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# 고상법에 의한 Dynorphin B 유도체의 합성

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# Synthesis of Dynorphin B Analogues by Solid-Phase Method

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**요 약.** Dynorphin B 유도체들인[Arg$^{11}$, D-Ala$^{12}$] dynorphin B, [D-Ala$^{2}$, Ala$^{6}$, Arg$^{11}$, D-Ala$^{12}$] dynorphin B 및 dynorphin B(1-11)를 고상법으로 합성하였다. 에탄올 용액에서 2% 다비닐벤젠으로 교차결합된 파라-염화메틸 폴리스티렌 수지와 Thr을 반응시켜 수지 1 g 당 1.20 mmol의 Thr을 치환시켰다. 모든 아미노산의 아미노기는 t-Boc기로 보호하였으며 Tyr과 Arg의 곁사슬은 2,6-디클로로벤질기와 니트로기로 각각 보호하였다. 각 유도체는 DCC와 HOBT를 짝지음 시약으로 사용하여 단계적 합성법으로 합성하였으며 생성물은 MeOH/MeCN (3/1)을 전개용매로 하여 Sephadex LH-20 column(2×50 cm)으로 정제하였으며 HPLC와 아미노산 분석기로 확인하였다.

**ABSTRACT.** Dynorphin B analogues, [Arg$^{11}$, D-Ala$^{12}$] dynorphin B, [D-Ala$^{2}$, Ala$^{6}$, Arg$^{11}$, D-Ala$^{12}$] dynorphin B, and dynorphin B (1-11) were synthesized by solid-phase method. A chloromethylated polystyrene resin cross-linked with 2% divinylbenzene was substituted with Thr in ethanol to contain 1.20 mmol Thr/g of resin. All amino groups of amino acids were protected with t-Boc group and 2,6-dichlorobenzyl and nitro groups were used to protect the side chains of Tyr and Arg, respectively. Stepwise synthetic method was applied for synthesis of the products. Dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) were used as the coupling reagents. The crude peptides were purified by gel filteration on Sephadex LH-20 column (2×50 cm) using MeOH/MeCN (3/1) and then characterized with HPLC, amino acid analyzer.

## INTRODUCTION

A number of endogeneous peptides with opiatelike activity have recently been isolated and characterized.[1-3] In addition to the opioid pentapeptides methionine-enkephalin ([Met] enkephalin) and leucine-enkephalin ([Leu] enkephalin),[4] there are large opioid peptides containing a [Met] enkephalin or [Leu] enkephalin sequence as a core fragment.

Dynorphin B is a endogeneous opioid tridecapeptide extracted from porcine brain by Goldstein *et al*[5] in 1982. In the GPI (Ginea Pig Ileum) assay,[6] it was about 5 times more potent than β-endorphin, about 40 times than [Leu] enkephalin, and about 30 times than normorphin. Dynorphin B (also called rimorphin) is most abundant in pituitary posterier lobe[7] and acts as an agonist on opioid κ-receptor,[8] produces catalepsy[9] and inhibits the vasopresin secrection.[10] Dynorphin B is also responsible for food ingestion and diuresis.[11]

In 1981 Mattice *et al.*[12] showed that anionic lipids induce conformational changes of endogeneous opioid peptides which contain the amino acid sequence of enkephalin. Anionic lipids have been suggested to be active component of the brain opiate receptor. All peptides examined were nearly devoid of helical content in water. However, appreciable helical content could be developed in the presence of anionic lipids.[13] The helical initiation and propagation of enkephalin containing

peptides were induced from arginyl, histidyl, and lysyl residue. It has been postulated that a dextrogyre amino acid in specific position might provoke a restriction in the conformational degree of freedom of peptide,[14] which results in the stabilization of conformations offering a higher affinity for the receptor. The peptides containing the dextrogyre amino acid are not cleaved by carboxypeptidase.

The structural features responsible for the high potency and opiate receptor specificity of dynorphin in the guinea pig ileum myenteric plexus were examined by Goldstein *et al.*[15] in 1981. Successive removal of COOH-terminal amino acids from dynorphin A demonstrated important contributions of lysine-13, lysine-11, and arginine-7 to the potency. Removal of the $NH_2$-terminal tyrosine abolished the biological activity. Several other structural modifications were shown to affect potency. Substitution of D-alanine for glycine-2 reduced the potencies of dynorphin (1-13) amide, -(1-11), and -(1-10), and methyl esterification of the COOH terminus enhanced the potency. Within the dynorphin A sequence, lysine-11 and arginine-7 were found to be important for selectivity of interaction with the dynorphin receptor, which is distinguishable from the μ receptor, in this tissue. Dynorphin B is less potent than dynorphin A. Amino acid sequence of dynorphin B differs from only 8-13. It is evidently responsible for the difference of potency between dynorphin A and B.

In order to investigate the relationship between dynorphin B and dynorphin receptor and to synthesize more powerful and potent analogues, it is essential to synthesize dynorphin B analogues. In this work we have synthesized dynorphin B analogues, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Arg-D-Ala-Thr (**I**), Tyr-D-Ala-Gly-Phe-Leu-Ala-Arg-Gln-Phe-Lys-Arg-D-Ala-Thr (**II**), and Tyr-Gly-Gly-Phe-Leu-Arg-Gln-Phe-Lys-Val (**III**) by solid phase method.

## RESULTS AND DISCUSSION

Amino groups of all amino acids were protected with *t*-butyloxycarbonyl (*t*-Boc). 2,6-Dichlorobenzyl, 2-chlorobenzyloxycarbonyl, and nitro group were used to protect the side chains of protection Tyr, Lys, and Arg respectively.

*Table* 1. Synthesis of N-protected amino acid with *t*-Boc-$N_3$

| Amino acid | Yield | Optical rotation $[\alpha]_D$ | c | solv | |
|---|---|---|---|---|---|
| D-Ala | 84 | +24.0 | 1 | H | 84 |
| Gly | 90 | | | | 98 |
| Phe | 86 | +24.7 | 1.5 | E | 92 |
| Val | 77 | -5.8 | 1 | H | 94 |
| Arg ($NO_2$) | 123-124 | -5.6 | 1 | D | 86 |
| Leu | 83 | -30.3 | 1 | H | 97 |
| Tyr (Dcb) | 110-111 | +21.0 | 2 | E | 65 |
| Gln-ONp | 149-150 | -35.0 | 1 | D | 58 |
| Lys | 42 | -11.8 | 2.5 | H | 95 |
| Thr (Obz) | 77 | +18.5 | 1.1 | M | 45 |

D=DMF, E=EtOH, H=HOAc, M=MeOH, All rotations are $[\alpha]_D$ ar r.t c; concentration (g/dL).

Boc-amino acid could be crystallized from petroleum ether and ethylacetate/hexane. Physical properties and yields of the synthesized Boc-amino acids are listed in *Table* 1.

A number of method[16] have been developed to synthesize the so-called Merrifield resin esters of Boc-amino acids, which was considered to be the key intermediate for solid phase synthesis. In our procedure, cesium salt method[16] was used. Stepwise method was applied for synthesis of the products. Dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotrizole (HOBT) was used as the coupling reagent.

Synthetic scheme is given in *Table* 2. When coupling and deprotection was finished, ninhydrin test was performed in every step to confirm the result. The Boc group was removed with 30% trifluoroacetic acid (TFA)-dichloromethane (MC) (3/1, v/v). A mixture of MC and DMF was used as the solvent in coupling reaction. To cleave the product from resin support, the polymer supported peptide was treated with TFA/30% HBr in acetic acid (1/1, v/v). Hydrogenolysis was performed to deprotect nitro group of the Arg residue.

TFA/30% HBr in acetic acid (1/1, v/v). Hydrogenolysis was performed to deprotect nitro gro-

*Table* 2. Schedule for DCC/HOBT coupling in solid phase peptide synthesis of dynorphin B analogues

| Step | Reagent | Vol. (mL) | Time (min) |
|---|---|---|---|
| Swelling | 1. DCM(wash) | 1×1 | 60 |
| Deprotection | 2. DCM(wash) | 15×4 | 2 |
| | 3. 30% TFA/DCM-Indole(prewash) | 15×2 | 2 |
| | 4. 30% TFA/DCM-Indole(deblock) | 15×1 | 30 |
| Washes | 5. DCM(wash) | 15×6 | 2 |
| Deprotonation | 6. 5% DIEA/DCM(prewash) | 15×2 | 2 |
| | 7. 5% DIEA/DCM(neutralization) | 15×1 | 10 |
| | 8. DCM(wash) | 15×8 | 2 |
| Coupling | 9. 3eq.DCC/6eq.HOBT/DMF | 1.0×1 | 10 |
| | 10. Boc-AA 3eq./DCM | 10×1 | 300 |
| Wash | 11. DCM(wash) | 15×3 | 2 |
| Deprotonation | 12. 5% DIEA/DCM(neutralization) | 15×1 | 2 |
| Wash | 13. DCM/DMF(2:1)(wash) | 15×2 | 2 |
| | 14. MeOH/DCM(2:1)(wash) | 15×2 | 2 |
| | 15. DCM(wash) | 15×5 | 2 |
| | 16. Test ninhydrin reaction | | 1 |

Double coupling repetition of (9)-(14)

DCM: Dichloromethane — TFA: Trifluoroacetic acid

DCC: Dicyclohexylcarbodiimide — DIEA: Diisopropylethylamine

HOBT: 1-Hydroxybenzotriazole — DMF: N,N-Dimethylformamide

up of the Arg residue.

The crude peptide was purified by gel filtration on sephadex LH-20 (2×50 cm) using MeOH/MeCN (3/1, v/v) and characterized with HPLC, amino acid analyzer.

## EXPERIMENTAL

All melting point were taken by the capillary method and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer. All of the amino acid derivatives were synthesized from commercial grade of amino acids. All reagents and solvents were reagent grade and used without further purification, except for TFA, DMF, acetic acid and MC, which were distilled before use by the literature procedure.[17]

Boc-amino acids were synthesized by using Schnabel[18] and Schwyzer[19] method.

The solid support used was polystyrene-2%-divinylbenzene resin (200-400 mesh 1.23 meq of Cl/g).

**Preparation of Boc-Val-$OCH_2$-$C_6H_4$-Resin**

Boc-Val-$O^-Cs^+$ (0.52 g, 1.48 mmol) was stirred with 1.0 g of chloromethylated resin of styrene-2% divinylbenzene copolymer in 10 mL of DMF for 16 hrs. The esterified resin was then collected and washed successively with DMF, DMF-$H_2O$, THF-$H_2O$, and MeOH. The product was 1.21 g (94%). Volhard titration indicated the presence of 1.16 mmol Val/g of resin.[20]

**Preparation of Boc-Thr-$OCH_2$-$C_6H_4$-Resin**

Boc-Thr-$OCH_2$-$C_6H_4$-Resin was prepared by the same procedure as above. The product was 1.33 g (97%). Volhard titration indicated the presence of 1.20 mmol Val/g of resin.

**Synthetic protocols**

Stepwise synthetic method was applied to the synthesis of Dynorphin B analogues. DCC and HOBT was used for coupling reagent.

**Cleavage of dynorphin B analogues from resin**

320 mg of polymer-supported peptides was treated with 4 mL of TFA/30% HBr in AcOH (1/1, v/v) and 10 mL of anisole as the nucleophilic scavenger. After 1hr at room temperature, the suspension

was filtered and washed with 2 mL portion of TFA/MC (1/1, v/v) and MC. After evaporation of solvent, the resulting oily residue was crystallized in ether. The ethanolic solution of the crystal was subjected to the hydrogenolysis in the presence of activated Pd catalyst to afford the white crystalline product.

**Isolation and purification of product**

The product was purified by gel chromatography (2.0×50 cm) on Sephadex LH-20 using MeOH/MeCN (3/1, v/v) as an eluent.

The product was hydrolyzed with 12 N HCl/AcOH/phenol (2/1/1, v/v) for 48 hrs at 110°C. The amino acid composition was confirmed by the amino acid analysis of the hydrolyzed product.

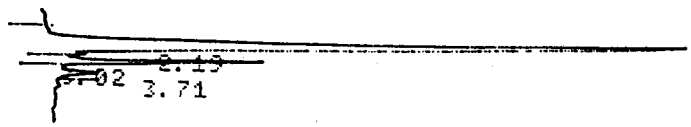
a) [Arg$^{11}$, D-Ala$^{12}$] dynorphin B

Total yield was 32 mg (7.5%). Amino acid composition is given in *Table* 3. HPLC[21] showed a main peak at a retention time 2.19 min (Purity 84%).

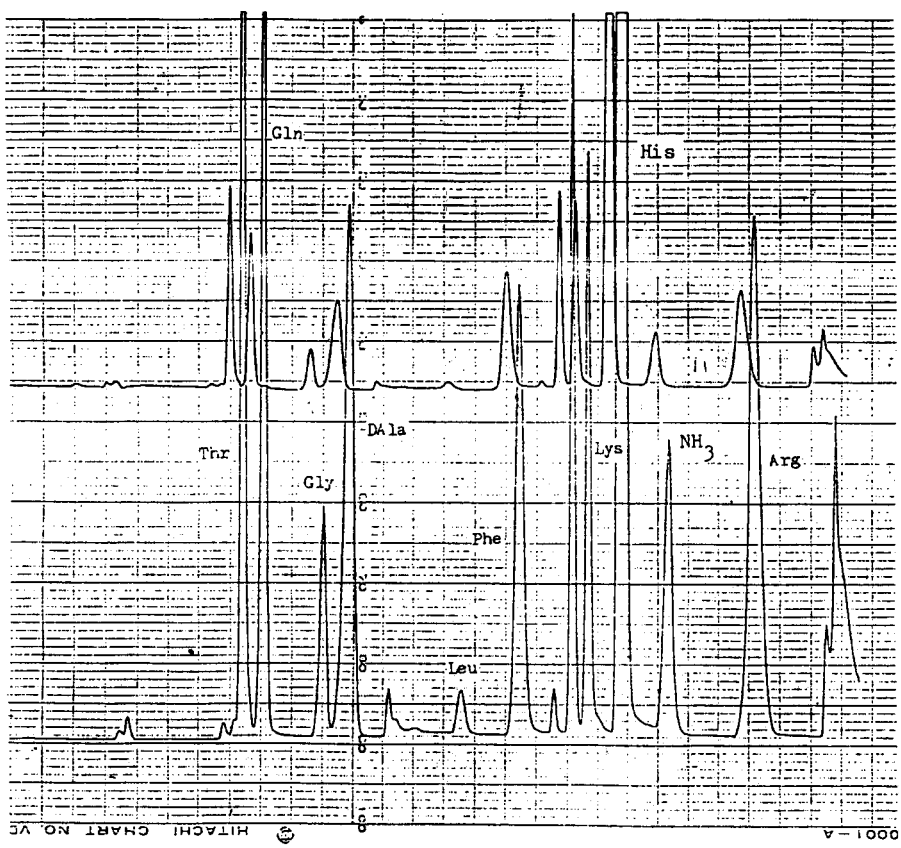
The spectrum of amino acid analysis[22] is given in *Fig.* 1.

b) [D-Ala$^{2}$, Ala$^{6}$, Arg$^{11}$, D-Ala$^{12}$] dynorphin B

Total yield was 53 mg (13%). Amino acid composition is given in *Table* 3. HPLC showed a main




(a)




(b)

*Fig.* 1. (a) HPLC profile and (b) Amino analysis of [Arg$^{11}$, D-Ala$^{12}$] dynorphin B(1-11).

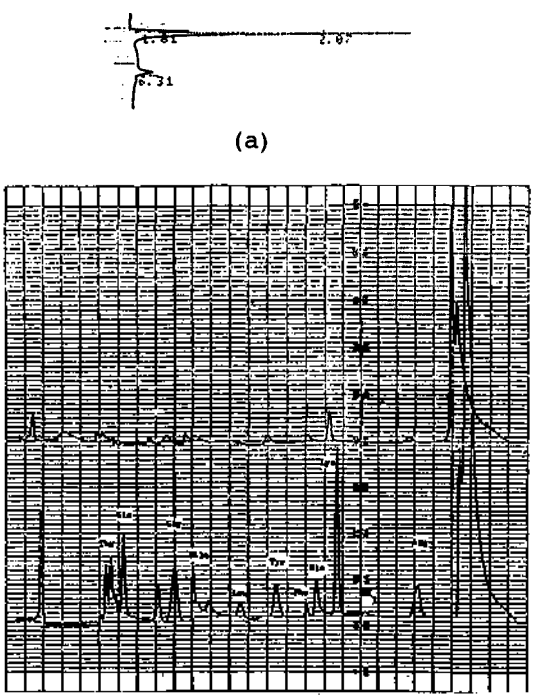


*Fig.* 2. (a) HPLC profile and (b) Amino analysis of [D-Ala$^2$, Ala$^6$, Arg$^{11}$, D-Ala$^{12}$] dynorphin B.

*Table* 3. Amino acid composition of the products

| Product | amino acid composition; amino acid experimental (theoretical) |
|---|---|
| I | Ttr 0.97(1), Gly 2.01(2), Phe 2.0(2), Leu 1.0(1), Arg 2.98(3), Gln 1.01(1) Lys 0.98(1), D-Ala 1.0(1) , Thr 1.0(1) |
| II | Tyr 1.0(1), Ala 2.98(3), Gly 1.0(1), Phe 2.0(2), Leu 1.2(1) Arg 1.8(2), Gln 1.0(1), Lys 1.01(1), Thr 0.97(1) |
| III | Tyr 1.0(1), Gly 2.02(2), Phe 2.01(2), Leu 1.0(1), Arg 1.96(2), Gln 0.88(1), Lys 1.10(1), Val 0.99(1) |

peak at a retention time 2.07 min (Purity 89%). The spectrum of amino acid analysis is given in *Fig.* 2.

c) Dynorphin B(1-11)

Total yield was 66 mg (18%). Amino acid composition is given in *Table* 3. HPLC showed a main peak at a retention time 2.08 min (Purity 91%). The spectrum of amino acid analysis is given in *Fig.* 3.

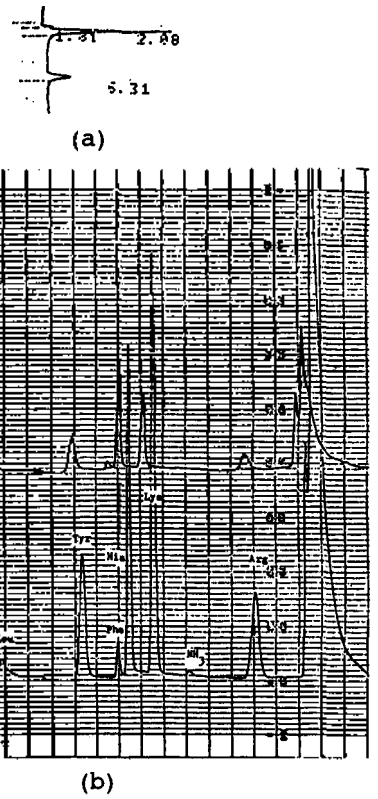


*Fig.* 3. (a) HPLC profile and (b) Amino analysis of dynorphin B(1-11).

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21. HPLC condition; μ-bondapak $C_{18}$ column (4×300 mm), detection at 210 nm, solvent MeOH/$H_2O$ (7/3, v/v), 0.08 absorbance scale, pressure 860 psi, sample injection 10 μL, flow rate 1.0 mL/min.
22. Amino acid analysis condition; standard column (2.6×150 mm) and ammonia filter column (4×50 mm), linear gradient of pH 3.3 to 5.9, flow rate 0.225 mL/min for buffer solution and 0.3 mL/min for ninhydrin, sample injection 16 μL, detection at 570 nm, chart speed 5 mm/min.