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Insights into the Biosynthesis of Dehydroalanines in Goadsporin

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Dehydroalanines in goadsporin are proposed to be formed by GodF and GodG, which show slight homology to the N-terminal glutamylation and C-terminal elimination domains, respectively, of LanB, a class I lanthipeptide dehydratase. Although similar, separated-type LanBs are conserved among thiopeptides and indispensable for their biosynthesis and biological activities, these enzymes had not yet been characterized. Here, we identified goadsporin B, which has unmodified Ser4 and Ser14, from both *godF* and *godG* disruptants. The *godG* disruptant also produced goadsporin C, a glutamylated-Ser4 variant of goadsporin B. These results suggested that dehydroalanines are formed by glutamylation and glutamate elimination. NMR analysis revealed for the first time that the glutamyl group was attached to a serine via an ester bond, by the catalysis of LanB-type enzymes. Our findings provide insights into the function of separated-type LanBs involved in the biosynthesis of goadsporin and thiopeptides.

Dehydroamino acids are often found in ribosomally synthesized, post-translationally modified peptides (RiPPs), such as lanthipeptides (e.g., nisin A, Scheme 1) and thiopeptides (e.g., nosiheptide, Scheme 1).[1] These non-proteinogenic amino acids are required for lanthionine formation in lanthipeptides and macrocyclization in thiopeptides, and to confer antibacterial activity on these compounds, and therefore their biosynthetic mechanism is of interest. Goadsporin (**1**, Scheme 1), a linear azole-containing peptide produced by *Streptomyces* sp. TP-A0584, is another RiPP decorated with dehydroamino acids.[2] Recently, NisB, a LanB (lanthionine biosynthetic enzyme B) involved in nisin biosynthesis, was biochemically and structurally characterized.[3] In the NisB-catalyzed reaction, the N-terminal domain catalyzes $tRNA^{Glu}$-dependent glutamylation of serines and threonines, and the C-terminal domain eliminates glutamate to form dehydroamino acids. In the biosynthetic gene clusters for **1**[4] and thiopeptides,[5] these N- and C-terminal domains of LanBs are encoded in two genes; in the case of **1**, these are *godF* and *godG* (Figure S1 and S2 A in the Supporting Information). However, the functions of these two enzymes (here called "separated-type LanBs") have not yet been determined. This is partly because inactivation of the corresponding genes abolishes production. For example, Liao et al. disrupted *tsrJ* (also known as *tsrC*)[6] and *tclE*, genes encoding separated-type LanBs involved in the biosynthesis of thiostrepton and thiocillin; however, metabolites related to thiopeptides were not identified in the disruptants.[7] Similar results were obtained in studies on nosiheptide[8] and lactazoles.[9] In addition, the relatively weak sequence similarity of GodF and GodG with known LanBs prevented us accurately predicting the functions of these enzymes (Figure S2 B and C).

In this study, in-frame deletions of *godD*, *godE*, *godF*, and *godG* were performed by a double crossover method in the chromosome of *Streptomyces lividans* TK23/pGSBC1, a recombinant strain that includes all of the biosynthetic genes for **1** (Figure S3).[4] In disruptants of *godD* and *godE*, which might be responsible for azole formation, the production of **1** was completely abolished, and compounds related to **1** were not detected (Figure 1). However, disruptants of *godF* and *godG*, which might be responsible for dehydroalanine formation, did not produce **1** but did accumulate **2** (Figure 1). In LC-MS analysis, this compound eluted at 16.7 min (representing *m/z* 1649 $[M+H]^+$). Therefore, its molecular mass was 36 Da greater than that of **1**. GodF and GodG were predicted to be LanB-like dehydratases, and thus this difference was attributed to two intact serine residues (Ser4 and Ser14) that avoided dehydration. In addition, we found **3** (*m/z* 1778 $[M+H]^+$) in the *godG* disruptant, although in smaller amounts. Its mass is greater than that of **1** by 129 Da, which corresponds to the addition of a glutamate, thus suggesting that glutamylation occurs in the biosynthesis of **1**, as in class I lanthipeptides.

For elucidation of the structure, we isolated 26.9 mg of the compound with *m/z* 1649 $[M+H]^+$ from a 20 L culture broth of the Δ*godF* mutant. The purified compound showed *m/z* 1646.6731 $[M-H]^-$ in HR-ESI-MS, thus indicating that the molecular formula was $C_{72}H_{101}N_{19}O_{22}S_2$ (calcd: *m/z* 1646.6737). In the ^{13}C NMR spectrum, the signals at 109.20 ppm and 108.02 ppm,[10] which are derived from the two β-carbons of two dehydroalanines (Dha4 and Dha14), were missing. Instead, new signals at 61.53 ppm (Ser4) and 61.60 ppm (Ser14) were observed when compared with the ^{13}C NMR spectrum of **1**. In the HSQC spectrum, these carbon signals correlated to proton signals at 3.74 and 3.68 ppm, thus suggesting the presence of two serine residues (NMR spectra, key correlations in 2D NMR, and chemical shifts in Figure S4–S9, S18, and Table S1). We also

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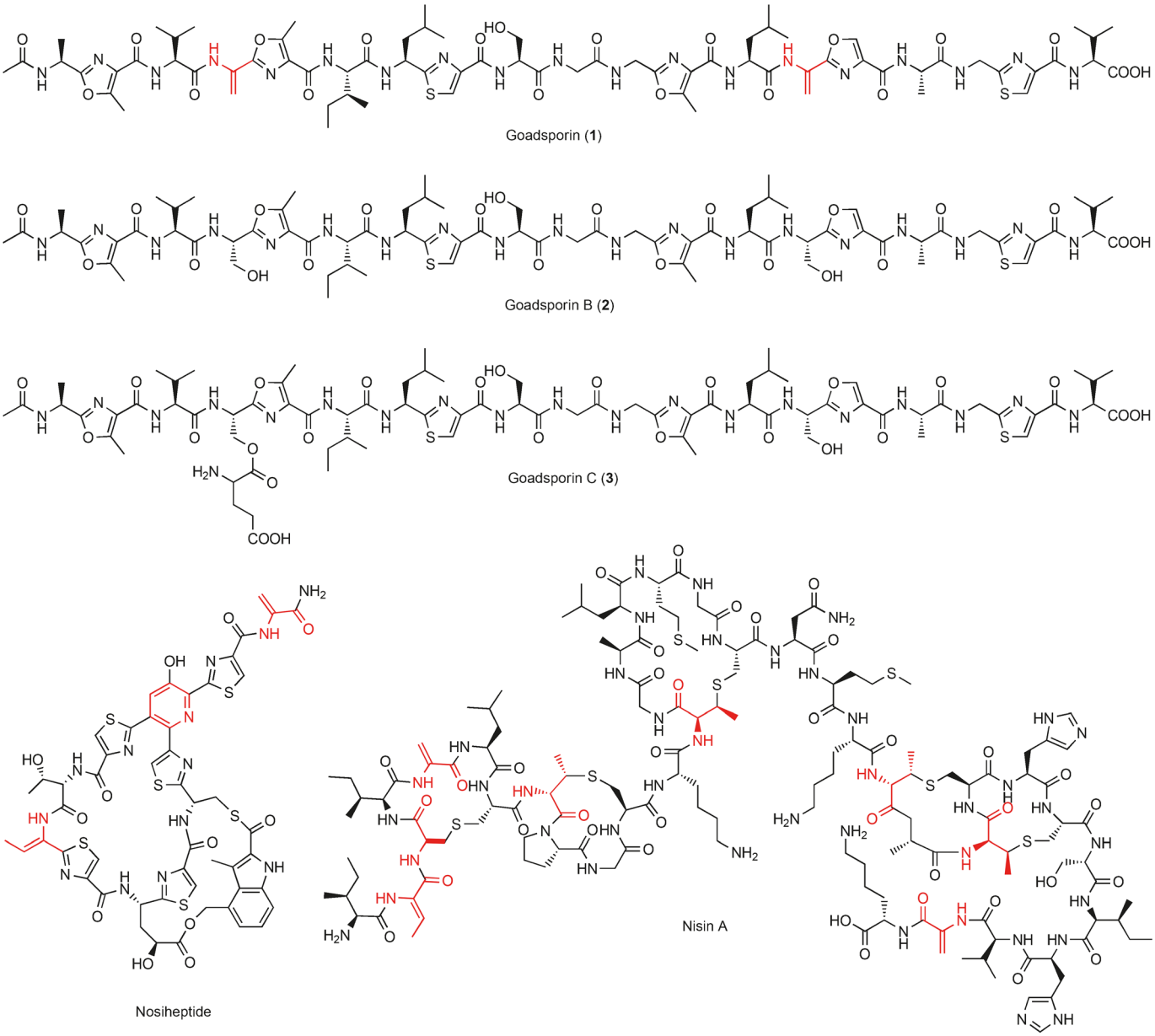


Scheme 1. Goadsporin, its derivatives isolated in this study, and natural products containing dehydroamino acids. Dehydroamino acids and structures derived from dehydroamino acids are shown in red.

compared the MS/MS spectrum of the isolated compound with that of **1** to confirm the structure (Figure 1B, 1C, and S19). In MS/MS analysis, the y16 fragment that showed *m/z* 1319 $[M+H]^+$ in **1** shifted to *m/z* 1355 $[M+H]^+$ in the isolated compound, thus indicating the addition of two water molecules in the region between Ser4 and Val19. In addition, an 18 Da shift of b13 and y6 indicated that the water molecules were at the regions between Ser4 and Leu13 and between Ser14 and Val19, respectively (Figures 1B and C, S19). Based on these observations, Ser4 and Ser14 were found to be unmodified. We term this compound "goadsporin B" (**2**, Scheme 1). As the two serine residues were unmodified in the structure of the isolated compound, the involvement of GodF and GodG in the dehydration of these residues was confirmed.

We then carried out bioconversion of **2** in the *godA* disruptant of strain TP-A0584,[4] which is deficient in the production of the precursor peptide but whose post-translational modification steps are intact (Table S2). If **2** is an intermediate in the biosynthesis of **1**, it will be converted into **1** in a *godA* disruptant. However, production of **1** was not restored, and **2** was still detected in the culture broth (Figure S20). As previously reported, the leader peptide of the precursor is required for substrate recognition by the N-terminal region of NisB.[3] Although incorporation efficiency should be considered, the absence of the leader peptide in **2** would explain why this compound was not transformed into **1** by the Δ*godA* mutant. Thus, we conclude that **2** is not an intermediate, but a product that skipped the steps of dehydroalanine formation in the biosynthesis of **1**.

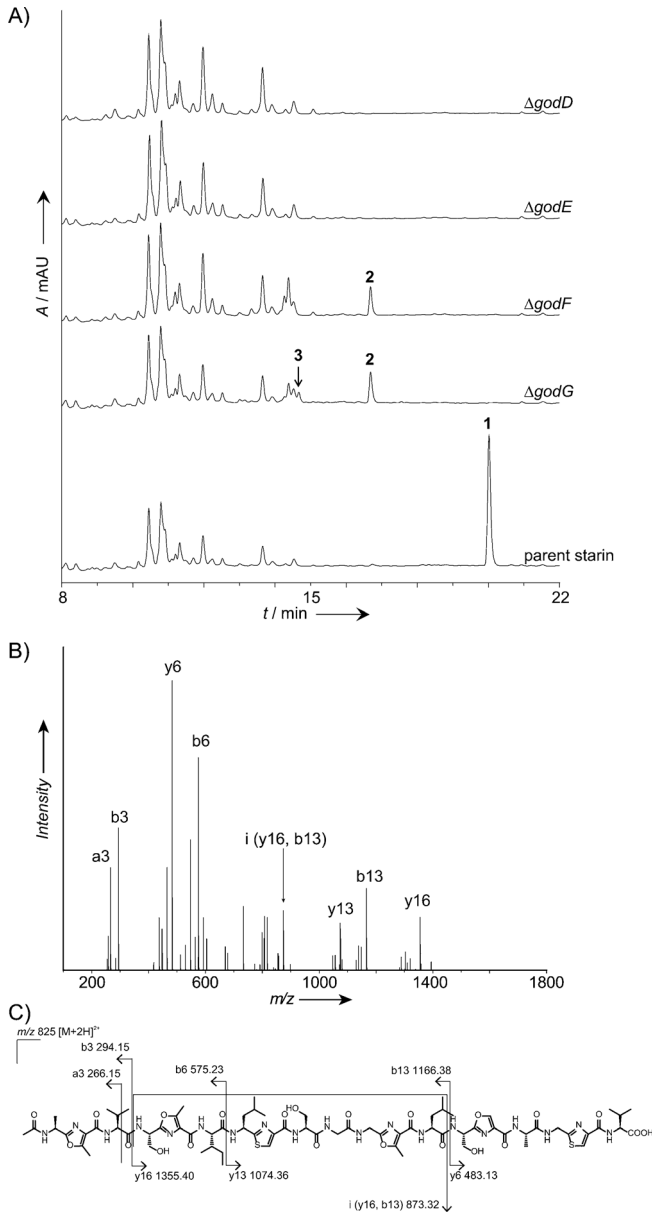


Figure 1. A) HPLC chromatograms of *S. lividans*/pGSBC1 and gene disruptants. Chromatograms were recorded at 254 nm. B) MS/MS spectrum of the signal representing *m/z* 825 $[M+2H]^{2+}$ derived from compound **2**. Identified a, b, and y fragments are labeled in the spectrum. The label, i (y16, b13) indicated the internal peptide fragment derived from fragments y16 and b13. C) Fragment assignment for **2**.

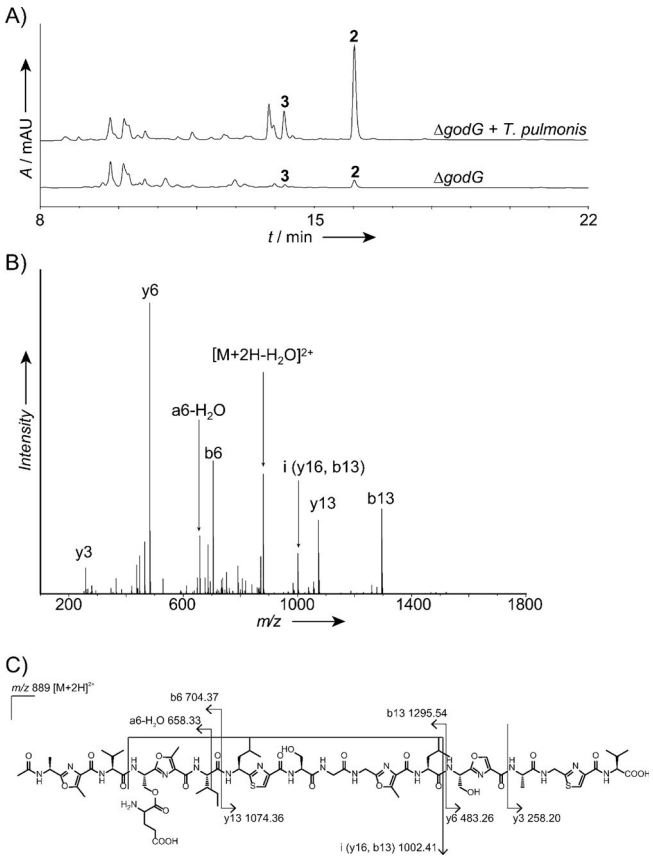


Figure 2. A) HPLC chromatograms of the pure culture of Δ*godG* strain and the combined-culture with *T. pulmonis*. Chromatograms were recorded at 254 nm. B) MS/MS spectrum of the signal representing *m/z* 889 $[M+2H]^{2+}$ derived from compound **3**. Identified a, b, and y fragments are labeled in the spectrum. The label, i (y16, b13) indicates the internal peptide fragment derived from fragments y16 and b13. C) Fragment assignment for **3**.

In addition, as **2** did not exhibit the antibiotic and morphogenic activities known for **1** (Figure S21), the two dehydroalanines are concluded to be important for the biological activity.

The *godG* disruptant produced compound **3** (*m/z* 1778 $[M+H]^+$; 14.7 min; Figure 1), albeit in trace amounts. As NisB glutamylates serine and threonine residues, this compound was expected to be a mono-glutamylated derivative of **2**. Based on MS/MS fragmentation analysis, glutamylation of Ser4 was indicated by the 129 Da shift of the b6 fragment (Figure 2B, 2C). A glutamylated-Ser14 product was not detected. A signal corresponding to a Ser4,Ser14-diglutamylated product (*m/z* 954 $[M+2H]^{2+}$) was detected, but the intensity was much lower than that of **3** (data not shown). This suggests that formation of Dha4 was required for the second glutamylation at Ser14. Although production was low in pure culture, it was significantly increased by co-culturing with *Tsukamurella pulmonis*, which we termed the method "combined-culture"[11] (Figure 2A), thus illustrating the effectiveness of this method for the isolation of a trace amount of metabolites. Approximately 5 mg of glutamylated-Ser4 goadsporin B was obtained from a 3 L culture; this compound is termed "goadsporin C" (**3**, Scheme 1). The purified compound showed *m/z* 1775.7157 $[M-H]^-$ in HR-ESI-MS, thus indicating that the molecular formula is $C_{77}H_{108}N_{20}O_{25}S_2$ (calcd: *m/z* 1775.7163). NMR analysis clearly indicated the structure shown in Scheme 1 (spectra in Figure S10–S17, Table S1). The ^{1}H NMR spectrum of the isolated compound was quite similar to that of **2**. However, additional signals at 1.90–2.00, 2.30–2.40, and 4.03 ppm in ^{1}H NMR and the corresponding signals in ^{13}C NMR suggest the presence of a glutamate. Notably in the HMBC experiment, β-protons of Ser4 weakly correlated to the α-carboxyl group of glutamate, which was newly attached (Figure S16–S18). Furthermore, in NOESY analysis, α- and β-protons of Ser4 weakly correlated to the α-proton of the glutamate (Figures S13, S14, and S18). The chemical shifts of the β-protons of Ser4 (3.74 and 3.68 ppm in

2) shifted to 4.59 and 4.42 ppm, which are typical for methylene protons adjacent to the ester bond (Table S1). These observations suggested that the glutamate is linked via an ester bond to the α-carboxyl moiety. To the best of our knowledge, this is the first elucidation of the position and connection of glutamylation catalyzed by LanB-type enzymes.

Finally, an advanced Marfey's method[12] was performed to determine the absolute configurations of **2** and **3**. By comparing the L- and DL-1-fluoro-2,4-dinitrophenyl-5-leuciamide (FDLA) derivatives of hydrolysates by LC-MS, Ala1, Val3, Ile6, Ser9, Leu13, Ala16, and Val19 in **2** and **3** were assigned to the L configuration (Figure 3). As L-Leu-Thz (thiazole) is known to elute earlier than D-Leu-Thz, Leu7 was determined to be in the L configuration.[10] In addition, Ser4 and Ser14 in both compounds were determined to be in the L configurations, because the amino acid adjacent to oxazole is converted into a normal amino acid in hydrolysis by hydrochloric acid.[13] However, an additional glutamate residue in **3** was found to be a mixture of L and D configurations (Figure 3B, see chromatogram extracted at *m/z* 442). Corresponding to this observation, the signals derived from Ser4 and glutamate were doubled in the ^{1}H NMR spectrum of **3**. As tRNA^{Glu} was proposed as a glutamate donor, the glutamate residue would originally be transferred in the L configuration, and racemization might occur during fermentation or purification. Nevertheless, we identified and structurally elucidated for the first time the metabolites that indicate the function of separated-type LanBs.

In summary, we performed in-frame deletion of four goadsporin biosynthetic genes in a heterologous host to characterize the pathway. Compound **2** and its glutamylated derivative **3** were identified from *godF* and *godG* disruptants, respectively. Structural elucidation included the absolute configuration. Although tRNA^{Glu}-dependent glutamylation catalyzed by NisB had already been reported, instability of the product prevented determination of the glutamylated position by tandem MS.[14] Our NMR and MS/MS analyses of **3** clearly indicate that the glutamate is attached at the serine residue for conversion into dehydroalanine. Furthermore, our findings reveal for the first time that the glutamate is attached via an ester bond. This result supports the proposed tRNA^{Glu}-dependent catalysis, where the α-carboxyl group of glutamate is activated by tRNA (Scheme 2). Because inactivation of azole-forming enzymes (GodD and GodE) did not result in the production of related metabolites, azoles are likely required for dehydroalanine formation (Scheme 2). Similarly to the biosynthesis of **1**, separated-type LanBs are widely found in the biosynthesis of structurally related thiopeptides. Therefore, our findings not only provide insights into the biosynthesis of **1**, but also suggest the universal use of glutamate in dehydroamino acid biosynthesis and aid an understanding of the biosynthesis of macrocyclic thiopeptides. Further in vitro study will reveal in more detail the biosynthetic mechanism for RiPPs highly decorated with both azoles and dehydroamino acids.

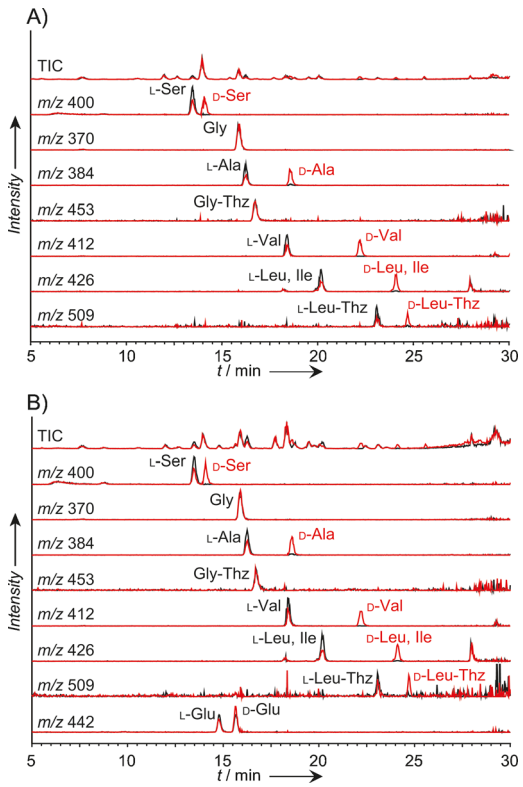
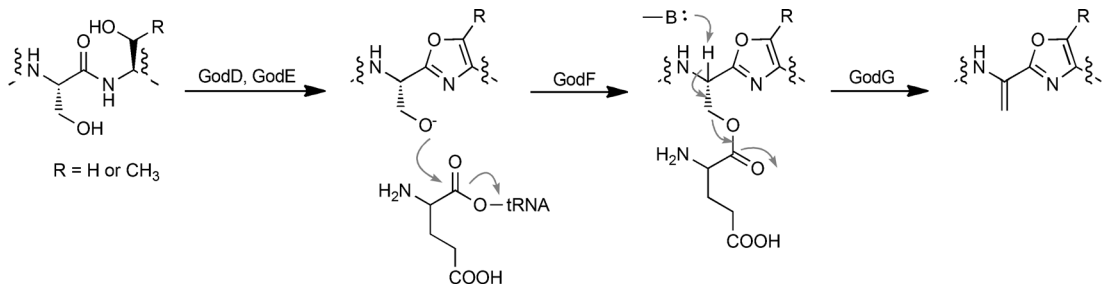


Figure 3. A) Mass chromatograms of the L- (——) and DL-FDLA (——) derivatives of hydrolysate of 2 by LC-MS in ESI positive mode. B) Mass chromatograms of the L- (——) and DL-FDLA (——) derivatives of hydrolysate of **3** by LC-MS in ESI positive mode. Elution profiles of L- and DL-FDLA derivatives of proteinogenic amino acids in **2** and **3** were compared with those of authentic samples (data not shown). TIC: total ion current; Thz: thiazole.



Scheme 2. Proposed pathway of azole and dehydroalanine biosynthesis.

Experimental Section

Disruption of goadsporin (1) biosynthetic genes: Strains, plasmids, and primers are listed in Table S2. *S. lividans* TK23/pGSBC1 was the parental strain for gene disruption. In-frame deletions of *godD*, *godE*, *godF*, and *godG* were performed by the double crossover method.[15] Gene regions including 2-kb upstream and downstream were amplified by PCR (primers in Table S2). The resulting DNA fragments were digested and cloned into the HindIII-XbaI and XbaI-EcoRI sites of vector pK18mob[16] to give plasmids pDgodD, pDgodE, pDgodF, and pDgodG. Gene disruption was performed as previously reported.[17] As previous annotations for *godD* (accession BAE46919) and *godG* (BAE46922) were proposed to be incorrect by recent bioinformatics analyses, we deposited new annotations for these genes at DDBJ.

Purification of compound 2: The Δ*godF* strain was cultivated in V-22 medium (soluble starch (1.0%), glucose (0.5%), NZ case (0.3%; Wako Pure Chemical), yeast extract (0.2%; Difco), Bacto Tryptone (0.5%), K_2HPO_4 (0.1%), $MgSO_4{\cdot}7H_2O$ (0.05%), and $CaCO_3$ (0.3%), pH 7.0) at 30 °C for 2 days. After cultivation, an aliquot (3 mL) was inoculated into A-3M production medium (100 mL; glucose (0.5%), glycerol (2.0%), soluble starch (2.0%), pharmamedia (1.5%; ADM, Chicago, IL), yeast extract (0.3%), and Diaion HP-20 adsorbent (1.0%; Mitsubishi Chemical), pH 7.0) in a 500 mL K1 flask and cultivated at 30 °C for 7 days. The A-3M medium (20 L) was extracted with an equal volume of *n*-butanol. The organic fraction was recovered to obtain 5.0 g of crude sample, which was then dissolved in ethanol. The soluble fraction was recovered and concentrated to obtain 3.5 g of crude sample, which was dissolved in methanol (60%) and extracted by dichloromethane. The dichloromethane fraction was recovered and dried. The resulting 2.9 g was dissolved in methanol (90%) and washed with *n*-hexane. The methanol fraction was then dried to obtain 0.71 g of extract. This sample was dissolved in a acetonitrile (50%)/formic acid (2%). The sample was subjected to LH-20 column chromatography and developed by using the same solution. Fractions containing **2** were collected and dried to obtain 0.24 g of sample. This fraction was subjected to octadecylsilyl column chromatography. The column was washed with acetonitrile (5, 15, 25, 40, 55, and 70%) containing formic acid (2%). The 40% fraction contained 84 mg of partially purified sample. This fraction was further purified by preparative HPLC with a Cosmosil $5C_{18}$ AR-II column (5 μm, 20×250 mm; Nacalai Tesque, Kyoto, Japan) and acetonitrile and formic acid (0.1%) as eluents (flow rate, 15 mL min^{-1}): 37% acetonitrile for 12 min, linear increase to 75% over 3 min, linear decrease to 37% over 6 min. The purified compound (26.9 mg) was subjected to spectral analysis for structural elucidation.

Purification of the compound 3: The Δ*godG* strain and the bacterium *T. pulmonis* (containing mycolic acid) were cultivated in V-22 medium at 30 °C for 2 days. Aliquots of the Δ*godG* culture (3 mL) and the *T. pulmonis* culture (1 mL) were inoculated into A-3M production medium (30×100 mL) in 500 mL K1 flasks and cultivated at 30 °C for 6 days. The medium was then extracted with an equal volume of *n*-butanol. The organic fraction (9 g of crude sample) was resuspended in water and washed with *n*-hexane. The aqueous fraction was recovered, washed with an equal volume of ethyl acetate, and lyophilized. This crude sample was dissolved in chloroform/methanol (10:1), and subjected to LH-20 column chromatography and developed by using the same solution. Fractions containing **3** were collected and dried, then subjected to a Cosmosil $75C_{18}$-PREP column (4.5×18 cm). The column was washed with acetonitrile (20, 30, 40, and 50%), and the 40% fraction was collected and lyophilized. The resulting powder was further purified by semi-preparative HPLC with a Cosmosil $5C_{18}$ AR-II column (5 μm, 10×250 mm). The column was eluted using 68% of 0.1% trifluoroacetic acid over 32% of acetonitrile (flow rate, 3.0 mL min^{-1}; column temperature, 40 °C; monitored at 254 nm).

Spectral analysis of isolated compounds: NMR spectra of **2** were obtained on an Avance 500 (Bruker) at 500 MHz (^{1}H) or 125 MHz (^{13}C). NMR spectra of **3** were obtained on an ECA-600 (JEOL) at 600 MHz (^{1}H) or 150 MHz (^{13}C). HR-ESI-MS data were collected on a micrOTOF spectrometer (Bruker) in negative mode. MS/MS analyses were performed on an amaZon SL ion-trap instrument (Bruker) equipped with a 1200 series HPLC system (Agilent Technologies) operating in MS/MS mode with positive ESI. Divalent parent ions were selected for each compound.

LC-MS: An amaZon SL ion-trap MS system was connected to a 1200 series HPLC system with a Cosmosil $5C_{18}$ AR-II column (5 μm, 2.0×150 mm) and acetonitrile and formic acid (0.1%) as eluents (column temperature 40 °C; flow rate 0.3 mL min^{-1}; 5% acetonitrile for 2 min, linear increase to 95% over 25 min, 95% for 5 min. MS and MS/MS analysis were performed with ESI in positive mode. Chromatograms were also monitored at 254 nm.

Procedure for the advanced Marfey's method: Compound **2** (1.8 mg) or **3** (0.8 mg) was hydrolyzed in HCl (500 μL, 6 N) at 100 °C for 1 h. The resulting hydrolysates were dried in vacuo, dissolved in water (100 μL), and separated into two fractions. $NaHCO_3$ (20 μL, 1 M) was added to each, then, L- or D-FDLA (100 μL, 0.1% in acetone) was added to the mixture for derivatization. After incubation at 40 °C for 1 h, the reactions were quenched by HCl (20 μL, 1 N) and diluted in acetonitrile (260 μL). Samples (1 μL) were injected into the LC-MS and column as described above (eluents: acetonitrile and formic acid (0.1%); column temperature 40 °C; flow rate 0.3 mL min^{-1}; 25% acetonitrile for 4 min, linear increase to 55% over 15 min, increase to 100% over 6 min, 100% for 8 min).

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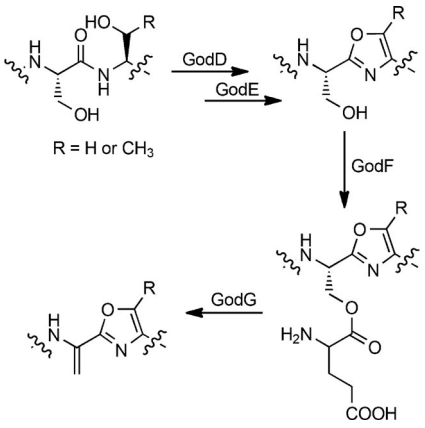
A clear route: The pathway of goadsporin biosynthesis is proposed following inactivation of the genes for azole and dehydroalanine formation. Notably, this study provides an insight into the structure of the glutamylated intermediate in the catalysis of LanB-type enzymes.



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