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PAPER

N-Activated β-lactams as versatile reagents for acyl carrier protein labeling†

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Acyl carrier proteins are critical components of fatty acid and polyketide biosynthesis. Their primary function is to shuttle intermediates between active sites *via* a covalently bound phosphopantetheine arm. Small molecules capable of acylating this prosthetic group will provide a simple and reversible means of introducing novel functionality onto carrier protein domains. A series of *N*-activated β-lactams are prepared to examine site-specific acylation of the phosphopantetheine-thiol. In general, β-lactams are found to be significantly more reactive than our previously studied β-lactones. Selectivity for the *holo* over *apo*-form of acyl carrier proteins is demonstrated indicating that only the phosphopantetheine-thiol is modified. Incorporation of an *N*-propargyloxycarbonyl group provides an alkyne handle for conjugation to fluorophores and affinity labels. The utility of these groups for mechanistic interrogation of a critical step in polyketide biosynthesis is examined through comparison to traditional probes. In all, we expect the probes described in this study to serve as valuable and versatile tools for mechanistic interrogation.

Introduction

Polyketide natural products are an enormous class of secondary metabolites with broad-spectrum applications as antibiotic, antitumor, and immunosuppressive agents.[1] The looming menace of accelerating drug resistance demands the continued production of new and diversified polyketides to retain this therapeutic efficacy. The genetic and chemical manipulation of existing polyketide systems provides a promising route to such next-generation products, but significant technical challenges remain. Simplified mechanistic and diagnostic tools are thus a major current objective in metabolic engineering.

Polyketides are biosynthesized by repeated decarboxylative condensation of malonate-derived extender units *via* collaborative enzyme clusters termed polyketide synthases (PKSs).[2–6] Each condensation step requires an extender unit-primed acyl carrier protein (ACP) and a ketosynthase (KS)-bound polyketide intermediate (Fig. 1). In both cases, the small molecule component is attached to the corresponding protein through a thioester linkage. While the KS active site harbors a typical cysteine residue for this purpose, the *apo* form of ACP is posttranslationally modified with a thiol-terminated phosphopantetheine (Ppant) group to generate the mature *holo*-ACP. These two sites serve as the primary points of covalent attachment throughout polyketide biogenesis.

Nearly all biosynthetic steps associated with modular PKS systems rely on acyl-ACPs. Therefore, functional analysis of each enzymatic event requires a robust method for both generating these entities and monitoring their fate. Traditionally, this has been accomplished through enzymatic incorporation of radiolabeled acyl-CoA substrates onto ACP domains. Unfortunately, this strategy is often fraught with difficult syntheses and very costly reagents. As genomics-based technologies continue to provide an abundance of new PKS systems, the need for complementary methods utilizing simple, reliable, and structurally flexible ACP-acylating agents has never been greater.

Our work in this area began with a simple idea of using electrophilic β-lactones as acylating agents for ACPs.[7] The resulting ACP-bound β-hydroxythioester represents a common structural motif in modular polyketide biosynthesis. Selectivity for the Ppant-thiol over competing nucleophiles, including the KS active site, was examined for a panel of substituted lactones. Although all structures investigated showed some level of Ppant-acylation, monosubstituted β-lactones were generally both more reactive and selective. However, even the best acylating agents required a very large excess to obtain complete loading of the ACPs. Therefore, we were compelled to consider alternate electrophiles for future experimentation.

We were initially worried that increased reactivity may result in decreased selectivity for the Ppant group when competing nucleophiles, most prominently the KS-active site, were present. However, recent work by Khosla and coworkers has helped to change the landscape of possibilities surrounding PKS interrogation.[8–11] Using limited proteolysis of intact modules, they were able to identify conserved regions that served as effective boundary points for each domain, thus affording "broken

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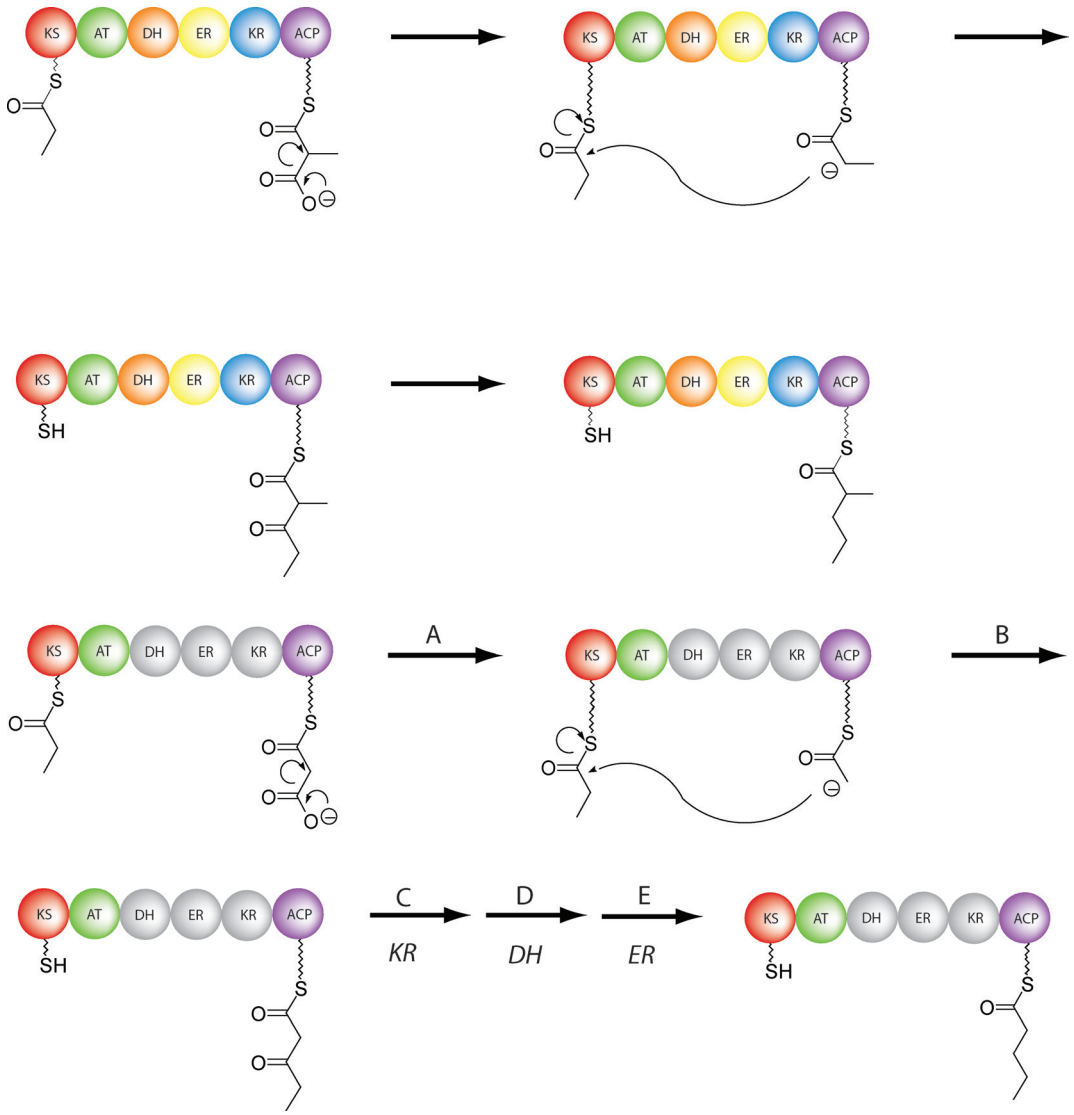

Fig. 1 Schematic diagram for polyketide formation in modular PKSs. (A) KS-mediated decarboxylation of ACP-bound malonate forms an ACP-bound enolate (B) Claisen-like condensation between the KS-bound chain and extender unit produces an ACP-bound β-ketothioester. (C) The ketoreductase domain reduces the β-ketothioester to a β-hydroxythioester. (D) The dehydratase domain dehydrates the β-hydroxythioester to an enoyl-thioester. (E) The enoyl reductase domain reduces the enoyl group to form a saturated acyl-chain. See text for abbreviations.

modules" where KS and ACP domains could be cloned and expressed separately. Most importantly, the separated domains retain their function when reintroduced *in vitro*. As a result, the challenge of designing Ppant-acylating agents is reduced to one of selectivity for *holo*-ACP over *apo*-ACP alone since the KS domain can be introduced after ACP-loading has occurred without limiting the scope of its utility.

Confident that all downstream applications of our probes would proceed smoothly using separated domains, we began to explore β-lactams as thiol-reactive acylating agents for isolated ACP domains. Relative to β-lactones, the amide nitrogen of β-lactams offers an additional site for ring substitution that is electronically coupled to the carbonyl, thus providing a reliable means of tuning electrophilicity. Herein, we describe our efforts to establish *N*-activated β-lactams as efficient and versatile acylating agents for ACP domains. The fluorescence and affinity labeling applications described set the stage for downstream studies aimed at increasing our understanding of KS-ACP recognition and intermodular polyketide transfer between these components.

Results and discussion

We expected that β-lactams could have a reactive advantage relative to β-lactones through appropriate activation of the ring nitrogen.[12] A variety of common nitrogen-protecting groups were introduced onto the parent azetidinone (compound **1**) *via* established methods to produce compounds **2**–**5** (Fig. 2).[13–16] Saturation curves were prepared by varying the number of equivalents of each lactam relative to isolated *holo*-ACP2 (module 2) and *holo*-ACP3 (module 3) from 6-deoxyerythronolide B synthase (DEBS) in analogous fashion to our previous work with β-lactones.[7] Briefly, lactam was added to a buffered solution of ACP and allowed to react for 1 h at room temperature. The mixture

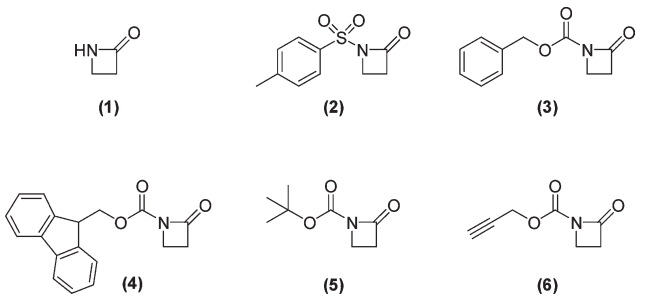

Fig. 2 Structures of *N*-activated β-lactams.

was subjected to trypsin digestion and subsequently quenched with formic acid. The resulting array of peptide fragments was analyzed by LC-MS. The extent of loading was estimated from the ratio of acylated Ppant-containing peptide to total Ppant-containing peptide, assuming similar ionization potentials.

To our satisfaction, compounds **2–5** exhibited excellent acylation efficiencies with both *holo*-ACPs. All but the Boc-activated lactam (compound **5**) showed around 80% loading at a mere 10 equivalents (Fig. 3). In addition, the thioester-ACP generated from compound 6 underwent a mere 10% hydrolysis after 24 h at room temperature, suggesting that the acyl-ACPs are suitably stable for all foreseeable applications (see supporting information). A direct competition between compound **6** and the most promising β-lactone from our previous study, 4-methyloxetan-2-one, reveals the substantial reactive advantage of β-lactams for acylation of *holo*-ACP (Fig. 4). Again, the Ppant-thiol is the only competent nucleophile present as these ACPs do not contain solvent-exposed cysteine residues. As expected, however, the general thiol-reactivity of these warheads renders them unsuitable for selective ACP-acylation in full modules due to competitive acylation of the KS-active site (see supporting information†).

To further examine selectivity for *holo*- over *apo*-ACP, we wished to employ a gel-based fluorescent assay. Based on work by Sieber and coworkers,[17] a [3 + 2] cycloaddition (click reaction) was planned for coupling acylated ACPs to a rhodamine-based fluorophore.[18–20] ACP2 and ACP3 (both *holo* and *apo*) were mixed with 1, 5, and 10 equivalents of **6** and allowed to react at room temperature for 1 h. Rhodamine azide was then added along with all necessary click reaction components and the resulting protein products were analyzed *via* SDS-PAGE. Bright bands were observed for the *holo*-ACPs while *apo*-ACPs provided only background fluorescence (Fig. 5). It should be noted that fluorescence can be readily observed at an impressive 5 equivalents of lactam for both ACPs.

To further explore Ppant selectivity and utility of the alkyne handle, we turned our focus to mixtures of *apo* and *holo* ACP2. In particular, we wanted to ask the question of whether *holo*-ACP2 could be isolated from a mixture of *apo* and *holo* by an affinity label conjugated to compound **6**. We chose a biotin-streptavidin interaction as the basis for ACP purification as this system (1) has been used extensively for protein separations[21–24] and (2) relies on a small molecule that we envisioned could readily be conjugated to the lactam warhead. As proof of principle and to assess the efficiency of each step, we began with samples of pure *holo*-ACP. Attempts to pre-conjugate compound

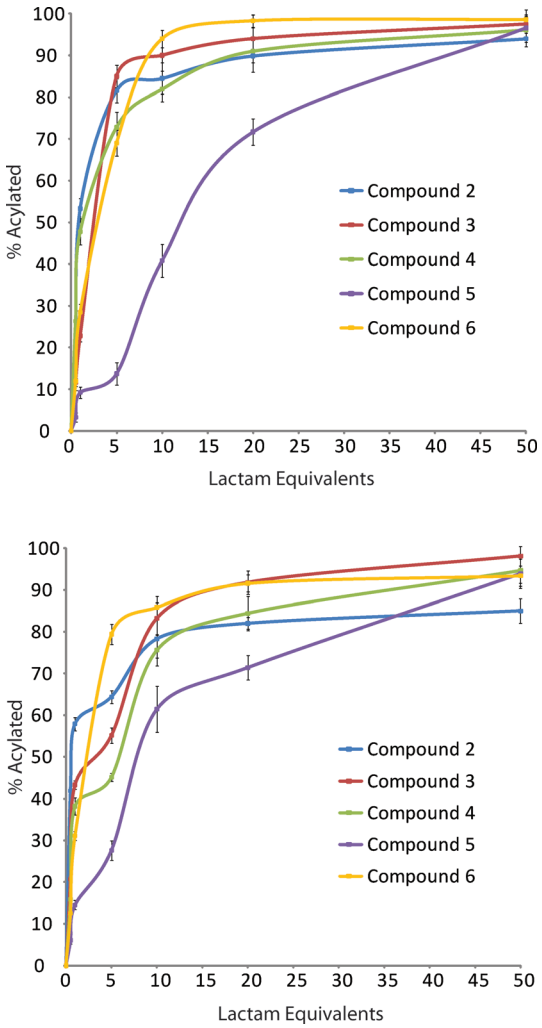

Fig. 3 Saturation curves as determined by tandem proteolysis-mass spectrometry for ACP2 (**Top**) and ACP3 (**Bottom**) with compounds **2–6**. Equivalents of lactam are per protein molecule. Lines are added for clarity. Error bars represent one standard deviation ($n = 3$).

6 with the biotin azide followed by 1 h reaction with the ACP resulted in low yields of the acylated protein. Competition from other proteinaceous nucleophiles was observed for longer reaction times which forced us to consider an alternative order of addition.

To our satisfaction, [3 + 2] cycloaddition of the biotin azide with pre-acylated *holo*-ACP resulted in 30% recovery following streptavidin–sepharose binding and subsequent treatment of the bound material with hydrazine, which restores the Ppant-thiol *via* nucleophilic attack on the thioester carbonyl. To remove excess lactam and biotin following the initial acylation and subsequent [3 + 2] cycloaddition, respectively, the samples were filtered twice prior to treatment with the streptavidin beads. Analysis of protein concentration at each stage revealed that the primary source of ACP loss in the process was these filtration steps. In fact, more than 60% of the protein subjected to the streptavidin was recovered upon treatment with hydrazine while almost half of the initial mixture is lost to the filtration membrane before this step (see supporting information†). We expect that upon scale-up, where small losses of protein to filtration are

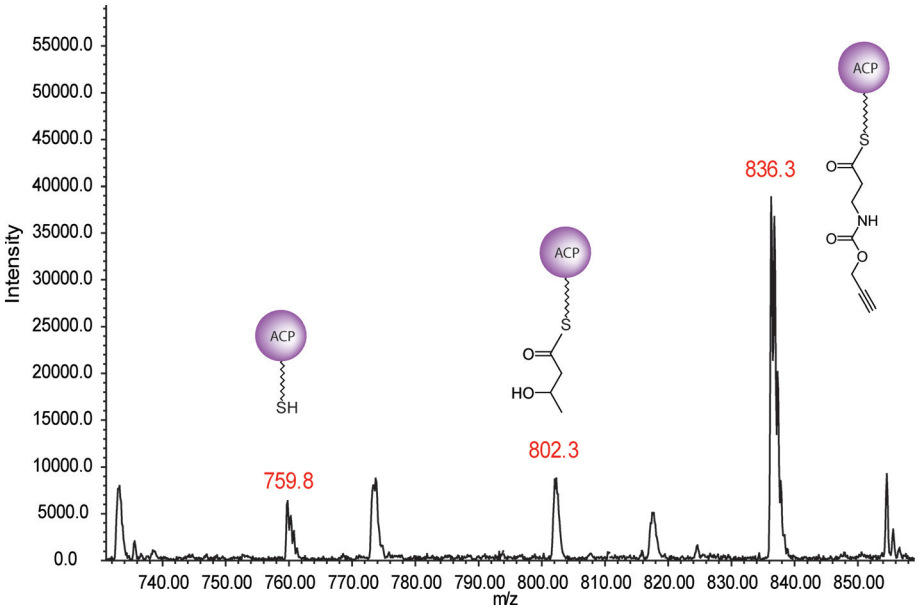

Fig. 4 β-lactam *vs.* β-lactone ACP-acylation. ACP2 was treated simultaneously with 10 equivalents of compound **6** and 4-methyloxetan-2-one for 1 h. The acylated-ACP was trypsinized and the resulting peptide fragments were analyzed by LC-MS. All labeled peaks arise from $m/z = +2$.

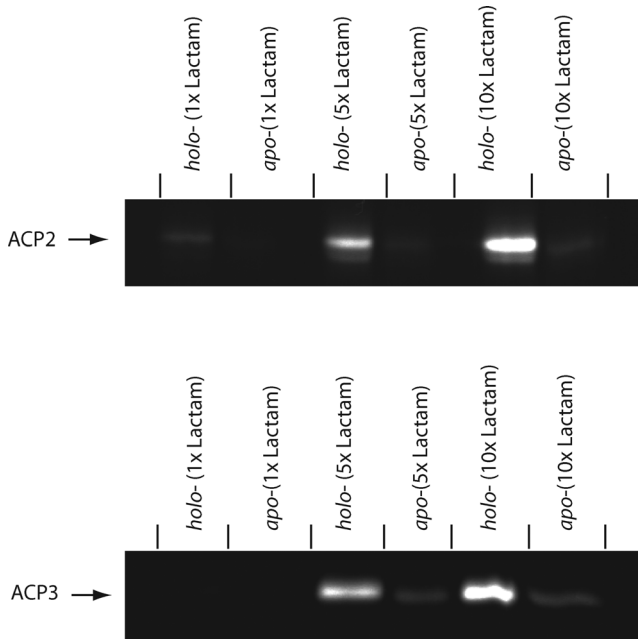

Fig. 5 Fluorescence SDS-PAGE analysis of DEBS ACP2 (*apo* and *holo*), and ACP3 (*apo* and *holo*) reaction with compound **6** and subsequent click reaction with rhodamine-azide. Lanes are marked above with the corresponding protein component and lactam equivalents (per protein molecule). Markers to the left indicate expected bands for the indicated species.

less significant, percent recovery will be drastically improved. As proof of principle, however, we were confident that these recovery levels would suffice for further experimentation.

Overexpression of ACP in BAP1, an engineered strain of *E. coli* with a phosphopantetheinyl transferase embedded in the genome, results in primarily the *holo* (Ppantylated)-form. However, as expression levels increase, the transferase struggles to keep up and mixtures of *holo*- and *apo*-ACP are often observed. To examine the viability of our approach for separating *holo*- from *apo*-ACP we prepared an 80 : 20 (*holo* : *apo*) mixture of ACP2, a typical ratio observed from overexpression of ACP in BAP1, and subjected it to the same process described above. Analysis of the final solution obtained from hydrazine treatment of the bound material by LC-MS revealed only *holo*-ACP2 (Fig. 6). Taken together with the fluorescence assay, these experiments nicely illustrate the remarkable Ppant-selectivity and versatility of compound **6**.

To confirm that the purification process did not alter the protein structure, the ability of eluted *holo*-ACP2 to accept a methylmalonyl-CoA unit from an acyltransferase was examined. To a buffered solution of DEBS module 6 ketosynthase-acyltransferase didomain[11,25–27] ([KS6AT6]) was added methylmalonyl-CoA and purified *holo*-ACP2. After 30 min of reaction time, the mixture was subjected to trypsinolysis and the resulting peptide fragments were separated and analyzed *via* LC-MS (Fig. 7). The prominent peak at 809.1 mass units suggests that the purified ACP remains competent for AT-ACP methylmalonate transfer as seen previously.[27]

It was now clear that carrier proteins could be efficiently acylated with β-lactams. However, to demonstrate the utility of these reagents for PKS examination, a direct comparison to traditional small molecule probes was necessary. In particular, we were interested in ACP to KS transfer of the β-aminothioester product (Fig. 8). As a primary determinant for proper flow of intermediates during polyketide biosynthesis, intermediate transfer between ACP and KS plays a critical role in product generation for all PKS systems. The most common method for direct acylation of KS active sites involves incubation of the KS with an *N*-acetylcysteamine thioester (SNAc) that mimics the terminal portion of the Ppant group.[28–31] To qualitatively determine the kinetic competency of our lactam-derived acyl-ACPs, relative to

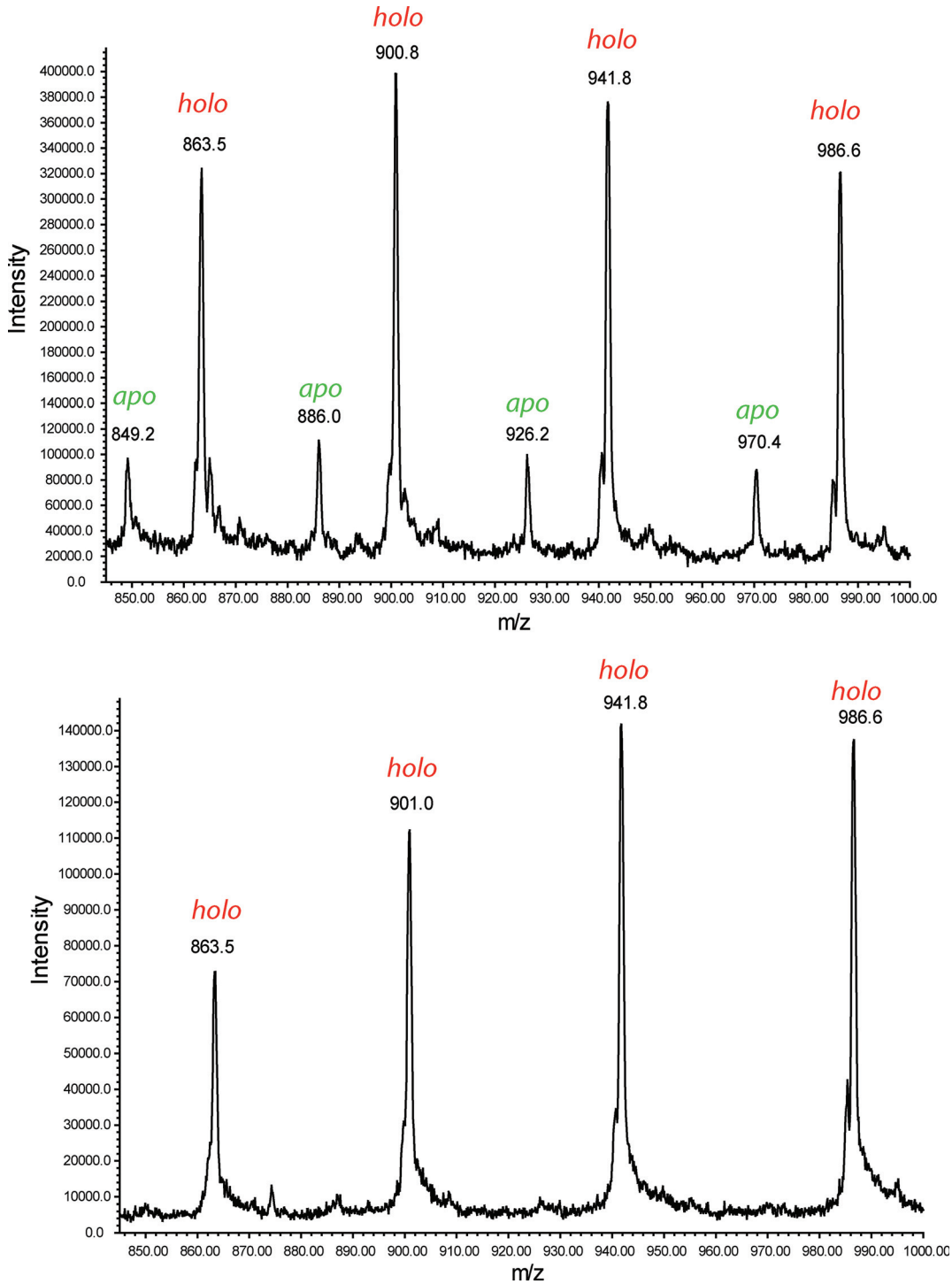

Fig. 6 Whole protein mass spectra of (top) an initial 80 : 20 (*holo* : *apo*) mixture of ACP2 and (bottom) the result of β-lactam based purification of *holo*-ACP2. The lack of *apo*-ACP2 in the latter indicates that the β-lactam (compound **6**) is highly selective for *holo*-ACP2. Peaks shown span *m*/*z* values from +21 through +24 for both species. See supporting information for full spectra.†

propionyl-SNAc and the potent KS-inhibitor, cerulenin,[32] a gel-based fluorescence assay was employed.

Acylation of *holo*-ACP2 was executed as before using 10 equivalents of compound **6** followed by Cu-mediated [3 + 2] cycloaddition with rhodamine azide. The resulting acyl-ACP was incubated with [KS6AT6] for 1 h at room temperature with or without competing KS-acylating/alkylating agents to explore ACP to KS transfer (Fig. 9). From these data, it is clear that fluorescence transfers from the ACP to KS in the absence and presence of both cerulenin and propionyl-SNAc, indicating that the lactam-derived acyl-ACPs are, at a minimum, competitive with traditional small molecule probes (Lanes 2, 4, and 6). The lack of KS-fluorescence observed when [KS6AT6] is pre-treated with each competitor (Lanes 3 and 5) confirms that transfer is

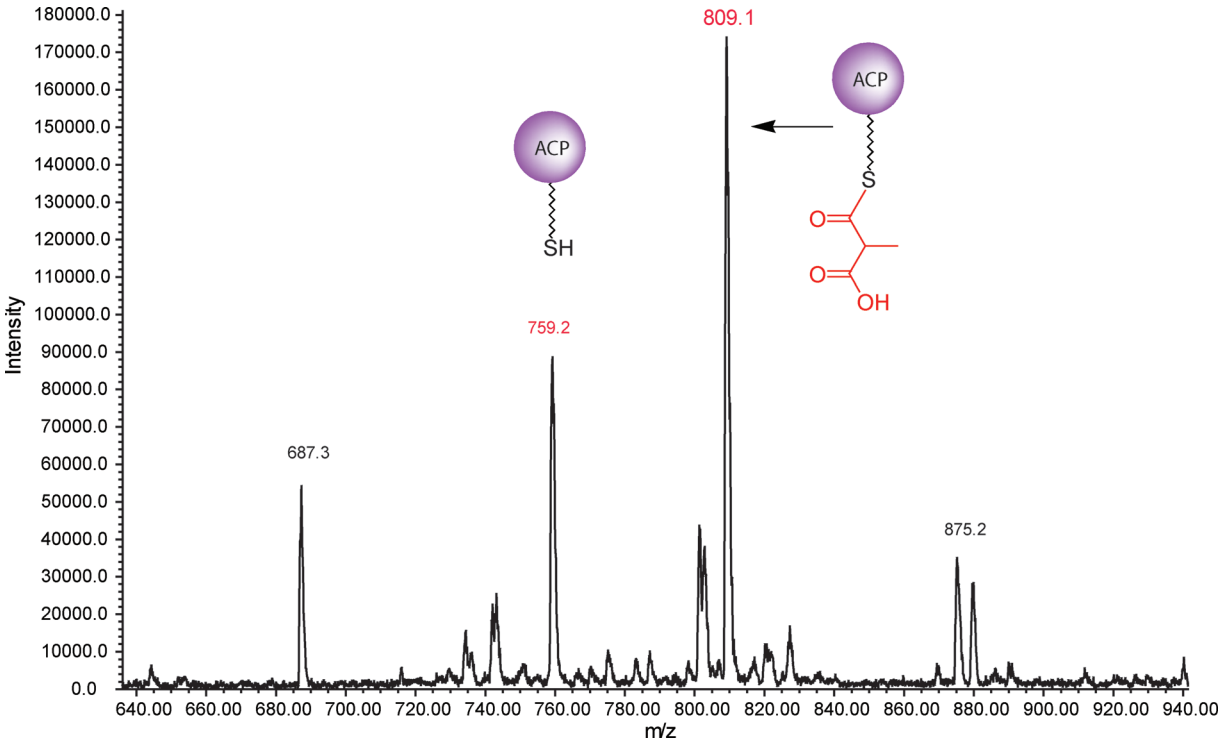

Fig. 7 Methylmalonyl-CoA transfer. Mass spectrum of purified *holo*-ACP2 digest following treatment with [KS6AT6] and methylmalonyl-CoA (see text). The prominent peak at 809.1 mass units confirms that the ACP functions properly after being subjected to β-lactam-based affinity purification. Both MS peaks correspond to $m/z = +2$.

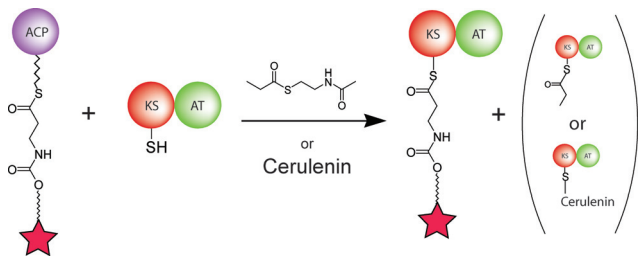

Fig. 8 Schematic diagram of ACP to KS acyl transfer. Fluorescently labeled ACP is mixed with a [KSAT] didomain in the presence or absence of competing KS probes and transfer of fluorescence from ACP to KS is monitored. The red star represents a rhodamine fluorophore.

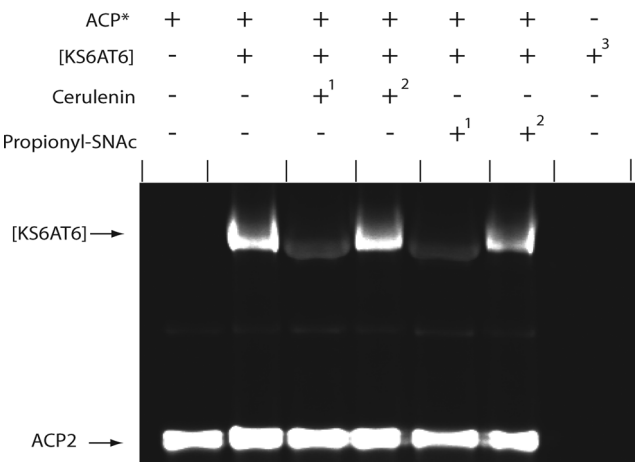

Fig. 9 Fluorescent competition assay for ACP to KS acyl transfer. Fluorescent ACP2 (ACP*) is prepared *via* click coupling of rhodamine azide with ACP2, preacylated with compound **6**. ACP2* is then incubated with [KS6AT6] under various conditions. Lanes are marked with the corresponding components for each reaction. [1][KS6AT6] was mixed with competitor (cerulenin or propionyl-SNAc) for 1 h prior to addition of ACP* (Lanes 3 and 5). [2]ACP* and competitor were introduced to [KS6AT6] simultaneously (Lanes 4 and 6). [3][KS6AT6] was mixed with 10 eq of the click product in the absence of ACP2 (Lane 7). SNAc = *N*-acetylcysteimine.

occurring between the Ppant arm and KS active site. Finally, the absence of a fluorescent band when [KS6AT6] is treated directly with the compound **6**-rhodamine click product implies that the ACP is required for transfer to occur (Lane 7). In all, these results suggest that β-lactam-derived, acyl-ACPs behave in analogous fashion to natural polyketide intermediates and are kinetically competitive with traditional PKS probes and inhibitors. Further experimentation to quantitatively determine the transfer rate is currently ongoing in the lab but, at this point, it is clear that activated β-lactams offer a greatly simplified and readily adaptable set of carrier protein modifiers.

Conclusion

In summary, we have demonstrated the utility of *N*-activated β-lactams for acylation of *holo*-ACPs. The structures examined are significantly more reactive than the β-lactones that we have previously reported and all are available in a single synthetic step from commercially available starting materials. Both carbamate and sulfamate moieties effectively activate the lactam ring

for ACP-acylation. The resulting acylation products maintain the thioester linkage common to all polyketide biosynthetic intermediates and the reaction can be readily reversed by treatment with hydrazine.

We are confident that the applications of β-lactams covered in this manuscript just begin to scratch the surface of their capabilities. The *N*-propargyloxycarbonyl group (compound **6**) provides a versatile chemical handle for nearly limitless elaboration of structure. Although we have focused exclusively on proteins involved in polyketide biosynthesis, we expect the general thiol-reactivity of these simple β-lactams to translate well to other systems bearing natural or engineered thiols.

Methods

General information

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. *n*-Butyl lithium was used as a 2.5 M solution in hexanes. Dichloromethane was distilled from calcium hydride and stored over activated molecular sieves. Acetonitrile was dried over activated molecular sieves for at least 24 h prior to use. Thin-layer chromatography (TLC) was conducted on glass-backed silica plates visualized with either UV illumination or basic permanganate stain. Flash chromatography was conducted on 60 Å silica gel.

Synthesis

1-[(4-Methylphenyl)sulfonyl]azetidin-2-one.[33] (2). 2-Azetidinone (72 mg, 1 mmol) was dissolved in THF (5 mL) at −78 °C. NaHDMS (367 mg, 2 mmol) dissolved in THF (1 mL) was added in portions. 4-Toluenesulfonyl chloride (763 mg, 4 mmol) dissolved in THF (5 mL) was added to the reaction mixture in portions slowly over 15 min. The reaction mixture was stirred until all the starting material was consumed (monitored by TLC), approximately 8 h. The reaction was washed with sodium bicarbonate (10 ml ×3) and extracted with DCM (25 mL ×3). The combined organic layers were dried over Na_2SO_4, and the solvent was removed by rotary evaporation. The crude material was purified by flash chromatography with 1 : 1 hexane : ethyl acetate. Yield: (178 mg, 0.79 mmol) 79% of final product as colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.9–7.87 (d, J = 8.29, 2H), 7.32–7.41 (d, J = 8.48, 2H), 3.67–3.64 (t, J = 5.2, 2H), 3.06–3.02 (t, J = 5.1, 2H), 2.46 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$) δ: 161.9, 143.4, 128.3, 125.7, 38.0, 34.9, 19.9.

IR: ν 2973, 1776, 1362, 1154, 682, 98 cm^{-1},

MS $[M + H]^+$ = 227

Benzyl 2-oxoazetidine-1-carboxylate.[14] (3). HDMS (250 mg, 1.5 mmol) was dissolved in THF (8 mL) and cooled to −78 °C. *n*BuLi (96 mg, 1.5 mmol) was added dropwise and stirred for 30 min. In a separate RBF, 2-azetidinone (106.6 mg, 1.5 mmol) was dissolved in 5 mL of THF at −78 °C. The contents of the first flask were transferred *via* cannula to the second flask and stirred for 1 h. Benzylchloroformate (256 mg, 1.5 mmol) was added in portions and stirred for 2 h at −78 °C. The reaction mixture was allowed to warm to ambient temperature and stirred for an additional 4 h. The reaction mixture was diluted with water and extracted with DCM (50 mL × 3). Combined organic phases were washed with brine and dried over Na_2SO_4. Solvent was removed by rotary evaporation. Purification of crude product by flash chromatography with 5 : 1 hexane : ethyl acetate yields (285 mg, 1.39 mmol) 93% of product as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.28–7.16 (m, 5H), 5.1 (s, 2H), 3.44–3.4 (t, J = 5.3, 2H), 2.85–2.81(t, J = 5.3, 2H).^{13}C NMR (400 MHz, $CDCl_3$) δ 23.7, 34.7, 36.1, 65.9, 126.1, 126.8, 147.1, 155.0, 167.8.

IR: ν 3040, 2985, 1811, 1724, 1388, 1330, 1214, 1177, 1120, 1044, 763, 699 cm^{-1},

MS $[M + H]^+$ = 206

(9*H*-Fluoren-9-yl)methyl 2-oxoazetidin-1-carboxylate (4). 2-Azetidinone (72 mg, 1 mmol) was dissolved in THF (5 mL), and LiHDMS (167 mg, 1 mmol) dissolved in THF (2 mL) was added dropwise at −78 °C over a 15 min period and stirred for an additional 30 min. 9-Fluorenylmethyl chloroformate (259 mg, 1 mmol) dissolved in THF (2 mL) was added dropwise at −78 °C. The resulting mixture was stirred for 1 h at −78 °C, then allowed to warm to R.T, and stirred for 1 h further at which point the starting material had been completely consumed (monitored by TLC). The reaction mixture was poured into water and extracted with DCM (3 × 25 mL). The combined extracts were washed with brine and dried over Na_2SO_4. After removal of the solvent by rotary evaporation, the crude reaction mixture was purified by flash chromatography with DCM : ethyl acetate, 95 : 5, producing the product as an off-white solid (62 mg, 0.62 mmol) in 86% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.13 (t, 2H), 3.68 (t, 2H), 4.32 (t, 1H), 4.47 (d, 2H), 7.26–7.80 (m, 8H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 162.1, 148.3, 141.3, 139.4, 126.4, 125.5, 123.5, 118.1, 66.8, 44.3, 34.8, 36.2

IR: ν 3015, 2922,1770, 1722, 1449, 1388, 1313,1119, 1044, 964, 738 cm^{-1}

HRMS (EI+) Calcd for $C_{18}H_{15}NO_3$, 293.1052; Found, 293.1046

***tert*-Butyl 2-oxoazetidine-1-carboxylate.[34] (5).** 2-Azetidinone (72 mg, 1 mmol) and DMAP (12.2 mg, 0.1 mmol) were dissolved in acetonitrile (5 mL) at 0 °C. Di-*tert*-butyl dicarbonate (218 mg, 1.1 mmol) was added to the reaction mixture in portions. The reaction mixture was stirred at 0 °C for 2 h, allowed to warm to ambient temperature and stirred overnight. The reaction mixture was diluted with ethyl acetate and washed with 1 N aq. HCl and brine. The aqueous layer was extracted with ethyl acetate (25 mL ×3). Combined organic layers were dried over Na_2SO_4 and the solvent was removed by rotary evaporation. The crude product was purified by flash chromatography using 2 : 1, hexanes : ethyl acetate (154 mg, 0.9 mmol) 90% of product as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.56–3.52 (t, J = 5.1, 2H), 3.03–2.87 (t, J = 5.2, 2H), 1.5 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ: 205.4, 162.1, 146.5, 81.2, 36.8, 35.0, 29.8, 26.2.

IR: ν 2984, 1795, 1719, 1331, 1157, 1043 cm^{-1},
MS $[M + H]^+ = 172$

Prop-2-yn-1-yl 2-oxoazetidine-1-carboxylate (6). 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 ×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 a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82–4.81(d, J = 23.3, 1H), 3.68–3.65 (t, J = 5.3, 2H), 3.10–3.07 (t, J = 5.3, 2H), 2.55–2.53 (t, J = 2.4, 1H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 34.9, 36.1, 51.8, 74.0, 146.3, 162.2.

IR: ν 3251, 2925, 2134, 1782, 1722, 1313, 1187, 1114, 1045, 615 cm^{-1}

HRMS (EI+) Calcd for $C_7H_7NO_3$, 153.0426; Found, 153.0402

Sulforhodamine-B azide (S1).[7] 3-Azido-1-aminopropane[35] (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.[36] *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.

NMR spectra are of poor quality due to long relaxation times (see supporting information†). Product is pure by LC-MS.

IR: ν 2975, 2100, 2590, 1339, 1180 cm^{-1}

HRMS (EI+) Calcd for $C_{30}H_{37}N_6O_6S_2$, 641.2210. Found, 641.2216.

Biotin azide (S2). D(+)-biotin (73 mg, 0.300 mmol) was dissolved in 3 mL of DMF by briefly heating the mixture with a heat gun. The mixture was cooled to 0 °C in an ice-water bath and 6-chloro-1-hexanol (120 mg, 0.900 mmol), and *N*,*N*-dimethylamino pyridine (73 mg, 0.600 mmol) were added. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (115 mg, 0.600 mmol) was added in portions over *ca.* 60 min and the reaction mixture was allowed to come to room temperature. Stirring was continued for 24 h. When biotin had been consumed (monitored by TLC), the reaction mixture was diluted with ethyl acetate (100 mL). The organic phase was washed with concentrated $NaHCO_3$ (3 × 50 mL), and pH 2 saturated Na_2SO_4 (5 × 50 mL portions). Solvent was removed by rotary evaporation and the material was used without further purification. The material from the ester coupling was dissolved in 5 mL of DMF and NaN_3 (40 mg, 0.600 mmol) and a catalytic amount of KI were added. A reflux condenser was attached and the reaction mixture was heated to 80 °C for 16 h. The material was diluted with 100 mL of ethyl acetate and washed with brine. The solvent was removed by rotary evaporation and the crude material purified by flash chromatography (5% MeOH in DCM) to yield 67 mg, 0.182 mmol of product (61%) as a waxy pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.32–1.52 (m, 6 H) 1.56–1.80 (m, 8 H) 2.33 (t, J = 7.45, 2 H) 2.74 (d, J = 12.76, 1 H) 2.91 (dd, J = 12.82, 4.99, 1 H) 3.16 (ddd, J = 8.34, 6.38, 4.74, 1 H) 3.28 (t, J = 6.88, 2 H) 4.06 (t, J = 6.63, 2 H) 4.31 (ddd, J = 7.67, 4.71, 1.14, 1 H) 4.51 (dd, J = 7.71, 5.05, 1 H) 5.67 (s, 1 H) 6.03 (s, 1 H).

^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 173.7, 163.7, 65.6, 61.2, 60.8, 56.1, 51.3, 40.5, 33.9, 29.9, 28.7, 28.4, 27.2, 26.3, 25.5, 24.8

IR: ν 3230, 2934, 2101, 1733,1700, 1264, 1175 cm^{-1}

HRMS (EI+) Calcd for $C_{16}H_{27}N_5O_3S$, 369.1848; Found, 369.1814

Propionyl-*N*-acetylcysteamine (Propionyl-SNAc)

To a solution of triethylamine (2.80 mmol) in dichlromethane (5 mL) was added propionic acid (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and N-acetyl cysteamine (SNAc)(1.35 mmol). The reaction mixture was stirred overnight. The organic layer was washed with saturated NaHCO3 solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated under vacuum, and purified by flash column chromatography (1 : 1 hexane : ethyl acetate) to provide compound (140 mg, 74%) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.86 (t, J = 7.58 Hz, 3H) 1.69 (s, 3H) 2.28 (q, J = 7.4 Hz, 2H) 2.72 (t, J = 6.8 Hz, 2H) 3.07 (q, J = 6.6 Hz, 2H) 7.39 (br s, 1H)

^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 199.4, 170.6, 39.0, 36.8, 28.0, 22.6, 9.4

IR: ν 3282,2979,1690,1650,1546,1373,1288,1090,935 cm^{-1}

MS $[M + H]^+ = 176$

Protein expression and purification

ACP2 was expressed from pNW06,[37] ACP3 was expressed from pVYA05.[11] [KS6AT6] was expressed from pAYC11.[26] *apo*-ACPs were harvested from *E. coli* BL-21 and *holo*-ACPs were harvested from *E. coli*. BAP-1[38] pNW6 and pVYA05 contain kanamycin resistant vectors.

General procedure for protein expression 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 3000 RPM for 10 min and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na_3VO_4, 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, average final concentration was approximately 3 mM. Proteins were flash frozen and stored at −80 °C until use.

Proteins acylation with β-lactams

Labeling reactions for gel assays were performed at 20 μL total volume, reactions for LC-MS were performed at 50 μL total volume. Final concentrations reported in procedure. Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM), and protein (0.025 mM) were reacted at ambient temperature for 15 min. β-Lactam was added to achieve the appropriate final concentration (0.5, 1, 5, 10, 20, 50x) with respect to protein. The mixture was equilibrated at ambient temperature for 60 min.

β-Lactam/β-lactone competition loading of ACP

10 equivalents of compound **6** and 4-methyloxetan-2-one were mixed and introduced to buffered *holo*-ACP2 for 1 h. The sample was trypsinized analyzed by LC-MS as described previously.[7]

Chromophore attachment

The reaction was carried out at 25 μL, final concentrations reported. **S1**, (2× with respect to **6**), 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 60 min.

Gel assay

Labeled samples were diluted to 35 μL with gel-loading buffer. ACP 2 was separated by 12% and ACP 3 with 15% SDS-PAGE, each with a 5% stacking gel run at 100 V, 50 mA, 135 min. Gels were developed in 10% acetic acid to visualize **S1**. Labeled proteins were imaged on a UV-transilluminator. Total protein was stained using Coomassie stain. For the ACP to KS transfer experiments (Fig. 8), proteins were separated using a 4–20% gradient HEPES-PAGE gel (100V, 50mA, 90 min).

Proteolysis

Sequencing grade modified trypsin was added to prepared samples so that the final trypsin : ACP ratio was 1 : 50 (w/w). The mixture was incubated at 30 °C for 18 h. Digestion was quenched by addition of an equal volume of 10% formic acid. Digests were flash frozen in liquid nitrogen and stored at −20 °C until analysis.

Affinity purification of *holo*-ACPs

Lactam modification of ACPs

Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. 1 μL of an 80 : 20 mixture of *holo* : *apo*-ACP2 (25 μM total protein) was equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. **6** (25× with respect to total ACP concentration) was added and the mixture was equilibrated at ambient temperature for 1 h. To remove excess **6**, the mixture was loaded into a 3kDa 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 3 times. Protein was removed from the concentrator by inverting it and spinning. The concentrator was then washed several times with phosphate buffer which was added to the protein sample. The filtered protein was reconstituted to 900 μL.

[*3 + 2*] *Cycloaddition*

In a separate microfuge tube, DMSO (100 μL), **S2** (50× with respect to protein), THPTA[39](1.1 mM), NaAsc (10 mM), and $CuSO_4$ (1 mM) were combined. Best results are obtained if these reagents are first combined in a separate container and then added to the protein. The combined click reagents are added to the protein from the previous step and allowed to react at ambient temperature for 6 h. Excess biotin reagent was removed using the same procedure for the removal of excess lactam. The modified protein was reconstituted to 1000 μL in phosphate buffer.

Immobilization of modified ACPs

Streptavidin Sepharose High Performance (10× with respect to protein) was washed 3× with 1000 μL of phosphate buffer and the supernatant was removed. The modified protein from the previous step was added to the beads, and the mixture equilibrated at ambient temperature for 60 min with agitation on an orbit shaker. The supernatant was removed and the beads were washed 5× with 1000 μL of phosphate buffer, 5× with 1000 μL of TRIS buffer (100 mM, pH 9.0), and once with 1000 μL of 100 mM TRIS, +10 mM N_2H_4, pH 9.0.

Elution of immobilized ACPs

The washed beads from the previous step were resuspended in 1000 μL of 100 mM ammonium formate containing 100 mM N_2H_4 (100 mM pH 9.0) and equilibrated overnight at 4 °C with agitation on an orbit shaker. Eluted protein was concentrated in a 3kDa NMW concentrator and subjected to buffer exchange for

further experiments, or combined with TCEP for direct infusion LC-MS experiments.

Fluorescent assay for ACP to KS transfer

Holo-ACP2 (25 μM) was labeled with 10 equivalents of **6** followed by [3 + 2] cycloaddition with **S2** as described above. For transfer of the fluorescent product, [KS6AT6] (25 μ M) was introduced to acyl-ACP2 (25 μM) and incubated for 1 h. For lanes 3 and 5 (Fig. 9), [KS6AT6] was pretreated with cerulenin (5 mM) or propionyl SNAc (10 mM) for 1 h before introducing it to acyl-ACP2. For lanes 4 and 6, acyl-ACP2 (25 μM) was mixed with cerulenin (5 mM) or propionyl-SNAc (10 mM) followed by 1 h incubation with [KS6AT6] (25 μM). The [KS6AT6] control (lane 7) was executed under the same acylation and click conditions as in other samples but without the ACP or small molecule competitors.

LC-MS

Separation was performed with a Waters 1525 system. The gradient employed was A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5–95% B over 60 min with a Waters XBridge C18 5u column (4.6 × 150 mm). Mass spectra were acquired with a Waters Micromass ZQ mass detector in EI+ mode: Capillary voltage = 3.50 kV, cone voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source T = 100 °C, desolvation T = 200 °C, desolvation gas = 300 L h^{-1}, desolvation gas = 0.0 L h^{-1} The system was operated by and spectra were processed using the Waters Empower software suite.

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