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Synthesis of Substance P Analogs with Arginine in Position Eleven[1)]

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Substance P analogs, [Arg^{11}]-SP and SP(1—10)-Arg–OH, were synthesized by the liquid phase method. These peptides were purified by ion-exchange chromatography and partition chromatography. The biological activity of the peptides was evaluated *in vitro* on guinea-pig ileum. Replacement of Met^{11} in SP with Arg diminished the potency of SP activity. The analogs were found to be inactive as antagonists.

Keywords——substance P; peptide synthesis; peptide analog; arginine replacement; guinea-pig ileum contraction; partition chromatography

Since the primary structure of substance P (SP: Arg^1–Pro^2–Lys^3–Pro^4–Gln^5–Gln^6–Phe^7–Phe^8–Gly^9–Leu^{10}–Met^{11}–NH_2) was elucidated,[2)] many analogs have been synthesized and their structure–activity relationships have been discussed.[3)] In particular, in studies on the C-terminal moiety of SP, [Nle^{11}]-SP,[4)] [Leu^{11}]-SP,[5)] [Ala^{11}]-SP,[6)] and [Des–Met^{11}]-SP[7)] have been synthesized, and all of these analogs retained their activity to some extent, except the last one, which was completely devoid of activity. Thus, the methionine site appears to be very important for activity, as does the hydrophobic nature of the side chain. In the present work, in order to determine the biological effect of replacement of methionine residue by a hydrophilic moiety, we synthesized two analogs, [Arg^{11}]-SP and SP(1—10)-Arg–OH. These peptides consequently contain two arginine residues, like bradykinin, which also has hypotensive activity.

Syntheses

The synthetic schemes are shown in Figs. 1 and 2. Z–Arg(NO_2)–Pro–Lys(Z)–Pro–OBzl was synthesized by the "hold-in-solution" method for rapid peptide synthesis in the liquid phase.[8)] Other fragments were synthesized stepwise by the mixed anhydride method[9)] and the active ester method.[10)] Two successive fragment condensations for the syntheses of hepta- and undecapeptide derivatives (**11**, **12**, **14**, and **15**) in each case were carried out by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCD·HCl) and HOBt as the coupling reagents. [Arg^{11}]-SP and SP(1—10)-Arg–OH were obtained from the protected undecapeptides **12** and **15**, respectively, by catalytic hydrogenation. These peptides were purified by chromatography on a carboxymethyl cellulose column with an ammonium acetate gradient, and further by partition chromatography on Sephadex G-25 with BuOH–pyridine–HOAc–H_2O. These compounds were shown to be homogeneous by thin layer chromatography (TLC) and gave the expected amino acid compositions and elemental analyses.

Structure–Activity Relationships

The relative potencies of synthetic analogs compared with that of authentic SP are shown in Table I. These potencies were much lower than that of bradykinin as well as SP. The hypotensive peptide bradykinin had approximately ten times less ability than SP to stimulate the contraction of guinea-pig ileum and actually induced relaxation of rat duodenum. [Arg^{11}]-SP and SP(1—10)-Arg–OH were classified as SP analogs because both analogs showed

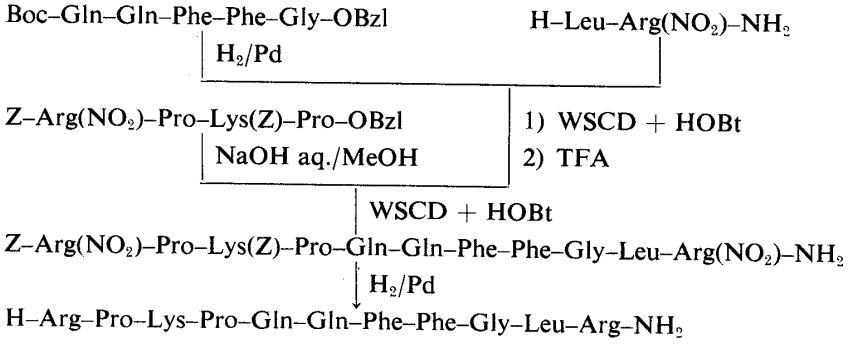

Fig. 1. Synthetic Scheme for [Arg[11]]-SP

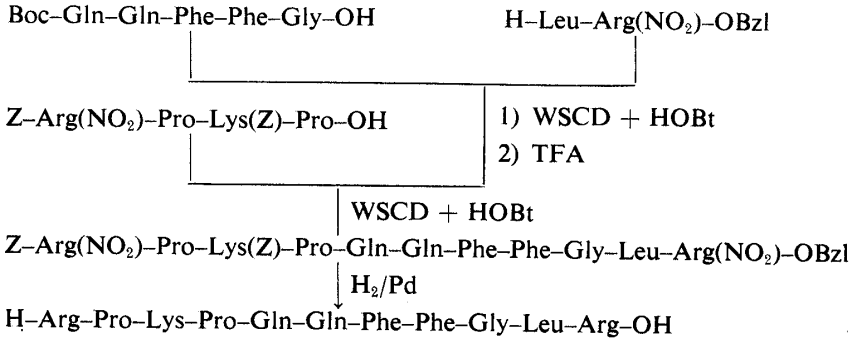

Fig. 2. Synthetic Scheme for SP(1—10)-Arg–OH

TABLE I. Activity to Contract Isolated Guinea-Pig Ileum[a)]

Compd.	Relative potency
SP[b)]	100
[Arg[11]]-SP	1
SP(1—10)-Arg–OH	0.1

a) The contractility of the ileum in response to added samples of peptide was measured in Tyrode's solution at 24 °C.
b) SP obtained from the Protein Research Foundation.

contraction of the duodenum, similarly to SP. Their relative potencies on the duodenum were similar to those on guinea-pig ileum, and these analogs did not exert any SP-antagonism on the duodenum. Replacement of Met^{11} in SP with arginine thus resulted in a 100-fold reduced activity. However, this C-terminal amide analog showed higher activity than the corresponding C-terminal free-carboxyl analog. These results suggest the importance of the presence of both a hydrophobic side chain in position eleven and a C-terminal amide for *in vitro* activity.

Experimental

The melting points are uncorrected. The homogeneity of the peptide derivatives was confirmed by TLC on Merck Silica gel 60 F_{254} plates with the following solvent systems (v/v): *Rf* (A), $CHCl_3$–MeOH (49 : 1); *Rf* (B), $CHCl_3$–MeOH (9 : 1); *Rf* (C), $CHCl_3$–MeOH (99 : 1); *Rf* (D), BuOH–HOAc–H_2O (4 : 1 : 1); *Rf* (E), CH_2Cl_2–EtOAc (6 : 1); *Rf* (F), $CHCl_3$–MeOH–HOAc (9 : 1 : 1); *Rf* (G), $CHCl_3$–MeOH (5 : 1). Avicel SF cellulose plates (Funakoshi, Tokyo) were also used with the following solvent systems (v/v): *Rf* (H), BuOH–pyridine–HOAc–H_2O (4 : 1 : 1 : 2); *Rf* (I), BuOH–pyridine–HOAc–H_2O (16 : 10 : 3 : 12); *Rf* (J), BuOH–pyridine–HOAc–H_2O (15 : 10 : 3 : 12). Amino acid analyses were performed with a JEOL automatic amino acid analyzer, after the samples had been hydrolyzed with constant-boiling HCl in evacuated sealed ampoules for 20 h at 110 °C.

Z–Arg(NO_2)–Pro–Lys(Z)–Pro–OBzl (1)——H–Pro–OBzl (5.0 mmol) was successively coupled with Boc–Lys(Z)–OH, Boc–Pro–OH and Z–Arg(NO_2)–OH by the "hold-in-solution" method,[8] using DCE as a solvent and WSCD as a coupling reagent. The final reaction mixture was washed successively with 0.1 M HCl (5 ml × 2), water (5 ml), 0.5 M Na_2CO_3 (5 ml × 2), and water (5 ml × 3). The DCE layer was concentrated to afford an oily residue which was crystallized by addition of ethyl ether. Yield 3.33 g (73% from H–Pro–OBzl·HCl); mp 83—85 °C; $[\alpha]_D^{21}$ −77.5° (c=0.9, DMF); TLC *Rf* (A) 0.17, *Rf* (B) 0.56. Amino acid ratio: Arg+Orn 0.74, Pro 2.20, Lys 1.06. *Anal.* Calcd for $C_{45}H_{57}N_9O_{11}\cdot 1/2H_2O$: C, 59.45; H, 6.43; N, 13.87. Found: C, 59.78; H, 6.61; N, 13.45.

Z–Arg(NO_2)–Pro–Lys(Z)–Pro–OH (2)——Compound **1** (5.70 g, 6.3 mmol) was saponified with alkali in the usual way to provide **2**. The crude product was purified on a Sephadex LH-20 column (3.5 × 95 cm), which was eluted with MeOH. The collected eluate was concentrated, and the residue was solidified with ethyl ether, yielding 4.2 g (82%) of pure **2**. mp 103.5—105 °C (lit.[3a] 101—103 °C); TLC *Rf* (B) 0.33.

Boc–Leu–Arg(NO_2)–OBzl (3)——Boc–Leu–OH (6.0 mmol) was coupled with H–Arg(NO_2)–OBzl (5.0 mmol) by the mixed anhydride method, using ethyl chloroformate as an activating reagent. Compound **3** was crystallized from EtOAc and ethyl ether. Yield 2.53 g (97%); mp 78—80 °C; $[\alpha]_D^{23}$ −33.9° (c=1.0, MeOH); TLC *Rf* (B) 0.50. *Anal.* Calcd for $C_{24}H_{38}N_6O_7$: C, 55.16; H, 7.33; N, 16.08. Found: C, 54.80; H, 7.65; N, 15.91.

Boc–Leu–Arg(NO_2)–NH_2 (4)——Compound **3** (1.2 g, 2.3 mmol) was treated with NH_3 in MeOH. The crude amide was purified by chromatography on a silica gel column (Wakogel C-200, 2.5 × 33 cm), which was developed successively with CH_2Cl_2 (100 ml), CH_2Cl_2–MeOH (20:1, v/v, 100 ml), and CH_2Cl_2–MeOH (15:1, v/v, 100 ml). Compound **4** was finally crystallized by treatment of the concentrate obtained from the column eluate with ethyl ether. Yield 0.67 g (68%); mp 119—120.5 °C; $[\alpha]_D^{23}$ −27.8° (c=0.7, MeOH); TLC *Rf* (B) 0.22. *Anal.* Calcd for $C_{17}H_{33}N_7O_6$: C, 47.32; H, 7.71; N, 22.72. Found: C, 47.21; H, 8.04; N, 22.46.

H–Phe–Gly–OBzl·HCl (5)——Boc–Phe–Gly–OBzl[11] (5.66 g, 13.7 mmol) was treated with HCl–dioxane as usual. After evaporation of the solvent, addition and evaporation of $CHCl_3$ were repeated twice. The product was obtained in the theoretical yield. TLC *Rf* (B) 0.39, *Rf* (D) 0.68.

Boc–Phe–Phe–Gly–OBzl (6)——Boc–Phe–OH (4.36 g, 16.4 mmol) was coupled with compound **5** (13.7 mmol) by the mixed anhydride method. The product was recrystallized from $CHCl_3$ and hexane. Yield 6.63 g (86%); mp 142 °C; $[\alpha]_D^{23}$ −21.1° (c=1.1, MeOH); TLC *Rf* (B) 0.95, *Rf* (E) 0.78. *Anal.* Calcd for $C_{32}H_{37}N_3O_6$: C, 68.67; H, 6.66; N, 7.51. Found: C, 68.39; H, 6.51; N, 7.44.

Boc–Gln–Phe–Phe–Gly–OBzl (7)——H–Phe–Phe–Gly–OBzl prepared from compound **6** (1.68 g, 3 mmol) by treatment with HCl–dioxane was coupled with Boc–Gln–ONO (1.44 g, 3.9 mmol) in DMF. The reaction mixture was concentrated and the residue was dissolved in aqueous BuOH (50 ml). The butanolic phase was washed with 0.5 M $NaHCO_3$ and water, and evaporated. The residual product was crystallized from DMF and ethyl ether. Yield 1.76 (85%); mp 211—212 °C; $[\alpha]_D^{23}$ −19.1° (c=0.6, DMF); TLC *Rf* (B) 0.54. *Anal.* Calcd for $C_{37}H_{45}N_5O_8$: C, 64.61; H, 6.59; N, 10.18. Found: C, 64.36; H, 6.65; N, 10.66.

Boc–Gln–Gln–Phe–Phe–Gly–OBzl (8)——H–Gln–Phe–Phe–Gly–OBzl obtained from compound **7** (0.88 g, 1.28 mmol) was coupled with Boc–Gln–ONO (0.61 g, 1.66 mmol) in DMF. Ethyl ether was added to the reaction mixture and the precipitated product was filtered off. The crude product was recrystallized from DMF and MeOH. Yield 0.66 g (63%); mp 231—232 °C; $[\alpha]_D^{25}$ −18.6° (c=0.9, DMF); TLC *Rf* (B) 0.21, *Rf* (F) 0.27. *Anal.* Calcd for $C_{42}H_{53}N_7O_{10}$: C, 61.83; H, 6.55; N, 12.02. Found: C, 61.63; H, 6.70; N, 12.42.

Boc–Gln–Gln–Phe–Phe–Gly–OH (9)——The benzyl group of compound **8** (2.18 g, 2.67 mmol) was removed by hydrogenolysis over Pd-black (100 mg) in DMF (100 ml) containing HOAc (1 ml). Recrystallization of the crude product from DMF and MeOH gave 1.2 g of **9**. Yield 62%; mp 242—244 °C; $[\alpha]_D^{23}$ −23.1° (c=0.5, DMF); TLC *Rf* (D) 0.63. *Anal.* Calcd for $C_{35}H_{47}N_7O_{10}$: C, 57.92; H, 6.53; N, 13.51. Found: C, 57.69; H, 6.63; N, 13.22.

H–Leu–Arg(NO_2)–NH_2·TFA (10)——Removal of the Boc group from compound **3** (0.35 g, 0.81 mmol) by treatment with TFA (5 ml) gave compound **10** in the theoretical yield. TLC *Rf* (G) 0.12.

Boc–Gln–Gln–Phe–Phe–Gly–Leu–Arg(NO_2)–NH_2 (11)——Compounds **10** (0.81 mmol) and **9** (0.37 g, 0.51 mmol) were dissolved in DMF (6 ml) containing Et_3N (0.12 ml). Coupling was performed with WSCD·HCl (0.17 g) and HOBt (0.12 g) at room temperature overnight. After evaporation of the DMF, the residue was washed successively with water, 0.1 M HCl, water, 0.5 M $NaHCO_3$ and water, then dried and chromatographed on a Sephadex LH-20 column (35 × 95 cm) with DMF. The product was crystallized from DMF and water, then dried. Yield, 320 mg (58%); mp 216—218.5 °C; $[\alpha]_D^{25}$ −30.2° (c=0.5, DMF); TLC *Rf* (D) 0.62. Amino acid ratio: Glu 2.00, Phe 1.95, Gly 1.04, Leu 1.02, Arg+Orn 0.80. *Anal.* Calcd for $C_{47}H_{70}N_{14}O_{13}\cdot 2H_2O$: C, 52.50; H, 6.94; N, 18.24. Found: C, 52.73; H, 6.83; N, 18.17.

Z–Arg(NO_2)–Pro–Lys(Z)–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Arg(NO_2)–NH_2 (12)——Compound **11** (275 mg, 0.256 mmol) was treated with TFA (5 ml), and the product was dissolved in DMF (5 ml), then compound **2** (430 mg, 0.53 mmol), Et_3N (0.08 ml), and HOBt (74 mg) were added. The coupling reaction was started by addition of WSCD·HCl (104 mg) and the mixture was stirred at room temperature overnight. After evaporation of the DMF, the residue was washed with water by decantation and dried. The crude product was purified by chromatography on a Sephadex LH-20 column (3.5 × 90 cm) with DMF, and recrystallized from 60% aqueous MeOH. Yield, 335 mg (74%); mp 139—141 °C; $[\alpha]_D^{25}$ −30.5° (c=0.5, DMF); TLC *Rf* (D) 0.55. *Anal.* Calcd for $C_{80}H_{111}N_{23}O_{21}\cdot 2H_2O$: C,

54.38; H, 6.56; N, 18.23. Found: C, 54.55; H, 6.43; N, 17.68.

H–Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Arg–NH_2·4HOAc (13)——Compound **12** (103 mg, 0.058 mmol) was hydrogenated over Pd-black. The product was purified by chromatography on a carboxymethyl cellulose column (Whatman CM52, 1.5 × 33 cm) with a gradient solvent system between 0.08 and 0.4 M NH_4OAc. The concentrated peak fractions were desalted by gel-filtration on a Sephadex G-10 column (1.7 × 42 cm; solvent, 5% HOAc). The product was further purified by partition chromatography on a Sephadex G-25 column (1.2 × 13 cm) with BuOH–pyridine–HOAc–H_2O (16 : 10 : 3 : 12). The collected fractions were lyophilized to give 18 mg (18%) of **13**. mp 164—167 °C (dec.); $[\alpha]_D^{23}$ −76.8° (c=0.4, H_2O); TLC *Rf* (H) 0.52, *Rf* (I) 0.58. Amino acid ratio: Arg 1.92, Pro 1.95, Lys 1.01, Glu 2.13, Phe 1.97, Gly 1.00, Leu 1.00. *Anal.* Calcd for $C_{64}H_{101}N_{21}O_{13}\cdot 4HOAc\cdot 5H_2O$: C, 50.78; H, 7.52; N, 17.27. Found: C, 50.86; H, 7.37; N, 16.63.

Boc–Gln–Gln–Phe–Phe–Gly–Leu–Arg(NO_2)–OBzl (14)——H–Leu–Arg(NO_2)–OBzl·TFA obtained from compound **3** (420 mg, 0.80 mmol) by treatment with TFA was dissolved in DMF (6 ml) containing Et_3N (0.12 ml), and coupled with compound **9** (370 mg, 0.51 mmol) by the use of HOBt (0.12 g) and WSCD·HCl (0.17 g) under the same conditions as used in the synthesis of **11**. The product was also purified as described for **11** to afford 414 mg (71%) of **14**: mp 217—219 °C; $[\alpha]_D^{23}$ −43.0° (c=0.6, DMF); TLC *Rf* (D) 0.78. Amino acid ratio: Glu 2.10, Phe 1.96, Gly 1.00, Leu 0.93, Arg+Orn 0.78. *Anal.* Calcd for $C_{54}H_{75}N_{13}O_{14}\cdot H_2O$: C, 56.48; H, 6.76; N, 15.86. Found: C, 56.50; H, 6.74; N, 15.73.

Z–Arg(NO_2)–Pro–Lys(Z)–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Arg(NO_2)–OBzl (15)——Compound **14** (344 mg, 0.30 mmol) was treated with TFA (5 ml). The dried residue was dissolved in DMF (3 ml), and Et_3N (0.042 ml) was added. After the addition of a solution of compound **2** (488 mg, 0.60 mmol), HOBt (82 mg) and WSCD·HCl (118 mg) in DMF (3 ml), the reaction mixture was stirred at room temperature for 2 h. Further Et_3N (0.042 ml) was added and stirring of the mixture was continued at room temperature overnight. After evaporation of the DMF, the residue was triturated with water and decanted. The dried crude product was purified by gel-filtration on a Sephadex LH-20 column (3.5 × 90 cm) with DMF. The residue obtained by evaporation of the eluent was solidified with ethyl ether and recrystallized from 60% aqueous MeOH to yield 432 mg (78%) of **15**: mp 128—131 °C; $[\alpha]_D^{25}$ −43.0° (c=0.6, DMF); TLC *Rf* (D) 0.76. *Anal.* Calcd for $C_{87}H_{116}N_{22}O_{22}\cdot 2H_2O$: C, 56.24; H, 6.51; N, 16.58. Found: C, 56.18; H, 6.58; N, 16.36.

H–Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Arg–OH·3HOAc (16)——Compound **15** (102 mg, 0.055 mmol) was hydrogenated in the presence of Pd-black. After removal of the catalyst, the filtrate was concentrated *in vacuo* and the residual product was purified by chromatography on a carboxymethyl cellulose column (Whatman CM52, 1.5 × 40 cm) with a gradient solvent system between 0.08 and 0.2 M NH_4OAc. After desalting with Sephadex G-10 in the same manner as with **13**, the product was chromatographed on a Sephadex G-25 column (1.3 × 16 cm) with BuOH–pyridine–HOAc–H_2O (15 : 10 : 3 : 12), and lyophilization of the eluate gave 30 mg (33%) of **16**. mp 170—174 °C (dec.); $[\alpha]_D^{23}$ −81.7° (c=0.4, H_2O); TLC *Rf* (H) 0.44, *Rf* (J) 0.59. Amino acid ratio: Arg 1.85, Pro 1.93, Lys 1.01, Glu 2.14, Phe 2.09. Gly 0.99, Leu, 0.99. *Anal.* Calcd for $C_{64}H_{100}N_{20}O_{14}\cdot 3HOAc\cdot 5H_2O$: C, 51.15; H, 7.48; N, 17.04. Found: C, 50.90; H, 7.11; N, 17.17.

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References and Notes

1) Abbreviations with no prefix indicate the L-amino acid residue and follow the IUPAC-IUB tentative nomenclature described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are: Z, benzyloxycarbonyl; Bzl, benzyl; Boc, *tert*-butoxycarbonyl; ONO, *o*-nitrophenoxy; TFA, trifluoroacetic acid; WSCD, water-soluble carbodiimide; HOBt, 1-hydroxybenztriazole; DCHA, dicyclohexylamine; THF, tetrahydrofuran; DMF, dimethylformamide; TsOH, *p*-toluenesulfonic acid; DCE, dichloroethane.

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