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Glycosidase Inhibitors of *gem*-Diamine 1-*N*-iminosugars: Structures in Media of Enzyme Assays

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Abstract—The relationships between structures and inhibitory activities of glycosidase inhibitors of *gem*-diamine 1-*N*-iminosugars in media of enzyme assays have been investigated. It has been proved that *gem*-diamine 1-*N*-iminosugar smoothly undergoes a structural change to a hydrated ketone or its derivative via a hemiaminal in the media (pH 5.0–6.3), and that the products generated in the media as well as the parent *gem*-diamine 1-*N*-iminosugars potently inhibit glycosidases.

Introduction

Glycosidase inhibitors are tools for unraveling the mechanism of action of glycosidases, and are of therapeutic and biotechnological relevance.[1] To this end, various types of natural and synthetic inhibitors have been developed in the last decade.[2] Our interest directed toward glycosidase inhibitor-based therapeutics modeled on natural siastatin B (**1**)[3] has led to a new class of glycosidase inhibitor, *gem*-diamine 1-*N*-iminosugars (**2**),[4] in which an anomeric carbon atom is replaced by a nitrogen, and of which protonated form may mimic hexopyranosyl cation (**3**) (Fig. 2). *gem*-Diamine 1-*N*-iminosugars have proved to be potent and specific glycosidase inhibitors.[4,5] In the course of our study for the mechanism of action of **2** against glycosidases, it has become apparent that **2** changes the structures dependently on pH value of solution. In this paper, we report the results of the structural changes of **2** in the media of enzyme assays and the inhibitory activities of the products generated in the media against enzymes.

Results and Discussion

In order to clarify the relationships between structures and inhibitory activities in media of enzyme assays, we examined the time-course alteration of structures and activities in media by ^{1}H NMR experiments and evaluation of the corresponding activities against enzymes. We chose the inhibitors of D-galacturonic acid-types (**4–6**)[4b,5b] and L-fucose-types (**7–9**)[5h,6] as the typical inhibitors of *gem*-diamine 1-*N*-imino-D- and L-sugars (**2**) for reasons of the potent competitive activities and different stabilities in media. Figure 4 shows a time-course alteration of the structure of the 2-trifluoroacetamide **4**, a strong inhibitor of β-glucuronidase in acetate buffer (pH 5.0) at 37 °C used for the enzyme assay. As shown in Figure 4, **4** has been proved to change its structure time-dependently to the unknown compounds (**10** and **11**) in the medium. Compound **4** has also shown the pH-dependent conversion of structure to **10** and **11**, while **4** is quite stable for several weeks in hydrochloric acid (pH < 1). Next, we undertook the isolation of compounds **10** and **11**. After adjustment of pH value of the aqueous solution of the hydrochloride of **4** to 5–6 by the weak basic resin, Dowex WGR, evaporation of the solvent gave the pure **10**. The large coupling constants ($J_{2ax,3} = 9.2$ and $J_{5,6} = 9.8$ Hz) and the small coupling constants ($J_{3,4} = J_{4,5} = 2.7$ Hz) in ^{1}H NMR spectrum of **10** are clearly indicative of the same configuration and $^4C_1$-conformation as those of **4**.[4b] The ^{13}C NMR spectrum of **10** shows the carbons at δ 38.74 (C-6), 43.50 (C-5), 68.99 (C-4), 71.50 (C-3), 173.41 (COOH) and a characteristic carbon at low-field of δ 79.14 (C-2) assigned to a hemiaminal carbon[7] instead of a carbon at δ 60.71 (C-2) assigned to a methanediamine carbon shown in that

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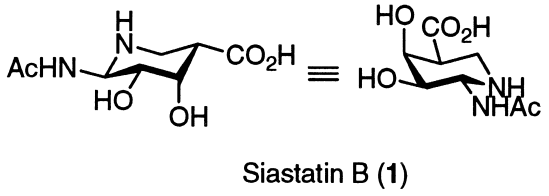
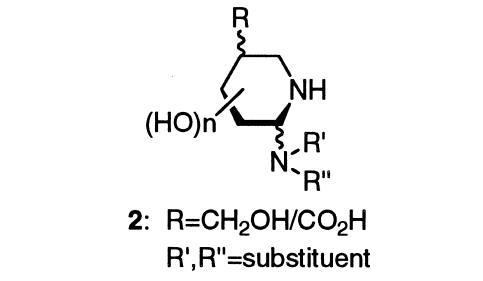
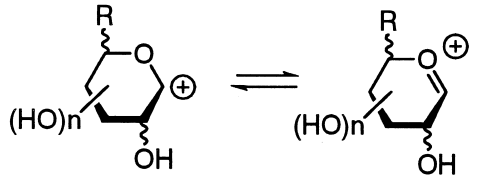


Siastatin B (**1**)

Figure 1. Structure of siastatin B (**1**).

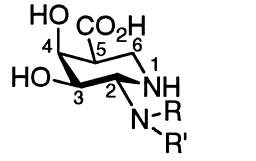


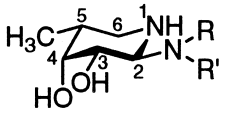
2: R=CH_2OH/CO_2H
R',R"=substituent



3: R=CH_2OH/CO_2H

Figure 2. *gem*-Diamine 1-*N*-iminosugars **2** and glycopyranosyl cation (**3**), the presumed transition state of enzymatic glycosidic hydrolysis.





4: R=H, R'=$COCF_3$
5: R=H, R'=$COCCl_3$
6: R,R'=

7: R=H, R'=$COCF_3$
8: R=H, R'=$COCCl_3$
9: R,R'=

Figure 3. *gem*-Diamine 1-*N*-iminosugars of D-galacturonic acid-types (**4**–**6**) and L-fucose-types (**7**–**9**).

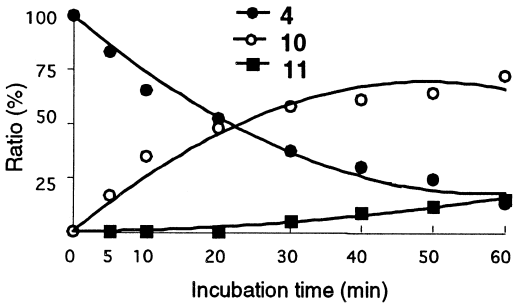
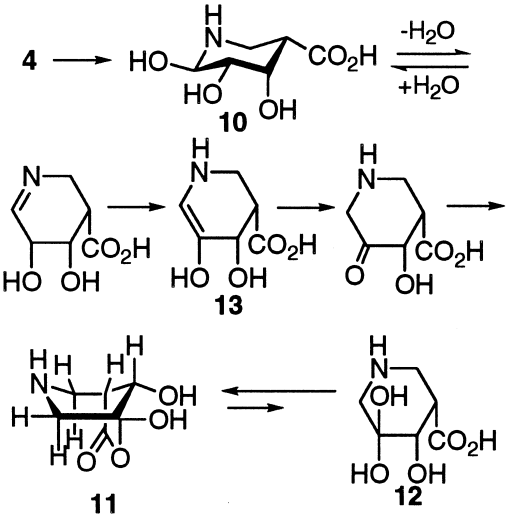


Figure 4. The time-course of alteration of **4** in 0.1 M acetate buffer (pH 5.0) at 37 °C.

of **4**. This low-field chemical shift of C-2 is in accordance with those of hemiaminal carbons of nojirimycin[8] and galactostatin.[9] The spectrum also shows no carbons at around δ 115.85 and 160.52 corresponding to the trifluoroacetamido groups. These results are indicative of the hemiaminal as the structure of **10** shown in Scheme 1. The mass spectra of **10** show *m*/*e* 178, 178 and 176 in FAB (positive), ESI (positive) and ESI (negative) modes, respectively, indicative of *m*/*e* 177 as a molecular ion of **10** and clearly supportive of the hemiaminal of **10** (MW = 177). On the other hand, adjustment of pH of the aqueous solution of the hydrochloride of **4** to pH 7–8 by Dowex WGR afforded the another product **11**. Compound **11** was also stable in an acidic aqueous solution. ^{1}H NMR spectrum of **11** in D_2O shows the methylene protons of the two aminomethyl groups at C-6 (2H, ABq, J = 12.4 Hz, δ = 2.98 and 3.05) and at C-2 (1H, t, $J_{2,2'} = J_{2,3}$ = 12.2 Hz, δ 3.06, H-2; 1H, dd, $J_{2,2'}$ = 12.2 and $J_{2',3}$ = 4.4 Hz, δ 3.21, H-2′), a proton at C-3 (1H, ddd, $J_{3,2}$ = 12.2 Hz, $J_{3,2'}$ = 4.4 Hz and $J_{3,4}$ = 1.2 Hz, δ 2.88), a proton at C-4 (1H, d, $J_{4,3}$ = 1.2 Hz, δ 3.98) and no proton at C-5, suggesting a boat-conformational lactal **11** shown in Scheme 1. The ^{13}C NMR spectrum of **11** in H_2O shows the carbons at δ 40.88 (C-2), 44.76 (C-3), 71.28 (C-4), 177.57 (−C(=O)−O−), and the two characteristic carbons at high-field of δ 47.26 (C-6) and at low-field of δ 92.47 (C-5) different from those of **10**, indicative of a lactal. The mass spectra of **11** of APCI (positive) mode and APCI (negative) mode show *m*/*e* 160 and 158, respectively, supportive of a formula of the cyclic lactal of **11** (MW = 159). Moreover, the methylene protons at C-2 of **11** were deuterated when the above adjustment of pH of the hydrochloride of **4** to pH 7–8 was carried out in D_2O. Evaporation of D_2O solution of **11** also deuterated the methylene protons at C-2.

These results confirmed the cyclic lactal as the structure of **11**, and also indicate that a sequence of dehydration of the aminal **10**, enolization to the enaminal **12** and protonation on C-6 of **12** would lead to the lactal **11** as shown in Scheme 1. This dehydration is commonly observed in



Scheme 1. Hemiaminal **10**, lactal **11**, hydrated carboxylic acid **12** and the putative enolate **13** generated from *gem*-diamine 1-*N*-iminosugar of D-galacturonic acid-type **4** in medium of acetate buffer (pH 5.0).

many hemiaminals, and the lactone **11** can be envisaged as the reaction product of a cyclic Amadori rearrangement of **10**.[10] Next, we attempted to clarify which species of **4**, **10** and **11** greatly inhibit β-glucuronidase in the medium. Firstly, the respective inhibitory-activities of **4**, **10** and **11** were assayed by the ordinary method of incubation of a substrate and a test sample with an enzyme in acetate buffer (pH 5.0) at 37 °C for 60 min. The IC_{50} values of **4**, **10** and **11** against β-glucuronidase were elucidated as 9.2×10^{-8} M (0.025 μg/mL), 1.6×10^{-7} M (0.029 μg/mL) and 3.1×10^{-6} M (0.5 μg/mL), respectively. Secondly, we attempted to evaluate the dynamic inhibitory-activity in a change of **4** on standing in the medium shown in Figure 4. The inhibitory activities of the mixtures over 20, 40 and 60 min in Figure 4 were selected as the typical ones for this purpose. In order to estimate these activities, the evaluation was carried out by the similar method mentioned above after pre-incubation of **4** in the medium for 20, 40 and 60 min. The IC_{50} values after pre-incubation for 20, 40 and 60 min were 0.022, 0.033 and 0.33 μg/mL, respectively, appearing to be the activities corresponding to the mixture of **4:10:11** = ca. 50:50:0, 28:65:7 and 15:70:15, respectively as shown in Figure 4. These results indicate that the 2-trifluoroacetamide of *gem*-diamine 1-*N*-iminosugar **4** and the hemiaminal **10** are the equally potent inhibitors against β-glucuronidase, and that both **4** and **10** contribute greatly to overall inhibitory activity in a change of **4** with the passage of time in the medium. Interestingly, the cyclic lactal **11** shows moderate inhibition against β-glucuronidase. It is likely that **11** may be slightly hydrolyzed in the medium and may be in equilibrium with the carboxylic acid **13**. Next, we examined a time-course conversion of the 2-trichloroacetamide analogue **5** in a medium of β-glucuronidase assay. Compound **5** itself has also proved to be very stable in a solution of strong acid (pH < 1). As shown in Figure 5, **5** is converted into the aminal **10** and the lactal **11** in a similar fashion as the alteration of **4** in Figure 4. The inhibitory activity (IC_{50}) of **5** was evaluated as 0.033 μg/mL, and IC_{50} values of the mixtures over 10, 20, 35, 60 and 90 min in Figure 5 were also elucidated to 0.033, 0.032, 0.036, 0.038 and 0.050 μg/mL, respectively.

These results are indicative of a strong inhibitory-activity of the 2-trichloroacetamide **5** as well as the 2-trifluoroacetamide **4**. Compound **5** has proved to be more stable than **4** in the medium of acetate buffer (pH 5.0). These results also indicate that both functional groups of 2-trifluoroacetamido and 2-trichloroacetamido assist the strong binding of *gem*-diamine 1-*N*-iminosugars of D-galacturonic acid-types to the active pocket of β-glucuronidase. We further examined the time-course transformation of the structure of the other *gem*-diamine 1-*N*-iminosugar, 2-phthalimide **6** in the medium. Compound **6**, in contrast to **4** and **5** has been ascertained to be unstable even in a solution of strong acid (pH < 1). The ^{1}H NMR spectrum showed the disappearance of **6** and the appearance of the aminal **10** for 3 h at 24 °C in hydrochloric acid (pH∼1). Interestingly, the hydrochloride of **6** in methanol converted into **10** within 10 min at 24 °C. Figure 6 shows the time-course conversion of **6** in the medium of enzyme assay (pH 5.0). As expected, **6** proved to be very unstable in the medium (acetate buffer, pH 5.0). Therefore, IC_{50} value (0.023 μg/mL) of **6** against β-glucuronidase should not be its own value, but the value of the aminal **10**. This result also supports that the aminal **10** itself is a strong inhibitor of β-glucuronidase. Next, we turned our attention to the another *gem*-diamine 1-*N*-iminosugars of L-fucose-types (**7–9**), the strong inhibitors of L-fucosidase. Figure 7 shows the time-course conversion of the structure of the 2-trifluoroacetamide **7** in citrate-phosphate buffer (pH 6.3) at 37 °C used for enzyme assay. As shown in Figure 7, it has become apparent that **7** rapidly converts the structure into the unknown compounds (**14** and **15**) in the medium. We speculated that the structural changes of **7** in citrate-phosphate buffer (pH 6.3) proceeded via the similar manner to those of the above *gem*-diamine 1-*N*-iminosugars of D-galacturonic acid-types (**4**, **5**, **6**) in acetate buffer (pH 5.0) to give the corresponding hemiaminal **14**

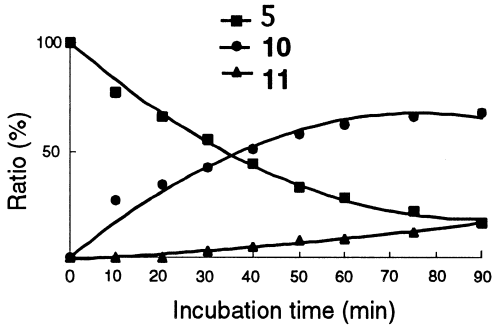


Figure 5. The time-course of alteration of **5** in 0.1 M acetate buffer (pH 5.0) at 37 °C.

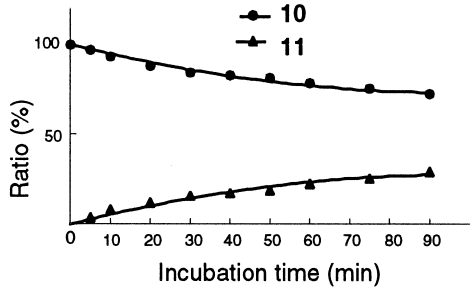


Figure 6. The time-course of alteration of **6** in 0.1 M acetate buffer (pH 5.0) at 37 °C.

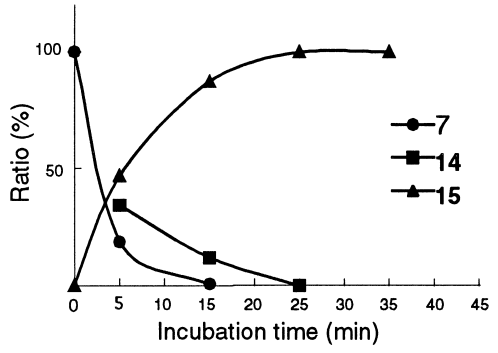
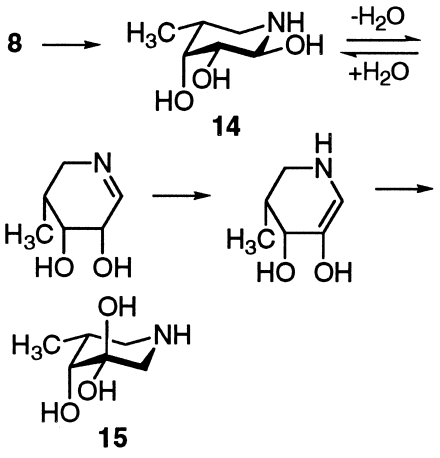


Figure 7. The time-course of alteration of **7** in 0.025 M citrate-phosphate buffer (pH 6.3) at 37 °C.

and hydrated ketone **15**. The ^{1}H NMR spectrum of dilute solution (1 mg/mL) of **7** in D_2O also shows a mixture of **14** and **15** in a ratio of 3:1 after standing for 2 days at room temperature. Next, we analyzed the structure of **14** utilizing this ^{1}H NMR and its ^{13}C NMR spectra. The large coupling constants ($J_{2,3}=9.3$ and $J_{5,6ax}=12.7$ Hz) and the small coupling constants ($J_{3,4}=J_{4,5}=2.9$ and $J_{5,6eq}=4.4$ Hz) in the ^{1}H NMR spectrum indicate clearly the same configuration and $^1C_4$-conformation as those of **7**.[5h,6] The ^{13}C NMR spectrum shows the carbons at δ 13.67 (CH_3), 32.45 (C-6), 43.37 (C-5), 71.61 (C-4), 72.15 (C-3) and a characteristic carbon at low-field of δ 78.85 (C-2) assigned to an aminal carbon[7–9] instead of a carbon at δ 61.99 assigned to a methanediamine carbon shown in that of **7**. The spectrum also shows no carbons at around δ 115.53 and 160.30 corresponding to the trifluoroacetamide group. These results indicate a cyclic aminal as the structure of **14** shown in Scheme 2, and consequently, imply that our previous wrong conclusion[5h] of generation of a cyclic methanediamine in the media of enzyme assay should be revised. On the other hand, the ^{1}H NMR spectrum of **7** in a D_2O solution of citrate-phosphate buffer (pH 6.3) shows only **15** on standing for 30 min at room temperature as shown in Figure 7. Therefore, we used this ^{1}H NMR and its ^{13}C NMR spectra for analysis of the structure of **15**. The ^{1}H NMR spectrum shows protons of C(2), C(4), C(5), C(6) and CH_3 at δ 3.17 and 3.15 (total 1H, s each), 3.66 (1H, d, $J_{4,5}=2.4$ Hz), 2.36 (1H, m), 3.12 (1H, dd, $J_{5,6eq}=4.9$ and $J_{6ax,6eq}=12.7$ Hz) and 2.85 (1H, t, $J_{5,6ax}=J_{6ax,6eq}=12.7$ Hz) and 1.03 (3H, d, $J=6.8$ Hz), respectively, and shows no proton corresponding to C(3), indicative of a $^1C_4$-conformational hydrated-ketone **15** bearing deuterated methylene protons shown in Scheme 2. On the other hand, the ^{13}C NMR spectrum shows the carbons at 14.10 (CH_3), 30.80 (C-5), 43.96 (C-6), 72.57 (C-4), and the two characteristic carbons at low-field of δ 92.30 (C-3) assigned to a hydrated-ketone and at high-field of δ 46.1–46.9 (m) (C-2) assigned to a deuterated methylene-carbon, clearly supportive of a hydrated-ketone **15**. This result is accordance with the structural alteration of **4** in the media mentioned above. In the



Scheme 2. Hemiaminal **14**, and keto-hydrate **15** generated from *gem*-diamine 1-*N*-iminosugar of L-fucose type **8** in medium of citrate-phosphate buffer (pH 6.3).

L-fucosidase inhibitors of *gem*-diamine 1-*N*-iminosugars, we attempted again to reveal which species significantly inhibit the enzyme in a change with the passage of time in the medium. It is already known that the 2-trifluoroacetamide **7**, 2-trichloroacetamide **8** and 2-phthalimide **9** inhibit very potently L-fucosidase as K_i and IC_{50} values of 5×10^{-9} M and 3 ng/mL,[5h] respectively, assayed by the usual manner at 37 °C. However, as the structural change of **7** in the medium proceeds rapidly as shown in Figure 7, so it has become apparent that the usual method provides only poorly characterized species which inhibit the enzyme. Therefore, we examined the relationship between the structure and the inhibitory activity in the medium at low temperature, utilizing **7** as a representative fucosidase-inhibitor. Figure 8 shows the time-course conversion of **7** into **14** and **15** in citrate-phosphate buffer (pH 6.3) at 23 °C, expressing the slow structure-change. Firstly, the fucosidase inhibition assay was carried out by incubation of a substrate and **7** with an enzyme in citrate-phosphate buffer (pH 6.3) at 23 °C for 60 min. The IC_{50} value was elucidated as 5 ng/mL, indicative of that of **7** itself as shown in Figure 8. Secondly, the inhibitory activity (IC_{50}) of the mixture of **7**, **14** and **15** (about 1:1:1) was evaluated as 4 ng/mL by the similar method after pre-incubation of **7** in the medium at 23 °C for 60 min as shown in Figure 8. Thirdly, the inhibitory activity (IC_{50}) of the hydrated-ketone **15** was determined as 14 ng/mL by the assay at 23 °C for 60 min after pre-incubation at 37 °C for 40 min as shown in Fig. 7. On the whole, these results indicate that **7**, **14** and **15** very strongly inhibit L-fucosidase, and that the hemiaminal **14** of these is proved to be the strongest inhibitor against the enzyme. These results also suggest that a hydroxyl group at C-2 play an important role for the interaction of active-site residues. It is known that a hydroxyl group at C-2 stabilize the transition state of enzyme-catalyzed glycoside hydrolysis by the interaction with the catalytic nucleophile (30–40 kJ mol^{-1}).[11]

In summary, glycosidase inhibitors of *gem*-diamine 1-*N*-iminosugars change the structures pH-dependently to the hydrated-ketones or their derivatives via the aminals in the media of enzyme assay. The products generated in the media as well as the parent gem-diamine 1-*N*-iminosugars are shown to have the potent inhibition against glycosidases. That these *gem*-diamine 1-*N*-iminosugars are potent inhibitors of glycosidases further supports the hypothesis of our design of the new type inhibitors.

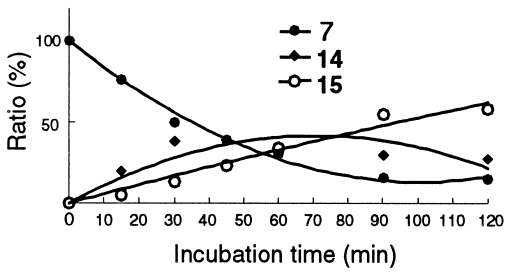


Figure 8. The time-course of alteration of **7** in 0.025 M citrate-phosphate buffer (pH 6.3) at 23 °C.

Experimental

General methods

^{1}H NMR and ^{13}C NMR spectra were recorded with JEOL GX-400 and α-500 spectrometers. Chemical shifts are expressed in δ values (ppm) with CD_2HOD (3.30) for CD_3OD and with HDO (4.65) for D_2O as an internal standard. The mass spectra were taken by JEOL SX102 for FAB and by Hitachi M-1200H for APCI (atmospheric pressure chemical ionization).

Materials

Compounds **4–7** were prepared by the methods previously developed by us.[4b,5b,5h,6] The enzymes of β-D-glucuronidase (bovine kidney) and α-L-fucosidase (bovine kidney), and the substrates of phenolphthalein mono-β-glucuronic acid and *p*-nitrophenyl α-L-fucopyranoside were purchased from Sigma Chemical Co.

General procedures for D-glucuronidase inhibition assay at 37°C

β-D-Glucuronidase inhibition was assayed using phenolphthalein mono-β-glucuronic acid (3.3×10^{-4} M) as a substrate at pH 5.0 (0.1M acetate buffer). The reaction mixture contained 0.075 mL of buffer, 0.01 mL of substrate solution and 0.05 mL of water or aqueous solution containing the test compound. The mixture was incubated at 37 °C, and 0.015 mL of enzyme was added. After 60 min of reaction, 0.15 mL of 0.6M glycine-sodium hydroxide buffer (pH 10.5) was added and the absorbance of the liberated phenolphthalein was measured at 570 nm. The percentage inhibition was calculated by the formula $(A-B)/A\times100$, where A is the phenolphtalein liberated by the enzyme without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% of enzyme activity.

General procedures for L-fucosidase inhibition assay at 23°C

α-L-Fucosidase inhibition was assayed using *p*-nitrophenyl α-L-fucopyranoside (1.5×10^{-3} M) as a substrate at pH 6.3 (0.025 M citrate-phosphate buffer). The reaction mixture contained 0.05 mL of buffer, 0.03 mL of substrate solution and 0.095 mL of water or aqueous solution containing the test compound. The mixture was incubated at 23 °C for 3 min, and 0.025 mL of enzyme was added. After 60 min of reaction, 0.1 mL of 0.6 M glycine-sodium hydroxide buffer (pH 10.5) was added and the absorbance of the liberated *p*-nitrophenol was measured at 405 nm. The IC_{50} value was elucidated by the same way as that of D-glucuronidase inhibition assay.

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