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Direct Acylation of Carrier Proteins with Functionalized β-Lactones

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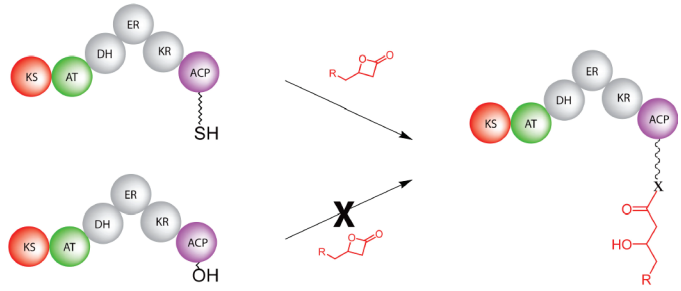
ABSTRACT



As the key component of many biosynthetic assemblies, acyl-carrier proteins offer a robust entry point for introduction of small molecule probes and pathway intermediates. Current labeling strategies primarily rely on modifications to the phosphopantetheine cofactor or its biosynthetic precursors followed by attachment to the *apo* form of a given carrier protein. As a greatly simplified alternative, direct and selective acylation of *holo*-acyl-carrier proteins using readily accessible β-lactones as electrophilic partners for the phosphopantetheine-thiol has been demonstrated.

Site-specific labeling of intact proteins remains a formidable challenge largely due to the multitude of potential nucleophilic sites in most biomacromolecules. β-Lactones have received significant attention as electrophilic tagging agents capable of reacting with typical amino acid functionalities.[1,2] Our interest in polyketide biosynthesis has spurred questions of whether these structures might be used to directly label acyl carrier proteins (ACPs) via the phosphopantetheine (Ppant)-thiol. β-Hydroxythioesters, formed by addition of the thiol to a β-lactone, are common components of polyketide intermediates rendering this strategy particularly appealing.

Acyl carrier proteins, the primary point of attachment for the growing polyketide chain, are inarguably the workhorse of modular polyketide synthases (PKSs).[3] They must recognize and properly associate with ketosynthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and in some cases thioesterase (TE) domains during polyketide maturation. To function, each *apo*-ACP is posttranslationally modified with a coenzyme A-derived PPant group to produce *holo*-ACP. The specific transferases responsible for this transformation have continually displayed broad tolerance for acyl groups on the terminal thiol.[4] As a result, several groups have taken advantage of this for loading ACPs with a variety of substrates. However, we reasoned that the Ppant group may be uniquely reactive,

(1) (a) Drahl, C.; Cravatt, B. F.; Sorensen, E. J. *Angew Chem., Int. Ed.* **2005**, *44*, 5788. (b) Kim, D. H.; Park, J. I.; Chung, S. J.; Park, J. D.; Park, N. K.; Han, J. H. *Bioorg. Med. Chem.* **2002**, *10*, 2553.

(2) Bottcher, T.; Sieber, S. A. *J. Am. Chem. Soc.* **2008**, *130*, 14400.

(3) Khosla, C.; Tang, Y.; Chen, A. Y.; Schnarr, N. A.; Cane, D. E. *Annu. Rev. Biochem.* **2007**, *76*, 195.

(4) (a) Mercer, A. C.; Meier, J. L.; Torpey, J. W.; Burkart, M. D. *ChemBioChem* **2009**, *10*, 1091. (b) Kapur, S.; Worthington, A.; Tang, Y.; Cane, D. E.; Burkart, M. D.; Khosla, C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3034. (c) Worthington, A. S.; Rivera, H.; Torpey, J. W.; Alexander, M. D.; Burkart, M. D. *ACS Chem. Biol.* **2006**, *1*, 687. (d) Meier, J. L.; Mercer, A. C.; Rivera, H.; Burkart, M. D. *J. Am. Chem. Soc.* **2006**, *128*, 12174. (e) Clarke, K. M.; Mercer, A. C.; La Clair, J. J.; Burkart, M. D. *J. Am. Chem. Soc.* **2005**, *127*, 11234. (f) Sieber, S. A.; Walsh, C. T.; Marahiel, M. A. *J. Am. Chem. Soc.* **2003**, *125*, 10862.

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given an appropriate electrophilic partner, and therefore, targetable for direct acylation. As we ideally wished to maintain polyketide-like functionality in the product, β-lactones seemed most suitable. The work described herein provides a greatly simplified alternative to traditional means of selectively loading ACPs.

The simplest test of selective Ppant acylation is to expose our reagents to isolated *holo*- and *apo*-ACP domains. Successful agents will exhibit selectivity for the *holo* structures, without nonspecific acylation of the *apo* ones. To begin assessing our direct acylation approach, an alkyne-bearing β-lactone, compound **1** (Figure 1), was prepared

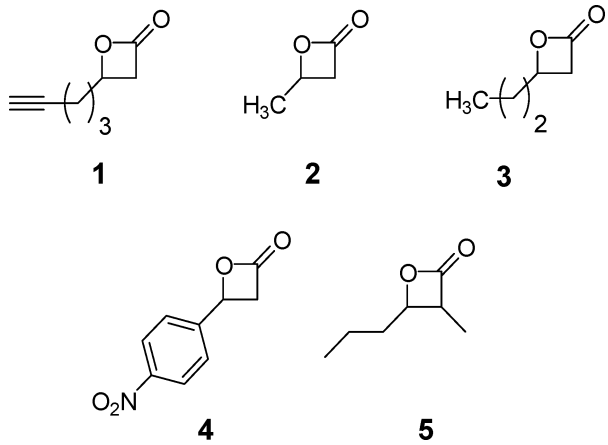

Figure 1. Structures of β-lactones prepared for direct acylation of acyl-carrier proteins.

according to the method of Nelson and co-workers.[5] Isolated ACP domains from modules 2 (ACP2) and 3 (ACP3) of the 6-deoxyerythronolide B synthase (DEBS) were overexpressed and purified in both *apo* and *holo* forms, using BL21 and BAP1 cells, repectively.

Of course, to be truly useful, our probes must avoid reaction with nucleophiles present on other common PKS domains. Since KS active site cysteines have been previously shown to be competent nucleophiles for β-lactone ring-opening,[6] a KS-AT didomain from DEBS module 6 (KS-AT6) was overexpressed and purified as previously reported.[7] We were encouraged by the previous report of Sieber and co-workers where minimal reactivity between compound **1** and the KS domain of bacterial fatty acid synthase was observed.[6] It remained to be seen, however, whether the purified DEBS KS domain would behave in a similar fashion. The stage was now set to examine both efficiency and selectivity of ACP-acylation with β-lactones.

Tandem proteolysis-mass spectrometry has been shown previously to be a powerful means of detecting PKS-bound small molecules in vitro.[8] Thorough trypsinolysis of *apo*- and *holo*-ACPs produced a series of peptide fragments including one containing the conserved DSL motif, to which the Ppant is attached in samples derived from BAP1 as determined by LC-MS (see the Supporting Information). Incubation of *holo*-ACP2 and -3 with varied equivalents of **1** at pH 7 for 1 h followed by exhaustive trypsinolysis and LC-MS, to determine the fraction acylated, produced saturation curves which indicated that a roughly 50-fold excess of lactone is required for complete acylation (Figure 2).

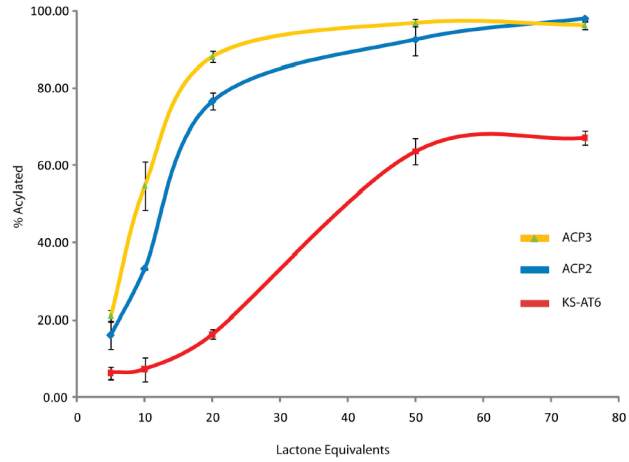

Figure 2. Saturation curves as determined by tandem proteolysis-mass spectrometry for ACP3 (yellow), ACP2 (blue), and KS-AT6 (red) with compound **1**. Equivalents of lactone are per protein molecule. Each data point is the average of three experiments. Standard deviations are shown as black error bars. Lines are added for clarity.

Gratifyingly, both *apo*-ACPs showed no acylation with 50 equiv of lactone, providing further evidence that the reaction is occurring on the Ppant arm as this is the only difference between the *apo* and *holo* forms of ACP (see the Supporting Information). The analogous saturation curve for KS-AT6 showed 25−50% less acylation than ACP for the range of lactone equivalents tested. Most importantly, at 10 lactone equivalents, the ACPs were approximately 35−50% loaded while the KS was mostly unreactive after 1 h of incubation. Standard deviations for these experiments were generally within 1−4% suggesting that the differential reactivity observed was genuine.

While tandem proteolysis/LC-MS confirms acylation of the PPant group, not all proteolysis products readily ionize under the conditions used. In addition, we could not rule out the potential for acylation of the peptide fragments over the intact protein. Therefore, an alternative, gel-based assay was designed according to previous work by Sieber and co-workers.[6] Each ACP (*apo* and *holo*) and KS-AT6 was

(5) Nelson, S. G.; Wan, Z.; Peelen, T. J.; Spencer, K. L. *Tetrahedron Lett.* **1999**, *40*, 6535–6539.

(6) Bottcher, T.; Sieber, S. A. *Angew. Chem., Int. Ed.* **2008**, *47*, 4600.

(7) (a) Kim, C.-Y.; Alekeyev, V. Y.; Chen, A. Y.; Tang, Y.; Cane, D. E.; Khosla, C. *Biochemistry* **2004**, *43*, 13892–13898. (b) Chen, A. Y.; Schnarr, N. A.; Kim, C.-Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2006**, *128*, 3067–3074.

(8) (a) Schnarr, N. A.; Chen, A. Y.; Cane, D. E.; Khosla, C. *Biochemistry* **2005**, *44*, 11836. (b) Hicks, L. M.; O'Connor, S. E.; Mazur, M. T.; Walsh, C. T.; Kelleher, N. L. *Chem. Biol.* **2004**, *11*, 327. (c) Hong, H.; Appleyard, A. N.; Siskos, A. P.; Garcia-Bernardo, J.; Staunton, J.; Leadlay, P. F. *FEBS J.* **2005**, *272*, 2373.

incubated for 1 h with 10 equiv of **1** followed by 1,3-dipolar cycloaddition ("click") reaction with rhodamine azide.[9] SDS-PAGE analysis of the resulting mixtures showed bright fluorescent bands corresponding to *holo*-ACP2 and -3 while the *apo*-ACPs and KS-AT6 displayed only background fluorescence (Figure 3). Not only does this provide initial

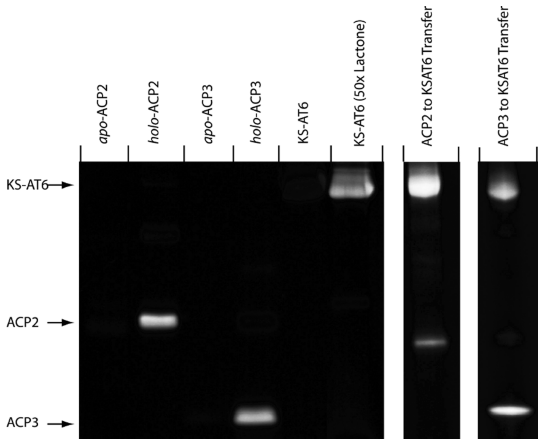

Figure 3. PAGE analysis of DEBS ACP3 (*apo* and *holo*), ACP2 (*apo* and *holo*), KS-AT3, and KS-AT6 reaction with compound **1** and subsequent click reaction with rhodamine-azide. Lanes are marked above with the corresponding protein component. Markers to the left indicate expected bands for the indicated species. The right two lanes depict transfer of the acylation product from preloaded ACP to KSAT indicating that the intermediates are functional. β-Lactone was applied at 10× relative to protein unless otherwise noted. ACP2 contains a C-terminal linker region accounting for its larger mass.

indication that β-lactones can be selective for ACP over KS but other potential competing residues such as surface cysteines, serines, and lysines can also be avoided.

To examine the functionality of the acyl-ACP intermediates and provide evidence of thioester over thioether formation, preloaded ACP2 and ACP3 (10× compound **1**) were mixed with KS-AT6. After 1 h, the click reaction was performed as before and the proteins separated by gel electrophoresis. Bright bands corresponding to the KS-AT6 didomain indicate that the β-hydroxythioester is efficiently transferred from the ACPs (Figure 3, right). It should be noted that direct KS-acylation is not observed under the conditions used in these experiments (Figure 3, left).

To examine β-lactone behavior with the next level of complexity, we overexpressed and purified *apo* and *holo* forms of the intact module 2 from spinosyn synthase (SpnB). This particular module was chosen because it contains KS, AT, DH, ER, KR, and ACP domains which represent the vast majority of synthase components present in a given assembly. Both *apo* and *holo* forms of spinosyn module 2 were incubated for 1 h with 10, 50, and 75 equiv of **1** followed by "click" reaction with rhodamine azide and PAGE analysis as before (Figure 4). Again, the only difference

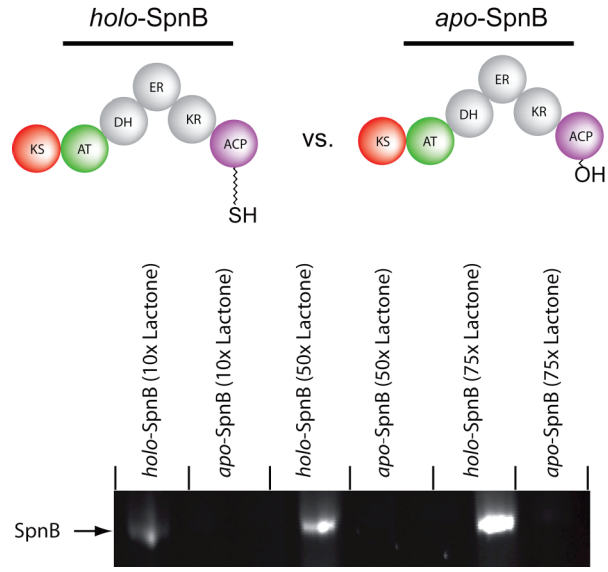

Figure 4. SDS-PAGE analysis of SpnB (*apo* and *holo*) reaction with 10, 50, and 75 equiv of compound **1** and subsequent click reaction with rhodamine-azide. Lanes are marked above with the corresponding protein component and equivalents of lactone.

between *apo* and *holo* forms was the absence or presence of the Ppant group, respectively. To our satisfaction, only the *holo*-protein showed significant fluorescence indicating that Ppant-thiol is the only competent partner for acylation among a multitude of alternative nucleophiles. Interestingly, minimal acylation of the *apo* form was observed even at very high equivalents of lactone.

With increased confidence that tandem proteolysis-mass spectrometry provides an accurate means of evaluating ACP and KS acylation, we began examination of a diverse panel of β-lactone structures. Compounds **2**−**5** were added to *holo*-ACP2 and -3 as well as KS-AT6 at 10- and 50-fold excess. Proteolysis and LC-MS anaylses were performed after 1 h of incubation as above. Acylation efficiencies, calculated as percent loading, were obtained from peak heights of acylated peptides divided by the total peptide (acylated plus unreacted) peak heights for a given LC-MS run (Table 1).

While all of the compounds tested acylate both ACP and KS to some degree, an interesting trend emerges from this study. The monosubstituted, alkyl-lactones, compounds **1**−**3**, are quite reactive toward both ACPs tested. In contrast, the disubstituted lactone, compound **5**, shows little ACP-acylation activity even at 50 equiv. KS-AT6, on the other hand, is more reactive than ACP2 and -3 toward compound **5** but significantly less reactive with compounds **1**−**3** than the ACPs. Finally, the monosubstitued, aromatic lactone, compound **4**, reacts slugishly with both ACP and KS. These data offer promising preliminary evidence that β-lactones can

(9) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021. (b) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053–1057. (c) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.

Table 1. Calculated Loading for ACP2, ACP3, and KS-AT6 with Compounds **1**−**5** at 10 and 50 equiv of Lactone

		loading, %		
compd	equiv of lactone	ACP2	ACP3	KSAT6
1	10	33	55	7
	50	93	97	64
2	10	78	64	26
	50	97	97	65
3	10	53	62	41
	50	96	94	62
4	10	8	13	6
	50	32	36	14
5	10	7	15	27
	50	28	35	58

provide an efficient and selective means of acylating carrier proteins. The differential reactivities observed for ACP versus KS suggest that future optimization of ACP-selectivity should focus on monosubstituted lactones. Efforts to further explore this structure-selectivity relationship are currently underway in our laboratory.

In summary, we have successfully demonstrated direct acylation of isolated *holo*-ACP domains with functionalized β-lactones. The *apo* form of this ACP is unreactive suggesting that β-hydroxythioester formation takes place on the Ppant arm of mature carrier proteins. In addition, ACPs can be selectively acylated in the presence of competing PKS active sites. This method offers a synthetically straightforward means of loading key PKS components with choice substrates.

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Supporting Information Available: Detailed descriptions of synthetic, molecular biology, and analytical procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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