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# An engineered aryl acid adenylation domain with a capacious active site microenvironment

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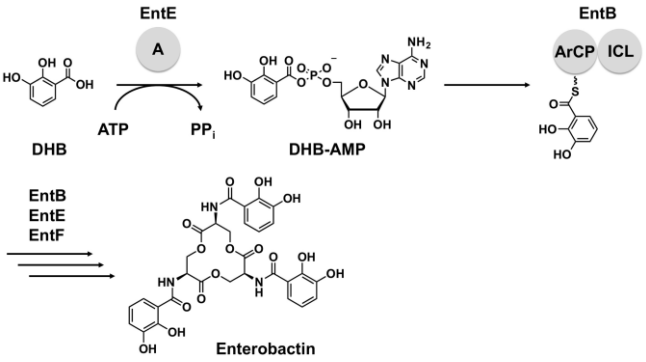
***Abstract:*** *Adenylation (A) domains act as the gatekeepers of non-ribosomal peptide synthetases (NRPSs) ensuring the activation and thioesterification of the correct amino acid/aryl acid building blocks. Aryl acid building blocks are most commonly observed in iron-chelating siderophores, but are not limited to them. Very little is known about the reprogramming of aryl acid A-domains. Here we show that a single asparagine-to-glycine mutation in an aryl acid A-domain creates novel enzyme specificities toward a wide range of non-native aryl acids. The engineered catalyst is capable of activating the non-native aryl acids functionalized with nitro, cyano, bromo, and iodo, even though no enzymatic activity of wild-type enzyme was observed toward these substrates. Co-crystal structures with non-hydrolysable aryl-AMP analogues revealed the origins of substrate promiscuity expansion, highlighting an enlarged substrate binding pocket of the enzyme. Our finding may be exploited to produce diversified aryl acid-containing natural products and serve as a template for further directed evolution in combinatorial biosynthesis.*

Peptide-based natural products known as non-ribosomal peptides (NRPs) are one of the most promising resources for drug discovery and development owing to a broad range of biologically important properties, including antimicrobial, anticancer, and immunosuppressive activities.[1] They are biosynthesized by multimodular enzymes known as non-ribosomal peptide synthetases (NRPSs).[2] Each module is responsible for selection, activation, and incorporation of a building block of the growing intermediate. Common to all NRPS machinery are the peptidyl carrier protein (PCP) and adenylation (A) domains; their activities are essential for incorporation of amino acid building blocks into the NRPS machinery. The A-domain activates an amino acid substrate at the expense of adenosine triphosphate (ATP) and facilitates covalent attachment of it to the *holo*-PCP at the thiol group of 4′-phosphopantetheine (Ppant), a cofactor post-translationally attached to a conserved Ser on the PCP. The A-domain catalyst serves as an entry point for an amino acid into NRPS machinery. Therefore, the A-domain makes them attractive targets for the engineering of NRPS machinery for the production of non-native NRPs.[3] Many strategies have been developed, including precursor-directed biosynthesis,[4] mutasynthesis,[5] active-site manipulation,[6] directed evolution,[7] and swapping domains and modules.[8,9]

Aryl acids such as the common 2,3-dihydroxybenzoic acid (DHB) and salicylic acid (Sal) of siderophores, are an important class of NRP building blocks,[10] but not limited to them. The unique building blocks including anthranilic acid (Ant), *p*-aminobenzoic acid, and 3-hydroxypicolic acid have been observed in acetylaszonalenin,[11] albicidin,[12] and pyridomycin,[13] respectively. Our knowledge about the non-ribosomal codes toward aryl acid building blocks is much more limited. Engineering efforts focusing on aryl acid A-domains are therefore limited so far. The recognition properties of DhbE, an aryl acid A-domain from the siderophore bacillibactin biosynthetic pathway, has been manipulated from DHB toward Ant with a 206-fold specificity switch via directed evolution based on yeast display technology.[14] The substrate specificity of a NRPS-like protein that can activate 5-methyl orsellinic acid has been engineered toward Ant with a 26-fold improvement in enzyme specificity via bioinformatics analysis and mutagenesis.[15] Aryl acid A-domains frequently display the ability to catalyze adenylation toward a range of structurally related aryl acids.[13,16] The substrate promiscuity would be afforded by a common benzoic acid (BA) recognition moiety. Presumably, the aryl acid A-domains form the fine-tuned active sites to accommodate the differences in substituents between aryl acid substrates by an appropriate cavity and/or a charge. In this study, we show that an engineered archetypal aryl acid A-domain (DHB-specific) exhibits a capacious active site which exhibits novel specificity toward aryl acids containing nitro, cyano, chloro, bromo, and iodo functionalities.




**Scheme 1.** Nonribosomal peptide biosynthesis of the siderophore enterobactin. DHB, 2,3-dihydroxybenzoic acid; ATP, adenosine triphosphate; $PP_i$, pyrophosphoric acid; AMP, adenosine monophosphate; A, adenylation domain; ArCP, aryl carrier protein; ICL, isochorismate lyase.

EntE is part of a three-module NRPS assembly line involving EntB, EntE and EntF; it is responsible for the biosynthesis of the siderophore enterobactin in *Escherichia coli*.[17] EntE is a stand-alone aryl acid A-domain that catalyzes the activation of DHB, and transfers it to the ArCP domain of EntB (Scheme 1). EntE displays the ability to catalyze adenylation toward a few number

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of non-native aryl acids.[18-20] The crystal structure of EntE complexed with the ArCP domain of EntB has been determined with a Sal-based vinylsulfonamide inhibitor (Figure S1A).[21] The crystal structure revealed that the hydroxyl group of the Sal-based inhibitor is hydrogen-bonded to the Asn235 side-chain, demonstrating an essential role for recognition of the 2-OH of aryl acid substrates (Figure S1A). The cocrystal structure of the homologous DhbE and DHB reveals that hydroxyl groups of DHB are hydrogen-bonded to the Ser240O$\gamma$ (3-OH) and Asn235N$\delta$2 (2- and 3-OH) (Figure S1B).[22] Since we intended to open up a cavity large enough to accommodate aryl acids bulkier than DHB and Sal, we focused on optimizing interactions between EntE and the C-2 functionalities in the aryl acid substrates. Replacing the Asn235 with smaller amino acids should overcome the limited size of the substrate binding-pocket. We therefore decided to generate variants (N235G, N235A, N235S, and N235T) (Figures S2-S6). The resulting single variants (N235G, N235A, N235S, and N235T) were individually tested against DHB and Sal substrates (Figures S7–S10, Table S1, and Supplementary Discussion). The N235G variant had a smaller effect on the $k_{cat}$ and $K_m$ values measured for DHB and Sal substrates, making it the most active catalyst toward both substrates in the mutant enzymes tested. It would be expected that the N235G mutation affords a capacious active site that is capable of accommodating a large variety of aryl acids with different functionalities bulkier than hydroxyl group at the 2-position of BA.

We next determined the substrate profiles of wtEntE and the N235G variant toward a group of 22 structurally related BA derivatives with different modifications at the 2- or 3-positions using a nonradioactive high-throughput malachite green colorimetric assay[23] (Figure 1 and Supplementary Discussion).

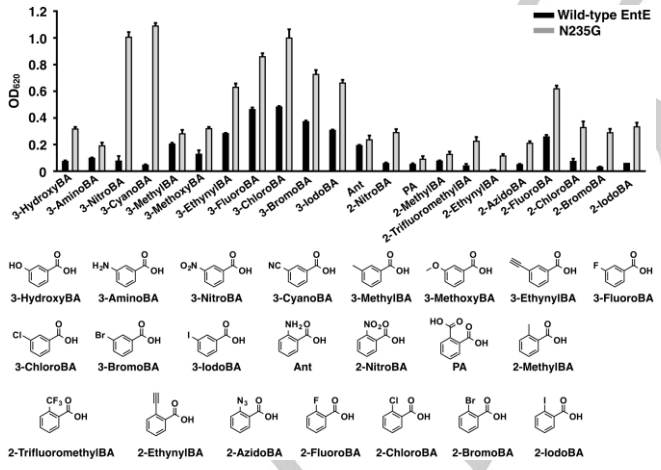


**Figure 1.** Substrate profiles of wtEntE and the N235G variant. Wild-type EntE and the mutant EntE (N235G) were used at 1 μM with 1 mM of aryl acid substrates described. Controls were treated identically except no substrates were added to the reaction buffer. To estimate the relative adenylation activities, we subtracted the $A_{620}$ value of the reaction mixture without substrate from the $A_{620}$ values of reaction mixtures containing substrate. Shown are average values from two independent experiments in duplicate. To be considered an active substrate, $OD_{620}$ values > 0.2 were required. It should be noted that all aryl acid substrates were active at least to some extent.

To obtain a detailed understanding of substrate selectivity and enzyme active-site microenvironments, we determined catalytic parameters of the adenylation reaction catalyzed by wtEntE and the N235G variant using 12 BA derivatives (Figures S11–S22 and Tables 1, S2, and S3). The members of group I were categorized as BA derivatives with modifications at the 3-position. wtEntE afforded calculated $k_{cat}/K_m$ values of 2.1, 9.9, 14, 3.2, and 1.3 $mM^{-1}$ $min^{-1}$ toward 3-ethynylBA, 3-fluoroBA, 3-chloroBA, 3-bromoBA, and 3-iodoBA, respectively. In contrast, the $k_{cat}/K_m$ values of the N235G variant with 3-ethynylBA, 3-fluoroBA, 3-chloroBA, 3-bromoBA, and 3-iodoBA were 16, 24, 13, 11, and 23 $mM^{-1}$ $min^{-1}$, respectively. These changes correspond to 30-, 10-, 3.8-, 14-, and 72-fold switch in substrate specificity relative to wtEntE. The N235G variant efficiently catalyzed the adenylation reaction of 3-nitroBA ($k_{cat}/K_m$ = 12 $mM^{-1}$ $min^{-1}$) and 3-cyanoBA ($k_{cat}/K_m$ = 18 $mM^{-1}$ $min^{-1}$), whereas wtEntE showed no catalytic activity toward these substrates. The members of group II were categorized as BA derivatives with modifications at the 2-position. The $k_{cat}/K_m$ value of the N235G variant with the 2-fluoro substituent was 5.7 $mM^{-1}$ $min^{-1}$ comparable to that of wtEntE, which resulted in a nearly 5-fold switch in substrate specificity. In contrast, the N235G variant displayed a lower $K_m$ value for 2-chloroBA ($K_m$ = 28 ± 6 μM), which is approximately 90-fold lower than that of wtEntE with 2-chloroBA. Our kinetics data indicate that the N235G variant switched the enzyme specificity 203-fold toward 2-chloroBA. Furthermore, the N235G variant afforded significant changes in substrate specificity toward 2-nitroBA ($k_{cat}/K_m$ = 2.4 $mM^{-1}$ $min^{-1}$), 2-bromoBA ($k_{cat}/K_m$ = 3.8 $mM^{-1}$ $min^{-1}$) and 2-iodoBA ($k_{cat}/K_m$ = 2.2 $mM^{-1}$ $min^{-1}$) with bulky 2-position substituents, albeit with wtEntE having no catalytic activity toward these substrates.

To test whether the N235G variant could interact with downstream domains and process non-native aryl acid substrates, we used an ArCP domain of EntB from the enterobactin synthetase (Scheme 1). We examined the transfer of 11 non-native aryl acids (3-nitroBA, 3-cyanoBA, 3-ethynylBA, 3-fluoroBA, 3-chloroBA, 3-bromoBA, 2-fluoroBA, 2-chloroBA, 2-bromoBA, 2-iodoBA, and 2-nitroBA) and 2 native substrates (DHB and Sal) to the Ppant group of the ArCP catalyzed by the N235G variant using MALDI-TOF-MS (Figures S23–S35). A MALDI assay showed that the N235G variant is capable of loading ≥ 90% of the ArCP domain of EntB with the substrates (DHB, Sal, 3-ethynylBA, 3-fluoroBA, 3-chloroBA, 2-fluoroBA, 2-chloroBA, 2-bromoBA, 2-iodoBA, and 2-nitroBA). In contrast, the N235G variant gave 50 to 70% loading of the ArCP domain of EntB with 3-nitroBA, 3-cyanoBA, and 3-bromoBA. This may be because ArCP-Ppant-aryl acid species could be easily hydrolyzed to ArCP-Ppant given by their instability resulting from electron-withdrawing substituents at the 3-position.

To understand the structural basis for this promising specificity, we solved X-ray crystal structures of the N235G variant complexed with non-hydrolysable aryl-AMP analogues (aryl-AMSs). We therefore synthesized a set of aryl-AMSs using DHB, Sal, 3-nitroBA, 3-cyanoBA, 2-nitroBA, 2-chloroBA, and 2-bromoBA as ligands (Scheme S1) and subsequently constructed inhibition profiles of wtEntE and the N235G variant (Figures S36–S43 and Table S4). We chose 3-cyanoBA-AMS and 2-nitroBA-AMS for co-crystallization with the N235G variant because of tight- and specific-binding properties toward the

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N235G variant, with calculated $K_i^{app}$ values of 183 ± 28 nM and 50 ± 3 nM, respectively (Figure 2A and Table S4), which should allow to provide a readout of the molecular basis for the altered specificity.

**Table 1.** Catalytic parameters of the adenylation reaction catalyzed by wtEntE and the N235G variant.[a]

| Vari. | Substr. | $K_m$ [μM] | $k_{cat}$ [$min^{-1}$] | $k_{cat}/K_m$ [$mM^{-1}$ $min^{-1}$] | Specificity [b] | Specificity switch[c] |
|---|---|---|---|---|---|---|
| WT[d] | DHB | 2.0 ± 0.8 | 2.6 ± 0.2 | 1311 | 1 | - |
| WT[d] | Sal | 28 ± 8 | 11 ± 0.9 | 412 | 0.31 | - |
| WT | 3-NitroBA | n.d.[e] | n.d.[e] | n.d.[e] | n.d.[e] | - |
| WT | 3-CyanoBA | n.d.[e] | n.d.[e] | n.d.[e] | n.d.[e] | - |
| WT | 3-EthynylBA | 1027 ± 221 | 2.2 ± 0.2 | 2.1 | $1.6\times10^{-3}$ | - |
| WT | 3-FluoroBA | 965 ± 129 | 9.5 ± 0.6 | 9.9 | $7.5\times10^{-3}$ | - |
| WT | 3-ChloroBA | 728 ± 58 | 10 ± 0.3 | 14 | $1.0\times10^{-2}$ | - |
| WT | 3-BromoBA | 738 ± 158 | 2.3 ± 0.2 | 3.2 | $2.4\times10^{-3}$ | - |
| WT | 3-IodoBA | 1987 ± 293 | 2.6 ± 0.2 | 1.3 | $1.0\times10^{-3}$ | - |
| WT | 2-NitroBA | n.d.[e] | n.d.[e] | n.d.[e] | n.d.[e] | - |
| WT | 2-FluoroBA | 388 ± 90 | 1.1 ± 0.09 | 2.8 | $2.2\times10^{-3}$ | - |
| WT | 2-ChloroBA | 2574 ± 1017 | 0.76 ± 0.19 | 0.30 | $2.3\times10^{-4}$ | - |
| WT | 2-BromoBA | n.d.[e] | n.d.[e] | n.d.[e] | n.d.[e] | - |
| WT | 2-IodoBA | n.d.[e] | n.d.[e] | n.d.[e] | n.d.[e] | - |
| NG[g] | DHB | 5.2 ± 0.9 | 1.7 ± 0.08 | 318 | 1 | - |
| NG[g] | Sal | 22 ± 4 | 3.4 ± 0.2 | 157 | 0.49 | 1.6 |
| NG[g] | 3-NitroBA | 544 ± 99 | 6.3 ± 0.4 | 12 | $3.7\times10^{-2}$ | -[f] |
| NG[g] | 3-CyanoBA | 471 ± 36 | 8.3 ± 0.2 | 18 | $5.6\times10^{-2}$ | -[f] |
| NG[g] | 3-EthynylBA | 95 ± 26 | 1.5 ± 0.1 | 16 | $4.9\times10^{-2}$ | 30 |
| NG[g] | 3-FluoroBA | 123 ± 21 | 2.9 ± 0.2 | 24 | $7.5\times10^{-2}$ | 10 |
| NG[g] | 3-ChloroBA | 176 ± 19 | 2.2 ± 0.07 | 13 | $4.0\times10^{-2}$ | 3.8 |
| NG[g] | 3-BromoBA | 196 ± 61 | 2.2 ± 0.2 | 11 | $3.5\times10^{-2}$ | 14 |
| NG[g] | 3-IodoBA | 60 ± 6 | 1.4 ± 0.03 | 23 | $7.2\times10^{-2}$ | 72 |
| NG[g] | 2-NitroBA | 241 ± 39 | 0.58 ± 0.03 | 2.4 | $7.5\times10^{-3}$ | -[f] |
| NG[g] | 2-FluoroBA | 265 ± 52 | 1.5 ± 0.1 | 5.7 | $1.8\times10^{-2}$ | 8.3 |
| NG[g] | 2-ChloroBA | 28 ± 6 | 0.41 ± 0.02 | 15 | $4.6\times10^{-2}$ | 203 |
| NG[g] | 2-BromoBA | 160 ± 38 | 0.60 ± 0.04 | 3.8 | $1.2\times10^{-2}$ | -[f] |
| NG[g] | 2-IodoBA | 354 ± 60 | 0.78 ± 0.04 | 2.2 | $6.9\times10^{-3}$ | -[f] |

[a] Catalytic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[24] For additional kinetics data and experimental errors, see Figures S11–S22 and Tables S2 and S3. [b] Specificity refers to the relative specificity for the indicated substrates with respect to DHB: $(k_{cat(substrate)}/K_{m(substrate)})/(k_{cat(DHB)}/K_{m(DHB)})$. [c] The specificity switch shows $specificity_{(N235G)}/specificity_{(wt)}$ for the indicated aryl acid. [d] Values taken from ref. [25]. [e] n.d.: not determinable. [f] We could not estimate specificity switch for these substrates because of the lack of wtEntE activities toward the corresponding substrates. [g] N235G.

The structures of EntE N235G proteins in complex with 3-cyanoBA-AMS (EntE $(N235G)_{3\text{-cyanoBA-AMS}}$, PDB 6IYL) and 2-nitroBA-AMS (EntE $(N235G)_{2\text{-nitroBA-AMS}}$, PDB 6IYK) have been determined at 2.56 Å and 2.45 Å resolution, respectively (Figures S44–S46 and Table S5). The overall structures of these EntE (N235G) complexes are almost identical to the EntE component of EntE-EntB (ArCP) complex (root mean square deviation of 0.44–0.45 Å for Cα atoms of chain A). Recent structural studies demonstrated that the multiple distinct conformations adopted by A-domains guide transfer of intermediates between domains in their catalytic cycles.[21,26,27] The N235G variant structures show a thioester-forming conformation that would be adopted to catalyze the covalent attachment of aryl acid units onto ArCP, as observed in the structure of the EntE-EntB (ArCP) complex (Figure S44). The N235G substitution enlarges the substrate binding site by ~25 $Å^3$ compared to that of wtEntE, providing a cavity for bulky substituents at 2- or 3-positions of BA (Figures 2B–D and S45). To obtain detailed insights into the substrate recognition mechanism of the N235G variant, we compared the positions of aryl-AMS ligands in these N235G variant structures. The AMS moieties of both aryl-AMS ligands occupy almost the same position in the nucleoside-binding pocket of wtEntE (Figures S46), whereas the position of the aryl acid moiety is slightly different between these two structures (Figure S46C). The position of the 2-nitroBA moiety in the EntE $(N235G)_{2\text{-nitroBA-AMS}}$ structure is similar to that of the Sal moiety in the EntE-EntB (ArCP) structure (Figure S46B), in which the 2-hydroxy group of the Sal moiety orients toward the Asn235 side-chain through formation of a hydrogen bond. The 2-nitro group of the 2-nitroBA moiety forms hydrogen bonding interactions with the backbone amide groups of Tyr236 and Ala335, and it orients toward the space created by the N235G substitution in the EntE $(N235G)_{2\text{-nitroBA-AMS}}$ structure. In contrast, the orientation of the 3-cyanoBA moiety in the EntE $(N235G)_{3\text{-cyanoBA-AMS}}$ structure is relatively different (Figure S46A). The aromatic ring of the 3-cyanoBA moiety rotates ~20º, probably because of steric hindrance with the side-chain of Ser240. This rotation allows the 3-cyano group of 3-cyanoBA moiety to orient toward the space created by the N235G substitution. Thus, the N235G substitution is sufficient to accommodate the bulky substituents at the 2- or 3-positions of BA.

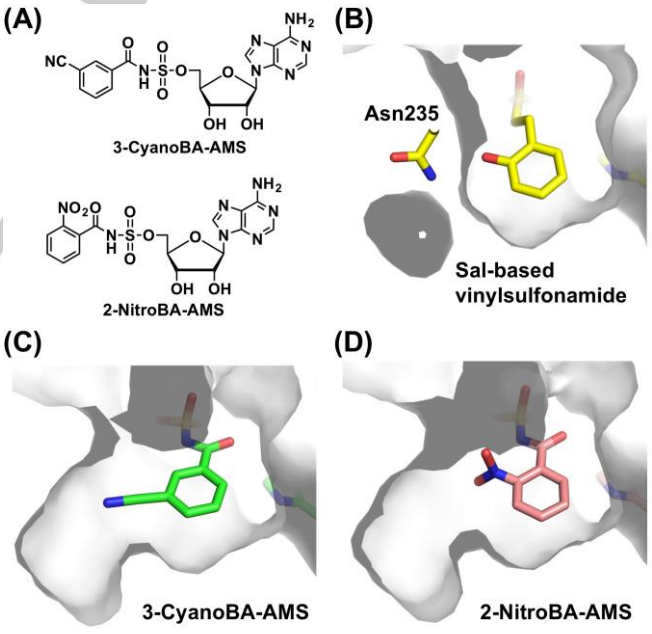


**Figure 2.** Structural analysis of the N235G variant. (A) Structures of Non-hydrolysable aryl-AMP analogues 3-cyanoBA-AMS and 2-nitroBA-AMS. (B) The substrate-binding pocket of the EntE-EntB (ArCP) complex (PDB code: 3RG2) using a Sal-based vinylsulfonamide inhibitor.[20] The Asn235 side-chain and Sal-based vinylsulfonamide inhibitor are shown as sticks. (C) The substrate-binding pocket of EntE $(N235G)_{3\text{-cyanoBA-AMS}}$ (PDB 6IYL) complexed with 3-cyanoBA-AMS. (D) The substrate-binding pocket of EntE $(N235G)_{2\text{-nitroBA-AMS}}$ (PDB 6IYK) complexed with 2-nitroBA-AMS. The 3-cyanoBA-AMS and 2-nitroBA-AMS molecules are shown as sticks.

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In this study, we describe the engineering of a DHB-activating A-domain EntE and biochemical and structural analysis of the N235G variant, a promiscuous aryl acid A-domain. We demonstrated that the N235G variant is capable of accepting a range of non-native aryl acids as a substrate and transferring them to the ArCP domain of EntB. Furthermore, crystal structures of the N235G variant complexed with aryl-AMSs revealed an enlarged recognition pocket for non-native aryl acids with diverse functionalities, providing new insight into the structural basis for creating enzyme substrate flexibility.

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** non-ribosomal peptide synthetase • aryl acid adenylation domain • engineering • mutagenesis • structural biology

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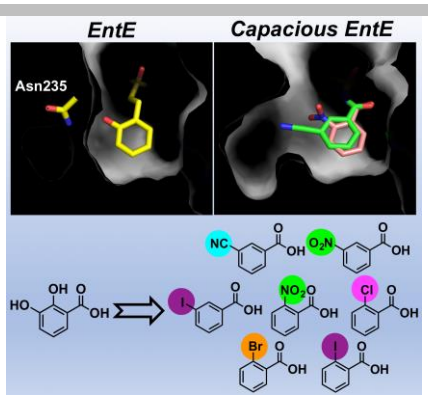
# COMMUNICATION

**A capacious pocket:** A single mutation in an aryl acid adenylation domain creates novel enzyme specificities toward a wide range of non-native aryl acids. In particular, the engineered variant is capable of activating the non-native aryl acids functionalized with cyano, nitro, bromo, and iodo, albeit with the wild-type enzyme having no activity toward these substrates. Co-crystal structures with non-hydrolysable aryl-AMP analogues revealed the origins of the substrate promiscuity, illuminating the enlarged active site of the variant.

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**An engineered aryl acid adenylation domain with a capacious active site microenvironment**

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