Effects of caffeine on stereoselectivities of high cell density biotransformations of cyclic β-keto esters with *Saccharomyces cerevisiae*

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Caffeine affects the stereoselectivity of microbial high cell density reductions with commercial grade *Saccharomyces cerevisiae* (Baker's yeast). Cyclic β-keto esters ethyl 2-oxocyclopentanoate (**1**) and ethyl 2-oxocyclohexanoate (**3**) were shown to be reduced with increased diastereoselectivity (**1**: 90.1 → 92.1% de, **3**: 75.0 → 90.0% de) after addition of caffeine. Effects on enantioselectivity were less pronounced (**1**: 97.3 → 98.5% ee, **3**: 90.1 → 92.1% ee). The observations are ascribed to the action of caffeine on cellular calcium homeostasis. These effects are accompanied by caffeine-induced cell-death, which preferably takes effect on pre-stressed cells which were found to decrease diastereoselectivity.

Introduction

The stereoisomerically pure cyclic *cis*-β-hydroxy esters **2** and **4** are important chiral pharmaceutical intermediates (CPI) for macrolide[1] and carbapeneme antibiotics,[2] chymotrypsin inhibitors,[3] HIV protease inhibitors[4] as well as for natural product synthesis.[5] Above all, they have received attention as promising chiral synthons for producing oxazolidinone antibiotics[6] for which reason their synthesis has been investigated thoroughly.[7] However, formation of the *cis*-configured species is thermodynamically unfavourable, since the standard free energy of formation ΔG° is higher for the respective *trans*-products (Fig. 1). This can easily be demonstrated by incubation of **2** and **4**, respectively, with 0.5 eq. NaOEt in ethanol at 60 °C, where 64% (**4**: 62%) isomerisation to the *trans*-isomer was observed within 1 h. Consequently, asymmetric catalytic hydrogenation mostly favours *trans*-product formation, and enantio- as well as diastereoselectivities differ greatly (Table 1, entries 3–5, 9, 10).[7c,7d] Where satisfactory diastereoselectivity is achieved, enantioselectivity drops to almost racemic mixtures (entries 6, 12).[7e]

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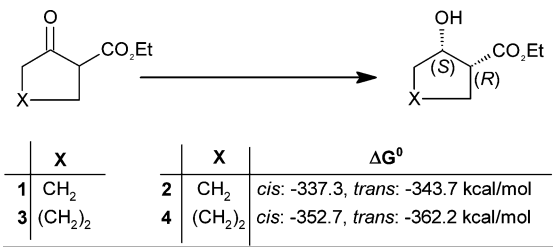

Fig. 1 Base catalysed isomerisation of cyclic *cis*-hydroxy esters to thermodynamically more stable *trans*-products.

Table 1 Synthetic methods for the production of stereoisomerically pure *cis*-(1*R*,2*S*)-β-hydroxy esters **2** and **4**

			Stereopurity (1*R*,2*S*)		
Entry	Product	Method	ee (%)	de (%)	Ref
1	**2**	Whole-cell-biotransformation (*Saccharomyces cerevisiae*)	97	97	9
2			n.d.[a]	60	7*a*
3		Whole-cells (*Daucus carota*)	97	n.d.	7*b*
4			96	−92	7*c*
5		Catalytic hydrogenation	95	−94	7*d*
6			11	94	7*e*
7		Enzymatic resolution of racemates	99	n.d.	7*f*
8	**4**	Whole-cell-biotransformation (*Saccharomyces cerevisiae*)	91	75	9
9			96	72	7*a*
10		Whole-cells (*Daucus carota*)	98	n.d.	7*c*
11		Catalytic hydrogenation	88	8	7*d*
12			14	94	7*e*
13		Enzymatic resolution of racemates	99	n.d.	7*f*
14			86	n.d.	7*g*
15			95	n.d.	7*h*

[a] n.d.—no data

Enzymatic variants are often tedious, some exhibit long reaction times (3–10 d), and conversions are mostly low (5–50%). While enzymatic resolutions of racemates, *e.g.* with *Candida antarctica* lipase B (CAL-B, entries 7, 13), proceed with high enantioselectivity, there are no data available for the diastereopurity of the obtained products.[7f,7g,7h,8]

Based on the literature data available (Table 1), the microbial reduction with *S. cerevisiae* appeared as the most promising method. And, in contrast to other variants, this methodology allows facile up-scaling into the gramme- to kilogramme-scale.[9] Furthermore, no limitations concerning microorganism availability are encountered, since Baker's yeast is produced commercially on a large scale.[10] Yet, a major drawback of whole-cell biotransformations of **2** and **4** with commercial grade yeast results from unsatisfactory stereopurity which, in addition, tends to vary from batch to batch.

Because of their significance as chiral synthons in the synthesis of pharmaceutically active compounds and natural products, attempts were undertaken to optimise the whole-cell biocatalytic syntheses of CPI **2** and **4** towards reproducibly good yields and stereoselectivities. In this article, we wish to report on the reasons for the varying product stereopurities and the outcome of caffeine addition as a remedy to overcome these limitations in microbial reductions of cyclic β-keto esters **1** and **3**.

Results and discussion

The microbial reduction is initiated by the highly substrate specific and highly diastereoselective reduction of the (1*R*)-configured β-keto ester substrate to give the (1*R*,2*S*)-hydroxy ester as a single product. After 18–20% conversion, (1*S*,2*S*)- and (1*S*,2*R*)-stereoisomers were also detectable in measurable amounts, while the (1*R*,2*R*)-diastereomer was not observed at all, even at the end of the bioprocess (Fig. 2).

The aim of these investigations was to optimise stereoselectivities of the whole-cell biotransformations, and the major challenge was to understand the underlying intracellular processes on a molecular level.

First indications were obtained from experiments that demonstrated that pure **4** is partially re-oxidised to give (*R*)-**3** (Fig. 3). After isomerisation of the latter to (*S*)-**3**, *trans*-isomer **11** was formed, which according to Jalloh, is 1.6 times less cytotoxic (maximal tolerated dose MTD(**4**) = 1.55 mmol g^{-1} cell dry weight, MTD(**11**) = 2.54 mmol g^{-1} cell dry weight).[11] Since **4** cannot be formed but by (2*S*)-carbinol selective reduction of (1*R*)-configured substrates, there must exist additional (*S*)-**3**-reducing enzyme activity contributing to product formation. As a consequence, not only re-oxidised material, but also unconverted substrate is reduced to undesired stereoisomers **11** and **12** (Fig. 3). Upon exposure to air and pure oxygen, **4** has proven chemically and configurationally stable.

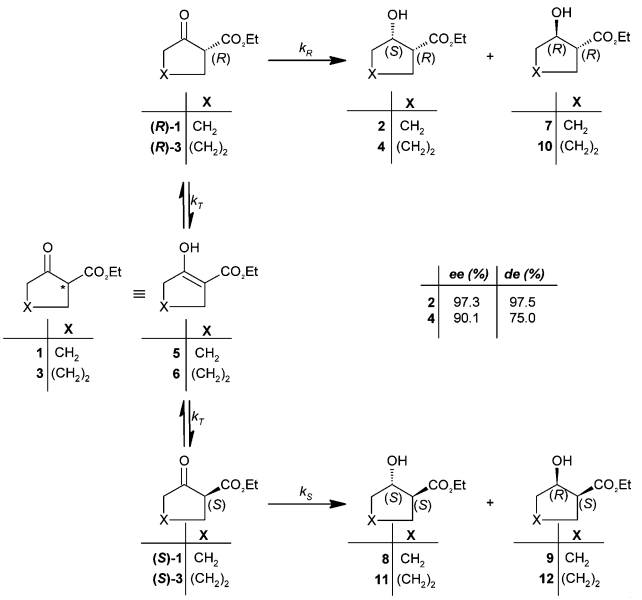

Fig. 2 Stereoselective reduction of cyclic β-keto esters. The chiral substrate exists in a tautomeric equilibrium consisting of the (1*R*)- and (1*S*)-configured optical antipodes and their enol. Enzyme substrate complex formation with the (1*R*)-substrate irrevocably determines configuration of the α-ester carbon in the product. Stereoselective reduction at C-2 furnishes a diastereomeric mixture of *cis*- and *trans*-hydroxy esters. Enantiomer formation requires enzyme substrate complex formation with the (1*S*)-substrate. For this reason, the biocatalytic approach to stereopure cyclic β-hydroxy esters is superior to conventional attempts—under the condition that there is no competing (1*S*)-specific enzyme activity as encountered in stressed cells.

Improving stereoselectivity

Initially, since not all enzymes involved in the stereoselective reduction of **1** and **3** have been identified and characterised yet,[12] established dehydrogenase inhibitors allyl alcohol,[13] methyl vinyl ketone (MVK),[14] allyl bromide[15] and ethyl chloroacetate[16] had been tested for their stereodirecting properties. However, no improvement of stereoisomer distribution was detected.

For that reason, an alternative concept was pursued, namely the perturbation of the intracellular enzyme network by a physiologically active substance (PAS). A living yeast cell can be understood in terms of a multi-enzyme system with different dehydrogenases acting on a substrate. And, as intracellular enzymes are highly counter-regulated, affecting one enzyme's activity may well be conceived to affect other enzyme's activities.[17] The observed stereoselectivity of a whole-cell biotransformation results from the sum of the overall dehydrogenase activity. Since altered growth conditions may greatly affect cell physiology, varying reaction conditions will have a considerable outcome on the complex cellular enzyme network and therefore on stereoisomer distribution

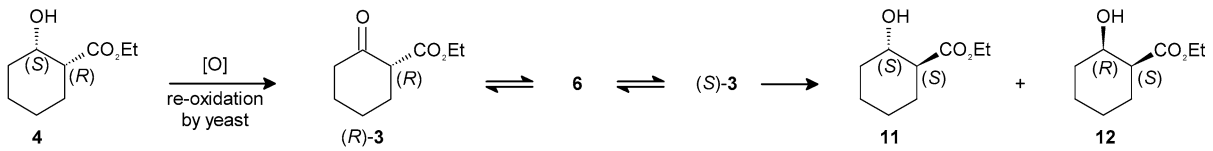

Fig. 3 Reoxidation of cyclic β-hydroxy esters and subsequent reduction cause competing stereoisomer formation.

Table 2 Effect of heat shock on stereoselectivities of the bioreductions **1** → **3** and **2** → **4**

Entry	*T*/°C	Product	ee (%)	de (%)
1	30	**2**	97.3	97.5
2	38		43.7	56.6
3	30	**4**	90.1	75.0
4	38		78.0	67.2

in the product.[18] Moreover, already the substrates themselves exert physiological effects because of which biotransformations of xenobiotics generally should be regarded as a major impact on cellular homeostasis. In other words, the cells experience stress, defined by Hohmann and Mager as 'any deviation from optimal life conditions for the cell'.[19] How deeply biotransformations of xenobiotics may interfere with cell physiology can be derived from Table 1, entries 1,2,8,9, where varying reaction conditions had a considerable outcome on stereoisomer distribution.

From earlier experiments, it is known that stressed cells produce the undesired (1*S*,2*S*)- and (1*S*,2*R*)-stereoisomers.[7i] Therefore, as a working hypothesis, we ascribed the differing product stereopurities to the presence of stress proteins with dehydrogenase activity. This interpretation was supported by heat shock experiments at 38 °C, which led to far lower stereoselectivities than bioconversions conducted at 30 °C (Table 2).

The participation of stress proteins with dehydrogenase activity is not surprising. In fact, we were able to demonstrate that also osmotic stress or acid stress did affect the stereoisomer distributions of whole-cell biotransformations.[20] Further, dehydrogenase activity of stress proteins is a common motif in stress responses.[21] For these reasons, the initial idea to improve stereoselectivities of the biotransformations of **1** and **3** was to impose additional stress on the whole-cell biocatalysts with the aim of completely interfering with the stress physiology of the cells. For these purposes, caffeine was chosen since this compound exhibits broad physiological activity.[22]

Influence of caffeine on stereoselectivity

Caffeine generally improved the stereoselectivity of *S. cerevisiae* mediated bioreductions. Enantiopurity of ethyl-2-oxocyclopentanoate (**1**) increased from 97.3 to 98.5% ee and that of ethyl-2-oxocyclohexanoate (**3**) from 90.8 to 92.1% ee. Diastereomeric excess of the reduction **1** → **2** increased (97.5 to 98.9% de). Likewise did the microbial conversion of the six-membered ring homologue **3** → **4**, where an even more substantial increase from 75.0 to 90.0% de was observed. Moreover, the effect of caffeine is concentration dependent with an optimal concentration of 50 mM. Above this value, cytotoxic effects of caffeine interfered with the biotransformation (Fig. 4), resulting in decreased diastereoselectivity and loss of cell vitality. From these results, it is evident that the perturbation concept proved successful and that caffeine-induced effects on eukaryotic cell physiology have an outcome on stereoselectivities of whole-cell biotransformations. Yet, substrate conversion and chemoselectivity of biotransformations of cyclic substrates **1** and **3** remained unaffected by caffeine.

However, although stereoselectivities of the microbial reductions **1** → **2** and **2** → **4** increased considerably by addition of caffeine, it has to be emphasised that improvements of bioreductions with additives mostly rely on empirical considerations. It must be, for this reason, the aim to establish a thorough understanding of microbial biotransformations on a molecular level. The latter would thus allow developing rational strategies to control the outcome of bioreductions and render biocatalytic processes more efficient. Therefore, further work was invested to investigate potential mechanisms underlying caffeine action on resting cells of *S. cerevisiae* in whole-cell biotransformations.

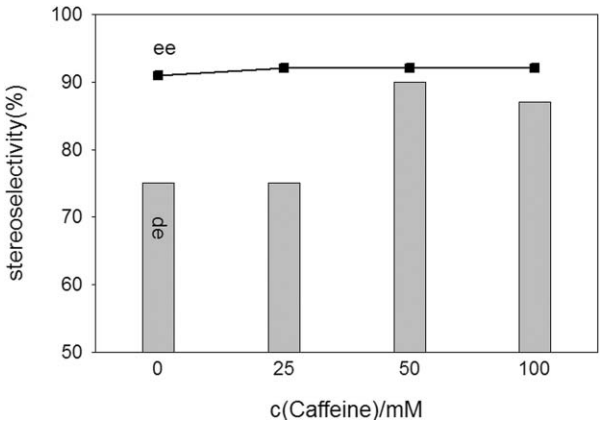

Fig. 4 Influence of caffeine on stereoselectivity of **3** → **4** whole-cell bioreduction with *S. cerevisiae*.

Influence of Ca^{2+} on stereoselectivity

As reported by Courchesne and Ozturk, caffeine affects calcium flux into yeast cells, for which reason altered intracellular Ca^{2+}-levels were the first candidates in the investigation of effects caused by caffeine.[23] This appeared reasonable, since intracellular Ca^{2+}-levels are known to considerably act on stereoisomer distribution in whole-cell biotransformations of β-keto esters.[24] Caffeine blocks Ca^{2+}-channels (*e.g.* through ion channels Mid1p and Cch1p) and therewith uptake of extracellular Ca^{2+}, but does not completely eliminate release of Ca^{2+} from intracellular stores.[23a] Hence, stereoselectivity of a microbial reduction can be conceived to be dependent on calcium homeostasis in yeast. In order to check whether these affects apply to the biotransformations of **1** and **3**, caffeine was added in different amounts to a medium containing 100 mM $CaCl_2$, the concentration of which had previously been shown to affect stereoselectivity.[24] As shown in Fig. 5, caffeine-fed cells are insensitive to $CaCl_2$, and $CaCl_2$ induced effects on stereoselectivity appear compensated by caffeine.

From this experiment, it is evident that in fact Ca^{2+}-uptake into cells is blocked by caffeine and that low intracellular Ca^{2+}-levels have an outcome on the diastereoselectivity of a biotransformation. Here, the effects favoured formation of the (1*R*,2*S*)-stereoisomer.

Influence of Mg^{2+} on stereoselectivity

Magnesium salts are reported to direct stereoselectivity of a microbial biotransformation in an opposite way to Ca^{2+}.[25] Therefore, with the purpose of further elucidating the stereodirecting effects of calcium ions, and in order to check for potential effects of Mg^{2+} on stereoselectivity, biotransformations were conducted with up to 100 mM $MgCl_2$ using MgO as a pH buffer instead

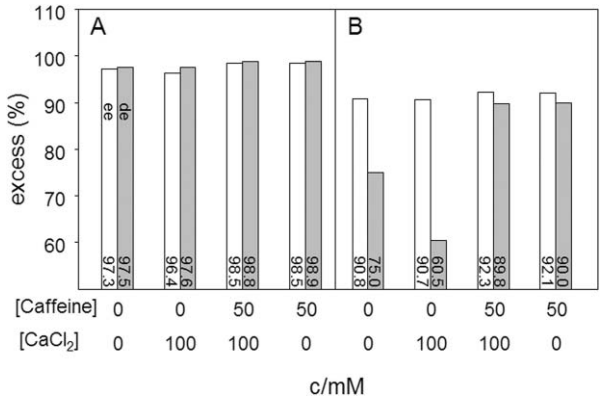

Fig. 5 Influence of caffeine and $CaCl_2$ on the stereoselectivity of **A**: **1** → **2** biotransformation, and **B**: **3** → **4** biotransformation.

of $CaCO_3$. However, stereoselectivity of the biotransformations was not significantly altered. This finding indicates that cellular ion homeostasis remained intact for the tested magnesium salt concentrations.[26]

Influence of caffeine and β-keto esters on cell vitality

β-Keto-esters **1** and **3**, as well as caffeine, impaired cell vitality of the biocatalyst *S. cerevisiae*, with **3** exhibiting a more pronounced effect (Fig. 6). Where caffeine was administered in combination with **1**(**3**) the fraction of vital cells was reduced by up to 20%. Although cell vitality decreased, conversion remained unaffected. Moreover, stereoselectivity was increasing to the abovementioned values (Fig. 4). What, at first sight, appeared surprising was finally the clue to the mode of action of caffeine in whole-cell biotransformations.

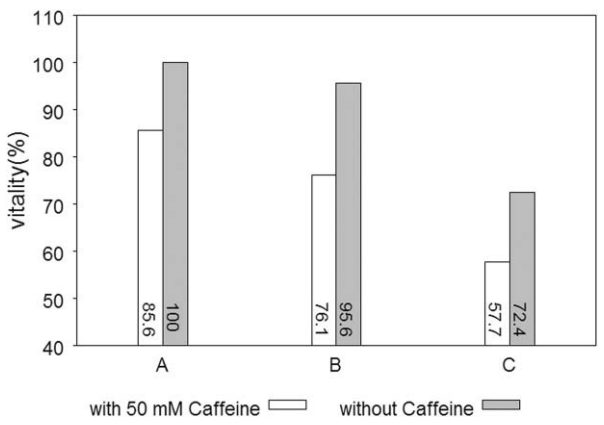

Fig. 6 Effect of β-keto esters **1**, **3** and caffeine on cell vitality of *S. cerevisiae*. **A**: Reference. **B**: Incubation of yeast cells with **1** for 24 h. **C**: Incubation of yeast cells with **3** for 24 h.

To start with, these results indicate an overall cell number sufficient to fully convert the β-keto esters. It has to be stressed at this point that the cells used were commercial grade. They were applied as supplied for the purpose of a biotransformation in order to reflect conditions in industrial biotechnology.[27] In fact, the whole-cell biocatalyst material consisted of intact cells that produce the desired (1*R*,2*S*)-diastereomer. A minor amount of cells, however, was capable of producing (1*S*,2*S*)- and (1*S*,2*R*)-stereoisomers. It appears reasonable that these cells have been pre-stressed by the environmental conditions in the course of manufacturing, transport and storage prior to use in a biotransformation. As caffeine is described to cause apoptosis in stressed cells,[28] it could have caused specific cell death of this pre-stressed yeast subpopulation finally resulting in higher stereoselectivities. Apoptosis will have an effect on the stereoselectivity of the biotransformation, because the capability of a cell to produce (1*S*)-specific reductase activity is the outcome of cell-stress.

In addition, caffeine exhibits broad physiological activity including inhibition of cAMP hydrolysing phosphodiesterase,[29] and phosphatidyl inositol kinase related protein kinases like the DNA-integrity checkpoint protein kinase Mec1p,[30] or the targets of rapamycin, Tor1p and Tor2p.[31] Since these caffeine-sensitive proteins are a part of signal transduction pathways (namely protein kinase A (PKA) and protein kinase C (PKC)/mitogen activated protein kinase (MAPK) pathways), all of their targets may also be affected, each of which increases the effects of caffeine on cell physiology. Last but not least, caffeine induces cell wall stress by activation of the cell integrity pathway.[31b]

For the purpose of determining the extent of a potential caffeine induced apoptosis in *S. cerevisiae*, flow cytometry experiments, in combination with fluorescent probes, were conducted in order to measure formation of reactive oxygen species (ROS), which constitute a key apoptotic feature.[32]

These investigations experienced further verification by tests with freshly prepared cultures. The stereoselectivities were in the range of those reported for caffeine addition. It has to be noted, however, that in commercial applications, cell densities $>10^{10}$ cells per mL come into use. This value is far beyond what can be achieved by regular cell growth, where 10^6–10^7 cells per mL are obtained. However, concentration processes in order to produce high density cell cultures for commercial scale applications provoke chemical (*e.g.* osmosis) and/or physical cell stress (shearing forces). Therefore, the addition of caffeine is an invaluable tool to increase product stereoselectivities of high cell density biotransformations with cells from whatever source.

Caffeine induced apoptosis

In order to characterise the observed cell death, DNA content and formation of reactive oxygen species (ROS) were measured by flow cytometry. The results of flow cytometric measurements of ROS formation in the course of the biotransformations of **1** and **3** are depicted in Fig. 7. The density plots show that cells exposed to **3** alone, or in combination with caffeine, form increased amounts of ROS, whereas less ROS are formed in the absence of caffeine, indicating the occurrence of an apoptotic like process. The same applied to **1** where effects were less pronounced, as this compound is a less potent ROS inducer. Consequently, apoptosis is more likely to occur with cells exposed to a combination of caffeine and β-keto ester. Treatment with caffeine or β-keto ester alone resulted in cell death without apoptotic markers.

During the cytometric analysis of ROS formation, counterstaining cells with propidium iodide (PI) served to assay cell vitality.[32b] Dead cells were expected to appear as a uniform peak with high fluorescence intensity. After 24 h, the histograms of samples treated with caffeine and either **1** or **3** showed PI-positive peaks with lower fluorescence intensity (Fig. 8) indicating less stained DNA. This

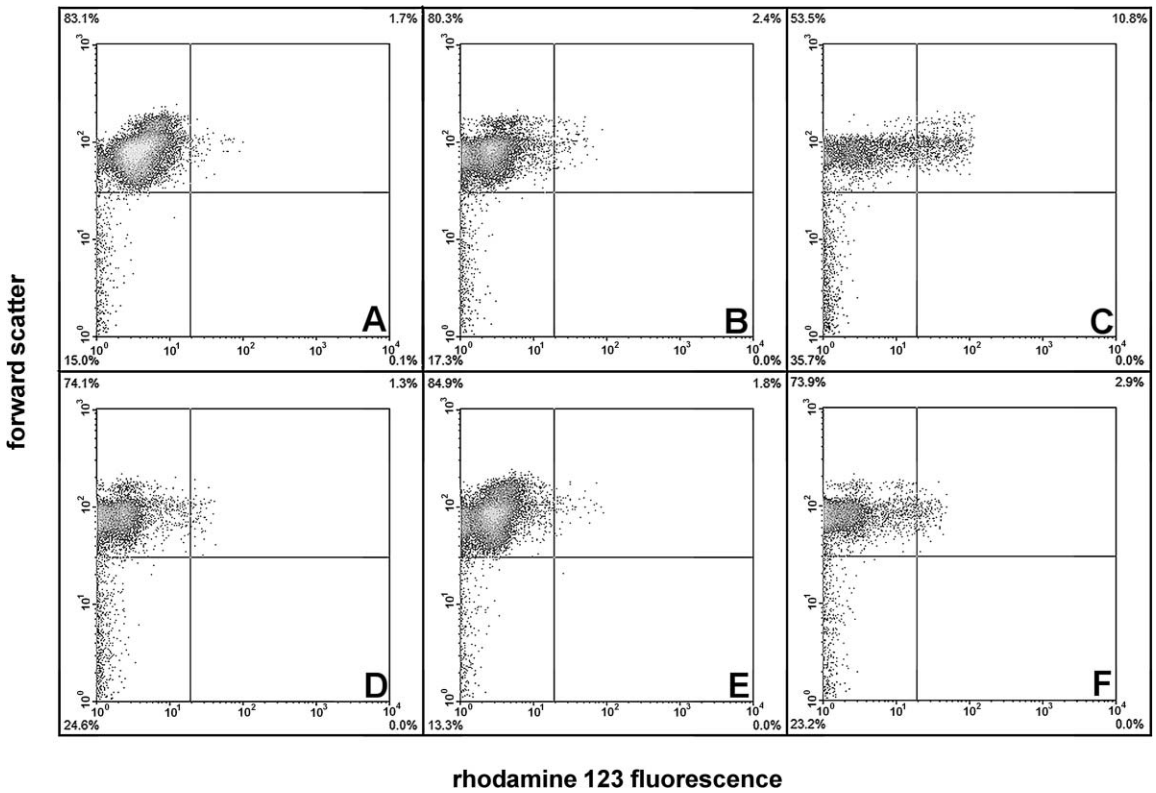

Fig. 7 Density plots obtained through flow cytometry of dihydrorhodamine 123 stained yeast cells, which were treated with or without caffeine in the presence of **1** or **3** for 5 h. (**A**) Reference. (**B**) Yeast cells treated with **3**. (**C**) Yeast cells treated with **3** and 50 mM caffeine. (**D**) Yeast cells treated with 50 mM caffeine. (**E**) Yeast cells treated with **1**. (**F**) Yeast cells treated with **1** and 50 mM caffeine. Quadrants were defined on the basis of reference (**A**).

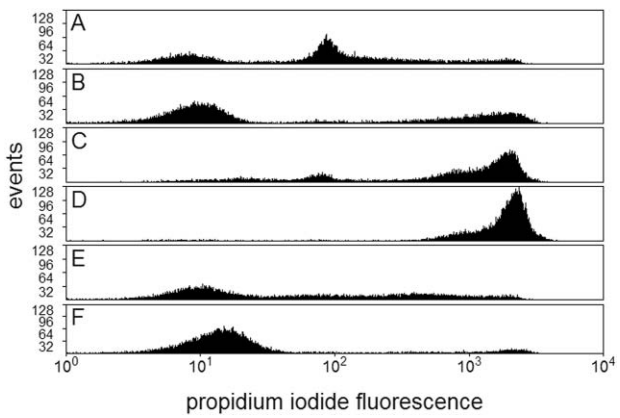

Fig. 8 Histograms obtained by flow cytometry of propidium iodide (20 μg mL^{-1}) stained yeast cells, which were treated with or without caffeine (50 mM) in the presence of **1**, **3** for 24 h. (**A**) Cells treated with **1** and caffeine. (**B**) Cells treated with **1**. (**C**) Cells treated with **3** and caffeine. (**D**) Cells treated with **3**. (**E**) Cells treated with caffeine. (**F**) Reference.

effect is either attributable to (i) the activation of endonucleases with subsequent loss of low molecular weight DNA fragments or (ii) a change in conformation of DNA (chromatin condensation) leading to reduced dye absorption capacity.[33] Appearance of the latter—a so called sub-G1 peak—provides evidence for apoptotic cells. Thus, these results support our hypothesis of apoptotic cell death induced by **1**, **3** in combination with caffeine.

The broad physiological activity of caffeine constitutes an adaptational challenge to yeast cells. It affects pre-stressed cells, and has an outcome on the bioreduction, where caffeine addition contributes to a decrease in the formation of undesired (1*S*,2*S*)- and (1*S*,2*R*)-stereoisomers by apoptotically eliminating the pre-stressed subpopulation, which exhibits the highest (1*S*)-specific reductase activity.

These results are in line with the finding that caffeine addition had no effect on the conversion rate, although the cell number was reduced. The biotransformatory capacity of pre-stressed cells spreads on the formation of three stereoisomers, *i.e.* they contribute to (1*R*,2*S*)-product formation only to a minor extent. Therefore, the caffeine-induced loss of these cells can be compensated by intact cells. The same applies for the simultaneous addition of caffeine and substrate, *i.e.* the biotransformation, where the fraction of vital cells was reduced by up to 20%.

Further characterisation of apoptotic cell-death in *S. cerevisiae* exposed to xenobiotics by determining phosphatidylserine translocation,[34] caspase activity[35] and mitochondrial function[34] are a matter of forthcoming investigations and beyond the scope of this contribution.

Conclusions

The biocatalytic synthesis of *cis*-configured cyclic β-hydroxy esters **2** and **4** with high cell density cultures of *S. cerevisiae* was improved by applying a new concept, namely the perturbation of the cells' enzymatic network by a physiological active substance

(PAS). Two different modes of action become effective upon caffeine addition: (i) pre-stressed cells are stimulated to commit apoptosis (programmed cell death). (ii) Intracellular calcium levels are decreased. The combination of both factors is responsible for the observed increase in stereoselectivity.

The former drawback of unsatisfactory diastereoselectivities could be overcome by a facile easy-to-use methodology. This way, batch to batch variations in bioreduction performance of commercial grade yeast can reproducibly be minimised.

In addition to controlling the outcome of whole-cell biotransformations, these results provide important information for xenobiotic metabolism *e.g.* in humans. Since pharmaceutically active compounds have to pass the gastro-intestinal microbial barrier prior to resorption in the gut, co-administration of caffeine with drugs may exert effects that require further investigation.

Experimental

General

Baker's yeast (*S. cerevisiae* L13) was the product of Societé industrielle de levure FALA, Strasbourg, France, and was obtained from the local subsidiary in Kesselsdorf, Germany. Chemicals and organic solvents were obtained from Fluka (Buchs, CH) and Acros (Geelen, Belgium). Sucrose was obtained from a local store.

Product identity was confirmed by NMR spectroscopy. ^{1}H-NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-500 spectrometer at 500 MHz. Chemical shifts are quoted in ppm from internal TMS or TSP. Absolute configuration was determined using a Carl Zeiss Jena polarimeter Polamat A ($c = 1.0$, $CHCl_3$) and by comparison of the obtained results with ref. 8*c*. All reactions were monitored by GC using an Analytik Jena Perichrom GC ST200. Conversions, yields and stereoisomer distributions were determined by GC.

Determination of the extent of conversion

The conversions were measured using a J&W Scientific DB-5 column (30 m, 0.25 mm I.D.). The pressure of N_2 gas was 1 bar; the temperatures of the injector and the detector were 250 °C and 260 °C, respectively. The percent conversions were determined using an integrator.

1 → **2**: Temperature programme: 50 °C to 115 °C with 5 K min^{-1}. Retention time of substrate **1** was 10.8 min. Products *cis*-**2** and *trans*-**2** were observed at a retention time of 10.3 and 11.3 min.

3 → **4**: Temperature programme: 50 °C to 100 °C with 10 K min^{-1}, then 10 min isothermal at 100 °C and to 200 °C with 30 K min^{-1}, 3 min isothermal at 200 °C. The substrate **3** and products *cis*-**4** and *trans*-**4** were observed at retention times of 11.6 min (ketone) and 12.2 min (enol), 10.9 min and 11.2 min.

Determination of optical purity

For the determination of enantiomeric excess (ee) and diastereomeric excess (de), the pure carbinols were converted into the corresponding trifluoroacetates by reaction with a 1.2 molar amount of trifluoroacetic acid anhydride in dry CH_2Cl_2 at 65 °C. After the reaction was complete, the volatile components were evaporated. GC analysis of the resulting trifluoroacetates was conducted using a Macherey & Nagel Lipodex E column (50 m, 0.25 mm I.D.). The trifluoroacetates of $NaBH_4$ reduced **1** and **3**, respectively, were used to find suitable conditions for chiral GC analysis. The pressure of N_2 gas was 1 bar; the temperatures of the injector and the detector were 240 °C and 250 °C, respectively. The relative amounts were determined using an integrator.

1 → **2**: Temperature programme: 80 °C to 160 °C with 3 K min^{-1}. Retention times: (1*R*,2*R*)-**2**: 14.7 min, (1*S*,2*S*)-**2**: 15.3 min, (1*S*,2*R*)-**2**: 17.3 min, (1*R*,2*S*)-**2**: 17.6 min.

3 → **4**: Temperature programme: 80 °C to 160 °C with 3 K min^{-1}. Retention times: (1*R*,2*R*)-**4**: 15.8 min, (1*S*,2*S*)-**4**: 16.0 min, (1*S*,2*R*)-**4**: 20.4 min, (1*R*,2*S*)-**4**: 21.5 min.

Cultivation and culture conditions

S. cerevisiae L13 was routinely grown in shake-flasks in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C, 150 rpm for 7 d (stationary-phase). Cells were harvested by centrifugation (3000 × g, 20 min, 4 °C) and washed with 0.9% aqueous solution of sodium chloride.

Whole-cell biotransformation

S. cerevisiae L13 (1.5 g dry weight) was suspended in a solution of sucrose (5.0 g) and caffeine (500 mg, 2.57 mmol) in tap water (50 ml) at 30 °C in a microaerated Erlenmeyer flask. The pH was kept at 6.3 by addition of $CaCO_3$ (0.75 g). The cells were incubated at 30 °C and shaken at 150 rpm for 30 min. β-Keto esters **1** and **3**, respectively, were added hourly over a period of 5 h (**1**: 500 µl, 0.54 g, 3.45 mmol; **3**: 500 µl, 0.55 g, 3.23 mmol). After that, the suspension was shaken for 19 more hours at 30 °C, resulting in an overall reaction time of 24 h. The biotransformation was monitored by GC. After 24 h, the reaction mixture was centrifuged and the yeast cells were washed with water. After incubation of the combined aqueous phases with 75 U *Bacillus subtilis* protease for 18 h (allowing phase separation according to the method described in ref. 36), the product was obtained through extraction (3×) with tertiary-butyl-methyl-ether (TBME). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. **2**: (0.45 g, 83%), 97.3% ee, 97.5% de. **4**: (0.43 g, 77%), 90.8% ee, 75.0% de.

Effects of caffeine on a whole-cell biotransformation

Biotransformations were conducted as described above with caffeine (486 mg, 250 µmol) added. **2**: (0.46 g, 84%), 98.5% ee, 98.9% de. **4**: (0.47 g, 85%), 92.1% ee, 90.0% de.

Effects of $CaCl_2$ on a whole-cell biotransformation

Biotransformations were conducted as described above. $CaCl_2$ was added as indicated in Fig. 5.

Effects of $MgCl_2$ on a whole-cell biotransformation

Biotransformations were conducted as described above. $MgCl_2$ was added up to 100 mM using adequate amounts of MgO as a pH buffer instead of $CaCO_3$.

Heat shock experiments

The whole-cell biotransformations were conducted as described above at 38 °C.

Determination of the cell vitality

Cell vitality was determined by propidium iodide (PI) staining. Therefore, an aliquot of cells was taken from the reaction mixture and diluted 1 : 500 with PBS (pH 7.0). Afterwards, cells were stained with 25 ml of a 1 mg mL^{-1} PI stock solution for 6 min in darkness at room temperature. The amount of dead cells was analysed by a CyFlow SL Blue flow cytometer (excitation: 20 mW solid sate laser at 488 nm; emission collected through a 630 nm high pass filter).

Determination of reactive oxygen species

1 ml was withdrawn from the reaction mixture and incubated for two hours with 25 μg ml^{-1} dihydrorhodamine 123 (DHR 123) in PBS, pH 7.0 at 30 °C and 150 rpm. Afterwards, cells were diluted 1 : 500 with PBS (resulting in approx. 10^6 yeast cells per ml) and incubated with 25 μg ml^{-1} PI for 6 min in darkness at room temperature. Samples were vortexed prior to flow cytometry (excitation: 20 mW solid sate laser at 488 nm; emission collected through a 530/20 nm band pass filter).

Force field calculations

Force field calculations were conducted on an IBM RS/6000 cluster with the software Molecular Simulations Cerius^2 3.5 using the Universal 1.02 force field and the charge-equilibriation module Qeq_charged 1.1. Minimisations were done according to the adopted basis Newton–Raphson (abnr) routine, and optimisations were performed till convergence at RMS force 0.001 Å.

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