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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.

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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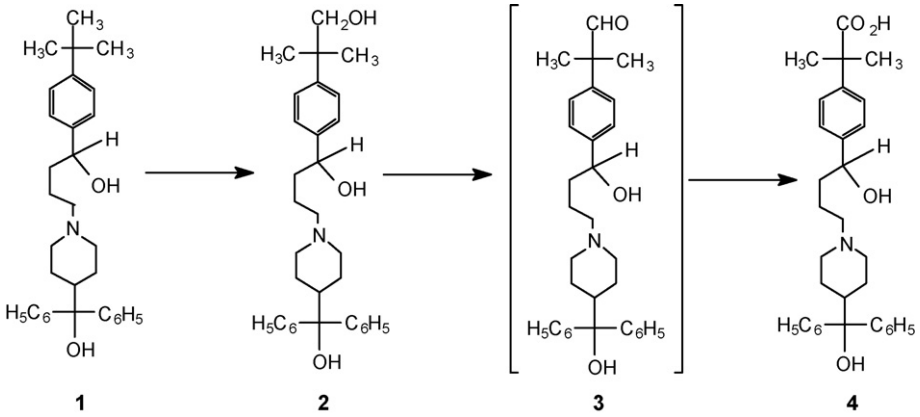

Fig. 1. Biotransformation of terfenadine.

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.

2.4. Incubation with cell-free extracts

S. platensis cells (500 ml) were cultivated in YMS medium in a 2-l Erlenmeyer flask. After 48 h, cells were collected by centrifugation. The bacterial pellets were suspended in TEG buffer (50 mM Tris–HCl pH 7.6, EDTA 1 mM, Glycerol 10%) containing chicken egg lysozyme and Dnase I. After 2 h of incubation at 30 °C, cells were subsequently disrupted by addition of detergent-based Bug Buster reagent, in the presence of protease inhibitors. The cells were then sonicated and centrifuged (7000 × *g*) at 4 °C to remove cells debris. The cloudy supernatant, which contained both cytosolic and membrane material, was fractionated by a centrifugation at 100 000 × *g* for 1 h, at 4 °C. The supernatant fraction, which contained the soluble proteins, was decanted. The pellet containing membrane proteins was resuspended in TEG buffer. Protein concentration of both cytosolic and membrane fractions was estimated using Bradfod method.

Substrate hydroxyterfenadine (10–50 μM) and soluble or membrane fractions (100–400 μg) were incubated in the presence of NAD^+ or $NADP^+$ (1 mM) in TEG buffer, in a total reaction volume of 400 μl. Reaction mixtures were incubated at 30 °C for up to 2 h, and methanol (1 volume) was added to quench the reaction. The precipitated proteins were removed by centrifugation (10 min, 10 000 × *g*) and the supernatants were analyzed by HPLC.

2.5. Incubations with whole cells

The biotransformations were performed in culture medium or in citrate buffer pH 5 at 30 °C in an orbital shaker (200 rpm) and substrate was added in DMF. Biotransformation was monitored as followed. Samples (800 μl) were diluted with methanol (700 μl), mixed vigorously and centrifuged at 10 000 × *g* for 5 min. The resulting supernatants were micro-filtered (0.45 μm) and analyzed by HPLC.

2.5.1. Incubations of hydroxyterfenadine in presence of terfenadine

S. platensis was grown in YM medium (500 ml), harvested, and resuspended in citrate buffer pH 5 (125 ml). Cells suspension was separate in 5 flasks and terfenadine was added in DMF (25 μl) to obtain final concentrations: 0, 0.01, 0.025, 0.05, 0.1 and 0.2 g l^{-1}. After 30 min, hydroxyterfenadine was added in DMF (final concentration 0.15 g l^{-1}). Biotransformation were monitoring as described above. After 20 h, samples (5 ml) of cells suspensions were taken and 1 ml of methanol was added. Mixtures were vigorously stirred, centrifuged and analyzed by HPLC.

2.5.2. Biotransformation under $^{18}O_2$ atmosphere

S. platensis was grown in YM or YMS medium (50 ml), harvested, and resuspended in citrate buffer pH 5 (50 ml) in which nitrogen was bubbled for 5 min. 2 ml of cell suspension were transferred in a 10-ml vial, which was sealed by turn off flange stopped. The remaining air was removed by vacuum using needle and replaced by nitrogen three times. Finally, the head space of vials were evacuated and replaced with pure oxygen enriched with $^{18}O_2$ (99 atom% ^{18}O), substrates were added by injection of 4 μl of solution in DMF (final concentration 0.2 g l^{-1}, 0.42 mM) and vials were placed in rotary shaker at 200 rpm and 30 °C. After 48 h (hydroxyterfenadine and aldehyde) or 120 h (terfenadine) of incubation, 400 μl of reaction mixtures were withdrawn, 350 μl of methanol was added. After vigorous agitation and centrifugation, supernatants were analyzed by LC–MS.

2.5.3. Effects of cytochrome P450 inhibitors on oxidation

S. platensis was grown in flask (500 ml) containing YM or YMS culture medium (250 ml). Culture was separated in 24× 10 ml in 25-ml flask. Inhibitors were added in solution in DMF (clotrimazole 20 μl, fluconazole 40 μl) to obtain final concentration (clotrimazole: 0.2, 0.3, 0.4, 0.5 and 0.7 mM; fluconazole: 10 and 15 mM). DMF was added in controls without inhibitor. Substrates were added in solution in DMF (20 μl) to obtain final concentration (0.2 g l^{-1}). Incubations were monitored by HPLC throughout 48 h. Experiments in presence of both inhibitors were conducted with final concentrations clotrimazole (0.5 mM) and fluconazole (15 mM) and hydroxyterfenadine (0.2 g l^{-1}) or 2-phenylpropionaldehyde.

2.6. Analyses of metabolites

Supernatants were analyzed by HPLC performed on a Gilson HPLC interfaced to computer using the Gilson Unipoint software. The system involved pump 305 and 306, gradient dynamic mixer 811B and autoinjector 234 and the detector was Shimadzu-SDP6A model.

The column (Agilent, C18, 5 μm (250× 4.6)) was in oven (Shimadzu CTO-10A model) at 40 °C and eluted (flow rate 1 ml/min) with a gradient solvent system: isocratic 70% (NH_4OAc 0.1 M/30% CH_3CN) for 7 min followed by gradient to 100% (40% NH_4OAc 0.1 M/60% CH_3CN) in 5 min and held 18 min. The detection was at UV 230 nm and sample volumes were 20 μl.

LC–MS data were performed with a Surveyor-LCQ Advantage mass spectrometer, using the same conditions except a flow rate of 0.2 ml/min and UV. The mass spectrometer was in ESI-positive mode, using a 4 kV capillary tube voltage and an inlet temperature of 275 °C.

3. Results and discussion

The main goal of our study is to obtain an effective method of fexofenadine production from terfenadine. This objective needs to confirm the chemical nature of the intermediates and to determine the type of enzyme catalyzing each reaction step.

3.1. Incubation with intermediate as substrate

The putative intermediates, alcohol **2** and aldehyde **3**, respectively were obtained by microbial hydroxylation as described [33] and oxidation of alcohol **2** as depicted in Section 2 (Section 2.2).

We showed that both intermediates were oxidized into fexofenadine by *S. platensis* NRRL 2364 cells cultured either in YM or YMS culture medium, and this result confirmed the oxidation pathway of terfenadine into fexofenadine (Fig. 1). The hydroxyterfenadine oxidation activity of YM-cells was surprising because incubations of terfenadine with cells obtained in these conditions afforded hydroxyterfenadine as the main product and a low amount of fexofenadine. To explain these results, we supposed an influence of terfenadine in the hydroxyterfenadine oxidation. To verify this hypothesis, cells produced in YM culture medium were incubated with hydroxyterfenadine (0.15 g l^{-1}) in the presence of terfenadine at various concentrations (0–0.2 g l^{-1}). Incubations were performed with 14 g of CDW l^{-1} in citrate buffer pH 5 at 30 °C for 20 h (Fig. 2). At low concentrations of terfenadine (0.01–0.025 g l^{-1}), fexofenadine formation increased with terfenadine concentration, while concentration of hydroxyterfenadine was lower than initial concentration. This is the result of hydroxylation of terfenadine in concentration-dependant manner and of oxidation of hydroxyterfenadine in concomitant reactions. For higher concentration of terfenadine (0.025–0.2 g l^{-1}), hydroxyterfenadine formation increased with terfenadine concentration, whereas fexofenadine formation decreased under these conditions. Therefore, at 0.2 g l^{-1} of terfenadine, no formation of fexofenadine was observed and the concentration of hydroxyterfenadine was higher than its initial concentration.

Table 1
Comparison of oxidative activity catalyzed by whole cells grown in YMS and YM culture medium.

Substrate	Terfenadine **1a**	Hydroxyterfenadine **1b**	Aldehyde **1d**
Product	Hydroxyterfenadine **1b**	Fexofenadine **1c**	Fexofenadine **1c**
R[a]	1,12	1,46	1,02

[a] Ratio of specific activity (YMS/YM).

Thus, cells grown in YM culture medium were able to oxidize hydroxyterfenadine to produce fexofenadine in two conditions either when hydroxyterfenadine is used as substrate or when terfenadine has disappeared more or less completely. We strongly suggest that this phenomenon is explained by terfenadine inhibition phenomenon. The same observation was described in the oxidation of toluene and pseudocumene by xylene monooxygenase [18].

The best oxidizing activities were observed with cells grown in YMS culture medium. On the one hand, these cells are able to produce fexofenadine in the presence of terfenadine. On the other hand, hydroxyterfenadine oxidation was approx. 1.5-fold higher than oxidation by cells grown in YM culture medium, while no significant difference between YM and YMS-cells was observed for terfenadine hydroxylation and aldehyde oxidation (Table 1). These results are in agreement with the soybean peptone-induction of alcohol-oxidative activity not inhibited by terfenadine.

3.2. Incubation with cell-free extracts

Experiments were conducted with cell-free extracts on terfenadine metabolism, in order to determine if flavin or P450 dependent monooxygenases were involved in the oxidation of the methyl group of the t-butyl moiety. *S. platensis* was cultivated in YMS culture medium and cells were disrupted as described in Section 2 (Section 2.6). Terfenadine was incubated with soluble or membrane fractions or a mixture of them, in the presence of the reduced form of the nicotinamide cofactor, NADH, H^+ or NADPH, H^+. No oxidation of terfenadine was observed with either compartment cells, in these conditions. Indeed, such a loss of enzymatic activity is often observed in the case of multiprotein complexes, as P450-dependent monooxygenases usually are. Therefore, this result indicated that P450s could be responsible for the first step of terfenadine oxidation, i.e. its hydroxylation.

In the same manner, experiments were conducted on hydroxyterfenadine metabolism, in order to determine if an alcohol dehydrogenase was involved in the oxidation of the alcohol into the corresponding aldehyde. Hydroxyterfenadine was incubated with soluble or membrane fractions or a mixture of them, in the presence of the oxidized form of the nicotinamide cofactor, NAD^+ or $NADP^+$. No oxidation of hydroxyterfenadine was observed in these conditions. On the contrary to flavin or P450-dependent monooxygenases which are known to be multi-protein systems, alcohol dehydrogenases do not require any other protein cofactor. Thus this result suggested that no alcohol dehydrogenase was involved in the transformation of the alcohol intermediate.

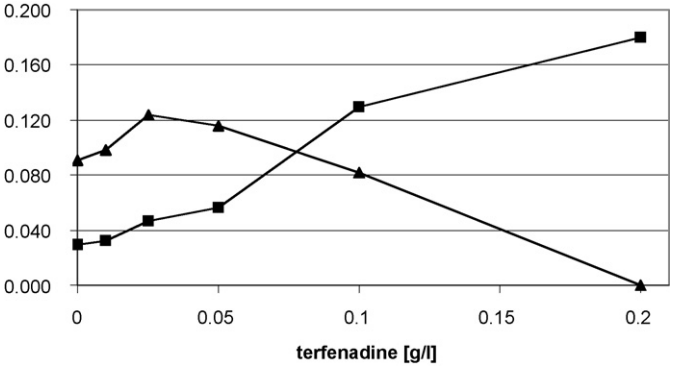

Fig. 2. Concentration of fexofenadine and hydroxyterfenadine after 20 h of incubation of hydroxyterfenadine (0.15 g l^{-1}) in presence of different concentrations of terfenadine. Biotransformations by *S. platensis*-whole cells grown in YM culture medium (65 h). Symbols: *square*, hydroxyterfenadine; *triangle*, fexofenadine.

3.3. Biotransformations under controlled atmosphere

These experiments were performed with *S. platensis* grown in YM or YMS culture media. Under argon, terfenadine was not oxidized into hydroxyterfenadine as expected when the reaction was catalyzed by a monooxygenase. In these conditions, when hydroxyterfenadine and aldehyde were added as substrates, there are no formations of aldehyde and/or acid, but the reduction of aldehyde was observed. These results showed survival of cells or active enzymes in these conditions and suggested that the oxidations of alcohol and aldehyde were dioxygen-dependant.

To investigate the dioxygen function in these reactions, terfenadine, hydroxyterfenadine and aldehyde were incubated with whole cells of *S. platensis* under $^{18}O_2$-enriched atmosphere (99 atom% ^{18}O). The reaction mixtures were analyzed by liquid chromatography–mass spectrometry in ESI-positive mode and the *m*/*z* values at 488, 486 and 502 Da for standard hydroxyterfenadine, aldehyde and fexofenadine correspond to $[M+H]^+$ molecular ions, respectively. The results are summarized in Table 2.

In both culture conditions, mass spectrum of hydroxyterfenadine formed from terfenadine showed incorporation of one atom of oxygen, with the normalized ratios of $[M+H]^+$ to $[M+H]^+ + 2$ molecular ion peaks of 10:90 and 20:80 for YM and YMS culture conditions, respectively (entries 1 and 2).

Further oxidations of hydroxyterfenadine afford fexofenadine, which is formed by incorporation of a second oxygen-atom. In monooxygenase-dependent reaction, this oxygen-atom is incorporated from molecular dioxygen whereas in dehydrogenase-dependent reaction, fexofenadine is formed from aldehyde by incorporation of one oxygen atom from water. Then, when terfenadine was added as a substrate in an $^{18}O_2$-enriched atmosphere, fexofenadine formed via monooxygenase catalyzed oxidation would give an $[M+H]^+$ ion at 506 Da, 4 Da greater than that of the standard, whereas fexofenadine formed via dehydrogenases (alcohol and aldehyde dehydrogenase) catalyzed oxidation would give an $[M+H]^+$ ion at 504 Da, 2 Da greater than that of the standard. In this study, fexofenadine (49%) was obtained after 120-h incubation of terfenadine with *S. platensis* whole cells grown in YMS-culture medium and mass spectrum showed a ratio of $[M+H]^+/[M+H]^+ + 2/[M+H]^+ + 4$ molecular ion peaks of 7:64:29 (entry 2). Thus, we observed an incorporation of two ^{18}O atoms into the carboxylic acid with a $[M+H]^+ + 4$ enrichment of 29%.

Mass spectra of fexofenadine formed when hydroxyterfenadine was added as substrate in incubations with *S. platensis* whole cells grown in YM and YMS-culture medium showed a ratio of $[M+H]^+/[M+H]^+ + 2/[M+H]^+ + 4$ molecular ion peaks of 5:84:11 and 6:80:14, respectively (entries 3 and 4). Fexofenadine obtained in incubations performed with aldehyde as substrate, was entirely mono-labeled (entries 5 and 6). Obviously, oxidation of aldehyde was catalyzed by monooxygenase(s).

Table 2
LC/MS analysis of hydroxyterfenadine **2** and Fexofenadine **4** formed from incubation of terfenadine **1** (120 h), hydroxyterfenadine **2** (48 h) and corresponding aldehyde **3** under $^{18}O_2$-atmosphere.

Substrate	Culture media	Incubation products			
		2		**4**	
		%[a]	$[M+H]^+/[M+H]^+ + 2$[b]	%[a]	$[M+H]^+/[M+H]^+ + 2/[M+H]^+ + 4$[b]
1	YM	80	10/90	0	
1	YMS	33	20/80	49	7/64/29
2	YM	15	90/10	85	5/84/11
2	YMS	10	86/14	90	6/80/14
3	YM	0		100	3/97/0
3	YMS	0		100	3/97/0

[a] Relative amounts of oxidation products (difference with 100% corresponding to terfenadine).
[b] Normalized ratios of isotopic compounds, representative of 3 experiments, except for aldehyde.

Since the incorporation of the second oxygen-atom occurs during the oxidation of aldehyde, the loss of labelling observed in fexofenadine from incubation of terfenadine could result from an exchange with water through equilibrium between aldehyde and hydrate form. We demonstrated this rapid exchange of oxygen atom by incubation of aldehyde in $^{18}OH_2$ and monitoring by mass spectrometry. After 30 min aldehyde was entirely labeled.

Several mechanisms have been proposed for aldehyde oxidation into carboxylic acid by monooxygenase, including hydroxylation of the aldehyde or of its hydrated form as described for acetaldehyde oxidation by cytochrome P450 2E1 [40]. However, study of oxidation of 11-oxo-Δ^8-tetrahydrocannabinol [41] rather showed the monooxygenation of the aldehyde. Results of this study were in agreement with oxidation of aldehyde form because the acid formed from the aldehyde was entirely labeled. Indeed, in mechanism involving the hydrated form, the mono-labeled ortho acid intermediate shall give unlabeled acid in large amount.

Fig. 3 shows the proposed pathway to explain the formation of doubly and mono-labeled fexofenadine from the oxidation of terfenadine, hydroxyterfenadine and aldehyde. The formation of a doubly labeled fexofenadine demonstrated that labeled aldehyde was stable enough to be oxygenated before exchange of oxygen atom. To explain this result it could be suggested that one enzyme catalyze the oxidation of alcohol and of aldehyde without its releasing from the active site.

3.4. Effects of cytochrome P450 inhibitors

The insertion of one atom of oxygen into a non-activated CH bond can be catalyzed by several heme or non-heme enzymes, as methane monooxygenases (MMO), flavin monooxygenases (FMO) or cytochromes P450-monooxygenases. *Streptomyces* species are well known to contain a wealth of cytochromes P450, most of them involved in the secondary metabolism [42]. According to this fact, the effect of cytochromes P450 selective inhibitors on terfenadine oxidative pathway was investigated.

Cytochrome P450 selective inhibitors are extensively used to determine which enzyme isoform catalyzes the oxidation of drugs [43]. Involvement of CYP3A4 [44] and 2D6 [45] in terfenadine metabolism was demonstrated by this method. The approach is also used to establish the participation of cytochrome P450 in microbial biotransformation because the direct demonstration of their contribution is difficult [36,39,46–48]. We tested five compounds (clotrimazole, fluconazole, methyrapone, menadione and SKF525A) from different classes of P450 inhibitors on the biotransformation of terfenadine, hydroxyterfenadine and aldehyde by *S. platensis* cells grown in culture medium containing or not soybean peptones. Inhibitors were added in solution in DMF to incubations (final concentration 0.5 mM except for fluconazole 15 mM) 30 min before substrate. The formation of oxidation product was monitoring by HPLC analysis during 24 h. All compounds showed an inhibition effect, but because problems of co-elution with substrate or oxidation products, only clotrimazole and fluconazole were used in further investigation.

Kinetic studies of the inhibition of *S. platensis* cells catalyzed oxidation of terfenadine **1**, hydroxyterfenadine **2** and aldehyde **3** by clotrimazole and fluconazole were performed at various clotrimazole (0–0.7 mM), fluconazole (0–15 mM) concentrations. Fig. 4 shows their inhibitory activity against *S. platensis* cells grown in YM (Fig. 4A) or in YMS (Fig. 4B) culture medium oxidation of three substrates. The inhibition effect was inhibitor concentration dependent. Clotrimazole caused the almost complete inhibition of terfenadine and aldehyde oxidation, and did not inhibit completely hydroxyterfenadine oxidation. The inhibitory activity of fluconazole depended on cells culture condition and substrate. Higher level of inhibition of terfenadine oxidation was observed

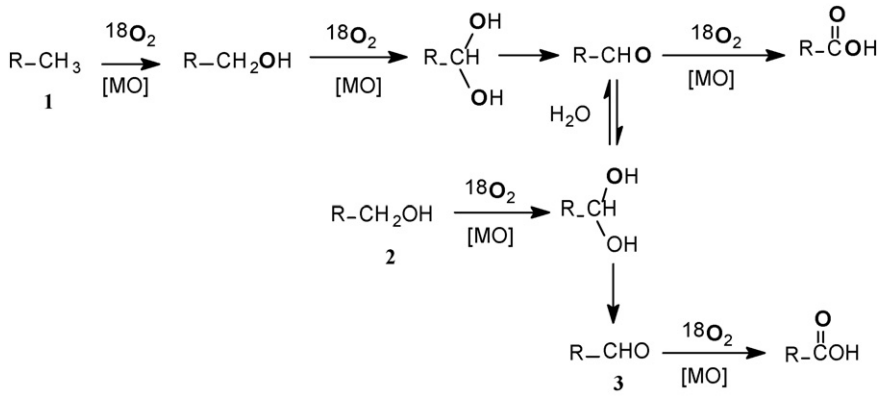

Fig. 3. Proposed pathways for oxidation of terfenadine **1**, hydroxyterfenadine **2** and aldehyde **3** under $^{18}O_2$-atmosphere.

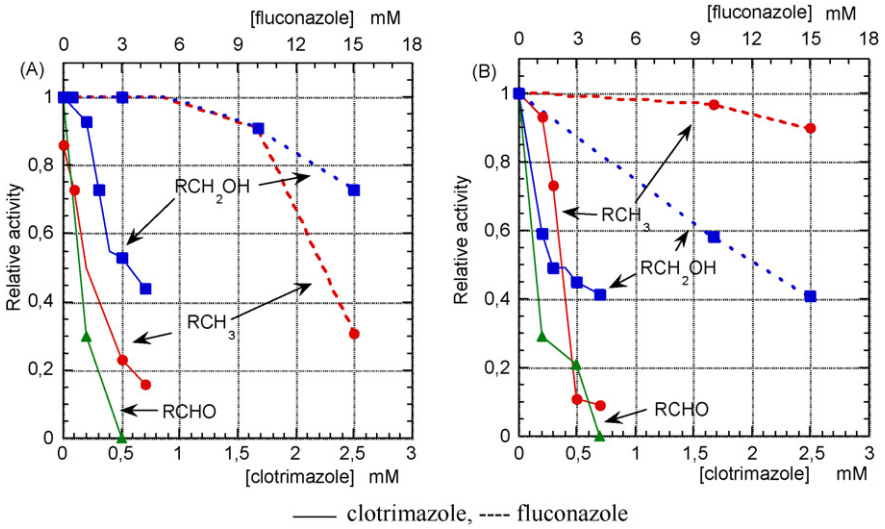

Fig. 4. Effect of clotrimazole and fluconazole on oxidative activities of *S. platensis*. (A) Cells grown in YM culture medium and (B) cells grown in YMS culture medium. Data are presented as the remaining relative activity as a function of inhibitor concentration. Control activities of oxidation were determined for each substrate. Calculations based on peak areas HPLC analysis of the main metabolite after 24 h incubation of three different substrates: terfenadine, RCH_3 (●); hydroxyterfenadine, RCH_2OH (■); aldehyde RCHO (▲).

against cells grown in YMS-culture medium than against cells grown in YM-culture medium while opposite effect was observed in hydroxyterfenadine oxidation. These results suggested that several monooxygenase activities were involved in hydroxyterfenadine oxidation, some of them being inhibited by clotrimazole and other by fluconazole. This hypothesis was confirmed by incubation of hydroxyterfenadine with *S. platensis* cells in presence of mixture of inhibitors, clotrimazole (0.5 mM) and fluconazole (15 mM). Incubations were performed with cells grown in YM and YMS culture medium, and biotransformation was widely (20% remaining activity) and almost completely inhibited, respectively (data no showed). The control of an absence of effect of inhibitors on other enzymatic activities and cells survival was accomplished. For that, we studied the biotransformation of 2-phenylpropionaldehyde by *S. platensis* in presence and in the absence of both inhibitors. Reduction was observed in both conditions in similar manner (data no showed) and we can therefore think reasonably that the observed decrease of oxidizing activities results from inhibition of cytochromes P450.

This study shows that *S. platensis* metabolism of terfenadine into fexofenadine involves cytochromes P450 in three oxidative reactions and could be compared to mammalian metabolism. It has been shown that CYP3A4 and CYP2J2 are responsible for the formation of fexofenadine [49] and carebastine [50].

Genomic analysis of different *Streptomyces* species revealed the presence of a large number of CYP genes: 18 CYP genes were found in *S. coelicolor* [51], 15 in *S. peucetius* [52] and 33 in *S. avermiticilis* [53]. In the vast majority of cases the physiological functions of these CYP are unknown. However, because of the various types of secondary metabolites with important medical application produced by *Streptomyces* species, it is envisaged that CYP enzymes may have roles in their biosynthesis [54]. For example, it was recently established that a CYP of *S. avermiticilis* (*PtlI*) can catalyze the two-step oxidation of methyl group of pentalenene to pentalen-3al, but the enzyme involved in the oxidation of aldehyde was not characterized yet [55]. Then, it would not be surprising that several cytochromes P450 are involved in the three-step oxidation of terfenadine, each reaction could be catalyzed by one or more enzymes. Consequently, the multiplicity of the necessary multi-component systems to produce fexofenadine from terfenadine makes difficult the improvement of an efficient biocatalytic system [56].

4. Conclusion

This study shows that *S. platensis* transforms terfenadine into fexofenadine via hydroxyterfenadine and corresponding aldehyde, which has been synthesized by a chemoenzymatic way. This metabolism involves cytochromes P450 in three oxidative reactions: fexofenadine obtained from terfenadine, hydroxyterfenadine and from aldehyde was labeled when incubations were performed in $^{18}O_2$-enriched atmosphere, and the reactions were inhibited by cytochromes P450 inhibitors. The best hydroxyterfenadine oxidation activity was obtained when *S. platensis* was grown in presence of soybean peptones. This is the first example in biocatalytic application using microbial wild type strain where involvement of monooxygenases was established in three-step oxidation of a methyl group into carboxylic acid. Work is in progress concerning the isolation, purification and cloning of one of the cytochrome P450 catalyzing these oxidations.

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