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RA-XXV and RA-XXVI, Bicyclic Hexapeptides from *Rubia cordifolia* L. Structure, Synthesis, and Conformation

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Abstract: Two new RA-series bicyclic hexapeptides, RA-XXV (**4**) and RA-XXVI (**5**), which have no *N*-methyl group at Tyr-5, were isolated from the roots of *Rubia cordifolia* L. Their amino acid compositions and sequences were determined by interpretation of MS and 1D and 2D NMR data and their relative structures were elucidated by X-ray crystallography of **4** and RA-XXVI acetate (**6**). The absolute stereochemistry of **4** was established by the total synthesis of **4**, and that of **5**, by the chemical correlation with **4**. Peptides **4** and **5** exhibited cytotoxicity toward human promyelocytic leukemia HL-60 (IC_{50}=0.062 and 0.066 μM, respectively) and human colonic carcinoma HCT-116 (IC_{50}=0.028 and 0.051 μM, respectively) cell lines. Analysis of the conformational structures of **4** and **6** in the crystalline state and those of **4** and **5** in solution revealed that the *N*-methyl group at Tyr-5 functions to make this series of peptides preferentially adopt the active conformation.

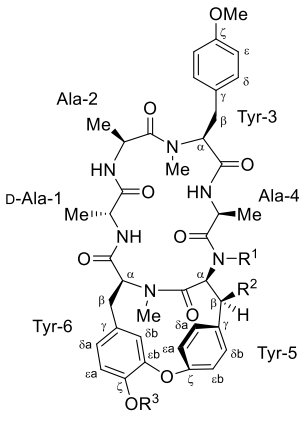
Introduction

RA-VII (**1**) is a bicyclic hexapeptide isolated from rubiaceous plants *Rubia cordifolia* L. and *R. akane* Nakai (Rubiaceae).[1,2] Structurally related peptides, bouvardin (NSC 259968, **2**) and deoxybouvardin (RA-V, **3**), were isolated from *Bouvardia ternifolia* (Cav.) Schltdl., a plant of the same family (Fig. 1).[3,4] These peptides have a structurally unique cycloisodityrosine unit in which the two phenyl rings of tyrosyl-tyrosine are connected by an ether linkage to form a 14-membered cyclophane macrocycle. These peptides exhibit potent antitumor activity; peptide **1** underwent phase I clinical trials as an anticancer drug in Japan from the late 80s to the early 90s.[5] The antitumor activity of these peptides is believed to be due to the inhibition of protein synthesis through interaction with eukaryotic ribosomes.[6] Peptide **1** was also shown to cause conformational changes in F-actin, which stabilize actin filaments and induce G2 arrest,[7] and peptide **3** was found to inhibit angiogenesis by down-regulating ERK1/2 phosphorylation in HUVEC and HMEC-1 endothelial cells.[8]

In the present study, we examined minor constituents in the methanol extracts of the roots of *R. cordifolia* L. We isolated two new bicyclic hexapeptides, RA-XXV (**4**) and RA-XXVI (**5**), and established their absolute structures. Their conformational structures in crystals were studied by X-ray diffraction analysis and those in solution were elucidated by ROESY experiments and computational methods. We also evaluated their cytotoxic activities on HL-60 and HCT-116 cells.

Results and Discussion

Isolation: A methanol extract obtained from the dried roots of *R. cordifolia* (50 kg) was partitioned between chloroform and water. The chloroform-soluble portion was subjected to a series of column chromatographic separations using silica gel, alumina, and then aminopropyl-bonded silica gel, employing a series of $CHCl_3$/MeOH mixtures as the eluent. After removal of the solvent, the residue of the $CHCl_3$ eluate and the residue of the $CHCl_3$/MeOH (9:1) eluate of the aminopropyl-bonded silica gel column chromatography were each crystallized from MeOH to give crystals of crude RAs and the mother liquors. By reversed-phase HPLC separation, the mother liquor from the $CHCl_3$ eluate gave RA-XXV (**4**, 16.9 mg, 3.4 × 10^{-5}%) and that from the $CHCl_3$/MeOH (9:1) eluate afforded RA-XXVI (**5**, 15.1 mg, 3.0 ×



	R^1	R^2	R^3
RA-VII (**1**)	Me	H	Me
bouvardin (**2**)	Me	OH	H
deoxybouvardin (**3**)	Me	H	H
RA-XXV (**4**)	H	H	Me
RA-XXVI (**5**)	H	H	H
RA-XXVI acetate (**6**)	H	H	Ac

Figure 1. Structures of RA-VII (**1**), bouvardin (**2**), deoxybouvardin (**3**), RA-XXV (**4**), RA-XXVI (**5**), and RA-XXVI acetate (**6**).

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Table 1 NMR data of RA-XXV (**4**) and RA-XXVI (**5**)[a]

		RA-XXV (**4**)				RA-XXVI (**5**)			
		conformer I		conformer II		conformer I		conformer II	
Residue		δ_H [ppm]	δ_C [ppm]	δ_H [ppm]	δ_C [ppm]	δ_H [ppm]	δ_C [ppm]	δ_H [ppm]	δ_C [ppm]
D-Ala-1	α	4.57 (quintet, 7.2)	47.2	4.33 (quintet, 6.9)	47.7	4.57 (quintet, 7.1)	47.3	4.34[b]	47.6
	β	1.22 (d, 7.2, 3H)	16.4	1.27 (d, 6.9, 3H)	16.03	1.22 (d, 7.1, 3H)	16.47	1.26 (d, 7.0, 3H)	16.03
	C=O		172.8		172.2		172.8		172.3
	NH	5.79 (brs)		6.52 (d, 6.9)		5.82 (brd, 6.0)		6.57[b]	
Ala-2	α	4.48 (quintet, 6.7)	46.1	4.46[b]	45.6	4.49 (quintet, 6.6)	46.2	4.43 (brm)	45.6
	β	1.46 (d, 6.9, 3H)	15.99	1.25 (d, 6.9, 3H)	15.99	1.47 (d, 6.9, 3H)	16.03	1.25 (d, 6.9, 3H)	15.96
	C=O		173.1		173.0		173.2		173.0
	NH	7.38 (d, 5.7)		n.o.		7.45[b]		n.o.	
Tyr-3	α	3.59 (brm)	69.3	3.58 (dd, 8.7, 7.1)	69.1	3.59 (brm)	69.2	3.58 (dd, 9.2, 6.6)	69.1
	βa	3.48[b]	32.6	3.40[b]	32.8	3.48[b]	32.6	3.40[b]	32.8
	βb	3.36 (dd, 13.4, 4.3)		3.40[b]		3.36 (dd, 13.9, 4.4)		3.40[b]	
	γ		130.4		130.9		130.2		130.85
	δ	7.11 (d, 8.6, 2H)	130.2[c]	7.04 (d, 8.6, 2H)	130.3[c]	7.11 (d, 8.6, 2H)	130.2[c]	7.04 (d, 8.5, 2H)	130.3[c]
	ε	6.85 (d, 8.6, 2H)	114.1[c]	6.813 (d, 8.6, 2H)	113.9[c]	6.85 (d, 8.6, 2H)	114.1[c]	6.81 (d, 8.5, 2H)	113.9[c]
	ζ		158.5		158.3		158.5		158.3
	C=O		170.0		170.2		170.0		170.2
	NMe	2.77 (s, 3H)	39.6	2.72 (s, 3H)	39.4	2.77 (s, 3H)	39.6	2.72 (s, 3H)	39.5
	OMe	3.80 (s, 3H)	55.3	3.78 (s, 3H)	55.2	3.80 (s, 3H)	55.3	3.78 (s, 3H)	55.2
Ala-4	α	3.69 (quintet, 7.1)	52.6	3.92[b]	52.5	3.71 (quintet, 7.1)	52.4	3.92 (brm)	52.4
	β	1.72 (d, 7.4, 3H)	16.5	1.61 (brd, 7.1, 3H)	17.6	1.70 (d, 7.4, 3H)	16.50	1.61 (brs, 3H)	17.6
	C=O		173.1		172.8		173.0		173.1
	NH	6.88 (brd, 6.2)		7.13 (d, 8.0)		6.88 (brd, 6.4)		7.15 (brd, 6.2)	
Tyr-5	α	5.17 (m)	51.7	4.81 (m)	52.5	5.17 (m)	51.5	4.80 (m)	52.6
	β (pro-*S*)	3.24 (t, 12.5)	36.9	3.48[b]	40.1	3.22 (t, 12.8)	37.0	3.50[b]	39.9
	β (pro-*R*)	3.34 (dd, 12.5, 5.1)		3.00[b]		3.32 (dd, 12.8, 5.4)		3.02[b]	
	γ		134.8		135.2		135.3		135.6
	δa	7.43 (dd, 8.3, 2.1)	131.6	7.26[b]	132.5	7.43 (dd, 8.2, 2.1)	131.7	7.26[b]	132.7
	δb	7.50 (dd, 8.6, 2.1)	131.5	7.46 (dd, 8.3, 2.1)	130.7	7.48 (dd, 8.6, 2.1)	131.6	7.44[b]	130.76
	εa	7.28 (dd, 8.3, 2.4)	127.4	6.90 (dd, 8.3, 2.4)	124.5	7.26 (dd, 8.2, 2.4)	127.2	6.85[b]	124.4
	εb	7.05 (dd, 8.6, 2.4)	123.7	7.18 (dd, 8.3, 2.4)	125.8	6.98 (dd, 8.6, 2.4)	123.5	7.13 (dd, 8.4, 2.4)	125.7
	ζ		157.6		158.2		157.3		157.8
	C=O		171.5		170.6		171.5		170.4
	NH	8.01 (brd, 7.0)		n.o.		7.95 (brd, 8.0)		n.o.	
Tyr-6	α	3.42 (dd, 10.8, 4.0)	67.4	4.36 (dd, 11.9, 3.7)	56.9	3.42 (dd, 10.4, 4.3)	67.1	4.36[b]	57.0
	β (pro-*S*)	3.02 (dd, 14.7, 4.0)	32.7	2.80[b]	33.2	3.00 (dd, 14.9, 4.3)	33.0	2.79[b]	33.3
	β (pro-*R*)	3.19 (dd, 14.7, 10.8)		3.38[b]		3.13 (dd, 14.9, 10.4)		3.34[b]	
	γ		129.7		127.9		129.0		127.4
	δa	6.63 (dd, 8.2, 1.9)	121.7	6.62[b]	121.2	6.57 (dd, 8.1, 1.9)	122.2	6.56[b]	121.9
	δb	4.87 (s)	119.1	4.30 (d, 1.9)	113.5	5.02 (s)	119.0	4.30 (d, 1.7)	113.1
	εa	6.75 (d, 8.2)	111.8	6.809 (d, 8.5)	112.2	6.78 (d, 8.1)	115.4	6.83 (d, 8.3)	115.7
	εb		152.0		153.1		149.8		151.1
	ζ		146.6		146.4		143.0		142.8
	C=O		169.5		170.4		169.4		170.4
	NMe	2.78 (s, 3H)	39.9	2.76 (s, 3H)	28.9	2.69 (s, 3H)	39.5	2.76 (s, 3H)	28.9
	OMe	3.93 (s, 3H)	56.11	3.94 (s, 3H)	56.14				
	OH					5.86[d]		5.86[d]	

[a] Recorded at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR in $CDCl_3$. Conformers I and II are the most populated and the second-most populated conformers in $CDCl_3$, respectively. *J*-values given in Hz in parentheses. n.o. = not observed. [b] Multiplicity not determined due to overlapping signals. [c] Two carbons. [d] Very broad signal.

10^{-5}%) along with known RA-series peptides.

Structures of RA-XXV (4) and RA-XXVI (5): RA-XXV (**4**), $[\alpha]_D^{25}$ =−155 (*c*=0.063 in MeOH), was obtained as colorless plates. Its molecular formula was determined to be $C_{40}H_{48}N_6O_9$ from the [*M*+Na^+] peak at *m/z* 779.3398 (calcd for $C_{40}H_{48}N_6O_9$+Na^+, 779.3380) in the high-resolution ESI-MS (HR-ESIMS). The ^{1}H NMR spectrum of **4** in $CDCl_3$ indicated the presence of four spectroscopically distinguishable conformers in the ratio of 69:25:3:3 at 300 K. The planar structure of **4** was determined by analyzing the NMR signals from the most populated conformer (conformer I).[9] The ^{1}H NMR, ^{13}C NMR, and HSQC spectra of **4** exhibited signals for three secondary methyl groups (δ_H/δ_C 1.22/16.4, 1.46/15.99, 1.72/16.5 ppm), two *N*-methyl groups (δ_H/δ_C 2.77/39.6, 2.78/39.9 ppm), two *O*-methyl groups (δ_H/δ_C 3.80/55.3, 3.93/56.11 ppm), three methylenes (δ_H/δ_C 3.02 and 3.19/32.7, 3.24 and 3.34/36.9, 3.36 and 3.48/32.6 ppm), six *N*-substituted methines (δ_H/δ_C 3.42/67.4, 3.59/69.3, 3.69/52.6, 4.48/46.1, 4.57/47.2, 5.17/51.7 ppm), eleven aromatic methines (δ_H/δ_C 4.87/119.1, 6.63/121.7, 6.75/111.8, 6.85/114.1 (×2), 7.05/123.7, 7.11/130.2 (×2), 7.28/127.4, 7.43/131.6, 7.50/131.5), seven aromatic quaternary carbons (δ_C 129.7, 130.4, 134.8, 146.6, 152.0 157.6, 158.5 ppm), and six carbonyl groups (δ_C 169.5, 170.0, 171.5, 172.8, 173.1 (×2) ppm) (Table 1).

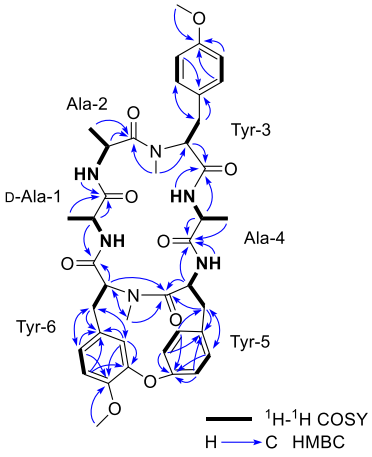

Figure 2. ^{1}H-^{1}H COSY and selected HMBC correlations for RA-XXV (**4**).

Inspection of the ^{1}H-^{1}H COSY and HMBC spectra and its molecular formula with 20 degrees of unsaturation suggested that peptide **4** had a bicyclic structure composed of three alanines, one tyrosine, and two *N,O*-dimethyltyrosines of which one had a 1,2,4-trisubstituted phenyl ring. Analysis of the HMBC correlations of the NH, NMe, and/or H_α proton of the amino acid residue (*i*) to the *i*−1 carbonyl carbon atom revealed that peptide **4** had the sequence shown in Figure 2. The presence of a cyclophane structure at Tyr-5 and Tyr-6 was suggested by the fact that the signals of the four aromatic methine groups of Tyr-5 had different chemical shifts in the NMR spectra. The $H_{\delta b}$ of Tyr-6 displayed a marked upfield signal at δ_H 4.87 ppm due to shielding by the Tyr-5 phenyl ring, which is characteristic of a 14-membered cycloisodityrosine structure of RA-series peptides having a diphenyl ether linkage between the phenolic oxygen of Tyr-5 and the $C_{\varepsilon b}$ of Tyr-6.[10,11] From these observations, the planar structure of **4** was determined as shown in Figure 2.

Although the planar structure of **4** corresponded to the des-*N*-methyl derivative of **1**, we could not elucidate its relative configuration by comparison of its NMR spectra with that of **1**,[11] because their spectra were dissimilar. The relative stereochemistry of **4** was determined by the X-ray diffraction analysis of **4** to be as shown in Figure 3,[12] revealing that peptide **4** has the same relative configuration as **1**–**3** at all the amino acid chiral centers.

RA-XXVI (**5**), $[\alpha]_D^{25}$=−170 (*c*=0.048 in MeOH), was obtained as a white amorphous solid. Its molecular formula, $C_{39}H_{46}N_6O_9$, which was deduced from the [*M*+H$^+$] peak at *m/z* 743.3405 (calcd for $C_{39}H_{46}N_6O_9$+H$^+$, 743.3399) in the HR-ESIMS, contained one methylene unit less than that of RA-XXV (**4**). Its ^{1}H NMR spectra measured in $CDCl_3$ showed that it was a mixture of four conformers in the 68:28:3:1 ratio. The ^{1}H and ^{13}C NMR spectra of **5** were very similar to those of **4**; the only differences were that the signal for the *O*-methyl group at Tyr-6 was missing and a phenolic hydroxyl signal newly appeared at δ_H 5.86 as a very broad signal in its ^{1}H NMR spectrum. These observations indicated that the methoxy group at Tyr-6 in **4** was replaced by a hydroxyl group in **5**. Treatment of **5** with acetic anhydride in pyridine gave crystalline acetate **6**, which was subjected to X-ray diffraction analysis to assign the relative configuration of **6** unambiguously (Fig. 3).[12] Thus, the structure and the relative configuration of RA-XXVI (**5**) were determined to be as shown in Figure 1.

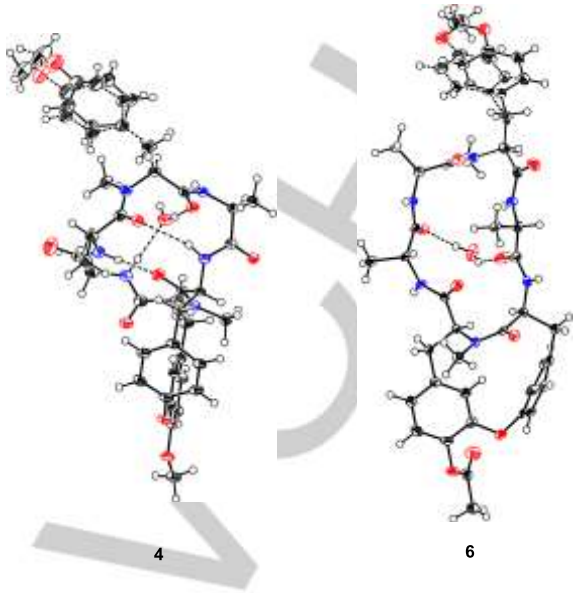

Figure 3. ORTEP representation of RA-XXV (**4**) and RA-XXVI acetate (**6**).

Absolute Configuration of RA-XXV (4) and RA-XXVI (5): The absolute structure of RA-XXV (**4**) was established by the total synthesis of this peptide with D-alanine at residue 1, L-alanines at residues 2 and 4, and modified L-tyrosines at residues 3, 5, and 6. We synthesized peptide **4** on the basis our previous synthesis of allo-RA-V,[13,14] a related compound but with a structurally different cycloisodityrosine unit in which a diphenyl ether bond was formed between the carbon atom at the ε position of Tyr-5 and the phenolic oxygen at the ζ position of Tyr-6. This synthesis was characterized by the diaryl ether formation of the core cycloisodityrosine unit by copper(II) acetate-mediated intramolecular phenol/arylboronic acid coupling[15,16] and the macrocyclization of the linear hexapeptide between the Tyr-6 and D-Ala-1 residues to construct the 18-membered cyclopeptide ring.

3-Iodo-L-tyrosine (**7**) was N-protected with a Cbz group (**8**) and subsequently treated with paraformaldehyde in the presence of *p*-toluenesulfonic acid in toluene to give crystalline oxazolidinone **9** (Scheme 1). Reductive cleavage of the oxazolidinone ring of **9** with triethylsilane in a trifluoroacetic acid-chloroform mixture[17] and subsequent (trimethylsilyl)diazomethane treatment gave the *N,O*-dimethyliodotyrosine derivative **10**. Compound **10** was converted into boronic acid **11** through the substitution of a pinacolboryl group for the iodine atom and subsequent hydrolysis of the boronic acid pinacol ester. Amine **12**, obtained by removal of the Cbz group from **11**, was coupled with Boc-L-tyrosine to give dipeptide **13**, which was then treated with copper(II) acetate and 4-(dimethylamino)pyridine in the

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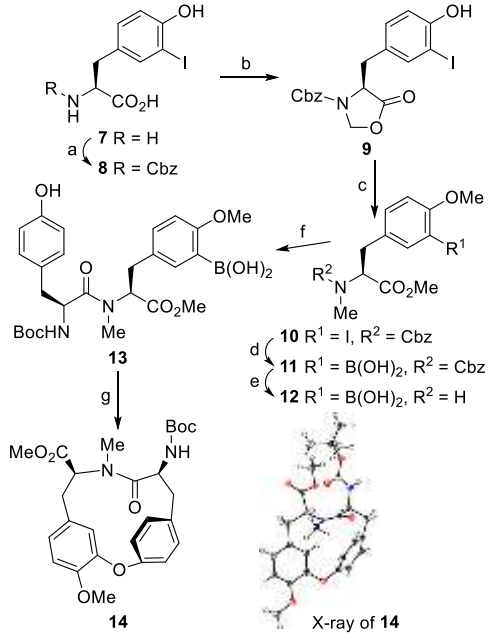
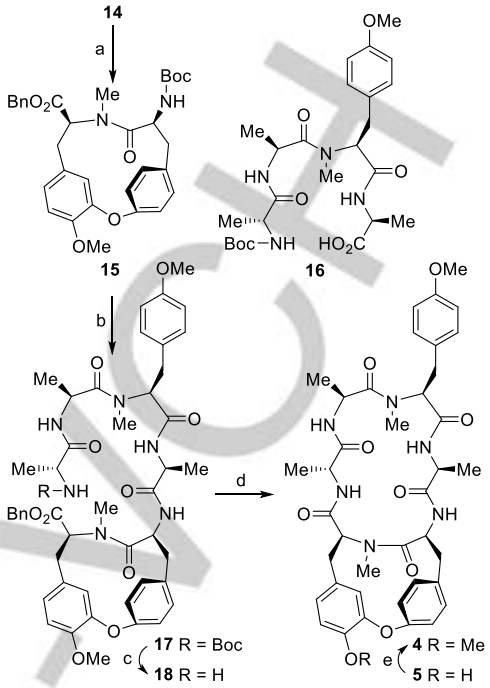


Scheme 1. Synthesis of cycloisodityrosine **14**. Reagents and conditions: a) Cbz-OSu, Et_3N, 1,4-dioxane/H_2O, 0 °C, 1 h, then RT, 17 h, 98%; b) paraformaldehyde, *p*-TsOH·H_2O, PhMe, RT, 3 d, 89%; c) (i) Et_3SiH, TFA, $CHCl_3$, RT, 3 d, (ii) (trimethylsilyl)diazomethane, MeCN/MeOH/Et_2O, RT, 4 d, 76%; d) (i) bis(pinacolato)diboron, $PdCl_2$(dppf)·CH_2Cl_2, KOAc, DMSO, 80 °C, 20 h, (ii) $NaIO_4$, NH_4OAc, acetone/H_2O, RT, 20 h, 94%; e) H_2, Pd/C, EtOH, RT, 20 min, 99%; f) Boc-L-Tyr-OH, EDC·HCl, HOAt, DMF, RT, 7 d, 85%; g) $Cu(OAc)_2$, DMAP, 4Å MS, CH_2Cl_2, RT, 2 d, 37%. For a list of abbreviations, see Ref. 23.



Scheme 2. Synthesis of RA-XXV (**4**). Reagents and conditions: a) (i) LiOOH, MeOH/H_2O, 0 °C, 1 h, then RT, 23 h, (ii) BnOH, diethyl azodicarboxylate, PPh_3, THF/toluene, 0 °C, 1 h, then RT, 10 h, 75%; b) (i) TFA, RT, 2 h, (ii) **16**, EDC·HCl, HOOBt, DMF, RT, 10 d, 70%; c) TFA, RT, 2.5 h, 77%; d) (i) H_2, Pd/C, EtOH, RT, 2.5 h, (ii) EDC·HCl, HOOBt, DMF, 0 °C, 2 h, then RT, 3 d, 42%; e) MeI, K_2CO_3, acetone/MeOH, 40 °C, 6 h, 94%. For a list of abbreviations, see Ref. 23.

presence of 4Å molecular sieves to afford cycloisodityrosine **14** in 37% yield. The structure of **14** was confirmed by X-ray crystallography.[12] As (*S*,*S*)-cycloisodityrosine is known to readily convert into its thermodynamically more stable epimer at the C-terminal residue under basic conditions, we converted at this stage the methyl ester group of **14** into a benzyl ester group that can be removed under neutral conditions at the final macrocyclization stage. This conversion was effected by treating **14** with lithium hydroperoxide and subsequent *O*-benzylation of the acid under Mitsunobu conditions (Scheme 2). The Boc group in **15** was removed and the resulting amine was coupled with tetrapeptide **16** to provide hexapeptide **17**. Deprotection of the N-terminus gave amine **18**. Removal of the benzyl protecting group of **18** by catalytic hydrogenolysis and subsequent cyclization with EDC·HCl (8 equiv) and HOOBt (8 equiv) in DMF under dilution conditions (0.0013 M) gave peptide **4** in 42% yield. Thus obtained **4**, $[\alpha]_D^{25}$=−152 (*c*=0.050 in MeOH), was shown to be identical to natural **4** by comparison of their ^1H and ^{13}C NMR spectra, IR spectra, mass spectra, and optical rotations. Accordingly, the absolute structure of RA-XXV (**4**) was determined to be as shown in Figure 1. Although the compound having structure **4** has already been reported as a synthetic des-*N*-methyl analogue of RA-VII,[18] the reported NMR data for this analogue did not match the data for our natural compound **4**. The reported analogue may be the diastereoisomer of **4**.[19]

The absolute configuration of RA-XXVI (**5**) was established by chemical correlation of **5** with **4**. Treatment of **5** with iodomethane and K_2CO_3 afforded a product, $[\alpha]_D^{25}$=−157 (*c*=0.020 in MeOH), that was shown to be identical to natural **4** on the basis of their spectroscopic data and optical rotations. Thus, peptide **5** was proved to have the same absolute configuration as **4** at all six amino acid chiral centers.

Structures of 4 and 6 in Crystals: The crystal structures of **4** and **6** exhibited disorder in the Tyr-3 residue (Fig. 3). The structure of **4** comprised two structures, **4A** and **4B**, with occupancies of 0.509(7) and 0.491(7), respectively, and that of **6** comprised two structures, **6A** and **6B**, with occupancies of 0.750(6) and 0.250(6), respectively. In each pair, the positions of the *p*-methoxyphenyl ring in Tyr-3 were somewhat different. Comparison of the crystal structures of **4** and **6** revealed that **4** and **6** have quite different structures in their peptide backbones in the crystalline state. The backbone dihedrals of the crystal structures of peptides **1**, **4**, and **6** are summarized in Table 2. In the crystal structure of **4**, all six peptide bonds are in *trans* conformation. This amide conformation pattern has not been observed in natural RA-series peptides with an *N*-methyl group at Tyr-5.[18] Figure 4 shows an overlay of the crystal structures of **1**, **4A**, and **6A**. Due to the *trans* amide bond between Tyr-5 and Tyr-6, peptide **4** has a different structure from peptide **1**.[11,20] It adopts a type II β-turn at Tyr-6 and D-Ala-1, which is stabilized

Table 2
Backbone dihedrals (degree) in RA-XXV (**4**), RA-XXVI acetate (**6**), and RA-VII (**1**)

residue		**4**[a]	**4**[b]	**6**[c]	**6**[d]	**1**[e]
D-Ala-1	φ	85.5(5)	99.5	106.1(4)		139.64(17)
	ψ	−10.6(6)	−38.9	−156.7(3)		−164.43(15)
	ω	178.2(4)	−169.3	−178.1(3)		−177.30(16)
Ala-2	φ	−135.9(4)	−78.3	−98.4(4)		−90.1(2)
	ψ	65.2(5)	109.8	121.3(3)		114.02(17)
	ω	−176.2(4)	−167.0	179.1(5)	−176.4(11)	−176.18(15)
Tyr-3	φ	53.5(5)	50.8	57.4(10)	46(3)	57.3(2)
	ψ	−121.9(4)	36.7	38.3(10)	49(2)	35.7(2)
	ω	−170.7(4)	−175.7	−169.1(5)	−173.9(10)	−173.67(16)
Ala-4	φ	−62.6(5)	80.6	−167.3(3)		−161.11(16)
	ψ	−29.3(5)	−24.4	165.6(3)		158.73(15)
	ω	170.0(4)	−172.3	170.9(3)		−178.87(15)
Tyr-5	φ	−86.6(4)	−80.9	−113.7(3)		−121.84(17)
	ψ	126.8(4)	107.8	118.5(3)		110.53(17)
	ω	−174.7(4)	−162.6	−25.9(5)		−13.4(2)
Tyr-6	φ	−43.2(5)	48.9	−68.8(4)		−79.9(2)
	ψ	131.3(4)	33.0	−163.3(3)		156.78(15)
	ω	178.2(4)	−179.3	170.6(3)		178.62(16)

[a] In crystal structure **4A**. [b] In the most stable structure found by Monte Carlo conformational search and optimized by DFT calculations. [c] In crystal structure **6A**. [d] In crystal structure **6B**. [e] In crystal structure, reference 13.

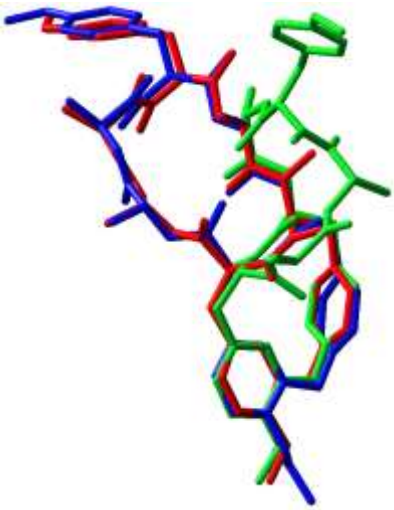


Figure 4. Overlay of crystal structures of RA-VII (**1**, red), RA-XXV (**4A**, green), and RA-XXVI acetate (**6A**, blue).

by an intramolecular hydrogen bond between Ala-2 NH and Tyr-5 C=O (N–O distance, 2.71 Å; N–H–O angle, 152°), and a type II′ β-turn at Tyr-3 and Ala-4. This crystal structure of **4** is also stabilized by a water molecule that forms hydrogen bonds with D-Ala-1 NH (N–O distance, 2.89 Å; N–H–O angle, 171°) and Tyr-3 C=O (O–O distance, 2.79 Å; O–H–O angle, 171°).

The structure of **6** has a *cis* amide between Tyr-5 and Tyr-6, and the other five peptide bonds are in all *trans* conformation. This amide conformation pattern is the same as that of the crystal structure of **1**, which was identified as the active conformation of peptide **1**.[16b,18,21] The backbone structure of **6** bears a good resemblance to that of **1**, namely, the three

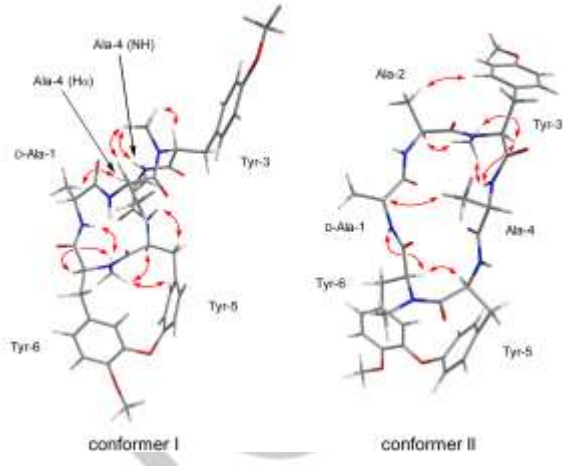


Figure 5. Key ROESY correlations observed in the $CDCl_3$ solution of **4**. The structure shown for conformer I represents the structure of the most stable conformer found in the Monte Carlo conformational search for **4**, and that shown for conformer II was built from the crystal structure of **6** by replacing the acetyl group by a methyl group.

tyrosine aromatic rings of both peptides occupy almost the same space.

Structures of 4 and 5 in Solution: The solution structures of peptides **4** and **5** were examined by analysis of the data from ROESY experiments (Fig. 5). In the ROESY spectrum of **4**, cross-peaks from the most populated conformer (conformer I) with population 69% were observed between Ala-2 H_α and Tyr-3 NMe and between Tyr-5 H_α and Tyr-6 NMe, indicating that this conformer had *trans* amide bonds between Ala-2 and Tyr-3 and between Tyr-5 and Tyr-6 as was observed in the crystal structure of **4** (Fig. 3). However, the crystal structure appeared not to reflect the solution structure of **4** in $CDCl_3$. There were some inconsistencies between the interproton distances of the crystal structure and the ROESY data. Although strong cross-peaks between D-Ala-1 $H_{3\beta}$ and Ala-4 H_α and between Tyr-3 NMe and Ala-4 NH were observed in the ROESY spectrum, the calculated distances from Ala-4 H_α to the closest proton of D-Ala-1 $H_{3\beta}$ and from Ala-4 NH to that of Tyr-3 NMe in the crystals were 8.20 Å and 5.25 Å, respectively, and were not sufficiently short to generate intense cross-peaks in the ROESY spectrum. The most stable conformation obtained from a Monte Carlo conformational search of the structure of **4** using MacroModel software and the MMFFs force field[22] was then subjected to geometry optimization at the B3LYP level of density functional theory (DFT) using the 6-31G(d) basis set. The thus-obtained structure is illustrated in Figure 5 with observed ROESY correlations designated by arrows. In this structure, the distances between those protons were estimated to be 2.32 Å and 2.80 Å, respectively. Other ROESY correlations observed were between D-Ala-1 NH and Tyr-6 NMe, Tyr-3 H_α and Tyr-3 NMe,Tyr-5 H_β (pro-*S*) and Tyr-5 NH, and Tyr-5 $H_{\delta b}$ and Tyr-6 NMe, and the distances between those protons were all less than 2.9 Å in the calculated most stable conformation. Thus, the observed ROE interactions for conformer I seemed to be

consistent with the conformation shown in Figure 5. This most stable conformation obtained from a Monte Carlo conformational search was stabilized by the intramolecular hydrogen bonds between Ala-2 NH and Tyr-5 C=O (N–O distance, 2.96 Å; N–H–O angle, 154°), between Ala-4 NH and D-Ala-1 C=O (3.18 Å; 155°), and between Tyr-5 NH and Ala-2 C=O (2.89 Å; 156°), and was calculated to be 4.8 kcal/mol more stable than the DFT-optimized crystal structure of **4**.

The ROESY cross-peaks derived from the second-most populated conformer (conformer II) with population 25% were observed between Ala-2 H_α and Tyr-3 NMe and between Tyr-5 H_α and Tyr-6 H_α, which indicated the presence of *trans* and *cis* amide bonds between Ala-2 and Tyr-3 and between Tyr-5 and Tyr-6, respectively. A weak ROESY cross-peak between D-Ala-1 H_α and Ala-4 $H_{3\beta}$ was observed. This transannular cross-peak was characteristic of the major conformer of RA-VII (**1**) and the distance between D-Ala-1 H_α and the nearest methyl proton of Ala-4 was estimated to be 2.92 Å in the crystal structure of **6**. Additional cross-peaks were observed between D-Ala-1 NH and Tyr-6 H_α, Ala-2 $H_{3\beta}$ and Tyr-3 $H_{2\delta}$, Tyr-3 H_α and Tyr-3 NMe, Tyr-3 H_α and Ala-4 NH, and Tyr-3 NMe and Ala-4 NH. The calculated distances between those protons in the crystal structure of **6** were all less than 3.0 Å. These observations indicated that the solution structure of conformer II resembled the crystal structure of **6**. These observations for RA-XXV (**4**) agreed with the results of a ROESY study of RA-XXVI (**5**). Thus, the conformational nature of **4** and **5** was found to be essentially identical as expected from the high similarity of the structures of **4** and **5**.

Cytotoxic activity: RA-XXV (**4**), RA-XXVI (**5**), and RA-XXVI acetate (**6**) were evaluated for their cytotoxic activity on human promyelocytic leukemia HL-60 and human colonic carcinoma HCT-116 cells with peptides **1** and **3** for comparison, and their IC_{50} values are summarized in Table 3. The cytotoxic activities of RA-XXV (**4**) and RA-XXVI (**5**) on those cell lines were significant but weaker than those of the corresponding *N*-methyl congeners. RA-XXV (**4**) was 7–19 times less cytotoxic than RA-VII (**1**), and RA-XXVI (**5**) was 7–12 times less cytotoxic than deoxybouvardin (**3**). RA-XXVI acetate (**6**) expressed almost the same activity as **5**. In addition to their structural differences, the weaker cytotoxic activity of **4** and **5** relative to that of **1** and **3** would partly be accounted for by their weaker tendency to adopt the active conformation. In $CDCl_3$ solution, RA-VII (**1**) adopted the active conformation, demonstrated by the crystal structure of **1**,[13] with population 83%. Peptides **4** and **5** adopted a similar conformation to the active conformation of **1** in $CDCl_3$ solution, which could be responsible for the activity, but their populations were only 25% and 28%, respectively.

Table 3

Cytotoxic activity[a]

Compound	HL-60	HCT-116
1	0.0032	0.0043
3	0.0053	0.0070
4	0.062	0.028
5	0.066	0.051
6	0.051	0.066

[a] IC_{50} (μM).

Conclusions

We have isolated two new RA-series peptides RA-XXV (**4**) and RA-XXVI (**5**) from the roots of *Rubia cordifolia* L. Their relative structures were determined on the basis of spectroscopic data and X-ray crystallography of RA-XXV (**4**) and RA-XXVI acetate (**6**), and their absolute stereochemistry was elucidated by the total synthesis of **4** and the chemical correlation between **4** and **5**, revealing that peptides **4** and **5** correspond to the des-*N*-methyl congeners of RA-VII (**1**) and deoxybouvardin (**3**) at Tyr-5, respectively. This is the first isolation of RA-series peptides lacking an *N*-methyl group at Tyr-5 residue from a natural source. Investigation of their conformational features showed that in solution, peptides **4** and **5** preferentially adopt a backbone conformation not seen in known natural peptides of this series, and the population of the active conformer is small. Thus, the *N*-methyl group at Tyr-5 is necessary for this series of peptides to take the active conformation preferentially. These findings will be useful to design the active synthetic analogues of peptide **1**.[18]

Experimental Section

General Experimental Procedures Melting points were determined on a Yanaco MP-3 apparatus and recorded uncorrected. Optical rotations were measured on a JASCO P-1030 digital polarimeter. NMR spectra were recorded on a Bruker AV-600, a DRX-500, or a DPX-400 spectrometer at 300 K unless otherwise indicated. The ^1H chemical shifts in $CDCl_3$, C_5D_5N, or CD_3OD were each referenced to the residual $CHCl_3$ (δ=7.26 ppm), C_5D_4HN (δ=7.21 ppm), or CD_2HOD (δ=3.31 ppm) resonance, and the ^{13}C chemical shifts to the solvent resonance (δ=77.03, 135.5, or 49.0 ppm). IR spectra were recorded on a JASCO FT/IR-620 spectrometer and UV spectra, on a JASCO V-530 spectrophotometer. 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 an SPD-10A UV detector (λ=254 nm) and a pre-packed ODS column (5 μm, 20 × 250 mm) eluted with a solvent mixture at the flow rate of 10 mL min^{-1}.

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

Extraction and Isolation The dried roots (50 kg) of *R. cordifolia* were 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 $CHCl_3$ eluate was crystallized from MeOH to give crystals of crude RAs (8.5 g) and the mother liquor (ML). After removal of the solvent, ML (10.2 g) was subjected to ODS HPLC using MeOH/H_2O (60:40, then 100:0) as eluent to give five fractions: M1 (0.42 g), M2 (2.44 g, mostly deoxybouvardin), M3 (0.33 g), M4 (0.11 g, mostly RA-VII), and M5 (1.12 g). Fraction M3 was separated by repeated ODS HPLC using MeCN/H_2O (30:70) as eluent to give RA-XXV (**4**, 16.9 mg). The residue obtained after removal of the solvent of the $CHCl_3$/MeOH (9:1) eluate from the aminopropyl-bonded silica gel CC was crystallized from MeOH to give crystals of crude RAs (2.4 g) and 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). Fraction F2 was subjected to ODS HPLC using MeCN/H_2O (28:72) and then MeOH as eluent to give nine fractions: F21 (45 mg), F22 (424 mg, RA-I), F23 (40 mg), F24 (209 mg), F25 (124 mg), F26 (37 mg), F27 (61 mg), F28 (28 mg), and F29 (MeOH eluate, 39 mg). Fractions F24 and F25 were each separated by ODS HPLC using MeOH/H_2O (55:45) as eluent to afford RA-XXVI (**5**, total 15.1 mg).

Characteristics of New Peptides

RA-XXV (4): Colorless plates; m.p. 293–295 °C (MeOH/H_2O); $[\alpha]_D^{25}$=−155 (*c*=0.063 in MeOH); IR (film): ν_{max}=3291, 3000, 2936, 2837, 1634, 1514, 1445, 1249, 1214, 1128, 1031, 754 cm^{-1}; UV (MeOH): λ_{max} (*ε*)=224sh (25100), 277 (3200), 282sh (2700 $mol^{-1}dm^3cm^{-1}$) nm; HR-ESIMS: *m/z* calcd for $C_{40}H_{48}N_6O_9$+Na^+: 779.3380 [*M*+Na^+]; found: 779.3398.

RA-XXVI (5): White amorphous solid; $[\alpha]_D^{25}$=−170 (*c*=0.048 in MeOH); IR (film): ν_{max}=3290, 2934, 1637, 1514, 1446, 1247 cm^{-1}; UV (MeOH): λ_{max} (*ε*)=225sh (26900), 277 (3700), 283sh (3300 $mol^{-1}dm^3cm^{-1}$) nm; HR-ESIMS: *m/z* calcd for $C_{39}H_{46}N_6O_9$+H^+: 743.3399 [*M*+H^+]; found: 743.3405.

Synthetic Study

RA-XXVI acetate (6): RA-XXVI (**5**) (4.6 mg, 0.0062 mmol) was treated with acetic anhydride (0.3 mL) in pyridine (0.6 mL) and the mixture was stirred at room temperature for 20 h. MeOH (1 mL) was added to the solution and the volatiles were removed in vacuo. The residue was crystallized from a MeOH/H_2O mixture to give acetate **6** (4.3 mg, 88%) as colorless plates: m.p. 277–281 °C; $[\alpha]_D^{25}$=−129 (*c*=0.085 in 1,4-dioxane); ^{1}H NMR (600 MHz, $CDCl_3$) conformer I: *δ*=8.01 (brd, *J*=7.6 Hz, 1H; Tyr-5 NH), 7.47 (dd, *J*=8.4, 2.1 Hz, 1H; Tyr-5 $H_{\delta b}$), 7.42 (dd, *J*=8.2, 2.1 Hz, 1H; Tyr-5 $H_{\delta a}$), 7.30 (d, *J*= 5.6 Hz, 1H; Ala-2 NH), 7.27 (dd, *J*=8.2, 2.4 Hz, 1H; Tyr-5 $H_{\varepsilon a}$), 7.11 (app d, *J*=8.6 Hz, 2H; Tyr-3 $H_{2\delta}$), 7.03 (dd, *J*=8.4, 2.4 Hz, 1H; Tyr-5 $H_{\varepsilon b}$), 6.90 (Ala-4 NH, overlapped), 6.89 (d, *J*=8.0 Hz, 1H; Tyr-6 $H_{\varepsilon a}$), 6.85 (app d, *J*=8.6 Hz, 2H; Tyr-3 $H_{2\varepsilon}$), 6.68 (dd, *J*=8.0, 1.9 Hz, 1H; Tyr-6 $H_{\delta a}$), 5.79 (brs, 1H; D-Ala-1 NH), 5.15 (m, 1H; Tyr-5 H_α), 5.07 (s, 1H; Tyr-6 $H_{\delta b}$), 4.57 (m, 1H; D-Ala-1 H_α), 4.47 (quintet, *J*=6.5 Hz; 1H, Ala-2 H_α), 3.81 (s, 3H; Tyr-3 OMe), 3.67 (quintet, *J*=7.2 Hz, 1H; Ala-4 H_α), 3.59 (brm, 1H; Tyr-3 H_α), 3.48 (Tyr-3 $H_{\beta a}$, overlapped), 3.41 (dd, *J*=11.1, 4.1 Hz, 1H; Tyr-6 H_α), 3.37 (dd, *J*=13.8, 4.3 Hz, 1H; Tyr-3 $H_{\beta b}$), 3.33 (dd, *J*=13.0, 5.3 Hz, 1H; Tyr-5 pro-*R* H_β), 3.22 (m, 2H; Tyr-5 pro-*S* H_β and Tyr-6 pro-*R* H_β), 3.07 (dd, *J*=14.6, 4.1 Hz, 1H; Tyr-6 pro-*S* H_β), 2.77 (s, 3H; Tyr-3 NMe), 2.73 (s, 3H; Tyr-6 NMe), 2.41 (s, 3H; Tyr-6 Ac), 1.72 (d, *J*=7.4 Hz, 3H; Ala-4 $H_{3\beta}$), 1.46 (d, *J*=6.9 Hz, 3H; Ala-2 $H_{3\beta}$), 1.22 ppm (d, *J*=7.1 Hz, 3H; D-Ala-1 $H_{3\beta}$); conformer II: *δ*=7.45 (dd, *J*=8.4, 2.1 Hz, 1H; Tyr-5 $H_{\delta b}$), 7.26 (Tyr-5 $H_{\delta a}$, overlapped), 7.17 (d, *J*=6.7 Hz, 1H; Ala-4 NH), 7.15 (dd, *J*=8.3, 2.4 Hz, 1H; Tyr-5 $H_{\varepsilon b}$), 7.04 (app d, *J*=8.6 Hz, 2H; Tyr-3 $H_{2\delta}$), 6.93 (d, *J*=8.2 Hz, 1H; Tyr-6 $H_{\varepsilon a}$), 6.90 (dd, *J*=8.4, 2.4 Hz, 1H; Tyr-5 $H_{\varepsilon a}$), 6.81 (app d, *J*=8.6 Hz, 2H; Tyr-3 $H_{2\varepsilon}$), 6.66 (dd, *J*=8.2, 1.9 Hz, 1H; Tyr-6 $H_{\delta a}$), 6.55 (d, *J*=7.0 Hz, 1H; D-Ala-1 NH), 4.79 (m, 1H; Tyr-5 H_α), 4.43 (d, *J*=1.9 Hz, 1H; Tyr-6 $H_{\delta b}$), 4.40 (m, 1H; Ala-2 H_α), 4.34 (Tyr-6 H_α, overlapped), 4.33 (quintet, *J*=7.0 Hz, 1H; D-Ala-1 H_α), 3.87 (brm, 1H; Ala-4 H_α), 3.78 (s, 3H; Tyr-3 OMe), 3.58 (dd, *J*=8.9, 6.8 Hz, 1H; Tyr-3 H_α), 3.50 (Tyr-5 pro-*S* H_β, overlapped), 3.45 (Tyr-6 pro-*R* H_β, overlapped), 3.40 (Tyr-3 $H_{2\beta}$, overlapped), 3.00 (Tyr-5 pro-*R* H_β, overlapped), 2.80 (Tyr-6 pro-*S* H_β, overlapped), 2.79 (s, 3H; Tyr-6 NMe), 2.71 (s, 3H; Tyr-3 NMe), 2.40 (s, 3H; Tyr-6 Ac), 1.66 (brd, *J*=6.9 Hz, 3H; Ala-4 $H_{3\beta}$), 1.27 (d, *J*=7.0 Hz, 3H; D-Ala-1 $H_{3\beta}$), 1.25 ppm (d, *J*=7.0 Hz, 3H; Ala-2 $H_{3\beta}$); ^{13}C NMR (150 MHz, $CDCl_3$) conformer I: *δ*=173.2 (2C; Ala-2 *C*=O and Ala-4 *C*=O), 172.8 (D-Ala-1 *C*=O), 171.5 (Tyr-5 *C*=O), 169.97 (Tyr-3 *C*=O), 169.4 (Tyr-6 *C*=O), 169.1 (Tyr-6 $COCH_3$), 158.5 (Tyr-3 C_ζ), 157.5 (Tyr-5 C_ζ), 153.9 (Tyr-6 $C_{\varepsilon b}$), 137.5 (Tyr-6 C_ζ), 135.9 (Tyr-6 C_γ), 135.2 (Tyr-5 C_γ), 131.7 (Tyr-5 $C_{\delta b}$), 131.4 (Tyr-5 $C_{\delta a}$), 130.4 (Tyr-3 C_γ), 130.2 (2C; Tyr-3 C_δ), 127.1 (Tyr-5 $C_{\varepsilon a}$), 123.5 (Tyr-5 $C_{\varepsilon b}$), 122.7 (Tyr-6 $C_{\varepsilon a}$), 121.8 (Tyr-6 $C_{\delta a}$), 120.5 (Tyr-6 $C_{\delta b}$), 114.1 (2C; Tyr-3 C_ε), 69.3 (Tyr-3 C_α), 67.4 (Tyr-6 C_α), 55.3 (Tyr-3 OMe), 52.7 (Ala-4 C_α), 51.6 (Tyr-5 C_α), 47.2 (D-Ala-1 C_α), 46.1 (Ala-2 C_α), 40.0 (Tyr-6 NMe), 39.6 (Tyr-3 NMe), 36.8 (Tyr-5 C_β), 33.2 (Tyr-6 C_β), 32.6 (Tyr-3 C_β), 20.82 (Tyr-6 $COCH_3$), 16.5 (Ala-4 C_β), 16.3 (D-Ala-1 C_β), 15.94 ppm (Ala-2 C_β); conformer II: *δ*=172.9 (Ala-2 *C*=O), 172.8 (Ala-4 *C*=O), 172.3 (D-Ala-1 *C*=O), 170.2 (2C; Tyr-5 *C*=O and Tyr-6 *C*=O), 170.03 (Tyr-3 *C*=O), 169.0 (Tyr-6 $COCH_3$), 158.3 (Tyr-3 C_ζ), 158.1 (Tyr-5 C_ζ), 155.2 (Tyr-6 $C_{\varepsilon b}$), 137.0 (Tyr-6 C_ζ), 135.5 (Tyr-5 C_γ), 134.1 (Tyr-6 C_γ), 132.6 (Tyr-5 $C_{\delta a}$), 130.9 (Tyr-3 C_γ), 130.8 (Tyr-5 $C_{\delta b}$), 130.3 (2C; Tyr-3 C_δ), 125.5 (Tyr-5 $C_{\varepsilon b}$), 124.4 (Tyr-5 $C_{\varepsilon a}$), 122.9 (Tyr-6 $C_{\varepsilon a}$), 121.6 (Tyr-6 $C_{\delta a}$), 114.6 (Tyr-6 $C_{\delta b}$), 113.9 (2C; Tyr-3 C_ε), 69.1 (Tyr-3 C_α), 56.8 (Tyr-6 C_α), 55.2 (Tyr-3 OMe), 52.9 (Ala-4 C_α), 52.5 (Tyr-5 C_α), 47.6 (D-Ala-1 C_α), 45.7 (Ala-2 C_α), 40.1 (Tyr-5 C_β), 39.4 (Tyr-3 NMe), 33.5 (Tyr-6 C_β), 32.8 (Tyr-3 C_β), 29.0 (Tyr-6 NMe), 20.79 (Tyr-6 $COCH_3$), 17.4 (Ala-4 C_β), 15.89 (Ala-2 C_β), 15.6 ppm (D-Ala-1 C_β); IR (film): ν_{max}=3296, 2985, 2934, 1766, 1661, 1631, 1512, 1246, 1200, 1113, 755 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{41}H_{48}N_6O_{10}$+H^+: 785.3510 [*M*+H^+]; found: 785.3528.

Cbz-3-iodo-L-tyrosine (8): Triethylamine (3.0 mL, 22.0 mmol) was added to a cooled solution (0 °C) of 3-iodo-L-tyrosine (**7**) (4.51 g, 14.7 mmol) in 1,4-dioxane/H_2O (1:1, 54 mL) and the solution was stirred at this temperature for 5 min. *N*-(Benzyloxycarbonyloxy)succinimide (4.03 g, 16.2 mmol) was added to the solution and the mixture was stirred at 0 °C for 1 h and then at room temperature for 17 h. The mixture was acidified (pH ~2) by adding aqueous $KHSO_4$ (5%) at 0 °C and then extracted with EtOAc (3 × 90 mL). The combined organic extracts were washed with brine (30 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was subjected to CC (silica gel, hexane/EtOAc/AcOH 15:15:1) to give **8** (6.36 g, 98%) as a white crystalline solid: m.p. 123–125 °C; $[\alpha]_D^{25}$=+14 (*c*=0.56 in MeOH); ^{1}H NMR (400 MHz, CD_3OD): *δ*=7.57 (d, *J*=1.9 Hz, 1H), 7.35–7.24 (m, 5H), 7.04 (dd, *J*=8.2, 1.9 Hz, 1H), 6.73 (d, *J*=8.2 Hz, 1H), 5.08 (d, *J*=12.5 Hz, 1H), 5.01 (d, *J*=12.5 Hz, 1H), 4.35 (dd, *J*=9.1, 4.9 Hz, 1H), 3.07 (dd, *J*=14.0, 4.9 Hz, 1H), 2.81 ppm (dd, *J*=14.0, 9.1 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): *δ*=175.0 (s), 158.4 (s), 156.8 (s), 141.0 (d), 138.2 (s), 131.40 (s), 131.38 (d), 129.5 (d, 2C), 128.9 (d), 128.5 (d, 2C), 115.6 (d), 84.4 (s), 67.5 (t), 56.8 (d), 37.2 ppm (t); IR (film): ν_{max}=3323, 3032, 2926, 1694, 1503, 1414, 1344, 1255, 1217, 1060, 752 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{17}H_{16}INO_5$+Na^+: 463.9971 [*M*+Na^+]; found: 463.9959; elemental analysis calcd (%) for $C_{17}H_{16}INO_5$: C 46.28, H 3.66, N 3.17; found: C 46.30, H 3.83, N 3.24.

Oxazolidinone 9: *p*-Toluenesulfonic acid monohydrate (157 mg, 0.825 mmol) was added to a suspension of Cbz-3-iodo-L-tyrosine (**8**) (1.82 g, 4.12 mmol) and paraformaldehyde (2.12 g, 70.6 mmol) in toluene (40

mL) and the mixture was stirred at room temperature for 3 days. The mixture was diluted with EtOAc (240 mL) and the solution was washed sequentially with saturated aqueous $NaHCO_3$ (3 × 40 mL), water (40 mL), and brine (40 mL), dried over $MgSO_4$, and filtered. The solvent was removed in vacuo and the residue was crystallized from EtOAc to give **9** (1.66 g, 89%) as colorless fine needles: m.p. 140–141 °C; $[\alpha]_D^{26}$=+158 (*c*=0.62 in $CHCl_3$); ^{1}H NMR (500 MHz, C_5D_5N, 360 K): *δ*=11.79 (brs, 1H), 7.86 (d, *J*=1.9 Hz, 1H), 7.50 (app d, *J*=7.5 Hz, 2H), 7.42 (app t, *J*=7.5 Hz, 2H), 7.35 (app t, *J*=7.5 Hz, 1H), 7.13 (dd, *J*=8.1, 1.9 Hz, 1H), 6.99 (d, *J*=8.1 Hz, 1H), 5.42 (d, *J*=4.0 Hz, 1H), 5.37 (d, *J*=13.0 Hz, 1H), 5.35 (d, *J*=13.0 Hz, 1H), 4.78 (d, *J*=4.0 Hz, 1H), 4.72 (m, 1H), 3.38 (dd, *J*=14.1, 5.7 Hz, 1H), 3.18 ppm (dd, *J*=14.1, 3.3 Hz, 1H); ^{13}C NMR (125 MHz, C_5D_5N, 360 K): *δ*=172.2 (s), 157.8 (s), 153.2 (s), 141.1 (d), 136.9 (s), 131.4 (d), 129.2 (d, 2C), 128.8 (d), 128.71 (d, 2C), 128.68 (s), 116.1 (d), 86.1 (s), 78.6 (t), 68.1 (t), 57.0 (d), 35.2 ppm (t); IR (film): ν_{max}=3362, 3031, 2919, 1800, 1712, 1693, 1418, 1359, 1129, 1051, 751 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{18}H_{16}INO_5$+Na$^+$: 475.9971 [*M*+Na$^+$]; found: 475.9976; elemental analysis calcd (%) for $C_{18}H_{16}INO_5$: C 47.70, H 3.56, N 3.09; found: C 47.61, H 3.69, N 3.16.

Cbz-3-iodo-*N,O*-dimethyl-L-Tyr-OMe (10): Triethylsilane (1.6 mL, 10 mmol) and TFA (21 mL) were added to a solution of **9** (1.55 g, 3.42 mmol) in $CHCl_3$ (21 mL), and the mixture was stirred at room temperature for 3 days. Volatiles were removed in vacuo and the residue was dissolved in MeCN/MeOH (9:1, 14 mL). To this, (trimethylsilyl)diazomethane (2.0 M in diethyl ether, 5.1 mL, 10.2 mmol) was added. The mixture was stirred at room temperature for 2 days and concentrated in vacuo. The residue was dissolved in MeCN/MeOH (9:1, 14 mL) again and treated with (trimethylsilyl)diazomethane (2.0 M in diethyl ether, 1.7 mL, 3.4 mmol). After stirring for 2 days at room temperature, the solution was concentrated in vacuo. The residue was subjected to CC (silica gel, hexane/EtOAc 7:2) to afford **10** (1.26 g, 76%) as a colorless amorphous solid: $[\alpha]_D^{25}$=−47 (*c*=0.63 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, mixture of two rotamers) major rotamer: *δ*=7.61 (s, 1H), 7.37–7.20 (m, 5H), 7.13 (d, *J*=8.3 Hz, 1H), 6.70 (d, *J*=8.3 Hz, 1H), 5.13 (d, *J*=12.6 Hz, 1H), 5.09 (d, *J*=12.6 Hz, 1H), 4.92 (dd, *J*=10.4, 5.5 Hz, 1H), 3.84 (s, 3H), 3.74 (s, 3H), 3.25 (dd, *J*=14.7, 5.5 Hz, 1H), 2.94 (dd, *J*=14.7, 10.4 Hz, 1H), 2.81 ppm (s, 3H); minor rotamer: *δ*=7.59 (s, 1H), 7.37–7.20 (m, 5H), 7.02 (d, *J*=8.3 Hz, 1H), 6.65 (d, *J*=8.3 Hz, 1H), 5.07 (d, *J*=12.5 Hz, 1H), 5.04 (d, *J*=12.5 Hz, 1H), 4.74 (dd, *J*=10.5, 4.8 Hz, 1H), 3.84 (s, 3H), 3.67 (s, 3H), 3.20 (dd, *J*=14.9, 4.8 Hz, 1H), 2.89 (dd, *J*=14.9, 10.5 Hz, 1H), 2.84 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of two rotamers) major rotamer: *δ*=171.2 (s), 157.0 (s), 156.6 (s), 139.8 (d), 136.6 (s), 131.3 (s), 129.9 (d), 128.5 (d, 2C), 127.9 (d), 127.6 (d, 2C), 110.9 (d), 85.7 (d), 67.3 (t), 60.3 (d), 56.3 (q), 52.3 (q), 33.5 (t), 31.8 ppm (q); minor rotamer: *δ*=171.0 (s), 157.0 (s), 155.8 (s), 139.7 (d), 136.3 (s), 131.3 (s), 130.0 (d), 128.5 (d, 2C), 128.1 (d), 128.0 (d, 2C), 110.8 (d), 86.0 (d), 67.5 (t), 60.6 (d), 56.3 (q), 52.3 (q), 33.9 (t), 32.1 ppm (q); IR (film): ν_{max}=3030, 3006, 2951, 2839, 1742, 1702, 1492, 1454, 1401, 1317, 1281, 1254, 1218, 1181, 1137, 1049, 1016, 699 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{20}H_{22}INO_5$+Na$^+$: 506.0440 [*M*+Na$^+$]; found: 506.0436.

Boronic acid 11: $PdCl_2(dppf)\cdot CH_2Cl_2$ (378 mg, 0.463 mmol) was added to a solution of **10** (1.49 g, 3.08 mmol), bis(pinacolato)diboron (1.02 g, 4.02 mmol), and potassium acetate (909 mg, 9.26 mmol) in dimethyl sulfoxide (21 mL) and the mixture was stirred at 80 °C for 20 h under an atmosphere of argon. Water (250 mL) was added to the solution, and the whole was extracted with EtOAc (4 × 36 mL). The combined organic extracts were washed with brine (36 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was subjected to CC (silica gel). After evaporation, the residue of the fraction eluted with hexane/EtOAc (3:1) was dissolved in acetone (140 mL) and to this, a solution of ammonium acetate (476 mg, 6.18 mmol) and sodium (meta)periodate (658 mg, 3.08 mmol) in water (70 mL) was added. After stirring at room temperature for 2 h, a solution of sodium (meta)periodate (658 mg, 3.08 mmol) in water (70 mL) was added and the mixture was stirred at room temperature for 18 h. The mixture was concentrated in vacuo to remove acetone and the residue was extracted with $CHCl_3$ (3 × 50 mL). The combined organic extracts were washed with brine (50 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was subjected to CC (silica gel, hexane/EtOAc 3:2) to afford **11** (1.16 g, 94%) as a colorless amorphous solid: $[\alpha]_D^{25}$=−55 (*c*=0.51 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, mixture of two rotamers) major rotamer: *δ*=7.68 (d, *J*=1.8 Hz, 1H), 7.34–7.18 (m, 6H), 6.80 (d, *J*=8.5 Hz, 1H), 6.30 (s, 2H), 5.11 (d, *J*=12.6 Hz, 1H), 5.07 (d, *J*=12.6 Hz, 1H), 5.00 (dd, *J*=10.9, 5.3 Hz, 1H), 3.87 (s, 3H), 3.74 (s, 3H), 3.31 (dd, *J*=14.6, 5.3 Hz, 1H), 3.01 (dd, *J*=14.6, 10.9 Hz, 1H), 2.82 ppm (s, 3H); minor rotamer: *δ*=7.68 (d, *J*=1.8 Hz, 1H), 7.34–7.18 (m, 5H), 7.15 (dd, *J*=8.5, 1.8 Hz, 1H), 6.75 (d, *J*=8.5 Hz, 1H), 6.27 (s, 2H), 5.05 (d, *J*=12.6 Hz, 1H), 5.02 (d, *J*=12.6 Hz, 1H), 4.80 (dd, *J*=10.8, 4.8 Hz, 1H), 3.87 (s, 3H), 3.67 (s, 3H), 3.26 (dd, *J*=14.4, 4.8 Hz, 1H), 2.96 (dd, *J*=14.4, 10.8 Hz, 1H), 2.84 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of two rotamers) major rotamer: *δ*=171.5 (s), 163.3 (s), 156.6 (s), 137.2 (d), 136.7 (s), 133.0 (d), 129.5 (s), 128.4 (d, 2C), 127.8 (d), 127.5 (d, 2C), 118.4 (s), 110.1 (d), 67.2 (t), 60.2 (d), 55.5 (q), 52.3 (q), 33.9 (t), 31.6 ppm (q); minor rotamer: *δ*=171.3 (s), 163.3 (s), 155.9 (s), 137.2 (d), 136.4 (s), 133.1 (d), 129.6 (s), 128.4 (d, 2C), 127.9 (d, 2C), 127.8 (d), 118.6 (s), 110.08 (d), 67.4 (t), 60.7 (d), 55.5 (q), 52.3 (q), 34.3 (t), 32.1 ppm (q); IR (film): ν_{max}=3408, 3030, 2952, 2842, 1742, 1698, 1607, 1493, 1421, 1331, 1236, 1042, 1008, 755, 698 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{20}H_{24}BNO_7$+H$^+$: 402.1724 [*M*+H$^+$]; found: 402.1707.

Amine 12: Palladium (10%) on charcoal catalyst (48.9 mg) was added to a solution of **11** (489 mg, 1.22 mmol) in EtOH (13 mL). Then, the reaction mixture was stirred at room temperature under an atmosphere of hydrogen for 20 min. The catalyst was filtered off, the filtrate was concentrated to dryness, and the residue was crystallized from EtOH to give **12** (291 mg) as a white crystalline solid. Chromatographic separation (silica gel, $CHCl_3$/MeOH 30:1) of ML gave additional **12** (30 mg, total 321 mg, 99%): m.p. 268–273 °C (decomp.); $[\alpha]_D^{25}$=+25 (*c*=0.72 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): *δ*=7.65 (d, *J*=2.3 Hz, 1H), 7.24 (dd, *J*=8.5, 2.3 Hz, 1H), 6.84 (d, *J*=8.5 Hz, 1H), 3.89 (s, 3H), 3.69 (s, 3H), 3.44 (t, *J*=6.7 Hz, 1H), 2.94 (dd, *J*=13.7, 6.4 Hz, 1H), 2.91 (dd, *J*=13.7, 6.9 Hz, 1H), 2.36 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ*=174.8 (s), 163.5 (s), 137.5 (d), 133.5 (d), 129.7 (s), 110.0 (d), 64.7 (d), 55.6 (q), 51.7 (q), 38.5 (t), 34.7 ppm (q); IR (film): ν_{max}=3515, 3321, 2950, 1734, 1605, 1582, 1492, 1418, 1335, 1236, 1177, 1046, 818, 756 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{12}H_{18}BNO_5$+H$^+$: 268.1356 [*M*+H$^+$]; found: 268.1368.

Dipeptide 13: EDC·HCl (290 mg, 1.51 mmol) was added to a solution of **12** (202 mg, 0.756 mmol), Boc-L-Tyr-OH (425 mg, 1.51 mmol), and HOAt (206 mg, 1.51 mmol) in DMF (3.7 mL) and the mixture was stirred at room temperature for 7 days. The solvent was removed in vacuo. The residue was treated with $CHCl_3$ (10 mL) and aqueous citric acid (10%, 20 mL) and extracted with $CHCl_3$ (3 × 20 mL). The combined organic extracts were washed with brine (20 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was subjected to HPLC (MeCN/H_2O 30:70) to give **13** (341 mg, 85%) as a colorless amorphous solid: $[\alpha]_D^{25}$=−48 (*c*=0.32 in $CHCl_3$); ^{1}H NMR (500 MHz, C_5D_5N, major rotamer): *δ*=11.29 (s, 1H), 9.17 (s, 2H), 8.22 (d, *J*=2.1 Hz, 1H), 8.10 (d, *J*=9.1 Hz, 1H), 7.37 (app d, *J*=8.4 Hz, 2H), 7.29 (dd, *J*=8.4, 2.1 Hz, 1H), 7.12 (app d, *J*=8.4 Hz, 2H), 6.84 (d, *J*=8.4 Hz, 1H), 5.22 (m, 1H), 5.20 (m, 1H), 3.64 (s, 3H), 3.60 (s, 3H), 3.48 (dd, *J*=14.2, 5.7 Hz, 1H), 3.39 (dd, *J*=13.7, 6.6 Hz, 1H), 3.28 (dd, *J*=14.2, 9.5 Hz, 1H), 3.18 (dd, *J*=13.7, 7.4 Hz, 1H), 3.04 (s, 3H), 1.45 ppm (s, 9H); ^{13}C NMR (125 MHz, C_5D_5N, major rotamer): *δ*=172.9 (s), 171.3 (s), 163.4 (s), 157.6 (s), 156.2 (s), 137.8 (d), 132.8 (d), 131.1 (d, 2C), 130.2 (s), 128.4 (s), 122.0

(s), 116.1 (d, 2C), 110.6 (d), 78.5 (s), 61.1 (d), 55.3 (q), 53.0 (d), 51.8 (q), 38.3 (t), 34.2 (q), 34.2 (t), 28.4 ppm (q, 3C); IR (film): ν_{max}=3346, 3013, 2978, 1740, 1695, 1635, 1613, 1517, 1494, 1420, 1367, 1238, 1170, 1046, 1020, 822, 757 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{26}H_{35}BN_2O_9$+Na^+: 553.2333 [*M*+Na^+]; found: 553.2333.

Cycloisodityrosine 14: A mixture of **13** (55.9 mg, 0.105 mmol), DMAP (64.1 mg, 0.525 mmol), 4Å powdered molecular sieves (300 mg) in CH_2Cl_2 (10 mL) was stirred at room temperature for 30 min. Copper(II) acetate (19.1 mg, 0.105 mmol) was added and the mixture was stirred at room temperature for 2 days. Insoluble materials were removed by filtration and aqueous $KHSO_4$ (5%, 10 mL) was added to the filtrate. The whole was extracted with $CHCl_3$ (3 × 10 mL). The combined extracts were washed with brine (10 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was separated by CC (silica gel, hexane/EtOAc 1:5) and then by HPLC (MeCN/H_2O 48:52) to give **14** (18.7 mg, 37%) as colorless prisms: m.p. 184–185 °C; $[\alpha]_D^{26}$=−103 (*c*=0.29 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$, major rotamer): *δ*=7.44 (dd, *J*=8.4, 2.2 Hz, 1H), 7.23 (dd, *J*=8.4, 2.2 Hz, 1H), 7.16 (dd, *J*=8.4, 2.4 Hz, 1H), 6.92 (dd, *J*=8.4, 2.4 Hz, 1H), 6.82 (d, *J*=8.2 Hz, 1H), 6.61 (app d, *J*=8.2 Hz, 1H), 5.12 (d, *J*=9.6 Hz, 1H), 4.45 (dd, *J*=12.1, 3.3 Hz, 1H), 4.41 (m, 1H), 4.33 (d, *J*=2.1 Hz, 1H), 3.94 (s, 3H), 3.70 (s, 3H), 3.26 (t, *J*=11.3 Hz, 1H), 3.22 (dd, *J*=17.9, 3.3 Hz, 1H), 3.06 (dd, *J*=11.7, 4.0 Hz, 1H), 2.99 (dd, *J*=17.9, 12.1 Hz, 1H), 2.61 (s, 3H), 1.44 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$, major rotamer): *δ*=173.8 (s), 171.4 (s), 158.2 (s), 154.6 (s), 153.1 (s), 146.3 (s), 134.9 (s), 132.4 (d), 130.5 (d), 127.9 (s), 125.9 (d), 124.7 (d), 120.6 (d), 113.6 (d), 112.3 (d), 79.8 (s), 57.4 (d), 56.2 (q), 53.1 (d), 52.7 (q), 40.8 (t), 32.6 (t), 28.4 (q, 3C), 28.2 ppm (q); IR (film): ν_{max}=3310, 2977, 2933, 2838, 1747, 1708, 1643, 1518, 1499, 1265, 1205, 1169, 1129, 754 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{26}H_{32}N_2O_7$+H^+: 485.2289 [*M*+H^+]; found: 485.2288; elemental analysis calcd (%) for $C_{26}H_{32}N_2O_7$: C 64.45, H 6.66, N 5.78; found: C 64.38, H 6.64, N 5.83.

Benzyl ester 15: A LiOOH solution [LiOH·H_2O (3.5 mg, 0.083 mmol) dissolved in aqueous H_2O_2 (35%, 0.1 mL)] was slowly added to a cooled (0 °C) solution of **14** (10.2 mg, 0.0211 mmol) in MeOH (0.4 mL). The solution was stirred at 0 °C for 1 h and then at room temperature for 23 h. Aqueous $NaHSO_3$ (5%, 0.5 mL) and aqueous citric acid (10%, 1 mL) were added to the solution at 0 °C. After stirring for 10 min, the mixture was extracted with $CHCl_3$ (3 × 10 mL). The combined organic extracts were washed with brine (5 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue, triphenylphosphine (22.0 mg, 0.0839 mmol), and benzyl alcohol (9.0 µL, 0.087 mmol) were dissolved in THF (0.4 mL) and to this, diethyl azodicarboxylate (2.2 M in toluene, 38 µL, 0.084 mmol) was slowly added at 0 °C. The mixture was stirred at 0 °C for 1 h and then at room temperature for 10 h. The solvent was removed in vacuo and the residue was subjected to HPLC (MeCN/H_2O 60:40) to give **15** (8.8 mg, 75%) as a white crystalline solid: m.p. 205–206 °C; $[\alpha]_D^{25}$=−82 (*c*=0.41 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$, major rotamer): *δ*=7.45 (dd, *J*=8.4, 2.2 Hz, 1H), 7.39–7.29 (m, 5H), 7.24 (dd, *J*=8.4, 2.2 Hz, 1H), 7.16 (dd, *J*=8.4, 2.4 Hz, 1H), 6.92 (dd, *J*=8.4, 2.4 Hz, 1H), 6.79 (d, *J*=8.4 Hz, 1H), 6.58 (dd, *J*=8.4, 1.9 Hz, 1H), 5.16 (d, *J*=9.6 Hz, 1H), 5.15 (d, *J*=12.0 Hz, 1H), 5.05 (d, *J*=12.0 Hz, 1H), 4.50–4.43 (m, 2H), 4.32 (d, *J*=1.9 Hz, 1H), 3.93 (s, 3H), 3.26 (t, *J*=11.3 Hz, 1H), 3.20 (dd, *J*=18.0, 2.9 Hz, 1H), 3.07 (dd, *J*=11.9, 4.0 Hz, 1H), 2.96 (dd, *J*=18.0, 12.1 Hz, 1H), 2.61 (s, 3H), 1.46 ppm (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$, major rotamer): *δ*=174.0 (s), 170.9 (s), 158.2 (s), 154.6 (s), 153.1 (s), 146.3 (s), 135.2 (s), 134.9 (s), 132.5 (d), 130.5 (d), 128.7 (d, 2C), 128.6 (d), 128.5 (d, 2C), 127.8 (s), 126.0 (d), 124.7 (d), 120.6 (d), 113.6 (d), 112.3 (d), 79.8 (s), 67.7 (t), 57.6 (d), 56.2 (q), 53.0 (d), 40.8 (t), 32.5 (t), 28.5 (q, 3C), 28.3 ppm (q); IR (film): ν_{max}=3436, 3315, 2977, 2932, 1746, 1707, 1644, 1518, 1498, 1280, 1265, 1218, 1200, 1172, 1129, 754 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{32}H_{36}N_2O_7$+Na^+: 583.2420 [*M*+Na^+]; found: 583.2411; elemental analysis calcd (%) for $C_{32}H_{36}N_2O_7$: C 68.55, H 6.47, N 5.00; found: C 68.61, H 6.47, N 4.98.

Hexapeptide 17: A solution of **15** (12.2 mg, 0.0218 mmol) in TFA (1.0 mL) was stirred at room temperature for 2 h. TFA was removed in vacuo. The residue was treated with $CHCl_3$ (5 mL) and saturated aqueous $NaHCO_3$ (10 mL) and the whole was extracted with $CHCl_3$ (3 × 10 mL). The combined $CHCl_3$ extracts were washed with brine (10 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue, **16** (17.1 mg, 0.0327 mmol), and HOOBt (5.3 mg, 0.032 mmol) were dissolved in DMF (0.5 mL) and to this, EDC·HCl (6.3 mg, 0.033 mmol) was added at 0 °C. The mixture was stirred at room temperature for 10 days. Saturated aqueous $NaHCO_3$ (10 mL) was added to the solution and the whole was extracted with $CHCl_3$ (3 × 10 mL). The combined organic extracts were washed sequentially with aqueous citric acid (10%, 2 × 10 mL) and brine (10 mL), dried over Na_2SO_4, and filtered, and the solvent was removed in vacuo. The residue was separated by CC (silica gel, hexane/$CHCl_3$/MeOH 20:20:1) and then by HPLC (MeCN/H_2O 55:45) to give **17** (14.6 mg, 70%) as a colorless amorphous solid: $[\alpha]_D^{26}$=−99 (*c*=0.13 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$, mixture of rotamers): *δ*=8.16 (d, *J*=8.3 Hz), 7.54–7.25 (m), 7.23 (dd, *J*=8.3, 1.9 Hz), 7.17 (d, *J*=8.2 Hz), 7.10 (d, *J*=8.4 Hz), 7.00 (d, *J*=8.4 Hz), 6.91 (d, *J*=8.3 Hz), 6.90 (d, *J*=8.3 Hz), 6.84 (d, *J*=8.5 Hz), 6.79 (d, *J*=8.3 Hz), 6.74 (d, *J*=8.1 Hz), 6.61–6.54 (m), 5.85 (brm), 5.57 (brm), 5.29 (brm), 5.13 (s), 5.02 (s), 4.84 (dd, *J*=11.3, 2.4 Hz), 4.84–4.76 (m), 4.74–4.66 (m), 4.60 (d, *J*=10.1 Hz), 4.54 (septet, *J*=7.8 Hz), 4.50 (d, *J*=10.8 Hz), 4.44–4.28 (m), 4.34 (s), 4.23 (brm), 3.93 (s), 3.78 (s), 3.74 (s), 3.41–3.18 (m), 3.08 (dd, *J*=11.8, 3.7 Hz), 3.02–2.87 (m), 2.89 (s), 2.86 (s), 2.59 (s), 2.57 (s), 1.44 (s), 1.43 (s), 1.40 (d, *J*=6.3 Hz), 1.36 (d, *J*=6.4 Hz), 1.30 (d, *J*=7.1 Hz), 0.47 ppm (d, *J*=6.6 Hz); ^{13}C NMR (125 MHz, $CDCl_3$, mixture of rotamers, selected data): *δ*=174.5 (s), 174.1 (s), 173.5 (s), 173.4 (s), 173.1 (s), 172.2 (s), 171.1 (s), 170.8 (s), 170.6 (s), 169.0 (s), 168.8 (s), 158.6 (s), 158.5 (s), 158.10 (s), 158.05 (s), 156.0 (s), 155.9 (s), 153.11 (s), 153.06 (s), 146.4 (s), 146.3 (s), 135.4 (s), 135.3 (s), 135.1 (s), 132.6 (d), 132.3 (d), 130.8 (d), 130.7 (d), 130.3 (d), 130.1 (d), 129.8 (s), 129.7 (s), 128.7 (d), 128.64 (d), 128.61 (d), 128.58 (d), 128.5 (d), 127.9 (s), 127.7 (s), 126.0 (d), 124.5 (d), 120.6 (d), 114.4 (d), 114.1 (d), 113.6 (d), 113.5 (d), 112.3 (d), 80.4 (s), 79.9 (s), 68.0 (t), 67.9 (t), 65.3 (d), 63.4 (d), 57.7 (d), 57.5 (d), 56.2 (q), 55.34 (q), 55.25 (q), 52.2 (d), 51.7 (d), 50.4 (d), 50.1 (d), 49.9 (d), 49.3 (d), 45.8 (d), 44.9 (d), 39.7 (t), 36.8 (q), 32.9 (t), 32.7 (t), 32.41 (t), 32.36 (t), 29.6 (q), 28.39 (q), 28.35 (q), 28.2 (q), 18.0 (q), 17.6 (q), 17.1 (q), 16.5 (q), 16.0 ppm (q); IR (film): ν_{max}=3301, 2978, 2933, 1742, 1635, 1515, 1249, 1177, 1029, 754 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{52}H_{64}N_6O_{12}$+Na^+: 987.4480 [*M*+Na^+]; found: 987.4496.

Amine 18: A solution of **17** (12.8 mg, 0.0133 mmol) in TFA (1.0 mL) was stirred at room temperature for 2.5 h. TFA was removed in vacuo. The residue was treated with $CHCl_3$ (5 mL) and saturated aqueous $NaHCO_3$ (5 mL) and the whole was extracted with $CHCl_3$ (3 × 10 mL). The combined $CHCl_3$ extracts were washed with brine (10 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was separated by CC (silica gel, $CHCl_3$/MeOH 20:1) to give amine **18** (8.8 mg, 77%) as a colorless amorphous solid: $[\alpha]_D^{26}$=−100 (*c*=0.09 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, major rotamer): *δ*=8.52 (d, *J*=8.9 Hz, 1H), 7.60 (d, *J*=4.8 Hz, 1H), 7.46 (dd, *J*=8.3, 2.3 Hz, 1H), 7.43–7.31 (m, 5H), 7.28 (d, *J*=9.1 Hz, 1H), 7.18 (dd, *J*=8.3, 2.3 Hz, 1H), 7.17 (dd, *J*=8.3, 2.3 Hz, 1H), 7.01 (app d, *J*=8.7 Hz, 2H), 6.89 (dd, *J*=8.3, 2.3 Hz, 1H), 6.79 (app d, *J*=8.7 Hz, 3H), 6.58 (dd, *J*=8.3, 2.1 Hz, 1H), 5.14 (s, 2H), 4.83 (dd, *J*=11.2, 3.1 Hz, 1H), 4.70 (m, 1H), 4.60 (dq, *J*=8.9, 7.3 Hz, and overlapping signal, 3H), 4.51 (dd, *J*=12.2, 3.3 Hz, 1H), 4.34 (d, *J*=2.1 Hz, 1H), 4.30 (qd, *J*=6.8, 4.8 Hz, 1H), 3.93 (s, 3H), 3.75 (s, 3H), 3.66 (q, *J*=7.0 Hz, 1H), 3.38 (dd, *J*=14.8, 3.0 Hz, 1H), 3.20 (m, 1H), 3.03–2.86 (m, 4H), 2.90 (s, 3H), 2.55 (s, 3H), 1.43 (d, *J*=7.3 Hz, 3H), 1.34 (d, *J*=7.0 Hz, 3H), 0.50 ppm (d, *J*=6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, major

rotamer): *δ*=177.5 (s), 173.4 (s), 172.9 (s), 172.3 (s), 171.1 (s), 168.9 (s), 158.6 (s), 158.0 (s), 153.0 (s), 146.3 (s), 135.5 (s), 135.3 (s), 132.4 (d), 130.7 (d), 130.3 (d, 2C), 130.1 (s), 128.6 (d, 4C), 128.4 (d), 127.8 (s), 126.1 (d), 124.4 (d), 120.7 (d), 114.3 (d, 2C), 113.5 (d), 112.2 (d), 68.0 (t), 63.5 (d), 57.4 (d), 56.2 (q), 55.3 (q), 52.0 (d), 50.5 (d), 50.4 (d), 44.6 (d), 39.7 (t), 32.8 (t), 32.4 (t), 29.5 (q), 28.2 (q), 21.1 (q), 18.2 (q), 15.8 ppm (q); IR (film): ν_{max}=3295, 2960, 2931, 1740, 1644, 1633, 1515, 1262, 1202, 1128, 1092, 1030, 803, 756 cm^{-1}; HR-ESIMS: *m/z* calcd for $C_{47}H_{56}N_6O_{10}$+Na^+: 887.3956 [*M*+Na^+]; found: 887.3956.

RA-XXV (4): Palladium (10%) on charcoal catalyst (17.6 mg) was added to a solution of **18** (8.8 mg, 0.010 mmol) in EtOH (2 mL). Then, the reaction mixture was stirred at room temperature under an atmosphere of hydrogen for 2.5 h. The catalyst was filtered off, the filtrate was concentrated to dryness, and the residue was dissolved in DMF (7.7 mL). To this solution were added HOOBt (13.1 mg, 0.0803 mmol) and EDC·HCl (15.3 mg, 0.0798 mmol) at 0 °C. After stirring at 0 °C for 2 h and then at room temperature for 3 days, the solvent was removed under reduced pressure. The residue was treated with $CHCl_3$ (5 mL) and aqueous citric acid (10%, 10 mL) and the whole was extracted with $CHCl_3$ (3 × 10 mL). The combined organic extracts were washed sequentially with saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried over $MgSO_4$, and filtered, and the solvent was removed in vacuo. The residue was separated by CC (silica gel, hexane/EtOAc 3:2, then $CHCl_3$/MeOH 5:1), and then by HPLC (MeOH/H_2O 58:42) to give a compound [3.2 mg, 42%, $[\alpha]_D^{25}$=−152 (*c*=0.050 in MeOH)] that was shown to be identical to natural **4** by comparison of their ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra, IR spectra, mass spectra, and optical rotations.

***O*-Methylation of RA-XXVI (5):** Iodomethane (4.5 µL, 0.072 mmol) and K_2CO_3 (2.0 mg, 0.014 mmol) were added to a suspension of RA-XXVI (**5**) (2.6 mg, 0.0035 mmol) in acetone/MeOH (3:1, 0.2 mL) and the mixture was stirred at 40 °C for 6 h. The mixture was passed through a CBA cartridge column with MeOH and the eluate was separated by HPLC (MeCN/H_2O 40:60) to give a compound [2.5 mg, 94%, $[\alpha]_D^{25}$=−157 (*c*=0.020 in MeOH)] that was shown to be identical to natural **4** by comparison of their ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra, IR spectra, mass spectra, and optical rotations.

Monte Carlo conformational search and DFT calculations

The software used for the Monte Carlo (MC) conformational search was MacroModel ver. 8.1 (Schrödinger, Portland, OR). The MC search was configured using the "automatic setup" routine in MacroModel with all amide configuration in the macrocycle fixed as determined by the ROESY experiments. Calculation consisted of 50000 MC steps with 500 iterations per step using the MMFFs force field and the PR conjugate gradient (PRCG) with no solvation. DFT calculations were performed using Gaussian 09W (revision B.01) software[24] with B3LYP functional and 6-31G(d) basis set.

Assays for Cytotoxic Activity

Assays for cytotoxic activity were performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. Human promyelocytic leukemia HL-60 cells were precultured in RPMI 1640 medium (Nissui Co. Ltd., Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (100 mg L^{-1}) in a humidified atmosphere of 93% air and 7% CO_2 at 37 °C. The cell suspension (3 × 10^4 cells mL^{-1}, 100 µL) was added to each well (3 × 10^3 cells $well^{-1}$) of a 96-microwell plate (flat-bottomed, polystyrene-treated) and incubated for 24 h. Test compound solutions in dimethyl sulfoxide (DMSO) (1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, 0.0001 µg mL^{-1}) were prepared and 10 µL of the test solution or DMSO (control) was added to each well. The plate was kept in an incubator for 48 h. After termination of the cell culture by adding 0.5% MTT in PBS (20 µL) to each well, the plate was kept in the incubator for 4 h. After addition of 100 µL of 10% SDS-0.01 N aqueous HCl to each well, the plate was read on a microplate reader (MPR A4i, Tosoh Co., Japan) at 550/700 nm. A dose-response curve was plotted for each compound and concentrations exhibiting 50% inhibition of cell growth (IC_{50}) were recorded.

The assay using human colon carcinoma HCT-116 cells was performed in the same manner except that McCoy's 5A medium (Invitrogen Co., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (100 mg L^{-1}) was used for preculture and that the cell suspension used was 3 × 10^5 cells mL^{-1}, 100 µL, i.e., 3 × 10^4 cells $well^{-1}$.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: *Rubia cordifolia* • RA-XXV • RA-XXVI • synthesis • conformation

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[23] Abbreviations: Cbz-OSu=*N*-(benzyloxycarbonyloxy)succinimide, DMAP=4-(dimethylamino)pyridine, EDC·HCl=1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride, HOAt=1-hydroxy-7-azabenzotriazole, HOOBt=3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one, $PdCl_2(dppf)\cdot CH_2Cl_2$=[1,1´-bis(diphenylphosphino)ferrocene]dichloropalladium(II) dichloromethane complex, TFA=trifluoroacetic acid.

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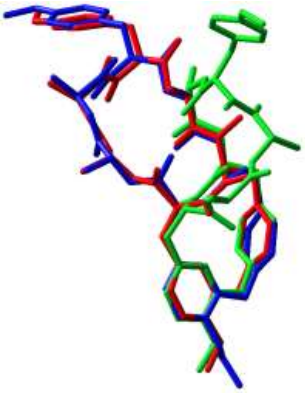
Layout 1:

FULL PAPER

Two new RA-series bicyclic hexapeptides, RA-XXV (**4**) and RA-XXVI (**5**), which have no *N*-methyl group at Tyr-5, were isolated from the roots of *Rubia cordifolia* L. and their absolute structures were established by synthetic methods. Investigation of their conformational property and cytotoxic acitivity revealed that the *N*-methyl group at Tyr-5 is necessary for this series of peptides to take the active conformation preferentially.



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RA-XXV and RA-XXVI, Bicyclic Hexapeptides from *Rubia cordifolia* L. Structure, Synthesis, and Conformation