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

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Tissue cultures of *Pimpinella anisum* and *P. major* were used to study the biosynthesis of pseudoisoeugenols. The putative precursors were labelled with ^{13}C with one exception that was labelled with ^{14}C. The incorporation of the label was controlled by ^{13}C NMR and liquid scintillation, respectively. The biosynthetic sequence found was L-phenylalanine, *t*-cinnamic acid, *p*-coumaric acid, *p*-hydroxycoumaric alcohol, *p*-methoxycoumaric alcohol and anethol. The incorporation rates ranged from 0.5% to 25%.

Introduction

During the last years phytochemical investigation of the roots of a variety of *Pimpinella* species [1–5] revealed the presence of unusually substituted phenylpropanoids. Because their basic skeleton is similar to isoeugenol we called their mother compound, 1-(*E*)-propenyl-2-hydroxy-5-methoxybenzene, pseudoisoeugenol [1].

As outlined in a previous paper [7], the unusual 2,5-dioxy substitution pattern requires its own biosynthetic explanation, different to that of ordinary phenylpropanoids. For the purpose of appropriate labelling experiments we maintain a leaf-differentiating callus culture of *Pimpinella anisum* which selectively accumulates epoxy-pseudoisoeugenol-(2-methylbutyrate), called EPB [6].

In a previous report we already described experiments to prove the sequence L-phenylalanine, *t*-cinnamic acid, and *p*-coumaric acid as starting point for the biosynthetic route [7]. In this paper we report experiments that clarify most of the biosynthetic steps leading from *p*-coumaric acid to pseudoisoeugenols.

Abbreviations: EPB, epoxy-pseudoisoeugenol-(2-methylbutyrate); EPT, epoxy-pseudoisoeugenol-tiglate; PAD-SH, tissue culture of *Pimpinella anisum;* PMD-SH, tissue culture of *Pimpinella major;* DIBAH, diisobutyl aluminium hydride.

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Results and Discussion

Labelling experiments

The fact that *p*-coumaric acid bears an oxygen substituent in the para position relative to the side chain whereas the pseudoisoeugenols do not was decisive for further biosynthetic considerations. Fig. 1 presents three routes that theoretically lead to the pseudoisoeugenol skeleton starting from *p*-coumaric acid. The problem of the final molecule lacking a *p*-hydroxy group is hypothetically solved by the assumption of a NIH shift of the side chain caused by the introduction of the second hydroxy group. The question arises at which stage of the biosynthesis this hydroxylation occurs. Route 1 postulates a previous partial or complete reduction of the side chain, whereas in the case of route 2 or 3 hydroxylation would take place with the acrylic acid side chain still present. Route 2 could be excluded because previous labelling experiments with 4-methoxy- and 2-hydroxy-5-methoxycinnamic acid yielded negative results. Missing incorporation of the latter compound also left little probability for route 3, because 2,5-dihydroxycinnamic acid with its reactive *p*-hydroquinone structure would have to be reduced at the side chain prior to methylation.

The precursors required for the experiments were labelled with ^{13}C either at position 2′ on the side chain or at the methoxy group and applied to the tissue culture.

According to Fig. 1 the sequence of assumptive precursors with the highest probability of incorporation appeared to be *p*-hydroxycinnamic aldehyde, *p*-hydroxycinnamic alcohol, anol and an-

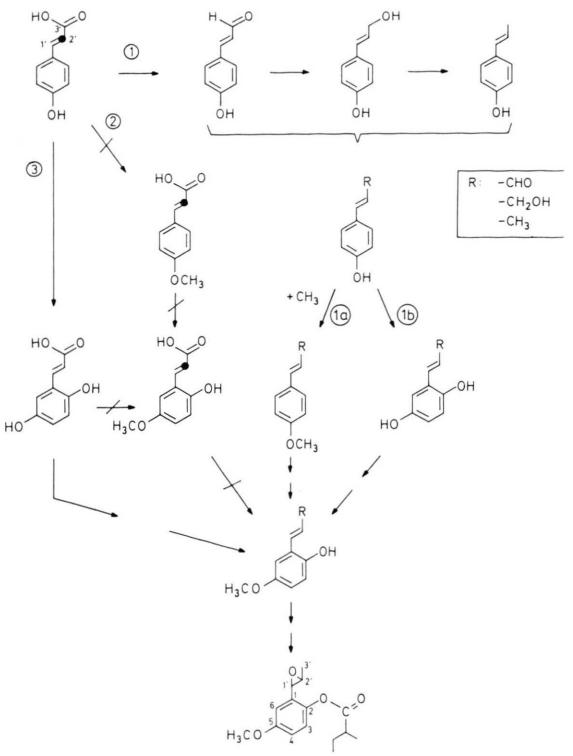

Fig. 1. Possible biosynthetic pathways for EPB starting from *p*-coumaric acid. ● indicates the site of the ^{13}C label in compounds already examined.

ethol. Due to the requirements of their synthesis, a deviation from this sequence appeared to be advantageous. The aldehyde was accessible by selective oxidation of the corresponding alcohol while labelled anol had to be synthesized by demethylation of anethol labelled at position 2′. This means that labelled *p*-hydroxycinnamic alcohol and anethol had to be used both as precursors and educts for syntheses.

As a result of both experiments remarkably high incorporation rates for *p*-hydroxycinnamic alcohol (5%) and anethol (25%) were observed. This unequivocally proved that they are precursors in the biosynthetic sequence examined. The incorporation of the alcohol together with the already known incorporation of *p*-coumaric acid made the additional synthesis of the respective aldehyde unnecessary, since there is no other way from an acid to the corresponding alcohol than *via* the aldehyde. The gap between *p*-hydroxycinnamic alcohol and anethol would be closed most likely by anol. This compound was synthesized from labelled anethol and fed to the culture. Very surprisingly, the resulting ^{13}C NMR spectrum of EPB did not show an appropriate signal enhancement.

To prove proper working conditions of the tissue culture while exposed to anol, a 1:1 mixture of [methyl-^{13}C]anethol and [2′-^{13}C]anol was offered in a subsequent experiment. In the spectrum of EPB (Fig. 2) only the signal of the methoxy group displayed substantial enhancement whereas that of C-2′ remained almost unaffected. This result indicates that only anethol is an obligatory intermediate in the biosynthesis of EPB.

The results obtained so far led to the conclusion that *p*-hydroxycinnamic alcohol was methylated at the phenolic hydroxy group prior to side chain reduction. In consequence, [methyl-^{13}C]*p*-methoxycinnamic alcohol was offered to the culture. The spectrum of the resulting EPB displayed, however, only moderate signal enhancement for C-2′, from which an incorporation rate of 0.5% was calculated, being one tenth of that of the unmethylated alcohol.

As a possible reason for this behaviour it could be argued that part of the methyl ether had been demethylated and then incorporated as *p*-coumaric alcohol *via* an unknown product not identical to anol. To check this possibility, the methyl ether with ^{13}C label at the methoxy group and the already described methyl ether with label at the 2′ position were applied in equal amounts to the same culture flasks. The ^{13}C spectrum of the resulting EPB displayed signal enhancements for both carbons, proving the hypothesis of demethylation to be wrong. In spite of the relatively low incorporation rate *p*-coumaric acid methyl ether has to be regarded as a potential precursor for EPB.

The origin of the methoxy group of EPB could easily be determined by offering commercially available L-[methyl-^{13}C]methionine, which was incorporated with a 0.9% yield.

To be absolutely sure that the non-incorporation of anol was not due to chemical instability towards the culture medium or to missing uptake by the tissue mass, both chemical stability and uptake were thouroughly examined. For this purpose, 4 mg of anol in DMSO was added to culture flasks either with culture medium and tissue mass or culture medium only. The result is shown in Fig. 3. As

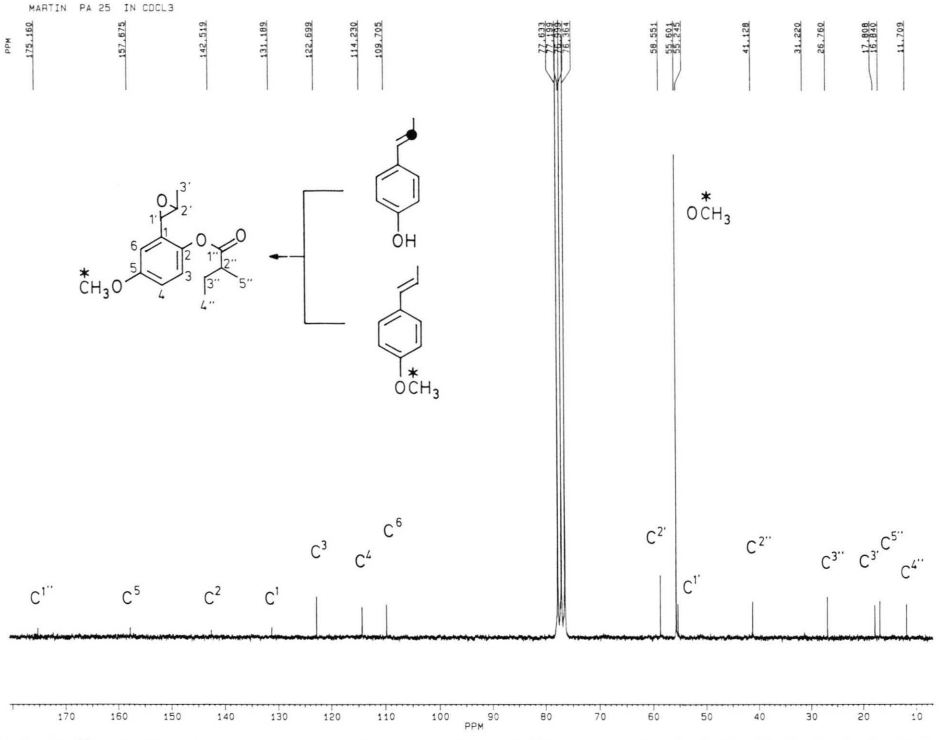

Fig. 2. ^{13}C NMR of EPB after offering a mixture of ^{13}C-labelled anethol and anol to the tissue culture. Only anethol is incorporated.

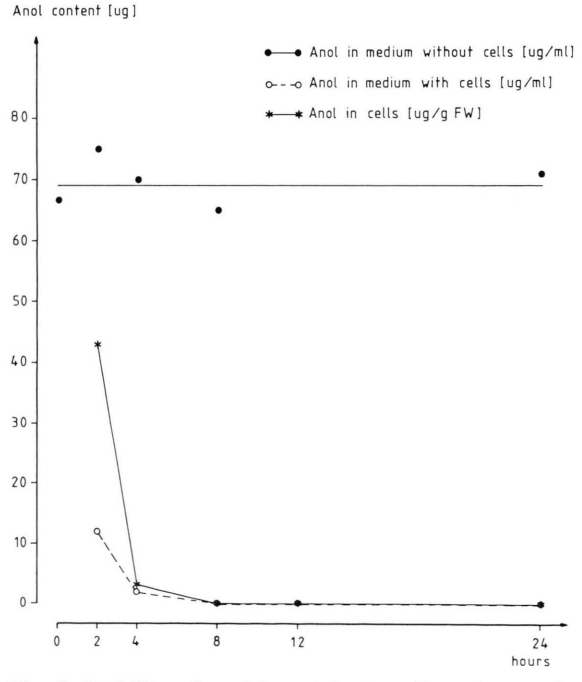

Fig. 3. Stability of anol in nutrient medium, its uptake and metabolism by the tissue.

can be seen, anol is stable in the medium for at least 24 h. The uptake by the tissue mass is almost complete within 4 h. Within the same time the concentration within the tissue mass also decreased drastically. This experiment clearly proves that anol is stable under the conditions employed. It is taken up by the plant tissue and metabolized, but not converted to EPB.

The availability of an analogous tissue culture of *Pimpinella major* that exclusively accumulates epoxy-pseudoisoeugenol-tiglate (EPT) [8] offered the possibility to check the possible incorporation of anethol into this molecule. Both isotopomeres of anethol were offered as described for *P. anisum*. The resulting ^{13}C NMR displayed respective signal enhancements also in this case with an incorporation rate of 20%. This result is particularly interesting since anethol has never been found in any part of *P. major* whole plants or in tissue culture. Only the fruits of *P. major* accumulate esters of anol with short chain fatty acids [8]. We conclude, therefore, that anethol is generally an obligatory precursor in the synthesis of pseudoisoeugenols.

The origin of the acyl moieties of EPB and EPT was clarified by feeding L-[U-^{14}C]isoleucine to both tissue cultures of *P. anisum* and *P. major*. The reason for choosing this amino acid was the known catabolic pathway of L-isoleucine, which leads over 2-methylbutyric and tiglic acid. The label was incorporated with 0.5% and 0.6%, respectively. This clearly showes the correctness of the assumption.

Conclusions

Based on our labelling experiments we propose a general biosynthetic scheme for pseudoisoeugenols which is given in Fig. 4. According to this scheme, the biosynthesis starts with L-phenylalanine which is converted to *t*-cinnamic acid and *p*-coumaric acid. Prior to the introduction of the second hydroxy group the carboxyl function of the side chain is reduced to the respective alcohol,

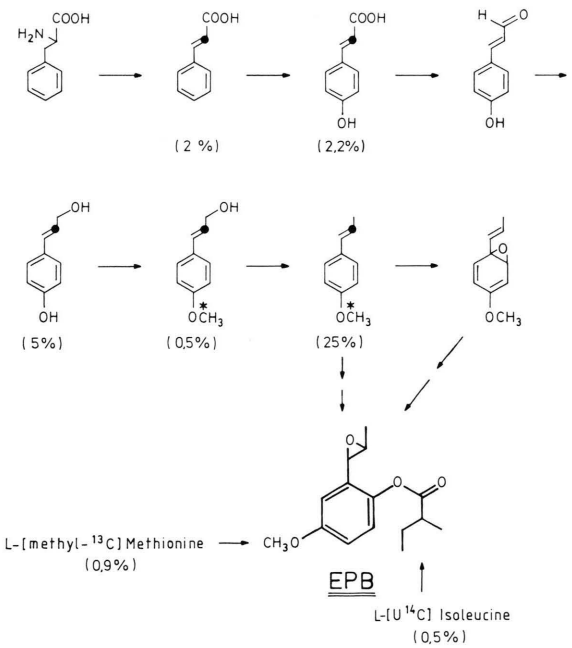

Fig. 4. Proposed biosynthetic pathway of EPB based on the conducted labelling experiments. ● and * indicate the site of the ^{13}C label. Formulas with both symbols indicate that each single labelled isotopomer has been fed separately. The incorporation rates into EPB are given in parentheses.

which is then methylated at the phenolic hydroxy group. The relatively low incorporation rate of this methyl ether might be explained by the assumption that both enzymes, the cinnamic alcohol methyl transferase and reductase, are situated next to each other and co-operate. Therefore, the channelling of an exogenous offered precursor into this enzyme system is more complicated. A more exact explanation of this stage of biosynthesis is not possible due to the fact that nor the enzyme neither the mechanism of this side chain reduction are known.

In opposition to *p*-methoxycinnamic alcohol, anol is not converted to EPB. Therefore, in the first instance we excluded anol as an obligatory precursor of anethol and the biosynthesis of pseudoisoeugenol, respectively. Nevertheless, we are aware of the fact that anol might be removed of the metabolism, *e.g.* by an oxidative polymerization. Further investigations have to clarify the fate of anol in the plant metabolism.

The compound that finally results from reduction is anethol. Anethol is unequivocally an important intermediate in the biosynthesis of EPB and the second hydroxy group must be introduced at this stage. The fact that both the methoxy label and the label in the side chain are retained indicates that one of the substituents has changed its position at the phenyl ring. Since the second hydroxy group is in juxtaposition to the side chain, it is concluded that the introduction of this hydroxy group caused the side chain to shift to the adjacent ring position, a metabolic process commonly known as NIH shift. This assumption is supported by the investigations of other authors who have studied the oxidation of a variety of aromatic compounds [9]. The successful labelling with anethol in *P. anisum* and *P. major* indicates a general biosynthetic scheme for all pseudoisoeugenols.

A final experiment to obtain certain proof of the occurrence of a NIH shift during hydroxylation of anethol is being undertaken and will be reported shortly.

Experimental

For description of the tissue cultures of *P. anisum* (PAD-SH) and *P. major* (PMD-SH) see ref. [6, 8]. Details of application of precursors, their isolation and ^{13}C NMR are given in ref. [7].

Stability and uptake of anol

This experiment was carried out using PAD-SH at day 14 of the culture period. 4 mg anol dissolved in 200 µl DMSO were added to each of five culture flasks. From a sixth flask the tissue mass was removed before the addition, this flask serving as control for the stability of anol. After 2, 4, 8, 12, 24 h one flask was harvested and the anol content in the medium and in the tissue quantified by HPLC. At the same time 1 ml of the control flask was also measured for anol.

Internal standard solution: 44.4 mg *p*-nitroacetophenone dissolved in 100 ml chloroform.

Extraction of medium: To 1.0 ml of culture medium (average volume/flask 25.5 ml) in a 10 ml separating funnel 50 µl (22.2 µg) of the internal standard solution was added and the mixture extracted three times with 3 ml chloroform. The chloroform was evaporated and the residue dissolved in 1 ml methanol and subjected to HPLC.

Extraction of tissue mass: The tissue was rinsed with water in a sieve and then dried using blotting paper. After weighing (average weight 17 g), 50 µl (22.2 µg) of internal standard solution was added and the tissue extracted with 20 ml of chloroform in a blender. The chloroform was filtered, dried with sodium sulphate, evaporated and the residue taken up in 1 ml methanol for HPLC analysis.

HPLC: Equipment as described in ref. [7]. Column: LiChroCart 125 × 4 mm, LiChroSpher 100 RP-18 5 µm. Solvent methanol/water/*o*-phosphoric acid 60:40:0.1, 1.2 ml/min. Detection wavelength 278 nm. Retention times: *p*-nitroacetophenone 2.3 min, anol 4.15 min.

Labelling with [U-^{14}C]isoleucine

To each flask of PAD-SH and PMD-SH was added 185 kBq (5 µCi) L-[U-^{14}C]isoleucine (specific activity >11 GBq/mmol), diluted with 1 mg of L-isoleucine. Work-up according to ref. [7].

1. [2′-^{13}C]p-hydroxycinnamic alcohol (p-coumaric alcohol)

a) [2′-^{13}C]*p*-coumaric acid methyl ester

30 mg [2′-^{13}C]*p*-coumaric acid (for synthesis see ref. [7]) was dissolved in 5 ml absolute methanol and 3 drops of conc. sulphuric acid added. The solution was refluxed overnight, poured into water after cooling to ambient temperature and extracted several times with ether. The combined extracts were dried with sodium sulphate and evaporated. The product was used for the next step without further purification.

b) [2′-^{13}C]*p*-hydroxycinnamic alcohol (*p*-coumaric alcohol)

In a 5 ml round bottom flask the ester was dissolved in 1 ml absolute THF, a stirring magnet added, the flask immersed in an ice bath and 1 ml diisobutylaluminium hydride (DIBAH) solution (1.5 molar in toluene) added with stirring. The ice bath was removed after 10 min and stirring continued for 1 h. After addition of 2 ml ether a 5% solution of ammonium chloride in water was added very carefully to decompose unreacted DIBAH and the aluminium salt of the product (*p*-coumaryl alcohol is acid sensitive and must not be treated with HCl). The mixture was transferred to a separating funnel, diluted with water and partitioned with ether. The combined ether fractions were dried with sodium sulphate, evaporated, and the residue taken up with dichloromethane. To remove the last traces of aluminium hydroxide, the solution was filtered through a column filled with silica gel (0.63–0.2 mm, 8 × 80 mm) by eluting first with a small amount of dichloromethane, then with dichloromethane/ethyl acetate 80:20. Completeness of the elution was checked by TLC (silica gel, dichloromethane/ethyl acetate 80:20), UV 254 nm. The yield of this reduction method is typically about 80% related to the amount of ester.

2. [2′-^{13}C]p-methoxycinnamic alcohol

From anise aldehyde and [2-^{13}C]malonic acid by Knoevenagel reaction [7], after esterification and reduction as described in 1.

3. [Methyl-^{13}C]p-methoxycinnamic alcohol

In a thick-walled screw cap tube with stirring magnet 30 mg *p*-coumaric acid methyl ester (prepared as given in 1. a) were dissolved in 1 ml dry acetone, finely powdered dry potassium carbonate (stored at 140 °C overnight) and 32 µl (72 mg, 0.5 mmol) [methyl-^{13}C]iodide were added. The bottle was sealed and kept at 60 °C with stirring for 5 h. The reaction mixture was poured into water in a separating funnel and extracted with

ether. After drying with sodium sulphate the ether was removed and the resulting ether reduced as described in 1.

4. [Methyl-^{13}C]anethol

a) Anol

Anol was synthesized by demethylation of commercially available anethol. In a screw cap flask with septum 2 g anethol was dissolved in 10 ml of *n*-heptane. 10 ml of DIBAH as 1.5 molar solution in toluene was added *via* a syringe and the needle left in the septum for pressure compensation [10]. The solution was kept at 90 °C for 18 h. After cooling, the reaction was stopped by careful addition of 5% citric acid, the mixture transferred to a separating funnel, diluted with water and extracted with ether. The combined ether extracts were reextracted with 3% NaOH to remove anethol still present. To the alkaline solution of anol some crushed ice was added and the solution acidified with 5% HCl. Anol was extracted with ether and finally purified over silica gel in analogy to 2., but only using pure dichloromethane. The yield was about 700 mg (39%), longer reaction times improve it.

Note: The usual procedure employing boron tribromide was not feasible since it did not yield any anol, nor could anethol be recovered, even at −78 °C reaction temperature. Most probably anethol is polymerized by this reagent.

b) [Methyl-^{13}C]anethol

Synthesis by the same procedure as in 3., starting with 100 mg (0.75 mmol) anol in 2 ml dry acetone, 200 mg potassium carbonate and 115 mg (0.8 mmol) [^{13}C-methyl]iodide. The reaction solution was poured into water, some NaOH added and the anethol extracted using *n*-pentane.

5. [2′-^{13}C]anethol

a) [1-^{13}C]ethyltriphenylphosphonium iodide

A mixture of 1.63 g (6.25 mmol) triphenylphosphine and 1 g (6.25 mmol) [1-^{13}C]ethyl iodide were kept at 120 °C overnight in a stainless steel pressure vessel. The product was dissolved with chloroform and the phosphonium salt precipitated by addition of ether. The liquid was carefully decanted, the product collected and dried overnight *in vacuo* in a desiccator to give 2 g (yield 76%) of white crystals.

b) [2′-^{13}C]anethol

In a 25 ml round bottom flask fitted with a septum locked adapter was added a stirring magnet, 418 mg (1 mmol) [1-^{13}C]ethyltriphenylphosphonium iodide, and 7 ml dry THF. The salt was dissolved by stirring while dry nitrogen was introduced. Addition of 1 mmol butyl lithium (0.65 ml 1.5 molar solution in hexane) formed the orange solution of the ylide. The flask was immersed in an ice bath and 136 mg anise aldehyde in 1 ml dry THF was added. After 20 min the white betain started to precipitate. After 90 min the septum adapter was replaced by a reflux condenser and the reaction mixture was refluxed at 80 °C for 2 h in an oil bath. At the end of this period, the mixture was only slightly turbid. After cooling to ambient temperature the solution was poured into a 50 ml volumetric flask containing 20 ml *n*-pentane whereupon triphenylphosphin oxide precipitated. The volume was completed to 50.0 ml by *n*-pentane and the anethol quantified by GLC (25 m OV 101 fused silica at 130 °C). The total amount of anethol was 106 mg (71.6% yield) with the ratio of *cis*/*trans* being 14.5:80. The decanted pentane solution was evaporated and the product purified by column chromatography: silica gel 63 to 200 µm, 2.5 × 15 cm, *n*-pentane. Gradient elution with 75 ml pentane, 100 ml pentane/dichloromethane 96.5:3.5, 300 ml pentane/dichloromethane 93:7. First fraction (No. 0) 75 ml, subsequent ones 25 ml each. Fraction 5 pure *cis* isomer, fr. 6 through 8 mixtures *cis* + *trans*, fr. 9 through 12 pure *trans* anethol. The fractions containing the *cis*/*trans* mixture were rechromatographed using the same system.

6. [2′-^{13}C]anol

From 30 mg [2′-^{13}C]anethol by demethylation as described in 3. a) using 1 ml DIBAH 1.5 molar in toluene.

Acknowledgement

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