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Letter

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One-pot Biocatalytic Synthesis of Substituted D-Tryptophans from Indoles Enabled by an Engineered Aminotransferase

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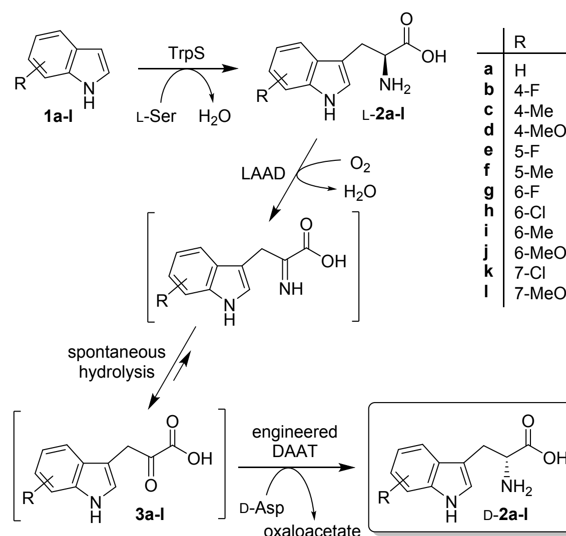
ABSTRACT: D-tryptophan and its derivatives are important precursors of a wide range of indole-containing pharmaceuticals and natural products. Here, we developed a one-pot biocatalytic process enabling the synthesis of D-tryptophans from indoles in good yields and high enantiomeric excess (91% to >99%). Our method couples the synthesis of L-tryptophans catalysed by *Salmonella enterica* tryptophan synthase with a stereoinversion cascade mediated by *Proteus myxofaciens* L-amino acid deaminase and an aminotransferase variant that we engineered to display native-like activity towards D-tryptophan. Our process is applicable to preparative-scale synthesis of a broad range of D-tryptophan derivatives containing electron-donating or withdrawing substituents at all benzene-ring positions on the indole group.

KEYWORDS: amino acids, biocatalysis, enzymes, organic synthesis, protein engineering, rational design

Introduction. The non-proteinogenic amino acid D-tryptophan is an important biosynthetic intermediate[1] and a precursor of a wide range of indole-containing pharmaceuticals and natural products including tadalafil,[2] lanreotide acetate,[3] skyllamycin,[4] macrolines,[5] and metalloprotease inhibitors for pain treatment.[6] Substituted D-tryptophans have also been employed in the preparation of analogues of these and other classes of chiral compounds. Although biocatalytic methods have been developed to synthesize optically pure D-amino acids using either asymmetric synthesis[7,8] or resolution/deracemization techniques,[9,10,11,12] to the best of our knowledge, fully biocatalytic approaches have not been applied to the synthesis of substituted D-tryptophan analogues from simple starting materials such as indoles. Recently, biocatalytic processes based on wild-type or engineered microbial tryptophan synthases (TrpS) have been developed for the synthesis of substituted L-tryptophans[13,14,15,16] and β-methyl-L-tryptophans[17,18] from indoles and L-serine or L-threonine. However, these methods are not applicable to the synthesis of D-tryptophans due to the strict stereoselectivity of TrpS for the L-enantiomer.[14,15] In this work, we develop a one-pot fully biocatalytic process that combines asymmetric synthesis of L-tryptophans from indoles using TrpS, with a stereoinversion cascade based on L-amino acid deaminase (LAAD) and an engineered D-alanine aminotransferase (DAAT) to allow efficient access to a library of D-tryptophans containing electron-donating or withdrawing substituents at all benzene-ring positions on the indole (Scheme 1).



Scheme 1. One-pot biocatalytic synthesis of D-tryptophans from indoles.

Results and discussion. Previous work on the deracemization of phenylalanine and its derivatives showed that complete stereoinversion of the L-enantiomer could be achieved by combining oxidative deamination of L-amino acids using *Proteus mirabilis* LAAD (*Pmir*LAAD) with transamination of D-amino acids using an engineered DAAT from *Bacillus* sp. YM-1 (DAAT-T242G).[9] In light of those results, we envisaged to broaden the applicability of our cascade to tryptophan derivatives, hoping to couple this stereoinversion process with the asymmetric synthesis mediated by TrpS.

As a first step in the development of our one-pot method, we measured D-tryptophan transamination activity of the DAAT V33G, S240G, and T242G single mutants that we previously engineered for enhanced transamination of D-phenylalanine.[9] These DAAT variants contain mutations to active-site residues (Fig. 1) that are postulated to reshape the substrate binding pocket for productive binding of bulky aromatic D-amino acids. Although specific activities of these mutants with the D-tryptophan donor substrate were 6–16-fold higher than that of the wild-type enzyme, they remained one or two orders of magnitude lower than those with the native substrate D-alanine (Supporting Information, Table S1). To maximize the efficiency of our proposed biocatalytic process, we aimed to further engineer DAAT to enhance its activity with D-tryptophan. We hypothesized that this could be achieved by combining the V33G, S240G and T242G mutations, which would increase space in the substrate binding pocket to accommodate the bulkier indole side chain of D-tryptophan. We thus generated all possible combinations of these mutations and measured D-tryptophan transamination activity for all resulting double and triple mutants (Table S1). Only the V33G/T242G and S240G/T242G double mutants displayed a significant increase in D-tryptophan transamination activity compared to the most active single mutant T242G. To evaluate the underlying reason for the increased D-tryptophan transamination activity of these two double mutants, we measured their kinetic parameters (Table 1). In both cases, K_M was significantly decreased (5.5–12-fold), in agreement with our hypothesis that combining these mutations would create a larger substrate binding pocket for the bulkier D-tryptophan substrate, leading to enhanced productive binding. The most active variant (DAAT-V33G/T242G) showed a 35-fold improvement to k_{cat}/K_M against D-tryptophan compared to the wild type, resulting in near-native catalytic efficiency with this non-native substrate (700 M^{-1} s^{-1}) that is higher than that of DAAT-T242G with D-phenylalanine (400 M^{-1} s^{-1}).[9]

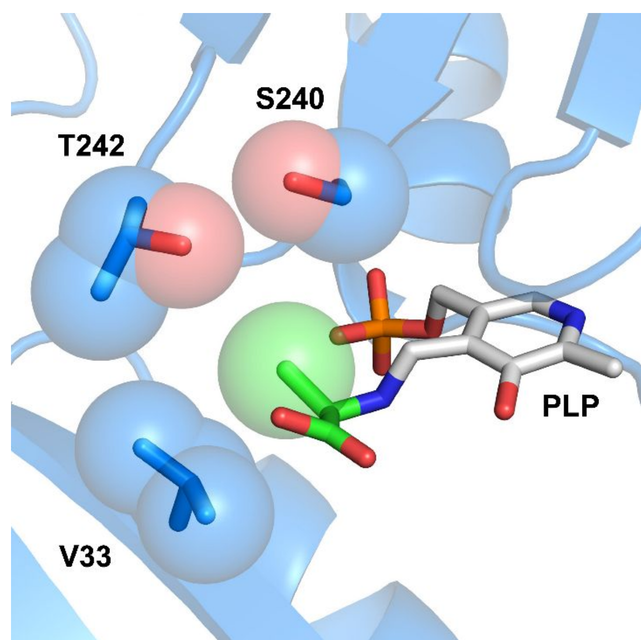


Figure 1. Crystal structure of *Bacillus* sp. YM-1 DAAT bound to its native substrate D-alanine (PDB ID: 3DAA). Residues V33, S240, and T242 in the substrate-binding pocket form a small cavity that can bind the side chain of D-alanine (green). It is unlikely that the bulky indole side chain of D-tryptophan could be accommodated in this small cavity without causing significant steric clashes.

Table 1. Apparent kinetic parameters of wild-type DAAT and its mutants for the transamination of D-tryptophan with oxaloacetate.[a]

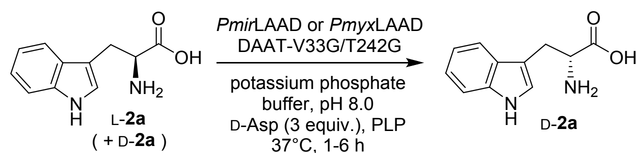
DAAT	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Wild type	10 ± 4	0.20 ± 0.06	20 ± 10
V33G/T242G	0.82 ± 0.05	0.57 ± 0.01	700 ± 40
S240G/T242G	1.8 ± 0.4	0.10 ± 0.01	60 ± 10

[a] Expt. cond.: 2 mU purified DAAT, 10 mM D-**2a**, 5 mM oxaloacetate, 16 µM PLP, 1 µM FAD, 0.5 mM *o*-dianisidine, 200 mU purified DDO, 5 U HRP, 100 mM potassium phosphate buffer, pH 8.0, 37°C. One unit (U) is defined as 1 µmol of product generated in 1 min. All reactions were performed in triplicate.

Having successfully engineered a DAAT variant with native-like catalytic efficiency towards D-tryptophan, we next tested stereoinversion/deracemization of this aromatic amino acid using DAAT-V33G/T242G in combination with *Pmir*LAAD, which we previously used for the deracemization of phenylalanine and its derivatives.[8,9,10] We also tested stereoinversion/deracemization using the LAAD from *Proteus myxofaciens* (*Pmyx*LAAD)[19,20] for comparison purposes. The reactions (Table 2) were carried out on both L- and *rac*-tryptophan, with D-aspartate as the amino donor of the transamination step. DAAT and variants have been shown to accept both D-aspartate and D-glutamate as amino donors, with slightly higher activity

against D-glutamate.[9] However, D-aspartate was used previously (and in this work) due to its lower cost and straightforward removal with D-aspartate oxidase (DDO). In addition, *E. coli* whole cells producing each biocatalyst

Table 2. Test reaction for the stereoinversion or deracemization of tryptophan.[a]



Substrate	LAAD	L-Ser (mM)	ee D-**2a** (%)[b]
L-**2a**	*Pmir*LAAD	0	76 (4 h)
			91 (6 h)
L-**2a**	*Pmyx*LAAD	0	78 (4 h)
			94 (6 h)
rac-**2a**	*Pmir*LAAD	0	96 (4 h)
			>99 (6 h)
rac-**2a**	*Pmyx*LAAD	0	98 (4 h)
			>99 (6 h)
L-**2a**	*Pmir*LAAD	40	23 (6 h)
L-**2a**	*Pmyx*LAAD	40	81 (6 h)

[a] Expt. cond.: 20 mM **2a**, 0–40 mM L-Ser, 60–180 mM D-Asp, 0.5 mM PLP, 5% *v/v* MeOH, 50 mg mL^{-1} LAAD wet cells, 50 mg mL^{-1} DAAT-V33G/T242G wet cells, 100 mM potassium phosphate buffer, pH 8.0, 37°C; [b] Determined by HPLC on a chiral stationary phase.

were used without lysis or protein purification, which is very convenient for large scale synthesis.

As shown in Table 2, all reactions reached good to excellent ee values after 6 hours, with complete deracemization of *rac*-**2a** being achieved. The high efficiency of these stereoinversion/deracemization cascades confirms the known high activity of both LAADs against L-tryptophan, [21] and the high catalytic efficiency that we measured for the engineered DAAT-V33G/T242G variant. Since the first step of our proposed one-pot biocatalytic synthesis of D-tryptophan would require at least a slight excess of L-serine to afford high conversion of indole (Scheme 1), we also tested the stereoinversion cascade in the presence of a two-fold excess of this polar amino acid. Stereoinversion of L-**2a** using *Pmyx*LAAD resulted in a 13% lower ee in the presence of excess L-serine. However, with *Pmir*LAAD the ee decreased by 68% (Table 2), a result that can be ascribed to higher reactivity of *Pmir*LAAD against L-serine relative to L-tryptophan compared to *Pmyx*LAAD.[21] Because of the observed reactivity of this stereoinversion system with L-serine, we elected to carry out our envisaged biocatalytic process (Scheme 1) as a one-pot telescopic system where biocatalysts for the stereoinversion cascade are introduced only after the TrpS-mediated L-tryptophan synthesis is completed.

Having demonstrated that the stereoinversion cascade worked satisfactorily, we turned our attention to the upstream synthesis of L-tryptophans from indoles (Scheme 1). Several TrpS enzymes have been previously described, in particular those from *Pyrococcus furiosus* and *Salmonella enterica* serovar *typhimurium*. While both of these enzymes work efficiently at pH 8.0 against a broad range of substrates,[14,15] we selected the *Salmonella* enzyme due to its high activity at ambient temperature (the TrpS from *P. furiosus* shows optimum activity at 75°C[13]). *Salmonella* TrpS is a heterodimeric enzyme composed of two subunits (α and β). The β subunit alone is sufficient for catalysis but its activity is severely attenuated in the absence of the α subunit, which acts as an allosteric actuator of the β subunit. Both subunits can be overproduced simultaneously in *E. coli* BL21(DE3) cells with the pSTB7 plasmid available from ATCC (product code: ATCC37845), affording active and soluble TrpS enzyme.[22]

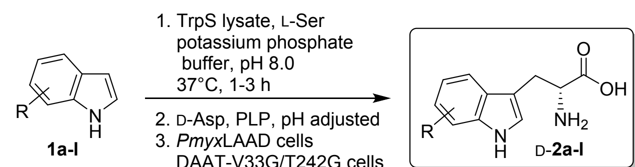
We first tested the TrpS reaction using 5 mM indole **1a**, 50 mM L-serine and variable catalyst loadings (10–100 mg mL^{-1} *E. coli* whole cells or 50–200 μL mL^{-1} crude lysate) under various conditions (pH, MeOH or DMSO cosolvent, and reaction time). MeOH and DMSO were selected as cosolvents due to their widespread use in TrpS literature.[14,16-18] As a general trend, conversions were higher at pH 8.0, using MeOH as cosolvent, and lysate instead of whole cells (detailed results of all the tests performed are reported in Table S2). Interestingly, we observed lower conversions in the presence of very high catalyst loadings or after prolonged incubation times, which suggests reversibility of the reaction. Nevertheless, at pH 8.0 and 5% MeOH, it was possible to achieve >99% conversion to L-**2a** in under 1 h, even with indole concentrations increased from 5 to 20 mM.

As noted earlier, the presence of a large excess of L-serine during stereoinversion of L-tryptophan leads to reduced ee. Thus, we evaluated the effect on conversion rate of the TrpS reaction under optimized conditions (pH, cosolvent) when different L-serine/indole ratios are used (Table S3). Almost complete conversion of **1a** could be obtained with L-serine/indole ratios as low as 2:1 or 1.5:1, confirming that a large excess of L-serine is not necessary to obtain high yields. To demonstrate the generality of the method, we tested a panel of substituted indoles containing electron-donating or withdrawing substituents at positions 4, 5, 6 or 7 (Scheme 1, **1b–l**). As shown on Table S3, some indoles afforded lower conversions with these ratios. Therefore, an L-serine/indole ratio of 3:1 was selected to achieve complete conversions with the full panel of indoles.

Having optimized both the TrpS reaction and LAAD/DAAT stereoinversion cascade separately, integration of these two steps was evaluated in a one-pot telescopic fashion on preparative scale (20 mM **1a**). This

multi-step process afforded 97% conversion of **1a** to D-**2a** (Table 3), demonstrating the feasibility of our biocatalytic approach for the synthesis of D-tryptophan from indole. To purify the D-tryptophan product, we considered using a similar procedure to the one that we previously developed to separate the D-phenylalanine product from the excess D-aspartate used as transamination donor, which

Table 3. Preparative scale one-pot synthesis of D-**2a–l** from indoles.[a]



Substrate	R	Conv. (%)[b]	Isol. Yield (%)	ee D-2a–l (%)[c]
1a	H	97	69	>99
1b	4-F	>99	68	97
1c	4-Me	95	64	>99
1d	4-MeO	>99	79	91
1e	5-F	>99	78	>99
1f	5-Me	81	63	>99
1g	6-F	>99	76	>99
1h	6-Cl	>99	68	>99
1i	6-Me	>99	74	97
1j	6-MeO	>99	71	94
1k	7-Cl	84	66	>99
1l	7-MeO	>99	76	97

[a] Expt. cond.: 20 mM **1a–l**, 60 mM L-Ser, 100 μL mL^{-1} TrpS lysate, 180 mM D-Asp, 0.5 mM PLP, 5% *v*/*v* MeOH, 50 mg mL^{-1} *Pmyx*LAAD wet cells, 50 mg mL^{-1} DAAT-V33G/T242G wet cells, 100 mM potassium phosphate buffer, pH 8.0, 37°C, total volume 4 mL; [b] Determined by HPLC on a non-chiral stationary phase; [c] Determined by HPLC on a chiral stationary phase.

involved incubation with D-aspartate oxidase (DDO) to convert this amino acid into oxaloacetate before purification with an ion-exchange resin.[9] However, this procedure is not suitable in the context of the D-tryptophan synthesis presented here because this product needs to be separated from not one but two amino acids used in excess (L-serine and D-aspartate). Thus, an oxidase that is strictly selective for L-serine would be required in addition to DDO in order to eliminate both undesired amino acids, which would be wasteful and impractical.

As an alternative, we considered adsorption on C_{18} reverse phase silica, exploiting the higher hydrophobicity of the D-tryptophan product compared to L-serine and D-aspartate. After optimization of the protocol, the preparative scale reaction containing 20 mM D-**2a** as well as residual L-serine and D-aspartate was dried in a centrifugal evaporator, acidified with diluted HCl, and loaded on C_{18} silica equilibrated with 0.5% *v*/*v* MeOH in water. Elution with 25% *v*/*v* MeOH afforded D-**2a** in 69% isolated yield (Table 3) and very high purity, as evaluated by NMR (Supporting Information).

Next, preparative scale reactions were set up with the full panel of substituted indoles (**1b–l**), affording a range of D-tryptophan derivatives in very high or quantitative conversion and 63–79% isolated yield (Table 3). Enantiomeric purity was shown to be excellent (≥ 97% ee) in most cases, with the exception of D-**2d** and D-**2j**, which could not be stereoinverted completely but still afforded good enantiopurity (91% and 94% ee, respectively). Furthermore, the same batch of C_{18} reverse phase silica could be recycled at least 3 times (with different derivatives) without losing efficiency or showing signs of cross contamination, helping to reduce costs and improve throughput.

Lastly, in order to demonstrate the applicability of our method to preparative scale synthesis, we scaled-up the protocol to 5 mmol of **1a** (corresponding to a theoretical yield of 1.02 g of D-**2a**), in a total volume of 200 mL. Indole concentration was increased to 25 mM and biocatalyst loadings were decreased to 40 μL mL^{-1} TrpS lysate and 25 mg mL^{-1} wet cells (full experimental details are provided in the Supporting Information, page S9). An overall yield of 66% D-**2a** was obtained, without significant differences in the work-up or isolation and without affecting the optical purity of the product (>99% ee).

Conclusion. In summary, we developed a novel one-pot fully biocatalytic procedure for the synthesis of a broad range of D-tryptophans from substituted indoles. The engineering of an aminotransferase displaying near-native catalytic efficiency towards D-tryptophan (DAAT-V33G/T242G) was key to improve the performance of this procedure, which compensates for the unavailability of a D-selective TrpS. Many of the substituted D-tryptophans that we synthesized in preparative scale using our one-pot procedure are useful synthetic building blocks for a broad range of target molecules such as mitragynine[23] and related 9-methoxy-substituted indole alkaloids[24] (from D-**2d**), prenylated tryptophans[25] (from D-**2f** and D-**2i**), inhibitors of breast cancer resistance protein[26] (from D-**2j**), and necrostatins[27] (from D-**2k**). The biocatalytic approach developed here allows for the efficient synthesis of useful chiral synthons for the preparation of complex natural products and pharmaceutical ingredients.

ASSOCIATED CONTENT

Supporting Information. Supporting figures and tables, experimental methods, product characterization data and copies of NMR and HRMS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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