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SYNTHESIS OF FRAGMENTS OF ANGLERFISH PRE-PROGLUCAGON I AND II (AGFI AND AGFII).

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Abstract - Four fragments of the two pre-proglucagons from the Anglerfish (*Lophius americanus*) have been synthesised. The fragments, AGFI (35-40) and (105-110), and AGFII (34-38) and (103-108) were prepared by solution peptide synthesis using a combination of hydrogenolysable and acid labile protecting groups. The pivalic mixed anhydride method was generally used for coupling although occasionally the DCCI/HOBt method was employed. Final removal of acid labile protecting groups was achieved using trifluoroacetic acid.

The hormone glucagon is a twenty-nine residue peptide which regulates blood glucose concentration.[1] It is a member of a family of structurally related hormones including vaso-intestinal peptide (VIP), gastric inhibitory peptide (GIP) and secretin.[2] Biosynthesis of these peptide hormones is from a very much larger pre-prohormone which is processed by enzymic cleavage at double basic residues to give the prohormone and ultimately the hormone itself.[3]

Recently, nucleotide sequencing of angler fish cDNAs showed that two distinct pre-proglucagons exist in the angler fish.[4-6] In fact similar duplication of pre-proglucagon is also seen in the hamster[7] and possibly other species. The primary sequences of the two angler fish pre-proglucagons (AGFI and AFGII, see Figure 1) are similar in that each pre-proglucagon is a protein containing two

AFGI ^{1}Signal peptide I ^{28} | ^{29} NH_2-peptide I ^{50} | ^{51}Lys ^{52}Arg | ^{53} Glucagon I ^{81} | ^{82}Lys ^{83}Arg |

AFGII ^{1}Signal peptide II ^{27} | ^{28} NH_2-peptide II ^{49} | ^{50}Lys ^{51}Arg | ^{52} Glucagon II ^{80} | ^{81}Lys ^{82}Arg |

AFGI ^{84}intervening peptide I ^{88} | ^{89} Lys.^{90}Arg | ^{91}Glucagon related CO_2H peptide I ^{124} |

AFGII ^{83}intervening peptide II ^{86} | ^{87} Arg.^{88}Arg | ^{89}Glucagon related CO_2H peptide II ^{122} |

FIGURE 1 Structure of Anglerfish pre-proglucagons

glucagon-related peptides arranged in tandem. The first peptide is the 29 amino acid glucagon sequence and the second, a 34 amino acid sequence showing close homology to glucagon and other peptides in the glucagon family. The two peptides are joined by an intervening penta or tetra-peptide and a double basic residue sequence which permits enzymic processing. Both the 29 amino acid sequence and the 34 amino acid sequence from the two precursors are highly homologous and it is clear that in the angler fish glucagon is synthesised by way of the expression of at least two genes.

In the present work we have synthesised fragments of the amino peptides I and II (see Figure 2) and fragments of the glucagon related carboxyl peptides I and II (see Figure 3). These peptide fragments have been selected as they exhibit minimum overlap between the AFGI and II sequences, and thus would provide the greatest chance of distinguishing between these peptides by

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	35 40	
AFGI	-Glu.Ala.Asp.Ser.Thr.Leu-	(6)
	34 38	
AFGII	-Leu.Asn.Glu.Asn.Ser-	(11)

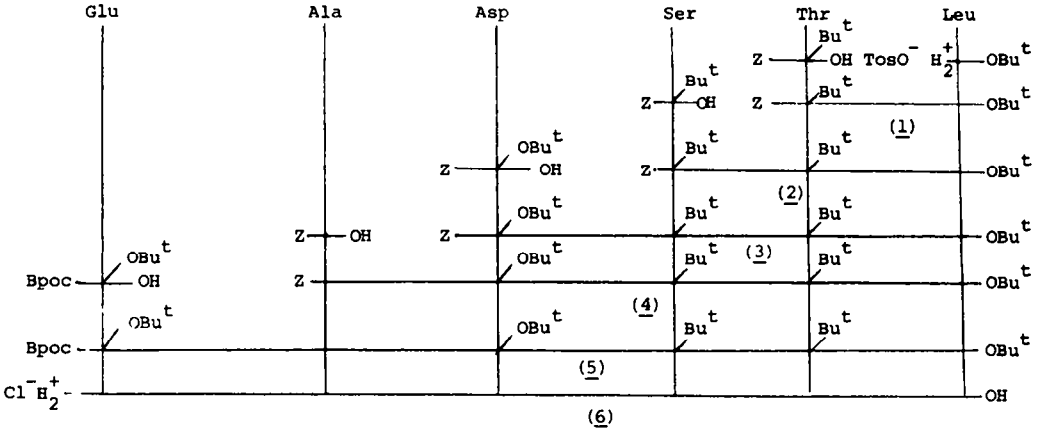
FIGURE 2 Fragments of NH_2-peptide I and II

	105 110	
AFGI	-Lys.Asp.Gln.Ala.Ile.Lys-	(17)
	103 108	
AFGII	-Gln.Asp.Gln.Ala.Ala.Lys-	(23)

FIGURE 3 Fragments of Glucagon related CO_2H peptide I and II.

immunological methods. The four sequences have been used to raise region specific antibodies to the various parts of the AFGI and AFGII chains: these antibodies will be used to investigate whether both proglucagons are present in islet and intestinal cells and to investigate whether there is tissue specific expression of the two genes. The immunological work will be reported elsewhere in collaboration with other workers.

The AFGI (35-40) fragment (6) was synthesised by the general route outlined in Scheme 1.†

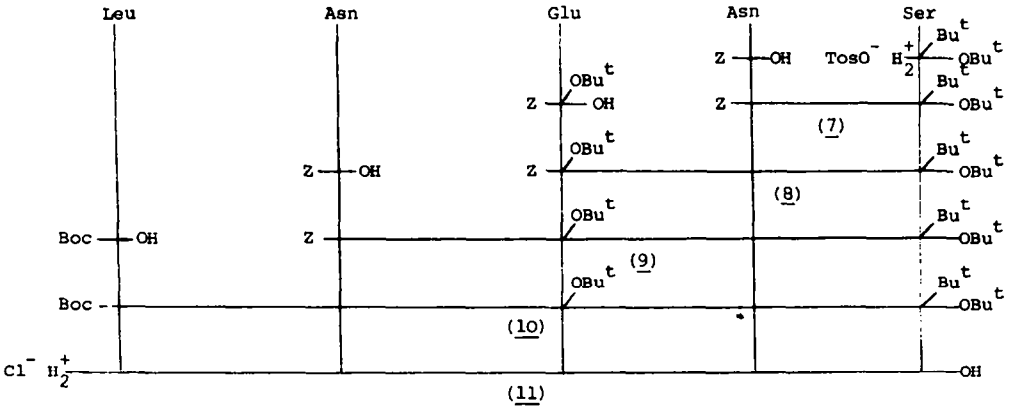


SCHEME 1 Synthesis of the Anglerfish pre-proglucagon AFGI (35-40) fragment (6).

In the assembly we have used t-butyl-based acid labile protecting groups for side chain and C-termina protection, and have extended the chain using benzyloxycarbonyl for intermediate amino group protection (see reference 8 for general aspects of peptide synthesis). The synthesis of the protected dipeptide (1) was carried out by the DCCI/HOBt[9] method and extension to the fully protected hexapeptide was carried out using pivalic mixed anhydrides for intermediate coupling. The amino terminal glutamic acid residue was N^{α}-protected with the acid labile Bpoc protecting group. Deprotection to yield the free peptide (6) was achieved using 90% TFA for one and a half hours. Anion exchange to give the corresponding hydrochloride was then carried out and final purification achieved by gel filtration on Sephadex G15. The free peptide was homogeneous by h.p.l.c., and gave a good amino acid analysis, the structure was also confirmed by positive ion FAB mass spectrometry.

The second fragment (11) of the amino peptide of AFGII as indicated in Figure 2 was synthesised by the route outlined in Scheme 2. The same general approach to the synthesis was used but on this occasion pivalic mixed anhydrides were used throughout for chain extension and the amino terminal residue (leucine) was incorporated as its Boc derivative. The fully

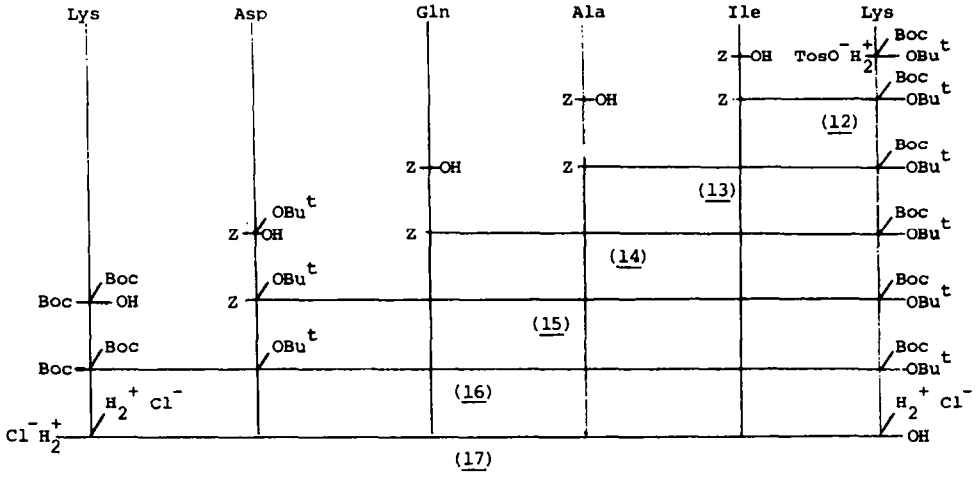
† The nomenclature and abbreviations used conform to the 1983 recommendations of IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN).



SCHEME 2 Synthesis of the Anglerfish pre-proglucagon AFGII (34-38) fragment (11)

protected peptide (10) was obtained in good yield and in a high state of purity. This peptide was then deprotected, again using 90% TFA, however on this occasion four hours were required for total deprotection. The trifluoroacetate was again exchanged for chloride anion and the product purified on Sephadex G10. The identity of the homogeneous product (11) was confirmed by amino acid analysis and FAB mass spectrometry.

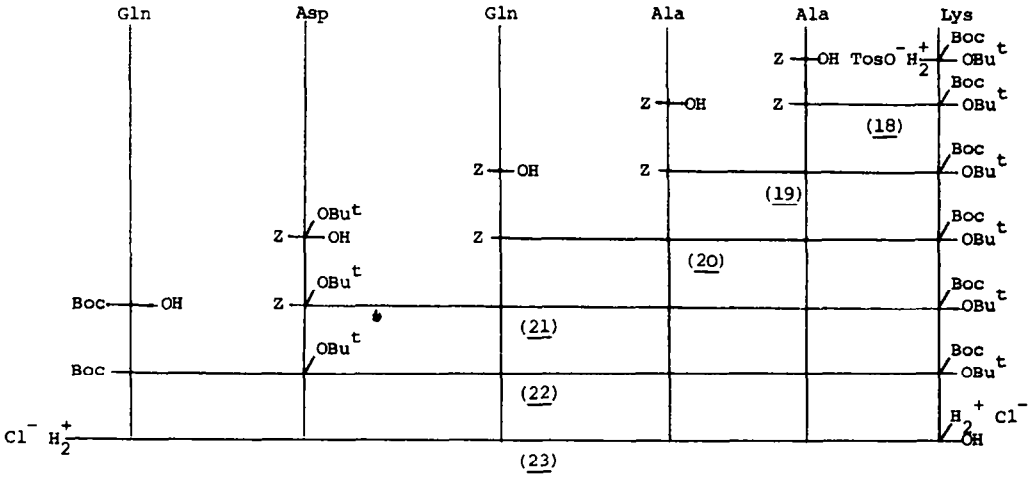
The 105 - 110 fragment (17) of the glucagon related carboxy peptide of AFGI indicated in Figure 3 was synthesised using the synthetic pathway outlined in Scheme 3. Protection of the basic



SCHEME 3 Synthesis of the Anglerfish pre-proglucagon AFGI (105-110) fragment (17).

side chain of lysine was achieved using the Boc group which allows acidolytic deprotection. The dipeptide (12) was synthesised by the DCCI/HOBt procedure and as in the case of the dipeptide (1) we found that this gave a much better yield than a mixed anhydride method. This was probably due to the fact that two sterically hindered residues were being coupled. Extension to the fully protected hexapeptide was achieved without complication using the pivalic mixed anhydride throughout. Deprotection of the peptide (16) using 90% TFA was achieved in 40 minutes and once again anion exchange gave the resulting hydrochloride (17) which was purified on Sephadex G15. The homogeneous product was obtained in reasonable yield and showed good amino acid analyses and FAB mass spectra.

The second glucagon related carboxyl peptide (23) shown in Figure 3 was prepared by the route outlined in Scheme 4. The same general approach was employed, with acid labile protecting groups



SCHEME 4 Synthesis of the Anglerfish pre-proglucagon AFGII (103-108) fragment (23)

being used to mask reactive functional groups. In the assembly of the protected hexapeptide (22) the pivalic mixed anhydride method was used throughout for carboxyl activation. Good yields were achieved and the final product (22) was obtained in a high state of purity. A one hour deprotection using 90% TFA followed by anion exchange gave the hydrochloride (23) in almost quantitative yield. The free peptide was homogeneous by h.p.l.c. and was characterised by amino acid analysis and FAB mass spectrometry.

Several interesting points emerged from this synthesis including the difficulty experienced when using pivalic mixed anhydrides to couple sterically hindered residues and the considerable variation in the ease of final deprotection of acid labile t-butyl-based protection with 90% TFA. In our work we routinely monitor such deprotections, as it is clear that there are no standard conditions which can be used to give an adequately pure product. Also, positive ion FAB mass spectroscopy was shown to be an ideal tool for examining the integrity of free peptides produced by synthesis.

The immunological work in which these peptides have been used to raise region specific antibodies is at present ongoing, and will be reported in due course.

EXPERIMENTAL

Melting points were determined on a Reichert-Koffler hot stage microscope, and are uncorrected. Optical rotations were measured using an NPL automatic polarimeter, type 243. Amino acid ratios were determined with a Jeol JLC-6AH amino acid analyser, after 24h hydrolysis in 6M HCl at 110°. Serine and threonine values are uncorrected. HPLC studies were performed with a Spectra Physics SP8700 solvent delivery system, a LiChrosorb 10 RP 18 column (250 x 4.6 mm), and an LC 871 variable-wavelength detector, operating at 230 nm. Unless otherwise stated, retention times refer to a single peak eluting on a 15 min. linear gradient from 100% water to 100% acetonitrile, flow rate 1 ml.min^{-1}. Water was glass distilled and HPLC-grade acetonitrile was purchased from Glenrothes Chemicals Limited, Fife, Scotland, being redistilled before use. Tlc plates were cut from Alugram Sil G/UV$_{254}$ sheets (Macherey-Nagel, Duren), and developed in the solvent system: A 95:5 CH_2Cl_2/MeOH; B 9:1 CH_2Cl_2/MeOH; C 3:1 CH_2Cl_2/MeOH; D 90:10:1 CH_2Cl_2/MeOH/AcOH; E 3:1:1 n-BuOH/AcOH/H_2O. The identity of all compounds was confirmed by ^{1}H-NMR at 220 MHz (Perkin Elmer R34 spectrometer: data not shown), and mass spectra were recorded on a VG 7070E instrument. DMF was distilled from CaH_2 and EtOAc distilled from K_2CO_3 before use. "Washed as usual" implies sequential washing with water, 10% citric acid, water, 5% $NaHCO_3$, water and brine, drying over Na_2SO_4, and filtration. The eluate from Sephadex LH-20 gel filtration columns was monitored by polarimetry and absorbance at 280 nm (Unicord II, LKB, Bromma, Sweden); that from Sephadex G-10 or Sephadex G-15 columns was monitored at 206 and 254 nm (Unicord III, LKB);

data was displayed on an LKB-6520 recorder.

Non-standard abbreviations:- DMF - dimethylformamide; NMM - N-methylmorpholine; N-methylpyrrolidone; PiviCl - Pivaloyl chloride; TFA - trifluoroacetic acid; TFE - trifluoroethanol.

GENERAL PROCEDURES

(a) Removal of benzyloxycarbonly groups (Z) by hydrogenolysis.

The N^{α}-Z-protected peptide (1 eq.) or amino acid (1 eq.) and p-toluenesulphonic acid (1 eq.) were dissolved in DMF (except for protected peptides (4) and (14) which were dissolved in NMP) and after purging with nitrogen, 10% Pd/C added (50 mg/mM). Hydrogen was passed through for approximately 5h. and then the catalyst removed by filtration through celite. The filtrate containing the p-toluenesulphonate was used directly in the coupling reaction without further purification.

(b) Coupling of protected amino acids.

The N-protected amino-acid (1.2* eq.) was dissolved in DMF and cooled to -15°, NMM (1.2* eq.) and pivaloyl chloride (1.15* eq.) were added and the solution stirred for 20 min. at 15° to form the mixed anhydride. (In the case of Z.Asn.OH and Z.Gln.OH, pyridine (1.2 eq.) was also added.) A pre-cooled solution of the peptide p-toluenesulphonate salt (1 eq.) in DMF or NMP was then added, along with NMM (1 eq.) and the reaction mixture stirred overnight with gradual warming to room temperature.

*In the syntheses of penta- and hexapeptides the ratios were altered slightly; using the N-protected amino-acid (1.5 eq.), NMM (1.5 eq.) and pivaloyl chloride (1.4 eq.).

AFGI (35-40) fragment (6)

Z.Thr(Bu^t).Leu.OBu^t (1) Z.Leu.OBu^t (4.85, 15.1 mM) was hydrogenolysed in the presence of Tos.OH.H_2O (2.87g, 15.1 mM) and 10% Pd/C (750 mg) in DMF (30 ml) employing the general method (a) above. Z.Thr(Bu^t).OH.DCHA (8.88g, 18.1 mM) was converted to the free acid using 10% citric acid. The resulting oil was extracted into EtOAc, dried over Na_2SO_4 and evaporated to give a dry foam. The foam was dissolved in the DMF solution from the hydrogenolysis and the resulting solution cooled to 0° prior to the addition of HOBt (4.9g, 36.2 mM), DCCI (4.1g, 19.9 mM) and NMM (1.7ml, 15.1 mM). The reaction mixture was stirred for 17h, whilst slowly being allowed to warm to room temperature.

The DMF was then evaporated *in vacuo* and the residue dissolved in EtOAc, washed with aqueous solutions of citric acid and $NaHCO_3$ then dried over Na_2SO_4. Evaporation of the solvent gave a yellow oil which was purified by chromatography on silica gel, eluting with CH_2Cl_2. The product (1) was subsequently crystallised from hexane, giving (4.05g, 56%); m.p. 91-92°, R_f (A) 0.8; HPLC (10 min. linear gradient; 60 - 100% MeCN in H_2O) 11.4 min.; $[\alpha]_D^{20}$ + 15.2° (C = 2, $CHCl_3$); $Thr_{0.82}Leu_{1.00}$; (Found: C, 65.36; H, 8.72; N, 6.10. $C_{26}H_{42}N_2O_6$ requires: C, 65.25; H, 8.85; N, 5.85%).

Z.Ser(Bu^t).Thr(Bu^t).Leu.OBu^t (2). The protected dipeptide (1) was hydrogenolysed by the general method (a) above, using (1) (2.22g, 4.6 mM), TosOH.H_2O (0.88g, 4.6 mM) and 10% Pd/C (230 mg) in DMF (10 ml). A mixed anhydride coupling (see (b) above) was carried out employing Z.Ser(Bu^t).OH (1.64g, 5.5 mM), NMM (0.623 ml, 5.5 mM) and Piv.Cl (0.657 ml, 5.3 mM). After overnight reaction the solvent was evaporated and the residue dissolved in EtOAc and this solution washed with aqueous solutions of citric acid and $NaHCO_3$. Washing with water, drying and evaporation gave an oil which was crystallised from hexane giving (2) (1.8g, 62%); m.p. 145 - 146°; R_f (A) 0.7; HPLC (10 min. linear gradient, 60 - 100% MeON in H_2O) 12.6 min; $[\alpha]_D^{20}$ + 19.9° (C = 2, $CHCl_3$); $Thr_{0.74}Ser_{0.52}Leu_{1.00}$; (Found: C, 63.56; H, 8.85; N, 6.76. $C_{33}H_{55}N_3O_8$ requires : C, 63.74; H, 8.92; N, 6.76%).

Z.Asp(OBu^t).Ser(Bu^t).Thr(Bu^t).Leu.OBu^t (3). Compound (2) (2.38g, 3.8 mM) in DMF (10 ml) was hydrogenolysed by the general method (a) in the presence of Tos.OH.H_2O (0.72g, 3.8 mM) and 10% Pd/C (190 mg). Z.Asp(OBu^t).OH.DCHA (2.51g, 4.9 mM) was converted to the corresponding free acid by the standard method employing 10% citric acid. This free acid was coupled to the amino-component

from the hydrogenolysis by a mixed anhydride coupling (see (b) above) using NMM (0.56 ml, 4.9 mM) and Piv.Cl (0.54 ml, 4.3 mM) in DMF (5 ml), and a further portion of NMM (0.428 ml, 3.8 mM) added. The reaction mixture was evaporated and taken up in EtOAc. This solution was washed in the usual way and evaporation of the EtOAc gave a white foam which crystallised from hexane yielding (3) (2.4g, 80%); m.p. 125 - 126°; R_f (A) 0.4; HPLC (10 min. linear gradient, 60 - 100% MeCN in H_2O), 13.6 min; $[\alpha]_D^{20}$ + 28.4° (C=1, $CHCl_3$); $Asp_{1.00}Thr_{0.83}Ser_{0.62}Leu_{1.02}$; (Found: C, 62.27; H, 8.85; N, 6.81. $C_{41}H_{68}N_4O_{11}$ requires: C, 62.10; H, 8.64; N 7.07%).

Z.Ala.Asp(OBu^t).Ser(Bu^t).Thr(Bu^t).Leu.OBu^t (4). The protected tetrapeptide (3) (1.19g, 1.5 mM) dissolved in DMF (5 ml) was hydrogenolysed using the general method (a) in the presence of Tos.OH.H_2O (0.29g, 1.5 mM) and 10% Pd/C (150 mg). The resulting amino-component was coupled to Z.Ala.OH (0.51g, 2.3 mM) using the pivalic mixed anhydride (see general method (b) above) employing NMM (0.25 ml, 2.3 mM) and Piv.Cl (0.26 ml, 2.1 mM) in DMF (5 ml). A second portion of NMM (0.17 ml, 1.5 mM) and $CHCl_3$ (10 ml) to maintain solubility were added prior to overnight reaction. Evaporation gave a foam which was dissolved in EtOAc and washed with acid and base in the usual way. The product crystallised from EtOAc/EtOH/hexane giving (1.1g, 83%); m.p. 218 - 220°; R_f (A) 0.3; HPLC (10 min. linear gradient, 60 - 100% MeCN in H_2O) 12.9 min; $[\alpha]_D^{20}$ + 15.6° (C = 0.5, $CHCl_3$); $Asp_{0.98}Thr_{0.80}Ser_{0.55}Ala_{1.00}Leu_{1.02}$; (Found: C, 61.35; H, 8.39; N, 8.14. $C_{44}H_{73}N_5O_{12}$ requires: C, 61.16; H, 8.52; N, 8.11%).

Bpoc.Glu(OBu^t).Ala.Asp(OBu^t).Ser(Bu^t).Thr(Bu^t).Leu.OBu^t (5). The compound (4) (1.05g, 1.2 mM) in NMP (30 ml) was hydrogenolysed by the general method (a) in the presence of Tos.OH.H_2O (0.23g, 1.2 mM) and 1% Pd/C (60 mg). Bpoc.Glu(OBu^t).OH (0.81g, 1.8 mM) in DMF (5 ml) was coupled by the mixed anyydride method (see (b) above) employing NMM (0.21 ml,1.8 mM) and Piv.Cl (0.15 ml, 1.7 mM). NMM (0.14 ml, 1.2 mM) was added and the reaction mixtured stirred for 20h, whilst gradually warming to room temperature. The reaction mixture was diluted with $CHCl_3$ and washed in the usual way. After evaporation of the $CHCl_3$, the crude product was subjected to gel filtration on Sephadex LH20, eluting with DMF. Evaporation of product fractions gave a pale yellow solid (0.95g, 68%); m.p. 214 - 215°; R_f (B) 0.7; HPLC (10 min. linear gradient, 60 - 100% MeCN in H_2O) 15.9 min; $[\alpha]_D^{20}$ + 0.4° (C = 1.5, $CHCl_3$); $Asp_{1.00}Thr_{0.86}Ser_{0.65}Glu_{0.92}Ala_{1.01}Leu_{1.04}$; (Found: C, 63.63; H, 8.70; N, 7.31. $C_{61}H_{96}N_6O_{15}$ requires: C, 63.52; H, 8.39; N, 7.29%).

$Cl^-H_2^+$.Glu.Ala.Asp.Ser.Thr.Leu.OH (6). The protected hexapeptide (5) (104 mg, 90 µmol) was stirred for 1½h. in 90% aqueous TFA (1 ml) at room temperature. The TFA was evaporated *in vacuo* and the residue twice taken up in 0.1 M hydrochloric acid and evaporated; a final evaporation from water was carried out to remove excess HCl.The resulting solid was dissolved in water,filtered t remove insoluble material, and applied to Sephadex G-15, eluting with water. Product fractions were lyophilised and again subjected to gel filtration on Sephadex G-15. Fractions from the centre of the product peak were lyophilised to give (6) as a white solid (37 mg, 52%). An analytical sample of this material, applied to a Sephadex G-15 column with high detector sensitivity, gave a single, symmetrical peak; FAB (positive ion) m/z: 661 (M + 2); $Asp_{1.02}Thr_{0.82}Ser_{0.60}Glu_{1.01}Ala_{0.97}Leu_{1.00}$.

AFGII (34-38) fragment (11)

Z.Asn.Ser(Bu^t).OBu^t (7). Z.Ser(Bu^t).OBu^t (8.8g, 25 mM) in DMF (150 ml) was hydrogenolysed according to the general method (a) in the presence of Tos.OH.H_2O (4.8g, 25 mM) and 10% Pd/C (1.25g). Mixed anhydride coupling (general method (b)) was carried out using Z.Asn.OH (8.0g, 30 mM), NMM (3.35 ml, 30 mM), pyridine (2.42 ml, 30 mM) and Piv.Cl (3.54 ml, 2.9 mM) in DMF (200 ml). The reaction mixture was stirred and attained room temperature overnight. The crude reaction mixture was poured into ice-cooled aqueous $NaHCO_3$ and the white precipitate filtered. This solid was washed thoroughly with water and dried then dissolved in warm EtOAc, some insoluble material was filtered and the solvent evaporated to leave a white solid (7.9g, 68%); m.p. 139 - 142°; R_f (A) 0.2; HPLC 15.3 min.; $[\alpha]_D^{20}$ + 6.8° (C = 1, TFE); $Asp_{1.00}Ser_{0.67}$; (Found: C, 59.23; H, 7.58; N, 9.06. $C_{23}H_{35}N_3O_7$ requires: C, 59.34; H, 7.58; N, 9.03%).

Z.Glu(OBu^t).Asn.Ser(Bu^t).OBu^t (8). The protected dipeptide (7) (4.7g, 10 mM) in DMF (80 ml) was hydrogenolysed in the presence of Tos.OH.H_2O (1.9g, 10 mM) and 10% Pd/C (0.5g) using the general method (a). Z.Glu(OBu^t).OH.DCHA (5.7g, 11 mM) was converted to the free acid by washing with citric acid solution in the usual manner. The free acid dissolved in DMF (80 ml) was coupled with the amino-component by the pivalic mixed anhydride method (see (b) above), using NMM (1.23 ml, 11 mM) and Piv.Cl (1.29 ml, 10.5 mM). A further addition of NMM (1.12 ml, 10 mM) was made and the reaction mixture stirred overnight, gradually warming to room temperature. The reaction mixture was evaporated and the residue redissolved in EtOAc. This solution was washed with 10% aqueous citric acid, 5% aqueous $NaHCO_3$, water and brine, dried over Na_2SO_4, filtered and evaporated. Recrystallisation from EtOAc/cyclohexane gave the product (8) as a white solid (3.7g, 57%); m.p. 150 - 157°; R_f (B) 0.32; HPLC 16.6 min.; $[\alpha]_D^{20}$ - 15.7° (C = 1, TFE); $Glu_{1.01}Asp_{1.00}Ser_{0.72}$; (Found: C, 59.07; H, 7.70; N, 8.59. $C_{32}H_{50}N_4O_{10}$ requires: C, 59.06; H, 7.74; N, 8.61%).

Z.Asn.Glu(OBu^t).Asn.Ser(Bu^t).OBu^t (9). Compound (8) (4.6g, 7.1 mM) in DMF (50 ml) was subjected to hydrogenolysis in the usual way (a) in the presence of Tos.OH.H_2O (1.3g, 7.1 mM) and 10% Pd/C (350 mg). Coupling (see (b) above) with Z.Asn.OH (2.3g, 8.5 mM) in DMF (60 ml) was carried out using NMM (0.95 ml, 8.5 mM), pyridine (0.686 ml, 8.5 mM) and Piv.Cl (1.00 ml, 8.1 mM) NMM (0.79 ml, 7.1 mM) was added and the reaction stirred overnight. The reaction mixture was poured into 3% aqueous $NaHCO_3$, the precipitate filtered, washed with water and dissolved in DMF. This solution was filtered and water added to reprecipitate the product which was filtered and dried; yielding (9) (3.5g, 65%); m.p. 218 - 220° ; R_f (C) 0.5; HPLC 15.2 min; $[\alpha]_D^{20}$ - 21.7° (C = 1, TFE); $Asp_{1.86}Glu_{1.00}Ser_{0.61}$; (Found: C, 56.48; H, 7.34; N, 11.11. $C_{36}H_{56}N_6O_{12}$ requires: C, 56.53; H, 7.38; N, 10.99%).

Boc.Leu.Asn.Glu(OBu^t).Asn.Ser(Bu^t).OBu^t (10). Hydrogenolysis of (9) (0.765g, 1 mM) was carried out in the presence of Tos.OH.H_2O (0.19, 1 mM) and 10% Pd/C (50 mg) in DMF (10 ml) in the usual way (a). Coupling by the mixed anhydride method (b) was carried out using Boc.Leu.OH (0.254g, 1.1 mM), NMM (0.123 ml, 1.1 mM), pyridine (0.089 ml, 1.1 mM) and Piv.Cl (0.129 ml, 1.05 mM). The reaction mixture was poured into 5% $NaHCO_3$ solution and the resulting solid filtered and redissolved in DMF prior to gel filtration on Sephadex LH20 eluting with DMF (Ve/Vt - 0.43). This gave the white solid (10) (0.531g, 63%); m.p. 230 - 232° (dec.); R_f (B) 0.3; HPLC 16.0 min.; $[\alpha]_D^{20}$ - 32.3° (C = 1, TFE); $Asp_{2.05}Ser_{0.55}Glu_{1.01}Leu_{1.00}$; (Found: C, 55.65; H, 8.45; N, 11.40. $C_{39}H_{69}N_7O_{13}$ requires: C, 55.50; H, 8.24; N, 11.62%).

$Cl^-H_2^+$.Leu.Asn.Glu.Asn.Ser.OH (11). The fully protected pentapeptide (10) (49 mg, 58 µM) was dissolved in 90% aqueous TFA (5 ml) and stirred 4h. at room temperature. Evaporation of the solvent gave a white powder (44 mg). This was twice taken up in 0.1M hydrochloric acid and evaporated, then evaporated from water to yield a hygroscopic white solid, which was purified by gel filtration on Sephadex G-10, eluting with water. The pure free peptide hydrochloride (11) (21 mg, 59%) was thus obtained; HPLC (eluted with water, 0.2 ml min^{-1} flow rate) 9.8 min.; FAB (positive ion) m/z 577 (M + 2); $Asp_{2.00}Ser_{0.72}Glu_{1.05}Leu_{1.00}$.

AFGI (105-110) fragment (17).

Z.Ile.Lys(Boc).OBu^t (12). Z.Lys(Boc).OBu^t (13.7g, 31.4 mM) in DMF (75 ml) was hydrogenolysed in the usual manner (a) in the presence of Tos.OH.H_2O (5.97g, 31.4 mM) and 10% Pd/C (1.57g). Z.Ile.OH.DCHA (16.8g, 37.7 mM) was converted to the free acid in the usual way and added to the DMF solution of the amino-component. After cooling to 0°, HOBt (10.2g, 75.4 mM), DCCI (8.6g, 41.5 mM) and NMM (3.51 ml, 31.4 mM) were added and the reaction mixture stirred at room temperature overnight. A few drops of acetic acid were added and the mixture cooled to -10° for 1h., filtered and evaporated The residue was dissolved in EtOAc and washed with solution of ice cold citric acid, sodium bicarbonate then washed with water and dried. The crude product was chromatographed on silica gel, eluting with CH_2Cl_2, then crystallised from hexane giving (12) (12.1g, 70%), m.p. 74 - 77°; R_f (B) 0.7; HPLC (10 min. gradient, 60 - 100% MeCN in H_2O) 8.8 min.; $[\alpha]_D^{20}$ - 3.7° (C = 4, MeOH); $Lys_{1.00}Ile_{0.91}$; (Found: C, 63.14; H, 8.71; N, 7.85. $C_{29}H_{47}N_3O_7$ requires: C, 63.36; H, 8.62; N, 7.64%).

Z.Ala.Ile.Lys(Boc).OBu^t (13). The protected dipeptide (12) (10.1g, 18.3 mM) in DMF (30 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (3.5g, 18.3 mM) and 10% Pd/C (0.9g). Coupling to Z.Ala.OH (4.9g, 22 mM) in DMF (10 ml) was carried out using the pivalic mixed anhydride method (b) employing NMM (2.5 ml, 22 mM) and Piv.Cl (2.6 ml, 21 mM). The reaction mixture was evaporated, and the residue taken up in EtOAc, washed with an ice cold solution of citric acid, $NaHCO_3$, water then dried. The product crystallised from EtOAc/hexane yielding (13) (7.1g, 63%); m.p. 119 - 125° (dec.); R_f (A) 0.2; HPLC (10 min. linear gradient, 60 - 100% MeCN), 7.8 min.; $[\alpha]_D^{20}$ - 8.6° (C = 2, TFE); $Lys_{1.08}Ala_{1.00}Ile_{0.95}$; (Found: C, 61.95; H, 8.48; N, 9.00; $C_{32}H_{52}N_4O_8$ requires: C, 61.91; H, 8.44; N, 9.03%).

Z.Gln.Ala.Ile.Lys(Boc).OBu^t (14). Compound (13) (4.5g, 7.3 mM) in DMF (20 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (1.4g, 7.3 mM) and 10% Pd/C (363 mg). Z.Gln.OH (2.4g, 8.7 mM) in DMF (20 ml) was then coupled by the mixed anhydride method (b) using NMM (0.973 ml, 8.7 mM), pyridine (0.708 ml, 8.7 mM) and Piv.Cl (1.03 ml, 8.3 mM). The gelatinous reaction mixture was poured into 3% aqueous $NaHCO_3$ with vigorous stirring and the resulting solid filtered. This material was washed thoroughly with water and dried. The product had very low solubility in DMF, but dissolved in TFE and was reprecipitated by the addition of water giving (14) (4.6g, 85%); m.p. 213 - 217°; R_f (B) 0.3; HPLC 15.4 min.; $[\alpha]_D^{20}$ - 54.3° (C = 1, TFE); $Lys_{1.02}Glu_{1.02}Ala_{0.98}Ile_{0.95}$; (Found: C, 59.24; H, 8.15; N, 11.41. $C_{37}H_{60}N_6O_{10}$ requires: C, 59.34; H, 8.08; N, 11.22%).

Z.Asp(OBu^t).Gln.Ala.Ile.Lys(Boc).OBu^t (15). The protected tetrapeptide (14) (3.7g, 5 mM) in NMP (50 ml) was hydrogenolysed by the standard method (a) in the presence of Tos.OH.H_2O (0.96g, 5mM) and 10% Pd/C (250 mg). Z.Asp(OBu^t).OH.DCHA (3.7g, 7.5 mM) was converted to the free acid in the usual way and coupled to the amino-component using the method (b) employing NMM (0.83 ml, 7.5 mM) and Piv.Cl (0.85 ml, 7.0 mM) with DMF (10 ml) as solvent. A further portion of NMM (0.56 ml, 5 mM) was added prior to overnight reaction. The crude product was precipitated by passing into 3% aqueous $NaHCO_3$, washed thoroughly with water, dried, and applied to Sephadex LH-20, eluting with DMF. Product fractions were evaporated to an off-white solid (2.9g, 45%); m.p. 219 - 221°; R_f (D) 0.4; HPLC 16.3 min.; $[\alpha]_D^{20}$ - 34.6° (C = 1, TFE); $Lys_{1.00}Asp_{0.96}Glu_{1.06}Ala_{1.00}Ile_{0.96}$; (Found: C, 58.78; H, 8.07; N, 10.42. $C_{45}H_{73}N_7O_{13}$ requires: C, 58.74; H, 8.00; N, 10.66%).

Boc.Lys(Boc).Asp(OBu^t).Gln.Ala.Ile.Lys(Boc).OBu^t (16). The compound (15) (0.683g, 0.74 mM) in DMF (10 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (0.141g, 0.84 mM) and 10% Pd/C (37 mg). Boc.Lys(Boc).OH.DCHA (0.587g, 1.11 mM) was converted to the free acid using citric acid by the standard method. This free acid was coupled to the amino-component by the mixed anhydride method (b) using NMM (0.124 ml, 1.11 mM) and Piv.Cl (0.128 ml, 0.7 mM). The crude product was precipitated by pouring into 3% aqueous $NaHCO_3$, then dried, redissolved in DMF, and applied to Sephadex LH-20, eluting with DMF. Evaporation of fractions containing product gave (16) as a white solid (0.583g, 70%); m.p. 218 - 221°; R_f (B) 0.4; HPLC 17.4 min; $[\alpha]_D^{20}$ - 40.2° (C = 1, TFE); $Lys_{2.01}Asp_{1.01}Glu_{1.01}Ala_{1.00}Ile_{1.00}$; (Found: C, 56.13; H, 8.43; N, 10.96. $C_{53}H_{95}N_9O_{16}.H_2O$ requires: C, 56.22; H, 8.63; N, 11.13%).

$Cl^-H_2^+$Lys(HCl).Asp.Gln.Ala.Ile.Lys(HCl).OH (17). The protected peptide (16) (280 mg, 251 μmol) was dissolved in 90% aqueous TFA (2 ml) at room temperature, stirred 40 min., then the solution evaporated *in vacuo*. Re-evaporation (x 2) from 0.1M hydrochloric acid, and once from water gave a white solid which was applied to Sephadex G-15, eluting with water. Product fractions were lyophilised, giving (17) (103 mg, 47%), rechromatography on Sephadex G-15 gave the pure material (85 mg, 39%) FAB (positive ion) m/z : 702 (M + 1); $Lys_{2.12}Asp_{1.00}Glu_{1.02}Ala_{0.99}Ile_{0.96}$.

AFGII (103 - 108) fragment (23).

Z.Ala.Lys(Boc).OBu^t (18). Z.Lys(Boc).OBu^t (7.5g, 17 mM) in DMF (10 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (3.26g, 17 mM) and 10% Pd/C (850 mg). Z.Ala.OH (4.55g, 20.4 mM) in DMF (15 ml) was then coupled using the pivalic mixed anhydride method (b) employing NMM (2.28 ml, 20.4 mM) and Piv.Cl (2.41 ml 19.5 mM); a further portion of NMM (1.87 ml, 17 mM) was added before overnight reaction. The reaction mixture was evaporated and redissolved in EtOAc. This solution was washed with 10% aqueous citric acid, water, 5% aqueous $NaHCO_3$, water and brine, dried over

Na_2SO_4 and evaporated to a yellow oil, which slowly solidified on standing. Recrystallisation from EtOAc/hexane gave a white solid, (6.04g, 70%); m.p. 125 - 128°; R_f (B) 0.5; HPLC 16.3 min.; $[\alpha]_D^{20}$ - 4.3° (C = 3.5, $CHCl_3$); $Lys_{1.00}Ala_{0.94}$; (Found: C, 61.52; H, 8.31; N, 8.24. $C_{26}H_{41}N_3O_7$ requires: C, 61.52; H, 8.14; N, 8.28%).

Z.Ala.Ala.Lys(Boc).OBu^t (19). Compound (18) (4.2g, 8.3mM) in DMF (10 ml) was subjected to hydrogenolysis (a) in the presence of Tos.OH.H_2O (1.57g, 8.3 mM) and 10% Pd/C (4.15 mg). Z.Ala.OH (2.22 g, 10 mM) was then coupled by method (b) using NMM (1.11 ml, 10 mM) and Piv.Cl (1.18 ml, 9.5 mM) with a second portion of NMM (0.913 ml, 8.3 mM) being added before overnight reaction. The reaction mixture was evaporated and the residue dissolved in EtOAc. This solution was washed with acid and base in the usual way then, after drying, crystallised by the addition of hexane giving (19) (3.02g, 63%); m.p. 110 - 114°; R_f (B) 0.3; HPLC 16.0 min.; $[\alpha]_D^{20}$ - 16.2° (C = 1, DMF); $Lys_{1.00}Ala_{1.97}$; (Found: C, 60.01; H, 8.18; N, 9.74. $C_{29}H_{46}N_4O_8$ requires: C, 60.19; H, 8.01; N, 9.68%).

Z.Gln.Ala.Ala.Lys(Boc).OBu^t (20). The protected peptide (19) (2.89g, 4.9 mM) in DMF (10 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (0.95g, 4.9 mM) and 10% Pd/C (250 mg). Z.Gln.OH (2.1g, 7.5 mM) in DMF (7 ml) was then coupled using the pivalic mixed anhydride method (b) employing NMM (0.839 ml, 7.5 mM), and Piv.Cl (0.718 ml, 7.5 mM), a second portion of NMM (0.559 ml, 4.19 mM) was then added. The reaction mixture was poured into 4% aqueous $KHCO_3$, and the resulting solid filtered. The solid was dissolved in DMF and this solution applied to Sephadex LH-20, eluting with DMF. Evaporation of the product fractions yielded the white solid (20) (2.7 g, 76%); m.p. 193 - 194°; R_f (B) 0.2; HPLC 14.5 min; $[\alpha]_D^{20}$ - 16.2° (C = 1, DMF); $Lys_{1.04}Glu_{1.00}Ala_{1.96}$; (Found: C, 58.05; H, 7.82; N, 11.77. $C_{34}H_{54}N_6O_{10}$ requires: C, 57.77; H, 7.70; N, 11.89%).

Z.Asp(OBu^t).Gln.Ala.Ala.Lys(Boc).OBu^t (21). The preceeding protected peptide (20) (2.7g, 3.8 mM), TosOH.H_2O (0.730g, 3.8 mM) and 10% Pd/C (190 mg) were added to DMF (20 ml) and hydrogenolysis (a) carried out. Z.Asp(OBu^t).OH.DCHA (2.9g, 5.7 mM) was converted to the corresponding free acid in the normal manner and this acid was coupled with the above amino-component by the mixed anhydride method (b) using NMM (0.637 ml, 5.7 mM) and Piv.Cl (0.656 ml, 5.3 mM), a second portion of NMM (0.425 ml, 3.8 mM) was then added. The crude product was precipitated by pouring into 3% aqueous $KHCO_3$. The resulting solid was redissolved in DMF, then precipitated by the addition of water yielding (21), (2.36g, 71%); m.p. 151 - 157°; R_f (B) 0.3; HPLC 15.5 min.; $[\alpha]_D^{20}$ - 17.3° (C = 1, DMF); $Lys_{1.00}Asp_{0.97}Glu_{1.00}Ala_{1.90}$; (Found: C, 56.97; H, 7.76; N, 10.82. $C_{42}H_{67}N_7O_{13}.\frac{1}{2}H_2O$ requires: C, 56.87; H, 7.73; N, 11.05%).

Boc.Gln.Asp(OBu^t).Gln.Ala.Ala.Lys(Boc).OBu^t (22). The protected pentapeptide (21) (1.5g, 1.7 mM) in DMF (10 ml) was hydrogenolysed (a) in the presence of Tos.OH.H_2O (0.325, 1.7 mM) and 10% Pd/C (85 mg). Boc.Gln.OH (0.628g, 2.6 mM) was coupled by the mixed anhydride method (b) using NMM (0.285 ml, 2.6 mM), pyridine (0.206 ml, 2.6 mM) and Piv.Cl (0.293 ml, 2.4 mM) in DMF (5 ml) with a second addition of NMM (0.19 ml, 1.7 mM). The crude product was precipitated with water, filtered and dried. The solid was dissolved in DMF and filtered to remove an insoluble residue. This solution was applied to Sephadex LH20 and eluted with DMF. Evaporation of the product fractions yielded (22) as a white solid (0.68g, 41%), m.p. 222 - 223° (dec.); R_f (E) 0.7; HPLC 13.9 min.; $[\alpha]_D^{20}$ - 37.8 (C = 1, TFE); $Lys_{1.00}Asp_{1.00}Glu_{1.97}Ala_{1.90}$; (Found: C, 54.13; H, 8.14; N, 12.88. $C_{44}H_{77}N_9O_{15}$ requires: C, 54.36; H, 7.98; N, 12.97%).

$Cl^-H_2^+$.Gln.Asp.Gln.Ala.Ala.Lys(HCl).OH (23). The fully protected hexapeptide (22) (2.68 mg, 2.75 μM) was dissolved in 90% TFA (2 ml) and stirred for 1h. at 0°. Evaporation *in vacuo* gave a gum which was twice taken up in 0.1M HCl and evaporated, the residue was evaporated from water. The crude product was purified by gel filtration on Sephadex G-15, eluting with water. Column fractions were analysed by HPLC, and those containing pure product (23) were lyophilised to a white solid (193 mg, 95%); HPLC 8.0 min. (5 min. linear gradient, 0 - 20% MeCN, 1 ml min^{-1}); FAB (positive ion) m/z : 661 (M + 2); $Lys_{1.03}Asp_{1.00}Glu_{1.97}Ala_{1.95}$.

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