CHEMBIOCHEM

DOI: 10.1002/cbic.200800480

Alkyne-Functionalized Chemical Probes for Assaying the Substrate Specificities of the Adenylation Domains in Nonribosomal Peptide Synthetases

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Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are two large classes of biosynthetic enzymes that produce medicinally active, natural product molecules with complex structures.[1–5] Adenylation (A) domains in NRPSs and acyltransferase (AT) domains in PKSs are the gate-keeping domains responsible for recognizing specific amino acid or carboxylic acid substrates for the enzymatic assembly of nonribosomal peptide or polyketide natural products.[6–9] To diversify the structures of these natural products further for drug development, the substrate specificities of A and AT domains need to be screened for their ability to incorporate non-native building block molecules into the NRPS and PKS assembly lines for the biosynthesis of natural product analogues.[10–21] Current methods for screening the substrate spectra of A or AT domains rely on radioactive assays by measurement of ATP/pyrophosphate (PPi) exchange at the active sites of the A domains[22,23] or by detection of the formation of covalent intermediates between radiolabeled substrates and the NRPS or PKS enzymes.[24] Recently, significant progress has been made in using mass spectrometry to characterize substrate loading in these enzymes.[25–28] Here we report a complementary method involving the use of alkyne-functionalized substrate analogues as chemical probes to assay the substrate specificities of the A domains of the NRPS enzymes. The development of these chemical probes provides an alternative strategy for the direct detection of substrate uptake and intermediate formation in the NRPS enzymes, and would be useful for identifying A domains or mutants capable of utilizing non-native substrate molecules for the biosynthesis of structurally diversified natural products.

The A domain catalyzes the formation of a thioester bond between the cognate amino acid substrate and the phosphopantetheinyl (Ppant) group appended to the neighboring peptidyl carrier protein (PCP) domain for substrate loading on the NRPS assembly lines (Scheme 1 A).[1,4] Because a covalent intermediate is formed between the amino acid substrate and the enzyme, we reasoned that a robust chemical method for detecting substrate attachment to PCP should be able to profile the catalytic activities of the A domains. In this strategy, potential substrates of the A domains are derivatized with bioorthogonal functional groups. In this way, substrate loading on PCP can be detected by conjugation of a reporter molecule such as biotin or a fluorophore to the substrate molecule attached to the Ppant group of PCP. This method would require the bioorthogonal functional group on the substrate molecule to be small in size for its accommodation at the active site of the A domain. Moreover, the conjugation reaction between the substrate and the reporter molecule would need to be highly specific and efficient for the sensitive detection of the covalent intermediate formed with the NRPS enzyme. To meet these requirements, we chose to use alkyne-functionalized substrate molecules as the chemical probes, as they can be conjugated with azide groups linked to biotin (biotin-azide **1**) or other reporter molecules through the use of the Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition reactions developed by Sharpless and colleagues (click chemistry; Scheme 1 B).[29] In this way, substrate loading catalyzed by an A domain is directly coupled to biotin conjugation with the carrier proteins embedded within the NRPS modules. Since biotin can be easily detected through its binding to streptavidin in a Western blot or by an enzyme-linked immunosorbent assay (ELISA), this method should provide a biochemical means for assaying substrate uptake and the formation of covalent intermediates by the NRPS enzymes. Alkyne or azide functionalities have recently been incorporated into various mechanism-based chemical probes and used for enzyme profiling through the use of the azide–alkyne cycloaddition reaction for reporter molecule conjugation.[30–35]

We first used the PCP domain of the NRPS module GrsA[36] to test whether an alkyne functionality attached to PCP can be detected by site-specific conjugation with biotin-azide **1**. GrsA was previously cloned by Marahiel and colleagues from *Bacillus brevis* as the initiation module of gramicidin S synthetase.[37] Coenzyme A-conjugated (CoA-conjugated) alkyne **2** (Figure 1) was synthesized as one of the reaction partners for azide–alkyne cycloaddition and was directly loaded by Sfp phosphopantetheinyl transferase[38–40] onto a serine residue of GrsA PCP (Figure 1 B). The resulting alkyne-modified PCP (alkyne-PCP) showed a molecular ion peak at *m/z* 11 746 as determined by MALDI-TOF (Figure 1 B), which is 421 Da larger than the mass of apo PCP (*m/z* 11 325; Figure 1 A) and matches the mass for the covalent attachment of alkyne-Ppant group ($M_W = 421$) to PCP. Alkyne-PCP was then incubated with biotin-azide **1** in the presence of CuBr for site-specific addition of **1** to the alkyne functional group on PCP. After 5 h at room temperature, complete conversion of alkyne-PCP into biotin-conjugated PCP (biotin-PCP) was observed, with a molecular ion peak at *m/z* 12 294 in the MALDI-TOF spectrum (Figure 1 C). This corresponds to a 548 Da increase in mass from alkyne-PCP, corresponding to the addition of biotin-azide **1** ($M_W = 552$). As is also shown in Figure 1 C, we did not observe any significant

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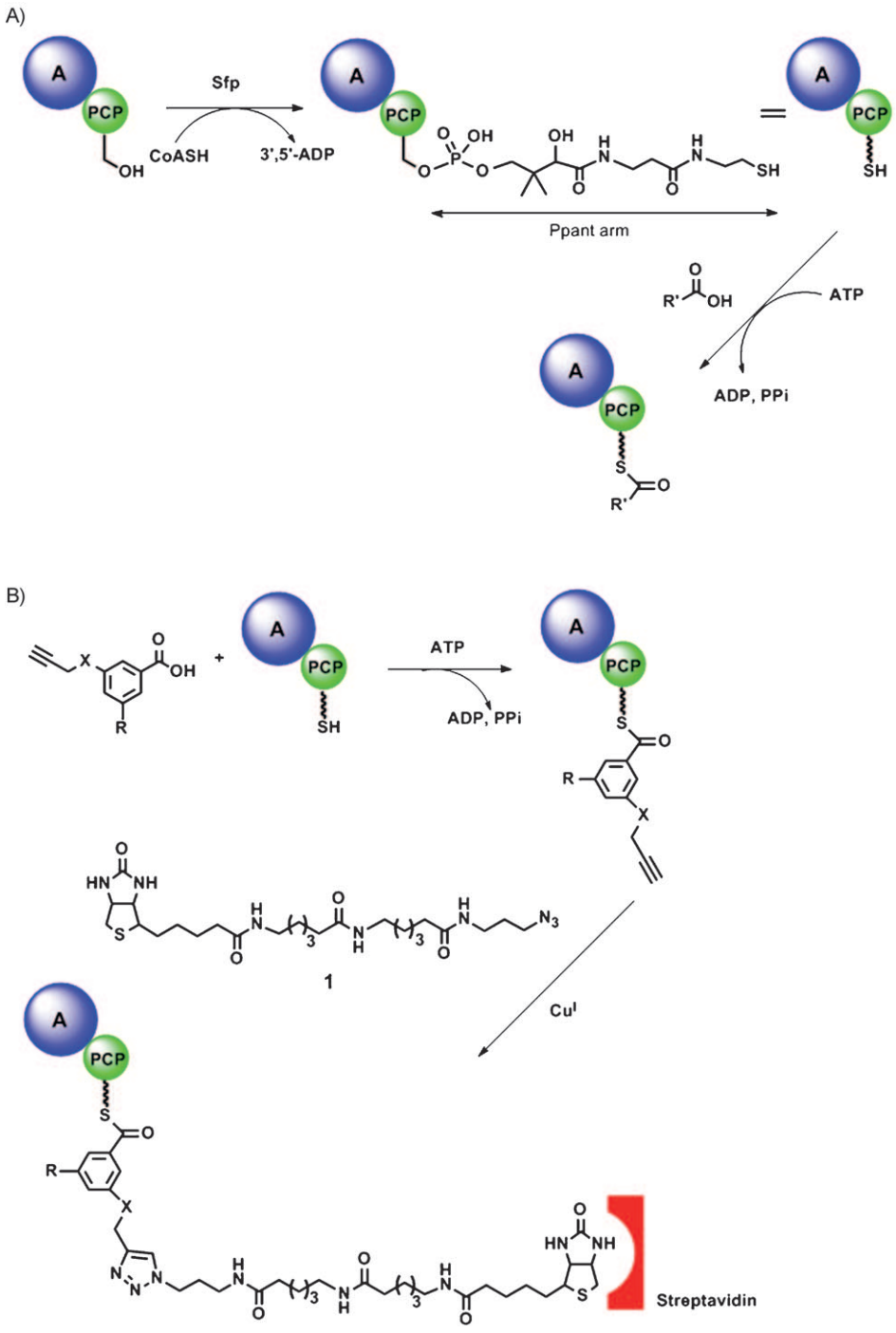

Scheme 1. General strategy for using chemical probes to profile the substrate spectra of the A domains in NRPS biosynthetic enzymes. A) PCP modification by Sfp leads to the covalent attachment of a phosphopantetheinyl prosthetic group (Ppant), which is followed by substrate loading to PCP catalyzed by the A domain. B) Loading of alkyne-functionalized substrates onto PCP is catalyzed by the A domain followed by subsequent conjugation of biotin-azide **1**. Biotin-labeled enzyme intermediates can then be detected by streptavidin binding.

mass signal corresponding to Ppant-modified PCP (M_W = 11 666) as the product of thioester hydrolysis of the alkyne-PCP. This suggests that the thioester linkage between the alkyne functional group and the Ppant arm on PCP is stable during the cycloaddition reaction. Biotin attachment to PCP was also confirmed by Western blot probed with streptavidin-linked horseradish peroxidase (HRP) and by ELISA with the binding of biotin-conjugated PCP to the streptavidin plate and the detection of immobilized PCP on the plate with an anti-His_6 tag antibody linked to HRP (Figures 1 D and E, lane 4). The apo PCP without alkyne modification was used as a control and was treated with biotin-azide **1** under the same conditions. No biotin-PCP was formed, as shown by the Western blot and ELISA (Figures 1 D and E, lane 2). We also found that more biotin-PCP was formed when CuBr was used instead of $CuSO_4$/TCEP as the Cu^I catalyst in the cycloaddition reaction (as suggested by the Western blot and ELISA Figures 1 D and E, lanes 3 and 4). These results demonstrate that Cu^I-catalyzed azide–alkyne cycloaddition reactions can be used to detect alkyne functionalities loaded onto the PCP domain.

We then tested whether alkyne-functionalized substrate analogues can be loaded onto the PCP domain by A domain catalysis with subsequent detection by biotin-azide conjugation targeting the alkyne group. The A domain in the NRPS-type loading module (LM) of RifA is specific for 3-amino-5-hydroxybenzoic acid (AHB, **3**, Figure 2) and has been shown to have substrate tolerance for benzoates substituted at the 3- and 5-positions.[41,42] RifA is the N-terminal component of rifamycin synthetase from *Amycolatopsis mediterranei*, which is responsible for the biosynthesis of proansamycin X, a precursor to antitubercular antibiotic rifamycin B.[43] The RifA LM has previously been cloned by Khosla and colleagues for expression and enzymatic studies.[42] To prepare alkyne-conjugated AHB analogues for probing of the substrate specificity of RifA LM, four alkyne-functionalized benzoate substrates (**4**–**7**), in which propargyl groups were linked to the benzoates through ether or amide bonds with the hydroxyl or amino substitutes on the phenyl rings, were synthesized (Figure 2).

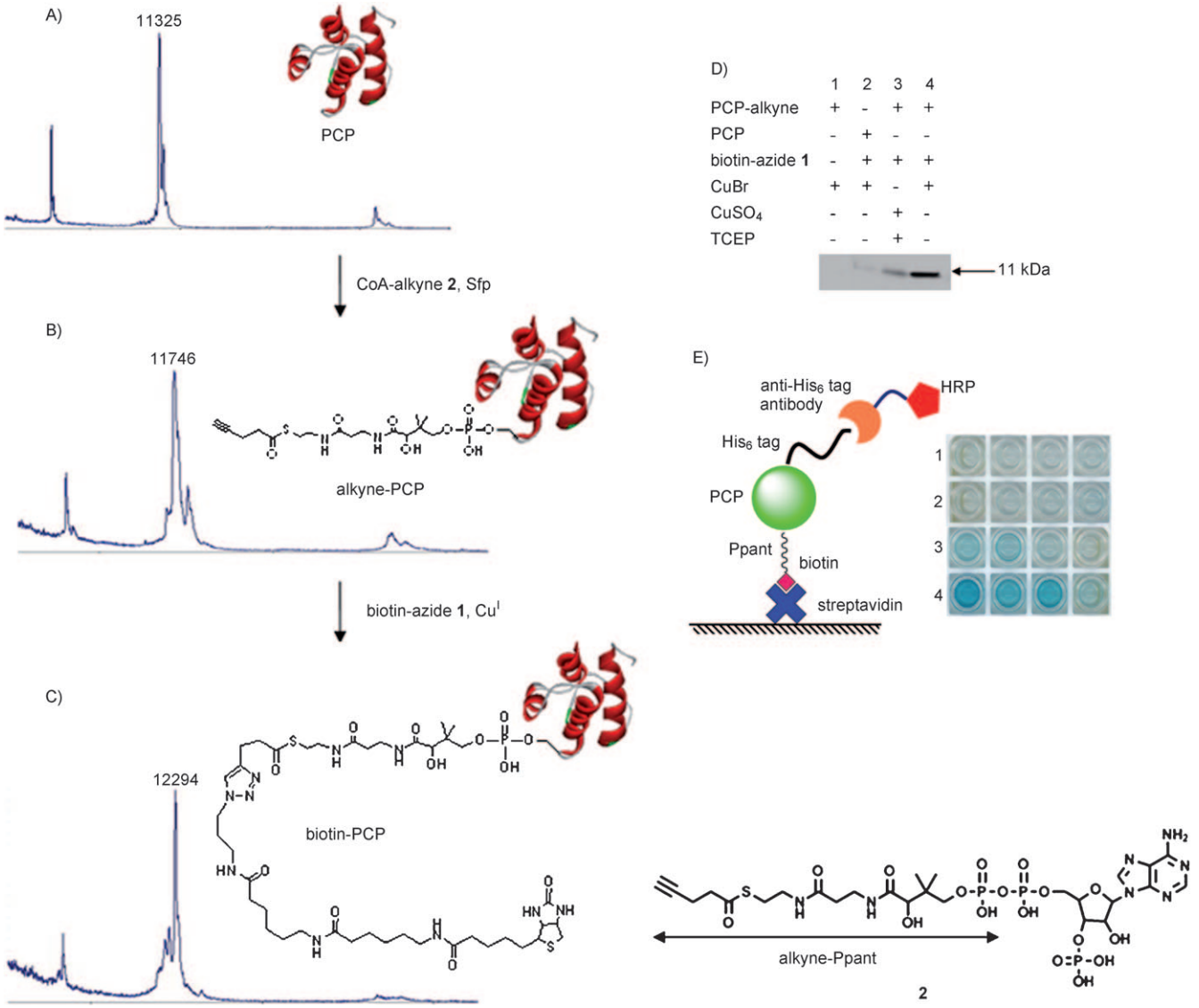

Figure 1. Coupling of alkyne-modified GrsA PCP with biotin-azide **1**. A) MALDI-TOF spectrum of apo PCP with *m/z* 11 325. B) MALDI-TOF spectrum of PCP loaded with alkyne-Ppant (alkyne-PCP) with *m/z* 11 746. C) MALDI-TOF spectrum of PCP conjugated with biotin-azide **1** (biotin-PCP) at *m/z* 12 294 after the azide–alkyne cycloaddition reaction. D) A Western blot of the coupling reaction of biotin-azide **1** with alkyne-loaded PCP. The Western blot was probed with streptavidin-HRP. E) ELISA of the coupling reactions from D, probed with an anti-His_6 tag antibody conjugated to HRP. The solution in the wells was diluted ten-fold across the plate from left to right. The lane assignment in E is the same as in D. The structure of CoA-alkyne **2** is also shown.

RifA LM was expressed as an A-PCP didomain with a C-terminal His_6 tag and was activated for substrate loading by CoA modification with Sfp for the installation of the Ppant group on the PCP domain. We first tested whether the alkyne-conjugated AHB analogues would be recognized by the A domain in RifA LM by means of an ATP/[^{32}P]PPi exchange assay. As shown in Figure 2, the alkyne-functionalized substrate analogues displayed activities for RifA LM that are competitive with the activity of the native substrate, **3**. Compound **4** is the 3,5-diamino analogue of **3**, and its activity for RifA LM-catalyzed ATP/[^{32}P]PPi exchange is about 67% of that of **3**. Compound **6** lacks the 5-amino group of **4** and was slightly less active than **4**, with 60% of the activity of **3**. The activity decreased more with the removal of the 3-amino group of **3** (compound **7**); compound **7**'s activity for ATP/[^{32}P]PPi exchange is only 25% of that of **3**. Introduction of a 5-hydroxyl group into **7** provided **5**, which has a slightly higher activity (29%) than **7** (Figure 2). To test whether the alkyne conjugation method can be used to assay A domain specificity with substrate analogues showing a wide range of activities, we chose **7**, a less active substrate, as the alkyne-functionalized chemical probe to detect substrate loading catalyzed by the A domain of RifA LM.

Probe **7** was incubated with the holo A-PCP didomain of RifA LM in the presence of ATP after the enzyme had been activated by Sfp for Ppant modification. The loading of **7** onto PCP was assayed by incubating the reaction mixture with biotin-azide **1** in the presence of either CuBr or $CuSO_4$/TCEP for biotin conjugation to **7** after substrate loading. The reaction mixtures were then assayed by Western blot, which was probed with streptavidin-HRP. The Western blot showed biotin

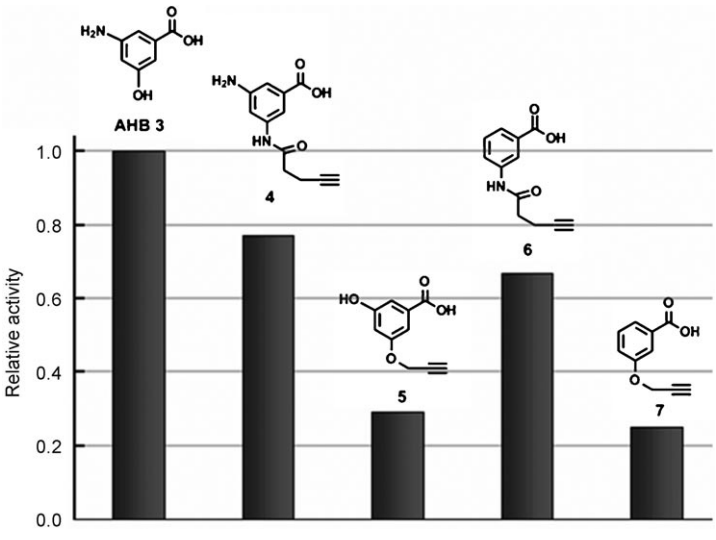

Figure 2. Relative activities of ATP/[^{32}P]PPi exchange catalyzed by the A domain of RifA LM with its native substrate AHB (**3**) and the alkyne-functionalized AHB analogues **4**–**7**.

labeling of RifA LM with a band at 67 kDa upon the addition of both biotin-azide **1** and the copper catalyst to the reaction mixtures (Figure 3 A, lanes 2 and 3). This suggests that the A domain of RifA LM can recognize probe **7** as a substrate and attach it to the Ppant arm on the neighboring PCP domain. Loading of **7** to RifA LM was strictly dependent on A domain catalysis, since RifA LM without Ppant activation was not labeled by biotin under the same conditions (Figure 3 A, lanes 7–9). Biotin conjugation to RifA LM was also dependent on the cycloaddition reaction between **7** loaded on the enzyme and biotin-azide **1**; this is shown by the control reactions in which no biotin labeling was observed on the Western blot without the addition of the copper reagent necessary for the cycloaddition reaction (Figure 3 A, lane 1) or without preloading of **7** on the enzyme (Figure 3 A, lanes 4–6). ELISA also showed similar results (Figure 3 B). These experiments validate our strategy for assaying substrate loading onto PCP that is catalyzed by the A domains through the use of an alkyne-functionalized substrate analogue as chemical probe. It also demonstrates that an alkyne moiety loaded onto a NRPS enzyme can be easily detected by efficient conjugation with a reporter molecule such as biotin-azide **1**.

We next used this strategy to assay substrate loading on RifA LM with alkyne-functionalized probes **4**–**6**. Again, the holo-A-PCP didomain was incubated with **4**–**6**, followed by conjugation with biotin-azide **1**. As shown in Figure 4 A, biotin labeling of RifA LM was detected with probes **4**–**6** on the Western blot probed with streptavidin-HRP. This suggests that these substrates can all be recognized by RifA LM for their incorporation into the biosynthetic assembly line. These results are also compatible with the ATP/[^{32}P]PPi exchange measurements, showing significant activities of the substrate analogues with RifA LM (Figure 2), and demonstrate that the alkyne functionality can be used as a convenient chemical label to screen the substrate specificities of the A domains with small-molecule building blocks of diverse structures.

Finally, we tested whether the alkyne-functionalized substrate probes can be used to detect the activity of RifA LM in cell lysates. *Escherichia coli* cells expressing RifA LM were lysed, and Sfp and CoA were added to the cell lysate for PCP modification with the Ppant group. Compound **7** was added to the reaction mixture, and the loading of the alkyne-functionalized substrate analogue on RifA LM was detected by copper-catalyzed cycloaddition with biotin-azide **1** (Figures 4 B and C, lane 1). Biotinylation of RifA LM was strictly dependent on the substrate loading on PCP catalyzed by the A domain, since no biotin conjugation was found when RifA LM was not activated by PCP phosphopantetheinylation (Figures 4 B and C, lane 3). Furthermore, when NovH (73 kDa), an A-PCP didomain from the NRPS enzyme for novobiocin biosynthesis,[44] was used for substrate loading in the cell lysate with **7**, no biotin labeling of NovH was observed on the Western blot

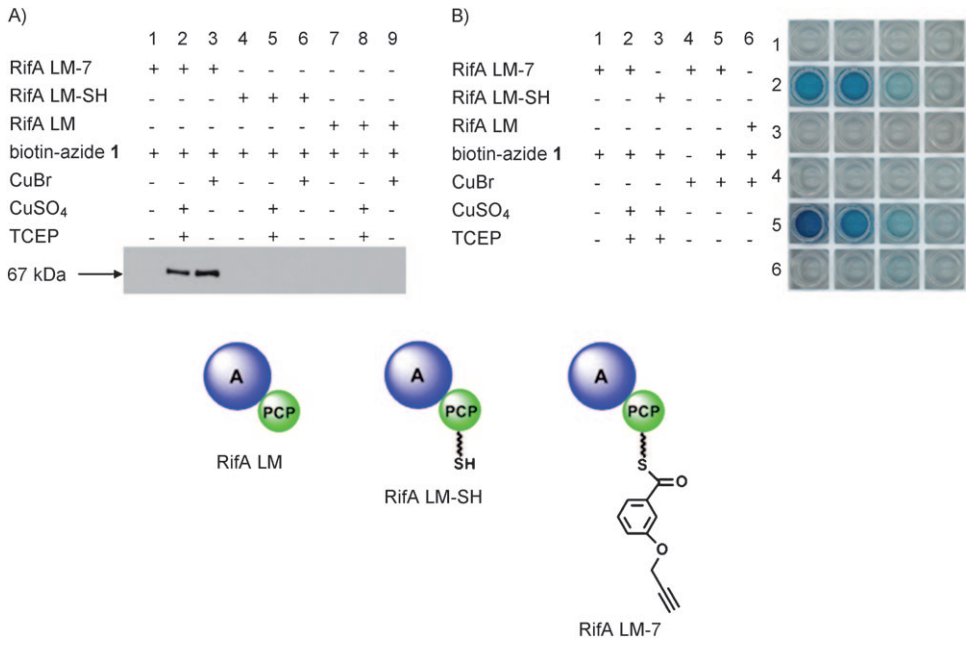

Figure 3. A) Western blot of the conjugation reactions between biotin-azide **1** and RifA LM loaded with **7**. The Western blot was probed with streptavidin-HRP. B) ELISA analysis of the biotin-azide conjugation reaction with RifA LM loaded with **7**. Loading reactions were assayed on a streptavidin plate probed with an anti-His_6 tag antibody conjugated with HRP. The solution in the wells was diluted ten-fold across the plate from left to right.

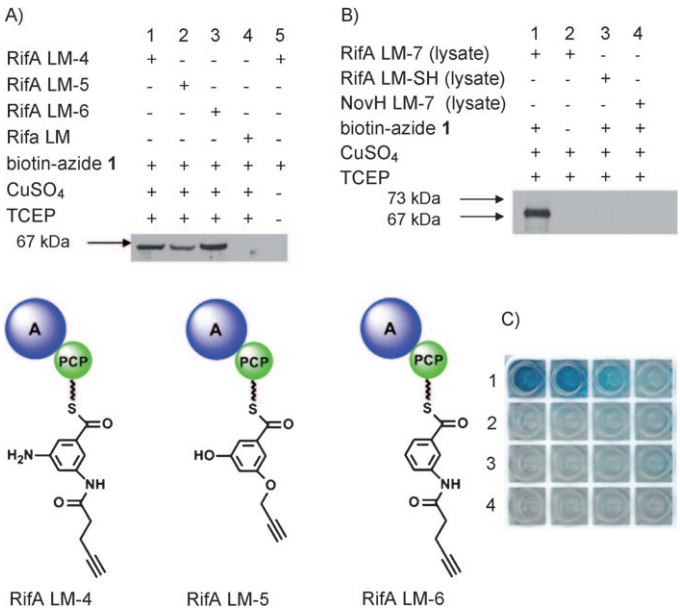

Figure 4. A) Western blot of the biotin-azide conjugation reactions with RifA LM for detection of substrate loading of **4**–**6**. The Western blot was probed with streptavidin-HRP. B) Compound **7** was used to detect the activity of RifA LM in cell lysates. A Western blot probed with streptavidin-HRP is shown. C) ELISA of the substrate loading reactions of **7**. Assay was completed on a streptavidin plate and detected with an anti-His_6 tag antibody conjugated with HRP. The solution in the wells was diluted ten-fold across the plate from left to right. Lane assignment of the ELISA plate in C is the same as in B.

after conjugation with biotin-azide **1** (Figures 4B and C, lane 4). This suggests that NovH, with its native substrate specificity for the amino acid tyrosine,[44] would not recognize **7** as a substrate. These results demonstrate the high sensitivity and specificity of the use of alkyne-functionalized substrate probes to detect A domain catalysis in cell lysates.

In summary, we have developed a simple and efficient method to screen the catalytic activities of NRPS A domains through the use of alkyne-functionalized substrate analogues as chemical probes. The detection of substrate loading onto PCP catalyzed by the A domain is highly sensitive, as it is based on efficient coupling of biotin-azide to chemical probes covalently attached to the PCP domain. This method can be used for screening the substrate spectra of the A domains in NRPS modules. We also expect that the same strategy should be applicable for assaying substrate loading in a PKS module through the use of alkyne-functionalized acyl CoA analogues, because an AT domain in a PKS should covalently attach substrates to a neighboring acyl carrier protein (ACP) domain.[2,5] Recently, precursor-directed biosynthesis[45,46] and mutational biosynthesis[47,48] have been used to screen cellular uptake of nonnative building block molecules for the enzymatic assembly of new natural product structures. The method in this report should be suitable for assaying the substrate tolerance of specific modules of the NRPS or PKS enzymes rather than the full biosynthetic pathway.

In comparison with those currently in use for assaying substrate loading on NRPS or PKS enzymes, the method reported here provides an efficient means for chemical detection of enzyme-attached substrates and does not require the use of radioactive substrates or sophisticated instruments for mass spectrometry. However, it requires the use of alkyne-functionalized substrates instead of native substrates. This method should thus be suitable for screening a panel of alkyne substrates for their uptake by a specific A or AT domain. We also showed that alkyne-functionalized substrate analogues can be used for detecting A domain activity in cell lysates. Cell lysate screening has greatly facilitated enzyme engineering through the expression of mutant enzymes in *E. coli* cells and the direct assay of the enzymatic activities in the cell lysates.[22,49–51] We thus envision that the chemical conjugation method reported here could be used to screen libraries of A or AT domains and their mutants for altered substrate specificity with alkyne-functionalized substrate analogues.

Experimental Section

General: Unless otherwise indicated, all reagents were obtained from commercial sources and used without further purification, and all reactions were performed under nitrogen. Anhydrous solvents were purchased from Acros. All other solvents were obtained either from Fisher Scientific or from VWR International (West Chester, USA). Column chromatography was carried out on silica gel 60 (EMD Chemicals, Cincinnati, USA) with elution solvents as described. ^{1}H NMR spectra were obtained with a Bruker Model DRX 400 NMR spectrometer (Bruker, Fallanden, Switzerland). MALDI-TOF spectra were acquired with a Voyager DE PRO MALDI mass spectrometer (PerSpective Biosystems, Foster City, USA). 2,5-Dihydroxybenzoic acid was used as the matrix for small-molecule samples, and sinapinic acid was used as the matrix for protein samples, unless otherwise indicated. HPLC purification was carried out on a POLARIS BioInert Gradient LC System (Varian, Walnut Creek, USA) with a reversed-phase Nucleodur C-18 column of 250 mm in length, 21 mm ID, and 10 μm particle size (Phenomenex, Torrance, USA).

Preparation of biotin azide 1: Azidoaminopropane (3.0 mg, 30 μmol) was added to a solution of *N*-hydroxysuccinimidyl-6′-(biotinamido)-6-hexanamido hexanoate (NHS-LC-LC-biotin, Pierce, 8.5 mg, 15 μmol) in DMF (500 μL), followed by the addition of *N*,*N*-diisopropylethylamine (5.8 mg, 45 μmol). The reaction was allowed to proceed at room temperature with stirring overnight. The reaction mixture was then purified by HPLC with a gradient of 0–65% acetonitrile in 0.1% TFA/water over 35 min at a flow rate of 10 mL min^{-1}. The purified compound was lyophilized, and its identity was confirmed by MALDI-TOF (positive mode): calcd for $C_{25}H_{45}N_8O_4S$: 553.33; found: 553.54.

Preparation of CoA-alkyne 2: Coenzyme-A (10 mg, 13 μmol) was dissolved in water, and *N*,*N*-diisopropylethylamine (20 μL) was added. The reaction was allowed to proceed at room temperature for 20 min. Pent-4-ynoic acid succinimidyl ester (3.3 mg, 17 μmol) was dissolved in DMSO, and this solution was added to the aqueous CoA solution. The reaction mixture was stirred at room temperature overnight. The crude product was purified by HPLC with a gradient of 0–65% acetonitrile in 0.1% TFA/water over 35 min at a flow rate of 10 mL min^{-1}. The purified compound was lyophilized, and its identity was confirmed by MALDI-TOF (positive mode): calcd for $C_{26}H_{41}N_7O_{17}P_3S$: 848.15; found: 848.44; MALDI-TOF (negative mode): calcd for $C_{26}H_{39}N_7O_{17}P_3S$: 846.13; found: 846.36.

Preparation of 3-amino-5-[(1-oxopent-4-ynyl)amino]benzoic acid (4): 3,5-Diaminobenzoic acid (93 mg, 0.61 mmol) was dissolved in DMF (10 mL). *N*,*N*-Diisopropylethyl amine (0.27 mL 197 mg, 1.53 mmol) was added to this solution, and the reaction mixture was stirred at room temperature for 30 min, after which pent-4-ynoic acid succinimidyl ester (100 mg, 0.51 mmol) was added. The reaction was allowed to proceed at room temperature with stirring overnight. The solvent was then removed by rotary evaporation, and the resulting crude product was purified by HPLC with a gradient of 0–100% acetonitrile in 0.1% TFA/water over 72 min at a flow rate of 10 mL min^{-1} to afford 3-amino-5-[(1-oxopentynyl)amino]benzoic acid (83 mg, 70%). ^{1}H NMR (CD_3OD): $\delta = 7.45$ (s, 1H), 7.32 (s, 1H), 7.13 (s, 1H), 6.36 (s, 1H), 2.57 (m, 4H), 2.30 ppm (s, 1H); MS (APCI): $[M+H]^+$ calcd for $C_{12}H_{13}N_2O_3$: 233.1; found: 233.1.

Preparation of 3-hydroxy-5-prop-2-ynyloxybenzoic acid (5): Bu_3NI (482 mg, 1.3 mmol) was added to a solution of methyl 3,5-dihydroxybenzoate (2.18 g, 13 mmol) and K_2CO_3 (3.60 g, 26 mmol) in DMF (40 mL), followed by the addition of propargyl bromide (1.19 g, 10 mmol). The reaction mixture was stirred at room temperature for 18 h. $NaHSO_4$ (1 M, 35 mL) was then added, and the reaction mixture was extracted with ethyl acetate (3×50 mL). The combined organic layer was washed with a saturated aqueous solution of $NaHCO_3$, water, and brine, dried with anhydrous magnesium sulfate, and concentrated under reduced pressure. Chromatography on silica gel with MeOH/DCM (1:80, *v*/*v*) as eluent gave 3-hydroxy-5-prop-2-ynyloxybenzoic acid (1.65 g, 80%). ^{1}H NMR ($CDCl_3$): $\delta = 7.28$ (s, 1H), 7.24 (s, 1H), 6.72 (s, 1H), 4.75 (s, 2H), 3.95 (s, 3H), 2.56 ppm (s, 1H).

3-Hydroxy-5-prop-2-ynyloxybenzoic acid (1.03 g, 5.0 mmol) was added to a solution of NaOH (0.5 g, 12.5 mmol) in MeOH (10 mL) and water (3 mL). After the system had been stirred for 2 h at room temperature, MeOH was removed, and the residue was acidified with HCl (2 N) and then purified by HPLC with a gradient of 0–100% acetonitrile in 0.1% TFA/water over 72 min at a flow rate of 10 mL min^{-1} to afford 3-hydroxy-5-prop-2-ynyloxybenzoic acid (0.95 g, 99%). ^{1}H NMR ([D_6]DMSO): $\delta = 7.03$ (s, 1H), 6.96 (s, 1H), 6.55 (s, 1H), 4.75 (s, 2H), 3.52 ppm (s, 1H); MS (APCI): $[M-H]^-$ calcd for $C_{10}H_7O_4$: 191.0; found: 191.1.

Preparation of 3-[(1-oxopent-4-ynyl)amino]benzoic acid (6): Compound **6** was prepared from methyl 3-hydroxybenzoate by the synthesis procedure described above. ^{1}H NMR (CD_3OD): $\delta =$ 8.24 (s, 1H), 7.86–7.76 (m, 2H), 7.45 (m, 1H), 2.63–2.57 (m, 4H), 2.31 ppm (s, 1H). MS (APCI): $[M+H]^+$ calcd for $C_{12}H_{12}NO_3$: 218.1; found: 218.1.

Preparation of 3-prop-2-ynyloxybenzoic acid (7): Compound **7** was prepared from methyl 3-hydroxybenzoate by the synthesis procedure used for 3-hydroxy-5-prop-2-ynyloxybenzoic acid (**5**). ^{1}H NMR ($CDCl_3$): $\delta = 7.62$–7.13 (m, 4H), 4.81 (s, 2H), 3.11 ppm (s, 1H); MS (APCI): $[M-H]^-$ calcd for $C_{10}H_7O_3$: 175.0; found: 175.0.

Protein expression and purification: pET plasmids (Novagen, Darmstadt, Germany) of pT2,[36] pSA8,[42] and pHC10[44] were kindly provided by Professor Christopher T. Walsh at Harvard Medical School for the expression of GrsA PCP, RifA LM, and NovH, respectively. Expression of the proteins followed reported protocols.[36,42,44] Briefly, plasmids harboring the genes of the C-terminal His_6-tagged fusion proteins were transformed into *E. coli* BL21-(DE3)pLysS chemically competent cells (Invitrogen). Cells were grown at 37 °C in Luria Broth (LB, 1 L) supplemented with ampicillin (100 mg mL^{-1}) to an optical density of 0.6 at 600 nm. Protein expression was then induced with the addition of isopropyl D-thiogalactopyranoside (IPTG, 1 mM) and the cell culture was grown at 15 °C overnight. The next day, the cells were harvested by centrifugation at 5000 rpm for 15 min and the cell pellets were resuspended in lysis buffer containing Tris (pH 8.0, 50 mM), NaCl (500 mM) and imidazole (5 mM) supplemented with DNAse I (1 unit mL^{-1}). The resuspended cells were disrupted with a French Press (Thermo Spectronic, Asheville, USA) with three passages at 12000 psi. Cell debris was removed by centrifugation at 16000 rpm for 30 min. The clarified cell extract was incubated with a suspension of Ni-NTA resin (Qiagen, 50%, 1 mL) for 3 h at 4 °C in a batch-binding format. The suspension was then loaded onto a gravity column and washed with the lysis buffer (20 mL). Protein bound to the column was eluted with elution buffer (6 mL) containing Tris (pH 8.0, 50 mM), NaCl (500 mM) and imidazole (250 mM). The purity of the fractions containing the protein of interest was checked by SDS-PAGE stained by Coomassie brilliant blue. Fractions with the desired purity were pooled and dialyzed against HEPES (50 mM, pH 7.5, 2×1 L), NaCl (100 mM), and glycerol (10%). Protein solutions were then aliquoted and stored at −80 °C.

ATP/[^{32}P]PPi exchange assay: The ATP/[^{32}P]PPi exchange assay was performed as previously reported.[44] Reaction mixtures (final volume 100 μL) contained Tris-HCl (75 mM, pH 7.5), $MgCl_2$ (5 mM), TCEP (5 mM), ATP (3.5 mM), substrate (1.5 mM), RifA LM enzyme (2 μM), and [^{32}P]pyrophosphate (1 mM, 17.86 Ci mol^{-1}, Perkin–Elmer). The reactions were initiated by the addition of RifA LM and allowed to proceed at 24 °C for 45 min before being quenched by addition of a stop mix (0.5 mL) containing activated charcoal (1.2%, *w*/*v*), tetrasodium pyrophosphate (0.1 M), and perchloric acid (0.35 M) in water. The charcoal was then pelleted by centrifugation and washed three times, each time with a solution (1 mL) containing tetrasodium pyrophosphate (0.1 M) and perchloric acid (0.35 M) in water. After the washes, the charcoal pellet was resuspended in water (0.5 mL) and added to liquid scintillation fluid (5 mL, Ultima Gold; Packard, Shelton, USA). The charcoal-bound radioactivity was determined by liquid scintillation counting with a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, USA).

Sfp-catalyzed CoA modification: In a total volume of 200 μL, Sfp phosphopantetheinyl transferase (2.0 μM), CoA (5 mM), and $MgCl_2$ (10 mM) in HEPES (50 mM, pH 7.5) were incubated with protein (20 μM) for 60 min. For alkyne-Ppant attachment to GrsA PCP, the same conditions were used with CoA-alkyne **2** (5 mM).

Substrate loading catalyzed by adenylation domains: Reactions were carried out with the following components: TrisHCl (75 mM, pH 7.5), dithiothreitol (DTT, 5 mM), ATP (3.5 mM), $MgCl_2$ (10 mM), holo-A-T didomain (20 μM), and the substrate (1.0 mM). After incubation for 60 min at 30 °C, the reaction mixture was applied to a protein desalting spin column (Pierce) preequilibrated with the reaction buffer. The protein component of the applied sample was eluted from the spin column according to the manufacturer's instructions. The eluted protein sample was used for biotin-azide **1** conjugation.

Cu^I-catalyzed azide–alkyne cycloaddition reaction: Biotin-azide **1** (3.0 mM) was added to alkyne-modified protein (30 μM) in PBS buffer (pH 7.4), followed by the addition of CuBr (1.0 mM) or $CuSO_4$ (1.0 mM)/tris(2-carboxyethyl)phosphine (TCEP, 2.0 mM) and tris(1-benzyl-1*H*-[1,2,3]-triazol-4-ylmethyl)amine (2.0 mM). The reaction was gently agitated at room temperature for 5 h. Protein samples were separated from excess reagents by centrifugation and analyzed by Western blot and ELISA.

Assaying the activities of the A domains in cell lysates: RifA LM and NovH were expressed as described in the section on "Protein expression and purification". Cell lysates collected after cell disrup-

tion with a French Press were directly used for Sfp-catalyzed CoA modification, substrate loading, and biotin-azide **1** conjugation as described in the previous sections.

ELISA and Western blot: A streptavidin-coated 96-well plate (Pierce) was first blocked with bovine serum albumin (BSA, 3%) in TBS buffer containing TrisHCl (25 mM, pH 7.5), NaCl (100 mM) at room temperature for 1 h. The blocking solution was then removed, and BSA in TBS buffer (1%, 90 μL) was added to each well. Loading reaction mixture (10 mL) was then added to the wells in the first column, and cross-plate tenfold dilution was performed. After incubation at room temperature for 1 hour, the plate was washed six times with Tween 20 (0.05%) and Triton X-100 (0.05%) in TBS and six times with TBS. BSA in TBS (1%, 100 μL) containing 1:1000 diluted anti-His_6 tag antibody conjugated to HRP (Santa Cruz Biotechnology) was added to each well, and the plate was incubated for 1 h at room temperature. The plate was again washed six times with Tween 20 (0.05%) and Triton X-100 (0.05%) in TBS and six times with TBS. After the washes, the bound peroxidase activity in each well was detected with a TMB substrate kit (Pierce).

For Western blot, labeling reaction mixture (20 μL) was loaded on a 4–15% SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with BSA in TBS (3%) for an hour, followed by incubation with BSA in TBS buffer (1%) containing $1/10^6$ diluted 1 mg mL^{-1} streptavidin-HRP conjugate (Pierce) for 1 hour. The membrane was then washed with Tween 20 (0.05%) and Triton X-100 (0.05%) in TBS (6×) and with TBS (6×) followed by detection by use of the ECL luminescent detection kit (Amersham Pharmacia).

Acknowledgements

This work was supported by a lab startup grant from the University of Chicago, the New Faculty Award from the Camille and Henry Dreyfus Foundation, and the NSFMRSEC program (DMR-0213745; J.Y.).

Keywords: adenylation domains · biosynthesis · Cu^I-catalyzed Huisgen cycloadditions · phosphopantetheinyl tranferase · screening

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Received: July 14, 2008
Published online on November 5, 2008

