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Isolation, Structure Determination, and Synthesis of Allo-RA-V and Neo-RA-V, RA-Series Bicyclic Peptides from *Rubia cordifolia* L.

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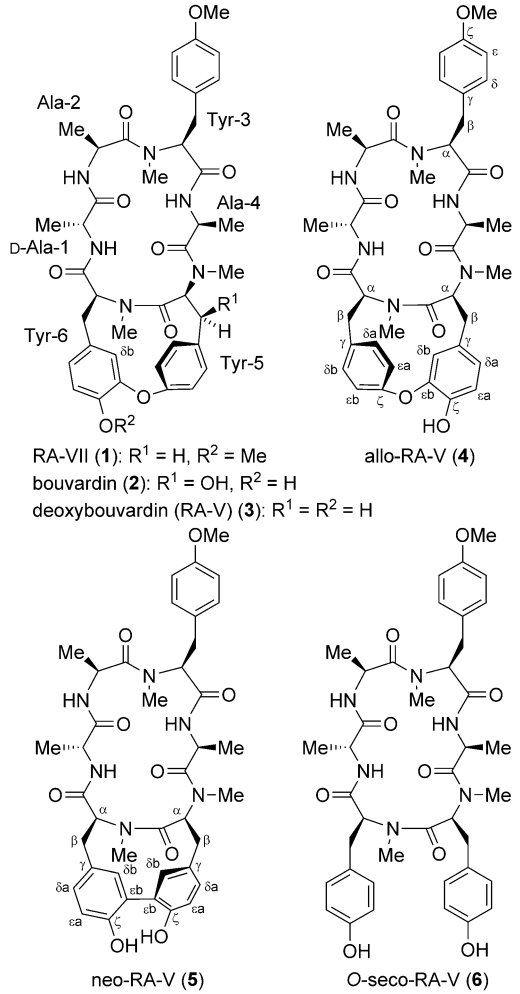
Abstract: Two bicyclic hexapeptides, allo-RA-V (**4**) and neo-RA-V (**5**), and one cyclic hexapeptide, *O*-seco-RA-V (**6**), were isolated from the roots of *Rubia cordifolia* L. Their gross structures were elucidated on the basis of spectroscopic analysis and X-ray crystallography of compound **5**. The absolute stereochemistry of compounds **4** and **5** were established by their total syntheses, and the absolute stereochemistry of compound **6** by chemical correlation with deoxybouvardin (**3**). Comparison of the 3D structures of highly active RA-VII (**1**) with less-active compounds **4** and **5** suggests that the orientation of the Tyr-5 and/or Tyr-6 phenyl rings plays a significant role in their biological activity. The isolation of peptides **4**–**6**, along with compound **3**, and the comparison of their structures seem to indicate that peptide **6** may be the common precursor to bicyclic peptides **3**–**5** in the plant.

Keywords: configuration determination · cytotoxicity · peptides · structure elucidation · total synthesis

Introduction

RA-VII (**1**), isolated from *Rubia cordifolia* L. and *R. akane* Nakai (Rubiaceae),[1,2] bouvardin (NSC 259968; **2**), isolated from *Bouvardia ternifolia* (Cav.) Schltdl. (Rubiaceae),[3] and deoxybouvardin (RA-V; **3**),[1–3] isolated from those three plants are bicyclic hexapeptides that are characterized by the presence of a unique strained 14-membered cycloisodityrosine unit in their structures (Scheme 1). These peptides possess promising antitumor activity, and from the late 1980s until the early 1990s, peptide **1** underwent phase I clinical trials as an anticancer drug in Japan.[4] The antitumor action of these peptides are believed to be due to inhibition of protein synthesis through interaction with eukaryotic ribosomes.[5,6] In addition, peptide **1** also causes conformational changes in F-actin, which stabilizes the actin filaments and induces G2 arrest.[7]

Herein, we report the isolation and structural elucidation of two bicyclic hexapeptides, allo-RA-V (**4**) and neo-RA-V (**5**), and one cyclic hexapeptide, *O*-seco-RA-V (**6**), along with the known RA-series peptides from the roots of *Rubia cordifolia* L. The absolute stereochemistry of compounds **4** and **5** were established by their total syntheses, and the stereochemistry of compound **6** was established by chemical



Scheme 1. Structures of RA-series peptides and bouvardins.

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correlation with deoxybouvardin (**3**). These peptides were also assayed for their cytotoxic activity against HL-60 and HCT-116 cell lines.

Results and Discussion

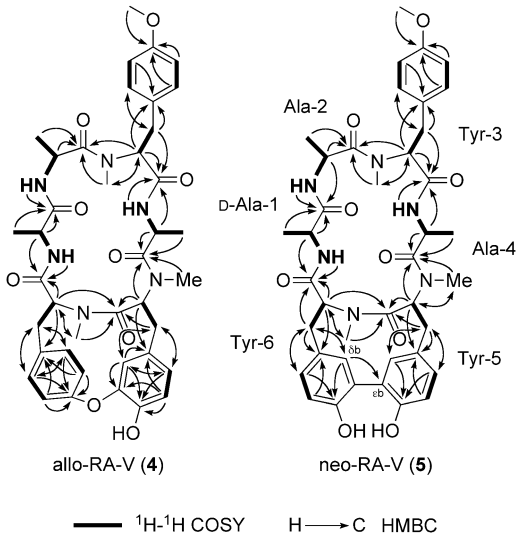
Isolation: A methanol extract obtained from dried roots of *R. cordifolia* (50 kg) gave four fractions by HP-20 column chromatography (H_2O/MeOH, 1:0, then 1:1, then 0:1, then acetone). Sequential column chromatography of the MeOH eluate on silica gel, charcoal, and aminopropyl-bonded silica gel with a series of solvent mixtures gave a fraction rich in RA compounds. The residue of this fraction, obtained after removal of the solvent, gave crystals of crude RAs on crystallization from MeOH/iPr_2O. Repeated reversed-phase HPLC (ODS) purifications of the mother liquor from the crystallization gave allo-RA-V (**4**) (2.2 mg), neo-RA-V (**5**) (6.3 mg), *O*-seco-RA-V (**6**) (42.7 mg), and six other known RA-series peptides, including deoxybouvardin.

Structures of allo-RA-V (4) and neo-RA-V (5): Allo-RA-V (**4**; $[\alpha]_D^{25} = -234$, $c = 0.13$, MeOH), was obtained as an amorphous solid. Its molecular formula was determined to be $C_{40}H_{48}N_6O_9$ from the $[M+Na]^+$ peak found at 779.3346 (calcd for $C_{40}H_{48}N_6O_9Na$: 779.3380) in the high-resolution mass spectrum (ESI). The ^1H NMR spectrum of compound **4** in $CDCl_3$ indicated the presence of three stable conformers in the ratio 86:7:7; the structure of the most-populated conformer was determined on the basis of NMR data. The ^1H and ^{13}C NMR spectra of compound **4** were similar to those of compound **3**, and ^1H-^1H COSY, HSQC, and HMBC analysis showed that peptide **4** consists of three alanine groups, one *N,O*-dimethyltyrosine group, and two *N*-methyltyrosine groups, one of which contains a 1,2,4-trisubstituted phenyl ring (Table 1, Scheme 2). Analysis of the HMBC spectrum revealed that peptides **3** and **4** have the same peptide sequence at residues 1–4, with a difference noted at residues 5 and 6. In peptide **3**, Tyr-5 has a *para*-substituted phenyl ring and Tyr-6 has a trisubstituted phenyl ring, whereas in peptide **4**, Tyr-5 has a trisubstituted phenyl ring and Tyr-6 a *para*-substituted phenyl ring. The chemical shift ($\delta_C = 150.8$ ppm) of the $C_{\varepsilon b}$ atom in Tyr-5 indicates that the oxygen atom is located on this carbon. The $H_{\delta b}$ atom of Tyr-5 in compound **4** showed a very upfield resonance in the ^1H NMR spectrum ($\delta_H = 3.96$ ppm), as did the $H_{\delta b}$ atom of

Table 1. NMR data of the major conformers of allo-RA-V (**4**) and neo-RA-V (**5**).[a]

Residue		**4**[b] δ_H [ppm]	**4**[b] δ_C [ppm]	**5**[c] δ_H [ppm]	**5**[c] δ_C [ppm]
D-Ala-1	α	4.36 (quintet, $J = 6.4$ Hz)	48.0	4.95[d]	48.1
	β	1.44 (d, $J = 6.9$ Hz, 3H)	20.0	1.66 (d, $J = 6.9$ Hz, 3H)	20.9
	C=O	--	173.1	--	173.2
	NH	7.05 (d, $J = 6.1$ Hz)	–	8.13 (d, $J = 6.6$ Hz)	–
Ala-2	α	4.65 (quintet, $J = 6.8$ Hz)	45.7	4.96[d]	45.3
	β	1.40 (d, $J = 6.8$ Hz, 3H)	16.0	1.47 (d, $J = 6.6$ Hz, 3H)	16.4
	C=O	--	172.3	--	173.3
	NH	6.31 (d, $J = 6.8$ Hz)	–	10.16 (d, $J = 7.4$ Hz)	–
Tyr-3	α	3.55 (dd, $J = 11.0$, 4.8 Hz)	68.2	4.07 (dd, $J = 10.7$, 4.7 Hz)	68.4
	βa	3.33[d]	32.8	3.86 (dd, $J = 13.8$, 10.7 Hz)	33.6
	βb	3.29 (dd, $J = 14.1$, 11.0 Hz)	–	3.80 (dd, $J = 13.8$, 4.7 Hz)	–
	γ	--	130.7	--	131.8
	δ	7.04 (d-like, $J = 8.6$ Hz, 2H)	130.3[e]	7.27 (d-like, $J = 8.6$ Hz, 2H)	130.8[e]
	ε	6.84 (d-like, $J = 8.6$ Hz, 2H)	114.0[e]	7.01 (d-like, $J = 8.6$ Hz, 2H)	114.4[e]
	ζ	--	158.4	--	158.8
	C=O	--	168.6	--	169.0
	NMe	2.87 (s, 3H)	39.9	3.13 (s, 3H)	39.9
	OMe	3.79 (s, 3H)	55.3	3.70 (s, 3H)	55.1
Ala-4	α	4.80 (dq, $J = 8.7$, 6.8 Hz)	46.4	5.22 (dq, $J = 8.5$, 6.6 Hz)	46.5
	β	0.97 (d, $J = 6.8$ Hz, 3H)	18.4	1.27 (d, $J = 6.6$ Hz, 3H)	18.9
	C=O	--	170.7	--	171.4
	NH	6.66 (d, $J = 8.7$ Hz)	–	7.29 (d, $J = 8.5$ Hz)	–
Tyr-5	α	5.23 (dd, $J = 10.6$, 1.5 Hz)	50.4	6.16 (d, $J = 9.9$ Hz)	54.6
	β (pro-*R*)	2.20 (d, $J = 16.0$ Hz)	33.2	2.36 (d, $J = 13.7$ Hz)	34.7
	β (pro-*S*)	3.71 (dd, $J = 16.0$, 10.6 Hz)	–	4.31 (dd, $J = 13.7$, 9.9 Hz)	–
	γ	--	129.4	--	129.2
	δa	6.52 (dd, $J = 8.3$, 1.8 Hz)	121.9	7.17 (dd, $J = 8.2$, 2.5 Hz)	128.8
	δb	3.96 (d, $J = 1.8$ Hz)	113.6	7.06 (d, $J = 2.5$ Hz)	140.8
	εa	6.76 (d, $J = 8.3$ Hz)	115.5	7.07 (d, $J = 8.2$ Hz)	115.7
	εb	--	150.8	--	129.3
	ζ	--	142.2	--	154.9
	C=O	--	169.5	--	171.8
	NMe	2.79 (s, 3H)	30.4	2.94 (s, 3H)	30.4
	OH	5.50 (br s)	–	11.26 (br s)[f]	–
Tyr-6	α	4.43 (dd, $J = 11.5$, 2.2 Hz)	64.1	5.61 (dd, $J = 11.5$, 4.1 Hz)	60.6
	β (pro-*R*)	3.33[d]	38.6	3.30 (dd, $J = 16.2$, 11.5 Hz)	35.8
	β (pro-*S*)	3.09 (dd, $J = 14.1$, 2.2 Hz)	–	3.50 (dd, $J = 16.2$, 4.1 Hz)	–
	γ	--	135.6	--	125.5
	δa	7.17 (dd, $J = 8.3$, 2.2 Hz)	130.6	7.11 (dd, $J = 8.5$, 1.9 Hz)	129.2
	δb	7.54 (dd, $J = 8.3$, 2.2 Hz)	130.8	6.77 (d, $J = 1.9$ Hz)	141.7
	εa	7.07 (dd, $J = 8.3$, 2.4 Hz)	124.9	7.06 (d, $J = 8.5$ Hz)	116.0
	εb	7.11 (dd, $J = 8.3$, 2.4 Hz)	126.7	--	128.6
	ζ	--	158.8	--	154.4
	C=O	--	169.6	--	170.8
	NMe	3.22 (s, 3H)	32.2	3.21 (s, 3H)	31.1
	OH	--	–	11.35 (br s)[f]	–

[a] ^1H NMR spectra recorded at 600 MHz and ^{13}C NMR spectra at 150 MHz. [b] In $CDCl_3$. [c] In [D_5]pyridine. [d] Multiplicity could not be determined owing to signal overlapping. [e] Two carbon atoms. [f] Assignments may be reversed.



Scheme 2. ^{1}H-^{1}H COSY and selected HMBC correlations for allo-RA-V (**4**) and neo-RA-V (**5**).

Tyr-6 in compound **1** ($\delta_H = 4.34$ ppm)[8] and the $H_{\delta b}$ atom in compound **3** ($\delta_H = 4.35$ ppm).[9] This unusual upfield shift of an aromatic proton in compounds **1** and **3** adjacent to the diphenyl ether bond is thought to be due to their location just above the phenyl ring of the other tyrosine in the 3D structure of the cycloisodityrosine.[8] From these observations, we concluded that peptide **4** has a cycloisodityrosine structure, as shown in Scheme 1, in which the phenolic oxygen of Tyr-6 is connected to the C_ε atom of Tyr-5 to form a diphenyl ether bond. Inequivalent chemical shifts of the $H_{\delta a}/C_{\delta a}$ and $H_{\delta b}/C_{\delta b}$ atoms, and of the $H_{\varepsilon a}/C_{\varepsilon a}$ and $H_{\varepsilon b}/C_{\varepsilon b}$ atoms of Tyr-6 in the ^{1}H and ^{13}C NMR spectra also indicated that this phenyl ring was involved in the formation of the cyclic diphenyl ether structure. The relative stereochemistry of compound **4** was deduced from the NMR data. The chemical shift of the proton and carbon signals of residues 1–4 were very similar to those of compounds **1** and **3**. In the NOESY spectrum, NOE correlations were observed between D-Ala-1 H_α/Ala-2 NH, Ala-2 H_α/Tyr-3 NMe, Tyr-3 NMe/Tyr-3 H_α, Tyr-3 H_α/Ala-4 NH, Ala-4 H_α/Tyr-5 NMe, Ala-4 $H_{3\beta}$/Tyr-5 NMe, Tyr-5 H_α/Tyr-6 H_α, Tyr-6 H_α/D-Ala-1 NH, and D-Ala-1 H_α/Ala-4 $H_{3\beta}$. These correlations are characteristic of the major conformer of peptides **1** and **3** in solution, where the molecule takes a type II β-turn at residues 1–4 and a type VI β-turn at residues 1 and 4–6.[8] Thus, we concluded that peptide **4** has the same relative configuration as peptides **1**–**3** at the amino acid chiral centers and that it adopts a very similar peptide backbone conformation to those of compounds **1**–**3** in solution.

Neo-RA-V (**5**), $[\alpha]_D^{25} = -290$ ($c = 0.14$, 1,4-dioxane), was obtained as colorless prisms, m.p. $>300\,^\circ$C. Its quasi-molecular ion peak at *m/z* 757.3596 ($[M+H]^+$; calcd for $C_{40}H_{49}N_6O_9$: 757.3561) in the HRMS (ESI) spectrum indicated that peptides **5** and **3** share a common molecular formula, $C_{40}H_{48}N_6O_9$. The ^{1}H NMR spectrum of compound **5** in [D_5]pyridine displayed two sets of signals in an 81:19 ratio, thereby implying that it consisted of two conformers. The ^{1}H and ^{13}C NMR spectra of compound **5** generally resembled those of compound **3**, thereby indicating that it consists of three alanine groups and three modified *N*-methyltyrosine groups (Table 1), and the HMBC spectrum revealed that its amino acid sequence is also the same as in compound **3** (Scheme 2). However, the phenyl rings of Tyr-5 and Tyr-6 in compound **5** were both 1,2,4-trisubstituted, whereas in compound **3**, only Tyr-6 was trisubstituted. The chemical shift of the $C_{\varepsilon b}$ signals in Tyr-5 and Tyr-6 ($\delta_C = 129.3$ and 128.6 ppm, respectively) indicated that no oxygen functionality was connected to those carbon atoms. A cross-peak was observed between the Tyr-5 $C_{\varepsilon b}$ and Tyr-6 $H_{\delta b}$ atoms in the HMBC spectrum (Scheme 2). Those NMR spectroscopic features, and the fact that compounds **5** and **3** had the same molecular formula, suggested that the Tyr-5 and Tyr-6 groups of compound **5** were linked to each other at their ε positions to form a biphenyl bond. The proximity of the Tyr-5 $H_{\delta b}$ and Tyr-6 $H_{\delta b}$ atoms was indicated by the cross-peak between those protons in the NOESY spectrum, which confirmed the presence of a biphenyl bond at this position. Accordingly, peptide **5** was determined to be a deoxybouvardin analogue in which the aromatic rings of Tyr-5 and Tyr-6 are connected at their ε positions to form a 12-membered cyclodityrosine unit. The relative stereochemistry of compound **5** was determined by X-ray crystallography to be as shown in Figure 1.[10] These data show that peptide **5** has the same relative configuration as compounds **1**–**3** at all the amino acid chiral centers.

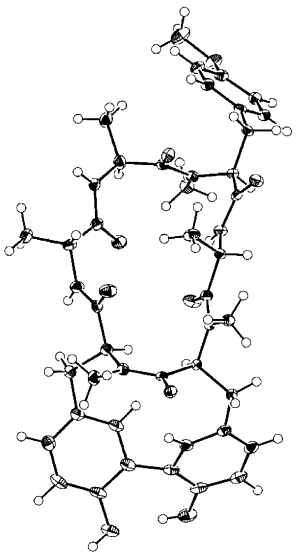


Figure 1. ORTEP of neo-RA-V (**5**).

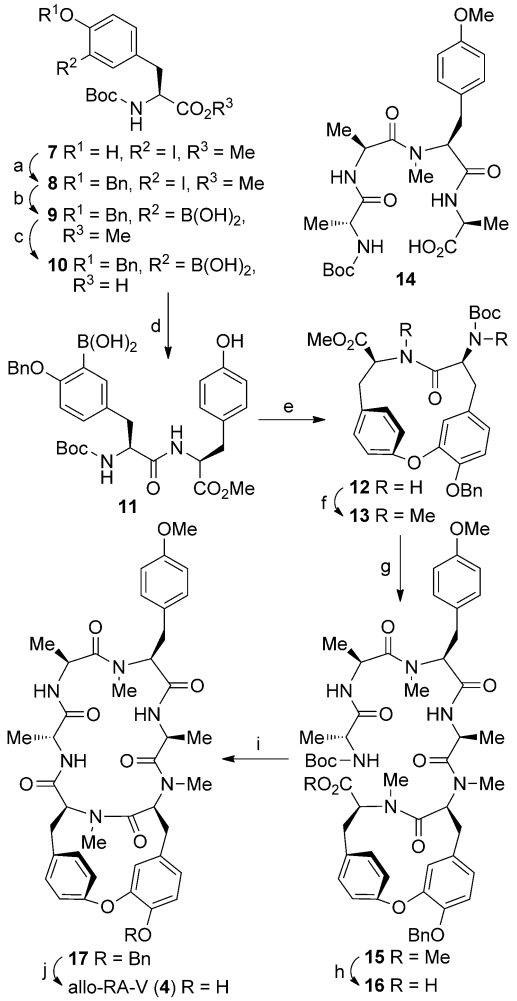
Synthesis of allo-RA-V (4) and neo-RA-V (5): The absolute structures of allo-RA-V (**4**) and neo-RA-V (**5**) were established by their total synthesis. In cyclopeptide synthesis, ring-closing at the correct position on the linear peptide is

essential for successful macrocyclization. Following the previous syntheses of RA-series peptides,[11–14] we attempted our ring formation by linking the Tyr-6 and D-Ala-1 groups to form an 18-membered macrocycle, because the NMR spectroscopic data and the crystal structure data of peptides **4** and **5** suggested that they have very similar conformational features to those of the previously synthesized compounds **1**–**3**.[8,9] Accordingly, the cycloisodityrosine unit (for compound **4**) and the cyclodityrosine unit (for compound **5**) were prepared first, to which the tetrapeptide segments, corresponding to residues 1–4, were connected to form hexapeptides that were then subjected to macrocyclization to construct the 18-membered cyclopeptide rings.

In the synthesis of allo-RA-V (**4**; Scheme 3), the formation of the 14-membered cycloisodityrosine unit was the major task. Several methods are known for its preparation, including: intramolecular phenolic oxidative coupling reactions with thallium(III) nitrate,[11,15] intramolecular Ullmann reactions,[12,16] and intramolecular S_NAr reactions.[13,17,18] We chose a copper-mediated etherification reaction with an arylboronic acid and a phenol.[19,20] Though low yields had been reported for the synthesis of cycloisodityrosines,[18d] we found that the use of 4-dimethylaminopyridine as a base instead of pyridine or triethylamine significantly improved the yield of the cyclic ether.[21] This method is attractive and is of value for the synthesis of cycloisodityrosines as it uses commercially available chiral amino acids and involves short reaction sequences.

Iodotyrosine derivative **8**, obtained by O-benzylation of *N*-Boc-3-iodo-L-tyrosine methyl ester (**7**), was converted into boronic acid **9**, which, after subsequent hydrolysis, gave carboxylic acid **10**. Acid **10** was linked to L-tyrosine methyl ester to give dipeptide **11**, which was treated with copper(II) acetate and 4-dimethylaminopyridine in the presence of 4 Å molecular sieves to afford the desired cycloisodityrosine (**12**) in 47% yield. N-Methylation of compound **12** under phase-transfer catalysis conditions[22] gave the fully functionalized cycloisodityrosine (**13**), which was usable for the synthesis of compound **4**. The Boc group in compound **13** was removed, and the resulting amine was coupled with tetrapeptide **14** provided hexapeptide **15**. Ester hydrolysis of compound **15** with in situ generated lithium peroxide gave acid **16**. Deprotection of the N-terminus of compound **16** and subsequent cyclization with EDC·HCl (8 equiv) and HOOBt (8 equiv) in DMF under dilute conditions (0.0013 M) gave bicyclic peptide **17** in 50% yield. Removal of the benzyl group of peptide **17** by hydrogenolysis gave the product ($[\alpha]_D^{25} = -221$, $c = 0.097$, MeOH), which was shown to be identical to natural product **4** by comparison of their ^1H and ^{13}C NMR spectra, IR spectra, mass spectra, and optical rotations. Thus, the absolute structure of allo-RA-V (**4**) was determined to be as shown in Scheme 1.

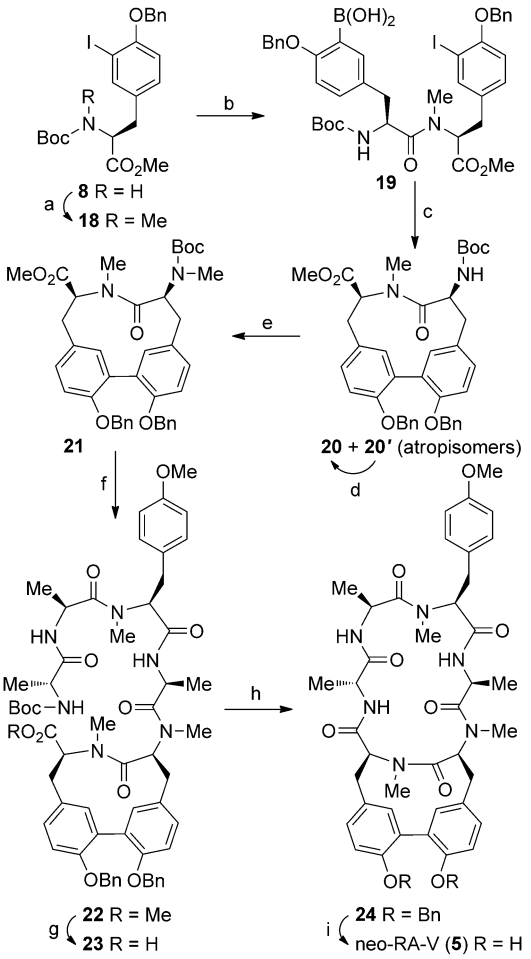
For the synthesis of neo-RA-V (**5**; Scheme 4), the preparation of the cyclodityrosine unit was the major problem because synthesis of this unit had not been reported before. We chose the intramolecular Suzuki–Miyaura cross-coupling reaction[23] of a modified tyrosyl–tyrosine dipeptide, where



Scheme 3. Synthesis of allo-RA-V (**4**). Reagents and conditions: a) BnOH, diethyl azodicarboxylate, PPh_3, THF/toluene 8:3, 0°C, 1 h, then RT, 3 h, 97%; b) 1) bis(pinacolato)diboron, $[PdCl_2(dppf)]\cdot CH_2Cl_2$, KOAc, DMSO, 80°C, 20 h, 2) $NaIO_4$, NH_4OAc, acetone/H_2O 1:1, RT, 17 h, 86%; c) LiOH, THF/MeOH/H_2O 3:3:1, 0°C, 1 h, then RT, 1 h, 88%; d) L-Tyr-OMe·HCl, EDC·HCl, HOAt, Et_3N, $CHCl_3$, 0°C, 1 h, then RT, 24 h, 95%; e) $Cu(OAc)_2$, DMAP, 4 Å molecular sieves, CH_2Cl_2, RT, 2 days, 47%; f) MeI, nBu_4NHSO_4, KOH, K_2CO_3, CH_2Cl_2, RT, 1 h, 72%; g) 1) TFA, 0°C, 1 h, 2) compound **14**, EDC·HCl, HOOBt, THF, RT, 2 days, 71%; h) LiOOH, MeOH/H_2O 6:1, 0°C, 1 h, then RT, 22 h, 92%; i) 1) TFA, $CHCl_3$, 0°C, 2 h, 2) EDC·HCl, HOOBt, DMF, 0°C, 1 h, then RT, 4 days, 50%; j) H_2, $Pd(OH)_2$/C, EtOH, RT, 3 h, 88%. For a list of abbreviations see Ref. [30].

the functionalized amino acid components used for the synthesis of compound **4** were exploited.

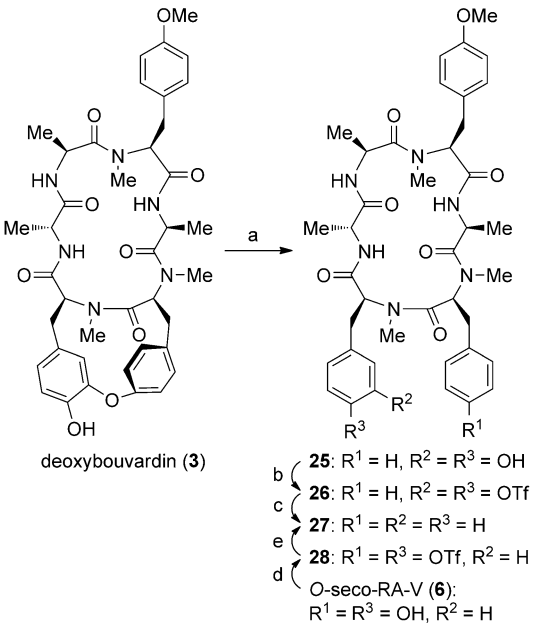
Iodotyrosine derivative **8** was N-methylated under phase-transfer catalysis conditions to give the *N*-methyltyrosine derivative **18**, which, after removal of the Boc group, was coupled with boronic acid derivative **10** to give dipeptide **19**. The $[Pd(PPh_3)_4]$-catalyzed intramolecular Suzuki–Miyaura coupling reaction of compound **19** efficiently gave cyclodityrosines **20** and **20′** in 50% and 14% yields, respectively.[24] Although the ^1H, ^{13}C, and 2D NMR data could not distinguish the structural differences between compounds **20** and



Scheme 4. Synthesis of neo-RA-V (**5**). Reagents and conditions: a) MeI, nBu$_4$NBr, NaOH, CH_2Cl_2, RT, 1 h, 88%; b) 1) TFA, $CHCl_3$, 0°C, 1 h, 2) compound **10**, EDC·HCl, HOAt, Et_3N, DMF, 0°C, 1 h, then RT, 4 days, 84%; c) [Pd(PPh$_3$)$_4$], Na_2CO_3, toluene/MeOH/H_2O 8:3:1, 90°C, 2 h, 50% yield of compound **20** and 14% yield of compound **20′**; d) toluene, 80°C, 20 h, 74% yield of compound **20** and 20% yield of compound **20′**; e) MeI, nBu$_4$NHSO$_4$, NaOH, CH_2Cl_2, RT, 2 h, 84%; f) 1) TFA, $CHCl_3$, 0°C, 1 h, 2) compound **14**, EDC·HCl, HOOBt, THF, RT, 3 days, 44%, g) LiOOH, MeOH/H_2O 3:1, 0°C, 1 h, then RT, 22 h, quant.; h) 1) TFA, $CHCl_3$, 0°C, 2 h, 2) EDC·HCl, HOOBt, DMF, 0°C, 1 h, then RT, 4 days, 60%; i) Pd/C, 1,4-cyclohexadiene, EtOH, RT, 7 days, 80%. For a list of abbreviations see Ref. [30].

20′, owing to line broadening of the signals and their complexity caused by *cis/trans* isomerization of the tertiary amide bond, we tentatively assigned compounds **20** and **20′** to be atropisomers. Although a biphenyl axis usually requires three or four *ortho* substituents for its conformers to become atropisomers,[25] their 12-membered ring structure and the two bulky benzyloxy groups might provide sufficient steric hindrance to the rotation about the C–C biphenyl bond. After heating the minor isomer (**20′**) in toluene at 80°C for 20 h, it yielded a 74% yield of compound **20** with 20% of starting isomer **20′**, thereby indicating that the major isomer (**20**) is thermodynamically more stable than the minor isomer (**20′**). Major isomer **20** was used in the remainder of the synthesis. N-Methylation of compound **20** gave compound **21**. After Boc deprotection, compound **21** was coupled with tetrapeptide **14** to afford hexapeptide **22**, which was then treated with lithium peroxide to give carboxylic acid **23**. After removal of the Boc group, compound **23** was subjected to macrocyclization at a concentration of 0.0013 M in DMF with EDC·HCl and HOOBt to afford bicyclic peptide **24** in 60% yield. However, debenzylation of compound **24** under standard catalytic hydrogenolysis conditions (H_2, Pd(OH)$_2$ or Pd/C, EtOH) did not afford compound **5** at all: it gave just a complex mixture of products. When compound **24** was treated with palladium on charcoal and 1,4-cyclohexadiene in EtOH,[26] it gave the desired deprotected compound in 80% yield, though the reaction was very sluggish. The thus obtained compound ($[\alpha]_D^{25} = -274$, $c = 0.09$, 1,4-dioxane), was shown to be identical to natural product **5**, by comparison of their ^{1}H and ^{13}C NMR spectra, IR spectra, mass spectra, and optical rotations. Accordingly, the absolute structure of neo-RA-V (**5**) was determined to be as shown in Scheme 1.

Structure of *O*-seco-RA-V (6): *O*-seco-RA-V (**6**), $[\alpha]_D^{25} = -82$ ($c = 0.42$, MeOH), was obtained as an amorphous solid and afforded a quasi-molecular ion peak at m/z 759.3685 $[M+H]^+$ in the high-resolution mass spectrum (ESI), which corresponded to a molecular formula of $C_{40}H_{50}N_6O_9$. The ^{1}H NMR spectra of compound **6** in CD_3OD showed that it was a mixture of four conformers in the ratio 54:42:3:1. 2D NMR spectroscopy revealed that peptide **6** was composed of three alanine groups and three *N*-methyltyrosine groups, of which one was O-methylated, and that the sequence of those alanine and *N*-methyltyrosine groups was the same as that in deoxybouvardin (RA-V, **3**), with the *N,O*-dimethyltyrosine as residue 3.[27] Thus, peptide **6** was shown to be a deoxybouvardin analogue with no diphenyl ether bond between Tyr-5 and Tyr-6. Because peptide **6** was considered to have the same configuration as compounds **3–5** at all of the amino acid residues, we tried to establish its absolute stereochemistry by chemical correlation with deoxybouvardin (**3**), whose absolute stereochemistry had been established by degradation studies[1] and by the total synthesis.[11,12a,c] As shown in Scheme 5, hydrogenolysis of compound **3** gave *O*-seco-derivative **25**, which was converted into ditriflate **26**. The palladium-mediated reduction[28] of compound **26** afforded *N*-methylphenylalanine derivative **27**. Analogously, peptide **6** was converted into ditriflate **28**, which gave compound **27** by subsequent reduction. Because the spectroscopic data (^{1}H and ^{13}C NMR, IR, and MS) and optical rotation ($[\alpha]_D^{25} = -85$, $c = 0.14$, MeOH) of compound **27** that was derived from compound **6** were the same as those ($[\alpha]_D^{25} = -86$, $c = 0.12$, MeOH) of compound **27** that was derived from deoxybouvardin (**3**), the structure of compound **6** was determined to be cyclo-(D-Ala-Ala-*N*-Me-Tyr(OCH$_3$)-Ala-*N*-Me-Tyr-*N*-Me-Tyr), that is, *O*-seco-RA-V (Scheme 1). Although peptide **6** has been previously reported as a synthesized monocyclic analogue of deoxybouvardin,[29] this is the first report on the isolation of compound **6** from nature.[30]



Scheme 5. Chemical correlation of *O*-seco-RA-V (**6**) with deoxybouvardin (**3**). Reagents and conditions: a) H_2, Pd/C, EtOH, 50 °C, 24 h, 78 %; b) Tf_2NPh, Et_3N, CH_2Cl_2, RT, 44 h, 91 %; c) $Pd(OAc)_2$, dppf, Et_3N, HCO_2H, DMF, 60 °C, 42 h, 75 %; d) Tf_2NPh, Et_3N, CH_2Cl_2, RT, 44 h, 98 %; e) $Pd(OAc)_2$, dppf, Et_3N, HCO_2H, DMF, 60 °C, 24 h, 75 %. For a list of abbreviations see Ref. [30].

Deoxybouvardin (RA-V, **3**), commonly detected in *Rubia akane*, *R. cordifolia*, and *Bouvardia ternifolia*, is composed of one D-alanine group, two L-alanine groups, and three modified *N*-methyl-L-tyrosine groups. One of those *N*-methyl-L-tyrosine groups is O-methylated, and the other two are connected to form a cycloisodityrosine. Cycloisodityrosines are tyrosyl–tyrosine dipeptide derivatives in which one of the two phenoxy groups forms an ether linkage with the aromatic carbon at the ε position of the other tyrosine. In peptides **1**–**3**, an ether linkage is formed between the hydroxy oxygen atom at the ζ position of Tyr-5 and the carbon atom at the ε position of Tyr-6, whereas in allo-RA-V (**4**), the ether linkage is formed between the carbon atom at the ε position of Tyr-5 and the hydroxy oxygen atom at the ζ position of Tyr-6. In neo-RA-V (**5**), those two tyrosines are linked by a C–C bond between the two ε positions to form an unusual 12-membered cycloditysosine structure.

The key question in the biosynthesis of RA-series peptides is whether the formation of the diphenyl ether linkage in the cycloisodityrosine moiety occurs prior to, or after, the formation of the cyclohexapeptide chain. Our isolation of *O*-seco-RA-V (**6**) along with peptides **3**–**5** from *Rubia cordifolia* may suggest that the cyclohexapeptide ring is formed first to produce compound **6**, which, by subsequent phenolic oxidative coupling reactions, generates bicyclic peptides **3**–**5** in the plant body.

Cytotoxic activities: Peptides **1** and **3**–**6** were evaluated for their cytotoxic activity against human promyelocytic leukemia HL-60 cells and human colon carcinoma HCT-116 cells, and their IC_{50} values are summarized in Table 2. The results indicated that only peptides **1** and **3** had potent cytotoxic activities. Superposition of the crystal structures of compounds

Table 2. Cytotoxic activity.[a]

Compound	HL-60	HCT-116
1	0.0049	0.0063
3	0.0060	0.0083
4	5.0	5.0
5	>100	>100
6	80	>100

[a] IC_{50} [μg mL^{-1}].

1 and **5**, and the energy-minimized structure of compound **4** (obtained by a Monte Carlo conformational search) is shown in Figure 2. This picture indicates that both the cyclo-

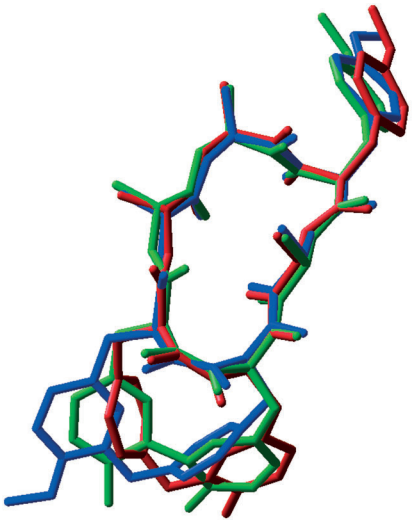


Figure 2. Superposition of the crystal structures of RA-VII (**1**, blue) and neo-RA-V (**5**, green), and the energy-minimized structure of allo-RA-V (**4**, red).

isodityrosine rings in compounds **1** and **4**, and the cycloditysosine ring in compound **5** have an apparently equal effect on the 18-membered cyclohexapeptide backbone, so that the 18-membered backbones of the three are almost superimposable. RA-series peptides adopt between two and three stable conformations in solution.[8] The-most populated conformer, represented by the crystal structure of compound **1** (Figure 2), has been identified as the active conformation,[12d,e,31] with the 4-methoxyphenyl ring of Tyr-3 also important for expressing the activity.[32] The phenyl rings of Tyr-5 and Tyr-6 in compounds **4** and **5** were not superimposable on those in the active compound **1**, thus suggesting that, in addition to the 4-methoxybenzyl side-chain of Tyr-3, a certain orientation of both or either of the phenyl rings of Tyr-5 and Tyr-6 is also required for expressing the activity.

Conclusion

Two bicyclic hexapeptides, allo-RA-V (**4**) and neo-RA-V (**5**), and a previously known cyclic hexapeptide, *O*-seco-RA-V (**6**), were isolated along with known RA-series peptides from the roots of *Rubia cordifolia*. The total synthesis of compounds **4** and **5** were performed by constructing the 14-membered cycloisodityrosine unit of compound **4** through an intramolecular phenol/arylboronic-acid coupling reaction mediated by copper(II) acetate, and the 12-membered cyclodityrosine unit of compound **5** through an intramolecular Suzuki–Miyaura cross-coupling reaction; these reactions unambiguously determined the absolute structures of compounds **4** and **5**. The absolute structure of compound **6** was established by chemical correlation with compound **3**. The fact that the 3D structures of the 18-membered cyclopeptide backbones of highly cytotoxic compound **1** and less-active compounds **4** and **5** are nearly superimposable and yet they show obviously different activities may suggest that, in addition to the proper alignment of the 4-methoxyphenyl ring of Tyr-3, the orientation of one or both of the Tyr-5 and Tyr-6 phenyl rings plays an essential role in expressing the activity. This information should be useful for designing structurally more simplified RA analogues. In this study, peptides **3**–**6** were isolated from the same plant source, *R. cordifolia*. This fact and the comparison of their structures may indicate that compound **6** is biosynthesized first, with subsequent oxidative coupling reactions producing peptides **3**–**5**.[33]

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[30] Abbreviations: BnOH = benzyl alcohol; DMAP = 4-dimethylaminopyridine; dppf = 1,1′-bis(diphenylphosphino)ferrocene; EDC·HCl = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOAt = 1-hydroxy-7-azabenzotriazole; HOOBt = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; $[PdCl_2(dppf)]\cdot CH_2Cl_2$ = [1,1′-bis(di-

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