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Microbial synthesis of (*R*)- and (*S*)-3,4-dimethoxyamphetamines through stereoselective transamination

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Abstract

Two soil isolates, *Arthrobacter* sp. KNK168 and *Pseudomonas* sp. KNK425, aminated 3,4-dimethoxyphenylacetone in presence of s*ec*-butylamine as an amino donor to yield 3,4-dimethoxyamphetamine (DMA) with different enantioselectivities. The former gave (*R*)-DMA (>99% e.e.) and the latter the (*S*)-isomer (>99% e.e.).

Introduction

Many pharmaceuticals and agricultural chemicals contain an amino-substituted chiral center, and the development of effective methods for the synthesis of optically active amino compounds as starting materials for their synthesis has been attempted. Enantiomers of amphetamine and related compounds, such as DMA, have distinctive pharmacological properties (Barfknecht *et al*. 1972, Miller *et al.* 1984) and are important compounds as intermediates for the synthesis of anti-diabetics and anti-obesity agents (Bloom *et al*. 1992). Preparation of optically active DMA and related compounds by enzymatic kinetic resolution using ω-amino acid aminotransferase (Stirling 1992) and amine oxidase (Ayse *et al*. 2000), has been reported. On the other hand, the asymmetric synthesis of the (*S*)-form of an amphtamine derivative, such as (*S*)-4-methoxyamphetamine, by *Brevibacterium linens* has been reported (Nakamichi *et al*. 1990). With the kinetic resolution process, the maximal obtainable yield of one isomer is 50%. However, asymmetric synthesis of an optically active amine from a prochiral precursor allows a theoretical yield of 100%.

In this paper, we describe screening for microorganisms having the ability to form (*R*)- or (*S*)-DMA from 3,4-dimethoxyphenylacetone through transamination, and some studies on the reaction (Figure 1).

Materials and methods

Chemicals

3,4-Dimethoxyphenylacetone and *sec*-butylamine were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade and commercially available.

*Screening for microorganisms producing (*R*) or (*S*)-DMA from 3,4-dimethoxyphenylacetone*

The following medium was used for the screening. *S* medium comprised 5 g glycerol, 1 g yeast extract, 3 g NaCl, 2 g KH_2PO_4, 2 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.004 g $FeSO_4 \cdot 7H_2O$, 0.0005 g $ZnSO_4 \cdot 7H_2O$, 0.0005 g $MnCl_2 \cdot 4H_2O$, 1.5 g pyruvic acid and 1 g (*R*)-DMA in 1 l of the medium, pH 7. Soil samples, 2 g, collected at various places were each suspended in 5 ml physiological saline. One ml of each supernatant was added to 4 ml of *S* medium in a test tube, followed by incubation at 30 °C with reciprocal shaking for 3 to 7 d. Each culture was spread on an S medium plate containing 1.5% agar, and then incubated at 30 °C.

Deaminating activity toward the (*R,S*)-DMA of the microorganisms isolated from the *S* medium plates was examined. Washed cells of each microorganism

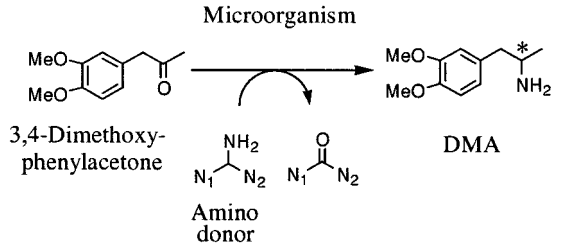

Fig. 1. The synthesis of (*R*)- and (*S*)-DMA.

obtained from 4 ml cultured broth with the same medium were incubated in 2 ml of a reaction mixture comprising 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 8.5), 0.4% (*R,S*)-DMA and 0.3% pyruvic acid in a test tube. The reaction was carried out at 30 °C for 1 d with shaking, and then the supernatant obtained from the reaction mixture was analyzed for the amount of 3,4-dimethoxyphenylacetone formed, and the amount and optical purity of DMA remaining by TLC and HPLC, respectively.

As the second screening, the reactivity and stereoselectivity for the amination of 3,4-dimethoxyphenylacetone of the selected microorgamisms were examined under the following conditions. Each reaction mixture, comprising cells from 2 ml cultured broth, 30 mM 3,4-dimethoxyphenylacetone and 60 mM *sec*-butylamine in 2 ml 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 8), was shaken in a test tube at 30 °C for 2 d. After the reaction, the amount and optical purity of DMA formed were determined by HPLC.

Identification of the microorganisms

Morphological, cultural and chemotaxonomic characteristics were examined. The morphological descriptions of KNK168 and KNK425 were from growth on Lab M nutrient agar. The organisms were identified according to Krieg & Holt (1984), and Bousfield *et al.* (1983).

Cultural conditions and preparation of washed cells

The *S* medium described above or the following P medium was used for the cultivation of *Arthrobacte*r sp. KNK168 and *Pseudomonas* sp KNK425. P medium comprised 20 g glycerol, 1 g yeast extract, 8 g Proekisu (Bansyu Chyoumiryou Co. Ltd., Japan), 5 g KH_2PO_4, 5 g K_2HPO_4, 1 g NaCl, 1 g $MgSO4 \cdot 7H_2O$, 0.004 g $FeSO_4 \cdot 7H_2O$, 0.0005 g $ZnSO_4 \cdot 7H_2O$, 0.0005 g $MnCl_2 \cdot 4H_2O$ and 2 g *sec*-butylamine in 1 l of the medium, pH 7. Five ml medium in a test tube or 100 ml medium in a shake-flask (500 ml) was inoculated from a slant culture, followed by incubation for 2 d at 30 °C with shaking. After cultivation, the cells were harvested by centrifugation and washed twice with 0.1 M Tris/HCl buffer (pH 8).

Reaction conditions for amination

The standard reaction condition for amination was as follows. The cells obtained from 2 ml cultured broth were added to 1 ml reaction mixture comprising 30 mM 3,4-dimethoxyphenylacetone and 45 mM amino donor in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 8), and then incubated in a test tube at 30 °C for 1–2 d with shaking.

Isolation and purification of DMA

The reaction mixture was adjusted at pH 2 with HCl and then extracted with toluene to remove 3,4-dimethoxyphenylacetone. The aqueous layer was adjusted at pH 12 with NaOH, and then re-extracted with toluene, and the organic layer was dried with Na_2SO_4 and then concentrated in vacuo. The residue was distilled to give DMA as colorless liquid (bp. 127 °C/4 mm Hg). ^{1}H-NMR ($CDCl_3$, 400 MHz): d: 6.81(1H, d), 6.71–6.74(2H, s, d), 3,87(3H, s), 3.86(3H, s), 3.1–3.18(1H, m), 2.67(1H, dd), 2.44(1H, dd), 1.41(2H, broad), 1.12(3H, d).

Analysis

Identification of DMA and 3,4-dimethoxyphenylacetone was carried out by TLC and HPLC. TLC was carried on Kieselgel $60F_{254}$ (developing solvent, diethyl ether/methanol/NH_4OH (27%) = 50:50:2, by vol.), and 3,4-dimethoxyphenylacetone was detected with 0.4% 2,4-dihydroxyphenylhydrazine and DMA with ninhydrin. HPLC was carried out on a Cosmosil AR C_{18} (4.6 × 250 mm) column (Nakalai Tesque, Japan) at room temperature at a flow rate of 1 ml min^{-1} (eluent: H_2O/acetonitrile/KH_2PO_4/sodium hexane sulfonate/H_3PO_4 = 1500 ml/300 ml/4.55 g/1.69 g/3 g). The absorbance of the eluate was monitored at 254 nm. DMA and 3,4-dimethoxyphenylacetone were eluted at 21 min and 43 min, respectively.

The optical purity of DMA was determined by HPLC analysis of the corresponding deastereomer with L-leucine *N*-carboxy anhydride (L-Leu-NCA). One mg DMA was reacted with 1 mg L-Leu-NCA

in 0.25 ml 0.45 M borate buffer (pH 10.4) with stirring vigorously, followed by incubation for 2 min at room temperature. The reaction solution was mixed with 0.1 ml 1 M HCl, and then filtered through a Cosmonice Filter W (Nacalai Tesque, Japan). The filtrate was analyzed by HPLC on a Cosmosil AR C_{18} (4.6 × 250 mm) column (Nakalai Tesque, Japan) at room temperature at a flow rate of 1 ml min^{-1} (eluent: H_2O/2-propanol/KH_2PO_4/H_3PO_4 = 82 ml/18 ml/0.25 g/0.1 g).

^{1}H-HMR spectrum was recorded with a FT-NMR, JNM-400 spectrometer (400 MHz; JEOL, Japan). Chemical shifts are expressed in parts/million (ppm), with tetramethylsilane as the internal standard. Optical rotations were determined with a digital polarimeter (SEPA-200; Horiba, Japan).

Results and discussion

Screening for microorganisms producing optically active DMA from 3,4-dimethoxyphenylacetone

First, we searched for microorganisms having stereoselective deamination activity on DMA in order to obtain microorganisms capable of forming optically active DMA from 3,4-dimethoxyphenylacetone in a similar manner to transamination. Deamination active microorganisms for (*R,S*)-DMA were screened from 492 atrains, which were isolated from plates containing (*R*)-DMA as a sole nitrogen source. Among them, 10 strains possessing (*R*)-selective deaminating activity and 19 strains possessing (*S*)-selective activity were obtained. Microorganisms having (*S*)-selective deamination activity were obtained in spite of the enrichment with (*R*)-DMA as a nitrogen source. Next, the strains obtained through the above procedure, which had steleoselective deamination activity, were tested for DMA formation from 3,4-dimethoxyphenylacetone. As a result, strain KNK168 among the 10 strains with (*R*)-selective deaminating activity, and strain KNK425 among the 19 strains with (*S*)-selective activity were found to form DMA from 3,4-dimethoxyphenylacetone. The DMA formed by KNK168 and KNK425 was the (*R*)-enantiomer and (*S*)-enantiomer, respectively, as described below.

Identification of strains KNK168 and KNK425

The morphological, cultural, physiological and chemotaxonomical characteristics of strains KNK168 and KNK425 were examined using standard procedures (Krieg & Holt 1984, Bousfield *et al.* 1983). Srain KNK168 was classified as belonging to the genus *Arthrobacter* and strain KNK425 to the genus *Pseudomonas*.

Table 1. Effects of amino compounds on the synthesis of DMA from 3,4-dimethoxyphenylacetone.

Amino donor	DMA formed (mM)	
	Arthrobacter sp. KNK168	*Pseudomonas* sp. KNK425
sec-Butylamine	15	17
DL-Alanine	0	0
DL-Lysine	0	0
DL-Ornithine	0	0
NH_4Cl	0	0
None	1	0

Reactions were carried out as described under Materials and methods, except that amino donors were used as indicated in the table. The reactions were carried out for 2 d.

Table 2. DMA formed by KNK168 and KNK425.

Microorganism	DMA formed (mM)	$[\alpha]_D^{21}$	e.e. (%)
Arthrobacter sp. KNK169	26	−32.1[a]	>99 (*R*)
Pseudomonas sp. KNK425	30	+31.6[b]	>99 (*S*)

The specific rotation of (*R*)-3,4-dimethoxyamphetamine is −30.9- (c = 4.13, chloroform) as described in the literature (Anthony 1957).
a) Measured in chloroform (c = 2.27).
b) Measured in chloroform (c = 2.14).

Synthesis of optically active DMA from 3,4-dimethoxyphenylacetone with Arthrobacter *sp. KNK168 and* Pseudomonas *sp. KNK425*

The effects of amino compounds as the amino donor on the synthesis of DMA from 3,4-dimethoxyphenylacetone were examined (Table 1). L-Alanine was reported to be a good amino donor for the amination of 3,4-dimethoxyphenylacetone by *Brevibacterium linens* IFO 12141 (Nakamichi *et al*. 1990). In the case of amination by both *Arthrobacter* sp. KNK168 and *Pseudomonas* sp. KNK425, α-amino acids such as DL-alanine were not used as amino donors but *sec*-butylamine proved to be a good amino donor. Therefore, *sec*-butylamine was used as the amino donor for further experiments.

The cells obtained from 100 ml cultured broth of *Arthrobacter* sp. KNK168 and *Pseudomonas* sp. KNK425 were incubated with 50 ml reaction mixture comprising 60 mM 3,4-dimethoxyphenylacetone, 90 mM *sec*-butylamine and 100 mM Tris/HCl buffer (pH 8.5) in a flask for 20 h at 30 °C with shaking (150 rpm). Table 2 shows the results of the reactions. With *Arthrobacter* sp. KNK168 and *Pseudomonas* sp. KNK425, 26 mM and 30 mM DMA were formed with a 43% and 50% conversion yields, respectively. The products were isolated and purified as described in Materials and methods, and specific rotation and enantiomeric purity of the isolates was measured (Table 2). DMA formed by *Arthrobacter* sp. KNK168 and *Pseudomonas* sp. KNK425 was of the (*R*) and (*S*)-configuration with a high enantiomeric excess, respectively. These results indicate that *Arthrobacter* sp. KNK168 has (*R*)-specific transamination activity and *Pseudomonas* sp. KNK425 has (*S*)-specific activity.

The enantioselective amination of a pro-chiral carbonyl compound using microorganisms and enzymes is a unique and significant method for the production of optically active amines from an economical point of view. Some ω-amino acid aminotransferases show stereoselective amination activity toward carbonyl compounds to form optically active secondary amines (Shin & Kim 1999). Shin & Kim (1999) reported the synthesis of (*S*)-α-methylbenzylamine from acetophenone and L-alanine. However, only the (*S*)-enantiomer could be produced with ω-amino acid transferase, because the ω-amino acid aminotransferase tested was (*S*)-enantioselective. In the present study, we found microorganisms, possessing (*R*) and (*S*)-selectivity in amination activity, in soil. These findings indicate the possibility of the preparation of both enantiomers of various optically active amine chemicals with these microorganisms. The reaction-conditions for amination by both strains, and the isolation and characterization of the enzymes involved in the reactions are now being investigated further.

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