# Studies on the Biosynthesis of Pseudoisoeugenols in Tissue Cultures of *Pimpinella anisum*

Rainer Martin*, Gerhard Schilling**, and Jürgen Reichling*

* Institut für Pharmazeutische Biologie der Universität Heidelberg, Im Neuenheimer Feld 364, D-6900 Heidelberg, Bundesrepublik Deutschland

** Organisch-Chemisches Institut der Universität Heidelberg, Im Neuenheimer Feld 270, D-6900 Heidelberg, Bundesrepublik Deutschland

Z. Naturforsch. **43c,** 328–336 (1988); received January 20, 1988

*Pimpinella anisum*, Umbelliferae, Pseudoisoeugenols, Tissue Culture, Biosynthesis

A leaf-differentiating tissue culture which produced substantial amounts of pseudoisoeugenol-(2-methylbutyrate) has been used to examine the origin of the pseudoisoeugenol skeleton. $^{14}$C- and $^{13}$C-labelling revealed L-phenylalanine, *trans*-cinnamic acid and *p*-coumaric acid as precursors. $^{13}$C-labelled precursors proved to be especially useful.

## Introduction

In a previous paper we reported the occurrence of several unusually substituted phenylpropanoids in three *Pimpinella* species which we called pseudoisoeugenols [1]. Other authors independently found the same class of compounds in different *Pimpinella* species and thus confirmed our results [2]. The unusual 2,5-dioxy substitution pattern of the pseudoisoeugenols is not consistent with the normally assumed phenylpropanoid pathway and requires its own explanation. In order to elucidate this by appropriate labelling experiments, we established a tissue culture of *Pimpinella anisum* which selectively promoted the production of epoxy-pseudoisoeugenol-(2-methylbutyrate), termed EPB [3]. Biosynthetic studies of this kind are normally performed employing $^{14}$C- or $^{3}$H-labelled precursors whereas labelling with stable isotopes, mainly $^{13}$C, is normally assumed to be ineffective for higher plants. This is due to poor incorporation rates and the low sensitivity of $^{13}$C NMR spectroscopy used for detection of the label. The aim of this report is to show that the biosynthesis of pseudoisoeugenols could be partially clarified using the above mentioned tissue culture in combination with $^{14}$C- and $^{13}$C-labelled precursors.

## Results and Discussion

The biosynthesis of pseudoisoeugenols cannot simply be explained with the normal phenylpropanoid pathway since this pathway involves a hydroxylation step in para-position relative to the $C_3$ unit. This position, however, is unsubstituted in the pseudoisoeugenols; they exhibit their typical 2-acetoxy-5-methoxy substitution pattern. This pattern can arise hypothetically from different biosynthetic paths as is shown in Fig. 1.

Since the pseudoisoeugenols are phenylpropanoids at least in the chemical sense, we decided to feed our tissue culture initially with uniformly $^{14}$C-labelled L-phenylalanine and L-tyrosine instead of acetate or gentisic acid.

Effective labelling experiments, mainly with a fast growing tissue culture like ours, also requires the correct choice of the point of time and duration for the experiment. As already described [3], our culture of *P. anisum* showed a remarkable increase in accumulation of EPB starting at the 15th day of the cultivation period. Most probably this behaviour was due to an increased production rate. Thus day 15 should be most promising for high incorporation rates while a duration of 24 h seemed to be adequate to keep randomization of the radioactivity low. These assumptions were proven by later experiments using $^{13}$C-labelled cinnamic and *p*-coumaric acid. As a result, prolongation of the incubation time to 48 h yielded improved signal intensity only in one single instance for cinnamic acid, but normally displayed neither higher incorporation rates nor randomization of the label.

The uptake of both $^{14}$C-labelled amino acids was observed by monitoring the decrease in radioactivity in the liquid nutrition medium, and proved to be between 96 and 98% after 24 h (see Experimental).

Reprint requests to Dr. R. Martin.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0500–0328 $ 01.30/0

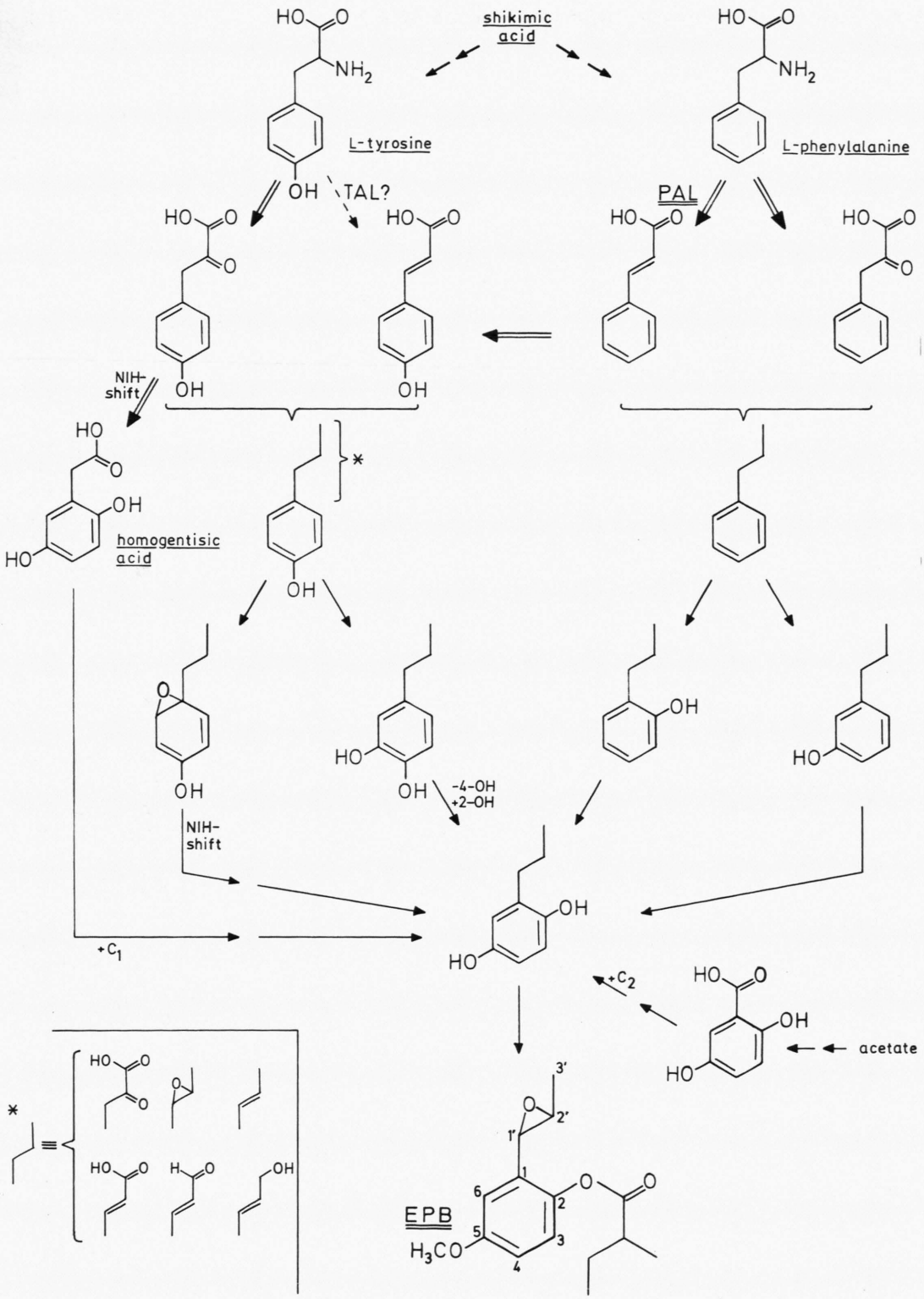


Fig. 1. Possible biosynthetic routes leading to the pseudoisoeugenoles. ⇒ indicates well established metabolic steps.

Because both uptake and incorporation rate may depend on the quantity of the compound offered, both precursors were diluted with different amounts of the corresponding unlabelled L-amino acid. As a result of this experiment, the optimum incorporation rate of phenylalanine was found to be 2.2% whereas that for tyrosine was only 0.02%, more than 100-fold lower. Provided that the radioactivity had not been extensively scrambled, phenylalanine seemed to be incorporated into the pseudoisoeugenol skeleton.

Based on the unexpectedly high incorporation rate, it was calculated that labelling with $^{13}C$ precursors and observation of the signal enhancement in the $^{13}C$ spectrum of the isolated EPB should be possible. This technique offers several advantages. The required precursors do not exhibit any hazard due to radioactivity which greatly faciliates their synthesis, application to the tissue culture and isolation of the product. Furthermore, the specific incorporation of the precursor applied does not require prove by chemical degradation but instead follows from the enhancement of the particular signal of the carbon observed by $^{13}C$ NMR spectroscopy. Due to the relative insensitivity of the method, however, feeding of the $^{13}C$-labelled precursors in the milligram range is required. Thus we examined the capacity of our tissue culture to convert amounts of up to 10 mg L-phenylalanine into EPB using L-[U-$^{14}C$]phenylalanine as a tracer. The results are shown in Fig. 2 and show a remarkable capacity for this conversion with an optimum of 4.57% incorporation rate at 2 mg L-phenylalanine/flask (ca. 10 g fresh tissue mass).

Before starting $^{13}C$-labelling, we examined the possible incorporation of $^{14}C$-labelled cinnamic acid. This compound is the most probable product arising from phenylalanine by the action of phenylalanine-ammonium-lyase (PAL) and is also commercially available. Since $^{13}C$-labelled cinnamic acid can be easily synthesized, the incorporation capacity for cinnamic acid was also examined as described for phenylalanine (Fig. 2). The incorporation rates strongly suggested cinnamic acid as a precursor for EPB and furthermore showed the capacity of the culture to be high enough for labelling with $^{13}C$-cinnamic acid. As a practical result, it was decided to feed always 3 mg of a probable precursor per flask, since this would not stress the metabolism to much but would offer a sufficient quantity of the label, as can be seen from the curve for the incorporated amount in Fig. 2. Regarding the amounts of EPB produced by a single flask [3], the collective amount of two flasks should always be sufficient to obtain $^{13}C$ spectra within an acceptable time.

Another basic requirement for the planned $^{13}C$-labelling experiments was an unequivocal assignment

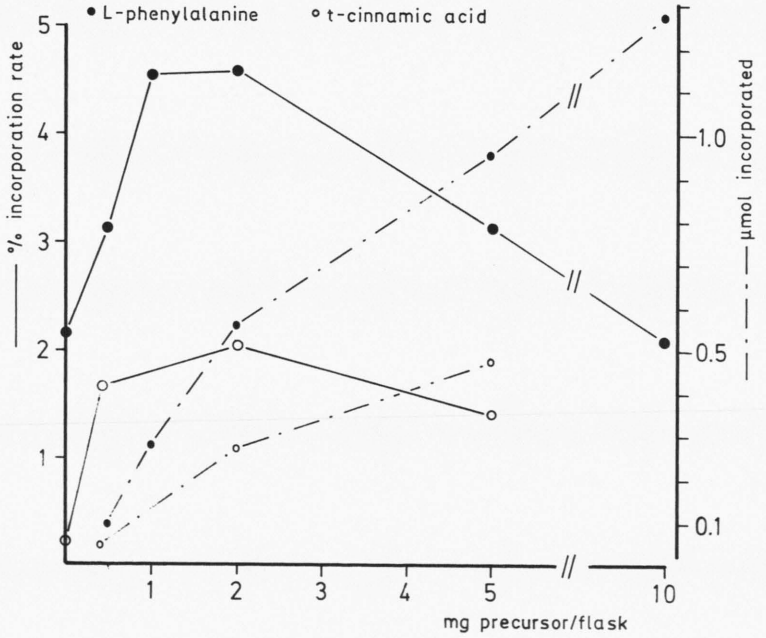


Fig. 2. Conversion capacity of the anise tissue culture for L-phenylalanine and cinnamic acid.

of all signals in the $^{13}C$ NMR. These assignments, especially for the ring atoms, have already been given in a previous paper [1]. However, the assignments for both carbons in the epoxy ring were based only on a comparison with literature data of similar epoxides since the NMR techniques available to us previously did not make them distinguishable. Modern C–H correlation NMR spectroscopy now enabled us to overcome this problem and revealed that our previous assignment for these two carbons had to be reversed, *i.e.* the absorbance for 2′-C is at 58.58 ppm and the signal of 1′-C at 55.25 ppm.

After feeding of [2′-$^{13}C$]cinnamic acid, EPB was isolated and the $^{13}C$ spectrum recorded. The spectrum displayed clear enhancement of the signal of 2′-C compared to the spectrum of EPB with natural $^{13}C$ abundance and, therefore, proved cinnamic acid to be a precursor for EPB.

Within the "normal" phenylpropanoid pathway the following question remains unanswered: is the carboxyl group of L-phenylalanine finally reduced to the terminal methyl group of the side chain or is it removed and the methyl group reintroduced later [4–7]? Being able to detect the $^{13}C$ label in such a convenient way, [carboxyl-$^{13}C$]cinnamic acid was also synthesized with the purpose of elucidating the origin of the terminal methyl group. In order to obtain unambiguous results, it seemed desirable to have an internal standard for this experiment to prove at least the proper working conditions of the tissue culture in the case that no labelling at 3′-C should be observed. Therefore 2 mg of the carboxyl-$^{13}C$ isotopomere together with 1 mg of [2′-$^{13}C$]cinnamic acid was applied to each of two flasks. After isolation, the $^{13}C$ spectrum clearly displayed a moderate signal enhancement for 2′-C of EPB and a strong enhancement for 3′-C, the terminal methyl group (Fig. 3). At least for the case of pseudoisoeugenols it is now indubitable that the carboxyl group of cinnamic acid remains in the molecule during biosynthesis and most probably the entire side chain remains intact. Since there is no indication of a loss of a carbon atom during the conversion of phenylalanine to cinnamic acid, this should also be true for phenylalanine. Furthermore, this experiment excluded homogentisic acid as an intermediate (Fig. 1) because of the observed retainment of 3′-C.

The next question concerns the following step: does hydroxylation of the cinnamic acid occur or does reduction of the side chain take place first? The easiest way to determine this fact was to feed the three possible monohydroxy cinnamic acids, *i.e.* *o*-, *m*- and *p*-coumaric acid as their [2′-$^{13}C$]isotopomeres. Surprisingly, it was *p*-coumaric acid that was incorporated into EPB (Fig. 3c), whereas the $^{13}C$ spectra obtained after application of both the other acids did not exhibit any signal enhancement.

Since *p*-coumaric acid was not synthesized as radiolabelled compound, incorporation rates could not be established by the same method as for phenylalanine and cinnamic acid. Determination of incorporation rates of $^{13}C$-labelled compounds can be achieved by quantification of the appropriate effects in the $^{13}C$ NMR or mass spectrum. Unless a special isotope mass spectrometer is available, these methods cannot furnish results with the same accuracy as measuring of radioactivity. But as shown in the experimental section for [2′-$^{13}C$]*p*-coumaric acid, a good estimation is possible using these methods, and proved the incorporation rate to be in the same range as for cinnamic acid.

Theoretically *p*-coumaric acid can be transformed to EPB either by removal of the *p*-hydroxy group after hydroxylation in the 2-position or by migration of the side chain when the second hydroxyl group is introduced into the phenyl ring (Fig. 1). The latter mechanism, known as the "NIH shift", has frequently been observed in many aryl hydroxylations of animals and microorganisms, more rarely in higher plants (*e.g.* ref. [8]). Rather often it is conceiled since only hydrogen atoms are involved giving no evidence for such a shift unless either deuterium or tritium labelling is employed. However, migration of the side chain of a phenylpropanoid takes place during the biosynthesis of homogentisic acid from tyrosine *via* *p*-hydroxyphenylpyruvate. In this case the migration is linked to chain shortening due to the loss of the carboxyl group. But, as already mentioned above, this was impossible during the biosynthesis of EPB.

There seemed to be several feasible methods to furnish proof of a NIH shift, which all required the synthesis of *p*-coumaric acid with a specifically labelled position. This could be either a distinct carbon or hydrogen of the phenyl ring or the oxygen of the *p*-hydroxy group. Since these syntheses are all fairly complicated, we decided to examine other possibilities first.

The possibility that *p*-coumaric acid could first be methylated to *p*-methoxy-cinnamic acid and then

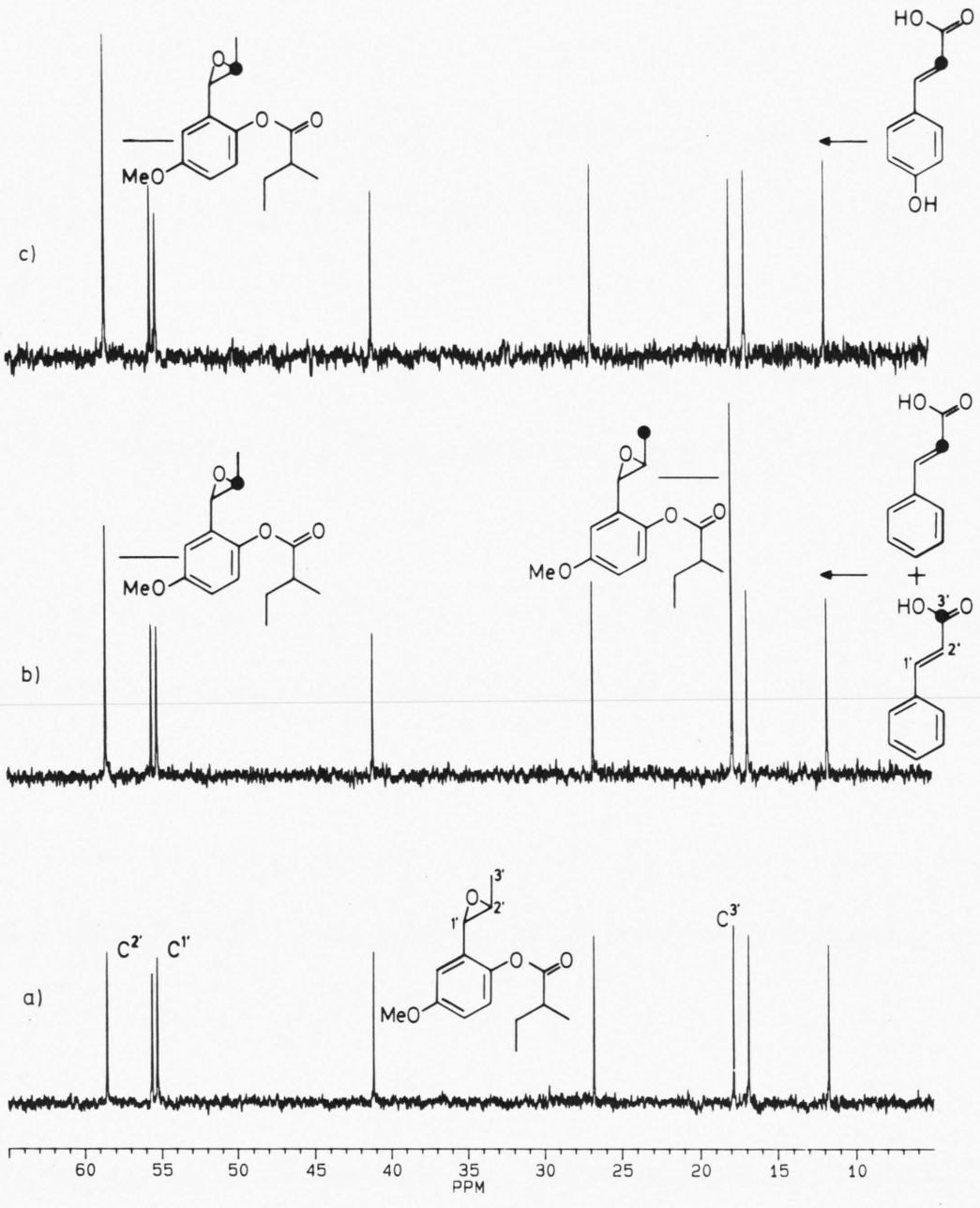


Fig. 3. Aliphatic portion of the $^{13}C$ NMR of EPB. a) unlabelled, b) feeding cinnamic acid, c) feeding *p*-coumaric acid. Emphasized position represents the site of the $^{13}C$-label.

undergo NIH shift, offered a convenient way of investigation by feeding the easily accessible [$^{13}C$]-methoxy isotopomere. Feeding of this compound did not show any incorporation.

Provided that reduction of the side chain takes place only after the substitution pattern of the phenyl ring (including methylation of the 5-OH group) has been completed, 2-hydroxy-5-methoxy-cinnamic

acid appeared to be a promising precursor. The compound, fed as the [2′-$^{13}$C]isotopomere, also showed no incorporation. This result suggested that methylation of the 5-OH group requires previous reduction of the side chain. For completeness, also [2′-$^{13}$C]-ferulic acid was applied and showed no incorporation.

Up to now $^{13}$C-labelled precursors seem to have been used predominantly for studies of the biosynthesis of secondary products in microorganisms which often exhibit impressive incorporation rates of more than 10% (*e.g.* ref. [9, 10]). Due to the relatively low incorporation rates of intact higher plants, this method seems to be restricted to the use of the derived tissue cultures which may exhibit higher incorporation rates if they show production of secondary products at all. Even then, investigations with $^{14}$C-labelling are numerous whereas $^{13}$C-labelling is rarely applied (*e.g.* ref. [11, 12]).

The results presented here show that biosynthetic studies in tissue cultures of higher plants need not be restricted to the use of radioactive precursors. Provided that the system is able to metabolize appropriate quantities of a precursor and incorporate it with at least 1%, labelling with $^{13}$C and detection of the label by $^{13}$C NMR is possible.

The results achieved by this method show that pseudoisoeugenols do have their origin in the phenylpropanoid pathway. As for many other phenylpropanoids, this pathway is followed at least up to *p*-coumaric acid. Most probably the "normal" pathway is left on the following steps to give rise to the unusual substitution pattern of the phenyl ring. The next question to answer will be the fate of the *p*-hydroxy group of *p*-coumaric acid. This can either be removed or virtually shifted to its neighbour position by NIH shift of the side chain. In order to elucidate this, experiments with specifically $^{13}$C-labelled compounds are in progress and will be subject of a separate report.

## Experimental

For culturing conditions of the anise culture see ref. [3].

### *Application of $^{14}$C-labelled precursors*

L-[U-$^{14}$C]phenylalanine and L-[U-$^{14}$C]tyrosine: specific activity 19.3 GBq (522 mCi)/mmol, 1.85 MBq (50 μCi)/1 ml solution in ethanol/water.

For initial experiments, 260.85 KBq (7.05 μCi) was added to each flask. The conversion capacity for phenylalanine was determined using 69.56 KBq (1.88 μCi)/flask. Unlabelled amino acids were added separately as appropriate volumes of aqueous stock solutions.

[1′-$^{14}$C]cinnamic acid: specific activity 2.07 GBq (56 mCi)/mmol, 1.85 MBq (50 μCi)/1 ml solution in toluene.

Addition of toluene to the tissue was avoided because of possible toxic effects. Instead, DMSO was used which normally does not affect cells in the concentrations used. From 40 μl (72.15 KBq (1.95 μCi) according to calibration) of the solution in toluene the solvent was removed by a gentle stream of nitrogen. To the vessel was added an appropriate amount of a solution of inactive cinnamic acid in DMSO (80 mg/10 ml), the volume completed to 250 μl by addition of pure DMSO. This solution was added to the tissue culture.

Uptake of $^{14}$C-labelled precursors was determined after 24 h by careful decantation of the cultivation medium from the tissue clumps and scintillation counting of 1.0 ml. By correction with the entire volume (25–30 ml) the total activity was calculated. This was always between 2 and 4% of the initial activity for L-phenylalanine, L-tyrosine and cinnamic acid which means uptake rates of at least 96%.

### *Isolation of $^{14}$C-labelled EPB*

Extraction of EPB was identical to that described for $^{13}$C labelling *(vide infra)*. Since the experiments only involved single flasks, the solutions of three consecutive extractions were collected without filtering in a 100 ml volumetric flask and filled up to 100.0 ml with chloroform. 50.0 ml of this solution (100.0 in the case of tyrosine labelling) was evaporated and to the residue 600 μl of MeOH was added. A white solid (phytosterols?) remained undissolved. EPB was isolated from the solution by HPLC without precleaning. HPLC conditions were identical to those as described later for $^{13}$C labelling, with the exception of the column which was of the same type but 4 × 250 mm and a flow of 1.5 ml/min.

Determination of radioactivity: The soln. of EPB in 80% MeOH/water obtained by HPLC was concentrated *in vacuo* to remove most of the methanol. The turbid mixture was transferred to a LSC tube with a pasteur pipette, the transfer completed by washing

with several small portions of MeOH. Scintillation cocktail was added which formed a gel and the tube subjected to liquid scintillation counting after about 30 min of equilibration time. The activity was calculated using a calibration curve based on the channel ratios and absolute activities of a set of calibration standards (Amersham).

*Application of $^{13}$C-labelled precursors*

The precursor (6 mg pure compound or 4 mg compound + 2 mg internal standard) was dissolved in 400 µl DMSO. Each 200 µl of this soln. were added to the two culture flasks designated for the experiment. The flasks were then kept on their normal places on the gyrotary shaker.

*Extraction and clean up of $^{13}$C-labelled EPB*

The fresh tissue masses of both equally labelled flasks were each transferred to a centrifuge tube of about 50 ml volume and extracted with 25 ml chloroform by thorough homogenizing with an appropriate blender (16,000 rpm). The suspensions were centrifuged for 3 min at 3500 rpm, the chloroform layers collected. Extraction was repeated twice, and all chloroform layers combined. A fourth extraction did not afford substancial amounts of EPB, as determined by analytical HPLC in one instance. The slightly turbid solution was filtered into a 250 ml volumetric flask and filled up to 250.0 ml with chloroform. 1 ml was kept for HPLC quantification of EPB, the remaining solution was evaporated almost to dryness *in vacuo* and redissolved in few milliliters of dichloromethane. This solution was separated on a silica gel column using dichloromethane as eluent. The fractions were checked by TLC (silicagel/dichloromethane, visualization with anisaldehyde/sulphuric acid). All fractions containing EPB were combined, evaporated *in vacuo* and to the residue 600 µl of MeOH was added.

HPLC of EPB: instrument (LDC/Milton-Roy): 2 pumps Constametric I and III, injector Rheodyne 7510 with 275 µl loop, detector Spectro Monitor D, computer MP3000E; column (Merck): LiChrospher 100 CH18/2, 5 µm, 10×250 mm; solvent: MeOH/water 80:20, flow 6.0 ml/min; detection wavelength 278 nm. The methanolic solution was injected in increasing portions and the eluting EPB was collected. The residue was re-extracted with a new 200 µl portion of MeOH which upon injection showed considerably lower peaks of EPB than was observed with the first extract. A third extraction of the residue furnished almost no additional amount of EPB and is unnecessary.

The solution of EPB in MeOH/water was concentrated *in vacuo* at a maximum temperature of 40 °C until it became turbid. After transfer to a separatory funnel it was diluted with the same volume of double distilled water and extracted three times with chloroform. The combined chloroform extracts were concentrated *in vacuo* to about 1 ml, transferred to a small glass cylinder and the chloroform removed by a stream of nitrogen (99.996% purity). The residue was redissolved in deuterated chloroform and subjected to $^{13}$C NMR spectroscopy (50 MHz) in tubes of 5 mm outer diameter. A proton spectrum (200 MHz) was recorded routinely to give additional prove for the purity of EPB.

Yields of EPB: The cumulative amounts of EPB in both parallel flasks was determined by direct weighing of the isolated compound and by HPLC-determination [3] using the 1 ml solution kept from the 250.0 ml stock solution. Comparison of both values revealed that 80% to 92% of the extracted EPB was finally isolated. The isolated amounts were in the range from 5.4 to 12 mg.

Estimation of incorporation rates: For $^{13}$C-labelled EPB, the incorporation rate can be estimated both by mass spectrometry and by quantifaction of the signal enhancement in the $^{13}$C NMR. A sample calculation for the feeding of [2′-$^{13}$C]*p*-coumaric acid based on both methods is given in the following:

MS (see ref. [1] for conditions): produced amount (HPLC-determination) 10.51 mg = 39.81 µmol (m.w. 264 u). Based on a natural abundance of $^{13}$C of 1.1% and 15 carbons in the molecule the sample should contain 6.57 µmol $^{13}$C. In the mass spectrum, the intensity of the isotope peaks should be $15 \times 1.1\% = 0.165$ times the intensity of the molecular peak, which proved to be true for an unlabelled sample displaying a deviation of only +1%. In the labelled EPB, the relative intensity of the mole peak was 52.61, the intensity of the isotope peak should therefore be 8.68 (natural abundance provided). Experimentally, it was 9.78 which is 12.6% higher. This sample of EPB therefore contained 7.39 µmol instead of 6.57 µmol, which means an incorporation of 0.82 µmol $^{13}$C from the precursor. The precursor itself was offered in an amount of 6.0 mg = 36.36 µmol (m.w. 165 µ, $^{13}$C-enrichment in position 2′: 92%). 0.82 µmol of the $^{13}$C-isotopomere make up

0.89 μmol of the entire precursor due to 92% $^{13}C$-enrichment. 0.89 μmol of 36.36 μmol represent 2.45% which is the absolute incorporation rate.

$^{13}C$ NMR: Based on the same assumptions as above, the $^{13}C$ content in position 2′ of 39.81 μmol EPB should be 0.438 μmol. The intensity in the $^{13}C$ NMR was 2.7-fold higher than with unlabelled EPB (the signal of 2′-C is normally as high as that of 1′-C). Position 2′ therefore contained $2.7 \times 0.438$ μmol = 1.18 μmol $^{13}C$ which means 0.74 μmol $^{13}C$ additionally. Corrected with 92% enrichment this makes up 0.8 μmol representing 2.2% of the offered 36.36 μmol precursor. The data based on independent spectroscopic methods are, therefore, in good agreement.

*Syntheses of $^{13}C$-labelled compounds*

1. [2′-$^{13}C$]cinnamic acid

In a 5 ml conical flask a solution of 60 mg (0.58 mmol) [2-$^{13}C$]malonic acid (92% $^{13}C$-enrichment), 60 mg benzaldehyde, 1 ml pyridine and 2 drops of piperidine was heated to 95 °C for 90 min. After cooling, 1.5 ml water was added, the mixture strongly acidified with conc. hydrochloric acid and extracted three times with chloroform. The combined chloroform extracts were dried with anhydrous sodium sulphate, the chloroform removed *in vacuo* and the residue recrystallized from *n*-hexane/chloroform. Yield 50 mg white needles (60% th.).

2. [2′-$^{13}C$]*m*-coumaric acid, [2′-$^{13}C$]*p*-coumaric acid and [2′-$^{13}C$]-ferulic acid

As given above for [2-$^{13}C$]cinnamic acid using 26 mg [2-$^{13}C$]malonic acid (0.25 mmol) and 31 mg of the appropriate hydroxy-benzaldehyde or 38 mg of vanilline in the case of ferulic acid. Reaction temp. 105 °C, recrystallization was omitted. For extraction, ether was employed instead of chloroform. Yield about 35 mg each (ca. 80% th.).

3. [2′-$^{13}C$]2-hydroxy-5-methoxy cinnamic acid

Procedure close to that described in ref. [13], adapted for microscale preparation.

a) 6-methoxy-[3-$^{13}C$]coumarin-3-carbonic acid ethyl ester

In a 5 ml conical flask 85 mg 2-hydroxy-5-methoxy-benzaldehyde, 81 mg (0.5 mmol) [2-$^{13}C$]-malonic acid diethylester and 1 drop piperidine were heated to 110 °C. After 3 min nitrogen was introduced until the mass was almost dry. Recrystallization from acetone in the same flask, finally at −18 °C, yielded 102 mg (82% th.) of yellow crystals.

b) 6-methoxy-[3-$^{13}C$]coumarin-3-carbonic acid

2 ml of a mixture of dioxane/HCl conc./water (5:3:2) was added to the flask with the ethyl ester and heated to 105 °C under reflux. After 15 min the acid started to precipitate. After 1.5 h the reflux condenser was removed and nitrogen introduced until the mixture remained only damp. Complete drying was achieved by evaporation *in vacuo*. The dark yellow crystal mass was twice recrystallized from acetone. Yield 74 mg (82% th.) of yellow crystal needles.

c) 6-methoxy-[3-$^{13}C$]coumarin

1 ml of a 14% aqueous sodium hydrogensulphite soln. was added to the acid from b) and warmed in a steam bath. The crystals dissolved under evolution of carbon dioxide. 0.8 ml conc. sulphuric acid was added and the soln. kept at 110 °C for 45 min. After cooling to ambient temp. 1 ml water was added and the precipitated product extracted three times with chloroform. After drying with anhydrous sodium sulphate the solution was transferred to a 10 ml conical flask and the solvent was removed by a gentle stream of nitrogen in a way that the product settled in the tip of the flask. Yield 42 mg (71% th.).

d) 2-hydroxy-5-methoxy-[2-$^{13}C$]cinnamic acid

To the flask from c) 1.5 ml of a 10% aqueous solution of sodium sulphite was added and the residue dissolved by gentle warming. 2 ml of 60% aqueous KOH was added and the solution kept at 130 °C under reflux. After cooling and acidifying with conc. hydrochloric acid, the precipitate of KCl was dissolved with 2 ml water. Repeated extraction with ether with subsequent drying over sodium sulphate yielded 32 mg (69% th.) of final product after evaporation.

4. [2′-$^{13}C$]*o*-coumaric acid

Synthesis as given for the analogous 5-methoxy derivative starting with 62 mg *o*-hydroxy-benzaldehyde and omitting the recrystallization of the initially formed ester.

5. [Carboxyl-$^{13}$C]cinnamic acid

Amounts were choosen according to the smallest quantity of commercially available $^{13}$C-carbon dioxide (99% $^{13}$C-enrichment) which was 100 ml gas at RT (= 4.46 mmol). The procedure employed is known to give only poor results due to extensive formation of side products (*e.g.* styrene, phenylacetylene etc.). Nevertheless, it appeared to be the cheapest way to yield the required amount of at least 4 mg of product.

a) Styryl magnesium bromide [14]

1 g Mg turnings were activated with iodine. In a 25 ml conical flask containing the magnesium and 5 ml of anhydrous THF, 763 mg bromstyrene was added, the reaction started by addition of 3 drops 1,2-dibromoethane and the flask kept in an ultrasonic bath for 1 h.

b) [Carboxyl-$^{13}$C]cinnamic acid

The carbon dioxide gas was frozen in the bottom of the shippingvessel by cooling with liquid nitrogen. The Grignard-solution was added, the vessel stoppered, transferred to a dry ice/acetone bath and allowed to stand overnight whereby the cooling bath evaporated.

The reaction mixture was transferred to a separating funnel, dilute hydrochloric acid added and extracted with ether. The combined ether extracts were re-extracted with 10% aqueous NaOH. After acidification of the NaOH with hydrochloric acid, the product was extracted with ether. Yield 90 mg (15% th.). $^1$H NMR of the crude product suggested that it was a mixture of 2 parts of the desired cinnamic acid and 1 part of phenylacetylenic acid. Separation of both was achieved on a silica gel column using dichloromethane with 2% acetic acid as eluent and furnished a fraction of 10 mg pure [carboxyl-$^{13}$C]cinnamic acid. The identity was proven by $^{13}$C NMR of a sample diluted with unlabelled cinnamic acid.

4-[methoxy-$^{13}$C]cinnamic acid: The compound was prepared from 4-hydroxy-cinnamic acid methyl ester by methylation with [$^{13}$C]methyliodide (99% $^{13}$C-enrichment) with subsequent alkaline hydrolysis. The $^1$H NMR spectrum exhibited the signal of one methoxy group at 3.85 ppm as a doublet with 144.4 Hz coupling constant.

*Acknowledgements*

We thank the Deutsche Forschungsgemeinschaft for financing this project.

[1] R. Martin, J. Reichling, and H. Becker, Planta med. **3,** 198 (1985).
[2] A. T. Bottini, V. Dev, D. J. Garfagnoli, C. S. Mathela, A. B. Melkani, A. A. Miller, and N. S. Sturm, Phytochemistry **25,** 207 (1986).
[3] J. Reichling, R. Martin, and U. Thron, Z. Naturforsch. **43c,** 42 (1988).
[4] L. Canonica, P. Manitto, D. Monti, and M. Sanchez, Chem. Comm. **1971,** 1108.
[5] P. Manitto, D. Monti, and P. Gramatica, J. Chem. Soc. Perk I, **1974,** 1727.
[6] P. Manitto, P. Gramatica, and D. Monti, J. Chem. Soc. Perk I, **1975,** 1548.
[7] M. Klischies, J. Stöckigt, and M. H. Zenk, J. Chem. Soc. Chem. Comm. **1975,** 879.
[8] B. B. Ellis and N. Amrhein: Phytochemistry **10,** 3069 (1971).
[9] H. G. Floss, P. J. Keller, and J. M. Beale, J. Natural Products **49,** 957 (1986).
[10] T. S. Wu, J. Cuncan, S. W. Tsao, C. J. Chang, P. J. Keller, and H. G. Floss, J. Natural Products **50,** 108 (1987).
[11] S. Seo, Z. Tomita, and K. Tori, J. Am. Chem. Soc. **103,** 2075 (1981).
[12] Ch. J. W. Brooks, D. G. Watson, D. S. Rycroft, and I. M. Freer, Phytochemistry **26,** 2243 (1987).
[13] F. Cramer and H. Windel, Chem. Ber. **89,** 354 (1956).
[14] T. Yoshino and Y. Manabe, J. Am. Chem. Soc. **85,** 2861 (1963).