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Regio and enantioselective reduction of *t*-butyl 6-chloro-3,5-dioxohexanoate with baker's yeast

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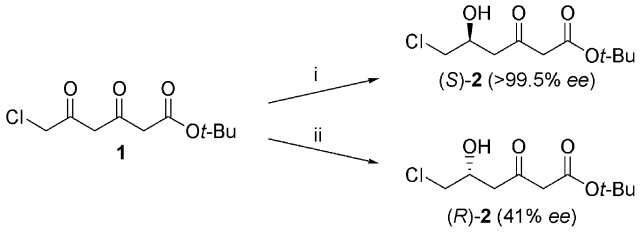
Abstract—Whole baker's yeast cells reduce *t*-butyl 6-chloro-3,5-dioxohexanoate regioselectively to the corresponding C5 hydroxy keto ester. While the (*R*)-alcohol was favored, the enantioselectivity was poor (41% ee). A variety of process conditions were evaluated in order to improve both the enantioselectivity and yield of this reduction. Including a nonpolar resin in the reaction mixture afforded the (*R*)-alcohol in 94% ee and 50% isolated yield. The enantioselectivity was further improved to >99% ee by substituting purified YGL157w in place of whole yeast cells. This reductase was identified by screening a collection of yeast enzymes uncovered by genome sequence analysis.

Optically active δ-hydroxy-β-keto esters are valuable chiral building blocks for the synthesis of chiral drugs and natural products of polyketide origin.[1,2] We recently described a synthesis of the densely functionalized, enantiopure hydroxy keto ester (*S*)-**2** that relies on a highly regio and enantioselective enzymatic reduction of diketo ester **1** (Scheme 1).[3] While the *Lactobacillus brevis* reductase has effectively solved the problem of preparing (*S*)-**2**, the impact of this strategy would be enhanced significantly by ready access to the antipode of this chiral building block in enantiomerically pure form.



Scheme 1. Reagents and conditions: (i) see Refs. 3 and 4; (ii) 20 mM **1**, BY (12 g/mmol **1**), sucrose (200 g L^{-1}), H_2O, 26 h, 20 °C.

This required a reduction biocatalyst with complementary enantioselectivity. Here, we disclose our results on the synthesis of (*R*)-**2**.[4]

A GC–MS-based screen of commercially available biocatalysts revealed that fermenting cells of pressed baker's yeast (BY, *Saccharomyces cerevisiae*) reduced diketo ester **1** at C_5 with high regioselectivity (Scheme 1). Reduction at C_3 occurred to a negligible extent (<5%). Unfortunately, both the enantiomeric excess and the reaction yield of (*R*)-**2** were disappointingly low (41% ee, 52% yield). We, therefore, investigated reaction parameters known to influence the performance of yeast-mediated ketone reductions (Table 1).[5]

Using resting, rather than fermenting yeast cells, provided a higher fractional conversion (72%); however, the enantiomeric excess of (*R*)-**2** increased only slightly (entry 1). By contrast, a biphasic water/*n*-hexane medium significantly improved the enantiomeric purity of (*R*)-**2**. Interestingly, the level of improvement depended on the water/alkane ratio (63–78% ee, entries 2–4). Formation of the desired product was depressed by the presence of the organic phase, however, and conditions providing the highest enantioselectivity (water/hexane = 25:75) afforded a fractional conversion of only 38%. Neither entrapping the biocatalyst in calcium alginate pellets (immobilized baker's yeast, IBY), adding sucrose as a source of reducing equivalents, nor including additives known to inhibit yeast reductase activities

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Table 1. Reduction of diketo ester **1** by baker's yeast (whole cells)

Entry	Yeast[a]	*c* (**1**) (mM)	BY:**1**[b]	H_2O:C_6[c]	H_2O:DBY[d]	Additive (concn)[e]	*t* (h)	(*R*)-**2**[f] (%)	Ee[g] (%)
1	BY	20	12	100:0	—	—	24	72	48
2	BY	18	12	90:10	—	—	22	76	63
3	BY	18	12	50:50	—	—	22	45	73
4	BY	18	12	25:75	—	—	22	38	78
5	IBY	20	12	0:100	—	—	78	40	75
6	IBY	18	12	90:10	—	Sucrose ($100\,g\,L^{-1}$)	29	66	57
7	IBY	20	12	100:0	—	—	6.5	74	49
8	IBY	18	12	90:10	—	—	29	84	61
9	IBY	18	12	90:10	—	$ClCH_2COOMe$ ($0.7\,g\,L^{-1}$)	15	50	66
10	IBY	18	12	90:10	—	$ClCH_2COOH$ ($0.7\,g\,L^{-1}$)	15	40	72
11	IBY	18	12	90:10	—	Crotonic acid ($3.0\,g\,L^{-1}$)	15	53	69
12	IBY	18	12	90:10	—	Allyl bromide ($4.0\,g\,L^{-1}$)	15	47	57
13	DBY1	20	5	100:0	10	—	19	79	69
14	DBY1	20	10	100:0	5	—	19	90 (8)	89
15	DBY1	20	10	90:10	4.5	—	19	89 (8)	80
16	DBY1	20	10	50:50	2.5	—	21	89 (10)	89
17	DBY1	20	10	35:65	1.8	—	19	78 (4)	88
18	DBY1	20	10	20:80	1.0	—	21	72	88
19	DBY1	10	10	25:75	2.5	—	19	67	92
20	DBY1	5	10	13:87	2.5	—	19	62	93
21	DBY2	20	10	50:50	2.5	—	19	84 (11)	72
22	DBY3	20	10	50:50	2.5	—	22	69 (30)	78
23	DBY1	20[h]	10	50:50	2.5	—	14 + 2	59 (6)	94
24	DBY1	20[h]	10	50:50	2.5	—	6 + 1	79 (7)	92.5
25	DBY1	20[h]	10	100:0	5	—	6 + 16	88 (10)	92
26	DBY1	21	10	100:0	4.7	XAD-7 ($22.5\,g\,L^{-1}$)	15	50[i]	94

[a] BY: 'Vital Gold' (Deutsche Hefewerke GmbH, local grocery); DBY1: 'Windhager' emulsifier-free (Bio-Zentrale GmbH, local grocery); DBY2: 'type II' (Sigma); DBY3: 'active dried' (ICN).
[b] Gram cell preparation per mmol **1**.
[c] Ratio water/hexane v/v.
[d] Milliliter water per gram cells.
[e] Preincubation time 0.5–3 h.
[f] Fractional conversion determined by GC–MS after extraction of aliquots with CH_2Cl_2 and derivatization with $(CF_3CO)_2O$/pyridine; numbers in brackets = percent dihydroxyhexanoate (if >5%).
[g] Determined by chiral stationary-phase-HPLC after extraction of aliquots with dichloromethane and cyclization with TFA (Merck ChiraSpher NT, MTBE/MeOH 98:2, 40 °C). Peak identity verified by comparison with both racemic **2** and (*S*)-**2**.
[h] Final concentration (feed).
[i] Isolated yield of purified product.

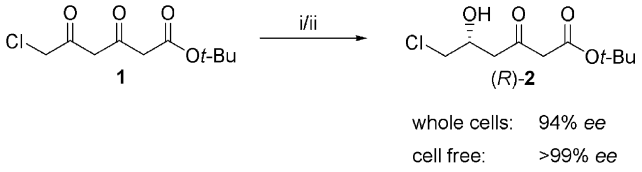
improved the situation. In all cases, increased enantioselectivity was accompanied by decreased formation of (*R*)-**2** (entries 5–12).

On the other hand, significant improvements were found by changing the biocatalyst source. Employing freeze-dried baker's yeast cells at a comparable cell/substrate ratio (DBY1) gave a fractional conversion of 79% along with an enantiomeric excess of 69% under single phase, aqueous conditions (entry 13). A further step forward was achieved by increasing the yeast cell/substrate ratio, which provided a fractional conversion of 90% and an ee of 89% (entry 14). Under these conditions, 8% of the doubly-reduced product *t*-butyl 6-chloro-3,5-dihydroxyhexanoate[4] was also observed (as a 1:1 mixture of diastereomers). Employing DBY1 cells under biphasic conditions did not improve the enantiomeric purity of (*R*)-**2** further (entries 15–18). Moreover, substrate conversion was decreased by low water content, and a minimum of 2.5 mL water per gram of dry cell mass was necessary to rehydrate the cells fully. This meant that the organic phase fraction could only be increased by lowering the substrate concentration if a constant yeast/substrate ratio was maintained. The enantiomeric excess of (*R*)-**2** improved to 93% ee under these conditions; however, the fractional conversion remained unsatisfactory (entries 19 and 20). Yeast cells from two additional suppliers were also examined (DBY2 and DBY3). Both performed worse in terms of enantioselectivity, however (entries 21 and 22). Moreover, DBY3 afforded 30% of the doubly-reduced product.

Based on the above mentioned results, we concluded that maximum enantioselectivity was observed when the aqueous phase concentration of **1** was kept low. Diketo ester **1** was, therefore, added over a period of 14 h, which improved the enantiomeric excess of (*R*)-**2** to 94%. Unfortunately, the fractional conversion was not enhanced (59%, entry 23). An acceptable conversion of 79% with an only slightly diminished ee of 92.5% was realized when the substrate was added over a 6 h period (entry 24). The fractional conversion was further improved by omitting the organic phase (88%, entry 25).

With the optimized reaction parameters in hand, the stage was set for preparative bioconversions (on 0.5–5.2 g scales). We were disappointed to discover that the isolated yields of (*R*)-**2** were limited to 21–34% under

these conditions, although the reductions proceeded with 90–91.5% ee. Product recovery was complicated by the presence of a rather stable emulsion consisting of *n*-hexane, water, and a large amount of cell material. Replacing the liquid organic phase with an adsorber resin (Amberlite™ XAD-7) solved this problem.[6,7] The diketo ester substrate was preadsorbed onto the resin, then it was added to an aqueous suspension of the yeast cells. Slow substrate addition was unnecessary since the adsorber resin acted as a reservoir to maintain a low steady-state aqueous concentration of **1**. Because the product (*R*)-**2** also adsorbed to the resin, it was easily separated from the cell suspension by filtration at the end of the reaction. The product (*R*)-**2** was isolated in a 50% yield after column chromatography under these conditions (94% ee, Scheme 2).[8–10] Control experiments showed that the modest yield was not due to product decomposition during the isolation and purification process.[11]



Scheme 2. Reagents and conditions: (i) 20 mM **1** (18% on XAD-7), DBY 1 (10 g/mmol **1**), H_2O, 20 h, 20 °C; (ii) 5 mM **1**, YGL157wp, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cyclodextrin, KPi buffer, pH 6, 6 h, 30 °C.

While the process optimization studies described above provided an acceptable solution to problems of yield and stereoselectivity in the reduction of **1**, our previous experience suggested that cell free conditions using isolated enzymes might provide an even cleaner bioprocess. The simultaneous operation of multiple yeast reductases with differing stereoselectivities is a major reason that reductions by whole baker's yeast cells often lead to multiple products. Moreover, the large number of potential yeast reductases encoded by the *S. cerevisiae* genome (ca. 50)[12] makes it difficult to eliminate unwanted competitors by selective inhibition[13,14] or gene knockout strategies.[15–17] This situation also makes it difficult to discover which yeast enzyme(s) participate in reducing specific substrates. Since the classical approach to isolating and identifying the yeast reductase(s) responsible for the (*R*)-selective conversion of diketo ester **1** was expected to be a troublesome and lengthy process, we instead took advantage of a library of baker's yeast ketone reductases that was assembled from an analysis of the fully sequenced yeast genome.[18,19] This collection contained 12 of the most promising reductases, each expressed as a fusion protein with glutathione *S*-transferase to allow one-step purification.

Screening our collection of 12 *S. cerevisiae* reductases revealed that three NADPH-dependent members of this library reduced diketo ester **1**: YGL157w, YOL151w, and YDR386w. All three enzymes provided the hydroxy keto ester **2** in the desired (*R*)-configuration and with enantiomeric excess values of >99%, 96.5%, and >99%, respectively (Scheme 2).[20] Enantiopure hydroxy keto ester (*R*)-**2** could be obtained on >0.5 g scales this way.[21]

In conclusion, we have demonstrated that baker's yeast is a favorable biocatalyst for the synthesis of the synthetically valuable hydroxy keto ester (*R*)-**2** by highly regio and enantioselective reduction of **1**. Under optimized reaction conditions involving whole yeast cells, the product (*R*)-**2** was obtained in 94% ee and in 50% isolated yield. The enantioselectivity could be improved even further by employing individual yeast reductases that were identified empirically following genome analysis. Both modes of reduction can be applied on preparative scales. Morever, we have shown that the ability to identify individual biocatalysts that cleanly carry out 'difficult' conversions, coupled with technologies for employing these enzymes under whole-cell[22] or cell-free conditions,[4] demonstrates how collaboration between chemical and biological methodologies can result in highly effective processes.

Acknowledgements

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10. Representative procedure: Commercially available Amberlite™ XAD-7 resin was washed prior to use with

water, acetone, and ethyl acetate, and dried under reduced pressure until the weight remained constant. Dry resin (9.0 g) was added to a solution of diketo ester **1** (2.00 g, 8.5 mmol) in ethyl acetate (70 mL), and the solvent was thoroughly evaporated under reduced pressure. The charged resin was added to a suspension of DBY-1 (85 g) in deionized water (400 mL), and the mixture was shaken at 20 °C (130 rpm, 1 L shake flask, horizontal shaker). After 15 h, the resin was collected on a sintered glass funnel (porosity 0), washed with a minimal amount of water, and extracted with acetone (4 × 50 mL) and ethyl acetate (50 mL). The extract was concentrated under reduced pressure, and the residue dissolved in ethyl acetate. The solution was washed with aq $NaHCO_3$ (5%) and brine, dried over $MgSO_4$, and the solvent evaporated under reduced pressure. Flash chromatography on silica gel (ethyl acetate/isohexane 40:60 v/v) gave hydroxy keto ester (*R*)-**2** as a pale yellow oil. Yield: 1.01 g (50%).

11. Two control experiments were carried out in order to show that neither the reaction nor isolation procedures diminished the yield or enantiomeric excess of (*R*)-**2**. To probe product stability under aqueous reaction conditions, a 50 mg portion of (*S*)-**2** (>99% ee) was dissolved in 1.5 mL of ethanol, then this was added to 50 mL of KP_i (pH 6.0) and the mixture was incubated at 30 °C for 18 h. After extraction with CH_2Cl_2, the combined organics were dried with $MgSO_4$ and concentrated to afford 49 mg of (*S*)-**2** (shown to be >99% ee by chiral-phase HPLC analysis). The stability of the product toward chromatography was demonstrated by filtering a 50 mg portion of (*S*)-**2** through a 1 × 5 cm silica column using 35:65 ethyl acetate/hexanes. The eluent was concentrated to yield 50 mg of (*S*)-**2** whose enantiomeric excess was >99% ee by chiral-phase HPLC analysis.
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20. The following yeast enzymes were screened (as GST fusion proteins): YOL151w, YDR368w, YDR541c, YGL039w, YGL157w, YDL124w, YJR096w, YCR107w, YNL331c, YBR149w, YNL274c and YPL113c. Assay mixtures contained $NADP^+$ (0.15 mg, 0.20 μmol), glucose-6-phosphate (4.3 mg, 14 μmol), glucose-6-phosphate dehydrogenase (Sigma type XV, 5 μg), 5 mM **1**, 1.0 equiv of hydroxypropyl-β-cyclodextrin and 100 μL of purified GST-fusion protein in a final volume of 1.0 mL of 100 mM KPi, pH 7. Reactions were incubated for 24 h at 30 °C and product formation was monitored by GC after extraction with CH_2Cl_2. The YOL151w, YDR368w and YGL157w GST-fusion proteins reduced **1**; none of the remaining nine displayed catalytic activity toward this substrate.
21. Representative procedure: Reaction mixtures contained $NADP^+$ (0.13 g, 0.17 mmol), glucose-6-phosphate (3.5 g, 10.4 mmol), glucose-6-phosphate dehydrogenase (Sigma type XV from baker's yeast, 2 mg), hydroxypropyl-β-cyclodextrin (9.6 g, 6.4 mmol), **1** (0.65 g, 2.8 mmol; dissolved in 50 mL of EtOH) and purified YGL157w fusion protein (180 mg) and 630 mL of 100 mM KPi, pH 6.0. The mixture was incubated at 30 °C for 6 h, then extracted with CH_2Cl_2 (3 × 600 mL). The combined organics were dried with $MgSO_4$ and concentrated in vacuo.
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