Journal of Molecular Catalysis B: Enzymatic 134 (2016) 310–316

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Chemodivergent fungal oxidation of isochroman

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ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 24 October 2016
Accepted 25 October 2016
Available online 29 October 2016

Keywords:
Biotransformation
Isochroman
Chemodivergent synthesis
Whole fungal cells

ABSTRACT

This work extends the present knowledge about the ability of filamentous fungi and Baker's Yeast to selectively transform oxygen-containing compounds. Previously, it has been demonstrated that several species of the *Aspergillus* genus are able to perform selective oxidation of benzopyrans. Isochroman or 3,4-dihydro-1*H*-benzopyran (**1**) was chosen as model substrate for the biotransformation since related motifs are often found in the structure of natural products with important biological and pharmacological activities. All the tested strains showed the ability to oxidize **1**. Chemodivergent reaction pathways between the employed microorganisms were observed. The use of cytochrome P450 enzyme inhibitors, and different oxygenation conditions allowed to inquire about the type of enzymes involved in the process. The results obtained were compared with chemical one-electron oxidation of compound **1** and thus, a metabolic pathway was proposed.

1. Introduction

Isochroman derivatives represent an important class of oxygenated heterocycles that naturally occur in many different organisms showing a broad range of biological activities [1]. Although no activity is reported on **1** their derivatives exhibited antifungal and cytotoxic activities [2]. These structural motifs are widespread elements in a plethora of different natural products including microorganisms [3], [4]; plants [5], [6]; and insect metabolites [7]. To date, several traditional chemical methodologies have been used for the preparation of new isochroman derivatives. However, environmentally safe, milder, energy saving and regio- and stereo- selective methods complementary to traditional approaches are required.

Yeasts have also been hugely significant in biotechnology. Its cells can be considered as an ideal catalyst, since they are widely available, inexpensive, non-pathogenic, and the reactions are easy to perform. *Saccharomyces cerevisiae* or baker's yeast has been extensively used as biocatalyst, in particular for its ability to catalyze the enantioselective reduction of carbonyl groups and electron deficient olefins, to perform the hydrolysis, oxidation and cyclization of different types of compounds [8,9].

On the other hand, *Aspergillus* genus has been historically used to perform biotransformation due its immense metabolic potential. At first glance, it is easy to assume that *Aspergilli* contain enzymatic machineries to detoxify compounds, transforming them into more hydrophilic and -often- less toxic metabolites. Such an ability to metabolize xenobiotic chemicals might be suitably exploited by organic chemists to generate interesting derivatives [10]. Among them, *A. niger* has been proved to be an important microorganism for the hydroxylation of many natural products [11,12].

In previous work, it has been demonstrated that several species of *Aspergillus* are able to perform selective redox transformations on benzopyranes [13,14]. This fact suggests that the microorganism can metabolize another benzopyran as **1**.

In this work, we report the metabolism of xenobiotic compound **1** by two fungal organisms, namely a yeast and a filamentous fungus in order to characterize and compare degradation pathways for the election of suitable biocatalysts.

2. Experimental

2.1. Chemicals and analytical methods

Compound **1**, Piperonyl butoxide (PB) (5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole) and

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http://dx.doi.org/10.1016/j.molcatb.2016.10.015
1381-1177/

Cerium (IV) Ammonium Nitrate (CAN) (ACS ≥98.5% of purity) were purchased from Sigma-Aldrich Argentina; solvents were distilled prior to use.

^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker AVANCE II 400 spectrometer (400 MHz for ^{1}H and 100.25 to ^{13}C). For structural elucidation the following 2D experiments were also used: COSY, HSQC, HMBC and DOSY. Chemical shifts in ppm are relative to TMS, coupling constants (*J*) are expressed in Hz.

Deuterated solvents used in ^{1}H NMR were $CDCl_3$, CD_3OD and $(CD_3)_2CO$ (all isotopically enriched in 99.80%); deuterium chloride (DCl – 99% D, 35% w/v in D_2O) and D_2O (99.97%) from Sigma-Aldrich. Analytical TLC were performed on Merck precoated silica gel 60 F254 plates. Column chromatography (CC) was carried out on Merck silica gel 60 (230 – 400 mesh). Solvent for TLC and CC were *n*-hexane: ethyl acetate mixtures. GC–MS analyses were performed on a GCQ-plus Finnigan MAT apparatus equipped with Restek 5-MS column 5% diphenyl – 95% dimethylpolysiloxane (30 m, 0.25 mm ID, 0.50 μm df). T1 = 100 °C for 3 min, (Δt = 15 °C/min), T2 = 300 °C, injector T = 250 °C; carrier gas: He 40 cm/s. The instrument was run in EI mode at energy of 70 eV (tune setting, trap off set between −10 and −20 V), ion source 183 °C, transfer line 250 °C, and mass range 50–400.

FT-IR spectra were obtained on a Nicolet Protegé 460 FTIR spectrophotometer.

High-resolution mass spectra (HR-MS) were recorded on a Bruker Daltonics, MicroTOF Q II spectrometer with an electrospray ionization (ESI) source operating in the positive mode, using nitrogen as nebulizing and drying gas and sodium formate 10 mM as internal calibrant.

2.2. Microorganisms

Fungal strain was obtained from the American Type Culture Collection (*Aspergillus niger* ATCC 11394). Fungi were maintained on Sabouraud – slants. Stock cultures of all fungal strains were stored in Petri dishes on solid Sabouraud or Czapeck media at 4 °C.

Lyophilized baker's yeast strain (*Saccharomyces cerevisiae*) from local market were grown in YPD liquid medium at 30 °C.

2.3. General procedures for biotransformation

2.3.1. Biotransformation by growing and resting cells systems of *A. niger*

2.3.1.1. Growing cells. A two-step-process was conducted. Spores of *A. niger* were inoculated into 30 mL Czapeck medium in 100 mL Erlenmeyer flasks (3) and incubated at 28 °C for 2 days on orbital shaker (180 rpm). Forty-eight-hour-old cultures (5 mL) were subcultured in 100 mL Erlenmeyer flasks containing 30 mL of fresh culture medium in a 10 pellets per batch relation.

Substrate **1** (10 mg/batch), dissolved in 100 μL of dimethylsulfoxide (DMSO), was added to the cultures 24 h later. The reaction mixtures were incubated at 28 °C on orbital shaker (180 rpm).

Bioconversion progress was monitored every day during a week and aliquots of the organic layers were analyzed by GC–MS (1 μL) and TLC analysis. Blank assays without substrate and without fungi were carried out in parallel. Experiments were performed in triplicate.

2.3.1.2. Resting cells. Pre-cultures of selected *Aspergillus* strains were prepared as described above. After incubation, fungal cells were harvested by centrifugation and washed with phosphate buffer. Cells (10 pellets per batch) were re-suspended in phosphate buffer (30 mL, pH 6.0, 0.1 M) in Erlenmeyer flasks (100 mL). Substrate **1** (10 mg/batch), dissolved in 100 μL of DMSO, was added and bio-reaction was incubated in orbital shaker (180 rpm) at 28 °C.

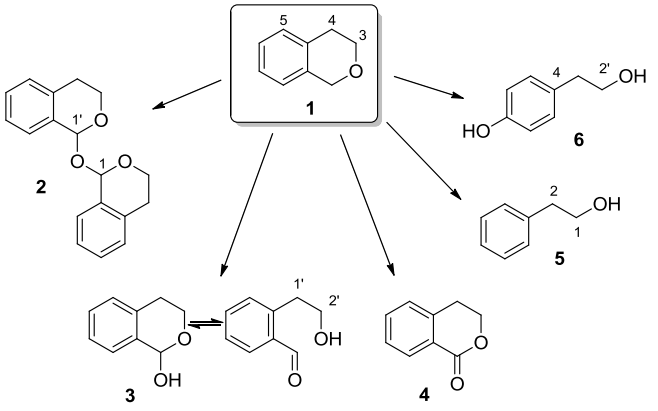

Fig. 1. Biotransformation studies of **1** using whole cells of *A. niger* and Baker's yeast.

Biotransformation progress and blank assays were prepared as describe above. Experiments were performed in triplicate.

2.3.2. Biotransformation by Saccharomyces cerevisiae (baker's yeast)

Lyophilized baker's yeast (2 g) was incubated in a nutrient medium containing sucrose (2 g), peptone (0.5 g), yeast extract (0.2 g) and $MgCl_2/ZnCl_2$ (0.1 g) dissolved in 0.1 mM sodium phosphate buffer, pH 6.5 (50 mL), and incubated at 28 °C for 24 h. To the reaction medium containing yeast cells, the substrate **1** (25 mg/batch), dissolved in 250 μL of DMSO, was added and incubated in an orbital shaker (180 rpm) for their optimal product transformation. For the biotransformation with isocroman-1-one (**4**), the amount of substrate added was 5 mg per batch dissolved in DMSO. Biotransformation progress and blank assays were prepared as describe above. Experiments were performed in triplicate.

2.4. Scaling up of the biotransformation

To determine the chemical identity of the biotransformation products it was necessary to scale up the process in order to isolate and purify each metabolite. Taking into account the importance of not modifying any assay condition, particularly oxygenation parameters, the scaling up was in fact a resizing of the experiment, performed simply by increasing the number of culture batches (10) in order to preserve all the biotransformation conditions. Incubation, substrate addition and work up procedures were carried out as described above for growing cell experiments.

2.5. Product isolation and purification

After incubation, the fermentation media were extracted three times with ethyl acetate. The organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure on a rotary evaporator which was then loaded on preparative TLC and eluted with *n*-hexane/ethyl acetate.

Incubation of **1** for 7 days with the fungus *A. niger* rendered three products namely 1,1′-oxodiisochromane (**2**; 88 mg, 37%), 3,4-dihydro-1*H*-isochromen-1-ol (**3**; 25 mg, 10%) and also 3,4-dihydro-1*H*-isochromen-1-one (**4**; 115 mg, 48%) as major metabolite. The metabolites of **1** obtained after 24 h incubation of baker's yeast were purified and identified based on spectroscopic data. Chromatographic separation of the crude extract resulted in the isolation of two metabolites namely 2-phenylethanol (**5**; 83 mg, 47%) and (2-(4-hydroxyphenyl) ethanol (**6**; 74.5 mg, 43%), see Fig. 1.

Supplementary evidence for identification of the compounds was obtained by comparing ^{1}H NMR and ^{13}C NMR spectral data of

metabolites with literature. The molecular structures of metabolites are shown in Fig. 1.

2.6. Time-course study of the biotransformation

In order to investigate the time course outcome of the different metabolites, sampling was carried out every 24 h during a week. The experiments were performed with growing cells as described above, by collecting a 30 mL batch assays. Through gas chromatography coupled to mass spectrometry (GC–MS), conversion plot of **1** were constructed as a function of time.

2.7. Solvolysis of diastereomeric mixture (**2**)

Four different procedures for the solvolysis of **2** were designed in order to demonstrate the existence of equilibrium between species.

a) *Experiment 1.* 10 mg of **2** were dissolved in 1 mL of tetrahydrofuran (THF). Then, 5 mL of HCl 0.1 M were added. The system was stirred and heated gradually to 50 °C in glycerine bath. The reaction was evaluated every 15 min by TLC until the product formation was detected.
b) *Experiment 2.* Following the above described procedure, the conditions were replicated in a NMR tube. In this case 10 mg of compound **2** dissolved in 600 μL of $CDCl_3$ in the NMR tube. Then, 100 μL of D_2O were added and then the system was stirred. Finally, 7 μL of DCI 35% w/v solution were added and the system mixed by vortex. ^{1}H NMR spectra of substrate and after addition of D_2O and DCI were recorded. After a few minutes at 25 °C (standard acquisition temperature) the temperature was raised up to 50 °C. A number of spectra were taken upon reaching such temperature (every 30 min for 6 h).
c) *Experiment 3.* 10 mg of compound **2** was dissolved in 1 mL of methanol (MeOH). Then 5 μL of HCl 0.1 M were added. The reaction mixture was stirred by magnetic stirring at room temperature. This procedure was evaluated every 15 min by TLC.
d) *Experiment 4.* In order to reproduce the above experiment to be monitored by NMR, 10 mg of **2** were dissolved in 600 μL of CD_3OD. Then, 5 μL of DCI 35% w/v solution were added and the system mixed by vortex.

2.8. Enzyme inhibition assay

Experiments were performed with resting cell systems in 30 mL phosphate buffer (pH 6) inoculated with fungal pellets harvested from 48 h-old *A. niger* cultures, with the addition of piperonyl butoxide (PB) dissolved in DMSO at a final concentration of 3 mM. Two hours later, 10 mg/batch of **1**, were added. Incubations and sample withdraws were carried out under the above described conditions.

2.9. Oxidation with CAN in methanol and water

Ceric ammonium nitrate (CAN) is a reagent widely used for qualitative analysis of alcohols, oxidation of phenols or methoxybenzenes to quinones and oxidation of benzyl alcohols to benzaldehydes [15]. A solution of the substrate (30 mg) and CAN (270 mg) in methanol or water (20 mL) was stirred for 30 min at room temperature and was evaluated every 5 min. At the end of the reaction it was directly extracted with methylene chloride, dried over anhydrous Na_2SO_4 and concentrated. Aliquots of the organic layers were analyzed by TLC with *n*-hexane/ethyl acetate. Oxidation products were assigned by ^{1}H NMR of the reaction crude.

3. Results and discussion

3.1. Biotransformation of **1** by growing and resting cells systems of A. niger

Firstly, a two-step biotransformation procedure was carried out in growing and resting cells conditions as described in the experimental section. By analyzing the chromatograms (TLC and GC–MS) obtained from samples harvested at every 24 h, it was evident the presence of a diversity of products that were absent in blank experiments.

Three compounds (**2**–**4**) were isolated and analyzed by ^{1}H and ^{13}C NMR and MS (Fig. 1). The results indicated that growing and resting cells systems rendered the same compounds but in the case of resting cells, with systematically lower yields.

The product **2** corresponds to the bisacetal (**2**), having a molecular ion M^+ 282, obtained as distereomeric mixture of homochiral and heterochiral bisacetal. The characterization of the mixture was carried out using several spectroscopic strategies. IR spectrum showed low intensity bands at 2820 cm^{-1}, characteristics for acetals (O—CH—O) thus confirming the presence of such functional group. By HRMS $[M^+ Na]^+$ it was possible to confirm the exact mass of compound (**2**) (calculated for $C_{18}H_{18}O_3Na$, 305.1148, found M+ 305.1194). ^{1}H and ^{13}C NMR spectra showed the existence of a mixture of compounds with closely related structure. By DOSY NMR, it was observed that all the signals of the mixture had equal diffusion coefficient as expected for a single compound.

The compound **3** has a hydroxyl group on C-1 (δ 100.8). The presence of a singlet at δ 6.06 in ^{1}H NMR spectrum allowed to deduce the presence of a hemiacetal from selective oxidation at C-1 corresponding to 3,4-dihydro-1*H*-isochromen-1-ol. The identity of lactol **3** was confirmed by comparing the spectroscopic data reported in literature [16]. Furthermore, as hemiacetal – aldehyde equilibria take place, a balance with the corresponding hydroxyl – aldehyde is proposed for this lactol [17] (Fig. 1). The product **3** is sought to undergo oxidation thus rendering lactone **4**. The latter contain a carbonyl group of δ-lactone at C-1 and was identified as 3,4-dihydro-1*H*-isochromen-1-one, previously obtained from **1** by photo-oxidation [18].

3.2. Biotransformation of **1** by baker's yeast growing cells

Following the procedure described in the experimental part, the products **5** and **6** were obtained (Fig. 1). Notheworthy, these compounds were not formed in biotransformations with filamentous fungi, *A. niger*.

In the ^{1}H NMR spectrum for compound **5**, it was remarkable the appearance of two triplets at δ 2.60 (J = 6.6 Hz) and 3.87 (J = 6.6 Hz), assigned to H-2 and H-1, respectively. No signals suggesting substitutions on the aromatic ring were detected. In the IR spectrum no signals that could be attributed to a carbonyl group were observed. Considering the M^+ 122, and spectroscopic data agreed with previously reported data [19], to this compound was assigned the structure of 2-phenylethanol. Furthermore, compound **5** was previously obtained by biotransformation of cinnamyl alcohol with the plant pathogenic fungus *Colletotrichum acutatum* [20]. It has been reported that 2-phenylethanol (**5**) can be prepared by biotransformation of L-phenylalanine using immobilized cells of yeast *S. cerevisiae* [21].

Compound **6** (2-(4-hydroxyphenyl) ethanol also known as tyrosol is likely formed by *para*-hydroxylation of compound **5**. This assumption lies on the analyses of time-course experiments in which **6** rises at the expense of **5**.

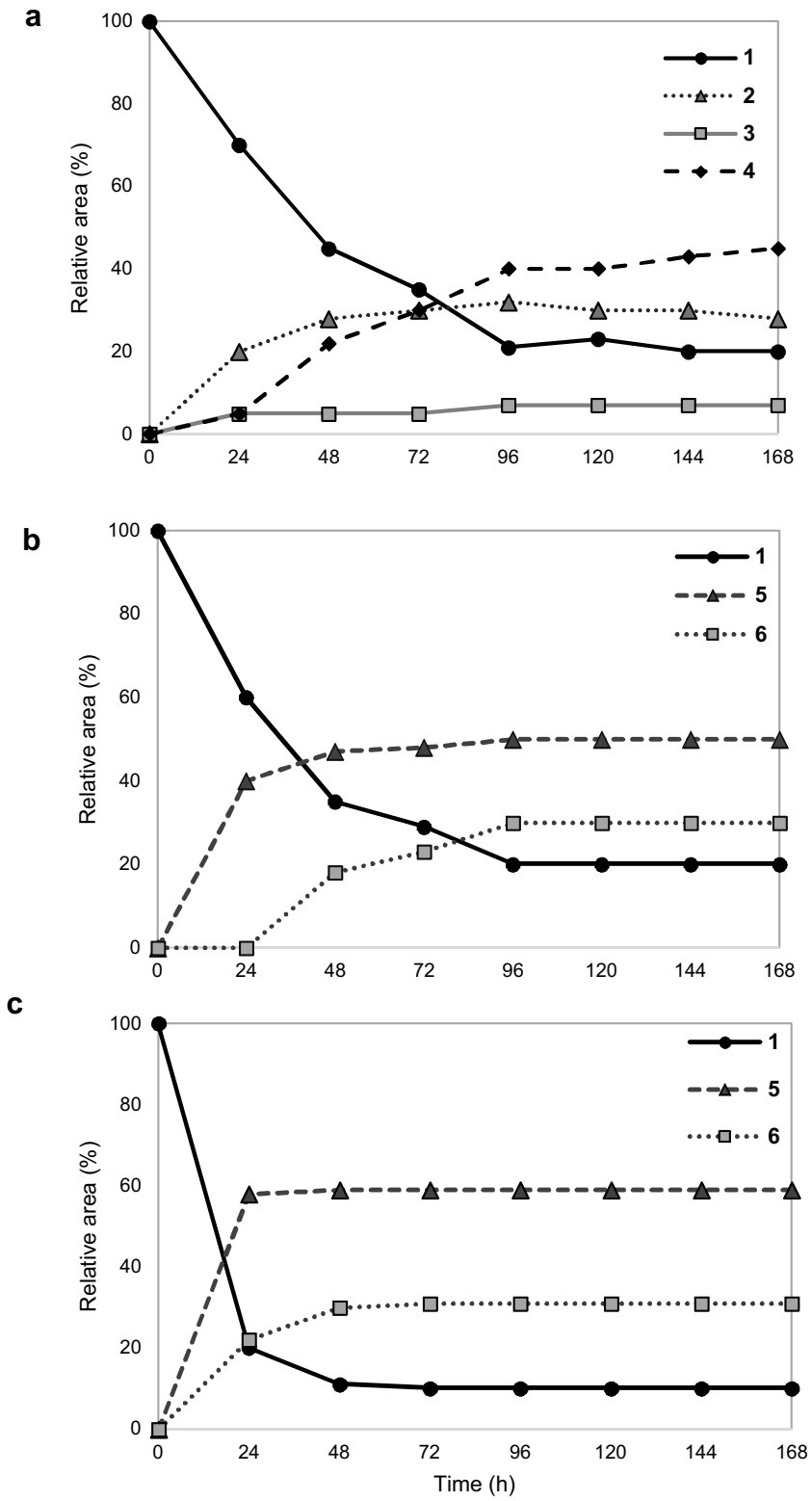

Fig. 2. Time course profile of **1** biotransformation by growing cells system (**a**) *A. niger;* (**b**) B. yeast; and time course profile of metabolite **4** by (**c**) B. yeast.

3.3. Time course study of the metabolites

A time course study was separately performed for each microorganism. From biotransformation of **1** by *A. niger* by growing cells system, after 24 h of incubation, 30% conversion of substrate was observed. The compounds **2**, **3** and **4** appeared in the broth from the first day. The biotransformation yield of **2** and **3** increased gradually and reaches the maximum amount after 4 days of incubation (Fig. 2a). Compound **4** was the major metabolite obtained with *A. niger* (45%).

The last time-course study was performed in order to investigate the bioconversion of **1** by *Baker's yeast*. As mentioned before, with yeast as biocatalyst, different products to those obtained by *A. niger*, were produced by bio-conversion. It was observed that

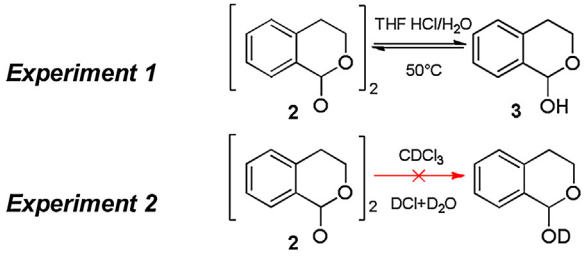

Fig. 3. Hydrolysis reactions performed on the diastereomeric mixture (**2**).

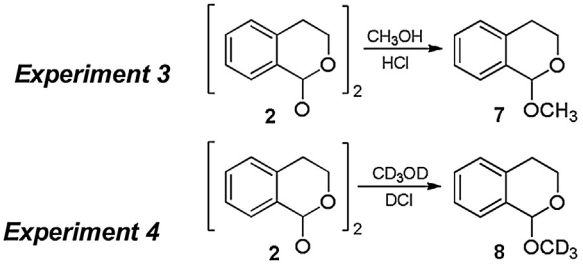

Fig. 4. Alcoholysis reactions performed on the diastereomeric mixture (**2**).

after 24 h the product **5** was formed and the amount of **5** increased slightly and began to form the compound **6** or tyrosol. The biotransformation products **5** and **6** reaches its maximum amount at 96 h. Afterward, there was no change in the amount of the metabolites (Fig. 2b).

To verify and rationalize the formation of compounds **5** and **6** a bio-conversion starting from **4** as substrate was performed with the baker's yeast. The results showed that at 24 h, 80% of the substrate was consumed forming compounds **5** and **6** with 60% and 20% relative abundance, respectively (Fig. 2c).

3.4. Solvolysis of mixture *2*

The Fig. 3 shows the proposed equilibrium between the metabolites **2** and **3**. This was evident from the solvolysis (hydrolysis or alcoholysis) experiments of the diastereomeric mixture (**2**). The design of four different procedures allowed to evaluate which methodology was the appropriated to put in evidence such an equilibrium.

When using *Experiment 1* on the mixture (**2**), it was observed that heating the system up to 50 °C the hydrolysis of the diastereomers (**2**) is favored. The increase of the amount of lactol **3** and the decrease of the dimer were observed. But, as the temperature decreased after the reaction was complete, the dimer was again the major product thus highlighting the existence of a balance between species (Fig. 3). In order to understand more clearly the balance between these species is that a new experience, *Experiment 2,* was designed, carried out in an NMR tube, to observe this phenomenon of interconversion; but unfortunately, no clear results were obtained since hydrolysis was barely affected by the acid catalyst likely due to the heterogeneous nature of the mixture (Fig. 3).

Considering these disadvantages of reactivity, a similar process was designed wherein the solvolysis reaction takes place in a homogeneous system that may subsequently be reproduced in the NMR tube. By *Experiment 3* using a mixture of methanol and hydrochloric acid, at room temperature, a rapid conversion of the compounds **2** to **7** was achieved. The latter would be the expected product of methanolysis (Fig. 4).

The results obtained in the above reaction allowed to reproduce the experiment in a homogeneous system in the NMR tube (*Experiment 4*). In this case, dissolving the mixture in deuterated methanol with the addition of deuterated hydrochloric acid in catalytic quantities, the same results were achieved as in *Experiment 3* (product **8**). As by definition, a homogeneous system has the same properties in all parts of the system, the stirring is no longer necessary for the reaction to occur rapidly (Fig. 4).

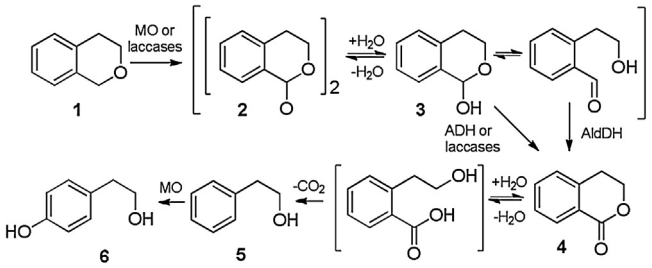

Fig. 5. Metabolic pathway from **1** by *A. niger* and Baker's Yeast.

Although interconvertion lactol-dimers was not observed it was evident the solvolysis of the **2** to form compound **7** and **8** respectively.

Solvolysis experiments confirmed the proposed balance between the found species, thus allowing to rationalize the results observed in metabolism of **1**.

3.5. Monooxigenases Cit-P450 enzymes inhibition assay by PB

In a previous study, various monoxygenases (MO) in *A. niger* have been reported [22], and hydroxylation can be catalized by MO such as cytochrome P450 and a flavin-containing monoxygenases (FMOs) [23]. To further elucidate the responsible enzyme in the biotransformation of **1** by *A. niger*, PB was added to the cultures. When PB, a known potent P450 inhibitor and a non-specific esterase inhibitor in filamentous fungi [24] was added to resting cells systems, a total inhibition of the formation of metabolites, was observed. Based on this result we can infer that this phenomenon may be caused by a strong inhibition by PB on P450-monooxigenases (which would be responsible for the formation of the oxidative metabolites). Other type of enzymes that could be present in the reaction medium and gave same results were the laccases although this cannot be inhibited by PB.

Previously, enzymatic oxidations on methylene groups to form ketones in one-pot and tandem reaction with MO and alcohol dehydrogenation (ADH) enzymes were proposed in compounds with similar molecular structure to **1** [25]. So, it would feasible that these reactions take place on our selected substrate (**1**).

3.6. Metabolic pathway

Taken together, the results of biotransformations (with the filamentous fungus and yeast) and in the aforementioned assays, a metabolic pathway to **1** was proposed. In Fig. 5 it is suggested which kind of enzymes/processes would be involved in oxidative and reductive metabolic routes.

In a first stage, the occurrence of compound **2**, which corresponds to a mixture of diastereomeric dimers would have possibly made up by monooxigenases or laccases enzymes activity from substrate.

Then, since the biohydroxylation at C-1 of **1** (possibly stereoselectively) gives rise to the hemiacetal (**3**), it can be expected that the enantiomeric excess of such hemiacetal was completely eroded by considering the equillibrium of the hemiacetal form with the hydroxyaldehyde open form. The formation of lactol **3** was little evident because rapid oxidation of the precursor probably occurs resulting compound **4.** In turn, the product **3** could be reoxidized

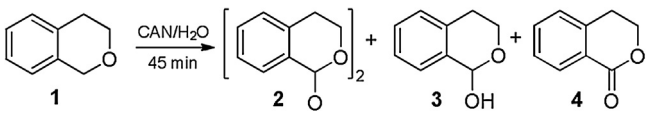

Fig. 6. Oxidation of **1** with CAN in water as solvent.

in two different fashions: by the action of alcohol dehydrogenase (ADH) [26] or through a laccase-catalyzed oxidation [27] to form **4.** Another possibility was analyzed, that **4** was formed from the aldehyde which was in equilibrium with the product **3**. Thus, the activity of the aldehyde dehydrogenase enzyme (AldDH) could explained the appearance of lactone (Fig. 5).

Besides, metabolic pathway of **1** was expanded, since metabolites **5** and **6** would arise through a decarboxylation reaction of the corresponding hydrolysis product of the lactone **4** (by *S. cerevisiae*). Once the compound **5** was formed, the reaction continued leading **6**. The product **6** would be obtained as a result of *para*-hydroxylation reaction of alcohol **5**, possibly due to the activity of MO enzymes (Fig. 5).

Moreover, the compounds obtained with yeast from **1** and lactone **4** allowed to complete the last part of the presented metabolic pathway.

Thereafter, according to our inhibition experiments, it is possible that metabolism of the compounds **2**, **3** and **4** from **1**, was mediated by MO Cit-P450 enzymes.

3.7. *Oxidation of **1** with cerium ammonium nitrate (CAN)*

Following the procedure described in the experimental section *2.9*, the compound **1** was reacted with CAN in methanol but no oxidation products were obtained. Instead, by conducting the oxidation in water, a heterogeneous system was set-up and the reaction proceeded rapidly reducing the cerium (IV) and forming the products **2** (2%), **3** (36%) and **4** (62%), being the latter the major product detected (Fig. 6). Although the relative amount of products is different as compared with the biocatalyzed reaction, this experiment demonstrate that besides oxidation, no other catalyzed reaction is needed to account for the formation of products **2**-**4**.

4. Conclusions

The fungal metabolism of **1** with a strain of *Aspergillus niger* and Baker's Yeast furnished five metabolites. Some of the products formed were recognized as dihydroisocoumarin (**4**) and tyrosol (**6**). Dihydroisocoumarin is an interesting motif since this type of natural products are isolated from various natural sources and have a wide range of biological activities such as; antifungal, phytotoxic, growth regulator, diuretic, antihypertensive and anti-tumor [28]. Besides, as far as we are aware, this is the first report the preparation of tyrosol, which is a powerful antioxidant [29], from biotransformation of **1**. Tyrosol is a known citoprotector agent and as such, has potential in the treatment of Alzheimer's and other neurodegenerative diseases where oxidative stress plays a major role in the pathology development and progression [30] [31].

Different experiments were performed in order to explain the formation of some key compounds and allowed to propose a diverging metabolic pathway of **1.**

From the results herein presented, it is demonstrated that by choosing the appropriate biocatalyst and tuning the conditions, different products can be obtained with this methodology, in chemodivergent fashion.

Acknowledgments

This work was supported by grants from CONICET (Argentina) (PIP 2012–2014, Resol. 1675/12), Ministerio de Ciencia y Tecnología de Córdoba (Argentina), SeCyT-UNC (2012-2013, 05/C608), and FONCYT (PICT 2775 and 0694). G.I.F. thanks CONICET (Argentina) for a fellowship. NMR assistance by G. Bonetto is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.10.015.

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