Biosynthesis and Incorporation of Nonstandard Amino Acids in Engineered Strains

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366 Colburn Laboratory | https://udel.zoom.us/j/93662154194 | Password: nsAA

Nonstandard amino acids (nsAAs) that are L-phenylalanine derivatives with aryl ring functionalization have long been harnessed in natural product synthesis, therapeutic peptide synthesis, and diverse applications of genetic code expansion. Yet, to date, these chiral molecules have often been the products of poorly enantioselective and environmentally harsh organic synthesis routes. Here, we reveal the broad specificity of multiple natural pyridoxal 5'-phosphate (PLP)-dependent enzymes, specifically, an L-threonine transaldolase, a phenylserine dehydratase, and an aminotransferase, towards substrates that contain aryl side chains with diverse substitutions. We exploit this tolerance to construct a one-pot biocatalytic cascade that achieves high-yield synthesis of diverse enantiopure L-phenylalanine derivatives from aldehydes under mild aqueous reaction conditions. We demonstrate the addition of a carboxylic acid reductase module to this cascade to enable the biosynthesis of L-phenylalanine derivatives from carboxylic acids, which may be less expensive and less reactive than the corresponding aldehydes. The economic feasibility and scalability of the polyspecific nsAA production platform is improved through the demonstration of both lysate-based and resting whole-cell based reactions, which remove the need for costly and time-intensive enzyme purification. This work offers an efficient, versatile, and scalable route with the potential to lower manufacturing cost and democratize synthesis for many valuable nsAAs.

To further improve translation of the platform for applications of genetic code expansion in live cells, we address a key limitation - the reliance on the external supplementation of nsAAs to cell culturing media. We investigate two alternatives to eliminate the need for the isolation of a synthesized nsAA: 1) the direct supplementation of reaction mixtures containing biocatalytically produced nsAAs to live bacterial cells for subsequent incorporation into a target protein and 2) the combination of phenylalanine derivative semi-synthesis and site-specific incorporation using an engineered bacterial host. Our system serves as a platform that exhibits broad substrate specificity towards commercially ubiquitous achiral building blocks of aryl aldehydes and carboxylic acids, producing the family of nsAAs that are most frequently used for genetic code expansion. These platforms allow for the rapid production and screening of industrially relevant and novel nsAAs for compatible orthogonal translation machinery. Additionally, we show that the combination of nsAA biosynthesis and incorporation steps can extend the chemical reach of the intrinsic biological containment strategy of synthetic auxotrophy from reliance on nsAAs to reliance on low-cost and achiral building blocks instead. We anticipate that our system will aid industrial-scale manufacturing of nsAA-containing peptides and proteins will facilitate access to expensive or commercially unavailable chemistries for labs that lack separations or synthesis expertise.

Lastly, we address the instability of nitroaromatic compounds in the presence of microbial cells, which has hindered the ability to biomanufacture these industrially and pharmaceutically relevant chemicals. Here, we perform a comprehensive analysis of bacterial nitroreductase (NTR)

activity by engineering strains of *Escherichia coli*, containing up to 15 knockouts of known and candidate NTR genes, and by evaluating the stability of over 20 nitroarenes exogenously supplemented to wild-type and engineered strains. For several chemistries, such as di-nitro compounds and nitro-aldehydes, our engineered nitroaromatic reductase knockout strains (NARKOS) enable first-time retention of compounds that are otherwise rapidly modified. We leverage these insights and engineered strains to improve or enable nitroarene biosynthesis, including the biocatalytic transformation of an amine precursor into a nitro compound and the biosynthesis of nitro-aldehydes. We implement our biosynthetic pathway for the production and site-specific incorporation of nitro-containing nsAAs, which are of interest as photolabile and immunogenic residues. This work establishes the foundation for integrating nitro functional group chemistry into biocatalysis, fermentation, and synthetic biology.