7-DEOXY-6-*EPI*-CASTANOSPERMINE, A TRIHYDROXYINDOLIZIDINE ALKALOID GLYCOSIDASE INHIBITOR FROM *CASTANOSPERMUM AUSTRALE*

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ABSTRACT.—A new indolizidine alkaloid has been isolated from the seeds of *Castanospermum australe* and identified as 7-deoxy-6-*epi*-castanospermine [1] by ms and ^{1}H- and ^{13}C-nmr spectroscopy. The alkaloid is the first trihydroxylated indolizidine to be isolated from this plant and may represent an intermediate in the biosynthetic pathway to the tetrahydroxy-indolizidines and -pyrrolizidines. It inhibits amyloglucosidase and yeast α-glucosidase but is significantly less active as a glycosidase inhibitor than its isomer swainsonine [2] and the tetrahydroxylated alkaloids castanospermine [3], 6-*epi*-castanospermine [4], and australine [5].

The Moreton Bay chestnut or black bean, *Castanospermum australe* A. Cunn. (Leguminosae), a large tree native to Australia, which has been introduced into many subtropical regions of the world as an ornamental, has proven to be a fruitful source of alkaloids of the polyhydroxy-indolizidine and -pyrrolizidine classes having novel biological properties (1). The ripe chestnut-like seeds have been shown to produce hemorrhagic gastroenteritis in cattle that consume them (2). One constituent of the seeds, castanospermine [3], is a tetrahydroxyindolizidine alkaloid (3) with potent α- and β-glucosidase inhibitory activity (4,5), which has generated intense interest because of its ability to inhibit replication of retroviruses (6), including the human immunodeficiency virus (HIV) (7). Its epimer, 6-*epi*-castanospermine [4], is more specific in its activity, inhibiting only α-glucosidase (8). Recently three additional α-glucosidase inhibitors have been isolated, namely australine [5] (9), 3-*epi*-australine (3,7a-di-*epi*-alexine) [6] (10), and 1-*epi*-australine [7] (11). These three compounds comprise a unique group of pyrrolizidine alkaloids, being the first tetrahydroxy derivatives of this class and having the hydroxymethyl group located at the 3-position rather than at the previously invariant 1-position (12). The co-occurrence of both indolizidine and pyrrolizidine alkaloids in *C. australe* raises interesting questions as to their biosynthesis. In addition, the glycosidase inhibitory activity of both classes establishes that ring size is not a significant restriction of biological activity for these fused bicyclic ring types (13).

All of the indolizidine and pyrrolizidine alkaloids isolated so far from Moreton Bay chestnut seeds have been tetrahydroxylated. However, less highly hydroxylated analogues having significant glycosidase inhibitory properties occur in other legume species. For example, the trihydroxyindolizidine alkaloid swainsonine [2], which has been isolated from *Swainsona, Astragalus,* and *Oxytropis* species and is responsible for the toxicity of the locoweeds, is the only known example of this class that inhibits α-mannosidase (14,15). Swainsonine exhibits significant antimetastatic activity towards melanoma cells in mice as a result of this property (16). Recently, a dihydroxyindolizidine alkaloid, lentiginosine [8], has been isolated from the spotted locoweed, *Astragalus lentiginosus* var. *diphysus*, together with its 2-epimer. Whereas lentiginosine is a good inhibitor of amyloglucosidase, the 2-epimer exhibits no significant glycosidase inhibitory activity (17).
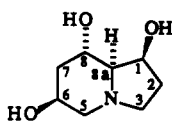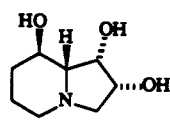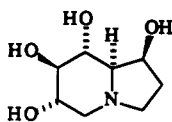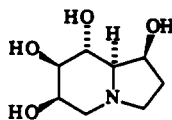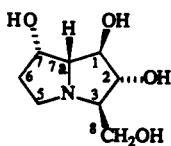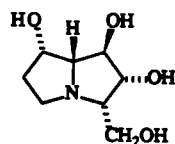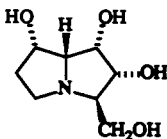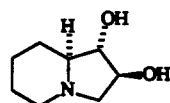
In order to establish the structural parameters that are necessary for the alkaloids to inhibit various glycosidases, we have attempted to isolate and evaluate less highly hydroxylated alkaloids than castanospermine and its isomers. Such compounds might also be expected to be precursors of the tetrahydroxy-indolizidines and -pyrrolizidines and therefore may provide information as to the biosynthetic pathway to the latter classes of alkaloids. Preparative tlc has now yielded a trihydroxyindolizidine alkaloid as a minor constituent of *C. australe* seeds. The structure was established as 7-deoxy-6-*epi*-castanospermine [1] by application of one- and two-dimensional high-field nmr techniques and by ms. This is the first trihydroxyindolizidine alkaloid to be isolated from *C. australe*. In contrast to the only other known naturally occurring trihydroxyindolizidine alkaloid, swainsonine [2], 7-deoxy-6-*epi*-castanospermine fails to inhibit α-mannosidase and is only a moderate inhibitor of amyloglucosidase.

RESULTS AND DISCUSSION

Preparative tlc of the alkaloidal mother liquors remaining after crystallization of castanospermine and separation of the australines gave a dextrorotatory, colorless oil that was more mobile on tlc than any of the above alkaloids and had an R_f value similar to that of swainsonine. Gc of the TMS ether derivative showed that the alkaloid was homogeneous. The material gave a purple spot on spraying the tlc plate with Ac_2O/ Ehrlich's reagent, indicative of an alkaloid having a pyrrolidine ring moiety with an unsubstituted position α to the nitrogen atom (18). The compound therefore cannot be an analogue of the australines but must be a polyhydroxyindolizidine.

The molecular formula was determined by hrms to be $C_8H_{15}NO_3$, possibly indicating a trihydroxyindolizidine alkaloid isomeric with swainsonine. However, the base

peak occurred at m/z 129, corresponding to a six-membered ring fragment bearing two hydroxyl groups, rather than a single hydroxyl group (m/z 113) as observed with swainsonine. Further fragmentation occurred to give a peak at m/z 112, due to loss of a hydroxyl group from this ion. A major peak was also observed at m/z 155, corresponding to loss of H_2O from the molecular ion. There was no evidence of loss of a hydroxymethyl group, which is a major fragmentation pathway for the australines (9). The alkaloid formed a triacetate derivative under mild acetylation conditions.

The ^{13}C nmr supported a trihydroxyindolizidine structure, showing two methylene groups adjacent to the nitrogen atom (δ 53.5 and 58.9) and a third (δ 33.4) having a chemical shift very similar to that of C-2 in castanospermine and 6-*epi*-castanospermine. However, in contrast to the latter alkaloids, an additional methylene group was observed having a chemical shift (δ 40.7) indicative of a location between two hydroxyl groups rather than adjacent to a single hydroxyl group. The remaining four signals corresponded to methine carbon atoms bearing hydroxyl groups or adjacent to the nitrogen atom. These data can only be accommodated by an indolizidine alkaloid structure having a 1,6,8-trihydroxy substitution pattern.

The high-resolution ^1H-nmr spectrum supported this distribution of hydroxyl groups around the bicyclic ring system, a ^1H-^1H homonuclear shift correlation experiment confirming the assignment of chemical shifts. Determination of the coupling constants by decoupling experiments established the stereochemistry of the hydroxyl groups. Thus, the coupling constants for H-1–H-8a and H-8–H-8a were 4.5 Hz and 9.3 Hz, respectively, being very close in value to those observed for castanospermine [3] (4.4 and 10 Hz) and 6-*epi*-castanospermine [4] (4.8 and 9.6 Hz), indicating that the stereochemistry of the hydroxyl groups adjacent to the bridgehead must be identical for all three compounds. The H-6 proton signal, located at δ 3.99, showed coupling constants of 5, 3, 2.5, and 1 Hz, with the coupling constant to the higher field H-7 proton at δ 1.38 being 3 Hz. The small values for all four coupling constants indicate that the H-6 proton must be equatorial, subtending similar angles to the protons at the 5 and 7 positions. This was confirmed by measurement of the appropriate angles with a molecular modeling program, all four values falling within the range of 52° to 67°. The hydroxyl group at the 6 position must then be β as in 6-*epi*-castanospermine. Because all of the previously isolated bicyclic alkaloids from *C. australe* have *R* bridgehead stereochemistry, we assume that this new alkaloid has the same configuration and is (1*S*, 6*S*, 8*S*, 8a*R*)-1,6,8-trihydroxyindolizidine [1].

The lesser degree of hydroxylation of 7-deoxy-6-*epi*-castanospermine relative to the previously isolated castanospermines 3 and 4 and australines 5–7 implies that it may be a biosynthetic intermediate on the pathway to the latter compounds. The existence of indolizidine alkaloids epimeric at the 6 position suggests that they may in turn be derived from a 6-keto intermediate. Such a compound, or its precursor, could then be the branch-point for biosynthesis of both polyhydroxy-indolizidine or -pyrrolizidine alkaloids. The isolation of 7-deoxy-6-*epi*-castanospermine may indicate appropriate biosynthetic experiments to elucidate the route to both classes of alkaloid.

The structural similarity of 7-deoxy-6-*epi*-castanospermine [1] to other polyhydroxylated indolizidine alkaloids suggested that this compound should have similar biological activity. Like castanospermine and 6-*epi*-castanospermine, 7-deoxy-6-*epi*-castanospermine was found to be a competitive inhibitor of amyloglucosidase, a fungal $\alpha 1 \mapsto 4$, $\alpha 1 \mapsto 6$ exoglucosidase (Figure 1), but required a concentration of 230 μM to achieve 50% inhibition of enzymatic activity. This concentration was much higher than that observed for either castanospermine (50% inhibition of enzymatic activity at a concentration of 7 μM) (Figure 1) or 6-*epi*-castanospermine (50% inhibition of enzymatic activity at a concentration of 20 μM) (8), indicating that hydroxylation at the C-7
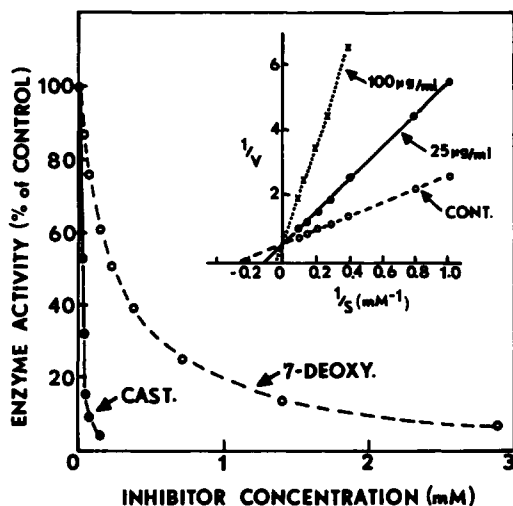
FIGURE 1. Inhibition of amyloglucosidase by 7-deoxy-6-*epi*-castanospermine [1] and castanospermine [3]. Activity is expressed relative to untreated enzyme. (○) 7-Deoxy-6-*epi*-castanospermine; (●) castanospermine. Inset shows kinetic analysis of 7-deoxy-6-*epi*-castanospermine against amyloglucosidase according to the method of Lineweaver and Burk.

position must be a requirement for potent inhibition of this exoglucosidase. Analogous reduction of glycosidase inhibitory activity has been observed for synthetic 1-deoxy-castanospermine derivatives (19,20), implying that a hydroxyl substituent at the 1 position is also an essential feature for such activity.

In addition to amyloglucosidase, 7-deoxy-6-*epi*-castanospermine [1] was tested against a number of other hydrolytic enzymes. The alkaloid showed no inhibitory effect on β-glucosidase, α-galactosidase, α- or β-mannosidase, β-N-acetylglucosaminidase B, β-glucuronidase, β-xylosidase, α-L-fucosidase, or the glycoprotein processing enzyme glucosidase II at concentrations up to 1 mM, but did inhibit β-galactosidase, intestinal sucrase, yeast α-glucosidase, and the glycoprotein processing enzyme glucosidase I at high levels. For example, at a concentration of 1 mM, 7-deoxy-6-*epi*-castanospermine inhibited the activities of yeast α-glucosidase, glucosidase I, intestinal sucrase, and β-galactosidase by 50%, 35%, 30%, and 20%, respectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The alkaloids were monitored for purity by tlc on 0.25 mm Si gel plates developed with $CHCl_3$-MeOH-NH_4OH-H_2O (50:26:2:2) and detected by spraying with Ac_2O followed by Ehrlich's reagent (18). Homogeneity was also checked by gc of the TMSi derivatives, prepared by treatment with 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 capillary column. Low resolution electron and ammonia chemical ionization mass spectra were obtained on a VG Micromass 7070 mass spectrometer; high resolution mass measurements were determined on the same instrument. Optical rotations were measured in a 1-dm cell on a Perkin-Elmer 241 automatic polarimeter. Nmr spectra were determined in CD_3OD on a Nicolet NTC 200FT spectrometer with software package at 200 MHz (^{1}H) and at 50.3 MHz (^{13}C) using TMS as an internal standard. Multiplicities for ^{13}C signals were determined by application of the carbon attached proton test (CAPT) sequence. Torsion bond angles were determined for an energy-minimized structure using the Alchemy II molecular modeling program.

PLANT MATERIAL.—Seeds of *C. australe* were collected from the Huntington Botanical Gardens, San Marino, California in September 1987. A voucher specimen is preserved at the Western Regional Research Center, Albany, California.

EXTRACTION AND FRACTIONATION.—Mature seeds were ground and extracted with MeOH in a Soxhlet apparatus, and the alkaloidal fraction was purified by ion-exchange chromatography on Dowex 50W-X8 (NH^+_4 form) (3). The eluate was repeatedly subjected to crystallization from MeOH until no additional castanospermine, mp 217–219°, could be isolated.

ISOLATION AND CHARACTERIZATION OF 7-DEOXY-6-*EPI*-CASTANOSPERMINE [1].—Preparative tlc yielded a colorless oil ($R_f = 0.53$), giving a purple-colored spot with Ac_2O/Ehrlich's reagent, with similar mobility to swainsonine ($R_f = 0.48$) but considerably more mobile than castanospermine ($R_f = 0.27$). The yield was approximately 0.005%. Gc analysis of the TMSi derivative gave a single peak with an Rt of 12.03 min; swainsonine showed Rt 13.67 min, while castanospermine had Rt 18.90 min. Optical rotation $[\alpha]^{26}_\lambda$ ($c = 0.712$, MeOH) +18.3° (589), +19.1° (578), +21.9° (546), +37.5° (436); ^{13}C nmr (50.3 MHz, CD_3OD) δ 75.4 (C-8a), 71.6 (C-1), 67.6 (C-6), 63.8 (C-8), 58.9 (C-5), 53.5 (C-3), 40.7 (C-7), 33.4 (C-2); ^1H nmr (200 MHz, CD_3OD) δ 4.31 (1H, ddd, $J_{1,2\alpha} = 6.5$ Hz, $J_{1,2\beta} = 1.5$ Hz, $J_{1,8a} = 4.5$ Hz, H-1), 4.12 (1H, ddd, $J_{7\alpha,8} = 11.5$ Hz, $J_{7\beta,8} = 5$ Hz, $J_{8,8a} = 9.3$ Hz, H-8), 3.99 (1H, m, $J = 5, 3$, 2.5, 1 Hz, H-6), 3.11 (1H, m, H-3β), 3.03 (1H, dd, $J_{5\beta,6} = 2$ Hz, $J_{5\alpha,5\beta} = -12$ Hz, H-5β), 2.12 (4H, m, H-2β, H-3α, H-5α, H-7β), 1.71 (1H, m, H-2α), 1.65 (1H, dd, $J_{1,8a} = 4.5$ Hz, $J_{8,8a} = 9.3$ Hz, H-8a), 1.38 (1H, ddd, $J_{6,7\alpha} = 3$ Hz, $J_{7\alpha,7\beta} = -13$ Hz, $J_{7\alpha,8} = 11.5$ Hz, H-7α); torsion bond angles H-6–H-5α 61.0°, H-6–H-5β −60.1°, H-6–H-7α −52.2°, H-6–H-7β 66.5°; eims *m/z* [M]$^+$ 173 (47), [M − H_2O]$^+$ 155 (84), 129 (100), 112 (100), 100 (92), 86 (100), 82 (99); hrms mass measurement [M]$^+$ *m/z* 173.1052 (calcd for $C_8H_{15}NO_3$, 173.1049).

PREPARATION OF 7-DEOXY-6-*EPI*-CASTANOSPERMINE TRIACETATE.—The alkaloid (10 mg) in dry pyridine (0.5 ml) was treated with Ac_2O (0.7 ml) and kept at room temperature for 18 h. The solution was poured into H_2O (20 ml), the aqueous mixture was extracted with $CHCl_3$ (3 × 10 ml), and the organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to yield a colorless oil (12 mg): ^1H nmr (200 MHz, $CDCl_3$) δ 5.43 (1H, ddd), 5.23 (1H, ddd), 5.20 (1H, m), 3.28 (2H, m), 2.40–1.90 (7H, m), 2.14 (3H, s, -OAc), 2.06 (3H, s, -OAc), 2.01 (3H, s, -OAc); ^{13}C nmr (50.3 MHz, $CDCl_3$) δ 170.7 (OCOMe), 170.6 (OCOMe), 169.8 (OCOMe), 71.7 (C-8a), 69.8 (C-1), 68.4 (C-6), 65.7 (C-8), 55.5 (C-5), 53.1 (C-3), 34.3 (C-7), 30.2 (C-2), 21.55 ($OCOCH_3$), 21.02 ($OCOCH_3$), 20.99 ($OCOCH_3$); eims *m/z* [M]$^+$ 299 (0.2), [M − Ac]$^+$ 256 (4), [M − HOAc]$^+$ 239 (11), [M − 2 × HOAc]$^+$ 179 (54), 178 (100), 154 (22), 128 (12); cims (NH_3) *m/z* [MH]$^+$ 300 (52), 256 (7), [MH − HOAc]$^+$ 240 (21), 239 (17), [MH − 2 × HOAc]$^+$ 180 (15), 179 (83), 178 (100), 177 (10); hrms mass measurement [MH]$^+$ *m/z* 300.1447 (calcd for $C_{14}H_{22}NO_6$, 300.1445).

GLUCOSIDASE INHIBITION.—Amyloglucosidase (from *Aspergillus niger*), α-glucosidase (from yeast), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), α-mannosidase (from jack bean), β-*N*-acetylglucosaminidase B (from bovine epididymis), α-glucuronidase (from bovine liver), β-xylosidase (from *A. niger*), α-L-fucosidase (from bovine kidney), and all *p*-nitrophenyl glycoside substrates were purchased from either Boehringer Mannheim Biochemicals or the Sigma Chemical Company. β-Mannosidase was purified from *A. niger* as previously described (21). Sucrase was purified to homogeneity from white rat (Sprague-Dawley) small intestine (22). Glucosidase I and glucosidase II were isolated from mung bean seedlings (23). [^3H]Glucose-labeled $Glc_3Man_9GlcNAc$-oligosaccharide was prepared as previously described (24).

The enzymatic activites of the arylglycosidases were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (13,25). Intestinal sucrase activity was determined by measuring the formation of reducing sugar from sucrose (13). Glucosidase I activity was determined by measuring the release of [^3H]glucose from [^3H]glucose-labeled $Glc_3Man_9GlcNAc$ as described (24). Glucosidase II activity was measured by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucoside (25).

ACKNOWLEDGMENTS

LITERATURE CITED

1.   A.D. Elbein and R.J. Molyneux, in: "Alkaloids: Chemical and Biological Perspectives." Ed. by S.W. Pelletier, Wiley, New York, 1987, Vol. 5, pp. 1–54.

2.   R.A. McKenzie, K.G. Reichmann, C.K. Dimmock, P.J. Dunster, and J.O. Twist, *Aust. Vet. J.*, **65**, 165 (1988).
3.   L.D. Hohenschutz, E.A. Bell, P.J. Jewess, D.P. Leworthy, R.J. Pryce, E. Arnold, and J. Clardy, *Phytochemistry*, **20**, 811 (1981).
4.   R. Saul, J.P. Chambers, R.J. Molyneux, and A.D. Elbein, *Arch. Biochem. Biophys.*, **221**, 593 (1983).
5.   R. Saul, R.J. Molyneux, and A.D. Elbein, *Arch. Biochem. Biophys.*, **230**, 668 (1984).
6.   P.S. Sunkara, T.L. Bowlin, P.S. Liu, and A. Sjoerdsma, *Biochem. Biophys. Res. Commun.*, **148**, 206 (1987).
7.   B.D. Walker, M. Kowalski, W.C. Rosen, L.R. Rohrschneider, W.A. Haseltine, and J. Sodroski, *Proc. Natl. Acad. Sci. USA*, **84**, 8120 (1987).
8.   R.J. Molyneux, J.N. Roitman, G. Dunnheim, T. Szumilo, and A.D. Elbein, *Arch. Biochem. Biophys.*, **251**, 450 (1987).
9.   R.J. Molyneux, M. Benson, R.Y. Wong, J.E. Tropea, and A.D. Elbein, *J. Nat. Prod.*, **51**, 1198 (1988).
10. R.J. Nash, L.E. Fellows, A.C. Plant, G.W.J. Fleet, A.E. Derome, P.D. Baird, M.P. Hegarty, and A.M. Scofield, *Tetrahedron*, **44**, 5959 (1988).
11. C.M. Harris, T.M. Harris, R.J. Molyneux, J.E. Tropea, and A.D. Elbein, *Tetrahedron Lett.*, **30**, 5685 (1989).
12. A.R. Mattocks, "Chemistry and Toxicology of Pyrrolizidine Alkaloids," Academic Press, London, 1986.
13. J.E. Tropea, R.J. Molyneux, G.P. Kaushal, Y.T. Pan, M. Mitchell, and A.D. Elbein, *Biochemistry*, **28**, 2027 (1989).
14. S.M. Colegate, P.R. Dorling, and C.R. Huxtable, *Aust. J. Chem.*, **32**, 2257 (1979).
15. R.J. Molyneux and L.F. James, *Science*, **216**, 190 (1982).
16. M.J. Humphries, K. Matsumoto, S.L. White, R.J. Molyneux, and K. Olden, *Cancer Res.*, **48**, 1410 (1988).
17. I. Pastuszak, R.J. Molyneux, L.F. James, and Alan D. Elbein, *Biochemistry*, **29**, 1886 (1990).
18. R.J. Molyneux, L.F. James, and K.E. Panter, in: "Plant Toxicology." Ed. by A.A. Seawright, M.P. Hegarty, L.F. James, and R.F. Keeler, Queensland Poisonous Plants Commission, Brisbane Australia, 1985, pp. 266–278.
19. D. Hendry, L. Hough, and A.C. Richardson, *Tetrahedron*, **44**, 6143 (1988).
20. D. Hendry, L. Hough, and A.C. Richardson, *Tetrahedron*, **44**, 6153 (1988).
21. A.D. Elbein, S. Ayda, and Y.C. Lee, *J. Biol. Chem.*, **252**, 2206 (1977).
22. J. Kolinska and G. Semenza, *Biochim. Biophys. Acta*, **146**, 187 (1967).
23. T. Szumilo, G.P. Kaushal, and A.D. Elbein, *Arch. Biochem. Biophys.*, **247**, 261 (1988).
24. T. Szumilo and A.D. Elbein, *Anal. Biochem.*, **157**, 32 (1985).
25. M. Rudick and A.D. Elbein, *J. Biol. Chem.*, **248**, 6506 (1973).