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# Enoyl acyl carrier protein reductase (FabI) catalyzed asymmetric reduction of the C=C double bond of α,β-unsaturated ketones: preparation of (*R*)-2-alkyl-cyclopentanones†

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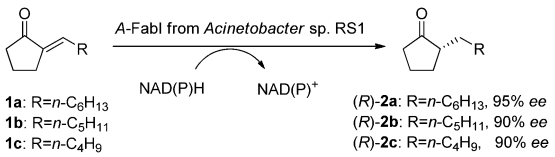
**Enoyl-ACP reductase (FabI) was identified as a non-OYE 'ene'-reductase for asymmetric reduction of the C=C double bond of α, β-unsaturated ketones. Reduction of several 2-alkylidenecyclopentanones with *A*-FabI and *E*-FabI gave (*R*)-2-alkylcyclopentanones in 95–90% and 70–81% *ee*, respectively. The product *ee* was improved to 99–98% in high yield by subsequent one-pot biooxidation.**

Enzymatic asymmetric reduction of C=C double bonds is a green and useful method for the preparation of enantiopure fine chemicals, pharmaceuticals, and aroma materials.[1] Thus far, old yellow enzymes (OYEs) have been used as the primary reductases for this type of reactions,[1,2] with their own substrate specificity and selectivity. Recently a few non-OYE reductases have been reported, including medium chain dehydrogenases/reductases (MDRs) and enoate reductases (EnoRs).[1c] The MDRs prefer the substrates containing strong electron-withdrawing groups such as enals and nitroalkenes,[3] and they are plant or mammalian enzymes. The EnoRs were discovered from anaerobes,[4] and they are oxygen sensitive. Therefore, the discovery of non-OYE reductases with novel substrate specificity, high enantioselectivity and easy utilization has remained a challenging and very important task. Here we report the identification of enoyl acyl carrier protein reductase (FabI) as a useful non-OYE reductase with unique substrate specificity and high enantioselectivity for the asymmetric reduction of the C=C double bond of α,β-unsaturated ketones.

Previously, we found *Acinetobacter* sp. RS1 catalyzing the asymmetric reduction of 2-alkylidenecyclopentanones **1a–b** to give the corresponding (*R*)-2-alkylcyclopentanones **2a–b** (Scheme 1), with good activity and enantioselectivity.[5] The reduction of substrates **1a–c** could not be achieved with OYEs, and the products were important intermediates for the preparation of chiral lactones, hydroxyl acids, and alcohols.[6] To identify the unique 'ene'-reductase from this strain, we purified the enzyme by using FPLC on anion exchange, hydrophobic interaction, and gel filtration columns. The purified enzyme displayed a specific activity of 110 U $g^{-1}$ protein for the reduction of **1b**, with a purification factor of 370. The *ee* of the produced (*R*)-**2b** was 83%, similar to the *ee* value obtained from the same reduction with a wild type strain.[5] The purified enzyme fraction had 60% purity, which is not pure enough for the determination of the terminal amino acid sequence. Nevertheless, MALDI-TOF analysis suggested a molecular weight of 27.5 KDa for the major protein in the active enzyme fraction.




Scheme 1 *A*-FabI-catalyzed asymmetric reduction of 2-alkylidenecyclopentanones **1a–c** to produce (*R*)-2-alkyl-cyclo-pentanones **2a–c**.

The genome of *Acinetobacter* sp. RS1 was then sequenced. By using bioinformatics tools, 7 possible C=C or C=N double bond reductases were predicted. Out of these reductases, the enoyl-ACP reductase (*A*-FabI) had a molecular weight of 28.6 kDa and thus was cloned. *E. coli* (*A*-FabI) expressing his-tagged *A*-FabI was engineered to produce the enzyme, and the enzyme was purified by affinity chromatography with 95% purity (ESI†). The purified *A*-FabI catalyzed the reduction of **1b** to give (*R*)-**2b** in 88% *ee* with a specific activity of 300 U $g^{-1}$ protein. Both NADH and NADPH were found to be the necessary cofactor for the reduction. *A*-FabI and the partially purified 'ene'-reductase from *Acinetobacter* sp. RS1 showed the same enantioselectivity, same cofactor dependence, and similar activity for the reduction, thus *A*-FabI was identified as the enzyme in *Acinetobacter* sp. RS1 responsible for the asymmetric reduction of the C=C double bond of **1b**.

The purified *A*-FabI was further examined in detail for the reduction of 2-alkylidenecyclopentanones **1a–c**, in the presence

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† Electronic supplementary Information (ESI) available: Experimental details of enzyme purification, identification, cloning and catalysis; molecular docking and HPLC chromatograms. See DOI: 10.1039/c4cc04150j

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Table 1 Asymmetric reduction of 2-alkylidenecyclopentanones **1a–c** with purified FabI

| Enzyme[a] | Sub. | Conc. (mM) | Prod. | Conv. (%) | Spec. Act.[b] (U $g^{-1}$) | *ee* (%) |
|---|---|---|---|---|---|---|
| *A*-FabI | **1a** | 10 | (*R*)-**2a** | 63 | 300 | 92 |
| | **1a** | 30 | (*R*)-**2a** | 27 | 400 | 95 |
| | **1b** | 10 | (*R*)-**2b** | 61 | 300 | 88 |
| | **1b** | 30 | (*R*)-**2b** | 31 | 300 | 90 |
| | **1c** | 10 | (*R*)-**2c** | 100 | 200 | 90 |
| | **1c** | 20 | (*R*)-**2c** | 75 | 200 | 89 |
| *E*-FabI | **1a** | 40 | (*R*)-**2a** | 100 | 800 | 79 |
| | **1a** | 50 | (*R*)-**2a** | 72 | 1200 | 81 |
| | **1b** | 40 | (*R*)-**2b** | 100 | 1200 | 70 |
| | **1b** | 50 | (*R*)-**2b** | 81 | 1500 | 70 |
| | **1c** | 15 | (*R*)-**2c** | 100 | 600 | 76 |
| | **1c** | 30 | (*R*)-**2c** | 53 | 900 | 75 |

[a] Biotransformation was conducted in 3 mL of Tris buffer (50 mM; pH = 7.5) containing 3 mg of purified FabI as well as the substrate and NADH in a molar ratio of 1 : 1.5 at 30 °C and 300 rpm for 1 h. [b] Specific activity was determined for the first 30 min reaction and given in U $g^{-1}$ protein.

of NADH or NADPH. As listed in Table 1, *A*-FabI gave a specific activity of 200–400 U $g^{-1}$ protein and a conversion of 61–100% for the reduction of 10 mM substrates **1a–c**, and it demonstrated high enantioselectivity to give (*R*)-**2a–c** in 90–95% *ee*. For practical applications, we are currently working on the development of immobilized *A*-FabI coupled with immobilized glucose dehydrogenase (GDH) for *in vitro* bioreduction with cofactor recycling.

Inspired by the discovery of *A*-FabI, another FabI enzyme was searched for the same type of reductions. The FabI enzyme (*E*-FabI) from *Escherichia coli* was found to show 60% sequence identity with *A*-FabI, and it was thus cloned, overexpressed in *E. coli*, and purified (ESI†). The purified *E*-FabI was found to catalyze the reductions of **1a–c** as well (Table 1), in the presence of NADH or NADPH. The reactions were also enantioselective, giving (*R*)-**2a–c** in 70–81% *ee*. Although *E*-FabI showed lower enantioselectivity than *A*-FabI, it gave much higher specific activity (900–1500 U $g^{-1}$ protein) and fully converted 40 mM **1a–b** and 15 mM **1c** to the corresponding products, respectively, within 1 h. The kinetics of *E*-FabI-catalyzed reduction of **1a–c** was determined (ESI†). The obtained $K_m$ (31–49 μM) and $k_{cat}$ (1.2–2.2 $s^{-1}$) were similar to those for the reduction of the natural enoyl thioester substrates (enoyl-ACP and enoyl-CoA).[7] The catalytic efficiency ($k_{cat}/K_m$) of 2.6–6.5 × $10^4$ $s^{-1}$ $M^{-1}$ suggested good application potential for *E*-FabI.

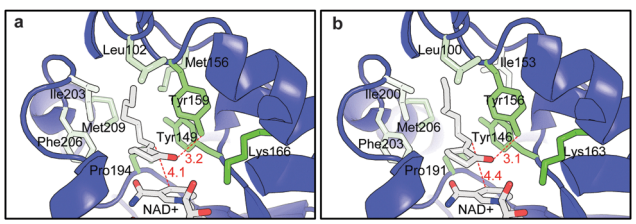
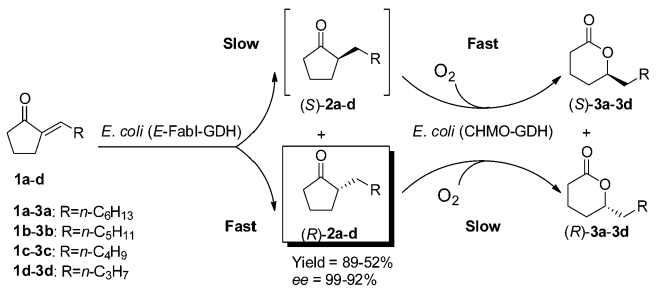



Fig. 1 Active docking pose of **1a** onto (a) the structure model of *A*-FabI and (b) the X-ray structure of *E*-FabI co-crystallized with $NAD^+$ (1MFP in PDB database). The color of the side chains of the amino acid residues indicates their degree of hydrophobicity from green (hydrophilic) to pale green (hydrophobic).

FabI belongs to metal-independent divergent short chain dehydrogenases/reductases. It is a key enzyme in biological type II fatty acid synthesis and has been a major target for antibacterial drug design.[7,8] The enzyme was firstly discovered from *E. coli* and more than 60 X-ray structures have been determined for the FabI enzymes from 10 different sources. So far the natural substrates (enoyl-ACP and enoyl-CoA) are the only reported substrates for the enzyme.[7] In contrast to the FMN-mediated *trans*-addition of OYEs,[1c] the catalysis of FabI does not require any prosthetic group. A hydride from the cofactor and a proton are directly added to the C=C double bond *via cis*-addition.[9] The natural substrates of FabI are enoyl thioesters with an ACP or a CoA moiety as the directing group in the enzyme active site. It was believed that the absence of such a directing group would eliminate the catalytic activity.[7] Meanwhile, the thioester group of the substrates forms hydrogen bonds within the active site to enhance the substrate binding.[7,9] However, non-natural substrates **1a–c** have no such functional groups. Molecular docking of **1a** to the homology structure model of *A*-FabI and the X-ray structure of *E*-FabI gave some insight into the binding of the substrates in the enzyme active site. As shown in Fig. 1, *A*-FabI and *E*-FabI have very similar binding pockets despite of only 60% sequence identity. **1a** adopted a similar 'U-shape' conformation as that recorded for the natural C16 substrate in the crystal structure of the FabI from *Mycobacterium tuberculosis*.[10] Due to the hydrophobic interaction, the side chain of **1a** settles down in a hydrophobic pocket consisting of Leu102, Met 156, Ile203, Phe206 and Met 209 in *A*-FabI (Fig. 1a). The carbonyl group of **1a** forms a hydrogen bond with Tyr159, which could stabilize the catalytic intermediate.[9] The plane of the conjugated C=O and C=C double bonds of **1a** is parallel to the nicotinamide ring of the cofactor $NAD^+$/NADH. A hydride is to be transferred from the reduced cofactor to the $C_\beta$ of the C=C double bond with a distance of 4.1 Å (Fig. 1a), while a proton from the solvent is to be added to the $C_\alpha$ *via* the reported *cis*-addition[9] to give (*R*)-**2a**. This explains the enantioselectivity outcome. The *cis*-addition of the enzyme is in contrast to the well-known *trans*-addition of OYEs.[1c] Similar explanations can be drawn from the active binding pose of **1a** in *E*-FabI (Fig. 1b) and of other substrates **1b–c** in *A*-FabI and *E*-FabI, respectively (ESI†). No other alternative active substrate pose was generated due to the




Scheme 2 One-pot sequential cascade biotransformation of 2-alkylidenecyclopentanones **1a–d** *via* *E*-FabI-catalyzed *R*-enantioselective reduction and CHMO-catalyzed *S*-enantioselective Baeyer–Villiger oxidation to prepare (*R*)-2-alkyl-cyclopentanones **2a–d** in high *ee*.


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Table 2 One-pot sequential cascade biotransformation of 2-alkylidenecyclopentanones **1a–d** by using resting cells of recombinant *E. coli* strains expressing *E*-FabI and CHMO, respectively, to prepare (*R*)-2-alkyl-cyclopentanones **2a–d**

| Sub. | Conc. (mM) | Catalyst A[a] | Vol. (mL) | Time (min) | Catalyst B[b] | Vol.[c] (mL) | Time (min) | Prod. | Yield[d] (%) | *ee* (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **1a** | 40 | *E. coli* (*E*-FabI–GDH) | 5 | 60 | *E. coli* (CHMO–GDH) | 1 | 60 | (*R*)-**2a** | 89 | 99 |
| **1b** | 40 | *E. coli* (*E*-FabI–GDH) | 5 | 60 | *E. coli* (CHMO–GDH) | 1 | 60 | (*R*)-**2b** | 82 | 98 |
| **1c** | 40 | *E. coli* (*E*-FabI–GDH) | 5 | 60 | *E. coli* (CHMO–GDH) | 5 | 60 | (*R*)-**2c** | 78 | 99 |
| **1d** | 30 | *E. coli* (*E*-FabI–GDH) | 5 | 180 | *E. coli* (CHMO–GDH) | 5 | 60 | (*R*)-**2d** | 52 | 92 |

[a] Cell density of 20 g cdw $L^{-1}$. [b] Catalyst B was added after the first step reduction. [c] The volume of the buffer containing catalyst B (8 g cdw $L^{-1}$) added after the first step reduction. [d] Analytical yield.

incompatible hydride transfer distance (>5.0 Å) in all docking cases of **1a–c**. Moreover, no alternative substrate pose that leads to the (*S*)-products was found in the docking of **1a** and **b** in *A*-FabI and *E*-FabI as well as **1c** in *A*-FabI. Docking of **1c** in *E*-FabI gave a substrate pose that would lead to the formation of (*S*)-**2c**. However, the distance for hydride transfer from the cofactor to the C=C double bond was 5.6 Å, which indicates a non-active pose (ESI†).

To enhance the product *ee* from *E*-FabI-catalyzed reduction, methods such as protein engineering could be applied.[11] Here we developed a cascade biocatalysis to improve the product *ee* by introducing a subsequent *S*-enantioselective Baeyer–Villiger oxidation in one pot to convert the minor *S*-enantiomer of ketones **2a–d** into the corresponding lactones **3a–d** (Scheme 2). *E. coli* (*E*-FabI–GDH) co-expressing *E*-FabI and GDH was engineered as a whole-cell biocatalyst for the reduction of **1a–d** with co-factor regeneration, and *E. coli* (CHMO–GDH) co-expressing cyclohexanone monooxygenase (CHMO) and GDH was constructed as the second whole-cell biocatalyst for the Baeyer–Villiger oxidation of **2a–d** with co-factor regeneration as well. The cascade reduction and oxidation were conducted in a sequential manner to avoid the oxidation of **1a–d** with CHMO. As shown in Table 2, cascade reduction and oxidation of 40 mM **1a–c** gave (*R*)-**2a–c** in 99–98% *ee* and 89–72% analytical yield. In the case of **1d**, the reduction with *E*-FabI showed low enantioselectivity to give (*R*)-**2d** in only 35% *ee*. Nevertheless, by introducing the subsequent Baeyer–Villiger oxidation, (*R*)-**2d** was obtained in 92% *ee*. For all these cascade reactions, the produced (*R*)-**2a–d** could be easily separated from the mixtures by fractional distillation due to the significant difference of the boiling points of ketones and lactones according to a previous study.[6a]

The time course of the one-pot sequential cascade biotransformation of **1a** to produce (*R*)-**2a** in high *ee* is shown in Fig. 2. The product concentration in the first reduction step with the resting cells of *E. coli* (*E*-FabI–GDH) increased linearly within 50 min, and all substrate **1a** was fully converted to (*R*)-**2a** in 80% *ee* after 50 min. In the subsequent oxidation with the resting cell of *E. coli* (CHMO–GDH), (*S*)-**2a** was rapidly oxidized to (*S*)-**3a** due to the *S*-enantioselectivity of CHMO. Consequently, the *ee* of (*R*)-**2a** increased with the time and reached 99% at 45 min. 89% analytical yield was achieved at 1 h. Similar reaction courses were also observed for the cascade biotransformation of **1b–d** with the same catalysts (ESI†).

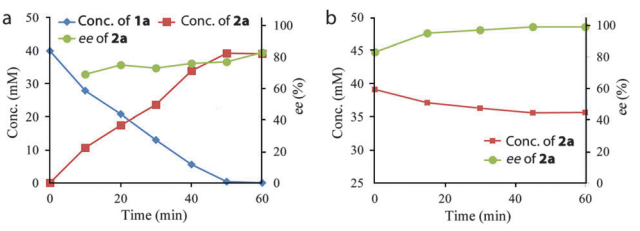


Fig. 2 Time course of the one-pot sequential cascade biotransformation of 2-heptylidenecyclopentanone **1a** to produce (*R*)-2-heptylcyclopentanone **2a** in high *ee*. (a) Reduction of **1a** with *E. coli* (*E*-FabI–GDH). (b) Oxidation of **2a** with *E. coli* (CHMO–GDH).

In conclusion, enoyl-ACP reductase (FabI), known for its role in cellular fatty acid synthesis, was discovered for the first time for the asymmetric reduction of C=C double bonds of non-natural substrates. As a non-OYE reductase *A*-FabI showed different substrate specificity to OYEs, reducing 2-alkylidene-cyclopentanones **1a–c** to give (*R*)-2-alkylcyclopentanones **2a–c** in 95–90% *ee* with high activity. *E*-FabI was also identified as a non-OYE reductase to catalyze the asymmetric reduction of **1a–c**, giving (*R*)-**2a–c** in 81–70% *ee* and showing even higher activity. Although the enantioselectivity of *E*-FabI is not excellent for these reductions, the product *ee* could be simply improved to 99–98% in 89–78% yield by introducing a subsequent *S*-enantioselective CHMO-catalyzed oxidation in one pot. Docking of substrates **1a–c** onto the structural model of *A*-FabI and the X-ray structure of *E*-FabI revealed similar binding patterns to that of natural thioester substrates in the FabI of *M. tuberculosis*, although **1a–c** do not contain the binding structure motifs of the natural substrates. Analysis of the active docking pose gave insight into the *R*-enantioselectivity. The discovery of FabI reductase for asymmetric reduction of C=C doubles bond of non-natural substrates could expand the toolbox for this challenging type of bioreduction in asymmetric synthesis. Currently we are developing immobilized FabI and GDH for *in vitro* reduction with cofactor regeneration as well as recombinant biocatalyst co-expressing *A*-FabI and GDH using a suitable host for whole-cell biotransformation. Further development of new FabI *via* protein engineering and/or enzyme evolution to achieve higher enantioselectivity and different substrate specificity is under way.

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