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SYNTHESIS OF IRIDODIAL BY CELL FREE EXTRACTS FROM RAUWOLFIA SERPENTINA CELL SUSPENSION CULTURES

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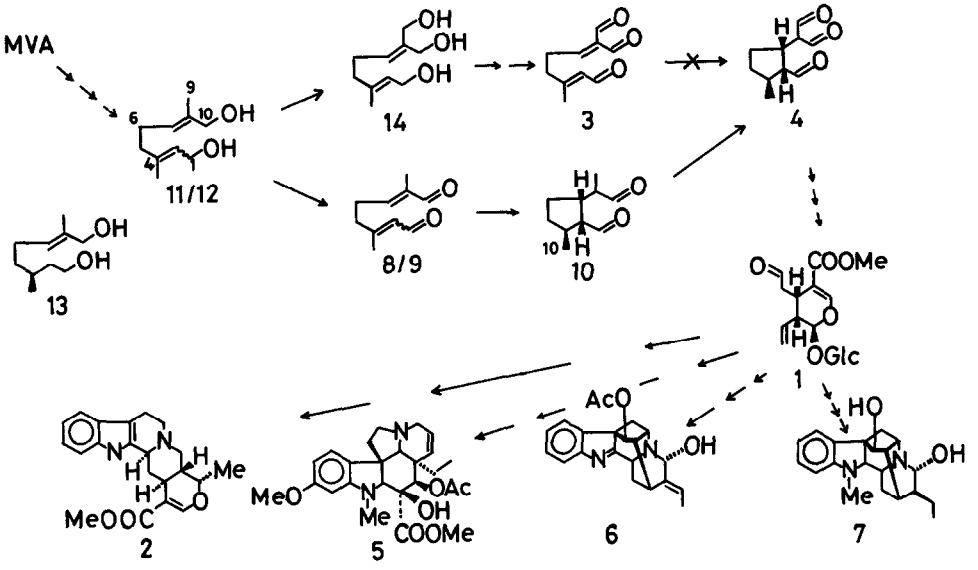
Summary: The cell free extracts of Rauwolfia serpentina cell suspension cultures converted 10-hydroxygeraniol/10-hydroxynerol into iridodial in the presence of oxidized and reduced pyridine nucleotides. However, neither 10-hydroxycitronellol nor 9,10-dihydroxygeraniol was transformed into monocyclic monoterpenes such as iridodial and iridotrial.

Regarding the formation of the iridane skeleton from acyclic monoterpenes in secologanin (**1**) and ajmalicine (**2**) biosynthesis, Balsevich and Kurz[1] proposed a mechanism involving the cyclization of 9,10-dioxogeranial (**3**) to iridotrial (**4**), in accordance with that assumed by Arigoni et. al.[2] In contrast, we have demonstrated through in vivo tracer studies that secologanin (**1**), vindoline (**5**), vomilenine (**6**) and ajmaline (**7**) are biosynthesized via cyclization of 10-oxogeranial (**8**)/10-oxoneral (**9**) to iridodial (**10**) in the plants of Catharanthus roseus[3,4], Lonicera morrowii[3], L. tatarica[4], Galium mollugo[5] as well as the cultured cells[4,6] of Rauwolfia serpentina.

Thus, for the unequivocal understanding of the iridane skeleton formation mechanism in the biosynthesis of the above-mentioned secoiridoid series compounds, enzymatic studies seem to be indispensable using an enzyme system capable of converting acyclic monoterpenes into monocyclic monoterpenes. We therefore attempted in vitro tracer experiments by preparing cell free extracts from R. serpentina cell suspension cultures[7], which produce a significant amount of indole alkaloids including **6** and **7**.

The cultured cells (fresh wt. 157 g) obtained 10 days after inoculation were homogenized in 0.1 M tris buffer (380 ml; pH 7.5, containing 5 mM mercaptoethanol) and centrifuged at 10^4 g. The supernatant (588 ml) was successively brought to 35% and 70% saturation with ammonium sulfate. The precipitate obtained by the 70% ammonium sulfate cut was dissolved in the above buffer and desalted by dialysis, leading to the cell free extract (11.6 ml, 10.3 mg/ml protein). Then, [1-^{3}H]-10-hydroxygeraniol (**11**)[3] (136 µl MeOH



solution, 200 µCi) was incubated in tris buffer (20 ml) (0.1 M, pH 7.5) containing each 40 µmol of ATP/Mg^{++}, NAD/NADH and NADP/NADPH at 25°C in the presence of 6.0 mg/ml protein. After 20 hr of incubation, the solution was extracted with CH_2Cl_2, and the residue of the CH_2Cl_2 extract was chromatographed on silica gel plates (20 x 20 cm) using CH_2Cl_2/MeOH (92:8). The radioactivities were located in bands (Rf 0.22 and 0.52) having the same Rf values as the starting material **11** and an enol-hemiacetal form of iridodial (**10**), respectively. Elution of the latter band with MeOH gave the conversion product (23.4 µCi, 11.7% of the radioactivity of the incubated [1-^{3}H]-**11**).

In order to examine the intermediary position of this product in the secoiridoid biosynthesis, an aliquot (6 µCi) was administered to the R. serpentina cell suspension cultures (1 flask, 20 ml medium) for 10 days. Vomilenine (**6**) and ajmaline (**7**) isolated from the cells (fresh wt. 15.92 g) were found to respectively retain 4.6% and 0.7% radioactivities of the compound fed. Thus, this conversion product is most likely a key intermediate formed by the cyclization of the acyclic monoterpene. The structure of the product was established in the following way: [4-^{13}C]-**11**[8] or [9-^{13}C]-10-hydroxynerol (**12**)[4] (each 0.2 mg) was incubated as described above in tris buffer (43 ml) containing 7.9 mg/ml protein. The CH_2Cl_2 soluble portion of the incubation mixture was subjected to GC-MS spectrometry. Fig. 1 illustrates the TIC of the CH_2Cl_2 soluble portion derived from the [4-^{13}C]-**11** incubation mixture. The peak at scan no. 249 was correlated to $(HOCH_2CH_2S)_2$ by its MS spectrum. Each of the peaks at approximately 329 was a mixture of unidentified compounds. The MS spectra (a and b) of the peak at 182 and the corresponding peak derived from

the [9-^{13}C]-**12** incubation mixture are presented in Fig. 2. For comparison, the MS spectrum (c) of a synthetic enol-hemiacetal form of iridodial (**10**)[9] is also shown in the same Figure. The spectrum (c) indicated a parent peak at m/z 168.1166 ($C_{10}H_{16}O_2$, Er. 1.6 MU), and the spectra (a and b) at m/z 169.1202 and 169.1183, respectively. Furthermore, in the ^{13}C NMR spectrum the CH_2Cl_2 soluble portion originating from the [4-^{13}C]-**11** incubation mixture showed a ^{13}C-enriched signal at 20.60 ppm, corresponding to the C-10 chemical shift of the synthetic iridodial (**10**). It was therefore deduced that the compound depicted to scan no. 182 could be the enol-hemiacetal form of ^{13}C-labelled iridodial (**10**) with the formula $C_9{}^{13}CH_{16}O_2$. Keeping this suggestion in mind,

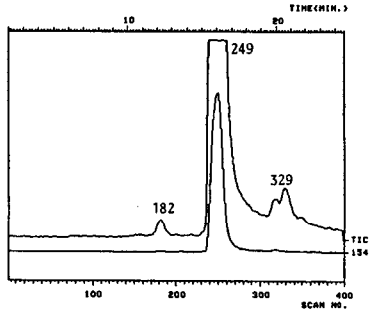


Fig. 1 TIC of the CH_2Cl_2 soluble portion of the [4-^{13}C]-**11** incubation mixture at 10^{-7} Torr.

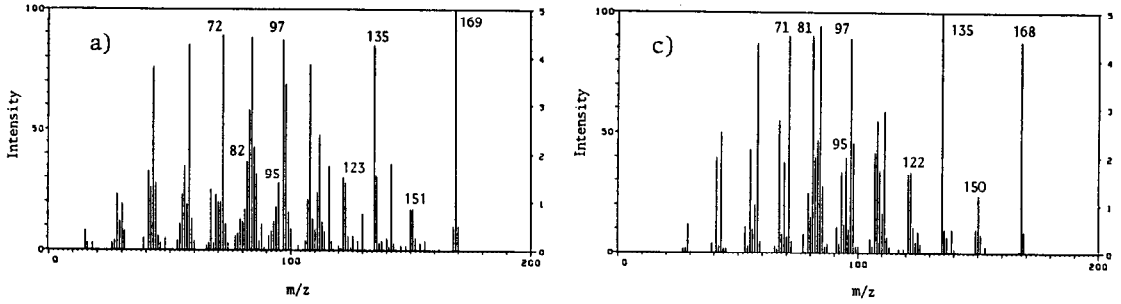


Fig. 2 GC-MS of the peak at 182 (a) in Fig. 1, the corresponding peak (b) derived from the [9-^{13}C]-**12** incubation mixture, and synthetic iridodial (10) (c).

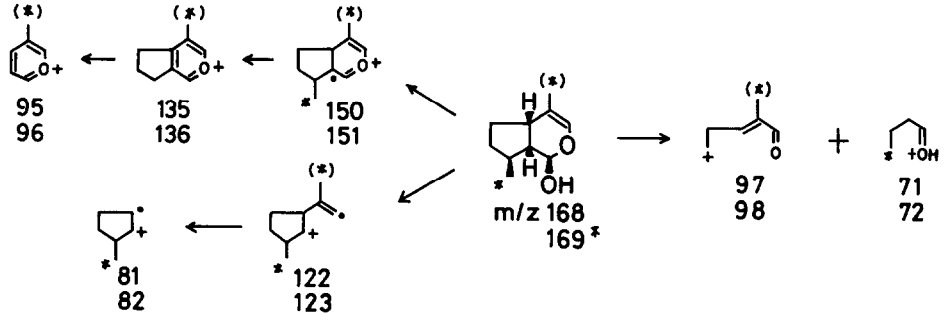


Fig. 3 Postulated fragmentation mechanism for iridodial (10).

the fragmentation patterns of the spectra (a and b) correspond with the mechanism postulated for the synthetic iridodial (**10**) shown in Fig. 3. Therefore, it was established that iridodial (**10**) is the intermediate precursor for **6** and **7** of the R. serpentina cell suspension cultures. It was also clarified that the enzyme system did not exhibit substrate specificity for the elaboration of **11** and **12** to **10**.

Additional incubation experiments afforded the following conclusion: i) neither (S)-(-)-[9-^{13}C]-10-hydroxycitronellol (**13**)[8] nor [2-^{13}C]-9,10-dihydroxygeraniol (**14**)[8] was converted into monocyclic monoterpenes such as labelled iridodial (**10**) and iridotrial (**4**) under the conditions described above, in accord with the evidence provided by our in vivo tracer studies[4,6]. ii) Even in the absence of ATP/Mg^{++} and with addition of ATPase to the usual incubation mixture (to decompose traces of endogenous ATP that may be present), the enzymatic conversion of **11** or **12** into **10** still proceeded. iii) Incubation of the cell free system containing both NAD/NADH and NADP/NADPH gave the higher ratio of conversion into **10** than any other combination of pyridine nucleotides. Thus, the cyclase involved was concluded to be a new type of monoterpenoid cyclase, differing from those so far known in that the former converts the oxo derivatives but not the pyrophosphates of the acyclic monoterpenes (**11** and **12**) into the monocyclic monoterpene (**10**).

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