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Isolation, structural elucidation, and synthesis of RA-XVII, a novel bicyclic hexapeptide from *Rubia cordifolia*, and the effect of side chain at residue 1 upon the conformation and cytotoxic activity

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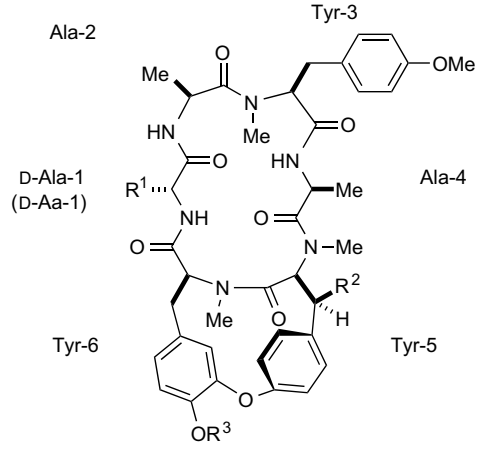
Abstract—A novel antitumor bicyclic hexapeptide RA-XVII was isolated from the roots of *Rubia cordifolia*. By spectral studies and synthetic approach, its structure was determined to be [D-2-aminobutyric acid-1]deoxybouvardin. Studies on the effect of side chain at residue 1 on cytotoxic activity and conformation showed that although it had little effect on the conformation of the molecule, it decreased the activity as it grew longer.

RA-VII (**1**) is an antitumor bicyclic hexapeptide from Rubiaceous plants. Seventeen congeners, RA-I–XVI[1,2] and RA-dimer-A,[3] have been isolated from *Rubia akane* Nakai or *R. cordifolia* L., and two congeners, bouvardin (NSC 259968) (**2**) and deoxybouvardin (RA-V) (**3**), from another Rubiaceous plant, *Bouvardia ternifolia* (Cav.) Schlecht.[4] These peptides possess a promising antitumor activity, and RA-VII (**1**) underwent phase I clinical trials as an anticancer drug in Japan.[5] In the present study, we isolated a novel bicyclic hexapeptide RA-XVII (**4**) as a minor peptide component from the roots of *R. cordifolia* L., which had D-2-aminobutyric acid as residue 1. In the present letter, we describe the isolation and structural elucidation of this new peptide, and the studies of the effect of the side chain at residue 1 upon the conformation and cytotoxic activity of RA series peptides.



R^1	R^2	R^3	
Me	H	Me	RA-VII (**1**)
Me	OH	H	bouvardin (**2**)
Me	H	H	deoxybouvardin (**3**)
Et	H	H	RA-XVII (**4**)
Et	H	Me	[D-2-aminobutyric acid-1]RA-VII (**5**)
n-Pr	H	Me	[D-norvaline-1]RA-VII (**6**)

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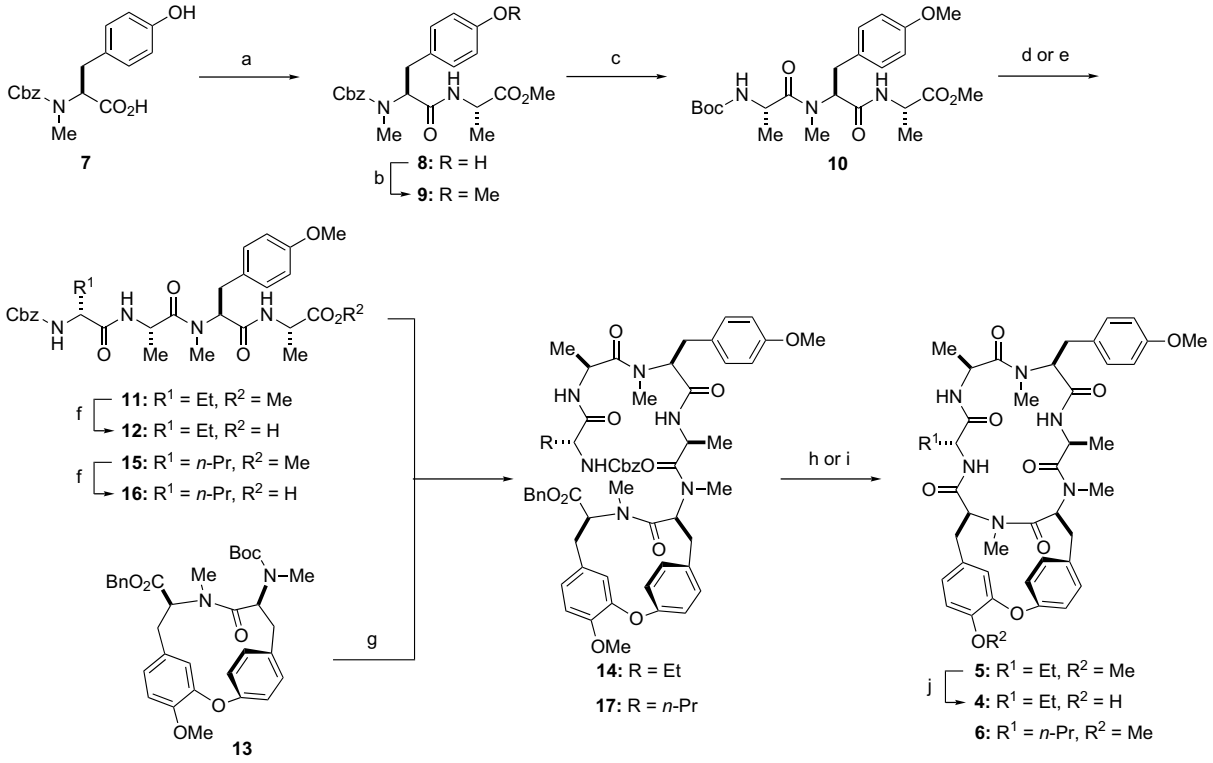
Methanol extract obtained from the dried roots of *R. cordifolia* (50 kg) was partitioned between chloroform and water. The chloroform-soluble portion was subjected to a series of column chromatographic studies using silica gel, alumina, and then aminopropyl-bonded silica gel, eluting with a chloroform/methanol mixture to give an RAs-rich fraction. The residue obtained after removal of the solvent of this fraction was crystallized from methanol to afford crystals of crude RAs (8.7 g). Separation of the crude RAs by reversed-phase HPLC (ODS) afforded RA-XVII (**4**, 0.24 mg, 4.8×10^{-7}% yield) as an amorphous powder, $[\alpha]_D^{24}$ −194° (*c* 0.01, $CHCl_3$). The molecular formula was determined to be $C_{41}H_{50}N_6O_9$ by HR-ESIMS (*m/z* 771.3737 $[M+H]^+$, for $C_{41}H_{51}N_6O_9$ $\Delta+1.9$ mmu). The quantity of the thus obtained peptide was very small, but it was sufficient for the 1D ^1H NMR, ^1H–^1H COSY, and NOESY spectral analysis.

As in the case of other related peptides of this series, peptide **4** was present in $CDCl_3$ as a mixture of one major (89% population) conformer and one minor conformer having a *trans* and *cis* amide bond between Ala-2 and Tyr-3 residues, respectively.[6] The ^1H NMR spectrum of **4** indicated a close resemblance between the structures of **4** and deoxybouvardin (**3**): characteristic resonances of O-methyl at Tyr-3 (δ 3.79, s), N-methyls at Tyr-3 (δ 2.85, s), Tyr-5 (δ 3.11, s), and Tyr-6 (δ 2.66, s), and alanyl methyls at Ala-2 (δ 1.36, d, $J=6.9$ Hz) and Ala-4 (δ 1.13, d, $J=6.7$ Hz) in **4** were almost identical to those in **3**.[7] The major differences between the spectra of **3** and **4** were that the spectrum of **4** had no doublet methyl resonance for D-Ala-1 observed in **3** and that, instead, it showed resonances of triplet methyl protons (δ 0.85, t, $J=7.4$ Hz) and methylene protons (δ 1.77, m and 1.58, m), all coupled to each other, were observed. Those methylene protons were also coupled to the methine proton (δ 4.33, m) assigned to the α-proton of residue 1. A transannular NOESY correlation between this α-proton and the methyl protons of Ala-4 residue indicated that the α-proton and the methyl group are located in proximity, thus suggesting that the configuration of the amino acid at residue 1 was D as in other peptides of this series. Accordingly, on the basis of those observations and the molecular formula derived from HR-ESIMS, we assigned the structure of **4** to a deoxybouvardin analogue in which D-Ala-1 of **3** was replaced by D-2-aminobutyric acid.

Confirmation of the assigned structure was performed by a synthetic approach shown in Scheme 1. The tetrapeptide fragment **12** consisting of residues 1–4 of **4** was synthesized by stepwise extension of Cbz-*N*-methyl-L-tyrosine (**7**).[8] Compound **7** was first coupled to L-alanine methyl ester, and subsequent O-methylation of **8** with diazomethane afforded dipeptide **9**. After removal of the Cbz protecting group, the less reactive *N*-methyl-dipeptide ester was coupled to Boc-L-alanine by using



Scheme 1. Reagents and conditions: (a) H-Ala-OMe·HCl, EDC, HOBt, Et_3N, CH_2Cl_2, 71%; (b) CH_2N_2, Et_2O–MeOH, 100%; (c) H_2, Pd/C, HCl, MeOH; Boc-Ala-OH, PyBOP, *i*-Pr_2NEt, CH_2Cl_2, 88%; (d) TFA; Cbz-D-2-aminobutyric acid, EDC, HOBt, $CHCl_3$, 89% for **11**; (e) TFA; Cbz-D-norvaline, EDC, HOBt, $CHCl_3$, 99% for **15**; (f) LiOH, THF–MeOH–H_2O, 92% for **12** and 99% for **16**; (g) TFA; **12** or **16**, EDC, HOObt, THF, 90% for both **14** and **17**; (h) H_2, Pd/C, EtOH; DPPA, Et_3N, 0.001 M, rt, 72 h, 44% for **5**; (i) H_2, Pd/C, EtOH; DPPA, Et_3N, 0.001 M, 0 °C, 72 h, 49% for **6**; (j) $AlCl_3$, CH_2Cl_2, 49%.

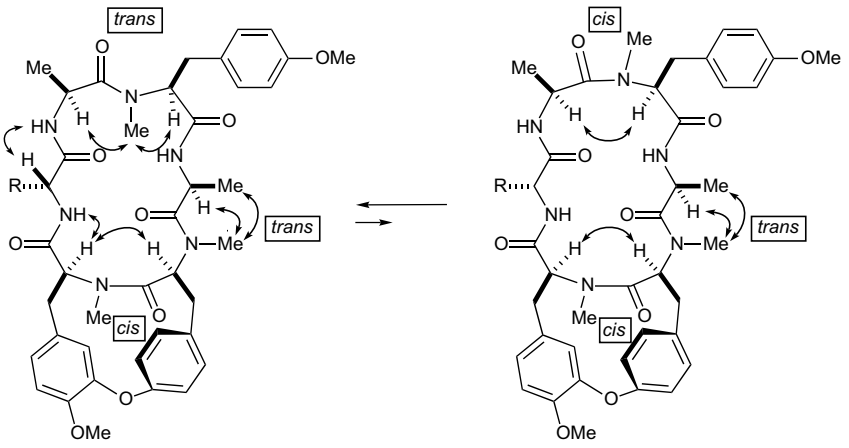


Figure 1. Key sequential NOESY correlations commonly observed in **1** (R = Me), **5** (R = Et), and **6** (R = *n*-Pr) in $CDCl_3$.

PyBOP (1*H*-benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) to afford tripeptide **10**. Further elongation of the peptide chain at the N-terminus with Cbz-D-2-aminobutyric acid gave tetrapeptide **11**, which, on treatment with lithium hydroxide, provided acid **12**.

Acid **12** was then subjected to the coupling reaction with N-deprotected **13**[9] by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt) to afford hexapeptide **14**. Macrocyclization of the peptide obtained by deprotection of **14** was performed by using diphenylphosphoryl azide (DPPA, 2 equiv) and triethylamine (10 equiv) in DMF (room temperature, 3 days, peptide concentration 0.001 M) to afford peptide **5**,[10] i.e. [D-2-aminobutyric acid-1]RA-VII. The yield of **5** from **14** was 44%. Selective O-demethylation at Tyr-6 residue afforded a compound,[11] which was shown to be identical to **4** isolated from *R. cordifolia*, by the comparison of their ^{1}H NMR and HR-ESI mass spectra, t_R of HPLC analysis, and optical rotations.

The amino acid at residue 1 of all the RA-series peptides isolated so far is D-alanine. RA-XVII (**4**) is the first example of RA-series peptides whose residue 1 amino acid is not D-alanine. The residue at 1 of the present peptide **4** is D-2-aminobutyric acid, which is not known in cyclic peptides of plant origin, though occasionally found in the cyclic peptides of microbial metabolites.[12]

Peptide **4** had an ethyl side chain at residue 1 and yet it was biologically active and gave an IC_{50} value of 0.028 μg/mL when tested on P-388 murine leukemia cells. Therefore, by a similar sequence of reactions employed for the synthesis of **5** (Scheme 1), we synthesized another peptide of this series, [D-norvaline-1]RA-VII (**6**),[13] in which the residue at 1 had an *n*-propyl group, and studied the influence of the side chain of residue 1 upon their conformation and cytotoxic activities. The NMR spectral features of **5** and **6** were very similar to those of **1**, showing that in $CDCl_3$, these three peptides were present in two conformers at the ratio of 89:11 at 300 K. Close similarity of the chemical shift values of the corresponding proton signals in their ^{1}H NMR spectra and observation of the same sequential NOESY correlations in their NOESY spectra suggested that the respective structures of the two conformers of peptides **1**, **5**, and **6** were practically identical (Fig. 1). The cytotoxic activities (IC_{50}) of RA-VII (**1**) and analogues **5** and **6** on P-388 cells were 0.0023, 0.0076, and 0.026 μg/mL, respectively. Thus, the results indicated that although the side chain at residue 1 had little effect on their conformation in solution, the longer carbon side chain at D-Ala-1 of peptide **1** decreased the activity.

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7. Data for **4**: ^{1}H NMR (500 MHz, $CDCl_3$, 300 K, major conformer, J in Hz) δ 7.42 (dd, 1H, $J = 8.4$, 2.3, Tyr-5 H-δ_b), 7.28 (dd, 1H, $J = 8.5$, 2.3, Tyr-5 H-δ_a), 7.21 (dd, 1H, $J = 8.4$, 2.4, Tyr-5 H-ε_b), 7.05 (d-like, 2H, $J = 8.6$, Tyr-3 H_2-δ), 6.84 (m, 1H, Tyr-5 H-ε_a), 6.84 (d-like, 2H, $J = 8.6$, Tyr-3 H_2-ε), 6.82 (d, 1H, $J = 8.2$, Tyr-6 H-ε_a), 6.70 (d, 1H, $J = 7.5$, Ala-4 NH), 6.53 (dd, 1H, $J = 8.2$, 2.0, Tyr-6 H-δ_a), 6.28 (d, 1H, $J = 7.2$, D-Abu-1 NH), 6.22 (d, 1H, $J = 8.5$, Ala-2 NH), 5.66 (br s, 1H, Tyr-6 OH), 5.39 (dd, 1H, $J = 11.4$, 3.0, Tyr-5 H-α), 4.89 (dq, 1H, $J = 8.5$, 6.9, Ala-2 H-α), 4.74 (dq, 1H, $J = 7.5$, 6.7, Ala-4 H-α), 4.60 (1H, dd, 11.5, 4.6, Tyr-6 H-α), 4.37 (d, 1H, $J = 2.0$, Tyr-6 H-δ_b), 4.33 (m, 1H, D-Abu-1 H-α), 3.79 (s, 3H, Tyr-3 OCH_3), 3.68 (dd, 1H, $J = 11.4$, 11.4, Tyr-5 H-β_b), 3.57 (dd, 1H, $J = 10.6$, 5.1, Tyr-3 H-α), 3.39 (dd, 1H, $J = 14.0$, 5.1, Tyr-3 H-β_a), 3.35 (dd, 1H, $J = 14.0$, 10.6, Tyr-3 H-β_b), 3.11 (s, 3H, Tyr-5 NCH_3), 3.05 (dd, 1H, $J = 17.9$, 11.5, Tyr-6 H-β_a), 3.00 (dd, 1H, $J = 17.9$, 4.6, Tyr-6 H-β_b), 2.85 (s, 3H, Tyr-3 NCH_3), 2.66 (s, 3H, Tyr-6 NCH_3), 2.63 (dd, 1H, $J = 11.4$, 3.0, Tyr-5 H-β_a), 1.77 (m, 1H, D-Abu-1 H-β_a), 1.58 (m, 1H, D-Abu-1 H-β_b), 1.36 (d, 3H, $J = 6.9$, Ala-2 H_3-β), 1.13 (d, 3H, $J = 6.7$, Ala-4 H_3-β), 0.85 (t, 3H, $J = 7.4$, D-Abu-1 H_3-γ); HPLC t_R 32.7 min (Inertsil ODS-3, 4.6×250 mm, 5 μm, 37% H_2O/MeOH, 0.6 mL/min, UV 254 nm).
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10. Data for **5**: white crystalline powder, mp 232–235 °C, $[\alpha]_D^{24}$ −169° (*c* 0.24, $CHCl_3$); HR-ESIMS *m/z* 807.3750 $[M+Na]^+$, calcd for $C_{42}H_{52}N_6O_9Na$ 807.3693.
11. Data for synthetic **4**: $[\alpha]_D^{26}$ −237° (*c* 0.20, $CHCl_3$); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K, major conformer, δ) D-Abu-1 (171.3, 53.0, 27.8, 9.3), Ala-2 (172.5, 44.5, 16.8), Tyr-3 (168.0, 158.4, 130.7, 130.2×2, 114.1×2, 68.4, 55.3, 39.7, 32.7), Ala-4 (171.7, 46.4, 18.5), Tyr-5 (169.3, 157.9, 135.7, 133.1, 131.0, 125.9, 124.2, 54.2, 36.9, 30.5), Tyr-6 (170.8, 151.1, 143.0, 127.6, 121.7, 115.7, 113.1, 57.7, 35.8, 29.3).
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13. Data for **6**: amorphous powder, $[\alpha]_D^{24}$ −237° (*c* 0.38, $CHCl_3$); HR-ESIMS *m/z* 821.3930 $[M+Na]^+$, calcd for $C_{43}H_{54}N_6O_9Na$ 821.3850.