A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* **2024**, e202410260

Link to VoR: https://doi.org/10.1002/anie.202410260

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10.1002/anie.202410260

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One-pot Biocatalytic Conversion of Chemically Inert Hydrocarbons into Chiral Amino Acids through Internal Cofactor and H_2O_2 Recycling

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Abstract: Chemically inert hydrocarbons are the primary feedstocks used in the petrochemical industry and can be converted into more intricate and valuable chemicals. However, two major challenges impede this conversion process: selective activation of C-H bonds in hydrocarbons and systematic functionalization required to synthesize complex structures. To address these issues, we developed a multi-enzyme cascade conversion system based on internal cofactor and H_2O_2 recycling to achieve the one-pot deep conversion from heptane to chiral (*S*)-2-aminoheptanoic acid under mild conditions. First, a hydrogen-borrowing-cycle-based NADH regeneration method and H_2O_2 *in situ* generation and consumption strategy were applied to realize selective C-H bond oxyfunctionalization, converting heptane into 2-hydroxyheptanoic acid. Integrating subsequent reductive amination driven by the second hydrogen-borrowing cycle, (*S*)-2-aminoheptanoic acid was finally accumulated at 4.57 mM with ee_p > 99%. Hexane, octane, 2-methylheptane, and butylbenzene were also successfully converted into the corresponding chiral amino acids with ee_p > 99%. Overall, the conversion system employed internal cofactor and H_2O_2 recycling, with O_2 as the oxidant and ammonium as the amination reagent to fulfill the enzymatic conversion from chemically inert hydrocarbons into chiral amino acids under environmentally friendly conditions, which is a highly challenging transformation in traditional organic synthesis.

Introduction

Hydrocarbons are the major constituents of crude oil and natural gas, which are the primary energy substances and essential feedstock for the petrochemical industry.[1] The petrochemical industry converts structurally simple and chemically inert hydrocarbons into structurally more complex and, therefore, more valuable intermediates/products, including diverse bulk, fine and specialty chemicals to realize comprehensive utilization of these hydrocarbons.[1] The deep conversion and comprehensive utilization of petroleum/natural gas-derived hydrocarbons holds great importance in achieving diversified molecular synthesis and expanding the petrochemical product landscape. However, there are two significant challenges to be addressed: the functionalization of chemically inert and unpolarized C-H bonds under mild conditions; and the design and construction of flexible and sustainable routes to effectively achieve these complicated conversions.

C-H bonds are ubiquitous and often chemically inert due to high bond energy and high bond-dissociation energy.[1a, 2] In organic synthesis, traditional methods with low selectivity are still prevalent for C-H bond functionalization, including transition metal-based or radical-mediated processes that require harsh conditions (*e.g.*, high temperatures and pressures). [2b, 3] On the contrary, enzymatic C-H bond functionalization is highly chemo-, regio-, and stereoselective but substrate-specific, operates under mild conditions in aqueous media, and shows significantly superior catalytic rates.[4] Representative monooxygenases[4b, 5] and peroxygenases[6] use clean oxidants (*e.g.*, O_2 or H_2O_2) to catalyze many selective C-H bond oxyfunctionalization under mild conditions. Thus, enzymatic C-H bond oxyfunctionalization is a more sustainable and eco-friendly alternative to traditional chemical methods. Cytochrome P450 monooxygenases (P450s or CYPs) are well-known bio-oxidation catalysts that catalyze C-H bond oxyfunctionalization of various organic substrates, which are widely used biocatalysts in biomanufacturing.[7] Some P450s, such as CYP152 peroxygenases, have evolved to use H_2O_2 as the sole source of oxygen and electrons, which allows them to bypass the need for redox partner(s) and expensive NAD(P)H via the peroxide shunt pathway.[8] Consequently, the H_2O_2-shunt reaction is an attractive candidate for monooxygenation reactions catalyzed by P450s.[9]

On the basis of effective C-H bond oxyfunctionalization, the subsequent multi-step reaction process design and construction are crucial for deep conversion of hydrocarbons and diverse target product synthesis. *In vitro* multi-enzyme cascade reactions are efficient and versatile for complex chemical transformations.[10] Enzymes obtained from different organisms can be employed to construct various artificial cascades to prepare diverse products in one pot, avoiding time-consuming purification of intermediates, tedious protection/deprotection steps, and functional group interconversions. [11] Most multi-enzyme cascades require expensive nicotinamide cofactors like

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NAD(P)H or NAD(P)$^+$ for stoichiometric reactions. Therefore, efficient regeneration of nicotinamide cofactors is important for reducing reaction costs and supplying required driving force to unidirectionally drive the cascade reactions. Traditional cofactor regeneration methods involve the use of additional oxidoreductases such as formate dehydrogenase, NADH oxidase, alcohol dehydrogenase, phosphate dehydrogenase, and glucose dehydrogenase, along with corresponding sacrificial substrates. The sacrificial substrates are either oxidized or reduced to regenerate the cofactors.[12] Notably, the biocatalytic hydrogen-borrowing strategy for cofactor regeneration has gained substantial popularity in recent years due to its outstanding atom efficiency.[13] Hydrogen-borrowing cofactor regeneration consists of two half cycles: oxidation and reduction. These two half cycles are coupled through the exchange of a hydride, which is facilitated by a cofactor. During the oxidation half cycle, the hydride is removed, while in the reduction half cycle, it is consumed without requiring additional oxidoreductase and sacrificial substrates.[14]

Chiral amino acids are chemicals that are extensively applied in food, agricultural, pharmaceutical, cosmetic, and feedstuff industries.[15] There have been significant advances in the synthetic methods used to produce chiral amino acids, due to their numerous practical applications. In recent years, enzymatic asymmetric synthesis has emerged as a popular method, thanks to its mild reaction conditions, high enantioselectivity, and impressive space-time yield. There are four main ways for the enzymatic asymmetric synthesis of chiral amino acids, including (1) asymmetric reductive amination of keto acids, (2) asymmetric transamination of keto acids, (3) enantioselective addition of ammonia to *α*,*β*-unsaturated acids, and (4) aldol condensation of an amino acid to aldehydes.[16] In addition, cascade C-H bond oxyfunctionalization at the *α*-carbon using fatty acids as starting substrates followed by reductive amination has also been reported as an alternative synthetic route.[6c, 17] Regardless of which method is mentioned above, chiral amino acids are synthesized from starting substrates that have activated functional groups (*e.g.*, carbonyl, carboxyl, aldehyde, and amino groups). However, if it were possible to directly synthesize chiral amino acids from chemically inert petroleum hydrocarbons without any activated functional groups, it would make amino acid synthesis more accessible and cost-effective. This breakthrough would also revolutionize organic synthesis methodology and allow for more comprehensive utilization of petroleum hydrocarbons.

In this study, we developed a multi-enzyme cascade conversion system that effectively addresses the challenges that are encountered during the deep conversion of chemically inert hydrocarbons. The system combines a two-step regioselective C-H bond oxyfunctionalization module with a asymmetric reductive amination module to construct an efficient conversion route, which enabled successful conversions of a number of chemically inert hydrocarbons into corresponding chiral amino acids. The reaction cascade consists of an alkane hydroxylase (AlkB, EC 1.14.15.3) from *Pseudomonas putida* GPO1,[4b] an engineered choline oxidase ($AcCO_6$, EC 1.1.3.17) from *Arthrobacter cholorphenolicus*,[18] an aldehyde dehydrogenase (EcALDH, EC 1.2.1.5) from *Escherichia coli* K-12,[19] a P450 peroxygenase ($P450_{CLA}$, EC 1.11.2.4) from *Clostridium acetobutylicum*,[17a, 20] a *D*-2-hydroxyisocaproate dehydrogenase (*D*-HIC-DH, EC 1.1.1.345) from *Lacticaseibacillus paracasei*,[21] an *L*-2-hydroxyisocaproate dehydrogenase (*L*-HIC-DH, EC 1.1.1.337) from *Weissella confuse,*[22] and a phenylalanine dehydrogenase (*L*-PheDH, EC 1.4.1.20) from *Rhodococcus sp.*[23] The entire conversion process was driven by hydrogen-borrowing cycle-based internal regeneration of NADH and *in-situ* generation and consumption of H_2O_2. This approach eliminated the need for adding sacrificial substrate, extra oxidoreductases, and external H_2O_2.

Results and Discussion

Design of the multi-enzymatic deep conversion system of heptane

Herein, the multi-enzymatic deep conversion of heptane was employed as a model reaction. As shown in **Figure 1**, the conversion from heptane to (*S*)-2-aminoheptanoic acid is realized through two successive functional modules. Module I is the two-step regioselective C-H bond oxyfunctionalization of heptane. In this module, heptane is first oxidized to 1-heptanol via the regioselective terminal C-H bond hydroxylation catalyzed by AlkB with the consumption of NADH. The resulting 1-heptanol is then oxidized to heptanal using $AcCO_6$, with O_2 as the oxidant and H_2O_2 as a byproduct. The heptanal is further oxidized by EcALDH to produce heptanoic acid. Simultaneously, NAD$^+$ generated from the C-H bond hydroxylation catalyzed by AlkB is reduced by EcALDH to regenerate NADH, forming the first hydrogen-borrowing cycle. H_2O_2, the byproduct of 1-heptanol oxidation catalyzed by $AcCO_6$, is utilized by $P450_{CLA}$ *in situ* to catalyze the regioselective C-H bond hydroxylation at the *α*-carbon of heptanoic acid, producing 2-hydroxyheptanoic acid. Module II is the asymmetric reductive amination of 2-hydroxyheptanoic acid followed by the generation of (*S*)-aminoheptanoic acid. In this module, both *D*- and *L*-Hi-CDH are used to catalyze the oxidation of 2-hydroxyheptanoic acid, which is generated in Module I. This oxidation produces 2-carbonylheptanoic acid and regenerates NADH. Then, *L*-PheDH consumes the regenerated NADH to further facilitate the asymmetric reductive amination of 2-carbonylheptanoic acid and the production of (*S*)-2-aminoheptanoic acid, forming the second cycle of hydrogen-borrowing. Driven by the two cycles of hydrogen-borrowing and *in situ* generation of H_2O_2, the chemically inert heptane is converted to chiral (*S*)-aminoheptanoic acid with O_2 as an oxidant and ammonium as an amino group donor under ambient conditions.

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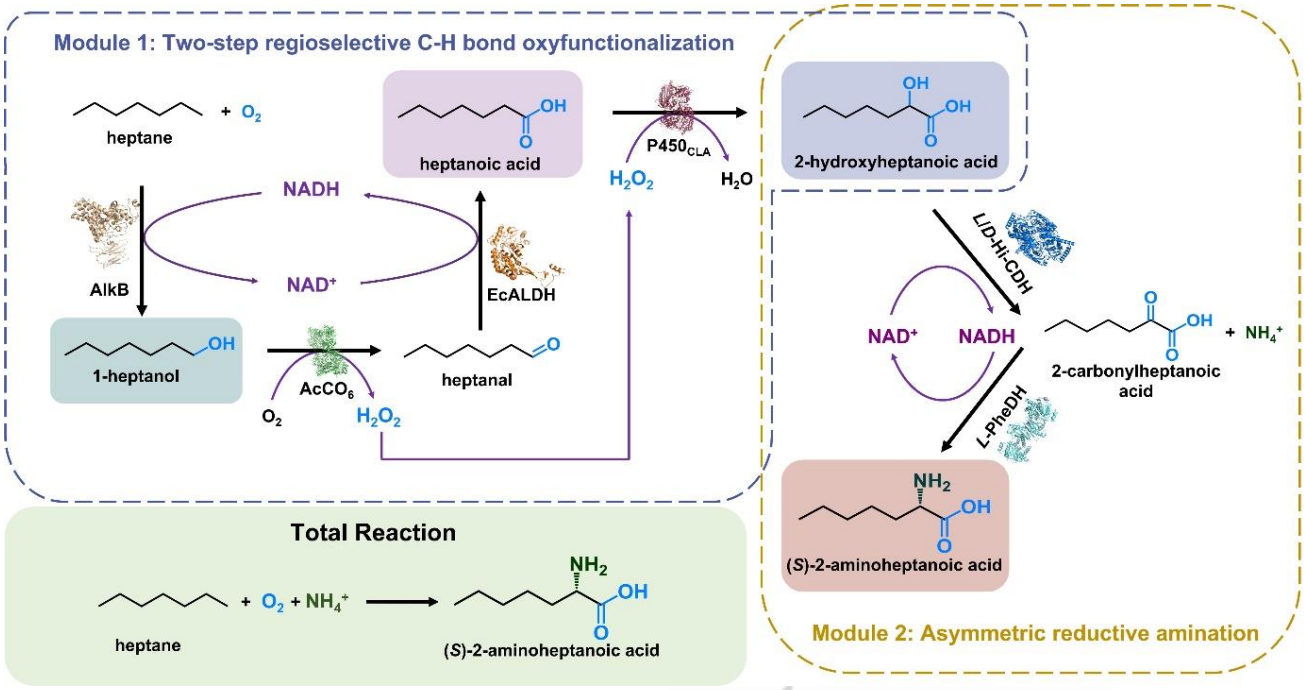

Figure 1. The schematic representation of the multi-enzyme hydrocarbon deep conversion system that converts heptane to (*S*)-aminoheptanoic acid. AlkB: alkane hydroxylase, $AcCO_6$: engineered choline oxidase, EcALDH: aldehyde dehydrogenase, $P450_{CLA}$: P450 peroxygenase, *D*-HIC-DH: *D*-2-hydroxyisocaproate dehydrogenase, *L*-HIC-DH: *L*-2-hydroxyisocaproate dehydrogenase, *L*-PheDH: phenylalanine dehydrogenase.

Module I: The optimization of heptane concentration

AlkB requires the assistance of redox partners, including rubredoxin (AlkG) and rubredoxin reductase (AlkT), to transfer electrons from NADH to the diiron cofactor of AlkB to catalyze the terminal C-H bond hydroxylation. As the expression level of AlkT in *E.coli* is low, a putidaredoxin reductase from *Pseudomonas putida* (PDR, Uniport: P16640) was used as the an alternative to AlkT.[4b] To achieve a relatively high accumulation concentration of 1-heptanol, the amount of initial heptane loading was first optimized with the addition of 3 mM NADH. As shown in **Figure 2**, it was observed that increasing the heptane loading had a positive impact on the concentrations of 1-heptanol and heptanal. The optimal concentration of heptane was found to be 1% (*v*/*v*), which led to the highest accumulated concentration of 1-heptanol (0.72 mM) and heptanal (0.22 mM) after 2-hour reaction.

To improve conversion efficiency, increasing heptane concentration is beneficial. However, the excess addition of heptane would form an aqueous-organic solvent two-phase system. As the two-liquid phase system must be intensively stirred to increase the interfacial area and enhance the mass transfer across the interphase, enzyme molecules adsorb at the interphase, which destabilizes the protein's electrostatic, hydrophobic, and hydrogen bond interactions, leading to irreversible denaturation.[24] In **Figure 2**, continuing to increase heptane concentration to 1.5% (*v*/*v*) and 2% (*v*/*v*) led to reduced accumulation of 1-heptanol and heptanal mainly due to the negative impact of excessive addition of heptane on AlkB activity. Meanwhile, heptane is volatile. When the initial loading was 1% (*v*/*v*, 68.4 mM), the heptane concentration decreased to approximately 15 mM after stirring for 2 hours. When the loading was reduced to 0.5% (*v*/*v*, 34.2 mM), the heptane concentration decreased to only 2.3 mM after 2 hours of stirring. For 1.5% (*v*/*v*, 102.6 mM) and 2% ((*v*/*v*, 136.8 mM) loading, the heptane concentration was 41 mM and 73 mM, respectively, after 2 hours of stirring (**Figure S9**). Therefore, a 1% (*v*/*v*) heptane loading was suitable for maintaining the appropriate heptane concentration during the reaction and preventing the adverse effect on enzyme activity due to excessive addition.

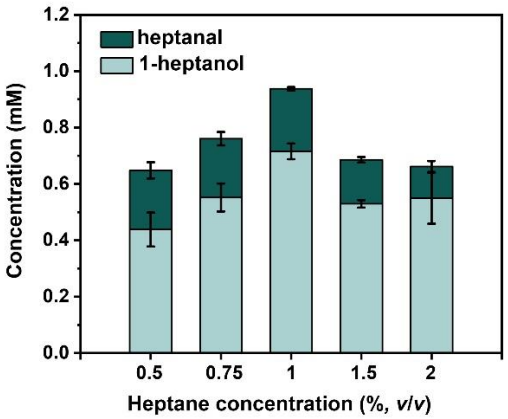

Figure 2. Optimization of heptane loading according to the concentration of generated 1-heptanol and heptanal. The reactions were performed in a 7 mL sealed vial with magnetic string at room temperature. The 1 mL reaction mixture contained 100 mM KPi buffer (pH=8.0), 0.42 mg/mL (0.15 U/mL) AlkB, 0.53 mg/mL AlkG and 2.62 mg/mL PDR (molar ratio of AlkB: AlkG: PDR = 1:3:6), 3 mM NADH and heptane at different concentrations of 0.5-2% (*v*/*v*) as substrate. The reactions were performed at room temperature for 2 h.

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Module I: The conversion from heptane to heptanoic acid

The conversion of heptane into 2-aminoheptanoic acid involves turning the terminal C-H bond into a carboxyl group, and then transforming the C-H bond at the *α*-carbon into an amino group. Therefore, heptanoic acid is the first key intermediate. As shown in **Figure 1**, heptane was successively converted to 1-heptanol, heptanal, and finally, heptanoic acid catalyzed by AlkB, $AcCO_6$, and EcALDH, respectively. Meanwhile, the reactions catalyzed by AlkB and EcALDH formed an integrated cycle of hydrogen borrowing, where NADH consumed by AlkB was regenerated by EcALDH. $AcCO_6$ is a highly active engineered choline oxidase variant with alcohol oxidase activity developed by Turner's group.[18] It enables specifically catalyzing the oxidation of primary alcohols to generate the corresponding aldehydes with O_2 as an oxidant and H_2O_2 as the byproduct.[18] In this study, $AcCO_6$ was used to catalyze the conversion from 1-heptanol to heptanal, which bridged the two half-cycles of the hydrogen-borrowing catalysis by AlkB and EcALDH.

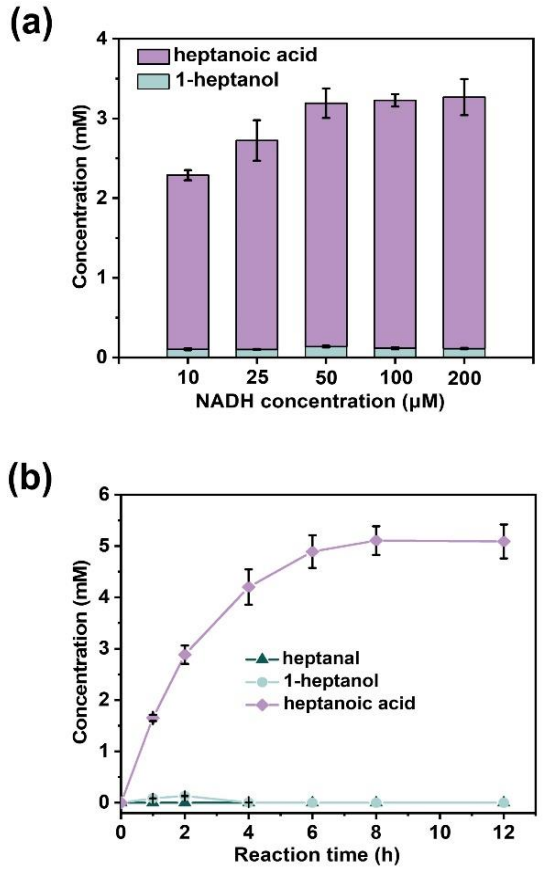

Figure 3. The conversion from heptane to heptanoic acid. (a) The optimization of initial NADH loading concentration. (b) The tracking of concentrations of 1-heptanol, heptanal, and heptanoic acid following time course. The reactions were performed in a 7 mL sealed vial with magnetic string at room temperature. The 1 mL reaction mixture contained 100 mM KPi buffer (pH=8.0), 0.42 mg/mL (0.15 U/mL) AlkB, 0.53 mg/mL AlkG and 2.62 mg/mL PDR (molar ratio of AlkB: AlkG: PDR = 1:3:6), 0.75 mg/mL (0.25 U/mL) $AcCO_6$, 0.5 mg/mL (0.35 U/mL) EcALDH, 50 μM NADH and 1% (*v*/*v*) heptane as substrate.

The initial concentration of NADH was first optimized. The results shown in **Figure 3a** demonstrated that increasing the initial NADH concentration from 10 μM to 50 μM enhanced the reaction efficiency and heptanoic acid accumulation. After a 2-hour reaction, 3.05 mM heptanoic acid and 0.14 mM 1-heptanol were produced with the addition of 50 μM NADH. However, adding more NADH did not further increase the concentration of heptanoic acid. A noteworthy result was that the NADH regeneration based on the hydrogen-borrowing cycle improved the efficiency of terminal C-H bond hydroxylation catalyzed by AlkB. As mentioned above, the conversion process driven by the cycle of hydrogen-borrowing resulted in the production of 3.05 mM heptanoic acid and 0.14 mM 1-heptanol (**Figure 3a**), indicating an activation of 3.19 mM heptane. However, when 3 mM NADH was directly added, only 0.72 mM 1-heptanol and 0.22 mM heptanal were generated (**Figure 2**), corresponding to an activation of only 0.94 mM heptane. This significant difference in C-H bond activation efficiency was probably due to the direct addition of a high concentration of reduced NADH (3 mM), which might consume the dissolved O_2 in the reaction solution. However, the conversion with a low initial addition (50 μM) and regeneration of NADH via the hydrogen-borrowing cycle significantly eliminated the consumption of dissolved O_2, thus providing a sufficient oxidant for the C-H bond hydroxylation catalyzed by AlkB. With 50 μM NADH addition, the accumulated concentrations of heptanoic acid kept increasing and achieved the highest level (5.1 mM) after 8 hours of reaction; and 1-heptanol only accumulated slightly during the first 2 hours of reaction (**Figure 3b**). Throughout the entire reaction process, heptanal did not accumulate. This suggested that the cycle of hydrogen-borrowing was efficient in regenerating NADH, which in turn drove the conversion from heptane to heptanoic acid.

Since AlkB catalyzed the first rate-limiting step of the multi-enzyme cascade, its catalytic efficiency is crucial for the performance of the entire conversion process. As with most monooxygenases, the efficiency of AlkB is hindered by two main issues. Firstly, the transfer of electrons from the redox partners (PDR and AlkG) to AlkB is not very effective.[25] Secondly, while oxygen is necessary for the reaction, it also reacts quickly with the reduced components of the electron transport chain, particularly NADH.[26] These inherent limitations make it challenging to improve the catalytic efficiency of AlkB. Maintaining the stability of AlkB is also essential for the entire conversion process. After 8 hours of incubation at room temperature, the specific activity of AlkB decreased to only 8.9% of the initial level (**Figure S10**). This limited activity is probably the reason why the conversion of heptane to heptanoic acid peaked after 8 hours of reaction and did not increase further. In the future, we will further improve the electron transfer efficiency and stability of AlkB through protein engineering to achieve more efficient conversion and utilization of chemically inert alkanes.

Module I: The conversion from heptane to 2-hydroxyheptanoic acid

After converting the terminal C-H bond of heptane to a carboxyl group, the oxyfunctionalization of the C-H bond at the *α*-carbon is also critical for the subsequent introduction of an amino group.

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$P450_{CLA}$ is a hydroxylase from the cytochrome P450 family 152 (CYP152).[27] Of note, $P450_{CLA}$ is distinct from typical P450s in that it can use H_2O_2 as an oxidant to improve atom economy (no requirement of redox partner proteins, recycling of NAD(P)H, and sacrificial co-substrates), system simplicity, and scalability of the reaction (no additional energy input required for gas to the liquid transfer of O_2).[17a, 28] In this study, $P450_{CLA}$ was supposed to utilize H_2O_2 generated *in situ* from the oxidation of 1-heptanol catalyzed by $AcCO_6$ to catalyze the *α*-hydroxylation of heptanoic acid (**Figure 1**).

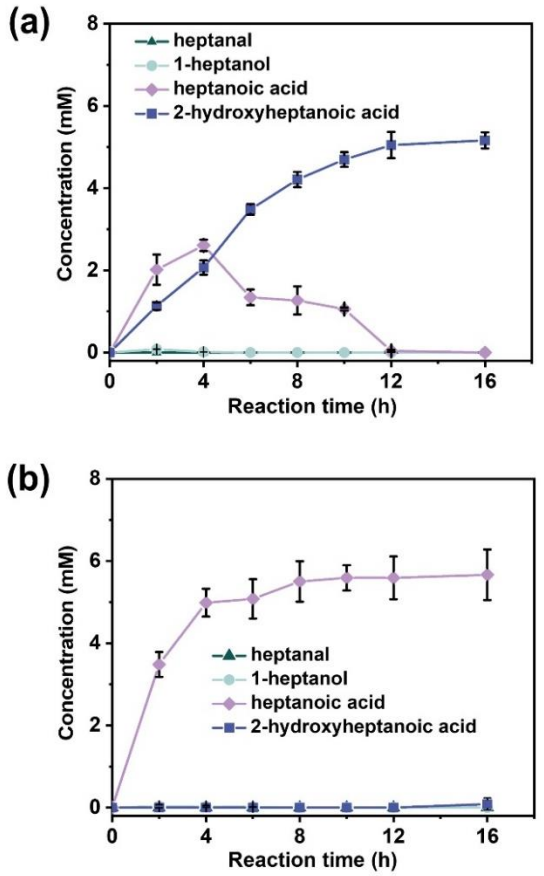

Figure 4. The conversion from heptane to 2-hydroxyheptanoic acid. (a) The tracking of concentrations of 1-heptanol, heptanal, heptanoic acid, and 2-hydroxyheptanoic acid following time course. (b) The tracking of concentrations of 1-heptanol, heptanal, heptanoic acid, and 2-hydroxyheptanoic acid following time course with the addition of 1.8 mg/mL CatA to savage the *in situ* generated H_2O_2. The reactions were performed in a 7 mL sealed vial with magnetic string at room temperature. The 1 mL reaction mixture contained 100 mM KPi buffer (pH = 8.0), 0.42 mg/mL (0.15 U/mL) AlkB, 0.53 mg/mL AlkG and 2.62 mg/mL PDR (molar ratio of AlkB: AlkG: PDR = 1:3:6), 0.75 mg/mL (0.25 U/mL) $AcCO_6$, 0.5 mg/mL (0.35 U/mL) EcALDH, 16 μM (0.92 U/mL) $P450_{CLA}$, 50 μM NADH and 1% (*v*/*v*) heptane as substrate.

As shown in **Figure 4a**, the concentration of 2-hydroxyheptanoic acid increased consistently and reached its highest level (5.16 mM) after 16 hours of reaction. The concentration of heptanoic acid kept increasing and reached a maximum level of approximately 2.61 mM after 4 hours. After that, it decreased continuously until all heptanoic acid was consumed after 12 hours of reaction. These results indicated that H_2O_2 generated *in situ* was sufficient to drive the *α*-hydroxylation of heptanoic acid catalyzed by $P450_{CLA}$. Moreover, the accumulation of 1-heptanol and heptanal was not observed during the entire conversion process. To further confirm that *in situ* generated H_2O_2 indeed drove the conversion from heptanoic acid to 2-hydroxyheptanoic acid, a catalase from *Bacillus subtilis* (CatA, Uniport: P26901) was added to savage H_2O_2. As expected, the addition of CatA completely inhibited the α-hydroxylation of heptanoic acid catalyzed by $P450_{CLA}$. As a result, 2-hydroxyheptanoic acid was undetectable throughout the entire conversion process due to the lack of H_2O_2, which was scavenged by CatA (**Figure 4b**).

In many previous studies, the external addition of H_2O_2 was required to initiate the C-H bond hydroxylation catalyzed by $P450_{CLA}$. However, the excessive addition of H_2O_2 would lead to enzyme deactivation. Therefore, it is crucial to have precise control over the amount of externally added H_2O_2.[17b] Two common strategies for controlling the amount of H_2O_2 include optimizing the initial addition amount and using fed-batch supplementation method are usually used to maintain a constant and reasonable level of H_2O_2.[17, 29] In this study, H_2O_2 used in the process was not externally added, but rather generated *in situ*, which was then consumed by $P450_{CLA}$ as an oxidant to facilitate the C-H bond hydroxylation. This approach allowed for simultaneous generation and consumption of H_2O_2, simplifying the process by eliminating the need for time-consuming optimization and complicated fed-batch addition to control H_2O_2 concentration. Additionally, this method improved the atom economy of the conversion.

Module II: The conversion from 2-hydroxyheptanoic acid to 2-aminoheptanoic acid

In Module I, the terminal and *α*-carbon C-H bonds were successively oxyfunctionalized to form carboxyl and hydroxyl groups. On the basis of the oxyfunctionalization, we sought to introduce an amino group to *α*-carbon to achieve the conversion from heptane to 2-aminoheptanoic acid. As shown in **Figure 1**, 2-hydroxyheptanoic acid generated in Module I was converted to 2-carbonylheptanoic acid via catalysis of *L/D*-Hi-CDH. Then, the generated 2-carbonylheptanoic acid was transformed to 2-aminoheptanoic acid via reductive amination catalyzed by *L*-PheDH. Simultaneously, the reactions catalyzed by *L/D*-Hi-CDH and *L*-PheDH formed the second cycle of hydrogen borrowing, where NADH generated by *L/D*-Hi-CDH was consumed by *L*-PheDH *in situ* to drive the reductive amination of 2-carbonylheptanoic acid.

As shown in **Figure 5**, the concentration of 2-aminoheptanoic acid increased and reached the highest level (4.57 mM) after 3 hours of reaction (**Figure S13-S16**). The conversion ratio from 2-hydroxyheptanoic acid to 2-aminoheptanoic acid achieved 88.57%. Furthermore, during the reaction in Module II, no accumulation of 2-carbonylheptanoic acid was detected. Several previous studies reported that *α*-keto acids could quickly undergo decarboxylation in the presence of oxidizing agents, such as H_2O_2, resulting in the formation of C1-truncated fatty acids. [14b, 29-30].

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Additionally, the concentration of hexanoic acid was monitored over time. We found that no hexanoic acid was produced (**Figure S11**), suggesting all H_2O_2 generated in Module I was completely consumed to prevent any side reactions from occurring in Module II.

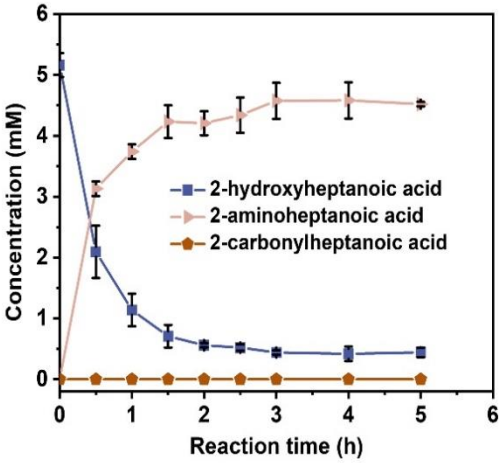


Figure 5. The tracking of concentrations of 2-hydroxyheptanoic acid, 2-carbonylheptanoic acid and 2-aminoheptanoic acid following time course. The reactions were performed in a 7 mL sealed vial with magnetic string at room temperature. The 1 mL reaction mixture contained 100 mM KPi buffer (pH = 8.0), 0.42 mg/mL (0.15 U/mL) AlkB, 0.53 mg/mL AlkG and 2.62 mg/mL PDR (molar ratio of AlkB: AlkG: PDR = 1:3:6), 0.75 mg/mL (0.25 U/mL) $AcCO_6$, 0.5 mg/mL (0.35 U/mL) EcALDH, 16 μM (0.92 U/mL) $P450_{CLA}$, 50 μM NADH and 1% (*v*/*v*) heptane as substrate. The reaction of Module II was started by adding 200 mM NH_4Cl, 20 mM $(NH_4)_2CO_3$, 0.83 mg/mL (6.16 U/mL) *L*-HiCDH, 0.5 mg/mL (157.61 U/mL) *D*-HiCDH, and 0.5 mg/mL (99.74 U/mL) *L*- PheDH to the reaction vessel. The pH value was adjusted to 8.5.

To further demonstrate the synthetic usefulness of this multi-enzyme cascade, we carried out a 50 mL semi-preparative scale synthesis of 2-aminoheptanoic acid using heptane as the initial substrate. In Module I, the concentration of 2-hydroxyheptanoic acid consistently increased, reaching 6.37 mM after 12 hours of the reaction (**Figure S12a**). In Module II, the highest concentration of 2-aminoheptanoic acid reached 5.52 mM (**Figure S12b**). As a result, the conversion ratio from 2-hydroxyheptanoic acid to 2-aminoheptanoic acid reached 86.65%.

Multi-enzyme cascade conversions of different chemically inert hydrocarbons to chiral amino acids.

Motivated by the successful one-pot heptane-to-2-aminoheptanoic acid conversion, we selected five aliphatic and one aromatic hydrocarbon as starting substrates to synthesize chiral amino acids. Upon a 15-hour reaction (Module I: 12h, Module II: 3h), all of the chemically inert hydrocarbons were converted to their corresponding amino acids, except for 2-methylpentane (**Table 1**). The concentrations of produced aliphatic amino acids, including 2-aminohexanoic acid (3.19 ± 0.16 mM), 2-aminoheptanoic acid (4.57 ± 0.30 mM), 2-aminooctanoic acid (4.21 ± 0.16 mM), and 2-amino-6-methylheptanoic acid (3.72 ± 0.24 mM) were virtually at the same level. The concentration of 2-amino-4-phenylbutanoic acid was 0.47 ± 0.05 mM, significantly lower than its aliphatic counterparts. This may be due to the lower activity of AlkB towards aromatic hydrocarbons compared to the aliphatic ones.[31] As the generated amino acid products are chiral, the optical purity was also determined (**Figure S17-S21**). Remarkably, the enantiomeric excess value (ee_p) of all the produced chiral amino acids were > 99% (**Table 1**).

As a proof-of-concept investigation, the results shown in **Table 1** confirmed the technical feasibility of our multi-enzyme cascade system for converting chemically inert hydrocarbons to chiral amino acids. The obtained chiral amino acids are important intermediates to synthesize various high value-added chemicals. The hydroxylated products of (*S*)-2-aminohexanoic acid (*L*-norleucine) are useful intermediates for production of omapatrilat, indospicines, siderophores, and peptide hormone analogs.[16, 32] (*S*)-2-aminoheptanoic acid (*L*-homonorleucine) can be used to modify vasopressin to synthesize *L*-homonorleucine-vasopressin, which has antidiuretic activity.[33] (*S*)-2-aminooctanoic acid is utilized to modify lactoferricin B peptide to improve its antimicrobial activity.[34] (*S*)-2-amino-4-phenylbutanoic acid (*L*-Homophenylalanine), is an essential building block of many chiral drugs, including angiotensin converting enzyme inhibitors, protease inhibitors, acetylcholinesterase inhibitors, neutral endopeptidase inhibitors, and β-lactam antibiotics.[35]

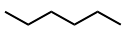
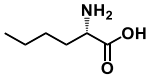
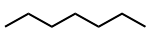
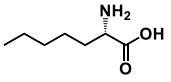
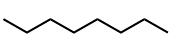
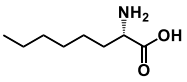
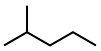
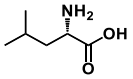
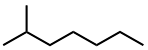
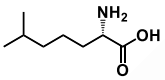
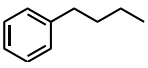
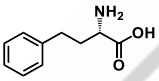
Conclusion

In this work, we present a novel multi-enzyme cascade conversion system in which the chemically inert hydrocarbons were successfully converted to chiral amino acids with high optical purity under mild conditions. Focusing on the two challenges for deep conversion of chemically inert hydrocarbons, C-H bond activation, and effective conversion pathway design, a hydrogen-borrowing cycle-based NADH regeneration method and H_2O_2 *in-situ* generation and consumption strategy were applied to realize C-H bond oxyfunctionalization at terminal and *α*-carbon of a select number of hydrocarbons. By integrating subsequent reductive amination module driven by the second hydrogen-borrowing-based NADH regeneration cycle, the chemically inert hydrocarbons were successfully converted to various chiral amino acids with high ee_p value and added-value. The challenging conversion was achieved with O_2 from the air as the oxidant and inexpensive ammonium as the amination reagent. The two hydrogen-borrowing cycles allowed for the internal regeneration of NADH without the need for a sacrificial substrate and an extra oxidoreductase. Additionally, the atom economy was improved by *in-situ* generation and consumption of H_2O_2, eliminating the need for external H_2O_2. We envision that the multi-enzyme cascade hydrocarbon deep conversion system developed in this study can comprehensively convert and utilize inert hydrocarbons from petroleum feedstock in an eco-friendly and gentle way. This system has the potential to outperform conventional organic synthesis methods and satisfy green chemistry requirements more effectively.

10.1002/anie.202410260

Considering the high cost of using purified enzymes, using whole-cell catalysis would be better option. Our next step involves constructing *E. coli* chassis cells with catalase and alkyl hydroperoxide reductase knocked out. We will then use genetic engineering methods to transfer the multi-enzyme cascade in this study into *E. coli* chassis cells to create engineered *E. coli* cells that can effectively catalyze the reaction, laying the foundation for the subsequent industrial application of preparing chiral amino acids from chemically inert alkanes.

Table 1. The conversion of various hydrocarbons to chiral amino acids[a]

Substrates[b]	Products	Concentration（mM）	Optical purity (ee_p%)
 hexane	 (*S*)-2-aminohexanoic acid	3.19 ± 0.16	>99%
 heptane	 (*S*)-2-aminoheptanoic acid	4.57 ± 0.30	>99%
 octane	 (*S*)-2-aminooctanoic acid	4.21 ± 0.16	>99%
 2-methylpentane	 *L*-leucine	n.d.[c]	n.d.[c]
 2-methylheptane	 (*S*)-2-amino-6-methylheptanoic acid	3.72 ± 0.24	>99%
 butylbenzene	 (*S*)-2-amino-4-phenylbutanoic acid	0.47 ± 0.05	>99%

[a] All product concentrations are presented as mean ± standard deviation (n=3). [b] The concentration of all substrates was 1% (*v*/*v*). [c] Not determined because of a very low conversion.

Acknowledgements

The authors thank the support from the National Natural Science Foundation of China (Grants 32371534). We also appreciate Prof. Yandong Zhang from Xiamen University for the helpful discussion.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the finding of this study are available in the supplementary material of this article.

Keywords: Biocatalysis • C-H activation • chiral amino acids • cofactor regeneration • inert hydrocarbons

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10.1002/anie.202410260

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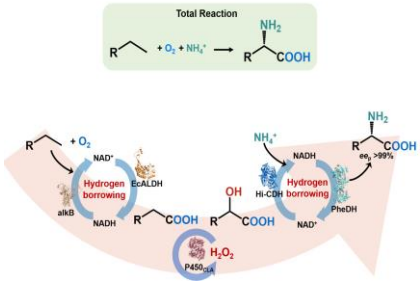

A multi-enzyme cascade conversion system was developed using O_2 as the oxidant and ammonium as the amination reagent. This system is based on internal cofactor and H_2O_2 recycling. It is designed to facilitate the one-pot enzymatic conversion from chemically inert hydrocarbons to chiral amino acids under environmentally friendly conditions, which is a highly challenging transformation in traditional organic synthesis.