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Iridoid Biosynthesis: 7-Deoxyloganetic Acid 1-*O*-Glucosyltransferase in Cultured *Lonicera japonica* Cells.

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Abstract

Iridoid 1-*O*-glucosylation enzyme activities of crude cell-free extracts prepared from loganin-producing plants and nonproducing cultured cells were comparatively examined. Crude cell-free extracts from *Lonicera japonica* cell suspension cultures glucosylated 7-deoxyloganetic acid, 7-deoxyloganetin, and loganetin, but not iridotrial, an intermediate just preceding 7-deoxyloganetic acid. Crude cell-free extracts from *Hydrangea macrophylla* young leaves also glucosylated 7-deoxyloganetin and loganetin, whereas those from cultured cells induced from iridoid-nonproducing plants did not show any iridoid glucosylation activity. The partially purified glucosyltransferase from *L. japonica* cells showed the highest glucosylation activity for loganetin. However, kinetic studies showed that K_m values for 7-deoxyloganetic acid, 7-deoxyloganetin, and loganetin were 106 μM, 561 μM, and 660 μM, respectively, indicating that the enzyme had the highest affinity to 7-deoxyloganetic acid. These data suggest the presence of a pathway for the biosynthesis of loganin, in which 7-deoxyloganetic acid is glucosylated at the 1-*O* position to give 7-deoxyloganic acid, which is further hydroxylated and methylated to produce loganin.

Key words: Biosynthesis, Caprifoliaceae, Cell suspension cultures, 7-Deoxyloganetic acid, Enzyme, Iridoid glucoside, Loganin, *Lonicera japonica*, UDP-glucose: iridoid 1-*O*-glucosyltransferase.

Introduction

Loganin is an iridoid glucoside derived from geraniol, and has 1-*O*-glucosyl, 7-hydroxyl, and 11-carboxymethyl groups on the iridodial skeleton. In the biosynthesis of monoterpenoid indole alkaloids, loganin is converted into secologanin, the final nonnitrogenous intermediate, which in turn is condensed with tryptamine to produce strictosidine, the central intermediate of indole alkaloids. It has been demonstrated that the terpenoid biosynthesis pathway leading to secologanin is the crucial and rate-limiting step in indole alkaloid biosynthesis (Dagnino *et al.*, 1995; Whitmer *et al.*, 1998; Morgan and Shanks, 2000), but the pathway and regulatory mechanism of secologanin biosynthesis remain poorly understood. In our investigation aimed at the effective production of iridoid glucosides and monoterpenoid indole alkaloids in plant cell cultures, we have investigated the production of secologanin in *Lonicera japonica* cell suspension cultures. *L. japonica* plants did not produce any indole alkaloids, but they contained loganin and several secoiridoid glucosides (Kawai *et al.*, 1988; Mehrotra *et al.*, 1988; Tomassini *et al.*, 1995), which would be suitable for simpler analyses of the turnover of the intermediates of loganin. Cultured cells of *L. japonica*, which did not produce any iridoid glucosides, do however have the ability to convert 7-deoxyloganin and loganin into secologanin (Yamamoto *et al.*, 1999). Using these cultured cells, we characterized two cytochrome P-450 monooxygenases, 7-deoxyloganin 7-hydroxylase, which is the enzyme catalyzing the conversion of 7-deoxyloganin to loganin (Katano *et al.*, 2001), and secologanin synthase, which catalyzes the oxidative cleavage of loganin to secologanin (Yamamoto *et*

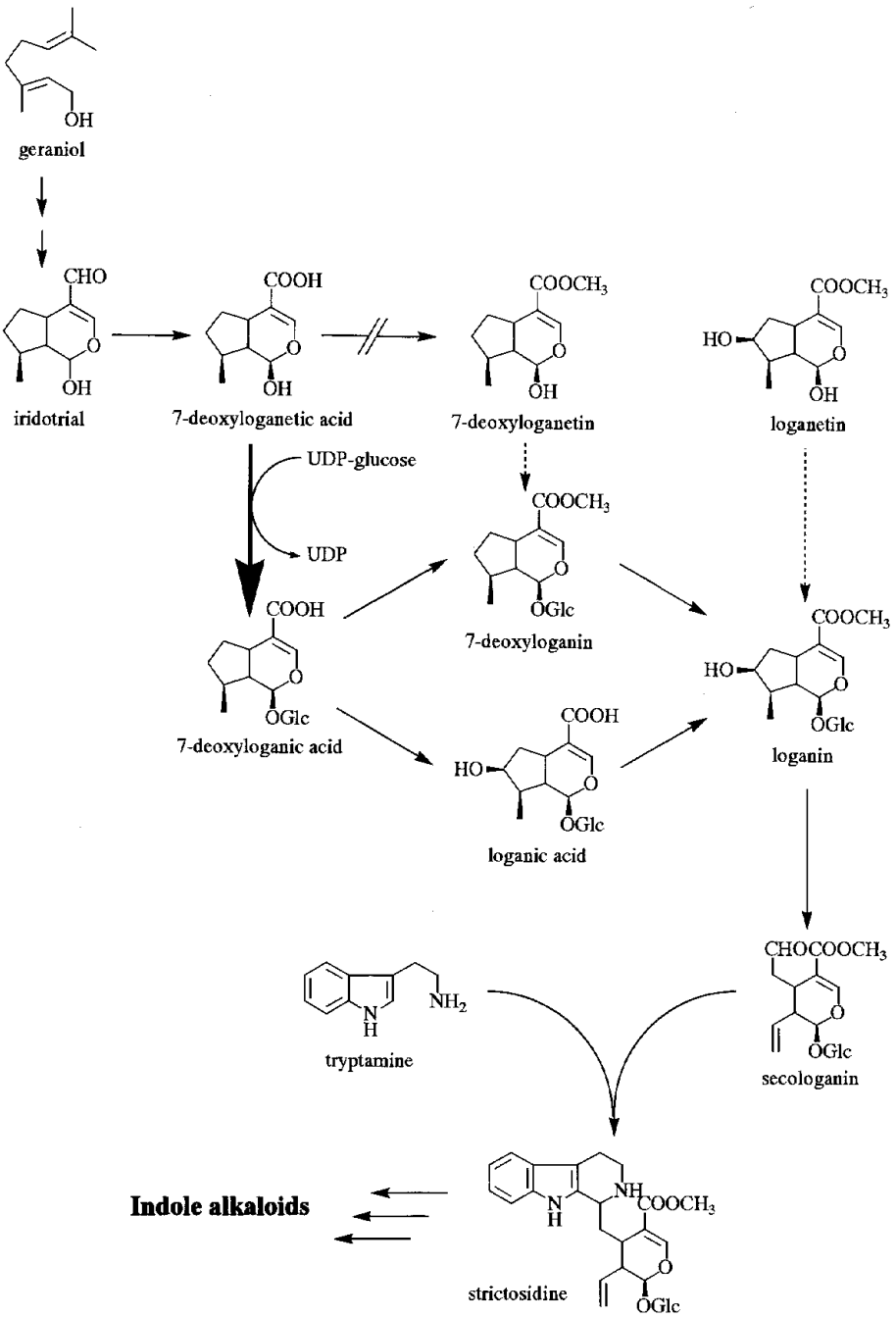

Fig. 1. A plausible pathway for the biosynthesis of loganin.

al., 2000a). Irmer *et al.* (2000) also detected these two enzymes in a microsomal preparation from *Catharanthus roseus* plant.

In the present study, we investigated the iridoid 1-*O*-glucosylating enzyme in *L. japonica* cells for the elucidation of the pathway for the biosynthesis of secologanin (see **Fig. 1**), the crucial intermediate of indole alkaloids, and found that the glucosyltransferase converted 7-deoxyloganetic acid into the corresponding 1-*O*-glucoside (7-deoxyloganic acid) more effectively than 7-deoxyloganetin.

Materials and Methods

Substrates

Loganetin, 7-deoxyloganetin, 7-deoxyloganetic acid, and iridotrial were enzymatically prepared from loganin, 7-deoxyloganin, 7-deoxyloganic acid and iridotrial glucoside, respectively. A 0.5 g aliquot of each glucoside was dissolved in 250 ml of 100 mM sodium citrate buffer (pH 5.0) containing 0.5 g Cellulase Onozuka RS (Yakult Pharmaceutical, Tokyo, Japan), and incubated for 4.5 h at 30 ℃ . After the incubation, the enzyme solution

was applied to Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan) equilibrated with H_2O. Absorbed aglucone was eluted by MeOH, evaporated, and separated by prep TLC (Silica gel 60 F_{254}, Merck, Darmstadt, Germany). $CHCl_3$:MeOH (97:3) was used as a solvent for purification of loganetin, 7-deoxyloganetin and iridotrial, and $CHCl_3$:MeOH: HCOOH (97:3:0.1) was used for 7-deoxyloganetic acid.

Plant materials and culture methods

The origin and subculturing methods of cell suspension cultures of *L. japonica* were as described in Yamamoto *et al.* (1999). Fresh cells (1.5 g) were transferred to 20 ml of Murashige-Skoog liquid medium (Murashige and Skoog, 1962) containing 10 μM naphthaleneacetic acid and 10 μM 6-benzylaminopurine, and agitated on a rotary shaker at a speed of 100 rpm at 23 ℃ in the dark, and subcultured every 2 weeks. Cell culture conditions are described elsewhere, for *Lithospermum erythrorhizon* cell strain M18TOM (Yamamoto *et al.*, 2000b), *Glehnia littoralis* (Kitamura *et al.*, 1998), and *Duboisia myoporoides* and *D. leichhardtii* (Kitamura, 1993).

Young leaves (ca. 1 cm long) of *L. japonica* and *Hydrangea macrophylla* were collected from plants grown in the Botanical Garden of the School of Pharmaceutical Sciences, Nagasaki University.

Enzyme preparation

All enzyme-preparation procedures were carried out at 4 ℃ . Thirty grams of *L. japonica* cells cultured for 7-9 days were ground in a mortar with 30 ml of 100 mM K-Pi buffer (pH 6.5) containing 10 mM dithiothreitol (DTT), 3 g of polyvinylpolypyrrolidone, and sea sand. The homogenate was centrifuged at 12,000 g for 20 min to remove cell debris. Crude cell-free extracts were obtained by passing the supernatant through a PD-10 gel filtration column (Amersham Pharmacia, Sweden) equilibrated with 100 mM Tris-HCl buffer (pH 8.5).

The glucosyltransferase was partially purified by ammonium sulfate precipitation followed by DEAE-Toyopearl column chromatography. For ammonium sulfate precipitation of proteins, solid $(NH_4)_2SO_4$ was added to the centrifuged supernatant up to a salt concentration of 40% saturation under continuous stirring. After centrifugation at 12,000 g for 20 min, $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 50% saturation. The solution was centrifuged again, and the precipitated protein was redissolved in 2.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM DTT, and desalted on a PD-10 column equilibrated with 10 mM Tris-HCl buffer. The desalted protein was applied to a DEAE-Toyopearl 650M column (3 × 20 cm, Tosoh, Tokyo, Japan) equilibrated with 10 mM Tris-HCl (pH 7.5). The proteins were eluted with a linear gradient from 0 to 0.25 M KCl in 10 mM Tris-HCl (pH 7.5) at a flow rate of 0.5 ml/min.

Fractions containing glucosyltransferase activity were precipitated with 50% saturated $(NH_4)_2SO_4$, and the protein pellets collected by centrifugation were desalted on a PD-10 column as above.

Crude cell-free extracts from other plant materials (5 g fresh weight each) were prepared in a same manner, except that 100 mM DTT and 1 g of polyvinylpolypyrrolidone were added to young leaves of *L. japonica* to avoid the oxidation of phenolics during the homogenization. Preparation of the microsomal fraction from *L. japonica* cells was carried out according to the method described in Katano *et al.* (2001).

Protein contents

Protein content was determined according to the method of Bradford (1976).

Enzyme assays

The standard glucosyltransferase assay solution consisted of 60 nmol iridoid aglucone dissolved in 10 μl of EtOH, 200 nmol UDP-glucose, 50 μl of the enzyme solution, and 100 mM Na-Borate buffer (pH 8.5), in a total volume of 200 μl. The reaction was initiated by the addition of iridoid aglucone to the mixture, and after incubation for 10 min at 30 ℃, terminated by the addition of 200 μl of EtOH. The mixture was centrifuged at 8,000 g for 5 min, and the supernatant was analyzed by HPLC. 7-Hydroxylase activity was analyzed using the HPLC assay described in Katano *et al.* (2001). All assays were performed in duplicate.

HPLC analysis

The amount of produced iridoid glucoside was determined by reversed-phase HPLC using a Hikarisil C18 column (5 μm, 4.6 × 250 mm, Asahi Kasei, Tokyo, Japan) in an oven at 40 ℃ , with a CH_3CN/H_2O linear-gradient solvent system containing 0.1% acetic acid and from 10% to 50% CH_3CN in 40 min at a flow rate of 1.0 ml/min, whilst monitoring the absorption at 240 nm. The quantities were calculated from the peak area at 240 nm recorded using a data processor (Chromatopac C-R4A, Shimadzu, Kyoto, Japan).

Identification of the reaction product

Reaction products were identified using an HPLC mass spectrometer (LCMS-2010, Shimadzu). The

HPLC conditions in the LC-MS system were as follows: column, Shim-pack VP-ODS (5 μm, 2.0 × 150 mm, Shimadzu); solvent, CH_3CN/H_2O containing 0.2% acetic acid; linear-gradient solvent system, from 10% to 30% CH_3CN in 30 min at a flow rate of 0.2 ml/min; absorption detection, at 240 nm; oven temperature, 40 ℃ ; negative FAB-MS, loganin: *m/z*; 389 ($[M-H]^-$), 7-deoxyloganic acid: 359 ($[M-H]^-$).

Results and Discussion

Detection of iridoid 1-O-glucosylation activity

Table 1 shows iridoid 1-*O*-glucosylation activities in crude cell-free extracts prepared from several iridoid-producing and nonproducing plant materials in the presence of UDP-glucose. The activities were examined for four conceivable intermediates in loganin biosynthesis; iridotrial, 7-deoxyloganetic acid, 7-deoxyloganetin, and loganetin, and the products were detected by HPLC as the corresponding glucosides. Iridoid 1-*O*-glucosylation activity was found in crude cell-free extracts from *H. macrophylla* young leaves, which are known to accumulate secologanin as the main iridoid glucoside (Inouye *et al.*, 1980), and in those from cell suspension cultures of *L. japonica*, which does not produce any iridoid derivatives but has the ability to convert loganin and 7-deoxyloganin into secologanin (Yamamoto *et al.*, 1999). Glucosylation activities in the extracts from *L. japonica* young leaves were barely detectable owing to the complete decomposition of the substrate during the incubation. The four other materials analyzed - crude cell-free extracts of cell suspension cultures established from iridoid-nonproducing plants - did not show any iridoid glucosylation activities.

It is noteworthy that in the crude cell-free extracts from *L. japonica* cells, the glucosylation activity for loganetin was the highest of those for the four aglucones examined. In contrast, iridotrial, which is known to be incorporated into 7-deoxyloganic acid in *Galium sputium* var. *echinosperum* (Inouye *et al.*, 1978), was barely glucosylated. Relative to that of loganetin, the glucosylation activities for 7-deoxyloganetin, 7-deoxyloganetic acid, and iridotrial were 38%, 15% and 3%, respectively. Similarly, crude cell-free extracts from *H. macrophylla* leaves glucosylated loganetin more strongly (at 40%) than 7-deoxyloganetin. Unfortunately, 7-deoxyloganetic acid and iridotrial glucosylation activities could not be detected in this enzyme source, because it still contained many impurities that interfere with the identification of glucosylated products, even after the removal of low-molecular-weight impurities by PD-10 treatment.

Table 1. Iridoid 1-*O*-glucosyltransfearse activities in cell-free extracts obtained from several iridoid glucoside-producing and nonproducing plant materials.

Plant materials	Activity (pkat/mg protein)			
	Loganetin	7-Deoxyloganetin	7-Deoxyloganetic acid	Iridotrial
Lonicera japonica (cultured cells)	0.71 (100%)[1]	0.26 (38%)	0.11 (15%)	0.02 (3%)
Lonicera japonica (young leaves)	- - -[2]	- - -	- - -	- - -
Hydrangea macrophylla (young leaves)	0.2 (100)	0.08 (40)	N.D.[3]	N.D.
Lithospermum erythrorhizon (cultured cells)	0.00	N.T.[4]	N.T.	N.T.
Glehnia littoralis (cultured cells)	0.00	N.T.	N.T.	N.T.
Duboisia myoporoides (cultured cells)	0.00	N.T.	N.T.	N.T.
Duboisia leichhardtii (cultured cells)	0.00	N.T.	N.T.	N.T.

[1] Relative glucosylation activity compared to loganetin glucosylation activity in each plant material.

[2] Substrate was decomposed.

[3] Not determined, because the product could not be identified.

[4] Not tested.

Biosynthetic route from 7-deoxyloganetic acid to loganin.

Crude cell-free extracts from *L. japonica* cells showed the highest glucosylation activity for loganetin, which was the most improbable intermediate of loganin biosynthesis based on previous results suggesting that 7-deoxyloganetic acid or iridotrial should be glucosylated followed by the oxidation and methylation to produce loganin (see the review by Inouye and Uesato, 1986). Thus, in order to solve this discrepancy, we attempted to characterize the iridoid glucosyltransferase in *Lonicera* cells.

Crude cell-free extracts from *L. japonica* cells were subjected to 40-50% saturated ammonium sulfate fractionation, which gave an enzyme solution with a 2.8-fold increase in specific activity. Purification of the enzyme by DEAE-Toyopearl chromatography produced a single active fraction, whose specific activity was almost the same or somewhat lower than that of the former step. In addition, only 11% of the glucosylation activity in the crude extracts was recovered after the DEAE-Toyopearl chromatography (data not shown). The stability of glucosyltransferase could not be improved by supplementing with 10% glycerol, 10 mM DTT, and 10 mM EDTA. Thus, the DEAE-Toyopearl-separated fraction was used for the characterization of the enzyme.

The iridoid 1-*O*-glucosyltransferase in the DEAE-Toyopearl fraction showed a broad optimum pH range of 8.0-9.0 in Tris-HCl buffer, and the half-maximal activities were observed at pH 6.5 (K-Pi buffer) and pH 9.7 (Borate buffer). Glycine-NaOH buffer strongly inhibited the glucosylation activity. When 7-deoxyloganetic acid or 7-deoxyloganetin were used as a glucosyl acceptor, similar pH-dependency profiles were obtained (data not shown). K_m values for 7-deoxyloganetic acid, 7-deoxyloganetin, and loganetin were 106, 561, and 660 μM, respectively, and their V_{max} values were 0.19, 0.58 and 3.17 pkat/mg protein, respectively (**Fig. 2**).

We also examined whether *L. japonica* cells have the hydroxylation activity of 7-deoxyloganetin to loganetin. However, no 7-deoxyloganetin hydroxylation activity could be detected in the microsomal fraction from *Lonicera* cells, whereas this membrane fraction hydroxylated 7-deoxyloganin - the glucoside of 7-deoxyloganetin - to produce loganin (7.0 pkat/microsomal protein, data not shown), as previously reported (Katano *et al.*, 2001).

The supply of secologanin has been demonstrated to be rate-limiting in indole alkaloid biosynthesis (Dagnino *et al.*, 1995; Whitmer *et al.*, 1998; Morgan and Shanks, 2000), but secologanin biosynthesis has not been investigated extensively. Feeding experiments with radioisotope-labeled precursors have shown that geraniol is metabolized to 7-deoxyloganic acid, *via* 10-hydroxygeraniol, 10-oxogeraniol, iridodial, and iridotrial, successively (see the review by Inouye and Uesato, 1986), although enzymatic studies have only been performed for geraniol 10-hydroxylase (Madyastha *et al.*, 1976) and 10-hydroxygeraniol dehydrogenase (Ikeda *et al.*, 1991). In contrast, the pathway after 7-

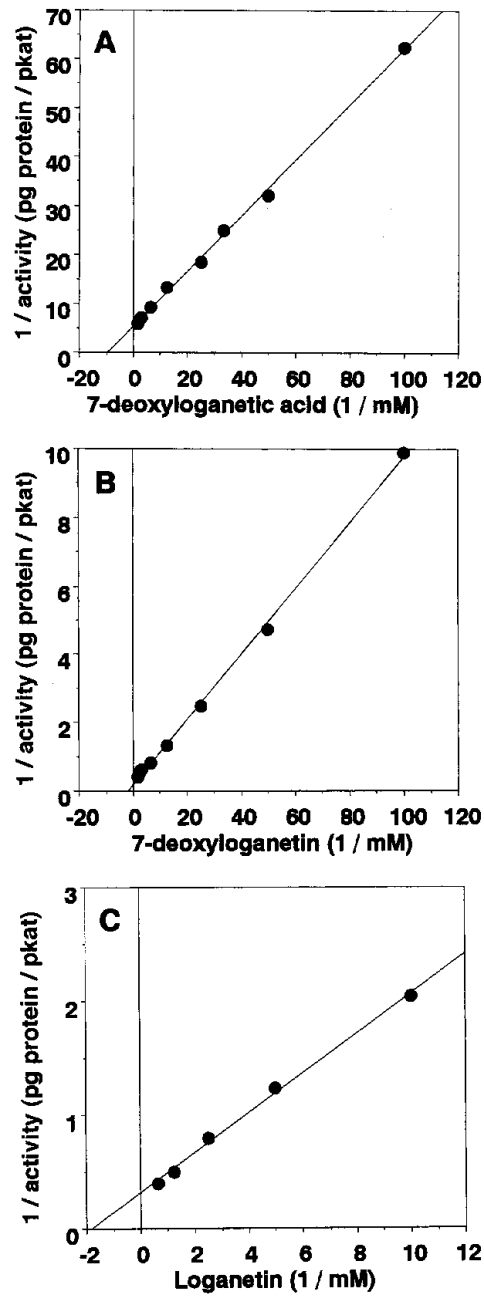

Fig. 2. Lineweaver-Burk plots for the calculation of K_m and V_{max} values for 7-deoxyloganetic acid (A), 7-deoxyloganetin (B), and loganetin (C). DEAE-Toyopearl-purified glucosyltransferase was used.

deoxyloganic acid in secologanin biosynthesis had been studied extensively. Battersby *et al.* (1970) demonstrated that 7-deoxyloganin - the next plausible metabolite of 7-deoxyloganic acid, in terms of its structural features - was incorporated into loganin and the indole alkaloids of *C. roseus*. Our recent results using *L. japonica* cells (Yamamoto *et al.*, 1999; Katano *et al.*, 2001) as well as an enzymatic study using *C. roseus* plants (Irmer *et al.*, 2000) also support that 7-deoxyloganic acid is methylated and hydroxylated, successively, to produce loganin. On the other hand, Uesato *et al.* (1986) used feeding experiments to show that the hydroxylation proceeds at the stage of 7-deoxyloganic acid, and Madyastha *et al.* (1973) have reported that the methyltransferase from *C. roseus* plants converted loganic acid into loganin, but not 7-deoxyloganic acid into 7-deoxyloganin. Thus, the pathway for the biosynthesis of loganin is yet to be established.

The present study demonstrates that crude cell-free extracts from *H. macrophylla* young leaves and from *L. japonica* cultured cells possess iridoid 1-*O*-glucosylation abilities. Furthermore, the partially purified enzyme fraction from *L. japonica* cells glucosylated not only 7-deoxyloganetic acid, a putative glucosyl acceptor suggested by Uesato *et al.* (1986), but also 7-deoxyloganetin and loganetin. Iridotrial, an intermediate preceding 7-deoxyloganetic acid, was not glucosylated. Surprisingly, the enzyme glucosylated loganetin more strongly than 7-deoxyloganetic acid, but kinetic studies showed that the affinity of the enzyme to 7-deoxyloganetic acid was about 6 times higher than that to loganetin. Moreover, the cells themselves did not show any ability to form loganetin. These results strongly suggest that 7-deoxyloganetic acid formed from iridotrial was glucosylated to 7-deoxyloganic acid, which was further metabolized to loganin. For the complete elucidation of secologanin biosynthesis, it is necessary to reinvestigate the iridoid 7-hydroxylation and 11-*O*-methylation steps in detail. The steps in the biosynthesis from 7-deoxyloganic acid to loganin are yet to be clarified, but the present results strongly suggest that the unidentified step(s) in the reaction series leading from geraniol to 7-deoxyloganetic acid is crucial for secologanin biosynthesis.

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