# Syntheses and Biological Activities of Neurokinin B Analogs Modified at Positions 2, 3, and 6

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**Synopsis.** Nine neurokinin B [NKB] related octa- and nona-peptides containing Arg and *N*-methylglycine, D-Ala, D-Phe, D-Trp, D-α-aminophenylacetic acid, D-Arg, D-Pro, D-homoglutamine, or D-homoglutamic acid instead of Met$^2$ or His$^3$, and Phe$^6$, respectively, in the original sequences were synthesized by standard solid phase method. The biological activities of the analogs were assayed on isolated guinea-pig ileum. [Arg$^3$, D-Ala$^6$]-NKB (3—10) was found to act as an antagonist of NKB.

Neurokinin B[1,2] (NKB) is a novel neuropeptide isolated from porcine spinal cord and sequenced as decapeptide amide (Fig. 1). NKB has close structural homology, particularly in the C-terminal sequence, with tachykinin such as substance P (SP).

The relationship study[3] between chain length and activity indicated that the contractile potency of NKB on guinea-pig ileum, rat vas deferens and rat duodenum remained nearly complete after removal of N-terminal tripeptide portion from the native peptide.

Our recent study[4] on the structure activity relationship of NKB revealed that the replacement of Phe$^6$ or Val$^7$ residue with Gly moiety brings drastic decrease of the contractile activities on isolated guinea-pig ileum and rat vas deferens, while the substitution of Gly for Phe$^5$ enhances their activities. Octapeptide analogs, [Gly$^6$]-NKB (3—10) **1** and [Gly$^7$]-NKB (3—10) **2**, have no agonistic activity on both assays.

Since our synthetic study on the structure activity relationship of NKB aimed the development of a specific and potent antagonist, the analogs **1** and **2** were examined for their antagonistic effects against NKB. The analog **1** was found to exert potent antagonistic effect, but analog **2** was not an antagonist. Based on this observation, a new series of octa-and nona-peptide analogs containing unusual amino acid at position 6 (Fig. 1) was designed in order to obtain more potent antagonist. *N*-methylglycine (sarcosine, Sar), D-Ala, D-Phe, D-Trp, D-α-aminophenylacetic acid (D-α-phenylglycine, Phg), D-Arg, D-Pro, D-homoglutamine (Hgn),[5] or D-homoglutamic acid (Hgu) residue was substituted for Phe$^6$. It has been observed that the replacement of natural residue with unusual amino acid in SP[6,7] changes the pharmacological spectrum of SP from that of an agonist to that of an antagonist. Arg moiety was incorporated into peptide chain to improve the solubility of the peptides in water according to SP antagonists.[7]

This paper describes the first application of a new amino acid D-Hgu to the synthetic study of bioactive peptide analogs. D-Hgu residue was incorporated into a peptide chain by the usual solid phase technique using Boc-D-Hgu(OBzl)-OH,[5] which was derived from the $N^{\alpha}$,$N^{ca}$-(Boc)$_2$-D-Hgn-OH[5,6] by the reaction with anhydrous sodium benzylate in benzyl alcohol.

The new NKB analogs were synthesized in the same way with the preparation of NKB analogs[4] on an automated peptide synthesizer by standard solid phase method[8] starting from *t*-butoxycarbonyl(Boc)-Met-benzhydrylamine-resin. Boc protection was used for α-amino function. The side-chain protective groups used were cyclohexyl for Asp, benzyl for D-Hgu, *p*-tolylsulfonyl for Arg and His, and Boc for the amide nitrogen ($N^{ca}$) of D-Hgn. After the completion of the chain elongation, the peptide resin was treated with liquid hydrogen fluoride[9] in the presence of anisole to cleave the peptide from the resin with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide. The synthetic products were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using 0.1% trifluoroacetic acid in acetonitrile as eluent. Highly purified peptides (Table 1) were obtained after gel filtration on Sephadex LH-20 column. Homogeneity of the peptides was demonstrated by analytical HPLC

| | | 1 2 3 6 7 10 |
|---|---|---|
| | NKB | H-Asp-Met-His-Asp-Phe — Phe-Val-Gly-Leu-Met-$NH_2$ |
| 1 | [Gly$^6$]-NKB (3—10) | H-His-Asp-Phe — Gly-Val-Gly-Leu-Met-$NH_2$ |
| 2 | [Gly$^7$]-NKB (3—10) | H-His-Asp-Phe — Phe-Gly-Gly-Leu-Met-$NH_2$ |
| 3 | [Arg$^3$, Sar$^6$]-NKB (3—10) | H-Arg-Asp-Phe — Sar-Val-Gly-Leu-Met-$NH_2$ |
| 4 | [Arg$^3$, D-Ala$^6$]-NKB (3—10) | H-Arg-Asp-Phe-D-Ala-Val-Gly-Leu-Met-$NH_2$ |
| 5 | [Arg$^3$, D-Phe$^6$]-NKB (3—10) | H-Arg-Asp-Phe-D-Phe-Val-Gly-Leu-Met-$NH_2$ |
| 6 | [Arg$^3$, D-Trp$^6$]-NKB (3—10) | H-Arg-Asp-Phe-D-Trp-Val-Gly-Leu-Met-$NH_2$ |
| 7 | [Arg$^3$, D-Phg$^6$]-NKB (3—10) | H-Arg-Asp-Phe-D-Phg-Val-Gly-Leu-Met-$NH_2$ |
| 8 | [Arg$^2$, D-Arg$^6$]-NKB (2—10) | H-Arg-His-Asp-Phe-D-Arg-Val-Gly-Leu-Met-$NH_2$ |
| 9 | [Arg$^2$, D-Pro$^6$]-NKB (2—10) | H-Arg-His-Asp-Phe-D-Pro-Val-Gly-Leu-Met-$NH_2$ |
| 10 | [Arg$^2$, D-Hgn$^6$]-NKB (2—10) | H-Arg-His-Asp-Phe-D-Hgn-Val-Gly-Leu-Met-$NH_2$ |
| 11 | [Arg$^2$, D-Hgu$^6$]-NKB (2—10) | H-Arg-His-Asp-Phe-D-Hgu-Val-Gly-Leu-Met-$NH_2$ |

Fig. 1. NKB and its analogs.

Table 1. Physical Properties and Yield of the Synthetic Analogs

| Analog | $[\alpha]_D^{18}$/° (*c* 0.1, DMF) | Retention time[a]/min | $R_f^1$ | $R_f^2$ | Yield/% |
|---|---|---|---|---|---|
| **3** | −13 | 14.0 | 0.09 | 0.62 | 22.9 |
| **4** | −10 | 13.8 | 0.11 | 0.64 | 12.6 |
| **5** | −11 | 18.4 | 0.14 | 0.67 | 15.9 |
| **6** | +3 | 18.0 | 0.14 | 0.66 | 11.2 |
| **7** | −28 | 17.8 | 0.14 | 0.68 | 15.3 |
| **8** | +6 | 12.7 | 0.03 | 0.62 | 17.7 |
| **9** | +7 | 16.7 | 0.06 | 0.64 | 29.5 |
| **10** | −16 | 12.8 | 0.04 | 0.61 | 23.4 |
| **11** | −1 | 13.9 | 0.05 | 0.61 | 20.6 |

a) Condition of analytical HPLC: column, Chemcosorb ODS; flow rate, 1 ml $min^{-1}$; Detection, 210 nm; eluent system, linear gradient from 14% to 35% $CH_3CN$ (15 min) in 20 mM phosphate buffer (pH 3.0).

and thin-layer chromatography (TLC). When single peak and single spot were observed for a peptide in all chromatographic systems, the peptide was considered appropriately pure for bioassay. The chromatographic data were reinforced by the amino acid analytical data.

Biological properties of the synthetic peptides were examined on contractile activities of isolated guinea-pig ileum. The agonistic effects of analogs **3**—**11** were compared with that of our synthetic NKB. Analogs **1**—**11** were tested for their antagonistic effects against NKB. The antagonistic activities of the analogs were evaluated in terms of $pA_2$ (negative concentration of antagonist that reduces the contractile response to a double dose of agonist in the presence of antagonist to that of a single dose without it), according to Schild.[10] The results are presented in Table 2.

Analogs **3**, **4**, **5**, **7**, **8**, **9**, and **11**, in which Phe[6] was replaced by Sar, D-Ala, D-Phe, D-Phg, D-Arg, D-Pro, or D-Hgu, had no agonistic activity on the ileum assay, and [Arg[3], D-Trp[6]]-NKB (3—10) **6** and [Arg[2], D-Hgn[6]]-NKB (2—10) **10** possessed a little contractile activity. It was observed that the replacement of Phe[6] with Gly or D-Ala changed the pharmacological spectrum of NKB from that of an agonist to that of an antagonist and the substitution by the unusual amino acide except D-Ala was unfavorable for antagonism to NKB. Analogs **1** and **4** exerted antagonistic effects against NKB showing similar $pA_2$ values. The results reveal that **1** and **4** are the first NKB analogs to act as fairly potent antagonists against NKB.

Table 2. Biological Activities of the Synthetic Analogs on Isolated Guinea-Pig Ileum

| Analog[a] | RA[b] | $pA_2$ |
|---|---|---|
| **1** | 0[c] | 5.82 |
| **2** | 0[c] | —[d] |
| **4** | 0 | 5.69 |
| **6** | <0.01 | — |
| **10** | <0.01 | — |

a) **3**, **5**, **7**, **8**, **9**, and **11** showed no agonistic and antagonistic activity. b) Relative contractile activity to NKB=1. c) Data from Ref. 4. d) —, no antagonistic activity.

## Experimental

Amino acid derivatives were purchased from Peptide Institute, Inc., Osaka, Japan and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol $g^{-1}$ of support) from Beckman Inc., Palo Alto, Calif. Optical rotations were measured in a 1 dm tube using a Nipponbunkoh DIP-4 Polarimeter. Amino acid analyses on samples previously hydrolyzed with 6 M HCl (1 M=1 mol $dm^{-3}$) (110 °C, 24 h) or on analog **6** with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (115 °C, 24 h) were performed on a Beckman System 7300 Amino Acid Analyzer. TLC were carried out on silica-gel plates (Merck). The following solvent systems were used and allowed to ascend for 10 cm: $R_f^1$, *n*-BuOH: AcOH: $H_2O$ (4:1:5, upper phase); $R_f^2$, *n*-BuOH: pyridine: AcOH: $H_2O$ (30:20:6:24). Analytical HPLC were effected on the following systems: column, Chemcosorb ODS (Chemco, 3.9×300 mm); flow rate, 1 ml $min^{-1}$; detection, 210 nm; eluent system, linear gradient for 15 min from 14% to 35% $CH_3CN$ in 20 mM phosphate buffer (pH 3.0).

**General Procedure for the Preparation of Analogs.** The solid phase synthesis was carried out using a Beckman System 990C Peptide Synthesizer as described previously.[4] Half gram of BHA-resin hydrochloride for each analog served as the solid support. A 2.5 fold excess of the amino acid derivative was used for all coupling. The coupling was affected with dicyclohexylcarbodiimide/1-hydroxybenzotriazole. The protected peptide resin was treated with anhydrous liquid HF containing 10% anisole. After evaporation of HF in vacuo under ice-cooling, the residue was washed with AcOEt and the peptide was extracted with 50% AcOH.

**Purification of the Peptides.** The crude peptide was subjected to HPLC as reported previously.[4] The apparatus was composed of a model 590 pump and a U6K injector (Waters) connecting with a column of Chemcosorb ODS (Chemco, 20×300 mm) or μ-Bondasphere $C_{18}$ (Waters, 19×150 mm). The eluates were monitored with a UV detector S-310A model-II (Soma) at 210 nm wavelength. $CH_3CN$-0.1% TFA solvent system was used as eluent at flow rate 10 or 7 ml $min^{-1}$. Each peptide was emerged at 40-60 min by isocratical elution with 15—26% $CH_3CN$ contents of the solvent system. The desired fraction was passed through a Sephadex LH-20 column (16×500 mm) eluted with aqueous DMF (90%) containing 0.02% 1,2-ethanedithiol. Homogeneity of the peptides was analyzed by analytical HPLC and TLC. Amino acid compositions of the acid hydrolysates of the synthetic peptides were consistent with the calculated values.

**Bioassay.** The agonistic activity of the synthetic analog was measured on ileum taken from guinea-pig, as described before.[4] The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with load of 2 g on a Servocorder (Watanabe Instruments, SR6204). Concentration-response curves were obtained using a

cumulative dose-assay, and the time between two consecutive dose-response curves was longer than 10 min. Though NKB at concentration $10^{-7}$ M induced maximal response, all of analogs were applied up to $10^{-6}$ M. In the tests for antagonistic activity on guinea-pig ileum, analog was added 10 min before NKB was added. The cumulative dose-response curve of NKB in the presence or the absence of the analog was obtained. In the presence of analogs at concentration $2\times10^{-6}$ M, the maximal response induced by NKB was almost the same response without analogs.

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