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Studies on Monoterpene Glucosides and Related Natural Products. XLII.[1] On the Possibility of the Intermediacy of 10-Hydroxyloganin in the Biosynthesis of Secologanin[2]

KENICHIRO INOUE, YOSHIO TAKEDA,[3] TAKAO TANAHASHI, and HIROYUKI INOUYE*

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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Administration of various ^3H labeled compounds, deoxyloganic acid (**6**), loganin (**1**), 10-hydroxyloganin (**3**) and 7-epi-10-hydroxyloganin (**5**) to *Lonicera morrowii* and *Adina pilulifera* suggested that, unlike the first two compounds, neither **3** nor **5** was a precursor of secologanin (**2**). This conclusion was supported by dilution analysis of **3** and **5** carried out during the same experiments.

Keywords——iridoid glucosides; biosynthesis; loganin; 10-hydroxyloganin; 7-epi-10-hydroxyloganin; secologanin; *Lonicera morrowii*; *Adina pilulifera*

In the preceding paper,[1] we reported the chemical conversion of geniposide (**4**) into 10-hydroxyloganin (**3**) which was presumed to be an intermediate in the process of the cyclopentane ring cleavage of loganin (**1**) to secologanin (**2**).[4]

This paper presents the results of an examination of the possible intermediacy of 10-hydroxyloganin (**3**) in the biosynthesis of secologanin (**2**).

In the course of studies on the synthesis of 10-hydroxyloganin (**3**), Tietze[5] found that, through a base-catalyzed reaction, the tosylate of 10-hydroxyloganin aglucone methyl ether gave an oxetane compound, whereas the corresponding 7-epi derivative afforded secologanin aglucone methyl ether. The possibility of 7-epi-10-hydroxyloganin (**5**) being a biosynthetic intermediate for **2**, therefore, does not seem to be eliminated. Furthermore, comparative experiments using **3** and **5** should be advantageous for the elucidation of the stereochemical course of the cleavage reaction.

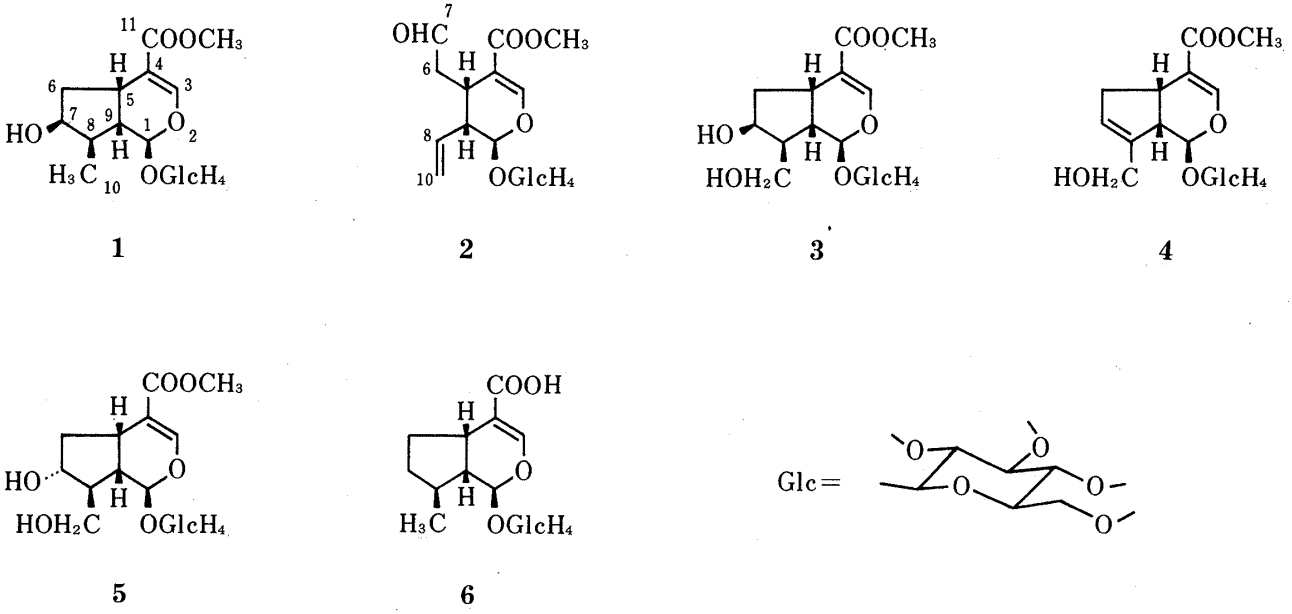


Fig. 1

Accordingly, we first prepared [7-^{3}H]-**3** and **5** and administered them to plants in order to examine their incorporation[6] into secologanin (**2**). Dilution analysis of **3** and **5** was also carried out after administration of ^{3}H labeled deoxyloganic acid (**6**) or loganin (**1**), both of which are precursors of secologanin (**2**).

The 7-dehydro compound (**7**), prepared from geniposide (**4**) through several steps of reaction, was reduced with NaB^2H_4 to [7-^{2}H]-4′,6′-O-benzylidene-2′,3′,10-tri-O-benzyl-7-epi-10-hydroxyloganin ([7-^{2}H]-**8**). The proton nuclear magnetic resonance (^{1}H NMR) spectrum of this compound lacked the signal due to the C-7 proton at δ 4.05 appearing in the corresponding $NaBH_4$ reduction product (**8**),[1] and showed double doublets of the C-6 protons at δ 1.54 and 2.48, which appeared as double triplets in the latter compound. Since the localization of deuterium on the C-7 position of **8** has thus been demonstrated, the 7-dehydro compound (**7**) was reduced with NaB^3H_4 to give [7-^{3}H]-4′,6′-O-benzylidene-2′,3′,10-tri-O-benzyl-7-epi-10-hydroxyloganin ([7-^{3}H]-**8**) in the same way. A part of this labeled compound was subjected to Jones oxidation to regenerate the 7-dehydro compound (**7**) with negligible radioactivity, showing the localization of the ^{3}H label on C-7. Another part of [7-^{3}H]-**8** was therefore subjected to hydrogenolysis over Pd–C to give [7-^{3}H]-7-epi-10-hydroxyloganin ([7-^{3}H]-**5**). The rest of [7-^{3}H]-**8** was converted into the tosylate ([7-^{3}H]-**9**). This was subjected to Walden inversion, affording the acetate ([7-^{3}H]-**10**), followed by Zemplén reaction, to give rise to [7-^{3}H]-4′,6′-O-benzylidene-2′,3′,10-tri-O-benzyl-10-hydroxyloganin ([7-^{3}H]-**11**). This compound was hydrogenated in the same way as above to furnish [7-^{3}H]-10-hydroxyloganin ([7-^{3}H]-**3**).

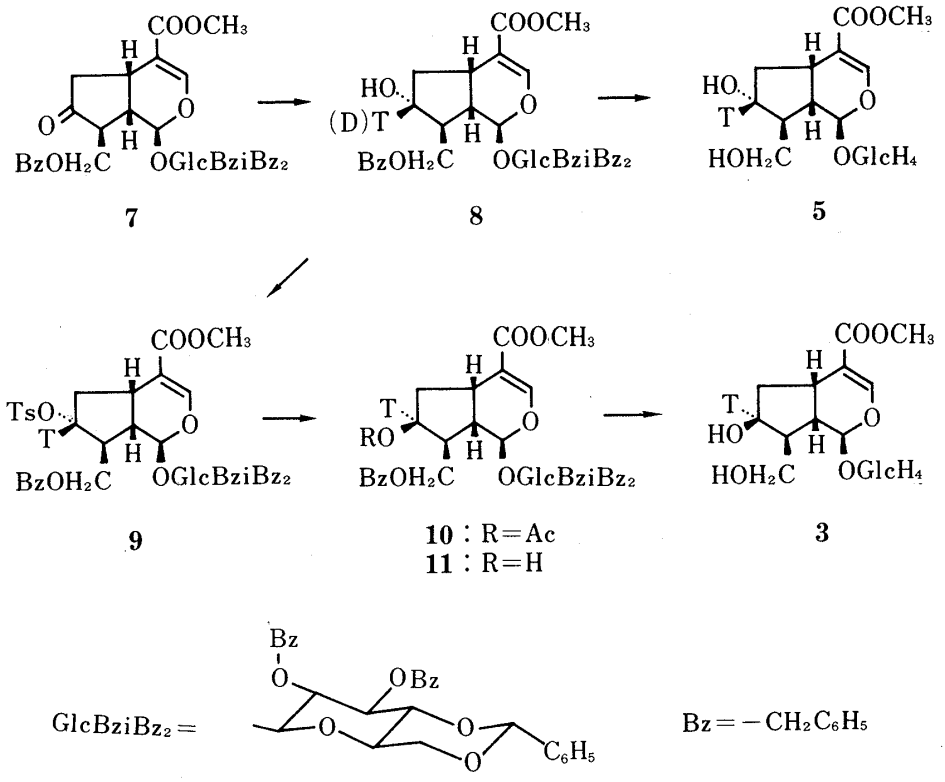
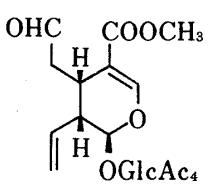


Fig. 2

[7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-**3**) and [7-^{3}H]-7-epi-10-hydroxyloganin ([7-^{3}H]-**5**) thus obtained were fed separately by the hydroponic method to twigs of *Lonicera morrowii* A. Gray which contains secologanin (**2**). After administration for 4 days, isolated secologanin (**2**) was converted into secologanin tetraacetate (**12**). A part of the acetate (**12**) was converted into sweroside tetraacetate (**13**) through treatment with $NaBH_4$, whereas the rest of **12** was transformed into secologanoside methyl ester tetraacetate (**14**) through Jones oxidation followed by

methylation. Both acetates **13** and **14** were purified to constant radioactivity. Table I shows the incorporation ratios of [7-^{3}H]-**3** and **5** into secologanin (**2**) as well as the ratio of the activity of ^{3}H label at C-7 of secologanin (**2**) to the total activity of the molecule which was calculated from the activities of **13** and **14**.



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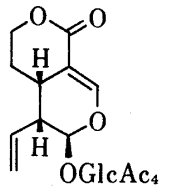

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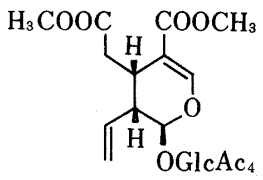

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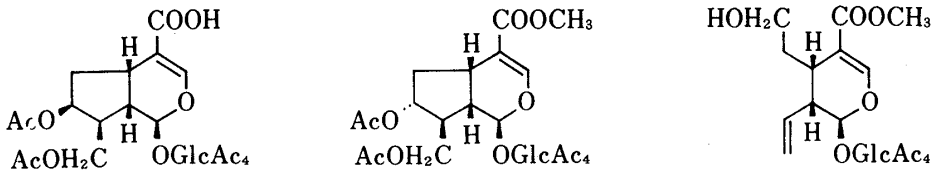
Fig. 3

TABLE I. Experiments on the Administration of [7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-**3**) and [7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-**5**) to *Lonicera morrowii*

Spec. act. (dpm/mmol) and (amount (mg)) of substance fed	Spec. act. (dpm/mmol) and (amount (mg)) of isolated secologanin (**2**)	Incoroporation (%) (Spec. incorp. (%))	Percentage of radioactivity at C-7
[7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-**3**) 6.21×10^{10} (10.2)	8.15×10^5 (289.2)	0.042 (0.00131)	57.7
[7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-**5**) 9.52×10^{10} (6.3)	3.56×10^5 (304.3)	0.019 (0.00037)	65.9

Next, [10-^{3}H]-deoxyloganic acid ([10-^{3}H]-**6**) and [7-^{3}H]-loganin ([7-^{3}H]-**1**)[7] were administered to *L. morrowii*, and dilution analyses of 10-hydroxyloganin (**3**) and 7-epi-10-hydroxyloganin (**5**) were carried out. After separate administration of [10-^{3}H]-**6** and [7-^{3}H]-**1** to the plant, the methanolic extract obtained in each experiment was divided into two halves, which were then mixed with authentic carriers **3** and **5**, and subjected to charcoal chromatography followed by preparative thin-layer chromatography (PLC). Of the reisolated 10-hydroxyloganin (**3**) and 7-epi-10-hydroxyloganin (**5**), the former was converted into 10-hydroxyloganic acid hexaacetate (**15**) through hydrolysis followed by acetylation, whereas the latter was transformed into the hexaacetate (**16**). Both acetates **15** and **16** were recrystallized to constant activity. On the other hand, secologanin (**2**) isolated after administration of [10-^{3}H]-**6** was worked up in the same way as in the administrations of [7-^{3}H]-**3** and **5** to give sweroside tetraacetate (**13**) and secologanoside methyl ester tetraacetate (**14**), whereas secologanin (**2**) isolated after administration of [7-^{3}H]-**1** was converted into the acetate (**12**) and reduced with $NaBH_4$ in dioxane saturated with CO_2 (by means of "dry ice") to give secologanol tetraacetate (**17**) and only a negligible amount of sweroside tetraacetate (**13**). The acetate (**17**) was subjected to Jones oxidation followed by methylation, yielding secologanoside methyl ester tetraacetate (**14**). Table II shows the incorporation ratios of the administered compounds into secologanin (**2**) as well as the ratio of the activity of ^{3}H label at C-7 of secologanin (**2**) to the total activity of the molecule which was calculated from the activities of **14** and **17**.

Analogous feeding experiments were conducted with *Adina pilulifera* (Lam.) Franch. [7-^{3}H]-**3** and **5** were separately administered by the cotton wick method to the twigs of *A. pilulifera*.[8] Secologanin (**2**) isolated in each experiment was acetylated to give secologanin tetraacetate (**12**), which in turn was converted into secologanoside methyl ester tetraacetate (**14**) *via* secologanol tetraacetate (**17**). A feeding experiment of [7-^{3}H]-**1** to the same plant was also carried out in the same way as that of [7-^{3}H]-**1** to *L. morrowii*.



15 **16** **17**

Fig. 4

TABLE II. Experiments on the Administration of [10-^{3}H]-Deoxyloganic Acid ([10-^{3}H]-**6**) and [7-^{3}H]-Loganin ([7-^{3}H]-**1**) to *Lonicera morrowii*

Substance isolated	Substance fed: [10-^{3}H]-Deoxyloganic acid ([10-^{3}H]-**6**)[a]		Substance fed: [7-^{3}H]-Loganin ([7-^{3}H]-**1**)[b]	
	Total act. (dpm) (Amount (mg))	Inc. (%) (Spec. inc. (%))	Total act. (dpm) (Amount (mg))	Inc. (%) Spec. inc. (%)
Secologanin (**2**)	(241.6)		(388.6)	
As sweroside tetraacetate (**13**)	1.33×10^8 [c]	30.4 (1.13)	—	—
As secologanol tetraacetate (**17**)	—	—	3.33×10^8	20.3 (0.84)
As secologanoside methyl ester tetraacetate (**14**)	1.35×10^8 [c]	30.8 (1.15)	$<2.78\times10^6$ [d]	—
10-Hydroxyloganin (**3**)	$<4.43\times10^4$	<0.010	$<1.63\times10^5$	<0.010
7-Epi-10-hydroxyloganin (**5**)	$<6.71\times10^4$	<0.015	1.50×10^4	0.005

a) [10-^{3}H]-**6** (4.37×10^8 dpm, 8.3 mg) and *b*) [7-^{3}H]-**1** (1.64×10^9 dpm, 16.2 mg) were administered.
c) These values are based on the assumption that 1/3 of the tritium labeling of **6** was lost during the ring cleavage.
d) This means that more than 99.2% of the radioactivity was located at C-7 of **2**.

TABLE III. Experiments on the Administration of [7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-**3**) and [7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-**5**) to *Adina pilulifera*

Spec. act. (dpm/mmol) and (amount (mg)) of substance fed	Spec. act. (dpm/mmol) and (amount (mg)) of isolated secologanin (**2**)	Incorporation (%) (Spec. incorp. (%))	Percentage of radioactivity at C-7
[7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-**3**) 2.02×10^{10} (10.6)	2.18×10^4 (342.0)	0.0036 (0.000108)	53.1
[7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-**5**) 2.61×10^{10} (10.7)	1.87×10^4 (274.5)	0.0019 (0.000072)	46.2

TABLE IV. Administration of [7-^{3}H]-Loganin ([7-^{3}H]-**1**) to *Adina pilulifera*

	Total activity (dpm) (Amount (mg))	Incorporation (%) (Spec. incorp. (%))
Substance fed		
[7-^{3}H]-Loganin([7-^{3}H]-**1**)	6.00×10^8 (10.6)	
Substance isolated		
Secologanin (**2**)	(229.0)	
As secologanol tetraacetate (**17**)	1.24×10^8	20.7 (0.95)
as secologanoside methyl ester tetraacetate (**14**)	$<8.20\times10^4$ [a]	—
10-Hydroxyloganin (**3**)	4.08×10^5	0.068
7-Epi-10-hydroxyloganin (**5**)	4.06×10^4	0.007

a) This means that more than 99.9% of the radioactivity was located at C-7 of **2**.

The results of these experiments (Table III, IV) indicate that both [7-^{3}H]-10-hydroxyloganin ([7-^{3}H]-**3**) and [7-^{3}H]-7-epi-10-hydroxyloganin ([7-^{3}H]-**5**) were incorporated into secologanin (**2**), though in very low ratios. In view of the much lower incorporation ratios of **3** and **5** into **2**, however, as compared with those of [10-^{3}H]-deoxyloganic acid ([10-^{3}H]-**6**) and [7-^{3}H]-loganin ([7-^{3}H]-**1**), it seems very improbable that **3** or **5** serves, like **1** and **6**, as an obligatory precursor of **2**. This view was supported by the following findings. i) There was no significant difference between the incorporation ratios of **3** and **5** into **2**. ii) The very low level of activity from [7-^{3}H]-**3** or **5**, which was incorporated into **2**, was distributed not only at C-7 but also at other positions with extensive randomization. iii) The incorporations ($<0.07\%$) of activity of compound **3** and **5** trapped in the dilution analyses were negligible in comparison to the total incorporations (*ca.* 21 and 31%) into secologanin (**2**) which was isolated in the same experiments. If **3** or **5** is an intermediate between loganin (**1**) and secologanin (**2**), the results obtained so far cannot be explained. We thus reached the conclusion that the intermediacy of 10-hydroxyloganin (**3**) in the cleavage reaction of loganin (**1**) to secologanin (**2**) could be excluded. As other possibilities, a mechanism involving the ring cleavage of loganin (**1**) initiated by the abstraction of hydride at the C-10 position, a mechanism involving the ring fission of **1** in a radical fashion and other mechanisms can be considered. Regarding the latter, it was reported[9] that an attempted chemical ring cleavage of loganin aglucone methyl ether and its three C-7 and C-8 stereoisomers failed to give the desired secologanin-type compound, in spite of evidence that the reaction proceeded in a radical fashion.

The above results alone, however, do not rule out the intermediacy of **3** (or **5**). An ambiguity in the present work arises because we did not carry out either administration or dilution analysis of the phosphate of **3** (or **5**). If 10-hydroxyloganin (**3**) is a biosynthetic intermediate, it is very probable that **3** undergoes phosphorylation prior to ring cleavage, as was proposed by Battersby.[4] As a result, the possibility remains that exogenous **3** (or **5**), failing to be phosphorylated, was not introduced into the metabolic process to secologanin (**2**). If so, this might be attributable to the occurrence of the ring cleavage reaction in loganin (**1**) initiated by hydroxylation on a multienzyme complex. Otherwise, the impermeability of the cell membrane to the administered compounds might hinder them from reaching a biosynthetic site. Examination of the precursor-capability of the phosphate of **3** (or **5**), as well as experiments at the enzymatic level, would be necessary to permit a clear-cut conclusion on the ring cleavage mechanism of loganin (**1**).

Experimental

Silica gel 60 F_{254} (Merck) was used for PLC and bands were detected under ultraviolet (UV) irradiation. Activated charcoal for chromatography (Wako) was used for column chromatography. Radioactivity was measured in a Beckmann liquid scintillation counter, model LS-230, with samples dissolved in a solution of 2,5-diphenyloxazole (PPO) (40 mg) and 2,2′-*p*-phenylenebis(5-phenyloxazole) (POPOP) (2 mg) in toluene (10 ml) or in a solution of naphthalene (1 g), PPO (20 mg), and POPOP (5 mg) in dioxane (10 ml). Specific activities are those before dilution. Radioactive chromatograms were monitored with an Aloka thin-layer chromatogram scanner, type JTC-201. Other procedures were as described in the preceding paper.[1]

Plant Materials——Twigs of *Lonicera morrowii* were collected at the Kyoto Botanical Garden and those of *Adina pilulifera* from the Botanical Garden of Osaka City University.

NaB^2H_4 Reduction of 7-Dehydro Compound (**7**)——A solution of **7** (70.9 mg) in dioxane (3 ml) was treated with NaB^2H_4 (9.3 mg), and the whole was stirred at room temperature for 90 min. After dropwise addition of a solution of phenylpropanal (119.3 mg) in dioxane (2 ml) under ice cooling, the whole was stirred for 1 hr. Next, H_2O (20 ml) and AcOH (3 drops) were added and the mixture was extracted with $CHCl_3$ (20 ml × 3). The $CHCl_3$ layer was washed with H_2O (20 ml), dried over $MgSO_4$ and concentrated *in vacuo*. The residue (74.3 mg) was subjected to PLC (benzene–Et_2O, 3:1, 4 developments) yielding crystalline [7-^{2}H]-4′,6′-*O*-benzylidene-2′,3′,10-tri-*O*-benzyl-7-epi-10-hydroxyloganin ([7-^{2}H]-**8**) (67.6 mg), mp 128—129°. NMR ($CDCl_3$) δ: 1.55 (1H, dd, J=13.5 and 7.0 Hz, 6-H), 1.88—2.17 (1H, m, 9-H), 2.03—2.27 (1H, m, OH, disappeared on treatment with D_2O), 2.50 (1H, dd, J=13.5 and 7.5 Hz, 6-H), 2.93 (1H, m, 5-H), 3.63 (3H, s, $COOCH_3$), 4.32 (1H, dd, J=10.0 and 5.0 Hz, 10-H),[10] 4.34 (1H, d, J=4.0 Hz, 1-H), 5.56 (1H, s, -CHPh) and 7.15—7.62

(21H, m, 3-H+arom. H_{20}).

NaB^3H_4 Reduction of 7-Dehydro Compound (7)——A solution of 7 (72.7 mg) in dioxane (3 ml) was treated with NaB^3H_4 (25 mCi, 4.2 mg), and the whole was stirred at room temperature for 45 min. After adding $NaBH_4$ (13.8 mg) and stirring for a further 45 min, a solution of phenylpropanal (241.7 mg) in dioxane (2 ml) was added dropwise to the reaction mixture under ice cooling and the whole was stirred for 1 hr. After the addition of AcOH (5 drops) and H_2O (10 ml), the mixture was extracted with $CHCl_3$ (10 ml×4) and the $CHCl_3$ layer was washed with H_2O, then dried over $MgSO_4$. The solvent was removed *in vacuo* to give rise to an oily residue. This substance was subjected to PLC (benzene–Et_2O, 3: 1, 3 developments) yielding crystalline [7-^{3}H]-4′,6′-O-benzylidene-2′,3′,10-tri-O-benzyl-7-epi-10-hydroxyloganin ([7-^{3}H]-**8**) (55.4 mg), which showed a single radioactive spot identical with that of an authentic sample (**8**) on TLC (benzene–Et_2O, 7: 3). Specific activity: 9.85×10^{10} dpm/mmol.

Jones Oxidation of [7-^{3}H]-4′,6′-O-Benzylidene-2′,3′,10-tri-O-benzyl-7-epi-10-hydroxyloganin ([7-^{3}H]-8)——Jones reagent was added to an ice-cold solution of [7-^{3}H]-**8** (96.0 mg, spec. activity 6.20×10^7 dpm/mmol) in acetone (6 ml) until the yellowish-brown color of the reagent persisted. After stirring for a further 45 min, the reaction was quenched by the addition of MeOH. The reaction mixture was diluted with H_2O (20 ml) and extracted with $CHCl_3$ (15 ml×4). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo* to afford a residue (93.5 mg). PLC (benzene–Et_2O, 4: 1) of the residue gave the 7-dehydro compound (**7**) (90.0 mg), which was recrystallized from Et_2O–pet. ether to a constant activity of 1.32×10^5 dpm/mmol, indicating the loss of more than 99.8% of the radioactivity of [7-^{3}H]-**8** through the oxidation.

Preparation of [7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-5)——A solution of [7-^{3}H]-**8** (12.7 mg, spec. activity 9.85×10^{10} dpm/mmol) in MeOH (8 ml) was stirred with a catalyst prepared from 5% $PdCl_2$–HCl solution (0.2 ml) and activated charcoal (Darco G-60, 50 mg) under a hydrogen atmosphere until the hydrogen uptake ceased. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo* to give [7-^{3}H]-**5** (6.3 mg). On TLC ($CHCl_3$–MeOH, 7: 3), this substance showed a single radioactive spot whose *Rf* value was identical with that of an authentic sample (**5**). Specific. activity: 9.52×10^{10} dpm/mmol.

Preparation of [7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-3)——TsCl (20.7 mg) was added to a solution of [7-^{3}H]-**8** (20.6 mg, spec. activity 9.85×10^{10} dpm/mmol) in pyridine (1 ml), and the mixture was stirred at room temperature for 43 hr. After the addition of ice-water (5 ml), the reaction mixture was extracted with benzene (5 ml×4). The benzene layer was washed successively with 1 N HCl, satd. aq. $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated *in vacuo*. The resulting tosylate ([7-^{3}H]-**9**), without further purification, was dissolved in dry acetone (2 ml) and a solution of Et_4NOAc (215.5 mg) in dry acetone (2 ml) was added. The mixture was refluxed for 24 hr. After further addition of a solution of Et_4NOAc (167.5 mg) in dry acetone (3 ml), the mixture was refluxed for a further 23 hr. The solvent was removed *in vacuo* and the residue was extracted with benzene (5 ml×4), washed successively with brine and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to PLC (benzene–Et_2O, 9: 1, 2 developments) giving rise to a colorless syrup (15.1 mg), which was identical with an authentic sample of acetate (**10**) on TLC (benzene–Et_2O, 4: 1). A solution of the acetate ([7-^{3}H]-**10**) (15.1 mg) and the carrier (8.0 mg) in anhyd. MeOH (1.5 ml) was treated with methanolic NaOMe (0.1 N, 0.12 ml), and the mixture was refluxed for 30 min. After the addition of H_2O (5 ml) and 1 N HCl (3 drops), the mixture was extracted with benzene (5 ml×4). The benzene layer was washed successively with satd. aq. $NaHCO_3$ and H_2O, then dried over $MgSO_4$. The solvent was removed *in vacuo* to give [7-^{3}H]-**11** (20.2 mg), which was dissolved in MeOH (8 ml) and hydrogenated over a catalyst prepared from 5% $PdCl_2$–HCl solution (0.2 ml) and activated charcoal (Darco G-60, 50 mg) until the hydrogen uptake ceased. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to give [7-^{3}H]-10-hydroxyloganin ([7-^{3}H]-**3**) as a colorless syrup (10.2 mg). On TLC ($CHCl_3$–MeOH, 7: 3), this substance showed a single radioactive spot whose *Rf* value was identical with that of an authentic sample of **3**. Specific activity: 6.21×10^{10} dpm/mmol.

Administration of [7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-5) to *Lonicera morrowii* and Isolation of Secologanin (2)——A solution of [7-^{3}H]-**5** (6.3 mg, spec. activity 9.52×10^{10} dpm/mmol) in H_2O (1 ml) was administered hydroponically to three twigs (20 cm in length) of *L. morrowii* in August. After 4 days, the plants (12.7 g) were cut into pieces and extracted with MeOH (200 ml×4) under reflux. After concentration of the MeOH extract *in vacuo*, the residue was dissolved in H_2O (30 ml) and insoluble materials were removed by filtration through a Celite layer. The Celite layer was washed with H_2O (total 50 ml). The filtrate and the washings were combined and concentrated *in vacuo* to give a residue (1.48 g), which was subjected to column chromatography on activated charcoal (5 g). The column was eluted successively with H_2O (350 ml), H_2O–MeOH 9: 1 (200 ml), 4: 1 (400 ml), 7: 3 (200 ml) and 1: 1 (400 ml) and MeOH (100 ml). On PLC ($CHCl_3$–MeOH, 7: 3, 2 developments), the combined residues (516.8 mg) of the fractions eluted with H_2O–MeOH (1: 1) and MeOH gave secologanin (**2**) (304.3 mg). An aliquot (203.5 mg) of this substance (**2**) was dissolved in 1.5 ml each of pyridine and Ac_2O and left overnight. After the addition of ice-water, the reaction mixture was extracted with $CHCl_3$ (8 ml×4). The $CHCl_3$ layer was washed successively with 1 N HCl, satd. aq. $NaHCO_3$, and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. PLC (Et_2O, 2 developments) of the residue afforded radioactive secologanin tetraacetate (**12**) (177.5 mg) as a colorless syrup. $NaBH_4$ (110.0 mg) and H_2O (3 drops) were added to a solution of an aliquot (112.2 mg) of the above radioactive **12** in dioxane (7 ml) and the whole was stirred at room temperature for 1.5 hr. The reaction was quenched with AcOH.

After the addition of H_2O, the reaction mixture was extracted with $CHCl_3$ (20 ml × 3). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to PLC (Et_2O, 2 developments), yielding a crystalline substance (40.9 mg) which was identical with an authentic sample of sweroside tetraacetate (**13**) on TLC (benzene–Et_2O, 1 : 1). This substance was diluted with carrier (40.2 mg) and recrystallized from EtOH to constant activity. Specific activity: 3.56×10^5 dpm/mmol. Jones reagent was added dropwise to a stirred solution of another aliquot (51.0 mg) of radioactive **12** in acetone (3.5 ml) under ice cooling until the yellowish brown color of the reagent persisted. Stirring was continued for a further 45 min. After decomposition of the excess reagent by adding MeOH, the solution was diluted with H_2O and extracted with $CHCl_3$ (8 ml × 4). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue (49.9 mg) was subjected to PLC (Et_2O) giving rise to an acidic substance (27.3 mg), which was methylated in the usual way with CH_2N_2–Et_2O. PLC of the reaction product (27.5 mg) yielded a crystalline substance (23.0 mg), which was identical with an authentic sample of secologanoside methyl ester tetraacetate (**14**) on TLC (Et_2O). This substance was diluted with carrier (38.6 mg) and recrystallized from EtOH to constant activity. Specific activity: 1.21×10^5 dpm/mmol.

Administration of [7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-3) to *L. morrowii* and Isolation of Secologanin (2)——A solution of [7-^{3}H]-3 (10.2 mg, spec. activity 6.21×10^{10} dpm/mmol) in H_2O (1 ml) was administered hydroponically to three twigs of *L. morrowii* (20 cm in length) in August. After 4 days, the plants (13.8 g) were worked up as in the case of the administration of [7-^{3}H]-**5** to give secologanin (**2**) (289.2 mg). A part (211.2 mg) of this substance was acetylated with pyridine and Ac_2O, yielding secologanin tetraacetate (**12**) (235.6 mg). An aliquot (153.8 mg) of this substance was reduced with $NaBH_4$ in the same way as above to yield sweroside tetraacetate (**13**), which was diluted with carrier (46.4 mg) and recrystallized from EtOH to constant activity. Specific activity: 8.15×10^5 dpm/mmol. Another aliquot (65.7 mg) of the radioactive **12** was subjected to oxidation followed by methylation as described above, yielding secologanoside methyl ester tetraacetate (**14**) (29.4 mg), which was diluted with carrier (30.1 mg) and recrystallized from EtOH to constant activity. Specific activity: 3.45×10^5 dpm/mmol.

Administration of [10-^{3}H]-Deoxyloganic Acid ([10-^{3}H]-6) to *L. morrowii*, Dilution Analysis of 10-Hydroxyloganin (3) and 7-Epi-10-hydroxyloganin (5) and Isolation of Secologanin (2)——A solution of [10-^{3}H]-**6** (8.3 mg, spec. activity 1.89×10^{10} dpm/mmol) in H_2O (1 ml) was administered hydroponically to three twigs (20 cm in length) of *L. morrowii* in August. After 4 days, the plants (10.9 g) were cut into pieces and extracted with MeOH (200 ml × 4) under reflux. The combined MeOH extracts were concentrated *in vacuo*. The residue was dissolved in MeOH–H_2O (2 : 1, 100 ml) and the solution was divided into two halves (designated as A and B).

A: After the addition of carrier **3** (30.4 mg), the solution A was concentrated *in vacuo*. The residue (0.68 g) was dissolved in H_2O (50 ml), and filtered through a Celite layer. The layer was washed with H_2O (10 ml × 3). The filtrate and the washings were combined and concentrated *in vacuo* to *ca.* 10 ml. The concentrated solution was subjected to column chromatography on activated charcoal (3.5 g). The column was eluted successively with H_2O (400 ml), H_2O–MeOH 95 : 5 (400 ml), 9 : 1 (400 ml), 8 : 2 (300 ml), 7 : 3 (200 ml), 6 : 4 (500 ml), 5 : 5 (100 ml) and MeOH (150 ml). The residue of the fraction eluted with H_2O–MeOH (6 : 4) was then subjected to PLC ($CHCl_3$–MeOH, 7 : 3). Bands around *Rf* 0.53 and 0.34 afforded secologanin (**2**) (63.7 mg) and 10-hydroxyloganin (**3**) (17.6 mg), respectively. The residue (122.5 mg) of the combined fractions eluted with H_2O–MeOH (5 : 5) and MeOH also gave secologanin (**2**) (57.9 mg) on PLC. A solution of **3** (17.6 mg) in 0.5 N NaOH (0.5 ml) was stirred at room temperature for 70 min and neutralized with Amberlite IR-120 (H^+-form). The resin was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was acetylated with pyridine and Ac_2O in the usual way, yielding 10-hydroxyloganic acid hexaacetate (**15**) (26.5 mg), which was diluted with carrier (57.3 mg) and repeatedly recrystallized from Et_2O. Total activity: less than 4.43×10^4 dpm. Substance **2** (121.6 mg) was acetylated to the tetraacetate (**12**) (95.9 mg). An aliquot (55.5 mg) of this substance was subjected to $NaBH_4$ reduction to yield sweroside tetraacetate (**13**) (14.3 mg), which was diluted with carrier (67.7 mg) and recrystallized from EtOH to constant activity. The rest of **12** (40.4 mg) was subjected to Jones oxidation followed by methylation yielding secologanoside methyl ester tetraacetate (**14**) (26.2 mg), which was diluted with carrier (73.7 mg) and recrystallized from EtOH to constant activity. Total activity: **13**, 1.33×10^8 dpm; **14**, 1.35×10^8 dpm. Specific activity: **13**, 2.14×10^8 dpm/mmol; **14**, 2.17×10^8 dpm/mmol.

B: After the addition of 7-epi-10-hydroxyloganin (**5**) (30.0 mg), solution B was concentrated *in vacuo* and the residue (0.66 g) was dissolved in H_2O (50 ml). The solution was filtered through a Celite layer and the layer was washed with H_2O. The filtrate and the washings were combined and concentrated *in vacuo* to *ca.* 10 ml. The concentrated solution was applied to an activated charcoal column (3.5 g) and eluted successively with H_2O (400 ml) and H_2O–MeOH 95 : 5 (400 ml), 9 : 1 (400 ml), 8 : 2 (300 ml), 7 : 3 (200 ml) and 6 : 4 (400 ml). The fraction eluted with H_2O–MeOH (6 : 4) was concentrated and the residue was subjected to PLC ($CHCl_3$–MeOH, 7 : 3). A band around *Rf* 0.25 gave 7-epi-10-hydroxyloganin (**5**) (16.0 mg), which was acetylated with pyridine and Ac_2O. The resulting 7-epi-10-hydroxyloganin hexaacetate (**16**) (24.7 mg) was diluted with carrier (113.9 mg) and recrystallized repeatedly from EtOH. Total activity: less than 6.71×10^4 dpm.

$NaBH_4$ Reduction of Secologanin Tetraacetate (12) to Secologanol Tetraacetate——Water (a few drops)

and small pieces of dry ice were added to a stirred solution of secologanin tetraacetate (**12**) (76.2 mg) in dioxane (5 ml). After disappearance of the dry ice, $NaBH_4$ (26.4 mg) was added to the reaction mixture and the whole was stirred at room temperature for a further 2 hr. After decomposition of the excess reagent with AcOH, the reaction mixture was diluted with H_2O (20 ml) and extracted with $CHCl_3$ (20 ml×3). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. PLC (Et_2O, *Rf* 0.21) of the residue yielded secologanol tetraacetate (**17**) (70.3 mg) which was repeatedly recrystallized from acetone–Et_2O–pet. ether giving rise to colorless needles (54.0 mg), mp 124—125°, $[\alpha]_D^{20}$ −94.4° (c=0.53, $CHCl_3$), IR ν_{max}^{KBr} cm^{-1}: 3480, 1745, 1710, 1700, 1695 and 1630. UV λ_{max}^{EtOH} nm (log ε): 231 (4.02). ^1H NMR ($CDCl_3$) δ: 1.96, 1.98, 2.00, 2.07 (each s, 4×$OCOCH_3$), 2.35 (1H, br s, disappeared on treatment with D_2O), 3.58 (2H, t, J=7.0 Hz, 7-H), 3.71 (3H, s, $COOCH_3$) and 7.39 (1H, s, 3-H). *Anal.* Calcd for $C_{25}H_{34}O_{14}$: C, 53.76; H, 6.14. Found: C, 53.63; H, 6.05.

Jones Oxidation of Secologanol Tetraacetate (17) and Methylation of the Oxidation Product——Jones reagent was added to an ice-cold solution of secologanol tetraacetate (**17**) (25.2 mg) in acetone (3 ml). After stirring for 1 hr, the reaction was quenched with MeOH and H_2O was added to the solution, which was then extracted with $CHCl_3$ (15 ml×3). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. An ethereal solution of the residue was treated with CH_2N_2–Et_2O under ice cooling. After standing for 10 min, the solution was concentrated *in vacuo*. The residue (30.3 mg) was subjected to PLC (benzene–Et_2O, 1: 1) yielding a crystalline substance (25.6 mg, *Rf* 0.35), which was recrystallized from Et_2O–pet. ether to give colorless needles. This substance was identical with an authentic sample of secologanoside methyl ester tetraacetate (**14**) on the basis of mixed mp, and IR and ^1H NMR spectra.

Administration of [7-^{3}H]-Loganin ([7-^{3}H]-1) to *L. morrowii*, Dilution Analysis of 10-Hydroxyloganin (3) and 7-Epi-10-hydroxyloganin (5) and Isolation of Secologanin (2)——A solution of [7-^{3}H]-**1** (16.2 mg, spec. activity 3.96×10^{10} dpm/mmol) in H_2O (2 ml) was administered hydroponically to five twigs of *L. morrowii* (*ca.* 20 cm in length) in September. After 4 days, the plants (15.5 g) were extracted and worked up in the same way as in the case of the administration of [10-^{3}H]-**6** and the resulting solution of the extract was divided into two halves, A and B.

A: After the addition of 10-hydroxyloganin (**3**) (51.2 mg), the solution A was concentrated *in vacuo*. The residue (1.43 g) was worked up in the usual way to give **3** (22.0 mg) and **2** (208.9 mg). Substance **3** (22.0 mg) was subjected to alkaline hydrolysis followed by acetylation. PLC (Et_2O) of the product yielded 10-hydroxyloganic acid hexaacetate (**15**) (25.5 mg), which was diluted with carrier (26.6 mg) and recrystallized repeatedly from Et_2O. Total activity: less than 1.63×10^5 dpm. Conventional acetylation of **2** (208.9 mg) with pyridine and Ac_2O afforded secologanin tetraacetate (**12**) (198.8 mg). A stirred solution of an aliquot (114.8 mg) of this substance in dioxane (8 ml) supplemented with H_2O (a few drops) was saturated with CO_2 by the addition of dry ice (a few pieces). $NaBH_4$ (60.7 mg) was then added to the mixture and the whole was stirred at room temperature for 3 hr. After decomposition of the excess reagent with AcOH, the reaction mixture was diluted with H_2O (20 ml) and extracted with $CHCl_3$ (20 ml×4). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue (116.2 mg) was subjected to PLC (Et_2O) yielding secologanol tetraacetate (**17**) (87.1 mg), which was recrystallized from acetone–Et_2O–pet. ether to constant activity. Total activity: 3.33×10^8 dpm. Specific activity: 3.33×10^8 dpm/mmol. Jones reagent was added to an ice-cold solution of **17** (79.2 mg) in acetone (8 ml), and the whole was stirred for 1 hr. After quenching of the reaction with MeOH, the reaction mixture was diluted with H_2O and extracted with $CHCl_3$ (10 ml×4). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was dissolved in Et_2O and methylated with CH_2N_2–Et_2O. PLC (Et_2O) of the reaction product (75.4 mg) afforded secologanoside methyl ester tetraacetate (**14**) (64.5 mg), which was repeatedly recrystallized from acetone–Et_2O–pet. ether. The specific activity was less than 2.78×10^6 dpm/mmol and thus less than 0.86% of that of **17**. Total activity: less than 2.78×10^6 dpm.

B: After addition of 7-epi-10-hydroxyloganin (**5**) (50.1 mg), solution B was concentrated *in vacuo*. The residue (1.23 g) was worked up in the usual way to reisolate **5** (25.4 mg). Conventional acetylation of this substance with pyridine and Ac_2O yielded 7-epi-10-hydroxyloganin hexaacetate (**16**) (38.1 mg), which was diluted with carrier (73.5 mg) and recrystallized from acetone–Et_2O–pet. ether to constant activity. Total activity: 8.50×10^4 dpm.

Identification of Secologanin of *Adina pilulifera*——Twigs of *A. pilulifera* (wet weight 3.88 g) were cut into pieces and extracted with MeOH (40 ml×3) under reflux for 20 min. The combined MeOH extracts were concentrated *in vacuo*. The residue was extracted five times with H_2O (total 30 ml) and the resulting solution was filtered through a Celite layer. The filtrate was concentrated *in vacuo* to give a residue (555.9 mg), which was subjected to column chromatography on activated charcoal (2 g). The column was eluted successively with H_2O (100 ml), H_2O–MeOH 95: 5 (100 ml), 9: 1 (100 ml), 85: 15 (100 ml) and 5: 5 (200 ml), and MeOH (100 ml). Combined fractions eluted with H_2O–MeOH (5: 5) and MeOH were concentrated and subjected to PLC ($CHCl_3$–MeOH, 7: 3) to give secologanin (**2**) (41.1 mg) as a white powder, which, on acetylation, gave secologanin tetraacetate (**12**). Both **2** and **12** were identified by comparison with authentic specimens (TLC and ^1H NMR data).

Administration of [7-^{3}H]-7-Epi-10-hydroxyloganin ([7-^{3}H]-5) to *A. pilulifera* and Isolation of Secologanin (2)——A solution of [7-^{3}H]-**5** (10.7 mg, spec. activity 2.61×10^{10} dpm/mmol) in H_2O (1 ml) was administered

by the cotton wick method to three twigs (*ca.* 25 cm in length) of *A. pilulifera* in July. After 4 days, the plants (21.1 g) were cut into pieces and extracted with MeOH (250 ml × 3) under reflux. The MeOH extract was concentrated *in vacuo*. The residue was dissolved in H_2O (10 ml) and insoluble materials were removed by filtration through a Celite layer. The Celite layer was washed repeatedly with H_2O (total 40 ml). The combined filtrate and washings were concentrated *in vacuo* and the residue (2.08 g) was subjected to column chromatography on activated charcoal (6 g). The column was eluted successively with H_2O (200 ml) and H_2O–MeOH 95: 5 (200 ml), 9: 1 (200 ml), 8: 2 (200 ml), 7: 3 (200 ml), 6: 4 (300 ml), 5: 5 (300 ml) and 4: 6 (200 ml). Fractions eluted with H_2O–MeOH (6: 4, 5: 5 and 4: 6) were combined and concentrated. The residue (365.2 mg) was subjected to PLC ($CHCl_3$–MeOH, 3: 1) to give secologanin (**2**) (274.5 mg), which was acetylated with pyridine and Ac_2O; the product was purified by PLC (Et_2O), giving rise to secologanin tetraacetate (**12**) (289.3 mg) as a colorless syrup. A few pieces of dry ice were added to a solution of this substance (**12**) (289.3 mg) in dioxane (15 ml) containing H_2O (10 drops) in order to saturate the solution with CO_2. $NaBH_4$ (108.9 mg) was then added to the mixture and the whole was stirred at room temperature for 2 hr. The reaction was quenched by adding AcOH, then the solution was diluted with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to PLC (benzene–Et_2O, 7: 3, 3 developments), yielding secologanol tetraacetate (**17**) (128.9 mg), which was recrystallized from acetone–Et_2O–pet. ether to constant activity. Specific activity: 1.87×10^4 dpm/mmol. Jones reagent was added to an ice-cold solution of the above-mentioned radioactive **17** (114.4 mg) in acetone (10 ml) and the solution was stirred for 1 hr. After decomposition of the excess reagent with MeOH, the solution was diluted with H_2O and extracted with $CHCl_3$ (15 ml × 4). The $CHCl_3$ layer was washed with H_2O, dried and concentrated *in vacuo*. The residue was dissolved in Et_2O and methylated with CH_2N_2 under ice cooling. The reaction product was subjected to PLC (benzene–Et_2O, 7: 3), yielding secologanoside methyl ester tetraacetate (**14**) (100.4 mg) as a colorless syrup, which was recrystallized alternately from EtOH and a mixture of acetone–Et_2O–pet. ether to constant activity. Specific activity: 1.00×10^4 dpm/mmol.

Administration of [7-^{3}H]-10-Hydroxyloganin ([7-^{3}H]-3) to *A. pilulifera* and Isolation of Secologanin (2)——A solution of [7-^{3}H]-**3** (10.6 mg, spec. activity 2.02×10^{10} dpm/mmol) in H_2O (1 ml) was administered by the cotton wick method to three twigs of *A. pilulifera* (*ca.* 25 cm in length) in July. After 4 days, the plants (24.7 g) were extracted with MeOH and the extract was worked up in the usual way to give secologanin (**2**) (342.0 mg), which was converted to colorless, syrupy secologanin tetraacetate (**12**) (297.0 mg). A solution of this substance in CO_2-saturated hydrous dioxane was treated with $NaBH_4$ in the manner described above, giving rise to secologanol tetraacetate (**17**) (147.5 mg), which was recrystallized from acetone–Et_2O–pet. ether to constant activity. Specific activity: 2.18×10^4 dpm/mmol. Jones oxidation of the above-described radioactive **17** (128.3 mg) followed by methylation yielded secologanoside methyl ester tetraacetate (**14**) (80.9 mg), which was recrystallized alternately from EtOH and a mixture of acetone–Et_2O–pet. ether to constant activity. Specific activity: 1.02×10^4 dpm/mmol.

Administration of [7-^{3}H]-1 to *A. pilulifera*, Dilution Analysis of 10-Hydroxyloganin (3) and 7-epi-10-Hydroxyloganin (5) and Isolation of Secologanin (2)——A solution of [7-^{3}H]-**1** (10.6 mg, spec. activity 2.21×10^8 dpm/mmol) in H_2O (1 ml) was administered by the cotton wick method to two twigs of *A. pilulifera* (*ca.* 30 cm in length) in August. After 4 days, the plants (21.7 g) were cut into pieces and extracted with MeOH (200 ml × 3) under reflux. After concentration of the MeOH extract *in vacuo*, the residue was dissolved in MeOH–H_2O (2: 1, 100 ml). The insoluble materials were washed repeatedly with H_2O (total 50 ml). The combined solution and washings were divided into two halves, A and B.

A: After the addition of 10-hydroxyloganin (**3**) (50.1 mg), part A was concentrated *in vacuo*, yielding a residue (1.40 g), which was dissolved in H_2O (50 ml). After removal of the insoluble materials through a Celite layer, the layer was washed with H_2O (10 ml × 3). The combined filtrate and washings were concentrated *in vacuo* to *ca.* 10 ml and subjected to column chromatography on activated charcoal (4 g). The column was eluted with H_2O (300 ml), H_2O–MeOH 9: 1 (200 ml), 8: 2 (300 ml), 7: 3 (200 ml), 6: 4 (400 ml), 5: 5 (400 ml) and 3: 7 (300 ml) and MeOH (100 ml). Fractions eluted with H_2O–MeOH (5: 5 and 3: 7) and MeOH were combined and concentrated to give a residue (262.4 mg). Repeated PLC ($CHCl_3$–MeOH, 7: 3) of the residue afforded secologanin (**2**) (107.5 mg) and 10-hydroxyloganin (**3**) (31.6 mg). After alkali hydrolysis of **3** (31.6 mg) followed by acetylation, the product was subjected to PLC (Et_2O), yielding 10-hydroxyloganic acid hexaacetate (**15**) (37.0 mg), which was diluted with carrier (128.4 mg) and repeatedly recrystallized from acetone–Et_2O–pet. ether to constant activity. Total activity: 4.08×10^5 dpm.

B: After the addition of 7-epi-10-hydroxyloganin (**5**) (50.4 mg), part B was concentrated *in vacuo*, yielding a residue (1.41 g), which was worked up in the same way as in the case of part A to isolate **2** (121.5 mg) and **5** (40.7 mg). After conventional acetylation of **5**, the product was subjected to PLC (benzene–Et_2O, 4: 6), affording 7-epi-10-hydroxyloganin hexaacetate (**16**) (56.4 mg), which was recrystallized alternately from EtOH and a mixture of acetone–Et_2O–pet. ether to constant activity. Total activity: 4.06×10^4 dpm. Combined specimens of **2** obtained from parts A and B were acetylated and the product was subjected to PLC (Et_2O), yielding secologanin tetraacetate (**12**) (206.9 mg). An aliquot (158.1 mg) of this substance was reduced with $NaBH_4$ in CO_2 saturated dioxane and the product was subjected to PLC (benzene–Et_2O, 7: 3) to yield secologanol tetraacetate (**17**) (91.4 mg), which was recrystallized from acetone–Et_2O–pet. ether to constant activity. Total activity: 1.24×10^8 dpm. Specific activity: 2.11×10^8 dpm/mmol. Substance

17 (48.8 mg) was subjected to Jones oxidation followed by methylation with CH_2N_2 and the product was purified by PLC (benzene–Et_2O, 7 : 3), yielding secologanoside methyl ester tetraacetate (**14**) (36.6 mg), which was diluted with carrier (24.0 mg) and recrystallized alternately from EtOH and a mixture of acetone–Et_2O–pet. ether. The specific activity of this substance was less than 0.07% of that of **17**. Total activity: less than 8.20×10^4 dpm. Specific activity: less than 1.39×10^5 dpm/mmol.

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