Pergamon 0031-9422(95)00880-2 Phytochemistry, Vol. 42, No. 2, pp. 397–403. 1996

0031-9422/96 $15.00 + 0.00

S-ADENOSYL-L-METHIONINE: ANOL-*O*-METHYLTRANSFERASE ACTIVITY IN ORGAN CULTURES OF *PIMPINELLA ANISUM*

BIRGITT KEMMERER and JÜRGEN REICHLING*

Institut für Pharmazeutische Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, W-69120 Heidelberg, Germany

(*Received in revised form* 10 *October* 1995)

Key Word Index—*Pimpinella anisum*; Apiaceae; biosynthesis; phenylpropanoids; epoxypseudoisoeugenol-2-methylbutyrate; S-adenosyl-L-methionine; anol-*O*-methyltransferase activity.

Abstract—The biosynthesis of epoxypseudoisoeugenol-2-methylbutyrate (EPB), a rare phenylpropanoid of the genus *Pimpinella*, was investigated *in vitro* by means of a leaf-differentiating callus culture of *Pimpinella anisum*. In an effort to corroborate an earlier proposed biosynthetic pathway of EPB, the step between *p*-coumaryl alcohol and (*E*)-anethole was reinvestigated. Further feeding experiments with ^{14}C-labelled precursors and preliminary studies at the enzyme level clearly revealed that anol, and not *p*-methoxycinnamyl alcohol as previously asserted, is an obligatory intermediate in EPB biosynthesis. S-Adenosyl-L-methionine: anol-*O*-methyltransferase, a key enzyme in EPB biosynthesis, was demonstrated and characterized for the first time.

INTRODUCTION

The genus *Pimpinella* contains phenylpropanoids with a rare 2,5-dioxysubstitution pattern at the phenyl ring [1–4]. The 1-(*E*)-propenyl-2-hydroxy-5-methoxy benzene skeleton of these compounds was named pseudoisoeugenol [1]. At first sight, the biosynthesis of pseudoisoeugenols cannot be simply explained by the normal phenylpropanoid pathway since this pathway involves a hydroxylation step at the *para*-position relative to the side-chain [5, 6]. This position is unsubstituted in pseudoisoeugenols. To learn more about the biosynthesis of these compounds, a leaf differentiating callus culture of *Pimpinella anisum* was established. Since *P. anisum* accumulates exclusively epoxypseudoisoeugenol-2-methylbutyrate (EPB), this compound served as a target molecule in all biosynthetic experiments conducted previously. Feeding experiments and studies on the entry enzymes [7] phenylalanine-ammonia-lyase (PAL) and cinnamic acid-4-hydroxylase (C4H) proved that, starting with L-phenylalanine (**1**), EPB (**10**) is synthesized via *trans*-cinnamic acid (**2**) and *p*-coumaric acid (**3**) which gives rise to *p*-coumaryl alcohol (**5**) and (*E*)-anethole (**8**) by stepwise reduction of the side-chain (Fig. 1). The biosynthetic step from (*E*)-anethole (**8**) to pseudoisoeugenol (**9**) involves migration of the side chain during the introduction of the second oxygen function into the phenyl ring [8]. Afterwards the pseudoisoeugenol is acylated and epoxidized to EPB [5, 6].

The gap between *p*-coumaryl alcohol (**5**) and (*E*)-anethole (**8**) (see Fig. 1) could be bridged in principle *via* anol (**7**) as well as *via* *p*-methoxycinnamyl alcohol (**6**). Unfortunately, in this case the ^{13}C-labelling experiments yielded no clear results. We have now studied this aspect of EPB biosynthesis by feeding [2′-^{14}C] anol and *p*-[*Me*-^{14}C] methoxycinnamyl alcohol to *P. anisum* as well as by enzyme studies.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Growth characteristics of P. anisum *and accumulation of EPB*

In order to study the biosynthetic pathway of pseudoisoeugenol derivatives, a leaf-differentiating callus culture of *P. anisum* was established some years ago [9]. This *in vitro* culture selectively carries out the synthesis of EPB. The growth of the *P. anisum* culture and EPB accumulation were monitored for 30 days [7]. The *P. anisum* culture achieved its maximum biomass (18 g fr. wt/flask) on the 24th day in culture. The increase in biomass and the accumulation of EPB rose in a more or less parallel fashion. Therefore, a maximum of about 70 mg/100 g fr. wt (equivalent to 12 mg EPB/50 ml nutrient medium) could be detected on day 28 of culture.

Stability of anol and p*-methoxycinnamyl alcohol in the cell culture medium*

In ^{13}C-labelling experiments *p*-methoxy [2′-^{13}C] cinnamyl alcohol was incorporated into EPB up to

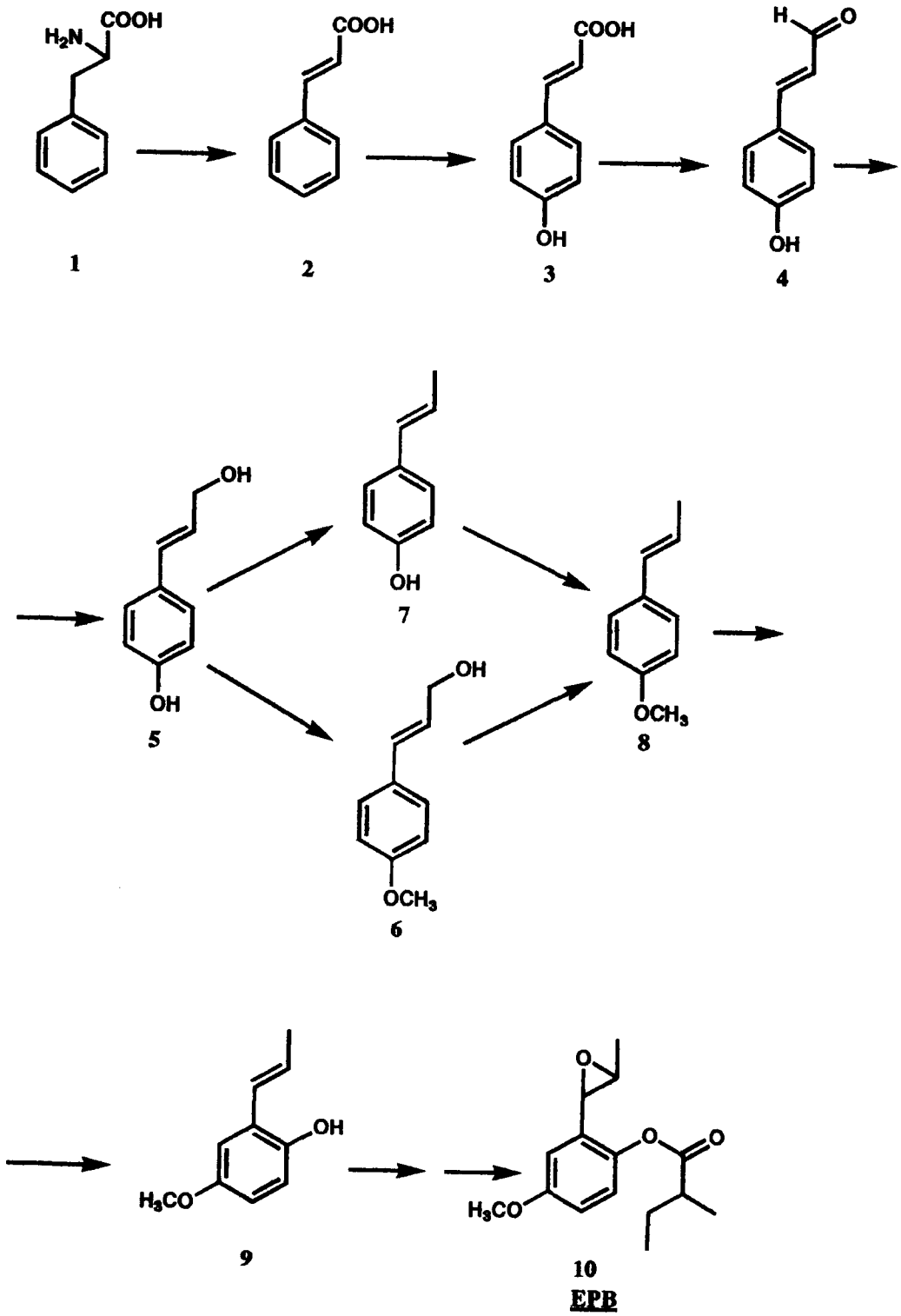

Fig. 1. Synthesis of EPB.

0.5%, whereas [2′-^{13}C] anol was not [6]. Therefore, we excluded anol as an obligatory precursor in the biosynthesis of EPB and pseudoisoeugenols, respectively. However, since we could not exclude the possibility that anol might be removed from cell metabolism by oxidative polymerization (e.g. catalysed by peroxidases), the stability of anol and *p*-methoxycinnamyl alcohol in the culture medium was examined.

First of all, we screened the culture medium from a *P. anisum* culture (at day 14) for peroxidase activity. It

was found to contain peroxidases that polymerize phenolic compounds e.g. guaiacol. Experiments with different incubation conditions showed that low temperatures (+10°) decreased peroxidase activity.

Studies on the stability of anol in the cell culture medium proved that anol was almost totally polymerized by perioxidases within 1 hr whenever a small amount of H_2O_2 was present (Figs 2(b) and 2(c)). No polymerization occurred when fresh nutrient medium was utilized (Fig. 2(a)). NaN_3, a potent inhibitor of peroxidases, prevented the polymerization to some degree (Fig. 2(d)). After addition of 0.1 M NaN_3 to the medium, 60% of the added anol was detectable after 1 hr, whereas 24 hr later only 26% was left. *p*-Methoxycinnamyl alcohol was much more stable in the culture medium than anol. After 24 hr, 62% of the compound remained unpolymerized. Addition of NaN_3 led to no loss of *p*-methoxycinnamyl alcohol. Since low temperatures and NaN_3 decreased the activity of the peroxidases, the effect of these conditions on feeding experiments was determined.

Feeding experiments

Feeding of [2'-^{14}C] anol and *p*-[*Me*-^{14}C] methoxycinnamyl alcohol led to different results. Under standard conditions (+26°, no NaN_3), both substances were converted to EPB. In parallel feeding experiments, the incorporation rate of anol amounted up to 1.2%, whereas only 0.5% of the *p*-methoxycinnamyl alcohol was converted to EPB. Modification of the incubation conditions (+10°, NaN_3) led to a remarkable observation. Under the new conditions the incorporation rate of anol increased up to 3.4%, whereas the incorporation rate of *p*-methoxycinnamyl alcohol was unchanged.

These experiments illustrate that peroxidases have a great influence on the incorporation of precursors bearing a free phenolic function, e.g. anol. So these results cast doubt on the former postulated biosynthetic pathway of EPB based on feeding ^{13}C-labelled precursors [6]. Based on the results of the tracer experiments with ^{14}C-labelled precursors, we suggest that anol and not *p*-methoxycinnamyl alcohol is the obligat-

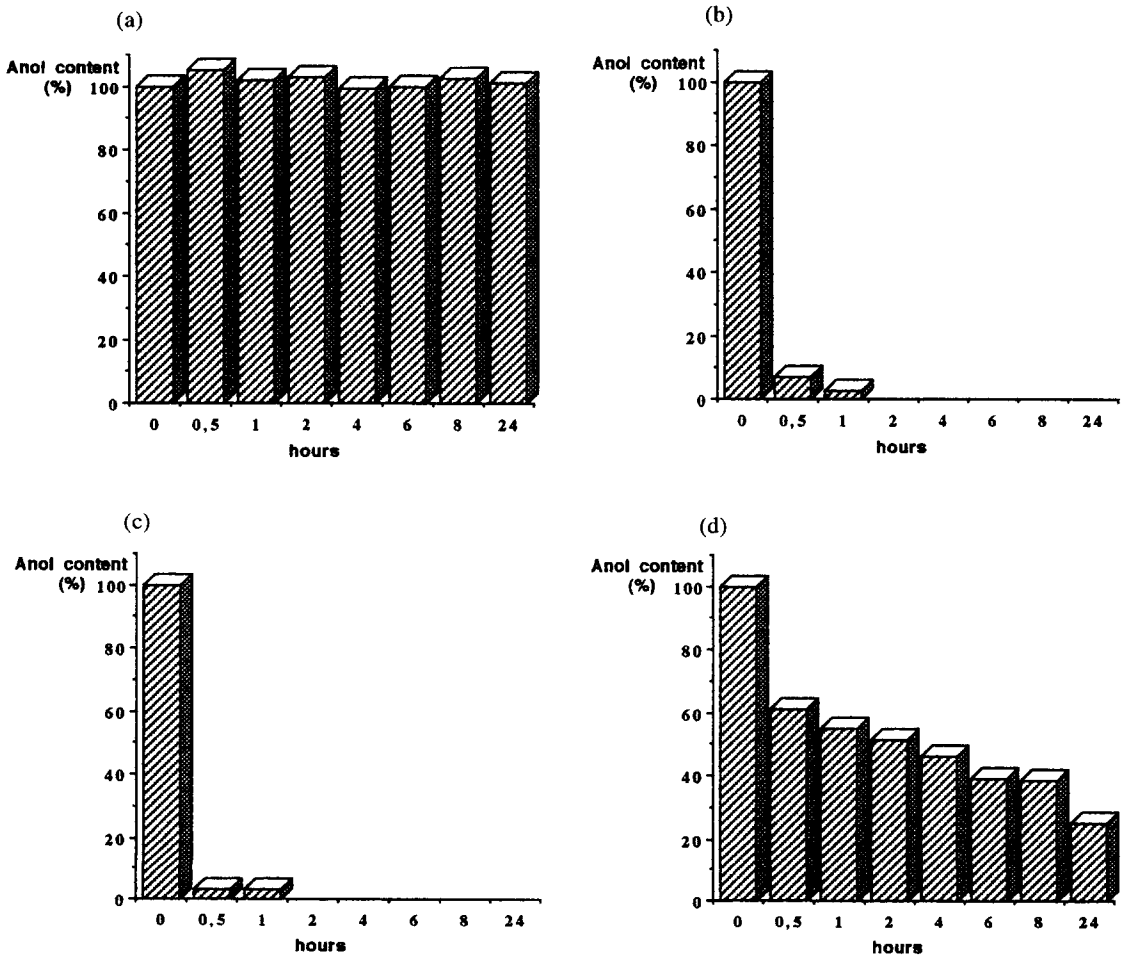

Fig. 2. Stability of anol in nutrient medium under different conditions. (a) Fresh nutrient medium; (b) nutrient medium after removing cells; (c) fresh nutrient medium with peroxidase from horseradish; and (d) nutrient medium after removing cells but with NaN_3.

ory intermediate in the biosynthesis of EPB. To confirm our findings we demonstrated the activity of an *O*-methyltransferase (AOMT) which converts anol to (*E*)-anethole.

Evidence of an S-adenosyl-L-methionine: anol-O-methyltransferase (AOMT)

Enzyme preparations obtained from $(NH_4)_2SO_4$ precipitation were incubated with S-adenosyl-L-[*Me*-^{14}C]methionine (SAM) and anol. HPLC and liquid scintillation counting of the phenylpropanoids extracted after a 2 hr incubation period revealed a prominent radioactive compound which had an R_f value identical to that of (*E*)-anethole. The formation of this compound showed a time dependent increase, whereas no formation took place in a boiled enzyme solution or in assays without added anol. About 30% of the methyl groups of SAM were transferred to anol. The identity of the radioactive (*E*)-anethole was demonstrated under different HPLC conditions and by comparison with authentic compound.

To confirm that AOMT is the only methylating enzyme in this biosynthetic sequence, we established that there was no S-adenosyl-L-methionine: *p*-coumaryl alcohol-*O*-methyltransferase (CoOMT) activity in *P. anisum*. Thus, neither in crude homogenates nor in $(NH_4)_2SO_4$-precipitates was *p*-coumaryl alcohol methylated to *p*-methoxy-cinnamyl alcohol.

Some properties of the S-adenosyl-L-methionine: anol-O-methyltransferase activity

The AOMT activity obtained from 30–70% $(NH_4)_2SO_4$-precipitation was used to determined some kinetic properties. The pH-optimum of the methyltransferase reaction was about pH 8.0. The activation (125%) of the enzyme by 6 mM DTE indicates the presence of labile SH-groups. The enzyme was sensitive to S-adenosyl-L-homocysteine (SAH), one of the products of transmethylation. 50% inhibition was observed with 20 mM SAH (Fig. 3). AOMT activity was stimulated by 8 mM $MgCl_2$ up to 175%. Furthermore, EDTA (8 mM) inhibited the rate of methylation by 40%. Apparent K_m-values were 7.8 mM for anol and 0.3 mM for SAM. The properties of the AOMT were in good agreement with those already reported for other plant *O*-methyltransferases [10]. To ensure that the AOMT was active during EPB production in *P. anisum*, the activity of the enzyme was examined over the whole growth cycle (Fig. 4). The enzyme activity varied in the range of 27 pkat g^{-1} protein at the beginning to 1 pkat g^{-1} protein at the end of the culture cycle. As has been reported for enzymes belonging to the secondary metabolism in plant cell cultures [11], the activity was induced shortly after subcultivation, perhaps by exposure of the tissue to the high nutrient concentration of fresh medium.

Substrate specifity of AOMT

The substrate specificity of the enzyme was tested using different phenylpropanoid derivatives (Table 1). The activity of the enzyme is *para*-specific. The substrates of the AOMT were those with a propenyl-side-chain without an oxygen function. Introduction of an oxygen function at the terminal carbon of the side-chain always caused a total loss of enzyme activity. Primary phenylpropanoids such as *p*-coumaric acid and *p*-coumaryl alcohol are not substrates for the AOMT.

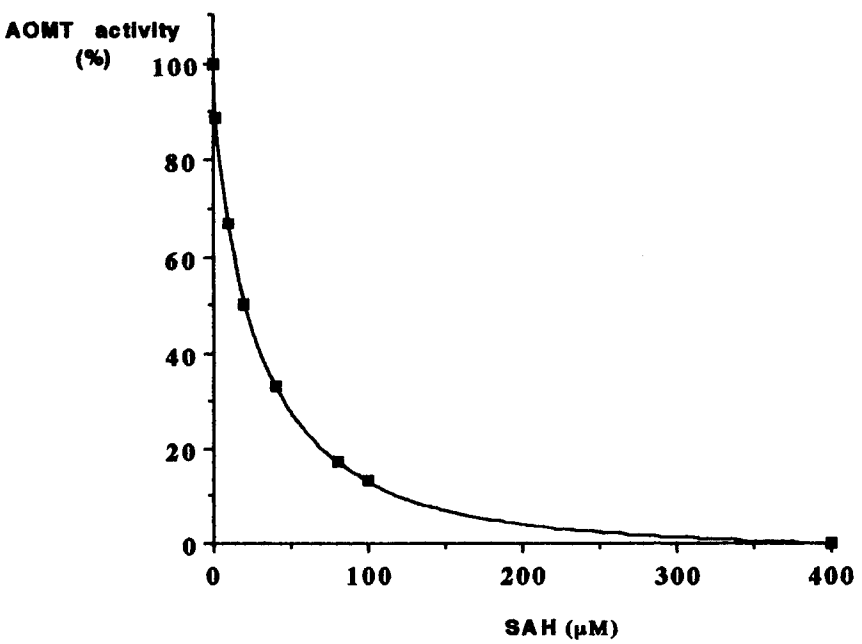

Fig. 3. Inhibition of AOMT activity with SAH.

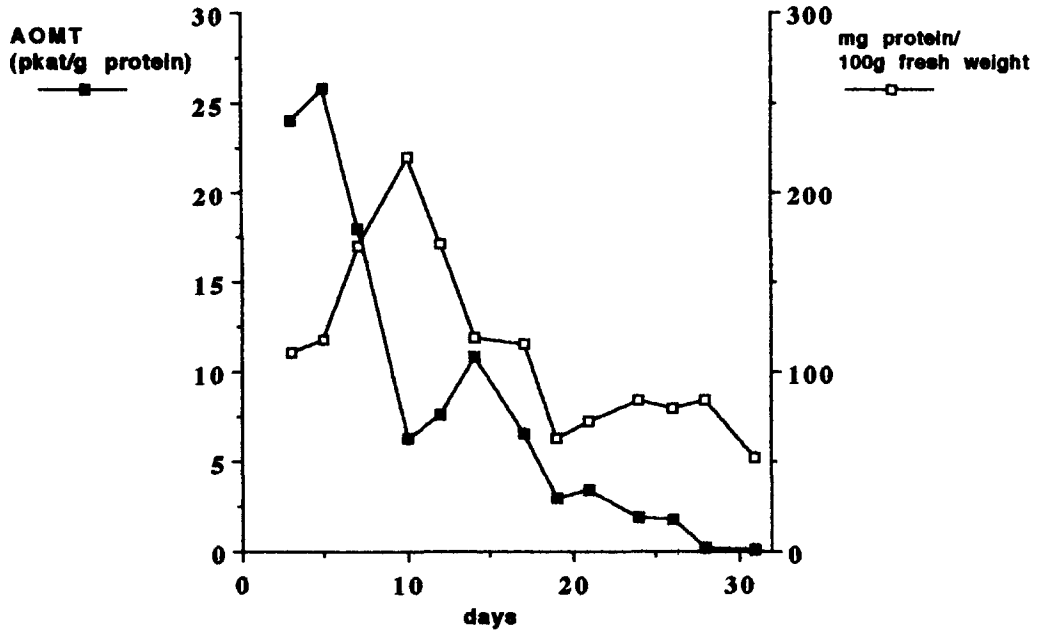

Fig. 4. Activity of the enzyme over the growth cycle.

The fact that the enzyme accepts only anol and no other metabolities of the biosynthesis of EPB proves that the obligatory metabolite between *p*-coumaryl alcohol and (*E*)-anethole is anol.

Purification of AOMT

The AOMT activity was enriched by fractional precipitation with $(NH_4)_2SO_4$. The highest enzyme activity per g protein was observed with the protein fraction precipitated between 60–70% saturation. Therefore this fraction was utilized for a gel filtration step. When the desalted $(NH_4)_2SO_4$-pellet was chromatographed on Sephadex G-250 three peaks were observed. AOMT activity was only detectable within peak 2. SDS-PAGE established that the apparent subunit molecular weight of the AOMT was 55 000 dalton.

Table 1. AOMT activity in relation to several compounds (100% = 15 pkat/g protein)

Compound	Rel. AOMT activity (%)
Anol	100
p-Coumaric acid	0
Caffeic acid	0
Ferulic acid	0
Isoferulic acid	0
p-Coumaryl alcohol	0
Coniferyl alcohol	0
Eugenol	94
Chavicol	49
Dihydro-anol	56
Vanillin	25
Isovanillin	0

CONCLUSIONS

Based on our labelling experiments as well as on enzyme studies, we propose that the biosynthesis of EPB proceeds as shown in Fig. 1. The biosynthesis starts with L-phenylalanine (**1**) which is converted to *trans*-cinnamic acid (**2**) by PAL and then to *p*-coumaric acid (**3**) by C4H. Prior to the introduction of the second hydroxyl group, the carboxyl function of the side-chain is reduced via the respective *p*-coumaryl alcohol (**5**) to anol (**7**). Anol is then methylated to (*E*)-anethole (**8**) by AOMT. Since we have not found an enzyme which methylates *p*-coumaryl alcohol to *p*-methoxycinnamyl alcohol, the alternative pathway via *p*-methoxycinnamyl alcohol (**6**) to (*E*)-anethole (**8**) must be rejected. The pseudoisoeugenol (**9**) emerging from hydroxylation of (*E*)-anethole (**8**) via an NIH shift is then acylated and epoxidized to EPB (**10**).

The second enzyme in the biosynthetic sequence between *p*-coumaryl alcohol and (*E*)-anethole reduces *p*-coumaryl alcohol to anol. In preliminary experiments, we have demonstrated *p*-coumaryl alcohol reductase activity, but further investigations are needed to characterize this enzyme. The presence of this enzyme activity accounts for the incorporation of *p*-methoxycinnamyl alcohol in feeding experiments.

The *p*-coumarylalcohol reductase is of great interest not only for proving the biosynthetic pathway of EPB in *P. anisum*. It is now possible to get information about the biosynthesis of propenyl- and allylphenols on

enzyme level. Since this problem has been discussed for more than 30 years [12] we undertook further experiments to get an answer to this general biological question.

EXPERIMENTAL

Pimpinella anisum cultures were cultured in a hormone-free M&S liquid medium and kept on a gyratory shaker (110 rpm) under permanent illumination (1500 erg sec^{-1} cm^{-2}). For more details, see refs [9, 13].

Peroxidase assay. 2 ml phosphate buffer (0.1 M; pH 7, 0), 50 μl guaiacol soln (18 mM), 40 μl H_2O_2 soln (8 mM) and 1 ml medium were pipetted into a cuvette and mixed. The increase in absorbance was monitored at 436 nm in a UV-VIS spectrometer. These experiments were carried out at 25° and 10°.

Stability of the precursors. 1 mCi [2′-^{14}C] anol (sp. act 0.275 mCi $mmol^{-1}$) or *p*-[*Me*-^{14}C]methoxycinnamyl alcohol (sp. act 0, 28 mCi $mmol^{-1}$) dissolved in 200 μl DMSO and 240 μl H_2O_2 soln were added to 10 ml fresh medium or to medium after removing the cells, respectively. The assays were kept under standard conditions (26°, continuous light). To inhibit the activity of the peroxidases 0.1 M NaN_3 was added to some assays. Within 24 hr samples of 0.5 ml were extracted with Et_2O, evapd, redissolved in MeOH and checked by HPLC to observe the decrease of anol and *p*-methoxycinnamyl alcohol, respectively. Assays with and without NaN_3 were compared. Additionally the instability of anol against peroxidases was tested in fresh nutrient medium which contained 1.6 μkat horseradish peroxidase per 10 ml of medium.

Feeding experiments. On the 12th day of the culture period the tissue mass and the medium was apportioned aseptically on Petri plates: about 3 g tissue mass and 10 ml medium per plate. 1 mCi [2′-^{14}C] anol or *p*-[*Me*-^{14}C]methoxycinnamyl alcohol (sp. act as described above) were dissolved in 200 μl DMSO. These solns were added to the Petri plates; in some experiments 0.1 M NaN_3 was added too. Afterwards the plates were kept under continuous light for 24 hr. The incubation temperature was 26° or 10°, respectively. [2′-^{14}C] anol was synthesized according to ref. [6]. *p*-[*Me*-^{14}C]methoxycinnamyl alcohol was a gift from Dr Leseticky, Prague.

Extraction of ^{14}C-labelled EPB. The tissue was harvested and extracted by $CHCl_3$, for more details see ref. [9]. EPB was isolated and quantified by HPLC, the incorporation of the label was determined by liquid scintillation counting.

Preparation of the enzyme extract. Unless otherwise indicated, all stages were carried out at 4°. 10 g tissue mass was homogenized with 10 ml 0.05 M Tris-HCl buffer (pH 8.0) containing 3 mM DTE. The homogenate was centrifuged for 20 min at 8000 *g*. The supernatant was brought to 30% saturation by addition of a satd soln of $(NH_4)_2SO_4$ over a period of 20 min and was then centrifuged at 34000 *g* for 20 min. The supernatant was then brought to 70% $(NH_4)_2SO_4$ saturation and centrifuged at 34000 *g* again. The pellet was resuspended in buffer and desalted on Sephadex G 25 columns, the resulting protein soln was used for enzyme assays. Protein fractions used for gel-chromatography were obtained from a precipitation with 60–70% $(NH_4)_2SO_4$ satn.

Protein determination was carried out according to Bradford [14]. Protein concentrations were determined in a dye-binding assay with Biorad-protein-assay. Bovine serum albumin was used as reference.

Enzyme assays. AOMT: The standard enzyme assay contained in a total vol. of 1 ml: 0.05 M Tris-HCl (pH 8.0), 6 mM DTE, 10 mM NaN_3, 75 mM anol, 1 mg protein and 1 mM S-adenosyl-L-[*Me*-^{14}C]methionine (SAM, sp. act 50 mCi $mmol^{-1}$) as the methyl donor. To determine the substrate specificity, anol was substituted with equimolar amounts of different phenylpropanoid derivatives. After incubation at 8° for 2 hr the reaction was terminated by adding 2 drops of H_3PO_4. (The unusual incubation temp was chosen because it gave the highest amounts of enzyme product.) The soln was directly applied onto Extrelut columns (Merck), and the phenylpropanoid derivatives eluted with Et_2O or Me_2CO. The extract was evapd to dryness and the residue was dissolved in 1 ml MeOH. 100 to 200 μl of this soln were fractionated by HPLC, and each fraction checked for radioactivity by liquid scintillation counting. To determine some properties of the AOMT activity the enzyme assay was varied as described in Results. Control assays contained boiled protein extracts or no phenylpropanoid substrate. CoOMT: The enzyme assay for *p*-coumaryl alcohol methyl-transferase activity was carried out as described for AOMT. Crude homogenates or $(NH_4)_2SO_4$- precipitations served as protein source.

Purification of the AOMT system. The desalted $(NH_4)_2SO_4$ pellet (obtained from a precipitation with 60–70% saturation) was chromatographed on a Sephadex G 250 column which had been equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 10% glycerol. Protein frs of the three eluted peaks were combined and precipitated with 70% $(NH_4)_2SO_4$ respectively. The three different protein pellets were desalted on Sephadex G-250 and assayed for AOMT activity against anol. The protein pellet obtained from the second peak contained AOMT activity and was used for SDS-PAGE.

PAGE. Proteins were separated under denaturating conditions using SDS-PAGE. Acrylamide gels were prepared as described in [15] with 3% stacking gels and 10% resolving gels. Samples and standard proteins were boiled in buffer. The proteins were stained with Coumassie Brilliant Blue. The M_r was determined with a standard protein kit.

HPLC analysis. LiChrosphere RP 18 (7 mm, Merck). Solvent for EPB: MeOH–H_2O (7:3) isocratic. Solvent for other phenylpropanoids: A: MeOH–H_2O (1:1) + 0.1% H_3PO_4; B: MeOH–H_2O (9:1) + 0.1% H_3PO_4. A linear gradient was run from 0% to 100% B in 20 min

at room temp; flow 1.2 ml min^{-1}. Injection: 250 μl. Detection: 258 nm.

Liquid scintillation counting. The fractions from HPLC were mixed with 10 ml Ultima Gold (Packard) as a scintillation cocktail and directly measured in a liquid scintillation counter (Rackbeta, LKB Wallac).

Acknowledgements—The financial support of our investigation by the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We thank Dr. Leseticky, University of Prague, for supplying us with *p*-[*Me*-^{14}C]methoxycinnamyl alcohol.

REFERENCES

1. Martin, R., Reichling, J. and Becker H. (1985) *Planta Med.* **51**, 198.
2. Kleimann, R., Plattner, R. D. and Weisleder D. (1988) *J. Nat. Prod.* **51**, 249.
3. Bottini, A. T., Dev, V., Garfagnoli, D. J., Mathela, C. S., Melkani, A. B., Miller, A. A. and Sturm, N. S. (1986) *Phytochemistry* **25**, 207.
4. Kubeczka, K. H., von Massow, F., Formacek, V. and Smith, M. A. (1976) *Z. Naturforsch.* **31b**, 283.
5. Martin, R., Schilling, G. and Reichling, J. (1988) *Z. Naturforsch.* **43c**, 328.
6. Reichling, J. and Martin, R. (1990) *Z. Naturforsch.* **45c**, 942.
7. Reichling, J., Kemmerer, B. and Sauer-Gürth, H. (1995) *Pharmacy World & Science* 4, **113**.
8. Martin, R. and Reichling, J. (1992) *Phytochemistry* **31**, 511.
9. Reichling, J., Martin, R. and Thron, U. (1988) *Z. Naturforsch.* **43c**, 42.
10. Poulton, J. E. (1981) in *The Biochemistry of Plants* (Conn E. E., ed.) **7**, 668.
11. Chapple, C. and Ellis, B. (1991) in *Biotechnology in Agriculture and Forestry, Medicinal and aromatic Plants* (Bajaj, Y. P. S., ed.) **15**, 478.
12. Birch, A. J. (1963) in *Chemical Plant Taxonomy* (Swain, T., ed.), 141. Academic Press, London.
13. Murashige, T. and Skoog, F. (1962) *Plant Physiol.* **15**, 473.
14. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
15. Laemmli, U. K. (1970) *Nature* **227**, 68.