

A mechanism-based fluorescence transfer assay for examining ketosynthase selectivity†

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Since their discovery, polyketide synthases have received massive attention from researchers hoping to harness their potential as a platform for generating new and improved therapeutics. Despite significant strides toward this end, inherent specificities within the enzymes responsible for polyketide production have severely limited these efforts. We have developed a mechanism-based, fluorescence transfer assay for a key enzyme component of all polyketide synthases, the ketosynthase domain. As demonstrated, this method can be used with both ketosynthase-containing didomains and full modules. As proof of principle, the ketosynthase domain from module 6 of the 6-deoxyerythronolide synthase is examined for its ability to accept a variety of simple thioester substrates. Consistent with its natural hexaketide substrate, we find that this ketosynthase prefers longer, α -branched thioesters and its ability to distinguish these structural features is quite remarkable. Substrate electronics are also tested *via* a variety of *p*-substituted aromatic groups. In all, we expect this technique to find considerable use in the field of polyketide biosynthesis and engineering due to its extraordinary simplicity and very distinct visible readout.

Introduction

Polyketide natural products are biosynthesized *via* successive Claisen-like condensation of malonate-derived units governed by multi-component enzyme systems termed polyketide synthases (PKSs).^{1–6} Over the past several decades, the field of polyketide engineering has faced a constant struggle to overcome often strict substrate selectivities of the critical biosynthetic enzymes.^{7–13} For modular PKS systems, the shuttling of intermediates between the acyl carrier protein (ACP) and ketosynthase (KS) domains dictates the overall flow of polyketide maturation along the assembly line (Fig. 1).^{14–17} As a result, KS substrate selectivity has been the focus of numerous studies with the hopes of both establishing a better understanding of natural systems and improving our ability to manipulate them.^{18–21} In its simplest form, this requires a means of accurately differentiating between substrates that acylate the KS active site-cysteine and those that do not. Unfortunately, the methods traditionally used to examine KS active site loading have significant drawbacks, including limited scope, reproducibility, and cost that can severely constrain the scope of these analyses. It is abundantly clear that techniques capable of providing easy and reliable

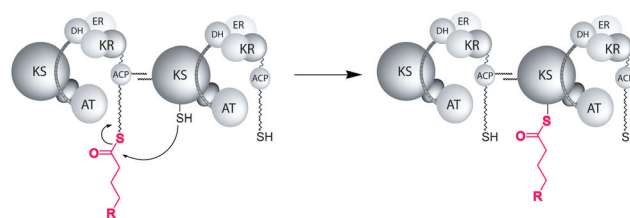


Fig. 1 Schematic diagram of intermodular transfer in modular polyketide synthases. A polyketide intermediate is passed from an upstream ACP to a downstream KS *via* the phosphopantetheine arm of *holo*-ACP. Often the substrate selectivity of the acceptor KS domain determines the efficiency of this process and controls the flow of intermediates down the assembly line. KS = ketosynthase, ACP = acyl carrier protein, AT = acyltransferase, DH = dehydratase, ER = enoyl reductase, KR = ketoreductase.

access to KS selectivity profiles are essential for continued progress in this field.

Early determinations of KS substrate selectivity relied heavily on radiolabeling of elongation products or, less commonly, the substrates themselves.^{15–17,21} Small molecules were generally delivered to the KS active site *via* a compatible ACP or as an *N*-acetylcysteamine (SNAc) thioester, a more synthetically accessible mimic of the phosphopantetheine (Pant) arm of *holo*-ACPs. Subsequent addition of radiolabeled extender units resulted in a single elongation event with the KS-bound substrate. The amount of labeled product excised from the protein provided an

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indirect measure of KS loading with each substrate. While very sensitive, the combination of high reagent cost and limited substrate availability has severely restricted the use of this method for medium and high-throughput applications.

More recently, a number of groups have developed mass spectrometry-based techniques for examining KS active site acylation.^{19,20,22} Many of these strategies utilize tandem proteolysis/LC-MS to tease out differences in loading propensities for various substrates. Despite the many advantages compared to radiolabeling, MS-based methods still suffer from a number of important shortcomings. Firstly, the extremely large size of intact PKS modules generally prohibits their use in these analyses. Smaller subunits such as KSAT didomains are often employed as substitutes for the full module and currently it is unclear what, if any, effect this has on substrate selectivity. In addition, the influence of various substrates on ionization potentials, especially when proteolysis is employed prior to analysis, has not been fully explored and may significantly impact data interpretation and reproducibility. Finally, when particular hydrophobic groups (*e.g.* octyl, naphthyl, cyclohexyl) are utilized as a substantial part of a given substrate, loss of signal for proteolysis fragments associated with KS domains is often observed, likely due to solubility issues.

As advances in genomic mining continue to uncover hosts of new biosynthetic systems, the need for simple, cost-effective means of characterizing them has never been greater. Consequently, we were compelled to develop an alternative means of directly determining KS substrate selectivity using readily accessible, inexpensive reagents and equipment. Such a method would greatly facilitate the process of high-throughput PKS investigation and allow for broadened exploration of the rules of substrate recognition that govern small molecule output.

Recently, our lab has developed a series of β -lactone and β -lactam probes capable of directly acylating the Ppant-thiol of *holo*-ACPs.^{23,24} Incorporation of an alkyne functionality at various ring positions provides a chemical handle for conjugation with fluorophores (Fig. 2). The thioester-ACP resulting from incubation of *holo*-ACP with a propargyloxycarbonyl (Poc)-activated β -lactam was shown to be competitive with traditional *N*-acetylcysteamine (SNAc) thioesters for KS acylation. Based on these results, it occurred to us that fluorescent thioester-ACPs, derived from an alkyne-bearing β -lactam, coupled with acyl-SNAC compounds, could provide a straightforward

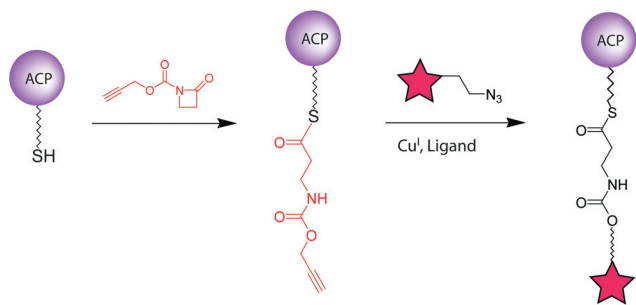


Fig. 2 Schematic representation of our method for site-selective ACP-acylation using *N*-activated β -lactams. Subsequent conjugation to a fluorophore (red star) uses standard click chemistry. The resulting fluorescent ACP is used as the fluorescence donor in the current study.

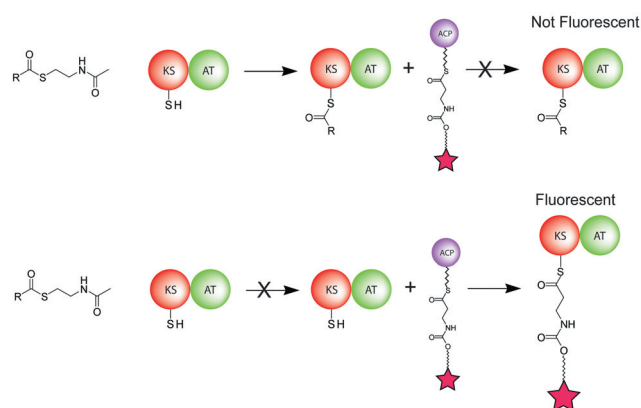


Fig. 3 Schematic diagram of a mechanism-based, fluorescence transfer assay for KS-selectivity. A given KS domain is incubated with acyl-SNAC followed by addition of fluorescently labeled ACP. The amount of fluorescence transferred from the ACP to the KS active site provides a direct readout of the substrate tolerance for each acyl-SNAC examined. KS = ketosynthase, ACP = acyl carrier protein, AT = acyltransferase, SNAC = *N*-acetylcysteamine.

means of examining KS selectivity in whole PKS modules. More specifically, the degree of KS acylation for a given SNAC substrate may be assayed by introducing a fluorescent ACP after incubation of KS and substrate and monitoring fluorescence transfer *via* gel electrophoresis. The amount of fluorescence transfer would therefore indicate to what extent the SNAC compound was accepted by the KS domain (Fig. 3). High KS-loading would block subsequent fluorescence transfer from ACP, and thus produce little to no fluorescent signal in the gel. Alternatively, insufficient KS-acylation with a SNAC compound would leave most KS active sites free to accept the fluorescent substrate from ACP resulting in a bright band. This technique would afford a direct measure of KS selectivity while circumventing the limitations of mass spectrometry methods described above. Most importantly, the use of mechanism-based fluorescence transfer from acyl-ACPs ensures that any fluorescence observed on the acceptor module results solely from the KS active site and not from competing, nucleophilic surface residues within the complex protein system.

We expect the straightforward nature of this method to permit all research labs to explore key features of polyketide construction. Studies ranging in scope from basic KS selectivity to more complex ACP/KS complementarity for designing hybrid systems will be greatly simplified, ultimately leading to significant advances in PKS engineering. Herein, we describe our initial work toward designing a mechanism-based, fluorescence transfer method for examining substrate selectivity in PKS assemblies.

Results and discussion

We knew from our previous work that the 6-deoxyerythronolide B synthase (DEBS) module 2 ACP (ACP2) could efficiently transfer a β -lactam-derived fluorescent probe to the KS active site of the DEBS module 6 KSAT²⁵ didomain (KSAT6).²⁴ As a result, we decided to begin with this ACP/KS pair for proof of principle studies. It should be noted that careful choice of the

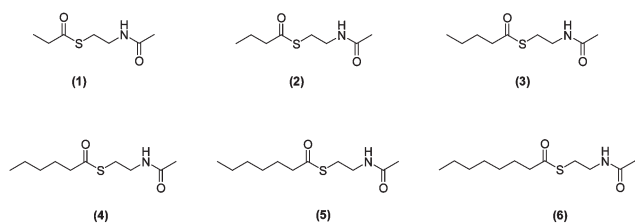


Fig. 4 Panel of SNAc thioesters used to probe chain-length selectivity.

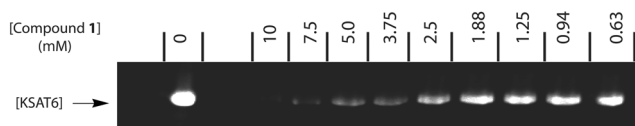


Fig. 5 SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT6 pretreated with variable concentrations of compound **1**. Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no compound **1** is added. Numbers at the top indicate the concentration of compound **1** added to KSAT6.

donor ACP is critical for success using this technique. We had previously determined that fluorescence transfer from ACP2 to KSAT6 was complete in as little as 30 min and no transfer occurred in the presence of the KS active site inhibitor cerulenin. The speed and specificity of this process prompted us to begin development of a mechanism-based fluorescence transfer assay capable of directly probing the influence of substrate structure (*i.e.* chain length, sterics, and electronics) on KS loading.

Based on the structure for the natural hexaketide recognized by module 6 KS, we hypothesized that longer chain substrates would be preferred.² In addition, module 5 incorporates a methylmalonyl-extendor unit which, following elongation, is passed to module 6. As a result, we further hypothesized that α -branched thioesters may be particularly well-suited for acylating the KS domain. Our fluorescence transfer method would allow us to readily observe these tendencies and, more importantly, provide high-resolution information as to their limits.

To examine chain length preferences for KSAT6, a panel of SNAc thioesters derived from straight-chain carboxylates was prepared (Fig. 4). Before we could begin, however, it was necessary to determine an appropriate acyl-SNac concentration where subtle differences in KS-loading could be readily observed. Fluorescently labeled ACP was prepared as previously described by reacting *holo*-ACP2 with *N*-propargyloxycarbonyl- β -lactam followed by Cu-catalyzed [3 + 2] cycloaddition with rhodamine azide (see Fig. 2).^{26–28} Excess click reagents were removed *via* filtration to avoid any complications during the assay. To determine the optimal substrate concentration for KS-selectivity studies, compound **1** was introduced to KSAT6 at concentrations ranging from 630 μ M to 10 mM. After 1 h incubation, a slight excess, relative to KSAT6, of fluorescent ACP2 was added for 30 min and the protein components were subsequently separated and analyzed by SDS-PAGE. From these data, it was determined that the inflection point between fully loaded and unloaded KS corresponded to a substrate concentration of roughly 2.5 mM for compound **1** (Fig. 5).

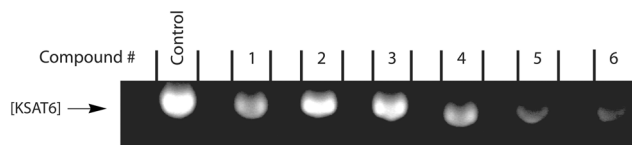


Fig. 6 SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT6 pretreated with compounds **1–6**. Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1–6**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP.

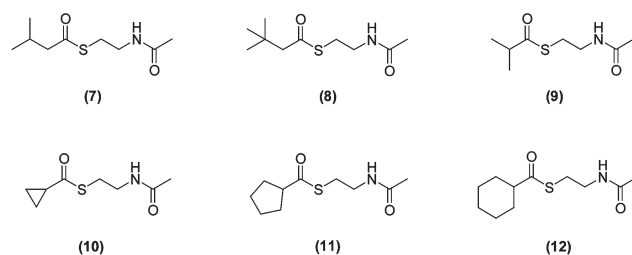


Fig. 7 Panel of SNAc thioesters used to probe α - and β -branching selectivity.

The variable chain length SNAc compounds (**1–6**) were subjected to the same analysis as above using 2.5 mM of each. To our satisfaction, a clear preference for longer chain substrates was observed (Fig. 6). Given that DEBS module 6 KS normally accepts a hexaketide intermediate from module 5, these results are consistent with what might be expected of modules further down the biosynthetic assembly line. Most importantly, the observed trend was reproducible over multiple, separate runs (see ESI[†]). It should be noted that previous attempts on our part to monitor KS-loading with compounds **5** and **6** *via* tandem proteolysis/mass spectrometry were unsuccessful most likely due to precipitation of the acylated KS active site fragment.

With a simple yet powerful method in hand, we were excited to examine the affects of more complex structural components on KS-loading. Specifically, we were interested in uncovering any substrate preferences for branched alkyl-SNac compounds. A small panel of these compounds was prepared with variable branching at both the α - and β -positions of the carbonyl (Fig. 7). Since the natural polyketide intermediate accepted by DEBS module 6 KS harbors an α -methyl group, it is not surprising that compounds **9**, **11**, and **12** appear to load KSAT6 quite efficiently while compounds **7** and **8** are not as effective (Fig. 8). The cyclopropyl-containing compound **10**, however, is very interesting. Despite the presence of an α -branch, this substrate clearly behaves more like those without α -substituents. It is tempting to speculate that this may arise from the extreme cyclopropane bond angles which might alter the side chain geometry to the point where the KS active site does not recognize any branching. Regardless of its origins, this is a rather remarkable response to very subtle differences in structure between isopropyl and cyclopropyl groups.

At this point the next logical step, in our minds, was to examine electronic aspects of KS-acylation using substrates

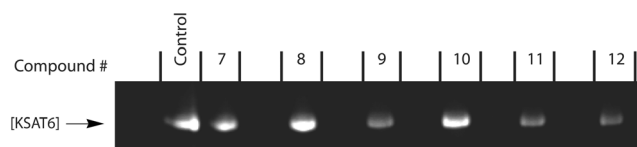


Fig. 8 SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT6 pretreated with compounds 7–12. Lack of fluorescence indicates near quantitative acylation of the KS active site with compound 1. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP.

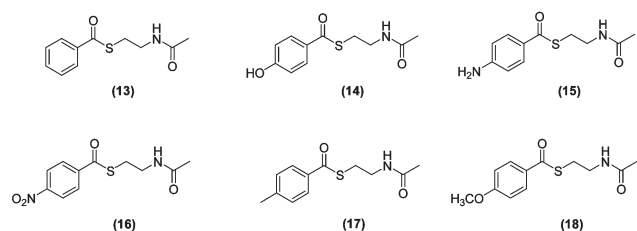


Fig. 9 Panel of SNac thioesters used to probe KS-selectivity for *p*-substituted aryl groups.

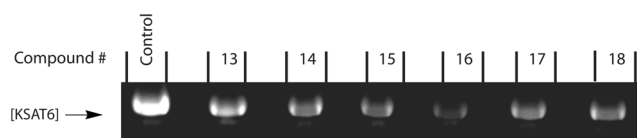


Fig. 10 SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT6 pretreated with compounds 13–18. Lack of fluorescence indicates near quantitative acylation of the KS active site with compound 1. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP.

bearing various electron withdrawing and donating aromatic groups. Therefore, a set of acyl-SNac compounds was prepared with an assortment of *p*-substituents for comparison to the parent phenyl ring of compound 13 (Fig. 9). From the data, it appears that all *p*-substituents confer at least a slight reactive advantage relative to the unsubstituted phenyl ring (Fig. 10). The nitrophenyl compound 16 is most reactive as might be expected from the increased carbonyl electrophilicity offered by the electron withdrawing nature of the nitro group. Clearly, the electronics of the thioester play a significant role in KS acylation, but without structural information regarding the active site binding interactions of each of these substrates, it is difficult to speculate further. Taken together with the results from the chain length and branching studies, this method is capable of generating a wealth of information regarding KS selectivity in short order with minimal cost and synthetic manipulation. However, to showcase its maximum value for examining substrate tolerance in polyketide biosynthesis, we felt it appropriate to move away from didomains in favor of full PKS modules.

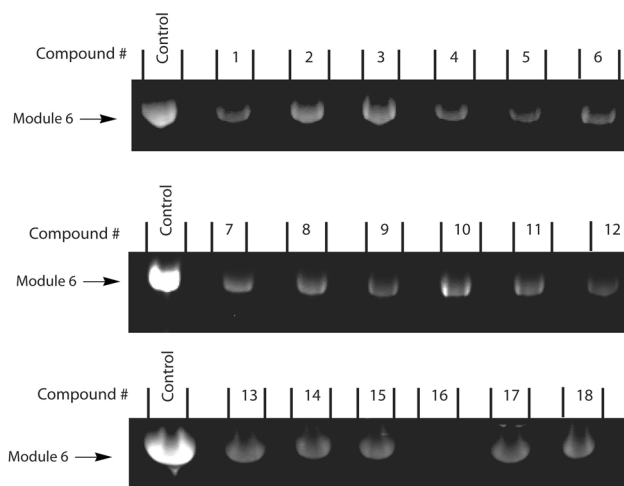


Fig. 11 SDS-PAGE analysis of fluorescence transfer from ACP2 to *apo*-module 6 pretreated with compounds 1–18. Lack of fluorescence indicates near quantitative acylation of the KS active site with compound 1. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top of each gel indicate which compound module 6 was treated with prior to addition of fluorescent ACP. Overall trends in each gel are consistent with what was observed for KSAT6 above indicating that the presence of DH, ER, KR, and ACP domains does not significantly alter KS-selectivity.

For comparison to the previous data sets, we reasoned that DEBS module 6 would be the most appropriate target for this study. This module contains a ketoreductase, acyl carrier protein, and thioesterase domain in addition to the KS and AT present in the didomain construct. To avoid any competition with the native ACP of this module, we opted to express the *apo* form which lacks the reactive Ppart arm. In addition, the potential for fluorescence transfer from ACP2 to the thioesterase domain, positioned at the C-terminus of DEBS module 6, prompted us to omit this entity from the construct used in this study. With the optimized full module in hand, the same 18 probes used to probe KSAT6 selectivity were applied.

To our satisfaction, all of the trends observed for KS-selectivity in the didomain precisely tracked those for the intact module although the contrast between “good” and “bad” substrates is less obvious (Fig. 11). The preference for both longer chain and α -branched substrates is again evident and the remarkable distinction between isopropyl and cyclopropyl groups appears consistent. The observed preference for the nitrophenyl-containing compound 16 over all other aryl-SNac substrates provides further credence to the role of electronics in KS-acylation. Taken together, these results offer a clear picture of the substrate scope tolerated by this KS in two unique environments. The fact that the observed trends are consistent between the didomain and intact module is encouraging as “broken modules” become increasingly popular for modern mechanistic studies of PKS systems.

Conclusion

With this work, we have successfully developed a mechanism-based, fluorescence transfer assay for substrate selectivity in KS

domains. The technique, in its current form, provides a simple, qualitative output without the need for expensive reagents or equipment. The experiments described above barely scratch the surface of what is possible with this method. We envision a number of diverse applications including: (1) further examination of substrate attributes (*i.e.* stereochemistry, heteroatom incorporation, *etc.*), (2) KS/ACP complementarity, (3) determining the impact of substrate binding pocket mutations within KS domains on substrate tolerance, and (4) assessing the influence of exogenous domains on KS selectivity in “chimeric” modules. The work described in this manuscript properly lays the necessary groundwork toward each of these endeavors. In all, we expect this method to significantly aid in the study of new and existing PKS systems leading to improved understanding of how these extraordinary biosynthetic machines function.

Methods

Materials

All moisture sensitive reactions were performed under argon in flame-dried glassware with Teflon-coated stir bars. Reagents and solvents for organic synthesis were used as received unless otherwise noted. Dichloromethane was distilled from calcium hydride and stored over activated molecular sieves. Thin-layer chromatography (TLC) was conducted on glass-backed silica plates visualized with either UV illumination or basic permanganate stain. Flash chromatography was carried out with 60 Å silica gel. ¹H and ¹³C NMR was performed on a 400 MHz Bruker NMR.

Protein expression

ACP2 was expressed from pNW06,¹⁴ ACP3 was expressed from pVYA05.²⁵ [KS6AT6] was expressed from pAYC11.¹⁸ *apo*-ACPs were harvested from *E. coli* BL-21 and *holo*-ACPs were harvested from *E. coli* BAP-1.³¹ pNW06 and pVYA05 contain kanamycin resistant vectors.

Cloning (pTL-DM6)

Cloning of DEBS-Mod 6 (without TE domain) was done by PCR on pRSG54 (a gift from Professor Khosla, which encodes for DEBS Mod 6+TE). Using the primer DEBS-M6-NdeI-F1: TTTTTCATATGGACCCGATCGCGATCGTCGGCATG, and DEBS-M6-HindIII-R1: AAAAAAAGCTTGAGCTGCTGTC-CTATGTGGTCGGC, PCR was performed in a 50 µL reaction mixture containing: 1 × Phusion GC buffer, 0.2 mM dNTP, 0.2 mM MgCl₂, 15% Glycerol, 1 µM of each primer, 1 U Phusion Hot Start II DNA polymerase (Thermo scientific), and approximately 300 ng of pRSG54 DNA. PCR amplicon of 4215 bp was obtained after 35 cycles PCR amplification. The DNA amplicons were excised and purified by using QIAquick Gel Extraction Kit (QIAGEN, Germany) and then directly subjected to double restriction enzyme digestion of NdeI and HindIII. The digested product was ligated to pre-digested pET-21b to obtain pTL-DM6.

Protein purification and isolation

Bacteria were grown in 1 L shake cultures of LB-antibiotic media at 37 °C in an incubating shaker until the OD₆₀₀ was between 0.6 and 0.8. Over expression was induced with 200 µL of 1 M IPTG (per liter of culture) and carried out at 18 °C for 18 h. After this point all work was carried out at 4 °C. Cells were pelleted by spinning at 5000 RPM for 10 min and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na₃VO₄, 1 µg mL⁻¹ leupeptin, pH 7.5). Cells were lysed using an ultrasonic converter. The lysate was pelleted at 10 000 rpm for 60 min. The supernatant was equilibrated with 3 mL of Ni-NTA slurry (per liter of culture) for 60 min by stirring with a PTFE-coated stir bar at minimal speed. The mixture was then poured into a fritted column and the supernatant eluted. The resin bed was washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with 3 mL (per liter of culture) of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was loaded into a 3 kDa NMW centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun until the final volume was ≤500 µL. Dilution and filtration was repeated a total of three times. Protein concentration was determined by Bradford assay with a BSA standard curve. Proteins were flash frozen and stored at -80 °C until use.

Fluorescence transfer assay

Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. ACP2 (25 µM total protein) was equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. Poc β-lactam (10× with respect to total ACP concentration) was added and the mixture was equilibrated at ambient temperature for 45 min. Rhodamine Azide (2× with respect to Poc β-lactam), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min. To remove excess Poc β-lactam and Rhodamine Azide, the mixture was loaded into a 3 kDa NMW concentrator and the volume reduced to 100 µL by spinning in a centrifuge cooled to 4 °C. The mixture was diluted to 500 µL with phosphate buffer, and then concentrated to 100 µL again. This process was repeated a total of 5 times. Protein was removed from the concentrator by inverting it and spinning. For transfer of the fluorescent product, [KSAT6] or the full module (25 µM) was pre-treated with respective SNAc (2.5 mM) derivatives for 1 h. This mixture was introduced to filtered acyl-ACP2 and incubated for 30 min. The [KSAT6] and *apo*-Mod6 control was executed under the same acylation and click conditions as in other samples but without the SNAc derivative and therefore represents full acyl transfer.

Chromophore attachment

The reaction was carried out at 25 µL, final concentrations reported. Rhodamine Azide, (2× with respect to Poc β-lactam),

sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min.

Gel assay

Labeled samples were diluted to 20 μ L with gel-loading buffer. Proteins were separated using a 4–20% gradient HEPES-PAGE gel (100 V, 50 mA, 70 min). Gels were developed in 10% acetic acid to visualize Rhodamine Azide. Labeled proteins were imaged on a UV-transilluminator. Total protein was stained using Coomassie stain.

Prop-2-yn-1-yl 2-oxoazetidine-1-carboxylate

2-Azetidinone (107 mg, 1.5 mmol) was dissolved in THF (7 mL) at -78 °C. LiHDMS (330 mg, 2 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture was stirred for 30 min. Propargyl chloroformate (181 mg, 1.5 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture was stirred at -78 °C for 2 h then allowed to warm to ambient temperature. The reaction mixture was diluted with water and extracted with DCM (50 mL \times 3). The combined organic layers were washed with brine and dried over Na_2SO_4 . Solvent was removed by rotary evaporation. Purification of crude product by flash chromatography with 95 : 5 DCM–Methanol yields (106 mg, 0.69 mmol) 46% of product as pale yellow solid.

Sulforhodamine-B azide

3-Azido-1-aminopropane²⁹ (22 mg, 0.22 mmol) and triethylamine (51 mg, 0.5 mmol) were dissolved in 1 mL of 5 : 1 DCM–DMF and cooled to 0 °C. Sulforhodamine B sulfonyl chloride [mixture of *ortho*- and *para*-sulfonyl chloride isomers] (115 mg, 0.20 mmol) was added portion-wise over *ca.* 30 min and stirred overnight at room temperature. Solvent was removed *in vacuo*, and the product was purified by flash chromatography over silica gel with 90 : 5 : 5 DCM–Acetonitrile–Methanol mobile phase. The *ortho* isomer was distinguished by its reversible color change in pH 9.0 buffer.³⁰ *Para* isomer: (22 mg 0.034 mmol) 17%, *ortho* isomer (18 mg, 0.028 mmol) 14%. Both obtained as a red solid with a metallic green luster. The *ortho* isomer was found to perform best in the click reaction and was utilized for labeling experiments.

General method for preparation of all *N*-acetylcysteamine (SNAc) thioester derivatives

To a solution of triethylamine (2.80 mmol) in dichloromethane (10 mL) was added the appropriate acid (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and *N*-acetylcysteamine (SNAc) (1.35 mmol) under argon. The reaction mixture was stirred overnight. The organic layer was washed with saturated NaHCO_3 solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated

under vacuum, and purified by flash column to provide the final product in pure form.

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