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Enzymatic reconstruction of the saframycin core scaffold defines dual Pictet-Spengler mechanisms

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Abstracts (70 words, 3 sentences)

Saframycin A is a potent antitumor antibiotic having unique pentacyclic tetrahydroisoquinoline scaffold. We found that the single nonribosomal peptide synthetase SfmC catalyzes a seven-step transformation from readily synthesized dipeptidyl substrates with long acyl chains into a complex saframycin scaffold. Based on series of enzymatic reactions, we propose a detailed mechanism involving the reduction of various peptidyl thioesters by an identical R-domain followed by C-domain-mediated Pictet-Spengler reactions in an iterative manner.

Saframycin A (**1**) from *Streptomyces lavendulae* is a representative tetrahydroisoquinoline antibiotic (**Fig. 1**).¹ Members of this family have been isolated from various soil bacteria and marine vertebrates, such as sponges and ascidians,² and exhibit potent antitumor activity derived from iminium ions *via* a carbinolamine moiety and its equivalents. The remarkably potent saframycin analog ecteinascidin 743 (ET-743) has been approved as an anticancer drug for use in patients with soft tissue sarcoma.³ Ecteinascidins share the central pentacyclic core with saframycins, except for the oxidation state of their terminal rings and the additional ten-membered macrolactone bridge found in ET-743. Due to the limited supply from natural sources, the production of ET-743 currently relies on a semisynthetic process⁴ requiring more than 25 transformation steps. Biosynthetic engineering⁵⁻⁷ may be an ideal alternative strategy for generating this important antibiotic from the viewpoint of efficiency and minimal environmental impact. Therefore, we examined the biosynthetic mechanism by which the pentacyclic tetrahydroisoquinoline scaffold is formed in these antitumor antibiotics.

A series of biosynthetic studies on **1** have previously established L-alanine, glycine, and two molecules of the modified amino acid **2**, derived from L-tyrosine, as its biosynthetic precursors.⁸⁻¹⁰ To date, three biosynthetic gene clusters of **1**, safracin B, and saframycin Mx1 have been identified, and analyses using bioinformatics methodologies indicated that the saframycin backbone is constructed by nonribosomal polypeptide synthetases (NRPSs).^{9,11,12} Recent report shows that the saframycin NRPSs (SfmA, SfmB, SfmC) assemble a putative tetrapeptidyl intermediate *via* an unusual biosynthetic

mechanism in the SfmC-catalyzed iterative reaction,¹² and that the subsequent unspecified cyclization reactions with modification enzymes form the pentacyclic skeleton. Although several groups proposed alternative biosynthetic schemes,^{9,12,13} no experimental data have yet been reported regarding biosynthetic intermediates or enzymatic conversions *in vitro*. Therefore, the detailed biosynthetic mechanism of this intriguing transformation remains unclear.

NRPSs are modular enzymes that generally produce a wide range of small molecule polypeptides with established reaction sequence.¹⁴ However, the structure of **1** lacks several carbonyl groups on the peptide backbone. Therefore, textbook knowledge cannot be applied to the saframycin NRPSs, and a novel mechanism must be involved in construction of the unique cyclic system. Here, we report the remarkable reaction mechanism of SfmC, which catalyzes seven transformation steps with sophisticated communication between the individual domains.

Many lipopeptides, such as mycosubtilin, daptomycin, and friulimicin, possess a long fatty acyl chain at their *N*-termini.^{15,16} The characteristics of the NRPSs responsible for producing these lipopeptides include a fatty acid-loading system, such as acyl-CoA ligase (AL), an acyl carrier protein (ACP), and the distinctive *N*-terminal condensation (C) domain of the first module of NRPS.¹⁵ Homologous AL-ACP didomains are present in the gene clusters of **1** (*S. lavendulae*) and saframycin Mx1 (*Myxococcus xanthus*), although their structures lack such fatty acyl chains. Although these domains have previously been overlooked as participating in the reaction pathway, we speculated that this cryptic long acyl chain¹⁷⁻¹⁹ may play a crucial role in saframycin biosynthesis. Based on the organization

of the second and third modules, which include the adenylation (A) domains specific for L-alanine and glycine, respectively, the NRPS-catalyzed reactions of the first three modules should yield an *N*-acyldipeptidyl intermediate **3** (**Fig. 1**). This leaves the last module, corresponding to SfmC, to catalyze the remaining considerable transformation from this intermediate to the pentacyclic skeleton.

To verify this hypothesis, we cloned and overexpressed *sfmC* encoding the last module consisting of the tetradomain C-A-peptidyl carrier protein (PCP)-reduction (R), in *Escherichia coli* as *N*- and *C*-terminal His₆-fusions (**Supplementary Fig. 1a**). Treatment of the purified *apo*-SfmC with recombinant *Bacillus subtilis* 4'-phosphopantetheinyltransferase (PPase) Sfp and coenzyme A provided the *holo*-SfmC. (**Supplementary Fig. 2**). Blue native PAGE analysis indicated that this enzyme existed as a homodimer (**Supplementary Fig. 1b – 1d**). The synthetic putative intermediate analog *N*-myristoyldipeptidyl-*S*-CoA **4b** (**Supplementary Fig. 3**) was subjected to recombinant SfmC-catalyzed conversion in the presence of **2**, ATP, Mg²⁺, and NADPH (**Fig. 2**). In the reaction with **4b**, LC-MS analysis of the reaction products indicated that four new products were generated, including the pentacyclic saframycin-type product **5b**, as well as three incomplete products **7b**, **8b**, and **9b** (**Fig. 2** and **Supplementary Fig. 4**). The structures of **8b** and **9b** were identical to those of the synthetic bicyclic tetrahydroisoquinoline product and the dipeptidylaldehyde (**Supplementary Fig. 4**), whereas **7b** was readily converted into **8b** by treatment with NaBH₄. As **8b** was presumably derived from the corresponding **7b** by enzymatic overreduction, **7b** and **9b** were regarded as biosynthetic intermediates. To unambiguously elucidate the structure of **5b**, we performed an enzymatic reaction with the

proposed intermediate **9b** in place of **4b**. The SfmC-catalyzed conversion with **9b** proceeded efficiently, leading to **5b**, and resulted in a marked acceleration of more than tenfold in comparison with the reaction employing **4b** (Supplementary Fig. 6). We additionally characterized the cyanoadduct **6** – created in a large-scale incubation followed by treatment with KCN – by various spectroscopic methods, including 2D-NMR analysis (Supplementary Fig. 7, Table 2). The relative stereochemistry of **6**, as a proxy for **5b**, which we named *N*-myristoylpresaframycin, was consistent with that of natural **1** (Fig. 1). It should also be noted that use of the coenzyme NADH significantly improved the production of **5b** up to a 23% overall yield based on **9b**, while the reaction utilizing NADPH resulted in the predominant formation of **8b** over **5b** (**5b**:**8b** = 1:2) (Supplementary Fig. 6).

Based on the results of the SfmC-catalyzed reaction, we proposed a plausible mechanism for assembly of the pentacyclic tetrahydroisoquinoline skeleton from **4b** (Fig. 1). The detection of **7b** and **9b** indicated that the pentacyclic skeleton is assembled in a stepwise manner. The growing polypeptide chains appended to PCP3 on SfmB and to PCP4 on SfmC could interact with the R-domain, which reductively releases **5b**, **7b**, **9b** and **10** as in the case of pikromycin thioesterase (Pik-TE) domain.²⁰ This mechanism explains not only the unusual reduction of the *N*-acyldipeptidyl intermediate but also the iterative use of the last module. Interestingly, this biosynthetic mechanism is closely related to that in the total synthesis reported by Myers and Kung.²¹

We further examined the role of the acyl chain with a series of synthetic precursors **4a-e** and **9a-c**. In contrast to the result with **4b**, no reduction or cyclization

products were observed in the case of the *N*-acetyl and the non-acylated CoAs **4d**, **4e**. In the experiments with CoA-esters and NADPH (3 mM), **4a** and **4b** with longer acyl chains were efficiently converted into **5a** and **5b** but the product ratios – and especially the amounts of overreduction products – were changed by the use of different substrates (**Supplementary Figs. 4, 8 and 9**). These products were not observed in the experiments of **4a** – **4c** with NADH. Similar results were obtained in the reactions with dipeptidylaldehydes (**Supplementary Fig. 10**). Interestingly, the reaction with *N*-lauroylpeptidylaldehyde **9c** gave products **5c**, **7c** and **8c** although the corresponding CoA-ester **4c** did not, indicating that the acyl chain is important for the substrate recognition of the first reduction (**Supplementary Figs. 9 and 10**).

To probe the stereospecificity of the R-domain reaction, the SfmC-catalyzed reactions in the presence of (*S*)- and (*R*)-[4-²H]NADPH were performed and analyzed by LC-MS. Only the experiment with (*S*)-[4-²H]NADPH showed the 3 Da-shifted fragment ion of **5b** thus obtained, as compared with the corresponding ion from the non-labeled **5b** (**Supplementary Fig. 11**), indicating that the deuterons on the three sp³ carbon centers of **5b** are introduced (**Fig. 1**). The combined experimental evidence thus shows that the SfmC R-domain has unusually broad substrate specificity and mediates the reduction of three distinct thioesters bound to PCPs into the corresponding aldehydes, yet maintains enough specificity to discriminate between the long acyl chains of **4a** and **4b** as compared to **4d** or **4e**. The full description of how this cryptic longer acyl chain influences substrate recognition remains to be seen.

To better understand the cofactor preferences of the reaction, we performed the

SfmC reaction in several time course experiments using either NADH or NADPH at different concentrations. A recent report on absolute metabolite concentrations in *Escherichia coli* showed that intracellular concentrations of NADPH and NADH are 120 and 86 μM , respectively.²² At the plausible physiological NADPH concentration (200 μM), the reaction with afforded **5b** as a predominant product while **5b** production was suppressed and accompanied with aberrant by-product formation at 2 mM NADPH (**Supplementary Fig. 12**). In the experiment with NADH (200 μM), **5b** formation was below 1 μM while at the high concentration (2 mM) formation of **5b** was increased to 18 μM (**Supplementary Fig. 12**). In addition, LC-MS data of the products in the competition experiments with *S*-[4-²H]NADPH and NADH (100 μM each) showed shifted molecular ion peaks at *m/z* 551.5 for **7b**, 707.6 for **5b** and 536.5 for **8b** while the products in the experiments with NADPH and *S*-[4-²H]NADH (100 μM each) gave no shifted peak, showing that SfmC uses NADPH preferentially (**Supplementary Fig. 13**). However, at high concentration (1 mM each) both labeled cofactors gave shifted peaks of all products, indicating that both cofactors were used with nearly equal efficiency. This suggests that efficient production is artificial event.

Finally, to determine the domain responsible for mediating the Pictet-Spengler reaction, we prepared two mutants (C-domain deletion: A-PCP-R, R-domain deletion: C-A-PCP) of SfmC (**Supplementary Fig. 1**). The enzymatic reaction of *N*-myristoyldipeptidyl-CoA **4b** with the C-domain deletion mutant simply yielded **9b**, but no cyclization product (**Supplementary Fig. 14**). Moreover, the reaction of **9b** with the R-domain deletion mutant, followed by reduction with NaBH₄, gave **8b** (**Supplementary**

Fig. 15), whereas none of this product was obtained in the reaction of **9b** with the C-domain deletion mutant. These observations indicated that this single-turnover reaction with the R-domain deletion mutant generates the Pictet-Spengler product bound to the PCP4 domain, which is reductively released with NaBH₄. Therefore, we conclude that the intriguing Pictet-Spengler reaction is catalyzed by the C-domain, designated here as the “PS-domain.” Additional function of C-domain is known in a Cy-domain which catalyzes similar multistep transformation including peptide bond formation and heterocycle formation between amide carbonyl and adjacent OH or SH group.²³ Sequence alignment and phylogenetic analyses of PS-domains showed modest identity (13% – 25%) to other C- and Cy-domains (**Supplementary Figs. 16 and 17**). Among the core consensus sequences of the C-domain, the highly conserved motif HHxxxDG in core 3 which plays important role for catalysis of condensation,²³ is replaced with the motif (HxxxxD) in the PS-domains, suggesting that this difference may be significant for the Pictet-Spengler reaction.

In conclusion, we have elucidated the detailed biosynthetic mechanism involved in the construction of saframycin pentacyclic tetrahydroisoquinoline scaffold, which involve sophisticated communication of the SfmC domains as well as the cryptic roles of the fatty acyl chain. The successful production of **5b** with SfmC from simple starting materials would set the stage for chemoenzymatic synthesis generating natural product analogs, including advanced intermediates in the semisynthesis of ecteinascidin 743.⁴

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AUTHOR CONTRIBUTIONS

H. Oikawa and K.W. designed the research, and H. Oikawa wrote the manuscript. K.K. and H.S. synthesized the substrates for the enzymatic reactions. K.W. cloned the *sfmC* gene and optimized the expression conditions. K.K. prepared the deletion enzymes and performed all enzymatic reactions. H. Oguri advised on the research and made a significant contribution to a proposal of the reaction mechanism.

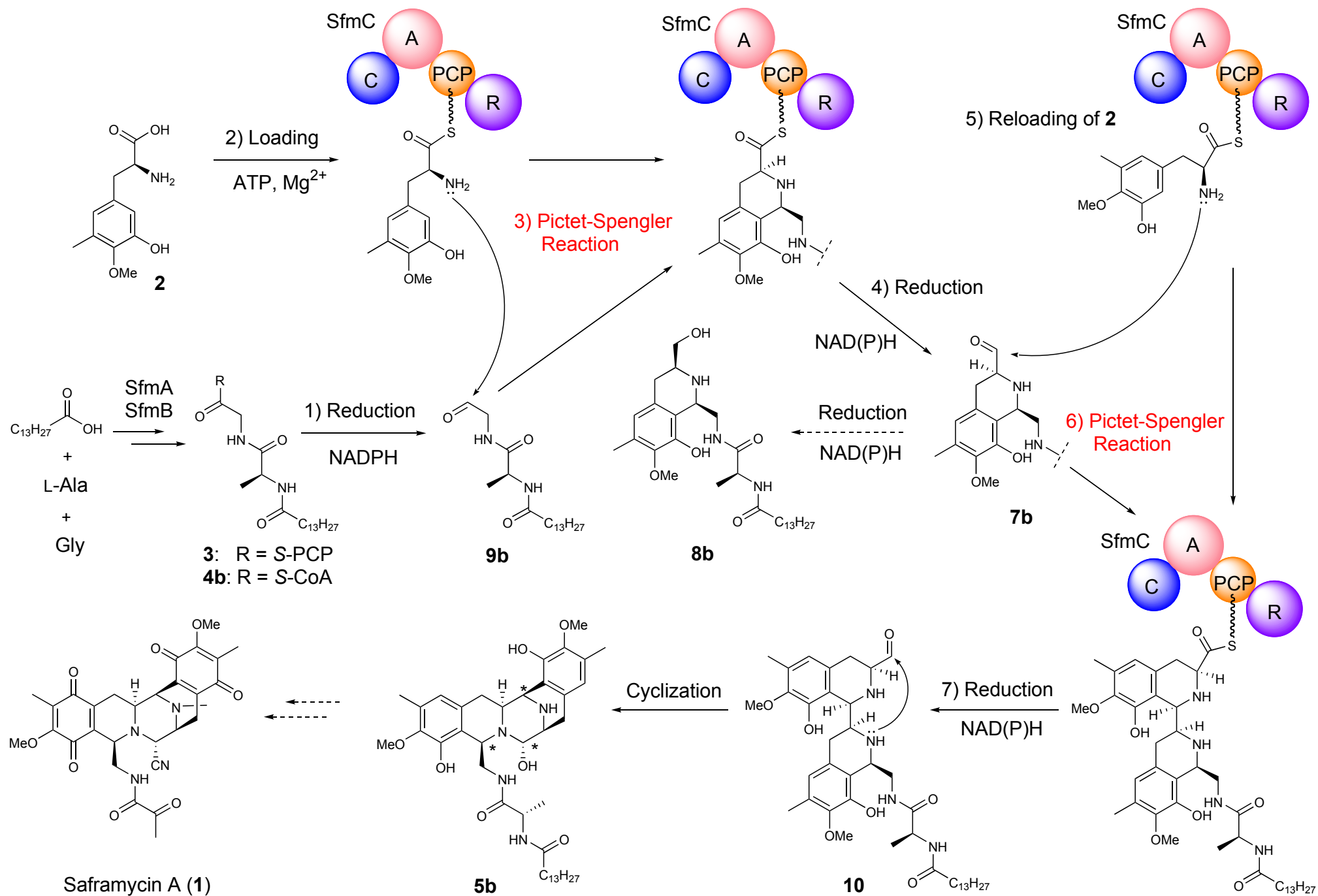
Competing interest statement. The authors declare no competing financial interests.

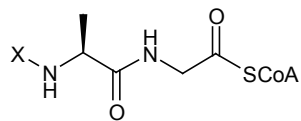
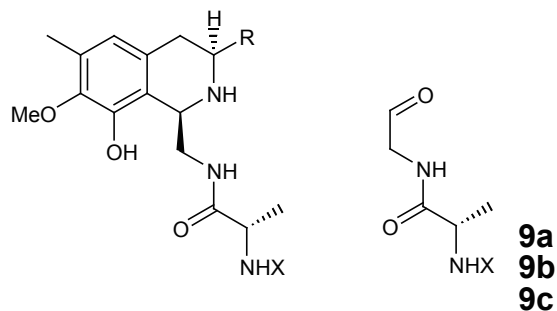
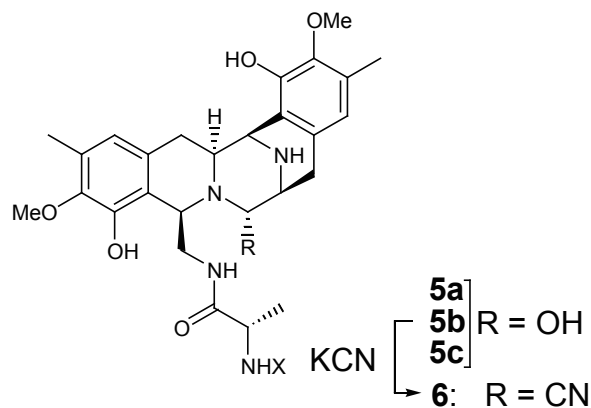
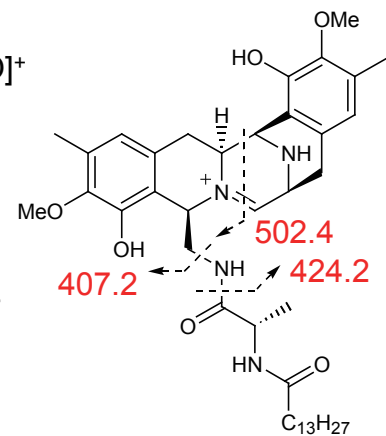
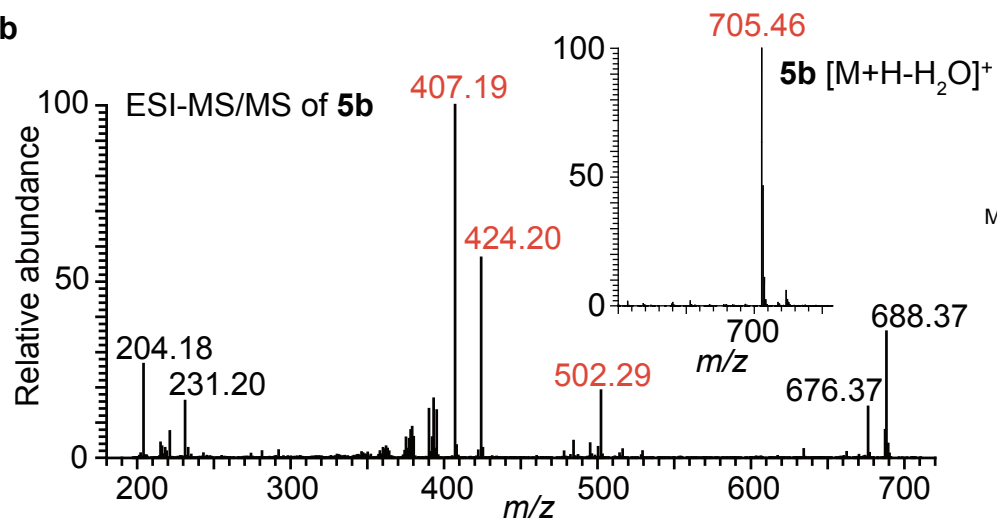
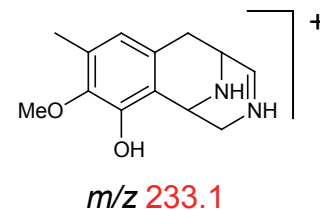
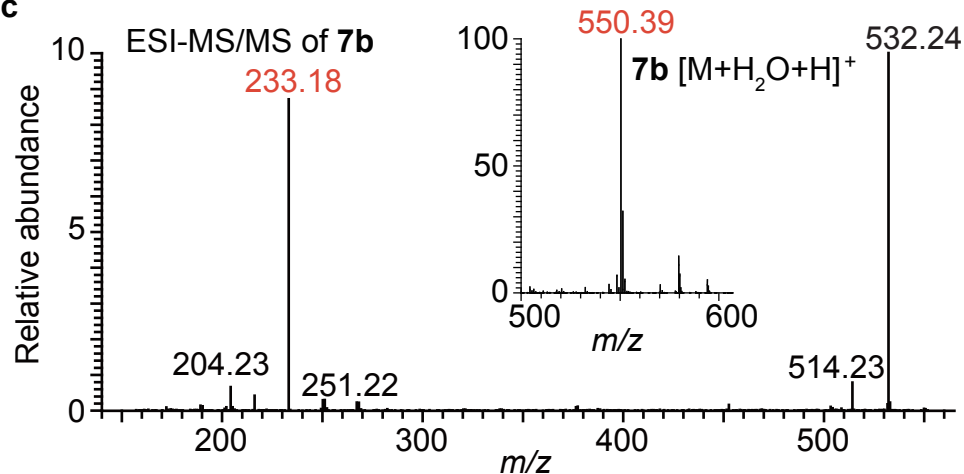
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Figure 1. Proposed overall scheme for the pentacyclic core skeleton assembly. The reactions proceed in the following sequence: (1) The 1st reduction of the thioester **4b**. (2) The 1st loading of **2**. (3) The 1st Pictet-Spengler reaction. (4) The 2nd reduction of the thioester intermediate. (5) The 2nd loading of **2**. (6) The 2nd Pictet-Spengler reaction. (7) The 3rd reduction of the thioester intermediate. The asterisks (*) in **5b** indicate the

deuterated positions in the experiment with (*S*)-[4-²H]NADPH.

Figure 2. (125 words) SfmC-catalyzed reaction with the various dipeptidyl-CoA substrates **4a – 4e**. **(a)** The scheme depicts the SfmC-catalyzed conversion of **4b** and **2** to the pentacyclic skeleton **5b**, the bicyclic aldehyde **7b**, the bicyclic alcohol **8b** and the *N*-myristoyldipeptidylaldehyde **9b**. **(b)** ESI-MS (inset) and ESI-MS/MS spectra of **5b**. **(c)** ESI-MS (inset) and ESI-MS/MS spectra of **7b**. **(d)** ESI-MS (inset) and ESI-MS/MS spectra of **8b**.



a**4a:** X = Palmitoyl ($\text{C}_{15}\text{H}_{31}-\text{CO}-$)**4b:** X = Myristoyl ($\text{C}_{13}\text{H}_{27}-\text{CO}-$)**4c:** X = Lauroyl ($\text{C}_{11}\text{H}_{23}-\text{CO}-$)**4d:** X = Acetyl ($\text{C}_1\text{H}_3-\text{CO}-$)**4e:** X = HSfmC, tyrosine derivative **2**ATP, Mg^{2+} , NADPH**b****c****d**