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Carbohydrate Research 293 (1996) 195–204

CARBOHYDRATE RESEARCH

Calystegine B_4, a novel trehalase inhibitor from *Scopolia japonica* [1]

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Received 30 May 1996; accepted 2 August 1996

Abstract

GLC–MS analysis has been developed for screening plants of the family Solanaceae for new calystegines. GLC–MS analyses of the extract of *Scopolia japonica* showed the presence of a new tetrahydroxy-*nor*-tropane alkaloid in addition to the known calystegines A_3, A_5, B_1, B_2, B_3, and C_1. We gave this new alkaloid the trivial name calystegine B_4. The structure of calystegine B_4 was determined as 1α,2β,3α,4α-tetrahydroxy-*nor*-tropane from a variety of NMR spectral data. Calystegines B_1, B_2, and C_1 are potent competitive inhibitors with K_i values ranging from 10^{-6} to 10^{-7} M for almond β-glucosidase, while calystegine B_4 inhibited this enzyme in a competitive manner, with a K_i value of 7.3 μM. Calystegine B_2 is also a potent inhibitor of green coffee bean α-galactosidase, whereas calystegine B_4 exhibited no significant activity for this enzyme. Among rat intestinal glycosidases, only trehalase was potently inhibited by calystegine B_4, with an IC_{50} value of 9.8 μM. Furthermore, calystegine B_4 potently inhibited pig kidney trehalase in a competitive manner, with a K_i value of 1.2 μM, but it was almost inactive against yeast and fungal trehalases. © 1996 Elsevier Science Ltd.

Keywords: *Scopolia japonica*; GLC–MS; Calystegine B_4; Trehalase inhibitor; Structure/activity relationships

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[1] The discoverers of this structural type of alkaloid named them calystegins. We decided to use calystegines instead, because they later used the name with an extra e, which brought it into line with names of most other alkaloids.

PII S0008-6215(96)00204-2

1. Introduction

The tropane alkaloids are bicyclic amines which combine a pyrrolidine (five-membered) and a piperidine (six-membered) ring. This class of alkaloid includes such important medicinal alkaloids as cocaine, scopolamine, and atropine. The genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia* of the Solanaceae are especially rich sources of hyoscyamine, or scopolamine, or both. Recently, polyhydroxylated nortropane alkaloids, named calystegines, have been isolated from plants in the Convolvulaceae, Solanaceae, and Moraceae families [1–5]. More recently, we reported the isolation of calystegines A_3, A_5, B_1, B_2, B_3 from *Physalis alkekengi* var. *francheti* (Solanaceae) [6], and calystegines A_3, A_5, A_6, B_1, B_2, B_3, and N_1 from *Hyoscyamus niger* [7]. Calystegine N_1 was a new type of calystegine with a bridgehead NH_2 group in the place of a bridgehead OH group in calystegine B_2.

The C-2, C-3, and C-4 OH groups and ring heteroatom in the six-membered ring of (+)-calystegine B_2 are lying in the same region of space as the C-4, C-3, and C-2 OH groups and ring heteroatom of 1-deoxynojirimycin (DNJ), respectively. These structural similarities suggest that calystegines might have glycosidase inhibitory activities. Molyneux et al. [8] and ourselves [4–6] have reported that calystegine B_2 is a potent competitive inhibitor of β-glucosidases and α-galactosidases, and calystegines B_1 and C_1 are potent competitive inhibitors of β-glucosidases, but are not inhibitors of α-galactosidases. In the present work, we adopted GLC–MS analysis for a search for

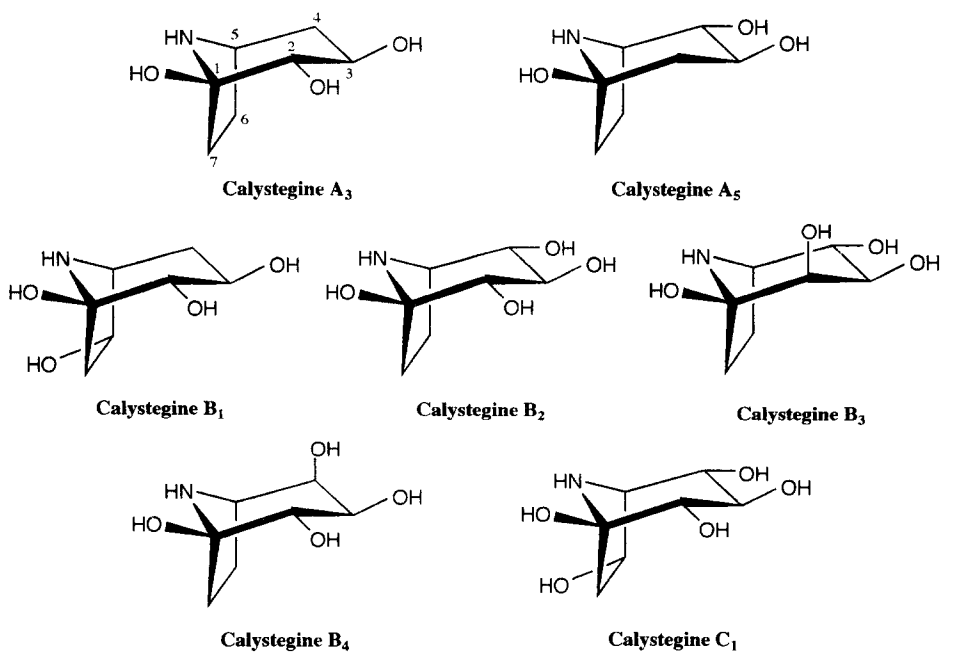

Fig. 1. Structures of calystegines isolated from *Scopolia japonica*.

new calystegines in plants of the family Solanaceae, and we found the new calystegine B_4 in addition to calystegines A_3, A_5, B_1, B_2, B_3, and C_1 from the roots of *Scopolia japonica* (Fig. 1). In this paper we reported the isolation of calystegine B_4, its structure determination and glycosidase inhibitory activities, and the structure–activity relationships considering the contribution of the chiral centers and substituents to the potency of the trehalase inhibition.

2. Results

GLC–MS analysis of the extract of S. japonica.—GLC analysis of the trimethylsilylated resin-treated extract of *S. japonica* is shown in Fig. 2. A mild silylation procedure using Sigma SIL-A left the secondary amino group underivatized. Calystegines B_1, B_2, and B_3 gave the tetra-*O*-trimethylsilyl (tetra-*O*-Me_3Si) derivative which had a molecular ion at *m/z* 463 and characteristic fragment ions at *m/z* 448 and 373, which are caused by loss of CH_3 and HO–$SiMe_3$, respectively. Calystegines B_2 and B_3 showed a base peak at *m/z* 217 due to Me_3Si–O–CH=CH–CH^+–O–$SiMe_3$ arising from three adjacent trimethylsilylated hydroxyl groups, while calystegine B_1 showed a base peak at *m/z* 244 due to a dihydropyrrolinium ion bearing one more trimethylsilylated hydroxyl group than that of calystegine A_3 arising from the five-membered ring moiety of calystegine B_1, as reported by Molyneux et al. [8], and Dräger [9]. Since the component

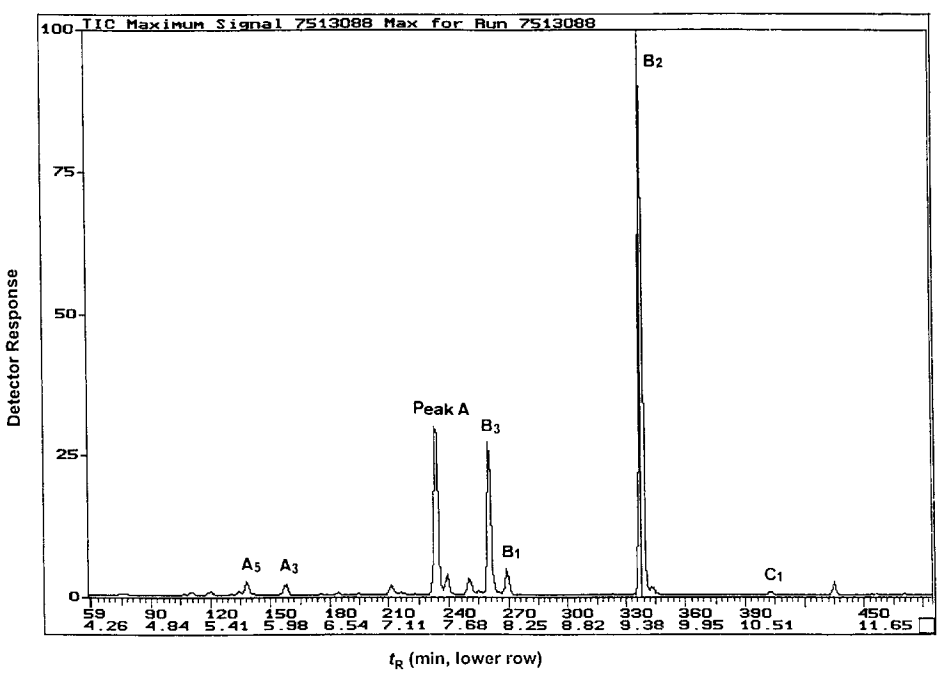

Fig. 2. GLC analysis of the *O*-trimethylsilylated resin-treated extracts of *Scopolia japonica*.

with a retention time of 7.57 min (Peak A in Fig. 2) did not correspond in retention time to any of the calystegine standards and had the same fragmentation pattern as those of calystegines B_2 and B_3, we designated this component calystegine B_4.

Isolation and purification of alkaloids.—The total alkaloid fraction was obtained by chromatography of the hot water extract of the roots of *S. japonica* (5 kg) on an Amberlite IR-120B (H^+) ion-exchange column. This fraction was further divided into three pools of A, B, and C in order of water elution from an Amberlite CG-50 (NH_4^+) column. Following column chromatography of each pool on Dowex 1-X2 (OH^-) and Amberlite CG-50 (NH_4^+), calystegines B_1, B_2, and C_1 were obtained from pool A, calystegines B_3 and B_4 from pool B, calystegines A_3 and A_5 from pool C. The ^1H and ^{13}C NMR spectra of calystegines A_3, A_5, B_1, B_2, B_3, and C_1 isolated from *S. japonica* were completely in accord with those of authentic samples isolated from *P. alkekengi* var. *francheti* [6] and *Morus alba* [5].

Structure determination of calystegine B_4.—The results of ^{13}C NMR and HRFABMS (*m/z* 176.0923, $[M + H]^+$; $C_7H_{14}O_4N$ requires 176.0923) analyses of calystegine B_4 indicate that it is a tetrahydroxy-*nor*-tropane. In the ^1H NMR spectrum in D_2O, the coupling pattern of H-4 (δ 3.77, t, $J_{3,4} = J_{4,5} = 2.9$ Hz) indicates that H-3 or H-4 is equatorial, or that they are both equatorial, but we could not determine its relative configuration from the spectrum in D_2O because of the complete overlapping of the H-2 and H-3 signals. In the ^1H NMR spectrum in the solvent system of 4:1 pyridine-d_5–D_2O, the H-2 signal was observed as a doublet of doublets with coupling constants of 1.7 and 8.8 Hz at δ 4.32. This large coupling constant (8.8 Hz) observed between H-2 and H-3 indicates that H-2 and H-3 are trans-diaxial, and, therefore, H-4 is equatorial. The stereoconfiguration of calystegine B_4 was corroborated by a definite NOE effect between H-4 and H-6*endo*. Consequently, the relative configuration of calystegine B_4 was shown to be 1α,2β,3α,4α-tetrahydroxy-*nor*-tropane.

Glycosidase inhibitory activities of calystegine B_4.—As previously reported [6], calystegines B_1 and C_1 are potent competitive inhibitors of β-glucosidase and β-galactosidase, and calystegine B_2 is a potent competitive inhibitor of β-glucosidase and α-galactosidase. Calystegine B_4 was a six-fold weaker inhibitor of almond β-glucosidase than calystegine B_2, and a ten-fold weaker inhibitor of *Aspergillus niger*

Table 1
Kinetic constants of calystegines for competitive inhibition of glycosidases

Enzyme	K_i (μM) for calystegines					
	A_3	B_1	B_2	B_3	B_4	C_1
β-Glucosidase						
almond	20	1.8	1.2	200	7.3	0.45
Caldocellum saccharolyticum	12	0.43	0.55	85	64	0.29
α-Galactosidase						
green coffee bean	20	NI [a]	0.86	NI	NI	90
Aspergillus niger	30	NI	2.3	33	22	14
β-Galactosidase						
bovine liver	30	1.6	46	NI	NI	3.6

[a] No inhibition.

Table 2
Concentration of calystegines giving 50% inhibition of rat digestive glycosidase activities

Substrate	IC_{50} (μM) of calystegines						
	A_3	A_5	B_1	B_2	B_3	B_4	C_1
Maltose	NI [a]	NI	NI	640	NI	NI	190
Sucrose	NI	NI	NI	500	NI	NI	160
Palatinose	NI	NI	NI	270	NI	NI	230
Trehalose	12	NI	260	9	92	9.8	740
Cellobiose	1000	NI	25	80	NI	380	6.6
Lactose	110	NI	2.6	7.8	NI	110	0.38

[a] No inhibition (less than 50% inhibition at 1000 μM).

α-galactosidase (Table 1). However, it had no inhibitory activity toward green coffee bean α-galactosidase and bovine liver β-galactosidase.

The IC_{50} values of calystegines toward rat digestive glycosidases are shown in Table 2. Calystegines B_1 and C_1 potently inhibited lactase, with IC_{50} values of 2.6 and 0.38 μM, respectively. Calystegine B_2 was a good inhibitor of trehalase and lactase, and potencies of calystegines A_3, B_2, and B_4 toward rat intestinal trehalase were almost the same, with IC_{50} values of 12, 9, and 9.8 μM, respectively. This indicates that the deoxygenation and epimerization at C-4 of calystegine B_2 have no significant effect for rat intestinal trehalase, and the C-4 epimerization appears to induce a shift in specific inhibition to trehalase.

The IC_{50} values of calystegines toward trehalases of various origins are shown in Table 3. Calystegines B_2 and B_4 had a very weak inhibitory activity toward the enzyme from the pathogenic fungus *Rhizoctonia solani*, and none of the calystegines exhibited any appreciable inhibition toward baker's yeast trehalase. On the other hand, calystegines A_3, B_2, and B_4 exhibited good inhibitory activities toward the last instar larvae midgut trehalases of *Bombyx mori* and *Spodoptera litura*. Calystegine B_4 was more effective for pig kidney trehalase than for rat intestinal trehalase, and inhibited pig

Table 3
Concentration of calystegines giving 50% inhibition of trehalase activities of various origins

Origin	IC_{50} (μM) of calystegines						
	A_3	A_5	B_1	B_2	B_3	B_4	C_1
Yeast and Fungus							
baker's yeast	NI [a]	NI	NI	NI	NI	NI	NI
Rhizoctonia solani	NI	NI	1000	700	NI	540	NI
Insect							
Bombyx mori	34	NI	1000	25	NI	19	NI
Spodoputera litura	50	NI	290	30	NI	40	NI
Mammal							
rat small intestine	12	NI	260	9	92	9.8	740
pig kidney	13	NI	NI	10	200	4.8	270

[a] No inhibition (less than 50% inhibition at 1000 μM).

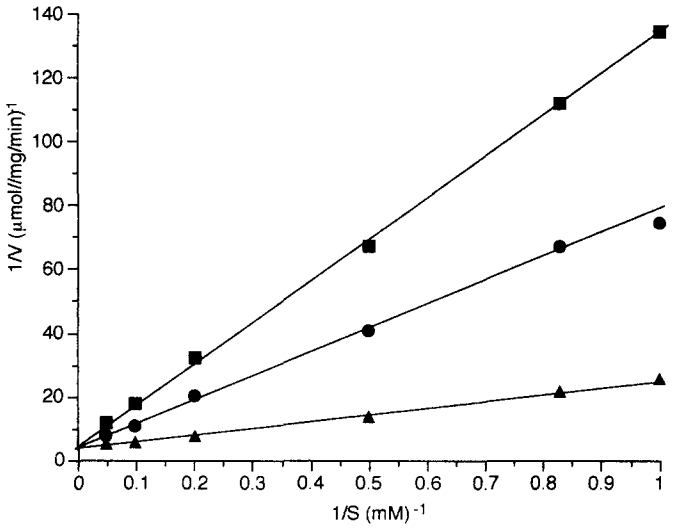

Fig. 3. Lineweaver–Burk plots of calystegine B_4 inhibition of pig kidney trehalase. The increasing concentrations of trehalose were used to determine the K_m and K_i values. Concentrations of calystegine B_4 were 0 (▲), 1 μM (●), and 5 mM (■). The D-glucose released was measured by the D-glucose oxidase–peroxidase method, and the data were plotted as $1/V$ against $1/S$. The calculated K_m of pig kidney trehalase was 5.5 mM.

kidney trehalase more potently than did calystegines A_3 and B_2. As seen in Fig. 3, calystegine B_4 was a competitive inhibitor of pig kidney trehalase with a K_i value of 1.2 μM and had a four-fold stronger affinity for the enzyme than calystegine B_2. As seen between calystegines B_1 and C_1, the introduction of the OH group into C-6*exo* significantly lowered the affinity for pig kidney trehalase.

3. Discussion

The structural basis of the inhibition of glycosidases by the calystegines is not obvious. Calystegine B_2 is a bicyclic amine that combines pyrrolidine ring and polyhydroxylated piperidine rings in a bridged structure and superimposes well on DNJ. However, their biological properties are quite different, that is, DNJ is a potent α-glucosidase inhibitor, while calystegine B_2 is a potent inhibitor of β-glucosidase and α-galactosidase [6]. Calystegine B_3 shows weak or no inhibitory activity toward α- and β-galactosidases, in spite of its good superimposition onto 1,5-dideoxy-1,5-imino-D-galactitol [10], which is an extremely powerful inhibitor of coffee bean α-galactosidase with a K_i value of 0.0016 μM. As seen in Fig. 4, calystegine B_4 superimposes well on *manno*-DNJ, but calystegine B_4 was quite inactive against α- and β-mannosidases and α-L-fucosidase (data not shown). Interestingly, the deoxygenation or epimerization at C-4 of calystegine B_2 completely retained the inhibitory potential toward rat intestinal trehalase, but it decreased the potency toward other glycosidases (Table 2). Calystegine

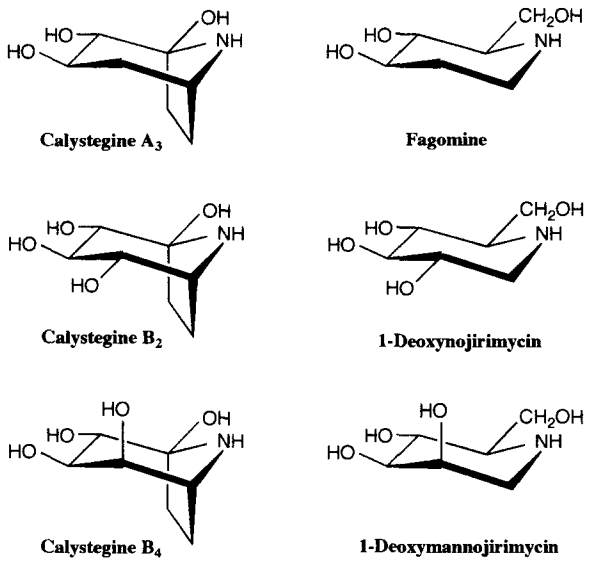

Fig. 4. Structures of calystegines A_3, B_2, and B_4, and of structurally related polyhydroxylated piperidine alkaloids.

B_4 appears to be more effective for pig kidney trehalase ($IC_{50} = 4.8$ μM) than for rat intestinal trehalase ($IC_{50} = 9.8$ μM) (Table 3). It can be seen in Table 3 that the order of sensitivity of various trehalases to calystegine B_4 was mammal > insect > fungus > yeast. Thus, the 4-epimerization of calystegine B_2 resulted in an inhibitor with increased potency and specificity for mammalian trehalase.

As shown in Fig. 4, calystegines A_3, B_2, and B_4 have structural similarities with fagomine, DNJ, and *manno*-DNJ, respectively. DNJ is a good competitive inhibitor of pig kidney trehalase with a K_i value of 8.5 μM, whereas fagomine and *manno*-DNJ are very weak competitive inhibitors of this enzyme, with K_i values of 680 and 390 μM, respectively. These results indicate that the equatorially oriented C-2 OH group of DNJ is an essential feature for strong binding with the active site of pig kidney trehalase, but it is impossible to predict the glycosidase inhibitory activities of the calystegines from the common topography with the polyhydroxylated piperidines corresponding to hexoses in the pyranose configuration.

There are marked differences between inhibitors in the specificity of inhibition of isoenzymes in the same cell or in the same organism or in different species. The disaccharide analogue trehalase inhibitors, such as validoxylamine A [11–13], trehalostatin [14], trehalozolin [15,16], and salbostatin [17], exhibit extremely potent inhibitory activity in a competitive manner against trehalases of all origins tested. On the other hand, the inhibition of trehalase by the monosaccharide analogues greatly depends on its origin. DNJ and 1,4-dideoxy-1,4-imino-D-arabinitol were good inhibitors of mammalian trehalases [18] but were poor inhibitors of insect ($IC_{50} = 250$ μM for *Bombyx mori*) and baker's yeast ($IC_{50} = 340$ μM) enzymes, respectively, while 2,5-dideoxy-2,5-imino-D-

mannitol (DMDP) is a good inhibitor of insect trehalase (IC_{50} = 55 μM) [19,20] and a potent inhibitor of bacterial trehalase (IC_{50} = 0.35 μM for *Corynebacterium* sp.) [21], but not of pig kidney trehalase [21]. Trehalamine [16] and calystegine B_4 are more specific inhibitors of mammalian trehalases, losing inhibitory activities against other glycosidases and trehalases of other origins. Glycosidase inhibitors can often provide valuable information about the mechanism of action, physiological role, and chemical topography of the active site of an enzyme. In higher animals, the enzyme trehalase is found at high levels in the brush border membranes of the small intestine [22] and the kidney proximal tubule [23]. Intestinal trehalase is most probably involved in the hydrolysis of ingested trehalose, but the physiological role of kidney trehalase is unknown. Specific trehalase inhibitors would be useful tools for studying the mechanism of action and function of this enzyme.

4. Materials and methods

General methods.—The purity of samples was checked by HPTLC Silica Gel-60F_{254} (E. Merck) using the solvent system 4:1:1 PrOH–AcOH–H_2O, and a chlorine–*o*-tolidine reagent was sprayed for detection. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-GX 400 spectrometer as indicated in D_2O using sodium 3-(trimethylsilyl)-propionate (TSP) and in 4:1 pyridine-d_5–D_2O using tetramethylsilane (Me_4Si) as the internal standard. ^{1}H NMR chemical shifts are reported as δ values in ppm, and the coupling constants (J) are given in Hz. Mass spectra were measured on JEOL JMS-SX 102A spectrometer.

Materials.—α-Glucosidases (from baker's yeast and rice), β-glucosidases (from almonds and *Caldocellum saccharolyticum*: recombinant), α-galactosidases (from green coffee beans and *Aspergillus niger*), β-galactosidase (from bovine liver), α-mannosidase (from jack beans), β-mannosidase (from snail), and trehalase (from pig kidney), and α-L-fucosidase (from bovine epididymis) and all *p*-nitrophenyl glycosides were purchased from Sigma Chemical Co. Disaccharides were purchased from Wako Pure Chemical Industries. Brush border membranes prepared from rat small intestine [24] were used as the source of rat digestive glycosidases. Yeast and fungal trehalases were partially purified from baker's yeast and *Rhizoctonia solani* according to the methods of Penek and Souza [25] and Asano et al. [11], respectively. Insect brush border membranes were prepared from midguts of last instar larvae of *Bombyx mori* and *Spodoputera litura* according to the literature [26] and used as the source of insect digestive trehalases. DNJ, fagomine, and 1-deoxymannojirimycin (*manno*-DNJ) were prepared according to published procedures [18].

GLC–MS analysis.—Samples were dried and silylated using 100 μL of Sigma SIL-A (Sigma Chemical Co.). The samples were heated at 30 °C for 30 min, and then 0.3 μL was injected directly into the GLC. The column was a 25 m × 0.25 mm BPX5 capillary column (SGE), and the 25 min temperature program ran from 180 to 300 °C with an initial rate of increase of 10 °C per min; the temperature was then held at 300 °C. The mass spectrometer was a QMASS 910 (Perkin–Elmer) with the EI mass range set at 100–650 mu.

Enzyme assays.—The activities of rice α-glucosidase, rat digestive glycosidases, and trehalases were determined using the appropriate disaccharide (25 mM) as substrate at the optimum pH of each enzyme. The incubations were performed for 10–60 min at 37 °C. The reaction was stopped by heating at 100 °C for 3 min. After centrifugation (600 *g*; 10 min), 0.05 mL of the supernatant was added to 3 mL Glucose B-test Wako (Wako Pure Chemical Industries). The absorbance at 505 nm was measured to determine the amount of the released D-glucose. Other enzyme activities were determined using the appropriate *p*-nitrophenyl glycosides as substrate at the optimum pH of each enzyme. The incubations were performed for 30 min at 37 °C. The reaction was stopped by adding 2 mL of 400 mM Na_2CO_3. The released *p*-nitrophenol was measured at 400 nm. Enzyme inhibition modes and K_i values of the calystegines were determined from the slope of Lineweaver–Burk plots.

Isolation of calystegines.—The roots of *S. japonica* (5 kg) purchased from a commercial source were extracted three times with hot water for 2 h. The total alkaloid fraction was obtained by chromatography of the hot water extract of *S. japonica* on an Amberlite IR-120B (H^+) ion-exchange column. This fraction was further divided into three pools of A, B, and C in order of water elution from an Amberlite CG-50 (NH_4^+) column. Following column chromatography of each pool on Dowex 1-X2 (OH^-) and Amberlite CG-50 (NH_4^+), calystegines B_1 (90 mg), B_2 (1.73 g), and C_1 (16 mg) were obtained from pool A, calystegines B_3 (503 mg) and B_4 (530 mg) from pool B, and calystegines A_3 (25 mg) and A_5 (35 mg) from pool C. The ^1H and ^{13}C NMR spectra of calystegines A_3, A_5, B_1, B_2, B_3, and C_1 isolated from *S. japonica* were completely in accord with those of authentic samples isolated from *P. alkekengi* var. *francheti* [6] and *Morus alba* [5].

Calystegine B_4 (1α,2β,3α,4α-tetrahydroxy-nor-*tropane).*—Calystegine B_4 was isolated as a colourless powder; $[\alpha]_D$ −63.0° (*c* 0.65, H_2O); HRFABMS: *m/z* 176.0923 $[M + H]^+$ ($C_7H_{14}NO_4$ requires 176.0923); ^1H NMR (400 MHz, 4:1 pyridine-d_5–D_2O) δ 1.35 (ddd, 1 H, $J_{6endo,6exo}$ 13.2, $J_{6endo,7endo}$ 9.9, $J_{6endo,7exo}$ 5.1 Hz, H-6*endo*), 1.98 (dddd, 1 H, $J_{2,7exo}$ 1.7, $J_{6endo,7exo}$ 5.1, $J_{6exo,7exo}$ 9.9, $J_{7endo,7exo}$ 13.2 Hz, H-7*exo*), 2.24 (dddd, 1 H, $J_{5,6exo}$ 7.8, $J_{6endo,6exo}$ 13.2, $J_{6exo,7endo}$ 4.4, $J_{6exo,7exo}$ 9.9 Hz, H-6*exo*), 2.43 (ddd, 1 H, $J_{6exo,7endo}$ 4.4, $J_{6endo,7endo}$ 9.9, $J_{7endo,7exo}$ 13.2 Hz, H-7*endo*), 3.71 (dd, 1 H, $J_{4,5}$ 2.9, $J_{5,6exo}$ 7.8 Hz, H-5), 4.02 (dd, 1 H, $J_{3,4}$ 4.4, $J_{4,5}$ 2.9 Hz, H-4), 4.04 (dd, 1 H, $J_{2,3}$ 8.8, $J_{3,4}$ 4.4 Hz, H-3), 4.32 (dd, 1 H, $J_{2,3}$ 8.8, $J_{2,7exo}$ 1.7 Hz, H-2); ^{13}C NMR (100 MHz, D_2O) δ 25.1 (C-6), 29.8 (C-7), 59.1 (C-5), 73.8 (C-3), 74.8 (C-4), 79.6 (C-2), 92.5 (C-1).

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