Biochemical Pharmacology, Vol. 39, No. 10, pp. 1537–1543, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

CASTANOSPERMINE-GLUCOSIDES AS SELECTIVE DISACCHARIDASE INHIBITORS

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(*Received* 24 *April* 1989; *accepted* 19 *September* 1989)

Abstract—Castanospermine (CS) is a potent but non-selective inhibitor of many glycohydrolases including the intestinal disaccharidases. Several CS-glucosides were synthesized to investigate the effect of an attached glucopyranosyl residue on the potency and selectivity of CS toward inhibition of intestinal disaccharidases. 8α-glucosyl-CS and 7α-glucosyl-CS were nearly as potent against sucrase activity as CS (IC_{50} values = 30, 40, and 20 nM respectively) but were 1/50 or less as potent as CS against lactase and trehalase activities. 8β-glucosyl-CS was 1/20 to 1/140 as potent as CS and 1α-glucosyl-CS was 1/57 to 1/1500 as potent as CS against disaccharidase activities. 1α-glc-CS was less selective than CS, whereas the other CS-glucosides were more selective. 7α-glc-CS and 8α-glc-CS were the most sucrase selective and were particularly ineffective against trehalase and lactase activities. 8β-glc-CS was similar to CS except for relatively weaker trehalase inhibition. In summary, selectivity toward certain disaccharidases was achieved by glucosylation of CS hydroxyls. However, a simple structural comparison of the CS-glucoside to a disaccharide substrate did not reliably predict which disaccharidase would be more inhibited by the CS-glucoside.

Potent glycohydrolase inhibitors have been isolated from many plant and microbial sources [1–13] or prepared synthetically [13–18]. With the exception of proteinaceous amylase inhibitors [3, 6], these glycohydrolase inhibitors contain 5, 6, or fused 5–6 membered rings with 3 or more attached hydroxyl groups and an amine function either as a member of the ring or directly attached to it. The stereochemistry of the hydroxyl groups appears to confer the necessary selectivity for the type of glycohydrolase inhibited. Potent inhibitors that have their hydroxyl stereochemistry mimicking the appropriate glycoside have been described for mannosidases [2, 8, 18], trehalase [7], glucosidase [8–17], galactosidase [5], and glucuronidase [4, 19]. Potential applications of these inhibitors include antibiotics [20], insecticides [21, 22], and therapeutic agents for diabetes [1, 15–17, 23, 24], obesity [25], cancer [26], and viral diseases [27, 29]. For diabetes, hyperlipidemia, obesity and related disorders associated with hyperglycemia, glycohydrolase inhibitors that inhibit intestinal disaccharidases are being evaluated clinically [23, 24].

One very potent glycohydrolase inhibitor occurs naturally in the Australian "black bean" tree, *Castanospermum australe* [30]. The inhibitor, castanospermine (CS) is a time-dependent, quasi-irreversible inhibitor of the disaccharidases, sucrase and isomaltase [31]. However, CS is non-selective in that it inhibits many other glycohydrolases including almond β-glucosidase [12], fungal amyloglucosidase [32], glycoprotein processing glucosidases I and II [33], lysosomal α-glucosidase [34, 35], and other disaccharidases [13]. CS, which shares structural similarity to another glucosidase inhibitor, deoxynojirimycin, can be described as a fused-ring aza-homolog of glucopyranose (Fig. 1). The glucosylation of CS, therefore, results in a pseudo-disaccharide. Although many N-alkyl derivatives of the glycohydrolase inhibitors have been synthesized and studied [14, 16, 17, 24], few studies have examined the inhibition characteristics of O-glucosyl derivatives. 4-*O*-α-glucosyl-Fagomine showed selection for lactase and galactosidase in comparison to fagomine [13] and 4-*O*-α-glucosyl-deoxynojirimycin showed selection for sucrase relative to maltase in comparison to deoxynojirimycin [36]. We have synthesized specific O-glucosylated derivatives of CS to determine the effect of the attached glucosyl residue on the selectivity of CS among the intestinal disaccharidases.

MATERIALS AND METHODS

CS [(1*S*,6*S*,7*R*,8*R*,8*aR*)-1,6,7,8-tetrahydroxyindolizidine] was isolated from seeds of *C. australe* [37]. 1-*O*-α-D-glucopyranosyl-CS (1α-glc-CS), 7-*O*-α-D-glucopyranosyl-CS (7α-glc-CS), 8-*O*-α-D-glucopyranosyl-CS (8α-glc-CS), and 8-*O*-β-D-glucopyranosyl-CS (8β-glc-CS) were synthesized as described by Liu *et al.* [38] and 1,5-dideoxy-1,5-imino-D-glucitol (deoxynojirimycin) was synthesized by the procedure of Junge *et al.* [39]. Other chemicals were obtained from commercial sources. Adult male 200–300 g Sprague–Dawley rats were obtained from Harlan Laboratories, Indianapolis, IN.

A disaccharidase preparation was obtained as previously described [37] from the intestinal mucosa of four rats. To examine the rate of sucrase inactivation, sucrase activity was measured by coupling the hydrolysis of sucrose to glucose dehydrogenase [40]. The reaction time–course monitored NADH accumulation by 340 nm absorbance. For this assay,

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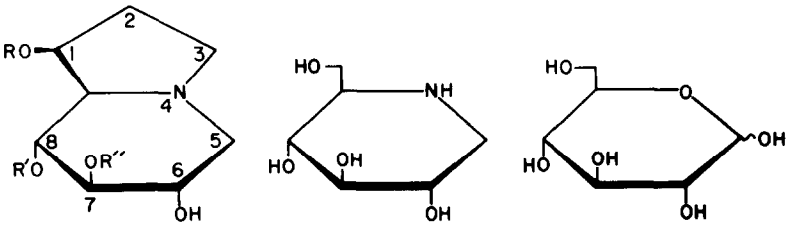

Compound	R	R′	R″
CS	H	H	H
1α-glc-CS	α-glc	H	H
7α-glc-CS	H	H	α-glc
8α-glc-CS	H	α-glc	H
8β-glc-CS	H	β-glc	H

deoxynojirimycin glucopyranose

Fig. 1. Structures of CS, CS-glucosides and deoxynojirimycin in comparison to glucopyranose.

20 μL of intestinal enzyme preparation containing 6 milliunits (6 nmol glucose produced/min) sucrase activity was mixed with 1.98 mL of 19 mM sucrose, 2.2 mM NAD, 13 units/mL glucose dehydrogenase (EC 1.1.1.47, Sigma G 9755), and 9 units/mL mutarotase (EC 5.1.3.3, Sigma M 4007) in 0.1 M sodium maleate, pH 5.9, 37°. Absorbance at 340 nm was monitored using a Varian DMS 200 Recording Spectrophotometer. After the reaction was started and an initial reaction rate determined, 40 μL inhibitor was added. Inactivation was determined by comparing the curves obtained with and without inhibitor.

To examine disaccharidase reactivation after inhibition, 20 μL containing 1–3 milliunits disaccharidase activity and the inhibitor at a concentration of 5× its IC_{50} (the inhibitor concentration causing a 50% decrease in enzyme activity) was incubated for 2 hr at 37°. Assay reagents were then added to dilute the inhibitor 100× to a concentration 1/20 its IC_{50}. Disaccharidase hydrolysis was then monitored for 1–2 hr by the coupled assay. This assay mixture was as described above except that the disaccharidase concentration was 100 mM and the starch concentration (for glucoamylase) was 0.6%. Assays were performed in duplicate or triplicate. Reactivation was determined by comparing the curves obtained with and without inhibitor.

To determine the IC_{50} of the inhibitor, the enzyme preparation and seven logarithmic inhibitor concentrations (1 nM to 1 mM) were incubated for 2 hr at 37° before substrate addition. One set of tubes was heat-inactivated at 90° for 2 min before adding substrate. Ten microliters containing 3.3 μmol of sucrose, maltose, lactose, isomaltose or trehalose (for the appropriate disaccharidase) or 0.5 mg of soluble potato starch (for glucoamylase) was added to a final assay volume of 100 μL in 0.1 M sodium maleate, pH 5.9. Additional incubation time at 37° depended upon the substrate added: lactose or starch, 60 min; sucrose or trehalose, 30 min; and maltose or isomaltose, 15 min. Reactions were terminated by heating at 90° for 2 min. Assays were performed in duplicate or triplicate. Glucose concentrations were determined using glucose dehydrogenase (Seradyn Inc., Indianapolis, IN). Glucose produced at each inhibitor concentration was plotted as percent of glucose produced with no inhibitor versus log inhibitor concentration. A line visually fit to the data was used to determine the IC_{50}.

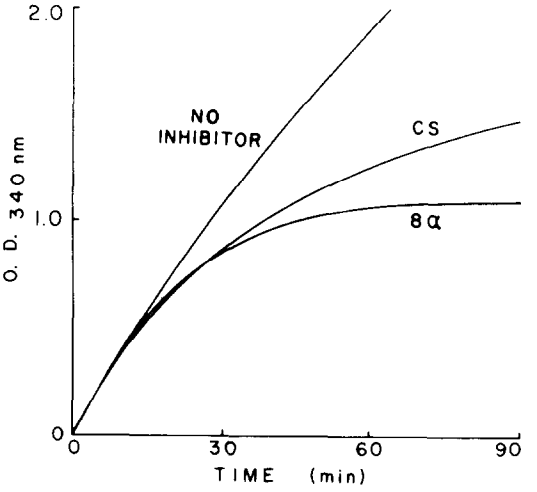

Fig. 2. Time–course of sucrase inactivation by CS or 8α-glc-CS in the presence of sucrose. Enzyme preparation containing 6 milliunits sucrase activity was mixed with 19 mM sucrose, 2.2 mM NAD, 26 units glucose dehydrogenase and 9 units mutarotase. After the reaction was allowed to proceed for 2 min, inhibitor was added to a final concentration of 20 nM CS or 20 μM 8α-glc-CS. Absorbance at 340 nm was monitored continuously.

RESULTS

CS and each CS-glucoside caused a very slow inactivation of intestinal sucrase, maltase, glucoamylase and isomaltase activities. Figure 2 illustrates the inactivation of sucrase by CS and by 8α-glc-CS. Except for the effect of 1α-glc-CS on isomaltase, reactivation of sucrase, maltase, glucoamylase, and isomaltase activities was not detectable following dilution of CS-inhibited or CS-glucoside-inhibited disaccharidase. Examples are shown for CS-inhibited

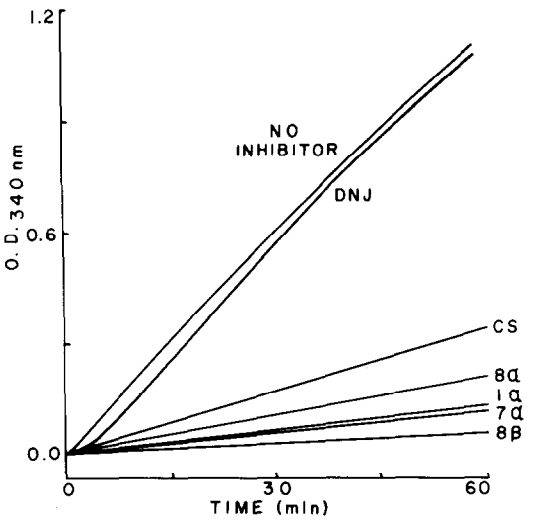

Fig. 3. Time–course of sucrase reactivation after inhibition and dilution. Enzyme preparation was preincubated with 5× the IC_{50} of the inhibitor for 2 hr at 37° and then was diluted 100-fold with 100 mM sucrose and reagents. See Materials and Methods for details. Final inhibitor concentration was 10 nM deoxynojirimycin (DNJ), 1 nM CS, 2 nM 8α-glc-CS (8α), 2 nM 7α-glc-CS (7α), 400 nM 1α-glc-CS (1α) or 20 nM 8β-glc-CS (8β).

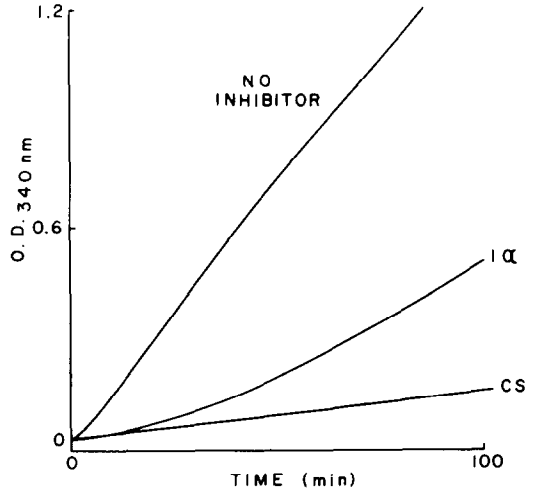

Fig. 4. Time–course of isomaltase reactivation after inhibition by 1α-glc-CS or CS and dilution. Enzyme preparation was preincubated with 5× the IC_{50} of the inhibitor for 2 hr at 37° and then was diluted 100-fold with 100 mM isomaltose and assay reagents. See Materials and Methods for details. Final inhibitor concentration was 3 μM 1α-glc-CS (1α) or 15 nM CS.

and CS-glucoside-inhibited sucrase following their dilution (Fig. 3). This figure also includes deoxynojirimycin-inhibited sucrase as an example of results obtained with a more readily reversibly sucrase inhibitor. Unlike CS and the other CS-glucosides, 1α-glc-CS was a reversible inhibitor of isomaltase activity with a half-reactivation time of 17 min (Fig. 4). The CS-glucosides and CS were all reversible inhibitors of trehalase activity with half-reactivation times of less than 2 min. Reactivation experiments with lactase activity were not performed because a contaminant in the coupled enzyme reagents generated 340 nm absorption.

Because the CS-glucosides, in most cases, gave no indication of reactivation following dilution, K_i was not determined. Instead the CS-glucosides were evaluated by comparing their IC_{50} values. With potent inhibitors, inhibitor concentration relative to enzyme concentration may not be in large excess in the assay. In this case, inhibitor binding to other disaccharidases in an intestinal mucosa preparation can result in a slightly higher IC_{50} value than would be determined using a purified enzyme. We have kept the contribution of other disaccharidases constant by always using the same amount of a single mucosa preparation. Although IC_{50} values determined may be higher with the mucosa preparation than with purified enzymes, the mucosa preparation may more closely reflect the *in vivo* interaction of inhibitor with intestinal disaccharidases.

The IC_{50} values of CS against intestinal disaccharidases are shown in column 1 of Table 1 with the enzyme activities listed in order from the most to least potently inhibited. To show the effect of each attached glucosyl on the potency of CS against each disaccharidase, the IC_{50} values of CS were set equal to 1 and relative IC_{50} values were obtained by dividing the IC_{50} of the CS-glucoside by the IC_{50} of CS (Table 1). Each CS-glucoside affected some intestinal disaccharidase activities more than others. 8β-glc-CS was a weaker inhibitor than CS of all disaccharidase activities, with trehalase activity being less potently inhibited than the other disaccharidases. 1α-glc-CS was a weaker inhibitor than 8α-glc-CS of all disaccharidase activities except trehalase. 7α-glc-CS retained CS's potent inhibition of sucrase activity, lost some potency against maltase, glucoamylase, and isomaltase activities, and lost much more potency against trehalase and lactase activities. 8α-glc-CS was similar to 7α-glc-CS, except that 8α-glc-CS was a slightly more potent inhibitor of lactase.

To evaluate the selectivity profile for each CS-glucoside, the IC_{50} for each disaccharidase was divided by the IC_{50} for the most potently inhibited disaccharidase, which was sucrase in every case (Table 2). Selectivity was then examined by comparing the profile of IC_{50} values of each CS-glucoside to the profile of CS. With the single exception of 1α-glc-CS's inhibition of trehalase, each CS-glucoside had the same relative order of disaccharidase inhibition as CS. Quantitatively, however, glucosylation of CS did not have an equal effect on inhibitory potency. 1α-glc-CS was less selective than CS, whereas the other CS-glucosides were more selective. 7α-glc-CS and 8α-glc-CS were the most sucrase selective and were particularly ineffective against trehalase and lactase activities. 8β-glc-CS was similar to CS except for relatively weaker trehalase inhibition.

DISCUSSION

CS is a potent but non-selective glycohydrolase inhibitor [12, 31–36]. Similar to deoxynojirimycin and related inhibitors, the amine of CS is probably

Table 1. Potency of CS and CS-glucosides against intestinal disaccharidase activites

Enzyme activity	CS IC_{50} (nM)	Relative IC_{50} values of each inhibitor compared to CS*				
		CS	1α-glc-CS	7α-glc-CS	8α-glc-CS	8β-glc-CS
Sucrase	20	1	400	2	1.5	20
Maltase	40	1	750	7.5	5	50
Glucoamylase	50	1	1200	8	4	60
Isomaltase	300	1	200	13	10	33
Lactase	600	1	1500	170	50	67
Trehalase	7000	1	57	>140†	>140†	140

* See Results for calculation of relative IC_{50} values.
† The highest inhibitor concentration used (1 mM) resulted in less than 50% inhibition.

Table 2. Selectivity of CS and CS-glucosides against intestinal disaccharidase activities

Enzyme activity	Relative IC_{50} values for each inhibitor among enzymes*				
	CS	1α-glc-CS	7α-glc-CS	8α-glc-CS	8β-glc-CS
Sucrase	1	1	1	1	1
Maltase	2	3.8	7.5	6.7	5
Glucoamylase	2.5	7.5	10	6.7	7.5
Isomaltase	15	7.5	100	100	25
Lactase	30	110	2500	1000	100
Trehalase	350	50	>25,000†	>33,000†	2500

* See Results for calculation of relative IC_{50} values.
† The highest inhibitor concentration used (1 mM) resulted in less than 50% inhibition.

responsible for the binding that results in inhibition [31], while the structural similarity of CS to a glucopyranoside appears relevant to CS's recognition by the glycohydrolase.

Part of the potency of CS relates to the time-dependent quasi-irreversible interaction observed with some of the glycohydrolases. This time-dependent inhibition is observed as a potency increase with preincubation [31, 41]; initial IC_{50} values [37] were generally larger than those determined following preincubation (Table 1). The quasi-irreversible inhibition of CS can be observed as either an undetectable or a very slow return of enzyme activity following dilution of the inhibited enzyme. Danzin and Ehrhard [31] calculated that the time required for half-reactivation of CS-inhibited sucrase was 54 hr. Reactivation of CS-inhibited isomaltase was not detected. Consistent with these findings, we observed no reactivation within 1 hr following dilution of CS-inhibited or CS-glucoside-inhibited sucrase, maltase, or glucoamylase activities and only one example of reactivation of isomaltase activity. This retention of inhibition suggests that glucosylation of the C-1, C-7, and C-8 hydroxyls of CS, though apparently important for the inhibitor's selectivity for a disaccharidase, is relatively unimportant to the quasi-irreversible character of CS. One observation contradicted this generalization: 1α-glc-CS-inhibited isomaltase could be reactivated with a half-reactivation time of 17 min, whereas isomaltase inhibited by CS or by the other CS-glucosides did not show reactivation. Why this particular derivative, which structurally resembles isomaltose, would have such an interaction with isomaltase is not known.

CS most likely inhibits disaccharidases by occupying the sub-site which would be normally occupied by the non-reducing glucopyranosyl of the substrate (Fig. 5, top) [31]. However, CS-glucosides could bind to the disaccharidase with either the glucoside unit (Fig. 5, middle) or the CS unit (Fig. 5, bottom) occupying this sub-site. In the former case, the CS-glucoside could theoretically be hydrolyzed, generating CS. We do not believe hydrolysis of the CS-glucoside is occurring to any significant extent because the inhibition profiles of the CS-glucosides and CS were distinctly different (Table 1). All IC_{50} determinations were performed with preincubation under the same conditions of time and disaccharidase concentration. Therefore, hydrolysis of a CS-glucoside would produce the same CS concentration with each disaccharidase activity determined. If the inhibition ascribed to a CS-glucoside actually resulted from CS present, the CS-glucoside's profile of disaccharidase inhibition would be the same as that of CS and the potency would reflect the amount of CS-glucoside hydrolyzed. Another possiblity to explain these results is that CS is generated but is retained within the active site of the disaccharidase. This possibility would be difficult to differentiate from a direct effect of intact CS-glucoside because such internal enzyme inactivation, likely self-limiting with each disaccharidase, could result in differences in inhibition profiles. However, with 1α-glc-CS, generation of CS even at the active site is inconsistent with a reversible inhibition of isomaltase activity

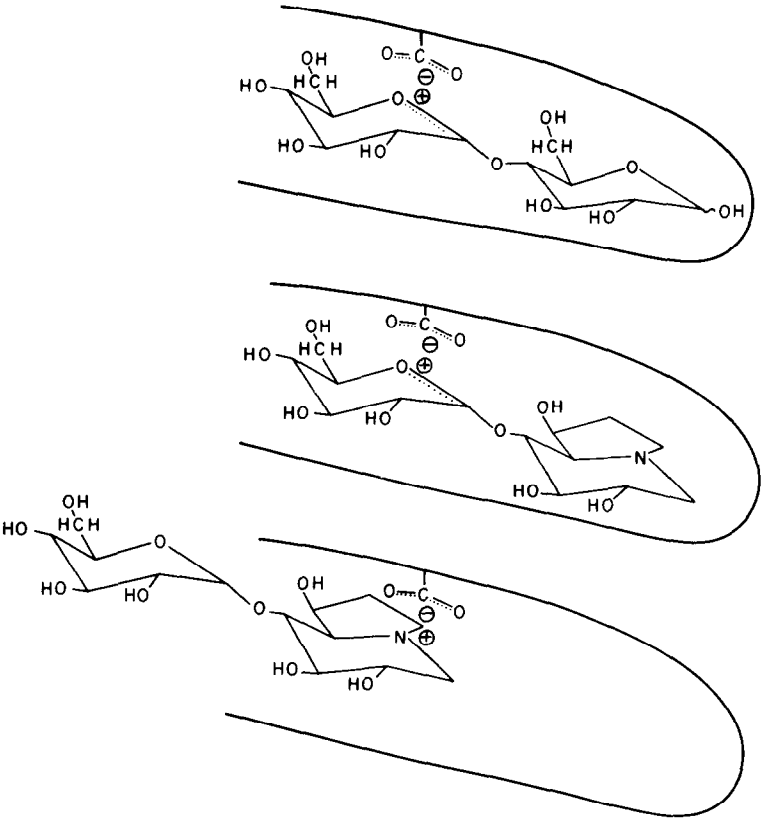

Fig. 5. Hypothetical binding positions of maltose or CS-glucoside within the active site of sucrase. Maltose (top) is shown as an example of a sucrase substrate. 8α-glc-CS is shown as an example of a CS-glucoside with either the glucoside (middle) or castanospermine (bottom) occupying the nonreducing glucopyranosyl subsite.

because the CS generated would result in irreversible inhibition.

With the glucoside unit of the CS-glucoside occupying the glucopyranosyl sub-site, comparison of the CS-glucosides to disaccharide substrates might predict which disaccharidase would be more inhibited. This predictability was observed only occasionally. 8α-glc-CS, which could be considered an analog of maltose, was a potent inhibitor of maltase, sucrase, and glucoamylase, enzymes which hydrolyze maltose. In contrast, 1α-glc-CS, an analog of isomaltose, showed a considerably reduced potency of disaccharidase inhibition and only a very slight selection for isomaltase compared to CS. However, the reversibility would partially account for its less potent isomaltase inhibition compared to CS and the other CS-glucosides. The profile of enzyme activities inhibited by 7α-glc-CS suggests that it was recognized by the same disaccharidases that recognize maltose even though 7α-glc-CS would seem a closer analog of nigerose. In this regard, nigerose was hydrolyzed by the intestinal preparation at a rate comparable to that of maltose. 8β-glc-CS, which could be considered an analog of cellobiose, showed little selection but the loss in potency is less than 1α-glc-CS.

If the CS unit of the CS-glucoside occupies the glucopyranosyl sub-site, inhibitory selectivity based upon a structural comparison of CS-glucoside to a disaccharidase substrate is irrelevant. The 4-*O*-α-glucosyl-deoxynojirimycins [36, 42] which share structural similarities to 8α-glc-CS are probably the closest literature precedents. Although their selectivity among the other intestinal disaccharidases was not reported, these deoxynojirimycin-glucosides also showed selectivity for sucrase compared to maltase [36].

In summary, selectivity was achieved by O-glucosylation of CS. A simple structural comparison of the CS-glucoside to a disaccharide substrate did not consistently predict which disaccharidase would be more inhibited by the CS-glucoside. 1α-glc-CS was unique from CS and the other CS-glucosides in its reversible inhibition of isomaltase activity.

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