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RA-III Lactone, a 19-Membered Ring Analogue of RAs, Antitumor Cyclic Hexapeptide

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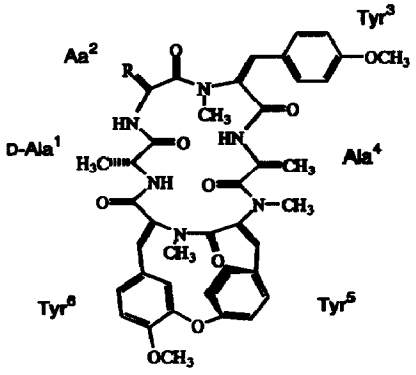
Key Words: RA; cyclic hexapeptide; N→O acyl rearrangement; conformation analysis; antitumor activity

Abstract: Antitumor cyclic hexapeptide RA-III (2) has been converted to a lactone analogue 3 which is the first analogue of RAs in which the 18-membered macro ring was modified. 3 was found to possess promising antitumor activity, and its solution conformation was established by NMR spectroscopy.

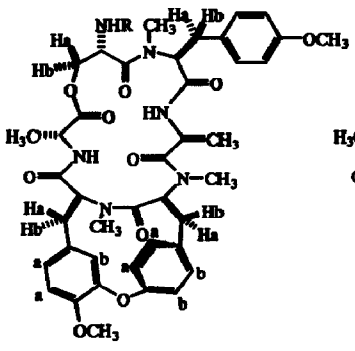
The RAs are a family of cyclic hexapeptides isolated from the roots of *Rubia akane* and *R. cordifolia* (Rubiaceae).[1] They exhibit promising antitumor activity against solid tumor cells, and RA-VII (1), one of the most potent congeners, is now under clinical trial in Japan as an anticancer agent.[2]

Because of chemically less accessible highly strained 14-membered ring structure incorporating isodityrosine unit and lack of suitable foothold for manipulation in 1, its structure-activity relationship study has been restricted to the aromatic ring substituents,[3] 2nd amino acid side chain, or rather simple isodityrosine mimics.[4] Natural RAs adopt two or three conformational states in solution,[5] which also hampered the determination of the bioactive conformation. In our way to clarify the relationship between the conformation and activity, we envisaged to modify the 18-membered macro ring structure of RAs without changing the configuration of the constituting amino acid residues.

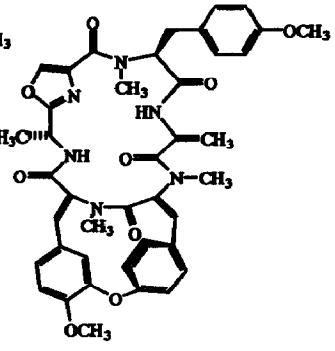
We chose RA-III (2), a minor congener of RAs, as a possible precursor for these modifications since 2 incorporates serine residue at position 2. Open chain peptides containing a β-hydroxy amino acid residue such as serine or threonine are readily susceptible to N→O acyl rearrangement under acidic conditions.[6] However, attempting this rearrangement for 2 using various acids (e.g. $BF_3 \cdot OEt_2$, HCl or H_2SO_4) was ineffective or caused decomposition, and only refluxing trifluoroacetic acid treatment for 96 h afforded desired RA-III lactone (3) in low yield (14%). The reluctance of the rearrangement could be attributed to the rigid framework of 2, which restricted adoption of a suitable conformation for the Ser^2 hydroxyl attacking to the D-Ala^1 carbonyl.[7] This conversion was ameliorated by the following procedures. Under the Mitsunobu conditions[8] (Ph_3P, DEAD, CH_2Cl_2, R.T., 48 h), 2 was converted to oxazoline 4[9] in 98% yield. Trifluoroacetic acid treatment of 4 at R.T. for 2 h smoothly opened the oxazoline ring, and successive neutralization with aq. $NaHCO_3$ afforded 3 as an amorphous solid in 89% yield. Structures of 3 and 4 were confirmed by IR, HR-FAB mass spectra and unambiguous assignments of their all proton and carbon resonances using a combination (H-H COSY, NOESYPH, HMBC and HMQC) of 2D NMR techniques.[10]



1: R = CH_3
2: R = CH_2OH



3: R = H
5: R = Ac



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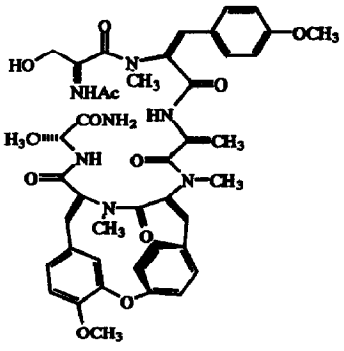

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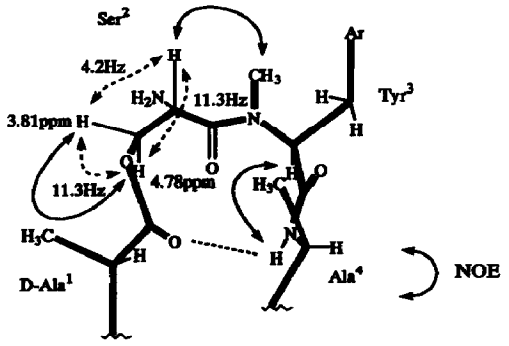

Fig.1. Selected NOESY correlations, chemical shifts and coupling constants of 3 in $CDCl_3$ at 303K.

Table 1. Cytotoxicity of Compounds 1, 2, 3 and 6 Against P388 and KB Cells.

#	IC_{50}(μg/ml) P388	KB
1	0.0013	0.0023
2	0.011	0.024
3	0.019	0.027
6	>10	>10

Table 2. Antitumor Activity of Compounds 1 and 3 Against P388 Leukemia in Mice.

# / dose[a]	T/C (%) 0.2	0.8	3.13	6.25	12.5	25.0
1	142	144	163	toxic		
3	129	133	158		176	170

[a]Dose administered i.p. on days 1-5 (mg/kg/day).

400 MHz ^{1}H-NMR spectrum showed that **3** adopts single conformational state in various solvents ($CDCl_3$, CD_3OD and DMSO-d_6), which enabled us to verify the solution conformation. ^{1}H and ^{13}C-NMR parameters of **3** including NOESYPH correlations around 14-membered cycloisodityrosine moiety are quite similar to those of **1** and **2**, suggesting little or no conformational change in this region, but 19-membered ring structure was altered by ring expansion. A large difference (Δ 0.97 ppm in $CDCl_3$) of chemical shifts between Ser^2 Hβ geminal protons suggested conformational homogeneity around this region.[11] One of these protons assignable to *proR* Hβ was coupled to vicinal Hα in 11.3 Hz, showing an *anti* relation (Fig. 1). Ser^2 Hα was strongly correlated to Tyr^3 NCH_3 in NOESYPH spectrum, which revealed the presence of a *trans* amide bond between Ser^2 and Tyr^3. The orientation of this *trans* amide bond is unique since it is reverse to that of natural RAs, Ala^2 CO pointing upside of the molecule. This structure well explains the low field shift of Tyr^3 Hα (2.0 ppm relative to that of **1**) and Ser^2 *proR* Hβ protons considering diamagnetic anisotropy of the Ser^2 carbonyl.

Internal hydrogen bond in peptides is important factor on characterizing the backbone conformation and its rigidity.[12] The temperature coefficients of the amide protons in DMSO-d_6 solution indicate the presence of two internal hydrogen bonds, between D-Ala^1 NH and Ala^4 CO ($\Delta\delta/\Delta T$=4.45x10^{-3} ppm/K); and Ala^4 NH and D-Ala^1 CO (2.01x10^{-3} ppm/K) in **3**. The latter strong bonding is also supported by small solvent induced shift of Ala^4 NH ($\delta_{DMSO\text{-}d6}$ - δ_{CDCl3}=-0.08 ppm) and calculated torsion angle of 137° for Tyr^3 C*H*-Ala^4 *NH* (J = 6.3 Hz) using modified Karplus equations,[13] indicative of suitable orientation of NH bond for the bonding.

Treatment of **3** with Ac_2O and pyridine at R.T. gave acetamide **5**[14] in 88% yield, which was subjected to ammonolysis (28% aq. NH_3-dioxane, 80°C, 12 h, scaled tube) to afford seco derivative **6**[15] in 50% yield.

The lactone **3** retained cytotoxicity against P388 and KB cells and expressed more promising in vivo anti-P388 activity than that of **1** or **2** in terms of T/C values, while seco derivative **6** showed no activity (Tables 1 and 2). The loss of activity in **6** can be interpreted as its no or little, if any, ability to adopt the bioactive conformation. Recently Boger et al suggested that in RAs 14-membered cycloisodityrosine moiety is a pharmacophore to express the activity.[16] However these results and recent our observations that substitution of the Tyr^3 methoxyl group of RAs with hydrogen (-H) or hydroxyl group (-OH) profoundly reduced (~1/100) or abolish activity in spite of no conformational change among those molecules[3b] demonstrate that *18- or 19-membered macro ring structures incorporatning modified O-methyltyrosine unit with particular conformation(s) are also important for the activity.* Further studies on the backbone modifications of RAs to determine the structural requirements for the activity and elucidate the bioactive conformation are currently undergoing in our laboratory.

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9. 4: A colorless powder. mp. 220-221°C; $[\alpha]_D$ -106.0° (*c*, 0.15, $CHCl_3$); HR-FABMS Calcd. for $C_{41}H_{49}N_6O_9[M+H]^+$:769.3561, Found:769.3655; ^{13}C-NMR (100 MHz, $CDCl_3$, major conformer, δ): 66.08, 69.53, 169.47 (oxazloine).
10. 3 is fairly stable under the neutral conditions. Heating DMSO-d_6 solution of 3 at 100°C for 2 h caused no detectable change in ^{1}H-NMR spectrum. Physical and spectral data for 3: $[\alpha]_D$ -207.9° (*c* 0.17, $CHCl_3$); HR-FABMS Calcd. for $C_{41}H_{51}N_6O_{10}[M+H]^+$:787.3667, Found: 787.3651; IR ν_{max} ($CHCl_3$): 3402, 1685, 1641 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.33 (3H, d, 7.0 Hz, D-Ala^{1}β), 1.37 (3H, d, 7.0 Hz, Ala^{4}β), 2.59 (3H, s, Tyr^{6}NMe), 2.67 (1H, dd, 11.5, 3.4 Hz, Tyr^{5}β$_a$), 2.86 (1H, dd, 14.7, 6.8 Hz, Tyr^{3}β$_{a\ or\ b}$), 2.96 (3H, s, Tyr^{3}NMe), 3.02-3.12 (2H, m, Tyr^{6}β$_{a,b}$), 3.19 (3H, s, Tyr^{5}NMe), 3.32 (1H, dd, 14.7, 9.0 Hz, Tyr^{3}β$_{b\ or\ a}$), 3.57 (1H, t, 11.5 Hz, Tyr^{5}β$_b$), 3.76 (3H, s, Tyr^{3}OMe), 3.81 (1H, dd, 11.3, 4.2 Hz, Ser^{2}β$_a$), 3.94 (3H, s, Tyr^{6}OMe), 3.99 (1H, dd, 11.3, 4.2 Hz, Ser^{2}α), 4.14 (1H, qd, 7.0, 4.9 Hz, Ala^{1}α), 4.35-4.40 (1H, m, Tyr^{6}α), 4.37 (1H, d, 1.7 Hz, Tyr^{6}δ$_b$), 4.69 (1H, qd, 7.0, 6.3 Hz, Ala^{4}α), 4.78 (1H, t, 11.3 Hz, Ser^{2}β$_b$), 5.29 (1H, dd, 11.5, 3.4 Hz, Tyr^{5}α), 5.59 (1H, dd, 9.0, 6.8 Hz, Tyr^{3}α), 6.18 (1H, d, 4.9 Hz, Ala^{1}NH), 6.60 (1H, dd, 8.4, 1.7 Hz, Tyr^{6}δ$_a$), 6.80 (2H, d, 8.5 Hz, Tyr^{3}ε), 6.82 (1H, d, 8.4 Hz, Tyr^{6}ε$_a$), 6.92 (1H, dd, 8.4, 2.4 Hz, Tyr^{5}ε$_a$), 7.05 (1H, d, 6.3 Hz, Ala^{4}NH), 7.14 (2H, d, 8.5 Hz, Tyr^{3}δ), 7.23 (1H, dd, 8.3, 2.4 Hz, Tyr^{5}ε$_b$), 7.28 (1H, dd, 8.4, 2.1 Hz, Tyr^{5}δ$_a$), 7.42 (1H, dd, 8.3, 2.1 Hz, Tyr^{5}δ$_b$); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): D-Ala^1 (19.27β, 49.43α, 172.52$_{co}$), Ser^2 (48.82α, 67.40β, 174.20co), Tyr^3 (30.41$_{NMe}$, 32.46β, 55.27$_{OMe}$, 57.14α, 113.82ε, 129.16γ, 130.15δ, 158.39ζ, 168.49$_{co}$), Ala^4 (18.58β, 46.74α, 171.33$_{co}$), Tyr^5 (30.50$_{NMe}$, 36.76β, 53.24α, 124.56$_{ea}$, 126.11$_{eb}$, 130.94$_{δb}$, 132.90$_{δa}$, 134.68γ, 158.30ζ, 170.47$_{co}$), Tyr^6 (28.70$_{NMe}$, 34.28β, 56.27$_{OMe}$, 58.03α, 112.52$_{ea}$, 113.68$_{δb}$, 120.66$_{δa}$, 127.98γ, 146.54ζ, 153.32$_{eb}$, 170.47$_{co}$).
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14. 5: An amorphous solid. $[\alpha]_D$ -264.3° (*c*, 0.03, $CHCl_3$); FABMS: 828 (M^+); ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.88 (3H, s, $COCH_3$), 5.83 (1H, d, 8.4Hz, NHAc).
15. 6: An amorphous solid. $[\alpha]_D$ -176.6° (*c*, 0.13, $CHCl_3$); HR-FABMS Calcd. for $C_{43}H_{56}N_7O_{11}[M+H]^+$:846.4038, Found: 846.4066 ^{1}H-NMR (400 MHz, CD_3OD, major conformer, δ): 1.23 (3H, d, 7.1 Hz, Ala^{4}β), 1.27 (3H, d, D-Ala^{1}β), 1.94 (3H, s, $COCH_3$), 2.62 (3H, s, Tyr^6$_{NMe}$), 2.84 (1H, dd, 11.5, 3.2 Hz, Tyr^{5}β$_b$), 2.96 (1, dd, 15.0, 10.8 Hz, Tyr^{3}β$_a$), 3.06 (1H, dd, 10.0, 5.7 Hz, Tyr^{6}β$_a$), 3.12 (3H, s, Tyr^3$_{NMe}$), 3.15 (1H, dd, 10.0, 3.1 Hz, Tyr^{6}β$_b$), 3.23 (3H, s, Tyr^5$_{NMe}$), 3.43 (1H, dd, 15.0, 5.2 Hz, Tyr^{3}β$_b$), 3.59 (1H, t, 11.5 Hz, Tyr^{5}β$_b$), 3.74-3.81 (2H, m, Ser^{2}β), 3.78 (3H, s, Tyr^3$_{OMe}$), 3.93 (3H, s, Tyr^6$_{OMe}$), 4.44 (1H, q, 7.0 Hz, Ala^{1}α), 4.57-4.66 (2H, m, Ala^{4}α and Tyr^{6}δ$_b$), 5.07 (1H, t, 7.8 Hz, Ser^{2}α), 5.46-5.52 (2H, m, Tyr^{3}α and Tyr^{5}α), 6.70-6.72 (1H, m, Ala^4$_{NH}$), 6.71 (1H, dd, 8.3, 1.8 Hz, Tyr^{6}δ$_a$), 6.82 (1H, dd, 8.3, 2.4 Hz, Tyr^{5}ε$_a$), 6.85 (2H, d, 8.7 Hz, Tyr^{3}ε), 6.94 (1H, d, 8.3 Hz, Tyr^{6}ε), 7.16 (2H, d, 8.7 Hz, Tyr^{3}δ), 7.27 (1H, dd, 8.4, 2.4 Hz, Tyr^{5}ε$_b$), 7.28 (1H, dd, 8.3, 2.2 Hz, Tyr^{5}δ$_a$), 7.53 (1H, dd, 8.4, 2.2 Hz, Tyr^{5}δ$_b$).
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