Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron 60 (2004) 789–797
Tetrahedron

Rapid identification of enantioselective ketone reductions using targeted microbial libraries

Michael J. Homann,* Robert B. Vail, Edward Previte, Maria Tamarez, Brian Morgan,† David R. Dodds‡ and Aleksey Zaks*

Schering-Plough Research Institute, 1011 Morris Avenue, Union, NJ 07083, USA

Received 18 August 2003; revised 10 October 2003; accepted 24 October 2003

Abstract—A collection of about 300 microbes was surveyed for the ability to generate chiral secondary alcohols by enantioselective reduction of a series of alkyl aryl ketones. Microbial cultures demonstrating utility in reducing model ketones were arrayed in multi-well plates and used to rapidly identify specific organisms capable of producing chiral alcohols used as intermediates in the synthesis of several drug candidates. Approximately 60 cultures were shown to selectively reduce various ketones providing both the *R* and *S* enantiomers of the corresponding alcohols in 92–99% ee with yields up to 95% at 1–4 g/L. An alternative approach to chiral alcohols based on selective microbial oxidation of racemic alcohols is also reported. This study provides a useful reference for generating chiral alcohols by selective microbial bioconversion.

1. Introduction

It is well recognized that the pharmacological activity of a drug often depends on the specific configuration of the substituent around its chiral center(s). During the early stages of drug candidate selection, the effect of chirality on biological activity must be ascertained, prompting the need to generate chiral intermediates such as secondary alcohols. Chiral alcohols are readily obtained by enantioselective reduction of ketones using both chemical and biological means. Although hydrogenation of ketones with Rh- and Ru-diphosphine catalysts has been frequently utilized for the synthesis of chiral alcohols on a large scale,[1] biological reductions catalyzed by isolated dehydrogenases or whole microbial cells continue to provide an attractive means to reduce a broad range of ketones selectively.[2] Indeed, biological reductions of carbonyl groups have found important applications in the synthesis of several drug intermediates including Taxol (anticancer),[3] Montelukast (anti-asthma),[4] Zetia (cholesterol absorption inhibitor),[5,6] Trimegestone (hormone mimetic),[7] and Trusopt (anti-glaucoma).[8] Despite a large number of examples of microbial ketoreductions reported in the literature (for reviews see refs. 2,9–14) the task of choosing a microbe capable of selectively reducing a particular intermediate is still largely based on screening hundreds of microbial cultures in flask fermentations. This time consuming process often prohibits practical integration of the bioreduction with process development. To avoid this drawback, the development of rapid screening and selection methodologies is essential. Identified in this study are approximately 60 cultures, selected from a library of over 300 bacteria, yeast and filamentous fungi, for their ability to reduce numerous model ketones in a highly efficient and enantioselective manner. The design of the culture library was based on a literature survey of microbial reductions of ketones reported in the last 20 years. Initial results of a subset of the collection were reported earlier by Dodds et al.[15] By using libraries of pre-selected organisms arrayed in multi-well plate formats, screens could be shortened significantly and the desired organism identified within days. Rapid identification of enantioselective cultures allowed for the successful utilization of a bioreduction step in the synthesis of several drug intermediates.

2. Results

The bioconversions of alkyl aryl substrates **1a**–**f**, **3a**–**d**, **5a**–**d**, **7**, **9**, **11a**–**b**, and **13** (Fig. 1) were carried out at 2 g/L with cultures grown in complex medium. The initial screen was focused on simply identifying active and enantioselective cultures. No systematic attempts were undertaken to maximize either enantioselectivity or efficiency, although it should be emphasized that in many cases the culture productivity and selectivity was affected by the substrate

Keywords: Enantioselective reductions; Chiral alcohols; Microbial bioconversion; Ketone reduction.
* Corresponding authors. Fax: +1-908-820-6096 (M.J.H.); (A.Z.);
e-mail addresses: michael.homann@spcorp.com; alex.zaks@spcorp.com
† Diversa Corp., 4955 Directors Place, San Diego, CA 92121.
‡ Dodds and Associates LLC, Manlius, NY, USA.

0040–4020/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2003.10.123

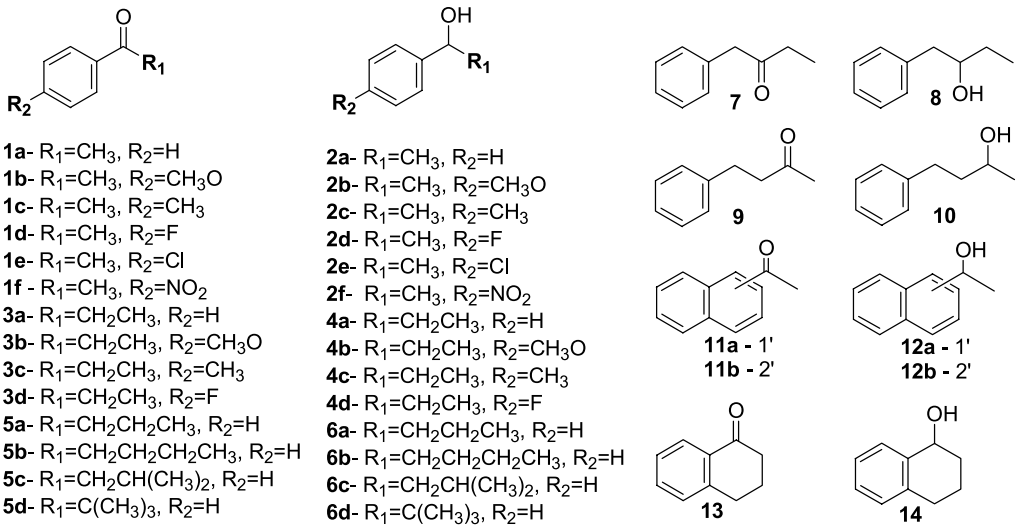


Figure 1. Ketones and alcohols used in microbial panel selection.

concentration, growth medium, and fermentation conditions. Cultures providing high selectivity and yield for each of the ketones surveyed were used to generate material in shake flask bioconversions for structural confirmation following purification (see Section 5).

Bioconversion of several 4′-substituted analogues of acetophenone and propiophenone were studied to investigate whether the presence of various substituents predictably alters the efficiency of microbial reduction. Cultures providing the highest enantioselectivity and yield are listed in Tables 1 and 2. Overall, cultures capable of reducing ketones without 4′-substituents were able to reduce the ketones with substituents in most cases. Not surprisingly, reduction of derivatives with electron withdrawing substituents such as nitro-, chloro- and fluoro- was more efficient than that of the derivatives with electron donating groups methyl- or methoxy. The effect of 4′-substitutions on enantioselectivity was culture specific and not predictable. Relatively few microbes were capable of selectively reducing aryl ketones with longer alkyl chain substituents (**5a**,**b**), branched alkyl substituents (**5c**,**d**), or tetralone (**13**) (Table 3). It is worth mentioning, however, that several cultures were identified that did reduce the highly hindered

Table 1. Selective microbial reduction of model 4′-analogues of acetophenone

Substrate	Yield (%)	ee (%)	Culture—ATCC
Acetophenone (**1a**)	59	>99 *S*−	*Rhodotorula glutinis 16740*
	24	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	24	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	39	97 *S*−	*Pichia subpelliculosa 16766*
	43	>99 *R*+	*Geotrichum klebahnii 20001*
	22	>99 *R*+	*Geotrichum candidum 34614*
4′-Methoxy- (**1b**)	61	>99 *R*+	*Yarrowia lipolytica 8661*
	37	>99 *R*+	*Pichia methanolica 58403*
	23	>99 *R*+	*Rhodotorula mucilaginosa 64684*
	20	>99 *R*+	*Mucor racemosus 7924*
	17	>99 *S*−	*Fusarium caucasicum 18791*
	15	>99 *S*−	*Aspergillus niveus 12276*
4′-Methyl- (**1c**)	53	>99 *S*−	*Pichia subpelliculosa 16766*
	77	73 *R*+	*Yarrowia lipolytica 8661*
4′-Fluoro- (**1d**)	75	>99 *S*−	*Pichia subpelliculosa 16766*
	62	>99 *S*−	*Rhodotorula glutinis 16740*
	68	95 *S*−	*Rhodotorula mucilaginosa 64684*
	79	97 *R*+	*Geotrichum candidum 34614*
	87	85 *R*+	*Yarrowia lipolytica 8661*
4′-Chloro- (**1e**)	80	>99 *S*+	*Rhodotorula glutinis 16740*
	75	>99 *S*+	*Rhodotorula mucilaginosa 64684*
	64	>99 *S*+	*Rhodotorula mucilaginosa 4056*
	52	>99 *S*+	*Aspergillus niveus 12276*
	48	>99 *S*+	*Aspergillus carneus 16798*
	39	>99 *S*+	*Pichia methanolica 58403*
	34	>99 *S*+	*Mucor racemosus 7924*
	86	>99 *R*−	*Yarrowia lipolytica 8661*
	34	>99 *R*−	*Fusarium caucasicum 18791*
4′-Nitro- (**1f**)	94	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	91	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	68	>99 *S*−	*Rhodotorula glutinis 16740*
	84	94 *R*+	*Geotrichum candidum 34614*

Table 2. Selective microbial reduction of model 4′-analogues of propiophenone

Substrate	Yield (%)	ee (%)	Culture—ATCC
Propiophenone (**3a**)	59	>99 *S*−	*Rhodotorula glutinis 16740*
	32	>99 *S*−	*Pichia subpelliculosa 16766*
	20	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	20	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	13	>99 *S*−	*Candida albicans 11006*
	22	97 *S*−	*Williopsis saturnus 20128*
	30	98 *R*+	*Debaryomyces hansenii 20220*
	19	87 *R*+	*Yarrowia lipolytica 8661*
4′-Methoxy- (**3b**)	25	>99 *S*−	*Pichia subpelliculosa 16766*
	17	>99 *S*−	*Aspergillus niveus 12276*
	10	>99 *R*+	*Yarrowia lipolytica 8661*
4′-Methyl- (**3c**)	31	>99 *S*−	*Mucor racemosus 7924*
	25	>99 *S*−	*Rhodotorula glutinis 16740*
	21	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	19	>99 *S*−	*Aspergillus carneus 20231*
	12	>99 *S*−	*Aspergillus carneus 16798*
	35	87 *R*+	*Yarrowia lipolytica 8661*
4′-Fluoro- (**3d**)	54	>99 *R*+	*Yarrowia lipolytica 8661*
	50	>99 *R*+	*Aspergillus carneus 20231*
	37	>99 *R*+	*Aspergillus carneus 16798*
	26	>99 *R*+	*Pichia methanolica 58403*
	20	>99 *S*−	*Rhodotorula mucilaginosa 4056*

Table 3. Microbial reduction of model phenone and naphthone substrates

Substrate	Yield (%)	ee (%)	Culture—ATCC
Butyrophenone (**5a**)	29	>99 *S*−	*Aspergillus niveus 12276*
	28	97 *S*−	*Williopsis saturnus 20128*
	19	>99 *S*−	*Aspergillus carneus 16798*
	15	>99 *S*−	*Mucor racemosus 7924*
Valerophenone (**5b**)	35	>99 *S*−	*Aspergillus niveus 12276*
	33	>99 *S*−	*Aspergillus carneus 16798*
	30	>99 *S*−	*Aspergillus carneus 20231*
	29	>99 *S*−	*Mucor circinelloides 7941*
	24	>99 *S*−	*Mucor racemosus 7924*
Isobutyrophenone (**5c**)	46	>99 *S*−	*Geotrichum klebahnii 20001*
	27	>99 *S*−	*Geotrichum candidum 34614*
	28	96 *S*−	*Fusarium caucasicum 18791*
	17	99 *S*−	*Geotrichum marinum 20614*
	12	>99 *S*−	*Pichia subpelliculosa 16766*
	18	>99 *S*−	*Candida albicans 20032*
	22	98 *R*+	*Debaryomyces hansenii 20220*
2,2-Dimethylpropiophenone (**5d**)	28	>99 *S*−	*Pichia subpelliculosa 16766*
	18	>99 *S*−	*Yamadazyma haplophila 20321*
	12	>99 *S*−	*Torulaspora delbrueckii 20100*
	9	>99 *R*+	*Debaryomyces hansenii 20220*
	17	75 *R*+	*Fusarium caucasicum 18791*
1-Phenyl-2-butanone (7)	56	>99 *S*+	*Pichia subpelliculosa 16766*
	14	>99 *S*+	*Candida albicans 11006*
	15	>99 *R*−	*Debaryomyces hansenii 34022*
	34	99 *R*−	*Mucor racemosus 7924*
	30	95 *R*−	*Geotrichum klebahnii 20001*
	33	94 *R*−	*Aspergillus niveus 12276*
Benzylacetone (**9**)	58	96 *S*+	*Zygosaccharomyces bailii 38924*
	20	96 *S*+	*Torulaspora delbrueckii 10662*
	28	>99 *R*−	*Trigonopsis variabilis 58377*
	15	>99 *R*−	*Candida parapsilosis 7330*
1-Acetonaphthone (**11a**)	79	>99 *S*−	*Rhodotorula glutinis 16740*
	66	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	61	>99 *S*−	*Geotrichum marinum 20614*
	61	99 *S*−	*Geotrichum candidum 34614*
	56	>99 *S*−	*Geotrichum klebahnii 20001*
	47	>99 *S*−	*Rhodotorula mucilaginosa 64684*
2-Acetonaphthone (**11b**)	41	>99 *S*−	*Rhodotorula glutinis 16740*
	39	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	36	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	21	>99 *S*−	*Pichia methanolica 58403*
α-Tetralone (**13**)	35	>99 *S*+	*Pichia methanolica 58403*
	14	>99 *S*+	*Rhodotorula glutinis 16740*
	13	>99 *S*+	*Williopsis saturnus 20128*

ketone **5d** with high enantioselectivity. Likewise, increasing the distance of the carbonyl moiety from the phenyl ring resulted in a significant decrease in the frequency and types of cultures identified which could selectively reduce phenones **7** and **9**. Carbonyl position had less influence on selective reduction of naphthones **11a** and **11b**. Cultures providing the highest selectivity and yield for reduction of aryl ketones **5a**–**d**, **7**, **9**, **11a**–**b** and **13** are listed in Table 3.

As presented in Tables 1–3, a higher number of cultures exhibited *pro-S* selectivity over *pro-R*. It was of interest, therefore, to examine whether alcohols with the opposite, (*R*)-stereochemistry, could be obtained by a selective oxidation of racemic alcohols. This approach has been found to be useful for the preparation of a variety of enantioenriched secondary alcohols.[16–18] A panel of microbes was screened for their ability to selectively oxidize racemates of **2a**, **4a**, **6a**–**d**, **8**, **10**, **12a**–**b** and **14** (Fig. 1) at 2 g/L. Bioconversions were conducted with cells suspended in TNC medium, ammonium acetate or sodium citrate buffers (see Section 5). Racemate resolution by oxidation was achieved with high selectivity for **2a**, **4a**, **10** and **14**. As expected, racemate resolution by oxidation resulted in the production of (*R*)-alcohols (with one exception) with good to excellent selectivity. Culture and media specific effects were observed and conditions affording maximum productivity are summarized in Table 4 (Fig. 1).

All cultures demonstrating selective conversion of one or more model compounds were incorporated into 24-well plate panels and used in reducing intermediates of several drug candidates—**15**, **17**, **19**, **21**, and **23** (Fig. 2). Remarkably, 5–25% of the cultures in the assembled panels reduced at least one of the five ketones with high selectivity, yielding the corresponding alcohols with ee>95%. A subset of cultures exhibiting high selectivity in reduction of the above ketones at 1 g/L is listed in Table 5. Ketones **15**, **17**, and **19** were reduced by a number of microbes into either the (*R*)- or (*S*)-enantiomer of the corresponding alcohol with varying yield and excellent enantioselectivity (ee 93–>99%). The yield and volumetric productivity were further improved by conducting the bioconversions with highly concentrated resting cells. For example, an 8 h

Table 4. Resolution of model racemic alkyl aryl alcohols by microbial oxidation

Racemate	Culture—ATCC	Media	Yield (%)	ee (%)
1-Phenyl-1-ethanol (**2a**)	*Yarrowia subpelliculosa* 16766	SCT	45	>99 *S*−
	Geotrichum candidum 34614	AMA	33	>99 *R*+
	Yamadazyma haplophila 20321	TNC	24	>99 *R*+
		SCT	48	95 *R*+
1-Phenyl-1-propanol (**4a**)	*Geotrichum candidum* 34614	TNC	24	96 *R*+
	Candida parapsilosis 16632	SCT	20	88 *R*+
1-Phenyl-3-butanol (**10**)	*Yarrowia lipolytica* 8661	AMA	22	95 *R*−
	Rhodotorula mucilaginosa 4056	TNC	41	89 *R*−
	Candida parapsilosis 16632	TNC	32	85 *R*−
α-Tetralol (**14**)	*Rhodotorula glutinis* 16740	SCT	34	98 *R*−
		TNC	37	95 *R*−
	Geotrichum candidum 34614	SCT	24	95 *R*−
		TNC	31	92 *R*−
	Rhodotorula mucilaginosa 4056	SCT	41	93 *R*−
		TNC	42	91 *R*−
	Pichia methanolitica 58403	SCT	33	91 *R*−

incubation of **15** (4 g/L) with *Candida apicola* 24616 cells (1 kg/L) in TNC medium at 30 °C produced (*R*)-**16** in 95% yield (ee>98%). Resting cells of *Debaryomyces hansenii* 10619 and *Candida bombicola* 22214 were also capable of reducing 4 g/L of **15** into (*R*)-**16** in 50–67% yield with high selectivity (ee 96–98%).

To prevent evaporation of the highly volatile ketone, **21**, a panel of 45 microbes selected from the initial plate screen was propagated in flasks and then screened in a resting state format employing screw cap tubes (Table 5). While a number of cultures produced **22** in the (*S*)-configuration in excellent yield and enantiomeric purity, the cultures with the *pro-R* preference were only moderately selective (ee of *R*-**22** was only in 84–92% range). In a similar trend, a number of cultures exhibited *pro-S* selectivity in the reduction of ketone **23**, while none was pro-*R* selective. Additionally, an increase in the substrate concentration up to 10 g/L did not affect enantioselectivity of several *pro*-(*S*) selective cultures. As a result, (*S*)-**24** was obtained in 66% yield (ee >99%) following a 48 h incubation of **23** at 10 g/L with resting cells of *M. plumbeus* 4740.

3. Discussion

Microbial fermentations provide a diverse renewable supply of dehydrogenase activities with inherent capabilities for cofactor regeneration. Numerous examples of selective microbial reductions have been reported for a wide variety of ketones (for recent examples see Refs. 11,19–28). Nevertheless, no algorithms have been developed yet which

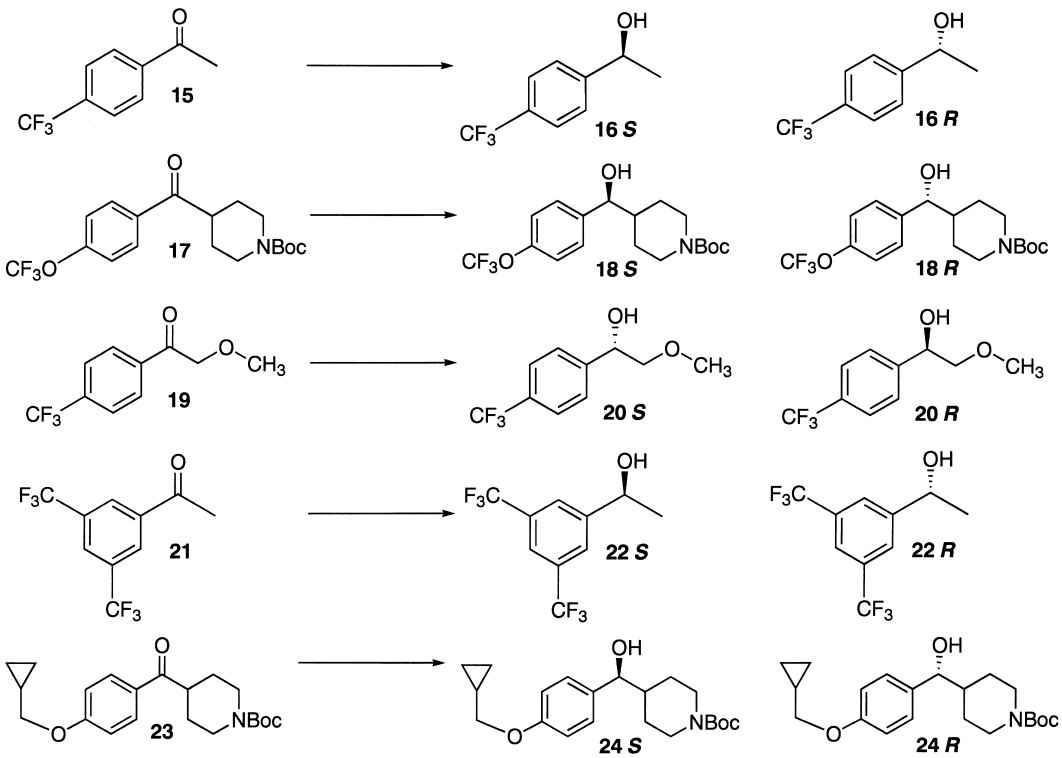


Figure 2. Selective reduction of ketone intermediates for synthesis of drug candidates.

Table 5. Generation of chiral alcohol intermediates by selective microbial reduction

Substrate	Yield (%)	ee (%)	Culture—ATCC
4′-Trifluoromethylacetophenone (**15**)	72	>99 *S*	*Rhodotorula glutinis*16740
	77	>99 *S*	*Rhodotorula mucilaginosa 4056*
	67	>99 *S*	*Rhodotorula pilimanae 32762*
	43	>99 *S*	*Torulaspora fermentati 46935*
	33	>99 *S*	*Torulaspora delbrueckii 20100*
	45	>99 *S*	*Saccharomyces cerevisiae 58520*
	50	96 *S*	*Aspergillus carneus 20231*
	40	95 *S*	*Mucor circinelloides 7941*
	22	98 *S*	*Mucor mucedo 20094*
	48	96 *S*	*Mucor hiemalis 16636*
	37	96 *S*	*Paecilomyces variotii 14572*
	56	95 *R*	*Yarrowia lipolytica 46482*
	72	>99 *R*	*Candida apicola 24616*
	38	99 *R*	*Candida bombicola 22214*
	66	96 *R*	*Candida parapsilosis 20224*
	81	98 *R*	*Debaryomyces hansenii 10619*
	88	95 *R*	*Trigonopsis variabilis 58377*
	88	96 *R*	*Torulaspora vanriji 56221*
4-(4-Trifluoromethoxy-benzoyl)-piperidine-1-carboxylic acid *tert*-butyl ester (**17**)	66	>99 *S*	*Candida guilliermondii 9058*
	46	>99 *S*	*Pichia anomala 66346*
	34	>99 *S*	*Torulopsis species 66815*
	33	>98 *S*	*Hansenula anomala 20211*
	87	97 *S*	*Candida fumata 26418*
	21	93 *R*	*Rhodotorula glutinis 26085*
	15	90 *R*	*Mortierella ramanniana 38191*
2-Methoxy-4′-trifluoromethylacetophenone (**19**)	85	87 *S*	*Debaryomyces hansenii 20220*
	60	88 *S*	*Candida polymorpha 20213*
	77	95 *S*	*Hansenula polymorpha 26012*
	71	99 *R*	*Rhodotorula mucilaginosa 64684*
	66	96 *R*	*Rhodotorula pilimanae 32762*
	66	95 *R*	*Schizosaccharomyces pombe 20130*
	79	97 *R*	*Williopsis saturnus 42306*
	73	99 *R*	*Trigonopsis variabilis 58536*
3′,5′-Bis-trifluoromethylacetophenone (**21**)	66	85 *R*	*Candida kefyr 14244*
	93	92 *R*	*Candida tropicalis 46491*
	>99	84 *R*	*Torulaspora etchellsii 20126*
	61	87 *R*	*Fusarium caucasicum 18791*
	64	86 *R*	*Trichosporon cutaneum 46446*
	66	>99 *S*	*Rhodotorula glutinis 26085*
	50	>99 *S*	*Rhodotorula mucilaginosa 64684*
	41	>99 *S*	*Rhodotorula pilimanae 32762*
	88	>99 *S*	*Williopsis saturnus 18119*
	97	95 *S*	*Aspergillus carneus 16798*
	70	>99 *S*	*Aspergillus terreus 24839*
	92	>99 *S*	*Geotrichum candidum 90685*
4-(4-Cyclopropylmethoxy-benzoyl)-piperidine-1-carboxylic acid *tert*-butyl ester (23)	37	>99 *S*	*Debaryomyces hansenii 20220*
	38	>99 *S*	*Rhodococcus species 21146*
	46	>99 *S*	*Fusarium caucasicum 18791*
	52	>99 *S*	*Geotrichum candidum 74487*
	47	>99 *S*	*Mortierella isabellina 44853*
	59	>99 *S*	*Mucor plumbeus 4740*

are capable of predicting the activity and selectivity of a culture on a ketone of interest with even moderate accuracy. The process of selecting a microbe for a particular biotransformation is still highly empirical and time consuming. A typical 1000-culture screen conducted in a classical flask fermentation format is estimated to take 4 weeks on average. The excessive amount of resources and the length of time required to complete such a screen often creates a barrier on the way to incorporating a biological step into a multi-step chemical synthesis. To overcome this limitation, an efficient approach allowing for the rapid identification of cultures with the desired activity was needed. In order to accelerate the microbial screening program we created a 'targeted' library of microbes enriched for the desired activity that was based on an extensive and critical evaluation of prior work on bioreductions. As a result, a collection of about 300 cultures representing 55 genera of bacteria, yeast and filamentous fungi, were assembled and tested against 19 commercially available UV active ketones.

Cultures demonstrating the highest selectivity and yield for each of the nineteen alkyl aryl ketones surveyed are listed in Tables 1–3. Notably absent from the compilation of the primary screen results were representative bacteria. Although various strains of *Lactobacilli*,[29–32] *Gluconobacter*,[33–35] and *Rhodococci*[19,36–39] have been shown to catalyze selective reductions of a variety of ketones, these cultures were not among the top performers in our library survey. In fact, the most efficient and selective cultures were

those of fungal origin (both yeast and filamentous fungi). Most cultures capable of reducing the alkyl aryl ketone series generated alcohols in the (*S*)-configuration, the pattern consistent with Prelog's Rule.[40] Nevertheless, the Prelog model was not a useful predictor for the outcome of whole cell bioconversions as was evident from the results presented in Tables 1–5. For example, *Yarrowia lipolytica*[16,17,41] and *Debaryomyces hansenii* were found to primarily generate (*R*)-alcohols. In the case where the ketoreduction failed to produce an alcohol in the (*R*)-configuration an alternative approach, based on resolution of the racemic alcohol by selective oxidation of one enantiomer proved to be quite useful (Table 4).

To demonstrate the utility of the targeted library approach, about 300 microorganisms were incorporated into a 24-well plate format and used to identify cultures capable of selective reduction of several target ketones (Table 5). The chiral alcohol products formed as a result of bioreductions of ketones **15**, **17** and **19** were used as chiral intermediates in the preparation of three antiviral CCR5 antagonists.[42–44] Selective reduction of **21** and **23** were investigated in conjunction with the synthesis of an antidepressant NK1 receptor antagonist[45–47] and an anti-muscarinic M2 receptor antagonist[48,49] respectively. The primary objective of these screens was to rapidly identify highly selective bioconversions to produce chiral alcohol intermediates for the synthesis of putative drug candidates. Since only gram quantities of the chiral alcohols were needed to support the synthesis, little optimization was conducted. Using multi-well plates prepared in advance containing grown cultures, the bioconversions of all five ketone intermediates were identified within just a few days, representing a 5–10-fold improvement in the average screen time (Table 5). Furthermore, by altering the time of substrate addition or by using high concentration of resting cells, further improvements in reaction productivity were achieved. For example, (*R*)-**16** was produced in 95% yield (ee >98%) in the presence of *Candida apicola* 24616 with 4 g/L of **15**; the yield of (*S*)-**18** (ee>99% ee) obtained with yeast *Pichia anomala* 66346 was improved from 46% to 71% and finally (*S*)-**24** (ee>99%) was obtained in 66% yield following the incubation of **23** at 10 g/L with *M. plumbeus* 4740.

4. Conclusion

Quick access to microbial bioreductions can prove to be useful at various stages of development, from supporting the synthesis of putative drug candidates at the laboratory level, to manufacturing intermediates and active pharmaceutical ingredients at commercial scale. This study has demonstrated the utility of using targeted libraries for rapid identification of microbial cultures capable of reducing a variety of ketones in a highly enantioselective manner. A library composed of about 300 commercially available cultures, arrayed in multi-well plates yielded a set of 60 microbes that reduced a series of 24 ketones with excellent selectivity. It is estimated that the described plate format afforded a 5–10-fold reduction in the average screen time compared to a conventional flask-based screening protocol. The abundance of microbial cultures capable of enantioselective reductions and their broad substrate tolerance provides a general and reliable approach to the synthesis of chiral alcohol enantiomers.

5. Experimental

5.1. Cultures, chemicals, media and laboratory ware

All microbial cultures used in this study were obtained from the American Type Culture Collection (Manassas, VA). Alkylaryl ketones (**1a–f**, **3a–d**, **5a–d**, **7**, **9**, **11a–b**, **13**, **15**, **21**) were purchased from Aldrich Chemicals (Milwaukee, WI). Most racemic alcohol-standards were either purchased from Aldrich or obtained by reducing the corresponding ketones with sodium borohydride or lithium aluminum hydride following standard procedures. Ketones **17**, **19**, and **23** and the corresponding racemic and chiral alcohols **18**, **20**, and **24** used as analytical standards were supplied by the Chemical Research Department, SPRI. Analytical grade solvents for extraction and HPLC were purchased from Fisher Scientific (Fairlawn, NJ). Medium components were purchased from Difco/Becton Dickson (Sparks, MD), Sensient (Juneau, WI), ICN Biomedical (Aurora, OH), Avebe (Veendam, Netherlands) and Fisher Scientific (Fairlawn, NJ). Silica Gel 60 was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) and TLC plates (Silica Gel GF plates 20×20 cm 1000u) from Analtech Inc. (Newark, DE). Polypropylene 24 and 384 deep well plates, 24-well cap mat and plastic lids were purchased from Whatman Inc. (Clifton, NJ). Slotted 384 well cap mats were purchased from Greiner Bio-One, Inc. USA (Longwood, FL).

5.2. Culture propagation, bioconversion and sample preparation

Filamentous fungi were grown in SIM-6 medium (35 g/L soy flour, 50 g/L white potato dextrin, 5 g/L dextrose, 2 mg/L cobalt chloride, 5 g/L calcium carbonate, pH 5.0). Yeast were grown in YPD (10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L dextrose, pH 5.5–6.0) or TNC (10 g/L Tastone 154, 20 g/L NZ-amine, 30 g/L cerelose at pH 5.0–5.5) media. Bacteria were grown in NYC medium (8 g/L nutrient broth, 11 g/L cerelose, 20 g/L Tastone 154, pH 7.0). Seed cultures were grown at 24–30 °C in 25 mL of medium in 125 mL flasks for up to 72 h with agitation (175–250 rpm). Seed cultures (1 mL) were transferred into a second stage flask fermentation for bioconversion. Cultures selected for panel screening were also transferred from the seed cultures into a series of 24-deep well plates along with glycerol (20% final conc.) and sealed with 24 well cap mats. The plates were stored at −80 °C. Frozen culture plates were thawed at room temperature followed by addition of 1.0 mL of pre-sterilized TNC or NYC media per well. The plate was covered with a loose fitting pre-sterilized plastic lid, followed by incubation at 24–30 °C for up to 72 h with agitation (180 rpm). Ketone or racemic alcohol substrates dissolved in methanol, ethanol or dimethyl sulfoxide were added to cultures in flasks or 24-deep well plates to a final concentration of 0.5–2 g/L following 24 h of culture growth. Flask fermentation

samples (2 mL) taken following 24–72 h of incubation, were extracted with 6 mL of MTBE. A 0.5 mL aliquot of extract was transferred into a 1 mL vial and analyzed by HPLC. For culture panels in screens, 2 mL bioconversions in 24 well plates were extracted by addition of 6 mL of MTBE per well. Sample plates were sealed with cap mats and a foil overlay and stored at −20 °C until sampled for HPLC analysis. Samples (0.3 mL) from each reaction extract were transferred and consolidated for analysis into 384 well plates using a MultiProbe EX II robotic liquid handling station (PerkinElmer Life Sciences, Boston, MA). Assay plates were sealed with 384-well cap mats prior to automated HPLC analysis using an Alliance 2795 fluidics module (Waters Corporation, Milford, MA) as described below. Evaluation of high cell density bioconversions was conducted by harvesting cells from standard flask fermentations by centrifugation and suspending the cell pellet in ammonium acetate buffer pH 7.0 (AMA; 30 g/L), sodium citrate buffer pH 7.0 (SCT; 30 g/L), TNC medium or 50 mM potassium phosphate buffer pH 7.0 containing glucose (100 g/L) at equal or reduced volumes to achieve the desired concentration of cells. Ketone or racemic alcohol substrates dispersed in ethanol were added to cells in flasks at a final concentration of 0.5–10 g/L. Reactions were conducted in flasks and sampled as described above.

5.3. Reverse phase and chiral HPLC analysis

All HPLC analyses were conducted with equipment from Waters Corporation (Milford, MA). Ketones and alcohols were analyzed using 10 μL injections, and monitoring UV at 200 −220 nm. Reverse phase assays were conducted using Symmetry™ C-18 (Waters Corporation, Milford, MA) or Luna™ C-18 (Phenomenex, Torrance, CA) 4.6×150 mm columns at 25–30 °C with isocratic elution using acetonitrile/water (60:40 or 70:30; 1–1.5 mL/min). Chiral assays separating alcohol enantiomers and the corresponding ketone substrates were conducted using Chiracel™ 4.6×250 mm columns (Chiral Technologies Inc., Exton, PA) at 25–30 °C with isocratic elution, employing mixed solvents as follows; OB-H column with hexanes/isopropyl alcohol (90/10), at 1 mL/min for bioconversions **1c** and **5a**; hexanes/isopropyl alcohol 96.5/3.5 (1–1.25 mL/min) for bioconversions **1d**, **3a**,**c**,**d**, **5c**, **11b**; hexanes/isopropyl alcohol 98/2, (1 mL/min) for bioconversions **5b**, **19**; *n*-heptane/ethanol 96:4, (1 mL/min) for bioconversions **1f**; *n*-heptane /ethanol 99/1, (1 mL/min) for bioconversion **15**; OD column with hexanes/isopropyl alcohol at 90/10, (1.5 mL/min) for bioconversion **11a**; hexanes/isopropyl alcohol 95/5 (1–1.25 mL/min) for **3b**, **5d**, and **9**; hexanes/isopropyl alcohol 98/2, (1–1.25 mL/min) for **1b**,**e**, **7** and **21**; OJ column with hexanes/isopropyl alcohol 98/2, (1.25 mL/min) for bioconversions **1a**; hexanes/isopropyl alcohol 95/5, (1.25 mL/min) for **13**; AD column with hexanes/ethanol 95/5, (1 mL/min) for bioconversion **17** and with *n*-heptane/ethanol 93:7 (1 mL/min) for **23**.

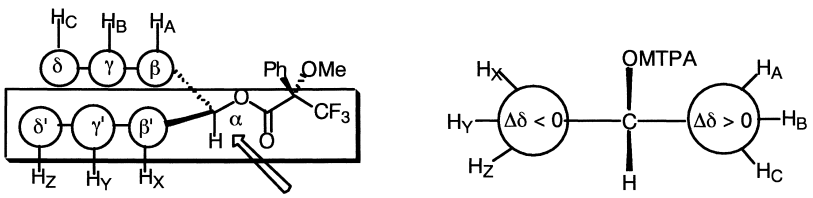
5.3.1. Synthesis of phenylalkanol by microbial bioconversion. The procedures used to generate and purify alcohol products were essentially identical except for the type of microorganism and propagation conditions (as specified above). A representative example based on the synthesis of (*S*)-4′-nitro-1-phenyl-1-ethanol (**2f**) is described. A seed culture of *R. muciaginosa* 4056 (formerly *Rhodotorula rubra*) was grown in a 300 mL flask containing 50 mL of YPD medium at 30 °C for 24 h with agitation (250 rpm). The entire amount of culture was then transferred into a 2 L flask containing 1 L of YPD medium and grown for 24 h at 30 °C with agitation (250 rpm). The substrate, 4′-nitroacetophenone **1f** (2 g) dissolved in methanol (4 mL) was then added and the mixture was incubated under the above conditions for another 48 h resulting in ~94% conversion of the ketone to alcohol product, **2f**. Fermentation broth was then centrifuged at 12,000*g* for 15 min to remove cells and the supernatant was extracted with MTBE (2×500 mL). Cell pellets were suspended in saturated NaCl solution (50 mL) and extracted with MTBE (2×50 mL). The MTBE layers were combined, dried with anhydrous $MgSO_4$, filtered and the solvent evaporated in vacuo. The crude product was purified by preparative TLC (Silica Gel GF plates; 30% EtOAc/hexanes) yielding **2f** as a yellow oil; [Found: C, 57.51; H, 5.43; N, 8.46. $C_8H_9NO_3$ calculated C, 57.48; H, 5.43; N, 8.38]; R_f (30% EtOAc/hexanes) 0.14; $[\alpha]_D^{24.5}=-29.7$ (*c* 2.59, EtOH); ν_{max} (liquid film) 3500–3200 (br), 2970, 1600, 1521, 1347, 1090 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.5 (3H, d, *J*=6.49 Hz, CH*Me*), 1.97–2.18 (1H, br, MeCHO*H*), 5.05 (1H, q, *J*=5.16 Hz, MeC*H*OH), 7.55 (2H, d, *J*=8.57 Hz, Ph-*H*), 8.20 (2H, d, *J*=8.72 Hz, Ph-*H*); δ_C (400 MHz, $CDCl_3$) 25.52, 69.51, 123.77, 126.13, 147.16, 153.10; *m*/*z* (CI, CH_4) 168 (M+1,100%), 150 (55%), 107 (6%).

5.3.2. Optical rotation determination. The specific rotation of each purified alcohol product was measured in EtOH (1.0–2.6 mg/mL) at the temperature indicated (°C). Alcohols generated using *P. subpelliculosa* 16766 (formerly *Hansenula subpelliculosa*) include; **2a** (*S*) $[\alpha]_D^{23.3}=-30.7$, **2c** (*S*) $[\alpha]_D^{23.5}=-40.0$, **2d** (*S*) $[\alpha]_D^{23.6}=-34.6$, **4a** (*S*) $[\alpha]_D^{24.7}=-34.3$, **4b** (*S*) $[\alpha]_D^{24.2}=-24.8$, **8** (*S*) $[\alpha]_D^{24.6}=+18.1$. Alcohols generated using *Y. lipolytica* 8661 include; **2b** (*R*) $[\alpha]_D^{23.7}=+27.7$, **2e** (*R*) $[\alpha]_D^{24.8}=+44.9$, **4d** (*R*) $[\alpha]_D^{24.4}=+33.9$. Alcohols generated using *R. glutinis* 16740 include; **4c** (*S*) $[\alpha]_D^{24.4}=-34.8$, **12a** (*S*) $[\alpha]_D^{25.0}=-79.4$ and **12b** (*S*) $[\alpha]_D^{23.6}=-43.1$. **2f** (*S*) $[\alpha]_D^{24.5}=-29.7$ was obtained using *R. mucilaginosa* 4056 as described above; **6a** (*S*) $[\alpha]_D^{24.6}=-42.5$ was obtained using *A. niveus* 12276; **6b** (*S*) $[\alpha]_D^{24.9}=-26.1$ was obtained using *A. carneus* 16798; **6c** (*R*) $[\alpha]_D^{23.4}=+25.0$ was obtained using *D. hansenii* 20220 (formerly *Torulaspora hansenii*) and **6c** (*S*) $[\alpha]_D^{24.2}=-26.4$ from *C. albicans* 20032; **6d** (*S*) $[\alpha]_D^{23.9}=-29.1$ was obtained using *Y. haplophila* 20321; **10** (*R*) $[\alpha]_D^{23.4}=-18.3$ was obtained with *T. variabilis* 58377 and **14** (*S*) $[\alpha]_D^{24.7}=+18.8$ was obtained using *P. methanolica* 58403.

5.4. Structure configuration of phenylalkanols by paired MTPA esters

The absolute configuration of **2b**–**f**, **4b**–**d**, **6b**,**d**, **8**, **10** was determined by the modified Mosher NMR method.[50] A single enantiomerically enriched alcohol was treated separately with the two enantiomers of the Mosher's acid chloride. The resulting pair of diastereomeric esters was analyzed by high field ^1H NMR in $CDCl_3$. The absolute configuration of the alcohol moiety was assigned on the

Mosher's Model



Difference in chemical shifts of corresponding resonances of the paired Mosher ester diastereomers.

Signal	$\Delta\delta = \delta_S-\delta_R$	Signal	$\Delta\delta = \delta_S-\delta_R$	Signal	$\Delta\delta = \delta_S-\delta_R$
(**2b**)		(**2c**)		(**2d**)	
1-CH, q	-0.039	1-CH, q	+0.028	1-CH, q	+0.039
2-CH_3, d	+0.042	2-CH_3, d	-0.075	2-CH_3, d	-0.058
4'-OCH_3,s	-0.028	4'-CH_3, s	+0.002	Phenyl, m	+0.053
Phenyl, m	-0.015	Phenyl, m	+0.012		+0.053
	-0.014		+0.023		+0.053
			+0.072		
(**2e**)		(**2f**)		(**4b**)	
1-CH, q	-0.054	1-CH, q	+0.043	1-CH, t	+0.067
2-CH_3, d	+0.049	2-CH_3, d	-0.041	2-CH, m	-0.046
Phenyl, m	-0.116	Phenyl, m	+0.069	2-CH, m	-0.044
	-0.106		+0.070	3-CH_3, t	-0.103
				4'-OCH_3, s	+0.003
				Phenyl, m	+0.050
					+0.057
(**4c**)		(**4d**)		(**6b**)	
1-CH, t	+0.061	1-CH, t	-0.074	1-CH, q	+0.068
2-CH, m	-0.050	2-CH, m	+0.034	2-CH_2, m	-0.046
2-CH, m	-0.032	2-CH, m	+0.269	3-CH_2, m	-0.031
3-CH_3, t	-0.111	3-CH_3, t	+0.093	4-CH, m	-0.110
4'-CH_3, s	+0.005	Phenyl, m	-0.064	4-CH, m	-0.065
Phenyl, m	+0.032		-0.064	5-CH_3, t	-0.054
	+0.030		-0.064	Phenyl, m	+0.050
			-0.064		+0.085
			-0.064		+0.084
					+0.083
					+0.082
					+0.077
					+0.064
					+0.057
(**6d**)		(**8**)		(**10**)	
1-CH, s	+0.134	1-CH_2, m	+0.079	1-CH, m	-0.062
2-CH_3, s	-0.052	2-CH, m	+0.040	2-CH, m	-0.036
Phenyl, m	+0.105	3-CH_2, m	-0.061	2-CH, m	-0.000
	+0.099	4-CH_3, t	-0.082	3-CH, m	+0.040
	+0.100	Phenyl, m	+0.115	4-CH_3, d	+0.108
	0.104		+0.128	Phenyl, m	-0.040
	+0.033		+0.117		-0.035

Figure 3. Structure configuration of phenylalkanols by paired MTPA esters.

basis of the difference in the chemical shifts of corresponding resonances of the paired diastereomers (see Fig. 3).

References and Notes

1. Blaser, H.-U.; Malan, C.; Pugin, B.; Spindler, F.; Steiner, H.; Studer, M. *Adv. Synth. Catal.* **2003**, *345*, 103–151.
2. Liese, A.; 2nd ed. *Enzyme Catalysis in Organic Synthesis*; 2002; Vol. 3. pp 1419–1459.
3. Patel, R. N.; Banerjee, A.; Howell, J. M.; McNamee, C. G.; Brozozowski, D.; Mirfakhrae, D.; Nanduri, V.; Thottathil, J. K.; Szarka, L. J. *Tetrahedron: Asymmetry* **1993**, *4*, 2069–2084.
4. Shafiee, A.; Motamedi, H.; King, A. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 709–717.
5. Homann, M. J.; Previte, E. Stereoselective microbial reduction for the preparation of 1-(4-fluorophenyl)-3(*R*)-[3(*S*)-hydroxy-3-(4-fluorophenyl)propyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone. U.S. Patent 6,133,001, 2000.
6. Homann, M. J.; Previte, E. Stereoselective microbial reduction of 5-fluorophenyl-5-oxopentanoic acid and a phenyloxazolidinone condensation product thereof. U.S. Patent 5,618,707, 1997.
7. Crocq, V.; Masson, C.; Winter, J.; Richard, C.; Lemaitre, G.; Lenay, J.; Vivat, M.; Buendia, J.; Prat, D. *Org. Proc. Res. Dev.* **1997**, *1*, 2–13.
8. Holt, R. A. *Chim. Oggi* **1996**, *14*, 17–20.
9. Kula, M. R.; Kragl, U. *Stereoselect. Biocatal.* **2000**, 839–866.

10. Chartrain, M.; Greasham, R.; Moore, J.; Reider, P.; Robinson, D.; Buckland, B. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 503–512.
11. Patel, R. N. *Enzyme Microb. Technol.* **2002**, *31*, 804–826.
12. Patel, R. N. *Adv. Appl. Microbiol.* **2000**, *47*, 33–78.
13. Patel, R. N.; Hanson, R. L.; Banerjee, A.; Szarka, *J. Am. Oil Chem. Soc.* **1997**, *74*, 1345–1360.
14. Hummel, W. *Adv. Biochem. Engng Biotechnol.* **1997**, *58*, 145–184.
15. Dodds, D. R.; Andrews, D. R.; Heinzelmann, C.; Homann, M. J.; Klesse, P.; Moran, W. B.; Previte, E.; Sudhakar, A.; Roehl, R. A.; Vail, R.; Zaks, A.; Zelazowski, A. Proceedings Chiral Europe'95. London, 1995. 1995, pp 55–62.
16. Fantin, G.; Fogagnolo, M.; Medici, A.; Pedrini, P.; Fontana, S. *Tetrahedron: Asymmetry* **2000**, *11*, 2367–2373.
17. Bortolini, O.; Fantin, G.; Fogagnolo, M.; Medici, A.; Pedrini, P. *Recent Res. Dev. Pure Appl. Chem.* **1999**, *3*, 137–152.
18. Fogagnolo, M.; Giovannini, P. P.; Guerrini, A.; Medici, A.; Pedrini, P.; Colombi, N. *Tetrahedron: Asymmetry* **1998**, *9*, 2317–2327.
19. Stampfer, W.; Kosjek, B.; Faber, K.; Kroutil, W. *J. Org. Chem.* **2003**, *68*, 402–406.
20. Groeger, H.; Hummel, W.; Buchholz, S.; Drauz, K.; Nguyen, T. V.; Rollmann, C.; Huesken, H.; Abokitse, K. *Org. Lett.* **2003**, *5*, 173–176.
21. Stampfer, W.; Kosjek, B.; Moitzi, C.; Kroutil, W.; Faber, K. *Angew. Chem., Int. Ed.* **2002**, *41*, 1014–1017.
22. Patel, R. N.; Chu, L.; Chidambaram, R.; Zhu, J.; Kant, J. *Tetrahedron: Asymmetry* **2002**, *13*, 349–355.
23. Haberland, J.; Hummel, W.; Daussmann, T.; Liese, A. *Org. Proc. Res. Dev.* **2002**, *6*, 458–462.
24. Goswami, A.; Mirfakhrae, K. D.; Totleben, M. J.; Swaminathan, S.; Patel, R. N. *J. Ind. Microb. Biotechnol.* **2002**, *28*, 349.
25. Garrett, M. D.; Scott, R.; Sheldrake, G. N. *Tetrahedron: Asymmetry* **2002**, *13*, 2201–2204.
26. Chin-Joe, I.; Haberland, J.; Straathof, A. J. J.; Jongejan, J. A.; Liese, A.; Heijnen, J. J. *Enzyme Microb. Technol.* **2002**, *31*, 665–672.
27. Hummel, W.; Riebel, B. *Biotechnol. Lett.* **2003**, *25*, 51–54.
28. Hummel, W.; Abokitse, K.; Drauz, K.; Rollmann, C.; Groger, H. *Adv. Synth. Catal.* **2003**, *345*, 153–159.
29. Hummel, W.; Riebel, B. Alcohol dehydrogenase of *Lactobacillus* and its use in the enzymic production of chiral alcohols. (Boehringer Mannheim Gmbh, Germany). Eur. Pat. Appl. EP. 796914, 1997.
30. Niefind, K.; Muller, J.; Riebel, B.; Hummel, W.; Schomburg, D. *J. Mol. Biol.* **2003**, *327*, 317–328.
31. Schubert, T.; Hummel, W.; Muller, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 634–637.
32. Bradshaw, C. W.; Hummel, W.; Wong, C. H. *J. Org. Chem.* **1992**, *57*, 1532–1536.
33. Kersters, K.; De Ley, J. *Methods Enzymol.* **1966**, *9*, 346–354.
34. Adlercreutz, P. *Enzyme Microb. Technol.* **1991**, *13*, 9–14.
35. Nanduri, V. B.; Banerjee, A.; Howell, J. M.; Brzozowski, D. B.; Eiring, R. F.; Patel, R. N. *J. Ind. Microb. Biotechnol.* **2000**, *25*, 171–175.
36. Ashraf, W.; Murrell, J. C. *Arch. Microbiol.* **1990**, *153*, 163–168.
37. Ludwig, B.; Akundi, A.; Kendall, K. *Appl. Environ. Microbiol.* **1995**, *61*, 3729–3733.
38. Krier, F.; Kreit, J.; Milliere, J. B. *Lett. Appl. Microbiol.* **1998**, *26*, 283–287.
39. Kreit, J.; Elalami, A. *J. Mol. Catal. B: Enzym.* **2002**, *19–20*, 253–259.
40. Prelog, V. *Pure Appl. Chem.* **1964**, *9*, 119–130.
41. Fantin, G.; Fogagnolo, M.; Giovannini, P. P.; Medici, A.; Pedrini, P.; Gardini, F.; Lanciotti, R. *Tetrahedron* **1996**, *52*, 3547–3552.
42. McCombie, S. W.; Tagat, J. R.; Vice, S. F.; Lin, S.-I.; Steensma, R.; Palani, A.; Neustadt, B. R.; Baroudy, B. M.; Strizki, J. M.; Endres, M.; Cox, K.; Dan, N.; Chou, C.-C. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 567–571.
43. Tagat, J. R.; Steensma, R. W.; McCombie, S. W.; Nazareno, D. V.; Lin, S.-I.; Neustadt, B. R.; Cox, K.; Xu, S.; Wojcik, L.; Murray, M. G.; Vantuno, N.; Baroudy, B. M.; Strizki, J. M. *J. Med. Chem.* **2001**, *44*, 3343–3346.
44. Palani, A.; Shapiro, S.; Josien, H.; Bara, T.; Clader, J. W.; Greenlee, W. J.; Cox, K.; Strizki, J. M.; Baroudy, B. M. *J. Med. Chem.* **2002**, *45*, 3143–3160.
45. Shih, N.-Y.; Shue, H.-J.; Reichard, G. A.; Paliwal, S.; Blythin, D. J.; Piwinski, J. J.; Xiao, D.; Chen, X. Preparation of 2-imidazolidinones and related compounds as selective neurokinin antagonists. (Schering Corporation, USA). PCT Int. Appl. WO 2001044200, 2001.
46. Duffy, R. A.; Varty, G. B.; Morgan, C. A.; Lachowicz, J. E. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 536–542.
47. Shih, N.-Y.; Albanese, M.; Anthes, J. C.; Carruthers, N. I.; Grice, C. A.; Lin, L.; Mangiaracina, P.; Reichard, G. A.; Schwerdt, J.; Seidl, V.; Wong, S.-C.; Piwinski, *J. Bioorg. Med. Chem. Lett.* **2002**, *12*, 141–145.
48. Wang, Y.; Chackalamannil, S.; Hu, Z.; Clader, J.; Greenlee, W. J.; Billard, W.; Binch, H., III; Crosby, G.; Ruperto, V.; Duffy, R.; Lachowicz, J.; Cohen-Williams, M.; Coffin, V.; Cox, K. A.; Grotz, D. E. *Abstracts of Papers*, 224th ACS National Meeting, Boston, MA, United States, Aug 18–22, 2002; MEDI-006.
49. Boyle, C. D.; Vice, S. F.; Campion, J.; Chackalamannil, S.; Lankin, C. M.; McCombie, S. W.; Billard, W.; Binch, H.; Crosby, G.; Williams, M.-C.; Coffin, V. L.; Cox, K. A.; Grotz, D. E.; Duffy, R. A.; Ruperto, V.; Lachowicz, J. E. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3479–3482.
50. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.