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STUDIES ON THE ANTITUMOR CYCLIC HEXAPEPTIDES OBTAINED FROM RUBIAE RADIX[1)]

Hideji Itokawa,*,[a] Koichi Takeya,[a] Kazuhiko Mihara,[a] Noboru Mori,[b] Toshinori Hamanaka,[b] Toru Sonobe,[b] and Yoichi Iitaka[c]

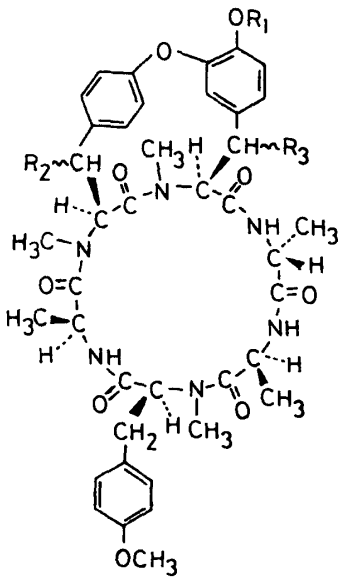
Tokyo College of Pharmacy,[a] Horinouchi 1432-1, Hachioji, Tokyo 192-03, Tobishi Pharmaceutical Co.,Ltd.,[b] Yurakucho 1, Chiyoda-ku, Tokyo 100 and Faculty of Pharmaceutical Science,[c] Univ. of Tokyo, Bunkyo-ku, Tokyo 113

Antitumor cyclic hexapeptides named RA-VII, V, IV and III were isolated from the MeOH extracts of Rubia cordifolia and R.akane.

KEYWORDS ——— Rubia cordifolia; Rubia akane; Rubiaceae; RA-VII; RA-V; RA-IV; RA-III; cyclic hexapeptide; antitumor activity

In the course of a continuing search for antitumor substances from crude drugs and collected plants,[2)] we found that the methanolic extract prepared from the roots of Rubia cordifolia L. (Rubiaceae) showed a significant antitumor activity against Sarcoma 180 ascites and P-388 leukemia in mice.[3)] In this paper, we present the isolation and structural elucidation of the active materials.

When the methanolic extract was fractionated by partitioning between benzene and water and then between ethyl acetate and water, the antitumor activity was concentrated in both the benzene and ethyl acetate extracts. The benzene extract was subjected to various kinds of column chromatography on Amberlite XAD-2, silica gel, Sephadex LH-20 and droplet counter-current chromatography (DCC), and was fractionated with the guidance of bioassay against Sarcoma 180A and P-388 leukemia in mice. Final purification of the active fraction with RP-18 Lobar column using the solvent system of MeOH-H_2O (4:1) gave four compounds RA-VII, RA-V, RA-IV and RA-III which showed an inhibitory activity against P-388 leukemia in mice. The active antitumor constituents of the ethyl acetate extract were also composed of the above compounds. From 220 kg of dried roots of R.cordifolia, 17.6 g of RA-VII, 10.4 g of RA-V, 42 mg of RA-IV and 60 mg of RA-III were isolated.



RA-VII : R_1=CH$_3$, R_2=R_3=H RA-IV : R_1=CH$_3$, R_2=H, R_3=OH
RA-V : R_1=R_2=R_3=H RA-III: R_1=CH$_3$, R_2=OH, R_3=H
RA-V-23: R_1=Ac, R_2=R_3=H

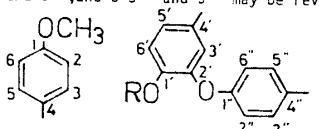
Fig.1. Structures of Cyclic Peptides from Rubia cordifolia
* RA-V-23 was obtained by the acetylation of RA-V.

RA-VII, colorless needles, mp > 300°C (from MeOH), $[\alpha]_D^{21}$ -229° (c=0.1 in $CHCl_3$), UV λ_{max}^{EtOH} nm(ε): 276(3800), 282(3200) had the molecular formula $C_{41}H_{50}O_9N_6$ (high MS m/z: Calcd 770.3635, Found 770.3588. Anal.Calcd for $C_{41}H_{50}O_9N_6 \cdot H_2O$: C, 62.42;

Table I. ^{13}C Chemical Shifts of Cyclic Peptides from Rubia cordifolia

assign-ments	RA-VII	RA-V	RA-V-23	RA-IV	RA-III
C-Me	16.61(q)	16.49(q)	16.49(q)	16.89(q)	18.57(q)
C-Me	18.51(q)	18.45(q)	18.45(q)	18.22(q)	20.93(q)
C-Me	20.76(q)	20.70(q)	20.70(q)	20.70(q)	29.35(q)*
Ac-Me	-	-	20.70(q)	-	-
N-Me	29.35(q)	29.41(q)	29.41(q)	30.44(q)	29.58(q)*
N-Me	30.56(q)	30.50(q)	30.50(q)	30.96(q)	30.50(q)
$-CH_2-$	32.63(t)	32.63(t)	32.63(t)	32.75(t)	32.58(t)
$-CH_2-$	35.57(t)	35.57(t)	35.86(t)	-	35.52(t)
$-CH_2-$	36.96(t)	36.84(t)	36.90(t)	36.90(t)	-
N-Me	39.84(q)	39.78(q)	39.78(q)	39.73(q)	40.13(q)
N-CH-CO	44.51(d)	44.57(d)	44.45(d)	44.40(d)	49.59(q)
N-CH-CO	46.36(d)	46.41(d)	46.30(d)	46.59(d)	46.24(d)
N-CH-CO	47.74(d)	47.68(d)	47.68(d)	47.80(d)	47.68(d)
N-CH-CO	54.31(d)	54.31(d)	54.26(d)	54.26(d)	54.26(d)
O-Me	55.24(q)	55.18(q)	55.18(q)	55.24(q)	55.18(q)
O-Me	56.16(q)	-	-	56.10(q)	56.10(q)
N-CH-CO	57.37(d)	57.43(d)	57.25(d)	73.34(d)	57.31(d)
N-CH-CO	68.27(d)	68.27(d)	68.21(d)	68.15(d)	68.67(d)
-CH(OH)-	-	-	-	62.85(d)	61.58(d)
C-1	158.32(s)	158.27(s)	158.27(s)	158.27(s)	158.32(s)
C-2	113.99(d)	113.93(d)	113.93(d)	113.99(d)	114.10(d)
C-3	130.19(d)	130.19(d)	130.13(d)	130.19(d)	130.13(d)
C-4	130.65(s)	130.54(s)	130.59(s)	130.65(s)	130.42(s)
C-5	130.19(d)	130.19(d)	130.13(d)	130.19(d)	130.13(d)
C-6	113.99(d)	113.93(d)	113.93(d)	113.99(d)	114.10(d)
C-1'	153.02(s)	151.17(s)	137.11(s)	153.08(s)	153.02(s)
C-2'	146.45(s)	143.05(s)	154.98(s)	148.41(s)	146.51(s)
C-3'	113.36(d)	115.95(d)	114.33(d)	114.28(d)	113.47(d)
C-4'	128.17(s)	127.42(s)	134.17(s)	132.09(s)	128.12(s)
C-5'	120.91(d)	121.54(d)	121.08(d)	122.52(d)	120.97(d)
C-6'	112.32(d)	113.18(d)	122.81(d)	112.26(d)	112.37(d)
C-1"	158.15(s)	157.92(s)	157.92(s)	158.91(s)	158.21(s)
C-2"	124.14(d)	124.08(d)	123.96(d)	123.96(d)	124.20(d)
C-3"	130.94(d)	130.94(d)	130.88(d)	129.85(d)	130.82(d)
C-4"	135.21(s)	135.50(s)	135.44(s)	135.04(s)	135.04(s)
C-5"	132.73(d)	132.90(d)	132.78(d)	133.77(d)	132.73(d)
C-6"	125.87(d)	125.81(d)	125.63(d)	125.98(d)	125.92(d)
C=O	168.07(s)	168.13(s)	168.01(s)	168.13(s)	167.84(s)
Ac-C=O	-	-	168.76(s)	-	-
C=O	169.28(s)	169.05(s)	169.11(s)	169.11(s)	169.11(s)
C=O	170.60(s)	170.55(s)	170.32(s)	169.75(s)	170.72(s)
C=O	171.65(s)	171.59(s)	171.59(s)	171.18(s)	171.30(s)
C=O	172.16(s)	172.22(s)	172.11(s)	172.28(s)	171.53(s)
C=O	172.45(s)	172.51(s)	172.39(s)	172.45(s)	172.51(s)

The measurements were made on a JEOL FX-100 spectrometer in $CDCl_3$ with TMS as an internal reference and are expressed in terms of ppm. The assignments of * marks, C-1 and 1", C-2" and 6", and C-3" and 5" may be reversed.



RA-VII : R=CH_3
RA-V : R=H
RA-V-23 : R=$COCH_3$
RA-IV : R=CH_3
RA-III : R=CH_3

Fig.2. Numbering System of Aromatic Carbons of Cyclic Peptides

H, 6.64; N, 10.65. Found: C, 62.39; H, 6.56; N, 10.98) and was assumed to be peptide from the IR spectrum ($\nu_{max}^{KBr}cm^{-1}$: 3380(NH), 1640(amide C=O)). In the ^1H-NMR spectrum, three methyl groups adjoining to methine at δ 1.09, 1.30 and 1.36(3H,d,J=7Hz,respectively), three N-methyl groups at δ 2.70, 2.87 and 3.13 (3H,s, resp.), two O-methyl groups at δ 3.79 and 3.94 (3H,s, resp.) and aromatic protons at δ 6.5-7.5 were observed. Also, as can be seen from Table I, the carbon signals due to three C-methyl, three methylene, three N-methyl, two O-methyl, six methine, six carbonyl and eighteen aromatic carbons (eleven tertiary and seven quarternary carbons) appeared in the ^{13}C-NMR spectrum.

RA-V, colorless powder, mp > 300°C (from MeOH), $[\alpha]_D^{21}$ -225° (c=0.3 in $CHCl_3$), UV $\lambda_{max}^{EtOH}nm(\varepsilon)$: 276(3800), 282 (3300) had the molecular formula $C_{40}H_{48}O_9N_6$ (high MS m/z: Calcd 756.3479, Found 756.3458. Anal.Calcd for $C_{40}H_{48}O_9N_6 \cdot 1\frac{1}{4}H_2O$: C, 61.64; H, 6.35; N, 10.78. Found: C 61.66; H, 6.28; N, 10.73). The IR spectrum of RA-V was similar to that of RA-VII. RA-V was acetylated to give mono-acetate (RA-V-23), colorless needles, mp 228-241°C (from MeOH), $[\alpha]_D^{21}$ -190° (c=0.26 in $CHCl_3$), UV $\lambda_{max}^{EtOH}nm(\varepsilon)$: 274(3400), 278(3400), 282(2500), MS m/z: 798(M^+), Anal.Calcd for $C_{42}H_{50}O_{10}N_6 \cdot 1\frac{1}{2}H_2O$: C, 61.07; H, 6.46; N, 10.17. Found: C, 61.07; H, 6.37; N, 10.00. RA-V was allowed to react with diazomethane to furnish RA-VII. Consequently, it was evident that one of two methoxyl groups in RA-VII was demethylating in RA-V. This conclusion was also supported by the fact that O-methyl signals at δ 3.94 and 56.16 in the ^1H- and ^{13}C-NMR spectra of RA-VII disappeared in those of RA-V.

In order to confirm the substituted mode of three benzene rings, which were presumed on the basis of eighteen aromatic carbon signals in the ^{13}C-NMR spectra of RA-VII, RA-V and RA-V-23, these chemical shifts were compared with the calculated chemical shifts obtained from the formula of empirical substituent increment in benzene derivates.[4] From the above result, the partial structure expressed in Fig.2 was derived and each aromatic carbon was assigned as shown in Table I. Next, in order to identify three kinds of N-methylamino acid, the hydrolysis of

RA-VII was carried out with 6N HCl for 24 hours at 100°C. When the reaction mixture was developed with 0.25 mm cellulose TLC plates using the solvent system of n-BuOH-AcOH-H_2O (4:1:1.7), five spots color-produced with ninhydrin were observed, Rf values: 0.33(K-1), 0.45(K-2), 0.56(K-3), 0.61(K-4) and 0.69(K-5). Each compound was isolated by the combined use of Sephadex G-10 column chromatography using the upper solvent of n-BuOH-AcOH-H_2O (4:1:5) and the DCC ascending method with n-BuOH-AcOH-H_2O (4:1:5). K-1, mp 265-268°C, $[\alpha]_D^{21}$+4.5° (c=0.2 in 5N HCl), CI-MS m/z: 90(M^++1) was identified as alanine by direct comparison with an authentic sample (IR,TLC) and its specific rotation was indicated to be a 2:1 ratio of L- to D-alanine (authentic L-alanine $[\alpha]_D^{21}$+11.9° (c=0.2 in 5N HCl)). This ratio also was confirmed by analyzing N-trifluoroacetylalanine-(-)-menthyl ester derived from K-1 using GLC.[5] K-2 was established as 3-[p-(2-N-methylamino-2-carboxyethyl) phenoxy]-4-hydroxy-N-methylphenylalanine by spectroscopic evidences; mp 235-239°C, CI-MS m/z: 301(base peak,M^++1-CH(NHMe)COOH), ^{1}H-NMR (20%DCl,δ,ppm): 2.81, 2.83(3H, s,NMe,resp.), 3.15-3.45(4H,Cβ-H), 4.2-4.5(2H,Cα-H), 6.9-7.4(aromatic H), its triacetyldimethylate, ^{1}H-NMR ($CDCl_3$,δ,ppm): 1.99, 2.02, 2.15(3H,s,Ac-Me,resp.), 2.80, 2.86(3H,s,N-Me,resp.), 2.95, 2.97(1H,dd,J=11,15Hz,Cβ-H,resp.), 3.29, 3.34(1H,dd, J=6,15Hz,Cβ-H,resp.), 3.70, 3.73(3H,s,COOMe,resp.), 5.08, 5.27(1H,dd,J=11,6Hz, Cα-H,resp.), 6.79(1H,d,J=2Hz,3-H), 6.87(1H,d,J=8Hz,6-H), 6.94(1H,dd,J=8,2Hz,5-H), 6.98(2H,d,J=9Hz,2'and 6'-H), 7.15(2H,d,J=9Hz,3'and 5'-H). K-3 was confirmed as 3-[p-(2-N-methylamino-2-carboxyethyl)phenoxy]-4-methoxy-N-methylphenylalanine by various spectral data; mp 240-244°C, CI-MS m/z: 315(base peak,M^++1-CH(NHMe)COOH), ^{1}H-NMR (20%DCl,δ,ppm): 2.83, 2.86(3H,s,NMe,resp.), 3.15-3.45(4H,Cβ-H), 3.82(3H,s, OMe), 4.2-4.5(2H,Cα-H), 6.9-7.4(aromatic H). K-4 was identified as N-methyl-L-tyrosine by direct comparison with an authentic sample (IR,TLC); mp 290°C, CI-MS m/z: 196(M^++1), $[\alpha]_D^{21}$+19° (c=0.1 in 5N HCl). K-5 was identified as p-methoxy-N-methyl-L-phenylalanine by direct comparison with an authentic sample (IR,TLC); mp 235-239°C, CI-MS m/z: 210(M^++1), $[\alpha]_D^{21}$+21.5° (c=0.2 in 5N HCl). From the previous description, it was evident that RA-VII and RA-V consisted of 2 mol of L-alanine, 1 mol of D-alanine, 1 mol of p-methoxy-N-methyl-L-phenylalanine and a kind of biphenyl having ether linkage. The similar antitumor substances bouvardin and deoxybouvardin have already been isolated from the methanolic extract of Bouvardia ternifolia (Rubiaceae).[6] But the physical data of RA-V corresponding to deoxybouvardin did not coincide with those in the literature.[6] Since it was not easy to find the amino acid sequence, we also carried out an X-ray analysis of p-bromobenzoate of RA-V.[1] This proved that RA-V was identical with deoxybouvardin. Consequently, the structures of RA-VII and RA-V were confirmed as shown in Fig.1.

RA-IV: colorless powder, mp 247-255°C (from MeOH), $[\alpha]_D^{28}$-126° (c=0.07 in $CHCl_3$), UV λ_{max}^{EtOH}nm(ε): 276(2600), 284(2000), MS m/z: 786(M^+), 768(M^+-H_2O).

RA-III: colorless needles, mp > 300°C (from MeOH), $[\alpha]_D^{28}$-199° (c=0.1 in $CHCl_3$), UV λ_{max}^{EtOH}nm(ε): 276(2300), 281(1800), MS m/z: 786(M^+), 768(M^+-H_2O).

The ^{1}H-NMR and IR spectra of RA-IV and RA-III were similar to those of RA-VII and their MS had the same molecular ion and exhibited a dehydration peak (M^+-18) at m/z 768. Therefore, RA-IV and RA-III were assumed to be mono-hydroxylated RA-VII. In order to determine the position of the hydroxyl group, the ^{13}C-NMR spectra of RA-IV and RA-III were compared with that of RA-VII. As can be seen from Table I, the carbon signal corresponding to δ 35.57 (t), one of three methylene

signals in RA-VII, disappeared in RA-IV. Also, the signal corresponding to δ 36.96 (t) in RA-VII diminished in RA-III. These phenomena showed that the new signals at δ 62.85 (d) in RA-IV and δ 61.58 (d) in RA-III originated from methine carbons which were formed by hydroxylation of each of the above methylenes. From the structural viewpoint of RA-VII, it was reasonable that the signals at δ 35.57 and 36.96 were assigned to methylene carbons having the diphenyl ether moieties containing cis peptide-linkages. Further, in the ^{13}C-NMR spectrum of RA-IV, the carbon signals due to 1,2,4-trisubstituted benzene and its Cα shifted to a lower field in comparison with those of RA-VII, but in that of RA-III, the shift of carbon signals due to the diphenyl ether moiety was hardly noticeable. Consequently, the hydroxyl group was confirmed to be linked to the Cβ position of amino acid moiety containing 1,2,4-trisubstituted benzene ring in RA-IV and to the Cβ position of amino acid moiety containing 1,4-disubstituted benzene ring of the diphenyl ether moiety in RA-III. The structures of RA-IV and RA-III are shown in Fig.1. On the other hand, it was assumed that the hydroxyl group in Fig.1 was the β-configuration in both RA-IV and RA-III, because in the ^{1}H-NMR spectra of these cyclic peptides, three N-methyl signals of RA-VII, RA-V and RA-III had almost the same chemical shifts, but one of the three N-methyl signals in RA-IV shifted to 0.32 ppm upper field in comparison with the N-methyl signal at δ 2.70 in RA-VII. These phenomena indicated that the deshielding range of the 1,2,4-trisubstituted benzene ring approximated the N-methyl group of the cis peptide-linkage moiety by forming a hydrogen bond between the β-hydroxyl and carbonyl groups in RA-IV, and the β-hydroxyl group in RA-III formed a hydrogen bond without the conformational change of the diphenyl ether moiety.

Antitumor activity of these cyclic hexapeptides were examined on a spectrum of experimental tumors in mice. The peptides exhibited a significant activity against leukemias and ascites tumors, P-388, L1210, B-16 melanoma and solid tumors, Colon 38, Lewis lung carcinoma and Ehrlich carcinoma.

RA-VII and RA-V obtained from R.cordifolia were also isolated from R.akane.

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