Peptide Synthesis: Synthesis of the Cysteine-containing Peptides of Biological and Pharmaceutical Interest, α-h-ANF and h-Big Endothelin

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The biologically important vasoactive peptides, α-h-atrial natriuretic factor (ANF) and h-Big endothelin (Big ET) have been synthesised using the solid-phase Fmoc strategy with reference to the choice of protecting group for cysteine residues. Selective disulfide bond formation was seen to be problematical in the synthesis of Big ET but use of the acetamidomethyl (Acm) group, removal with the silver ions and oxidation using the mixed disulfide method proved successful.

Solid-phase peptide synthesis (SPPS) has furnished the biochemist and pharmacologist with a wide array of peptides and peptide analogues for structure/activity studies leading towards an understanding of the active sites of enzymes and receptors with a view to the discovery of drug candidates which may be peptide or non-peptide. Considerable challenges still face the peptide chemist from three particular standpoints. Firstly, the design and incorporation of suitable amino acid analogues and isosteres which may be used to increase activity and resistance to degradation in peptide or peptoid models. Secondly, the synthesis of large polypeptides, *e.g.* greater than 50 amino acids, has by no means been optimised and has led to the necessity of protracted purification procedures with resultant low final yields. The third challenge, and that which is of immediate concern here, is the general synthesis of bioactive peptides containing specific disulfide bonds, notwithstanding the pioneering work of the CIBA group on the synthesis of insulin [1] in which the disulfide bonds were formed selectively. The choice of the protecting group for cysteine appears to be fairly random with little concensus on the merits or otherwise of available methodology.

Atrial natriuretic factor (α-h-ANF) **1** is the simplest of a family of peptides derived from a common precursor.[2] It exhibits diuretic, natriuretic and vasodilator activities. Endothelins, ET1 **2**, ET2 **3** and ET3 **4**, are a range of closely related peptides consisting of a bicyclic 21 amino acid sequence derived from vascular endothelial cells. They are similarly related to a number of other peptides including snake venom toxins and mouse VIC, **5**–**9**[3] (Fig. 1).

H–Ser–Leu–Arg–Arg–Ser–Ser–Cys–Phe–Gly–Gly–Arg–Met–Asp–Arg–
Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys–Asn–Ser–Phe–Arg–Tyr–OH

α-Human atrial natriuretic factor (α-h-ANF) **1**

The endothelins have remarkable vasoconstrictor properties and have similar effects in non-vascular smooth muscle. They have a wide range of associated physiological effects and have stimulated a large body of research.[4] ET1 and the other endothelins are processed from a putative 203 amino acid precursor to Big ET (1–38) **10** by endopeptidase activity specific for a dibasic pair of residues. Big ET is further processed by an unusual cleavage at the Trp–Val bond, catalysed by a cathepsin-like proteinase,[5] to ET1 **2** which has a 140-fold gain in potency. Inhibition of this event would seem to be one of the myriad of possibilities for controlling the physiological effects of the endothelins in a therapeutic context. In order to examine the structural dependence of Big ET and its processing there is a requirement for the efficient synthesis of Big ET and its analogues.

1 5 10 15
H–Cys–Ser–Cys–Ser–Ser–Leu–Met–Asp–Lys–Glu–Cys–Val–Tyr–Phe–Cys–
20 25 30
His–Leu–Asp–Ile–Ile–Trp–Val–Asn–Thr–Pro–Glu–His–Val–Val–Pro–Tyr–
35 38
Gly–Leu–Gly–Ser–Pro–Arg–Ser–OH

h-Big ET 1-38 **10**

Peptide Synthesis.—The classic choice between Fmoc/Bu^t and BOC/benzyl strategies was addressed by Wade[6] in a comparative synthesis of α-h-ANF by both techniques. Although these results afforded the conclusion that both techniques gave broadly similar results, the Fmoc synthesis suffered from the fact that a suitable protecting group for arginine (of which there are five) was not then available. The 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group which was employed required deprotection for an extended period (18 h). The design of the more acid labile 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) group[7] should be expected to lead to an improved product.

The continuous-flow polyamide strategy as used by Wade[6] and advocated by Sheppard[8] was not used, rather we selected the batch system based on the trifluoroacetic acid (TFA) labile Wang linker[9] attached to a polystyrene support which has worked well in our hands[10] using the Applied Biosystems ABI 430A peptide synthesiser.

H–Cys–Ser–Cys–Ser–Ser–Leu–Met–Asp–Lys–Glu–Cys–Val–Tyr–Phe–Cys–His–Leu–Asp–Ile–Ile–Trp–OH							**2**
	Trp–Leu						**3**
Thr	Phe–Thr–Tyr–Lys			Tyr			**4**
	Lys–Asp–Met–Thr		Leu–Asn		Gln	Val	**5**
	Lys–Asp–Met–Thr		Leu		Gln	Val	**6**
Thr	Asn–Asp–Met–Thr	Glu	Leu–Asn		Gln	Val	**7**
Thr	Lys–Asp–Met–Thr		Leu–Asn		Gln		**8**
	Asn Trp–Leu						**9**

Fig. 1 Endothelins, sarafotoxins and related peptides: **2** h,p-ET1, **3** h-ET2, **4** h,r-ET3, **5** sarafotoxin S6a, **6** S6b, **7** S6c, **8** S6d, **9** mouse vasoactive intestinal constrictor

In order to obtain real-time information about cycle efficiency an in-line deprotection monitoring system has been developed. This system involves withdrawal of a sample of the deprotection solution (20% piperidine in DMF) which is then diluted with DMF and sent directly through the UV detector by means of DMF from the solvent reservoirs. This gives a single peak which can be directly integrated to give a measure of the amount of Fmoc present. In-line monitoring, which is also available using continuous flow polyamide technology, should be used in conjunction with a capping cycle in order to remove unchanged α-amino groups remaining after incomplete coupling. Thus, the course of a synthesis can be monitored and any obvious sequence failures may be observed. Similarly, the loading of an amino acid or peptide on a resin can be quantified by deprotection of an analytical sample and recording the UV spectrum. The total chain assembly may thus be monitored in real-time and assessed.

The choice of protecting group for cysteine seemed likely to be one of the most critical decisions to be taken before the onset of synthesis. The trityl group[11] appears to provide the simplest strategy being cleaved simultaneously with the other protecting groups. It does, however, suffer from a tendency to re-attach itself to the peptide even in the presence of scavengers and, in our experience, has given poor results with peptides of any complexity.

The acetamidomethyl (Acm) group[12] has been applied widely in recent years, however its removal with mercuric ions, or direct iodine oxidation, can be troublesome. The recently advocated *tert*-butylthio (SBu^t) group[13] appeared rather more attractive and had been used with success in our laboratory in the synthesis of a signal peptide.[14] This group is stable to TFA but cleaved by tributylphosphine or thiols.

We sought to investigate the relative merits of the Acm and SBu^t protection of cysteine residues using as synthetic targets α-h-ANF **1** and h-Big ET 1–38 **10**. In the latter case, having two S–S links, it was also the intention to compare the directed oxidation to give specific S–S links with the equilibrium method leading to a thermodynamic mixture of two possible S–S links followed by separation of the two products.

Synthesis of α-h-ANF **1**.—The synthesis of α-h-ANF was carried out on the ABI 430A using protocols already established with a wide range of peptides. Assembly of the protected sequence, in which the Cys thiol groups were protected by SBu^t, appeared to be satisfactory from considerations of the UV deprotection monitoring in addition to amino acid analysis of the assembled Fmoc-peptide resin (Table 1). Initial cleavages on small amounts of resin were used to establish optimum deprotection conditions but variation of scavengers and cleavage time did not give any single identifiable product. Direct cleavage of the Fmoc-peptide gave a similar HPLC profile with increased retention times due to the presence of the *N*-terminal Fmoc group, the profile being virtually identical at 214 nm (amide bonds) and 266 nm (Fmoc group). It was suspected that the array of peaks in the HPLC was due to partially deprotected ANF peptides rather than truncated sequences. Analysis of cleaved peptides by diode array detector showed the absence of any peaks at 252 nm indicating that no Pmc groups remained attached to the product. Thus, the recalcitrant protection was likely to be of the *tert*-butyl type. The deprotection was left for extended times but no change was observed after 5 h.

The crude deprotected peptide, after precipitation and isolation, was further treated with fresh scavengers and TFA for 2 h to give, after work-up, a material that proved much more promising with one peak of greater intensity appearing at the expense of more hydrophobic peaks. Gel filtration and preparative HPLC gave the 7,23-di-SBu^t-α-h-ANF **11**.

Removal of the SBu^t groups was achieved with tributylphosphine in trifluoroethanol (95%)/water (5%) and the crude product then purified by gel filtration. Aerial oxidation at 0.1 mmol dm^{-3}, pH 8 gave the required α-h-ANF **1** together with minor quantities of another component. Separation of these two peptides by preparative HPLC enabled the major product to be identified as α-h-ANF **1** while the minor compound was found to be the corresponding sulfoxide by mass spectrometry.

Synthesis of h-Big ET 1–38 **10**.—A synthesis of h-Big ET 1–38 **10** was attempted using selective protection for cysteine (1,15-Acm), (3,11-SBu^t). While the chain assembly appeared to proceed smoothly, problems were encountered with the cleavage and deprotection. Repeat acid treatment, as had proved successful with ANF, did not improve matters and the procedure of Eritja *et al.*[15] was employed where the *tert*-butylthio groups are removed on the resin-bound peptide with mercaptoethanol and the disulfide bond formed *in situ* by oxidation with ferricyanide. Subsequent cleavage of the peptide from the resin gave no improvement in the HPLC profile. A synthesis of ET1 **2**, carried out by the same strategy, did not appear to be any more promising.

Because of problems with deprotection, a synthesis of Big ET 1–38 was carried out in which all four cysteines were protected by the Acm group. This proved much more promising. Cleavage gave the tetra-Acm peptide **12** in *ca.* 80% yield after gel filtration and HPLC purification.

Reports of disulfide bond formation in the endothelins[16] agree that the correct disulfides (1–15, 3–11) are preferred over the incorrect form (1–11, 3–15) in a ratio of 3:1 with no detectable quantities of the third possible isomer (1–3, 11–15). There were indications that separation of the isomer mixture might be troublesome requiring isocratic HPLC conditions and there is evidence that changing amino acids to give analogues may have a dramatic effect on the isomer distribution.[17] The facility of synthesis and ease of handling of intermediates, nevertheless, rendered this the most promising approach. Direct cleavage/oxidation with iodine in acetic acid was attempted but the reaction appeared fairly sluggish and a number of side products were formed. The problems of iodine oxidation in the presence of tryptophan and methionine are well known and have been studied recently in connection with the endothelins.[18]

Standard mercuric ion deprotection of Acm can often give incomplete cleavage particularly with multiple cysteine-containing sequences[19] and, instead, we were prompted to investigate the use of silver trifluoromethanesulfonate in TFA as advocated by Fujii.[20] Deprotection proceeded smoothly as monitored by HPLC but isolation of the fully reduced peptide by lyophilisation gave a material which could not be readily solubilised at pH 8 to allow oxidation to take place. Aerial oxidation for 24 h gave only low yields of Big ET (1–38). Isolation and lyophilisation gave the product which under isocratic and shallow gradient HPLC conditions gave the characteristic isomer ratio of 3:1, the more abundant component co-eluting with commercial Big ET.*

Two modifications of the isolation procedure have improved considerably the yield of Big ET. Firstly, the fully reduced peptide was kept in solution and directly diluted in the oxidation mixture at pH 8. Secondly, the mixed disulfide oxidation method of Tam *et al.*[21] with reduced and oxidised glutathione in guanidine hydrochloride (1 mol dm^{-3}) was employed and the oxidation monitored by HPLC. The oxidation proved extremely rapid with the peptide remaining in solution throughout. Filtration and direct application of the oxidation solution to a C_8 HPLC column, followed by washing

* Samples of h-Big ET were purchased from Novabiochem and Peptide Institute Inc.

and slow gradient elution, gave the crude oxidised peptide which, after lyophilisation, was separated into the bis-disulfide isomers by preparative HPLC using a shallow gradient. The two isomers have been characterised by amino acid analysis, mass spectrometry, HPLC comparison with authentic material.

Conclusions.—This study has compared the application of various protecting groups for cysteine in the synthesis of two important pharmacologically active peptides. The choice of protecting groups for cysteine depends on a number of factors, *e.g.* the size, hydrophobicity and chemical nature of the peptide and requirements for selective disulfide bond formation.

Our previous experience with the application of Fmoc.Cys-(Trt) to the synthesis of α-h-ANF and Big ET, as well as many other peptides, has not been encouraging. Slow couplings of Fmoc.Cys(Trt) and great heterogeneity of the crude peptide products caused us to look carefully at the characteristics of other *S*-protecting groups.

The chemical advantages of the *tert*-butylthio protection group are counterbalanced by the hydrophobic character which this group imparts on the peptide. A synthesis of ET1 **2** carried out with this protecting group for all four cysteines yielded an *S*-protected peptide which could not be dissolved in any of the traditional solvents for gel filtration or HPLC. This situation was not as pronounced with Big ET in which the C-terminus imparts some hydrophilic character. The general hydrophobic nature of these peptides leads to worrying sample loss during purification techniques such as gel filtration and preparative HPLC purification.

The choice of scavengers is also restricted by certain cysteine-protecting groups, *i.e.* Acm is partially cleaved by thioanisole (a well known aid to cleavage acceleration in Fmoc/Bu^t chemistry) and SBu^t is neither compatible with thioanisole nor EDT.[22] Therefore, there is still a need for the further development of cysteine-protecting groups and it would be particularly useful if these were hydrophilic and cleaved under mild conditions while allowing as wide an array of scavengers as possible for troublesome acidolytic deprotections.

The optimum synthesis, cleavage and disulfide bond-forming conditions for α-h-ANF and h-Big ET (1–38) have been studied and these are being applied to a series of biologically interesting analogues.

Experimental

The Wang resin [9] and Fmoc-amino acids of the L-configuration were purchased from Novabiochem. Peptide synthesis solvents DMF, dioxane and piperidine were purchased from Rathburn Chemicals, and were of peptide synthesis grade. Dichloromethane (DCM) was purchased from Fisons and was HPLC grade. Diisopropylcarbodiimide (DIC), acetic anhydride and pyridine were purchased from Aldrich and used as supplied. *N*,*N*-Dimethylaminopyridine (DMAP) and *N*-hydroxybenzotriazole (HOBt) were purchased from Fluka. 95% TFA represents TFA (95%)–H_2O (5%).

Loading of the First Amino Acid.—The Wang resin (1.30 g, 1.0 m equiv.) was swollen in the minimum volume of DMF and DMAP (0.1 mmol) was added to it. The Fmoc-AA-OH (4 mmol) was dissolved in DMF (10 cm^3) and DIC (2 mmol) was added to it. The activated amino acid solution was added to the resin and sonicated for 3 h. Filtration, washing with DMF and DCM followed by drying *in vacuo* afforded the loaded resin.

A small sample of resin (2–5 mg) was made up to 10 cm^3 with piperidine (20%) in DMF and sonicated for 15 min. The UV spectrum of the supernatant was recorded (290–320 nm) and the UV absorption at 300 nm indicated the resin loading (ε = 9000, standardised with Fmoc-Gly-OH).

If the resin loading was found to be satisfactory the resin was swollen in DMF and free hydroxy groups capped by the addition of pyridine (1 cm^3) and benzoyl chloride (1 cm^3) for 30 min before filtering, washing and drying as above. The loading was rechecked and this value taken as the basis for the synthesis.

Peptide Synthesis.—Peptide synthesis[10] was carried out on the ABI 430A using double couple cycles incorporating UV deprotection monitoring; the scale used was 0.5 mmol or 0.25 mmol.

Capping. The synthesis cycle commenced with a capping step in order to allow interrupts to be set at the end of cycles after coupling. DMF (5 cm^3) was added to the peptide-resin followed by pyridine (0.5 mol dm^{-3}; 1 cm^3) and acetic anhydride (0.5 mol dm^{-3}; 1 cm^3) in DMF. The capping step was carried out for 2 min followed by washing and a repeat capping for 4 min. The resin was washed again.

Deprotection. The peptide-resin was treated with piperidine for 3 min. The solution was drained to waste *via* a loop, the content of which was then isolated and transferred to the concentrator and then diluted with DMF. After washing the valve blocks, the concentrator was drained through the loop again and the loop contents passed through a pre-primed line to the UV by means of the pressure from the DMF bottles. The absorption at 300 nm was integrated and after washing a further deprotection was carried out for 1 min as before. In a normal synthesis the second deprotection was insignificant compared to the first one though occasionally larger second deprotection peaks can be observed (though not with ANF or endothelins) which are considered to be due to a slow, difficult deprotection. It was considered that this could be due to steric hindrance which would be indicative of a difficult coupling for the next residue and in these cases, the resin was checked after coupling by the Kaiser test before continuing with the synthesis.

Coupling. (1) Preformed symmetrical anhydride. The amino acid (2 × 1 mmol) was dissolved in DMF (5 cm^3) and DIC (0.5 mol dm^{-3}; 2 cm^3) in dioxane added to it. Activation proceeded for 15 min and the solution transferred to the reaction vessel. Coupling proceeded for 30 min (normal) or 45 min (extended).

(2) HOBt active ester. The amino acid (1 × 1 mmol) was dissolved in DMF (4 cm^3) and HOBt (0.5 mol dm^{-3}; 2 cm^3) added to it, followed by DIC (0.5 mol dm^{-3}; 2 cm^3) in dioxane. The activation was carried out for 30 min and coupled as with (1) above.

In 0.25 mmol scale syntheses the quantity of amino acid and the concentrations of DIC and HOBt were halved.

Cleavage. The Fmoc-peptide resin was suspended in piperidine (20%) in DMF (10 cm^3) sonicated for 10 min, filtered and washed with DMF and DCM.

The scavengers were added to the resin and stirred for 10 min followed by TFA (95%) (10 cm^3) in a N_2 atmosphere. The deprotection mixture was stirred for the prescribed time, the solution filtered and the resin washed with TFA and DCM. The solution was evaporated under reduced pressure and further DCM added to it. Evaporation and DCM addition was repeated and the crude peptide precipitated by the addition of ether. The solid was filtered and washed with ether.

In order to determine the optimum time for deprotection, this was monitored by withdrawing 0.5 cm^3 aliquots of the solution and quenching in cold ether. The solid was centrifuged, washed with ether, dissolved in acetic acid (30%), and the solution filtered and analysed by HPLC.

Purification. The crude precipitated peptide (500 mg) was dissolved in acetic acid (30%) and passed down a Sephadex column, G25 (ANF) or G50 (Big ET) by means of a peristaltic pump. Elution was monitored by UV at 254 and 280 nm (LKB

Table 1 Amino acid analyses in the α-h-ANF synthesis

Amino acid	Reqd.	Resin	di-SBu^t peptide **11**	h-ANF **1**
Asp	2	1.9	2.1	2.4
Ser	5	4.3	4.2	4.1
Glu	1	1.0	1.1	1.3
Gly	5	4.8	4.7	5.0
Ala	1	1.1	1.2	1.0
Cys	2	1.1	1.0	1.1
Met	1	0.8	0.8	0.7
Ile	1	0.9	1.0	0.9
Leu	2	1.9	1.9	1.8
Tyr	1	1.0	0.9	0.9
Phe	2	1.7	1.8	1.9
Arg	5	4.2	4.7	4.2

2138 Uvicord-S). The appropriate fractions containing peptide were pooled and lyophilised.

HPLC was carried out using ABI equipment, *e.g.* 2 × 1406A solvent delivery systems, a 1480A injector/mixer and a 1783A detector/controller. Analytical columns were Aquapore RP300 C_8 or C_{18} (7 μm spherical silica) 4.6 × 220 mm (long) or 4.6 × 100 mm (medium) or Vydac C_{18} (5 μm, 218TP54) 4.6 × 250 mm at 1 cm^3 min^{-1}, 214 nm. A 2 cm^3 injection loop was used.

Preparative HPLC was carried out on Aquapore RP300 Prep-10 C_8 or C_{18} columns 10 × 250 mm (long), 10 × 100 mm (medium) at 5 cm^3 min^{-1} or a Vydac C_{18} (10 μm, 218TP1022), 22 × 250 mm at 10 cm^3 min^{-1}, 229 or 240 nm. A 5 cm^3 injection loop was used. Solvents were A – H_2O (0.1% TFA), B – MeCN (0.1% TFA).

Amino acid analysis was carried out on a LKB 4151 Alpha-plus instrument following sealed tube acid (6 mol dm^{-3} HCl) hydrolysis at 110 °C for 24 or 36 h. Mass spectrometry (FAB) was performed on a Kratos MS50TC using thioglycerol or 3-nitrobenzyl alcohol (NOBA) as matrix. UV spectra were recorded on a Cary 210 spectrophotometer. Simultaneous multiple wavelength detection was carried out using an LKB Pharmacia Diode Array Detector and data processor.

Synthesis of α-h-ANF **1**.—A 0.5 mmol scale was employed starting with Fmoc-Tyr(Bu^t) loaded on the Wang resin (0.52 mmol g^{-1}). The first half of the synthesis was carried out in the absence of dioxane, *i.e.* couplings in DMF. All amino acids were double coupled except Gly and *tert*-butyl side-chain protection used except for Asn(Mbh), Gln(Mbh), Arg(Pmc) and Cys(SBu^t). After coupling of Arg(14), half of the resin was removed and the synthesis was continued using the same quantities of amino acids. The synthesis was very encouraging from the chain-assembly point of view, 1.71 g of dry peptide resin being obtained.

A sample (100 mg) of ANF(14–28)-resin after Fmoc deprotection was cleaved with TFA (95%, 10 cm^3), anisole (0.5 cm^3), and ethyl methyl sulfide (0.5 cm^3) for 1 h giving a product containing considerable quantities of impurities.

The synthesis was carried out on the remainder of the resin using DMF/dioxane (1 : 1) for coupling (DIC was 0.5 mol dm^{-3} in dioxane), 1.21 g of resin being produced. The synthesis again appeared very efficient there being very little difference between the two batches.

Cleavage experiments were carried out on 100 mg samples of the resin as before using: (a) TFA (95%; 10 cm^3), ethyl methyl sulfide (0.5 cm^3) and anisole (0.5 cm^3); (b) TFA (95%; 10 cm^3), ethyl methyl sulfide (0.5 cm^3), anisole (0.5 cm^3) and phenol (100 mg); (c) TFA (95%; 10 cm^3), Ac-Met-OEt (100 mg) and Ac-Tyr-OEt (100 mg).

Cleavage appeared to be extremely sluggish and none of these deprotections gave a reasonable HPLC profile. Cleavage with TFA, PhSMe and ethyl methyl sulfide, *i.e.* the conditions used by Wade,[6] failed to yield any isolable material.

Preparation of (7,23-*bis*-SBu^t)-α-*h-ANF* **11**.—A sample of the dry resin (500 mg) after removal of the Fmoc group was cleaved under conditions (a) above. Samples (0.5 cm^3) were removed every hour and worked up as normal. HPLC of the crude peptide indicated that no further change could be effected after 5 h. A further 500 mg of resin was cleaved for 5 h and the two materials combined as the crude peptide (440 mg). Gel filtration yielded the crude peptide (280 mg) of similar HPLC profile to that obtained with the analytical cleavages above.

The crude peptide was treated with fresh deprotection reagents for 2 h after which the HPLC of the crude product looked much more promising with a single peak much enhanced and most of the more hydrophobic materials correspondingly absent. Gel filtration and lyophilisation gave the crude peptide (165 mg).

The protected peptide **11** was purified by preparative HPLC on an Aquapore C_{18} column, 229 nm, 3AUFS, 10–60% B in 30 min, peptide concentration 10 mg cm^{-3}. The appropriate fractions were pooled and lyophilised to give pure **11** (98 mg). HPLC t_R = 11.3 min, 20–50% B in 20 min. A suitable mass spectrum could not be obtained using thioglycerol or NOBA as matrix.

Preparation of α-h-ANFSP **1**.—The protected peptide **11** (98 mg, 30 mmol) was dissolved in TFE (95%, 5 cm^3) under N_2 and stirred whilst Bu_3P (75 mm^3, 0.3 mmol) was added to it; stirring was continued for 3 h. The TFE was removed under reduced pressure and the oil precipitated with methyl *tert*-butyl ether–ethyl acetate (3 : 1). Filtration and washing was followed by dissolution in TFE and work-up as before until the solid was free of Bu_3P. The crude reduced peptide was dissolved in water to a concentration of 0.1 mmol (300 cm^3) and the pH adjusted to 8 with NH_4OH. The oxidation mixture was stirred for 24 h and the progress monitored by HPLC and the Ellman test for sulfydryl groups. The single peak corresponding to the reduced material was transformed into the characteristic peak of α-h-ANF **1** with a small amount of an impurity.

Concentration and lyophilisation gave the crude ANF (50 mg) which was purified by preparative HPLC (conditions as with **11** above) giving h-ANF **1** (15 mg, 4.9 μmol), HPLC t_R = 10.0 min 20–50% B in 20 min; *m/z* (FAB) 3080.455 89 ($C_{127}H_{204}N_{45}O_{39}S_3$ requires 3080.455 76), < 1 ppm followed by an impurity (3 mg) which was found to be the corresponding sulfoxide; *m/z* (FAB) 3098 (3096), t_R (HPLC) 10.6 min 20–50% B in 20 min.

Attempted Synthesis of Big ET (1–38) **10** *and ET*1 **2** *using Differential S Protection.*—These syntheses were carried out in an identical manner from Fmoc-Ser(Bu^t) and Fmoc-Trp loaded on the Wang resin respectively on a 0.25 mmol scale using the same protecting group strategy as with ANF except that the Asn side chain was unprotected and Cys residues 1 and 15 used the Acm group while residues 3 and 11 were protected with the SBu^t group. Chain assembly appeared to take place without problems. Trial cleavage studies were carried out as with ANF under a variety of conditions, *e.g.*: (a) TFA (95%, 10 cm^3), EDT (0.25 cm^3), Ac-Trp-OMe (100 mg) and phenol (500 mg); (b) TFA (95%, 10 cm^3), anisole (0.5 cm^3), EMS (0.5 cm^3), phenol (100 mg) and 3-methylindole (100 mg); (c) TFA (95%, 10 cm^3), anisole (0.5 cm^3), EDT (0.5 cm^3), EMS (0.15 cm^3) and 3-methylindole (100 mg).

None of these cleavage studies gave satisfactory crude peptide and repeat deprotections, which had been successful with ANF, showed no improvement.

Table 2 Amino acid analyses in the big endothelin synthesis

Amino acid	Reqd.	1,15-Acm 3,11-SBu^t Resin	1,2,11,15-Acm-Resin Big ET	1,3,11,15-Acm **12**	Big endothelin **10**	
					Sample A	Sample B
Asp	3	2.79	2.87	3.06	2.80	3.17
Thr	1	0.84	0.84	0.87	0.85	1.02
Ser	5	3.27	3.87	3.60	3.63	3.91
Glu	2	2.20	2.12	2.38	2.12	2.21
Pro	3	3.57	5.27	7.98	2.88	2.55
Gly	2	1.94	2.01	2.07	2.16	2.25
Cys	4	1.40	0.67	0.51	2.73	3.48
Val	4	3.79	3.81	3.70	3.41	4.05
Met	1	0.88	0.65	0.94	1.00	0.77
Ile	2	1.39	1.15	1.34	1.87	2.18
Leu	3	3.46	2.98	3.00	2.61	3.38
Tyr	2	0.98	1.82	1.95	2.28	2.00
Phe	1	1.11	1.06	1.05	0.85	1.02
His	2	2.00	1.97	2.04	1.93	2.12
Lys	1	0.96	1.00	1.02	0.97	1.12
Arg	1	0.95	1.06	1.01	0.95	0.95

Attempted Formation of the 3–11 *Disulfide on the Resin-bound Peptide.*—The procedure of Eritja[15] was followed. The Fmoc protected peptide resin (100 mg) was suspended with sonication in DMF–mercaptoethanol (1:1) (5 cm^3) for 5 h and then washed well with DMF and DCM. The resin was suspended in DMF (10 cm^3) and aqueous potassium ferricyanide (1 mol dm^{-3}; 1 cm^3) added with vigorous agitation followed by sonication overnight. The resin was washed as before, Fmoc deprotected and cleaved as in (c) above. The HPLC profile looked similar to that obtained with the fully protected peptide and the product was not further investigated.

Synthesis of Big ET (1–38) *using* 4 × *Cys(Acm).*—This synthesis was performed on a 0.5 mmol scale using Acm for protection of all four cysteine residues. Triple coupling was carried out on each residue (except Gly where an extra HOBt ester coupling was used). Acidolytic deprotection of the assembled resin (2 × 500 mg deprotections) with TFA (95%; 10 cm^3) anisole (1 cm^3), EDT (1 cm^3), EMS (0.5 cm^3) and 3-methylindole (100 mg) for 3 h gave good quality crude tetra-Acm protected peptide **12** (657 mg). Gel filtration (497 mg) followed by preparative HPLC gave pure **12** (150 mg); t_R (HPLC) 16.44 min, 20–50% B in 25 min; *m/z* (FAB) 4571.124 01 ($C_{201}H_{307}N_{52}O_{60}S_5$ requires 4571.123 91).

Oxidation of Big ET (1–38) (1,3,11,15-*tetrakis-S-Acm*) **12** *with Iodine.*—The above compound (10 mg, 0.022 mmol) was dissolved in acetic acid (80%; 20 cm^3) containing 3-methylindole (10 mg) and a solution of iodine (2 mmol dm^{-3}; 33 cm^3, 0.066 mmol) in acetic acid (80%) was added to it. The reaction mixture was stirred at room temperature and monitored by HPLC. The conversion of the *S*-protected material to the native Big ET proved to be surprisingly slow with *ca.* 50% conversion being seen after 2 days. The reaction mixture was diluted with water (200 cm^3) and extracted several times with carbon tetrachloride and lyophilised. The presence of iodine in the lyophilised material, the absence of any peptidic material and the presence of solid in the carbon tetrachloride extract suggested that an alternative work-up procedure should be adopted.

This procedure was repeated but after 2 days the conversion was again only *ca.* 50% and therefore a further 5 mg of the peptide was treated with iodine in the absence of the indole scavenger. Since the HPLC profile after 1 day showed no marked improvement, the two reaction mixtures were combined and zinc powder added to them. The mixture was stirred for 1 h after which it was diluted with water (200 cm^3), filtered and concentrated to 50 cm^3. A slight colouration of iodine was seen in the filtered solution and more zinc was added and stirred for a further hour. Attempts were made to extract the peptide by use of Sep-Pak C_{18} cartridges but no peptidic material could be isolated. This method was abandoned in favour of milder oxidation methods.

Attempted Synthesis of Big ET (1–38) *from the Acm Peptide.*—The Acm peptide (100 mg, 0.022 mmol) was dissolved in TFA (5 cm^3) and anisole (96 mm^3, 40 equiv.) added to the solution. After saturation of the latter with nitrogen, silver trifluoromethanesulfonate (226 mg, 0.88 mmol, 40 equiv.) was added to it and the mixture stirred at 0 °C for 3 h. The TFA was partially removed under reduced pressure and the silver complex precipitated by addition of ether. The solid was filtered off, dissolved in acetic acid (50%; 15 cm^3) and DTT (339 mg, 2.2 mmol, 100 equiv.) added to the solution which was then stirred for 3 h. The solid was removed by centrifugation and the supernatant subject to gel filtration (G25). The peptide fractions were combined and lyophilised to give the fully reduced peptide (80 mg). This was dissolved with difficulty in acetic acid (10%; 30 cm^3) and diluted to 200 cm^3 with water (10^{-4} mol dm^{-3} peptide). The pH was adjusted to 8 with ammonia, and oxidation was allowed to proceed for 24 h with HPLC monitoring. Concentration and filtration followed by lyophilisation gave crude Big ET (1–38) (4 mg) which was purified by preparative HPLC (20–60% B in 20 min 5 cm^3 min^{-1}, Vydak C_{18}) (3.2 mg). Coinjection with commercial Big ET gave a single peak but the peak due to synthetic Big ET was rather broad. Isocratic analytical HPLC (C_{18} short, 24% B) showed the presence of two isomers in a 3:1 ratio.

Modification of Synthesis of Big ET (1–38) **10** *using the Mixed Disulfide Method.*—The above procedure was followed on a 50 mg sample of the tetra-Acm peptide until the gel filtration stage. The relevant fractions were pooled, diluted to 1100 cm^3 (10^{-5} mol dm^{-3} peptide) with guanidine hydrochloride (1 mol dm^{-3}), and the pH adjusted to 8 with ammonia. Thereafter, glutathione (338 mg, 1.1 mmol) and oxidised glutathione (67 mg, 0.11 mmol) were added to the mixture. HPLC immediately showed the presence of a peak corresponding to Big ET (1–38) and the absence of a peak for the reduced peptide. Since being stirred for 24 h the solution showed no further change, it was acidified to pH 4, filtered (Millipore HV, 45 nm) and then applied directly through the A pump to an Aquapore C_8 short prep column. The column was washed with solvent A and then

eluted with a gradient of 0–80% B in 60 min. The broad peak corresponding to Big ET was lyophilised to give the crude peptide (25 mg).

Examination of the crude peptide under isocratic conditions % B = 28% showed two broad peaks in a ratio of approximately 3 : 1 which became sharper when a shallow gradient was employed. Preparative HPLC, using a shallow gradient, resolved the peptide into two components (A 12 mg) and (B 5 mg) which were compared under a variety of analytical HPLC conditions with commercial Big ET samples. A and B had identical molecular weights and amino acid analyses and A was co-eluted with commercial Big ET and the major component from the previous synthesis.

Compound A *m/z* (FAB) 4283.8 (4283.0), t_R (HPLC) 39.6 min 20–50% B in 120 min. Vydac C_{18}, identical with natural Big ET; *m/z* 4282.944 31, < 1 ppm ($C_{189}H_{283}N_{48}O_{56}S_5$ requires 4282.944 17).

Compound B *m/z* (FAB) 4281.8 (4283.0), t_R (HPLC) 37.9 min (Found: *m/z* 4282.944 31; therefore < 1 ppm).

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