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


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


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. Our side-by-side comparison of inhibition potency and spectrum against each enzyme. Castanospermine (10) and 1-deoxynojirimycin (DNJ) have a common a-gluco configuration, and they showed the expected similar inhibition potency and spectrum. In sharp contrast, 6-epi-castanospermine (12) and 1-deoxynojirimycin (manno-DNJ) both have the a-manno configuration but the x-mannoside 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 complete loss of x-galactosidase A inhibition. This behavior against x-galactosidase A is similar to that observed for 1-epi-australine (6) and other-DMDP.

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. A large number of iminosugars have been discovered from plants and microorganisms. The polyhydroxylated indolizidine alkaloid castanospermine (10) has attracted special interest as a potent inhibitor of various glycosidases such as glycoprotein processing ER x-glucosidases (Pan et al., 1983; Sasak et al., 1985; Szumilo et al., 1986; Elbein, 1991) and lysosomal x-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-castanospermines has been shown to be effective in vivo.
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; Durante, 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 (Rithiwigrom 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 13C NMR spectra of alkaloid 1 were in accord with those of the polyhydroxylated pyrrolizidines, australine, 1-epi-australine, 2,3-diepi-australine, 2,3,7-triepi-australine, and the polyhydroxylated indolizidines, castanospermine, castanospermine-8-o-glucoside, 6-epi-castanospermine isolated earlier from C. austral (Molyneux et al., 1986, 1988; Kato et al., 2003). The 1H NMR and 13C 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 (+)-tetrac acid derivative (Liu et al., 2010).

The dried leaves (360 g) of C. austral 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 13C 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 C8H15NO4 by HRFABMS. The 13C 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 signals to those of 7-deoxy-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 (GDGP), 2,5-dideoxy-2,5-imino-D-altritol (aldo-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-Deoxyronojirimycin (altro-DNJ) was isolated from Scilla sibirica (Hyacinthaceae) (Yamashita et al., 2002), 1-Deoxytalonojirimycin, talo-DNJ, was prepared from readily available D-glucoronolactone 15 (Weymouth-Wilson et al., 2009; Martinez 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 cycloto-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.
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-6 in 7-epi-australine to give casuarine significantly enhanced its inhibition toward each enzyme, 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 glycosidase activities and α-mannosidases. Intestinal maltase, isomaltase, and sucrase are target enzymes against postprandial hyperglycemia. Lysosomal acid α-glucosidase 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 epidiymal 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, α-glucosidase 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 altreo-DMDP and aldo-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

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<th>Enzyme</th>
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Table 1: Concentration of pyrrolidine- and pyrrolizidine-type iminosugars giving 50% inhibition of various glycosidases.

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

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Table 2

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<th>Concentration of piperidine- and indolizidine-type iminosugars giving 50% inhibition of various glycosidases.</th>
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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 C50 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 of 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.
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-di-O-mannosidase, (c) castanospermine isomers (12, 13, and 6,7-di-O-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.

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-di-O-mannosidase, (c) castanospermine isomers (12, 13, and 6,7-di-O-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 13C 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 D2O as internal standard. The assignment of proton and carbon NMR signals was determined from extensive homonuclear decoupling experiments, and the DEPT, 1H-13C COSY, HMOC, and HMBD spectroscopic data. FABMS 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 PrOH:AcOH:H2O (4:1:1), and a chlorine-α-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 (D2O: δH = 4.79 ppm) (Gottlieb et al., 1997). In the case of 13C spectra of D2O samples, acetone was added as a reference (acetone: δMe = 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 (deg cm2 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, University.
4.3. Extraction and isolation

The seeds (5.8 kg) of C. australis were extracted with 50% aqueous EtOH. The filtrate was applied to a column of Amberlite IR-120B (2000 mL, H\textsuperscript{+} form). The 0.5 M NH\textsubscript{4}OH eluate was concentrated to give a brown oil (49.2 g), which was applied to Dowex 1-X2 (OH\textsuperscript{-} form) to remove amino acids and pigments, and eluted with H\textsubscript{2}O. This eluate was concentrated and chromatographed over an Amberlite CG-50 column (2000 mL, NH\textsubscript{4}H\textsubscript{2}O form) with H\textsubscript{2}O as eluant (fraction size 500 mL). The H\textsubscript{2}O 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 NH\textsubscript{4}OH eluate from the same column was designated pool III (fractions 2–8, 208 mg). Each pool was further chromatographed over an Amberlite CG-50 column (2.0 × 95 cm, NH\textsubscript{4}H\textsubscript{2}O form) with H\textsubscript{2}O as eluant to give alkaloids castanospermine (10) (1.54 g), castanospermine-8-β-D-glucoside epistarospermine (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-hydroxypropylidine (C\textsubscript{2}-B) (2.40 g), fagomine (2) (255 mg), 3-epi-fagomine (3) (69 mg), 2,3-di-epi-australine (8) (208 mg), and 2,3,7-tri-epi-australine (9) (15 mg) from pool III.

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

4.4.1. 1-Deoxynaltosojirimycin (talo-DNJ)

The enzymes α-mannosidase (from jack bean, pH 4.5), p-nitrophenyl glycosides, and various disaccharides were purchased from Sigma–Alrich Co. Brush border membranes were prepared from the rat small intestine according to the method of Kesler 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 was 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 α-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 Na\textsubscript{2}CO\textsubscript{3}. The released p-nitrophenol was measured spectrophotometrically at 400 nm. Human lysosomal alglucosidase alfa, β-glucocerebrosidase, and α-galactosidase A were obtained from Genzyme (Boston, MA) and their activities were determined using 4-methylumbelliferone as substrate at the optimum pH of each enzyme. The reaction mixture contained 2 mM of the substrate and the appropriate amount of enzyme. 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glucose levels were measured by a portable kit, StatStrip Xpress®.

(Nova Biochemical Co. Ltd. MA, USA).

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References


