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Enzymatic synthesis of the glycosides of calystegines B_1 and B_2 and their glycosidase inhibitory activities

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Abstract

Several glycosides of calystegines B_1 and B_2 were synthesized by use of rice α-glucosidase and the whole cells of *Rhodotorula lactosa*, and their glycosidase inhibitory activities were investigated. Incubation of a mixture of calystegine B_1 and maltose with rice α-glucosidase gave 3-*O*-α-D-glucopyranosylcalystegine B_1 (**2**, 11.3%). An enzymatic β-transglucosylation reaction of calystegines B_1 or B_2 with cellobiose using the whole cells of *R. lactosa* gave 3-*O*-β-D-glucopyranosylcalystegine B_1 (**1**) (0.9%) or 4-*O*-β-D-glucopyranosylcalystegine B_2 (**3**, 11.2%), respectively, while a similar β-transgalactosylation of calystegine B_2 from lactose gave 4-*O*-β-D-galactopyranosylcalystegine B_2 (**4**, 10.1%). The glycosylation of calystegines B_1 and B_2 markedly decreased or abolished their inhibition against β-glucosidase, α- or β-galactosidase. Compound **4** however retained more or less the potency of calystegine B_2 against trehalase. Interestingly, compound **1** was a noncompetitive inhibitor of rice α-glucosidase, with a K_i value of 0.9 ± 0.1 μM.

Keywords: Calystegine; Transglycosylation; Rice α-glucosidase; *Rhodotorula lactosa*; α-Glucosidase inhibition

1. Introduction

Recently, the first glycoside of a polyhydroxylated nortropane alkaloid (calystegine) was isolated from *Nicandra physalodes* Boehm (Solanaceae) fruits and the structure was determined to be 3-*O*-β-D-glucopyranosylcalystegine B_1 (**1**) [1]. Calystegine B_1 is a potent inhibitor of almond β-glucosidase and bovine liver β-galactosidase, with K_i values of 1.8 and 1.6 μM, respectively [2], but the biological activity of **1** has not been reported. We recently found that potatoes, reported to contain calystegines A_3 and B_2 [3], synthesize the 4-*O*-α-D-galactopyranoside (**5**) of calystegine B_2 on cold storage.

The enzymatic synthesis of the α- and β-gluco-

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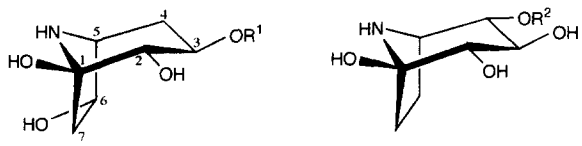

Calystegine B_1 : R^1 = H

1 : R^1 = β-D-glucopyranosyl

2 : R^1 = α-D-glucopyranosyl

Calystegine B_2 : R^2 = H

3 : R^2 = β-D-glucopyranosyl

4 : R^2 = β-D-galactopyranosyl

5 : R^2 = α-D-galactopyranosyl

Scheme 1. Structures of calystegines B_1 and B_2 and their glycosides.

sides of 1-deoxynojirimycin and their glycosidase inhibitory activities were previously reported in our laboratory, and it has been found that 3-*O*-α-D-glucopyranosyl-1-deoxynojirimycin is more effective than 1-deoxynojirimycin against rice α-glucosidase [4]. The enzymatic synthesis of glycosides of calystegines B_1 and B_2 (Scheme 1), their structural determination and glycosidase inhibitory activities are now reported in order to investigate the effect of glycosylation on the inhibition of glycosidases.

2. Results and discussion

Preparation of glycosides and structural determination.—A transglucosylation reaction using commercially available rice α-glucosidase was performed with maltose as D-glucose donor and calystegine B_1 or B_2 as acceptor. The time course of the reaction was monitored by HPTLC on Silica Gel (E. Merck). The glucose transfer to calystegine B_1 was observed and the formation of the product reached a maximum after 8–10 h of incubation, whereas that to calystegine B_2 was not observed. Incubation of a mixture containing maltose (2.5 g), calystegine B_1 (100 mg), and rice α-glucosidase (168 units) at 37 °C for 9 h gave 21.7 mg (11.3%) of a major glucoside and a trace amount of one more glucoside. The structure of the major glucoside isolated from the reaction mixture was determined to be 3-*O*-α-D-glucopyranosylcalystegine B_1 (**2**) on the basis of ^1H and ^{13}C NMR spectroscopy, including ^1H–^{13}C-COSY and heteronuclear multiple bond correlation (HMBC) spectral data. The complete carbon and hydrogen atom connectivity of both the aglycon and glycon was defined. The HMBC spectrum showed a correlation peak between the anomeric proton of the glycon and the aglycon C-3 carbon atom, defining the linkage site. The coupling constant of H-1′ ($J_{1',2'}$ 3.9 Hz) and a 8.5 ppm downfield shift for C-3 in the ^{13}C NMR spectrum compared to the free calystegine were also consistent with a 3-*O*-α-linkage (Table 1).

A β-transglucosylation reaction using the whole cells of *Rhodotorula lactosa* was performed with cellobiose as D-glucose donor and calystegine B_1 or B_2 (65 mg) as acceptor. According to our previous paper [5], the reaction mixtures were incubated at 27 °C for 48 h to regioselectively give the glucoside of calystegine B_1 or B_2 with respective yields of 0.9% and 11.2%. The ^1H- and ^{13}C NMR spectra of the calystegine B_1 β-D-glucoside were completely in accord with those of 3-*O*-β-D-glucopyranosylcalystegine B_1 (**1**) isolated from *N. physalodes* fruits. The calystegine B_2 β-D-glucoside was determined to be 4-*O*-β-D-glucopyranosylcalystegine B_2 (**3**) from the correlation peak between the anomeric proton and the C-4 carbon atom in the HMBC spectrum. This structure was corroborated by a definite NOE effect

Table 1
^{13}C NMR chemical shifts[a] for calystegines B_1 and B_2 and their glycosides

Carbon	Calystegine B_1	1	2	Calystegine B_2	3	4	5
C-1	93.8	93.7	93.6	93.2	93.0	93.1	93.4
C-2	81.3	79.3	80.3	80.4	80.4	80.3	80.4
C-3	72.7	80.3	81.2	77.7	76.7	76.7	76.4
C-4	38.9	36.2	38.2	77.6	87.4	87.2	81.7
C-5	62.9	62.7	62.9	58.6	57.8	57.9	54.8
C-6	76.0	75.7	75.9	24.5	24.9	24.9	24.7
C-7	43.6	43.5	43.6	31.5	31.5	31.5	31.6
C-1′		102.9	102.5		106.3	106.9	98.5
C-2′		75.8	74.4		76.2	73.9	71.2
C-3′		78.4	75.7		78.5	75.5	72.0
C-4′		72.5	72.5		72.5	71.3	72.1
C-5′		78.7	75.0		78.7	77.9	73.6
C-6′		63.6	63.4		63.5	63.7	63.9

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

between H-1′ and H-4, and a 9.8 ppm downfield shift for C-4 in the ^{13}C NMR spectrum.

Although a β-transgalactosylation reaction was performed with lactose as D-galactose donor and calystegine B_1 or B_2 as acceptor in a similar manner, the galactose transfer to calystegine B_1 was not observed. The β-D-galactoside of calystegine B_2 was regioselectively synthesized in a yield of 10.1% and the site of the galactosidic linkage was determined to be at C-4 from the correlation peak in the HMBC spectrum and a 9.6 ppm downfield shift for C-4 in the ^{13}C NMR spectrum. Therefore, the structure of this compound was shown to be 4-*O*-β-D-galactopyranosylcalystegine B_2 (**4**).

Glycosidase inhibitory activities.—The IC_{50} values of the glycosides against various glycosidases are shown in Table 2. Among the calystegines isolated to date, only calystegine B_2, the C-2, C-3, and C-4 OH groups of which are lying in the same region of space as the C-4, C-3, and C-2 OH groups of 1-deoxynojirimycin, is known to be a good inhibitor of rice α-glucosidase, while calystegine B_1 is inactive against this enzyme [2]. The introduction of a glycosyl residue to calystegines B_1 and B_2 resulted in a significant decrease of inhibitory activity against β-glucosidase, α- or β-galactosidase, and β-xylosidase. Since calystegines B_1 and B_2 are competitive inhibitors of these enzymes [2] and can be considered to interact with their glycon binding site, this is to be expected because the glycosyl groups are likely to interfere with this interaction in exoglycosidases, as seen in castanospermine glucosides [6]. Calystegine glycosides might be an inhibitor of some endoglycanases because 4-*O*-β-D-glucopyranosyl-1,6-dideoxynojirimycin is active against some cellulase from the cellulolytic bacterium *Thermomonospora fusca* [7]. Interestingly, the 3-*O*-β-D-glucoside (**1**) of calystegine B_1, but not the 3-*O*-α-D-glucoside (**2**) of calystegine B_1 nor the 4-*O*-β-D-glucoside (**3**) of calystegine B_2, exhibited a potent inhibitory activity against rice α-glucosidase, with an IC_{50} value of 1.9 μM. To determine the K_i value and mode of inhibition of this glucoside, rice α-glucosidase activity was assayed at varying substrate and inhibitor concentrations and the data analyzed by Lineweaver–Burk plots (Fig. 1). The glucoside **1** inhibited this enzyme in a noncompetitive manner, with a K_i value of 0.9 ± 0.1 μM. The 4-*O*-β-D-galactoside (**4**) of calystegine B_2 retained potency against trehalase.

We previously reported the enzymatic synthesis of the 2-*O*-α, 3-*O*-α-, 4-*O*-α-, 2-*O*-β-, and 4-*O*-β-D-glucosides of 1-deoxynojirimycin [4] and the isolation of 3-*O*-β-D-glucopyranosyl-1-deoxynojirimycin from *Morus alba* [8]. 1-Deoxynojirimycin is a powerful inhibitor of rice α-glucosidase, with an IC_{50} value of 50 nM, and the introduction of the α-glucopyranosyl residue to the C-3 position enhances its inhibition towards this enzyme. However, the β-glucosylation of 1-deoxynojirimycin markedly lowers its inhibition. This trend in inhibitory activity is quite

Table 2
Concentration of calystegines and their glycosides giving 50% inhibition of glycosidase activities

Enzyme	IC_{50} (μM)					
	Calystegine B_1	**1**	**2**	Calystegine B_2	**3**	**4**
α-Glucosidase						
Rice	NI[a]	1.9	NI	70	NI	NI
β-Glucosidase						
Almond	4	460	NI	2.6	480	NI
Caldocellum saccharolyticum	1	300	NI	2.4	340	NI
α-Galactosidase						
Green coffee bean	NI	NI	NI	1.9	26	80
Aspergillus niger	NI	NI	NI	3.9	NI	NI
β-Galactosidase						
Bovine liver	9.8	NI	NI	240	880	NI
Trehalase						
Porcine kidney	NI	NI	NI	10	440	34
β-Xylosidase						
A. niger	22	NI	NI	NI	NI	NI

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

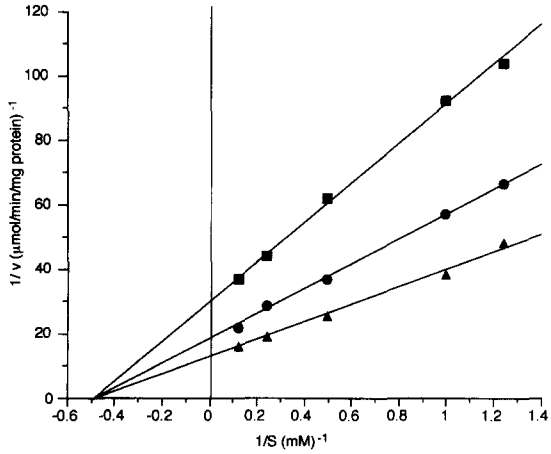

Fig. 1. Lineweaver–Burk plots for 3-*O*-β-D-glucopyranosylcalystegine B_1 (**1**) inhibition of rice α-glucosidase. Increasing concentrations of maltose were used to determine the K_m and K_i values. Concentrations of **1** were 0 μM (▲), 0.5 μM (●), and 1.0 μM (■). The D-glucose released was measured by the D-glucose oxidase–peroxidase method, and the data were plotted as $1/V$ vs. $1/S$. The calculated K_m and K_i values were 2.2 mM and 0.9 ± 0.1 μM, respectively.

different from that shown by the calystegine B_1 glucosides. It would be of value to have more calystegine glucosides in order to understand the contribution of the glucosyl residue in calystegines to glycosidase inhibition.

Calystegines B_1 and B_2 are inhibitors of β-glucosidase of *R. lactosa*, with IC_{50} values of 9.8 and 62 μM, respectively. The potent inhibitory activity of calystegine B_1 may be due to the protonation of the C-6 *exo* OH group, in place of the β-glucoside oxygen of the substrate, by an acidic group within the active site of β-glucosidase [2]. This could explain why such a low yield (0.9%) of the β-glucoside of calystegine B_1 was obtained when the whole cells of *R. lactosa* were used for the glucosylation reaction. The lack of α-glucosyl transfer to calystegine B_2 and β-galactosyl transfer to calystegine B_1 could be due to the inhibition of rice α-glucosidase by calystegine B_2 and yeast β-galactosidase by calystegine B_1, respectively. When the whole cells of *R. lactosa* were used in a transglycosylation reaction, the overall yield of starting material and product was not equal to 100%. This was due to the partial degradation of calystegine B_1 and B_2 by the whole cells because some degradation products were detected by TLC on incubation of calystegines with the cell suspensions.

We recently found that Solanaceae species like *Atropa belladonna* and *Solanum dulcamara* contain glycosides of calystegines A_3 and B_1. The glycoside of calystegine A_3 is under structural determination but the B_1-glycoside has been identified as **1**. Isolation and structural determination of these glycosides will be reported elsewhere.

3. Experimental

Materials.—The enzymes α-glucosidase (EC 3.2.1.20, from rice), β-glucosidases (EC 3.2.1.21, from almonds and *Caldocellum saccharolyticum*: recombinant), α-galactosidases (EC 3.2.1.22, from green coffee beans and *Aspergillus niger*), β-galactosidase (EC 3.2.1.23, from bovine liver), trehalase (EC 3.2.1.28, from porcine kidney), β-xylosidase (EC 3.2.1.37, from *A. niger*), and *p*-nitrophenyl glycoside and disaccharide substrates were purchased from Sigma. Yeast β-glucosidase was partially purified from *R. lactosa* IFO 1424 by the procedure previously reported [9]. Calystegines B_1 and B_2 were isolated from the roots of *Scopolia japonica* according to the literature [10].

Analytical methods.—The purity of samples were checked by HPTLC on 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. ^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.

Enzyme assays.—The activities of rice α-glucosidase (pH 5.0), porcine kidney trehalase (pH 6.5), and β-glucosidase of *R. lactosa* (pH 6.0) were assayed at 37 °C in a total vol of 0.2 mL containing 25 mM maltose, or α,α-trehalose, or cellobiose, respectively, and an appropriate amount of enzyme. After incubation for 10–30 min, the reaction 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). The absorbance at 505 nm was measured to determine the amount of D-glucose released. 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 an appropriate amount of enzyme. The incubations were performed for 30 min at 37 °C. The reaction was stopped by adding Na_2CO_3 (400 mM, 2 mL). The *p*-nitrophenol released was measured at 400 nm. The enzyme inhibition mode and K_i value

of **1** were determined from the slope of Lineweaver–Burk plots.

Transglycosylation reactions.—Enzymatic α-glucosylation of calystegine B_1. A mixture containing calystegine B_1 (100 mg), maltose (2.5 g), 0.05 M acetate buffer (ph 5.0 (25 mL)), and rice α-glucosidase (168 units) was incubated at 37 °C for 9 h. The reaction mixture was applied to a column of Dowex 50W-×2 (30 mL, H^+ form) and eluted with 0.5 M NH_4OH. The eluate was concentrated and chromatographed on a Dowex 1-×2 column (1.2 × 66 cm, OH^- form) with water as eluent (fraction size 10 mL) to give calystegine B_1 (Fractions 19–24, 75.5 mg) and 3-*O*-α-D-glucopyranosylcalystegine B_1 (**2**) (Fractions 35–41, 21.7 mg).

β-Transglucosylation of calystegines B_1 and B_2 using the whole cells. The whole cells of *R. lactosa* were collected according to our previous paper [5]. A mixture containing calystegine B_1 or B_2 (65 mg), cellobiose (6.5 g) 0.05 M phosphate buffer (pH 6.0 (65 mL)), and the whole cells (12 g, wet weight) was incubated at 27 °C for 48 h with shaking. The reaction mixture was centrifuged and the supernatant was applied to a column of Dowex 50W-×2 (30 mL, H^+ form) and eluted with 0.5 M NH_4OH. A concd eluate was chromatographed on a Dowex 1-×2 column (1.5 × 30 cm, OH^- form) with water as eluent (fraction size 7.5 mL) to give calystegine B_1 (Fractions 14–18, 55 mg) and 3-*O*-β-D-glucopyranosylcalystegine B_1 (**1**) (Fractions 30–39, 1.1 mg) or calystegine B_2 (Fractions 11–14, 37.6 mg) and 4-*O*-β-D-glucopyranosylcalystegine B_2 (**3**) (Fractions 25–33, 14 mg).

β-Transgalactosylation of calystegine B_2 using the whole cells. A reaction mixture containing calystegine B_2 (200 mg), lactose (20 g), 0.05 M phosphate buffer (pH 6.0 (180 mL)) and the whole cells (35 g, wet wt) was incubated at 27 °C for 72 h with shaking. The reaction mixture was centrifuged and the supernatant was applied to a column of Dowex 50W-×2 (H^+ form, 50 mL) and eluted with 0.5 M NH_4OH. A concd eluate was chromatographed on a Dowex 1-×2 column (OH^- form, 1.5 × 62 cm) with water as eluent (fraction size 10 mL) to give calystegine B_2 (Fractions 19–25, 134 mg) and 4-*O*-β-D-galactopyranosylcalystegine B_2(**4**) (Fractions 40–50, 39 mg).

3-O-α-D-glucopyranosylcalystegine B_1 (**2**).—$[d]_D$ +77.6° (*c* 0.35, H_2O); ^1H NMR (400 MHz, D_2O): δ 1.422 (ddd, 1 H, $J_{2,7exo}$ 1.7, $J_{6,7exo}$ 2.7, $J_{7endo,7exo}$ 14.4 Hz, H-7 *exo*), 1.621 (ddd, 1 H, $J_{3,4ax}$ 10.8, $J_{4ax,5}$ 3.9, $J_{4ax,4eq}$ 13.4 Hz, H-4 *ax*), 2.155 (ddd, 1 H, $J_{3,4eq}$ 6.4, $J_{4eq,5}$ 2.6 Hz, H-4 *eq*), 2.549 (dd, 1 H, $J_{6,7endo}$ 7.3 Hz, H-7 *endo*), 3.268 (m, 1 H, H-5), 3.396 (dd, 1 H, $J_{3',4'}$ 9.0, $J_{4',5'}$ 9.8 Hz, H-4′), 3.496 (ddd, 1 H, $J_{2,3}$ 8.3 Hz, H-3), 3.527 (dd, 1 H, $J_{1',2'}$ 3.9, $J_{2',3'}$ 9.8 Hz, H-2′), 3.551 (dd, 1 H, H-2), 3.702 (dd, 1 H, H-3′), 3.748 (dd, 1 H, $J_{5',6'a}$ 5.6, $J_{6'a,6'b}$ 12.0 Hz, H-6′a), 3.847 (dd, 1 H, $J_{5',6'b}$ 2.2 Hz, H-6′b), 4.146 (dd, 1 H, H-6), 5.162 (d, 1 H, H-1′); ^{13}C NMR data, see Table 1.

4-O-β-D-glucopyranosylcalystegine B_2 (**3**).—$[\alpha]_D$ +4.6° (*c* 0.39, H_2O); ^1H NMR (400 MHz, D_2O): δ 1.532 (m, 1 H, H-7 *exo*), 1.790 (m, 1 H, H-6 *endo*), 1.954 (m, 1 H, H-6 *exo*), 2.015 (m, 1 H, H-7 *endo*), 3.306 (dd, 1 H, $J_{1',2'}$ 8.1, $J_{2',3'}$ 9.1 Hz, H-2′), 3.378 (dd, 1 H, $J_{3',4'}$ 9.1, $J_{4',5'}$ 9.8 Hz, H-4′), 3.450 (dd, 1 H, $J_{2,7exo}$ 1.7, $J_{2,3}$ 8.3 Hz, H-2), 3.459 (ddd, 1 H, $J_{5',6'a}$ 5.9, $J_{5',6'b}$ 2.5 Hz, H-5′), 3.504 (t, 1 H, H-3′), 3.526 (t, 1 H, $J_{3,4}$ 8.3 Hz, H-3), 3.594 (dd, 1 H, $J_{4,5}$ 3.9, $J_{5,6exo}$ 6.8 Hz, H-5), 3.662 (dd, 1 H, H-4), 3.706 (dd, 1 H, $J_{6'a,6'b}$ 12.5 Hz, H-6′a), 3.904 (dd, 1 H, H-6′b), 4.620 (d, 1 H, H-1′); ^{13}C NMR data, see Table 1.

4-O-β-D-Galactopyranosylcalystegine B_2 (**4**).—$[\alpha]_D$ +29.2° (*c* 0.67, H_2O); ^1H NMR (400 MHz, D_2O): δ 1.536 (m, 1 H, H-7 *exo*), 1.810 (m, 1 H, H-6 *endo*), 1.960 (m, 1 H, H-6 *exo*), 2.024 (m, 1 H, H-7 *endo*), 3.456 (dd, 1 H, $J_{2,7exo}$ 1.8, $J_{2,3}$ 8.4 Hz, H-2), 3.532 (t, 1 H, $J_{3,4}$ 8.4 Hz, H-3), 3.548 (dd, 1 H, $J_{1',2'}$ 7.8, $J_{2',3'}$ 9.8 Hz, H-2′), 3.613 (dd, 1 H, $J_{4,5}$ 4.0, $J_{5,6exo}$ 7.0 Hz, H-5), 3.659 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3′), 3.667 (dd, 1 H, H-4), 3.68 (m, 1 H, H-5′), 3.734 (dd, 1 H, $J_{5',6'a}$ 4.6, $J_{6'a,6'b}$ 11.8 Hz, H-6′a), 3.773 (dd, 1 H, $J_{5',6'b}$ 7.5 Hz, H-6′b), 3.920 (dd, 1 H, $J_{4',5'}$ 1.0 Hz, H-4′), 4,553 (d, 1 H, H-1′); ^{13}C NMR data, see Table 1.

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