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Insights into β -ketoacyl-chain recognition for β -ketoacyl-ACP utilizing AHL synthases†

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Abstract

Beta-ketoacyl-ACP utilizing enzymes in fatty acid, polyketide and acyl-homoserine lactone biosynthetic pathways are important targets for developing antimicrobial, anticancer and antiparasitic compounds. Published reports on successful isolation of beta-ketoacyl-ACPs in a laboratory remain scarce to date and thus most beta-ketoacyl-ACP utilizing enzymes are routinely characterized using small molecule substrates in lieu of the bonafide 3-oxoacyl-ACPs. We report the systematic investigation into the electronic, geometric and spatial aspects of beta-ketoacyl-chain recognition to develop 3-oxoacyl-ACP substrate mimics for two beta-ketoacyl-ACP utilizing quorum signal synthases.

Beta-ketoacyl-acyl carrier proteins are important cellular metabolites synthesized by a ketosynthase catalyzed Claisen condensation of malonyl-acyl carrier protein and an unsubstituted acyl-acyl carrier protein during fatty acid and polyketide biosynthesis.¹ β -Ketoacyl-ACP utilizing enzymes in these pathways are prime targets for developing medicines to treat bacterial infections, parasitic infections, cancer, obesity *etc.*² Most β -ketoacyl-ACP utilizing enzymes are often characterized using *N*-acetylcysteamine or CoA-based small molecule substrates in lieu of ACP-based substrates, perhaps due to the relatively easier access (cost, time and yield) to these compounds compared to protein-based substrates. Unfortunately, biochemical investigations on this class of enzymes with small molecule substrates miss out valuable information on the role of protein-protein recognition and dynamics in enzyme catalysis. Despite more than three decades of literature on the synthesis of unsubstituted acyl-ACPs, peer-reviewed publications on the successful isolation of 3-oxoacyl-ACPs, especially the long-chain β -ketoacyl-ACPs, are surprisingly hard to find.³ In our own laboratory, despite multiple attempts, we were unable to synthesize β -ketoacyl-ACPs in the amounts required for routine enzyme assays. Although we do not

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Conflicts of interest

There are no conflicts of interests to declare.

claim that beta-ketoacyl-ACPs are impossible to make, based on the observations discussed above, we surmise that the direct functionalization of coenzyme A derivatives with an enolizable β -keto moiety will be a synthetically challenging task to accomplish. We therefore decided to circumvent this issue by (a) not installing the " β -keto" moiety but instead mimic this functionality in the acyl-chain of β -ketoacyl-ACPs or (b) suppress enolization by masking the α -hydrogens. To develop stable and catalytically active β -ketoacyl-ACP mimics, we describe the design, synthesis and characterization of a library of molecular probes to systematically investigate the electronic, geometric and spatial characteristics of the " β -keto" functional group recognition for two β -ketoacyl-ACP utilizing enzymes.

The following guiding principles were considered while designing the β -ketoacyl-ACP mimics: (i) presence of a hetero-atom at or around the β -carbon in the acyl-chain, (ii) reduction or elimination of β -ketoenolate tautomer formation, and (iii) commercial availability or ease of synthesis of the carboxylic acid starting materials. Based on the limited structural information on β -ketoacyl-substrate utilizing enzymes available to date, it appears that the active site usually has a complementary hydrogen bond donor residue such as threonine to specifically aid in β -keto moiety recognition.⁴ We therefore placed high emphasis to include a heteroatom at or around the β -carbon in the acyl-chain of the substrate (Fig. 1). For instance, 2-furanacetyl-ACP (**10-ACP**) includes an oxygen atom attached to C3 that is also sp^2 hybridized. So, we hypothesized this substrate would most likely be a close mimic of a β -ketoacyl-ACP. Nevertheless, we were concerned about the effect of having one of the lone pairs of electrons on oxygen becoming involved in the $(4n + 2)$ aromatic system and whether its participation in π -electron delocalization would potentially disrupt the ability of the remaining ring oxygen atom lone pair to hydrogen bond with a complementary active site residue. Furthermore, this substrate should reveal the importance of enolizability of the β -keto oxygen in enzyme-substrate recognition. In the fully reduced ring of 2-tetrahydrofuranacetyl-ACP (**11-ACP**), the oxygen lone pair is not delocalized; however, the β -carbon is sp^3 hybridized. The sulfur atom in 2-thiopheneacetyl-ACP (**12-ACP**) has a larger van der Waals radius (longer C-S bond), increased electron pair delocalization and decreased hydrogen bonding potential compared to oxygen in 2-furanacetyl-ACP.⁵ Unlike thiophene, the nitrogen atom in 2-pyridylacetyl-ACP (**13-ACP**) is part of a larger 6-membered ring and since the nitrogen lone pair is not delocalized, it can participate in hydrogen bonding interactions with a suitable hydrogen bond donor in the active site. 2-Benzofuranacetyl-ACP (**15-ACP**) includes a second ring intended to gain additional binding energy with enzymes that have a deeper acyl-chain pocket. In order to assess the importance of the position of oxygen atom in the acyl-chain, we synthesized the 4-oxoacyl-ACP (g-position; substrates **6-ACP** and **7-ACP**), 5-oxoacyl-ACP (δ -position; substrates **8-ACP** and **9-ACP**) and 2-furoyl-ACP (α -position; **14-ACP**) substrates. The pK_a of α -hydrogens in α -ketoacyl(thio)esters are reportedly lower compared to simple oxo or thioesters.⁶ Under soft enolization conditions, we wondered if the enolate ion could promote intermolecular displacement of the thiol (over a period of time) to decrease substrate stability? We therefore replaced the two α -hydrogen atoms with two methyl-groups in substrates **4-ACP** and **5-ACP**. Unsubstituted acyl-ACPs (**1-ACP**, **2-ACP** and **3-ACP**) were included as a control in this study to determine the effect of removing the β -keto oxygen on substrate activity.

The *N*-acyl-L-homoserine lactone synthases aka AHL synthases in Gram-negative bacteria use acyl-ACP or acyl-CoA as the acyl-substrate and S-adenosyl-L-methionine as the acyl-acceptor to synthesize AHL quorum sensing signals (Fig. 2).⁷ Since signal synthesis play a vital role in microbial communication, AHL synthase enzymes are bonafide targets for inhibiting quorum sensing in several pathogenic bacteria that use a β -ketoacyl-ACP utilizing 3-oxo-AHL synthase. Some of these enzymes include 3-oxododecanoyl-ACP utilizing *Pseudomonas aeruginosa* LasI (causative agent for pneumonia, cystic fibrosis, bacterial meningitis *etc.*), 3-oxooctanoyl-ACP utilizing *Yersinia pestis* YspI (bubonic plague pathogen) and 3-oxohexanoyl-ACP utilizing *Pantoea stewartii* EsaI (plant pathogen that causes Stewart's wilt and leaf blight disease).⁴ With the exception of a nice paper from the Greenberg laboratory on a few YspI inhibitors discovered through a high throughput screen, there is little progress in AHL synthase inhibitor discovery for this class of enzymes.⁸ This deficiency in the literature could be attributed to most 3-oxo-AHL synthases still remaining uncharacterized, perhaps due to challenges in the successful isolation of β -ketoacyl-ACPs and its analogs, discussed above. To test the feasibility of our approach towards developing stable and active β -ketoacyl-ACP mimics that can be readily synthesized in sufficient yields for routine enzymological investigations, we synthesized and tested the activity of analogs **1-ACP-15-ACP** on EsaI and YspI AHL synthases.

Tables S1 and S2 (ESI[†]) summarizes the kinetic data for EsaI and YspI determined using lactonization (MTA) and acylation (holo-ACP) HPLC assays (Fig. 2). The catalytic efficiencies of these substrates with EsaI and YspI were measured independently using both HPLC assays as described in the ESI[†] section of this paper (Fig. S75 and S76).⁹ The kinetic constants obtained from both acylation and lactonization assays were comparable, which further validates the robustness of the HPLC assay used in this study. The kinetic constants for β -ketoheptanoyl-ACP mimics with EsaI are represented in Table S1 (see also Fig. S77 and S78, ESI[†]). The acyl-chain binding pocket in 3-oxo-AHL synthases typically have a complementary hydrogen bond donor residue (such as Thr140 in EsaI) to recognize the β -keto oxygen. As discussed earlier, 2-furanacetyl-ACP (**10-ACP**) has an sp^2 hybridized oxygen atom at the β -position and therefore is the likely candidate to mimic the native substrate. Indeed, this substrate has the highest k_{cat}/K_m with EsaI among all substrates in our focused library ($0.37 \pm 0.05 \text{ mM}^{-1} \text{ min}^{-1}$; Table S1, ESI[†]). This result also seem to suggest that enolization of the " β -keto" moiety is not essential for substrate recognition in EsaI. Although the lone pair of electrons on the heteroatom could donate two electrons towards the 6π electrons in the ring, it seems the reduced aromatic character of furan compared to other 5-membered heterocycle such as pyrrole or thiophene could have contributed towards enhancing the activity of this substrate. 2-Tetrahydrofuranacetyl-ACP is almost as active as 2-furanacetyl-ACP despite sp^3 hybridization at the β -position ($k_{cat}/K_m = 0.27 \pm 0.04 \text{ }\mu\text{M}^{-1} \text{ min}^{-1}$). Although the above result seems to hint that EsaI doesn't appear to discriminate between sp^2 and sp^3 hybridized centers at the β -carbon, this outcome must be interpreted with caution because the substrate selectivity differences between furan and tetrahydrofuran side-chains with EsaI may not truly resemble the reactivity differences between 3-oxoacyl-ACP and 3-hydroxyacyl-ACPs with the enzyme. While the K_m is unaltered between 2-

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pyridylacetyl-ACP and 2-furanacetyl-ACP, the loss of substrate activity is due to a lower k_{cat} value, which indicate that [E 2-pyridylacetyl-ACP SAM] ternary complex is in a non-optimal, less-productive conformation compared to [E 2-furanacetyl-ACP SAM] for the chemistry and/or product release steps in AHL synthesis. The catalytic efficiencies of 2-furoylacetyl-ACP, 4-oxohexanoyl-ACP and 5-oxohexanoyl-ACP are about 4–20 fold lower than 2-furanacetyl-ACP with EsaI highlighting the importance of the heteroatom position in the acyl-chain. Displacement of the heteroatom from β to α , γ , and δ positions in the acyl-chain increased the K_m and decreased k_{cat} relative to the most active substrate, 2-furanacetyl-ACP. The α -dimethylated substrate was the second most active substrate in the series suggesting that the methyl substitution doesn't seem to affect the substrate's catalytic efficiency ($k_{\text{cat}}/K_m = 0.30 \pm 0.05 \mu\text{M}^{-1} \text{min}^{-1}$).

EsaI has a narrow acyl-chain pocket that restricts acyl-chain lengths to 6 carbons or less.^{4a} The low catalytic efficiencies for larger/longer acyl-chain substrates (2-benzofuranacetyl-ACP, octanoyl-ACP, 4-oxooctanoyl-ACP and 5-oxooctanoyl-ACP) indicate that larger substrates are probably occluded from the narrow acyl-chain cavity in EsaI. Hexanoyl-ACP, a 6-carbon substrate was one of the worst substrates for EsaI wildtype enzyme clearly underscoring the importance of a heteroatom at the β -position. Furthermore, the 50–60 fold increase in the activity of hexanoyl-ACP with EsaI T140A mutant over wild-type enzyme reiterates the role of T140 as a gatekeeper residue in ensuring the selective recognition of 3-oxoacyl-ACP over unsubstituted acyl-ACP (Fig. S79, ESI†).¹⁰

Substrate activity trends in YspI resemble the trends in substrate catalytic efficiencies with EsaI. Since YspI synthesizes 3-oxooctanoyl-homoserine lactone autoinducers for *Y. pestis*, its preference for longer acyl-chains is clearly seen in substrate activity trends shown in Table S2 (Fig. S80 and S81, ESI†).^{4c} For instance, while EsaI's most active substrate is 2-furanacetyl-ACP, the two best substrates for YspI are 2-benzofuranacetyl-ACP and 2,2-dimethyl-3-oxooctanoyl-ACP (Tables S1, S2 (ESI†) and Fig. 3). The 2-pyridylacetyl-ACP substrate is more active with YspI suggesting the larger pyridine ring faced less steric clashes with a deeper acyl-chain pocket of YspI. Substrates with heteroatoms at α , γ and δ positions are far worse active in YspI compared to EsaI revealing a more stringent requirement for the heteroatom position in this enzyme. Irrespective of acyl-chain lengths, unsubstituted acyl-ACPs fare worse again reiterating the importance of a hydrogen bond acceptor at the b-position. Overall, it appears YspI is more selective in substrate preference compared to EsaI.

Finally, 2-thiopheneacetyl-ACP is the only substrate in our library to display substrate inhibition with both EsaI and YspI (Fig. S77, S78, S80 and S81 (ESI†); see Fig. S77 (ESI†) legend for details on substrate inhibition mechanism). The K_m of 2-thiopheneacetyl-ACP with both enzymes is also the lowest among all substrates in our library suggesting a tightly bound [enzyme-substrate] complex for this substrate (Tables S1 and S2, ESI†). Although commonly found in proteins, hydrogen bonding interactions with sulfur are proposed to be weaker compared to oxygen and the increased aromatic character of thiophene should further diminish the potential of sulfur atom to accept a hydrogen bond from a donor residue in the active site.¹¹ Nevertheless, sulfur also participates in "sigma hole" interactions with electronegative atoms such as N, O *etc.*¹² Quantum mechanical calculations reveal that the

magnitude of interaction energies at the sigma hole could match typical hydrogen bond energies (22 to 30 kJ mol⁻¹).¹³ In addition, the size of two sigma holes on the divalent sulfur are larger on aromatic rings such as thiophene.¹⁴ It appears these additional sigma hole interactions for sulfur could have likely contributed to the low K_m of 2-thiopheneacetyl-ACP with EsaI and YspI. In addition, we observe that the apparent $\langle MI \rangle$ is about 3-fold lower when the fixed substrate is 2-thiopheneacetyl-ACP relative to the most active acyl-ACP substrate with both EsaI and YspI (Table S3, ESI[†]). Interestingly, the k_{cat} is also 4–5 fold lower when the acyl-substrate is 2-thiopheneacetyl-ACP compared to 2-furanacetyl-ACP for EsaI or 2-benzofuranacetyl-ACP for YspI (Tables S1–S3, ESI[†]). The above observations suggest that the [E 2-thiopheneacetyl-ACP SAM] ternary complexes in both enzymes are perhaps in a non-optimal mode for chemistry and product release steps in AHL synthesis. The ability of the thio-analog to bind tighter, albeit in a less-productive mode for substrate turnover should inform future design of inhibitors for this class of enzymes.

The small library of β -ketoacyl-ACP mimics described in this work was designed to evaluate key structural features essential for substrate activity with β -ketoacyl-ACP utilizing AHL synthase enzymes such as EsaI and YspI. In this study, we have uncovered 2-furanacetyl-ACP and 2-benzofuranacetyl-ACP, respectively, as the most active β -ketoacyl-ACP mimic for 3-oxohexanoyl-ACP utilizing EsaI and 3-oxooctanoyl-ACP utilizing YspI enzymes. The bar diagram in Fig. 3 reveals that the catalytic efficiencies of 2-furanacetyl-ACP (EsaI) and 2-benzofuranacetyl-ACP (YspI) are at least similar or in some cases, even higher than the catalytic efficiencies of native substrates with several well-characterized AHL synthases.⁹ This data seems to indicate that the activity of 2-furanacetyl-ACP with EsaI and 2-benzofuranacetyl-ACP with YspI should closely mirror the activity of 3-oxoacyl-ACPs (if they can be successfully isolated) with these enzymes.

The flexibility to load a wide range of carrier proteins on to the beta-ketoacyl-chain mimics described in this study should open new avenues for mechanistic investigation of beta-ketoacyl-ACP utilizing enzymes in therapeutically important biosynthetic pathways.² While β -ketoacyl-ACP mimics could be explored as ketosynthase product inhibitors, the inert, ring substrate analogs such as 2-furanacetyl-ACP could be used to develop competitive inhibitors for ketoreductase. The potential of β -ketoacyl-ACP mimics to inhibit both ketosynthase and ketoreductase is particularly appealing to develop potent fatty acid synthase inhibitors, develop mechanistic probes that could arrest polyketide synthesis at specific steps and discover novel antivirulent compounds that inactivate 3-oxoacyl-homoserine lactone synthases from pathogenic bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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3-oxoacyl-ACPs (Native) **Unsubstituted acyl-ACPs** **C₂-Dimethylation** **Position of oxygen** **Cyclic side chains** **Fig. 1.**

Chemical structures of β -ketoacyl-ACP substrate analogs used in this study. The structural attributes of each substrate is described in Fig. S84 (ESI[†]).

Fig. 2.

Catalytic efficiencies of β -ketoacyl-ACP substrate analogs in 3-oxo-AHL synthesis. (A) The native substrate for *P. stewartii* EsaI and *Y. pestis* YspI, respectively, are 3-oxohexanoyl-ACP and 3-oxooctanoyl-ACP. (B) Catalytic efficiencies of β -ketoacyl-ACP substrate analogs with EsaI and YspI enzymes.

Fig. 3.

The β -ketoacyl-ACP mimic for EsaI and YspI. (A) Bar diagram comparing the catalytic efficiencies of 2-furanacetyl-ACP substrate for EsaI and 2-benzofuranacetyl-ACP for YspI (both shown in red bars) with native substrates of a few well-characterized AHL synthases. (B) Structural similarities between 2-furanacetyl-ACP (EsaI)/2-benzofuranacetyl-ACP (YspI) and β -ketoacyl-ACPs. The part of the substrate that closely mimics the β -ketoacyl-ACPs is drawn in red.