Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 3034–3038
Bioorganic & Medicinal Chemistry Letters

Mechanism based protein crosslinking of domains from the 6-deoxyerythronolide B synthase

Shiven Kapur,[a] Andrew Worthington,[b] Yinyan Tang,[a] David E. Cane,[c] Michael D. Burkart[b] and Chaitan Khosla[a,d,e,*]

[a]*Department of Chemistry, Stanford University, Stanford, CA 94305, USA*
[b]*Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA*
[c]*Department of Chemistry, Brown University, Box H, Providence, RI 02912-9108, USA*
[d]*Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA*
[e]*Department of Biochemistry, Stanford University, Stanford, CA 94305, USA*

Received 13 October 2007; accepted 16 January 2008
Available online 24 January 2008

Abstract—The critical role of protein–protein interactions in the chemistry of polyketide synthases is well established. However, the transient and weak nature of these interactions, in particular those involving the acyl carrier protein (ACP), has hindered efforts to structurally characterize these interactions. We describe a chemo-enzymatic approach that crosslinks the active sites of ACP and their cognate ketosynthase (KS) domains, resulting in the formation of a stable covalent adduct. This process is driven by specific protein–protein interactions between KS and ACP domains. Suitable manipulation of the reaction conditions enabled complete crosslinking of a representative KS and ACP, allowing isolation of a stable, conformationally constrained adduct suitable for high-resolution structural analysis.
© 2008 Elsevier Ltd. All rights reserved.

Since the discovery of colinearity between gene sequence and product structure of the 6-deoxyerythronolide B synthase,[1,2] there has been considerable effort towards harnessing the programmable biochemistry of multimodular polyketide synthases (PKSs). More recently, there has been a growing realization of the importance of protein–protein interactions in the successful engineering of these megasynthases.[3–5] A key challenge encountered in studying these protein–protein interactions is their inherent weakness ($K_d > 1$ μM in most cases). Here we describe the development of a new method for studying the weak but crucial non-covalent interaction between the acyl carrier protein (ACP) and the ketosynthase (KS) domains of a modular PKS.

The acyl carrier protein (ACP) is a small (~10 kDa), highly conserved domain found in PKSs as well as the related fatty acid synthases and non-ribosomal peptide synthases. Although it lacks intrinsic catalytic activity, it is involved in every step of polyketide biosynthesis, collaborating with enzymes involved in building block incorporation (the acyl transferase, AT), β-carbon processing (the ketoreductase, KR, dehydratase, DH, and enoyl reductase, ER), and finally chain termination (the thioesterase, TE). The specificity of these protein–protein interactions has been noted,[6–10] and the high-resolution structure of a prototypical ACP domain from module 2 of the 6-deoxyerythronolide B synthase (DEBS) has also been reported.[11] However, elucidating the structure of a complex between an ACP domain of a modular PKS and its partner domains has been challenging, presumably due to the exceptionally high mobility of this domain.

To directly visualize the interactions between the KS domain of a modular PKS and its cognate ACP partner, we have taken the approach of crosslinking the two domains using chemically synthesized coenzyme A (CoA) analogues. Although 1,3-dibromopropanone has been used as a crosslinking reagent to study FASs,[12] we found this approach to be unsuitable for the problem at hand due to low yields of the crosslinked product. In contrast, the mechanism-based crosslinking method

Keywords: Acyl carrier protein; Ketosynthase; Crosslinking; Polyketide synthase; Protein–protein interaction.
* Corresponding author. Tel.: +1 650 7236538; fax: +1 650 7257294; e-mail: khosla@stanford.edu

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.01.073

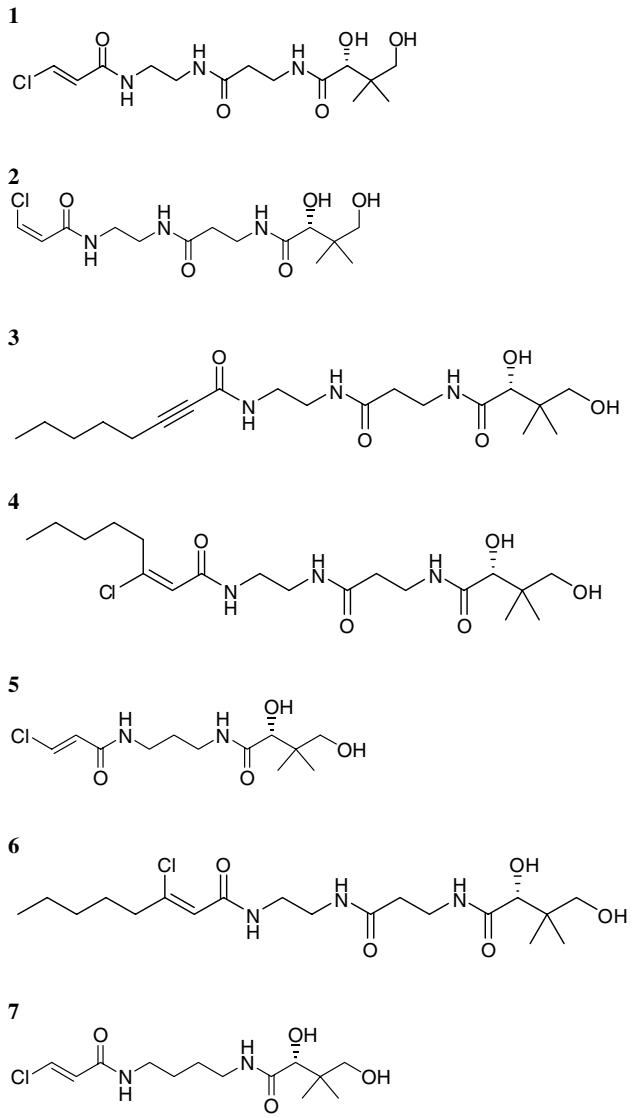


Figure 1. Structures of crosslinkers used in this study.

of Burkart and coworkers[13] yielded more promising results, as described in this report. Briefly, this method harnesses enzymes from the CoA biosynthetic pathway to yield CoA analogues that can be attached to the active site serine of an ACP using a non-specific phosphopantetheinyl transferase (PPTase). The crosslinkers of greatest interest harbor an electrophilic warhead in place of the nucleophilic cysteamine moiety, and are therefore able to react irreversibly with the active site cysteine of the KS domains. Several CoA analogues capable of crosslinking the KS and ACP proteins of the *Escherichia coli* fatty acid synthase were reported by Burkart and coworkers.[13,14]

Given the mechanistic and structural similarity between fatty acid synthases and PKSs, we screened compounds **1**–**7** (Fig. 1) for their ability to crosslink the didomain [KS][AT] proteins of DEBS modules 3 and 5 with their cognate stand-alone ACP domains. The X-ray crystal structures of both these didomain proteins have been solved to atomic resolution.[15,16] A one-pot reaction was performed containing compounds **1**–**7** individually, along with three CoA biosynthetic enzymes (CoaA, CoaD and CoaE), the *Sfp* PPTase and either ACP3 or ACP5 in phosphate buffer. Thereafter, the [KS3][AT3] protein or the [KS5][AT5] protein was added, and the reaction products were analyzed by SDS–PAGE (Fig. 2).[17] In the presence of the electrophilic CoA analogues, bands with apparent molecular weights corresponding to a [KS][AT]–ACP adduct were observed with both modules. Interestingly, compound **1** showed the highest efficiency for crosslinking the DEBS proteins, whereas its geometric isomer **2** was inactive. Compound **1** was also the most effective in crosslinking the *E. coli* fatty acid synthase proteins.[13] However, unlike the latter system, where all compounds showed reasonable crosslinking efficiency, the DEBS proteins were much more selective. Analysis of reaction mixtures incubated without [KS3][AT3] by matrix-assisted laser desorption/ionization–time of flight mass spectrometry showed greater than 90% conversion of *apo* to *crypto-*

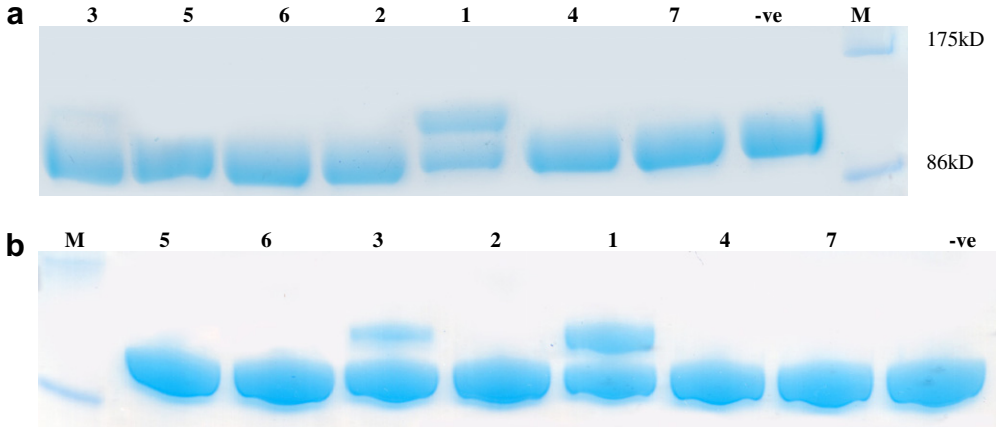


Figure 2. (a) SDS–PAGE analysis of crosslinking reaction mixtures containing compounds **1**–**7**, [KS3][AT3] and ACP3. For compound identity refer to Figure 1. (b) SDS–PAGE analysis of crosslinking reaction mixtures containing compounds **1**–**7**, [KS5][AT5] and ACP5. For compound identity refer to Figure 1.

ACP[18] for compounds **1**–**7**, eliminating differences in pantetheinylation as a possible cause of the observed selectivity. Structural or computational modeling studies with these alternative CoA analogues docked in the active site of the KS may offer some insight into the mechanistic origins of this discriminative power.

The ACP construct utilized in this study was engineered with the FLAG epitope[19] (DYKDDDDK) the presence of which can be easily detected by Western blot analysis utilizing commercially available antibodies. SDS–PAGE followed by Western blot analysis[20] (Fig. 3a) revealed that the observed higher molecular weight band was indeed the expected [KS][AT]–ACP adduct.

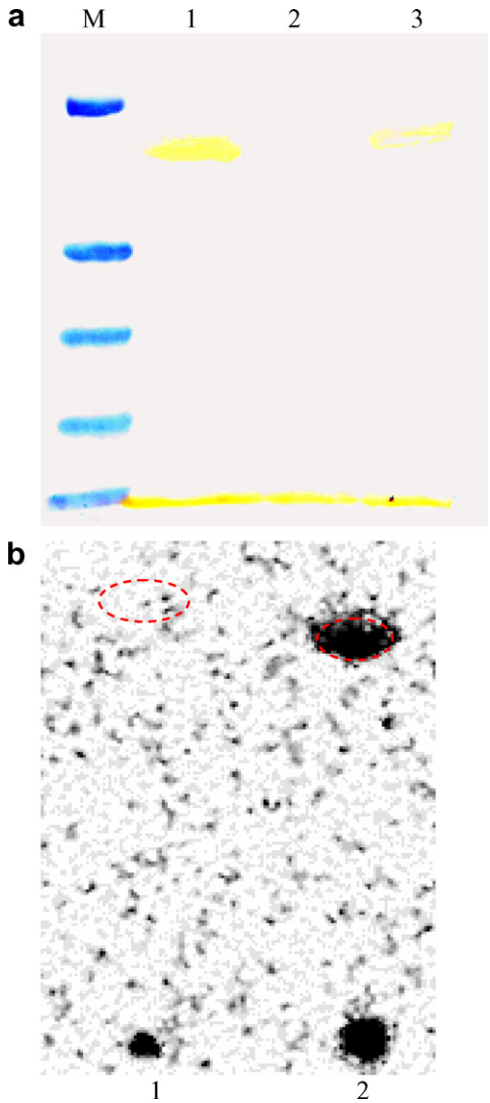


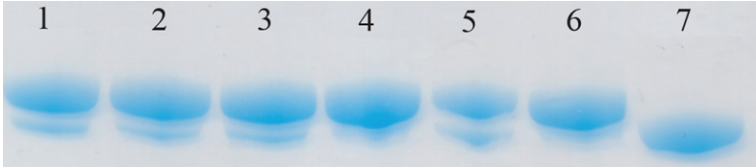
Figure 3. (a) Western blot analysis of lane 6 from Figure 2a with anti-FLAG M2 antibody confirming formation of [KS3][AT3]–ACP3 crosslinked adduct (lane 1). Negative control (lane 2) contained no crosslinker. Bands visible at the bottom are due to unreacted ACP3. Lane 3 is a 2-fold dilution of lane 1. (b) Radio SDS–PAGE image of [^{14}C](2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester labeling of purified crosslinked adduct (lane 1) and [KS3][AT3] (lane 2). Red dashed ellipses indicate the position of the bands on the SDS–PAGE gel visualized by Coomassie blue staining.

The active site cysteine of a KS domain can be selectively acylated by a suitably loaded *N*-acetylcysteamine thioester which functions as a surrogate for the phosphopantetheinyl arm of the ACP.[3,9] When incubated with [^{14}C](2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester, the crosslinked [KS][AT]–ACP adduct could not be labeled, whereas the control [KS][AT] protein was readily labeled (Fig. 3b).[21] This observation confirmed that the active site cysteine of the KS domain was the site of crosslinking between the [KS][AT] and the ACP.

Earlier studies have demonstrated that KS–ACP interactions in DEBS are specific.[9] To investigate whether this differential specificity could affect KS–ACP crosslinking, [KS3][AT3] was crosslinked with ACP3 and ACP5 utilizing **1** as the linker. The KS demonstrated a clear preference for its cognate ACP, as evidenced by the higher intensity of crosslinked band (Fig. 5). A similar experiment performed with [KS5][AT5] using linkers **1** and **3** confirmed the hypothesis that protein–protein interactions drive crosslinking (Fig. 5).

Structural studies have verified that the active [KS][AT] didomain protein exists as a homodimer with two active sites per complex.[15,16] Thus, it can be deduced that an incompletely crosslinked [KS][AT] protein exists in solution as three distinct species with 0, 1 or 2 ACP domains covalently attached to the [KS][AT] homodimer. Although the presence of orthogonal purification tags (FLAG on ACP and hexa-His on [KS][AT]) can be exploited to purify the crosslinked species from the two starting materials via affinity chromatography, incompletely crosslinked homodimers cannot be separated from 2:2 complexes in a straightforward fashion. This represents a major roadblock for obtaining a homogeneous preparation of the desired species for X-ray crystallography. To minimize this potential problem, we optimized the crosslinking reaction so that it would proceed to completion. A number of variables including temperature, incubation time, and relative and absolute concentrations of reactants and buffer conditions were evaluated (Fig. 4). The temperature and absolute concentration of reactants (particularly [KS][AT] and ACP) were the most critical variables. It should be noted that optimal crosslinking of [KS5][AT5] with ACP5 occurred using different conditions compared to the optimal crosslinking of [KS3][AT3] with ACP3 (data not shown), suggesting that each protein pair must be optimized individually.

In summary, we have demonstrated the utility of a chemo-enzymatic methodology to crosslink ACP and KS domains from two DEBS modules in a site-specific manner, exploiting the native reactivity of these domains. Further experiments established the role of protein–protein interaction in this process and suitable manipulation of the reaction conditions allowed us to obtain homogeneous crosslinked protein. Our results demonstrate the value of this approach as a route to engineer a functionally relevant, relatively rigid KS–ACP complex for high-resolution structural studies. They also highlight the value of this approach for studying other



	1	2	3	4	5	6	7
[KS][AT]	40	40	40	60	30	75	40
ACP3	120	120	120	180	90	225	120
CROSSLINKER	360	360	360	540	270	675	0
CoaA,CoaD,CoaE	3	3	3	3	3	3	3
Sfp	10	10	10	15	7.5	18.75	10
Temp(°C)	37	30	23	23	23	23	23
Incubation Time(h)	2	2	2	3	3	4	3

Figure 4. SDS–PAGE gel showing variability of [KS3][AT3] crosslinking to ACP3 as a function of alternative reaction conditions (numbers refer to table below). Lower temperatures allowed access to longer incubation times without inducing protein precipitation observed at higher temperatures.

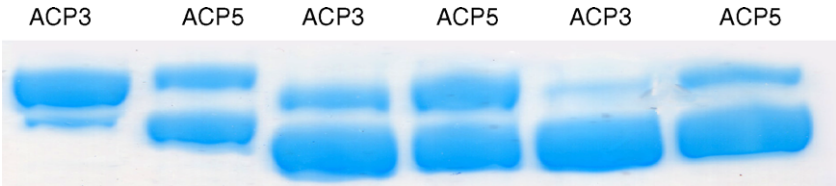


Figure 5. SDS–PAGE analysis of reaction mixtures containing [KS3][AT3] (lanes 1 and 2) or [KS5][AT5] (lanes 3–6) and alternative ACP domains. Crosslinker **1** was used in lanes 1–4, whereas compound **3** was used in lanes 5 and 6. Reaction conditions were identical to those described for lane 3 in Figure 4.

transient interactions involving ACP domains in multimodular PKSs.

Acknowledgments

This work was supported by Grants from the NIH (CA 66736 to C.K., GM 22172 to D.E.C. and GM 75797 to M.D.B.).

References and notes

1. Cortes, J.; Haydock, S. F.; Roberts, G. A.; Bevitt, D. J.; Leadlay, P. F. *Nature* **1990**, *348*, 176.
2. Donadio, S.; Staver, M. J.; Mcalpine, J. B.; Swanson, S. J.; Katz, L. *Science* **1991**, *252*, 675.
3. Gokhale, R. S.; Tsuji, S. Y.; Cane, D. E.; Khosla, C. *Science* **1999**, *284*, 482.
4. Tsuji, S. Y.; Wu, N.; Khosla, C. *Biochemistry* **2001**, *40*, 2317.
5. Khosla, C.; Tang, Y.; Chen, A. Y.; Schnarr, N. A.; Cane, D. E. *Annu. Rev. Biochem.* **2007**, *76*, 195.
6. Tsuji, S. Y.; Wu, N.; Khosla, C. *Biochemistry* **2001**, *40*, 2326.
7. Wu, N.; Cane, D. E.; Khosla, C. *Biochemistry* **2002**, *41*, 5056.
8. Wu, N.; Tsuji, S. Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2001**, *123*, 6465.
9. Chen, A. Y.; Schnarr, N. A.; Kim, C. Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2006**, *128*, 3067.
10. Chen, A. Y.; Cane, D. E.; Khosla, C. *Chem. Biol.* **2007**, *14*, 784.
11. Alekseyev, V.; Liu, C.; Puglisi, J.; Khosla, C. *Protein Sci.* **2007**, *16*, 2093.
12. Stoops, J. K.; Henry, S. J.; Wakil, S. J. *J. Biol. Chem.* **1983**, *258*, 12482.
13. Worthington, A. S.; Rivera, H.; Torpey, J. W.; Alexander, M. D.; Burkart, M. D. *ACS Chem. Biol.* **2006**, *1*, 687.
14. Worthington, A. S.; Hur, G. H.; Meier, J. L.; Burkart, M. D., in preparation.
15. Tang, Y.; Kim, C. Y.; Mathews, I. I.; Cane, D. E.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11124.
16. Tang, Y.; Chen, A. Y.; Kim, C. Y.; Cane, D. E.; Khosla, C. *Chem. Biol.* **2007**, *14*, 931.
17. A typical 10 μL reaction mixture was set up as follows: to a 100 mM potassium phosphate (pH 7.2), buffer with 12.5 mM $MgCl_2$ and 8 mM ATP were added ACP3 (5 μg, 25 μM, 5 equiv), CoaA (1.5 μM), CoaD (1.5 μM), CoaE (1.5 μM), *Bacillus subtilis* Sfp (20 μM) and crosslinker (200 μM, 40 equiv). The mixture was incubated at 37 °C for 1.5 h [KS3][AT3] (9.1 μg, 5 μM, 1 equiv) was then added and the mixture was further incubated at 30 °C for 2 h. Incubation for longer periods of time resulted in precipitation of [KS][AT]. All gels were run under the following conditions: 7.5% Tris–HCl, 10 well, 30 μL, Bio-Rad, Ready gels. 200 V, 150 mA, 100 W.
18. Data obtained from MALDI–TOF mass analysis of *apo*-ACP and *crypto*-ACP domains is as follows: (figures in parentheses refer to calculated molecular weights for $[M+H]^+$-methionine. For compound identity refer to Fig. 1). *Apo*-ACP: 12891.6(12884.2). **1**-*Crypto*-ACP: 13304.15(13295). **2**-*Crypto*-ACP: 13303.78(13295). **3**-*Crypto*-ACP: 13345.35(13328.67). **4**-*Crypto*-ACP: 13382.18(13365.13). **5**-*Crypto*-ACP: 13254.26(13237.95). **6**-*Cryp-*

to-ACP: 13383.04(13365.13). **7**-*Crypto*-ACP: 13270.63 (13251.98).

19. For the expression of ACP3 with an internal FLAG epitope, pYYT01 was constructed as follows. The DNA sequence encoding DEBS ACP3 was amplified by PCR as an NdeI-EcoRI fragment using primers 5′-CATATG GACTACAAGGACGACGACGACAAGCGGCTCGCG GGGCTTTCCCCGGACGAGCAG-3′ and 5′-CGCGG CGAATTCTTAGGCGTCACCGACGAGCCGGGC-3′. This NdeI-EcoRI fragment was cloned into pET28a expression vector to yield plasmid pYYT01, encoding DEBS ACP3 with N-terminal His_6-tag and an internal FLAG tag.
20. Western blot with anti-FLAG M2 monoclonal antibody(Sigma) was performed as per the manufacturer's instructions using nitrocellulose membrane.
21. Purified crosslinked adduct (HiTrap-Q anion-exchange column, GE Healthcare) or [KS3][AT3] (10 μM, in 100 mM phosphate at pH 7.2) was incubated with 50 μM [^{14}C](2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester for 2 h at room temperature. Samples were quenched with SDS–PAGE loading buffer lacking any reducing agents and loaded directly onto a SDS–PAGE gel. The gel was dried using a Bio-Rad gel-drying system and analyzed using a phosphorimager.