Efficient Access to β-Branched Noncanonical Amino Acids via Transaminase-Catalyzed Dynamic Kinetic Resolutions Driven by a Lysine Amine Donor

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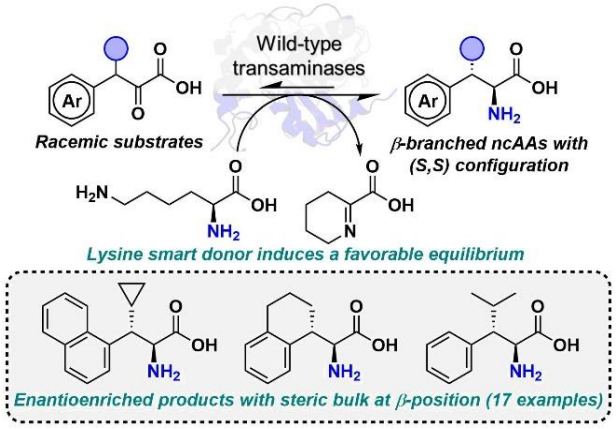
KEYWORDS: Biocatalysis, Transaminase, Smart donor, Dynamic kinetic resolution, Noncanonical amino acid

ABSTRACT: Transaminases are choice biocatalysts for the synthesis of chiral primary amines, including amino acids bearing contiguous stereocenters. In this study, we employ lysine as a "smart" amine donor in transaminase-catalyzed dynamic kinetic resolution reactions to access β-branched noncanonical arylalanines. Our mechanistic investigation demonstrates that, upon transamination, the lysine-derived ketone byproduct readily cyclizes to a six-membered imine, driving the equilibrium in the desired direction and thus alleviating the need to load superstoichiometric quantities of the amine donor or deploy a multi-enzyme cascade. Lysine also shows good overall compatibility with a panel of wild-type transaminases, a promising hint of its application as a smart donor more broadly. Indeed, with this discovery in hand, we furnished a broad scope of β-branched arylalanines, including some bearing hitherto intractable cyclopropyl and isopropyl substituents, with high yields and excellent selectivities.



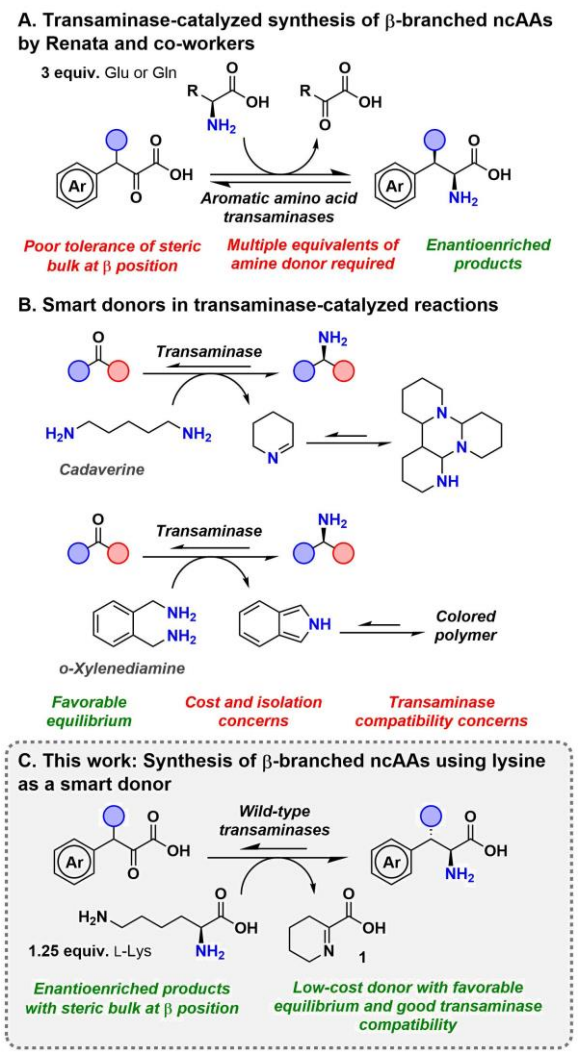
INTRODUCTION

Transaminases are pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze the reversible transfer of an amine from a donor substrate to a ketone or aldehyde via a ping-pong mechanism.[1,2] The synthetic value of transaminases is derived from their ability to furnish enantioenriched primary amines,[3–5] and this transmission of chiral information from catalyst to product has been extended to adjacent stereocenters via dynamic kinetic resolutions (DKRs, **Scheme 1A**).[6–10] Because transaminase-catalyzed reactions are reversible, several strategies to drive equilibria toward the product have been explored. One approach is to load a vast excess of amine donor, such as isopropylamine (IPAm), but this often requires protein engineering to bolster the enzyme's tolerance to the donor or its resultant ketone.[11,12] Another approach is to dynamically remove the donor byproduct by either evaporation or enzymatic degradation, but these strategies increase the operational complexity.[3] A third approach, and a promising alternative to the former two, is to deploy "smart" amine donors that, upon

transamination, undergo further thermodynamically favored cyclization reactions (**Scheme 1B**). Two prominent examples are cadaverine, whose byproduct undergoes cyclization and then trimerization,[13] and *ortho*-xylenediamine, which forms isoindoles that further polymerize.[14] While others have also been described,[15,16] each suffers from one or more of the following drawbacks: high cost, complications to product isolation, and low transaminase compatibility.[3] Consequently, the identification of a smart donor that bypasses these obstacles would be of great value.

In this study, we leverage lysine as a smart donor in transaminase-catalyzed DKR reactions to effect the synthesis of β-branched noncanonical amino acids (ncAAs, **Scheme 1C**), privileged building blocks that can imbue proteolytic stability and enhance binding interactions of bioactive peptides with a biological target.[17,18] Although lysine is the native substrate of several wild-type enzymes,[19,20] it has not, to our knowledge, been recognized nor leveraged as a smart donor in synthetic biocatalysis. Our mechanistic investigation suggests that spontaneous cyclization of the 6-amino-2-oxohexanoic acid byproduct to the corresponding imine **1** drives the equilibrium, enabling significantly reduced amine donor stoichiometry and thus a straightforward isolation procedure at scale. We accessed a suite of β-branched ncAAs in high yield, overcoming substrate limitations previously encountered,[10] with diastereo- and enantioselectivities that exceed the traditional chemical methods.[21–24] Furthermore, we show that lysine exhibits promising transaminase compatibility, rendering this inexpensive, readily available proteinogenic amino acid an exceedingly practical smart donor.

Scheme 1. Transaminases in chiral amine synthesis

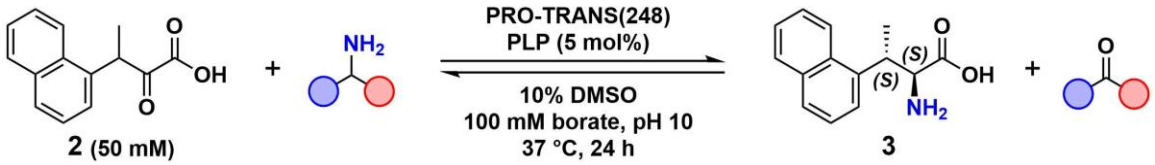


RESULTS AND DISCUSSION

Reaction Optimization by a Lysine Amine Donor. (2*S*,3*S*)-alkyl substituted arylalanines are critical β-branched ncAAs in ongoing peptide drug discovery efforts, but their chemical synthesis via cross-electrophile coupling requires

protecting groups and a challenging separation of β-stereoisomers.[25–27] For a sustainable and more efficient route to these compounds, we reasoned that a transaminase could generate the desired ncAA in high enantioselectivity from the corresponding keto acid and an appropriate amine donor. Furthermore, if the keto acid substrate's β-stereocenter racemizes under sufficiently alkaline conditions, we could leverage high enzyme substrate specificity in a DKR from a readily prepared racemate. For our initial studies, we chose the transformation of 3-(naphthalen-1-yl)-2-oxobutanoic acid (**2**) to (2*S*,3*S*)-2-amino-3-(naphthalen-1-yl)butanoic acid (**3**).

Table 1. Selected optimization parameters of the L-Lys-promoted synthesis of **3**.



Entry	Donor (equiv.)	PRO-TRANS(248) (wt%)	Conversion (%)
1	L-Ala (4.0)	50	32
2	L-Ala (4.0)	300	27
3	L-Ala (10)	50	42
4	L-Ala (50)	50	48
5	L-Lys (1.3)	50	81
6	L-Lys (4.0)	50	99
7[a]	L-Lys (1.5)	75	99
8[b]	L-Lys (3.5)	32	98 (100 g scale)

[a]Reaction proceeded for 48 h. [b]Reaction proceeded for 66 h.

In an initial screen of commercial wild-type transaminases using L-alanine (L-Ala) as an amine donor,[28] PRO-TRANS(248) provided **3** in >99% enantioselectivity, albeit with low conversion (**Table 1**, entry 1). Neither increasing enzyme loading (entry 2) nor L-Ala stoichiometry (entries 3 and 4) resulted in conversions greater than 50%, suggesting that the amine transfer equilibrium favors L-Ala and ketoacid **2** (K_{eq} ~0.046, *vide infra*). Encouraged by the promiscuity of PRO-TRANS(248) toward other amino acid donors, we hypothesized that lysine could induce a more favorable equilibrium via spontaneous cyclization of the 6-amino-2-oxohexanoic acid byproduct. Indeed, use of only 1.3 and 4.0 equiv. L-lysine (L-Lys) achieved 81% and 99% conversion, respectively, over 24 h (entries 5 and 6). Increasing enzyme loading to 75 wt% and increasing reaction time by an additional 24 h led to full conversion (entry 7). The preparative value of this reaction was demonstrated on 100 g scale using 32 wt% PRO-TRANS(248) to afford **3** as a single isomer in 80% isolated yield after a simple precipitation from the reaction mixture (entry 8, see *Supporting Information* for details).

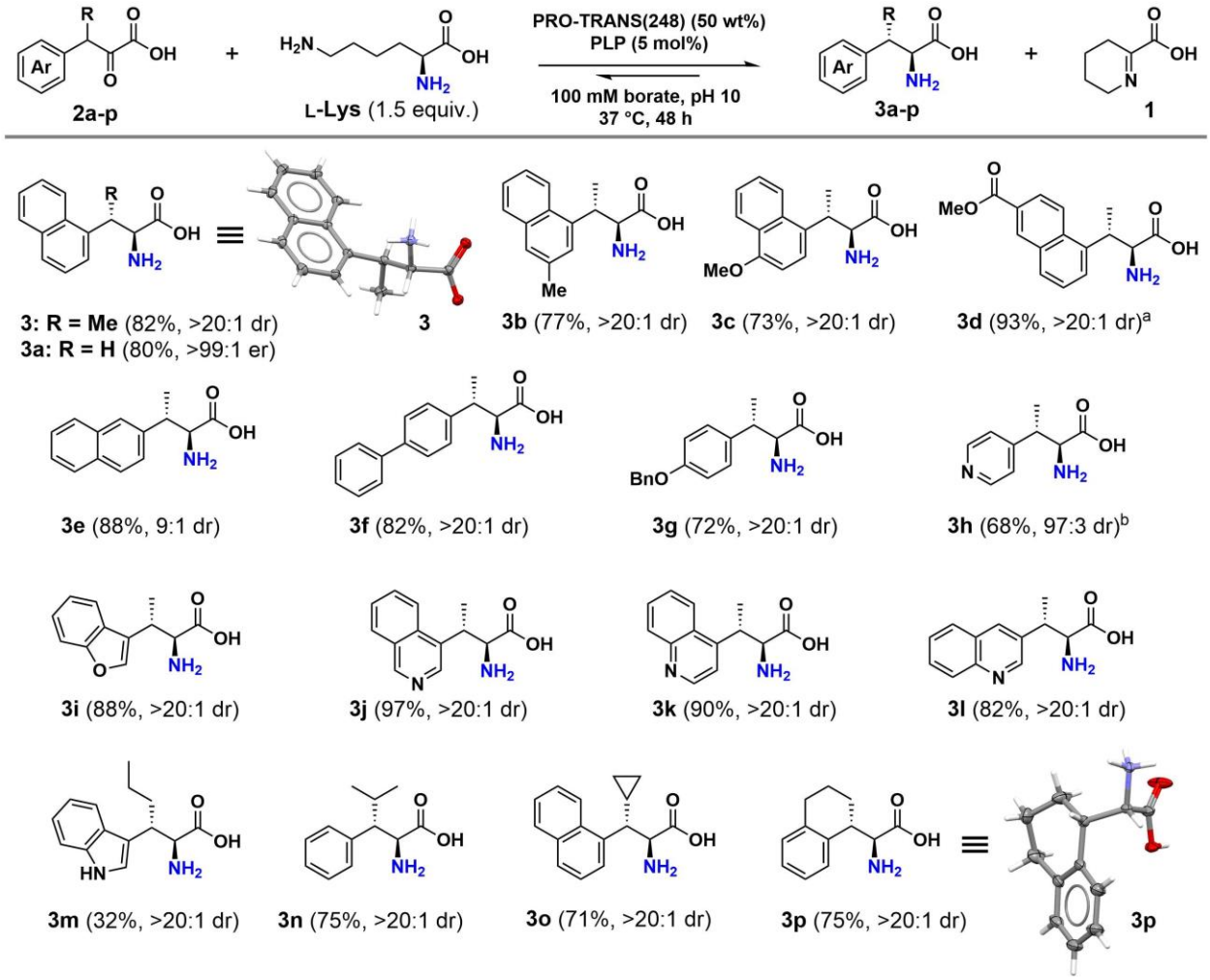


Figure 1. Substrate scope of PRO-TRANS(248). Reactions were performed at 0.5-2.0 mmol scale at 20 g/L substrate loading with 1.5 equiv. L-Lys and 50 wt% Pro-TRANS(248) in 100 mM borate buffer at 37 °C for 48 h. In parentheses: yields refer to the isolated product after purification by reverse phase column chromatography (see *Supporting Information* for details). The single crystal of **3** was grown as a dihydrate; water was omitted from ORTEP for clarity. The single crystal of **3p** was grown as an HCl salt; the chloride was omitted from ORTEP for clarity. [a]Reaction was conducted at pH 8.2 to avoid ester hydrolysis. [b]Product was isolated as the Fmoc derivative for purification purposes.

Substrate Scope. With optimized conditions identified for the DKR of **2** to **3** (82% AY, >20:1 dr), we evaluated the α-ketoacid substrate scope of PRO-TRANS(248) to provide access to β-naphthyl alanine (**3a**) and a variety of β-branched ncAAs on 0.5-2.0 mmol scale (**Figure 1**). The enzyme is tolerant of substitution on the β-naphthyl ring (**3b – 3d),** affording (*S,S*)-ncAAs with >20:1 dr and moderate to excellent yields (see *Supporting Information* for selectivity analysis); moving the ncAA branch point to another naphthyl position (2-naphthyl, **3e**) led to a measurably lower dr (9:1), despite good yield. High diastereoselectivities (>20:1) were restored for substrates with substituted β-phenyl rings (**3f, 3g**), and β-heteroaryl groups (**3h-3l**). Impressively, PRO-TRANS(248) is tolerant of larger β-alkyl functionality, including n-propyl (**3m**), isopropyl (**3n**),

cyclopropyl (**3o**), and a propylene bridge (**3p**) to the β-aryl substituent, providing complex (*S,S*)-ncAAs with excellent diastereoselectivities (>20:1). The stereochemical assignment was confirmed using single crystal X-ray diffraction of **3** as well as the tetrahydronapthyl-substituted glycine analog **3p**.

Effects of Amine Donors on Reaction Equilibria. To support our hypothesis that transamination with L-Lys is driven by byproduct cyclization, we compared the thermodynamics of amine transfer to **3** from other commonly deployed amine donors by means of the equilibrium constant (K_{eq}), which directly reflects the ratio of products to substrates of a closed system that has reached a steady state.[29] We calculated the apparent Keq (K_{eq}^{app}) of individual PRO-TRANS(248) reaction mixtures with **2** and a collection of amine donors by measuring the [**3**] at equilibrium (see *Supporting Information* for details), which we determined to be established through regular sampling (**Figure 2**). In the case of lysine, spontaneous byproduct cyclization should favor the formation of **3** compared to those donors that lack such a capacity. Indeed, the K_{eq}^{app} calculated from reactions with L-Lys (5.9) is more than fourteenfold greater than those with other commonly deployed proteinogenic amino acids, such as L-Ala (0.046), L-glutamic acid (L-Glu, 0.060), L-glutamine (L-Gln, 0.42), L-aspartic acid (L-Asp, 0.0080),

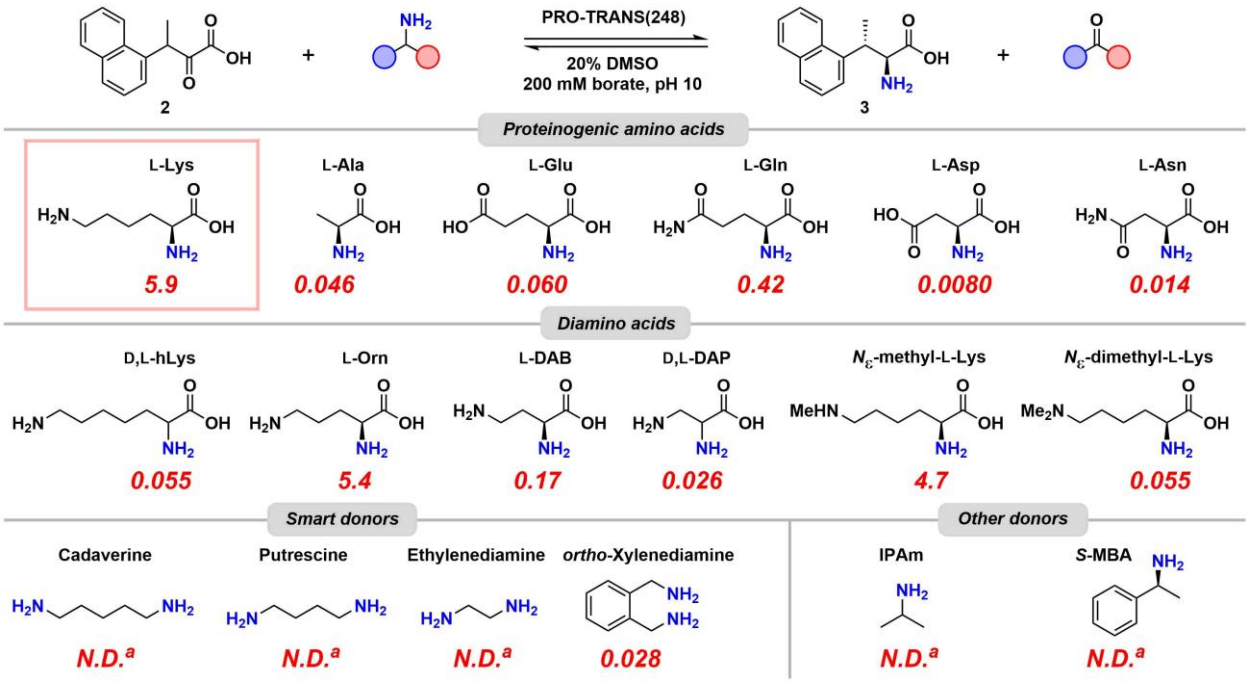


Figure 2. Effects of amine donors on PRO-TRANS(248) reaction equilibria at 30 °C. The reaction was prepared to the following conditions: 33 mM **2**, 40 mM amine donor, 24 g/L PRO-TRANS(248) fermentation powder, and 12 mol% PLP in 20 vol% DMSO and 80 vol% 200 mM borate, pH 10. The values in red represent the K_{eq}^{app} as determined by the method described in the *Supporting Information*. [a]Value was not determined (N.D.) because the reaction did not proceed, likely because the amine donor was incompatible with the enzyme.

and L-asparagine (L-Asn, 0.014). Notably, L-Gln, which has likewise been leveraged as a smart donor because of its resulting ketone's propensity to cyclize,[30] also elicits an elevated K_{eq}^{app}, lending further credence to the proposed mechanism.

Next, we compared L-Lys to other diamino acids whose varied side-chain lengths would govern the ring size of their purported imine byproducts, and by extension the relative distribution of their byproducts' linear and cyclized forms (**Figure 2**, *Diamino acids*). If imine formation is truly what drives the change in reaction equilibria, as we hypothesize, then we would expect those diamino acids that engender 6- and 5-membered cyclic imines to elicit superior K_{eq}^{app} values than those that promote less-favored configurations.[31] Indeed, the K_{eq}^{app} calculated from reactions with L-Lys (5.9) and L-ornithine (L-Orn, 5.4) are more than thirtyfold greater than those with D,L-homolysine (D,L-hLys, 0.055), L-2,4-diaminobutyric acid (L-DAB, 0.17), and D,L-2,3-diamopropionic acid (D,L-DAP, 0.026). By similar reasoning, we also probed the effects of ε-amine methylation using N_{ε}-methyl-L-Lys and N_{ε}-dimethyl-L-Lys as donors. Interestingly, the singly methylated N_{ε}-methyl-L-Lys (4.7) exhibits only a modest decrease in K_{eq}^{app} compared to L-Lys (5.9), which could be driven by the formation of either an enamine or hemiaminal. With N_{ε}-dimethyl-L-Lys (0.055), however, the K_{eq}^{app} drops to a value commensurate with L-Glu (0.060), as the ε-amine is now restricted from participating in any subsequent cyclization reactions, congruent with our hypothesis.

Finally, we explored the effects of other commonly deployed amine donors on our model reaction (**Figure 2**, *Smart donors* and *Other donors*). Of cadaverine, putrescine, ethylenediamine, and *ortho*-xylenediamine (0.028), which have all been leveraged as smart donors,[13,14] only the latter elicited a reaction, but it proved much less effective than L-Lys (5.9). In the former cases, it is likely that sluggish kinetics stemming from incompatibility with PRO-TRANS(248) prevented the thermodynamic equilibria from being achieved, thus rendering a head-to-head comparison with L-Lys unfeasible. Likewise, IPAm and *S*-methylbenzylamine (*S*-MBA) also appeared to be incompatible.

Differentiating the Transferred Amine. To establish whether PRO-TRANS(248) transfers the α- or ε-amine of the donor, we prepared reactions with $^{15}N_{\alpha}$-L-Lys and tracked the isotopic label by UPLC-MS (**Figure S2**). Indeed, a +1 shift in the *m/z* value of **3** compared to that produced in reactions with unlabeled L-Lys was observed, confirming the transfer of the α-amine in preference to the ε-amine.

Reaction Progress Curves Monitored by NMR Spectroscopy. To gain a deeper understanding of the reaction mechanism and speciation, we conducted a study on the amination of ^{13}C-**2** using ^{13}C NMR spectroscopy. In a reaction that proceeds to 75% conversion after 50 h (**Figure 3**), we observed excellent mass balance in the conversion of ^{13}C-**2** to ^{13}C-**3** (purple line). Importantly, the major ^{13}C-L-Lys-derived species (red) contained a nitrogen with an ^{15}N chemical shift of 293.9 ppm, which is characteristic of an imine species. This detection was achieved using a ^{1}H–^{15}N HMBC experiment at natural abundance, and correlations from the methylene ^{1}H resonances at 2.26 ppm and 2.19 ppm to the imine nitrogen were

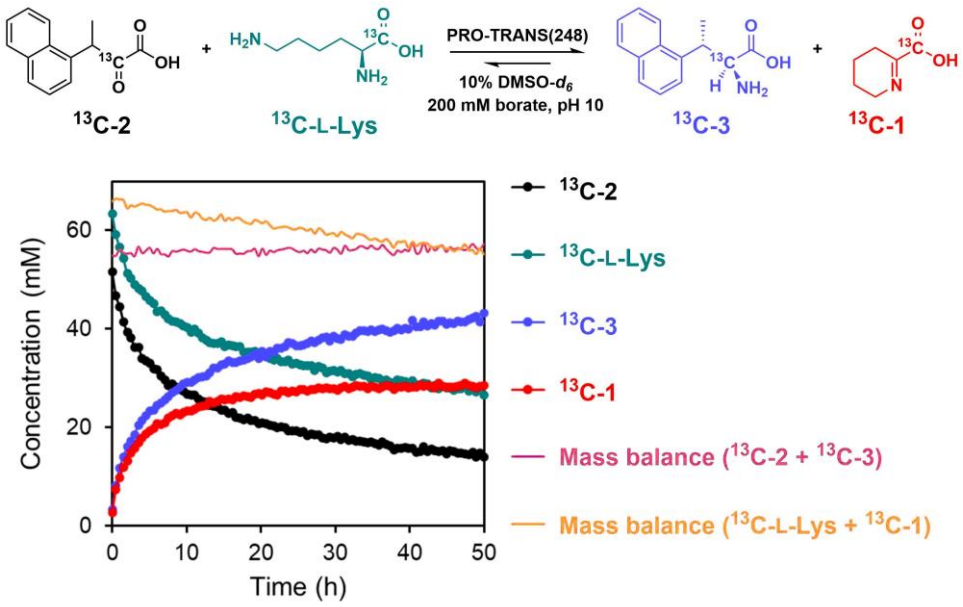


Figure 3. Reaction progress curves of the PRO-TRANS(248)-catalyzed amination of ^{13}C-**2** tracking ^{13}C-**2** (black), ^{13}C-L-Lys (teal), ^{13}C-**3** (blue), and ^{13}C-**1** (red) by ^{13}C NMR at 25 °C. The purple and orange lines depict the mass balances of ^{13}C-**2** + ^{13}C-**3** and ^{13}C-L-Lys + ^{13}C-**1**, respectively. The reaction was prepared to the following conditions: 55 mM ^{13}C-**2**, 66 mM ^{13}C-L-Lys, 50 wt% PRO-TRANS(248) fermentation powder, and 6 mol% PLP in 10 vol% DMSO-d_6 and 90 vol% 200 mM borate, pH 10.

observed (see *Supporting Information* for details), consistent with the formation of the 3,4,5,6-tetrahydropyridine isomer ^{13}C-**1**.[32] This cyclic byproduct results from the spontaneous cyclization of its purported linear counterpart, 6-amino-2-oxohexanoic acid, which, according to our mechanistic hypothesis, plays a significant role in driving the equilibrium toward the formation of **3**. Other unidentified species are also observed during the reaction that contribute to the slight decrease in mass balance between ^{13}C-**1** and ^{13}C-L-Lys (orange line, see *Supporting Information* for details).

Influence of Amine Donor Loadings on Reaction Kinetics. To enable reaction optimization, we examined the effects of L-Lys, L-Orn, and L-Ala loadings on the rate of **3** formation in PRO-TRANS(248)-catalyzed reactions (**Figure 4**, see *Supporting Information* for details). Applying the Michaelis-Menten equation to the experimental data, we calculated the corresponding Michaelis constants (K_M), or the concentration of each donor required to achieve one half of the initial rate (V_0) at which the

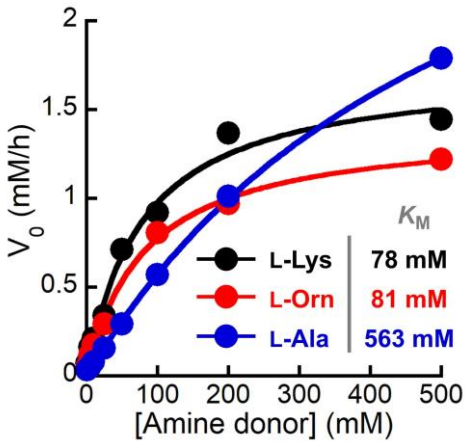


Figure 4. Michaelis-Menten plot showing the change initial rate (V_0) as a function of L-Lys (black), L-Orn (red), and L-Ala (blue) concentrations. V_0 and K_M values were extracted from the nonlinear regression analyses described in the *Supporting information* section.

enzyme is saturated.[33] Interestingly, both L-Lys and L-Orn elicit a lower K_M than L-Ala (78 mM, 81 mM, and 563 mM, respectively), suggesting that the former two are better suited for amine transfer by PRO-TRANS(248) under practical reaction conditions, although L-Ala does indeed elicit faster initial reaction rates at concentrations exceeding ~200–300 mM. Mindful of the fact that K_M is dependent on both the substrate and the enzyme, and thus subject to change in the context of a different

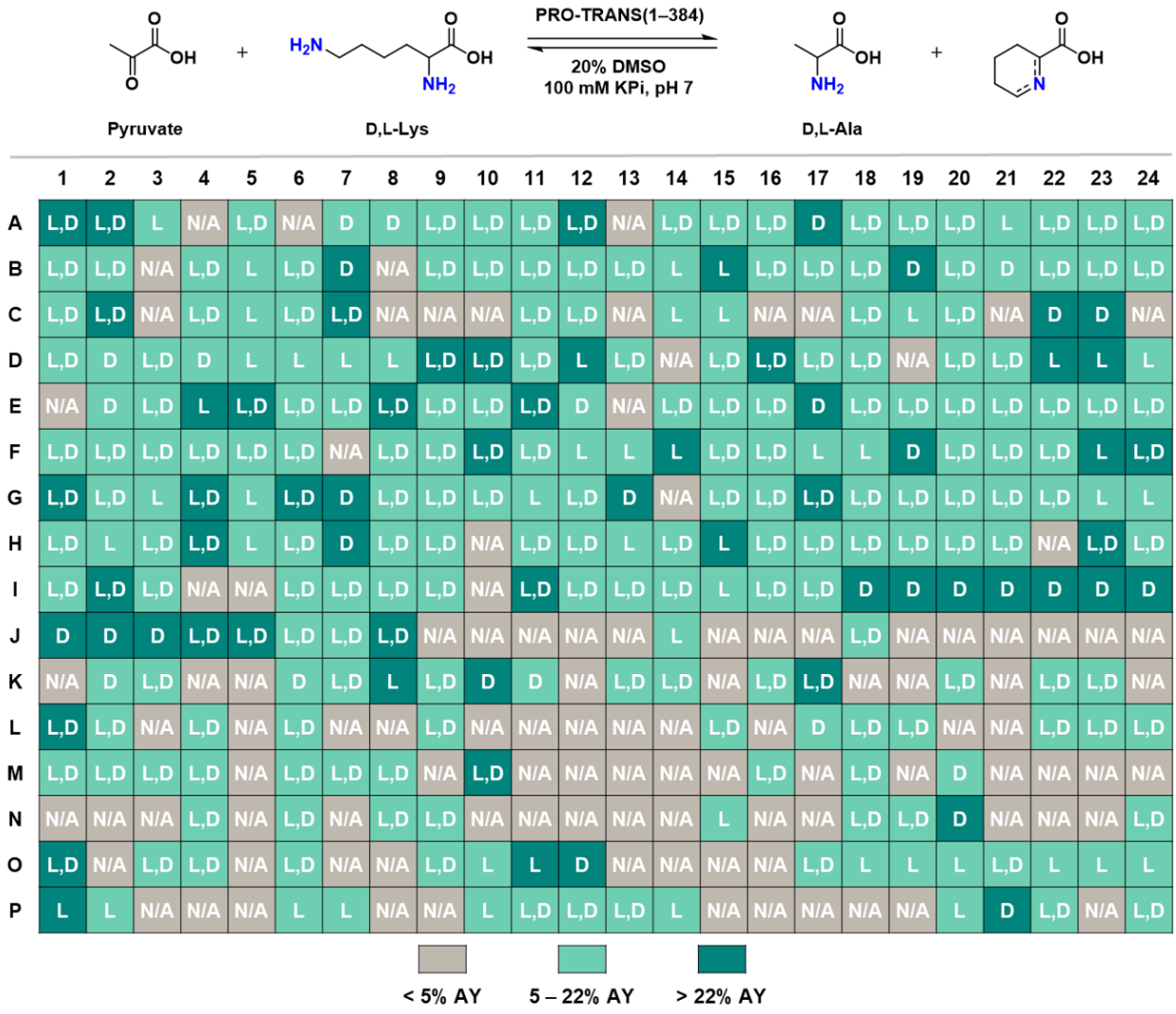


Figure 5. Lysine compatibility with PRO-TRANS(1–384) at 30 °C. Reactions were prepared in individual wells to the following concentrations: 20 mM sodium pyruvate, 100 mM L- or D-lysine, 20 g/L PRO-TRANS fermentation powder, and 20 mol% PLP in 20 vol% DMSO and 80 vol% 100 mM potassium phosphate, pH 7.0. Gray wells indicate an assay yield (AY) of alanine of <5%, light teal wells an AY of 5–22%, and dark teal wells an AY of >22%. L and D labels signify a preference for L-Lys or D-Lys, respectively, as defined by a 2 or more fold difference in activity for one enantiomer over the other after 24 h reaction time. L,D labels indicate that both lysine enantiomers were accepted as substrates, as defined by differences in activity for each enantiomer of less than 2 fold after 24 h reaction time. N/A labels were ascribed to those wells whose overall activity was <5%.

transaminase, we anticipate that this analysis will prove nonetheless insightful when contemplating initial reaction conditions for future endeavors involving these smart donors.

Lysine Compatibility with Wild-Type Transaminases. To assess whether lysine is accepted as a substrate by transaminases more broadly, we subjected a 384-member panel of wild-type transaminases to reactions with L- and D-Lys separately, monitored the conversion of pyruvate to alanine, and compiled our findings into a single heat map depicting the assay yield (AY) of alanine in each enzymatic reaction as well as each enzyme's preference (or lack thereof) for a particular lysine enantiomer (**Figure 5**, **Figures S6**–**S8**). We chose pyruvate as the amine acceptor because of its generally broad transaminase compatibility,[28] as this would then minimize the number of low-yielding reactions that would otherwise result from incompatibility with the acceptor rather than with lysine. We categorized the results as follows: >22% AY (dark teal)—the activity of PRO-TRANS(248) under these unoptimized conditions—indicates good compatibility with lysine; 5–22% AY (light teal) suggests promising activity that may require rigorous condition optimization or protein engineering to achieve high yields; and <5% AY (gray) suggests incompatibility, at least in the context of a practical kinetic regime. By these criteria, 72% of the transaminases examined showed at least promising activity, indicating that lysine may indeed be well tolerated as an amine donor by transaminases more broadly.

CONCLUSIONS

Lysine is an inexpensive, abundantly available smart donor in transaminase-catalyzed reactions that can be leveraged for the synthesis of chiral amines using wild-type enzymes. We have shown its utility across an array of transaminases and demonstrated mechanistic evidence for its impact on the reaction equilibrium. Using lysine as an amine donor, we prepared previously inaccessible ncAAs, including derivatives of phenylalanine, tyrosine, and tryptophan, with generally high yields and excellent selectivities. Furthermore, we demonstrated the scalability and practicality of this method by conducting the reaction on large scale to give stereochemically pure product under mild, environmentally friendly conditions.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, characterization of new compounds, X-ray crystal structure data, NMR spectra, and UPLC/HPLC chromatograms.

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Funding Sources

This work was funded by Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Omer Ad for assistance with HRMS experiments, and Dr. Ania Fryszkowska, Dr. Danielle Schultz, Dr. Ian Mangion, and Dr. Rebecca Ruck for editing the manuscript.

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