MICROBIOLOGICAL SYNTHESIS OF 7-BROMO-5-(o-CHLOROPHENYL)-3-HYDROXY-1,2-DIHYDRO-3H-1,4-BENZODIAZEPIN-2-ONE BY IMMOBILIZED ACTINOMYCETES

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UDC 577.154

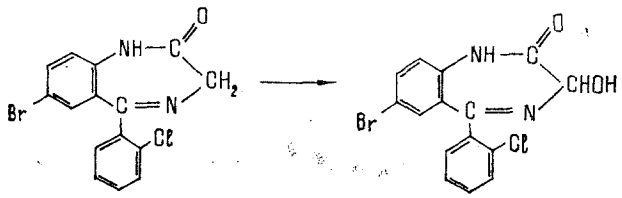
It has been shown that the highest yields (15%) of 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one are obtained by the use of the cells of actinomycetes immobilized in poly(vinyl alcohol) in the presence of the cosubstrate 7-bromo-5-(o-chlorophenyl)-3-methyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one.

It has been shown previously that phenazepam [7-bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one] possesses a unique physiological activity, being a sedative, soporific, and antispasmodic agent [1]. In order to study the metabolism of phenazepam and the pharmacaological properties and toxicity of its hydroxy derivative, it appeared of interest to synthesize 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one.

A one-stage process of introducing a hydroxy group into position 3 of diazepam and of triazolobenzodiazepines using potassium tert-butanolate in a mixture of tert-butanol, tetrahydrofuran, dimethylformamide, 1,2-dimethoxyethane, and trimethoxyphosphine is known [2]. However, the preparative difficulties of this method and, particularly, the narrow range of compounds capable of direct oxidation has led to the development of a multistage synthesis of 3-hydroxy derivatives of 1,4-benzodiazepin-2-ones [3] for which a low yield of the final products and the formation of a large amount of by-products difficult to separate are characteristic.

The aim of the present work was to ascertain the possibility of the microbiological synthesis of 3-hydroxy derivatives of phenazepam by immobilized actinomycetes.

In the study of the microbiological transformation of phenazepam by the actinomycetes *Actinomyces roseochromogenes*, VKM A-612, and *Streptomyces viridis* VKM A-607, we showed the possibility of the formation of 3-hydroxyphenazepam with a tield of 20-30% by the following scheme:



IR, UV, and mass spectra, and also the results of elementary analysis, confirmed the formation of the hydroxy product.

The IR spectrum showed the stretching vibrations of free and associated hydroxy groups in the 3600-3480 cm^{-1} region, the bands of free and associated —N—H groups at 3390 and 3180 cm^{-1}, a strong band of the stretching vibrations of a carbonyl group at 1692 cm^{-1}, and a weaker band of a —C=N bond at 1610 cm^{-1}.

The UV spectrum of the compound is characterized by the presence of an absorption band with λ_{max} 230-231 nm.

The mass spectrum confirmed the molecular weight of the compound — 365.

The substance is optically active: $[\alpha]_D^{20}$ +103°.

Physicochemical Institute, Academy of Sciences of the Ukrainian SSR, Odessa. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 779-783, November-December, 1981. Original article submitted July 25, 1981.

0009-3130/81/1706-0571$07.50

TABLE 1. Results of the Immobilization of Actinomycetes Hydroxylating 1,4-Benzodiazepin-2-ones

Days	Yield of the 3-hydroxy derivative of 1,4-benzodiazepin-2-one, %									
	cells in a polyacrylamide gel		cells in an agar gel		cells in a poly(vinyl alcohol)		cells in poly(vinyl alcohol) with blowing by O_2		cells in poly(vinyl alcohol) with a cosubstrate	
	I	II	I	II	I	II	I	II	I	II
1	1,2	0,9	0,6	0,5						
2	1,3	1,4	1,0	0,6	3,4	2,4	6,4	5,2	7,4	11,2
3	1,5	2,1	1,0	0,8						
4	1,3	1,6	1,1	0,9	3,4	3,6	6,7	5,4	7,6	15,0
5	1,0	1,3								
6	1,2	1,1	0,9	0,8	2,9	3,1	5,8	6,1	6,8	8,3
7	1,2	1,1								
8	1,2	1,0	0,5	0,8	2,3	2,6	5,1	4,8	6,1	5,3
10	1,0	0,9			1,4	2,5	3,2	4,1	4,5	4,9

Note. I — *Actinomyces roseochromogenes*, VKM A-612; II — *Streptomyces viridis*, VKM A-607.

TABLE 2. Storage of Actinomyces Hydroxylating 7-Bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one, the Cells being Immobilized in Poly(vinyl alcohol)

Days of storage	Yield of 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, %	
	Actinomyces roseochromogenes, VKM A-612	Streptomyces viridis, VKM A-607
4	100	100
8	82	59
10	61	41
16	59	31
20	52	39
30	26	19
40	16	11

The results of the hydroxylation of fenazepam by actinomycetes immobilized in polyacrylamide, agar gel, and poly(vinyl alcohol) are given in Table 1. As follows from this table, the yields of the 3-hydroxy products are low, but they are 2-3 times higher for the cells immobilized in poly(vinyl alcohol) than in the other gels. Consequently, forced aeration was carried out: the cells immobilized in poly(vinyl alcohol) were placed in a flask with distilled water, and oxygen was passed through the solution for 3 h. Then cultivation was carried out on a shaking machine at 28°C for 1-10 days. As a result it was possible to raise the yield of hydroxy product.

With the aim of further increasing the activity of the immobilized cells they were immobilized in poly(vinyl alcohol) in the presence of a cosubstrate [7-bromo-5-(o-chlorophenyl)-3-methyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one] and microbial synthesis was carried out. On the fourth day with the immobilized cells, 15% of hydroxy product was formed.

The reason for such an increase in the yield of the desired product is not yet clear. Some role is probably played by the spatial arrangement of the cells in the presence of the cosubstrate.

The cells of *Actinomyces roseochromogenes*, VKM A-612, and *Streptomyces viridis*, VKM A-607, immobilized in poly(vinyl alcohol) retain their capacity for transforming 7-bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one on storage (4°C) for 40 days, *Actinomyces roseochromogenes* VKM A-612, retaining 50% of its activity, and *Streptomyces viridis* VKM A-607, 11% (Table 2).

As has been shown previously [4], the microsomal fraction of animal livers also catalyzes the hydroxylation of 7-bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one with a yield of 3.6% of hydroxy product. However, when this was included in polyacrylamide gel the activity of the immobilized fraction of microsomes fell by 63% (at 37°C). It was possible to carry out the hydroxylation process three times with one sample but then the final activity had fallen by as much as 50%.

Thus, the results of the investigations that we have performed indicate the possibility of the microbiological synthesis of 3-hydroxyphenazepam by immobilized actinomycetes and the highly promising nature of the use for this purpose of immobilized cells rather than microsomal fractions.

EXPERIMENTAL

Actinomyces roseochromogenes, VKM A-612, and *Streptomyces viridis*, VKM A-607, from the collection of the Institute of Microbiology of the Academy of Sciences of the USSR, were used.

The experimental procedure was as follows: a culture with an agarized medium containing 1% of maize extract, 1% of starch, 3% of $(NH_4)_2SO_4$, 3% of NaCl, 2.5% of $CaCO_3$, and 2% of agar was placed in test-tubes with 10 ml of a liquid medium containing 0.5% of maize extract, 0.25% of glucose, 0.25% of NaCl, 0.2% of $(NH_4)_2SO_4$, 0.25% of $CaCO_3$, and 0.75% of starch, pH 6.0-7.0, and cultivation was carried out on a shaking machine (220 rpm) at 28°C. Then the contents of the test-tubes were transferred to an erlenmeyer flask that had been charged with 100 ml of a medium consisting of 1% of maize extract, 0.5% of glucose, 0.5% of NaCl, 0.35% of $(NH_4)_2SO_4$ 0.5% of $CaCO_3$, and 1.5% of starch, pH 6.8-7.0, and cultivation was continued under the same conditions for another 24-36 h.

The cells were collected by centrifugation (2.5 thousand rpm) and were washed three times in distilled water.

The composition of the polymerization mixture for the inclusion of the cells in polyacrylamide gel was as follows: 10 ml of 0.1 M potassium phosphate buffer, pH 7.5, prepared in a 20% solution of glycerol containing 1 mM EDTA and 1 mM dithiothreitol with 7.5 g of acrylamide, 0.6 g of N,N'-methylenebisacrylamide, 5 ml of a 5% solution of tetramethylenediamine, and 5 ml of a 2.5% solution of ammonia persulfate dissolved in it. To eliminate oxygen during polymerization, the solution was purged with nitrogen, and all the operations were performed at 0-2°C. Polymerization took 3-5 min at room temperature. The block of gel was dispersed and was passed through a sieve. The resulting granules were washed with water.

To include the cells in an agar gel, 5 g of agar was soaked in 100 ml of water for 2 h. Then it was heated to 80°C and rapidly mixed with a suspension of the cells. The gel obtained was dispersed.

To immobilize the cells in poly(vinyl alcohol), a 10% solution of poly(vinyl alcohol) in distilled water was prepared and the mixture was poured into petri dishes. The films were dried, cut into fragments with dimensions of 100-200 mm^2, and used for the transformation.

The immobilized cells were transferred into an erlenmeyer flask containing 100 ml of distilled water, and 3-5 mg of 7-bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one in solution in 0.1 ml of dimethyl sulfoxide or 1.0 ml of ethanol was added. Incubation was carried out on a shaking machine (220 rpm) at 28°C. After 1-10 days, the contents of the flasks were extracted with three volumes of chloroform. The chloroform layer was evaporated to dryness. The residue was dissolved in chloroform and was deposited on a Silufol UV-254 plate which was developed in the chloroform—hexane—acetone (3:2:1) solvent system, and then the layer of Silufol with the 3-hydroxy product was removed. It was placed in 4 ml of ethanol and, after centrifugation (2000 rpm, 15 min), the product was determined spectrometrically at 230-231 nm. The quantitative yield of substances with respect to the substrate introduced into the synthesis was determined from a calibration graph drawn up beforehand.

The 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one was isolated to determine its spectrochemical characteristics either by recrystallization from ethanol of the residue after the evaporation of the chloroform or on a column of neutral (pH 7.0) alumina containing 6% of water. The 7-bromo-5-(o-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepin-2-one was eluted with chloroform—acetone—ethanol (17:2:1) and the 3-hydroxy

derivative with methanol or ethanol—acetic acid (250:1). According to UV spectroscopy, this method permits the isolation of 90-100% of the untransformed 1,4-benzodiazepin-2-one and 60% of the desired product formed. The UV spectra of the 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one were recorded on an SF-16 spectrophotometer in ethanol, the IR spectra of a Perkin-Elmer 577 instrument in KBr tablets, and the mass spectra on an MKh-1303 instrument with a system for direct introduction at an ionizing energy of 50 eV, an emission current of 1.5 μA, and an inlet temperature 20-40°C below the melting point of the substance.

SUMMARY

1. *Actinomyces roseochromogenes*, VKM A-612, and *Streptomyces viridis* VKM A-607 effect the microbiological hydroxylation of phenazepam.

2. Cells of the actinomycetes immobilized in poly(vinyl alcohol) in the presence of a cosubstrate perform the microbiological synthesis with a 15% yield of 3-hydroxyphenazepam.

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LOW-MOLECULAR-WEIGHT METABOLIES OF WHEAT.

I. COMPONENTS OF AN ETHEREAL EXTRACT OF WHEAT LEAVES

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UDC 63.311+547.992

The chromatographic separation of the components of an ethereal extract of the leaves of "Mironovskaya 808" wheat has been performed. The presence in wheat of phthalic acid and its dimethyl, diethyl, and dibutyl esters has been shown for the first time. Substituted benzoic and cinnamic acids, p-hydroxybenzaldehyde, vanillin, 6-methoxybenzoxazolone, tricin, and aconitic and fumaric acids have also been isolated. The structures of the compounds isolated have been confirmed by IR, UV, mass, and NMR spectra.

Questions of the chemical regulation of the productivity of agricultural crops are connected primarily with an understanding of the inherent endogenous regulation of a plant. It is necessary to know the complex of low-molecular-weight metabolites characteristic for each plant and the changes in its composition taking place in the process of growth and development and to understand the role of each component of this complex. With respect to the most important agricultural crop — wheat — so far the attention of workers has been attracted mainly by questions of changing the composition of this biologically active substances in various periods of ontogenesis (without studying their structure, solely on the basis of histograms; see, for example, [1, 2]). The participation of known compounds widely distributed in plants has also been investigated. Thus, the dynamics of the levels of the most important phytohormones in wheat shoots in vernalization [3], in the developing heads [4], and in the seeds [5] has been studied. It has been shown that the compositions and amounts of free and bound forms of phenolic compounds are linked with lignification processes (see, for example, [6]), with the resistance of the plants to stem rust [7], and with drought resistance [8]. There are reports of considerable fluctuations in the amount

M. M. Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences of the USSR, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 783-789, November-December, 1981. Original article submitted April 23, 1981.

 0009-3130/81/1706-0574$07.50