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Biocatalyzed asymmetric reduction of benzils to either benzoins or hydrobenzoins: pH dependent switch†

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Enantiopure benzoins and hydrobenzoins are precursors of various pharmaceuticals and biologically active compounds. In addition, hydrobenzoins are precursors of chiral ligands and auxiliaries in stereoselective organic synthesis. Biocatalytic reduction of benzils is a straightforward approach to prepare these molecules. However, known methods are not selective and lead to formation of a mixture of benzoin and hydrobenzoin, requiring expensive separation procedures. Here, we describe an enzyme system *Talaromyces flavus*, which exhibited excellent pH dependent selectivity for the conversion of benzil to either benzoin or hydrobenzion in high ee. Thus, (*S*)-benzoin was the exclusive product at pH 5.0 (ee >99%), whereas at pH 7.0, (*S*,*S*)-hydrobenzoin (ee >99%, *dl*/*meso* 97 : 3) was the exclusive product. The observed pH dependent selectivity was shown to be due to the presence of multiple enzymes in *Talaromyces flavus*, which specifically accepted either benzil or benzoin as a substrate and exhibited different pH profiles of their activity. The biocatalyst efficiently reduced a variety of symmetrical and unsymmetrical benzils. Moreover, a 36.4 kDa benzoin reductase was purified, the N-terminal sequence of which did not show a significant similarity to any of the known reductase/dehydrogenase in the database. The protein therefore appears to be a novel reductase.

Introduction

Enantiopure benzoins (α-hydroxyketones) are important intermediates for various pharmaceuticals, such as antifungal agents, antitumor antibiotics (olivomycin A, chromomycin A3, and epothilones),[1] selective inhibitors of amyloid-β-protein (for Alzheimer's disease treatment),[2] farnesyltransferase inhibitors kurasoin A and B,[3] antidepressants (bupropion and its metabolites),[4] urease inhibitors[5] and heterocycles.[6] In addition, these are very interesting molecules from a synthetic chemistry point of view as these are precursors of amino alcohols and 1,2-diols, which in turn are precursors of various biologically active compounds as well as chiral ligands and auxiliaries in stereoselective organic synthesis. Because of the importance of α-hydroxyketones in organic synthesis and as intermediates of various bioactive molecules, many chemical approaches have been developed for their synthesis. These include α-hydroxylation of ketones and Sharpless asymmetric dihydroxylation of the silyl enol ether of the corresponding ketone,[7] ketohydroxylation of olefins,[8] asymmetric monooxidation of 1,2-diols,[9] oxidative kinetic resolution of racemic α-hydroxyketones,[10,11] organocatalytic strategies involving asymmetric α-oxygenation of ketones in the presence of proline or alanine[12,13] and traditional benzoin condensation carried out stereoselectively by means of optically active catalysts such as chiral thiazolium and triazolium salts, in a biomimetic fashion.[14,15] These reported chemical methods are successful in producing a variety of molecules but suffer from drawbacks, such as large number of steps, rare high ee, low overall yields and too much waste.

Biocatalytic ketone reduction combined with economic and environmentally friendly reaction conditions is an effective strategy to overcome these limitations.[16–20] *Penicillium claviforme*,[21] *Pichia glucozyma*,[22] *Aspergillus oryzae*, *Fusarium roseum*,[23] *Bacillus cereus*[24] and *Xanthomonas oryzae*[25] have been used for the preparation of benzoins by asymmetric reduction of benzils. *Cryptococcus macerans*,[26] yeast strains, *Saccharomyces uvarum*, *Saccharomyces montanus*[27] and

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† Electronic supplementary information (ESI) available: Fig. S1–3 showing determination of **2a**/**3a** ratios, *dl*/*meso* ratios and ee, Fig. S4 showing the fractionation scheme for proteins of *T. flavus*, Fig. S5–7 showing the protein purification profile on various columns and Table S1 showing the summary of protein purification steps. Tables S2–4 showing HPLC traces of racemic and biocatalyzed benzoins and hydrobenzoins. NMR images of all relevant compounds. See DOI: 10.1039/c5cy00158g

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Rhizopus sp.[28] and alcohol dehydrogenases from *Thermoanaerobacter* sp., *Lactobacillus brevis* and *Ralstonia* sp.[29] have been used for enantio- and diastereo-selective synthesis of hydrobenzoins.

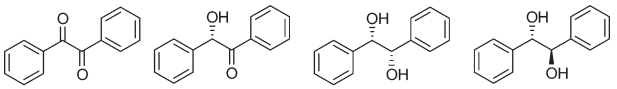
In principle, chiral benzoins as well as chiral hydrobenzoins are easily accessible *via* biocatalytic reduction of the corresponding benzils. However, there is a dearth of biocatalysts which accept such bulky–bulky substrates.[29] Moreover, known biocatalysts are not selective and bring about the formation of a mixture of benzoin and hydrobenzoin, requiring expensive separation procedures, which hampers the acceptability of these biocatalysts for application in technical scale preparations.

Herein, we describe an enzyme system *Talaromyces flavus*, which exhibited excellent pH dependent selectivity for the conversion of benzil to either benzoin or hydrobenzion in high ee. Thus, (*S*)-benzoin was the exclusive product at pH 5.0 (ee >99%), whereas at pH 7.0, (*S*,*S*)-hydrobenzoin (ee >99%, *dl*/*meso* 97/3) was the exclusive product. We have demonstrated that the pH dependent selectivity of *T. flavus* is due to the presence of multiple enzymes in the system, which specifically accepted either benzil or benzoin as a substrate and exhibited different pH profiles of their activity. The biocatalyst *T. flavus* accepted a variety of symmetrical and unsymmetrical benzils as substrates. Moreover, a 36.4 kDa benzoin reductase was purified from *T. flavus.* The N-terminal sequence obtained from the purified protein by Edman degradation did not show a significant similarity to any of the known reductases/dehydrogenases in the database. The protein therefore appears to be a novel reductase.

Results and discussion

Strain selection

Enantiopure benzoins and hydrobenzoins are intermediates for various bioactive molecules. They also serve as chiral ligands in stereoselective organic synthesis. In our previous report, we have described three biocatalysts *Penicillium* sp. MTCC 10832, *Alternaria alternata* MTCC 10833 and *Talaromyces flavus* MTCC 10834 for enantioselective reduction of methyl heteroaryl and aryl heteroaryl ketones to their corresponding (*S*)-heteroaryl alcohols in >99% ee.[30] We tested these strains for enantioselective reduction of benzil (**1a**). The fungal strains were grown as described in the experimental section. The cells were isolated by centrifugation, washed with phosphate buffer (0.2 M, pH 7.0) and resuspended in the same buffer at a concentration of 100 g L^{-1}. Benzil (**1a**; 50 mg, 0.24 mmol) was added to a suspension of 5 g of cells in 50 mL of phosphate buffer and the contents were shaken at 200 rpm and 30 °C until complete consumption of the substrate occurred. The progress of the reaction was monitored by TLC using a commercially available standard sample of *rac*-benzoin (**2a**). At 100% conversion, cells were removed from the reaction mixture by centrifugation and the contents were extracted in ethyl acetate. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate and solvents evaporated under reduced pressure to yield a residue which was analysed by ^{1}H NMR. All the three strains were able to reduce **1a** to a mixture of benzoin (**2a**) and hydrobenzoin (**3a**) in varying ratios (Table 1). The ratio of **2a**/**3a** was determined by ^{1}H NMR (Fig. S1, ESI†). The single methine proton of pure *rac*-**2a** appeared as a bs (broad singlet) at δ 5.9. Hydrobenzoin **3a** prepared by sodium borohydride reduction of **2a** showed two bs at δ 4.7 and 4.8, corresponding to two methine protons of the *dl* and *meso* pairs. The ratio of **2a**/**3a** of the biocatalyzed product in the reaction mixture was determined from integral values of the resonance peaks at δ 5.9, 4.7 and 4.8. The ee of **2a** was determined by chiral HPLC using a Chiralcel OD-H column. Elution was carried out with hexane : isopropanol (90/10) at a flow rate of 1 ml min^{-1}. Detection was performed at λ_{254}. In contrast to two enantiomers of *rac*-**2a** eluted at 11.9 and 17.4 min, the biocatalyzed product showed the predominant presence of one enantiomer eluted at about 11.9 min (Fig. S2, ESI†). Ee was calculated from the relative area under the two peaks. *T. flavus* gave an ee of >99%, whereas *Penicillium* sp. and *A. alternata* gave ees of 95% and 91%, respectively. The absolute configuration of the *T. flavus* catalyzed product **2a** was assigned as (*S*) based on the comparison of the sign of optical rotation ($[\alpha]_D^{25}$ = +115.2 (c 1.5, acetone)) with that in the literature (lit. ref. 31, $[\alpha]_D^{25}$ = +115.0 (c 1.5, acetone)). Accordingly, the enantiomer eluted at 11.9 min by HPLC of (*S*)-**2a** corresponds to an (*S*)-enantiomer and that at 17.4 min to an (*R*)-enantiomer.



1a (*S*)-**2a** (*S*,*S*)-**3a** (meso)-**3a**

The *dl*/*meso* ratio of **3a** was determined by HPLC and ^{1}H NMR. A sample of *dl*/*meso*-**3a** prepared by sodium borohydride reduction of **2a** on a chiral HPLC using a Chiralcel OJ column was resolved into three peaks with retention times of 16.2, 18.8 and 22.6 min (Fig. S3, ESI†). The peaks at 16.2 and 18.8 min appeared in an almost 1 : 1 ratio and were assigned to the *dl* pair of **3a**. The peak eluting at 22.6 min was therefore assigned to *meso*-**3a**. The ratio of *dl*/*meso* in the borohydride reaction was found to be 5 : 95, indicating the predominant formation of the *meso* product in the reaction. A similar ratio was also obtained by comparison of integral values of the resonance peaks at δ 4.84 and 4.72, corresponding to methine protons of the *meso* and *dl* pairs, respectively, in the ^{1}H NMR spectra of **3a** obtained by borohydride reduction of **2a** (Fig. S1, ESI†). Thus, the *dl*/*meso* ratio in all subsequent studies was calculated by ^{1}H NMR. In contrast to the predominant formation of the *meso* product in borohydride reduction, the biocatalyzed reduction produced *dl* as the major product. The ratios of *dl*/*meso* obtained from *Penicillium* sp., *A. alternata* and *T. flavus* were 65/35, 94/6 and 80/20, respectively. The ee of the *dl* pair of **3a** was determined by chiral HPLC using a Chiralcel OJ column (Fig. S3, ESI†). *T. flavus*

Table 1 Biocatalyzed reduction of benzil

Entry	Strain	Conv.[b] (%)	Time (h)	2a : 3a[a]	(*S*)-2a[b,c] Ee (%)	(*S*,*S*)-3a[b,c] *dl*/*meso*[b]	Ee (%)
1	*Penicillium* sp.	100	24	1 : 0.21	95	65/35	93
2	*A. alternata*	100	18	1 : 1.53	91	94/6	85
3	*T. flavus*	100	37	1 : 0.94	>99	80/20	99

[a] The conversion, ratio of **2a** : **3a** and *dl*/*meso* ratio were determined by ^{1}H NMR based on integral values of specified peaks. [b] Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Daicel, Japan) columns, respectively. [c] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature.

gave an ee of 99% while ees of 93% and 85% were obtained in the case of *Penicillium* sp. and *A. alternata*, respectively. The absolute configuration of *T. flavus* catalyzed **3a** was assigned as 1*S*,2*S* based on the sign of optical rotation $[\alpha]_D^{25}$ = −91.0 (c 1.05, ethanol) [lit. ref. 32 and 33, $[\alpha]_D^{25}$ = +91.6 (c 1.05, ethanol), 99.9% ee (*R*,*R*)]. Thus, in the *dl* pair, the peak eluting at 16.2 min corresponds to a (1*S*,2*S*) configuration.

Exclusive preparation of (*S*)-benzoin and (*S*,*S*)-hydrobenzoin with *T. flavus*: pH dependent reduction of benzil

T. flavus was found to be the best biocatalyst for the enantioselective preparation of (*S*)-benzoin and (*S*,*S*)-hydrobenzoin with >99% ee. However, it produced a mixture of **2a** and **3a**, which limits its scope for the preparation of these molecules in high chemical purity. Demir *et al.* have reported an interesting effect of pH on *Rhizopus oryzae* catalyzed reduction of benzil/benzoin to give (*R*)-**2a** (>99% ee) at pH 6.5–8.5 and (*S*)-**2a** (80% ee) at pH 4.2–5.0 in 5–6 days. Continuation of the reaction for 21 days at pH 6.5–8.5 resulted in the formation of **3a** with a *dl*/*meso* ratio of 99/1 and an ee of >99%.[28,34] Although a high ee was observed, this method suffered from low yields and very long reaction time. Also, the reactions were carried out with a growing culture, where media components and secondary metabolites are likely to contaminate the product. Moreover, no attempt was made to provide a rationale for the effect of pH on the course of reaction.

In view of these observations, we studied the effect of pH on *T. flavus* catalyzed asymmetric reduction of **1a**. Biocatalytic reduction of **1a** was carried out from pH 4.0–9.0 as

Table 2 pH dependent asymmetric reduction of benzil by *Talaromyces flavus*

Entry	pH	Time (h)	Conv.[b] (%)	2a : 3a[a]	ee[b,c] (%) (2a) (config)	*dl* : *meso*[a] (3a)	Ee[b,c] (%) (3a) (config)
1	4.0	14	22	1 : 0	97 (*S*)	n.d.	n.d.
2		37	29	1 : 0	97 (*S*)	n.d.	n.d.
3		144	90	1 : 0.025	86 (*S*)	33/67	33.3 (*R*,*R*)
4		264	100	1 : 0.08	70 (*S*)	25/75	45.9 (*R*,*R*)
5	5.0	14	66	1 : 0	97 (*S*)	n.d.	n.d.
6		**37**	**100**	**1 : 0.035**	**99 (*S*)**	**43/57**	**91 (*S*,*S*)**
7		144	100	1 : 0.94	96 (*S*)	47/53	2.5 (*S*,*S*)
8		264	100	1 : 1.3	74 (*S*)	52/48	35 (*R*,*R*)
9	6.0	14	80	1 : 0.07	>99 (*S*)	56/44	>**99** (*S*,*S*)
10		37	100	1 : 0.393	>99 (*S*)	75/25	>99 (*S*,*S*)
11		144	100	1 : 1.2	96 (*S*)	88/12	>99 (*S*,*S*)
12		264	100	1 : 1.6	78 (*S*)	88/12	97.2 (*S*,*S*)
13	7.0	14	50	1 : 0.392	>99 (*S*)	80/20	>99 (*S*,*S*)
14		37	100	1 : 0.94	>99 (*S*)	80/20	>99 (*S*,*S*)
15		144	100	1 : 1.76	82 (*S*)	82/18	>99 (*S*,*S*)
16		264	100	1 : 2.14	81 (*S*)	84/16	97.6 (*S*,*S*)
17[d]		**18**	**100**	**0 : 1**	—	**97/3**	**>99 (*S*,*S*)**
18	8.0	14	65	1 : 0.174	>99 (*S*)	77/23	>99 (*S*,*S*)
19		37	100	1 : 0.441	>99 (*S*)	78/22	>99 (*S*,*S*)
20		144	100	1 : 1.07	80 (*S*)	83/17	96.2 (*S*,*S*)
21		264	100	1 : 1.26	76 (*S*)	88/12	63.7 (*S*,*S*)
22	9.0	14	65	1 : 0.25	>99 (*S*)	85/15	>99 (*S*,*S*)
23		37	98	1 : 0.51	>99 (*S*)	80/20	96 (*S*,*S*)
24		144	100	1 : 0.95	76 (*S*)	80/30	93.7 (*S*,*S*)
25		264	100	1 : 1	71 (*S*)	86/14	78.2 (*S*,*S*)

[a] The % conversion, ratio of **2a** : **3a** and *dl*/*meso* ratio were determined by ^{1}H NMR based on integral values of specified peaks. [b] Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Daicel, Japan) columns, respectively. [c] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature. [d] Substrate concentration was 2 mM in this case. n.d. = not detected.

described in the experimental section using appropriate buffers. Aliquots were drawn at 14, 37, 144 and 264 h and analyzed for % conversion, product ratio and ee determination as described above for strain selection. The results are summarized in Table 2. It was observed that (i) at pH 4.0–6.0, (*S*)-**2a** was the major product in high ee at 100% conversion (entries 4, 6, and 10; Table 2). (ii) The amount of **3a** increased with increasing pH. At pH 7.0, an almost 1 : 1 ratio of **2a** to **3a** was observed at 100% conversion in 37 h. While the ee of (*S*)-**2a** was >99%, **3a** was also produced in >99% ee (entry 14; Table 2). (iii) A decrease in the ee of (*S*)-**2a** was observed at pH 5.0 to 9.0 when the reaction was continued for longer duration and (iv) at pH 4.0 and 5.0, not only a diol was formed with a low ratio, but also a low *dl*/*meso* ratio was obtained. Also, the diol was formed in low ee. The *dl* product had an (*R*,*R*) configuration, whereas, at pH 6.0–8.0, diol **3a** was produced in high ee and had an (*S*,*S*) configuration.

In summary, pH 5.0 was found to be optimal for production of (*S*)-**2a** (entry 6; Table 2). Similarly, pH 7.0 was found to be optimal for production of (*S*,*S*)-**3a** (entry 15; Table 2). Next, we reduced the substrate concentration from about 5 mM to 2 mM. We were pleased to note that at pH 7.0 and a substrate concentration of 2 mM, (*S*,*S*)-**3a** was obtained as the exclusive product in a *dl*/*meso* ratio of 97/3 and >99% ee (entry 17; Table 2). Thus, depending on the pH and concentration, either (*S*)-**2a** or (*S*,*S*)-**3a** can be selectively obtained in high ee and high *dl*/*meso* ratio. In contrast to *Rhizopus oryzae*, we did not observe pH dependent complete reversal of enantioselectivity with *T. flavus*. However, we did observe a drop in the ee of (*S*)-**2a** when the reaction was continued for a longer period of 144–264 h. Optically pure (*S*)-**2a** did not show any racemization at pH 4.0 to 9.0 in the absence of the biocatalyst even after prolonged incubation for 264 h, indicating the presence of racemase activity in *T. flavus.*

We also studied the reduction of *rac*-**2a** with *T. flavus* as the biocatalyst at various pH values in the range of 4.0–9.0 in appropriate buffers. The results are summarized in Table 3. The following observations may be highlighted: (i) whereas no reaction occurred at pH 4.0, the reaction at pH 5.0 was very sluggish. (ii) At pH 6.0–9.0 and about 50% conversion, (*S*)-**2a** was recovered in ~30% ee and **3a** was obtained in a *dl*/*meso* ratio of approximately 3/7 (entries 5, 8, 11, and 14; Table 3). These results suggest that both (*S*)- and (*R*)-**2a** are substrates for the diol reductase enzyme, but the (*R*)-enantiomer reacts faster than the (*S*)-enantiomer, which made partial resolution of *rac*-**2a** possible at about 50% conversion. (iii) It was interesting to note that after 24 h at pH 7.0–9.0, almost complete conversion of **2a** to **3a** occurred to give a *dl*/*meso* ratio of 3 : 7 (entries 9, 12, and 15; Table 3). However, upon continuation of the reaction for 96 h, the ratio of *dl*/*meso* changed to 1 : 1; the enantiomeric excess of the *dl* form increased to 96–98% (entries 7, 10, 13, and 16; Table 3). Continuation of the reaction for longer times did not result in any further change in the *dl*/*meso* ratio of **3a**. These results may be explained by the presence of oxidase activity, which converted *meso*-**3a** to **2a** which was re-reduced to (*S*,*S*)-hydrobenzoin.

pH dependent selectivity of *T. flavus* due to the presence of multiple dehydrogenases

T. flavus catalyzed bioreduction of **1a** resulted in the formation of (*S*)-**2a** in >99% ee at pH 5.0 and (*S*,*S*)-**3a** in >99% ee at pH 7.0. In principle, these results may be explained by applying a single enzyme which has pH dependent substrate specificity or by applying a two-enzyme system, each of which has a different pH profile of activity. In addition to the above pH dependent selectivity, conversion of *meso*-**3a** to *dl*-**3a** was also observed, which could be explained based on the oxidation of a hydroxyl group to the keto group to generate **2a** as the intermediate. Put together, these results indicate the presence of more than one reductase in *T. flavus*, which are

Table 3 pH dependent asymmetric reduction of *rac*-benzoin (**2a**) by *Talaromyces flavus*

Entry	pH	Time (h)	Conv.[a] (%)	Ee[b,c] (%) (residual (*S*)-**2a**)	*dl* : *meso* of **3a**[a]	Ee[b,c] (%) ((*S*,*S*)-**3a**)
1	4	24	0	n.d.	n.d.	n.d.
2		96	0	n.d.	n.d.	n.d.
3	5	24	33	15	26/74	78.5
4		96	48	22	30/70	85
5	6	12	50	32	30/70	86
6		24	100	—	23/77	93.5
7		**96**	**100**	—	**50/50**	**97.9**
8	7	12	48	30	28/72	84.6
9		24	92	—	30/70	95.4
10		**96**	**100**	—	**50/50**	**98.5**
11	8	12	53	29	26/64	85.9
12		24	100	—	28/72	93.9
13		**96**	**100**	—	**47/53**	**96.7**
14	9	12	47	31	26/64	83.8
15		24	100	—	28/72	94.2
16		96	100	—	50/50	95.6

[a] The % conversion and *dl*/*meso* ratio were determined by ^{1}H NMR based on integral values of specified peaks. [b] Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Daicel, Japan) columns, respectively. [c] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature. n.d. = not detected.

involved in the reduction of **1a**. Thus, we fractionated proteins of *T. flavus* in an attempt to isolate proteins with various reductase activities.

The cell-free extract of *T. flavus*, designated as fraction F1 (see Fig. S4, ESI†) was obtained by disruption of cells in Dyno Mill (KDL, Switzerland). *T. flavus* was mixed with glass beads (0.5–0.75 μm) in a 1 : 1 ratio (wt/wt) and disruption was conducted at 4 °C for 15 min followed by centrifugation at 20 000*g* for 20 min at 4 °C to remove cell debris. The cell-free extract was subjected to ammonium sulfate fractionation, maintaining the pH and temperature constant at 7.0 and 4 °C, respectively. Total activity was present in 40–80% ammonium sulphate pellets. The protein was redissolved in phosphate buffer (10 mM, pH 8.0) and desalted using a Sephadex G-25 column. The desalted protein was loaded into a reactive red column. The column was washed with the same buffer and the unbound protein fraction was assayed for activity. The bound proteins were eluted with a 0–1.5 M NaCl gradient and different fractions were assayed for activity. Both unbound and bound fractions of proteins were found to be active. The active fractions from the eluent were combined and subjected to ion-exchange chromatography over a Q-sepharose column followed by size-exclusion chromatography on a Superdex S-200 column. The active protein thus obtained exhibited only one homogeneous band at ~40 kDa in SDS-PAGE, run under the reducing conditions (Fig. 1b). It was designated as fraction F3 (Fig. S4, ESI†). Approximately 20-fold purification was achieved in a 5-step purification procedure (see Table S1 and Fig. S5–7 in the ESI† for the summary of protein purification steps and purification profiles on various columns).

The pure protein fraction (F3) showed a specific activity of 4.75 μmol per min per mg of protein. The purified protein was subjected to MALDI analysis. The MALDI of the native protein gave a molecular weight of ~36.4 kDa. Glycosylation is a key feature of post-translational modification for protein stability, secretion and activity in fungi.[35] Accordingly, glycosylation of the *T. flavus* enzyme reported herein is possible, but its investigation was considered beyond the scope of this study. The partial N-terminal amino acid sequence was determined by using an automated Edman degradation procedure. The N-terminal amino acid sequence was determined after blotting the enzyme onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot cell. The obtained N-terminal sequence, APTNQ_AYDHSV, was then searched for homologous counterparts using the BLAST server at www.ncbi.nlm.nih.gov. The retrieved hits for the N-terminal sequence did not show significant homology to any of the known benzil/benzoin reductases or dehydrogenases in the database. Therefore, the protein appears to be a novel reductase.

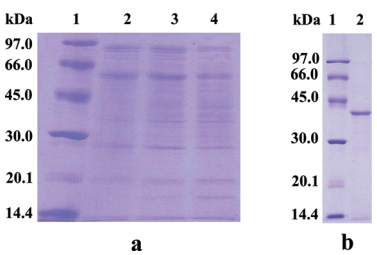


Fig. 1 SDS-PAGE of fractions F2 and F3 run under reducing conditions: (a) fraction 2; lane 1: molecular wt. markers, lanes 2, 3 and 4: different fractions from the size exclusion chromatography step and (b) fraction 3; lane 1: molecular wt. markers, lane 2: purified protein.

Similarly, the unbound protein fraction of the reactive red step was also subjected to ion-exchange chromatography over Q-sepharose followed by size-exclusion chromatography on a Superdex S-200 column. The active fractions were pooled and designated as fraction F2. However, a homogeneous preparation could not be obtained in this case as revealed by SDS-PAGE of F2 run under reducing conditions (Fig. 1a). The summary of fractionation of proteins of *T. flavus* is shown in Fig. S4, ESI.†

The cell-free extract (F1), partially purified protein (F2) and purified protein (F3) were studied for asymmetric reduction of **1a**, *rac*-**2a** and (*S*)-**2a** at pH 5.0 and 7.0 as described in the experimental section. The results are summarized in Table 4. The presence of the following enzyme activities was observed: *(i) (S)-selective benzil reductase activity*: fraction F2 converted **1a** to (*S*)-**2a** at pH 5.0 as well as pH 7.0 (entries 6 and 7; Table 4). No trace of hydrobenzoin (**3a**) could be detected in these reactions, which clearly shows that F2 is devoid of any benzoin reductase activity. However, a lower ee of 91% was obtained with F2 compared to 99% obtained with whole cells at pH 5.0. The improved ee in whole cells could be due to the conversion of (*R*)-**2a** to *meso*-**3a** by benzoin reductase activity present in whole cells, but absent in fraction F2; *(ii) (R,R)-selective benzoin reductase activity*: the pure protein (F3) showed (*R*,*R*)-selectivity for reduction of **1a** at pH 7.0 (entries 9 and 10; Table 4). F3 produced only (*R*,*R*)-**3a** and no trace of (*S*,*S*)-**3a** or *meso*-**3a** could be detected either by NMR or HPLC. However, the conversion rate for this enzyme was very low compared to that for whole cells, requiring 24 h for 25% conversion. Also, the (*R*,*R*)-selectivity was not strict, because when (*S*)-**2a** was used as the substrate, (*S*,*S*)-**3a** was produced in 53% ee at 50% conversion (entry 11; Table 4). F3 did not show any appreciable activity at pH 5.0; *(iii) (S)-benzoin to (R)-benzoin epimerase activity*: when the pure protein fraction F3 catalyzed conversion of (*S*)-**2a** (>99% ee) to **3a** was stopped at 50% conversion, the ee of the remaining (*S*)-**2a** was found to be only 32% (entry 11; Table 4). This clearly suggested that the pure protein in addition to benzoin reductase activity also has *S*-to-*R* epimerase activity. However, the conversion rate for this activity was also very low; *(iv) (S,S)-selective benzoin reductase activity*: the whole cell system of *T. flavus* contains a very high (*S*,*S*)-selective benzoin reductase activity at pH 7.0, but not at pH 5.0. However, this activity could not be recovered from any of the fractions except F1 (entry 5; Table 4), which showed considerably lower activity compared to the whole cell system, indicating that the enzyme responsible for this activity is unstable outside the

Table 4 Asymmetric reduction of benzil (**1a**) and benzoin (**2a**) with protein fractions of *Talaromyces flavus*

			Benzil (**1a**) as substrate				Benzoin (**2a**) as substrate[a]			
Entry	Fraction	pH	Conv.[b] (%)	Ee[c,d] (%) (**2a**) (config)	% **3a** (*dl*/*meso*)[b]	Ee[c,d] (%) (*dl*-**3a**) (config)	Conv.[b] (%)	Ee[c,d] (%) (**2a**) (config)	% **3a** (*dl*/*meso*)[b]	Ee[c,d] (%) (*dl*-**3a**) (config)
1	Whole cells	5.0	100	99 (*S*)	~3	—	48	28 (*S*)	26/74	75 (*S*,*S*)
2		7.0	100	—	100 (97/3)	>99 (*S*,*S*)	100	n.d.	50/50	97 (*S*,*S*)
3		7.0	—	—	—	—	80[c]	100 (*S*)	80/20	>99
4	F1	5.0	20	90 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5		7.0	40	86 (*S*)	7 (78/22)	80 (*S*,*S*)	15	11 (*S*)	16/84	86 (*S*,*S*)
6	F2	5.0	50	91 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7		7.0	50	96 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	F3	5.0	<5	48 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9		7.0	16	75 (*S*)	20 (100 : 0)	>99 (*R*,*R*)	20	36 (*S*)	99/1	>99 (*R*,*R*)
10		7.0	25	6 (*R*)	33 (100 : 0)	>99 (*R*,*R*)	50	25 (*S*)	100/0	>99 (*R*,*R*)
11		7.0	—	—	—	—	50[c]	32 (*S*)	100/0	53 (*S*,*S*)

[a] Benzoin was racemic except in entries 3 and 11 where (*S*)-benzoin was used as the substrate. [b] The % conversion and *dl*/*meso* ratio were determined by ^{1}H NMR based on integral values of specified peaks. [c] Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Daicel, Japan) columns, respectively. [d] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature. n.d. = not detected.

cell, in spite of the presence of protease inhibitors in the buffer at the time the cell-free extract was prepared from whole cells. Several attempts to isolate the protein corresponding to this activity were unsuccessful.

T. flavus catalyzed asymmetric monoreduction of various symmetrical benzils (1a–1g)

In order to study the scope of *T. flavus* catalyzed monoreduction of benzils in general, various symmetrical benzils **1a–1g** were synthesized and subjected to treatment with the biocatalyst at pH 5.0 under the reaction conditions described in the experimental section. Purification of the products was carried out by flash chromatography over silica gel. The desired products **2a–2g** were obtained in 54–90% yield and 30–99% ee. The results are summarized in Table 5 (entries 1–7). These results suggested that (i) the substitution of a phenyl ring with a +I or −I substituent considerably slowed down the reaction. Whereas benzil was reduced in 30 h, substituted benzils typically required about 96 h for the reaction to go to completion. No reduction occurred in the case of the 4-ethoxy substituent (entry 6; Table 5). (ii) The substitution of electron donating methoxy or electron withdrawing chloro groups resulted in reduced enantioselectivity (entries 2 and 7; Table 5). The loss of enantioselectivity was considerably higher in the case of chloro substitution compared to methoxy substitution. (iii) The ee in the case of methoxy substitution followed the order: 4-methoxy > 3-methoxy > 2-methoxy (entries 2–4; Table 5). It indicates that, in addition to electronic effects, steric effects in the vicinity of the carbonyl group also play a role. Thus, the ee increased from 42% to 77% as the methoxy group was moved from the 2-position to the 3-position of the phenyl group. The ee was 82% when the methoxy group was moved to the 4-position. (iv) An interesting observation was the reversal of stereoselectivity in the case of 2-chloro substitution (entry 7; Table 5). Whereas unsubstituted benzil and methoxy or methyl substituted benzil produced the corresponding benzoin predominantly in the (*S*)-configuration, 2-chloro substituted benzil produced the corresponding benzoin in the (*R*)-configuration. (v) The substitution of the methyl group at the 4-position of the phenyl group had a minimal

Table 5 Enantioselective reduction of symmetrical and unsymmetrical benzils to benzoins by *Talaromyces flavus* at pH 5.0

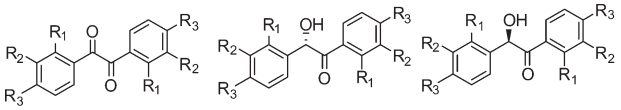
Entry	Substrate	Product	Ee[a] (%) (config)	Yield[d] (%)	Time (h)
1	**1a**	**2a**	99 (*S*)[b]	90	30
2	**1b**	**2b**	42 (*S*)[b]	90	96
3	**1c**	**2c**	77 (*S*)[b]	85	96
4	**1d**	**2d**	82 (*S*)[b]	54	96
5	**1e**	**2e**	97 (*S*)[b]	84	96
6	**1f**	**2f**	n.r.	n.r.	n.r.
7	**1g**	**2g**	30 (*R*)[b]	62	96
8	**4a**	**5a**	89 (*S*)[c]	91	17
9	**4b**	**5b**	96 (*S*)[c]	88	17
10	**4c**	**5c**	>99 (*S*)[c]	84	24
11	**4d**	**5d**	96 (*S*)[c]	80	96
12	**4e**	**5e**	>99 (*S*)[c]	85	48

[a] Ee was determined by chiral HPLC on a Chiralcel OD-H (Daicel, Japan) column. [b] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature. [c] Configuration tentative. n.r. = no reaction. [d] Yield of isolated product.

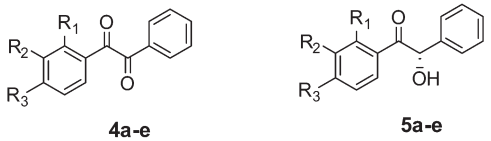
effect on the enantioselectivity of the biocatalyst and (vi) no trace of hydrobenzoin was observed at 100% conversion with any of the substrates under these reaction conditions.

T. flavus catalyzed enantio- and regio-selective mono-reduction of various unsymmetrical benzils (4a–4e)

In order to study the scope of *T. flavus* for regioselective reduction of benzils, various unsymmetrical benzil derivatives **4a–4e** were synthesized and subjected to treatment with the biocatalyst at pH 5.0 under the reaction conditions described in the experimental section. Purification of the products was carried out by flash chromatography over silica gel. The desired products **5a–5e** were obtained in 80–91% yield. The results are summarized in Table 5 (entries 8–12). In summary, the results indicate that (i) in all the examples studied, the carbonyl α to the unsubstituted phenyl group was regioselectively reduced. (ii) Substitution had no effect on the enantioselectivity as an ee of 96–99% was obtained in all examples studied except when the methoxy group was present at the 2-position of the phenyl ring, which gave an ee of 89% (entry 8; Table 5). This is in contrast to symmetrical benzils, where substitution had a detrimental effect on the ee of the products. (iii) *N*-substituents were well tolerated as the dimethylamino group at the 4-position of the phenyl ring produced the desired product in >99% ee and 85% yield (entry 12; Table 5). (iv) No hydrobenzoin was produced in any of the examples studied even when the reactions were carried out for longer times and (v) the ee and regioselectivity obtained in the *T. flavus* catalyzed reaction for these examples were higher compared to any report in the literature to date. In the literature. *Saccharomyces cerevisiae* catalyzed regioselective reduction of these substrates has been reported but the ee of their products was not calculated.[36] With *Xanthomonas oryzae*, a mixture of both regioisomers was obtained.[25]



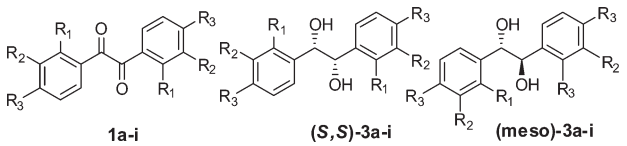
a, $R_1=R_2=R_3=H$; **b**, $R_1=OCH_3$, $R_2=R_3=H$; **c**, $R_1=R_3=H$, $R_2=OCH_3$; **d**, $R_1=R_2=H$, $R_3=OCH_3$; **e**, $R_1=R_2=H$ $R_3=CH_3$; **f**, $R_1=R_2=H$ $R_3=OEt$; **g**, $R_1=Cl$, $R_2=R_3=H$



a, $R_1=OCH_3$, $R_2=R_3=H$; **b**, $R_1=R_2=H$, $R_3=OCH_3$; **c**, $R_1=R_2=H$ $R_3=OCH_2CH_3$; **d**, $R_1=R_2=OCH_3$ $R_3=H$; **e**, $R_1=R_2=H$,$R_3=NMe_2$

T. flavus catalyzed asymmetric reduction of various benzils (1a–1i) to their corresponding 1,2-diols

We have already demonstrated that biocatalytic reduction of benzils by *Talaromyces flavus* at pH 7.0 leads to the predominant formation of chiral 1,2-diols. We extended this study to various derivatives of benzil in order to study the effect of the substituent on the conversion rate and ee of the product. *T. flavus* catalyzed reduction of benzil derivatives (**1a–1i**) at pH 7.0 produced the corresponding 1,2-diols in 85–89% yield (Table 6). The following observations are worth noting. (i) The presence of a substituent at the 2- or 4-position of the phenyl ring slowed down the reaction, whereas a substituent at the 3-position had much less influence on the conversion rate. No reaction occurred when a OCH_3 group was present at the 2-position of benzil (entry 2; Table 6), whereas the 2-chloro derivative required 168 h for the reaction to go to completion (entry 7; Table 6). 4-Methoxy and 4-methyl derivatives also required about 168 h for the reaction to go to completion (entries 4 and 5; Table 6). Surprisingly, no reaction occurred with the 4-ethoxy substituted derivative (entry 6; Table 6). (ii) The substitution of +I methoxy or −I chloro groups resulted in a reduced *dl*/*meso* ratio. Whereas benzil gave a *dl*/*meso* ratio of 97/3, the 3-methoxy and 4-methoxy derivatives gave *dl*/*meso* ratios of 89/11 and 38/62, respectively (entries 3 and 4; Table 6). The derivative with a chloro substituent at the 2-position gave a *dl*/*meso* ratio of 10/90 (entry 7; Table 6). (iii) The presence of a methoxy or methyl substituent at the 4-position resulted in a decreased ee of 78–84% (entries 4 and 5; Table 6), while with a 2-chloro substituent, an ee of >99% was obtained (entry 7; Table 6).



a, $R_1=R_2=R_3=H$; **b**, $R_1=OCH_3$, $R_2=R_3=H$; **c**, $R_1=R_3=H$, $R_2=OCH_3$; **d**, $R_1=R_2=H$, $R_3=OCH_3$; **e**, $R_1=R_2=H$ $R_3=CH_3$; **f**, $R_1=R_2=H$ $R_3=OEt$; **g**, $R_1=Cl$, $R_2=R_3=H$; **h**, $R_1=R_3=H$ $R_2=Cl$; **i**, $R_1=R_2=H$ $R_3=Cl$

Conclusions

We have shown that by modulating the pH in *T. flavus* catalyzed reduction of benzil, the reaction can be controlled to obtain either (*S*)-benzoin (**2a**) or (*S*,*S*)-hydrobenzoin (**3a**) as the exclusive product in high ee. Two prominent enzymes involved in the pH dependent selectivity are (i) (*S*)-selective benzil reductase, which is active at pH 5.0 and 7.0 and (ii) (*S*, *S*)-selective benzoin reductase, which is active at pH 7.0, but has no activity at pH 5.0. In addition, a novel dual-activity (*R*, *R*)-selective benzoin reductase and benzil epimerase protein was also purified. The (*R*,*R*)-selectivity of this enzyme was not strict because it also converted (*S*)-benzoin to (*S*,*S*)-hydrobenzoin. Benzoins were obtained in high ee when one or both phenyl groups of benzil were unsubstituted. Except for **3a**, all other hydrobenzoins were obtained in moderate de. However, considering the facts that (i) these bulky–bulky 1,2-diketones are very tough substrates for enzymes and (ii) the ee and de obtained in benzil reduction with *T. flavus* compare quite favorably with those of any of the known biocatalysts in the

Table 6 Enantio- and diastereo-selective reduction of 1,2-diketones to chiral 1,2-diols by *Talaromyces flavus* at pH 7.0

Entry	Substrate	Product	*dl*/*meso*[a]	ee[b,c] (%) (config)	Yield[d,e] (%)	Time (h)
1	**1a**	**3a**	97/3	>99 (*S*,*S*)	86	56
2	**1b**	**3b**	n.r.	n.r.	n.r.	n.r.
3	**1c**	**3c**	89/11	n.d.	88	48
4	**1d**	**3d**	38/62	84 (*S*,*S*)	89	168
5	**1e**	**3e**	67/33	78.5 (*S*,*S*)	87	168
6	**1f**	**3f**	n.r.	n.r.	n.r.	n.r.
7	**1g**	**3g**	10/90	>99 (*S*,*S*)	85	168
8	**1h**	**3h**	70/30	n.d.	88	48
9	**1i**	**3i**	67/33	>99%(*S*,*S*)	86	168

[a] The *dl*/*meso* ratio was determined by ^{1}H NMR based on integral values of specified peaks. [b] Ee of the *dl* pair was determined by chiral HPLC on a Chiralcel OJ (Daicel, Japan) column. [c] Absolute configuration was assigned based on comparison of the sign of optical rotation with that in the literature. [d] Combined yield for *dl* and *meso*. [e] Yield of isolated product. n.r. = no reaction, n.d. = not determined.

literature. Therefore, *T. flavus* expands and enriches the biocatalytic toolbox for asymmetric reduction of benzils.

Experimental

General

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

General procedure for pH dependent asymmetric reduction of benzil and benzoin by *Talaromyces flavus*

A 100 mL round bottomed flask was charged with cells (5 g, wet weight) suspended in 50 ml of an appropriate 0.2 M buffer (acetate buffer for pH 4.0 and 5.0; phosphate buffer for pH 6.0–8.0; Tris-HCl buffer for pH 9). The substrate (50 mg, 0.24 mmol) was added to the cell suspension and the mixture was shaken on an orbital shaker at 30 °C and 200 rpm. During the course of reaction, the pH of the reaction mixture was maintained by addition of 1 N NaOH or 1 N HCl. 10 ml of the sample from each reaction was taken after different time intervals (14, 37, 144 and 264 h for benzil and 12, 24 and 96 h for benzoin) and centrifuged (9000*g*, 4 °C, 15 min) to remove cells. The cell-free broth was then extracted with ethyl acetate (3 × 10 ml), washed with brine (1 × 5 mL) and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure using a rotary evaporator to leave a residue. The conversion, ratio of benzoin to hydrobenzoin and *dl*/*meso* ratio of hydrobenzoin were calculated by comparison of integral values of the corresponding peaks from ^{1}H NMR and the ee of the products was determined by HPLC.

General procedure for enantioselective reduction of benzil and benzoin by protein fractions of *T. flavus*

Enantioselective reduction of benzil, (*S*)-benzoin and *rac*-benzoin by protein fractions from *T. flavus* was carried out at pH 5.0 in 50 mM acetate buffer or pH 7.0 in 50 mM phosphate buffer. A solution of $NADP^+$ (784 nmol), glucose dehydrogenases (14 units), glucose (0.5 mmol), the protein fraction (8 units of dehydrogenase activity) and the substrate (0.02 mmol) in 4 mL of appropriate buffer was energetically mixed using a magnetic stirrer. After 16 h, the products were extracted in ethyl acetate (3 × 4 ml) and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure to leave a residue. The conversion, ratio of benzoin/hydrobenzoin and *dl*/*meso* ratio of hydrobenzoin were calculated as described in preceding section.

General procedure for microbial monoreduction of symmetrical and unsymmetrical benzils by *Talaromyces flavus*

A 100 mL round bottomed flask was charged with cells (5 g, wet weight) suspended in 50 mL of acetate buffer (0.2 M, pH 5.0). The substrate (0.24 mmol, 50–72 mg depending upon the molecular mass of the substrate) was added and the mixture was shaken on an orbital shaker at 30 °C and 200 rpm till complete consumption of the starting material occurred. The reaction mixture was then centrifuged (9000*g*, 4 °C, 15 min) to remove cells. The supernatant was extracted with ethyl acetate (3 × 25 mL), washed with saturated brine (10 mL) and dried over anhydrous sodium sulfate. Solvents were

removed under reduced pressure to leave a residue which was purified by flash chromatography (silica, methanol/chloroform 10 : 90). Ee was determined by using HPLC (system 1). The yield, ee, NMR and HPLC data for the products are given below.

(*S*)-2-Hydroxy-1,2-diphenylethanone (2a). Yield: 45.2 mg (90%); 99% ee; $[\alpha]_D^{25}$ = +115.2 (c 1.5, acetone) [lit. ref. 31, $[\alpha]_D^{25}$ = +115.0 (c 1.5, acetone), >99% ee, (*S*,*S*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.54 (bs, 1H), 5.95 (s, 1H), 7.23–7.34 (m, 5H), 7.36–7.41 (m, 2H), 7.49–7.54 (m, 1H), 7.89–7.92 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 76.3, 127.8, 128.7, 129.2, 133.6, 134.0, 139.0, 199.0; HPLC data (HPLC analysis, system 1): retention time = 11.9 min (major) and 17.4 min (minor).

(*S*)-2-Hydroxy-1,2-bis(2-methoxyphenyl)ethanone (2b). Yield: 58.3 mg (90%); 42% ee; $[\alpha]_D^{25}$ = +52.0 (c 0.9, $CHCl_3$) [lit. ref. 37, $[\alpha]_D^{25}$ = −125.0 (c 0.9, $CHCl_3$), >99% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.70 (s, 3H), 3.71 (s, 3H), 4.46 (bs, 1H), 6.09 (s, 1H), 6.73–6.77 (m, 2H), 6.80–6.85 (m, 1H), 6.89–6.94 (m, 1H), 7.12–7.20 (m, 2H), 7.32–7.38 (m, 1H), 7.65–7.69 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.2, 55.3, 75.9, 110.9, 111.2, 120.5, 125.3, 127.6, 129.6, 130.0, 130.7, 133.9, 157.3, 158.2, 201.7; HPLC data (HPLC analysis, system 1): retention time = 21.7 min (minor) and 31.2 min (major).

(*S*)-2-Hydroxy-1,2-bis(3-methoxyphenyl)ethanone (2c). Yield: 55 mg (85%); 77% ee; $[\alpha]_D^{25}$ = +119.0 (c 1.0, CH_3OH) [lit. ref. 37, $[\alpha]_D^{25}$ = −156.0 (c 1.0, CH_3OH), >99% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 (s, 3H), 3.75 (s, 3H), 5.90 (s, 1H), 6.77–6.80 (m, 1H), 6.86–6.87 (m, 1H), 6.91–6.93 (m, 1H), 7.02–7.05 (m, 1H), 7.18–7.28 (m, 2H), 7.45–7.49 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.3, 55.4, 76.3, 113.3, 113.5, 114.2, 120.2, 120.5, 121.8, 129.7, 130.2, 134.8, 140.5, 159.8, 160.2, 198.8; HPLC data (HPLC analysis, system 1): retention time = 19.2 min (major) and 27.0 min (minor).

(*S*)-2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone (2d). Yield: 35 mg (54%); 82% ee; $[\alpha]_D^{25}$ = +73.8 (c 1.0, CH_3OH) [lit. ref. 37, $[\alpha]_D^{25}$ = −90.4 (c 1.0, CH_3OH), >99% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.74 (s, 3H), 3.81 (s, 3H), 5.84 (s, 1H), 6.82–6.86 (m, 4H), 7.24 (d, *J* = 8.6 Hz, 2H), 7.89 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.3, 55.5, 75.3, 114.0, 114.6, 126.4, 129.1, 131.6, 131.9, 159.7, 164.0, 197.4; HPLC data (HPLC analysis, system 1): retention time = 42.0 min (major) and 44.4 min (minor).

(*S*)-2-Hydroxy-1,2-di-*p*-tolylethanone (2e). Yield: 48 mg (84%); 97% ee; $[\alpha]_D^{25}$ = +144.6 (c 0.7, CH_3OH) [lit. ref. 37, $[\alpha]_D^{25}$ = −150 (c 0.7, CH_3OH), >99% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.28 (s, 3H), 2.34 (s, 3H), 5.89 (s, 1H), 7.11 (d, *J* = 8.2 Hz, 2H), 7.16–7.23 (m, 4H), 7.82 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.2, 21.8, 76.8, 127.8, 129.4, 129.5, 129.9, 131.0, 136.4, 138.4, 145.0, 198.7; HPLC data (HPLC analysis, system 1): retention time = 9.2 min (major) and 11.6 min (minor).

(*R*)-1,2-Bis(2-chlorophenyl)-2-hydroxyethanone (2g). Yield: 41.5 mg (62%); 30% ee; $[\alpha]_D^{25}$ = −14.1 (c 1.0, $CHCl_3$) [lit. ref. 37, $[\alpha]_D^{25}$ = −46.0 (c 1.0, $CHCl_3$), 97% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.53 (bs, 1H), 6.34 (s, 1H), 7.11–7.17 (m, 2H), 7.18–7.25 (m, 3H), 7.27–7.32 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 75.5, 126.6, 127.4, 129.1, 129.4, 130.0, 130.1, 131.6, 132.4, 133.9, 134.9, 135.6, 200.9; HPLC data (HPLC analysis, system 1): retention time = 11.9 min (minor) and 15.0 min (major).

(*S*)-2-Hydroxy-1-(2-methoxyphenyl)-2-phenylethanone (5a). Yield: 52.4 mg (91%); 89% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.79 (s, 3H), 4.64 (bs, 1H), 6.28 (s, 1H), 6.82–6.89 (m, 2H), 7.16–7.24 (m, 2H), 7.29–7.35 (m, 2H), 7.41–7.46 (m, 1H), 7.91–7.94 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.6, 71.1, 111.6, 121.3, 127.7, 127.8, 128.6, 128.8, 128.9, 129.2, 130.1, 133.7, 156.6, 199.6; HPLC data (HPLC analysis, system 1): retention time = 19.4 min (major) and 21.7 min (minor). Configuration was assigned tentatively as *S* based on the elution order.

(*S*)-2-Hydroxy-1-(4-methoxyphenyl)-2-phenylethanone (5b). Yield: 51 mg (88%); 96% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.81 (s, 3H), 4.64 (d, *J* = 5.16 Hz, 1H), 5.88 (d, *J* = 5.16 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 2H), 7.23–7.33 (m, 5H), 7.90 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.8, 75.8, 114.0, 126.3, 127.8, 128.5, 129.2, 131.7, 139.6, 164.2, 197.2; HPLC data (HPLC analysis, system 1): retention time = 19.1 min (major) and 21.2 min (minor). Configuration was assigned tentatively as *S* based on the elution order.

(*S*)-1-(4-Ethoxyphenyl)-2-hydroxy-2-phenylethanone (5c). Yield: 52 mg (84%); >99% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.36 (t, *J* = 6.87 Hz, 3H), 3.99 (q, *J* = 6.87 Hz, 2H), 5.90 (s, 1H), 6.82 (d, *J* = 8.9 Hz, 2H), 7.21–7.36 (m, 5H), 7.89 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.6, 63.9, 75.8, 114.4, 126.1, 127.8, 128.7, 129.1, 131.7, 139.7, 163.6, 197.3; HPLC data (HPLC analysis, system 1): retention time = 15.7 min (major) and 16.3 min (minor). Configuration was assigned tentatively as *S* based on the order of elution in HPLC.

(*S*)-2-Hydroxy-1-(2,3-dimethoxyphenyl)-2-phenylethanone (5d). Yield: 52 mg (80%); 96% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.77 (s, 3H), 3.78 (s, 3H), 4.71 (s, 1H), 5.86 (s, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 7.17–7.31 (m, 5H), 7.43–7.48 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.9, 56.0, 75.8, 110.1, 111.2, 124.3, 126.4, 127.7, 128.5, 129.1, 139.9, 149.0, 153.9, 197.2; HPLC data (HPLC analysis, system 1): retention time = 35.3 min (major) and 41.9 min (minor). Configuration was assigned tentatively as *S* based on the order of elution in HPLC.

(*S*)-1-(4-(Dimethylamino)phenyl)-2-hydroxy-2-phenylethanone (5e). Yield: 52 mg (85%); >99% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.01 (s, 6H), 4.80 (d, *J* = 5.85 Hz, 1H), 5.84 (d, *J* = 5.85 Hz, 1H), 6.56 (d, *J* = 8.9 Hz, 2H), 7.23–7.36 (m, 5H), 7.83 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 40.1, 75.3, 110.7, 120.8, 127.7, 128.2, 129.0, 131.6, 140.6, 153.8, 196.0; HPLC data (HPLC analysis, system 1): retention time = 54.4 min (major) and 56.4 min (minor). Configuration was assigned tentatively as *S* based on the order of elution in HPLC.

General procedure for microbial reduction of benzils to hydrobenzoins by *Talaromyces flavus*

The procedure was the same as described above for the monoreduction of benzils except that the phosphate buffer

(0.2 M, pH 7.0) was used instead of the acetate buffer. Ee was determined by using HPLC (system 2). The ee, NMR and HPLC data for the products are given below.

(1*S*,2*S*)-1,2-Diphenylethane-1,2-diol ((*S*,*S*)-3a). >99% ee; $[\alpha]_D^{25}$ = −91.0 (c 1.05, ethanol) [lit. ref. 32, $[\alpha]_D^{25}$ = +91.6 (c 1.05, ethanol), 99.9% ee, (*R*,*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.72 (s, 2H), 7.11–7.15 (m, 4H), 7.21–7.25 (m, 6H) ppm; HPLC data (HPLC analysis, system 2): retention time = 16.2 min (*S*,*S*), 18.8 min (*R*,*R*) and 22.6 min (*meso*).

(1*S*,2*S*)-1,2-Bis(3-methoxyphenyl)ethane-1,2-diol ((*S*,*S*)-3c). Ee not determined. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 (s, 6H), 4.68 (s, 2H), 6.71–6.80 (m, 5H), 7.12–7.26 (m, 3H) ppm.

(1*S*,2*S*)-1,2-Bis(4-methoxyphenyl)ethane-1,2-diol ((*S*,*S*)-3d). 84% ee; $[\alpha]_D^{25}$ = −99.1 (c 1.0, EtOH) [lit. ref. 38, $[\alpha]_D^{25}$ = −118.3 (c 1.0, EtOH), 99% ee, (*S*,*S*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.75 (s, 6H), 4.63 (s, 2H), 6.76 (d, *J* = 8.9 Hz, 4H), 7.0 (d, *J* = 8.9 Hz, 4H) ppm.

(1*S*,2*S*)-1,2-Di-*p*-tolylethane-1,2-diol ((*S*,*S*)-3e). 78.5% ee; $[\alpha]_D^{25}$ = −89 (c 1.0, ethanol) [lit. ref. 38, $[\alpha]_D^{25}$ = −102.5 (c 1.0, ethanol), 99% ee, (*S*,*S*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.29 (s, 6H), 4.67 (s, 2H), 7.13 (d, *J* = 8.9 Hz, 4H), 7.19 (d, *J* = 8.9 Hz, 4H) ppm; HPLC data (HPLC analysis, system 2): retention time = 12.2 min (*S*,*S*), 14.6 min (*R*,*R*) and 15.6 min (*meso*).

(1*S*,2*S*)-1,2-Bis(2-chlorophenyl)ethane-1,2-diol ((*S*,*S*)-3g). >99% ee. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.36 (s, 2H), 7.16–7.18 (m, 5H), 7.25–7.28 (m, 3H) ppm; HPLC data (HPLC analysis, system 2): retention time = 26.3 min (*meso*), 50.9 min (*S*,*S*) and 57.6 min (*R*,*R*).

(1*S*,2*S*)-1,2-Bis(3-chlorophenyl)ethane-1,2-diol ((*S*,*S*)-3h). Ee not determined. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.64 (s, 2H), 6.91 (d, *J* = 7.2 Hz, 2H), 7.12–7.26 (m, 6H) ppm.

(1*S*,2*S*)-1,2-Bis(4-chlorophenyl)ethane-1,2-diol ((*S*,*S*)-3i). >99% ee; $[\alpha]_D^{25}$ = −91.0 (c 1.0, $CHCl_3$) [lit. ref. 38, $[\alpha]_D^{25}$ = +93 (c 1.0, $CHCl_3$), 99% ee, (*R*,*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.63 (s, 2H), 7.0 (d, *J* = 8.6 Hz, 4H), 7.21 (d, *J* = 8.6 Hz, 4H) ppm; HPLC data (HPLC analysis, system 2): retention time = 44.9 min (*S*,*S*), 47.0 min (*R*,*R*) and 55.0 min (*meso*).

Materials and methods

Source of materials and microorganisms

Symmetrical and unsymmetrical benzils were synthesized by benzoin condensation followed by oxidation with pyridinium chlorochromate.[39] Culture media, TSB (tryptone soya broth) and PDB (potato dextrose broth) were purchased from HiMedia (Mumbai, India). Sephacryl S-200, bovine serum albumin (BSA), β-mercaptoethanol, sodium dodecyl sulfate (SDS), Reactive Red 120-agarose type 3000-CL, and molecular weight standards were from Sigma Chemical Company, USA. Q-Sepharose Fast Flow and Phenyl Sepharose CL-4B were from Pharmacia (Freiburg, Germany). *Penicillium* sp., *Alternaria alternata* and *Talaromyces flavus* are our own isolates and were described previously (Pal *et al.*, 2012). These have been deposited with Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh (http://mtcc.imtech.res.in) with accession numbers MTCC 10832, MTCC 10833 and MTCC 10834, respectively.

Growth conditions

Fungal strains were maintained as 25% glycerol stocks at −78 °C. A 100 mL Erlenmeyer flask was charged with 20 mL of sterile PDB media (24 g L^{-1}; pH 5.2–5.3). After inoculation with fungi, the flask was incubated with shaking at 200 rpm in an incubator shaker at 30 °C for 24 h. This was used to inoculate a 2 L Erlenmeyer flask containing 400 mL of the same media. The flask was then incubated at 30 °C with shaking at 200 rpm on an orbital shaker for 72 h. Microbial cells were isolated by centrifugation at 9000*g* for 15 min at 4 °C. Composition of PDB: potato infusions (200 g L^{-1}) and dextrose (3 g L^{-1}).

Preparation of cell-free extract

T. flavus was grown in several 2 L flasks, as described in preceding section. Cells of *T. flavus* (75 g, wet weight) were suspended in 200 mL of phosphate buffer (50 mM, pH 7.0) containing 1 mM protease inhibitor cocktail (dithiothreitol, phenylmethylsulfonyl fluoride and 1,10-phenanthroline) and cooled to 4 °C. The suspension was disrupted by glass beads (0.5–0.75 μm) in a 1:1 (wt/wt) ratio at 4 °C for 15 min in Dyno Mill. The cell-free extract was obtained by centrifugation at 20 000*g* for 20 min at 4 °C

Ammonium sulfate fractionation

Solid ammonium sulfate was added in small portions to the cell-free extract at 4 °C to 40% saturation. The pellet was removed by centrifugation and the supernatant was brought to 80% saturation with solid ammonium sulfate while maintaining the temperature at 4 °C and the pH at 7.0. The precipitated proteins were collected by centrifugation. The pellet was re-dissolved in phosphate buffer (10 mM, pH 8.0) and centrifuged to remove any precipitate.

Affinity chromatography

The supernatant from the above step was desalted using a Sephadex G-25 column. This solution was applied on a Reactive Red 120-agarose type 3000-CL column (2 × 13 cm) pre-equilibrated with phosphate buffer (10 mM, pH 8.0). The column was washed with the same buffer until all unbound proteins were completely removed. An increasing linear gradient of NaCl (0–1.5 M) in phosphate buffer (10 mM, pH 6.5) at a flow rate of 60 mL h^{-1} was used to elute the proteins. The active fractions were pooled and concentrated by ultrafiltration (Millipore-Amicon Ultra-15 centrifugal filter units with a 10 kDa membrane). Desalting was carried out by repeated dilution with Tris-HCl buffer (10 mM, pH 8.0) and the sample was finally concentrated to 1 mL volume.

Ion-exchange chromatography

The desalted and concentrated sample (1 mL) obtained from Reactive Red chromatography was loaded into a Q-Sepharose Fast Flow column (2 × 5 cm) pre-equilibrated with Tris-HCl buffer (10 mM, pH 8.0). The column was then washed with the same buffer and the enzyme was eluted with thirty column volumes of an increasing linear gradient of NaCl (0–0.5 M) in Tris-HCl buffer (10 mM, pH 8.0) at a flow rate of 60 mL h^{-1}. The active fractions were pooled and concentrated by ultrafiltration (Millipore-Amicon Ultra-15 centrifugal filter units with a 10 kDa membrane). Desalting was carried out by repeated dilution with Tris-HCl buffer (10 mM, pH 8.0) and the sample was concentrated to a final volume of 0.5 mL.

Gel filtration chromatography

The desalted and concentrated sample (0.5 mL) obtained from Q-Sepharose chromatography was loaded into a Sephacryl S-200 column (2 × 60 cm, 43 mL bed volume) previously equilibrated with Tris-HCl buffer (50 mM, pH 7.5) containing 0.15 M NaCl at a flow rate of 30 mL h^{-1}. The active fractions were pooled and concentrated in an ultrafiltration cell using a 10 kDa membrane.

Enzyme assay method

The reductase activity was determined by monitoring the decrease in absorbance of NADPH at 340 nm (molar absorption coefficient of 6800 M^{-1} cm^{-1}) as described previously.[40] The standard reaction mixture (1 mL) contained 0.1 mM NADPH, 50 μl of the cell-free extract and 2.5 mM substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute under specified conditions. Protein concentration was estimated by the Coomassie Brilliant Blue-G method of Bradford using bovine serum albumin (BSA) as the standard.[41]

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed in 12.5% (w/v) gels according to the procedure of Laemmli.[42] Prior to loading into the gel, the samples were heated in a boiling water bath for 5 min. The discontinuous gel system usually had 5% stacking and 12.5% resolving gels. Electrophoresis was carried out using Laemmli buffer at a constant current of 15 mA first till the samples entered the gel and then at 20 mA till completion. Upon completion of electrophoresis, the gel was immersed in 0.05% Coomassie Blue R-250 in methanol : acetic acid : water (4 : 1 : 5) with gentle shaking and was then destained in a destaining solution (staining solution without dye) till the background was clear. The protein standards used were phosphorylase b, rabbit muscle (97 000 Da), albumin, bovine serum (66 000 Da), ovalbumin, chicken egg white (45 000 Da), carbonic anhydrase, bovine erythrocyte (30 000 Da), trypsin inhibitor, soybean (20 100 Da), and α-lactalbumin, bovine milk (14 400 Da).

N-terminal sequencing and bioinformatics studies

Purified protein was run on SDS-PAGE. The gel was washed once with water and equilibrated with 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffer, pH 11.0 containing 0.037% (w/v) SDS and 10% methanol for 15 min. A polyvinylidene difluoride (PVDF) membrane of gel size was cut and wetted with methanol for 1 min, rinsed once with water and equilibrated with the above buffer for 15 min. Proteins were electrophoretically transferred onto the PVDF membrane at 300–350 mA for 1 h at 4 °C. The extent of transfer was visualized by staining with 0.1% (w/v) amido black (in 1% acetic acid) for 5 min. Destaining was carried out in 50% (v/v) methanol. The transferred protein of interest was excised and washed once with 20% (v/v) methanol for 1 h at RT. Thereafter, cut-out PVDF bands were washed thoroughly (10 times) with double distilled water to remove traces of methanol. These PVDF bands were dried on Whatman sheets and stored at 4 °C until analysis using an Applied Biosystems 476 A protein sequencer following the Edman degradation protocol. The obtained N-terminal sequence, APTNQ_AYDHSV, was then searched for homologous counterparts using the BLAST server at www.ncbi.nlm.nih.gov.

MALDI-TOF mass spectral analysis

Purified enzyme (10 μM) and fresh matrix (3,5-dimethoxy-4-hydroxycinnamic acid, sinapinic acid, 10 mM) solutions were premixed in a small Eppendorf tube and applied directly to the sample support (*i.e.*, sample plate). The sample was allowed to evaporate in air, irradiated with a nanosecond laser pulse and analyzed using a MALDI-TOF Voyager DE-STR instrument.

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