

Synthesis of Substance P Analogs and Agonistic and Antagonistic Activities

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Fourteen analogs of substance P (SP), six previously synthesized by a solution method and eight newly synthesized by a solid-phase technique, have been tested for agonistic and antagonistic activities utilizing the isolated guinea pig ileum. The primary objective is to achieve effective inhibitors of SP. These analogs had agonistic activities ranging from negligible to that equivalent to SP. Six of the fourteen analogs had some degree of antagonistic activity [D-Leu⁶, D-Phe⁶]-SP is an analog which constitutes a lead to new substitutions, because it had antagonistic activity, but only negligible agonistic activity. One concept for effective antagonistic activity requires negligible or no agonistic activity.

Investigations of the neurotransmitter activities of peptide hormones can be facilitated by the availability of specific antagonists. However, the design, synthesis, and assay to achieve potent and specific antagonists of such peptide hormones is currently conducted in the absence of reliable rules which are applicable to diverse peptide hormones. For example, after the synthesis of perhaps a thousand or more analogs of the luteinizing hormone-releasing hormone (LH—RH) by many investigators on an international basis, potent antagonists of LH—RH finally were achieved, which could completely inhibit ovulation in rats at a dose level of 200 μ g.¹ Apparently, the limited number of known antagonists to the thyrotropin releasing hormone (TRH) are active only in concentrations of 10⁴-times that of TRH.²

To date, there is no known highly potent and specific peptide antagonist to substance P (SP).

Some eledoisin analogs with D-amino acid substitutions have been reported to have low antagonistic activity.³ Baclofen [β -(4-chlorophenyl)aminobutyric acid] has been reported to have antagonistic activity to SP⁴. However, the stimulating effect of SP on DOPA formation in the rat brain was not influenced by baclofen, and there was no apparent action by baclofen on the behavior of animals, according to Carlsson *et al.*⁵

Yamaguchi *et al.*⁶ and Rackur *et al.*⁷ have reported that [D-Phe⁷]-SP had low antagonistic activity, and that [Ile⁶]-SP and the heptapeptide, D-Arg-Gln-Phe-Ile-Gly-Leu-Met-NH₂, had both weak antagonistic activity and high agonistic activity. [D-Arg¹]-SP and the heptapeptide, D-Arg-Gln-Phe-Ile-Gly-Leu-Met-NH₂, were previously reported^{7,9} to have no antagonistic activity. Later, tests (Table I) on [D-Arg¹]-SP and unpublished data disclosed that both of these analogs have weak antagonistic activity.

Six analogs of SP had been available from previous synthesis by the solution method.⁸ Eight more analogs of SP have now been synthesized by a solid-phase technique.

All fourteen analogs have been bioassayed for agonistic and antagonistic activities by utilizing a biological system of the isolated guinea pig ileum.

BIOLOGICAL METHODS

The agonistic activities of the synthetic analogs and of substance P were compared using the

Table I. Data on agonist and antagonist activities.

No.		% of activity of SP on the guinea pig ileum	Antagonist activity Conc., 10 ⁻⁶ M
SP	Substance P		
1	[D-Leu ⁸ , D-Phe ⁹]-SP	< 0.03	+ ^{a,c}
2	[D-Arg ¹]-SP	103	+
3	[des- α -amino-Arg ¹]-SP	109	+
4	[Ile ⁷]-SP	0.15	- ^b
5	[Tyr ⁷ , Gly ⁸ , Phe ¹⁰]-SP	< 0.01	-
6	D-Arg-Gln-Tyr-Gly-Gly-Phe-Met-NH ₂	< 0.03	-
7	D-Arg-Gln-Phe-D-Ala-Gly-Leu-Leu-NH ₂	< 0.03	-
8	D-Arg-Gln-D-Phe-Ile-Gly-Leu-Leu-NH ₂	< 0.03	-
9	< Glu-Gln-Phe-Phe-Gly-Leu-Met-NH ₂	11	+
10	Phe-Phe-Gly-Leu-Met-NH ₂	< 0.05	-
11	Phe-Gly-Leu-Met-NH ₂	< 0.02	-
12	[Leu ¹¹]-SP	26	+
13	[des-Arg ¹]-SP	35	+
14	[des-Met ¹¹]-SP	< 0.03	-

^a +, positive. ^b -, =negative. ^c + at 10⁻⁴ M; - at 10⁻⁶ M.

terminal portion of the guinea pig ileum. Concentration-response curves were obtained by adding the analog cumulatively so that the concentration in the bath was increased by a factor of 2 whenever a steady response to the previous concentration had been reached. In the tests for antagonistic activity, the analog of substance P was added ten minutes before substance P was added, as described by Rosell *et al.*⁹ and to be described in greater detail in the future.

EXPERIMENTAL

The peptides were synthesized by the solid phase method¹⁰ and couplings were performed with a Beckman Model 990 Peptide Synthesizer using a program as described.⁶ The benzhydrylamine (BHA) resin was used as a solid support.¹¹ The α -amino functions were protected by Boc groups except in the case of D-Arg where the Z group and Arg where the Aoc group was used. The side chain functionalities were protected by Tos(Arg), NO₂(D-Arg), 2-Cl-Z(Lys) and 2-Br-Z(Tyr).

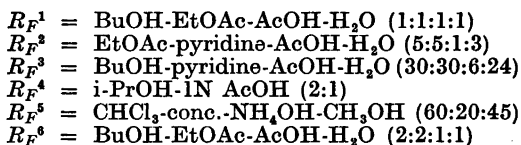
Amino acids and the BHA resin were purchased from Beckman, Inc., Palo Alto, California, or from Bachem Inc., Marina del Rey, California.

The first Boc amino acid was coupled to the BHA resin by the DCC method. After the initial amino acid was coupled to the BHA resin, the product was used without amino acid

analysis. The BHA resin as purchased was specified to contain 0.47 mequ. N/g. A complete coupling of the amino acid derivative, as proved by the ninhydrin color test,¹² was reached in every case.

The following Boc amino acid derivatives were attached to the resin by the DCC method except for Gln where the active ester method (Boc-Gln-ONP) was used. The peptides were cleaved and deblocked by liquid HF.⁶

Homogeneity of the peptides was demonstrated by TLC on silica gel plates, using the following solvent systems:



All peptides showed one spot in all systems, detected with the ninhydrin and chlorine-oxidine reagents, and after an acceptable amino acid analysis, they were considered as pure.

For amino acid analysis, samples were hydrolyzed in 6N HCl in evacuated sealed glass ampoules at 110°C for 18 h. The hydrolysate was evaporated to a residue which was dissolved in a sodium citrate buffer (pH 2.2). This solution was injected into a Beckman Model 119 Amino Acid Analyzer equipped with an Infotronics Model CRS-210 digital integrator.

Arg-Pro-Lys-Pro-Gln-Phe-D-Leu-D-Phe-Leu-Met-NH₂ or [D-Leu⁸, D-Phe⁹]-SP (1).

Starting from 2 g BHA resin (HCl salt), 2.8 g of Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-

Phe-D-Leu-D-Phe-Leu-Met-BHA resin was prepared. Cleavage by HF yielded 600 mg of the crude unprotected undecapeptide.

A portion of 250 mg was purified by partition chromatography on Sephadex G-25 with 0.1 % AcOH-BuOH-pyridine (10:7:3), and 120 mg of almost pure undecapeptide was recovered. Another purification by the same method yielded 65.5 mg of the pure peptide.

Amino acid analysis: Glu 0.98 × 2; Pro 1.00 × 2; Met 1.01; Leu 0.96 × 2; Phe 0.99 × 2; Lys 1.11; Arg 1.00. R_F Values: R_{F^1} = 0.51, R_{F^2} = 0.78, R_{F^3} = 0.39, R_{F^4} = 0.13, R_{F^5} = 0.31.

D-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ or [D-Arg¹]-SP (2).

Starting with 5 g of BHA resin (HCl salt), 7.18 g of Boc-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-BHA resin was prepared. To a portion of 3.28 g, Z-D-Arg(NO₂) was coupled after deprotection and 3.4 g of the protected undecapeptide resin was obtained. Cleavage by HF yielded 910 mg of crude peptide. A portion of 400 mg was purified by gel filtration chromatography on Bio-Gel P2, eluted with 1.3 % acetic acid, and the main peak yielded 288 mg of partially purified peptide. One half of this product was further purified by partition chromatography on Sephadex G-25 with the system 1 % AcOH-BuOH-pyridine (10:7:3) and the most pure fractions yielded 63 mg of the almost pure peptide. Repeating the same column yielded 42 mg pure [D-Arg¹]-SP.

Amino acid analysis: Glu 1.15 × 2; Pro 1.04 × 2; Gly 0.85; Met 0.87; Leu 0.92; Phe 1.08 × 2; Lys 0.93; Arg 0.90. R_F Values: R_{F^1} = 0.07, R_{F^2} = 0.49; R_{F^3} = 0.02; R_{F^4} = 0.13.

des-α-Amino-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ or [des-α-NH₂-Arg¹]-SP (3).

Five grams of BHA resin (HCl salt) afforded 7.18 g of Boc-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-BHA resin. To a portion of 3 g, des-α-amino-Arg(NO₂) was coupled and 3.16 g of the protected undecapeptide resin was obtained. Cleavage by HF yielded 1.08 g of crude peptide. It was subjected to a gel filtration chromatography on Bio-Gel P2 with 1.3 % AcOH, and 460 mg of partially purified peptide was recovered. A portion of 108 mg was further purified by partition chromatography on Sephadex G-25 with 0.1 % AcOH-BuOH-pyridine (11:5:3) and yielded 22 mg pure undecapeptide.

Amino acid analysis: Glu 1.15 × 2; Pro 0.90 × 2; Gly 0.87; Leu 0.93; Phe 1.13 × 2; Lys 0.92. R_F Values: R_{F^1} = 0.61, R_{F^2} = 0.52; R_{F^3} = 0.18; R_{F^4} = 0.21.

Arg-Pro-Lys-Pro-Gln-Gln-Ile-Phe-Gly-Leu-Met-NH₂ or [Ile⁷]-SP (4). Starting from 2.5 g BHA resin (HCl salt), 4.5 g Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Ile-Phe-Gly-Leu-Met-BHA resin was prepared. Cleavage by HF yielded 1.4 g crude peptide. An aliquot of 400 mg was first purified by partition chromatography on Sephadex G-25 eluting with 1 % AcOH-

BuOH-pyridine (10:7:3) and 240 mg of partially purified material was obtained. Further purification on Sephadex LH-20 with H₂O-BuOH (100:6) yielded 60 mg of pure [Ile⁷]-SP.

Amino acid analysis: Glu 2 × 1.10; Pro 2 × 1.18; Gly 1.05; Met 0.85; Ile 0.87; Leu 0.95; Phe 0.89; Lys 0.96; Arg 0.89. R_F Values: R_{F^1} = 0.23; R_{F^2} = 0.70; R_{F^3} = 0.59; R_{F^4} = 0.04; R_{F^5} = 0.11.

Arg-Pro-Lys-Pro-Gln-Gln-Tyr-Gly-Gly-Phe-Met-NH₂ or [Tyr⁷, Gly⁸, Phe¹⁰]-SP (5).

Five grams of BHA resin (HCl salt) afforded 5.6 g of Boc-Met-BHA resin. One gram was used to prepare 1.3 g of Boc-Gln-Tyr(BrZ)-Gly-Gly-Phe-Met-BHA resin. From a portion of 560 mg of this protected hexapeptide resin, 740 mg of Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Tyr-Gly-Gly-Phe-Met-BHA resin was synthesized. Cleavage by HF gave 215 mg crude peptide. Purification by partition chromatography on Sephadex G-25 with 0.1 % AcOH-BuOH-pyridine (11:5:3) yielded 70 mg of the pure peptide.

Amino acid analysis: Glu 1.17 × 2; Pro 0.97 × 2; Gly 1.01 × 2; Met 0.87; Tyr 0.96; Phe 0.98; Lys 0.95; Arg 0.92. R_F Values: R_{F^1} = 0.22, R_{F^2} = 0.86, R_{F^3} = 0.18, R_{F^4} = 0.22, R_{F^5} = 0.09.

D-Arg-Gln-Tyr-Gly-Gly-Phe-Met-NH₂ (6).

To 740 mg of Boc-Gln-Tyr(BrZ)-Gly-Gly-Phe-Met-BHA resin, Aoc-Arg(Tos) was coupled, to yield 770 mg of the protected heptapeptide resin. Cleavage by HF yielded 170 mg of the crude unprotected peptide. It was purified by partition chromatography on Sephadex G-25 with 0.1 % AcOH-BuOH-pyridine (10:7:3). The main fractions yielded 100 mg of product which was further purified by the same method to yield 42.5 mg of the pure peptide.

Amino acid analysis: Glu 1.0; Gly 1.08 × 2; Met 0.93; Tyr 0.99; Phe 0.99; Arg 0.94. R_F Values: R_{F^1} = 0.17; R_{F^2} = 0.82; R_{F^3} = 0.52, R_{F^4} = 0.63; R_{F^5} = 0.18.

D-Arg-Gln-Phe-D-Ala-Gly-Leu-Leu-NH₂ (7).

Four grams of BHA resin (HCl salt) were used to prepare 5 g of Boc-Gly-Leu-Leu-BHA resin. From a portion of 2.4 g of this resin, 2.5 g Boc-D-Ala-Gly-Leu-Leu-BHA resin was made. After dividing it into two equal parts, 1.42 g of Z-D-Arg(NO₂)-Gln-Phe-D-Ala-Gly-Leu-Leu-BHA resin was synthesized. Cleavage by HF yielded 195 mg of the crude peptide. A portion of 100 mg was purified by chromatography on Sephadex LH-20 and the product was eluted with BuOH-H₂O (6:100) and by adding AcOH in gradient from 1 to 5 %. From the main peak, 44.5 mg of the pure peptide was recovered.

Amino acid analysis: Glu 1.00; Gly 0.95; Ala 0.92; Leu 1.12 × 2; Phe 0.97; Arg 0.92. R_F Values: R_{F^1} = 0.72, R_{F^2} = 0.89, R_{F^3} = 0.59, R_{F^4} = 0.50, R_{F^5} = 0.27.

D-Arg-Gln-D-Phe-Ile-Gly-Leu-Leu-NH₂ (8).

A portion of 1.2 g of Boc-Gly-Leu-Leu-BHA resin from the synthesis of 7 was used for the preparation of 1.45 g of Z-D-Arg(NO₂)-Gln-D-Phe-Ile-Gly-Leu-Leu-BHA resin. Cleavage from

the resin and deprotection yielded 210 mg of crude peptide. Purification by partition chromatography on Sephadex G-25 with 0.1% AcOH-BuOH-pyridine (11:5:3) yielded a slightly impure material. Another partition chromatography on Sephadex G-25 with 0.1% AcOH-BuOH-pyridine (11:5:3) gave 35 mg of the pure peptide.

Amino acid analysis: Glu 1.01; Gly 0.95; Ile 0.88; Leu 1.12×2 ; Phe 0.97; Arg 0.93. R_F Values: $R_{F^1} = 0.58$, $R_{F^2} = 0.87$, $R_{F^3} = 0.60$, $R_{F^4} = 0.69$, $R_{F^5} = 0.37$.

RESULTS AND DISCUSSION

Analogs 5 and 6 contain the sequence of Met-enkephalin. Kosterlitz *et al.*¹³ suggested that the enkephalins may be inhibitory neurotransmitters. Therefore, it was desirable to test SP analogs containing an enkephalin-like structure for antagonistic activity. Neither of these analogs had antagonistic activity to SP.

To investigate the importance of the L-configuration and the α -amino groups of Arg¹, [D-Arg¹]-SP 2 and [des- α -NH₂-Arg¹]-SP 3 were synthesized. Both peptides showed weak antagonistic activity which classifies these peptides as partial antagonists. These two substitutions of Arg¹ gave a differentiation of SP activities. Both peptides have the same activities as SP on the guinea pig ileum and on blood pressure; however, their activities to lower body temperature were 1/20–1/10 that of SP.¹⁴

It has been reported that [Ile⁶]-SP has activity⁷ comparable with SP, but [Ile⁷]-SP 4 has a relative activity of only 0.15 on the guinea pig ileum. [Ile⁷]-SP was also relatively inactive on monoamine synthesis in the brain, but it moderately increased locomotor activity according to Carlsson.¹⁵ The two C-terminal SP-like octapeptides, 7 and 8, had very low agonistic activity on the guinea pig ileum.

[D-Leu⁸, D-Phe⁹]-SP is an important analog which constitutes a lead to new substitutions. This peptide had antagonistic activity to SP by assay on the guinea pig ileum, and it had very low agonistic activity.

[D-Leu⁸, D-Phe⁹]-SP was found by Carlsson *et al.*¹⁵ to show a potent effect on DOPA formation in the brain. They also found that it induced a very potent change in the behavior of the rats with a complete loss of the muscle tone over long-lasting barrel rotation.

The agonistic activities of the peptides 3, 10, 11, 12, 13, and 14 were in good agreement with the data from another laboratory.⁸ Only our data for the heptapeptide, 9, are quite different from the previous data;⁸ *i.e.*, 10% *vs. ca.* 100%, which could be explained by a difficulty in the solubility of this peptide in the assay systems.

It is important that [Leu¹¹]-SP has antagonistic activity and that [des-Met¹¹]-SP does not. Furthermore, it is significant that analogs with changes in position 1 may lead to antagonistic activity. The promising data on [D-Leu⁸, D-Phe⁹]-SP encourages the synthesis of SP analogs with double D-amino acid substitutions in the C-terminal part.

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