Efficient bioreduction of bicyclo[2.2.2]octane-2,5-dione and bicyclo[2.2.2]oct-7-ene-2,5-dione by genetically engineered *Saccharomyces cerevisiae*

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A screening of non-conventional yeast species and several *Saccharomyces cerevisiae* (baker's yeast) strains overexpressing known carbonyl reductases revealed the *S. cerevisiae* reductase encoded by YMR226c as highly efficient for the reduction of the diketones **1** and **2** to their corresponding hydroxyketones **3**–**6** (Scheme 1) in excellent enantiomeric excesses. Bioreduction of **1** using the genetically engineered yeast TMB4100, overexpressing YMR226c, resulted in >99% ee for hydroxyketone (+)-**4** and 84–98% ee for (−)-**3**, depending on the degree of conversion. Baker's yeast reduction of diketone **2** resulted in >98% ee for the hydroxyketones (+)-**5** and (+)-**6**. However, TMB4100 led to significantly higher conversion rates (over 40 fold faster) and also a minor improvement of the enantiomeric excesses (>99%).

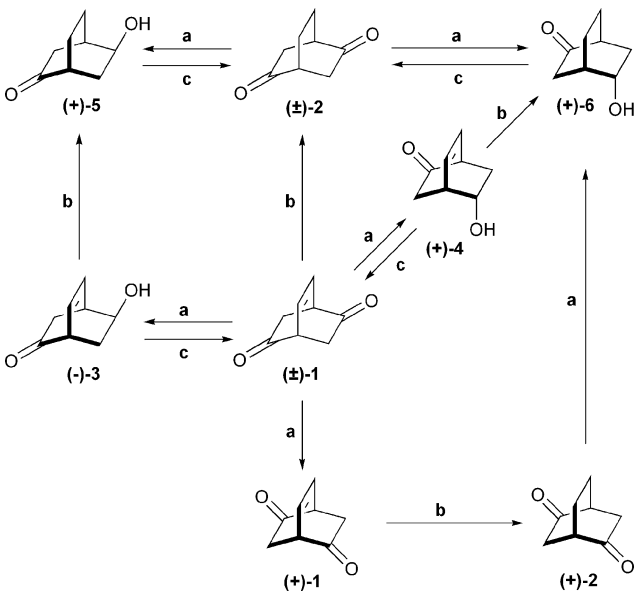
Introduction

Optically active bicyclo[2.2.2]octane derivatives have attracted interest as rigid building blocks in natural product synthesis[1–3] and have recently been investigated as therapeutic agents for cocaine abuse.[4]

The diketones **1** and **2** (Scheme 1) have been used as starting materials for the synthesis of CNS-modulators,[5] and optically active **2** for the synthesis of chiral ligands.[6,7]

Optically active **2** has previously been obtained by resolution of the racemate by fractional recrystallization of its dihydrazone of (*R*)-5-(1-phenylethyl)semioxamazide,[8] by a 1,2-carbonyl transposition route from (1*R*,4*S*,6*S*)-6-hydroxybicyclo[2.2.2]octan-2-one[9] or from its corresponding enantiomer of **1**.[10,11]

The racemic diketone **1** has been resolved as diastereomeric diethyl(*R*,*R*)-(+)-tartrate acetals using HPLC[10] and as an inclusion complex with (*S*)-(−)-10,10-dihydroxy-9,9-biphenantryl.[11] In the work of Hill *et al.*[10] and Lightner *et al.*[12] the diacid **7** (Scheme 2) was also resolved as its brucine salt followed by electrolysis to give (−)-**1** of 90.4% ee, however in low total yield. For the synthesis of the diketones **1** and **2** in multigram quantities of high optical purity we did not find any of these routes satisfactory. Interestingly, Lightner *et al.* also used baker's yeast for the resolution of (±)-**1**, to give hydroxyketone (−)-**3** and unreacted diketone (+)-**1** of varying ee values depending on the incubation time. Although baker's yeast, *Saccharomyces cerevisiae*, is readily available, inexpensive and non-pathogenic,[13] drawbacks such as low reductase activities towards xenobiotic compounds, low yields and low ee values due to competing enzymes with overlapping activities, have often limited its applicability.[14,15] However, the use of recombinant DNA technology can deal with many of these shortcomings. Overexpression of relevant reductases not only increases reaction rates but also reduces the fraction of unwanted products from competing enzymes and yeast metabolism thereby increasing both yield and ee.[15] The reductions can often be achieved with smaller amounts of yeast in a shorter time, thus facilitating product recovery. Moreover, by employing a strain with a decreased phosphoglucose isomerase (PGI) activity, the glucose consumption can be decreased several fold without affecting the supply of the needed NADPH cofactor.[16,17] This decreases the formation of ethanol and other metabolic by-products and prolongs the activity of the yeast by keeping the concentration of inhibitory metabolites low and by delaying glucose depletion. Previous work has shown that the open reading frames (ORFs) YMR226c,



Scheme 1 Reagents and conditions: (a) yeast transformation; (b) H_2, Pd/C, EtOH, 1 atm, rt, 3 h; (c) TPAP, NMO, 3 Å molecular sieves, CH_2Cl_2, rt, 2 h.

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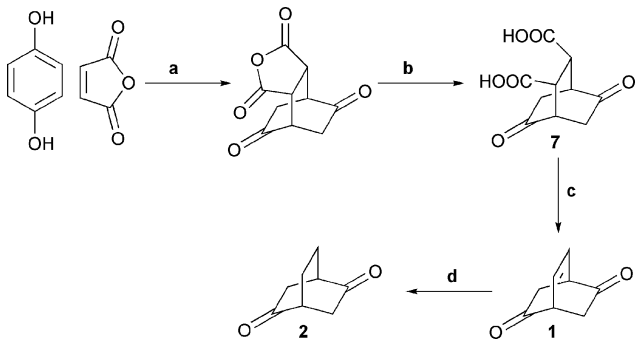


Scheme 2 Reagents and conditions (Paquette *et al.*[10,12]): (a) 210 °C, 2 h (15%); (b) H_2O, 80 °C (90–95%); (c) pyridine, H_2O, NEt_3, 45 V (42%); (d) H_2, Pd/C (10 wt%), EtOH, 1 atm, 3 h (80–100%).

YDR368w (encoding the *YPR1* gene) and YOR120w (*GCY1*) displayed high activities towards bicyclo[2.2.2]octane-2,6-dione,[17] which is structurally similar to **1** and **2**. Also, YOL151w (*GRE2*) has been shown to reduce many dicarbonyls, often with a different selectivity, generating other diastereomers and enantiomers than normally obtained in baker's yeast catalysed reductions.[15] It is also known that non-conventional yeast species can generate other products or reduce compounds not accepted by *S. cerevisiae*[15] and it has been seen in screenings of non-conventional yeast libraries that in particular species of *Rhodoturula* and *Candida* tend to give rise to different stereoisomers.[18,19] Based on these findings we thus anticipated that the bioreduction of (±)-**1** could be improved and that bioreduction of (±)-**2** could be achieved. We here report the screening of several yeast species, both *S. cerevisae* strains with overexpressed reductases and non-conventional yeast strains, for the stereoselective reduction of the diketones (±)-**1** and (±)-**2** to form the hydroxyketones (−)-**3**, (+)-**4**, (+)-**5** and (+)-**6** (Scheme 1) in excellent enantiomeric excesses. It should be noted that these hydroxyketones are not easily obtained directly by conventional Diels–Alder routes and although seemingly uncomplicated structures, (+)-**4** has not previously been reported in the literature and **5** only as a racemic mixture of isomers.[20]

Results and discussion

The racemic diketone **1** was prepared by the method of Lightner *et al.*[12] (Scheme 2) with minor modification. Although the yield in the first step was low (15%), the starting materials are cheap, purification easy and the reaction could be performed in large scale. Racemic diketone **2** has been reported to be available *via* a Diels–Alder reaction of (a) the lithium enolate of cyclohexenone and 2-(*N*-methylanilino)acrylonitrile,[21] (b) 2-((trimethylsilyl)oxy)-1,3-cyclohexadiene and α-acteoxyacrylonitrile[22] or (c) 2-((trimethylsilyl)oxy)-1,3-cyclohexadiene and 2-chloroacrylonitrile.[9] However the dienophile in route (a) requires multistep synthesis and when routes (b) and (c) were attempted, the yields were low (typically varying between 10–25%). For multigram quantities, racemic diketone **2** was most conveniently prepared from **1** by catalytic hydrogenation of the double bond.[11]

In the work of Lightner *et al.*, the best yeast reduction of (±)-**1** (preparative scale) afforded (−)-**3** of 100% ee (12% yield) and (+)-**1** of 85% ee (28% yield) in a reduction period of 5 d.

In a similar experiment, using approximately twice the amount of baker's yeast and an incubation time of 2.5 d, we obtained a somewhat different result. The hydroxyketone (−)-**3** was isolated in 45% yield and 80% ee, the recovered diketone (+)-**1** in 40% yield and 94% ee and the hydroxyketone (+)-**4** was formed in 12% yield and 80% ee. Also, further reduction experiments revealed, in agreement with Lightner *et al.*, that the ee values were depending on the incubation time. Long incubation time led to a higher ee (and lower yield) of the recovered diketone (+)-**1** and a lower ee (and higher yield) of (−)-**3** while the opposite was true for a short incubation time.

To improve the enantioselectivity and possibly the diastereoselectivity in the baker's yeast reduction and to achieve a higher reproducibility of the enantiomeric excesses of the isolated products, a screening of yeast species for the asymmetric reduction of (±)-**1** was performed (Table 1). Non-conventional yeasts and *S. cerevisiae* strains expressing various reductase genes were compared to the control strain TMB4094 and to regular baker's yeast. In addition to the hydroxyketones (−)-**3** and (+)-**4**, small amounts of diols due to over-reduction of the hydroxyketones were observed for several of the recombinant strains. Excellent ee values (>99%) and high yields were obtained for (+)-**4** using the yeasts TMB4100 and TMB4091, both expressing the ketoreductase encoded by YMR226c. However, the overexpression of YDR368w and YOR120w resulted in lower ee values as compared to the control strain. This is most likely the cause for the low enantioselectivity observed in the reduction with baker's yeast, which should contain a higher relative proportion of these reductases as compared to YMR226c. The results for TMB4097 was very similar to baker's yeast and the control strain TMB4094, indicating that the overexpressed YOL151w has very little or no activity towards diketone **1**.

For the hydroxyketone (−)-**3**, a larger variation of the ee values was observed as compared to (+)-**4**. The best ee values were obtained with baker's yeast, TMB4097 (YOL151w) and TMB4094 (control). TMB4100 and TMB4091, both expressing YMR226c, displayed different ee values of the major enantiomer (−)-**3**, varying conversions and amounts of by-products. The ee dependence on reduction time and conversion could be explained if **3** was further reduced to form diols. To investigate this hypothesis, the ee of (−)-**3** was monitored during a prolonged reduction of **1** with TMB4100 (Fig. 1).

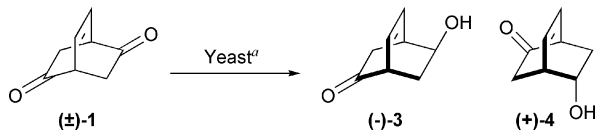
The yield of (−)-**3** reached a maximum of 42% after 3–4 h after which it steadily decreased along with the concomitant formation of diols and a marked drop in ee from 99 to 87%. The yield of ketoalcohol (+)-**4** increased on the other hand until it reached its theoretical maximum of 50% after about 24 h with excellent ee (>99%).

Reductions with non-conventional yeasts showed no improvement in ee for (+)-**4** as compared to baker's yeast, though several *Candida* strains reached a higher conversion. For (−)-**3**, all non-conventional yeasts displayed a lowered ee. A reversed diastereoselectivity, favoring the formation of (+)-**4** over (−)-**3**, was observed for *Candida intermedia*, *Rhodoturula gracilis* and *Rhodoturula mucilaginosa.*

For the generation of (+)-**4** and (−)-**3** a bioreduction of (±)-**1** was performed in a preparative scale (5 g) (Fig. 2).

TMB4100 overexpressing YMR226c and having a 1% PGI activity was deemed the most appropriate strain. The lowered

Table 1 Screening of yeast species for the stereo-selective reduction of (±)-**1**



Yeast strain	(−)-**3** : (+)-**4** ratio	Conversion of (±)-**1** (%)	(−)-**3** ee (%)[b]	(+)-**4** ee (%)[b]
***Saccharomyces cerevisiae* strains**				
Baker's yeast	84 : 16	27	97	81
TMB4100 (1% PGI, YMR226c)	45 : 55	95 (8)[c]	84	>99
TMB4091 (YMR226c)	63 : 37	76 (3)[c]	93	>99
TMB4093 (YDR368w)	52 : 48	49 (<1)[c]	67	16
TMB4096 (YOR120w)	60 : 40	49 (<1)[c]	64	48
TMB4097 (YOL151w)	84 : 16	22	96	77
TMB4094 (control strain, empty plasmid)	90 : 10	25	96	86
Non-conventional yeasts				
Candida boidini CBS8030	86 : 14	<2	69	77
Candida intermedia PYCC4715	43 : 57	8	84	31
Candida tropicalis CBS094	54 : 46	30	76	33
Candida wickerhamii	50 : 50	21	56	72
Rhodoturula gracilis	40 : 60	3	65	54
Rhodoturula mucilaginosa	38 : 62	4	66	69
Pichia pastoris GS115	61 : 39	4	78	72

[a] 5 g l^{-1} dw yeast, 60 g l^{-1} glucose, 100 mM citric acid buffer pH 5.5, 24 h. [b] Determined by GC on an α-DEX capillary column. [c] Figures in parentheses represent the amount of diols formed.

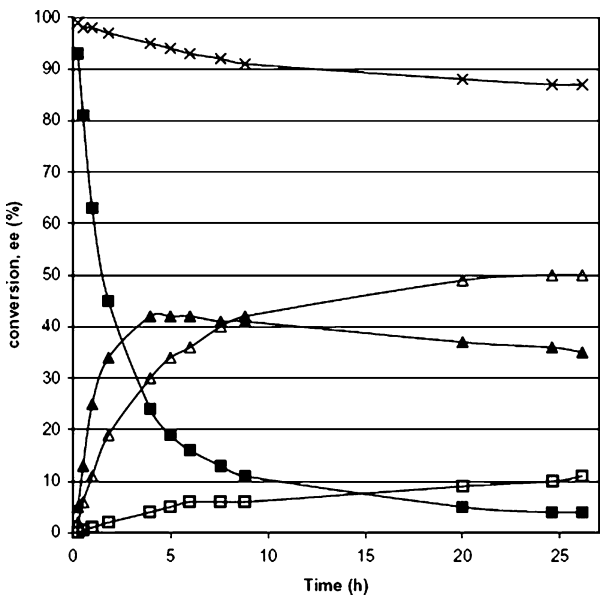


Fig. 1 Bioreduction of 5 g l^{-1} diketone **1** to (−)-**3** and (+)-**4** with 15 g l^{-1} dw TMB4100 in 5 ml scale (X = enantiomeric excess of (−)-**3**; ■ = **1**; ▲ = (−)-**3**; △ = (+)-**4**; □ = diols).

PGI activity in TMB4100 decreases the glucose consumption and ethanol formation rates thereby delaying glucose depletion and allowing NADPH dependent bioreductions to continue to higher product titers. The reaction was run in semi fed-batch mode to decrease possible substrate inhibitory effects, which had been observed for the reduction of a similar bicyclic diketone.[16] Reduction of (−)-**3** proceeded rapidly while (+)-**4** lagged behind as observed in the previous experiment. After about 15 h, most diketone was consumed and another batch was added. Formation of hydroxyketone (−)-**3** soon reached its maximum, while (+)-**4** slowed to a halt after 50–60 h at a concentration of about 15 g l^{-1} (theoretical maximum 20 g l^{-1}). However, a similar experiment using half the concentration of **1** (10 + 10 g l^{-1}) in 100 mg scale showed an almost full conversion to (+)-**4** after 65 h, demonstrating that the substrate concentration is of importance for the conversion. In this experiment a decrease of (−)-**3** was observed in the end due to diol formation (data not shown). The observed difference in conversion to (+)-**4** for the two reductions was likely caused by the inability of the aging yeast to continue to reduce the substrate for the additional time required when the higher substrate concentration was used. This effect could be more pronounced if **1**, (−)-**3** or (+)-**4** have toxic effects on the yeast.

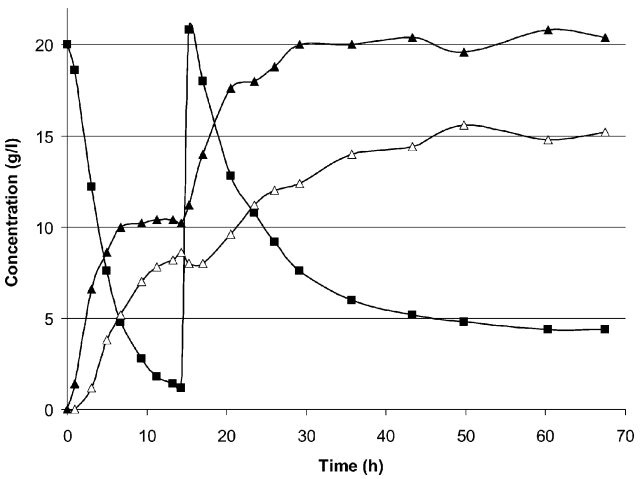


Fig. 2 Semi fed-batch reduction of diketone **1** (■) with 15 g l^{-1} dw TMB4100 to (−)-**3** (▲) and (+)-**4** (△).

The diastereomers (−)-**3** and (+)-**4** were easily separated by column chromatography. Thus, hydroxyketone (+)-**4** of >99% ee was obtained in 32% isolated yield and (−)-**3** of 94% ee in 38%

isolated yield. The diketone (+)-**1** of 98% ee was recovered in 10% yield. A higher total yield of the hydroxyketones was obtained using TMB4100 (70%) as compared to the baker's yeast (62%), but the total amount of recovered material (including unconverted **1**) after work up and purification was lower. In this case 80% for TMB4100 as compared to 97% for baker's yeast. This difference in material recovery is most likely caused by over-reduction of the hydroxyketones to diols, which is more pronounced with TMB4100, which has a higher conversion rate than baker's yeast. Extraction of the diols from the aqueous phase, especially in the preparative scale, was difficult due to their high water solubility. This implies that diols may be present in larger amounts than was observed by GC also for the screening experiments, although a proportionally larger amount of solvent was used for the extraction of those samples than for the preparative scale experiments. For the best possible yields of the hydroxyketones (−)-**3** and (+)-**4** to be obtained, further optimization of the process would be required.

The screening of yeast species for the asymmetric reduction of the racemic diketone **2** (Table 2) revealed several strains able to generate (+)-**5** and (+)-**6** with excellent ee values (>98%). The best results were again obtained using TMB4100 and TMB4091, both overexpressing YMR226c. These two strains also displayed a somewhat changed diastereoselectivity, favouring (+)-**6** over (+)-**5**, as compared to the other *S. cerevisiae* strains. TMB4093 and TMB4096 expressing YDR368w and YOR120w, respectively, showed a decreased ee for (+)-**6**. Baker's yeast and the control strain also produced >98% ee for both diastereoisomers, but had a poorer conversion. Also in these cases, diol formation was observed during prolonged incubation times. However, in contrast to the diastereomer (−)-**3** (from the diketone **1**), over-reduction did not affect the ee of the corresponding isomer (+)-**5**.

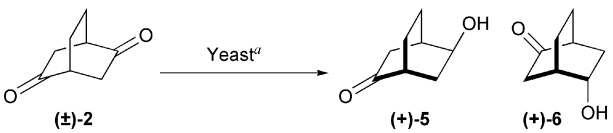
The screening of the non-conventional yeasts did not reveal any strain with improved properties as compared to baker's yeast. Although *Candida boidini* had a somewhat higher conversion of **2**, it also showed a marked decrease in ee for both (+)-**5** and (+)-**6**. None of the other non-conventional yeasts showed any improvement of neither ee nor conversion. Similarly as for diketone **1**, a reversed diastereoselectivity (favoring (+)-**6** over (+)-**5**) was observed with several strains.

Corresponding to diketone **1**, reductions of **2** using baker's yeast and TMB4100 were performed in a preparative scale. The hydroxyketones (+)-**5** and (+)-**6** were isolated as diastereomeric mixtures in 86 and 72% total yield for the baker's yeast and TMB4100, respectively, with ee values similar to those in the screening experiments (Table 2). In the baker's yeast reduction a higher isolated yield was obtained even though full conversion of the starting material was not achieved as was the case for TMB4100. This result can again be explained by the larger amounts of diols formed as a result of over-reduction using the more efficient strain TMB4100. The best yields of the diastereomeric mixture of (+)-**5** and (+)-**6** were obtained at approximately 90% conversion of the diketone. Comparing the conversion rates (g diketone reduced/g yeast × h) TMB4100 was found to be >40-fold faster. In both experiments, small amounts of the diols were isolated; predominantly the *endo*/*exo*-diol, as confirmed by ^{1}H NMR.

Separation of the diastereomeric mixture (+)-**5** and (+)-**6** was not easily achieved. Separation was unsuccessful using column chromatography and the separation by preparative HPLC was insufficient. Several ether and ester derivatives of the mixture were synthesized without improving the ease of purification. Eventually the diasteroisomers were separated by column chromatography as their acetal acetates **9a** and **9b** (Scheme 3).

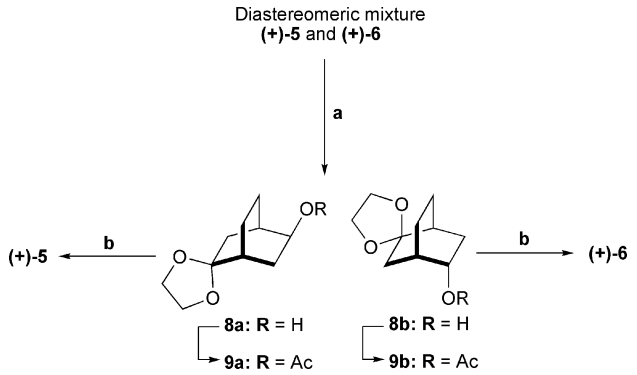
Ketalization followed by esterification (step a) of the diastereomeric mixture of (+)-**5** and (+)-**6** and deprotection (step b) of the separated diastereomers **9a** and **9b** were performed sequentially without purification of the intermediate hydroxy acetal mixtures **8a** and **8b** to give (+)-**5** and (+)-**6** in total

Table 2 Screening of yeast species for the stereoselective reduction of (±)-**2**



Yeast strain	(+)-**5** : (+)-**6** ratio	Conversion of (±)-**2** (%)	(+)-**5** ee (%)[b]	(+)-**6** ee (%)[b]
***Saccharomyces cerevisiae* strains**				
Baker's yeast	58 : 42	26 (<1)[c]	99	99
TMB4100 (1% PGI, YMR226c)	48 : 52	91 (7)[c]	>99	>99
TMB4091 (YMR226c)	45 : 55	98 (12)[c]	>99	98
TMB4093 (YDR368w)	57 : 43	28 (<1)[c]	98	91
TMB4096 (YOR120w)	51 : 49	38 (<1)[c]	98	95
TMB4097 (YOL151w)	58 : 42	28	>99	98
TMB4094 (control strain, empty plasmid)	61 : 39	26	98	99
Non-conventional yeasts				
Candida boidini CBS8030	55 : 45	39	86	88
Candida intermedia PYCC4715	56 : 44	8	98	90
Candida tropicalis CBS094	30 : 70	7	97	66
Candida wickerhamii	48 : 52	19	86	95
Rhodoturula gracilis	22 : 78	6	87	78
Rhodoturula mucilaginosa	25 : 75	4	85	88
Pichia pastoris GS115	28 : 72	10	81	81

[a] 5 g l^{-1} dw yeast, 60 g l^{-1} glucose, 100 mM citric acid buffer pH 5.5, 24 h. [b] Determined by GC on an α-DEX capillary column. [c] Figures in parentheses represent the amount of diols formed.



Scheme 3 Separation of the diastereoisomers (+)-**5** and (+)-**6**. Reagents and conditions: (a) (i) ethylene glycol, PTS, benzene, reflux, 12 h; (ii) acetic anhydride, pyridine, 24 h and (iii) separation of **9a** and **9b** by column chromatography; (b) (i) K_2CO_3, MeOH, H_2O, 24 h and (ii) 2M HCl, acetone, 2 h.

yields of 39 and 38%, respectively, over the entire separation sequence.

As we needed large amounts of the hydroxyketone (+)-**6** for future applications, it was desirable to avoid this separation sequence. Therefore the following route was applied. Hydroxyketone (+)-**6** of >99% ee was obtained in 99% yield by catalytic hydrogenation of (+)-**4** and also by baker's yeast reduction of optically pure (+)-**2** (obtained by resolution with baker's yeast of (+)-**1** followed by catalytic hydrogenation of the double bond). Treating the diketone (+)-**2** with 12 g l^{-1} dw TMB4100 for 6 h selectively gave (+)-**6** of >99% ee in 85% yield. In this experiment, the *endo*,*endo*-diol was also isolated in 5% yield. Treating optically pure (+)-**2** with baker's yeast resulted in slow conversion of the starting material. After a reduction period of 7 d with yeast and glucose additions every 48 h, the reaction was stopped and (+)-**6** was isolated in only 65% yield. It should be noted that although the diketone (+)-**1** obtained from the baker's yeast reduction is not optically pure, the enantiomeric excess could be increased to >99% ee by recrystallization from diisopropyl ether. Although these routes resulted in hydroxyketone (+)-**6**, the most efficient one was to reduce diketone (±)-**1** to (+)-**4** using TMB4100 followed by catalytic hydrogenation of the double bond (Scheme 1).

The formation of only the diastereoisomer (+)-**6** from (+)-**2** also confirms the absolute configuration of (+)-**6**. Lightner *et al.* previously determined the absolute configuration of (−)-**3** and thus the absolute configuration of (+)-**5** was confirmed by hydrogenation of the double bond of (−)-**3**.

Oxidation of the hydroxyketones **3**–**6** using tetrapropylammoniumperruthenate (TPAP) smoothly afforded the optically active diketones (+)-**1**, (−)-**1**, (+)-**2** and (−)-**2** in 91–100% yield with ee values corresponding to their precursor hydroxyketones (Scheme 1).

Conclusions

The screening of the non-conventional and genetically modified yeasts identified several yeast strains that markedly improved the bioconversions of the diketones **1** and **2** to their corresponding hydroxyketones. The genetically modified yeast TMB4100 was successfully applied for the synthesis of the optically active hydroxyketones **3**–**6** with high ee values. These compounds are otherwise not straightforwardly obtained *via* baker's yeast reduction due to poor conversion rates and, for (−)-**3** and (+)-**4**, only moderate ee values were obtained. In this screening, the selection of yeast strains was based on the previous knowledge that certain yeast reductases were able to reduce the related bicyclic compound bicyclo[2.2.2]octane-2,6-dione.[17] As for **1** and **2** in this work, overexpression of YMR226c was found to give the highest reduction rate. However, for bicyclo[2.2.2]octane-2,6-dione, YDR368w gave the highest optical purity, >98%, compared to 93% for YMR226c.[17] This demonstrates that even though reductases may have different selectivities for related substrates, a limited screening of modified yeast strains could be sufficient to quickly identify a suitable strain when reductases accepting similar compounds are already known.

This work also presents a route to the diketones **1** and **2** in high enantiomeric excesses through the oxidation of their corresponding hydroxyketones. The results presented herein may allow for improvements of conversion rates, yields and enantiomeric excesses for substrates where regular baker's yeast previously has been used with limited success.

Experimental

General

Compressed commercial baker's yeast (Kronjäst from Jästbolaget AB, Sollentuna, Sweden), was purchased from a local distributor. *Saccharomyces cerevisiae* CEN.PK113-7A [*MAT a*, *MAL-8c*; *SUC2*; *his3-Δ1*] was a gift from Dr P. Kötter, Institute of Microbiology, Frankfurt, Germany. *S. cerevisiae* strains overexpressing YMR226c (TMB4091), YDR368w (TMB4093),[23] YOR120w (TMB4096)[17] and a control with an empty plasmid (TMB4094)[23] were previously constructed from a CEN.PK background. TMB4100 (YMR226c) was previously constructed from *S. cerevisiae* RBY 7–1 (ENY.WA-1A, with 1% PGI activity, encoded by YBR196c) as described elsewhere.[17]

The non-conventional yeast strains used in the screening were the following: *Candida wickerhamii* obtained from the Yeast Culture Collection of the University of the Free State, Bloemfontein, South Africa, *Candida tropicalis* CBS094, *Candida boidini* CBS8030 *Candida intermedia* PYCC4715, *Rhodotorula mucilaginosa* and *Rhodotorula gracilis* obtained from the Division of Food Biotechnology and Microbiology, Warsaw Agricultural University, Poland and *Pichia pastoris* GS115 (Invitrogen). *Escherichia coli* DH5α (Life Technologies, Rockville, MD) was used for all subcloning.

GLC (Perkin-Elmer AutoSystem XL) was performed with a Factor Four capillary column (Varian, 30 m × 0.25 mm, 0.25 μm film thickness) and with an α-DEX 120 permethylated α-cyclodextrin fused silica capillary column (Supelco, 30 m × 0.25 mm, 0.25 μm film thickness) for determination of enantiomeric compositions. The samples were analysed at 100 °C (60 min) −2 °C min^{-1} −180 °C. The retention times were: 79.34 min ((+)-**3**), 79.64 min ((−)-**3**), 83.84 min ((−)-**4**), 84.17 min ((+)-**4**), 84.45 min ((−)-**5**), 84.80 min ((+)-**5**), 86.67 min ((−)-**6**) and 86.98 min for (+)-**6**.

HPLC analyses were performed on a Chiralcel OD–H column (250 × 4.6 id, 5 μm) (Daicel) on a Varian Prostar system equipped with a PDA detector. Flow rate: 1.0 ml min^{-1}, detection at 220 nm.

Solvent: hexane–2-propanol 90 : 10, 13.6 min ((−)-**1**), 19.3 min ((+)-**1**).

All NMR spectra were recorded on a Bruker ARX 300 spectrometer, unless otherwise indicated, using $CDCl_3$ ($CHCl_3$ δ 7.26 (^{1}H) and 77.9 (^{13}C)) or C_6D_6 (C_6H_6 δ 7.16 (^{1}H) and 128.39 (^{13}C)) as solvents. Column chromatography was performed on Matrex (25–70 μm) silica gel. TLC was carried out on silica gel (60 F_{254}, Merck), the plates were impregnated with a solution of $KMnO_4$ (10 g), K_2CO_3 (50 g), 5% NaOH (20 ml) and H_2O (900 ml) and the compounds visualized upon heating. All R_f values are given using heptane–EtOAc 1 : 1 as the mobile phase. Melting points were taken on a Sanyo Gallenkamp melting point apparatus (MPD.350.BM3.5) and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 22 °C and are given in 10^{-1} deg cm^{2}g^{-1}. Elemental analyses were performed by H. Kolbe Mikroanalytisches Laboratorium. Organic extracts were dried using Na_2SO_4 throughout.

General description 1

For the baker's yeast catalysed reductions performed on a preparative scale, the following procedure was applied: Sucrose (35.5 g) and Baker's yeast (35.5 g compressed yeast corresponding to 9.6 g dry weight) was added to a solution of diketone (22.0 mmol) in water (185 ml). The resulting suspension was slowly stirred at 30 °C for 60 h ((±)-**1**) (48 h for (±)-**2** and 7 d for (+)-**2**) and then filtered through a pad of Celite. The resulting yeast cake was incorporated in the Celite by stirring with a spatula to remove lumps before it was carefully rinsed with EtOAc. The aqueous phase was extracted with EtOAc (5 × 150 ml) and dried. The organic phase was filtered and the solvent removed at reduced pressure.

General description 2

For the preparative scale reductions using the genetically engineered yeast TMB4100, the following procedure was applied: bioreductions were performed at 30 °C in 100 mM citric acid buffer, pH 5.5 supplemented with 60 g l^{-1} of glucose. Reductions in 5 ml scale were run in glass vials with rubber stoppers equipped with magnetic stirrers and syringes for outlet gas and sampling. Reductions in 90 and 120 ml scale were performed in a 150 ml water tempered fermentor equipped with a magnetic stirrer and a cotton plugged syringe for outlet gas. Cell free medium from the reductions was collected by centrifugation at 4000 g for 5 min. The reductions were performed with 15 g l^{-1} dry weight TMB4100 except for the reduction of (+)-**2** which used 12 g l^{-1} dw. The reduction times were 67.5 h ((±)-**1**) (6 h for (±)-**2** and 6.5 h for (+)-**2**). The yeast was separated from the reaction mixtures by centrifugation and the resulting yeast pellets were washed once with a small amount of Milli-Q water (10% of the reaction volume). The wash water was combined with the reaction medium and the combined aqueous phase was repeatedly extracted with EtOAc and dried. The organic phase was filtered and the solvent removed at reduced pressure.

General description 3

For the screening of yeast species for asymmetric reduction of **1** and **2** to give **3**–**6**, the following procedure was applied: Screening for whole-cell reduction of (±)-**1** and (±)-**2** was made with 5 g l^{-1} dry weight yeast in 1 ml medium composed of 100 mM citric acid buffer, pH 5.5 and 60 g l^{-1} of glucose using 5 mg of (±)-**1** or (±)-**2**. The experiments were carried out at 30 °C on a rocking table in 1.5 ml Eppendorf tubes provided with syringe holes for outlet gas for 24 h. The reaction mixtures were extracted with EtOAc (5 ml), dried (Na_2SO_4), filtered and analyzed by GC.

Molecular biology methods

Plasmid DNA was prepared with the Biorad miniprep kit (Hercules, CA). DNA enzymes were obtained from Fermentas (St. Leon-Rot, Germany) or Life Technologies (Rockville, MD). DNA extractions from PCR reactions and agarose gels were made using the QIAquick extraction kit (Qiagen, Hilder, Germany).

Competent DH5α *E. coli* cells were prepared using the method of Inoue *et al.*[24] Yeast transformation was made as described elsewhere.[25] *E. coli* transformants were selected on Luria–Bertani (LB) medium[26] plates with 100 μg ml^{-1} ampicillin (IBI Shelton Scientific, Inc., Shelton, CT). *E. coli* transformants were grown over night in LB medium with 100 μg ml^{-1} ampicillin. *S. cerevisiae* transformants were selected on minimum medium agar plates containing 40 g l^{-1} glucose, 6.7 g l^{-1} nitrogen base and 20 g l^{-1} agar.

Construction of strain TMB4097

The YOL151w (GRE2) gene was amplified from chromosomal *S. cerevisiae* DNA using primers containing specific restriction sites (Table 3). The shuttle vector p423-GPD (with *HIS3* marker) and the YOL151w PCR product were digested with *Bam*HI, *Spe*I and ligated at 16 °C overnight. The resulting plasmid p423GPD-YOL151w (*HIS3*) was amplified in *E. coli* DH5α and used to transform *S. cerevisiae* CEN.PK113-7A generating TMB4097.

Yeast growth

Yeast strains were kept at −80 °C and streaked on rich agar plates containing 20 g l^{-1} glucose, 20 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 15 g l^{-1} agar and 100 mM phosphate buffer pH 6.3, except TMB4100 which was streaked on the same medium but with 20 g l^{-1} fructose and 1 g l^{-1} glucose. Colonies were taken from the agar plates and used to inoculate 25–200 ml medium in shake flasks with sterile cotton applicators. Non-conventional yeasts were grown in

Table 3 Primers and restriction sites (bold) for the construction of the *S. cerevisiae* strain TMB4097 overexpressing the reductase encoded by YOL151w

Primer for amplification of YOL151w (GRE2)	Restriction site
5′-AA**ACTAGT**AACAGATAGCAGTATCACACGCCCGTAAAT-3′	*Spe*I
5′-AA**GGATCC**GAAGAGAAAAATGCGCAGAGATGTACTAGATGAT-3′	*Bam*HI

20 g l^{-1} glucose, 20 g l^{-1} tryptone, 10 g l^{-1} yeast extract in 100 mM phosphate buffer pH 6.3. TMB-strains were grown in 20 g l^{-1} glucose and 7 g l^{-1} yeast nitrogen base in 100 mM phosphate buffer pH 6.3, except TMB4100 which was grown in the same medium but with 20 g l^{-1} fructose, 1 g l^{-1} glucose and supplemented with 50 mg l^{-1} of tryptophan, uracil and leucine. The cells were grown over-night at 30 °C and 200 rpm, except TMB4100 which was grown for 40 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed once with Milli-Q water. Cell concentration was determined by dry weight. Culture broth (1–5 ml) was filtered and the filter (0.45 μm membrane filter, Pall) was washed with 3 volumes of distilled water and dried in a microwave oven to constant weight (7 min, 350 W). Dry weight measurements were carried out in duplicate.

(±)-Bicyclo[2.2.2]oct-7-ene-2,5-dione (1). Compound **1** was prepared by modification of the literature procedure[12] as follows. A solution of (±)-5,7-dioxobicyclo[2.2.2]octan-2,3-dicarboxylic acid (18.5 g, 81.8 mmol) in pyridine (150 ml), water (150 ml) and triethylamine (25 ml) was electrolyzed using Pt electrodes at 45 mV (0.8 A) in an open water-cooled (5–10 °C) vessel for 6 h. The reaction mixture was concentrated at reduced pressure to give a black tar where after aqueous sat. NaCl (100 ml) was added. The resulting mixture was acidified to pH 1 with conc. HCl and worked up as follows: extraction with EtOAc (3 × 100 ml) followed by drying, filtration and removal of the solvent at reduced pressure. The residue was column chromatographed (SiO_2, heptane–EtOAc 2 : 1) and the fractions containing **1** (R_f 0.46) were concentrated to give impure **1** as a yellow oil. Recrystallization from isopropyl ether gave **1** (4.70 g, 42%) as white needles: mp 95–98 °C (lit.[10]: mp 95–99 °C). ^{1}H NMR data were consistent with those reported.[10]

Synthesis of (−)-3 and (+)-4 by baker's yeast reduction

Following the general description 1, (±)-**1** (3.00 g, 22.0 mmol) was reduced with baker's yeast. The residue was purified by column chromatography (SiO_2, heptane–EtOAc 4 : 1 until (+)-**1** eluted and then 1 : 1, TLC R_f 0.46 for (+)-**1**, 0.21 for (+)-**4** and 0.11 for (−)-**3**) to give recovered (+)-**1** (1.19 g, 40%) of 94% ee (HPLC, Chiralcel OD–H) as a white solid (mp 97–100 °C; $[\alpha]_D$ +1116 (*c* 0.22, $CHCl_3$)) (lit.[12]: mp 98–100 °C; $[\alpha]_D$ +1005 (*c* 0.107, $CHCl_3$)).

(1*R*,4*R*,5*S*)-5-Hydroxybicyclo[2.2.2]oct-7-en-2-one ((+)-4). Hydroxyketone (+)-**4** (0.38 g, 12%) of 80% ee (GC, α-DEX 120) was obtained as a pale yellow semi-solid; ($[\alpha]_D$ +397 (*c* 1.00, *t*BuOMe)). IR (KBr) 3426, 1715 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 6.39 (1 H, t, *J* 7.3), 6.19 (1 H, t, *J* 7.1), 4.10 (1 H, m), 3.07 (1 H, m), 2.96 (1 H, m), 2.65 (1 H, dd, *J* = 18.5 and 1.3), 2.14 (2 H, m), 1.94 (1 H, dd, *J* 18.5 and 1.8), 1.51 (1 H, dt, *J* = 14.0 and 2.5). ^{13}C NMR ($CDCl_3$) δ 212.5, 135.3, 129.8, 67.2, 49.0, 40.5, 33.3, 32.7. HRMS (FAB$^+$, direct inlet) [M + H] calcd for $C_8H_{11}O_2$: 139.0759; found 139.0743. (Found: C, 69.68; H, 7.24%. $C_8H_{10}O_2$ requires C, 69.54; H, 7.30%).

(1*S*,4*S*,5*S*)-5-Hydroxybicyclo[2.2.2]oct-7-en-2-one ((−)-3). Hydroxyketone (−)-**3** (1.37 g, 45%) of 80% ee (GC, α-DEX 120) was obtained as a white solid; (mp 149–158 °C; $[\alpha]_D$ −463 (*c* 0.27, $CHCl_3$)) (lit.[12]: 140–157 °C; $[\alpha]_D$ −528 (*c* 0.123, $CHCl_3$)). ^{1}H NMR data were consistent with those reported.[12]

Synthesis of (−)-3 and (+)-4 by reduction with the genetically engineered yeast TMB4100

Following general description 2, (±)-**1** (5.00 g, 36.7 mmol) was reduced with TMB4100. The reduction was performed in semi fed-batch mode with a starting concentration of 20 g l^{-1} of (±)-**1** (125 ml scale). Another batch of substrate together with 60 g l^{-1} of glucose was added when the reaction had slowed down and most diketone was reduced. The residue was purified as described for the baker's yeast reduction above to give recovered (+)-**1** (0.49 g, 10%) of 98% ee (HPLC, Chiralcel OD–H) as a white solid (mp 88–96 °C; $[\alpha]_D$ +1252 (*c* 0.55, $CHCl_3$)), (+)-**4** (1.60 g, 32%) of >99% ee (GC, α-DEX 120) as a white solid (mp 149–152 °C; $[\alpha]_D$ +542 (*c* 0.52, *t*BuOMe)) and (−)-**3** (1.91 g, 38%) of 94% ee (GC, α-DEX 120) as a white solid (mp 163–165 °C; $[\alpha]_D$ −554 (*c* 0.41, $CHCl_3$)). ^{1}H NMR data were consistent with those reported for the baker's yeast reduction above.

Synthesis of (+)-5 and (+)-6 by baker's yeast reduction

Following general description 1, **2** (2.50 g, 18.1 mmol) was reduced with baker's yeast. The residue was purified by column chromatography (SiO_2, heptane–EtOAc 1 : 1, TLC R_f 0.15) to give an inseparable mixture of the diastereomers (+)-**6** and (+)-**5** (2.19 g, (86%) as a white solid; (mp 235–238 °C; $[\alpha]_D$ +5.99 (*c* 1.84, EtOH)).

Separation of the diastereoisomers (+)-5 and (+)-6 *via* the acetals (+)-9a and (+)-9b

Ethylene glycol (12.0 ml, 214 mmol) and PTS (140 mg, 810 μmol) was added to a mixture of (+)-**5** and (+)-**6** (2.00 g, 14.3 mmol) in benzene (50 ml) under an argon atmosphere in a Dean–Stark apparatus. Water removal was performed at reflux temperature over night and then cooled to ambient temperature where after diethyl ether (150 ml) was added. The resulting solution was washed sequentially with sat $NaHCO_3$ (2 × 30 ml) and water (30 ml) followed by drying, filtration and removal of the solvent at reduced pressure to give a diastereomeric mixture of hydroxy acetals **8a** and **8b** (2.20 g, 84%) as a transparent oil; TLC R_f 0.25, which was used directly in the next step. An analytical sample was column chromatographed (pentane–diethyl ether 3 : 2) and a pure fraction of the hydroxy acetal (+)-**8a** was obtained as a transparent oil.

(1*R*,2*S*,4*R*)-[Spiro[bicyclo[2.2.2]octan-5,2′-[1,3]dioxolane]]-2-ol ((+)-8a). $[\alpha]_D$ +11.8 (*c* 2.99, *t*BuOMe). IR (NaCl) 3387 cm^{-1}. ^{1}H NMR (C_6D_6) δ 4.04 (1 H, m), 3.63 (2 H, m), 3.50 (2 H, m), 2.46 (1 H, m), 2.20–1.95 (4 H, m), 1.89 (1 H, dt, *J* 14.3 and 2.8), 1.76 (1 H, q, *J* 3.1), 1.70–1.61 (2 H, m), 1.55–1.32 (2 H, m). ^{13}C NMR (C_6D_6) δ 110.9, 68.5, 64.3, 64.3, 39.7, 34.9, 33.7, 33.6, 21.9, 18.1. HRMS (FAB$^+$, direct inlet) [M + H] calcd for $C_{10}H_{16}O_3$: 184.1099; found 184.1111. (Found: C, 65.03; H, 8.76%. $C_{10}H_{16}O_3$ requires C, 65.19; H, 8.75%).

Acetic anhydride (3.50 ml, 36.9 mmol) was added to a solution of the crude mixture of **8a** and **8b** (1.70 g, 9.23 mmol) in pyridine (7.8 ml) under an argon atmosphere. The resulting solution was kept at room temperature for 24 h and then filtered through a 10 cm plug of silica (heptane–EtOAc 9 : 1) for removal of excess pyridine. The solvent was removed at reduced pressure and the

residue was column chromatographed (SiO_2, heptane–EtOAc 87 : 13) to give (+)-**9a** (1.01 g, 50%) as an oil (TLC R_f 0.63) and (+)-**9b** (0.94 g, 47%) as an oil (TLC R_f 0.57).

(1*R*,2*S*,4*R*)-[Spiro[bicyclo[2.2.2]octan-5,2′-[1,3]dioxolane]]-2-yl acetate ((+)-9a). $[\alpha]_D$ +9.1 (*c* 0.55, *t*BuOMe). IR (KBr) 2949, 2874, 1734, 1367, 1248, 1121, 1020 cm^{-1}. ^1H NMR (C_6D_6) δ 5.10 (1 H, m), 3.57–3.32 (4 H, m), 2.49 (1 H, m), 2.00–1.74 (5 H, m), 1.69 (3 H, s), 1.54 (1 H, p, *J* 3.1), 1.52–1.42 (1 H, m), 1.35 (2 H, m). ^{13}C NMR (C_6D_6) δ 170.1, 110.4, 72.0, 64.4, 64.3, 39.4, 33.4, 31.6, 31.0, 21.7, 21.2, 18.8. HRMS (FAB^+, direct inlet) [M + H] calcd for $C_{12}H_{19}O_4$: 227.1283; found 227.1276. (Found: C, 63.78; H, 7.94%. $C_{12}H_{18}O_4$ requires C, 63.70; H, 8.02%).

(1*S*,2*S*,4*S*)-[Spiro[bicyclo[2.2.2]octan-5,2′-[1,3]dioxolane]]-2-yl acetate ((+)-9b). $[\alpha]_D$ +17 (*c* 0.90, *t*BuOMe). IR (KBr) 2939, 2872, 1732, 1371, 1242, 1132, 1024 cm^{-1}. ^1H NMR (400 MHz, C_6D_6) δ 4.86 (1 H, m), 3.51 (2 H, m), 3.37 (2 H, m), 2.39 (1 H, m), 1.98 (2 H, m), 1.88 (1 H, m), 1.81 (1 H, m), 7.73 (1 H, ddd, *J* 14.3, 3.3 and 1.6), 1.67 (3 H, s), 1.53 (1 H, p, *J* 3.0), 1.48 (1 H, m), 1.26 (1 H, m), 1.05 (1 H, m). ^{13}C NMR (C_6D_6) δ 170.3, 110.5, 71.6, 64.4, 64.2, 35.8, 33.7, 32.0, 31.6, 22.6, 21.2, 20.8. HRMS (FAB^+, direct inlet) [M + H] calcd for $C_{12}H_{19}4_2$: 227.1283; found 227.1286. (Found: C, 63.49; H, 7.88%. $C_{12}H_{18}O_4$ requires C, 63.70; H, 8.02%).

Deprotection of (+)-9a and (+)-9b to give (+)-5 and (+)-6. K_2CO_3 (2.26 g) was added to a mixture of (+)-**9a** (1.13 g, 5.00 mmol) (or (+)-**9b** (1.00 g, 4.42 mmol)) in MeOH (29 ml) and water (11 ml). The resulting mixture was stirred at room temperature for 24 h where after the MeOH was removed at reduced pressure. The mixture was concentrated to approximately 29 ml followed by acidification with 2 M HCl where after acetone (29 ml) was added. The reaction mixture was stirred at room temperature for 2 h before it was concentrated to approximately 35 ml and then extracted with EtOAc (5 × 50 ml). Drying of the organic extracts, filtration and removal of the solvent at reduced pressure was followed by column chromatography (SiO_2, heptane–EtOAc 1 : 2).

(1*R*,4*R*,5*S*)-5-Hydroxybicyclo[2.2.2]octan-2-one ((+)-5). Hydroxyketone (+)-**5** of >99% ee (GC, α-DEX 120) was obtained as a white solid in 92% yield. TLC R_f 0.14; mp 241–242 °C (melt and subl.); $[\alpha]_D$ +4.15 (*c* 2.73, EtOH). IR (KBr) 3426, 1727 cm^{-1}. ^1H NMR ($CDCl_3$) δ 4.11 (1 H, m), 2.40–2.10 (6 H, m), 2.06 (1 H, br s), 1.93 (1 H, m), 1.78 (1 H, m), 1.62 (1 H, m), 1.49 (1 H, m). ^{13}C NMR ($CDCl_3$) δ 216.6, 68.0, 42.6, 41,7, 35.6, 33.9, 23.1, 17.5. HRMS (FAB^+, direct inlet) [M + H] calcd for $C_8H_{13}O_2$: 141.0916; found 141.0917. (Found: C, 68.42; H, 8.70%. $C_8H_{12}O_2$ requires C, 68.54; H, 8.63%).

(1*S*,4*S*,5*S*)-5-Hydroxybicyclo[2.2.2]octan-2-one ((+)-6). Hydroxyketone (+)-**6** of >99% ee (GC, α-DEX 120) was obtained as a white solid in 96% yield. TLC R_f 0.14; mp 151 °C (subl.); $[\alpha]_D$ +13.1 (*c* 2.70, EtOH). ^1H NMR data were consistent with those reported.[9]

Synthesis of (+)-5 and (+)-6 by reduction with the genetically engineered yeast TMB4100

Following the general description 2, (±)-**2** (100 mg, 0.72 mmol) was reduced with TMB4100. The reduction was performed in batch mode at a concentration of 20 g l^{-1} of (±)-**2** (5 ml scale). The residue was purified as described for the baker's yeast reduction above to give an inseparable mixture of the diastereomers (+)-**5** and (+)-**6** (73 mg, (72%), both of >99% ee (GC, α-DEX 120), as a white solid; (mp 243–244 °C; $[\alpha]_D$ +8.55 (*c* 1.45, EtOH)).

Synthesis of (+)-6 by baker's yeast reduction

Following the general description 1, (+)-**2** (1.23 g, 8.91 mmol) was reduced with baker's yeast. Additional yeast (12.5 g) and sucrose (14 g) was added every 48 h. The residue was purified by column chromatography (SiO_2, heptane–EtOAc 1 : 1, TLC R_f 0.14) to give (+)-**6** (807 mg, (65%) of >99% ee (GC, α-DEX 120) as a white solid; (mp 151 °C (subl.); $[\alpha]_D$ +13.1 (*c* 3.57, EtOH).

Synthesis of (+)-6 by reduction with the genetically engineered yeast TMB4100

Following the general description 2, (+)-**2** (1.80 g, 13.0 mmol) was reduced with TMB4100. The reduction was performed in batch mode at a concentration of 20 g l^{-1} of (+)-**2** (90 ml scale).

The residue was purified as described for the baker's yeast reduction above to give (+)-**6** (1.56 g, 85%) of >99% ee (GC, α-DEX 120) as a white solid (mp 151 °C (subl.); $[\alpha]_D$ +13.6 (*c* 2.52, EtOH)) and the *endo*,*endo*-bicyclo[2.2.2]octane-2,5-diol (87 mg, 5%) (as determined by comparison to an authentic sample by ^1H and ^{13}C NMR[9]).

Synthesis of (−)-1, (+)-1, (−)-2 and (+)-2 by TPAP oxidation

N-Methylmorpholine-*N*-oxide (51 mg, 0.43 mmol), 3 Å crushed molecular sieves (150 mg) and tetrapropylammonium perruthenate (3.8 mg, 5 mol%) were added to a solution of the hydroxyketones (**3**–**6**) (0.22 mmol) in CH_2Cl_2 (5 ml) under an argon atmosphere. The resulting mixture was stirred at room temperature for 2 h and then filtered through a pad of silica (bottom layer) and celite (top layer), rinsing with EtOAc. The solvent was removed at reduced pressure and the residue was column chromatographed (SiO_2, heptane–EtOAc 1 : 1) to give (−)-**1**, (+)-**1**, (−)-**2** and (+)-**2** in 91–100% yield. For (−)-**1** (from (−)-**3** of 94% ee): mp 101–103 °C; $[\alpha]_D$ −1222 (*c* 0.55, $CHCl_3$), (+)-**1** (from (+)-**4** of >99% ee): mp 89–94 °C; $[\alpha]_D$ +1256 (*c* 0.55, $CHCl_3$), (−)-**2** (from (+)-**5** of 88% ee): mp 208–213 °C (melt and subl.); $[\alpha]_D$ −48 (*c* 0.42, $CHCl_3$) and (+)-**2** (from (+)-**6** of >99% ee): mp 212–213 °C (melt and subl.); $[\alpha]_D$ +50 (*c* 0.44, $CHCl_3$), respectively.

Synthesis of (+)-5 and (+)-6 by catalytic hydrogenation

The hydrogenation catalyst, 10 wt% Pd/C (327 mg) was added to a mixture of (−)-**3** (100 mg, 0.72 mmol) (or (+)-**4** (300 mg, 2.17 mmol)) in ethanol (10 ml for (−)-**3**) or 30 ml for (+)-**4**) at room temperature. The resulting slurry was stirred under a hydrogen atmosphere at room temperature at atmospheric pressure for 3 h and then filtered through a pad of silica (bottom layer) and celite (top layer). The solvent was removed at reduced pressure and the residue purified by column chromatography (SiO_2, heptane–EtOAc 1 : 1) to give (+)-**5** (or (+)-**6**) in 99% yield. For (+)-**5** (from (−)-**3** of 80% ee): mp 238–239 °C; $[\alpha]_D$ +1.45 (*c* 1.45, EtOH) and (+)-**6** (from (+)-**4** of >99% ee): mp 151 °C (subl.); $[\alpha]_D$ +13.6 (*c* 2.61, EtOH).

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