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# Mechanistic investigations of PoyD, a radical SAM enzyme catalyzing iterative and directional epimerizations in polytheonamide A biosynthesis

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**ABSTRACT:** Ribosomally-synthesized and post-translationally-modified peptides (RiPPs) are a growing family of bioactive peptides. Among RiPPs, the bacterial toxin polytheonamide A is characterized by a unique set of post-translational modifications catalyzed by novel radical SAM enzymes. Here we show that the radical SAM enzyme PoyD catalyzes *in vitro* polytheonamide epimerization in a *C*-to-*N* directional manner. By combining mutagenesis experiments with labeling studies and investigating the enzyme's substrate promiscuity, we deciphered in details the mechanism of PoyD. We notably identified a critical cysteine residue as a likely key H-atom donor and demonstrated that PoyD belongs to a distinct family of radical SAM peptidyl epimerases. In addition, our study shows that the core peptide directly influences the epimerization pattern allowing for production of peptides with unnatural epimerization patterns.

## INTRODUCTION

Ribosomally-synthesized and post-translationally-modified peptides (RiPPs) form an expanding family of natural products which was recently unified[1-2]. This large family of natural products contains diverse classes of peptides including lanthipeptides, thiopeptides and microcins which have relevant biological properties including notably antibiotic and anticancer activities. These fascinating biological properties are one of the reasons behind the renewed interest in RiPPs. Indeed, RiPPs appear as promising natural products to address the antibiotic resistance crisis but also as a source of novel molecules to regulate the human microbiota[3-5].

RiPPs are produced according to a simple biosynthetic logic, a precursor peptide containing a leader or a follower sequence is synthesized and modified to various extent by tailoring enzymes, before being generally secreted and the leader (or follower) cleaved off[6-7]. RiPPs have been shown to contain a wealth of post-translational modifications such as thioether[8-9] and carbon-carbon[10-12] bonds, unusual C-methylation[13-16] and epimerization[5, 17]. In a unique manner, the so-called radical SAM enzymes, an emerging superfamily of metallo-enzymes[4, 18-19] have been shown to catalyze all these various and chemically unrelated modifications[2]. Indeed, radical SAM enzymes, despite a core mechanism involving the coordination of *S*-adenosyl-L-methionine (SAM) to an $[4Fe\text{-}4S]^{2+/1+}$ cluster in a bidendate fashion[20-21] and the generation of the 5'-deoxyadenosyl radical (5'-dA$^{\bullet}$)[22] to initiate catalysis, have evolved an unsurpassed but still ill-understood diversity of mechanisms and reactions.

Among RiPPs, polytheonamide A is so far unique by requiring three radical SAM enzymes (PoyB, PoyC and PoyD) to introduce two types of post-translational modifications (*i.e.* methylation and epimerization)[17]. Another fascinating feature of polytheonamide A is the extent of post-translational modifications introduced by these three enzymes. Indeed, the two $B_{12}$-dependent radical SAM enzymes: PoyC and PoyB, have been recently shown *in vitro*[15] and *in vivo*[23] to be responsible for the formation of the thirteen $C_{\beta}$ methylation and the *N*-terminal *ter*-butyl group (**Figure 1a**). By co-expressing PoyD with various truncated forms of the precursor peptide PoyA in *E. coli*[17, 23], it has been shown that PoyD catalyzes the eighteen epimerizations found in polytheonamide A in a likely *C*-to-*N* directionality (**Figure 1**). Based on its sequence and these unique properties, PoyD has been predicted to form a distinct class of radical SAM enzymes[2, 5, 24]. To understand the mechanism of this enzyme and unravel how it introduces a unique pattern of epimerizations within a peptide backbone, we undertook the biochemical characterization of the radical SAM enzyme PoyD.

## RESULTS

**PoyD is a radical SAM enzyme catalyzing *in vitro* peptide epimerization**. PoyD was expressed as a Strep-tag fusion protein in *E. coli* (**Figure 2a**). The purified protein exhibited the typical brownish color of iron-sulfur enzymes and, after anaerobic iron-sulfur reconstitution, UV-visible analysis showed an increase in the absorption bands at 320 nm and 420

nm, consistent with an increase of the iron-sulfur cluster content of the protein (**Figure 2b**). Determination of the iron content indicated that as-purified PoyD contained 1.1± 0.1 mol of Fe per polypeptide. After anaerobic reconstitution, PoyD contained 4.1± 0.4 mol of Fe per polypeptide. These results supported that PoyD contained one [4Fe-4S] cluster per monomer.

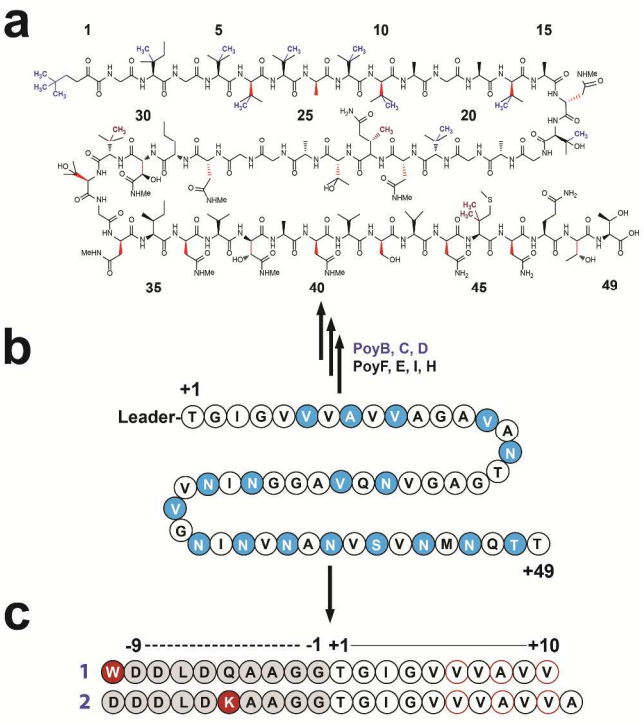


**Figure 1 – Structure of Polytheonamide A and peptide substrates designed to investigate PoyD mechanism**. (**a**) **Structure of polytheonamide A**. Numbers indicates amino acid residues location. Methyl groups labeled in blue are inserted by the radial SAM enzyme PoyC, while methyl groups labeled in purple have been proposed to be inserted by the radical SAM enzyme PoyB. Red labels are D-amino acid residues formed by the radical SAM enzyme PoyD. (**b**) **Sequence of PoyA, the peptide precursor of polytheonamide A**. Circles filled in blue indicate the amino acid residues epimerized in mature polytheonamide A. The enzymes responsible for post-translational modifications of PoyA are indicated next to the arrows. (**c**) **Sequence of peptides 1 & 2 used as substrates**. Circles filled in grey indicate amino acid residues from the leader sequence while circles filled in white indicate amino acids from the core sequence. White circles with a red line are residues epimerized in polytheonamide A. Circles filled in red indicate amino acid residues introduce in the sequence for analytical purpose. Numbers are relative to PoyA sequence with positive numbers for the core peptide and negative numbers for the leader-peptide sequence.

To assess the activity of PoyD, we tried to produce PoyA, the polytheonamide A precursor and proposed substrate of PoyD (**Figure 1b**), in *E. coli*. Previous studies have pointed out that PoyA cannot be expressed in the absence of PoyD, suggesting a role of foldase/chaperone for this latter[17, 23]. We thus expressed PoyA as a His-tag fusion protein in the presence of PoyD. However, in order to obtain an unmodified PoyA, we also attempted to express PoyA in the absence of PoyD. PoyA was then purified under denaturating conditions (See Supplementary Methods) and analyzed by gel electrophoresis and mass spectrometry. As shown, we were able to express and purify PoyA even in the absence of PoyD, with purity similar to previous reports[17, 23] (**Supplementary Figure S1**). LC-MS/MS analysis of the amino acid content of PoyA, after acid hydrolysis and derivatization with *N*-α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) (see Supplementary Methods) showed, as expected, that PoyA contained D-amino acid residues when co-expressed with PoyD. We notably identified D-Asn (converted as D-Asp during the hydrolysis process) and D-Val, which are characteristic of polytheonamide A[17] (**Figures 2c**). PoyA, expressed in the absence of PoyD, did not contained D-amino acid residues. Unfortunately, unmodified PoyA exhibited very poor solubility in aqueous buffers and proved to be an impracticable substrate for the *in vitro* study of PoyD.

We have recently shown that a peptide derived from the core sequence of PoyA (residues 1 to 49, **Figures 1a & b**) and containing the residues +1 to +15, could serve as substrates for PoyC, the $B_{12}$-dependent radical SAM enzyme catalyzing valine *C*-methylation[15]. However, because of the high content of hydrophobic residues (*i.e.* Ile, Val and Ala), we had to insert an *N*-terminus stretch of Lys residues to obtain a soluble substrate. With this substrate, PoyC catalyzed methylation of Val-14 but not of the five other Val residues located between positions +5 to +10, presumably because of the presence of the Lys-stretch.

Interestingly, the leader sequence of PoyA contains charged amino acid residues (Asp) that could be exploited to improve the solubility of the peptides and make them more suitable for LC-MS analysis. We thus synthesized PoyA derivatives containing residues -9 to -1 from the leader sequence and residues +1 to +10 from the core region (**Figure 1c**). To simplify the detection and analyses by HPLC and LC-MS, we also introduced either one *N*-terminal Trp residue (**Peptide 1**) or substituted the residue Gln -5 by a Lys moiety (**Peptide 2**) (**Figure 1c**). Each peptide was assayed with PoyD under anaerobic and reducing conditions in the presence of the *S*-adenosyl-L-methionine (SAM) cofactor.

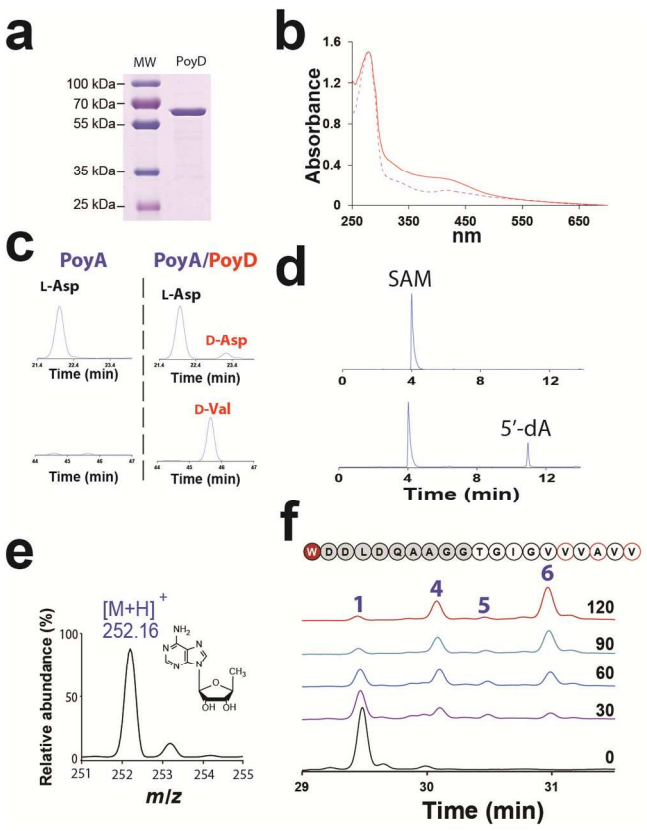


**Figure 2** – ***In vitro*** **characterization of PoyD**. (**a**) **Gel electrophoresis analysis of purified PoyD expressed in *E. coli***. MW: Molecular weight markers. (**b**) **UV-visible spectrum of as-purified (dotted line) and anaerobically reconstituted PoyD (plain line)**. (**c**) **HPLC analysis of the amino acid content of PoyA after its *in vivo* expression in *E. coli* alone or in the presence of PoyD** (left and right panels respectively). Amino acids were analyzed after acid hydrolysis and derivatization with *N*-α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) and their retention times compared with authentic standards. (**d**) **HPLC analysis of SAM incubated with PoyD**. Traces indicate incubation under anaerobic conditions in the presence of sodium dithionite at t=0 and after 90 min. As shown, during incubation *S*-adenosyl L-methionine (SAM) is cleaved in 5'-deoxyadenosine (5'-dA). (**e**) **LC-MS analysis of 5'-deoxyadenosine (5'-dA) produced by PoyD**. (**f**) **Activity of PoyD toward peptide 1**. HPLC Analyses were performed at t=0, 30, 60, 90 and 120 min (lower to upper traces respectively). See Supplementary Information for experimental conditions.

In each condition, PoyD catalyzed the reducing cleavage of SAM into 5'-dA (**Figure 2d & e**; $[M+H]^+ = 252.1$) demonstrating its activity as a radical SAM enzyme. Incubation of PoyD with **peptide 1** led to the formation of three peptides (**Peptides 4**, **5** & **6**, eluting at 30.1, 30.4 & 30.9 min, respectively) (**Figure 2f**). Mass spectrometry analysis revealed no mass difference between **peptide 1** and the products formed (**Supplementary Figure S2**). In order to ascertain the nature of the modification and to identify the modified residues, we performed the reaction in deuterated buffer. Indeed, *vivo*[23-24] and *in vitro*[5] investigations of radical SAM epimerases have shown that they introduce solvent derived H-atoms into their products. Under these conditions, the peptides produced had their masses shifted from $[M+2H]^{2+} = 969.6$ to $[M+2H]^{2+} = 970.6$ for **peptides 4** & **5** and to $[M+2H]^{2+} = 971.1$ for **peptide 6** (**Figure 3a**).

These results indicated that two deuterium atoms were introduced into **peptides 4** & **5** while three deuterium atoms were introduced into **peptide 6** during the reaction. LC-MS/MS analysis of **peptide 4** showed that one deuterium atom was located in Ala-8 and another one in Val-10 (**Figure 3b**, **Supplementary Figure S3 & Tables S1 & S2**). Analysis of **peptide 5** revealed a different pattern with one deuterium atom introduced into Val-9 and one into Val-7 (**Supplementary Figure S4a & Table S3**). Finally, **peptide 6** proved to contain three deuterium atoms located in Val-5, Val-7 and Val-9 (**Supplementary Figure S4b & Table S4**). The modified peptides were further purified by HPLC and their amino acid content analyzed by LC-MS/MS (see Supplementary Methods). Comparison with authentic standards showed that, in each peptide produced (*i.e.* **peptides 4**, **5** & **6**), D-Val and D-Ala residues were present (**Figure 3c & Supplementary Figure S5**). These results established that **peptides 4**, **5** and **6** are diastereoisomers of **peptide 1** and that PoyD is a peptidyl epimerase which requires only an [4Fe-4S] cluster and the SAM cofactor to convert *in vitro* L-Val and L-Ala into their D-configured counterparts.

**PoyD substrate specificity**. As shown above, incubation of **peptide 1** led to the formation of three peptides with two epimerization patterns (*i.e.* natural pattern: Ala-8 and Val-10 and unnatural pattern: Val-5, Val-7 and Val-9) involving either the *C*-terminal or the penultimate residue with alternating epimerizations, one of two residues (**Figures 3a & b**). With **peptide 2**, only one new peptide was produced, albeit at low level (**Supplementary Figure S6**). This novel peptide (**peptide 3**) had a mass increment of +3 Da when the reaction was performed in deuterated buffer, consistent with the incorporation of three deuterium atoms. LC-MS/MS analysis allowed to position deuterium incorporation into Val-6, Ala-8 and Val-10 (*i.e.* the natural epimerization pattern) (**Figure 3b**, **Supplementary Figure S7 & Tables S5 & S6**).

In order to determine the influence of the leader peptide on the activity and specificity of PoyD, we synthesized a peptide containing the residues +3 to +10 (**Peptide 7**, **Figure 3d**). With this peptide, devoid of residues from the leader peptide, PoyD catalyzed the formation of five peptides (**Peptides 8**, **9**, **10**, **11** & **12**) (**Figure 3d**). LC-MS/MS analysis showed that the main products formed were two peptides with three epimerized residues (*i.e.* Val-5, Val-7 and Val-9 or Val-6, Ala8 and Val-10**, Peptides 11** & **12**, respectively), two peptides with two epimerized residues (*i.e.* Val-7 and Val-9 or Ala-8 and Val-10, **Peptides 10** & **9**, respectively) and a very low amount of a mono-epimerized peptide (**Peptide 8**) (see **Supplementary Figures S8-S13 & Supplementary Tables S7-S12**).

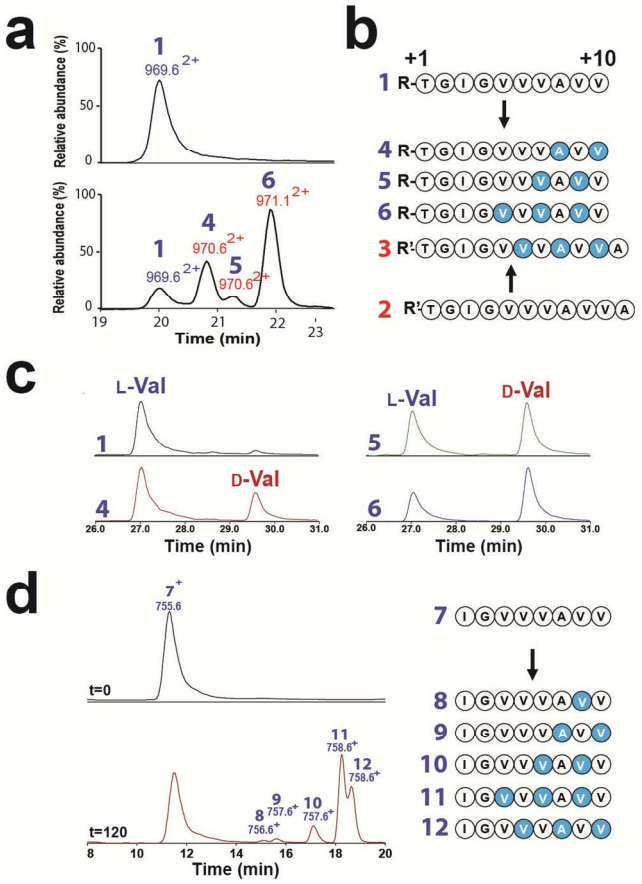


**Figure 3 – Characterization of the products formed *in vitro* by PoyD. (a) LC-MS/MS analysis of peptide 1 incubated with PoyD.** Upper trace peptide 1. Lower trace, peptide 1 incubated with PoyD for 2 hours, in deuterated buffer. See Supplementary Information for experimental conditions. Numbers refer to the corresponding peptides. (**b**) **Sequence of the different products formed by PoyD *in vitro* after incubation with peptide 1 or peptide 2**. See Figure 1 for the amino acids residues corresponding to R & R' and Figures S3-4 and S6 and Supplementary Tables S1-S6 for complete peptide assignment. (**c**) **LC-MS/MS analysis of the L-/D-Val content in peptide 1 and the products: peptides 4, 5 & 6 obtained after incubation with PoyD.** Amino acids were analyzed after hydrolysis and derivatization by L-FDVA and detected by LC-MS after ion current extraction in MS/MS experiments using the transition 398>352 for Val-FDVA derivatives. Numbers indicate peptides analyzed. (**d**) **LC-MS/MS analysis of peptide 7 incubated with PoyD.** Analysis was performed at t=0 and after 2 hours incubation, upper and lower traces, respectively. Sequences of the peptides produced are indicated. See Supplementary Figures S8-13 and Supplementary Tables S7-S12 for full peptide assignment. Numbers refer to the corresponding peptides.

Thus, this short substrate, despite lacking residues from the leader peptide, recapitulated the different epimerization patterns obtained with **peptides 1** & **2**.

Collectively, these data showed that subtle variations in the sequences of the substrates led to the formation of peptides with the epimerization pattern found in polytheonamide A (*i.e.* **peptides 3**, **4**, **9** & **12**) but also peptides with an unnatural epimerization pattern (*i.e.* **peptides 5**, **6**, **8**, **10** & **11**). However, PoyD always catalyzed epimerization of residues from the core sequence at 1,3-positions but never of residues from the leader sequence. In addition, these experiments support that the activity of PoyD is largely independent of the leader peptide. Of note, PoyD produced several peptides with epimerization located on the last residue (*i.e.* **peptides 4**, **9** & **12**), in sharp contrast with a recent *in vivo* study which suggested that PoyD cannot modify the last residue of truncated PoyA peptides[23]. Finally, because the post-translational modifications accumulated at the *C*-terminal end of the various peptides assayed, our results were consistent, as recently suggested by *in vivo* experiments, that PoyD has a directional activity from the *C*-terminal toward the *N*-terminal end of the peptide.

To date, several radical SAM enzymes such as AlbA[8-9] and YydG[5] have been shown to introduce multiple post-translational modifications in their substrate, *in vitro*. However, there is no evidence that one molecule of enzyme is responsible for the insertion of several post-translational modifications on one molecule of substrate. Only for the radical SAM enzyme lipoyl synthase, it has been shown that one molecule of enzyme introduces sequentially two modifications in its substrate (*i.e.* insertion of two sulfur atoms into its fatty acyl substrate)[25]. However, the recently solved structure of lipoyl synthase has shown that the incorporation of the two sulfur atoms is part of the same catalytic event[26] and does not imply enzyme motion. The production by PoyD of peptides containing several epimerized residues indicated either the combined action of several enzymes on a same peptide backbone or a processive activity of PoyD.

**Mechanistic investigation of PoyD**. Having developed an *in vitro* assay for PoyD, we were able to interrogate its mechanism. MS analysis of the epimerized amino acid residues (*i.e.* D-Val & D-Ala) produced by PoyD in deuterated buffer showed a mass shift of +1 Da compared to their L-configured counterparts (**Figure 4a** & **Supplementary Figure S5).** This result, in line with *in vivo* studies[23-24], confirmed that one solvent-derived deuterium atom was incorporated during catalysis. However, MS analysis of the 5'-dA produced ($[M+H]^+$: 252.1) showed no deuterium incorporation consistent with PoyD abstracting a substrate non-exchangeable H-atom (**Figure 4b**). To further validate this conclusion, we incubated PoyD in deuterated buffer but omitted the peptide substrate. Under these conditions, the molecular weight of 5'-dA shifted to $[M+H]^+$: 253.1 (**Figure 4b**, middle panel) indicating that in absence of its substrate, PoyD still generated 5'-dA$^\bullet$ but that this latter reacted with buffer components (**Figure 4b**).

Kinetic experiments performed with **peptide 1** showed that formation of **peptide 4** stopped after 90 minutes (**Figure 4c**) while **peptide 6** was produced over three hours with an estimated kcat (per epimerization) of 0.02 $min^{-1}$ and 0.03 $min^{-1}$, respectively (**Figure 4c**). Thus, **peptide 6**, with the unnatural epimerization pattern, was the most efficiently produced peptide, *in vitro*. Interestingly, after an initial accumulation, **peptide 5** tended to disappear while production of **peptide 6** still proceeded. This result suggested that **peptide 5** could serve as substrate for PoyD and further converted into **peptide 6**. Since **peptides 4** and **5** have two modifications and **peptide 6** has three modifications, production of 5'-dA (~1100 µM) and the three epimerized peptides (**peptide 4** (157 µM), **peptide 5** (23µM) and **peptide 6** (248 µM)) indicated a good correlation between epimerization events and SAM consumption (**Figure 4c & d**). In addition, LC-MS analysis of 5'-dA produced overtime in deuterated buffer exhibited no deuterium incorporation. Only when the substrate became limiting (after one hour), we monitored <10% deuterium incorporation in 5'-dA

(**Figure 4b**, lower trace) while no labeling was measured in the remaining SAM. Altogether, these results were consistent with one molecule of SAM being used by epimerization event.

**Identification of a potential critical H-atom donor**. We have recently discovered a peptidyl epimerase in *Bacillus subtilis*. This enzyme called YydG possesses, in addition to the radical SAM cluster, an additional [4Fe-4S] cluster[5]. This auxiliary cluster has been proposed to assist radical quenching during catalysis[5]. Interestingly, the only other radical SAM epimerase characterized *in vitro*, NeoN[27] which epimerizes the C-5''' of neomycin, also contains an additional [4Fe-4S] cluster[27] in a SPASM-like domain[12, 28-29].

Sequence analysis of PoyD revealed no obvious motif for the coordination of an additional [4Fe-4S] cluster among the 10 cysteine residues present within the protein sequence. To confirm this hypothesis, we replaced the cysteine residues of the radical SAM motif (CxxxCxxC) by alanine residues and probed for the presence of additional [4Fe-4S] clusters. After purification and anaerobic reconstitution, the triple Cys→Ala mutant (**A3** mutant) exhibited a distinct UV-visible spectrum from the wild-type enzyme (**Figures 5a & b**). Determination of the iron content indicated that the **A3** mutant contained 0.3± 0.2 mol of Fe per polypeptide consistent with its UV-visible spectrum showing the absence of iron-sulfur clusters. To assay its activity, we co-expressed *in vivo* the **A3** mutant with PoyA. As shown (**Figure 5c**), no epimerized residues could be identified in PoyA supporting the critical role of the radical SAM cluster for PoyD activity.

In several radical SAM enzymes such as spore photoproduct lyase[30-34], PolH[35], NeoN[27] or YydG[5], it has been shown that a cysteine residue is used as a critical H-atom donor. However, because of the lack of significant homology between PoyD and these enzymes, we could not identify a putative H-atom donor. We thus aligned the sequence of PoyD with several PoyD-homologs recently identified[24]. In addition to the three cysteine residues from the radical SAM motif, only one cysteine residue (**Cys-372**) was conserved among these enzymes (**Figure 5d**). We further searched for homolgs in protein databases and identified 67 homologs (sequence identity> 25%) mostly in Proteobacteria. Sequences alignment confirmed that beside the cysteines from the radical SAM motif, only one cysteine residue (*i.e.* Cys-372 in PoyD) was conserved among these proteins (**Supplementary Figure S14**).

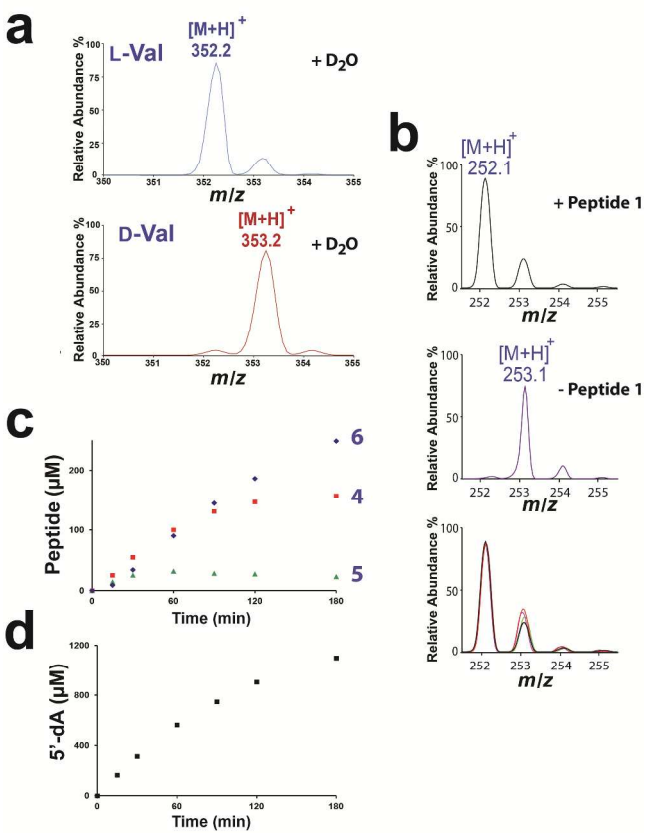


**Figure 4 – Mechanistic and kinetic analysis of the reaction catalyzed by PoyD**. (**a**) **MS/MS spectra of L-Val (upper traces) and D-Val (lower traces) obtained after incubation of PoyD with peptide 1 in deuterated buffer**. Peptide products were purified and after acid hydrolysis, amino acids were derivatized with L-FDVA and analyzed by LC-MS/MS. See Supplementary Information for experimental conditions. (**b**) **MS spectra of 5'-dA produced by PoyD**. PoyD was incubated in deuterated buffer under anaerobic and reducing conditions in the presence (upper trace) or in the absence of peptide 1 (middle trace). The lower trace shows MS spectra of 5'-dA produced over time (from t=0 to t=240 min) in the presence of peptide 1 in deuterated buffer. **Production of epimerized peptides** (**c**) **and 5'-dA** (**d**) **by PoyD**. PoyD was incubated in the presence of peptide 1 under anaerobic conditions with sodium dithionite and SAM. Numbers refer to the corresponding peptides formed.

To probe for the function of Cys-372, we performed its Cys→Ala replacement and co-expressed the corresponding mutant (**C372A** mutant) *in vivo* with PoyA. Interestingly, the **C372A** mutant failed to epimerize PoyA (**Figure 5c**). However, analysis of the purified **C372A** mutant showed that its iron content increased from 0.2± 0.1 to 3.5± 0.2 mol of Fe per polypeptide after anaerobic reconstitution, similarly to the wild-type enzyme.

We further assayed the activity of the **C372A** and **A3** mutants against **peptide 1**, *in vitro* (**Figure 5e**). As shown, the **A3** mutant had no enzymatic activity in-line with the *in vivo* experiments. In contrast, the **C372A** mutant proved to produce a novel peptide (**peptide 13**) eluting at 29.4 min and distinct from **peptides 4**, **5** & **6**, produced by the wild-type enzyme.

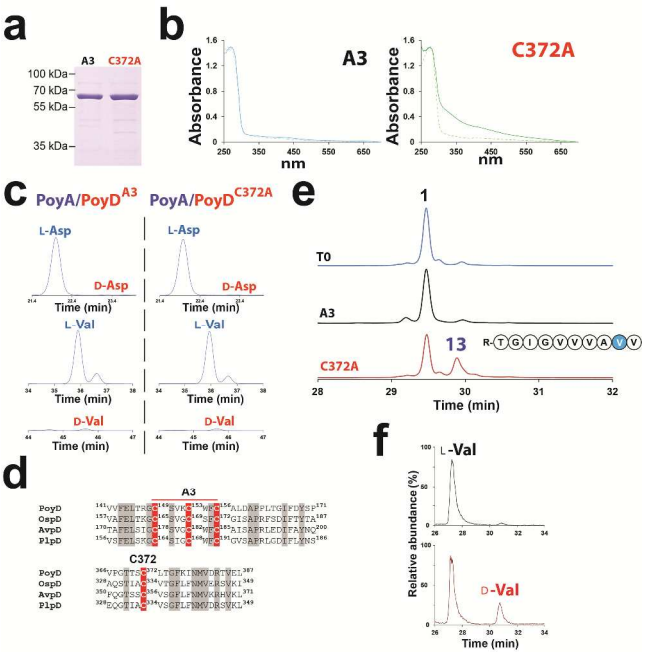


**Figure 5 – Identification of a potential H-atom donor in PoyD.** (**a**) **Gel electrophoresis analysis of the A3 and C372A mutants expressed in *E. coli*.** (**b**) **UV-visible spectrum of the A3 and C372A mutants before (dotted line) and after anaerobic reconstitution (plain line).** (**c**) **HPLC analysis of the amino acid content of PoyA after its *in vivo* expression in *E. coli* with the A3 or the C372A mutants** (left and right panels, respectively). Amino acids were analyzed after acid hydrolysis and derivatization with L-FDVA and their retention times compared with authentic standards. See Supplementary Information for experimental conditions. (**d**) **Sequence alignment between PoyD and other proteusin epimerases OspD, AvpD and PlpD**. Strictly conserved residues are highlighted in grey or red (cysteine residues). Numbers refers to amino acid residues location in the respective sequences. (**e**) **HPLC analysis of peptide 1 incubated in the presence of the A3 or C372A mutant**. Upper trace: HPLC analysis of peptide 1 at t=0. Middle trace: HPLC analysis of peptide 1 after 120 min incubation with the A3 mutant. Lower trace: HPLC analysis of peptide 1 after 120 min incubation with the **C372A** mutant. The sequence of the product formed by the **C372A** mutant is indicated. See Supplementary Figure S15 and Supplementary Table S13 for full assignment. (**f**) **LC-MS/MS analysis of the L-/D-Val content of peptides 1 & 13**. Upper trace corresponds to peptide 1 and lower trace to peptide 13 produced by the **C372A** mutant. LC MS/MS experiments were performed using the transition 412>366.

LC-MS/MS analysis of this novel peptide showed the penultimate valine residue, Val-9, to be epimerized (**Supplementary Figure S15**). This result was further confirmed by amino acid analysis which confirmed the presence of D-valine in **peptide 13** with a D-Val/L-Val ratio of ~20%, consistent with the modification of one valine residue out of 5 (**Figure 5f**). Thus, contrary to *in vivo* conditions, *in vitro* experiments showed that the **C372A** mutant is able to catalyze peptide epimerization.

Such apparent discrepancies, between *in vivo* and *in vitro* activities, have been reported during the investigation of another radical SAM enzyme, the spore photoproduct lyase[36], for which mutation of the H-atom donor (*i.e.* Cys-141)[30, 32-33] has been shown to impair the DNA repair activity in spores but not the ability of the enzyme to repair the spore photoproduct *in vitro*[30, 33, 37]. Altogether, these results support that Cys-372 fulfills an important function likely as a critical H-atom donor. However, we cannot rule out that other residues are involved in this process notably tyrosine residues, as shown for carbapenem synthase[38].

**Processivity of PoyD**. The fact that the **C372A** mutant produced peptide with only one epimerized residue, while the wild-type enzyme systematically produced peptides with multiple epimerizations, prompted us to assay the activity of PoyD against the mono-epimerized **peptide 13**. Indeed, the failure of the **C372A** mutant to catalyze multiple epimerizations suggested that only peptides containing L-amino acid residues could serve as substrates for PoyD. We thus incubated **peptide 1** with the **C372A** mutant and purified **peptide 13** (**Figure 6a**). This peptide was then further incubated with the wild-type enzyme and the reaction analyzed by HPLC and LC-MS. In contrast to **peptide 1**, incubation of **peptide 13** with the wild-type enzyme led to the formation of only two peptides (**peptides 5** and **6**). The implication of this result is twofold: It demonstrates that epimerized peptides are substrates for PoyD and, more importantly, that the first epimerization event guides and restricts the following epimerization events in order to preserve the strict 1,3-pattern of epimerization.

To try to discern between processivity and cooperativity, we further performed kinetic experiments in the presence of an equal amount of wild-type (**peptide 1**) and mono-epimerized peptide (**peptide 13**). As shown, **peptide 13** was converted three-times faster than **peptide 1** (**Figure 6b**). In agreement with this result, **peptide 5**, which was a minor species when PoyD was incubated with **peptide 1** alone (**Figure 4c**), was the dominant product formed during the first 30 minutes of the reaction (**Figure 6c**). As the reaction proceeded, **peptide 5** was then further converted into **peptide 6** containing three D-amino acid residues (**Figure 6c**). These results demonstrate that PoyD has a better activity on a peptide containing an epimerized residue rather than on a peptide containing only L-amino acid residues. However, in contrast to **peptide 1** which was converted into peptides with different epimerization patterns, **peptide 13** was converted only into **peptide 5** and ultimately **peptide 6** (**Figure 6**). This suggests that binding and positioning of the substrate is determined, in part, by the presence of epimerized residues.

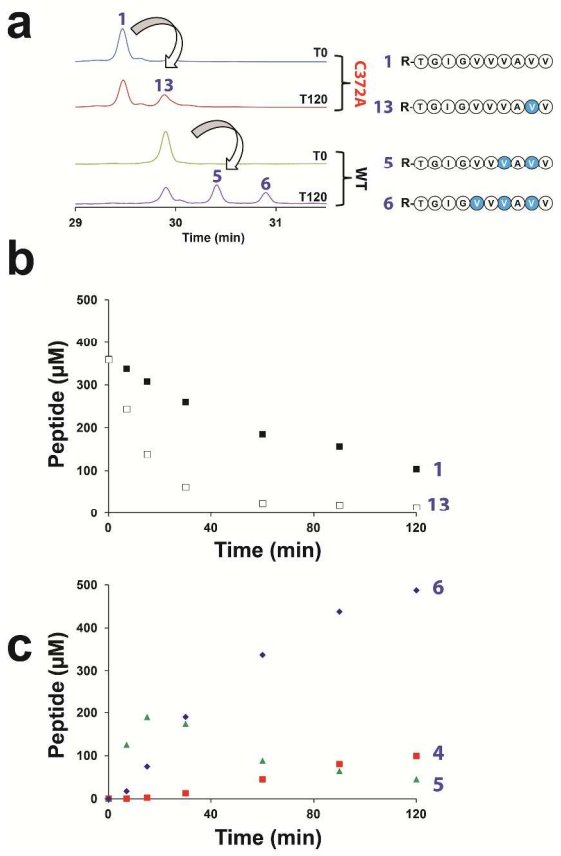


**Figure 6 – (a) HPLC analysis of peptide 1 after incubation with the C372A mutant and wild-type PoyD**. Peptide 1 was incubated with the C372A mutant and analyzed at t=0 (upper blue trace) and t=120 (red trace). After purification, peptide 13 was incubated with PoyD and analyzed by HPLC at t=0 (green trace) and t=120 min (purple lower trace). Numbers refer to the corresponding peptides. (**b**) **Consumption of peptides 1 and 13 during incubation with PoyD. (c) Production of epimerized peptides by PoyD**. PoyD was incubated in the presence of peptide 1 under anaerobic conditions with sodium dithionite and SAM. Numbers refer to the corresponding peptides formed.

Interestingly, the transient accumulation of **peptide 5**, in the range of the enzyme concentration (~150µM), is consistent with at least a partially processivity of PoyD, as recently shown for lanthipeptide synthetases[39-40]. Further studies will be required to definitively address this question.

## DISCUSSION

Epimerization reactions were predicted to be catalyzed by radical SAM enzymes more than a decade ago, consecutively to the investigation of the avilamycin A biosynthetic pathway[41]. *In vivo* studies have shown that a radical SAM enzyme, AviX12, was responsible for a critical C-2 epimerization of a glucose moiety, essential to obtain the active form of this antibiotic. Similarly, radical SAM epimerases have been identified in the biosynthetic pathways of several RiPPs including the bacterial toxin polytheonamide A[17, 23]. However, it is only recently that mechanistic insights have been gained on these novel enzymes. The first radical SAM epimerase characterized at the biochemical level, was the carbohydrate epimerase NeoN which converts neomycin C into neomycin B[27]. More recently, while investigating YydG, a radical SAM enzyme of unknown function from *Bacillus subtilis*, we demonstrated this enzyme to be a peptidyl epimerase[5] converting L-Val and L-Ile into their epimers, during the biosynthesis of the so-called epipeptides[2, 5].

Interestingly, despite being active on similar hydrophobic amino acid residues, YydG is unrelated, at the sequence level, to PoyD. Notably, YydG is devoid of the RiPP precursor peptide recognition element (*i.e.* RRE or PqqD-like domain)[42], characteristic of PoyD and many RiPP modifying enzymes[2]. In addition, our study shows that PoyD, in contrast to YydG, contains only one [4Fe-4S] cluster.

Epimerized peptides produced *in vitro* contained modifications only in the *C*-terminal region strongly supporting a *C*-to-*N* directionality for the enzyme, as recently suggested by *in vivo* experiments. Interestingly, the recent *in vitro* study of PoyC has demonstrated that it catalyzes methyl transfer to the *C*-terminal end of a synthetic peptide[15]. We can thus speculate that PoyC, like PoyD, introduces post-translational modifications with a similar *C*-to-*N* directionality. Definitive proofs of the directionality of PoyD came from the investigation of the **C372A** mutant. Indeed, this mutant produced only a mono-epimerized peptide. The implications here are twofold: first, it unveiled the initiation site of the peptide modification and second, it suggests a processive mode of action of the enzyme. Indeed, we did not evidence the production of other mono-epimerized products (*i.e.* peptides epimerized on other residues) or the formation of peptides with several epimerizations, as expected in case of the action of several molecules of enzyme.

Interestingly, contrary to recent *in vivo* studies[23, 43], peptides produced *in vitro* by PoyD contained either the same epimerized residues than the ones found in polytheonamide A (*i.e.* natural pattern of epimerization: **peptides 3**, **4**, **9** & **12**) or unnatural epimerizations involving amino acid residues not epimerized in polytheonamide A (*i.e.* **peptides 5**, **6**, **8**, **10** & **11**).

Of note, we were able to obtain these epimerization patterns using either peptides containing a portion of the leader peptide (*i.e.* **peptides 1** & **2**) or peptides containing only residues from the core sequence (*i.e.* **peptide 7**). Hence, the activity of PoyD is largely independent of the leader peptide, as shown for other radical SAM enzymes catalyzing peptide post-translational modifications such as YydG[5], AlbA[9] and PoyC[15], but in contrast to other enzymes such as the KW_cyclase[10, 12].

Based on these results, we can propose the first mechanism for PoyD and proteusin epimerases in general. Following the reductive cleavage of SAM, PoyD generates 5'-dA$^{\bullet}$ which abstracts a substrate $C_{\alpha}$ H-atom leading to the formation of 5'-dA and a carbon-centered radical (**Figure 7**). After the loss of the stereochemistry, one solvent exchangeable H-atom is transferred from an H-atom donor (likely Cys-372) to the radical intermediate to produce an epimerized amino acid residue and a thiyl radical on the protein.

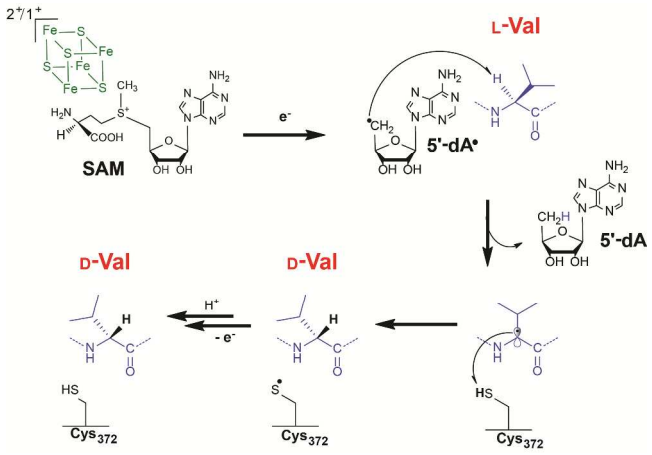


**Figure 7 – Proposed mechanism for the radical SAM peptide epimerase PoyD**. After the reducing SAM cleavage, PoyD generates a 5′-dA• which abstracts the amino acid $C_\alpha$ H-atom. A carbon-centred radical is formed and quenched by the thiolate H-atom of Cys-372 leading to the formation of a D-amino acid residue. Reduction of the thiyl radical is likely assisted by other amino acid residues from PoyD similarly to ribonucleotide reductase or spore photoproduct lyase for the next catalytic cycle.

In the absence of an additional [4Fe-4S] cluster, the most likely hypothesis is that Cys-372 is regenerated by the reduction of the thiyl radical by another cysteine residue like in ribonucleotide reductase[44]. However, we did not identify an obvious candidate to fulfill this function. Therefore, other residues such as a tyrosine residue[32-34, 37] may also be involved in the regeneration of Cys-372, as suggested for the radical SAM enzyme spore photoproduct lyase[30, 36, 45].

To conclude, our study establishes that PoyD constitutes a distinct group of peptidyl epimerases within the superfamily of radical SAM enzymes. We also demonstrate here that the pattern of epimerization is likely an intrinsic property of the enzyme which always produces epimerized peptides on the 1,3-positions. Surprisingly, the enzyme is able to recognize a peptide already containing one epimerized residue and to catalyze the next epimerization event, preserving the 1,3 epimerization pattern. The function of the leader peptide, even if not essential, is likely to guide the positioning of PoyA within PoyD active site for the first epimerization event. Further studies should allow to decipher precisely how PoyD interacts with its substrate and what are the molecular determinants of its apparent processivity.

## MATERIALS AND METHODS

**Expression and purification of PoyD**. The PoyD gene was optimized for expression in *E. coli* and synthesized by Life Technologies. The synthesized PoyD gene was inserted between the NdeI and XhoI restriction sites of a $pASK_{17}^+$ plasmid with a Strep-tag fusion. The plasmid was then used to transform *E. coli* BL21 (DE3) star cells. An overnight culture of a single colony of *E. coli* BL21 (DE3)/$pASK_{17}^+$-Strep-tag-PoyD was used to inoculate 9 L of LB medium containing ampicillin (100 µg/L). Cells growth was carried out at 37°C and 180 rpm until the OD at 600 nm reached ~0.7. Protein expression was performed by adding anhydrotetracycline (400 µM) and iron citrate (250 µM). Cells were harvested by centrifugation (5 000 x g for 15 minutes at 4°C) after an incubation time of 20 hours at 21°C and disrupted by ultra-sonication on ice in buffer A (Tris 50 mM, KCl 300 mM, pH 8) supplemented with protease inhibitor cocktail (EDTA-free), 1% Triton 100X and 0.2% 2-mercaptoethanol**.** Cells debris were removed by centrifugation at 45,000 x g for 1.5 hours and the protein supernatant was loaded onto a Streptactin high capacity gel (IBA) previously equilibrated with buffer A. The gel was washed with 5 column volumes of buffer A and the PoyD protein was eluted with 6 mL of buffer A containing desthiobiotine (3 mM) and dithiotreitol (DTT, 3 mM). The purified protein was then concentrated with Amicon concentrator (molecular cut-off of 10 kDa), aliquoted and stored at -80°C and the protein purity was assayed on a 12% SDS–PAGE.

**Production of the PoyD mutant proteins**. The $pASK_{17}^+$-Strep-tag-PoyD plasmid served as template for site-directed mutagenesis using this pair of primers:5′-ACA ACC AGC GCT CTG ACC GGC-3′ /5′-GCC GGT CAG AGC GCT GGT TGT-3′ to introduce an alanine at position 372. The triple mutant C149A/C153A/C156A was obtained by two site-directed mutagenesis. First, the C149A mutant was obtained using the $pASK_{17}^+$-Strep-tag-PoyD plasmid as template and the primers: 5'-ACC CGT GGT GCT AGC GTT AAA-3' and 5'-TTT AAC GCT AGC ACC ACG GGT-3. The plasmid $pASK_{17}^+$-Strep-tag-PoyD-C149A was then used as DNA template to introduce alanine mutations at positions 153 and 156 using the primers 5'-CGT GGT GCT AGC GTT AAA GCT TGG TTT GCT GCA CTG-3' and 5'-CAG TGC AGC AAA CCA AGC TTT AAC GCT AGC ACC ACG -3'. Clones were selected on LB agar plate containing ampicillin (100 µg/L) and DNA sequencing was performed to check the sequence of the mutants. The plasmid was then used to transform *E. coli* BL21 (DE3) star cells for protein expression. The expression and purification of all mutant proteins were conducted in similar conditions to the WT protein.

**Enzyme reconstitution**. Reconstitution of the [4Fe-4S] cluster of PoyD and mutants, was achieved in a glove box under strictly anaerobic conditions (< 1 ppm $O_2$). Typically, enzymes were reduced with 3mM DTT for 15 min prior rapid addition of 6 molar equivalent of ammonium iron sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2$) and sodium sulfide ($Na_2S$) followed by overnight incubation at 4°C. Excess unbound iron and sulfur was removed by desalting proteins on Sephadex G25 column against buffer A. Proteins was concentrated with Amicon concentrator and concentration measured on Nanodrop by measuring the absorbance at 280 nm with an extinction coefficient value of 57,870 $M^{-1}.cm^{-1}$.

**Enzyme assays**. Enzyme assays were performed at 25°C under strictly anaerobic conditions in buffer A. Deuterated buffer was obtained by several cycles of freeze-drying in $D_2O$. Otherwise stated, 150 µM PoyD protein (WT or mutants after anaerobic reconstitution), 2 mM SAM and 330 µM substrate were mixed and the reaction initiated by adding 6 mM sodium dithionite (DTN). For enzyme kinetics reactions, 10 µl aliquots were sampled overtime for analysis and analyzed by HPLC.

**Amino-acids enantiomer analysis after L-FDVA derivatization**. After reaction with PoyD, peptides were purified by HPLC and hydrolyzed in DCl (or HCl, 6N) under vacuum conditions at 110°C for 18h. Samples were dried using a centrifugal vacuum concentrator and dissolved in 10 µL MilliQ water. Reaction mixtures were incubated 1 hour at 42 °C after addition of 10 µl $NaHCO_3$ 1M and 25 µL *N*α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA, suspended in 0.5%

acetone). The derivatization reaction was stopped by addition of 10 µL of HCl 2N. The mixture was diluted 1/10 in 20% acetonitrile containing 0.1% formic acid before analysis. A similar protocol was used to analyze the amino acid content of PoyA expressed alone or with PoyD and PoyD mutants.

**HPLC analysis**. An Agilent 1200 series infinity equipped with a reversed phase column (LiChroCART RP-18e 5 µm, Merck Millipore) was used to perform HPLC analysis. Samples were diluted 10-fold in 0.1% trifluoroacetic acid solution. The column was equilibrated with solvents A (H2O, 0.1% TFA) and the solvent B (80 % CH3CN, 19.9 % H2O, 0.1% TFA) was applied as follow: 0-1 min: 0% B; 1-20 min: linear gradient with 1.2% B and 20-40 min: linear gradient with 3% B at a flow rate of 1 min/L. Samples were diluted 10-fold before analysis. Detection was performed with a diode array detector at 257, 278 and 340 nm and by fluorescence (ex/em: 278/350 nm).

**Liquid chromatography - mass spectrometry analysis**. Mass spectrometry analysis was performed using an LTQ standard mass spectrometer (Thermo Fisher Scientific) coupling to a nano-HPLC system (Ultimate 3000, Dionex thermo fisher Scientific) with a nano-electrospray source. Samples were inject onto a Pepmap100 C18 column (0,075 X 150 mm, 100A, 3µm ; Dionex) or a Proswift RP4H polymeric nano-column (0.1 X 250 mm, 1000A, Dionex) (for PoyA), at a flow rate of 0.3 µl/min and 0.45µl/min, respectively. The following buffer system was used: Buffer A: formic Acid 0.1% and buffer B: 80% acetonitrile, 0.1%formic Acid. Several linear gradients were used according the molecules to analyze: 25 to 75% buffer B for peptide analysis; 0 to 20% buffer B for 5'-dA; 30-100% buffer B for L-FDVA derivatives analysis and 0 to 60 % buffer B for PoyA analysis. Mass detection was realized in positive enhanced resolution. The doubly charge ions for peptides 1 to 13 were fragmented at 35% NCE and spectra acquired in profile and enhanced resolution mode to locate deuterium incorporation. For L-FDVA derivatives, we extracted the ion current corresponding to the most intense ion daughter detected in MS/MS spectrum (respectively the transition 398>352 and 370>324 for Val-FDVA and Ala-FDVA were used).

## ASSOCIATED CONTENT

### Supporting Information

Supporting Information document includes experimental procedures, Figures S1-S14 and Table S1-13.
This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

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## ABBREVIATIONS

SAM, S-adenosyl-L-methionine; 5'-dAdo•, 5'-deoxyadenosyl radical. RiPP: Ribosomally-synthesized and post-translationally-modified peptide.

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Table of Contents Artwork