METABOLISM OF ACETOPHENONE COMPOUNDS OF THE FUNGUS *Sporotrichum pulverulentum*

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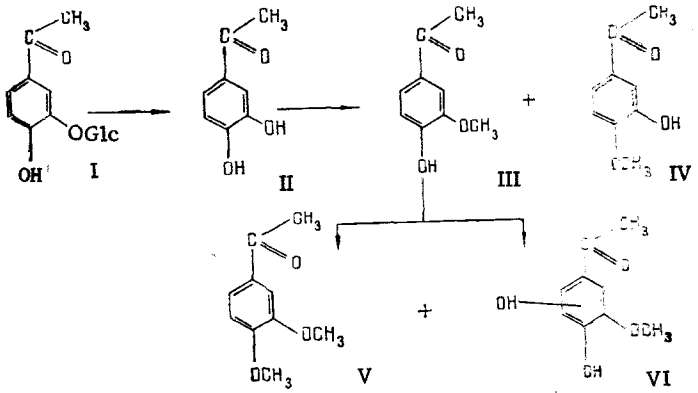
With the aim of revealing the specific reactions of biotransformation under lignolytic conditions, the metabolic transformation by Sporotrichum pulverulentum of a number of acetophenone compounds not previously investigated that model fragments of the lignin molecule has been studied. It has been established that the metabolism of 4-hydroxyacetophenone 3-O-β-D-glucopyranoside takes place as a sequence of processes involving deglucosylation, the methylation of free phenolic hydroxy groups with the formation of methylated intermediates, and the further hydroxylation of the m-methylated metabolite.

One of the promising approaches to a study of the metabolic pathways of the biodegradation of lignin is the investigation of the biotransformation reactions of compounds of simple structure reproducing fragments of the lignin molecule. The most active microorganisms breaking down lignin are recognized to be the storage rot fungi [1, 2], a presentative of which is Sporotrichum pulverulentum.

The aim of the present work was to study the biotransformation reactions of a number of acetophenone compounds of natural origin modeling the monomeric unit of lignin by the storage rot fungus Sporotrichum pulverulentum.

The metabolic transformations of 4-hydroxyacetophenone 3-O-β-D-glucopyranoside (I) under the conditions of the expression of the lignolytic system were investigated for Sporotrichum pulverulentum, strain F-1764.

When Sp. pulverulentum F-1764 was incubated with (I), the metabolites 3,4-dihydroxyacetophenone (II), 4-hydroxy-3-methoxyacetophenone (III), 3-hydroxy-4-methoxyacetophenone (IV), 3,4-dimethoxyacetophenone (V), and a dihydroxy-3-methoxyacetophenone (VI) were detected, showing the occurrence of deglucosylation, methylation, and hydroxylation processes [3, 4].



To establish the sequence of metabolic reactions, Sp. pulverulentum F-1764 was incubated with the intermediates (II), (III), and (IV). Analysis of the culture liquid of the fungus on incubation of (II) as substrate showed the presence of (III), (IV), (V), and (VI). When (III) was incubated, the metabolites (V) and (VI) were detected. The substrate (IV) was

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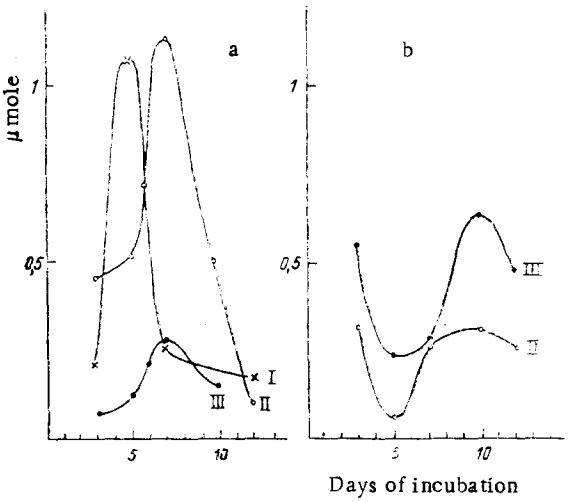

Fig. 1. Amounts of metabolites in the culture filtrate of Sp. pulverulentum on the incubation of 4-hydroxyacetophenone 3-O-β-D-glucopyranoside (a) and of 3,4-dihydroxyacetophenone (b): I) 3,4-dihydroxyacetophenone; II) 3-hydroxy-4-methoxyacetophenone; III) 4-hydroxy-3-methoxyacetophenone.

not transformed by the fungus. The metabolic products detected and their stability shown on their incubation in the culture medium without the fungus enabled us to suggest a sequential scheme of the methanolic reactions of Sp. pulverulentum F-1764.

An investigation of the in vitro reaction of a culture filtrate of the fungus with (I) led to the appearance of the aglycon (II) and to the absence of methylated compounds. When the culture filtrate was first boiled in the water bath for several minutes, no (II) was found from the in vitro reaction. These facts show that the process of deglucosylation has an enzymatic nature and was carried out by a secretory protein. It is known Sp. pulverulentum secretes β-glucosidase into the culture medium in the presence of cellulose [5]. In view of this, it may be assumed that β-glucosidase takes place in the deglucosylation of (I).

As our experiments showed, not all the free hydroxy groups undergo methylation. The OH groups in structures (II) and (III) were methylated but not the OH group in the structure of (IV) which can be explained by the substrate specificity of the working O-methyl transferase. The glycoside (I), itself, is not methylated, either. This indicates that the first stage in the biotransformation is a deglucosylation process, which is followed by methylation. The further transformation of the substrates is connected with the hydroxylation of the methylated intermediates.

To determine the degree of toxicity of the intermediate for Sp. pulverulentum, curves of inhibition by compounds (II), (III), and (IV) were plotted. It was found that, even in a concentration of 12 μmole per 12 ml of agarized medium, (III) had a substantial inhibiting effect on the growth of the fungus, and the area of mycelium of which under these conditions amounted to only 2% of the area of the mycelium in a control. The same concentration of (II) and (IV) had no appreciable inhibiting influence on the growth of the fungus.

A more detailed investigation of the metabolic transformations of (II) and (I) was carried out with the recording of the temporal dynamics of the transformation of the initial substrates by the strain F-1764. The results of the investigation are shown in Fig. 1. The deglucosylation of substrate (I) proceeded most intensively in the first five days of incubation (Fig. 1a). Then the methylation of the phenolic hydroxyls of (II) took place, this being predominantly at the 4-OH group with the formation of the relatively nontoxic (IV). The maximum amounts of (III) and (IV) were found on the seventh day of incubation. In the transformation of (III) there were substantial differences from that of (I). In the first few days the methylation of (II) took place with the formation of (III) and (IV), but in this case methylation at the 3-OH group predominated, with the formation of the more toxic (III), which was then methylated to the nontoxic (V). An increase in the amounts of (III) and (IV) was observed on the 10th day of incubation (Fig. 1b).

The difference in the quantitative formation of methylated intermediates in the transformation of (I) and (II) is probably connected with the presence of chemically bound glucose in the structure of (I) and its inclusion in a catabolic process.

For comparison, we investigated the metabolic transformation of (I) by another three strains of Sp. pulverulentum (F-1765, F-1766, and F-1767) under the same conditions as for F-1764. All the strains metabolized the glycoside (I) in a similar manner to F-1764, with the formation of cmpounds (II), (III), (IV), (V), and (VI).

At the present time, individual facts showing the possibility of the methylation of phenolic substrates by fungi have been reported. Thus, for the fungus Lentinus lepideus a capacity for methylating the methyl esters of cinnamic acids, but not of benzoic acids and their esters, has been shown [6, 7], while the p-activity of the intracellular methyltransferases fell with an increase in the number of hydroxy groups in the substrate.

A culture of Phanerochaete chrysosporium under lignin-degrading conditions methylated ferulic and vanillic acids and acetovanillone to the corresponding 3,4-dimethoxy derivatives [8].

The results that we have obtained showed the detection of 3- and 4-O-methylating activity of Sp. pulverulentum on a substrate with two free hydroxy groups (compound (II)) with the formation of two isomers ((III) and (IV)) and also the possibility of the further transformation of the intermediate (III) by the p-methylation route (metabolite (V)) and the hydroxylation route (metabolite (VI)). It may be assumed that the hydroxylation of a methylated intermediate will lead to a decrease in its stability and to the subsequent breakdown of the aromatic ring.

The role of the methylation of phenolic substrates and its link with the biodegradation of lignin require further study.

EXPERIMENTAL

Cultivation. Four strains of Sp. pulverulentum (F-1764, F-1765, F-1766, and F-1767) obtained from the collection of the Institute of the Biochemistry and Physiology of Microorganisms of the USSR Academy of Sciences were used.

Cultivation was performed in a liquid culture medium necessary, according to the literature, for the expression of the lignolytic enzymatic system under the conditions of a deficiency of nitrogen (2.4 mmole N) with the use as the source of carbon of microcrystalline cellulose (5 g/liter, Serva) [9]. The volume of the medium was 30 ml in 200-ml flat-bottomed flasks. For the growth of an inoculate, the culture was transferred to agarized must after incubation for three days. The inoculate was seeded into the culture medium from a 10-day fungal culture. Inoculation was carried out in an amount of 10^8 spores per 1 ml of medium. The fungal cultures were incubated at 28°C with shaking for 3, 5, 7, 10, and 12 days with the substrates mentioned below taken in an amount of 60 μmole per 30 ml of medium.

For the in vitro reaction, the culture liquid of the fungus was sterilized by filtration through Millipore filters (0.6 μ, Sweden) and 6.5 μmole of the substrate was incubated with 3 ml of culture filtrate at 38°C for 3 days. The curves of the inhibition of the fungus by the substrates were plotted from the areas of the fungal mycelium. An agarized block of fungus was placed in the center of a Petri dish on agarized nutrient medium with the addition and without the addition of the substrates. Incubation was carried out at 28°C for 2 days.

Synthesis of the Substrates Isolated. Compounds (I) and (II) were isolated from the extractive substances of the needles of Picea obovata [10], and (III) and (IV) were synthesized from guaiacol [11] with subsequent purification by chromatograhic methods. Compound (V) was synthesized from veratrole using acetic acid [12].

Isolation of the Metabolites. After the end of incubation, the mycelium was eliminated from the culture medium by filtration. The filtrate, after being acidified to pH 3.5-2, was extracted three times with an equal volume of diethyl ether. The combined ethereal extract was washed with distilled water, dried over anhydrous Na_2SO_4, and evaporated in a rotary evaporator in vacuum. The dry residue was recrystallized from acetone. The acetone extract was analyzed, and the metabolites were identified by comparison with standard compounds.

Mass Spectrometry. Mass spectra were taken on a MAT-212 mass spectrometer (Varian) using a system for direct introduction at an energy of the ionizing electrons of 70 eV, and also on a chromato-mass spectrometer with a capillary column coated with the stationary phases SE-50. The temperature was programmed from 80 to 240°C at the rate of 14°C/min.

Gas-liquid chromatography was performed on a LKhM 8 MD(5) chromatograph using a FID with steel column (0.3 × 300 cm) filled with 3% of OV-17 on Chromaton N Super (0.125-0.160 mm) with a programmed rise in temperature from 50 to 300°C at the rate of 6°C/min, and also under isothermal conditions at 200°C with an evaporator temperature of 300°C and a rate of flow of the carrier gas, nitrogen, of 30 ml/min.

Qualitative identification was made by comparing retention times and elution temperatures of standards with the components of the mixture being analyzed, and also by the marker method. The quantitative calculation of the amounts of compounds identified was performed by the internal-standard method with the use of correction factors. In parallel, the amounts of metabolites were determined by UV spectroscopy. The mixture of metabolites was first separated by GLC on Silufol plates as described below. The quantitative calculation was made from calibration curves plotted for each compound at the wavelength corresponding to the absorption maximum in the spectrum. The sorbent with the substance detected in UV light was collected, and the substance was eluted with methanol. The spectrum was recorded on a Specord spectrophotometer.

TLC was performed on Silufol (UV-254) plates using the CCl_4–acetone (3:1) and (4:1) solvent systems as the mobile phases. The compounds were detected in UV light and from the reaction with an alkaline solution of diazotized sulfanilic acid.

SUMMARY

1. The metabolic transformation of 4-hydroxyacetophenone 3-O-β-D-glucopyranoside takes place as a sequence of deglucosylation and methylation reactions with the formation of two methylated isomers and the hydroxylation of the m-methylated metabolite.

2. A 3- and 4-O-methylating activity of the fungus Sporotrichum pulverulentum has been detected for the first time on a substrate with two free hydroxy groups — 3,4-dihydroxyacetophenone — with the formation of two isomers: 4-hydroxy-3-methoxyacetophenone and 3-hydroxy-4-methoxyacetophenone.

3. The possibility has been shown of the transformation of 4-hydroxy-3-methoxyacetophenone by the p-methylation and hydroxylation pathway, and a resistance of the isomer 3-hydroxy-4-methoxyacetophenone to biotransformation has been demonstrated.

4. A comparative investigation of the metabolic transformation of glycoside (I) and its aglycon (II) has shown that the ratios of the isomeric intermediates differ, which is probably due to the effect of the catabolism of the glucose when (I) is used as the initial substrate.

5. Information has been obtained on the degree of inhibition of the mycelial growth of the fungus by substrates of the acetophenone series.

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TANNINS FROM LEAVES OF Hippophaë rhamnoides

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Four individual hydrolyzable tannins have been isolated for the first time from the leaves of the common sea buckthorn, two of which have been identified as strictinin and isostrictinin. The structure of the new tannin hipporhamnin has been established (6-O-galloyl-1,3-O-hexahydroxydiphenoyl-β-D-glucose). It has been shown that the free gallic acid in the leaves of the common sea buckthorn is an artifact.

The chemical composition of the leaves of the common sea buckthorn is complex. In them have been found flavonoids [1-4], carotenoids [6], amino acids [6], triterpene compounds [7, 8], sterols [7], coumarins [3], alkaloids [9], quebrachitol [11], gallic acid [3], and tannins [3, 10]. The amount of tannin substance in sea buckthorn leaves exceeds 10% [10, 12, 13].

From the tannin fraction of the leaves of Hippophaë rhammoides (common sea buckthorn) we have isolated four individual substances belonging to the group of hydrolyzable gallo-ellagotannins which give a positive reaction with ferric chloride and with sodium nitrite for bound ellagic (hexahydroxydiphenic) acid [15, 16]. Under the action of a dilute mineral acid, these substances hydrolyze with the formation of gallic and ellagic acids and glucose. In other compounds, the ratio of the components mentioned was 1:1:1. This ratio was confirmed in each case by the ^{1}H NMR spectrum. Tannins isolated from various plants with the same ratio of gallic and hexahydroxydiphenyl groups and glucose are known [17-27]. Compound (I) was close in the value of its specific optical rotation and the rate of hydrolysis to juglanin, the structure of which has not been determined [26].

In the ^{1}H NMR spectrum of (I) (deuteroacetone, TMS), the protons of the gallic acid residue gave a signal at 7.12 ppm (s, 2 H) and two protons of the hexahydroxydiphenyl residue gave signals at 6.50 and 6.78 ppm (singlets, 1 H). The four signals of the protons of the glycoside moiety of (I), the assignment of which was made with the aid of double resonance, were present at 5.65 ppm, $J_{1,2}$ = 4.8 Hz (H_1); 4.70 ppm, $J_{2,3}$ = 2 Hz (H_2); 4.08 ppm, $J_{6,5}$ = 2.9 Hz; $J_{6,6'}$ = 13.3 Hz; and 4.82 ppm, $J_{6',5}$ < 1 Hz (H_6 and $H_{6'}$). The signals of the remaining three protons (H_3, H_4, and H_5) were observed in a narrow region of the spectrum (5.2-5.4 ppm). The signals of two protons (H_4 and H_5) could be approximated by an AB quartet ($J_{4,5}$ = 9.7 Hz), the components of which were separated as the result of the interaction of the H_4 proton (5.43 ppm, $J_{4,3} \approx 2.0$ Hz) with the H_3 proton (5.40 ppm) and of the H_5 proton (5.30 ppm) with the proton of a methylene group giving a signal in the weak field. The assignment of the signal at 5.43 ppm (H_4), was made on the basis of the results of an analysis of the intensities of the lines of this signal ($\Delta\nu_{3,4} \approx J_{3,4}$). Below, we give the chemical shifts δ_i (ppm) and coupling constants $J_{i,i+1}$ (Hz) of the protons in the glucopyranose moiety of compound (I):

H	1	2	3	4	5	6	6′
δ_i	5.65	4.70	5.40	5.43	5.30	4.08	4.82
$\delta_{i,i+1}$	1,2	2,3	3,4	4,5	5,6	6,6′	6′,5
J, Hz	4.8	9.0	2.0	9.7	2.9	13.3	0

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