Oxidation of nitrogen-containing heterocycles using biocatalysts

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A method for stereospecific hydroxylation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones using free and immobilized cells of actinomycetes as biocatalysts was developed. The hydroxylation under the action of yeast results in the formation of racemates. Actinomyces do not hydroxylate quinazolinones, quinoxalinones, and tetrahydro-1,5-benzodiazepin-2-ones; derivatives of 1,2,3,4-tetrahydro-1,5-benzodiazocin-2-ones are transformed into 2-[*N*-(3-acetylaminopropionyl)amino]benzophenones.

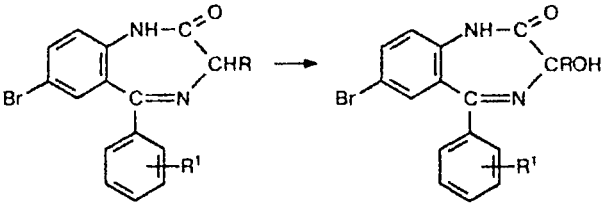
Key words: 1,4-benzodiazepin-2-ones, quinazolinones, quinoxalinones, 1,5-benzodiazepin-2-ones, 1,5-benzodiazocin-2-ones, hydroxylation, actinomycete and yeast cells, immobilization in carrageenan.

Oxidation reactions using biocatalysts are under intense study; however, specific introduction of the hydroxy group into molecules of both aromatic and heterocyclic organic compounds remains an important problem of preparative chemistry. 3-Hydroxy-derivatives of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones, *viz.*, oxazepam, temazepam, and lorazepam, possess interesting pharmacological properties and low toxicity. However, wider use of 3-hydroxy-derivatives is hindered due to such problems as the multi-stage character of synthesis and the low yield of final products.[1,2] In this case, the use of various systems modeling enzymatic ones based on Fe^{II} tetraporphyrin, iodosobenzene, $Fe(ClO_4)_3$, EDTA, ascorbic acid, and the Fenton systems was unsuccessful. Therefore, in this work, we consider the oxidation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones by actinomycetes and yeast cells as biocatalysts. In addition, in an attempt to disclose the specificity of oxygenases of actinomycetes, we studied microbiological transformations of six-membered (quinozalinones and quinoxalinones), seven-membered (tetrahydro-1,5-benzodiazepin-2-ones), and eight-membered (1,2,3,4-tetrahydro-1,5-benzodiazocin-2-ones) nitrogen-containing heterocycles, which are of pharmacological interest. It was anticipated that optically active compounds will be obtained.

Results and Discussion

The study of hydroxylation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones by representatives of taxonomic groups of bacteria *Pseudomonas fluorescens* (5 strains), *Bacillus subtilis* (4 strains), *Escherichia coli* (3 strains), *Aspergillus niger* (5 strains), *Aspergillus oryzae* (5 strains) and yeast *Saccharomyces cerevisiae* (6 strains) and *Actinomyces* (138 strains) showed that the yield of 3-hydroxy-derivatives is higher in the case of *Actinomyces* and *Saccharomyces*, and *Streptomyces viridis* VKMA-607, *Streptoverticillium cinnamoneum* VKMA-608, *Actinomyces roseochromogenes* VKMA-612, and *Saccharomyces cerevisiae* VKMU-379 are the most active. Actinomycete and yeast cells heated at 100 °C for 1 h are inactive. This indicates that the hydroxylation process is enzymatic, and the absence of hydroxylating activity in the incubation medium after separation of the cells is evidence for its intracellular character.

Optimization of the incubation medium, the study of the effect of pH, temperature, and duration of hydroxylation, and the influence of surfactants made it possible to develop a general method for transformation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones with the formation of their 3-hydroxy-derivatives as the single product in 40—50% yield.



For compounds with *ortho*-substituents in the phenyl ring, the yields of 3-hydroxy-derivatives are higher (~50%), and in the case of the *meta*- and *para*-substituted derivatives, the products of transformation are formed in equal (~40%) yields. 3-Hydroxy-derivatives were isolated by extraction with organic solvents and purified by recrystallization of the residue from ethanol or by chromatography on a column with neutral Al_2O_3.

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It is known that derivatives of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones can be obtained by the Polonovsky rearrangement of the corresponding 4-oxides.[3] This suggests that *N*-oxides can participate as intermediates in the hydroxylation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones; however, we did not observe their formation in either actinomycetes or yeast. 7-Bromo-5-(*o*-chlorophenyl)-, 7-bromo-5-(*m*-chlorophenyl)-, and 7-chloro-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one 4-oxides added to *Actinomyces roseochromogenes* VKMA-612 and *Saccharomyces cerevisiae* VKMU-379 cultures were not transformed into 3-hydroxy-derivatives.

The structure of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one derivatives (Table 1) was confirmed by IR, UV, and mass spectra and elemental analysis data. For example, the IR spectrum of 7-bromo-5-(*o*-chlorophenyl)-3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one exhibits stretching vibrations of the free and associated hydroxy groups in the region of 3600—3480 cm^{-1} and free and associated NH groups at 3390 and 3180 cm^{-1}. The spectrum also contains an intense band of stretching vibrations of the carbonyl group at 1692 cm^{-1} and a lower-intensity band of the —C=N— bond at 1610 cm^{-1}; a series of bands in the 1300—900 cm^{-1} range most likely corresponds to deformation vibrations of C—H of the benzene rings (1300—1000 cm^{-1}, planar vibrations, and 1000—900 cm^{-1}, extraplanar vibrations).

The UV spectra of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones are characterized by the absorption bands with λ_{max} 230—231 and 290—330 nm.

The mass spectra confirmed the molecular masses of the compounds (see Table 1). The mass spectrum of 7-bromo-5-(*o*-chlorophenyl)-3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (3-hydroxyphenazepam) contains, along with a low-intensity peak of the molecular ion (*m/z* 364), intense peaks of $[M-H_2O]^+$ ions with *m/z* 346 and $[M-HCO]^+$ ion with *m/z* 335, which indicates that the benzodiazepine system has the hydroxy group at the C(3) atom.

3-Hydroxy-derivatives were obtained in an optically active form (see Table 1). 7-Bromo-5-(*o*-chlorophenyl)-3-methyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one recovered after hydroxylation also possessed optical activity, $[\alpha]_D^{20}$ −57° (*c* 0.094, EtOH).

However, fermentation with *Saccharomyces cerevisiae* VKMU-379 did not result in stereoselective hydroxylation, and only 7-bromo-(*o*-chlorophenyl)-3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one was obtained in the optically active form ($[\alpha]_D^{20}$ +17° (*c* 0.10, EtOH)), whereas other 3-hydroxy-derivatives were optically inactive.

That yeast do not manifest stereodirected activity is not exception. The formation of a racemic product was also observed in the reduction of 3-methoxy-8,14-seco-D-homo-$\Delta^{1,3,5,(10),9(11)}$-estratetraene-14,17-dione[4] under the action of *Saccharomyces oviformis* VKMU-499 and *Candida robusta* VKMU-367.

A hydroxylating system usually contains several electron carriers: reductase of the flavoprotein nature, a protein containing non-heme iron, and hydroxylase, which is often represented by the heme-containing protein cytochrome P-450.[5] Hydroxylase systems of various microorganisms differ in composition of the components. Since no data on the hydroxylase system that oxidizes 1,4-benzodiazepin-2-ones are available, we obtained yeast protoplasts, a cell-free extract, microsomes,

Table 1. Properties of 7-bromo-3-R-5-(halophenyl)-3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones obtained by microbiological hydroxylation

R	Halogen	M.p. /°C	Found / Calculated (%)			Molecular formula	Molecular mass	
			C	H	N		Calculated	Found from mass spectrum[a]
H	*o*-Cl[b]	160—162	49.3 / 49.2	2.6 / 2.7	7.7 / 7.7	$C_{15}H_{10}BrClN_2O_2$	365.6	364
H	*m*-Cl[c]	193—194	49.4 / 49.2	2.3 / 2.7	7.5 / 7.7	$C_{15}H_{10}BrClN_2O_2$	365.6	364
H	*p*-Cl[d]	205—206	49.1 / 49.2	2.4 / 2.7	7.4 / 7.7	$C_{15}H_{10}BrClN_2O_2$	365.6	364
Me	*o*-Cl[e]	246—248	50.3 / 50.6	3.0 / 3.2	7.1 / 7.4	$C_{16}H_{12}BrClN_2O_2$	379.6	378
Me	*m*-Cl	Oil	50.4 / 50.6	2.9 / 3.2	7.2 / 7.4	$C_{16}H_{12}BrClN_2O_2$	379.6	378
Me	*p*-Cl	Oil	50.7 / 50.6	3.1 / 3.2	7.0 / 7.4	$C_{16}H_{12}BrClN_2O_2$	379.6	378
H	*o*-Br	171—172	43.6 / 43.9	2.1 / 2.5	6.5 / 6.8	$C_{15}H_{10}Br_2N_2O_2$	410.1	408
H	*m*-Br	105—206	43.5 / 43.9	2.3 / 2.5	6.7 / 6.8	$C_{15}H_{10}Br_2N_2O_2$	410.1	408

[a] Molecular masses of the peaks corresponding to ^{35}Cl and ^{79}Br are presented. [b] $[\alpha]_D^{20}$ +103° (*c* 0.097, ethanol). [c] $[\alpha]_D^{20}$ +93° (*c* 0.087, ethanol). [d] $[\alpha]_D^{20}$ +97° (*c* 0.11, ethanol). [e] $[\alpha]_D^{20}$ +91° (*c* 0.093, ethanol).

and a supernatant by the known procedures.[6] Cytochrome P-450 and the activity of NADPH-cytochrome-*c*-reductase were determined in cell fractions.

Data on the formation of 3-hydroxyphenazepam upon hydroxylation of phenazepam by different cell fractions of *Saccharomyces cerevisiae* are presented below.

Cell fraction	3-Hydroxyphenazepam (% of maximum)
Cell-free extract	30
Microsomes	100
Supernatant	0

The content of cytochrome P-450 (*m*/nmol (g of protein)$^{-1}$) and the specific activity of NADPH—cytochrome-*c*-reductase (*a*/nmol cytochrome *c* min^{-1} (mg of protein)$^{-1}$) in different cell fractions of *Saccharomyces cerevisiae* are presented below.

Cell fraction	*m*	*a*
Microsomes	52	131
Supernatant	0	0
Cell-free extract	Not determined	15

The results presented indicate that the enzymatic activity is related to the microsomal fraction. The content of cytochrome P-450 in growing cells correlated with the formation of 3-hydroxyphenazepam (Fig. 1).

The pharmacological activities of the optical isomers of 1,4-benzodiazepin-2-ones usually differ, which was shown for oxazepam hemisuccinate.[7] For example, the (+)-isomer of oxazepam hemisuccinate, unlike the (−)-form, exhibits a pronounced effect on the central nervous system. It is established[8] that the D- and L-forms of 7-chloro-5-phenyl-3-propyl-1,4-benzodiazepin-2-ones differ in activity.

(+)-3-Hydroxyphenazepam obtained by us upon transformation with *A. roseochromogenes* VKMA-612 is distinguished by a more pronounced activity as compared to Corazole, and potentiation of hexenal sleeping is higher than that of the racemate (Table 2).

Using the actinomycete strains that were the most active in hydroxylation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones, we incubated under the same conditions substituted quinazolines, 6-halogeno-4-(R-phenyl)-quinazolin-2-ones (halogen is Cl, Br; R = H, *o*,*m*,*p*-Cl, *o*-Br, and *p*-Me), and quinoxalinones, 4-(2-R-benzoyl)-3,4-dihydroquinoxalin-2-ones (R = H, Cl, Br, and Me) for 1 week with daily analysis. We observed no transformations of these compounds.

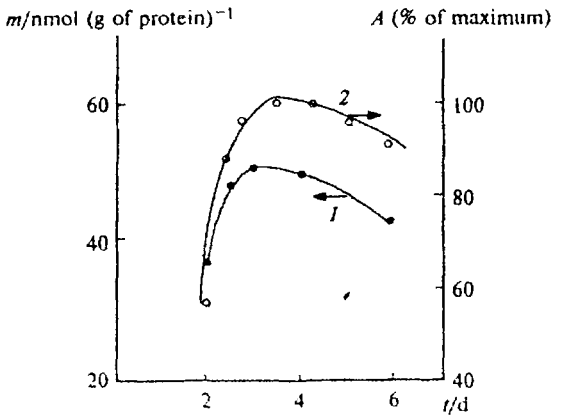


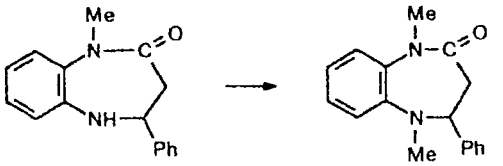
Fig. 1. Accumulation of cytochrome P-450 (*1*) and 3-oxyphenazepam (*2*) in growing *Saccharomyces cerevisiae* VKMU-379.

Table 2. Psychopharmacological properties of 3-hydroxyphenazepam (ED_{50}/mg kg^{-1})[a]

Isomer	Antagonism with Corazole	Potentiation of hexenal sleep
Racemate	0.017 (0.012—0.034)	0.084 (0.062—0.11)
(+)	0.008 (0.006—0.011)	0.047 (0.039—0.069)

[a] ED_{50} are effective doses for experimental animals (white mice). Figures in parentheses are confidence intervals of ED_{50} at $P = 0.05$.

As is known, *N*-substituted tetrahydro-1,5-benzodiazepin-2-ones exhibit antitumor and fungicidal activities, their 5-acyl- and 1,5-diacyl-derivatives possess high antiinflammatory activity, and 1- or 5-alkyl-derivatives retard[9] the growth of pulmonary carcinoma by 46.5 and 41.1%, respectively. However, attempts to obtain their hydroxy derivatives by actinomycetes did not give positive results, and only 1-methyl-4-phenyltetrahydro-1,5-benzodiazepin-2-one gave 1,5-dimethyl-4-phenyltetrahydro-1,5-benzodiazepin-2-one in 40% yield, which was identified by physicochemical methods.



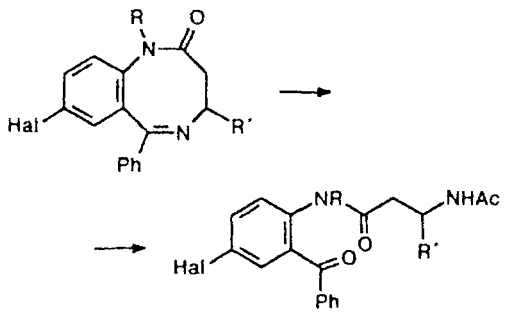
According to the mass spectrometric data, the molecular mass of the product is 266. The UV spectrum of the compound obtained contains absorption bands with λ_{max} 238, 256, and 336 nm. The IR spectrum exhibits bands of C=O stretching vibrations in the region of 1670—1660 cm^{-1}. Signals of two methyl groups at δ 3.4 are observed in the ^{1}H NMR spectrum.

The elemental analysis data confirmed the formation of 1,5-dimethyl-4-phenyl-2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-2-one. It is noteworthy that methylation by microorganisms is a rather rare process. Only *N*-methylation of aniline by *Mycobacterium tuberculosis* culture and *N*-1-methylation of α-carbolines under the action of *Streptomyces sp.* are known.[10]

Taking into account that derivatives of 1,2,3,4-tetrahydro-1,5-benzodiazocin-2-ones are of pharmacological interest because of their hypnosedative, antispasmodic,

and myorelaxant properties and low toxicity, we studied their transformations during incubation with actinomyces. However, in this case, 2-[*N*-R-*N*-(3-acetylaminopropionyl)amino]-5-halobenzophenones are formed, and in the case of *N*-methyl- and *N*-benzyl-derivatives, the eight-membered ring opening is accompanied by *N*-dealkylation (Scheme 1).

Scheme 1



Hal = Cl, Br; R = H, Me, $PhCH_2$; R' = H, Ph

The physicochemical properties of the products of microbiological transformation of 1,5-benzodiazocin-2-ones are presented in Table 3. Experiments with heated cells confirmed the assumption on the enzymatic character of scission of the 1,5-benzodiazocine ring.

In the case of the transformation of 8-chloro-1-methyl-4,6-diphenyl-1,2,3,4-tetrahydro-1,5-benzodiazocin-2-one, optically active amidobenzophenones $C_{24}H_{21}ClN_2O_3$ ($[\alpha]_D^{20}$ +51° (*c* 0.19, EtOH)) and $C_{25}H_{23}ClN_2O_3$ ($[\alpha]_D^{20}$ +62° (*c* 0.16, EtOH)) are formed (see Table 3).

Thus, of the nitrogen-containing heterocycles studied only 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones undergo hydroxylation under the action of actinomycetes. It is noteworthy that the formation of products of aromatic hydroxylation was observed for none of the compounds tested.

Since the enzymatic complex performing hydroxylation is intracellular, we considered the possibility of the use of immobilized actinomycetes. Immobilization was performed in polyacrylamide, agar, and carrageenan gels and in polyvinyl alcohol.

The carrageenan gel was found to be the most promising: 3-hydroxy-derivatives were formed in a yield up to 65% of that obtained with free cells. On storage of the immobilized cells for 50 days at 0—2 °C, 100% of the initial activity was retained. In the column regime (37 °C, blowing of sterile oxygen, elution rate 1 mL min^{-1}), stable operation is possible during 20 days. Carrageenan-immobilized actinomycete cells were "activated" during the work of the column (after 60 h), which is possibly related to cell multiplication inside and on the surface of the granules.

Experimental

We studied 138 strains of actinomycetes kindly provided by Prof. V. D. Kuznetsov (Institute of Microbiology, Russian Academy of Sciences) and 6 strains of *Saccharomyces cerevisiae* (Collection of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences). Substituted 1,4-benzodiazepin-2-ones were provided by Dr. A. S. Yavorskii and Dr. L. N. Yakubovskaya, 1,5-benzodiazocin-2-ones were provided by Dr. V. V. Danilin (A. V. Bogatsky Physico-

Table 3. Properties of 2-[*N*-R-*N*-(3-acetylaminopropionyl)amino]-5-halobenzophenones

R	R'	Halogen	M.p. /°C	Yield (%)	Found / Calculated (%)			Molecular formula	Molecular mass	
					C	H	N		Calculated	Found from the mass spectrum[a]
H	H	Cl	151—152	32	62.7 62.7	4.5 4.9	8.4 8.1	$C_{18}H_{17}ClN_2O_3$	344.8	344
Me	H	Cl	117—118	54	53.3 63.6	5.1 5.3	7.9 7.8	$C_{19}H_{19}ClN_2O_3$	358.8	356
H	H	Br	155—156	10	55.7 55.5	4.2 4.4	7.0 7.2	$C_{18}H_{17}BrN_2O_3$	389.2	389
Me	H	Br	147—148	70—80	56.7 56.6	4.5 4.7	6.7 6.9	$C_{19}H_{19}BrN_2O_3$	403.2	402
CH_2Ph[b]	H	Cl	Oil	50	69.3 69.0	5.0 5.3	6.1 6.4	$C_{25}H_{23}ClN_2O_3$	434.9	432
H	Ph	Cl	163—165	15	68.5 68.3	5.0 4.8	6.6 6.9	$C_{24}H_{21}ClN_2O_3$		
Me	Ph	Cl	175—176	30	69.0 69.3	5.3 5.0	6.4 6.6	$C_{25}H_{23}ClN_2O_3$		

[a] Molecular masses of the peaks corresponding to ^{35}Cl and ^{79}Br are presented.
[b] 2-(β-Acetylaminopropionylamino)-5-chlorobenzophenone (30%) is also formed.

Chemical Institute, National Academy of Sciences of Ukraine) and 1,5-benzodiazepin-2-ones were provided by B. A. Puodzhyunaite (Institute of Biochemistry, Vilnius, Lithuania).

Specific optical rotation was determined on a Perkin—Elmer 141 spectropolarimeter. IR spectra were recorded on a Specord IR-75 spectrometer, and UV spectra were recorded on an SF-46 spectrophotometer in ethanol. Mass spectra were obtained on a Varian MAT-112 mass spectrometer under the following conditions: ionizing voltage 70 V, temperature of the ionization chamber 180—200 °C, resolution 700—800. Samples were introduced into the ionization chamber by the direct inlet system. ^{1}H NMR spectra were recorded on a Tesla BS-487C spectrometer (80 MHz). Melting temperatures were determined using the Kofler hot stage.

1,5-Dimethyl-4-phenyl-2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-2-one was obtained as follows. A two-week culture of *Streptomyces viridis* VKMA-607 from the agarized medium was transported in tubes containing the nutrient medium (10 mL). The tubes were incubated on a shaker for 2 days (28—30 °C) with rotation velocity 220 rpm. Then the seed material was transferred into 750-mL flasks with a medium (100 mL) containing (g per L of water): corn extract, 10; glucose, 5; $(NH_4)_2SO_4$, 3.5; $CaCO_3$, 5; starch, 15; and NaCl, 5. The flasks were incubated for 2 days. Then 1-methyl-4-phenyl-2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-2-one (40—50 mg) in dimethylsulfoxide was added to the flasks, and after three-day incubation the products were extracted with chloroform. The solvent was evaporated, and the title product was isolated by column chromatography (Al_2O_3).

The optimum procedure for the preparation of **amidobenzophenones** is as follows. A seed culture of *Actinomyces roseochromogenes* VKMA-612 was grown as described above. Then the contents of the tubes were transferred into Erlenmeyer 750-mL flasks with a medium (100 mL) containing (g per L of water): starch, 15; corn extract, 10; glucose, 5; $(NH_4)_2SO_4$, 3; and $CaCO_3$, 5. The flasks were incubated under the same conditions for 2 days. Then 1,5-benzodiazocin-2-ones (20—30 mg) in dimethylsulfoxide was introduced into the flasks. Two days later, the products were extracted with chloroform, and the extract was evaporated to dryness. Amidobenzophenones were obtained after recrystallization from ethanol.

Hydroxylation of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones. *A.* A two-week culture of *Actinomyces roseochromogenes* VKMA-612 from the agarized medium (oats, 2%; yeast extract, 0.25%; agar, 2%) was transferred into 20 tubes with a liquid medium (10 mL) containing (g per L of water): starch, 7; corn extract, 5; NaCl, 5; $(NH_4)_2SO_4$, 3.5; $CaCO_3$, 5; and glucose, 5. The incubation was conducted at 28 °C (220 rmp) for 2 days. The contents of the tubes was transferred into 750-mL Erlenmeyer flasks with a medium (100 mL) containing (g per L of water): starch, 24; NaCl, 1.5; $(NH_4)_2SO_4$, 3; $CaCO_3$, 5; glucose, 5; and corn extract, 10. The incubation was conducted under the same conditions for 2 days. Then 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones (50 mg) in dimethylsulfoxide were added to each flask. In addition, hydroxylation was performed in a 10-L flask (4 L of the medium), in this case, the load was 3 g. The incubation was conducted at 28 °C for 3 days, and the rotation velocity of the shaker was 220 rpm, pH 7.2—7.4. Then the products were isolated by extraction with organic solvents (chloroform, ethyl acetate, light petroleum, or a mixture of dichloroethane and ethyl acetate) and purified by recrystallization from ethanol or column chromatography on neutral Al_2O_3 with a water content of 6%. The starting compounds were eluted by a chloroform—acetone—ethanol (17 : 2 : 1) mixture, and 3-hydroxy-derivatives were eluted by a mixture of methanol or ethanol with acetic acid (250 : 1).

B. Saccharomyces cerevisiae were grown at 33 °C on a medium of the following composition (g per L of water): yeast extract, 10; molasses, 20; and glucose, 20. The transformation was carried out in Rider's medium containing (g per L of water): $(NH_4)_2SO_4$, 3; $MgSO_4$, 0.7; NaCl, 0.5; KH_2PO_4, 1; and K_2HPO_4, 0.1. Sucrose (5%) was added to the medium, pH 6.6—6.8, 37 °C, incubation time 2—3 days, rotation velocity of shaker 130 rpm. Substrates were added as solutions in ethanol or dimethylsulfoxide. The products were isolated as described above.

Cell fractions of *Saccharomyces cerevisiae* VKMU-379. Protoplasts were obtained by the previously described method,[6] using the complex of enzymes from the freeze-dried gastric juice of the snail *Helix pomatia* as the lytic factor. The enzyme complex was isolated by the known procedure.[11] To obtain the cell-free extract, protoplasts were destroyed using a pistil-type homogenizer by 20 movements of the pistil with a velocity of 800 rpm at 4 °C. The homogenate was separated from the nondestroyed cells by centrifugation at 6000 rpm for 15 min. The supernatant was centrifuged for 15 min at 10000 *g* to precipitate mitochondria, and then the supernatant was used as the cell-free extract. A portion of the cell-free extract was divided into microsomal and supernatant fractions by ultracentrifugation at 165000 *g* for 60 min. The activity of NADPH-cytochrome-*c*-reductase was determined in three fractions obtained by the previously described procedure.[12] The specific activity of the enzyme was calculated using the molar extinction coefficient for cytochrome *c*, which was equal to 21000 L mol^{-1} cm^{-1}.[13] Cytochrome P-450 in the cell fractions was determined by the known procedure:[14] a fraction of microsomes in a 0.1 *M* KH_2PO_4—NaOH buffer (pH 7.4) in 20% glycerol was introduced in two cells of a spectrophotometer. Several crystals of sodium dithionite were added to the control cell, and a weak CO flow was passed for 1 min through the working cell. Then sodium dithionite was also added, and 3—5 min later, photometry was performed. The content of cytochrome P-450 was determined from the difference in the optical density at 450 and 420 nm using the molar extinction coefficient $91 \cdot 10^3$ L mol^{-1} cm^{-1}. Protein was determined according to Lowry.[15]

Immobilization. The composition of the polymerization mixture for immobilization of actinomycetes into polyacrylamide gel was as follows: acrylamide (7.5 g) and *N*,*N*-methylenebisacrylamide (0.6 g) in 10 mL of an 0.1 *M* potassium phosphate buffer (pH 7.5) prepared in 20% glycerol containing 1 *mM* EDTA and 1 *mM* dithiothreitol, 5 mL of 5% tetramethylethylenediamine, and 5 mL of 2.5% ammonium persulfate. Nitrogen was flushed through the solution during polymerization to remove oxygen, and all procedures were carried out at 0—2 °C. Polymerization lasted for 3—5 min. The block of the gel was dispersed by pressing through a sieve. The granules obtained were washed with water.

For incorporation of cells into the agar gel, agar (5 g) was kept in water (100 mL) for 2 h, then heated to 80 °C to obtain the homogeneous mass, cooled to 40 °C, and mixed rapidly with a suspension of actinomycete cells. The gel obtained was dispersed.

For immobilization of actinomycetes into polyvinyl alcohol, 10% solution of polyvinyl alcohol in distilled water was prepared, then an aqueous suspension of cells was added, and the mixture was poured into Petri dishes. The films obtained were dried, cut into fragments 100×200 mm in size, washed with distilled water for 2 h, and used for transformation.

Immobilized cells were transferred into Erlenmeyer flasks with distilled water (100 mL), and a solution of 7-bromo-5-(*o*-chlorophenyl)-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one

(3—5 mg) in dimethylsufoxide or ethanol was added. Incubation was conducted on a shaker (220 rpm) at 28 °C. Two days later, the products were extracted with chloroform. The chloroform was distilled off. The residue was recrystallized from a mixture of chloroform and ethyl acetate (1 : 1). The yield of 3-hydroxy-derivatives was determined as follows: the residue after distillation of chloroform was dissolved in chloroform and applied onto a Silufol UV-254 plate. The plates were eluted with a chloroform—hexane—acetone (3 : 2 : 1) solvent system. The yield was determined spectrophotometrically with respect to the substrate using a calibration plot. The yield was 15%.

Cells were immobilized in carrageenan as follows: carrageenan (3 g) was dissolved in distilled water (160 mL) at 70—80 °C. Then the temperature was decreased to 40—42 °C, and the solution was mixed with a cell suspension of actinomycetes (40 mL) containing 20 g of cells (wet weight). For strengthening, the block was kept in a 0.3 *M* solution of KCl. Then the block obtained was cut into cubes (3×3×3 mm), which were used to fill as column.

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