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Enzyme Catalysis

Dynamic Kinetic Resolution for Asymmetric Synthesis of L-Non-canonical Amino Acids from D-Ser Using Tryptophan Synthase and Alanine Racemase

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Abstract: L-Ser is often used to synthesize some significant L-noncanonical α-amino acids(L-ncAAs), which are the prevalent intermediates and precursors for functional synthetic compounds. In this study, threonine aldolase from *Escherichia coli* k-12 MG1655 has been used to synthesize L-Ser. In contrast to the maximum catalytic capacity (20 g/L) for L-threonine aldolase(LTA), D-Ser was synthesized with high yield (240 g/L) from cheap Gly and paraformaldehyde using D-threonine aldolase (DTA) from *Arthrobacter sp* ATCC. In order to fully utilize D-Ser and expand the resource of L-Ser, a dynamic kinetic resolution system was constructed to convert D/DL-Ser to L-Ser through combining alanine racemase (Alr) from *Bacillus subtilis* with L-tryptophan synthase (TrpS) from *Escherichia coli* k-12 MG1655, and L-ncAAs including L-Trp and L-Cys derivatives were synthesized with excellent enantioselectivity and in high yields. The results indicated L-ncAAs could be efficiently synthesized from D-Ser using this original and green dynamic kinetic resolution system, and the reliable L-Ser resource has been established from simple and achiral substrates.

Introduction

L-noncanonical α-amino acids (L-ncAAs) are often used as intermediates in biosynthesis and are prevalent precursors for functional synthetic compounds, including over 12 % of the 200 top-selling pharmaceuticals, since they possess, at minimum, two reactive functional groups (the amine and carboxylic acid) and typically have at least one stereocenter. Therefore, increasing attention has been devoted to the efficient synthesis of L-ncAAs in recent years.[1]

Enzymes are widely applied to synthesize L-ncAAs since they not only can catalyze the reactions in mild conditions but also could maintain highly stereoselective and obviate the need for protecting groups, thereby decreasing the number of synthetic steps.[2] Among these enzymes, tryptophan synthase (TrpS, E.C. 4.2.1.20) is a pyridoxal 5-phosphate (PLP)-dependent enzyme that catalyzes the synthesis of L-Trp from indole and L-Ser.[3] Frances H. Arnold et al. has reported that TrpS could react with myriad indole analogs to provide a direct biocatalytic route to L-Trp derivatives, a kind of L-ncAAs.[4]

L-Ser as an essential substrate for TrpS is widely employed in cosmetics, pharmaceuticals, food industries and also utilized as a building block chemical. The approaches to produce L-Ser involve the extraction from keratin protein hydrolysates (e.g. hair and feather) or poultry waste hydrolysates,[5] the fermentation with sugar substrates (e.g. glucose and sucrose), the chemical synthesis from α-haloacrylic acid derivatives,[6] and the enzymatic synthesis from Gly and formaldehyde using serine hydroxymethyltransferase.[7] However, some disadvantages make them less attractive such as the complicated process of separation and purification, low productivity and stereoselectivity, and the expensive cofactor (e.g. tetrahydrofolate) for serine hydroxymethyltransferase catalysis.[7] Among these approaches, an alternative enzyme has been sought to synthesize L-Ser due to the advantages of enzymatic synthesis. Threonine aldolase has attracted considerable attention recently since it shows good activity for preparation of β-hydroxy-α-amino compounds from Gly and the corresponding aldehydes.[8] Since Ser possesses the characteristic of β-hydroxy-α-amino, we designed to synthesize L-Ser using L-threonine aldolases (LTA) from *E. coli* k-12 MG1655, and yet it showed weak activity only with the maximum catalytic capacity 20 g/L. In contrast to LTA, D-threonine aldolase (DTA) could synthesize D-Ser in excellent yield (240 g/L).[9] Therefore, how to efficiently convert D/DL-Ser to L-Ser and make full use of D/DL-Ser should be considered for the better economic value of L-Ser.

In the past decades, kinetic resolution (KR) has been widely studied through using stereoselective lipase, acylase, amidase, and carbamoylase to hydrolyze the racemates to obtain optically pure compounds. However, the theoretical yield of product (≤50 %) for KR limits its further application in industry, and consequently dynamic kinetic resolution (DKR) has been developed.[10] Compared with the traditional KR, DKR can provide

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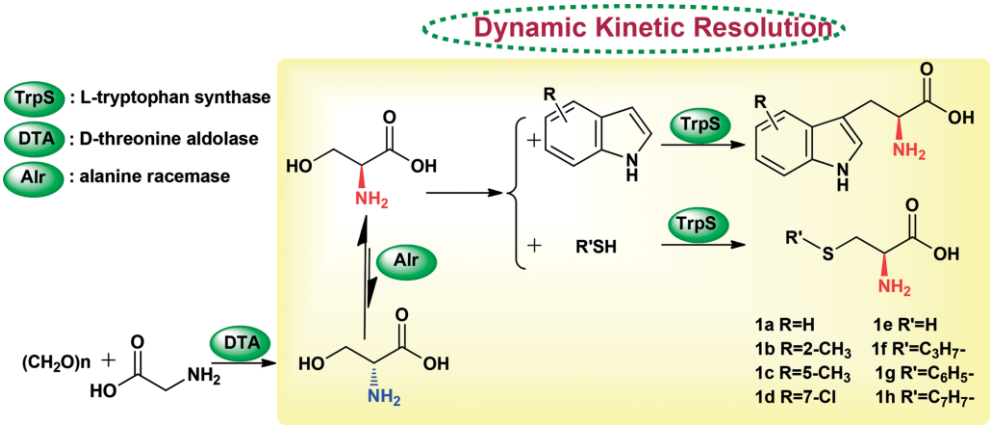


Scheme 1. Enzymatic synthesis of L-ncAAs from Gly and $(CH_2O)_n$ using DTA, Alr and TrpS.

complete conversion to enantiomerically pure product by combining the classical KR with continuous racemization of the unreacted enantiomer, and now DKR is one of the most powerful and elegant methods for efficient synthesis of one enantiomer from racemic starting materials.[11]

Racemases have attracted considerable attention recently because of their remarkable potency of racemization in DKR. As one of the members of racemase family, alanine racemase (Alr, EC 5.1.1.1) possesses the similar characteristics of racemase, which is PLP-dependent enzyme that catalyze the reversible interconversion of D- and L-forms of alanine.[12] Considering that the structures of Ser and alanine are similar, we attempted to perform the racemization of D-Ser to D,L-Ser using Alr, simultaneously with the conversion of L-Ser to L-Trp and L-Cys derivatives using TrpS. We expected that the source of L-Ser will be expanded by DKR using the combination of two enzymes.

In this report, we focused on D-Ser as starting compounds for L-ncAAs synthesis through DKR because D-Ser can be easily obtained from the cheap Gly and paraformaldehyde ($(CH_2O)n$) using DTA. By combining Alr with TrpS in a one-pot reaction, D-Ser was racemized to D, L-Ser to synthesize eight L-α-amino acids including L-Trp, L-Cys and their corresponding derivatives (Scheme 1). The aim of this study was to develop a green, economic enzymatic synthesis route of L-ncAAs from simple and achiral substrates.

Results and Discussion

Enzymatic Synthesis of Serine Using Threonine Aldolase

Enzymatic activities can be affected by many environmental factors, such as pH, substrate concentration, and temperature. Herein the enzymatic activities were tested by varying one of the bioconversion conditions at a time while keeping others constant to investigate the influential factors systematically.[13] All the enzymatic reactions were optimized and the data weren't shown except DTA since the synthesis of D-Ser by DTA was rarely reported.

For DTA, the reaction conditions including pH (5–10), temperature (20–50 °C) and substrate concentration (0.5–4 M) were

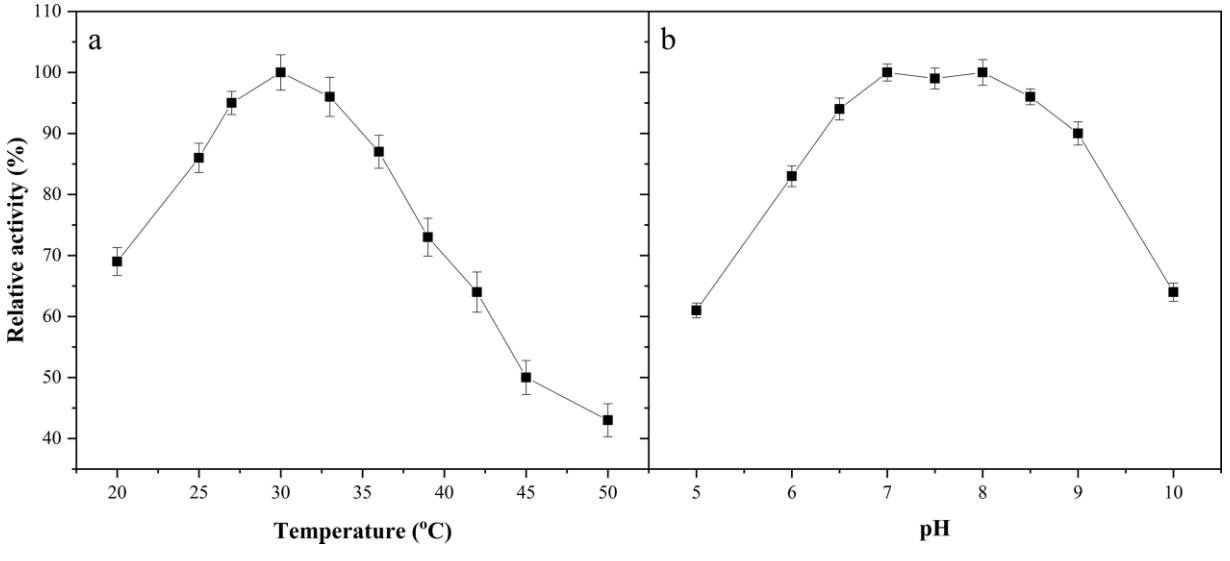


Figure 1. The effect of pH and temperature on DTA activity. The reaction (volume: 10 mL) was carried out at 200 rpm, in which DTA dry whole cell (5 mg), Gly (2.5 M), $(CH_2O)n$ (2.5 M) and PLP (0.4 mM) were contained. The mixture was shaken for 2 h at 30 °C for determination of the optimal pH and was shaken at pH 7 for determination of the optimal temperature. 100 % relative activity denoted that 16 % conversion of Gly or $(CH_2O)n$.

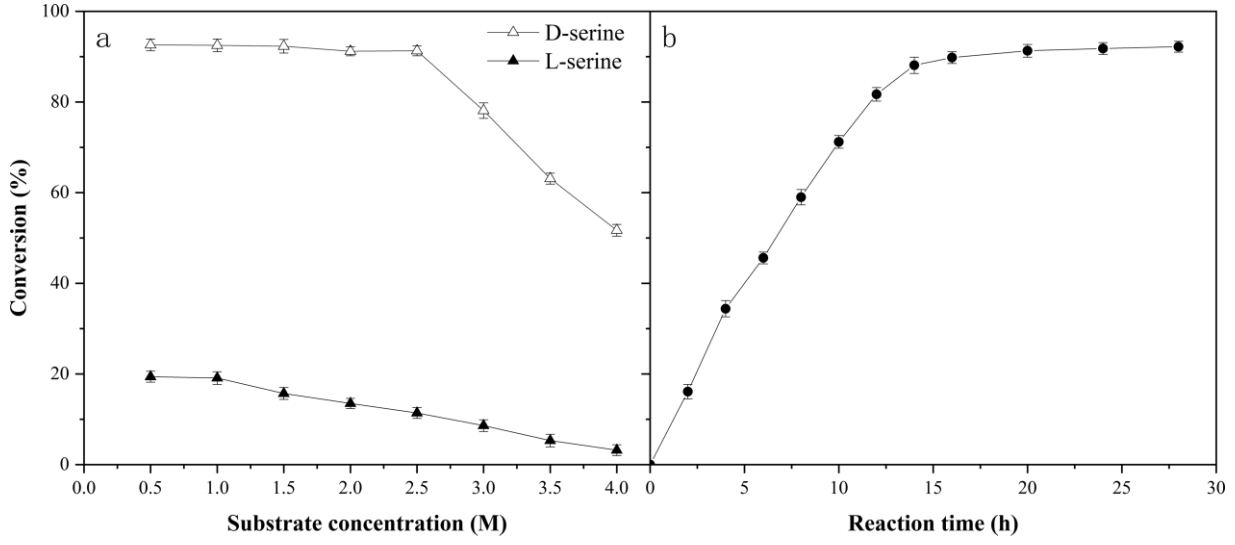


Figure 2. a) The effect of substrate concentration on threonine aldolase activity. The mixture (volume: 10 mL) including threonine aldolase dry whole cell (5 mg), Gly, $(CH_2O)n$, PLP (0.4 mM) was reacted for 20 h at 40 °C, pH 8.0 for LTA and at 30 °C, pH 7.0 for DTA. The mol ratio of Gly and $(CH_2O)n$ was fixed at 1:1, the substrate concentrations varied as shown. b) Time course for the production of D-Ser from Gly and $(CH_2O)n$ using DTA. The reaction (volume: 100 mL, pH 7.0) was carried out at 30 °C, 200 rpm, in which DTA dry whole cell (50 mg), Gly (2.5 M), $(CH_2O)n$ (2.5 M) and PLP (0.4 mM) were contained.

optimized. The pH was fixed at the designated value by constantly adding sodium hydroxide solution (30 %, m/v) since pH would gradually decrease during the reaction. Figure 1 and Figure 2a show that the optimal pH, temperature and substrate concentration for the reaction catalyzed by DTA were 7.0, 30 °C and 2.5 M, respectively.

As described in Figure 2a, all conversions reached above 91 % between 0.5 M and 2.5 M, and then the conversions decreased while the substrate concentration increased from 3.0 M to 4.0 M for DTA catalysis. In the case of LTA, the highest conversion was only 19 % (that was 20 g/L for 1.0 M), and the conversion decreased above 1 M substrate concentration. The enzyme activities of DTA and LTA were 6.7 U/mg and 0.43 U/mg respectively (Table 1), which indicated that LTA exhibited a weak activity towards Gly and $(CH_2O)n$ compared with DTA. Therefore, DTA was used to synthesize D-Ser, and the reaction was scaled up to 100 mL to obtain D-Ser as the starting material for the following DKR process. Figure 2b shows that D-Ser was mainly accumulated in 14 h and increased very little after the subsequent 14 h, which indicated D-Ser could be efficiently synthesized by DTA in high yield after appropriate reaction time.

In terms of the reaction catalyzed by threonine aldolase, formaldehyde as the substrate will result in the inhibition of DTA to some extent, and the inhibition has been overcome through adding formaldehyde by fed batch. Compared with formaldehyde, $(CH_2O)n$ could slowly release formaldehyde in solution, which was equal to the fed batch. The scale-up reaction described that $(CH_2O)n$ was a good substrate for DTA since the conversion of $(CH_2O)n$ (91.7 %, that was 240 g/L) was satisfactory after 28 h (Figure 2b).

D- Ser Racemization with Alr

Though D-Ser could be synthesized in a good yield using DTA, it is still not meaningful for the enzymatic reactions with L-Ser

Table 1. Enzymes activities.

Substrate	Concentration	Reaction	Enzyme	Enzyme[a]
	[mM]	time [min]		activity(U/mg)
C_8H_7N(1a) + L-Ser	20	15	TrpS	1.2
2-CH_3-C_8H_6N(1b) + L-Ser	20	15	TrpS	1.5
5-CH_3-C_8H_6N(1c) + L-Ser	20	15	TrpS	1.4
7-Cl-C_8H_6N(1d) + L-Ser	20	15	TrpS	0.46
NaSH(1e) + L-Ser	20	15	TrpS	5.4
n-C_3H_7SH(1f) + L-Ser	20	15	TrpS	5.3
C_6H_5SNa(1g) + L-Ser	20	15	TrpS	3.7
$C_6H_5CH_2SH$(1h) + L-Ser	20	15	TrpS	4.5
D-Ser	20	15	Alr	9.4
+ Gly + $(CH_2O)n$	20	15	DTA	6.7
Gly+$(CH_2O)n$	20	15	LTA	0.43

[a] One unit (U) of enzyme activity was defined as the amount of dry whole cells catalyzing the conversion of substrate at a rate of 1 μmol/min.

as substrate if D-Ser couldn't be effectively converted to L-Ser. Therefore, an enzyme was needed to racemize D-Ser to D,L-Ser and simultaneously avoid racemizing the amino acid products. Serine racemase was firstly considered, however, according to the reported protocol,[14] serine racemase from *Dictyostelium discoideum* could catalyze not only serine racemization but also serine dehydration (α,β-elimination) to yield pyruvate and ammonia, which brings the consumption of D-Ser and produces the side-product, consequently, decreases the conversion of D-Ser to the target L-ncAAs.

Another amino acid racemase (AAR) from *Pseudomonas putida* KT2440 (ATCC 47054) was shown to convert D-Ser to L-Ser,[15] nevertheless, it also could racemize the other amino acids including the products, which would influence not only the purity of products but also the dynamic kinetic resolution process. Alr was selected because its substrate alanine is similar to Ser and it does not catalyze racemization of the products. It was first used to racemize D-Ser and synthesize L-Trp, L-Cys and their corresponding derivatives through DKR.

Figure 3 shows that the racemization had been almost completed within 1 h by Alr for low concentration D-Ser (e.g. 0.2 and 0.4 M), and there was no inhibition over time even for high concentration D-Ser (e.g. 1.5 M) since its optical rotation steadily reached to zero after 10 h. Alr (9.4 U/mg) could catalyze the racemization quickly for low concentration D-Ser and has the potential to racemize high concentration D-Ser. On the other hand, Alr is inactive with the target. Therefore, Alr was chosen as the optimal racemase to catalyze the racemization of D-Ser in DKR system.

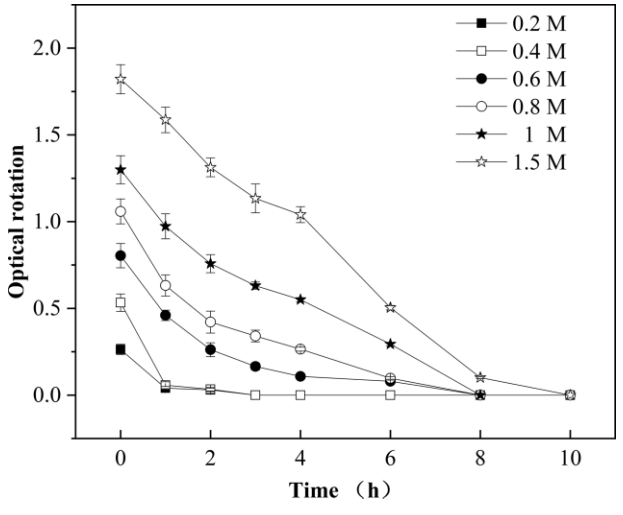
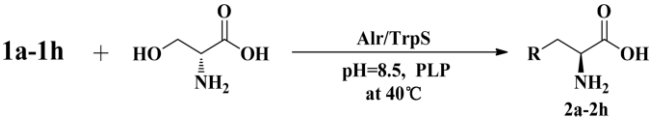


Figure 3. The effect of D-Ser concentration on Alr activity. The mixture (100 mL, pH 8.5) containing D-Ser, Alr dry whole cell (50 mg) and PLP (0.4 mM) reacted at 40 °C, 200 rpm.

Considering insolubility of the substrates (**1a**–**1d**), low concentration substrate (0.2 M) was used to synthesize L-ncAAs while investigating DKR system. Thereby D-Ser (0.2 M) was used according to the optimal reaction conditions and long reaction time was avoided since Alr could almost completely catalyze the racemization of low concentration D-Ser within 1 h (Table 2).

Table 2. Enzymatic synthesis of L-cystine, L-Trp and their derivatives.[c]



Substrate (**1a**–**1h**)	Concentration	Reaction	Conversion[a]	*ee*[b]
	(M)	time(h)	[%]	(% ee)
C_8H_7N (1a)	0.2	14	>77	>99
2-CH_3-C_8H_6N (1b)	0.2	6	>85	ND[d]
5-CH_3-C_8H_6N(1c)	0.2	6	>84	>99
7-Cl-C_8H_6N(1d)	0.2	24	>34	>99
NaSH(1e)	0.2	24	>20	>99
n-C_3H_7SH (1f)	0.2	4	>95	>99
C_6H_5SNa(1g)	0.2	8	>93	>99
$C_6H_5CH_2SH$(1h)	0.2	8	>94	>99

[a] The conversions were determined by HPLC and calculated according to the substrates (**1a**–**1h**) concentration. [b] The enantiomeric excesses were determined by the derivation of amino acid products with the Marfey's reagent. [c] The reactions (volume: 10 mL, pH 8.5) were carried out at 40 °C and catalyzed by Alr (5 mg) and TrpS (5 mg) dry whole cells, in which 0.2 M D-Ser, PLP (0.4 mM) and 0.2 M of each substrate was added. [d] Not detected.

Enzymatic Synthesis of L-2a, L-2b, L-2c, L-2d

As shown in Figure 4, the concentration of D-Ser was the same as L-Ser (0.084 M) in the mixture after 1 h, and the racemization was well performed in accordance with converting D-Ser only by Alr (Figure 3). On the other hand, L-Trp concentration increased slowly which was still approximately 0.06 M after 6 h and subsequently almost kept unchanged. The low concentration of L-Trp in solution was attributed to two factors, one was the poor solubility of indole which made the efficiency of TrpS decrease, the other was the low solubility of L-Trp at pH 8.5 which quickly precipitated to decrease its concentration in solution and shift the reaction equilibrium toward the product formation. Finally, the total yield of L-Trp was 65 % after the separation and purification.

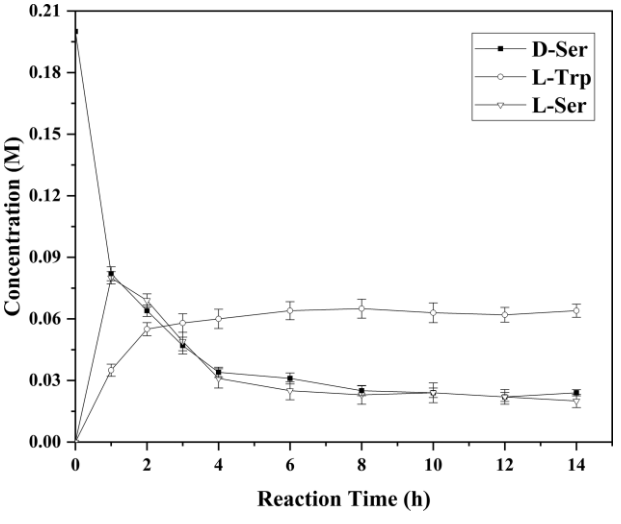


Figure 4. Time course for the enzymatic synthesis of L-Trp using Alr and TrpS. The reaction was performed as described in Table 2.

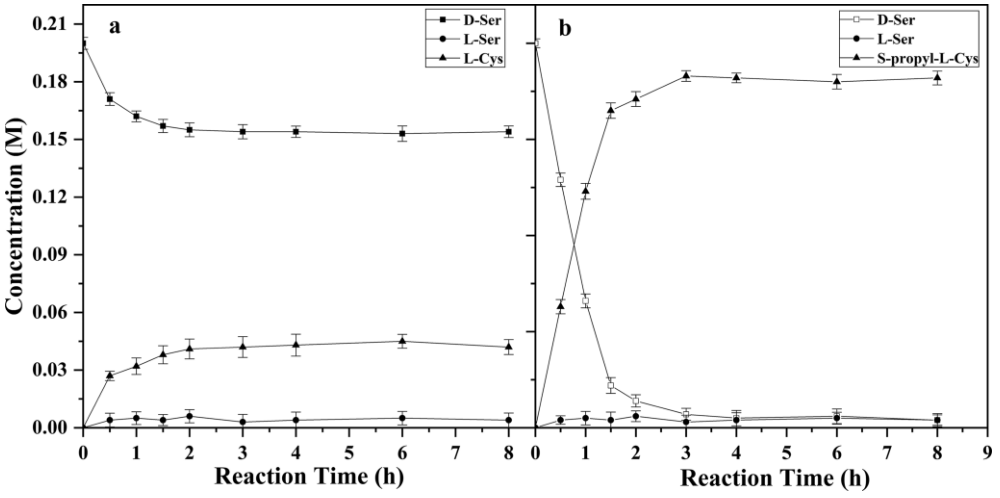


Figure 5. Time course for the enzymatic synthesis of **L-2e** (a), **L-2f** (b). The reactions were performed as described in Table 2.

As observed for L-Trp, the other L-ncAAs (**L-2b, L-2c L-2d)** all quickly precipitated during the reactions and were synthesized from **1b**– **d** in a similar procedure, so their time courses weren't shown again. The indole derivatives, except **L-1d** (>34 %), were all well converted with the excellent enantiomeric purities (Table 2).

Enzymatic Synthesis of L-2e, L-2f, L-2g, L-2h

In this study, L-Cys derivatives could be synthesized through DKR system with high yields, which showed that TrpS could act on the wide range of mercaptans as substrates (Table 2). The mercaptan derivatives are the important chiral intermediates to prepare a variety of pharmaceuticals including HIV protease inhibitors, anti-inflammatory analgesics, and oxytocin, therefore, it is important to efficiently synthesize these compounds.

In contrast to **L-2f**, **L-2g,** and **L-2h**, **L-2e** was produced in low yield by DKR with sodium hydrosulfide as sulfur donating reagent. As indicated in Figure 5a, at the beginning of the reaction, D-Ser decreased from 0.2 M to 0.17 M with L-Cys increasing to 0.029 M at half an hour, but after then the conversion increased slowly, even if the reaction time extended to 8 h, the conversion only increased from 15 % to 22.5 %. Moreover, after 2 h, Alr showed the difficulty in racemizing D-Ser with keeping it at 0.154 M from 2 h to 8 h, and thereby the conversion of L-Cys wasn't very good in this DKR system. Though TrpS activity (5.4 U/mg) for sodium hydrosulfide (**1e**) was the highest among the substrates (**1a**–**1h**) and Alr (9.4 U/mg) could racemize D-Ser well (Table 1), the preparation of L-Cys by combining TrpS with Alr form D-Ser couldn't be smoothly performed.

As described in Figure 5b, **1f** was quickly converted into **L-2f** with high conversion (>95 %) and *ee* value (>99 %) after 4 h. Since **L-2g** and **L-2h** also continuously precipitated during the reactions, the time courses for **L-2g** and **L-2h** were similar to that of **L-2a** and not described again.

Analysis of the Amino Acids' Enantiomeric Purity

New chiral stereocenters are generally formed under the catalysis of the enzymes, so it is necessary to evaluate the enzyme selectivities by quantifying the enantiomeric purity of the products. In this study, analysis of the amino acids' enantiomeric purity was divided into two parts including natural and unnatural amino acids. The enantiomers of natural amino acids could be obtained commercially, so we determined their enantiomeric purities by HPLC through modifying the conversion solution with Marfey's reagent and then comparing with the standards.[16]

For the L-ncAAs, it was difficult to assay their optical purities because they cannot be commercially bought as standards especially D-isomers. Fortunately, we found amino acid racemase *Pseudomonas putida* KT2440 (ATCC 47054) could racemize the L-ncAAs products, [15] which made it unsuitable to catalyze the racemization of D-Ser in DKR**.** Therefore, the products were racemized by amino acid racemase and subsequently modified as diastereomers with Marfey's reagent. The D- and L-amino acid derivatives were separated by HPLC and the L-amino acid derivative was usually eluted before the corresponding D-isomer,[16,17] and thereby the absolute configuration was determined. The related results were shown in supporting information, which indicated all the analyzed products were L-configuration with >99 % *ee* (Figure S8-S22). The absolute configuration of the product **2b** was not detected since it couldn't be racemized by amino acid racemase (Table 2).

Conclusions

L-Ser is not only an important amino acid but also a key building block, so it is significant to obtain it economically and environmentally friendly. In this study, we have synthesized D-Ser with high yield (240 g/L) from Gly and $(CH_2O)n$ using DTA. Herein a DKR system was reported through using the combination of Alr and TrpS to convert D/DL-Ser to L-Ser and simultaneously convert L-Ser to other L-ncAAs, which could make full use of D-Ser and provide a desirable source of L-Ser. Furthermore, some important L-Trp and L-Cys derivatives could be efficiently synthesized with excellent enantiomeric purities and in good yields by the DKR system.

This work highlights not only an improved and complementary route to the conversion of D-Ser to L-Ser, but also the effective enzymatic synthesis of L-Trp, L-Cys and their derivatives by DKR. In addition, the DKR strategy reported in this study would encourage us to uncover more DKR systems in which L-Ser as a key substrate is used to efficiently synthesize optical purity canonical or L-ncAAs.

Experimental Section

Chemicals: Lactose and PLP were purchased from Sigma (St. Louis, Mo. USA). Silica gel GF254 plate (5.0 × 10.0 cm) was purchased from Haiyang Chemical Co. Ltd. (Qingdao, China). All other chemicals and reagents used in this work were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Restriction enzymes and kits for genetic manipulation were purchased from Takara Bio (Dalian, China).

Microorganisms and Shake Flask Fermentation: LTA (Uniprot ID: P75823) was amplified from genomic DNA of *E. coli* str. K-12 substr. MG1655, two primers were designed as follows: forward primer is: 5′-TGGGGATCCATGCAATTCTCTCGC-3′ (*BamHI* site underlined); reverse primer is: 5′-GGGGAATTCTTAGTCAACCAGGATTTCGG-3′ (*EcoRI* site underlined). The PCR fragment was inserted into pETDuet plasmid to construct the recombinant plasmid pETDuet-LTA.

DTA from *Arthrobacter sp* ATCC 21022 (Uniprot ID: O82872) and amino acid racemase from *Pseudomonas putida* KT2440 (ATCC 47054) (Uniprot ID: B8K1S4) in pETDuet plasmid, and Alr from *Bacillus subtilis* (Uniprot ID: P10725) in pGEX-KG plasmid were all total synthesized by Genscript Biotech Co. (Nanjing, China) with codons optimized for expression in *E. coli*. The recombinant plasmids pETDuet-DTA, pETDuet-AAR, pGEX-KG-Alr, and pETDuet-LTA were all transformed into *E. coli* BL21 (DE3) for the expression of DTA, AAR, Alr and LTA respectively. TrpS from *E. coli* k-12 MG1655 (Uniprot ID: P0A879) in pET28a plasmid was obtained according to our previous studies.[3a]

All the media used for inoculums preparation were adjusted to pH 7.0 before autoclaving. For DTA, AAR, Alr, and LTA, a loopful of strain was inoculated in a 100 mL Erlenmeyer flask containing 30 mL of LB medium in which ampicillin (50 mg/L) was added in advance. For TrpS, kanamycin (50mg/L) was added into LB medium. These flasks incubated on a rotary shaker at 200 rpm and 30 °C for 12 h.

The obtained bacterial strains in LB medium were inoculated and cultivated according to our previous protocols.[18] And then fermented broth was collected at the end of the fermentation process and centrifuged at 10000 rpm and 4 °C for 10 min to obtain wet cells, which were lyophilized and stored at −20 °C for further study.[18]

Definition of DTA and LTA Activity: Activities of DTA and LTA were measured by detecting the formation of D-Ser and L-Ser respectively. The standard assay solution (final volume, 10 mL) containing 20 mM Gly, 20 mM paraformaldehyde, PLP (0.4 mM) and dry whole cell (5 mg) was incubated for 15 min at 40 °C, pH 8.0 for LTA and at 30 °C, pH 7.0 for DTA. One unit (U) of enzyme activity was defined as the amount of dry whole cells catalyzing the conversion of substrate to the product at a rate of 1 μmol/min.

Definition of Alr Activity: Activity of Alr was measured by detecting the concentrations of D-Ser and L-Ser. The standard assay solution (final volume, 10 mL) containing 20 mM D-Ser, PLP (0.4 mM) and dry whole cell (5 mg) was incubated at 40 °C, pH 8.5 for 15 min. One unit (U) of enzyme activity was defined as the amount of dry whole cells catalyzing the conversion of substrate at a rate of 1 μmol/min.

Definition of TrpS Activity: TrpS activity was determined by detecting the concentrations of L-Trp, L-Cys and their corresponding derivatives. The mixture (final volume, 10 mL) containing 20 mM L-Ser, 20 mM the other corresponding substrates, PLP (0.4 mM) and 5 mg TrpS whole cell was performed at 40 °C, pH 8.5 for 15 min. One unit (U) of enzyme activity was defined as the amount of dry whole cells catalyzing the conversion of substrate at a rate of 1 μmol/min.

Enzymatic Synthesis of D- Ser: The reaction conditions were optimized as shown in Figure 1 and Figure 2a. The scaled-up synthesis of D-Ser was operated as follows: DTA dry whole cells (50 mg) were mixed with a solution (100 mL) containing Gly (18.8 g), $(CH_2O)n$ (7.5 g) and PLP (0.4 mM) under pH 7.0, 30 °C at 200 rpm for 16 h. The reaction mixture was centrifuged to remove bacteria cells. And then, the supernatant was concentrated under vacuum and the residue was recrystallized from water/ethanol (1:2 v/v). D-Ser (22 g) was obtained as white crystals in >99 % *ee* and 84 % yield.

Enzymatic Synthesis of L-Ser: L-Ser was prepared from Gly and $(CH_2O)n$ using LTA, the mixture (volume: 10 mL) containing LTA dry whole cell (5 mg), Gly (1.88 g), $(CH_2O)n$ (0.75 g) and PLP (0.4 mM) was reacted at 40 °C, pH 8.0 and 200 rpm.

General Scaled-up Procedure for DKR Employing Alr and TrpS Whole Cells: The mixture (100 mL, pH 8.5) containing D-Ser (0.2 M), the other corresponding substrates (0.2 M), PLP (0.4 mM), Alr (50 mg), and TrpS dry whole cells (50 mg) was shaken at 200 rpm and 40 °C. Finally, the reaction was quenched by adding ammonia water. ^{1}H-NMR and ^{13}C-NMR of the products were all provided in supporting information (Figure S23-S38).

Enzymatic Synthesis of L-Tryptophan and its Derivatives: The reaction solution was quenched by adjusting pH to 11 using ammonia water (25 %–28 %) and then centrifuged at 10000 rpm for 10 min. The supernatant was decolorized by active charcoal (1 g) at 50 °C for 20 min and subsequently filtered. The filtrate was concentrated under vacuum, and the crude product was recrystallized with water to obtain L-Trp as white crystals in >99 % *ee* and 65 % yield. The spectral characterization of L-Trp was shown as follows: ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 7.62 (d, 1H, *J* =8.0 Hz), 7.39 (d, 1H, *J* =8.2 Hz), 7.11–7.14 (m, 2H), 7.05 (dd, 1H, *J* =7.3 Hz, 7.7 Hz), 3.45 (dd, 1H, *J* =5.4 Hz, 7.1 Hz), 3.06 (dd, 1H, *J* =5.3 Hz, 14.5 Hz), 2.91 (dd, 1H, *J* =7.2 Hz, 14.5 Hz). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 182.9, 136.1, 127.2, 124.2, 121.7, 119.0, 118.8, 111.7, 110.5, 56.5, 30.3.

The other L-Trp derivatives were obtained in the similar operation.

2-CH_3-L-tryptophan: white powder, yield, 74 %. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 7.50 (d, 1H, *J* =7.7 Hz), 7.27 (d, 1H, *J* = 8.0 Hz), 6.97–7.04 (m, 2H), 3.39 (dd, 1H, *J* =5.9 Hz, 7.3 Hz), 2.98 (dd, 1H, *J* =5.7 Hz, 14.5 Hz), 2.81 (dd, 1H, *J* =7.5 Hz, 14.4 Hz), 2.24 (s, 3H). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 183.0, 135.1, 134.5, 128.4, 120.7, 118.9, 117.9, 110.7, 106.3, 56.8, 29.4, 10.7.

5-CH_3-L-tryptophan: light gray solid, yield, 75 %. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 7.42 (s, 1H), 7.28 (d, 1H, *J* =8.3 Hz), 7.07 (s, 1H), 6.98 (d, 1H, *J* =8.3 Hz), 3.44 (dd, 1H, *J* =5.3 Hz, 7.2 Hz), 3.03 (dd, 1 H, *J* = 5.2Hz, 14.4 Hz), 2.85 (dd, 1 H, *J* =7.4 Hz, 14.4 Hz), 2.32 (s, 3H). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 182.9, 134.4, 128.7, 127.5, 124.4, 123.2, 118.1, 111.5, 110.1, 56.5, 30.3, 20.4.

7-Cl-L-tryptophan: white solid, yield, 20 %. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 11.25 (s, 1H), 7.55 (d, 1H, *J* =7.9 Hz), 7.28 (d, 1H, *J* =2.0 Hz), 7.15 (d, 1H, *J* =7.5 Hz), 6.99 (t, 1H, *J* =7.7 Hz), 3.42

(dd, 1H, *J* =4.0 Hz, 8.5 Hz), 3.27 (dd, 1H, *J* =4.0 Hz, 15 Hz), 2.99 (dd, 1H, *J* =8.6 Hz, 15 Hz). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 170.0, 133.5, 129.8, 125.9, 120.9, 119.8, 118.1, 116.2, 111.6, 55.1, 27.5.

Enzymatic Synthesis of L-Cys: The reaction mixture was centrifuged and the supernatant was adjusted to pH 2.0 with HCl (6 M) to convert the excessive sodium hydrosulfide to hydrogen sulfide, which was released from the solution and was trapped with NaOH (6 M). Due to the instability of L-Cys in the air, L-Cys was completely oxidized into L-cystine to calculate its yield. After hydrogen sulfide was exhausted, the acidic solution was adjusted to pH 4.6 with ammonia water, and hydrogen peroxide solution (2 mL, 6 %, m/v) was subsequently added to oxidize L-Cys to L-cystine, which precipitated and was collected by centrifugation. L-cystine was purified as follows: it was dissolved in HCl solution (pH 0.5) and decolorized with active carbon at 70 °C for 30 min, followed by filtration. The filtrate was neutralized to pH 4.6 with ammonia water to precipitate L-cystine again.[18] L-cystine was obtained as white crystals in *ee*>99 % and 13 % yield. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 3.47 (dd, 2H, *J* =4.7 Hz, 7.6 Hz), 3.01 (dd, 2H, *J* =4.7 Hz, 13.6 Hz), 2.80 (dd, 2H, *J* =7.6 Hz, 13.6 Hz) (m, 4H). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 180.8, 54.8, 43.5.

Enzymatic Synthesis of S-Propyl-L-cysteine: The mixture was centrifuged and the supernatant was concentrated under vacuum. The residue was recrystallized with water-acetone (1:1, v/v). S-propyl-L-cysteine was obtained as white crystals in *ee*>99 % and 84 % yield. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 3.31 (dd, 1H, *J* =5.1 Hz, 6.7 Hz), 2.76 (dd, 1H, *J* =5.0 Hz, 13.4 Hz), 2.67 (dd, 1H, *J* =6.8 Hz, 13.4 Hz), 2.46 (t, 2H, *J* =7.3 Hz), 1.46–1.53 (m, 2H), 0.85 (t, 3H, *J* =7.4 Hz). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 181.2, 55.1, 36.8, 33.7, 22.3, 12.6.

Enzymatic Synthesis of S-Phenyl- and S-Benzyl-L-cysteine: After the reaction mixture had been incubated whilst stirring at 40 °C for 8 h, the pH of the reaction solution was adjusted to **11** to make the product dissolve completely, and then the mixture was centrifuged to remove the whole cells and the other insoluble impurities. The supernatant was concentrated under vacuum and the residue was recrystallized with water/methanol (1:1, v/v). S-phenyl-L-cysteine was obtained as white crystals in *ee*>99 % and 76 % yield. ^{1}H-NMR (D_2O) (Bruker DRX600, Germany) δ: 7.35 (d, 2H, *J* =7.7 Hz), 7.26 (t, 2H, *J* =7.6 Hz), 7.18 (t, 1H, *J* =7.4 Hz), 3.29 (dd, 1H, *J* =4.9 Hz, 7.3 Hz), 3.21 (dd, 1H, *J* =4.8 Hz, 13.4 Hz), 2.98 (dd, 1H, *J* =7.4 Hz, 13.4 Hz). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 180.6, 134.8, 129.6, 129.2, 126.8, 55.1, 39.0.[18]

Based on the similar operation, S-benzyl-L-cysteine was obtained as white crystals in >99 % *ee* and 83 % yield, and its structure was confirmed by ^{1}H-NMR and ^{13}C-NMR. 7.19–7.28 (m, 5H), 3.66 (s, 2H), 3.26 (dd, 1H, *J* =5.3 Hz, 6.7 Hz), 2.65 (dd, 1H, *J* =5.2 Hz, 13.5 Hz), 2.58 (dd, 1H, *J* =6.8 Hz, 13.5 Hz). ^{13}C-NMR (D_2O, Bruker DRX600, Germany) δ: 181.0, 138.6, 129.0, 128.8, 127.3, 54.8, 36.3, 35.4.

Analytical Methods

Detection of the Product Concentrations by HPLC: Products except cysteine could be directly analyzed by HPLC using Waters e2695 separations module coupled with Waters 2998 photodiode array detector, and XBridge C18 column (250 × 4.6 mm, 5 μm) at 210 nm and 30 °C. The mobile phase composed of 30 % v/v methanol and 70 % v/v 0.05 M potassium dihydrogen phosphate solution at a flow rate of 0.8 mL/min. The HPLC chromatograms were shown in Figure S1-S7.

Analysis of the Enantiomeric Purities of the Products: The enantiomeric purities of products were determined by derivation with Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA).[16,17a] To a solution (1 mL, pH 8.5) of amino acid racemase dry whole cells (1 mg) and PLP (0.4 mM), the unnatural amino acid products (10 mg) were added respectively. After the reaction mixture had been incubated whilst stirring at 40 °C for 6 h, the reactions were centrifuged to remove bacteria cells, and then the supernatants were derivatized with Marfey's reagent. The derivatives with FDAA were prepared according to the previously reported protocol.[16]

The diastereomers with FDAA were analyzed by the same Waters e2695 instrument at 335 nm and 30 °C using 40 % v/v CH_3CN in H_2O (0.1 % v/v trifluoroacetic acid) as the mobile phase with a flow rate of 1 mL/min. The HPLC chromatograms were shown in Figure S8-S22.

The concentration and *ee* value of L-Cys and D/L-Ser were all detected by HPLC through modifying with Marfey's reagent.[18] In this study, all assays were performed in triplicates.

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