

Selected Papers

Glycomonomeric and Glycopolymeric Inhibitors for β-Glucuronidase. π–π Stacking Interaction, Polymeric Effect, and Plausible Conformation

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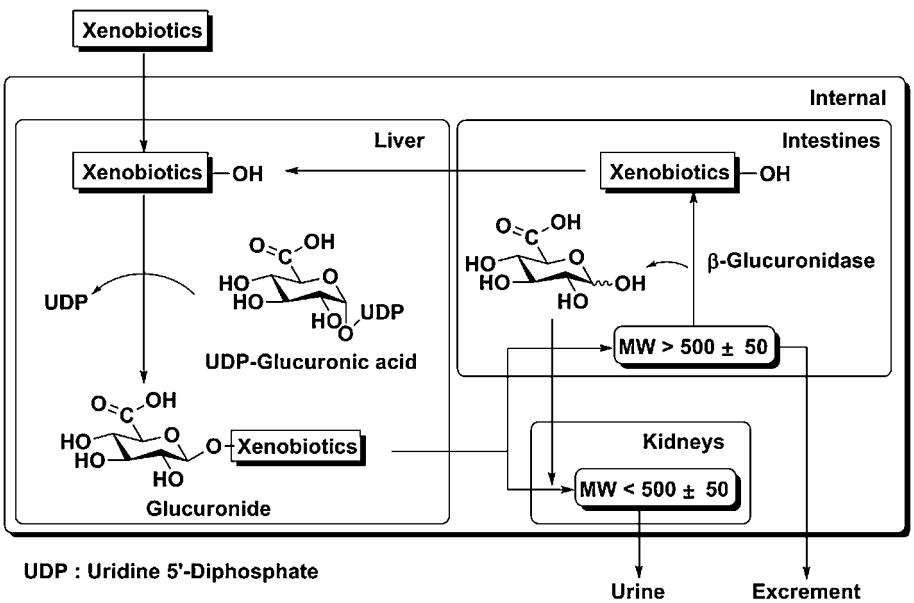
N-*p*-Vinylbenzyl-6-D-glucaramic acid (VB-6-D-GlcaH, **1**) and its corresponding polymer, P(VB-6-D-GlcaH-*co*-AAm) (**2**), were found to inhibit β-glucuronidase activity more efficiently than D-glucaro-6,3-lactone, while the inhibition ability of *N*-butyl-6-D-glucaramic acid (Butyl-6-D-GlcaH, **3**) on the enzyme activity was seriously lower than that of the lactone. The π–π stacking interaction between the styryl part of **1** and Trp_{587} of β-glucuronidase was confirmed by the blue shift detected on fluorescence spectrophotometry and the observation of the 3D motif for the active site with the glycomonomer, which must enhance the inhibition ability of the enzyme. In the case of **2**, however, such a shift was not observed, which indicated that the effective inhibition of **2** was induced not by the π–π stacking interaction but a polymeric effect. Based on these results and our previous work, plausible conformations of **1** and **2** fitting in β-glucuronidase were proposed.

When xenobiotics, such as medicines and toxins, are absorbed in the human body, they are sent to the liver to be oxidized and converted to their glucuronides. This metabolism is referred to as detoxication.[1] If the molecular weight of the glucuronides is less than 500 ± 50, they are sent to the kidneys and excreted in urine. On the other hand, when their molecular weight is more than 500 ± 50, they are sent to the intestines and discharged as feces.[2] However, almost all glucuronides are hydrolyzed to the free xenobiotics and glucuronic acid with bacteria-derived β-glucuronidase in the intestine. Therefore the free xenobiotics are sent back to the liver via the portal vein and metabolized again.[1] Such behavior is well-known as enterohepatic circulation (Scheme 1). If the xenobiotics are medicines, such circulation could be helpful because the medicinal effects would be prolonged. In contrast, if they are toxic, a serious condition could be caused by this circulation. Indeed, irinotecan, an anticancer drug, has been reported to cause serious diarrhea by its side effect as a result of this circulation.[3–7] Therefore, occasional inhibition of β-glucuronidase activity could be beneficial for maintaining health.[8–13]

Several saccharic acids, metal ions, and amino acids have been used as candidates for inhibitors of β-glucuronidase.[14–21]



Scheme 1. Xenobiotics in the human body and enterohepatic circulation.

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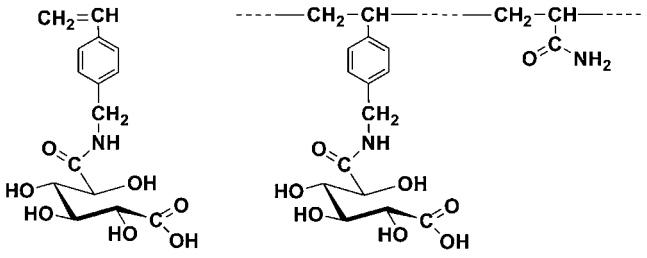


Chart 1.

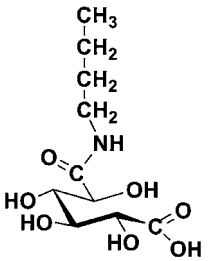


Chart 2.

Among them, the saccharic acids were the most effective inhibitors for the enzyme.[15,19,20] If administrated orally however, most of them are absorbed as nutritive substances, before they reach the intestine to work as inhibitors. Therefore, the exclusive transportation of the inhibitors into the intestines is important for clinical research on their activity in the human body.

We have already reported that the styryl monomers containing glucaric (VB-6-D-GlcaH, **1**) (Chart 1), xylaric (VB-D,L-XylaH), tartaric (VB-L-TartH), and mannaric (VB-D-ManaH) moieties were synthesized, and subsequently copolymerized with acrylamide to give the water-soluble glycopolymers, respectively [P(VB-6-D-GlcaH-*co*-AAm) (**2**), P(VB-D,L-XylaH-*co*-AAm), P(VB-L-TartH-*co*-AAm), and P(VB-D-ManaH-*co*-AAm)].[8–10,20,21] The all saccharic acid-derived polymers prepared in our previous work were found to inhibit β-glucuronidase activity in vitro much more efficiently than not only the corresponding glycomonomers but also the saccharic acids and lactones respectively, especially at lower concentration ($<0.1\,\mathrm{mol\,L^{-1}}$). Additionally, the gluconic macromolecular analogs were also synthesized and found not to inhibit the enzyme effectively. Therefore, the free carboxy group in the saccharic moiety was revealed to be essential for the inhibition.[9]

During our previous work,[20,21] the following unexpected phenomenon was observed. All glycomonomers bearing a free carboxy group always inhibited the enzyme activity more effectively than the corresponding saccharic acids or lactones. Such behavior was observed especially in the cases of the tartaric and mannaric series. Whether the polymerizable styryl group exists or not is the biggest structural difference between our glycomonomers and the corresponding saccharic acids or lactones. Therefore, in this article, the effect of the styryl group was first investigated by comparing the inhibition abilities and the inhibition behavior of VB-6-D-GlcaH (**1**) with those of *N*-butyl-6-D-glucaramic acid (Butyl-6-D-GlcaH (**3**)) (Chart 2) bearing a hydrophobic but not aromatic group. After the effect of the styryl group was confirmed more clearly by fluorescence spectrophotometry, a plausible conformation of VB-6-D-GlcaH (**1**) and P(VB-6-D-GlcaH-*co*-AAm) (**2**) fitting in the active site of β-glucuronidase was proposed by computer simulation.

Experimental

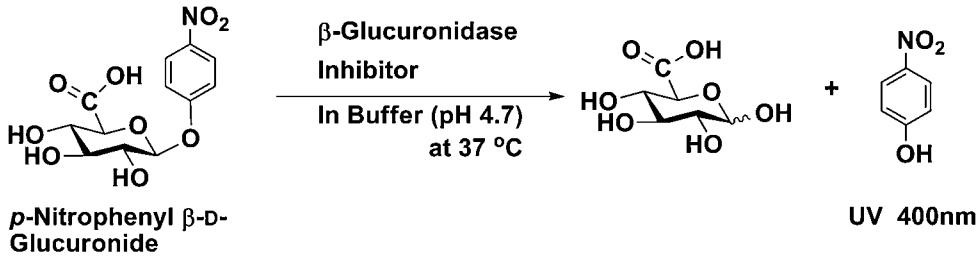
Materials. D-Glucose, butylamine, and acetic anhydride were purchased from Kanto Kagaku Co. (Tokyo, Japan). D-Glucaro-6,3-lactone was prepared from D-glucose according to the literature.[22] *p*-Vinylbenzylamine was prepared from *p*-vinylbenzyl chloride, which was kindly supplied by AGC Seimi Chemical Co., Ltd. (Chigasaki, Kanagawa, Japan).[23] β-Glucuronidase (bovine liver) was purchased from Sigma (MO, USA) and used as-received. *p*-Nitrophenyl β-D-glucuronide was purchased from Nacalai Tesque (Kyoto, Japan) and used without further purification. VB-6-D-GlcaH (**1**) was prepared through the reaction of D-glucaro-6,3-lactone with *p*-vinylbenzylamine.[8] Butyl-6-D-GlcaH (**3**) was prepared by a similar method to that of VB-6-D-GlcaH (**1**) using butylamine and D-glucaro-6,3-lactone (see Supporting Information for the specific method and physical data). P(VB-6-D-GlcaH-*co*-AAm) (**2**) was prepared by copolymerization of VB-6-D-GlcaH (**1**) with acrylamide.[8]

Instruments. ^{1}H NMR and ^{13}C NMR spectra were taken with a JEOL JNM-ECX-400 Fourier transform high-resolution spectrometer. A Molecular devices SPECTRA$^{TM}_{MAX}$ 190 microplate spectrophotometer was used for the determination of *p*-nitrophenol generated during the hydrolysis test. Elemental analysis was carried out with a CHN/O Analyzer 2400II (Perkin-Elmer). The amino-acid sequence of bovine β-glucuronidase by BLAST was obtained from UniPort and the 3D motif of the human enzyme was obtained from Protein Data Bank Japan. The Fluorescence spectrometry was measured with F-3010 Fluorescence Spectrophotometer (Hitachi Co., Ltd.).

Inhibition Test for β-Glucuronidase Activity. A model compound for the β-D-glucuronide conjugates of xenobiotics, *p*-nitrophenyl β-D-glucuronide, was hydrolyzed with β-glucuronidase in the absence or presence of the resulting inhibitors (Scheme 2). The amount of *p*-nitrophenol liberated from the glucuronide was determined by spectroscopy. The inhibition value (%) was calculated from the hydrolytic rates of the substrate in the absence and presence of the inhibitors. A Lineweaver–Burk plot was constructed using data determined in 0.4–1.4 mM of *p*-nitrophenyl β-glucuronide in the presence of different amounts of the inhibitors.[24,25]

3D Computer Graphical Observation of the β-Glucuronidase Active Site. Discovery Studio Visualizer ver. 2.0 (DS Visualizer, Accelrys®) was used to observe the 3D motif of the human β-glucuronidase active site, which was reported by Jain.[26] The 3D motif of the human β-glucuronidase was obtained from Protein Data Bank Japan (PDBj). Since Discovery Studio Visualizer ver. 2.0 is the viewer software for the protein, plausible structures were illustrated manually.

Measurement of the Tryptophanyl Fluorescence Spectra. Tryptophanyl fluorescence spectra were measured in the range of 300–400 nm employing an excitation wavelength of 285 nm



Scheme 2. Inhibition test for β-glucuronidase activity by inhibitors.

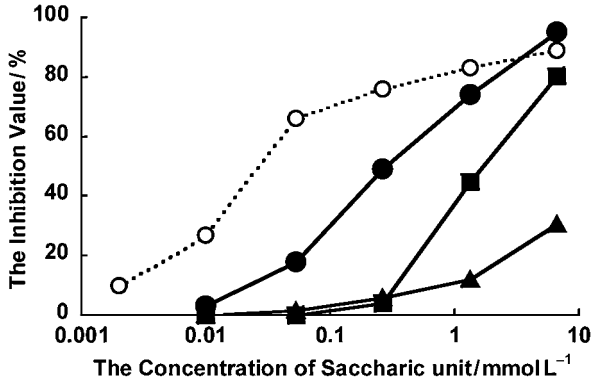


Figure 1. The Inhibition of β-glucuronidase activity by different glucaric inhibitors at 37 °C (*p*-nitrophenyl β-D-glucuronide, 7 mmol L^{-1}; β-glucuronidase, 14 IU L^{-1}). ○; P(VB-6-D-GlcaH-*co*-AAm) (**2**):[9] ●; VB-6-D-GlcaH (**1**): ■; D-Glucaro-6,3-lactone: ▲; Butyl-6-D-GlcaH (**3**).

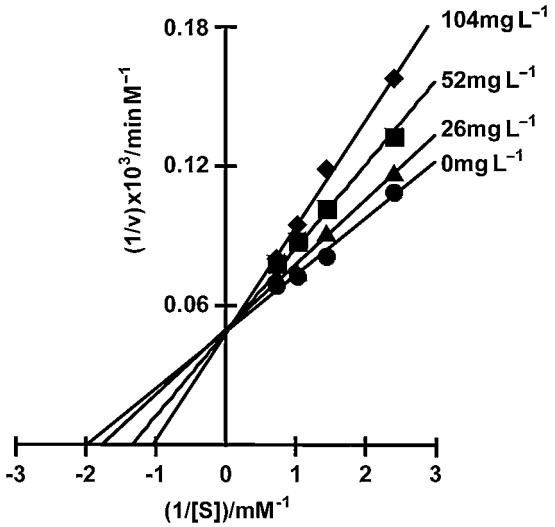


Figure 2. A Lineweaver–Burk plot for kinetic data of the hydrolysis of *p*-nitrophenyl β-D-glucuronide by β-glucuronidase in the presence of Butyl-6-D-GlcaH (**3**).

and slit widths of 5 nm with F-3010 Fluorescence Spectrophotometer (Hitachi Co., Ltd.). The β-glucuronidase solutions mixed with VB-6-D-GlcaH (**1**), P(VB-6-D-GlcaH-*co*-AAm) (**2**), and Butyl-6-D-GlcaH (**3**) were prepared by adding the enzyme (80 mg) with the corresponding inhibitors (0.12 mmol) in 10 mL of acetic buffer, and compared with the blank solution (the enzyme 80 mg in 10 mL).

Results and Discussion

Whether the polymerizable styryl group exists or not was the biggest structural difference between our glycomonomers and the corresponding saccharic acids or lactones. Therefore, a new inhibitor with an aliphatic moiety, Butyl-6-D-GlcaH (**3**), was synthesized in order to compare its inhibition ability of the β-glucuronidase activity with that of VB-6-D-GlcaH (**1**) having the styryl group which is a typical competitive inhibitor.[10] A model compound for the β-D-glucuronide conjugates of xenobiotics, *p*-nitrophenyl β-D-glucuronide, was hydrolyzed with β-glucuronidase in the absence or presence of VB-6-D-GlcaH (**1**), Butyl-6-D-GlcaH (**3**), or D-glucaro-6,3-lactone, that was kinetically traced by spectroscopy. The inhibition value (%) was calculated from the hydrolytic rates of the substrate in the absence and presence of the above-mentioned saccharic derivatives (v_0 and v, respectively), as shown in eq 1.

$$\text{Inhibition value } (\%) = [(v_0 - v)/v_0] \times 100 \qquad (1)$$

As shown in Figure 1, the inhibition ability of VB-6-D-GlcaH (**1**) increased to 95% at the 6.67 mmol L^{-1} saccharic concentration and was higher than that of D-glucaro-6,3-lactone at all concentrations, although it was lower than that of the corresponding glycopolymer **2**. In contrast, the inhibition ability of Butyl-6-D-GlcaH (**3**) was much lower than those of VB-6-D-GlcaH (**1**) and D-glucaro-6,3-lactone, especially in the range of 1.37–6.67 mmol L^{-1}. Additionally, the inhibition behavior of Butyl-6-D-GlcaH (**3**) was confirmed to be competitive from the Lineweaver–Burk plot in which all extrapolating lines intersected on the vertical axis (Figure 2). Therefore, the inhibition type was found not to change from competitive to the any other else by introducing the *n*-butyl group instead of the styryl group to the glucaric moiety. These two results suggest that the styryl moiety assists the inhibition indirectly as the aromatic part rather than as the hydrophobic part.

π–π stacking interaction is a well-known aromatic interaction. In addition, some researchers have reported that this interaction enhances the ability of inhibitors.[27–29] Moreover, aromatic amino acid residues in enzymes such as tryptophan and phenylalanine have been confirmed to interact with the aromatic groups in the inhibitors by this interaction. Therefore, if this interaction assisted the inhibition ability of VB-6-D-GlcaH (**1**) for β-glucuronidase activity, the corresponding amino acid residue should exist around the active site in the enzyme, because VB-6-D-GlcaH (**1**) is a typical competitive inhibitor.

Since only the human β-glucuronidase 3D motif has been reported, the active site of human enzyme was used for our discussion instead of that of the bovine β-glucuronidase. The

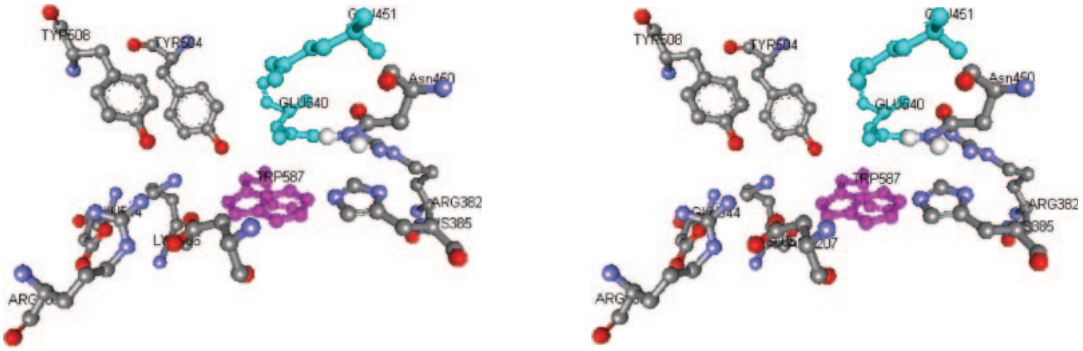


Figure 3. A stereo view of the active center of β-glucuronidase.

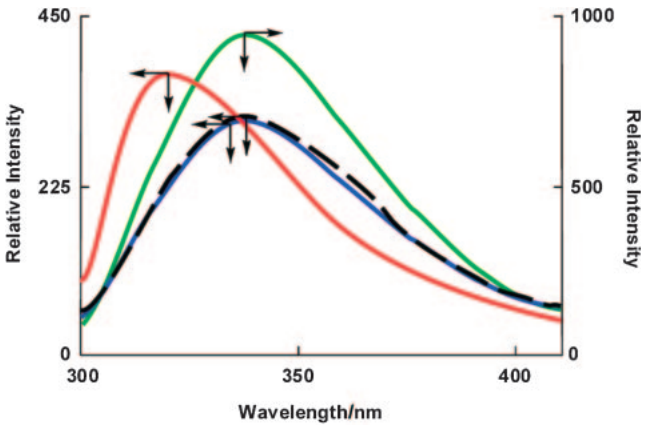


Figure 4. The fluorescence emission spectra of β-glucuronidase (black chain line), β-glucuronidase with Butyl-6-D-GlcaH (**3**) (blue line), β-glucuronidase with P(VB-6-D-GlcaH-*co*-AAm) (**2**) (green line), and β-glucuronidase with VB-6-D-GlcaH (**1**) (red line).

amino-acid sequence homologies of the human and bovine β-glucuronidase were confirmed to ca. 80% and the active sites were almost completely the same (Figure S1). Islam et al. reported that Glu_{451} and Glu_{540} were essential catalytic residues (illustrated in light blue) in β-glucuronidase as shown in Figure 3.[30] As illustrated in pink in Figure 3, there was Trp_{587} on the straight line of the catalytic residues. So, we expected that Trp_{587} interacts with the styryl group in the glycomonomeric inhibitor by π–π stacking. It is well-known that the λ_{max} of tryptophan residual fluorescence emission becomes shorter with increasing hydrophobicity of the tryptophan environment.[31–33] Thus, tryptophanyl fluorescence emission spectra of four systems (β-glucuronidase only, the enzyme with VB-6-D-GlcaH (**1**), the enzyme with Butyl-6-D-GlcaH (**3**), and the enzyme with P(VB-6-D-GlcaH-*co*-AAm) (**2**)) were investigated and the results are illustrated in Figure 4 and each λ_{max} is summarized in Table 1. The λ_{max} in the systems of the enzyme with Butyl-6-D-GlcaH (**3**) and P(VB-6-D-GlcaH-*co*-AAm) (**2**) were hardly changed from that of the enzyme only. However, the intensity increased in the latter system with **2**, which was caused by the styryl moiety of the polymer side chain. Therefore, these results revealed that the π–π stacking interaction did not occur between Trp_{587} and both inhibitors. In contrast, in the case of the enzyme with VB-6-D-GlcaH (**1**), the λ_{max} was found to be 16 nm shorter, which indicates that the

Table 1. The Wavelength of Maximal Fluorescence Emission[a] of β-Glucuronidase in the Presence of the Inhibitors

System	λ_{max}/nm	Relative intensity
β-Glucuronidase (E)	337.2	313
E + Butyl-6-D-GlcaH (**3**)	337.8	311
E + P(VB-6-D-GlcaH-*co*-AAm) (**2**)	337.0	983
E + VB-6-D-GlcaH (**1**)	321.2	373

a) Excitation λ, 285 nm.

tryptophan environment in the enzyme becomes hydrophobic in the presence of VB-6-D-GlcaH (**1**). Therefore, this is consistent with the Trp_{587} residue interacting with the styryl group through π–π stacking and the VB-6-D-GlcaH (**1**) being localized around the active site. Consequently, the inhibition ability by VB-6-D-GlcaH (**1**) was inferred to be higher than those of the corresponding saccharic acid or lactones.

Our previous and present work has so far elucidated that 1) the free carboxy group in the saccharic moiety is essential for the inhibition of β-glucuronidase activity,[9] 2) the inhibition behaviors of VB-6-D-GlcaH (**1**) and P(VB-6-D-GlcaH-*co*-AAm) (**2**) are competitive, and 3) the styryl group interacts with Trp_{587} residue through π–π stacking. Additionally, although no crystal structure of the substrates and/or inhibitors in β-glucuronidase has been reported, Asn_{450} was predicted to interact with the hydroxy group of saccharide moiety.[34,35] Based on these results, plausible conformations of VB-6-D-GlcaH (**1**) and P(VB-6-D-GlcaH-*co*-AAm) (**2**) in the enzyme are illustrated in Figure 5. Since the styryl group overlaps with Trp_{587} by π–π stacking interaction and the glucaric moiety occupies the active site, VB-6-D-GlcaH (**1**) (as illustrated in yellow in Figure 5A) seems to be consequently bent in the enzyme. However, in the case of P(VB-6-D-GlcaH-*co*-AAm) (**2**), the styryl moiety would not be located near the active cite in the enzyme because of steric hindrance. Hence, it is easily supposed that P(VB-6-D-GlcaH-*co*-AAm) (**2**) inhibits the β-glucuronidase activity by the simple insertion of the glucaric moiety in the active site (Figure 5B). This inference suggests also that the efficient inhibition ability of P(VB-6-D-GlcaH-*co*-AAm) (**2**) is induced only by a polymeric effect. Since glucaric units are linked as pendant groups along the polymer chain, the inhibitor concentration may become higher locally, even at lower concentration. As a side note, these plausible conformations were illustrated manually, so the uncertain interaction between the hydroxy group and amino acid residues will not be discussed here.

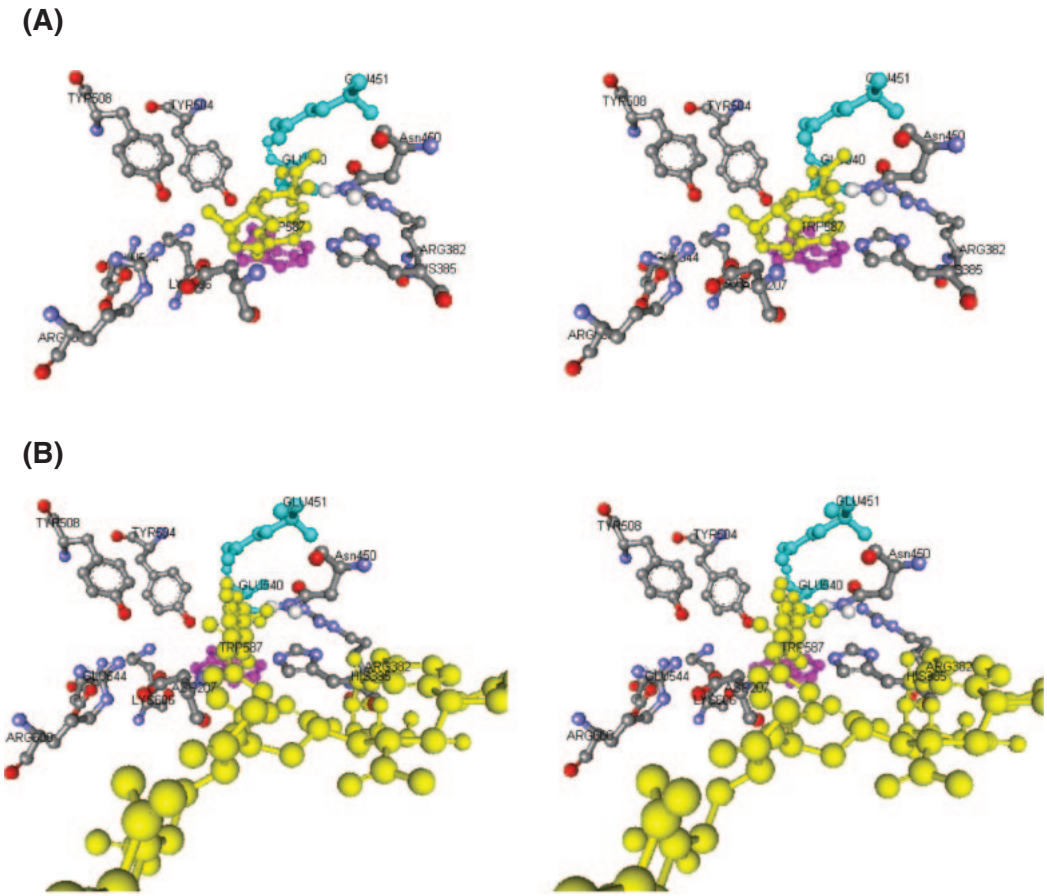


Figure 5. Stereo views for plausible structures of (A) VB-6-D-GlcaH (**1**) and (B) P(VB-6-D-GlcaH-*co*-AAm) (**2**) in β-glucuronidase.

Summary

In summary, the comparison of the inhibition abilities of VB-6-D-GlcaH (**1**) for β-glucuronidase with that of Butyl-6-D-GlcaH (**3**) and their tryptophanyl fluorescence spectra revealed the π–π stacking interaction between the styryl group in the inhibitor and Trp_{587} in the enzyme, which assisted indirectly the inhibition of the β-glucuronidase activity in the case of monomeric inhibitors. On the other hand, in the case of polymeric inhibitor, the π–π stacking interaction does not contribute to the inhibition of the enzyme because the polymer chain itself creates steric hindrance. Plausible structures of VB-6-D-GlcaH (**1**) and P(VB-6-D-GlcaH-*co*-AAm) (**2**) fitting in the enzyme were proposed, which had not yet been confirmed by X-ray crystallography. Investigation and confirmation of the structures would be helpful for the design of new inhibitors.

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Supporting Information

This file contains as follows: A) the synthesis method and the physical data of Butyl-6-D-GlcaH (**3**), B) the figure of the amino-acid sequence homologies of the human and bovine β-glucuronidase. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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