A GLYOXALASE I INHIBITOR OF A NEW STRUCTURAL TYPE PRODUCED BY *STREPTOMYCES*

TOMIO TAKEUCHI, HIDEO CHIMURA, MASA HAMADA and HAMAO UMEZAWA

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo, Japan

OSAMU YOSHIOKA, NORIKO OGUCHI, YUKIO TAKAHASHI and AKIRA MATSUDA

Research Laboratories, Nippon Kayaku Co., Ltd., Tokyo, Japan

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Many streptomyces strains produced an inhibitor of crude glyoxalase prepared from rat liver which did not inhibit glyoxalase I prepared from yeast. Another inhibitor, $C_{11}H_{14}O_6$, which inhibited glyoxalases prepared from both rat liver and yeast was obtained from a cultured broth of *Streptomyces griseosporeus* and crystallized. Preincubation of this inhibitor with reduced glutathione increased its inhibitory activity, which suggested its reaction with reduced glutathione. It showed a strong inhibition of growth of HeLa cells and inhibition of EHRLICH ascites carcinoma by daily injection. It also showed weak inhibition of the solid type of EHRLICH carcinoma and prolonged the survival period of mice inoculated with L-1210 cells.

Methylglyoxal which is widely distributed in living cells reacts with sulfhydryl group of reduced glutathione and may have a role in the regulation of cell growth.[1,2] Thus, α-ketoaldehydes and glyoxalase inhibitors have been studied as potential anticancer agents,[3,4,5] though it is not certain whether glyoxalase is involved in the main metabolic degradation of methylglyoxal. Another enzyme, α-ketoaldehyde dehydrogenase, oxidizes this toxic metabolite.[6] In these circumstances, we were interested in glyoxalase inhibitors produced by microorganisms, because we thought that such inhibitors of new structural types produced by living organisms might be useful in analysing the role of glyoxalase in cells and might exhibit antitumor effect. In this paper, we report the isolation of an inhibitor, 2-crotonyloxymethyl-(4R,5R,6R)-4,5,6-trihydroxycyclohex-2-enone, from a streptomyces. This inhibitor exhibited antitumor effects. Structure elucidation will be reported in another paper.

Glyoxalase consists of two enzymes, glyoxalase I (S-lactoyl-glutathione methylglyoxal-lyase, EC 4.4.1.5) and glyoxalase II (S-2-hydroxyacrylglutathione hydrolase, EC 3.1.2.6), plus the reduced glutathione (GSH), and catalyzes the conversion of α-ketoaldehydes to hydroxy acids. Methylglyoxal is converted to D-lactic acid in the following successive reactions catalyzed by these enzymes.

$$CH_3COCHO+GSH \xrightarrow{I} CH_3CHOHCOSG \xrightarrow{II,\ H_2O} CH_3\text{-}CHOH\text{-}COOH+GSH$$

ALEXANDER and BOYER[7] developed a rapid assay method for glyoxalase activity in crude tissue extracts. In this method, methylglyoxal is converted to its disemicarbazone and determined by reading the absorbance at 286 nm. This method was modified and employed to

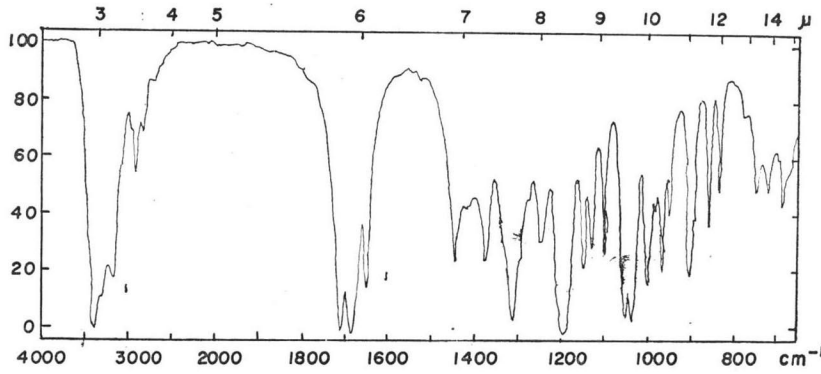
determine the activity of streptomyces culture filtrates in inhibiting glyoxalase. Many strains produced active agents in culture filtrates obtained by the shaking culture in a medium containing soybean meal and carbon sources. These active agents were adsorbed on Amberlite XAD-II, eluted with 50 % aqueous acetone, and after evaporation of the solvent extracted into *n*-butanol. They showed Rf 0.18, 0.25, 0.34 on thin-layer chromatography with silica gel and butanol - acetic acid - water (4 : 1 : 1, v/v). These active agents did not inhibit yeast glyoxalase I. However, besides these active agents, one of the strains designated MD287-CF4 produced an active compound which inhibited both glyoxalases prepared from rat liver and yeast. This strain was isolated from a soil sample collected in Tokyo and taxonomic studies suggested that it can be classified as *Streptomyces griseosporeus* NIIDA and OGASAWARA (ISP type culture No. 5562), though there were the following minor differences: MD287-CF4 strain produced white aerial mycelium and ISP 5562 produced white and light gray aerial mycelium on glycerol-asparagine agar (ISP medium 5); MD287-CF4 strain produced brownish gray to light gray aerial mycelium on yeast maltose agar (ISR ISP 2 medium) and ISP 5562 produced white to grayish white aerial mycelium; MD287-CF4 produced colorless to light yellow aerial mycelium with reddish tinge on oatmeal agar (ISP medium 3) and ISP 5562 produced light gray aerial mycelium; ISP 5562 produced taitomycin but MD287-CF4 did not.

As described above, this strain produced two types of glyoxalase-inhibiting agents in media containing various carbon and nitrogen sources, and the following medium was selected for the production of the compound which inhibited both glyoxalases: glucose, 1.0 %; starch, 1.0 %; soybean meal, 1.5 %; NaCl, 0.3 %; KH_2PO_4, 0.1 %; $FeSO_4 \cdot 7H_2O$, 0.0001 %; $MnCl_2 \cdot 4H_2O$, 0.0008 %; $ZnSO_4 \cdot 7H_2O$, 0.0002 %; adjusted to pH 7.2 before sterilization. Under shake-culture conditions on a reciprocating shaker, the activity of the culture filtrate in inhibiting crude rat liver glyoxalase reached its maximum after 4 days when addition of 15~30 μl of the filtrate to the glyoxalase reaction mixture showed about 50~80 % inhibition.

However, most of the activity of the culture filtrate was due to the compounds which showed inhibition against the crude glyoxalase of rat liver but not against yeast glyoxalase I. As described later, the other compound which also inhibits yeast glyoxalase I could be measured by silica gel thin-layer chromatography. Therefore, the production of this active agent probably reaches a maximum value after 4 days of shake culture.

The active agent which inhibited both glyoxalases existed chiefly in the aqueous phase of the culture broth and was adsorbed on carbon and eluted with acidic 50 % methanol or 50 % aqueous acetone. It was also adsorbed on Amberlite XAD-II and eluted with 50 % acetone. The latter method gave a higher yield. After evaporation of the acetone, the eluate was transferred into butanol at pH 2.0. After evaporation of the solvent to dryness under reduced pressure, the residue was dissolved in methanol, and 4 volumes of chloroform were added. The active compounds which showed inhibition against the crude glyoxalase of rat liver but not against yeast glyoxalase I remained undissolved. The methanol-chloroform solution was evaporated under reduced pressure, and the active compound thus obtained was purified by silica gel column chromatography with chloroform-methanol (20 : 1 or 10 : 1, v/v). It was further purified by Sephadex LH-20 column chromatography with methanol. Recrystallization from chloroform-methanol (10 : 1) yielded colorless needle cystals of the inhibitor of glyoxalase I, m.p. 181°C.

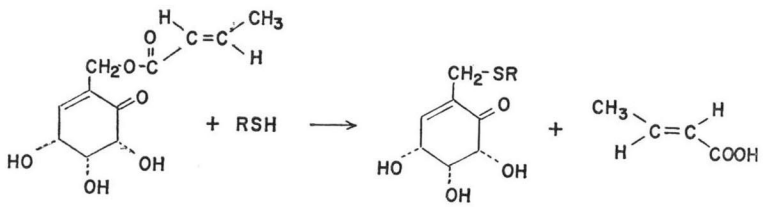
Fig. 1. Infrared absorption spectrum of glyoxalase I inhibitor (KBr).



The inhibitor shows levorotation: $[\alpha]_D^{24}$ $-109°$ (*c* 1.5, methanol). Elemental analysis: Calcd. for $C_{11}H_{14}O_6$: C 54.54, H 5.79, O 39.67; found: C 54.42, H 5.85, O 38.97. This formula was supported by 14 protons in pmr spectrum (dissolved in hexadeuterodimethylsulfoxide). It gives positive ferric chloride (purple), 2,3,5-triphenyltetrazolium chloride (pinkish red) and 2,4-dinitrophenylhydrazine (yellow) reactions, but negative ninhydrin, TOLLENS and FEHLING reactions. The following maxima are present in the ultraviolet spectrum: 211 nm (ε 19400) and 312 nm (ε 52) in ethanol; 213 nm (ε 21800) and 310 nm (ε 56) in water. The infrared spectrum in potassium bromide shows the following bands (Fig. 1): 3400, 3200, 2930, 2800, 1710, 1690, 1650, 1445, 1385, 1310, 1245, 1195, 1150, 1130, 1100, 1055, 1040, 1000, 965, 905, 860, 840, 750, 720 and 690 cm^{-1}.

The inhibitor is soluble in water, methanol, ethanol, dimethylsulfoxide, slightly soluble in butanol, ethyl acetate, butyl acetate and insoluble in chloroform, acetone, *n*-hexane, benzene, ethyl ether, and petroleum ether. Rf values on silica gel thin-layer chromatography are as follows: 0.75 with chloroform - methanol (10 : 1); 0.73 with *n*-butanol - ethanol - water (4 : 1 : 1). Rf on Avicel thin-layer chromatography is 0.74 with *n*-butanol - ethanol - water (4 : 1 : 1). The spot can be shown by spraying 2,3,5-triphenyltetrazolium chloride reagent. Thin-layer chromatography, especially that using silica gel and chloroform - methanol (10 : 1) can be employed for chemical determination of the active agent. When thin-layer chromatography is used for determination of the active agent in culture filtrates, the active agent is extracted by Amberlite XAD-II process before chromatography.

As described in another paper, the active agent was shown to be 2-crotonyloxymethyl-4(R),5(R),6(R)-trihydroxycyclohex-2-enone which reacts with sulfhydryl compounds in pH 7.4 phosphate buffer as follows:



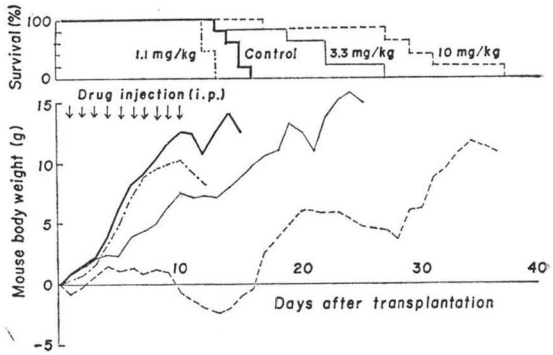
Inhibition of glyoxalase I is due to this reaction. In a reaction mixture containing 1.59 mM

Table 1. Effect of the glyoxalase I inhibitor on subcutaneous solid tumors of EHRLICH carcinoma.

Dose mg/kg/day for 10 days (i.p.)	Tumor weight* (mg)	Toxicity (animals dead/total)
10	680±250	0/5
5	980±150	0/5
2.5	1,110±130	0/5
1.25	1,040±100	0/5
0.63	1,070±200	0/5
0.32	1,530±330	0/5
0	1,780±110	0/15

* Solid tumors were taken at the 15th day of the inoculation and weighed.

Fig. 2. The effect of the glyoxalase I inhibitor on EHRLICH ascites tumor.



of reduced glutathione, and preincubated for 3 minutes before addition of a crude glyoxalase, 1.8 mM of this compound showed 50 % inhibition. When the preincubation time of the inhibitor with reduced glutathione was increased, inhibition increased: 2.5 mM of the inhibitor showed 65 % inhibition after 3-minute preincubation and 91 % inhibition after 20-minute preincubation. Preincubation of the inhibitor with methylglyoxal or glyoxalase I does not have any influence on inhibition.

The inhibitor shows no antibacterial activity at 200 μg/ml except against *Xanthomonas oryzae* and *Aeromonas punctata*. The former was inhibited at 100 μg/ml and the latter at 200 μg/ml. However, the inhibitor is active against animal cells. Fifty percent inhibition of growth of HeLa cells in cell culture was observed at 18.0 μg/ml (7.25×10^{-5} M) of the active agent. It inhibits EHRLICH ascites carcinoma. As shown in Fig. 2, when 10^6 cells of EHRLICH carcinoma were intraperitoneally inoculated into a mouse and the inhibitor was intraperitoneally injected every day for 10 days starting 24 hours after the inoculation of the carcinoma cells, 3.3 mg/kg/day and 10 mg/kg/day prolonged the survival period. However, even in the case of 10 mg/kg/day, the ascites started to increase 7 days after the last injection of the inhibitor. The results suggest that 10 mg/kg daily for 10 days would be enough to react with the sulfhydryl compounds in cancer cells, and during several days after the last injection the inhibitor would be completely washed out. The daily intraperitoneal injection of 10 mg/kg gave inhibition of growth of subcutaneous solid tumor of EHRLICH carcinoma as shown in Table 1. It showed also a weak inhibition against L-1210 leukemia. When 10^5 cells of L-1210 were intraperitoneally inoculated into BDF_1 mice, and the daily intraperitoneal injection of the inhibitor was started 24 hours after the inoculation and continued for 7 days, prolongation of the survival period was observed. The ratio of survival time (days) in treated animals to survival time of the control without treatment was as follows: 177 % at 35 mg/kg/day, 140 % at 25 mg/kg/day, 127 % at 15 mg/kg/day, 124 % at 10 mg/kg/day. The dose of 50 mg/kg/day was toxic and four out of five animals died. One of 5 mice administered 35 mg/kg/day also died from toxicity.

From the results observed against EHRLICH carcinoma and L-1210, the inhibitor has a relatively low toxicity: LD_{50} to mice was about 90 mg/kg by intravenous injection.

Experimental

Method of determining the inhibition of a crude glyoxalase.

The method described by ALEXANDER and BOYER[7] was modified as follows: To 70 μl of 10 mM methylglyoxal and 2.5 mM of reduced glutathione in 0.086 M potassium phosphate buffer (pH 7.4), 30 μl of the same buffer with or without the test material were added. After 3 minutes at 37°C, 10 μl of enzyme solution were added and the reaction mixture incubated for 10 minutes at 37°C. To 110 μl of the reaction mixture, 3 ml of 0.8 M semicarbazide-HCl in 0.1 M potassium phosphate buffer (pH 7.4) were added. After the residual methylglyoxal was converted to the disemicarbazone for 20 minutes at room temperature, the optical density at 286 nm of the reacted solution which was diluted 20 times with distilled water was determined. The percent inhibition of the test sample was calculated by the formula $(A-B)-(I_a-I_b)/(A-B)\times 100$, wherein A is the optical density obtained from the reaction mixture without addition of the enzyme and a test material, B is the optical density in presence of the enzyme but without the test material ($A-B$ is the amount of methylglyoxal consumed in the absence of the test material), I_a is the optical density obtained from the reaction solution with the test sample but without addition of the enzyme, I_b was that with both the test material and the enzyme (I_a-I_b is the amount of methylglyoxal consumed in the presence of the test material). In the absence of the test material, $A-B$ was in the range of 0.140 ± 0.01.

The crude glyoxalase was prepared as follows:[7] liver from 5 rats was homogenized with 4 volumes of 0.25 M sucrose in a Teflon-glass homogenizer. The homogenate was centrifuged successively at 15,000 g for 15 minutes and at 105,000 g for one hour. The supernatant thus obtained contained glyoxalases I and II. For the assay, 1 ml of this solution was diluted with 50 ml of distilled water. It was designated crude glyoxalase.

Method of testing the inhibition of glyoxalase I.

In the previous method, instead of the crude glyoxalase solution, glyoxalase I solution was added. This enzyme solution was prepared as follows:[8] 100 ml of 105,000 g centrifuged supernatant of rat liver was passed through a column (3.0×44.0 cm) of SP-Sephadex C-25 equilibrated and developed with 50 mM acetate buffer (pH 5.6). The effluent (270 ml) was passed through a column (2.0×31.0 cm) of CM-cellulose equilibrated with the same buffer; 260 ml of the active fraction were subjected to column (2.0×50.0 cm) on DEAE-cellulose equilibrated with the 25 mM Tris-HCl buffer (pH 7.8) and eluted with a linear gradient of $0\sim 0.5$ M NaCl in the same buffer; the active fractions (36 ml) were collected and concentrated to 5.0 ml using a collodion bag. For glyoxalase I assay, 1 ml of this solution was diluted with 25 ml of distilled water. This enzyme solution was designated rat liver glyoxalase I.

Yeast glyoxalase I, which was purchased from SigmaChemical Co. was dissolved at a concentration of 10 mcg protein/ml (distilled water) and used for the assay.

Chemical detection of the active compound inhibiting both glyoxalases of rat liver and yeast.

The test material dissolved in methanol was applied to the thin-layer silica gel plates (60F254, purchased from Merck Co.) and developed with chloroform - methanol (10 : 1). The spot was demonstrated by spraying with 2,3,5-triphenyltetrazolium chloride solution (2.0 % in 0.5 N sodium hydroxide in methanol). The glyoxalase I inhibitor showed a spot of Rf 0.75.

Fermentation and isolation of the inhibitor.

One hundred and twenty five ml of the medium which was described in the previous section was placed in a 500-ml of several flasks, and inoculated with strain MD287-CF4. Forty flasks containing the medium thus inoculated were placed on a reciprocating shaker (130 strokes per minute) at 27°C for 4 days. The pH of the cultured broth was as follows: 7.0 before inoculation, 6.5 after 2 days, 6.8 after 3 days, and 7.2 after 4 days. The culture broths of all flasks were combined, filtered to give 4,000 ml of filtrate. Addition of 15 μl of the filtrate to the reaction mixture for assay of glyoxalase showed 50 % inhibition. However, most of this activity was due to compounds which showed inhibition against crude glyoxalase of rat liver but not against yeast gloxalase I.

The filtrate was passed through a column of Amberlite XAD-II (800 ml) and after a wash with 5,000 ml of distilled water the active agent was eluted with 50 % aqueous acetone. The active eluate was concentrated under reduced pressure to 300 ml. The active agent in the concentrated solution was extracted twice with 300 ml of *n*-butanol at pH 2.0. Thus, 80 % of the activity against crude glyoxalase was transferred into the solvent. The extract was evaporated under reduced pressure to dryness (2.5 g of brown powder). The powder was dissolved in 100 ml of methanol and 400 ml of chloroform were added. The insoluble material was removed. The compounds which inhibited the crude glyoxalase of rat liver but not yeast glyoxalase I mostly remained in the insoluble material. To the soluble part, 10.0 g of silica gel (silicic acid, Mallinckrodt) were added and the mixture dried under reduced pressure. This solid was placed on a silica gel column (3×80 cm) which was prepared from 125 g of silica gel and chloroform-methanol (10 : 1, v/v), and the column was developed with chloroform-methanol (10 : 1). The eluate was collected as 20-g fractions. The inhibitor of both glyoxalases which could be identified by thin-layer chromatography appeared from the 50th to the 120th fractions. The fractions 50 to 80 were combined and evaporation yielded 520 mg of a brown powder. This powder was dissolved in 100 ml of methanol and after stirring with 52 mg of active carbon, was filtered. The filtrate was concentrated under reduced pressure to 10 ml. This concentrated solution was subjected to column chromatography on Sephadex LH-20 (300 ml) and developed with methanol. Then, the eluate (30 ml) which showed the presence of the active agent by thin-layer chromatography was evaporated, yielding 150 mg of a white powder. The powder was dissolved in 5.0 ml of methanol and after addition of 50 ml of chloroform the solution kept at 4°C overnight, yielding 82 mg of colorless needle crystals of the inhibitor of glyoxalase I.

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