Total Synthesis of Urogastrone (Human Epidermal Growth Factor, h-EGF). Part 2.[1] Synthesis of Urogastrone having the Structure Proposed for the Natural Compound

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Deprotection of the fully protected urogastrone was carried out by a two-step deprotection strategy. In the first step the protecting groups of all side-chain functional groups except for the cysteine thiol were removed by treatment with HF and the resulting $(S\text{-Acm})_6$-urogastrone was purified by ion-exchange chromatography prior to the disulphide bond formation. In the second step the six Acm groups of the purified $(S\text{-Acm})_6$-urogastrone were removed by $Hg(OAc)_2$. After formation of disulphide bonds by air oxidation, the synthetic urogastrone was purified by ion-exchange chromatography and gel filtration. The structure of the synthetic urogastrone was further characterized by amino acid analysis, tryptic peptide mapping, thermolytic peptide mapping, and fast atom bombardment mass spectroscopy.

In view of the outstanding biological activity of urogastrone (human epidermal growth factor, h-EGF), a polypeptide of 53 amino acid residues with three disulphide bonds[2] (Figure 1), we have undertaken its chemical synthesis in order to obtain sufficient to allow detailed biological and biochemical studies. In the preceding paper,[1] we described the synthesis of the protected polypeptide (**20**) corresponding to the full sequence of urogastrone. This paper is concerned with removal of the protecting groups in (**20**) and formation of the disulphide bonds to complete the synthesis of urogastrone.

As described in the preceding paper, we adopted a two-step deprotection strategy applying a maximum protection procedure for our synthesis of urogastrone. The cysteine thiol groups were protected with the Acm[3] group which could be removed by treatment with $Hg(OAc)_2$ and the other side-chain functional groups were masked with the protecting groups removable by treatment with HF.

Urogastrone contains one Met, two Trp, and five Tyr residues, which are likely to suffer side reactions (alkylation) during the deprotections. In order to minimize these side reactions, we adopted a procedure whereby an excess of Met (for protection of Met in the sequence) and an excess of *p*-cresol[4] (for Tyr) were present during the treatment with HF; the Boc group was removed by treatment with TFA before the HF treatment.[5]

In order to avoid alkylation of Trp,[6] it was protected by formylation of the indole nitrogen[7,8] (see the preceding paper). The deprotection of this formyl group was found to be complete by addition of EDT,[9] Me_2S,[10] and Met on treatment with HF. This was verified by using the fragment, Boc-(40—53)-OBzl as follows. When it was treated with HF in the presence of *p*-cresol and EDT (HF:*p*-cresol:EDT = 10:1:1), the product was accompanied by a minor product in which the Trp residues still carried the formyl group (Figure 2). In contrast, the addition of Me_2S and Met to this mixture (HF:*p*-cresol:EDT:Me_2S:Met = 20:2:2:1:20 equiv.) completed the deprotection of the formyl group in the Trp residues (Figure 2).

A further tedious problem in the synthesis of urogastrone, which contains two sequences of Asp-Gly and one sequence of Asp-Ser, is the succinimide formation in these sequences during the HF treatment.[11] Fortunately, adoption of the Chx[12] group for the Asp protection (as described in the preceding paper) was found to keep this side reaction to a minimum. When the fragments Boc-(1—12)-OH (**17**) and Boc-(13—26)-OH (**16**)

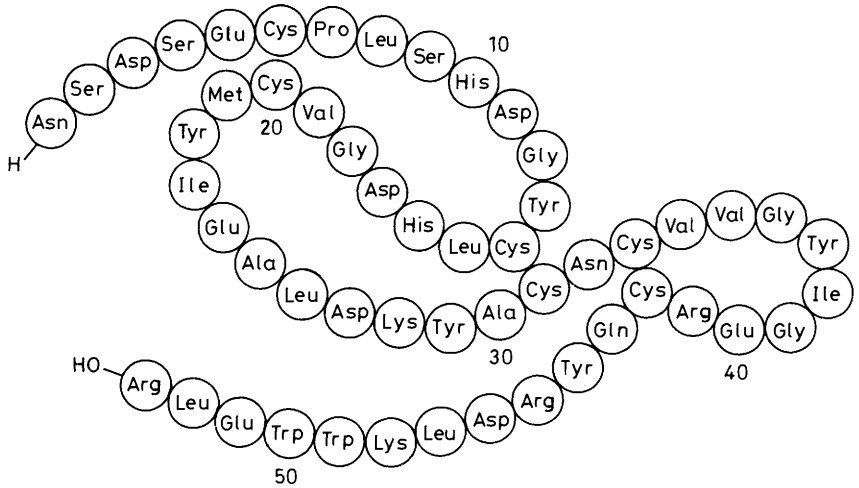


Figure 1. Structure of urogastrone (h-EGF)

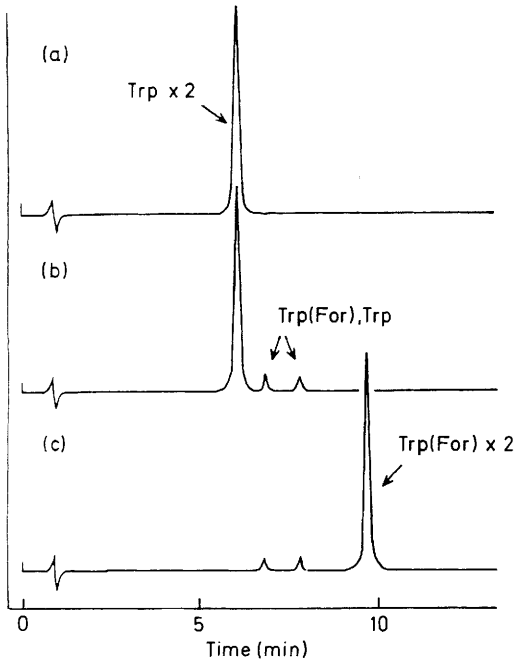


Figure 2. H.p.l.c. profile of the deprotected fragment (40—53). (a) HF–*p*-cresol–EDT–Me_2S–Met (20:2:2:1:20 equiv., 0 °C, 1 h); (b) HF–*p*-cresol–EDT (10:1:1, 0 °C, 1 h); (*c*) HF–*p*-cresol (10:1, 0 °C, 1 h). Column: Nucleosil $5C_{18}$ (4 × 150 mm; eluant; 23% MeCN in 0.1M phosphate buffer (pH 4.8); flow rate: 1.0 ml/min; detection: absorbance at 210 nm

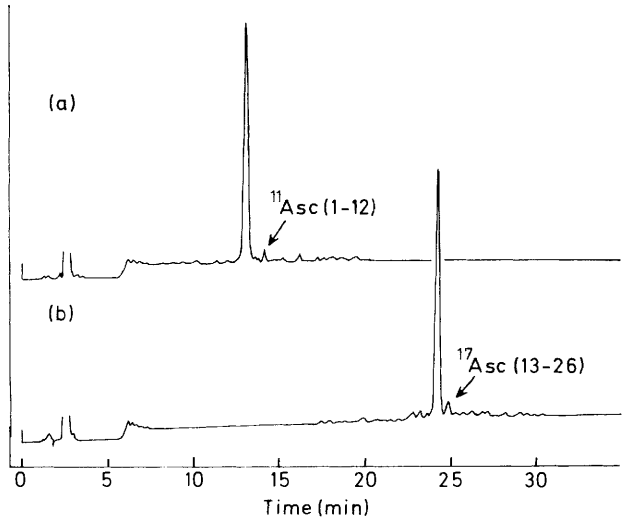
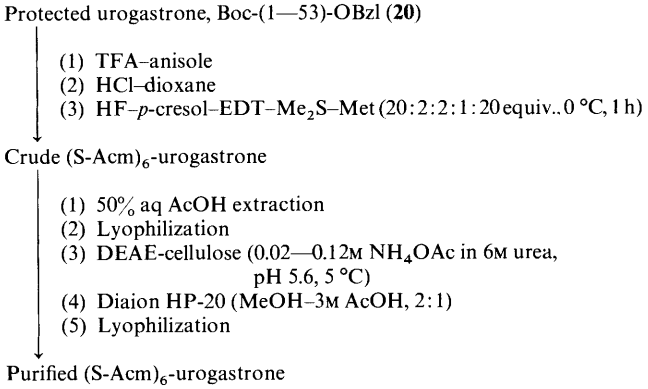


Figure 3. H.p.l.c. profile of the deprotected fragment (1—12) (a) and (13—26) (b). Column: Nucleosil $5C_{18}$ (4 × 150 mm); eluant: 0.1% aqueous TFA–MeCN (99:1)/0.1% aqueous TFA–MeCN (50:50) (linear gradient, 30 min); flow rate: 1.0 ml/min; detection: absorbance at 210 nm.

containing one Asp-Gly sequence, respectively, were treated with HF in the presence of *p*-cresol, EDT, Me_2S, and Met (the above ratio), the succinimide formations were suppressed to only 1.2 and 2.1%, respectively, as shown in Figure 3.

With these preliminary experimental results in hand, we prepared the linear $(S\text{-}Acm)_6$-urogastrone according to Scheme 1. After the Boc group in the fully protected urogastrone (**20**) was removed by treatment with TFA–anisole, the peptide was treated with HF in the presence of *p*-cresol, EDT, Me_2S, and Met (HF:*p*-cresol:EDT:Me_2S:Met = 20:2:2:1:20 equiv.) at 0 °C for 1 h. Removal of HF under reduced pressure, followed



Scheme 1. Synthesis and purification of $(S\text{-}Acm)_6$-urogastrone

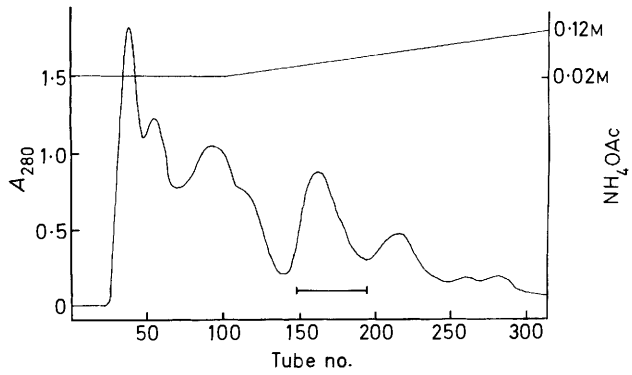


Figure 4. Purification of the crude $(S\text{-}Acm)_6$-urogastrone by ion-exchange chromatography on DEAE-cellulose. Column: 3.5 × 50 cm; eluant: 0.02M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 000 ml) and a linear gradient starting from 0.02M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 200 ml) to 0.12M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 200 ml); detection: absorbance at 280 nm. The $(S\text{-}Acm)_6$-urogastrone fraction is indicated by a bar

by trituration with ether gave a powder, which was dissolved in 50% aqueous AcOH and, after removal of the insoluble materials by filtration; the filtrate was lyophilized to give crude $(S\text{-}Acm)_6$-urogastrone. This product was dissolved in 0.02M NH_4OAc buffer (pH 5.6) containing 6M urea and applied to a column of DEAE cellulose eluting with 0.02M–0.12M NH_4OAc (linear gradient) and 6M urea at 5 °C. The eluants were monitored by observation of the u.v. absorbance at 280 nm (Figure 4). The fraction (tube No. 148—195) was desalted by chromatography on Diaion HP-20 and lyophilized to give pure $(S\text{-}Acm)_6$-urogastrone in 9.8% yield. The h.p.l.c. profile of the crude and the purified products are shown in Figure 5.

The homogeneity and the primary structure of the synthetic $(S\text{-}Acm)_6$-urogastrone were confirmed by amino acid analysis and peptide mapping of a tryptic digestion on h.p.l.c. The amino acid composition after acidic hydrolysis with 6M HCl containing 5% phenol at 110 °C for 24 h and enzymic hydrolysis with trypsin, α-chymotrypsin, and leucine aminopeptidase M at 37 °C for 24 h were in agreement with the calculated value (Table 1).

The digestion of $(S\text{-}Acm)_6$-urogastrone by TPCK-trypsin in a 0.1M NH_4HCO_3 solution at 37 °C for 30 min produced seven peptide fragments, which were isolated by semipreparative h.p.l.c. and characterized by amino acid analysis (Figure 6, and Table 2). The amino acid compositions of T-7 (1—28), T-5 (29—41), T-3 (42—45), T-1 (46—48), and T-6 (49—53) were consistent with the theoretical values of the tryptic peptides expected from the structure of $(S\text{-}Acm)_6$-urogastrone. T-4 (29—

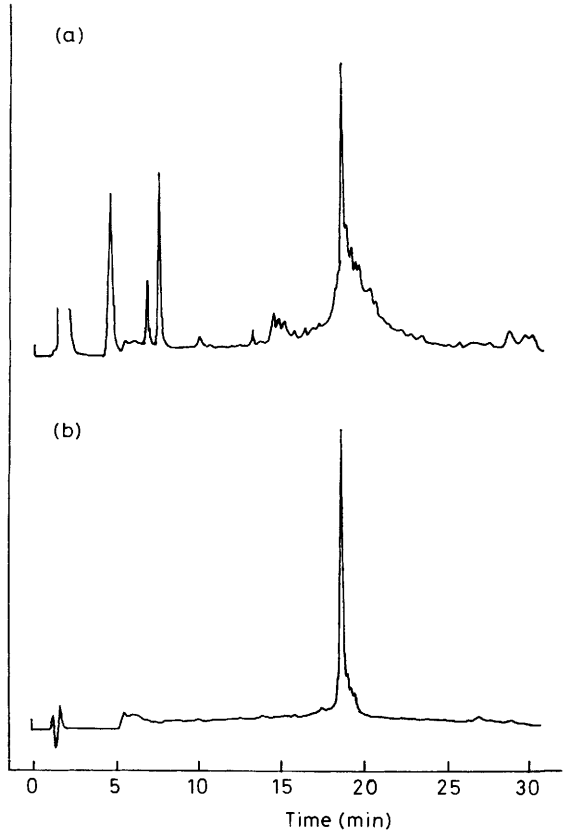


Figure 5. H.p.l.c. profile of the $(S\text{-}Acm)_6$-urogastrone. (a) the crude $(S\text{-}Acm)_6$-urogastrone, (b) DEAE-cellulose purified $(S\text{-}Acm)_6$-urogastrone. Column; Nucleosil $5C_{18}$ (4 × 150 mm); eluant: 0.1% aqueous TFA–MeCN (90:10)/0.1% aqueous TFA–MeCN (30:70) (linear gradient, 30 min); flow rate: 1.0 ml/min; detection: absorbance at 210 nm

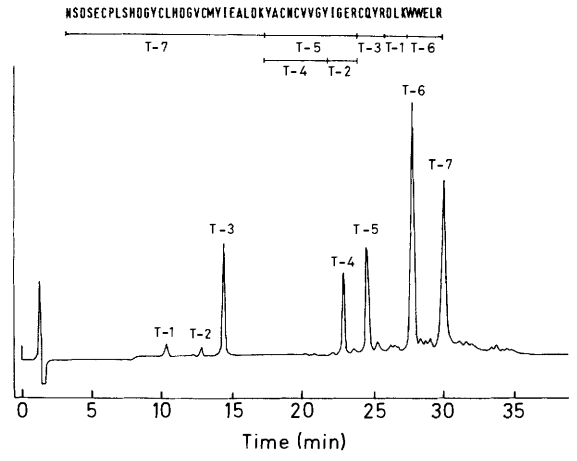


Figure 6. Analytical h.p.l.c. profile of tryptic fragments of purified $(S\text{-}Acm)_6$-urogastrone. Column: Nucleosil $5C_{18}$ (4 × 150 mm); eluant: 0.1% aqueous TFA–MeCN (99:1)/0.1% TFA–MeCN (60:40) (linear gradient, 40 min); flow rate: 1.0 ml/min; detection; absorbance at 210 nm

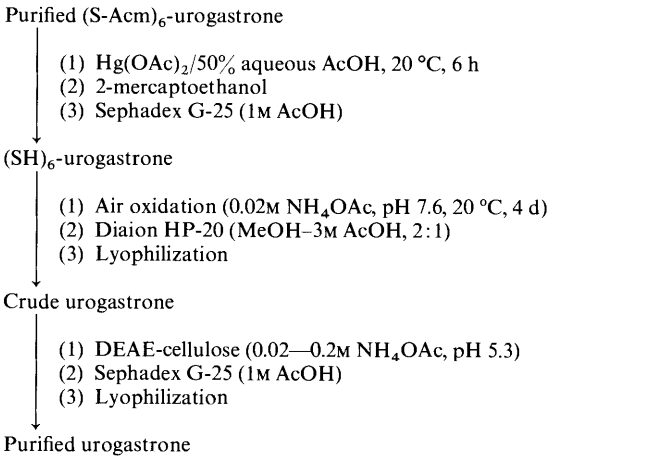
37) and T-2 (38—41) were probably generated from T-5 by a residual α-chymotrypsin activity of the TPCK-trypsin. Thus, the primary structure of the purified $(S\text{-}Acm)_6$-urogastrone was shown to be identical with that of the natural urogastrone.

We next focused on the deprotection of the Acm group in the Cys residues and formation of the disulphide bonds (Scheme 2). The purified linear $(S\text{-}Acm)_6$-urogastrone was treated with

Table 1. Amino acid analysis of purified $(S\text{-}Acm)_6$-urogastrone

Residue	Acid hydrolysis	Enzymic hydrolysis
Asp	6.90 (7)	4.92 (5)
Asn, Gln		3.26 (3)
Ser	2.79 (3)	3.48 (3)
Glu	4.97 (5)	4.14 (4)
Pro	1.12 (1)	1.08 (1)
Gly	3.93 (4)	4.28 (4)
Ala	2.25 (2)	2.58 (2)
Cys	1.14 (6)[a]	
Val	2.84 (3)	3.30 (3)
Met	0.91 (1)	0.99 (1)
Ile	1.88 (2)	2.11 (2)
Leu	5.00 (5)	5.00 (5)
Tyr	5.01 (5)	4.84 (5)
Lys	2.05 (2)	2.19 (2)
His	2.17 (2)	*b*
Arg	2.80 (3)	2.50 (3)
Trp	1.15 (2)[a]	1.92 (2)
Cys(Acm)		4.98 (6)

[a] The lower values for Cys(Acm) and Trp may be due to their decompositions during the acid hydrolysis. [b] The value of His was not determined because of its overlap with the NH_3 peak.



Scheme 2. Synthesis and purification of urogastrone

$Hg(OAc)_2$ (18-fold excess) in 50% aqueous AcOH at 20 °C for 6 h in order to remove the Acm group;[3] after removal of the mercury ions by treatment with a large excess of 2-mercaptoethanol, the product was purified by gel-filtration on Sephadex G-25 (1M aqueous AcOH). The resulting $(SH)_6$-urogastrone was successively subjected to aeriol oxidation at a concentration of 4.5×10^{-6}M in a 0.02M NH_4OAc buffer (pH 7.6) at 20 °C for 4 days with gentle stirring. The progress of the disulphide bond formation was monitored by using the Ellman reagent[13] and h.p.l.c. The crude urogastrone was isolated by chromatography on Diaion HP-20 and lyophilization in 55.8% yield from $(S\text{-}Acm)_6$-urogastrone. The h.p.l.c. profile of the reduced form of urogastrone and the crude urogastrone were shown in Figure 7. The crude peptide was purified by chromatography on DEAE-cellulose eluting with 0.02—0.2M NH_4OAc (linear gradient).[14] After the elution curve had been monitored by observation of the u.v. absorption at 280 nm and analysed by h.p.l.c., the fractions (tubes nos. 197—218, Figure 8) was pooled, desalted by gel-filtration on Sephadex G-25 (1M aqueous AcOH), and lyophilized. The yield of the purified urogastrone was 25.2% from $(S\text{-}Acm)_6$-urogastrone.

The structure of the synthetic urogastrone was further con-

Table 2. Amino acid compositions of tryptic fragments of purified (S-Acm)$_6$-urogastrone

Position / Residue	T-1 (46—48)	T-2 (38—41)	T-3 (42—45)	T-4 (29—37)	T-5 (29—41)	T-6 (49—53)	T-7 (1—28)
Asp	1.00						3.78
Asn				1.01	0.53		0.78
Ser							2.93
Glu		1.07			1.13	1.01	2.00
Gln			0.85				
Pro							0.98
Gly		1.09		1.37	2.12		1.81
Ala				1.48	1.33		1.06
Val				2.00	2.00		1.00
Met							0.88
Ile		1.00			1.08		0.97
Leu	1.03					1.00	2.80
Tyr			1.00	1.77	1.92		1.80
Lys	0.97						0.99
His							2.19
Arg		0.98	0.96		0.98	0.88	
Trp						1.72	
Cys(Acm)			1.21	1.82	2.43		2.32

Table 3. Amino acid analysis of purified urogastrone

Residue			
Asp	6.82 (7)	Ile	1.77 (2)
Ser	3.11 (3)	Leu	5.00 (5)
Glu	4.69 (5)	Tyr	4.65 (5)
Pro	1.19 (1)	Lys	1.91 (2)
Gly	4.17 (4)	His	2.20 (2)
Ala	2.09 (2)	Trp	1.89 (2)
Val	2.34 (2)	Arg	2.89 (3)
Met	0.83 (1)	Cya	5.91 (6)[a]

[a] The cysteine residues were independently quantified as cysteic acid (Cya).

Table 4. Amino acid compositions of thermolytic fragments of purified urogastrone

Th-1: H-Tyr-Cys-OH H-Ala-Cys-OH

Residue	Th-1
Cya	2.00 (2)
Ala	0.88 (1)
Tyr	0.75 (1)

Th-2: H-Asn-Cys-OH H-Gly-Glu-Arg-Cys-OH

Residue	Th-2
Cya	2.00 (2)
Asp	0.89 (1)
Glu	0.95 (1)
Gly	0.85 (1)
Arg	0.77 (1)

Th-3: H-Asn-Ser-Asp-Ser-Glu-Cys-Pro-OH H-Val-Cys-Met-Tyr-OH

Residue	Th-3	Residue	Th-3
Cya	2.00 (2)	Pro	1.16 (1)
Asp	2.18 (2)	Val	1.21 (1)
Ser	2.20 (2)	$MetO_2$	1.16 (1)
Glu	1.19 (1)	Tyr	0.67 (1)

firmed by amino acid analysis and fast atom bombardment mass (f.a.b.m.s.) spectroscopy as follows. The amino acid analysis of the synthetic urogastrone was carried out after acid hydrolysis with 4M methanesulphonic acid containing 2% tryptamine at 110 °C for 24 h in order to recover the Trp residues. The cysteine residues were independently quantified as cysteic acid after performic acid oxidation followed by acid hydrolysis. Its amino acid ratios were in agreement with the expected values as shown in Table 3. To make sure that the synthetic urogastrone is a monomeric form and has the structure shown in Figure 1, its molecular weight was measured by f.a.b.m.s. (ZAB-SE). Figure 9 shows the spectrum in which the protonated molecular ion appeared at 6 216.74 corresponding to the calculated chemical mass of 6 216.95 ($C_{270}H_{395}N_{73}O_{83}S_7$ + H).

To confirm that the synthetic urogastrone has the correct disulphide linkages, it was digested with thermolysin in 0.1M pyridine–AcOH buffer (pH 6.5) at 45 °C for 24 h. All of the peptide fragments obtained from this thermolytic digestion were isolated by h.p.l.c. (Figure 10) and submitted to amino acid analysis after performic acid oxidation. Their amino acid compositions indicated that the cystine-containing peptides were Th-1 (tetrapeptide, ^{14}Cys-^{31}Cys), Th-2 (hexapeptide, ^{33}Cys-^{42}Cys), and Th-3 (undecapeptide, ^{6}Cys-^{20}Cys) as shown in Table 4 and the disulphide bonds were formed at the desired positions. The synthetic urogastrone was thus shown to have the structure proposed for the natural urogastrone by Gregory *et al.*[2]

We have thus been able to synthesize urogastrone chemically by a method which produces significant amounts for biological and biochemical evaluation. The synthetic urogastrone showed receptor binding affinity on rat liver cell membranes, it stimulated DNA synthesis of gastric cells, and it inhibited gastric acid secretion stimulated by gastrin in Heidenhain pouch dog. Details will be reported in due course.

Experimental

Optical rotation was determined on a JASCO DIP-140 polarimeter. The amino acid compositions of acidic and enzymic hydrolysates were determined with a Hitachi model 835 amino acid analyser and are not corrected for amino acid destruction. U.v. absorption was measured by a Hitachi model 220 spectrophotometer. Fast atom bombardment mass spectroscopy was measured on a ZAB-SE (VG anaytical Co.).

H.p.l.c. was carried out on a Hitachi model 655 liquid

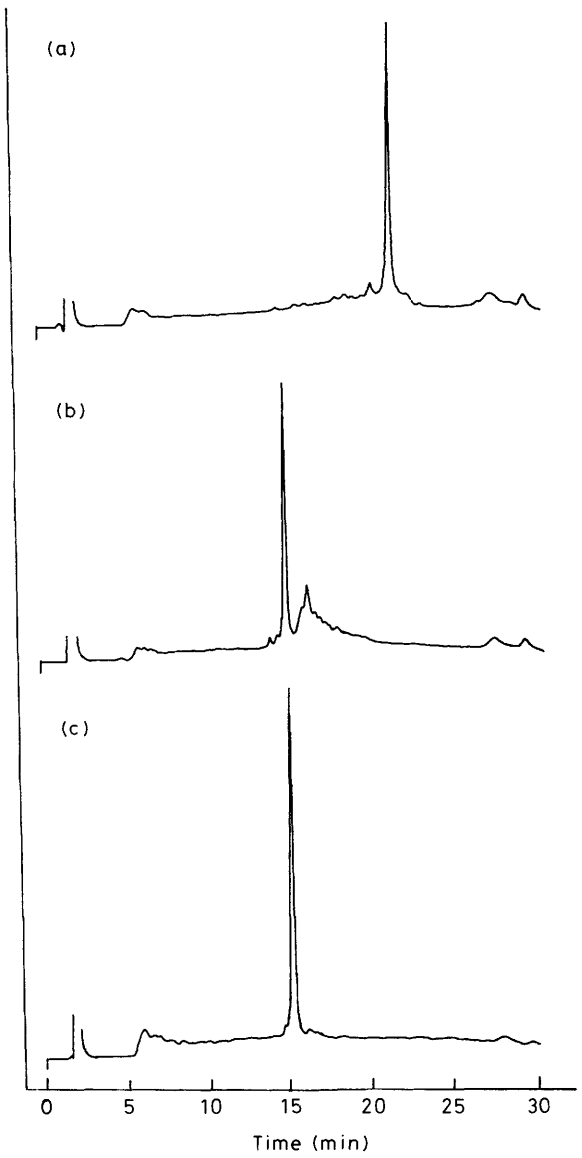


Figure 7. H.p.l.c. profile of synthetic urogastrone: (a) the reduced form of urogastrone, (b) the crude urogastrone, (c) the DEAE-cellulose purified urogastrone. Column: Nucleosil $5C_{18}$ (4 × 150 mm); eluant: 0.1% aqueous TFA–MeCN (90:10)/0.1% aqueous TFA–MeCN (30:70) (linear gradient, 30 min); flow rate: 1.0 ml/min; detection: absorbance at 210 nm

chromatography system by using a Nucleosil $5C_{18}$ (4 × 150 mm) column or a Cosmosil $5C_{18}$ (10 × 250 mm) column with u.v. detection at 210 nm. The solvent system were as follows: eluant A, 23% MeCN in 0.1M phosphate buffer (pH 4.8); eluant B, 0.1% aqueous TFA–MeCN (99:1)/0.1% aqueous TFA–MeCN (50:50) (linear gradient); eluant C, 0.1% aqueous TFA–MeCN (90:10)/0.1% aqueous TFA–MeCN (30:70) (linear gradient); eluant D, 0.1% aqueous TFA–MeCN (99:1)/0.1% aqueous TFA–MeCN (60:40) (linear gradient).

Trypsin, α-chymotrypsin, and leucine aminopeptidase M were purchased from Shigma chemical Co. TPCK-trypsin was purchased from Worthington Biochemical Co. Thermolysin was obtained from Seikagaku Kogyo Co.

Effect of Additives on Removal of the Formyl Groups in Fragment (40—53).—Fragment Boc-(40—53)-OBzl (60 mg) was divided into three equal parts and each was treated with (1)

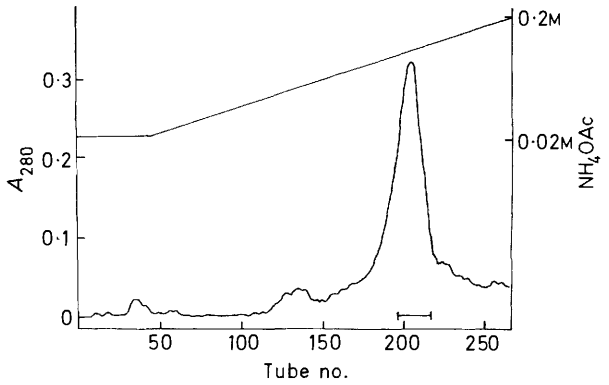


Figure 8. Purification of the crude urogastrone by ion-exchange chromatography on DEAE-cellulose. Column: 2.2 × 50 cm; eluant: 0.02M NH_4OAc buffer (pH 5.3; 200 ml) and a linear gradient starting from 0.02M NH_4OAc buffer (pH 5.3; 500 ml) to 0.2M NH_4OAc buffer (pH 5.3; 500 ml); detection: absorbance at 280 nm

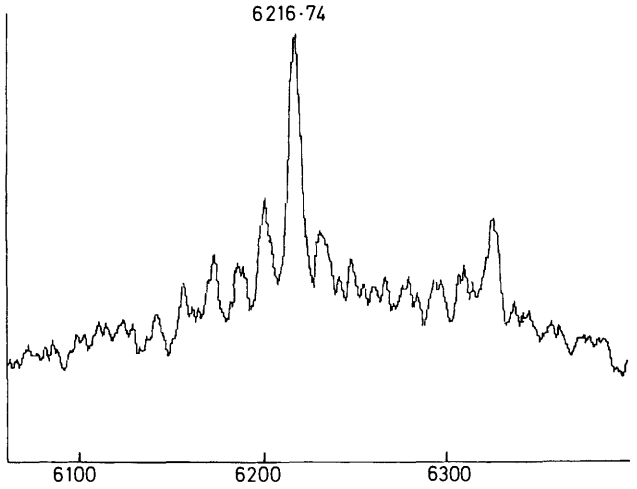


Figure 9. Molecular ion for purified urogastrone obtained by f.a.b.m.s. on a VG ZAB SE mass spectrometer

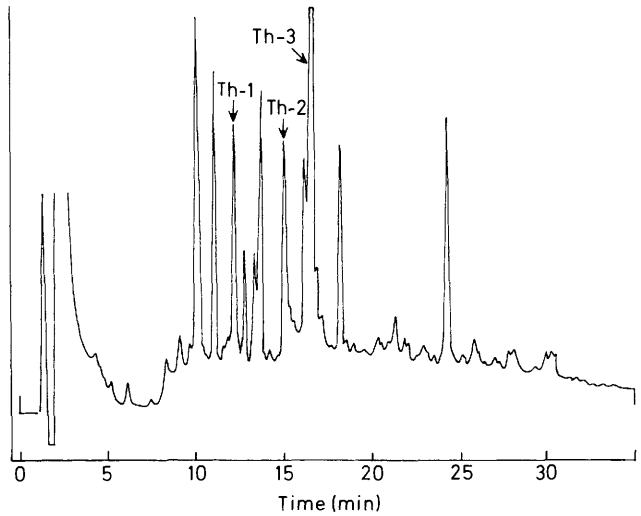


Figure 10. Analytical h.p.l.c. profile of thermolytic fragments of purified urogastrone. Column: Nucleosil $5C_{18}$ (4 × 150 mm); eluant: 0.1% aqueous TFA–MeCN (99:1)/0.1% aqueous TFA–MeCN (60:40) (linear gradient, 30 min); flow rate: 1.0 ml/min; detection: absorbance at 210 nm

HF–*p*-cresol (10:1, v/v, 1 h, 0 °C), (2) HF–*p*-cresol-EDT (10:1:1, v/v, 1 h, 0 °C), and (3) HF–*p*-cresol–EDT–Me_2S–Met (20:2:2:1:20 equiv., v/v, 1 h, 0 °C). After removal of HF, the residue were triturated with ether to give a crude powder. Each unpurified peptide was analysed by h.p.l.c. on a Nucleosil $5C_{18}$ (4.0 × 150 mm) column. The results are summarized in Figure 2. The retention time of the peptides in which the two Trp residues were both deprotected was 6.1 min; those of the peptides in which one was deprotected and one remained unchanged were 6.9 and 7.8 min; that of the peptide in which both remained unchanged was 9.7 min (solvent system A).

Examination of Aminosuccinimide (Asc) Formation during Deprotection in Fragment (1—12) *and* (13—26).—Fragment Boc-(1—12)-OH·HCl (**17**) (100 mg) and Boc-(13—26)-OH·HCl (**16**) (100 mg) were treated with HF–*p*-cresol–EDT–Me_2S–Met (20:2:2:1:20 equiv., v/v, 1 h, 0 °C), respectively. After removal of HF, each residue was triturated with ether to give a precipitate. Each product was then dissolved in 50% aqueous AcOH and analysed by h.p.l.c. on a Nucleosil $5C_{18}$ (4.0 × 150 mm) column (solvent system B). The results are shown in Figure 3. The retention times were as follows: fragment (1—12) (Asp) 13.1 min and (Asc) 14.0 min; fragment (13—26) (Asp) 24.0 min and (Asc) 24.7 min.

Deprotection of the Boc Group of the Protected Urogastrone.—The Boc group of the protected urogastrone (5.00 g, 0.525 mmol) was removed by treatment of the latter with a mixture of TFA (50 ml) and anisole (5 ml) in an ice-bath for 1 h. After removal of TFA, 5.9M HCl in dioxane (0.53 ml, 3.13 mmol) was added and the mixture was stirred for 10 min. After concentration of the mixture under reduced pressure the residue was triturated with dry ether and the resulting solid was dried over KOH pellets *in vacuo* to give the protected urogastrone, 3HCl·H-(1—53)-OBzl (4.99 g, 99.8%).

Deprotection of the Partially Deprotected Urogastrone.—The partially deprotected urogastrone, 3HCl·H-(1—53)-OBzl (500 mg, 0.052 mmol) was treated with HF (25 ml) in the presence of *p*-cresol (2.5 ml), EDT (2.5 ml), Me_2S (1.25 ml), and methionine (155 mg, 1.04 mmol) at 0 °C for 1 h. After removal of HF, the crude product was triturated with dry ether, and the resulting powder was washed with dry ether and dried over KOH pellets *in vacuo*. This procedure was repeated twice more and the resulting three batches were combined, dissolved in 50% aqueous AcOH (100 ml), and filtered to remove insoluble materials. Lyophilization of the filtrate gave the crude $(S\text{-Acm})_6$-urogastrone as a powder (1.11 g). The analytical h.p.l.c. profile of this compound is shown in Figure 5.

Purification of the Crude (S-Acm)$_6$-Urogastrone.—The crude urogastrone (1.11 g) was dissolved in 0.02M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 400 ml) and insoluble materials were filtered off. The filtrate was applied to a column of DEAE-cellulose (DE-52, Whatman, 3.5 × 50 cm), which was first washed with 0.02M aqueous NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 000 ml) and then eluted with a linear gradient starting from 0.02M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 200 ml) to 0.12M NH_4OAc buffer in 6M aqueous urea (pH 5.6; 1 200 ml) at 5 °C. The u.v. absorption at 280 nm in each fraction (12 g) was determined. The fractions (tubes Nos. 148—195, Figure 4) containing the main peak on h.p.l.c., were combined and applied to a column of Diaion HP-20 (Mitsubishi Kasei Co., 3 × 15 cm) eluting with a mixture of MeOH and 3M aqueous AcOH (2:1, v/v). The eluant was concentrated and lyophilized to give purified $(S\text{-Acm})_6$-urogastrone as a white, fluffy powder (101.2 mg, 9.8%). Analytical h.p.l.c. of this compound is shown in Figure 5: Nucleosil $5C_{18}$ (4 × 150 mm); solvent system C; flow rate 1 ml min^{-1}; detection, u.v. 210 nm; retention time, 19.7 min. Acid hydrolysis was carried out by treating the peptide (1.19 mg) with 6M HCl containing 5% phenol for 24 h at 110 °C. Enzymic hydrolysis was carried out by treating the peptide (1.16 mg) with trypsin (25 μg), α-chymotrypsin (20 μg), and leucine aminopeptidase M (245 μg) for 24 h at 37 °C. Amino acid analysis of the acidic hydrolysate and the enzymic hydrolysate are summarized in Table 1. The lower values for Cys(Acm) and Trp may be due to their decompositions during the acid hydrolysis. The value of His was not determined because of its overlap with the NH_3 peak.

Tryptic Mapping of Purified (S-Acm)$_6$-Urogastrone.—$(S\text{-Acm})_6$-urogastrone (1.94 mg) was digested with TPCK-trypsin (80 μg) in 0.1M aqueous NH_4HCO_3 (970 μl) for 30 min at 37 °C. The tryptic fragments were isolated by h.p.l.c. using a semi-preparative Cosmosil $5C_{18}$ (10 × 250 mm) column eluting with solvent system D; flow rate, 3.0 ml min^{-1}. Analytical h.p.l.c. of the tryptic fragments is shown in Figure 6. The isolated tryptic fragments were subjected to amino acid analysis after 24 h of hydrolysis at 37 °C by α-chymotrypsin and leucine aminopeptidase M in 0.1M aqueous NH_4HCO_3. The results (Table 2) are as follows: T-1 urogastrone (46—48), Asp(1.00)-Leu(1.03)-Lys(0.97); T-2 urogastrone (38—41), Gly(1.09)-Glu(1.07)-Ile(1.00)-Arg-(0.98); T-3 urogastrone (42—45), Cys(Acm)(1.21)-Gln(0.85)-Tyr(1.00)-Arg(0.96); T-4 urogastrone (29—37), Cys(Acm)-(1.82)-Asn(1.01)-Gly(1.37)-Ala(1.48)-Val(2.00)-Tyr(1.77); T-5 urogastrone (29—41), Cys(Acm)(2.43)-Asn(0.53)-Glu-(1.13)-Gly(2.12)-Ala(1.33)-Val(2.00)-Ile(1.08)-Tyr(1.92)-Arg(0.98); T-6 urogastrone (49—53), Glu(1.01)-Leu(1.00)-Trp(1.72)-Arg(0.88); T-7 urogastrone (1—28), Cys-(Acm)-(2.32)-Asp(3.78)-Asn(0.78)-Ser(2.93)-Glu(2.00)-Pro-(0.98)-Gly(1.81)-Ala(1.06)-Val(1.00)-Met(0.88)-Ile(0.97)-Leu-(2.80)-Tyr-(1.80)-Lys(0.99)-His(2.19).

Synthesis of Urogastrone: Deprotection of the Acm Group in (S-Acm)$_6$-Urogastrone and Aerial Oxidation.—$(S\text{-Acm})_6$-urogastrone (65.8 mg, 9.9 μmol) was dissolved in a degassed 50% aqueous AcOH (10 ml) and $Hg(OAc)_2$ (5.68 mg, 178 μmol) was added under N_2 at room temperature. The reaction mixture was stirred for 5 h at the same temperature and 2-mercaptoethanol (1.63 ml, 23.8 mmol) was added. After being stirred for 20 h, the mixture was passed through a Sephadex G-25 column (2.4 × 60 cm) equilibrated with a 1.0M aqueous AcOH and monitored at 280 nm. The fractions corresponding to the reduced urogastrone were combined and diluted with water (2 200 ml). The solution was adjusted to pH 7.6 with 0.5M aqueous NH_4OH, and stirred gently at 5 °C for 4 days. The progress of aerial oxidation reaction was assured by an Ellman test and analytical h.p.l.c. (Figure 7). The entire solution was applied to a column of Diaion HP-20 (3.0 × 12.0 cm) eluting with a mixture of MeOH and 3M aqueous AcOH (2:1, v/v). After concentration of the eluant, the residue was lyophilized to give a fluffy powder (34.3 mg, 55.8%).

Purification of the Crude Urogastrone.—The crude product (32.0 mg, 5.15 μmol) was dissolved in 0.02M NH_4OAc buffer (pH 5.3; 20 ml) and the solution was applied to a column of DEAE-cellulose (2.2 × 50 cm) equilibrated with 0.02M NH_4OAc buffer (pH 5.3). After being washed with the above buffer (200 ml), the column was eluted with a linear gradient starting from 0.02M NH_4OAc buffer (pH 5.3; 500 ml) to 0.02M NH_4OAc buffer (pH 5.3; 500 ml). Each fraction (4 ml) was analysed by u.v. absorption (280 nm) and analytical h.p.l.c. The fractions (tubes 197—218, Figure 8) corresponding to the main peak were combined. After concentration, it was applied to a column of

Sephadex G-25 (2.2 × 50 cm) equilibrated with 1.0M aqueous AcOH and eluted with the same solvent. Lyophilization gave the product as a white fluffy powder (8.1 mg, 25.2%). Analytical h.p.l.c. profile of this compound is shown in Figure 7: Nucleosil $5C_{18}$ (4 × 150 mm); solvent system C; flow rate, 1 ml min^{-1}; detection, u.v. 210 nm; retention time, 15.2 min. $[\alpha]_D^{23}$ −90.65° (*c*, 0.46 in 1% AcOH). Amino acid analysis: Asp(7), 6.82; Ser(3), 3.11; Glu(5), 4.69; Pro(1), 1.19; Gly(4), 4.17; Ala(2), 2.09; Val(3), 2.34; Met(1), 0.83; 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), 2.89; Cys(6), 5.91 (as cysteic acid) [Found: *m/z* (f.a.b.) 6 216.74 Calc. for: 6 216.95 ($C_{270}H_{395}N_{73}O_{83}S_7$ + H)].

Digestion of Urogastrone with Thermolysin.—The purified urogastrone (0.26 mg, 42 nmol) was digested with thermolysin (8.7 μg) in 0.1M pyridine–AcOH buffer (pH 6.5;120 μl) for 24 h at 45 °C. A 60 μl portion of the mixture was fractionated by h.p.l.c. using a Nucleosil $5C_{18}$ (4.0 × 150 mm) column eluting with solvent system D; flow rate, 1.0 ml min^{-1}. An analytical h.p.l.c. profile of the thermolytic fragments is shown in Figure 10. After lyophilization, the isolated fragments were subjected to performic acid oxidation [formic acid (2.5 μl)–performic acid (20 μl), 0 °C, 2 h]. These peptides were hydrolysed with 6M HCl containing 2% phenol at 110 °C for 24 h. The amino acid compositions of three cysteic acid-containing fragments were as follows: Th-1 urogastrone (13—14)-(30—31), Cya(2.00)-Ala-(0.88)-Tyr(0.75); Th-2 urogastrone (32—33)-(39-42), Cya(2.00)-Asp(0.89)-Glu(0.95)-Gly(0.85)-Arg(0.77); Th-3 urogastrone (1—7)-(19—22), Cya(2.00)-Asp(2.18)-methionine sulphone-(1.16)-Ser(2.20)-Glu(1.19)-Val(1.21)-Tyr(0.67)-Pro(1.16).

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