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Studies on Peptides. CXXXIX.[1,2] Solution Synthesis of a 42-Residue Peptide Corresponding to the Entire Amino Acid Sequence of Human Glucose-Dependent Insulinotropic Polypeptide (GIP)

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Eight peptide fragments were prepared by known amide-forming reactions as building blocks for the solution synthesis of the dotetracontapeptide corresponding to the entire amino acid sequence of human intestinal GIP (gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide). Besides Lys(Z), Trp(Mts) and Gln–OBzl, two new amino acid derivatives, Asp(OChp) and Glu(OChp) [Mts = mesitylenesulfonyl, Chp = cycloheptyl], were employed to suppress various side reactions. These fragments were successively assembled by the azide procedure to minimize racemization and all protecting groups employed were removed from the protected GIP by using 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. Synthetic GIP exhibited a significant glucose-dependent insulinotropic activity in dogs, but failed to produce any notable anti-gastric activity in rats.

Keywords——human glucose-dependent insulinotropic polypeptide synthesis; β-cycloheptylaspartate; γ-cycloheptylglutamate; N^{in}-mesitylenesulfonyltryptophan; 2,2,2-trichloroethyloxycarbonylhydrazine; trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; insulinotropic activity; gastric inhibition

We wish to report the solution-phase synthesis of human GIP (hGIP; GIP stands for gastric inhibitory polypeptide[3] or glucose-dependent insulinotropic polypeptide[4]). At present, GIPs isolated from porcine,[5] bovine[6] and human intestinal mucosa[7] have been chemically characterized. hGIP has the same 42 amino acid residues as porcine and bovine GIPs, except for replacement of two or three residues at positions 18, 34 and 37, as shown in Fig. 1.

H-Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-
Lys-Ile-(18)-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln-Lys-
Gly-Lys-Lys-(34)-Asp-Trp-(37)-His-Asn-Ile-Thr-Gln-OH

	18	34	37
human	His	Asn	Lys
porcine	Arg	Ser	Lys
bovine	Arg	Ser	Ile

Fig. 1. Structure of GIP

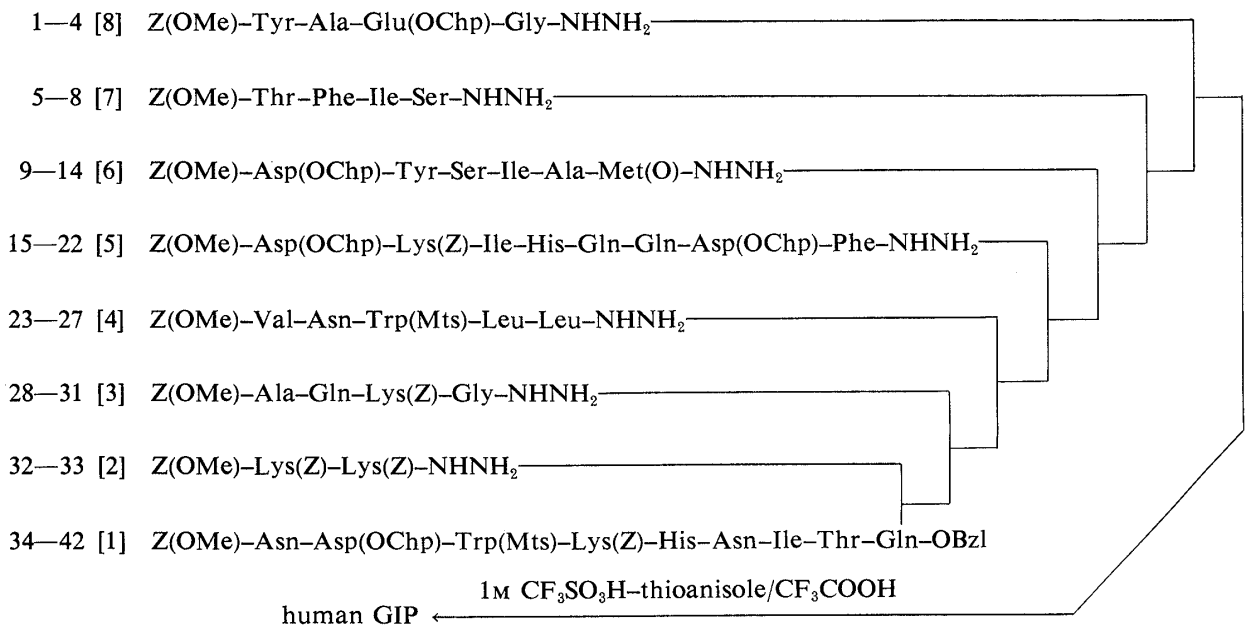

Fig. 2. Synthetic Route to Human GIP

Our synthetic route to hGIP is illustrated in Fig. 2, which shows eight fragments selected as building blocks to construct the entire amino acid sequence of hGIP. Of these, fragments [2] and [7] are known fragments.[8,9] The Z(OMe) group,[10] removable by TFA, was adopted as a temporary N^{α}-protecting group for every intermediate.

Our previous synthesis of porcine GIP[9] (Brown's 1971 formula[11]) was performed by the HF deprotecting strategy.[12] In the present synthesis, the thioanisole-mediated deprotecting procedure[13] was employed. Several advantageous features of this acidolytic deprotection were reviewed recently.[14] Besides Lys(Z), Trp(Mts)[15] and two new amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[16] were employed to suppress various side reactions; *i.e.*, Trp(Mts)[15] for suppression of indole-alkylation during N^{α}-TFA deprotection,[17] Asp(OChp)[18] for suppression of base-catalyzed succinimide formation[19] and Glu(OChp) for suppression of base-catalyzed pyrrolidone formation.[20] Z(OMe)–Glu(OChp)–OH was prepared at first to make the protecting groups as uniform as possible with Asp(OChp), but later it was found that this derivative is less susceptible to base-catalyzed pyrrolidone formation than Glu(OBzl). This new compound was prepared by two alternative routes (Fig. 3) starting with the known Glu half-ester, *i.e.*, by esterification of the α-Bzl ester of Z–Glu–OH[21] with Chp–OH followed by hydrogenolysis and subsequent *p*-methoxybenzyloxycarbonylation (route 1) and by esterification of the α-*p*NB ester of Z(OMe)–Glu–OH with Chp–OH followed by reductive removal of the *p*NB ester (route 2). The corresponding Boc-derivative was also prepared as a reference.

The Met residue was reversibly protected as its sulfoxide[22] in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air oxidation during the synthesis. The substituted hydrazine, Troc–$NHNH_2$,[23] was employed for the preparation of three fragments containing the Asp(OChp) or the Glu(OChp) residue. This Troc group is known to be cleaved by Zn[24] or Cd[25] in AcOH or Zn in the presence of NH_4Cl[26] without affecting other functional protecting groups.

The C-terminal nonapeptide, Z(OMe)–Asn–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl [1], was synthesized according to the scheme shown in Fig. 4. Starting with H–Gln–OBzl, two available dipeptides, Z(OMe)–Ile–Thr–$NHNH_2$[9] and Z(OMe)–Lys(Z)–His–$NHNH_2$,[9] were successively introduced into the peptide chain by

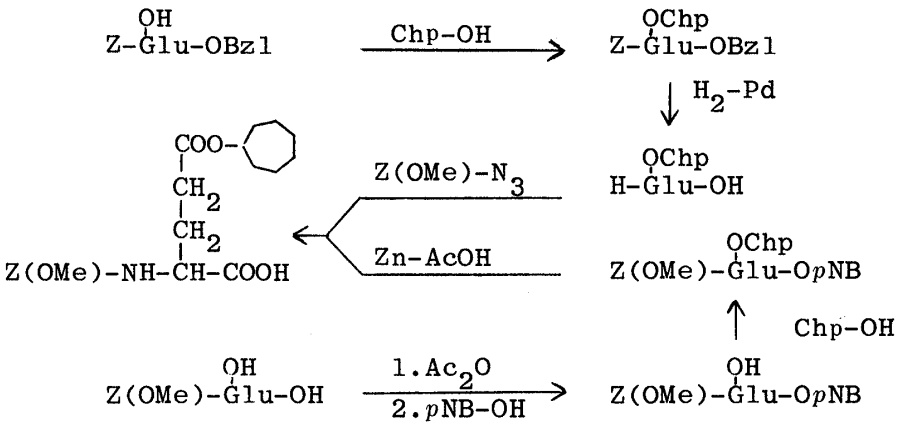

Fig. 3. Preparation of Z(OMe)–Glu(OChp)–OH

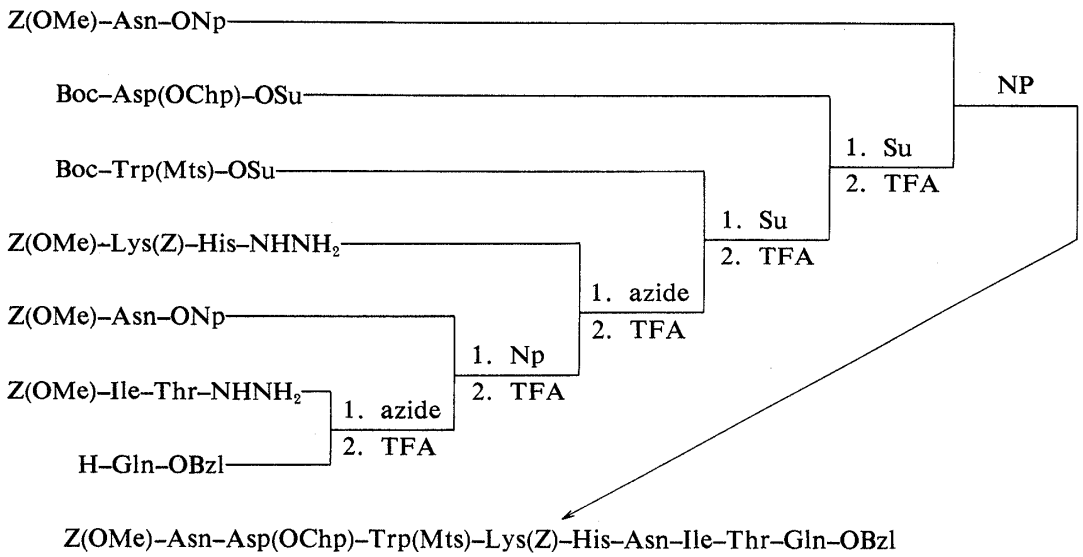

Fig. 4. Synthetic Scheme for the Protected Nonapeptide Ester, Z(OMe)–(hGIP 34—42)–OBzl [1]

Honzl and Rudinger's azide procedure[27] and the rest of the amino acid residues, including Asp(OChp), in a stepwise manner by an active ester procedure, such as the Su[28] or the Np procedure.[29] Homogeneity of fragment [1] was confirmed by thin layer chromatography (TLC) and amino acid analysis after 6 N HCl hydrolysis, as was done with other fragments.

Fragment [3], Z(OMe)–Ala–Gln–Lys(Z)–Gly–$NHNH_2$, was prepared starting with Z(OMe)–Gln–Lys(Z)–Gly–OMe, an intermediate of our previous synthesis of porcine GIP.[9] The tripeptide, after TFA treatment, was condensed with Z(OMe)–Ala–OH by the Np method and the resulting protected tetrapeptide ester was converted to [3] by the usual hydrazine treatment.

Fragment [4], Z(OMe)–Val–Asn–Trp(Mts)–Leu–Leu–$NHNH_2$, was prepared in a stepwise manner starting with Z(OMe)–Leu–Leu–OMe.[30] The respective amino acid residues were introduced by an active ester procedure, *i.e.*, the Su method for Trp(Mts) and the Np method for Asn and Val, as shown in Fig. 5. For introduction of the Val residue, HOBt was employed as an accelerator.[31] The resulting pentapeptide ester was smoothly converted to [4] by the usual hydrazine treatment.

Fragment [5], Z(OMe)–Asp(OChp)–Lys(Z)–Ile–His–Gln–Gln–Asp(OChp)–Phe–

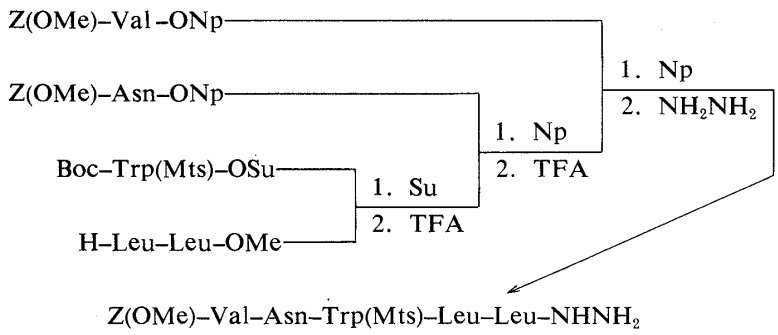

Fig. 5. Synthetic Scheme for the Protected Pentapeptide Hydrazide Z(OMe)–(hGIP 23—27)–$NHNH_2$ [4]

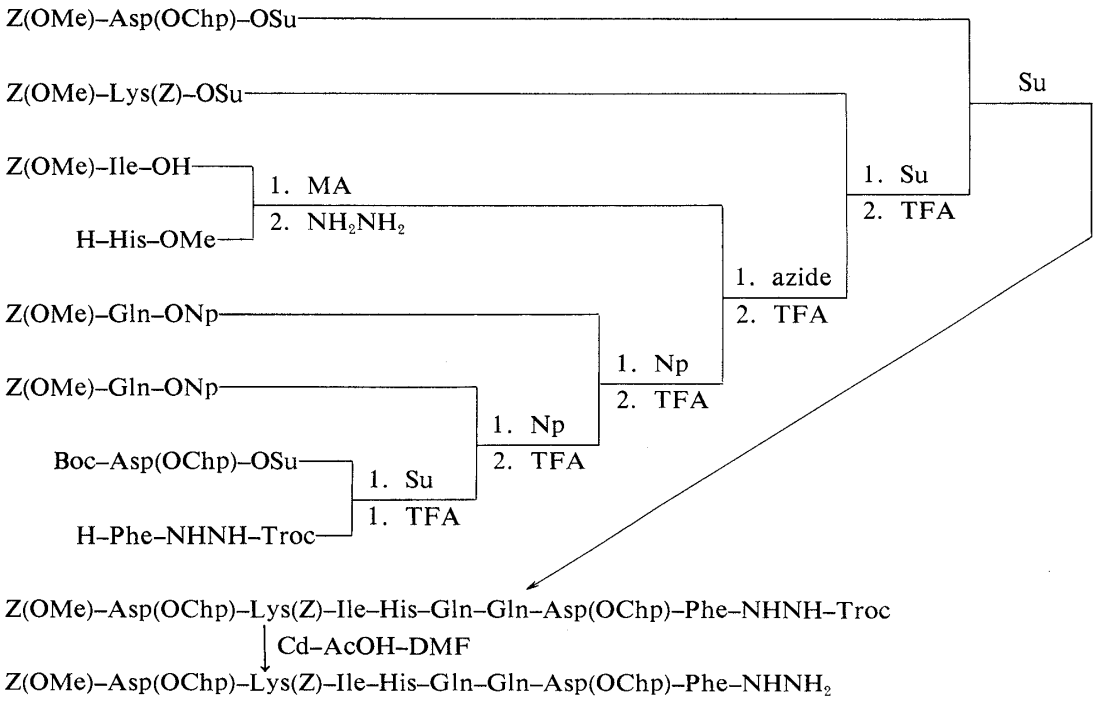

Fig. 6. Synthetic Scheme for the Protected Octapeptide Hydrazide, Z(OMe)–(hGIP 15—22)–$NHNH_2$ [5]

$NHNH_2$ containing two Asp(OChp) residues, was prepared as shown in Fig. 6. First, Z(OMe)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc was prepared in a stepwise manner. The Su method was employed for introduction of Boc–Asp(OChp)–OH and the Np method for the two Gln residues. In these Np steps, HOBt was used as an accelerator. Next, Z(OMe)–Ile–His–$NHNH_2$, prepared by the mixed anhydride method (MA)[32] followed by hydrazinolysis, was condensed with a TFA-treated sample of the above tetrapeptide ester *via* the azide. The resulting protected hexapeptide still showed fairly good solubility in DMF. Thus we decided to elongate the peptide chain to [5] by successive Su condensations of Z(OMe)–Lys(Z)–OH and Z(OMe)–Asp(OChp)–OH. From the resulting protected octapeptide derivative, the Troc group was removed by using Cd[25] in AcOH–DMF, to give the hydrazide [5].

Fragment [6], Z(OMe)–Asp(OChp)–Tyr–Ser–Ile–Ala–Met(O)–$NHNH_2$, was prepared as shown in Fig. 7. Starting with H–Met(O)–NHNH–Troc,[33] Z(OMe)–Tyr–Ser–Ile–Ala–Met(O)–NHNH–Troc was prepared by successive azide condensations of two dipeptide

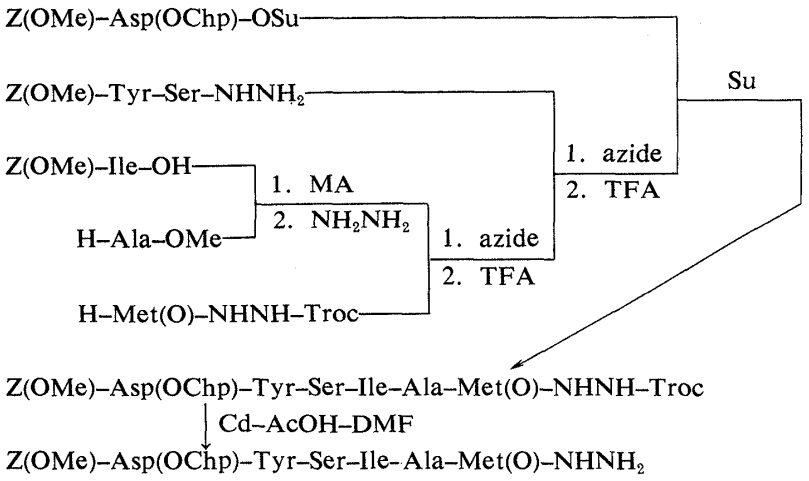

Fig. 7. Synthetic Scheme for the Protected Hexapeptide Hydrazide, Z(OMe)-(hGIP 9—14)-$NHNH_2$ [6]

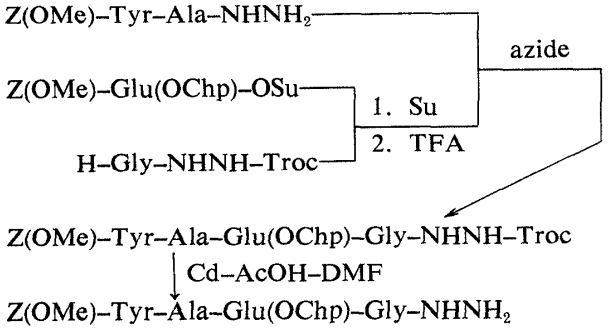

Fig. 8. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hGIP 1—4)-$NHNH_2$ [8]

hydrazides, Z(OMe)-Ile-Ala-$NHNH_2$ and Z(OMe)-Tyr-Ser-$NHNH_2$. The former was prepared by the MA procedure followed by the usual hydrazinolysis. The latter is a known derivative.[9] Finally, Z(OMe)-Asp(OChp)-OH was condensed with a TFA-treated sample of the above pentapeptide derivative by the Su method as performed above. From the resulting protected hexapeptide derivative, the Troc group was removed by Cd, as described in the preparation of fragment [5].

As mentioned above, fragment [7] is a known compound.[9] Fragment [8], Z(OMe)-Tyr-Ala-Glu(OChp)-Gly-$NHNH_2$ was prepared starting with H-Gly-NHNH-Troc, as shown in Fig. 8. Z(OMe)-Glu(OChp)-OH prepared as described above was condensed with H-Gly-NHNH-Troc[34] by the Su method and the resulting dipeptide derivative, after TFA-treatment, was condensed with Z(OMe)-Tyr-Ala-$NHNH_2$[9] *via* the azide. As performed in the preparations of fragments [5] and [6], the Troc group was removed from the resulting protected tetrapeptide derivative by treatment with Cd in AcOH-DMF.

The eight fragments thus prepared were assembled successively by Honzl and Rudinger's azide procedure[27] (Fig. 2) in order to minimize racemization. The condensations from fragments [1] to [3] proceeded satisfactorily as usual. However, the subsequent azide condensations of fragments from [4] to [8] had to be performed at lower temperature (−18 °C) than usual (4 °C) in order to minimize the Curtius rearrangement.[35] Otherwise, an acid hydrolysate of each product gave a low recovery of the amino acid located at the C-terminus of each fragment. The amount of the acyl component was increased from 1.5 to 4 eq as the chain was elongated. Each protected product was purified by either precipitation from DMF or DMSO with appropriate solvents, such as methanol or AcOEt or by gel-filtration on Sephadex LH-60 using DMF as an eluant.

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic hGIP and Its Intermediates

	Protected peptides							Syn. hGIP	Residue
	32—42	28—42	23—42	15—42	9—42	5—42	1—42		
Asp	2.99	2.97	4.27	6.18	7.27	6.97	6.73	6.86	(7)
Thr	1.00	1.00	1.00	1.00	1.00	2.00	2.00	2.00	(2)[a]
Ser					1.12	1.92	1.97	1.97	(2)
Glu	1.05	2.09	2.10	4.31	4.42	4.01	5.38	5.24	(5)
Gly		1.06	1.06	1.11	1.09	0.97	2.15	2.08	(2)
Ala		1.08	1.09	1.07	2.29	2.19	3.51	3.21	(3)
Val			0.94	0.89	0.86	0.78	1.08	0.98	(1)
Met					1.08	0.80	0.98	0.84	(1)[b]
Ile	1.01	1.02	1.00	1.65	3.33	3.77	3.99	3.90	(4)
Leu			1.95	2.17	2.05	1.90	2.20	2.18	(2)
Tyr					1.27	0.96	2.11	1.96	(2)
Phe				0.98	1.04	1.91	2.16	2.07	(2)
Trp	0.66	0.78	1.38	1.29	1.43	1.51	1.45	N.D.	(2)[c]
Lys	3.02	4.13	3.99	5.06	4.98	4.61	5.30	5.18	(5)
His	1.03	1.04	1.03	1.76	1.76	1.76	1.89	1.91	(2)
Recov. (%)	86	87	77	92	89	89	88	89	

a) Diagnostic amino acid. *b*) Met + Met(O). *c*) 4 N MSA hydrolysate.

Throughout this synthesis, Thr was used as a diagnostic amino acid. Each intermediate was subjected to acid hydrolysis and the recovery of Thr was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each condensation (Table I). The homogeneity of each product was further ascertained by elemental analysis and TLC.

In the final step, the protected hGIP thus obtained was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and ethanedithiol in an ice-bath for 180 min to remove all the protecting groups employed, except for the Met(O) residue. The Met(O) residue is known to be partially reduced under this thioanisole-mediated condition.[36] *m*-Cresol is known to be effective to suppress partial O-sulfonation at the Tyr residue[37] and ethanedithiol to suppress indole-sulfonation at the Trp residue.[38] The deprotected peptide was treated with dil. ammonia to reverse any possible N→O shift[39] at the Ser and Thr residues and then incubated with 2-mercaptoethanol at 37 °C for 20 h to ensure the complete reduction of the Met(O) residue. The reduced product, after gel-filtration on Sephadex G-50, was purified by ion-exchange chromatography on carboxymethyl(CM)-cellulose using gradient elution with ammonium acetate buffer (from 0.01 to 0.2 M). The product was finally purified by high performance liquid chromatography (HPLC) on Nucleosil 5C18 using gradient elution with acetonitrile (from 27 to 37%) in 0.1% TFA.

The purified peptide exhibited a sharp single spot on TLC in two different solvent systems and a single band in disk isoelectrofocusing. Its purity was further confirmed by amino acid analysis after acid hydrolysis and leucine-aminopeptidase (LAP) digestion. This peptide is very sensitive to air-oxidation. When the HPLC-purified sample was rechromatographed, two small peaks were always observed in front of the main peak. When this sample was exposed to hydrogen peroxide, the amounts of these two peaks increased, with a corresponding decrease of the main peak. Thus, these two peaks seem to be due to the stereoisomers of the Met(O) residue.

Our synthetic GIP (20 μg/kg) exhibited marked insulinotropic activity in dogs under background infusion of glucose as shown in Fig. 9, but it (4—64 μg/kg/h) failed to show any significant anti-gastric activity against acid-secretion stimulated by pentagastrin (1.5 μg/kg/h)

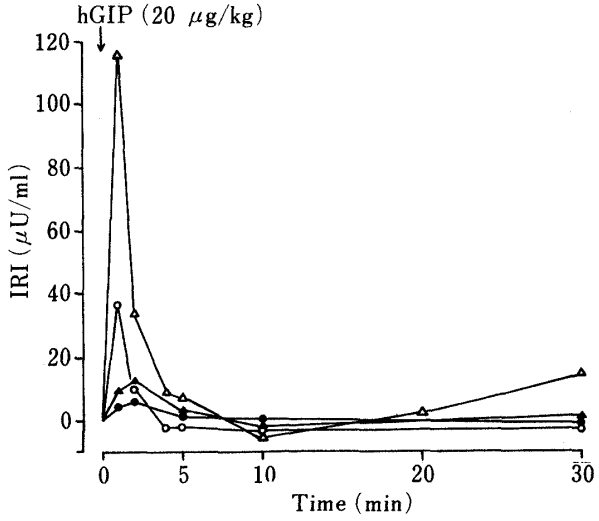

Fig. 9. Insulinotropic Activity of Synthetic hGIP

GIP + glucose (8 g/h): △—△, portal; ▲—▲, peripheral. GIP alone: ○—○, portal; ●—●, peripheral.

in rats ($n=4$).

Experimental

General experimental methods employed here are essentially the same as described in Part CXXXIII[40] of the present series.

N^{α}-Deprotection——The N^{α}-protecting group, Z(OMe) or Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of anisole (2 eq or more) at ice-bath temperature for 60 min. After evaporation of TFA *in vacuo* at 15—20 °C, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Condensation Reaction——The DCC and the active ester condensation reactions were performed at room temperature. Each hydrazide was converted to the corresponding azide by using isoamyl nitrite and the azide reaction was performed at 4 °C. A mixed anhydride was prepared using isobutyl chloroformate and allowed to react with an amino component in an ice-bath for 5 h.

Purification——Unless otherwise mentioned, products were purified by one of the following procedures. Procedure A: For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized or precipitated from appropriate solvents. Procedure B: For purification of protected peptides less soluble in AcOEt, the crude 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 or precipitated from appropriate solvents. For purification of His-containing peptides, 5% $NaHCO_3$ and H_2O were used for washing.

TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v): Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_3 $CHCl_3$–MeOH (10:0.5), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1), Rf_6 $CHCl_3$.

HPLC was conducted with a Waters 204 compact model. Optical rotation and ultraviolet (UV) absorption were measured with a Union PM 101 instrument and a Hitachi model 100-20 spectrometer respectively. Infrared (IR) spectra were measured with a Hitachi 215 grating IR spectrometer.

Z–Glu(OChp)–OBzl——DCC (4.85 g, 1.3 eq) was added to a mixture of Z–Glu–OBzl[21] [prepared from 10.0 g (18.1 mmol) of the DCHA salt], Chp–OH (3.26 ml, 1.5 eq) and DMAP (0.22 g, 0.1 eq) in THF (100 ml) and the solution, after being stirred overnight, was filtered and concentrated. The product was purified by procedure A, followed by recrystallization from AcOEt and *n*-hexane; yield 6.35 g (75%), mp 38—40 °C, $[\alpha]_D^{28}$ −18.9° ($c=1.6$, MeOH), Rf_6 0.64. *Anal.* Calcd for $C_{27}H_{33}NO_6$: C, 69.36; H, 7.11; N, 3.00. Found: C, 69.29; H, 7.10; N, 3.20.

H–Glu(OChp)–OH——Z–Glu(OChp)–OBzl (6.0 g, 12.83 mmol) in MeOH (50 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 24 h. The catalyst was removed by filtration, the filtrate was concentrated and the residue was recrystallized from MeOH and AcOEt; yield 2.37 g (76%), mp 184—186 °C, $[\alpha]_D^{28} \fallingdotseq 0°$ ($c=0.7$, MeOH), Rf_1 0.30. *Anal.* Calcd for $C_{12}H_{21}NO_8 \cdot 1/2H_2O$: C, 57.12; H, 8.79; N, 5.55. Found: C, 57.21; H, 8.64; N, 5.82.

Z(OMe)–Glu–O*p*NB·DCHA——A mixture of Z(OMe)–Glu-anhydride [prepared from 10.0 g (32.1 mmol) of Z(OMe)–Glu–OH] and *p*NB–OH (5.4 g, 1.1 eq) in AcOEt (100 ml) was stirred at room temperature overnight. The

solution was washed with H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was dissolved in ether and DCHA (6.4 ml, 1.1 eq) was added to form a solid, which was recrystallized from MeOH and ether; yield 13.0 g (65%), mp 145—147 °C, $[\alpha]_D^{22} \fallingdotseq 0°$ ($c=0.2$, MeOH), Rf_2 0.67. *Anal.* Calcd for $C_{33}H_{45}N_3O_9$: C, 63.14; H, 7.23; N, 6.69. Found: C, 63.26; H, 7.31; N, 6.89.

Z(OMe)–Glu(OChp)–O*p*NB——DCC (5.14 g, 1.3 eq) was added to a mixture of Z(OMe)–Glu–O*p*NB [prepared from 12.0 g (19.1 mmol) of the DCHA salt], Chp–OH (3.46 ml, 1.5 eq) and DMAP (0.23 g, 0.1 eq) in THF (100 ml) and the solution, after being stirred overnight, was concentrated. The product was purified by procedure A, followed by recrystallization from AcOEt and ether–*n*-hexane (1 : 1); yield 6.97 g (67%), mp 43—45 °C, $[\alpha]_D^{22} -15.3°$ ($c=0.6$, MeOH), Rf_6 0.18. *Anal.* Calcd for $C_{28}H_{34}N_2O_9$: C, 61.98; H, 6.32; N, 5.16. Found: C, 62.23; H, 6.30; N, 5.32.

Z(OMe)–Glu(OChp)–OH·DCHA——A: According to Weygand and Hunger,[10] H–Glu(OChp)–OH (0.90 g, 3.70 mmol) was converted to the corresponding Z(OMe)-derivative and its DCHA salt was recrystallized from MeOH and ether; yield 1.96 g (90%), mp 127—129 °C, $[\alpha]_D^{20} -2.1°$ ($c=0.5$, MeOH), Rf_1 0.70, Rf_2 0.65. *Anal.* Calcd for $C_{33}H_{52}N_2O_7$: C, 67.32; H, 8.90; N, 4.76. Found: C, 67.53; H, 9.08; N, 4.87. B: In the presence of Zn powder (7.8 g, 10 eq), a solution of Z(OMe)–Glu(OChp)–O*p*NB (6.5 g, 12.0 mmol) in AcOEt–90% AcOH (40 ml–20 ml) was stirred in an ice-bath for 5 h, then filtered. The filtrate was washed with 0.5 N HCl, H_2O–NaCl, 3% EDTA and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was converted to the corresponding DCHA salt as stated above; yield 4.97 g (71%), mp 127—129 °C, $[\alpha]_D^{22} -1.8°$ ($c=0.5$, MeOH), Rf_1 0.70, Rf_2 0.65. Its IR spectrum was superimposable on that of the sample obtained in A.

Boc–Glu(OChp)–OH·DCHA——According to the procedure of Itoh *et al.*,[41] H–Glu(OChp)–OH (0.60 g, 2.47 mmol) was converted to the corresponding Boc-derivative with 2-*tert*-butyloxycarbonyloxyimino-2-phenylacetonitrile. The product was converted to the DCHA salt, which was recrystallized from MeOH and ether; yield 1.06 g (82%), mp 117—119 °C, $[\alpha]_D^{22} \fallingdotseq 0°$, Rf_1 0.71. *Anal.* Calcd for $C_{29}H_{52}N_2O_6$: C, 66.38; H, 9.99; N, 5.34. Found: C, 66.44; H. 10.25; N, 5.56.

Treatment of H–Glu(OR)–OH (R = Bzl and Chp) with Et_3N——In the presence of Et_3N (1 eq), each γ-ester (0.86 mmol) in DMF–H_2O (1 : 1, 4 ml) was incubated at 40 °C for 20 h and an aliquot was examined by TLC. In each case, three compounds (Rf_4) were detected by $Ce(SO_4)_2$; pyro-Glu–OH (0.50), H–Glu–OH (0.38; formed partially by saponification) and H–Glu(OChp)–OH (0.80) or H–Glu(OBzl)–OH (0.67). For quantification, the percentages of H–Glu–OH and H–Glu(OR)–OH were determined on an amino acid analyzer, in which an equal amount of Gly was used in each case as an internal standard for comparison. The percentages of pyro-Glu–OH thus calculated by subtraction of the sum of H–Glu–OH and H–Glu(OR)–OH were 4.1% for H–Glu(OChp)–OH and 88.3% for H–Glu(OBzl)–OH.

Z(OMe)–Gln–OBzl——Z(OMe)–Gln–OH was esterified with benzyl bromide with the aid of DCHA as usual; yield 81%, mp 120—124 °C, $[\alpha]_D^{23} -5.3°$ ($c=0.8$, DMF), Rf_1 0.75. *Anal.* Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 63.07; H, 6.03; N, 7.02.

Z(OMe)–Ile–Thr–Gln–OBzl——The azide [prepared from 5.42 g (13.2 mmol) of Z(OMe)–Ile–Thr–$NHNH_2$[9]] in DMF (50 ml) and Et_3N (1.83 ml, 13.2 mmol) were added to an ice-chilled solution of H–Gln–OBzl [prepared from 4.42 g (11.0 mmol) of the Z(OMe)-derivative] in DMF (40 ml). After being stirred for 14 h, the solution was concentrated and the product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 4.14 g (61%), mp 220—222 °C, $[\alpha]_D^{20} +1.5°$ ($c=0.7$, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{31}H_{42}N_4O_9 \cdot 1/2H_2O$: C, 59.70; H. 6.95; N, 8.98. Found: C, 59.70; H, 6.83; N, 9.12.

Z(OMe)–Asn–Ile–Thr–Gln–OBzl——A TFA-treated sample of Z(OMe)–Ile–Thr–Gln–OBzl (4.14 g, 6.73 mmol) was dissolved in DMF–DMSO (2 : 1, 50 ml) together with Et_3N (1.87 ml, 13.5 mmol), Z(OMe)–Asn–ONp (3.37 g, 8.07 mmol) and HOBt (107 mg, 0.67 mmol) and the mixture, after being stirred for 2 h, was concentrated. The product was purified by procedure B, followed by precipitation from DMSO with MeOH; yield 4.20 g (86%), mp 235—240 °C, $[\alpha]_D^{20} -13.7°$ ($c=0.9$, DMSO), Rf_1 0.56. *Anal.* Calcd for $C_{35}H_{48}N_6O_{11}$: C, 57.68; H, 6.64; N, 11.53. Found: C, 57.58; H, 6.69; N, 11.59.

Z(OMe)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl——The azide [prepared from 5.15 g (8.65 mmol) of Z(OMe)–Lys(Z)–His–$NHNH_2$[9]] in DMF (50 ml) and NMM (0.87 ml, 8.65 mmol) were added to an ice-chilled solution of H–Asn–Ile–Thr–Gln–OBzl [obtained from 4.20 g (5.77 mmol) of the Z(OMe)-derivative] in DMF (40 ml), and the mixture was stirred for 14 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.15 g (79%), mp 228—230 °C, $[\alpha]_D^{20} -13.6°$ ($c=0.7$, DMF), Rf_1 0.69. *Anal.* Calcd for $C_{55}H_{73}N_{11}O_{15} \cdot H_2O$: C, 57.63; H, 6.60; N, 13.44. Found: C, 57.50; H, 6.68; N, 13.63.

Boc–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl——A mixture of a TFA-treated sample of the above hexapeptide (5.15 g, 4.57 mmol), Et_3N (1.90 ml, 13.70 mmol) and Boc–Trp(Mts)–OSu (4.00 g, 6.85 mmol) in DMF (50 ml) was stirred for 2 h and concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.80 g (58%), mp 232—234 °C, $[\alpha]_D^{20} -13.8°$ ($c=1.0$, DMF), Rf_1 0.68. *Anal.* Calcd for $C_{71}H_{93}N_{13}O_{17}S \cdot 3/2H_2O$: C, 58.42; H, 6.63; N, 12.48. Found: C, 58.39; H, 6.65; N, 12.65.

Boc–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl——A mixture of a TFA-treated sample of the above heptapeptide (3.80 g, 2.65 mmol), Et_3N (1.11 ml, 7.95 mmol) and Boc–Asp(OChp)–OSu (1.70 g, 3.98 mmol) in DMF (40 ml) was stirred for 5 h and concentrated. The product was purified by procedure B, followed by

recrystallization from MeOH; yield 2.63 g (60%), mp 228—230 °C, $[\alpha]_D^{20} - 15.3°$ ($c = 0.7$, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{82}H_{110}N_{14}O_{20}S \cdot H_2O$: C, 59.26; H, 6.79; N, 11.80. Found: C, 59.36; H, 6.82; N, 11.91.

Z(OMe)–Asn–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl, Z(OMe)–(hGIP 34—42)–OBzl [1]——A mixture of a TFA-treated sample of the above octapeptide (2.63 g, 1.60 mmol), Et_3N (0.67 ml, 4.80 mmol), HOBt (22 mg, 0.16 mmol) and Z(OMe)–Asn–ONp (1.00 g, 2.40 mmol) in DMF (30 ml) was stirred for 5 h and concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.00 g (69%), mp 227—229 °C, $[\alpha]_D^{20} - 13.0°$ ($c = 0.9$, DMF), Rf_1 0.57. Amino acid ratios in 6 N HCl hydrolysate; Asp 2.94, Thr 1.00, Glu 1.05, Ile 0.94, Trp N. D., Lys 1.04, His 1.03 (recovery of Thr 85%). *Anal.* Calcd for $C_{90}H_{116}N_{16}O_{23}S$: C, 59.32; H, 6.42; N, 12.30. Found: C, 59.18; H, 6.53; N, 12.14.

Z(OMe)–Ala–Gln–Lys(Z)–Gly–OMe——A mixture of a TFA-treated sample of Z(OMe)–Gln–Lys(Z)–Gly–OMe[9] (5.00 g, 7.77 mmol), Et_3N (2.14 ml, 15.54 mmol) and Z(OMe)–Ala–ONp (4.36 g, 11.70 mmol) in DMF–DMSO (5 : 1, 50 ml) was stirred overnight and concentrated. The product was purified by procedure B, followed by precipitation from DMF–DMSO (5 : 1) with MeOH; yield 4.85 g (87%), mp 219—221 °C, $[\alpha]_D^{20} - 11.0°$ ($c = 0.8$, DMSO), Rf_1 0.72. *Anal.* Calcd for $C_{34}H_{46}N_6O_{11}$: C, 57.13; H, 6.49; N, 11.76. Found: C, 57.17; H, 6.57; N, 11.59.

Z(OMe)–Ala–Gln–Lys(Z)–Gly–NHNH$_2$, Z(OMe)–(hGIP 28—31)–NHNH$_2$ [3]——The above tetrapeptide ester (4.60 g, 6.44 mmol) in HMPA–DMF (1 : 10, 50 ml) was treated with 80% hydrazine hydrate (2.01 ml, 5 eq) overnight. The solution was concentrated *in vacuo* and MeOH was added to form a powder, which was precipitated from DMSO with MeOH; yield 3.73 g (81%), mp 214—216 °C, $[\alpha]_D^{20} - 8.2°$ ($c = 0.6$, DMSO), Rf_1 0.57. Amino acid ratios in 6 N HCl hydrolysate: Glu 0.99, Gly 1.00, Ala 1.06, Lys 0.96 (recovery of Gly 74%). *Anal.* Calcd for $C_{33}H_{46}N_8O_{10}$: C, 55.45; H, 6.49; N, 15.68. Found: C, 55.28; H, 6.48; N, 15.48.

Boc–Trp(Mts)–Leu–Leu–OMe——A mixture of a TFA-treated sample of Z(OMe)–Leu–Leu–OMe[30] (12.58 g, 29.8 mmol), Et_3N (8.4 ml, 59.6 mmol) and Boc–Trp(Mts)–OSu (17.38 g, 29.8 mmol) in DMF (200 ml) was stirred for 14 h, then concentrated. The product was purified by procedure A, followed by column chromatography on silica gel (7.2 × 20 cm) using $CHCl_3$ as an eluant. The product was recrystallized from AcOEt and *n*-hexane; yield 10.95 g (51%), mp 102—104 °C, $[\alpha]_D^{20} - 34.2°$ ($c = 0.6$, MeOH), Rf_6 0.12. *Anal.* Calcd for $C_{38}H_{54}N_4O_8S$: C, 62.78; H, 7.49; N, 7.71. Found: C, 62.67; H, 7.69; N, 7.61.

Z(OMe)–Asn–Trp(Mts)–Leu–Leu–OMe——A mixture of a TFA-treated sample of the above tripeptide (8.00 g, 11.0 mmol), Et_3N (3.23 ml, 23.1 mmol) and Z(OMe)–Asn–ONp (5.05 g, 12.1 mmol) in DMF (50 ml) was stirred overnight, then concentrated. The product was purified by procedure B, followed by recrystallization from MeOH and EtOH; yield 6.10 g (61%), mp 232—234 °C, $[\alpha]_D^{20} - 48.6°$ ($c = 0.6$, MeOH), Rf_3 0.36. *Anal.* Calcd for $C_{46}H_{60}N_6O_{11}S$: C, 61.04; H, 6.68; N, 9.29. Found: C, 60.80; H, 6.75; N, 9.36.

Z(OMe)–Val–Asn–Trp(Mts)–Leu–Leu–OMe——A mixture of a TFA-treated sample of the above tetrapeptide (5.00 g, 5.52 mmol), Et_3N (1.62 ml, 11.61 mmol), HOBt (0.37 g, 2.74 mmol) and Z(OMe)–Val–ONp (2.45 g, 6.09 mmol) in DMF (50 ml) was stirred overnight, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 3.98 g (72%), mp 215—217 °C, $[\alpha]_D^{20} - 31.8°$ ($c = 0.8$, DMF), Rf_3 0.34. *Anal.* Calcd for $C_{51}H_{69}N_7O_{12}S$: C, 61.00; H, 6.93; N, 9.76. Found: C, 60.72; H, 6.97; N, 9.57.

Z(OMe)–Val–Asn–Trp(Mts)–Leu–Leu–NHNH$_2$, Z(OMe)–(hGIP 23—27)–NHNH$_2$ [4]——The above protected pentapeptide ester (3.50 g, 3.49 mmol) in DMF–MeOH (1 : 1, 30 ml) was treated with 80% hydrazine hydrate (3.27 ml, 15 eq) overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with MeOH; yield 2.85 g (81%), mp 273—275 °C, $[\alpha]_D^{20} - 10.4°$ ($c = 1.0$, DMF), Rf_1 0.69. Amino acid ratios in 4 M MSA hydrolysate: Asp 0.98, Val 0.89, Leu 2.00, Trp 0.83 (recovery of Leu 91%). *Anal.* Calcd for $C_{50}H_{69}N_9O_{11}S$: C, 59.80; H, 6.93; N, 12.55. Found: C, 59.58; H, 6.95; N, 12.47.

Boc–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of Z(OMe)–Phe–NHNH–Troc[42] (5.81 g, 11.2 mmol), Et_3N (3.11 ml, 22.4 mmol) and Boc–Asp(OChp)–OSu (4.78 g, 11.2 mmol) in DMF (50 ml) was stirred for 14 h, then concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 4.47 g (60%), mp 206—208 °C, $[\alpha]_D^{20} - 29.5°$ ($c = 0.5$, DMF), Rf_2 0.92, Rf_3 0.52. *Anal.* Calcd for $C_{28}H_{39}Cl_3N_4O_8$: C, 50.49; H, 5.90; N, 8.41. Found: C, 50.53; H, 6.07; N, 8.53.

Z(OMe)–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above dipeptide derivative (4.47 g, 6.70 mmol), Et_3N (1.86 ml, 13.4 mmol), HOBt (0.10 g, 0.74 mmol) and Z(OMe)–Gln–ONp (3.18 g, 7.37 mmol) in DMF (40 ml) was stirred for 3 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.23 g (91%), mp 221—223 °C, $[\alpha]_D^{20} + 1.7°$ ($c = 0.6$, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{37}H_{47}Cl_3N_6O_{11}$: C, 51.78; H, 5.52; N, 9.79. Found: C, 51.61; H, 5.52; N, 9.87.

Z(OMe)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above tripeptide derivative (5.23 g, 6.08 mmol), Et_3N (1.69 ml, 12.2 mmol), HOBt (82 mg, 0.61 mmol) and Z(OMe)–Gln–ONp (2.89 g, 6.69 mmol) in DMF (50 ml) was stirred for 5 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.56 g (93%), mp 224—226 °C, $[\alpha]_D^{20} - 21.7°$ ($c = 0.8$, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{42}H_{55}Cl_3N_8O_{13}$: C, 51.14; H, 5.62; N, 11.36. Found: C, 51.38; H, 5.78; N, 11.41.

Z(OMe)–Ile–His–OMe——A mixed anhydride [prepared from 8.76 g (29.7 mmol) of Z(OMe)–Ile–OH] in AcOEt (80 ml) was added to an ice-chilled solution of H–His–OMe [prepared from 7.18 g (29.7 mmol) of the

dihydrochloride] in DMF (70 ml) and the mixture, after being stirred for 3 h, was concentrated. The product was purified by procedure B, followed by recrystallization from MeOH and ether; yield 5.64 g (43%), mp 163—165 °C, $[\alpha]_D^{20}$ −14.0° (c=0.6, MeOH), Rf_1 0.73. *Anal.* Calcd for $C_{22}H_{30}N_4O_6$: C, 59.18; H, 6.77; N, 12.55. Found: C, 58.88; H, 6.74; N, 12.47.

Z(OMe)–Ile–His–NHNH$_2$——Z(OMe)–Ile–His–OMe (5.64 g, 12.6 mmol) in MeOH (50 ml) was treated with 80% hydrazine hydrate (3.95 ml, 5 eq) overnight. The solvent was removed by evaporation and the residue was treated with isopropanol to form a powder, which was precipitated from MeOH with isopropanol; yield 4.79 g (85%), mp 158—160 °C, $[\alpha]_D^{20}$ −34.1° (c=0.6, MeOH), Rf_1 0.66. *Anal.* Calcd for $C_{21}H_{30}N_6O_5 \cdot 1/2H_2O$: C, 55.36; H, 6.86; N, 18.45. Found: C, 55.55; H, 6.90; N, 18.16.

Z(OMe)–Ile–His–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——The azide [prepared from 1.81 g (4.05 mmol) of Z(OMe)–Ile–His–NHNH$_2$] in DMF (10 ml) and NMM (0.41 ml, 4.05 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc (2.66 g, 2.70 mmol) in DMF (20 ml) containing Et_3N (0.38 ml, 2.70 mmol) and the mixture, after being stirred for 14 h, was concentrated. The product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 3.27 g (98%), mp 248—250 °C, $[\alpha]_D^{20}$ −15.4° (c=0.7, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{54}H_{73}Cl_3N_{12}O_{15}$: C, 52.45; H, 5.95; N, 13.59. Found: C, 52.16; H, 5.99; N, 13.50.

Z(OMe)–Lys(Z)–Ile–His–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above hexapeptide (3.27 g, 2.64 mmol), Et_3N (1.10 ml, 7.93 mmol) and Z(OMe)–Lys(Z)–OSu (2.15 g, 3.96 mmol) in DMF (30 ml) was stirred for 3 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 3.00 g (76%), mp 248—250 °C, $[\alpha]_D^{20}$ −18.1° (c=0.7, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{68}H_{91}Cl_3N_{14}O_{18}$: C, 54.49; H, 6.12; N, 13.08. Found: C, 54.24; H, 6.09; N, 12.86.

Z(OMe)–Asp(OChp)–Lys(Z)–Ile–His–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above heptapeptide (3.06 g, 2.04 mmol), Et_3N (0.85 ml, 6.13 mmol) and Z(OMe)–Asp(OChp)–OSu (1.50 g, 3.06 mmol) in DMF (30 ml) was stirred for 13 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.41 g (69%), mp 248—250 °C, $[\alpha]_D^{20}$ −9.8° (c=0.6, DMF), Rf_1 0.61. *Anal.* Calcd for $C_{79}H_{108}Cl_3N_{15}O_{21} \cdot 2H_2O$: C, 54.34; H, 6.47; N, 12.03. Found: C, 54.01; H, 6.24; N, 12.36.

Z(OMe)–Asp(OChp)–Lys(Z)–Ile–His–Gln–Gln–Asp(OChp)–Phe–NHNH$_2$, Z(OMe)–(hGIP 15—22)–NHNH$_2$ [5]——The above protected octapeptide derivative (2.41 g, 1.41 mmol) in DMF–AcOH (20 ml–2 ml) was treated with Cd powder (3.17 g, 20 eq) at room temperature overnight. The precipitate formed during the reaction was dissolved with the aid of DMSO and the solution was filtered. The filtrate was concentrated and the residue was treated with 5% EDTA to form a powder, which was washed with 5% $NaHCO_3$ and H_2O and precipitated from DMSO with MeOH; yield 1.67 g (77%), mp 245—250 °C, $[\alpha]_D^{20}$ −48.0° (c=0.5, DMSO), Rf_1 0.57. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.02, Glu 2.22, Ile 0.83, Phe 1.00, Lys 1.05, His 0.92 (recovery of Phe 76%). *Anal.* Calcd for $C_{76}H_{107}N_{15}O_{19} \cdot 3H_2O$: C, 57.45; H, 7.17; N, 13.23. Found: C, 57.33; H, 6.95; N, 13.31.

Z(OMe)–Ile–Ala–OMe——The title compound was prepared by the MA procedure and purified by procedure A, followed by recrystallization from MeOH and ether; yield 16.21 g (57%), mp 155—157 °C, $[\alpha]_D^{20}$ −39.1° (c=0.2, MeOH), Rf_1 0.92. *Anal.* Calcd for $C_{19}H_{28}N_2O_6$: C, 59.98; H, 7.42; N, 7.36. Found: C, 59.93; H, 7.51; N, 7.37.

Z(OMe)–Ile–Ala–NHNH$_2$——The above dipeptide (10.42 g, 27.4 mmol) in MeOH (100 ml) was treated with 80% hydrazine hydrate (8.6 ml, 5 eq) overnight. The solvent was evaporated and the residue was treated with EtOH to form a powder, which was precipitated from MeOH with EtOH; yield 7.27 g (70%), mp 230—232 °C, $[\alpha]_D^{20}$ −27.1° (c=1.0, MeOH), Rf_1 0.71. *Anal.* Calcd for $C_{18}H_{28}N_4O_5$: C, 56.82; H, 7.42; N, 14.73. Found; C, 57.06; H, 7.36; N, 14.75.

Z(OMe)–Ile–Ala–Met(O)–NHNH–Troc——The azide [prepared from 5.97 g (15.7 mmol) of Z(OMe)–Ile–Ala–NHNH$_2$] in DMF (50 ml) and Et_3N (2.18 ml, 15.7 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Met(O)–NHNH–Troc[33] (8.14 g, 15.7 mmol) in DMF (80 ml) containing Et_3N (2.18 ml, 15.7 mmol), and the mixture was stirred for 24 h, then concentrated. The product was purified by procedure B, followed by recrystallization from MeOH and ether; yield 7.96 g (72%), mp 209—211 °C, $[\alpha]_D^{20}$ +4.2° (c=0.5, MeOH), Rf_1 0.67. *Anal.* Calcd for $C_{26}H_{38}Cl_3N_5O_9S \cdot 1/2H_2O$: C, 43.85; H, 5.52; N, 9.84; Found: C, 43.70; H, 5.80; N, 9.90.

Z(OMe)–Tyr–Ser–Ile–Ala–Met(O)–NHNH–Troc——The azide [prepared from 3.12 g (6.98 mmol) of Z(OMe)–Tyr–Ser–NHNH$_2$[9]] in DMF (30 ml) and Et_3N (0.97 ml, 6.98 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tripeptide derivative (4.91 g, 6.98 mmol) in DMF (50 ml) containing Et_3N (0.97 ml, 6.98 mmol), and the mixture was stirred for 24 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 4.71 g (71%), mp 202—204 °C, $[\alpha]_D^{20}$ +6.2° (c=1.4, DMF), Rf_1 0.42. *Anal.* Calcd for $C_{38}H_{52}Cl_3N_7O_{13}S$: C, 47.87; H, 5.50; N, 10.29. Found: C, 47.88; H, 5.74; N, 10.59.

Z(OMe)–Asp(OChp)–Tyr–Ser–Ile–Ala–Met(O)–NHNH–Troc——Z(OMe)–Asp(OChp)–OSu (2.78 g, 5.66 mmol) and NMM (0.57 ml, 5.66 mmol) were added to a solution of a TFA-treated sample of the above pentapeptide derivative (4.50 g, 4.72 mmol) in DMF (40 ml) containing Et_3N (0.66 ml, 4.72 mmol), and the mixture was stirred at 4 °C for 3 h, then concentrated. The product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 2.97 g (54%), mp 205—207 °C, $[\alpha]_D^{20}$ +2.8° (c=0.7, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{49}H_{69}Cl_3N_8O_{16}S \cdot H_2O$: C, 49.76; H, 6.05; N, 9.48, Found: C, 49.96; H, 5.94; N, 9.62.

Z(OMe)–Asp(OChp)–Tyr–Ser–Ile–Ala–Met(O)–$NHNH_2$, Z(OMe)–(hGIP 9—14)–$NHNH_2$ [6]——The above protected hexapeptide derivative (2.73 g, 2.34 mmol) was treated with Cd powder, and the product was isolated as described in the preparation of fragment [5]. However, DMSO was unnecessary in this case. The hydrazide thus obtained was precipitated from DMF with EtOH; yield 1.58 g (68%), mp 231—233 °C, $[\alpha]_D^{20}$ −2.8° (c=1.4, DMF), Rf_1 0.56. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Ser 0.93, Ala 1.06, Met+Met(O) 0.92, Ile 1.00, Tyr 0.96 (recovery of Ile 80%). *Anal.* Calcd for $C_{46}H_{68}N_8O_{14}S \cdot 3/2H_2O$: C, 54.37; H, 7.04; N, 11.03. Found: C, 54.32; H, 6.97; N, 11.06.

Z(OMe)–Glu(OChp)–Gly–NHNH–Troc——A mixture of a TFA-treated sample of Z(OMe)–Gly–NHNH–Troc[34] (4.37 g, 10.2 mmol), Et_3N (2.60 ml, 18.7 mmol) and Z(OMe)–Glu(OChp)–OSu (oil, 4.28 g, 8.49 mmol) in DMF (40 ml) was stirred at 4 °C overnight, then concentrated. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 4.33 g (65%), mp 62—64 °C, $[\alpha]_D^{20}$ −6.3° (c=0.5, MeOH), Rf_3 0.32. *Anal.* Calcd for $C_{26}H_{35}Cl_3N_4O_9$: C, 47.75; H, 5.39; N, 8.57. Found: C, 47.66; H, 5.60; N, 8.69.

Z(OMe)–Tyr–Ala–Glu(OChp)–Gly–NHNH–Troc——The azide [prepared from 1.42 g (3.30 mmol) of Z-(OMe)–Tyr–Ala–$NHNH_2$[9]] in DMF (15 ml) and Et_3N (0.46 ml, 3.30 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above dipeptide derivative (2.16 g, 3.30 mmol) in DMF (20 ml) containing Et_3N (0.46 ml, 3.30 mmol), and the mixture was stirred for 24 h, then concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and EtOH; yield 2.41 g (82%), mp 148—151 °C, $[\alpha]_D^{20}$ −14.2° (c=0.4, MeOH), Rf_1 0.73. *Anal.* Calcd for $C_{38}H_{49}Cl_3N_6O_{12} \cdot H_2O$: C, 50.36; H, 5.67; N, 9.27. Found: C, 50.56; H, 5.40; N, 9.41.

Z(OMe)–Tyr–Ala–Glu(OChp)–Gly–$NHNH_2$, Z(OMe)–(hGIP 1—4)–$NHNH_2$ [8]——The above protected tetrapeptide derivative (2.41 g, 2.72 mmol) was treated with Cd powder and the product was isolated as described in the preparation of fragment [5]. The hydrazide thus obtained was recrystallized from MeOH; yield 1.45 g (75%), mp 215—217 °C, $[\alpha]_D^{20}$ −8.2° (c=0.5, DMF), Rf_1 0.69. Amino acid ratios in 6 N HCl hydrolysate: Glu 1.00, Gly 1.00, Ala 1.07, Tyr 0.92 (recovery of Gly 100%). *Anal.* Calcd for $C_{35}H_{48}N_6O_{10}$: C, 58.97; H, 6.79; N, 11.79. Found: C, 58.69; H, 6.81; N, 11.56.

Synthesis of the Protected hGIP——Successive azide condensations of the eight fragments were carried out according to the Scheme (Fig. 2). Prior to condensation, the Z(OMe) group was removed from the amino component by TFA treatment as described in the general experimental procedure. A TFA-treated sample was precipitated with ether, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF, except for the condensation reaction of fragment [4]. In this case, DMF–DMSO (1 : 1) was employed. The solution was neutralized with Et_3N. The azide (1.5 to 4 eq according to the degree of chain elongation) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at 4 °C (for fragment condensations of [1], [2] and [3]) or −18 °C (from [4] to [8]) for 14 to 36 h, depending upon the conditions, until the solution became ninhydrin-negative. H_2O was added to the solution and the resulting powder was purified by either precipitation from DMF with MeOH (procedure B-1) or from DMSO with MeOH (procedure B-2), or by gel-filtration on Sephadex LH-60 (procedure C). In the latter case, DMF was used as an eluant and eluates were examined by measuring the UV absorption at 280 nm. The fractions corresponding to the front main peak were combined, the solvent was removed by evaporation and the residue was treated with AcOEt to form a powder. The purification procedure, physical constants and analytical data of the protected hGIP and its intermediates are listed in Table II.

H–Tyr–Ala–Glu–Gly–Thr–Phe–Ile–Ser–Asp–Tyr–Ser–Ile–Ala–Met–Asp–Lys–Ile–His–Gln–Gln–Asp–Phe–Val–Asn–Trp–Leu–Leu–Ala–Gln–Lys–Gly–Lys–Lys–Asn–Asp–Trp–Lys–His–Asn–Ile–Thr–Gln–OH (hGIP)——The protected hGIP (50 mg, 73.9 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (108 μl, 140 eq) and EDT (43 μl, 70 eq) in an ice-bath for 180 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O (5 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH and after 30 min, to 5.0 with 1 N AcOH. 2-Mercaptoethanol (207 μl, 400 eq) was added and the solution, after being incubated at 37 °C for 20 h, was applied to a column of Sephadex G-50 (2.3 × 142 cm), which was eluted with 1 N AcOH. The fractions (6 ml each, monitored at 280nm) corresponding to the front main peak (tube Nos. 41—64) were combined and the solvent was removed by lyophilization to give a powder; yield 36 mg (98%).

The crude product thus obtained (11 mg) was dissolved in 0.01 N AcOH (25 ml) and the solution, after being adjusted the pH to 6.4 with 5% NH_4OH, was applied to a column of CM-cellulose (0.9 × 14 cm), which was eluted with 0.2 M $AcONH_4$ (pH 6.4, 250 ml) through a mixing flask containing 0.01 M $AcONH_4$ (pH 6.4, 150 ml). The fractions (4 ml each, monitored at 280 nm) corresponding to the main peak (tube Nos. 29—38) were combined (Fig. 10). The solvent and the salt were removed by repeated lyophilization to give a white powder; yield 2.3 mg (21%). The rest of the sample was similarly purified; total yield 7.4 mg (20%).

Subsequent purification was performed by reversed-phase HPLC on a Nucleosil 5C18 column (10 × 250 mm). A part of the above CM-purified sample (2.0 mg) was dissolved in 0.1% TFA (200 μl) and the solution was applied to the column, which was eluted with a gradient of acetonitrile (27 to 37% in 1.5 h) in 0.1% TFA at a flow rate of 1.5 ml per min (Fig. 11-a). The eluate corresponding to the main peak (retention time 64.2 min) was collected and the solvent was removed by lyophilization to give a fluffy powder; yield 1.0 mg. The rest of the CM-purified sample was similarly

TABLE II. Characterization of the Protected hGIP and Its Intermediates

	Puri. proc. Yield (%)	Rf_1	mp (°C)	$[\alpha]_D^{20}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)–(32—42)–OBzl	B-1 87	0.72	243—245	−23.1	$C_{118}H_{152}N_{20}O_{29}S \cdot 2H_2O$	59.60 (59.48)	6.53 6.60	11.90 11.76)
Z(OMe)–(28—42)–OBzl	B-2 89	0.67	254—256	−18.5	$C_{142}H_{186}N_{26}O_{36}S \cdot 4H_2O$	57.91 (58.06)	6.63 6.66	12.39 12.40)
Z(OMe)–(23—42)–OBzl	C 77	0.64	128—130	−5.7	$C_{183}H_{243}N_{33}O_{44}S_2 \cdot 2H_2O$	59.16 (59.25)	6.66 6.71	12.30 12.46)
Z(OMe)–(15—42)–OBzl	C 78	0.69	135—137	+9.9	$C_{250}H_{346}N_{46}O_{60}S_2 \cdot 4H_2O$	58.85 (59.06)	6.79 6.86	12.73 12.68)
Z(OMe)–(9—42)–OBzl	C 74	0.58	138—140	≒0	$C_{287}H_{394}N_{52}O_{71}S_3 \cdot 7H_2O$	58.18 (58.12)	6.76 6.93	12.00 12.28)
Z(OMe)–(5—42)–OBzl	C 75	0.56	133—135	+20.7	$C_{309}H_{426}N_{56}O_{77}S_3 \cdot 9H_2O$	57.80 (57.85)	6.76 6.98	12.17 12.23)
Z(OMe)–(1—42)–OBzl	C 48	0.59	155—158	−21.0	$C_{335}H_{462}N_{60}O_{84}S_3 \cdot 12H_2O$	57.47 (57.59	6.78 7.01	11.80 12.03)

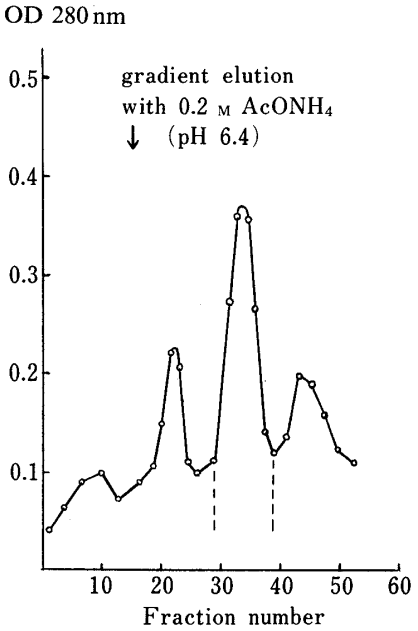

Fig. 10. CM-Cellulose Purification of Synthetic hGIP

purified; yield 3.7 mg. The overall yield from the protected hGIP was 10%. $[\alpha]_D^{15} -39.7°$ ($c=0.1$, 1 N AcOH), Rf_4 0.26, Rf_5 0.11. The retention time of the main peak [with two small side peaks due to Met(O)-derivative] was 30.5 min in HPLC on an analytical Nucleosil 5C18 column (4 × 150 mm) by gradient elution with CH_3CN (27% to 37%, 60 min) in 0.1% TFA at a flow rate of 0.6 ml per min (Fig. 11-b). A single band was seen in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 7.3 cm) containing Pharmalyte (pH 3.0—10.0): mobility 4.2 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin toward the cathodic end of the gel after running at 200 V for 4 h (Fig. 12). The amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in an LAP digest (numbers in parentheses are theoretical): Asp 3.76 (4), Thr 1.72 (2), Ser 1.57 (2), Glu 0.94 (1), Gly 2.00 (2), Ala 3.39 (3), Val 1.19 (1), Met 0.76 (1), Ile 3.88 (4), Leu 2.20 (2), Tyr 1.79 (2), Phe 2.03 (2), Trp 1.91 (2), Lys 5.17 (5), His 2.01 (2) (recovery of Gly 78%, Gln and Asn were not determined).

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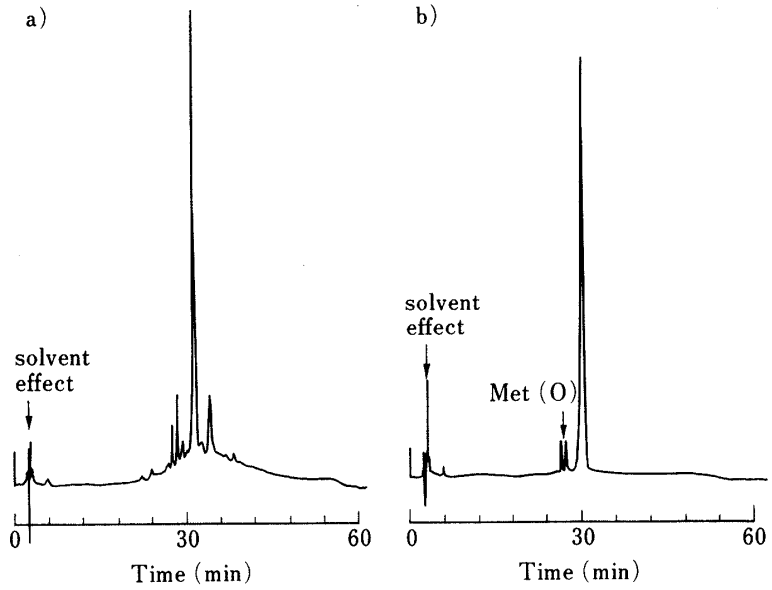

Fig. 11. HPLC of Synthetic hGIP

a) CM-purified sample. b) Purified synthetic hGIP.

Fig. 12. Disk Isoelectrofocusing of Synthetic hGIP

References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. The following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, Mts=mesitylene-2-sulfonyl, Bzl=benzyl, *p*NB=*p*-nitrobenzyl, Chp=cycloheptyl, Troc=2,2,2-trichloroethyloxycarbonyl, DCC=dicyclohexylcarbodiimide, Su=*N*-hydroxysuccinimidyl, Np=*p*-nitrophenyl, HOBt=*N*-hydroxybenzotriazole, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, TFMSA=trifluoromethanesulfonic acid, EDT=ethanedithiol, DCHA=dicyclohexylamine, NMM=*N*-methylmorpholine, EDTA=ethylenediaminetetraacetic acid disodium salt, DMF=dimethylformamide, THF=tetrahydrofuran, DMSO=dimethylsulfoxide, HMPA=hexamethylphosphoramide, DMAP=4-dimethylaminopyridine.
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