# Neurokinin B Analogs Substituted with Glycine

Tadashi HASHIMOTO,* Tadashi MORO, and Yoshiki UCHIDA
School of Pharmacy, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920-11
(Received July 2, 1986)

**Synopsis.** Four Neurokinin B analogs substituted with glycine at position 4, 5, 6, or 7 were synthesized by Merrifield's solid phase method. Pharmacological property of the analogs is described. The results suggest that Phe$^6$ and Val$^7$ residues may be essential for intrinsic activity on smooth muscle.

Neurokinin B (NKB, also called neuromedin K) is a novel neuropeptide isolated from porcine spinal cord. Structure elucidation[1] revealed that NKB has close structural homologies with tachykinin such as substance P (SP), kassinin and physalaemin (Fig. 1). NKB, decapeptide amide, is very similar to the undecapeptide SP, particularly in the sequence between Phe$^6$ and Met$^{10}$ residues of NKB, and between Phe$^7$ and Met$^{11}$, and has the same C-terminal pentapeptide sequence of dodecapeptide kassinin.

The structural determination of NKB led us to undertake systematic studies on structure-activity relationship by means of highly purified synthetic peptides related to the neuropeptide. This paper describes synthesis and biological property of four kinds of NKB analogs.

In study[3] of the relationships between chain length and activity, it is revealed that the removal of the N-terminal two amino acids from NKB increases the contractile activity on rat vas deferens as the peptide chain is shortened and NKB (5—10) shows unequivocal loss of activity. In the present investigation, in order to examine the biological effect of the substitution of Gly moiety for Asp$^4$, Phe$^5$, Phe$^6$, and Val$^7$ residues, we synthesized [Gly$^4$]-, [Gly$^5$]-, [Gly$^6$]-, and [Gly$^7$]-NKB (3-10) (Fig. 2), and submitted them for biological assay to compare with our synthetic NKB.

Synthesis of NKB and the related peptides used in this study was performed on a Beckman System 990C Peptide Synthesizer by standard solid phase method[4] 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 and *p*-toluenesulfonyl for His. Deprotection and dicyclohexylcarbodiimide (DCC) coupling were programed as given in Table 1. For second coupling, a program without steps 1—4 was used. A 2.5 fold excess of amino acid derivatives was used for all couplings. The second coupling was repeated with additive of one equivalent of 1-hydroxybenzotriazole (HOBt). The coupling reaction was monitored by the ninhydrin color test procedure of Kaiser et al.[5] A trace of unreacted amino group was acetylated. After the completion of the chain elongations, the peptide resin was treated with liquid hydrogen fluoride (HF)[6] in the presence of anisole for 1 h at 0 °C to cleave the peptide from the resin with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide. The excess HF was removed in vacuo, and the resulting material was washed with ethyl acetate prior to extraction of the peptide with aq acetic acid (50%). The crude product was purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using dil trifluoroacetic acid (0.1%) in acetonitrile as eluent. The desired fractions were collected and desalted on a column of Sephadex LH-20 eluted with aq *N*,*N*-dimethylformamide (90%), giving pure peptide. The yield was sacrificed for the purity of the fractions selected. Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC) (Table 2). When single peaks and single spots were observed for a peptide in all chromatographic systems, the sample was considered

| | 1 | | 10 |
|---|---|---|---|
| NKB | H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-$NH_2$ | | |
| | 1 | | 11 |
| SP | H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$ | | |
| | 1 | | 12 |
| Kassinin | H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-$NH_2$ | | |
| | 1 | | 11 |
| Physalaemin | pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ | | |

Fig. 1. Amino acid sequences[2] of tachykinin peptides.

| | 3 | 10 | |
|---|---|---|---|
| [Gly$^4$]-NKB (3—10) | H-His-Gly-Phe-Phe-Val-Gly-Leu-Met-$NH_2$ | | **1** |
| [Gly$^5$]-NKB (3—10) | H-His-Asp-Gly-Phe-Val-Gly-Leu-Met-$NH_2$ | | **2** |
| [Gly$^6$]-NKB (3—10) | H-His-Asp-Phe-Gly-Val-Gly-Leu-Met-$NH_2$ | | **3** |
| [Gly$^7$]-NKB (3—10) | H-His-Asp-Phe-Phe-Gly-Gly-Leu-Met-$NH_2$ | | **4** |

Fig. 2. Synthetic NKB analogs.

Table 1. Program for Solid Phase Synthesis

| Step | Reagent | Operation | Mix time/min |
|---|---|---|---|
| 1 | $CH_2Cl_2$ | 3×Wash | 1.5 |
| 2 | TFA (25% in $CH_2Cl_2$) | Prewash | 1.5 |
| 3 | TFA (25% in $CH_2Cl_2$) | Deprotection | 30 |
| 4 | $CH_2Cl_2$ | 3×Wash | 1.5 |
| 5 | $CH_2Cl_2$ | 3×Wash | 1.5 |
| 6 | TEA (10% in $CH_2Cl_2$) | 3×Neutralization | 1.5 |
| 7 | $CH_2Cl_2$ | 6×Wash | 1.5 |
| 8 | Boc-Amino Acid (in $CH_2Cl_2$) | Mix | 5 |
| 9 | DCC | Mix | 120 |
| 10 | $CH_2Cl_2$ | 6×Wash | 1.5 |

Table 2. Physical Properties and Yield of the Peptides

| Analog | $[\alpha]_D^{22}$/° (*c* 0.1, DMF) | Retention time[a)]/min | $R_f^I$ | $R_f^{II}$ | Yield/% |
|---|---|---|---|---|---|
| **1** | −36.0 | 17.5 | 0.15 | 0.75 | 39.2 |
| **2** | −33.0 | 15.1 | 0.10 | 0.68 | 6.3 |
| **3** | −83.0 | 14.8 | 0.10 | 0.68 | 10.5 |
| **4** | −47.0 | 16.9 | 0.10 | 0.68 | 38.0 |

a) Condition of analytical HPLC: column, NOVA-PAK $C_{18}$; flow rate, 1 ml $min^{-1}$; Detection, 210 nm; eluent system, linear gradient from 7% to 42% $CH_3CN$ (15 min) in 20 mM phosphate buffer (pH 3.0).

Table 3. Amino Acid Analyses of the Peptides

| Analog | Found (Calcd) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | His | Asp | Gly | Val | Met | Leu | Phe | $NH_3$ |
| **1** | 0.93 (1) | — | 2.04 (2) | 1.16 (1) | 0.98 (1) | 0.94 (1) | 1.90 (2) | 1.79 (1) |
| **2** | 1.04 (1) | 1.19 (1) | 1.87 (2) | 1.03 (1) | 0.89 (1) | 1.09 (1) | 0.84 (1) | 1.27 (1) |
| **3** | 1.33 (1) | 1.21 (1) | 1.80 (2) | 0.94 (1) | 0.84 (1) | 0.90 (1) | 1.03 (1) | 1.67 (1) |
| **4** | 1.08 (1) | 1.23 (1) | 1.95 (2) | — | 0.93 (1) | 1.00 (1) | 1.82 (2) | 1.57 (1) |

Table 4. Relative Potencies of Synthetic NKB Analogs

| Analog | GPI[a)] | RVD[a)] |
|---|---|---|
| **1** | 0.28 | 0.63 |
| **2** | 2.0 | 1.58 |
| **3** | 0 | 0 |
| **4** | 0 | 0 |

a) Relative activity accepted NKB as 1.

appropriately pure for bioassay. These chromatographic data were reinforced by the amino acid analytical data (Table 3).

Biological properties of the synthetic NKB analogs in this study were examined on the isolated rat vas deferens (RVD) and the guinea pig ileum (GPI). The contractile activities of the analogs were compared with those of synthetic NKB. The results are shown in Table 4.

The potencies of analog **2** were higher than the activities of NKB itself on both assays. Analog **1** showed reduced potency, while the peptides **3** and **4** possessed no activity. Thus, these results suggest that $Phe^6$ and $Val^7$ residues of NKB may be essential for the intrinsic 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 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) were performed on a Hitachi KLA-5 Amino Acid Analyzer. TLC was carried out on silica-gel plates (Merck). The following solvent systems were used and allowed to ascend for 10 cm: $R_f^I$, *n*-BuOH : AcOH : $H_2O$ (4 : 1 : 5, upper phase); $R_f^{II}$, *n*-BuOH : pyridine : AcOH : $H_2O$ (30 : 20 : 6 : 24). Analytical HPLC was effected on the following systems: column, NOVA-PAK $C_{18}$ (3.9×150 mm); flow rate, 1 ml $min^{-1}$, detection, 210 nm; eluent system, linear gradient of $CH_3CN$ in 20 mM phosphate buffer (pH 3.0).

**General Procedure of Analog.** The first Boc-amino acid was coupled as follows to the BHA-resin (0.60 mequiv N $g^{-1}$). The BHA-resin hydrochloride was treated with a solution of 10% (by volume) triethylamine (TEA) in dichloromethane (10 ml $g^{-1}$ of resin) for 4.5 min. Then the resin was washed with 10 ml of dichloromethane per gram. For half gram of the resin, 2.5 equiv of Boc-Met-OH 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 second coupling, the unreacted amino

groups were blocked by acetylation using $Ac_2O$ (5 equiv) and pyridine (1 equiv) in dichloromethane for 20 min. For the coupling reactions, using 2.5 equiv of DCC in dichloromethane, the amino acid derivatives were dissolved in dichloromethane. Deprotection and DCC coupling were programed as given in Table 1. For second coupling, a program without steps 1—4 was used. A 2.5 fold excess of the amino acid derivative was used for all couplings. One equivalent of HOBt was used only for the second DCC-coupling. The protected peptide resin was simultaneously cleaved and deblocked with anhydrous liquid HF containing 10% anisole. Approximately 5 ml of liquid HF was used for 0.25 g resin, and the reaction time was 1 h at 0 °C. After evaporation of HF, the peptide-resin mixture was washed with AcOEt. The peptide was extracted with three portions of 50% AcOH and the extracts combined were lyophilized.

**Purification of the Peptides.** The crude peptide was subjected to HPLC which is composed of a model 590 pump and a U6K injector (Waters) connecting with a column (20×300 mm) of Chemcosorb ODS (Chemco). 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 ml $min^{-1}$. Each peptide was emerged at 40—60 min by isocratical elution with 22—28% $CH_3CN$ contents of the solvent system. The desired fraction was desalted on a Sephadex LH-20 column (15×500 mm) eluted with DMF-$H_2O$ (9:1) and the product was lyophilized. Homogeneity of the peptides was analyzed by analytical HPLC on a column of NOVA-PAK $C_{18}$ (3.9×150 mm) with gradient elution using 20 mM phosphate buffer (pH 3.0)/$CH_3CN$ and by TLC on silica-gel plate. The peptides on silica- gel plate were detected with ninhydrin and chlorine-*o*-tolidine reagents. When single peaks and single spots were observed for a peptide in all chromatographic systems and amino acid composition after hydrolysis of the peptides were consistent with the calculated values, the sample was submitted for bioassay.

**Bioassay.** The bioactivities of NKB analogs were measured on the isolated rat vas deferens and guinea pig ileum. Male rat (weighing 250—300 g) and male guinea pig (weighing 200—250 g) were stunned by a blow to the neck and exsanguinated. Vas deferens and ileum were rapidly isolated and kept for dissection in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.4 mM $NaH_2PO_4$, 12 mM $NaHCO_3$, 5.6 mM glucose) at room temperature. Both organs were suspended in a 30 ml organ bath containing Tyrode's solution thermostated at 30 °C and bubbled with 95% $O_2$ and 5% $CO_2$ gas for 1 h. The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with load of 1 or 2 g (for vas deferens and ileum respectively) on a Servocorder (Watanabe Instruments, SR6204). Concentration-response curves were obtained using a cumulative dose-assay, the time between two consecutive dose-response curves longer than 10 min.

## References

1) S. Kimura, M. Okada, Y. Sugita, I. Kanazawa, and E. Munekata, *Proc. Jpn. Acad.*, **59B**, 101 (1983); H. Kangawa, N. Minamino, A. Fukuda, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, **114**, 533 (1983).

2) Amino acids, peptides and their derivatives mentioned in this paper are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9 (1984).

3) F. Osakada, K. Kubo, K. Goto, I. Kanazawa, and E. Munekata, *Eur. J. Pharmacol.*, **120**, 201 (1986).

4) R. B. Merrifield, *Adv. Enzymol.*, **32**, 221 (1969).

5) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).

6) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).