# MECHANISM FOR IRIDANE SKELETON FORMATION IN THE BIOSYNTHESIS OF SECOLOGANIN AND INDOLE ALKALOIDS IN *LONICERA TATARICA, CATHARANTHUS ROSEUS* AND SUSPENSION CULTURES OF *RAUWOLFIA SERPENTINA**

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**Abstract**—Feeding experiments of various $^3$H-, $^2$H- and $^{13}$C-labelled compounds to plants of *Lonicera tatarica*, and *Catharanthus roseus* (hybrid) and to suspension cultures of *Rauwolfia serpentina* corroborated that secologanin, vindoline, ajmaline and vomilenine in these plants and cultured cells are biosynthesized via cyclization of 10-oxogeranial/10-oxoneral to iridodial in the same way as secologanin and vindoline in plants of *L. morrowii* and *C. roseus*. Therefore, this cyclization mechanism seems to be common in plants containing secoiridoids and indole alkaloids.

## INTRODUCTION

In the previous papers [1,2], we demonstrated by feeding experiments with $^3$H-labelled monoterpenes that secologanin (1) and vindoline (2) in *Lonicera morrowii* and *Catharanthus roseus* are biosynthesized via geraniol, 10-oxogeranial (3)/10-oxoneral (4) and iridodial (5); this route is different from that proposed by Balsevich and Kurz [3], who suggested the intermediacy role of 9,10-dioxogeranial (6) and iridotrial (7) for the biosynthesis of ajmalicine in *C. roseus* suspension cultures.

Therefore, our attention was directed toward the examination of the validity of the route via 3/4 to 5 in other plants as well as cultured cells. The present paper deals with the administrations of $^3$H-, $^2$H- and $^{13}$C-labelled putative precursors to the plants of *L. tatarica* and *C. roseus*, and suspension cultures of *Rauwolfia serpentina*.

## RESULTS AND DISCUSSION

*Administration of* [1-$^3$H]-10-*hydroxygeraniol* (8) *and* [1-$^3$H-9,10-*dihydroxygeraniol* (9) *to plants of* L. tatarica *and* C. roseus

[1-$^3$H]-10-Hydroxygeraniol (8) and [1-$^3$H]-9,10-dihydroxygeraniol (9) prepared according to the previously reported method [2] were administered hydroponically to the twigs of *L. tatarica* on Aug. 18 (Expt 1) and on Aug. 30 (Expt 2). Radioactive secologanin (1) was

isolated and converted through sodium borohydride reduction and acetylation to secologanol pentaacetate (10). Similarly, [1-$^3$H]-8 and [1-$^3$H]-9 were fed to the hydrid [4, 5] of *C. roseus* producing a significant amount of vindoline (2) (Expt 3) on Aug. 20 (the same season as in our earlier experiments with *C. roseus* [1, 2]) and radioactive 2 was isolated by dilution analysis.

In Expt 1, the incorporation rate (Table 1) of 8 into 1 was *ca* seven times higher than that of 9. However, in Expt 2, the former rate was reduced to one-tenth, whereas the latter rate decreased to one-third. This finding, together with the results obtained in *L. morrowii* [1,2], led to the conclusion that iridane skeleton formation proceeds via 10-hydroxygeraniol (8)/10-hydroxynerol (11), 3/4 and 5 in the biosynthesis of secoiridoids in *Lonicera* plants. The observed low incorporations of 9 into 1 suggested that 9 does not contribute to the main biosynthetic route of secologanin (1) in these plants.

The marked difference in the incorporation rates of 8 between Expts 1 and 2 indicated that the enzyme system functioning for the biosynthesis of secoiridoids is particularly activated during a short period of development. This observation is consistent with the result reported by Damtoft *et al.* [6] who found that 30% of $^2$H-labelled deoxyloganin was incorporated into dihydrocornin and cornin when applied to *Verbena officinalis* plants before flowering, but the value decreased considerably during the flowering period. This phenomenon was also observed for the incorporation of 9 into 1 although the difference between the incorporation ratios in Expts 1 and 2 was very small. Thus, the efficiency of the exogenously applied precursors to be metabolized to iridoids seems different depending on the developmental stage of the plants.

In the experiment with the hybrid of *C. roseus* (Expt 3), the incorporation ratios of [1-$^3$H]-8 into 2 was five times higher than that of [1-$^3$H]-9, in accordance with the result

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Table 1. Administration of [1-³H]-10-hydroxygeraniol (8) and [1-³H]-9,10-dihydroxygeraniol
(9) to *L. tatarica* and *C. roseus* plants

| Plant (compound isolated) | | Date | Metabolic period (hr) | Total incorporation (%)* [1-³H]-8 | [1-³H]-9 |
|---|---|---|---|---|---|
| *L. tatarica* | Expt 1 | (Aug. 18) | 27 | 17.4 | 2.7 |
| (secologanin, 1) | Expt 2 | (Aug. 30) | 28 | 1.7 | 0.9 |
| *C. roseus* | Expt 3 | (Aug. 20) | 28 | 0.5 | 0.1 |
| (vindoline, 2) | | | | | |

*Calculated based on the assumption that one half of the ³H label of 8 and 9 remained in 1 and 2.

obtained in our earlier experiments [1, 2]. It is therefore demonstrated that the monoterpene moiety of vindoline (2) in *C. roseus* plants is formed by the same cyclization process as secologanin (1) in *Lonicera* plants (Scheme 1).
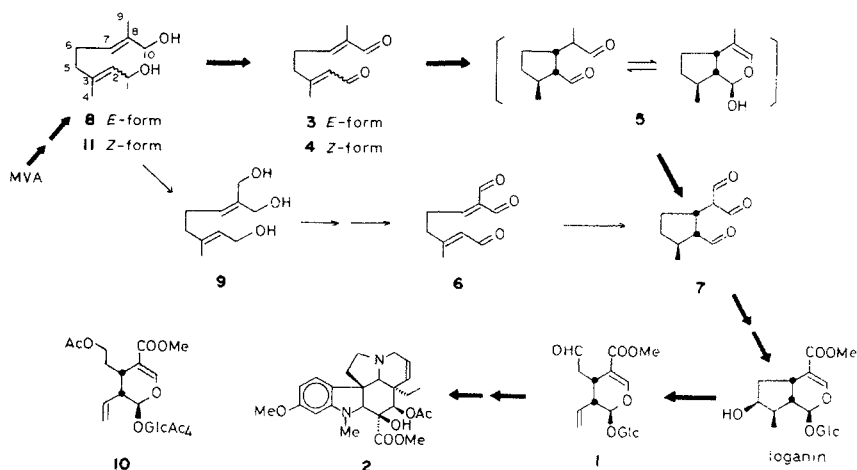
*Administration of* [4-¹³C]-**8**, [9-¹³C]-**11**, [2-¹³C]-**9** *and* [10-²H₂]-**5** *to Rauwolfia serpentina suspension cultures*

Among the above labelled monoterpenes, **5**, **8** and **9** were prepared by our previously reported methods [7, 8], whereas **11** was prepared by Wittig reaction of (α-carboethoxy methyl-[¹³C]etylidene)triphenylphosphorane [7] and (Z)-6-acetoxy-4-methyl-4-hexenal followed by lithium aluminium hydride–aluminium chloride reduction of the resultant condensation product. A mixture of [4-¹³C]-**8**, [9-¹³C]-**11**, [2-¹³C]-**9** and [10-²H₂]-**5** was administered to *R. serpentina* suspension cultures [9]

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*The configuration at the 20-position of dihydrovomilenine (**14**) was established as *S* on the basis of ¹³C NMR. The inversion to the *R*-configuration at C-20 of ajmaline-type alkaloids is known to cause an upfield shift of C-14 and a downfield shift of C-16. Libot, F., Kunesch, N. and Poisson, J. (1980) *Phytochemistry* **19**, 989; Danieli, B., Palmisano, G. and Ricea, G. S. (1981) *Tetrahedron Letters* 4007.

5 days after inoculation and incubation was continued for 8 days. Ajmaline (**12**) and vomilenine (**13**) were isolated, and the latter was converted to the more stable dihydro-vomilenine (**14**)* through Pd–C catalysed reduction. The ²H NMR spectra were taken under usual conditions (see Experimental), and the incorporation ratios of ²H-labelled **5** into **12** and **14** were calculated using the percentage enrichments of the ²H-18 signal in **12** and **14**, which were normalized relative to the peaks of the natural C²HCl₃ in CHCl₃ used as solvent. On the other hand, the ¹³C NMR spectra were recorded by means of a gated decoupling-without-NOE technique [10] (see Experimental) in order to integrate each signal intensity accurately. The incorporation ratios of ¹³C-labelled **8**, **11** and **9** into **12** and **14** were calculated using the percentage enrichments of the C-18, C-17 and C-20 signals in **12** and **14**, which were normalized relative to the peaks of the C-14 signal.

As shown in Table 2, the incorporation rates of irido-dial (**5**) into **12** and **14** was highest, and those of 10-hydroxygeraniol (**8**) and 10-hydroxynerol (**11**) were next. However, the incorporation rates of **9** were six–seven times less than those of **8** and **11**. This finding clearly indicated that **5**, **8** and **11** but not **9** should be considered as intermediates. It was therefore concluded that indole alkaloids including ajmaline (**12**) and vomilenine (**13**) in *R. serpentina* suspension cultures are biosynthesized via



Scheme 1. Biosynthetic pathway of secologanin (1) and vindoline (2) in *Lonicera tatarica* and *Catharanthus roseus*.

Table 2. Incorporation of $^2$H- and $^{13}$C-labelled putative precursors into ajmaline (12) and vomilenine (13) determined as dihydrovomilenine (14) in *Rauwolfia serpentina* suspension cultures

| Compound fed [amount (mg)] | | $^2$H and $^{13}$C chemical shifts (ppm) | | (Spec. Incorp., %) | |
|---|---|---|---|---|---|
| | | 12 | 14 | 12 | 14 |
| [4-$^{13}$C]-10-Hydroxygeraniol (8) (15.4) | C-18: | 12.2 | 11.9 | 2.3 (0.8) | 7.1 (1.8) |
| [9-$^{13}$C]-10-Hydroxynerol (11) (16.0) | C-17: | 77.7 | 78.6 | 1.9 (0.7)* | 7.2 (1.9)* |
| [2-$^{13}$C]-9,10-Hydroxygeraniol (9) (19.4) | C-20: | 48.0 | 47.0 | 0.3 (0.1) | 1.2 (0.3) |
| [10-$^2$H$_2$]Iridodial (5) (18.3) | 18-$^2$H: | 0.9 | 1.0 | 14.4 (6.0) | 10.7 (3.2)† |

\* Estimated based on the presumption that scrambling of carbons corresponding to C-9 and C-10 of 11 takes place during the biosynthesis of 12 and 13.

† The values seem to be somewhat low as compared with the values for ajmaline (12). This may be due to the elimination of deuterium which would take place during the catalytic reduction of vomilenine (13) to dihydrovomilenine (14).
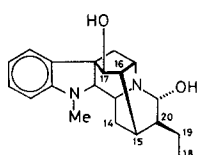


12
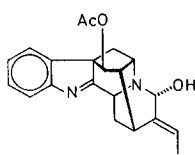
13
14  19, 20–dihydro

the same cyclization process as secologanin (1) and vindoline (2) in *Lonicera* and *Catharanthus* plants.

So far as our experiments are concerned, the incorporation rates of 10-hydroxygeraniol (8) and 10-hydroxynerol (11) into secoiridoids and indole alkaloids were always much higher than those of 9,10-dihydroxygeraniol (9). Thus, the route involving cyclization of 10-oxogeranial (3)/10-oxoneral (4) to iridodial (5) is likely to be common in plants containing secoiridoids and indole alkaloids.

## EXPERIMENTAL

*General.* Silica SIL G/UV$_{254}$ (Machery–Nagel) Polygram and silica gel 60 GF$_{254}$ (Merck) were used for TLC and prep. TLC, respectively. $^1$H NMR were taken at 199.50 MHz in CDCl$_3$ with TMS as int. standard. $^2$H NMR spectra were recorded at 30.63 MHz: 8 K data points, 90° pulse, pulse delay 100 msec, aquisition time 2.048 sec, line broadening 0.5 Hz. $^{13}$C NMR spectra were measured at 50.23 MHz with TMS as int. standard under the following conditions: 16 K data points, 45° pulse, pulse delay 35.000 sec, aquisition time 0.8190 sec (gated decoupling-without-NOE); 16 K data points, 45° pulse, pulse delay 1.5 sec, aquisition time 0.8190 sec (complete decoupling). Radioactive samples were dissolved in liquid scintillator (10 ml) and $^3$H activity was measured by liquid scintillation counting. Activities were corrected by the int. standard method for self absorption. The rel. efficiencies were obtained by counting [U-$^3$H]-toluene standards (Amersham). TLC plates were scanned with a radioscanner.

*Administration of* [1-$^3$H]-10-*hydroxygeraniol* (8) *to* L. tatarica. *Expt* 1. A soln of [1-$^3$H]-8 (69.4 μCi) in MeOH (32 μl) was dil with H$_2$O (1.5 ml) and administered hydroponically to the twigs (1 g) of *L. tatarica* L. sp. pl. plants on Aug. 18, 1983. After 27 hr, the plants were extracted with 80% EtOH (10 ml × 2)

under reflux. The combined extracts were concd *in vacuo*. The residue was subjected to prep. TLC with MeOH–CH$_2$Cl$_2$ (3:17) to afford radioactive secologanin (1, 21 mg). This substance was dissolved in MeOH (5 ml) and stirred together with NaBH$_4$ (15 mg) at 0° for 15 min. After neutralization with 10% HOAc–MeOH, the soln was concd *in vacuo* to give a residue, whose acetylation yielded secologanol pentaacetate (10). The acetate was purified by prep. TLC (MeOH–CH$_2$Cl$_2$, 1:49) and recrystallization from MeOH to constant radioactivity (1.33 × 10$^7$ dpm). *Expt* 2. A soln of [1-$^3$H]-8 (89 μCi) in MeOH (46 μl) was diluted with H$_2$O (1.5 ml) and administered hydroponically to the twigs (2 g) of *Lonicera* plants on Aug. 30, 1983. After 28 hr, the plants were worked up in the same manner as above to give radioactive 1 (53.9 mg). This compound was converted into 10, which was recrystallized from MeOH to constant radioactivity (1.71 × 10$^6$ dpm).

*Administration of* [1-$^3$H]-9,10-*dihydroxygeraniol* (9) *to* L. tatarica. *Expt* 1. A soln of [1-$^3$H]-9 (58.9 μCi) in MeOH (19.6 μl) was diluted with H$_2$O (1.5 ml) and administered hydroponically to twigs (0.9 g) of *L. tatarica* plants for 27 hr on Aug. 18, 1983. Usual work-up afforded radioactive secologanin (1, 26 mg), which was converted into 11 (total activity 1.53 × 10$^6$ dpm). *Expt* 2. A soln of [1-$^3$H]-9 (91.0 μCi) in MeOH (33 μl), diluted with H$_2$O (1.5 ml), was administered hydroponically to the twigs (1.2 g) of *L. tatarica* plants for 28 hr on Aug. 30, 1983. Work-up gave radioactive 1 (43.3 mg), which was converted into 11 (total activity 9.36 × 10$^5$ dpm).

*Administration* [1-$^3$H]-10-*hydroxygeraniol* (8) *and* [1-$^3$H]-9,10-*dihydroxygeraniol* (9) *to hybrid of* C. roseus. *Expt* 3. A soln of [1-$^3$H]-8 (69.4 μCi) in MeOH (32 μl), diluted with H$_2$O (1.5 ml), was administered hydroponically to the terrestrial parts (1.5 g) of *C. roseus* (L.) G. Don plants at the flowering period on Aug. 20, 1983. After 28 hr, the plants were extracted with 80% EtOH (10 ml × 2) under reflux. The combined extracts, after addition of carrier vindoline (2, 11.8 mg), were concd *in vacuo*. The residue was submitted to prep. TLC with Me$_2$CO–petrol–Et$_2$NH (2:7:1). The band showing the same $R_f$ value as that of an authentic sample of 2 gave radioactive vindoline (2), which was recrystallized from Et$_2$O to constant radioactivity (3.99 × 10$^5$ dpm). A soln of [1-$^3$H]-9 (58.9 μCi) in MeOH (19.6 μl) was diluted with H$_2$O (1.5 ml) and administered hydroponically to the terrestrial parts (1.9 g) of *C. roseus* plants at the flowering period on August 20, 1983. After 28 hr, the plants were extracted with 80% EtOH (10 ml × 2). The combined extracts, after addition of carrier (10.5 mg), were concd *in vacuo*. The residue was worked up in the same manner as above to afford radioactive

**2**, which was recrystallized from Et$_2$O to constant radioactivity (7.02 × 10$^4$ dpm).

*Preparation of* [9-$^{13}$C]-10-*hydroxynerol* (**11**). A soln of neryl acetate (608 mg) in dry CH$_2$Cl$_2$ (30 ml) containing pyridine (0.25 ml) was cooled to −78° and treated with a stream of ozonized O$_2$ until the starting material became undetectable on TLC. After addition of Zn dust (1.42 g) and HOAc (5.5 ml), the reaction mixture was stirred for 2 hr. The solid material was filtered off and washed with *n*-hexane. The combined filtrate and washings were shaken successively with 10% NaHCO$_3$ and H$_2$O, dried and concd *in vacuo*. The residue was subjected to vacuum distillation to give (Z)-6-acetoxy-4-methyl-4-hexenal (157 mg) as a colourless oil. The soln of this compound in dry CH$_2$Cl$_2$ (2 ml) was added dropwise to (α-carboethoxy methyl-[$^{13}$C] etylidene)triphenylphosphorane (410 mg) under a N$_2$ atmosphere and the mixture stirred at room temp. for 16 hr. The soln was concd *in vacuo* and the residue purified by prep. TLC (*n*-hexane-Et$_2$O, 6 : 1) to furnish an oily substance, ethyl-(2E, 6Z)-8-acetoxy-2,6-dimethyl-2,6-octadienoate (200 mg). An aliquot (97 mg) of this compound was dissolved in dry Et$_2$O (5 ml) and added to a stirred suspension of LiAlH$_4$ (74 mg) and AlCl$_3$ (88 mg) in dry Et$_2$O (27 ml) at −15°. Stirring was continued for a further 1.5 hr at elevated temp. (−15°–0°) under N$_2$. After decomposition of excess reagent with H$_2$O, the mixture was neutralized with 5% HCl and extracted with Et$_2$O (5 ml × 4). The combined extracts were washed with brine, dried and concd *in vacuo*. The residue was purified by prep. TLC in Et$_2$O to give [9-$^{13}$C]-**11** (57 mg) as a colourless oil. Its IR and $^1$H NMR spectra were identical with those of an authentic sample.

*Administration of* [4-$^{13}$C]-10-*hydroxygeraniol* (**8**), [9-$^{13}$C]-10-*hydroxynerol* (**11**), [2-$^{13}$C]-9,10-*dihydroxygeraniol* (**9**) *and* [10-$^2$H$_2$]*iridodial* (**5**) *to* R. serpentina *suspension cultures*. A mixture of [4-$^{13}$C]-**8** (15.4 mg), [9-$^{13}$C]-**11** (16.0 mg), [2-$^{13}$C]-**9** (19.4 mg), and [10-$^2$H$_2$]-**5** (18.3 mg) was dissolved in 70% EtOH (12 ml) and administered to R. serpentina Benth. suspension cultures (eight flasks, each 280 ml medium). After 8 days of incubation, the cells (965.3 g) were frozen in liquid N$_2$ and extracted with EtOAc (1.5 l.) containing Pi buffer (1 M, pH 8.5; 100 ml) with stirring for 18 hr. The insoluble materials were filtered off and washed with EtOAc (400 ml). The combined EtOAc layers were extracted with 2% H$_2$SO$_4$ (400 ml × 2). The acid layer was basified with 10% NH$_4$OH at *ca* 5° and extracted with CHCl$_3$ (500 ml × 2). The CHCl$_3$ layer was washed with H$_2$O (100 ml × 2), dried and concd *in vacuo* to give an alkaloid mixture (481 mg), which was subjected to prep. TLC in Me$_2$CO–petrol–Et$_2$NH (2:10:1). Of two main bands, the more mobile band gave ajmaline (**12**, 85 mg) as colourless needles

(recrystallized from MeOH), whereas the less mobile one afforded vomilenine (**13**, 127 mg) as a pale-yellow amorphous powder.

*Catalytic hydrogenation of* [$^2$H, $^{13}$C]*vomilenine* (**13**). A soln of [$^2$H,$^{13}$C]-**13** (70 mg) in EtOH (3 ml) was hydrogenated over 10% Pd–C (100 mg) for 6 hr. The catalyst was filtered off and the soln concd *in vacuo* to give a residue, which after prep. TLC (Me$_2$CO–petrol Et$_2$NH, 2:10:1) yielded dihydrovomilenine (**14**, 16 mg) as a pale-yellow amorphous powder. $^1$H NMR (CDCl$_3$): δ0.98 (br t, J = 7 Hz, 8-H$_3$), 2.81 (dd, J = 12, 5 Hz, H-6), 4.35 (m, H-3), 5.02 (br s, H-7), 7.2–7.7 (m, aromatic H$_4$). $^{13}$C NMR (CDCl$_3$): δ11.9 (q, C-18), 21.2 (q, COMe ), 25.9 (t, C-19), 27.5 (d, C-15), 27.7 (t, C-14), 37.4 (t, C-6), 42.0 (d, C-16), 47.0 (d, C-20), 49.7 (d, C-3), 54.7 (d, C-5), 65.0 (s, C-7), 78.6 (d, C-17), 87.4 (d, C-21), 121.1, 123.7, 125.4 and 128.7 (d, C-9, C-10, C-11 and C-12), 136.1 (s, C-8), 156.6 (s, C-13), 169.9 (s, COMe), 182.9 (s, C-2). HRMS: 352.1776, Calc. for C$_{21}$H$_{24}$N$_2$O$_3$ (M$^-$) 352.1786.

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