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Synthesis of [Gly-1]RA-VII, [Gly-2]RA-VII, and [Gly-4]RA-VII. Glycine-Containing Analogues of RA-VII, an Antitumor Bicyclic Hexapeptide from *Rubia* Plants

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Received September 23, 2003

Three analogues of RA-VII (**1**), an antitumor bicyclic hexapeptide from *Rubia* plants, were synthesized. Three analogues, [Gly-1]RA-VII (**4**), [Gly-2]RA-VII (**5**), and [Gly-4]RA-VII (**6**), in which one of the three alanine residues in **1** was replaced by a glycine residue, were prepared by linking of the cycloisodityrosine unit, obtained by degradation of **1**, to three different glycine-containing tetrapeptides followed by macrocyclization. Of these three analogues, analogue **4** showed the highest cytotoxic activity. The NMR study revealed that in solution the conformer structures and their ratios of analogue **4** were very similar to those of natural peptide **1**, suggesting that the methyl groups at Ala-2 and Ala-4 should be essential for producing the bioactive conformation, whereas that at D-Ala-1 is not essential.

Introduction

An antitumor bicyclic hexapeptide RA-VII (**1**) and its 16 congeners have been isolated from *Rubia akane* and *Rubia cordifolia* (Rubiaceae),[1−3] and two related peptides, bouvardin (NSC 259968) (**2**) and deoxybouvardin (**3**), were isolated from another plant of this family, *Bouvardia ternifolia*.[4] The structures of the peptides of this series are characterized by two macrocyclic rings, i.e., an 18-membered cyclohexapeptide ring and a 14-membered cycloisodityrosine ring, fused together to form a 26-membered ring. The antitumor action of **1** is considered to be due to the inhibition of protein synthesis through its interaction with eukaryotic 80S ribosomes,[5,6] and the cycloisodityrosine ring is proposed to be the pharmacophore in this type of peptides because cycloisodityrosine itself shows a potent cytotoxic activity.[7] However, the role of the 18-membered ring moiety in the activity should also be vital because some cycloisodityrosine-containing RA-VII analogues, in which the tetrapeptide fragment of the 18-membered ring is modified by reduction,[8] by cleavage of the 18-membered ring,[9] or by replacement with a polyglycine unit,[10] show a very weak or no cytotoxic activity. The configuration of certain amino acid residues in cyclic peptides takes part in deciding the conformation of peptide rings[11] and thus should affect the conformation of the whole molecule. In the present study, we studied the effect of the three alanyl methyls of the 18-membered ring in **1** upon its conformation and cytotoxic activity by syntheses of RA-VII analogues in which one of the three alanines was replaced by glycine. Information obtained by this study may provide a useful knowledge for designing more simple and more active analogues of RA-VII with a reduced number of chiral centers.

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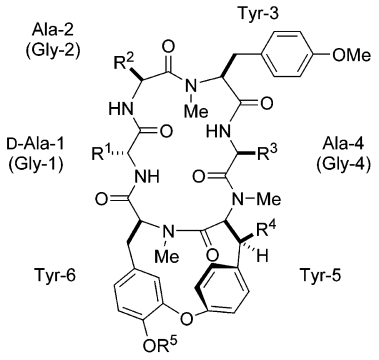
10.1021/jo030293p CCC: $27.50
Published on Web 02/03/2004

Synthesis of such analogues inevitably demands efficient preparation of a cycloisodityrosine unit of definite stereochemistry. However, all the methods reported for preparation of cycloisodityrosines are not satisfactory in this regard. They involve a multistep sequence of reactions with elaboration of unusual amino acids,[7b,c,12−15] and the reaction conditions usable for the formation of the diphenyl ether linkage and further transformations are strictly limited because of the easily epimerizable properties of the C-terminal residue of cycloisodityrosines. Thus, preparation of cycloisodityrosines in quantity has been an obstacle to the synthesis of RA-VII analogues with modified 18-membered rings.[10,16]

In the present study, first, we developed an easier and more efficient means for preparation of the protected cycloisodityrosines via partial degradation of natural RA-VII (**1**) and then by using this unit prepared three analogues of **1** with modified 18-membered rings, i.e., [Gly-1]RA-VII (**4**), [Gly-2]RA-VII (**5**), and [Gly-4]RA-VII (**6**). Their conformational properties in solution were studied by NMR analysis, and the relationship between the conformation and their cytotoxic activity on P-388 cells was discussed.

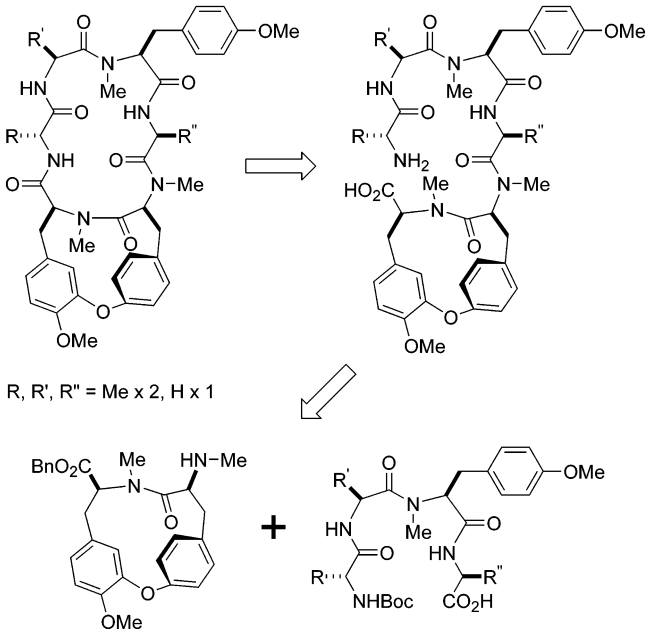


	R^1	R^2	R^3	R^4	R^5
RA-VII (**1**)	Me	Me	Me	H	Me
bouvardin (**2**)	Me	Me	Me	OH	H
deoxybouvardin (**3**)	Me	Me	Me	H	H
[Gly-1]RA-VII (**4**)	H	Me	Me	H	Me
[Gly-2]RA-VII (**5**)	Me	H	Me	H	Me
[Gly-4]RA-VII (**6**)	Me	Me	H	H	Me

Results and Discussion

Synthetic Plan for the Macrocycle. The synthetic approach to the present analogues **4**−**6** is illustrated

SCHEME 1



retrosynthetically in Scheme 1. Cyclization is generally the limiting step in the synthesis of cyclic peptides. The yield of cyclized products is known to depend strongly upon the sites involved in the cyclization. In all three reported RA-VII syntheses, the macrocyclization is performed between Tyr-6 and D-Ala-1 residues.[12a,13a,14c] Thus, in our present synthesis of these analogues, following their strategy we linked the cycloisodityrosine unit to the modified tetrapeptide fragments and subsequently performed macrocyclization by linking the Tyr-6 and D-Ala-1 (Gly-1) residues.[17]

Cycloisodityrosine from RA-VII. The procedure is shown in Scheme 2. The most abundant congeners of bicyclic hexapeptides of this series in *R. akane* and *R. cordifolia* are RA-VII (**1**) and deoxybouvardin (**3**), and **3** is easily converted into **1** by O-methylation. Therefore, the most readily available peptide **1** was used for the degradation study. To obtain cycloisodityrosines from peptide **1**, the peptide bonds between Ala-4 and Tyr-5 and between Tyr-6 and D-Ala-1 must be selectively

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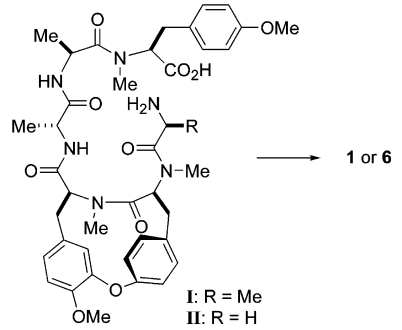
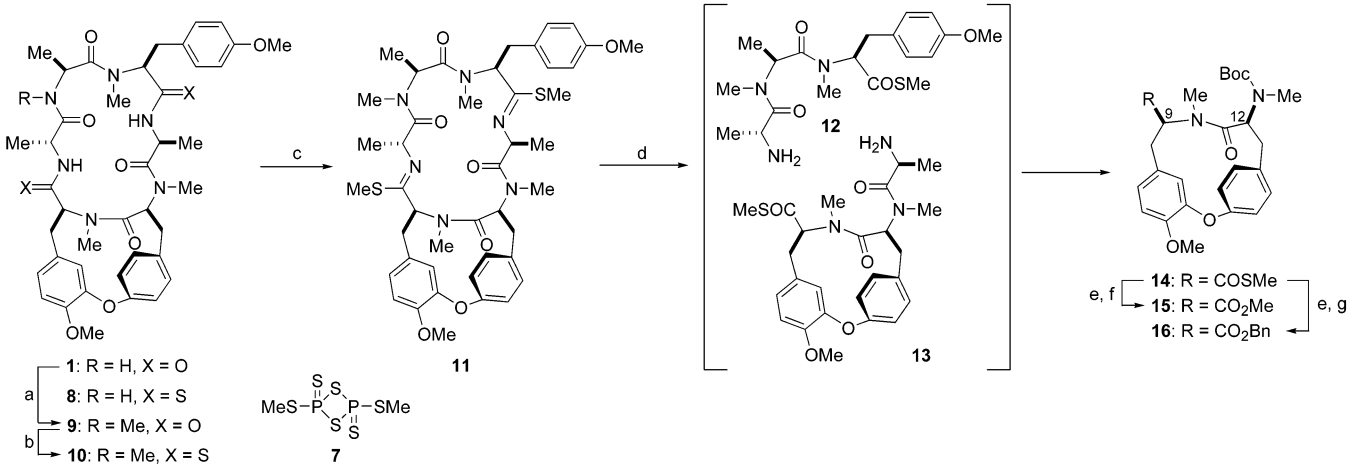
(17) To evaluate the efficacy of the macrocyclization at different locations, we also attempted the cyclization of peptide **I** by linking the Tyr-3 and Ala-4 residues in DMF by using the reagents A−D of Table 1. Reagents A, B, and D did not afford cyclized products, whereas reagent C [HBTU (4 equiv), HOBt (4 equiv), and DMAP (8 equiv)] gave **1** in 13% yield. By the same method, peptide **II** afforded **6** in 17% yield. The results indicated that cyclization at this position is feasible, but we did not consider that this strategy is more advantageous over the scheme described in the text.

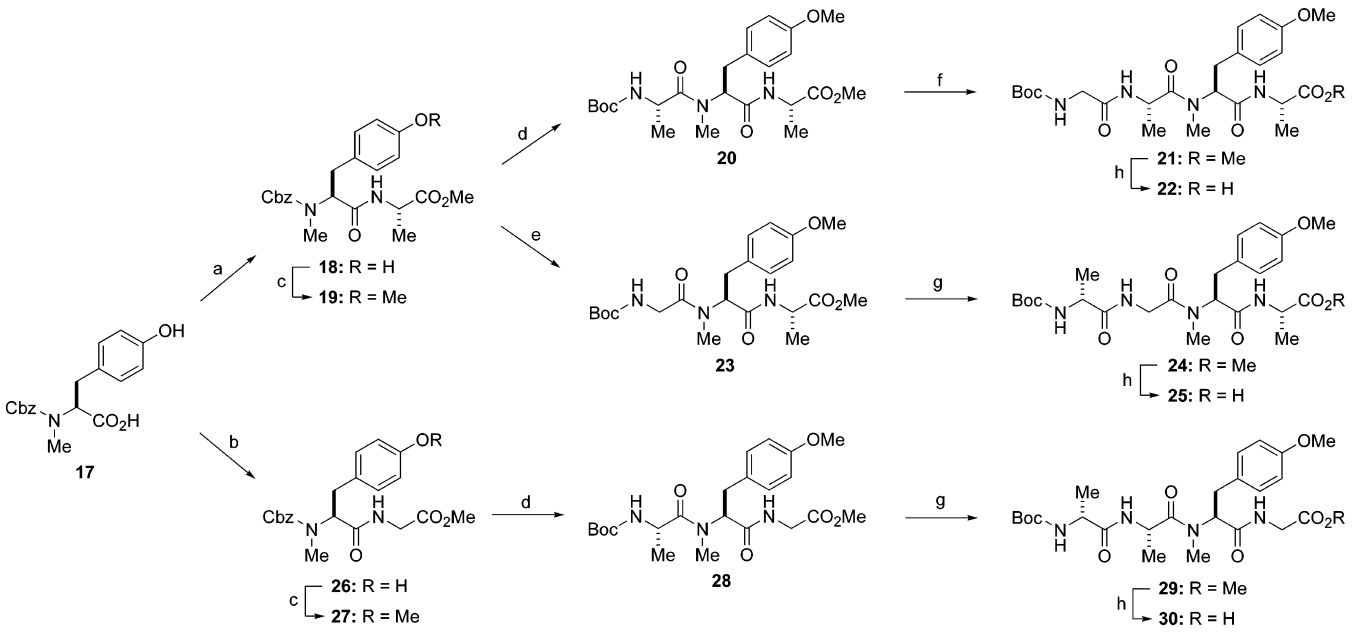


SCHEME 2[a]



[a] Reagents and conditions: (a) see ref 18; (b) **7**, dioxane, 91%; (c) MeI, K_2CO_3, acetone; (d) 6 M HCl, MeCN; neutralized with 1 M K_2CO_3; phenyl isothiocyanate; 6 M HCl, MeCN; neutralized with 1 M K_2CO_3; Boc_2O, 78% from **10**; (e) LiOH, H_2O_2, THF−H_2O; (f) (trimethylsilyl)diazomethane, MeCN−MeOH, 97% from **14**; (g) benzyl alcohol, DEAD, Ph_3P, THF, 90% from **14**.

SCHEME 3[a]



[a] Reagents and conditions: (a) H-Ala-OMe·HCl, EDC, HOBt, Et_3N, CH_2Cl_2, 71%; (b) H-Gly-OMe·HCl, EDC, HOBt, Et_3N, CH_2Cl_2, 79%; (c) CH_2N_2, Et_2O−MeOH, 100%; (d) H_2, Pd/C, HCl, MeOH; Boc-Ala-OH, PyBOP, *i*-Pr_2NEt, CH_2Cl_2, 88% for **20** and 73% for **28**; (e) H_2, Pd/C, HCl, MeOH; Boc-Gly-OH, PyBOP, *i*-Pr_2NEt, CH_2Cl_2, 96%; (f) TFA; Boc-Gly-OH, EDC, HOBt, CH_2Cl_2, 95%; (g) TFA; Boc-D-Ala-OH, EDC, HOBt, CH_2Cl_2, 84% for **24** and 79% for **29**; (h) LiOH, THF−MeOH−H_2O, 99% for **22**, 98% for **25** and 93% for **30**.

cleaved. Previously, we reported that, when treated with 2,4-bis(methylthio)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Davy-Reagent-Methyl) (**7**) at room temperature, peptide **1** gives bis(thioamide) **8** in 38% yield, with accompanying thioamides and other bis(thioamides).[8] Bis(thioamide) **8** should be a suitable substrate for the present degradation. However, even when the reaction conditions were modified, the yield of **8** was not successfully increased beyond 54%. On the other hand, when [*N*-methyl-Ala-2]RA-VII (**9**), which was readily prepared from peptide **1** in 97% yield,[18] was thionated by using the same procedure, bis(thioamide) **10** possessing thioamide bonds at the same positions as in **8** was produced in 91% yield. This marked enhancement of the yield of the bis(thioamide) may be attributed to the fact that the introduction of a methyl group into the Ala-2 amide nitrogen makes the D-Ala-1/Ala-2 peptide bond a less reactive tertiary amide and thus stabilizes the structure of **9**. As a result, the two reactive secondary amide carbonyls at Tyr-3 and Tyr-6 were selectively thionated to afford **10** in a high yield. Bis(thioamide) **10** was then converted into bis(thioimidate) **11** by using iodomethane and potassium carbonate, and the subsequent treatment of **11** with 6 M HCl in acetonitrile caused the cleavage

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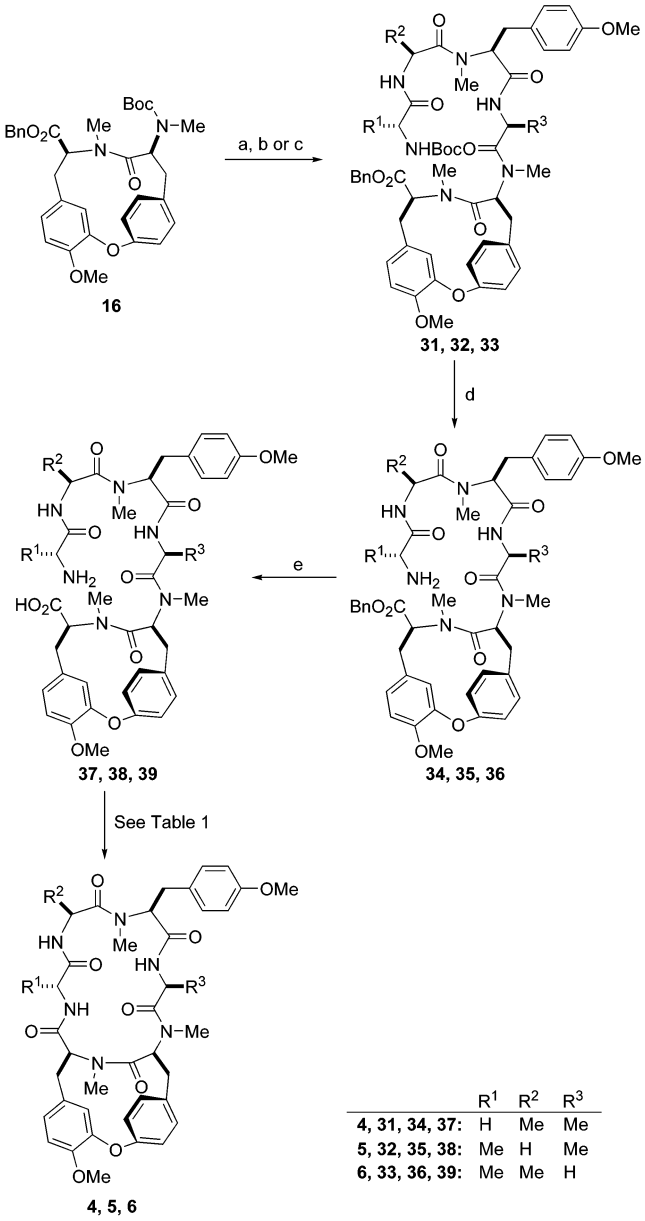
of the two thioimidate linkages to produce a mixture of two tripeptide segments **12** and **13**. Without separation, the mixture of **12** and **13** was subjected to Edman degradation with phenyl isothiocyanate. Treatment of the resultant crude thiourea with 6 M HCl and successive neutralization and N-protection using di-*tert*-butyl dicarbonate afforded cycloisodityrosine thioester **14** in 78% yield from bis(thioamide) **10**. In regard to the stereochemistry at the α-carbon of the C-terminal amino acid residue, the natural (9*S*,12*S*)-cycloisodityrosine esters are known to epimerize readily to become thermodynamically more stable (9*R*,12*S*)-cycloisodityrosine esters.[13c−e,14b] However, in thioester **14**, both of the stereocenters at 9 and 12 were confirmed to be *S* by X-ray crystallography.[19] When thioester **14** was hydrolyzed with lithium hydroxide in THF−H_2O, a considerable amount of epimerized acid contaminated the product to demonstrate facile epimerization of the compound. Accordingly, hydrolysis of **14** was performed with more nucleophilic and less basic lithium hydroperoxide prepared in situ. Subsequent treatment with (trimethylsilyl)diazomethane or benzylation using Mitsunobu conditions[20] afforded methyl ester **15** (97%) or benzyl ester **16** (90%), respectively, in an isomerically pure form. The spectroscopic data of **15** were in good agreement with those of the synthetic **15** reported.[13d,14b] The series of chemical conversions described in Scheme 2 proceeded efficiently, and the overall yields of cycloisodityrosines **15** and **16** from peptide **1** were 67% and 62%, respectively. In the subsequent reactions for the analogue syntheses, benzyl ester **16** was used.

Synthesis of Tetrapeptides. The tetrapeptide fragments of **4**, **5**, and **6**, each consisting of residues 1−4, were synthesized by the stepwise extension of Cbz-*N*-methyl-L-tyrosine (**17**)[12a,b] as shown in Scheme 3. For the preparation of the fragment for **4**, **17** was first coupled to L-alanine methyl ester and subsequently O-methylated with diazomethane to afford dipeptide **19** (Scheme 3). The Cbz protecting group was removed to give a less reactive dipeptide ester having an *N*-methyl group at the N-terminus, which was then coupled to Boc-L-alanine by using 1*H*-benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) to afford tripeptide **20** in good yield. Further elongation of the peptide chain of **20** at the N-terminus with Boc-glycine gave tetrapeptide **21**, which on treatment with lithium hydroxide provided acid **22**. The tetrapeptide fragments **25** and **30** for the synthesis of analogues **5** and **6**, respectively, were prepared analogously following the processes illustrated in Scheme 3 via tripeptides **23** and **28**, respectively.

Coupling Reaction of Tetrapeptide to Cycloisodityrosine Unit. The coupling reaction and subsequent macrocyclization are shown in Scheme 4. Tetrapeptides **22**, **25**, and **30**, obtained as described above, were then subjected to the coupling reaction with N-deprotected cycloisodityrosine derived from **16** by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt) (Scheme 4). When the tetrapeptides employed were **22** and **25**, a significant amount (28−34%)

SCHEME 4[a]



[a] Reagents and conditions: (a) TFA; **22**, EDC, HOObt, THF, 97% for **31**; (b) TFA; **25**, EDC, HOObt, THF, 79% for **32**; (c) TFA; **30**, EDC, HOAt, THF, 92% for **33**; (d) TFA, 95% for **34**, 98% for **35** and 94% for **36**; (e) H_2, Pd/C, EtOH.

of isomeric hexapeptides were produced, which were probably the epimers at the Ala-4 residue. This epimerization during the coupling process was effectively suppressed by using 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt) as the additive to afford desired hexapeptides **31** and **32** in good yields.

Cyclization of Hexapeptides. The macrocyclization of the cycloisodityrosine-containing linear peptides **37**, **38**, and **39**, obtained by debenzylation of **34**, **35**, and **36**, respectively, was performed in the presence of a coupling reagent (Scheme 4). The effects of the reagents diphenylphosphoryl azide (DPPA), pentafluorophenyl diphenylphosphinate (FDPP), *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and EDC on the macrocyclization were examined. All the

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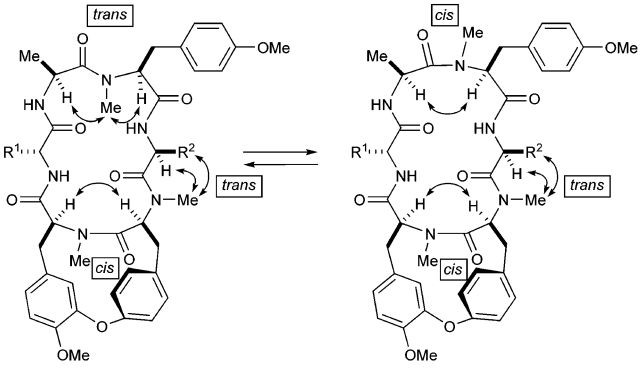
TABLE 1. Cyclization of 37−39[a]

entry	substrate	reagent[b]	solvent	product	yield[c] (%)
1	**37**	A	DMF	**4**	0
2	**37**	B	DMF	**4**	32
3	**37**	C	DMF	**4**	0
4	**37**	D	DMF	**4**	29
5	**37**	D	dioxane	**4**	18
6	**38**	A	DMF	**5**	0
7	**38**	B	DMF	**5**	0
8	**38**	C	DMF	**5**	0
9	**38**	D	DMF	**5**	10
10	**38**	D	dioxane	**5**	0
11	**39**	A	DMF	**6**	0
12	**39**	B	DMF	**6**	18
13	**39**	C	DMF	**6**	12
14	**39**	D	DMF	**6**	54
15	**39**	D	dioxane	**6**	0

[a] All reactions were carried out for 4 days at room temperature at a concentration of 0.0013 M. [b] Conditions: (A) DPPA (2 equiv), Et_3N (10 equiv); (B) FDPP (3 equiv), *i*-Pr_2NEt (3 equiv); (C) HBTU (4 equiv), HOBt (4 equiv), DMAP (8 equiv); (D) EDC (8 equiv), HOOBt (8 equiv). [c] Two-step yield from benzyl ester **34**, **35**, or **36**.

reactions were performed at room temperature for 4 days, the concentration of peptides being 0.0013 M. The results are summarized in Table 1. The results showed that the macrocyclization was to be governed by the coupling reagents and the solvent used. EDC generally favored the macrocyclization of this series, and when used in DMF it gave a yield of as high as 54% to **6** (entry 14), 29% to **4** (entry 4), and 10% to **5** (entry 9). The effect of solvent on the reaction may not be ignored. For macrocyclization of **38** and **39**, EDC in dioxane gave no positive result, but for the reaction of **37** the combination produced the desired product (entries 5, 10, and 15). FDPP in DMF also worked as a reagent for macrocyclization (entries 2 and 12). The most commonly used coupling reagent for macrocyclization of linear peptides, DPPA in DMF, which was used in the synthesis of RA-VII (**1**),[13a,14c] bouvardin (**2**),[13b] and some related analogues,[7b,10,16] apparently did not work on peptides **37**, **38**, and **39** (entries 1, 6, and 11). HBTU appeared to be unsuitable for the cyclization of these peptides either; only **39** cyclized to **6** in 12% yield (entries 3, 8, and 13).

Solution Conformation of Analogues 4−6. The biological activity is considered to be related to the conformation of molecules in solution. Therefore, we studied the conformer structures of those peptide analogues in solution by NMR spectroscopy to examine the effect of each alanyl methyl group of the peptide molecule. The results were compared with those of the parent active peptide **1**, whose solution form is well studied. The most distinctive features which characterize the conformation of cyclic peptides are their backbone amide configurations. Peptide **1** possesses three modified *N*-methyltyrosines at residues 3, 5, and 6, and the amide bonds between Ala-2/Tyr-3, Ala-4/Tyr-5, and Tyr-5/Tyr-6 can be either cis or trans. Peptide **1** is known to adopt two stable conformations in $CDCl_3$ and CD_3OD solutions in ratios of 89:11 and 84:16, respectively. The major conformer 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, whereas the minor conformer t-c-t-t-c-t configurations, as characterized by



compd	R^1	R^2	solvent	population	
				t-t-t-t-c-t	t-c-t-t-c-t
RA-VII (**1**)	Me	Me	$CDCl_3$	89%	11%
RA-VII (**1**)	Me	Me	CD_3OD	84%	16%
[Gly-1]RA-VII (**4**)	H	Me	$CDCl_3$	89%	11%
[Gly-4]RA-VII (**6**)	Me	H	CD_3OD	28%	72%

FIGURE 1. Conformer population and key sequential NOESY/ROESY correlations commonly observed in **1**, **4**, and **6** in solution.

X-ray crystallography, NOESY experiments, and computational methods.[21]

The ^1H NMR spectrum of **4** in $CDCl_3$ was very similar to that of peptide **1**. It demonstrated the presence of two conformers in a ratio of 89:11 at 300 K, and their structures were analyzed by the ROESY spectra. The major conformer showed ROESY correlations between Ala-2 H_α/Tyr-3 NMe, Tyr-3 NMe/Tyr-3 H_α, Ala-4 H_α/Tyr-5 NMe, Ala-4 Me/Tyr-5 NMe, and Tyr-5 H_α/Tyr-6 H_α, whereas the minor one between Ala-2 H_α/Tyr-3 H_α, Ala-4 H_α/Tyr-5 NMe, Ala-4 Me/Tyr-5 NMe, and Tyr-5 H_α/Tyr-6 H_α (Figure 1). These correlations demonstrated that the major conformer of analogue **4** possessed t-t-t-t-c-t amide configurations as the major conformer in peptide **1** and that the minor one in **4** had t-c-t-t-c-t configurations as the minor conformer in **1**. In addition to the above ROESY correlations, the very similar chemical shifts of the proton signals in the spectrum of **1** and that of **4** indicated that the structures of these two conformers in the two peptides were almost identical.

In the case of analogue **5**, the ^1H NMR signals in $CDCl_3$ and DMSO-d_6 at 300 K and at higher temperatures were too broad to allow reliable conformational analysis. At a lower temperature (283 K), the ^1H NMR spectrum was very complex, suggesting that it was a mixture of three or more conformers. Thus, the conformational analysis of **5** by NMR studies was not possible in the present experiment, and we concluded that analogue **5** existed in a mixture of three or more unstable conformers in solution.

NMR spectra of analogue **6** were studied in CD_3OD because of its poor solubility in $CDCl_3$. In this solvent, analogue **6** was noted to consist of two conformers in a ratio of 72:28 at 300 K. The NOESY correlations demonstrated that the major conformer of **6** had t-c-t-t-c-t amide configurations and was identical to the minor conformer of **4** or **1** and that the minor conformer of **6** having t-t-t-t-c-t amide configurations was identical to

(21) Morita, H.; Kondo, K.; Hitotsuyanagi, Y.; Takeya, K.; Itokawa, H.; Tomioka, N.; Itai, A.; Iitaka, Y. *Tetrahedron* **1991**, *47*, 2757−2772.

TABLE 2. Cytotoxicity of RA-VII (1) and Its Analogues 4−6 against P-388 Leukemia Cells

compd	IC_{50} (μg/mL)
RA-VII (**1**)	0.0038
[Gly-1]RA-VII (**4**)	0.014
[Gly-2]RA-VII (**5**)	0.032
[Gly-4]RA-VII (**6**)	0.16

the major conformer of **4** or **1** (Figure 1). The similarity of the chemical shifts of proton signals for each conformer positively supported that the major conformer in peptide **6** was almost identical to the minor conformer of **1** and the minor conformer in **6** was the major conformer of **1**.

Cytotoxicity of Analogues 4−6. Cytotoxicity of analogues **4**−**6** was evaluated by using P-388 murine leukemia cells, and the results are shown in Table 2. RA-VII (**1**) was also assayed for comparison (Table 2). Of those three glycine analogues, analogue **4** was the most active, which may be due to its having the conformational features very similar to those of **1**. Both of the two conformers of **6** were found in **1** and **4**, yet it was 11 times less cytotoxic than **4**. It may support the suggestion that the major conformer of **1** and **4** or the minor one of **6** having t-t-t-t-c-t amide configurations is the bioactive conformer of this peptide series.[22] Thus, the reduced activity of analogue **6** may be due to the low population of this active conformer. A difference is noted between the cytotoxic activities of **1** and **4**, though their bioactive conformer structures are about the same. No further investigation was performed as to the cause of difference in cytotoxic activity, but the alanyl methyl group at 1 may play some role in the interaction with 80S ribosomes.

(22) Itokawa, H.; Kondo, K.; Hitotsuyanagi, Y.; Isomura, M.; Takeya, K. *Chem. Pharm. Bull.* **1993**, *41*, 1402−1410.

Conclusion

We prepared three analogues **4**−**6** of RA-VII (**1**), in which one of the three alanine residues was replaced by glycine, and demonstrated the possible role of the methyl groups in the three alanine residues upon their conformational features and accordingly upon the cytotoxic activity. The methyl group of D-Ala-1 residue apparently affects neither the backbone structure nor the population of the conformers in solution, whereas the methyl group of Ala-2 residue seemed to be essential for maintaining the stable conformation. The methyl group of Ala-4 residue does not affect the backbone structures of the conformers, but affects the ratio of those conformers. Thus, the presence of the methyl groups of both Ala-2 and Ala-4 residues is necessary for producing the conformational property of RA-VII (**1**) responsible for the activity.

Supporting Information Available: Experimental details, ^{1}H NMR spectra of **4**−**6**, **10**, **14**−**16**, and **18**−**36**, and X-ray data of **14**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO030293P