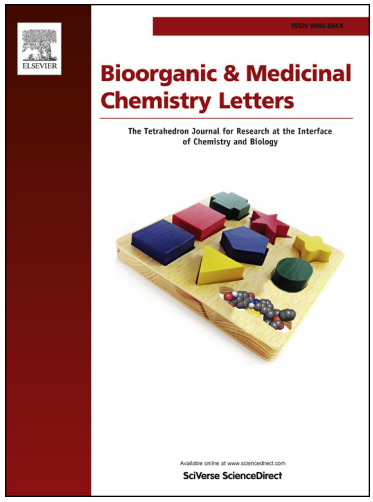

Chemical Structure and Biological Activity of a Quorum Sensing Pheromone from *Bacillus subtilis* subsp. *natto*

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Chemical Structure and Biological Activity of a Quorum Sensing Pheromone from *Bacillus subtilis* subsp. *natto*

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Key words: *Bacillus subtilis*; *natto*; posttranslational modification; quorum sensing.

Abstract

Bacillus subtilis subsp. *natto* secrets a peptide pheromone, named $ComX_{natto}$ pheromone, as an inducer for biofilm formation containing poly-γ-glutamic acid. Recently, the $ComX_{natto}$ pheromone was identified to be a hexapeptide with an amino acid sequence of Lys-Trp-Pro-Pro-Ile-Glu, and the tryptophan residue was post-translationally modified with a farnesyl group. In order to determine the precise modification of the tryptophan residue, $ComX_{natto}$ pheromone was synthesized using solid-phase peptide synthesis. Biological activity of the $ComX_{natto}$ pheromone was then investigated. It was demonstrated that poly-γ-glutamic acid production were accelerated by $ComX_{natto}$ pheromone at more than 1 nM in *natto*.

Quorum sensing is a system to activate specific gene expression dependent on cell density.[1] In quorum sensing, specific pheromones were constantly secreted as an inducer. The pheromone increases with cell population and triggers gene expression to stimulate various phenomena such as acquisition of virulence, antibiotic production, biofilm formation, bioluminescence, conjugation, genetic competence, sporulation, and so on. Since each phenomenon is specifically regulated in response to each quorum sensing pheromone, quorum sensing is an attractive target for drug discovery. For instance, an inhibitor of quorum sensing pheromones derived from pathogenic bacteria must be a promising drug candidate as an anti-pathogenic drug.

Bacillus subtilis secrets ComX pheromone in the control of quorum sensing to stimulate natural genetic competence.[2] ComX pheromone is an oligopeptide synthesized from ComX through posttranslational modification catalyzed by ComQ and unknown processing system of N-terminal side. In the sufficient presence of the ComX pheromone, a receptor histidine kinase, ComP, autophosphorylates and donates phosphate to a response regulator, ComA, via a two-component-system to activate competence gene expression.[3] Striking polymorphism is exhibited in the amino acid sequence of the ComX pheromone variants in *Bacillus* strains, but each possesses a modified tryptophan residue at either the 3rd or the 4th residue from the C-terminal.[4] The tryptophan residue is modified with either a geranyl group or a farnesyl group at its γ-position, resulting in the formation of a tricyclic structure.[5]

B. subtilis subsp. *natto* is well known as a seed of fermented soybeans food in Japan. *Natto* also possesses the genes cluster, $comQXPA_{natto}$, but the $ComX_{natto}$ contains a tryptophan residue at neither the 3rd nor the 4th from the C-terminal, but at only the 54th among 73 amino acids.[6] Recently, $ComX_{natto}$ pheromone was identified as a hexapeptide having an amino acid sequence of Lys-Trp-Pro-Pro-Ile-Glu, corresponding to from the 53th to the 58th residue of $ComX_{natto}$, and the 2nd tryptophan residue is posttranslationally modified with a farnesyl group.[7] $ComX_{natto}$ pheromone is unique in the senses that C-terminal residues of $ComX_{natto}$ are processed as well as N-terminal ones in biosynthesis of $ComX_{natto}$ pheromone and the 5th tryptophan

residue from the C-terminal of $ComX_{natto}$ pheromone is farnesylated. In addition, it was demonstrated that $ComX_{natto}$ pheromone stimulated biosynthesis of poly-γ-glutamic acid (γ-PGA) involved in biofilm formation in *natto*. Since γ-PGA is water-soluble, edible, and highly sticky polymer utilized to develop medical applications, $ComX_{natto}$ pheromone will attract attention as a promising promoter for production of γ-PGA in *natto*. Here we report the chemical synthesis of $ComX_{natto}$ pheromone with solid-phase peptide synthesis including improvement method for cleavage from the resin to confirm the structure $ComX_{natto}$ pheromone. Subsequently, using synthetic $ComX_{natto}$ pheromone, inducing activity of poly-γ-glutamic acid production in *natto* was evaluated.

According to our previous studies, the tryptophan residue of $ComX_{natto}$ pheromone is probably modified with a farnesyl group at its γ-position, resulting in the formation of a tricyclic structure in the same manner of $ComX_{168}$ pheromone and $ComX_{RO\text{-}C\text{-}2}$ pheromone.[5] However, it was difficult to purify enough $ComX_{natto}$ pheromone for NMR analysis because of instability. In order to confirm the precise chemical structure of $ComX_{natto}$ pheromone, we synthesized the modified hexapeptide candidate corresponding to $ComX_{natto}$ pheromone. According to the synthesis of ComX pheromones, we synthesized the modified tryptophan residue from Bz-Trp-OMe **1**, as shown in Figure 1A.[5,8] The synthetic protocols of **2-5** were mainly based on the reference 5c and 8b. Notably, we chose lithium borohydride as a reduction reagent in imine-selective reduction step.[9] We used catecholborane in previous report but it was difficult to purify the product from reaction mixture containing catechol.[5c, 8b] After deprotection of the Fmoc group from the amino compounds, resulting each diastereomeric mixture was easily separated by column chromatography using mixed solvents of chloroform and methanol to afford optically pure Trp*(Far)-OMe **4**. Desired Fmoc-Trp*(Far) **5** for solid-phase peptide synthesis was easily prepared from Trp*(Far)-OMe **4**. Subsequently, Fmoc-Glu(Clt) **7** were prepared in 2 steps using commercially available Fmoc-Glu-OAll **6**, as shown in Figure 1B.[10] As shown Figure 1C, peptide bond formation was accomplished with solid-phase peptide synthesis, and then both Clt resin and the Clt group were cleaved under extremely mild condition by treatment with silica gel in methanol

at 37 °C for 24 h.[11] HPLC purification was carried out to give the synthetic $ComX_{natto}$ pheromone candidate.[12] Other cleavage or purification trials under acidic conditions were failed. For instance, $ComX_{natto}$ pheromone was completely decomposed under 1% trifluoroacetic acid (data not shown). It was thus demonstrated that the improved method by using silica gel in methanol was very effective against cleavage of Clt resin and deprotection of Clt groups in Fmoc solid phase peptide synthesis.

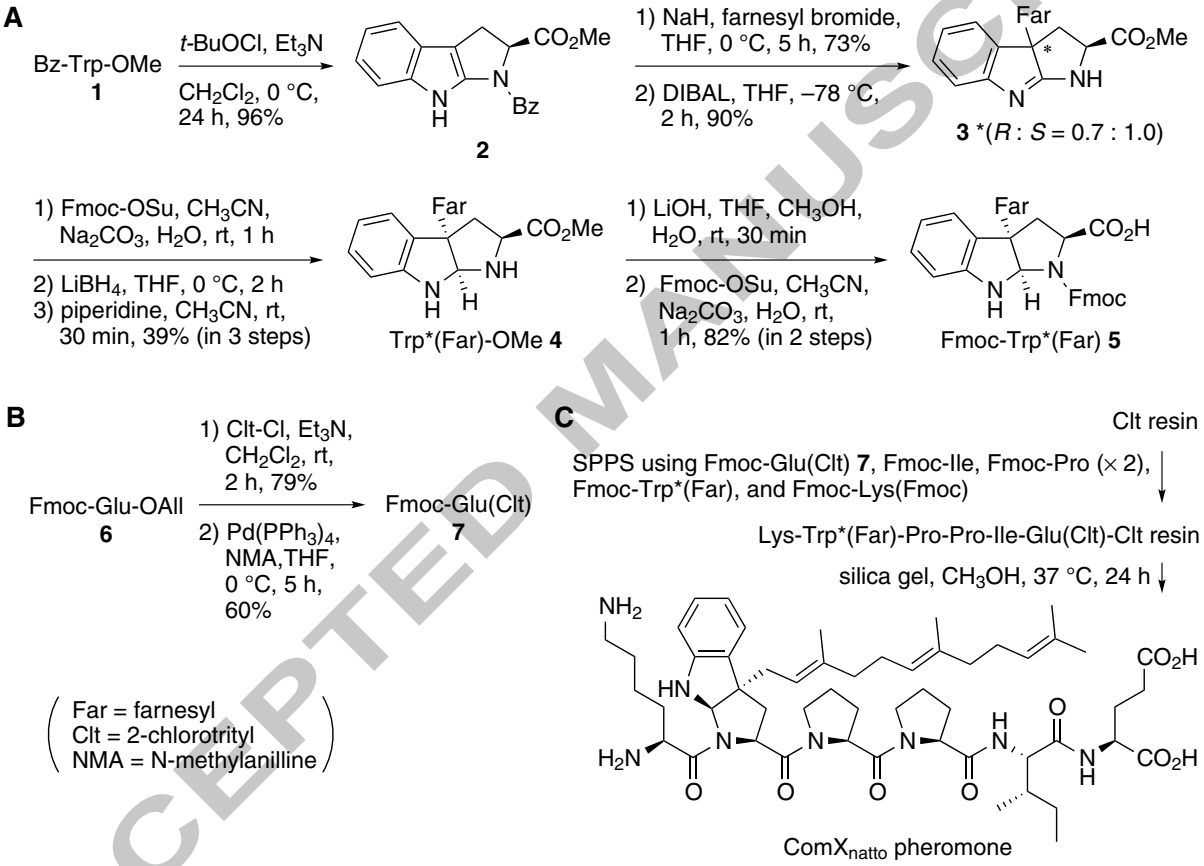

Figure 1. Synthetic scheme of $ComX_{natto}$ pheromone.

A, Synthesis of Fmoc-Trp*(Far) **5**. The synthetic protocols were mainly based on the reference 5c and 8b. The synthetic protocol for the reduction by $LiBH_4$ was described in footnotes 9; B, Synthesis of Fmoc-Glu(Clt) **7**. The synthetic protocol was described in footnotes 10; C, Synthesis of $ComX_{natto}$ pheromone.

We compared the natural $ComX_{natto}$ pheromone with the synthetic $ComX_{natto}$ pheromone by LC-MS/MS, as

shown in Figure 2.[11] The synthetic $ComX_{natto}$ peptide exhibited the same retention time as the natural pheromone by LC-MS monitoring at *m/z* 973.6, as shown Figure 2a and 2b. In addition, the MS/MS spectrum of the synthetic $ComX_{natto}$ pheromone was identical to that of the natural $ComX_{natto}$ pheromone, as shown Figure 2c and 2d. Both peptides exhibited a significant signal at *m/z* 455.3 (or 455.2) as a y_4 ion of the fragment ion series of the parent ion at *m/z* 973.7 (or 973.6). Consequently, the precise structure of $ComX_{natto}$ pheromone was determined to be identical to the synthetic $ComX_{natto}$ pheromone.

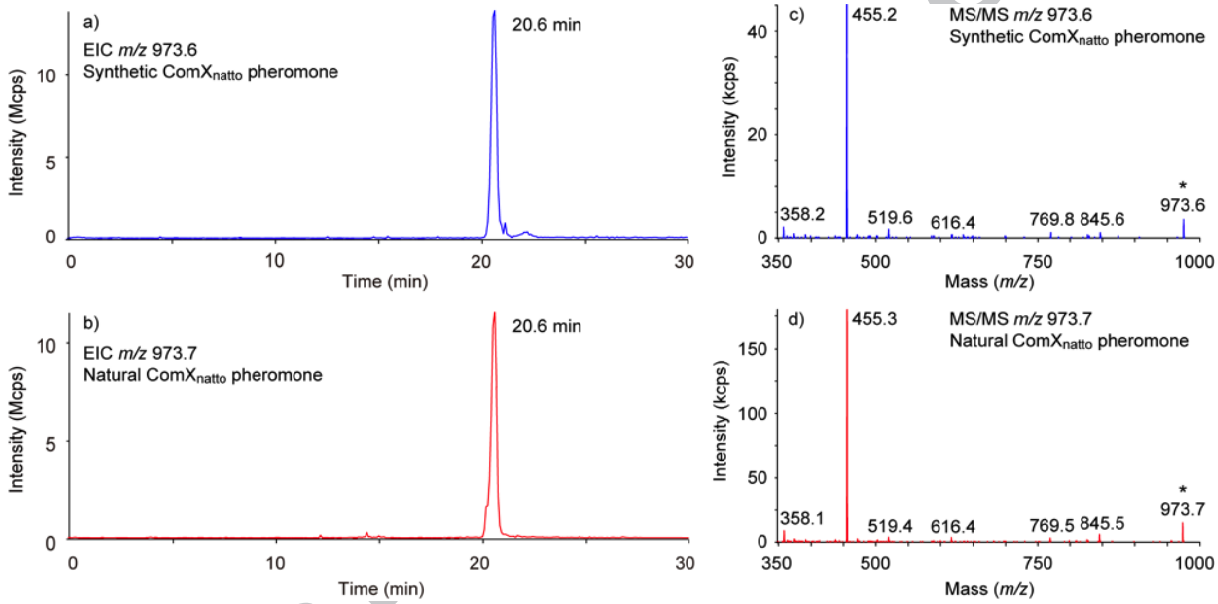

Figure 2. Analyses of the Natural $ComX_{natto}$ Pheromone and the Synthetic $ComX_{natto}$ pheromone.

a) EIC *m/z* 973.6 from LC-MS analysis for synthetic $ComX_{natto}$ pheromone; b) EIC *m/z* 973.7 from LC-MS analysis for natural $ComX_{natto}$ pheromone; c) MS/MS *m/z* 973.6 for synthetic $ComX_{natto}$ pheromone. An asterisk represents a parent ion. Each fragment ion corresponds to the cleavage of a peptide bond (b_n and y_n) or a farnesyl group (–Far), 845.5 (y_5), 769.5 (-Far), 616.4 (b_3), 519.6 (b_2), 455.3 (y_4), 358.1 (y_3); d) MS/MS *m/z* 973.7 for natural $ComX_{natto}$ pheromone. An asterisk represents a parent ion. Each fragment ion corresponds to the cleavage of a peptide bond (b_n and y_n) or a farnesyl group (–Far), 845.5 (y_5), 769.5 (-Far), 616.4 (b_3), 519.6 (b_2), 455.3 (y_4), 358.1 (y_3).

Successively, we investigated the effect of the $ComX_{natto}$ pheromone on γ-PGA production of *natto* , as shown in Figure 3. After the synthetic $ComX_{natto}$ pheromone (or other samples) was added to a liquid medium, *natto* was cultured for 3 days, and the amount of γ-PGA in the broth was measured.[12] It was demonstrated that γ-PGA production were accelerated by pre-addition of $ComX_{natto}$ pheromone at more than 1 nM in contrast to a plain peptide, Lys-Trp-Pro-Pro-Ile-Glu. The natural $ComX_{natto}$ pheromone at 10 nM based on LC-MS analysis exhibited almost same as the synthetic one at 10 nM. Pre-addition of 100 nM of the synthetic $ComX_{natto}$ pheromone stimulated increase of the yield of γ-PGA more than 2 times compared to 10 μL pre-addition of water as a negative control. The final concentration of the $ComX_{natto}$ pheromone in the culture broth of the negative control was calculated to be 13 nM based on LC-MS analysis. Together with previous genetic studies, these findings suggest that $ComX_{natto}$ pheromone plays a crucial role in γ-PGA production as a quorum sensing pheromone in *natto*.

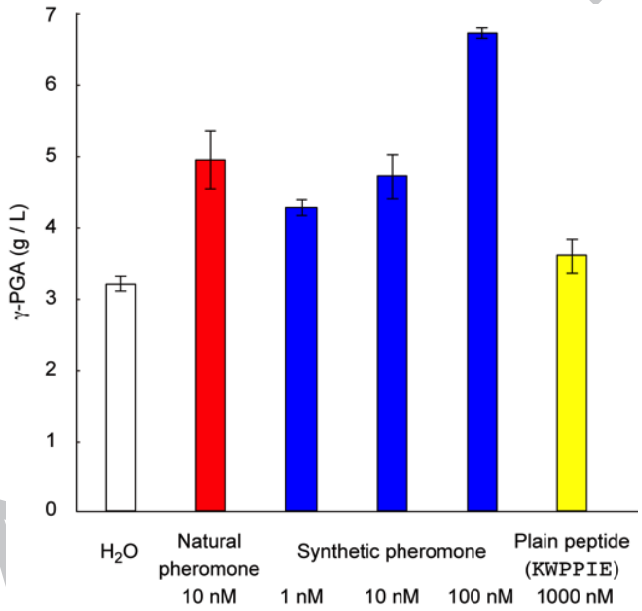

Figure 3. Effects of $ComX_{natto}$ pheromone on γ-PGA production of *natto*.

Bars represent the means of triplicate samples on γ-PGA production of *natto*: H_2O (white), natural $ComX_{natto}$ pheromone (red), synthetic $ComX_{natto}$ pheromone (blue), and plain peptide Lys-Trp-Pro-Pro-Ile-Glu

(yellow). Error bars represent standard deviation.

It is notable that $ComQ_{natto}$ catalyzed farnesylation of the tryptophan residue up to at least the 5th position from the C-terminal while other known ComQ variants can catalyze isoprenylation from only the 2nd to the 4th tryptophan residue from the C-terminal, as previously reported.[13] One possibility is that $ComX_{natto}$ possesses a particular sequence. ComX pheromone variants among *Bacillus* 6 strains possess a modified tryptophan residue at the 3rd from the C-terminal, except $ComX_{RO\text{-}B\text{-}2}$ pheromone that possesses a modified tryptophan residue at the 4th from the C-terminal.[2,4] Comparing $ComX_{natto}$ pheromone and $ComX_{RO\text{-}B\text{-}2}$ pheromone with the other five ComX pheromone variants, only the two pheromones possess a proline residue at 2 residue away from the C-terminal side of the modified tryptophan residue. ComQ will probably receive tryptophan-containing peptides that possess a proline residue on 2 residue away from the C-terminal side of the tryptophan residue as a substrate, but further studies must be needed to elucidate the details. Another point to note is that C-terminal residues of ComX were processed as well as N-terminal ones to produce $ComX_{natto}$ pheromone. So far, it is not clear which step occurred first, farnesylation or N- or C-truncation of amino acid residues, but this study has demonstrated that posttranslational farnesylation could have occurred at the internal tryptophan residue of a substrate in *Bacillus* species and related bacilli as well as at that near the C-terminus one.

In conclusion, we synthesized extremely acid-labile $ComX_{natto}$ pheromone with improved synthetic methodology in this study to confirm the precise chemical structure of $ComX_{natto}$ pheromone. In addtion, γ-PGA production were accelerated in *natto* by pre-addition of $ComX_{natto}$ pheromone at more than 1 nM. $ComX_{natto}$ pheromone will attract attention as a promising additive for improving the production of γ-PGA by *natto*.

Acknowledgments

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9. A typical protocol is follows: To a solution of the imine in THF (0.1 M) was added 0.5 equivalent of

lithium borohydride at 0 °C. After stirring for 2 h, the reaction mixture was poured into 0.1 M phosphate buffer (pH 7.0). The mixture was extracted with EtOAc and the organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography to give diasteromers of Fmoc-Trp*(Far)-OMe. Since the compound was unstable, so they were carried on to the next step immediately.

10. A typical protocol is follows: To a solution of Fmoc-Glu-OAll **6** and 2.5 equivalent of N-ethyldiisopropylamine (DIPEA) in CH_2Cl_2 (0.1 M) at room temperature under nitrogen was added 1.1 equivalent of 2-chlorotrityl chloride (Clt-Cl). After the reaction mixture was stirred for 2 h, the reaction mixture was washed with saturated aqueous NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography eluted with 0.5% DIPEA containing solvent mixture to prevent from Clt deprotection, to give Clt ester of Fmoc-Glu-OAll [Fmoc-Glu(Clt)-OAll] in 79% overall yield. Since the Clt ester was unstable, they were carried on to the next step immediately. To a solution of Fmoc-Glu(Clt)-OAll and 10 equivalent of NMA in THF (0.1 M) at 0 °C under nitrogen was added 0.01 equivalent of $Pd(PPh_3)_4$. After the reaction mixture was stirred at 0 °C for 5 h, the organic layer was washed with 5% aqueous $KHSO_4$ and saturated aqueous NaCl, and dried over Na_2SO_4. After evaporation of the solution, the residue was recrystallized from $CHCl_3$/Et_2O to give Fmoc-Glu(Clt) **7** as a pale yellow powder in 60% overall yield. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.76 (m, 2H), 7.59 (m, 2H), 7.40-7.20 (m, 18H), 5.48 (br, 1H), 4.50-4.40 (m, 3H), 4.23 (m, 1H), 2.72 (m, 1H), 2.34 (m, 1H), 1.28 (m, 1H), 0.90 (m, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$): 171.4, 170.6, 155.8, 143.7, 143.5, 142.3, 141.1, 139.4, 133.5, 131.6 131.3, 131.2, 129.1, 127.8, 127.6, 127.4, 126.9, 125.8, 125.0, 119.8, 119.0, 90.2, 67.0, 66.1, 53.4, 47.1, 30.9, 27.5. The ^{1}H and ^{13}C NMR spectra can be found in Supplementary Information.

11. HPLC conditions were as follows: X-select CSH C18 column (Waters) with linear gradient from 60% to 98% methanol in 10 mM of aqueous ammonium formate.

12. After the sample was added to 10 mL of liquid medium, *natto* was statically incubated in the medium for

72 h at 37 °C. After sodium chloride (585 mg, 10 mmol) was added, the broth was centrifuged and filtrated. After 20 mL of ethanol was added to the filtrate, the suspension was centrifuged to collect precipitate. The pellet was resolved in water, and the solution was filtrated through an ultrafiltration membrane (100 kDa) to afford almost pure γ-PGA as a filtration residue. After the freeze-drying, the weight of γ-PGA was measured. ^{1}H NMR (D_2O, *t*-BuOH: δ1.23): δ4.15 (m, 1H; relative), 2.34 (m, 2H; relative), 2.05 (m, 1H; relative), 1.91 (m, 1H; relative). The ^{1}H NMR spectrum can be found in Supplementary Information.

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Legends for Figures.

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Graphical Abstract

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$ComX_{natto}$ pheromone

KW*(Far)PPIE