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Reprogramming Nonribosomal Peptide Synthetases for "Clickable" Amino Acids**

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Abstract: *Nonribosomal peptide synthetases (NRPSs) are multifunctional enzymes that produce a wide array of bioactive peptides. Here we show that a single tryptophan-to-serine mutation in phenylalanine-specific NRPS adenylation domains enables the efficient activation of non-natural aromatic amino acids functionalized with azide and alkyne groups. The resulting 10^5-fold switch in substrate specificity was achieved without appreciable loss of catalytic efficiency. Moreover, the effective communication of the modified A domains with downstream modules in dipeptide synthetases permitted incorporation of O-propargyl-L-tyrosine into diketopiperazines both in vitro and in vivo, even in the presence of competing phenylalanine. Because azides and alkynes readily undergo bioorthogonal click reactions, reprogramming NRPSs to accept non-natural amino acids that contain these groups provides a potentially powerful means of isolating, labeling, and modifying biologically active peptides.*

Methods for chemoselectively labeling biomolecules have become indispensible in modern chemical biology.[1] Bioorthogonal reactions, such as copper(I)-catalyzed and strain-promoted Huisgen cyclizations between alkynes and azides, also known as "click" reactions,[2] are particularly useful in this regard. In addition to being selective and high-yielding, such reactions tolerate a broad range of functional groups. They are consequently widely used to isolate, visualize, and otherwise modify individual molecules in complex biological samples.

Diverse strategies have been developed to equip biomolecules with alkyne and azide functionalities. For example, codon reassignment and nonsense suppression enable efficient ribosomal production of proteins containing amino acids with azide or alkyne side chains.[3] Click building blocks have also been incorporated into DNA and cell-surface glycans.[4] Even complex, drug-related alkaloid or polyketide natural products can be endowed with "clickable" alkyne groups,[5] thereby facilitating biological studies and the search for new drug candidates.[6]

Nonribosomal peptides (NRPs), an important source of antibiotics and other biologically active agents, comprise a particularly diverse class of natural products. They are produced by mega-enzymes, called nonribosomal peptide synthetases (NRPSs), that function as biosynthetic assembly lines (Figure 1).[7] NRPSs consist of dedicated protein modules, strung together like beads on a string, which are responsible for activating the amino acid building blocks and incorporating them into the growing peptide chain. The number, nature, and order of the individual modules determine the length and composition of the final natural product. Such modularity has fueled efforts to produce novel bioactive peptides by combinatorial biosynthesis, for example by engineering, exchanging, or concatenating individual NRPS domains or entire modules.[8]

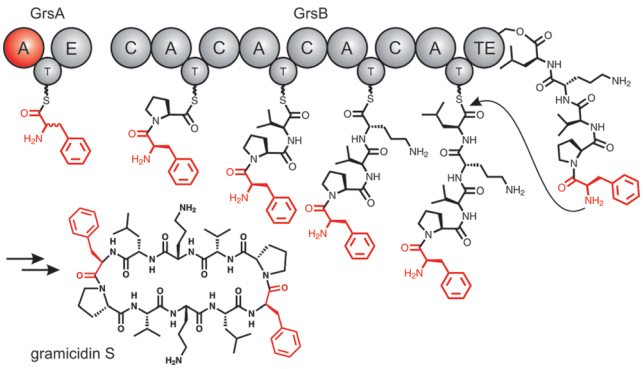

Figure 1. Nonribosomal synthesis of the antibiotic gramicidin S. A = adenylation domain; T = thiolation domain; E = epimerization domain; C = condensation domain; TE = thioesterase domain.[7] The phenylalanine residue encoded by A domain $GrsA_A$ in the first module is highlighted in red.

Although alkyne-functionalized chemical probes have been used to assay the substrate specificities of NRPS adenylation (A) domains,[9] a general method for click functionalization of NRPs does not exist. As A domains dictate molecular recognition in these systems, they could conceivably be adapted for this purpose. Indeed, sequence patterns deduced by comparisons of homologous active sites have proven useful for predicting as well as altering A domain specificity.[8b,10] Some conservative changes in substrate preference, such as L-Asp to L-Asn or 3-methyl-Glu to 3-methyl-Gln, are relatively easy to achieve by targeted mutagenesis of

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residues lining the substrate binding pocket.[8b,10b,d] However, redesigning A domains for recognition of non-cognate amino acids that exhibit larger differences in size or charge is generally more difficult, and adenylation efficiency often drops substantially.[10e,f] These challenges notwithstanding, we show here that only minimal changes are needed to reengineer phenylalanine-specific A domains for the efficient incorporation of aromatic amino acids containing alkyne and azide functional groups into NRPs.

To expand the recognition properties of $GrsA_A$, a phenylalanine-specific A domain (PheA) from gramicidin S synthetase (Figure 1),[7b,11] eight active-site residues known to correlate with substrate specificity[10a,b] were chosen for cassette mutagenesis (Figure 2 A,B). The resulting single-mutant libraries were individually screened against the twenty proteinogenic amino acids using a microtiter-plate-based pyrophosphate exchange assay for adenylation activity.[10e,12] Replacing Trp239, located at the bottom of the PheA recognition pocket, with smaller amino acids afforded promising changes in substrate specificity. Substituting tryptophan with serine (W239S), for example, resulted in an approximately three times higher preference for L-Tyr over L-Phe, which corresponds to an 800-fold switch in specificity relative to wild-type (wt) $GrsA_A$. The k_{cat}/K_M value for L-Tyr activation by this variant is $600\ mM^{-1}min^{-1}$, only 40 times below the k_{cat}/K_M determined for L-Phe with the wild-type enzyme (Table 1).

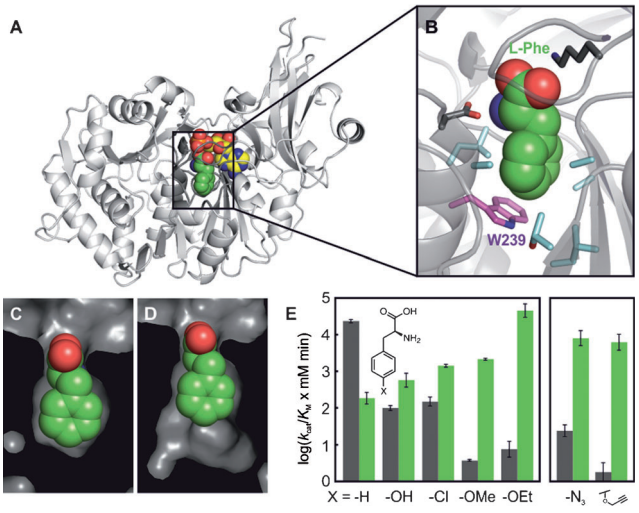

Figure 2. Engineering the $GrsA_A$ binding pocket. A) Crystal structure of $GrsA_A$ (PDB code 1AMU)[11] with bound L-Phe (green carbon atoms) and adenosine monophosphate (yellow carbon atoms). B) Residues lining the L-Phe binding pocket are shown as stick models. The amino acids in cyan and magenta (Ala236, Trp239, Thr278, Ile299, Ala301, Ala322, Ile330, and Cys331) were individually subjected to cassette mutagenesis; Cys331 is hidden behind the substrate. Asp235 and Lys517 (gray), important for catalysis, were not mutated. Adenosine monophosphate was omitted for clarity. C) Cut-away view of the binding pocket in the plane of the phenyl ring. D) PyMOL model of the W239S variant. E) Adenylation kinetics of $GrsA_A$ (gray) and $GrsA_A$-W239S (green) with a range of aromatic amino acid substrates, including the click amino acids *p*-azido-L-Phe and *O*-propargyl-L-Tyr. See Table 1 and Table S1 in the Supporting Information for details.

Table 1: Catalytic parameters of the adenylation reaction catalyzed by $GrsA_A$ and $GrsA_A$-W239S.[a]

Variant	Substrate	k_{cat} [min^{-1}]	k_{cat}/K_M [$mM^{-1}\ min^{-1}$]	Specificity[b]	Specificity switch[c]
wt	L-Phe	300	25 000	1	1
wt	*p*-azido-L-Phe	200	25	10^{-3}	–
wt	*O*-propargyl-L-Tyr	30	2	10^{-5}	–
W239S	L-Tyr	230	600	3.2	800
W239S	*p*-azido-L-Phe	200	9000	47	5×10^4
W239S	*O*-propargyl-L-Tyr	190	7000	37	5×10^5

[a] Catalytic parameters were determined by a pyrophosphate exchange assay.[10e,12] For additional kinetic data and experimental errors see Table S1. [b] Specificity: k_{cat}/K_M (target substrate)/k_{cat}/K_M (L-Phe). [c] Specificity switch: $(specificity)_{mut}/(specificity)_{wt}$ for the indicated amino acid.

Simple modeling suggested that the W239S mutation opens up a cavity large enough to accommodate amino acids even bulkier than tyrosine (Figure 2 C and D). Although L-Trp was inefficiently adenylated ($k_{cat}/K_M = 36\ mM^{-1}min^{-1}$), phenylalanine derivatives bearing bulky *para* substituents were excellent substrates. Adenylation rates increased with the size of the *para*-substituent in the order H < OH < Cl < OMe < OEt (Figure 2 E; Table S1). The *p*-ethoxy substituent afforded remarkably high activity ($k_{cat}/K_M = 50000\ mM^{-1}min^{-1}$), greater than that of wt $GrsA_A$ with L-Phe (k_{cat}/K_M=25 000 $mM^{-1}min^{-1}$). The variations in adenylation activity manifest in these k_{cat}/K_M values largely reflect differences in K_M for the different amino acids. Under saturating conditions at high substrate concentration, all turnover numbers, including those with L-Trp, were in the range of $GrsA_A$ with L-Phe, i.e. 10^2 to $10^3\ min^{-1}$ (Table S1).

The capacious $GrsA_A$-W239S binding site also supported efficient activation of click amino acids containing azide and alkyne functionality. Thus, *p*-azido-L-Phe and *O*-propargyl-L-Tyr, which are poorly processed by wt $GrsA_A$, serve as excellent substrates for the mutant PheA domain (Figure 2 E). For example, $GrsA_A$-W239S adenylated *O*-propargyl-L-Tyr 10 times more rapidly than L-Tyr, the best naturally occurring amino acid substrate, and 40 times faster than L-Phe. These changes, corresponding to a 5×10^5-fold switch in enzyme specificity (Table 1), were achieved with little loss in catalytic efficiency, judging from the steady-state parameters for the mutant enzyme with the non-natural substrate ($k_{cat} = 190\ min^{-1}$ and $k_{cat}/K_M = 7000\ mM^{-1}min^{-1}$) relative to wt $GrsA_A$ with L-Phe ($k_{cat} = 300\ min^{-1}$ and $k_{cat}/K_M = 25000\ mM^{-1}min^{-1}$).

Adenylation activity alone is insufficient for nonribosomal peptide synthesis with unnatural building blocks; permissivity of downstream domains is also essential. To test whether the modified $GrsA_A$ domain can communicate with other domains and modules, we employed a truncated GrsA/GrsB1 dipeptide synthetase excised from the gramicidin S NRPS (Figure 3).[13,14] In addition to the PheA domain, the complete GrsA initiation module contains a thiolation (T) and an epimerase (E) domain, whereas GrsB1 is a truncated elongation module containing a proline-specific A domain, a T domain, and a condensation (C) domain. The wild-type dipeptide synthetase loads L-Phe and L-Pro onto their

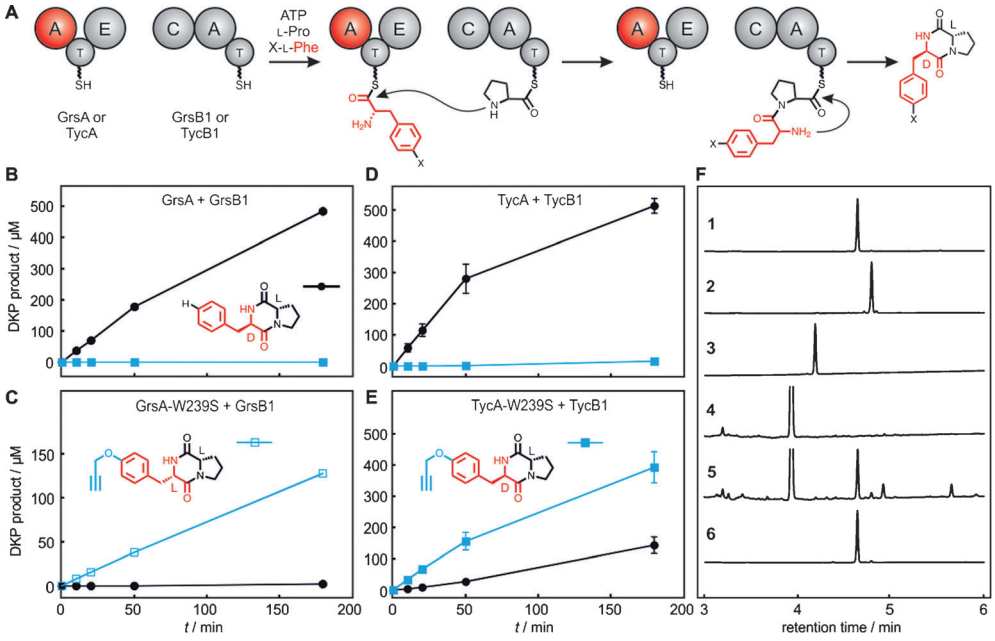

Figure 3. Nonribosomal synthesis of propargylated DKP. A) Mechanism of nonribosomal DKP formation by the truncated NRPSs. B–E) Kinetics of DKP formation from 500 µM L-Pro, L-Phe, and *O*-propargyl-L-Tyr as competing substrates were determined with different dimodular synthetases: B) GrsA/GrsB1, C) GrsA-W239S/GrsB1, D) TycA/TycB1, and E) TycA-W239S/TycB1. k_{obs} values are summarized in Table S2. Concentrations of D-Phe-L-Pro DKP (black circles), *O*-propargyl-L-Tyr-L-Pro DKP (open cyan squares), and *O*-propargyl-D-Tyr-L-Pro DKP (cyan squares) were determined by UPLC. F) UPLC traces of DKP standards (1: *O*-propargyl-D-Tyr-L-Pro DKP, 2: *O*-propargyl-L-Tyr-L-Pro DKP, 3: D-Phe-L-Pro DKP) and propargyl DKP produced in vivo (4: reaction t = 0 h, 5: t = 24 h, 6: purified product). The peak at 3.9 minutes corresponds to *O*-propargyl-L-Tyr. Traces were normalized to the height of the DKP peak.

respective T domains and, following epimerization of phenylalanine and peptide bond formation at the active site of the C domain, affords a D-Phe-L-Pro thioester that is spontaneously released as a D-Phe-L-Pro diketopiperazine (DKP; Figure 3A).[13,14]

Phosphopantetheinylated GrsA-W239S and GrsB1 modules were produced in *E. coli* strain HM0079, which endogenously expresses phosphopantethein transferase Sfp.[15] The proteins were purified by NiNTA affinity chromatography and assayed for DKP synthesis. In the presence of equimolar amounts of *O*-propargyl-L-Tyr, L-Phe, and L-Pro, the GrsA-W239S/GrsB1 synthetase generated propargylated DKP only 4.5 times more slowly than wt GrsA/GrsB1 produced D-Phe-L-Pro DKP under identical conditions (Figure 3B,C). However, in contrast to the 98:2 preference for the D,L over the L,L product exhibited by the wild-type synthetase,[16] the variant afforded predominantly L,L-configured DKP. Only around 10% of the D,L product isomer was observed, indicating inefficient epimerization of *O*-propargyl-L-Tyr by the initiation module. The propargyloxy substituent may interfere with binding to the epimerase or, alternatively, override the inherent stereochemical preference of the downstream C domain for a D-configured aminoacyl thioester.[13] Damage to the E domain can be ruled out by the fact that, in the absence of *O*-propargyl-L-Tyr, GrsA-W239S/GrsB1 converts L-Phe and L-Pro quantitatively to the corresponding epimerized product within one day. Despite only partial epimerization, these results clearly demonstrate that the mutant A domain is competent for peptide chain elongation. Moreover, the specificity observed for the isolated domain was maintained in the context of the model synthetase. When both amino acids were present in equal amounts under competitive conditions, the mutant synthetase preferentially incorporated *O*-propargyl-L-Tyr into the DKP product by a factor of roughly 70:1, exceeding the adenylation specificity observed with the isolated $GrsA_A$-W239S domain.

As Trp239 is a common residue in PheA domains (Table S3), the specificity switch achieved by mutating this residue is potentially general. The portability of the W239S substitution was tested with the homologous TycA protein from tyrocidine synthetase,[17] which shares 62% sequence identity with GrsA. The resulting variant displayed high turnover with *O*-propargyl-L-Tyr in the pyrophosphate exchange assay (k_{cat} = 280 min^{-1}), but its catalytic efficiency, judged by the k_{cat}/K_M parameter (850 $mM^{-1}min^{-1}$), was somewhat lower than that with $GrsA_A$-W239S (Table S1). Nevertheless, TycA-W239S exhibited an eight-fold preference for *O*-propargyl-L-Tyr over L-Phe. Moreover, it successfully communicated with the downstream proline-specific TycB1 module to produce propargylated DKP, even in the presence of competing L-Phe (Figure 3D,E). The initial rate of DKP formation was four times faster than for GrsA-W239S/GrsB1 and only 1.7 times slower than the natural reaction sequence catalyzed by wt TycA/TycB1 (Table S2). In this case, the unnatural substrate was properly epimerized by the TycA E domain prior to chain elongation to give propargylated DKP in a D,L/L,L ratio of 95:5, similar to that observed for the wild-type dimodule.[16]

Intracellular peptide biosynthesis constitutes the most stringent test for incorporation of non-natural building blocks into NRPs. Despite potential interference from competing endogenous amino acids, the activity and selectivity of the engineered TycA-W239S/TycB1 synthetase enabled efficient in vivo production of propargylated peptide. Following 24 hour incubation with *O*-propargyl-L-Tyr (500 µM) and L-Pro (500 µM) at 37 °C, propargylated DKP was isolated from a bacterial culture (1 L) co-expressing TycA-W239S and TycB1. Purification of the organic extract by HPLC afforded 10 mg of the D,L-configured product. The biosynthetic DKP was identical to a chemically synthesized standard, as corroborated by its UPLC retention time (Figure 3F) and

^{1}H NMR spectrum (Figure S1). Its yield also compared favorably with the 9 mg L^{-1} of D-Phe-L-Pro DKP produced by the wild-type enzyme under similar conditions.[15] As D-Phe-L-Pro DKP was barely detectable in the sample, the intracellular concentrations of *O*-propargyl-L-Tyr were evidently high enough to outcompete endogenous L-Phe. Efficient incorporation of this non-standard amino acid into an NRP provides a potentially general means to attach fluorescent labels, simplify purification, and modulate bioactivity through postsynthetic click functionalization.

The W239S mutation is a strikingly non-invasive, yet powerful way to repurpose PheA domains. This single active site substitution creates an enlarged binding pocket that accommodates the unnatural amino acid *O*-propargyl-L-Tyr ($K_M = 30$ μM) without compromising catalysis. Both the activity and selectivity of the mutant for the new substrate are almost on par with the wild-type enzyme. Importantly, too, the downstream thioesterification, epimerization (in one case), and condensation steps readily accommodate the modified substrate. Achieving efficient activation of a clickable amino acid in two homologous synthetases highlights the potential generality of this strategy. Similar reprogramming of other synthetases should be possible, as many NRPSs share the phenylalanine recognition motif utilized by $GrsA_A$ and $TycA_A$. Synthetases that produce the natural products thaxtomin, glycopeptidolipids, barbamides, virginiamycin, mannopeptimycin, and aureobasidin contain homologous PheA domains, as do many synthetases from as yet unassigned gene clusters (Table S3).

Successful reprogramming of $GrsA_A$ and $TycA_A$ through a single active site mutation recalls early studies on phenylalanyl-tRNA synthetase (PheRS), in which the substrate recognition pocket was mutationally enlarged for halogenated phenylalanine analogues by a single alanine-to-glycine mutation.[18] Since then, laboratory evolution of aminoacyl-tRNA synthetases has substantially expanded the ribosomal code for phenylalanine derivatives functionalized for click reactions and other bioorthogonal chemistries.[19] Our results suggest that analogous experiments with NRPS A domains, aided by powerful computational[10f,20] and evolutionary[10e,21] approaches, could similarly expand the nonribosomal code. Tailoring A domain specificity without sacrificing catalytic efficiency should greatly enrich the chemical biology toolkit, enabling production of NRPs containing diverse reactive handles, photoactivatable groups, and spectroscopic probes.

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