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6,7-Di*epi*castanospermine, a Tetrahydroxyindolizidine Alkaloid Inhibitor of Amyloglucosidase†

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ABSTRACT: A tetrahydroxyindolizidine alkaloid, 6,7-di*epi*castanospermine, was isolated from the seeds of *Castanospermum australe* by extraction with methanol and purified to homogeneity using ion-exchange, preparative thin-layer, and radial chromatography. A very low yield of a pyrrolidine alkaloid, *N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine, was also obtained by analogous methods. The purity of both alkaloids was established by gas chromatography of their trimethylsilyl (TMS) derivatives as better than 99%. The molecular weight of each alkaloid was established as 189 and 161, respectively, by mass spectrometry, and the structure of each was deduced from their ^1H and ^{13}C NMR spectra. The structure of the pyrrolidine alkaloid is suggestive of a possible biosynthetic route to the polyhydroxyindolizidine and polyhydroxypyrrolizidine alkaloids which co-occur in *C. australe*. 6,7-Di*epi*castanospermine was found to be a moderately good inhibitor of the fungal α-glucosidase, amyloglucosidase ($K_i = 8.4 \times 10^{-5}$ M) and a relatively weak inhibitor of β-glucosidase. It failed to inhibit α- or β-galactosidase, α- or β-mannosidase, or α-L-fucosidase. Comparison of its inhibitory activity toward amyloglucosidase with those of its isomers, castanospermine and 6-*epi*castanospermine, demonstrated that epimerization of a single hydroxyl group can produce significant alteration of such inhibitory properties.

During the past decade a number of polyhydroxyindolizidine and polyhydroxypyrrolizidine alkaloids have been isolated from certain members of the plant family Leguminosae. All of these alkaloids have been shown to be inhibitors of glycosidases, exhibiting various degrees of potency and specificity (Elbein & Molyneux, 1987; Elbein, 1987). The most fruitful source of polyhydroxyalkaloids has been the Moreton Bay chestnut, or Black Bean (*Castanospermum australe*), a large leguminous tree native to northeastern Australia, which has been introduced into other subtropical areas of the world for its ornamental qualities. However, the large chestnut-like seeds produce hemorrhagic gastroenteritis when consumed by cattle (McKenzie et al., 1988) and on occasion have proved fatal to humans (Everist, 1974). The major alkaloidal constituent of the seeds, castanospermine (**1**) (Figure 1) (Hohenschutz et

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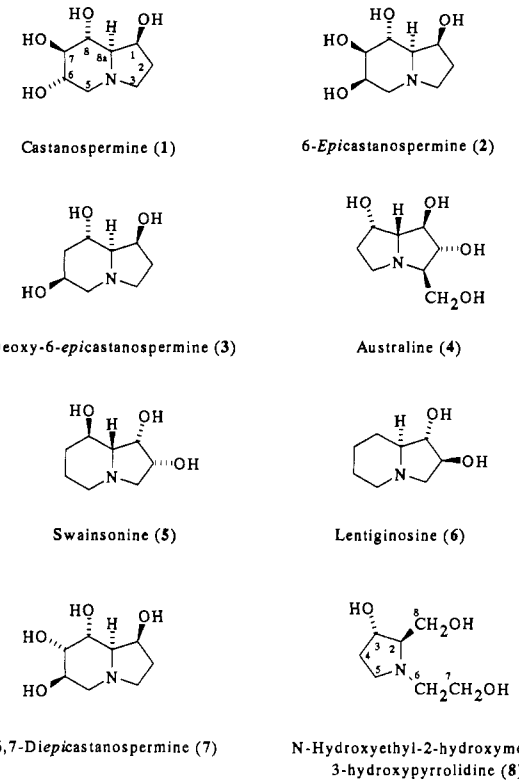

FIGURE 1: Structures of polyhydroxyindolizidine and polyhydroxypyrrolizidine alkaloids of *C. australe* and *A. lentiginosus*.

al., 1981), is a tetrahydroxyindolizidine alkaloid which possesses potent α- and β-glucosidase inhibitory activity (Saul et al., 1983, 1984) and also inhibits processing of N-linked glycoproteins at the glucosidase I stage. In cultured cells, the presence of castanospermine in the medium results in the accumulation of glycoproteins with $Glc_3Man_9(GlcNAc)_2$ structures (Pan et al., 1983), and the alkaloid has proven to be a useful tool for investigating the effects of alterations in glycoprotein processing. Considerable interest in castanospermine has been generated by its ability to inhibit the infectivity of retroviruses (Sunkara et al., 1987), especially the human immunodeficiency virus (HIV) (Gruters et al., 1987; Tyms et al., 1987; Walker et al., 1987), and to alter glycogen storage (Saul et al., 1985).

Crystallization of the majority of the castanospermine from the alkaloid extract of *C. australe* seeds yields a residual fraction in which a considerable number of minor alkaloids can be detected by thin-layer or gas chromatographic analysis. Several of these constituents have recently been isolated and structurally identified, encompassing alkaloids of both the polyhydroxyindolizidine and polyhydroxypyrrolizidine classes. These include 6-*epi*castanospermine (**2**) (Molyneux et al., 1986), 7-deoxy-6-*epi*castanospermine (**3**) (Molyneux et al., 1990), australine (**4**) (Molyneux et al., 1988), and the 1-, 3-, and 7-*epi*australines (Harris et al., 1989; Nash et al., 1988, 1990). The alkaloids all show varying degrees of inhibitory activity toward amyloglucosidase, but unlike castanospermine they show no activity toward β-glucosidase. Both castanospermine and australine inhibit glycoprotein processing at the glucosidase I stage, leading to accumulation of $Glc_3Man_{7-9}(GlcNAc)_2$ structures in influenza virus infected cell cultures (Tropea et al., 1989), thereby establishing that inhibitory activity is not restricted solely to the indolizidine ring system.

The trihydroxyindolizidine alkaloid, 7-deoxy-6-*epi*castanospermine (**3**), is significantly less active as a glycosidase inhibitor than its tetrahydroxy analogues. However, another trihydroxyindolizidine, swainsonine (**5**), has been isolated from *Swainsona* species of Australia (Colegate et al., 1979) and *Astragalus* and *Oxytropis* species of western North American (Molyneux & James, 1982; Davis et al., 1984; Molyneux et al., 1985), rangeland plants which are toxic to grazing animals. This alkaloid has been shown to be a potent and specific inhibitor of lysosomal α-mannosidase (Dorling et al., 1980) and other arylmannosidases (Kang & Elbein, 1983a). Swainsonine also inhibits the glycoprotein processing mannosidase II (Tulsiani et al., 1982) and in cell culture leads to formation of N-linked glycoproteins possessing hybrid types of oligosaccharides (Elbein et al., 1981; Arumughan & Tanzer, 1983; Kang & Elbein, 1983b; Tulsiani et al., 1984). Recently two dihydroxyindolizidine alkaloids, lentiginosine (**6**) and 2-*epi*lentiginosine, have been isolated from spotted locoweed (*Astragalus lentiginosus*). Whereas lentiginosine was found to be a good inhibitor of amyloglucosidase, its 2-epimer showed no significant activity against this or any other glycosidase (Pastuszak et al., 1990).

These results show that glycosidase inhibitory activity is not restricted solely to the more highly hydroxylated alkaloids. Specificity and potency appear to be controlled by both position and stereochemistry of the individual hydroxyl group substituents of the bicyclic ring systems. Attempts to elucidate such structure–activity relationships have been driven by the intense interest in certain of these alkaloids as potential chemotherapeutic agents. Thus, in addition to activity against retroviruses, both castanospermine and swainsonine exhibit significant antimetastatic, antiproliferative, and immunomodulatory properties (Humphries et al., 1988; Hino et al., 1985).

In this paper we report the isolation and purification, from a methanolic extract of *C. australe* seeds, of a new tetrahydroxyindolizidine alkaloid that inhibits amyloglucosidase. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry established the structure of this compound as 6,7-di*epi*castanospermine (**7**). The alkaloid also showed moderate inhibitory activity toward β-glucosidase but was inactive against other glycosidases. Comparison of its inhibition of amyloglucosidase with that of castanospermine (**1**), 6-*epi*castanospermine (**2**), and 7-deoxy-6-*epi*castanospermine (**3**) indicated that the presence of a 7-hydroxyl substituent is required for significant inhibitory activity but that such activity is decreased by α-orientation of this group. A minor amount of a monocyclic alkaloid, *N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine (**8**), was also isolated from the extract. This compound is a putative biosynthetic precursor of both the castanospermine and australine groups of alkaloids.

Experimental Procedures

Materials. The enzymes amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), α-L-fucosidase (from bovine kidney), and α-mannosidase (from jack beans) and all the *p*-nitrophenyl glycoside substrates were obtained from Sigma Chemical Co. β-Mannosidase was purified from *A. niger* as previously described (Elbein et al., 1977). Glucosidase I and mannosidase I were purified from mung bean seedlings (Szumilo et al., 1986). [^{3}H]Glucose-labeled $Glc_3Man_9GlcNAc$ oligosaccharide was isolated from influenza virus infected MDCK cells grown in the presence of castanospermine and labeled with [6-^{3}H]galactose, as previously described (Szumilo & Elbein, 1985).

Enzyme Assays. (*a*) *Arylglycosidases.* The enzymatic activities of amyloglucosidase, β-glucosidase, α- and β-galactosidase, α-L-fucosidase, and α- and β-mannosidase were determined colorimetrically by monitoring the release of *p*-

nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (Rudick & Elbein, 1974). All reaction mixtures contained 50 μmol of sodium acetate buffer, pH 5.0, 1.5 μmol of *p*-nitrophenyl glycoside, and enzyme in a final volume of 0.5 mL. Incubations were for 15 min at 37 °C, and the reactions were terminated by the addition of 2.5 mL of 0.4 M glycine buffer, pH 10.4. The *p*-nitrophenol liberated in the reaction was measured at 410 nm. Assays were done under conditions where the amount of *p*-nitrophenol released was linear with both time and protein concentration.

(*b*) *Glucosidase I and Mannosidase I.* Glucosidase I activity was determined by measuring the release of [^{3}H]glucose from [^{3}H]glucose-labeled $Glc_3Man_9GlcNAc$, while mannosidase I activity was measured by following the release of [^{3}H]mannose from [^{3}H]mannose-labeled $Man_9GlcNAc$, as described previously (Szumilo & Elbein, 1985). The incubation mixture for both enzymes contained 50 mM MES buffer, pH 6.5, 0.1% Triton X-100, enzyme, and substrate (25 000 cpm of $Glc_3Man_9GlcNAc$ for glucosidase I or $Man_9GlcNAc$ for mannosidase I) in a final volume of 0.25 mL. A typical incubation was for 1 h at 37 °C, and the release of radioactive glucose or mannose was linear with both time and protein concentration for the duration of the assay.

Isolation of 6,7-Diepicastanospermine (**7**) *and N-(Hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine* (**8**). Seeds of *C. australe* were obtained from commercial sources in Australia. The seeds were ground and extracted with methanol, and the extract was purified by ion-exchange chromatography as previously described (Molyneux et al., 1986). Castanospermine was removed from the extract by fractional crystallization. The residual material was then fractionated by a combination of ion-exchange chromatography, thin-layer chromatography, and preparative centrifugal TLC (Molyneux et al., 1988) to give three fractions which were examined for purity by GC of their trimethylsilyl (TMS) ethers. Three pure fractions were obtained with GC retention times of 10.98 min [*N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine], 15.74 min (6,7-di*epi*castanospermine), and 17.49 min (6-*epi*castanospermine).

Structural Elucidation of Alkaloids. Thin-layer chromatography (TLC) was performed on Merck 0.25-mm silica gel 60, precoated, glass-backed plates developed with $CHCl_3$:CH_3OH:NH_4OH:H_2O (70:26:2:2). Detection was achieved by spraying the plates with 10% acetic anhydride in benzene, followed by Ehrlich's reagent (Molyneux et al., 1991). The alkaloids were monitored for homogeneity by gas chromatography (GC) of the TMS ether derivatives, prepared by treatment with *N*-methyl-*N*-(trimethylsilyl)fluoroacetamide (MSTFA) in pyridine, on a Hewlett-Packard 5830 instrument, equipped with a flame ionization detector, on-column injector, and a 30-m × 0.32-mm i.d. SE-30 fused silica column. Low-resolution electron-impact mass spectra (MS) were obtained on a VG Micromass 7070 mass spectrometer; high-resolution mass measurements were determined on the same instrument. Nuclear magnetic resonance (NMR) spectra were determined on a Nicolet NT 200 FT spectrometer, with software package, at 200 MHz (^{1}H) and at 50.3 MHz (^{13}C), using solutions in deuterium oxide with 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) as an internal standard or in deuteriochloroform with tetramethylsilane (TMS) as an internal standard. Multiplicities for ^{13}C signals were determined by application of the carbon-attached proton test (CAPT) sequence. Two-dimensional spectra were obtained by homonuclear ^{1}H–^{1}H shift spectroscopy (COSY) and by heteronuclear ^{1}H–^{13}C shift correlations (CSCM). Optical rotations were measured in methanol or chloroform solution in a 1-dm cell on a Perkin-Elmer 241 automatic polarimeter. Torsion bond angles were determined for an energy-minimized structure using the Alchemy II molecular modeling program.

6,7-Diepicastanospermine (**7**). The alkaloid was obtained as a clear oil, which failed to crystallize, with a retention time on GC of the TMS ether of 15.74 min: EIMS *m/z* 189 (M, 39%), 172 (25%), 171 (18%), 154 (11%), 145 (100%), 128 (25%), 116 (12%), 100 (18%), 86 (88%); high-resolution MS mass measurement [M^+] *m/z* 189.0995 (calcd. for $C_8H_{15}NO_4$ 189.1001); ^{1}H NMR (D_2O) δ 4.44 (ddd, $J_{1,2\alpha}$ = 6.5, $J_{1,2\beta}$ = 1.5, $J_{1,8\alpha}$ = 4, H1), 4.08 (dd, $J_{7,8}$ = 2, $J_{8,8a}$ = 10, H8), 3.98 (2 H, dd, $J_{5\alpha,6}$ = $J_{5\beta,6}$ = 1.5, $J_{6,7}$ = $J_{7,8}$ = 2, H6 and H7), 3.25 (m, H3β), 3.08 (br dd, $J_{5\beta,6}$ = 1.5, $J_{5\alpha,5\beta}$ = 12.5, H5β), 2.65 (br dd, $J_{5\alpha,6}$ = 1.5, $J_{5\alpha,5\beta}$ = 12.5, H5α), 2.48 (dd, $J_{8a,1}$ = 4, $J_{8a,8}$ = 10, H8a) 2.38 (m, H3α), 2.28 (m, H2β), 1.73 (m, H2α); ^{13}C NMR (D_2O) δ 72.9 (C7), 72.4 (C1), 71.9 (C6), 69.2 (C8a), 67.8 (C8), 54.94 (C5), 54.92 (C3), 34.2 (C2). The ^{1}H–^{1}H COSY NMR spectrum is illustrated in Figure 2. Torsion bond angles: H5α–H6 +60.9°, H5β–H6 −60.0°, H6–H7 +66.7°, H7–H8 +47.6°, H8–H8a −172.3°, $[\alpha]^{27}_D$ +42.7°, $[\alpha]_{578}$ +44.6°, $[\alpha]_{546}$ +50.5°, $[\alpha]_{436}$ +86.4° (c 0.675, MeOH).

N-(Hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine (**8**). The alkaloid was isolated as a colorless oil, with a retention time on GC of the TMS ether of 10.98 min. EIMS *m/z* 161 (M, 1%), 144 (1%), 131 (7%), 130 (100%), 98 (4%); CIMS (isobutane) *m/z* 162 (MH, 100%), 160 (7%), 144 (7%), 130 (17%); high-resolution MS mass measurement [MH^+] *m/z* 162.1126 (calcd. for $C_7H_{16}NO_3$ 162.1130); ^{1}H NMR (D_2O) δ 4.18 (m, H3), 3.73 (2 H, dd, $J_{6,7}$ = $J_{6',7}$ = 6.5, H7 and H7′), 3.67 (dd, $J_{2,8}$ = 5, $J_{8,8'}$ = 11.7, H8), 3.55 (dd, $J_{2,8'}$ = 6.7, $J_{8,8'}$ = 11.7, H8′), 3.14 (m, H5), 3.03, (dd, $J_{6,7}$ = 6.5, $J_{6,6'}$ = 13, H6), 2.72 (dd, $J_{6',7}$ = 6.5, $J_{6,6'}$ = 13, H6′), 2.64 (m, H5′), 2.62 (m, H2), 1.97 (br m, H4), 1.80 (br m, H4′); ^{13}C NMR (D_2O) δ 76.8 (C2), 76.1 (C3), 64.2 (C8), 62.1 (C7), 59.6 (C6), 54.8 (C5), 34.9 (C4).

Results

Isolation and Purification of Alkaloids. Castanospermine (**1**) was crystallized from the basic fraction obtained by chromatography of the crude methanolic extract of *C. australe* seeds on a Dowex 50 NH_4^+ ion-exchange column (Hohenschutz et al., 1981). Examination of the residual mother liquors by TLC indicated the presence of additional alkaloids responding to Ehrlich's reagent as well as the previously reported 6-*epi*castanospermine (**2**) and 7-deoxy-6-*epi*castanospermine (**3**). Following ion-exchange chromatography on Dowex 50 with ammonium hydroxide gradient elution, the residue was subjected to preparative TLC, and a broad band which contained alkaloids having R_f values greater than that of castanospermine was removed from the plate. Further purification by radial chromatography gave a series of fractions, three of which were shown to be pure by gas chromatography of their trimethylsilyl ether derivatives. One of these, which eluted last from the radial chromatogaphy, had a GC retention time of 17.49 min and was shown to be 6-*epi*castanospermine (**2**) by comparison of its ^{1}H and ^{13}C NMR spectra with an authentic sample (Molyneux et al., 1986) and by gas chromatographic coinjection. Another fraction from the radial chromatogram preceeded the 6-*epi*castanospermine, and its TMS derivative had a retention time of 15.74 min on the GC. In addition, another pure fraction, which eluted earliest, had a GC retention time of 10.98 min.

Structural Characterization of 6,7-Diepicastanospermine (**7**). The GC retention time of 15.74 min for the alkaloid

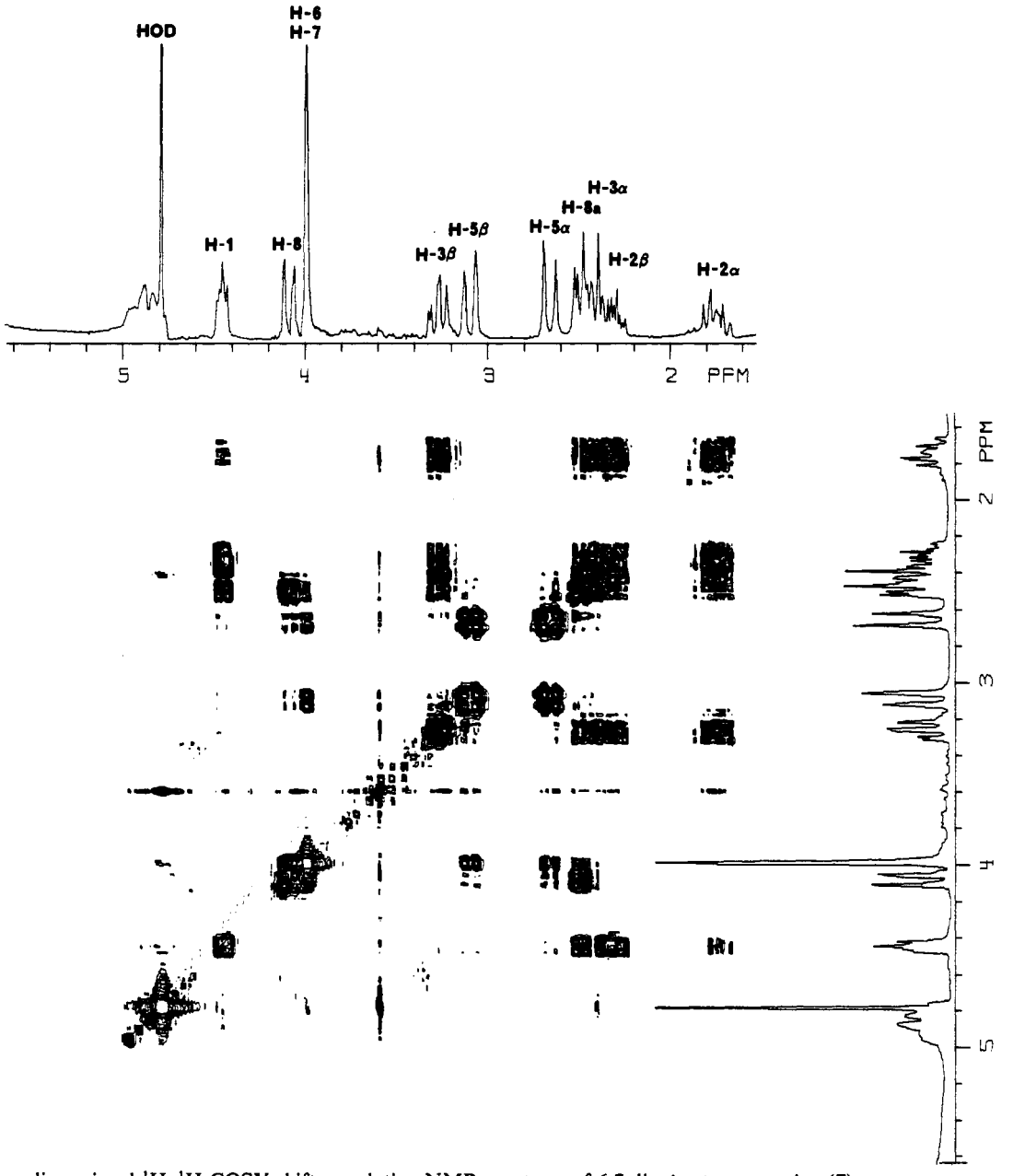

FIGURE 2: Two-dimensional ^1H–^1H COSY shift correlation NMR spectrum of 6,7-di*epi*castanospermine (7).

which eluted just before 6-*epi*castanospermine on radial chromatogaphy was closer to that of 6-*epi*castanospermine (17.49 min) than to that of 7-deoxy-6-*epi*castanospermine (12.03 min). This property was indicative of a tetrahydroxylated alkaloid. This was confirmed by the high-resolution mass spectrum, which established the molecular formula as $C_8H_{15}NO_4$, and which was virtually identical with those of castanospermine and 6-*epi*castanospermine. The lack of a fragment ion corresponding to loss of a $-CH_2OH$ group (31 amu) showed that the compound was not a pyrrolizidine alkaloid of the australine class (Molyneux et al., 1988) and could only be an indolizidine alkaloid. The parent ion at m/z 145 established a trihydroxylation pattern for the six-membered ring moiety of the indolizidine ring system, and the alkaloid was therefore a stereoisomer of castanospermine.

This was confirmed by the ^{13}C NMR spectrum, which showed strong similarities to the spectra of castanospermine and its 6-epimer. The only difference that was immediately apparent was that the signals corresponding to the C3 and C5 methylene groups, which are typically separated by ca. 4 ppm, occurred as a single signal of doubled intensity at 54.9 ppm. These were subsequently resolved into two individual signals at 54.92 and 54.94 ppm, respectively, by detailed analysis of the cross signals in the CSCM spectrum. The latter spectrum also revealed a significant shift of ca. 5 ppm for the C7 methine signal relative to that for the same carbon atom in castanospermine, whereas the other signals differed by only 3 ppm or less.

Analysis of the ^1H NMR spectrum showed many similarities to those of castanospermine and 6-*epi*castanospermine. In particular, the bridgehead proton (H8a) showed similar coupling constants to H1 and H8 for all three alkaloids, indicating a common stereochemical configuration for the two hydroxyl groups adjacent to the bridgehead. However, certain signals in the spectrum of the unknown alkaloid were less complex. Both of the H5 protons occurred as broad doublets, each having small coupling constants (1.5 Hz) to H6, indicative of a similar dihedral angle, approaching 60°, between the vicinal protons. The hydroxyl group at C6 must therefore have a β configuration. Analogous small couplings (2 Hz) between H6 and H7, and H7 and H8, also imply bond angles close to 60° between these protons. Such values can only be accommodated

Table I: Effect of 6,7-Di*epi*castanospermine on Activity of Various Glycosidases

concn of 6,7-di*epi*castanospermine (μg/mL)	OD 410 nm						
	amyloglucosidase	β-glucosidase	α-galactosidase	β-galactosidase	α-mannosidase	β-mannosidase	α-L-fucosidase
0	2.24 (100)[a]	1.95 (100)	1.06 (100)	1.14 (100)	2.06 (100)	2.75 (100)	0.63 (100)
2.5	1.97 (88)	2.01 (102)	1.11 (105)	1.17 (102)	2.80 (135)	2.77 (101)	0.61 (97)
5.0	1.67 (74)	1.91 (98)	1.16 (109)	1.11 (97)	2.81 (136)	2.79 (101)	0.67 (106)
10.0	1.37 (61)	1.74 (89)	1.17 (110)	1.15 (101)	2.81 (136)	2.69 (98)	0.64 (102)
25.0	0.84 (37)	1.45 (74)	1.31 (123)	1.08 (95)	2.65 (128)	2.66 (96)	0.61 (97)
50.0	0.43 (19)	1.12 (57)	1.41 (132)	1.07 (94)	2.71 (131)	2.67 (97)	0.66 (105)

[a] Numbers in parentheses indicate percentage of control.

if H7 and H8 both have a β orientation, and the structure of the unknown alkaloid was therefore established as 6,7-di*epi*-castanospermine (**7**). Calculation of the torsion bond angles for an energy-minimized structure with a molecular modeling program yielded values compatible with the observed coupling constants, according to the Karplus equation. Decoupling experiments and a ^1H–^1H COSY NMR spectrum (Figure 2) confirmed the complete connectivity and substitution pattern of the alkaloid as (1*S*,6*R*,7*S*,8*R*,8a*R*)-tetrahydroxyindolizidine (**7**) or its enantiomer. On the basis of biosynthetic considerations we favor the structure shown.

Structural Characterization of N-(Hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine (**8**). The mass spectrum of the alkaloid with a GC retention time of 10.98 min established the molecular formula as $C_7H_{15}NO_3$. The chemical ionization mass spectrum showed fragment ions corresponding to loss of H_2O (*m/z* 144) and loss of a protonated CH_2OH group (*m/z* 130) from the molecular ion (*m/z* 162). The GC retention time for the TMS derivative was consistent with a trihydroxylated alkaloid.

The ^{13}C NMR spectrum showed the presence of two methine carbon signals, both of which were adjacent to either an oxygen or nitrogen atom, and five methylene carbon resonances. Only one of the latter occurred sufficiently upfield (δ 34.9) for it to lack an oxygen or nitrogen substituent. Two of the remaining signals had values (δ 64.2 and 62.1) characteristic of $-CH_2OH$ groups. The total of seven carbon atoms was insufficient to accommodate a bicyclic ring system, and the alkaloid therefore had to be of the pyrrolidine or piperidine type. The two methine carbon atoms were consistent only with a disubstituted pyrrolidine ring system, bearing a tertiary substituent on the nitrogen atom.

Analysis of the ^1H NMR spectrum, together with decoupling experiments, established the presence of a single hydroxyl substituent on the ring. The methine proton at δ 2.62 had a chemical shift typical of one adjacent to the nitrogen atom and a hydroxyl group and was coupled to the methine proton at δ 4.18 and to one of the $-CH_2OH$ groups. The alkaloid was therefore a 2-(hydroxymethyl)-3-hydroxypyrrolidine, analogous to that previously reported by Nash et al. (1985), but bearing an additional $-CH_2CH_2OH$ substituent. The absence of additional coupling to the methylene group, other than that of the $-CH_2OH$ group, established that this hydroxyethyl substituent was attached to the pyrrolidine nitrogen atom. Two-dimensional ^1H–^1H and ^1H–^{13}C NMR shift correlation spectra confirmed the structure of the alkaloid as *N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine. The stereochemical relationship between the –OH and $-CH_2OH$ groups could not be unequivocally determined from the coupling constant, but the trans orientation is favored on the basis of the previously isolated simpler alkaloid (Nash et al., 1985).

Glycosidase-Inhibitory Activity of 6,7-Diepicastanospermine. The isolation of a number of polyhydroxyindolizidine alkaloids with glycosidase inhibitory properties from plant sources, and the synthesis of analogues which also show such activity, suggests that glycosidase inhibition is a general property of this class of compounds. However, a predictive structure–activity correlation has so far proved impossible to derive. It was therefore of interest to test the pure 6,7-di*epi*castanospermine in order to determine its type and specificity of inhibition, together with its potency relative to structurally similar alkaloids.

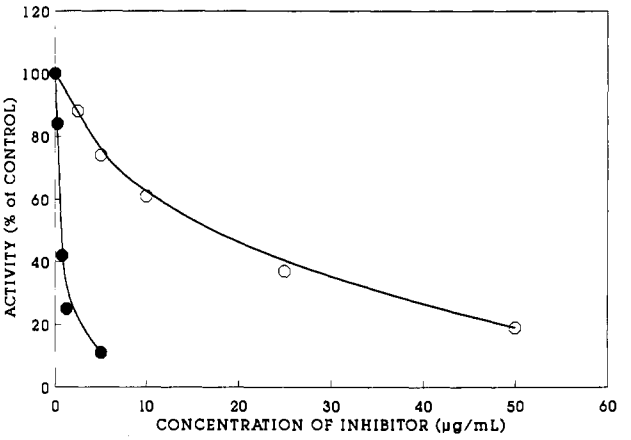

FIGURE 3: Effects of inhibitor concentration on the activity of amyloglucosidase. Incubation mixtures contained 50 μmol of acetate buffer, pH 5.0, 1.5 μmol of *p*-nitrophenyl α-D-glucopyranoside, and various amount of inhibitors, castanospermine, or 6,7-*epi*castanospermine, in a final volume of 0.5 mL. Reactions were terminated by the addition of 2.5 mL of 0.4 M glycine buffer, pH 10.4, and the amount of *p*-nitrophenol released was measured at 410 nM. Results are presented as percent of control. Curves are as follows: (solid symbols) castanospermine and (open symbols) 6,7-di*epi*castanospermine.

6,7-Di*epi*castanospermine was shown to be a fairly good inhibitor of amyloglucosidase, showing 50% inhibition at 16 μg/mL. The alkaloid was also slightly inhibitory toward β-glucosidase, with 43% inhibition occurring at 50 μg/mL (Table I). Figure 3 shows a concentration curve comparing the degree of inhibition of amyloglucosidase by various amounts of castanospermine and 6,7-di*epi*castanospermine. It can be seen that castanospermine is the more potent inhibitor, producing 50% inhibition at 1.5 μg/mL, as compared to 16 μg/mL for its 6,7-diepimer.

To determine whether 6,7-di*epi*castanospermine was a competitive or noncompetitive inhibitor of the amyloglucosidase, a series of experiments were done where the substrate (*p*-nitrophenyl α-D-glucose) concentration was varied, and several different concentrations of the alkaloid were used. When the data from these experiments was plotted by the method of Lineweaver and Burk, the intercept of $1/V$ versus $1/S$ was the same in the presence or absence of the inhibitor, indicating that the inhibition is of the competitive type. Castanospermine, 6-*epi*castanospermine and 7-deoxy-6-*epi*-

castanospermine were all previously shown to be competitive inhibitors of amyloglucosidase. As shown in Table I, 6,7-di*epi*castanospermine was inactive against β-galactosidase, β-mannosidase, and α-L-fucosidase but may have caused slight stimulation of α-galactosidase and α-mannosidase.

6,7-Di*epi*castanospermine was also tested for inhibitory activity against the glycoprotein processing enzymes, glucosidase I and mannosidase I. It was shown to inhibit glucosidase I at very high concentrations but to be inactive against mannosidase I (data not shown).

Discussion

The results presented herein describe the structural elucidation of an additional indolizidine alkaloid from *C. australe*. This compound has the configuration (1*S*,6*R*,7*S*,8*R*,8a*R*)-tetrahydroxyindolizidine, i.e. 6,7-di*epi*castanospermine (**7**). In addition we have isolated and characterized the alkaloid, *N*-(hydroxyethyl)-2-(hydroxymethyl)-3-hydroxypyrrolidine (**8**). The latter compound was isolated in very low yield, and the stereochemical structure could not be determined unequivocally from the NMR data, due to the known sensitivity of coupling constants to slight variations in bond angles in five-membered ring systems. However, a pyrrolidine alkaloid lacking the *N*-(hydroxyethyl) substituent has previously been isolated from the same plant source (Nash et al., 1985) and its structure determined by X-ray crystallography. Since this alkaloid was shown to have the 2-hydroxymethyl and 3-hydroxyl substituents in a trans configuration, it seems probable that the alkaloid which we have isolated has a similar orientation and we therefore propose structure **8** in Figure 1. Confirmation of this configuration must await either isolation of sufficient material to prepare a crystalline derivative for X-ray crystallography or its synthesis.

In spite of this stereochemical uncertainty, the alkaloid presents an intriguing insight into a putative biosynthetic pathway to *C. australe* alkaloids of both the indolizidine (castanospermine) and pyrrolizidine (australine) types. Insertion of an additional carbon atom, possibly acetate derived, between the 2-(hydroxymethyl) group and the terminal carbon atom of the *N*-(hydroxyethyl) group would yield an indolizidine alkaloid ring system. In contrast, insertion of a carbon atom between the 2-(hydroxymethyl) group and the methylene group adjacent to the nitrogen atom of the *N*-(hydroxyethyl) group would lead to a pyrrolizidine alkaloid. Such condensations might well occur with more highly oxidized pyrrolidine alkaloid precursors than those which we isolated. Nevertheless, these proposed routes may suggest appropriate precursors for biosynthetic labeling experiments. Circumstantial support for such pathways is the isolation of minor alkaloids epimeric at the 6-position, deoxygenated at the 7-position, and in this report, epimeric at both 6- and 7-positions. Such a biosynthetic pathway to castanospermine and its congeners differs markedly from that of swainsonine, which has been shown to derive from a preformed six-membered ring, pipecolic acid, the five-membered ring moiety being generated by subsequent addition of a two-carbon fragment (Harris et al., 1988).

The isolation and structural identification of 6,7-di*epi*castanospermine (**7**) provides an opportunity to investigate the effect of subtle structural changes upon the glycosidase inhibitory potency of the *C. australe* alkaloids. All of the indolizidine alkaloids isolated so far from this plant have proven to be competitive inhibitors of amyloglucosidase (Table II). Among these castanospermine is superior, showing 50% inhibition of enzymatic activity at a concentration of 8 μM. Epimerization of the 6-hydroxyl group, as in 6-*epi*castanospermine, results in a loss in activity such that a concentration of 20 μM is necessary to achieve 50% inhibition. Additional epimerization of the 7-hydroxyl group to give 6,7-di*epi*castanospermine further reduces the biological activity to give a K_i of about 84 μM, a level of activity about 10 times less than that of castanospermine. Furthermore, 7-deoxy-6-*epi*-castanospermine, which lacks a 7-hydroxyl group entirely, shows considerably reduced activity, with 50% inhibition occurring at 230 μM, a concentration approximately 29-fold higher than that required for castanospermine. In this series therefore, loss of a hydroxyl group decreases the level of inhibitory activity, as does an increasing degree of epimerization relative to castanospermine.

Table II: Concentration of Polyhydroxyindolizidine or Polyhydroxypyrrolizidine Alkaloid To Produce 50% Inhibition of Amyloglucosidase

alkaloid	concn (μM)	alkaloid	concn (μM)
castanospermine (**1**)	8	lentiginosine (**6**)	32
6-*epi*castanospermine (**2**)	20	australine (**4**)	6
6,7-di*epi*castanospermine (**7**)	84	1-*epi*australine	26
7-deoxy-6-*epi*-castanospermine (**3**)	230		

A similar gradation occurs in the pyrrolizidine alkaloid series, wherein australine (**4**) produces 50% inhibition of amyloglucosidase at a concentration of 6 μM, and 1-*epi*australine requires a concentration of 26 μM to produce the same effect, while the 3- and 7-epimers are very poor inhibitors of amyloglucosidase.

In addition to its inhibitory activity toward amyloglucosidase, 6,7-di*epi*castanospermine is also a moderate inhibitor of β-glucosidase, a property which it shares with castanospermine. In contrast, 6-*epi*castanospermine shows no activity against the latter enzyme. The stereochemical disposition of the hydroxyl substituents of the six-membered ring moiety of 6,7-di*epi*castanospermine is identical with that in deoxyfuconojirimycin, which is a potent inhibitor of α-L-fucosidase (Winchester et al., 1990) and is enantiomeric with respect to the same groups in 1-deoxygalactostatin, an inhibitor of β-galactosidase (Miyake & Ebata, 1988). However, 6,7-di*epi*castanospermine was found to be inactive against α-L-fucosidase and both α- and β-galactosidases. An analogous situation occurs with 6-*epi*castanospermine which has an identical stereochemical configuration of its hydroxyl substituents to deoxymannojirimycin, an excellent inhibitor of α-mannosidase. Nevertheless, no inhibition of either α- or β-mannosidase is produced by 6-*epi*castanospermine.

The growing body of evidence provided by this and previous studies of glycosidase inhibition by indolizidine alkaloids indicates that such activity not only is dependent on the number of hydroxyl groups present but also is sensitive to their position on the indolizidine ring and their relative stereochemistry. It should be noted, however, that no definitive conclusions can be drawn regarding the extent of hydroxylation necessary for inhibitory activity. Thus, lentiginosine (**6**) an indolizidine alkaloid having merely two hydroxyl substituents, isolated from the spotted locoweed (*A. lentiginosus*), produces 50% inhibition of amyloglucosidase at 32 μM (Pastuszak et al., 1990), demonstrating that this inhibitor is only one-fourth as active as castanospermine but is intermediate in activity between 6-*epi*castanospermine and 6,7-di*epi*castanospermine. However, 2-*epi*lentiginosine is completely inactive against the same enzyme even at concentrations as high as 6 mM. It is apparent that a predictive model for glycosidase inhibition can only be developed by sophisticated molecular modeling methods based upon data obtained from both natural and synthetic poly-

hydroxyindolizidine alkaloids.

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