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Towards Structural Determination of the ComX Pheromone: Synthetic Studies on Peptides Containing Geranyltryptophan

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Bacteria produce and respond to signal molecules depending on their cell density. This process is called "quorum sensing". The ComX pheromone, controlled by quorum sensing, activates natural genetic competence in *Bacillus subtilis*. ComX is an oligopeptide with a posttranslational modification. It has been suggested that ComX pheromone is modified with an isoprenoid at its tryptophan residue, but the complete chemical structure is unknown. We first determined the molecular formula of $ComX_{RO\text{-}E\text{-}2}$, a competence factor for *B. subtilis* strain RO-E-2. Then we synthesized putative pheromones with 1-, 2-, 4-, 5-, 6-, or 7-geranyl substituted tryptophan residues. The regio- and stereoselective synthesis of the geranyl tryptophans was successful, and we prepared the six peptides with modified tryptophan residues. These peptides had the same molecular formula and showed similar hydrophobicity to the natural $ComX_{RO\text{-}E\text{-}2}$ in LC–MS analysis. But, none of them showed the same retention time as the natural pheromone and none exhibited its biological activity. These results suggest that the isoprenoid modification pattern of the tryptophan residue is more complex than postulated.

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

Bacteria monitor their environments, allowing adaptation to changing conditions. Among the sensor systems employed for this purpose, quorum sensing is a mechanism that determines cellular behavior in response to cell density.[1)] To accomplish this, quorum sensing detects the concentrations of pheromones secreted into the external medium. Among the responses governed by quorum sensing are biofilm formation, sporulation, conjugation, bioluminescence, genetic competence, and the acquisition of virulence. The pheromones of Gram-negative bacteria are generally *N*-acylhomoserine lactones with species-specific acyl chain lengths. In the case of Gram-positive bacteria, the quorum sensing pheromones are usually oligopeptides.

Bacillus subtilis is a Gram-positive bacterium that secretes several quorum sensing pheromones, including ComX, which stimulates natural genetic competence. The ComX pheromone of *B. subtilis* 168, the commonly used laboratory strain, is an oligopeptide possessing 10 amino acids, derived from the *C*-terminal codons of the *comX* gene.[2)] The purified peptide has the same amino acid sequence as that determined from the *comX* DNA sequence except that a predicted tryptophan residue is not recovered by Edman degradation. The molecular weight of the pheromone is 206 Daltons higher than that of the simple peptide, suggesting that it receives posttranslational modification at the position of the tryptophan residue. It has been shown that ComQ, the enzyme responsible for modifying ComX, resembles isoprenyl transferase in its primary sequence,[3)] and that tritium labeled mevalonic acid was incorporated into the ComX pheromone molecule.[4)] Hence the modification of the strain 168 ComX pheromone was predicted to be a farnesyl chain, in accordance with the molecular weight of the modification. It appears likely that an isoprenoid chain modifies the ComX pheromone, although its complete structure is unknown.

To investigate the chemical structure of the ComX pheromone, we first determined its molecular formula by obtaining a high-resolution mass spectrum (HRMS). From the results obtained, we estimate that the isoprenoidal moiety is simply substituted for a proton in the tryptophan residue. We have considered two approaches for determining the structure. The first approach is to obtain an NMR spectrum of a purified ComX pheromone and the other is to synthesize peptides with all the possible simple structures of the natural ComX pheromone. The preparation of enough natural ComX pheromone for NMR analysis proved to be difficult

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Abbreviations: Clt, 2-chlorotrityl; DIPEA, *N*,*N*′-diisopropylethylamine; dppf, diphenylphosphinoferrocene; Ger, geranyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; LDA, lithium diisopropylamide; Phth, phthaloyl; Pp, 2-phenyl-2-propyl; Su, succinimide; TMEDA, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine

because the concentration of secreted pheromone in the medium is low. Consequently, the latter approach was adopted, and we have elected to study the simplest pheromone, produced by *B. subtilis* strain RO-E-2. $ComX_{RO\text{-}E\text{-}2}$ contains only six amino acid residues and is predicted to have a geranyl moiety, based on arguments analogous to those described above for the $ComX_{168}$ pheromone.[4)]

Materials and Methods

General. High performance liquid chromatography was performed on an HPLC system equipped with Jasco PU-980 pumps and a Jasco UV-980 UV detector or with Shimadzu LC-6A pumps and a Shimadzu SPD-6A UV detector. HRMS (ESI-TOF, positive) was recorded on a Mariner system (Applied Biosystems) using either an angiotensin/bradykinin/neurotensin mixture or a polypropylene glycol solution as a calibration standard. LC–MS spectra (ESI-Q, positive) were recorded on an API2000 mass spectrometer (Applied Biosystems) and an HPLC (Hewlett-Packard Agilent LC system). The optical density was measured with an AE-15F photoelectric colorimeter (Erma).

Bacterial strains, pheromone production, and biological activity. Escherichia coli $ComX_{RO\text{-}E\text{-}2}$ producer strain (ED413) and *B. subtilis* tester strain (BD3020) were prepared as described previously.[4,5)] Pheromone production was as performed previously.[4)] Biological activity was measured by a β-galactosidase assay.[2,5)]

Pheromone purification. Partial purification was performed as described previously.[4)] The supernatant obtained after centrifugation of conditioned medium was filtered through a membrane filter (0.22 μm). The filtrate was loaded onto a C-18 Sep-Pak cartridge (Waters) in 20% aqueous CH_3CN and 0.1% trifluoroacetic acid (TFA). ComX pheromone was eluted with a step gradient of CH_3CN (20, 50, and 80% in 0.1% aqueous TFA). The 50% eluant mainly contained ComX pheromone. The solution of ComX-RO-E-2 was neutralized with aqueous ammonium, then concentrated and dissolved in 50% aqueous CH_3CN (about 1 ml). The solution was purified by HPLC on an ODS column (4.6 × 250 mm ID, Develosil ODS-HG-5, Nomura Chemical) at a flow rate of 0.8 ml/min, with a gradient of 20–60% CH_3CN in 0.1% aqueous ammonium acetate over 45 min. The most active fraction was collected and purified by HPLC on a cyanopropyl column (4.6 × 250 mm ID, Develosil CN-UG-5, Nomura Chemical) at a flow rate of 1.0 ml/min, with a gradient of 20–32% CH_3CN in 0.1% aqueous ammonium acetate for 24 min to give the $ComX_{RO\text{-}E\text{-}2}$ solution (about 0.1 ml; yield about 1 μg from 1 l broth).

HRMS analysis. The solution of $ComX_{RO\text{-}E\text{-}2}$ (10 μl) was added to a polypropylene glycol solution (20 μM, 10 μl). The mixture was introduced for ESI (positive)–TOF mass spectroscopy with aqueous 50% CH_3CN and 0.05% formic acid at a flow rate of 5 μl/min and recorded. Two polypropylene glycol fragment ions (889.6458 and 947.6877 $[M + NH_4]^+$) were used as an internal standard for calibration. The $ComX_{RO\text{-}E\text{-}2}$ ion $[M + H]^+$ was found at m/z 915.4983, which was assigned as $C_{48}H_{67}N_8O_{10}$ (Calcd. 915.4975) with an error of +0.83 ppm.

Synthesis of putative pheromones.

General. All starting materials and reagents were purchased from commercial sources (Aldrich, Sigma, Wako Pure Chemicals, Watanabe Chemical, Nacalai Tesque, Fluka, Tokyo Chemical Industry, Kanto, Peptide Institute, Applied Biosystems) and used without purification. Organic solvents were purchased as anhydrous grade except for the following solvents, which were freshly distilled prior to use: tetrahydrofuran (THF) and diethyl ether were dried by distillation from Na and benzophenone ketyl, and dichloromethane was dried by distillation from calcium hydride. Analytical TLC was conducted with silica gel 60 F254 plates (Merck). Air-sensitive reactions were carried out under a dry nitrogen atmosphere in well-dried equipment with a tightly fitted rubber septum. Open column chromatography was performed using silica gel BW-300 (Fuji silysia) or ODS Cosmosil 140C18-OPN (Nacalai Tesque). NMR spectra were recorded on a Bruker ARX-400 (^1H; 400 MHz, ^{13}C; 100 MHz) or a Bruker AMX-600 (^1H; 600 MHz, ^{13}C; 150 MHz) instrument. NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_H 7.26 and δ_C 77.0 for $CDCl_3$, δ_H 2.04 and δ_C 29.8 for CD_3COCD_3, δ_H 3.30 and δ_C 49.0 for CD_3OD, and δ_H 1.98 and δ_C 1.3 for CD_3CN. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Solid-phase peptide synthesis was performed with a model 433A peptide synthesizer (Applied Biosystems). Amino acid sequencing was performed with a 490 Procise protein sequencer (Applied Biosystems).

N-Phthaloyl-1-geranyl-L-tryptophan methyl ester ***2****.* To a solution of *N*-phthaloyl-L-tryptophan methyl ester **1** (1.55 g, 4.45 mmol) in *N*,*N*-dimethylformamide (DMF) (45 ml) at 0 °C under nitrogen was added NaH (60% oil suspension, 265 mg, 6.63 mmol). After stirring at 0 °C for 1 h, geranyl bromide (1.0 ml, 5.02 mmol) was added to the mixture at 0 °C. The reaction mixture was stirred and warmed to room temperature for 4 h. The reaction mixture was quenched with 5% aqueous $KHSO_4$ (50 ml), and then the aqueous layer was extracted with Et_2O (4 × 50 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 10/1→5/1) to give *N*-phthaloyl-1-geranyl-L-tryptophan methyl ester **2** (1.32 g, 2.72 mmol, 61%) as a yellow oil. $[\alpha]^{27}_D$ +0.20° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 1.60 (3H, s), 1.62 (3H, s), 1.68 (3H, s), 1.96–2.08 (4H, m), 3.75 (2H, d,

$J = 7.6$ Hz), 3.80 (3H, s), 4.55 (2H, d, $J = 6.8$ Hz), 5.05 (1H, t, $J = 6.8$ Hz), 5.20 (1H, t, $J = 6.8$ Hz), 5.28 (1H, t, $J = 7.2$ Hz), 6.91 (1H, s), 7.05 (1H, t, $J = 7.2$ Hz), 7.12 (1H, t, $J = 7.2$ Hz), 7.22 (1H, d, $J = 7.2$ Hz), 7.61 (1H, d, $J = 7.2$ Hz), 7.66 (2H, m), 7.76 (2H, m). NMR (100 MHz) δ_C ($CDCl_3$) 16.2, 17.6, 24.7, 25.6, 26.2, 39.3, 43.8, 52.6, 52.8, 109.4, 118.5, 118.9, 119.5, 119.7, 121.4, 123.2, 123.2, 123.3, 123.6, 123.7, 125.8, 127.8, 131.7, 133.9, 136.2, 139.6, 167.4, 169.6. *Anal.* Found: C, 74.38; H, 6.74; N, 5.88. Calcd. for $C_{30}H_{32}N_2O_4$: C, 74.36; H, 6.66; N, 5.78.

*Fmoc-Trp(1-Ger) **3***. To a solution of *N*-phthaloyl-1-geranyl-L-tryptophan methyl ester **2** (820 mg, 1.69 mmol) in 1:1 CH_2Cl_2/CH_3OH (20 ml) at 40 °C under nitrogen was added hydrazine hydrate (80%, 0.11 ml, 116 mg, 1.85 mmol). The reaction mixture was stirred at 40 °C for 24 h. The reaction mixture was poured into water (30 ml), and extracted with Et_2O (4 × 30 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/acetone 8/1→5/1) to give 1-geranyl-L-tryptophan methyl ester (494 mg, 1.39 mmol, 83%) as a colorless oil. To a solution of 1-geranyl-L-tryptophan methyl ester (375 mg, 1.06 mmol) in THF (5 ml) was dropped 1 M aqueous LiOH (5 ml) at room temperature. After the reaction mixture was stirred for 1 h, it was neutralized with 5% aqueous $KHSO_4$, and H_2O (10 ml) was added. After THF was removed, the precipitate was filtered and washed with H_2O (2 × 5 ml). The precipitate was dissolved in dioxane (20 ml) and 1 M aqueous Na_2CO_3 (10 ml). To the solution was added Fmoc-OSu (460 mg, 1.36 mmol) at room temperature. After the reaction mixture had been stirred for 3 h, the reaction was quenched with 5% aqueous $KHSO_4$ (50 ml). The reaction mixture was extracted with EtOAc (4 × 30 ml). The organic layer was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography ($CHCl_3$/CH_3OH 100/1→80/1) to give Fmoc-Trp(1-Ger) **3** (514 mg, 0.913 mmol, 87%) as a white powder. $[\alpha]^{22}_D$ +1.6° (*c* 1.0, CH_3OH). NMR (600 MHz) δ_H (CD_3OD) 1.47 (3H, s), 1.55 (3H, s), 1.70 (3H, s), 1.88–1.91 (2H, m), 1.97–2.01 (2H, m), 3.13 (1H, dd, $J = 8.4$, 14.4 Hz), 3.28 (1H, dd, $J = 4.8$, 14.4 Hz), 4.07 (1H, t, $J = 7.2$ Hz), 4.15 (1H, dd, $J = 7.2$, 10.8 Hz), 4.24 (1H, dd, $J = 7.2$, 10.8 Hz), 4.51 (1H, q, $J = 4.8$, 8.4 Hz), 4.56 (1H, d, $J = 6.6$ Hz), 4.93 (1H, t, $J = 6.6$ Hz), 5.19 (1H, t, $J = 6.6$ Hz), 7.00 (1H, s), 7.01 (1H, t, $J = 7.2$ Hz), 7.10 (1H, t, $J = 7.2$ Hz), 7.17 (1H, d, $J = 7.2$ Hz), 7.22 (2H, m) 7.31 (2H, m), 7.51 (2H, d, $J = 7.2$ Hz); 7.59 (1H, d, $J = 7.2$ Hz) 7.71 (2H, d, $J = 7.2$ Hz). NMR (150 MHz) δ_C (CD_3OD) 16.4, 17.7, 25.8, 27.3, 28.7, 40.4, 44.8, 68.0, 110.7, 110.7, 119.6, 119.9, 120.9, 121.5, 122.4, 124.9, 126.2, 126.3, 127.5, 128.1, 128.7, 129.7, 132.5, 137.8, 140.3, 142.5, 145.2, 145.2, 158.3, 175.6. *Anal.* Found: C, 76.86; H, 6.98; N, 4.83. Calcd. for $C_{36}H_{38}N_2O_4$: C, 76.84; H, 6.81; N, 4.98.

*1-Triisopropylsilyl-4-geranylgramine **6***. To a solution of 1-(triisopropylsilyl)gramine **5** (4.98 g, 15.1 mmol) in Et_2O (78 ml) at −78 °C under nitrogen was added *t*-BuLi (1.7 M in heptane, 10 ml, 17 mmol). After stirring at 0 °C for 1 h, geranyl bromide (2.4 ml, 12.1 mmol) was dropped at 0 °C. The reaction mixture was stirred at 0 °C overnight. The reaction mixture was quenched with H_2O (50 ml), and then the aqueous layer was extracted with Et_2O (4 × 50 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 50/1→20/1) to give 1-(triisopropylsilyl)-4-geranylgramine **6** (3.07 g, 6.59 mmol, 44%) as a pale yellow oil. NMR (400 MHz) δ_H ($CDCl_3$) 1.15 (18H, d, $J = 7.6$ Hz), 1.61 (3H, s), 1.69 (3H, s), 1.76 (3H, s), 2.05–2.17 (4H, m), 2.25 (6H, s), 3.59 (2H, s), 3.94 (2H, d, $J = 6.8$ Hz), 5.14 (1H, t, $J = 6.0$ Hz), 5.46 (1H, t, $J = 6.4$ Hz), 6.91 (1H, d, $J = 7.8$ Hz), 7.04 (1H, t, $J = 7.8$ Hz), 7.09 (1H, s), 7.32 (1H, d, $J = 7.8$ Hz). NMR (100 MHz) δ_C ($CDCl_3$) 12.8, 16.3, 17.7, 18.2, 25.7, 26.7, 31.6, 39.7, 44.9, 56.6, 111.7, 115.9, 119.8, 121.4, 124.4, 124.5, 129.0, 131.2, 131.4, 134.8, 135.1, 142.2. *Anal.* Found: C, 76.98; H, 11.24; N, 5.84. Calcd. for $C_{30}H_{50}N_2Si$: C, 77.19; H, 10.80; N, 6.00.

*Compound **8***. To a solution of 1-Boc-4-geranylgramine **7** (685 mg, 1.67 mmol) in toluene (15 ml) at room temperature under nitrogen was dropped ethyl chloroformate (0.18 ml, 1.89 mmol). After the reaction mixture had been stirred for 15 min, the solvent was evaporated. The residue was dissolved in THF (15 ml) under nitrogen and used without any further purification. To a solution of Schollköpf chiral auxiliary **4** (414 mg, 1.95 mmol) in THF (20 ml) was dropped *n*-BuLi (1.7 M in hexane, 1.3 ml, 2.2 mmol) at −78 °C under nitrogen. After stirring at −78 °C for 60 min, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) (0.33 ml, 2.19 mmol) was added at −78 °C. After stirring for 30 min at −78 °C, the solution prepared above was dropped in at −78 °C. The reaction mixture was stirred and warmed to 0 °C for 18 h. It was poured into H_2O. (20 ml), and then the aqueous layer was extracted with Et_2O (4 × 20 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 50/1) to give compound **8** (756 mg, 1.31 mmol, 78%) as a pale yellow oil. $[\alpha]^{27}_D$ −11° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 0.72 (3H, d, $J = 7.2$ Hz), 1.03 (3H, d, $J = 7.2$ Hz), 1.21 (3H, t, $J = 7.2$ Hz), 1.30 (3H, t, $J = 7.2$ Hz), 1.60 (3H, s), 1.65 (9H, s), 1.69 (3H, s), 1.76 (3H, s), 2.07–2.14 (4H, m), 2.25 (1H, m), 3.12 (1H, dd, $J = 7.2$, 14.8 Hz), 3.55 (1H, dd, $J = 4.6$, 14.8 Hz), 3.77 (1H, t, $J = 3.4$ Hz), 3.84–4.28 (7H, m), 5.13 (1H, t, $J = 7.0$ Hz), 5.34 (1H, t, $J = 6.5$ Hz), 7.01 (1H, d, $J = 7.8$ Hz), 7.19 (1H, t, $J = 7.8$ Hz), 7.45 (1H, s), 8.08 (1H, d, $J = 7.8$ Hz). NMR (100 MHz) δ_C ($CDCl_3$) 14.3, 16.3, 16.7, 17.7, 19.0, 25.7, 26.7, 28.2, 31.3, 31.8, 31.9, 39.7, 56.5, 60.5, 60.6, 60.7, 82.9, 113.0, 117.6, 123.1, 123.6, 123.9, 124.3, 124.6, 129.0, 131.4, 135.0, 135.8,

135.9, 149.6, 162.8, 163.2. *Anal.* Found: C, 72.76; H, 9.13; N, 7.26. Calcd. for $C_{35}H_{51}N_3O_4$: C, 72.75; H, 8.90; N, 7.27.

*Compound **12***. To a solution of 1-Boc-gramine **9** (9.35 g, 34.1 mmol) in toluene (200 ml) at room temperature under nitrogen was dropped ethyl chloroformate (3.5 ml, 36.9 mmol). After the reaction mixture had been stirred for 15 min, the solvent was evaporated. The residue was recrystallized from hexane/benzene to give 1-Boc-3-(chloromethyl)indole (7.80 g, 29.4 mmol, 86%) as a white crystalline solid. To a solution of Schollköpf chiral auxiliary **4** (1.82 g, 8.57 mmol) in THF (80 ml) was dropped *n*-BuLi (1.7 M in hexane, 5.5 ml, 9.35 mmol) at −78 °C under nitrogen. After stirring at −78 °C for 60 min, TMEDA (1.7 ml, 11.3 mmol) was added to the mixture at −78 °C. After stirring for 60 min at −78 °C, 1-Boc-3-(chloromethyl)gramine (2.95 g, 11.1 mmol) in THF (20 ml) was dropped into the mixture at −78 °C. The reaction mixture was stirred and warmed to 0 °C for 18 h. It was poured into H_2O (50 ml), and the aqueous layer was extracted with Et_2O (4 × 100 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 100/1→20/1) to give compound **12** (3.35 g, 7.56 mmol, 88%) as a white powder. $[\alpha]^{27}_D$ +52° (*c* 1.0, CH_3OH). NMR (600 MHz) δ_H ($CDCl_3$) 0.67 (3H, d, J = 6.8 Hz), 0.95 (3H, d, J = 6.8 Hz), 1.24 (3H, t, J = 7.1 Hz), 1.33 (3H, t, J = 7.1 Hz), 1.65 (9H, s), 2.18 (1H, m), 3.17 (1H, dd, J = 5.8, 14.3 Hz), 3.21 (dd, 1H, J = 3.4, 14.3 Hz), 3.56 (t, 1H, J = 3.3 Hz), 3.78 (d, 1H, J = 6.0 Hz), 4.02 (1H, m), 4.09–4.18 (3H, m), 4.29 (1H, m), 7.18 (1H, t, J = 7.8 Hz), 7.25 (1H, t, J = 7.8 Hz), 7.37 (1H, s), 7.59 (1H, d, J = 7.8 Hz), 8.09 (1H, br). NMR (150 MHz) δ_C ($CDCl_3$) 14.4, 16.6, 19.0, 28.2, 29.2, 31.7, 56.0, 60.4, 60.5, 60.6, 83.1, 114.9, 116.7, 119.6, 122.0, 123.9, 124.2, 131.4, 135.1, 149.7, 162.3, 163.5. *Anal.* Found: C, 59.61; H, 6.85; N, 8.77. Calcd. for $C_{25}H_{35}N_3O_4$: C, 59.61; H, 6.88; N, 8.69.

*Intermediate **15***. To a solution of compound **12** (910 mg, 2.06 mmol) in Et_2O (15 ml) was dropped lithium diisopropylamide (LDA) (0.5 M in Et_2O, 10 ml, 5.00 mmol) at −78 °C under nitrogen. After stirring at −78 °C for 90 min, CuCN (185 mg, 2.06 mmol) was added to the mixture at −78 °C. After stirring for 90 min at −78 °C, geranyl bromide (547 mg, 2.52 mmol) was added to the mixture at −78 °C. The reaction mixture was stirred overnight. The reaction mixture was quenched with H_2O (10 ml), and the aqueous layer was extracted with Et_2O (4 × 50 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 100/1→50/1) to give intermediate **15** (798 mg, 1.38 mmol, 67%) as a yellow oil. $[\alpha]^{22}_D$ +29° (*c* 1.0, $CHCl_3$). NMR (600 MHz) δ_H ($CDCl_3$) 0.64 (3H, d, J = 6.6 Hz), 0.96 (3H, d, J = 6.6 Hz), 1.20 (3H, t, J = 7.2 Hz), 1.33 (3H, t, J = 7.2 Hz), 1.56 (3H, s), 1.64 (3H, s), 1.65 (9H, s), 1.74 (3H, s), 1.95–2.04 (4H, m), 2.17–2.23 (1H, m), 2.94 (1H, dd, J = 7.8, 14.4 Hz), 3.30 (1H, dd, J = 4.2, 14.4 Hz), 3.61 (1H, t, J = 6.6 Hz), 3.78 (1H, d, J = 6.0 Hz), 4.02–4.10 (3H, m), 4.19–4.25 (2H, m), 5.06 (1H, t, J = 6.0 Hz), 5.20 (1H, t, J = 6.0 Hz), 7.16 (1H, t, J = 7.2 Hz), 7.20 (1H, t, J = 7.2 Hz), 7.54 (1H, d, J = 7.2 Hz), 8.06 (1H, d, J = 7.2 Hz). NMR (150 MHz) δ_C ($CDCl_3$) 14.4, 16.5, 16.5, 17.6, 19.1, 25.6, 26.0, 26.6, 28.1, 29.4, 31.4, 39.6, 56.9, 60.4, 60.4, 60.7, 83.3, 115.0, 115.4, 119.0, 121.8, 122.1, 123.2, 124.2, 130.6, 131.4, 135.2, 136.0, 137.9, 150.5, 162.8, 163.2. *Anal.* Found: C, 72.75; H, 8.95; N, 7.30. Calcd. for $C_{35}H_{51}N_3O_4$: C, 72.75; H, 8.90; N, 7.27.

*Intermediate **18***. To a solution of diethylbutanoyl derivative **17** (419 mg, 0.896 mmol) and TMEDA (0.27 ml, 1.79 mmol) in Et_2O (10 ml) was dropped *s*-BuLi (1.3 M in Et_2O, 0.49 ml, 0.637 mmol) at −78 °C under nitrogen. After stirring at −78 °C for 1 h, geranyl bromide (0.09 ml, 98.1 mg) was added to the mixture. The reaction mixture was stirred at −78 °C for 5 h. It was quenched with H_2O (5 ml), and the aqueous layer was extracted with Et_2O (3 × 20 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 100/1→50/1) and ODS column chromatography (CH_3CN/*i*-PrOH 100/0→9/1) to give intermediate **18** (171 mg, 0.366 mmol, 41%) as a yellow oil. $[\alpha]^{24}_D$ +43° (*c* 0.10, $CHCl_3$). NMR (600 MHz) δ_H ($CDCl_3$) 0.67 (3H, d, J = 7.2 Hz), 0.88 (9H, t, J = 7.2 Hz), 0.94 (3H, d, J = 7.2 Hz), 1.23 (3H, t, J = 7.2 Hz), 1.30 (3H, t, J = 7.2 Hz), 1.61 (3H, s), 1.69 (3H, s), 1.70 (3H, s), 1.74 (6H, q, J = 7.2 Hz), 2.05–2.22 (5H, m), 3.17–3.25 (2H, m), 3.33–3.42 (2H, m), 3.51 (1H, t, J = 3.6 Hz), 3.93–4.04 (1H, m), 4.05–4.10 (1H, m), 4.11–4.20 (2H, m), 4.34 (1H, m), 5.14 (1H, t, J = 6.6 Hz), 5.31 (1H, t, J = 6.6 Hz), 7.16 (1H, t, J = 7.2 Hz), 7.19 (1H, t, J = 7.2 Hz), 7.37 (1H, s), 7.44 (1H, d, J = 7.2 Hz). NMR (150 MHz) δ_C ($CDCl_3$) 8.7, 14.3, 14.4, 15.2, 16.1, 16.6, 17.7, 19.0, 25.7, 26.6, 27.9, 29.1, 31.6, 32.9, 39.8, 52.4, 55.8, 60.5, 60.6, 116.7, 116.8, 122.5, 122.8, 123.3, 124.4, 125.3, 129.1, 131.4, 131.8, 135.6, 136.8, 162.2, 163.7, 176.0. *Anal.* Found: C, 75.58; H, 9.45; N. 6.77. Calcd. for $C_{38}H_{57}N_3O_3$: C, 75.58; H, 9.51; N, 6.96.

*Pinacol borate **19***. To a suspension of compound **13** (1.97 g, 3.78 mmol), $PdCl_2(dppf)\cdot CH_2Cl_2$ (155 mg, 0.190 mmol) and triethylamine (1.6 ml, 1.16 g, 11.5 mmol) in toluene (38 ml) at 90 °C under nitrogen was dropped pinacolborane (0.84 ml, 741 mg, 5.79 mmol). The reaction mixture was stirred for 9 h at 90 °C. The reaction mixture was filtered through a glass filter and the residue was washed with EtOAc (3 × 20 ml). The combined filtrate was washed with H_2O and sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 20/1→12/1) to give pinacol borate **19** (1.91 g, 3.36 mmol, 89%) as a white powder. $[\alpha]^{25}_D$ +23° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 0.66 (d, 3H,

$J = 6.8$ Hz), 0.93 (d, 3H, $J = 6.8$ Hz), 1.22 (t, 3H, $J = 7.1$ Hz), 1.30–1.35 (m, 15H), 1.64 (s, 9H), 2.16 (m, 1H), 3.15–3.29 (m, 2H), 3.50 (t, 1H, $J = 3.5$ Hz), 4.02 (m, 1H), 4.07–4.18 (m, 3H), 4.31 (m, 1H), 7.33 (s, 1H), 7.71 (dd, 1H, $J = 1.1$, 8.3 Hz), 8.06–8.09 (m, 2H). NMR (100 MHz) δ_C ($CDCl_3$) 14.4, 14.4, 16.6, 19.0, 24.8, 25.0, 28.1, 29.1, 31.7, 56.0, 60.4, 60.5, 60.6, 83.2, 83.5, 114.1, 117.0, 124.2, 127.1, 130.4, 131.0, 137.3, 149.6, 162.2, 163.5. *Anal.* Found: C, 65.54; H, 8.23; N, 7.51. Calcd. for $C_{36}H_{38}N_2O_4$: C, 65.61; H, 8.17; N, 7.40.

*Pinacol borate **20***. $[\alpha]^{26}_D$ +24° (*c* 1.0, $CHCl_3$). NMR (600 MHz) δ_H ($CDCl_3$) 0.66 (3H, d, $J = 7.2$ Hz), 0.95 (3H, d, $J = 7.2$ Hz), 1.24 (3H, t, $J = 7.2$ Hz), 1.32 (3H, t, $J = 7.2$ Hz), 1.64 (9H, s), 2.17 (1H, m), 3.16 (2H, d, $J = 4.8$ Hz), 3.56 (1H, t, $J = 3.6$ Hz), 3.98–4.18 (4H, m), 4.27 (1H, m), 7.30 (2H, m), 7.44 (1H, d, $J = 8.4$ Hz), 8.30 (1H, br). NMR (100 MHz) δ_C ($CDCl_3$) 14.4, 16.6, 19.0, 28.1, 29.1, 31.9, 55.9, 60.4, 60.5, 60.7, 83.7, 116.6, 117.7, 118.1, 120.8, 125.0, 125.2, 130.3, 135.8, 149.3, 162.0, 163.6. *Anal.* Found: C, 57.76; H, 6.62; N, 7.96. Calcd. for $C_{25}H_{34}BrN_3O_4$: C, 57.69; H, 6.58; N, 8.07.

*Intermediate **21***. To a suspension of pinacol borate **19** (155 mg, 0.273 mmol) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (23.0 mg, 0.0282 mmol) in THF (6.8 ml) and 2 M aqueous KOH (1.37 ml) at 60 °C under nitrogen was dropped geranyl bromide (0.16 ml, 174 mg, 0.803 mmol). The reaction mixture was stirred for 48 h at 60 °C. It was filtered through a glass filter and the residue was washed with Et_2O (3 × 20 ml). The combined filtrate was washed with H_2O and sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 100/1→30/1) to give intermediate **21** (93.0 mg, 0.161 mmol, 59%) as a pale yellow oil. $[\alpha]^{25}_D$ +15° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 0.67 (3H, d, $J = 6.8$ Hz), 0.95 (3H, d, $J = 6.8$ Hz), 1.23 (3H, t, $J = 7.1$ Hz), 1.32 (3H, t, $J = 7.1$ Hz), 1.61 (3H, s), 1.64 (9H, s), 1.69 (3H, s), 1.74 (3H, s), 2.03–2.21 (5H, m), 3.12–3.20 (2H, m), 3.43 (2H, d, $J = 7.1$ Hz), 3.56 (1H, t, $J = 3.4$ Hz), 3.98–4.20 (4H, m), 4.29 (1H, m), 5.11 (1H, m), 5.37 (1H, m), 7.09 (1H, d, $J = 8.4$ Hz), 7.32 (1H, s), 7.38 (1H, s), 7.93 (1H, d, $J = 8.4$ Hz). NMR (100 MHz) δ_C ($CDCl_3$) 14.4, 16.2, 16.7, 17.7, 19.0, 25.7, 26.7, 28.2, 29.2, 31.7, 34.3, 39.8, 56.1, 60.5, 60.6, 82.9, 114.7, 116.6, 118.9, 123.9, 124.3, 124.6, 131.4, 131.7, 135.5, 149.7, 162.3, 163.5. *Anal.* Found: C, 72.65; H, 9.13; N. 7.23. Calcd. for $C_{35}H_{51}N_3O_4$: C, 72.75; H, 8.90; N, 7.27.

*Intermediate **22***. $[\alpha]^{27}_D$ +16° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 0.66 (3H, d, $J = 6.8$ Hz), 0.95 (3H, d, $J = 6.9$ Hz), 1.23 (3H, t, $J = 7.0$ Hz), 1.32 (3H, t, $J = 7.1$ Hz), 1.60 (3H, s), 1.64 (9H, s), 1.68 (3H, s), 1.74 (3H, s), 2.02–2.14 (4H, m), 2.18 (1H, m), 3.12–3.20 (2H, m), 3.46 (2H, d, $J = 7.2$ Hz), 3.57 (1H, t, $J = 3.4$ Hz), 3.98–4.20 (4H, m), 4.28 (1H, m), 5.10 (1H, m), 5.39 (1H, t, $J = 7.2$ Hz), 7.03 (1H, d, $J = 8.0$ Hz), 7.29 (1H, s), 7.47 (1H, d, $J = 8.0$ Hz), 7.93 (1H, br). NMR (100 MHz) δ_C ($CDCl_3$) 14.4, 16.2, 16.6, 17.7, 19.0, 25.7, 26.7, 28.2, 29.3, 31.7, 34.7, 39.8, 56.0, 60.4, 60.5, 60.6, 82.9, 114.6, 116.7, 119.3, 122.7, 123.7, 123.7, 124.3, 129.5, 131.4, 135.6, 138.0, 149.8, 162.3, 163.4. *Anal.* Found: C, 72.61; H, 8.99; N. 7.15. Calcd. for $C_{35}H_{51}N_3O_4$: C, 72.75; H, 8.90; N, 7.27.

*Compound **23***. According to the procedure described for compound **16**, intermediate **15** (47.0 mg, 0.0813 mmol) in THF (0.18 ml), and NaOMe (28% in CH_3OH, 0.08 ml, 22.4 mg, 0.415 mmol) gave compound **23** (33.5 mg, 0.0701 mmol, 86%) as a colorless oil. $[\alpha]^{22}_D$ +35° (*c* 1.0, $CHCl_3$). NMR (600 MHz) δ_H ($CDCl_3$) 0.61 (3H, d, $J = 6.6$ Hz), 0.91 (3H, d, $J = 6.6$ Hz), 1.20 (3H, t, $J = 7.2$ Hz), 1.34 (3H, t, $J = 7.2$ Hz), 1.63 (3H, s), 1.74 (6H, s), 2.09–2.18 (5H, m), 3.15 (1H, dd, $J = 6.0$, 14.4 Hz), 3.22 (1H, t, $J = 3.6$ Hz), 3.29 (1H, dd, $J = 4.2$, 14.4 Hz), 3.48 (1H, d, $J = 6.6$ Hz), 3.99–4.05 (1H, m), 4.08–4.12 (2H, m), 4.17–4.24 (1H, m), 4.28–4.30 (1H, m), 5.12 (1H, t, $J = 6.6$ Hz), 5.31 (1H, t, $J = 6.6$ Hz), 7.01 (1H, t, $J = 7.2$ Hz), 7.06 (1H, t, $J = 7.2$ Hz), 7.21 (1H, d, $J = 7.2$ Hz), 7.56 (1H, d, $J = 7.2$ Hz) 7.74 (1H, s). NMR (150 MHz) δ_C ($CDCl_3$) 14.3, 14.4, 16.1, 16.4, 17.7, 19.1, 25.0, 25.8, 26.4, 29.0, 30.9, 39.6, 57.3, 60.1, 60.3, 60.5, 107.2, 109.9, 118.7, 119.0, 120.6, 120.7, 124.1, 129.7, 131.8, 135.0, 135.5, 138.1, 162.9, 163.4. *Anal.* Found: C, 75.44; H, 8.95; N, 8.55. Calcd. for $C_{30}H_{43}N_3O_2$: C, 75.43; H, 9.07; N, 8.80.

*Compound **24***. $[\alpha]^{26}_D$ +5.2° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 0.66 (3H, d, $J = 6.8$ Hz), 0.97 (3H, d, $J = 6.9$ Hz), 1.16 (3H, t, $J = 7.1$ Hz), 1.26 (3H, t, $J = 7.1$ Hz), 1.58 (3H, s), 1.67 (3H, s), 1.74 (3H, s), 2.05–2.12 (4H, m), 2.19 (1H, m), 3.18 (1H, dd, $J = 6.6$, 14.8 Hz), 3.55–3.61 (2H, m), 3.84 (1H, m), 3.91–4.08 (4H, m), 4.20 (1H, m), 4.27 (1H, m), 5.12 (1H, m), 5.38 (1H, m), 6.85 (1H, d, $J = 7.6$ Hz), 6.97 (1H, d, $J = 2.3$ Hz), 7.03 (1H, t, $J = 7.6$), 7.15 (1H, d, $J = 7.6$ Hz), 7.99 (1H, br). NMR (100 MHz) δ_C ($CDCl_3$) 14.3, 16.3, 16.6, 17.7, 19.1, 25.7, 26.7, 31.4, 31.6, 32.1, 39.8, 57.3, 60.5, 60.6, 109.0, 112.8, 119.6, 121.6, 123.0, 123.9, 124.4, 126.0, 131.3, 134.8, 135.4, 136.5, 163.0, 163.3. *Anal.* Found: C, 75.44; H, 9.31; N, 8.77. Calcd. for $C_{30}H_{43}N_3O_2$: C, 75.43; H, 9.07; N, 8.80.

*Ethyl ester **28***. To a solution of compound **23** (32.1 mg, 0.0672 mmol) in THF (0.65 ml) at 0 °C was dropped 1.5 M aqueous HCl (0.44 ml). The reaction mixture was stirred at 0 °C for 2 h. It was quenched with sat. aqueous $NaHCO_3$ (5 ml), and then the aqueous layer was extracted with EtOAc (4 × 10 ml). The organic extract was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography ($CHCl_3/CH_3OH$ 50/1) to give ethyl ester **28** (20.0 mg, 0.0543 mmol, 81%) as a yellow oil. $[\alpha]^{25}_D$ +2.2° (*c* 0.50, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 1.23 (1H, t, $J = 7.2$ Hz), 1.63 (3H, s), 1.73 (3H, s), 1.75 (3H, s), 2.08–2.20 (4H, m), 2.97 (1H, dd, $J = 8.3$, 14.3 Hz), 3.28 (1H, dd, $J = 5.1$, 14.3 Hz), 3.51 (2H, d, $J = 7.2$ Hz), 3.79 (1H, m), 4.15 (2H, m), 5.11 (1H, m), 5.32 (1H, t, $J = 7.3$ Hz), 6.98 (1H, s), 7.08 (1H, t, $J = 7.2$ Hz), 7.12 (1H, t, $J = 7.2$ Hz), 7.27 (1H, d,

$J = 7.2$ Hz), 7.54 (1H, d, $J = 7.2$ Hz), 7.84 (1H, br). NMR (100 MHz) δ_C ($CDCl_3$) 14.1, 16.2, 17.8, 25.0, 25.8, 26.4, 30.2, 39.6, 55.4, 60.9, 106.5, 110.4, 118.2, 119.4, 120.0, 121.2, 124.0, 128.9, 131.9, 135.2, 135.7, 138.6, 175.4. *Anal.* Found: C, 74.96; H, 8.75; N, 7.75. Calcd. for $C_{23}H_{32}N_2O_2$: C, 74.96; H, 8.75; N, 7.60.

Ethyl ester ***29***. $[\alpha]^{24}_D$ +17° (*c* 1.0, $CHCl_3$). NMR (400 MHz) δ_H ($CDCl_3$) 1.23 (3H, t, $J = 7.1$ Hz), 1.59 (3H, s), 1.68 (3H, s), 1.74 (3H, s), 2.04–2.13 (4H, m), 2.97 (1H, dd, $J = 8.9$, 14.6 Hz), 3.50 (1H, dd, $J = 4.5$, 14.6 Hz), 3.71–3.82 (3H, m), 4.18 (2H, q, $J = 7.1$ Hz), 5.12 (1H, t, $J = 6.2$ Hz), 5.38 (1H, t, $J = 6.2$ Hz), 6.91 (1H, d, $J = 7.6$ Hz), 6.99 (1H, s), 7.08 (1H, t, $J = 7.6$ Hz), 7.17 (1H, d, $J = 7.6$ Hz), 8.47 (1H, br). NMR (100 MHz) δ_C ($CDCl_3$) 14.1, 14.2, 16.3, 17.6, 25.6, 26.6, 31.9, 33.0, 39.6, 55.7, 60.8, 109.3, 111.8, 119.8, 122.1, 123.4, 123.5, 124.3, 125.1, 131.3, 134.5, 135.9, 137.1, 175.2. *Anal.* Found: C, 74.97; H, 8.89; N, 7.56. Calcd. for $C_{23}H_{31}N_2O_2$: C, 74.96; H, 8.89; N, 7.60.

Fmoc-Trp(2-Ger) ***33***. To a solution of ethyl ester **28** (267 mg, 0.725 mmol) in THF (5 ml) was dropped 1 M aqueous LiOH (5 ml) at room temperature. After the reaction mixture had been stirred for 1 h, it was neutralized with 5% aqueous $KHSO_4$, and H_2O (10 ml) was added. The reaction mixture was extracted with EtOAc (5 × 20 ml). The organic solvent was removed by evaporation. The residue was dissolved in dioxane (10 ml) and 1 M aqueous Na_2CO_3 (10 ml). To the solution was added Fmoc-OSu (337 mg, 1.00 mmol) at room temperature. After the reaction mixture had been stirred for 3 h, the reaction was quenched with 5% aqueous $KHSO_4$ (10 ml). The reaction mixture was extracted with EtOAc (4 × 30 ml). The organic layer was washed with sat. brine, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography ($CHCl_3/CH_3OH$ 100/1→ 50/1) to give Fmoc-Trp(2-Ger) **33** (246 mg, 0.437 mmol, 60%) as a yellow oil. $[\alpha]^{23}_D$ −0.62° (*c* 1.0, CH_3OH). NMR (600 MHz) δ_H (CD_3COCD_3) 1.54 (3H, s), 1.61 (3H, s), 1.76 (3H, s), 2.01–2.14 (4H, m), 3.26 (1H, dd, $J = 7.7$, 14.6 Hz), 3.39 (1H, dd, $J = 5.6$, 14.6 Hz), 3.55–3.62 (2H, m), 4.16 (1H, t, $J = 7.2$ Hz), 4.24 (1H, d, $J = 7.4$ Hz), 5.08 (1H, m), 5.41 (1H, t, $J = 6.8$ Hz), 6.52 (1H, d, $J = 8.5$ Hz), 6.99 (1H, t, $J = 7.0$ Hz), 7.01 (1H, t, $J = 7.0$ Hz), 7.25–7.30 (3H, m), 7.38 (2H, t, $J = 7.4$ Hz) 7.61–7.65 (3H, m) 7.82 (2H, d, $J = 7.6$ Hz). NMR (150 MHz) δ_C (CD_3COCD_3) 16.2, 17.5, 25.6, 25.6, 27.0, 27.5, 29.2, 40.2, 47.7, 55.6, 67.0, 106.4, 111.2, 118.5, 119.4, 120.5, 121.1, 121.7, 124.8, 125.9, 127.7, 129.6, 131.6, 136.6, 137.2, 137.3, 141.8, 144.8, 144.8, 156.6, 173.6. *Anal.* Found: C, 76.67; H, 6.93; N, 5.20. Calcd. for $C_{36}H_{38}N_2O_4$: C, 76.84; H, 6.81; N, 4.98.

Solid-phase peptide synthesis.

Peptide bond formation. Solid-phase peptide synthesis was performed on a peptide synthesizer using Fmoc amino acid synthesis protocol with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) reagent, except that geranyl substituted for Fmoc-tryptophan, which was conducted manually as follows: To a suspension of Glu(Pp)-Gln-Clt resin (0.10 mmol), which was synthesized with peptide synthesizer, in *N*-methyl-2-pyrrolidinone (NMP) (1 ml) under nitrogen was added Fmoc-Trp(n-Ger) (85 mg, 0.15 mmol) in NMP (2 ml), diisopropylethylamine (DIPEA) (0.40 mmol) in DMF (2 ml), and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (0.15 mmol) in DMF (2 ml) at room temperature under nitrogen. The reaction mixture was shaken overnight. It was filtered with membrane filter (PTFE, 0.45 μm), washed with CH_3CN (2 × 5 ml) and dried *in vacuo* to give Fmoc-Trp(n-Ger)-Glu(Pp)-Gln-Clt resin. The resin was treated with peptide synthesizer again to give resin attached to the full length amino acid residues.

Cleavage, deprotection, and purification.

1-Geranyl ***38***. To a resin (67 μmol), 4% TFA in CH_2Cl_2 (0.44 ml) was dropped. The reaction mixture was stirred at room temperature for 1 h. It was filtered with plug of cottons, and washed with 50% aqueous CH_3CN (4 × 10 ml). The filtrate was neutralized with 1% aqueous ammonium, concentrated *in vacuo* to remove organic solvent, and the aqueous residue was freeze-dried. The residue was dissolved with 50% aqueous CH_3CN and filtered with membrane filter (PTFE, 0.45 μm). The filtrate was purified by HPLC on an ODS column (10 × 250 mm ID, Develosil ODS-HG-5, Nomura Chemical) at a flow rate of 3.0 ml/min, with a gradient of 20–60% CH_3CN in 0.1% aqueous ammonium acetate over 45 min. After freeze-drying, the putative peptide **38** (2.4 mg, 2.6 mmol, 4.0%) was obtained as a white powder. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.55 (3H, d, $J = 6.6$ Hz), 0.70 (3H, t, $J = 7.2$ Hz), 0.91 (1H, m), 1.15 (1H, m), 1.50 (3H, s), 1.56 (3H, s), 1.59 (1H, m), 1.79 (3H, s), 1.79–1.85 (3H, m), 1.94–2.20 (9H, m), 2.71 (1H, dd, $J = 8.4$, 14.4 Hz), 2.89 (1H, dd, $J = 4.8$, 14.4 Hz), 3.08 (1H, dd, $J = 8.4$, 14.4 Hz), 3.19 (2H, s), 3.27 (1H, dd, $J = 4.8$, 14.4 Hz), 3.98 (1H, d, $J = 7.2$ Hz), 4.08 (1H, dd, $J = 4.8$, 8.4 Hz), 4.22 (1H, dd, $J = 4.8$, 9.0 Hz), 4.44 (1H, dd, $J = 4.8$, 9.6 Hz), 4.59 (1H, dd, $J = 4.8$, 9.6 Hz), 4.69 (2H, d, $J = 6.6$ Hz), 4.98 (1H, m), 5.25 (1H, m), 7.02–7.03 (2H, m), 7.03–7.09 (2H, m), 7.14–7.18 (4H, m), 7.29 (1H, d, $J = 7.8$ Hz), 7.56 (1H, d, $J = 8.4$ Hz). HRMS *m*/*z* ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.4971.

2-Geranyl ***39***. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.60 (3H, d, $J = 6.6$ Hz), 0.73 (3H, t, $J = 7.2$ Hz), 0.96 (1H, m), 1.23 (1H, m), 1.51 (3H, s), 1.57 (3H, s), 1.59 (1H, m), 1.82 (3H, s), 1.82–1.85 (3H, m), 1.97–2.18 (9H, m), 2.79 (1H, dd, $J = 8.4$, 14.4 Hz), 2.90 (1H, dd, $J = 4.8$, 14.4 Hz), 3.06 (1H, dd, $J = 8.4$, 14.4 Hz), 3.15 (1H, dd, $J = 8.4$, 14.4 Hz), 3.23 (2H, s), 3.38 (2H, d, $J = 7.4$ Hz), 3.97–3.99 (2H, m), 4.16 (1H, dd, $J = 4.8$,

9.0 Hz), 5.07 (1H, m), 5.28 (1H, m), 7.02–7.09 (4H, m), 7.21–7.25 (3H, m), 7.30 (1H, d, $J = 7.9$ Hz), 7.38 (1H, d, $J = 7.6$ Hz). HRMS m/z ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.4966.

4-Geranyl ***40***. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.62 (3H, d, $J = 6.6$ Hz), 0.72 (3H, t, $J = 7.2$ Hz), 0.95 (1H, m), 1.21 (1H, m), 1.55 (3H, s), 1.62 (3H, s), 1.63 (1H, m), 1.68 (3H, s), 1.78–1.81 (3H, m), 1.96–2.16 (9H, m), 2.82 (1H, dd, $J = 9.6$, 14.4 Hz), 3.02 (1H, dd, $J = 8.4$, 14.4 Hz), 3.17–3.21 (3H, m), 3.38 (1H, dd, $J = 7.2$, 14.4 Hz), 3.71 (2H, t, $J = 6.0$ Hz), 4.01–4.05 (2H, m), 4.21 (1H, dd, $J = 4.8$, 8.4 Hz), 4.55–4.60 (2H, m), 5.09 (1H, m), 5.30 (1H, m), 6.81 (1H, d, $J = 7.2$ Hz), 7.00–7.03 (2H, m), 7.12–7.14 (2H, m), 7.18–7.24 (4H, m). HRMS m/z ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.5000.

5-Geranyl ***41***. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.53 (3H, d, $J = 6.7$ Hz), 0.72 (3H, t, $J = 7.3$ Hz), 0.91 (1H, m), 1.14 (1H, m), 1.46 (1H, m), 1.56 (3H, s), 1.62 (3H, s), 1.71 (3H, s), 1.78–1.87 (3H, m), 1.91–2.22 (9H, m), 2.82 (1H, dd, $J = 9.4$, 14.0 Hz), 3.02 (1H, dd, $J = 5.4$, 14.0 Hz), 3.08 (1H, dd, $J = 7.1$, 14.4 Hz), 3.07–3.10 (3H, m), 3.41 (2H, d, $J = 7.1$ Hz), 3.97 (1H, dd, $J = 7.3$, 12.6 Hz), 4.07 (1H, d, $J = 8.3$ Hz), 4.48 (1H, dd, $J = 5.4$, 9.4 Hz), 4.58 (1H, dd, $J = 6.2$, 7.1 Hz), 5.08 (1H, m), 5.35 (1H, m), 6.95 (1H, d, $J = 8.3$ Hz), 7.07–7.09 (3H, m), 7.17–7.22 (3H, m), 7.30 (1H, d, $J = 8.3$ Hz), 7.36 (1H, s). HRMS m/z ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.4952.

6-Geranyl ***42***. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.47 (3H, d, $J = 6.6$ Hz), 0.69 (3H, t, $J = 7.3$ Hz), 0.89 (1H, m), 1.13 (1H, m), 1.38 (1H, m), 1.54 (3H, s), 1.63 (3H, s), 1.71 (3H, s), 1.82–1.88 (3H, m), 1.95–2.22 (9H, m), 2.73 (1H, dd, $J = 9.0$, 14.4 Hz), 2.91 (1H, dd, $J = 5.4$, 14.4 Hz), 3.06 (1H, dd, $J = 8.4$, 15.0 Hz), 3.21–3.27 (3H, m), 3.40 (2H, d, $J = 7.8$ Hz), 3.92 (1H, d, $J = 7.8$ Hz), 4.08 (1H, dd, $J = 9.4$, 13.8 Hz), 4.48 (1H, dd, $J = 5.4$, 9.0 Hz), 4.59 (1H, dd, $J = 5.4$, 8.4 Hz), 5.09 (1H, m), 5.36 (1H, m), 6.89 (1H, d, $J = 7.8$ Hz), 7.04 (1H, s), 7.07 (2H, d, $J = 6.6$ Hz), 7.16 (1H, s), 7.16–7.23 (3H, m), 7.36 (1H, d, $J = 7.8$ Hz). HRMS m/z ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.4981.

7-Geranyl ***43***. NMR (600 MHz) δ_H (CD_3CN, D_2O) 0.46 (3H, d, $J = 6.6$ Hz), 0.84 (3H, t, $J = 7.3$ Hz), 0.89 (1H, m), 1.19 (1H, m), 1.51 (1H, m), 1.53 (3H, s), 1.64 (3H, s), 1.69 (3H, s), 1.79–1.85 (3H, m), 1.95–2.22 (9H, m), 2.70 (1H, dd, $J = 8.4$, 13.6 Hz), 2.88 (1H, dd, $J = 5.4$, 13.6 Hz), 3.06 (1H, dd, $J = 8.4$, 15.0 Hz), 3.21–3.27 (3H, m), 3.51 (2H, d, $J = 6.6$ Hz), 3.93 (1H, d, $J = 7.8$ Hz), 4.09 (1H, dd, $J = 4.8$, 13.2 Hz), 4.47 (1H, dd, $J = 5.4$, 8.4 Hz), 4.61 (1H, dd, $J = 5.4$, 8.4 Hz), 5.09 (1H, m), 5.39 (1H, m), 6.93 (1H, d, $J = 7.8$ Hz), 7.00 (1H, d, $J = 7.8$ Hz), 7.05 (2H, d, $J = 6.6$ Hz), 7.12 (1H, s), 7.16–7.22 (3H, m), 7.31 (1H, d, $J = 7.8$ Hz). HRMS m/z ($[M + H]^+$): Calcd. for $C_{48}H_{67}N_8O_{10}$ 915.4975, found 915.4994.

LC–MS analysis. A cyanopropyl column (2.0 × 250 mm ID, Develosil CN-UG-5, Nomura Chemical) was attached to a HPLC system and equilibrated with 35% CH_3CN in 0.05% aqueous formic acid over 10 min before injection. The peptides (synthetic peptides; 10 μM in 10 μl, isolated peptide was 5 ml broth in 10 μl) were eluted at a flow rate of 0.2 ml/min with a gradient of 35–45% CH_3CN in 0.05% aqueous formic acid for 20 min. The elution was recorded into mass spectroscopy with Q1MI scan mode (m/z value 915.5).

Results

The molecular formula of ComX$_{RO-E-2}$

The partial purification of $ComX_{RO-E-2}$ was carried out as described previously.[4] Further purification was performed by ODS HPLC with gradient elution of CH_3CN in 0.1% aqueous ammonium acetate. The most active fraction, determined by the β-galactosidase assay, which uses a *srfA–lacZ* reporter construct,[2,5] was applied to a cyanopropyl column, and the peak detected by UV absorbance at 220 nm was collected. This fraction was directly injected into an ESI–TOF mass spectrometer to obtain a HRMS. The mass of the observed $[M + H]^+$ ion was 915.4983, assigned to the composition $C_{48}H_{67}N_8O_{10}$, and the molecular formula of $ComX_{RO-E-2}$ was therefore estimated to be $C_{48}H_{66}N_8O_{10}$. The amino acid sequence of $ComX_{RO-E-2}$ determined from DNA and peptide sequencing was GIFWEQ, calculated to be $C_{38}H_{50}N_8O_{10}$.[4] The remaining $C_{10}H_{16}$ indicated an isoprenoidal group. Based on this result, we estimated that the modified tryptophan in the peptide was simply the substitution for a tryptophanyl proton by a geranyl group.

Geranyl tryptophan synthesis

In order to determine the complete structure of $ComX_{RO-E-2}$, we attempted to synthesize peptides that possess the amino acid sequence of $ComX_{RO-E-2}$ with a geranyl tryptophan (TrpGer) residue. We synthesized six geranyl substituted tryptophan derivatives: 1-geranyl tryptophan [Trp(1-Ger)], 2-geranyl tryptophan [Trp(2-Ger)], 4-geranyl tryptophan [Trp(4-Ger)], 5-geranyl tryptophan [Trp(5-Ger)], 6-geranyl tryptophan [Trp(6-Ger)], and 7-geranyl tryptophan [Trp(7-Ger)]. We then synthesized each geranyl substituted Fmoc-tryptophan [Fmoc-Trp(n-Ger)]. Finally, peptide bond formation was carried out either with a peptide synthesizer or by manual synthesis, with an Fmoc solid-phase peptide synthesis strategy. Protecting groups and resin were cleaved, and HPLC purification was used to isolate the desired products.

For the synthesis of Trp(1-Ger), an appropriate protected tryptophan was used as starting material with the synthetic route shown in Fig. 1. Treatment of N^{α}-phthaloyl tryptophan methyl ester **1** with geranyl bromide and NaH as base in DMF gave the intermediate **2**. The phthaloyl group was cleaved with hydrazine, the

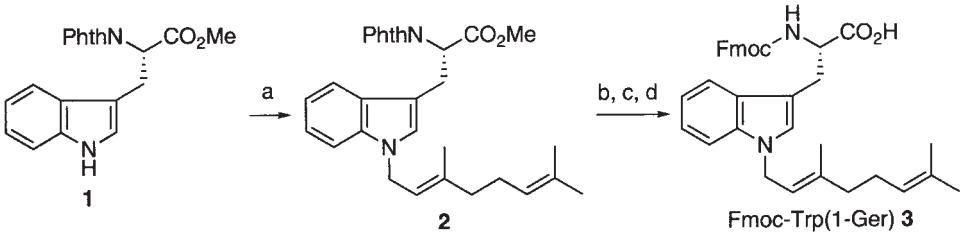

Fig. 1. Synthesis of the Fmoc-Trp(1-Ger) **3**.
Reaction conditions: (a) NaH, geranyl bromide, DMF, 0 °C, 61%. (b) NH_2NH_2, CH_2Cl_2/CH_3OH, 40 °C, 83%. (c) 1 M aqueous LiOH, THF. (d) Fmoc-OSu, 1 M aqueous Na_2CO_3, CH_3CN, 87% in 2 steps.

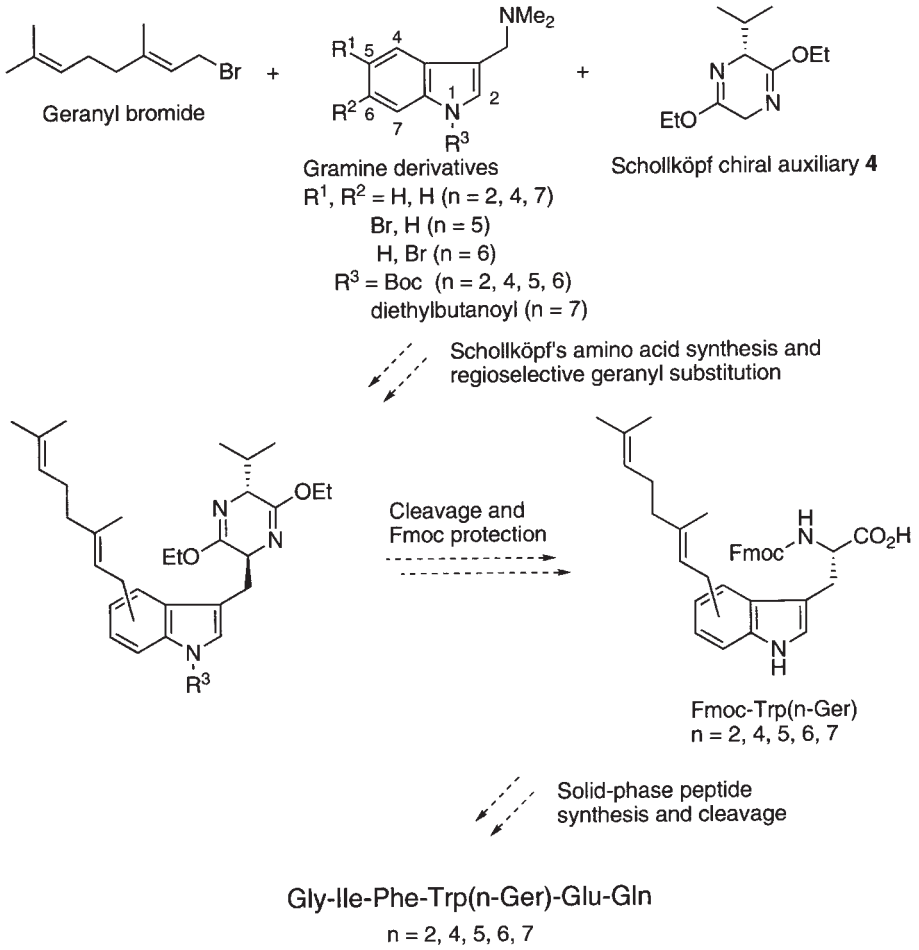

Fig. 2. Synthetic Strategy of Putative Peptides.

methyl ester was hydrolyzed under basic conditions, and an Fmoc group was introduced by a conventional method to give Fmoc-Trp(1-Ger) **3**.

For the other five compounds, we chose convergent and short step synthesis of TrpGer due to its efficiency and versatility. An outline of the synthetic route is illustrated in Fig. 2. We selected geranyl bromide, the Schollköpf chiral auxiliary **4**,[6,7] and suitable gramine derivatives as starting materials. TrpGer derivatives were synthesized with Schollköpf's asymmetric amino acid synthesis and lithium-mediated or palladium-catalyzed geranyl substitution.

The intermediate **8** of Trp(4-Ger) was prepared by regioselective geranyl substitution of 1-(triisopropylsilyl)gramine **5** on the 4-position, as reported by Shinohara *et al.*[8] before Schollköpf's amino acid synthesis, as shown in Fig. 3. Treatment of 1-(triisopropylsilyl)gramine **5** with *t*-BuLi in ether at 0 °C for 1 h was mediated directly by lithiation of gramine at the 4-position assisted by its amino functionality. The resulting species was reacted with geranyl bromide to give the compound **6**. Replacement of the protecting group in 2 steps from triisopropylsilyl (TIPS) to a Boc group was followed by treatment with ethyl chloroformate to give

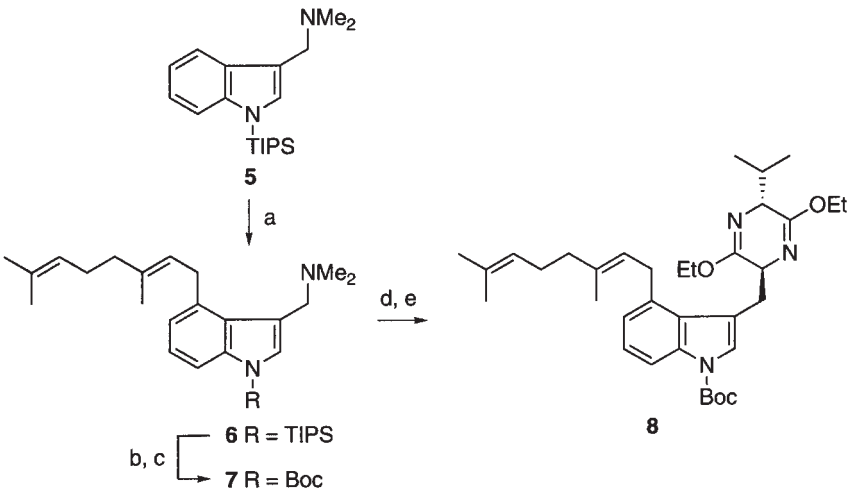

Fig. 3. Synthesis of the Intermediate **8**.
Reaction conditions: (a) *t*-BuLi, Et_2O, DMF, $-78 \rightarrow 0\,^{\circ}C$, 1 h, then geranyl bromide, 44%. (b) TBAF, THF, 77%. (c) $(Boc)_2O$, Et_3N, DMAP, THF, 93%; (d) ethyl chloroformate, toluene. (e) *n*-BuLi, TMEDA, **4**, THF, $-78 \rightarrow 0\,^{\circ}C$, 78% from **7**.

the chloride, which was used in the next step without purification because of its instability. Metallation of the Schollköpf chiral auxiliary **4** with *n*-BuLi in THF was followed by alkylation of the chloride in the presence of TMEDA to provide the intermediate **8** in moderate yield (78%). The addition of TMEDA improved the diastereomeric ratio up to 30:1 (*trans*:*cis*) detected by ^{1}H-NMR and NOESY analysis.

The precursors **12**, **13**, or **14** of regio-selective geranyl substitution at the 2, 5, 6, and 7-position were also synthesized along with Schollköpf's amino acid synthesis, as shown in Fig. 4. Treatment of 1-Boc-gramine **9**, 1-Boc-5-bromogramine **10**, or 1-Boc-6-bromogramine **11** with ethyl chloroformate gave the respective chlorides. These chlorides, except for the 6-bromo compound, were relatively stable, and could be purified by recrystalization. Alkylation of Schollköpf chiral auxiliary **4** with these chlorides furnished compounds **12**, **13**, or **14** in higher yield (88~97%) compared to that of **8** and with a similar *trans* diastereoselectivity.

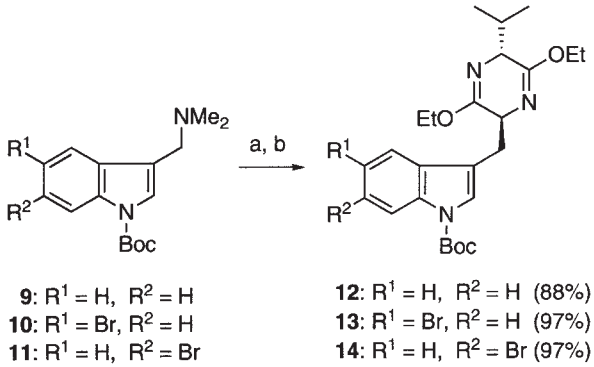

Fig. 4. Synthesis of the Intermediates **12**, **13**, and **14**.
Reaction conditions: (a) ethyl chloroformate, toluene. (b) *n*-BuLi, TMEDA, **4**, THF, $-78 \rightarrow 0\,^{\circ}C$.

The intermediate **15** of Trp(2-Ger) was synthesized from **12** by treatment with LDA, geranyl bromide, and CuCN in moderate yield (67%), as shown in Fig. 5. Due to a steric effect and acidity, LDA was deprotonated only at the 2-position of the indole ring,[9] and the formation of an organocopper complex mediated by CuCN improved the yield of **15**. On the other hand, changing the protecting group from Boc to a diethylbutanoyl group enabled deprotonation at the 7-position of the indole ring, as discussed by Fukuda *et al.*[10] The Boc group was cleaved with $NaOMe/CH_3OH$ to give compound **16**, followed by treatment with NaH and diethylbutanoyl chloride to give the diethylbutanoyl derivative **17**. Since the 2-position of the indole ring was sterically hindered and the neighboring diethylbutanoyl group participated in the reaction, the treatment with *s*-BuLi and TMEDA to the diethylbutanoyl derivative **17** gave only the 7-position deprotonated compound, which was reacted with geranyl bromide to give the intermediate **18**. The intermediates **21** of Trp(5-Ger) and **22** of Trp(6-Ger) were synthesized with Suzuki coupling, as shown in Fig. 5, because transmetallation of the halogen mediated by various alkyllithum reagents failed.[11] The bromide **13** or **14** was treated with catalytic $PdCl_2$(dppf), triethylamine, and pinacolborane to give the borate **19** or **20** respectively.[12] The borate **19** or **20** was reacted with geranyl bromide under Suzuki coupling conditions using 2 M aqueous KOH in THF to give the intermediate **21** or **22** respectively. Reverse geranyl substitution was observed as a byproduct from the borate **19** or **20**, but the desired intermediate **21** or **22** was obtained in moderate yield after the byproduct was separated carefully by column chromatography. Employment of $Pd(PPh_3)_4$ and $Pd(Pt\text{-}Bu_3)_2$ as a palladium catalyst gave undesired reactions such as reductive elimination, and under anhydrous conditions using K_2CO_3 or triethylamine as base in DMF, the starting materials were recovered.[13]

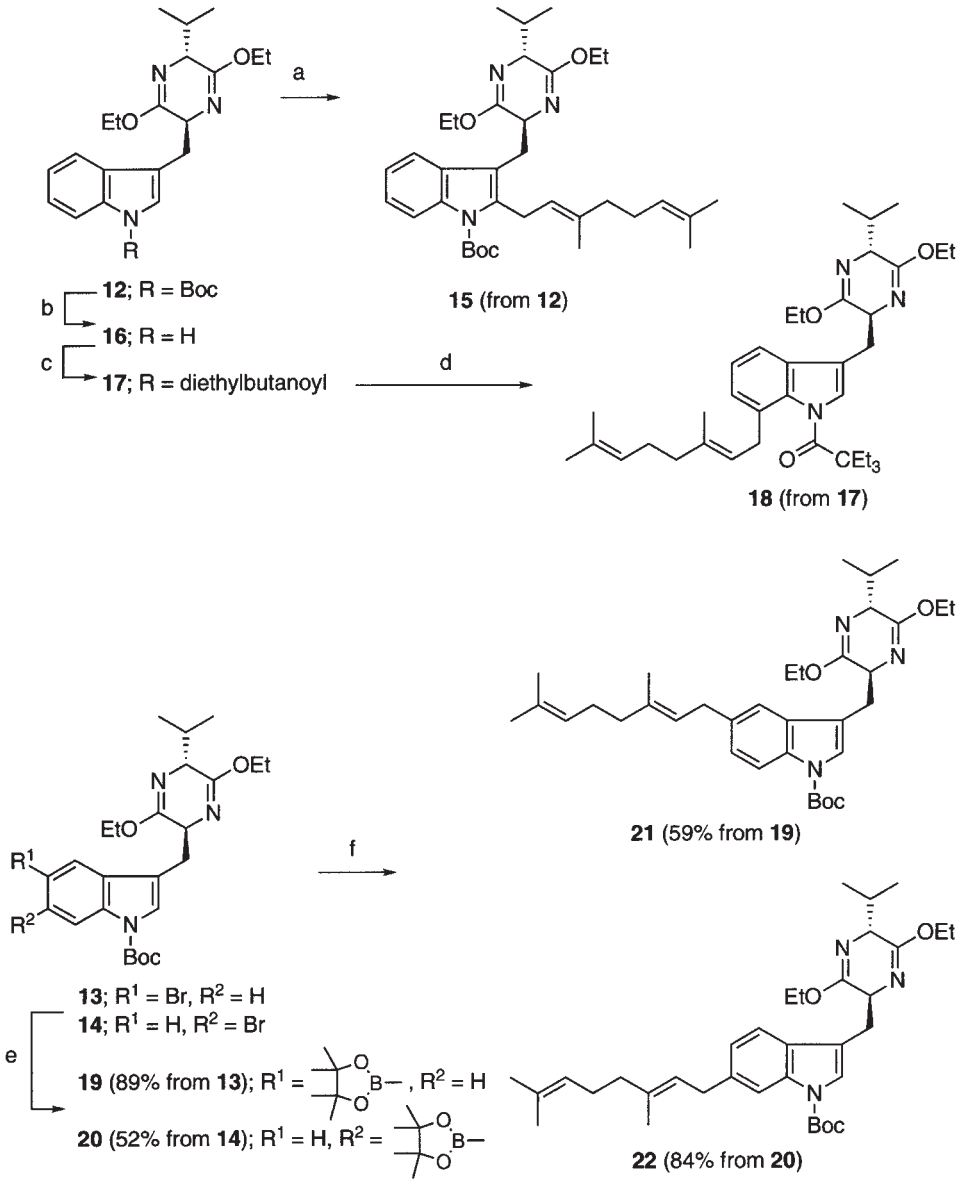

Fig. 5. Synthesis of the Intermediates **18**, **21**, and **22**.

Reaction conditions: (a) LDA, CuCN, geranyl bromide, Et_2O, −78 °C, 67%. (b) NaOMe/CH_3OH, THF, 97%. (c) NaH, diethylbutanoyl chloride, THF, 0 °C, 63%. (d) *s*-BuLi, TMEDA, geranyl bromide, Et_2O, −78 °C, 41%. (e) $PdCl_2$(dppf), Et_3N, pinacolborane, toluene, 90 °C. (f) $PdCl_2$(dppf), geranyl bromide, 2 M aqueous KOH, THF, 60 °C.

All of the geranyl substituted intermediates **8**, **15**, **18**, **21**, **22** were treated under similar conditions to give geranyl substituted Fmoc-tryptophans **33**∼**37**, as shown in Fig. 6. Boc and diethylbutanoyl groups were cleaved with NaOMe/CH_3OH in THF. Cleavage of the diethylbutanoyl group required a much longer time (2 d) than the Boc group (3 h). When *t*-BuOK/H_2O was employed in place of NaOMe/CH_3OH, removal of the diethylbutanoyl group occurred quickly, but racemization occurred. Acidic hydrolysis was carefully operated at 0 °C to give ethyl esters **28**∼**32**, followed by basic hydrolysis to give free Trp(n-Ger). Finally Fmoc protection was achieved under general conditions to give the desired geranyl substituted tryptophans **33**∼**37**.

Peptide synthesis

For solid-phase peptide synthesis as shown in Fig. 7, we chose a 2-chlorotrityl (Clt) resin, Gln without a protecting group, and Glu protected by a 2-phenyl-2-propyl (Pp) group on the side chain because all of the geranyl substituted tryptophans were acid labile. These resin and protecting groups can be cleaved under extremely mild acidic conditions. After the first peptide bond formation in the peptide synthesizer, the resin was removed from the machine. The resin was manually treated with each Fmoc-Trp(Ger) prepared, using HATU and diisopropylethylamine (DIPEA) in NMP. Then the manually treated resin was remounted in the automatic peptide synthesizer and the remaining three amino acid residues were coupled. Crude peptides were obtained

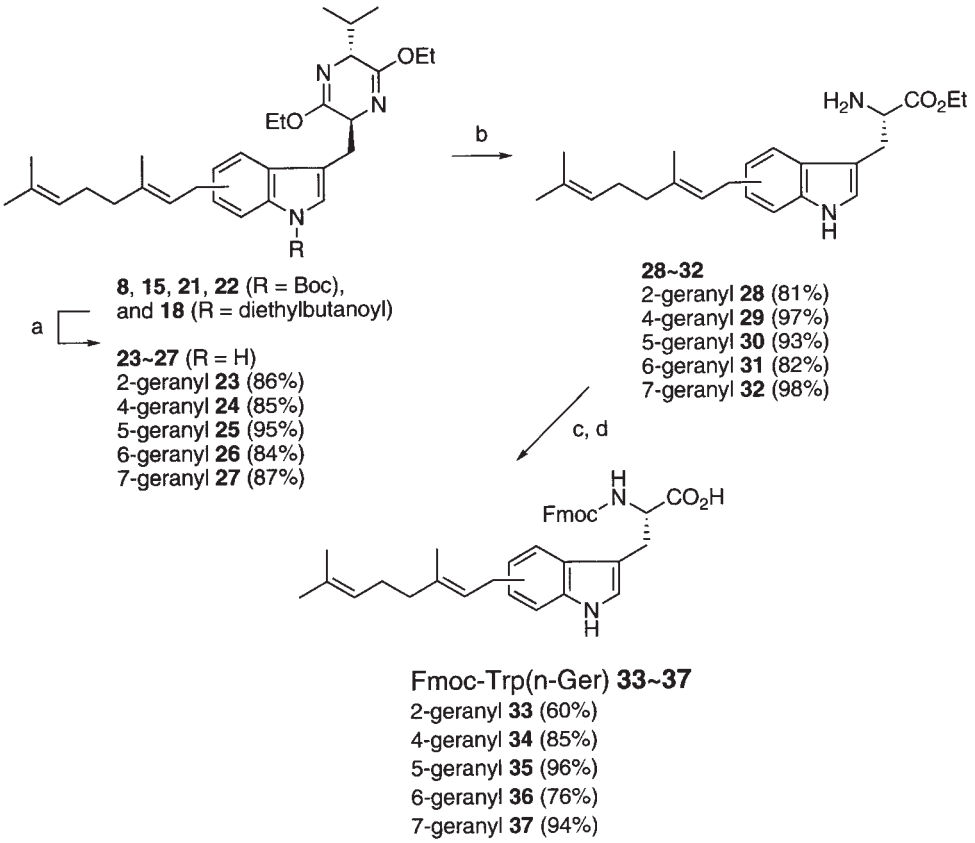

Fig. 6. Synthesis of Fmoc-Trp(n-Ger) **33**~**37**.
Reaction conditions: (a) NaOMe/CH_3OH, THF. (b) 1.5 M aqueous HCl, THF, 0 °C. (c) 1 M aqueous LiOH, THF. (d) Fmoc-OSu, 1 M aqueous Na_2CO_3, CH_3CN.

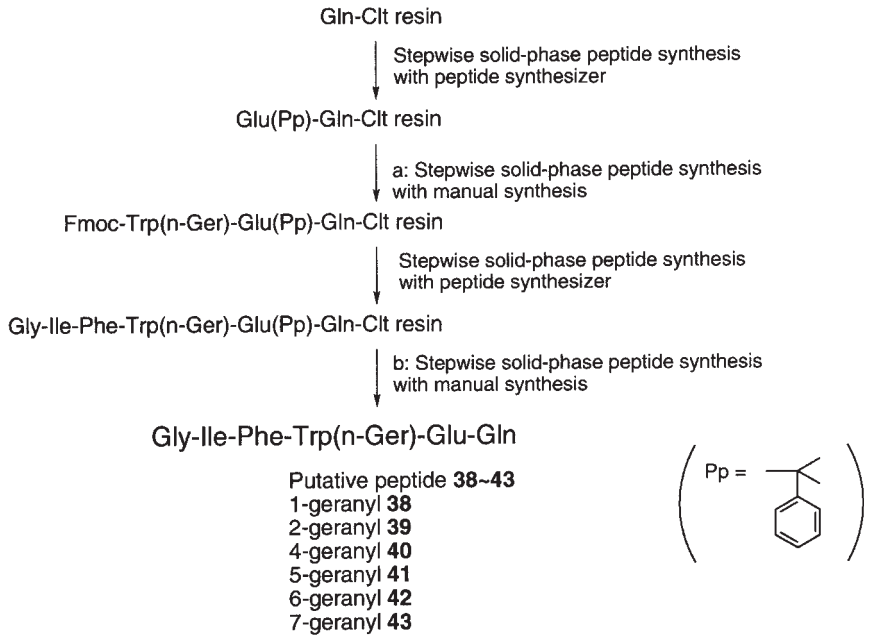

Fig. 7. Synthesis of the Putative Peptides **38**~**43**.
Reaction conditions: (a) HATU, DIPEA, NMP, Fmoc-Trp(n-Ger) **3**, **33**~**37**. (b) 4% TFA, 2.5% mercaptoethanol, CH_2Cl_2.

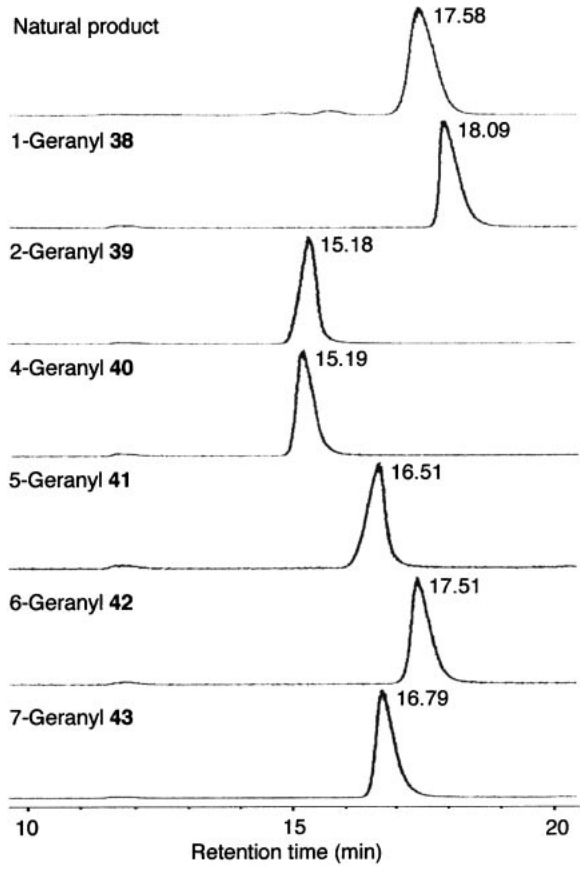

Fig. 8. LC–MS Analysis of Natural Product and Synthetic Peptides.

after the cleavage of protecting groups and resin with 4% TFA in CH_2Cl_2 for 1 h. Then each crude peptide was purified by HPLC, and we finally obtained the desired six peptides **38**∼**43**. We confirmed that all peptides had the proper structures by ^{1}H-NMR and HRMS analysis and by protein sequencing.

Comparison of synthetic peptides and ComX$_{\text{RO-E-2}}$

Using LC–MS, we compared the synthetic peptides and the natural pheromone. When an HPLC system was equipped with a cyanopropyl column, all the synthetic peptides showed distinct retention times, as shown in Fig. 8. During the analysis of synthetic peptides by LC on a cyanopropyl column with CH_3CN and formic acid, the intensity of the ion at m/z 915.5 was monitored by MS. Compared to ComX$_{\text{RO-E-2}}$, all six peptides prepared showed similar hydrophobicity, and the 6-geranyl substituted peptide **42** had almost the same retention time. However, using a different solvent system (CH_3CN–0.1% NH_4OAc) for HPLC, the natural peptide (Rt = 22.80) and **42** (Rt = 26.31) were completely distinguishable. Thus, none of the peptides synthesized was identical to the natural ComX pheromone.

We investigated the biological activity of the synthesized peptides by a β-galactosidase assay.[2,5] The natural pheromone showed biological activity at about 1 nM, although none of the synthetic peptides showed activity up to 3 μM. Thus the synthetic peptides were different from the natural pheromone with respect to both biological activity and physicochemical properties. These results suggest that the manner of modification of the tryptophan residue in ComX pheromone is more complex than expected.

Discussion

From previous reports, we believed that the ComX pheromone is isoprenylated, but the possibility remained that the tryptophan residue was not only isoprenylated but also reduced.[2] We have now investigated the HRMS and the molecular formula of ComX$_{\text{RO-E-2}}$, and confirmed that ComX$_{\text{RO-E-2}}$ possesses a non-reduced geranylated tryptophan residue.

Based on the hypothesis that ComX$_{\text{RO-E-2}}$ is simply geranylated at a position on the indole ring, we synthesized regio- and stereo-selective geranyl tryptophans. Our method should be widely applicable to synthesis of regio- and stereo-selective functionalized tryptophans such as farnesyl and geranylgeranyl tryptophans. The 5- and 6-substituted tryptophans were prepared from 5- and 6-bromoindole, because direct geranylation as used for formation of other geranyl tryptophans was impossible. After several trials, we finally found that Suzuki coupling gave a satisfactory result. This procedure should be useful for the introduction of alkyl chains to the indole 5- or 6-positions.

Using geranyltryptophan, six putative ComX$_{\text{RO-E-2}}$ candidates were synthesized by solid-phase peptide synthesis. We found that the natural pheromone is acid labile, because its biological activity decreased after concentrating the HPLC eluant containing 0.1% TFA and CH_3CN at room temperature under vacuum. Hence, we chose the Clt resin, which is cleaved under mild acidic conditions. We also used Gln without a protecting group and Glu with Pp protection. Peptide bond formation between Trp(Ger) and Glu was carried out manually to save the synthesized Fmoc-Trp(Ger) and to obtain a high reaction yield. We used 1.5 equivalents of Fmoc-Trp(Ger) and HATU as the coupling reagent in the manual reaction, in contrast to 10 equivalents of Fmoc-amino acids and HBTU reagent in the automatic peptide synthesizer. The cleavage reaction was carried out carefully under mild acidic conditions and for a short time. The isolated yield of purified peptides was approximately 5%, which is very low compared with that of the simple peptide having the ComX$_{\text{RO-E-2}}$ sequence without geranyl modification.

After purification of the crude peptides by HPLC, the purity was high enough for NMR and MS analysis. We could identify the substitution pattern of Trp in each peptide, although only a small amount of these peptides could be dissolved in NMR solvent. The molecular weights of these six peptides and the natural one were exactly the same as measured by HRMS.

We then established the LC–MS analysis conditions to separate all six peptides synthesized. Based on this LC–MS analysis, only peptide **42**, having a 6-geranyl tryptophan residue, showed the same retention time as the natural pheromone. However, this peptide showed a completely different retention time relative to the natural pheromone by HPLC using a different solvent system. Thus all synthetic peptides had a different structure from the natural ComX peptide. Since the synthetic peptides were acid labile and exhibited hydrophobicity similar to the natural pheromone, we expected that some of these peptides would show weak biological activity. We investigated the biological activities of the synthetic peptides, but none of them showed activity, even at very high concentrations. These results indicate that the manner of modification of the tryptophan residue is not as simple as we expected.

There are four examples of naturally occurring posttranslational modification on a tryptophan residue. The first is a cross-link between two tryptophan residues, in which one is modified to indole-6,7-dione and attached at its 4-position to the 2-position of the other.[14] The second is *C*2-mannopyranosylated tryptophan.[15,16] The third is 6-bromotryptophan,[17] and the last is kynurenine [2-amino-4-(2-aminophenyl)-4-oxobutanoic acid], formed by oxidative modification.[18,19] These four examples, however, do not appear to help in the structural determination of the modified tryptophan residue in ComX. Natural alkaloids substituted by isoprenylation derived from tryptophan have been reported.[20] Most of them, such as tryprostatin, are simply prenylated on their indole rings, as are the compounds we synthesized in this study.

Isoprenoidal peptides were first discovered as sex pheromones of basidiomycetous yeasts.[21,22] These peptides have cysteine residues at their carboxy termini with modification of their sulfhydryl groups by farnesylation. This type of farnesylation and geranylgeranylation of thiols is now well studied and is known in many proteins.[23] The posttranslational farnesyl or geranylgeranyl modification is important for the function of these proteins. The geranyl or farnesyl groups in ComX are also essential for the function of this pheromone, because the unmodified peptide shows no pheromone activity,[2,4] and the similar peptides described in the present study also lacked biological activity. Determination of the ComX structure is essential to clarify its biological role.

In this study, we synthesized six peptides for which expected a high possibility for the chemical structure of ComX pheromone. The possible structure remaining is a tryptophan residue geranylated at the alpha carbon or nitrogen, or substituted by two isoprenes. We cannot exclude the possibility that the ComX pheromone has D-amino acid(s), although the peptide is coded on DNA.

Now we are attempting to synthesize peptides having possible ComX pheromone structure and to purify a natural pheromone from microbial fermentation. We believe that the chemical structure of ComX pheromone will be determined in the near future.

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