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Biochemical Characterization of β-Amino Acid Incorporation in Fluvirucin B₂ Biosynthesis

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Abstract

Naturally occurring lactams, such as the polyketide-derived macrolactams, provide a diverse class of natural products that could enhance existing chemically produced lactams. While β-amino acid loading in the fluvirucin B₂ polyketide pathway has been 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 B₂ 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 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.

Keywords
polyketides, biosynthesis, lactams, fluvirucin, transferase

Abbreviations
Polyketide (PK), Polyketide synthase (PKS), Fluvirucin (Flv), Acyl carrier protein (ACP), Pyridoxal-phosphate (PLP)

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, rifamycin and geldanamycin.[4,7,8] Fluvirucin B₂ is a 14-membered macrolactam that shows both antifungal and antiviral activity,[4,9] and one of the main structural features of fluvirucin B₂ is the β-alanine starter unit of the polyketide skeleton (Figure 1). The biosynthetic nature of polyketide synthases and their potential to be re-programmed could enhance existing chemically produced lactams analogs, such as valerolactam, caprolactam, and laurolactam (Figure 1). Previous work on the origin of the β-alanine moiety in fluvirucin B₂ suggests it originates from L-aspartate via decarboxylation of its α-carboxyl group.[3] More recently, the putative biosynthetic gene cluster of fluvirucin B₂ from *Actinomadura fluva subsp. indica* ATCC 53714 and its corresponding β-amino acid loading pathway were annotated.[10] In this presumptive fluvirucin B₂ 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**) (Figure 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 (Figure 1, Figure S1).[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 homologs were expressed in *Escherichia coli* as N-terminal His₆-tagged proteins from codon-optimized synthetic genes (Figure S2, Table S1) and reconstituted *in vitro.* All ACP-tethered intermediates were initially monitored via MALDI TOF, 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 Figure 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, FlvL with all the necessary cofactors in the presence of 2mM L-aspartate. The mass corresponds to aspartyl-FlvL (**1**) (Figure 2A-B). This initial experiment supported the previously proposed selectivity of FlvN for L-aspartate 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 2C). Surprisingly, we also observed FlvN selectivity for L-asparagine and transfer to FlvL (Figure 2B, Table S2) with a comparable mass shift to the aspartyl-FlvL (**1**) in our initial experiment with L-aspartate (Figure 2B, 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 the FlvO to the 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 2A-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. Since 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 = 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, FlvM in the presence of various amino acids and short chain acids (Figure 3A-C). FlvM demonstrated substrate promiscuity, aminoacylating the β-alanyl-FlvL (**2**) intermediate with L-alanine (**3a**), L-serine (**3b**) and glycine (**3c**) (Figure 3). Partial aminoacylation was also observed with L-leucine and L-isoleucine (Figure 3C, Figure S3J-K). A closer inspection at 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 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 suggest L-alanine as the preferred substrate over other amino acids. Unexpectedly, FlvM displays a large amino acid substrate flexibility, which has never been described in this type of β-amino acid loading pathway.

The final step in 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 at the FlvK (GenBank: BAV56001.1) sequence suggest FlvK contains an AMP binding domain and is closely related to adenylate-forming domains (AFD) class 1 superfamily. This was further supported by the lack of transfer observed in our *in vitro* reconstitution experiments of FlvK. A closer inspection at the nucleotide sequence of the fluvirucin biosynthetic gene cluster 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*, FlvP1 ACP$_L$ and monitored dipeptidyl transfer from the FlvL to the FlvP1 ACP$_L$. The native FlvK* displayed promiscuity to transfer all three dipeptide intermediates from FlvL to the FlvP1 ACP$_L$. The dipeptidyl-FlvL intermediate with L-alanine (**3a**), L-serine (**3b**), and glycine (**3c**) were transferred to the FlvP1 ACP$_L$, generating (**4a**), (**4b**), and (**4c**) (Figure 4A-B, Table S3). Partial transfer by FlvK* was observed with the unprotected β-alanyl (**2**) FlvL intermediate (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 (**2**) FlvL intermediate. We focused on the previously identified AT homologs 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 to transfer all three dipeptide intermediates from the FlvL to the FlvP1 ACP$_L$(Figure 4, Figure S5). Surprisingly, the AT VinK from vicenistatin biosynthesis demonstrated selectivity not only for the L-alanine aminoacylated (**3a**) but the unprotected β-alanyl (**2**) FlvL intermediates as well (Figure 4, Table S3, Figure S5). 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 AT's from SceG and VinK to be selective 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 stand-alone FlvL ACP and the FlvP1 ACP$_L$ share a sequence similarity of 71.9% in a 32-amino acid overlap region where the conserved "DSL" motif is located (Figure S7D). To this end, we reconstituted FlvN, 2mM L-aspartate and replaced the stand-alone FlvL with an excised version of FlvP1 ACP$_L$. The reaction was monitored for L-aspartate addition via 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 an hour time point, this was utilized as a point of reference. Partial loading of L-aspartate by FlvN onto the FlvP1 ACP$_L$was observed after an 8 hour incubation. The majority of the 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 on to the 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 present a detailed biochemical description of β-amino acid selection, incorporation and promiscuity in the fluvirucin B_2 loading pathway. The loading pathways ability to utilize 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 on to 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.

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

**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	6.64 ± 1.05	10.96 ± 0.49	1.165
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	10.69 ± 1.42	18.92 ± 0.79	1.77

Figure 1. β-amino acid loading platform involved in fluvirucin B₂ biosynthesis.
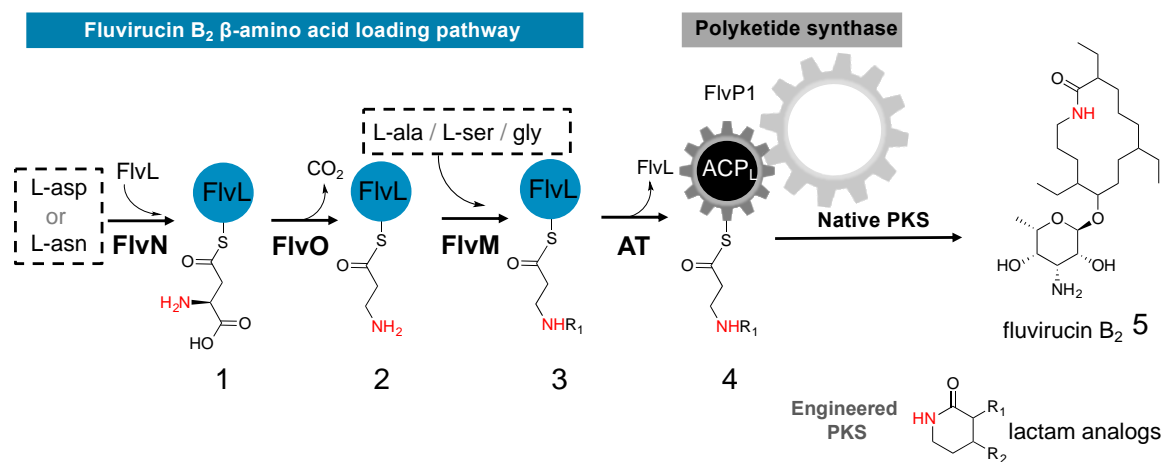
Figure 2. A) Schematic representation of FlvN activation and transfer of L-aspartate and L-asparagine onto FlvL and subsequent decarboxylation by FlvO and B) the corresponding MALDI TOF traces of the FlvL modifications. C) A list of the various substrates tested.
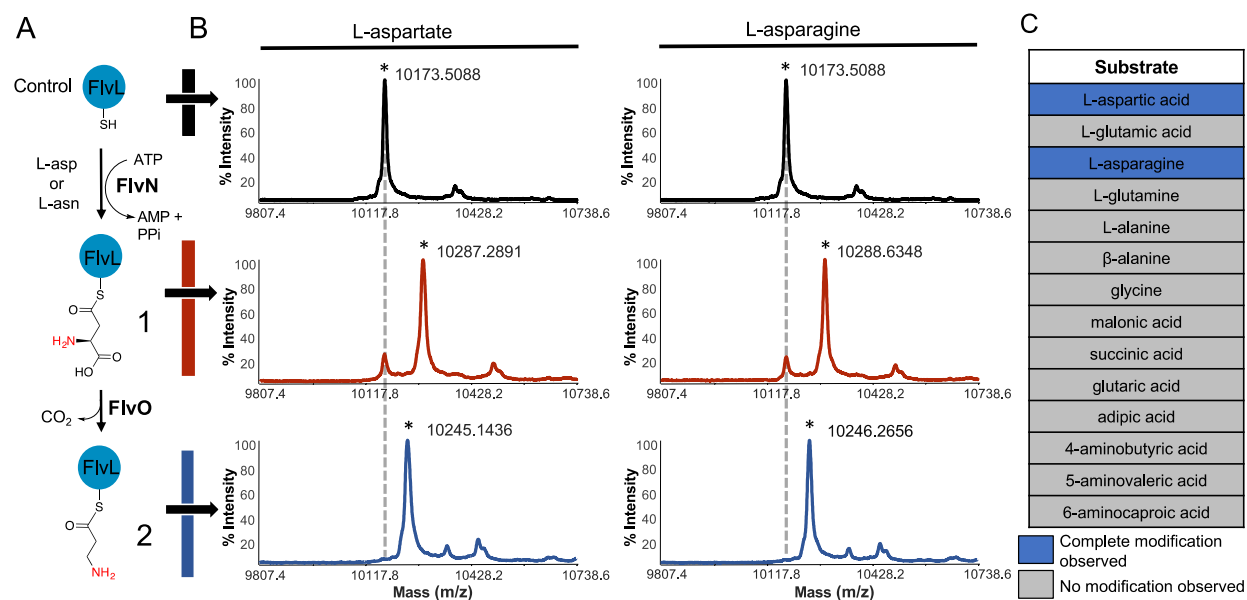
Figure 3. A) Schematic representation of FlvM aminoacylation of the β-alanyl-FlvL intermediate (2) and B) the corresponding MALDI TOF traces of the FlvL modifications. C) A list of the various substrates tested with FlvM. Partial aminoacylation was also observed with L-leucine and L-isoleucine (yellow), as depicted by the MALDI TOF traces in Figure S3J-K.
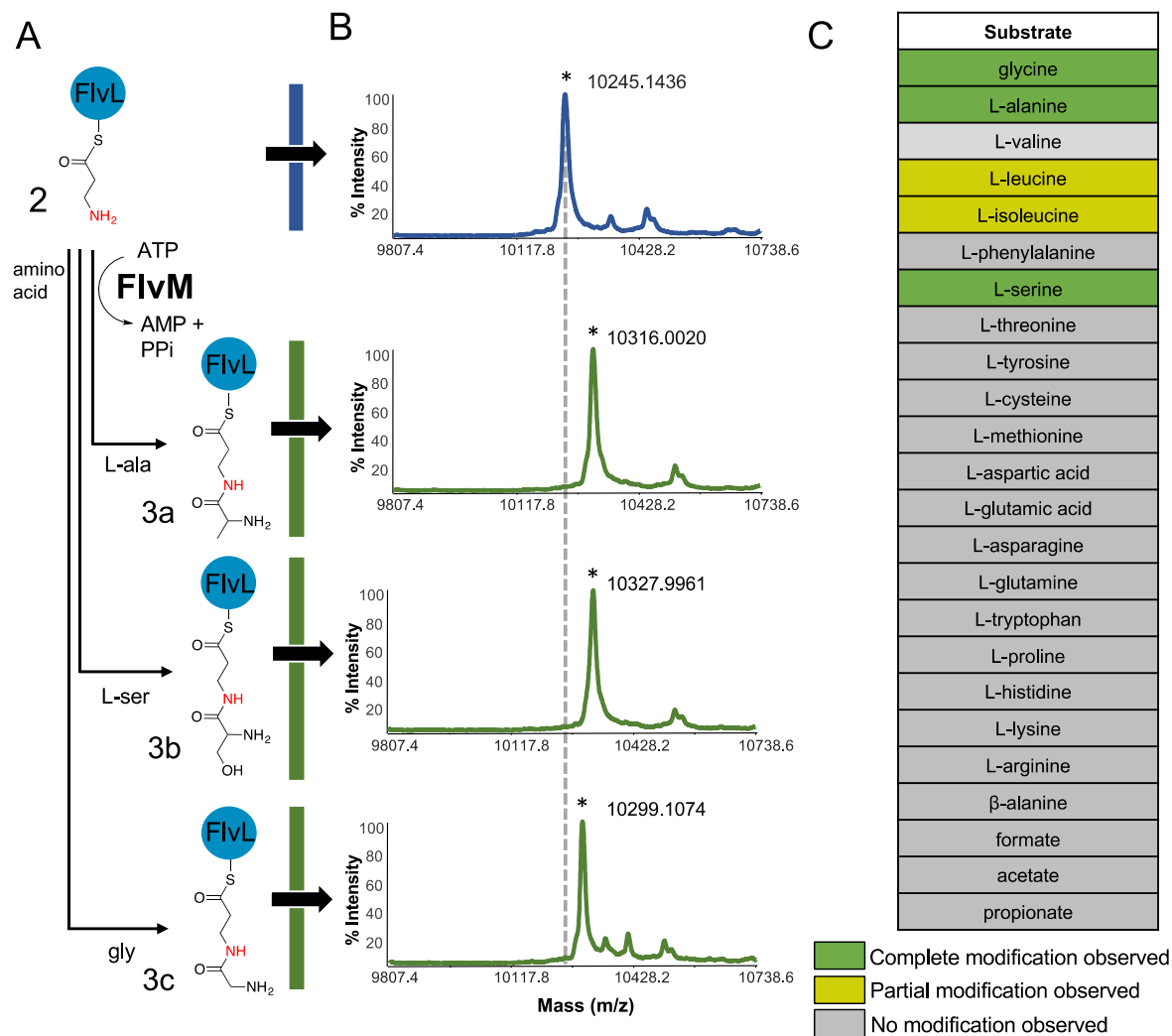
Figure 4. A) Schematic representation of dipeptide transfer from FlvL to the FlvP1 ACP by the native FlvK*, the non-native acyl transferases VinK and SceG. B) The corresponding MALDI TOF traces focused on the FlvP1 ACP. VinK also transfers **3a** to generate **4a** as depicted in Figure S5. The non-native acyl transferase SceG also selectively transfers intermediates **3a-c** to generate **4a-c**. MALDI TOF traces for SceG and VinK that are in parenthesis can be found in Figure S5.
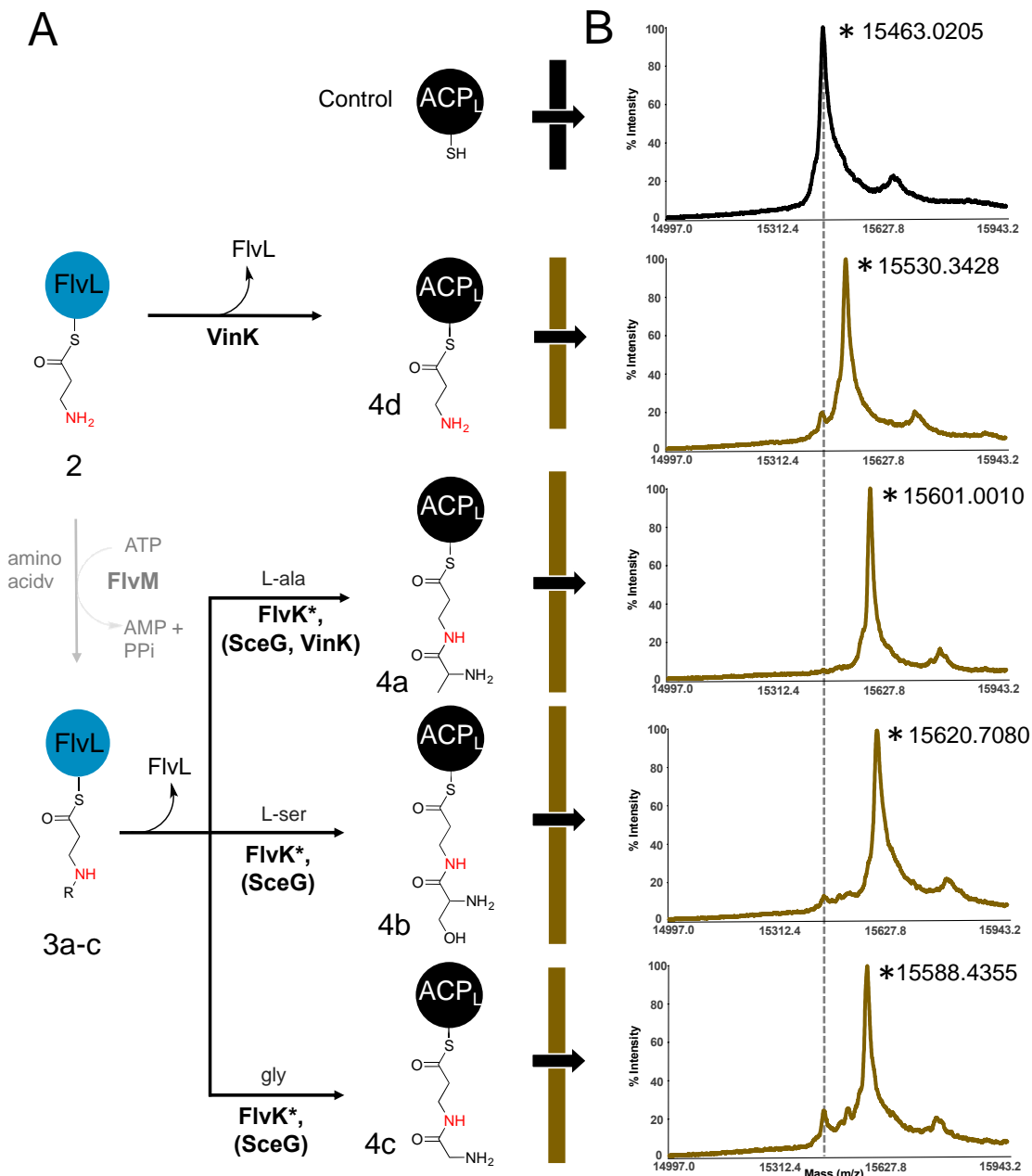
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