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NMR Analysis of Quinocycline Antibiotics: Structure Determination of Kosinostatin, an Antitumor Substance from *Micromonospora* sp. TP-A0468

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A quinocycline antibiotic, kosinostatin, was isolated from the culture broth of *Micromonospora* sp. TP-A0468 along with isoquinocycline B. Structure of kosinostatin was determined to be the stereoisomer of isoquinocycline B regarding to the stereochemistry at the C-2′ spiro carbon by NMR analysis. Kosinostatin isomerizes to isoquinocycline B through the inversion of the stereocenter at C-2′. Comparison of physico-chemical properties indicated that kosinostatin is presumably identical with quinocycline B isolated by CELMER *et al.* from *Streptomyces aureofaciens*.

During the course of our screening for new antibiotics from rare actinomycetes, kosinostatin (**1**) was isolated from *Micromonospora* sp. TP-A0468. Taxonomy and fermentation of the producing strain and isolation and biological properties of **1** were described in the preceding paper[1]. We herein report on the physico-chemical properties and structure determination of **1** in comparison with isoquinocycline B[2] (**2**) (Fig. 1). This paper presents the detailed NMR analysis of two quinocycline antibiotics, kosinostatin and isoquinocycline B.

Results and Discussion

Physico-chemical Properties

The physico-chemical properties of kosinostatin (**1**) are summarized in Table 1. **1** was obtained as a dark yellow powder with the decomposition point of 155°C. It was soluble in DMSO and methanol and slightly soluble in chloroform and ethyl acetate. The presence of amino functionality was indicated by the extraction test where **1** was extracted by ethyl acetate at pH 7~8 but not at pH 3~6. **1** showed the UV-vis spectrum similar to that of the anthracycline compounds with the absorption maxima at 228, 258, 291 and 423 nm. The FAB-MS positive mode measurement of **1** gave the parent ion peak $[M+H]^+$ at *m/z* 617. The molecular formula of **1** was determined to be $C_{33}H_{32}N_2O_{10}$ on the basis of the high resolution FAB-MS spectrum (617.2143, Δ +0.7 mmu) and NMR spectra.

The most notable property of **1** was the isomerization to isoquinocycline B (**2**). The isomerization took place both in solid state and in solution irreversibly. The halflife of **1** was *ca.* 3 days in methanol at 25°C and **1** completely isomerized to **2** in a few months.

Structure Determination

NMR Analysis of the Aglycons of Kosinostatin (3) and Isoquinocycline B (4)

The NMR spectra of **1** suggested the presence of a deoxysugar moiety. The acid-catalyzed methanolysis of **1** afforded the aglycon (**3**) and the mixture of α- and β-methyl glycosides. Comparing to the chemical yield of **3**, that of the glycoside was poor due to the instability in acidic condition. In the FAB-MS measurement, **3** showed the parent ion peak of *m/z* 445 $[M+H]^+$. The molecular formula of **3** was thus determined to be $C_{25}H_{20}N_2O_6$ based on the high resolution FAB-MS (445.1393, Δ −0.7 mmu).

The ^{13}C NMR spectrum of **3** confirmed the presence of 25 carbons and the HMQC spectrum established all one-

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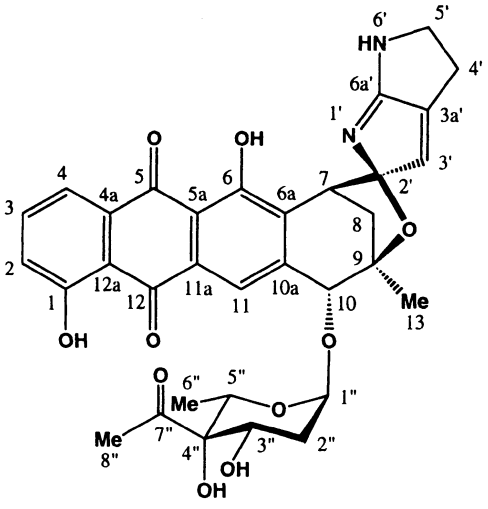
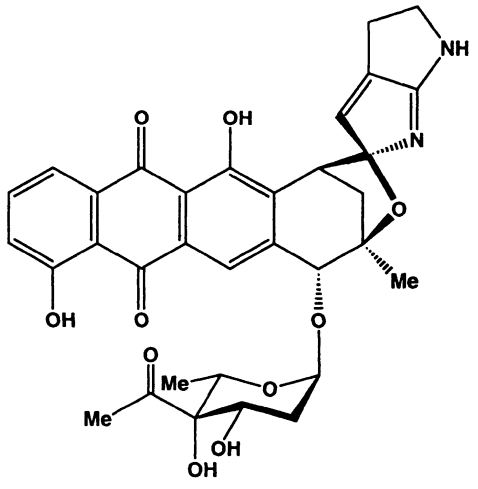
Fig. 1. Structure of kosinostatin and isoquinocycline B.



Kosinostatin



Isoquinocycline B

Table 1. Physico-chemical properties of kosinostatin.

Appearance	Dark yellow powder
MP	>155°C (dec)
$[\alpha]_{Hg546}^{25}$	+116.7 (*c* 0.11, MeOH-HCl)
HRFAB-MS	
Found:	617.2143 $[M+H]^+$
Calcd:	617.2136 for $C_{33}H_{33}N_2O_{10}$
Molecular formula	$C_{33}H_{32}N_2O_{10}$
UV λ_{max}^{MeOH} nm (log ε)	
in MeOH	228 (4.76), 258 (4.09), 291 (4.09), 423 (4.20)
in 0.01N HCl-MeOH (1:9)	229 (4.59), 257 (4.40), 290 (3.91), 423 (4.03)
in 0.01N NaOH-MeOH (1:9)	231 (4.57), 252 (4.49), 287 (4.02), 316 (3.62), 418 (3.79), 506 (3.78)
IR ν_{max} (cm^{-1})	3340, 2950, 1720, 1630, 1590
Solubility	
soluble in	MeOH, DMSO
slightly soluble in	$CHCl_3$, EtOAc
TLC[a] Rf	0.33
HPLC[b] Rt	18.6 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)
[b] HPLC conditions: Cosmosil AR-II (250 × 4.6 mm, i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (30:70), flow rate: 0.7 ml/min, detection: UV-254 nm.

bond ^1H-^{13}C connectivities (Table 2). The aromatic protons, H-2, H-3 and H-4, were assigned to a part of anthracyclinone moiety based on their ^1H-^1H coupling patterns. The assignment of the quaternary carbons in anthraquinone moiety and the substitution of two phenolic hydroxyl groups at C-1 and C-6 were established by the HMBC correlations from H-4 to C-5 and C-12a, from H-3 to C-1, C-12a and C-4a, from H-2 to C-1 and C-12a, and from H-11 to C-5a, C-6, C-6a, C-10a, C-11a and C-12. Long-range couplings of H-7 to C-6a and C-6 and that of H-8 to C-6a confirmed the connectivity between C-6a and C-7. In addition, the bonding between C-10 and C-10a was established by the long-range coupling of H-10 to C-6a, C-10a and C-11. Formation of another six-membered ring fused to the anthraquinone was deduced by the HMBC correlation of the C-13 methyl group to C-8, C-9 and C-10, those of H-8 and H-10 to C-9, and that between H-8 and H-10.

The remaining atoms must make up a pyrrolopyrrole ring. Evidence that the pyrrolopyrrole ring was linked to the anthracyclinone moiety *via* the C-2′ spiro carbon at C-7 and the oxygen atom at C-9 was provided by the LC-MS/MS experiment (Fig. 2). Fragment ions at *m/z* 321 and *m/z* 123 derived from the bond cleavage between C-7 and C-2′ and that between C-9 and the oxygen atom at C-9 were detected. In addition, a fragment of pyrrolopyrrole ring arising from the bond dissociation between C-2′ and

Table 2. NMR data for aglycons of kosinostatin (**3**) and isoquinocycline B (**4**) in DMSO-d_6.

Position	**3**		**4**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	161.73		161.69	
2	124.76	7.44 (1H, dd, 1.2 and 8.4)	124.68	7.42 (1H, dd, 1.2 and 8.4)
3	137.42	7.86 (1H, t, J=8.0 Hz)	137.28	7.85 (1H, dd, 7.6 and 8.4)
4	119.06	7.81 (1H, dd, J=1.2 and 7.6 Hz)	118.98	7.78 (1H, dd, 1.2 and 7.2)
4a	132.97		133.05	
5	187.28		187.40	
5a	113.89		113.83	
6	158.89		158.09	
6a	133.40		133.48	
7	40.51	3.86 (1H, d, 3.6)	43.34	3.85 (1H, d, 3.6)
8	34.20	1.98 (1H, d, 11.6)	33.49	2.02 (1H, d, 11.6)
		2.30 (1H, dd, 3.6 and 12.4)		2.46 (1H, dd, 4.4 and 12.4)
9	82.40		83.91	
10	73.09	4.21 (1H, s)	72.53	4.18 (1H, s)
10a	148.24		147.92	
11	121.39	7.90 (1H, s)	121.03	7.88 (1H, s)
11a	131.28		131.41	
12	187.28		187.18	
12a	115.68		115.81	
13	22.86	1.43 (3H, s)	22.21	1.49 (3H, s)
2'	112.79		112.51	
3'	129.79	6.41 (1H, br.s)	126.89	5.15 (1H, br.s)
3a'	140.32		142.39	
4'	21.56	2.59 (2H, m)	21.66	2.51 (2H)*
5'	58.07	3.94 (2H, m)	57.44	3.98 (2H, m)
6a'	170.17		169.49	

^{1}H and ^{13}C NMR were measured at 400 MHz and 100 MHz respectively.
The DMSO-d_6 signals (2.50 ppm for ^{1}H; 39.5 ppm for ^{13}C) were used as references.
Integral, multiplicities and the coupling constants (Hz) are in parentheses.
* overlapped with the solvent signal.

C-7 and between C-2′ and the C-9 oxygen was observed at *m/z* 108. Since few significant long-range ^{13}C-^{1}H correlations around the pyrrolopyrrole ring were detected in the HMBC spectrum of **3**, the assignment of the pyrrolopyrrole moiety was achieved by comparing the NMR data with those of the aglycon of isoquinocycline B (**4**), which afforded the HMBC spectrum amenable to the complete assignment (Fig. 3). The connectivity from C-3′ to C-5′ *via* C-3a′ was revealed by ^{1}H-^{1}H correlation and allylic four-bond coupling between H-4′ and H-3′ and HMBC correlations of H-3′ and H-4′ to C-3a′. Long-range couplings of H-4′ and H-5′ to the quaternary sp^2 carbon C-6a′ at 169.49 ppm confirmed its bondings to two nitrogen atoms. The X-ray crystallographic study of isoquinocycline A showed that the carbon-nigtrogen bond was delocalized between two nitrogens[3]. For clarification, one of the possible resonance structures is depicted in this paper. The linkage of the pyrrolopyrrole ring at C-7 to the anthracycline moiety was deduced by the HMBC correlations from H-3′, H-7 and H-8 to C-2′. As summarized in Table 2, the NMR assignment of **3** and **4** confirmed that they possessed the identical planar structure.

Stereostructure of Kosinostatin Aglycon (**3**)

NOESY experiments were performed to analyze the stereochemical differences between **3** and **4** (Fig. 4).

Fig. 2. MS/MS fragmentation of the aglycon of kosinostatin.

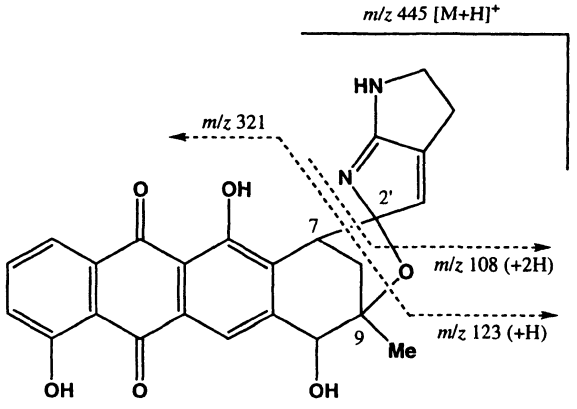


Fig 3. HMBC correlations observed with aglycon of isoquinocycline B.

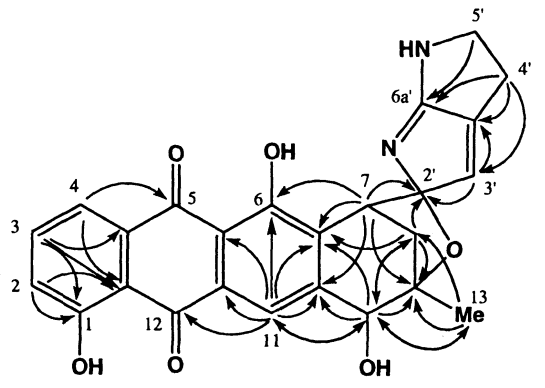
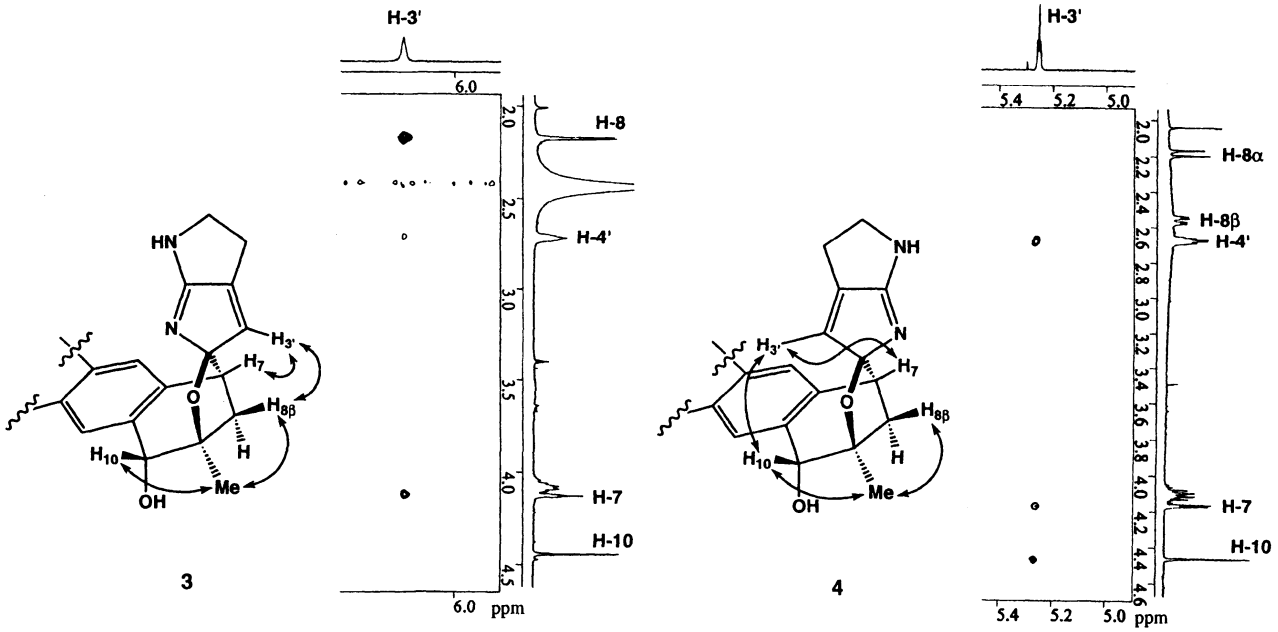


Fig. 4. Comparison of NOEs observed in the NOESY spectrum of aglycons of kosinostatin (**3**) and isoquinocycline B (**4**).



Preferable results were obtained in $CDCl_3$ with the mixing time of 800 msec. The ^1H NMR data in $CDCl_3$ for **3** and **4** were given in Experimental. The most significant NOESY correlation was observed between H-3′ and H-8β in **3** and between H-3′ and H-10 in **4**. In the both NOESY spectra, the correlation was detected between H-3′ and H-7, H-8β and the C-13 methyl group, and the methyl group and H-10, but not between H-10 and H-8α. These results lead to the conclusion that **3** is the stereoisomer of **4** at C-2′ as shown in Fig. 4. According to this model, the downfield shift of H-3′ and H-8β in **4** could be explained reasonably: H-3′ of **4** is located in the shielding field induced by ring current whereas that of **3** is away from the aromatic ring; the electronegative nitrogen atom at 1′-position is spatially much closer to H-8β in **4** than **3**.

Structure of Kosinostatin (1)

As discussed above, because the isomerization of **1** to **2** is based on the inversion at the C-2′ spiro carbon in the aglycon, the sugar moiety of **1** and **2** was concluded to be identical. The ^{1}H NMR spectrum of the methyl glycoside obtained by the methanolysis of **1** was in good accordance with that of methyl 4-*C*-acetyl-2,6-dideoxy-*xylo*-hexopyranoside previously reported[3]. The NMR assignment of the deoxysugar moiety is summarized in Table 3. The glycosylation site at the C-10 hydroxyl group was confirmed by the long-range coupling between the anomeric proton H-1″ and H-10. The anomeric

Table 3. NMR data of kosinostatin (**1**) and isoquinocycline B (**2**) in DMSO-d_6.

Position	1		2	
	^{13}C	^{1}H	^{13}C	^{1}H
1	161.69		161.58	
2	124.95	7.44 (1H, dd, 1.2 and 8.0)	124.78	7.42 (1H, dd, 1.2 and 8.4)
3	137.49	7.86 (1H, t, J=8.0 Hz)	137.29	7.84 (1H, t, 8.0)
4	119.15	7.80 (1H, dd, J=1.2 and 8.0 Hz)	119.03	7.77 (1H, dd, 1.2 and 7.6)
4a	132.86		132.94	
5	187.45		187.53	
5a	114.69		114.58	
6	158.98		158.11	
6a	133.48		133.71	
7	40.65	3.89 (1H, d, 3.6)	43.47	3.90 (1H, d, 3.6)
8	34.93	2.09 (1H, d, 12.0)	34.33	2.15 (1H, d, 11.6)
		2.39 (1H, dd, 3.6 and 12.0)		2.53 (1H)*
9	81.67		83.14	
10	77.09	4.44 (1H, s)	76.41	4.43 (1H, s)
10a	144.74		144.31	
11	120.28	7.78 (1H, s)	119.97	7.76 (1H, s)
11a	131.47		131.47	
12	187.12		186.99	
12a	115.64		115.75	
13	23.18	1.51 (3H, s)	22.54	1.58 (3H, s)
2'	113.07		112.75	
3'	130.20	6.50 (1H, br.s)	126.71	5.09 (1H, br.s)
3a'	140.32		142.39	
4'	21.46	2.62 (2H, m)	21.61	2.52 (2H)*
5'	57.70	3.94 (2H, m)	57.28	3.98 (2H, m)
6a'	169.97		169.43	
1"	95.88	5.37 (1H, t, 4.8)	95.68	5.33 (1H, t, 4.8)
2"	32.93	1.71 (1H, ddd, 4.8, 8.0 and 14.4)	32.84	1.70 (1H, ddd, 4.4, 7.2 and 14.4)
		2.05 (1H, dt, 14.4 and 4.8)		2.04 (1H, ddd, 4.4, 5.6 and 14.4)
3"	70.19	3.77 (1H, m)	70.08	3.77 (1H, m)
4"	80.85		80.73	
5"	66.27	4.48 (1H, q, 6.4)	66.11	4.49 (1H, q, 6.4)
6"	14.61	1.03 (3H, d, 6.4)	14.55	1.04 (3H, d, 6.8)
7"	210.87		210.76	
8"	26.78	2.15 (3H, s)	26.69	2.15 (3H, s)
3"-OH		4.95 (1H, br.s)		4.91 (1H, br.s)
4"-OH		5.52 (1H, br.s)		5.49 (1H, br.s)

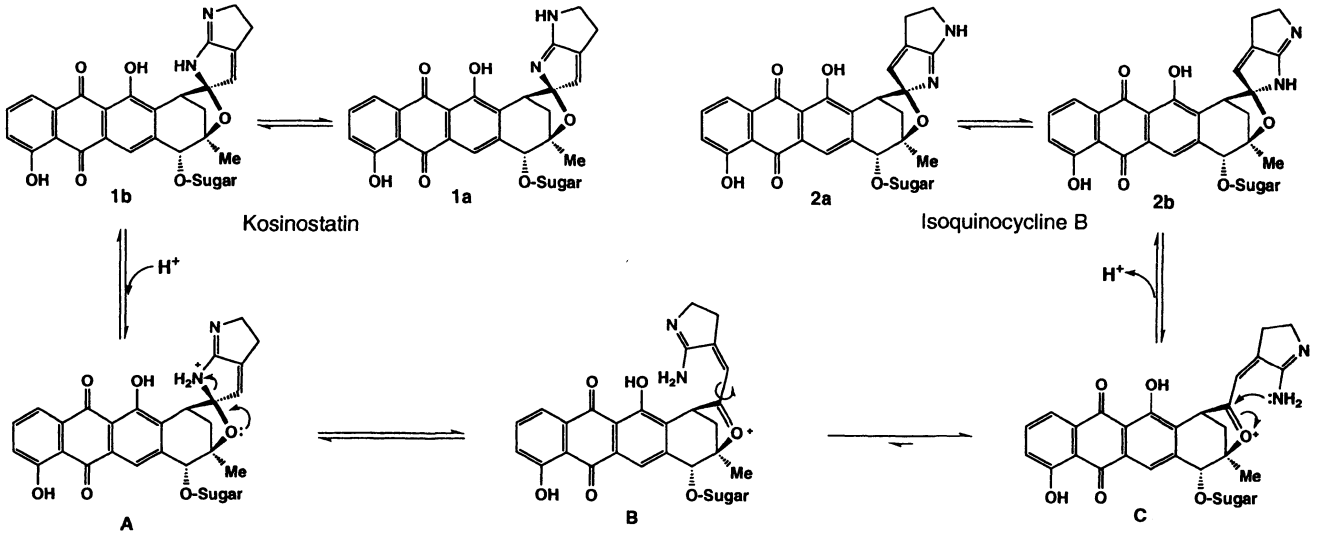
^{1}H and ^{13}C NMR were measured at 400 MHz and 100 MHz respectively.

The DMSO-d_6 signals (2.50 ppm for ^{1}H; 39.5 ppm for ^{13}C) were used as references.

Integral, multiplicities and the coupling constants (Hz) are in parentheses.

* overlapped with the solvent signal.

Fig. 5. Plausible mechanism of isomerization from kosinostatin to isoquinocycline B.



configuration was determined to be α on the basis of the coupling constants $J_{1'',2''eq}=J_{1'',2''ax}=4.8$ Hz. We, therefore, determined the structure of kosinostatin (**1**) to be the stereoisomer of isoquinocycline B (**2**) regarding to the C-2′ spiro carbon (Fig. 1).

Isoquinocycline B was originally isolated from *Streptomyces aureofaciens* along with quinocyclines A and B, and isoquinocycline A by CELMER *et al.*[2] Whereas the structures of quinocyclines are not yet elucidated, that of isoquinocycline A was determined by X-ray crystallography[3] and isoquinocycline B was identified as its congener by degradation study[4]. CELMER reported that quinocyclines and isoquinocyclines showed the identical UV-vis spectra and quinocyclines A and B isomerized to isoquinocyclines A and B, respectively. These properties common to quinocycline B and kosinostatin prompted us to investigate the identity between them. The only physico-chemical data for quinocycline B available to the comparison was the optical rotation value, $[\alpha]_{Hg}+140$ (MeOH), though the wavelength and concentration was not indicated[2]. We first examined the optical rotation of isoquinocycline B isolated by us, and found that it showed similar value $[\alpha]$ +26 to the reported value $[\alpha]$ +24[2] when measured at 546 nm. At the same wavelength, kosinostatin showed $[\alpha]$ +116 which was a little smaller than the optical rotation of quinocycline B, $[\alpha]$ +140. Although these findings indicated that kosinostatin is identical with quinocycline B, direct comparison was not possible because the authentic sample of quinocycline B could not be obtained.

The mechanism of isomerization is proposed although no experimental evidence is available (Fig. 5). As described above, kosinostatin exists in two resonance structures, **1a** and **1b**. The first step is the protonation of the nitrogen at 1′-position of **1b** with the aprotic solvents, followed by the formation of the oxonium intermediate (**A**→**B**). Owing to the sp^2 character of the C-2′ carbon in this intermediate, the bond between C-2′ and C-3′ must exist in the plain made by C-7, C-2′, C-9 oxygen and C-9, that results in the steric repulsion between the 1′-amino group and the anthracyclinone plain. To avoid the repulsion, the amino group is then positioned in the opposite side of the anthracyclinone moiety *via* the rotation of the bond between C-2′ and C-3′ (**B**→**C**). Then, the nucleophilic attack of the amino group at C-2′ carbon terminates the reaction sequence with the release of the proton (**C**→**2b**). The reverse reaction practically does not occur because the equilibrium prefers **C** to **B**.

In summary, we determined the structure of kosinostatin, a quinocycline antibiotic, as a stereoisomer of isoquinocycline B regarding to the C-2′ spiro carbon using spectroscopic techniques. Comparison of physico-chemical properties indicated that kosinostatin is presumably identical with quinocycline B isolated by CELMER *et al.* from *Streptomyces aureofaciens*.

Experimental

Instrumental Analysis

Melting points were determined on a Yanagimoto apparatus and are uncorrected. NMR experiments were performed on a JEOL GSX-400 NMR spectrometer in the solvents specified. The FABMS spectra were measured on JEOL DX303 and JMS-HX110A spectrometers. The LC-MS/MS experimets were performed on a Hewlett Packard HP-1050 system with Finnigan MAT TSQ7000 mass spectrometer. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Shimadzu FT IR-300 spectrophotometer. The reported optical rotation values for quinocycline B and isoquinocycline B were measured with their hydrochloride salts. To adjust the condition, the measurement was performed in methanol containing a small amount of hydrochloric acid on a JASCO DIP-370 polarimeter using Hg lamp.

Isoquinocycline B (**2**)

Yellow orange powder; m.p. >155°C (dec); $[\alpha]^{25}_{Hg546}$ +26 (*c* 0.11, HCl-MeOH); HR-FABMS *m/z* 617.2138 $[M+H]^+$ (calcd *m/z* 614.2136 for $C_{33}H_{32}N_2O_{10}$).

Aglycon of Kosinostatin (**3**)

1 (8 mg, 13 μmol) was dissolved in 0.4 N HCl in methanol (8 ml). After incubating at 32°C for 3 hours, the solution was diluted with excess ice-water and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give the methyl glycoside of deoxysugar as a mixture of α- and β-isomer (0.8 mg, 30% yield). The water layer was adjusted to pH 7.0 with diluted $NaHCO_3$ solution, extracted with ethyl acetate, dried over anhydrous Na_2SO_4 and evaporated to dryness. This was further purified by ODS column chromatography as described in the preceding paper[1] to afford the aglycon of kosinostatin (4 mg, 70% yield) as a dark yellow powder: m.p. >155°C (dec); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57 (3H, s, H-13), 2.17 (2H, d, 2.5 Hz, H-8), 2.72 (2H, m, H-4′), 4.08 (2H, m, H-5′), 4.13 (1H, t, 2.5 Hz, H-7), 4.44 (1H, s, H-10), 6.19 (1H, br.s, H-3′), 7.33 (1H, dd, 1 and 8.5 Hz, H-2), 7.70 (1H, dd, 7.5 and 8.5 Hz, H-3), 7.86 (1H, dd, 1 and 7.5 Hz, H-4), 8.01 (1H, s, H-11); HRFAB-MS *m/z* 445.1393 $[M+H]^+$ (calcd *m/z* 445.1400 for $C_{25}H_{21}N_2O_6$).

Aglycon of Isoquinocycline B (**4**)

In the same manner as described above, HCl treatment of **3** (4 mg) in methanol gave **4** (2 mg) as a yellow orange powder: m.p. >155°C (dec); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.69 (3H, s, H-13), 2.18 (1H, d, 12.5 Hz, H-8), 2.56 (1H, dd, 3.5 and 12.5 Hz, H-8), 2.68 (2H, m, H-4′), 4.10 (2H, m, H-5′), 4.10 (1H, d, 4 Hz, H-7), 4.46 (1H, s, H-10), 5.25 (1H, t, 3 Hz, H-3′), 7.35 (1H, dd, 1 and 8.5 Hz, H-2), 7.71 (1H, dd, 7.5 and 8.5 Hz, H-3), 7.86 (1H, dd, 1 and 7.5 Hz, H-4), 8.00 (1H, s, H-11); HRFAB-MS *m/z* 445.1404 $[M+H]^+$ (calcd *m/z* 445.1400 for $C_{25}H_{21}N_2O_6$).

Acknowledgments

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References

1) FURUMAI, T.; Y. IGARASHI, H. HIGUCHI & T. OKI: Kosinostatin, a quinocycline antibiotic with antitumor activity from *Micromonopora* sp. TP-A0468. J. Antibiotics 55: 128~133, 2002
2) CELMER, W. D.; K. MURAI, K. V. RAO, F. W. TANNER, Jr. & W. S. MARSH: The quinocycline complex. I. Isolation and characterization. *In* Antibiotics Annual 1957–1958, pp. 484~492, New York, Medical Encyclopedia, Inc., 1958
3) TULINSKY, A.: The structure of isoquinocycline A. An X-ray crystallographic determination. J. Am. Chem. Soc. 86: 5368~5369, 1964
4) MATERN, U.; H. GRISEBACH, W. KARL & H. ACHENBACH: Structure of the sugar components of the quinocycline complex. Eur. J. Biochem. 29: 1~4, 1972