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CALYSTEGINE ALKALOIDS FROM *DUBOISIA LEICHHARDTII*

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Key Word Index—*Duboisia leichhardtii*; Solanaceae; calystegine C_1; calystegine C_2; pentahydroxy-*nor*-tropane; α-mannosidase inhibitor.

Abstract—Pentahydroxy-*nor*-tropane alkaloids, calystegines C_1 and C_2, were isolated from the leaves of greenhouse-cultivated *Duboisia leichhardtii*, together with calystegines B_1, B_2 and B_4. Calystegine C_1 is a potent inhibitor of β-glucosidase and β-galactosidase, whereas calystegine C_2 exhibited no significant inhibitory activity against these enzymes. Only calystegine C_2, the 2-epimer of calystegine C_1, among calystegines isolated to date exhibited an inhibitory activity against all α-mannosidases tested. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The tropane alkaloids occur mainly in the Solanaceae family and are also found in the Convolvulaceae, Erythroxylaceae, Proteaceae, Rhizophoraceae, Euphorbiaceae and Cruciferae families [1]. Recently, a novel structural type of polyhydroxylated alkaloids has been isolated from many plants in the Convolvulaceae, Solanaceae and Moraceae [2–6]. These alkaloids have been named calystegines, which are polyhydroxy-*nor*-tropanes with a bridgehead OH group. We have recently reported the occurrence of calystegines A_3, A_5, B_1, B_2 and B_3 in *Physalis alkekengi* var. *francheti* (Solanaceae) [7], and of calystegines A_3, A_5, A_6, B_1, B_2, B_3 and N_1 in *Hyoscyamus niger* [8]. The genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* of the Solanaceae are especially rich sources of hyoscyamine, or scopolamine, or both. Among these species, *Atropa belladonna* [2, 9–11], *Datura wrightii* [4] and *Scopolia carniolica* [9] are known to produce calystegines. We have examined the occurrence of calystegines in the roots of *Scopolia japonica* using GC-mass spectrometry and have found a new tetrahydroxy-*nor*-tropane alkaloid, calystegine B_4, in addition to the known calystegines A_3, A_5, B_1, B_2, B_3 and C_1 [12]. In this paper, we report the occurrence of calystegines in *Duboisia leichhardtii*, the structure determination of a new calystegine C_2, and the glycosidase inhibitory activities of the isolated alkaloids.

RESULTS AND DISCUSSION

The alkaloid fraction was obtained by chromatography of the hot water extract of *Duboisia leichhardtii* (1.4 kg of dry leaves) on an Amberlite IR-120 (H^+ form) ion-exchange column. The alkaloid fraction was divided into two pools (A and B) in order of elution with water as an eluant from an Amberlite CG-50 (NH_4^+ form) column. Further chromatography of pool A on a Dowex 1-X2 (OH^- form) column gave calystegines B_2 (34 mg), B_1 (4 mg), C_2 (9 mg) and C_1 (1.2 g) in order of elution. By the same chromatography using Dowex 1-X2 (OH^- form), calystegine B_4 (31 mg) was obtained from pool B.

The structures of five calystegines isolated from *D. leichhardtii* were determined by combined ^1H and ^{13}C NMR spectroscopy, including extensive homonuclear decoupling experiments, NOE enhancements, and two-dimensional HMQC experiments. The ^1H and ^{13}C NMR spectra of calystegines B_1, B_2, B_4 and C_1 isolated from *D. leichhardtii* were completely in accord with those of authentic samples isolated from *P. alkekengi* var. *francheti* [7], *Scopolia japonica* [9] and *Morus alba* [6].

High-resolution FAB-mass spectrometry (glycerol) of calystegine C_2 gave an $[M+H]^+$ ion at m/z 192.0871 ($C_7H_{14}O_5N$ requires 192.0872). The ^{13}C NMR (100 MHz) spectra in D_2O of calystegine C_2 gave seven resonances at δ 45.7 (C-7), 66.9 (C-5), 70.2 (C-6), 72.8 (C-3), 73.4 (C-4), 76.3 (C-2), and 93.2 (C-1). The ^1H NMR spectroscopic data, together with information from extensive decoupling experiments and two-dimensional HMQC spectral data, defined the complete connectivity of the carbon and hydrogen atoms.

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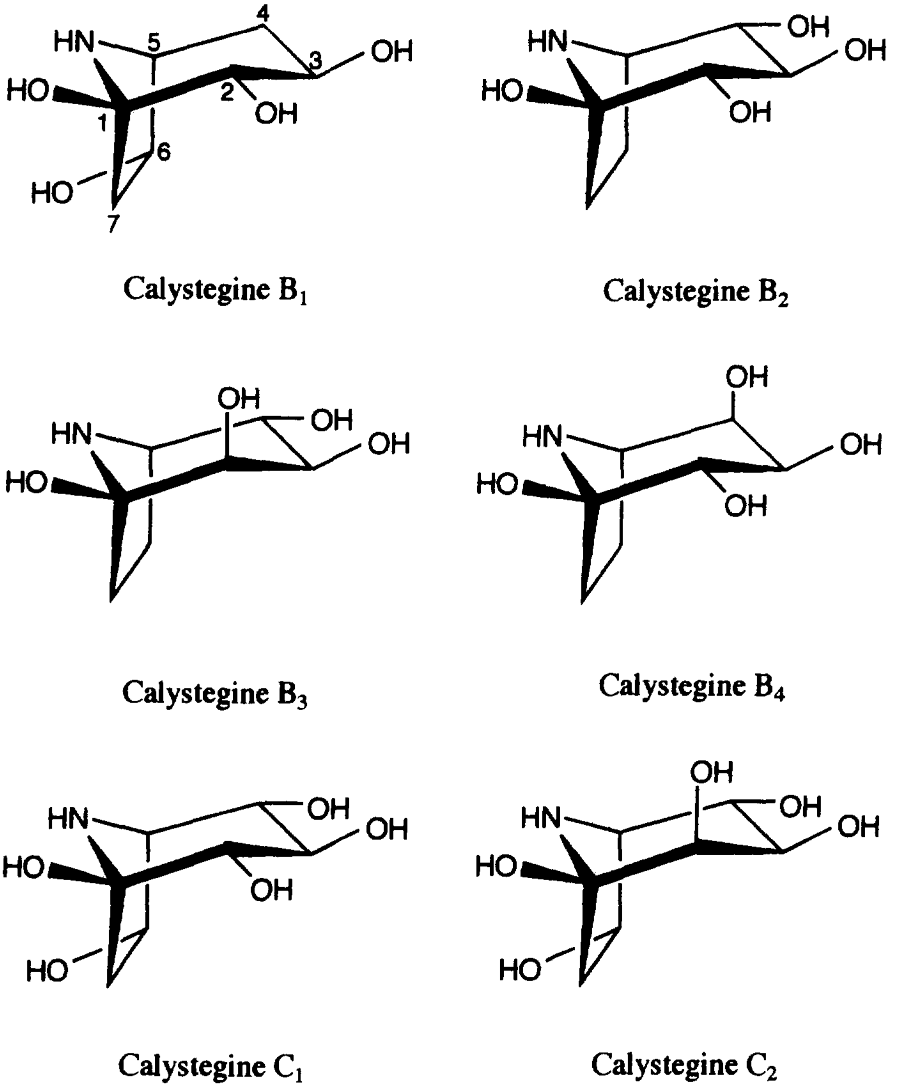


Calystegine B_1 Calystegine B_2

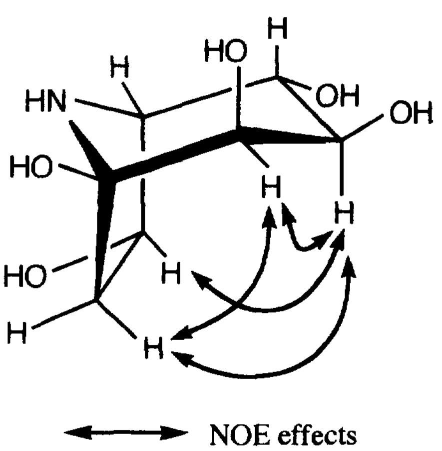
Calystegine B_3 Calystegine B_4

Calystegine C_1 Calystegine C_2

Fig. 1. Structure of tetra- and pentahydroxy-*nor*-tropanes.

In the ^1H NMR spectrum, the coupling patterns of H-2 (*d*, $J_{2,3} = 4.0$ Hz), H-3 (*dd*, $J_{2,3} = 4.0$, $J_{3,4} = 9.9$ Hz), and H-4 (*dd*, $J_{3,4} = 9.9$, $J_{4,5} = 4.0$ Hz) indicate that H-2, H-3 and H-4 are equatorial, axial and axial, respectively. Furthermore, the absence of a W-path long-range coupling between H-2 and H-7*exo* is consistent with an axial OH at C-2. The H-6 signal was observed as a doublet of doublets ($J_{6,7endo} = 7.3$, $J_{6,7exo} = 2.9$ Hz), and there was no resolved coupling between H-5*eq* and H-6*endo*, indicating a dihedral angle close to 90° between them. The NOE effects as shown in Fig. 2 provided further configurational support. Therefore, the relative configuration of calystegine C_2 was shown to be 1α,2α,3α,4β,6α-pentahydroxy-*nor*-tropane.

The IC_{50} values of calystegines C_1, C_2, and the structurally related calystegines against various glycosidases are shown in Table 1. As previously reported [5–7], calystegine B_2 is a potent inhibitor of β-glucosidase, α- and β-galactosidases, and trehalase, and calystegine C_1 is a more potent inhibitor than caly-



Calystegine C_2

Fig. 2. NOE effects of calystegine C_2.

Table 1. Concentration of calystegines giving 50% inhibition (molar) of various glycosidases

Enzyme	IC_{50} (M) for Calystegine B_2	Calystegine B_3	Calystegine C_1	Calystegine C_2
α-Glucosidase				
Rice	7.5×10^{-5}	NI*	4.2×10^{-4}	NI
Rat intestine maltase	6.4×10^{-4}	NI	1.9×10^{-4}	NI
β-Glucosidase				
Almond	2.6×10^{-6}	7.2×10^{-4}	8.2×10^{-7}	1.7×10^{-4}
Caldocellum saccharolyticum	2.4×10^{-6}	3.9×10^{-4}	8.6×10^{-7}	9.0×10^{-5}
Rat intestine cellobiase	8.0×10^{-5}	NI	6.6×10^{-6}	NI
α-Galactosidase				
Coffee beans	1.9×10^{-6}	NI	3.6×10^{-4}	NI
Aspergillus niger	3.9×10^{-6}	2.2×10^{-4}	4.4×10^{-4}	NI
β-Galactosidase				
Bovine liver	2.4×10^{-4}	NI	1.6×10^{-5}	NI
Rat intestine lactase	7.8×10^{-6}	NI	3.8×10^{-7}	NI
α-Mannosidase				
Almond	NI	NI	NI	6.8×10^{-4}
Jack beans	NI	NI	NI	4.6×10^{-4}
Rat liver soluble	NI	NI	NI	6.0×10^{-5}
Rat liver lysosome	NI	NI	NI	2.4×10^{-4}
Rat epididymis	NI	NI	NI	1.0×10^{-4}
β-Mannosidase				
Rat epididymis	NI	NI	NI	NI
Trehalase				
Rat intestine	9.0×10^{-6}	9.2×10^{-5}	7.4×10^{-4}	6.0×10^{-4}
Porcine kidney	1.0×10^{-5}	2.0×10^{-4}	2.7×10^{-4}	4.6×10^{-4}

*NI = no inhibition (less than 50% inhibition at 1.0×10^{-3} M).

stegine B_2 of β-glucosidase and β-galactosidase, but not against α-galactosidase. Calystegine C_2, the 2-epimer of calystegine C_1, was over 100-fold weaker as an inhibitor of β-glucosidase than calystegine C_1 and had no inhibitory activity against α- and β-galactosidases. Only calystegine C_2 exhibited a moderate inhibitory activity against all α-mannosidases tested and it was not an inhibitor of β-mannosidase. On the other hand, other calystegines exhibited no significant inhibitory activity against α-mannosidases even at a concentration of 1 mM.

Duboisia, a small genus within the Solanaceae native to Australia, consists of three species. *D. leichhardtii* and *D. myoporoides* are important commercial sources of the tropane alkaloids, hyoscyamine and scopolamine. The second pentahydroxy-*nor*-tropane alkaloid, calystegine C_2, was isolated from the leaves of greenhouse-cultivated *D. leichhardtii*, and calystegine C_1 was found to be present at 0.09% w/w (relative to dry leaf) in this plant. Calystegines have been found to occur in the genera *Atropa* [2, 9–11, 13], *Datura* [4], *Hyoscyamus* [8, 9], and *Scopolia* [9, 12], which are major sources of the anticholinergic alkaloids, hyoscyamine and scopolamine. Thus, calystegines are widely distributed in the Solanaceae family plants, especially in the tropane-producing genera. Highly water soluble tropane alkaloids, such as the calystegines, may be widely distributed in many solanaceous species and have escaped detection in the past because of commonly used purification procedures such as chloroform extraction.

The structural basis of the inhibition of glycosidases by the calystegines is not obvious. The C-2, C-3, C-4 OH groups and ring heteroatom of calystegine B_2 lie in the same region of space as the C-4, C-3, C-2 OH group and ring heteroatom of 1-deoxynojirimycin, that is, calystegine B_2 superimposes well on 1-deoxynojirimycin. Calystegines B_3 and B_4 superimpose well on 1,5-dideoxy-1,5-imino-D-galactitol and 1-deoxymannojirimycin, respectively. However, the biological properties of calystegines and piperidine alkaloids are quite different. Calystegine B_2 is a potent inhibitor of β-glucosidase and α-galactosidase, but not α-glucosidase. Calystegine B_3 shows no significant inhibitory activity against α- and β-galactosidases, and calystegine B_4 is a specific inhibitor of mammalian trehalase [9], but not α-mannosidase. The introduction of a hydroxyl group at the C-6*exo* position of calystegine B_2 enhanced the inhibitory potential toward β-glucosidase and β-galactosidase, while the epimerization at C-2 of calystegine B_2 reduced its inhibition of all glycosidases tested. Interestingly, the introduction of a hydroxyl group at the C-6*exo* position of calystegine B_3 produced inhibitory activity against α-mannosidases. It would be of value to measure inhibitory activities of a much larger set of natural or synthetic isomers and epimers against glycosidases to understand better the structural requirements for glycosidase inhibition.

EXPERIMENTAL

Analytical methods. The purity of samples was checked by HPTLC Silica Gel-60F_{254} (Merck): solvent system PrOH–AcOH–H_2O (4:1:1); detection with chlorine-*o*-tolidine reagent. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra recorded on a Jeol JNM-GX 400 spectrometer. Chemical shifts expressed in ppm downfield from internal sodium 3-(trimethylsilyl)propionate (TSP) in D_2O. Mass spectra measured on Jeol JMS-SX 102A spectrometer.

Materials. Enzymes α-glucosidase (from rice), β-glucosidases (from almonds and *Caldocellum saccharolyticum*: recombinant), α-galactosidases (from green coffee beans and *Aspergillus niger*), β-galactosidase (from bovine liver), α-mannosidases (from almonds and jack beans), and trehalase (from porcine kidney), and *p*-nitrophenyl glycoside and disaccharide substrates from Sigma Chemical Co. Brush border membranes prepd from rat intestine [14] were used as the source of rat intestinal glycosidases. Epididymal fluid prepd from rat epididymis [15] was used as the source of rat epididymis α- and β-mannosidases. Soluble α-mannosidase was prepd from the soluble fr. of rat liver homogenates according to method of Bishoff and Kornfeld [16], and lysosomal α-mannosidase was prepd from rat liver homogenates according to method of Opheim and Touster [17].

Enzyme assays. Activities of rice α-glucosidase (pH 5.0), porcine kidney trehalase (pH 6.5), and rat intestinal glycosidases (pH 5.8) were assayed at 37 in a total vol. of 0.2 ml containing 25 mM of appropriate disaccharide and appropriate amount of enzyme. After 10–30 min incubation, the reaction mix. was stopped by heating at 100 for 3 min. After centrifugation, 0.05 ml of the supernatant was added to 3 ml of Glucose B-test Wako (Wako Pure Chemical Industries). A 505 nm was measured to determine amount of released D-glucose. Other enzyme activities were assayed using the appropriate *p*-nitrophenyl glycoside as the substrate at the optimum pH of each enzyme. The reaction mixture (1 ml) contained 2 mM of the substrate and the appropriate amount of enzyme. The incubations were performed for 30 min at 37 . The reaction was stopped by adding 2 ml of 400 mM Na_2CO_3. The *p*-nitrophenol released was measured at 400 nm.

Isolation of calystegines. The dried leaves (1.4 kg) of *D. leichhardtii* were extracted 3 × with hot H_2O for 2 hr. After the hot H_2O extract (30 l) was filtered through Celite, the filtrate was applied to a column of Amberlite IR-120B (H^+, 1 l) and eluted with 0.5 M NH_4OH. The brown oil (25 g) obtained by concn of an eluate was applied to a Dowex 1-X2 column (2.9 × 62 cm, OH^- form) and eluted with H_2O (fr. size 15 ml). Frs 10–30 were concd to give a powder (12 g). This powder was further chromatographed with an Amberlite CG-50 column (3.8 × 90 cm, NH_4^+ form) and eluted with H_2O (fr. size 15 ml). The H_2O eluate was divided into two pools: A (frs 25–48, 1.4 g) and B (frs 60–85, 182 mg). Pool A was chromatographed on a Dowex 1-X2 column (1.9 × 92 cm, OH^- form) with water as an eluant (fr. size 15 ml) to give calystegine B_2 (34 mg), B_1 (4 mg), C_2 (9 mg), and C_1 (1.2 g) in order of elution. Pool B was also chromatographed on the same column with H_2O as an eluant to give calystegine B_4 (31 mg).

Calystegine C_2 (*1α,2α,3α,4β. 6α-pentahydroxy*-nor*tropane*). $[\alpha]_D$ -40.6 (c 0.32, H_2O); HRFAB-MS m/z 192.0871 $[M+H]^+$ ($C_7H_{14}O_5N$ requires 192.0872); ^{1}H NMR (400 MHz, D_2O): δ 1.648 (*br d*, 1H, H-7*exo*), 2.371 (*dd*, 1H. $J_{6,7endo} = 7.3$, $J_{7endo,7exo} = 14.7$ Hz, H-7*endo*), 3.171 (*br d*, 1H, $J_{4,5} = 4.0$ Hz, H-5), 3.476 (*dd*, 1H, $J_{2,3} = 4.0$, $J_{3,4} = 9.9$ Hz, H-3), 3.618 (*dd*, 1H, $J_{3,4} = 9.9$, $J_{4,5} = 4.0$ Hz, H-4), 3.783 (*d*, 1H, $J_{2,3} = 4.0$ Hz, H-2), 4.276 (*dd*, 1H, $J_{6,7endo} = 7.3$, $J_{6,7exo} = 2.9$ Hz, H-6); ^{13}C NMR (100 MHz, D_2O): δ 45.7 (C-7), 66.9 (C-5), 70.2 (C-6), 72.8 (C-3), 73.4 (C-4), 76.3 (C-2), 93.2 (C-1).

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