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Synthesis of hydrophobic peptides: An Fmoc "Solubilising Tail" method

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Abstract: The development of an Fmoc method for synthesis and purification of hydrophobic peptides using a "solubilising tail" strategy is described. Peptide-constructs of the form hydrophobic peptide-[4-Hmb ester]-solubilising peptide were synthesised. Procedures for forming the 4-Hmb ester linkage, and sequences of solubilising peptides suitable for use with Fmoc SPPS, were investigated. The method was used to synthesise two model peptides and three designed transmembrane peptides.

In a recent paper we described a strategy for the synthesis and purification of hydrophobic peptides using Boc SPPS methodology.[1] In this strategy, a peptide-construct of the general form (hydrophobic peptide-[orthogonal linker]-solubilising peptide) was synthesised. The sequence of the peptide at the C-terminus of the construct was intended to assist in solubilising the construct, while the orthogonal linker was chosen to be stable to all of the conditions of peptide synthesis, cleavage, and purification, but then to be readily cleaved once the total peptide-construct had been purified. As part of a study into the synthesis of designed transmembrane helices, we have developed an Fmoc variation of the Boc solubilising strategy.

The first requirement of this strategy was the synthesis of a homogeneous solubilising tail peptide. Although the solubilising peptide $Gly(ArgGly)_4$ was readily synthesised by optimised Boc SPPS methodology,[2] electrospray mass spectrometric analysis of $Gly(ArgGly)_4$-amide synthesised by Fmoc methodology showed significant quantities of mono, di, and tri -Arg-containing peptides. An investigation of the Fmoc synthesis[3] of other possible solubilising tail peptides showed $Gly(ArgGlyGly)_3$ and $Gly(LysGly)_6$ could be synthesised in high purity.

An ester linkage based on 4-hydroxymethyl benzoic acid (4-Hmb)[4] was used between the tail peptide and the target peptide in this study rather than the glycolamide ester used in the Boc method;[1] however, the glycolamide ester may also be suitable for use with Fmoc SPPS.[5] The 4-Hmb ester could be formed using either the cesium salt method[6,7] or the anhydride method.[8] Consequently, peptide-constructs of the form (hydrophobic peptide-[4-Hmb linker]-solubilising peptide) (Figure 1) were synthesised.

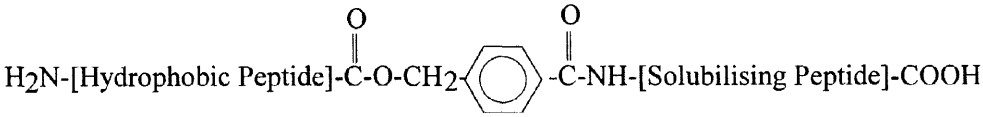

Figure 1. General form of Peptide-Constructs synthesised in this study

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Five peptide-constructs of the form shown in Figure 1 were synthesised by standard Fmoc SPPS methods.[3] The peptide-construct sequences and details of the syntheses are given in Table 1.

Table 1. Peptide-Constructs Synthesised Using Fmoc SPPS Chemistry

Peptide-Construct Sequence	Eq. Fmoc-AA per Coupling	Single/Double Coupling	Mass of Major HPLC peak*	Calculated Mass
1$^{\chi}$ (NAc)CEWNSAHFIAYK-[4-Hmb]-G$(RGG)_3$-amide	4	Double for C,E,W,H,F,R only	2511 Da	2511 Da
2$^{\chi}$ A_{12}-[4-Hmb]-G$(KG)_5$-amide	20	Single	1987 Da	1989 Da
3$^{\chi}$ (NAc)C(acm)GGSPDQVWLNVLVSLLNVLV SLYTAQKAKNC -[4-Hmb]-G$(KG)_6$-amide	10	Single	4850 Da	4849 Da
4$^{\chi}$ EAELENAVYLNALVSLLNALVSLWTAKNP GAA -[4-Hmb]-G$(KG)_6$ -amide	10	Single	4657 Da	4657 Da
5$^{\alpha}$ LLNVLVSLYTAQKAKNGGDAELENAVYL NALVSLLNALVSLWTAKNPG-[4-Hmb]-G$(KG)_6$-amide	10 (first 30 aa) 20 (last 18 aa)	Single	6375 Da	6375 Da

χ 4-Hmb ester formed using the cesium salt method

α 4-Hmb ester formed using the anhydride method

* The masses shown are those of the major peaks from HPLC analyses (peaks marked * in Figures 2 and 3).

The peptide-constructs were analysed by HPLC (Figures 2 and 3), and the masses of the main peaks from these HPLC analyses were determined by Electrospray or MALDI-TOF mass spectrometry.

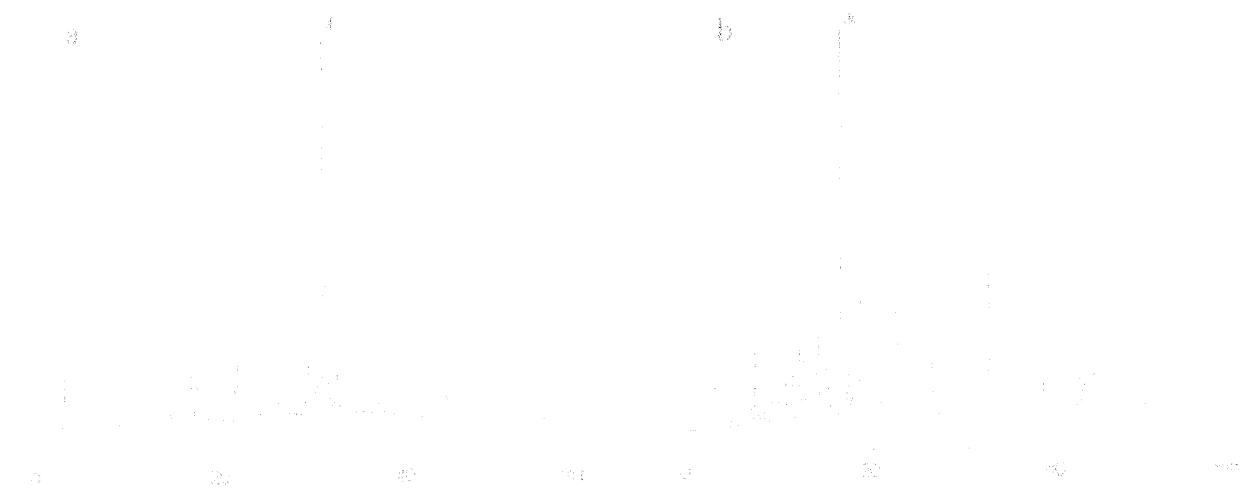

Figure 2. HPLC chromatograms of peptide-constructs **1** (Figure 2a) and **2** (Figure 2b).
HPLC conditions: Vydac C4, 4.6 X 250 mm, 0-60% B over 60 minutes at 1 ml/min.
A = 0.1% TFA; B = 0.1% TFA, 10% water, 90% acetonitrile.

The model peptide-construct **1** N-Ac-CEWNSAHFIAYK-[4-Hmb]-G(RGG)$_3$-amide, containing amino acids bearing many of the side-chain protecting groups used in Fmoc SPPS, was first synthesised. HPLC analysis (Figure 2a) and mass analysis (Table 1, entry **1**) of the crude cleaved peptide-construct showed that this strategy could be used to synthesise a complex peptide in reasonable purity. It was found (data not shown) that the 4-Hmb ester of HPLC-purified peptide-construct **1** was hydrolysed in less than two minutes using 0.1M NaOH solution, thus separating the target and solubilising tail peptides.

Peptide-construct **2**, A$_{12}$-[4-Hmb]-G(KG)$_5$-amide, was readily soluble, showing that the solubilising tail peptide G(KG)$_5$-amide resulted in the solubilisation of Ala$_{12}$ as a (hydrophobic peptide-[4-Hmb]-solubilising peptide) type peptide-construct. HPLC analysis (Figure 2b) showed that that the Ala$_{12}$ peptide-construct was well separated from a number of other impurities. This poor synthesis of Ala$_{12}$ as shown by HPLC analysis is perhaps not surprising, given the known difficulty in synthesising poly-Ala sequences using both Fmoc[9] and standard Boc[10] SPPS methodology. The Ala$_{12}$ peptide-construct was not further purified, as we have already shown in a previous paper that HPLC purification of an Ala$_{12}$ peptide-construct is readily accomplished once the hydrophobic peptide has been solubilised.[1]

Although an Ala$_{12}$-containing peptide-construct, (A$_{12}$-[glycolamide ester]-G(RG)$_4$), was readily synthesised using the Boc solubilising methodology,[1] such a facile synthesis of an Ala$_{12}$-bearing peptide-construct could not be repeated using the Fmoc solubilising method. It may be that a combination of the use of this solubilising strategy with 2-hydroxy 4-methoxybenzyl (Hmb) protected amino acids[11] may help to overcome problems in the Fmoc synthesis and purification of difficult, insoluble peptides.

Peptide-constructs **3-5**, designed transmembrane peptides, were also synthesised. HPLC chromatograms of the crude cleaved peptide-constructs **3-5** are shown in Figures 3a-c respectively. The masses of the major peaks from HPLC analyses of peptide-constructs **3-5** showed that they had the expected molecular weights (Table 1). The HPLC chromatograms show that the desired peptide constructs were well separated from impurities, which would allow easy purification by HPLC. These data also show that the synthesis, in reasonable purity, of relatively long peptide-constructs (e.g. **5**: 48 amino acid target peptide, a total of 61 amino acids) was possible using standard Fmoc SPPS chemistry. These peptide-constructs will be studied by CD and other techniques, both before and after cleavage of the solubilising tail peptides.

Figure 3. HPLC chromatograms of peptide-constructs **3-5** (Figures 3a-c respectively).
HPLC conditions: Vydac C4, 4.6 X 250mm, 20% B 0-10 minutes, 20-100% B from 10-90 minutes at 1 ml/min. Solvents A and B as above.

The difficulties encountered in purifying hydrophobic synthetic peptides due to poor solubility are well known.[12] We have demonstrated modifications of the original Boc solubilising tail method[1] that enable soluble (hydrophobic peptide-[4-Hmb linker]-solubilising peptide) constructs to be synthesised by Fmoc SPPS methodology. Following standard HPLC purification of the peptide-construct, the 4-Hmb ester linkage is cleaved by aqueous base to liberate the desired hydrophobic peptide. The 4-Hmb ester linker used in this study is also stable to the conditions used for synthesis and cleavage of peptides made using Boc SPPS methods, including liquid HF,[4a] and has the added advantage of being easily formed by the anhydride method. Thus the 4-Hmb linkage may serve as an alternative to the glycolamide ester linkage in the original Boc solubilising tail method.[1]

References and Notes

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3. A Perkin Elmer ABI 433A peptide synthesiser using Fastmoc® peptide synthesis protocols was used for peptide synthesis. Amino acid side chain protecting groups used were Cys(Trt), Cys(acm) His(Trt), Lys(Boc), Ser(tBu), Arg(Pmc), Asp(OtBu), Glu(OtBu), Asn(Trt), Gln(Trt), Tyr(tBu), Thr(tBu). Unprotected Trp was used in the synthesis of peptide **1**, whereas peptides **3-5** were synthesised using Trp(Boc). Novabiochem amide linker resin was used for all syntheses. Dried peptide-resin was cleaved with a mixture of TFA:water:thioanisole: EDT:phenol 40:2:2:1:3 v/v/v/v/w (**1**); water: TFA 5:95 v/v (**2**), or water:EDT: TFA 2.5:2.5:95 v/v/v (**3-5**). The crude ether-precipitated peptides were worked up using normal procedures.
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7. The anhydride of 4-bromomethylbenzoic acid was coupled to the amine of the resin-bound tail peptide. The 4-Hmb ester linkage was formed by reacting overnight 4 eq of the cesium salt of Fmoc-amino acid with the bromomethyl-group of the linker. Ninhydrin test of the resulting resin showed partial removal of the Fmoc group, however HPLC analysis of crude cleaved peptides after the syntheses generally revealed only low levels of unwanted side products.
8. To H-Gly(LysGly)$_6$-amide linker-resin was added 1.1 eq 4-hydroxymethylbenzoic acid and 1.1 eq HBTU in a minimum volume of DMF. DIEA (1.3 eq) was added to initiate the reaction. After 2 hours reaction MALDI-TOF mass analysis of a sample cleaved with 95%TFA: 5% water showed no free H-Gly(LysGly)$_6$-amide; there was, however, some di-addition of the 4-Hmb linker. The second 4-Hmb molecule was removed by treating the resin twice for 5 minutes with a mixture of 2M NaOH: methanol: dioxane (1:4:15 v:v:v, cleavage confirmed by MALDI-TOF mass analysis of a 95% TFA cleaved sample). It may be possible to avoid di-addition of 4-hydroxymethyl benzoic acid by coupling using 4-hydroxymethyl benzoic acid - 2,4,5 Trichlorophenyl ester.[4a] Reaction of Fmoc-Ala anhydride (4 eq) with DMAP catalyst (0.1 eq relative to resin-bound peptide) for 30 minutes resulted in quantitative formation of Fmoc-Ala-[4-Hmb]-Gly(LysGly)$_6$-amide linker resin (MALDI-TOF analysis of the aqueous solution resulting after cleavage and workup of a sample of this resin showed only Fmoc-Ala-[4-Hmb]-Gly(LysGly)$_6$-amide, mass 1612.7 Da, to be present).
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