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MICROBIOLOGICAL TRANSFORMATION OF PIPERIDINE AND PYRIDINE DERIVATIVES

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Optically active 2-(4-hydroxyphenylcarbamoyloxymethyl)-1-methyl-1,2,5,6-tetrahydropyridine is obtained on microbiological transformation of 1-methyl-2-(N-phenyl-carbamoyloxymethyl)-1,2,5,6-tetrahydropyridine with a growing culture of the fungus C. verticillata VKPM F-430. Transformation of 2-(2-phenylethyl)pyridine by a methanoxidating culture of Methylococcus occurs either by hydroxylation of the side chain or conversion of the substrate into N-oxide, depending on the process conditions.

Derivatives of pyridine and piperidine containing hydroxyl groups in the heterocycle and/or in the side chain have recently attracted the attention of synthetic chemists and pharmacologists [1-3]. It was shown previously that certain cultures of mycelial fungi are capable of hydroxylating heterocyclic carbon atoms of derivatives of piperidine [4], tetrahydropyridine [5], a series of bicyclic piperidine derivatives [6,7], and also side chain carbon atoms of alkylpyridines [8].

Certain strains of bacteria and mycelial fungi obtained from collections of microorganisms in Russia and in the USA served as subjects of the investigation.

The following fungus strains were investigated for possible microbiological oxidation of the phenylurethane – 1-methyl-2-(N-phenylcarbamoyloxymethyl)-1,2,5,6-tetrahydropyridine (I): *C. verticillata* VKPM F-430, *B. bassiana* VKM F-3111D, *B. bassiana* ATCC 7159, *A. niger* VKM F-1119, *A. niger* KM-11, *A. niger* NRRL 3228, *F. culmorum, F. solani, F. sporotrichiela* var. *poal, F. moniliforma, F. microcera* var. *orthoconium, F. oxysporum, F. kuhnii, F. heterosporium,* and *F. averaceum* var. *herbarum.* It was established that all strains of *Beauveria bassiana* and *Aspergillus niger* proved to be inactive towards this substrate. All fungi of the genus *Fusarium* effected only hydrolysis of the carbamoyl group and only the initial substrate (I) and methyl-2-hydroxymethyl-1,2,5,6-tetrahydropyridine (II) were present in the incubation mixture according to chromato-mass spectrometry. Only on transformation of the given substrate by *Cunninghamella verticillata* VKPM F-430 an optically active transformation product was successfully isolated from the reaction mixture in 50% yield. The structure of 2-(4-hydroxyphenylcarbamoyloxymethyl)-1,2,5,6-tetrahydropyridine (III) was assigned on the basis of spectral data (Scheme 1). In its IR spectrum two new (compared with compound I) broad absorption bands appeared at 3280-3680 cm^{-1}, corresponding to the stretching vibrations of hydroxyl group free and bound in a hydrogen bond. A peak for a molecular ion of mass 262 (16 units greater than that of the initial compound I) was observed in the mass spectrum of this compound, which indicates the presence of one more oxygen atom in the composition of product III. Analysis of the fragmentation patterns of the molecular ion confirmed the presence of oxygen atom in the benzene fragment of the molecule (Scheme 1).

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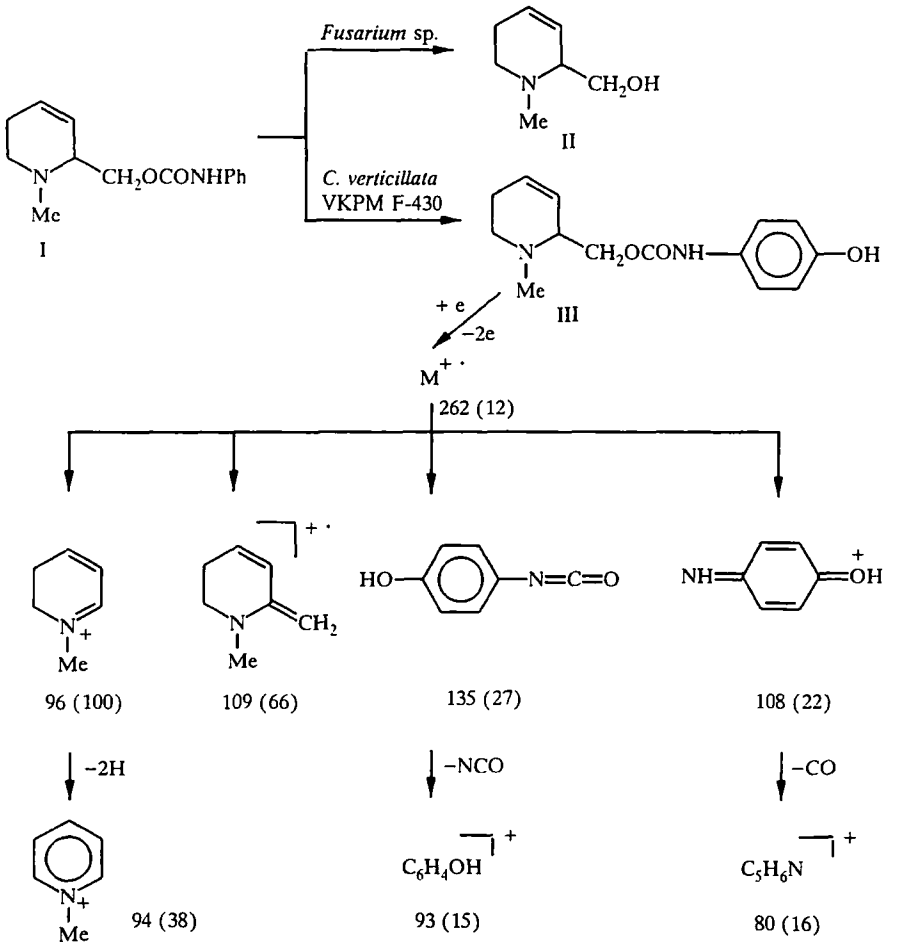
Scheme 1



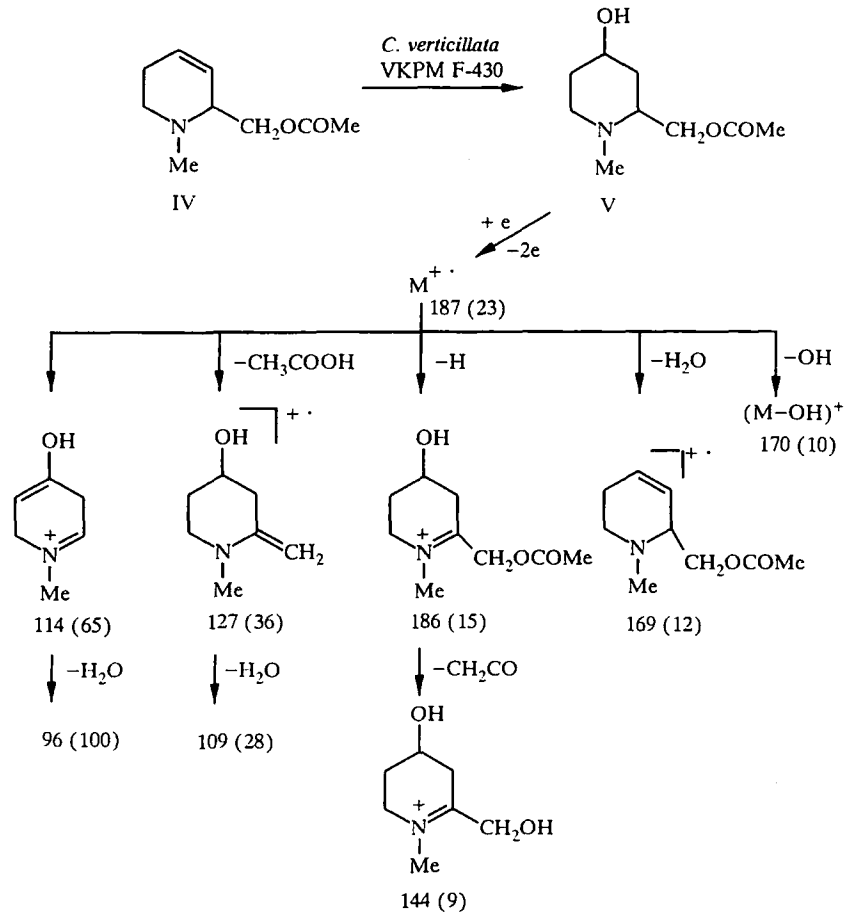
In reality the presence of intense ion peaks* (*m/z* 96 and 109) in the mass spectrum, also a characteristic of the mass spectrum of the initial urethane I, confirmed the absence of changes in the structure of the tetrahydropyridine fragment of the molecule. However an intense peak for the phenyl isocyanate ion (119) was present in the mass spectrum of compound I, but was absent in the spectrum of compound III in which an ion peak (135) appeared.

In the ^{1}H NMR spectrum of compound III two single-proton multiplets were observed at 5.3 and 6.2 ppm, indicating retention of the multiple bond, in addition to a three-proton singlet for the methyl group at position 1 (2.15), a two-proton doublet for the CH_2–O group (4.2), and a multiplet for the five alicyclic protons at positions 2,5, and 6 of the heterocycle. The same picture was characteristic of the ^{1}H NMR spectrum of compound I, however the character of the signals in the weak field region was different in the spectrum of compound III. In place of the five-proton multiplet for compound I two two-proton doublets were observed at 6.75 and 7.15 ppm, which indicate *para*-substitution. In addition to a broadened signal for the N–H proton at 7.2 ppm a signal appeared at 7.4 ppm which may be explained by the presence of a phenolic hydroxyl. An analogous process of hydroxylation of the carbamoyl part of the molecule was noted previously [9].

Replacement of the phenylcarbamoyl group by acetyl group precluded the possibility of side chain hydroxylation. However none of the cultures used transformed 2-acetoxymethyl-1-methyl-1,2,5,6-tetrahydropyridine (IV) with the exception of *C. verticillata* VKPM F-430, which hydrated this substrate to an insignificant extent (Scheme 2). Consequently on chromato-mass spectrometric analysis of chloroform extract of the obtained culture fluid, in addition to the peak of the unchanged substrate (retention time 8 min 45 sec, mol. mass 169), there was a peak for compound V (11 min 28 sec) the area of which was 1/10 the area of the compound IV peak.

* Here and subsequently values of *m/z* are given for ion peaks.

Scheme 2



Besides the molecular ion peak (187) in the mass spectrum of compound V peaks were observed for ions M–OH (170) and M–H_2O (169) indicating hydration of the substrate on transformation. The presence of an ion peak (114) in the spectrum confirmed that the hydroxyl group was located in the heterocycle. The processes forming these and several other ions most important for establishing the structure are represented in Scheme 2, and these enable the structure of the compound obtained to be confirmed with a high degree of probability.

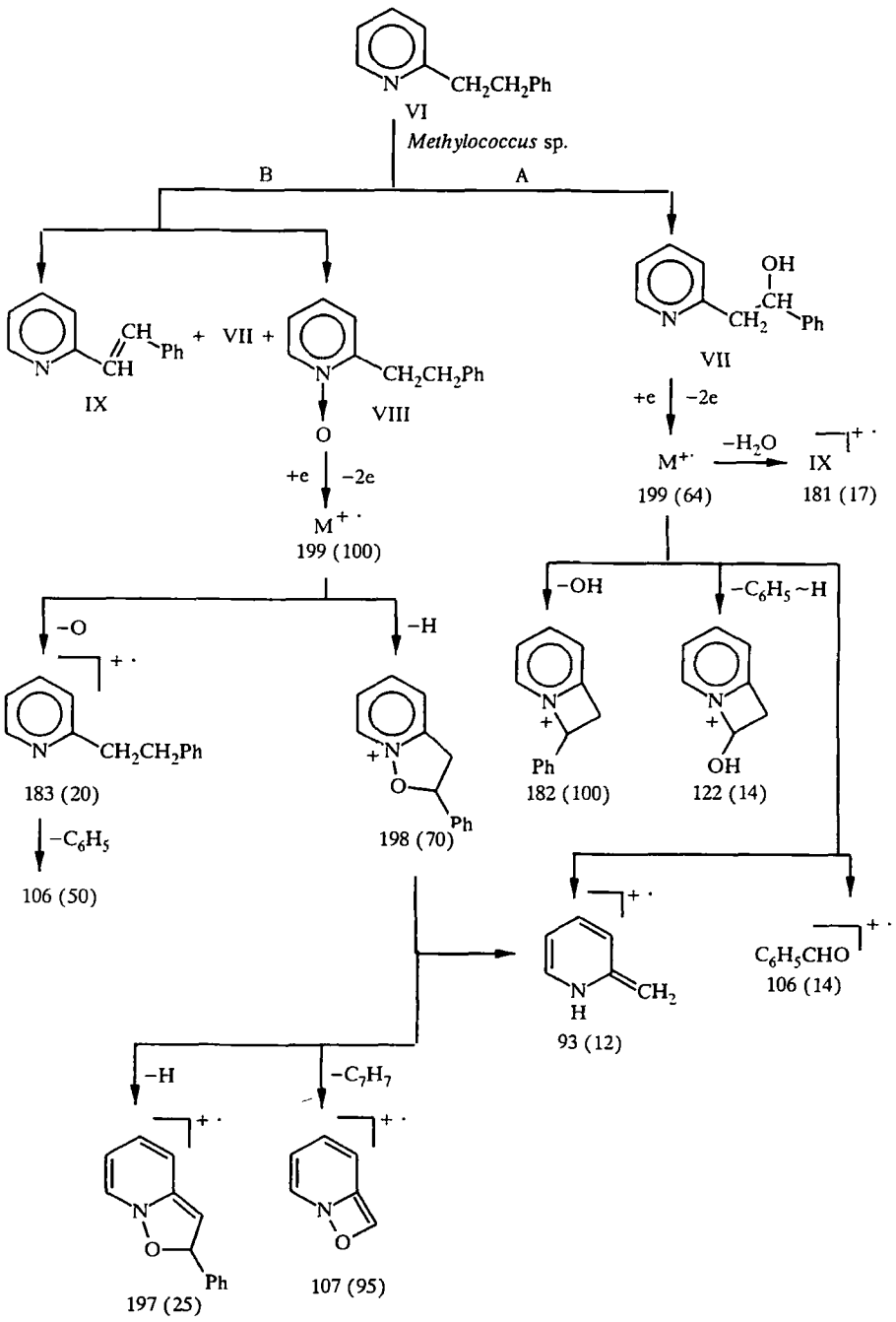
Regretably we were unable to isolate compound V in the pure state. The earlier observed formation of products of dihydroxylation of the multiple bond of tetrahydropiperidine derivatives [5] by culture of *C. verticillata* VKPM F-430 was not confirmed in the case of compounds I and IV.

Previously we have shown that cultures of the fungi *Beauveria bassiana* ATCC 7159, *Aspergillus terreus*, and *Penicillium pusillum* were able to hydroxylate with varying degrees of selectivity the "benzyl" position of the isomeric 2- and 3-ethylpyridines [10], while cells of *Nocardia*, *Arthrobacter* and several other microorganisms converted 3-methylpyridine directly into nicotinic acid under cooxidative (see Experimental) conditions [11].

As part of the search for cultures of microorganisms capable of selective hydroxylation of the position 2 (with respect to the pyridine ring) of the ethyl fragment of 2-(2-phenylethyl)pyridine (VI) we have studied the transformation of this compound by mixed methanoxidating culture of *Methylococcus*, known for its high hydroxylating capability in relation to hydrocarbons [12]. The process was carried out in two ways: A, with a suspension of nonmultiplying cells and B, with a growing culture under cooxidative conditions.

One substance was isolated in the first case from the culture fluid by preparative TLC (R_f 0.25) in 40% yield and was assigned the structure of 2-(2-hydroxy-2-phenylethyl)pyridine (VII) on the basis of spectral data (Scheme 3).

Scheme 3



In the second case also by TLC method compounds VII (R_f 0.25) and VIII (R_f 0.6) were isolated in approximately equal yields (about 5%) from chloroform extract of the culture fluid. A broad absorption band was present at 3150-3500 cm^{-1} in the IR spectrum of the first. This band is characteristic of a hydrogen-bonded hydroxyl group and was absent from the IR spectrum of the second compound. However an intense peak was present at 1250 cm^{-1} in the spectrum of the second compound and suggests the presence of N-oxide grouping [13]. In the mass spectra of both compounds peaks were observed for molecular ions of the same mass number 199, however the characters of their fragmentation differed markedly. In the case of compound VII the molecular ion efficiently eliminated hydroxyl and phenyl group (Scheme 3), or underwent fission at the C–C bond with migration of hydrogen atom and formation of ions of *m/z* 106 and 93. The most specific for the dissociative ionization process of compound VIII (Scheme 3) was the loss of oxygen atom or one or two hydrogen atoms from its highly

stable molecular ion. The process in the case of compound VIII is characteristic of the N-oxides of azines, while for compound VII it is known that similar processes have been observed many times previously on fragmentation of molecular ions of 2-aryl- and arylalkylpyridines [13]. Chromato-mass spectrometric analysis of the same chloroform extract showed the presence, apart from the initial substrate VI (t_r = 6 min 25 sec), of alcohol VII (t_r = 15 min 7 sec), N-oxide VIII (t_r = 12 min 43 sec), and stilbazole IX (t_r = 9 min 39 sec) with area ratios of the chromatographic peaks of 29:9:8:1 respectively. The mass spectra of compounds VII and VIII corresponded with those described above but the mass spectra of compounds VI and IX were identical with those published in [13,14].

The processes of microbiological hydroxylation of 1,2,5,6-tetrahydropyridines and of arylalkylpyridines are therefore very sensitive both to the presence of new substituents in the molecule and to the conditions of culturing the microorganisms.

EXPERIMENTAL

Strains of the fungi *Aspergillus niger* VKM F-1119 and *Beauveria bassiana* VKM F3111D were obtained from the All-Russian Collection of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Academy of Sciences of the Russian Federation, strains of *Cunninghamella verticillata* VKPM F-430 and *Rhizopus oryzae* VKPM F-431 – from the All-Russian Collection of Industrial Microorganisms of the All-Russian Research Institute for Genetics, two strains of the fungi *Aspergillus niger* KM-11 and *Penicillium simplicissimum* KM-16 – from the microorganism collection of the Department of Microbiology of the Biological Faculty of Moscow State University, nine species of fungi of the genus *Fusarium* (*F. culmorum, F. solani, F. sporotrichiela* var. *poal, F. monoliforma, F. microcera* var. *orthoconium, F. oxysporum, F. kuhnii, F. heterosporium,* and *F. averaceum* var. *herbarum*) were submitted by the Department of Lower Plants of the Biological Faculty of Moscow State University. The strain of *Aspergillus niger* NRRL 3228 was obtained from the collection of the Northern Regional Research Laboratories of the USA, and the fungus *Beauveria bassiana* ATCC 7159 – from the American Type Culture Collection. The biomass of the mixed culture of methanoxidating bacteria of the genus *Methylococcus* was obtained from the All-Russian Research Institute for the Biosynthesis of Proteins (VNII BBV).

The substrates for transformation, *viz.* 1-methyl-2-(N-phenylcarbamoyloxymethyl)-1,2,5,6-tetrahydropyridine (I) and 2-acetoxymethyl-1-methyl-1,2,5,6-tetrahydropyridine (IV) were synthesized in the Chemical Faculty of Moscow State University, 2-(2-phenylethyl)pyridine (VI) was kindly given to us by Professor L. D. Smirnov.

The UV spectra were measured on a Cary 219 (Varian) spectrometer in ethanol. The ^{1}H NMR spectra were recorded on a Tesla BS-467 instrument, operating frequency 60 MHz, in DMSO-d6, internal standard was TMS. The mass spectra were obtained on MX-1321A and M-25 (Kratos) mass spectrometers at ionization energy of 70 eV with direct insertion of samples into the ion source or through the chromatograph. Specific rotation was measured on a Perkin-Elmer 141 spectropolarimeter. Chromatographic resolution of transformation products was performed on Kieselgel 60-F 254 plates (Merck, 0.25 mm) in the solvent systems petroleum ether–ethyl acetate, 1:1 (N1), and hexane–ethyl acetate–ethanol, 10:10:2 (N2).

1-Methyl-2-(N-phenylcarbamoyloxymethyl)-1,2,5,6-tetrahydropyridine (I) was obtained in mixture with its Δ^4 isomer by the reaction of phenyl isocyanate with mixture of 2-hydroxymethyl-1-methyl-1,2,5,6- and 1,2,3,6-tetrahydropyridine and was isolated in the pure state (oil) by high-performance column flash chromatography on silica gel, eluting with solvent system N1. R_f 0.37. IR spectrum: 1730 (C=O), 3200, 3330 cm^{-1} (N–H). ^{1}H NMR spectrum: 2.10 (2H, m, 5-CH_2); 2.38 (3H, s, N–CH_3); 2.67 (2H, m, 6-CH_2); 2.80 (1H, m, 2-CH); 3.57 (2H, d, 2'-CH_2); 5.52 (1H, m, 4-CH); 5.89 (1H, dd, 3-CH); 6.75-7.25 ppm (5H, m, C_6H_5). On analysis by HPLC (Millichrome instrument, UV detector, wavelength 220 nm) it had retention time 1 min 45 sec. Mass spectrum (I, %): 246(1), 119(34), 109(12), 96(100), 94(10), 93(13), 77(4), 66(3), 65(5).

All the strains of mycelial fungi investigated were maintained on solid medium of the following composition (g/liter): glucose 10.0, corn extract 20.0, agar 20.0, tap water, pH of the medium 5.0.

A liquid medium of the following composition (g/liter) was used for incubating fungi and carrying out the transformation reaction: glucose 20.0, corn extract 10.0, peptone 5.0, KH_2PO_4 5.0, tap water, pH 5.0. The cultures of microscopic fungi were incubated on agar medium for 10-14 days (until abundant spore formation) at 28°C. Washings from the surface of the medium were then made by adding sterile tap water (5 ml), and the inoculate was then transferred to a 750 ml flask containing liquid medium (about 100 ml).

The fungi were incubated on a rotary rocker (200-220 rpm) at 28°C and the biomass obtained was used as the inoculum which was introduced (5% by vol.) into the medium. Microscopic fungi were also incubated for 96 h on a rotary rocker in 750 ml flasks with 100 ml of medium, introducing the substrate to be transformed (300 mg/liter, dissolved in 3 ml of ethanol) after 24 h. The density of the raw biomass of cells was 30.0-33.0 g/liter.

When transforming with nonmultiplying cells of *Methylococcus* the biomass obtained from VNIII BBV was resuspended in phosphate buffer (1000 ml: 0.01 M, pH 7.0). The density of the raw biomass of cells in buffer was about 30 g/liter. The substrate being transformed (50 mg/liter) was added to the cell suspension in buffer, after which the reaction mixture was incubated on the rocker for 24-28 h at 40°C in 750 ml flasks (100 ml of mixture in each flask).

Transformation with bacteria of the genus *Methylococcus* under cooxidative conditions was effected in 7 flasks (750 ml). Phosphate buffer (600 ml, 0.01 M, pH 7.2) was used when carrying out the transformation process. To the buffer solution the living cells (obtained from VNII BBV, 30-35 mg/liter) and substrate (420 mg, 60 mg dissolved in 2 ml of alcohol in each flask) were added. A stream of mains gas was passed through the flasks containing the reaction mixture for 20-25 sec, after which the flasks were hermetically sealed with plugs and shaken on the rotary rocker for 24 h at 37-39°C.

At the end of the transformation process the bacterial cells were separated by centrifugation, the fungal biomass was filtered off on a Buchner funnel through a paper filter into a Bunsen flask. The filtrates were made alkaline to pH 7.5, extracted with hot chloroform for 30 h (in extractors for heavy liquids), the extracts were evaporated to dryness in vacuum, the residue dissolved in methanol (0.5 ml) and analyzed by GC-MS or TLC. The transformation products were subsequently separated by chromatographic methods.

An oil (25 mg, R_f 0.37) was isolated on transformation of 1-methyl-2-(N-phenylcarbamoyloxymethyl)-1,2,5,6-tetrahydropyridine (I) (50 mg) with strain of *Fusarium*. Only two peaks were detected by chromato-mass spectrometric analysis (area ratio 1 : 3). The first corresponded to compound II (t_r 3 min 27 sec), mass spectrum: 127(55) M^+ , 126(22) (M-H), 109(18) (M-H_2O), 96(100) (M-CH_2OH), 94(17) (M-CH_2OH-H_2), 84 (M-C_2H_5N), 66(12) (M-H_2O-C_2H_5N). The second compound (t_r 11 min 27 sec) was the initial substrate I (see above for mass spectrum).

Two compounds were isolated after transforming compound I (100 mg) with the strain of *C. verticillata* VKPM F-430 and chromatographic resolution (system N1) of the extract obtained. One was the initial substrate I (34 mg, R_f 0.37). The other was compound III (53 mg, 50%) as an oil (R_f 0.28). The IR, ^1H NMR, and mass spectra are given above, $[\alpha]_D^{20}$ -6.50 (concn. 1.85, ethanol).

On transforming compound VI (50 mg) with nonmultiplying *Methylococcus* cells compound VII (20 mg, 37%) was isolated chromatographically (system N2) from the chloroform extract. It was an oil (R_f 0.25), UV spectrum (methanol), λ_{max} 216, 262 nm (IR and mass spectra are given above).

On transforming compound VI (420 mg) with the same strain but under cooxidative conditions, an oil (230 mg) was isolated from the organic extract. Two transformation products were isolated from the oil by TLC (system N2), *viz.* substance VII (20 mg), R_f 0.25, λ_{max} 216 and 262 nm and substance VIII (18 mg), R_f 0.6, λ_{max} 257 and 268 nm. Data of IR, mass and chromato-mass spectrometry are given above.

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