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A novel bicyclic hexapeptide, RA-XVIII, from *Rubia cordifolia*: Structure, semi-synthesis, and cytotoxicity

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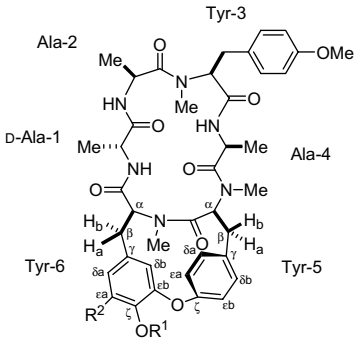
Abstract—A new bicyclic peptide of RA-series, RA-XVIII (**3**), was isolated from the roots of *Rubia cordifolia* L. Its structure was established to be a hydroxylated derivative of RA-VII by the semi-synthesis of **3** from deoxybouvardin, and its cytotoxicity against P-388 cells was 0.012 μg/mL.

Natural antitumor bicyclic hexapeptides, RA-VII (**1**)[1] and deoxybouvardin (**2**),[2] and their congeners,[3] characterized by unique structural features of having a 14-membered strained cycloisodityrosine unit, have attracted considerable attention recently (Fig. 1). They are provided with unique biological actions including inhibition of protein synthesis through interaction with eukaryotic ribosomes,[4,5] and peptide **1** was recently shown to cause conformational changes of F-actin and stabilization of actin filaments to induce G_2 arrest.[6] As part of our series of studies on the RA-series peptides from *Rubia cordifolia* L.,[7] the present paper describes the isolation and structure elucidation of a new peptide, RA-XVIII (**3**). Semi-syntheses of **3** and its analogues from **2** and **1**, and the cytotoxicity of representative analogues of this series against P-388 leukemia cells are also described.



RA-VII (**1**): R^1 = Me, R^2 = H
deoxybouvardin (**2**): R^1 = R^2 = H
RA-XVIII (**3**): R^1 = Me, R^2 = OH

Figure 1.

Peptide **3** was prepared from a chloroform–methanol (10:1)-soluble portion of a methanol extract of the dried roots of *R. cordifolia* (55 kg), by a series of column chromatography using silica gel, alumina, and then aminopropyl-bonded silica gel eluting with a chloroform–methanol mixture. The resulting RAs-rich fraction obtained after removal of the solvent was crystallized from methanol to give a crude crystalline RA mixture, which was subjected to separation by reversed-phase HPLC (ODS) to afford RA-XVIII (**3**)[8] [4.8 mg, 8.7×10^{-6}%, amorphous solid, $[\alpha]_D^{25}$ −222 (*c* 0.13, MeOH)]. The molecular formula $C_{41}H_{50}N_6O_{10}$ of **3** was obtained from the $[M+H]^+$ ion, *m/z* 787.3677 (calcd for $C_{41}H_{51}N_6O_{10}$, 787.3667), in HR-ESI-MS. The ^1H and ^{13}C NMR spectra of **3** in $CDCl_3$ (Table 1) showed signals typical of an RA-series peptide, demonstrating the presence of two conformers in a ratio of 89:11.[9,10] The structure of **3** was determined by using the resonances caused by the major conformer, which included signals for three secondary methyl groups (δ_H/δ_C 1.11/18.5, 1.30/20.7, 1.35/16.6), three *N*-methyl groups (δ_H/δ_C 2.69/29.3, 2.86/39.8, 3.11/30.5), two *O*-methyl groups (δ_H/δ_C 3.79/55.3, 4.09/61.4), six *N*-substituted methines (δ_H/δ_C 3.58/68.4, 4.36/47.9, 4.51/57.3, 4.74/46.4, 4.84/44.6, 5.39/54.2), a

Keywords: RA-XVIII; Deoxybouvardin; Cytotoxicity; *Rubia cordifolia*; Synthesis.
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Table 1. NMR data for the major conformer of RA-XVIII (**3**) in $CDCl_3$ at 300 K

Position		δ_H[a]	δ_C[b]	Position		δ_H[a]	δ_C[b]
D-Ala-1	α	4.36 (qd, 7.0, 6.6)	47.9		βa	2.63 (dd, 11.4, 3.1)	37.0
	β	1.30 (d, 6.8, 3H)	20.7		βb	3.67 (t, 11.4)	
	C=O		172.3		γ		135.4
	NH	6.44 (d, 6.6)			δa	7.25 (dd, 8.4, 2.2)	132.7
Ala-2	α	4.84 (dq, 8.4, 7.0)	44.6		δb	7.41 (dd, 8.4, 2.2)	131.0
	β	1.35 (d, 7.0, 3H)	16.6		εa	6.85 (dd, 8.4, 2.5)	124.1
	C=O		172.6		εb	7.18 (dd, 8.4, 2.5)	125.8
	NH	6.35 (d, 8.4)			ζ		158.0
Tyr-3	α	3.58 (dd, 10.6, 5.0)	68.4		C=O		169.2
	βa	3.38 (dd, 14.1, 5.0)	32.7		NMe	3.11 (s, 3H)	30.5
	βb	3.34 (dd, 14.1, 10.6)		Tyr-6	α	4.51 (dd, 11.9, 3.7)	57.3
	γ		130.7		βa	3.01 (dd, 18.2, 11.9)	35.9
	δ	7.05 (d-like, 8.6, 2H)	130.2[c]		βb	2.88 (dd, 18.2, 3.7)	
	ε	6.83 (d-like, 8.6, 2H)	114.1[c]		γ		131.2
	ζ		158.4		δa	6.24 (d, 1.9)	107.5
	C=O		168.0		δb	3.89 (d, 1.9)	106.0
	NMe	2.86 (s, 3H)	39.8		εa		149.4
	OMe	3.79 (s, 3H)	55.3		εb		155.9
Ala-4	α	4.74 (dq, 7.7, 6.6)	46.4		ζ		133.9
	β	1.11 (d, 6.6, 3H)	18.5		C=O		170.6
	C=O		171.8		NMe	2.69 (s, 3H)	29.3
	NH	6.71 (d, 7.7)			OMe	4.09 (s, 3H)	61.4
Tyr-5	α	5.39 (dd, 11.4, 3.1)	54.2		OH	5.91 (br s)	

[a] ^1H spectrum recorded at 500 MHz referenced to residual C*H*Cl_3 (7.26 ppm); *J*-values given in Hz in parentheses.
[b] ^{13}C spectrum recorded at 125 MHz referenced to *C*DCl_3 (77.03 ppm).
[c] 2C.

1,4-substituted benzene ring (δ_H/δ_C 6.83/114.1 × 2, 7.05/130.2 × 2), two aromatic rings, methylenes of the three tyrosine residues (δ_C 32.7, 35.9, 37.0), six amide carbonyl groups (δ_C 168.0, 169.2, 170.6, 171.8, 172.3, 172.6), and three amide protons (δ_H 6.35, 6.44, 6.71). The ^1H NMR data of **3** were very similar to those of **1**[10] except that the H-δa signal of Tyr-6 in **3** was at δ_H 6.24 as a *meta*-coupled doublet (J = 1.9 Hz), whereas that in **1** was at δ_H 6.57 as a doublet of doublets (J = 8.3, 2.0 Hz). Consequently, **3** was suggested to be an analogue of **1** with a modified aromatic ring of Tyr-6. The molecular formula of **3** showed that it was equivalent to that of **1** with one additional oxygen. The additional oxygen atom in **3** indicated the presence of a hydroxyl group in **3**; the proton signal of which was observed as a broad singlet at δ_H 5.91 in the ^1H NMR spectrum. HMBC correlations from the hydroxyl proton to C-δa, C-εa, and C-ζ (Fig. 2) placed this hydroxyl group at the εa-position of Tyr-6 in **3**, which was verified by the chemical shift value of the C-εa signal, δ_C 149.4. Therefore, **3** was concluded to be an analogue of **1** with a hydroxyl group at the εa-position of Tyr-6, which was further confirmed by the semi-synthesis of **3** from **2**.

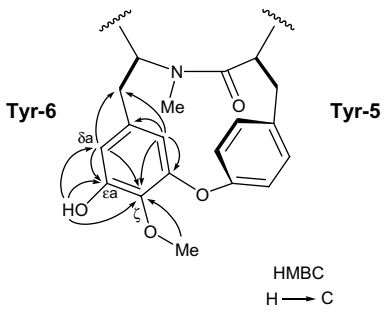
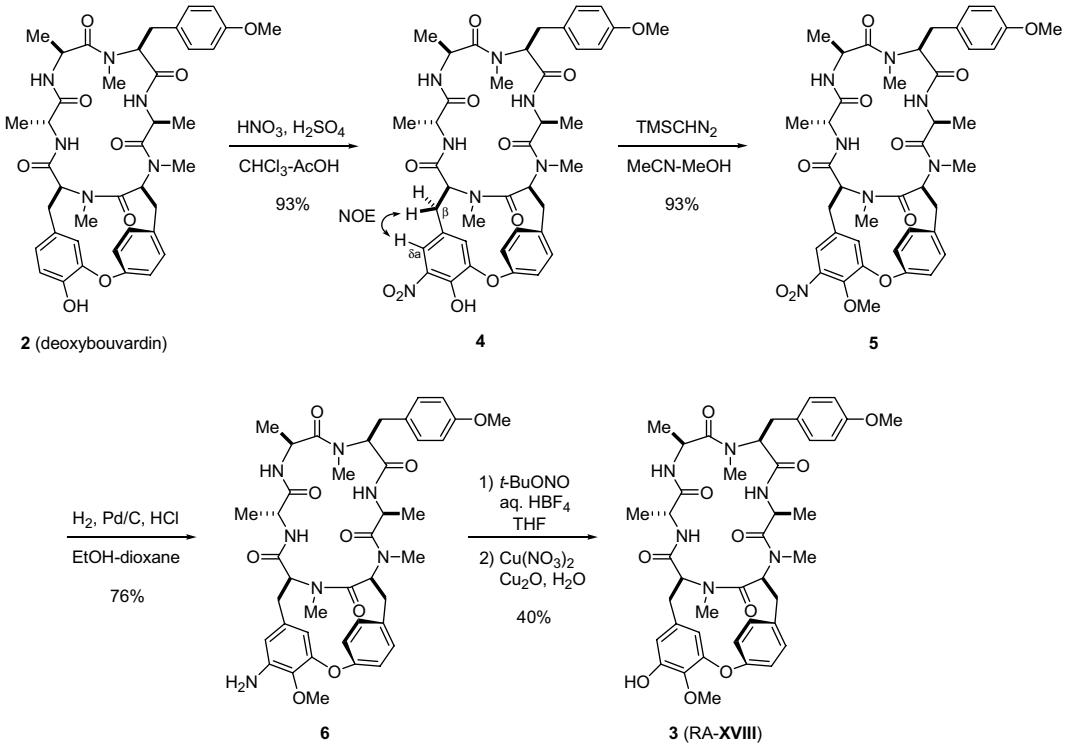


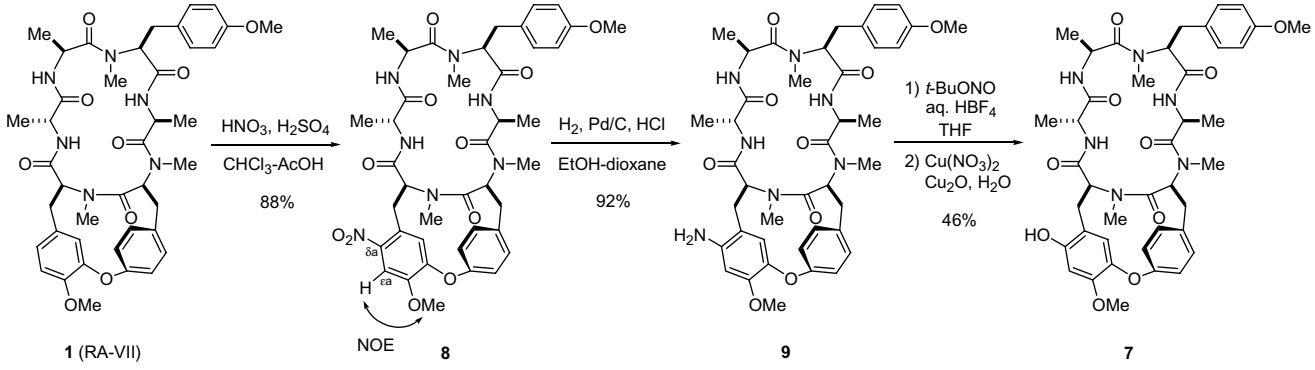
Figure 2. Key HMBC correlations for **3**.

Semi-synthesis of **3** from **2** was performed as shown in Scheme 1. Thus, treatment of **2** with nitric acid and sulfuric acid in a chloroform–acetic acid mixture afforded nitro compound **4**.[11] The position of the nitro group was confirmed by observation of the H-δa of Tyr-6 as a singlet signal (δ_H 7.46) which showed an NOE correlation with H-βa. *O*-Methylation of **4** with (trimethylsilyl)diazomethane gave methyl ether **5**, which gave, by subsequent catalytic hydrogenation over Pd/C, amine **6**. Amine **6** was then converted into a diazonium intermediate, which, on treatment with copper(II) nitrate and copper(I) oxide in an aqueous media,[12] produced a compound, $[\alpha]_D^{25}$ −223 (*c* 0.12, MeOH), which was found to be identical to natural **3** by comparison of their optical rotations, ^1H and ^{13}C NMR, and mass spectra. Accordingly, the absolute structure of **3** was determined to be as shown in Figure 1.

In this series of peptides, hydroxylation of the Tyr-6 aromatic ring apparently increases the electron density of this ring and changes the polarity of the molecules, which may affect their biological activities. To investigate such a positional effect of the hydroxyl group on the cytotoxic activity in the peptides of this series, an isomeric δ-hydroxide, **7**, was prepared from **1** by using a similar protocol employed in the synthesis of **3** from **2** (Scheme 2). Nitration of **1** gave nitro compound **8**, whose nitro group was confirmed to be at the δa position of Tyr-6 by observation of H-εa of Tyr-6 as a singlet signal (δ_H 7.74) and an NOE correlation of this signal with the methoxyl proton of Tyr-6 (δ_H 4.03).[11]



Scheme 1.



Scheme 2.

Then, **8** was subjected to catalytic hydrogenation to yield amine **9**, which was then converted into phenol **7**.

The present new peptide **3**, its analogues **4**–**9**, and as references, **1** and **2**, were evaluated for their cytotoxicity against P-388 leukemia cells. The results are summarized in Table 2. The IC_{50} value of **3** was 0.012 μg/mL, indicating that its cytotoxicity was one-fourth of that of **1**, though slightly higher than that of **2**. Analogues **4**–**9** were all less cytotoxic than peptide **1**, thus suggesting that introduction of a hydroxyl, a nitro, or an amino group at the δa or the εa position of Tyr-6 causes a decrease in the cytotoxic activity. As regards the effect of a substituent at different positions of Tyr-6, introduction of a substituent at the δa position seemed to decrease the activity more than that at the εa position: the activities of **3**, **5**, and **6** tended to be higher than those of the corresponding analogues **7**, **8**, and **9**, respectively.

Table 2. Cytotoxicity of RA-VII (**1**), deoxybouvardin (**2**), RA-XVIII (**3**), and their analogues **4**–**9** against P-388 leukemia cells

Compound	IC_{50} (μg/mL)
1	0.0030
2	0.014
3	0.012
4	0.014
5	0.010
6	0.0071
7	0.093
8	0.056
9	0.70

RA-XVIII (**3**) is a natural peptide of the RA-series in which the εa position of Tyr-6 is hydroxylated. Since cytotoxic peptides **3** and **6**, having an additional hydroxyl or amino group on the Tyr-6 aromatic ring, are readily prepared from **2**, the most accessible congener in

nature, these peptides may be expected to be useful starting materials for producing new modified RA-series peptides.

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11. The location of the nitro group in the resulting nitration reaction product is apparently controlled by the nature of the substituent at the ζ position of Tyr-6. In **2**, the hydroxyl group at ζ having a strong electron-donating effect causes the nitro group to be introduced to εa to produce an ortho-nitrated compound, **4**, whereas, in the case of **1**, the methoxyl group at ζ sterically inhibits the introduction of a nitro group to the corresponding position, so that the nitro group is introduced to the less-hindered δa position, which is also activated by the diphenyl ether oxygen, and the nitration product is **8**.
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