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BIOSYNTHESIS OF C-NOR-D-HOMO-STEROIDAL ALKALOIDS FROM ACETATE-1-^{14}C, CHOLESTEROL-4-^{14}C AND CHOLESTEROL-26-^{14}C IN *VERATRUM GRANDIFLORUM*

KEI KANEKO, HIROSHI MITSUHASHI, KOICHIRO HIRAYAMA and NAOTOSHI YOSHIDA

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

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Abstract—The incorporations of acetate-1-^{14}C, cholesterol-4-^{14}C, and cholesterol-26-^{14}C into the jerveratrum and ceveratrum alkaloids were shown in *Veratrum grandiflorum* Loesen. fil. The biosynthetic activity of veratramine was affected by the concentration of jervine in the plant organ which synthesizes the steroidal alkaloids.

INTRODUCTION

Veratrum plants contain five types of steroidal alkaloids, the solanidanine alkaloids (solanidine,[1] rubijervine,[2] isorubijervine,[3] and veralobine),[4] jerveratrum alkaloids (jervine (IV),[5,7] veratramine (V),[6,7] veratrobasine,[7] and verarine),[8] ceveratrum alkaloids (veratroylzygadenine[9] (X), veramarine,[10] etc.), 22,26-epiminocholestane alkaloid (verazine),[11] and 22,26-epimino-17β-methyl-18-nor-cholestane alkaloids (veralinine,[12] veralkamine,[13] and veramine).[14] The jerveratrum alkaloid and ceveratrum alkaloid have the naturally abnormal C-nor-D-homo-steroidal skeleton which is found only in some genera of Liliaceae.

The formation of the C-nor-D-homo-steroidal skeleton from normal steroids was established by Hirschmann[15] and Mitsuhashi,[16] but the biological rearrangement of *Veratrum* alkaloids from normal steroidal skeleton has not been established up to the present.

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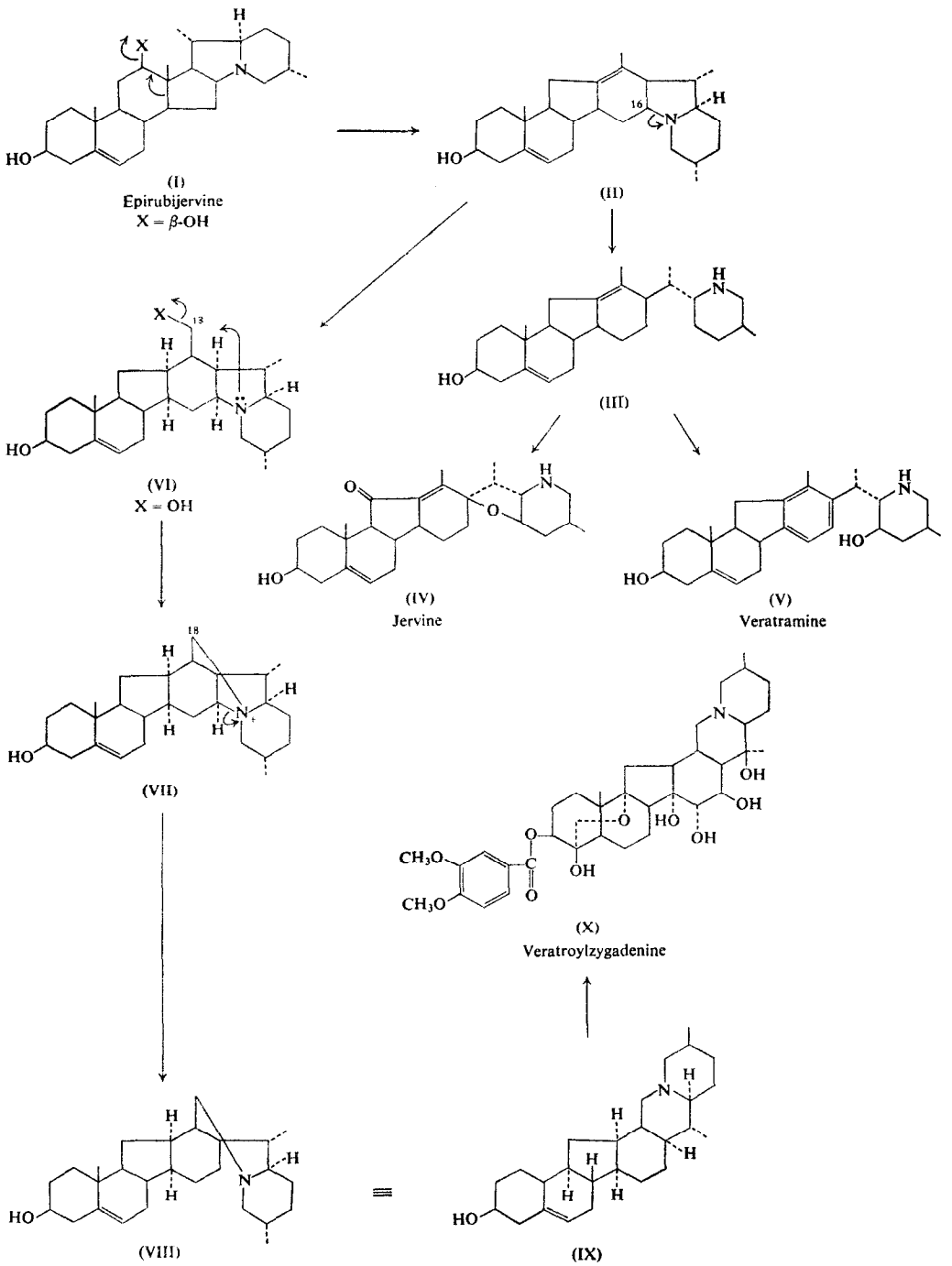

CHART I. HYPOTHETICAL PATHWAY OF *Veratrum* ALKALOID BIOSYNTHESIS.

Narayanan[17] has postulated that an equatorial leaving group or the carbonium ion at C-12 can rearrange epirubijervine (I) to a C-nor-D-homo-steroidal alkaloid (II) and the reductive cleavage of the C-16—N bond gives the skeleton of jerveratrum alkaloid (III), while the removal of leaving group from C-18 (VI) would introduce a C-18—N bond (VII), and the reduction of the quaternary salt so formed, via cleavage of the C-16—N bond, would readily produce the skeleton of ceveratrum alkaloid (VIII, IX) by the chemical rearrangement described by Hirschmann[15] and Pelletier.[18] The same mechanisms for the formation of C-nor-D-homo-steroidal skeleton may occur *in vivo*.

On the other hand, Tschesche[19–21] and Heftmann[22,23] have reported that cholesterol is incorporated into spirostane and spirosolane derivatives and solanidine. Thus, cholesterol is probably an important precursor in the biosynthesis of steroidal sapogenins and alkaloids in the plant kingdom. Therefore it is likely that cholesterol is a precursor in the biosynthesis of *Veratrum* alkaloids.

We have therefore decided to study three points in the biosynthesis of *Veratrum* alkaloids; (1) the conversion of acetate and cholesterol to solanidanine alkaloids, (2) the rearrangement of solanidanine alkaloid to the C-nor-D-homo-steroid skeleton, and (3) the formation of jerveratrum alkaloid and ceveratrum alkaloid after the C-nor-D-homo rearrangement. This report describes the incorporation of acetate-1-^{14}C, cholesterol-4-^{14}C, and cholesterol-26-^{14}C into jerveratrum alkaloids and ceveratrum alkaloids in *Veratrum grandiflorum*.

RESULTS

Incorporation of Acetate-1-^{14}C at Different Growing Stages

Veratrum rhizome stored at 0° after budding was cultivated in quarter strength Hoagland solution under constant aeration. At four different stages, 50 μc of acetate-1-^{14}C was fed to plant by the improved cotton-wick method, and the plant was allowed to grow another 10

TABLE 1. INCORPORATION OF ACETATE-1-^{14}C AT DIFFERENT GROWING STAGES

	Incorporation ratio (%) cultivation time before feeding with acetate-1-^{14}C (weeks)			
Plant parts	1	2	3	4
Root	0·01	0·003	0·005	0·007
Leaf	0·87	0·024	0·043	0·09
Rhizome	0·99	0·30	0·32	0·38
Whole plant	1·87	0·33	0·37	0·48

The incorporation ratio is expressed as $\frac{\text{radioactivity of crude alkaloid}}{\text{radioactivity of the fed acetate-1-}^{14}\text{C}} \times 100$. The plants were cultivated for 10 days after feeding with acetate-1-^{14}C.

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days. The plant was harvested, dried, and the crude alkaloid was isolated by the method of Jacobs.[25]

The maximum incorporation of acetate-1-^{14}C into the crude alkaloid was found in plants which had been kept in cultivation for 1 week. Since these plants are too soft and small for administration of radioactive compound, plants which had been cultivated for 10 days were subsequently used for all feeding experiments.

Biosynthesis of Veratrum *Alkaloids from Acetate*-1-^{14}C

From 22 *Veratrum* plants which were fed with a total of 1·1 mc of acetate-1-^{14}C (50 μc/plant), a crude alkaloid mixture was isolated and purified by the method of Jacobs.[25] Acetate-1-^{14}C was incorporated into solanidanine, jerveratrum, and ceveratrum alkaloids in almost the same order. However, the incorporation ratio of acetate-1-^{14}C was somewhat different between jervine (IV) and veratramine (V) when the experiment was performed with a single plant, as shown in Table 3.

TABLE 2. INCORPORATION OF ACETATE-1-^{14}C INTO *Veratrum* ALKALOID

Alkaloid	Yield (mg)	Radioactivity (dis/min/mmole × 10^{-5})
Rubijervine	7	9·2
Jervine (IV)	700	5·2
Veratramine (V)	677	4·0
Veratroylzygadenine (X)	110	2·2

Twenty-two *Veratrum* plants were cultivated for 10 days, allowed to grow 10 days fed with 1·1 mc of acetate-1-^{14}C.

TABLE 3. DIFFERENCE IN THE INCORPORATION OF ACETATE-1-^{14}C INTO JERVINE (IV) AND VERATRAMINE (V)

Expt. No.	Alkaloid	Radioactivity (dis/min/mmole × 10^{-5})
1	Jervine (IV)	12·30
	Veratramine (V)	5·40
2	Jervine (IV)	6·93
	Veratramine (V)	2·68

Difference in Incorporation Ratio Between Jervine and Veratramine Biosynthesis

Veratrum rhizomes harvested in summer are in the resting stage, when the stem and the leaf are withered and decayed, and contain almost equal amounts of jervine (IV) and veratramine (V), whereas *Veratrum* rhizomes and young leaves in the early spring contain much more of (IV) than of (V). Since the biosynthetic route to these alkaloids is mainly the same as that to the common steroids, differences in the incorporation rate would be expected with the age of the rhizome. Indeed, feeding acetate-1-^{14}C to rhizomes at different stages, led to an inversion of the incorporation ratios (Table 4). Since the content of alkaloid, i.e. mainly reserve alkaloid, in the rhizome changes with the weight of the rhizome, the difference in the radioactivity in both alkaloids in the two experiments may be due to dilution by the reserve alkaloid.

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TABLE 4. CHANGE IN V/J RATIO WITH THE PERIOD OF CULTIVATION

Precursor	μc	Feeding period (days)	Radioactivity (dis/min/mmole × 10^{-5})		V/J ratio
Acetate-1-^{14}C	50	10	Jervine (IV)	6·93	0·38
			Veratramine (V)	2·68	
Acetate-1-^{14}C	50	28	Jervine (IV)	2·74	1·43
			Veratramine (V)	3·93	

The incorporation ratio of acetate-1-^{14}C into jervine (IV) and veratramine (V) changed following a long period of administration with acetate-1-^{14}C. Moreover, the administration of acetate-1-^{14}C with additional jervine stimulated synthesis of veratramine (V), as shown in Table 5.

TABLE 5. CHANGE OF V/J RATIO BY THE ADMINISTRATION OF JERVINE

Expt. No.	Composition of feeding solution	Feeding period (days)	Radioactivity (dis/min/mmole × 10^{-5})		V/J ratio
1	50 μc of acetate-1-^{14}C	10	Jervine (IV)	6·93	0·38
			Veratramine (V)	2·68	
2	50 μc of acetate-1-^{14}C plus 5 mg of Jervine	10	Jervine (IV)	1·40	2·34
			Veratramine (V)	3·42	
3	50 μc of acetate-1-^{14}C plus 10 mg of Jervine	10	Jervine (IV)	3·56	12·20
			Veratramine (V)	44·60	

It appears that the newly synthesized jervine (IV) from acetate-1-^{14}C, i.e. radioactive jervine (IV), is diluted with the fed jervine with acetate-1-^{14}C, but in Expt. 3 the V/J ratio increased about 32-fold over Expt. 1. These results indicate that the elevation of the V/J ratio is due to the increase in synthesis of veratramine (V) and not to the dilution effect of the fed jervine.

Biosynthesis of Veratrum *Alkaloid from Cholesterol*

The administration of cholesterol to a plant is difficult because it is quite water insoluble. Heftmann[23] sprayed cholesterol-4-^{14}C on the plant leaf as a 0·05% solution in Tween 20 and he succeeded in getting an incorporation of about 0·8% cholesterol into steroidal sapogenins in *Lycopersicon pimpinellifolium*.

Comparison was made on the use of cholesteryl-4-^{14}C-3-phosphate in 5% Tween 80 solution and cholesterol-26-^{14}C in 5% Tween 80 solution. 5% Tween 80 solution did not cause morphological damage nor decrease the rate of biosynthesis of steroidal alkaloid in *Veratrum* plant.

TABLE 6. INCORPORATION OF CHOLESTEROL INTO JERVINE (IV) AND VERATRAMINE (V)

Precursors	Radioactivity of alkaloids (dis/min/mmole × 10^{-4})	
	Jervine	Veratramine
Cholesterol-4-^{14}C-3-phosphate, 4·6 × 10^{7} dis/min	1·3	2·0
Cholesterol-26-^{14}C, 1·1 × 10^{8} dis/min	1·1	1·4

The incorporation of cholesterol-^{14}C was about 0·01 % for the two alkaloids, but some questions arise as to (1) how much cholesterol-^{14}C is able to reach the synthetic organ of the alkaloid in the leaf, because of its insolubility in plant sap, and (2) whether cholesterol is capable of becoming the direct precursor in *Veratrum* or not, because some intermediates in cholesterol biosynthesis are probably converted into *Veratrum* alkaloids.

DISCUSSION

Tschesche and Heftmann observed that cholesterol-4-^{14}C is incorporated into spirostane[19,23] and spirosolane[22] derivatives, and solanidine.[20] This observation suggests that the synthetic pathway of cholesterol from acetate or mevalonate occupies the main route to natural steroidal alkaloid or sapogenin. It is reasonable to assume that the *Veratrum* plant synthesizes jerveratrum alkaloid and ceveratrum alkaloid from acetate or cholesterol. As acetate-1-^{14}C was incorporated into jerveratrum, ceveratrum, and solanidanine alkaloids in the same order, these steroidal alkaloids seem to be synthesized by the common route of steroidal biosynthesis.

The incorporation of acetate-1-^{14}C into jervine is higher than that into veratramine in short-term feeding experiments, while veratramine biosynthesis surpasses jervine biosynthesis in long-term experiments. Such a phenomenon suggests that the product of the C-nor-D-homo rearrangement from epirubijervine (I) is converted into a key intermediate, which is then converted into jervine (IV) and veratramine (V) by separate pathways. This hypothesis is supported by the results of the exchange of V/J ratios obtained from the incorporation of acetate-1-^{14}C when plants were fed acetate-1-^{14}C with jervine. Thus the biosynthesis of jervine is controlled by the amount of jervine present in the plant; this is the same as the allosteric effect known in animals and in microorganisms. A result reported in the following paper supports more directly the presence of a key intermediate, because 11-deoxojervine-^{14}C is incorporated into jervine but not into veratramine. However, we have not yet succeeded in isolating this compound from *Veratrum*.

EXPERIMENTAL

Plant Material

The rhizomes of *Veratrum grandiflorum* Loesen. fil. were harvested at Teine, Hokkaido, Japan, in summer. The plant was already withered except for the rhizome. The rhizomes were washed with the tap-water, surrounded with horticulture moss (commercial), and stored at 1–3° in a cold room. After 5 months, the rhizomes are capable of budding when transferred to a quarter strength Hoagland solution, at 15–25° with forced aeration. The bud can grow in this solution at the same rate as in nature, whereas it grows very slowly in soil. After cold treatment, the rhizomes are covered with cracked ice and stored in the cold room to suppress budding; they have the ability to grow, for several months. When cultivating the rhizomes, they were irradiated for 12 hr/day with artificial light placed 20 cm from each plant.

Administration of the Radioactive Compound

The young bud which will grow next season is located on the top of rhizome, and surrounded by the base of the leaves which connect with the rhizome at the foot of the young bud. From the surface of rhizome, a fine hole was made to its center from beneath the young bud, using a fine drill. Two or three cotton-wicks were passed through a moistened polyethylene tube (No. 25) into the plant. A solution of 50 μc of acetate-1-^{14}C was dissolved in 0·5 ml of H_2O and fed through the wick from a small test-tube. This solution was absorbed by the plant in 1 day, and for the complete absorption of the tracer, 1 ml of H_2O was then fed in. 10 mg of jervine was dissolved in 2·0 ml of 0·2 M citric acid and adjusted to pH 4·5 by the dropwise addition of 0·2 M triammonium citrate. When some turbidity appeared, the solution was clarified with a few drop of 5% Tween 80 solution, and final vol. was made to 5·0 ml. The solution was placed into the feeding tube. When the plant absorbed 0·5 vol. of this solution, 50 μc of acetate-1-^{14}C was added to the tube.

Synthesis of Cholesteryl-4-^{14}C-3-phosphate[24]

A solution of 10 mg of cholesterol (100 μc) dissolved in 0·17 ml of pyridine was mixed with 0·07 ml of a solution of 0·09 ml of $POCl_3$ and 0·6 ml pyridine, and the mixture was stored overnight at 0°. The reaction mixture was poured into 5 ml of water, refluxed for 1·5 hr, and then concentrated under a reduced pressure. The residue was dissolved in benzene and purified by column chromatography on silica gel (200 mg). Elution with benzene gave 2·9 mg of cholesteryl-4-^{14}C-3-phosphate (yield, 25%). A solution of 2·9 mg of cholesteryl-4-^{14}C-3-phosphate dissolved in 0·1 ml of 0·35 N KOH in 5% Tween 80 solution and cholesterol-26-^{14}C dissolved in 5% Tween 80 solution were placed into the feeding tube.

Isolation and Purification of Radioactive Alkaloids

Isolation of *Veratrum* alkaloids mainly followed the method of Jacobs.[25] The dried rhizomes from twenty-two plants, weighing 880 g, were extracted with ammoniacal CH_2Cl_2–MeOH(1:1), yielded 80 g of crude extract in CH_2Cl_2. This solution was extracted with 1·2 l. 5% tartaric acid, the aqueous phase was made alkaline with NaOH, and the alkaline solution was reextracted with CH_2Cl_2. This extract dissolved in 6·8 ml of AcOH was added to a mixture of 27 ml of H_2O and 20 ml of saturated $(NH_4)_2SO_4$. The precipitate was washed with 10 ml of saturated $(NH_4)_2SO_4$ solution and then suspended with 20 ml of water. The suspension was extracted with $CHCl_3$ after being made alkaline with 10% NaOH, the resulting residue gave 4 g. The residue was placed on an Al_2O_3 column, and eluted in turn with 700 ml of benzene, 300 ml of 10% ether in benzene, 400 ml of 20% ether in benzene. Jervine was obtained from the fraction of 10% ether in benzene, then crystallized from MeOH, gave 700 mg, m.p. 243–245°. Veratramine was obtained from the fraction of 20% ether in benzene and crystallized from MeOH, gave 677 mg, m.p. 205–207°.

The soluble fraction with $(NH_4)_2SO_4$ gave 5 g of alkaloid fraction, which was purified by alumina column chromatography. The benzene fraction gave 1·7 g of rubijervine fraction and the methanol fraction gave veratroylzygadenine fraction. The benzene fraction was purified by repeated column and TLC and 7·0 mg of rubijervine, m.p. 240–242°, was isolated. Veratroylzygadenine fraction was purified by the repeated column and TLC and gave 110 mg of veratroylzygadenine (X), m.p. 260–263°.

The crude alkaloid fraction, when radioactive compound was fed to a single plant, was purified by alumina chromatography at first and then by repeated TLC on Silica Gel HF_{254} (*n*-hexane–EtOH–Et_2NH, 8:1:1) to separate jervine (IV) and veratramine (V).

Determination of Radioactivity

The radioactivities of all the samples were determined by liquid scintilation counter (Aloca LSC 501 Liquid Scintillation Counter, Tokyo, Japan) in toluene scintillator. Activity due to ^{14}C was determined simultaneously, by external standardization counting with ^{60}Co.