THRAZARINE, A NEW ANTITUMOR ANTIBIOTIC

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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A new antitumor antibiotic thrazarine was soluble in water and positive to anisaldehyde-sulfuric acid and ninhydrin color reactions. The absolute structure of thrazarine was determined to be *O*-((3*R*)-2-diazo-3-hydroxybutyryl)-L-serine by acid hydrolysis, spectroscopic analysis and X-ray crystallographic analysis. Structurally, thrazarine was a new member of azaserine group antibiotics.

Thrazarine, isolated from a culture broth of *Streptomyces coerulescens* MH802-fF5, is a new antitumor antibiotic which sensitizes tumor cells to macrophage-mediated cytolysis. We have reported the taxonomy of the producing organism and the isolation and biological properties of thrazarine in the preceding paper.[1]

In this report, we describe the physico-chemical properties and the structure determination of thrazarine, which belongs to a group of azaserine.[2,3]

Results and Discussion

Thrazarine (Fig. 1) was obtained as light yellow crystals; mp 135~139°C (dec), $[\alpha]_D^{22}$ +13° (*c* 1.0, water). It was soluble in water, slightly soluble in methanol and ethanol and hardly soluble or insoluble in other organic solvents. The UV spectra of thrazarine are as follows: UV λ_{max} (1/15 M phosphate buffer, pH 6.8) nm (ε) 257 (11,600); λ_{max} (0.1 M HCl) nm (ε) 235 (sh, 200); λ_{max} (0.1 M NaOH) nm (ε) 257 (11,600), 216 (14,300). Thrazarine was positive to anisaldehyde-sulfuric acid and ninhydrin color reactions. It gave a single spot at Rf 0.43 on the silica gel TLC (chloroform - methanol - water, 5:5:1). The molecular formula of thrazarine was established as $C_7H_{11}N_3O_5$ (MW 217) by elemental analysis and secondary ion mass spectrometry (SI-MS), *Anal* calcd for $C_7H_{11}N_3O_5 \cdot \frac{1}{3}H_2O$: C 37.67, H 5.25, N 18.83; found: C 37.68, H 5.13, N 18.73; SI-MS *m/z* 218 (MH^+). The

Fig. 1. Structure of thrazarine (1).

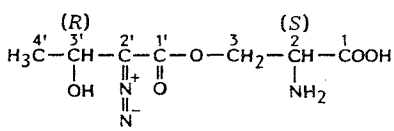

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Fig. 2. IR spectrum of thrazarine (KBr).

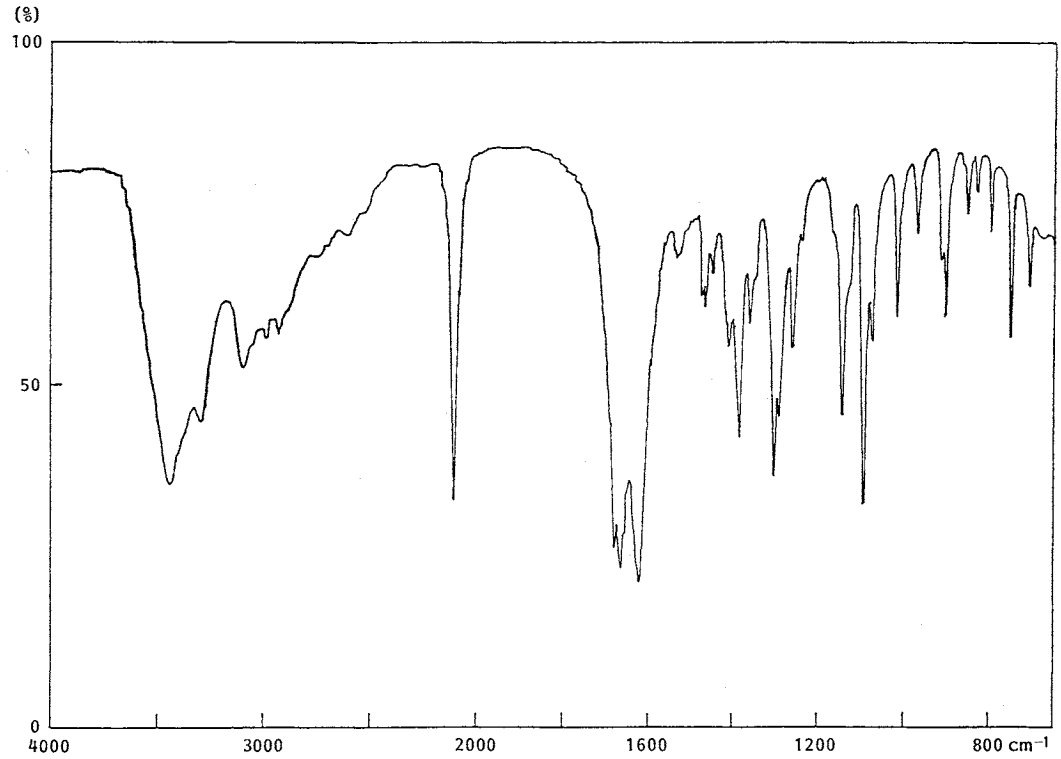


Table 1. ^1H NMR data of thrazarine in D_2O (400 MHz).

Proton	Chemical shift (δ value in ppm) and coupling constants (Hz)
2	4.11 (1H, br t)
3	4.63 (2H, m)
3′	4.88 (1H, q, J=6.7)
4′	1.43 (3H, d, J=6.7)

Table 2. HPLC of diastereomeric thiourea derivatives of serine with GITC* as a chiral reagent.

Compound	Retention time (minutes)
Ser derived from hydrolysate of **1**	6.82
D-Ser	7.19
L-Ser	6.82

* 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate.

IR spectrum (Fig. 2) showed a characteristic conjugated diazo-ester absorption (2120, 1680 and 1660 cm^{-1}).

The ^{13}C NMR spectrum (D_2O, 100 MHz) of the antibiotic showed only 6 carbon signals at δ 171.6 (COOH), 168.0 (C(=O)O, weak and broad), 64.0 (CH_2), 62.7 (CH), 54.6 (CH) and 20.0 (CH_3) compared with the expected 7 carbons.

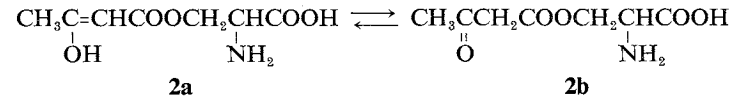
The ^1H NMR spectral data (400 MHz, D_2O, Table 1) indicated the presence of one methyl group, one methylene group and two methine groups. Spin decoupling experiments indicated the presence of two kinds of carbon chain in the structure, CH_3CH and CH_2CH.

Degradation studies of thrazarine were carried out to determine the structure. Strong acid hydrolysis of thrazarine with 6 M HCl at 100°C for 3 hours in a sealed tube gave serine, and no other amino acid was obtained. The absolute configuration of the serine was determined as L-form by HPLC for enantiomeric resolution of amino acid[4] in comparison with authentic L-serine and its D-enantiomer

(Table 2). From this result, a carbon chain, CH_2CH, presumed from the ^1H NMR spectrum was assigned to the L-serine moiety in the structure.

It has been reported that the decomposition of diazo compounds in various solvents gave olefin derivatives.[5~7]

Thrazarine released nitrogen by the mild acid hydrolysis with 50% formic acid at room temperature for 1 hour to afford a β-keto-ester (**2**), 7 carbons of which were observed in its ^{13}C NMR spectra. The ^{13}C NMR spectrum of **2** in D_2O showed six signals at 207.4, 171.5, 169.4, 64.3, 54.2 and 30.6 ppm and one quintet ($J_{C,D}$=20.0 Hz) at 49.2 ppm. On the other hand, in DMSO-d_6 seven carbons of **2** resonated at 169.7, 168.8, 153.2, 84.4, 65.5, 58.5 and 23.2 ppm. These data indicated that compound **2** had a dideuterated keto-form (**2b'**) in D_2O and a enol-form (**2a**) in DMSO-d_6.



2a **2b**

2b'

Although we could not observe the carbon bearing the diazo group in the ^{13}C NMR spectrum of thrazarine (in general, the carbon attached to the diazo group in the disubstituted diazoalkanes resonated at 45~65 ppm in the ^{13}C NMR spectrum[8]), we found the corresponding carbon signal in the degradation product (**2**).

From all results mentioned above, we proposed the structure of thrazarine to be *O*-(2-diazo-3-hydroxybutyryl)-L-serine. The configuration of C-3' of thrazarine was determined by the X-ray crystallographic analysis.

The crystals were grown in a methanol solution as yellow plates. A small crystal of approximate dimensions 0.6×0.45×0.07 mm was cut and coated with vaseline to avoid deterioration due to the loss of solvent in the crystal. Cell dimensions and intensity data were measured on a Philips PW-1100 diffractometer using CuKα radiation monochromated by a graphite plate. Crystal data are as follows: Thrazarine methanol solvate $C_7H_{11}N_3O_5 \cdot CH_3OH$, FW=249.3. Monoclinic, space group $P2_1$, Z=2. Cell dimensions, a=12.190(7), b=9.780(5), c=5.072(3) Å, β=95.62(5)°, U=601.8 Å^3. D_{calc}=1.375 gcm^{-3}, μ for CuKα=9.8 cm^{-1}.

A total of 1267 reflections out of 1340 possible ones were measured as above the 2σ(I) level up to the 2θ angle of 156°. Scans were repeated twice when the total counts measured for a single scan were less than 3000. Scan speed was 6°2θ/minute and the mode of scan was 2θ-ω. No absorption correction was applied. The intensity fall-off for 20 hours to measure the whole data was about 7% and the integrated intensities were corrected for the intensities of three standard reflections measured every 1 hour. The R(F) value for 118 symmetry related reflections was 3.9%.

The crystal structure was solved by the direct method using MULTAN.[9] One of the 128 phase sets gave a reasonable E-map which subsequently refined by block-diagonal-matrix least-squares and Fourier calculations. Eleven out of 15 hydrogen atoms were found on a difference electron-density map. The four hydrogen atoms belonging to the solvate molecule were not included in the refinement. The final R value was 8.6% for seventeen C, O and N and eleven H atoms. The atomic parameters are listed in Table 3.

Table 3. The positional parameters and equivalent thermal parameters with estimated standard deviations in parentheses.

Atom	X ($\times 10^4$)	Y ($\times 10^4$)	Z ($\times 10^4$)	B_{eq} (Å^2)
C1	183 (7)	7818 (8)	3704 (14)	3.11 (0.11)
C2	103 (6)	6333 (0)	2672 (14)	2.75 (0.10)
C3	1247 (7)	5701 (9)	2808 (17)	3.62 (0.12)
O4	1966 (5)	6687 (6)	1659 (11)	3.75 (0.09)
C5	2625 (7)	6267 (9)	−118 (17)	3.54 (0.12)
C6	3205 (7)	7365 (10)	−1124 (17)	3.86 (0.13)
C7	3071 (7)	8855 (11)	−631 (20)	4.61 (0.15)
C8	4142 (9)	9687 (13)	−651 (26)	6.36 (0.21)
O9	−56 (6)	8769 (6)	2145 (12)	4.35 (0.10)
O10	513 (5)	7936 (7)	6149 (10)	3.99 (0.09)
N11	−401 (5)	6347 (6)	−127 (12)	3.06 (0.09)
O12	2745 (6)	5049 (6)	−697 (13)	4.97 (0.11)
N13	3807 (6)	7005 (9)	−3093 (17)	4.95 (0.13)
N14	4325 (8)	6730 (13)	−4708 (19)	7.07 (0.19)
O15	2298 (6)	9527 (8)	−2528 (17)	6.26 (0.13)
O(Me)	2568 (9)	2471 (11)	1586 (30)	12.33 (0.29)
C(Me)	2802 (11)	2511 (17)	4412 (24)	8.66 (0.28)

Atom	X ($\times 10^3$)	Y ($\times 10^3$)	Z ($\times 10^3$)	B_{eq} (Å^2)
H(C2)	−47 (6)	609 (8)	410 (14)	3 (2)
H(C3)	164 (8)	550 (11)	492 (18)	6 (2)
H′(C3)	156 (7)	467 (11)	213 (18)	7 (3)
H(C7)	282 (7)	893 (11)	166 (17)	6 (2)
H(C8)	406 (7)	1068 (10)	31 (16)	5 (2)
H′(C8)	437 (9)	966 (14)	−292 (22)	9 (3)
H″(C8)	472 (10)	946 (14)	71 (22)	9 (3)
H (N11)	7 (6)	680 (9)	−157 (14)	4 (2)
H′(N11)	−56 (8)	551 (11)	−134 (18)	7 (3)
H″(N11)	−125 (8)	657 (12)	−38 (19)	8 (3)
H(O15)	158 (7)	882 (10)	−317 (16)	5 (2)

Since L-serine was isolated as the hydrolysis product, the atomic coordinates and the drawing of the molecule shown in Table 3 and Fig. 3 were referred to L-serine. From these data, we decided that C-3′ (C7 in Fig. 3) of thrazarine had *R*-configuration. Bond lengths and bond angles are listed in Table 4. The values were normal and agreed in general with those found in related molecules. In the amino acid group, the twist angle of the bond C2-N11 to C1-O9 was −6.6(7)° and the butyrate group extended nearly *trans* to the C^{α}-C^{β} bond [C2-C3-O4-C5 = −132.4(5)°]. However, the molecule as a whole took rather folded conformation. The diazo group was oriented *cis* to the carbonyl group [N13-C6-C5-O12 = 8.9(11)°] and nearly perpendicular to the hydroxyl group [N13-C6-C7-O15 = 76.1(9)°].

The molecules were bound together with hydrogen bonds from amino nitrogen N11 to carbonyl O10 (at $\theta, \theta, -1$) and O9 (at $x, -1/2+y, z$) and to O(CH_3) with distances 2.763(9), 2.798(9) and 2.890(12) Å, respectively. Hydroxyl O15 formed a hydrogen bond to O10 [at $\theta, \theta, -1$, with 2.705(10) Å] and carbonyl O12 formed another bond to O(CH_3) with the distance 2.791(13) Å.

From the above hydrolysis experiments and X-ray crystallographic analysis, the absolute structure of thrazarine was determined to be *O*-((3*R*)-2-diazo-3-hydroxybutyryl)-L-serine.

Fig. 3. Molecular structure of thrazarine.

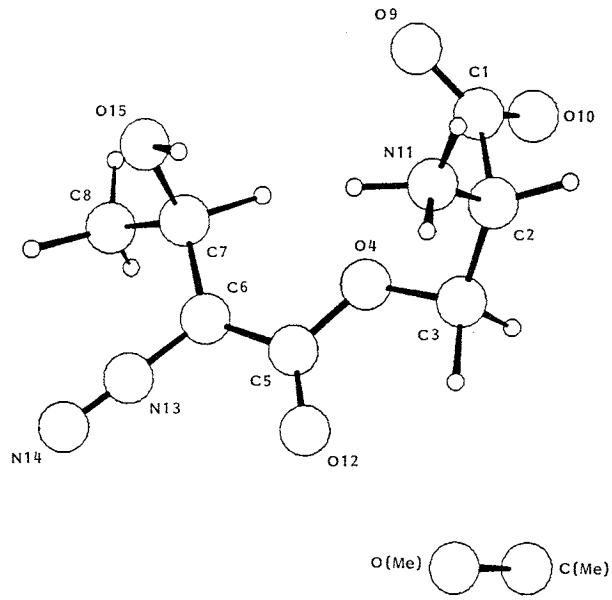


Table 4. Bond distances (Å) and angles (°).

Distances		Angles	
C1-C2	1.543 (8)	C2-C1-O9	119.2 (7)
C1-O9	1.237 (10)	C2-C1-O10	114.9 (6)
C1-O10	1.271 (9)	O9-C1-O10	125.9 (7)
C2-C3	1.521 (11)	C3-C2-C1	109.8 (6)
C2-N11	1.491 (9)	C3-C2-N11	109.7 (6)
C3-O4	1.461 (11)	C1-C2-N11	108.7 (5)
O4-C5	1.329 (11)	O4-C3-C2	107.3 (6)
C5-C6	1.408 (13)	C5-O4-C3	119.5 (7)
C5-O12	1.240 (11)	C6-C5-O4	111.6 (7)
C6-C7	1.491 (14)	C6-C5-O12	124.9 (8)
C6-N13	1.343 (13)	O4-C5-O12	123.4 (8)
C7-C8	1.540 (15)	C7-C6-C5	128.1 (8)
C7-O15	1.438 (12)	C7-C6-N13	117.2 (8)
N13-N14	1.115 (13)	C5-C6-N13	113.6 (8)
O(Me)-C(Me)	1.434 (19)	C8-C7-C6	114.1 (9)
		C8-C7-O15	104.8 (8)
		C6-C7-O15	114.2 (8)
		N14-N13-C6	178.4 (10)

Recently, LEE and co-workers have reported the isolation of a new azaserine-group antibiotic, LL-D05139β.[10]

Experimental

General

MP was determined with a Yazawa mp apparatus and was uncorrected. Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. IR and UV spectra were recorded with a Hitachi 260-10 IR spectrophotometer and a Hitachi 220S spectrophotometer, respectively. The ^{1}H

and ^{13}C NMR spectra were measured with a Jeol JNM-GX400 spectrometer. The mass spectra were recorded with a Hitachi M-80H mass spectrometer. TLC was performed on a Silica gel plate (Kieselgel 60 F_{254}, Merck).

Hydrolysis of Thrazarine with Hydrochloric Acid and HPLC for Enantiomeric Resolution of Amino Acid

A solution of thrazarine (1 mg) in 1 ml of 6 M HCl was refluxed for 3 hours. After removal of excess hydrochloric acid with evaporation, TLC was performed on a silica gel developed with a mixture of BuOH - AcOH - water (2 : 1 : 1) for detection of amino acid in thrazarine with the result to give a single ninhydrin-positive spot, Rf 0.40, which was identical with serine. The hydrolysate was dissolved in 1 ml of 50% aq acetonitrile containing 0.4% (w/v) triethylamine and to 50 μl of the solution was added 50 μl of 0.2% (w/v) 2,3,4,6-*tetra*-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[11] in acetonitrile. The mixed solution was allowed to stand at room temperature for 30 minutes. Thus obtained thiourea derivative was analyzed by reversed phase HPLC (ODS-1151-SH, Senshu Science Co., 4.6×150 mm; mobile phase, 10 mM phosphate buffer (pH 2.8) - MeOH - CH_3CN (70 : 15 : 15); detection UV 250 nm; flow rate 0.9 ml/minute) with comparison to authentic L- and D-serine. As shown in Table 2, serine derived from thrazarine hydrolysate was found to have the L-configuration.

Hydrolysis of Thrazarine with Formic Acid

A solution of 10 mg of thrazarine in 0.4 ml of 50% formic acid was allowed to stand at room temperature for 1 hour. The solution was then lyophilized to give 8 mg of white powder (**2**): SI-MS *m/z* 190 (MH^+); ^1H NMR (400 MHz, D_2O) δ 2.32 (s, CH_3), 4.10 (dd, J=3.6 and 4.6 Hz, CH), 4.59 (ABX, J=11.9, 3.6 and 4.6 Hz, CH_2); ^{13}C NMR (100 MHz, D_2O) δ 207.4 (C=O), 171.5 (COOH), 169.4 (C(=O)O), 64.3 (OCH_2), 54.2 (CH), 49.2 (CD_2, $J_{C,D}$=20 Hz), 30.6 (CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.7 (COOH), 168.8 (C(=O)O), 153.2 (OC=), 84.2 (CH=), 65.5 (OCH_2), 58.5 (CH), 23.2 (CH_3); IR (KBr) cm^{-1} 1760, 1720, 1640, 1600, 1520.

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