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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Communication

Biosynthetic Functionalization of Nonribosomal Peptides

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Cite This: J. Am. Chem. Soc. 2021, 143, 2736−2740
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ABSTRACT: Nonribosomal peptides (NRPs) are a therapeutically important class of secondary metabolites that are produced by modular synthetases in assembly-line fashion. We previously showed that a single Trp-to-Ser mutation in the initial Phe-loading adenylation domain of tyrocidine synthetase completely switches the specificity toward clickable analogues. Here we report that this minimally invasive strategy enables efficient functionalization of the bioactive NRP on the pathway level. In a reconstituted tyrocidine synthetase, the W227S point mutation permitted selective incorporation of Phe analogues with alkyne, halogen, and benzoyl substituents by the initiation module. The respective W2742S mutation in module 4 similarly permits efficient incorporation of these functionalized substrate analogues at position 4, expanding this strategy to elongation modules. Efficient incorporation of an alkyne handle at position 1 or 4 of tyrocidine A allowed site-selective one-step fluorescent labeling of the corresponding tyrocidine analogues by Cu(I)-catalyzed alkyne−azide cycloaddition. By combining synthetic biology with bioorthogonal chemistry, this approach holds great potential for NRP isolation and molecular target elucidation as well as combinatorial optimization of NRP therapeutics.

Genetic code expansion with engineered aminoacyl-tRNA synthetases (aaRSs) has enabled myriad novel chemistries in polypeptides, including site-specific incorporation of bioorthogonal functional groups.[1,2] These chemical handles have since become indispensable tools for studying and modifying peptides and proteins.[3] Similarly, bioorthogonal functionality in nonribosomal peptides (NRPs) would greatly facilitate chemical derivatization of this therapeutically important class of secondary metabolites.[4,5] Unlike ribosomal polypeptides, however, NRPs are produced by a series of multidomain modules that control amino acid loading, modification, and concatenation (Figure 1).[6] The aaRS equivalents in these modular assembly lines are adenylation (A) domains that activate amino acids and tether them to the synthetase. For the biosynthesis of NRPs with bioorthogonal groups, A domains thus need to be repurposed to process the corresponding functionalized building blocks.

In previous work, we showed that a single Trp-to-Ser mutation in the active site of the Phe-specific A domains of GrsA (W239S) and TycA (W227S) switches the substrate preference from native L-Phe to the clickable analogue *O*-propargyl-L-Tyr.[7] To map the potential of the enlarged binding pocket of W227S TycA for accommodating additional bioorthogonal functionalities, we used a pyrophosphate exchange assay[8] to determine the steady-state kinetics for the adenylation of various para-substituted Phe analogues (Figure 2). Beyond clickable amino acids, we tested *p*-bromo-L-Phe and *p*-iodo-L-Phe bearing halogen substituents for palladium-catalyzed cross-coupling reactions[9] as well as the photoreactive amino acid *p*-benzoyl-L-Phe for nonspecific cross-linking to molecular targets and binding partners.[10] For all of these substrates, the W227S point mutation in TycA afforded substantial specificity switches, in particular for the halogenated analogues. Even the bulkiest substrate, *p*-benzoyl-L-Phe, was activated as efficiently as native L-Phe by the variant (Table S1). Although the specific activity decreased substantially in some cases, it is generally not rate-limiting in tyrocidine biosynthesis.

To test the incorporation of these bioorthogonal functionalities into the antibiotic tyrocidine A (**1**), we reconstituted the full nonribosomal synthetase encompassing the initiation module TycA (123 kDa), trimodular TycB (405 kDa), and hexamodular TycC (724 kDa) in vitro (Figure 1).[11] The corresponding genes were cloned by Gibson assembly[12] from the natural producer *Brevibacillus parabrevis* into IPTG-inducible vectors under the control of the strong P_{trc} promoter. The resulting His_6-tagged constructs were produced in *Escherichia coli* HM0079,[13] which additionally harbors the *Bacillus subtilis* 4′-phosphopantetheine transferase Sfp for cofactor loading, and purified by Ni-NTA affinity chromatography (Figure S2). When the recombinant synthetase was incubated in vitro with ATP, Mg^{2+}, and all of the substrate amino acids, HPLC analysis revealed efficient biosynthesis of **1** with a k_{obs} of 3.4 ± 0.5 min^{-1} (Figure S3) and 750 ± 50 total turnovers. The high turnover allowed facile in vitro production and purification of **1** on a preparative scale. The macrocyclic peptide was thoroughly characterized by mass spectrometry (Figure S5) and NMR spectroscopy (see the Supporting Information) and shown to exhibit the expected antibiotic activity.[14,15]

Received: January 25, 2021
Published: February 11, 2021

https://dx.doi.org/10.1021/jacs.1c00925
J. Am. Chem. Soc. 2021, 143, 2736−2740

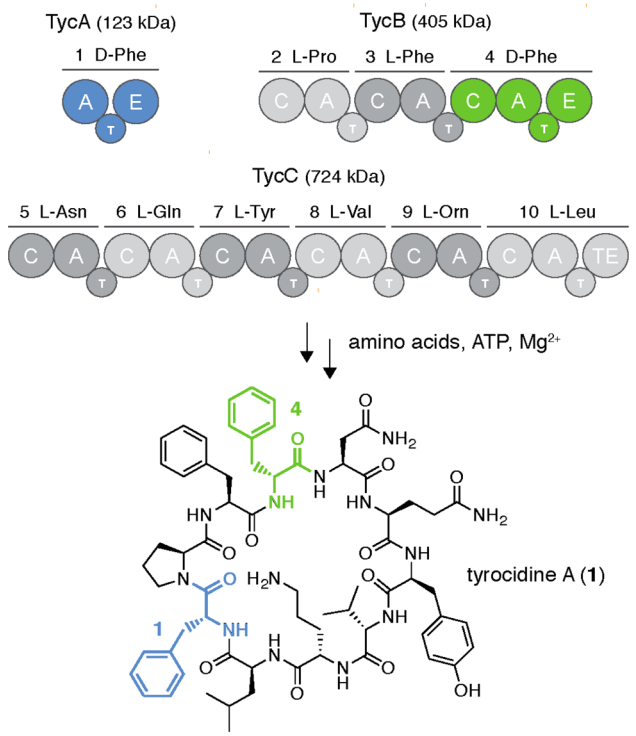

Figure 1. Tyrocidine synthetase assembles the macrocyclic decapeptide antibiotic tyrocidine A via 10 modules distributed over three proteins, TycA, TycB, and TycC. Modules 1 (blue) and 4 (green) activate L-Phe for incorporation into the NRP and epimerize it via the associated epimerization (E) domain. A, T, C, and TE refer to the adenylation, thiolation, condensation, and thioesterase domains, respectively.

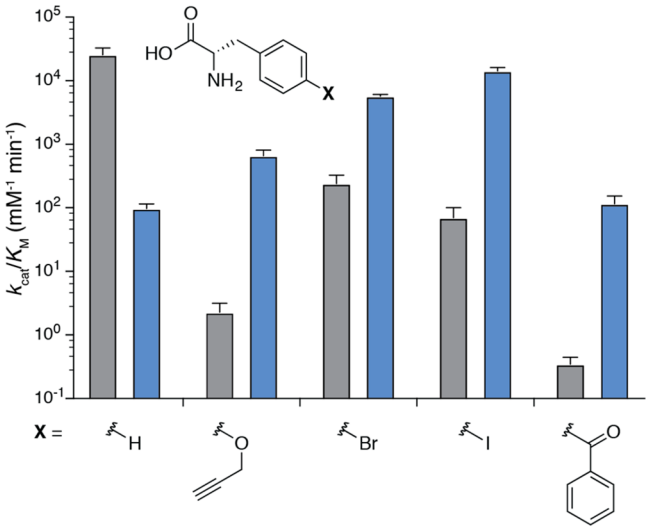

Figure 2. Adenylation kinetics for Phe and four noncanonical analogues determined with TycA (gray) and W227S TycA (blue) using a pyrophosphate exchange assay. See Table S1 for details.

In vitro reconstitution of tyrocidine synthetase provides a versatile platform for testing reprogrammed A domains in the context of a full nonribosomal peptide synthetase (NRPS) assembly line. To assess incorporation of functionalized amino acids at position 1 of tyrocidine A, we assembled a reprogrammed synthetase consisting of the initiation module W227S TycA and wild-type TycB and TycC. All four of the tested substrate analogues—*O*-propargyl-L-Tyr, *p*-bromo-L-Phe, *p*-iodo-L-Phe, and *p*-benzoyl-L-Phe—were incorporated at position 1 to afford tyrocidine analogues **2**–**5**, highlighting the remarkable tolerance of the nine downstream modules (Figure 3A,B and Table S2). The propargylated derivative **2**, for example, is produced efficiently with a k_{obs} of 1.1 ± 0.2 min^{-1} (Figure S3) and 220 ± 70 total turnovers.

Given the importance of the initial Phe residue for macrocyclization of the linear precursor,[16] the ability of the terminal thioesterase (TE) domain of TycC to cyclize the modified decapeptides is particularly notable. In no case was the hydrolyzed linear decapeptide observed. Depending on the substrate analogue, however, the reprogrammed synthetase does produce some diketopiperazine (DKP) by premature termination of the growing peptide chain as well as tyrocidines **1** and **6** by incorporation of L-Phe or L-Tyr instead of the Phe derivative. Formation of the latter side products correlates with the adenylation specificity of W227S TycA (Table S1) and could be suppressed by increasing the concentration of the functionalized substrate analogue (Figure S4).

Many Phe-activating A domains share the conserved amino acids that line the substrate binding pocket of the TycA A domain, including the delimiting Trp residue (Table S4). These residues constitute a nonribosomal code that is diagnostic for substrate specificity.[17,18] The nonribosomal code for the Phe-specific A domain of the third TycB module, for instance, differs from that of TycA at only two of eight positions (Table S4). To test the incorporation of modified amino acids by an elongation module, the point mutation W2742S, analogous to W227S TycA, was introduced into the corresponding A domain, and the reprogrammed synthetase TycA/W2742S TycB/TycC was assembled in vitro. Upon supplementation with amino acids, ATP, and Mg^{2+}, this synthetase efficiently produced functionalized tyrocidine analogues **7**–**10** with *O*-propargyl-L-Tyr, *p*-bromo-L-Phe, *p*-iodo-L-Phe, and *p*-benzoyl-L-Phe, respectively, at position 4 (Figure 3A,C and Table S3). In contrast to the W227S TycA synthetase, DKP formation was negligible, and only small amounts of wild-type **1** and the corresponding Tyr-containing variant **11** were formed, indicating more specific building block selection. Indeed, the reprogrammed synthetase incorporated *O*-propargyl-L-Tyr with a rate constant of 2.1 ± 0.2 min^{-1} and 560 ± 70 total turnovers, which approach the values seen for the wild-type synthetase (TycA/TycB/TycC) with its native substrate L-Phe (Figure S3). The derivatized building blocks introduced by the reprogrammed A domain are efficiently processed by the upstream C domain and all of the downstream domains of the tyrocidine NRPS. Unlike position 1, position 4 is not important for tyrocidine cyclization,[16] which may explain the higher turnover and decreased formation of side products. Modifications of Phe1 could conceivably cause stalling at other points along the assembly pathway, but given the tolerance of modules 4–10 to the same modifications of Phe4, this seems unlikely.

For detailed characterization and subsequent chemical modification of the bioactive core, biosynthesis was scaled up to produce preparative amounts of pure tyrocidine analogues **2** and **7** with an alkyne handle at position 1 or 4, respectively. Both modified peptides retained full antimicrobial activity (Table 1). Harnessing the bioorthogonal handle, **2** and **7** were then coupled to 6-TAMRA-azide (**12**) by copper(I)-catalyzed alkyne–azide cycloaddtion[19,20] to give the fluorescent tyrocidine analogues **13** and **14** (Figure 4). While the bulky

https://dx.doi.org/10.1021/jacs.1c00925
J. Am. Chem. Soc. 2021, 143, 2736–2740

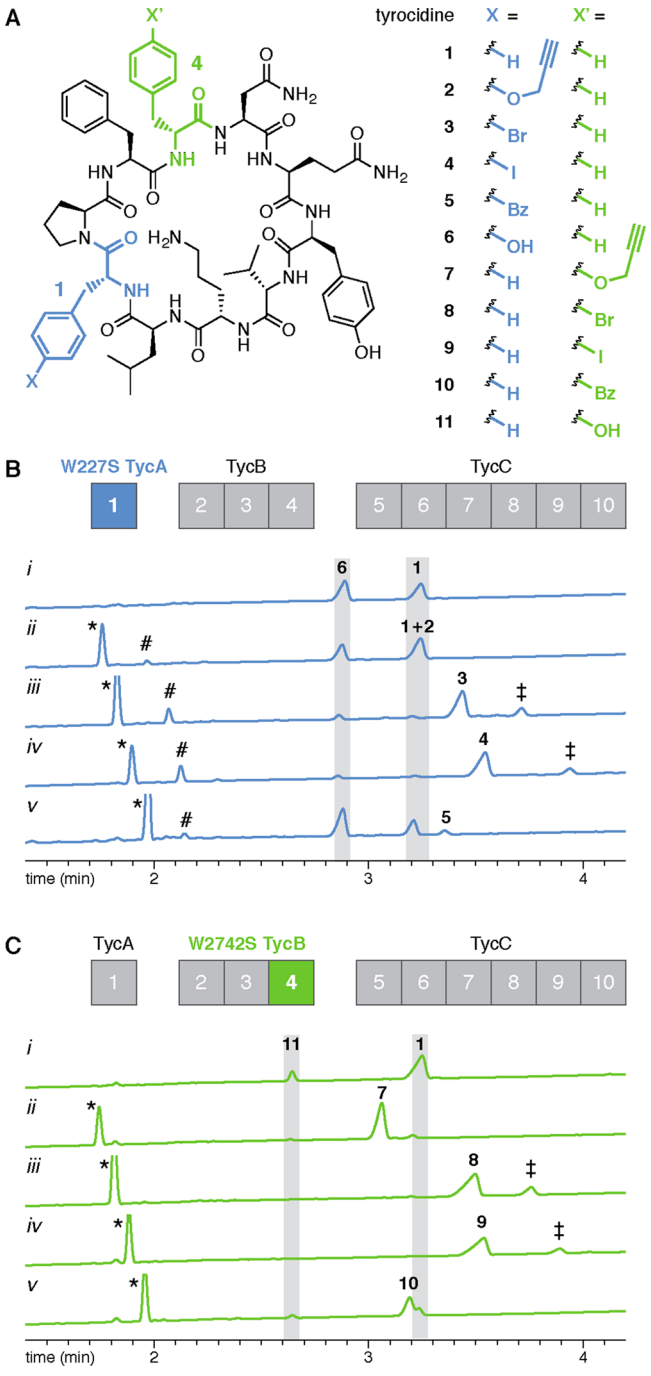

Figure 3. Nonribosomal synthesis of functionalized tyrocidines. (A) Tyrocidine analogues produced by the reprogrammed tyrocidine synthetases. (B) HPLC chromatograms of in vitro reactions with W227S TycA/TycB/TycC containing all wild-type amino acids and supplemented with (*i*) additional L-Phe, (*ii*) *O*-propargyl-L-Tyr, (*iii*) *p*-bromo-, (*iv*) *p*-iodo-, or (*v*) *p*-benzoyl-L-Phe. (C) HPLC chromatograms of in vitro reactions with TycA/W2742S TycB/TycC containing all wild-type amino acids and supplemented with (*i*) additional L-Phe, (*ii*) *O*-propargyl-L-Tyr, (*iii*) *p*-bromo-, (*iv*) *p*-iodo-, or (*v*) *p*-benzoyl-L-Phe. * indicates the supplemented amino acid, # the DKP side product, and ‡ a tyrocidine analogue containing two noncanonical amino acids. Canonical amino acids elute together in the injection peak, which is not shown.

fluorophore completely abolished bioactivity when introduced at position 4, installation of the same modification at position 1 led to more nuanced modulation of the therapeutic properties,

Table 1. Bioactivities of Tyrocidine A (1) and Analogues

	MIC/MHC (μM)[a]				
strain	**1**	**2**	**13**	**7**	**14**
E. coli HM0079	>100	>100	38[b]	>100	>100
B. subtilis JH642	8	4	3[b]	5	>100
A. baumannii	8	5	38[b]	6	>100
S. aureus	8	5	>100[b]	5	>100
erythrocytes	4	3[c]	38[b]	3[c]	56[c]

[a]Minimal inhibitory concentrations (MICs) of tyrocidine A and analogues were determined for both Gram-positive and Gram-negative bacterial strains. Minimal hemolytic concentrations (MHCs) were determined using human erythrocytes. Unless otherwise indicated, three biological replicas were performed. [b]This value was determined only once. [c]Based on two independent measurements.

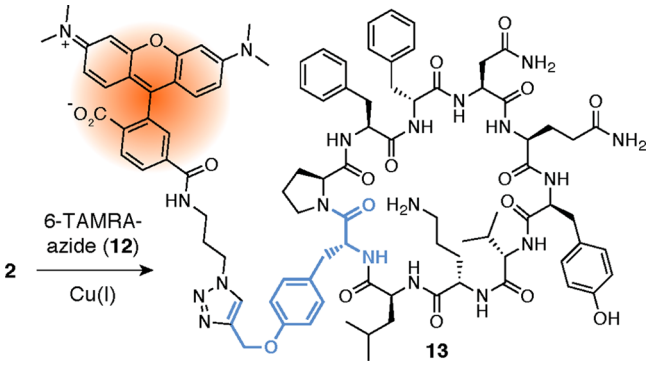

Figure 4. Click modification of tyrocidine. The clickable handle in tyrocidine **2** enabled one-step modification with 6-TAMRA-azide (**12**) by Cu(I)-catalyzed alkyne–azide cycloaddition to give **13**. Tyrocidine **7** was modified analogously to give the fluorescent derivative **14**.

imparting, for example, modest antibiotic activity against Gram-negative *Escherichia coli* and reduced hemolytic activity (Table 1). Much more drastic effects could conceivably be achieved by harnessing the biosynthetically installed handles for combinatorial optimization of the bioactive core.

Diverse strategies for altering the recognition properties of A domains have been reported,[21−28] including a high-throughput platform that we recently established to enable general A-domain engineering.[29] The Trp-to-Ser mutation provides a minimally invasive yet versatile strategy for repurposing Phe-specific A domains for a variety of functionalized building blocks. Importantly, these noncanonical amino acids are efficiently processed on the pathway level to produce functionalized, bioactive NRPs. Tailoring disparate nonribosomal codes for functionalized analogues will allow the site-selective incorporation of these bioorthogonal groups at any peptide position to integrate synthetic biology and bioorthogonal chemistry for the isolation, visualization, and combinatorial optimization of NRP therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c00925.

Complete experimental procedures and additional kinetic and characterization data, including Tables S1–S4 and Figures S1–S5 (PDF)

https://dx.doi.org/10.1021/jacs.1c00925
J. Am. Chem. Soc. 2021, 143, 2736−2740

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by ETH Zurich and the Swiss National Research Foundation. The authors thank Prof. M. A. Marahiel for supplying strain HM0079 and plasmid pSU18_tycA, Prof. J. Piel for the *A. baumannii* and *S. aureus* strains, Dr. M.-O. Ebert and the staff of the NMR Service of the Laboratory of Organic Chemistry at ETH Zurich for support with the NMR analyses, H.-M. Fischer for assistance with radiochemical experiments, L. Bertschi from the Mass Spectrometry Service of the Laboratory of Organic Chemistry at ETH Zurich for technical support, and D. A. Hansen, A. Camus, H. Kries, and S. Studer for helpful discussions.

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