STUDIES ON RELATIONSHIPS BETWEEN THE STRUCTURE AND THE GLYCOSIDASE-INHIBITING ACTIVITY OF HPAAO AND ITS ANALOGS

TADAHIKO HAZATO*, TAKAAKI AOYAGI and HAMAO UMEZAWA

Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan
*The Tokyo Metropolitan Institute of Medical Science
Honkomagome, Bunkyo-ku, Tokyo, Japan

(Received for publication May 27, 1978)

p-Hydroxyphenylacetaldoxime (HPAAO) (*anti* and *syn* forms) obtained by fermentation and its analogs chemically synthesized were tested for their activities to inhibit various glycosidases. HPAAO inhibited bovine liver β-galactosidase in a competitive manner at pH 7.0 with an apparent *Ki* value of 8×10^{-8} M. HPAAO also inhibited various mammalian β-glycosidases which had pH optima between 6.0 and 8.0. The *syn* form of HPAAO was found to be more active than the *anti* form against bovine liver neutral β-galactosidase. It was concluded that the oxime moiety of HPAAO and its analogs was essential for their enzyme-inhibiting activity and the activities of aromatic or aliphatic oxime derivatives were dependent on the number of carbon atoms in their alkyl-chains.

It has been demonstrated by many other researchers that the structural changes in the glycoproteins or glycolipids on the surface of mammalian cells results in a significant modulation of physiological function[1,2]. Specific enzyme inhibitors have been evaluated in their utility on these studies[3].

We have, therefore, searched for a specific glycosidase inhibitor in culture filtrates of actinomycetes. As reported in a previous paper[4], an active compound inhibiting β-galactosidase was found in a culture of *Streptomyces nigellus* and identified to be *p*-hydroxyphenylacetaldoxime (HPAAO).

This paper describes the structure-activity relationship of HPAAO and its analogs.

Materials and Methods

Materials

p-Nitrophenyl β-D-galactopyranoside and *o*-nitrophenyl β-D-galactopyranoside were purchased from BDH Chemical Ltd. (England). Other substrates were also obtained from commercial sources as described below, *m*-nitrophenyl β-D-galactopyranoside from Fluka AG. (Switzerland), phenolphthalein β-D-glucuronide, *p*-nitrophenyl N-acetyl-β-D-glucosaminide, *p*-nitrophenyl β-D-fucoside, *p*-nitrophenyl β-D-galactoside and 6-bromo-2-naphthyl β-D-galactopyranoside from Sigma Chemical Co.(U.S.A.), phenyl β-D-galactopyranoside from Nakarai Chemicals Co. (Kyoto, Japan). Other chemicals were purchased from Tokyo Kasei Co. (Tokyo, Japan).

Bovine liver and *Escherichia coli* β-galactosidases were purchased from Sigma Chemical Co., charonia lamps and sweet almond β-glucosidases were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), human liver and small intestine were kindly supplied by Dr. Y. SEYAMA (Dept. of Biochemistry, School of Medicine, Univ. of Tokyo), human placenta was given by Dr. S. HAZATO (Dept. of Gynecology, Medical School of Toho Univ.), pig and bovine organs were obtained from Shibaura Slaughterhouse (Tokyo), rats (Wister strain) were obtained from Shizuoka Experimental Animal Co. (Shizuoka, Japan).

Bovine liver neutral β-galactosidase was purified by the method of CHYTIL[5]. Other enzymes that were not obtained from commercial sources were used after 25~80% ammonium sulfate precipitation step from above-mentioned respective organs.

β-Galactosidase assay

The method of DAHLQVIST and ASP[6] was used with a slight modification. The reaction mixture consisted of 0.4 ml of a buffer, selected 0.05 M Tris-HCl buffer (pH 6.0~7.0) or a 0.05 M citrate-phosphate buffer (pH 3.5~5.0), 0.05 ml of 5 mM *p*-nitrophenyl β-D-galactopyranoside and 0.04 ml of distilled water with or without a test material. It was incubated for 3 minutes at 37°C, then 0.01 ml of an enzyme solution was added. After incubated for 15 minutes at 37°C, the reaction was stopped by the addition of 2 ml of 0.4 M glycine-NaOH buffer, pH 10.5. The amount of the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm after centrifugation at 1,500 *g* for 10 minutes.

β-Glucuronidase assay

Liberation of phenolphthalein from 10 mM phenolphthalein β-D-glucuronide in 0.1 M citrate-NaOH buffer (pH 4.8) was measured according to the method of CONCHIE *et al.*[7].

N-Acetyl-β-D-glucosaminidase assay

Liberation of *p*-nitrophenol from 25 mM *p*-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M citrate-NaOH buffer (pH 4.5) was measured by the procedure of ASP[8].

Other glycosidase assay: β-Galactosidase, β-glucosidase and β-fucosidase activities were measured according to the procedures described by CONCHIE *et al.*[9].

Preparation of oxime derivatives

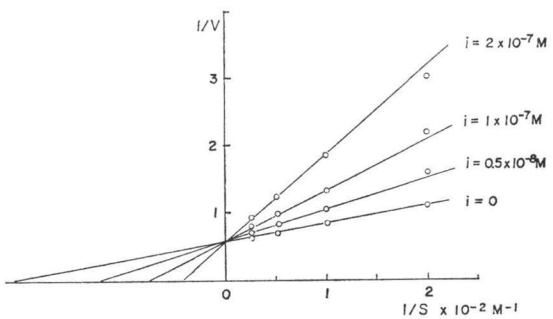
HPAAO containing the *anti* form at 80% was prepared by fermentation of *Streptomyces nigellus*. HPAAO containing the *syn* form 70% was chemically synthesized as described in our previous paper[4]. Various oxime derivatives were prepared by reacting respective aldehydes with hydroxylamine hydrochloride by the method of BOUSQUET[10]. N-(2-Hydroxylamino-3-methylbutyl)crotonamide, an intermediate in the chemical synthesis of dopastin[13], was a kind gift from Dr. H. IINUMA of the Institute of Microbial Chemistry. *Anti* form and *syn* form of oxime compounds were distinguished by the methylene and methine proton signals of their NMR spectrum.

Results and Discussion

Various glycosidases were prepared from liver (bovine, pig, rat, human), small intestinal mucosa (bovine, pig, rat, human), kidney (bovine, pig, rat), placenta (human), charonia lamps, *E. coli* and sweet almond. As shown in Table 1, only mammalian β-galactosidase, β-glucosidase and β-fucosidase, which have pH optima in the neutral region (pH 6~8), were strongly inhibited by HPAAO.

LINEWEAVER-BURK plots for the action of HPAAO (about 80% *anti* form) to inhibit bovine liver neutral β-galactosidase are shown in Fig. 1. The substrate (*p*-nitrophenyl β-D-galactopyranoside) concentration was changed from 5 mM to 40 mM. It was demonstrated that HPAAO inhibited β-galactosidase in a competitive manner. The apparent *Ki* value was calculated to be 8×10^{-8} M. All the oxime analogs synthesized also inhibited β-galactosidase competitively.

Fig. 1. LINEWEAVER-BURK plots for the inhibition of bovine liver neutral β-galactosidase by HPAAO.
The concentration of *p*-nitrophenyl β-D-galactopyranoside varied from 5 mM to 40 mM. Bovine liver neutral β-galactosidase was purified according to the method of CHYTIL[5].
37°C, pH 7.0, 0.05 M Tris-HCl buffer. $Km=3.0\times10^{-3}$ M, $Ki=8.0\times10^{-8}$ M.



The activity of HPAAO to inhibit bovine liver neutral β-galactosidase was measured using various substrates. As shown in Table 2, HPAAO inhibited the hydrolysis of *p*-nitrophenyl β-D-galactopyranoside, *o*-nitrophenyl β-D-galac-

Table 1. Inhibitory activities of *p*-hydroxyphenylacetaldoxime (HPAAO)* against various glycosidases.

Enzymes	Sources		Substrates***	pH optima	Inhibition % ($\times 10^{-5}$ M)
β-Galactosidase	Bovine	Liver	PNP-β-Gal	7.0	89
		S. intes. muc.**		4.5	26
		Kidney		4.0	10
	Pig	Liver		8.0	84
		S. intes. muc.**		7.5	86
		Kidney		8.0	90
	Rat	Liver		3.5	0
		S. intes. muc.**		4.5	0
		Kidney		4.5	0
	Human	Liver		4.5	0
		S. intes. muc.**		4.5	0
		Placenta		4.5	0
	Charonia lamps			4.0	0
	E. coli			4.2	0
	Streptomyces			7.0	0
α-Galactosidase	Bovine liver		PNP-α-Gal	6.0	0
β-Glucosidase	Bovine liver		PNP-β-Gal	6.5	82
	Sweet almond			5.0	0
β-Glucronidase	Bovine liver		PPGA	4.8	0
β-Fucosidase	Bovine liver		PNP-β-Fuc	7.0	80
N-Acetyl β-D-glucosaminidase	Bovine liver		Ph-β-GlcNAc	4.2	0

* The ratio of the *anti* to the *syn* form; 8: 2.

** Small intestinal mucosa

*** PNP-; *p*-Nitrophenyl-, PPGA; phenolphthalein β-D-glucuronic acid
Ph-β-GlcNAc; phenyl-N-acetyl β-D-glucosaminide

Table 2. Inhibitory activity of HPAAO* against bovine liver neutral β-galactosidase using various substrates.

The activities to inhibit the hydrolysis of *p*-nitrophenyl β-D-galactopyranoside (5×10^{-3} M), *o*-nitrophenyl β-D-galactopyranoside (5×10^{-3} M), *m*-nitrophenyl β-D-galactopyranoside (5×10^{-4} M), phenyl β-D-galactopyranoside (2×10^{-3} M), and 6-bromo-2-naphthyl β-D-galactopyranoside (4×10^{-3} M) by bovine liver neutral β-galactosidase in 0.05 M phosphate buffer at pH 7.0 was measured.

IC_{50} was calculated as described previously[12].

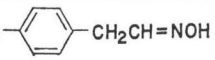
Substrates	IC_{50} (μM)
p-Nitrophenyl β-D-galactopyranoside	0.10
o-Nitrophenyl β-D-galactopyranoside	0.15
m-Nitrophenyl β-D-galactopyranoside	0.35
Phenyl β-D-galactopyranoside	1.80
6-Bromo-2-naphthyl β-D-galactopyranoside	320

* The ratio of the *anti* to the *syn* form; 8: 2.

topyranoside, *m*-nitrophenyl β-D-galactopyranoside and phenyl β-D-galactopyranoside by β-galactosidases but not the hydrolysis of 6-bromo-2-naphthyl β-D-galactopyranoside[11].

As shown in Table 3, the elimination of the aromatic hydroxyl group of HPAAO did not change markedly the original inhibitory activity. However, when the oxime moiety of HPAAO was replaced by other functional groups such as nitrile, aldehyde, carboxyl, hydroxymethyl, aminomethyl and

Table 3. Activities of HPAAO* and its analogs to inhibit bovine liver neutral β-galactosidase.

Compounds	Structures	IC_{50} (μg/ml)
p-Hydroxyphenylacetaldoxime		0.015
Phenylacetaldoxime	$PhCH_2CH{=}NOH$	0.016
Propionaldoxime	$CH_3CH_2CH{=}NOH$	4
Phenylacetonitrile	$PhCH_2CN$	>100
Phenylacetaldehyde	$PhCH_2CHO$	>100
Phenylacetic acid	$PhCH_2COOH$	>100
β-Phenylethylalcohol	$PhCH_2CH_2OH$	>100
β-Phenylethylamine	$PhCH_2CH_2NH_2$	>100
β-Phenylethylchloride	$PhCH_2CH_2Cl$	>100
Phenylacetaldoxime acetate	$PhCH_2CH{=}NOCOCH_3$	>100

* The ratio of the *anti* to the *syn* form; 8: 2.

Table 4. The activity of *syn* and *anti* oxime compounds to inhibit bovine liver neutral β-galactosidase.

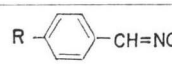
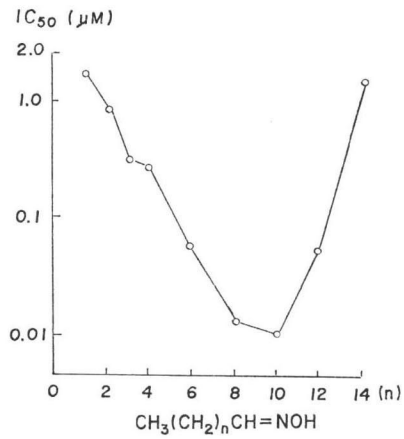
Compounds	Type of oxime	IC_{50} (μg/ml)
		
R=H	*Syn*	1.1
R=H	*Anti*	3.2
R=OH	*Syn*	3.2
$R=CH_3$	*Syn*	2.2
$R=NO_2$	*Syn*	1.5
R=Br	*Syn*	0.5
$Ph(CH_2)_nCH{=}NOH$		
n=1	*Syn*	0.016
n=2	*Syn*	0.004

Fig. 2. The activities of *n*-alkylaldoximes to inhibit bovine liver neutral β-galactosidase.



chloromethyl groups, the enzyme-inhibiting activity was eliminated. The O-acetylation of the oxime moiety also eliminated the activity. Thus, the oxime moiety of HPAAO was shown as the key structure required for the inhibition of β-galactosidase. Whether the aromatic ring of HPAAO should contribute to the activity or not was studied by testing the activity of chemically synthesized aliphatic oximes. As shown in Table 3, the aromatic ring was not essential for the action to inhibit β-galactosidase. In addition to the aldoxime compounds shown in Table 3, ketoxime compounds inhibited β-galactosidase. Namely, N-(2-hydroximino-3-methylbutyl)crotonamide and acetoxime inhibited bovine liver neutral β-galactosidase with IC_{50} values 4.6 and 6.0 μg/ml, respectively.

The chemically synthesized *anti* and *syn* forms of benzaldoxime and *syn*-HPAAO derivatives were measured for their activities to inhibit bovine liver neutral β-galactosidase. The results are shown in Table 4. In case of HPAAO, it was proved that the *syn* form was more active than the *anti* form. Also *syn*-benzaldoxime showed a stronger inhibitory activity than *anti*-benzaldoxime. The compound having phenethyl group was shown to be more active than the one having benzyl group. This result and the data shown in Table 3 suggested that there was a relationship between the length of

the alkyl chain and the activity. As shown in Fig. 2, the activities of alkylaldoximes were dependent on the number of carbon atoms in their alkyl chains. Among these compounds the one having 12 carbon atoms in the alkyl chain was found to be the strongest inhibitor. Its IC_{50} value was calculated as 0.002 μg/ml (1×10^{-8} M) and was 10 times lower than the IC_{50} value of *anti*-HPAAO (1.1×10^{-7} M).

Acknowledgements

This work was partly supported by a contract research fund from the Division of Cancer Treatment, the National Cancer Institute, U.S.A., NO1-CM-57009 and a grant-in-aid for Cancer Research 51-11 from the Ministry of Health and Welfare.

References

1) BRYAN, M. T.; N. G. BERATIS & K. HIRSCHHORN: Cell-specific differences in membrane β-glucosidase from normal and GAUCHER cells. Biochim. Biophys. Acta 480: 442~449, 1977
2) CHRISTIAN, H. B.; P. VESCHER, H. GRÜNHOLZ & W. REUTTER: Glycosyltransferases and glycosidases in MORRIS hepatomas. Cancer Res. 37: 1513~1518, 1977
3) NEROME, K.; M. KUMAGAI & T. AOYAGI: Effect of panosialin on myxoviruses. Arch. Ges. Virusforsh. 39: 353~359, 1972
4) HAZATO, T.; M. KUMAGAI, H. NAGANAWA, T. AOYAGI & H. UMEZAWA: *p*-Hydroxyphenylacetaldoxime, an inhibitor of β-galactosidase, produced by actinomycetes. J. Antibiotics 32: 91~93, 1979
5) CHYTIL, F.: Mammalian β-galactosidase. Biochem. Biophys. Res. Commun. 19: 630~636, 1965
6) DAHLQVIST, A. & G. ASP: Rat small-intestinal β-galactosidase, influence of pH on the hydrolysis of different substrate. Biochem. J. 103: 83~89, 1967
7) CONCHIE, J.; A. J. HAY & G. A. LEVVY: Mammalian glycosidase. 3. The intracellular localization of β-glucuronidase in different mammalian tissues. Biochem. J. 79: 324~330, 1961
8) ASP, N. G.: Improved method for the assay of phenylglycosidase activity with a 4-aminoantipyrine reagent. Anal. Biochem. 40: 281~286, 1971
9) CONCHIE, J.; J. FINDLAY & G. A. LEVVY: Mammalian glycosidase, distribution in the body. Biochem. J. 71: 318~325, 1959
10) BOUSQUET, E. W.: Organic Synthesis. Collective volume 2, pp. 313~315, *Ed.* by A. H. BLATT, John Wiley & Sons, 1943
11) DAHLQVIST, A.; B. BULL & B. E. GUSTAFSSON: Rat intestinal 6-bromo-2-naphthyl-glycosidase and disaccharidase activities. I. Enzymic properties and distribution in the digestive tract of conventional and germ-free animals. Arch. Biochem. Biophys. 109: 150~158, 1965
12) UMEZAWA, H.: Enzyme Inhibitors of Microbial Origin. pp. 11~13, University of Tokyo Press, Tokyo, 1972
13) IINUMA, H.; M. MATSUZAKI, T. NAGATSU, T. TAKEUCHI & H. UMEZAWA: Biochemical and biological studies on dopastin, an inhibitor of dopamine β-hydroxylase. Agr. Biol. Chem. 38: 2099~2105, 1974