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Reduction of acetophenones with methyl fluorines and a bulky group on the aromatic ring using microorganisms and related enzymes

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ABSTRACT

Whole-cell yeasts and mold-catalyzed reduction of two fluorinated acetophenone derivatives with very bulky substituents on *ortho* position of aromatic ring, (±)-1′-(2-*tert*-butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′-difluoroethanone and (±)-1′-(2-*tert*-butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′,2′-trifluoroethanone were examined. On the former substrate, *Geotrichum candidum* NBRC 5767 showed high *re*-facially selective attack of hydride, while with *Pichia angusta* JCM 3620, complementary *si*-facially selective attack proceeded. *G. candidum* NBRC 5767 was revealed to be potent biocatalyst which provides (1′*S*)-alcohols from both substrates in a highly facially selective manner. Some unknown reductases were suggested responsible for those reductions, other than so far having been reported acetophenone reductase and trifluoromethyl ketone reductase from *G. candidum*, comparing the results obtained by applying those enzymes.

1. Introduction

Asymmetric reduction of ketones with cultured whole-cell biocatalysts, such as strains of *Geotrichum candidum* (NBRC 4597 and 5767), have been studied intensively [1–3]. Their activity and enantioselectivity in asymmetric reduction are extremely variable. For example, *G. candidum* NBRC 4597 reduces acetophenone (**1**) and 2′-fluoroacetophenone (**3**) to yield (*S*)-**2** and (*R*)-**4** in the same enantiofacial selectivity. The enantiofacial preference changes, with increasing the number of fluorine atoms on the methyl groups. When using difluoro derivative **5** as the starting material, the enantioselectivity in **6** is lost and, eventually, in 2′,2′,2′-trifluoroacetonphenone (**7**), the selectivity was completely inversed to give (*S*)-**8** [4,5].

When starting with (±)-**9**, which contains a unique acetal on the aromatic ring, the reduction with *G. candidum* NBRC 4597 was very slow. This result agreed well with studies showing that the introduction of a bulky substituent on the *ortho* position suppressed reduction by this microorganism [6]. The enantiofacially selective reduction, however, was proceeded by applying cultured cells of strain *G. candidum* NBRC 5767 [7] to give mainly (2*S*,1′*S*)- and (2*R*,1′*S*)-**10a**. Based on the results from ketones **1**, **3**, and **5**, the effect of the fluorine-containing substituents in **9** and **11** on reactivity and stereoselectivity was investigated. This report presents the difference in selectivity of the cultured whole-cell catalyzed reduction of trifluoromethyl ketone **9** and difluoromethyl ketone **11** from eight yeasts and fungi strains, including *G. candidum*. Scheme 1.

2. Experimental

IR spectra were measured as films for oils or KBr disks of solids on a Jasco FT/IR-410 spectrometer, and as ATR on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ at 270 MHz on a Jeol JNM EX-270 or at 400 MHz on an Agilent 400-MR spectrometer. ^{13}C NMR spectra were measured in $CDCl_3$ at 68 MHz on a Jeol JNM EX-270 or at 100 MHz on an Agilent 400-MR or at 125 MHz on an Agilent INOVA-500 spectrometer. HPLC data were recorded on Jasco MD-2010 or SHIMADZU SPD-20A multi-channel detectors. Merck silica gel 60 F_{254} thin-layer plate (1.05715, 0.25 mm thickness) was used for thin-layer chromatographic analysis. Merck silica gel 60 F_{254} thin-layer plates (1.05744, 0.5 mm thickness) and silica gel 60 (spherical and neutral; 100–210 μm, 37560-79) from Kanto Chemical Co., Inc. were used for preparative thin-layer chromatography and column chromatography, respectively. Yeast strains are available from Japan Collection of Microorganisms; Riken Bioresource Center, Planning

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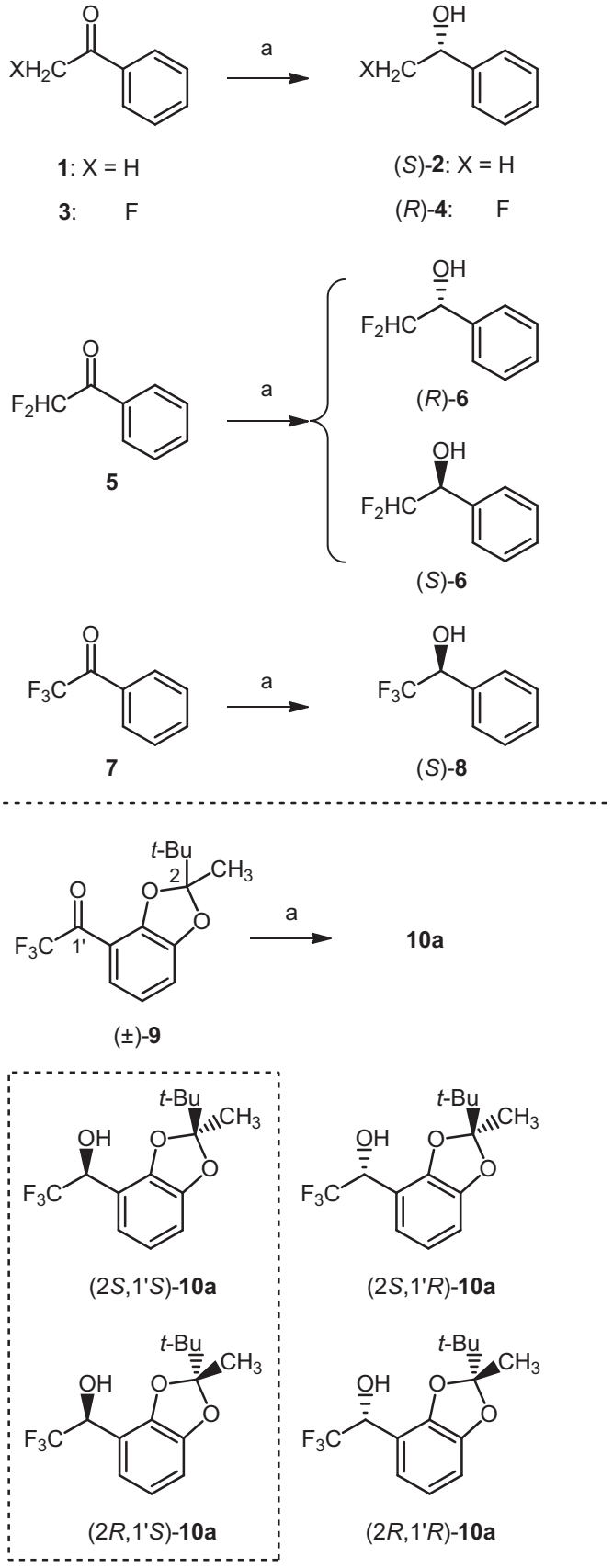


Scheme 1. Reagents and conditions: (a) *Geotrichum candidum*.

Section, Research Promotion Division, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan, and to NITE Biological Resource Center; Department of Biotechnology, National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan. Peptone, malt extract and yeast extract were purchased from Kyokuto Pharmaceutical Industrial Co., Ltd.

*2.1. (±)-1′-(2-tert-Butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′-difluoroethanone (**11**)*

In a similar way for the preparation of **9** [7], a solution of lithiated form of 2-*tert*-butyl-2-methyl-1,3-benzodioxole (1.00 g, 5.2 mmol), which was made with *n*-butyllithium (2.6 M in hexane, 3.0 mL, 1.5 equiv.) and TMEDA (1.1 mL, 1.5 equiv.) was treated with F_2HCCO_2Et (1.4 mL, 13 mmol, 2.5 equiv.) at −50 °C. Similar workup [7] and the purification of the residue by silica gel column chromatography (100 g) by the elution with hexane/AcOEt = 40:1 to 20:1 provided ketone **11** (1.17 g, 83%) as yellow oil. ^{1}H NMR: δ 1.08 (s, 9H, *tert*-Bu), 1.62 (s, 3H, Me), 6.48 (t, $J_{2',F}$ = 53.6 Hz, 1H, H2′), 6.85 (dd, $J_{5,6}$ = 8.2 Hz, $J_{6,7}$ = 7.6 Hz, 1H, H6), 6.94 (dd, $J_{5,7}$ = 1.4 Hz, 1H, H7), 7.36 (dd, 1H, H5); ^{13}C NMR: δ 20.2, 24.4, 39.6, 109.1 (t, $J_{2',F}$ = 989.4 Hz, C2′), 111.6, 113.3, 120.4, 121.4, 126.0, 149.3, 149.3, 184.8 (t, $J_{1',F}$ = 96.8 Hz, C1′), the signals 24.4 included totally three carbons; IR: 2978, 1709, 1460, 1142, 854, 725 cm^{-1}.

2.2. (2R,1′R*)-(±)-1′-(2-tert-Butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′-difluoroethanol (**12a**) and (2R*,1′S*)-(±)-1′-(2-tert-butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′-difluoroethanol (**12a**)*

To a solution of **11** (15.9 mg, 0.059 mmol) in EtOH (590 μL) were treated with $NaBH_4$ (6.7 mg, 0.176 mmol, 3.0 equiv.) at room temperature. The same workup and the purification of the residue by preparative TLC [developed with hexane/AcOEt = 4:1] to afford (2*R**,1′*R**)-**12a** and (2*R**,1′*S**)-**12a** (15.0 mg, 94%) as pale yellow oil as a mixture. The diastereomeric ratio between (2*R**,1′*S**)- and (2*R**,1′*R**)-**12a** was determined to be 3:2 judging from their NMR spectrum: δ 5.88 for (2*R**,1′*S**)- and δ 5.92 for (2*R**,1′*R**)-**12a**. A small portion was separated by a preparative HPLC [column, Kanto Chemical Co., Inc. Mightysil 60, 1 cm × 25 cm; hexane/AcOEt = 15:1); flow rate 5 mL/min]: t_R (min) = 6.6 [(2*R**,1′*R**)-**12a**], 6.8 [(2*R**,1′*S**)-**12a**] and the former was isolated in pure state. (2*R**,1′*R**)-**12a**: ^{1}H NMR: δ 1.05 (s, 9H, *tert*-Bu), 1.55 (s, 3H, Me), 4.86 (ddd, $J_{1',2'}$ = 4.1 Hz, $J_{1',F}$ = 9.8, 13.9 Hz, 1H, H1′), 5.92 (dt, $J_{2',F}$ = 55.8 Hz, 1H, H2′), 6.70–6.83 (aromatic, 3H); ^{13}C NMR: δ 20.0, 24.5, 39.4, 70.7 (t, $J_{1',F}$ = 99.8 Hz, C1′), 108.4, 115.1 (t, $J_{2',F}$ = 976.0 Hz, C2′), 116.5, 119.6, 121.2, 124.4, 145.8, 148.1, the signals 24.5 included totally three carbons. (2*R**,1′*S**)-**12a**: ^{1}H NMR: δ 1.05 (s, 9H, *tert*-Bu), 1.55 (s, 3H, Me), 4.93 (dt, $J_{1',2'}$ = 4.3 Hz, $J_{1',F}$ = 10.6 Hz, 1H, H1′), 5.88 (dt, $J_{2',F}$ = 55.9 Hz, 1H, H2′), 6.70–6.83 (aromatic, 3H); ^{13}C NMR: δ 20.0, 24.4, 39.5, 69.8 (q, $J_{1',F}$ = 101.2 Hz, C1′), 108.3, 115.2 (t, $J_{2',F}$ = 976.0 Hz, C2′), 116.5, 119.0, 121.2, 124.3, 145.8, 148.0, the signals 24.4 included totally three carbons. HPLC [column, Daicel Chiralcel OJ-H, 0.46 cm × 25 cm; hexane/*i*-PrOH = 40:1; flow rate 0.5 mL/min]: t_R (min) = 18.3 [(2*R*,1′*R*)-**12a**], 20.4 [(2*S*,1′*S*)-**12a**], 22.4 [(2*R*,1′*S*)-**12a**], 30.5 [(2*S*,1′*R*)-**12a**]. The assignment of relative and absolute configurations as above were described in detail, in Sections 2.7 and 2.8.

*2.3. Screening of microorganisms for the reduction of **9** and **11***

The microorganisms from stock culture samples were incubated in glucose medium [8–10] [containing glucose (5.0 g), peptone

Table 1
Whole cell microorganism-catalyzed reduction of (±)-**11** and (±)-**9**.

Entry	Microorganisms (JCM No.)	X[a]	Conv.[b] (%)	Product ratio[c]			
				(2*R*,1′*R*)	(2*S*,1′*S*)	(2*R*,1′*S*)	(2*S*,1′*R*)
1	*Pichia minuta* (3622)	H	75	0	52	40	8
2	*P. minuta* (3622)	F	80	10	20	47	23
3	*Trichosporon cutaneum* (1534)	H	66	28	4	1	67
4	*T. cutaneum* (1534)	F	76	31	11	17	41
5	*Candida floricola* (9439)	H	22	9	65	9	17
6	*C. floricola* (9439)	F	66	27	26	31	16
7	*Williopsis californica* (3600)	H	89	9	50	14	27
8	*W. californica* (3600)	F	19	30	12	20	38
9	*Candida nitratophila* (9856)	H	11	12	31	14	43
10	*C. nitratophila* (9856)	F	24	30	13	22	35
11	*Pichia angusta* (3620)	H	23	40	2	0	58
12	*P. angusta* (3620)	F	0	–	–	–	–
13	*Geotrichum candidum* (4597)[d]	H	4	13	14	25	48
14	*G. candidum* (4597)[d]	F	3	12	13	33	42
15	*G. candidum* (5767)[d]	H	86	1	48	50	1
16	*G. candidum* (5767)[d]	F	91	0	49	50	1

[a] For substituents, as shown in Scheme 2.
[b] Determined by ^{1}H NMR spectrum of the crude product, as shown in Section 2.3.
[c] Determined by HPLC analysis, as shown in Sections 2.2 and 2.3.
[d] NBRC No.

(2.0 g), yeast extract (0.5 g), KH_2PO_4 (0.3 g), K_2HPO_4 (0.2 g), at pH 6.5, total volume of 100 mL, in the Erlenmeyer cultivating flasks] for 2 days at 30 °C. Pre-cultivation of *G. candidum* NBRC 4597 and 5767 were described later in Section 2.6.

The wet cells in the broth (100 mL) were harvested by centrifugation (3000 rpm) and 500 mg of cells were re-suspended in phosphate buffer (0.1 M, pH 6.0, 5 mL) in a test tube, together with a substrate **9** or **11** (25 mg) and glucose (250 mg), and shaken on a reciprocal shaker (210 cpm) for 2 days at 30 °C. The extractive work-up and the analysis of stereoisomers were performed in the similar manner as described later in Section 2.6. The conversion was estimated by the NMR spectra of the crude products. The ratio of area for signals δ 5.88 [dt, 1H, H2′ for (2*R**,1′*S**)-**12a**, 5.92 [dt, 1H, H2′ for (2*R**,1′*R**)-**12a**], and 6.48 (t, 1H, H2′ for **11**) were calculated. Stereoisomeric ratio of **10a** was determined HPLC analysis [column, Daicel Chiralcel OJ-H, 0.46 cm × 25 cm; hexane/*i*-PrOH = 40:1; flow rate 0.5 mL/min]: t_R (min) = 13.2 [(2*R*,1′*R*)-**10a**], 13.9 [(2*R*,1′*S*)-**10a**], 14.9 [(2*S*,1′*S*)-**10a**], 20.8 [(2*S*,1′*R*)-**10a**] [7]. The results are summarized in Table 1.

2.4. Acetophenone reductase-catalyzed reduction of *9* and *11*

To a solution of the partially purified acetophenone reductase from *G. candidum* NBRC 4597 [11] which contains 64 units (one unit of enzyme activity was defined as the micromoles of **2** released by the reduction of **1** per minute) in a HEPES buffer [0.1 M, pH, 7.2, containing DTT (1 mM) and PMSF (1 mM), total 2.53 mL] were added ketone **9** or **11** (20 mg, *ca.* 0.07 mmol), NAD^+ (30 mg, 0.04 mmol), and 2-propanol (0.45 mL, 15%, v/v). The mixture was reciprocally shaken at 250 cpm and 40 °C for 20 h and saturated with NaCl. After filtration with Celite, the filtrate was extracted three times with AcOEt. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The conversion was estimated in the same manner as Section 2.3. The alcohols were further purified by preparative TLC [developed with hexane/AcOEt = 4:1)].

Reduction of **9**: conversion: 4%, HPLC analysis of **10a**: t_R (min) = 13.2 [17.1%, (2*R*,1′*R*)-], 13.9 [31.9%, (2*R*,1′*S*)-], 14.9 [17.6%, (2*S*,1′*S*)-], 20.8 [33.4%, (2*S*,1′*R*)-].

Reduction of **11**: conversion: 3%, HPLC analysis of **12a**: t_R (min) = 18.3 [83.7%, (2*R*,1′*R*)-], 20.4 [0.7%, (2*S*,1′*S*)-], 22.4 [1.8%, (2*R*,1′*S*)-], 30.5 [13.8%, (2*S*,1′*R*)-].

2.5. Trifluoromethyl ketone reductase-catalyzed reduction of *9* and *11*

Trifluoromethyl ketone reductase was partially purified as follows, according to the reported procedure [5]. Cell free extract of *G. candidum* NBRC 5767 was firstly fractionated with $(NH_4)_2SO_4$, and the desired fraction was sequentially submitted to chromatography with anion exchange, hydrophobic, hydroxyapatite, and finally anion exchange column. To a mixture of the enzyme (0.4 units; one unit of enzyme activity was defined as the micromoles of **8** released by the reduction of **7** per minute) in HEPES buffer (0.1 M, pH, 7.2, 13.3 mL) were added NADPH (80 mg, 0.10 mmol) and ketone **9** or **11** (10 mg, *ca.* 0.04 mmol) dissolved in ethanol (0.70 mL). The mixture was reciprocally shaken at 250 cpm and 40 °C for 20 h. The extractive work-up and evaluation of the products were as in the same manner described in Section 2.4.

Reduction of **9**: conversion: 7%, HPLC analysis of **10a**: t_R (min) = 13.2 [5.3%, (2*R*,1′*R*)-], 13.9 [48.9%, (2*R*,1′*S*)-], 14.9 [8.7%, (2*S*,1′*S*)-], 20.8 [37.1%, (2*S*,1′*R*)-].

Reduction of **11**: conversion: 12%, HPLC analysis of **12a**: t_R (min) = 18.3 [10.1%, (2*R*,1′*R*)-], 20.4 [11.6%, (2*S*,1′*S*)-], 22.4 [50.4%, (2*R*,1′*S*)-], 30.5 [27.9%, (2*S*,1′*R*)-].

2.6. Whole cell-catalyzed reduction of (±)-*11* with G. candidum NBRC 5767

A loopful of the strain grown on the agar-plate culture was aseptically inoculated to a glucose medium [glucose (20.0 g), peptone (8.0 g), yeast extract (2.0 g), KH_2PO_4 (1.2 g), K_2HPO_4 (0.8 g), at pH 6.2, total volume of 400 mL] in four 500-mL baffled Erlenmeyer cultivating flasks. The flask was shaken on a gyratory shaker (180 rpm) for 24 h at 30 °C. The wet cells were harvested by filtration with a rough paper filter and washed with water. The weight of combined wet cells was *ca.* 22.2 g from 400 mL of the broth.

Above-mentioned harvested cells of *G. candidum* (15.0 g) were re-suspended in phosphate buffer solution (0.2 M, 50 mL, pH 6.0), together with a substrate **11** (0.5 g, 1.9 mmol) and glucose (7.5 g), in a 200 mL round-bottomed flask which was loosely capped with sterilized filter paper for a plenty of oxygen supply. The mixture was stirred at 30 °C for 24 h. The mixture was centrifuged (3000 rpm) to

remove cell mass and the supernatant was further filtered through a pad of Celite. The filtrate was saturated with NaCl and extracted with AcOEt. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by a silica gel column chromatography (30 g). Elution with hexane/AcOEt = 20:1 to 5:1 afforded **12a** (330.8 mg, 64%) as colorless solid. HPLC analysis was performed under the same conditions in Section 2.2. t_R (min) = 18.3 [0.6%, (2*R*,1′*R*)-**12a**], 20.4 [47.8%, (2*S*,1′*S*)-**12a**], 22.4 [50.3%, (2*R*,1′*S*)-**12a**], 30.5 [1.3%, (2*S*,1′*R*)-**12a**].

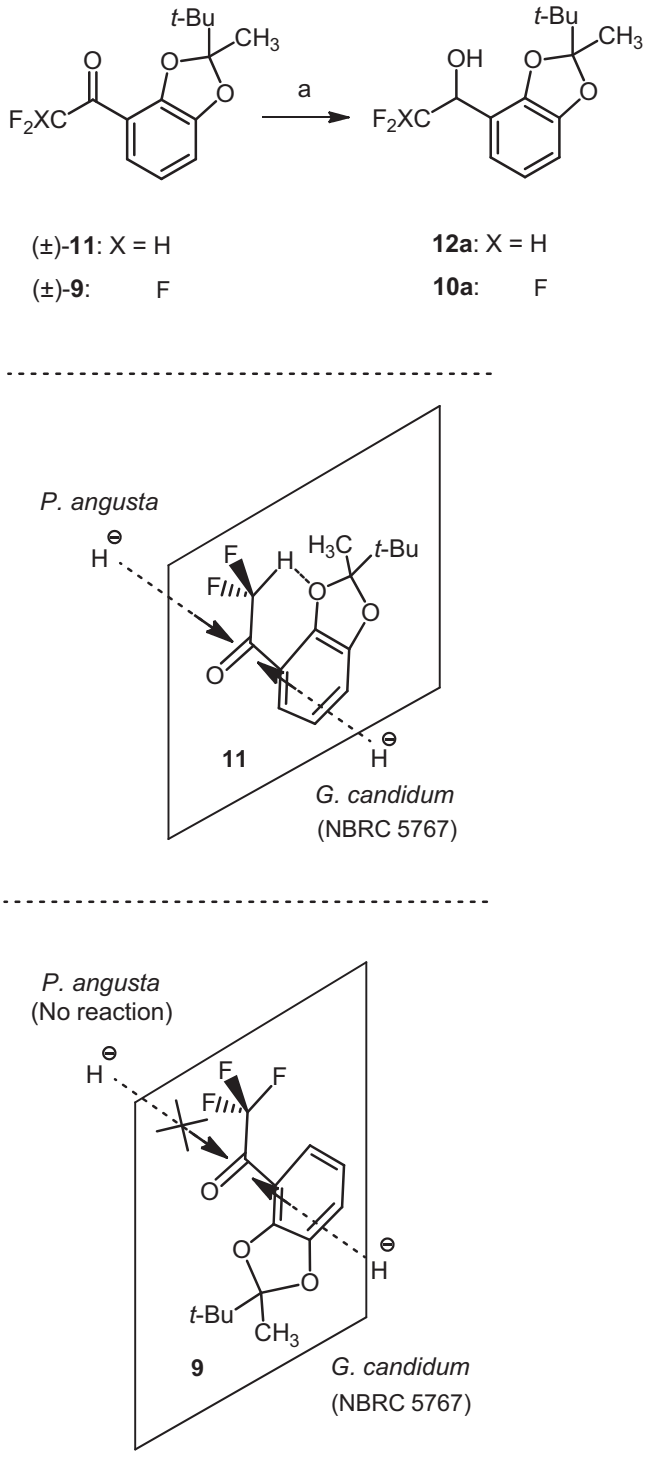
2.7. MTPA esters of (2S,1′S)-1′-(2-tert-butyl-2-methyl-1,3-benzodioxol-4-yl)-2′,2′-difluoroethanol (*12b* and *12c*)

A small portion of the product mixture in Section 2.3 was separated by a preparative HPLC using Mightysil 60 under the conditions in Section 2.2, and the diastereomerically pure (2*S*,1′*S*)-**12a** was obtained. HPLC analysis using Chiralcel OJ-H under the conditions in Section 2.2: t_R (min) = 18.3 [1.2%, (2*R*,1′*R*)-**12a**], 20.4 [98.8%, (2*S*,1′*S*)-**12a**]. A mixture of this purified alcohol (2.4 mg, 0.01 mmol), (*R*)-α-methoxy-α-trifluoromethylphenylacetyl chloride [(*R*)-MTPA chloride, 5.5 mg, 0.03 mmol], pyridine (100 μL), and catalytic amount of DMAP was stirred at room temperature for 30 min. The reaction was quenched by the addition of water, and the organic materials were extracted with AcOEt. The combined organic layer was washed with water, hydrochloric acid (1 M), saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by preparative TLC [developed with hexane/AcOEt = 4:1)] to afford (*S*)-MTPA ester **12b** (2.5 mg, 58%) as colorless oil. ^{1}H NMR: δ ^{1}H NMR: δ 1.04 (s, 9H, *tert*-Bu), 1.41 (s, 3H, Me), 6.13 (dt, $J_{1',2'}$ = 5.3 Hz, $J_{2',F}$ = 55.3 Hz, 1H, H2′), 6.14 (ddd, $J_{1',F}$ = 5.9, 10.8 Hz, 1H, H1′), 6.61 (dd, $J_{5,7}$ = 1.8 Hz, $J_{5,6}$ = 7.5 Hz, 1H, H5), 6.69 (dd, $J_{6,7}$ = 7.5 Hz, 1H, H6), 6.72 (dd, 1H, H7).

In the similar manner, (*R*)-MTPA ester **12c** was obtained (2.2 mg, 61%) as colorless oil. ^{1}H NMR: δ 1.05 (s, 9H, *tert*-Bu), 1.52 (*s*, 3H, Me), 6.08 (dt, $J_{1',2'}$ = 5.3 Hz, $J_{2',F}$ = 55.0 Hz, 1H, H2′), 6.23 (ddd, $J_{1',F}$ = 7.6, 9.8 Hz, 1H, H1′), 6.70–6.83 (aromatic, 3H).

2.8. Authentic samples of the mixture of (2R,1′R)-*12a* and (2R,1′S)-*12a*

According to the reported procedure [12], to slurry of magnesium turnings (4.2 mg, 0.173 mmol) in anhydrous THF (1.5 mL) was added chlorotrimethylsilane (44 μL, 0.346 mmol) and the mixture was stirred 5 min. Then a solution of (*R*)-**9** [7] (50.0 mg, 0.173 mmol) in anhydrous THF (2.2 mL) was added dropwise. After stirring for 2 h at 0 °C, the mixture was concentrated *in vacuo*. The residue was mixed with hexane and filtered with filter paper. The filtrate was concentrated *in vacuo* and the residue was dissolved in MeOH (2.0 mL). To the solution was added tetra-*n*-butylammonium fluoride (1.0 M solution in THF, 173 μL, 0.173 mmol), immediately followed by $NaBH_4$ (13 mg, 0.346 mmol), and the mixture was stirred at room temperature for 30 min. The mixture was cooled with ice-water bath and the reaction was quenched by the addition of water and acetic acid, and the organic materials were extracted with AcOEt. The combined organic layer was washed with water, saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by preparative TLC [developed with hexane/AcOEt = 4:1)] to afford a mixture of (2*R*,1′*R*)-**12a** and (2*R*,1′*S*)-**12a** (22.3 mg, 48%) as colorless oil. HPLC analysis using Chiralcel OJ-H under the conditions in Section 2.2: t_R (min) = 18.3 [37.8%, (2*R*,1′*R*)-**12a**], 22.4 [62.2%, (2*R*,1′*S*)-**12a**].



Scheme 2. Reagents and conditions: (a) cultured whole-cell microoganisms.

3. Results and discussion

3.1. Stereochemical preference of whole-cell biocatalysts on *9* and *11*

Results for reduction of (±)-**9** and (±)-**11** to obtain **10a** and **12a**, respectively, by the cultured whole-cell biocatalyst are summarized in Table 1, and characteristic examples are shown in Scheme 2. For difluoromethyl ketone **11**, *Pichia angusta* JCM 3620 and *G. candidum* NBRC 5767 showed complementary enantiofacial

preference (Table 1, entries 11 and 15) as shown in Scheme 2. *G. candidum* NBRC 5767 showed similar reactivity and selectivity toward both substrates **9** and **11** (entries 15 and 16), while *P. angusta* JCM 3620 reduced **9** to a negligible extent (entry 12). The contrast in behavior between difluoromethyl (**11**) and trifluoromethyl ketone (**9**) with *P. angusta* (entries 11 and 12) is interesting. The downfield H2′ chemical shift of **11** (δ: 6.48) compared with that in a simple non-substituted 2′,2′-difluoroacetophenone (δ: 6.29) was examined. This shift suggests hydrogen bonding between the acetal oxygen atom and proton in difluoromethyl group to give a very compact conformation of **11**, which would be advantageous for a carbonyl reductase in *P. angusta*. In contrast, the trifluoromethyl group of **9** can rotate freely between CF_3-CO, and the CF_3 group is much bulkier, which is a disadvantage to the reductase (Scheme 2).

3.2. Reduction of *9* and *11* with enzymes involved in G. candidum

As shown in the entries 13–16 in Table 1, although *G. candidum* NBRC 4597 and 5767 are the same species, these possess differing reactivity to ketones **9** and **11**. Two types of enzymes have been reported [5,11,13] from these strains. The enzyme from *G. candidum* NBRC 4597 is an "acetophenone reductase [11]," which is responsible for the production of (*S*)-**2** from **1**. In contrast, the enzyme from *G. candidum* NBRC 5767 is a "trifluoromethyl ketone reductase [5]," which reduces **7** to (*S*)-**8**. We became interested in the activity of the above two reductases, toward **9** and **11**.

When using **11** as the substrate, kinetic resolution of the remote chiral center on the acetal (C2) with acetophenone reductase-catalyzed reduction proceeded with a preference for (*R*)-**11**, resulting in generation of (2*R*,1′*R*)-**12a** (83.7% of the four stereoisomers, as shown in Section 2.4) *via si*-facial attack of the hydride on the carbonyl group. Although the activity of acetophenone reductase was very low toward both substrates **9** and **11** (4% and 3% conversion, respectively), it contributed to the formation of (2*R*,1′*R*)- and (2*S*,1′*R*)-**12a** from **11** in the whole cell-mediated *G. candidum* NBRC 4597 reduction with (entry 13 in Table 1).

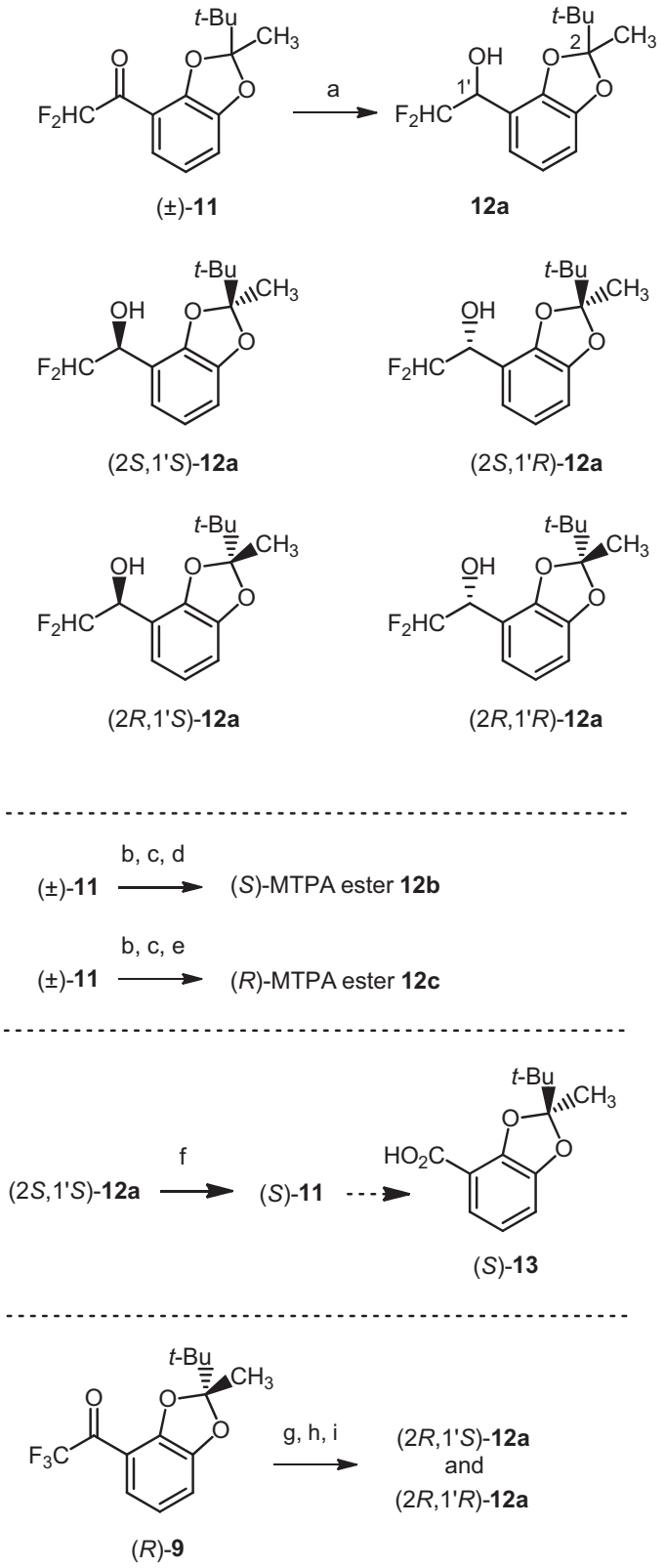
For trifluoromethyl ketone reductase, the activity increased slightly compared with the acetophenone reductase, but was still low (7% conversion for **9** and 12% for **11**, respectively). The products (2*R*,1′*S*)-**12a** (50.4% of the four stereoisomers, as shown in Section 2.5) from **11** and (2*R*,1′*S*)-**10a** (48.9% of the four stereoisomers) from **9** were obtained through *re*-facial selectivity. As mentioned earlier, the major products in the whole cell-mediated *G. candidum* reduction, however, were almost exclusively the stereoisomers (2*R*,1′*S*) and (2*S*,1′*S*) (entries 15 and 16 in Table 1). Upon application of trifluoromethyl ketone reductase, other diastereomers [(2*S*,1′*R*)-**12a** (27.9%) and (2*S*,1′*R*)-**10a** (37.1%)] were formed with the reverse enantiofacial selectivity. These results suggest that unknown reductases exist that are responsible for efficient enantiofacially selective reduction of **9** and **11** by *G. candidum* NBRC 5767.

3.3. Stereochemical assignment of four stereoisomers of *12a*

Because the alcohol **12a** had not been described previously, the stereochemistry in its two chiral centers was determined as shown below, including the Kusumi–Mosher method, for the evidence of

Table 2
Kusumi–Mosher ^{1}H NMR calculation on MTPA esters.

Proton	$\Delta\delta$ H [**12c–b**]	$\Delta\delta$ H [**10c–b**] [7]
H5	+0.16	+0.23
H6	+0.08	+0.13
H7	+0.05	+0.06
H2′	−0.05	–



Scheme 3. Reagents and conditions: (a) $NaBH_4$, EtOH, quant; (b) *Geotrichum candidum* NBRC 5767, 64%; (c) chromatographic separation of diastereomers; (d) (*R*)-MTPA chloride, DMAP, pyridine; (e) (*S*)-MTPA chloride, DMAP, pyridine; (f) 1-Me-AZADO, NaOCl, KBr, CH_2Cl_2, aq. $NaHCO_3$, quant; (g) Mg, TMSCl, THF; (h) TBAF, MeOH; and (i) $NaBH_4$, MeOH, 48%.

the discussion in Sections 3.1 and 3.2. First, the non-stereoselective reduction of racemic difluoromethyl ketone (±)-**11** with $NaBH_4$ was performed to give four possible stereoisomers of **12a**, which produced four independent peaks on HPLC with a Chiralcel OJ-H column.

Reduction with *G. candidum* NBRC 5767 afforded two main isomers of **12a**, which were related to the second and third peaks in the elution order among the four peaks observed using the Chiralcel OJ-H column. The products were diastereomers, as determined by NMR. The less polar isomer related to the second peak was isolated, and derived to the corresponding (*S*)- and (*R*)-α-methoxy-α-trifluoromethylphenylacetate (MTPA) esters **12b** and **12c**, respectively. Upfield signals for H5-7 or aromatic ring and downfield signal for H2′ of difluoromethyl group in the NMR spectra of (*S*)-**12b** compared to (*R*)-**12c** were observed. The two aromatic rings proximate to each other and a difluoromethyl group is separated from the MTPA aromatic ring in **12b** indicated that the absolute configuration of secondary alcohol in **12a** (C1′) obtained by *G. candidum*-mediated reduction was (*S*). The differences in chemical shifts together with (*S*)-**10b** and (*R*)-**10c** [7] are summarized in Table 2. Thus, the mixture of (2*R*,1′*S*)- and (2*S*,1′*S*)-**12a** was revealed to be the second and third peaks, respectively, on HPLC (Scheme 3).

Determination of the absolute configuration of C-2 of (1′*S*)-**12a** was first attempted, by converting it to the (*S*)-TBMB carboxylic acid **13** with known absolute configuration [7] *via* oxidation of the secondary alcohol with 1-Me-AZADO [14] and subsequent haloform reaction of the CHF_2−CO moiety in (*S*)-**11**. Enolate formation prior to halogenation of the difluoromethyl ketone under basic conditions, however, was very slow. Alternatively, an authentic sample of trifluoromethyl ketone (*R*)-**9** [7] was treated under reductive defluorination conditions [12]. Deprotection of the resulting enol trimethylsilyl ether and subsequent reduction of the resulting ketone with $NaBH_4$ provided an authentic mixture of (2*R*,1′*R*)- and (2*R*,1′*S*)-**12a**. This mixture contained the first and third peaks from the HPLC analysis. Combination of all of this information allowed unambiguous determination of the retention times of the four stereoisomers as follows: t_R (min) = 18.3 [(2*R*,1′*R*)-**12a**], 20.4 [(2*S*,1′*S*)-**12a**], 22.4 [(2*R*,1′*S*)-**12a**], 30.5 [(2*S*,1′*R*)-**12a**]. The enantiomeric relation between the isomers of the first and second peaks was further supported by HPLC and NMR analyses of the pure racemic sample [(2*R**,1′*R**)-**12a**], which was obtained from the four stereoisomers.

4. Conclusion

Unique biocatalysts consisting of cultivated yeasts and molds reduced 2′,2′,2′-trifluoro- and 2′,2′-difluoroacetophenone derivatives having very bulky *ortho*-substituents (**9** and **11**) with high catalytic activity and stereoselectivity. New enzymes are likely to be isolated from *P. angusta* JCM 3620 and *G. candidum* NBRC 5767, which demonstrated highly selective *si*-facial and *re*-facial attacks of hydrides, respectively, on newly designed substrates. The advantages of these methods, using whole-cell biocatalysts (regeneration of the reduced form of coenzymes, enzymes immobilized in cell materials, use of biomass as a sink for substrates and products) or isolated enzymes (high concentration of enzymes, clean products), can contribute to the large-scale synthesis of enantiomerically enriched compounds.

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