# A Synthetic Method Suitable for the Rapid Preparation of $^{13}$N-Labeled Dermorphin Analogue, H–Tyr–D-Met(O)–Phe–Gly–$NH_2$ (SD-62)[1)]

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**A synthetic method suitable for the preparation of $^{13}$N-labeled dermorphin analogue, H–Tyr–D-Met(O)–Phe–Gly–$NH_2$ (SD-62), was established; *i.e.*, SD-62 was synthesized by a 5 min treatment of the active ester precursor with ammonia. When the $^{13}$N-labeled SD-62, prepared by this method with [$^{13}$N]ammonia, was administered into mice, the time profile of the radioactivity accumulation in the brain paralleled well that of the analgesic activity.**

**Keywords** positron emission tomography; dermorphin analogue; peptide synthesis; $^{13}$N ammonia; active ester ammonolysis; brain uptake; analgesic activity

Recently, the positron emission tomography (PET) technique has been applied to study of the *in vivo* behavior of endogenous opioid peptides.[2)] Nagren *et al.*[3)] reported $^{11}$C-labeled methionine-enkephalin and its analogues as the labeled peptides suitable for the PET technique. However, the labeling method was limited to methionine-containing peptides, and the labeled peptide showed rapid degradation *in vivo*. We now report a synthetic method suitable for the preparation of a $^{13}$N-labeled dermorphin analogue, H–Tyr–D-Met(O)–Phe–Gly–$NH_2$ (SD-62), which can be easily applied to the PET technique. SD-62 has much higher opioid activity *in vitro* and *in vivo* than morphine or Met-enkephalin, as well as high stability in enzymatic digestion.[4)]

Our synthetic scheme for the preparation of $^{13}$N-labeled SD-62 is illustrated in Fig. 1. We employ [$^{13}$N]ammonia as a labeling reagent for the ammonolysis of the precursor peptide, since [$^{13}$N]ammonia can be prepared by a fully automated system.[5)] As the precursor peptide for ammonolysis, an active ester form is employed. In this labeling method, no condensation reagent is necessary, which makes the following purification step easy, and no racemization occurs during ammonolysis since SD-62 possesses a Gly at its C-terminus.

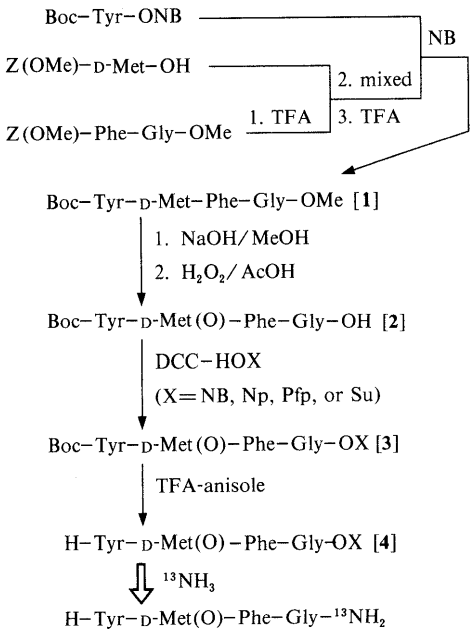


Fig. 1. Synthetic Scheme for the Preparation of $^{13}$N-Labeled SD-62

The precursor of SD-62 [**4**] was prepared according to the scheme shown in Fig. 1. The published synthetic route to SD-62 starting from Gly–$NH_2$[6)] is not applicable to our labeling method because of the limited amount of $^{13}NH_3$ available. Starting with H–Phe–Gly–OMe prepared by the usual TFA treatment of a known dipeptide ester, Z(OMe)–Phe–Gly–OMe,[7)] $N^{\alpha}$-protected D-Met and Tyr were condensed successively by the mixed anhydride[8)] and the NB active ester[9)] methods to give Boc–Tyr–D-Met–Phe–Gly–OMe [**1**]. The tetrapeptide methyl ester [**1**] was converted to tetrapeptide [**2**] by ordinary hydrolysis with NaOH in methanol followed by oxidation with $H_2O_2$ in AcOH. As the active ester of the precursor peptide [**4**], the NB, Np,[10)] Pfp,[11)] and Su[12)] esters were selected and the yield of ammonolysis of the peptide active ester was examined as described later. Each precursor peptide [**4**] was prepared by the condensation of tetrapeptide [**2**] and the corresponding alcohol using the dicyclohexylcarbodiimide (DCC) method followed by usual TFA treatment.

Next, ammonolysis of the precursor peptide was examined using nonradioactive $NH_3$. The Np active ester precursor [**4**] was treated with 28% aq. $MH_3$ and the product was purified by gel-filtration followed by FPLC (Fast Protein Liquid Chromatography, Pharmacia). The purified SD-62 had the same elution time on analytical high performance liquid chromatography (HPLC) as an authentic sample prepared by the known method[6)] and produced amino acids in the ratios predicted by theory.

In practical radiosynthesis with [$^{13}$N]ammonia, however, the labeled peptide should be prepared within a short time due to the short half-life of $^{13}$N (9.96 min). Thus, the yield of ammonolysis of each precursor active ester peptide [**4**] in 5 min was examined using nonradioactive $NH_3$. The

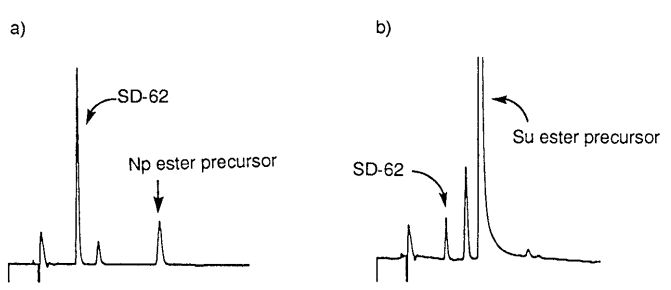


Fig. 2. Ammonolysis of the Active Ester Precursor [**4**] with aqueous $NH_3$

a, ammonolysis of the Np ester precursor; b, ammonolysis of the Su ester precursor.

TABLE I. Yield of Ammonolysis of Precursor Peptide [4]

| Precursor [4] | Yield (%) |
|---|---|
| Np ester | 25 |
| NB ester | 29 |
| Pfp ester | 22 |
| Su ester | 5 |

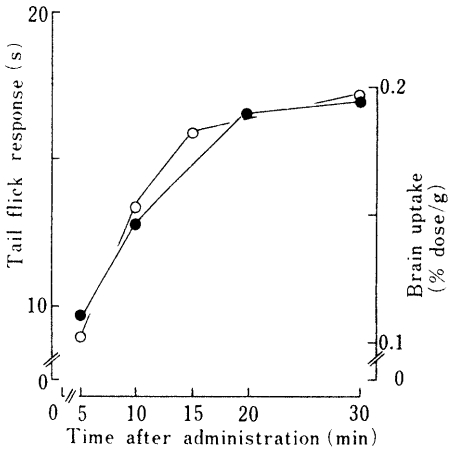


Fig. 3. Correlation of Brain Uptake and Analgesic Activity

●, brain uptake of $^{13}$N-SD-62 (1.5 mg/kg, i.v.); ○, analgesic activity assayed by the tail flick test (0.8 mg/kg, i.v.).

conversion yield of each reaction mixture was estimated from the peak area of the resulting SD-62 on HPLC (Fig. 2). As shown in Table I, nearly the same conversion yields (22—29%) were obtained for all active ester peptides except for the Su ester precursor which gave a very low yield (5%). Each yield obtained with Np, NB, or Pfp ester precursor is moderate and suggests that the method can be applicable for radiosynthesis using [$^{13}$N]ammonia since $^{13}$N nuclide has high specific radioactivity (0.053 pmol/mCi). In addition, the desired product can easily be purified from the reaction mixture using HPLC within 15 min.

From the experimental data described above; we concluded that our synthetic method is suitable for the preparation of [$^{13}$N]-labeled SD-62. When the [$^{13}$N]-labeled SD-62, prepared by our method, was administered into mice, the time profile of the radioactivity uptake into the brain closely paralleled that of analgesic activity (Fig. 3). The details of these biological results will be published elsewhere.

## Experimental

Thin layer chromatography (TLC) was performed on silica gel (Kiesel-gel 60 $F_{254}$, Merck). *Rf* values refer to the following v/v solvent system: $Rf_1$ $CHCl_3$–MeOH–$H_2O$ (8:3:1, lower phase), $Rf_2$ $CHCl_3$–MeOH (10:0.5), $Rf_3$ *n*-BuOH–AcOH–pyridine–$H_2O$ (4:1:1:2). Analytical HPLC was conducted with a Hitachi 655A. Amino acid analysis was conducted with a Hitachi L-8500.

**Z(OMe)-D-Met–Phe–Gly–OMe** Z(OMe)–Phe–Gly–OMe[7] (5.0 g, 12.5 mmol) was treated with TFA–anisole (8.8 ml–2.5 ml) in an ice-bath for 60 min, then TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (15 ml) containing $Et_3N$ (1.8 ml, 12.5 mmol). A mixed anhydride [prepared from 4.7 g (15.0 mmol) of Z(OMe)–D-Met–OH] in DMF (10 ml) was added to the above ice-chilled solution and the mixture was stirred for 1.5 h. The solvent was removed by evaporation and the product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$ and recrystallized from DMF with ether; yield 4.67 g (70%), mp 165—167 °C, $[\alpha]_D^{23}$ −20.2° ($c$=0.5, MeOH), $Rf_2$ 0.52. *Anal.* Calcd. for $C_{26}H_{33}N_3O_7S$: C, 58.74; H, 6.26; N, 7.91. Found: C, 58.19; H, 6.32; N, 7.78.

TABLE II. Yield and Physical Constants of the Active Ester [3]

| X | Yield (%) | mp (°C) | $[\alpha]_D^{21}$ (°) | $Rf_1$ |
|---|---|---|---|---|
| Np | 59 | 203—205 | −15.0 ($c$=0.6, DMF) | 0.51 |
| NB | 35 | 134—136 | −36.0 ($c$=0.5, DMF) | 0.44 |
| Pfp | 32 | 106 (dec.) | −28.0 ($c$=0.5, DMF) | 0.67 |
| Su | 40 | 111—114 | −28.0 ($c$=0.5, DMF) | 0.40 |

**Boc–Tyr–D-Met–Phe–Gly–OMe [1]** The above protected tripeptide ester (4 g, 7.5 mmol) was treated with TFA–anisole–EDT (8.5 ml–2.3 ml–0.63 ml) and the $N^{\alpha}$-deprotected peptide isolated as stated above was dissolved in DMF (15 ml) containing $Et_3N$ (1.1 ml, 7.5 mmol). Boc–Tyr–ONB [prepared by DCC (2.23 g, 10.8 mmol) coupling of Boc–Tyr–OH (2.5 g, 9 mmol) and HONB (1.77 g, 9.9 mmol) in tetrahydrofuran (THF) (10 ml)] and $Et_3N$ (1.1 ml, 7.5 mmol) were added to the above solution and the mixture was stirred at 25 °C overnight. After evaporation of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$–NaCl, then dried over $Na_2SO_4$ and concentrated. The residue was recrystallized from MeOH with ether; yield 3.83 g (81%), mp. 173—175 °C, $[\alpha]_D^{22}$ +1.2° ($c$=1.0, MeOH), $Rf_1$ 0.74. *Anal.* Calcd. for $C_{31}H_{42}N_4O_8S$: C, 59.03; H, 6.71; N, 8.88. Found: C, 59.20; H, 6.90; N, 8.68.

**Boc–Tyr–D-Met(O)–Phe–Gly–OH [2]** To an ice-chilled solution of the above tetrapeptide ester [1] (3.5 g, 5.6 mmol) in MeOH (30 ml) was added 1 N NaOH (6.7 ml), and the mixture was stirred for 2 h at an ice-bath temperature. The pH of the mixture was adjusted to 7 with citric acid, then the methanol was removed by evaporation. The resulting aqueous solution was acidified with 1 N HCl and extracted with AcOEt. The organic extract was washed with $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. The residue was reprecipitated from MeOH with ether to give Boc–Tyr–D-Met–Phe–Gly–OH; yield 2.60 g (76%), $Rf_1$ 0.61. 35% $H_2O_2$ (0.51 ml, 4.5 mmol) was added to an ice-chilled solution of the above tetrapeptide (2.5 g, 4.1 mmol) in AcOH (10 ml) and the mixture was stirred at 25 °C for 1 h. The solvent of the mixture was removed by evaporation and the residue was recrystallized from MeOH with ether; yield 2.31 g (90%), mp. 134—136 °C, $[\alpha]_D^{22}$ +2.1° ($c$=1.0, MeOH), $Rf_1$ 0.24. Amino acid ratios in 6 N HCl hydrolysate; Gly 1.00, Met 0.92, Tyr 1.02, Phe 0.99 (recovery of Gly 89%). *Anal.* Calcd. for $C_{30}H_{40}N_4O_9S \cdot 2H_2O$: C, 53.88; H, 6.63; N, 8.38. Found: C, 53.61; H, 6.64; N, 8.40.

**Boc–Tyr–D-Met(O)–Phe–Gly–OX (X=Np, NB, Pfp or Su) [3]** Boc–Tyr–D-Met(O)–Phe–Gly–OH was dissolved in THF together with a coresponding alcohol (1.1 eq) and DCC (1.1 eq) and the mixture was stirred for 12 h at 25 °C. The solution was filtered and the solvent of the filtrate was removed by evaporation. The residue was reprecipitated from THF with AcOEt; yield and physical constants of each active ester peptide are listed in Table II.

**H–Tyr–D-Met(O)–Phe–Gly–$NH_2$ (SD-62)** Boc–Tyr–D-Met(O)–Phe–Gly–ONp (100 mg, 0.13 mmol) was treated with TFA–anisole (2 ml–72 μl) for 1 h at an ice-bath temperature. The TFA of the mixture was removed by evaporation and the residue was triturated with ether. The resulting powder was dissolved in DMF (2 ml) and 28% $NH_4OH$ (47 μl, 0.66 mmol) was added to the solution. After stirring for 1 h, the mixture was concentrated and the solution was applied to a column of Sephadex G-15 (3.2×45 cm), which was eluted with 1 N AcOH. The ultraviolet (UV) absorption at 280 nm was determined in each fraction (6 ml). The fractions corresponding to the front main peak were combined and the solvent was removed by lyophilization. The resulting powder was dissolved in 0.1% TFA (3 ml) and applied to a column (1.6×50 cm) packed with YMC-gel ODS-AQ 120A S-50, which was eluted with a linear gradient of $CH_3CN$ (0—60%, 400 min) in 0.1% TFA at a flow rate of 3.0 ml/min. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization. The purified peptide exhibited a single peak (retention time 5.62 min, peak area 24467/nmol, determined by UV absorption measurement at 280 nm) on a Cosmosil 5C18ST (4.6×150 mm) column eluted with 14% $CH_3CN$ in 0.1 M NaCl–HCl (pH 2.0) at a flow rate of 0.7 ml/min: yield 30 mg (43%), $[\alpha]_D^{24}$ +25.0° ($c$=0.5, 0.5 N AcOH), $Rf_3$ 0.53. Amino acid ratios in 6 N HCl hydrolysate; Gly1.00, Met 0.81, Tyr 1.00, Phe 1.02 (recovery of Gly 92%).

**Ammonolysis of Precursor Peptide [4]** Each precursor active ester [4] prepared as stated above (5 mg) was dissolved in DMF–$H_2O$ (0.1 ml–

0.5 ml), and 28% aqueous $NH_3$ (4 $\mu$l, 3 eq) was added to the solution. After 5 min, 10 $\mu$l of each mixture was applied to a Cosmosil 5C18ST (4.6 × 150 mm) column, which was eluted with 14% $CH_3CN$ in 0.1 M NaCl–HCl (pH 2.0) at the flow rate of 0.7 ml/min. The amount of each resulting SD-62 (elution time 5.62 min) was estimated from the peak area. The typical elution profiles are shown in Fig. 2 and each conversion yield is lited in Table I.

**References and Notes**


1) The following abbreviations are used: Boc = *tert*-butyl oxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, NB = 5-norbornene-2,3-dicarboximidyl, Np = *p*-nitrophenyl, Pfp = pentafluorophenyl, Su = succinimidyl, TFA = trifluoroacetic acid, DMF = dimethylformamide, EDT = ethanedithiol.
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