Chem. Pharm. Bull.
30(5)1738—1746(1982)

# Studies on Peptides. CX.[1,2] Solution Synthesis of the Tetratriacontapeptide Amide corresponding to Positions 1 to 34 of Human Parathyroid Hormone

SUSUMU FUNAKOSHI[a], HARUAKI YAJIMA,*,[a] CHOHEI SHIGENO[b], ITSUO YAMAMOTO,[b] RIKUSHI MORITA[b] and KANJI TORIZUKA[b]

*Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto, 606, Japan, and School of Medicine, Kyoto University,[b] Shogoin, Sakyo-ku, Kyoto, 606, Japan*

(Received November 13, 1981)

The tetratriacontapeptide amide corresponding to positions 1 to 34 of human parathyroid hormone, H-(hPTH 1—34)-$NH_2$, was synthesized by the solution method. As a deprotecting reagent, 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid was employed. When mouse bone adenyl cyclase activity was measured, our synthetic active fragment of human PTH exhibited an activity of 3380—4400 IU/mg.

**Keywords**——solution synthesis of an active fragment of human parathyroid hormone; $N^G$-mesitylenesulfonylarginine; β,β,β-trichloroethyloxycarbonylhydrazine; 1 M trifluoromethanesulfonic acid in TFA as a deprotecting reagent; thioanisole as a cation scavenger; mouse bone adenyl cyclase activity

In 1976, Bader *et al.*[3] reported that careful Edman degradation studies on the active fragment of human parathyroid hormone, H-(hPTH 1—34)-$NH_2$, prepared by the solid phase method[4] revealed the presence of *ca.* 20% impurities derived from incomplete condensation of incorporated amino acids. Such contamination was also noted in the active fragment of bovine parathyroid hormone, H-(bPTH 1—34)-OH, prepared by a similar solid phase method.[5] The recorded activity of H-(hPTH 1—34)-$NH_2$ (1574 IU/mg) seems lower than that of H-(hPTH 1—34)-OH (2457 IU/mg) synthesized by a conventional solution method by Takai *et al.*[6] In order to clarify the ambiguity over the potency of this human-type active fragment, we undertook the synthesis of H-(hPTH 1—34)-$NH_2$ in a conventional manner.

The synthetic scheme for H-(hPTH 1—34)-$NH_2$, illustrated in Fig. 1, is essentially the same as that of our previous synthesis of H-(hPTH 1—38)-OH.[1] Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole/TFA[7] were employed, *i.e.*, Arg(Mts),[8] Lys(Z), Glu(OBzl) and Asp(OBzl). In the present synthesis, the Met residue was

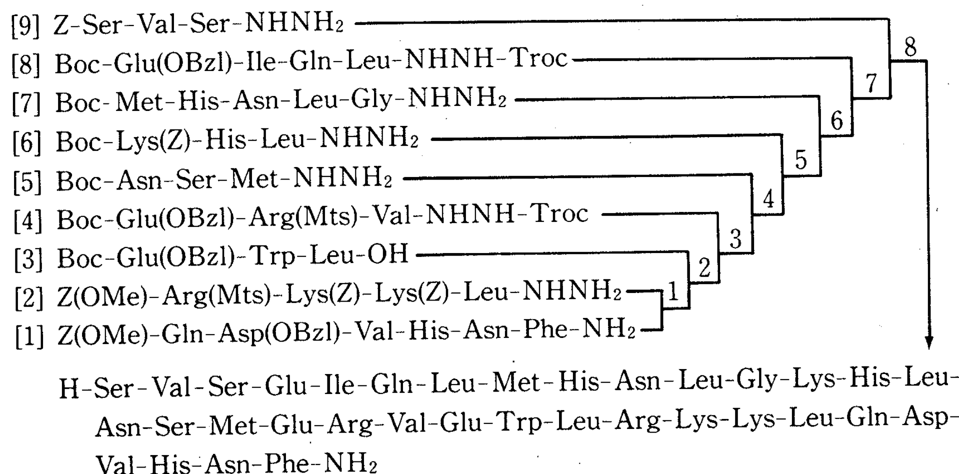


Fig. 1. Synthetic Scheme for the Human Parathyroid Hormone (1—34) Amide, H-(hPTH 1—34)-$NH_2$

NII-Electronic Library Service

not protected as its sulfoxide,[9] since thioanisole was found to prevent *S*-alkylation at the Met residue during the deprotection.[10] Thus, after deprotection, a rather prolonged reduction step of the Met sulfoxide residue was eliminated. To prepare Trp-containing peptides, the Boc group was adopted for $N^{\alpha}$-protection, since much less side reaction[11] is involved than with the Z(OMe) group during the TFA deprotection.[12] Of the nine fragments employed in this synthesis, six fragments, [2], [3], [4], [6], [8] and [9], were identical with those employed for our previous synthesis of H–(hPTH 1—38)–OH.[1,13] The other three fragments, the *C*-terminal hexapeptide [1] and two Met-containing fragments [5] and [7], were newly synthesized.

The *C*-terminal hexapeptide amide, Z(OMe)–Gln–Asp(OBzl)–Val–His–Asn–Phe–$NH_2$ [1], was prepared according to the scheme shown in Fig. 2. The known hydrazide, Z(OMe)–Val–His–$NHNH_2$,[14] was condensed *via* the azide[15] with H–Asn–Phe–$NH_2$ obtained by the *p*-nitrophenyl ester procedure,[16] followed by TFA treatment, to afford the protected tetrapeptide, Z(OMe)–Val–His–Asn–Phe–$NH_2$. Next, Z(OMe)–Asp(OBzl)–OH and Z(OMe)–Gln–OH were condensed stepwisely with this tetrapeptide by the *p*-nitrophenyl ester procedure. Every reaction proceeded smoothly to afford the desired fragment [1] without particular difficulty.

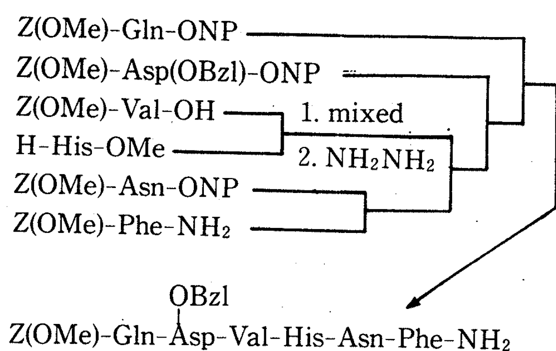


Fig. 2. Synthetic Scheme for the Protected Hexapeptide Amide, Z(OMe)–(hPTH 29—34)–$NH_2$ [1]

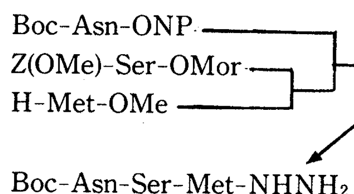


Fig. 3. Synthetic Scheme for the Protected Tripeptide Hydrazide, [5] Boc-(hPTH 16—18)–$NHNH_2$

The fragment Boc–Asn–Ser–Met–$NHNH_2$ [5] was prepared by condensation of Boc–Asn–ONP with H–Ser–Met–OMe derived from the known Z(OMe)–derivative,[17] followed by the usual hydrazine treatment. Some impurity formed during the TFA deprotection of Z(OMe)–Ser–Met–OMe, presumably the *S*-alkylated compound, was removed during recrystallization of Boc–Asn–Ser–Met–OMe. Every reaction was performed under a nitrogen atmosphere to prevent partial air oxidation of the Met residue. This was also done in later reactions.

The next Met-containing fragment, Boc–Met–His–Asn–Leu–Gly–$NHNH_2$ [7], was prepared easily, as shown in Fig. 4, by addition of Boc–Met–OH to the known tetrapeptide ester, H–His–Asn–Leu–Gly–OMe,[1] by the *N*-hydroxysuccinimide procedure[18] followed by the usual hydrazine treatment.

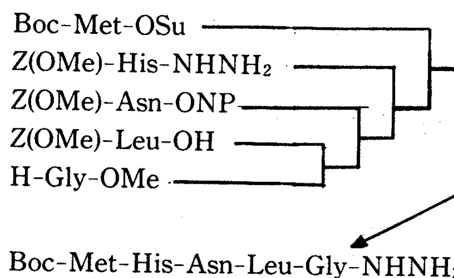


Fig. 4. Synthetic Scheme for the Protected Pentapeptide Hydrazide, Boc–(hPTH 8—12)–$NHNH_2$ [7]

The nine fragments thus available were then assembled in a stepwise manner by the azide procedure, except for fragment [3]. This fragment was condensed by the DCC–HOBT

NII-Electronic Library Service

procedure[19] to suppress possible racemization. After incorporation of the Trp residue with fragment [3] into the chain, the Boc group was cleaved, prior to the next coupling, by TFA in the presence of anisole containing 2% EDT[20] to prevent possible indole-alkylation, as performed in our previous synthesis.[1]

For the purification of protected intermediates, precipitation was occasionally useful, but column chromatography on silica gel was required for purification of the product in step 3 (Fig. 1), and gel-filtration on Sephadex LH-60 for the products in steps 6 and 7. The final protected tetratriacontapeptide amide was purified by repeated gel-filtration on Sephacryl S-200[21] using DMF containing 5% water as the eluent. Throughout the synthesis, the recovery of Phe in an acid hydrolysate of each product was selected as the basis of calculation. This amino acid is located at the *C*-terminus and occurs only once in this active fragment. With this standard, newly incorporated amino acids could be accurately determined after each condensation reaction. The data obtained are listed in Table I.

TABLE I. Amino Acid Analysis of Z–(hPTH 1—34)–$NH_2$ and Its Intermediates

| | 29—34 | 25—34 | 22—34 | 19—34 | 16—34 | 13—34 | 8—34 | 4—34 | 1—34 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Asp | 2.06 | 1.96 | 1.96 | 2.07 | 3.17 | 3.10 | 4.27 | 4.24 | 4.21 | 4 |
| Ser | | | | | 0.90 | 0.79 | 0.81 | 0.83 | 2.78 | 3 |
| Glu | 1.06 | 0.98 | 1.99 | 3.06 | 2.98 | 2.99 | 3.03 | 4.93 | 5.10 | 5 |
| Gly | | | | | | | 1.23 | 1.22 | 1.08 | 1 |
| Val | 1.00 | 0.97 | 0.95 | 1.87 | 1.92 | 1.96 | 2.00 | 2.04 | 3.18 | 3 |
| Met[a] | | | | | 0.70 | 0.78 | 1.61 | 1.75 | 1.49 | 2 |
| Ile | | | | | | | | 1.07 | 1.07 | 1 |
| Leu | | 0.97 | 1.92 | 2.03 | 2.01 | 3.12 | 4.36 | 5.41 | 5.18 | 5 |
| Phe | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1 |
| Lys | | 2.02 | 2.12 | 2.16 | 2.14 | 3.12 | 3.15 | 3.00 | 2.91 | 3 |
| His | 0.83 | 0.79 | 0.93 | 0.99 | 0.96 | 2.18 | 3.06 | 2.92 | 2.45 | 3 |
| Arg | | 1.19 | 0.98 | 2.08 | 2.12 | 1.99 | 2.00 | 1.94 | 1.91 | 2 |
| Trp | | | ND | ND | ND | ND | ND | ND | ND | 1 |
| Rec. | 70% | 75% | 79% | 70% | 82% | 79% | 78% | 80% | 79% | |

*a*) Met+Met(O).

In the final step, all the protecting groups employed were removed by 1 M TFMSA–thioanisole/TFA in the presence of *m*-cresol and the deprotected product was subsequently purified as follows. 1, Conversion to the corresponding acetate by Amberlite CG-4B. 2, Dilute ammonia treatment for reversal of possible N→O shift.[22] 3, Gel-filtration on Sephadex G-50. 4, Ionexchange chromatography on CM-cellulose. These procedures were essentially the same as those employed for the synthesis of H–(hPTH 1—38)–OH, except for the sulfoxide-reduction step, as described previously. The product thus obtained was incubated with dithiothreitol at 30°C to ensure complete reduction of Met sulfoxide possibly formed during the above purification steps, then finally purified by gel-filtration on Sephadex G-25. The purity of the product was assessed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), disc-isoelectrofocusing,[23] acid hydrolysis, enzymic digestion and elemental analysis.

When mouse bone adenyl cyclase activity was assayed according to Luben *et al.*,[24] using calvaria cells obtained from newborn mouse, our peptide, H–(hPTH 1—34)–$NH_2$, synthesized by the solution method exhibited an activity of 3380—4400 IU/mg, and the results were reproducible within this range. Thus, we conclude that the active fragment of human parathyroid hormone, H–(hPTH 1—34)–$NH_2$, possesses a much higher activity than that reported by Bader *et al.*

NII-Electronic Library Service

## Experimental

General experimental methods employed in this investigation were essentially the same as described in Part LXXXVIII of this series.[14] Condensations of peptides containing Met were performed under nitrogen gas. HPLC was performed using a Waters Compact Model with a μ Bondapak $C_{18}$ (0.25″×1′) column. Gradient elution starting from acetonitrile–0.1 M $H_3PO_4$ in 0.1% AcOH (30: 70) to (50: 50) was carried out at a flow rate of 1 ml/min. TLC was performed on silica gel (Kiesel gel G, Merck) and *Rf* values refer to the following solvent systems: $Rf_1$ $CHCl_3$–MeOH–$H_2O$ (8: 3: 1), $Rf_2$ $CHCl_3$–MeOH–AcOH (9: 1: 0.5), $Rf_3$ *n*-BuOH–AcOH–pyridine–$H_2O$ (4: 1: 1: 2). TLC of the synthetic H-(hPTH 1—34)-$NH_2$ was performed on DC-Alufolien Cellulose (Merck) and *Rf* values refer to the following solvent systems; $Rf_{I}$ *n*-BuOH–AcOH–pyridine–$H_2O$ (4: 1: 1: 2), $Rf_{II}$ *n*-BuOH–AcOH–AcOEt–$H_2O$ (1: 1: 1: 1), $Rf_{III}$ *n*-BuOH–pyridine–AcOH–$H_2O$ (5: 3: 0.1: 11, upper phase).

**Z(OMe)-Phe-NH$_2$**——A mixed anhydride [prepared from 32.90 g, (0.1 mol) of Z(OMe)–Phe–OH] was added to an ice-chilled solution of 28% $NH_4OH$ (21 ml) and the mixture was stirred in an ice-bath for 4 h. The resulting solid was collected by filtration, washed with 5% $NH_4OH$ and $H_2O$ and then recrystallized from dioxane–MeOH and AcOEt; yield 28.20 g (86%), mp 180—182°C, $[\alpha]_D^{20}$ −17.4° (*c*=0.9, DMF), $Rf_1$ 0.74. *Anal.* Calcd for $C_{18}H_{18}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 65.76; H, 6.28; N, 8.44.

**Z(OMe)-Asn-Phe-NH$_2$**——Z(OMe)–Phe–$NH_2$ (5.88 g, 17.9 mmol) was treated with TFA–anisole (15.6 ml–3.9 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 (60 ml) together with $Et_3N$ (5.02 ml, 35.8 mmol) and Z(OMe)–Asn–ONP (7.47 g, 17.9 mmol). After being stirred at room temperature for 20 h, the solution was concentrated and the residue was treated with AcOEt and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $Na_2CO_3$ and $H_2O$ and recrystallized from DMSO and MeOH; yield 6.01 g (76%), mp 243—245°C, $[\alpha]_D^{25}$ −19.3° (*c*=0.9, DMSO), $Rf_1$ 0.60, $Rf_2$ 0.15. *Anal.* Calcd for $C_{22}H_{26}N_4O_6$: C, 59.72; H, 5.92; N, 12.66. Found: C, 59.43; H, 5.93; N, 12.63.

**Z(OMe)-Val-His-Asn-Phe-NH$_2$**——Z(OMe)–Asn–Phe–$NH_2$ (6.00 g, 13.6 mmol) was treated with TFA–anisole (17.72 ml—4.4 ml) as usual, 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 DMSO–DMF (1: 1, 50 ml) containing $Et_3N$ (1.90 ml, 13.6 mmol). The azide [prepared from 7.04 g (16.3 mmol) of Z(OMe)–Val–His–$NHNH_2$[14]] in DMF (30 ml) and $Et_3N$ (2.51 ml, 17.9 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 48 h, while the product began to precipitate. The solvent was evaporated off and the residue was treated with AcOEt and 5% AcOH. The resulting powder was washed with 3% AcOH, 5% $Na_2CO_3$ and $H_2O$ and precipitated from DMSO with MeOH; yield 6.22 g (65%), mp 204—207°C, $[\alpha]_D^{25}$ −13.9° (*c*=1.1, DMSO), $Rf_1$ 0.30. Amino acid ratios in a 6 N HCl hydrolysate: Val 1.02, His 0.95, Asp 0.98, Phe 1.00 (recovery of Phe 87%). *Anal.* Calcd for $C_{33}H_{42}N_8O_8\cdot 1.5H_2O$: C, 56.16; H, 6.43; N, 15.88. Found: C, 55.90; H, 6.14; N, 15.70.

**Z(OMe)-Asp(OBzl)-Val-His-Asn-Phe-NH$_2$**——Z(OMe)–Val–His–Asn–Phe–$NH_2$ (6.00 g, 8.84 mmol) was treated with TFA–anisole (11.5 ml–2.9 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 3 h and then dissolved in DMF (50 ml) together with $Et_3N$ (3.69 ml, 26.5 mmol) and Z(OMe)–Asp(OBzl)–ONP (5.39 g, 10.6 mmol). During stirring at room temperature for 18 h, the solution became pasty. The solution was concentrated and the residue was treated with 3% AcOH and AcOEt. The resulting powder was washed as described above and precipitated from DMF with MeOH; yield 6.00 g (77%), mp 236—237°C, $[\alpha]_D^{25}$ −16.3° (*c*=1.0, DMSO), $Rf_1$ 0.39. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.01, Val 1.00, Phe 1.00, His 0.91 (recovery of Phe 85%). *Anal.* Calcd for $C_{44}H_{53}N_9O_{11}$: C, 59.78; H, 6.04; N, 14.26. Found: C, 60.06; H, 6.25; N, 14.37.

**Z(OMe)-Gln-Asp(OBzl)-Val-His-Asn-Phe-NH$_2$, Z(OMe)-(hPTH 29—34)-NH$_2$ [1]**——The above protected pentapeptide amide (5.00 g, 5.66 mmol) was treated with TFA–anisole (12.3 ml–3.1 ml) as usual, then dry ether was added. The powder thus isolated was dissolved in DMF (50 ml) together with $Et_3N$ (2.53 ml, 18.1 mmol) and Z(OMe)–Gln–ONP (2.93 g, 6.8 mmol). During stirring at room temperature for 48 h, the solution became pasty. The solution was concentrated and the product was purified by washing as described above, followed by precipitation twice from DMSO and MeOH; yield 5.11 g (90%), mp 234—238°C, $[\alpha]_D^{25}$ −22.6° (*c*=1.1, DMSO), $Rf_1$ 0.31. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.06, Asp 2.06, Val 1.00, Phe 1.00, His 0.83 (recovery of Phe 70%). *Anal.* Calcd for $C_{49}H_{61}N_{11}O_{13}\cdot H_2O$: C, 57.13; H, 6.16; N, 14.96. Found: C, 57.39; H, 6.08; N, 14.76.

**Boc-Asn-Ser-Met-OMe**——Z(OMe)–Ser–Met–OMe[17] (18.65 g, 45 mmol) was treated with TFA–anisole (39.1 ml–9.8 ml) as usual, then dry ether was added. Treatment of the oily residue with ether afforded a gummy precipitate, which was dried over KOH pellets *in vacuo* for 3 h then dissolved in DMF (90 ml) together with $Et_3N$ (13.2 ml, 95 mmol) and Boc–Asn–ONP (17.5 g, 49.5 mmol). After being stirred for 16 h, the solution was concentrated and an oily residue was purified by the usual extraction procedure with AcOEt. The extract was concentrated and the residue was triturated with ether and recrystallized from MeOH and ether. Some impurity due to the deprotection of Z(OMe)–Ser–Met–OMe by TFA was removed at this stage; yield 12.96 g (62%), mp 133—135°C, $[\alpha]_D^{25}$ −31.8° (*c*=0.9, DMF), $Rf_1$ 0.51, $Rf_2$ 0.24. *Anal.* Calcd for $C_{18}H_{32}N_4O_8S$: C, 46.54; H, 6.94; N, 12.06. Found: C, 46.77; H, 6.89; N, 11.98.

NII-Electronic Library Service

**Boc-Asn-Ser-Met-NHNH$_2$, Boc-(hPTH 16—18)-NHNH$_2$** [5]——Boc–Asn–Ser–Met–OMe (4.00 g, 8.6 mmol) in MeOH (50 ml) was treated with 80% hydrazine hydrate (2.15 ml, 5 eq) overnight. The resulting solid was triturated with EtOH and precipitated from DMF with EtOH; yield 2.89 g (72%), mp 212—216°C, $[\alpha]_D^{25}$ −26.4° ($c$=1.0, DMF), $Rf_1$ 0.32, $Rf_2$ 0.10. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.00, Ser 0.88, Met+Met(O) 0.80 (recovery of Asp 93%). *Anal.* Calcd for $C_{17}H_{32}N_6O_7S$: C, 43.95; H, 6.94; N, 18.09. Found: C, 43.86; H, 7.02; N, 17.87.

**Boc-Met-His-Asn-Leu-Gly-OMe**——Z(OMe)–His–Asn–Leu–Gly–OMe[1] (5.08 g, 8.22 mmol) was treated with TFA–anisole (12 ml–4.0 ml) as usual, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (50 ml) together with $Et_3N$ (3.42 ml, 24.6 mmol) and Boc–Met–OSu (2.85 g, 8.2 mmol). After being stirred for 24 h, the solution was concentrated and the residue was extracted with *n*-BuOH. The extract was washed with $H_2O$ and concentrated. The oily residue was triturated with ether and the resulting powder was recrystallized from MeOH and ether; yield 2.57 g (46%), mp 122—125°C, $[\alpha]_D^{25}$ −26.7° ($c$=0.7, DMF), $Rf_1$ 0.59. *Anal.* Calcd for $C_{29}H_{48}N_8O_9S \cdot H_2O$: C, 49.56; H, 7.07; N, 15.94. Found: C, 49.60; H, 7.02; N, 15.78.

**Boc-Met-His-Asn-Leu-Gly-NHNH$_2$, Boc-(hPTH 8—12)-NHNH$_2$** [7]——The above protected pentapeptide ester (3.27 g, 4.78 mmol) in MeOH (30 ml) was treated with 80% hydrazine hydrate (1.20 ml, 5 eq) overnight. The solvent was evaporated off and the residue was treated with EtOH to form a powder, which was recrystallized twice from MeOH and EtOH; yield 2.98 g (91%), mp 184—186°C, $[\alpha]_D^{25}$ −33.1° ($c$=0.9, DMF), $Rf_1$ 0.31. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.95, Gly 1.01, Met 0.92, Leu 1.00, His 0.95 (recovery of Leu 90%). *Anal.* Calcd for $C_{28}H_{48}N_{10}O_8S$: C, 49.11; H, 7.07; N, 20.46. Found: C, 49.04; H, 7.14; N, 20.23.

**Z(OMe)-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-NH$_2$, Z(OMe)-(hPTH 25—34)-NH$_2$**——Z(OMe)–(hPTH 29—34)–$NH_2$ [1] (5.78 g, 5.71 mmol) was treated with TFA–anisole (12.4 ml–3.1 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (30 ml) containing $Et_3N$ (1.59 ml, 11.42 mmol). The azide [prepared from 8.03 g (6.85 mmol) of Z(OMe)–Arg(Mts)–Lys(Z)–Lys(Z)–Leu–$NHNH_2$[13]] in DMF (20 ml) and $Et_3N$ (1.15 ml, 8.22 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 18 h. The additional azide [prepared from 2.00 g (1.71 mmol) of the hydrazide] in DMF (10 ml) and $Et_3N$ (0.29 ml, 2.05 mmol) were added and stirring was continued for an additional 18 h. After neutralization with a few drops of AcOH, the solution was concentrated and the residue was treated with 3% AcOH and AcOEt. The resulting powder was washed with 3% AcOH, 5% $NaHCO_3$ and $H_2O$ and precipitated three times from DMSO with MeOH; yield 9.17 g (81%), mp 226—230°C, $[\alpha]_D^{25}$ −17.8° ($c$=1.0, DMSO), $Rf_1$ 0.40. *Anal.* Calcd for $C_{98}H_{130}N_{20}O_{23}S$: C, 59.20; H, 6.59; N, 14.09. Found: C, 59.09; H, 6.69; N, 14.02.

**Boc-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-NH$_2$, Boc-(hPTH 22—34)-NH$_2$**——Z(OMe)–(hPTH 25—34)–$NH_2$ (7.00 g, 3.52 mmol) was treated with TFA–anisole (15.3 ml–3.8 ml) as described above and *n*-hexane was added. The resulting oily precipitate was triturated with 5% $NaHCO_3$ and ether to afford a powder, which was dried over KOH pellets *in vacuo* for 8 h and then dissolved in HMPA–DMSO–DMF (1:1:1, 30 ml), together with HOBT (0.86 g, 6.3 mmol), *N*-methylmorpholine (0.7 ml, 6.3 mmol) and Boc–Glu(OBzl)–Trp–Leu–OH [3][13] [prepared from 3.36 g (5.28 mmol) of the DCHA salt as usual]. Under cooling with ice, DCC (1.31 g, 6.34 mmol) was added and the mixture was stirred at 4°C for 18 h. Further DCC (0.22 g, 1.1 mmol) was added and stirring was continued for an additional 18 h. The solution was concentrated and the residue was treated with 3% AcOH and AcOEt. The resulting powder was washed as described above and precipitated four times from DMF with MeOH; meanwhile, DC-urea was removed by filtration; yield 5.01 g (58%), mp 282°C (dec.), $[\alpha]_D^{25}$ −19.4° ($c$=0.8, DMSO), $Rf_1$ 0.43. *Anal.* Calcd for $C_{123}H_{164}N_{24}O_{27}S$: C, 60.47; H, 6.77; N, 13.76. Found: C, 60.88; H, 6.78; N, 13.88.

**Boc-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-NH$_2$, Boc-(hPTH 19—34)-NH$_2$**——Boc–(hPTH 22—34)–$NH_2$ (4.80 g, 1.96 mmol) was treated with TFA (12.8 ml)–anisole containing 2% EDT (3.2 ml) in an ice-bath for 60 min and the $N^\alpha$-deprotected peptide, precipitated with ether as a powder, was dissolved in DMF (30 ml) containing $Et_3N$ (0.55 ml, 3.92 mmol). The azide [prepared from 2.32 g (2.94 mmol) of Boc–Glu(OBzl)–Arg(Mts)–Val–$NHNH_2$[13]] in DMF (10 ml) and *N*-methylmorpholine (0.78 ml, 7.06 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4°C for 48 h, was concentrated. The residue was treated with 3% AcOH and AcOEt and the resulting powder was purified by washing as described above followed by precipitation from DMF with AcOEt. The product was dissolved in $CHCl_3$–MeOH–$H_2O$ (8:3:1, 5 ml) and the solution was applied to a column of silica gel (Kiesel gel 60 Merck, 2.3×50 cm), which was eluted with the same solvent system. Two fractions with different $Rf_1$ values, 0.51 and 0.41, were isolated. The fractions with $Rf_1$ 0.41 were combined and the solvent was removed by evaporation. Treatment of the residue with AcOEt afforded a powder; yield 4.07 g (67%), mp 255°C (dec.), $[\alpha]_D^{25}$−1.0° ($c$=1.0, DMF), $Rf_1$ 0.41, $Rf_3$ 0.73. *Anal.* Calcd for $C_{155}H_{208}N_{30}O_{34}S_2 \cdot 1.5H_2O$: C, 59.54; H, 6.80; N, 13.44. Found: C, 59.24; H, 6.68; N, 13.37.

**Boc-Asn-Ser-Met-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp-(OBzl)-Val-His-Asn-Phe-NH$_2$, Boc-(hPTH 16—34)-NH$_2$**——Boc–(hPTH 19—34)–$NH_2$ (0.50 g, 0.16 mmol) was treated with TFA (1.4 ml)–anisole containing 2% EDT (0.4 ml) and the $N^\alpha$-deprotected peptide isolated

NII-Electronic Library Service

as described above was dissolved in DMF (5 ml) containing $Et_3N$ (45.2 µl, 0.32 mmol). The azide [prepared from 0.15 g (0.32 mmol) of Boc–Asn–Ser–Met–$NHNH_2$] in DMF (3 ml) and $Et_3N$ (54.2 µl, 0.39 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 48 h. After neutralization with a few drops of AcOH, the solution was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$ and precipitated five times from DMF with AcOEt; yield 0.46 g (84%), mp 143—144°C, $[\alpha]_D^{25}$ −2.7° ($c$=0.8, DMF), $Rf_1$ 0.57, $Rf_3$ 0.79. *Anal.* Calcd for $C_{167}H_{228}N_{34}O_{39}S_3 \cdot 4H_2O$: C, 57.24; H, 6.79; N, 13.59. Found: C, 57.34; H, 6.59; N, 13.47.

**Boc-Lys(Z)-His-Leu-Asn-Ser-Met-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-$NH_2$, Boc-(hPTH 13—34)-$NH_2$**——Boc–(hPTH 16—34)–$NH_2$ (0.44 g, 0.13 mmol) was treated with TFA (1.6 ml)–anisole containing 2% EDT (0.4 ml) and the $N^\alpha$-deprotected peptide isolated as described above was dissolved in DMF (5 ml) containing $Et_3N$ (35 µl, 0.25 mmol). The azide [prepared from 0.16 g (0.25 mmol) of Boc–Lys(Z)–His–Leu–$NHNH_2$[1]] in DMF (3 ml) and $Et_3N$ (43 µl, 0.31 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4°C for 60 h, was concentrated. Treatment of the residue with 3% AcOH and ether afforded a powder, which was washed with 3% AcOH, 5% $NaHCO_3$ and $H_2O$ and then precipitated five times from DMF with MeOH; yield 0.48 g (96%), mp 118—120°C, $[\alpha]_D^{25}$ −2.7° ($c$=1.1, DMF), $Rf_1$ 0.38, $Rf_3$ 0.75. *Anal.* Calcd for $C_{193}H_{264}N_{40}O_{44}S_3 \cdot 6H_2O$: C, 57.20; H, 6.86; N, 13.83. Found: C, 57.07; H, 6.58; N, 13.67.

**Boc-Met-His-Asn-Leu-Gly-Lys(Z)-His-Leu-Asn-Ser-Met-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg-(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-$NH_2$, Boc-(hPTH 8—34)-$NH_2$**——Boc–(hPTH 13—34)–$NH_2$ (0.48 g, 0.12 mmol) was treated with TFA (1.8 ml)–anisole containing 2% EDT (0.5 ml) and the $N^\alpha$-deprotected peptide isolated as described above was dissolved in DMF (5 ml) containing $Et_3N$ (51 µl, 0.37 mmol). The azide [prepared from 0.17 g (0.24 mmol) of Boc–Met–His–Asn–Leu–Gly–$NHNH_2$] in DMF (3 ml) and $Et_3N$ (41 µl, 0.29 mmol) were added and the mixture was stirred at 4°C for 48 h. After addition of a few drops of AcOH, the solution was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was washed as described above and precipitated five times from DMF with AcOEt. The product was dissolved in DMF (10 ml) and the solution was applied to a column of Sephadex LH-60 (3.3×140 cm), which was eluted with the same solvent. Individual fractions (9 ml each) were collected and the absorbancy at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 43—55) were collected, the solvent was evaporated off, and the residue was treated with AcOEt to afford a powder; yield 0.36 g (67%), mp 135—137°C, $[\alpha]_D^{25}$ −4.7° ($c$=0.6, DMF), $Rf_1$ 0.34, $Rf_3$ 0.69. *Anal.* Calcd for $C_{216}H_{300}N_{48}O_{50}S_4 \cdot 5H_2O$: C, 56.55; H, 6.81; N, 14.66. Found: C, 56.32; H, 6.66; N, 14.77.

**Boc-Glu(OBzl)-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys(Z)-His-Leu-Asn-Ser-Met-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-$NH_2$, Boc-(hPTH 4—34)-$NH_2$**——Boc–(hPTH 8—34)–$NH_2$ (0.36 g, 79.4 µmol) was treated with TFA (1.4 ml)–anisole containing 2% EDT (0.35 ml) and the $N^\alpha$-deprotected peptide isolated as described above was dissolved in DMF (5 ml) containing $Et_3N$ (44.3 µl, 0.32 mmol). The azide [prepared from 0.11 g (0.16 mmol) of Boc–Glu(OBzl)–Ile–Gln–Leu–$NHNH_2$[1]] in DMF (1 ml) and $Et_3N$ (26.6 µl, 0.19 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 48 h. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was treated with 5% citric acid and ether. The resulting powder was washed as described above and precipitated twice from DMF with AcOEt. The product was dissolved in DMF (5 ml) and the solution was applied to a column of Sephadex LH-60 (3.3×140 cm), which was eluted with the same solvent. Individual fractions (9 ml each) were collected and the absorbancy at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 54—61) were collected, the solvent was evaporated off, and the residue was treated with AcOEt to afford a powder; yield 0.24 g (60%), mp 138—141°C, $[\alpha]_D^{25}$ −4.0° ($c$=0.5, DMF), $Rf_1$ 0.43, $Rf_3$ 0.77. *Anal.* Calcd for $C_{245}H_{343}N_{53}O_{57}S_4 \cdot 7H_2O$: C, 56.62; H, 6.92; N, 14.28. Found: C, 56.47; H, 6.91; N, 14.29.

**Z-Ser-Val-Ser-Glu(OBzl)-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys(Z)-His-Leu-Asn-Ser-Met-Glu(OBzl)-Arg(Mts)-Val-Glu(OBzl)-Trp-Leu-Arg(Mts)-Lys(Z)-Lys(Z)-Leu-Gln-Asp(OBzl)-Val-His-Asn-Phe-$NH_2$, Z-(hPTH 1—34)-$NH_2$**——Boc-(hPTH 4—34)-$NH_2$ (0.24 g, 47.4 µmol) was treated with TFA (0.84 ml)-anisole containing 2% EDT (0.21 ml) and the $N^\alpha$-deprotected peptide isolated as described above was dissolved in DMF (5 ml) containing $Et_3N$ (24.6 µl, 0.19 mmol). The azide [prepared from 41.7 mg (94.8 µmol) of Z–Ser–Val–Ser–$NHNH_2$[1]] in DMF (1 ml) and $Et_3N$ (15.9 µl, 0.11 mmol) were added and the mixture was stirred at 4°C for 48 h. After addition of a few drops of AcOH, the solvent was evaporated off, and the residue was treated with 3% AcOH and ether. The resulting powder was washed as described above and precipitated three times from DMF with AcOEt. The product was dissolved in DMF (10 ml) and the solution was applied to a column of Sephacryl S-200 (3.4×140 cm), which was eluted with DMF containing 5% $H_2O$. Individual fractions (10 ml each) were collected and the absorbancy at 280 nm was determined. The fractions corresponding to the main peak (Fig. 5-a, tube Nos. 75—89) were combined, concentrated and rechromatographed on the same column. A symmetrical single peak was detected (Fig. 5-b). The product fractions (tube Nos. 76—88) were collected and the solvent was evaporated off. Treatment of the residue with AcOEt afforded a powder; yield 111 mg (44%), mp 145—148°C, $[\alpha]_D^{25}$−3.5° ($c$=0.3, DMF), $Rf_1$ 0.60, $Rf_3$ 0.81. *Anal.* Calcd for $C_{259}H_{360}N_{56}O_{62}S_4 \cdot 7H_2O$: C, 56.51; H, 6.85; N, 14.25. Found: C, 56.55; H, 6.81; N, 13.98.

NII-Electronic Library Service

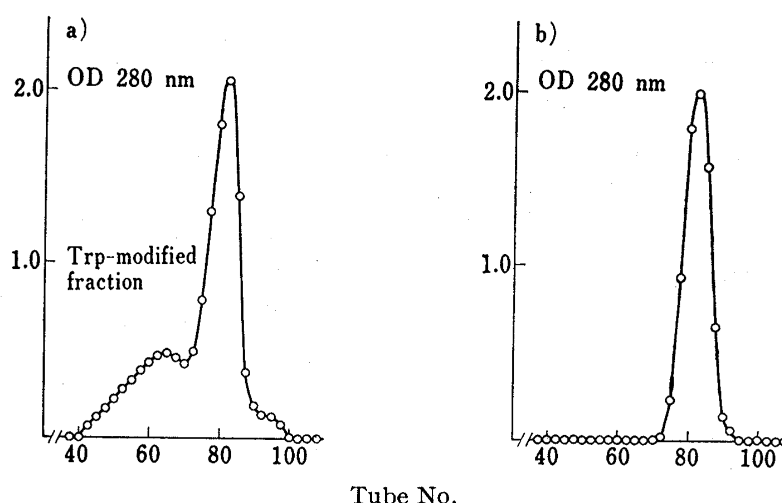


Fig. 5. Purification of Z–(hPTH 1→34)–$NH_2$ by Gel-filtration on Sephacryl S-200

a) Sample: 200 mg. Column: 3.4×140 cm. Solvent: 95% DMF($H_2O$). Flow rate: 50 ml/h. Fraction: 10 ml/tube.
b) Re-chromatography of the main fraction. Sample: 141 mg.

**H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-$NH_2$**——The protected tetratriacontapeptide amide, Z–(hPTH 1—34)–$NH_2$ (200 mg, 37.2 μmol) was treated with 1 M TFMSA–thioanisole in TFA (7.44 ml) in the presence of *m*-cresol (0.78 ml, 7.44 mmol) in an ice-bath for 2 h and then *n*-hexane was added. The resulting oily precipitate was washed with *n*-hexane and washed with ether (stored over $FeSO_4$). The resulting powder was collected by filtration and dried over KOH pellets *in vacuo* for 30 min. The product was again treated with 1 M TFMSA–thioanisole in TFA under identical conditions to ensure complete deprotection. The product isolated as described above was dissolved in $H_2O$ (10 ml) and the solution was treated with Amberlite CG-4B type II (acetate form, approximately 2 g) for 30 min. After filtration, the filtrate was adjusted to pH 10 with 5 N $NH_4OH$ and stirred in an ice-bath for 30 min. The pH of the solution was adjusted to 6 with a few drops of AcOH and the solution was lyophilized to give a white fluffy powder. The product was dissolved in 1 N AcOH (5 ml) and the solution was applied to a column of Sephadex G-50 (2.8×143 cm), which was eluted with 1 N AcOH. Individual fractions (10 ml, each) were collected and the absorption at 280 nm was determined. The main peak fractions (tube Nos. 58—72) were collected and the solvent was removed by lyophilization; yield 140 mg (81%). The product was next dissolved in 0.01 M ammonium acetate buffer in 8 M urea (pH 5.1) (5 ml) and the solution was applied to a column of CM-cellulose (2.4×5 cm, Whatmann CM-52). The column was eluted first with 0.01 M ammonium acetate buffer (pH 5.1) (40 ml) and then with a linear gradient from the starting buffer (300 ml) to 0.3 M ammonium acetate buffer (300 ml). Individual fractions (5 ml each) were collected and the absorption at 280 nm was determined. The main peak fractions

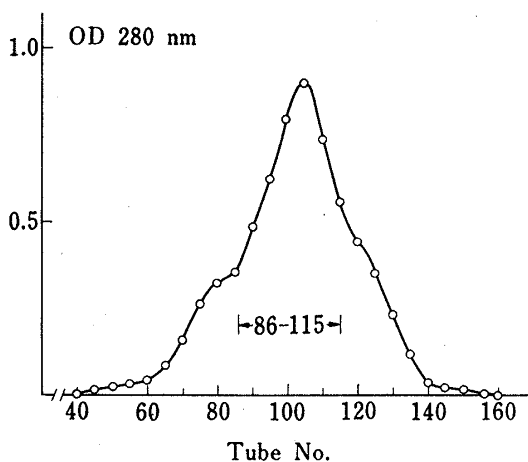


Fig. 6. Purification of H–(hPTH 1—34)–$NH_2$ by Column Chromatography on CM-cellulose

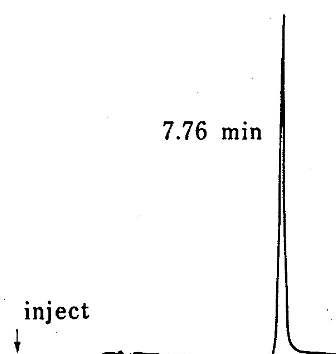


Fig. 7. HPLC of Synthetic H–(hPTH 1—34)–$NH_2$

NII-Electronic Library Service

(Fig. 6. tube Nos. 86—115) were collected and the solvent was removed by lyophilization to constant weight; yield 41.5 mg. The CM-purified sample was dissolved in $H_2O$ (5 ml) and incubated with dithiothreitol (53.0 mg, 40 eq) under nitrogen gas at 30°C for 48 h. The solution was then applied to a column of Sephadex G-25 (1.8×140 cm), which was eluted with 1 N AcOH. The desired fractions (tube Nos. 27—38) were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 38.6 mg (22%), $[\alpha]^{24}_D$ −55.2° ($c$=0.2, 0.1 N AcOH), $Rf_{I}$ 0.57, $Rf_{II}$ 0.67, $Rf_{III}$ 0.48. The product exhibited a single peak on HPLC at a retention time of 7.76 min (Fig. 7) and a single band on disc-isoelectrofocusing on 8 M urea-acrylamide gel (0.5×6.0 cm) at pH 3—10 (Pharmalyte preparation). Its mobility was 0.75 cm from the origin toward the acidic end of the gel, after running at 1 mA per tube for 1 h and then at 200 V for 3 h. Amino acid ratios in a 4 N MSA hydrolysate: Asp 4.11; Ser 2.97, Glu 4.86; Gly 1.05, Val 3.19, Met 1.55, Ile 1.01, Leu 5.12, Phe 1.00, Trp 0.84, Lys 3.20, His 2.76, Arg 2.00 (recovery of Phe 81%). Amino acid ratios in leucine aminopeptidase (Sigma, Lot. No. L-6007) digest: Asp 0.92, Asn 2.88, Ser 2.91, Glu 2.93, Gln 1.91, Gly 1.03, Val 3.11, Met 1.84, Ile 0.94, Leu 4.95, Phe 1.00, Trp 0.78, Lys 3.25, His 2.85, Arg 1.99 (recovery of Phe 70%). *Anal.* Calcd for $C_{181}H_{292}N_{56}O_{50}S_2 \cdot 9CH_3COOH \cdot 15H_2O$: C, 48.50; H, 7.32; N, 15.92. Found: C, 48.55; H, 7.65; N, 15.79.

**Acknowledgement** This investigation was supported in part by a grant (No. 56470118) from the Ministry of Education, Science and Culture. The authors are grateful to Drs. Susumu Watanabe and Shigeo Katuragi, Toyo Jozo Co. Research Laboratories for biological assay.

### References and Notes

1) Studies on Peptides. CIX: S. Funakoshi, N. Fujii, H. Yajima, C. Shigeno, I. Yamamoto, R. Morita, and K. Torizuka, *Chem. Pharm. Bull.*, **30**, 1706 (1982).
2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2846 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972): Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, Troc=$\beta,\beta,\beta$-trichloroethyloxycarbonyl, NP=*p*-nitrophenyl, Su=*N*-hydroxysuccinimide, Mor=*N*-hydroxymorpholinyl, DCC=dicyclohexylcarbodiimide, HOBT=*N*-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, HMPA=hexamethylphosphoric triamide, EDT=ethanedithiol, DCHA=dicyclohexylamine, MSA=methanesulfonic acid, TFMSA=trifluoromethanesulfonic acid.
3) C.A. Bader, J.D. Monet, P. Rivaille, C.M. Gaubert, M.S. Moukhtar, G. Milhaud, and J.L. Funck-Brentano, *Endocr. Res. Comm.*, 3, 167 (1976); J.D. Monet, C.A. Bader, E. Herbigny, and J.L. Funck-Brentano, *FEBS Lett.*, **96**, 76 (1978).
4) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); *idem*, *Adv. Enzymol*, **32**, 221 (1969); P. Rivaille, A. Robinson, M. Kamer, and G. Milhaud, *Helv. Chim. Acta*, **54**, 2772 (1971).
5) G.W. Tregear, J. van Rietschoten, R. Sauer, H.D. Niall, H.T. Keutmann, and J.T. Potts, Jr., *Biochem.*, **16**, 2817 (1977).
6) M. Takai, Y. Kurano, H. Kimura, and S. Sakakibara, "Peptide Chemistry," ed. by H. Yonehara, Protein Res. Found., Osaka, 1979, p.187.
7) H Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, **1974**, 107; H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *ibid.*, **1976**, 922; N. Fujii, S. Funakoshi, T. Sasaki, and H. Yajima, *Chem. Pharm. Bull.*, **25**, 3096 (1977); Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and H. Moritoki, *J. Chem. Soc., Chem. Commun.*, **1979**, 971.
8) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, **1978**, 482; H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3572 (1978).
9) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
10) Y. Kiso, K. Ito, S. Nakamura, K. Kitagawa, T. Akita, and H. Moritoki, *Chem. Pharm. Bull.*, **27**, 1472 (1979).
11) H. Ogawa, T. Sasaki, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 3144 (1978).
12) Yu. B. Alakhov, A.A. Kiryushkin, V.M. Lipkin, and G.W.A. Milne, *J. Chem. Soc., Chem. Commun.*, **1970**, 406; M. Löw, L. Kisfaludy, E. Jaeger, P. Thamm, S. Knof, and E. Wüsnch, *Z. Physiol. Chem.*, **359**, 1637 (1978); M. Löw, L. Kisfaludy, and P. Sohar, *ibid.*, **359**, 1643 (1978); Y. Mori, Y. Matsuda, S. Aimoto, Y. Shimo-nishi, and M. Yamamoto, *Chem. Lett.*, **1976**, 805; K. Hashizume and Y. Shimonishi, "Peptide Chemistry," ed. by H. Yonehara, Protein Res. Found., Osaka, 1979, p. 77; Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **53**, 464 (1980).
13) S. Funakoshi and H. Yajima, *Chem. Pharm. Bull.*, **30**, 1697 (1982).
14) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 789.
15) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
16) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

NII-Electronic Library Service

17) K. Okamoto, K. Yasumura, S. Shimamura, M. Nakamura, A. Tanaka, and H. Yajima, *Chem. Pharm. Bull.*, **27**, 499 (1979).
18) G.W. Anderson, J.E. Zimmerman, and F. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963); *idem, ibid.*, **86**, 1839 (1964).
19) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
20) J.J. Sharp, A.B. Robinson, and M.D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973); H. Yajima, H. Ogawa, M. Kubota, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, H. Adachi, H. Imura, and T. Taminato, *ibid.*, **97**, 5593 (1975).
21) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 804; *idem, ibid.*, **1981**, 831.
22) J. Lenard and G.P. Hess, *J. Biol. Chem.*, **239**, 3275 (1964); S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 1, ed. by B. Weinstein, Marcel Dekker, New York, 1971, p. 51; M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 539 (1978).
23) G.R. Finlayson, *Anal. Biochem.*, **40**, 292 (1971).
24) R. Luben, G. Wong, and D.V. Cohn, *Endocrinol.*, **99**, 526 (1976).

NII-Electronic Library Service