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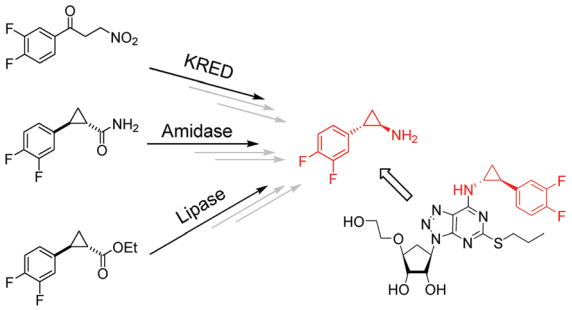
Three different enzymatic routes were employed to produce a precursor of the important antithrombotic agent Ticagrelor with high ee.



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PAPER

Biocatalytic approaches to a key building block for the anti-thrombotic agent ticagrelor†

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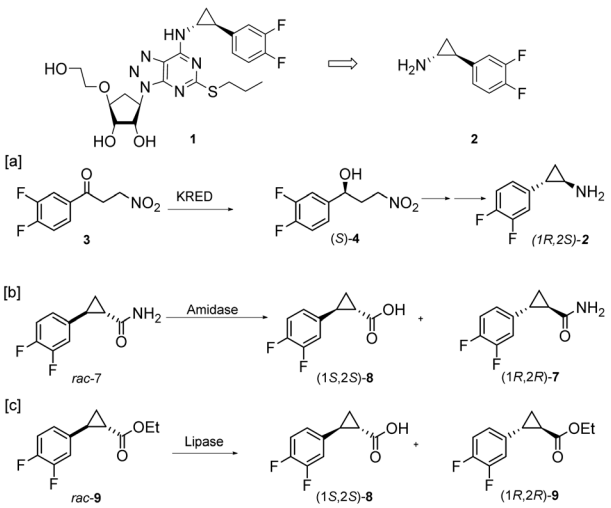
Three complementary biocatalytic routes were examined for the synthesis of the cyclopropyl amine (1*R*,2*S*)-**2**, which is a key building block for the anti-thrombotic agent ticagrelor **1**. By employing either a ketoreductase, amidase or lipase biocatalyst, the key building blocks for synthesis of the amine **2** were obtained in 99.9, 94.6 and 48.8% ee, respectively.

Introduction

During the past two decades, the pharmaceutical industry has sought to address environmental challenges associated with manufacturing by embracing the principles of green chemistry.[1] One of these guidelines is directed towards reducing the waste[2] while another addresses the use of reagents from sustainable sources to render a process more environmentally sustainable. In this context biocatalysis represents an attractive option in that enzymes offer the possibility of highly selective (enantio-, regio-, chemo-) transformations based upon sustainable catalysts with high catalytic rates of turnover. Ticagrelor **1** is one of the most potent drugs on the market for the treatment of acute coronary syndrome. The cyclopropyl amine **2** represents a challenging target, in particular to explore and ultimately compare alternative biocatalytic routes for its synthesis. Previously reported routes to the amine **2** include the use of chiral auxiliaries (*e.g.* ruthenium based chiral ligands,[3] Oppolzer's chiral camphorlactam,[4,5] or menthol[6,7]) as well as kinetic resolution of the corresponding racemate by formation of diastereomeric salts.[4,6] We opted to employ a biocatalytic retrosynthetic approach[8] in order to develop and evaluate potential routes towards the target compound (Scheme 1).



Scheme 1 Biocatalytic approaches towards the target cyclopropylamine **2**.

Results and discussion

The first approach envisaged was to explore asymmetric reduction of ketone **3** to either (*R*)- or (*S*)-**4** using a ketoreductase (KRED) (Scheme 1a). Actavis recently reported[9] enantioselective reduction of ketone **3** to alcohol (*R*)-**4** using CBS-borane. Subsequent cyclopropyl ring formation *via* a Mitsunobu type reaction yielded the nitrocyclopropane (1*R*,2*S*)-**5** which was then reduced to the desired amine **2** with an ee = 99% (Scheme 2). The (*S*)-alcohol has also been converted to the target amine (1*R*,2*S*)-**2** *via* initial bromination to give the bromide **6** followed by cyclopropane formation with DBU and reduction of the nitro group.

We initially screened a number of ketoreductases from the Codexis Codex® KRED Screening Kit as well as the RasADH

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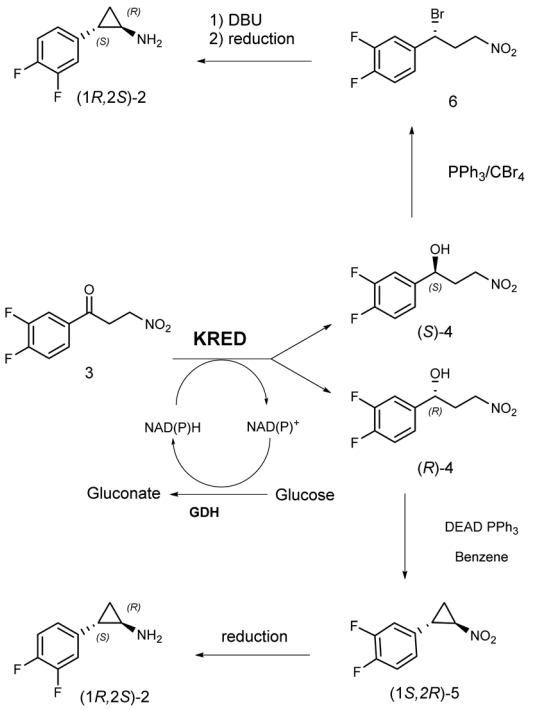


Scheme 2 Ketoreductase mediated preparation of (*R*)- and (*S*)-alcohol **4**.

from *Ralstonia* sp.[10,11] for activity towards **3**. We were able to identify biocatalysts for generation of both the (*R*)- and (*S*)-alcohol **4** with good to excellent ee. (Table 1). KRED-130 (Codexis) proved to be the best candidate (entry 1) yielding the (*S*)-enantiomer with an enantiomeric excess of 99.9% and 100% conversion after 48 h. The use of 10% DMSO enhanced the solubility of the substrate and although alternative systems for NADPH co-factor recycling were examined, the use of glucose/glucose dehydrogenase (GDH) proved to be optimal (data not shown). RasADH was also found to yield (*S*)-**4** although with lower ee. Using KRED 110 (Codexis), the (*R*)-alcohol **4** can also be prepared but with low conversion (23%) and modest ee (71.6%). Although the enantioselectivities and conversions obtained with the KRED were good, the catalyst loading in this approach suggested further optimisation would be required. In contrast to a theoretical 100% yield in an asymmetric approach such as a, hydrolase-mediated approaches are limited to a 50% yield maximum. However we weighted catalyst loading against theoretical yields and shifted our attention to approaches b and c (Scheme 1).

Table 1 KRED mediated reduction of ketone **3**

Entry	Enzyme	T [°C]	t [h]	Conv.	ee (Config.)
1	KRED-130	30	48	100	99.9 (*S*)
2	KRED-110	30	48	23	71.6 (*R*)
3	RasADH	30	48	34	91.6 (*S*)

For subsequent approaches to amine **2**, hydrolase mediated kinetic resolutions of suitable precursors[12] were explored to access the acid **8** (Scheme 1b and c). The (1*R*,2*R*)-acid **8** has previously been reported to undergo a stereospecific Curtius rearrangement to yield (1*R*,2*S*)-**2**.[6,13] The microorganism *Rhodococcus rhodochrous* has previously been shown to hydrolyse cyclopropyl-containing carboxamides with high enantioselectivity[14–16] and thus we prepared racemic amide **7** and tested it as a substrate using whole cells of *R. rhodochrous* IFO 15564. In agreement with previous studies the amidase was found to exhibit (*S*)-selectivity towards **7** yielding (1*S*,2*S*)-acid **8**.

In order to optimise conversion, the effect of different cell densities (OD600), co-solvents and temperature were examined (see Table 2). Amide **7** dissolved readily in methanol and ethanol, but partially precipitated after coming into contact with the aqueous reaction medium. The precipitate disappeared over the course of reaction as the amide was hydrolysed by the amidase. In the absence of co-solvent, the reaction medium formed a suspension between the cell suspension and the hydrophobic amide. Under these conditions, it is likely that only small amounts of substrate will have dissolved, thereby creating a substrate/catalyst ratio similar to that attained in a reaction performed at high cell density.

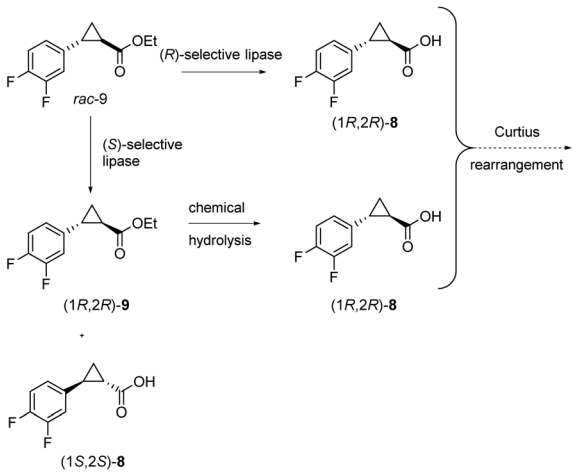
The data in Table 2 shows an inverse relationship between the enantioselectivity of the hydrolysis and the cell loading. A possible explanation for this phenomenon is related to the uptake of the substrate. Resting cells are employed in these experiments, therefore the observed amidase activity has to be due to an intracellular enzyme (extracellular enzymes are removed through several washing steps). Although we were able to attain both amide and carboxylic acid in high enantiomeric purity and with a conversion close to the theoretical 50% **8** (Table 2 entry 4) *via* this approach, the comparably lower yields obtained prevented further scale-up of this method.

As a third approach, lipase mediated hydrolysis of the corresponding racemic ethyl ester **9** (Scheme 1c) was investigated, with the goal of identifying either an (*R*)- or (*S*)-selective biocatalyst for the generation of the corresponding enantiomerically pure recovered ester or carboxylic acid product (Scheme 3). In either case the target would be the carboxylic acid (1*R*,2*R*)-**8** which as before could be converted to the target amine **2** *via* a Curtius rearrangement.

Table 2 Hydrolysis of amide *rac*-**7**

Entry	OD 600	T [°C]	t [h]	ee_s	ee_p	Conv.	E (Config.)
1	1.5	26	23	97.8	43.2	69.4	10 (*S*)
2	1	26	23	96.8	60.2	61.7	16 (*S*)
3	0.5	26	23	98.0	60.0	62.0	17 (*S*)
4	0.25	26	20	92.5	86.6	51.6	46 (*S*)
5[a]	0.25	26	20	11.8	88.7	11.7	19 (*S*)
6	0.25	4	26	32.0	94.9	25.2	*52.8 (S)*
7[b]	0.25	4	16.7	95.3	14.9	48.8	*16.7 (S)*

[a] No co-solvent. [b] Methanol (10% v/v) was used as a co-solvent.



Scheme 3 lipase mediated hydrolysis of precursor ester **9**.

A number of lipases and esterases from different commercial sources (Almac, Johnson Matthey) were screened, most of which showed no activity and/or enantioselectivity towards **9**. However, the lipase from *Thermomyces lanuginosus* (TlL) showed good activity on an initial small scale screen (5 mg ester *rac*-**9**, 5 wt% lipase in 1 mL MTBE, 48 h at 30 °C orbital shaking) and yielded the desired (1*R*,2*R*)-ester **9** and (1*S*,2*S*)-acid **8** with ee_s = 14.7 and ee_p = 99.9 (conv. = 12.8%, E > 200). Based upon these initial results the reaction was subsequently scaled up and optimised with respect to co-solvents (Table 3).

Lipase activity is known to be enhanced by interfacial activation[17] and hence the reactions were performed in 10% water-immiscible co-solvents.[18] In ethyl acetate no hydrolysis of ester **9** was observed. Addition of 10% (v/v) methyl *tert*-butyl ether (MTBE) had a negative influence on the reaction compared to a biotransformation performed only in buffer (*cf.* Table 3, entries 2 and 4). Finally, addition of cyclohexane seemed to have a beneficial influence on the conversion of the biotransformation. The degree of conversion was double compared to the conversion obtained in a buffered system without co-solvent (*cf.* Table 3, entry 4).

Experimental

Ketoreductases

Conditions for the KRED-130 (Codexis): reactions were performed in 100 mM potassium phosphate buffer (pH 7) with 5 g L^{-1} ketone **3** in 10% DMSO, 5 g L^{-1} ketoreductase, 1 g L^{-1} GDH, 1 g L^{-1} NADPH, 1.25 molar equivalents of glucose. The reaction was kept stirring (magnetic agitation 500 rpm) at 30 °C for 48 h. Conditions for the RasADH (W. Kroutil, I. Lavandera): the lyophilised enzyme was rehydrated in buffer for 30 min prior to the addition of the substrate. RasADH screens were performed in 50 mM Tris HCl buffer pH 7.5 with 8 g L^{-1} ketone 3, 37 g L^{-1} ketoreductase, 0.7 g L^{-1} NADPH, 1 g L^{-1}, 2 molar equivalents of glucose. The reaction was kept stirring (magnetic agitation 500 rpm) at 30 °C for 48 h. Workup: all reactions were stopped in all cases by extraction of the aqueous phase with two volumes of MTBE. The organic phases were combined and dried over $MgSO_4$ and the crude was analysed *via* HPLC and NMR.‡

Table 3 Use of co-solvents in TlL mediated hydrolysis of *rac*-**9**

Entry	Co-solvent	ee_s	ee_p	Conv.	E (Config.)
1	AcOEt	0	0	0	0
2	MTBE	3.1	76.5	3.9	8 (*S*)
3	Cyclohexane	46.3	96.9	32.3	101 (*S*)
4	Buffer	18.4	99.1	15.6	>100 (*S*)

Hydrolases

Amidase-mediated hydrolysis of compound **7**: a suspension of arrested cells in a 0.1 M phosphate buffer at pH 7.0 was used for all biotransformations performed. The bacterial solutions employed showed an OD650 of 1.5, 1.0, 0.5 and 0.25, respectively. Amide rac-**7** was added to the bacterial solution in 10% (w/v) ethanol to obtain a final concentration of 0.1% (w/v). The reactions were shaken for 22 h (orbital shaker, temperatures as indicated) and were then stopped by centrifugation and decantation of the supernatant. The resulting aqueous solution was basified with $NaHCO_3$ and extracted with dichloromethane by shaking and centrifugation. Subsequently the resulting aqueous phase was acidified (1 N HCl) and again extracted with AcOEt. The organic phases were dried over $MgSO_4$ and the solvent evaporated in a rotary evaporator. The compounds were dried under vacuum and could be isolated in pure state with yields of ≥90%. Lipase-mediated ester hydrolysis of compound rac-**9**: 1 ml Screens were performed in MTBE in a 2 mL Eppendorf tube. The substrate ester rac-**9** was added to the solvent (1 mL) as a 0.5% (w/v; 5 mg) solution. The enzyme was added to give a 5% (w/v; 50 mg enzyme suspension) solution. The reactions were left to shake in an orbital incubator at 30 °C for 48 h. Medium scale reactions were performed in phosphate buffer 0.1 M, pH 7.0 (5 mL) and 10% co-solvent in a 10 mL flask. The substrate ester rac-**9** was added as a 1% (w/v; 50 mg) solution to the solvent. The enzyme was added to give a 2% (w/v; 100 mg enzyme suspension) solution. The reactions were stirred at room temperature (18 °C) for 48 h.

Conclusion

Three complementary biocatalytic approaches have been examined towards the key intermediate cyclopropylamine **2** used in the synthesis of Ticagrelor. While the KRED-mediated approach afforded alcohol-**4** with good to excellent enantioselectivities, the catalyst loading was somewhat high. The hydrolase-mediated approaches closely met the theoretical

‡ ee_s and ee_p were determined by chiral HPLC, conversion was determined as $c = \frac{ee_s}{ee_s + ee_p}$, and enantioselectivity E determined from ee_s and ee_p.[19]

50% yield. The amidase from *R. rhodochrous* yielded the target amide (1*R*,2*R*)-**7** in acceptable enantiomeric purity, although the comparably lower yields obtained prevented further scale-up of this method. The enantioselectivities and conversions obtained in the lipase-mediated approach show high potential for further process optimisation and thus represent a key step in applying enzyme technologies for the synthesis of the anti-thrombotic agent Ticagrelor.

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