Enzymatic Synthesis of 4′- and 3′,4′-Hydroxylated Flavanones and Flavones with Flower Extracts of *Sinningia cardinalis*

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Flowers of *Sinningia* (syn. *Rechsteineria*) *cardinalis* contain glycosides of the flavones apigenin (4′-OH) and luteolin (3′,4′-OH) respectively, and of the related 3-deoxyanthocyanidins apigeninidin and luteolinidin. Studies on substrate specificity of the key enzyme of flavonoid biosynthesis, chalcone synthase, revealed that the 3′,4′-hydroxylated flavonoids are formed by hydroxylation of flavonoid compounds rather than by incorporation of caffeoyl-CoA into the flavonoid skeleton during the condensation reaction. In fact, flavonoid 3′-hydroxylase activity could be demonstrated in the microsomal fraction of the flower extracts. The enzyme catalyses hydroxylation of naringenin and apigenin in the 3′-position to eriodictyol and luteolin, respectively, with NADPH as cofactor. Besides flavanone 3′-hydroxylase a further NADPH-dependent enzyme activity (flavone synthase II) was observed in the microsomal fraction catalysing the oxidation of naringenin to apigenin and of eriodictyol to luteolin. The Cytochrome P-450 inhibitor ancymidol was found to abolish completely flavone synthase II activity, whereas flavonoid 3′-hydroxylase activity was not impaired.

Introduction

Flowers of the Gesneriaceae *Sinningia* (syn. *Rechsteineria*) *cardinalis* are known to contain glycosides of the 4′- and 3′,4′-hydroxylated 3-deoxyanthocyanidins apigeninidin and luteolinidin, respectively and of the related flavones apigenin and luteolin [1, 2]. Using flower extracts of this plant we could recently demonstrate for the first time an enzyme which catalyses reduction of the flavanones naringenin and eriodictyol to the respective flavan-4-ols apiforol and luteoforol. The latter compounds are the immediate precursors for the 3-deoxyanthocanidins apigeninidin and luteolinidin (Fig. 1).

Now we investigated the enzymatic formation of apigenin and luteolin and the determination of the B-ring hydroxylation pattern of both 3-deoxyanthocyanidins and flavones.

Two types of enzymes are known to catalyse flavone formation from flavanones. The enzyme from parsley cell suspension cultures requires 2-oxoglutarate, Fe^{2+} and possibly ascorbate as co-factors for this reaction classifying it as belonging to the 2-oxoglutarate-dependent dioxygenases [3]. In contrast, in flowers of a range of plants [4–6] and in osmotically stressed soybean cell cultures [7] flavone formation is catalyzed by an NADPH-dependent microsomal enzyme now called flavone synthase II.

There are also two different ways for the determination of the B-ring hydroxylation pattern of flavonoids. In most cases the 3′-hydroxyl group is introduced by hydroxylation of flavonoid compounds (mainly naringenin and dihydrokaempferol) the reaction being catalyzed by flavonoid 3′-hydroxylase, an NADPH-dependent microsomal enzyme [3, 5, 8–13]. But there are also a few examples [5, 14, 15], where the key enzyme of flavonoid biosynthesis, chalcone synthase, uses caffeoyl-CoA besides *p*-coumaroyl-CoA as substrate for the condensation reaction leading to the formation of eriodictyol (3′,4′-OH) besides naringenin (4′-OH) (Fig. 1).

In the present paper we report that both flavone formation and introduction of the 3′-hydroxyl group are catalyzed by NADPH-dependent microsomal enzymes. The use of suitable substrates and of the cytochrome P-450 inhibitor ancymidole allowed a separation of both enzyme activities.

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Materials and Methods

Plant material

The scarlet flowers of the commercial strain "Feuerschein" (Fa. Walz, Stuttgart) of the Gesneriaceae *Sinningia* (syn. *Rechsteineria*) *cardinalis* were used for the investigations. The plants were cultivated in a greenhouse.

Chemicals and synthesis of substrates

Naringenin, eriodictyol, apigenin and luteolin were obtained from Roth (Karlsruhe, FRG). 4-Coumaroyl-CoA were from our laboratory collection. [2−^{14}C]Malonyl-CoA (2.22 GBq/mmol) was obtained from Amersham-Buchler (Braunschweig, FRG) and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. [4a,6,8−^{14}C]Naringenin (3.09 GBq/mmol) was prepared from [^{14}C]malonyl-CoA and 4-coumaroyl-CoA with enzyme preparation from parsley containing chalcone synthase and chalcone isomerase activity [3, 16]. Further transformation to [^{14}C]apigenin was achieved by addition of the co-factors 2-oxoglutarate and Fe^{2+} for flavone formation with parsley extracts to the chalcone synthase assay [3]. Labelled naringenin and apigenin were purified by TLC on cellulose plates with 15% acetic acid. Hydroxylation of labelled naringenin in 3′-position to eriodictyol was performed with partially purified enzyme preparations from *Petunia hybrida* as described earlier [12, 17]. Ketoconazole, ancymidol and tetcyclacis were kind gifts from H. Grisebach (Freiburg, FRG).

Preparation of enzyme extracts

All steps were carried out at 4 °C. Four g flowers (1.5−2 cm long, top still closed) were homogenized in a prechilled mortar together with 2 g PVP, 2 g quartz sand and 12−16 ml K-Pi buffer, pH 7.5, containing 10% saccharose and 7 mM 2-mercaptoethanol. The homogenate was centrifugated for 20 min at 12,000 × *g*. The clear supernatant was used as crude extract and for the preparation of the microsomal fraction by Mg^{2+}-precipitation according to ref. [8, 12].

In case of chalcone synthase, saccharose and 2-mercaptoethanol in the buffer were replaced by 20 mM Na-ascorbate [16].

Enzyme assays

Standard procedures were used to measure the enzyme activity and the pH optimum of chalcone synthase [10, 18], flavonoid 3′-hydroxylase [8, 12] and flavanone oxidase [4]. The assay systems for the latter two enzymes contained 0.06 nmol (167 Bq) radioactive substrate (naringenin, eriodictyol or apigenin).

After chromatographic separation of substrates and products, radioactivity was localized by scanning the plates (TLC analyzer, Berthold, Wildbad, FRG) and enzyme activity was determined by integration of the peak areas of the substrate and the product. In case of chalcone synthase, the radioactive zone containing the flavanone was scraped off and counted in Unisolve in a scintillation counter. Because no by-products were formed, the amount of flavanone could also be measured directly in the ethyl acetate extracts in Unisolve.

Analytical methods

Protein content of both crude extract and microsomal fraction was determined by a modified Lowry procedure [17].

Flavonoids (substrates and products) were separated on precoated cellulose TLC plates (Merck, Darmstadt, FRG) using the following solvent systems: (1) chloroform/acetic acid/water (10:9:1, v/v/v), (2) 30% acetic acid, (3) acetic acid/HCl/water (30:3:10, v/v/v) and (4) n-butanol/acetic acid/water (4:1:5, v/v/v, upper phase). The reaction products formed were identified by co-chromatography with authentic samples.

Flavonoids were detected under UV-light, after fuming with ammonia and by spraying the plates with 0.1% fast blue B salt and subsequent exposure to ammonia vapors. Flavanones were also detected by reduction with borohydride and subsequent exposure to HCl fumes [19].

Results

I. Determination of the B-ring hydroxylation pattern

Flavonoids with 3′,4′-hydroxylation pattern can be formed either by incorporation of caffeic acid during synthesis of the flavonoid skeleton catalyzed by chalcone synthase or by hydroxylation of flavonoids in the 3′-position catalyzed by flavonoid 3′-hydroxylase. The investigation of both enzyme reac-

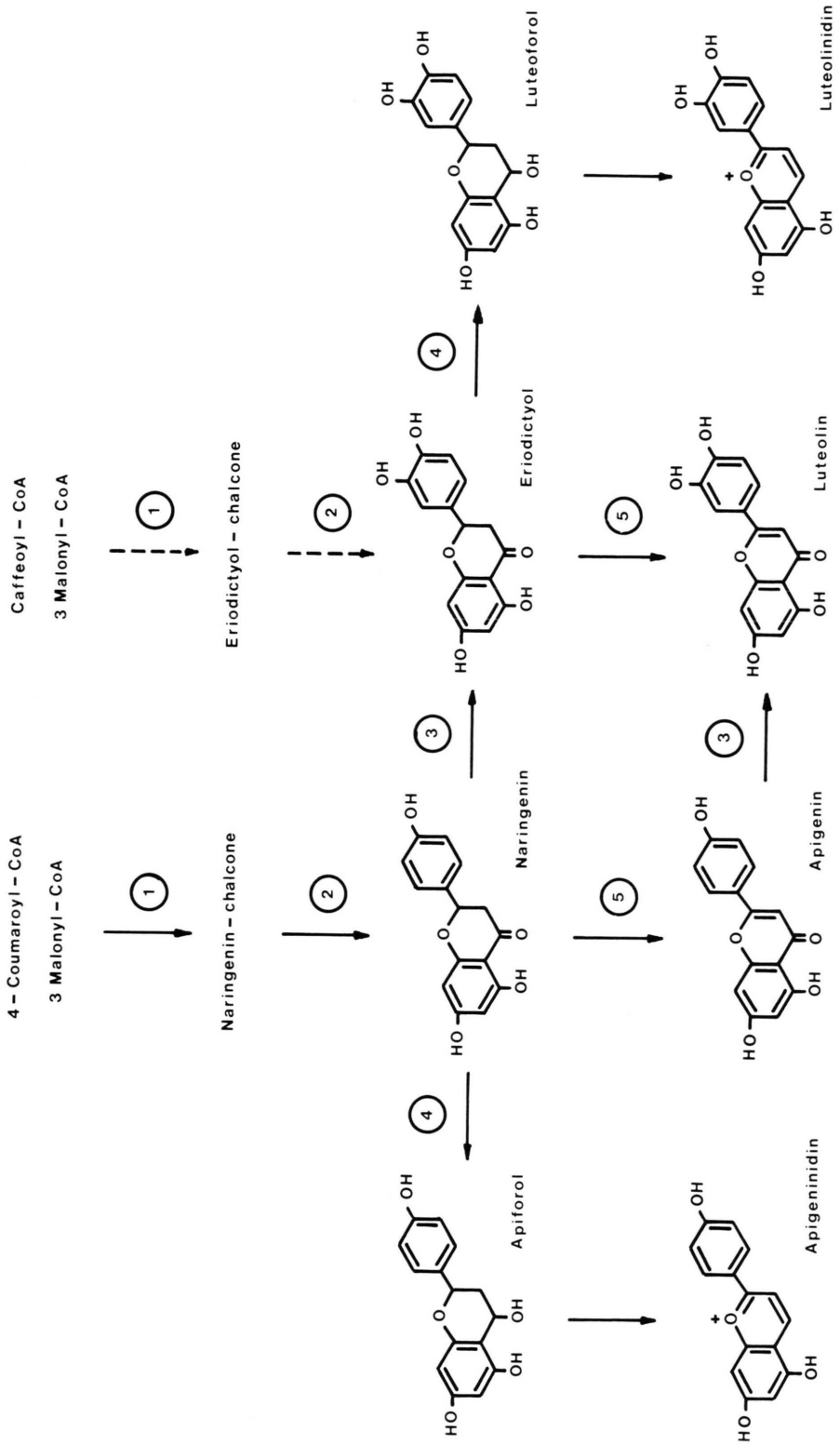

Fig. 1. Structural formulas and the biosynthetic pathway of flavonoids in flowers of *S. cardinalis*. **1** Chalcone synthase; **2** chalcone isomerase; **3** flavonoid 3′-hydroxylase; **4** flavanone 4-reductase; **5** flavanone oxidase (flavone synthase II).

tions should reveal at what stage of flavonoid biosynthesis the introduction of the 3′-hydroxyl group is achieved.

Chalcone synthase reaction

Incubations of flower extracts from *S. cardinalis* with [^{14}C]malonyl-CoA and 4-coumaroyl-CoA led to the formation of a reaction product which corresponded to authentic naringenin (Fig. 1) in four different solvent systems (Table I). Because chalcone isomerase activity is also present in the flower extracts, the flavanone and not the isomeric chalcone is found in the reaction mixture. Formation of by-products was not observed. This allowed a direct measurement of naringenin synthesis in the ethyl acetate extracts of the reaction mixture.

Highest formation of naringenin was found at pH 8.1. When caffeoyl-CoA was used as substrate instead of 4-coumaroyl-CoA in enzyme assays of pH 8.1, no reaction product was found to be formed. In agreement to other reports on substrate specificity of chalcone synthase [5, 10, 20], however, product formation from caffeoyl-CoA could be observed at lower pH with a maximum at pH 6.6. The product was identified as eriodictyol (Fig. 1) by co-chromatography with the authentic compound (Table I).

Similar to chalcone synthase from other plants, naringenin formation was about 1.6 times higher than eriodictyol synthesis at the respective pH optima and at pH 6.6 formation of naringenin was only somewhat lower than that of eriodictyol. Moreover, in the presence of both activated cinnamic acids in an enzyme assay, about 90% naringenin and only 10% eriodictyol were found to be formed at pH 6.6 and no eriodictyol synthesis was again observed at pH 8.1.

Demonstration of flavonoid 3′-hydroxylase

The weak eriodictyol formation by chalcone synthase in particular in the presence of both activated cinnamic acids suggests an introduction of the 3′-hydroxyl group by a specific hydroxylase at the stage of flavonoid compounds. This reaction is known to be catalyzed by the NADPH-dependent microsomal enzyme flavonoid 3′-hydroxylase.

When [^{14}C]naringenin was incubated with microsomal preparation from flowers of *S. cardinalis* in the presence of NADPH, three radioactive products were observed on radiochromatograms of the reaction mixture (Fig. 2). The main product was identified as apigenin (Fig. 1) by co-chromatography (Table I). The other two products corresponded in four solvent systems to eriodictyol and luteolin, respectively (Fig. 1, Table I). This compounds are obviously synthesized by a combined action of flavonoid 3′-hydroxylase and the enzyme for flavone formation, flavanone oxidase, which has the same subcellular localisation and co-factor requirement as the flavonoid 3′-hydroxylase (see below).

Besides naringenin apigenin was found to be hydroxylated in the 3′-position leading to luteolin. This allowed investigation of the 3′-hydroxylation reaction separately from the relatively high enzyme activity for flavone formation. As expected, flavonoid 3′-hydroxylase activity was localized in the microsomal fraction (Table II). In crude extracts, nearly no product formation could be found. The reaction was strictly dependent on NADPH. Substitution

Table I. R_f-values (× 100) of substrates and products of the enzymatic reaction.

Compound	Solvent systems			
	1	2	3	4
Naringenin	78	59	91	92
Eriodictyol	56	52	81	87
Apigenin	69	18	76	85
Luteolin	37	9	58	83

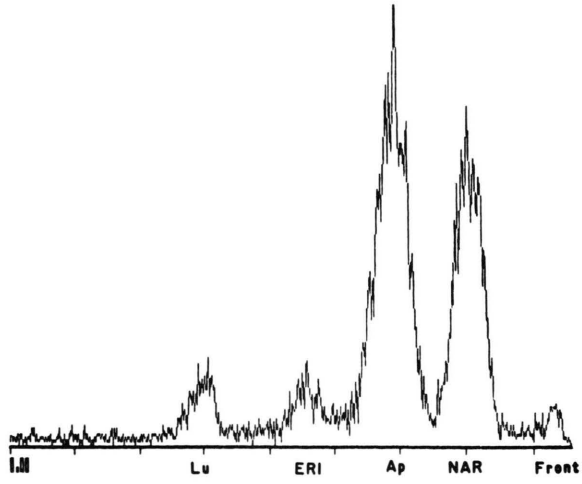

Fig. 2. Radioscan of TLC on cellulose with solvent system 1 from incubation of [^{14}C]naringenin in the presence of NADPH with microsomal fraction from flowers of *S. cardinalis*. NAR, Naringenin; Ap, Apigenin; ERI, Eriodictyol; Lu, Luteolin.

Table II. Subcellular localization and co-factor requirement of flavonoid 3′-hydroxylase activity and flavone synthase II activity with apigenin and eriodictyol, respectively, as substrate.

Enzyme source	Co-factor added	Formation of luteolin by	
		3′-Hydroxylase (μkat/kg protein)	Flavanone oxidase (μkat/kg protein)
Crude extract	none	0	0.02
	NADPH	0.01	0.59
Supernatant of microsomal pellet	NADPH	0.01	0.39
Microsomal pellet	none	0	0
	NADPH	0.19	4.54
	NADH	0.01	0.40

of NADPH by NADH strongly reduced the enzyme activity (Table II).

At 25 °C and with apigenin as substrate, the reaction was linear with protein concentration up to about 30 μg microsomal protein per assay. Linearity with time was observed for at least 30 min. Similar to flavonoid 3′-hydroxylase from other plants [8–13], the pH optimum of the reaction was at pH 7.5. Microsomal preparations containing 10% saccharose could be frozen in liquid nitrogen and stored at −80 °C without loss of enzyme activity.

Enzymatic 3′-hydroxylation was partially inhibited by KCN, *p*-chloromercuribenzoate N-ethylmaleimide and diethylpyrocarbonate (Table III). Complete and nearly complete inhibition was found with the cytochrome P-450 inhibitors ketoconazole and tetcyclacis, respectively. Surprisingly, however, the cytochrome inhibitor ancymidole did not influence 3′-hydroxylation activity but inhibited completely flavone formation (Table III). Thus, in the presence of 50 μM ancymidole in the enzyme assays, 3′-hydroxylation of naringenin to eriodictyol could be measured uninfluenced by flavanone oxidase. Under this condition eriodictyol synthesis from naringenin as substrate was found to be about 1.4 times higher than luteolin formation from apigenin.

II. Formation of flavones

Flavanone oxidase activity was found to accompany 3′-hydroxylation activity which, in contrast to the first enzyme, could not specifically be abolished by one of the inhibitors tested (Table III). But studies on substrate specificity revealed that besides naringenin eriodictyol is a suitable substrate for flavone formation leading to luteolin. This allowed a separate characterisation of the flavanone oxidase reaction.

The enzyme activity for flavone formation is clearly located in the microsomal fraction with the reaction requiring NADPH as co-factor. In the presence of NADH instead of NADPH as co-factor, the enzyme activity is drastically reduced (Table II).

Table III. Effect of inhibitors on flavanoid 3′-hydroxylase activity and flavanone oxidase activity.

Additions	3′-Hydroxylase [%]	Flavanone oxidase [%]
none	100.0	100
5 mM KCN	54.3	79.3
2 mM EDTA	94.2	101.3
100 μM *p*-Chloromercuribenzoate	66.0	54.2
2 mM N-Ethylmaleinimide	72.5	56.5
2 mM Diethyldithiocarbamate	89.1	114.4
2 mM Diethylpyrocarbonate	13.5	21.4
50 μM Ketoconazole	5.9	0.0
50 μM Ancymidol	99.2	0.0
50 μM Tetcyclacis	1.9	17.6

Moreover, with the co-factors 2-oxoglutarate, Fe^{2+} and ascorbate for the flavone forming enzyme of parsley, flavone synthesis could be observed neither in crude extracts nor in microsomal preparations.

For eriodictyol as substrate a pH optimum of about pH 7.0 was determined. At 25 °C luteolin formation increased linear with protein concentration up to at least 10 µg microsomal protein per assay and with time for about 15 min. No appreciable loss of enzyme activity occurred during storage of the microsomal fraction containing 10% saccharose at −80 °C. Flavone formation was completely or strongly inhibited by addition of the Cytochrome P-450 inhibitors ancymidole, ketoconazole and tetcyclacis to the enzyme assay. Partial inhibition was observed with *p*-chloromercuribenzoate, N-ethylmaleimide and diethylpyrocarbonate (Table III).

Discussion

Flowers of *S. cardinalis* form 4′-hydroxylated (apigenin and apigeninidin) and 3′,4′-hydroxylated (luteolin and luteolinidin) flavonoids in comparable high amounts. Such a pattern is expected if both 4-coumaroyl-CoA and caffeoyl-CoA are equivalent substrates for chalcone synthase leading to the formation of naringenin and eriodictyol, respectively. In fact, chalcone synthase of *S. cardinalis* accepted both activated hydroxycinnamic acids as substrates. But the use of caffeoyl-CoA was found to be restricted to pH values around 6.6 and even at this optimal pH almost only naringenin was formed in incubations containing both caffeoyl-CoA and 4-coumaroyl-CoA. Naringenin but not eriodictyol was also found to be present naturally in the flowers [2]. Moreover, flavonoid 3′-hydroxylase activity could be demonstrated in the microsomal preparation from flowers of *S. cardinalis*. All these results suggest that, as have been found in most plants, 4-coumaroyl-CoA is the true physiological substrate for the formation of the flavonoid skeleton and that the 3′-hydroxyl group of flavonoids is introduced by action of flavonoid 3′-hydroxylase [8–10, 12, 21, 22].

Subcellular localisation, co-factor requirement and pH optimum of the 3′-hydroxylase from *S. cardinalis* flowers and the influence of some inhibitors on enzyme activity correspond to the respective enzyme from other plants [5, 8–13]. But in comparison to those, 3′-hydroxylase activity of *S. cardinalis* flowers is rather low. Under this condition only a part of the available amounts of 4′-hydroxylated flavonoids can additionally be hydroxylated in the 3′-position. This fact explains the cocomitant presence of flavonoids with 4′- and 3′,4′-hydroxylation pattern of the B-ring.

Luteolin cannot only be formed by desaturation of eriodictyol but also by hydroxylation of apigenin in the 3′-position. 3′-Hydroxylation of apigenin to luteolin has been shown with enzyme preparations from parsley cell cultures [11] and with enzyme extracts from *Verbena* flowers [5].

For the formation of the 3′,4′-hydroxylation pattern of luteolinidin also exist different possibilities. But, although the 3′-hydroxylation of the flavan-4-ol apiforol to luteoforol or of the respective 3-deoxyanthocyanidin apigeninidin to luteolinidin can not be excluded, the 3′,4′-hydroxylation pattern of luteolinidin is most probably established already at the flavanone stage by hydroxylation of naringenin to eriodictyol. Thus, both flavanones were found to be equivalent substrates for reduction to apiforol and luteoforol, respectively [2]. Moreover, in case of the biosynthesis of the common anthocyanidins like cyanidin, introduction of the 3′-hydroxyl group was reported to be restricted to naringenin and dihydrokaempferol [23].

Flavone formation in *S. cardinalis* flowers is clearly not catalyzed by the soluble 2-oxoglutarate-dependent dioxygenase found in parsley [3] but by an NADPH-dependent microsomal enzyme activity. The latter type of flavone synthesis has been found in osmotically stressed soybean cell cultures [7] and in particular in enzyme preparations from flavone-containing flowers of all plants tested as yet [4–6]. The significance of this enzyme activity for flavone formation is further underlined by the fact that flowers, which naturally lack flavones, also lack this NADPH-dependent microsomal enzyme activity [4].

In soybean cell cultures, flavone synthase II was studied in more detail [7]. Subcellular localization, co-factor requirement, inhibition by carbon monoxide and cytochrome *c* and in particular the fact that Cytochrome P-450 inhibitors such as ketoconazole, ancymidole and tetcyclacis [24, 25] strongly interfere with enzyme activity suggest that this enzyme, like cinnamate 4-hydroxylase and flavonoid 3′-hydroxylase [11] belongs to the Cytochrome P-450-dependent monooxygenases. Surprisingly, flavonoid 3′-hydroxylase of *S. cardinalis* was not influenced by ancymidole but only by tetcyclacis and ketoconazole.

More detailled studies including measurement of the influence of these inhibitors on flavonoid 3′-hydroxylase and flavone synthase II from other plants are necessary in order to generalize and to explain this observation. Probably the use of these inhibitors allows not only in *S. cardinalis* but also in other plants a separation of both enzyme activities. Up to now, separate investigations of flavone formation was only possible on mutants lacking 3′-hydroxylation activity and of both enzymes by the use of suitable substrates.

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