New Cytotoxic Bicyclic Hexapeptides, RA-XXIII and RA-XXIV, from *Rubia cordifolia* L.

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Two new bicyclic hexapeptides, RA-XXIII and RA-XXIV, were isolated from the roots of *Rubia cordifolia* L. (Rubiaceae). Their structures were determined by the analysis of their 2D NMR spectra, chemical methods, and X-ray crystallography. The IC_{50} values of RA-XXIII and RA-XXIV against P-388 leukemia cells were 0.16 and 0.48 μg/ml, respectively.

Key words *Rubia cordifolia*; RA-XXIII; RA-XXIV; peptide; cytotoxicity; X-ray crystallography

A number of antitumor bicyclic peptides of RA-series (RAs) have been isolated from the plants, *Rubia cordifolia* L. and *R. akane* Nakai (Rubiaceae).[1)] Of them, RA-VII (**1**),[2)] showing the most potent antitumor activity, was subjected to clinical trials in Japan as a possible potential anti-cancer drug in the 1980—90s.[3)] Their antitumor action is considered to be due to inhibition of protein synthesis through interaction with eukaryotic ribosomes,[4,5)] and peptide **1** is known to cause conformational changes in F-actin and stabilization of actin filaments to induce G2 arrest.[6)] In the present study, two new RA-series peptides, RA-XXIII (**2**) and RA-XXIV (**3**), were further isolated from the roots of *R. cordifolia*. This paper describes their isolation, structure determination, and cytotoxicity against P-388 cells.

A methanol extract obtained from dried roots of *R. cordifolia* (50 kg) was partitioned between chloroform and water. The chloroform-soluble portion was subjected to a series of column chromatography using silica gel, alumina, and then aminopropyl-bonded silica gel, eluting with a series of chloroform/methanol mixtures, to give a fraction rich in peptides of RAs. The residue of this fraction, obtained after removal of the solvent, was crystallized from methanol to give crystals of crude RAs and a mother liquor. The mother liquor afforded, after separation by reversed-phase HPLC (ODS) and subsequent crystallization from methanol, RA-XXIII (**2**, 141 mg, 2.8×10^{-4}%) and RA-XXIV (**3**, 281 mg, 5.6×10^{-4}%).

RA-XXIII (**2**) was obtained as colorless fine needles. Its molecular formula was determined to be $C_{43}H_{53}N_7O_{10}$ from the $[M+H]^+$ peak at *m/z* 828.3997 (Calcd for $C_{43}H_{54}N_7O_{10}$, 828.3932) in the HR-ESI-MS. The ^{1}H- and ^{13}C-NMR spectra of **2** in pyridine-d_5 (Table 1) showed signals typical of an RA-series peptide, and also demonstrated the presence of two conformers in a ratio of 79 : 21.[7,8)] The structure of **2** was determined by the analysis of the resonances caused by the major conformer, which included signals for two secondary methyl groups (δ_H/δ_C 1.38/19.1, 1.53/21.7), three *N*-methyl groups (δ_H/δ_C 3.01/29.7, 3.02/30.3, 3.31/40.1), two *O*-methyl groups (δ_H/δ_C 3.65/55.1, 3.86/56.2), five methylenes (δ_H/δ_C 2.46—2.59/27.5; 2.61—2.74/31.8; 2.62, 3.65/36.8; 3.36, 3.56/36.1; 3.89/33.9), six N-substituted methines (δ_H/δ_C 4.10/68.8, 5.00/57.7, 5.10/46.8, 5.15/48.3, 5.47/48.9, 5.75/54.4), two 1,4-disubstituted benzene rings (δ_C 132.0, 158.8 and δ_H/δ_C 7.01/114.5×2, 7.35/130.8×2; δ_C 136.1, 158.7 and δ_H/δ_C 6.94/126.4, 6.96/124.5, 7.21/131.0, 7.46/133.5), one 1,2,4-trisubstituted benzene ring (δ_C 129.9, 147.1, 153.6 and δ_H/δ_C 4.59/114.8, 6.78/121.5, 6.97/113.5), seven amide carbonyl groups (δ_C 168.7, 169.9, 171.2, 172.1, 172.7, 172.9, 174.4), and five amide protons (δ_H 7.38, 7.71, 8.07, 8.70, 9.81). The analysis of its ^{1}H–^{1}H COSY, HMBC, and NOESY spectra and their comparison with the data of the known peptide **1** revealed the features of the component amino acid units and the amino acid sequence of **2** (Fig. 2). The ^{1}H–^{1}H COSY correlations revealed that the C-α of amino acid at position 2 was connected to an ethylene unit (C-β and C-γ). HMBC correlations from H_2-β (δ_H 2.46—2.59) and H_2-γ (δ_H 2.61—2.74) to the carbonyl carbon at δ_C 174.4, and NOE correlations between the amide protons at δ_H 7.71 and 8.07 and H_2-γ suggested that C-δ constituted a carboxamide group. The profiles of the chemical shifts of the ^{13}C-NMR signals and NOE correlations due to the peptide backbone in **1** and **2** were very similar to each other, indicating that **1** and **2** shared the same relative configuration. These observations suggested that peptide **2** was an analogue of **1** whose Ala-2 was replaced by glutamine, which was verified by the preparation of peptide **2** by ammonolysis of RA-IX (**4**), another RA-series peptide obtained from the same plant source previously.[9)] The structure of **2** was confirmed to be as shown in Fig. 1 by X-ray crystallography of bromide **2a** derived from **2** (Fig. 3). The absolute structure parameter (Flack parameter) was 0.040 with an esd of 0.012, which indicated that the absolute structure shown in the figure represents the correct enantiomer.

RA-XXIV (**3**) was obtained as colorless needles. Its molecular formula was determined to be $C_{42}H_{51}N_7O_{10}$ from the $[M+H]^+$ peak at *m/z* 814.3829 (Calcd for $C_{42}H_{52}N_7O_{10}$, 814.3776) in the HR-ESI-MS. The ^{1}H- and ^{13}C-NMR spectra of **3** were very similar to those of **2**, except that the ^{1}H-NMR spectrum of **3** had only one methoxyl signal with a newly appeared phenolic hydroxyl signal at δ_H 11.72. This phenolic hydroxyl signal showed a cross-peak with H-εa in the NOESY spectrum, thus indicating that in **3**, a hydroxyl group substituted for the methoxyl group at the ζ-position of Tyr-6 in **2**. A downfield shift of the ^{13}C-NMR signal for C-εa in Tyr-6, from δ_C 113.5 for **2** to δ_C 117.8 for **3**, also explains the replacement of the *O*-methyl group by a hydroxyl group. Treatment of **3** with (trimethylsilyl)diazomethane afforded a product which was shown to be identical to natural **2** by their spectroscopic data and optical rotations. Thus, the absolute structure of **3** was determined to be as shown in Fig. 1.

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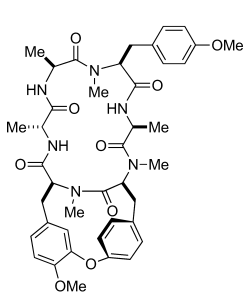
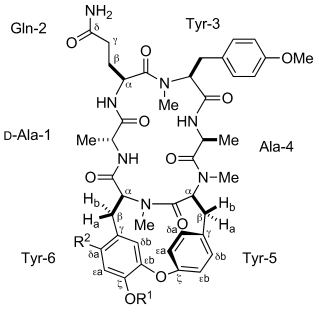
Table 1. NMR Data for the Major Conformers of RA-XXIII (**2**) and RA-XXIV (**3**) in Pyridine-d_5 at 300 K

Position		**2**		**3**	
		δ_H [a]	δ_C [b]	δ_H [a]	δ_C [b]
D-Ala-1	α	5.15 (m)	48.3	5.16 (m)	48.2
	β	1.53 (d, 6.9, 3H)	21.7	1.52 (d, 7.1, 3H)	21.7
	C=O		172.7		172.8
	NH	8.70 (d, 8.0)		8.73 ([c])	
Gln-2	α	5.47 (m)	48.9	5.47 (m)	48.9
	β	2.46—2.59 (m, 2H)	27.5	2.47—2.60 (m, 2H)	27.5
	γ	2.61—2.74 (m, 2H)	31.8	2.62—2.7 (m, 2H)	31.8
	$CONH_2$	7.71, 8.07 (br s)	174.4	7.73, 8.10 (br s)	174.4
	C=O		172.9		172.9
	NH	9.81 (d, 8.5)		9.83 (d, 8.3)	
Tyr-3	α	4.10 (t, 7.5)	68.8	4.09 (t, 7.5)	68.8
	β	3.89 (d, 7.5, 2H)	33.9	3.89 (d, 7.5, 2H)	33.9
	γ		132.0		132.0
	δ	7.35 (d-like, 8.6, 2H)	130.8[d]	7.35 (d-like, 8.6, 2H)	130.8[d]
	ε	7.01 (d-like, 8.6, 2H)	114.5[d]	7.01 (d-like, 8.6, 2H)	114.5[d]
	ζ		158.8		158.8
	C=O		168.7		168.7
	NMe	3.31 (s, 3H)	40.1	3.31 (s, 3H)	40.1
	OMe	3.65 (s, 3H)	55.1	3.64 (s, 3H)	55.1
Ala-4	α	5.10 (m)	46.8	5.11 (m)	46.8
	β	1.38 (d, 6.7, 3H)	19.1	1.38 (d, 6.6, 3H)	19.1
	C=O		172.1		172.1
	NH	7.38 (d, 7.8)		7.39 (d, 7.6)	
Tyr-5	α	5.75 (dd, 11.5, 3.3)	54.4	5.77 (dd, 11.5, 3.2)	54.5
	βa	2.62 (dd, 11.5, 3.3)	36.8	2.63 (dd, 11.5, 3.2)	36.8
	βb	3.65 (t, 11.5)		3.66 (t, 11.5)	
	γ		136.1		136.1
	δa	7.46 (dd, 8.3, 2.2)	133.5	7.46 (dd, 8.3, 2.2)	133.5
	δb	7.21 (dd, 8.2, 2.2)	131.0	7.22 ([c])	130.9
	εa	6.96 (dd, 8.3, 2.4)	124.5	6.92 (dd, 8.3, 2.4)	124.6
	εb	6.94 (dd, 8.2, 2.4)	126.4	6.97 (dd, 8.3, 2.4)	126.5
	ζ		158.7		158.9
	C=O		169.9		169.9
	NMe	3.02 (s, 3H)	30.3	3.02 (s, 3H)	30.3
Tyr-6	α	5.00 (dd, 12.0, 3.5)	57.7	5.02 (dd, 11.7, 3.5)	57.9
	βa	3.36 (dd, 18.0, 12.0)	36.1	3.34 (dd, 17.9, 11.7)	36.3
	βb	3.56 (dd, 18.0, 3.5)		3.56 (dd, 17.9, 3.5)	
	γ		129.9		128.1
	δa	6.78 (dd, 8.3, 1.9)	121.5	6.77 (dd, 8.1, 1.7)	122.1
	δb	4.59 (d, 1.9)	114.8	4.66 (d, 1.7)	115.0
	εa	6.97 (d, 8.3)	113.5	7.23 (d, 8.1)	117.8
	εb		153.6		152.7
	ζ		147.1		145.4
	C=O		171.2		171.3
	NMe	3.01 (s, 3H)	29.7	3.04 (s, 3H)	29.7
	OMe	3.86 (s, 3H)	56.2		
	OH			11.72 (br s)	

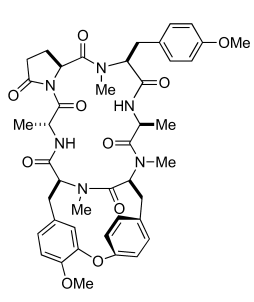
a) ^{1}H spectrum recorded at 500 MHz, chemical shifts referenced to residual pyridine-d_4 (7.21 ppm). *J*-values given in Hz in parentheses. *b*) ^{13}C spectrum recorded at 125 MHz, chemical shifts referenced to the solvent (135.5 ppm). *c*) Multiplicity patterns were unclear due to signal overlapping. *d*) Two carbons.



1, RA-VII



2, RA-XXIII: R^1 = Me, R^2 = H
2a: R^1 = Me, R^2 = Br
3, RA-XXIV: R^1 = R^2 = H



4, RA-IX

Fig. 1

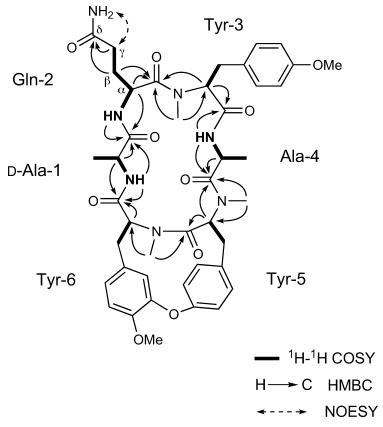


Fig. 2. Key ^{1}H–^{1}H COSY, HMBC, and NOESY Correlations for Determination of the Peptide Sequence of **2**

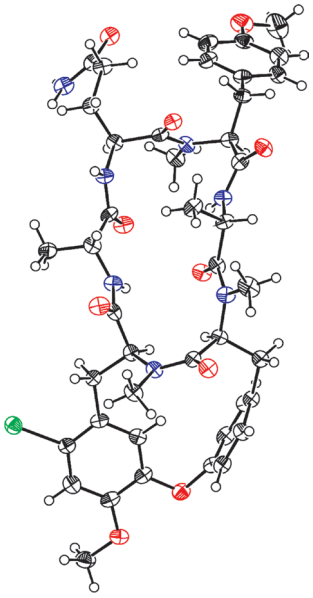


Fig. 3. Crystal Structure of Compound **2a**

Peptides **2** and **3** both showed moderate cytotoxicity against P-388 murine leukemia cells with IC_{50} values of 0.16 and 0.48 μg/ml, respectively.

Experimental

General Experimental Procedures Melting points were determined on a Yanaco MP-3 apparatus and are recorded uncorrected. Optical rotations were measured on a JASCO P-1030 digital polarimeter, IR spectra on a JASCO FT/IR 620 spectrophotometer, and UV spectra on a JASCO V-530 spectrophotometer. NMR spectra were measured on a Bruker DRX-500 spectrometer at 300 K. The ^{1}H chemical shifts in pyridine-d_5 were referenced to the residual pyridine-d_4 (7.21 ppm), and the ^{13}C chemical shifts to the solvent (135.5 ppm). Mass spectra were obtained with a Micromass LCT spectrometer. Single-crystal X-ray analysis was carried out on a Bruker AXS APEX II ULTRA CCD area detector diffractometer with a rotating anode source (MoKα radiation, λ=0.71073 Å). Preparative HPLC was carried out on a Shimadzu LC-6AD pump unit equipped with a SPD-10A UV detector (λ 254 nm) and a pre-packed ODS column (5 μm, 20×250 mm), eluting with a MeOH/H_2O mixture at a flow rate of 10 ml/min.

Plant Material The roots of *Rubia cordifolia* L. were commercially obtained in Tokyo in March 2004. The material was identified by Prof. Koichi Takeya, and a voucher specimen (Tko-0403-01) has been deposited at the Herbarium of Tokyo University of Pharmacy and Life Sciences.

Extraction and Isolation The dried roots (50 kg) of *R. cordifolia* was extracted with MeOH (3×175 l). After removal of MeOH under reduced pressure, the residue (3.6 kg) was partitioned between chloroform and water. The chloroform-soluble portion (993 g) was placed on a column of silica gel (Merck, 70—230 mesh, 3.6 kg) and eluted with $CHCl_3$ (9 l), EtOAc (18 l), and $CHCl_3$/MeOH (9 : 1, 27 l), sequentially to give three fractions. After removal of the solvent, the residue of the $CHCl_3$/MeOH (9 : 1) fraction (152 g) was subjected to alumina (Merck, 3 kg) column chromatography (CC) eluting sequentially with $CHCl_3$ (2 l) and $CHCl_3$/MeOH (9 : 1, 12 l). After evaporation, the $CHCl_3$/MeOH (9 : 1) fraction (41.9 g) was subjected to aminopropyl-bonded silica gel (Chromatorex, 200—350 mesh, 300 g) CC eluting sequentially with $CHCl_3$ (6 l) and $CHCl_3$/MeOH (9 : 1, 1 l). The residue obtained after removal of the solvent of the $CHCl_3$/MeOH (9 : 1) eluate was crystallized from methanol to give crystals of crude RAs (2.4 g) and mother liquor (ML). After removal of the solvent, ML (6.2 g) was subjected to ODS HPLC (MeOH/H_2O 60 : 40) to give four fractions, F1 (0.52 g), F2 (1.07 g), F3 (0.31 g), and F4 (0.17 g). F1 and F3 were each crystallized from methanol to give RA-XXIV (**3**) (281 mg, 5.6×10^{-4}%) and RA-XXIII (**2**) (141 mg, 2.8×10^{-4}%), respectively.

RA-XXIII (**2**): Colorless fine needles, 254—256 °C (MeOH), $[\alpha]_D^{26}$ −184.2° (c=0.12, $CHCl_3$). IR (film) ν_{max} 3376, 2930, 1660, 1634, 1513, 1415, 1025 cm^{-1}. UV (MeOH) λ_{max} (log ε) 203 (4.53), 224sh (4.15), 277 (3.27) nm. ^{1}H- and ^{13}C-NMR, a mixture of two conformers in a ratio of 79 : 21 in pyridine-d_5 at 300 K. For the data of the major conformer, refer to Table 1. HR-ESI-MS m/z 828.3997 ([M+H]$^+$, Calcd for $C_{43}H_{54}N_7O_{10}$, 828.3932).

RA-XXIV (**3**): Colorless needles, 258—261 °C (MeOH), $[\alpha]_D^{26}$ −168.5° (c=0.09, MeOH). IR (film) ν_{max} 3366, 2930, 1653, 1635, 1507, 1417, 1026 cm^{-1}. UV (MeOH) λ_{max} (log ε) 205 (4.82), 224sh (4.53), 277 (3.64) nm. ^{1}H- and ^{13}C-NMR, a mixture of two conformers in a ratio of 79 : 21 in pyridine-d_5 at 300 K. For the data of the major conformer, refer to Table 1. HR-ESI-MS m/z 814.3829 ([M+H]$^+$, Calcd for $C_{42}H_{52}N_7O_{10}$, 814.3776).

Ammonolysis of 4 Saturated aqueous ammonia solution (0.5 ml) was added to a solution of **4** (5.0 mg, 0.0062 mmol) in MeOH (0.5 ml) in a pressure tube. The tube was sealed and immersed in an oil bath preheated to 50 °C. After heating (50 °C) for 23 h, the volatiles were evaporated and the residue was subjected to ODS HPLC (MeOH/H_2O 35 : 65). The compound obtained [1.3 mg, 25%, $[\alpha]_D^{26}$ −185.8° (c=0.06, $CHCl_3$)] was shown to be identical to natural **2** by comparison of their ^{1}H- and ^{13}C-NMR spectra, mass spectra, and optical rotations.

Bromination of 2 Pyridinium hydrobromide perbromide (*ca.* 85% purity, 13.7 mg, 0.0364 mmol) and sodium acetate (3.0 mg, 0.037 mmol) were added to a solution of **2** (20.1 mg, 0.0243 mmol) in MeOH/EtOAc (1 : 1, 1 ml) at 0 °C. The mixture was stirred at 0 °C for 1 h, and then at room temperature for 16 h. The mixture was diluted with chloroform (20 ml), washed sequentially with 5% aqueous sodium sulfite (5 ml) and brine (10 ml), dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure, and the residue was separated by ODS HPLC (MeOH/H_2O 35 : 65) to afford **2a** (15.7 mg, 71%) as colorless needles, mp 256—259 °C (MeOH). ^{1}H-NMR (500 MHz, pyridine-d_5, 300 K, major conformer) δ: 9.80 (1H, d, J=8.3 Hz, Gln-2 NH), 8.90 (1H, d, J=8.1 Hz, D-Ala-1 NH), 8.09 and 7.72 (each 1H, br s, Gln-2 $CONH_2$), 7.47 (1H, d, J=8.3, 2.1 Hz, Tyr-5 H-δa), 7.39 (1H, d, J=7.8 Hz, Ala-4 NH), 7.34 (2H, d-like, J=8.3 Hz, Tyr-3 H_2-δ), 7.34 (1H, s, Tyr-6 H-εa), 7.25 (1H, dd, J=8.3, 2.1 Hz, Tyr-5 H-δb), 7.03 (1H, dd, J=8.3, 2.1 Hz, Tyr-5 H-εa), 7.00 (2H, d-like, J=8.3 Hz, Tyr-3 H_2-ε), 6.84 (1H, dd, J=8.3, 2.1 Hz, Tyr-5 H-εb), 5.75 (1H, dd, J=11.5, 3.2 Hz, Tyr-5 H-α), 5.48 (1H, m, Gln-2 H-α), 5.18 (1H, m, D-Ala-1 H-α), 5.11 (1H, m, Ala-4 H-α), 4.89 (1H, dd, J=11.5, 3.3 Hz, Tyr-6 H-α), 4.53 (1H, s, Tyr-6 H-δb), 4.09 (1H, t, J=7.8 Hz, Tyr-3 H-α), 3.89 (2H, d, J=7.8 Hz, Tyr-3 H_2-β), 3.85 (3H, s, Tyr-6 OMe), 3.64 (1H, t, J=11.5 Hz, Tyr-5 H-βb), 3.64 (3H, s, Tyr-3 OMe), 3.52 (1H, dd, J=18.5, 11.5 Hz, Tyr-6 H-βa), 3.45 (1H, dd, J=18.5, 3.3 Hz, Tyr-6 H-βb), 3.29 (3H, s, Tyr-3 NMe), 3.04 (3H, s, Tyr-5 NMe), 3.00 (3H, s, Tyr-6 NMe), 2.73—2.60 (3H, m, Gln-2 H_2-γ and Tyr-5 H-βa), 2.60—2.45 (2H, m, Gln-2 H_2-β), 1.54 (3H, d, J=6.8 Hz, D-Ala-1 H_3-β), 1.39 (3H, d, J=6.8 Hz, Ala-4 H_3-β); ^{13}C-NMR (125 MHz, pyridine-d_5, 300 K, major conformer, δ) D-Ala-1 (172.7 C=O, 48.3 α, 21.7 β), Gln-2 (174.4 δ, 172.9 C=O, 48.8 α, 31.8 γ, 27.6 β), Tyr-3 (168.7 C=O, 158.8 ζ, 132.0 γ, 130.8 δ×2, 114.5 ε×2, 68.8 α, 55.1 OMe, 40.1 NMe, 33.9 β), Ala-4 (172.2 C=O, 46.9 α, 19.1 β), Tyr-5 (170.2 C=O, 157.7 ζ, 136.4 γ, 133.3 δa, 131.6 δb, 125.9 εb, 124.2 εa, 54.4 α, 36.9 β, 30.4 NMe), Tyr-6 (170.8 C=O, 153.1 εb, 147.7 ζ, 128.6 γ, 116.9 εa, 115.1 δb, 114.7 δa, 57.9 α, 56.4 OMe, 37.2 β, 29.5 NMe). HR-ESI-MS m/z 928.2991 ([M+Na]$^+$, Calcd for $C_{43}H_{52}N_7O_{10}NaBr$, 928.2857).

X-Ray Crystallographic Study of 2a $C_{43}H_{52}BrN_7O_{10}\cdot3(O)$, M= 954.83, 0.38×0.05×0.02 mm, monoclinic, $P2_1$, a=11.424(5) Å, b= 12.540(5) Å, c=17.173(7) Å, β=106.607(4)°, V=2357.5(16) Å^3, Z=2, D_x=1.345 Mg m^{-3}, μ(MoKα)=0.941 mm^{-1}, 25646 reflections collected,

10355 unique, $R_{int}=0.0647$, $R1=0.0677$, $wR2=0.1727$ (observed data), GOF=0.823; $R1=0.1094$, $wR2=0.2083$ (all data), absolute structure parameter 0.040(12). The structure was solved by direct methods using SHELXS-97,[10] and refined by full-matrix least-squares on F^2 using SHELXL-97.[11]

CCDC 672443 contains the supplementary crystallographic data for compound **2a** reported in this paper. These data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/data_request/cif, or by e-mailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, U.K.; fax: +44 1223 336033.

O-Methylation of 2 (Trimethylsilyl)diazomethane (117 μl of a 2.0 M solution in diethyl ether, 0.234 mmol) was added to a stirred solution of **3** (5.3 mg, 0.0065 mmol) in MeCN/MeOH (9 : 1, 0.5 ml) at room temperature. After stirring at room temperature for 2 d, the mixture was concentrated *in vacuo*. The residue was subjected to ODS HPLC (MeOH/H_2O 35 : 65) to provide a compound [3.4 mg, 63%, $[\alpha]_D^{26}$ −169.1° ($c=0.17$, $CHCl_3$)], which was shown to be identical to natural **2** by comparison of their ^{1}H- and ^{13}C-NMR spectra, mass spectra, and optical rotations.

Assay for Cytotoxic Activity The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed on a 96-well plate. Murine P-388 leukemia cells (3×10^3 cells) in 100 μl of RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% fetal calf serum (Mitsubishi Chemical Industry Co., Ltd., Tokyo, Japan) and kanamycin (100 μg/ml) were placed into each well and incubated at 37 °C in a humidified atmosphere of 7% CO_2. After 24 h incubation, samples of test compounds at various concentrations (10 μl) were added to the cultures, and the mixtures were incubated for 48 h at 37 °C. Then, 20 μl of an MTT solution (5 mg/ml) was added to each well. After a further incubation for 4 h, 100 μl of 10% sodium dodecyl sulfate–0.01 M HCl solution was added to each well and the formazan crystals formed in each well were dissolved by stirring with a pipette. Optical density was recorded on a microplate reader (Tosoh MPR-A4i) at 550 nm. The cytotoxic activities represent the average of three replicate measurements for each test.

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