Reduction of Nitroolefin Using Microorganisms

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Microbial reduction of 1-phenyl-2-nitro-1-propene (3) was carried out using 57 strains of yeast, 40 strains of aerobic and facultatively anaerobic bacteria and 40 strains of strictly anaerobic bacteria. Nine strains of yeast (*Candida tropicalis*, etc.) had the ability to reduce (3) to 1-phenyl-2-nitropropane (1) (94.1%—60.3% yield). The ability of the aerobic and anaerobic bacteria was weaker than yeast (35.6%—14.0% and less than 5%, respectively). When 11 strains of strictly anaerobic bacteria (*Clostridium innocuum* etc.) were used, a final reduced product like amphetamine (2) was detected, although the efficiency of reduction was very poor.

Keywords nitroolefin; amphetamine; microbial transformation; intestinal bacteria; yeast

It is well known that many organic compounds are converted into optically active substances by microorganisms such as yeast, fungi and actinomycetes. The function of intestinal microorganisms should be investigated in detail, since they are known to metabolize many compounds¹⁾; however, the use of these microorganisms is still limited, mainly because of experimental difficulties. We reported previously that racemic 1-phenyl-2-nitropropane (*R,S*)-1 was resolved into the optically active (*S*)-amphetamine (*S*)-2 and (*R*)-unchanged 1 by *Clostridium perfringens*, a typical intestinal bacterium.²⁾ It is reported that the 1,2-substituted nitroolefins are reduced by yeast to optically active saturated nitro compounds.³⁾ However, the optical purity of the reduction products is generally low (less than 30% enantiomeric excess). In this paper, we report the reduction of 1-phenyl-2-nitro-1-propene 3 by aerobic or anaerobic microorganisms to the optically active nitro compound 1 or amphetamine 2, respectively.

Materials and Methods

Chemicals 1-Phenyl-2-nitro-1-propene (*E* form) 3 was synthesized by the methods reported previously.^{4,5)}

Microorganisms The 57 strains of yeast, 40 strains of aerobic and facultatively anaerobic bacteria and 40 strains of strictly anaerobic bacteria used were obtained from the culture collection of the Animal and Cellular Systems Laboratory and the Synthetic Organic Chemistry Laboratory of the Institute of Physical and Chemical Research (Riken Institute).

Medium Three types of medium were used for cultivation.

(A) The medium for yeast was prepared by the previously reported method.⁶⁾

(B) Composition of the medium for aerobic and facultatively anaerobic bacteria was as follows: Yeast extract 0.5%, casamino acid 0.5%, glucose 0.5%, K₂HPO₄ 0.2%, vitamin sol⁷⁾ 0.5% in distilled water, pH 7.0.

(C) M10 broth was used for the culture of strictly anaerobic bacteria.⁸⁾

Nuclear Magnetic Resonance (NMR) Measurement The proton NMR spectra were obtained with a JEOL (JNM-GX400) 400-MHz ¹H-NMR spectrometer using deuteriochloroform as solvent and tetramethylsilane (TMS) as internal standard.

Chromatography Conditions Gas chromatography (GC) conditions were as follows: A Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector had a glass column (2 m × 3 mm inner diameter) which was packed with 3% OV-17 on 80/100-mesh Chromosorb W HP. The carrier gas was nitrogen (50 ml/min). The column temperature was 150 °C, and the temperature of both the injection port and detector was 200 °C. Sample size was 2 μl. Gas chromatography and gas chromatography-mass spectrometry (GC-MS) conditions for the amphetamine analysis were reported previously.²⁾

Conditions for high-performance liquid chromatography (HPLC) were as follows: A Jasco trirotar HPLC equipped with an refraction index detector was used. A stainless steel column (250 mm × 8 mm inner diameter) was packed with silica gel (Senshu-pack; Nucleosil, 5 μm), with the solvent

system hexane-ethyl acetate (10:1, v/v) and a flow rate of 3 ml/min.

Screening Method The microorganism was cultured in 10 ml of liquid medium in a test tube. Precultivation of aerobic and anaerobic bacteria was carried out for 1 d at 37 °C. Precultivation of yeast was done for 3 d at 30 °C. After each precultivation, about 10 mg of 3 was added to each medium. Then the mixture was shaken on a rotary shaker for 3 d at 37 °C for bacteria or 30 °C for yeast. The cultivation of strictly anaerobic bacteria was carried out under anaerobic conditions. After completion of the cultivation, the medium was extracted with 20 ml of ethyl acetate, and the extracts were subjected to GC analysis.

Preparative Method In the screening experiment, 8 strains of yeast and 4 strains of aerobic and facultatively anaerobic bacteria were found to be effective for the reduction of the double bond of 3. In order to obtain a substantial amount of the reduced product 1, the above precultivated medium (10 ml) was transferred to 1000 ml of the same medium in a 2-l bottle. After cultivation for 3 d, 100 mg of substrate 3 was added to the reaction bottle and the cultivation was continued for an additional 3 d under the same conditions as above. In the case of baker's yeast, reduction was done by incubating a mixture of sugar (10 g), baker's yeast (10 g) and substrate 3 (100 mg) in distilled water (50 ml) for 12 h at 30 °C.

Separation of Products After the cultivation, the products were extracted with ethyl acetate and concentrated by a rotary evaporator. The residue was purified by silica gel column chromatography (Wako gel, C-200; hexane:ethyl acetate=49:1) and then preparative HPLC.

Estimation of Reduced Products The absolute configuration and optical purity of the reduced products were determined by the method reported previously.²⁾

Results and Discussion

The preliminary screening test revealed that 19 strains of yeast and 21 strains of aerobic and facultatively anaerobic bacteria were active in reducing 1-phenyl-2-nitro-1-propene 3 to 1-phenyl-2-nitropropane 1. Among these effective microorganisms, 13 strains were selected for a preparative experiment and the absolute configuration and optical purity of reduced product 1 were examined. The results are shown in Table I. In the case of yeast, the chemical yield of 1 was fairly high (61%—94%) but its optical purity was not very satisfactory. Only when *Candida tropicalis* was used was the optical purity (51.6% ee) of 1 much improved over the value reported previously (29% ee) by Sakai *et al.*³⁾ In cases where aerobic and facultatively anaerobic bacteria were used, both the chemical yield and optical purity were generally low.

In a screening experiment using strictly anaerobic bacteria under anaerobic cultivation conditions, chemical yield of 1 was less than 5%. However, among 40 kinds of strictly anaerobic bacteria, 11 strains were found to produce amphetamine 2 directly, showing that a nitro group as well as a double bond were reduced in these cases, although the

TABLE I. Preparative Microbial Reduction of 1-Phenyl-2-nitro-1-propene Using Microorganisms

Microorganism		Yield (%)	Optical purity (% ee)
Yeast			
<i>Candida tropicalis</i>	JCM 1541	61.9	51.6 (S)
<i>Candida lusitanae</i>	JCM 1814	71.1	1.6 (R)
<i>Candida albicans</i>	JCM 1543	94.1	10.1 (S)
<i>Candida parapsilosis</i>	JCM 1784	69.4	17.2 (S)
<i>Saccharomyces delbrueckii</i>	KSY 15	71.7	15.8 (S)
<i>Saccharomyces fermentati</i>	KSY 16	85.1	18.4 (S)
<i>Torulopsis famata</i>	KSY 27	61.1	1.7 (R)
<i>Rhodotorula rubra</i>	KSY 33	65.4	4.1 (S)
Baker's yeast		60.3	5.5 (R)
Bacteria			
<i>Klebsiella</i> sp.	11	15.1	8.9 (R)
<i>Klebsiella pneumoniae</i>	28	14.0	11.7 (R)
<i>Bacillus subtilis</i>	H 17	16.2	0.4 (R)
<i>Escherichia coli</i>	H 140	35.6	7.6 (S)

TABLE II. Formation of Amphetamine in the Culture Broth of Strictly Anaerobic Bacteria in the Presence of Benzaldehyde and Nitroethane

Effective strictly anaerobic bacteria			
<i>Clostridium difficile</i>	O	<i>Mitsuokella multiacidus</i>	VI 70
<i>Clostridium perfringens</i>	C-01	<i>Eubacterium limosum</i>	E-1
<i>Clostridium butyricum</i>	V5	<i>Eubacterium aerofaciens</i>	S-12
<i>Clostridium innocuum</i>	M-51	<i>Eubacterium multiforme</i>	X-8
<i>Clostridium clostridiforme</i>	U-111	<i>Peptostreptococcus anaerobius</i>	X-36
<i>Clostridium</i> sp2	B-7		

efficiency of reduction was very poor.

Encouraged by the results of the above screening, we examined whether amphetamine **2** could be obtained directly by incubating a mixture of benzaldehyde and nitroethane with strictly anaerobic bacteria, since nitroolefin **3** can be synthesized from benzaldehyde and nitroethane under weak basic or neutral conditions.

A mixture of benzaldehyde (10 mg) and nitroethane (10 mg) was added to the precultivated strictly anaerobic bacteria in M10 broth (10 ml) and the mixture was incubated at 37 °C for 5 d. The crude amine fraction obtained in the same way as described for the microbial reduction of the

nitro group to the amino group²⁾ was analyzed by GC. It was found that 11 strains of strictly anaerobic bacteria produced amphetamine **2** directly from this mixture, as shown in Table II.

Preparative cultivation using *Clostridium innocuum* was carried out to isolate the products, because this was found to be the most effective strictly anaerobic bacterium in the screening experiment. A mixture of benzaldehyde (158 mg) and nitroethane (112 mg) was added to the precultivated *Clostridium innocuum* in M10 broth (1000 ml) and the mixture was incubated under the same conditions as described above. The ethyl acetate extract from M10 broth was separated into basic and neutral fractions by the method reported previously.²⁾ From the basic fraction, 11 µg of amphetamine **2** was detected by GC and GC-MS analysis. On the other hand, from the neutral fraction, three products, nitroolefin **3**, the saturated nitro compound **1** (0.52 mg) and a diastereomeric mixture of the nitroalcohol **4** (0.13 mg) were obtained by preparative HPLC. The structures of these compounds were individually confirmed by 400-MHz ¹H-NMR and high-resolution MS analysis and direct comparison with each authentic sample.

We have shown that conjugated nitroolefinic compounds such as 1-phenyl-2-nitro-1-propene **3** are reduced to a double bond reduced product (1-phenyl-2-nitropropane **1**) by yeast and bacteria. Yeast, aerobic and facultatively anaerobic bacteria had no ability to reduce **1** to amphetamine **2**. However, strictly anaerobic bacteria such as *Clostridium innocuum* did have the ability to reduce **3** to **2**, although the efficiency of reduction was very poor. Consequently, the absolute configuration and the optical purity of **2** could not be estimated because the amount of **2** (11 µg) was insufficient for NMR analysis.

These results suggested that when benzaldehyde and nitroethane were mixed in the M10 broth with *Clostridium innocuum*, aldol condensation and subsequent dehydration took place, initially yielding the nitroolefin **3**, which was then reduced to the saturated nitro compound **1** and an amphetamine **2**. In fact, the amount of **3** and nitroalcohol **4** in the culture medium was decreased significantly in comparison with that of **3** and **4** in the absence of microorganisms. It is therefore concluded that some kinds of strictly anaerobic bacteria such as *Clostridium innocuum* reduce both the carbon-carbon double bond of nitroolefin and the nitro group, yielding an aliphatic amino compound.

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