

Structure of the *Bacillus subtilis* quorum-sensing peptide pheromone ComX

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The ComX pheromone is an extracellular signaling molecule that stimulates natural competence in response to crowding in the Gram-positive bacterium *Bacillus subtilis*. The pheromone is formed by isoprenylation of an inactive precursor peptide, but its precise structure is not known. Here we report the structure of the ComX pheromone, showing that addition of a geranyl group to a tryptophan residue results in the formation of an unusual ring structure.

In a process called quorum sensing, bacteria produce signaling molecules that induce a response at a high cell density. ComX pheromone, a post-translationally modified peptide, activates natural genetic competence in *B. subtilis* in response to crowding¹. Natural isolates of bacilli show marked polymorphism in the sequence of the corresponding gene, *comX*^{2,3}; however, each possesses an invariant tryptophan residue²⁻⁴, which is modified by isoprenylation to form the active ComX pheromone^{2,5}.

To determine the precise nature of the ComX modification, we chose to work with the pheromone from *B. subtilis* strain RO-E-2, because this molecule has only six amino acid residues² (Fig. 1a). The molecular formula of the ComX_{RO-E-2} pheromone indicated that the peptide modification might be a simple substitution of a tryptophanyl proton by a geranyl group⁶. In previous work, we had synthesized peptides that had the amino acid sequence of the ComX_{RO-E-2} pheromone but contained six kinds of geranylated tryptophans (1-, 2-, 4-, 5-, 6- and 7-geranyltryptophan). Although the resulting peptides all had the same molecular weight as the natural ComX_{RO-E-2} pheromone, none had biological activity⁶.

Using a previously reported *Escherichia coli* expression system^{2,5} (see **Supplementary Methods** online), we optimized the culture and purification conditions and were able to obtain 0.20 mg of pure ComX_{RO-E-2} pheromone from 5 liters of culture broth (see **Supplementary Methods** and **Supplementary Scheme 1** online). Purified ComX_{RO-E-2} pheromone was difficult to dissolve in DMSO, water or other protic solvents. Moreover, this peptide was unstable under acidic condition at room temperature. We used 60% CD₃CN in D₂O as the NMR solvent but achieved saturation with only 550 μM. We obtained ¹H NMR, DQF-COSY (double-quantum filtered-correlation spectroscopy) and ROESY (rotating-frame Overhauser enhancement spectroscopy) spectra (see **Supplementary Figs. 1–3**

online), but could not use ¹³C or ¹H-¹³C two-dimensional NMR spectroscopy because of the low sample concentration.

We analyzed the ¹H NMR spectrum and assigned signals to each amino acid residue by reference to the DQF-COSY and ROESY spectra (**Supplementary Methods**). The proton signals of the amino acid residues glycine, isoleucine, phenylalanine, glutamic acid and glutamine showed insignificant changes compared with the simple peptide GIFWEQ and the peptides containing geranyl tryptophan that had been synthesized previously⁶. The geranyl group in the ComX_{RO-E-2} pheromone showed no evidence of isomerization or molecular weight change resulting from oxidation or reduction (see **Supplementary Table 1** online). The chemical shift and coupling pattern of the 1' methylene (2.11 p.p.m., d, *J* = 7.4 Hz, 2H) in the geranyl side chain indicated that the geranyl group was connected to a quaternary carbon but not to a heteroatom. Because four aromatic coupling protons of the indole ring were observed, the benzene ring of the tryptophan residue seemed to be preserved. A substantial difference in chemical shift was observed for the beta protons (1.00 and 2.28 p.p.m.) and for the methyne proton (5.06 p.p.m.) at the 2 position of tryptophan. The signals of these protons in the ComX_{RO-E-2} pheromone were shifted to a higher field compared to those of tryptophan residues in related peptides (see **Supplementary Table 1**). These up-field shifts were explained by a loss of aromaticity in the pyrrole ring of tryptophan. These results led us to conclude that the geranyl group was connected to the 3 position of tryptophan. The molecular formula requires that the tryptophan 2 position be a methyne carbon. Furthermore, an imine formation between N-1 and C-2 of the indole ring is ruled out on chemical shift grounds. Considering the molecular weight and the observed chemical shift of the methyne proton at the 2 position of tryptophan, we propose that the tryptophan residue in the ComX_{RO-E-2} pheromone is modified to the tricyclic ring structure.

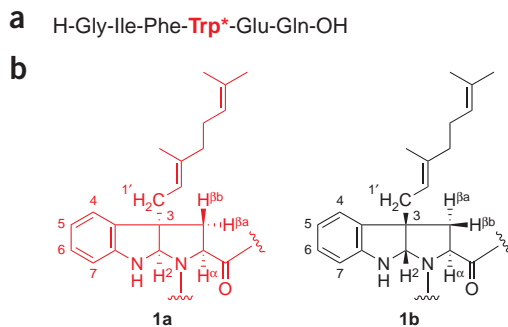


Figure 1 Amino acid sequence of the ComX_{RO-E-2} pheromone and structures of the modified tryptophan residue in the ComX_{RO-E-2} pheromone. (a) Amino acid sequence of the ComX_{RO-E-2} pheromone. Trp* is modified by a geranyl group. (b) The possible absolute structures of the modified L-tryptophan residues in **1a** and **1b**.

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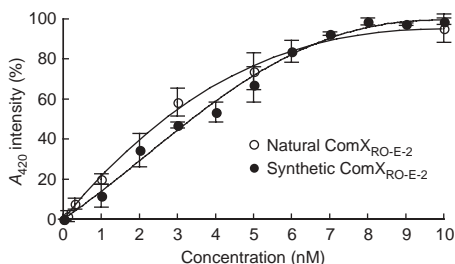


Figure 2 Dose-response curves obtained using the natural ComX_{RO-E-2} pheromone and the synthetic peptide **1a**. Error bars, s.d. of the means of triplicate samples.

We also analyzed the stereochemistry of the modified tryptophan residue. As we observed a nuclear Overhauser effect (NOE) between H-2 and H-1', H-2 and H-βa, and H-βa and H-1' in the ROESY spectrum, H-2, H-βa and the geranyl group should be on the same side of the ring. Considering these data and the L-configuration of the amino acid, we propose the absolute structure of the modified tryptophan to be **1a** or **1b** (Fig. 1b). Because the NOE between H-α and H-1' or H-2 was not observed, we carried out a conformational search to select an appropriate structure. The alpha proton (H-α, 3.45 p.p.m., d, *J* = 8.5 Hz) is coupled to H-βa (1.00 p.p.m., dd, *J* = 8.5, 12.7 Hz) but not to H-βb (2.28 p.p.m., d, *J* = 12.7 Hz). This observation indicated that the dihedral angle between H-α and H-βb is fixed near 90° by the newly formed five-membered ring. A conformational simulation carried out with a Monte Carlo protocol showed that only structure **1a** could have a dihedral angle near 90° in the stable conformation (Supplementary Methods and Supplementary Fig. 4 online). These results also reasonably explain the NOE data mentioned above. Consequently, we proposed the structure of modified tryptophan residue to be **1a** (Fig. 1b).

To confirm this chemical structure, we synthesized the modified tryptophan residue in **1a** and the corresponding ComX_{RO-E-2} peptide (see Supplementary Methods and Supplementary Scheme 2 online). The synthetic peptide showed an ¹H NMR spectrum identical to that of the natural peptide pheromone. We also observed that the synthetic peptide showed a very similar biological activity to that of the ComX_{RO-E-2} pheromone (Fig. 2 and Supplementary Methods). Successively, we also synthesized the modified tryptophan residue in **1b** and two isomers having the D-configuration, as well as the corresponding peptides, using similar synthetic methods (data not

shown). These three peptides showed no biological activity at up to 1.0 μM and their NMR spectra differed from that of the natural pheromone. We have thus succeeded in determining the structure of the ComX_{RO-E-2} pheromone to be **1a** (Fig. 1b) with the amino acid sequence shown in Figure 1a.

Other ComX pheromones—such as ComX₁₆₈ and ComX_{RO-C-2}, produced by strains 168 and RO-C-2 of *B. subtilis*, respectively—are predicted to be modified by farnesyl groups, based on the mass of their modifications^{1,2}. Given the role of ComQ in the isoprenylation of each ComX variant⁵, the structures of these pheromones may be similar to that described here. Early work on the farnesyl modification of the C-terminal cysteine in peptides was reported for the sex pheromones of basidiomycete yeasts⁷. This type of post-translational modification is now recognized as quite common and is important for peptide and protein function⁸. Because the modification of tryptophan markedly alters the hydrophobicity and conformation of the unmodified peptide precursor, the elucidation of the pheromone structure will permit investigations of pheromone interactions with the cognate ComP receptors⁹ and the determinants of specificity in these interactions. Although several secondary metabolites of isoprenylated tryptophan derivatives are known, the ring structure we determined for the isoprenyl-modified tryptophan residue in the ComX pheromone is a unique post-translational modification.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Magnuson, R., Solomon, J. & Grossman, A.D. *Cell* **77**, 207–216 (1994).
- Ansaldo, M., Marolt, D., Stebe, T., Mandic-Mulec, I. & Dubnau, D. *Mol. Microbiol.* **44**, 1561–1573 (2002).
- Ansaldo, M. & Dubnau, D. *J. Bacteriol.* **186**, 15–21 (2004).
- Tortosa, P. *et al.* *J. Bacteriol.* **183**, 451–460 (2001).
- Bacon Schneider, K., Palmer, T.M. & Grossman, A.D. *J. Bacteriol.* **184**, 410–419 (2002).
- Okada, M. *et al.* *Biosci. Biotechnol. Biochem.* **68**, 2374–2387 (2004).
- Sakagami, Y., Yoshida, M., Isogai, A. & Suzuki, A. *Science* **212**, 1525–1527 (1981).
- Clarke, S. *Annu. Rev. Biochem.* **61**, 355–386 (1992).
- Piazza, F., Tortosa, P. & Dubnau, D. *J. Bacteriol.* **181**, 4540–4548 (1999).