Development of a versatile and efficient C-N lyase platform for asymmetric hydroamination via computational enzyme redesign

Yinglu Cui
Institute of Microbiology

Yinghui Wang
Institute of Microbiology

Wenya Tian
Institute of Microbiology

Yifan Bu
Institute of Microbiology

Tao Li
Institute of Microbiology

Xuexian Cui
Institute of Microbiology

Tong Zhu
Institute of Microbiology

Ruifeng Li
Institute of Microbiology

Bian Wu (✉️ wub@im.ac.cn)
Institute of Microbiology  https://orcid.org/0000-0002-6524-2049

Article

Keywords: C-N lyase platform, computational enzyme redesign, asymmetric hydroamination

DOI: https://doi.org/10.21203/rs.3.rs-92435/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Although C-N bonds are ubiquitous in natural products, pharmaceuticals, and agrochemicals, biocatalysts that forge these bonds with high atom efficiency and enantioselectivity have primarily been limited to a few select enzymes. In particular, the use of ammonia lyases has emerged as a powerful strategy to access C-N bond formation through hydroamination reactions, which has no counterpart in traditional synthetic chemistry. However, the broad utility of ammonia lyases is rather restricted due to their narrow synthetic scope, and the conjugate addition of a matrix of nucleophilic donors to electrophilic acceptors remains a longstanding challenge. Herein, we report the computational redesign of aspartase, a highly specific ammonia lyase, to yield C-N lyases with unprecedented cross-compatibility of nonnative nucleophiles and electrophiles. A wide range of noncanonical amino acids (ncAAs) are afforded with excellent conversion (up to 99%), regioselectivity > 99%, and product enantiomeric excess > 99%, and the process is scalable under industrially relevant protocols (demonstrated in kilogram-scale synthesis). Furthermore, the redesigned enzymes can be facilely integrated in cascade reactions, as we demonstrated the synthesis of β-lactams with different substitution patterns at the N-1 and C-4 positions in a one-pot reaction. This versatile and efficient C-N lyase platform supports the preparation of diverse libraries of ncAAs and their derivatives and will present opportunities in medicinal chemistry and synthetic biology.

Introduction

Outside of the twenty proteinogenic amino acids that serve as the foundational building blocks of life, there are manifold noncanonical amino acids (ncAAs) that exhibit diverse physiological functions and are extensively used as intermediates for bioactive products. An analysis of FDA-approved medicines reveals that approximately 12% of the 200 top-grossing drugs contain at least one ncAA building block. The implementation of ncAAs in synthetic pipelines can bypass many difficulties in installing challenging pharmaceutical functionalities, such as chiral amines and unprotected polar groups. Moreover, the physical and chemical properties of synthetic peptides and proteins can be selectively manipulated through the incorporation of ncAAs, which has contributed to understanding biological macromolecules and the development of novel therapeutics and high-performing biocatalysts. In a broader sense, the biotechnological application of ncAAs would benefit synthetic biology’s central goal: to create novel life forms and functions. However, despite the impressively wide perspectives of ncAAs, their synthesis remains a challenge because stringent stereoselectivity and functional-group compatibility are required. Consequently, few chiral ncAAs are readily available, and the full potential of this class of compounds has not been realized. Thus, simple, sustainable, cost-effective, and scalable processes for ncAAs are highly desirable.

Given the apparent advantages in terms of selectivity, sustainability, and evolvability, biocatalytic preparation of ncAAs has attracted increasing interest. From a retrosynthetic perspective, the utilization of coupling reactions imparts excellent benefits as it introduces quite a few atom economical
reactions that allow multistep procedures and purifications to be avoided to provide a target product\(^\text{11}\). In this respect, chiral ncAAs can be obtained through the hydroamination of carbon-carbon double bonds, one of the top aspirational reactions in synthetic chemistry\(^\text{12}\). Asymmetric C-N addition enables an enantioselective and atom-economic synthesis in which easily accessible prochiral compounds are conjugated to form an optically pure ncAA with 100% theoretical yield, albeit typically with a requirement for tedious protection-deprotection steps and expensive catalysts or chiral auxiliaries via metal catalysis or organocatalysis\(^\text{13,14}\). These problems compound when ncAAs contain additional reactive functional groups and need to fit the stringent criteria for industrial applications. In this context, nature circumvents such challenges by using ammonia lyases (ALs) to catalyze reversible C-N bond cleavage and formation\(^\text{15-18}\) (Fig. 1A). Native reactions mediated by aspartase and phenylalanine AL (PAL) exemplify the enzymes’ advantages perfectly, which have been exploited to synthesize \(\text{L-}\)aspartic acid and \(\text{L-}\)phenylalanine on the thousand-ton scale since the 1980s\(^\text{18}\) (Fig. 1B). More recently, a rapid increase in C-N lyase applications in biocatalytic and therapeutic fields has benefited from the advancement of discovery and engineering technologies to improve these enzymes’ functional properties and offer expanded synthetic scopes\(^\text{19-25}\)(Fig. 1C and 1D). The growing toolbox of C-N lyases enables syntheses of a range of high-value-added products, with prominent examples including cypermethrin by engineered PAL\(^\text{19}\), a series of valuable aspartic acid derivatives by two methylaspartate AL (MAL) variants\(^\text{21}\), and the fungal natural product aspergillomarasmine A (AMA) by ethylenediamine-N, \(\text{N'-}\)disuccinic acid lyase (EDDS)\(^\text{24}\).

Although inspiring advances have been made, C-N lyases are not considered to tap all of the catalytic potential within their scaffold. The cross addition of nonnative nucleophilic amines to substituted alkene partners remains elusive, with no success in simultaneously diversifying the electrophile and nucleophile substrate scope of C-N lyases. To endow C-N lyases with broader synthetic utility, there are several issues to be considered in addition to the inherent difficulties in asymmetric hydroamination, such as asymmetric induction and compatibility problems (Fig. 1E). First, such a transformation requires that both amines and alkenes can access the enzyme active site and are stabilized at applicable positions to maintain enantioselectivity. Second, the high regio- and enantioselectivities achieved by C-N lyases rely mainly on the precise hydrogen-bonding network or aromatic microenvironment of these enzymes, wherein residues enabling exquisite hydrogen bond networks to stabilize nucleophiles and electrophiles are interlaced, which inherently limits the substrate scope. Third, within a compact active site (an average volume of 1072 Å\(^3\) calculated from the Catalytic Site Atlas database\(^\text{26}\)), approximately 10 key residues (the van der Waals volumes of proteinogenic amino acids range from 67–163 Å\(^3\)\(^\text{27}\)) need to be screened for the whole active site and \(10! \approx 10^6\) possible combination paths linking these single mutations. The vast majority of mutational pathways to new properties would be tortuous in the presence of epistatic effects, and the challenge lies in identifying an efficient path to the desired function along the rugged fitness landscape.
In this study, encouraged by our recent success in redesigning an aspartase from *Bacillus* sp. YM55-1 (AspB)\(^{20}\), we attempted to tackle the longstanding challenge of creating C-N lyases with cross-compatibility of nonnative substrates by performing computational protocols that allow large jumps in function to traverse inactive sequence space along the fitness landscape. We envisioned that the AspB variants with a thoroughly reshaped active site could achieve cross addition of a large variety of nucleophilic amines to unsaturated acids, thus providing a C-N lyase platform that possesses unique and attractive properties for the biocatalytic preparation of a myriad of practically relevant ncAAs and their demand-tailored derivatives.

**Results**

**Computational redesign of AspB.**

We commenced our study by defining calculation criteria based on the detailed enzymatic mechanism. Although thermodynamically feasible, hydroamination reactions generally have a high activation barrier due to the repulsion of the electron-rich π-system of the alkene substrate and the electron pair on the amine nitrogen atom\(^{28}\). However, the entropic penalty can conceivably be overcome by preorganization in an enzyme reactive site\(^{15}\). The reaction mechanism of AspB involves the abstraction of the pro-R proton from the C\(_β\) atom of substrate aspartate by the general base Ser318 within the SS loop\(^{29}\). The carboxylate group of the formed enolate anion intermediate is stabilized through a network of hydrogen bonds including residues Thr101, Ser140, Thr141, and Ser319, with the amino group being easily accommodated in the nucleophile binding pocket by hydrogen-bonding interactions of the side chains of Thr101, Asn142, and His188\(^{30}\). The original α-carboxylate binding pocket consists of residues Thr187, Met321, Lys324, and Asn326, which were substituted to target different electrophiles in our previous work\(^{20}\). Initial experiments on the asymmetric hydroamination reaction of amine groups revealed that Asn142 served as a dispensable residue, whereas Thr101 and His188 functioned as crucial residues, i.e., substitutions of Thr101 and His188 to alanine provoked dramatic reductions in catalytic activities. From the structural point of view, residues Ala99, Leu358, and Glu362 create a hindered environment to suppress the amine group from entering its ideal reactive pose. Motivated by these findings, we performed computational redesign to expand the nucleophile spectrum of AspB. Specifically, Ala99, Asn142, Leu358, and Glu362 were envisioned to be mutated into less-bulky residues while the global folding of the enzyme was maintained. In addition, the hydrogen-bonding interaction network of the β-carboxylate was preserved in the near-attack conformation during the design process (Fig. 2).

**Conjugate addition of a matrix of nucleophilic donors to electrophilic acceptors by computationally redesigned AspB.**

We first evaluated the asymmetric hydroamination of crotonic acid (1) with amine derivatives. As the benchmark reactions for computational design, substrates that provides an exceptional opportunity for late-stage diversification (allylamine, h) or bears relatively larger substituents (cyclopropylamine, j) were chosen. A mutant library of the B19 enzyme\(^{20}\) was generated *in silico* by simultaneous substitutions of
Ala99, Asn142, Leu358, and Glu362 to less-bulky residues (A99 to G, N142 to GAVSTC, L358 to GAVIMSTCDNH, and E362 to GAVLIMSTCDNH). Experimental validation of a small set of 22 designs for $1\text{h}$ and 16 designs for $1\text{j}$ resulted in the identification of 37 mutants, among which the referred BA15 design (containing A99G-N142S-T187C-M231I-K324L-N326A-L358V-E362M mutations) showed the highest specific activity for syntheses of both $1\text{h}$ and $1\text{j}$ as ascertained by HPLC with a chemically prepared authentic standard. Excellent conversions (> 99%) were achieved within 2 h with only < 0.005 mol% biocatalyst, and control experiments showed that the amines did not react with crotonic acid in the absence of the enzymes. The successful design encouraged the evaluation of a broad spectrum of compounds (a–n), in which most of the amines were efficiently converted by BA15 to afford the respective optically pure products (> 99% e.e.) with > 96% conversions (Fig. 3C) at a substrate loading up to 150 g/L. Notably, sterically hindered amines (c and e), which were problematic substrates for previously reported enzymatic hydroamination reactions, were successfully catalytically incorporated. For strong nucleophile ethylenediamine (m), weak spontaneous reaction occurred, which gave a slight drop of product enantioselectivity to 90% e.e. Structural analysis showed that A99G-N142S-L358V-E362M mutations would afford an enlarged amine binding pocket that would retain the van der Waals interactions and permit the binding of bulkier amine groups in different orientations (Fig. 3A). Hence, nonnative amines with charged or large substituents might be accepted in addition to simple ammonia. More importantly, the conformational change of the nucleophilic pocket was considered to not conflict with the electrophilic pocket, which raised the possibility of direct combination of the nucleophilic and electrophilic pockets without multiple rounds of design.

To evaluate the compatibility of the nucleophilic pocket and designed electrophilic pockets, the redesigned nucleophile binding pocket was introduced into the AspB wild type (yielded AA15 design containing A99G-N142S-L358V-E362M mutations) and its engineered enzymes P1 (yielded PA15 design containing A99G-N142S-T187C-M231I-K324L-N326C-L358V-E362M mutations) and F29 (yielded FA15 design containing A99G-N142S-T187C-M231V-K324I-N326C-L358V-E362M mutations) to catalyze the conjugate addition of a matrix of diverse nucleophilic donors to electrophilic acceptors. As anticipated, the unsaturated amino acids bearing an ethyl group efficiently underwent a hydroamination reaction with the evaluated amines to afford the corresponding products in excellent conversions (> 94%) and enantioselectivities (> 99% e.e., expect for 2m). The substrate tolerances peaked at concentration up to 100 g/L. For charged substrate fumaric acid, most of the substituted amines with aliphatic, unsaturated, or charged groups were efficiently processed, providing the desired products > 90% conversions and excellent stereoselectivity (> 99% e.e., expect for 3m) at a high substrate loading of 80–130 g/L, except for isopropylamine and cyclobutylamine, which gave moderate conversions (88% and 79%, respectively). For aromatic substrates, a lower concentration (7.5 g/L) had to be used due to their low solubility. Amines with relatively small substituents exhibited low conversions. Nonetheless, cinnamic acid was proven to be a competent coupling partner with methoxamine, providing the product with 97% conversion. Aromatic acrylates bearing electron-withdrawing/-donating groups also afforded desired products with satisfactory conversions, which demonstrates the compatibility of the redesigned enzyme to tolerate functionalized groups typically encountered in pharmaceutical agents.
For a few ncAAs bearing valuable scaffolds or providing opportunities for further functionalization, preparative reactions were performed. As summarized in Fig. 3C, various ncAA products were successfully synthesized by the corresponding redesigned AspBs on ten to hundred gram scales to afford the products in good to excellent isolated yields (74–93%). Remarkably, propargylamine was efficiently converted to give the corresponding product 1i in excellent isolated yield (93%, 131 g), leaving the alkynyl group available for potential downstream synthetic manipulation. With a 1.5-fold molar equiv. of propargylamine over crotonic acid, the reactions were complete within 1 h at 50 °C, providing the space-time yield of 131 g/L/h, which is, to our knowledge, the highest value reported to date for C-N lyases. Within the rather broad substrate spectrum, we next examined substrates with long aliphatic chains that are precursors of aspartame derivatives. Derivatization of the artificial dipeptide sweetener aspartame with N-alkyl groups can generate even sweeter compounds, such as the approved food additive neotame, which is 7000–13000 times sweeter than sucrose31. In this study, N-butyl-L-aspartic acid (3f), which is the precursor to neotame analog, was synthesized on a kilogram scale using whole cells fermented from merely 2 L medium with excellent conversion (> 97%), isolated yield (92%, 1.4 kg), and stereoselectivity (> 99% e.e.) (Fig. 3D), demonstrating the great potential of the redesigned AspBs to offer alternative synthetic options for the industrial preparation of valuable ncAA products.

One-pot chemoenzymatic synthesis of β-lactam compounds.

The significantly broader substrate scope of the redesigned C-N lyases has also raised the possibility of building entirely new synthetic pathways for valuable precursors to pharmaceuticals. By harnessing the elegance of biocatalysis and the robustness of chemical catalysis, efficient routes toward the β-lactam heterocycle, one of the most acclaimed pharmacophoric moieties32, from simple starting materials were examined. With the enzymatically prepared ncAAs in hand, we subsequently performed the cyclization reaction in the same pot without purification, accomplishing the full conversion for the second cyclization step. The substrate cyclopropylamine (j), which could be efficiently converted by BA15 with 99% conversion, was chosen for our initial investigation. The one-pot chemoenzymatically prepared β-lactam product 1jc was isolated with moderate overall yield (63%) and excellent optical purity (e.e. > 99%) and without racemization of the potentially sensitive Cβ stereogenic center (Fig. 4A). To further demonstrate the synthetic usefulness of this one-pot synthetic strategy, 1 g, 1i, 1 k, 2 h, 2i, and 2 k, which were well accepted substrates of the redesigned enzymes, were chosen and provided good overall isolated yields (46–71%). The results demonstrated a simplified practical procedure toward optically pure β-lactam heterocycles through a one-pot chemoenzymatic synthesis route.

It is noteworthy that alkenes and alkynes are highly versatile synthetic handles suitable for elaboration into a wide variety of useful functional groups. The modification of the alkyne or alkene tag in β-lactam heterocycles via well-established click chemistry allows the introduction of bulky reporter groups (e.g., rhodamine). Therefore, the corresponding probes are useful tools to study enzyme activity, function, and assembly33. To our delight, the use of 2ic as the alkyne donor was feasible, and the corresponding azole product was obtained in 3 h as ascertained by ESI-MS (Fig. 4B). The successful attempt to synthesize
chemoenzymatic β-lactam heterocycles via click chemistry may further provide access to tailor-made enzyme inhibitors and provide potential molecular probes to unravel the activity and function of proteins.

Discussions

Through evolution, nature has fashioned a plethora of enzymes to catalyze diverse reactivities that make life possible. The conventional views on enzyme limitations in synthetic applications often assume that an enzyme’s exquisite activity comes at the cost of strict selectivity for accepted substrates. Nevertheless, a majority fraction of enzymes do not make use of all the possible chemistries that are accessible by their scaffold, which has fueled efforts to tailor the performance of existing enzymes and, more ambitiously, to create new reactions. Exploring a fitness landscape via traditional laboratory evolution relies on the presence of generalists as starting points. It is an arduous task that does not involve knowledge of what determines new activity, and the quality of the said starting point is unknown since iterative rounds of mutagenesis target sites scattered throughout the global protein structure. An alternative avenue in engineering existing enzymes has proven successful in enhancing our ability to recognize the origin of enzymes’ remarkable performances using theoretical and computational methods. By exploring a small fraction of the vast sequence space, successful examples of studies focused on reshaping active sites that accommodate individual substrates have been reported.

However, a fantastic array of biocatalysts, such as C-N lyases, imine reductases, and aldolases, catalyze the cross-coupling of multiple substrates. Consequently, there raised the question of whether rational computational modeling could be applicable for more challenging tasks, where manipulations of enzymes are no longer limited to a small set of residues but are expanded to deal with collective mutations synchronously lining the whole active site.

Here, we addressed this possibility by dramatically transforming the substrate recognition pattern of the extremely specific enzyme AspB. This goal was achieved using mechanism-based computation protocols, dissecting nearby interactions relevant for binding and catalysis, retrieving essential catalytic geometric criteria from structural analyses and MD simulations, and allowing large jumps in sequence space while accommodating interactions between multiple simultaneous mutations and substrate. The significant advantage of this strategy lies in minimalizing experimental efforts while maintaining the exploration of synergisms between mutations. As such, desired enzymes possessing up to eight mutations at spatially adjacent positions in the exquisite active site were obtained by the screening of only a handful of variants. Without the assistance of computation, such extensive sculpting of the enzyme’s active site would be either formidable by rational inspection or extremely labor intensive via experimental molecular evolution. The redesigned enzymes successfully permitted a wide range of aliphatic, aromatic, and charged ncAAs to be prepared. This enzymatic route also highlights the power of the redesigned C-N lyase to precisely access the desired stereoisomer of the product with excellent regioselectivity. Remarkably, these nonnatural biotransformations can be exceptionally efficient, even able to fulfill industrial requirements for substrate loading, product yield and profile, and scalability. Further advantages are that the redesigned C-N lyases were suitable for cascade reactions, enabling
sequential one-pot transformations that produce other high-value-added products, such as β-lactam heterocycles. As the computational redesign workflow is well established, we expect that this protein scaffold may be further exploited to tackle more challenging transformations, such as the exploration of unactivated alkenes without carboxyl groups, the anti-Markovnikov hydroamination of terminal alkenes to generate linear aliphatic amines, and the synthesis of ncAAs with double carbon stereocenters.

In summary, we presented an unprecedented versatile C-N lyase platform tuned by computation to directly conjugate a matrix of nucleophilic amines and unsaturated acids through asymmetric hydroamination, which has no counterparts in traditional synthetic chemistry. The results provide convincing support for the notion that computational tools that efficiently navigate large regions of sequence space to propose beneficial mutants hold promise for tackling the challenges in biocatalysis in general. We anticipate that further development of this effective biocatalyst platform may open up new opportunities to allow stepwise economic connections of structurally diverse building blocks through C-N links for the synthesis of ncAAs and their derivatives, not only providing utilities in synthetic and medicinal chemistry but also laying molecular building blocks for future synthetic biology development.

Declarations

Acknowledgments

This work is supported by the National Key R&D Program of China (2018YFA0901600), the National Natural Science Foundation of China (31822002), the Biological Resources Programme (KFJ-BRP-009) and the Key Research Program of Frontier Sciences (ZDBS-LY-SM014) from the Chinese Academy of Sciences.

Author Contributions

B.W. initiated the project. Y.C. performed the computational work. Y.W., W.T., T.L., X.C., and R.L. performed biocatalytic experiments. B.Y. and T.Z. performed preparative-scale synthesis of amino acids and derivatives. Y.C. and B.W. provided supervision and input on experimental design and wrote the manuscript, which was revised and approved by all authors. Y.C., Y.W. and W.T. contributed equally to this work.

Conflict of Interest

The authors declare no conflicts of interest.

Data and materials availability

All data are available in the main text or the supplementary materials.

References
1. Young, T. S. & Schultz, P. G. Beyond the canonical 20 amino acids: Expanding the genetic lexicon. *J. Biol. Chem.* **285**, 11039–11044 (2010).

2. McGrath, N. A., Brichacek, M. & Njardarson, J. T. A graphical journey of innovative organic architectures that have improved our lives. *Chem. Educ.* **87**, 1348–1349 (2010).

3. Young, D. D. & Schultz, P. G. Playing with the molecules of life. *ACS Chem. Biol.* **13**, 854–870 (2018).

4. Blaskovich, M. A. Unusual amino acids in medicinal chemistry. *J. Med. Chem.* **59**, 10807–10836 (2016).

5. Agostini, F., Völler, J. S., Koksch, B., Acevedo-Rocha, C. G., Kubyshkin, V. & Budisa, N. Biocatalysis with unnatural amino acids: enzymology meets xenobiology. *Angew. Chem. Int. Ed.* **56**, 9680–9703 (2017).

6. Burke, A. J., Lovelock, S. L., Frese, A, Crawshaw, R., Ortmayer, M., Dunstan, M., Levy, C. & Green A. P. Design and evolution of an enzyme with a non-canonical organocatalytic mechanism. *Nature* **570**, 219–223 (2019).

7. Blaskovich, M. A. Handbook on syntheses of amino acids: General Routes for the syntheses of amino acids, Oxford University Press: New York, (2010).

8. Almhjell, P. J., Boville, C. E. & Arnold, F. H. Engineering enzymes for noncanonical amino acid synthesis. *Chem. Soc. Rev.* **47**, 8980–8997 (2018).

9. Xue, Y. P., Cao, C. H., & Zheng, Y. G. Enzymatic asymmetric synthesis of chiral amino acids *Chem. Soc. Rev.* **47**, 1516–1561 (2018).

10. Zou, H., Li, L., Zhang, T., Shi, M., Zhang, N., Huang, J. & Xian, M. Biosynthesis and biotechnological application of non-canonical amino acids: Complex and unclear. *Biotechnol. Adv.* **36**, 1917–1927 (2018).

11. Honig, M., Sondermann, P., Turner, N. J. & Carreira E. M. Enantioselective chemo- and biocatalysis: Partners in retrosynthesis. *Angew. Chem. Int. Ed.* **56**, 8942–8973 (2017).

12. Constable, D. J. C., Dunn, P. J., Hayler, J. D., Humphrey, G. R., Leazer, J. L., Linderman, R. J., Lorenz, K., Manley, J., Pearlman, B. A., Wells, A., Zaks, A. & Zhang, T. Y. Key green chemistry research areas - a perspective from pharmaceutical manufacturers. *Green Chem.* **9**, 411–420 (2007).

13. Huang, L., Arndt, M., Gooßen, K. Heydt, H. & Gooßen, L. J. Late transition metal-catalyzed hydroamination and hydroamidation. *Chem. Rev.* **115**, 2596–2697 (2015).

14. Weiner, B., Szymański, W., Janssen, D. B., Minnaard, A. J. & Feringa, B. L. Recent advances in the catalytic asymmetric synthesis of β-amino acids. *Chem. Soc. Rev.* **39**, 1656–1691 (2010).

15. Turner, N. J. Ammonia lyases and aminomutases as biocatalysts for the synthesis of α-amino and β-amino acids. *Curr. Opin. Chem. Biol.* **15**, 234–240 (2011).

16. Heberling, M. M., Wu, B., Bartsch, S. & Janssen, D. B. Priming ammonia lyases and aminomutases for industrial and therapeutic applications. *Curr. Opin. Chem. Biol.* **17**, 250–260 (2013).

17. Parmeggiani, F., Weise, N. J., Ahmed, S. T., & Turner, N. J. Synthetic and therapeutic applications of ammonia-lyases and aminomutases. *Chem. Rev.* **118**, 73–118 (2018).
18. Zhang, J., Abidin, M. Z., Saravanan, T., & Poelarends, G. J. Recent applications of carbon-nitrogen lyases in asymmetric synthesis of noncanonical amino acids and heterocyclic compounds. *ChemBioChem* **21**, 2733–2742 (2020).

19. de Lange, B., Hyett, D., Maas, P. J. D., Mink, D., van Assema, F. B. J., Sereinig, N., de Vries, A. H. M. & de Vries, J. G. Asymmetric synthesis of (S)-2-indoline carboxylic acid by combining biocatalysis and homogeneous catalysis. *ChemCatChem* **3**, 289–292 (2011).

20. Li, R., Wijma, H. J., Song, L., Cui, Y., Otzen, M., Du, J., Niu, D., Chen, Y., Feng, J., Han, J., Chen, H., Tao, Y., Janssen, D. B. & Wu, B. Computational redesign of enzymes for regio- and enantioselective hydroamination. *Nat. Chem. Biol.* **14**, 664–670 (2018).

21. Raj, H., Szymański, W., de Villiers, J. Rozeboom, H. J., Veetil, V. P., Reis, C. R., de Villiers, M., Dekker, F. J., de Wildeman, S., Quax, W. J., Thunnissen, A. M. W. H., Feringa, B. L., Janssen, D. B. & Poelarends, G. J. Engineering methylaspartate ammonia lyase for the asymmetric synthesis of unnatural amino acids. *Nat. Chem.* **4**, 478–484 (2012).

22. Weise, N. J., Parmeggiani, F., Ahmed, S. T. & Turner, N. J. The bacterial ammonia lyase EncP: A tunable biocatalyst for the synthesis of unnatural amino acids. *J. Am. Chem. Soc.* **137**, 12977–12983 (2015).

23. Ahmed, S. T., Parmeggiani, F., Weise, N. J. Flitsch, S. L. & Turner, N. J. Engineered ammonia lyases for the production of challenging electron-rich L-phenylalanines. *ACS Catal.* **8**, 3129–3132 (2018).

24. Fu, H., Zhang, J., Saifuddin, M. Cruiming, G., Tepper, P. G. & Poelarends, G. J. Chemoenzymatic asymmetric synthesis of the metallo-β-lactamase inhibitor aspergillomarasmine A and related aminocarboxylic acids. *Nat. Catal.* **1**, 186–191 (2018).

25. Zhang, J., Grandi, E., Fu, H. Saravanan, T., Bothof, L., Tepper, P. G., Thunnissen, A. M. W. H. & Poelarends, G. J. Engineered C-N Lyase: Enantioselective synthesis of chiral synthons for artificial dipeptide sweeteners. *Angew. Chem. Int. Ed.* **59**, 429–435 (2020).

26. David, J. M., Jie, L. & Bob, E. Ionizable side chains at catalytic active sites of enzymes. *Eur. Biophys.* **41**, 449–460 (2012).

27. Darby, N. J. & Creighton, T. E. *Protein Structure*. Oxford: Oxford University Press; (1993).

28. Shaolin, Z., Nootaree, N. & Stephen, L. B. Enantio- and regioselective CuH-catalyzed hydroamination of alkenes. *J. Am. Chem. Soc.* **135**, 15746–15749 (2013).

29. Fibriansah, G., Veetil, V. P., Poelarends, G. J. & Thunnissen, A. M. Structural basis for the catalytic mechanism of aspartate ammonia lyase. *Biochemistry* **50**, 6053–6062 (2011).

30. Zhang, J. & Liu, Y. A QM/MM study of the catalytic mechanism of aspartate ammonia lyase. *J. Mol. Graph. Model.* **51**, 113–119 (2014).

31. Nofre, C. & Tinti, J. Neotame: discovery, properties, utility. *Food Chem.* **69**, 245–257 (2000).

32. Pitts, C. R. & Lectka, T. Chemical synthesis of β-lactams: Asymmetric catalysis and other recent advances. *Chem. Rev.* **114**, 7930–7953 (2014).
33. Bottcher, T. & Sieber, S. A. β-Lactams and β-lactones as activity-based probes in chemical biology. *Med. Chem. Commun.* 3, 408–417 (2012).

34. Reetz, M. T. What are the limitations of enzymes in synthetic organic chemistry? *Chem. Rec.* 16, 2449–2459 (2016).

35. Leveson-Gower, R. B., Mayer, C. & Roelfes, G. The importance of catalytic promiscuity for enzyme design and evolution. *Nat. Rev. Chem.* 3, 687–705 (2019).

36. Tracewell, C. A. & Arnold, F. H. Directed enzyme evolution: Climbing fitness peaks one amino acid at a time. *Curr. Opin. Chem. Biol.* 13, 3–9 (2009).

37. Wrenbeck, E. E., Azouz, L. R. & Whitehead, T. A. Single-mutation fitness landscapes for an enzyme on multiple substrates reveal specificity is globally encoded. *Nat. Commun.* 8, 15695 (2017).

38. Welborn, V. V. & Headgordon, T. Computational design of synthetic enzymes. *Chem. Rev.* 119, 6613–6630 (2019).

39. Huang, P., Boyken, S. E. & Baker, D. The coming of age of de novo protein design. *Nature* 537, 320–327 (2016).

40. Himo, F. Recent trends in quantum chemical modeling of enzymatic reactions. *J. Am. Chem. Soc.* 139, 6780–6786 (2017).

41. Mirts, E. N., Petrik, I. D., Hosseinzadeh, P., Nilges, M. J. & Lu, Y. A designed heme-[4Fe-4S] metalloenzyme catalyzes sulfite reduction like the native enzyme. *Science* 361, 1098–1101 (2018).

42. Jindal, G., Slanska, K., Kolev, V., Damborsky, J., Prokop & Warshel, A. Exploring the challenges of computational enzyme design by rebuilding the active site of a dehalogenase. *Proc. Natl. Acad. Sci. U. S. A.* 116, 389–394 (2019).

43. Alonso, S., Santiago, G., Cea-Rama, I., Fernandez-Lopez, L., Coscolín, C., Modregger, J., Ressmann, A. K., Martínez-Martínez, M., Marrero, H., Bargiela, R., Pita, M., Gonzalez-Alfonso, J. L., Briand, M. L., Rojo, D., Barbas, C., Plou, F. J., Golyshin, P. N., Shahgaldian, P., Sanz-Aparicio, J., Guallar, J. & Ferrer, M. Genetically engineered proteins with two active sites for enhanced biocatalysis and synergistic chemo- and biocatalysis. *Nat. Catal.* 3, 319–328 (2020).

44. Liu, D. S., Nivón, L. G., Richter, F., Goldman, P. J., Deerinck, T. J., Yao, J. Z., Richardson, D., Pipps, W. S., Ye, A. Z., Ellisman, M. H., Drennan, C. L., Baker, D. & Ting, A. Y. Computational design of a red fluorophore ligase for site-specific protein labeling in living cells. *Proc. Natl. Acad. Sci. U. S. A.* 111, E4551-E4559 (2014).

45. Santiago, G., de Salas, F., Lucas, M. F., Monza, E., Acebes, S., Martinez, A. T., Camarero, S. & Guallar, V. Computer-aided laccase engineering: toward biological oxidation of arylamines. *ACS Catal.* 6, 5415–5423 (2016).

46. He, J., Huang, X., Xue, J. & Zhu, Y. Computational redesign of penicillin acylase for cephradine synthesis with high kinetic selectivity. *Green Chem.* 20, 5484–5490 (2018).

47. Lu, X., Liu, Y., Yang, Y., Wang, S., Wang, Q., Wang, X., Yan, Z., Cheng, J., Liu, C., Yang, X., Luo, H., Yang, S., Gou, J., Ye, L., Lu, L., Zhang, Z., Guo, Y., Nie, Y., Lin, J., Li, S., Tian, C., Cai, T., Zhuo, B., Ma, H., Wang,
W., Ma, Y, Liu, Y., Li, Y. & Jiang, H. Constructing a synthetic pathway for acetyl-coenzyme A from one-carbon through enzyme design. *Nat. Commun.* **10**, 1378 (2019).