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Spacer Molecules in Peptide Sequences: Incorporation into Analogues of Atrial Natriuretic Factor

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Abstract: In the present study, 10 modified human atrial natriuretic factor (hANF) analogues were designed using solid phase synthesis. This was carried out by replacing 'alkyl or glycol' spacer with octapeptide sequence within the cyclic portion of hANF in each analogue synthesised. The unnatural amino acid spacers (1b) and (2d) have been synthesised using solution chemistry. The latter spacers were successfully incorporated into the peptide structure, using solid phase synthesis assembly. Amongst the ten analogues, thus prepared, two in which the alkyl spacer was used to substitute amino acid residues Gly_{15} to Gly_{22} and Arg_{14} to Leu_{21} to give 4a and 4b, successively. In the purification process of the latter analogues (4a, 4b), problems of their severe solubility were encountered. The eight glycol-spaced analogues (5a-5h) were successfully synthesised and purified using HPLC. The structure of (5a-5h) was confirmed by the presence of mass ion peaks in the atom bombardment mass spectroscopy (FAB MS) and by NMR. The latter analogues were tested, *in vivo*, for their ability to bind to specific hANF receptors, as agonists or antagonists. The biological results have showed that none of these analogues (5a-5h) were active. © 1997 Elsevier Science Ltd.

1. Introduction:

Atrial Natriuretic Factors (ANF) are a group of cyclic peptides which was discovered and isolated, from the atrium of the heart.[1, 2] It has been found to consist of three major groups: Atrial Natriuretic Peptide(ANP), B-form Natriuretic Peptide (BNP), and C-Natriuretic Peptide(CNP).[3] It is a 28 amino acid polypeptide hormone which originated from a 126 amino acid ANF-Prohormone[4] and the human hormone consists of amino acid sequence of 99-126 amino acids, as shown in figure 1.[5]

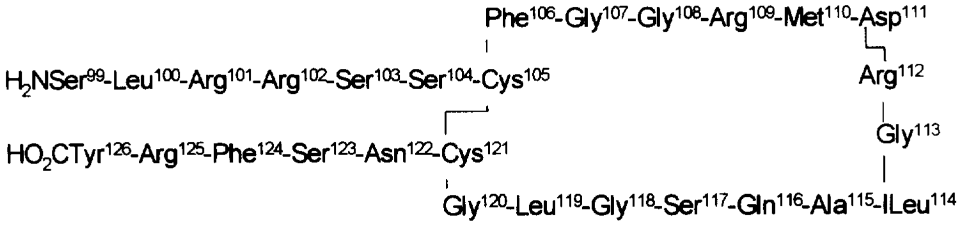


Figure 1. Human Atrial Natriuretic Factor (h-ANF)

ANF is primarily produced and secreted by cardiac atrial myocytes in response to atrial stretch[6-10]. ANF as well as BNP are continuously released from the heart, but appropriate mechanical or neuroendocrine stimuli increase their rate of release with or without concomitant increase in synthesis.[10]

This peptide hormone exerts potent diuretic, natriuretic, vascular smooth muscle relaxing activity and complex interactions with the hormonal and nervous system[11-12]. It acts on the kidney to increase sodium excretion and GFR, to antagonise renal vasoconstriction, and to inhibit renin secretion.[3,13] In the cardiovascular system, ANF antagonizes vasoconstriction , and shifts fluid from the intravascular to the interstitial compartment. In the adrenal gland , ANF is a powerful inhibitor of aldesterone synthesis.[8,13-14]

It participates importantly in the natriuretic response to acute and chronic volume overload. ANF's property of shifting fluid from the vascular to the interstitial compartment ANF acts as a buffering device. guarding against excessive plasma volume expansion in face of an increased total extracellular fluid volume. It is also a physiological modulator of GFR, and mediates nephron hyperfiltration and natriuresis when salt excretion is threatened by a reduction in the number of nephrons[8]. ANF is found to be a potent inhibitor of angiotensin II (ANG II) induced stimulation of transport by a mechanism that requires phosphorylation mediated by cGMP-dependent protein kinase subsequent to a decrease of PKA activity.[15]

ANF and its analogues have been shown to inhibit corticotrophin-releasing hormone (CRH) induced adrenocoticotrophin (ACTH) release, both in vivo and in vitro and it has been suggested that ANF may be a, or the, physiological ACTH-inhibitory factor, to determine whether ANF is relevant in the regulation of ACTH secretion in the ovine foetus. Mcfarlane, et al[16], studies, in this case, have examined the effect of ANF on CRH-stimulated ACTH release in the mature ovine foetus. their results suggested that, ANF does not acutely modulate basal or CRH-stimulated ACTH and cortisol release in the mature ovine foetus.

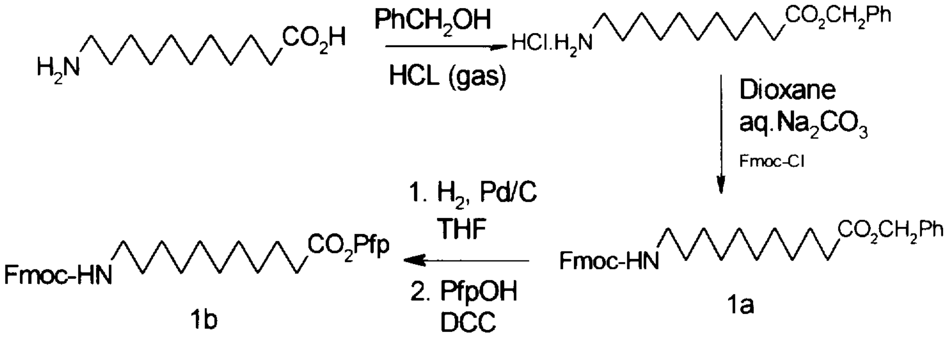
ANF is a short acting agent, susceptible to a rapid inactivation in circulation by neutral endopeptidase resulting in major product cleaved between amino acid residues Cys_7 and Phe_8.[17] Neutral endopeptidase present in high abundance in the kidney, has been implicated in terminating the activity of several peptide hormones, including ANF.[18-19] The unique pharmacological profile of ANF had drawn considerable interest and we sought to prepare structurally related ANF analogues. The main objective, in this study, is to report the synthesis of the unnatural amino acid spacers (**1b & 2d**) and the analogues of the cyclic portion of hANF (**4a, 4b & 5a-5h**).

2. Discussion:

2.1. Discussion of the synthesis of the spacers

2.1.1. Alkyl Spacer

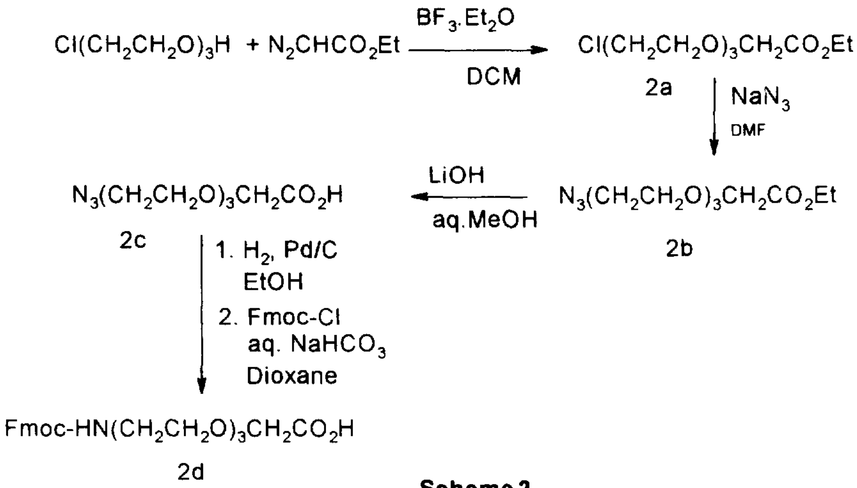
Attempts to N-protect 11-aminoundecanoic acid by reaction with 9-fluorenylmethyl chloroformate in aqueous dioxane[20] were unsuccessful due to the poor solubility of the amino acid in any suitable reaction solvent, or combination of solvents. Therefore the carboxylic acid has to protected, using benzyl alcohol and hydrochloric acid gas, to obtain the ester hydrochloride which was reacted with 9-fluorenylmethyl chloroformate to give Fmoc-aminoundecanoic acid benzyl ester (**1a**) in high yield. This was deprotected by hydrogenolysis on palladium/charcoal in THF to give the Fmoc-N-protected undecanoic acid.[21] The pentafluorophenyl ester (**1b**) was then synthesised, in 78% yield, by reacting the N-protected amino acid with pentafluorophenol in the presence of dicyclohexylcarbodiimide (DCC) in ethyl acetate.[22]



Scheme 1

2.1.2. Glycol Spacer

Initial efforts at synthesising the glycol amino acid were based on coupling triethylene glycol with bromoacetic acid to be followed by esterification.[23] However, this lengthy and low yielding process was replaced by a more efficient coupling reaction. This was carried out by reacting 2-(chloroethoxy)ethoxyethanol and ethyl diazoacetate in the presence of boron trifluoride etherate[24] to give ethyl-2{2-[2(chloroethoxy)ethoxy]ethoxy} acetate (**2a**) in 85% yield. The latter compound was reacted with activated sodium azide[25] in dry DMF to give the azido product (**2b**), in quantitative yield, which in turn was treated with lithium hydroxide in aqueous methanol to result the free acid (**2c**). The azido group was then reduced to amino group, by pressurised hydrogenolysis, followed by the protection of the resulted amine, using 9-fluorenylmethyl chloroformate under standard conditions, to give (**2d**).

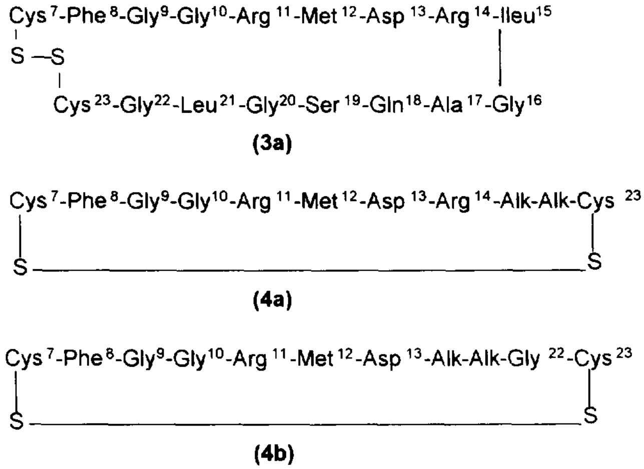


Scheme 2.

2.2. Discussion of the synthesis of ANF analogues:

2.2.1. Lipophilic Spacer Analogues

The alkyl spacer (Alk) was assembled into two peptides, ANF(7-14,Alk, Alk,23) (**4a**) and ANF(7-13,Alk, Alk,22-23) (**4b**), as shown in scheme 3.



(3a)

(4a)

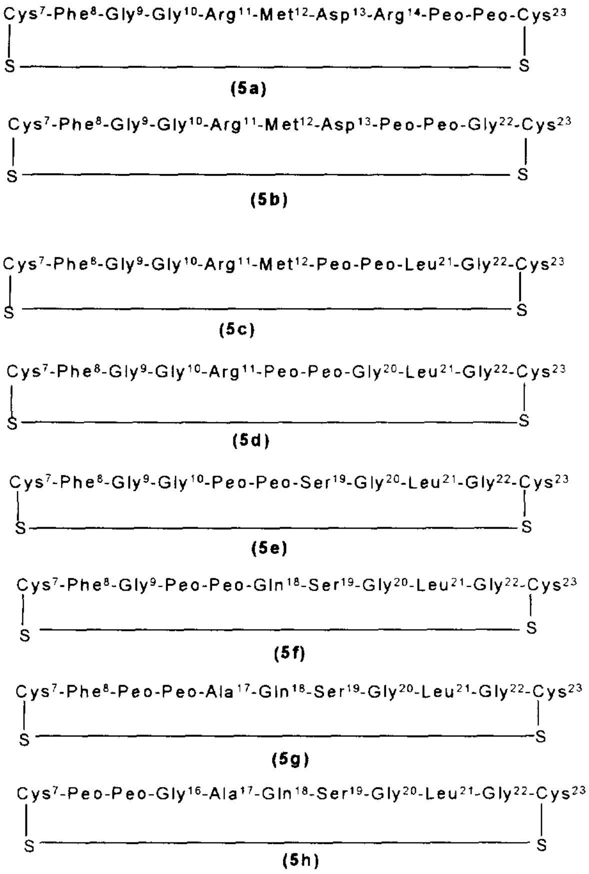
(4b)

Scheme 3.

Assemblies were performed on a CRB Pepsyn II using KA resin and Fmoc/Pfp ester protected amino acids. After cleavage of protecting groups with trifluoroacetic acid (TFA) in the presence of ethanedithiol and phenol as scavengers. After the usual work-up, the product was cyclised to 4a and 4b. Both products isolated were found to be insoluble in most solvents and purification by HPLC was impossible. An alternative isolation using ether precipitation also gave an insoluble solid, in either case, which could not be purified although the correct masses were detected using FAB-MS. The severe solubility problems of these analogues led us to abandon synthesis of other planned alkyl-spaced peptides in this sequence.

2.2.2. Hydrophilic Spacer Analogues

The corresponding two peptides incorporating the polyethyleneoxy spacer (Peo), (**5a**) and (**5b**), were assembled on a Milligen 9050 apparatus by the same chemistry except that the spacer molecule was reacted as the free acid (2d) in the presence of diisopropylcarbodiimide and hydroxybenzotriazole (HOBt). Deprotection and cleavage was carried out as before, with evaporative work-up, gave crude water soluble peptide together with significant amounts of insoluble solid which was shown by ^{1}Hnmr to be polymeric ethanedithiol. Ether precipitation was found to be inefficient due to the solubility of peptide in ether /TFA mixtures. An alternative isolation procedure has evolved which seems to be of general application and superior to other methods. This was carried out by diluting the filtered TFA solution with 5-10 times its volume of petroleum ether with continual swirling. The petroleum layer is decanted and the residue washed with further petroleum ether. Careful washing with diethyl ether by gentle swirling and decantation leaves a residue of peptide essentially free of scavengers and artefacts. Following this process the peptides were dissolved in nitrogen purged 0.1M acetic acid then the pH was adjusted to 7.5 with NH_4OH and the solutions left standing in full conical flasks for cyclisation to occur by air oxidation. After cyclisation, peptidic material was isolated by solid-phase extraction using C_{18} Bondelut tubes. The peptides were then purified using reverse-phase HPLC on Vydac C_{18} using water/acetonitrile gradients with 0.1%TFA. The two peptides, **5a** and **5b**, were characterised by FAB-MS and following their successful preparation the remaining sequences, **5c** to **5h**, were similarly synthesised using the parallel assembly facility of the Milligen 9050. Post-assembly treatment was as before except that phenol was omitted from the deprotection mixture with peptides that did not contain arginine. All the peptides (**5a-5h**), as described in scheme 4, were single peaks by analytical HPLC and showed only the expected ion masses with FAB-MS[26].



Scheme 4

Biological activity of the analogues (5a-5h) and a general review

From early studies, on structure activity relationship to ANF analogues, Berman et al.,[27] (review)[28] have prepared the retro-inverso isomeric of ANF in effort to provide metabolically stabilised biologically active analogues. They have found their compounds were not active, indicating the importance of peptide backbone direction in determining the conformation and hydrogen bond donor/acceptor pattern required in the interaction of truncated ANF sequences with ANF receptor.

Dong Li. Song et al.,[29] investigated the structure function relationships of ANF to develop more potent, longer acting and more specific ANF analogues for future clinical application. They found their compounds, Des-Ser$_{103,104}$, [Mpr$_{105}$, D.Ala$_{107,114}$]APII-amide, more active than human ANF(99-126) in lowering blood pressure and causing natriuresis in urethane anaesthetised rats by 2.5 and 2.2 fold.

For the structural variants of ANF most structure-activity studies have concentrated on deletions from within the ring leading to compounds with substantially reduced binding affinity to bovine *zona glomerulosa* cell membranes.[30] In a review by Watanabe et al.[31] reported that the disulphide bridge is vital for maintenance of the bioactive conformation , however, its replacement by an ethylene linkage gives an analogue that has 20% of normal activity.[32] Ring contractions by loss of one or more residues have a major impact on the activity.[33] Aminopeptidase resistant analogues have been synthesised by replacement of Cys-7 with HS(CH2)xCO2H; with x=2 (analogues to Cys) there was a little loss of activity (95% original), but when x=1 a dramatic reduction in activity was observed (15% original).[34] Replacement of Asp_{13} or Phe_8 with the corresponding D-amino acid leads to almost complete loss of activity (0.3% and 4.4% respectively), but Ala_9 can be inverted with little effect (98%).[30] The replacement of Arg_{27} by lysine gives an analogue with 3.4% of original activity, this indicates that an important interaction may exist involving the arginine guanidinium group.[35]

2D ^{1}Hnmr studies of rat ANF(1-23) suggest that a *B*-type structure is adopted (due to the presence of several glycines),[36] although Theriault and co-workers (1987)[37] reported that no defined secondary or tertiary structure in pH range 2.73-5.21 for rat ANF(3-28). IR observations of monomeric ANF aqueous solutions in the amide region point to a random structure, but gradual self association occurs to give a *B*-sheet assembly[38]. This ordering was also observed in ANF binding to bilayer vesicles of dimyristyl phosphatidylglycerol, a possible mimic of receptor interactions.[39]

Most previous studies on the atrial natriuretic factor structure activity, suggest that, the cyclic portion of ANF, thought to be the most important part in the activity of ANF. In addition to a therapeutic standpoint, the endogenous 28 residue peptide (ANF) is not orally active and it has a short duration of biological action. Within these constraints, we prepared 10 modified cyclic analogues, of the cyclic portion of hANF, in which octapeptide sequences from residues Phe_8 to Gly_{22} are replaced by unnatural amino acid spacers, ranging from the lipophilic, alkyl spacer $-HN(CH_2)_{10}-COHN(CH_2)_{10}-CO-$, to the more hydrophilic, glycol spacer $HN(CH_2CH_2O)_3CH_2CO-NH(CH_2CH_2O)_3CH_2CO-$. The purified analogues (**5a-5h**) were tested for their ability to bind to the ANF receptors. The results suggest that none of the synthetic analogues showed any agonist or antagonist properties in the isolated rabbit renal artery preparation. This might be due, by incorporating the spacer, to the removal of some the hydrogen bonding possibilities and the introduction of more flexibility within the cyclic portion of ANF. This could make a big alteration on the binding site of the analogues. On the other hand, the removal of eight side chains of amino acids, at once, might effect binding affinity.

In summary, we have developed a new hydrophilic spacer unit, and demonstrated its ready incorporation into cyclic peptide analogues of ANF, although with loss of biological activity. This spacer should find further applications in solution and solid phase synthesis, and, potentially, in the construction of peptoid libraries.

Experimental Work:

Instrumentation and Experimental Techniques:

Infrared spectra were recorded in the range of 4000-600 cm^{-1} , using a perkin Elmer 1600 series FTIR. Proton NMR was recorded on a Joel GNM GX FT 270 spectrometer. The spectra were recorded in $CDCl_3$, and are expressed in parts per million (δ). Tetramethylsilane was used as an internal standard. Multiplicities are given as follows: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m).

Thin layer chromatography (TLC) was used extensively as qualitative guide during the preparation of spacers. All the reagents and solvents were purified and dried when required.

1. Synthesis Of Fmoc-$HN(CH_2CH_2CH_2)_3CH_2CO_2$Pfp (1b)

Benzyl-11-aminoundecanoate hydrochloride.[40] A current of dry hydrochloric acid gas was passed through a suspension of 11-aminoundecanoic acid (20g, o.1 mol) in benzyl alcohol (200ml) until saturated. The resulting mixture was heated at 80°C for 2hrs and the water formed was removed by azeotropic distillation, followed by removal of excess benzyl alcohol. Diethyl ether was added to the cooled mass to effect crystallisation, and the product was recrystallised from absolute ethanol to give (19.8g, 68%) colourless crystals with m.p., 106-108°C (Lit, 113°C). ^{1}Hnmr (60MHz, D_2O) δ 7.15(m, 5H, Ar-H), 4.95(s, 2H, $PhCH_2O$), 3.0(t, 2H, J= 5.15Hz, D_2NCH_2-), 2.2(t, 2H, J= 5.10Hz, -CH_2CO-), 1.2(m, 16H, -$(CH_2)_8$-); Anal. Calcd.for $C_{18}H_{29}NO_2.HCl$ (343.88): C, 62.90; H, 8.80; N, 4.10. Found: C, 63.40; H, 9.20; N, 4.10.

Benzyl 11-(9-Fluorenylmethyloxycarbonyl-amino)undecanoate (1a).[20] To a suspended solution of Benzyl 11-aminoundecanoate hydrochloride y (5.8g, 0.02mol) in 10% sodium carbonate (150ml) and dioxane (170ml), 9-Fluorenylmethyl chloroformate (5.2g, 0.02mol) in dioxane(50ml) was dropwise added over a period of 1hr at 0°C. The resulting suspension was vigorously stirred for further 3hrs at room temperature. The mixture was then filtered and the solvent was removed under reduced pressure. The residue was taken up in dichloromethane (300ml), washed with 2N.hydrochloric acid (100ml), dried over $MgSO_4$, filtered and evaporated. Product was recrystallised from dichloromethane on addition of petroleum ether (b.p.=60-80°C) to give benzyl 11-(Fmoc-amino)undecanoate (9.5g, 93%) as a colourless needles with mp. 86-87°C. ^{1}Hnmr (270 MHz, $CDCl_3$) δ 7.28-7.77(m, 13H, Ar-H), 5.11(s, 2H, $PhCH_2O$-), 4.78(brs, 1H, NH), 4.34(d, 2H, J=6.6Hz, Fmoc-H), 4.21(t, 1H, J=6.6Hz, Fmoc-H), 3.18(m, 2H, $HNCH_2$-), 2.33(t, 2H, J=5.10Hz, -CH_2CO-), 1.28(m, 16H, -$(CH_2)_8$-). FAB.MS; 514(M^{+1}). Found: C,76.80; H, 7.70; N, 2.70; $C_{33}H_{39}NO_4$ (513.65), requires: C, 77.00; H, 7.65; N, 2.70.

11-(9-Fluorenylmethyloxycarbonyl-amino)undecanoic acid. Benzyl-11-(Fmoc-amino)undecanoate (9.25g, 0.018mol), in THF (150ml) was treated with 10% palladium on charcoal (120mg), and placed on an atmospheric pressure hydrogenator overnight. The mixture was filtered through celite and solvent was removed in vacuo. Product was recrystallised from ethyl acetate to give 11-(Fmoc-amino)undecanoic acid (6.7g, , 88%), as colourless crystals with mp. 118-120°C. ^{1}Hnmr (270MHz, $CDCl_3$:$DMSO^{-d}_6$,1:1) δ 11.6(brs, 1H, OH), 7.36-7.73(m, 8H, Fmoc-H), 6.58(brs, 1H, NH), 4.34(d, 2H, J= 6.6Hz, Fmoc-H), 4.23(t, 1H, J=6.6Hz, Fmoc-H), 3.08(t, 2H, J= 5.2Hz, $-HNCH_2-$), 2.26(t, 2H, J=5.15Hz, $-CH_2CO$), 1.27-1.29(m, 16H, $-(CH_2)_8-$); Found: C,73.40; H, 7.95, N, 3.25; $C_{26}H_{33}NO_4$ (423.53) requires: C, 73.70; H, 7.85; N, 3.30.

Pentafluorophenyl 11-(9-Fluorenylmethyloxycarbonyl-amino)undecanoate (1b).[22] 11-(Fmoc-amino) undecanoic acid (6.35g, 0.015mol) in dry THF (125ml) was treated with pentafluorophenol (2.76g, 0.015mol) and dicyclohexylcarbodiimide (DCC) (2.9g, 0.015mol) at 0°C. After stirring at room temperature for 4hrs, precipitated urea (DCU) was filtered and solvent was removed in vacuo. Solid residue was recrystallised from petroleum ether (bp.60-80°C) to give Pentafluorophenyl 11-(9-Fluorenylmethyloxycarbonyl-amino)undecanoate (7.9g, 89%), as white crystals, with mp. 64-66°C; ^{1}Hnmr (270MHz, $CDCl_3$), as the previous(z) except δ 2.26 triplet moved to δ 2.75; MS, CI, m/z 590(M^{+1}, 100%). Found: C, 65.50; H,5.50; N, 2.40; $C_{32}H_{32}F_5NO_4$ (589.57) requires; C, 65.20; H, 5.50; N, 2.40.

2. Synthesis of Fmoc-NH$(CH_2CH_2O)_3CH_2CO_2H$ (2d)

Ethyl 2-{2-[2-(chloroethoxy)ethoxy]ethoxy}acetate (2a).[2] To a mixture of 2-(chloroethoxy)ethoxy-ethanol (16.57g, 0.098mol) and ethyl diazoacetate (11.21g, 0.098mol) in dry dichloromethane (270ml) at 0°C under nitrogen, 6-7 drops of freshly distilled boron trifluoride etherate ($BF_3.Et_2O$) (ex CaH_2) was added. The resulting mixture was stirred until bright yellow colour had virtually disappeared (over weekend). Couple drops of $BF_3.Et_2O$ were added, no N_2 evolution was observed. 0.2N NaOH (29ml) was then added and the mixture was rapidly stirred for 10mins.. After separating the two layers, the organic phase was washed with water (35ml), the aqueous layer was extracted with DCM (35ml). The combined organic solution was dried over Sodium sulphate, filtered and evaporated in vacuo to give a pale yellow oil. The latter oil was distilled under vacuum at 120-124°C/0.2-0.3mmHg to obtain Ethyl 2-{2-[2-(chloroethoxy)ethoxy]ethoxy}acetate (21.27g, 85%), in a pure form; ^{1}Hnmr (270MHz, $CDCl_3$) δ 4.18(q, 2H, J=7.10Hz, $-COOCH_2-$), 4.15(s, 2H, OCH_2CO-), 3.68(m, 12H, $Cl(CH_2\ CH_2O)_3$), 1.28(t, 3H, J=7.10Hz, $-CH_3$); MS-CI; m/z 255(M^{+1}, 100%); Found: C, 47.14; H, 7.80; Cl, 13.90; Calcd.for $C_{10}H_{19}ClO_5$ (254.35); C, 47.15; H, 7.72; Cl, 14.20; ν_{max} (liquid film), 2860 (CH), 1730(CO), 1120-1200cm^{-1} (CO-OEt).

Ethyl 2-{2-[2-(azidoethoxy)ethoxy]ethoxy}acetate(2b).[25] Ethyl 2-{2-[2-(chloroethoxy)ethoxy]ethoxy} acetate (8.8g, 0.0345mol) and activated sodium azide (3.25g, 0.050mol) in dry DMF (86ml) were heated together at 100°C for 5hrs after which the mixture was filtered and the filtrate was concentrated in vacuo. The residue was taken up in dichloromethane (50ml) and washed with water (2x30ml), dried over sodium sulphate, filtered and evaporated to give a pure product (8.7g, 96%). ^{1}Hnmr (270MHz, CDCL$_3$) δ 4.21(q, 2H, J=7.10Hz, -COOCH$_2$-), 4.15(s, 2H, OCH$_2$CO-), 3.67(m, 10H, 5xCH$_2$), 3.39(t, 2H, J= 5.0Hz, N$_3$CH$_2$-), 1.28(t, 3H, J=7.10Hz, -CH$_3$); MS-CI; m/z 262(M^{+1}. 100%); Found: C, 46.15; H, 7.40; N, 15.90; $C_{10}H_{19}N_3O_5$ (261.27) requires; C, 46.00; H, 7.30; N, 16.10; ν_{max} (liquid film), 2860(CH), 2080(N$_3$), 1730 cm^{-1}(CO).

2-{2-[2-(azidoethoxy)ethoxy]ethoxy}acetic acid (2c). Ethyl 2-{2-[2-(azidoethoxy)ethoxy]ethoxy} acetate (7.6g, 0.029mol) and lithium hydroxide (2.78g, 0.066mol) in 66% aqueous methanol (60ml) were stirred overnight at room temperature. Volatiles were removed in vacuo and the residue was acidified with conc. hydrochloric acid. The product was extracted with dichloromethane (2x50ml), dried over sodium sulphate, filtered and evaporated to give a pale yellow oil (6.10g, 90%). ^{1}Hnmr (270 MHz, CDCL$_3$) δ 8.64(brs, 1H, -CO$_2$H), 4.19(s, 2H, OCH$_2$CO-), 3.66(m, 10H, 5xCH$_2$), 3.39(t, 2H, J= 5.10Hz, N$_3$CH$_2$-) ; MS-CI; m/z 234(M^{+1}. 100%); Found: C, 40.80; H, 6.60; N, 18.20; $C_8H_{15}N_3O_5$ (233.22) requires; C, 40.10; H, 6.50; N, 18.00; ν_{max} (liquid film), , 2105cm^{-1} (N$_3$).

2-{2-[2-(aminoethoxy)ethoxy]ethoxy}acetic acid. 2-{2-[2-(azidoethoxy)-ethoxy]ethoxy} acetic acid (7g, 0.030mol) in absolute ethanol (125ml) was treated with 10% palladium on charcoal catalyst (500mg), degassed with nitrogen and placed on a pressurised hydrogenator overnight at 110p.s.i. The solution was then filtered through celite and concentrated in vacuo to give a pale yellow syrup (6.2g, 100%) with no trace of azide (by IR), and giving a positive test with ninhydrin reagent. The crude product was used in the next step without further purification.

2-{2-[2-(Fmoc-aminoethoxy)ethoxy]ethoxy}acetic acid (2d). 2-{2-[2-(amino-ethoxy)ethoxy]ethoxy} acetic acid (12.9g, 0.062mol) in water (125ml) at 0°C was treated with 10% sodium bicarbonate (125ml) and 9-fluorenylmethyl chloroformate (11.20g, 0.068mol) in dioxane (125ml), added dropwise over 3hrs, and the resulting suspension stirred at 0°C for 2hrs and at room temperature for 48hrs. Volatiles were then removed, the residue was dissolved in 2N.hydrochloric acid and extracted with dichloromethane (3x150ml). The organic phase was dried over magnesium sulphate, filtered and evaporated. The resulting oil was purified by dry flash column chromatography using progressively increasing percentage of acetic acid and ethyl acetate, combining relevant fractions, and removing residual acetic acid by azeotropy with dioxane to give (13.36g, 50%) of a pale yellow oil. ^{1}Hnmr (270MHz, CDCl$_3$) δ 10.88(brs, 1H, CO$_2$H), 7.73(d, 2H, J= 7.7Hz, Fmoc-H), 7.58(d, 2H, J = 7.30Hz, Fmoc-H), 7.24-7.39(m, 4H, Fmoc-H), 5.65(brs, 1H, NH), 4.37(d, 2H, J= 6.6Hz, Fmoc-H), 4.22(t, 1H, J= 6.6Hz, Fmoc-H), 4.19(s, 2H, OCH$_2$CO$_2$H), 3.60-3.62(m, 10H, 5x-CH$_2$-), 3.35-3.37(m, 2H, NCH$_2$-); FAB MS, m/z 430(M^{+1}, 100%); Found: C, 64.00; H, 6.40; N, 3.10; $C_{23}H_{27}NO_7$ (429.45) requires; C, 64.30; H, 6.30; N, 3.30.

Synthesis, purification and analysis of ANF analogues

Materials and Methods

Assembly of the spacer group nonpeptides was carried out using a continuos flow variant of the 9-fluorenylmethyloxycarbonyl (Fmoc)/ polyamide solid phase method on the 'miligen automaticpepsynthesiser apparatus', which was connected to a 'PCV', with continuos U.V. monitoring of the various steps at 213-217nm.

For hyroxylmethylphenoxyacetyl-norleucyl-polydimetylacrilamide-Keiselghur supported resin (Pepsyn KA) and all the Fmoc-amino acids and active esters were obtained from Cambridge research biochemical (CRB), whereas the glycol (Peo) spacer unit (free carboxylic acid end) was prepared.

Appropriate active side chains were protected as follows: Arg as the N-methoxytrimethylbenzenesulphonyl (Mtr), derivative, Asp as the t-Butyl ester (t-BuO) and Cys as trityl (Trt) thioester. Intermediate Fmoc-peptide resins were deprotected with 20% piperidine/dimethylformamide (DMF) solution. DMF was freshly distilled under reduced pressure.

Reverse phase HPLC (analytical and preparative) was performed with an LKB bromma system incorporating gradient elution and diode array detector and LDC/Milton Roy system.

Trifluoroacetic acid (TFA) for HPLC was purified by distillation from solid potassium permanganate, water was double distilled and acetonitrile was far U.V. transparent HPLC grade. FAB Mass Spectrometry was routinely applied to confirm the composition of each peptide, although purity was established by HPLC.

Reverse phase HPLC column was used to analyse and purify the crude peptide. The analytical chromatography was performed on a 5µ C_{18} Vydac packed column (4.6 mm x 250mm) with flowrate of 1ml/min. Whereas, the preparative chromatography was performed on a wider pore 10µ C_{18} Vydac packed column (10mm x 250mm) with flowrate of 3ml/min. Solvents mixture used in the purification of the synthesised analogues are: Solvent A= 0.1%TFA in Water; Solvent B= 0.1%TFA in Acetonitrile/ Water (9:1)

Synthesis of Cys(trt)-Pepsyn-KA. Pepsyn-KA resin (2g, 0.2meq/g) was swollen with DMF then washed clean of fine material by several cycles of swirling and decantation. The resin was then transferred to the reaction columns (1g in each). Fmoc-Cys(trt)-OPfp (4equi) was dissolved in a minimum of DMF, in the presence of 4-dimethylaminopyridine (DMAP), (5mg, 0.04mmol) was added. After 4hrs recirculating the solution, the column was washed with DMF and the esterification repeated with fresh Fmoc-Cys(Trt)-OPfp, and DMAP. A quantitative U.V. test confirmed nearly 100% loading of the active sites on the resin. The Fmoc group was cleaved from the washed peptide resin with 20% piperidine /DMF. The Cys(Trt)-OPepsyn KA-resin was washed, dried and stored in the freezer to be used in the synthesis of ANF analogues.

Synthesis of linear ANF analogues. Pepsyn KA resin which had previously derivitised with Cys(Trt)(0.10meq) was placed in the reaction column. To this the desired Fmoc-protected amino acid pentafluoro-ester (4 equi.) dissolved in a minimum amount of DMF, in the presence of 1-hydroxybenzotriazole (HOBt) (4 equi.) was added. After 30mins. Of recirculation, a Kaiser colour test indicated there was no free amine present. The Fmoc group was cleaved from the peptide-bound resin with 20% piperidine/DMF and the synthesis was continued using active PfpO esters of protected Fmoc-amino acids and the Fmoc-spacer substituent (4 equi.) together with equimolar additions of HOBt. After the peptide has been assembled and the final Fmoc protecting group removed it was washed with t-amyl alcohol (50ml), glacial acetic acid (50ml), dichloromethane (100ml) and finally diethyl ether (100ml) then dried in a stream of nitrogen to leave peptide bound-resin.

ANF(7-14, Alk, Alk,23) (4a). To a cooled sample of peptide-bound resin (30mg), a cooled mixture of scavenger; TFA (2.90ml), phenol (0.19g, 5%), deionised water (0.18ml, 5%), thioanisol (0.18ml, 5%) and ethanedithiol (0.09ml, 2.5%) was added. The resulting mixture was stirred on a stir plate and allowed to warm up to room temperature for 2hrs. The reaction mixture was then filtered and to the filtrate a cold solution of diethyl ether (15ml) was added . The linear peptide had precipitated and the resulting mixture was centrifuged. This operation was repeated several times to give a white solid. The latter product was analysed by HPLC using the same column chromatography as above but the solvent gradient profile used was, 5min. At 20% B followed by a gradient of 1% B/min. To 100% B. The solvent composition was maintained at this value for 5 min. then adjusted to 20% B over 16 min. period. Finally, it was kept at 20% B for 10 min (overall run time 126 mins.). Peak detection was carried out at 213-217nm and 252-254nm. No peak was detected on the chromatogram, this could be due to the severe solubility of the peptide in the solvent used.

ANF(7-13, Peo, Peo, 22-23) (5b). A sample of the peptide-bound resin (59mg) was added to a degassed solution of TFA (10ml), ethanedithiol (0.20ml, 2.5%) and phenol (0.25g, 2.5%). After standing overnight (16hrs) at room temperature, the mixture was filtered, washed with TFA (5ml) and concentrated in vacuo to leave a yellow liquid (2ml). The latter was dissolved in water (15ml) and washed with t-butyl methyl ether (2 x 10ml), separated and lyophilised overnight to give crude linear ANF(7-13, Peo, Peo, 22-23) (24mg) as a while fluffy solid.

The crude product, thus prepared was dissolved in a degassed solution of 5% B in a 100ml conical flask. To this, a degassed solution of 2.5% of acetic acid in water was added. The resulting mixture was purged with nitrogen while pH of the solution was adjusted to pH 7.5 using NH_4OH solution. The reaction mixture was then left to stand to an open air for 5 days by which time a free thiol test proved negative. The reaction mixture was then passed through a C_{18} Bond Elut column and by washing by 5% B to remove all buffer solution and any remaining scavenger. The crude cyclic peptide was collected by washing the Bond Elut by

60% B (20ml). After removing the organic solvent, the aqueous solution was lyophilised to dryness to give a fluffy white solid (18 mg). The latter peptide was purified using the same solvent gradient profile for both columns (analytical and preparative): 5min at 5% B followed by a gradient of 1% B/ min to 25% B, 0.5%B/min to 40% B, 1%B/min to 60% B, 5min at 60% B, then 5%B/min to 5% B and 10min at 5% B (overall run time 110min). Peak detection was carried out at 213-217nm and 252-254nm. The crude peptide was purified in three batches each of approximately 6mg (2mg/ml). The major peak was collected on an automated fraction collector, and had a retention time of 24 min (24% B). The fraction from the above purification pooled and evaporated to dryness on the freeze drier to yield a white fluffy solid (6.2 mg). FAB MS; 1322 (M^{+1}, 100%), Calculated 1321.52. 2mg were send for biological test and no activity was shown by the latter analogue.

ANF(7-14, Peo, Peo, 23) (5a), ANF(7-11, Peo, Peo, 20-23) (5d), and ANF(7-12, Peo, Peo, 21-23) (5c) were treated in the same way and at similar conditions as the above analogue (5X). After purification the above analogues were obtained in a pure form, their retention times and FABMS results are as follows: (5a); 23-24 min (23-24% B); FAB MS, 1421(M^{+1}, 100%), calculated 1420.60, (5d); 39 min (32% B); FAB MS; 1246(M^{+1}, 100%), Calculated 1245.56 and (5c) 32 min (28.5% B); FAB MS, 1320(M^{+1}, 100%), Calculated 1319.58.

ANF(7, Peo, Peo, 16-23) (5h). Peptide-bound resin (1.36g) was added to a solution of TFA (40ml) and 5% ethanedithiol. The resulting mixture was stirred on a stir plate for 3hrs at 20-25°C and then filtered. To the filtrate, a distilled petroleum (bp.60-80°C) (500ml, more than 10 times of the volume of scavenger) was added. The resulting mixture was shaken for period of time and then the solution was decanted. A same volume of petroleum was added to the residue to make sure all the scavenger was removed. After the petroleum ether was decanted, a solution of cold diethyl ether (200ml) was added to the residue, which was precipitated. The latter operation was repeated several times (from 3 to 4) and then the crude linear peptide was dissolved in a degassed solution of 5% B in a 100ml conical flask. To this, a degassed solution of 2.5% of acetic acid was added. The resulting mixture was purged with nitrogen while pH of the solution was adjusted to pH 7.5 using NH_4OH solution. After 3 days standing at open air. The reaction mixture was then passed through a C_{18} Bond Elut column and by washing by 5% B to remove all buffer solution and any remaining scavenger. The crude cyclic peptide was collected by washing the Bond Elut by 60% B (20ml). The organic solvent was removed, the aqueous solution was lyophilised to dryness to give a fluffy white solid (120 mg). The latter peptide was analysed and purified (using the same columns, same packing material as before). The solvent gradient profile used was: 5(5), 15(15), 45(30), 60(60), 65(60), 75(5), 86 min (5% B). The product was collected and had a retention time of 20-21 min (17.5-18.5% B). After the evaporation of the organic solvent and freeze-dried the water solution, a pure peptide was obtained as a white solid (11mg), FAB MS, 1171(M^{+1}, 100%), calculated 1170.5.

ANF(7-8, Peo, Peo, 17-23) (5g), ANF(7-9, Peo, Peo, 18-23) (5f), and ANF(7-10, Peo, Peo, 19-23) (5e) were prepared and purified in the similar manner and under the same conditions as the above peptide analogue (5X) and their retention times and FABMS results are successively shown; (18.3mg) 35-36 min (25-26% B) and FAB MS; 1262(M^{+1}, 100%), calculated 1261.54, (21mg) 29-30 min (22-23% B) and FAB MS; 1248(M^{+1}, 100%), calculated 1247.29, and (19mg) 46.5-47 min (33-34% B) and FAB MS; 1177(M^{+1}, 100%), calculated 1176.49

Biological Activity

Method. Male New Zealand white rabbits (3-3.5Kg) were killed by intravenous pentobarbitone and the left renal artery was rapidly dissected. The vessel was cleaned of any excess tissue and rings of 2-3mm cut. Each ring was placed onto a transducer hook in Kreb's solution with indomethacin (3μM) present and gassed with 95% O_2, 5% CO_2. Each tissue was washed and tensioned to 2g, left for 5mins. And then retensioned a total of four times over 15mins.

After an incubation period of 30mins, the tissue was challenged with KCl 80mM to contract it, and then relaxed with acetyl choline 10^{-5}M to confirm that endothelium was present. After washing 4 times over 15mins and leaving a further 30mins to settle the tissue, phenylepherine (PE) 10^{-4}M was added and then washed out as before. A further 30mins later, each tissue was contracted with 3 x 10^{-6}M PE and left until a steady state response was recorded. The tissue was then relaxed with Human ANF (hANF) followed by sodium nitroprusside (SNP) 10^{-4} to give a maximum relaxation.

After washing, the tissue was left a further 30mins before being contracted again with PE 3 x 10^{-6}M . When a steady state response had been achieved, the tissue was given the synthetic analogue. If the analogue had no effect on tissue, then hANF was given to see if the analogue was acting as an antagonist to ANF. At the end of the hANF curve, SNP 10^{-4}M was again given.

Method of preparation of analogues. Each analogue was dried under nitrogen. The vial was then weighed and 0.1ml of 0.1% TFA in water added to dissolve the peptide. This was then removed to another container and the vial rinsed out with 0.4ml acid/alcohol which was added to the 0.1ml TFA. The vial was then dried and reweighed. The difference in weights gave the amount of peptide in each vial. Each was then made up to 10^{-3} or 10^{-4}M, depending on amount available.

Results

Change in tension induced by drug is expressed as a percentage of the contraction to PE 3 x 10^{-6}M when a steady state response has been established .

Acetyl choline relaxed the KCl contracted tissue by 64.3% ±4.2 (n=6) showing that the endothelium was still intact.

Phenylepherine 3 x 10^{-6}M caused 74.1± 4.0% (n=7) of maximum contraction (10^{-4}M) in the first curve, and 79.0±3.1% (n=7) in the second.

SNP 10^{-4}M gave 96.2±2.2% relaxation of the first PE curve and 96.4±0.3% of the second (n=7).

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