# INHIBITORY ACTIVITY OF PYRIDINDOLOL ON β-GALACTOSIDASE

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The activity of pyridindolol in inhibiting β-galactosidases obtained from various sources has been studied. Whereas acid bovine liver β-galactosidase (optimal pH 4.0) was not affected by this compound, neutral bovine liver β-galactosidase (pH-optimum =7.0) was inhibited by pyridindolol in reaction mixtures of pH 4.0~5.0. There was no inhibition at pH 7.0. The type of inhibition is non-competitive by formation of a pyridindolol-enzyme complex. Since β-galactosidases from other sources are not affected by pyridindolol, the inhibitory action of this compound seems to be rather specific for neutral bovine liver β-galactosidase.

β-Galactosidase (Lactase, EC 3.2.1.23) is a glycosidase which hydrolyzes β-galactoside bonds under releasing of galactose and is widely distributed among microorganisms, plants and animals. It was recently reported that β-galactosidase activity in fibroblasts shows a marked increase after transformation by oncogenic viruses,[1] suggesting an involvement of this enzyme in oncogenicity.

There are many reports on β-galactosidase from *Escherichia coli*,[2~5] but there are very few on the action mechanism of this enzyme of animal origin.

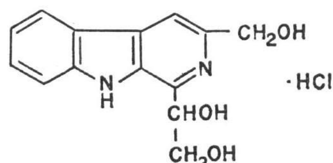
As previously reported,[6,7] we isolated pyridindolol from *Streptomyces alboverticillus* culture filtrates as a specific inhibitor of neutral bovine liver β-galactosidase. In this paper, we report the action of this compound in detail and discuss its mechanism.

## Materials and Methods

Pyridindolol (1-[1(*R*), 2-dihydroxyethyl]-3-hydroxymethyl-9*H*-pyrido (3, 4-*b*) indole) hydrochloride as shown below was prepared according to the method described in a previous paper.[6,7]

Chemicals:

*p*-Nitrophenyl β-D-galactopyranoside (PNPG) and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were purchased from BDH Chemical Ltd. (Poole, England), phenyl β-D-galactopyranoside from Nakarai Chemicals (Kyoto, Japan), 6-bromo-2-naphthyl β-D-galactopyranoside and harman·HCl from Sigma Chemical Co. (St. Louis, U. S. A.) and harmine·HCl from Aldrich Chemical Co. (Milwaukee, U. S. A.).




Enzymes:

Two types of bovine liver β-galactosidases which have reaction optima in acid or in neutral media were prepared according to the method described by CHYTIL[8] from the commercial preparation of bovine liver β-galactosidase purchased from Sigma. β-Galactosidases from other animals were prepared by methods described by HAY *et al.*[9]: Fresh bovine and pig organs were obtained from the slaughter-house; rats of the Wistar strain were decapitated and their organs were cooled at 0°C. Human liver and small intestine were supplied by Dr. Y. SEYAMA,

Department of Biochemistry, School of Medicine, the University of Tokyo; human placenta and amnion were kindly given by Dr. S. HAZATO, Department of Gynecology, Medical School of Toho University. The organs were minced, homogenized in cold water and subsequently incubated for 1 hour in acetate buffer of pH 5.2 at 37°C. The clear extracts were fractionated by addition of $(NH_4)_2SO_4$. Fractions which precipitated at 25~80% saturation were collected and kept frozen at −20°C until used.

Assay of enzyme activities:

The following buffers were employed for reactions at various pH-values: Phthalate-hydrochloric acid buffer[10] (0.2 M $KHC_8H_4O_4$+0.2 M HCl, diluted two fold) for pH 2.2~2.4; citrate-phosphate buffer[10] (0.1 M citric acid+0.2 M $Na_2HPO_4$, diluted two fold) for pH 2.4~7.0; phosphate buffer[10] (0.2 M $NaH_2PO_4$+0.2 M $Na_2HPO_4$, diluted two fold) for pH 7.2~8.0; tris-acetate buffer[4] (0.05 M) for pH 4.2~7.5.

When PNPG or ONPG was used as substrate, the assay was carried out as described by ASP *et al.*[11]: Each tube contained 0.05 ml of PNPG or ONPG (0.05 M), 0.4 ml of buffer and 0.025 ml water or inhibitor solution. After 3-minute preincubation at 37°C, the reaction was started by addition of 0.025 ml of enzyme solution. After suitable time of incubation (5~60 minutes) at 37°C, 2 ml of 0.4 M glycine-NaOH buffer of pH 10.5 were added and the amount of liberated *p*- or *o*-nitrophenol was measured spectrophotometrically at 400 nm or 420 nm, respectively.

When phenyl β-D-galactopyranoside was used as substrate, the assay was carried out as described by ASP.[12] The incubation mixture contained 0.05 ml of phenyl β-D-galactopyranoside (0.020 M), 0.05 ml buffer or buffer containing an inhibitor. After 3-minute incubation at 37°C, the reaction was started by addition of 0.4 ml of an enzyme solution preincubated in buffer. After 20 minutes at 37°C, 0.02 ml of 1 N HCl was added. The mixture was heated at 100°C for 2 minutes, neutralized by addition of 0.02 ml of 1 N NaOH and was subsequently prepared for the color reaction by addition of 1.5 ml of 1 M tris-HCl buffer (pH 8.5) containing 4-aminoantipyrine (0.2 mg/ml) and ethanol (1.0%) followed by addition of 0.5 ml of 0.4% potassium ferricyanide. The absorbance was read at 510 nm.

When 6-bromo-2-naphthyl β-D-galactopyranoside was used as substrate,[13] the reaction mixture consisting of 0.01 ml of 0.04 M 6-bromo-2-naphthyl β-D-galactopyranoside and 0.06 ml of buffer or buffer containing an inhibitor was preincubated at 37°C for 3 minutes. The reaction was started by addition of 0.03 ml of the enzyme solution. After 60 minutes at 37°C, the reaction was stopped by heating at 100°C for 2 minutes. The reaction mixture was cooled and 0.02 ml of 1 M tris-HCl buffer (pH 7.7) and 0.02 ml of fast blue B salt solution (1 mg/ml) were added. After further addition of 0.04 ml of 80% trichloroacetic acid solution, the mixture was shaken with 2 ml of chloroform. After centrifugation at 3,000 rpm for 3 minutes, the chloroform layer was pippetted and the absorbance was read at 580 nm.

Enzyme-inhibitor interaction:

The interaction between pyridindolol and the enzyme was studied by the gel filtration method of FRUTON *et al.*[14]: A Sephadex G-15 column (0.5×150 cm) was equilibrated with 0.05 M citrate-phosphate buffer (pH 4.2 or 7.0) containing $2\times10^{-7}$ M pyridindolol. Five mg of a purified neutral bovine liver β-galactosidase were dissolved in 1.0 ml of the same buffer and after 30 minutes at room temperature, the enzyme solution was applied to the column. The elution was carried out with the same buffer and fractions of 1 ml each were collected. Each fraction was diluted to 3 ml with 0.1 N HCl and the fluorescence intensity at 450 nm of each fraction was measured with a Hitachi 203 fluorescence spectrophotometer with exciting light of 380 nm. The fluorescence is due to the presence of pyridindolol.

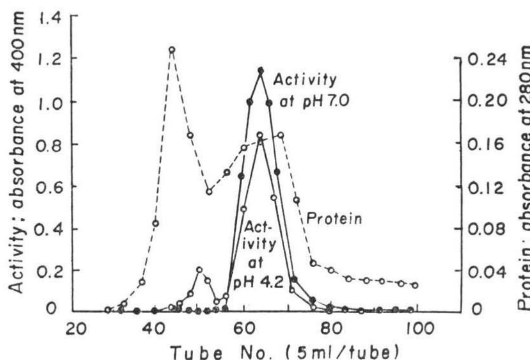
## Results

In the previous report we employed a commercial preparation of bovine liver β-galactosidase.

In order to elucidate the inhibition mechanism by pyridindolol, it was necessary to use a more purified enzyme. When the commercial bovine liver β-galactosidase obtained from Sigma was applied to a Sephadex G-100 column, and the enzymatic activity of eluted fractions was measured at pH 4.2, using PNPG as substrate, two peaks appeared in fr. No. 48~59 and fr. No. 59~78. The latter peak, representing neutral β-galactosidase, showed a higher activity than the former peak of acid β-galactosidase (Fig. 1). The activity of the neutral enzyme peak at pH 7.0 is also shown in Fig. 1. This enzyme was rechromatographed on a Sephadex G-200 column.

Fig. 1. Separation of the β-galactosidases of bovine liver on a Sephadex G-100 column

The enzymes were eluted from the column by 0.05 M tris-HCl buffer, pH 7.0, containing 0.1 M KCl. Enzyme activity was measured at pH 4.2 with 0.05 M citrate-phosphate buffer (incubation time 1 hour) and at pH 7.0 with 0.05 M phosphate buffer (incubation time 0.5 hour) using *p*-nitrophenyl β-D-galactopyranoside as substrate. Absorbance at 280 nm was measured as the protein concentration.

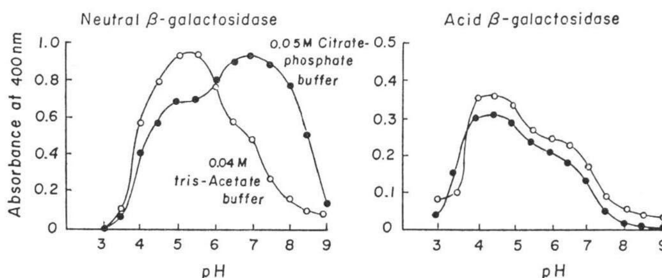



## pH Dependence of Enzyme Activity

The pH activity relationship of the two β-galactosidases described above, using tris-acetate buffer and citrate-phosphate buffer is

Fig. 2. pH Dependence of acid and neutral bovine liver β-galactosidases

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactopyranoside ($5\times10^{-3}$ M) in 0.04 M tris-acetate buffer and 0.05 M citrate-phosphate buffer.




shown in Fig. 2. The acid β-galactosidase was found to have an optimum pH of 4.2 in both buffers. On the other hand, it was found that neutral β-galactosidase has different optimal pH values depending on buffers; pH 5.0 in tris-acetate buffer and pH 6.5~7.0 in citrate-phosphate buffer.

## Inhibition of Neutral Bovine Liver β-Galactosidase by Pyridindolol

Fig. 3 shows the inhibition of neutral β-galactosidase by various concentrations of pyridindolol at pH 4.5 and 7.0; $7.4\times10^{-6}$ M of this compound showed 50% inhibition of the enzyme activity in the acid assay system. However, the activity of β-galactosidase in the neutral medium was inhibited only weakly by pyridindolol.

## Influence of pH on the Inhibiting Activity of Pyridindolol

As shown in Fig. 4, the pH dependence curve of the inhibiting activity of pyridindolol is

Fig. 3. Inhibition of neutral bovine liver β-galactosidase by varying concentrations of pyridindolol

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactopyranoside ($5\times10^{-3}$ M) in 0.04 M tris-acetate buffer at pH 4.5 and at pH 7.0. Percent inhibition was calculated as follows:

$$\text{Inhibition } (\%)=[1-(I-I_0/S-S_0)]\times100$$

$S$: Absorbance at 400 nm without pyridindolol
$S_0$: Absorbance at 400 nm without pyridindolol blank
$I$: Absorbance at 400 nm within pyridindolol
$I_0$: Absorbance at 400 nm within pyridindolol blank

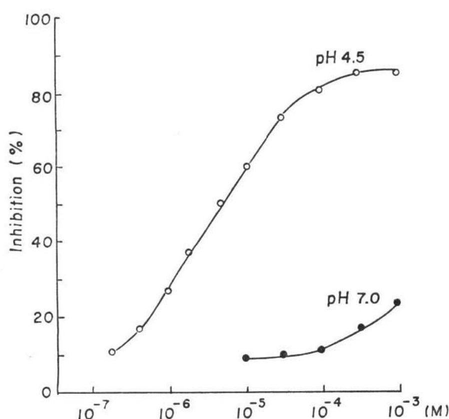
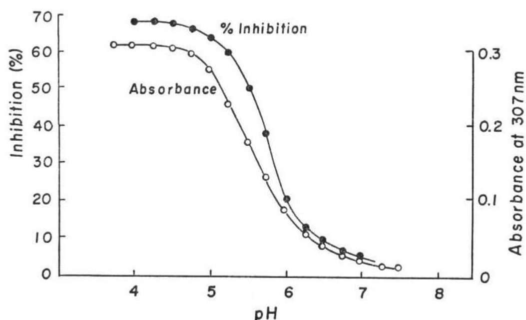



Fig. 4. The influence of pH on the inhibitory activity of pyridindolol against the bovine liver β-galactosidase and titration curve of pyridindolol

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactopyranoside with ($2\times10^{-5}$ M) or without pyridindolol·HCl in 0.04 M tris-acetate buffer. Percent inhibition was calculated as shown in Fig. 3. Titration curve was drawn by determination of pyridindolol absorbance at 307 nm in 0.05 M citrate-phosphate buffer.



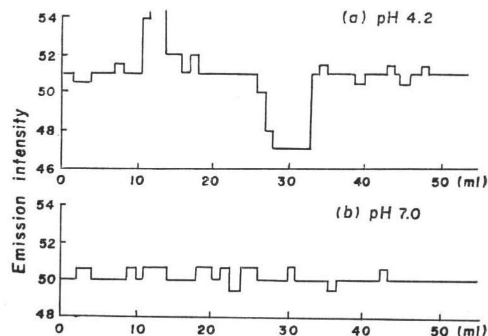

compared with the pH titration curve of the inhibitor, which indicates the relative amounts of the protonated and the base form of pyridindolol. The two curves closely resemble each other, indicating the same inflection point at pH 5.4~5.6 which corresponds to a pKá value (5.35) of pyridindolol. These results suggest that the protonated pyridindolol is more potent as an inhibitor than its unchanged base.

Enzyme-Inhibitor Interaction

The interaction between neutral bovine liver β-galactosidase and pyridindolol was studied by the gel filtration method. Fig. 5a shows the elution diagram at pH 4.2. The β-galactosidase was eluted in the 12 ml~16 ml fractions, and the fluorescence intensity of pyridindolol decreased in the 27~37 ml fractions. The decrease in the pyridindolol concentration suggests a binding of pyridindolol to the enzyme and an elution of this compound together with the enzyme. Fig. 5b represents the elution diagram at pH 7.0. In this case the enzyme was eluted at the same fraction as in the experiment at pH 4.2, but a decrease

Fig. 5. Elution diagrams of pyridindolol and β-galactosidase system from Sephadex G-15 upon pH 4.2 and pH 7.0

The interaction between the neutral bovine liver β-galactosidase and pyridindolol was carried out as described in the text. The elution diagram was obtained by analysis of individual fractions of effluent solution diluted 3 fold with 0.1 N HCl. The fluorescence emission intensity ($\lambda_{em}$ 450 nm) was determined by use of exciting light of 380 nm.



in the fluorescence was not observed, indicating a much weaker binding of pyridindolol to the enzyme at pH 7.0 than at pH 4.2.

## Effect of Pyridindolol on Other β-Galactosidase

Other β-galactosidases were prepared from small intestinal mucosa (human, bovine, pig, rat), kidney (bovine, pig), ovary (bovine, pig), heart (bovine), placenta (human) and amnion (human) and used for the study of the inhibiting effect of pyridindolol on the activities of these enzymes. As shown in Table 1, pyridindolol has no effect on these β-galactosidases except for the bovine liver β-galactosidase. Thus, pyridindolol is a specific inhibitor for neutral bovine liver β-galactosidase in acid reaction mixtures.

Table 1. Inhibitory effects of pyridindolol on various β-galactosidases

| Sources | | Inhibition (%) | | | |
|---|---|---|---|---|---|
| Organs | Animals | pH 4.2 | | pH 7.0 | |
| | | $10^{-3}$ M | $10^{-5}$ M | $10^{-3}$ M | $10^{-5}$ M |
| Liver | Human | — | — | — | — |
| | Bovine | 85 | 60 | 23 | 10 |
| | Pig | — | — | — | — |
| | Rat | — | — | — | — |
| Small intestinal mucosa | Human | — | — | — | — |
| | Bovine | — | — | 10 | — |
| | Pig | — | — | — | — |
| | Rat | — | — | — | — |
| Kidney | Bovine | — | — | — | — |
| | Pig | — | — | — | — |
| Ovary | Bovine | — | — | — | — |
| | Pig | — | — | — | — |
| Heart | Bovine | — | — | — | — |
| Placenta | Human | — | — | — | — |
| Amnion | Human | — | — | — | — |

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactopyranoside ($5\times10^{-3}$ M) in 0.05 M pH 4.2 or pH 7.0 citrate-phosphate buffer. Percent inhibition was calculated as described in Fig. 3. (—) indicates that the inhibition was less than 10%.

Table 2. Inhibitory activities of pyridindolol on the enzymatic hydrolysis of four substrates at pH 4.2

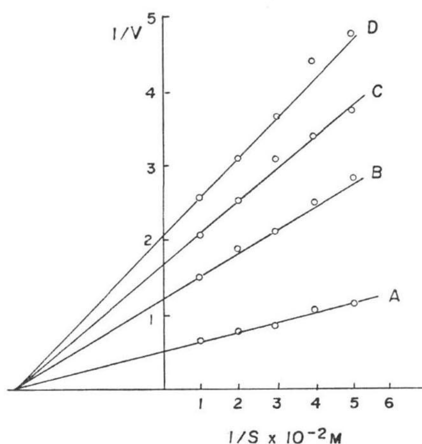
| Substrates | Inhibition | |
|---|---|---|
| | % with $10^{-5}$M | $I_{50}$ M |
| *p*-Nitrophenyl β-D-galactopyranoside | 60 | $7.4\times10^{-6}$ |
| *o*-Nitrophenyl β-D-galactopyranoside | 56 | $7.6\times10^{-6}$ |
| Phenyl β-D galactopyranoside | 26 | $5\times10^{-2}$ |
| 6-Bromo-2-naphthyl β-D-galactopyranoside | — | — |

Enzyme activities were measured with *p*-nitrophenyl β-D-galactopyranoside ($5\times10^{-3}$ M), *o*-nitrophenyl β-D-galactopyranoside ($5\times10^{-3}$ M), phenyl β-D-galactopyranoside ($2\times10^{-3}$ M) and 6-bromo-2-naphthyl β-D-galactopyranoside ($4\times10^{-3}$ M) in 0.05 M citrate-phosphate buffer at pH 4.2. Percent inhibitions were calculated as shown in Fig. 3. $I_{50}$ (M) were derived graphically. (—) indicates that the inhibition observed was less than 10%.

Fig. 6. Reciprocal plots for the inhibitory effect of pyridindolol on neutral bovine liver β-galactosidase

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactopyranoside ($10^{-2}$ M, $5\times10^{-3}$ M, $3.33\times10^{-3}$ M, $2.5\times10^{-3}$ M, and $2\times10^{-3}$ M) in the presence of pyridindolol·HCl ($D=5\times10^{-6}$ M, $C=2.5\times10^{-6}$ M, $B=1.25\times10^{-6}$ M and $A=0$) in 0.04 M tris-acetate buffer pH 4.5.

$Km=2.5\times10^{-3}$ M, $Ki=2\times10^{-6}$ M



## Effect of Substrates on the Inhibitory Activity of Pyridindolol

Various kinds of substrates were examined for their inhibition of pyridindolol action. Table 2 shows the percent inhibition of the enzymatic activity by pyridindolol at the concentration of $10^{-5}$ M and the $I_{50}$ values which indicate the molar concentration of the inhibitor for 50% inhibition. When PNPG or ONPG were employed, the $I_{50}$ values were approximately $7.5\times10^{-6}$ M. But, when phenyl β-D-galactopyranoside was used as a substrate, the $I_{50}$ increased to the order of $10^{-2}$ M.

## Kinetic Parameters

A LINEWEAVER-BURK plot of the inhibitory action of pyridindolol on neutral bovine liver β-galactosidase using pH 4.5 tris-acetate buffer is shown in Fig. 6. As the concentration of substrate increased, the slope of the line also increased. Therefore, it is clear that pyridindolol inhibits β-galactosidase in a non-competitive manner. The Km and Ki values with PNPG as substrate are $2.5\times10^{-3}$ M and $2\times10^{-6}$ M, respectively.

Table 3. Inhibition of β-galactosidase by pyridindolol and related compounds

| Compound | Structure | MW | pKá | Inhibition % | |
|---|---|---|---|---|---|
| | | | | $10^{-3}$ M | $10^{-5}$ M |
| Pyridindolol·HCl | $CH_2OH$, N, ·HCl, N H, CHOH, $CH_2OH$ | 294.8 | 5.35 | 85 | 57 |
| Pyridindolol triacetate | $CH_2OAc$, N, N H, CHOAc, $CH_2OAc$ | 384.8 | | — | — |
| Harman·HCl | N, ·HCl, N H, $CH_3$ | 218 | 6.10 | 21 | — |
| Harmine·HCl·$H_2O$ | $CH_3O$, N H, N, ·HCl, ·$H_2O$, $CH_3$ | 248 | 6.50<br>10.70 | 17 | — |
| Harmol·HCl·$H_2O$ | HO, N H, N, ·HCl, ·$H_2O$, $CH_3$ | 252.7 | 6.70<br>11.20 | — | — |
| 2, 6-Pyridine dimethanol | $HOH_2C$, N, $CH_2OH$ | 139.2 | 4.35 | — | — |
| Ethylene glycol | $HOCH_2-CH_2OH$ | 62.1 | | — | — |
| Propylene glycol | $CH_3CH(OH)-CH_2OH$ | 76.1 | | — | — |

Enzyme activities were measured by hydrolysis of *p*-nitrophenyl β-D-galactoside ($5\times10^{-3}$ M) in the presence ($10^{-3}$ M and $10^{-5}$ M) and in the absence of compounds in 0.05 M pH 4.2 citrate-phosphate buffer. Percent inhibitions were calculated as described in Fig. 3.

The Activity of Pyridindolol Analogs

The inhibitory activities of pyridindolol analogs in concentrations of $10^{-3}$ M and $10^{-5}$ M at pH 4.2, their chemical structure and pKá values are shown in Table 3. The results suggest that both hydroxy groups and the β-carboline skeleton are essential for the activity of pyridindolol in inhibiting neutral β-galactosidase in acid reaction media.

Influences of Metal Ions, Cysteine and Chelating Agents

Studies on the effects of various kinds of metal ions on the inhibitory activity of pyridindolol against the neutral bovine liver β-galactosidase revealed that generally metal ions ($10^{-4}$ M) did not interfere with the action of pyridindolol, except that mercuric ions inhibited the β-galactosidase. L-Cysteine ($10^{-4}$ M) and EDTA ($10^{-4}$ M) had also no effect on the inhibitory activity of pyridindolol.

## Discussion

The data described above indicate that hydroxy groups and the β-carboline skeleton of pyridindolol are essential for the inhibitory activity on the enzymatic action of neutral bovine liver β-galactosidase. But, at present, we cannot explain why pyridindolol is a specific inhibitor of only neutral bovine liver β-galactosidase and why it has no effect on the enzymes for other sources. HAY *et al.*[9] reported that bovine liver β-galactosidase and kidney β-galactosidase are similar and classified these enzymes into the same category. However, the data described in this paper indicate that bovine liver β-galactosidase, at least the neutral enzyme, differs from bovine kidney β-galactosidase in its behavior against the inhibitor.

WALLENFELS *et al.*[5] summarized that the hydrolysis of ONPG by β-galactosidase from *E. coli* K-12 is inhibited competitively by D-galactose, lactose and some other β-D-galactosides. They also summarized that phenyl thiogalactoside has almost the same affinity for the enzyme of *E. coli* ML as phenyl β-D-galactoside. HAY *et al.*[9] reported that galactono (1→4) lactone inhibits the β-galactosidase competitively. LEE *et al.*[16] described that D-galactal inhibits various β-galactosidases and that its inhibition is competitive or of a type of hyperbolic competition. These inhibitors are all substrate analogs. WALLENFELS *et al.*[5] suggested that the inhibitory actions of these compounds are probably due to the formation of a half-chair conformation and inhibitors in this conformation resemble the carbonium ion of the substrate in the transition state. The inhibitors are supposed to bind to a negative charge group in the active site of the enzyme.

Although pyridindolol cannot form a half-chair conformation for its very rigid structure, the data described above indicate that the protonated pyridindolol is active and binds to a negative group in the neutral bovine liver β-galactosidase. Similar observations were made in the case of the eserine, which is a competitive inhibitor of acetylcholine esterase.[17] The non-competitive type of inhibition and the behavior on gel filtration of the neutral bovine liver β-galactosidase-pyridindolol mixture indicates the formation of a pyridindolol-enzyme complex.

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