Total Synthesis of Urogastrone (Human Epidermal Growth Factor, h-EGF)

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The first total synthesis of urogastrone (h-EGF) has been achieved by the segment condensation method in solution applying the maximum protection strategy.

Urogastrone (human epidermal growth factor, h-EGF, Figure 1), isolated from human urine as a potent inhibitor of gastric acid secretion, is a polypeptide of 53 amino acid residues with 3 disulphide bonds.[1] This polypeptide has received a renewed interest in recent years since it was found that mouse EGF,† structurally related to h-EGF with 70% homogeneity (37 of 53 amino acids), stimulates the proliferation and differentiation of cells of ectodermal and mesodermal origin.[3] However, the biological evaluation of this peptide has been hampered in the past owing to a great difficulty in isolating it from human urine. Herein we report the first total synthesis of urogastrone, which is capable of providing the amounts necessary for biological and biochemical studies.

† The synthesis of m-EGF has recently been reported by Yajima *et al.* (ref. 2).

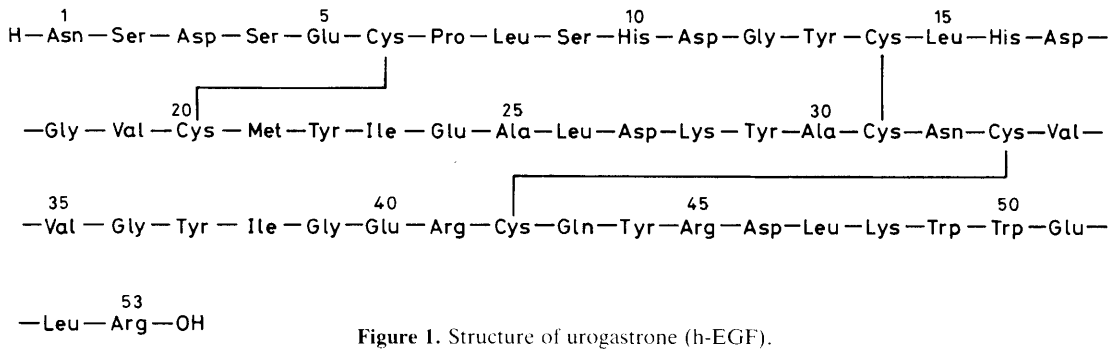
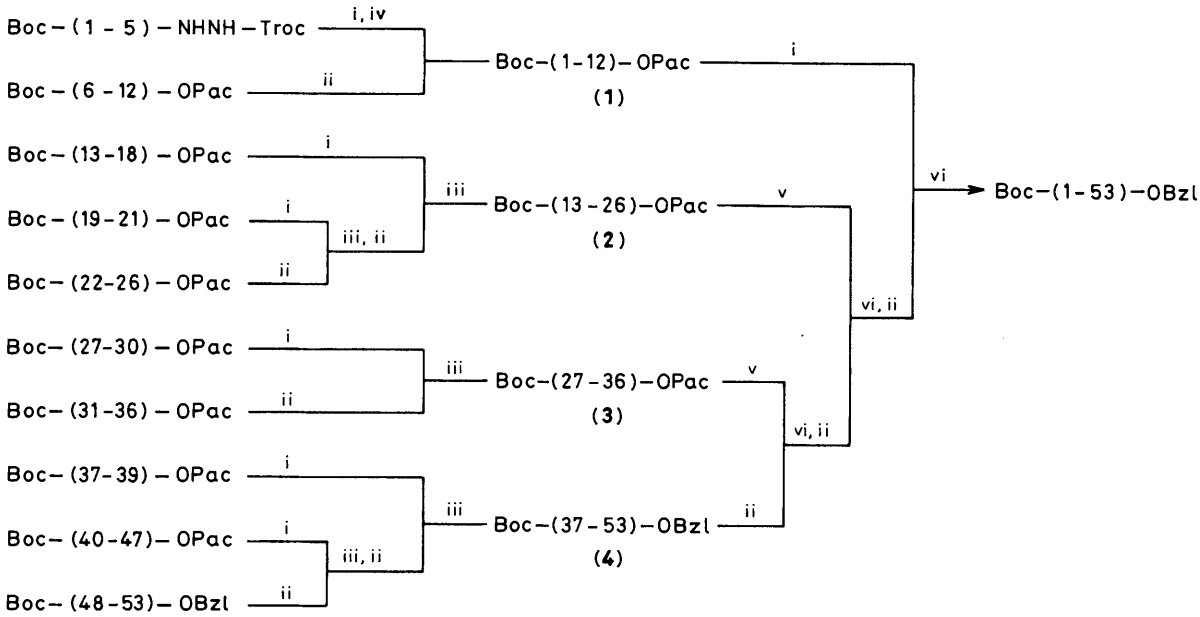


Figure 1. Structure of urogastrone (h-EGF).



Scheme 1. Synthetic scheme of the protected urogastrone. *Reagents:* i, Zn–AcOH/DMF; ii, (1) TFA–anisole, (2) HCl/dioxane; iii, WSCD–HOBt/DMF; iv, Bu^{t}ONO/DMF; v, Zn–anthranilic acid–pyridine/NMP–DMSO; vi, WSCD–HOBt/NMP.

The polypeptide chain was constructed from 10 segments as indicated in Scheme 1. These segments were synthesised by the conventional solution method using t-butoxycarbonyl (Boc) for the amino protecting group and phenacyl (Pac) for the carboxy protecting group except for the segment (48–53) which was protected as the benzyl (Bzl) ester and segment (1—5) used as the 2,2,2-trichloroethoxycarbonyl (Troc) hydrazide.[4] All the side-chain functional groups were protected as follows: Glu(OBzl), Asp(OcHex: cyclohexyl), Ser(Bzl), His(Tos: tosyl), Arg(Tos), Tyr(Cl_2Bzl: 2,6-dichlorobenzyl), Lys(ClZ: 2-chlorobenzyloxycarbonyl), and Trp(CHO). Cys was protected with acetamidomethyl (Acm)[5] which could be cleaved with $Hg(OAc)_2$ without affecting the other groups. The Tos group for His was cleaved with pyridine·HCl[6] after incorporation into the peptides.

These small segments were then coupled, after removal of the Boc group with trifluoroacetic acid (TFA)–anisole and the Pac and Troc groups with Zn–AcOH/dimethylformamide (DMF), to make 4 larger segments [(1—12) (**1**), (13—26) (**2**), (27—36) (**3**), and (37—53) (**4**)]. The condensation reactions were carried out in DMF using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSCD)–1-hydroxybenzotriazole (HOBt) method[7] for (**2**), (**3**), and (**4**) and the azide method for (**1**) in satisfactory yields, respectively. Racemization did not occur (h.p.l.c.) during these condensation reactions.

These large segments were further coupled to make the full sequence of urogastrone after removal of the Pac ester groups in (**1**), (**2**), and (**3**) as follows. The ester group in (**1**) was

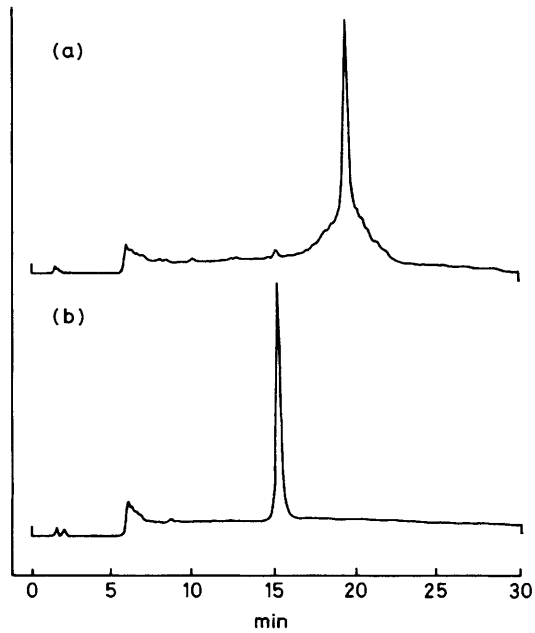
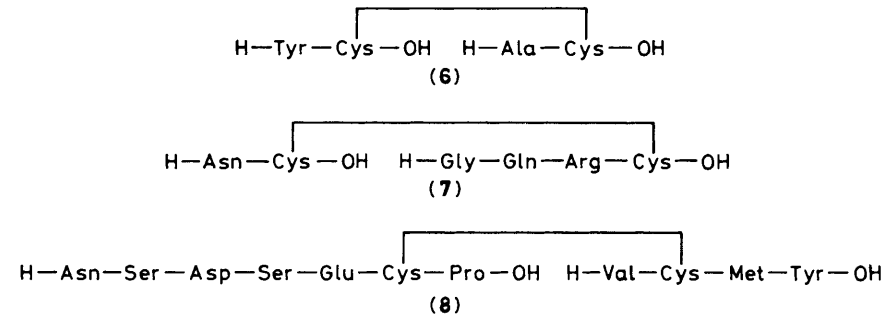


Figure 2. H.p.l.c. profile of the purified [Cys(Acm)]$_6$-urogastrone (a) and the purified urogastrone (b). Column: Nucleosil 5C_{18} (4 × 150 mm); eluant: 0.1% aqueous TFA, in which acetonitrile concentration was increased linearly within 30 min from 10% to 70%; flow rate: 1.0 ml/min; detection: absorbance at 210 nm.



removed with Zn–AcOH/DMF to give its free acid in 88% yield, although the applications of this method to (**2**) and (**3**) were unsuccessful owing to their poor solubility in DMF. It was found, however, that a method using Zn–anthranilic acid/pyridine was effective for the removal of the Pac groups in (**2**) and (**3**) in a mixture of *N*-methylpyrrolidone (NMP) and dimethyl sulphoxide (DMSO).‡ Thus, the ester group in (**2**) was removed in a 20 : 5 : 1 ratio of NMP–DMSO–pyridine at 35 °C for 6 h in 92% yield,§ while (**3**) was cleaved in a 2 : 1 : 1 ratio of the same solvents at 50 °C for 2 h in 86% yield. The resulting free acids of (**1**), (**2**), and (**3**) were in turn coupled to (**4**) also by the WSCD–HOBt method in NMP to afford the fully protected urogastrone in 83% overall yield from (**4**).

On treatment with liquid HF in the presence of *p*-cresol, ethane-1,2-dithiol (EDT), Me_2S (10 : 1 : 1 : 0.5 v/v), and Met (20 equiv.) at 0 °C for 1 h, the protected urogastrone was converted into [Cys(Acm)]$_6$–urogastrone (**5**) (in crude form).¶ Purification on DEAE-cellulose (0.02—0.2 M $AcONH_4$, 8 M urea, pH 5.6) and Diaion HP-20AG gave (**5**) (26% from the protected urogastrone), whose h.p.l.c. is shown in Figure 2(a). The byproducts, such as aminosuccinyl and alkyl products, arising from the HF treatment were removed during these purification processes.[7] The amino acid sequence of (**5**) was confirmed by peptide mapping of a tryptic digestion, which gave, on h.p.l.c., 7 expected peptides including those split at the ^{37}Tyr–^{38}Ile linkage.[1d] These peptides were isolated and characterized by amino acid analysis. Amino acid analyses of (**5**) itself by acid (6 M HCl, 2% phenol, 110 °C, 24 h) and enzyme (aminopeptidase M and α-chymotrypsin) were also in good agreement with the theoretical values, thus confirming the structure of (**5**).

Finally, the Acm groups of (**5**) were cleaved with $Hg(OAc)_2$ in 50% AcOH for 6 h and, after removal of the Hg ion with 2-mercaptoethanol, the product was purified by gel-filtration on Sephadex G-25 (1 M AcOH). The resulting cysteine form of urogastrone was successively subjected to air oxidation in a highly diluted solution (4.5×10^{-6} M, 0.01 M $AcONH_4$, pH 7.6) to provide urogastrone (in crude form). Purification of this material by preparative h.p.l.c. (or DEAE-cellulose[8]) afforded, in 10% yield (7.9 mg) from (**5**) (83 mg), pure urogastrone [$C_{270}H_{395}N_{73}O_{83}S_7$; fast atom bombardment mass spectrum (ZAB-SE): obs., 6216.74; calc. 6219.95 (MH^+); amino acid analysis (4 M $MeSO_2OH$, tryptamine, 110 °C, 24 h): Asp(7) 6.82, Cys(6) 5.91, Met(1) 0.83, Ser(3) 3.11, Glu(5) 4.69, Gly(4) 4.17, Ala(2) 2.09, Val(3) 2.34, Ile(2) 1.77, Leu(5) 5.00, Tyr(5) 4.65, Lys(2) 1.91, His(2) 2.20, Trp(2) 1.89, Arg(3) 2.87, Pro(1) 1.19 (Cys was quantified by amino acid analysis after performic acid oxidation)], whose h.p.l.c. is shown in Figure 2(b). Enzymic digest (thermolysin, 45 °C, pH 6.5) gave 3 cystine-containing peptides (**6**)—(**8**), which were isolated by h.p.l.c. and characterized by amino acid analysis, proving that the disulphide bonds were formed at the desired positions. The synthetic product was thus shown to have the structure proposed for natural urogastrone.[1d]

The synthetic urogastrone which could be produced in significant amounts showed receptor binding affinity on rat liver cell membranes, it stimulated DNA synthesis of gastric cells (measured by ^{3}H-thymidine incorporation), and it inhibited gastric acid secretion stimulated by gastrin in Heidenhain pouch dog.

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‡ This particular method could be fully applicable for removal of the Pac group in sparingly soluble peptides. Details will be reported elsewhere.

§ The formation of the aminosuccinyl peptide was minimized by limiting the ratio of pyridine.

¶ An examination revealed that the presence of both EDT and Me_2S is effective for the complete deprotection of the formyl group of Trp. The alkylations of Tyr were kept to a minimum by the addition of *p*-cresol. The formation of aminosuccinyl compounds especially at the Asp–Gly residues was also minimised under these conditions.

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