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Stereoselective reduction of flavanones by marine-derived fungi

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ABSTRACT

Biotransformation is an alternative with great potential to modify the structures of natural and synthetic flavonoids. Therefore, the bioreduction of synthetic halogenated flavanones employing marine-derived fungi was described, aiming the synthesis of flavan-4-ols **3a-g** with high enantiomeric excesses (*ee*) of both *cis*- and *trans*-diastereoisomers (up to >99% *ee*). Ten strains were screened for reduction of flavanone **2a** in liquid medium and in phosphate buffer solution. The most selective strains *Cladosporium* sp. CBMAI 1237 and *Acremonium* sp. CBMAI1676 were employed for reduction of flavanones **2a-g**. The fungus *Cladosporium* sp. CBMAI 1237 presented yields of 72–87% with 0–64% *ee cis* and 0–30% *ee trans* with diastereoisomeric ratio (*dr*) from 52:48 to 64:36 (*cis:trans*). Whereas *Acremonium* sp. CBMAI 1676 resulted in 31% yield with 77–99% *ee* of the *cis* and 95–99% *ee* of the *trans*-diastereoisomers **3a-g** with a *dr* from 54:46 to 96:4 (*cis:trans*). To our knowledge, this is the first report of the brominated flavon-4-ols **3e** and **3f**. The use of fungi, with emphasis for these marine-derived strains, is an interesting approach for enantioselective reduction of halogenated flavanones. Therefore, this strategy can be explored to obtain enantioenriched compounds with biological activities.

1. Introduction

Flavonoids possess a basic C6-C3-C6 flavan skeleton that presents two aromatic rings linked by a heterocyclic pyran ring [1]. Based on their chemical structure, degree of oxidation, linking chain, and unsaturation, flavonoids can be further classified into 6 major groups: isoflavonoids, flavanones, flavanols, flavonols, flavones and anthocyanidins [2].

Among these types of flavonoids, flavone and isoflavone derivatives deserve to be highlighted due to occurrence in nature and stablished biological activities [3]. However, flavanones also deserve attention due to antioxidant, antiviral, anticancer, anti-inflammatory, and anti-estrogenic activities [4,5]. Moreover, these compounds are present at high concentrations in different fruits, such as oranges, grapefruit, lemons, limes, and some aromatic herbs like oregano, rosemary, and peppermint [6].

Synthetic flavonoids have also been studied for their biological activities [7,8]. In this context, halogenated chalcones and flavanols presented better anticancer activity than the natural quercetin, promoting interest for the obtention of these compounds [9]. Also, brominated and chlorinated flavanones, flavones and catechins showed antifungal activities against potential pathogens for humans [10], and halogenated derivatives of 5,7-dihydroxyflavanone presented increased activity against bacteria and fungi [11].

Flavanones present a heterocyclic ring C with no conjugation between both rings A and B (Fig. 1C), and different strategies have been employed to obtain new derivatives [12]. Biocatalysis has had an impact in the conversion of starting materials and preparation of different compounds [13]. Therefore, biotransformation is an alternative with great potential to modify the structures of natural and synthetic flavonoids [14]. Different biocatalytic approaches were already employed, such as microbial biotransformation, enzymatic engineering, metabolic engineering, and the use of plant cells [15–19].

Biotransformation reactions were carried out by different biocatalysts aiming to overcome the cell toxicity of flavonoid aglycones and producing novel bioactive molecules with interesting activities [20]. For

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example, dihydrochalcones and an alcohol product of 4′-methylchalcones biotransformation presented interesting bactericidal activity [21].

Engineered microorganisms have also been employed for biotransformation of flavanones. Quercetin was converted to quercetin 3-*O*-gentiobioside by *Escherichia coli* expressing two uridine diphosphate dependent glycosyltransferases and two nucleotide sugar biosynthetic genes [22], and the cytochrome P450 CYP52G3 from *Aspergillus oryzae* expressed in *E. coli* converted flavanone into 4′-hydroxyflavanone and 6-hydroxyflavanone [23]. Moreover, some native bacterial strains were employed for flavanones biotransformation [24].

For reduction reactions, promising results have been presented with the screening of fungi from distinct environments with substrates like flavanone [25], 7-hydroxyflavanone [26], 6-hydroxyflavanone [27], chalcones [28], 4-chromanol, 4-flavanol, xanthydrol [29], and glabranin [30]. Moreover, it is important to emphasize the successful use of marine-derived fungi for biotransformation of flavonoids like chalcones [31] and 2′-hydroxychalcones [32].

In this work, the biotransformation of synthetic halogenated flavanones employing marine-derived fungi aiming to obtain flavan-4-ols with high enantiomeric excesses (*ee*) of both *cis*- and *trans*-diastereoisomers was reported for the first time.

2. Material and methods

2.1. Reagents, solvents and culture media

The reagents 2-hydroxyacetophenone (99%), benzaldehyde (99%), 3-bromobenzaldehyde (97%), 4-bromobenzaldehyde (99%), 3-chlorobenzaldehyde (97%), 3-fluorobenzaldehyde (97%), 4-fluorobenzaldehyde (98%) and $NaBH_4$ were obtained from Sigma-Aldrich. 4-Methoxybenzaldehyde (98%) was purchased from Vetec. Sodium hydroxide (97%) and hydrochloric acid (37%) were acquired from Quemis (Brazil). The solvents ethyl acetate (PA) and hexane (PA) were purchased from Synth. Chromatographic grade solvents (hexane, methanol and 2-propanol) were obtained from Panreac. All reagents were used without prior purification.

The flash silica gel with pore diameter of 6 nm (0.035–0.070 nm) used in the purifications of the compounds by column chromatography was obtained from Acros Organics. The salts used in the preparation of synthetic seawater were obtained from Synth. Agar and malt extract were purchased from Kasvi. The deuterated solvents, DMSO-d_6 (99.9%) and $CDCl_3$ (99.9%) were purchased from Cambridge Isotope Laboratories. L-proline and anhydrous Na_2SO_4 were obtained from Synth. The sterile and disposable Petri plates were obtained from Olen.

2.2. Synthesis of chalcones *1a-g*

The chalcones 1**a-g** were synthesized in a 125 mL round-bottom flask with 50 mL of ethanol, 2-hydroxyacetophenone (0.01 mol) and benzaldehyde derivatives **a-g** (0.01 mol). Then, 5 mL of NaOH (5 M) was added dropwise, and the reaction mixture was stirred for 12–24 h. The reactions were monitored by Thin Layer Chromatography (TLC) (Macherey-Nagel, 20 × 20 cm, silica gel 60, G / UV 254 nm). After that period, the reaction mixture was transferred to a beaker flask (500 ml), and 5 mL of HCl (10%) was added slowly. The precipitate was filtered and washed 4 times with cold distilled water [31,32]. The yields were determined after purification by column chromatography using hexane and ethyl acetate, ratio of 9.5:0.5 (Fig. 1A).

2.3. Synthesis of (±)-flavanones *2a-g*

In a 25 mL flask, 2′-hydroxychalcones **1a-g** (0.45 mmol), KOH (8 M, 1 mL), L-proline (0.01 g) and distilled water (5.0 mL) were added. The suspension was kept under magnetic stirring at room temperature for 1–24 h. Then, the formed precipitate was collected by filtration, washed

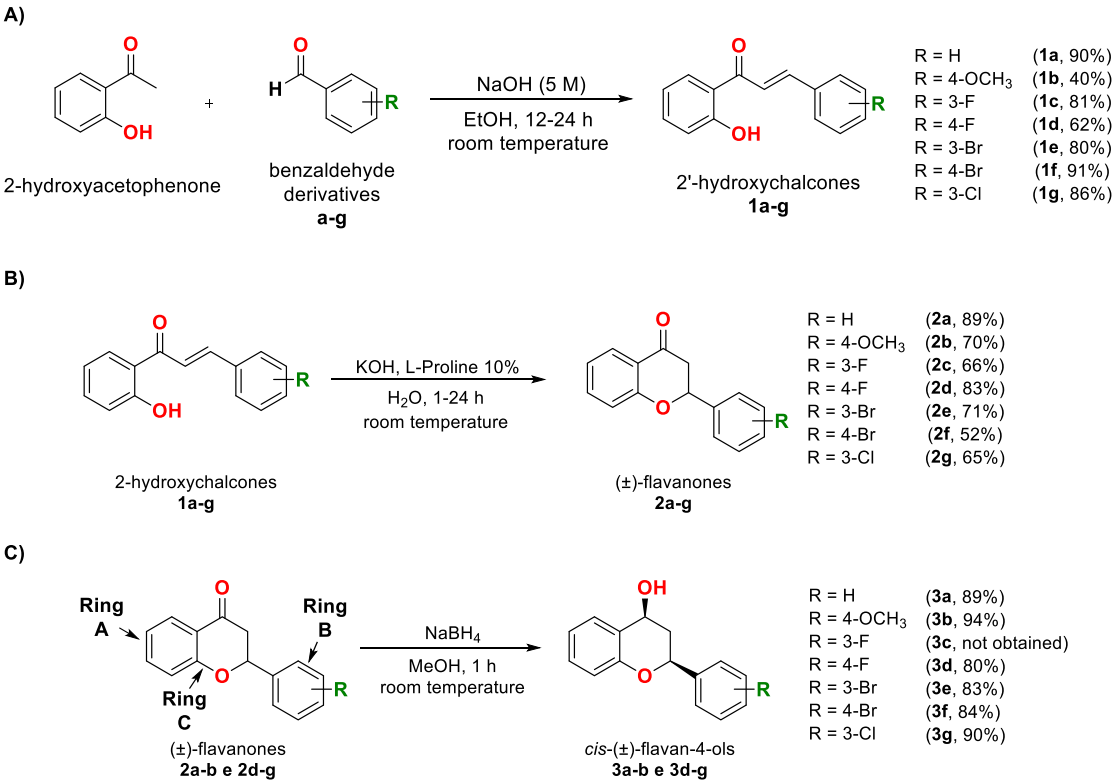


Fig. 1. (A) Synthesis of 2′-hydroxychalcones derivatives **1a-g** by Claisen-Schmidt aldolic condensation; (B) Synthesis of flavanones **2a-g** from 2′-hydroxychalcones in basic medium; (C) Synthesis of flavan-4-ols **3a-g** from the reduction of (±)-flavanones with $NaBH_4$.

with distilled water and dried at room temperature [33]. When necessary, purification was performed by column chromatography using hexane and ethyl acetate (9.5:0.5). Products **2a-g** were characterized by IV, MS, NMR (^{1}H and ^{13}C) and their yields determined (Fig. 1B).

2.4. Synthesis of (±)-flavan-4-ols *3a-g*

In a 25 mL flask, 1 mmol of the flavanone **2a-g** and 10 mL of ethanol were added. The mixture was placed in an ice bath under magnetic stirring for 5 min. Then $NaBH_4$ was added with an excess of 0.5 mmol in relation to (±)-flavanone. The reaction was monitored by TLC until the complete conversion of the reagents. After the reaction, methanol was removed in a rotoevaporator and a solid was obtained. Then, 10 mL of distilled water were added, and a liquid-liquid extraction was performed with ethyl acetate (3 × 10 mL). Anhydrous sodium sulfate was added to the organic phase and the solvent was filtered and evaporated under reduced pressure. Subsequently, the sample was purified by column chromatography, dried and its yield was determined (Fig. 1C). The (±)-flavan-4-ols **3a-b** and **3d-g** were characterized by IR, MS and NMR (^{1}H and ^{13}C). The employed methodological procedure was not suitable for the synthesis of flavan-4-ol **3c**, so, it was not obtained by chemical synthesis. However, **3c** was obtained by biotransformation and characterized.

2.5. Marine-derived fungi

The strains of marine-derived fungi employed for the biotransformation of flavanones **2a-g** were already available at the Group of Organic Chemistry and Biocatalysis (colony images shown in Supporting Material (SM), Item 1). The fungi were isolated from marine sponges and provided by Prof. Roberto G. S. Berlinck (IQSC / USP) and Prof. Mirna H. R. Seleghim (DBE / UFSCar) in previous studies [34–36]. These strains were selected for reduction of flavanones based on good results presented in the biotransformation and biodegradation of xenobiotics described in the literature [37,38].

These strains are deposited in the Brazilian Collection of Microorganisms from the Environment and Industry (CBMAI - http://webdrm.cpqba.unicamp.br/cbmai/, WDCM823), deposited as *Fusarium* sp. CBMAI 1830, *Acremonium* sp. CBMAI 1676, *Aspergillus* sp. CBMAI 1829, *Aspergillus sydowii* CBMAI 935, *Penicillium oxalicum* CBMAI 1996, *Penicillium citrinum* CBMAI 1186, *Penicillium raistrickii* CBMAI 931, *Cladosporium* sp. CBMAI 1237, *Mucor racemosus* CBMAI 847 and *Westerdykella* sp. CBMAI 1679.

2.6. Culture medium

Marine-derived fungi were cultivated in 2% malt agar prepared with 20 g of malt extract and 15 g of Agar added to 1 L of synthetic seawater composed of: $CaCl_2{\bullet}2H_2O$ (1.36 g), $MgCl_2{\bullet}6H_2O$ (9.68 g), KCl (0.61 g), NaCl (30 g), Na_2HPO_4 (0.014 mg), Na_2SO_4 (3.47 g), $NaHCO_3$ (0.17 g), KBr (100 mg), $SrCl_2{\bullet}6H_2O$ (40 mg), and H_3BO_3 (30 mg). The pH was adjusted to 7.0 with KOH 0.5 mol L^{-1}. Then, the medium was sterilized in autoclave for 20 min (121 °C, 1 atm) and poured into disposable Petri plates under aseptic conditions [39].

2.7. Biotransformation reactions by marine fungi

The screening of biocatalysts for biotransformation of **2a** was performed with 10 different strains of marine-derived fungi. Each strain was cultivated in five 250 mL flasks with 100 mL of 2% malt broth covered with cotton plugs. All sets were sterilized in autoclave (121 °C, 15 min, 1 atm) and inoculated with seven slices of microbial inoculum previously prepared in malt agar during 7 days at 32 °C. After growth (32 °C, 130 rpm, 7 days), the cells were harvested by filtration using a Buchner apparatus and employed for biotransformation in two different conditions, A and B.

Condition A: reaction by 2.5 g of wet cells of fungi in 50 mL of malt broth, and Condition B: reaction by 2.5 g of wet cells of fungi in 50 mL of phosphate buffer solution (Na_2HPO_4/KH_2PO_4 0.1 mol L^{-1}, pH 7.0). Both were carried out in sterilized 125 mL Erlenmeyer flasks employing cotton plugs with the addition of 25 mg of **2a-g** dissolved in 400 μL of DMSO. The screening reactions were performed for 7 days, but different periods were tested further.

Control experiments employing each of the fungus strains were performed since the production of compounds with similar structure can occur as natural products. Moreover, experiments in abiotic conditions were carried out to evaluate the occurrence of spontaneous reactions with the employed substrates in the experimental conditions.

2.8. Extraction of the biotransformation reactions

The samples were extracted by the addition of ethyl acetate in the proportion of 1:1 in relation to the amount of reaction medium. Then, the flasks were stirred magnetically for 30 min, and the mixture was centrifuged (10,000 rpm, 20 min) in a Hitachi CR22GIII. The liquid phase (organic phase and aqueous phase) was then transferred to a separating funnel. A liquid-liquid extraction was performed with two following steps by the addition of 25 mL ethyl acetate. After that, the organic phase was collected and anhydrous sodium sulfate was added, then filtered, and the solvent was evaporated under reduced pressure.

2.9. Chromatographic analyses

The Gas Chromatography-Mass Spectrometry analyzes were performed in a Shimadzu GC2010plus coupled to a selective mass detector (Shimadzu MS2010plus) in Electronic Impact mode (E.I., 70 eV) with a DB-5MS column (30 m x 0.25 mm x 0.25 μm, Agilent J & W Advanced) as already described in the literature [32].

High-Performance Liquid Chromatography (HPLC-UV) was performed in a Shimadzu 2010 chromatograph equipped with a CBM-20A controller, LC-20AT pump, DGU-20A5 degasser, SIL-20AHT sampler, CTO-20A oven and SPD-M20A UV–VIS detector. The oven temperature was 40 °C. The analysis for determination of diastereoisomeric ratios (*dr*) of the flavan-4-ols **3a-g** were performed on isocratic reverse mode with a Luna C18 column (0.46 × 25 cm; 5 μm) equipped with a pre-column. The measurements of racemic and/or enantiomerically enriched flavanones **2a-g** and flavon-4-ols **3a-g** were performed using a Chiralcel OD-H column (0.46 × 25 cm; 5 μm) in normal phase and isocratic mode. All methods employed for the analysis of the compounds in C18 column and in the chiral OD-H column are described in SM Item 2.

2.10. Product characterization

The determination of the melting point of the solid compounds was performed using closed capillaries in a Fisatom device (model 431). The absorption spectra in the infrared region were obtained using a Shimadzu IRAffinity-1 spectrometer operating with Fourier Transform. The analyzes were performed using KBr tablets at wavelengths in the region of 400 to 4000 cm^{-1}.

The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) analyzes were performed on an Agilent Technologies 500/54 Premium Shielded or on an Agilent Technologies 400/54 Premium Shielded spectrometer. Deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6) were used to solubilize the samples. Moreover, tetramethylsilane (TMS) was used as reference signal. The chemical shifts were expressed in parts per million (ppm) and referenced to the internal standard TMS and the deuterated solvent used, DMSO-d_6 (δ_H 2.50, δ_C 39.52) and $CDCl_3$ (δ_H 7.26, δ_C 77.16). The coupling constants (*J*) values were reported in Hz.

Optical rotation measurements were performed using a Jasco polarimeter model P-2000 equipped with a sodium lamp ($\lambda = 589$ nm). The measurements were performed at 23 °C using a 1.0 dm long cell.

Samples were diluted in spectroscopic grade chloroform with concentrations expressed in g/100 mL.

The description of compounds characterization is presented in SM Item 3, whereas an additional discussion was presented in SM Item 4 and spectra at SM Item 5. The obtained data was in accordance with the literature [40–48]. To our knowledge, this is the first report of compounds **3e** and **3f**.

3. Results and discussion

3.1. Screening of marine-derived fungi for biotransformation of flavanone 2a

Flavanones possess different biotransformation sites for obtaining interesting products, such as alcohols and hydroxylated compounds [14]. In this sense, a screening was carried out with ten fungi from marine environment for biotransformation employing the substrate **2a** as model. For each strain, the biotransformation was performed in malt medium (Condition A) and phosphate buffer solution 0.1 M pH 7.0 (Condition B).

The analyses of the samples by HPLC-UV and GC–MS revealed that different products were obtained depending on the microorganism employed as biocatalyst, resulting in reactions of reduction, hydroxylation, and cleavage of the heterocyclic ring (Fig. 2a). An abiotic control for spontaneous reactions assessment was prepared in both conditions, and no transformation product was observed after a period of 7 days. Moreover, in the biotic controls, in which the fungi biocatalyst without substrate addition was used, no natural flavonoids were identified by the employed methods. Chromatograms were presented in SM Item 6.

The obtention of dihydrochalcone **4a** by marine-derived fungi was explored in a previous study [32], and hydroxylation reactions for synthesis of dihydroxy-dihydrochalcone **5a** will be further investigated. In this manuscript, the enantioselective reduction of flavanone **2a** to flavan-4-ol **3a** was the main objective.

Flavanone **2a** possess a stereogenic center, and consequently, a pair of (*S*)−**2a** and (*R*)−**2a** enantiomers. Moreover, this compound also presents a pro-stereogenic ketone in its structure, being able to provide a new chirality center in the molecule generating a pair of diastereoisomers and two pairs of enantiomers. The produced flavan-4-ol **3a** can present the (2*R*,4*R*)-*cis*-**3a** and (2*S*,4*S*)-*cis*-**3a** and/or (2*R*,4*S*)-*trans*-**3a** and (2*S*, 4*R*)-*trans*-**3a** stereoisomers, where the ratio of the stereoisomeric products will be determined by the selectivity of the enzymes involved in the reduction process, Fig. 2B.

The screened strains can be classified into three groups according to the products from the biotransformation of flavanone **2a**. In Group I, which resulted in the production of the dihydrochalcone **4a** as unique product, is the fungus *P. raistrickii* CBMAI 931. In Group II were *Fusarium* sp. CBMAI 1830 and *A. sydowii* CBMAI 935 that produced the dihydrochalcone **4a** and dihydroxy-dihydrochalcone **5a**. Most of strains, *Westerdykella* sp. CBMAI 1679, *Acremonium* sp. CBMAI 1676, *Cladosporium* sp. CBMAI 1237, *Aspergillus* sp. CBMAI 1829, *P. oxalicum* CBMAI 1996, *P. citrinum* CBMAI 1186 and *M. racemosus* CBMAI 847 were at Group III, in which flavan-4-ol **3a** was the main product (Table 1). Chromatograms in SM Item 6 Fig. 6.1.

P. raistrickii CBMAI 931 (Group I) promoted the opening of ring C of flavanone **3a** producing dihydrochalcone **4a** in both conditions A and B. In general, the opening of ring C of flavanones leads to the formation of chalcones [49], however, the formation of chalcone **1a** was not observed, probably because of a reduction reaction catalyzed by fungal ene-reductases. Dihydrochalcone **4a** was isolated with 30% yield in condition A.

It is also observed a greater selectivity for the consumption of (*S*)−**2a** for the formation of dihydrochalcone **4a**. After 7 days of reaction, (*R*)−**2a** was obtained with an enantiomeric excess of 57% and 56% *ee* in conditions A and B, respectively. Chiral chromatograms are in SM Item 6 Fig. 6.2. In the literature, the production of dihydrochalcone from flavanone was reported with 13% yield by the bacteria *Gordonia* sp. DSM 44456 [50] The biotransformation reactions by *Fusarium* sp. CBMAI

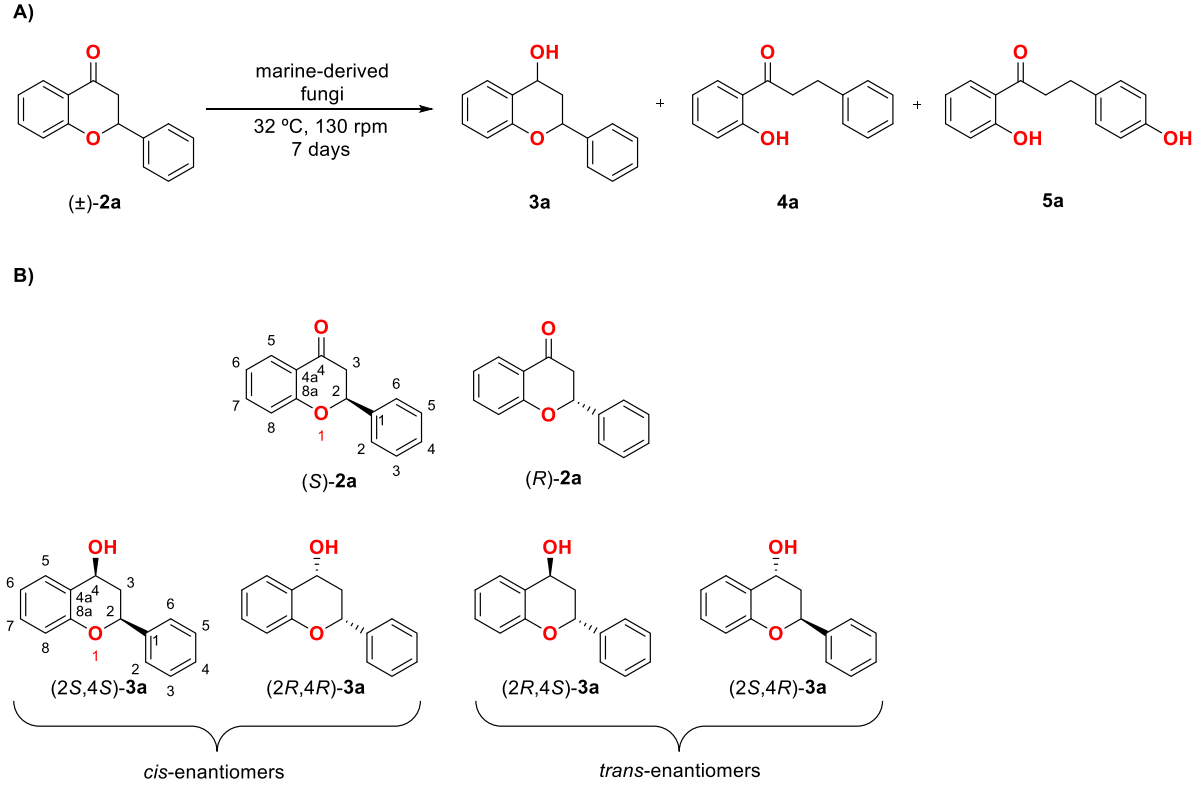
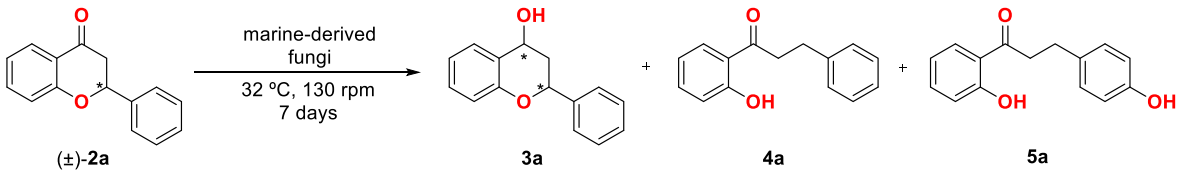


Fig. 2. A) Biotransformation of flavanone **2a** to obtain flavan-4-ol **3a**, dihydrochalcone **4a**, and/or dihydroxy-dihydrochalcone **5a** by fungi from marine environment. B) Structures of the stereoisomers of flavanone **2a** and flavan-4-ol **3a**.

Table 1
Biotransformation of (±)-flavanone **2a** by marine derived fungi (32 °C, 130 rpm, 7 days).



Strain	Condition	Remaining **2a** (%)	*ee* (%)	**3a** (%)	(*cis:trans*)−**3a** *dr*	*ee* (%) of **3a** *cis* for (2*S*,4*S*)	trans	Isolated Yield of **3a**	**4a** (%)	**5a** (%)
Group I										
P. raistrickii CBMAI 931	A	45	57 (*R*)	–	–	–	–		28	–
	B	45	56 (*R*)	–	–	–	–		30	–
Group II										
Fusarium sp. CBMAI 1830	A	57	23 (*R*)	–	–	–	–		6	34
	B	7	12 (*R*)	–	–	–	–		5	64
A. sydowii CBMAI 935	A	10	22 (*R*)	–	–	–	–		3	11
	B	7	–	–	–	–	–		6	30
Group III										
Westerdykella sp. CBMAI 1679	A	14	0	57	82:18	13	49	63%	–	–
	B	39	12 (*R*)	36	72:28	29	43	–	–	–
Acremonium sp. CBMAI 1676	A	28	28 (*R*)	11	85:15	96	98	–	–	–
	B	62	27 (*R*)	22	91:9	97	88	31%	–	–
Cladosporium sp. CBMAI 1237	A	13	91 (*R*)	25	42:58	54	24	–	–	–
	B	11	38 (*R*)	35	44:56	49	24	83%	–	–
Aspergillus sp. CBMAI 1829	A	75	–	8	78:22	–	–	–	–	–
	B	71	–	<2	68:32	–	–	–	–	–
P. oxalicum CBMAI 1996	A	68	2 (*R*)	<2	47:53	–	–	–	–	–
	B	75	1 (*R*)	<2	30:70	–	–	–	–	–
P. citrinum CBMAI 1186	A	72	1 (*R*)	<2	26:74	–	33	–	–	–
	B	76	3 (*R*)	<2	33:67	–	−19	–	–	–
M. racemosus CBMAI 847	A	62	1 (*R*)	<2	22:78	–	23	–	–	–
	B	62	7 (*R*)	6	29:71	–	3	–	–	–

Condition A: biotransformation in malt 2% medium; Condition B: biotransformation in phosphate buffer (Na_2HPO_4/KH_2PO_4 0.1 mol L^{-1}, pH 7.0).

1830 and *A. sydowii* CBMAI 935 led to the formation of two main products, dihydrochalcone **4a** and 2′,4-dihydroxy-dihydrochalcone **5a**. For *Fusarium* sp. CBMAI 1830, (*S*)−**2a** consumption was favored in both experimental conditions, leading to the opening of ring C of flavanone **2a** and hydroxylation of ring B to obtain **4a** and **5a**, respectively. Enantiomeric excesses of 23 and 12% were obtained for unreacted (*R*)−**2a** in conditions A and B, respectively. Chiral chromatograms are in SM Item 6 Fig. 6.3.

Westerdykella sp. CBMAI 1679 promoted the formation of flavan-4-ol **3a** from flavanone (±)−**2a** in a mixture of diastereoisomers. The diastereoisomeric ratio for flavan-4-ol **3a** in condition A was 82:18 (*cis:trans*) and in condition B was 72:28 (*cis:trans*). The enantiomeric excesses in conditions A (13% *ee* for peak 2, 42.4 min, *cis*-**3a**; 49% *ee* for peak 1, 26.2 min, *trans*-**3a**) and B (29% *ee* for peak 2, 42.4 min, and later defined as (2*S*,4*S*)-*cis*-**3a**; 43% *ee* for peak 1, 26.2 min, *trans*-**3a**) were similar. It is important to emphasize that the absolute configuration of the *trans*-**3a** enantiomers was not assigned.

In condition A, *Westerdykella* sp. CBMAI 1679 presented a higher

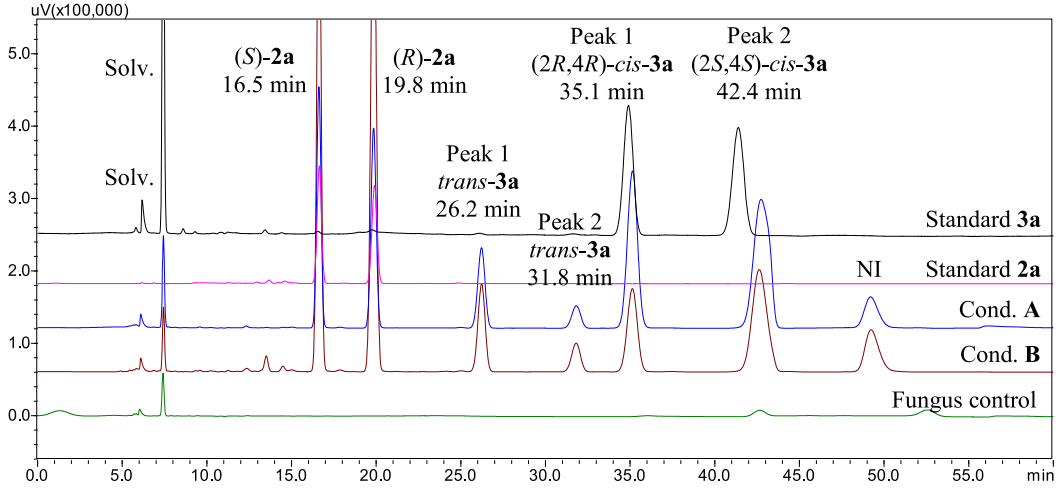


Fig. 3. Chromatograms obtained by HPLC-UV with chiral column for biotransformation by *Westerdykella* sp. CBMAI 1679 (32 °C, 130 rpm, 7 days). Analysis conditions: HPLC-UV with chiral column Chiralcel OD-H (0.46 cm x 25 cm; 5 μm), isocratic elution with hexane and isopropanol (95:5) at a flow of 0.5 mL min^{-1}, total analysis time of 60 min. Chromatograms obtained at wavelength of 215 nm. Retention times: (*S*)−**2a** (RT = 16.5 min), (*R*)−**2a** (RT = 19.8 min), *trans*-**3a** (RT = 26.2 min and 31.8 min), *cis*-**3a** (RT = 35.1 min for 2*R*,4*R*-**3a** and 42.4 min for 2*S*,4*S*-**3a**). Solv. = solvent residue of ethyl acetate (RT = 7.0 min) and isopropanol (RT = 3.8 min). NI = not identified.

conversion of **2a** (c = 83%) than condition B (c = 59%). Possibly, the malt broth provided a higher concentration of enzymes and more effectiveness of the natural regeneration system of cofactors due to the increased availability of nutrients. The flavan-4-ol **3a** formed in the reaction of **2a** was isolated under condition A with a yield of 63% and 49% *ee* for *trans*-**3a** and 13% *ee* for *cis*-**3a**. However, it was not possible to isolate the *cis*-**3a** and *trans*-**3a** diastereoisomers separately by column chromatography because the bands were too close. Therefore, only a mixture of the diastereoisomers was obtained. Chiral chromatograms were presented in Fig. 3.

Although reactions with *Acremonium* sp. CBMAI 1676 presented decreased yield of **3a**, this biocatalyst was more selective than *Westerdykella* sp. CBMAI 1679 and *Cladosporium* sp. CBMAI 1237. The diastereoselectivity of flavan-4-ol **3a** formed in condition A was 85:15 (*cis:trans*), while for condition B was 91:9 (*cis:trans*). For the diastereoisomer *cis*-**3a**, 96% *ee* and 97% *ee* (peak 2, 42.4 mim, later defined as 2*S*,4*S*) were obtained in conditions A and B, respectively. The enantiomeric excesses for the *trans*-**3a** diastereoisomer pair in conditions A and B were 98% *ee* and 88% *ee* (peak 1, 26.2 min), respectively. Flavan-4-ol **3a** was isolated from the reaction at condition A with 31% yield and an enantiomeric excess of 98% for *trans*-**3a** and 96% *ee* for *cis*-**3a**. See chiral chromatograms in SM Item 6 Fig. 6.4.

The unreacted flavanone **2a** was isolated with an enantiomeric excess for (*R*)−**2a** which had $[\alpha]^{23,}{}_{589}$ + 4.2° (*c* 1.00, $CHCl_3$, 28% *ee*) and was designated by comparison with the literature $[\alpha]^{23,}{}_{589}$ + 54.8° (*c* 0.80, $CHCl_3$, 85% *ee*) [51]. The measurement of optical rotation for flavan-4-ol **3a** isolated from the reaction with the fungus *Acremonium* sp. CBMAI 1676 was determined as $[\alpha]^{23,}{}_{589}$ + 8.1° (*c* 1.00, $CHCl_3$, 97% *ee*) and compared with the rotation presented in the literature $[\alpha]^{23,}{}_{589}$ + 59.8 (*c* 1.20, $CHCl_3$, 95% *ee*) for the *cis*-(2*S*,4*S*)−**3a** [51]. In view of the optical rotation values, the diastereoisomeric ratio, the enantiomeric excess, the preferential consumption of (*S*)−**2a** and the fact that this stereogenic center was not the target of the transformation, it can be suggested that the *cis*-(2*S*,4*S*)−**3a** was the major product from the biotransformation of **2a** by the fungus *Acremonium* sp. CBMAI 1676.

The biotransformation of **2a** by *Cladosporium* sp. CBMAI 1237 resulted in the formation of *cis* and *trans* flavan-4-ol **3a** with a discrete selectivity for the *trans*-**3a** diastereoisomer. The *dr* of **3a** was 42:58 (*cis:trans*) in condition A and 44:56 (*cis:trans*) in condition B. In condition A with *Cladosporium* sp. CBMAI 1237 the enantioselectivity for the *cis*-**3a** enantiomer pair was 54% *ee* (peak 2, 42.4 min, 2*S*,4*S*) and for the *trans*-**3a** diastereoisomer was 24% *ee* (peak 1, 26.2 min). In condition B, the enantioselectivity for the *cis*-**3a** enantiomer pair was 49% *ee* (peak 2, 42.4 min, 2*S*,4*S*) and for the *trans*-**3a** enantiomers was 24% *ee* (peak 1, 26.2 min)

Despite having lower diastereoselectivity, condition B showed a conversion of 90%, which was slightly higher when compared to condition A with 87% conversion. Flavan-4-ol **3a** was then isolated from condition B as a mixture of diastereoisomers with 83% yield. Chiral chromatograms are presented in SM Item 6 Fig. 6.5.

In biotransformation reactions using the fungi *Acremonium* sp. CBMAI 1676, *Westerdykella* sp. CBMAI 1679 and *Cladosporium* sp. CBMAI 1237, the formation of the *cis*-**3a** product was predominant. The same pattern was observed in the reactions of synthesis of flavan-4-ols using $NaBH_4$ and methanol, where the product *cis*-(±)−**3a** was obtained in 95:5 in relation to the product *trans*-**3a**.

The major formation of the *cis*-**3a** product suggests that the hydrogen approached by the face with reduced steric impediment. Therefore, the attack must be carried out from the opposite face of the bulkier substituting group (ring B) located in the stereogenic center of flavanone **2a**. Thus, the nucleophilic attack on the carbonyl group of the *R* enantiomer of flavanone occurs through the *Si* face, while the nucleophilic attack on the *S* enantiomer occurs through the *Re* face of the pro-stereogenic ketone (Fig. 4).

The reactions with *Fusarium* sp. CBMAI 1830, *Aspergillus* sp. CBMAI 1829, *A. sydowii* CBMAI 934, *P. citrinum* CBMAI 1186, *M. racemosus* CBMAI 847 and *A. sclerotiorum* CBMAI 849 showed formation of flavan-4-ol **3a** with low conversion. Despite the reduced production of **3a,** the reactions with *P. citrinum* CBMAI 1186 and *M. racemosus* CBMAI 847 presented an interesting characteristic, the formation of the *trans* configuration of flavan-4-ol **3a** was favored in relation to the *cis* configuration. The diastereoisomeric ratio obtained with *M. racemosus* CBMAI 847 were 22:78 and 29:71 (*cis:trans*) in conditions A and B, respectively.

However, the *ee* values of the *trans*-**3a** diastereoisomer were low for both conditions A and B with 27% and 3% (peak 1, 26.2 min), respectively. The determination of *cis*-**3a** *ee* was not possible because of the low **2a** conversion to this diastereoisomer (8–10%). For reaction with *P. citrinum* CBMAI 1186, the diastereoisomeric ratios were 26:74 (*cis:trans*) in condition A and 33:67 (*cis:trans*) in condition B, presenting 33% *ee* (peak 1, 26.2 min) in condition A and 19% *ee* (peak 2, 31.8 min) in condition B for the *trans*-**3a** diastereoisomer.

The biotransformation of flavanone **2a** has been reported in the literature describing the bioreduction by yeasts of the genera *Rhodotorula, Candida* and *Saccharomyces* to form flavan-4-ol **3a** [51]. The authors observed that the bioreduction of the *R* enantiomer of flavanone

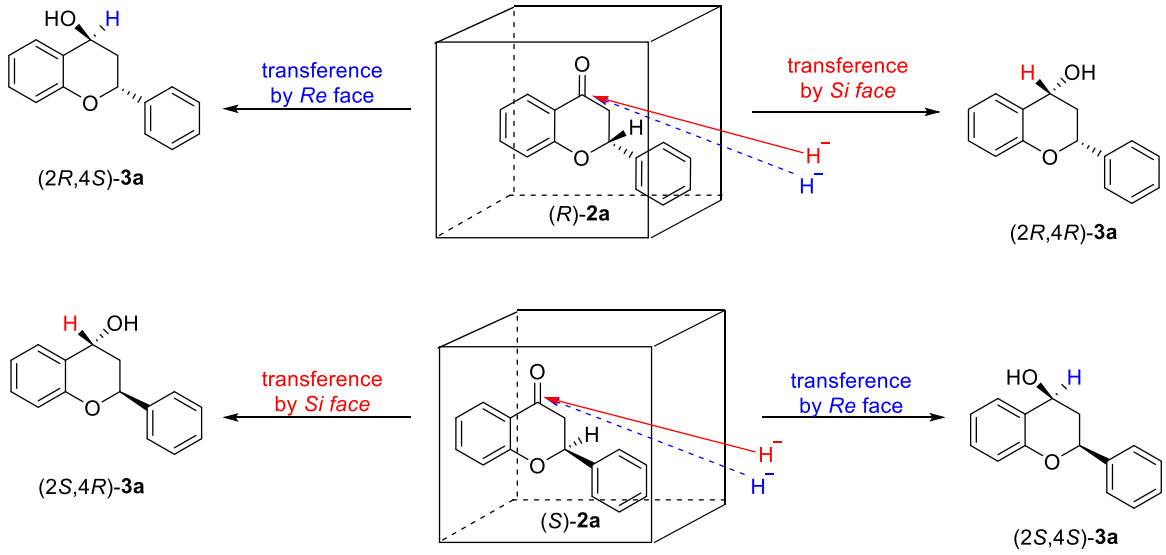


Fig. 4. Representation of the nucleophilic attack of the hydride ion by the *Re* and *Si* faces of the pro-stereogenic ketone of flavanone **2a** to form the flavan-4-ol **3a**.

was favored in most of cases. However, *Rhodotorula* yeasts were more selective for the formation of *trans*-**3a** enantiomers with high enantiomeric excesses (> 99% *ee*) for *trans*-(2*R*,4*S*)−**3a**, while *cis*-**3a** enantiomers showed selectivity for *cis*-(2*S*,4*S*)-flavan-4-ol **3a**.

In the literature, the reactions promoted by fungi of the *Aspergillus* genus in general were selective for the formation of the *cis* enantiomers of flavan-4-ols. The terrestrial fungus *Aspergillus niger* KB reduced the carbonyl group of racemic 7-methoxyflavone and led to the formation of (±)−2,4-*cis*-7-methoxyflavane-4-ol [26]. *A. niger* KB also promoted the biotransformation of flavanone and 6-hydroxyflavanone to obtain (±)-*cis*-flavan-4-ol and (±)−2,4-*cis*-6-hydroxiflavan-4-ol, respectively [25]. Also, the derivatives 6-acetoxy-, 6-propionoxy and 6-butyryloxy-flavanones were hydrolyzed and enantioselective hydroxylated to produce (*S*)−6,4-dihydroxyflavanone [27].

In another example, the natural flavanone Bavachinin, that can be found in species of the genus *Psoralea* (*P. corylifolia, P. corylifoliaos*) and be useful for treatment of asthma and diarrhea, was biotransformed by the terrestrial fungus *P. raistrickii* ATCC 10490 resulting in a high selective reduction for the production of (2*S*,4*R*)−2-(4-hydroxyphenyl)−7-methoxy-6-(3-methylbut-2-en-1-yl)chromen-4-ol with 73% yield [52].

Alcohol dehydrogenases (ADH) are the enzymes responsible for the reduction of aldehydes and ketones to obtain enantiomerically pure or enriched alcohols. ADH enzymes are dependent on cofactors, such as NAD^+ or $NADP^+$, which in their respective reduced form (NADH/NADPH) possess hydride ions that can be transferred to another molecule [53,54]. However, these coenzymes are expensive and therefore must be accompanied of a recycling system using an easily accessible co-substrate, for example, 2-pronanol. Moreover, promiscuous reactions are also a possibility [55].

In the use of whole cells of microorganisms as in this work, the addition of cofactors and/or co-substrates was not necessary, since fungi produce the cofactors and the metabolic pathways for their regeneration, maintaining the enzymatic catalysis that can accept natural and synthetic substrates. Multiple dehydrogenase enzymes can be produced by a living organism, each of them can have different specificities for the substrate enantiomers [56]. Thus, in the biotransformation reactions of **2a** that resulted in both *cis*-**3a** and *trans*-**3a** isomers, it was not possible to establish that a single oxidoreductase was responsible for the transformation of the substrate.

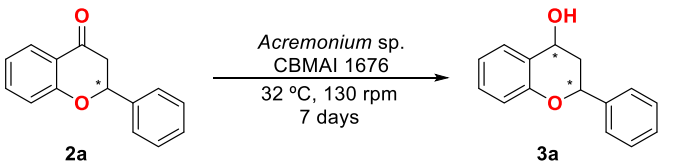
3.2. Biotransformation of **2a** over time by Acremonium sp. CBMAI 1676

The **2a** biotransformation reaction by the fungus *Acremonium* sp. CBMAI 1676 was monitored using the condition A for periods of 1, 3, 5, 7 and 14 days for evaluation of the reaction course and product formation. The consumption of flavanone **2a** and formation of flavan-4-ol **3a** was determined (Table 2).

The biotransformation of **2a** by *Acremonium* sp. CBMAI 1676 promoted the formation of **3a** in the first 24 h with a diastereoisomeric ratio of 95:5 (*cis:trans*) and 99% *ee* for the *cis* and *trans* enantiomers. Over time, there was an increase from 14% to 36% of conversion for 7 days of reaction and the diastereoselectivity remained high during this period. After 14 days, the conversion continued to increase to 47%, but there was a decrease in the diastereoisomeric ratio to 85:15 (*cis:trans*), as well as in the *ee* of the *cis* (96% *ee*) and *trans* (93% *ee*) enantiomers of flavan-4-ol **3a**.

Despite the progressive increase in the conversion of flavanone **2a** in reactions with the fungus *Acremonium* sp. CBMAI 1676, selectivity loss was observed over time. Thus, it was decided to carry out the biotransformation of flavanones **2b-g** by the fungus *Acremonium* sp. CBMAI 1676 in the condition A with 7 days of incubation. The fungus *Cladosporium* sp. CBMAI 1237 was also used in the biotransformation of flavanones **2b-g** because of the high yields determined in the formation of *cis*-**3a** and *trans*-**3a** isomers (*Section 3.1*).

Table 2
Bioreduction of flavanone **2a** by *Acremonium* sp. CBMAI 1676 over time (32 °C, 130 rpm, 1–14 days).



2a **3a**

Time (days)	**2a** c (%) remaining	*ee* (%)	**3a** *dr* (*cis:trans*)	*ee* (%) de **3a** *cis* (2*S*,4*S*)	*trans*
1	86	4 (*R*)	95:5	>99	–
3	80	13 (*R*)	95:5	99	>99
5	70	17 (*R*)	95:5	99	>99
7	64	26 (*R*)	94:5	>99	>99
14	53	27 (*R*)	85:15	96	93

c = remaining **3a** concentration determined by analytical curve using HPLC-UV employing C18 column.
ee = enantiomeric excess determined by chiral HPLC-UV analysis.
dr = diastereoisomeric ratio determined by HPLC-UV analysis employing C18 column.

3.3. Biotransformation of flavanones **2b-g** by Acremonium sp. CBMAI 1676 and Cladosporium sp. CBMAI 1237

Racemic flavanones **2b-g** were submitted to biotransformation with the fungi *Acremonium* sp. CBMAI 1676 and *Cladosporium* sp. CBMAI 1237 for the formation of the respective flavan-4-ols **3b-g**(Table 3). These products were obtained in reactions with the fungus *Cladosporium* sp. CBMAI 1237 with good isolated yields (67–87%), and those obtained from reactions with *Acremonium* sp. CBMAI 1676 presented lower isolated yields (15–46%) but higher enantioselectivities (up to 99% *ee*). Chromatograms are shown in the SM Item 7 Fig. 7.1.

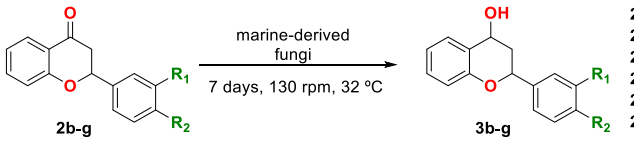
The diastereoisomeric ratios were low in reactions with the fungus *Cladosporium* sp. CBMAI 1237, but the compounds with smaller substituents **2a** (-H), **2c** (F) and **2d** (-F) and **2 g** (-Cl) showed some predominance for the formation of *trans* stereoisomers, while flavanones with bulkier substituents **2b** (–OCH_3), **2e** (Br) and **2f** (-Br) favored the formation of the *cis* stereoisomers. In the literature, a study with whole cells of yeast presented the *cis* preference in substrates with small substituents, but the opposite diastereoselectivity was observed when bulky substituents were tested [57].

Among the flavan-4-ols obtained in the biotransformation reactions promoted by *Cladosporium* sp. CBMAI 1237, flavan-4-ol **3c** showed the highest diastereoselectivity and enantioselectivity with a 35:65 *dr* (*cis:trans*) with 64% *ee* in the *cis*-**3c** and 30% *ee* in the *trans*-**3c**. The reactions with flavanone **2c** using *Acremonium* sp. CBMAI 1676 presented an opposite diastereopreference in flavan-4-ol **3c** 66:34 (*cis: trans*) when compared to that obtained with the fungus *Cladosporium* sp. CBMAI 1237, showing 77% *ee* in the *cis*-**3c** and >99% *ee* in the *trans*-**3c**. Chiral chromatograms for product **3c** are presented in SM Item 7 Fig. 7.2.

The biotransformation reaction of flavanone **2e** employing *Acremonium* sp. CBMAI 1676 showed greater selectivity than by *Cladosporium* sp. CBMAI 1237. Flavan-4-ol **3e** was isolated from the reaction with *Acremonium* sp. CBMAI 1676 as a mixture of diastereoisomers with 15% yield and diastereoisomeric ratio of 81:19 (*cis:trans*). Chiral chromatograms for product **3e** are presented in SM Item 3 Fig. 7.3.

The *trans*-**3e** and *cis*-**3e** diastereoisomers were produced with 97% and 95% *ee*, respectively, highlighting that the enzymes of the fungus *Acremonium* sp. CBMAI 1676 showed selectivity for the transfer of the hydride ion over the *Si* face of flavanone **2e**. Flavan-4-ol **3e** was obtained by the reaction with *Acremonium* sp. CBMAI 1676 with 67% yield and 52:48 diastereoisomeric ratio (*cis:trans*), but with low enantiomeric excesses (<4% *ee*). To our knowledge, this is the first report of compound **3e**.

Table 3
Bioreduction of flavanones **2b-g** promoted by the fungi *Cladosporium* sp. CBMAI 1237 and *Acremonium* sp. CBMAI 1676 (32 °C, 130 rpm, 7 days).



2b and **3b** (R_1 = H; R_2 = OCH_3)
2c and **3c** (R_1 = F; R_2 = H)
2d and **3d** (R_1 = H; R_2 = F)
2e and **3e** (R_1 = Br; R_2 = H)
2f and **3f** (R_1 = H; R_2 = Br)
2g and **3g** (R_1 = Cl; R_2 = H)

Product	Isolated yield (%)	*dr* (*cis:trans*)	*ee* (%)	
			*cis**	*trans***
Cladosporium sp. CBMAI 1237[a]				
3a	83	44:56	49 (peak 2)	24 (peak 1)
3b	87	61:39	–	–
3c	65	35:65	64 (peak 2)	30 (peak 2)
3d	82	42:58	–	–
3e	67	52:48	4	3
3f	77	64:36	22 (peak 2)	29 (peak 2)
3g	72	45:55	4	2
Acremonium sp. CBMAI 1676[b]				
3a	31	85:15	96 (peak 2)	>99 (peak 1)
3b	46	96:4	94 (peak 2)	>99 (peak 2)
3c	44	66:34	77 (peak 2)	>99 (peak 2)
3d	29	76:24	–	–
3e	15	81:19	97 (peak 2)	95 (peak 2)
3f	19	54:46	95 (peak 2)	>99 (peak 2)
3g	25	75:25	93 (peak 2)	97 (peak 2)

Reaction conditions:.
[a] Fungal fungal cells (5 g) and flavanones **3a-g** (50 mg) in phosphate buffer (Na_2HPO_4 / KH_2PO_4 0.07 M, 100 mL, pH 7) incubated in an orbital shaker (32 °C, 130 rpm, 7 days);.
[b] Wet fungal cells (5 g) and flavanones **3a-g** (50 mg) in culture broth (100 mL) incubated on orbital shaker (7 days, 130 rpm, 32 °C).
ee = enantiomeric excess determined by chiral HPLC-UV analysis.
dr = diastereoisomeric ratio determined by HPLC-UV analysis.
4a: *cis** = Peak 2 (42.4 min) and *trans*** = peak-1 (26.2 min); **4c**: *cis** = peak 2 (46.8 min) and *trans*** = peak 2 (28.8 min); **4e**: *cis** = peak 2 (55.6 min) and *trans*** = peak 2 (32.0 min); **4f**: *cis** = peak 2 (52.4 min) and *trans*** = peak 2 (36.4 min); **4 g**: *cis** = peak 2 (45.3 min) and *trans*** = peak 2 (28.7 min).

The bioreduction of flavanone **2f** by *Cladosporium* sp. CBMAI 1237 followed the same behavior observed in the previous biotransformation reactions promoted by this fungus. Flavan-4-ol **3f** was obtained as a mixture of diastereoisomers with 77% yield, 64:36 *dr* (*cis:trans*) and 22% *ee* (peak 2) of *cis*-**3f** and 29% *ee* of *trans*-**3f** diastereoisomer.

A lower selectivity in the formation of diastereoisomers was observed for reactions with *Acremonium* sp. CBMAI 1676 when compared to other flavan-4-ols, obtaining a *dr* of 54:46 (*cis:trans*) with 19% isolated yield. However, enantiomeric selectivity was not affected, as flavan-4-ol **3f** was obtained with > 99% *ee* in the *cis*-(2*S*,4*S*)−**3f** and, 95% *ee* in the *trans*-**3f** diastereoisomer. Chiral chromatograms for product **3f** are presented in SM Item 7 Fig. 7.4. To our knowledge, this is the first report of compound **3f**.

Flavan-4-ols obtained with *Cladosporium* sp. CBMAI 1237 were isolated as a mixture of the two diastereoisomers *cis*-**3g** and *trans*-**3g** in 72% yield. However, flavan-4-ol **3g** showed low diastereoselectivities 45:55 (*cis:trans*) and enantioselectivities (2 and 4% *ee*).

The **2g** biotransformation reaction by *Acremonium* sp. CBMAI 1676 showed a lower isolated yield for **3g** (25%) when compared to that obtained with the fungus *Cladosporium* sp. CBMAI 1237. However, the reaction was more selective in relation to both diastereoselectivity and enantioselectivity. Flavan-4-ol **3g** obtained from reactions with *Acremonium* sp. CBMAI 1676 was isolated as a mixture of *cis* and *trans* diastereoisomers (75:25 *dr*) with high *ees* in the *trans*-**3 g** (97% *ee*) and *cis*-**3g** (93% *ee*). Chiral chromatograms for product **3g** are presented in SM Item 7 Fig. 3.5.

In general, the biotransformation reactions of flavanones **2a-g** by the marine-derived fungus *Cladosporium* sp. CBMAI 1237 resulted in good conversions of the substrates in their respective flavan-4-ols **3a-g** with different diastereoselectivities and enantioselectivities. The low selectivity may be due to the presence of different enzymes capable of carrying out the same reaction, thus, flavan-4-ols **3a-g** were not necessarily produced by the action of a single alcohol dehydrogenase.

This feature is one of the disadvantages of using whole cells of microorganisms, since more than one enzyme can catalyze the same type of transformation. Experiments with prebiotics and lactic bacteria employing hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, hesperetin, and naringenin showed that different classes of enzymes can act on the substrate, promoting ring fission and consumption of the substrate as energy source [58,59].

For products **3c-g,** *Acremonium* sp. CBMAI 1676 presented low diastereoselectivity, differently from the sample of **3a** and **3b**. Therefore, high diastereoselective or diastereopure products of **3c-g** were not obtained, and it was not possible to separate these diastereoisomers by chromatographic column. Consequently, the absolute configuration of the obtained compounds was not assigned. Although optical rotation measurements were presented in SM Item 8.

In summary, different strains of marine-derived fungi biotransformed flavanone **2a** to dihydrochalcone **4a**, 2′,4-dihydroxy-dihydrochalcone **5a** and/or flavan-4-ol **3a** with enantioselectivity and diastereoselectivity, showing the potential of these biocatalysts for reduction of this class of compounds. *Acremonium* sp. CBMAI 1676 and *Cladosporium* sp. CBMAI 1237 reduced flavanones **2a-g** to the respective flavan-4-ols **3a-g** with good yields, enantioselectivity and diastereoselectivty, which were defined by the action of the alcohol dehydrogenases produced by each fungus strain. Indicating that this bioreduction process can be further explored employing enzyme isolation, characterization and genetic engineering.

The fungus *Cladosporium* sp. CBMAI 1237 can be an important source of alcohol dehydrogenases. In addition, *Acremonium* sp. CBMAI 1676 was the most enantioselective biocatalyst of this study presenting values of 99% *ee* for both diastereoisomers of flavanones. Also, the presence of substituents on the substrates did not inhibited the enantioselectivity of the alcohol dehydrogenases present in these biocatalysts. Moreover, the obtained products can be assessed for biological activities in future studies.

4. Conclusion

Fungi derived from the marine environment presented potential for asymmetric reduction of flavanones with a pro-stereogenic center. The employed strains reduced flavanones **2a-g** with *Cladosporium* sp. CBMAI 1237 presenting high yields (about 80%), and *Acremonium* sp. CBMAI 1676 resulting in up to 99% *ee* in the *cis* and *trans* diastereoisomers. The use of fungi, with emphasis for these marine-derived strains, was an interesting approach for enantioselective reduction of halogenated flavanones. To our knowledge, this was the first report of brominated flavan-4-ols. Therefore, this strategy can be important for obtaining enantioenriched compounds with biological activities.

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CRediT authorship contribution statement

Iara L. de Matos: Conceptualization, Methodology, Validation, Software, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Willian G. Birolli:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization. **Darlisson de A. Santos:** Methodology, Writing – review & editing. **Marcia Nitschke:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **André Luiz M. Porto:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mcat.2021.111734.

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