Article

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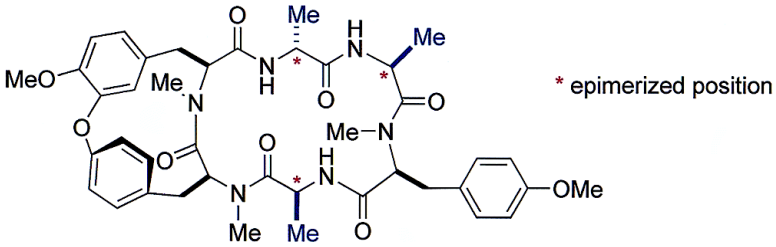

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Synthesis of [L-Ala-1]RA-VII, [D-Ala-2]RA-VII, and [D-Ala-4]RA-VII by Epimerization of RA-VII, an Antitumor Bicyclic Hexapeptide from *Rubia* Plants, through Oxazoles

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Abstract: Three epimers of a natural cyclic hexapeptide RA-VII were prepared via formation of oxazoles from thioamides or thioimidates of RA-VII followed by hydrolysis. They are the epimers at L-Ala-1, D-Ala-2, and D-Ala-4, respectively. The one having L-Ala-1 adopted *trans-cis-trans-trans-trans-trans* (*t-c-t-t-t-t*) amide configurations in the crystal, a type-VI β-turn for residues 1−4 stabilized by one intramolecular hydrogen bond between Ala-4 N*H* and L-Ala-1 C = *O*, and in $CDCl_3$ existed as a mixture of six conformers, of which the major conformer was very similar to that in the crystal, but quite different from that of RA-VII in solution. The second epimer, having D-Ala-2 had in the crystalline state *t-t-t-t-c-t* amide configurations, a γ-turn at Tyr-3 stabilized by two intramolecular hydrogen bonds between D-Ala-2 N*H* and Ala-4 C = *O* and between Ala-4 N*H* and D-Ala-2 C = *O*, and existed in $CDCl_3$ as a single conformer, the structure of which was very similar to its crystal structure, and to the crystal structure of peptide **25** except for the backbone and the side chains at residues 1 and 2. The third epimer, having D-Ala-4 had *t-c-t-t-c-t* amide configurations in the crystal, a type-VI β-turn for residues 1−4 as observed in the first epimer, and in $CDCl_3$ existed in three conformers, of which the major one was similar to that in the crystal but different from that of RA-VII in solution. The three epimers showed very weak cytotoxicity on P-388 leukemia cells, which may be because of their conformational differences from the active conformation of RA-VII.

Introduction

RA-VII (**1**) is an antitumor bicyclic hexapeptide isolated from *Rubia akane* Nakai and *R. cordifolia* L.[1,2] with structural homology to bouvardin (NSC 259968) (**2**) from *Bouvardia ternifolia* (Cav.) Schlecht.[3] Both cyclic peptides show potent antitumor activity, and their mode of action is considered to be inhibition of protein synthesis through interaction with eukaryotic 80S ribosomes.[4,5] Peptide **1** exists as a mixture of two to three stable conformers in solution.[6] The major conformer has been characterized in the *trans-trans-trans-trans-cis-trans* (*t-t-t-t-c-t*) amide configurations and proposed to be the active conformer.[7] The configuration of the amino acid residues in cyclic peptides is known to affect their conformation.[8] Because the conformation of RA-VII may determine its ability to interact with the ribosome and exhibit biological activity, the roles of the configuration of each alanine residue on the conformation and biological activity of peptide **1** have been explored by the preparation of three epimers of **1**: [L-Ala-1]RA-VII (**3**), [D-Ala-2]RA-VII (**4**), and [D-Ala-4]RA-VII (**5**). Their crystal and solution structures were examined by X-ray and NMR studies and their cytotoxicities were also assayed.

Total synthesis of analogues of **1** is difficult due to the highly strained 14-membered cycloisodityrosine unit[9−12] and the 18-membered cyclopeptide ring. A more efficient method for preparing epimeric analogues of cyclic peptides was investigated using selective epimerization of **1** via oxazole intermediates

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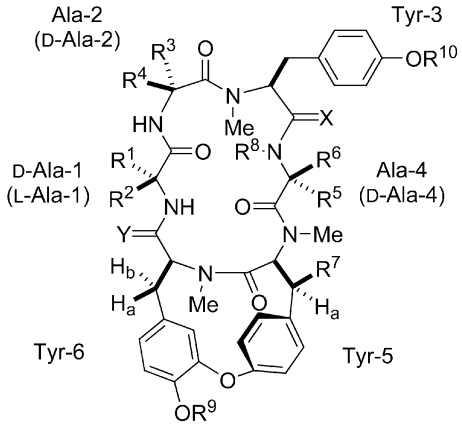
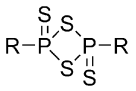
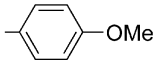


	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	X	Y
1	Me	H	H	Me	H	Me	H_b	H	Me	Me	O	O
2	Me	H	H	Me	H	Me	OH	H	H	Me	O	O
3	H	Me	H	Me	H	Me	H_b	H	Me	Me	O	O
4	Me	H	Me	H	H	Me	H_b	H	Me	Me	O	O
5	Me	H	H	Me	Me	H	H_b	H	Me	Me	O	O
7	Me	H	H	Me	H	Me	H_b	H	Me	Me	S	O
8	Me	H	H	Me	H	Me	H_b	H	Me	Me	S	S
9	Me	H	H	Me	H	Me	H_b	H	Me	Me	O	S
11	Me	H	H	Me	H	Me	H_b	H	Me	Me	NOH	S
14	Me	H	H	Me	H	Me	H_b	Ac	Me	Me	O	O
25	Me	H	H	Me	H	Me	H_b	H	Me	H	O	O
26	Me	H	H	Me	H	Me	H_b	H	*p*-BrBz	Me	O	O



6: R = 

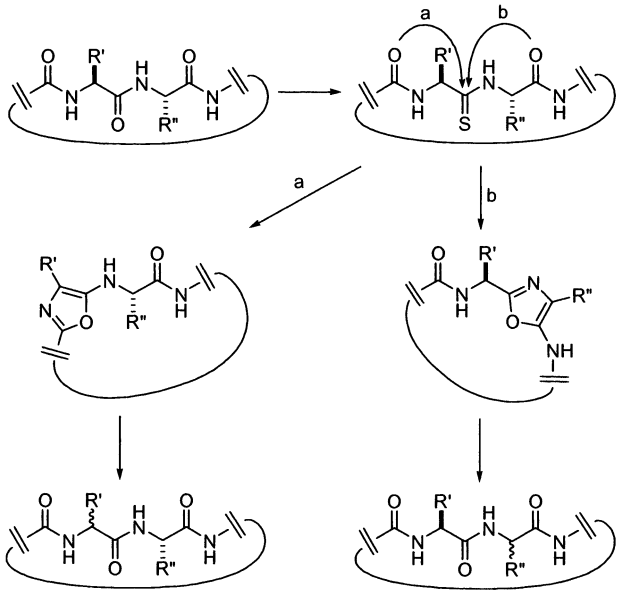
10: R = SMe

followed by partial hydrolysis and chromatographic separation of the desired epimeric peptides.[13]

Results and Discussion

Preparaion of Cyclic Thionopeptides. Thionopeptides may be suitable precursors for oxazole intermediates and readily prepared by treating cyclic peptides with a thionating reagent, such as Lawesson's reagent (**6**). Normally only one or two particular peptide bonds are thionated on the basis of their structural and conformational features.[14] Treatment of RA-VII (**1**) with **6** in dioxane at 50 °C afforded thioamide **7** in which Tyr-3 was thionated and bis(thioamide) **8** in which both Tyr-3 and Tyr-6 were thionated in yields of 80% and 3%, respectively.[15]

Scheme 1



The isomeric thioamide **9** in which Tyr-6 was specifically thionated could not be obtained in quantity from thionation of **1**, because the amide carbonyl group of Tyr-3 was more reactive toward the thionating reagent than that of Tyr-6. Thioamide **9** was synthesized by first preparing bis(thioamide) **8**[16] in 54% yield on treatment of **1** with Davy-Reagent-Methyl (**10**), followed by reaction with hydroxylamine hydrochloride, triethylamine and $Hg(OAc)_2$, and subsequent treatment of hydroxylamidine **11** (88%) with nitrous acid, which afforded **9** in 83% yield. A more effective alternative involved oxidation of **8** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in aqueous dioxane to give **9** in 79% yield.

Preparation of Oxazoles from Thionopeptides. When thionopeptides are treated with a thiophilic reagent, condensation of the thioamide group with an adjacent peptide bond may occur to produce an oxazole ring as shown in Scheme 1. For example, oxazoles **12** and **13** were formed on treating thioamide **7** with two molar equivalents of a thiophilic reagent, $AgBF_4$ or $Hg(OAc)_2$, in THF under an argon atmosphere at room temperature for 20 h, and the products were separated by HPLC (Table 1). Different thiophilic reagents gave different results. Reaction of thioamide **7** with $AgBF_4$ in THF gave oxazole **12** in 24% yield (entry 1), and with $Hg(OAc)_2$ in the same solvent afforded oxazole **13** as a major product (45%) along with acetimide **14** (14%) and oxazole **12** (3.9%) (entry 5). Yields were also greatly influenced by the reaction solvent employed when $AgBF_4$ was used as a thiophilic reagent. Better yields were obtained in THF

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Scheme 2

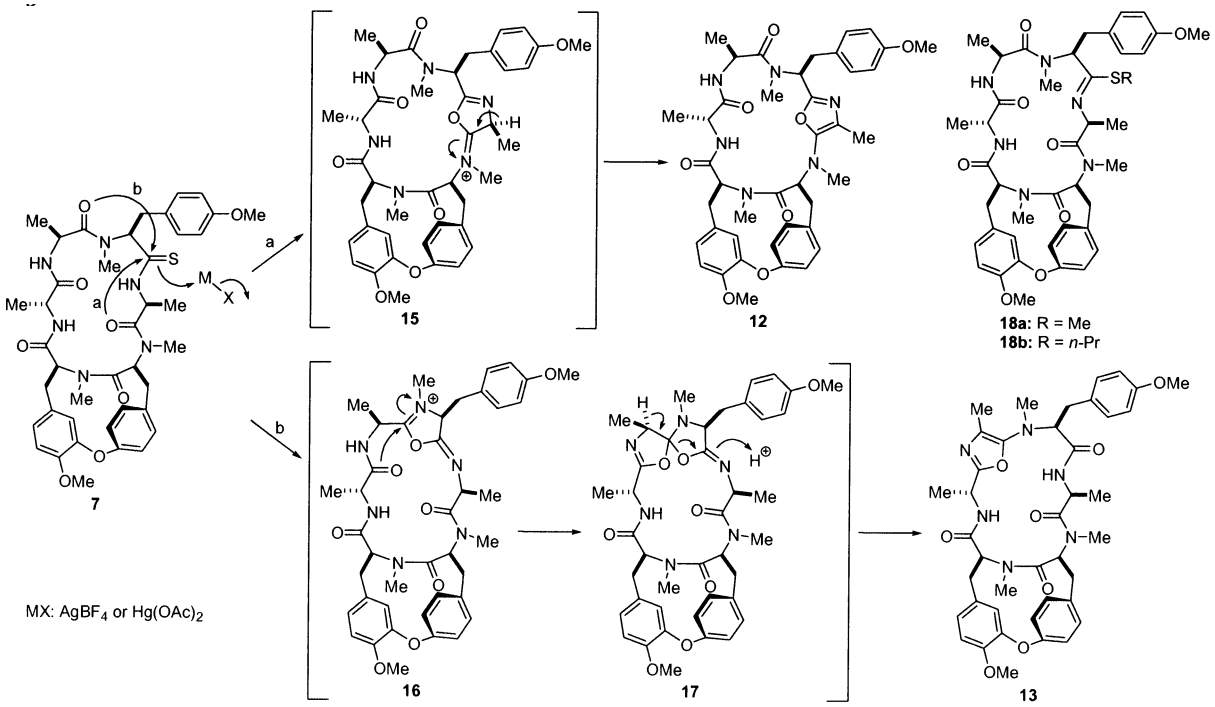


Table 1. Reaction of Thioamide 7 with Thiophilic Reagents

			yield (%)				
entry	reagent[a]	solvent	**1**	**12**	**13**	**14**	**7**[b]
1	A	THF	23	24	0	0	9.1
2	A	DME	24	24	1.0	0	18
3	A	MeCN	7.7	1.0	0	0	81
4	A	$CHCl_3$	35	7.3	0	0	41
5	B	THF	9.3	3.9	45	14	0
6	B	DME	14	8.7	42	8.2	0
7	B	MeCN	22	26	32	4.2	0
8	B	$CHCl_3$	29	4.1	44	6.2	0

[a] Two molar equiv of reagent were used. A: $AgBF_4$, B: $Hg(OAc)_2$. [b] Recovered starting material.

(entry 1) and in DME (entry 2) relative to in MeCN (entry 3) and in $CHCl_3$ (entry 4) from which 81% and 41% of the starting material **7** was recovered, respectively. On the other hand, solvent composition had little influence on the reaction with $Hg(OAc)_2$ (entries 5−8).

The formation of oxazoles **12** and **13** was established by the observation of the quasi-molecular ion peak $[M+H]^+$ at *m*/*z* 753 in the FABMS and of a singlet methyl signal assignable to the alanine methyl group incorporated in the oxazole ring in their ^1H NMR spectra. The position of the oxazole ring in **12** and **13** was determined by the observation of HMBC correlations between C-5 of the oxazole and the *N*-methyl protons of Tyr-5 and Tyr-3, respectively. The structure of acetimide **14** was elucidated by the analysis of its HMBC spectra. Furthermore, the production of **1** when **14** was treated with methanolic sodium hydroxide at room temperature suggested acetylation of an amide nitrogen.

Possible mechanisms for the formation of oxazoles **12** and **13** from **7** are shown in Scheme 2. The amide oxygen of Ala-4 may attack the carbon atom of the thioamide group to produce oxazoline **15**, which on isomerization affords oxazole **12** (path a). The attack of the amide oxygen of Ala-2 on the carbon atom of the thioamide group may produce oxazoline **16** which may

Table 2. Reaction of Thioimidates 18a,b and 19a,b with Thiophilic Reagents[a]

				time	yield (%)[c]				
entry	substrate	reagent[b]	solvent	(h)	**1**	**12**	**13**	**14**	**20**
1	**18a**	A	DME	12	7.5	87	0.8	0	0
2	**18a**	A	DMF	24	16	61	6.1	0	0
3	**18a**	A	MeCN	24	25	32	1.0	0	0
4	**18a**	A	CH_2Cl_2	24	4.2	24	1.9	0	0
5	**18a**	B	DME	12	7.6	1.6	41	18	0
6	**18a**	B	DMF	24	14	48	32	2.8	0
7	**18a**	B	MeCN	24	12	68	12	0	0
8	**18a**	B	CH_2Cl_2	24	18	5.2	38	17	0
9	**18b**	A	DME	12	6.1	84	1.1	0	0
10	**18b**	B	DME	12	7.5	3.0	55	21	0
11	**19a**	A	THF	24	9.4	9.8	11	0	0
12	**19a**	A	DME	24	6.6	8.4	9.8	0	8.7
13	**19a**	A	MeCN	24	no reaction[d]				
14	**19b**	A	THF	20	0	8.8	13	0	19
15	**19b**	A	DME	24	0	12	9.0	0	0
16	**19b**	A	MeCN	21	0	0	0	0	6.7[e]
17[f]	**19b**	A	MeCN	15	0	0	4.1	0	27[g]

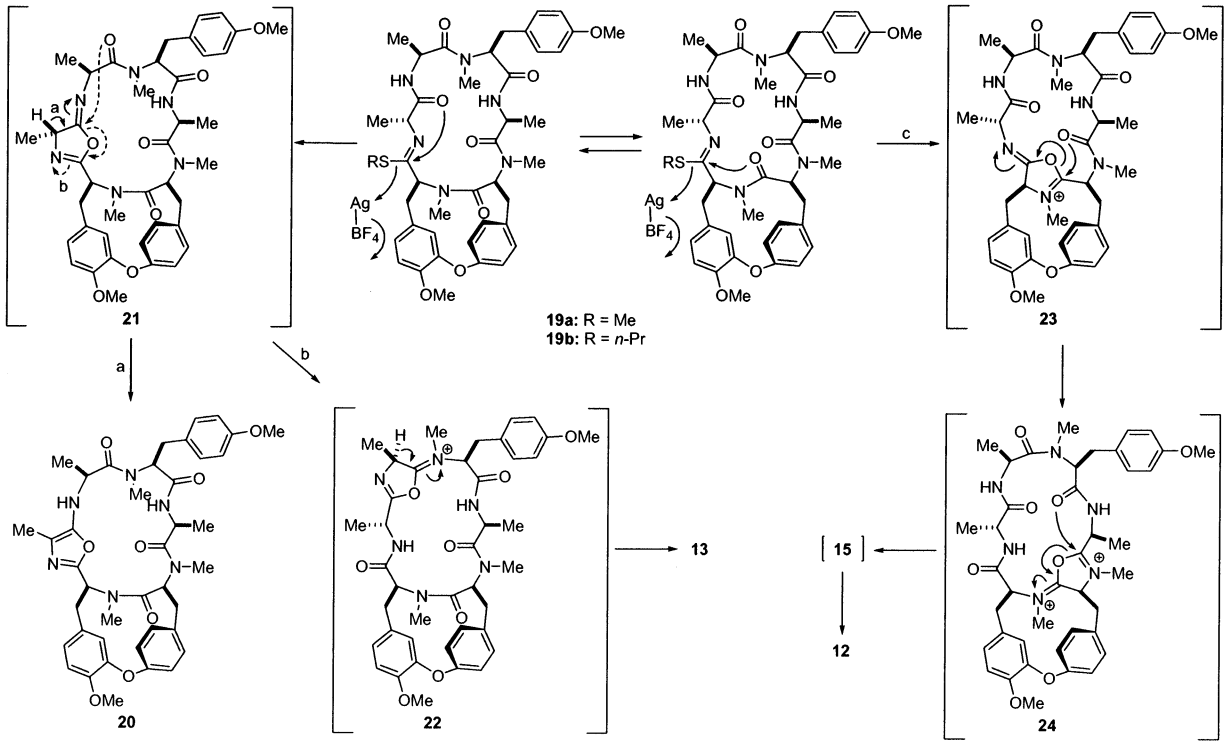
[a] This table continues in Table S1. See Supporting Information. [b] Two molar equiv of reagent were used. A: $AgBF_4$, B: $Hg(OAc)_2$. [c] Two-step yields from thioamide **7** or **9**. [d] 88% of the starting material **19a** was recovered. [e] 56% of the starting material **19b** was recovered. [f] The reaction was carried out at 40 °C. [g] 30% of the starting material **19b** was recovered.

then convert into oxazole **13** via spirocyclic intermediate **17** (path b).[13,17] Although the formation of oxazole **13** via this route may be unusual, the tertiary amide between Ala-2 and Tyr-3 may hamper the formation of an oxazole between these residues (path a in Scheme 1) and thus favor formation of **13**.

Preparation of Oxazoles via Thioimidates. To enhance the reactivity of the substrate, thioamide **7** was converted into *S*-methyl- and *S*-propylthioimidates, **18a** and **18b**, on treatment with K_2CO_3 and iodomethane or 1-iodopropane, respectively. Without further purification, these thioimidates were reacted

(17) Involvement of spirocyclic intermediates in the hydrolysis of 5-amino-oxazoles has been reported. Zhao, G.; Sun, X.; Bienaymé, H.; Zhu, J. *J. Am. Chem. Soc.* **2001**, *123*, 6700−6701.

Scheme 3



with a thiophilic reagent in different solvents (DME, dioxane, THF, DMF, MeCN, and CH_2Cl_2, Tables 2 and S1). The reaction proceeded efficiently; no substrate, **18a** nor **18b**, was recovered in any run. As in the case of thioamide **7**, the reaction of **18a** and **18b** with $AgBF_4$ tended to produce oxazole **12** rather than **13**. When DME was used as a solvent, the yields of **12** via **18a** and **18b** (entries 1 and 9) were 87% and 84%, respectively. Oxazole **12** was also formed from the reaction of **18a** in MeCN in 32% yield (entry 3), in contrast to the 1.0% yield of **12** when thioamide **7** was used as a substrate (Table 1, entry 3). When $Hg(OAc)_2$ was used in ethers and CH_2Cl_2, **18a** and **18b** produced oxazole **13** as the major product (entries 5, 8 and 10). The best yield of 55% for **13** was obtained when the reaction was carried out in DME (entry 10). On the contrary, in DMF and MeCN, oxazole **12** was obtained as a major product using $Hg(OAc)_2$ in yields of 48% and 68%, respectively (entries 6 and 7). Similarly, the proportion of **12** relative to **13** was increased in MeCN when thioamide **7** was used as a substrate (Table 1, entry 7).

Analogously, thioamide **9**, in which Tyr-6 was thionated, was converted into *S*-methyl- and *S*-propylthioimidates, **19a** and **19b**, and reacted with $AgBF_4$ in THF, DME, and MeCN (Table 2). Oxazole yields were generally low with **19** relative to **18** and influenced by the structure of the thioimidate. Oxazole **20** was best prepared by reacting **19b** with $AgBF_4$ in MeCN at 40 °C albeit in 27% yield (entry 17).

A mechanism to explain the formation of oxazoles **13** and **20** from **19a** and **19b** involves the amide oxygen of D-Ala-1 attacking the carbon atom of the thioimidate group to produce oxazoline **21** (Scheme 3). Tautomerization would afford oxazole **20** (path a). Oxazole **13** may be formed via oxazoline **22** (path b), and oxazole **12** may be produced through sequential formations of three oxazoline intermediates **23**, **24**, and **15** (path c).

Table 3. Hydrolysis of Oxazoles 12, 13, and 20

entry	oxazole	catalyst	solvent[a]	temp. (°C)	product (yield, %)
1	**12**	*p*-TsOH	A	50	**5** (13), **1** (74)
2	**12**	$BF_3{\cdot}OEt_2$	B	rt	**5** (16), **1** (64)
3	**12**	$BF_3{\cdot}OEt_2$	B	50	**5** (26), **1** (74)
4	**12**		C	90	no reaction
5	**13**	$BF_3{\cdot}OEt_2$	B	50	**4** (24), **1** (60)
6	**13**		C	90	**4** (50), **1** (38)
7	**20**	$BF_3{\cdot}OEt_2$	B	rt	**3** (61), **1** (33)
8	**20**		C	90	**3** (45), **1** (38)

[a] A: THF−H_2O (9:1); B: MeCN−H_2O (9:1); C: THF−AcOH−H_2O (5:2:1).

Hydrolysis of Oxazoles. Oxazoles **12**, **13**, and **20** were hydrolyzed under mild acidic conditions to afford epimers **5**, **4**, and **3**, respectively (Table 3). The stability of each oxazole in solution appears to be different. Although oxazoles **13** and **20** were slowly degraded on standing in aqueous solution at room temperature, oxazole **12** was stable in a THF−AcOH−H_2O mixture at 90 °C (entry 4).[18,19]

Conformation of Epimers 3−5 in the Crystalline State. X-ray analyses of epimers **3**, **4**, and **5** as well as RA-II (**25**), another cyclic hexapeptide from the same plant source,[20] all were successful (Figure 1, Table 4); however, RA-VII (**1**) did not give crystals suitable for analysis. The backbone structures of epimers **3**−**5** were compared with that of **25** which should be essentially the same as that of **1**, because their differences resided only at the *para* substituent of the phenyl ring. The data

(18) 5-(Acylamino)oxazoles were used as a dipeptide precursor for the synthesis of a naturally occurring cyclopeptide analogue. (a) Lipshutz, B. H. *Chem. Rev.* **1986**, *86*, 795−819. (b) Lipshutz, B. H.; Huff, B. E.; McCarthy, K. E.; Miller, T. A.; Mukarram, S. M. J.; Siahaan, T. J.; Vaccaro, W. D.; Webb, H.; Falick, A. M. *J. Am. Chem. Soc.* **1990**, *112*, 7032−7041.
(19) A similar approach towards an epimeric analogue of a cyclodepsipeptide has been reported. Oberhaauser, B.; Baumann, K.; Grohmann, B.; Sperner, H. *Synlett.* **1999**, 893−896.
(20) Itokawa, H.; Takeya, K.; Mori, N.; Sonobe, T.; Mihashi, S.; Hamanaka, T. *Chem. Pharm. Bull.* **1986**, *34*, 3762−3768.

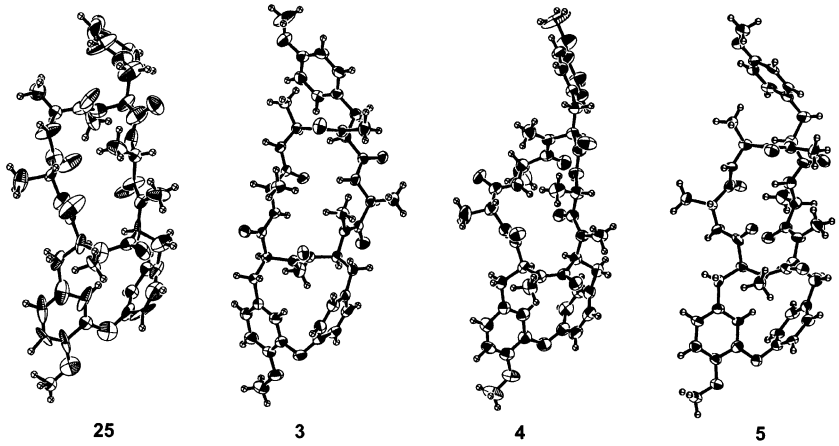


Figure 1. Crystal structures of RA-II (**25**) and epimers **3**−**5**.

Table 4. Backbone Torsion Angles (deg) in the X-ray Crystal Structures of Bouvardin (2), [L-Ala-1]RA-VII (3), [D-Ala-2]RA-VII (4), [D-Ala-4]RA-VII (5), RA-II (25), and Deoxybouvardin *p*-Bromobenzoate (26)

residue	torsion angle	2[a]	3	4	5	25	26[b]
D-Ala-1	ϕ	134	−172	140	67	148	138
(L-Ala-1)	ψ	−164	162	−42	−148	−175	−170
	ω	−176	169	−179	−172	166	−175
Ala-2	ϕ	−87	−65	147	−97	−70	−83
(D-Ala-2)	ψ	117	126	−79	121	119	121
	ω	−177	−9	170	7	174	−178
Tyr-3	ϕ	56	−127	−93	−124	57	54
	ψ	37	59	52	69	41	39
	ω	−172	180	−168	167	−166	−168
Ala-4	ϕ	−156	−159	−180	153	−160	−159
(D-Ala-4)	ψ	164	−53	151	−75	166	171
	ω	179	−171	172	−164	171	174
Tyr-5	ϕ	−121	−134	−117	−127	−138	−137
	ψ	101	93	102	110	108	102
	ω	−8	−171	4	−32	−12	2
Tyr-6	ϕ	−84	50	−90	−72	−79	−92
	ψ	165	39	164	−176	157	163
	ω	176	177	164	171	177	171

[a] Taken from ref 3. [b] Taken from ref 6.

for deoxybouvardin *p*-bromobenzoate (RA-V *p*-bromobenzoate) (**26**)[1a,6] and bouvardin (**2**)[3] whose crystal structures are very similar to that of **25** are also presented in Table 4 to show that differences in side chains had little effect on the peptide backbone. The most distinctive feature which characterizes the structure of cyclic peptides is their backbone amide configurations. Peptides **25** and **3**−**5** possess three *N*-methyltyrosine residues at positions 3, 5 and 6, the amides of which can adopt both cis and trans isomers. Peptide **25** possesses *trans-trans-trans-trans-cis-trans* (*t-t-t-t-c-t*) amide configurations between D-Ala-1/Ala-2, Ala-2/Tyr-3, Tyr-3/Ala-4, Ala-4/Tyr-5, Tyr-5/Tyr-6, and Tyr-6/D-Ala-1, respectively.[6] Epimers **3**−**5** possess *t-c-t-t-t-t*, *t-t-t-t-c-t*, and *t-c-t-t-c-t* configurations, respectively.

The trans amide configuration between Tyr-5/Tyr-6 observed in the crystal structure of **3** is not adopted in the natural peptides of RA-series. The conformation of **3** was also characterized by a type-VI β-turn comprising residues 1−4, stabilized by an intramolecular hydrogen bond between the Ala-4 N*H* and the L-Ala-1 C = *O* (N···O distance 2.92 Å). This type of turn was also observed by NMR spectroscopy in the minor conformer of peptide **1** in solution. The type-VI β-turn is a relatively rare secondary structure usually featuring an amide cis isomer

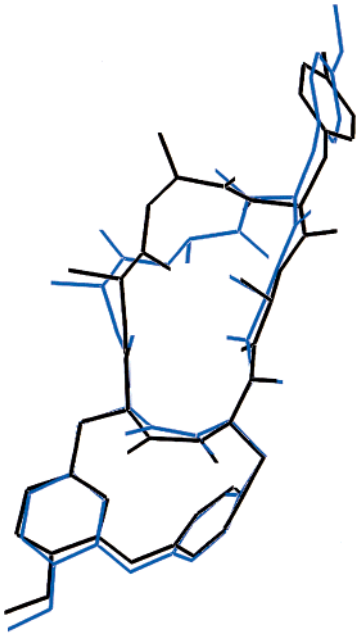


Figure 2. Superposition of the crystal structures of RA-II (**25**) (black) and [D-Ala-2]RA-VII (**4**) (blue).

N-terminal to a proline residue situated at the $i + 2$ position of the peptide bend.

Peptide **25** and epimer **4** both adopted the *t-t-t-t-c-t* amide configurations despite their configurational difference at the α carbon of residue 2. Peptide **25** adopted a type II β-turn comprising residues 1−4. On the other hand, epimer **4** adopted a γ-turn conformation centered at Tyr-3 stabilized by two weak intramolecular hydrogen bonds between the D-Ala-2 N*H* and the Ala-4 C = *O* (N···O distance 3.10 Å) and between the Ala-4 N*H* and the D-Ala-2 C = *O* (N···O distance 3.06 Å). Despite different turn structures, the superposition of the crystal structures of **4** and **25** (Figure 2) showed that the backbone about residues 3−6 and the locations of aromatic rings of the three tyrosines were superimposable. Differences were noted in the backbone structure about the D-Ala-1 and D-Ala-2 residues and the locations of their side chains. Such similarity of the backbone structure and the topology of the three tyrosine aromatic rings was not observed between **25** and **3** nor between **25** and **5**.[21]

Epimer **5** possessed the *t-c-t-t-c-t* amide configurations, which was observed as a minor conformer of natural RA-series

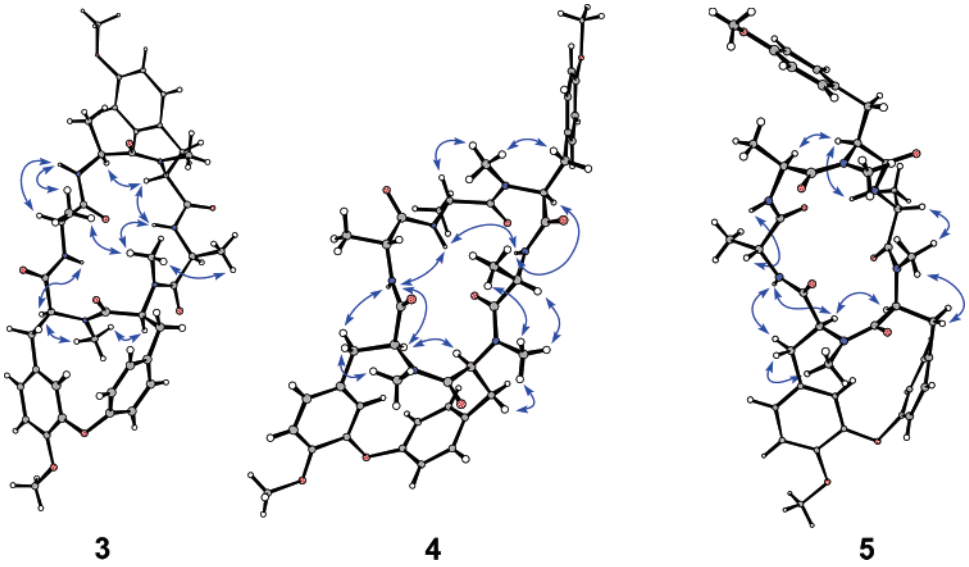


Figure 3. Selected NOESY/ROESY correlations observed in the major conformers of **3**, **4**, and **5**.

Table 5. NOEs Observed in the Major Conformers of Epimers 3−5 in $CDCl_3$ Which Characterize the Amide Configurations of the Peptide Backbones

	NOE intensity		
proton(s)	**3**	**4**	**5**
Ala-1 H_α/Ala-2 NH	medium	weak	medium
Ala-1 Me/Ala-2 NH	medium	weak	weak
Ala-2 H_α/Tyr-3 H_α	strong		strong
Ala-2 H_α/Tyr-3 NMe		strong	
Tyr-3 NMe/Tyr-3 H_α	weak	medium	
Tyr-3 H_α/Ala-4 NH	medium	medium	medium
Ala-4 H_α/Tyr-5 NMe	medium	strong	strong
Ala-4 Me/Tyr-5 NMe	medium	medium	medium
Tyr-5 H_α/Tyr-6 H_α		strong	strong
Tyr-5 H_α/Tyr-6 NMe	strong		
Tyr-6 NMe/Tyr-6 H_α	strong	weak	
Tyr-6 H_α/Ala-1 NH		weak	medium

peptides by NMR spectroscopy in solution. As in the case of epimer **3**, this conformation exhibited a type-VI β-turn comprising residues 1−4 stabilized by an intramolecular hydrogen bond between the D-Ala-4 N*H* and the D-Ala-1 C = *O* (N···O distance 2.85 Å).

Conformation of Epimers 3−5 in Solution. The parent peptide **1** has been studied by NMR spectroscopy[6,22,23] and shown to adopt two stable conformations in $CDCl_3$ solution in a ratio of 89:11. The major *t-t-t-t-c-t* conformer is similar to the conformation of **26** in the crystalline state. The minor *t-c-t-t-c-t* conformer was characterized by NOESY experiments and computational methods.[6] Analogously, the solution forms of epimers **3**−**5** were examined by NOESY and ROESY experiments (Figure 3).

In $CDCl_3$, epimer **3** existed as a mixture of six conformers. The major conformer (68%) showed NOESY correlations consistent with the *t-c-t-t-t-t* amide configurations, as observed in the crystal (Table 5). In addition, NOESY correlations were also observed between L-Ala-1 Me/Tyr-5 NMe, and Ala-4 NH/Tyr-5 NMe. The spacings between these pairs of protons were calculated to be less than 2.8 Å in the crystal. The methyl signal of Ala-2 was also observed at δ 0.38, due likely to its close proximity to the aromatic ring of Tyr-3, as observed in the crystal (Figure 1). A similar upfield shift of the methyl signal was also observed for the minor conformer of **1**, which exhibited a cis amide bond between Ala-2/Tyr-3. These observations indicated that the structure of the major conformer of **3** in solution is very similar to that in the crystalline state. The characterization of the minor conformers of **3** by the NMR analysis was not possible because of overlapping resonances.

In $CDCl_3$, epimer **4** existed as a single *t-c-t-t-t-t* conformer as in the crystal (Table 5). A close relationship between the conformation in solution and in the crystalline state was strongly suggested by the fact that the NOESY correlations were observed between D-Ala-1 NH/D-Ala-2 NH, D-Ala-2 NH/Ala-4 NH, Tyr-5 NMe/Tyr-5 $H_{\beta b}$, Tyr-6 NMe/Tyr-6 $H_{\beta a}$, and Tyr-6 $H_{\beta b}$/D-Ala-1 NH in solution, and the spacings between the protons of each pair were less than 2.7 Å in the crystal.

Epimer **5** existed as a 91:5:4 mixture of three stable conformers in $CDCl_3$. The structure of the major conformer (91%) was analyzed by a ROESY experiment which indicated that the major conformer of epimer **5** possessed *t-c-t-t-c-t* amide configurations as in its crystalline state (Table 5). In the crystal, the methyl of Ala-2 of **5** is located just above the aromatic ring of Tyr-3 as in the case of **3**. This explains the shielding effect of the Tyr-3 aromatic ring on the methyl signal at Ala-2 to give it an upfield shift (δ 0.97). The similarity of the structures in solution and in the crystal was further supported by the presence of ROESY correlations between Tyr-5 NMe/Tyr-5 $H_{\beta b}$, Tyr-6 NMe/Tyr-6 $H_{\beta a}$, and Tyr-6 $H_{\beta b}$/D-Ala-1 NH. The spacings between the protons of each pairs in the crystal were calculated to be less than 2.7 Å.

Apparently, the methyl group of Ala residues plays a crucial role in determining the conformation of the 18-membered cyclic backbone of the peptide of this series. The inversion of the alanyl chiral center induces steric repulsion between the alanyl methyl group and the neighboring groups, which does not exist in the original peptide **1**. For example, when epimer **3** adopts the same backbone conformation as that of the major conformer of **1**,

(21) Superposition of the crystal structures of **3**, **5**, and **25** is depicted in Figure S1. See Supporting Information.
(22) Bates, R. B.; Cole, J. R.; Hoffmann, J. J.; Kriek, G. R.; Linz, G. S.; Torrance, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1343−1347.
(23) Boger, D. L.; Patane, M. A.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7357−7363.

Table 6. Calculated Distances (Å) between the Atoms Participating the Steric Repulsion

compd	relevant groups	distance[a]	A	B	C
3	L-Ala-1 CH_3/L-Ala-4 CH_3	C···C	3.14	6.90	5.37
	L-Ala-1 CH_3/Tyr-6 C = *O*	C···O	2.39	3.13	3.56
4	D-Ala-2 CH_3/D-Ala-1 C = *O*	C···O	2.61	3.43	4.08
	D-Ala-2 CH_3/Tyr-3 NCH_3	C···C	2.36	4.41	3.91
5	D-Ala-4 CH_3/Tyr-3 C = *O*	C···O	2.63	3.43	3.46
	D-Ala-4 CH_3/Tyr-5 NCH_3	C···C	2.51	4.39	3.24

[a] A: The calculated values when epimers **3**−**5** are assumed to adopt the same backbone structure as that of crystalline RA-II (**25**); B: Actual distance in the crystalline state; C: The distance between the corresponding atoms in crystalline RA-II (**25**).

the spacings between L-Ala-1 *Me* and Ala-4 *Me* and between L-Ala-1 *Me* and Tyr-6 C = *O* are calculated to be 3.14 Å (C···C) and 2.39 Å (C···O), respectively, by using the atomic coordinates of RA-II (**25**) (Table 6). These values are much too short compared with the corresponding atom spacings in RA-II (**25**); which are 5.37 and 3.56 Å, respectively. Thus, a strong steric repulsion is expected to be induced between the relevant groups. To avoid the steric repulsion, the molecule should go through conformational changes, which include inversion of the amide configuration between Ala-2 and Tyr-3 and between Tyr-5 and Tyr-6. Thus, **3** takes the unique conformation nonexistent in the natural peptide **1**. As shown in Table 6, in the conformer that **3** assumes in the crystal, the spacings are 6.90 and 3.13 Å, respectively.

Similarly, when epimers **4** and **5** adopt the same backbone conformation as that of the major conformer of **1**, strong steric repulsion is expected between D-Ala-1 C = *O* and D-Ala-2 *Me* (2.61 Å) and between D-Ala-2 *Me* and Tyr-3 N *Me* (2.36 Å) for **4**, and between Tyr-3 C = *O* and D-Ala-4 *Me* (2.63 Å) and between D-Ala-4 *Me* and Tyr-5 N*Me* (2.51 Å) for **5**. These repulsions make them take more stable conformations as observed in the crystal and in solution.

Cytotoxicity of RA-VII (1) and Epimers 3−5. The major conformer of peptide **1** having *t*-*t*-*t*-*t*-*c*-*t* amide configurations has been identified as an active one. The methoxy group on Tyr-3 residue is also known to be essential for expression of activity in the RA-series of cyclic peptides; hydrogen and hydroxyl substituents cause reduced cytotoxicity (1/100−1000 relative to the parent methoxy compound).[24] RA-VII (**1**) and its epimeric analogues [L-Ala-1]RA-VII (**3**), [D-Ala-2]RA-VII (**4**), and [D-Ala-4]RA-VII (**5**) were evaluated using P-388 murine lymphocytic leukemia cells and exhibited IC_{50} values of 0.0023, 0.31, 8.5 and 8.1 μg/mL, respectively. The reduced activity of epimers **3** and **5** may be explained by their *t*-*c*-*t*-*t*-*t*-*t* and *t*-*c*-*t*-*t*-*c*-*t* amide configurations, respectively, which produce conformational features very different from that adopted by **1** when interacting with 80S ribosomes. Epimer **4** has the same amide configurations as the major conformer of **1** and **25**, *t*-*t*-*t*-*t*-*c*-*t*. In the crystal structures of **4** and **25**, the backbones at residues 3−6 place the tyrosine aromatic side-chains in similar positions. The loss of activity of **4** may thus be attributed to the difference in the peptide structure about residues 1 and 2, which may hinder the interaction of the peptide with 80S ribosomes.

Conclusion

Three analogues of RA-VII (**1**) were synthesized via oxazole intermediates. Modification of peptide **1** by epimerization of alanine residues 1, 2, and 4, respectively, caused decreased cytotxic activity relative to the original peptide. X-ray crystallographic and NMR spectroscopic analyses of the conformation of the epimers in the solid-state and in solution demonstrated that the major conformer in solution corresponded to the conformational structures as those of the respective ones in the crystals. The reduction of cytotoxic activity was correlated to the configurational and conformational changes in these peptide analogues. The active conformer of peptide **1** was shown to require the D, L, L-configurations for alanines 1, 2, and 4, respectively, as well as the *t*-*t*-*t*-*t*-*c*-*t* amide configurations.

Supporting Information Available: Further discussion of the solvent effects on the oxazole formation, Table S1, experimental details, ^{1}H NMR spectra and assignments of ^{1}H and ^{13}C NMR signals for **3**−**5**, **11**−**14** and **20**, X-ray data of **3**−**5** and **25**, and superposition of the crystal structures of **3**, **5**, and **25** are available free of charge via the Internet at http://pubs.acs.org.

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