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Calystegin N_1, a novel nortropane alkaloid with a bridgehead amino group from *Hyoscyamus niger*: structure determination and glycosidase inhibitory activities

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Abstract

Seven calystegins were isolated from the whole plant of *Hyoscyamus niger*. They are calystegins A_3, A_5, A_6, B_1, B_2, B_3, and N_1. The structure of new calystegin A_6 was determined as $1\alpha,2\beta,7\alpha$-trihydroxy-*nor*-tropane, and another new calystegin N_1 was elucidated to be a trihydroxy-*nor*-tropane alkaloid with a bridgehead NH_2 group in the place of a bridgehead OH group in calystegin B_2. Very interestingly, on storage for a half year in a refrigerator, approximately 40% of calystegin N_1 was nonenzymically converted into calystegin B_2. Calystegin N_1 was a weaker inhibitor of glycosidases compared to calystegin B_2 but with the same inhibitory spectrum. However, calystegin N_1 inhibited porcine kidney trehalase in a noncompetitive manner, whereas calystegin B_2 inhibited this enzyme in a competitive manner. © 1996 Elsevier Science Ltd.

Keywords: *Hyoscyamus niger*; Calystegin; Structure determination; Bridgehead amino group; Glycosidase inhibitor

1. Introduction

A novel structural type of polyhydroxylated alkaloids has recently been isolated from many plants in the Convolvulaceae, Solanaceae, and Moraceae families [1–5]. These

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alkaloids, calystegins A_3, B_1, B_2, and C_1, are tri-, tetra- or penta-hydroxy-*nor*-tropanes and have proved to be potent inhibitors of β-glucosidases and/or α-galactosidases [4–6]. More recently we have reported the isolation of calystegins A_3, A_5, B_1, B_2, and B_3 from *Physalis alkekengi* var. *francheti* (Solanaceae) and that calystegin B_1 is a potent competitive inhibitor of β-glucosidase and β-galactosidase, and calystegin B_2 is a potent competitive inhibitor of β-glucosidase and α-galactosidase [7]. Furthermore, new calystegins A_5 and B_3, which are the 2-deoxy derivative and 2-epimer of calystegin B_2, respectively, show either a lack of or a marked decrease in inhibitory activity toward any glycosidases tested.

The genuses *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia*, which belong to the Solanaceae, are especially rich sources of hyoscyamine, or scopolamine, or both. Among these species, *Atropa belladonna* [1] and *Datura wrightii* [3] are known to produce calystegins. We have examined the occurrence of calystegins in plants of Solanaceae and have found that calystegins are widely distributed in this family [7]. In this paper, we report the isolation of seven calystegins including two more new calystegins A_6 and N_1 from this plant, their structure determination, and glycosidase inhibitory activities.

2. Results

Isolation and purification of calystegins.—The alkaloid fraction was obtained by chromatography of the hot water extracts of *Hyoscyamus niger* (whole plant) on an Amberlite IR-120 (H^+ form) ion-exchange column. The alkaloid fraction was divided into four pools, A, B, C, and D, in order of elution with water as the eluant from an Amberlite CG-50 (NH_4^+ form) column. Further chromatography of pool A on a Dowex 1-X2 (OH^- form) column gave calystegins N_1, B_2, and B_1 in order of elution. Chromatography on Dowex 1-X2 (OH^- form) and Amberlite CG-50 (NH_4^+ form) resulted in the isolation of calystegin B_3 from pool B, calystegins A_5 and A_6 from pool C, and calystegin A_3 from pool D.

The structures of seven calystegins isolated from *H. niger* (see Fig. 1) were determined by combined ^1H and ^{13}C NMR spectroscopy, including extensive homonuclear decoupling experiments, NOE enhancements, two-dimensional ^1H–^{13}C COSY, HMQC and HMBC experiments. The ^1H and ^{13}C NMR spectra of calystegins A_3, A_5, B_1, B_2, and B_3 isolated from *H. niger* were completely in accord with those of authentic samples isolated from *P. alkekengi* var. *francheti* [7].

Structure determination of calystegin A_6.—The ^{13}C NMR (100 MHz) spectral analysis of calystegin A_6 revealed the presence of three methines, three methylenes, and a quaternary carbon. The results of ^{13}C NMR and FABMS (m/z 160, $[M+H]^+$) analyses indicated that this calystegin was a trihydroxy-*nor*-tropane. The ^1H NMR data, together with information from extensive decoupling experiments and two-dimensional ^1H–^{13}C COSY spectral data, defined the complete connectivity of the carbon and hydrogen atoms. In the ^1H NMR spectrum, the coupling pattern of H-2 (δ 3.697, dd, $J_{2,3ax}$ 11.4, $J_{2,3eq}$ 6.2 Hz) indicated an axial orientation of H-2. Furthermore, the absence of a W-path long-range coupling between H-2 and H-7 suggested an *endo*

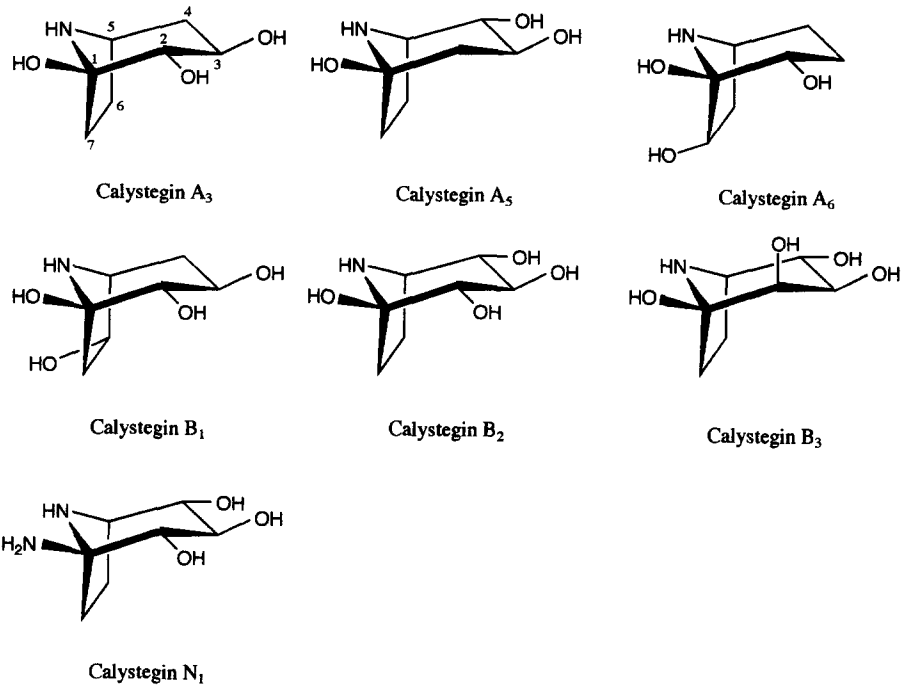

Fig. 1. Structure of calystegins isolated from *Hyoscyamus niger*.

orientation of H-7. This was corroborated by a definite NOE effect between H-3*ax* and H-7. Therefore, the relative configuration of calystegin A_6 was shown to be 1α,2β,7α-trihydroxy-*nor*-tropane.

Structure determination of calystegin N_1.—The ^{13}C NMR (100 MHz) spectral analysis of calystegin N_1 revealed the presence of four methines, two methylenes, and a quaternary carbon. However, the chemical shift (δ 78.3) of a quaternary carbon of calystegin N_1 differed significantly from those of any calystegins (δ 93–94) isolated to date. The ^1H NMR spectral data, combined with extensive decoupling experiments and two-dimensional HMQC and HMBC spectral data, defined the complete connectivity of carbon and hydrogen of calystegin N_1. Interestingly, the chemical shifts of any other carbons besides C-1 were very close to those of calystegin B_2 (Table 1). As shown in Fig. 2, the ^1H NMR spectrum (400 MHz) of calystegin N_1 is very similar to that of calystegin B_2, except for the upfield shifts of H-2 and H-7*exo*. The large J values ($J_{2,3} = J_{3,4} = 8.5$ Hz) seen in H-2 and H-3, and the H-4 signals of calystegin N_1 indicate all trans-axial orientations of H-2, H-3, and H-4. From comparison of FABMS data of calystegin N_1 (m/z 175, $[M + H]^+$) and calystegin B_2 (m/z 176, $[M + H]^+$), calystegin N_1 was presumed to bear a bridgehead NH_2 group in the place of a bridgehead OH group of calystegin B_2. To confirm this, we prepared its acetyl derivative and analyzed it by ^1H NMR spectroscopy and FABMS. As shown in Fig. 3, acetylation of calystegin N_1 with acetic anhydride in pyridine afforded an *N,O*-pentaacetate (acetyl resonances at

Table 1
^{13}C NMR chemical shifts [a] of calystegins B_2 and N_1

Carbon	Calystegin B_2	Calystegin N_1
C-1	93.2 (s)	78.3 (s)
C-2	80.4 (d)	80.7 (d)
C-3	77.7 (d)	77.7 (d)
C-4	77.6 (d)	77.5 (d)
C-5	58.6 (d)	59.5 (d)
C-6	24.5 (t)	25.8 (t)
C-7	31.5 (t)	32.9 (t)

[a] Chemical shifts are expressed in ppm downfield from internal TSP in D_2O.

δ 1.913, 2.022, 2.043, 2.080, and 2.264 in $CDCl_3$). The broad singlet at δ 8.401 disappeared on deuterium oxide exchange and was assigned to a bridgehead amide proton. Furthermore, a definite NOE effect was observed between this amide proton and H-2. These results and FABMS data of this acetyl derivative (m/z 385, $[M + H]^+$) confirmed the presence of a bridgehead NH_2 group in calystegin N_1. Thus, the relative configuration of calystegin N_1 was shown to be 1α-amino-2β,3α,4β-trihydroxy-*nor*-tropane.

Glycosidase inhibitory activities.—Calystegin A_6 was a weak inhibitor of porcine kidney trehalase (IC_{50} = 210 μM) but exhibited no significant inhibitory activity against any other glycosidases tested.

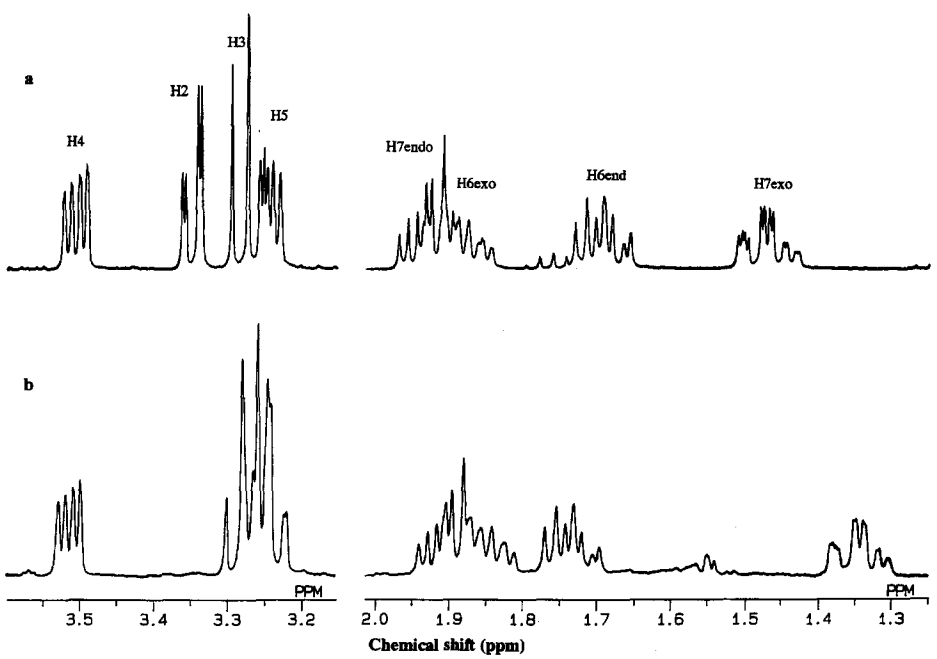

Fig. 2. ^1H NMR (400 MHz) spectra of calystegins B_2 (a) and N_1 (b) in D_2O.

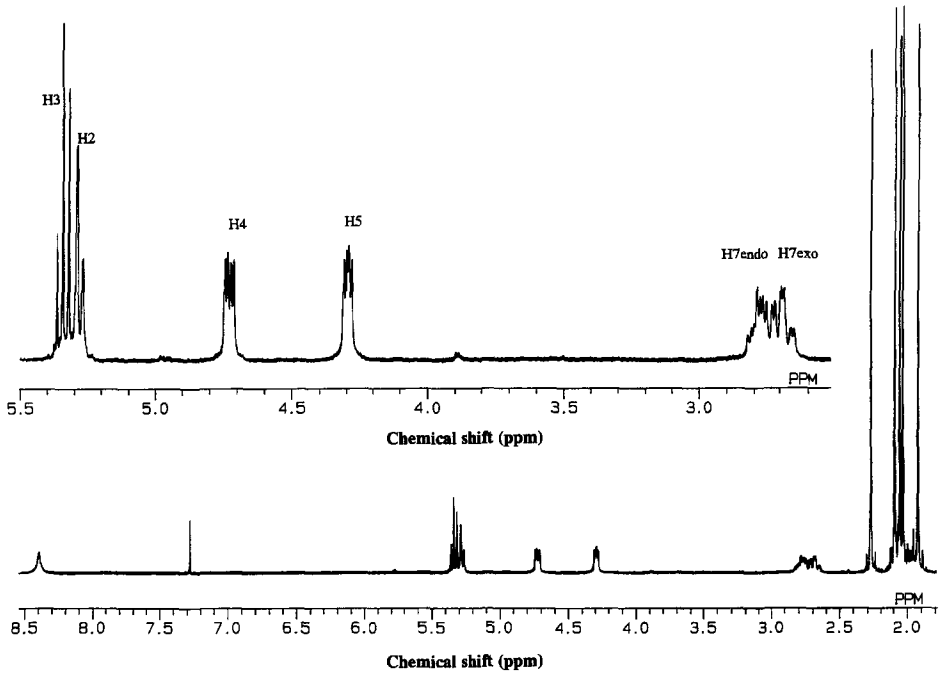

Fig. 3. ^{1}H NMR (400 MHz) spectrum of calystegin N_1 *N,O*-pentaacetate in $CDCl_3$.

The IC_{50} values of calystegins B_2 and N_1 against rat digestive glycosidases are shown in Table 2. Calystegin N_1, compared to calystegin B_2, showed a lower inhibition toward all rat digestive glycosidases, although calystegin N_1 relatively retained a potency for only cellobiase.

The IC_{50} and K_i values, and modes of inhibition of calystegin B_2 and N_1 for a variety of glycosidases are shown in Table 3. Calystegin N_1 is a good inhibitor of β-glucosidases, α-galactosidases, and porcine kidney trehalase, but it exhibited weaker inhibitory activities than did calystegin B_2. Calystegin N_1 inhibited β-glucosidases and

Table 2
Concentration of calystegins B_2 and N_1 giving 50% inhibition of rat digestive glycosidase activities

Substrate	IC_{50} (M) for	
	Calystegin B_2	Calystegin N_1
Maltose	6.4×10^{-4}	NI [a]
Sucrose	5.0×10^{-4}	1.0×10^{-3}
Palatinose	2.7×10^{-4}	NI
Trehalose	9.0×10^{-6}	2.1×10^{-4}
Cellobiose	8.0×10^{-5}	9.7×10^{-5}
Lactose	7.8×10^{-6}	3.6×10^{-4}

[a] No inhibition (less than 50% inhibition at 1.0×10^{-3} M).

Table 3
Measured IC_{50} and K_i values, and modes of inhibition

Enzyme	Calystegin B_2			Calystegin N_1		
	IC_{50} (M)	K_i (M)	Inhibition	IC_{50} (M)	K_i (M)	Inhibition
α-Glucosidase						
Rice	7.0×10^{-5}	ND [a]		NI [b]	NI	
β-Glucosidase						
Almond	2.6×10^{-6}	1.2×10^{-6}	C [c]	3.4×10^{-5}	1.4×10^{-5}	C
C. saccharolyticum [d]	2.4×10^{-6}	5.5×10^{-7}	C	1.4×10^{-5}	5.5×10^{-6}	C
Trehalase						
Porcine kidney	1.0×10^{-5}	5.3×10^{-6}	C	1.0×10^{-4}	6.2×10^{-5}	NC [e]
α-Galactosidase						
Coffee beans	1.9×10^{-6}	8.6×10^{-7}	C	2.6×10^{-4}	7.5×10^{-5}	C
Aspergillus niger	3.9×10^{-6}	2.3×10^{-6}	C	1.0×10^{-4}	6.8×10^{-5}	C
β-Galactosidase						
Bovine liver	2.4×10^{-4}	4.5×10^{-5}	C	NI	NI	
β-N-Acetylglucosaminidase						
Rat epididymis	NI	NI		NI	NI	

[a] ND = not determined.
[b] NI = no inhibition (less than 50% inhibition at 1.0×10^{-3} M).
[c] C = competitive.
[d] *Caldocellum saccharolyticum* (Recombinant).
[e] NC = noncompetitive.

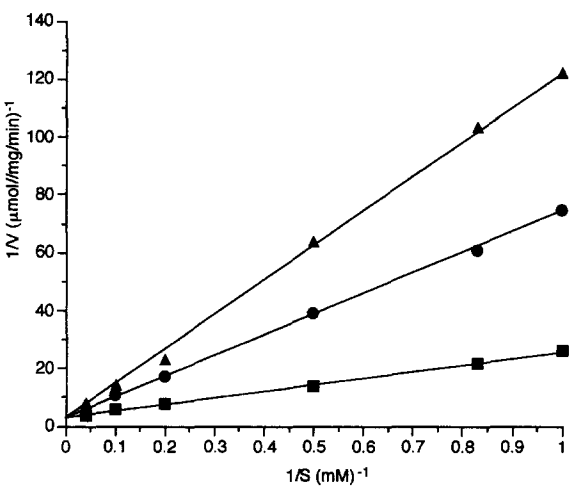

Fig. 4. Lineweaver–Burk plots of calystegin B_2 inhibition of porcine kidney trehalase. The increasing concentrations of trehalose were used to determine the K_m and K_i values. Concentrations of calystegin B_2 were 0 (■), 10 (●), and 25 μM (▲). The D-glucose released was measured by the glucose oxidase–peroxidase method, and the data were plotted as $1/V$ against $1/S$. The calculated K_m of porcine kidney trehalase was 5.5 mM.

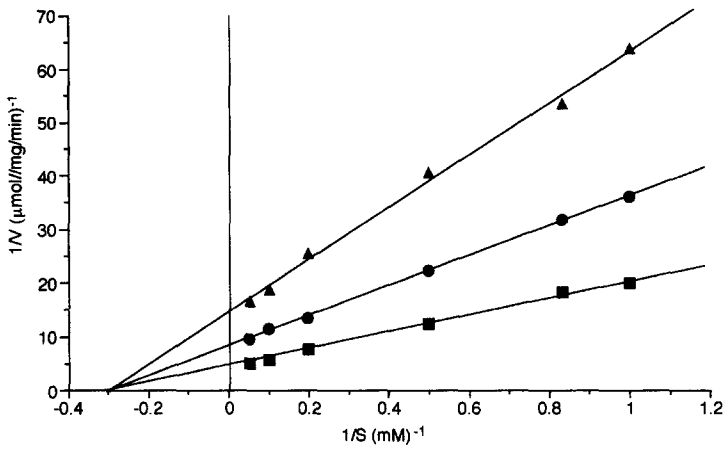

Fig. 5. Lineweaver–Burk plots of calystegin N_1 inhibition of porcine kidney trehalase. Concentrations of calystegin N_1 were 0 (■), 50 (●), and 100 μM (▲).

α-galactosidases in a competitive manner. Interestingly, calystegin B_2 inhibited porcine kidney trehalase in a competitive manner, with a K_i value of 5.3 μM (Fig. 4), whereas calystegin N_1 inhibited this enzyme in a noncompetitive manner, with a K_i value of 62 μM (Fig. 5).

3. Discussion

In a search for plant metabolic mediators in the rhizosphere, Tepfer and co-workers [1,2] discovered polyhydroxy-*nor*-tropane alkaloids, which have been given the trivial names calystegins. These compounds are abundant in the underground organs and root exudates of *Calystegia sepium*, *Convolvulus arvensis* (Convolvulaceae), and *Atropa belladonna* (Solanaceae) [1]. Three (A_3, B_1, and B_2) of the six calystegins in *C. sepium* were structurally characterized by the use of ^1H and ^{13}C NMR spectroscopy and mass spectroscopy [2]. In the course of the purification of nitrogen-in-the-ring sugars from *Morus* sp., we found calystegin B_2 and new calystegin C_1, which is the first naturally occurring pentahydroxy-*nor*-tropane alkaloid [4,5]. The isolation of these calystegins prompted us to carry out a search for new calystegins, and we found five calystegins (A_3, A_5, B_1, B_2, and B_3) from the roots of *Physalis alkekengi* var. *francheti* (Solanaceae) [7].

The known calystegins (A_3, A_5, B_1, B_2, B_3, and C_1) and calystegin A_6 in this report have a bridgehead OH group (or an aminoketal function). This aminoketal system does not exist as an equilibrium mixture of 1-hydroxynortropane and 5-aminocycloheptanone, but exists entirely in bicyclic form. Among a large number of tropane and nortropane alkaloids, calystegins, physoperuvine [8], and 1-hydroxytropacocaine [9] are the only ones bearing a bridgehead OH group. More interestingly, approximately 40% of calystegin N_1 had been converted into calystegin B_2 on storage at 4 °C in a refrigerator

for six months. The biosynthetic pathways of 1-hydroxynortropane and 1-aminonortropane alkaloids are extremely interesting problems.

The replacement of an OH group at the bridgehead in calystegin B_2 by an NH_2 group lowered a potency toward all enzymes tested about ten times. It is noteworthy that calystegin N_1 inhibited porcine kidney trehalase in a noncompetitive manner, whereas calystegin B_2 inhibited this enzyme in the same competitive manner as 1-deoxynojirimycin and castanospermine [10]. In higher animals, the trehalase is distributed at high levels in the brush border membranes of the small intestine [11] and kidney proximal tubule [12]. Intestinal trehalase is most probably involved in the hydrolysis of ingested trehalose, but the physiological role of kidney trehalase remains unresolved. Calystegins B_2 and N_1 will be useful tools to study the mechanism of action and function of this enzyme.

4. Materials and methods

Analytical 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.

^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Jeol JNM-GX 400 spectrometer. Chemical shifts are expressed in ppm downfield from internal sodium 3-(trimethylsilyl)propionate (TSP) in D_2O and internal Me_4Si (TMS) in $CDCl_3$. Mass spectra were measured on Jeol JMS-SX 102A spectrometer.

Materials.—The 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), and trehalase (from porcine kidney), and *p*-nitrophenyl glycoside and disaccharide substrates were purchased from Sigma Chemical Co. Brush border membranes prepared from the rat intestine [13] were used as the source of rat intestinal glycosidases. Epididymal fluid prepared from rat epididymis [14] was used as the source of rat β-*N*-acetylglucosaminidase.

Enzyme assays.—The 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 °C in a total vol of 0.2 mL containing 25 mM of the appropriate disaccharide and the appropriate amount of enzyme. After incubation for 10–30 min, the reaction mixture was stopped by heating at 100 °C 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). The absorbance at 505 nm was measured to determine the amount of the released D-glucose. Other emzyme 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 °C. The reaction was stopped by adding 2 mL of 400 mM Na_2CO_3. The *p*-nitrophenol released was measured at 400 nm.

Kinetics of inhibition.—Enzyme inhibition modes and K_i values for the calystegins were evaluated from the slope of Lineweaver–Burk plots.

Isolation of calystegins.—The dried whole plant (2 kg) of *H. niger* was extracted three times with hot water for 2 h. After cooling, an equiv vol of EtOH was added to this extract. After centrifugation, the supernatant was applied to a column of Amberlite IR-120B (H^+, 1 L) prepared in 50% EtOH. A 0.5 M NH_4OH eluate was concentrated to give a brown oil (18 g) that was chromatographed over Amberlite CG-50 (3.8 × 90 cm, NH_4^+ form) and eluted with water (fraction size 20 mL). The water eluate was divided into four pools, A (fractions 12–32, 2.93 g), B (fractions 36–46, 77 mg), C (fractions 54–71, 288 mg), and D (fractions 72–100, 144 mg). Pool A was rechromatographed on the same column with the same fraction size, and fractions 30–47 were concentrated to give a brown powder (620 mg). This powder was further chromotographed on a Dowex 1-X2 column (1.9 × 92 cm, OH^- form) with water as an eluant (fraction size 20 mL) to give calystegin N_1 (fractions 16–19, 12 mg), calystegin B_2 (fractions 22–36, 243 mg), and calystegin B_1 (fractions 40–45, 63 mg), respectively. Pools B and D were further chromatographed on a column of Dowex 1-X2 column (1.9 × 92 cm, OH^- form) with water as an eluant (fraction size 20 mL) to give calystegin B_3 (fractions 7–11, 7 mg) and calystegin A_3 (fractions 7–12, 108 mg), respectively. Pool C was also chromatographed on a column of Dowex 1-X2 column (1.9 × 92 cm, OH^- form) with water as an eluant (fraction size 10 mL) to give calystegin A_3 (fractions 16–19, 38 mg), calystegin A_5 (fractions 22–30, 42 mg), and calystegin A_6 (fractions 33–35, 6 mg), respectively, in order of elution.

*Calystegin A_6 (1α,2β,7α-trihydroxy-*nor*-tropane).*—Physicochemical data: $[\alpha]_D$ −27.6° (*c* 0.37, H_2O); FABMS *m/z* 160 $[M+H]^+$; ^1H NMR (400 MHz, D_2O): δ 1.277 (m, 1 H, H-3*ax*), 1.50 (m, 1 H, H-4*eq*), 1.56 (m, 1 H, H-4*ax*), 1.866 (dddd, 1 H, $J_{4,6exo}$ 1.5, $J_{5,6exo}$ 7.7, $J_{6exo,7}$ 3.3, $J_{6endo,6exo}$ 14.3 Hz, H-6*exo*), 1.974 (m, 1 H, H-3*eq*), 2.116 (dd, 1 H, $J_{6endo,6exo}$ 14.3, $J_{6endo,7}$ 7.7 Hz, H-6*endo*), 3.450 (m, 1 H, H-5), 3.697 (dd, 1 H, $J_{2,3ax}$ 11.4, $J_{2,3eq}$ 6.2 Hz, H-2), 4.076 (dd, 1 H, $J_{6endo,7}$ 7.7, $J_{6exo,7}$ 3.3 Hz, H-7); ^{13}C NMR (100 MHz, D_2O): δ 28.6 (C-3), 32.5 (C-4), 40.6 (C-6), 53.7 (C-5), 70.2 (C-7), 74.5 (C-2), 93.1 (C-1).

*Calystegin N_1 (1α-amino-2β,3α,4β-trihydroxy-*nor*-tropane).*—Physicochemical data: $[\alpha]_D$ +59.4° (*c* 0.18, H_2O); FABMS *m/z* 175 $[M+H]^+$; ^1H NMR (400 MHz, D_2O): δ 1.342 (m, 1 H, H-7*exo*), 1.732 (m, 1 H, H-6*endo*), 1.850 (m, 1 H, H-6*exo*), 1.910 (m, 1 H, H-7*endo*), 3.232 (dd, 1 H, $J_{2,3}$ 8.5, $J_{2,7exo}$ 1.5 Hz, H-2), 3.263 (dd, 1 H, $J_{4,5}$ 4.0, $J_{5,6exo}$ 7.0 Hz, H-5), 3.281 (t, 1 H, $J_{2,3} = J_{3,4} = 8.5$ Hz, H-3), 3.515 (dd, 1 H, $J_{3,4}$ 8.5, $J_{4,5}$ 4.0 Hz, H-4); ^{13}C NMR (100 MHz, D_2O): δ 25.8 (C-6), 32.9 (C-7), 59.5 (C-5), 77.5 (C-4), 77.7 (C-3), 78.3 (C-1), 80.7 (C-2).

Acetylation of calystegin N_1.—A solution of calystegin N_1 (4 mg) in pyridine (1 mL) and Ac_2O (0.5 mL) was stirred overnight at 4 °C and then evaporated. The residue was chromatographed on a short column of silica gel with 2:1 toluene–acetone as eluant to give a colorless solid (6 mg); FABMS *m/z* 385 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 1.913 (s, 3 H, CH_3CO), 1.93–2.02 (m, 2 H, H-6*endo*, H-6*exo*), 2.022 (s, 3 H, CH_3CO), 2.043 (s, 3 H, CH_3CO), 2.080 (s, 3 H, CH_3CO), 2.264 (s, 3 H, CH_3CO), 2.667 (m, 1 H, H-7*exo*), 2.765 (m, 1 H, H-7*endo*), 4.282 (dd, 1 H, $J_{4,5}$ 4.0, $J_{5,6exo}$ 7.3 Hz, H-5), 4.709 (dd, 1 H, $J_{3,4}$ 8.4, $J_{4,5}$ 4.0 Hz, H-4), 5.266 (dd, 1 H, $J_{2,7exo}$ 1.5, $J_{2,3}$ 8.4 Hz, H-2), 5.329 (t, 1 H, $J_{2,3} = J_{3,4} = 8.4$ Hz, H-3), 8.401 (br s, 1 H, NHCO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.7 ($2 \times CH_3CO$), 20.8 (CH_3CO), 21.1 (C-6), 23.8

(CH_3CO), 24.4 (CH_3CO), 29.5 (C-7), 56.3 (C-5), 70.5 (C-3), 73.6 (C-4), 74.0 (C-2), 78.1 (C-1), 169.7, 169.8, 170.4, 170.8, 172.7 (CH_3CO).

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