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Communication

Chemical Structure of Posttranslational Modification with A Farnesyl Group on Tryptophan

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***Bacillus subtilis* and related bacilli produce a posttranslationally modified oligopeptide, the ComX pheromone, that stimulates natural genetic competence controlled by quorum sensing. The $ComX_{RO\text{-}C\text{-}2}$ pheromone from strain RO-C-2 must be modified with a farnesyl group on the Trp residue, but the precise structure is not known. Here we report the precise nature of posttranslational farnesylation of $ComX_{RO\text{-}C\text{-}2}$ pheromone on the Trp residue, resulting in the formation of a tricyclic structure. The $ComX_{168}$ pheromone, produced by the standard laboratory strain used in the study of *B. subtilis*, is also posttranslationally farnesylated according to phylogenetic resemblance.**

Key words: *Bacillus*; ComX; posttranslational modification; quorum sensing; tryptophan

In quorum sensing, bacteria regulate their gene expression by secreting specific extracellular signaling molecules.[1,2] *Bacillus subtilis* and related bacilli produce a posttranslationally modified oligopeptide that stimulates natural genetic competence,[3,4] termed the ComX pheromone.[5] Natural isolates of bacilli exhibit striking polymorphism in the amino acid sequence of ComX, but each possesses an invariant Trp residue.[6,7] Previous studies have indicated that the Trp residue is modified with an isoprenoid, which is essential for pheromone activity, to form the mature ComX pheromone.[6,8,9] The $ComX_{RO\text{-}E\text{-}2}$ pheromone from *B. subtilis* strain RO-E-2 was recently found to have a unique modified Trp residue with a geranyl group at the 3 position of its indole ring, resulting in the formation of a tricyclic structure.[10,11] Successively, the $ComX_{RO\text{-}H\text{-}1}$ pheromone from *B. mojavensis* strain RO-H-1 has also been confirmed to have the geranyl modified Trp residue in the same manner as the $ComX_{RO\text{-}E\text{-}2}$ pheromone.[12] The $ComX_{168}$ and $ComX_{RO\text{-}C\text{-}2}$ pheromones, produced by strains 168 and RO-C-2 respectively, are modified with a farnesyl group, because the increase in molecular weight matches the farnesyl modification detected by MS analyses.[5,6] Given the role of the modification in each ComX variant, the structure of the farnesyl modification might be similar to that of geranylation but proof of the precise structure is unavailable. Since each ComX pheromone shows group-specific bioactivity, determination of the nature of the modifications is necessary in order to correlate group-specific structures and activities, but sufficient preparation of purified natural ComX pheromone has proven to be difficult because the concentration of secreted pheromone in the medium was low and the ComX pheromone is highly unstable.[5,10,12,13] Hence, we considered doing a synthesis of a possible ComX peptide possessing the farnesyl modified Trp residue and a comparison with the synthetic peptide and culture broth containing the natural ComX pheromone. Here we report the precise structure of the $ComX_{RO\text{-}C\text{-}2}$ pheromone, providing the first example of the chemical structure of a naturally occurring farnesyl group on a Trp residue.

We prepared a modified Trp residue with a farnesyl group based on synthesis of the $ComX_{RO\text{-}E\text{-}2}$ pheromone, which possesses a geranyl modified Trp residue (Scheme 1A).[10,11] *C*-Farnesylation of tricyclic compound **1** using farnesyl bromide and NaH in THF afforded diastereomixture of **2** in 73% yield and 1:1.6 (α-farnesyl:β-farnesyl) diastereomeric ratio. The yield and the ratio of the desired α-farnesyl compound were lower than those of *C*-geranylation, but employment of *C*-farnesylation in NMP greatly diminished the ratio, to 1:9. After reductive deprotection of a Bz group chemoselectively, the diastereomixture was easily separated by column chromatography using a solvent system of

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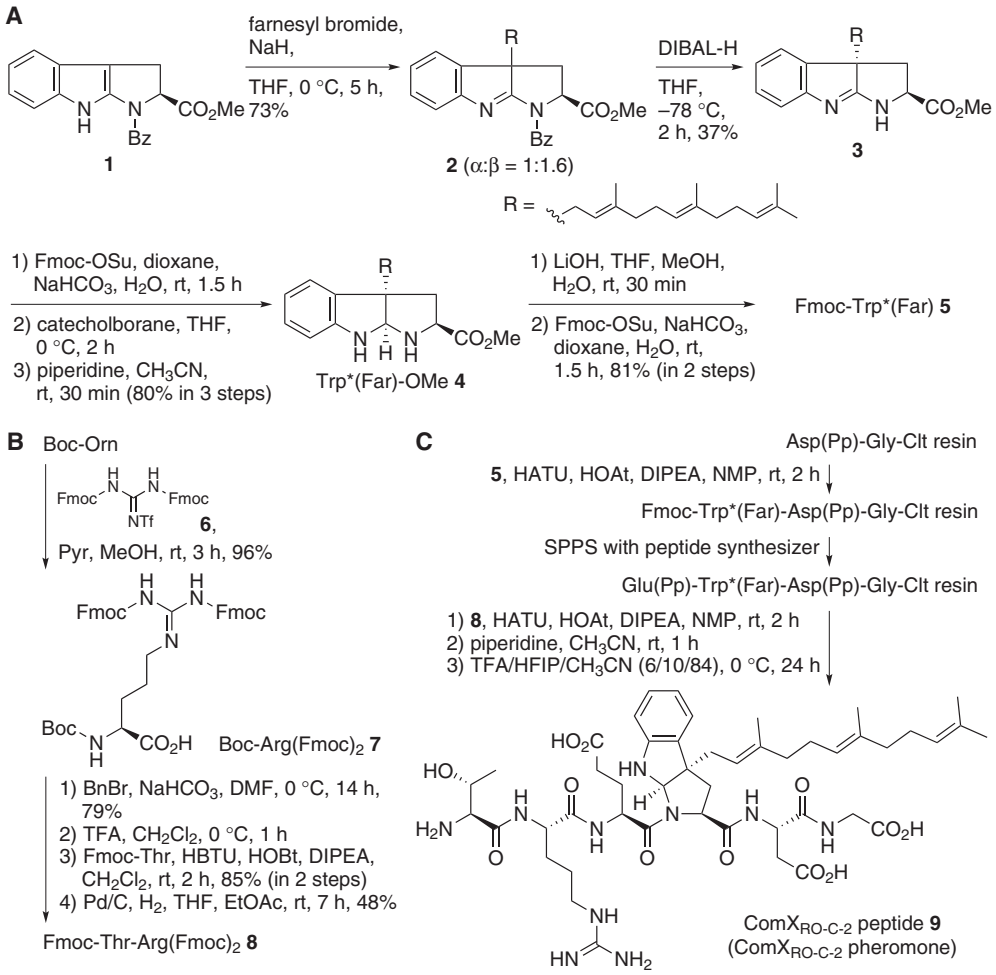
Abbreviations: Clt, 2-chlorotrityl; Far, farnesyl; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl; Pp, 2-phenyl-2-propyl



Scheme 1. Synthesis of $ComX_{RO\text{-}C\text{-}2}$ Peptide **9** (the $ComX_{RO\text{-}C\text{-}2}$ Pheromone).

chloroform and methanol to afford optically active **3**. Fmoc protection, chemoselective double-bond reduction with catecholborane, and Fmoc deprotection gave Trp*(Far)-OMe **4**.[*1] The resulting **4** had only a *cis* configuration between the newly added H-2 proton and the farnesyl group based on NOE analysis. Hydrolysis and Fmoc protection gave Fmoc-Trp*(Far) **5** as two conformational isomers of the amide bond in approximately 1:1 ratio.

Fmoc-Agr(Pbf) is generally employed in solid-phase peptide synthesis, but deprotection of the Pbf group did not proceed with 7% TFA at 0 °C for 24 h, the same condition as the final deprotections used in the synthesis of the $ComX_{RO\text{-}H\text{-}1}$ pheromone.[12] Hence we prepared Boc-Arg(Fmoc)$_2$ **7** for solid-phase peptide synthesis of the $ComX_{RO\text{-}C\text{-}2}$ pheromone according to the synthesis of Fmoc-Arg(Boc)$_2$ (Scheme 1B).[14,15] Treatment of Boc-Orn with the guanidinylation reagent **6** in MeOH afforded the desired Boc-Arg(Fmoc)$_2$ **7**.[*2] The dipeptide, Fmoc-Thr-Arg(Fmoc)$_2$ **8**, was prepared *via* condensation with Thr derivative in four steps.

After the formation of peptide bonds with a combination of peptide synthesizer and manual synthesis, as previously reported,[10–13,16] both 2-chlorotrityl (Clt) resin and protecting groups such as a 2-phenyl-2-propyl (Pp) group were cleaved under mild acidic conditions by treatment with 6% TFA at 0 °C for 24 h (Scheme 1C). After HPLC purification, the structure of $ComX_{RO\text{-}C\text{-}2}$ peptide **9** was confirmed by ESI-HRMS.[*3]

We compared the natural $ComX_{RO\text{-}C\text{-}2}$ pheromone in

[*1] Chemical data of Trp*(Far)-OMe **4**: ^{1}H-NMR (400 MHz, $CDCl_3$): $\delta = 1.56$ (s, 3H), 1.58 (s, 3H), 1.60 (s, 3H), 1.68 (s, 3H), 1.94–2.07 (m, 8H), 2.34–2.80 (m, 4H), 3.34 (s, 3H), 3.88 (dd, 1H, $J = 7.4$, 7.7 Hz), 4.81 (s, 1H), 5.06–5.11 (m, 2H), 4.81 (s, 1H), 5.15 (m, 1H), 6.54 (dd, 1H, $J = 0.8$, 7.5 Hz), 6.69 (dd, 1H, $J = 0.8$, 7.4 Hz), 6.98–7.03 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 16.3, 17.7, 25.7, 26.5, 26.8, 36.5, 39.7, 39.9, 41.1, 52.8, 57.7, 60.1, 82.5, 109.5, 118.8, 119.6, 123.8, 124.1, 124.3, 128.1, 131.3, 133.4, 135.1, 138.1, 149.4, 174.4; ESI-HRMS m/z ($[M + H]^+$) Calcd. for $C_{27}H_{36}N_2O_2$: 421.2850, Found 421.2847.

[*2] Chemical data of Boc-Arg(Fmoc)$_2$ **7**: ^{1}H-NMR (400 MHz, CD_3OD): $\delta = 1.40$ (s, 9H), 1.75–1.82 (m, 3H), 1.95 (m, 1H), 3.55 (m, 1H), 4.23 (m, 1H), 4.27–4.37 (m, 4H), 4.56 (d, 1H, $J = 6.8$ Hz), 7.32 (t, 4H, $J = 7.4$ Hz), 7.38–7.43 (m, 4H), 7.66 (d, 2H, $J = 7.4$ Hz), 7.72 (d, 2H, $J = 7.4$ Hz), 7.85 (d, 4H, $J = 7.4$ Hz).

[*3] HRMS data of $ComX_{RO\text{-}C\text{-}2}$ peptide **9**; ESI-HRMS m/z ($[M + 2H]^{2+}$) Calcd. for $C_{48}H_{68}N_8O_{10}$: 458.2524, Found 458.2525.

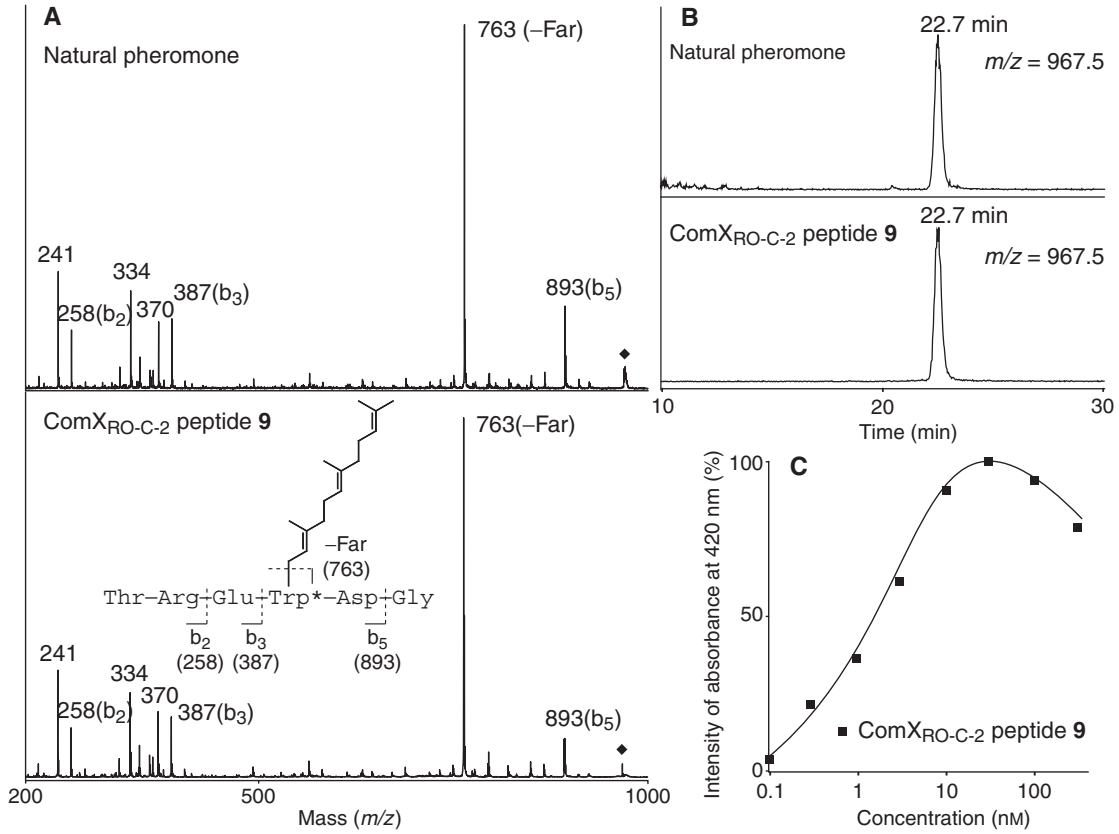


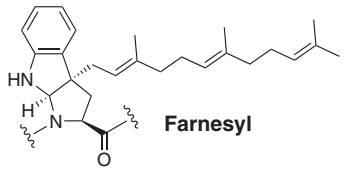
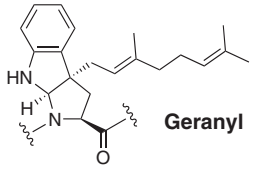
Fig. 1. Analyses of the Natural $ComX_{RO\text{-}C\text{-}2}$ Pheromone and the Synthetic $ComX_{RO\text{-}C\text{-}2}$ Peptide **9**.

A, MALDI-TOF-MS/MS analyses of the natural $ComX_{RO\text{-}C\text{-}2}$ pheromone and the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9**. MALDI-TOF-MS/MS were acquired using an 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Squares represent the precursor ions at m/z 967.5. B, LC/ESI-MS analyses of the natural $ComX_{RO\text{-}C\text{-}2}$ pheromone and the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9**. LC/ESI-MS were acquired using an API2000 LC/ESI-MS System (Applied Biosystems, Foster City, CA) in Q1MI scan mode (m/z value 967.5). A toriacontyl column (250 × 4.6 mm ID, Develosil C30-UG-5, Nomura Chemical, Tokyo) was attached to a HPLC system and the sample solutions were eluted at a flow rate of 1.0 ml/min with 25% CH_3CN in 0.1% aqueous ammonium acetate for 2 min and then a gradient of 25–55% CH_3CN in 0.1% aqueous ammonium acetate for 30 min. C, Concentration-response curve using synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9**. The curve represents the means of duplicate samples.

the culture broth with the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9** by MALDI-TOF-MS. They had the same molecular composition possessing the expected ion at m/z 967.5. The MS/MS spectrum of the natural $ComX_{RO\text{-}C\text{-}2}$ pheromone was identical to that of the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9** by analysis of the precursor ion at m/z 967.5 (Fig. 1A). Furthermore, the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9** showed the same retention time as the natural pheromone by LC-ESI-MS monitoring at m/z 967.5 (Fig. 1B). We then investigated the biological activity of the synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9** for *B. subtilis* strain RO-C-2 using the *B. subtilis* tester strain, which responds to the $ComX_{RO\text{-}C\text{-}2}$ pheromone, by monitoring the expression of an *srfA-lacZ* fusion.[6] β-Galactosidase assay was carried out by measurement of the intensities of absorbance at 420 nm by a standard method with ONPG, as previously reported (Fig. 1C). Peptide **9** showed potent biological activity, with an EC_{50} value of 2 nM. This value was reasonable for comparing the observed biological activity of the culture broth with the equivalent concentration of the natural $ComX_{RO\text{-}C\text{-}2}$ pheromone in the culture broth calculated by the intensity of LC-ESI-MS. Additionally, it has been found that the precisely-modified Trp residue is essential to biological activity,[16] and that synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9** has biological activity approximately equal to other natural ComX pheromones, such as the $ComX_{RO\text{-}E\text{-}2}$ pheromone.[5,10–12] Thus the structure of the $ComX_{RO\text{-}C\text{-}2}$ pheromone was confirmed to be synthetic $ComX_{RO\text{-}C\text{-}2}$ peptide **9**, and the posttranslational modification was farnesylation on the Trp residue at the 3 position of its indole ring, resulting in the formation of a tricyclic structure.

We have prepared six recombinant mature ComX variants using an *Escherichia coli* expression system.[6] These previous results, together with the present findings, reveal that posttranslational isoprenoidal modification in the ComX pheromones can be classified into two types, with geranyl and farnesyl modifications respectively (Table 1). For instance, the $ComX_{168}$ pheromone, produced by the standard laboratory strain used in the study of *B. subtilis*, is also modified with a farnesyl group as the $ComX_{RO\text{-}C\text{-}2}$ pheromone. This similarity corresponds to the phylogenetic resemblance

Table 1. Posttranslational Modifications of ComX Variants

Bacillus Strain	Sequence	Chemical Structure of **W***
168	ADPITRQ**W***GD	
RO-C-2	TRE**W***DG	Farnesyl
RO-E-2	GIF**W***EQ	
RO-H-1	MLD**W***KY	Geranyl
RS-B-1	MMD**W***HY	
RO-B-2	YTNGN**W***VPS	

of the modifying enzymes (ComQ) and the ComX precursors of the two strains.[6,7] Although the precise modification of the $ComX_{NAF4}$ pheromone from *B. natto*, which we were unable to produce from *E. coli*, is unknown, our chemical synthetic methodology can potentially clarify the structure of this pheromone in the near future.

Our results suggest that the isoprenyl side chain is an influential determinant of group specificity, since the geranyl and the farnesyl modified Trp residues have similar chemical structures, differing only in their side-chain lengths. Exchanging residues other than the conserved Trp for Ala revealed that these were not absolutely required for the activity of the $ComX_{RO\text{-}E\text{-}2}$ pheromone.[16] Furthermore, the synthetic $ComX_{RO\text{-}C\text{-}2}$ pheromone showed significant activity for tester strain 168, with an EC_{50} value of 6 nM, but no activity for the tester strain RO-E-2 up to 300 nM. A previous study showed a similar specific pattern using conditioned media of producer strains and partially purified ComX variants.[6] Gram negative bacteria produce *N*-acyl-L-homoserine lactones as quorum sensing pheromones, and the length of their acyl side chains is responsible for their pheromone group specificities. The alteration of the length of lipophilic side chain in quorum sensing pheromones might be a fundamental strategy for the acquisition of group specificity in bacteria.

In conclusion, we identified the precise nature of posttranslational modification in the $ComX_{RO\text{-}C\text{-}2}$ pheromone. The Trp residue was modified with a farnesyl group, resulting in the formation of a tricyclic structure. This is the first example of naturally occurring post-translational farnesylation on a Trp residue.

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References

1) Bassler, B. L., and Losick, R., Bacterially speaking. *Cell*, **125**, 237–246 (2006).
2) Williams, P., Winzer, K., Chan, W., and Cámara, M., Look who's talking: communication and quorum sensing in bacterial world. *Philos. Trans. R. Soc. B*, **362**, 1119–1134 (2007).
3) Tortosa, P., and Dubnau, D., Competence for transformation: a matter of taste. *Curr. Opin. Microbiol.*, **2**, 588–592 (1999).
4) Hamoen, L. W., Venema, G., and Kuipers, O. P., Controlling competence in *Bacillus subtilis*: shared use of regulators. *Microbiology*, **149**, 9–17 (2003).
5) Magnuson, R., Solomon, J., and Grossman, A. D., Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. *Cell*, **77**, 207–216 (1994).
6) Ansaldi, M., Marolt, D., Stebe, T., Mandic-Mulec, I., and Dubnau, D., Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants. *Mol. Microbiol.*, **44**, 1561–1573 (2002).
7) Ansaldi, M., and Dubnau, D., Diversifying selection at the *Bacillus* quorum-sensing locus and determinants of modification specificity during synthesis of the ComX pheromone. *J. Bacteriol.*, **186**, 15–21 (2004).
8) Tortosa, P., Logsdon, L., Kraigher, B., Itoh, Y., Mandic-Mulec, I., and Dubnau, D., Specifity and genetic polymorphism of the Bacillus competence quorum-sensing system. *J. Bacteriol.*, **183**, 451–460 (2001).
9) Schneider, K. B., Palmer, T. M., and Grossman, A. D., Characterization of *comQ* and *comX*, two genes required for production of ComX pheromone in *Bacillus subtilis*. *J. Bacteriol.*, **184**, 410–419 (2002).
10) Okada, M., Sato, I., Cho, S. J., Iwata, H., Nishio, T., Dubnau, D., and Sakagami, Y., Structure of the *Bacillus subtilis* quorum-sensing peptide pheromone ComX. *Nat. Chem. Biol.*, **1**, 23–24 (2005).
11) Okada, M., Sato, I., Cho, S. J., Dubnau, D., and Sakagami, Y., Chemical synthesis of ComX pheromone and related peptides containing isoprenoidal tryptophan residues. *Tetrahedron*, **62**, 8907–8918 (2006).
12) Okada, M., Yamaguchi, H., Sato, I., Tsuji, F., Qi, J., Dubnau, D., and Sakagami, Y., Acid labile ComX pheromone from *Bacillus mojavensis* RO-H-1. *Biosci.*

Biotechnol. Biochem., **71**, 1807–1810 (2007).
13) Okada, M., Sato, I., Cho, S. J., Suzuki, Y., Ojika, M., Dubnau, D., and Sakagami, Y., Towards structural determination of the ComX pheromone: synthetic studies on peptides containing geranyltryptophan. *Biosci. Biotechnol. Biochem.*, **68**, 2374–2387 (2004).
14) Feichtinger, K., Zapf, C., Sings, H. L., and Goodman, M., Diprotected triflylguanidines: a new class of guanidinylation reagents. *J. Org. Chem.*, **63**, 3804–3805 (1998).
15) Antopolsky, M., and Azhayev, A., Stepwise solid-phase synthesis of peptide-oligonucleotide phosphorothioate conjugates employing Fmoc peptide chemistry. *Tetrahedron Lett.*, **41**, 9113–9117 (2000).
16) Okada, M., Yamaguchi, H., Sato, I., Cho, S. J., Dubnau, D., and Sakagami, Y., Structure-activity relationship studies on quorum sensing $ComX_{RO\text{-}E\text{-}2}$ pheromone. *Bioorg. Med. Chem. Lett.*, **17**, 1705–1707 (2007).