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PAPER

Application of biocatalysis towards asymmetric reduction and hydrolytic desymmetrisation in the synthesis of a β-3 receptor agonist

Matthew Badland,[a] Michael P. Burns,[b] Robert J. Carroll,*[a] Roger M. Howard,[a] Daniel Laity[a] and Nathan J. Wymer[b]

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Chemoenzymatic syntheses of two key intermediates in the preparation of a potent β-3 receptor agonist **1** are described. A lipase-catalysed hydrolytic desymmetrisation is employed in a new synthesis of intermediate **7**, which avoids the use of alkyl-tin reagents. A second biotransformation delivers chiral chlorohydrin **5** from its parent ketone in greater enantiomeric excess than the previously-described Noyori-reduction process. A brief discussion of the enantioselectivity of a set of single-point mutants of *Sporobolomyces salmonicolor* aldehyde reductase in this bioreduction is also presented.

Introduction

The use of β-3 agonists for the treatment of a variety of diseases and disorders has been of interest to medicinal chemistry for many years. In particular, many of the larger pharmaceutical companies have progressed candidates into development for obesity and Type 2 diabetes.[1] More recently, groups have targeted β-3 agonists for the treatment of urinary incontinence.[2] We now wish to report an alternative method of preparation of **1**, a potent β-3 agonist for the treatment of overactive bladder (OAB).

The synthesis of **1** has been described elsewhere.[3] In our original synthesis, the target compound **1** was synthesised by coupling epoxide **2** and amine **3**, which were themselves generated by Noyori asymmetric ketone reduction and Ritter chemistries, respectively. The route suffered from two key issues. The first of these was the relatively low e.e. (77%) achieved for the Noyori asymmetric reduction[4] of **4** to **5**. Due to the sub-optimal enantiomeric excess, preparative reversed-phase chiral HPLC was required for purification of chlorohydrin **5** to 99.6% e.e resulting in an overall yield of only 42%. Alternative reduction conditions, including homogeneous asymmetric hydrogenations, CBS-type and DIP-Cl asymmetric reductions, were attempted without any improvement.[5]

The second issue was the use of undesirable tin chemistry in the synthesis of alcohol **7**, as detailed in Scheme 2. The original synthesis involved a Stille coupling of isopropenyl acetate with **11** to provide ketoester, **12**. Treatment of acid **13** with excess methyl Grignard afforded **7** in high yield. Tertiary alcohol **7** was then coupled with the commercially available amino isoxazole **8**, prior to conversion to amine **3** by means of a Ritter reaction (see Scheme 1). Whilst this sequence was high yielding, it relied on a tin reagent in the second step, with the associated difficulties of handling, cleaning and waste disposal when utilizing such reagents.

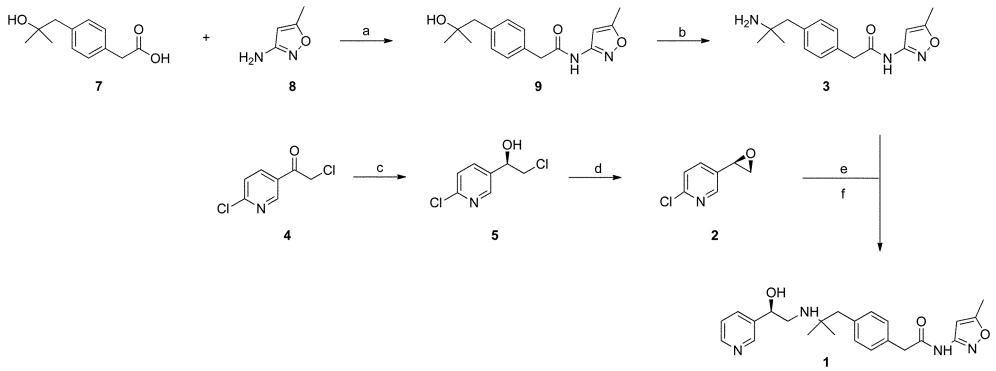
In order to tackle these limitations, we took the opportunity to apply biocatalysis to the synthesis in the form of a ketoreductase-catalyzed asymmetric ketone reduction of ketone **4** and a lipase-catalysed desymmetrisation-incorporating chemoenzymatic route to alcohol **7**, with our results described herein.

Results and discussion

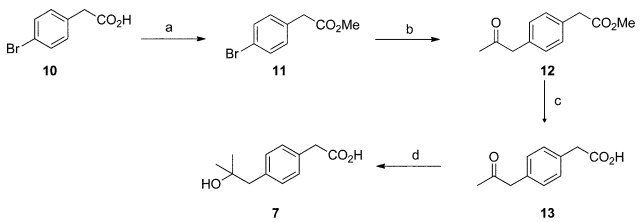
Bioreduction of ketone 4 to (*R*)-chlorohydrin 5

A collection of 260 ketoreductases was screened for the bioreduction of ketone **4** to chlorohydrin **5**. Many enzymes were identified as being highly selective for the generation of either the desired (*R*)-chlorohydrin **5** or for the undesired (*S*)-chlorohydrin **6** (Table 1). The best candidate ketoreductases based upon high conversion to product (>70%) and e.e. (generating >90% e.e. (*R*)-**5** or >99% e.e. (*S*)-**5**) included biocatalysts from commercial sources (Codexis, Julich and Daicel) as well as ketoreductases that had been cloned into *E. coli* previously by our group. Our in-house candidates included *Candida parapsilosis* conjugated polyketone reductase C2,[6,7] *Candida magnoliae* carbonyl reductase S1 [8,9] and single-point mutants of the *Sporobolomyces salmonicolor* aldehyde reductase (SSAR),[10,11] as prepared by the reported method.[12]

[a]*Pfizer Worldwide Research and Development, Sandwich Laboratories, Ramsgate Road, Sandwich, Kent, United Kingdom, CT13 9NJ. E-mail: Robert.carroll@pfizer.com*
[b]*Pfizer Worldwide Research and Development, Chemical Research and Development, Eastern Point Road, Groton, CT, 06340, USA*



Scheme 1 Chemical synthesis of **1**. *Reagents and conditions*: (a) 3-Amino-5-methylisoxazole, EtOAc, CDI, 50–55 °C, 80%; (b) $ClCH_2CN$, TFA, 50 °C, 83%, then thiourea, AcOH, MeOH, 60 °C, 81%; (c) $(RuCl_2(Cymene)_2)_2$, DMF, HCO_2H, TEA, TBME, 25 °C, 42%, 99.6% e.e. (post chiral chromatography); (d) NaOH, 2-MeTHF, 10 min, 92%; (e) **2** + **3**, H_2O, 70 °C, 48%; (f) Zn, AcOH/H_2O/MeOH 10 : 10 : 1, 25 °C, 6 h, 93%.



Scheme 2 Initial preparation of the tertiary alcohol **21** *via* organotin reagents. *Reagents and conditions*: (a) AcCl, MeOH, rt, 16 h, 100%; (b) $(Bu)_3SnOMe$, isoprenyl acetate, $Pd(OAc)_2$, $(o\text{-tolyl})_3P$, PhMe, 100 °C, 98%; (c) LiOH (2 M), THF, 0 °C, 89%; (d) MeMgBr, Et_2O, THF, rt, 73%.

Table 1 Ketoreductase (KRED) catalyzed reduction of 2-chloro-1-(6-chloropyridin-3-yl)ethanone **4**

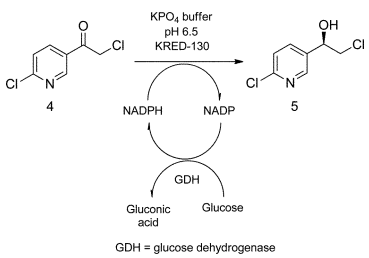
Entry	source	enzyme	% conversion	% e.e.
1	Codexis	Julich ADH-T	83	98 (*R*)
2	Diacel	Diacel E048	98	97 (*R*)
3	Codexis	KRED-142	92	95 (*R*)
4	Pfizer	SSAR Q245P[a]	80	95 (*R*)
5	Pfizer	SSAR Q245K[a]	74	95 (*R*)
6	Codexis	KRED-132	95	94 (*R*)
7	Codexis	KRED-130	95	90 (*R*)
8	Codexis	EXP-A1E	87	99 (*S*)
9	Pfizer	*Candida parapsilosis* cpr-C1	87	99 (*S*)
10	Codexis	EXP-B1K	86	99 (*S*)
11	Codexis	KRED-107	85	99 (*S*)
12	Codexis	EXP-A11	88	99 (*S*)
13	Pfizer	*Candida magnoliae* CRS1	92	99 (*S*)
14	Codexis	EXP-B1E	87	99 (*S*)
15	Codexis	EXP-B1H	85	99 (*S*)
16	Codexis	EXP-A1F	86	99 (*S*)
17	Codexis	Julich CDX013	73	99 (*S*)

[a] Mutants of *Sporobolomyces salmonicolor* aldehyde reductase. Reagents and conditions: 10 mg mL^{-1} **4**; 40 mg mL^{-1} cofactor (NADH or NADPH); ~10 mg mL^{-1} biocatalyst; 100 mM phosphate buffer, pH 7.0, 30 °C; 24 h. Reactions run in 96-well plate format with reaction volume of 0.1 mL.

Interestingly, the results obtained from our collection of SSAR single-point mutants demonstrate the power of enzyme engineering to affect the stereoselectivity of an enzyme. Not only did the mutants show significant variation in product enantiopurity; when the amino acid residue at position 245 was changed from the wild-type's glutamine (Q) to either proline (P) or lysine (K) the enzyme's enantioselectivity completely reversed, switching from (*S*)- to (*R*)-selectivity, as illustrated in Fig. 1.

Following screening, the development of a bioreduction process employing either Julich ADH-T or a *Sporobolomyces salmonicolor* aldehyde reductase mutant (either Q245P or Q245K in particular) was attractive to us due to the availability, high enantioselectivity and low cost of these biocatalysts. Our initial investigations into a preparative bioreduction using Julich ADH-T and NADPH co-factor recycling with glucose dehydrogenase (Julich CDX-901) were encouraging (data not shown). However, on preparative scale the reaction stalled at 60% conversion, despite being operated at the relatively modest substrate loading of 10 g L^{-1}. The use of this biocatalyst was not pursued further.

Our focus shifted to Codexis KRED-130, which had shown higher conversion to product in screening, albeit in modest enantiomeric excess (Table 1, entry 7). A KRED-130-catalysed reduction was carried out on 1.0 g substrate at 50 g L^{-1} concentration, employing a glucose dehydrogenase (Julich CDX-901) co-factor recycling system and complete conversion was observed within 5 h. The resulting (*R*)-chlorohydrin **5** was isolated in a yield of 97% with a better-than-expected e.e. of 95% (Scheme 3).



Scheme 3 Formation of the (*R*)-chlorohydrin **5**.

Furthermore, we repeated the bioreduction on 1.0 g scale (100 mL volume), this time employing our *Sporobolomyces salmonicolor* aldehyde reductase mutant Q245K (1.8 g of wet

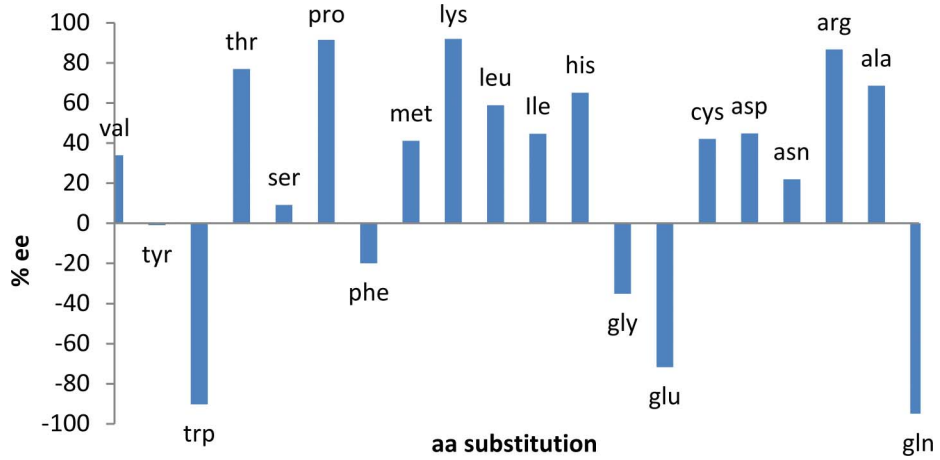
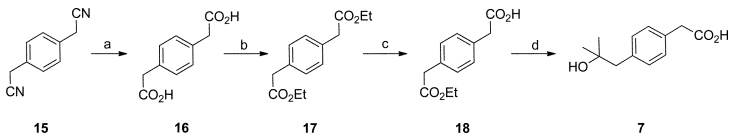


Fig. 1 Selectivity of SSAR mutants at position 245 (positive values for % ee denote (*R*)-selectivity).



Scheme 4 Preparation of alcohol **7** by a chemoenzymatic route. *Reagents and conditions*: (a) H_2O, HCl, 100 °C, 88%; (b) EtOH, H_2SO_4, 100 °C, 99%; (c) Lipozyme TL 100 L, pH 6.0 calcium acetate buffer (0.2 M), MeCN, 35 °C, 94%; (d) THF, MeMgBr, 0 °C, 54%.

cell paste, NADPH and glucose dehydrogenase co-factor recycle system). The transformation was successful with the desired (*R*)-chlorohydrin recovered in 85% yield, 98% chemical purity (as determined by HPLC) and 97% enantiomeric excess, the highest achieved to date for a preparative synthesis of this compound.

We believe that the proof-of-concept scale procedures described herein constitute a process suitable for the delivery of at least multi-hundred gram quantities of (*R*)-chlorohydrin **5** upon scale-up, thus eliminating the need for a Noyori reduction with chromatographic purification.

Chemoenzymatic synthesis of alcohol 7

As an alternative to the route shown in Scheme 2, it was envisaged that commercially available dinitrile **15** could be the starting point for a chemoenzymatic synthesis of alcohol **7** (Scheme 4). Hydrolysis of the dinitrile to di-acid **16** was easily achieved and subsequent esterification in the same vessel furnished the corresponding diester **17** in 88% yield over two steps. Successful hydrolytic desymmetrisation of **17** would provide monoester **18** which, upon treatment with excess Grignard reagent, could be converted into dimethyl-hydroxy acid **7** in 54% yield.

Literature precedent for a chemical desymmetrisation to **18** by reacting the diacid **16** and diester **17** together with hydrochloric acid at 140 °C was noted.[13] However, a milder and more environmentally friendly synthesis was considered desirable and for this reason the use of a hydrolase-catalysed process was investigated.

A general high-throughput screening protocol[14] was employed, which identified fifteen commercially available hydrolases capable of selectively hydrolysing **17** to **18**.[15] It was noted that small amounts of diacid **16** (<10%) were generated by some of these hydrolases.

One of the most promising hits, Meito lipase MY (*Candida cylindracea* lipase), was investigated at preparative scale. Desymmetrisation of diester **17** with Meito lipase MY (100 wt% loading) was attempted under pH control (reaction maintained at pH 6.5 by automatic titration with 1 M NaOH). After 21 h, analysis of the reaction liquors by HPLC showed 94% conversion to mono-acid **18**. Unfortunately, work-up by extraction with ethyl acetate at pH <4.0 provided the isolated product in only 49% yield. The loss of material was tentatively attributed to product entrainment within the biocatalyst residues and no further investigations were pursued with this lipase.

Novozymes Lipozyme TL 100 L (*Thermomyces lanuginosus* lipase) had also been identified as a potential biocatalyst for the desymmetrisation and milligram-scale studies confirmed that it could be employed without product entrainment during work up. A screen of organic co-solvents with pH 6.0 calcium acetate buffer (0.2 M) was conducted to optimize the degree of conversion to mono-acid **18** with suppression of di-acid **16** formation.[16] A range of co-solvents were identified as being suitable for the reaction, with acetonitrile (10–30 vol%) giving excellent conversion as well as selectivity towards mono- over di-acid (Fig. 2). The optimized desymmetrisation reaction in aqueous acetonitrile was successfully carried out on 200 g scale generating mono-acid **18** in 94% yield (Scheme 4). The route to **18** is operationally-simple and does not require any chromatographic purifications or highly toxic reagents, making it an obvious choice to replace the original tin-mediated synthetic route.

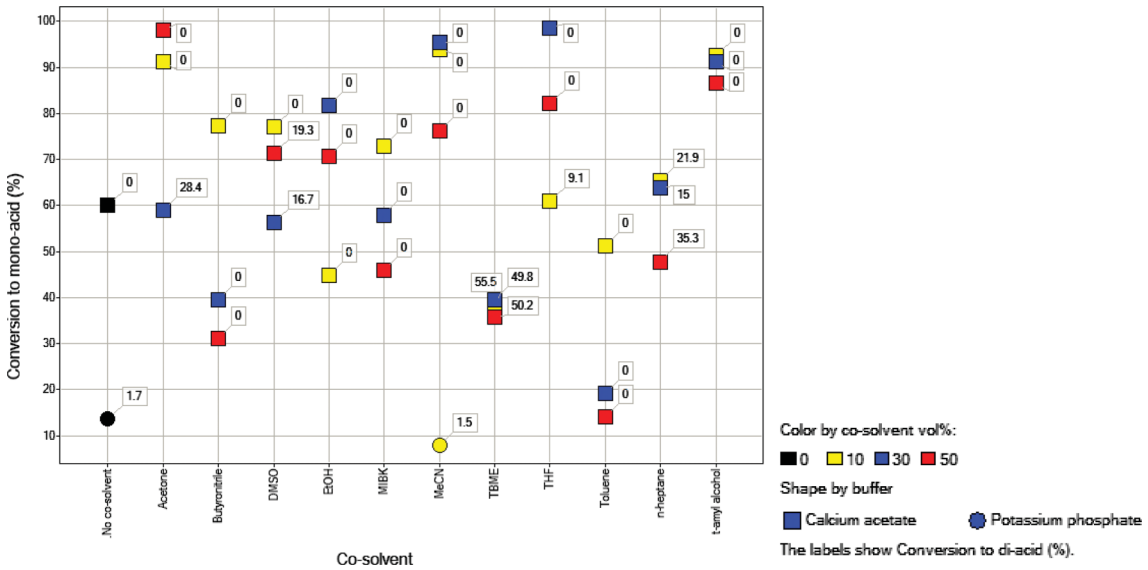


Fig. 2 Co-solvent screen in the enzymatic desymmetrisation of diester **17**. *Reagents and conditions*: 40 mg mL^{-1} **17**; 40 mg mL^{-1} Lipozyme TL 100L; 0.2 M $Ca(OAc)_2$ or pH 7.0 0.1 M potassium phosphate buffer; organic co-solvent as specified, 40 °C, 900 RPM, 24 h.

Conclusions

In summary, to overcome the poor selectivity of a Noyori asymmetric hydrogenation of **4**, a highly selective and high-yielding bioreduction was demonstrated with both a commercially available and a custom-made ketoreductase. Elimination of difficult-to-handle tin reagents through the development of an operationally-simple chemoenzymatic route to **7** was also achieved, employing *Thermomyces lanuginosus* lipase in the key enzymatic desymmetrisation step. Process Mass Intensity (PMI) is a common green chemistry metric, widely applied, to enable benchmarking of processes. It is a measure of the mass of raw materials, reagents, solvents, *etc*. used to provide the mass of API synthesized, *i.e.* kg input/kg output. Calculation of PMI[17] for the epoxide **5** *via* our previous chemical route[3] gives a value of 3051 (impacted by column chromatography) *vs.* 395 using the above enzymatic reduction (individual reagent, solvent and water PMI contributions are given in Table 2). The synthesis of acid **7** under the organotin route[3] results in a PMI of 214 *vs.* 300 using the hydrolytic desymmetrisation approach. This surprisingly high PMI for the bio-catalytic synthesis of **7** is predominantly the result of using relatively large amounts of aqueous media.[18] No doubt this could be optimized to improve the efficiency of the process.

Both of these processes should be capable of providing multi-hundred gram to kilogram quantities of the desired materials.

Experimental

General experimental

Commercial grade reagents and solvents were purchased from Sigma-Aldrich and used without further purification. The commercially available ketoreductase enzymes were purchased from Codexis (Redwood City, CA, USA) and Daicel Chemical Industries (Japan).

Table 2 PMI values comparing chemical and enzymatic synthesis. All values are kg input to kg product

Synthesis	Reagent	Solvent	Water	Total PMI
Enzymatic route to **5**	18	265	112	395
Chemical route to **5**	33	2983	35	3051
Enzymatic route to **7**	32	101	167	300
Chemical route to **7**	35	127	52	214

Ketoreductases cloned into *E. coli*

Candida parapsilosis conjugated polyketone reductase C2 and *Candida magnoliae* carbonyl reductase S1 were cloned into *E. coli* BL21 Gold(DE3) cells (Agilent 230132) and expressed using Overnight Express Instant TB medium (Novagen 71491) at 30 °C.

SSAR-Q245x mutants

SSAR-Q245x mutant preparation. The gene for the *S. salmonicolor* AR2 enzyme was optimized for *E coli* expression and synthesized *de novo* (Blue Heron, Bothell, WA, USA). This gene was then cloned into a pET28b expression plasmid (Stratagene, Santa Clara, CA, USA) between the 5′-NdeI and 3′-EcoRI restriction sites. The mutants were prepared using the QuickChange mutagenesis protocol (Stratagene). The complementary DNA primers listed in the appendix were used to prepare the mutants individually.

Into each PCR tube, SSAR-pET28 plasmid (1 µL, ~50 ng µL^{-1} stock), primers (1 µL each, 10 µM stock), dNTP's (1 µL, 10 µM combination stock), Pfu buffer (5 µL, 10× stock), Pfu Turbo polymerase (1 µL, Stratagene) and water (40 µL). The following PCR profile was used: initial (1 cycle): 2 min at 95 °C; heat cycles (16 cycles): 30 s at 95 °C, 30 s at 50 °C, 6 min at 68 °C; polish (1 cycle): 10 min at 68 °C; hold at 4 °C. To each reaction, DpnI restriction enzyme (1 µL, Stratagene) was added and the reaction mixture incubated at 37 °C for 1 h. The digested samples were then electroporated into Stbl4

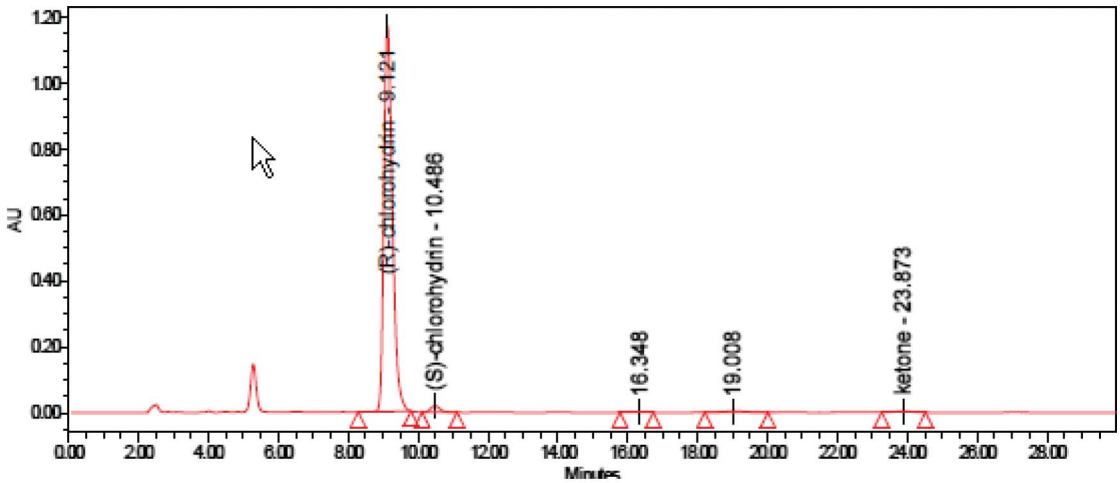


Fig. 3 The chiral HPLC spectrum of **5**.

cells (Invitrogen, Carlsbad, CA) following normal procedures, plated onto LB medium containing kanamycin and incubated overnight at 37 °C. Single colonies were picked for each mutant and incubated overnight at 37 °C/210 RPM. These overnight colonies were submitted to plasmid DNA isolation (miniprep, Qiagen, Hilden, Germany) and submitted for DNA sequencing. Only those plasmids containing the proper mutations were carried forward for expression and activity testing.

SSAR-Q245x mutant expression. The SSAR-Q245x library plasmids were transformed using normal procedures into chemically competent *E. coli* strain BL21 Gold(DE3) cells (Stratagene) for protein expression, plated onto LB medium containing kanamycin and incubated overnight at 37 °C. Single colonies were picked for each mutant into 2 mL LB medium containing kanamycin and incubated overnight at 37 °C/210 RPM. Samples (500 μL) of overnight culture were dispensed into autoinduction medium (25 mL, Overnight Express TB from Novagen, Gibbstown, NJ, USA) containing kanamycin in 125 mL baffled flasks (4116-0125, Nalgene, Rochester, NY, USA). The cultures were incubated overnight at 30 °C/210 RPM. To analyze protein expression, overnight culture (1 mL) was pelleted with centrifugation and the medium decanted. The cell pellet was lysed with BugBuster (1 mL, Novagen) and the cell debris pelleted. The soluble protein expression was analyzed using standard SDS-PAGE techniques with Coomassie staining. All of the SSAR-Q245x mutants showed consistent, high-level soluble expression. The remaining overnight expression cultures were pelleted with centrifugation, the medium decanted and the wet cell pellets stored at –80 °C until needed.

Preparation of 5

To a 250 mL Erlenmeyer flask was charged 80 mL of 100 mM potassium phosphate buffer; pH 7.5, 9.5 mL of 20% aqueous glucose solution; 210 mg $NADP^+$ disodium (Oriental Yeast Company, Japan, cat # 44300900); 200 mg glucose dehydrogenase (Codexis, Redwood City, CA, USA, cat # CDX-901); 9.0 mL of *Sporobolomyces salmonicolor* Q245K wet cell paste as a previously frozen 200 mg mL^{-1} suspension in 100 mM potassium phosphate buffer, pH 7.0; and a solution of ketone **4** (1.0 g) in DMSO (2 mL). The flask was covered with Parafilm and placed on a rotary shaker with a 50 mm throw and shaken at 210 RPM at 30 °C for 4 h. The reaction mixture was extracted with 3 volumes of MTBE and the combined organic layers filtered through Celite. The organic layers were then dried under reduced pressure to yield crude chlorohydrin **5** (0.85 g, 85%). The product's analytical data was consistent with the desired structure and literature precedent.[3]

Chiral HPLC for the analysis of ketone 4 chlorohydrin 5

The bioreduction of ketone **4** to chlorohydrin **5** was followed by chiral HPLC using a ChiralPak AS-RH, 4.6 × 150 mm, 5 micron column (Chiral Technologies, Japan) eluted with water : acetonitrile:TFA (80 : 20 : 0.1, v/v/v). Detection was by photodiode array with quantitation using 270 nm. A representative chromatogram showing a bioreduction using *S. salmonicolor* AR Q245K is shown above (Fig. 3).

Preparation of 16

To a suitably sized vessel was charged **15** (1.25 mol, 196.5 g, 1.0 eq) followed by H_2O (786 mL, 4 mL g^{-1}) and the resulting slurry stirred at room temperature under $N_{2(g)}$. To the slurry was charged conc. HCl (~36%, 4 mL g^{-1}, 786 mL). A small exotherm was noted (internal temperature rise to 35 °C). The resulting slurry was heated to reflux (100 °C) for 12 h. The reaction slurry was cooled to room temperature and isolated by filtration. The filter cake was washed with water (3 mL g^{-1}, 588 mL), the solid collected and dried in vacuum oven for 8 h at 45 °C to yield **16** (1.1 mol, 214.2 g, 88% yield). The product's analytical data was consistent with the desired structure and literature precedent.[19]

Preparation of 17

To a suitably sized vessel was charged **16** (1.02 mol, 200.0 g) followed by ethanol (5 mL g^{-1}, 1.0 L) and the resulting slurry stirred at room temperature under $N_{2(g)}$. To the slurry was added

conc. H_2SO_4 (0.5 mL g^{-1}, 100 mL) and an exotherm was observed (internal temperature rise to 45 °C). The resulting slurry was heated to reflux (80 °C) to give a clear solution. The solution was maintained at 80 °C for 12 h before cooling to room temperature and removing the volatiles *in vacuo*. The resulting residue was dissolved in toluene (6 mL g^{-1}; 1.2 L) and $NaHCO_{3(aq)}$ (30% solution) was slowly added (off-gassing observed) to achieve pH 8.0 in the aqueous layer. The solution was transferred to a separating funnel, the aqueous layer removed and the organic layer washed with water (4 mL g^{-1}, 800 mL) and again separated. The organic phase was then dried over anhydrous $MgSO_4$, filtered and concentrated to dryness to provide **17**, which crystallized upon standing (1.02 mol, 255.8 g, 99% yield). The product's analytical data was consistent with the desired structure and literature precedent.[20]

Preparation of 18

To a suitably sized vessel was charged $Ca(OAc)_2 \cdot H_2O$ (0.91 mol, 160 g) followed by water (4.50 L) and stirred until a solution was obtained. To this was added Lipozyme TL 100 L (100 mL, *Thermomyces lanuginosus* lipase from Novozymes, USA) and the resulting mixture stirred at 35 °C. A pre-formed solution of **17** (0.79 mol, 200 g) in MeCN (2.5 mL g^{-1}, 500 mL) was added to the reaction mixture to give a hazy suspension and an autotitrator was employed to maintain pH 6.5–6.6 with addition of 1.0 M NaOH (~800 mL added over 12 h). Once full conversion to mono-acid **18** was achieved, the reaction was acidified to pH < 2.0 with the addition of 2 M HCl (~1.30 L). The reaction mixture was extracted with EtOAc (10 mL g^{-1}, 2.0 L) and the resulting biphasic mixture filtered through a bed of Celite before being transferred to a separating funnel and the lower aqueous layer removed. The organic phase was washed with water (5 mL g^{-1}, 1.0 L) and then brine (5 mL g^{-1}, 20% w/w, 1.0 L). Finally, the aqueous layers were discarded and the organic phase was concentrated to dryness to yield **18** (0.74 mol, 164.5 g, 94%). The product's analytical data was consistent with the desired structure and literature precedent.[20]

Preparation of 7

To a suitably sized vessel was charged **18** (0.67 mol, 150.0 g) followed by anhydrous THF (10 mL g^{-1}, 1.5 L) and the resultant mixture stirred until a solution was obtained. The solution was cooled to 0 °C and MeMgBr in 75% toluene–25%THF (1.4 M, 2.69 mol, 4 eq.) added dropwise whilst maintaining the temperature below 10 °C. During the addition the solution became a thick suspension and an exotherm and gas evolution were observed. After complete addition of the Grignard reagent, the reaction was stirred for a further 30 min, maintaining the reaction temperature below 10 °C. The reaction was quenched by slow addition of HCl (2.0 M, 10 mL g^{-1}, 1.5 L) maintaining a reaction temperature of less than 10 °C (exotherm and gas evolution observed). The reaction was then warmed to 25 °C, stirred for 15 min and the resultant clear biphasic solution was transferred to a separating funnel. The aqueous layer was discarded and the organic layer washed with water (5 mL g^{-1}, 750 mL) and then brine (5 mL g^{-1}, 750 mL). The organic phase was concentrated to dryness and the crude residue granulated in TBME (7 mL g^{-1}, 1 L) for 2 h prior to isolation of the solid by filtration to yield **7** (0.31 mol, 65.2 g 54% yield). The product's analytical data was consistent with the desired structure and literature precedent.[20]

Appendix

Complementary DNA primers used for mutant creation

Q245A	CCT GCC CTT GCC TTA ATG CCC CCA GCG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Ar	CCA ATA TCT ACG GCG CTG ACA TAA TAC GCT GGG GGC ATT AAG GCA AGG GCA GG
Q245R	CCT GCC CTT GCC TTA ATG CCC CCA CGT TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Rr	CCA ATA TCT ACG GCG CTG ACA TAA TAA CGT GGG GGC ATT AAG GCA AGG GCA GG
Q245N	CCT GCC CTT GCC TTA ATG CCC CCA AAC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Nr	CCA ATA TCT ACG GCG CTG ACA TAA TAG TTT GGG GGC ATT AAG GCA AGG GCA GG
Q245D	CCT GCC CTT GCC TTA ATG CCC CCA GAC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Dr	CCA ATA TCT ACG GCG CTG ACA TAA TAG TCT GGG GGC ATT AAG GCA AGG GCA GG
Q245C	CCT GCC CTT GCC TTA ATG CCC CCA TGC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Cr	CCA ATA TCT ACG GCG CTG ACA TAA TAG CAT GGG GGC ATT AAG GCA AGG GCA GG
Q245E	CCT GCC CTT GCC TTA ATG CCC CCA GAG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Er	CCA ATA TCT ACG GCG CTG ACA TAA TAC TCT GGG GGC ATT AAG GCA AGG GCA GG
Q245G	CCT GCC CTT GCC TTA ATG CCC CCA GGT TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Gr	CCA ATA TCT ACG GCG CTG ACA TAA TAA CCT GGG GGC ATT AAG GCA AGG GCA GG
Q245H	CCT GCC CTT GCC TTA ATG CCC CCA CAC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Hr	CCA ATA TCT ACG GCG CTG ACA TAA TAG TGT GGG GGC ATT AAG GCA AGG GCA GG
Q245I	CCT GCC CTT GCC TTA ATG CCC CCA ATC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Ir	CCA ATA TCT ACG GCG CTG ACA TAA TAG ATT GGG GGC ATT AAG GCA AGG GCA GG
Q245L	CCT GCC CTT GCC TTA ATG CCC CCA CTG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Lr	CCA ATA TCT ACG GCG CTG ACA TAA TAC AGT GGG GGC ATT AAG GCA AGG GCA GG
Q245K	CCT GCC CTT GCC TTA ATG CCC CCA AAA TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Kr	CCA ATA TCT ACG GCG CTG ACA TAA TAT TTT GGG GGC ATT AAG GCA AGG GCA GG
Q245M	CCT GCC CTT GCC TTA ATG CCC CCA ATG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Mr	CCA ATA TCT ACG GCG CTG ACA TAA TAC ATT GGG GGC ATT AAG GCA AGG GCA GG
Q245F	CCT GCC CTT GCC TTA ATG CCC CCA TTC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Fr	CCA ATA TCT ACG GCG CTG ACA TAA TAG AAT GGG GGC ATT AAG GCA AGG GCA GG
Q245P	CCT GCC CTT GCC TTA ATG CCC CCA CCG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Pr	CCA ATA TCT ACG GCG CTG ACA TAA TAC GGT GGG GGC ATT AAG GCA AGG GCA GG
Q245S	CCT GCC CTT GCC TTA ATG CCC CCA TCT TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Sr	CCA ATA TCT ACG GCG CTG ACA TAA TAA GAT GGG GGC ATT AAG GCA AGG GCA GG
Q245T	CCT GCC CTT GCC TTA ATG CCC CCA ACC TAT TAT GTC AGC GCC GTA GAT ATT GG

Q245Tr	CCA ATA TCT ACG GCG CTG ACA TAA TAG GTT GGG GGC ATT AAG GCA AGG GCA GG
Q245W	CCT GCC CTT GCC TTA ATG CCC CCA TGG TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Wr	CCA ATA TCT ACG GCG CTG ACA TAA TAC CAT GGG GGC ATT AAG GCA AGG GCA GG
Q245Y	CCT GCC CTT GCC TTA ATG CCC CCA TAC TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Yr	CCA ATA TCT ACG GCG CTG ACA TAA TAG TAT GGG GGC ATT AAG GCA AGG GCA GG
Q245V	CCT GCC CTT GCC TTA ATG CCC CCA GTT TAT TAT GTC AGC GCC GTA GAT ATT GG
Q245Vr	CCA ATA TCT ACG GCG CTG ACA TAA TAA ACT GGG GGC ATT AAG GCA AGG GCA GG

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16 Co-solvent screen of enzymatic desymmetrisation of diester (**17**). Conditions: diester **17** (20 mg) was charged to a set of reaction vials and co-solvent (0–250 μL) added, followed by pH 6.0 calcium acetate buffer (0.2 M) to make the total solvent volume up to 0.5 mL. Finally, a solution of Lipozyme TL 100 L (20 μL) was charged to each vial, the reaction vials were crimped and placed into an Eppendorf thermomixer orbital shaker and shaken at 900 RPM at 40 °C over 24 h.

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