Highly regio- and enantioselective multiple oxy- and amino-functionalizations of alkenes by modular cascade biocatalysis

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New types of asymmetric functionalizations of alkenes are highly desirable for chemical synthesis. Here, we develop three novel types of regio- and enantioselective multiple oxy- and amino-functionalizations of terminal alkenes via cascade biocatalysis to produce chiral \(\alpha\)-hydroxy acids, 1,2-amino alcohols and \(\alpha\)-amino acids, respectively. Basic enzyme modules 1–4 are developed to convert alkenes to (\(S\))-1,2-diols, (\(S\))-1,2-diols to (\(S\))-\(\alpha\)-hydroxy acids, (\(S\))-1,2-diols to (\(S\))-amino alcohols and (\(S\))-\(\alpha\)-hydroxy acids to (\(S\))-\(\alpha\)-amino acids, respectively. Engineering of enzyme modules 1 & 2, 1 & 3 and 1, 2 & 4 in \textit{Escherichia coli} affords three biocatalysts over-expressing 4–8 enzymes for one-pot conversion of styrenes to the corresponding (\(S\))-\(\alpha\)-hydroxy acids, (\(S\))-amino alcohols and (\(S\))-\(\alpha\)-amino acids in high e.e. and high yields, respectively. The new types of asymmetric alkene functionalizations provide green, safe and useful alternatives to the chemical syntheses of these compounds. The modular approach for engineering multi-step cascade biocatalysis is useful for developing other new types of one-pot biotransformations for chemical synthesis.

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Alkenes are readily available and excellent starting materials for chemical synthesis. Asymmetric functionalization of alkenes is of great importance in the synthesis of enantiopure chemicals for pharmaceutical manufacturing. Thus far, many metal catalysts have been developed for this, including the well-known Sharpless epoxidation, dihydroxylation amino hydrogenation, Jacobsen epoxidation and palladium-catalysed asymmetric alkene functionalization. On the other hand, enzyme catalysts could provide green alternatives due to the non-toxicity, high selectivity and mild reaction conditions. Despite these achievements, the development cyclopropanation and aziridination with engineered P450 catalysed asymmetric alkene functionalization5. On the other hand, enzyme catalyses could provide green alternatives due to the non-toxicity, high selectivity and mild reaction conditions.6–8. However, enzyme catalyses could provide green alternatives due to the non-toxicity, high selectivity and mild reaction conditions.6–8. Nevertheless, the epoxidation–hydrolysis cascade for asymmetric trans-dihydroxylation of alkenes37–39 recently developed by us is the only known biocatalytic cascade for asymmetric functionalization of alkenes. Thus far, most of the reported cascade biocatalysis enables only two to three relatively simple enzymatic reactions. It is very challenging to engineer the efficient cascade system containing more than four enzymatic reactions.

We have keen interest in developing new types of asymmetric alkene functionalizations and engineering enzyme cascade catalysing more than four enzymatic reactions. One-pot regio- and stereoselective multiple oxy- and amino-functionalizations of terminal alkenes to produce chiral α-hydroxy acids, 1,2-amino alcohols and α-amino acids, respectively, are designed as the target reactions (Fig. 1a). Enantiopure α-hydroxy acid40, 1,2-amino alcohol41 and α-amino acid42 are the three very important groups of chiral chemicals with broad applications in chiral pharmaceuticals and asymmetric syntheses. The designed new transformations could provide green, safe and complementary alternatives to the toxic cyanide-based asymmetric synthesis of chiral α-hydroxy acid (via cyanohydrin)43 and α-amino acid (Strecker reaction)44 and the osmium-based asymmetric synthesis of chiral amino alcohols43,45. Herein, we report the development of the three new types of asymmetric functionalizations of terminal alkenes, the modular approach for engineering efficient cascade biocatalysis containing more than four concurrent reactions, and the simple and green syntheses of α-hydroxy acids, 1,2-amino alcohols and α-amino acids in high enantiomeric excess (e.e.) and high yield.

**Figure 1** | Regio- and enantioselective multiple oxy- and amino-functionalizations of terminal alkenes by modular cascade biocatalysis. (a) One-pot conversion of terminal alkenes to chiral α-hydroxy acid, 1,2-amino alcohol and α-amino acid with *E. coli* cells containing multiple basic enzyme modules, respectively. (b) Four general basic enzyme modules and their cascade biotransformations. Module 1: epoxidase (EP) and epoxide hydrolase (EH) for epoxidation-hydrolysis of terminal alkenes to 1,2-diol; Module 2: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) for terminal double oxidation of 1,2-diol to α-hydroxy acid; Module 3: ADH, α-transaminase (α-TA) and alanine dehydrogenase (AlaDH) for oxidation-transamination of 1,2-diol to 1,2-amino alcohol; Module 4: hydroxide oxidase (HO), α-transaminase (α-TA), catalase (CAT) and glutamate dehydrogenase (GluDH) for oxidation-transamination of α-hydroxy acid to α-amino acid.
Results
Design of modular biocatalysis for cascade reactions. To realize the targeted asymmetric alkene functionalizations (Fig. 1a), we designed microbial cells containing two to three basic enzyme modules, each of them catalysing two to four enzymatic reactions (Fig. 1b), based on biocatalytic retrosynthesis analysis26,27. The basic modules were designed by using the following criteria: (a) each module utilizes a stable input, such as alkene, diol and hydroxy acid, and gives a stable output, such as diol, hydroxy acid, amino alcohol and amino acid; (b) each module enables fast conversion of unstable or toxic intermediates, such as epoxide, hydroxy aldehyde and keto acid, to minimize their accumulation and side reactions. Assemblies of module 1 and 2 in one cell, module 1 and 3 in one cell and module 1, 2 and 4 in one cell gave rise to whole-cell catalysts for one-pot transformations of terminal alkene to chiral α-hydroxy acid, 1,2-amino alcohol and α-amino acid, respectively (Fig. 1a). To demonstrate the concept, we chose the biotransformations of styrenes 1a–k to (S)-α-hydroxy acids 5a–k, (S)-1,2-amino alcohols 6a–k and (S)-α-amino acids 8a–k, respectively, as the representative examples of the three types of asymmetric reactions (Fig. 2). While the styrenes are easily available substrates, the (S)-α-hydroxy acids, (S)-1,2-amino alcohols and (S)-α-amino acids are highly valuable chiral chemicals with many applications (Supplementary Table 1).

Engineering of basic enzyme modules. Enzyme module 1 for the conversion of alkene to diol was engineered according to our previously reported method39. Escherichia coli (R-M1) containing gene module 1 on plasmid pRSFDuet-1 (Table 1) was constructed to co-express styrene monooxygenase (SMO)47 and epoxide hydrolase (SpEH)48 (Fig. 2a). As shown in Fig. 3a, 5 g cdw1−1 of E. coli (R-M1) cells efficiently transformed 50 mM styrene 1a to 46 mM (S)-1-phenyl-1,2-ethanediol 3a in 5 h, without significant accumulation of (S)-styrene oxide 2a (<1%). For further assembly of multiple basic modules and optimization of enzyme expression in one E. coli strain, gene module 1 was sub-cloned into other three different but compatible plasmids, pACYCDuet-1, pCDFDuet-1 and pETDuet-1, to generate three new recombinant plasmids, A-M1, C-M1 and E-M1, respectively (Table 1).

To engineer enzyme module 2 for the conversion of diol to α-hydroxy acid, many commercially available alcohol dehydrogenases (ADH), cloned ADHs and wild-type strains collected in our laboratory (Supplementary Table 2) were screened for the terminal oxidation of (S)-1-phenyl-1,2-ethanediol 3a to identify a highly regioselective enzyme for the first reaction of the module (Fig. 2a). AlkJ from Pseudomonas putida GPo1 (ref. 49), a membrane-associated non-canonical ADH, was found to oxidize 3a at the terminal position to give mandelaldehyde 4a and mandelic acid 5a with S-enantioselectivity. Phenylacetalddehyde dehydrogenase (EcALDH, encoded by padA) from E. coli was then found to fully oxidize α-hydroxy aldehyde 4a to give 5a, the second reaction of the module. Thus, the genes of AlkJ and EcALDH were genetically engineered into a non-natural operon as gene module 2 on plasmid pRSFDuet-1 (R-M2, Table 1). E. coli (R-M2) cells expressed both AlkJ and EcALDH very well (Fig. 3b) and catalysed the highly regioselective terminal oxidation of 50 mM (S)-3a to give 49 mM (S)-5a in 8 h (Fig. 3b), without the accumulation of intermediate (S)-4a. Similarly, gene module 2 was also sub-cloned to the three plasmids to generate new recombinant plasmids, A-M2, C-M2 and E-M2, respectively (Table 1).

Enzyme module 3 is for the conversion of diol to α-amino alcohol. AlkJ-catalysed highly regioselective oxidation of (S)-3a to (S)-4a is the first reaction of module 3 (Fig. 2b). The α-transaminase from Chromobacterium violaceum (CvoTA, encoded by cv_2025)51 was chosen for the transamination of (S)-4a, the second reaction of the module. An E. coli strain was
engineered to coexpress AlkJ and Cv\textsubscript{2025} and CvoTA and for the biotransformation of 45 mM (S)-3a with 200 mM L-alanine as amine donor. While the desired product (S)-phenylethanolamine 6a was produced (50% yield; 22 mM), some (S)-4a (14 mM) and (S)-5a (5 mM) remained in the system (Supplementary Fig. 1). To increase the formation of (S)-6a in the reversible transamination reaction and utilize the cellular L-alanine and pyruvate, L-alanine dehydrogenase (AlaDH, encoded by \textit{ald}, Genbank ID 936557) from \textit{Bacillus subtilis} was used to regenerate L-alanine from cellular pyruvate with ammonia as amine donor\textsuperscript{52}. A non-natural operon (gene module 3) containing the genes of AlkJ, CvoTA and AlaDH was cloned into the plasmid pRSFDuet-1 (R-M3, Table 1). The \textit{E. coli} (R-M3) strain coexpressed the three enzymes well and catalysed the biotransformation of 50 mM (S)-3a to afford 35 mM (S)-6a in 8 h by using 200 mM ammonia without addition of \textit{L}-alanine (Fig. 3c). Substrate (S)-3a, intermediate (S)-4a, and by-product (S)-5a remained in relatively low amount (3, 5 and 2 mM, respectively). This \textit{in vivo} amination with coexpressed \textit{cvo}TA and \textit{ala}DH is complementary to the recently developed \textit{in vitro} system\textsuperscript{26,52}. Similarly, gene module 3 was sub-cloned into other three plasmids to generate A-M3, C-M3 and E-M3 (Table 1).

Enzyme module 4 is for the conversion of \textit{\alpha}-hydroxy acid to \textit{\alpha}-amino acid. To engineer this module, mandelate dehydrogenase\textsuperscript{53} and hydroxymandelate oxidase (HMO, encoded by \textit{sco3228} from \textit{Streptomyces coelicolor} A3(2)\textsuperscript{54}) were cloned into \textit{E. coli} and examined for the oxidation of (S)-5a, respectively (Fig. 2c). HMO was found to be more efficient than the dehydrogenase (Supplementary Fig. 2), thus being chosen as the first enzyme of module 4. For the enantioselective amination of 7a to (S)-8a, an amino acid dehydrogenase and four \textit{\alpha}-transaminases (\textit{\alpha}-TA) were screened with either ammonia or glutamate as amine donor (Supplementary Fig. 3). EcxTA, the branch chain amino acid transaminase from \textit{E. coli} (encoded by \textit{ilvE}, Genbank ID 948278), was found to give the best results. Glutamate dehydrogenase (GluDH, encoded by \textit{gdhA}, Genbank ID 946802) was then coexpressed with EcxTA in \textit{E. coli} to enable the regeneration of glutamate by using ammonia during the transamination (Supplementary Fig. 4). Since HMO is a H\textsubscript{2}O\textsubscript{2}-generating oxidase, a catalase (CAT, encoded by \textit{katE}, Genbank ID 946234) was used to decompose H\textsubscript{2}O\textsubscript{2} to improve the biotransformation (Supplementary Fig. 5). The genes of HMO, EcxTA, GluDH and CAT were thus engineered on plasmid pRSFDuet-1 (R-M4, Table 1) to construct module 4. \textit{E. coli} (R-M4) coexpressed the four enzymes well (Fig. 3d) and converted 50 mM (S)-5a to 45 mM (S)-8a within 26 h (Fig. 3d). Gene module 4 was sub-cloned into three plasmids to give A-M4, C-M4 and E-M4, respectively (Table 1).

### Engineering of catalyst to convert alkene to \textit{\alpha}-hydroxy acid

Module 1 and 2 were assembled together in \textit{E. coli} cells as the catalyst (Fig. 2a). To explore the optimal combination, four module 1 plasmids (A-M1, C-M1, E-M1 and R-M1) and four module 2 plasmids (A-M2, C-M2, E-M2 and R-M2) were combinatorially combined and transformed into \textit{E. coli}. Since plasmids with the same backbone (for example, A-M1 and A-M2) are not compatible with each other, 12 \textit{E. coli} strains were obtained (Table 1), each co-expressing SMO, SpEH, AlkJ and EcALDH. These \textit{E. coli} strains grew well in M9-glucose medium and expressed the desired enzymes. The collected cells were examined for the biotransformation of 100 mM styrene 1a in a two-liquid-phase system (buffer and n-hexadecane; 1:1) containing 0.5% glucose. As shown in Fig. 4a, all strains were able...

### Table 1 | Genetic construction of recombinant \textit{E. coli} strains containing different enzyme modules.

| Genetic construction of modules\textsuperscript{*} | Plasmids containing modules\textsuperscript{+} | Recombinant \textit{E. coli} strains containing different modules |
|--------------------------------------------------|--------------------------------------------------|----------------------------------------------------------------|
| Module 1 (M1) | A-M1 C-M2, A-M1 E-M2, A-M1 R-M2, C-M1 A-M2, C-M1 E-M2, C-M1 R-M2, E-M1 A-M2, E-M1 C-M2, E-M1 R-M2, R-M1 A-M2, R-M1 C-M2, R-M1 E-M2 | E. coli (A-M1), E. coli (C-M1), E. coli (E-M1), E. coli (R-M1) |
| Module 2 (M2) | A-M1 C-M3, A-M1 E-M3, A-M1 R-M3, C-M1 A-M3, C-M1 E-M3, C-M1 R-M3, E-M1 A-M3, E-M1 C-M3, E-M1 R-M3, R-M1 A-M3, R-M1 C-M3, R-M1 E-M3 | E. coli (A-M1), E. coli (A-M1 E-M2), E. coli (A-M1 M2), E. coli (A-M1 R-M2), E. coli (E-M1 C-M2), E. coli (E-M1 M2), E. coli (E-M1 R-M2), E. coli (R-M1 A-M2), E. coli (R-M1 C-M2), E. coli (R-M1 E-M2) |
| Module 3 (M3) | A-M1 C-M4, A-M1 E-M4, A-M1 R-M4, C-M1 A-M4, C-M1 E-M4, C-M1 R-M4, E-M1 A-M4, E-M1 C-M4, E-M1 R-M4, R-M1 A-M4, R-M1 C-M4, R-M1 E-M4 | E. coli (A-M1), E. coli (A-M1 E-M2), E. coli (A-M1 R-M2), E. coli (C-M1 A-M2), E. coli (C-M1 E-M2), E. coli (C-M1 R-M2), E. coli (E-M1 A-M2), E. coli (E-M1 C-M2), E. coli (E-M1 R-M2), E. coli (R-M1 A-M2), E. coli (R-M1 C-M2), E. coli (R-M1 E-M2) |
| Module 4 (M4) | A-M1 C-M5, A-M1 E-M5, A-M1 R-M5, C-M1 A-M5, C-M1 E-M5, C-M1 R-M5, E-M1 A-M5, E-M1 C-M5, E-M1 R-M5, R-M1 A-M5, R-M1 C-M5, R-M1 E-M5 | E. coli (A-M1), E. coli (A-M1 E-M2), E. coli (A-M1 M2), E. coli (A-M1 R-M2), E. coli (C-M1 A-M2), E. coli (C-M1 E-M2), E. coli (C-M1 R-M2), E. coli (E-M1 C-M2), E. coli (E-M1 M2), E. coli (E-M1 R-M2), E. coli (R-M1 A-M2), E. coli (R-M1 C-M2), E. coli (R-M1 E-M2) |

\textsuperscript{*} \textit{ilvA}, \textit{clyB} and \textit{ald} are the genes of SMO and SpEH, respectively; \textit{alkJ} and \textit{padA} are the genes of AlkJ and EcALDH, respectively; \textit{cly2025} and \textit{ald} are the genes of \textit{AlkJ}, CvoTA and AlaDH, respectively; \textit{sco3228}, \textit{ilvE}, \textit{gdhA} and \textit{katE} are the genes of HMO, EcTA, GluDH and CAT, respectively.

\textsuperscript{+} 1A-M1–4 using plasmid pACYC-Duet-1; C-M1–4 using plasmid pCDF-Duet-5; E-M1–4 using plasmid pET-Duet-1; R-M1–4 using plasmid pRSFDuet-1.
to convert \(1a\) to \((S)-5a\) (21–83 mM). Among them, three strains gave \((S)-5a\) in 71–83 mM, and \(E. coli\) (A-M1-R-M2) is the best one to produce 83 mM \((S)-5a\) (83% conversion) in 20 h together with 9 mM \((S)-3a\). SDS–PAGE analysis of the cell proteins of the 12 strains (Supplementary Fig. 6) revealed that the three good strains exhibited a relatively balanced expression of the four enzymes, whereas several strains with lower productivity expressed much less AlkJ and EcALDH (module 2) than SMO and SpEH (module 1). The whole-cell activities of \(E. coli\) (A-M1-R-M2) towards \(1a\), \((S)-2a\), \((S)-3a\) and \((S)-4a\) were determined to be 43, 220, 280 and 120 mM \((g\text{ cdw})^{-1}\), respectively. \(E. coli\) (A-M1-R-M2) was used to transform 100–150 mM \(1a\) to \((S)-5a\), and the highest product concentration was observed with 120 mM \(1a\) (Supplementary Fig. 7a). The time course of biotransformation of 120 mM \(1a\) with 15 g cdw\(^{-1}\) resting cells of \(E. coli\) (A-M1-R-M2) was shown in Fig. 4b. A total of 94 mM (14.2 g l\(^{-1}\)) \((S)-5a\) was produced in 98% e.e. and 78% conversion in 22 h. The unreacted substrate \(1a\) and intermediate \((S)-3a\) were found at a relatively low level (9 and 12 mM, respectively).

**Engineering of catalyst to convert alkene to amino alcohol.** Module 1 and module 2 were combined for the asymmetric aminohydroxylation of alkenes (Fig. 2b). Combinatorial assembly of module 1 plasmids (A-M1, C-M1, E-M1 and R-M1) and module 3 plasmids (A-M3, C-M3, E-M3 and R-M3) led to 12 different \(E. coli\) strains (Table 1), each co-expressing SMO, SpEH, AlkJ, CvoTA and AlaDH. Biotransformation of 50 mM \(1a\) was examined with resting cells of each \(E. coli\) strain in a two-liquid-phase system for 10 h (Fig. 4c). All strains produced \((S)-6a\) (1–28 mM), and three of them produced \((S)-6a\) in 26–28 mM. The best one, \(E. coli\) (A-M1-E-M3), gave 28 mM \((S)-6a\) (56% conversion) with the accumulation of \((S)-3a\) (2 mM), \((S)-4a\) (2 mM) and \((S)-5a\) (5 mM). The reaction buffer and temperature were then optimized to improve the final product yield (Supplementary Fig. 8). \(E. coli\) (A-M1-E-M3) was chosen as the catalyst for this type of biotransformations. The specific activities of \(E. coli\) (A-M1-E-M3) towards \(1a\), \((S)-2a\), \((S)-3a\) and \((S)-4a\) were 45, 280, 39 and 11 U \((g\text{ cdw})^{-1}\), respectively. The biotransformation was examined with styrene \(1a\) at 50–80 mM, and 60 mM substrate was found to give the highest concentration of \((S)-6a\) (Supplementary Fig. 7b). Figure 4d depicted the time course of the reaction of 60 mM \(1a\) with 15 g cdw\(^{-1}\) resting cells: 42 mM (5.8 g l\(^{-1}\)) \((S)-6a\) was produced in 98% e.e. and 70% conversion in 12 h. Unreacted substrate \(1a\), intermediates \((S)-3a\) and \((S)-4a\), and by-product \((S)-5a\) remained at low concentrations (0.2, 0.1 and 4 mM, respectively). The cascade biocatalysis did not produce phenylglycinol, suggesting the excellent regioselectivity of the aminohydroxylation.

**Engineering of catalyst to convert alkene to \(\alpha\)-amino acid.** Modules 1, 2 and 4 were assembled together as the catalyst (Fig. 2c). Instead of combinatorial assembly of the basic modules, module 4 on four different plasmids (A-M4, C-M4, E-M4 and R-M4) was transformed into the existing best four recombinant \(E. coli\) strains containing module 1 and 2, \(E. coli\) (A-M1-E-M2), \(E. coli\) (A-M1-R-M2), \(E. coli\) (C-M1-E-M2) and \(E. coli\) (R-M1-E-M2). This generated eight different \(E. coli\) strains, each containing module 1, 2 and 4 on different plasmids (Table 1). The eight strains were individually examined for biotransformation of 50 mM styrene \(1a\) to \((S)-\alpha\)-phenylglycine \(8a\) (Fig. 4e). All strains produced \((S)-8a\) (15–40 mM), and five of them produced 37–40 mM \((S)-8a\). \(E. coli\) (A-M1-R-M2-C-M4) showed the highest productivity, generating 40 mM \((S)-8a\) (80%) conversion) in 24 h together with \((S)-3a\), \((S)-5a\) and \(7a\) (1 mM each). This
Figure 4 | Regio- and enantioselective multiple oxy- and amino-functionalizations of styrene 1a with E. coli strains containing multiple enzyme modules. (a) Product concentration of biotransformation of 100 mM 1a to (S)-5a with twelve E. coli strains (10 g cdw l\(^{-1}\)), each containing both enzyme module 1 and 2, respectively. (b) Time course of biotransformation of 120 mM 1a to (S)-5a with E. coli (A-M1,R-M2) cells (15 g cdw l\(^{-1}\)) in a two-liquid-phase system (KP buffer containing 0.25% glucose and n-hexadecane; 1:1) at 30 °C. (c) Product concentration of biotransformation of 50 mM 1a to (S)-6a with twelve E. coli strains (10 g cdw l\(^{-1}\)), each containing both enzyme module 1 and 3, respectively. (d) Time course of biotransformation of 60 mM 1a to (S)-6a with E. coli (A-M1,E-M3) cells (15 g cdw l\(^{-1}\)) in a two-liquid-phase system (NaP buffer containing 1% glucose and 200 mM NH\(_3\)/NH\(_4\)Cl and n-hexadecane; 1:1) at 25 °C. (e) Product concentration of biotransformation of 50 mM 1a to (S)-8a with eight E. coli strains (10 g cdw l\(^{-1}\)), each containing enzyme module 1, 2 and 4, respectively. (f) Time course of biotransformation of 60 mM 1a to (S)-8a with E. coli (A-M1,R-M2,C-M4) cells (15 g cdw l\(^{-1}\)) in a two-liquid-phase system (KP buffer containing 0.5% glucose and 100 mM NH\(_3\)/NH\(_4\)Cl and n-hexadecane; 1:1) at 30 °C (arrow: adding additional 0.5% glucose and 100 mM NH\(_3\)/NH\(_4\)Cl at 20 h). All biotransformations were performed in triplicate, and error bars show ± s.d.
strain was chosen for this type of biotransformations. The specific activities of *E. coli* (A-M1-R-M2_C-M4) towards 1a, (S)-2a, (S)-3a, (S)-4a, (S)-5a and 7a, were determined to be 20, 75, 11, 16, 10 and 16 U (g cdw)⁻¹, respectively. Biotransformations of 50–80 mM 1a to (S)-8a were examined, and 60 mM 1a was found to give the highest final product concentration (Supplementary Fig. 7c). As shown in Fig. 4f, biotransformation of 60 mM 1a with 15 g cdw l⁻¹ resting cells gave 33 mM (S)-8a at 20 h, together with 8 mM 1a and 16 mM 7a. By the additional feeding of 0.5% glucose and 100 mM NH₃/NH₄Cl at 20 h, enantiopure (S)-8a was produced in 48 mM (7.3 g l⁻¹) and 80% conversion at 24 h, with intermediates (S)-3a, (S)-5a and 7a at low concentrations (3, 0.5 and 1 mM, respectively).

### Table 2 | Regio- and enantioselective functionalization of terminal alkenes 1a-k to (S)-α-hydroxy acids 5a-k via cascade biocatalysis.

| Substrate* | R group | Product | Conversion (%) | e.e. (%) |
|------------|---------|---------|---------------|---------|
| 1a         | H       | (S)-5a  | 95            | 98      |
| 1b         | o-F     | (S)-5b  | 90            | 98      |
| 1c         | m-F     | (S)-5c  | 99            | 99      |
| 1d         | p-F     | (S)-5d  | 94            | >99     |
| 1e         | m-Cl    | (S)-5e  | 83            | >99     |
| 1f         | p-Cl    | (S)-5f  | 83            | 99      |
| 1g         | m-Br    | (S)-5g  | 71            | 99      |
| 1h         | p-Br    | (S)-5h  | 69            | 99      |
| 1i         | m-Me    | (S)-5i  | 86            | 99      |
| 1j         | p-Me    | (S)-5j  | 78            | 96      |
| 1k         | m-OMe   | (S)-5k  | 97            | 98      |

*Reactions were conducted with 1a-k (20 mM in organic phase) and resting cells of *E. coli* (A-M1-R-M2) (10 g cdw l⁻¹) for 12 h in a two-liquid-phase system (KP buffer and n-hexadecane; 1:1) (Table 2). Five (S)-α-hydroxy acids (5a-d and 5k) were produced in 90–99% conversion, and six (S)-α-hydroxy acids (5e-j) were produced in 69–86% conversion. The (S)-configurations of 5a-k were established by comparing the bioproducts with the commercially available enantiopure standards (5a, 5d-f and 5j) or derived from the previously established (S)-configurations of the diol intermediates (3b, 3c, 3g-i and 3k). Ten chiral α-hydroxy acids were examined, and 60 mM 1a were produced in 90–99% conversion, and six (S)-α-hydroxy acids (5e-j) were produced in 69–86% conversion. The (S)-configurations of 5a-k were established by comparing the bioproducts with the commercially available enantiopure standards (5a, 5d-f and 5j) or derived from the previously established (S)-configurations of the diol intermediates (3b, 3c, 3g-i and 3k).

### Table 3 | Regio- and enantioselective functionalization of terminal alkenes 1a-k to 1,2-amino alcohols 6a-k with *E. coli* (A-M1-R-M2) via cascade biocatalysis.

| Substrate* | R group | Product | Conversion (%) | e.e. (%) |
|------------|---------|---------|---------------|---------|
| 1a         | H       | (S)-6a  | 86            | 98      |
| 1b         | o-F     | (S)-6b  | 65            | >99     |
| 1c         | m-F     | (S)-6c  | 71            | 97      |
| 1d         | p-F     | (S)-6d  | 78            | 91      |
| 1e         | m-Cl    | (S)-6e  | 20            | 99      |
| 1f         | p-Cl    | (S)-6f  | 36            | >99     |
| 1g         | m-Br    | (S)-6g  | 16            | 99      |
| 1h         | p-Br    | (S)-6h  | 26            | 96      |
| 1i         | m-Me    | (S)-6i  | 81            | >99     |
| 1j         | p-Me    | (S)-6j  | 69            | 96      |
| 1k         | m-OMe   | (S)-6k  | 81            | 98      |

*Reactions were conducted with 1a-k (20 mM in organic phase) and resting cells of *E. coli* (A-M1-E-M3) (10 g cdw l⁻¹) in NaP buffer (200 mM, pH 8.0, 1% glucose, 200 mM NH₃/NH₄Cl) and n-hexadecane (1:1) at 30 °C for 24 h. Conversion was determined by reversed phase HPLC analysis of the final product 6a-k. The values are averages of two experiments. Enantiomeric excess (e.e.) was determined by chiral HPLC analysis. The values are averages of two experiments.
(5a–i and 5k) were produced in 98–99% e.e., and 5j was obtained in 96% e.e. The high product e.e. values were generated by the highly enantioselective transamination of prochiral α-keto acids 7a–k with EcxTA.

Preparative biotransformations. The synthetic application of the three new types of asymmetric functionalizations of alkenes was demonstrated by the preparative biotransformations to produce two (S)-α-hydroxy acids, two (S)-α-amino alcohols and two (S)-α-amino acids. The ratio of aqueous buffer and n-hexadecane of the two-phase system was examined for the biotransformations, and 1–5:1 ratios gave similar high conversion for all three types of reactions (Supplementary Fig. 9). Thus, the preparative biotransformations were performed at 5:1 ratio of aqueous buffer: n-hexadecane to reduce the use of organic solvent. Biotransformations of alkenes 1a (100 mM) and 1d (50 mM) with resting cells of E. coli (A-M1-R-M2) (20 g cdw l$^{-1}$) gave (S)-α-hydroxy acids 5a and 5d in 83 and 80% conversion at 24 h, respectively. Simple extraction and crystallization gave (S)-5a (98% e.e.) and (S)-5d (98% e.e.) in 72 and 61% yield, respectively. Similarly, biotransformations of alkenes 1a (50 mM) and 1i (25 mM) with resting cells of E. coli (A-M1-E-M3) (20 g cdw l$^{-1}$) afforded (S)-α-amino alcohols 6a and 6i in 71 and 63% conversion at 24 h, respectively. Extraction and flash chromatography afforded (S)-6a (98% e.e.) and (S)-6i (98% e.e.) in 62% and 55% yield, respectively. Finally, biotransformations of alkenes 1a (50 mM) and 1d (25 mM) were carried out with resting cells of E. coli (A-M1-R-M2-C-M4) (20 g cdw l$^{-1}$) for 24 h to give (S)-α-amino acids 8a and 8d in 81 and 79% conversion, respectively. Simple extraction and evaporation afforded enantiopure (S)-8a and (S)-8d in 70 and 59% yield, respectively.

Discussion
Cascade biocatalysis is of great importance in green synthesis of chemicals, since it could avoid the waste-generating, time-consuming, and costly separation and purification of intermediates in traditional multi-step process. However, efficient non-natural cascades with more than four enzymatic reactions are rare, and their development is challenging. The modularization concept reported here provides a useful tool for the engineering of complex cascade biocatalysis, which is different from the classical cascade biocatalysis. The methodology reported here provides a useful tool for the engineering of complex cascade biocatalysis.
from the modularization in synthetic biology to build complex genetic circuits and in metabolic engineering to optimize production of certain metabolites. In the modular cascade biocatalysis, the appropriate basic modules are designed and engineered to ideally give full conversion with no accumulation of the intermediates. In these aspects, modules 1, 2 and 4 are excellent: they produced 90–98% of the final products in high e.e. from the starting materials, with no accumulation of the intermediates (Fig. 3a,b,d). These results were achieved by using enzymes having relatively high activities and using the second enzyme with much higher activity than the first enzyme (SpEH versus SMO; EcALDH versus AlkJ; EcZTA versus HMO) in the module (Supplementary Table 3). In module 3, the conversion of the starting material to the final product reached 70%. Overall, 10% of the intermediate remained, which is possibly due to the relatively low activity of CvoTA for the second reaction, the transamination (Fig. 3c). Further improvement of module 3 might be achieved by using other enzymes with higher amination activity.

Efficient cascade catalysis systems consisting of multiple basic modules were developed by the combinatorial assembly of the basic modules on different plasmids to adjust the expression level of the enzymes. This method led to the development of E. coli (A-M1-R-M2), E. coli (A-M1-E-M3) and E. coli (A-M1-R-M2-C-M4) as powerful catalysts for the asymmetric functionalizations of alkenes to (S)-α-hydroxy acids, (S)-1,2-amino alcohols and (S)-α-amino acids, respectively. These catalysts enabled the biotransformation of 120 mM 1a to (S)-5a in 78% conversion, 60 mM 1a to (S)-6a in 70% conversion and 60 mM 1a to (S)-8a in 80% conversion, respectively, with low accumulation of the intermediates (Fig. 4b,d). Since some oxidoreductases were used in the cascade biocatalysis, cofactor recycling has to be considered for efficient biotransformations. While SMG47 and AlaDH52 are nicotinamide adenine dinucleotide (NADH)-dependent, GluDH is nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and EcALDH is NAD + -dependent. On the other hand, SpEH48, HMO54, EcZTA, CAT and CvoTA51 are independent of nicotinamide cofactors, and AlkJ is a non-canonical ADH coupling to the bacterial respiratory chain instead of nicotinamide cofactors49. Therefore, there is no net consumption of the nicotinamide cofactor in the functionalization of styrenes to (S)-hydroxy acids (Fig. 2a). However, 2 moles NADH are needed for producing 1 mole (S)-amino alcohol from styrene (Fig. 2b), and 1 mole NADPH is required for producing 1 mole (S)-amino acid from styrene (Fig. 2c). For these two types of biotransformations with whole-cell biocatalysts, the regeneration of NAD(P)H was achieved via cell metabolism of glucose. This was clearly demonstrated in Fig. 4f: feeding of additional glucose at 20 h significantly improved the conversion of the final product (S)-8a. Future improvement of these reactions might be achieved by co-expressing a NAD(P)H-regenerating enzyme in the recombinant biocatalysts.

An important parameter in catalysis is the total turnover number (TTN). We calculated the TTN of individual enzymes in the biotransformations based on the amount of the enzymes inside three whole-cell biocatalysts estimated by separation and analysis of the proteins with SDS–PAGE and densitometer (Supplementary Fig. 10; Supplementary Table 4). SMO, SpEH, AlkJ and EcALDH were expressed in E. coli (A-M1-R-M2) at 10, 6.0, 23 and 14 mg protein (g cdw)–1, respectively, and gave a TTN of 58,000, 95,000, 26,000, 38,000 and 24,000, respectively, in the biotransformation of 1a to (S)-8a (Fig. 4f). The good TTN values of these key enzymes indicate the high efficiency of their catalysis in the cascade biotransformations.

On the basis of the specific activities of the three whole-cell biocatalysts towards substrate 1a and the corresponding intermediates (Supplementary Table 5), the following bottlenecks could be deduced: AlkJ-catalysed oxidation of (S)-3a in the transamination of 1a to (S)-5a; CvoTA-catalysed transamination of (S)-4a in the conversion of 1a to (S)-6a; and AlkJ-catalysed oxidation of (S)-3a and HMO-catalysed oxidation of (S)-5a in the transamination of 1a to (S)-8a. These bottlenecks were also confirmed by some accumulation of (S)-3a (Fig. 4b), (S)-5a (Fig. 4d, possibly due to the oxidation of (S)-4a to (S)-5a) by other enzymes inside the E. coli cells, and both (S)-3a and (S)-5a at early reaction stage (Fig. 4f) in the corresponding biotransformations. On the basis of the determined specific activities of AlkJ, EcALDH, CvoTA1 and EC1 (0.19, 1.1, 12 and 14 U (mg protein)–1, respectively) (Supplementary Table 3; Supplementary Fig. 11) and the reported specific activities of SMO,48, SpEH48 and HMO59 (2.1, 16 and 1.8 U (mg protein)–1, respectively), AlkJ and CvoTA are not very active. Since their expression in the whole-cell catalysts is not low, these two enzymes might be replaced with more active enzymes selected from natural sources or enzyme engineering60–62 to improve the efficiency of the cascade catalysis. On the other hand, HMO has a relatively high activity, but its expression level is low (too low to be estimated). Thus, future improvement might focus on the enhancement of HMO or replacement of HMO by other enzymes with higher activity and easier expression. In general, the expression of all involved enzymes might be fine-tuned to a high and balanced level by altering other genetic elements, such as promoters and ribosome-binding sites.

The engineered whole-cell biocatalysts accept a group of styrene derivatives as substrates for the three types of asymmetric alkene functionalizations. E. coli (A-M1-R-M2) catalysed the biotransformations of eleven alkenes (1a–k) to produce the corresponding (S)-α-hydroxy acids (5a–k) in good conversion (90–99% for five products and 69–86% for six products) and high e.e. (98–99% for ten products and 96% for one product). Biotransformation of the same eleven alkenes (1a–k) with E. coli (A-M1-E-M3) gave the corresponding (S)-1,2-amino alcohols (6a–k) in high e.e. (96–99% for ten products and 91% for one product) with good conversion (65–86%) for seven products (6a–d and 6i–k) and lower conversion (16–36%) for four products (6e–h). E. coli (A-M1-R-M2-C-M4) catalysed the reaction of alkenes 1a–k to produce (S)-α-amino acids 8a–k in enantiopure form (all ≥99%e) with good conversion (88–91% for two products and 55–76% for eight products) except (S)-8h (28%). The regio- and enantioselectivity of three types of alkene functionalizations are outstanding. For the low-conversion biotransformations of 1c–h to (S)-6e–h and of 1h to (S)-8h, 42–63% of unreacted alkenes remained in the reaction mixture. Thus, the SMO-catalysed epoxidation is the main bottleneck in these reactions, which was possibly caused by (a) the relatively low epoxidation activity of SMO towards those styrenes containing a bulky or electron-withdrawing group, (b) the relatively low expression of SMO in E. coli (A-M1-E-M3) and E. coli (A-M1-R-M2-C-M4) and (c) the inefficient cofactor supply and regeneration in the biotransformations. The improvement of these bioconversions might be achieved by enhancing the expression of SMO, co-expressing a NADH-regenerating enzyme, and/or using an engineered SMO with higher activity towards the alkene substrates.
The synthetic application of the developed whole-cell biocatalysts and three new types of asymmetric functionalizations of alkenes were clearly demonstrated in the preparation of two (S)-hydroxy acids, two (S)-amino alcohols and two (S)-amino acids. Biotransformations, workup and purification are straightforward, affording 208–1,095 mg (55–72% yield) of (S)-5a, (S)-5d, (S)-6a, (S)-6i, (S)-8a and (S)-8d in 98–99% e.e.

The biocatalytic syntheses utilize non-toxic and biodegradable catalysts, consume inexpensive and green stoichiometric reagents (O₂, NH₃ or glucose) and operate under mild reaction conditions and is greener and safer than the cyanide-based Strecker synthesis involving many isolation and purification steps. The biocatalytic synthesis is greener and safer than the cyanide-based synthesis using cyanohydrin. It gives high product yield and e.e. from the low-cost alkenes, being more attractive than the reported kinetic resolution (maximum yield: 50%) and asymmetric reduction of β-keto esters (substrates are not cheap). The one-pot asymmetric functionalization of alkenes to give chiral α-amino acids has also no chemical counterpart and is greener and safer than the cyanide-based Streecker synthesis of chiral α-amino acids. It enables high product yield and e.e. from inexpensive alkenes, being advantageous over the kinetic resolution (maximum yield: 50%) and asymmetric hydrogenation (substrates are not cheap) approaches. The one-pot conversion of alkenes to chiral 1,2-amino alcohols is a new type of biotransformation and offers an alternative or even better method in some cases to the existing chemical asymmetric aminohydroxylation (oxygenation). The biocatalytic aminohydroxylation produces primary amines of the amino alcohols by utilizing ammonia as the nitrogen source, while chemical aminohydroxylation has difficulty in using ammonia. It could also provide much better regio- and enantioclectivity than the chemical methods. As an example for comparison, biotransformation of styrene 1ɑ afforded (S)-phenylethanolamine 6ɑ in 98% e.e. with 100% regioselectivity, while Sharpless asymmetric aminohydroxylation of styrene ɑ gave (R)-phenylethanolamine (as 7-toluenesulfonyl derivative) in 55% e.e. together with (S)-phenylglycinol (as 4-toluenesulfonyl derivative).

From biochemical point of view, our synthetic routes from terminal alkenes to chiral ω-hydroxy acids, 1,2-amino alcohols and ω-amino acids are three novel non-natural pathways containing four to eight reactions, which are unambiguously distinguished from the natural aromatic or aliphatic alken degradation pathways reported so far. Nevertheless, the three synthetic pathways were successfully engineered in microbial cells by modular approach and catalysed well the desired non-natural reactions. In comparison with in vitro cascade biocatalysis, the whole-cell approach with a single recombinant strain enables the easy production of the multiple enzymes and the cost-effective biotransformation. This approach opens new possibility of engineering cells for one-pot multi-step biotransformations to manufacture different types of chemicals in a green, selective and cost-effective manner.

In summary, we successfully developed three new types of one-pot asymmetric oxy- and amino-functionalizations of terminal alkenes by cascade biocatalysis, simple and green syntheses of a group of useful and valuable (S)-ω-amino acids, (S)-ω-amino alcohols and (S)-ω-amino acids in high e.e. and high yields, and a modular approach for engineering efficient one-pot cascade biocatalysis containing more than four concurrent enzymatic reactions.

Methods

General procedure to engineer recombinant E. coli strains. E. coli 77 expression strain (an E. coli B strain derivative) was purchased from New England Biolabs (#C25663) and used as host strain for all molecular cloning and biocatalysis experiments. The gene module 1 comprising of styA, sty7 and spEH was constructed previously. Alix gene was amplified from the OCT megaplasmid extracted from P. putida GI01 as reported. Genes of pdaA, dhbA, fadA and katE were amplified from the genomic DNA extracted from S. cerevisiae (M28256) with genomic DNA Purification Kit (Thermo Scientific). Ald gene was amplified from the genomic DNA extracted from B. subtilis str.168 with genomic DNA Purification Kit. CoE1-optimized cv2025 gene was synthesized from GenScript based on the sequence from the strain of C. violaceum DSM30191 (ref. 51). CoE1-optimized sch2 gene was synthesized from GenScript based on the C. violaceum A3(2)54 (see Supplementary Methods for codon-optimized sequences).

All genetic constructions were carried out by using standard molecular biology techniques with Plusion DNA polymerase, FastDigest restriction enzymes and T4 DNA ligase (all from Thermo Scientific). PCR primers were synthesized from IDT for DNA Technologies (see Supplementary Table 6 for a full list of key primers). Purification of DNA after electrophoresis or enzyme digestion was performed with Z.N.A. Gel Extraction Kit (Omega Biotek), and extraction of plasmids was performed with AxyPrep Plasmid Miniprep Kit (Axygen). Basic gene modules 1–4 were constructed on a set of compatible plasmids pACYC-Duet1, pCDFT-Duet1, pETDuet-1 and pRSFDuet-1 (Novagen) as individual artificial genetic module(s). Further transformation of these genetic module(s) to a constructed E. coli strain or two basic modules gave an E. coli strain containing two or three basic modules for the desired asymmetric alken functionalizations (see Supplementary Methods for details and Supplementary Fig. 10 for enzyme expression).

General procedure to grow E. coli strains. Recombinant E. coli strain was first inoculated in 1 ml LB medium containing appropriate antibiotics (50 mg l⁻¹ chloramphenicol, 50 mg l⁻¹ streptomycin, 100 mg l⁻¹ ampicillin, 50 mg l⁻¹ kanamycin). E. coli was grown at 37 °C for 2 h. The culture was then transferred into 25 ml M9 medium containing glucose (20 g l⁻¹), yeast extract (6 g l⁻¹) and appropriate antibiotics in a 125 ml tri-baffled flask. The cells were grown at 37 °C and 300 r.p.m. for about 2 h to reach an OD₆₀₀ of 0.6, followed by the addition of IPTG to 0.5 mM to induce the enzyme expression. The cells were shaken at 12–13 °C at 250 r.p.m. for 16 h, centrifuged (5,000 g, 10 min) and the cell pellets were resuspended in an appropriate buffer to the desired density as resting cells for biotransformation.

General procedure to convert 1ɑ-k to (S)-5ɑ-k. Overview, 2 ml suspension (10 g cdw⁻¹) of freshly prepared E. coli (A-M1-R-M2) cells in KP buffer (200 mM, pH 8.0) containing glucose (0.5%, w/v), w/v) were mixed with 2 ml n-hexadecane containing one of alkenic substrates 1ɑ-k (20 mM). The mixture was shaken at 300 r.p.m. and 30 °C for 12 h, and 150 μl aliquots of each phase were taken out at different time points for following the reaction. For organic phase, 100 μl n-hexadecane were separated after centrifugation (13,000 × g, 2 min), diluted with 900 μl n-hexane (containing 2 mM benzyl alcohol as internal standard) and analysed by normal phase HPLC for quantifying alkenes 1ɑ-k and possible epoxides 2ɑ-k. For aqueous phase, 100 μl supernatant were separated after centrifugation (13,000 × g, 2 min), diluted with 400 μl TFA solution (0.5%) and 500 μl acetonitrile (containing 2 mM benzyl alcohol as internal standard) and then analysed by reverse phase HPLC for quantifying hydrophilic products 3ɑ-k, 4ɑ-k and 5ɑ-k. To determine the e.e. of 5ɑ-k, the remaining aqueous phase at the end of reaction was separated after centrifugation (13,000 × g, 2 min), acidified with TFA and saturated with NaCl, followed by extraction with ethyl acetate and dry over Na₂SO₄. After evaporation of ethyl acetate, the residue was dissolved in solvent (n-hexane: IPA = 9:1) for chiral HPLC analysis (see Supplementary Table 7; Supplementary Figs 19–29; Supplementary Methods for analytic methods).

General procedure to convert 1ɑ-k to (S)-6ɑ-k. Overview, 2 ml suspension (10 g cdw⁻¹) of freshly prepared E. coli (A-M1-E-M3) cells in NaPF₆ (sodium phosphate) buffer (200 mM, pH 6.0) containing glucose (0.5%, w/v) and NH₄H₂SO₄ (200 mM, NH₃:NH₄Cl = 1:1) were mixed with 2 ml n-hexadecane containing one of the alkenic substrates 1ɑ-k (20 mM). The mixture was shaken at 300 r.p.m. and 25 °C for 24 h. At 12 h, additional glucose (0.5%, w/v) and NH₄H₂SO₄ (100 mM) were added. Samples were taken at different time points and prepared for analysis according to the procedure described above in the conversion of 5ɑ-k to 5ɑ-k. Alkenes 1ɑ-k and possible epoxides 2ɑ-k were analysed by normal phase HPLC, and hydrophilic products 3ɑ-k, 4ɑ-k, 5ɑ-k and 6ɑ-k were determined by reverse phase HPLC. To determine the e.e. of 6ɑ-k, the remaining aqueous phase at the end of reaction was separated after centrifugation (13,000 × g, 2 min), acidified with TFA, separated and diluted with 900 μl TFA solution (0.1%) for chiral HPLC analysis (see Supplementary Table 7; Supplementary Figs 30–40; Supplementary Methods for analytic methods).
Biotransformation of 1a or 1d to prepare (S)-5a or (S)-5d. Overall, 100 ml suspension of E. coli (A-M1_R-M2) cells (20 g cdw 1⁻¹) in KP buffer (200 mM, pH 8.0) containing glucose (0.5%, w/v) and NH₄Cl (200 mM, NH₄NH₄Cl = 1:1) was mixed with 20 ml n-hexane containing one of alkene substrates 1a–k (20 or 5 mM). The reaction mixture was shaken at 300 r.p.m. and 30 °C for 24 h. At 20 h, additional glucose (0.5%, w/v) and NH₄Cl/NH₄NH₄Cl (100 mM) were added. 300 ml aliquots of the mixture (150 ml of each phase) were taken out at different time points for reversed phase HPLC analysis to follow the reaction. At 12 h, additional glucose (1%, w/v) and NH₄Cl/NH₄NH₄Cl (200 mM) were added. After 24 h, the reaction mixture was subjected to centrifugation (4,000 g, 15 min) to remove the cells and organic phase. The collected aqueous phase was filtered to further remove solid impurities, followed by washing with ethyl acetate (2 × 25 ml) to remove organics impurities to pH 7 with NaOH (10 M), the aqueous solution was concentrated to about 15 ml by evaporation to precipitate the acid. The solid was collected by filtration, washed with cold water and EtOH and dried overnight under vacuum. (S)-2-Amino-2-phenylacetic acid 8a white solid; 528 mg; yield: 70%; e.e.: 99%; [α]D²⁵ = +168° (c 1.0, 1 M HCl) [literature 70]; [α]D²⁵ = +130° (c 1.0, 1 M HCl), 99% e.e.). ¹H NMR (400 MHz, D₂O containing 2% H₂SO₄): δ = 7.36–7.30 (m, 1H), 5.04 (s, 1H) p.p.m.; ¹³C NMR (100 MHz, D₂O containing 2% H₂SO₄): δ = 148.3, 131.5, 130.4, 129.7, 129.1, 128.1, 128.9, 116.7, 116.4, 58.5 p.p.m. (Supplementary Fig. 18).

Biotransformation of 1a or 1d to prepare (S)-5a or (S)-5d. Overall, 100 ml suspension of E. coli (A-M1_R-M2) cells (20 g cdw 1⁻¹) in KP buffer (200 mM, pH 8.0) containing glucose (0.5%, w/v) and NH₄Cl/NH₄NH₄Cl (100 mM, NH₄NH₄Cl = 1:1) was mixed with 20 ml n-hexane containing one of alkene substrates 1a–k (5 mmol, 2.5 ml). The reaction mixture was shaken at 300 r.p.m. and 30 °C, and 100 μl aliquots of the aqueous phase were taken out at different time points for reversed phase HPLC analysis to follow the reaction. After 24 h, the reaction mixture was subjected to centrifugation (4,000 g, 15 min) to remove the cells and organic phase. The aqueous phase was collected, saturated with NaCl, adjusted to pH > 12 with NaOH (10 M) and washed with ethyl acetate two times (2 × 25 ml) to remove n-hexane and other organic impurities. The aqueous phase was adjusted to pH < 2 with HCl (10 M) and extracted with ethyl acetate (3 × 100 ml). The organic phase was collected and dried over Na₂SO₄. After filtration, the organic phase was subjected to evaporation by using a rotary evaporator (Buchi Rotavapor R-215) to remove the solvent. The crude hydroxy acid was purified by crystallization in ethyl acetate through normal phase HPLC are the same as the above mentioned for the isolation of 1a–k. The obtained crystals were washed with 2 ml of ethyl acetate and air-dried. (Supplementary Table 7; Supplementary Figs 41–51; Supplementary Methods for analytic methods).

Biotransformation of 1a or 1d to prepare (S)-5a or (S)-5d. Overall, 100 ml suspension of E. coli (A-M1_R-M2) cells (20 g cdw 1⁻¹) in NaP (sodium phosphate) buffer (200 mM, pH 8.0) containing glucose (0.5%, w/v) and NaH₂PO₄/Na₂HPO₄ (200 mM, Na₂H₂PO₄/Na₂HPO₄ = 1:1) were mixed with 20 ml n-hexane containing one of alkene substrates 1a–k (5 mmol, 2.5 ml) or 1d (5 mmol, 2.5 ml). The reaction mixture was shaken at 300 r.p.m. and 30 °C, and 100 μl aliquots of the aqueous phase were taken out at different time points for reversed phase HPLC analysis to follow the reaction. After 24 h, the reaction mixture was subjected to centrifugation (4,000 g, 15 min) to remove the cells and organic phase. The aqueous phase was collected, saturated with NaCl, adjusted to pH > 12 with NaOH (10 M) and washed with ethyl acetate (2 × 25 ml) to remove n-hexane and other organic impurities. The aqueous phase was adjusted to pH < 2 with HCl (10 M) and extracted with ethyl acetate (3 × 100 ml). The organic phase was collected and dried over Na₂SO₄. After filtration, the organic phase was subjected to evaporation by using a rotary evaporator (Buchi Rotavapor R-215) to remove the solvent. The crude hydroxy acid was purified by crystallization in ethyl acetate through normal phase HPLC are the same as the above mentioned for the isolation of 1a–k. The obtained crystals were washed with 2 ml of ethyl acetate and air-dried. (Supplementary Table 7; Supplementary Figs 41–51; Supplementary Methods for analytic methods).
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Author contributions
S.W. designed and performed most of the experiments. S.W. and Y.Z. carried out preparative biotransformations, enzyme purification and enzyme activity determination. T.W. cloned AlkJ. H.-P.T. provided helpful discussion and lab facilities at the late stage of the project. D.I.C.W. co-advised some research work of S.W. Z.L. supervised the entire research project. S.W. and Z.L. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: Z.L. and S.W. are the co-inventors on two patent applications: 'Production of enantiopure \( \alpha \)-hydroxy carboxylic acids from alkenes by cascade biocatalysis' PCT application number PCT/SG2014/000221; and 'Production of chiral 1,2-amino alcohols and \( \alpha \)-amino acids from alkenes by cascade biocatalysis' US provisional application 62/283,508.

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