Biosynthesis of Acidic Iridoid Monoterpene Glucosides in *Vinca rosea*[1a]

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Abstract: The occurrence and metabolism of a group of acidic iridoid glucosides have been demonstrated in the higher plant, *Vinca rosea*. Both loganic acid, **1**, and secologanic acid, **3**, have been identified as major constituents of the plant and feeding experiments with [2-^{14}C]mevalonate and [2-^{3}H,2-^{14}C]mevalonate followed by degradation of labeled glucosides established the monoterpenoid origin of their aglucones. A ^{14}C-labeled loganic acid (**1**) derived biosynthetically was administered and shown to be converted to loganin (**2**), secologanic acid (**3**), and secologanin (**4**) whereas a similarly derived [^{14}C]loganin was incorporated into secologanic acid, **3**. Administered [3′-^{14}C]3-hydroxy-3-methylglutarate was not converted directly to mevalonic acid. Another new seco acid, secologanoside, was isolated as its methyl ester tetraacetate, **13**, and its structure elucidated. Loganic acid (**1**) was also found to the extent of 1% in seeds of both *V. rosea* and *Strychnos nux vomica*.

Although loganin (**2**) and secologanin (**4**) have been implicated as the salient iridoid and secoiridoid glucoside precursors of the nontryptamine segment of indole alkaloids, their pool sizes are relatively low in many higher plants which are active in this metabolic process.[2] For example, in *Vinca rosea*, the presence of the two glucosides **2** and **4** was initially established by isotope dilution studies, suggesting they undergo a rapid turnover.[2] In examining a series of *Strychnos* species for glucosides, Jaminet[3] found certain tissue contained loganin (**2**) but almost all possessed large amounts of a compound which he termed loganic acid (**1**). His structure assignment was based upon uv spectra, chromatographic mobility, a color test, and generation of the acid **1** by saponification of loganin (**2**) which was believed to be a lactone at that time. Having identified loganic acid (**1**) as a key intermediate in secoiridoid biosynthesis in *Swertia caroliniensis*,[4,5] we raised the question of its metabolic role in indole alkaloid-synthesizing plants. Loganic acid was discovered in substantial amounts in both *V. rosea* and *Strychnos nux vomica*. Secologanic acid (**3**) has also been isolated as a free intermediate in *V. rosea* and tracer studies reported herein reveal the biosynthesis and metabolic interconversion of the acids and their methyl esters **1**–**4**. Preliminary communications of these investigations have appeared.[6]

Experimental Section

General methods and conditions of spectral analyses have been reported previously.[4,5] Satisfactory elemental analyses were obtained for polyacetates of loganic acid, loganin, secologanic acid (**11**), and secologanoside methyl ester (**13**).

Substrates. Labeled mevalonates were purchased from Amersham Searle. [3′-^{14}C]3-Hydroxy-3-methylglutaric acid was obtained from New England Nuclear.

Plant Material. *Vinca rosea*. Linn (*Catharanthus roseus* G. Donn) were grown from seeds (purchased from Atlee Burpee Co., Philadelphia, Pa.) in the Missouri Botanical Garden greenhouses. *Strychnos nux vomica* seeds were purchased from S.B. Penick and Co., New York, N. Y.

Incorporation. Application of radioactive precursor to 2–8 month-old *V. rosea* plants was achieved by the cotton wick technique.[4] The cotton thread was drawn through the stem 0.5 cm below meristematic tissue. Incorporation into 60 day-old seedlings was accomplished hydroponically by immersing their roots (previously pierced with a fine needle) into the radioactive tracer solution. In tracer experiments with germinating seedlings, seeds were germinated between filter papers (Whatman No. 1) and when the *radical* appeared they were transferred onto a watch-glass containing labeled precursor.

Isolation and Characterization of Loganic Acid (1). Loganic acid (**1**) was isolated as previously described[4] and converted to loganin (**2**), loganic acid pentaacetate, and loganin pentaacetate. The derivatives possessed superimposable spectra with those previously described from *Swertia caroliniensis*.[4] This included optical rotation, uv, ir, nmr, and mass spectrometry. Melting points of admixtures of loganin or loganin pentaacetate with authentic samples were not depressed.

In the time course experiments (Figure 1) generally sufficient amounts of seedlings were used to isolate loganin and loganic acid (which was converted to loganin) and to ascertain homogeneity on the basis of melting point and mixture melting point and uv and ir spectral analyses.

Isolation and Characterization of Secologanic Acid (3). Methanolic extracts of *V. rosea* were evaporated to dryness, and the residue was washed with hexane. The extract was redissolved in water and subjected to ion-exchange column chromatography on Dowex 1-X8 (200–400 mesh) formate form (200× the theoretical milliequivalents). After washing the column with water to remove sugars and other neutral substances, elution with 0.1 *N* formic acid was initiated. Fractions 1–3 contained unidentified carboxylic acids. Secologanic acid was the major component of fractions 4–6 giving an R_f 0.6 on plates coated with silica gel GF_{254} and developed with ethyl acetate–methanol–formic acid (4:1:0.5). Loganic acid was eluted in fractions 8 and 9. Further purification of fractions 4–6 by ion-exchange chromatography using a linear gradient elution ($H_2O \rightarrow$ 0.1 *N* formic acid) led to isolation of **3** in pure form which upon evaporation under reduced pressure formed a foamy white residue: $[\alpha]D$ −115° (*c* 1, CH_3OH); uv λ_{max}^{EtOH} 239 nm (log ϵ 3.97); ir λ_{max}^{Nujol} 3400, 1710, 1690, 1622 cm^{-1}; nmr (D_2O) at δ 9.9 (t, CHO), 7.17 (d, H-3).

Secologanic Acid Pentaacetate (11). The free glucoside (100 mg) was acetylated yielding 160 mg of crude product. Preparative tlc on plates coated with silica gel GF_{254} and developed with benzene–ethyl acetate (2:1) gave 110 mg of a colorless oil: $[\alpha]D$ −146° (*c* 1, $CHCl_3$); uv λ_{max}^{EtOH} 243 nm (log ϵ 3.97); ir $\lambda_{max}^{CHCl_3}$ 3010, 2940, 1760, 1720, 1640, 1010, 910 cm^{-1}; nmr δ 7.55 (d, *J* =

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(1) (a) This research was supported in part by NSF research Grant GB 17957 and NIH Research Career Development Award to C. J. Coscia. (b) In partial fulfillment of the requirement for the Ph.D. degree, St. Louis University.

(2) A. R. Battersby, R. T. Brown, R. S. Kapil, J. A. Knight, J. A. Martin, and A. O. Plunkett, *Chem. Commun.*, 890 (1966); P. Loew and D. Arigoni, *ibid.*, 137 (1968); A. R. Battersby, A. R. Burnett, and P. G. Parsons, *J. Chem. Soc.*, 1187 (1969).

(3) Fr. Jaminet, *Lejeunia*, **15**, 23 (1951); *J. Pharm. Belg.*, **35**, 339 (1953).

(4) C. J. Coscia, R. Guarnaccia, and L. Botta, *Biochemistry*, **8**, 5036 (1969).

(5) C. J. Coscia, L. Botta, and R. Guarnaccia, *Arch. Biochem. Biophys.*, **136**, 498 (1970).

(6) R. Guarnaccia, L. Botta, and C. J. Coscia, *J. Amer. Chem. Soc.*, **92**, 6098 (1970); R. Guarnaccia and C. J. Coscia, *ibid.*, **93**, 6320 (1971).

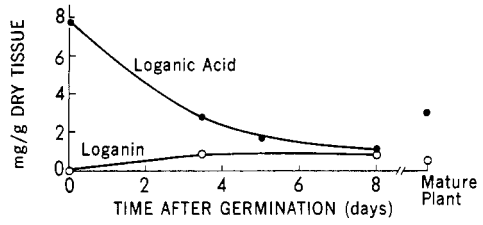


Figure 1.

2.0 Hz, H-3), 6.4 and 6.5 (m, H-7), 5.0–5.5 (m, 8 H), 4.3 (dd, CH_2 of glucose), 3.7 (m, 1 H), 2.13, 2.10, 2.03, 2.00, 1.98 (each s, 5 CH_3CO).

Isolation and Characterization of Secologanin (4). Fresh *V. rosea* plants were homogenized and extracted in the usual manner. After solvent removal, the dried methanolic extract was first washed with hexane and then with chloroform. The remaining residue was subjected to column chromatography on silica gel G, and the column was eluted with chloroform–methanol (9:1). The fractions rich in secologanin were combined and rechromatographed as above. Secologanin (**4**) was obtained as an amorphous white powder: $[\alpha]D$ −96° (*c* 1, CH_3OH); uv λ_{max}^{EtOH} 237 nm (log ϵ 3.93); ir $\lambda_{max}^{CHCl_3}$ at 3400, 1705, 1632 cm^{-1}. Acetylation of the free glucoside yielded crude secologanin tetraacetate. This was purified by preparative tlc on plates coated with silica gel GF_{254} and developed with benzene–hexane–methanol (4.5:4.5:1). Recrystallization from ethanol–hexane afforded white needles: mp 120°; $[\alpha]D$ −98° (*c* 1, $CHCl_3$) (lit.[2] −100°); uv λ_{max}^{EtOH} 233 nm (log ϵ 4.02); ir $\lambda_{max}^{CHCl_3}$ 3000, 1755, 1720, 1700, 1632, 1370, 1260–1212 cm^{-1}; nmr at δ 9.9 (t, C-7–H), 7.4 (d, C-3–H, J = 2 Hz), 5.5–5 (m, 8 H), 4.24 (dd, CH_2OAc of glucose), 3.7 (s, OCH_3), 2.10, 2.03, 2.00, 1.9 (each s, 4 CH_3CO).

Sweroside Tetraacetate (12) from Secologanic Acid (3). Secologanic acid (200 mg) was dissolved in 15 ml of methanol and sodium borohydride (120 mg) was added in portions. After stirring for 5 hr at room temperature, the reaction mixture was acidified to pH 5 with 6 *N* hydrochloric acid and evaporated to dryness. Acetylation gave 320 mg of crude acetate which was purified on preparative tlc plates coated with silica gel GF_{254} and developed with benzene–ethyl acetate (2:1). This afforded 198 mg of sweroside tetraacetate (**12**) which was recrystallized from ethanol. Melting point and mixture melting point with authentic sample 167°; $[\alpha]D$ −171° (*c* 1, $CHCl_3$) (lit.[7] −177°); uv λ_{max}^{EtOH} 243 nm (log ϵ 3.99); ir $\lambda_{max}^{CHCl_3}$ at 3000, 2940, 1755, 1710, 1622 cm^{-1}; nmr at 7.55 (d, C-3–H, J = 2 Hz), 4.9–5.5 (m, 8 H), 4.4 (m, C-7–H), 4.2 (dd, CH_2OAc of glucose), 3.77 (m, 1 H), 2.8 (m, 2 H), 2.10, 2.05, 2.02, 1.95 (each s, 4 CH_3CO) 1.7 (m, C-6–H).

Sweroside Tetraacetate (12) from Secologanin (4). In a typical experiment 50 mg of secologanin was dissolved in 10 ml of methanol and 10 ml of 0.1 *N* sodium hydroxide. After adding 30 mg of sodium borohydride the mixture was stirred for 12 hr at room temperature. Upon acidification to pH 5 with 6 *N* hydrochloric acid, the reaction mixture was evaporated to dryness and acetylated. This provided 49 mg of crystalline sweroside tetraacetate, mp and mmp 167°. This sample was spectrally and chromatographically indistinguishable from the material prepared from secologanic acid and from an authentic specimen of sweroside tetraacetate (kindly provided by Dr. H. Linde).

Isolation and Characterization of Secologanoside Methyl Ester Tetraacetate 13. The acidic components from *V. rosea* plants (1 kg), obtained by ion-exchange chromatography as described above, were subjected to diazomethylation. Most of the loganin obtained from loganic acid was recrystallized from acetone and the mother liquors were subjected to silica gel G column chromatography, the eluting solvent being chloroform–methanol (9:1). Fractions less polar than loganin were pooled and acetylated with an equal volume of acetic anhydride and pyridine. After the usual work-up, the residue was purified on preparative silica gel GF_{254} plates, benzene–hexane–methanol (4.5:4.5:1). The band having an R_f of 0.48 was rechromatographed with benzene–ethyl acetate (2:1) as a developing solvent. The major band was extracted from the silica gel with acetone to afford after evaporation to dryness 60 mg of residue which was recrystallized from ethanol: mp 140.5°; $[\alpha]D$ −99° (*c* 1 $CHCl_3$); uv λ_{max}^{EtOH} 230 nm (log ϵ 4.06); ir $\lambda_{max}^{CHCl_3}$ at 3010, 2940, 1755, 1740 (sh), 1710, 1630, 1440, 1370, 1285, 1250, 1230, 1080, 1065, 1040, 1015, 950, 905 cm^{-1}; nmr at δ 7.4 (d, C-3–H, J = 2 Hz), 5.5–5 (8 H), 4.2 (dd, CH_2OAc of glucose), 3.73 (s, OCH_3), 3.77 (s, OCH_3), 2.13, 2.06, 2.02, 1.95 (each s, 4 CH_3CO).

Secologanoside Methyl Ester Tetraacetate 13 from Secologanic Acid (3) or Secologanin (4). In a typical experiment a solution containing silver nitrate (0.88 mmol) and sodium hydroxide (0.16 mmol) was combined with 0.04 mmol of secologanic acid (**3**) or secologanin (**4**) in 15 ml of H_2O. The reaction mixture was stirred for 3 hr at room temperature. After filtration, the filtrate was neutralized with 2 *N* hydrochloric acid and subjected to ion-exchange chromatography on Dowex 1-X8 (formate form). After washing the column with water, it was eluted with 0.5 *N* formic acid, to afford the acidic components. The latter were brought to dryness by coevaporation with benzene. The residue was redissolved in methanol treated with an ethereal solution of diazomethane and dried. Usual acetylation and work-up provided a yellow semicrystalline material which was further purified on GF_{254} silica gel plates, benzene–ethyl acetate (2:1), benzene–hexane–methanol (4.5:4.5:1), and finally crystallized from ethanol affording 15 mg of secologanoside methyl ester tetraacetate (**13**), mp 140.5°.

Glucosidase Treatment of Loganin. Loganin (30 mg) was subjected to emulsin treatment as described previously.[8]

Aglucone Isolation. Ethyl acetate extraction of the incubation mixture afforded 18 mg of the oily loganetin, which after two successive elutions on preparative thin layer chromatography plates of silica gel GF_{254} with ethyl–acetate–benzene (1:1 v/v) showed no change in specific activity.

Glucose Isolation. Addition of an equal volume of ethanol to the ethyl acetate extracted incubation mixture resulted in precipitation of the protein and some citrate. After centrifugation, the supernatant was concentrated and subjected to preparative thin layer chromatography on silica gel GF_{254} eluting with chloroform–methanol (7:3 v/v). (Visualization of glucose under uv light was not possible and markers on both sides of the plate were used. After placing a glass plate over the band to be isolated, the marker was visualized by iodine staining.) The recovered glucose (13 mg) was allowed to stand overnight in a pyridine–acetic anhydride mixture (1:1). Following the usual work-up, the mixture of α- and β-glucose pentaacetate (19 mg) was recrystallized from ethanol.

Loganin Decarboxylation. The decarboxylation was carried out in a two-neck 15-ml pear-shaped flask to which a reflux condenser and a gas tube were attached. CO_2-free nitrogen gas was bubbled through the reaction flask through the condenser into a gas train of three tubes containing saturated barium hydroxide with 2% $BaCl_2$. The reaction mixture, containing 8 mg of loganin dissolved in 2.5 ml of 6 *N* HCl, was refluxed and within 10 min CO_2 evolution began. After 2 hr the barium carbonate was collected by centrifugation and washed with CO_2-free water three times and twice with acetone. The barium carbonate was dried in an oven at 100° to constant weight (3 mg). Specific activities were determined by liquid scintillation spectrometry of weighed samples of $BaCO_3$ suspended in a Cab-O-Sil system.[4] Under identical conditions glucose did not afford any detectable CO_2.

Results

Isolation and Biosynthesis of Loganic Acid. Indole alkaloid synthesizing plants were examined for loganic acid (**1**) using chromatographic techniques previously developed for the isolation of the polar iridoid glucoside carboxylic acids from *Swertia caroliniensis*.[4] Seeds of both *V. rosea* and *S. nux vomica* contained loganic acid (**1**) in amounts up to 1% of their total weight. Only a small proportion if any of the acid occurs in the seed coat. Upon germination of *V. rosea* seeds, the concentration of loganic acid (**1**) decreased and approached a value of 0.4% on a dry weight basis in the mature plant (Figure 1). Simultaneously, loganin (**2**), which was not detectable in seeds, rises to a level of about 0.1% in the first 8 days of germination and the *S*-adenosyl-L-methionine:loganic acid methyl transferase exhibits a relatively high activity during this period.[9]

(7) H. Inouye, S. Ueda, and Y. Nakamura, *Chem. Pharm. Bull.*, **18**, 1856 (1970).

(8) G. Buchi and R. E. Manning, *Tetrahedron*, **18**, 1049 (1962).

(9) K. Madyastha, R. Guarnaccia, C. Baxter, and C. J. Coscia, *J. Biol. Chem.*, **248**, 2497 (1973).

Table I. Utilization of [2-^{14}C]Mevalonic Acid in *V. rosea*[a]

Expt	Age of plant	Method of administration	% incorporation			Length of exposure, hr
			Loganic acid	Loganin	Secologanic acid	
1	GS 1 day	H	0.36			24
2	S 60 days	H	0.52	0.013		27
3	S 60 days	H	1.2		0.1	72
4	FP	W	1.16	0.009		72
5	FP 11 months	W	0.22		0.11	168

[a] Abbreviations: G = germinating, S = seedlings, FP = flowering plant, H = hydroponics, W = cotton wick.

De novo synthesis of loganic acid (**1**) from [2-^{14}C]-mevalonate was observed in seedlings at various stages of maturation as well as in flowering plants (Table I). Feeding experiments were carried out in essentially two different ways. Germinating seedlings were allowed to absorb water containing tracer (hydroponically), whereas mature plants were fed by the cotton wick technique.[4] Variations in methods and conditions prevent a comparison of rates of synthesis *versus* age of plant or length of exposure.

After addition of 15 mg of carrier **1** to the methanol extract of the plant, loganic acid (**1**) was isolated by the ion-exchange method. The crude acid was methylated and acetylated. The resulting loganin pentaacetate was recrystallized to constant radioactivity. Saponification[9] with stoichiometric amounts of barium hydroxide in methanol afforded loganin **2**. The product was again recrystallized with no change in molar specific activity. Degradation of purified loganin (**2**) by β-glucosidase catalyzed hydrolysis provides the aglucone, loganetin, and glucose. Determination of their respective activities established exclusive incorporation of [2-^{14}C]mevalonate into the isoprenoid moiety (experiments 1 and 4, Table II). Decarboxylation studies on

Table II. Degradation of Labeled Loganic Acid from *in vivo* Experiments in *V. rosea*

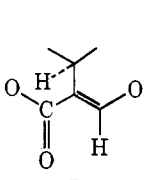
Expt	Compd counted	Specific activity, μCi/mol	Relative molar activities
1	Loganic acid (as loganin)	51	1
	$BaCO_3$ from C-11	10	0.20
4	Loganic acid (as loganin)	360	1
	Aglucone	320	0.91
	Glucose	0	0
	$BaCO_3$ from C-11	70	0.20
6[a]	Loganic acid (as loganin)	38	1
	Aglucone	31	0.81
	Glucose	4.5	0.12
	CH_3COOH (from C-10 and C-8)	8.1	0.21

[a] Precursor: [3′^{14}*C*]3-hydroxy-3-methylglutaric acid; 0.01% incorporation.

loganin indicated that 22% of the label in the aglucone is at C-11. This is consistent with expected randomization of the original terminal dimethyl groups of geraniol at some point in the biosynthesis beyond ω-hydroxylation of the acyclic monoterpene alcohols.[10]

[3′-^{14}C]3-Hydroxy-3-methylglutaric acid was also incorporated into loganic acid by the cotton wick technique, and its (**1**) degradation revealed considerable randomization of label (experiment 6, Table II). Hence the glucose contained 13% of the total radioactivity of loganic acid, whereas carbon 8 and 10 isolated as acetic acid after Kuhn Roth oxidation, possessed 23% of the total activity. Had 3-hydroxy-3-methylglutaric acid been converted directly to mevalonate, C-10 should have possessed 50% of the label of the aglucone. The breakdown of this precursor is consistent with findings on isoprenoid biosynthesis from acetate fragments in mammalian and bacterial systems as well as in other plants.[11] The extent of label at C-10 is too high to be reconciled with complete degradation of 3-hydroxy-3-methylglutarate to acetate but may reflect partial utilization of acetoacetate in its CoA form, the latter arising by a thiophorase-catalyzed reaction. Thus, it appears that there may be little if any access to a thiokinase which can catalyze the conversion of 3-hydroxy-3-methylglutarate to its coenzyme A derivative, a direct precursor of mevalonate.

Identification of Secologanic Acid. Fractionation of methanolic extracts of *V. rosea* plants by gradient elution ion-exchange chromatography resulted in the isolation of an amorphous white compound in pure form possessing a chromophore λ_{max} (EtOH) 239 nm (log ϵ 3.9). From the uv spectra as well as an nmr signal at 7.57 ppm (doublet) and ir absorption band at 1622 cm^{-1}, an enol ether conjugated carbonyl (**7**) is suggested.



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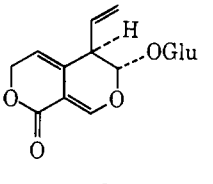

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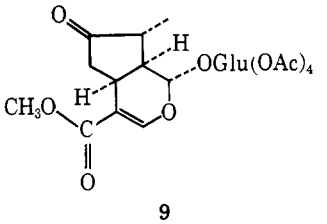

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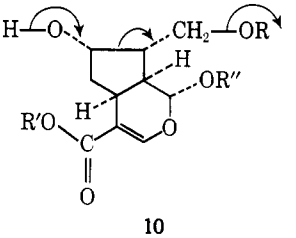

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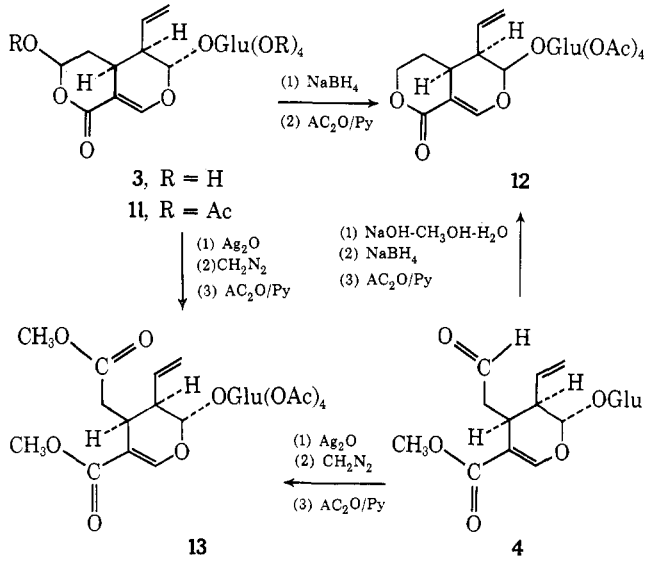
Upon acetylation the nmr spectra exhibited five acetoxy singlets between 2 and 2.10 ppm, a new pair of multiplets at 6.4 and 6.5 ppm, and no OMe signal. When the nmr spectra of the sodium salt of the free glucoside was taken, a signal at 9.9 ppm (triplet) was observed, indicating the presence of an aldehyde function.

(10) S. Escher, P. Loew, and D. Arigoni, *Chem. Commun.*, 823 (1970); A. R. Battersby, S. H. Brown, and T. G. Payne, *ibid.*, 827 (1970); T. D. Meehan and C. J. Coscia, *Biochem. Biophys. Res. Commun.*, **53**, 1043 (1973).

(11) C. M. Hepper and B. G. Audley, *Biochem. J.*, **114**, 379 (1969).

Identification of secologanic acid was secured with the interlocking chemical evidence outlined in Scheme I.

Scheme I



Sodium borohydride reduction of the free glucoside followed by acetylation and purification of the acetate derivative by preparative tlc gave tetra-*O*-acetyl sweroside (**12**) in good yield. The latter **12** was identified by direct comparison with an authentic sample[12] (kindly provided by Dr. Linde) and possessed superimposable spectral properties and the same chemical characteristics as the compound **12** obtained from secologanin isolated from *V. rosea* plants (Scheme I).[13]

Silver oxide oxidation of secologanic acid (**3**) followed by ion-exchange chromatographic purification, methylation, and acetylation afforded a diester tetraacetate (**13**). Upon subjecting secologanin (**4**) to the same chemical sequence of reactions an identical compound was afforded. On the basis of chemical and spectral evidence, structure **13** was assigned and given the common name of secologanoside methyl ester tetraacetate. It follows therefore from the chemical and physical data presented that the glucoside termed secologanic acid possesses structure **3**, and the pair of multiplets in the nmr of penta-*O*-acetyl secologanic acid (**11**) at 6.4 and 6.5 ppm can be attributed to the C-7 hydrogen of the two epimeric forms generated by acetylation of this center. Secologanic acid pentaacetate obtained by chemical degradation of foliamenthin was characterized by the Arigoni and Battersby groups and the chemical and spectroscopic data described here are in complete agreement with reported values.[14]

Isolation and Structure Elucidation of Secologanoside. In the course of our isolation studies another new previously undescribed congener was obtained which possessed a carboxyl group at C-7, *i.e.*, at the location of the free aldehyde in secologanic acid (**3**) or secologanin (**4**) and the primary alcohol function in sweroside (**5**), gentiopicroside (**8**), and swertiamarin. Upon methylation the new glucoside was found to be less polar than loganin (**2**), whereas its tetraacetate behaved similarly to loganin pentaacetate on thin layer chromatography. The melting points of both compounds were almost identical (140 ± 1°); however, mixture melting points showed 15° depression. Although a similarity was also observed in the ultraviolet absorption spectra, the nonidentity of the new compound and loganin pentaacetate was clear. The optical activity of the isolated material was significantly different from that of loganin pentaacetate, and its infrared spectra showed bands at 1755 cm^{-1} which could be assigned to the acetate carbonyl functions, 1740 and 1710 cm^{-1} indicative of an aliphatic and a conjugated carbonyl group, respectively. The presence of a typical enol ether conjugated double bond system was revealed by both absorptions in the uv (λ_{max} 230 nm) and ir (1630 cm^{-1}). The nmr spectra revealed characteristic secoiridoid resonance signals. Two OCH_3 ester functions were indicated by the two singlets at 3.73 and 3.77 ppm. Moreover physical and spectral properties of this new compound were found to be in complete agreement with those of partially synthesized compound **13** prepared as described in Scheme I. The common name secologanoside was designated. The possibility that **13** may be an artifact of the isolation procedure arising by oxidation of secologanic acid (**3**) or secologanin (**4**) cannot be discounted.

Biosynthesis of Secoiridoids in *V. rosea*. To demonstrate the mevalonoid origin of secologanic acid (**3**), experiments were undertaken using [2-^{14}C,2-^{3}H]-mevalonate. In similar studies with *Swertia carolinien-sis* plants, it was established that C-3 and C-7 of loganic acid (**1**) contain tritium from [2-^{3}H]mevalonate, and in experiments with asymmetrically labeled [2-^{3}H]MVA, the same $^{3}H/^{14}C$ ratio for the carboxylic acid **1** as well as for gentiopicroside **8** was observed.[4,6] In all cases an enrichment of tritium at C-3 occurs and may be ascribed to an isotope effect at some step in the biosynthesis. Because of their metabolic relationship it was assumed that tritium was present at C-7 and C-3 in the secoiridoid (**8**). However, the position of the label was not investigated.

The $^{3}H/^{14}C$ ratio of metabolites in such a double labeled experiment is also an index of the biosynthetic sequence or sequences. Similar $^{3}H/^{14}C$ ratios for loganic acid (**1**), secologanic acid (**3**), loganin (**2**), and secologanin (**4**) would suggest metabolic interrelationship and a common biogenetic pathway, particularly if an isotope effect is encountered.

Three days after feeding [2-^{14}C,2-^{3}H]mevalonate to 2 month-old *V. rosea* seedlings by the hydroponic method, the plants were harvested and carrier loganic acid, secologanic acid, loganin, and secologanin were added during the isolation, to facilitate the purification of these glucosides. Anion ion-exchange chromatography was utilized to separate the neutral from the acidic components and the mixture of the former was further purified by repetitive preparative tlc.

Loganin was crystallized to constant radioactivity and to determine the $^{3}H/^{14}C$ ratio of the tetraacetate (**9**) it was then subjected to Jones oxidation and subsequent acetylation (Table III). Crystallization of this deriva-

(12) H. Inouye, S. Ueda, and Y. Nakamura, *Tetrahedron Lett.*, 5229 (1966); H. A. Linde and M. S. Ragab, *Helv. Chim. Acta*, **50**, 991 (1967); H. Inouye, T. Yoshida, Y. Nakamura, and S. Tobita, *Tetrahedron Lett.*, 4429 (1968).

(13) Sweroside **5** was also isolated from *V. rosea* plants as its tetraacetate and identified by melting point and chromatographic properties.

(14) P. Loew, Ch. V. Szczepanski, C. J. Coscia, and D. Arigoni, *Chem. Commun.*, 1276 (1968); A. R. Battersby, A. R. Burnett, G. D. Knowles, and P. G. Parsons, *ibid.*, 1277 (1968).

Table III. Radioactivity of Monoterpene Derivatives and Degradation Products from *in Vivo* Experiments in *Vinca rosea*

Precursor	Monoterpene/derivatives	^{14}C % inc	$^{3}H/^{14}C$ radioactivity ratio	$^{3}H/^{14}C$ atomic ratio—Obsd	$^{3}H/^{14}C$ atomic ratio—Theory
[2-^{14}C,2-^{3}H]MVA			3.0		2:1
	Loganin (**2**) from loganic acid (**1**)	0.25	0.9	1.35:2	1.33:2
	7-Oxologanin (**9**) from **1**		0.35	0.52:2	0.33:2
	Loganin (**2**)	0.02	0.94	1.41:2	1.33:2
	7-Oxologanin (**9**) from **2**		0.38	0.57:2	0.33:2
	Sweroside tetraacetate (**12**) from **3**	0.17	1.1	1.65:2	1.33:2
	Secologanoside (**13**) from **3**		0.5	0.75:2	0.33:2
	Sweroside tetraacetate (**12**) from Secologanin (**4**)	0.2	0.9	1.35:2	1.33:2
	Secologanoside (**13**) from **4**		0.36	0.54:2	0.33:2

tive gave a ratio from which the tritium content of loganin at C-7 could be calculated.

To determine the $^{3}H/^{14}C$ ratio in secologanin (**4**) an easily recrystallizable derivative was needed. Therefore, a portion of this glucoside was converted to sweroside tetraacetate (**12**) (Scheme I). To determine the tritium content at C-7 of secologanin (**4**) the remainder of the biosynthetically derived secoiridoid was subjected to silver oxide oxidation followed by methylation and acetylation to afford secologanoside methyl ester tetraacetate (**13**) (Scheme I).

The acidic fraction from anion-exchange chromatography contained primarily loganic acid (**1**) and secologanic acid (**3**) which were rechromatographed by ion-exchange chromatography using the gradient elution technique. Loganic acid (**1**) was methylated and the resulting **2** purified, counted, and then converted to 7-oxologanin tetraacetate (**9**). The $^{3}H/^{14}C$ ratio in **3** was determined by converting part of this carboxylic acid to sweroside tetraacetate (**12**). The tritium content at C-7 was obtained by oxidizing the remaining portion with silver oxide and further conversion to **13** (Scheme I). As shown in Table III the $^{3}H/^{14}C$ ratios for the metabolites, **1–4**, were essentially identical. The same $^{3}H/^{14}C$ is observed for **13** derived from **3**. Moreover, the $^{3}H/^{14}C$ ratio for the oxidized iridoids and secoiridoids reveals the tritium content at C-7 was comparable.

Differences in the $^{3}H/^{14}C$ ratios between derivatives of secologanic acid **3** and those of other monoterpenes may not be significant. Due to relatively low yields of **3**, a greater dilution of the labeled compound was made thereby affording lower and less accurate counts.

Conversion of Iridoid to Secoiridoids. A sample of loganic acid was isolated from *V. rosea* plant fed [2-^{14}C]mevalonate and then purified as previously described.[6] Its specific activity was established by methylation to **2** followed by crystallization to constant radioactivity. An aliquot of this derivative was also converted to loganin pentaacetate and crystallized with no change in molar specific activity. Loganic acid was regenerated by saponification of **2** and its pentaacetate with barium hydroxide and the acid purified again by ion-exchange chromatography. This rigorously purified material (20 mg, 6.75×10^5 dpm/mmol) was administered to *V. rosea* plants by the cotton wick technique. After ten days the plant material was harvested and extracted with methanol. Carriers for **2–4** were added and the metabolites were resolved and then converted to crystalline derivatives as indicated in Table IV.

Table IV. Interconversion of Monoterpene Glucosides in *V. rosea*

Precursor	Isolated monoterpene	Derivative recrystallized	% incorporation
[^{14}C]Loganic acid (**1**)	Loganin (**2**)	Loganin (**2**)	1.0
	Secologanin (**4**)	Sweroside tetraacetate	8.8
	Secologanic acid (**3**)	Sweroside tetraacetate	6.7
[^{14}C]Loganin (**2**)	Secologanic acid (**2**)	Secologanoside methyl ester tetraacetate	2.0

A sample of loganin (31 mg, 8.05×10^5 dpm/mmol), prepared from [2-^{14}C]mevalonate-derived **1**, was similarly purified and administered to a *V. rosea* plant. After 10 days the plant was harvested and extracted with methanol. Carrier **3** was added to facilitate its purification by conversion to secologanoside methyl ester tetraacetate (**13**) (Scheme I). Results demonstrate loganin (**2**) indeed served as a precursor for secologanic acid (**3**). Insufficient amounts of loganic acid (**1**) and secologanin (**4**) were recovered to determine their specific activity.

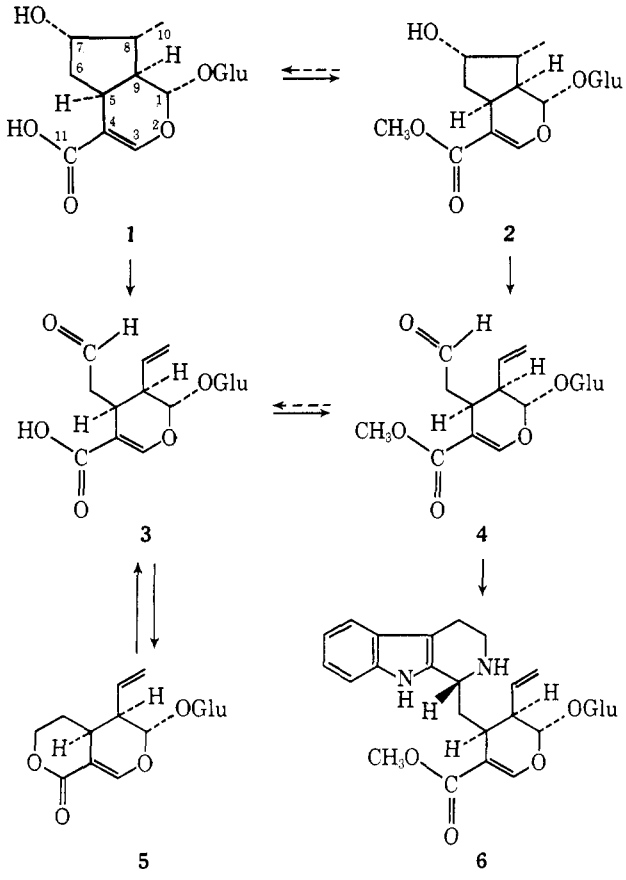
Discussion

The occurrence of relatively large amounts of loganic and secologanic acids in comparison to their methyl esters in *V. rosea* and *S. nux vomica* seeds suggests the importance of methylation to alkaloid synthesis. The possibility may be raised that loganic acid is metabolically inert having been simply laid down in the seed as an excretion product[15] (or as a bitter principle if one wishes to consider an allelochemical role). But this is not consistent with its decrease in concentration on a dry weight basis with concomittant increase in loganin levels during the first week of germination (Figure 1). Furthermore, the relatively high levels of *S*-adenosyl-L-methionine:loganic acid methyl transfer-

(15) K. Mothes, *Experientia*, **25**, 225 (1969).

ase activity in seedlings of the same age[9] as well as active alkaloid synthesis[16] all suggest a rapid utilization of loganic acid. In fact, the possibility of a sparing effect by loganic acid also presents itself. Thus if isoprenoid synthesis of primary metabolites, *e.g.*, sterols for cell membranes, were derepressed, loganic acid might serve as the source of the nontryptamine segment of the indole alkaloid. At this stage of development, this would prevent a drain on isoprenoid primary metabolism assuming a common pathway. The fact that mevalonate is readily incorporated into loganic acid within the first 24 hr of germination belies this hypothesis at least to the extent that *de novo* synthesis of loganic acid is not completely curtailed. To answer the question of whether the rate of iridoid glucoside synthesis is reduced would take experiments under more rigorously controlled conditions. The occurrence and metabolism of secologanic acid, sweroside, and possibly secologanoside suggests a dual pathway wherein metabolic conversions can occur *via* free acids or methyl esters with methylation being a critical step for alkaloid synthesis (Scheme II). In substrate specificity studies

Scheme II



with the partially purified methyl transferase we have established that this can occur both with loganic and secologanic acids as substrate.[9] A metabolic grid is also supported by the double labeling studies with [2-^{3}H,2-^{14}C]mevalonate (Table III). Thus ratios of $^3H/^{14}C$ for loganic acid, secologanic acid, loganin, and secologanin were quite similar as was the extent of labeling at C-7 which one would expect from metabolites being formed in the same pathway.

(16) A. I. Scott, P. B. Reichardt, M. B. Slaytor, and J. G. Sweeny, *Bioorg. Chem.*, **1**, 157 (1971).

A reversible oxidoreductase-catalyzed reaction between secologanic acid (**3**) and sweroside (**5**) would explain Inouye's discovery[17] that the latter is an excellent precursor for the indole alkaloid vindoline in *V. rosea*. It is thus envisaged that sweroside undergoes oxidation to secologanic acid and methylation to afford secologanin which by Mannich condensation with tryptamine affords the indolic glucoside, vincoside (**6**) (Scheme II). This pathway is supported by *in vitro* studies wherein loganic acid methyl transferase was shown to methylate secologanic acid to secologanin as it does loganic acid.

Conversion of cyclopentano glucosides to their seco derivatives appears to occur by a mechanism with some precedence in nature.[18] Ostensibly such reactions are reminiscent of the C_3–C_4 bond breaking encountered in ring A of polycyclic di- and triterpenes.[19] Such cleavages may differ mechanistically, however, as in the case of the beyerene–diterpenes.[20] Potentially a number of mechanisms can be invoked in the present instance even taking into account the mechanistic implication of retention of the C-7 proton in the transformation.

One of the most attractive mechanisms presently under consideration involves a 10-hydroxyloganic acid or methyl ester intermediate **10**. This 1,3-diol with a suitable leaving group at the C-10 hydroxyl should readily undergo fragmentation. Recently a chemically induced cleavage has been accomplished with a synthetic 10-hydroxyloganin derivative[21] **10**. However, this occurred only when the reacting centers on the cyclopentane ring were anticoplanar. Thus, 7-epihydroxyloganin underwent cleavage but the compound **10** having the same configuration at C-7 as the naturally occurring glucoside failed to fragment under the same conditions. This has raised the possibility of the intermediacy of a 7-epihydroxyloganin which would have to be reconciled with retention of the C-7 proton. However, we have found that 7-epiloganic acid is not methylated by the loganic acid transmethyase *in vitro*.[9] Inouye and coworkers have observed that both loganin (**2**) and 7-epiloganin were precursors of another cyclopentano monoterpene glucoside, asperuloside, but only the epimer of loganin lost its tritium at C-7 in the conversion. Finally an enzymatic cleavage of 10-hydroxyloganin (**10**) in this manner need not occur by a concerted mechanism. Interaction with an electrophillic group at C-8 of **10** could facilitate appropriate product formation in the same manner as nucleophillic X group participation is believed to direct the stereochemical course of the prenyl transferase reaction.[22]

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(19) D. Arigoni, D. H. R. Barton, R. Bernasconi, C. Djerassi, J. S. Mills, and R. Wolff, *J. Chem. Soc.*, 1900 (1960); G. H. Whitman, *ibid.*, 2016 (1960).

(20) H. J. Bakker, E. L. Ghisalberti, and P. R. Jefferies, *Phytochemistry*, **11**, 2221 (1972).

(21) L. F. Tietze, *J. Amer. Chem. Soc.*, **96**, 946 (1974).

(22) G. Popjak and J. W. Cornforth, *Biochem. J.*, **101**, 553 (1966).