The Synthesis of a Heavy-Atom Derivative of Gramicidin S (GS), [D-Phe(4-Br)$^{4,4'}$]-GS, by a Novel Method

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Synopsis. A new approach for the preparation of cyclic peptides was successfully applied to the synthesis of *cyclo*(-Val-Orn-Leu-D-Phe(4-Br)-Pro-Val-Orn-Leu-D-Phe (4-Br)-Pro-) ([D-Phe(4-Br)$^{4,4'}$]-GS). [D-Phe(4-Br)$^{4,4'}$]-GS showing antibacterial activity was obtained as crystals.

Cyclic peptides show interesting functions, such as acting as ionophores or antibiotics, and also serve as good model compounds for the basic study of peptide and protein conformation. Thus, many cyclic peptides have been synthesized, but mostly by the solution method which takes much time and effort. To overcome these difficulties, a new, simple, and time-saving method was developed. The present paper describes how it was used to prepare [D-Phe(4-Br)$^{4,4'}$]-GS and also reports some characteristics of the compound.

Results and Discussion

Synthesis of [D-Phe(4-Br)$^{4,4'}$]-GS (7). [D-Phe(4-Br)$^{4,4'}$]-GS was synthesized according to the scheme shown in Fig. 1. The chain assembly of the peptide was carried out by the standard Merrifield solid-phase method[1] using Boc-amino acid derivatives of Val, Orn(Z), Leu, D-Phe(4-Br) and Pro for the chain elongation and Troc-Val for the introduction of N-terminal Troc-Val. The Troc group was used to block the α-amino group of the peptide because it is stable under acid-treatment conditions.[2-4] The weight increase in the protected-peptide resin **2** was 0.48 g, based on 1.54 g of Boc-Pro-$OCH_2C_6H_4$-resin (**1**). The HF treatment[5] of 1.05 g of Compound **2** gave 275 mg of a crude product **3**. This peptide was very soluble in aq acetonitrile and could be easily purified by reversed-phase high-performance liquid chromatography (HPLC), for it had no extra-protecting groups except for the Troc group. The purified Compound **3** (261 mg) was confirmed by fast-atom-bombardment (FAB) mass measurement. The Boc group was introduced to 200 mg of Compound **3** with the aid of *N*-(*t*-butoxycarbonyloxy)succinimide (Boc-ONSu) in the presence of triethylamine (TEA). The resultant material was purified by crystallization and gave 175 mg of Compound **4**. The Troc group on Compound **4** (84.5 mg) was cleaved by zinc-dust treatment in 90% aq acetic

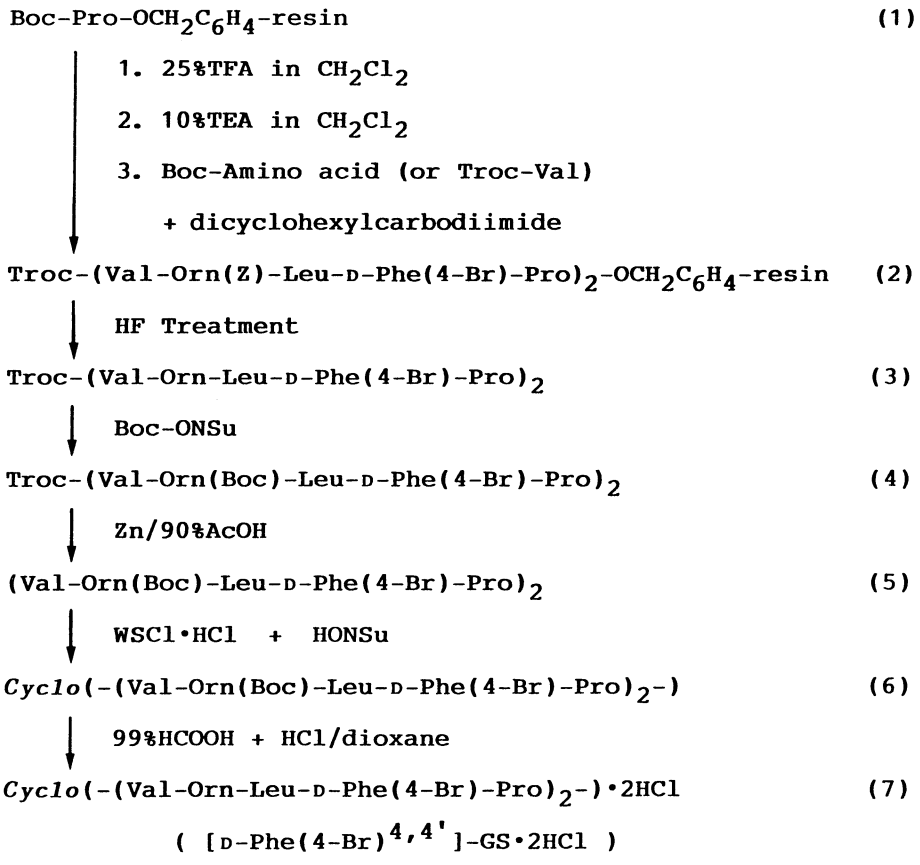

Fig. 1. Synthetic scheme of [D-Phe(4-Br)$^{4,4'}$]-GS.

acid.[2] After the removal of the zinc dust and zinc ions, the peptide solution was freeze-dried to a powder. Compound **5** (64.1 mg) was cyclized by adding about a twofold excess of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCI·HCl) and *N*-hydroxysuccinimide (HONSu) in a mixture of *N*,*N*-dimethylformamide (DMF)-CH_2Cl_2 at about 2.5 mM (1 M=1 mol dm^{-3}). One-pot cyclization proceeded, with a yield of about 80%, judging from the elution profile of the reaction mixture on HPLC. This yield was almost equal to those described by two other research groups.[6,7] No cyclic dimer was detected in the reaction mixture by FAB mass measurements. Compound **6** was purified by crystallization to give 36 mg of crystals in a 50% yield, based on Compound **4**. Compound **6** was treated with a mixture of 99% formic acid and HCl in dioxane to remove the Boc groups. After the separation of minor impurities from the main product by means of HPLC, the final product **7** was obtained in an 80% yield, based on Compound **6**. Each of the steps gave good yields, and the purities of the intermediates and the final products were satisfactory. This new approach was successfully applied to the synthesis of [D-Phe(4-Br)$^{4,4'}$]-GS. Starting from Boc-Pro-O$CH_2C_6H_4$-resin (**1**), the final product **7** was obtained as crystals in 2 weeks.

Biological Activity. The minimum concentration of [D-Phe(4-Br)$^{4,4'}$]-GS needed to completely inhibit the growth of several microorganisms was determined by a dilution method using nutrient agar. [D-Phe(4-Br)$^{4,4'}$]-GS showed antibacterial activity. The bromine atom on the phenyl group of D-Phe affected the biological activity of GS, but not seriously.

Crystallization. We attempted to prepare crystals of [D-Phe(4-Br)$^{4,4'}$]-GS under several conditions. The trifluoroacetate or hydrochloride gave fine needle crystals in aq EtOH, aq MeOH, or aq DMF. The perchlorate gave thick needle crystals in dimethyl sulfoxide. These crystals will be analyzed in a subsequent X-ray crystallographic study.

Experimental

The melting points were determined by the capillary method and are given as uncorrected values. The Pro, Val, Orn, and Leu used were of the L-configuration. High-performance liquid chromatography (HPLC) was carried out on a handmade column of the YMC-GEL ODS S5 100 Å Type (8×300 mm) (Yamamura Chemical Laboratory Co., Ltd., Kyoto, Japan), with the acetonitrile concentration increasing from 40 to 80% in 0.05% aq trifluoroacetic acid (TFA) at a flow rate of 2 ml min^{-1}.

Troc-Val-Orn-Leu-D-Phe(4-Br)-Pro-Val-Orn-Leu-D-Phe(4-Br)-Pro (3). The chain elongation of a peptide was carried out on a Beckman Model 990E synthesizer (Palo Alto, Ca, USA) according to the standard Merrifield solid-phase method. Boc-Pro-O$CH_2C_6H_4$-resin (**1**) (1.54 g), containing 0.4 mmol of Boc-Pro was used as the starting material. For the deprotection of the Boc group, the resin was treated twice with 25% TFA in CH_2Cl_2, for 1 min and then for 30 min. The resin was treated twice with 10% TEA in CH_2Cl_2, for 2 min and then for 5 min, to neutralize the resin. In each coupling reaction, 1.2 mmol of Boc-amino acid or Troc-Val was mixed with a peptide resin in the presence of dicyclohexylcarbodiimide (250 mg, 1.2 mmol) for 2 h. The Boc-amino acids used were Boc-Val, Boc-Orn(Z), Boc-Leu, Boc-D-Phe(4-Br), and Boc-Pro. Weight increase, 480 mg. The protected peptide resin **2** (1.05 g) was treated with anhydrous hydrogen fluoride (10 ml) containing anisole (1 ml) at 0 °C for 70 min. After the evaporation of the hydrogen fluoride, a peptide was extracted with 50% aq acetonitrile. The solution was passed through a column of Amberlite IR-45 (acetate form) (3.5×8 cm). All of the eluent was then collected and freeze-dried. The crude material was purified on HPLC. Lyophilization of the main fractions gave 261 mg of Compound **3**. Found: *m/z* 1489.2 $(M+H)^+$. Calcd for $C_{63}H_{94}O_{13}N_{12}Br_2Cl_3$: *m/z* 1489.5 $(M+H)^+$.

Troc-Val-Orn(Boc)-Leu-D-Phe(4-Br)-Pro-Val-Orn(Boc)-Leu-D-Phe(4-Br)-Pro (4). Compound **3** (200 mg, ca. 130 μmol) was dissolved in DMF (1.7 ml) containing 85 μl (ca. 600 μmol) of TEA. Boc-ONSu (110 mg, 512 μmol) was added to the solution, which was then stirred at room temperature for 1 h. The reaction mixture was poured into a dilute aqueous solution of citric acid. The precipitate thus formed was collected, dried over diphosphorus pentaoxide in vacuo, and crystallized from MeOH-EtOAc to give 175 mg of crystals **4** (ca. 79%): mp 228—229 °C, $[\alpha]_D^{24}$ −108° (*c* 0.5, EtOH). Found: *m/z* 1690.1 $(M+H)^+$. Calcd for $C_{73}H_{110}O_{17}N_{12}Br_2Cl_3$: *m/z* 1689.7 $(M+H)^+$. Found: C, 51.33; H, 6.56; N, 9.69; Br, 9.42; Cl, 6.27%. Calcd for $C_{73}H_{109}O_{17}N_{12}Br_2Cl_3 \cdot H_2O$: C, 51.24; H, 6.54; N, 9.82; Br, 9.34; Cl, 6.22%.

Val-Orn(Boc)-Leu-D-Phe(4-Br)-Pro-Val-Orn(Boc)-Leu-D-Phe(4-Br)-Pro (5). Compound **4** (84.5 mg, 49.4 μmol) in 90% acetic acid (5 ml) was treated with zinc dust (100 mg) while being stirred at room temperature for 5 h. After the removal of the remaining zinc dust and zinc ions, the peptide solution was freeze-dried to give 64.1 mg of Compound **5** (ca. 85%). Found: *m/z* 1515.4 $(M+H)^+$. Calcd for $C_{70}H_{109}O_{15}N_{12}Br_2$: *m/z* 1515.7 $(M+H)^+$.

***cyclo*(-Val-Orn(Boc)-Leu-D-Phe(4-Br)-Pro-Val-Orn (Boc)-Leu-D-Phe(4-Br)-Pro-) (6).** Compound **5** (64 mg, ca. 42 μmol) was dissolved in DMF (10 ml) containing 11.5 mg (100 μmol) of HONSu. Into the solution, 19.2 mg (100 μmol) of WSCI·HCl in CH_2Cl_2 (10 ml) was added at 0 °C. The solution was stirred at 0 °C for 1 h and at room temperature for 24 h. After the concentration of the reaction mixture in vacuo, the residual mass was solidified with water, collected by centrifugation, and dried over diphosphorus pentaoxide in vacuo. The solid material was crystallized from EtOAc-MeOH; yield, 36 mg (ca. 57%); mp 231—232 °C; $[\alpha]_D^{21}$ −277° (*c* 0.5, AcOEt). Found: *m/z* 1497.5 $(M+H)^+$. Calcd for $C_{70}H_{107}O_{14}N_{12}Br_2$: *m/z* 1497.7 $(M+H)^+$. Found: C, 54.70; H, 7.12; N, 10.91; Br, 10.46%. Calcd for $C_{70}H_{106}O_{14}N_{12}Br_2 \cdot 2H_2O$: C, 54.75; H, 7.22; N, 10.91; Br, 10.41%.

***cyclo*(-Val-Orn-Leu-D-Phe(4-Br)-Pro-Val-Orn-Leu-D-Phe(4-Br)-Pro-)·HCl·3H$_2$O (7).** Compound **6** (76 mg, 50 μmol) in 99% formic acid (2 ml) and 6.72 M HCl in dioxane (0.2 ml) was stirred for 20 min at room temperature. After the evaporation of the solvent, the crude product was purified on HPLC and lyophilized from dilute hydrochloric acid. The powder thus obtained was crystallized from MeOH-ether; yield, 57.8 mg (81%); mp 266—270 °C (decomp), $[\alpha]_D^{20}$ −284° (*c* 0.15, MeOH). Found: *m/z* 1297.3 $(M+H)^+$. Calcd for $C_{60}H_{91}O_{10}N_{12}Br_2$: *m/z* 1297.6 $(M+H)^+$. Found: C, 50.94; H, 6.80; N, 11.54%. Calcd for $C_{60}H_{90}O_{10}N_{12}Br_2 \cdot 2HCl \cdot 3H_2O$: C,50.52; H, 6.93; N, 11.79%.

Crystallization of [D-Phe(4-Br)$^{4,4'}$]-GS Perchlorate. [D-Phe(4-Br)$^{4,4'}$]-GS trifluoroacetate or hydrochloride was dissolved in a small amount of methanol. To the solution, dilute perchloric acid was added until a precipitate formed. This precipitate was collected by centrifugation, washed with distilled water, and dried; then a minimum amount of dimethyl sulfoxide was added until the precipitate had dissolved completely. The solution in the uncovered test tube

was left standing. Crystals appeared after 3 d and were allowed to grow for one week. The crystal dimensions were 0.1×0.1×1.0 mm.

Microbiological Assays. The minimum inhibitory concentrations of [D-Phe(4-Br)$^{4,4'}$]-GS·2HCl·3H_2O for several microorganisms were determined by a dilution method using nutrient agar. The minimum inhibitory concentrations of the compound were 12.5 μg ml^{-1} on *Staphylococcus aureus* FDA 209P and *Staphylococcus aureus* 1840, 25 μg ml^{-1} on *Staphylococcus aureus* 308A-1, and 6.25 μg ml^{-1} on *Bacillus subtilis* PCl 219.

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