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

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The dotetracontapeptide corresponding to the revised amino acid sequence of porcine GIP (glucose-dependent insulinotropic polypeptide; originally named gastric inhibitory polypeptide) was synthesized by assembling eight peptide fragments of established purity, followed by thioanisole-mediated deprotection with trifluoromethanesulfonic acid in trifluoroacetic acid. The cycloheptyl esters of aspartic acid and glutamic acid were employed to suppress base-catalyzed side reactions. When tested in dogs, the synthetic peptide produced a significant increase of immunoreactive insulin in blood under background infusion of glucose.

Keywords——β-cycloheptylaspartate; γ-cycloheptylglutamate; N^{G}-mesitylenesulfonylarginine; N^{in}-mesitylenesulfonyltryptophan; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; pyrrolidone formation; succinimide formation; insulinotropic activity; gastric inhibition

The structure of porcine GIP (pGIP, glucose-dependent insulinotropic polypeptide; originally named gastric inhibitory polypeptide), proposed first by Brown and Dryburgh[3] in 1971, was reinvestigated by Jörnvall *et al.*[4] in 1981 and one internal Gln residue was eliminated from the earlier formula.

Following the synthesis of human GIP (hGIP),[5] we wish to report the synthesis of a 42 residue peptide corresponding to the revised amino acid sequence of pGIP. As in the case of the synthesis of hGIP, the recently introduced ω-cycloheptyl esters of Asp and Glu, Asp(OChp)[6] and Glu(OChp),[5] were employed to suppress base-catalyzed side reactions, *i.e.*, succinimide formation[7] and pyrrolidone formation[8] respectively. pGIP differs from hGIP in the replacement of two amino acid residues: His (position 18) and Asn (position 34) in hGIP are replaced by Arg and Ser in pGIP, respectively. Thus, of eight fragments used for the synthesis of hGIP, two fragments, [1] and [5] which cover the areas of species variation, were newly synthesized (Fig. 1).

Fragment [1], Z(OMe)–Ser–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl (positions 34—42), was easily prepared by the azide condensation[9] of Z(OMe)–Ser–$NHNH_2$ with the available amino octapeptide component used in our previous synthesis of fragment [1] of hGIP.[5] Fragment [5], Z(OMe)–Asp(OChp)–Lys(Z)–Ile–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–$NHNH_2$ (positions 15—22), was prepared according to the Scheme shown in Fig. 2. Starting from a TFA-treated sample of the available protected tetrapeptide derivative, Z(OMe)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc,[5] the respective amino acids were introduced in a stepwise manner by the mixed anhydride procedure[10] or an active ester procedure, such as the Np[11] or the Su procedure.[12] From the resulting protected octapeptide

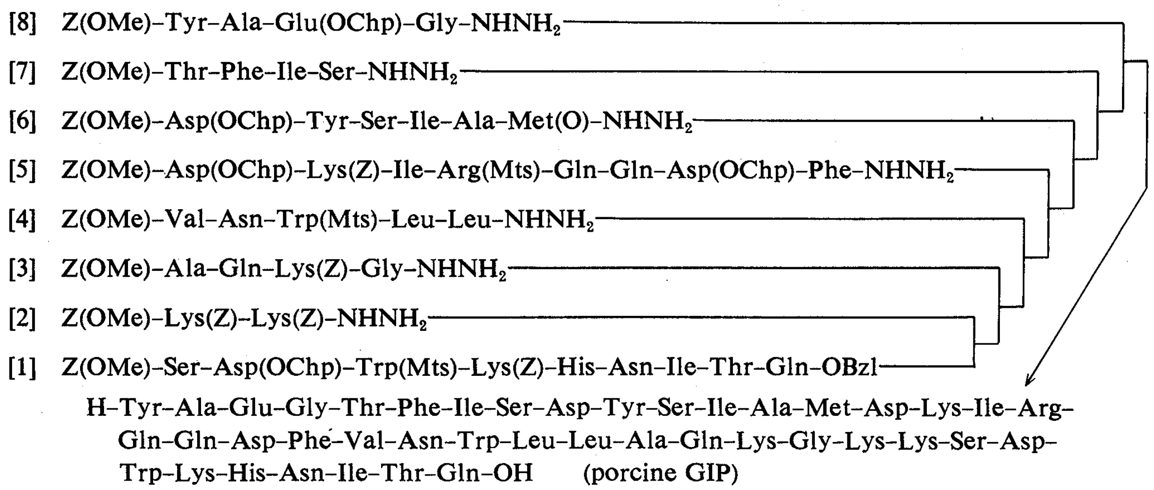

Fig. 1. Synthetic Route to Porcine GIP

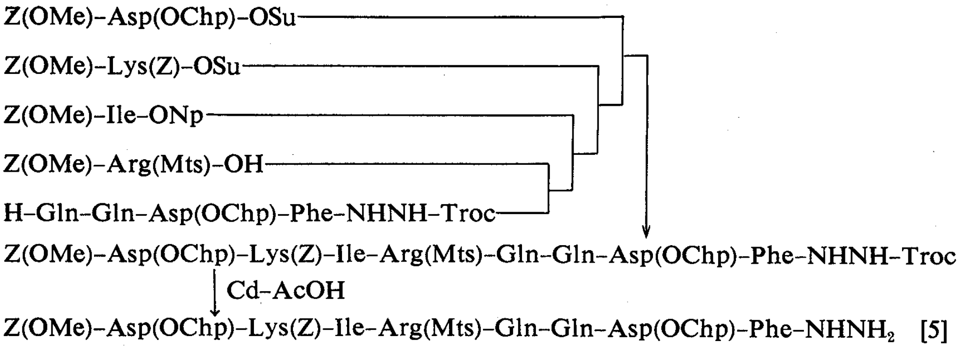

Fig. 2. Synthetic Scheme for the Protected Octapeptide Hydrazide, Z(OMe)-(pGIP 15—22)-NHNH$_2$

derivative, the Troc group was removed by treatment with Cd.[13] The purities of fragments [1] and [5] were ascertained by thin layer chromatography (TLC), elemental analysis, and amino acid analysis after acid hydrolysis.

The two fragments, [1] and [5] prepared as stated above, and the available six fragments were then assembled successively as shown in Fig. 1 by the azide procedure to minimize racemization. The amount of an acyl component employed in the condensation was increased from 1.5 to 4 equivalents as elongation of the chain progressed. The condensations from [1] to [3] were performed as usual, then the subsequent azide condensations of fragment [4] to [8] were performed at a lower temperature (−16 °C) than usual (4 °C) in order to minimize the possible Curtius rearrangement.[14] Each protected product was purified either by precipitation from DMF with MeOH or by gel-filtration on Sephadex LH-60 using DMF as an eluant.

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

In the final step, the protected pGIP was treated with 1 M TFMSA–thioanisole in TFA[15] in the presence of *m*-cresol and EDT to remove all protecting groups employed, except Met(O). Met(O) is known to be partially reduced in this thioanisole-mediated process.[16] The deprotected peptide was purified in the same manner as described in the final step of the hGIP synthesis: 1. Treatment with dil. ammonia to reverse any possible N→O shift.[17] 2. Incubation

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic pGIP and Its Intermediates

	Protected peptides							Syn. pGIP	Residue
	32—42	28—42	23—42	15—42	9—42	5—42	1—42		
Asp	1.72	1.80	2.27	4.28	5.96	5.21	5.85	5.42	(6)
Thr	1.00	1.00	1.00	1.00	1.00	2.00	2.00	2.00	(2)
Ser	0.96	1.00	1.00	1.01	2.13	2.81	3.02	3.00	(3)
Glu	1.07	2.31	2.11	4.22	4.66	3.97	5.51	5.25	(5)
Gly		1.23	1.08	1.12	1.15	0.93	2.20	2.21	(2)
Ala		1.29	1.08	1.20	2.34	2.01	3.58	3.16	(3)
Val			0.85	0.85	0.95	0.88	1.10	1.05	(1)
Met					1.06	0.95	0.60	0.98	(1)[b]
Ile	1.00	0.99	0.97	1.97	2.97	4.10	4.29	3.95	(4)
Leu			1.88	2.26	2.25	1.85	2.22	2.05	(2)
Tyr					1.09	1.09	2.17	2.05	(2)
Phe				0.94	1.08	2.27	2.31	2.03	(2)
Trp	0.85	0.99	2.31	1.96	1.69	1.92	2.18	N.D.	(2)[a]
Lys	2.82	4.12	4.16	4.97	5.36	4.30	5.20	5.08	(5)
His	1.00	1.02	1.08	1.02	1.00	0.87	0.98	1.03	(1)
Arg				0.94	0.95	1.04	1.11	0.91	(1)
Rec. %	83	86	86	85	78	81	76	82	

a) 4 N CH_3SO_3H hydrolysate. *b*) Met+Met(O).

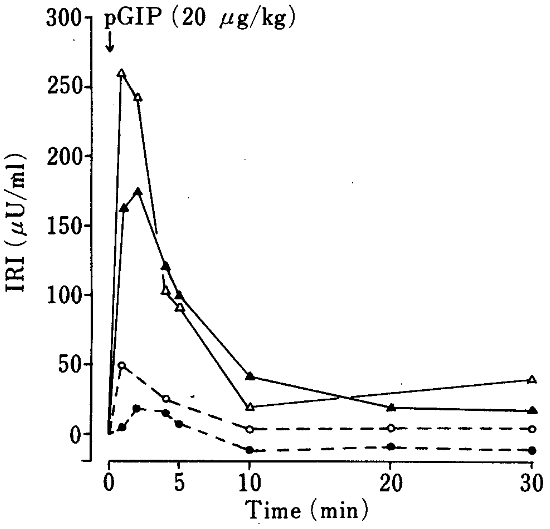

Fig. 3. Insulinotropic Activity of Synthetic pGIP

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

with 2-mercaptoethanol to ensure the complete reduction of Met(O). 3. Gel-filtration on Sephadex G-50. 4. Ion exchange chromatography on CM-cellulose. 5. High performance liquid chromatography (HPLC) on Cosmosil 5C18. The purified peptide exhibited a sharp single spot on TLC in two different solvent systems and a single band in disk isoelectrofocusing (Pharmalyte pH 3—10). Its purity was further confirmed by amino acid analysis after acid hydrolysis and leucine aminopeptidase (LAP) digestion.

Our synthetic pGIP (20 μg/kg) induced a rapid and significant increase of immunoreactive insulin (IRI) in both peripheral and portal blood in dogs under background infusion of glucose (8 g/h) as shown in Fig. 3, and its potency was judged to be higher than that of synthetic hGIP.[5)] However, like hGIP, our synthetic pGIP (dose 4—64 μg/kg) showed no significant inhibition of gastric acid secretion stimulated by pentagastrin (1.5 μg/kg) in rats.

Recently, Kumagaya *et al.*[18)] reported solution syntheses of hGIP and pGIP, conducted by the HF procedure.[19] Shortly after Jörnvall's publication,[4] we synthesized pGIP using Asp(OBzl). A LAP hydrolysate of synthetic pGIP obtained after TFMSA–thioanisole deprotection gave a low recovery of Asp (*ca.* 77%), indicating contamination with the succinimide derivative. In the present synthesis, a satisfactory recovery of Asp (96%) was obtained in the LAP digest. We conclude that an unequivocal synthesis of pGIP, as well as hGIP, was thus achieved by the use of Asp(OChp).

Experimental

General experimental methods employed here are essentially the same as described in our hGIP synthesis.[5] HPLC was conducted with a Waters 204 compact model. 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 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2) and Rf_3 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1).

Z(OMe)–Ser–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl [1]——The azide [prepared from 0.52 g (1.83 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (5 ml) and Et_3N (0.25 ml, 1.83 mmol) were added to an ice-chilled solution of a TFA-treated sample of Boc–Asp(OChp)–Trp(Mts)–Lys(Z)–His–Asn–Ile–Thr–Gln–OBzl[5] (2.00 g, 1.22 mmol) in DMF (20 ml) containing Et_3N (0.34 ml, 2.44 mmol) and the mixture was stirred at 4 °C for 14 h, then concentrated. H_2O (200 ml) was added and the resulting powder was precipitated from DMF with MeOH; yield 1.68 g (80%), mp 224—226 °C, $[\alpha]_D^{15}$ −12.7 ° (*c* = 0.4, DMF). Rf_1 0.53. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.65, Thr 1.00, Ser 0.81, Glu 1.08, Ile 1.01, Lys 1.08, His 1.05 (recovery of Thr 78%). *Anal.* Calcd for $C_{89}H_{115}N_{15}O_{23}S \cdot H_2O$: C, 58.96; H, 6.50; N, 11.59. Found: C, 58.79; H, 6.42; N, 11.46.

Z(OMe)–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixed anhydride [prepared from 4.50 g (8.64 mmol) of Z(OMe)–Arg(Mts)–OH] in THF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc[5] (4.26 g, 4.32 mmol) in DMF (40 ml) containing Et_3N (0.60 ml, 4.32 mmol) and the mixture was stirred at 4 °C for 14 h, then concentrated. Treatment of the residue with H_2O afforded a powder, which was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and precipitated from DMF with MeOH; yield 4.39 g (77%), mp 198—200 °C, $[\alpha]_D^{15}$ −12.7 ° (*c* = 0.7, DMF). Rf_1 0.57. *Anal.* Calcd for $C_{57}H_{77}Cl_3N_{12}O_{16}S$: C, 51.68; H, 5.86; N, 12.69. Found: C, 51.92; H, 6.07; N, 12.93.

Z(OMe)–Ile–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above pentapeptide derivative (4.20 g, 3.17 mmol), Et_3N (1.10 ml, 7.93 mmol), HOBT (43.2 mg, 0.32 mmol) and Z(OMe)–Ile–ONp (1.98 g, 4.76 mmol) in DMF (20 ml) was stirred at room temperature for 14 h and concentrated. Treatment of the residue with H_2O (200 ml) afforded a powder, which was washed as stated above and precipitated from DMF with MeOH; yield 3.94 g (86%), mp 225—228 °C, $[\alpha]_D^{15}$ −17.2 ° (*c* = 0.6, DMF). Rf_1 0.57. *Anal.* Calcd for $C_{63}H_{88}Cl_3N_{13}O_{17}S$: C, 52.62; H, 6.17; N, 12.66. Found: C, 52.84; H, 6.22; N, 12.68.

Z(OMe)–Lys(Z)–Ile–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above hexapeptide derivative (3.80 g, 2.64 mmol), Et_3N (0.92 ml, 6.60 mmol) and Z(OMe)–Lys(Z)–OSu (2.14 g, 3.96 mmol) in DMF (30 ml) was stirred at room temperature for 14 h and concentrated. The product was purified as stated above; yield 3.26 g (73%), mp 229—232 °C, $[\alpha]_D^{15}$ −16.8 ° (*c* = 0.4, DMF). Rf_1 0.48. *Anal.* Calcd for $C_{77}H_{106}Cl_3N_{15}O_{20}S$: C, 54.39; H, 6.28; N, 12.36. Found: C, 54.61; H, 6.40; N, 12.50.

Z(OMe)–Asp(OChp)–Lys(Z)–Ile–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–NHNH–Troc——A mixture of a TFA-treated sample of the above heptapeptide derivative (3.00 g, 1.76 mmol), Et_3N (0.61 ml, 4.40 mmol) and Z(OMe)–Asp(OChp)–OSu (1.29 g, 2.64 mmol) in DMF (30 ml) was stirred at room temperature for 14 h and concentrated. The product was purified as stated above; yield 2.81 g (84%), mp 233—236 °C, $[\alpha]_D^{15}$ −13.4 ° (*c* = 0.4, DMF). Rf_1 0.55. *Anal.* Calcd for $C_{88}H_{123}Cl_3N_{16}O_{23}S \cdot H_2O$: C, 54.78; H, 6.53; N, 11.62. Found: C, 54.75; H, 6.59; N, 11.66.

Z(OMe)–Asp(OChp)–Lys(Z)–Ile–Arg(Mts)–Gln–Gln–Asp(OChp)–Phe–$NHNH_2$ [5]——The above protected octapeptide derivative (2.50 g, 1.31 mmol) dissolved in DMF–HMPA–AcOH (10 ml–5 ml–3 ml) was treated with Cd powder (2.94 g, 20 eq) at room temperature overnight. The solid product formed during the treatment was dissolved by addition of DMSO (20 ml) and the solution was filtered to remove Cd powder. The filtrate was concentrated and the residue was treated with H_2O. The resulting powder was washed with 5% EDTA, 5% $NaHCO_3$ and H_2O and precipitated from DMSO with MeOH; yield 1.96 g (86%), mp 252—255 °C, $[\alpha]_D^{15}$ −28.3 ° (*c* = 0.5, DMSO). Rf_1 0.56. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.99, Glu 2.01, Ile 0.91, Phe 1.00, Lys 0.99, Arg 0.96 (recovery of Phe 85%). *Anal.* Calcd for $C_{85}H_{122}N_{16}O_{21}S \cdot H_2O$: C, 58.20; H, 7.12; N, 12.78. Found: C, 58.23; H, 6.91; N, 12.53.

Synthesis of the Protected pGIP——Successive azide condensations of eight fragments were carried out according to the Scheme (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 1 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and

TABLE II. Characterization of the Protected pGIP and Its Intermediates

	Puri. Proc. (Yield %)	Rf_1	mp (°C)	$[\alpha]_D^{15}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(32—42)-OBzl	A (83)	0.45	235—238	−16.5°	$C_{117}H_{151}N_{19}O_{29}S \cdot H_2O$	60.11 (60.11	6.74 6.60	11.42 11.39)
Z(OMe)-(28—42)-OBzl	A (79)	0.63	235—238	−22.1°	$C_{141}H_{185}N_{25}O_{36}S \cdot 3H_2O$	58.31 (58.55	6.53 6.66	12.37 12.11)
Z(OMe)-(23—42)-OBzl	B (55)	0.67	126—129	−58.5°	$C_{182}H_{242}N_{32}O_{44}S_2 \cdot 4H_2O$	58.88 (58.79	6.73 6.78	12.13 12.06)
Z(OMe)-(15—42)-OBzl	B (49)	0.47	123—127	−11.4°	$C_{258}H_{352}N_{46}O_{62}S_3 \cdot 6H_2O$	58.31 (58.53	6.80 6.93	12.32 12.17)
Z(OMe)-(9—42)-OBzl	B (65)	0.55	131—135	−17.0°	$C_{295}H_{408}N_{52}O_{73}S_4 \cdot 10H_2O$	57.36 (57.52	6.73 7.00	11.54 11.83)
Z(OMe)-(5—42)-OBzl	B (70)	0.61	133—138	−13.5°	$C_{317}H_{440}N_{56}O_{79}S_4$	58.99 (59.23	6.90 6.90	12.26 12.20)
Z(OMe)-(1—42)-OBzl	B (61)	0.63	137—140	−15.8°	$C_{343}H_{476}N_{60}O_{86}S_4 \cdot 6H_2O$	58.53 (58.41	6.90 6.98	11.87 11.92)

A, precipitation from DMF with MeOH. B, gel-filtration on Sephadex LH-60.

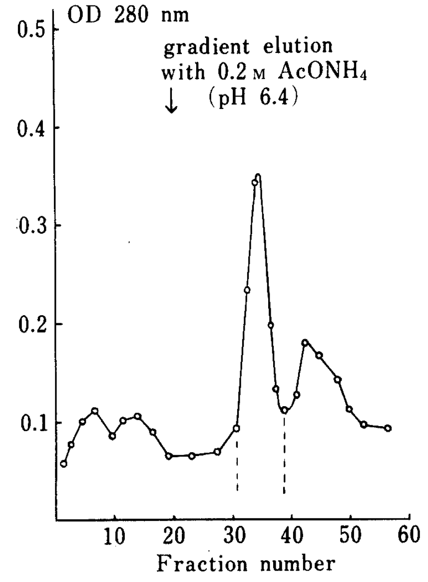

Fig. 4. Ion-Exchange Chromatography of Synthetic pGIP on CM-Cellulose

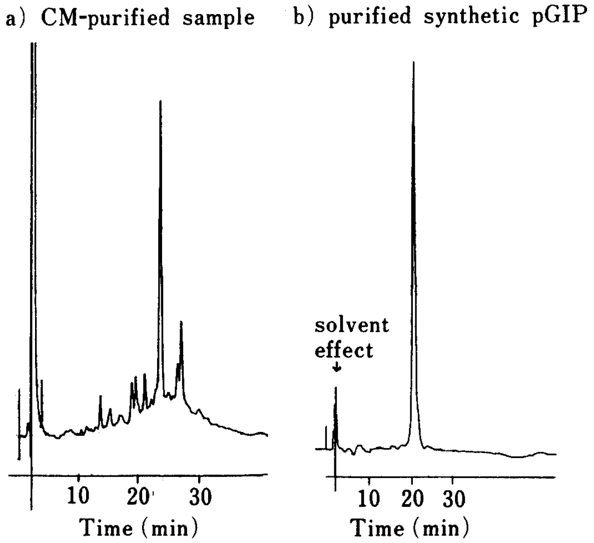

Fig. 5. HPLC of Synthetic pGIP

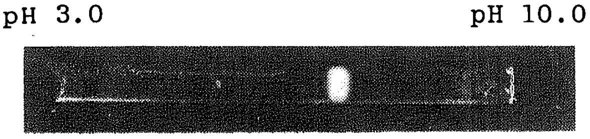

Fig. 6. Disk Isoelectrofocusing of Synthetic pGIP

dissolved in DMF containing Et_3N (1 eq). The corresponding azide (the amount was increased from 1.5 to 4 eq as chain elongation progressed) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at 4 °C (condensations from [1] to [3]) or −16 °C (condensations from [4] to [8]) as described in the text, until the solution became negative to the ninhydrin test. H_2O was added and the resulting powder was purified by either one of the following procedures. A: Precipitation from DMF with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF as an eluant. In procedure B, eluates (10 ml each) were examined by measuring the ultraviolet (UV) absorption at 280 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to form a powder. Purification procedure, yield, physical constants and analytical data of the protected pGIP 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-Arg-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln-Lys-Gly-Lys-Lys-Ser-Asp-Trp-Lys-His-Asn-Ile-Thr-Gln-OH (pGIP)——The protected pGIP (42 mg) was treated with 1 M TFMSA–thioanisole in TFA (2.5 ml) in the presence of *m*-cresol (96 μl, 150 eq) and EDT (38 μl, 75 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 (171 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 (4 ml each, monitored at 280 nm) corresponding to the front main peak (tube Nos. 52—76) were combined and the solvent was removed by lyophilization to give a powder; yield 32 mg (= 100%). The crude product thus obtained (10 mg) was dissolved in 0.01 N AcOH (25 ml) and the solution, after being adjusted to pH 6.4 with 5% NH_4OH, was applied to a column of CM-52 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 $AcONH_4$ (150 ml). The fractions (4 ml each, monitored at 280 nm) corresponding to the main peak (tube Nos. 31—39) were combined (Fig. 4). The solvent and the salt were removed by repeated lyophilization to give a white powder; yield 3.0 mg. The rest of the sample was similarly purified; total yield 8.9 mg (29%).

Subsequent purification was performed by reversed-phase HPLC on a Cosmosil 5C18 column (10 × 250 mm). A part of the above CM-purified sample (3.0 mg) was dissolved in 0.1% TFA aq (300 μl) and the solution was applied to a column, which was eluted with a gradient of CH_3CN (28% to 35% in 2 h) in 0.1% TFA aq. at a flow rate of 1.5 ml per min (Fig. 5a). The eluate corresponding to the main peak (retention time 57 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 0.67 mg. The rest of the CM-purified sample was similarly purified; total yield 2.02 mg. Overall yield from the protected pGIP was 6.7%. $[\alpha]_D^{17}$ −49.9° (c = 0.04, 0.1 N AcOH). Rf_2 0.22, Rf_3 0.28; retention time, 21 min in HPLC on an analytical Nucleosil 5C18 column (4 × 150 mm) by gradient elution with CH_3CN (28% to 35%, 30 min) in 0.1% TFA aq. at a flow rate of 0.8 ml per min (Fig. 5b); a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 6.0 cm) containing Pharmalyte (pH 3.0—10.0): mobility 3.8 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin to ward the cathodic end of the gel, after running at 200 V for 4 h (Fig. 6). 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 3.82 (4), Thr 2.00 (2), Ser 3.00 (3), Glu 1.12 (1), Gly 2.00 (2), Ala 3.04 (3), Val 1.10 (1), Met 0.91 (1), Ile 3.92 (4), Leu 1.94 (2), Tyr 1.88 (2), Phe 2.14 (2), Lys 4.70 (5), His 0.94 (1), Arg 1.01 (1), Trp 1.87 (2), Asn (2) and Gln (4) were not determined (recovery of Thr 76%).

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References and Notes

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2) Amino acids used in this study are of the L-configuration. The following abbreviations were used: Z = benzyloxycarbonyl, Bzl = benzyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylenesulfonyl, Boc = *tert*-butoxycarbonyl, Troc = 2,2,2-trichloroethyloxycarbonyl, Su = *N*-hydroxysuccinimidyl, Np = *p*-nitrophenyl, HOBT = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, EDT = ethanedithiol, EDTA = ethylenediaminetetraacetic acid disodium salt, DMF = dimethylformamide, THF = tetrahydrofuran, DMSO = dimethylsulfoxide, HMPA = hexamethylphosphoramide, CM = carboxymethyl.
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