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Running Title: Inhibition of α-glucosidase by *Morus australis* extracts

Kinetic analysis of inhibition of α-glucosidase by leaf powder from *Morus australis* and its component iminosugars

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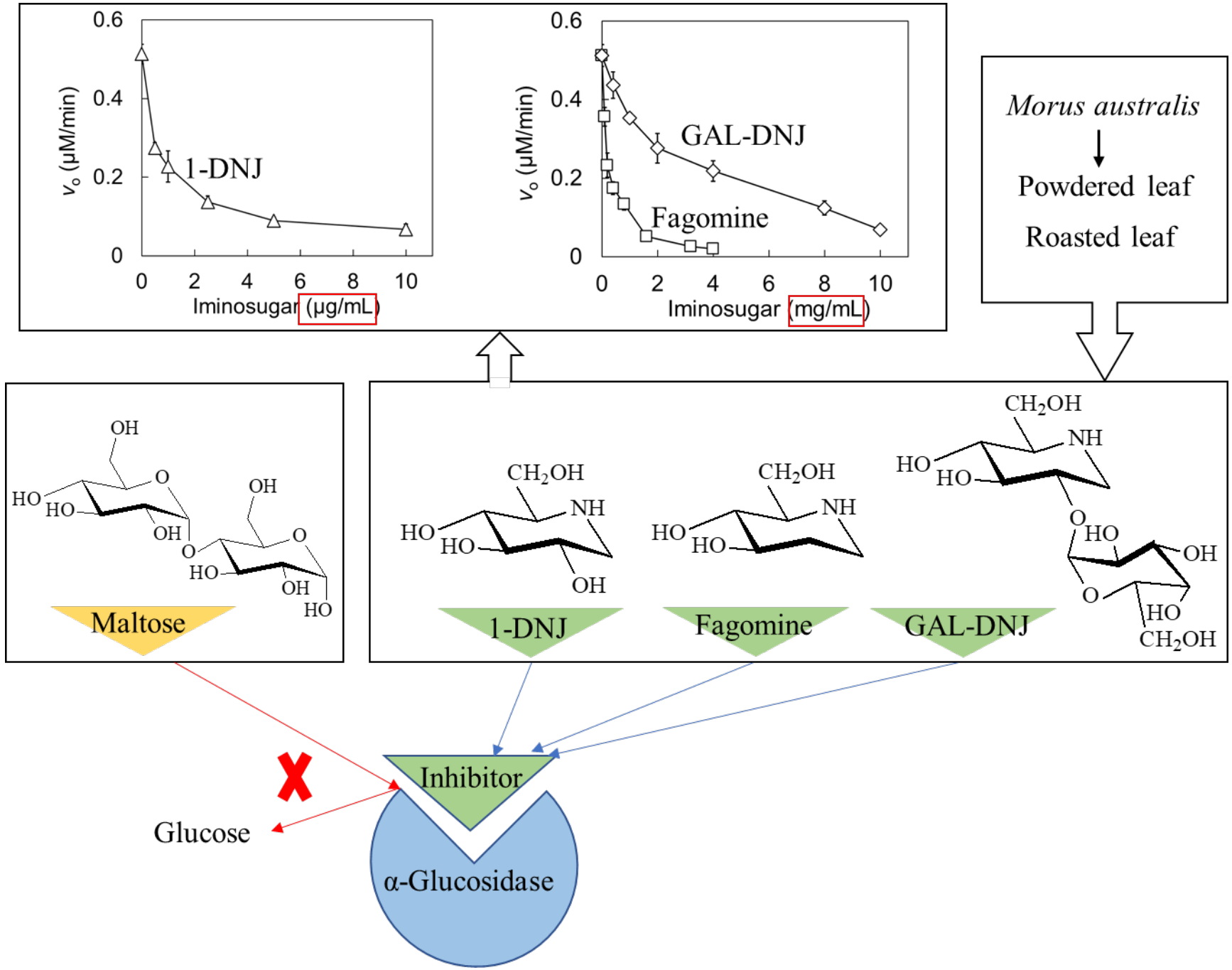
Abbreviations: 1-DNJ, 1-deoxynojirimycin; GAL-DNJ, 2-*O*-α-D-galactopyranosyl-DNJ


0.6
0.4
0.2
0
v_o (μM/min)
1-DNJ
0 2 4 6 8 10
Iminosugar (μg/mL)
GAL-DNJ
Fagomine
Iminosugar (mg/mL)
Morus australis
Powdered leaf
Roasted leaf
Maltose
1-DNJ
Fagomine
GAL-DNJ
Inhibitor
Glucose
α-Glucosidase

Mulberry leaves contain iminosugars, such as 1-deoxynojirimycin (1-DNJ), fagomine, and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ) that inhibit α-glucosidase. In this study, we quantified iminosugars in *Morus australis* leaves and made the kinetic analysis in the hydrolysis of maltose by α-glucosidase. By LC-MS/MS, the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the powdered leaves were 4.0, 0.46, and 2.5 mg/g, respectively, and those in the roasted ones were 1.0, 0.24, and 0.73 mg/g, respectively, suggesting that the roasting process degraded iminosugars. Steady-state kinetic analysis revealed that the powdered and roasted leaves exhibited competitive inhibition. At pH 6.0 at 37ºC, the IC_{50} values of the extracts from the boiled powdered or roasted leaves were 0.36 and 1.1 mg/mL, respectively. At the same condition, the IC_{50} values of 1-DNJ, fagomine, and GAL-DNJ were 0.70 μg/mL, 0.18 mg/mL, and 2.9 mg/mL, respectively. These results suggested that in *M. australis*, 1-DNJ is a major inhibitor of α-glucosidase.

Key words: α-glucosidase; fagomine; iminosugar; 1-DNJ; *Morus australis*.

Introduction

α-Glucosidase [EC 3.2.1.20] catalyzes the hydrolysis reaction of α-1,4 glycosidic bonds at the non-reducing end of the substrate. It is involved in the digestion of sugars. One of the strategies to treat type-2 diabetes is inhibition of α-glucosidase. Acarbose, miglitol, and voglibose are clinically used for this purpose.[1)] 1-Deoxynojirimycin (1-DNJ) strongly inhibits α-glucosidase activity and is anticipated for clinical use.[2)] In addition, various α-glucosidase inhibitors have been identified in plants. They include alkaloids such as vasicine,[3)] flavonoids such as quercetin,[4)] polyphenols such as *p*-hydroxycinnamic acid and protocatechuic acid,[5)] and iminosugars such as 1-DNJ,[2,6-9)] fagomine,[7)] and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ).[7)]

Morus (commonly known as mulberry) is distributed in temperate regions in the world. It is cultivated as a crop for silkworm feed, fruit, and timber. *Morus* contains various α-glucosidase inhibitors, among which 1-DNJ is the most characterized.[7-10)] *Morus australis* is distributed in Ryukyu islands and is widely cultivated in Urasoe-shi, Okinawa, Japan. It was reported that the 1-DNJ concentrations in *M. australis* leaves were 4 to 9-fold higher than those in *Morus alba* leaves distributed in Japanese mainland.[10,11)] Powdered and roasted *M. australis* leaves were developed as a tea with a function to inhibit α-glucosidase activity. The ingestion of the tea suppressed sucrose-induced elevation of blood glucose and insulin levels, indicating that the tea inhibited postprandial elevation of blood glucose.[11)] In the inhibition of the hydrolysis of sucrose by sucrase from rat intestine, boiled water extract of powdered *M. australis* leaves exhibited the IC_{50} value of 12 ± 1.3 μg/mL.[11)] These results suggest that a high intake of *M. australis* tea might be effective for the prevention of type-2 diabetes. Recently, various physiological activities of 1-DNJ have been reported, such as suppression of the elevation of

postprandial blood glucose,[13] postprandial glycemic control in subjects with impaired glucose metabolism,[14] stimulation of adiponectin and GLUT4 expressions adipocytes,[15] hypoglycemic effect,[16] prevention of diet-induced obesity through increases in adiponectin,[17] and suppression of lipid accumulation through activation of the β-oxidation system.[18] To pursue the application of *M. australis* leaves, enzyme chemical analysis of the inhibitory effects of *M. australis* leaves and its inhibitory components toward α-glucosidase is required. In this study, we selected three iminosugars 1-DNJ, fagomine, and GAL-DNJ as a research target. We quantified their concentrations in *M. australis* leaves by LC-MS/MS and made the kinetic analysis of their inhibitory effects toward the hydrolysis of maltose by α-glucosidase.

Materials and methods

Materials. Rat intestinal acetone powder was purchased from Sasaki Chemical (Kyoto, Japan) and used as the preparation of α-glucosidase. Maltose, 1-DNJ, and Glucose CII test were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Fagomine and GAL-DNJ were purified from mulberry leaves as reported previously.[9,12] Powdered and roasted *M. australis* leaves were purchased from Urasoe-shi silver human resources center (Urasoe, Japan).

Preparation of extracts of M. australis leaves. Boiled water (50 mL) was added to powdered or roasted *M. australis* leaves (500 mg) and stirred for 5 min. The solution was suction-filtrated, and the filtrate was collected and used as the leaf extract.

LC-MS/MS analysis. LC-MS/MS analysis was performed as previously described.[12] Briefly, 50 mg of powdered or roasted *M. australis* leaves were dissolved in 5.0 mL of 50% ethanol. After sonication for 5 min, the mixture was centrifuged at 10,000

x *g* for 5 min at 4ºC. The supernatant was separated and filtered (0.2 μm pore size; Sartorius Stedim Biotech, Goettingen, Germany). The filtrate was diluted with 0.1% formic acid, 50% acetonitrile to be 1% v/v for the quantification of 1-DNJ and GAL-DNJ and to be 10% for the quantification of fagomine. Standard 1-DNJ, fagomine, and GAL-DNJ were dissolved in 0.1% formic acid, 50% acetonitrile to be 200–1,000 ng/mL.

Conditions of LC-MS/MS are as follows: apparatus, LC-20A and LCMS-8045 (Shimadzu, Kyoto, Japan); column, TSK gel Amide-80 (particle size 5 μm; 100 mm × 2.0 mm i.d., Tosoh, Tokyo, Japan); column oven temperature, 40ºC; mobile phase, 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B); mobile phase flow rate, 0.2 mL/min; injection volume, 5 μL; ion source, electrospray ionization (positive mode); drying gas, nitrogen (180ºC, 7 L/min); nebulizing gas, nitrogen (1.6 bar); capillary voltage, -4,500 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, nitrogen (1.6 bar); collision energy, 10 eV; collision RF, 100 Vpp; and mass range, *m/z* 50–1500. Elution gradients are as follows: 0–2.0 min, 20%–60% B; 2.0–5.5 min, 60% B; 5.5–5.6 min, 60%–20% B; and 5.6–8.0 min, 20% B. 1-DNJ, fagomine, and GAL-DNJ were detected individually in the postcolumn by MS/MS with multiple reaction monitoring (MRM) for transition of the parent ions to the product ions. The concentrations of 1-DNJ, fagomine, and GAL-DNJ in powdered and roasted *M. australis* leaf extracts were calculated from calibration curves using standard 1-DNJ, fagomine, and GAL-DNJ.

Preparation of crude α-glucosidase solution. Rat intestinal acetone powder (0.5 g) was suspended with 5 mL of water followed by sonication. The solution was centrifuged at 10,000 x *g* for 15 min at 4ºC, and the supernatant was collected. Then, the solution was diluted with water to be 35 mg/mL in protein concentration and used as the α-glucosidase

106 solution.

107 *Measurement of α-glucosidase activity.* 1-DNJ, fagomine, and GAL-DNJ were
108 dissolved in water. Protocol for the reaction at pH 6.0 at 37ºC is as follows. Pre-incubation
109 (90 μl) was initiated by mixing 80 μl of the maltose solution in 0.1 M maleate buffer (pH
110 6.0) and 10 μl of the extracts of powdered (0–10 mg/mL) or roasted (0–20 mg/mL) *M.*
111 *australis* leaves or 10 μl of the 1-DNJ (0–1 mg/mL), fagomine (0–40 mg/mL), and GAL-
112 DNJ (0–100 mg/mL) solution in water. After the pre-incubation at 37ºC for 10 min, the
113 reaction was initiated by adding 10 μl of α-glucosidase solution and continued at 37ºC.
114 Blank solution was prepared by adding 10 μl of 0.1 M maleate buffer (pH 6.0). After 1 or
115 2 min, the solution was boiled for 5 min to stop reaction. To 13.3 μl of the solution, 200
116 μl of the coloring solution in Glucose CII test was added. The absorbance at 505 nm was
117 measured with an EnSight multimodal plate reader (PerkinElmer, Waltham, MA). From
118 the reaction rate, the Michaelis constant in the absence of inhibitor (K_m), the Michaelis
119 constant in the presence of inhibitor (K_{mapp}), and maximum velocity (V_{max}) were
120 calculated based on using Hanes-Woolf plot with Microsoft Excel.

121 The inhibitor constant (K_i) of reversible competitive inhibitor was calculated by the
122 following equation:

123

$$\frac{K_{mapp}}{K_m} = 1 + \frac{[I]_o}{K_i} \quad (1)$$

124

125 where $[I]_o$ is the initial inhibitor concentration.

126

127 *Thermodynamic analysis.* The enthalpy change, ΔH^o, of the dissociation of the

complex of α-glucosidase and inhibitor was determined from a van't Hoff plot according to Eq. 2, as described previously.[19] The Gibbs free energy change of dissociation, ΔG^o and the entropy change of dissociation, ΔS^o, at certain temperature were determined according to Eqs. 3 and 4, respectively.

$$\ln(K_i) = A - (\Delta H^o/R)(1/T) \quad (2)$$

$$\Delta G^o = -RT \ln K_i \quad (3)$$

$$\Delta S^o = (\Delta H^o - \Delta G^o) / T \quad (4)$$

where A, R, and T are the constant term, the gas constant (= 8.314 J K^{-1} mol^{-1}), and absolute temperature in degrees Kelvin.

Results and discussion

Quantification of iminosugars in M. australis leaves by LC-MS/MS

For quantification of iminosugars in *M. australis* leaves, we applied a standard 1-DNJ, fagomine, and GAL-DNJ to LC-MS/MS to make a calibration curve. The total ion scanning profile showed an intense molecular ions at *m/z* 164.20 $[M+H]^+$ for 1-DNJ, 148.20 $[M+H]^+$ for fagomine, and 326.20 $[M+H]^+$ for GAL-DNJ. The product ion scanning for these ions showed intense molecular ions at *m/z* 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (Fig. S1a), at *m/z* 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (Fig. S1b), and at *m/z* 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (Fig. S1c). The MRM chromatogram of the total of these three ions showed a peak at 5.4 min for 1-DNJ (Fig. S2a), at 4.0 min for fagomine (Fig. S2b), and at 4.3 min for GAL-DNJ (Fig. S2c).

The peak intensity increased linearly with increasing concentration of 1-DNJ (0–1,000 ng/mL) (Fig. S3a), fagomine (0–1,000 ng/mL) (Fig. S3b) or GAL-DNJ (0–1,000 ng/mL) (Fig. S3c).

We applied the extracts of powdered or roasted *M. australis* leaves to determine the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the extracts of powdered (Fig. 1a–c) and roasted (Fig. 1d–f) leaves. The MRM chromatogram showed one peak at 5.4 min for the analysis of 1-DNJ (Fig. 1a and d), two peaks at 3.4 and 4.0 min, respectively, for the analysis of fagomine (Fig. 1b and e), and one peak at 4.3 min for the analysis of GAL-DNJ (Fig. 1c and f). In the analysis of fagomine, the peak at 4.0 min was used (Fig. 1b and e), according to the MRM chromatogram of standard fagomine (Fig. S2b). The structure of the substance corresponding to the peak at 3.4 min is unknown. Based on the calibration curves (Fig. S3), the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the powdered leaf extracts were calculated to be 40, 4.6, and 25 μg/mL, respectively, and those in the roasted leaf extracts were calculated to be 10, 2.4, and 7.3 μg/mL, respectively. Thus, the concentrations of these three iminosugars in the leaves were calculated to be 4.0, 0.46, and 2.5mg/g, respectively, for the powdered ones and 1.0, 0.24, and 0.73 mg/g, respectively, for the roasted ones. These results indicated that the iminosugar concentrations in the roasted leaves were 25–52% of those in the powdered leaves, suggesting that the roasting process considerably degraded iminosugars. These results also indicated that the 1-DNJ concentration in the leaves was 1.6–8.7 and 1.4–4.2 fold higher than the fagomine and GAL-DNJ concentrations, respectively.

The 1-DNJ concentrations of mulberry leaves in *M. alba and M. bombysis* were reported to be 1.0–1.4 mg/g, and those of mulberry leaf products on the market were 1.3–4.8 mg/g.[10] Thus, it seems that the 1-DNJ concentration of *M. australis* leaf is

176 relatively high. However, it should be noted that 1-DNJ concentration in mulberry leaf
177 varies depending on seasons and collection site.[19)]
178
179 *Kinetic analysis of inhibition of α-glucosidase by the extracts of M. australis leaves*
180 The extract we prepared from rat intestinal acetone powder and used as the α-
181 glucosidase solution was thought to contain maltase-glucoamylase and sucrase-
182 isomaltase.[20)] In this study, we characterized the inhibitory effects of the extracts of *M.*
183 *australis* leaves and imminosugars toward the maltase-mediated maltose-hydrolyzing
184 activity and regarded the effects toward α-glucosidase activity.
185 We examined the reaction rates of α-glucosidase in the hydrolysis of 50 mM
186 maltose in the presence of various concentrations of the extracts of powdered (0–1.0
187 mg/mL) or roasted (0–2.0 mg/mL) *M. australis* leaves at pH 6.0 at 37ºC (Fig. 2a). The
188 reaction rate decreased with increasing the concentration of each extract. The IC_{50}
189 values, which were determined using intersection at 50% activity in line graphs, of the
190 extract of powdered leaves and that of roasted leaves were 0.36 and 1.1 mg/mL,
191 respectively, indicating that the inhibitory effect of the powdered leaves was 3-fold
192 higher than that of the roasted leaves.
193 We measured the reaction rates of α-glucosidase in the hydrolysis of various
194 concentrations (0–75 mM) of maltose in the presence of 0.6 mg/mL of powdered or
195 roasted leaf extract at pH 6.0 at 37ºC. All plots showed saturated profiles (Fig. 2b).
196 Hanes-Woolf plot showed parallel lines intersecting at the Y-axis (Fig. 2c), indicating
197 that the manner of inhibition was competitive.
198
199 *Kinetic analysis of inhibition of α-glucosidase by 1-DNJ, fagomine, and GAL-DNJ*

Figure 3a and b show the reaction rates of α-glucosidase in the hydrolysis of 50 mM maltose in the presence of various concentrations of 1-DNJ (0–10 μg/mL), fagomine (0–4 mg/mL), and GAL-DNJ (0–8 mg/mL) at pH 6.0 at 37ºC. The reaction rates decreased with increasing the concentration of each iminosugar. The activity was almost completely inhibited by 1-DNJ and fagomine, while 20% activity remained by GAL-DNJ, suggesting that the inhibition by GAL-DNJ is partial. The IC_{50} values were 0.70 μg/mL for 1-DNJ, 0.18 mg/mL for fagomine, and 2.9 mg/mL for GAL-DNJ, indicating that the inhibitory effect of 1-DNJ was 250- and 4,000- fold higher than that of fagomine or GAL-DNJ, respectively.

Figure 3c shows the reaction rates of α-glucosidase in the hydrolysis of various concentrations (0–75 mM) of maltose in the presence of 0.25 μg/mL of 1-DNJ, 0.1 mg/mL of fagomine, or 1.5 mg/mL of GAL-DNJ at pH 6.0 at 37ºC. Like the case with the reaction in the presence of the leaf extracts, all plots showed saturated profiles (Fig. 2b). Hanes-Woolf plot showed parallel lines intersecting at the Y-axis (Fig. 3d), indicating competitive inhibition. The K_m value was calculated to be 1.9 mM in the absence of iminosugar, and the K_{mapp} values were calculated to be the 9.0 mM in the presence of 0.25 μg/mL 1-DNJ, 16 mM in the presence of 0.1 mg/mL fagomine, and 30 mM in the presence of 1.5 mg/mL GAL-DNJ.

The K_i values of 1-DNJ (molecular weight: 163), fagomine (147), and GAL-DNJ (330) calculated from the K_m and K_{mapp} values using Eq. 1 were 0.068 μg/mL (= 4.1×10^{-7} M), 14 μg/mL (= 9.2×10^{-5} M), and 102 μg/mL (= 3.1×10^{-4} M), respectively. The K_i values of 1-DNJ and fagomine thus obtained were similar to those previously reported (1.12×10^{-6} M for 1-DNJ and 3.24×10^{-4} M for fagomine).[21)]

Insight into the contribution of 1-DNJ in M. australis leaves to the inhibition of α-glucosidase

As described above, the concentrations of IC_{50} value of the powdered leaf extract, roasted leaf extract, and 1-DNJ were 360, 1,100, and 0.70 μg/mL, respectively, and the concentrations of 1-DNJ in the powdered and roasted leaves were 4.0 and 1.0 mg/g, respectively. Nakanishi et al. showed that dependences on season of 1-DNJ amount and the α-glucosidase inhibitory activity of mulberry leaves exhibited similar profiles.[22)] In this study, the inhibitory effects of fagomine and GAL-DNJ to α-glucosidase were thought to be marginal compared with 1-DNJ. Assuming that 1-DNJ is a sole component that inhibits α-glucosidase in mulberry leaf, it was calculated that 360 μg/mL powdered leaf extract contained 1.4 μg/mL 1-DNJ, and 1,100 μg/mL roasted leaf extract contained 1.1 μg/mL 1-DNJ. These concentrations correspond to 200% and 150%, respectively, of the IC_{50} value of 1-DNJ (0.70 μg/mL), suggesting that in *M. australis*, the inhibitory effects of 1-DNJ were suppressed in the powdered and roasted leaf extracts. We speculate that GAL-DNJ, which exhibited partial inhibition, might suppress the inhibitory effect of 1-DNJ.

Effects of temperature and pH on the inhibition of α-glucosidase by the extracts of M. australis leaves and 1-DNJ

To explore the role of 1-DNJ on the inhibition of α-glucosidase by the extracts of *M. australis* powdered leaves, we examined the effects of temperature (27–47ºC) and pH (6.0–8.0) on the inhibition. Figure S4 shows the effects of reaction temperature and pH on the α-glucosidase activity. The highest activity was observed at 62ºC (Fig. S4a), and the relatively high activity was observed at pH 5–8 (Fig. S4b), suggesting that α-

glucosidase did not lose activity at pH 6.0–8.0 or at 27–47ºC. Figure S5a–d show the reaction rates of α-glucosidase in the hydrolysis of various concentrations (0–75 mM) of maltose in the presence of 0.15 mg/mL of the powdered leaf extracts or 0.25 μg/mL 1-DNJ at pH 6.0–8.0, at 27–47ºC. All plots showed saturated profiles.

The K_i values were calculated from Eq. 1. Figure 4a and b show the dependence of K_i values at pH 6.0 on reaction temperature and the dependence of K_i values at 37ºC on pH, respectively. The profiles of the extracts of powdered leaves and 1-DNJ were very similar, suggesting that 1-DNJ is a major inhibitory component in the powdered leaves.

The K_i values of the dissociation depended on temperature (Fig. 4a). Figure 4c shows van't Hoff plot of K_i values. Enthalpy changes (ΔH^o) at pH 6 were calculated from the slope to be -87.6 ± 6.4 kJ mol^{-1} for the extracts of powdered leaves and -72.8 ± 12.9 kJ mol^{-1} for 1-DNJ. The negative ΔH^o values of the dissociation indicate that the dissociation was exothermic. For 1-DNJ, Gibbs free energy change of dissociation (ΔG^o) at pH 6.0 at 37ºC were calculated be 38.1 kJ mol^{-1} for 1-DNJ, and entropy change of dissociation (ΔS^o) at pH 6.0 at 37ºC were calculated to be -351.7 J mol^{-1} K^{-1} from Eq. 3. Since ΔH^o is negative and $-T\Delta S^o$ is positive, the dissociation is enthalpy-driven. The K_i values of the dissociation also depended on pH (Fig. 4b). This might be due to that changes in ionization state of the amino acid residues of α-glucosidase involved in the binding.

In conclusion, *M. australis* leaves contain 1-DNJ a lot and strongly inhibit α-glucosidase activity. The K_i values of the extract of *M. australis* powdered leaves and 1-DNJ are temperature- and pH-dependent. By combining LC-MS/MS, the kinetic analysis of leaf extracts and its components might be valuable to evaluate the inhibitory activity toward α-glucosidase of various plant products.

Author contribution

M.I., T.K. and K.Y. designed research; Y.Q., J.N., and K.Y. performed research; Y.Q., J.N., T.I., M.I., T.K., K.K., T.T, and K.Y. analyzed data; Y.Q., M.I., T.K., and K.Y. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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354 **Figure Legends**

355

356 Fig. 1. MRM chromatogram of the extracts of *M. australis* leaves.

357 Notes: The extracts of powdered (a–c) or roasted (d–f) leaves were applied. MRM
358 chromatogram of the total of ions at *m/z* 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20
359 $[M+H-H_2O]^+$ for 1-DNJ (a, d), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15
360 $[M+H-H_2O]^+$ for fagomine (b, e), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and
361 164.20 $[M+H-162]^+$ for GAL-DNJ (c, f) are shown.

362

363 Fig. 2. Effects of the extracts of *M. australis* leaves on the hydrolysis of maltose by α-
364 glucosidase.

365 Notes. (a) Dependence on the concentration of leaf extracts on the reaction rate.
366 The reaction was carried out with 50 mM maltose and varying concentrations of
367 powdered (open triangle) or roasted (open square) leaf extracts at pH 6.0 at 37ºC. (b, c)
368 Dependence on the substrate concentration of the reaction rate. The reaction was carried
369 out with varying concentrations of maltose in the absence of iminosugar (open circle) or
370 in the presence of 0.6 mg/mL powdered leaf extracts (open triangle) or roasted leaf
371 extracts (open square) at pH 6.0 at 37ºC. (b) Reaction rate (v_o) *vs.* maltose concentration
372 ([maltose]). Solid line represents the best fit to the experimental data using the
373 Michaelis-Menten equation using Microsoft Excel. (c) Hanes-Woolf plot ([maltose] /v_o
374 *vs.* [maltose]). The K_m and V_{max} values were calculated to be 1.9 mM and 0.48 μM/min,
375 respectively, in the absence of leaf extracts, and the K_{mapp} and V_{max} values were
376 calculated to be 30 mM and 0.37 μM/min, respectively, in the presence of 0.6 mg/mL of
377 powdered leaf extract, and 18 mM and 0.45 μM/min, respectively, in the presence of 0.6

mg/mL of roasted leaf extract. Error bars indicate SD values. The average of triplicate determination is shown.

Fig. 3. Effects of 1-DNJ, fagomine, and GAL-DNJ on the hydrolysis of maltose by α-glucosidase.

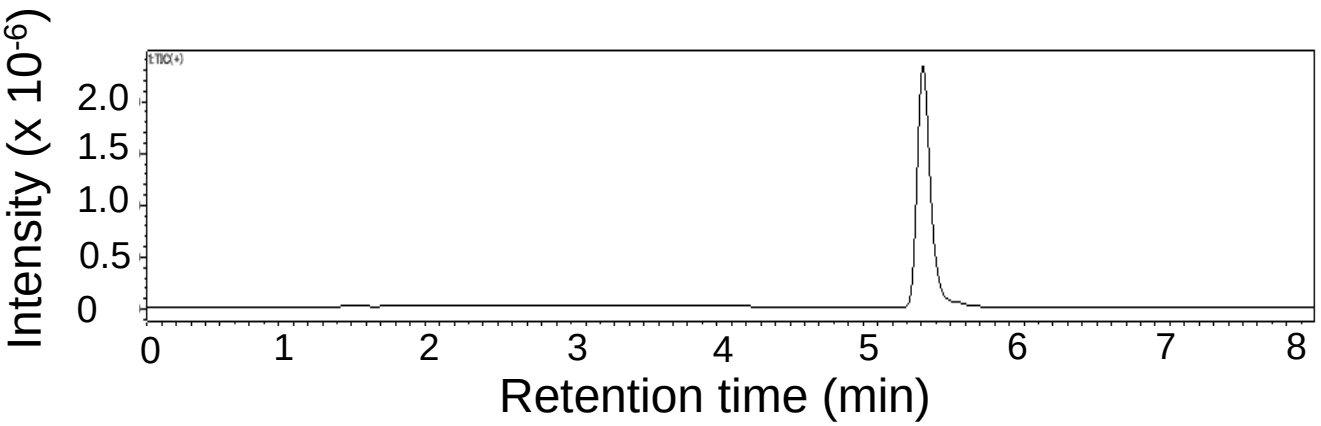
Notes. (a, b) Dependence on the concentration of 1-DNJ, fagomine, and GAL-DNJ on the reaction rate. The reaction was carried out with 50 mM maltose and varying concentrations of 1-DNJ (open triangle) (a), fagomine (open square) (b), or GAL-DNJ (open diamond) (b) at pH 6.0 at 37ºC. (c, d) Dependence on the substrate concentration of the reaction rate. The reaction was carried out with varying concentrations of maltose in the absence of iminosugar (open circle) or in the presence of 0.25 μg/mL of 1-DNJ (closed triangle), 0.1 mg/mL of fagomine (closed square), or 1.5 mg/mL of GAL-DNJ (closed diamond) at pH 6.0 at 37ºC. (d) v_o *vs.* [maltose]. Solid line represents the best fit to the experimental data using the Michaelis-Menten equation using Microsoft Excel. (d) Hanes-Woolf plot ([maltose] /v_o *vs.* [maltose]). The K_m and V_{max} values were calculated to be 1.9 mM and 0.48 μM/min, respectively, in the absence of iminosugar, and the K_{mapp} and V_{max} values were calculated to be 9.0 mM and 0.44 μM/min, respectively, in the presence of 0.25 μg/mL of 1-DNJ, 16 mM and 0.44 μM/min, respectively, in the presence of 0.1 mg/mL of fagomine, and 30 mM and 0.46 μM/min, respectively, in the presence of 1.5 mg/mL of GAL-DNJ. Error bars indicate SD values. The average of triplicate determination is shown.

Fig. 4. Effects of reaction temperature and pH on the inhibitory effects of the extracts of *M. australis* powdered leaves and 1-DNJ toward the hydrolysis of maltose by α-

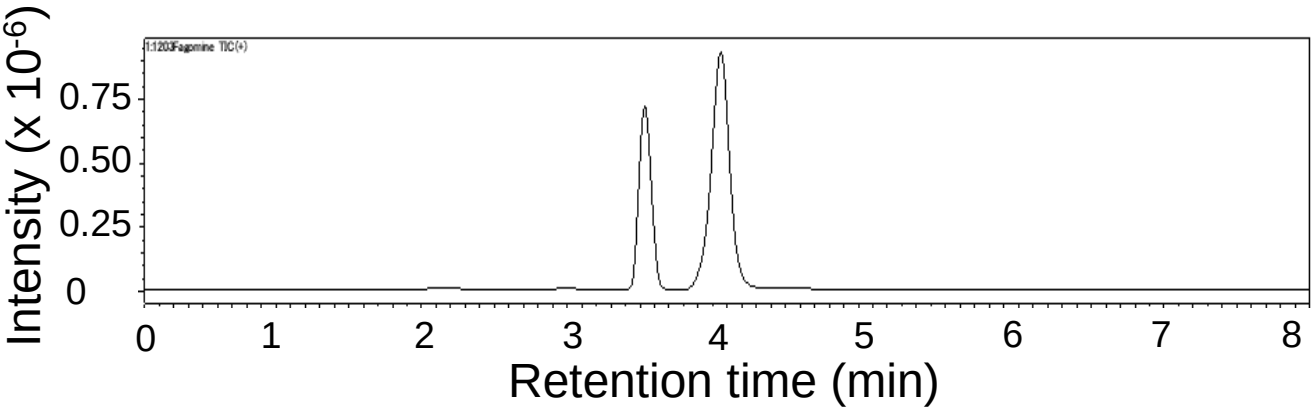
glucosidase.

Notes: (a) Dependence of K_i values at pH 6.0 on reaction temperature. The K_i values at 27ºC, 37ºC, and 47ºC are 0.21 ± 0.01, 0.040 ± 0.002, and 0.024 ± 0.003 mg/mL for the extracts of powdered leaves and 0.22 ± 0.02, 0.067 ± 0.001, and 0.035 ± 0.008 μg/mL for 1-DNJ. (b) Dependence of K_i values at 37ºC on pH. The K_i values at pH 6, 7, and 8 are 0.04 ± 0.01, 0.049 ± 0.001, and 0.18 ± 0.01 mg/mL for the extracts of powdered leaves and 0.067 ± 0.001, 0.098 ± 0.003, and 0.30 ± 0.05 μg/mL for 1-DNJ. (c) van't Hoff plot of K_i values. K_i values were plotted against the reciprocal of the absolute temperature. Enthalpy changes (ΔH^o) were calculated from the slope to be −87.6 ± 6.4 kJ mol^{-1} for the extracts of powdered leaves and −72.8 ± 12.9 kJ mol^{-1} for 1-DNJ. Error bars indicate SD values. The average of triplicate determination is shown.

a


Intensity (x 10-6)
2.0
1.5
1.0
0.5
0
0
1
2
3
4
5
6
7
8
Retention time (min)

b


Intensity (x 10-6)
0.75
0.50
0.25
0
0
1
2
3
4
5
6
7
8
Retention time (min)

c

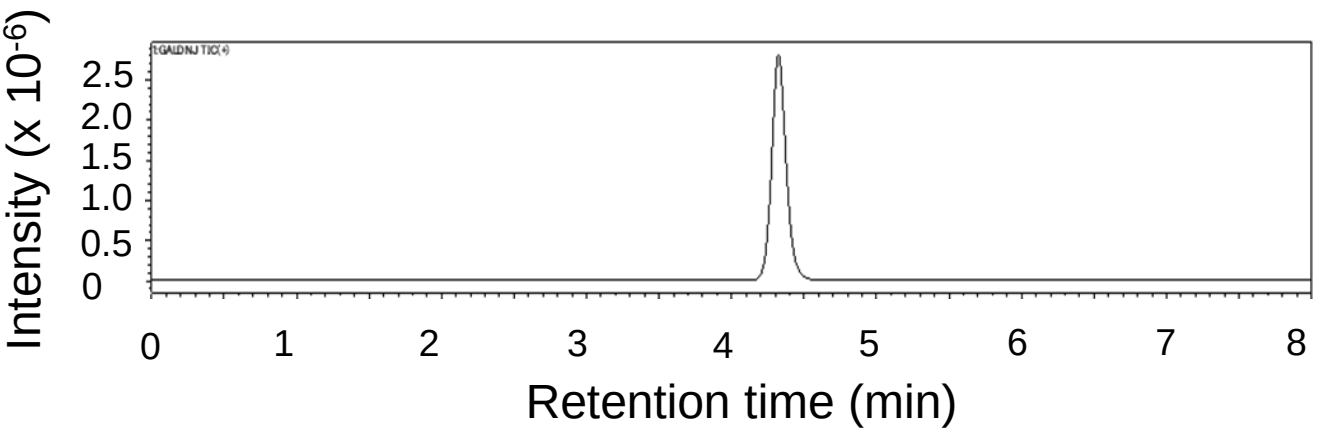
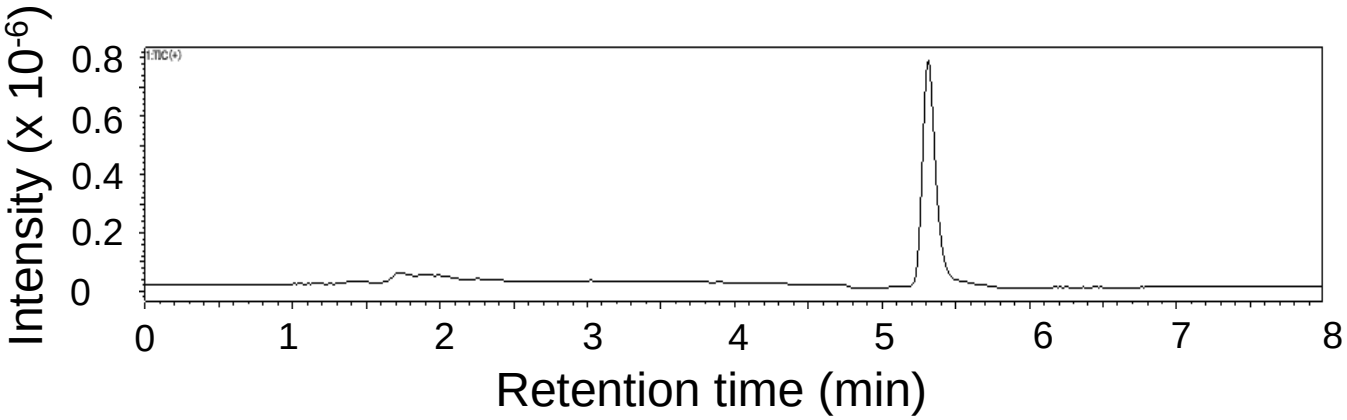
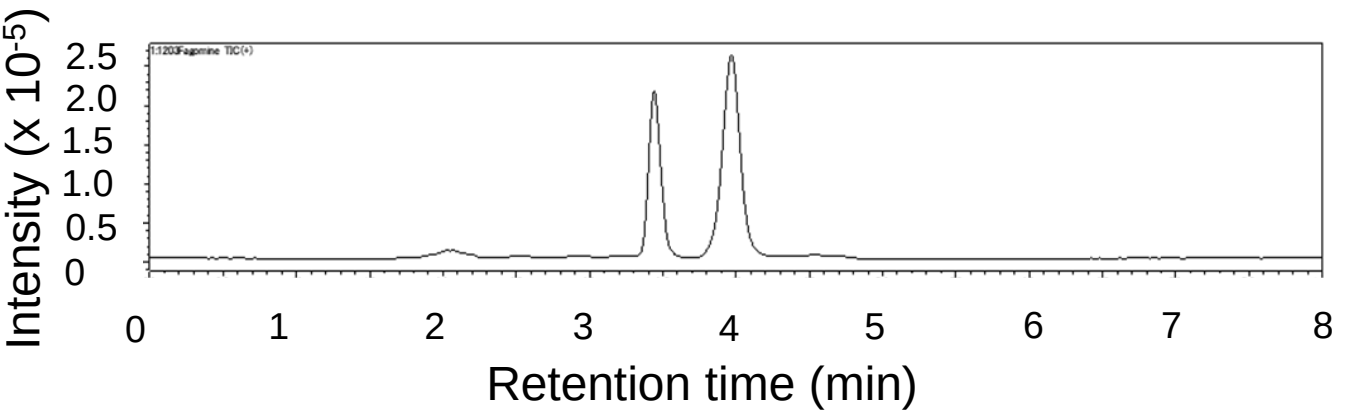

Intensity (x 10-6)
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
4
5
6
7
8
Retention time (min)

Fig. 1

d


1:TIC(+)
Intensity (x 10-6)
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
Retention time (min)

e


1:1203Fagomine TIC(+)
Intensity (x 10-5)
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
4
5
6
7
8
Retention time (min)

f

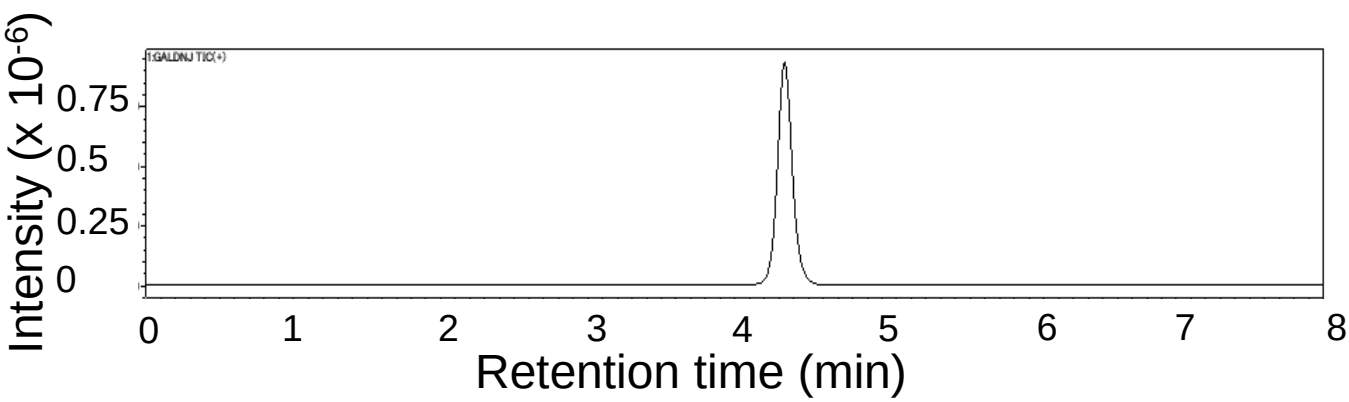
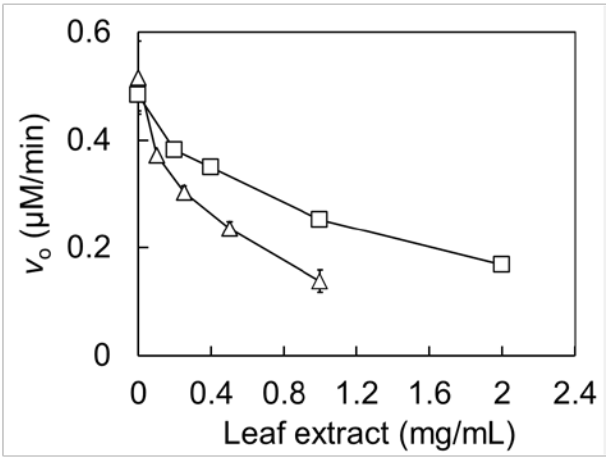
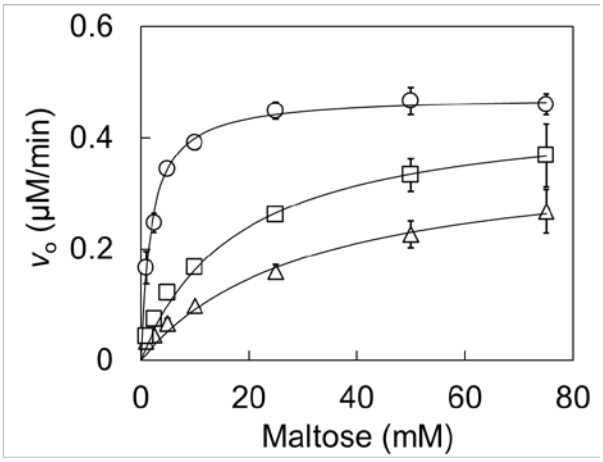

1:GALDNJ TIC(+)
Intensity (x 10-6)
0.75
0.5
0.25
0
0
1
2
3
4
5
6
7
8
Retention time (min)

Fig. 1

a


0.6
0.4
0.2
0
v_o (μM/min)
0
0.4
0.8
1.2
1.6
2
2.4
Leaf extract (mg/mL)

b


0.6
0.4
0.2
0
v_o (μM/min)
0
20
40
60
80
Maltose (mM)

c

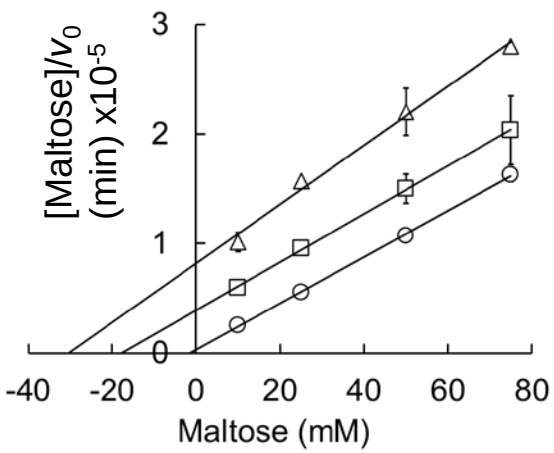

3
2
1
0
[Maltose]/v_0 (min) x10^{-5}
-40
-20
0
20
40
60
80
Maltose (mM)

Fig. 2

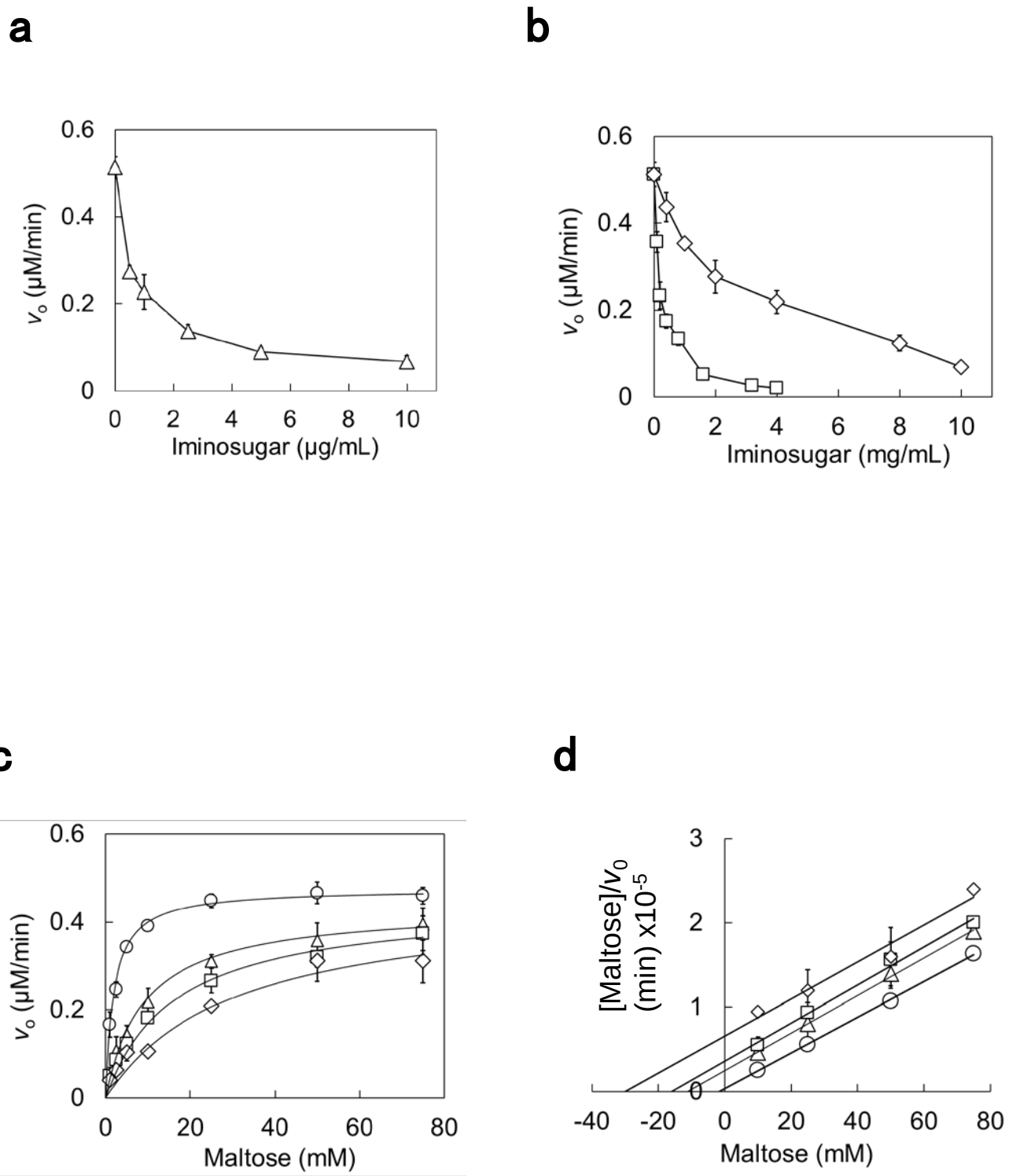

a
b
c
d
0.6
0.4
0.2
0
v_o (μM/min)
0
2
4
6
8
10
Iminosugar (μg/mL)
Iminosugar (mg/mL)
20
40
60
80
Maltose (mM)
3
2
1
-40
-20
[Maltose]/v_0
(min) x10^{-5}

Fig. 3

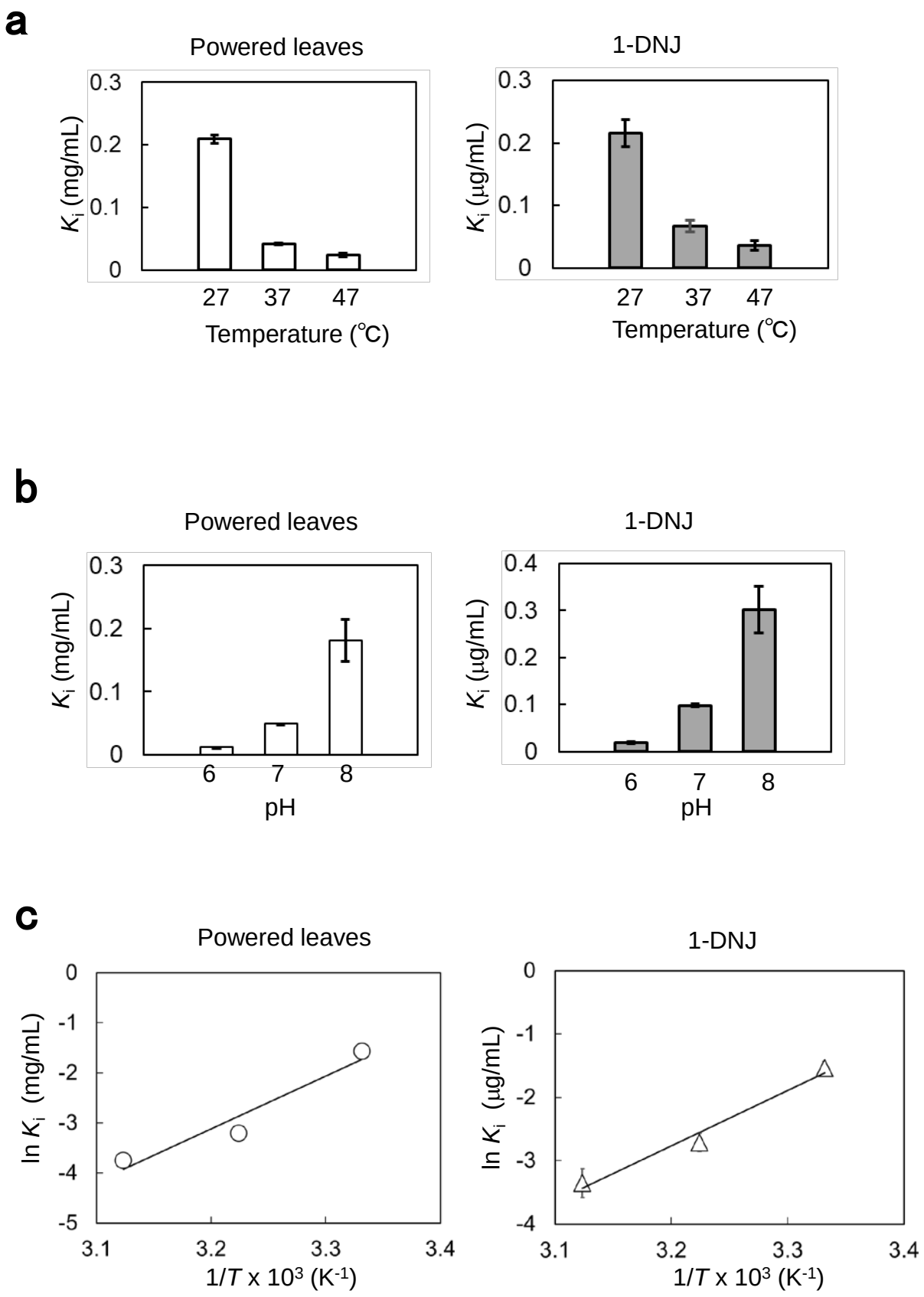

a
Powered leaves
1-DNJ
0.3
0.2
0.1
0
K_i (mg/mL)
K_i (μg/mL)
27
37
47
Temperature (℃)
b
Powered leaves
1-DNJ
0.4
6
7
8
pH
c
Powered leaves
1-DNJ
ln K_i (mg/mL)
ln K_i (μg/mL)
-1
-2
-3
-4
-5
3.1
3.2
3.3
3.4
1/T x 10^3 (K^{-1})

Fig. 4

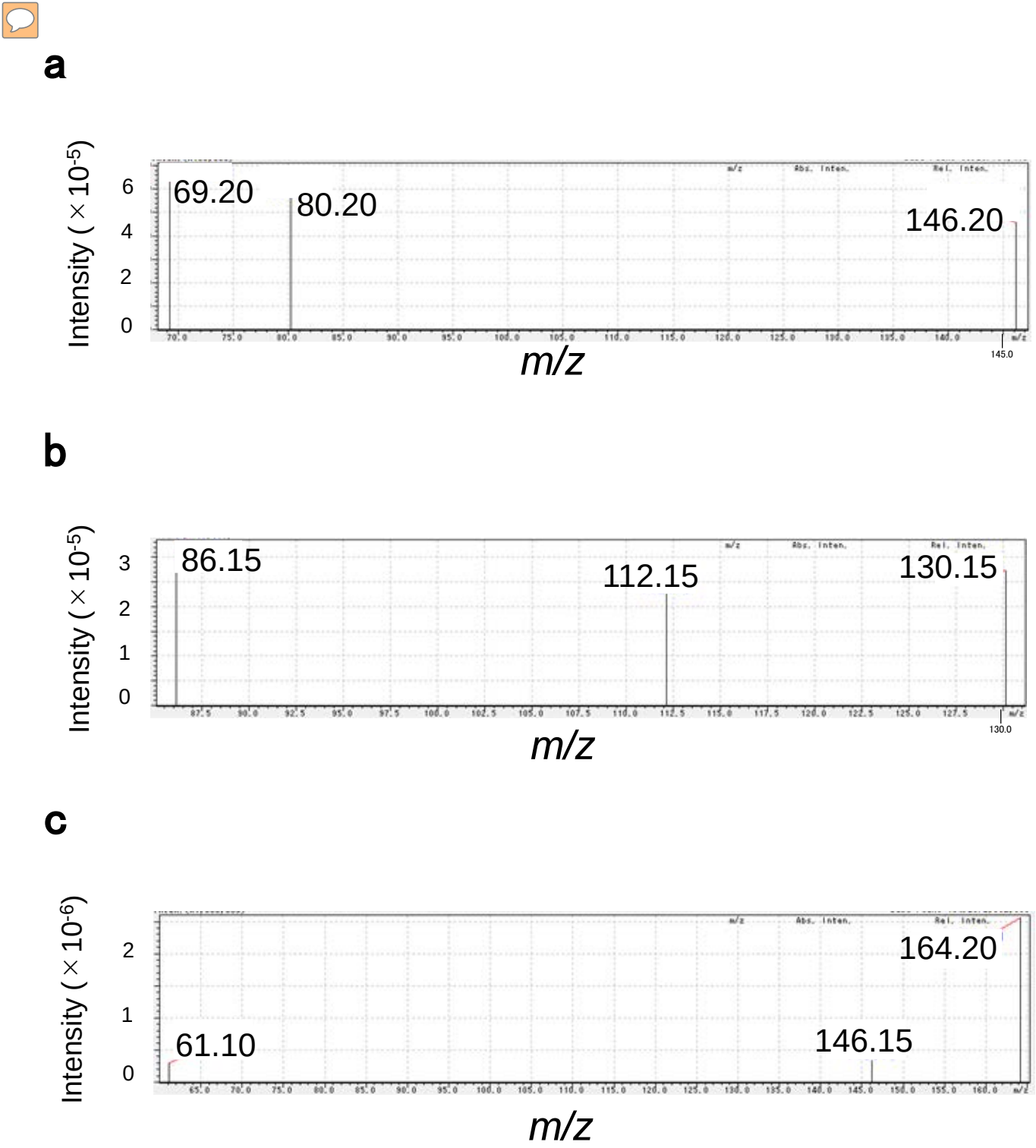


Fig. S1. Product ion spectra of 1-DNJ, fagomine, and GAL-DNJ.

Notes: Standard 1-DNJ (5 μL in 1.0 μg/μL) (a), fagomine (5 μL in 1.0 μg/μL) (b), GAL-DNJ (5 μL in 1.0 μg/μL) (c) were applied. Product ion spectra of the ions at *m/z* 164.20 $[M+H]^+$ for 1-DNJ (a), at *m/z* 148.20 $[M+H]^+$ for fagomine (b), and at *m/z* 326.20 $[M+H]^+$ for GAL-DNJ (c) are shown.

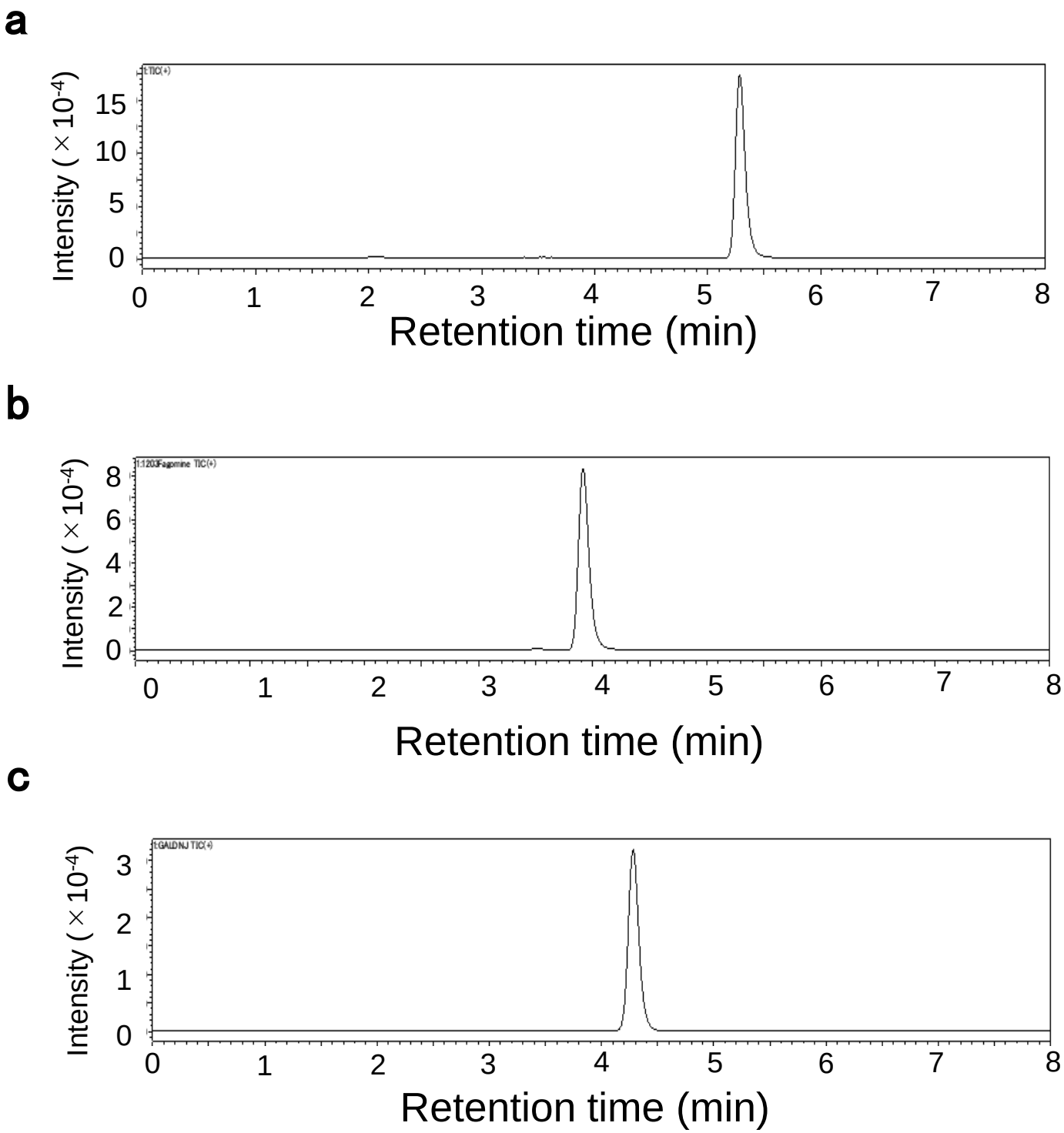


Fig. S2. MRM chromatogram of 1-DNJ, fagomine, and GAL-DNJ

Notes: Standard 1-DNJ (5 μL in 1.0 μg/μL) (a), fagomine (5 μL in 1.0 μg/μL) (b), GAL-DNJ (5 μL in 1.0 μg/μL) (c) were applied. MRM chromatogram of the total of ions at *m/z* 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (a), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (b), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (c) are shown.

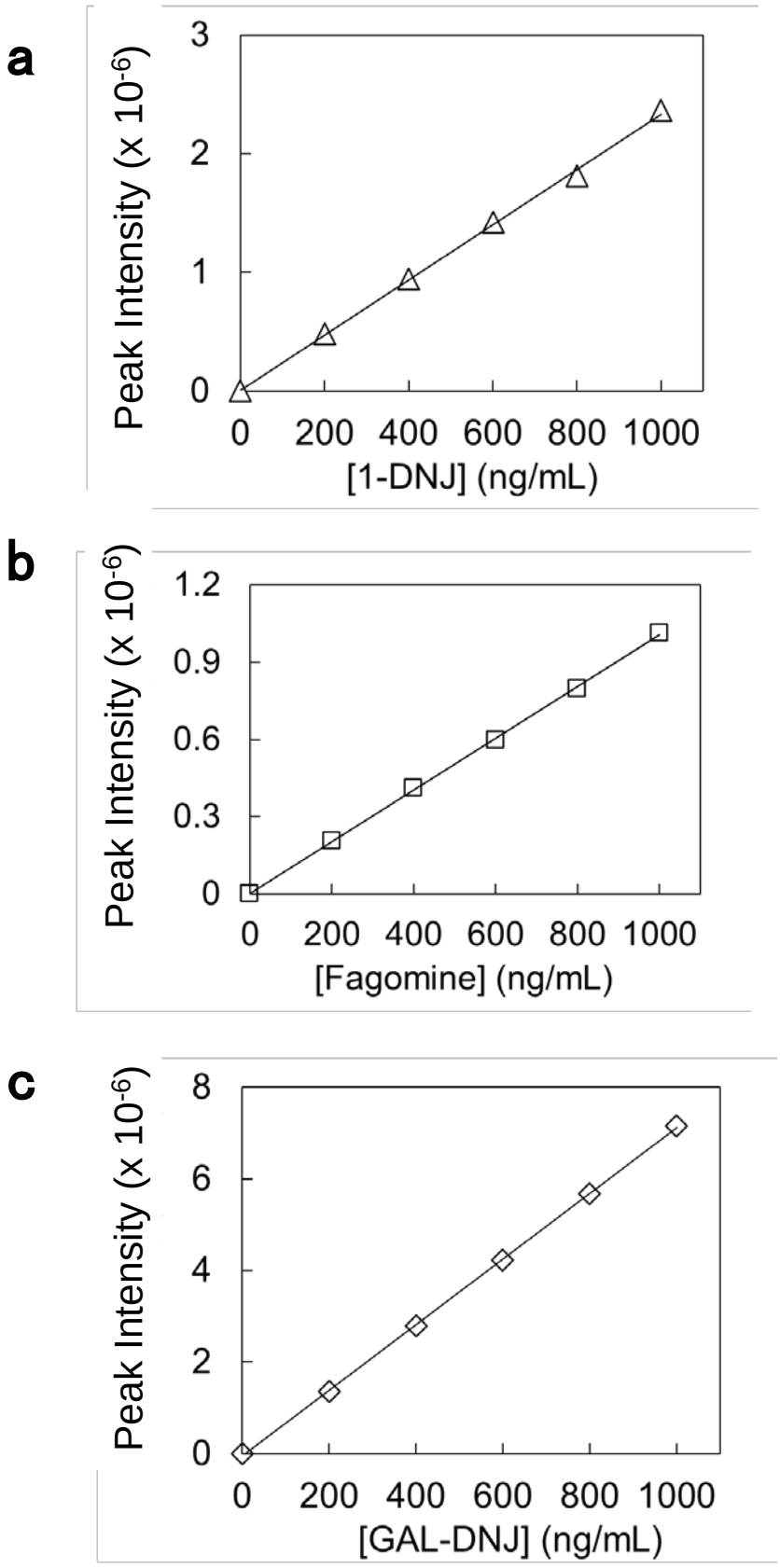


Fig. S3. Calibration curves of 1-DNJ, fagomine, and GAL-DNJ.
Notes: Calibration curves of 1-DNJ (0–1000 ng/mL) (a), fagomine (0–1000 ng/mL) (b), and GAL-DNJ (0–1000 ng/mL) (c) are shown.

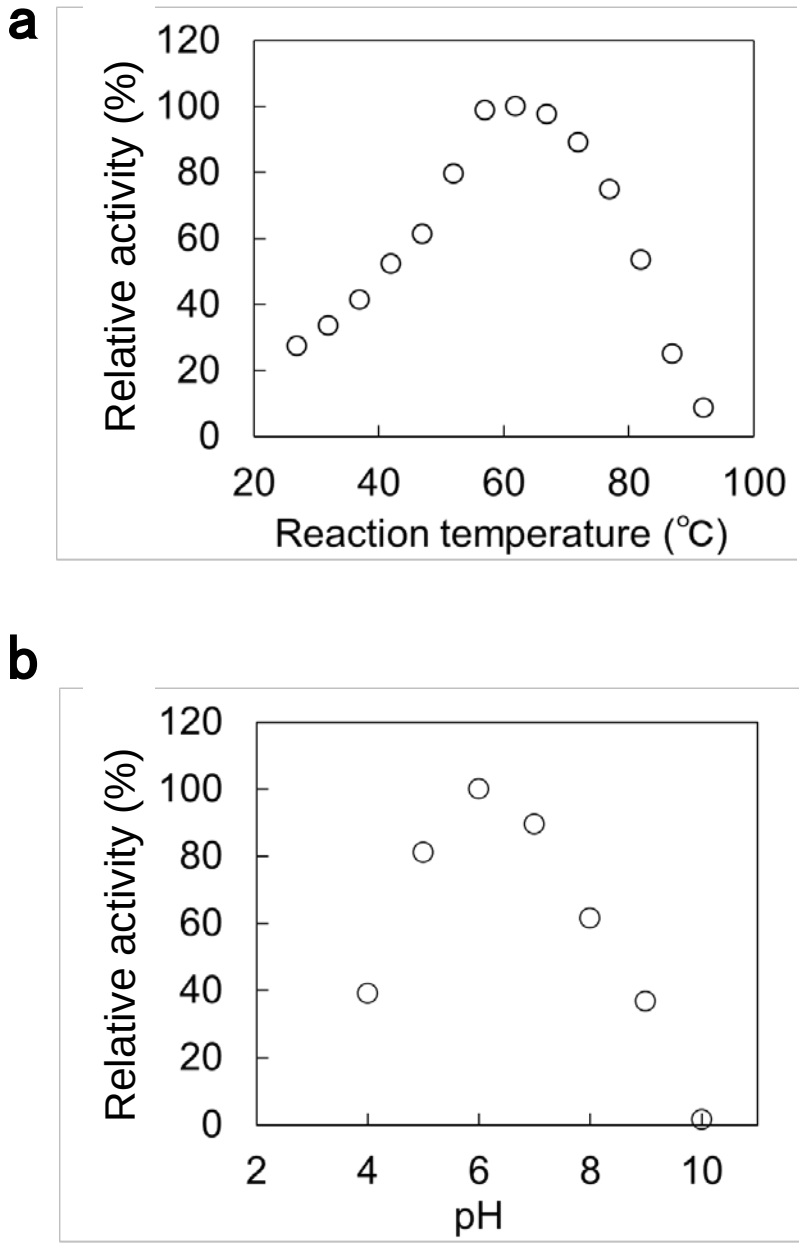


Fig. S4. Effects of reaction temperature and pH on the α-glucosidase activity.

Notes: (a) Temperature dependence. The activity at 62ºC was defined as 100%. The reaction was carried out with 3.5 mg/mL extract from rat acetone powder, 50 mM maltose at pH 6 at 27–92ºC. (b) pH dependence. The activity at pH 6 was defined as 100%. The reaction was carried out with 3.5 mg/mL extract, 50 mM maltose at pH 4–6 at 37ºC.

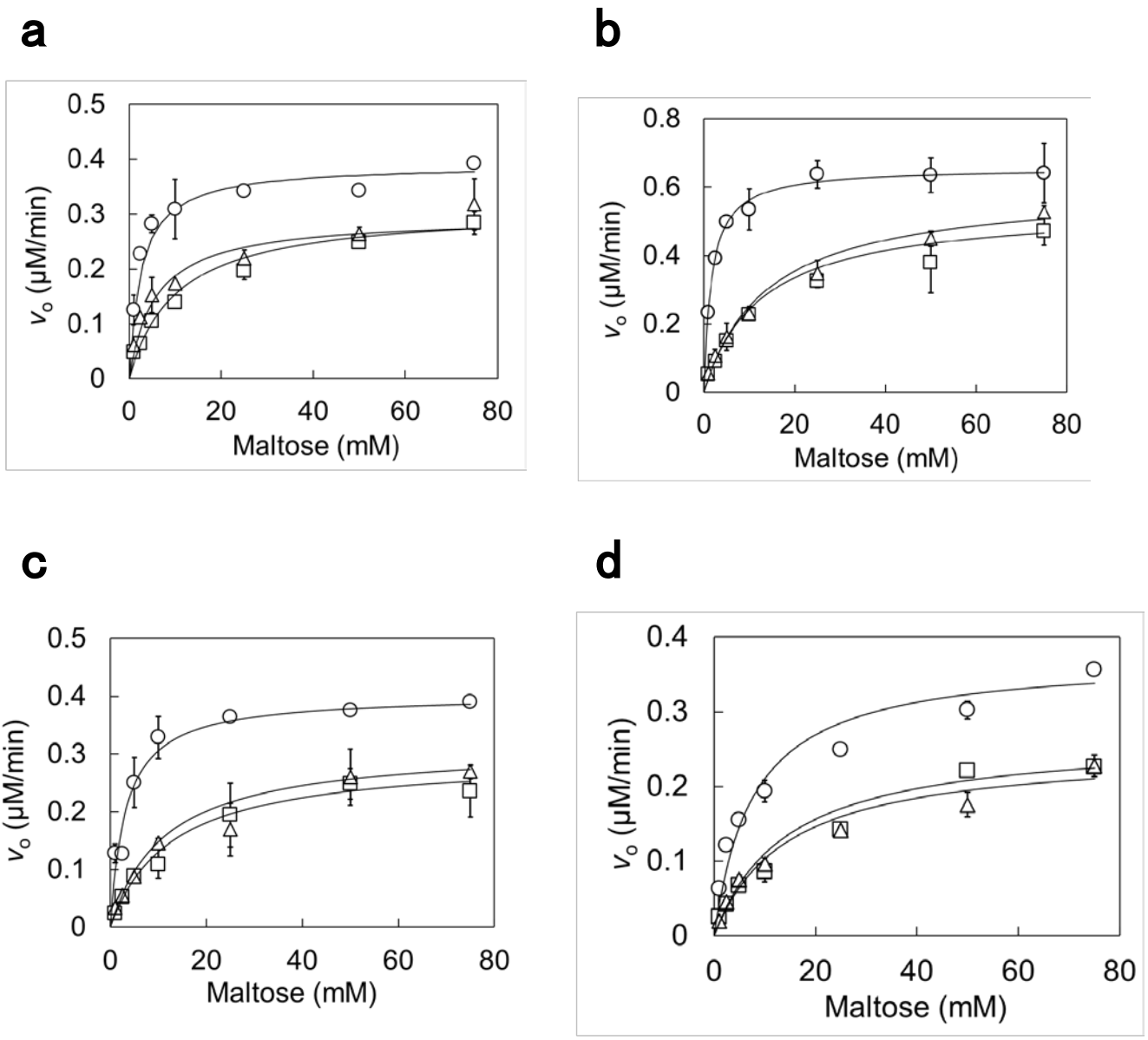


Fig. S5. Effects of reaction temperature and pH on the inhibitory effects of the extracts of *M. australis* powdered leaves and 1-DNJ toward the hydrolysis of maltose by α-glucosidase.

Notes: (a–d) Dependence on the substrate concentration of the reaction rate. The reaction was carried out with varying concentrations of maltose in the absence of the extracts of *M. australis* leaves or 1-DNJ (open circle) or in the presence of 0.15 mg/mL powdered leaf extracts (open sqaure) or 0.25 μg/mL 1-DNJ (open triangle) at pH 6.0 at 27ºC (a), at pH 6.0 at 47ºC (b), at pH 7.0 at 37ºC (c), and at pH 8.0 at 37ºC (d). Solid line represents the best fit to the experimental data using the Michaelis-Menten equation with the nonlinear least-squares methods. Error bars indicate SD values.