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Biooxidation of methyl group: Part 2. Evidences for the involvement of cytochromes P450 in microbial multistep oxidation of terfenadine

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ABSTRACT

The actinomycete *Streptomyces platensis* grown in culture medium containing soybean peptones can transform terfenadine, an antihistamine drug, into its active metabolite fexofenadine. The microbial oxidation of methyl group of terfenadine into carboxylic acid could be an alternative to chemical ways to produce fexofenadine. This bioconversion requires three oxidation steps: a hydroxylation of one methyl group followed by the oxidation of the corresponding alcohol into the aldehyde and finally its oxidation into the carboxylic acid. The oxidation reaction of each step has been studied. Terfenadine and intermediates incubated with whole cells were not oxidized under argon whereas their biotransformation under $^{18}O_2$-enriched atmosphere gave labeled fexofenadine. P450 inhibitors, such as clotrimazole or fluconazole, inhibited oxidation activity of each step. While the two last steps could be catalyzed by dehydrogenases or oxidases, this study strongly demonstrates the role of at least one, or possibly several cytochromes P450, in the oxidation of terfenadine into fexofenadine by *S. platensis* cells. To our knowledge, this is one of the few examples of involvement of P450s in such three steps oxidation of a xenobiotic.

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1. Introduction

Biocatalysis and biotransformation have become a commonly used tool in organic chemical synthesis, in particular to produce synthons and metabolites of xenobiotics. They are used for the synthesis of chiral molecules but also of achiral products where a chemical process would not be possible. For example, chemical oxidations of heteroaromatic molecules and non-activated carbon-atom give side-products and are generally unspecific. On the contrary, some examples describe the specific hydroxylation of methyl and methylene groups by microorganisms [1]. The most appropriate activity could be obtained by screening isolated enzymes or microorganisms. While some enzymes are commercially available (hydrolases, reductases), oxygenases are generally provided by microorganisms and used in whole cells [2]. However, for preparative scale, an improvement of the biocatalyst is often necessary [3].

We are interested in the chemoenzymatic synthesis of fexofenadine, an antihistamine drug devoid of cardiotoxicity [4,5] which is the main mammalian metabolite of terfenadine. Because fexofenadine chemical synthesis is laborious, microbial hydroxylation of the easily accessible terfenadine has been considered as the first step. Moreover, according to Microbial Model of Mammalian Drug Metabolism it was expected to biotransform terfenadine into fexofenadine.

It was found that several microorganisms are able to catalyze the multistep oxidation of terfenadine (Fig. 1) [6,7] and analogs [8]. Among them, we showed [9] that the fungus *Absidia corymbifera* and the bacterium *Streptomyces platensis* are the most efficient in fexofenadine synthesis. However, the activity per gram of cells is too low for a scale-up process and fexofenadine production must be optimized by proteins engineering. This scale-up requires the precise knowledge of the enzymatic activity(ies) involved in this multistep oxidation and the cloning of the proteins corresponding genes [10].

While whole microbial cells contain a wealth of enzymes holding different redox activities, various enzymatic systems can catalyze these three reactions: the first step, hydroxylation of a methyl group can only be catalyzed by monooxygenases, whereas the two following oxidation steps could be catalyzed by either dehydrogenases, oxidases or monooxygenases (a hem and non-hem enzymes) [11–13]. Dehydrogenases are the most often cited in biotechnological applications. For example, a recent work describes [14] an efficient oxidation of alcohol into the corresponding acid using whole cells of *Brevibacterium* sp. or a three enzymes system,

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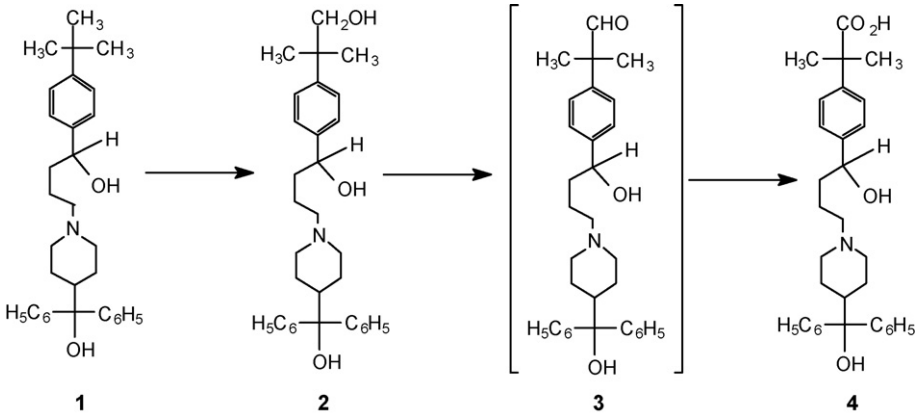

Fig. 1. Biotransformation of terfenadine.

2-phenylethanol dehydrogenase, phenyl acetaldehyde dehydrogenase and NADH oxidase to regenerate the NAD^+. In an industrial process, the oxidation of methyl groups on aromatic heterocycles to the corresponding carboxylic acids were achieved by enzymes from wild type *Pseudomonas putida*, xylene monooxygenase (XMO), benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase [15,16]. However, Schmid and coworkers showed [17] that XMO has an alcohol and an aldehyde oxidizing activities, and used it in synthesis of aryl carboxylic acids [18]. There are only few examples of involvement of microbial cytochrome P450 in the formation of carboxylic acid, including oxidation of 2-ethylhexanol by CYP101 from *P. putida* [19], and formation of dicarboxylic acid from alkanes in *Candida tropicalis* [20]. However, three fatty alcohol oxidases from the strain *C. tropicalis*, which could be involved, were also characterized [21]. Some aldehyde oxidases are characterized including one from a *Streptomyces* strain [22] and one from a *Brevibacillus* sp. that is used to remove glutaraldehyde, a potential environmental pollutant [23]. Finally, some fungal extracellular hem thiolate peroxidases are capable of performing oxidation of methyl groups leading to the corresponding acids [24].

In some cases, there is a lack of knowledge of the enzymes involved in the preparation of acids by microbial oxidation [25–30]. Schwartz et al. reported the oxidation of ebastine to carebastine by *C. blabesleena* and suggested that the two alcohol oxidation steps were catalyzed by oxido-reductases [31].

Contrary to Schwartz suggestion, we obtained some results in agreement with a monooxygenase-dependent mechanism for the three steps oxidation involved in the fexofenadine formation by *S. platensis*. Firstly, oxidation of hydroxyterfenadine to fexofenadine occurs with cells grown in culture medium containing soybean peptone, which are known to induce cytochrome P450 in *Streptomyces griseus* [32]. Secondly, oxidation of hydroxyterfenadine in $^{18}O_2$-enriched atmosphere lead to labeled fexofenadine, resulting to incorporation of one atom of dioxygen [33].

Then, prior to endeavor to purify the enzymes involved in the multistep oxidation of terfenadine by *S. platensis* cells, it was necessary to identify the precise class of these oxidizing enzymes. We describe here the investigations performed to inquire the type of enzyme implicated in each oxidation step. Alcohol dehydrogenase and monooxygenase-dependent oxidative activities were seeked in vitro with cell-free extracts. However, as P450-monooxygenases dependent activities that result from a multi-component systems with a relatively low natural level of expression [34], are rarely preserved during preparation of cell-free extract, indirect approaches are often used to decipher the precise type of enzymes involved in oxidation activities [35–39]. Using this strategy, we performed the biotransformation of terfenadine, and of the corresponding alcohol and aldehyde intermediates under $^{18}O_2$ atmosphere or in the presence of P450 inhibitors. Our results suggest that the overall biotransformation arise from three cytochrome P450 dependent oxidation steps.

2. Materials and methods

2.1. Chemicals

Terfenadine was purchased from Sigma, hydroxyterfenadine was prepared as previously reported [33] and the corresponding aldehyde was obtained as described in this work. Dioxygen $^{18}O_2$ (99% atom ^{18}O) and $H_2{}^{18}O$ was purchased from Cortecnet (Paris) and Euriso-top, respectively. Clotrimazole is obtained from Sigma chemicals (Lyon, France) and fluconazole from Pfizer (Orsay, France).

2.2. Preparation of aldehyde **3**

Hydroxyterfenadine **2** (96.6 mg, 0.2 mmol), TEMPO (31.2 mg, 0.2 mmol) and tetrabutylammonium chloride (22.2 mg, 0.08 mmol) were dissolved in CH_2Cl_2 and buffer ($NaHCO_3/K_2CO_3$ pH 8.3, 2 ml). The mixture was stirred at room temperature and N-chlorosuccinimide (53.4 mg, 0.4 mmol) was added in four portions (2 h). After 1 h, aqueous layer was extracted with CH_2Cl_2 (two times). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on silica gel (dichloromethane/methanol: 97/3) to afford **3** in 45% yield. ^1H RMN ($CDCl_3$, 500 MHz): $\delta = 9.50$ (s, 1 H, C*H*O), 7.53–7.20 (m, 14 H, *H*ar), 4.67 (m, 1H, C*H*OH), 3.26–2.97 (m, 2H, C*H*NC*H*′), 2.72 (m, 2H, $CH_2(CH_2)_2CHOH$), 2.56 (t, H, $J = 5.00$ Hz, C*H*), 2.51–2.34 (m, 2H, C*H*NC*H*′), 2.03–1.99 (m, 2H, $CHCH_2NCH_2CH'$), 1.91–1.76 (m, 4H, CH_2CH_2CHOH), 1.60–1.49 (m, 2H, $CHCH_2NCH_2CH'$), 1.47 (s, 6H, 2 CH_3). RMN ($CDCl_3$, 500 MHz) $\delta = 203.7$ (CHO), 146.9, 145.2, 141.4, 129.8, 128.2, 127.7, 127.0, 80.5, 74.1, 58.2, 54.5, 51.6, 44.2, 38.3, 25.4, 23.9, 23.1. HRMS *m*/*z* calcd for $C_{32}H_{40}NO_3$ $[M+H]^+$ 486.3008, Found. 486.3010.

2.3. Bacteria and culture conditions

S. platensis NRRL 2364 cultures were maintained on agar slants (ISP medium 2) and stored at 4 °C. Liquid culture media containing (per l) glucose 16 g, yeast extract (DIFCO) 4 g, malt extract (DIFCO) 10 g (YM medium), and glucose 16 g, yeast extract 4 g, malt extract 10 g and soybean peptones (Organotechnie) 5 g (YMS medium) were sterilized without glucose at 120 °C for 20 min. Microorganism was cultivated at 30 °C for 48 h in an orbital shaker (200 rpm).

Cell dry weight (CDW) was obtained by centrifugation of 100 ml culture medium and biomass was dried at 100 °C for 24 h.

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