THE STRUCTURE OF A GLYOXALASE I INHIBITOR AND ITS CHEMICAL REACTIVITY WITH SH-COMPOUNDS

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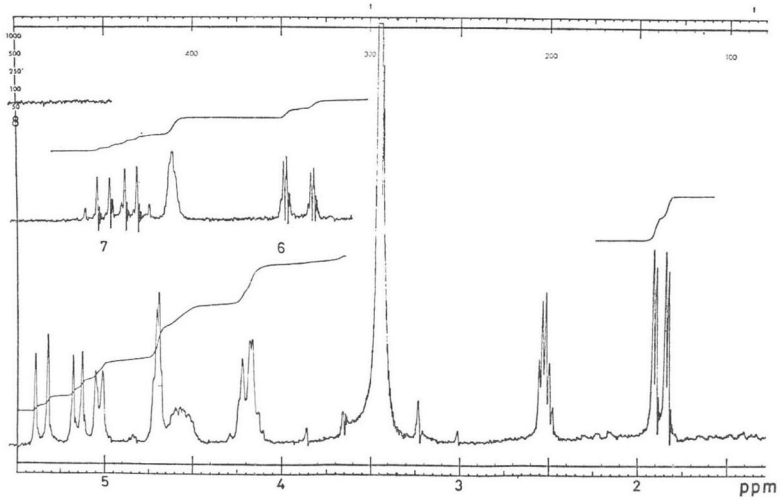
The structure of a glyoxalase I inhibitor (**I**), isolated from a cultured broth of *Streptomyces griseosporeus*, was found to be 2-crotonyloxymethyl-4,5,6-trihydroxy-cyclohex-2-enone by chemical studies. Stereochemistry and absolute configuration were determined to be 4R, 5R and 6R by X-ray crystallographic analysis of a bromine-containing crystalline derivative. The crotonyloxy group of **I** shows a surprising proclivity to be displaced by SH-compounds. This property is shown to be the basis for its biological activity.

The isolation, physicochemical properties and biological activities of a glyoxalase I inhibitor (**I**) were reported in the preceding paper[1]. In this paper, structural determination by chemical studies and X-ray crystallographic analysis, and its facile and novel reaction with SH-compound are presented.

Chemical Studies

Compound I is obtained as colorless needles from chloroform - methanol (10 : 1). m.p. 181°C, $[\alpha]_D^{24}$ −109° (*c* 1.5, methanol), $\lambda_{max}^{H_2O}$ 213 (ε 21800) and 310 nm (ε 56), $\nu_{C=O}^{KBr}$ 1715 (conjugated carboxylic acid ester) and 1690 cm^{-1} (α,β-unsaturated ketone). The molecular formula was established as $C_{11}H_{14}O_6$ by elemental analysis and PMR spectrometry. It showed positive color reactions with ferric chloride (purple), 2,3,5-triphenyltetrazolium chloride (pinkish red) and 2,4-dinitrophenylhydrazine. The PMR spectrum in DMSO-d_6 is shown in Fig. 1. A double doublet signal at δ 1.87 (3H, *J* 2 and 7 Hz) coupled with two double quartet signals at δ 5.90 (1H, *J* 2 Hz) and 6.93 (1H, *J* 7 Hz). The latter two signals are coupled with each other with a large coupling constant (15.5 Hz). These chemical shifts and coupling patterns suggested the presence of the crotonate moiety. A broad peak of another olefinic proton at δ 6.61 was coupled with a nonequivalent methylene proton signal centered at δ 4.70 with small coupling constant (about 1.5 Hz). There were three methine proton signals appeared at δ 4.19(×2) and 4.57, which were coupled with OH protons at δ 5.03 (*J* 4 Hz), 5.15 (*J* 5 Hz) and 5.35 (*J* 7 Hz), respectively. The methine protons at δ 4.19 also were coupled with the olefinic proton at δ 6.61, the methylene

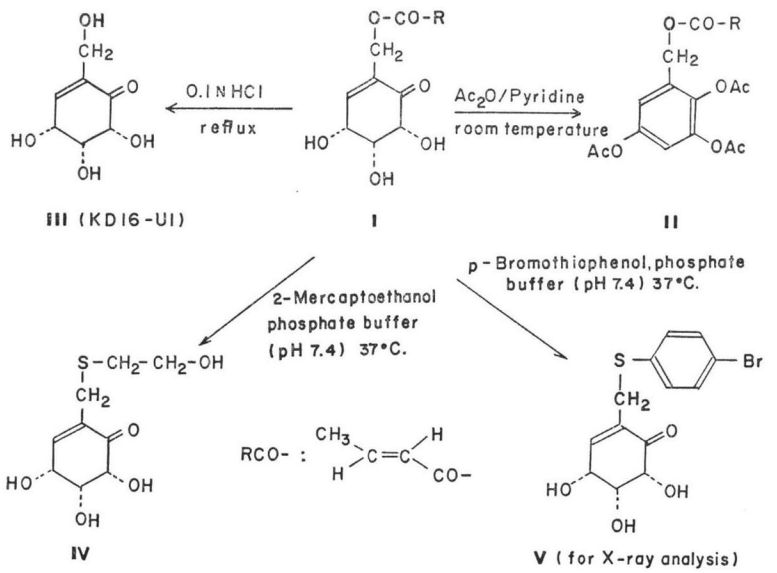
Fig. 1. PMR spectrum of a glyoxalase I inhibitor (I) in DMSO-d_6 (100 MHz).



protons at δ 4.70 and the methine proton at δ 4.57 with small coupling constants. These coupling relations, together with information obtained from the IR and UV spectra, suggested that **I** should have the structure, 3-crotonyloxymethyl-4,5,6-trihydroxyl-cyclohex-2-enone, though there is no information about stereochemistry.

Compound **I** was acetylated with acetic anhydride and pyridine at room temperature yielding a triacetyl derivative (**II**). M^+: *m/e* 350.1024. Calcd. for $C_{17}H_{18}O_8$: 350.1001. m.p. 95°C, $\nu_{C=O}^{KBr}$ 1770 (phenol acetae) and 1720 cm^{-1} (crotonate). The PMR spectrum in $CDCl_3$ showed the presence of three acetyl groups (δ 2.24, 2.26 and 2.28), crotonyloxymethyl moiety [δ 1.88 (3H, double doublet, *J* 2 and 7 Hz), 5.84 (1H, double quartet, *J* 2 and 15.5 Hz), 6.97 (1H, double quartet, *J* 7 and 15.5 Hz), and 5.10 (2H, singlet)] and two meta-coupled aromatic protons at

Chart 1.



δ 7.05 and 7.09 (J 2.5 Hz). Aromatization during the acetylation was clearly indicated by the ^{13}C-NMR studies. In the ^{13}C-NMR spectrum of **I**, there were five peaks (δ 18.50, 61.76, 69.98, 76.38 and 77.23) in the region higher than δ 100, (tetramethylsilane as the internal reference), while there were only two peaks (δ 17.89 and 60.30) in this region in the spectrum of **II**, except three acetyl-methyl peaks (δ 19.98, 20.44 and 20.80) which were introduced by the acetylation. These results indicate that the three peaks of **I** at δ 69.98, 76.38 and 77.23, assigned to the three aliphatic carbons of the cyclohexenone ring, were shifted to less than δ 100 due to aromatization. In fact, there were 12 peaks in the lower field of the spectrum of **II**.

At this stage, we found that KD16-U1 under study by TATSUTA *et al.* is identical with decrotonyl **I** (**III**). These authors reported its structure as 2-hydroxymethyl-4α,5α,6?-trihydroxycyclohex-2-enone or its enantiomer[2].

Decrotonation of **I** was achieved by mild acid hydrolysis (0.1 N HCl, reflux for 30 minutes). Decrotonyl **I** was identical with KD16-U1 by comparison of their IR spectra, melting points, 112~113°C (Lit.[2] 113~114°C) and optical rotations, $[\alpha]_D^{20}-170°$ (c 1.0, H_2O). Lit.[2] ($[\alpha]_D^{20}-168°$ (c 1.0, H_2O)). Ester bounding between **III** and crotonic acid in **I** was found to be the hydroxymethyl group of **III** by PMR analysis, because the methylene proton (δ 4.70) of **I** was significantly deshielded compared with the hydroxymethyl group of **III** (δ 4.27). Thus, the structure of **I** was determined to be 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone.

X-Ray Structure Determination

The stereochemistry of KD16-U1 is not yet completely elucidated, and the absolute configuration remains to be solved. To determine the stereochemistry and absolute configuration of **I** by the X-ray diffraction method, a crystalline derivative containing a heavy atom was needed. The reaction product of **I** and *p*-bromothiophenol (see Reaction with SH-Compounds) gave colorless well-developed platy crystals from acetone. The crystals contain acetone as a solvent of crystallization and easily deteriorate on loss of solvent. X-Ray diffraction measurements were therefore carried out with a crystal sealed in a thin-walled glass

Fig. 2. Bond lengths and angles of compound **V** (viewed along C-axis).

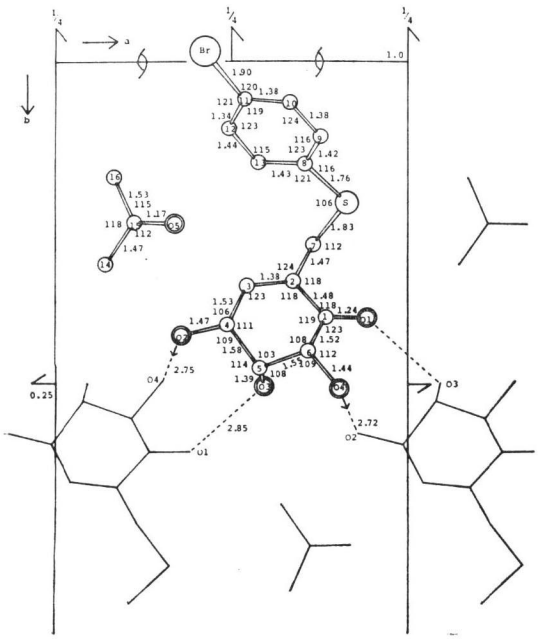
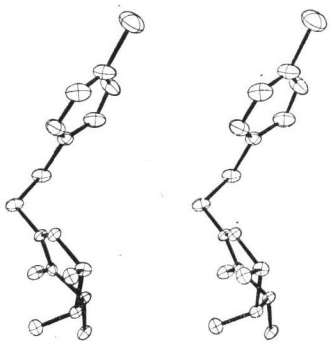


Fig. 3. Stereodiagram of compound **V**.



capillary. Intensity data were collected with a Philips four-circle X-ray diffractometer using CuKα radiation monochromated by a graphite plate. Integrated intensities were measured by a $\theta-2\theta$ scan method with a scan speed of 4°/min in θ. Background was measured at both ends of the scan. The size of the crystal was about $0.13\times0.02\times0.5$ mm.

Crystal data:

2-*p*-bromophenylthiomethyl-4,5,6-trihydroxy-cyclohex-2-enone

$C_{13}H_{13}O_4BrS\cdot(CH_3)_2CO$ F.W.=403.3

Orthorhombic, $P2_12_12_1$, Z=4, $D_x=1.512$ gcm^{-3}

a=10.055(5), b=37.57(2), c=4.691(2) Å

U=1772.2 Å^3

Of the possible 1846 reflexions within θ of 65°, 1227 reflexions were measured with intensities greater than the 2σ level. Friedel pairs of reflexions hkl and $h\bar{k}l$ were measured at θ angles less than 40°. No absorption correction was applied for the intensity data. The structure was determined by the heavy atom method and refined by the block-diagonal least-squares calculations to an R value of 0.09. In the latter calculations, the unit weight was applied for each reflexion and anisotropic temperatuer factors were assumed for each atom. No hydrogen atoms were assigned.

Absolute configuration was determined by use of the anomalous dispersion of CuKα radiation by the bromine and sulphur atoms. Forty six pairs of reflexion clearly indicated the

Table 1. Final atomic parameters ($\times10^4$) with estimated standard deviations in parentheses. Temperature factors are of the form $T=\exp[-(\beta_{11}h^2+\beta_{22}k^2+\beta_{33}l^2+2\beta_{12}hk+2\beta_{13}hl+2\beta_{23}kl)]$. To represent the correct absolute configulation the x, y, z coordinates should be referred to the left-handed coordinate system.

	x	y	z	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
BR	4250(3)	−77(1)	5472(7)	191(3)	14(0)	999(19)	−21(1)	35(9)	27(2)
S	8267(4)	1087(1)	355(13)	63(4)	5(0)	680(32)	4(1)	−7(13)	−1(3)
O 1	8846(9)	1979(2)	203(30)	49(10)	6(1)	556(79)	−2(2)	39(29)	−17(8)
O 2	3588(9)	2122(3)	1284(25)	47(10)	6(1)	340(69)	0(2)	−26(23)	2(6)
O 3	5940(8)	2510(2)	−403(25)	37(9)	6(1)	321(60)	1(2)	4(25)	5(7)
O 4	8074(9)	2532(2)	3678(23)	35(10)	6(1)	333(61)	−3(2)	−11(22)	−15(6)
C 1	7693(14)	1972(4)	1118(35)	63(15)	5(1)	230(87)	−4(4)	−27(34)	6(9)
C 2	6778(12)	1697(3)	4(37)	46(12)	4(1)	293(84)	−3(3)	30(38)	−11(9)
C 3	5445(12)	1731(3)	643(36)	40(12)	4(1)	284(87)	2(3)	−28(33)	−7(9)
C 4	4893(14)	2033(4)	2482(40)	29(13)	5(1)	442(99)	3(3)	5(33)	2(9)
C 5	5805(14)	2373(3)	2325(33)	40(14)	4(1)	290(84)	0(3)	12(35)	−6(8)
C 6	7182(13)	2235(4)	3324(36)	34(13)	4(1)	325(90)	0(3)	−25(32)	−9(9)
C 7	7334(15)	1410(4)	−1762(43)	64(16)	4(1)	569(124)	5(4)	15(43)	−12(10)
C 8	7087(16)	792(4)	1808(40)	91(20)	4(1)	433(104)	4(4)	−13(41)	−1(10)
C 9	7530(19)	577(4)	4096(60)	130(24)	6(1)	909(189)	3(5)	−7(67)	1(14)
C10	6665(19)	312(5)	4945(54)	135(23)	10(2)	640(151)	−8(6)	−38(66)	40(16)
C11	5368(19)	287(5)	4029(56)	137(24)	7(1)	862(178)	−8(5)	−31(64)	6(15)
C12	4923(18)	515(5)	2046(58)	79(19)	8(2)	1000(186)	1(5)	−58(56)	−4(15)
C13	5761(20)	779(4)	716(49)	138(26)	7(1)	661(137)	−4(5)	38(65)	13(13)
C14	1444(20)	1570(6)	5677(78)	118(26)	12(2)	1642(292)	2(6)	−90(87)	46(25)
C15	2241(18)	1245(7)	6075(66)	59(18)	20(3)	1164(226)	−9(6)	−66(64)	−15(25)
C16	1692(26)	898(6)	4846(95)	202(37)	13(2)	2077(354)	−8(8)	−159(135)	−79(29)
O 5	3395(19)	1253(5)	6369(55)	220(27)	21(2)	1925(221)	16(7)	−273(74)	−86(20)

absolute configulation as shown in Fig. 2, that is, 4R, 5R and 6R. Table 1 lists the final atomic parameters and their estimated standard deviations. The bond lengths and angles are shown in Fig. 2. These values are consistent with those expected for the chemical structure **V**. The standard deviations were estimated to be σ(Br–C)=0.02 Å, σ(S–C)=0.02 Å, σ(C–O)=0.02 Å, σ(C–C)=0.02~0.03 Å, σ(Br–C–C)=2°, σ(C–S–C)=1°, σ(S–C–C)=1°, σ(C–C–O)=1°, σ(C–C–C)=1~2°. The conformation of the molecule may be clearly seen Fig. 3. The atoms O(1), C(1), C(2), C(3), C(4), and C(6) lie nearly on a plane which comprise the planar part of the cyclohexenone ring. The ring is puckered at C(5) and the two substituent hydroxyl groups O(2)H and O(4)H extend in an equatorial direction while O(3)H is in an axial direction. The torsion angle C(2)–C(7)–S–C(8) is 82° and the *p*-bromobenzene group is oriented neither *trans* nor *cis* with respect to the cyclohexenone ring.

The configuration of C_6 of **III** elucidated in this paper is different from that of the C_5 of shikimic acid. This suggests that although the biosynthesis of **III** through sikimic acid route was suspected by TATSUTA *et al.*, **I** may be biosynthesized from acetate *via* tetraketide like epoxydon[3].

Reaction with SH-Compounds

During the study of mechanism of the action of **I** against glyoxalase, we found that **I** reacts with glutathione (a cofactor of glyoxalase) and other SH-compounds such as cysteine, 2-mercaptoethanol or thiophenol. The reaction product with 2-mercaptoethanol was studied in detail. Compound **I** reacted easily with 2-mercaptoethanol in 0.1 M phosphate buffer (pH 7.4) at 37°C. The reaction was completed within 20 minutes. The reaction product (**IV**) was crystallized from methanol. m.p. 147°C. $\nu_{C=O}^{KBr}$ 1670 cm^{-1} (α,β-unsaturated ketone). It has the molecular formula $C_9H_{14}O_5S$. The IR spectrum suggested lack of the ester function existed in **I**. The PMR spectrum showed the presence of an ethylenic carbon chain originating from mercapthoethanol [δ 2.49 (2H, triplet, J 7 Hz) and 3.51 (2H, triplet, J 7 Hz)] and the absence of the crotonate moiety. The chemical shift of the methylene proton (δ 3.24) attached to the C-2 position of the cyclohex-2-enone ring suggested that **IV** was derived by substitution with 2-hydroxyethylthio-group for the crotonate moiety of **I**. The structure of the displacement product with SH-compound was confirmed by the X-ray structure determination described earlier. This facile and novel displacement reaction could not be observed with **III**. Compounds **III**, **IV** and **V** did not show any biological activity. Thus, the susceptibility to nucleophilic displacement of **I** was shown to be the principle of its action against glyoxalase reaction in which glutathione is involved as a cofactor.

Experimental

Infrared (IR) spectra were measured on a Hitachi EPI-S2 Spectrometer with potassium bromide discs. Ultraviolet (UV) spectra were measured on a Hitachi 124 Spectrometer. Proton nuclear magnetic resonance (PMR) spectra were measured on Varian A-60D and HA-100D spectrometers and are given in parts per million (δ) downfield from internal tetramethylsilane. Carbon-13 nuclear magnetic resonance (CMR) spectra were measured on a Varian XL-100 spectrometer and are given in parts per million (δ) downfield from internal tetramethylsilane. Mass spectra (MS) were measured on Hitachi RMU-6M and -7M (high resolution MS) spectrometers and were taken with direct inlet electron impact ionization. Thin-layer chromatography (TLC)

was performed with silica gel (precoated Kieselgel $60F_{254}$, E. Merck Co., 0.25 mm and 2 mm) and cellulose powder (precoated Avicel SF, Funakoshi Yakuhin Co., 0.25 mm).

Compound I

Compound I used for the present investigation was isolated by the procedure described in the preceding paper[1]. m.p. 181°C.

Acetylation of I (II)

To 1 ml pyridine solution containing 96.0 mg of I was added dropwise 5 ml of acetic anhydride under agitation and ice-cooling. After reaction at room temperature for 20 hours, the solvent was removed under reduced pressure. The residue was dissolved in 130 ml of $CHCl_3$ and washed three times with 130 ml of water, and evaporated to give 90.1 mg of oily substance. Compound II was isolated by preparative silica gel TLC developed with benzene - acetone (10 : 1). It was crystallized from ethyl ether - petroleum ether (1 : 10). Needle crystal, m.p. 95°C. M^+ (*m/e*): 350.1024, Calcd. for $C_{17}H_{18}O_8$: 350.1001. $\nu_{C=O}^{KBr}$ (cm^{-1}): 1770 (phenol acetate), 1720 (crotonate).

Hydrolysis of I (III)

A 100 mg sample of I was dissolved in 50 ml of 0.1 N HCl and refluxed for 30 minutes. After neutralization with Amberlite IR 44 (OH^- form), 71.6 mg of dried material was obtained. It was dissolved in 5 ml of acetone and purified by preparative silica gel TLC developed with *n*-butanol - ethanol - water (4 : 1 : 1) to yield 27.3 mg. It was crystallized twice from ethyl acetate giving 12.6 mg. Needle crystal, m.p. 112~113°C, $[\alpha]_D^{20}$ −170° (*c* 1.0, H_2O). $\lambda_{max}^{H_2O}$ nm (ε): 229 (9800), 311(60). $\nu_{C=O}^{KBr}$ (cm^{-1}): 1690 (conjugated ketone).

Preparation of IV

To 150 ml of 0.1 M phosphate buffer (pH 7.4) solution containing 99 mg (0.41 m mole) of I was added 10 ml of phosphate buffer solution containing 36.2 mg (0.41 m mole) of 2-mercaptoethanol. After storage at 37°C for 20 minutes, the reaction mixture was charged on a column (200 ml) of Amberlite XAD-2. After washing with water, the reaction product was eluted with 50 % methanol and gave 92.0 mg of pale yellow powder. It was purified by preparative silica gel TLC developed with *n*-butanol - ethanol - water (4 : 1 : 1), and crystallized from methanol to give 38.0 mg of platy crystal, m.p. 147°C. Anal. Calcd. for $C_9H_{14}O_5S$: C, 46.14; H, 6.02; S, 13.69; O, 34.15. Found: C,46.25; H, 6.08; S, 13.45; O, 34.09. $\lambda_{max}^{H_2O}$ nm (ε): 232(9400), 300~315(57.) $\nu_{C=O}^{KBr}$ (cm^{-1}): 1670 (conjugated ketone).

Preparation of V for X-ray analysis

To 30 ml of 0.1 M phosphate buffer (pH 7.4) containing 60.5 mg of I was added 0.5 ml of methanol solution containing 50.0 mg of *p*-bromothiophenol (purchased from Aldrich Chemical Co.). After keeping at 37°C for 10 minutes, the precipitate was collected by filtration with glass filter and washed with water to yield 76.0 mg. After crystallization with acetone, 47.1 mg of II was obtained. Recrystallization with acetone gave colorless well-developed platy crystals. m.p. 166~167°C.

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