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Synthesis of the Docosapeptide corresponding to the Amino Acid Sequence of Salmon Corticotropin-like Intermediate Lobe Peptide(CLIP)[1]

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The docosapeptide H–Arg–Pro–Val–Lys–Val–Tyr–Thr–Asn–Gly–Val–Glu–Glu–Gln–Ser–Ser–Glu–Gly–Phe–Pro–Ser–Glu–Met–OH, corresponding to the amino acid sequence of salmon corticotropin-like intermediate lobe peptide (CLIP), was synthesized, with the use of 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid as a deprotecting reagent.

Keywords——salmon pituitary peptide; salmon corticotropin-like intermediate lobe peptide; trifluoromethanesulfonic acid–thioanisole deprotection; *m*-cresol as a scavenger; mesitylene-2-sulfonylarginine; oxidation of Met with *N*-chlorosuccinimide; HPLC of synthetic salmon CLIP

In 1973, Scott and Lowry *et al.* demonstrated the presence of a docosapeptide corresponding to positions 18 to 39 of adrenocorticotropin (ACTH) in the pars intermediate lobe of pig pituitary,[2] and later in dogfish,[3] and suggested that this peptide (termed corticotropin-like intermediate lobe peptide, CLIP) is formed together with α-melanocyte-stimulating hormone (α-MSH) by intracellular cleavage of a common precursor, ACTH.

In 1979, Kawauchi *et al.*[4] isolated a similar docosapeptide (CLIP) from the pituitary of salmon (*Oncorhynchus keta*). This CLIP differs from the avian[5] and mammalian[2,6] variants by replacement of several amino acid residues (from six to nine residues), and from dogfish CLIP[3] by twelve residues.

Following the synthesis[7] of salmon α-MSH II,[8] we wish to report the synthesis of salmon CLIP. As shown in Fig. 1, we selected three fragments to construct the entire sequence of salmon CLIP, *i.e.*, Z–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–$NHNH_2$ (III), Z(OMe)–Thr–Asn–Gly–OH (II) and Z(OMe)–Val–Glu(OBzl)–Glu(OBzl)–Gln–Ser–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl (I). In the present sythesis, trifluoromethanesulfonic acid (TFMSA)–thioanisole in TFA[9] was adopted as a deprotecting reagnt in the final step of the

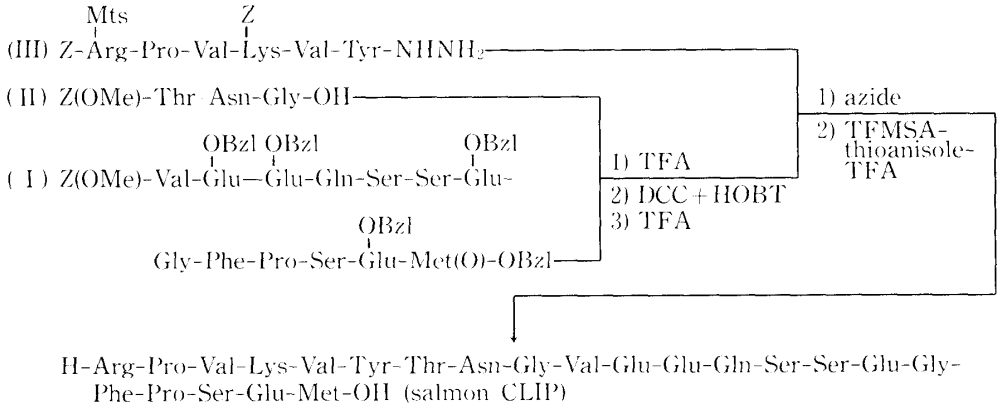

Fig. 1. Synthetic Route to Salmon CLIP

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synthesis. Thus, amino acid derivatives bearing protecting groups removable by the combination of these two reagents were adopted; *i.e.*, Arg(Mts),[10] Lys(Z) and Glu(OBzl). The α-amino function of intermediates was protected by the TFA-labile Z(OMe) group.[11] In addition, the Met residue was protected as its sulfoxide[12] to prevent partial oxidation during the synthesis.

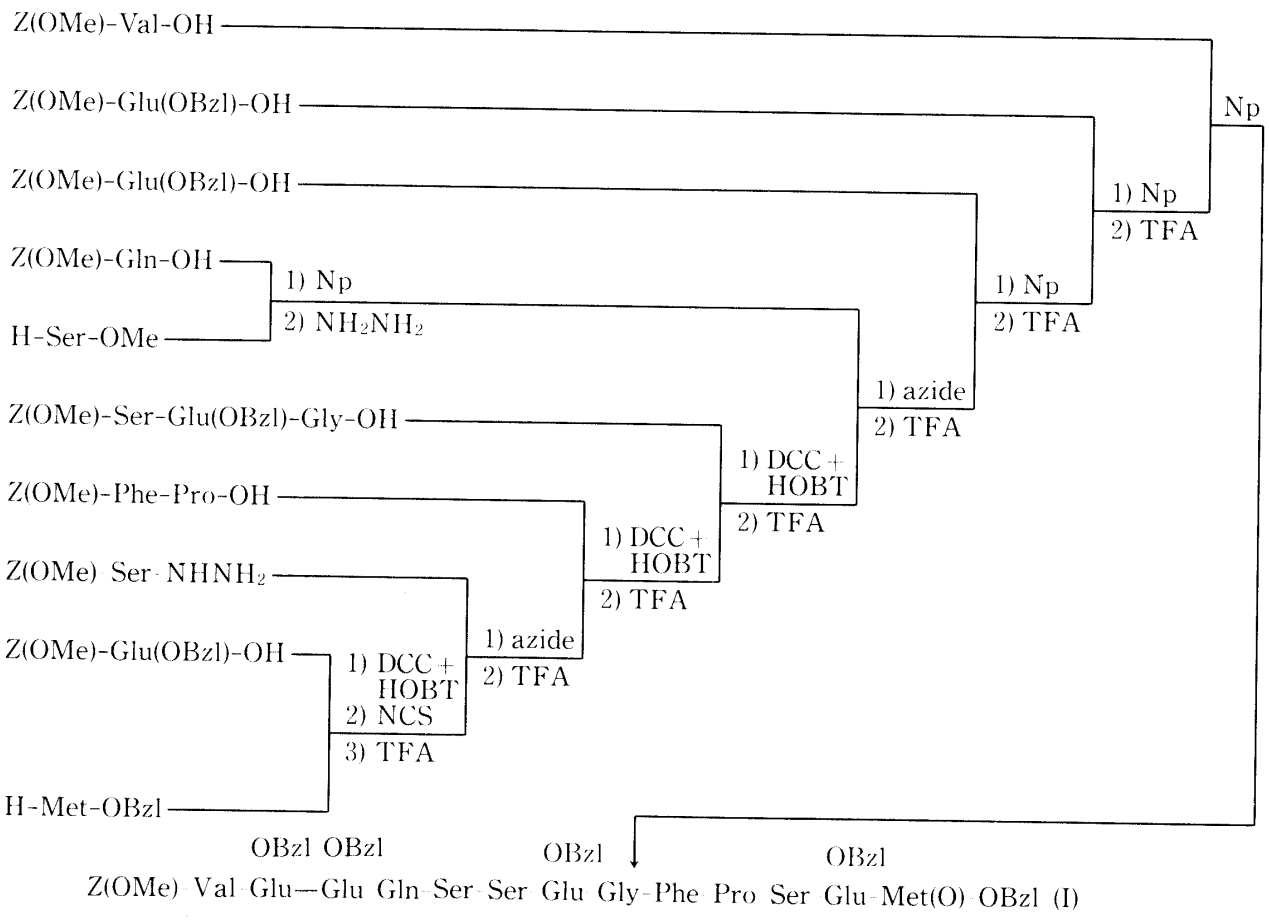

Fig. 2. Synthetic Route to the C-Terminal Tridecapeptide Ester (I)

The synthetic route to the C-terminal protected tridecapeptide ester (I) is illustrated in Fig. 2. Z(OMe)–Glu(OBzl)–Met–OBzl was first prepared by the DCC/HOBT procedure[13] and subsequently oxidized with *N*-chlorosuccinimide[14] to give the sulfoxide, Z(OMe)–Glu(OBzl)–Met(O)–OBzl, in nearly quantitative yield. This, after the usual TFA treatment, was condensed with Z(OMe)–Ser–$NHNH_2$ by the modified azide procedure[15] to afford the protected tripeptide ester, Z(OMe)–Ser–Glu(OBzl)-Met(O)–OBzl. A TFA-treated sample of this protected tripeptide ester was then condensed successively with Z(OMe)–Phe–Pro–OH[16] and Z(OMe)–Ser–Glu(OBzl)–Gly–OH[16] by the DCC/HOBT procedure to form the protected octapeptide ester, Z(OMe)–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl. Next, Z(OMe)–Gln–Ser–$NHNH_2$ prepared by the Np method,[17] followed by the usual hydrazine treatment, was condensed with the TFA-treated sample of the above protected octapeptide ester to give the protected decapeptide ester, Z(OMe)–Gln–Ser–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl. Next, the Np method was employed to introduce stepwise two residues of Z(OMe)–Glu(OBzl)–OH, and one residue of Z(OMe)–Val–OH to afford the desired fragment (I). All intermediates and the fragment (I) were purified by either recrystallization or precipitation from appropriate solvents.

The next fragment, Z(OMe)–Thr–Asn–Gly–OH (II), was prepared by the modified azide

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condensation of Z(OMe)–Thr–$NHNH_2$ with H–Asn–Gly–OH[18] without particular difficulty.

The synthetic route to the *N*-terminal protected hexapeptide hydrazide (III) is illustrated in Fig. 3. Z(OMe)–Val–Tyr–OMe was first prepared by the DCC/HOBT procedure, and after the usual TFA treatment, condensed successively with Z(OMe)–Val–Lys(Z)–$NHNH_2$[19] and Z–Arg(Mts)–Pro–OH[16] by the modified azide procedure and the DCC/HOBT method respectively. The resulting protected hexapeptide ester was converted to III by the usual hydrazine treatment.

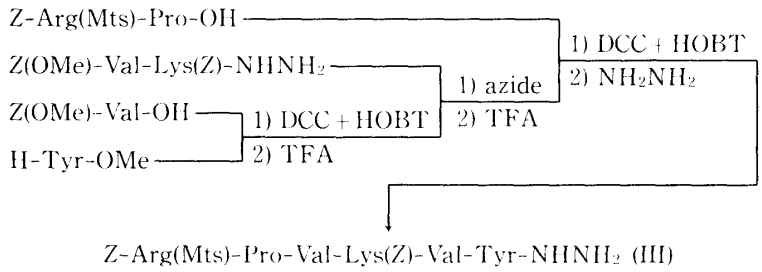

Fig. 3. Synthetic Route to the N-Terminal Hexapeptide Hydrazide (III)

The three fragments thus obtained were then assembled according to the route illustrated in Fig. 1. For the condensation of II and I, the Z(OMe) group of the latter was cleaved by the usual TFA treatment and the resulting TFA salt was converted to the free base with Et_3N. This N^{α}-deprotected tridecapeptide ester was coupled with II by the DCC/HOBT procedure to give protected hexadecapeptide ester, Z(OMe)–Thr–Asn–Gly–Val–Glu(OBzl)–Glu(OBzl)–Gln–Ser–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl, which was purified by precipitation from DMF with ether. This protected hexadecapeptide ester, after the usual TFA treatment, was then condensed with III by the modified azide procedure. The resulting protected docosapeptide ester, Z–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–Thr–Asn–Gly–Val–Glu(OBzl)–Glu(OBzl)–Gln–Ser–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl, was purified by column chromatography on silica and its homogeneity was confirmed by three criteria; *i.e.*, thin-layer chromatography (TLC), elemental analysis and amino acid analysis after hydrolysis with 4 N MSA.[20]

Finally, the protected docosapeptide ester obtained above was treated with 1 M TFMSA–thioanisole in TFA in an ice-bath for 2 h to remove all protecting groups. *m*-Cresol was employed as an additional scavenger to suppress a possible side reaction at the Tyr residue, *i.e.*, *O*-mesitylene-2-sulfonylation.[10] TLC examination revealed that most of the Met(O) residue was reduced back to Met by this acid treatment with thioanisole. The deprotected peptide, after conversion to the corresponding acetate with Amberlite IRA-400 (acetate form), was purified by ion-exchange chromatography on DEAE-cellulose using gradient elution with 0.1 M ammonium acetate buffer, pH 7.0. The DEAE-cellulose-purified sample was next incubated with 2-mercaptoethanol at 50°C for 15 h in order to ensure the complete reduction of the Met(O) residue. The reduced sample was further purified by gel-filtration on Sephadex G-25 and finally lyophilized to give a fluffy powder. The absorbancy at 275 nm due to the Tyr residue was used to monitor these purifications. The homogeneity of the synthetic salmon CLIP thus obtained was assessed by TLC, elmental analysis, amino acid analysis after 4 N MSA hydrolysis, and further high-performance liquid chromatography (HPLC). Despite the presence of the Pro residues,[21] complete digestion of this synthetic peptide with commercial aminopeptidase (AP-M)[22] was achieved and the presence of Asn and Gln residues in the product was thus confirmed.

The physiological role of CLIP is not clear at the present time. Our synthetic salmon CLIP may be useful for studies of its biological function.

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Experimental

Melting points are uncorrected. Rotations were measured with a Union automatic polarimeter, model P-101 (cell length: 1 cm). TLC was performed on silica gel (Kieselgel 60 F 254, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (29: 1), Rf_2 $CHCl_3$–MeOH (9: 1), Rf_3 $CHCl_3$–MeOH–H_2O (18: 3: 1, lower phase), Rf_4 $CHCl_3$–MeOH–H_2O (8: 3: 1, lower phase), Rf_5 *n*-butanol–AcOH–pyridine–H_2O (4: 1: 1: 2), and Rf_6 *n*-butanol–AcOH–pyridine–H_2O (5: 1: 5: 4). High-performance liquid chromatography (HPLC) was conducted on a Waters Associates liquid chromatograph with a Cosmosil (5C18) (4.6 × 150 mm) column using 0.02 M, pH 3.0 triethylammonium phosphate–CH_3CN (80: 20) as the eluent (HPLC conditions: flow rate, 1.0 ml/min; detection, ultraviolet (UV) at 275 nm).

Z(OMe)-Glu(OBzl)-Met-OBzl——Z(OMe)–Glu(OBzl)–OH (20.1 g), DCC (10.3 g), and HOBT (7.3 g) were added to a solution of H–Met–OBzl (prepared from 20.6 g of the tosylate and 7.0 ml of Et_3N) in THF (100 ml) under cooling with ice. The mixture was stirred in an ice-bath for 2 h and at room temperature for 20 h, then filtered. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The organic solution was washed with 0.5 M citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residual solid was recrystallized from AcOEt and petroleum ether; yield 25.8 g (83%), mp 88—89°C, $[\alpha]_D^{24}$ −12.8° (c=0.78, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{33}H_{38}N_2O_8S$: C, 63.65; H, 6.15; N, 4.50. Found: C, 63.44; H, 6.12; N, 4.54.

Z(OMe)-Glu(OBzl)-Met(O)-OBzl——A mixture of Z(OMe)–Glu(OBzl)–Met–OBzl (23.0 g) in AcOEt (250 ml) and *N*-chlorosuccinimide (5.4 g) in H_2O (250 ml) was stirred efficiently under cooling with ice for 30 min; after this time, the spot corresponding to the starting material had disappeared on TLC. The organic phase was separated, washed with H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residual solid was recrystallized from AcOEt and petroleum ether; yield 22.3 g (95%), mp 104—106°C, $[\alpha]_D^{24}$ −8.66° (c=1.16, DMF), Rf_1 0.27. *Anal.* Calcd for $C_{33}H_{38}N_2O_9S$: C, 62.05; H, 6.00; N, 4.39. Found: C, 61.88; H, 5.95; N, 4.26.

Z(OMe)-Ser-Glu(OBzl)-Met(O)-OBzl——Z(OMe)–Glu(OBzl)–Met(O)–OBzl (25.0 g) was treated with TFA–anisole (50 ml–12 ml) in an ice-bath for 60 min, then the excess TFA was evaporated off *in vacuo*. The resulting oily product was washed with ether, dried over KOH pellets *in vacuo* and then dissolved in DMF (50 ml). The solution, after neutralization with Et_3N (5.4 ml), was kept under cooling with ice. The azide [prepared from 11.1 g of Z(OMe)–Ser–$NHNH_2$] in DMF (50 ml) and Et_3N (5.4 ml) were added to the above ice-chilled solution, and the mixture was stirred at 4°C for 24 h, then filtered. The filtered solution was concentrated *in vacuo* and the residue was dissolved in AcOEt (50 ml). The solid formed on standing in a refrigerator was washed with 0.5 M citric acid, 5% $NaHCO_3$ and H_2O and then recrystallized from EtOH and ether; yield 25.3 g (89%), mp 109—112°C, $[\alpha]_D^{24}$ −19.3° (c=1.35, DMF), Rf_2 0.49. *Anal.* Calcd for $C_{36}H_{43}N_3O_{11}S \cdot 0.5H_2O$; C, 58.84; H, 6.04; N, 5.72. Found: C, 58.84; H, 5.95; N, 5.98.

Z(OMe)-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——Z(OMe)–Ser–Glu(OBzl)–Met(O)–OBzl (25.0 g) was treated with TFA–anisole (50 ml–10 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in 3.2 N HCl/DMF (20 ml). After evaporation of the solvent, the oily residue was washed with ether, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (50 ml). Et_3N (4.8 ml), Z(OMe)–Phe–Pro–OH (16.1 g), DCC (8.5 g) and HOBT (6.0 g) were added to the above ice-chilled solution and the mixture was stirred at room temperature for 24 h, then filtered. The filtrate was concentrated *in vacuo* and the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O–NaCl, dried over Na_2SO_4, and then concentrated *in vacuo*. Petroleum ether was added to the residue to form a solid, which was recrystallized from AcOEt and ether; yield 29.4 g (88%), mp 94—98°C, $[\alpha]_D^{24}$ −36.6° (c=0.82, DMF), Rf_2 0.45. *Anal.* Calcd for $C_{50}H_{59}N_5O_{13}S$: C, 61.91; H, 6.13; N, 7.22. Found: C, 61.96; H, 6.13; N, 7.25.

Z(OMe)-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——Z(OMe)–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl (9.7 g) was treated with TFA–anisole (20 ml–5.0 ml) in an ice-bath for 60 min, and the N^{α}-deprotected peptide, after conversion to the corresponding hydrochloride as stated above, was dissolved in DMF (20 ml). To this ice-chilled solution were added Et_3N (1.4 ml), Z(OMe)–Ser–Glu(OBzl)–Gly–OH (6.0 g), DCC (2.7 g) and HOBT (1.9 g), and the mixture was stirred at room temperature for 20 h, then filtered. The filtrate was concentrated *in vacuo* and the product was purified by the extraction procedure described above followed by precipitation from THF with ether; yield 9.8 g (74%), mp 94—98°C, $[\alpha]_D^{24}$ −25.9° (c=0.43, DMF), Rf_3 0.33. Amino acid ratios in 4 N MSA hydrolysate: Ser 1.85, Glu 2.08, Gly 1.00, Phe 0.95, Pro 1.13, Met+Met(O) 0.98 (recovery of Gly, 84.0%). *Anal.* Calcd for $C_{67}H_{80}N_8O_{19}S$: C, 60.35; H, 6.05; N, 8.40. Found: C, 60.24; H, 5.95; N, 8.33.

Z(OMe)-Gln-Ser-OMe——Z(OMe)–Gln–ONp (4.1 g) and Et_3N (1.3 ml) were added to a solution of H–Ser–OMe (prepared from 1.5 g of the hydrochloride) in DMF (30 ml) and the mixture was stirred at room temperature for 68 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the resulting solid was recrystallized from DMF and ether; yield 3.7 g (95%), mp 198—200°C, $[\alpha]_D^{15}$ +3.2° (c=0.63, DMF), Rf_2 0.24. *Anal.* Calcd for $C_{18}H_{25}N_3O_8$: C, 52.55; H, 6.13; N, 10.21. Found: C, 52.56; H, 5.95; N, 10.26.

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Z(OMe)-Gln-Ser-NHNH$_2$——Z(OMe)–Gln–Ser–OMe (8.5 g) in DMF (40 ml) was treated with 80% hydrazine hydrate (5.0 ml) at room temperature for 24 h. The resulting solid was collected by filtration and washed with MeOH; yield 8.1 g (95%), mp 226—227°C (dec.), $[\alpha]_D^{15}$ −2.67° (c=2.25, 90% aqueous DMSO), Rf_5 0.55. *Anal.* Calcd for $C_{17}H_{25}N_5O_7$: C, 49.63; H, 6.13; N, 17.02. Found: C, 49.36; H, 5.95; N, 16.87.

Z(OMe)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——The above protected octapeptide ester, Z(OMe)–Ser–Glu(OBzl)–Gly–Phe–Pro–Ser–Glu(OBzl)–Met(O)–OBzl (7.7 g) was treated with TFA–anisole (20 ml–4.0 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then dissolved in DMF (10 ml) containing Et_3N (0.8 ml). The azide [prepared from 2.6 g of Z(OMe)–Gln–Ser–$NHNH_2$] in DMF (10 ml)–DMSO (5 ml) and Et_3N (0.9 ml) were added to the above ice-chilled solution, and the mixture was stirred at 4°C for 48 h, then filtered. The filtrate was concentrated *in vacuo* and H_2O was added to the residue. The resulting powder was collected by filtration and recrystallized from THF and ether; yield 7.8 g (87%), mp 120—123°C, $[\alpha]_D^{24}$ −33.1° (c=0.61, DMF), Rf_3 0.18, Rf_4 0.57. Amino acid ratios in 4 N MSA hydrolysate: Glu 3.08 Ser 2.76, Gly 1.00, Phe 0.95, Pro 1.11, Met(O) 0.98 (recovery of Gly, 86.5%). *Anal.* Calcd for $C_{75}H_{93}N_{11}O_{23}S\cdot 3H_2O$: C, 56.21; H, 6.23; N, 9.61. Found: C, 55.93; H, 6.11; N, 9.45.

Z(OMe)-Glu(OBzl)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——The above protected decapeptide ester (7.6 g) was treated with TFA–anisole (16.0 ml–4.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (30 ml) together with Et_3N (1.4 ml) and Z(OMe)–Glu(OBzl)–ONp (3.1 g). After being stirred at room temperature for 48 h, the solution was concentrated *in vacuo* and the residue was treated with H_2O. The resulting powder was recrystallized twice from THF and ether; yield 6.3 g (72%), mp 111—115°C, $[\alpha]_D^{24}$ −23.9° (c=1.22, DMF), Rf_3 0.16, Rf_4 0.64. Amino acid ratios in 4 N MSA hydrolysate: Glu 3.96, Ser 2.64, Gly 1.00, Phe 0.94, Pro 1.06, Met(O) 1.00 (recovery of Gly, 85.7%). *Anal.* Calcd for $C_{87}H_{106}N_{12}O_{26}S\cdot H_2O$: C, 58.51; H, 6.10; N, 9.41. Found: C, 58.40; H, 5.84; N, 9.43.

Z(OMe)-Glu(OBzl)-Glu(OBzl)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——In the usual manner, the above protected undecapeptide ester (6.0 g) was treated with TFA–anisole (15 ml–3.0 ml), and the resulting N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (15 ml), together with Et_3N (0.9 ml) and Z(OMe)–Glu(OBzl)–ONp (2.1 g). The mixture was stirred at room temperature for 48 h, then the solvent was evaporated off *in vacuo* and the residue was treated with H_2O. The resulting powder was recrystallized twice from THF and ether; yield 6.5 g (96%), mp 149—151°C, $[\alpha]_D^{33}$ −20.5° (c=1.17, DMF), Rf_3 0.24. Amino acid ratios in 4 N MSA hydrolysate: Glu 5.35, Ser 2.78, Gly 1.00, Phe 0.97, Pro 1.11, Met(O) 1.01 (recovery of Gly, 87.6%). *Anal.* Calcd for $C_{99}H_{119}N_{13}O_{29}S$: C, 59.84; H, 6.04; N, 9.16. Found: C, 59.58; H, 6.33; N, 8.93.

Z(OMe)-Val-Glu(OBzl)-Glu(OBzl)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl (I)——The above protected dodecapeptide ester (6.4 g) was treated with TFA–anisole (15 ml–3.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (20 ml), together with Et_3N (1.0 ml) and Z(OMe)–Val–ONp (1.5 g). The mixture was stirred at room temperature for 40 h, then the solvent was evaporated off *in vacuo* and the residue was treated with ether. The resulting powder was washed with MeOH and then precipitated from DMF with ether; yield 5.6 g (84%), mp 201—204°C, $[\alpha]_D^{33}$ −21.6° (c=0.88, DMF), Rf_3 0.25. Amino acid ratios in 4 N MSA hydrolysate: Val 0.94, Glu 5.17, Ser 2.70, Gly 1.00, Phe 1.00, Pro 1.09, Met(O) 0.95 (recovery of Gly, 95.1%). *Anal.* Calcd for $C_{104}H_{128}N_{14}O_{30}S$: C, 59.87; H, 6.18; N, 9.40. Found: C, 59.71; H, 6.23; N, 9.44.

Z(OMe)-Thr-Asn-Gly-OH (II)——The azide [prepared from 9.4 g of Z(OMe)–Thr–$NHNH_2$] in DMF (60 ml) and Et_3N (3.3 ml) were added to a solution of H–Asn–Gly–OH (3.0 g) and Et_3N (2.2 ml) in H_2O (30 ml), and the mixture was stirred at 4°C for 48 h. The solvent, after addition of a few drops of AcOH, was evaporated off *in vacuo* and the residue was dissolved in 3% NH_4OH. The aqueous solution was washed with AcOEt, acidified with citric acid and then kept in a refrigerator overnight. The resulting gelatinous solid was collected by filtration, washed with cold H_2O and then recrystallized from MeOH and ether; yield 4.2 g (58%), mp 163—167°C (dec.), $[\alpha]_D^{33}$ +3.6° (c=1.1, DMF), Rf_4 0.05, Rf_5 0.46. *Anal.* Calcd for $C_{19}H_{26}N_4O_9\cdot 0.5H_2O$: C, 49.24; H, 5.87; N, 12.09. Found: C, 49.28; H, 6.03; N, 12.37.

Z(OMe)-Val-Tyr-OMe——A solution of Z(OMe)–Val–OH (12.2 g) in THF (80 ml), DCC (9.8 g) and HOBT (6.9 g) were added to an ice-chilled solution of H–Tyr–OMe (prepared from 10.0 g of the hydrochloride) in THF (80 ml). The mixture was stirred in an ice-bath for 2 h and at room temperature for 46 h, then filtered. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was recrystallized from THF and petroleum ether; yield 16.1 g (81%), mp 170—173°C, Rf_1 0.46, Rf_2 0.83, $[\alpha]_D^{16}$ +8.55° (c=1.76, DMF). *Anal.* Calcd for $C_{24}H_{30}N_2O_7$: C, 62.87; H, 6.60; N, 6.11. Found: C, 62.79; H, 6.72; N, 6.13.

Z(OMe)-Val-Lys(Z)-Val-Tyr-OMe——Z(OMe)–Val–Tyr–OMe (2.6 g) was treated with TFA–anisole (5.0 ml–1.3 ml) as usual, then *n*-hexane was added. The resulting oily precipitate was treated with ether to form a powder, which was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (10 ml). The solution, after neutralization with Et_3N (0.8 ml), was added to a solution of the azide [prepared from 2.6 g of Z(OMe)–Val–Lys(Z)–$NHNH_2$] in DMF (10 ml). After further addition of Et_3N

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(0.7 ml), the mixture was stirred at 4°C for 48 h, then poured into H_2O (500 ml). The resulting gelatinous solid was collected by filtration, washed with 0.5 M citric acid and H_2O, and then recrystallized from DMF and ether; yield 3.4 g (90%), mp 223—225°C, $[\alpha]_D^{16}$ −12.0° (c=1.25, DMF), Rf_1 0.23. *Anal.* Calcd for $C_{43}H_{57}N_5O_{11}$: C, 62.99; H, 7.01; N, 8.54. Found: C, 62.85; H, 7.14; N, 8.28.

Z-Arg(Mts)-Pro-Val-Lys(Z)-Val-Tyr-OMe——Z(OMe)–Val–Lys(Z)–Val–Tyr–OMe (3.2 g) was treated with TFA–anisole (10 ml–1.0 ml) in the usual manner, then dry ether was added. The resulting powder was collected by filtration and dissolved in DMF (5.0 ml) containing Et_3N (0.5 ml). Addition of ether afforded a powder, which was collected by filtration, dried over KOH pellets *in vacuo* and again dissolved in DMF (5 ml). To this solution, Z–Arg(Mts)–Pro–OH (2.8 g), DCC (1.0 g) and HOBT (0.7 g) were added, and the mixture was stirred at room temperature for 48 h. The solution was filtered and the filtrate was concentrated *in vacuo*. The powder formed by addition of ether was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O and then precipitated from $CHCl_3$ with petroleum ether; yield 3.9 g (81%), mp 156—159°C, $[\alpha]_D^{15}$ −19.1° (c=1.05, DMF), Rf_2 0.47. *Anal.* Calcd for $C_{62}H_{84}N_{10}O_{14}S\cdot H_2O$: C, 59.89; H, 6.97; N, 11.26. Found: C, 60.14; H, 6.87; N, 11.18.

Z-Arg(Mts)-Pro-Val-Lys(Z)-Val-Tyr-NHNH$_2$ (III)——Z–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–OMe (3.5 g) in MeOH (30 ml) was treated with 80% hydrazine hydrate (0.7 ml) at room temperature for 24 h. The resulting solid was collected by filtration, washed with MeOH and H_2O and then precipitated from DMF with MeOH; yield 3.3 g (94%), mp 225—227°C, $[\alpha]_D^{15}$ −25.0° (c=0.60, DMF), Rf_4 0.58. Amino acid ratios in 4 N MSA hydrolysate: Arg 0.94, Pro 1.10, Val 2.00, Lys 0.95, Tyr 0.91 (recovery of Val 82.3%). *Anal.* Calcd for $C_{61}H_{84}N_{12}O_{13}S$: C, 59.79; H, 6.91; N, 13.72. Found: C, 59.49; H, 6.92; N, 13.59.

Z(OMe)-Thr-Asn-Gly-Val-Glu(OBzl)-Glu(OBzl)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met-(O)-OBzl——The above protected tridecapeptide ester (I) (5.4 g) was treated with TFA–anisole (15 ml–3.0 ml) as usual. The powder formed by addition of dry ether was collected by filtration and dissolved in DMF (10 ml) containing Et_3N (0.4 ml). Ether was added to this solution and the resulting free amino component, obtained as a powder, was collected by filtration and dried over KOH pellets *in vacuo*. Next, DCC (0.8 g) and HOBT (0.5 g) were added to an ice-chilled solution of Z(OMe)–Thr–Asn–Gly–OH (1.3 g) in DMF (20 ml) and the mixture was stirred in an ice-bath for 30 min. The above N^{α}-deprotected peptide ester was added and the mixture was stirred at room temperature for 65 h. The mixture was filtered and the filtrate was poured into ether (100 ml). The resulting powder was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O, and precipitated from DMF with ether; yield 4.7 g (77%), mp 208—211°C, $[\alpha]_D^{33}$ −16.0° (c=1.57, DMF), Rf_3 0.11, Rf_4 0.56. Amino acid ratios in 4 N MSA hydrolysate: Thr 0.81, Asp 0.87, Gly 1.89, Val 1.00, Glu 5.26, Ser 2.80, Phe 0.96, Pro 1.02, Met(O) 1.00 (recovery of Val, 91.7%). *Anal.* Calcd for $C_{114}H_{144}N_{18}O_{35}S$: C, 58.05; H, 6.15; N, 10.69. Found: C, 57.86; H, 6.41; N, 10.87.

Z-Arg(Mts)-Pro-Val-Lys(Z)-Val-Tyr-Thr-Asn-Gly-Val-Glu(OBzl)-Glu(OBzl)-Gln-Ser-Ser-Glu(OBzl)-Gly-Phe-Pro-Ser-Glu(OBzl)-Met(O)-OBzl——The above protected hexadecapeptide ester (0.32 g) was treated with TFA–anisole (2.0 ml–0.1 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (3.0 ml) containing Et_3N (0.02 ml). The azide [prepared from 0.34 g of Z–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–$NHNH_2$] in DMF (3.0 ml) and Et_3N (0.04 ml) were added to the above ice-chilled solution. The mixture was stirred at 4°C for 48 h, then filtered, and the filtrate was poured into H_2O (50 ml). The resulting powder was purified by column chromatography on silica (5×15 cm) using $CHCl_3$–MeOH–H_2O (18: 3: 1, lower phase) as an eluent. The product was finally precipitated from DMF with ether; yield 0.29 g (62%), mp 248—250°C (dec.), $[\alpha]_D^{15}$ −7.25° (c=0.69, DMF), Rf_3 0.16, Rf_4 0.63. Amino acid ratios in 4 N MSA hydrolysate: Arg 0.94, Pro 2.07, Val 3.00, Lys 0.96, Tyr 0.92, Thr 1.03, Asp 1.10, Gly 2.16, Glu 5.31, Ser 3.04, Phe 1.10, Met(O) 0.97 (recovery of Val, 66.5%). *Anal.* Calcd for $C_{166}H_{216}N_{28}O_{45}S_2\cdot 2H_2O$: C, 58.23; H, 6.48; N, 11.45. Found: C, 57.99; H, 6.55; N, 11.78.

H-Arg-Pro-Val-Lys-Val-Tyr-Thr-Asn-Gly-Val-Glu-Glu-Gln-Ser-Ser-Glu-Gly-Phe-Pro-Ser-Glu-Met-OH (salmon CLIP)——The above protected docosapeptide ester (58.3 mg) was treated with 1 M TFMSA–thioanisole (1: 1) in TFA (3.44 ml) in the presence of *m*-cresol (0.14 ml) in an ice-bath for 2 h, then *n*-hexane was added. The precipitate was washed repeatedly with *n*-hexane and dissolved in H_2O (10 ml)–AcOH (2 ml). The solution was treated with Amberlite IRA-400 (acetate form, approximately 3 g) for 30 min with stirring, then the resin was removed by filtration. The filtrate was lyophilized and the residue was dissolved in H_2O (15 ml). The solution was applied to a column of DEAE-cellulose (1.4×13 cm), which was eluted with H_2O (50 ml) and then with a gradient formed from 0.1 M ammonium acetate buffer (pH 7.0) through a mixing flask containing H_2O (100 ml). Individual fractions (5 ml each) were collected and the absorbancy at 275 nm was determined. After elution of a front peak (tube Nos. 3—12) consisting of scavengers and several ninhydrin-positive products, the main peak (tube Nos. 36—45) and two minor peaks were detected. The fractions corresponding to the main peak were combined and the solution was concentrated *in vacuo* to approximately 5 ml. This solution was incubated with 2-mercaptoethanol (1 ml) at 50°C for 15 h and then applied to a column of Sephadex G-25 (2×150 cm), which was eluted with 3% AcOH. The UV absorption of each fraction (5 ml) at 275 nm was measured. A single symmetrical peak (tube Nos. 39—45) was detected. These desired fractions were pooled and the solvent was removed by lyophilization to give a fluffy white powder; yield 17.5 mg (37%), $[\alpha]_D^{14}$ −88.9° (c=0.09, 3% AcOH), Rf_6 0.41. Amino acid ratios in 4 N MSA hydrolysate: Arg 0.93, Pro 1.91, Val 3.00, Lys 1.01, Tyr 0.97, Thr 1.01, Asp 1.05, Gly 2.07,

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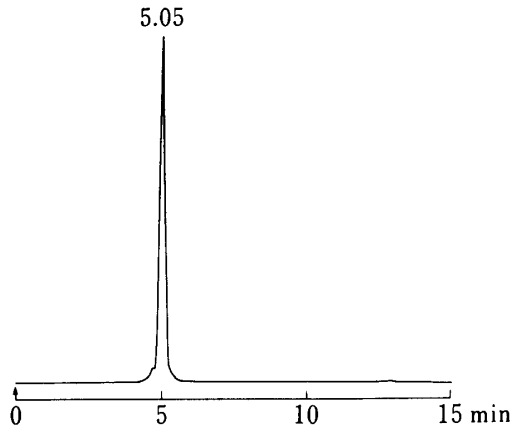

Fig. 4. HPLC Examination of Synthetic Salmon CLIP

Glu 4.96, Ser 2.73, Phe 1.00, Met 0.98 (recovery of Val, 82.7%). Amino acid ratios in aminopeptidase (AP-M, Merck, lot No. 0040347) digest (numbers in parentheses are theoretical values): Arg 1.06 (1), Pro 2.03 (2), Val 3.00 (3), Lys 1.03 (1), Tyr 1.07 (1), Thr+Gln 1.90 (1+1, Calcd as Thr), Asn+Ser 3.91 (1+3, Calcd as Ser), Gly 2.01 (2), Glu 4.24 (4), Phe 1.01 (1), Met 1.01 (1) (recovery of Val, 80.8%). HPLC: retention time, 5.05 min.(Fig. 4) *Anal.* Calcd for $C_{106}H_{164}N_{28}O_{38}S \cdot 2CH_3COOH \cdot 6H_2O$: C, 48.95; H, 6.87; N, 14.53. Found: C, 49.10; H, 6.76, N, 13.90.

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References and Notes

1) Amino acids, peptides and their derivatives (except glycine) mentioned in this paper are of the L-configuration. Abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, OBzl=benzyl ester, Np=*p*-nitrophenyl ester. Other abbreviations used are: Mts=mesitylene-2-sulfonyl, DCC=*N*,*N*′-dicyclohexylcarbodiimide, HOBT=*N*-hydroxybenzotriazole, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, NCS=*N*-chlorosuccinimide, DMF=dimethylformamide, DMSO=dimethyl sulfoxide, THF=tetrahydrofuran.
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