Synthesis and Biological Activity of Analogs of Substance P, Modified for Conformational Information by D-Amino Acids

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Eight analogs of substance P (SP) were synthesized by an automated solid phase-technique. Three analogs are decapeptides, as is SP, and five are heptapeptides. Phe of SP is significant for activity, but Phe is not. Gly of SP, when replaced by D-Leu, resulted in a desirable loss of activity toward the design of antagonists. Three of the five heptapeptides have single D-amino acid substitution, and the other two have two D-amino acid substitutions. Those three heptapeptides, having a D-amino acid in the position corresponding to Gly of SP, had the desirable lowest agonist activity. [D-Phe]-SP had antagonist activity which was weak, probably because it also had low agonist activity.

The biological activity of substance P (SP) was discovered in 1931 by von Euler and Gaddum. Chang and Leeman isolated a sialogogic peptide from bovine hypothalami, and found that the biological properties were indistinguishable from those described for substance P. This peptide was sequenced by Chang et al., and synthesized by Tregear et al. and by Fisher et al. A survey was published in 1976.

Some investigators have presumed substance P to be a neurotransmitter in sensory pathways.

In the mammalian gut, immunoreactivity of SP has been found in the nerve fibers of the outer smooth muscle layer. Immunoreactive endocrine cells are found in the epithelium. Such a distribution indicates that SP would have several sites of action in the gastrointestinal tract.

Chemically, SP has been classified with the tachykinins. Six naturally occurring tachykinins have been characterized from vertebrates and invertebrates as given in Scheme 1.

Since the sequences of eledoisin and that of physalemin were established in 1962 and 1964, respectively, by Ersparer et al., a large number of synthetic analogs related to these two tachykinins and later to SP had been synthesized and biologically studied before the advent of the sequence and synthesis of SP. Consequently, the literature on structure-activity relationships for the tachykinins is extensive; Ersparer et al.

Bury and Mashford reviewed the pharmacology of substance P and its physiological roles, and included the activities of analogs, and a background on the sedative effect. Magnusson et al. reported on the effect of substance P in monoamnergic mechanisms in the brain.

\[ \text{Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH}_2 \]
\[ < \text{Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH}_2 \]
\[ \text{Asp-Val-Pro-Lys-Ser-Asp-Glu-Phe-Val-Gly-Leu-Met-NH}_2 \]
\[ < \text{Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH}_2 \]
\[ < \text{Glu-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH}_2 \]
\[ < \text{Glu-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH}_2 \]

Scheme 1.

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One of our goals is to achieve an effective inhibitor of substance P. There are no reliable guidelines to modify synthetically the agonist activity of a peptide hormone to give an effective inhibitor of the hormone. One may first study which amino acids of a peptide are essential for recognition, binding, and activation as such aspects are applicable to substance P. Since acquisition of knowledge on the receptor-bound conformation is so difficult, the acquisition of biological data on analogs is a feasible initial study. The insertion of D-amino acids was emphasized in the research by many investigators on antagonists of the luteinizing hormone-releasing hormone (LH-RH), and it was found that such substitutions at critical positions in the sequence of LH-RH could confer antagonistic activity. Since it is known that heptapeptides are essentially as active as the undecapeptide sequence of SP, we have synthesized three undecapeptides and five heptapeptides with emphasis on D-amino acids for conformational aspects, and have tested them for agonist and antagonist activity.

BIOLOGICAL METHODS

The activities of the synthetic analogs and the activity of substance P were compared using the terminal portion of the guinea pig ileum in an organ bath (Table 1). 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 10 min before substance P was added. The details of the protocol for studies emphasizing antagonistic activity will be described after this research, to achieve an effective antagonist has further progressed, but the basis of the method is evident from the published details of the assay for agonist activity.

CHEMICAL METHODS AND MATERIALS

Amino acids and the BHA resins were purchased from Beckman Inc., Palo Alto, Calif., and from Bachem Inc., Marina de Rey, Calif. For amino acid analyses, samples were hydrolyzed in 6 N 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 119 Amino Acid Analyzer equipped with an Infotronics Model CRS-210 digital integrator. After the initial amino acid is coupled to the benzhydrylamine (BHA) resin, the product is used without amino acid analysis when the test with ninhydrin is negative. The BHA resin as purchased was specified to contain 0.47 meq.N/g.

During chromatography-fractionation on Sephadex monitoring was carried out at 254 nm using an ISCO UV monitor. The fractions of the major peak were checked for purity by TLC using the best system for each peptide. Those fractions showing only one spot by TLC were combined and lyophilized. The yield was sacrificed for the purity of the fractions selected.

Table 1. Data on agonist and antagonist activities.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Potency a</th>
<th>Antagonist activity con., 1-10 M b</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SP)</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₄ (Substance P)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>(I)</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-d-Phe-Phe-Gly-Leu-Met-NH₄ [d-Phe⁷]-SP</td>
<td>2</td>
<td>pos.</td>
</tr>
<tr>
<td>(II)</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-d-Leu-Met-NH₄ [d-Leu⁷]-SP</td>
<td>0.5</td>
<td>n.t.</td>
</tr>
<tr>
<td>(III)</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₄ [d-Phe⁷, d-Phe⁷]-SP</td>
<td>0.3</td>
<td>n.t.</td>
</tr>
<tr>
<td>(IV)</td>
<td>&lt; Glu-Gln-Phe-Phe-d-Leu-Leu-Met-NH₄</td>
<td>&lt; 0.02</td>
<td>neg.</td>
</tr>
<tr>
<td>(V)</td>
<td>&lt; Glu-Gln-d-Phe-Phe-Gly-Leu-Met-NH₄</td>
<td>0.25</td>
<td>neg.</td>
</tr>
<tr>
<td>(VI)</td>
<td>&lt; Glu-Gln-d-Phe-Phe-Gly-Leu-Met-NH₄</td>
<td>0.11</td>
<td>neg.</td>
</tr>
<tr>
<td>(VII)</td>
<td>Lys-Gln-Phe-Phe-d-Leu-Phe-Leu-Met-NH₄</td>
<td>&lt; 0.06</td>
<td>neg.</td>
</tr>
<tr>
<td>(VIII)</td>
<td>Lys-Gln-Phe-Ile-d-Phe-Leu-Met-NH₄</td>
<td>&lt; 0.07</td>
<td>neg.</td>
</tr>
</tbody>
</table>

a Relative to SP isolated from guinea pig ileum. b pos., positive; n. t., not tested, neg., negative.

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Table 2. Program for double deprotection and DCC coupling.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Operations</th>
<th>Mix Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CH₃Cl₂</td>
<td>3 x Wash</td>
<td>2</td>
</tr>
<tr>
<td>2. TFA (30 % in CH₃Cl₂)</td>
<td>Prewash</td>
<td>2</td>
</tr>
<tr>
<td>3. TFA</td>
<td>Deprotection</td>
<td>30</td>
</tr>
<tr>
<td>4. CH₃Cl₂, i-Prop, CH₃Cl₂</td>
<td>3 x Wash each</td>
<td>2</td>
</tr>
<tr>
<td>5. TFA</td>
<td>Prewash</td>
<td>2</td>
</tr>
<tr>
<td>6. TFA</td>
<td>Double deprotection</td>
<td>30</td>
</tr>
<tr>
<td>7. CH₃Cl₂, i-Prop, CH₃Cl₂</td>
<td>3 x Wash each</td>
<td>2</td>
</tr>
<tr>
<td>8. Et₃N (10 % in CH₃Cl₂)</td>
<td>2 x Prewash</td>
<td>2</td>
</tr>
<tr>
<td>9. Et₃N</td>
<td>Neutralization</td>
<td>5</td>
</tr>
<tr>
<td>10. Boc-Asp (in CH₃Cl₂ or DMF)</td>
<td>Delivery, transfer, and mix</td>
<td>2</td>
</tr>
<tr>
<td>11. DCC (10 % in CH₃Cl₂)</td>
<td>Delivery and mix</td>
<td>180-600</td>
</tr>
<tr>
<td>12. CH₃Cl₂, DMF and CH₃Cl₂</td>
<td>3 x Wash</td>
<td></td>
</tr>
</tbody>
</table>

Homogeneity of the peptides was demonstrated by thin-layer chromatography on silica gel plates, using the following six solvent systems: Rₚ(power 1), n-BuOH-EtOAc-AcOH-H₂O (1:1:1:1); Rₚ(power 4), EtOAc-pyridine-AcOH-H₂O (5:5:1:3); Rₚ(power 8), n-BuOH-pyridine-AcOH-H₂O (30:30:6:24); Rₚ(power 4), i-Prop-1 N AcOH (2:1); Rₚ(power 8), CHCl₃-conc NH₄OH-CH₃OH (60:20:45); Rₚ(power 4), n-BuOH-EtOAc-AcOH-H₂O (2:2:1:1). The peptide spots were detected with the ninhydrin and chlorine-o-tolidine reagents. When single spots were observed for a peptide in all six chromatographic systems, the sample was considered appropriately pure for bioassay. These Rₚ data were reinforced by the amino acid analytical data.

The analogs were synthesized by the solid-phase method using a Beckman Model 990 Peptide Synthesizer. The BHA resin was used as a solid support.

The α-amino functions were protected by the tert Boc group, except for the Arg group for Arg, and the Z group for <Glu. The first Boc amino acid was coupled as follows to the BHA resin (0.47 meq N/g). The BHA-resin hydrochloride was treated with a solution of 25 % (by volume) triethylamine in methylene chloride (25 ml/g of resin) for 10 min. Then, it was washed with 25 ml methylene chloride per gram. For one gram of the resin, 2.5 meq. of the C-terminal Boc-amino acid and DCC were used. The quantitation of coupling reaction was revealed by a negative ninhydrin color test. When the ninhydrin test was positive, even after 3 or 4 couplings, the unreacted amino groups were blocked by acetylation. The side chain functionalities were protected by Tosyl (Arg) and 2-Cl-Z (Lys).

For the coupling reactions, using 10 % DCC in methylene chloride, the amino acid derivatives were dissolved in methylene chloride, except the Aoc-Arg (Tos) and the Z <Glu which were dissolved in DMF (distilled over ninhydrin). For the coupling of Gln, the active ester, Boc-Gln-ONp, dissolved in DMF (distilled over ninhydrin) was used.

Double deprotection and DCC coupling were programmed as given in Table 2: For double coupling, a program without steps 1-6 was used. The active ester coupling was performed with a program without step 11. A 2.5-fold excess of the amino acid derivative was used for the first coupling and one equivalent was used for the second DCC-coupling. A three-fold excess of Boc-Gln-ONp was used for the first active ester single coupling, and a two-fold excess was used for the double coupling. Coupling times usually ranged from 3 to 5 hours for the DCC, and 10 to 12 hours for the active ester coupling.

The protected peptide resin was simultaneously cleaved and deblocked with anhydrous (dried over CoCl₂) liquid HF containing 10-20 % anisole. Approximately 10 ml of liquid HF was used for 1 g resin, and the reaction time was 1 h at 0°C. The HF was removed under reduced pressure. Then the peptide-resin mixture was washed with ethyl acetate and the peptide was extracted with either 1 or 10 % acetic acid. After lyophilization, the crude peptide was purified.

PURIFICATION OF THE PEPTIDES

[d-Leu^{5}]-SP (II) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-d-Leu-Leu-Met-NH_{4}). Two grams of BHA-resin (HCl salt) afforded 2.7 g of Boc-Gln-Phe-Phe-d-Leu-Leu-Met-BHA, and 1.2 g of this resin was used for the preparation of 1.7 g of Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Phe-Phe-d-Leu-Leu-Met-BHA resin. Cleavage by HF gave 410 mg of the crude peptide, and a portion of 150 mg was purified by partition chromatography on Sephadex G-25 with 0.1 % AcOH—n-BuOH—pyridine (10:7:3), and 61 mg of the pure peptide was obtained. Amino acid analysis: Glu 1.22 x 2, Pro 1.05 x 2, Met 0.91, Leu 0.95 x 2, Phe 0.87 x 2, Lys 0.92, Arg 0.90; R_{F} values: R_{F}^{1} = 0.16, R_{F}^{2} = 0.52, R_{F}^{3} = 0.61, R_{F}^{4} = 0.04, R_{F}^{5} = 0.17.

[d-Phe^{1}]-SP (I) (Arg-Pro-Lys-Pro-Gln-d-Phe-Phe-Gly-Leu-Met-NH_{4}). Two grams of BHA-resin (HCl salt) were used to prepare 2.86 g of Boc-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin. From 1.36 g of this hexapeptide resin, 1.77 g of Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin was obtained. Cleavage by HF yielded 465 mg of the crude peptide. Purification by gel filtration chromatography on Bio-Gel P2 with 1.3 % AcOH gave 356 mg partially purified peptide. From 258 mg of this material, by partition chromatography on Sephadex G-25 which was eluted with 0.1 % AcOH—n-BuOH—pyridine (10:7:3), 187 mg of pure [d-Phe^{1}]-SP was obtained. Amino acid analysis: Glu 1.20 x 2, Pro 1.08 x 2, Gly 0.91, Met 0.90, Leu 0.88, Phe 0.98 x 2, Lys 0.91, Arg 0.90; R_{F} values: R_{F}^{1} = 0.13, R_{F}^{2} = 0.45, R_{F}^{3} = 0.51, R_{F}^{4} = 0.04, R_{F}^{5} = 0.13.

[d-Phe^{1}, d-Phe^{3}]-SP (III) (Arg-Pro-Lys-Pro-Gln-Gln-d-Phe-Phe-Gly-Leu-Met-NH_{4}). Three grams of BHA-resin (HCl salt) afforded 4.05 g of Boc-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin. From 1.9 g of this protected hexapeptide resin, 2.5 g of Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin were obtained. Cleavage by HF gave 720 mg of crude peptide. A portion (185 mg) of the peptide was subjected to a partition chromatography on Sephadex G-25 which was eluted with 0.1 % AcOH—n-BuOH—pyridine (10:7:3), and 10 mg of the pure peptide was obtained. Amino acid analysis: Glu 1.18 x 2, Pro 1.07 x 2, Gly 0.93, Met 0.80, Leu 0.92, Phe 0.99 x 2, Lys 0.92, Arg 0.89; R_{F} values: R_{F}^{1} = 0.10, R_{F}^{2} = 0.44, R_{F}^{3} = 0.51, R_{F}^{4} = 0.04, R_{F}^{5} = 0.13.

< Glu-Gln-Phe-Phe-d-Leu-Leu-Met-NH_{4},(IV). Z<Glu was attached to 1.5 g of Glu-Gln-Phe-Phe-d-Leu-Leu-Met-BHA resin from the synthesis of (II), and 1.52 g of Z<Glu-Gln-Phe-Phe-d-Leu-Leu-Met-BHA was obtained. After cleavage with HF, the peptide resin mixture was washed with ethyl acetate (100 ml), then extracted with 10 % AcOH (100 ml), afterwards with the lower phase (100 ml) of 0.1 % AcOH—n-BuOH—pyridine (10:7:3), and at last with the upper phase (100 ml) of 0.1 % AcOH—n-BuOH—pyridine (10:7:3). From the extract with 10 % AcOH, 71 mg crude peptide and from that of the lower phase of 0.1 % AcOH—n-BuOH—pyridine (10:7:3) 145 mg of crude peptide were obtained. The extract of the upper phase of 0.1 % AcOH—n-BuOH—pyridine (10:7:3) yielded 23 mg of the peptide. This fraction was pure in all TLC systems. Amino acid analysis: Glu 1.04 x 2, Met 0.85, Leu 1.05 x 2, Phe 1.07 x 2; R_{F} values: R_{F}^{1} = 0.78, R_{F}^{2} = 0.93, R_{F}^{3} = 0.84, R_{F}^{4} = 0.79, R_{F}^{5} = 1.0, R_{F}^{6} = 0.70.

< Glu-Gln-d-Phe-Phe-Gly-Leu-Met-NH_{4},(V). Glu-d-Phe-Phe-Gly-Leu-Met-BHA resin (1.5 g prepared in the synthesis of (I) was used to prepare 1.53 g of Z<Glu-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin. Cleavage by HF yielded 370 mg crude peptide. Purification by partition chromatography on Sephadex G-25 with n-BuOH—AcOH—H_{2}O (4:1:5) gave 73 mg of the pure peptide. Amino acid analysis: Glu 1.10 x 2, Gly 0.86, Met 0.85, Leu 0.89, Phe 1.14 x 2; R_{F} values: R_{F}^{1} = 0.74, R_{F}^{2} = 0.89, R_{F}^{3} = 0.81, R_{F}^{4} = 0.76, R_{F}^{5} = 1.0, R_{F}^{6} = 0.59.

< Glu-Gln-d-Phe-Phe-Gly-Leu-Met-NH_{4},(VI). Glu-d-Phe-Phe-Gly-Leu-Met-BHA resin prepared in the synthesis of (III) was used to prepare 2.2 g of Z<Glu-Gln-d-Phe-Phe-Gly-Leu-Met-BHA resin. Cleavage by HF yielded 310 mg crude peptide, and 110 mg of this material were purified by partition chromatography on Sephadex G-25 with n-BuOH—AcOH—H_{2}O (4:1:5) to yield 75 mg pure peptide. Amino acid analysis: Glu 1.19 x 2, Gly 0.88, Met 0.85, Leu 0.92, Phe 1.06 x 2; R_{F} values: R_{F}^{1} = 0.72, R_{F}^{2} = 0.76, R_{F}^{3} = 0.74, R_{F}^{4} = 0.59.

Lys-Gln-Phe-Phe-d-Leu-Leu-Met-NH_{4},(VII). From 1.5 g of BHA resin (HCl salt) 1.9 g

of Boc-D-Phe-Leu-Met-BHA resin was prepared; 1.2 g of this product was used to prepare 1.32 g of Boc-Gln-Phe-D-Leu-D-Phe-Leu-Met-BHA resin. A portion of 600 mg of this protected peptide resin was used to obtain 630 mg of Boc-Lys(2-Cl-Z)-Gln-Phe-D-Leu-D-Phe-Leu-Met-BHA resin. Cleavage by HF resulted in 133 mg of the crude peptide. It was purified by partition chromatography on Sephadex G-25 with the elute 0.1 % AcOH — n-BuOH — pyridine (10:7:3), and 70 mg of partially purified peptide were recovered. Further purification of 40 mg of this material on Sephadex LH-20 with n-BuOH — H₂O — AcOH (6:90:10) yielded 19.8 mg of the pure peptide. Amino acid analysis: Glu 0.95, Met 0.93, Leu 1.08 × 2, Phe 1.05 × 2, Lys 0.87; RF values: RF² = 0.78, RF³ = 0.83, RF⁴ = 0.59, RF⁵ = 0.59, RF⁶ = 0.88, RF⁷ = 0.31.

Lys-Gln-Phe-Ileu-D-Phe-Leu-Met-NH₄ (VIII). Starting with 700 mg of Boc-D-Phe-Leu-Met-BHA resin, which was prepared for the synthesis of (VII), 770 mg of Boc-Lys(2-Cl-Z)-Gln-Phe-Ileu-D-Phe-Leu-Met-BHA resin were synthesized. Cleavage by HF yielded 167 mg crude peptide. It was subjected to a partition chromatography on Sephadex G-25 with 0.1 % AcOH — n-BuOH — pyridine (11:5:3), and 55.7 mg of partially purified peptide were recovered. Further purification with a second column G-25 using 0.1 % AcOH — n-BuOH — pyridine (10:7:3) yielded 47 mg almost pure peptide. Another chromatography on LH-20 eluted with n-BuOH — H₂O — AcOH (6:90:10) provided 43.6 mg of pure peptide. Amino acid analysis: Glu 1.04, Met 0.95, Ileu 0.87, Leu 0.98, Phe 1.09 × 2, Lys 0.99. RF values: RF² = 0.75, RF³ = 0.93, RF⁴ = 0.59, RF⁵ = 0.88, RF⁶ = 0.28.

RESULTS AND DISCUSSION

In analog I, the Phe⁷ of SP has been replaced with D-Phe⁷, and this undecapeptide had a relative activity of only 2 % in comparison to SP showing again the importance of Phe⁷ in SP for biological activity. It is notable that Phe is in position 7 of the 6 tachikinins, and counting from the C-terminal, Phe is in position 5 of all 6 tachikinins.

In analog II, Gly⁷ of SP is replaced by D-Leu, and this substitution resulted in a very low activity (0.5 %). However, the substitution of Gly⁷ in edelosin by certain L-amino acids did not reduce the activity.²¹ The introduction of D-Leu into position 9 of SP may have resulted in a conformational change, which is particularly detrimental for activity.

In analog III, the Phe⁴-D-Phe⁷ moiety of SP has been replaced by D-Phe⁴-D-Phe⁷; the relative activity of 0.3 % confirms the importance of Phe in position 7 of SP and the relative unimportance of Phe in position 8, because analogs I and III have comparable low activities.

Analogos IV—VIII are heptapeptides, and analogs, IV, V, VIII contain one D-amino acid moiety, and analogs VI and VII contain two D-amino acid moieties.

The heptapeptides IV, VII and VIII, of the five heptapeptides, had comparable lowest agonist activities. In each of these three analogs of such low activity, the Gly⁷ of SP has been replaced with a D-amino acid, either D-Leu or D-Phe. Consequently, position 9 as well as position 7 are important for continuing studies on agonist and antagonist activities.

In this research, structural substitutions to achieve substantial loss of agonist activity may be a desirable step toward effective antagonists, because effective antagonists may have very little, or no agonist activity. The design of an inactive analog of an agonist does not necessarily also provide an effective antagonist. However, Analog I, which is an undecapeptide with the important Phe⁷ of SP replaced by D-Phe⁷ did show antagonist activity, but the effect was weak. Such low antagonism may be expected, because this analog also showed agonist activity (2 %). These data may be a basis for the design of new analogs having agonist and antagonist activities.

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REFERENCES


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