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RADIOIMMUNOASSAY FOR THE DETERMINATION OF LOGANIN AND THE BIOTRANSFORMATION OF LOGANIN TO SECOLOGANIN BY PLANT CELL CULTURES

TAKAO TANAHASHI, NAOTAKA NAGAKURA, HIROYUKI INOUYE* and MEINHART H. ZENK

Lehrstuhl Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-8000 München 2, West Germany; *Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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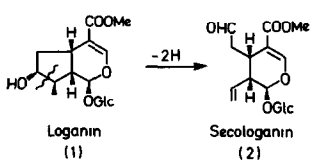
Key Word Index—*Lonicera*; Caprifoliaceae; radioimmunoassay; loganin; secologanin; biotransformation; cell cultures.

Abstract—A radioimmunoassay technique has been developed for the quantitative measurement of loganin in crude extracts from both fresh and dried material of whole plants and cultivated plant cells. The assay makes use of 6′-carboxyloganin which is rendered immunogenic through linkage to bovine serum albumin. The tracer molecule was synthesized via periodate opening of the glucose moiety of loganin and subsequent reduction with sodium borotritide of high specific activity. The rabbit antibodies had a high affinity ($K_a = 1.6 \times 10^9$ l/mol) for loganin and permitted the detection of as little as 0.1 ng per 0.05 ml of sample. The antiserum was highly specific for loganin and its aglycone, with only 10-hydroxyloganin and 7-epi-loganin showing a substantial cross reactivity. A number of cell cultures of the Caprifoliaceae were tested for their ability to transform added loganin to secologanin. By labelled precursor feeding experiments members of the genera *Weigelia*, *Lonicera*, *Hydrangea* and *Symphoricarpus* were found to open the cyclopentane ring of loganin. The time course of the biotransformation of loganin was monitored using radioimmunoassays for both loganin and secologanin and cell cultures of *Lonicera tatarica* as biological material.

INTRODUCTION

The monoterpene glucoside secologanin (**2**) is the key intermediate in the formation of a multitude of indole as well as ipecac alkaloids and represents the prototype of secoiridoids [1]. Loganin (**1**) has been proven to be the immediate precursor of **2** [2, 3]. While the synthesis of a number of indole alkaloids starting from secologanin and tryptamine has now been clarified at the enzymatic level [4, 5], the *in vitro* formation of **1** or **2** is completely unknown [1]. The formation of **2** from **1** involves the oxidative opening of the methylcyclopentane ring by a radical or ionic mechanism. There seems to be no direct parallel for this interesting reaction in organic chemistry. The *in vivo* and *in vitro* transformation of **1** to **2** is therefore of considerable theoretical interest. In order to monitor the formation and metabolism of **1** it was decided to develop a radioimmunoassay for this compound. This assay principle which has been introduced by us into phytochemistry [6, 7] is extremely useful in that it allows the quantitation of a given compound in crude plant extracts and enzyme incubation mixtures with high specificity, precision and possibility for automation. A radioimmunoassay for **1** could be used to survey plants for new sources of **1**, to follow *in vivo* and *in vitro* the metabolism of loganin and to purify the enzymes involved. A general method is needed to link the glucoside through the 6′-position of the sugar moiety to the protein carrier. This procedure should expose the aglycone in such a predictable way that cross reactivities of the antibodies towards chemically related molecules should be minimal.



Loganin (1) Secologanin (2)

RESULTS AND DISCUSSION

General assay parameters

The rabbits immunized with the synthesized BSA-conjugate of 6′-carboxyloganin (Fig. 1) developed anti-loganin antibodies. All of the animals produced satisfactory levels of specific antibodies and titer. The sera were pooled and used for the present study. At a final assay dilution of 1:450 this serum bound 35% of an added 2.7 ng (60 000 dpm, 9000 cpm) of the borotritide reduction product of periodate oxidized loganin (compound **7**, Fig. 1), and the antibody bound tracer was readily displaced by unlabelled loganin. The antibody-antigen reaction was stable over a wide pH-range (pH 6.5–8) and optimal results were obtained with phosphate-buffered physiological saline pH 7.4, as incubation buffer. A selective separation of antibody-bound antigen from free antigen was achieved by precipitation of the immunoglobulin fraction with 49% ammonium sulphate at room temperature. Under the conditions employed, unspecific binding was 0.9–1.1%. The antigen antibody reaction was at equilibrium after a 1 hr incubation, a time period which was subsequently routinely used.

Assay sensitivity is documented by a typical standard curve which is depicted in Fig. 2. The range of the assay

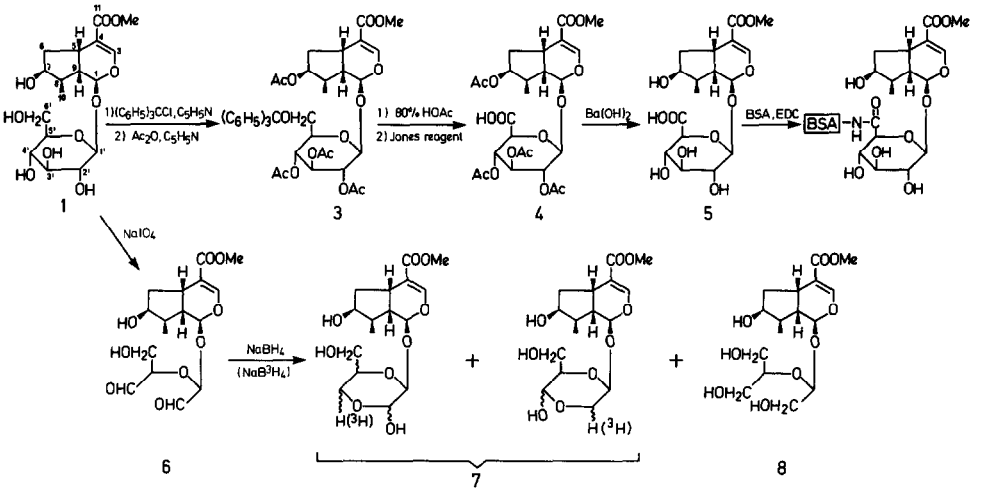


Fig. 1. Synthesis of immunogenic 6'-carboxyloganin–bovine serum albumin conjugate and a tritium labelled loganin derivative of high specific activity.

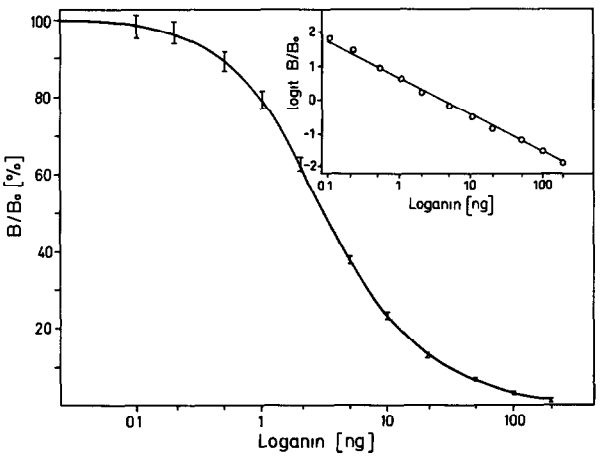


Fig 2 Standard curve for loganin radioimmunoassay showing standard deviations and linearization after logit–transformation (insert)

Table 1. Assay parameters of loganin radioimmunoassay

Maximum affinity constant of serum	1.6×10^9 l/mol
Total number of binding sites in serum	1.4×10^{-7} mol/l
Serum titer (binding 30% of total radioactivity, final dilution)	1:450
Amount of tracer per assay	2.7 ng
Specific radioactivity of tracer	3.51 Ci/mmol
Unspecific binding	0.9–1.1%
Detection limit	0.69 pmol; 0.27 ng
Linear range of logit/log plot	0.5–100 ng
Midrange	8.6 pmol; 3.4 ng
Slope of logit/log plot	−1.1

extends from 0.5 to 100 ng of loganin, well over three orders of magnitude, and the detection limit of the assay at the 99.5% confidence limit is 0.27 ng (0.69 pmol) of loganin. For ease of orientation, the principal assay parameters are summarized in Table 1.

Assay specificity

Since the assay was designed for application to crude plant extracts, checks of assay specificity were carried out using iridoids of potential cross reactivity with the loganin antibody. Cross reactivity was determined as previously[6]. A total of 22 iridoids have been evaluated. Table 2 summarizes the cross reactivity of these components. Substantial cross reactivity (30%) is shown only by loganin aglycone (**11**) which is in agreement with theoretical considerations [6], by 10-hydroxyloganin (**20**) which is not known to be a natural product [1] and by 7-epi-loganin which again has not yet been found in nature. 7-Dehydrologanin (**22**), mussaenoside (**16**) and dihydrocornin (**15**) all of which have been isolated from plant sources only show marginal cross reactivity in the 1–3% range, thus indicating that the 7-hydroxy group of loganin is an essential feature and is fully recognized by the loganin-antibodies. The construction of the conjugate by linking the haptene to the protein carrier at the remote 6' carbon atom of the glucose part proved fully satisfactory and the aglycone moiety is exposed in such a manner that the antibodies directed against the aglycone part can recognize stereochemical alterations, as for instance in 8-epi-loganin (**14**) or minor structural alterations as in deoxyloganin (**12**), gardoside methyl ester (**18**) or 7-dehydrologanin. Most important however, is the discrimination of secologanin (**2**) by a factor of 1000 through the loganin directed antibodies which makes the loganin radioimmunoassay a useful tool for metabolic studies.

Biotransformation of loganin

About 5 years ago, we have established cell cultures of species belonging to four genera of the Caprifoliaceae. Leaf material of all four genera was found to contain secologanin in substantial amounts. The cultures were

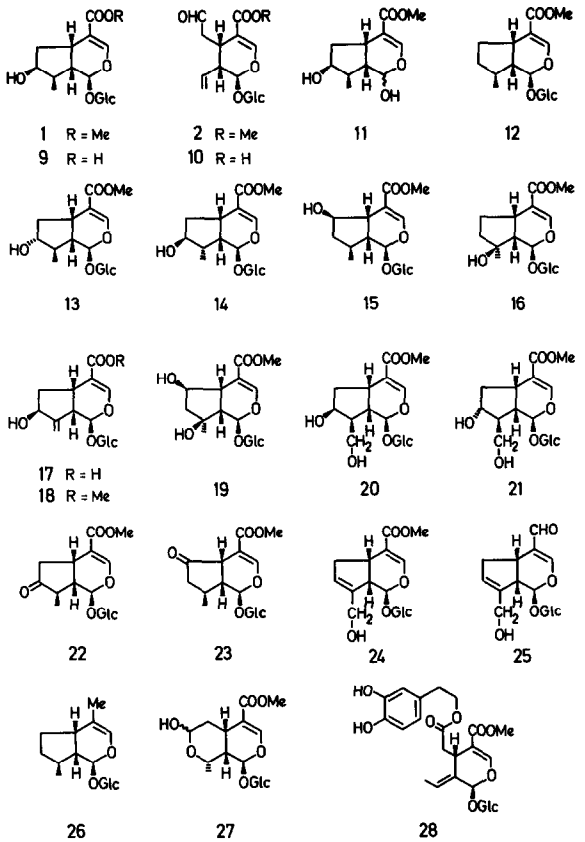


Table 2. Cross-reactions of anti-loganin antiserum on a molar basis

Compound	Cross-reaction (%)
Loganin (1)	100
Secologanin (2)	0.07
Loganic acid (9)	0
Secologanic acid (10)	0.2
Loganin aglycone (11)	30
Deoxyloganin (12)*	0.8
7-Epi-loganin (13)	8
8-Epi-loganin (14)	0.6
Dihydrocornin (15)	1 3
Mussaenoside (16)	1.7
Gardoside (17)	0
Gardoside methyl ester (18)	0.03
Shanzhiside methyl ester (19)	0.05
10-Hydroxyloganin (20)	25
7-Epi-10-hydroxyloganin (21)	0.2
7-Dehydrologanin (22)	3 1
Verbenalin (23)	0
Geniposide (24)	0.12
Tarennoside (25)	0
Iridodial glucoside (26)	0
Morroniside (27)	0.13
Oleuropein (28)	0.12

*Contains 5% of 8-epimer.

grown and maintained on 4X medium [8], a modification of Gamborg's B5 [9]. Cell suspension cultures were allowed to grow for a period of 18 days. Cells were harvested, extracted (80% ethanol) and analysed for their content of either **1** or **2**. Both compounds were analysed by radioimmunoassay. While **1** was measured by the assay described here, secologanin (**2**) was determined by a yet unpublished [Weiler, E. W. and Zenk, M. H., unpublished results] radioimmunoassay procedure which involved the construction of the antigen through the aldehyde function of the secoiridoid onto BSA and subsequent reduction of the Schiff-base thus formed by $NaBH_3CN$. [^{3}H]-Secologanol served as tracer [10]. This radioimmunoassay for **2** showed a 20% cross reactivity towards loganin but in spite of this, proved useful for this study. Application of both extremely sensitive radioimmunoassays directed against **1** and **2** toward the search for these iridoids in Caprifoliaceae cell cultures gave absolutely negative results. There was no indication of the formation of these iridoids by the cell cultures down to a limit of detection of 2×10^{-5}% in spite of the fact that the original whole plants contained secologanin in the > 1% range. In order to test the potential ability of these cultures to carry out the oxidative opening of the methylcyclopentane ring of **1** to yield **2**, the cultures were supplemented with [O-Me-^{3}H]-**1** of varying specific activity. As shown in Table 3 all 11 cell cultures of the four genera had the ability to take up appreciable amounts of the glucoside **1** and also to transform it into **2**. Thus in spite of the fact that these cultures were not able to synthesize *de novo* either compounds **1** or **2**, they still contained the enzyme system necessary to transform **1** to **2**. This conversion is best documented in Fig. 3. In this case, carrier free [O-Me-^{3}H]-**1** was transformed by *Weigelia gigantiflora* cell cultures into **2** in almost quantitative yield. In no case during all these investigations was there any indication of the formation of a stable and separable intermediate between **1** and **2**. *L. tatarica* proved to combine good growth with good transformation characteristics. This culture was therefore selected to study in more detail the conversion of **1** to **2**. Varying the concentration of the precursor in the cell culture medium over a 100-fold range showed that **1** was taken up almost quantitatively by the cells and that **1** was transformed within the cells in a yield between 60 and 70% into **2** regardless of the initial precursor concentration. No secologanin (**2**) was found in the ambient medium which demonstrates that **1** has to be transported into the cells and is subsequently oxidized internally, unlike the *Digitalis* system [11], where digitoxin is hydroxylated to digoxin which accumulates in the medium. The transformation capacity of the *Lonicera* culture is 'considerable' in that per liter of medium out of 300 mg of **1** 200 mg of **2** can be easily formed within a fermentation period of 12 days, the rest being unchanged **1**. Thus, the more readily available **1** (from *Strychnos* seeds) can be transformed into **2** for use in preparative synthesis [12].

The time course of the biotransformation of **1** to **2** catalysed by cultivated *L. tatarica* cells is shown in Fig. 4. It can be seen that **1** is rapidly and, without an appreciable lag period, taken up by the cells. Loganin is virtually absent from the medium after the 14th day of incubation. However, about one third of the amount of **1** taken up by the tissue is found unchanged within the cells. Most probably this amount of **1** escapes transformation by being metabolically inactivated through storage in the

Table 3. Administration of [O-Me-^{3}H]-loganin to various suspension cell cultures

Cell cultures	Expt	Dry wt (g/l)	Uptake (%)	Peak intensities by radio-chromatoscanner (%) secologanin	loganin	Incorporation into secologanin (%)
Weigelia japonica	a	7 9	15.7	28.6	60.3	4.5
	b	9.8	22.1	57.3	18 9	12.7
W middendorfiana	b	4.0	45.2	28.4	46.4	12.8
W florida	a	15 0	3 2	42 2	40.4	1.4
	b	8.8	24.2	53.2	24 0	12.9
W gigantiflora	a	1 0	1 7	22.3	77 7	0 4
Lonicera korolkowii	a	14.2	42.7	33.3	50 9	14 2
	b	3 7	40.1	29.6	22.8	11.9
L morrowii	a	10.9	20.6	37.1	23 8	7.6
	b	4 4	47.9	39 0	19 8	18.7
L. minutiflora	a	14.8	31.4	31.4	46.2	9 9
	b	11.9	51.6	37.9	33.0	19.6
L. glaucescens	a	19.6	23 3	44.1	40.6	10 3
L. tatarica	b	14 2	44 6	55 0	21.5	24 5
Hydrangea macrophylla	a	10.7	30.2	26.0	58 7	7 8
	b	5 0	48.8	17.1	68.8	8.3
Symphoricarpus orbiculatus	a	7.5	55.1	18 4	60.0	10 2

(a) [O-Me-^{3}H]-Loganin (15 μCi) and unlabelled loganin (1 mg) in H_2O (1 ml) were administered to cell cultures (5 ml); (b) [O-Me-^{3}H]-loganin (27 μCi, 55 9 mCi/mmol) was administered to cell cultures (10 ml).

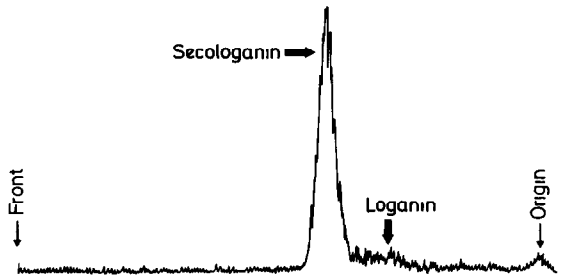


Fig. 3. Scanning record of a TLC chromatogram of the alcoholic extract of *Weigelia gigantiflora* cell cultures supplemented with [O-Me-^{3}H]-loganin.

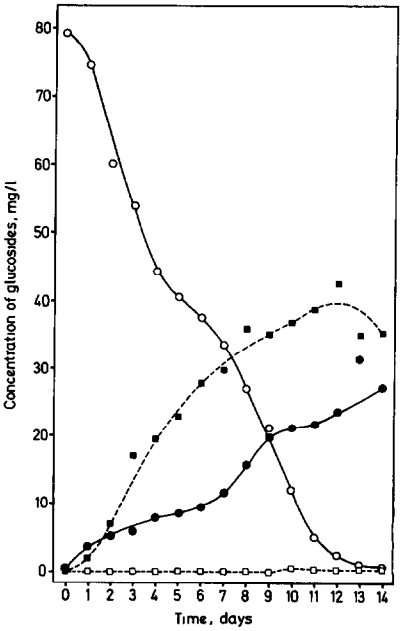


Fig. 4. Time course of uptake and biotransformation of loganin by *L. tatarica* cell cultures. (○) Loss of loganin from medium; (●) loganin accumulation in cells, (■) secologanin formed within cells; (□) secologanin released into medium.

vacuole of the cells. About 50 per cent of added **1** is transformed into the secoiridoid **2**. There is a lag phase in the transformation kinetics which could possibly be interpreted as an induction phase for the enzyme(s) involved in this process. No trace of transformed **2** is released into the growth medium, up to two weeks of incubation. During this period of time, cells increase from 1.5 g dry wt/l (inoculum) to 10 g/l at day 10 of the incubation. Growth under these conditions follows the classic growth curve pattern. The lag period in fresh weight increase is about 2 days followed by a logarithmic phase from day 3 to 12 and, from that time, fresh weight stays constant. Biotransformation of **1** to **2** is independent of the growth phase.

The radioimmunoassay described here has proven to be a valuable tool for the quantitative determination of loganin in biological samples. The Caprifoliaceae tissue cultures should be excellent sources to further investigate the biological transformation of **1** to **2**. It is hoped that a cell-free preparation can be obtained from these tissues, catalysing the opening of the cyclopentane ring of **1**, thus making this intriguing reaction mechanism accessible for further studies. Furthermore the loganin RIA presented here should be valuable in the search for enzymes

Table 4. Biotransformation of loganin to secologanin by cell suspension cultures of *L. tatarica*

Concn of loganin fed (mg/l)	Loganin recovered (%)		Secologanin formed (%)	
	medium	extract	medium	extract
3	0.42	11.20	1.37	77.87
10	0.51	16.64	0 96	73.12
30	1.92	20.16	0.45	62.85
100	4.51	21.47	0.12	57.47
300	8 34	23.14	0 10	66.24

hydroxylating deoxyloganin in position 7 to yield loganin. Caprifoliaceae tissue cultures should also lend themselves as valuable material for studying the biosynthetic transformation of more distant potential precursors at the level of geraniols or iridoids to **2**. By means of such studies the point of repression of secologanin biosynthesis in these cell cultures should also be pinpointed and valuable insight into the mechanism of regulation of a secondary pathway should thus be gained.

EXPERIMENTAL

Mps are uncorr. ^{1}H NMR spectra were measured at 200 MHz. Chemical-ionization mass spectra were run with iso-butane as the reagent gas Silica gel GF_{254} (Merck) and SIL G/UV_{254} plates (Macherey and Nagel) were employed for prep. TLC.

Plant material. All cell cultures were maintained as 70 ml batches in 4X-medium on a gyratory shaker (100 rpm) in continuous light (750 lux) at 24° and transferred to fresh medium at weekly intervals.

Chemicals and immunochemicals. NaB^3H_4 was purchased from Amersham. BSA (100% pure) was obtained from Behringwerke, Marburg, and bovine serum from Mediapharm. Complete Freund's adjuvant was obtained from Difco.

Preparation of 6'-carboxyloganin from loganin. Trityl chloride (1.37 g) was added to a soln of loganin (**1**) (1.00 g) in C_5H_5N (8 ml) and the mixture allowed to stand at room temp. for 4 days. After addition of Ac_2O (6 ml), the mixture was set aside overnight at room temp. The reaction mixture was worked up in the usual way. The resulting residue was chromatographed on silica gel (45 g) and eluted with C_6H_6–EtOAc with an increasing EtOAc content. Elution with C_6H_6–EtOAc (9:1 and 22:3) gave 2',3',4',7-tetra-*O*-acetyl-6'-*O*-trityl-loganin (**3**) (1.70 g), which was crystallized from Me_2CO–Et_2O–*n*-hexane. Mp 200–201°. IR ν_{max}^{KBr} cm^{-1}: 1750, 1725, 1705, 1630, 1440, 695; ^{1}H NMR ($CDCl_3$): δ 1.07 (3H, *d*, J = 6.7 Hz, 10-H), 1.74, 1.93, 2.00, 2.05 (each *s*, 4 × OCOMe), 3.08 (1H, *dd*, J = 4.7 and 10.6 Hz, 6'-H), 3.31 (1H, *dd*, J = 2.1 and 10.6 Hz, 6'-H), 3.58 (1H, *m*, 5'-H), 3.71 (3H, *s*, COOMe), 4.82–5.22 (4H, *m*, 2',3',4',7-H), 5.33 (1H, *d*, J = 2.9 Hz, 1-H), 7.19–7.47 (16H, *m*, 3-H and arom. H).

A soln of 2',3',4',7-tetra-*O*-acetyl-6'-*O*-trityl-loganin (**3**) (565 mg) in 80% HOAc (18 ml) was heated at 80° for 40 min. After cooling, the reaction was diluted with H_2O and extracted with $CHCl_3$ (3 × 50 ml). The $CHCl_3$ layer was washed successively with satd $NaHCO_3$ aq. soln and H_2O, dried and concd *in vacuo*. The crystalline residue was dissolved in Me_2CO (15 ml) and treated with Jones reagent for 1 hr. After addition of *iso*-PrOH the soln was diluted with H_2O and extracted with $CHCl_3$ (3 × 50 ml). The combined $CHCl_3$ layers were concd to 50 ml and extracted with satd $NaHCO_3$ aq. soln (5 × 30 ml). Aq. layers were slightly acidified with 1 N HCl and extracted with $CHCl_3$ (3 × 50 ml). The H_2O washed and dried $CHCl_3$ extract was concd *in vacuo* to give a crystalline residue. This was recrystallized from Me_2CO–Et_2O–*n*-hexane to afford 2',3',4',7-tetra-*O*-acetyl-6'-carboxyloganin (**4**) (327 mg) as colorless needles, mp 220–221°. $[\alpha]_D^{14}$ −75.6° ($CHCl_3$, *c* 0.58); UV λ_{max}^{MeOH} nm (log ε): 233 (4.04); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1730, 1705, 1630; ^{1}H NMR ($CDCl_3$): δ 1.02 (3H, *d*, J = 6.8 Hz, 10-H), 1.93, 2.03, 2.05, 2.06 (each *s*, 4 × OCOMe), 3 01 (1H, *m*, 5-H), 3.70 (3H, *s*, COOMe), 4.15 (1H, *d*, J = 9.7 Hz, 5'-H), 4.93–5.26 (4H, *m*, 1',2',3',4'-H), 5.15 (1H, *br t*, J = 4.5 Hz, 7-H), 5.28 (1H, *d*, J = 3.5 Hz, 1-H), 5.80–6.10 (1H, *m*, COOH), 7.29 (1H, *d*, J = 0.9 Hz, 3-H). (Found: C, 52.23; H, 5.85. Calc. for $C_{25}H_{32}O_{15}$: C, 52.45; H, 5.63%).

2',3',4',7-Tetra-*O*-acetyl-6'-carboxyloganin (**4**) (150 mg) was dissolved in a mixture of MeOH (30 ml), a satd $Ba(OH)_2$ methanolic soln (1.0 ml) and a few drops of H_2O. The whole was stirred at room temp. for 4 hr. After neutralization of the mixture with Amberlite IR-120 (H^+ form), the resin was filtered off and the filtrate was concd to give 6'-carboxyloganin (**5**) (104 mg) as an amorphous powder. IR ν_{max}^{KBr} cm^{-1}. 3420–3280, 1705, 1690, 1625; ^{1}H NMR (D_2O): δ 1.06 (3H, *d*, J = 6.8 Hz, 10-H), 1 68–2.24 (4H, *m*, 6, 8, 9-H), 3.07 (1H, *br q*, J = 7.6 Hz, 5-H), 3.30–3.64 (3H, *m*, 2', 3', 4'-H) 3.73 (3H, *s*, COOMe), 4.00 (1H, *d*, J = 9.7 Hz, 5'-H), 4.14 (1H, *br t*, J = 4.0 Hz, 7-H), 5.36 (1H, *d*, J = 3.5 Hz, 1-H), 7.43 (1H, *br s*, 3-H). This product was over 95% pure and used for the coupling reaction without further purification because of its instability on silica gel.

Linking of 6'-carboxyloganin to BSA. To a soln of 6'-carboxyloganin (**5**) (53 mg) in 50% aq. C_5H_5N (5 ml) was added a soln of *N*-ethyl-*N'*-(3-dimethyl)-aminopropylcarbodiimide hydrochloride (55 mg) in 50% aq. C_5H_5N (2.5 ml) with stirring. After 10 min a soln of BSA (87 mg) in H_2O (5 ml) was added dropwise. The whole was stirred at room temp. for 20 hr under nitrogen. The reaction mixture was dialysed against H_2O for 4 days and finally lyophilized, giving 6'-carboxyloganin–BSA conjugate (75 mg) as a powder.

Treatment of loganin with $NaIO_4$. A stirred soln of loganin (200 mg) in 67% EtOH (15 ml) was treated with 0.4 M aq. $NaIO_4$ soln (5 ml) at room temp. for 2 hr. Ethylene glycol was then added to the soln, and the mixture was stirred for further 30 min. After removal of insoluble material by filtration, the filtrate was concd *in vacuo* and subjected to prep. TLC ($CHCl_3$–MeOH, 9.1), giving **6** (155 mg) as a white powder. IR ν_{max}^{KBr} cm^{-1}· 3370–3430, 1705, 1685, 1630; ^{1}H NMR (D_2O): δ 1.06, 1.11 (altogether 3H, each *d*, J = 7.8 Hz, 10-H), 1.66–2.28 (4H, *m*, 6, 8, 9-H), 3.11 (1H, *br q*, J = 8.2 Hz, 5-H), 3.73 (3H, *s*, COOMe), 4.16 (1H, *m*, 7-H), 4.49 (0.7H, *s*, HC<O–O–), 5 05 (0.7H, *s*, HC<O–O–), 5 16, 5.27 (altogether 1H, each *d*, J = 4.3 and 4.1 Hz respectively, 1-H), 7.46, 7.47, 7.49 (altogether 1H, each *br s*, 3-H); MS *m/z*: 349 $[M + H]^+$ This compound may exist in soln as a mixture of inter- and/or intramolecular hemiacetal and hydrate forms.

$NaBH_4$ *reduction of compound* **6**. To a soln of compound **6** (67 mg) in EtOH (15 ml) was added $NaBH_4$ (2.6 mg) under ice cooling. After stirring for 2 hr, the reaction mixture was concd *in vacuo* and the resulting residue was purified by prep. TLC ($CHCl_3$–MeOH, 9·1). Of the two bands, the more mobile one gave compound **7** (33 mg) as a white powder. The less mobile band afforded compound **8** (10 mg) as a white powder Reduction of compound **6** (50 mg) with excess $NaBH_4$ (5 4 mg) gave only compound **8** (34 mg)

Compound **7**. IR ν_{max}^{KBr} cm^{-1}. 3380, 1700, 1690, 1630; ^1H NMR (CD_3OD): δ 1.13, 1 14, 1.18*, 1.20 (altogether 3H, each *d*, J = 7.0 Hz, 10-H), 1 62 (1H, *ddd*, J = 5.0, 9.0 and 14.0 Hz, 6-H), 1.91 (1H, *m*, 9-H), 2.06 (1H, *dt*, J = 5.0 and 9.0 Hz, 6-H), 2.33 (1H, *m*, 8-H), 3.75, 3.77* (altogether 3H, each *s*, COOMe), 4 12 (1H, *m*, 7-H), 4.75 (0.8H, *s*, $\underline{HC}<^{OH}_{O-}$) 4.95 (0.8H, *s*, $HC<^{O-}_{O-}$), 5.14, 5.19* 5.31, 5.33 (altogether 1H, each *d*, J = 4.5 Hz, 1-H), 7.41, 7.43, 7.45*, 7 46 (altogether 1H, each *d*, J = 1.0 Hz, 3-H), MS *m/z*: 351 $[M+H]^+$. (* represents major signals (*ca* 80%)) This compound exists in soln as a mixture of intramolecular hemiacetal forms.

Compound **8**. ^1H NMR (D_2O)· δ 1.07 (3H, *d*, J = 7.0 Hz, 10-H) 1.68–2 26 (4H, *m*, 6, 8, 9-H), 3 06 (1H, *q*, J = 7.9 Hz, 5-H), 3.60–3.75 (4H, *m*, 2',4',6'-H), 3.73 (3H, *s*, COOMe), 3.86 (1H, *m*, 5'-H), 4.15 (1H, *br t*, J = 4.1 Hz, 7-H), 5 15 (1H, *t*, J = 4.9 Hz, 1'-H), 5.41 (1H, *d*, J = 3 8 Hz, 1-H), 7.45 (1H, *d*, J = 1.1 Hz, 3-H); MS *m/z*: 353 $[M+H]^+$.

Tracer synthesis. To a soln of compound **6** (10 mg) in EtOH (0.3 ml) was added a suspension of NaB^3H_4 (50 mCi) in EtOH (0.3 ml) under ice cooling and the whole was stirred for 2 hr. The reaction mixture was purified by prep. TLC (1 Me_2CO–$CHCl_3$–H_2O, 70:30:5; 2· EtOAc–EtOH, 8·1) to give [^{3}H]-**7** (29.6 mCi, sp. act. 3.51 Ci/mmol).

Immunization and antiserum production The conjugate was administered to rabbits as an 1 : 1 emulsion in complete Freund's adjuvant. After 4 weekly intradermal immunizations, intramuscular booster injections were given monthly and blood was collected 1 and 2 weeks after each booster. After removal of the whole cells by centrifugation, the antiserum was collected and stored at −18°.

Performance of RIA. Triplicate determinations were performed throughout. Diluted sample or standard (0.05 ml) was added to glass tubes. Then, Pi-buffered saline (0.5 ml, 0.01 M phosphate, 0.15 M NaCl, pH 7.4), dilute bovine serum (0.1 ml), dilute tracer (0.1 ml) and H_2O were added, the tubes were mixed again and incubated for 1 hr at room temp. followed by the addition of a freshly prepared $(NH_4)_2SO_4$ soln (1 ml, 10 vol. satd soln plus 1 vol. H_2O) and mixing. The samples were incubated for 1 hr at room temp. and centrifuged The pellets were washed once with 49% $(NH_4)_2SO_4$ soln (1 ml), dissolved in H_2O (0 25 ml) and mixed with scintillation fluid (1 ml). The tubes were counted for radioactivity with punched tape output. Calculations were done on a programmed off-line calculator using the spline approximation method

Conversion of loganin into secologanin by cell suspension cultures of L tatarica *Time course*. Cell suspension cultures of *L. tatarica* (70 ml) were transferred to a fresh 4X-medium (300 ml) containing loganin (29.3 mg, 0.075 mmol) and agitated on a gyratory shaker Duplicate samples (each 3 ml) were withdrawn at daily intervals. Cells and medium were separated by suction filtration. The fresh cells were washed with H_2O and weighed. One sample was used for dry weight determination and another was extracted with 80% EtOH (2 × 5 ml). The extract and medium were analysed for their loganin and secologanin contents by RIA

Capability of conversion. All experiments were conducted in the same way except for the concn of loganin. Cell suspension cultures (2.5 ml) were transferred to a fresh 4X-medium (12.5 ml) containing loganin and agitated. After 10 days, cultures were harvested and analysed for loganin and secologanin content by RIA.

Preparation of [O-Me-^{3}H]-*loganin* [13]. Loganic acid (504 mg) was dissolved in dry DMF (5 ml) and tritiated water (0.15 ml, 750 mCi). After 30 min, dry CH_2N_2–Et_2O was introduced into the soln. The reaction mixture was allowed to stand at room temp for 30 min. After removal of tritiated water with a stream of N_2 gas, the resulting viscous residue was subjected to prep. TLC. The plates were washed with $CHCl_3$ and then developed with Me_2CO–$CHCl_3$–H_2O (80:20·5) to afford [O-Me-^{3}H]-loganin (42.9 mCi, sp act. 55.9 mCi/mmol).

Administration of [O-Me-^{3}H]-*loganin to various suspension cell cultures*. Administration of [O-Me-^{3}H]-loganin was conducted in the following two types of experiments. (a) [O-Me-^{3}H]-loganin (15 μCi) and unlabelled loganin (1 mg) in H_2O (1 ml) were administered to cell cultures in 4X-medium (5 ml). Cell cultures were allowed to grow for 18 days and separated to cells and medium by suction filtration. Cell materials were extracted with boiling 80% EtOH The total activity of the extract was measured by scintillation counter. The activity of loganin and secologanin was determined by radiochromatoscanner. (b) Cell suspension cultures (1 ml) were transferred to a fresh 4X-medium (10 ml) containing [O-Me-^{3}H]-loganin (27 μCi, sp. act. 55.9 mCi/mmol), and agitated (100 rpm) for 14 days. The cultures were worked up in the same way as expt (a).

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