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

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Abstract: *The spacer reagents FmocHN($CH_2CH_2CH_2$)$_3CH_2COOPfp$ (**1b**) and FmocHN(CH_2CH_2O)$_3CH_2COOPfp$ (**2d**) have been prepared and used to substitute for tetra-residue sequences in the cyclic portion of Atrial Natriuretic Factor (ANF) by solid phase peptide assembly.*

A useful strategy in the pursuit of structure-activity correlation in peptides is that of aminoacid deletion and substitution. This can enable the regions of the peptide important for binding to be identified, as distinct from those necessary merely for maintenance of gross structure. It is often important that the substituent species should readily mimic the backbone secondary structure of the replaced aminoacid sequence in both conformation and general lipophilicity, and a range of conformationally locked systems have been introduced. We have had interests in flexible mimetics for incorporation into peptide sequences.[1] Our recent studies have been extended to the production of lipophilic and hydrophilic 'spacer' molecules that might be of general application in a form appropriate for incorporation by solid-phase peptide methods. We report herein our preliminary spacer synthetic studies, and exemplification of the methodology by incorporation of our spacers into ANF sequences.

7-Aminoheptanoic acid had been used by us[2] during our studies of Melanin Concentrating Hormone, as a linear replacement for cystine. More usually, it is required to substitute for a sequential series of residues. After considering the availability of suitable starting materials and the solubilities of intermediates it was decided most appropriate to aim for substitution of blocks of four residues or multiples thereof. The simple alkyl chain would require 11-aminoundecanoic acid (**1a**), but reaction of this with 9-fluorenylmethyl chloroformate[3] in a variety of solvent systems was problematical due to poor solubility of the substrate. The corresponding benzyl ester however, did react smoothly in aqueous dioxane to give the Fmoc derivative in high yield. Hydrogenolysis over palladium/charcoal in THF, followed by reaction with pentafluorophenol in ethyl acetate with dicyclohexylcarbodiimide[4] gave the required Pfp ester (**1b**) as a colourless crystalline solid with mp = 64-66°C in 78% overall yield.

$RHN(CH_2CH_2CH_2)_3CH_2COOX$ (1)

a) R=H; X=H
b) R=Fmoc; X=Pfp

It was recognised that an alkyl spacer would be too lipophilic for many applications so a molecule based on polyethylene glycol was proposed (**2**). Initial effort at synthesising the glycol aminoacid was based on coupling triethylene glycol with bromoacetic acid to be followed by esterification.[5] However, this lengthy and low yielding process was replaced by the more efficient coupling[6] of triethylene glycol with ethyl diazoacetate in the presence of boron trifluoride etherate. This was further improved by using chloroethylene glycol to give (**2a**) in yields greater than 85%. Conversion to the azide (**2b**) using "activated" sodium azide[7] in dry DMF was almost quantitative. Catalytic hydrogenation of the azide gave low yield of the required amine, presumably due to polymerisation and lactam formation. Prior hydrolysis of the ester function with lithium hydroxide followed by hydrogenation over palladium/charcoal in ethanol gave the aminoacid (**2c**) which was converted to the Fmoc-Pfp derivative (**2d**) in the usual way.

$R(CH_2CH_2O)_3CH_2COOX$ (2)

a)R=Cl; X=Et
b)R=N_3; X=Et
c)R=NH_2; X=H
d)R=FmocHN; X=Pfp
e)R=FmocHN; X=H

During these studies a report was published[8] of the incorporation of 8-aminooctanoic acid as a three aminoacid surrogate in Atrial Natriuretic Factor. As ANF(7-23)(**3**) was the planned target peptide for our

(3)

Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Met^{12}-Asp^{13}-Arg^{14}-Ile^{15}
S—S
Cys^{23}-Gly^{22}-Leu^{21}-Gly^{20}-Ser^{19}-Gln^{18}-Ala^{17}-Gly^{16}

studies, it was decided to use two spacers to substitute for sequences of eight aminoacids at a time in order to minimise duplication. 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**). Assemblies were performed on a CRB Pepsyn II using KA resin and Fmoc/Pfp ester chemistry. After cleavage and deprotection with trifluoroacetic acid, using ethanedithiol and phenol as scavengers, the product isolated on evaporation was found to be insoluble in most solvents and chromatography was not possible. An alternative isolation using ether precipitation also gave an insoluble solid which could not be purified, although the correct mass was detected using FAB-MS. The severe solubility problems of these products led us to abandon synthesis of other alkyl-spaced peptides in this sequence.

The corresponding two peptides incorporating the polyethyleneoxy spacer (Peo), **5a** and **5b**, were assembled on a Milligen 9050 using the same chemistry except that the spacer molecule was reacted as the free acid (**2e**) in the presence of diisopropylcarbodiimide and hydroxybenzotriazole.

(4a) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Met^{12}-Asp^{13}-Arg^{14}-Alk-Alk-Cys^{23}

(4b) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Met^{12}-Asp^{13}-Alk-Alk-Gly^{22}-Cys^{23}

(5a) Cys_{7}-Phe_{8}-Gly_{9}-Gly_{10}-Arg_{11}-Met_{12}-Asp_{13}-Arg_{14}-Peo-Peo-Cys_{23}

(5b) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Met^{12}-Asp^{13}-Peo-Peo-Gly^{22}-Cys^{23}

(5c) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Met^{12}-Peo-Peo-Leu^{21}-Gly^{22}-Cys^{23}

(5d) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Arg^{11}-Peo-Peo-Gly^{20}-Leu^{21}-Gly^{22}-Cys^{23}

(5e) Cys^{7}-Phe^{8}-Gly^{9}-Gly^{10}-Peo-Peo-Ser^{19}-Gly^{20}-Leu^{21}-Gly^{22}-Cys^{23}

(5f) Cys^{7}-Phe^{8}-Gly^{9}-Peo-Peo-Gln^{18}-Ser^{19}-Gly^{20}-Leu^{21}-Gly^{22}-Cys^{23}

(5g) Cys^{7}-Phe^{8}-Peo-Peo-Ala^{17}-Gln^{18}-Ser^{19}-Gly^{20}-Leu^{21}-Gly^{22}-Cys^{23}

(5h) Cys^{7}-Peo-Peo-Gly^{16}-Ala^{17}-Gln^{18}-Ser^{19}-Gly^{20}-Leu^{21}-Gly^{22}-Cys^{23}

Deprotection and cleavage as before, with evaporative work-up, gave crude water-soluble peptide together with significant amounts of insoluble solid which was shown by NMR to be polymeric ethane dithiol. Precipitation

with ether 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. The filtered TFA solution is slowly diluted with 5-10 times its volume of petroleum ether with continual swirling. The petroleum layer is removed by decantation 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 artifacts. 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 (3 days), peptidic material was isolated by solid-phase extraction using C_{18} Bondelut tubes. The peptides were then purified by 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 were single peaks by analytical HPLC and showed only the expected ion masses with FAB-MS.[9]

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9. Analytical HPLC was performed on Vydac 218TPB10 (C_{18}, 10μ, 300Å pore) with a gradient between water and acetonitrile (with added 0.1% TFA) of 0.5% per minute over the elution window. FAB-MS were measured at the SERC Mass Spectrometry Centre, Swansea, on a VG-ZAB instrument using a NOBA matrix.

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