ChemPubSoc Europe

DOI: 10.1002/cbic.201800169

CHEMBIOCHEM Communications

Biochemical Characterization of β-Amino Acid Incorporation in Fluvirucin B_2 Biosynthesis

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Naturally occurring lactams, such as the polyketide-derived macrolactams, provide a diverse class of natural products that could enhance existing chemically produced lactams. Although β-amino acid loading in the fluvirucin B_2 polyketide pathway was proposed by a previously identified putative biosynthetic gene cluster, biochemical characterization of the complete loading enzymes has not been described. Here we elucidate the complete biosynthetic pathway of the β-amino acid loading pathway in fluvirucin B_2 biosynthesis. We demonstrate the promiscuity of the loading pathway to utilize a range of amino acids and further illustrate the ability to introduce non-native acyl transferases to selectively transfer β-amino acids onto a polyketide synthase (PKS) loading platform. The results presented here provide a detailed biochemical description of β-amino acid selection and will further aid in future efforts to develop engineered lactam-producing PKS platforms.

Lactams are an important class of chemicals used in the production of polymers, pharmaceuticals, and insecticides.[1–5] In nature, the polyketide-derived macrolactams are a clade of natural products containing unique β-amino acids as part of the core macrolide structure.[6] Notable macrolactams include the fluvirucins (Flvs), rifamycin, and geldanamycin.[4,7,8] Fluvirucin B_2 is a 14-membered macrolactam that shows both antifungal and antiviral activity,[4,9] and one of its main structural features is the β-alanine starter unit of the polyketide skeleton (Scheme 1). The biosynthetic nature of polyketide synthases and their potential to be reprogrammed could enhance existing chemically produced lactam analogues, such as valerolactam, caprolactam, and laurolactam (Scheme 1). Previous work on the origin of the β-alanine moiety in fluvirucin B_2 suggests it originates from L-aspartate through decarboxylation of its α-carboxyl group.[3] More recently, the putative biosynthetic gene cluster of fluvirucin B_2 from *Actinomadura fluva* subsp. *indica* (ATCC 53714) and its corresponding β-amino acid loading pathway were annotated.[10] In this presumptive fluvirucin B_2 loading pathway, L-aspartate is first activated by the adenylation enzyme FlvN and ligated with the standalone acyl carrier protein (ACP) FlvL to give aspartyl-ACP (**1**; Scheme 1). Then, the pyridoxal-phosphate (PLP)-dependent decarboxylase FlvO removes the α-carboxy group of the aspartyl-FlvL (**1**), resulting in a β-alanyl-FlvL intermediate (**2**). It has been proposed that the resulting β-alanyl-FlvL (**2**) is further aminoacylated with L-alanine by another adenylation enzyme, FlvM, to give a dipeptidyl-FlvL (**3**). The dipeptidyl-FlvL intermediate is then transferred to the loading ACP domain of the fluvirucin polyketide synthase (PKS) FlvP1 (**4**) by the amino acyltransferase (AT) FlvK (Scheme 1 and Figure S1 in the Supporting Information).[10] Similar loading pathways have been proposed in other β-amino-acid-priming PKS products, such as vicenistatin and sceliphrolactam.[11–13] In our efforts to understand the entire biochemical pathway, all Flv enzymes and related homologues were expressed in *Escherichia coli* as N-terminal His_6-tagged proteins from codon-optimized synthetic genes (Figure S2, Table S1) and were reconstituted in vitro. All ACP-tethered intermediates were initially monitored by MALDI-TOF spectrometry, as it allowed us to robustly test a wide range of substrates and conditions with accurate resolution on the entire ACP. Here, we describe the results from each biochemical step in order, as depicted in Scheme 1.

At the first step of β-amino acid loading, Miyanaga and coworkers proposed selection of L-aspartate by the ATP-dependent adenylation enzyme FlvN, which ligates the amino acid onto the stand-alone ACP FlvL to generate aspartyl-FlvL (**1**).[10] We reconstituted FlvN and FlvL with all the necessary cofactors in the presence of 2 mM L-aspartate. The mass corresponded to aspartyl-FlvL (**1**; Figure 1A–B). This initial experiment supported the previously proposed selectivity of FlvN for L-aspar-

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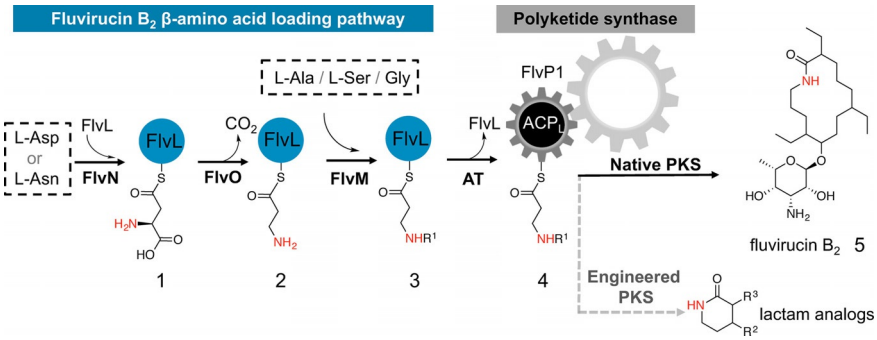

Scheme 1. β-Amino acid loading platform involved in fluvirucin B_2 biosynthesis.

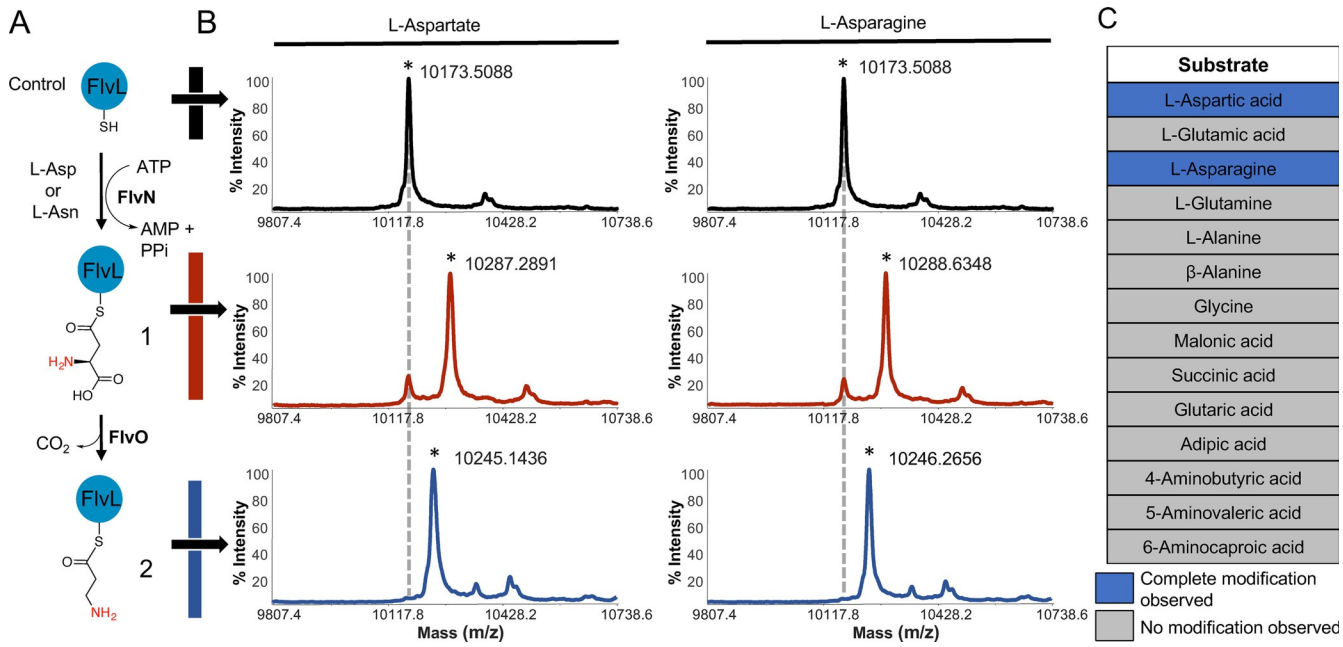

Figure 1. A) Schematic representation of FlvN activation and transfer of L-aspartate and L-asparagine onto FlvL and subsequent decarboxylation by FlvO, and B) corresponding MALDI-TOF traces of FlvL modifications. C) List of the various substrates tested.

tate and provided direct evidence of the aspartyl-FlvL (**1**) intermediate. We repeated the same experiment and tested a variety of amino acids, dicarboxylic acids, and ω-amino fatty acids at the same 2 mM concentration (Figure 1 C). Surprisingly, we also observed FlvN selectivity for L-asparagine and transfer to FlvL, with a comparable mass shift to the aspartyl-FlvL (**1**) in our initial experiment with L-aspartate (Figure 1 B, Table S2). This suggested that L-asparagine selection and ligation to FlvL by the adenylating enzyme, FlvN, produced the same aspartyl-FlvL (**1**) intermediate. To further test this hypothesis, we introduced the second enzyme in the pathway, the PLP-dependent decarboxylase FlvO. It has been proposed that FlvO removes the α-carboxy group of the aspartyl-ACP (**1**), resulting in a β-alanyl-ACP intermediate (**2**). If both L-aspartate and L-asparagine are activated and ligated to FlvL to form the same aspartate-FlvL (**1**) intermediate, decarboxylation by FlvO would be equally observed with either starting amino acid and display the same mass shift. To this end, we introduced FlvO to reactions containing either L-aspartate or L-asparagine. Both reactions displayed a mass shift corresponding to the β-alanyl-ACP intermediate (**2**), indicating successful decarboxylation by FlvO (Figure 1 A–B, Table S2). These results further supported our hypothesis that both L-aspartate and L-asparagine can be selected by FlvN to generate the same aspartate-FlvL (**1**) intermediate, which is subsequently decarboxylated by FlvO.

As both L-aspartate and L-asparagine could be utilized by FlvN, we next investigated the substrate preference and catalytic properties of FlvN. As FlvN has an ATP-dependent adenylation domain, we anticipated that the FlvN-catalyzed reaction would require ATP hydrolysis to activate the amino acid prior to being transferred onto the 4′-phosphopantetheine of the holo-FlvL. We tested FlvN against L-aspartate and L-asparagine by using an ATP-PPi release assay previously reported by Duckworth et al. and Wilson et al.[14,15] The ATP-PPi release assay allowed us to measure FlvN's ability to activate L-aspartate and L-asparagine with ATP. Overall, FlvN demonstrated much higher catalytic activity on L-aspartate (k_{cat}/K_m = 221.27 min^{-1} mM^{-1}) compared to L-asparagine (k_{cat}/K_m =

Table 1. Steady-state kinetic parameters of FlvN and FlvM.

Enzyme	Substrate	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1}/mM^{-1}]
FlvN	L-aspartate	0.055 ± 0.006	12.17 ± 0.22	221.27
FlvN	L-asparagine	6.640 ± 1.050	10.96 ± 0.49	1.17
FlvM	L-alanine	0.189 ± 0.024	10.95 ± 0.23	57.78
FlvM	L-serine	2.749 ± 0.429	10.15 ± 0.38	3.69
FlvM	glycine	10.690 ± 1.420	18.92 ± 0.79	1.77

1.165 $min^{-1}\,mM^{-1}$; Table 1, Figure S4A). These results suggest L-aspartate is the preferred amino acid in the fluvirucin β-amino acid loading pathway.

We then focused on testing aminoacylation of the β-alanyl-FlvL (**2**) intermediate by the ATP-dependent FlvM. It has been proposed that the β-alanyl-ACP (**2**) is further aminoacylated with L-alanine to give a dipeptidyl-ACP intermediate (**3**). Using the same in vitro reconstitution strategy, in parallel with MALDI-TOF, we reconstituted 2 mM L-aspartate with FlvL, FlvN, FlvO, and FlvM in the presence of various amino acids and short chain acids (Figure 2A–C). FlvM demonstrated substrate promiscuity, aminoacylating the β-alanyl-FlvL (**2**) intermediate with L-alanine (**3a**), L-serine (**3b**), and glycine (**3c**; Figure 2). Partial aminoacylation was also observed with L-leucine and L-isoleucine (Figures 2C and S3J, K). A closer inspection of the substrate preference of FlvM by leveraging the previously mentioned ATP-PPi release assay demonstrated preference for L-alanine ($k_{cat}/K_m = 57.78\ min^{-1}\,mM^{-1}$), followed by L-serine ($k_{cat}/K_m = 3.69\ min^{-1}\,mM^{-1}$), and then glycine ($k_{cat}/K_m = 1.77\ min^{-1}\,mM^{-1}$; Table 1, Figure S4B). Both MALDI-TOF and the ATP-PPi release assays of FlvM suggested L-alanine as the preferred substrate over other amino acids. Unexpectedly, FlvM displayed a large amino acid substrate flexibility, which has never been described in this type of β-amino acid loading pathway.

The final step in the β-amino acid loading pathway is the AT-catalyzed transfer of the dipeptidyl from FlvL onto the loading ACP from the fluvirucin PKS (FlvP1 ACP_L). In the fluvirucin B_2 pathway, the putative AT (FlvK) has been proposed to conduct such transfer.[10] However, a closer inspection of the FlvK sequence (GenBank: BAV56001.1) suggested FlvK contains an AMP-binding domain and is closely related to the class 1 superfamily of adenylate-forming domains (AFD). This was further supported by the lack of transfer observed in our in vitro reconstitution experiments for FlvK. Closer inspection of the nucleotide sequence of the fluvirucin biosynthetic gene cluster

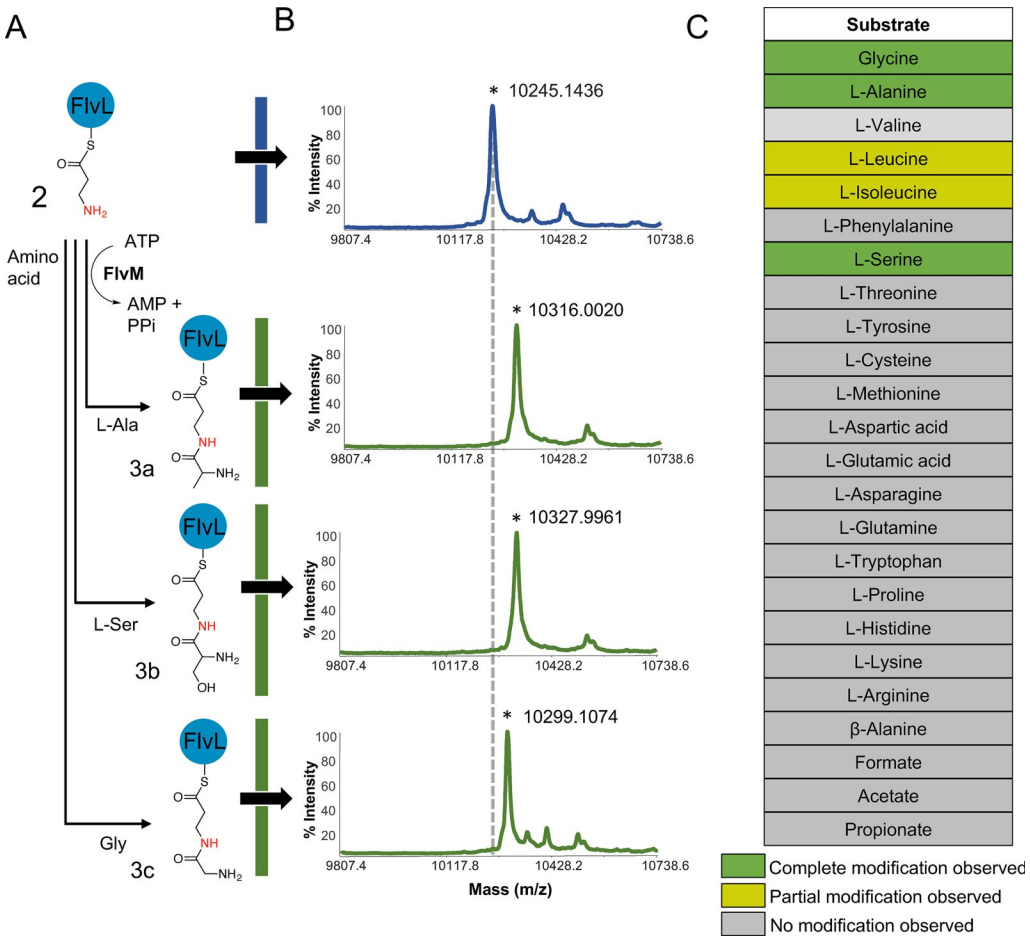

Figure 2. A) Schematic representation of FlvM aminoacylation of the β-alanyl-FlvL intermediate (**2**), and B) corresponding MALDI-TOF traces of FlvL modifications. C) List of the various substrates tested with FlvM. Partial aminoacylation was also observed with L-leucine and L-isoleucine (yellow), as depicted by the MALDI-TOF traces in Figure S3J–K.

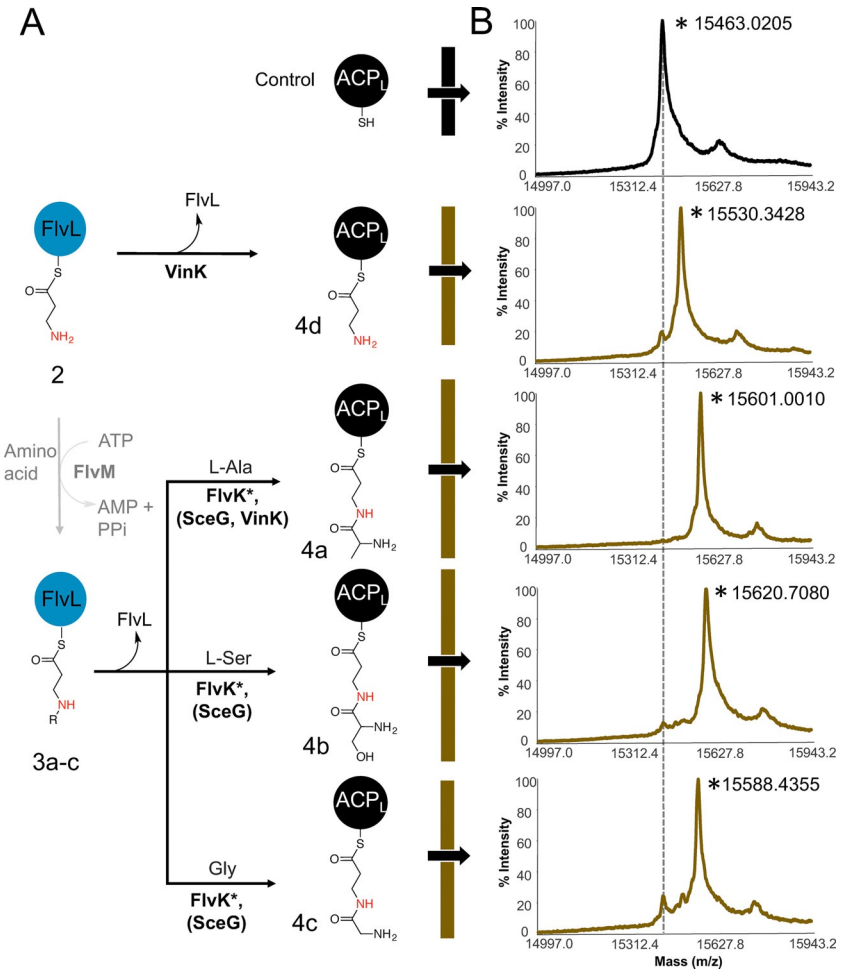

Figure 3. A) Schematic representation of dipeptide transfer from FlvL to FlvP1 ACP by the native FlvK* and the non-native acyl transferases VinK and SceG. B) The corresponding MALDI-TOF traces focused on FlvP1 ACP. VinK also transfers **3a** to generate **4a**, as depicted in Figure S5. The non-native acyl transferase SceG also selectively transfers intermediates **3a–c** to generate **4a–c**. MALDI-TOF traces for SceG and VinK in parentheses can be found in Figure S5.

using antiSMASH revealed a stand-alone AT, as predicted by Miyanaga et al.[10,16,17] To this end, we cloned and expressed the newly annotated FlvK (FlvK*). We reconstituted the entire β-amino acid pathway with FlvK* and FlvP1 ACP$_L$ and monitored dipeptidyl transfer from the FlvL to FlvP1 ACP$_L$. The native FlvK* displayed promiscuity, transferring all three dipeptide intermediates from FlvL to FlvP1 ACP$_L$. The dipeptidyl-FlvL intermediates with L-alanine (**3a**), L-serine (**3b**), and glycine (**3c**) were transferred to FlvP1 ACP$_L$, generating **4a**, **4b**, and **4c** (Figure 3A–B, Table S3). Partial transfer by FlvK* was observed with the unprotected β-alanyl FlvL intermediate (**2**, Figure S5).

In addition, we aimed to test the compatibility of non-native ATs to transfer the various FlvL intermediates, primarily the unprotected β-alanyl FlvL intermediate (**2**). We focused on the previously identified AT homologues from the vicenistatin pathway (VinK) and the sceliphrolactam pathway (SceG; Figure S7C).[11–13] Similar to FlvK*, the AT SceG from sceliphrolactam biosynthesis demonstrated promiscuity, transferring all three dipeptide intermediates from FlvL to FlvP1 ACP$_L$ (Figures 3 and S5). Surprisingly, the AT VinK from vicenistatin biosynthesis demonstrated selectivity not only for the L-alanine aminoacylated (**3a**) but for the unprotected β-alanyl (**2**) FlvL intermediates as well (Figures 3 and S5, Table S3). VinK selectively transferred both of these intermediates to generate **4a** and **4d**. These results illustrate the flexibility of non-native AT domains to be compatible in the fluvirucin biosynthetic pathway. Equally important, these results demonstrate the selectivity of ATs from SceG and VinK towards different ACP-tethered intermediates, providing key gatekeeping insights into β-amino acid priming in lactam-producing PKSs.

Having biochemically characterized the Flv β-amino acid loading enzymes on the stand-alone FlvL ACP, we wanted to investigate if the FlvL ACP could be bypassed, and all loading/modifications could be directly accomplished on the PKS-tethered loading FlvP1 ACP$_L$. The standalone FlvL ACP and FlvP1 ACP$_L$ share a sequence similarity of 71.9% in a 32-residue overlap region where the conserved DSL motif is located (Figure S7D). To this end, we reconstituted FlvN in 2 mM L-aspartate and replaced the stand-alone FlvL with an excised version of FlvP1 ACP$_L$. The reaction was monitored for L-aspartate addition by MALDI-TOF at various time points (Figure S6). Given that almost complete conversion of holo-FlvL to aspartyl-FlvL (**1**) by FlvN was observed at a 1 h time point, this was used as a point of reference. Partial loading of L-aspartate by FlvN onto

FlvP1 ACP_L was observed after an 8 h incubation. The majority of FlvP1 ACP_L, was present in the holo form, suggesting a lower efficiency by FlvN to transfer the L-aspartate to the loading ACP. No aspartate decarboxylation was observed in the presence of FlvO. This evidence suggests that the stand-alone FlvL is required for adequate L-aspartate loading and processing, prior to being transferred onto FlvP1 ACP_L. Moreover, it provides evidence that other factors, such as FlvN and FlvL protein–protein interactions, may play an important role.

In this report, we have presented a detailed biochemical description of β-amino acid selection, incorporation, and promiscuity in the fluvirucin B_2 loading pathway. The ability of the loading pathway to use various amino acids as starter units and aminoacylating groups provides new insights into the flexibility of β-amino acid selection in polyketide biosynthesis. The range of amino acids that can be utilized in the β-amino acid loading platform presented in this study provides a roadmap for future metabolic engineering efforts to establish a host with the appropriate amino acid precursor pools. Moreover, this work illustrates the ability to mix-and-match compatible AT domains for selective β-amino acid transfer onto a native or potentially engineered PKS. With a growing interest in the renewable production of lactams, a broader understanding of β-amino acid selection in naturally existing macrolactam biosynthetic pathways will further aid in future efforts to develop engineered lactam-producing PKS platforms.

Acknowledgements

We thank Sam Curran and Mitchel Thompson for helpful discussions. This work was part of the Department of Energy (DOE) Joint BioEnergy Institute (https://www.jbei.org) supported by the U. S. DOE, Office of Science, Office of Biological and Environmental Research, and was part of the Agile BioFoundry (https://agilebiofoundry.org) supported by the U.S. DOE, Energy Efficiency and Renewable Energy, Bioenergy Technologies Office, through contract DE-AC02-05CH11231 to the Lawrence Berkeley National Laboratory. The views and opinions of the authors expressed herein do not necessarily state or reflect those of the U.S. Government or any agency thereof. Neither the U.S. Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility or the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

Conflict of Interest

J.D.K. has financial interests in Amyris, Lygos, Constructive Biology, Demetrix, Napigen, and Maple Bio.

Keywords: biosynthesis · fluvirucin · lactams · polyketides · transferases

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Manuscript received: March 28, 2018
Accepted manuscript online: March 30, 2018
Version of record online: May 14, 2018