The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture

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Abstract Secologanin is the iridoid building block of the majority of the terpenoid indole alkaloids. In the biosynthesis of secologanin, mevalonate was considered to be the exclusive precursor of isopentenyl diphosphate. After [1-^{13}C]glucose feeding to a cell culture of *Catharanthus roseus*, its incorporation into secologanin was studied by ^{13}C NMR spectroscopy. The data showed that the novel triose phosphate/pyruvate and not the mevalonate pathway was the major route for the biosynthesis of secologanin.

Key words: Secologanin; Iridoid; Alkaloid; Biosynthesis; Isopentenyl diphosphate; 1-Deoxy-D-xylulose

1. Introduction

Isoprenoids comprise the largest class of secondary metabolites found in higher plants. It was generally assumed that mevalonate was the exclusive precursor of these substances, although mevalonate and acetate were poorly incorporated into some mono- and diterpenes [1]. Recently, an alternative pathway for isoprenoid biosynthesis was discovered and partially elucidated in bacteria [2,3] and was found to be also operative in higher plants [4]. In this alternative, triose phosphate/pyruvate pathway, 1-deoxy-D-xylulose and not mevalonate is the precursor involved in the formation of isopentenyl diphosphate.

Secologanin is a biogenetic key isoprenoid glucoside, a polyfunctional molecule which occupies a central position in several biosynthetic pathways and acts as a starting compound for a multitude of other natural products. It is the ultimate precursor of the C9-C10 moiety common to the majority of the indole alkaloids and of some quinoline and isoquinoline alkaloids. Secologanin is of value not only because of its importance in alkaloid biosynthesis, but also as a synthon for the synthesis of compounds of pharmacological interest. So far, the isolation from plant materials (e.g. *Lonicera* sp., *Symphoricarpus* sp.) constitutes the major source of secologanin [5]. In *Catharanthus roseus* cell cultures, the biosynthesis of secologanin is usually limiting for alkaloid accumulation [6].

The terpenoid origin of secologanin was deduced from feeding experiments in which mevalonate was used in the late 1960s, while its biosynthesis from geraniol has been investigated in a number of in vivo tracer experiments and enzymatic studies [6–8]. Nowadays it is accepted that the pathway passes through 10-hydroxygeraniol, 10-oxogeranial, iridodial, iridotrial, loganic acid and loganin [9]. In this pathway, substantial data from tracer experiments for the intermediacy of mevalonate were obtained, although with low incorporation rates [10–12]. However, it was recently shown that in some essential oils, geraniol was not of mevalonoid origin but derived from 1-deoxy-D-xylulose [13]. 1-Deoxy-D-xylulose was also the predominant precursor of phytol, β-carotene and lutein whereas the mevalonate metabolic products were preferentially channeled into the biosynthesis of sitosterol in *C. roseus* [14]. These data indicate that also for the biosynthesis of secologanin, the involvement of the alternative pathway should be considered.

This paper describes the studies with a cell suspension culture of *C. roseus* on the possible involvement of the triose phosphate pathway in the biosynthesis of secologanin. This particular cell culture was used because of its high level of secologanin accumulation. After growth in a [1-^{13}C]glucose enriched medium, secologanin was isolated and analyzed by ^{13}C NMR spectroscopy. It could thus be concluded whether under the conditions utilized, the biosynthesis of secologanin was from the mevalonate pathway, the triose phosphate pathway, or possibly from the two pathways.

2. Materials and methods

2.1. Cell suspension culture

Cell suspension cultures of *C. roseus* (cell line A_{11}) accumulating secologanin [15] were maintained in B5 medium (B5 salts [16], 0.1 g/l *myo*-inositol, 10 mg/l thiamine, 1 mg/l pyridoxine, 1 mg/l niacin, 1.86 mg/l NAA, 20 g/l sucrose). They were subcultured every 3 weeks by inoculation of 8–9 g of biomass in 250 ml flasks containing 50 ml of medium. Culture conditions: temperature 25 ± 1°C, light 2800 lux 24 h/day on gyratory shaker at 80 rpm. For the experiment, 12 g per flask of biomass was subcultured on 50 ml of the same medium, but containing 10 g/l sucrose and 10 g/l glucose instead of 20 g/l of sucrose. The content of secologanin decreased after the first subculture in the new medium, as measured by HPLC [15]. However, after six subcultures, the initial content of secologanin gradually recovered (ca. 87 μg secologanin/g fresh weight) and became stable. No secologanin was ever detected in the medium. After the stabilization of the content of secologanin, two batches of 20 flasks grown for 15 days in non-labelled medium were utilized as controls. For the incorporation experiment, one batch of 20 flasks grown under the same conditions but in medium containing 10 g/l sucrose and 10 g/l [1-^{13}C]glucose (Isotec, Miamisburg, OH) was used.

2.2. Isolation of secologanin

Cells were separated from the medium by filtration and 460 g of fresh biomass was extracted exhaustively with acetone. The volume of the extract was reduced to approximately 100 ml under reduced pressure at 40°C. This extract was saturated with sodium chloride and put on ice for 15 min. The non-polar fraction was precipitated and the

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Abbreviations: MVA-5PP, mevalonate-5 diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate

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aqueous phase was separated by filtration on glass-fiber filter 200 (Schleicher and Schuell, Dassel, Germany). The aqueous phase was extracted exhaustively with a mixture of chloroform/methanol (3:1 v/v). The organic phase was evaporated to dryness and the residue was dissolved in methanol and mixed with 500 mg of kieselgel 60 (70–230 mesh) at 40°C until dryness. This material was applied on a column (1.2×11 cm) pre-packed with 5 g kieselgel 60 (70–230 mesh) in ethyl acetate. The column was eluted with 10 ml ethyl acetate and 10 ml of each of the following acetone mixtures in ethyl acetate: 5%, 10%, 30%, 50% (v/v). These fractions were discarded and secologanin was obtained from a subsequent elution with 30 ml of 50% (v/v) acetone in ethyl acetate. The secologanin fraction was dried and the residue was redissolved in 2 ml of water and freeze-dried. The yield of pure secologanin was 3 mg, corresponding to a recovery of about 7.5% of the total content of secologanin present in the fresh weight extracted.

2.3. NMR spectroscopy

^{1}H and ^{13}C NMR spectra of secologanin were recorded in D_2O using a Bruker DPX 300 spectrometer equipped with an Indy (Silicon graphics) computer. ^{13}C NMR spectra were measured as follows: 38° pulse (4 μs); repetition time, 2 s; spectral width, 18.83 kHz; data set, 32 kilo-words; temperature, 20°C; line broadening, 2.5 Hz; ^{1}H decoupling by WALTZ 16 during acquisition and relaxation. Two-dimensional experiments and data processing routines were performed according to standard Bruker software (XWINNMR 2.1). All ^{13}C NMR signals of secologanin were assigned unequivocally on the basis of two-dimensional $^{1}H^{13}C$ correlation experiments in conjunction with ^{1}H homocorrelation experiments. ^{1}H decoupled ^{13}C NMR spectra of the sample from the incorporation experiment and of controls were recorded under identical conditions. In the incorporation experiment, the ^{13}C abundance of the carbon showing the lowest intensity was assumed to have the same intensity as found in the controls. Both were set at 1, the other signals were expressed as relative to these signals.

3. Results and discussion

The mevalonate and the alternative pathway each will result in a specific labelling pattern of IPP and DMAPP when feeding experiments with [1-^{13}C]glucose are performed [2–4]. On the basis of these data, the labelled positions of secologanin from [1-^{13}C]glucose can be predicted (Fig. 1).

Coscia et al. [10,11] suggested that randomization of the terminal dimethyl groups of DMAPP in loganic acid occurs after geranyl diphosphate formation from [2-^{3}H,2-^{14}C]-evalonate. The label found in the corresponding positions of loganic acid was not, however, equally distributed between the two positions; it was thought that the equilibrium was age dependent or would occur for only a fraction of the molecules in the pool, causing a preferential label. Also in the case of intermediacy of 1-deoxy-D-xylulose for the biosynthesis of secologanin, this effect would produce a ^{13}C label in both C9 and C10 positions of secologanin in different proportions.

An important step in the formation of secologanin is the enzymatic methylation of loganic acid to loganin, being the methyl group donated by *S*-adenosyl methionine. In the biosynthesis of methionine, the 1-^{13}C from glucose is incorporated into the β-carbon of serine which is first transferred to tetrahydrofolate and then to homocysteine to form methionine [17]. According to this mechanism, the carbon 11 of secologanin should thus also be enriched.

For the glucosyl moiety of secologanin, by the activities of aldolase and triose phosphate isomerase, two positions are expected to be enriched, namely carbons 1′ and 6′ [17]. Carbon 1′ is expected to have the highest ^{13}C enrichment concerning the direct incorporation of the [1-^{13}C]glucose into the iridoid pathway.

The results obtained from the control experiments and from the [1-^{13}C]glucose incorporation experiment are presented in Table 1.

The enrichments obtained were in accordance with the predictions for the intermediacy of 1-deoxy-D-xylulose, although some discrepancies among the incorporation values were observed.

In the control experiments, the signals of the glucosyl moiety showed higher intensities than those of the iridoid moiety (Table 1). This was caused by the fact that this iridoid contains an aldehyde function which can undergo reversible hydration in aqueous solution; also a reversible enolization of the aldehyde function can take place. Thus, not one but several interconvertible forms of secologanin exist in solution, which will have different shifts for the carbon signals but will all share the same signals for the glucosyl moiety.

If secologanin was formed exclusively from mevalonate, the carbon atoms 1, 3, 6 and 8 should not or only slightly be enriched. However, their relative intensities varied between 1.6 and 7.0. The carbons which would present enrichments only from the mevalonate pathway showed relative intensities of 2.1 (C2), 2.5 (C5) and 1.0 (C7), while the carbons which would present enrichments only from the alternative pathway showed relative intensities of 7 (C1) and 4.7 (C6) (Table 1).

From the incorporation experiment, two unforeseen positions of the glucosyl moiety of secologanin were significantly labelled, namely positions 3′ and 4′. The incorporation of intermediates of the citric acid cycle into glucose by gluconeogenesis can explain this event. This is considered the main pathway for the net synthesis of glucose after the direct gluconeogenesis from pyruvate [17]. In the citric acid pathway, oxaloacetate formed from the pyruvate obtained after the glucolysis of [1-^{13}C]glucose would form malate labelled in either of the two carbonyl groups. Malate can leave the mitochondria and undergo oxidation to oxaloacetate in the cytoplasm, where the formation of phosphoenol pyruvate takes place, regenerating glucose labelled in positions 3 and 4. If glucose labelled in these positions was able to be attached as

Table 1
^{13}C NMR analysis of secologanin from *C. roseus* cells after growth in non-labelled glucose or [1-^{13}C]glucose enriched medium

Position	ppm	Relative intensity of the signal			Increase in relative intensity (= I.E./C)
		C	ΔC	I.E.	
1	97.74	1.28	0.16	7.00	**5.48**
2	44.51	1.45	0.14	2.13	1.47
3	133.59	1.27	0.00	2.42	1.90
4	121.51	1.19	0.10	6.04	**5.08**
5	207.60	1.32	0.04	2.49	1.89
6	44.53	1.24	0.12	4.70	**3.79**
7	27.46	1.00	0.00	1.00	1.00
8	109.52	1.02	0.08	1.57	1.54
9	153.94	1.43	0.12	5.24	**3.66**
10	170.20	0.90	0.04	4.19	**4.66**
11	52.52	1.42	0.04	7.20	**5.06**
1′	99.48	1.40	0.02	10.39	**7.42**
2′	73.27	2.12	0.20	2.75	1.30
3′	76.32	1.95	0.32	4.27	2.18
4′	70.24	2.24	0.14	5.04	2.25
5′	77.04	2.44	0.52	2.74	1.12
6′	61.37	1.93	0.28	9.45	**4.91**

C: average from two control experiments with non-labelled glucose; ΔC: difference between the two control experiments; I.E.: incorporation experiment with [1-^{13}C]glucose.

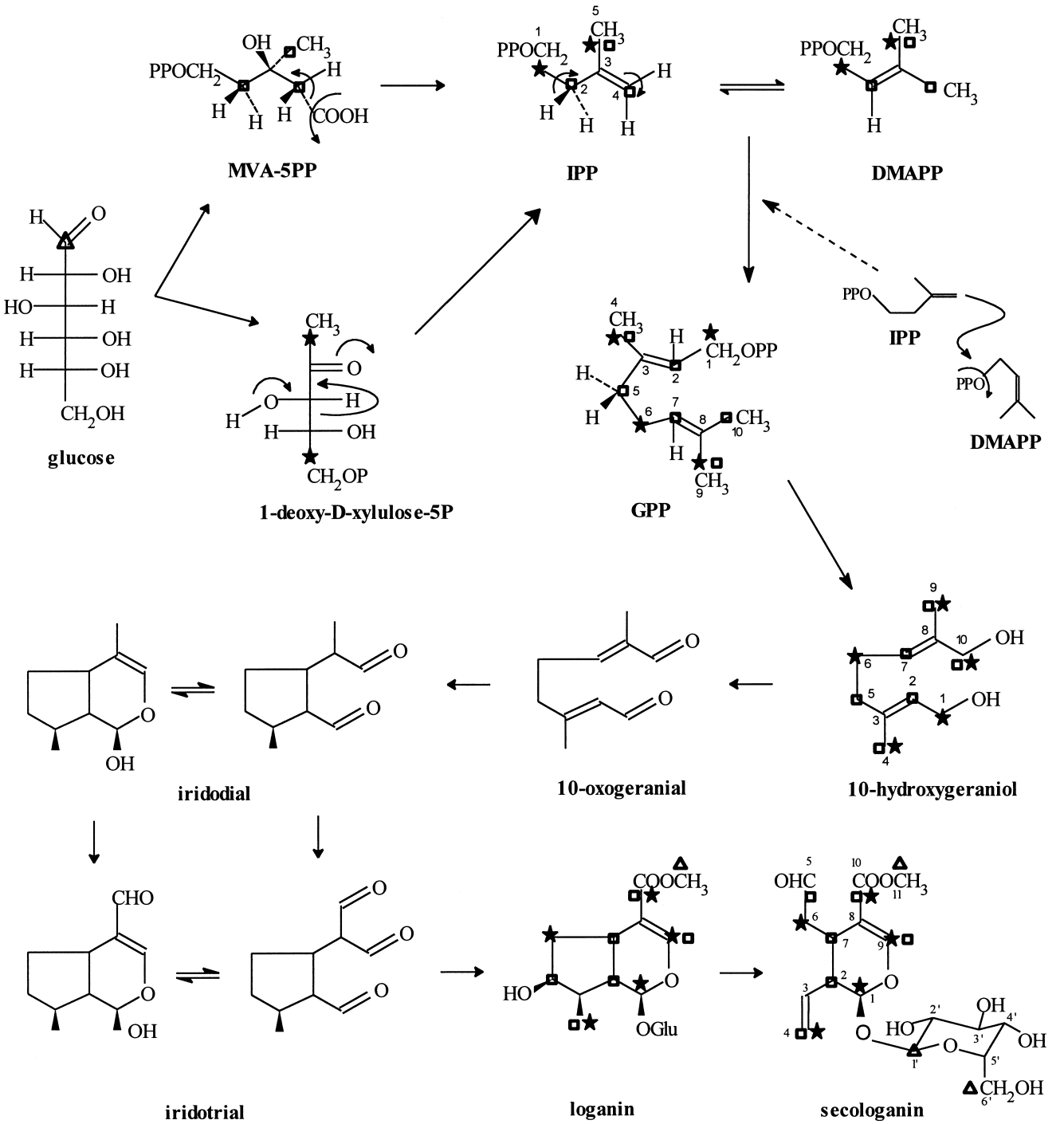

Fig. 1. Biosynthesis of secologanin from [1-^{13}C]glucose (△). In the case of intermediacy of mevalonate, carbons (□) are enriched. In the case of intermediacy of 1-deoxy-D-xylulose, carbons (★) are enriched.

such into secologanin, also new formation of IPP from [1,3,4,6-^{13}C]glucose could take place. Consequently, IPP formed from 1-deoxy-D-xylulose would also be labelled in position 4, though to a lesser extent. If the label in the acetyl-CoA which also enters the citric acid pathway was considered, positions 2 and 5 of glucose would be enriched. However, this effect was observed only in a minor degree, suggesting that the acetyl-CoA label is more extensively randomized prior to its reincorporation into glucose.

In conclusion, the data obtained clearly demonstrate the major participation of the new triose phosphate/pyruvate and not of the mevalonate pathway for the biosynthesis of secologanin in our *C. roseus* cell culture. However, one cannot ignore the extensive data indicating mevalonate as a minor precursor. The operation of each of the two pathways may depend on a particular physiological state of the cells. It still needs to be studied whether the availability of IPP from either mevalonate or 1-deoxy-D-xylulose determines which precursor is used by the cells for iridoid biosynthesis. Studies with different cell lines and under different conditions could help us to

attain a better understanding of the co-existence and possible interaction of the two pathways, as described for phytol, β-carotene, lutein and phytosterols in *C. roseus* cultured cells [14], for bisaboloxide A and chamazulene in chamomile [18], and for chlorophyll and β-carotene in cultured cells of liverworts [19].

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