HR-ESI-TOF-MS obsd *m*/*z* 359.2224 $[M + H]^+$, $C_{22}H_{30}O_4$ + H requires 359.2224. See Tables 2 and 3 for NMR data.

Indoxamycin D (4): colorless oil, $[\alpha]_D$ −36.9 (*c* 0.10, MeOH); IR (KBr) 3420, 2965, 2929, 2869, 1637, 1569, 1457, 1396, 1314, 1191, 1097, 1042 cm^{-1}; HR-ESI-TOF-MS obsd *m*/*z* 359.2224 $[M + H]^+$, $C_{22}H_{30}O_4$ + H requires 359.2224. See Tables 2 and 3 for NMR data.

Indoxamycin E (5): colorless oil, $[\alpha]_D$ −30.6 (*c* 0.10, MeOH); IR (KBr) 3410, 2968, 2930, 2878, 1647, 1559, 1448, 1394, 1314, 1217, 1085 cm^{-1}; HR-ESI-TOF-MS obsd *m*/*z* 351.2084 $[M + H - OH]^+$, $C_{22}H_{30}O_4$ + H requires 351.2038. Also a very small parent ion was observed. See Tables 2 and 3 for NMR data.

Indoxamycin F (6): colorless oil, $[\alpha]_D$ −55.8 (*c* 0.10, MeOH); IR (KBr) 3420, 2970, 2926, 2870, 1647, 1559, 1448, 1397, 1314, 1251, 1086, 1033 cm^{-1}; HR-ESI-TOF-MS obsd. *m*/*z* 359.2223 $[M + H^+]$, $C_{22}H_{30}O_4$ + H requires 359.2224. See Tables 2 and 3 for NMR data.

Preparation of (*R*)-MTPA Ester 1a and (*S*)-MTPA Ester 1b. A solution of **1** (2.0 mg, 5.8 μmol) in MeOH (0.3 mL) was added to trimethylsilyldiazomethane (44 μL, 87 μmol, 2.0 M in hexane solution) at room temperature. After being stirred at room temperature for 20 min, the reaction mixture was concentrated under reduced pressure to give the methyl ester. A mixture of methyl ester (2.1 mg, 5.8 μmol) in *t*-BuOH/acetone/H_2O (1:2:1, 0.32 mL) was added to a solution of 4-methylmorpholine *N*-oxide (0.68 mg, 5.8 μmol) in H_2O (20 μL) and a solution of OsO_4 in *t*-BuOH (30 μL) at 0 °C. After being stirred for 15 min, the reaction mixture was warmed to room temperature and stirred for an additional 30 min. The reaction was quenched by the addition of $Na_2S_2O_3$ after 1 h, followed by the addition of EtOAc. The aqueous layer was extracted with EtOAc three times and the combined organic layer was concentrated under reduced pressure. Purification by TLC ($CHCl_3$/MeOH = 9:1) afforded 1.2 mg of tetraol analogue as a colorless oil along with 1.1 mg of diol analogue as a colorless oil. Tetraol was divided into two portions. A solution of tetraol (0.6 mg, 1.4 μmol) in CH_2Cl_2 (0.2 mL) was added to (*R*)-MTPA (6.8 mg, 29 μmol), DCC (3.7 mg, 18 μmol), and DMAP (1.6 mg, 13 μmol) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. Purification by TLC (Hex/EtOAc = 2:1) afforded 0.9 mg of **1a** as a colorless oil. (*S*)-MTPA ester **1b** was obtained by treating tetraol (0.6 mg, 1.4 μmol) as described for Mosher ester **1a**. **(*R*)-MTPA ester 1a:** ^{1}H NMR (400 MHz, CD_3OD) δ 7.64−7.60 (m, 2H), 7.51 (dd, *J* = 7.3, 2.0 Hz, 2H), 7.49−7.40 (m, 6H), 7.12 (d, *J* = 16.0 Hz, 1H, H-1′), 5.74 (d, *J* = 16.0 Hz, 1H, H-2′), 5.44 (q, *J* = 6.8, 1H, H-2″), 5.42 (s, 1H, H-6), 5.24 (s, 1H, H-3), 3.74 (s, 3H,

3′-OMe), 3.72 (s, 1H, H-7a), 3.69 (s, 1H, H-2), 3.58 (s, 3H), 3.49 (s, 3H), 2.44 (s, 1H, H-4a), 1.64 (s, 3H, H-9), 1.48 (s, 3H, H-10), 1.41 (d, J = 6.8 Hz, 3H, H-3″), 1.28 (s, 3H, H-8), 1.23 (s, 3H, H-4″), 1.18 (s, 3H, H-12), 1.18 (s, 3H, H-11). (***S*)-MTPA ester 1b:** ^{1}H NMR (400 MHz, CD_3OD) δ 7.71−7.66 (m, 2H), 7.51 (dd, J = 7.5, 2.0 Hz, 2H), 7.49−7.40 (m, 6H), 7.08 (d, J = 16.1 Hz, 1H, H-1′), 5.64 (d, J = 16.1 Hz, 1H, H-2′), 5.45 (q, J = 6.5 Hz, 1H, H-2″), 5.40 (s, 1H, H-6), 5.26 (s, 1H, H-3), 3.77 (s, 1H, H-7a), 3.74 (s, 3H, 3′-OMe), 3.70 (s, 1H, H-2), 3.63 (s, 3H), 3.59 (s, 3H), 2.42 (s, 1H, H-4a), 1.63 (s, 3H, H-9), 1.37 (s, 3H, H-10), 1.34 (s, 3H, H-4″), 1.29 (s, 3H, H-11), 1.28 (d, J = 6.5 Hz, 3H, H-3″), 1.27 (s, 3H, H-8), 1.18 (s, 3H, H-12).

Preparation of (*R*)-MTPA Mosher Ester 6a and (*S*)-MTPA Ester 6b. A solution of **6** (5.0 mg, 14 μmol) in MeOH (0.75 mL) was added to trimethylsilyldiazomethane (0.14 mL, 0.28 mmol, 2.0 M in hexane solution) at room temperature. After being stirred at room temperature for 20 min, the reaction mixture was concentrated under reduced pressure to give the methyl ester. A solution of methyl ester (1.5 mg, 4.0 μmol) in 0.2 mL of CH_2Cl_2 was added to (*R*)-MTPA (10.0 mg, 43 μmol), DCC (8.0 mg, 39 μmol), and DMAP (1.0 mg, 8.2 μmol) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. Purification by TLC (Hex/EtOAc = 5:1) afforded 2.0 mg of **6a** as a colorless oil. (*S*)-MTPA ester **6b** was obtained by the same experimental procedure as described for Mosher ester **6a**. **(*R*)-MTPA Mosher ester 6a:** ^{1}H NMR (400 MHz, CD_3OD) δ 7.60−7.53 (m, 2H), 7.48−7.46 (m, 3H), 7.17 (d, 1H, J = 16.0 Hz, H-1′), 5.98 (d, 1H, J = 16.0 Hz, H-2′), 5.49 (q, 1H, J = 6.8 Hz, H-2″), 5.46 (s, 1H, H-6), 5.46 (s, 1H, H-11), 5.28 (s, 1H, H-11), 5.19 (s, 1H, H-3), 3.99 (s, 1H, H-2), 3.82 (s, 1H, H-7a), 3.71 (s, 3H, 3′-OMe), 3.69 (s, 3H), 2.25 (s, 1H, H-4a), 1.63 (d, 3H, J = 6.8 Hz, H-3″), 1.62 (s, 3H, H-9), 1.56 (s, 3H, H-4″), 1.42 (s, 3H, H-10), 0.73 (s, 3H, H-8), 0.69 (s,3H, H-12). **(*S*)-MTPA ester 6b:** ^{1}H NMR (400 MHz, CD_3OD) δ 7.48−7.46 (m, 2H), 7.45−7.43 (m, 3H), 7.17 (d, 1H, J = 16.0 Hz, H-1′), 5.92 (d, 1H, J = 16.0 Hz, H-2′), 5.59 (s, 1H, H-6), 5.56 (s, 1H, H-11), 5.53 (q, 1H, J = 6.8 Hz, H-2″), 5.40 (s, 1H, H-11), 5.22 (s, 1H, H-3), 4.36 (s, 1H, H-7a), 4.07 (s, 1H, H-2), 3.71 (s, 3H, 3′-OMe), 3.52 (s, 3H), 2.07 (s, 1H, H-4a), 1.97 (d, 3H, J = 6.8 Hz, H-3″), 1.66 (s, 3H, H-4″), 1.64 (s, 3H, H-9), 1.14 (s, 3H, H-10), 0.98 (s, 3H, H-12), 0.82 (s, 3H, H-8).

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Note Added after ASAP Publication: Scheme 1 was incorrect in the version published July 2, 2009; The correct version was published ASAP July 9, 2009.

Supporting Information Available: The ^{1}H, ^{13}C, NOE/NOESY, and HR-ESI-TOF-MS spectra of **1**−**6** and CD spectra of **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.