ENZYMIC SYNTHESIS OF LOGANIN BY CARBOXYL GROUP METHYLATION OF LOGANIC ACID*

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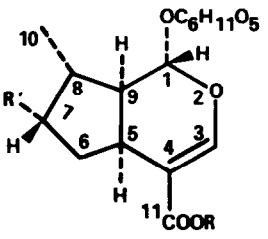
1. Introduction

We have found that mevalonic acid-2-^{14}C is incorporated into the aglucone moiety of the iridoid glucoside, loganic acid (fig. 1), in both flowering *Vinca rosea* and germinating seedlings [1]. Loganin, its methyl ester, serves as a precursor of the nontryptophan moiety of three different classes of indole alkaloids in the same plant [2, 3]. However, the occurrence of 7-deoxyloganin in *V. rosea* has also been reported and this glucoside was found to be hydroxylated to loganin in vivo. Since tracer studies in a related species, *Menyanthes trifoliata,* have revealed that the *O*-methyl group of loganin is derived from the methyl of methionine [4], it was of interest to determine which monoterpene, loganic acid or 7-deoxyloganic acid, is methylated prefentially in *V. rosea.*

* Part IV in a series on "Monoterpene Biosynthesis"; Part III: ref. [1].



Loganic Acid R=H, R'=OH
Loganin R=CH_3, R'=OH
7-Deoxyloganic Acid R=R'=H
7-Deoxyloganin R=CH_3, R'=H

Fig. 1.

2. Materials and methods

Tender leaves and shoots from 4–8 month old *V. rosea* plants were used for the preparation of cell-free extracts. Loganic acid was isolated from *Swertia caroliniensis* and purified as previously described [6].

Plant material was ground in liquid nitrogen and extracted with 3 volumes of 0.2 M phosphate buffer, pH 7.2 containing 0.01 M sodium metabisulfite and 1 mM dithiothreitol (DDT). To this Polyclar AT (tissue: Polyclar AT, 4:1 w/w) was added and the extract squeezed through two layers of silk [7]. After centrifugation of the homogenate at 10,000 *g* for 20 min, the supernatant was fractionated on a column of Bio-gel P_{10} eluting with 0.1 M phosphate buffer, pH 7.2, containing 1 mM DTT. To the first eluant after the void volume, 3 volumes of acetone were added at −15°. The precipitate thus obtained was freed from acetone and dissolved in 0.1 M phosphate buffer, pH 7.2, containing 1 mM DTT. Denatured protein was removed by centrifugation and the clear supernatant was used for the enzymatic assays. Protein concentration was measured by the Lowry method [8].

Assays were conducted as mentioned in table 1 and the reaction was terminated by addition of 1 ml of methanol. Carrier loganin (0.5 mg) was added and the mixture centrifuged. The aqueous supernatant was subjected to preparative thin-layer chromatography [5], ion-exchange chromatography [5] and descending paper chromatography (Whatman no. 1 paper, developed with butanol–acetic acid–water, 15:1:4 v/v/v). In the latter loganin (R_f 0.56) was visualized with a vanillin reagent [9] and radioactive peaks were

Table 1
Incorporation of methyl-^{14}C of *S*-adenosyl-L-methionine into loganin.

Experiment	Loganin formed (dpm/mg prot./hr)	
	Crude cell-free extract	Acetone-treated enzyme
Complete system	1707	3250
plus boiled enzyme	155	126
less DTT	620	–
DTT replaced by NADPH+FAD (2.5 μmoles each)	1122	–

The assay mixture contained potassium phosphate buffer, pH 7.1, 25 μmoles; loganic acid, 2.0 μmoles; DTT, 4.0 μmoles; $MgCl_2$, 1.0 μmole; S-AM, 200 nmoles, 140,000 dpm and enzyme (crude cell-free extract, 450 μg, or acetone-treated enzyme, 300 μg) in total volume of 450 μl. The reaction mixture was incubated at 32–33° for 3 hr.

located using a Packard Radiochromatogram Scanner Model 7201. For quantitative assessment the radioactive peak corresponding to loganin was cut out and counted in a Packard Liquid Scintillation Counter Model 3380.

Initially the identity of the reaction product as loganin-^{14}C was further established by crystallization to constant specific activity after addition of carrier loganin (10 mg). In a representative experiment the specific activities of loganin in the third, fourth and fifth recrystallizations were 590, 567 and 570 dpm/mg respectively (2.22×10^5 dpm/mmole). The recrystallized loganin-^{14}C was also acetylated and recrystallized to constant activity as loganin pentaacetate (356, 354 and 359 dpm/mg in the second, third and fourth recrystallizations respectively, 2.14×10^5 dpm/mmole).

3. Results

As seen in table 1, cell-free extracts of *V. rosea* contain a methyl transferase capable of converting loganic acid into loganin in the presence of *S*-adenosyl-L-methionine (S-AM). The latter may be replaced by methionine and ATP although a smaller fraction of acid is methylated. Cell-free extracts prepared in the presence of DTT were nearly four times more active than extracts isolated in the presence of sodium metabisulfite alone. In the assay equimolar amounts of mono-sulfhydryl group reagents such as mercaptoethanol, reduced glutathione or cysteine were not capable of replacing DTT, whereas a combination of NADPH and FAD could partially substitute for the dithiol with the crude cell-free extract (table 1). Under these same assay conditions no significant methylation of 7-deoxyloganic acid was observed.

Fig. 2(a) reveals the effect of pH on the enzymatic formation of loganin. The crude methyl transferase has a pH optimum between 6.9 and 7.4. Fig. 2(b) shows the time course of incorporation of methyl-^{14}C of *S*-adenosylmethionine into loganin. Maximum incorporation occurs in about 3 hr.

4. Discussion

To our knowledge this is the first methyl transferase capable of converting the carboxyl group of a low molecular weight compound into a methyl ester in higher

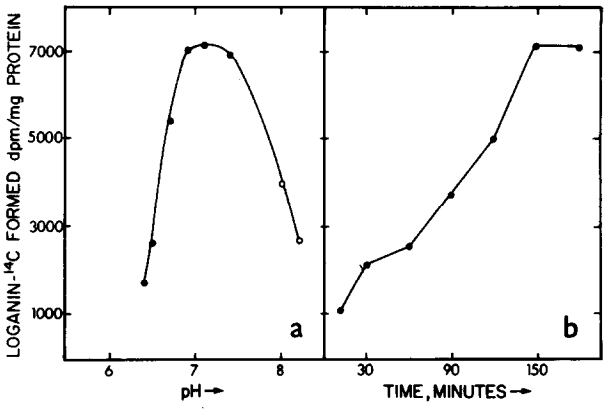


Fig. 2(a). The effect of pH on methyl transferase activity. The assay mixture contained in a total volume of 450 μl: buffer, 30 μmoles (pH as indicated ○—○ tris-HCl; ●—● phosphate); DTT, 4 μmoles; $MgCl_2$, 1.0 μmole; S-AM 200 nmoles (140,000 dpm); loganic acid, 2.0 μmoles and acetone precipitated enzyme, 1.5 mg; incubated at 32–33° for 3 hr. (b) Time course of methylation of loganic acid. The assay mixture contained in a total volume of 450 μl: phosphate buffer, pH 7.2, 30 μmoles; $MgCl_2$, 1.0 μmole; DTT, 4.0 μmoles; loganic acid, 2.0 μmoles; S-AM, 200 nmoles (140,000 dpm) and acetone-treated enzyme, 500 μg; incubated at 32–33° for different time intervals.

plants. The methylation of the carboxyl groups of pectin by *S*-adenosyl-L-methionine has been shown to occur on the polysaccharide [10]. Recently synthesis of methyl esters of fatty acids by extracts of *Mycobacterium phlei* with *S*-adenosyl-L-methionine serving as methyl donor has been observed [11].

The evidence presented above suggests the importance of the presence of reduced sulfhydryl groups for enzymatic activity. This is supported by preliminary results indicating enzyme inhibition by *p*-chloromercuribenzoate, iodoacetamide and *N*-methylmaleimide. The specific requirement for DTT may be due to its strong reducing potential. DTT has been found to be the most suitable reducing agent for other methyl transferases [12]. Alternatively the enzyme may contain sulfhydryl groups which can form disulfide bridges and thus are more facilely reduced by dithiols. Reduced lipoic acid also restores methyl transferase activity. Preliminary studies indicate that the effect of FAD and NADPH decreases with increasing purification of the enzyme. This is not observed with DTT or lipoic acid. Further studies on the role of sulfhydryl groups are in progress.

In summary the ability of partially purified transferase to methylate loganic acid but not 7-deoxyloganic acid re-emphasizes the intermediacy of the former in indole alkaloid biosynthesis.

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