Preparation and Cytotoxicity of Cyclic Hexapeptides, RA Derivatives[1)]

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Several aromatic ring substituent modified RA derivatives were prepared from RA-VII (1), RA-V (8) and RA-II (11), and evaluated for cytotoxicity against P388 leukemia and KB cells. In terms of IC_{50} values, the C_ζ methoxyl group of Tyr-3 greatly influenced the activities, while the substituents at the C_ζ position of Tyr-6 were less important. One of the derivatives, Tyr-6-C_ζ-deoxyRA-V (9, P388, IC_{50}, 0.0025 μg/ml) was nearly as active as RA-VII (1, 0.0013 μg/ml), and also expressed promising anti-P388 *in vivo* activity (test/control = 171%, at 25 mg/kg).

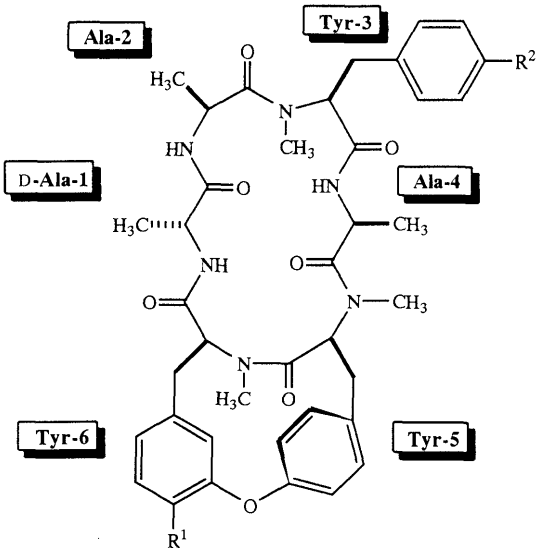
Keywords RA-VII; cytotoxicity; antitumor activity; cyclic hexapeptide; demethoxyRA; de-*O*-methylRA

Introduction

RA series bicyclic hexapeptides originally isolated from the roots of *Rubia akane* and *R. cordifolia*[2)] showed significant antileukemic and antitumor activities. As shown in Fig. 1, their structures are characterized by both 18- and 14-membered ring systems with unique isodityrosine structure. Their structure elucidation,[3)] physiological activities,[4)] total synthesis,[5)] conformational analysis[6)] and modifications on Tyr-6 aromatic ring of RA-V[7)] have been reported. A report of an RA derivative lacking a diphenyl ether linkage with no antitumor activity by Bates *et al.*,[8)] and recent synthetic studies of RA analogues by Boger *et al.*[9)] suggested the 14-membered ring moiety to be biologically important. In view of these results, we investigated modifications on the aromatic ring of Tyr-3 and/or Tyr-6 of RAs to elucidate the minimum structure requirements in the aromatic ring moiety for their cytotoxicities and antitumor activity.

This paper describes the preparation and cytotoxic activities against P388 leukemia and KB cells of aromatic ring substituent modified RA derivatives.

Chemistry Treatment of RA-VII (**1**) with boron tribromide (BBr_3) in dichloromethane (CH_2Cl_2) afforded the dide-*O*-methylRA-VII (**2**) in 66% yield. Treatment of **2** with *N*-phenyltrifluoromethanesulfonimide (Tf_2NPh, 3 eq) in CH_2Cl_2, followed by reduction of the resulting ditriflate (**3**) with formic acid (HCOOH) in the presence of palladium acetate ($Pd(OAc)_2$)[10)] gave didemethoxyRA-VII (**4**) in 88% (from **2**) yield. In order to obtain the demethoxyRA-V (**7**), **2** was triflated under a controlled condition with 1.5 eq of Tf_2NPh to afford the desired dide-*O*-methylRA-VII Tyr-3-*O*-triflate (**5**, 27%, yield), along with **3** (34%), dide-*O*-methylRA-VII Try-6-*O*-triflate (**6**, 19%), and recovered **2** (20%). The triflated position was determined by a comparison of proton nuclear magnetic resonance (^{1}H-NMR) spectra of the products. Resonances of Tyr-3 C_ε protons of **5** were shifted downfield by *ca.* 0.4 ppm relative to those of **2** and **6** due to deshielding effect of the triflate group, with no significant change in resonances of Tyr-6 aromatic protons. Reduction of **5** with HCOOH–$Pd(OAc)_2$ system furnished demethoxyRA-V (**7**) in 72% yield.



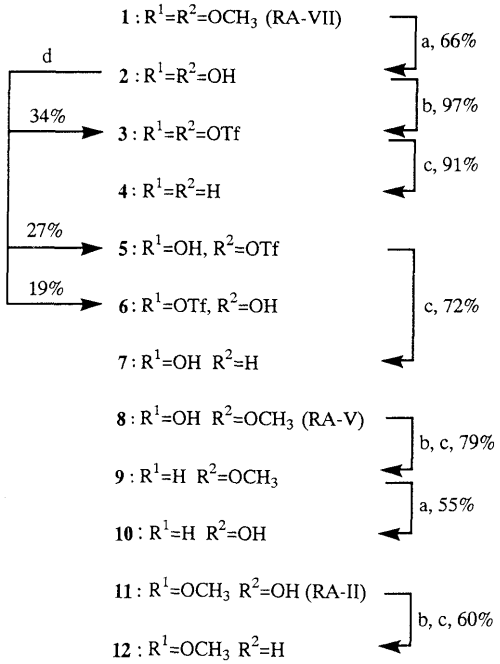


Fig. 1. Structure and Synthetic Scheme of RA Derivatives; Ala = L-alanine, Tyr = *N*-methyl-L-tyrosine, D-Ala = D-alanine
a) BBr_3, CH_2Cl_2, −78 °C—r.t. b) Tf_2NPh (3 eq), Et_3N, CH_2Cl_2, r.t. c) $Pd(OAc)_2$, DPPF, Et_3N, HCOOH, DMF, 60 °C. d) Tf_2NPh (1.5 eq), Et_3N, CH_2Cl_2, r.t.

TABLE I. ^{13}C-NMR Spectral Data of RA Derivatives (in $CDCl_3$, Major Conformer, 100 MHz, δ-Values)

Compound Amino acid	Carbon	2[a]	4	7	9	10	12
D-Ala-1	C_α	47.91	47.89	47.88	47.89	47.88	47.91
	C_β	20.66	20.66	20.66	20.67	20.63	20.66
Ala-2	C_α	44.71	44.58	44.63	44.56	44.65	44.58
	C_β	16.01	16.57	16.55	16.61	16.47	16.62
Tyr-3	C_α	68.59	68.32	68.34	68.37	68.35	68.31
	C_β	32.92	33.66	33.68	32.70	32.72	33.66
	C_γ	129.50	138.90	138.92	130.75	130.12	138.93
	C_δ	130.53	129.33	129.34	130.23	130.38	129.33
	C_ε	115.84	128.64	128.66	114.08	115.67	128.64
	C_ζ	156.08	126.69	126.71	158.45	155.07	126.68
	C_N	40.11	39.71	39.71	39.76	39.84	39.67
	C_O				55.27		
Ala-4	C_α	46.82	46.47	46.49	46.43	46.95	46.47
	C_β	18.25	18.49	18.51	18.50	18.46	18.51
Tyr-5	C_α	54.88	54.30	54.34	54.29	54.37	54.27
	C_β	37.02	36.99	36.93	36.99	36.97	37.01
	C_γ	135.52	135.18	135.68	135.18	135.13	135.16
	$C_{\delta a}$	133.06	132.79	133.04	132.78	132.77	133.40
	$C_{\delta b}$	131.06	131.06	131.05	131.04	131.05	130.99
	$C_{\varepsilon a}$	124.52	124.23	124.19	124.21	124.24	124.25
	$C_{\varepsilon b}$	126.35	125.85	125.89	125.83	125.90	125.91
	C_ζ	158.88	158.22	157.98	158.21	158.23	158.28
	C_N	30.73	30.53	30.54	30.52	30.55	30.52
Tyr-6	C_α	57.86	57.23	57.53	57.22	57.25	57.42
	C_β	35.94	36.13	35.66	36.13	36.15	35.53
	C_γ	127.53	137.04	127.67	137.02	136.98	128.21
	$C_{\delta a}$	116.76	114.41	115.77	114.40	114.45	112.40
	$C_{\delta b}$	121.81	120.97	121.68	120.96	121.00	120.93
	$C_{\varepsilon a}$	114.02	112.65	113.04	112.64	112.65	113.46
	$C_{\varepsilon b}$	143.89	164.23	143.04	164.22	164.23	153.18
	C_ζ	152.08	129.26	151.12	129.25	129.29	146.57
	C_N	29.72	29.32	29.37	29.32	29.40	29.29
	C_O						56.20
$C_{C=O}$		169.43	167.94	167.98	168.04	168.27	167.92
		169.99	169.27	169.18	169.26	169.34	169.34
		171.31	170.68	170.66	170.67	170.76	170.71
		172.03	171.78	171.78	171.78	171.76	171.78
		172.57	172.27	172.32	172.23	172.27	172.22
		173.43	172.63	172.65	172.52	172.80	172.53

a) CD_3OD–$CDCl_3$ (3 : 1).

A similar scheme was also applied for the preparation of Tyr-6-C_ζ-deoxyRA-V (**9**) and demethoxyRA-II (**10**) starting from RA-V (**8**), and of Tyr-3-C_ζ-deoxyRA-II (**12**) from RA-II (**11**). Deoxygenation of aromatic ring was confirmed by mass and NMR spectroscopy, and the appearance of an additional aromatic proton resonance and the upfield (*ca.* 20—30 ppm) of deoxygenated C_ζ resonance in ^{13}C-NMR spectrum were especially characteristic (Table I).

Biological Results and Discussion

The results of the cytotoxic activities of the prepared RA derivatives against P388 leukemia and KB cells are shown in Table II. Standard reference antitumor agent RA-VII (**1**) was included in each experimental run for purposes of comparison. Clear structure–activity relationships were observed in terms of IC_{50} values. In the Tyr-3 aromatic ring modified derivatives, the difference of the substituents considerably affected the IC_{50} values. Tyr-6-C_ζ-deoxyRA-V (**9**) lacking the Tyr-6 C_ζ oxygen function was nearly as effective as RA-VII (**1**), while demethoxyRA-II (**10**), in which the methoxyl group of **9** was replaced by hydroxyl group, was inactive, and didemethoxyRA-VII (**4**), the corresponding demethoxyl derivative of **9** was much less potent than **9**.

TABLE II. Cytotoxicity of RA Derivatives on P388 Leukemia and KB Cells

Compound	IC_{50} (μg/ml)	
	P388	KB
1 (RA-VII)	0.0013	0.0023
2	>10	7.81
4	0.37	0.84
7	0.031	0.36
8 (RA-V)	0.0027	0.0038
9	0.0025	0.0063
10	>10	>10
12	0.22	0.42

TABLE III. Antitumor Activity of RA-VII (**1**) and **9** on P388 Leukemia in Mice

Compound	T/C (%)						
	Dose (mg/kg)						
	0.4	0.8	1.6	3.13	6.25	12.5	25.0
1 (RA-VII)	144	144	152	163	Toxic		
9	119		131		150	161	171

On the other hand, a series of Tyr-6 modified derivatives **4**, **7** and **12**, which corresponded to the analogues in which each Tyr-3 unit was replaced by phenylalanine, showed weak activity. Another series of Tyr-6 modified derivatives RA-VII (**1**), RA-V (**8**) and **9**, possessing methoxyl group at Tyr-3 C_ζ position, retained high activity.

Tyr-6-C_ζ-deoxyRA-V (**9**) which expressed the most promising cytotoxicity among the derivatives prepared in this work was evaluated against P388 leukemia in mice (Table III). The *in vivo* antileukemic activity of **9** was rather weak compared to that of RA-VII (**1**), however the maximum test/control (T/C) value was superior. The less toxic property of **9** might offer a helpful suggestion on designing more promising RA derivatives as antitumor drugs.

Based on these results and the previous study[7] which showed several Tyr-6-*O*-alkylated RA-V derivatives expressing high cytotoxic activity, the following conclusion can be postulated. It is notable that the methoxyl group at Tyr-3 C_ζ position greatly enhanced the cytotoxicities, and the hydroxyl group profoundly reduced them compared with derivatives having no methoxyl or hydroxyl group at the same position, while the substituent at Tyr-6 C_ζ position little affected the activities. Furthermore, the observation that any series of derivatives having the same functions at the Tyr-3 C_ζ position showed a similar degree of activity in each group might suggest that the Tyr-3 portion participates in the cytotoxic effects.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Perkin Elmer 1710 spectrometer. Optical rotations were measured with a

JASCO DIP-4 automatic digital polarimeter, $[\alpha]_D$ values are given in 10^{-1} deg·cm^2 g^{-1}. The proton and carbon nuclear magnetic resonance spectra were recorded on a Bruker AM-400 spectrometer. Chemical shifts were expressed in ppm with tetramethylsilane as an internal standard. The mass spectra (MS) were taken with a Hitachi M-80 and a VG AutoSpec spectrometer. The ultraviolet (UV) and visible absorption spectra were recorded on a Shimadzu UV-240 spectrophotometer. Silica gel column chromatography was performed with a CIG column system (22 i.d. × 100 mm, Kusano Scientific Co., Tokyo) prepacked with 10 μ silica gel.

Dide-*O*-methylRA-V (2) BBr_3 (1.0 M in CH_2Cl_2, 2.5 ml, 2.5 mmol) was dropped to a solution of **1** (385.4 mg, 0.50 mmol) in CH_2Cl_2 (10 ml) at −78 °C. After stirring at room temperature for 24 h, the mixture was diluted with CH_2Cl_2, washed successively with H_2O, 1 N HCl, sat. $NaHCO_3$ and brine, and dried. The solvent was evaporated off *in vacuo* to leave a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–AcOEt–MeOH (15 : 2 : 1), followed by recrystallization from MeOH–isopropyl ether to give **2** (244.5 mg, 66%) as a colorless powder, mp 264 °C (dec.), $[\alpha]_D$ −189.3° (c = 0.15, MeOH). IR ν ($CHCl_3$): 3399, 1673 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 220 (4.49), 278 (3.66). High-resolution FAB-MS Calcd for $C_{39}H_{47}N_6O_9$: 743.3405 $[M+H]^+$. Found: 743.3388. MS m/z (%): 742 (20, M^+), 119 (100). ^{1}H-NMR ($CDCl_3$: CD_3OD = 3 : 1, major conformer) δ: 1.09 (3H, d, J = 6.7 Hz, Ala-4-H$_\beta$), 1.29 (3H, d, J = 7.0 Hz, Ala-1-H$_\beta$), 1.33 (3H, d, J = 6.9 Hz, Ala-2-H$_\beta$), 2.66 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_{\beta a}$), 2.69 (3H, s, Tyr-6-NMe), 2.92 (3H, s, Tyr-3-NMe), 2.99 (1H, dd, J = 18.8, 5.1 Hz, Tyr-6-H$_{\beta a}$), 3.05 (1H, dd, J = 18.8, 10.9 Hz, Tyr-6-H$_{\beta b}$), 3.10 (3H, s, Tyr-5-NMe), 3.26—3.29 (2H, m, Tyr-3-H$_\beta$), 3.62—3.68 (1H, m, Tyr-3-H$_\alpha$), 3.65 (1H, t, J = 11.3 Hz, Tyr-5-H$_{\beta b}$), 4.30—4.40 (Ala-1-H$_\alpha$ and CD_3OH), 4.43 (1H, d, J = 1.9 Hz, Tyr-6-H$_{\delta a}$), 4.63 (1H, dd, J = 10.9, 5.1 Hz, Tyr-6-H$_\alpha$), 4.74 (1H, dq, J = 7.8, 6.9 Hz, Ala-2-H$_\alpha$), 4.76 (1H, dq, J = 8.0, 6.7 Hz, Ala-4-H$_\alpha$), 5.44 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_\alpha$), 6.52 (1H, dd, J = 8.2, 1.9 Hz, Tyr-6-H$_{\delta b}$), 6.77 (1H, d, J = 8.2 Hz, Tyr-6-H$_{\varepsilon a}$), 6.78 (2H, d, J = 8.5 Hz, Tyr-3-H$_\varepsilon$), 6.86 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\varepsilon a}$), 6.90 (1H, d, J = 8.0 Hz, Ala-4-NH), 6.97 (2H, d, J = 8.5 Hz, Tyr-3-H$_\delta$), 7.05 (1H, d, J = 7.8 Hz, Ala-2-NH), 7.22—7.25 (2H, m, Tyr-5-H$_{\delta a}$ and Tyr-5-H$_{\varepsilon b}$), 7.43 (1H, dd, J = 8.4, 2.2 Hz. Tyr-5-H$_{\delta b}$), 8.28 (1H, d, J = 7.3 Hz, Ala-1-NH).

DidemethoxyRA-VII (4) A mixture of **2** (14.8 mg, 0.021 mmol), Tf_2NPh (21.4 mg, 0.060 mmol), Et_3N (8.4 μl, 0.062 mmol) and dry CH_2Cl_2 (2 ml) was stirred at room temperature for 48 h. The mixture was diluted with CH_2Cl_2, washed successively with H_2O, 1 N HCl and brine, and dried. The solvent was evaporated off *in vacuo* to leave a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–AcOEt–MeOH (15 : 2 : 1) to give ditriflate (**3**) (19.4 mg, 97%), as an amorphous powder. IR ν ($CHCl_3$): 3425, 1675, 1145 cm^{-1}. CI-MS m/z (%): 1007 (10, $M+1^+$), 135 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: Tyr-3 [3.43 (1H, dd, J = 13.9, 10.6 Hz, H$_{\beta a}$), 3.50 (1H, dd, J = 13.9, 5.0 Hz, H$_{\beta b}$), 3.61 (H, dd, J = 10.6, 5.0 Hz, H$_\alpha$), 7.22—7.23 (4H, m, H$_\delta$ and H$_\varepsilon$)]; Tyr-6 [2.98 (1H, dd, J = 18.0, 3.0 Hz, H$_{\beta a}$), 3.17 (1H, dd, J = 18.0, 12.0 Hz, H$_{\beta b}$), 4.47 (1H, d, J = 1.7 Hz, H$_{\delta a}$), 4.55 (1H, dd, J = 12.0, 3.0 Hz, H$_\alpha$), 6.65 (1H, dd, J = 8.4, 1.7 Hz, H$_{\delta b}$), 7.11 (1H, d, J = 8.4 Hz, H$_\varepsilon$)].

A mixture of **3** (19.4 mg, 0.019 mmol), $Pd(OAc)_2$ (1.7 mg, 0.0075 mmol), 1,1′-bis(diphenylphosphino)ferrocene (8.9 mg, 0.016 mmol), Et_3N (16 μl, 0.11 mmol), HCOOH (3.5 μl, 0.076 mmol) and *N*,*N*-dimethylformamide (DMF) (1 ml) was stirred for 18 h at 60 °C. The mixture was concentrated *in vacuo*, and the residue was dissolved in CH_2Cl_2, washed successively with H_2O, 1 N HCl and brine, and dried. The solvent was evaporated off *in vacuo* to leave a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–AcOEt–MeOH (15 : 2 : 1), followed by recrystallization from MeOH–isopropyl ether to give **4** (12.5 mg, 91%) as a colorless powder, mp >300 °C, $[\alpha]_D$ −206.7° (c = 0.09, $CHCl_3$). IR ν ($CHCl_3$): 3425, 1670 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 226 (4.34), 272 (3.45), 279 (3.46). High-resolution MS Calcd for $C_{39}H_{46}N_6O_7$: 710.3428. Found: 710.3434. MS m/z (%): 710 (20, M^+), 134 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: 1.12 (3H, d, J = 6.7 Hz, Ala-4-H$_\beta$), 1.31 (3H, d, J = 7.0 Hz, Ala-1-H$_\beta$), 1.35 (3H, d, J = 6.9 Hz, Ala-2-H$_\beta$), 2.64 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_{\beta a}$), 2.70 (3H, s, Tyr-6-NMe), 2.83 (3H, s, Tyr-3-NMe), 2.99 (1H, dd, J = 18.2, 3.8 Hz, Tyr-6-H$_{\beta a}$), 3.13 (3H, s, Tyr-5-NMe), 3.16 (1H, dd, J = 18.2, 11.9 Hz, Tyr-6-H$_{\beta b}$), 3.40 (1H, dd, J = 13.8, 10.9 Hz, Tyr-3-H$_{\beta a}$), 3.46 (1H, dd, J = 13.8, 4.7 Hz, Tyr-3-H$_{\beta b}$), 3.63 (1H, dd, J = 10.9, 4.7 Hz, Tyr-3-H$_\alpha$), 3.68 (1H, t, J = 11.3 Hz, Tyr-5-H$_{\beta b}$), 4.34 (1H, d, J = 2.4 Hz, Tyr-6-H$_{\delta a}$), 4.36 (1H, qd, J = 7.0, 6.7 Hz, Ala-1-H$_\alpha$), 4.58 (1H, dd, J = 11.9, 3.8 Hz, Tyr-6-H$_\alpha$), 4.75 (1H, dq, J = 7.6, 6.7 Hz, Ala-4-H$_\alpha$), 4.84 (1H, dq, J = 8.5, 6.9 Hz, Ala-2-H$_\alpha$), 5.42 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_\alpha$), 6.36 (1H, d, J = 8.5 Hz, Ala-2-NH), 6.46 (1H, d, J = 6.7 Hz, Ala-1-NH), 6.61 (1H, d, J = 7.7 Hz, Tyr-6-H$_\zeta$), 6.72 (1H, d, J = 7.6 Hz, Ala-4-NH), 6.84 (1H, dd, J = 8.4, 2.4 Hz, Tyr-6-H$_{\delta b}$), 6.98 (1H, dd, J = 8.4, 2.4 Hz, Tyr-5-H$_{\varepsilon a}$), 7.12—7.17 (2H, m, Tyr-3-Ar-H), 7.15 (1H, m, Tyr-6-H$_{\varepsilon a}$), 7.17 (1H, dd, J = 8.4, 2.4 Hz, Tyr-5-H$_{\varepsilon b}$), 7.23—7.31 (3H, m, Tyr-3-Ar-H), 7.26 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\delta a}$), 7.42 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\delta b}$).

DemethoxyRA-V (7) A mixture of **2** (14.6 mg, 0.020 mmol), Tf_2NPh (10.7 mg, 0.030 mmol), Et_3N (5 μl, 0.036 mmol) and dry CH_2Cl_2 (2 ml) was stirred at room temperature for 72 h. The reaction mixture was diluted with CH_2Cl_2, washed successively with H_2O, 1 N HCl and brine, and dried. The solvent was evaporated off *in vacuo* to leave a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–AcOEt–MeOH (15 : 2 : 1) to give **3** (7.0 mg, 34%), **5** (4.8 mg, 27%), and **6** (3.2 mg, 19%) together with recovered **2** (3.0 mg, 20%). **5**: an amorphous powder. IR ν ($CHCl_3$): 3425, 1675, 1145 cm^{-1}. CI-MS m/z (%): 875 (20, $M+1^+$), 119 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: Tyr-3 [3.45 (1H, dd, J = 14.0, 10.5 Hz, H$_{\beta a}$), 3.52 (1H, dd, J = 14.0, 5.0 Hz, H$_{\beta b}$), 3.62 (1H, dd, J = 10.5, 5.0 Hz, H$_\alpha$), 7.23—7.24 (4H, m, H$_\delta$ and H$_\varepsilon$)].

A mixture of **5** (4.8 mg, 0.0054 mmol), $Pd(OAc)_2$ (0.9 mg, 0.0040 mmol), 1.1′-bis(diphenylphosphino)ferrocene (4.4 mg, 0.0080 mmol), Et_3N (8.4 μl, 0.062 mmol), HCOOH (1.5 μl, 0.033 mmol) and DMF (1 ml) was stirred for 72 h at 60 °C. The mixture was concentrated *in vacuo*, and the residue was diluted with CH_2Cl_2, and washed successively with H_2O, 1 N HCl and brine, and dried. The solvent was evaporated off *in vacuo* to leave a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–AcOEt–MeOH (15 : 2 : 1), followed by recrystallization from MeOH–isopropyl ether to give **7** (2.9 mg, 72%) as a colorless powder, mp 246—247 °C, $[\alpha]_D$ −198.3° (c = 0.11, $CHCl_3$). IR ν ($CHCl_3$): 3397, 1673 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 280 (3.59), 288 (3.55). High-resolution FAB-MS Calcd for $C_{39}H_{47}N_6O_8$: 727.3455 $[M+H^+]$. Found: 727.3495. MS m/z (%): 726 (10, M^+), 119 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: 1.11 (3H, d, J = 6.7 Hz, Ala-4-H$_\beta$), 1.30 (3H, d, J = 6.9 Hz, Ala-1-H$_\beta$), 1.35 (3H, d, J = 6.9 Hz, Ala-2-H$_\beta$), 2.63 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_{\beta a}$), 2.69 (3H, s, Tyr-6-NMe), 2.83 (3H, s, Tyr-3-NMe), 2.90 (1H, m, Tyr-6-H$_{\beta a}$), 3.04 (1H, m, Tyr-6-H$_{\beta b}$), 3.11 (3H, s, Tyr-5-NMe), 3.40 (1H, dd, J = 13.9, 10.8 Hz, Tyr-3-H$_{\beta a}$), 3.46 (1H, dd, J = 13.9, 5.0 Hz, Tyr-3-H$_{\beta b}$), 3.63 (1H, dd, J = 10.8, 5.0 Hz, Tyr-3-H$_\alpha$), 3.69 (1H, t, J = 11.3 Hz, Tyr-5-H$_{\beta b}$), 4.35 (1H, d, J = 2.0 Hz, Tyr-6-H$_{\delta a}$), 4.37 (1H, qd, J = 6.9, 6.8 Hz, Ala-1-H$_\alpha$), 4.54 (1H, dd, J = 11.8, 4.0 Hz, Tyr-6-H$_\alpha$), 4.76 (1H, dq, J = 7.7, 6.7 Hz, Ala-4-H$_\alpha$), 4.82 (1H, dq, J = 8.4, 6.9 Hz, Ala-2-H$_\alpha$), 5.41 (1H, dd, J = 11.3, 3.0 Hz, Tyr-5-H$_\alpha$), 6.38 (1H, d, J = 8.4 Hz, Ala-2-NH), 6.44 (1H, d, J = 6.8 Hz, Tyr-6-NH), 6.51 (1H, dd, J = 8.3, 2.0 Hz, Tyr-6-H$_{\delta b}$), 6.72 (1H, d, J = 7.7 Hz, Ala-4-NH), 6.81 (1H, d, J = 8.3 Hz, Tyr-6-H$_{\varepsilon a}$), 6.84 (1H, dd, J = 8.4, 2.4 Hz, Tyr-5-H$_{\varepsilon a}$), 7.13—7.15 (2H, m, Tyr-3-Ar-H), 7.20 (1H, dd, J = 8.4, 2.4 Hz, Tyr-5-H$_{\varepsilon b}$), 7.23—7.32 (4H, m, Tyr-3-Ar-H and Tyr-5-H$_{\delta a}$), 7.42 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\delta b}$).

Tyr-6-C$_\zeta$-deoxyRA-V (9) This compound was prepared from **8** in a similar manner to that described for **4** in 79% yield as a colorless powder, mp >300 °C (MeOH–isopropyl ether), $[\alpha]_D$ −215.8° (c = 0.20, $CHCl_3$). IR ν ($CHCl_3$): 3425, 1675 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 223 (4.34), 272 (3.46), 279 (3.46). High-resolution MS Calcd for $C_{40}H_{48}N_6O_8$: 740.3534. Found: 740.3537. MS m/z (%): 740 (20, M^+), 164 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: 1.11 (3H, d, J = 6.7 Hz, Ala-4-H$_\beta$), 1.30 (3H, d, J = 6.9 Hz, Ala-1-H$_\beta$), 1.35 (3H, d, J = 6.9 Hz, Ala-2-H$_\beta$), 2.64 (1H, dd, J = 11.3, 2.8 Hz, Tyr-5-H$_{\beta a}$), 2.69 (3H, s, Tyr-6-NMe), 2.86 (3H, s, Tyr-3-NMe), 2.99 (1H, dd, J = 18.3, 3.7 Hz, Tyr-6-H$_{\beta a}$), 3.12 (3H, s, Tyr-5-NMe), 3.13 (1H, dd, J = 18.3, 11.9 Hz, Tyr-6-H$_{\beta b}$), 3.33 (1H, dd, J = 13.8, 10.6 Hz, Tyr-3-H$_{\beta a}$), 3.37 (1H, dd, J = 13.8, 5.1 Hz, Tyr-3-H$_{\beta b}$), 3.58 (1H, dd, J = 10.6, 5.1 Hz, Tyr-3-H$_\alpha$), 3.67 (1H, t, J = 11.3 Hz, Tyr-5-H$_{\beta b}$), 3.79 (3H, s, Tyr-3-OMe), 4.33 (1H, br s, Tyr-6-H$_{\delta a}$), 4.37 (1H, qd, J = 6.9, 6.8 Hz, Ala-1-H$_\alpha$), 4.58 (1H, dd, J = 11.9, 3.7 Hz, Tyr-6-H$_\alpha$), 4.76 (1H, dq, J = 7.7, 6.7 Hz, Ala-4-H$_\alpha$), 4.84 (1H, dq, J = 8.3, 6.9 Hz, Ala-2-H$_\alpha$), 5.42 (1H, dd, J = 11.3, 2.8 Hz, Tyr-5-H$_\alpha$), 6.44 (1H, d, J = 8.3 Hz, Ala-2-NH), 6.46 (1H, d, J = 6.8 Hz, Ala-1-NH), 6.61 (1H, d, J = 7.6 Hz, Tyr-6-H$_\zeta$), 6.72 (1H, d, J = 7.7 Hz, Ala-4-NH), 6.83 (2H, d, J = 8.6 Hz, Tyr-3-H$_\varepsilon$), 6.84 (1H, d, J = 7.6 Hz, Tyr-6-H$_{\delta b}$), 6.97 (1H, dd, J = 8.4, 2.3 Hz, Tyr-5-H$_{\varepsilon a}$), 7.04 (2H, d, J = 8.6 Hz, Tyr-3-H$_\delta$), 7.14 (1H, t, J = 7.6 Hz, Tyr-6-H$_{\varepsilon a}$), 7.17 (1H, dd, J = 8.4, 2.3 Hz, Tyr-5-H$_{\varepsilon b}$), 7.26 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\delta a}$), 7.42 (1H, dd, J = 8.4, 2.2 Hz, Tyr-5-H$_{\delta b}$).

DemethoxyRA-II (10) This compound was prepared from **9** in a similar manner to that described for **2** in 55% yield. A colorless powder, mp 258—259 °C (MeOH), $[\alpha]_D$ −118.9° (c = 0.17, $CHCl_3$). IR ν ($CHCl_3$): 3390, 1668 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 225 (4.37), 273 (3.51), 279 (3.55). High-resolution FAB-MS Calcd for $C_{39}H_{47}N_6O_8$: 727.3455 $[M+H^+]$. Found: 727.3483. MS m/z (%): 726 (10, M^+), 119 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: 1.09 (3H, d, J = 6.6 Hz, Ala-4-H$_\beta$), 1.31 (3H, d,

$J=7.0$ Hz, Ala-1-H_β), 1.33 (3H, d, $J=7.0$ Hz, Ala-2-H_β), 2.64 (1H, dd, $J=11.3$, 3.5 Hz, Tyr-5-$H_{\beta a}$), 2.70 (3H, s, Tyr-6-NMe), 2.87 (3H, s, Tyr-3-NMe), 2.99 (1H, dd, $J=18.3$, 3.5 Hz, Tyr-6-$H_{\beta a}$), 3.10 (3H, s, Tyr-5-NMe), 3.15 (1H, dd, $J=18.3$, 11.8 Hz, Tyr-6-$H_{\beta b}$), 3.32 (1H, dd, $J=14.1$, 10.0 Hz, Tyr-3-$H_{\beta a}$), 3.36 (1H, dd, $J=14.1$, 5.4 Hz, Tyr-3-$H_{\beta 2}$), 3.59 (1H, dd, $J=10.0$, 5.4 Hz, Tyr-3-H_α), 3.67 (1H, t, $J=11.3$ Hz, Tyr-5-$H_{\beta b}$), 4.34 (1H, br s, Tyr-6-$H_{\delta a}$), 4.39 (1H, dq, $J=8.1$, 7.0 Hz, Ala-1-H_α), 4.59 (1H, dd, $J=11.8$, 3.5 Hz, Tyr-6-H_α), 4.75 (1H, dq, $J=8.3$, 6.6 Hz, Ala-4-H_α), 4.82 (1H, qd, $J=7.0$, 6.7 Hz, Ala-2-H_α), 5.42 (1H, dd, $J=11.3$, 3.5 Hz, Tyr-5-H_α), 6.51 (1H, d, $J=6.7$ Hz, Ala-2-NH), 6.61 (1H, d, $J=7.6$ Hz, Tyr-6-H_ζ), 6.65 (1H, d, $J=8.1$ Hz, Ala-1-NH), 6.76 (1 , d, $J=7.6$ Hz. Tyr-6-$H_{\delta b}$), 6.79 (2H, d, $J=8.3$ Hz, Tyr-3-H_ε), 6.83 (1H, dd, $J=8.3$, 2.3 Hz, Tyr-5-$H_{\varepsilon a}$), 6.97 (3H, d, $J=8.3$ Hz, Ala-4-NH and Tyr-3-H_δ), 7.13 (1H, t, $J=7.6$ Hz, Tyr-6-$H_{\varepsilon a}$), 7.17 (1H, dd, $J=8.4$, 2.3 Hz, Tyr-5-$H_{\varepsilon b}$), 7.24 (1H, dd, $J=8.3$, 2.1 Hz, Tyr-5-$H_{\delta a}$), 7.41 (1H, dd, $J=8.4$, 2.1 Hz, Tyr-5-$H_{\delta b}$).

Tyr-3-C_ζ-deoxyRA-II (12) This compound was prepared from **11** in a similar manner to that described for **4** in 60% yield. A colorless powder, mp 242—243 °C (MeOH). $[\alpha]_D$ −171.4° ($c=0.14$, $CHCl_3$). IR ν ($CHCl_3$): 3425, 1675 cm^{-1}. UV λ_{max}^{EtOH} nm (log ε): 224 (4.59), 293 (4.29). High-resolution FAB-MS Calcd for $C_{40}H_{49}N_6O_8$: 741.3612 [M+H$^+$]. Found: 741.3589. MS m/z (%): 740 (10, M$^+$), 134 (100). ^{1}H-NMR ($CDCl_3$, major conformer) δ: 1.13 (3H, d, $J=6.7$ Hz, Ala-4-H_β), 1.30 (3H, d, $J=6.9$ Hz, Ala-1-H_β), 1.35 (3H, d, $J=6.9$ Hz, Ala-2-H_β), 2.64 (1H, dd, $J=11.4$, 3.1 Hz, Tyr-5-$H_{\beta a}$), 2.69 (3H, s, Tyr-6-NMe), 2.82 (3H, s, Tyr-3-NMe), 2.95 (1H, dd, $J=18.3$, 3.7 Hz, Tyr-6-$H_{\beta a}$), 3.10 (1H, dd, $J=18.3$, 11.9 Hz, Tyr-6-$H_{\beta b}$), 3.13 (3H, s, Tyr-5-NMe), 3.40 (1H, dd, $J=13.9$, 10.9 Hz, Tyr-3-$H_{\beta a}$), 3.46 (1H, dd, $J=13.9$, 4.9 Hz, Tyr-3-$H_{\beta b}$), 3.63 (1H, t, $J=11.4$ Hz, Tyr-5-$H_{\beta b}$), 3.63 (1H, dd, $J=10.9$, 4.9 Hz, Tyr-3-H_α), 3.93 (3H, s, Tyr-6-OMe), 4.32—4.39 (1H, m, Ala-1-H_α), 4.35 (1H, d, $J=2.0$ Hz, Tyr-6-$H_{\delta a}$), 4.54 (1H, dd, $J=11.9$, 3.7 Hz, Tyr-6-H_α), 4.75 (1H, dq, $J=7.6$, 6.7 Hz, Ala-4-H_α), 4.84 (1H, dq, $J=8.5$, 6.9 Hz, Ala-2-H_α), 5.42 (1H, dd, $J=11.4$, 3.1 Hz, Tyr-5-H_α), 6.19 (1H, d, $J=8.5$ Hz, Ala-2-NH), 6.42 (1H, d, $J=6.7$ Hz, Ala-1-NH), 6.57 (1H, dd, $J=8.3$, 2.0 Hz, Tyr-6-$H_{\delta b}$), 6.69 (1H, d, $J=7.6$ Hz, Ala-4-NH), 6.80 (1H, d, $J=8.3$ Hz, Tyr-6-$H_{\varepsilon a}$), 6.87 (1H, dd, $J=8.4$, 2.4 Hz, Tyr-5-$H_{\varepsilon a}$), 7.12—7.15 (2H, m, Tyr-3-Ar-H), 7.22 (1H, dd, $J=8.4$, 2.4 Hz, Tyr-5-$H_{\varepsilon b}$), 7.23—7.31 (4H, m, Tyr-3-Ar-H and Tyr-5-$H_{\delta a}$), 7.42 (1H, dd, $J=8.4$, 2.2 Hz, Tyr-5-$H_{\delta b}$).

Cytotoxicity MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate.[11] The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Human KB oral epidermoid carcinoma cells (1×10^4 cells/ml) or mouse P388 leukemia cells (2×10^4 cells/ml) were inoculated in each well with 100 μl of RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, UK), 100 units/ml of penicillin and 100 μg/ml of streptomycin. After overnight incubation (37 °C, 5% CO_2), 100 μl of sample solution was added to each well and the plates were incubated for 3 d (KB cells) or 2 d (P388). Then, 50 μl of MTT (200 μg/ml PBS) was added to each well and the plates were inclubated for a further 4 h. The resulting formazan was dissolved in 150 μl of DMSO. The plate was placed on a plate shaker for 5 min and read immediately at 540 nm. The IC_{50} (μg/ml) value was defined as that concentration of sample which achieved 50% reduction of growth in sample-treated cells with respect to the controls. IC_{50} was calculated by using the probit test.

Antitumor Activity P388 murine leukemia cells (1×10^6 cells) were inoculated i.p. into female CDF_1 mice (6—7 weeks old; test $n=8$, control $n=16$) on day 0. Samples, suspended in 0.5% gum arabic–saline solution, were administered i.p. on days 1—5. The antitumor activity was estimated according to the NCI tumor panel screening method.[12]

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References and Notes

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