Biocatalytic synthesis of non-standard amino acids by a decarboxylative aldol reaction

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Enzymes are renowned for their catalytic efficiency and selectivity. Despite the wealth of carbon–carbon bond-forming transformations in traditional organic chemistry and nature, relatively few C–C bond-forming enzymes have found their way into the biocatalysis toolbox. Here we show that the enzyme UstD performs a highly selective decarboxylative aldol addition with diverse aldehyde substrates to make non-standard γ-hydroxy amino acids. We increased the activity of UstD through three rounds of classic directed evolution and an additional round of computationally guided engineering. The enzyme that emerged, UstD2.0, is efficient in a whole-cell biocatalysis format. The products are highly desirable, functionally rich bioactive γ-hydroxy amino acids that we demonstrate can be prepared stereoselectively on the gram scale. The X-ray crystal structure of UstD2.0 at 2.25 Å reveals the active site and provides a foundation for probing the UstD mechanism.

Major advances have been made in the practical use of enzymes for enantioselective functional group manipulations. For example, the asymmetric reduction of ketones and enantiospecific hydrolysis of racemic esters are now routine in process chemistry. Also, impressive strides have been made in enzymatic C–H activation. However, the development of enzymes to form C–C bonds on a preparative scale lags behind that of traditional synthetic organic methodology. Although nature is ripe with C–C bond-forming enzymes, these catalysts often have substantial limitations, such as a limited substrate scope or poor heterologous expression. Engineering can overcome these challenges, but a more severe limitation is thermodynamic in nature: reactions that form carbon nucleophiles via C–H deprotonation, such as classic aldol transformations, are typically irreversible. In nature, metabolic flux drives reactions and preserves the stereochemical purity of the products. Laboratory approaches mimic nature by coupling reversible biocatalytic C–C bond-forming reactions to a thermodynamic sink, such as a subsequent transformation or selective crystallization.

Although these advances are substantial, the potential of biocatalytic enzymes in assembling carbon chains is still hindered by the simple lack of high-quality exergonic transformations. Hence, development of scalable and thermodynamically favourable C–C bond-forming reactions may open diverse avenues of biocatalytic synthesis.

To fill this gap, we were drawn to a recently described pyridoxal 5′-phosphate (PLP)-dependent enzyme involved in the biosynthesis of Ustiloxin B, an inhibitor of microtubulin polymerization (Fig. 1a)19. This enzyme, UstD, decarboxylates the side chain of L-aspartate (1) to form a putative nucleophilic enamine intermediate (Fig. 1b). This enamine then attacks an aliphatic aldehyde appended to a cyclic tetrapeptide, which results in the formation of a γ-hydroxy amino acid side chain. The loss of CO2 renders this enantioselective C–C bond-forming reaction effectively irreversible. This decarboxylative aldol addition mechanism is distinct from the classic aldolases, transketolases and PLP-dependent Thr aldolases, which catalyse the tautomerization of an imine to form an enamine nucleophile14,15. It has been shown that the transketolase catalytic cycle can be non-natively entered through decarboxylation, and that the reactions initially proceed to a high conversion. However, the native proton transfer machinery eventually breaks down the product into an equilibrium mixture with starting materials10. Although the detailed mechanism of this UstD addition has not yet been explored, Ye et al. reported that the UstD reaction cannot be initiated from L-Ala, which indicates that enamine formation through tautomerization is not viable. Therefore, UstD is mechanistically distinct from classic aldolases and may have unique properties as a biocatalyst.

The native substrate for UstD is a complex, cyclic peptide, and it was not known if this enzyme would react promiscuously with alternative substrates. If so, the enzyme would directly produce γ-hydroxy amino acids (Fig. 1b). Such non-standard amino acids (nsAAs) are found in bioactive natural products, such as caspofungin and clavamid (Fig. 1a)21. Although nature employs side-chain hydroxylation to tune bioactivity, these nsAAs are virtually absent from medicinal chemistry because they require multistep synthesis. The need for multistep synthesis to prepare these nsAAs has begun to be addressed by biocatalysis, in which an elegant multi-enzyme cascade was recently developed by Clapés and co-workers to access γ-hydroxy nsAAs22. However, the ability to use a single enzyme to produce the same motif offers a greater practical utility and versatility. Beyond their use in pharmaceuticals, nsAAs can be enabling for a host of synthetic and chemical biology applications. Therefore, the development of UstD for organic synthesis would introduce a valuable and much-needed enantioselective C–C bond-forming enzyme into the biocatalytic toolbox and provide direct access to a structurally complex synthon.

Here we show that the enzyme UstD performs a highly selective decarboxylative aldol addition with diverse aldehyde substrates to make non-standard γ-hydroxy amino acids. We increased the activity of UstD through three rounds of classic directed evolution and an additional round of computationally guided engineering. The enzyme that emerged, UstD2.0, is efficient in a whole-cell biocatalysis format, which circumvents the need for enzyme purification, and thereby facilitates its use in traditional organic settings on a gram scale. The X-ray crystal structure of UstD2.0 at 2.25 Å reveals the active site and the molecular basis for the promiscuity of this catalyst.

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**Results**

**Initial characterization of UstD.** We expressed C-His-UstD (wild-type (wt)-UstD) in *Escherichia coli* (Supplementary Fig. 1), but were uncertain whether molecular recognition for the structurally complex native substrate would be required for catalytic activity. We therefore assessed the reactivity of wt-UstD with benzaldehyde 2a and were pleased to observe a successful decarboxylative aldol addition to afford the ω-hydroxy nsAA 3a by ultra-high pressure liquid chromatography–mass spectrometry (UPLC–MS) (Supplementary Fig. 2). A preparative scale reaction with 0.125 mol% catalyst gave the product in 43% yield, and analysis by NMR spectroscopy indicated a single diastereomer predominated (d.r. >98:2). To determine the absolute stereocchemical preference for the enzyme, we analysed the product from a reaction with 4-bromobenzaldehyde (2b). The crystal structure of the product (3b) revealed that the aldol addition occurred with the same stereocchemical outcome as that of the native reaction (Supplementary Fig. 2). These transformations indicated that wt-UstD has the potential for organic synthesis, but the comparatively modest activity (<1,000 turnovers with the initial reaction conditions) and low catalyst expression would hinder routine use of the natural enzyme. Given the inherent structural differences between the native tetrapeptide substrate and simpler commercially available aldehydes (such as 2a), we hypothesized that directed evolution and reaction-condition optimization could be used to increase the catalytic efficiency of UstD towards non-native substrates.

**Directed evolution of UstD for improved catalytic activity.** To inform our engineering process, we used a homology model of wt-UstD derived from a distantly related cysteine desulfurase (27% identity)\(^2\). Six residues in the predicted active site were chosen for saturation mutagenesis, and we used benzaldehyde (2a) as a model substrate for the directed evolution (Fig. 2a). Mutation at positions predicted to form direct contacts with the cofactor resulted in inactivation of the catalyst, a common trend among PLP-dependent enzymes\(^3\). Nevertheless, these libraries yielded a single variant in a putative loop region that flanked the substrate binding site, C392L, with a 2.3-fold boost in activity (Fig. 2b). Concurrently, we employed global random mutagenesis on wt-UstD to search throughout the protein sequence for activating mutations. A second activating mutation was discovered, L393M, immediately adjacent to Cys392. We combined these mutations to yield the double variant UstD TLM, which had a further increase in activity to 4.9-fold above the wild type (Supplementary Fig. 3). It is common for the mutation of neighboring residues to display cooperativity\(^26,27\), and we chose to test additional mutations in this region of the sequence (Fig. 2b). We used a degenerate codon mutagenesis strategy on four contiguous residues from Ile391 to Ala394. We restricted the sequence space to residues commonly found among UstD homologues, which provided a good structural diversity in a focused set of mutations (see Supplementary Information for the details). Screening this library revealed that mutation of Ala394 was generally deleterious. However, multiple highly active variants retained Ala394 and contained mutations at Ile391, Cys392 and Leu393. To best capture the relative rate effects of mutations, catalysts were compared under dilute conditions. Variants UstD\(^{TLM}\) and UstD\(^{FVF}\) (the superscript refers to the identity of the residues at positions 391–393) had a 5.1-fold and 4.1-fold increase in activity relative to wt-UstD, respectively.

We next optimized the reaction conditions for the most active variant, UstD\(^{TLM}\). Reaction mixtures were initially coloured yellow (Supplementary Fig. 1) by the presence of PLP that co-purified with the enzyme, but became colourless over time, which suggests the cofactor degraded during the reaction. Gratifyingly,
supplementation of PLP led to a large increase in product formation (Supplementary Fig. 4). We did not observe a notable change when the concentration of 1 was increased (Supplementary Fig. 4). However, we observed the formation of L-Ala in the reactions, which indicates some 1 was lost to a non-productive protonation of the nucleophilic enamine intermediate\textsuperscript{11}. We therefore used aldehyde as the limiting reagent and 2 equiv. 1 for subsequent experiments, which identified an optimal initial pH of 7.0 (Supplementary Fig. 2). For reactions with Marfrey’s reagent to provide a uniform chromophore for the quantitative measurement of turnover and selectivity via UPLC–MS\textsuperscript{30}. Product formation was observed with virtually every substrate tested from the large and hydrophobic biphenyl aldehyde (2g) to the small and hydrophilic glycolaldehyde (2p) (Fig. 2c). Generally, the variant UstDTLM performed the most turnovers and displayed an excellent diastereoselectivity, typically forming a d.r. of 95:5. Although UstD\textsuperscript{FW} usually performed fewer turnovers than UstDTLM with most substrates, UstD\textsuperscript{FW} generally had a higher selectivity than wt-UstD or UstDTLM (Supplementary Table 1). Reactions with p-substituted aromatic aldehydes exhibited a Hammett-like reactivity trend: more product was formed as the aldehyde electrophilicity increased. Activity was lowest with the electron rich p-anisaldehyde (2c), but a high activity was observed for the electron deficient p-NO\textsubscript{2}-benzaldehyde (2d) with both engineered enzymes. To better capture the maximum turnover number (TON) with 2d, we repeated the reactions at lower catalyst loadings, which revealed that the engineered variants can perform ~34,000 turnovers (Supplementary Fig. 6). Active-site mutagenesis had little apparent impact on reactions with some highly hydrophobic substrates, such as 3,4-dichlorobenzyl (2f) and 4,5-dichlorophenyl (2k) aldehydes; reactivity in these cases may be limited by poor aqueous solubility (Supplementary Fig. 2c). In contrast, the reactivity of o-toluic acid (2h) and thiophene-3-carboxaldehyde (2i) increased dramatically during evolution. UstDTLM displayed a ninefold increase in activity on 2i and a remarkable 23-fold increase in turnovers with 2h compared with those of wt-UstD. Activity with the imidazole substrate
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Clavalanine (Fig. 1b)\(^\text{17}\), an antibiotic that inhibits the biosynthesis of was identified as a key intermediate in the synthesis of to yield the dihydroxylated amino acid. Previously, a protected 3p, the probability of crystallization\(^\text{35}\). However, we found that the Cys residues can increase the soluble expression and increase known among protein crystallographers that removing surface details relied on purified protein for preparative-scale reactions. Linear regression guided protein engineering. The above studies relied on purified protein for preparative-scale reactions. However, access to enzymes in sufficient quantity is a common and often underappreciated limitation of biocatalysis. As is observed for many proteins, UstD had a relatively low expression titres in E. coli (8 mg l\(^{-1}\) culture) due to poor solubility (Supplementary Fig. 1). Although enzyme immobilization can be used to increase the utility of purified protein catalysts\(^\text{34}\), a complementary synthetic methodology would use whole-cell preparations of UstD; this latter approach is attractive to process chemists\(^\text{41}\). Whole-cell catalysts are operationally simple to generate, stable over long periods and obviate the need for expensive protein purification.

We sought to further engineer UstDTLM to increase the soluble heterologous expression in E. coli for whole-cell biocatalysis. This enzyme contains nine Cys residues, and our homology model suggested five are surface exposed (Supplementary Fig. 8). It is well known among protein crystallographers that removing surface Cys residues can increase the soluble expression and increase the probability of crystallization\(^\text{49}\). However, we found that the mutation of all five putative surface Cys residues to Ala eliminated catalytic activity. To identify mutations that would retain the activity while increasing the soluble expression, we performed sequence-similarity network analysis to identify non-Cys residues at these positions common among UstD homologues. Based on this analysis, we constructed a five-site degenerate codon library (Fig. 3a and Supplementary Fig. 8).

To efficiently navigate this sequence space, we employed linear regression modelling to predict sequence–activity relationships\(^\text{46}\). We hypothesized that this simple computational approach would be effective because the target residues are dispersed throughout the protein, which should make non-linear, pairwise mutational effects unlikely. We screened and sequenced 176 random clones from this library for increased activity in lysate, which is sensitive to changes in both soluble enzyme expression and enzymatic efficiency. Although most variants in this library were inactive, we were heartened to observe several apparently improved variants (Fig. 3a). Linear regression model testing using leave-one-out cross-validation of the full dataset indicated a poor predictive behaviour of the model for high-activity variants (Supplementary Fig. 9). We suspected that the model quality was diminished by the abundance of inactive variants, for which activity measurements are indistinguishable from experimental noise. We therefore restricted our analysis to variants for which bona fide activity could be measured, which left just 26 sequence–activity relationships. Despite the sparsity of these data (~5% of the sequence space), leave-one-out cross-validation showed the model was dramatically improved (see Supplementary Information for details).

We evaluated the three most active variants predicted by the model, UstD\(^{TLM-ACASC}\), UstD\(^{TLM-ASCSC}\) and UstD\(^{TLM-ASASC}\). Comparisons of expression and whole-cell activity were made between these variants, the parent enzyme and the most active variant identified from screening, UstD\(^{TLM-ACASC}\). We were delighted to find the expression titre increased relative to that for UstD\(^{TLM}\) for all the variants, up to 48 mg protein l\(^{-1}\) culture (Supplementary Fig. 10). Although purified enzyme activity is slightly decreased for the new variants, their overall activity in whole cells is substantially improved (Fig. 3a and Supplementary Fig. 10). Tests at the analytical scale showed, at a 0.25% w/v cell loading, that UstD\(^{TLM}\) formed 3a with just a 13% yield, which highlights the challenges associated with translating in vitro activity into large-scale reaction formats. In contrast, the variant with the highest whole-cell activity, the computationally predicted UstD\(^{TLM-ACASC}\) (designated UstD\(^{TLM-29}\)), produced 3a in a 31% yield, a 2.4-fold boost over that of UstD\(^{TLM}\) and a cumulative 15-fold boost over the wild type. Higher conversions were achieved by increasing the cell loading of UstD\(^{TLM}\) to 1% w/v, which afforded 3a in 78% yield on an analytical scale (Fig. 3a). To demonstrate the utility of UstD\(^{TLM}\), large-scale reactions were carried out with 2a and 2d. The reaction with 2a at a 0.5% w/v catalyst loading afforded 0.80 g of 3a in a 77% isolated yield with pristine stereoselectivity after purification by reverse-phase chromatography. The reaction with 2d at just a 0.1% w/v catalyst loading provided 1.4 g of 3d in a 98% isolated yield with a high stereoselectivity (see Supplementary Information for details). Notably, these cell loadings are sufficient for process-scale biocatalytic reactions\(^\text{49}\), which illustrates that UstD\(^{TLM}\) can operate on the scale needed to meet the demands of practical organic synthesis.

Crystallography of UstD\(^{TLM}\). Although the engineering we report here produced a generalist variant of UstD, structural information could guide more targeted engineering for the production of specific y-hydroxy nsAAs. Despite extensive efforts, we were unable to produce crystals of wt-UstD. In contrast, UstD\(^{TLM}\) readily crystallized, which we attribute to the decrease in surface Cys residues. The 2.25 Å crystal structure of UstD\(^{TLM}\) was determined using experimental phases from a Au(III) derivative (Fig. 3b, Protein Data Bank ID
This structure revealed an active site at the dimer interface, which is common among fold-type I PLP-dependent enzymes. The internal aldimine that involves a Schiff base linkage to Lys258 and a salt bridge between the pyridinium N1 and Asp232 is clearly resolved in the active site. The 391–393 loop, which harbours the activating TLM mutations, projects over the top of the active site that forms part of the substrate binding pocket. The remainder of the pocket appears to be solvent exposed, which explains the tolerance of UstD for diverse aldehyde substrates (Supplementary Fig. 11).

In the future, we envision engineering UstD for increased activity with non-aldehyde substrates. As an initial demonstration, we showed that purified UstDv2.0 performs ~50 turnovers with the ketone substrate trifluoroacetone to produce a nsAA that bears a tertiary alcohol side chain (Supplementary Fig. 12). The comparatively low turnover highlights the challenges associated with aldol addition into ketones. When nucleophilic attack is sufficiently slow, irreversible protonation of the enamine can quench the reactive intermediate and, indeed, we observed substantial accumulation of l-Ala in this reaction. A similar scenario was observed with the hydrodysls of an electrophilic PLP intermediate formed by TrpB and the reactions with attenuated substrates were enabled by directed evolution that increased the lifetime of the reactive intermediate.

Hence, future engineering to decrease the rate of enamine protonation in UstDv2.0 may further expand the substrate scope.

**Discussion**

Here we improved a C–C bond-forming enzyme, UstD, that catalyses a decarboxylative aldol addition using the loss of CO2 from L-Asp as a thermodynamic driving force to produce γ-hydroxy amino acids. This mechanism of action and the innate tolerance of diverse aldehydes marked UstD as a candidate for directed evolution into a versatile catalyst for organic synthesis. To screen for improved catalysts, we used a combination of globally random, site-saturation and degenerate codon mutagenesis libraries. We illustrated the engineering potential of the active site with two variants, UstDFVF and UstDTLM, that share no mutations in common and display a commensurate or superior activity to wt-UstD with the vast majority of aldehydes tested. We demonstrated how a simple regression-modelling approach to protein engineering can increase protein-soluble expression and crystallizability. The evolved variant, UstDv2.0, is poised to deliver desirable nsAA precursors for medicinal chemistry, and the crystal structure will facilitate future work to explore the mechanism and reactivity of this intriguing enzyme.
Methods
All chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich, VWR, Chem-Impex International, Alfa Aesar, Combi-blocks and Oakwood Products) at the highest quality available and used without further purification unless stated otherwise. Genes were purchased as gBlocks from Integrated DNA Technologies. E. coli cells were electroporated with an Eppendorf E-porator at 2,500 V. New Brunswick 126R shaker incubators (Eppendorf) were used for cell growth. Cell disruption via sonication was performed with a Sonic Dismembrator 550 (Fisher Scientific) sonicator. Ultraviolet–visible spectrophotometric measurements were collected on a UV-2600 Shimadzu spectrophotometer. Optical density measurements were collected using an optical density reader (Amerham Biosciences). UPLC–MS data were collected on an Acquity UPLC (Waters) equipped with an Acquity PDA and QDA MS detector using either a BEH C18 column (Waters) for the substituted benzaldehyde reactions, or an IntraNano Amino column (Imtakt) for the aliphatic aldehyde reactions. All UPLC–MS data were processed using Empower 3 (Waters). Preparative column separations were performed on an Isolera One Flash Purification system (Biotage). NMR data were collected on Bruker 400 or 500 MHz spectrometers equipped with BBFO and DCH cryoprobe, respectively. All NMR chemical shifts were referenced either to a residual solvent peak or tetramethylsilane internal standard. Spectra recorded using DMSO-d6 were referenced to the residual DMSO signal at 2.5 ppm for H and 39.52 ppm for 13C NMR analysis. Spectra recorded using CDCl3 were referenced to the residual CHCl3 peak at 7.26 ppm for H and 77.16 ppm for 13C NMR spectroscopy. Spectra recorded using CD3OD were referenced to the CH3OD residual solvent peak at 3.33 ppm for H and 49.00 ppm for 13C NMR analysis. Spectra recorded using D2O-acetonitrile-d6 as the solvent were referenced to the residual H2O signal at 4.79 ppm for H and absolute reference to the H spectrum for 13C NMR analysis. Signal positions were recorded in ppm with the abbreviations s, t, q, dd and m denoting singlet, triplet, quartet, doublet of doublets and multiple, respectively. All the coupling constants / were measured in Hertz. High-resolution mass spectrometry data were collected with a Q Exactive Plus Orbitrap (Thermo Scientific) equipped with samples ionized by electrospray ionization.

Cloning of wt-UstD. A codon-optimized copy of the Aspergillus flavus UstD gene was purchased as a gBlock from Integrated DNA Technologies. This DNA fragment was inserted into a PET-22b(+) vector by the Gibson Assembly method41. The DNA product via electroporation. After 45 min of recovery in LB media that contained 0.4% glucose at 37 °C, cells were plated onto LBplates and incubated overnight. Single colonies were used to inoculate 5 ml LBplates, which were grown overnight at 37 °C. Cells were then harvested by centrifugation and were there 1–2 coding mutations for both the concentrations of MnCl2.

Protein engineering (library expression, screening and validation). Electrocomptent BL21(DE3) cells were transformed with mutagenized plasmid DNA and allowed to recover for 45 min in 800 µl of TB. After recovery, the cells were grown on 1 ml of Luria Broth (LB) plus 25 µl of isopropyl β-D-thiogalactopyranoside. The cultures were allowed to react at 37 °C for 16 h. Afterwards, each reaction was quenched with 200 µl of water and the plate was subsequently lysed for 1 h at 37 °C. The lysate was pelleted at 4,000 g for 30 min. Clarified lysate was added to a 96-well plate in which each well contained a master mix solution, such that the end reaction concentrations were 25 mM aldehyde, 25 mM L-Asp, PLP and buffer (100 mM KPi + NaCl, pH 7.0). The ratio of clarified lysate to reaction master mix was varied over the course of the engineering to maintain a reasonable product measurement dynamic range. The reactions were allowed to incubate overnight at 37 °C, and were subsequently quenched with 100 µl of acetone/DMSO (ACN) and pelleted at 4,000 g for 30 min. The cleared reaction mixture was transferred to a 0.2 µm centrifugation filter plate (PALL) and filtered at 1,500g for 10 min into a clean 96-well plate before being sealed prior to analysis by UPLC–MS.

The relative amount of product formed in the reactions compared with that in the positive control reaction was measured by absorbance at 210 nm via UPLC–MS. Given the relatively high variability in the parent signal in this assay, wells typically required an apparent 1.5-fold increase in product compared with that of the positive control reaction. New glycerol stocks from the starter culture plate (described above), the wells of interest could be streaked on to a fresh LBplates for subsequent screening and validation.

Every mutant of interest was validated by heterologous expression and Ni-NTA purification, which accounted for changes in the soluble enzyme concentration as well as changes in the activity of the enzyme. The activity was measured over the course of engineering, each key variant in the evolutionary lineage was expressed and purified in tandem, as described above (Supplementary Fig. 3). Parallel triplicate 200 µl reactions that contained 25 mM benzaldehyde, 50 mM L-Asp sodium salt monohydrate, 2.5 µl PLP and 0.25 µl UstD variant (0.001 mol% catalyst, 100,000 maximum TON) were allowed to react at 37 °C for 16 h (Table 1). Each reaction was terminated with 50 µl of a solution that contained 1 mM trypsin as an internal standard, and the reaction mixtures were analysed by UPLC–MS. A standard curve was made using previously purified 3a to
facilitate the TTN calculations. The variants were also trialed against several other α-hydeydes, which included biphenyl-4-carboxaldehyde (20,000 maximum TON), p-anisaldehyde (20,000 maximum TON) and glycolaldehyde (100,000 maximum TON). Reactions were run using the same reaction conditions and procedure, with catalyst loading changed to match the indicated maximum TON. Simple fold-response measurements were used to quantify the activity differences between variants (Supplementary Fig. 5).

UstD reaction condition optimization. All the optimization reactions were conducted in triplicate on an analytical scale (100 μL). PLP and L-Asp stock solutions were made with a 100 mM potassium phosphate buffer that contained 100 mM sodium chloride (reaction buffer) at the indicated pH. Postreaction quenching was done by adding 100 μL of 99% ACN ethanol with 1 μM trypamine as an internal standard. Quenched reactions were then centrifuged at 15,000 g to remove aggregated proteins, and diluted with 200 μL of 1:1 water:ACN. Quantification was performed by UPLC–MS analysis. Measurements of the internal standard, benzaldehyde and product concentrations was done by injection of 5 μL of the reaction mix (1 equiv., 0.5 mM final amine from unreacted L-Asp and formed L-Asp, and γ-hydroxy amino acid product) was added to a solution of 144 μL of 10.41 mM NaHCO₃ (10 equiv., 5 mM final concentration) and 0.21 mM of either L-Arg (0.1 mM final concentration, α-amino acids), or L-Asp (2.5 mM final concentration, aldehydes 2a–2k) or tryptamine (0.1 mM final concentration, aldehydes 2a–2k), followed by the addition of 150 μL of 5 mM L-FDAA (Marfey’s reagent) dissolved in ACN (5 equiv., 2.5 mM final concentration) to bring the total reaction volume to 300 μL. Each reaction vial was sealed with a pieceable LC vial cap, placed in a dark 37 °C incubator for 18 h and then quenched with 300 μL of 1:1 ACN:60 mM HCl (15 mM postquench). Quenched reaction mixtures were analysed by UPLC–MS no later than 24 h after quenching; the results are shown in Supplementary Table 1 and Supplementary Figs. 13–28.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The structure of UstD is available through the Protein Data Bank ID 7MKV. The sequence-activity data used for linear regression modelling is available through GitHub. All the other data are available from the authors upon reasonable request.

Code availability
The linear regression modelling code used during the final round of protein engineering is available through GitHub under the MIT License.

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Author contributions

A.R.B. and J.M.E. conceptualized the goals and aims of the project. J.M.E., M.E.C., P.K., E.P.G., C.A.B. and A.R.B. carried out the development of the chemistry and enzymes. J.M.E. developed the code for data analysis and developed the linear regression model. J.M.E. and M.E.C. verified the results. J.M.E., M.E.C., P.K. and A.R.B. prepared the figures and data visualizations. A.R.B. secured funding for the project that led to this publication. A.R.B. coordinated team members for the development of the chemistry and enzyme evolution. C.A.B. supervised the data acquisition of protein crystals that led to the resolved crystal structure. A.R.B. supervised the research activity planning and execution. J.M.E., M.E.C. and A.R.B. prepared the initial manuscript. J.M.E., M.E.C., P.K. and A.R.B. reviewed and edited the initial manuscript and provided critical commentary and revisions.

Competing interests

A.R.B., J.M.E. and P.K. have a patent pending on the use of engineered UstD for the synthesis of nsAAs, US Patent application no. 20210115480A1. All other authors declare no competing interests.

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