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ELUCIDATION OF IRIDODIAL FORMATION MECHANISM - PARTIAL PURIFICATION AND CHARACTERIZATION OF THE NOVEL MONOTERPENE CYCLASE FROM RAUWOLFIA SERPENTINA CELL SUSPENSION CULTURES

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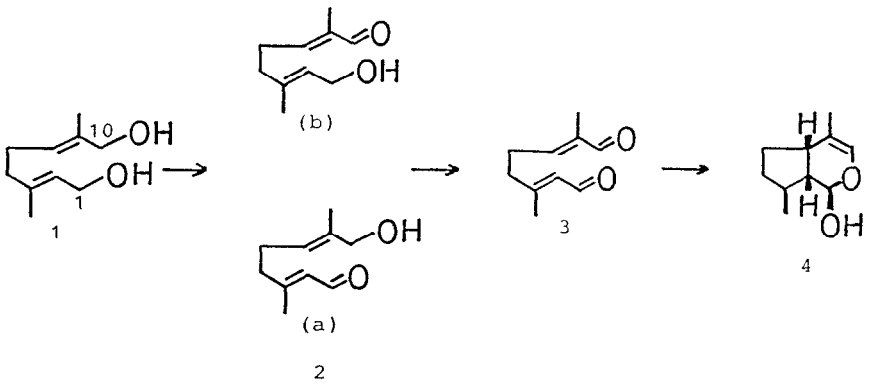
Summary: From the incubation system of 10-hydroxygeraniol and crude enzyme extracts from Rauwolfia serpentina cell suspension cultures, 10-oxogeraniol, 10-hydroxygeranial and 10-oxogeranial were isolated. A precursory role in the biosynthesis of iridodial has been demonstrated for these three compounds. This fact unequivocally substantiates our previous claim for the biosynthesis of iridodial. In addition, a novel monoterpene cyclase catalyzing the iridodial formation was partially purified and characterized.

In the preceding paper[1] we demonstrated that crude enzyme extracts from Rauwolfia serpentina cell suspension cultures[2] convert 10-hydroxygeraniol (**1**) into iridodial (**4**) in the presence of oxidized and reduced pyridine nucleotides. Thus, the enzyme involved in the above cyclization process was deduced to be a novel type of monoterpene cyclase differing from those thus far known in that it converts the aldehyde derivative 10-oxogeranial (**3**), but not pyrophosphate of the acyclic monoterpene 10-hydroxygeraniol (**1**), into the cyclic monoterpene iridodial (**4**)[1,3].

During the purification of the cyclase, we succeeded in trapping three intermediates between (**1**) and (**4**). We present here a demonstration of the intermediary roles of these compounds as well as a characterization of the partially purified cyclase.

[1-^{3}H]-10-Hydroxygeraniol (**1**) was incubated with the crude enzyme extract prepared according to the earlier reported procedure[1] in the presence of NAD and NADH at pH 6.5 (Expt. 1). After 2 hr, the incubation mixture was subjected to TLC on SiO_2 (CH_2Cl_2-EtOH, 95 : 5). The radiochromatogram showed a new peak in addition to those of the starting material (**1**) and iridodial (**4**). This new substance (**2**) was tentatively identified as the 1- or 10-monoaldehyde derivative of (**1**) or a mixture of both by comparison with the authentic samples on

TLC. Moreover, when [1-^{3}H]-(**1**) was incubated with the crude enzyme extract in the presence of only NAD at pH 6.5 (Expt. 2), not only (**2**) but also another conversion product identified by TLC as 10-oxogeranial (**3**) was formed. However (**4**) was not yielded. On addition of $NaBH_4$ to this incubation mixture, the peaks of (**2**) and (**3**) disappeared and instead the peak of (**1**) appeared again on a radiochromatogram. In order to demonstrate the intermediary positions of (**2**) and (**3**)[4] in the biosynthesis of iridodial (**4**), compounds (**2**) and (**3**) isolated from a large scale incubation were re-incubated with the crude enzyme extracts in the presence of NAD and NADH at pH 7.2, respectively (Expts. 3 and 4). In either incubation, radioactive iridodial (**4**) was formed. These results led to the conclusion that 10-hydroxygeraniol (**1**) is oxidized via (**2**) to (**3**), which then undergoes the reductive cyclization to iridodial (**4**) (Scheme 1).



Scheme 1. Biotransformation pathway from 10-hydroxygeraniol to iridodial.

The remaining enigma regarding the above transformation process is which of the two hydroxy groups of (**1**) is oxidized to the monoaldehyde derivative. This was approached in the following way. Non-labelled (**2**) obtained from the incubation of (**1**) under the same condition as in Expt. 2 was reduced by NaB^2H_4 to produce deuterium labelled (**1**). The ^{2}H-NMR spectrum ($CHCl_3$) showed two signals at δ 4.12 and δ 3.97 with an intensity ratio of 9 : 7, corresponding to 1-C^{2}HH and 10-C^{2}HH of (**1**). This result indicated that the monoaldehyde consists of 10-hydroxygeranial (**2a**) and 10-oxogeraniol (**2b**)[4,5] and suggested that the alcohol dehydrogenase involved in the crude enzyme extract has no specificity for oxidation of the hydroxy group of (**1**).

Subsequently the cyclase converting (**3**) into iridodial (**4**) was partially purified in the following manner for purposes of characterization. The crude enzyme extract obtained by the 35-70% $(NH_4)_2SO_4$ cut of a protein solution (in 20 mM Tris-HCl buffer containing 5 mM mercaptoethanol; pH 7.2) from Rauwolfia serpentina cell suspension cultures was subjected to ion exchange chromatog-

raphy on DEAE-Sephacel (with 0-500 mM NaCl gradient). The pooled active fractions were further fractionated by fast protein liquid chromatography (FPLC) on Phenyl-Superose (0-30% ethyleneglycol gradient) and an anion exchanger (Mono Q, 0-500 mM NaCl gradient) to yield a partially purified monoterpene cyclase. Fig. 1 illustrates the time course of the transformation of (3) into (4) by this enzyme. The specific activity of the enzyme was estimated at 7.90 nKat/mg protein. Thus, the enzyme was purified about 440-fold as compared with the crude enzyme extract obtained from the $(NH_4)_2SO_4$ treatment (spec. act. 0.018 nKat/mg protein). Examination of pyridine nucleotide specificity of the reductive cyclization (Table 1) resulted in the conclusion that NADPH most effectively accelerates the cyclization. Furthermore, as shown in Figs. 2 and 3, the optimal pH was found to be pH 7.0 and the optimal temperature 37 °C. The molecular weights of the cyclase and its subunit were determined to be 118,000 and 28,700 by means of gel filtration on a Sephadex G-100 superfine and SDS/polyacrylamide gel electropholysis, respectively.

From the evidence obtained so far, the mechanism for the iridane skeleton formation from an acyclic monoterpene in the biosynthesis of indole alkaloids of the Rauwolfia serpentina cell suspension cultures is becoming clear due to the use of enzyme preparations.

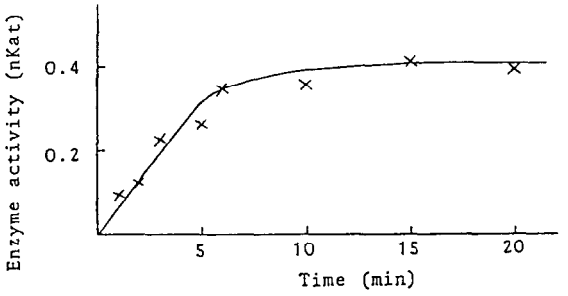


Fig. 1. Time course of the transformation of 10-oxogeranial into iridodial.

Substrate (5 µCi, 60 nmol) was incubated with the enzyme (0.45 µg/ml protein) in the presence of 20 mM Tris-HCl (pH 7.2) containing 5 mM mercaptoethanol and 0.25 µmol NADPH at 25°.

Table 1. Pyridine nucleotide specificity of the cyclization.

Coenzyme	NAD	NADH	NADP	NADPH
% Activity	3.6	53.0	4.1	100.0*

* One hundred percent activity represents 0.858 nKat/mg protein.

Assay was performed in the same manner as described in Fig. 1.

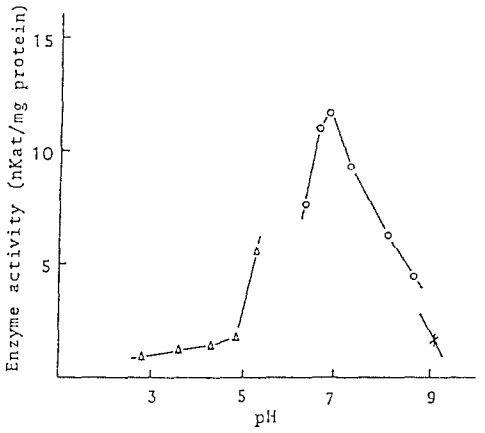


Fig. 2. The effect of pH on the cyclase activity. Buffers used: 0.1 M glycine buffer (-*-*-), 0.1 M Tris-HCl buffer (-o-o-) and 0.1 M citrate buffer (-Δ-Δ-).

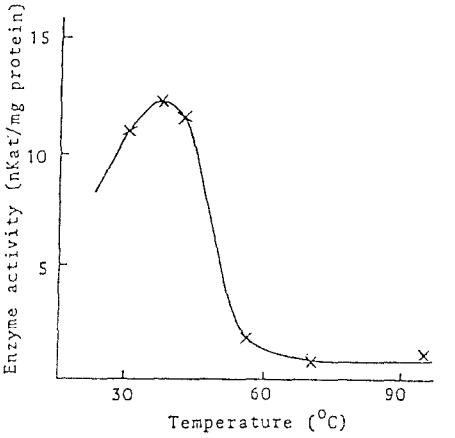


Fig. 3. Dependence of the cyclase activity on temperature.

Assay was performed in a 20 mM tris-HCl buffer at pH 7.2.

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