

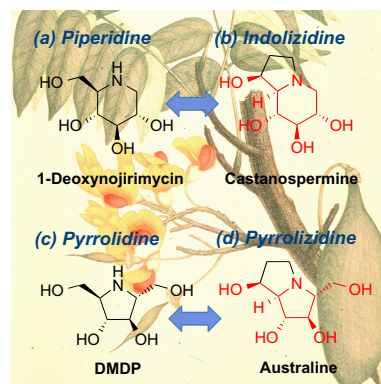
Graphical abstract

Isolation and SAR studies of bicyclic iminosugars from *Castanospermum australe* as glycosidase inhibitors

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Atsushi Kato*, Yuki Hirokami, Kyoko Kinami, Yutaro Tsuji, Shota Miyawaki, Isao Adachi, Jackie Hollinshead, Robert J. Nash, J.L. Kiappes, Nicole Zitzmann, Jin K. Cha, Russell J. Molyneux,
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Fourteen iminosugars were isolated from *Castanospermum australe*. Our side-by-side comparison between bicyclic and corresponding monocyclic iminosugars revealed that inhibition potency and spectrum are clearly changed by their core structures against each enzyme.



Highlights

- The isolation and structure determination of 14 iminosugars from *C. australe*.
- New alkaloid was determined to be 6,8-diepi-castanospermine.
- Focus on the difference in the inhibition spectrum of mono- and bi-cyclic iminosugars.
- Castanospermine (CST) showed strong anti-hyperglycemia effects than that of miglitol.
- CST isomers were better α -glucosidase inhibitors than the corresponding piperidines.



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ABSTRACT

We report the isolation and structural determination of fourteen iminosugars, containing five pyrrolizidines and five indolizidines, from *Castanospermum australe*. The structure of a new alkaloid was elucidated by spectroscopic methods as 6,8-diepi-castanospermine (**13**). Our side-by-side comparison between bicyclic and corresponding monocyclic iminosugars revealed that inhibition potency and spectrum are clearly changed by their core structures against each enzyme. Castanospermine (**10**) and 1-deoxynojirimycin (DNJ) have a common *D*-gluco configuration, and they showed the expected similar inhibition potency and spectrum. In sharp contrast, 6-*epi*-castanospermine (**12**) and 1-deoxymannojirimycin (*manno*-DNJ) both have the *D*-manno configuration but the α -mannosidase inhibition of 6-*epi*-castanospermine (**12**) was much better than that of *manno*-DNJ. 6,8-Diepi-castanospermine (**13**) could be regarded as a bicyclic derivative of *talo*-DNJ, but it showed a complete loss of α -galactosidase A inhibition. This behavior against α -galactosidase A is similar to that observed for 1-*epi*-australine (**6**) and *altro*-DMDP.

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1. Introduction

Glycosidases are involved in a wide range of important biological processes, such as intestinal digestion, post-translational processing of glycoproteins, their quality control mechanisms, and lysosomal catabolism of glycoconjugates. Thus, glycosidase inhibitors could have beneficial effects as therapeutic agents for diabetes, viral infections, and as pharmacological chaperones for various lysosomal diseases (Davis, 2009; Asano, 2009; Nash et al., 2011; Horne et al., 2011). Among them, iminosugars are an important class of sugar-mimics that inhibit various glycosidases in a reversible and competitive manner because of their structural resemblance to the terminal sugar moiety of the natural substrates. A

large number of iminosugars have been discovered from plants and microorganisms (Asano et al., 2000a; Watson et al., 2001).

The polyhydroxylated indolizidine alkaloid castanospermine (**10**) has attracted special interest as a potent inhibitor of various glycosidases such as glycoprotein processing ER α -glucosidases (Pan et al., 1983; Sasak et al., 1985; Szumilo et al., 1986; Elbein, 1991) and lysosomal α -glucosidase (Saul et al., 1985). This iminosugar was first isolated in 1981 from the seeds of the Australian legume *Castanospermum australe* (Hohenschutz et al., 1981). Although about 30 years have passed since this classical glycosidase inhibitor was discovered in this plant, its derivatives and stereoisomers are still a popular synthetic target (Jensen et al., 2009; Bowen and Wardrop, 2010; Liu et al., 2010), and it has been investigated for the treatment of various diseases such as cancer (Allan et al., 2013; Sanchez-Fernandez et al., 2010), hepatitis C virus (HCV), parainfluenza virus, dengue virus (DENV) (Watanabe et al., 2012), and lysosomal diseases (Luan et al., 2009; Li et al., 2013). In particular, combination therapy of 6-O-butanoyl-cast-

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anospermine (Celgosivir) with peginterferon and/or ribavirin has been in Phase II clinical trials for the treatment of patients with chronic HCV (Whitby et al., 2004; Durantel, 2009), and a Phase 1b trial of Celgosivir as a monotherapy for DENV has been recently reported (Low et al., 2014).

Despite almost all classic pyrrolizidine alkaloids bearing a carbon substituent at C-1, alexine (Nash et al., 1988) and australine (5) (Molyneux et al., 1988) were the first examples of pyrrolizidine alkaloids with a carbon substituent at the C-3 position. The alexines and australines have been reported in only two small genera of the Leguminosae, and the highly polyhydroxylated pyrrolizidine casuarine occurs only in related genera in the Casuarinaceae and Myrtaceae (Nash et al., 1996). Consequently, the search for lead compounds of this type from natural sources tends to be constrained compared to other compound classes. Recently, however, other polyhydroxylated pyrrolizidines, namely uniflorine A (Matsumura et al., 2000) and the hyacinthacines (Asano et al., 2000b; Yamashita et al., 2002; Kato et al., 2007) have been isolated from genera of the Myrtaceae and Hyacinthaceae and have become popular synthetic targets (Ritthiwigrom et al., 2012).

Following on from our recent work concerning the structure–activity relationships of monocyclic iminosugars (Kato et al., 2005; Ayers et al., 2012, 2014; Jenkinson et al., 2013), we turned our attention to the bicyclic australine and castanospermine isomers. In this paper, we describe the isolation, structural determination, and glycosidase inhibitory activity of fourteen iminosugars from *C. australe* (Leguminosae). Furthermore, we performed side-by-side comparison between monocyclic and bicyclic iminosugars and elucidated the difference of inhibition potency and spectrum.

2. Results and discussion

2.1. Isolation and characterization of iminosugars from *C. australe*

The seeds (5.8 kg) of *C. australe* were extracted with 50% aqueous EtOH. The chromatographic separation of the extract using various ion-exchange resins led to isolation of eleven alkaloids (1–3, 5, 6, 8–12, and 14). The ^1H NMR and ^{13}C NMR spectra of alkaloid 1 were in accord with those of 2*R*-hydroxymethyl-3*S*-hydroxypyrrolidine (CYB-3), which has been isolated previously from the same plant *C. australe* (Nash et al., 1985; Kato et al., 2003). The biosynthesis of the bicyclic indolizidine and pyrrolizidine iminosugars has not been elucidated yet, but CYB-3 (1), *N*-hydroxyethyl-2-hydroxymethyl-3-hydroxypyrrolidine, and *N*-methyl-*trans*-4-hydroxy-*L*-proline (Molyneux et al., 1991; Haraguchi et al., 2003) can be speculated as being important intermediate compounds because the distributions of these pyrrolidines and of castanospermine are closely related and appear to be restricted to a small taxonomic group. Alkaloids 2 and 3 were identified as fagomine and 3-*epi*-fagomine, respectively, from an analysis of their ^1H NMR and ^{13}C NMR spectroscopic data. Fagomine (2) was first isolated from buckwheat seeds (*Fagopyrum esculentum*) (Koyama and Sakamura, 1974). We have previously reported that fagomine (2) and 3-*epi*-fagomine (3) occur abundantly in the leaves and roots of *Xanthocercis zambesiaca*, from southern Africa (Kato et al., 1997). The ^1H and ^{13}C NMR spectroscopic data of alkaloids 5, 6, and 8–12 were in accord with those of the polyhydroxylated pyr-

rolizidines, australine, 1-*epi*-australine, 2,3-*diepi*-australine, 2,3,7-*triepi*-australine, and the polyhydroxylated indolizidines, castanospermine, castanospermine-8-*O*- β -*D*-glucoside, 6-*epi*-castanospermine isolated earlier from *C. australe* (Molyneux et al., 1986, 1988; Kato et al., 2003). The ^1H NMR and ^{13}C NMR spectra of alkaloid 14 were in accord with those of 7-deoxy-6-*epi*-castanospermine (Molyneux et al., 1990). Recently, this compound has been synthesized in ten steps from a common chiral building block (+)-tetramic acid derivative (Liu et al., 2010).

The dried leaves (360 g) of *C. australe* were extracted with 50% aqueous MeOH. The chromatographic separation of the extract using various ion-exchange resins led to isolation of nine alkaloids (1, 2, 4–7, 10, 12, and 13). Alkaloids 4 and 7 were identified as 3,4-*diepi*-fagomine and 3-*epi*-australine, respectively, from an analysis of their ^1H NMR and ^{13}C NMR spectroscopic data (Kato et al., 1997, 2003). The structural determination of the new alkaloid 13 is described below.

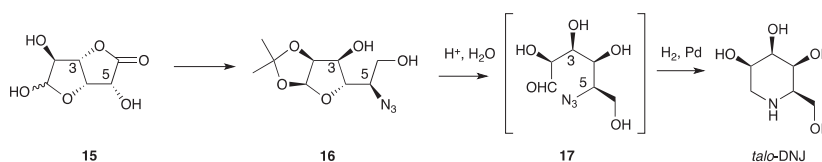
Alkaloid 13 was determined to have the molecular formula $\text{C}_8\text{H}_{15}\text{NO}_4$ by HRFABMS. The ^{13}C NMR spectroscopic data of 6,8-*diepi*-castanospermine were closely related to those of 6-*epi*-castanospermine. In the ^1H NMR spectrum, the H-1 showed similar coupling constants to H-2 and H-8a as those of 6-*epi*-castanospermine, indicating a common stereochemical configuration for H-1 and H-8a. The stereo configurations of 6,8-*diepi*-castanospermine were corroborated by definite NOEs between H-7 and H-8a or H-5ax and by the presence of a W-path long-range coupling between H-6 and H-8. Hence, alkaloid 13 was shown to be 6,8-*diepi*-castanospermine.

2.2. Preparation of corresponding DMDP and DNJ isomers

2,5-Dideoxy-2,5-imino-*D*-mannitol (DMDP), 2,5-dideoxy-2,5-imino-*D*-altritol (*altro*-DMDP), 2,5-dideoxy-2,5-imino-*D*-glucitol (DGDP), 2,5-dideoxy-2,5-imino-*D*-allitol (*allo*-DMDP) were prepared according to our previous reports (Ayers et al., 2012). 1-Deoxynojirimycin (DNJ) and 1-deoxymannojirimycin (*manno*-DNJ) were isolated from *Baphia nitida* Lodd. (Fabaceae) (Kato et al., 2008). 1-Deoxyaltronojirimycin (*altro*-DNJ) was isolated from *Scilla sibirica* (Hyacinthaceae) (Yamashita et al., 2002). 1-Deoxytalonojirimycin, *talo*-DNJ, was prepared from readily available *L*-glucuronolactone 15 (Weymouth-Wilson et al., 2009; Martínez et al., 2014). 15 was converted to the protected *talo*-azide 16 by introduction of nitrogen at C5 with inversion of configuration and inversion of configuration at C3 (Best et al., 2010; Ayers et al., 2012). Acid hydrolysis of 16 by Dowex 50WX8 removed the isopropylidene protecting group to give 17 which, on hydrogenation in the presence of palladium on carbon afforded an amine which underwent reductive amination to produce cyclo-DNJ [83% yield from 16]. (Scheme 1: Synthesis of *talo*-DNJ).

2.3. Comparison of australine and DMDP isomers as glycosidase inhibitors

Pyrrolizidine iminosugars are generally moderate and broad inhibitors of glycosidases (Kato et al., 1999, 2007; Asano et al., 2000b; Yamashita et al., 2002). Furthermore, it is not usually easy to predict whether they will inhibit a particular glycosidase from



Scheme 1. Synthesis of *talo*-DNJ.

Table 1
Concentration of pyrrolidine- and pyrrolizidine-type iminosugars giving 50% inhibition of various glycosidases.

Enzyme	IC ₅₀ (μM)	5	6	7	alatro-DMDP	DGDP	8	allo-DMDP
Rat intestinal glycosidases								
Maltase	228		NI (28.1%)		NI (31.8%)	138	NI (7.3%)	NI (45.0%)
Isomaltase	45		NI (11.7%)		NI (13.8%)	382	NI (0.5%)	841
Sucrase	26		443		32	190	NI (0%)	47
Lactase	253		16		41	30	NI (19.6%)	115
Trehalase	358		NI (0%)		NI (28.3%)	NI (49.0%)	NI (47.0%)	NI (23.9%)
Human lysosomal acid glycosidases								
Alglucosidase alfa	239		NI (0%)		NI (0%)	591	NI (0%)	NI (9.0%)
β-Glucocerebrosidase	665		NI (27.9%)		NI (28.1%)	NI (34.2%)	NI (9.7%)	NI (1.1%)
α-Galactosidase A	^a NI (1.6%)		NI (0%)		4.3	NI (0.8%)	NI (0.4%)	40
α-Mannosidases								
Jack bean	NI (12.4%)		NI (3.1%)		NI (47.6%)	NI (4.0%)	NI (11.6%)	NI (26.2%)
Rat epididymis	NI (34.2%)		NI (8.3%)		NI (45.7%)	NI (14.2%)	NI (21.3%)	NI (35.0%)

^a NI: No inhibition (less than 50% inhibition at 1000 mM).^b (): Inhibition% at 1000 mM.

the configuration of the hydroxy groups on the pyrrolizidine ring. For example, 7-*epi*-australine showed very weak inhibition toward rat intestinal maltase, porcine kidney trehalase, and *Aspergillus niger* amyloglucosidase, with IC₅₀ values of 310, 310 and 92 μM, respectively. However, the introduction of the OH group to C-6α in 7-*epi*-australine to give casuarine significantly enhanced its inhibition toward each enzymes, with IC₅₀ values of 0.7, 12, and 0.7 μM, respectively (Kato et al., 2003). Furthermore, the 6-O-α-D-glucoside of casuarine retained the potency toward *A. niger* amyloglucosidase (IC₅₀ = 1.1 μM) and increased inhibitory activity toward porcine kidney trehalase (IC₅₀ = 0.34 μM), but markedly lowered toward maltase (IC₅₀ = 260 μM). This is because the pyrrolizidine ring can be considered as two pyrrolidines, either of which may be a mimic of a monosaccharide. Australine (5) can be regarded as a derivative of DMDP with an ethylene bridge between the hydroxymethyl group and the ring nitrogen. However, from the view point of biosynthesis, it seems that both iminosugars are not clearly connected to each other because the distribution of australine (5) and its epimers appears to be restricted to the limited Leguminosae family (Nash et al., 1996), whereas DMDP would appear to be a fairly widespread secondary metabolite, as it has been reported from plant species of quite unrelated families and *Streptomyces* (Watson et al., 2001). For the purpose of clarifying the difference of the inhibition spectrum between pyrrolizidine and pyrrolidine iminosugars, we first compared the activity of the isolated australine isomers (5–8) and their related DMDP isomers towards various glycosidases. Table 1 shows the 50% inhibitory concentrations (IC₅₀) of these compounds against rat intestinal glycosidases, human lysosomal acid glycosidases and α-mannosidases. Intestinal maltase, isomaltase, and sucrase are target enzymes against postprandial hyperglycemia. Lysosomal acid alglucosidase alfa, β-glucocerebrosidase, and α-galactosidase A are key enzymes for pharmacological chaperone therapy of Pompe disease, Gaucher disease, and Fabry disease, respectively (Fan et al., 1999; Butters, 2007; Ishii, 2012). Jack bean α-mannosidase catalyzes the hydrolysis of glycoproteins and epididymal α-mannosidase is related to fertilization (Skudlarek et al., 1992). We found that the inhibitory potency and spectrum of australine (5) was very similar to DMDP, except for lactase inhibition. Australine (5) showed moderate inhibition against isomaltase and sucrase with IC₅₀ values of 45 and 26 μM, respectively. This compound also showed broad inhibition toward maltase, lactase, trehalase, alglucosidase alfa, and β-glucocerebrosidase, with IC₅₀ values in the 200–700 μM range. In sharp contrast, 3-*epi*-australine (7) and 2,3-*diepi*-australine (8) were completely inactive on all glycosidases tested even at concentrations as high as 1000 μM. These results clearly suggested that the orientation of the C3 hydroxymethyl group in the pyrrolizidine ring is one of the essential features for recognition and strong binding by the active site of glycosidases. None of the australine isomers obtained in the present work showed significant inhibitory activity toward human lysosomal α-galactosidase A, whereas alatro-DMDP and allo-DMDP showed potent inhibition with IC₅₀ values of 4.3 and 40 μM, respectively.

2.4. Comparison of castanospermine and DNJ isomers as glycosidase inhibitors

Indolizidine iminosugars can be considered as fused pyrrolidine and piperidine rings and castanospermine may be regarded as a bicyclic derivative of DNJ, with an ethylene bridge between the hydroxymethyl group and the ring nitrogen. The hydroxylation of the piperidine moiety in castanospermine and its isomers resemble the pyranose form of the corresponding hexose. For the purpose of clarifying the correlation of inhibition spectrum between piperidine and indolizidine iminosugars, we next elucidated the isolated castanospermine isomers (10, 12, 13) and their related DNJ isomers towards various glycosidases. 6,7-Diepi-castanospermine

Table 2
Concentration of piperidine- and indolizidine-type iminosugars giving 50% inhibition of various glycosidases.

Enzyme	IC ₅₀ (μM)	10	DNJ	12	manno-DNJ	13	talo-DNJ	6,7-diepi-CST	altro-DNJ
Rat intestinal glycosidases									
Maltase	0.49	0.12	89	NI (37.2%)	25	NI (46.0%)	228	NI (21.5%)	NI (7.4%)
Isomaltase	4.2	0.53	20	548	ND	NI (17.6%)	NI (41.7%)	NI (7.4%)	NI (18.2%)
Sucrase	0.23	0.19	9.4	337	63	1000	74	208	NI (27.3%)
Lactase	1.1	40	NI (27.0%)	NI (42.9%)	250	100	NI (14.6%)	NI (7.3%)	NI (6.9%)
Trehalase	14	80	NI (38.6%)	NI (0%)	ND	NI (8.7%)	NI (41.0%)	NI (0%)	NI (4.3%)
Human lysosomal acid glycosidases									
α-Glucosidase alfa	1.6	0.23	506	NI (20.5%)	85	NI (40.1%)	NI (32.0%)	NI (1.6%)	NI (7.7%)
β-Glucocerebrosidase	50	505	NI (7.5%)	NI (0%)	236	NI (37.0%)	NI (38.0%)	NI (6.0%)	NI (5.0%)
α-Galactosidase A	^a NI (2.2%)	^a NI (46.7%)	NI (5.7%)	NI (14.3%)	NI (8.7%)	NI (37.0%)	NI (38.0%)	NI (6.0%)	NI (1.8%)
α-Mannosidases									
Jack bean	NI (6.0%)	NI (42.7%)	93	774	NI (5.4%)	NI (37.0%)	NI (38.0%)	NI (6.0%)	NI (7.7%)
Rat epididymis	NI (27.9%)	NI (49.4%)	13	NI (44.9%)	NI (5.8%)	NI (37.0%)	NI (38.0%)	NI (6.0%)	NI (1.8%)

^a NI: No inhibition (less than 50% inhibition at 1000 mM).^b (): Inhibition% at 1000 mM.^c ND: Not determined.

was used as a reference compound and was isolated in 1991 from the seeds of *C. australe* (Molyneux et al., 1991). 6,7-Diepi-castanospermine was prepared as previously described (Kim et al., 1993, 1994). Table 2 shows the 50% inhibitory concentrations (IC₅₀) of these compounds against rat intestinal glycosidases, human lysosomal acid glycosidases and α-mannosidases. Castanospermine (10) has the D-gluco configuration in the piperidine ring and the inhibitory potency and spectrum was very similar to DNJ. Both compounds showed powerful inhibition towards rat intestinal maltase, isomaltase, sucrase, and human lysosome acid alglucosidase alfa, with IC₅₀ values in the 0.1–5.0 μM range. However, castanospermine also showed potent inhibition against β-glycosidases such as lactase and lysosomal β-glucocerebrosidase. It is noteworthy that DMJ has the D-manno configuration in the piperidine ring; however, the α-mannosidase inhibition was very weak (IC₅₀ = 774 μM for the Jack bean enzyme and no inhibition at 100 μM for the rat epididymal enzyme). In sharp contrast, 6-epi-castanospermine (12), which has the D-manno configuration in the piperidine part, showed much better inhibition against these enzymes, with IC₅₀ values of 93 and 13 μM, respectively. The comparison of the inhibition potency of castanospermine isomers (12, 13, and 6,7-diepi-castanospermine) and the corresponding DNJ isomers (manno-DNJ, talo-DNJ, and altro-DNJ) revealed that the indolizidine ring is much more suitable than the piperidine ring for intestinal α-glucosidase (maltase, isomaltase, and sucrase) inhibition. Notably, their sucrase inhibition was significantly increased over that of DNJ isomers. However, it seems that this tendency does not apply to every glycosidase because the inhibition potency of 6,8-diepi-castanospermine (13) against α-galactosidase is much weaker than for the corresponding talo-DNJ (Fig. 1).

2.5. Carbohydrate loading test

On the basis of these findings, it clearly appeared that the indolizidine ring is much more suitable than the piperidine ring for inhibition of intestinal α-glucosidases. Among them, castanospermine (10) and 6-epi-castanospermine (12) showed potent inhibition against sucrase, with IC₅₀ values of 0.23 and 9.4 μM, respectively. We then investigated the influence of castanospermine (10), 6-epi-castanospermine (12) and miglitol, which was used as positive control, on blood glucose levels after an *in vivo* sucrose-loading (Fig. 2). The control group was loaded with saline only. The “second generation” α-glucosidase inhibitor miglitol differs from acarbose and voglibose as it is rapidly and completely absorbed in the upper region of the small intestine (Arh et al., 1997). This pharmacokinetic property consequently enables the strong and early-phase suppression of postprandial hyperglycemia, with no or minimal gastrointestinal complications. The administration of sucrose (2.5 g/kg body weight p.o.) to fasted mice resulted in a rapid increase in blood glucose concentrations from 78 ± 4 to a maximum of 182 ± 2 mg/dL after 15 min. Thereafter blood glucose levels recovered to the pretreatment level at 120 min. When compared to the saline control, the positive control miglitol caused significantly lower blood glucose levels at 15 and 30 min (Fig. 2). In sharp contrast, the remaining blood glucose levels were slightly higher with miglitol than with the placebo at 60 and 120 min. This behavior with strong and early-phase suppression of postprandial hyperglycemia is a superior feature of miglitol. Castanospermine (10) and 6-epi-castanospermine (12) resulted in significantly lower blood glucose levels at 15, 30, and 60 min than with placebo administration (Fig. 2). The suppressive effect of castanospermine (10) is clearly superior to that for miglitol. However, their suppression curve did not show the delayed-phase curve as did miglitol. This result may suggest that castanospermine (10) and 6-epi-castanospermine (12) were not as readily absorbed in the upper region of the small intestine.

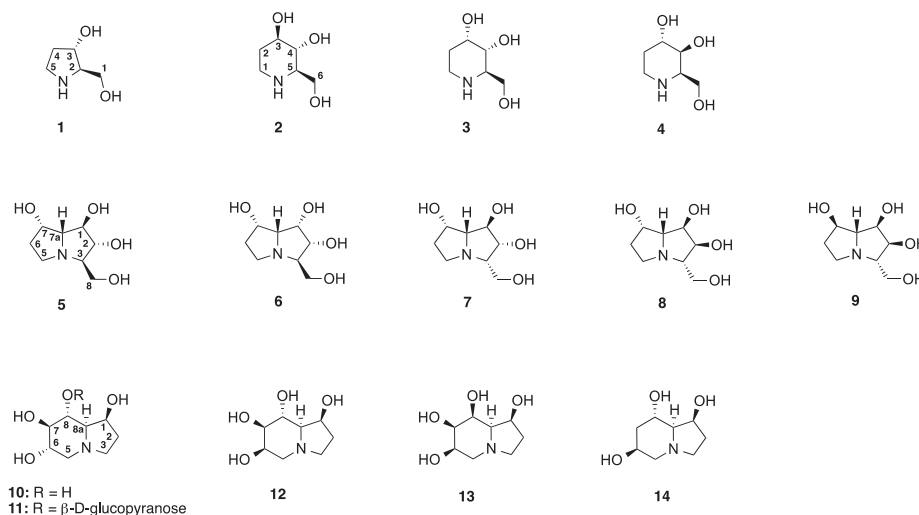


Fig. 1. Structures of iminosugars isolated from *Castanospermum australe*.

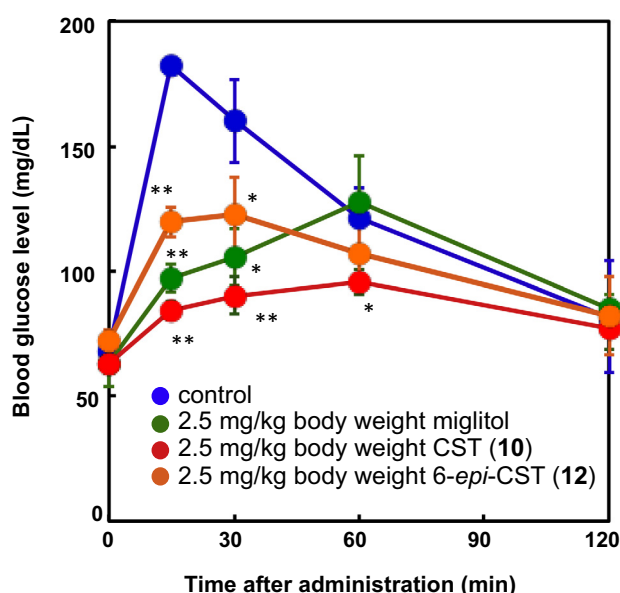


Fig. 2. Effects of miglitol, castanospermine (**10**: CST), and 6-*epi*-castanospermine (**12**: 6-*epi*-CST) on blood glucose levels. Blood glucose concentrations of male C57BL/6J mouse after an oral load with sucrose, 2.5 g/kg body weight, with 2.5 (green) mg/kg body weight miglitol, 2.5 (red) mg/kg body weight castanospermine, 2.5 (orange) mg/kg body weight 6-*epi*-castanospermine. The control group was loaded with saline (blue). Each value represents the mean \pm SEM ($n = 5$). Significant difference **($p < 0.01$) and *($p < 0.05$) compared with the control.

3. Conclusion

In this study, we describe the isolation and structure determination of fourteen iminosugars (one pyrrolidine, three piperidines, five pyrrolizidines, and five indolizidines) from *C. australe*. The structure of a new alkaloid was elucidated by spectroscopic methods as 6,8-*diepi*-castanospermine (**13**). Previous reports with limited structural diversity have alluded to bicyclic iminosugars being generally moderate and broad inhibitors, and it is not usually easy to predict whether they will inhibit a particular glycosidase from the configuration of the hydroxy groups. For the purpose of clarifying the difference in inhibition spectrum between monocyclic and bicyclic iminosugars, we performed side-by-side comparison. The main features are as follows: (a) the orientation of the C3

hydroxymethyl group in the pyrrolizidine ring is the essential feature for recognition and strong binding by the active site of glycosidases, (b) bicyclic 6-*epi*-castanospermine (**12**) was a much stronger inhibitor than monocyclic *manno*-DNJ against α -mannosidases, (c) castanospermine isomers (**12**, **13**, and 6,7-*diepi*-castanospermine) showed better inhibition than corresponding DNJ isomers against intestinal α -glucosidases, and (d) the suppressive effect of castanospermine (**10**) *in vivo* is clearly better than that of miglitol. However, the curve did not show the delayed-phase curve as with miglitol.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter (Tokyo, Japan). ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker DRX500 spectrometer. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate (TSP) in D_2O as internal standard. The assignment of proton and carbon NMR signals was determined from extensive homonuclear decoupling experiments, and the DEPT, ^1H - ^{13}C COSY, HMQC, and HMBC spectroscopic data. FAB/MS were measured using glycerol as a matrix on a JEOL JMS-700 spectrometer. The purity of samples was checked by HPTLC on silica gel 60F₂₅₄ (E. Merck) using the solvent system $\text{PrOH-AcOH-H}_2\text{O}$ (4:1:1), and a chlorine-*o*-tolidine reagent or iodine vapor was used for detection. As for the synthesis of 1-deoxytalonojirimycin (*talo*-DNJ), NMR spectra were recorded on a Bruker AVII-400 instrument and calibrated using residual undeuterated solvent as an internal reference (D_2O : $\delta_{\text{H}} = 4.79$ ppm) (Gottlieb et al., 1997). In the case of ^{13}C spectra of D_2O samples, acetone was added as a reference (acetone: $\delta_{\text{Me}} = 30.89$ ppm). High-resolution mass spectra (HRMS) were recorded using electrospray ionization (ESI, 4000 V) and a time-of-flight (TOF) mass analyzer. Optical rotations were recorded on a Perkin-Elmer Model 341 polarimeter at 589 nm, and are reported in units of $10^{-1} (\text{deg cm}^2 \text{g}^{-1})$, with the corresponding sample concentrations (c) reported in g/100 mL.

4.2. Plant material

The seeds of *C. australe* were purchased in June 2006 from a flower shop in Japan. A voucher specimen (AK20060102) is deposited in the Herbarium of the Medicinal Plants Garden, Hokuriku University. *C. australe* was grown at the Medicinal Plants Garden,

Hokuriku University, Japan and collected in August 2008. A voucher specimen (NA20080062) is deposited in the Herbarium of the Medicinal Plants Garden, Hokuriku University.

4.3. Extraction and isolation

The seeds (5.8 kg) of *C. australe* were extracted with 50% aqueous EtOH. The filtrate was applied to a column of Amberlite IR-120B (2000 mL, H⁺ form). The 0.5 M NH₄OH eluate was concentrated to give a brown oil (49.2 g), which was applied to Dowex 1-X2 (OH⁻ form) to remove amino acids and pigments, and eluted with H₂O. This eluate was concentrated and chromatographed over an Amberlite CG-50 column (2000 mL, NH₄⁺ form) with H₂O as eluant (fraction size 500 mL). The H₂O eluate was divided into two pools: I (fractions 1–3, 36.5 g) and II (fractions 4–6, 3.17 g). The 0.5 M NH₄OH eluate from the same column was designated pool III (fractions 2–8, 5.5 g). Each pool was further chromatographed with Dowex 1-X2 (OH⁻ form) with H₂O as eluant and/or Amberlite CG-50 column (2.0 × 95 cm, NH₄⁺ form) with H₂O as eluant to give alkaloids castanospermine (**10**) (1.54 g), castanospermine-8-O-β-D-glucoside 6-*epi*-castanospermine (**12**) (813 mg), and 7-deoxy-6-*epi*-castanospermine (**14**) (7 mg) from pool I, australine (**5**) (599 mg) and 1-*epi*-australine (**6**) (1.36 g) from pool II, and 2R-hydroxymethyl-3S-hydroxypyridine (CYB-3: **1**) (2.40 g), fagomine (**2**) (255 mg), 3-*epi*-fagomine (**3**) (69 mg), 2,3-diepi-australine (**8**) (208 mg), and 2,3,7-triepi-australine (**9**) (15 mg) from pool III.

A 50% aqueous MeOH extract of the dried leaves (360 g) of *C. australe* was applied to a column of Amberlite IR-120B (250 mL, H⁺ form). The 0.5 M NH₄OH eluate was concentrated to give a brown oil (7.2 g). The oil was chromatographed over an Amberlite CG-50 column (2.0 × 95 cm, NH₄⁺ form) with H₂O as eluant (fraction size 15 mL). The H₂O eluate was divided into three pools: I (fractions 18–27, 4.6 g), II (fractions 28–34, 320 mg), and III (fractions 35–62, 540 mg). The 0.1 M NH₄OH eluate from the same column was divided into three pools: IV (fractions 15–60, 880 mg), V (fractions 72–84, 200 mg), and VI (85–100, 115 mg). Each pool was further chromatographed with Dowex 1-X2 (OH⁻ form) with H₂O as eluant and/or CM-Sephadex C-25 (NH₄⁺ form) with 0.01 M NH₄OH as eluant to give castanospermine (**10**) (1.04 g), 6-*epi*-castanospermine (**12**) (41 mg), and 6,8-diepi-castanospermine (**13**) (7 mg) from pool I, australine (**5**) (123 mg) from pool II, 1-*epi*-australine (**6**) (200 mg) from pool III, 3-*epi*-australine (**7**) (335 mg) from pool IV, fagomine (**2**) (15 mg) and 3,4-diepi-fagomine (**4**) (84 mg), and 2R-hydroxymethyl-3S-hydroxypyridine (CYB-3: **1**) (98 mg) from pool V.

4.3.1. 6,8-Diepi-castanospermine (**13**)

Colorless oil; $[\alpha]_D^{25} +3.3$ (c 0.2, H₂O); ¹H NMR (500 MHz, D₂O) δ 1.73–1.80 (1H, m, H-2), 2.15 (1H, dd, *J* = 1.0, 5.0 Hz, H-8a), 2.20 (1H, dd, *J* = 9.2, 9.2 Hz, H-3), 2.32 (1H, dd, *J* = 2.1, 12.4 Hz, H-5), 2.37 (1H, m, H-2), 3.13 (1H, dd, *J* = 1.8, 9.2 Hz, H-3), 3.29 (1H, dd, *J* = 3.2, 12.4 Hz, H-5), 3.68 (1H, dd, *J* = 3.2, 3.2 Hz, H-7), 3.97 (1H, m, H-6), 4.41 (1H, dd, *J* = 1.0, 3.2 Hz, H-8), 4.56 (1H, ddd, *J* = 2.7, 5.0, 7.4 Hz, H-1); ¹³C NMR (125 MHz, D₂O) δ 36.3 (C-2), 54.2 (C-3), 59.0 (C-5), 70.4 (C-8a), 72.5 (C-6), 72.8 (C-7), 73.8 (C-8), 75.8 (C-1); HRFABMS *m/z* 190.1079 [M+H]⁺ (calcd for C₈H₁₆NO₄ 190.1080).

4.4. Preparation of 1-deoxytalonojirimycin (talo-DNJ)

5-Azido-5-deoxy-1,2-O-isopropylidene-α-D-talofuranose **16** (Best et al., 2010; Ayers et al., 2012) (1.50 g, 6.12 mmol, 1.0 equiv) was dissolved in H₂O (20 mL). After addition of Dowex 50WX8 resin (H⁺ form, 2.50 g), the suspension was heated to 70 °C and stirred at the same temperature for 3 h. The mixture was cooled to 25 °C and filtered. The resin was washed with a further portion

of H₂O (20 mL). To the filtrate was added Pd/C (10% Pd, 0.625 g, 50 weight-%), and the reaction vessel was evacuated and flushed with argon. After evacuating the vessel a second time, it was flushed with H₂ and stirred at 25 °C for 24 h under H₂. The reaction mixture was filtered through glass microfiber (GF/B) and concentrated under reduced pressure. The residue was purified by ion exchange chromatography (Dowex 50WX8, H⁺ form), washing with H₂O (20 column volumes) and subsequently eluting with aq. NH₄OH (7%, 2 column volumes). The ammoniacal fractions were collected and concentrated under reduced pressure to provide 1-deoxytalonojirimycin (826.6 mg, 5.07 mmol, 83% yield) as a colorless oil.

4.4.1. 1-Deoxytalonojirimycin (talo-DNJ)

Colorless oil; $[\alpha]_D^{20} = -22.5$ (H₂O, *c* = 1.0) {Ref. (Liu et al., 1991)} $[\alpha]_D^{23} = -22.4$ (MeOH, *c* = 1.6); ¹H NMR (400 MHz, D₂O): δ 3.34 (dd, *J* = 13.8, 2.0 Hz, 1 H), 3.48 (td, *J* = 6.9, 1.7 Hz, 1 H), 3.61 (dd, *J* = 13.8, 2.9 Hz, 1 H), 3.92 (t, *J* = 3.4 Hz, 1 H), 3.96 (d, *J* = 6.9 Hz, 2 H), 4.25–4.22 (m, 1 H), 4.34–4.30 (m, 1 H) ppm; ¹³C NMR (100 MHz, D₂O): δ 49.6, 59.6, 62.0, 69.8, 69.9, 70.2 ppm; HRMS (ESI-TOF) calcd for C₆H₁₃NO₄ [M+H]⁺: 164.0923 found 164.0916.

4.5. Biological assays

The enzymes α-mannosidase (from Jack bean, pH 4.5), *p*-nitrophenyl glycosides, and various disaccharides were purchased from Sigma–Aldrich Co. Brush border membranes were prepared from the rat small intestine according to the method of Kessler et al. (1978) and were assayed at pH 6.8 for rat intestinal maltase, isomaltase, sucrase, lactase, and trehalase using the appropriate disaccharides as substrates. For rat intestinal glycosidase activities, the reaction mixture contained 25 mM substrate and the appropriate amount of enzyme, and the incubations were performed for 10–30 min at 37 °C. The reaction was stopped by heating at 100 °C for 3 min. After centrifugation (600g; 10 min), 0.05 mL of the resulting reaction mixture were added to 3 mL of the Glucose CII-test Wako (Wako Pure Chemical Ind., Osaka, Japan). The absorbance at 505 nm was measured to determine the amount of the released D-glucose. The rat epididymal fluid was purified from epididymis according to the method of Skudlarek et al. (1992). The purified epididymal fluid and Jack bean α-mannosidase activities were determined using a *p*-nitrophenyl-α-D-mannopyranoside as substrate at the optimum pH of each enzyme. The reaction mixture contained 2 mM of the substrate and the appropriate amount of enzyme. The reaction was stopped by adding 2 mL of 400 mM Na₂CO₃. The released *p*-nitrophenol was measured spectrometrically at 400 nm. Human lysosomal α-glucosidase alfa, β-glucocerebrosidase, and α-galactosidase A were obtained from Genzyme (Boston, MA) and their activities were determined using 4-methylumbelliferyl α-D-glucoside, β-D-glucoside and α-D-galactoside (Sigma–Aldrich Co.), respectively, as substrates. Liberated 4-methylumbelliferone was measured (excitation 362 nm, emission 450 nm) with a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

4.6. Disaccharide loading test

The animal experimental protocols in this study were approved by the Animal Experiments Committee of the University of Toyama (A2013UH-3). Male C57BL/6J mice (30–35 g) after an overnight fast were used for acute disaccharide loading tests. Sucrose (2.5 g/kg body weight) as well as the inhibitors, miglitol, were dissolved in 0.9% NaCl solution and administered to mice *via* a stomach tube. A control group was loaded with saline only. Blood samples for glucose measurements were obtained from the tail vein at 0, 15, 30, 60, and 120 min after sucrose-loading. The blood

glucose levels were measured by a portable kit, StatStrip Xpress® (Nova Biochemical Co. Ltd. MA, USA).

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