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Enantio- and chemoselective oxidation of omeprazole sulfide to enantiopure (*S*)-omeprazole with whole-cells of *Aspergillus carbonarius*

Shefali Sangar[1], Bhavna Vaid, Ravinder S. Jolly*

CSIR-Institute of Microbial Technology, Sector 39, Chandigarh 160036, India

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ABSTRACT

Development of a biocatalytic method for the preparation of (*S*)-omeprazole (esomeprazole) in enantiomerically pure form is in great demand in pharmaceutical industry. A newly isolated fungal strain has been described, which converted omeprazole sulfide (5-methoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]thio-1H-benzoimidazole) to (*S*)-enantiomer of omeprazole (5-Methoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl] sulfinyl-1H-benzimidazole) in > 95% e.e. The fungal strain was identified as *Aspergillus carbonarius*. A conversion of 91.8% was achieved at substrate concentration of 0.05 gmL^{-1} with the suspension of cells of *A. carbonarius* in phosphate buffer pH 8.0 at 30 °C. The bi°catalyst was chemoselective for oxidation of sulfide to sulfoxide as no trace of corresponding sulfone could be detected during the reaction.

1. Introduction

Omeprazole is a highly potent gastric acid secretion inhibitor, which acts by irreversibly blocking the H^+/K^+ ATPase enzyme system (proton pump) of the gastric parietal cells (Sachs et al., 2006; Sakai et al., 2016). Its (*S*)-enantiomer, also known as Esomeprazole exhibited superior clinical efficacy than racemic omeprazole, which was ascribed to its higher and more consistent bioavailability compared to omeprazole (Lindberg et al., 2003; Olbe et al., 2003). (*S*)-Omeprazole can be obtained either by resolution strategies or by the oxidation of the corresponding prochiral sulfide. The resolution methods include inclusion complexation with a chiral host compound, preferential crystallization of conglomerates and diastereomeric salts formation (Hein et al., 2013; Stabile, 2016). Direct enantioselective oxidation of prochiral sulfide offers a most effective route to (*S*)-omeprazole. Chemical routes to enantioselective oxidation of prochiral sulfide include use of tailor-made ligands and application of the Kagan-Modena modification of the Sharpless enantioselective epoxidation protocol (Delsarte et al., 2018; Mahale et al., 2010; Song et al., 2015; Talsi et al., 2015). Currently, (*S*)-omeprazole is commercially produced by application of the Kagan-Modena modification of the Sharpless enantioselective epoxidation protocol employing titanium based catalyst. However, optimization of the process remained complicated, which prompted Delsarte et al. to explore a metal free approach using Davis oxaziridine in an attempt to develop an improved process (Delsarte et al., 2018). The results of these efforts, which were published in March 2018 showed remarkable improvement in the process, however some complications remain unresolved. Enantioselective oxidation resulted in only 85% yield and 76% e.e. The e.e. of the product was improved in a separate step utilizing methanol reslurry method, which under the optimized conditions produced (*S*)-omaprazole in 99.6% e.e. and 60% yield. Overall, the process remained tedious and resulted in moderate overall yield.

The enantioselective microbial oxidation of prochiral sulfide offers a cost effective green route to chiral sulfoxides and accordingly has received considerable attention in the recent years (Thomas et al., 2002). Whole-cell oxidation is preferred over the isolated enzymes to avoid the addition of expensive cofactor and cofactor recycling system (Carballeira et al., 2009; Legros et al., 2005). However, efforts made at finding a suitable biocatalyst for producing (*S*)-omeprazole gave miserable results (Holt et al., 1998; Yoshida et al., 2001), except one recent report by Babiac et al., which described a bacterium belonging to genus *Lysinibacillus* that produced (*S*)-omeprazole in 100% e.e. with conversion of 77% (Babiak et al., 2011). The conversion was achieved with the growing cells of bacterium, wherein the presence of media components and secondary metabolites in the reaction mixture are likely to complicate purification process for the product.

Here, we report the isolation of a fungal strain, identified as *Aspergillus carbonarius* that catalyzed enantioselective oxidation of omeprazole sulfide to (*S*)-omeprazole in > 95% e.e. and 91.8% conversion (Fig. 1). The cells of *A. carbonarius* were isolated in stationary

* Corresponding author.
E-mail address: jolly@imtech.res.in (R.S. Jolly).
[1] Present Address: PG Department of Chemistry, MCM DAV College for Women, Sector 36, Chandigarh 160036, India.

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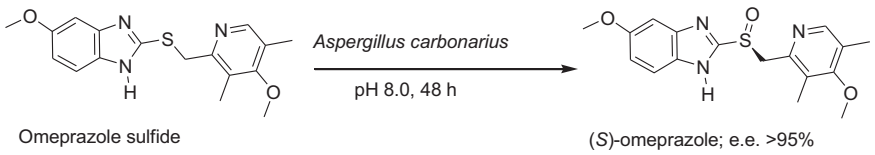

Fig. 1. Conversion of omeprazole sulfide to (*S*)-omeprazole with *Aspergillus carbonarius*.

phase and prewashed with buffer to remove media components and secondary metabolites before use in the reaction. No concomitant oxidation of omeprazole to sulfone was observed during the conversion. To the best of our knowledge this is the first example of a fungal strain catalyzed oxidation of omeprazole sulfide to (*S*)-omeprazole in > 95% e.e. The unique characteristics of catalyst offer an excellent opportunity for the development of a sustainable green process for the preparation of enantiopure (*S*)-omeprazole.

2. Materials and methods

2.1. Chemicals and microorganisms

Benzyl phenyl sulfide was purchased from Lancaster, UK. Its sulfoxide and sulfone were synthesized in our laboratory as described below. Omeprazole sulfide and standard sample of omeprazole were gift from Reddys Lab, Hyderabad, India. Culture media, TSB (tryptone soya broth) and PDB (potato dextrose broth) were purchased from Hi Media (Mumbai, India). Pure cultures of microorganisms were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-IMTECH, Chandigarh, India (https://www.mtccindia.res.in/). These were single colony isolates from soil and water samples from a variety of niches in India.

2.2. Media and culture conditions

Bacteria were grown in TSB media prepared by dissolving TSB (30 g) in distilled water (1 l). Composition of TSB: pancreatic digest of casein (17 gL^{-1}), papaic digest of soybean meal (3 gL^{-1}), NaCl (5 gL^{-1}), K_2HPO_4 (2.5 gL^{-1}) and dextrose (2.5 gL^{-1}). Fungi were grown in PDB media prepared by dissolving PDB (24 g) in distilled water (1 l). Composition of PDB: potato infusions (200 gL^{-1}) and dextrose (3 gL^{-1}). Marine isolates were grown in artificial sea water (ASW); composition: NaCl (28.13 gL^{-1}), KCl (0.77 gL^{-1}), $CaCl{\cdot}2H_2O$ (1.6 gL^{-1}), $MgCl_2{\cdot}6H_2O$ (4.8 gL^{-1}), $NaHCO_3$ (0.11 gL^{-1}) and $MgSO_4{\cdot}7H_2O$ (3.5 gL^{-1}). The cultures of bacteria were grown at 37 °C, pH 7.0–7.5 for 24 h. Fungal strains were grown at 30 °C, pH 5.2–5.3 for 72 h. Microbial cells were isolated by centrifugation at 9000 × *g* for 15 min at 4 °C.

2.3. Analytical methods

^{1}H NMR were obtained at 300 MHz and referenced to TMS (0.0 ppm) or the residual solvent peak ($CHCl_3$ 7.26 ppm). Chemical shifts are reported as parts per million (ppm) using the δ scale. ^{13}C NMR spectra were recorded at 75 MHz and referenced either to TMS (0.0 ppm) or internal solvent ($CDCl_3$ 77.0 ppm). Thin layer chromatography (TLC) were performed on Merck silica gel DC Alurolle Kieselgel $60F_{254}$ plates and visualized under UV lamp and/or with 0.25% w/v $KMnO_4$ and 2% $NaHCO_3$ solution in distilled water. Flash column chromatography was carried out using silica gel (200–400 mesh). Analytical HPLC analyses were performed on a system equipped with high pressure gradient dual pump, auto injector, variable temperature column compartment and PDA detector. E.e. was determined by HPLC using one of the following systems. HPLC System 1 - Column: 250 × 4.6 mm CHIRALCEL® OB-H (Daicel, Japan); detection UV at 302 nm; elution 10% 2-propanol in hexane at flow rate of 1 mL min^{-1}; temperature 25 °C (Bonato, 2002). HPLC System 2 - Column: 250 × 4.6 mm CHIRALCEL®OB-H (Daicel, Japan) column; detection 254 nm; Elution hexane:2-propanol 98:2, initial period 2 min and then a gradient during 2–40 min to hexane:2-propanol 75:25 at a flow rate of 0.8 mL min^{-1}; temperature 25 °C.

2.4. Enzyme assays

The peroxidase activity assay mixture contained 50 mM phosphate buffer pH 6.8, 0.025% (w/w) H_2O_2, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 50 µL of cell-free extract in a total volume of 1 mL. The consumption of reduced ABTS was followed at 436 nm.

The monooxygenase activity assay mixture contained 5 mM omeprazole sulfide, 0.1 mM NADPH in 50 mM phosphate buffer pH 7.0 and limiting amounts of enzyme in a total volume of 1 mL. The consumption of reduced coenzyme was followed by spectrophotometer at 340 nm using molar absorption coefficient of 6800 M^{-1} cm^{-1} for calculation. (1 unit = the amount of enzyme which catalyzed the reduction of 1 µmol of NADPH per min under specified conditions).

2.5. Amplification of ITS region and D1/D2 domain of 26S rRNA gene

The internal transcribed spacer region, comprising of ITS1, 5.8 S *r*RNA gene and ITS2 was amplified with primers pITS1 and pITS4, which were from the conserved regions of 18S and 26S *r*RNA genes respectively (White et al., 1990). The D1/D2 domain of 26 S *r*RNA gene was amplified with primers NL1 and NL4 (Kurtzman and Robnett, 1998).

Genomic DNA from fresh mycelia was isolated using DNA isolation kit (Zymo Research, catalog number D6005) following the manufacture's protocol and stored at −20 °C. The genomic DNA was visualized on a 1% tris-acetate EDTA agarose gel stained with ethidium bromide (0.5 µg/mL) at 100 V, 400 mA for 45 min. Polymerase chain reaction (PCR) was done in 50 µL reaction volume. Each reaction tube contained 3 µL of total genomic DNA, 10 µL of 5X GoTaq green buffer (Promega), 1.5 µL each of 10 µM primers (pITS1 F and pITS4 R) (White et al., 1990), 3 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 0.25 µL of 5U Go Taq polymerase enzyme and 29.75 µL of sterile water. The PCR reactions were carried out in Eppendorf master cycler with following cycling parameters. Initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 50 s and extension at 72 °C for 1 min. Final extension was done at the end of the PCR amplification at 72 °C for 10 min and reaction was held at 4 °C until further processed. The PCR products were run on tris-acetate EDTA agarose gel (1%) stained with ethidium bromide (0.5 µg/mL) at 100 V, 400 mA for 45 min to check the presence of desired band of ~500 bp. The PCR products were further cleaned with QIAquick PCR purification kit (QIAGEN, catalog number 28106) according to the manufacturer's protocol. The purified PCR products were quantified using nanodrop spectrophotometer ND-1000 (Thermo). The PCR products were sequenced using pITS1 and pITS4 primers with ABI Big Dye v3.1. terminator ready reaction cycle sequencing kit (Applied Biosystem) using manufacturers protocol. The samples were purified to remove excess salt, denatured with HiDi-formamide at 95 °C for and analyzed using 3730 DNA analyzer (Applied Biosystems) at the central DNA sequencing facility available at MTCC and gene bank, CSIR-IMTECH, Chandigarh, India (http://www.mtccindia.res.in).