Synthesis of a 53-Residue Peptide with EGF Activity

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Mouse epidermal growth factor (EGF), which consists of 53 amino acids with 3 disulphide bridges, was synthesized in a conventional manner by assembling 15 peptide fragments of established purity followed by thioanisole-mediated deprotection with trifluoromethanesulphonic acid in trifluoroacetic acid and subsequent air oxidation.

Epidermal growth factor (EGF) isolated from male mouse submaxillary glands is a peptide consisting of 53 amino acids with 3 disulphide bridges[1] and is known to be a powerful inhibitor of gastric acid secretion,[2] as is urogastrone,[3] a structurally related peptide isolated from human urine.

Based on the early observation that air-oxidation of reduced EGF regenerated the full biological activity with reconstitution of the 3 disulphide bridges,[4] we undertook the synthesis of a linear 53-residue peptide corresponding to the reduced form of EGF with 6 cysteine residues (Figure 1) and were able to isolate, after air-oxidation, a peptide with antigastric activity equivalent to that of natural EGF.

For this synthesis we employed a combination of the TFA (trifluoroacetic acid)-labile N^{α}-Boc (t-butoxycarbonyl) or Z(OMe) (4-methoxybenzyloxycarbonyl) group, and the TFMSA (trifluoromethanesulphonic acid)/TFA[5]-labile protected amino acid derivatives Glu(OBzl), Asp(OBzl), Arg(Mts),[6] Cys[Bzl(OMe)], or Ser(Bzl) [Bzl = benzyl, Mts = mesitylenesulphonyl, Bzl(OMe) = 4-methoxybenzyl]. Of these, the Bzl ester groups of the two Asp residues in positions 11 and 40 were purposely removed by hydrogenolysis at the stage of fragment synthesis, in order to avoid base-catalysed ring-closure of Asp(OBzl), since this side reaction is known to be sequence dependent.[7] The hydroxy groups of the five Ser residues located near the unmasked Asp and His residues were protected as the Bzl ether derivatives since in the synthesis of chicken VIP (vasoactive intestinal polypeptide)[8] Honzl and Rudinger's azide reaction[9] did not proceed satisfactorily, unless the hydroxy group of the Ser residue was masked.

Fifteen relatively small peptide fragments and Boc-His-$NHNH_2$ were selected to construct the linear peptide backbone of EGF, *i.e.*, the C-terminal tripeptide ester [1], the next di-Trp-fragment [2], and thirteen Boc-peptide hydrazides. These fragments were prepared by the known amide-forming procedures. Of these, two hydrazides, [3] and [10], containing a Glu(OBzl) or Asp(OBzl) residue, were prepared with

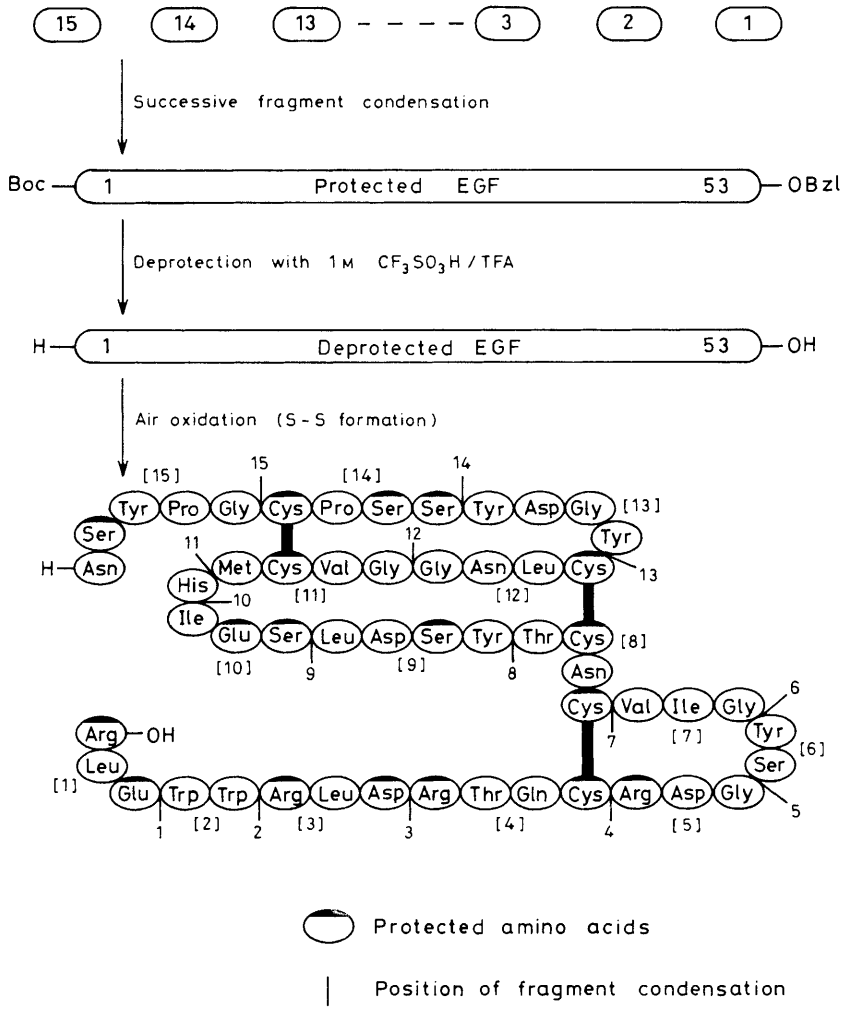

Figure 1. Synthetic scheme for EGF. The protected amino acid derivatives used were: Cys[Bzl(OMe)], Ser(Bzl), Glu(OBzl), Asp(OBzl), and Arg(Mts).

the aid of Troc-$NHNH_2$ (Troc = 2,2,2-trichloroethyloxycarbonyl),[10] the protecting group of which can be removed by Zn[11] or Cd[12] in acetic acid. Fragment [12] possessing the base-sensitive Asn-Gly sequence[13] was also prepared using this substituted hydrazine.

Fragments [1] and [2] were condensed using DCCI (dicyclohexylcarbodi-imide) in the presence of *N*-hydroxybenzotriazole,[14] then the remaining fragments were successively introduced by means of Honzl and Rudinger's azide procedure, in order to minimize racemization. After incorporation of the Trp residues into the chain, the N^{α}-Boc group was removed by TFA in the presence of anisole containing ethanedithiol[15] in order to suppress alkylation at its indole moiety.[16] The His residue (position 22) had to be introduced as one unit since, for example, the azide condensation of Boc-His-Ile-Glu(OBzl)-Ser(Bzl)-$NHNH_2$ was disappointing, because of poor incorporation of the acyl component. A similar tendency has been reported in the synthesis of the structurally related compound, urogastrone.[17]

The protected intermediates (with the exception of two compounds) were purified by precipitation from DMF (dimethylformamide) or a mixture of DMF and DMSO (dimethyl sulphoxide) with appropriate solvents, such as MeOH or AcOEt. The protected peptide (positions 14—53) and the protected EGF were purified by gel-filtration on Sephadex LH-60 using DMSO–DMF (3:7) as an eluant. Throughout this synthesis, Leu was used as a diagnostic amino acid: each intermediate was subjected to acid hydrolysis and the recovery of Leu was compared with those of the newly added amino acids, in order to ascertain satisfactory incorporation, after each condensation. The homogeneity of every intermediate was further ascertained by elemental analysis and t.l.c.

In the final step, the Cys[Bzl(OMe)] sulphoxide,[18] as well as the Met sulphoxide,[19] which had partially formed during the synthesis, were reduced by benzenethiol.[18] The protected EGF thus obtained was treated with 1 M TFMSA–thioanisole/TFA in the presence of *m*-cresol (0 °C for 120 min). This thioanisole-mediated treatment[20] was repeated twice more to ensure complete deprotection. The deprotected peptide was reduced with dithiothreitol in 6 M guanidine–HCl buffer at pH 8.0 and, after gel-filtration on Sephadex G-10, was submitted to air oxidation to form 3 disulphide bridges according to Taylor *et al.*[4] A diluted solution (peptide concentration 0.07 mg/ml) in 0.20 M $AcONH_4$ buffer at pH 8.2 was allowed to stand at 25 °C for 7 days and the progress of the reaction was monitored by Ellman's test.[21] The crude product was then purified by ion-exchange chromatography on

DEAE-cellulose followed by h.p.l.c. on Cosmosil $5C_{18}$ according to the methods of Savage *et al.*[1] and Matrisian *et al.*[22] respectively.

The purified peptide (yield 4.1% from the reduced form of the protected EGF) exhibited a single peak with the same retention time (12.5 min) as that of natural EGF in h.p.l.c. on a Cosmosil $5C_{18}$ (4.6 × 160 mm) column using isocratic elution (flow rate, 0.4 ml per min) with 30% acetonitrile in 0.05 M AcOH–Et_3N (pH 5.6) at 40 °C, and a single band with the same mobility as that of natural EGF in disk isoelectrofocusing (Pharmalite pH 3—10). It also exhibited a sharp immunodiffusion band against anti-natural EGF sera, and its amino acid ratios in 6 M HCl hydrolysate were in excellent agreement with those predicted by theory.

When tested in Shild rats (sample size 6), our synthetic peptide (30 μg/kg) suppressed the acid output stimulated by histamine (taken as 100% using 500 μg/kg) to 66.5% (68.6% using the same dose of natural EGF).

It is interesting that when air oxidation was performed in the presence of glutathione according to Scheraga *et al.*,[23] a relatively stable glutathione adduct (2 mol of glutathione per mol of EGF) was isolated. The anti-gastric activity of this adduct was one fifth of that of natural EGF.

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