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Expediting the Fmoc Solid Phase Synthesis of Long Peptides Through the Application of Dimethyloxazolidine Dipeptides

PETER WHITE,[a]* JOHN W. KEYTE,[b] KEVIN BAILEY[b] and GRAHAM BLOOMBERG[c]

[a] Novabiochem, Merck Biosciences Ltd, Padge Road, Beeston NG9 2JR, UK
[b] School of Biomedical Sciences, University of Nottingham NG7 2UH, UK
[c] Department of Biochemistry, University of Bristol BS8 1TD, UK

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Abstract: This paper describes the step-wise Fmoc solid phase synthesis of a 95-residue peptide related to FAS death domain. Attempts to prepare this peptide employing conventional amino acid building blocks failed. However, by the judicious use of dimethyloxazolidine dipeptides of serine and threonine, the peptide could be readily prepared in remarkable purity by applying single 1 h coupling reactions.

Keywords: peptide aggregation; difficult peptides; pseudoproline dipeptide; oxazolidine dipeptide

INTRODUCTION

Boc chemistry often gives superior results to Fmoc chemistry in the solid phase synthesis of long peptides, as the current strategy of using neat TFA for Boc group removal and *in situ* neutralization during coupling is extremely effective at overcoming peptide aggregation during chain extension [1]. Whilst methods exist for Fmoc SPPS, such as Hmb backbone protection [2] and the use of chaotropic reagents [3,4] and complex solvents mixtures [5], none have found widespread acceptance owing either to their lack of general applicability, partial effectiveness or incompatibility with standard protocols. Therefore, no general measures are taken to avoid aggregation during routine Fmoc SPPS of long peptides. As a consequence, the results in comparison with those obtained with Boc chemistry are often disappointing and the products obtained can be quite heterogenous, containing truncation and deletion sequences arising from incomplete acylation and deprotection reactions. The latter is a particular problem in Fmoc chemistry [3]; similar difficulties are not observed in Boc synthesis, owing to the excellent solvent properties of TFA for protected peptides.

Of the various solutions advocated to the problem of aggregation in Fmoc SPPS, Mutter's [6,7] dimethyloxazolidine dipeptides (Figure 1) appear to hold the most promise. They have been shown by us [8] and others [9] to be very powerful tools for facilitating the synthesis of 'difficult peptides' [10], and their use involves no modification of existing synthetic protocols. Dimethyloxazolidine dipeptides introduce into the peptide chain a pseudoproline residue, derived from either Ser or Thr, which disrupts peptide chain aggregation in the same manner as proline [11,12]. Since the pseudoproline residue is incorporated as a part of a pre-formed dipeptide unit, the problems involved in acylation of *N*-alkylamino acid residues that severely limit the utility of the Hmb backbone protection strategy are avoided. Regeneration of the Ser or Thr residue from the oxazolidine occurs during the course of the normal 95% TFA-mediated cleavage reaction.

Abbreviations: AM, aminomethyl polystyrene; Hmb, 2-hydroxy-4-methoxybenzyl; TOF, time-of-flight.

* Correspondence to: Dr Peter White, Merck Biosciences Ltd, Padge Road, Beeston NG9 2JR, UK;
e-mail: peter.white@merckbiosciences.co.uk

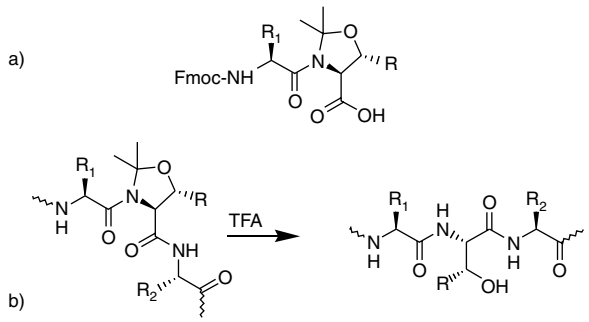

Figure 1 R = H, Ser; R = Me, Thr. (a) Fmoc-protected oxazolidine dipeptide building block; (b) TFA-mediated ring opening of oxazolidine to regenerate Ser or Thr.

As a result of our earlier success [8] in using oxazolidine dipeptides, they are now used prophylactically, whenever the opportunity allows, in order to reduce the number of sequences that require resynthesis. It has been observed that their routine use not only facilitates the production of difficult sequences, but also appears to provide significant improvements in coupling and deprotection kinetics for 'standard' peptides. This led us to consider whether this approach could also benefit the synthesis of long peptides/small proteins by Fmoc chemistry.

As a model for our studies, a 95-residue peptide related to FAS death domain (FDD) [13] was selected. Binding of the FAS ligand to FAS initiates the signal transduction cascade that leads ultimately to cell death. One of the key events is the interaction of the death domain of FAS with a complementary death domain of FAS associated protein, which results in recruitment of caspase-8 to the complex and subsequent autoactivation by proteolytic processing [14]. FDD consists of six helical regions and its structure is represented schematically in Figure 2.

The target peptide, as shown in Figure 3, includes five of the helices plus the c-myc antigen immunological marker [16] at the *C*-terminus. The sequence has plenty of opportunities to employ oxazolidine dipeptides as it contains numerous well-distributed

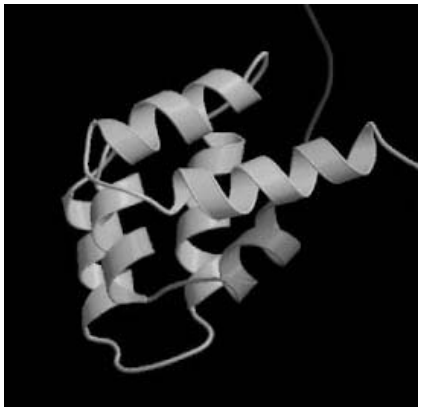

Figure 2 Schematic representation of FAS death domain [15].

Ser or Thr residues. The optimum spacing of pseudoprolines is six residues, as the effects of proline substitution on aggregation during chain assembly have been shown to last for a minimum of six amino acid addition cycles [17]. The sites selected for oxazolidine dipeptide substitution are shown in Figure 3. Residue 63, which in the native sequence is Cys, was substituted for Ser so as to avoid problems with disulfide bond formation and also to provide an additional site for the use of pseudoprolines. Small aliquots of resin were removed after assembly of each helical region, at 30, 46, 64 and 80 residues as marked in Figure 3, and the intermediate peptides were cleaved and isolated. Analysis of these shorter peptides would provide an insight into the efficiency of the synthesis. To evaluate the effects of pseudoproline insertion on synthetic efficiency, the peptide was also prepared in an identical manner using only standard Fmoc amino acid derivatives.

MATERIALS AND METHODS

Materials

The resin, all amino acids and dimethyloxazolidine dipeptides were purchased from Novabiochem

H-(***Leu-Ser***-Gln-Val-Lys-Gly-Phe-Val-Arg-Lys)-Asn-Gly-Val-Asn-Glu-(Ala^{80}-Lys-Ile-Asp-Glu-Ile-Lys-Asn-Asp-Asn)-Val-Gln-***Asp-Thr***-Ala-Glu-(Gln^{64}-Lys-Val-Gln-Leu-Leu-Arg-Asn-Trp-His)-Gln-Leu-His-Gly-Lys-Lys-Glu-Ala-(Tyr^{46}-***Asp-Thr***-Leu-Ile-Lys-Asp-Leu-Lys-Lys)-Ala-Asn-Leu-***Ser*-Thr***-Leu-(Ala^{30}-Glu-Lys-Ile-***Gln-Thr***-Ile-Ile-Leu-Lys-Asp-Ile-***Thr)-Ser***-Asp-Ser-Glu-***Asn-Ser***-Asn-Glu-His-Lys-Leu-***Thr-Ser***-Glu-Lys-Asp-Leu-NH_2

Figure 3 Sequence of 95-residue peptide. c-Myc antigen sequence underlined. Helical regions are enclosed by parentheses. Sites substituted with oxazolidine dipeptides are in bold and italicized. Ser* indicates Cys residue in native sequence replaced by Ser at this point. The numbers of the synthesis cycles where resin samples were removed are marked in superscript.

(Merck Biosciences AG, Läufelfingen, Switzerland). HATU and peptide synthesis solvents (DMF, DCM, DIPEA, piperidine) were supplied by Applied Biosystems (Foster City, USA). All other solvents were of analytical or biochemical grade and were obtained from Aldrich (Gillingham, UK).

Peptide Synthesis

All the peptides were assembled on an Applied Biosystems Inc. 431 peptide synthesizer using modified Fastmoc cycles. Coupling reactions were performed using Fmoc amino acids (10 eq.) activated with HATU (10 eq.) and DIPEA (20 eq.) for 1 h. Fmoc removal was effected by treating the resin for 2×5 min with 20% piperidine in DMF. The side-chains of trifunctional amino acids were protected as follows: Arg(Pmc), Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu).

Rink amide AM resin (0.6 mmol/g) was loaded automatically by coupling a mixture of Fmoc-Leu-OH/Boc-Leu-OH (1 : 2) as described above. This gave a resin with an Fmoc content, as determined by the Fmoc UV assay, of 0.2 mmol/g [18].

Cleavage of peptides from the resin with concomitant side-chain deprotection was effected by treating peptidyl resins with TFA/water/triisopropylsilane (95 : 2.5 : 2.5 v/v) for 3 h. Following removal of the TFA and scavengers by evaporation under vacuum, the peptides were precipitated by the addition of diethyl ether. The peptides were isolated by filtration and washed three times with clean ether.

HPLC Analysis

The crude peptides were analysed by reverse phase HPLC using a Merck HPLC system equipped with either a Vydac C18 300 Å (Hichrom, Reading, UK), Merck Chromolith C18 (VWR International, Poole, UK), or Brownlee Aquapore C18 (Perkin Elmer, Beaconsfield, UK) columns. The gradient elution system for the Vydac and Merck columns was 0.1% TFA in water (buffer A) and acetonitrile/water/TFA (90 : 10 : 0.1) (buffer B). The Vydac column was eluted with a gradient from 5% to 100% of buffer B in 40 min at a flow rate of 1 ml/min. The Chromolith column was eluted with a gradient from 5% to 100% buffer B in 6 min at 5 ml/min. The gradient elution system for the Brownlee column was 0.1% formic acid (buffer A) and acetonitrile/water/formic acid (70 : 10 : 0.1) (buffer B). The column was eluted with a gradient from 5% to 100% of buffer B in 30 min at 1 ml/min. The peaks were detected at 220 nm. Samples were either dissolved in water or 6 M guanidinium chloride prior to injection.

ESI-TOF Measurements

All mass spectra were recorded using a Micromass Q-TOF II spectrometer operating under control of Masslynx 3.5 software (Micromass UK Ltd, Manchester, UK).

Tryptic Digest

The digestion was performed by dissolving approximately 1 mg of peptide in 200 mM ammonium bicarbonate (1 ml) and adding trypsin (0.1 mg, Promega sequencing grade, Promega, Southampton, UK) and leaving it to stand overnight at room temperature. The mixture was desalted on C18 Ziptip (Millipore Corp., Watford, UK) prior to injection in the mass spectrometer.

RESULTS AND DISCUSSION

Our strategy was to prepare the peptide in a conventional manner on a standard automated synthesizer using single 1 h coupling reactions with no capping step. To minimize overcrowding in the resin beads during the assembly of such a long peptide, the loading of commercially available Rink AM amide resin was reduced from 0.6 mmol/g to 0.2 mmol/g by coupling a mixture of Fmoc-Leu-OH/Boc-Leu-OH (1 : 2) as the first cycle. HATU [19] was chosen as the activation reagent because of the excellent reactivity of OAt esters.

As mentioned above, aliquots of resin were removed after 30, 46, 64 and 80 amino acid additions; these were cleaved and the intermediate peptides isolated and characterized. The sequences of these peptides are shown in Table 1.

Cleavage of the peptides from the resin was carried out using TFA/water/triisopropylsilane, which has been shown to be a highly effective cleavage cocktail for peptides containing a mixture of Arg(Pmc) and Trp(Boc) [20].

The progress of the synthesis was assessed by UV monitoring of the Fmoc deblocking reaction. After each addition of piperidine/DMF to the reaction vessel, a small aliquot of the reaction mixture was passed through the flow cell of a spectrophotometer and the optical density measured. The piperidine treatment was carried out twice. In a non-aggregated

Table 1 FAS Death Domain-related Peptides Prepared in this Study

Peptide	Sequence
1	H-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2
2	H-Tyr-Asp-Thr-Leu-Ile-Lys-Asp-Leu-Lys-Lys-Ala-Asn-Leu-Ser-Thr-Leu-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2
3	H-Gln-Lys-Val-Gln-Leu-Leu-Arg-Asn-Trp-His-Gln-Leu-His-Gly-Lys-Lys-Glu-Ala-Tyr-Asp-Thr-Leu-Ile-Lys-Asp-Leu-Lys-Lys-Ala-Asn-Leu-Ser-Thr-Leu-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2
4	H-Ala-Lys-Ile-Asp-Glu-Ile-Lys-Asn-Asp-Asn-Val-Gln-Asp-Thr-Ala-Glu-Gln-Lys-Val-Gln-Leu-Leu-Arg-Asn-Trp-His-Gln-Leu-His-Gly-Lys-Lys-Glu-Ala-Tyr-Asp-Thr-Leu-Ile-Lys-Asp-Leu-Lys-Lys-Ala-Asn-Leu-Ser-Thr-Leu-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2
5	H-Leu-Ser-Gln-Val-Lys-Gly-Phe-Val-Arg-Lys-Asn-Gly-Val-Asn-Glu-Ala-Lys-Ile-Asp-Glu-Ile-Lys-Asn-Asp-Asn-Val-Gln-Asp-Thr-Ala-Glu-Gln-Lys-Val-Gln-Leu-Leu-Arg-Asn-Trp-His-Gln-Leu-His-Gly-Lys-Lys-Glu-Ala-Tyr-Asp-Thr-Leu-Ile-Lys-Asp-Leu-Lys-Lys-Ala-Asn-Leu-Ser-Thr-Leu-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2
6	H-Lys-Lys-Glu-Ala-Tyr-Asp-Thr-Leu-Ile-Lys-Asp-Leu-Lys-Lys-Ala-Asn-Leu-Ser-Thr-Leu-Ala-Glu-Lys-Ile-Gln-Thr-Ile-Ile-Leu-Lys-Asp-Ile-Thr-Ser-Asp-Ser-Glu-Asn-Ser-Asn-Glu-His-Lys-Leu-Thr-Ser-Glu-Lys-Asp-Leu-NH_2

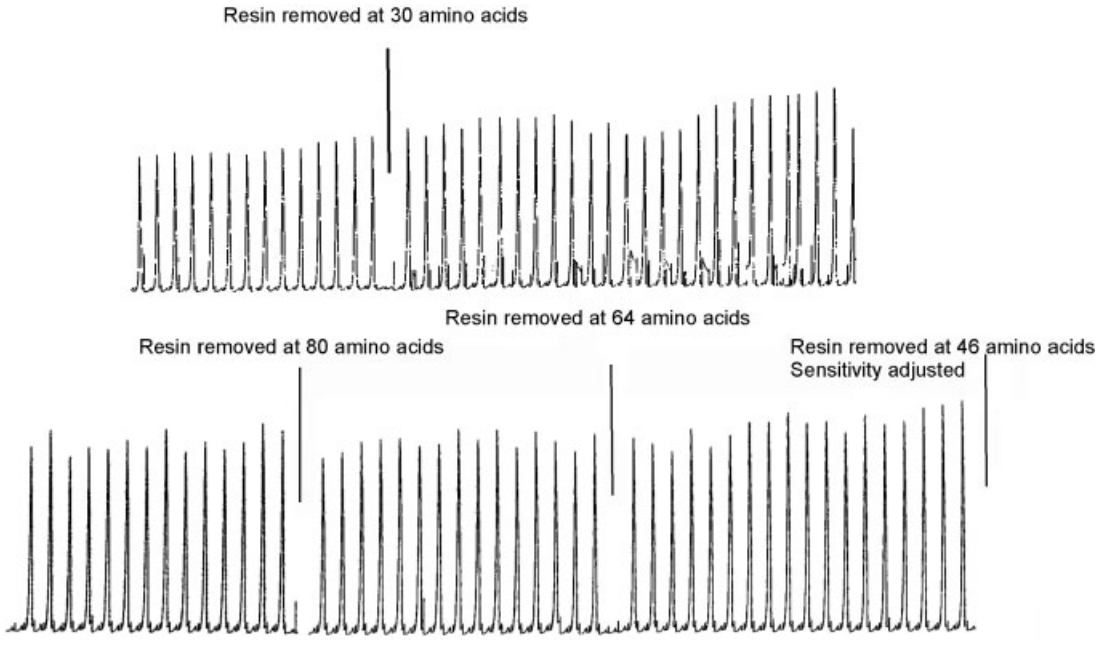

Figure 4 UV monitoring trace of deprotection reactions.

system, where deprotection was rapid, a large peak was observed for the first deblocking reaction, followed by a very much smaller peak for the second. When aggregation occurred, the height of the first peak reduced dramatically with a concomitant increase in the height of the second. Figure 4 shows the UV monitoring traces obtained for the complete assembly of the 95mer using oxazolidine dipeptides. These results gave a strong indication that the synthesis had proceeded smoothly and without significant aggregation. For the authors what was most striking about these results was

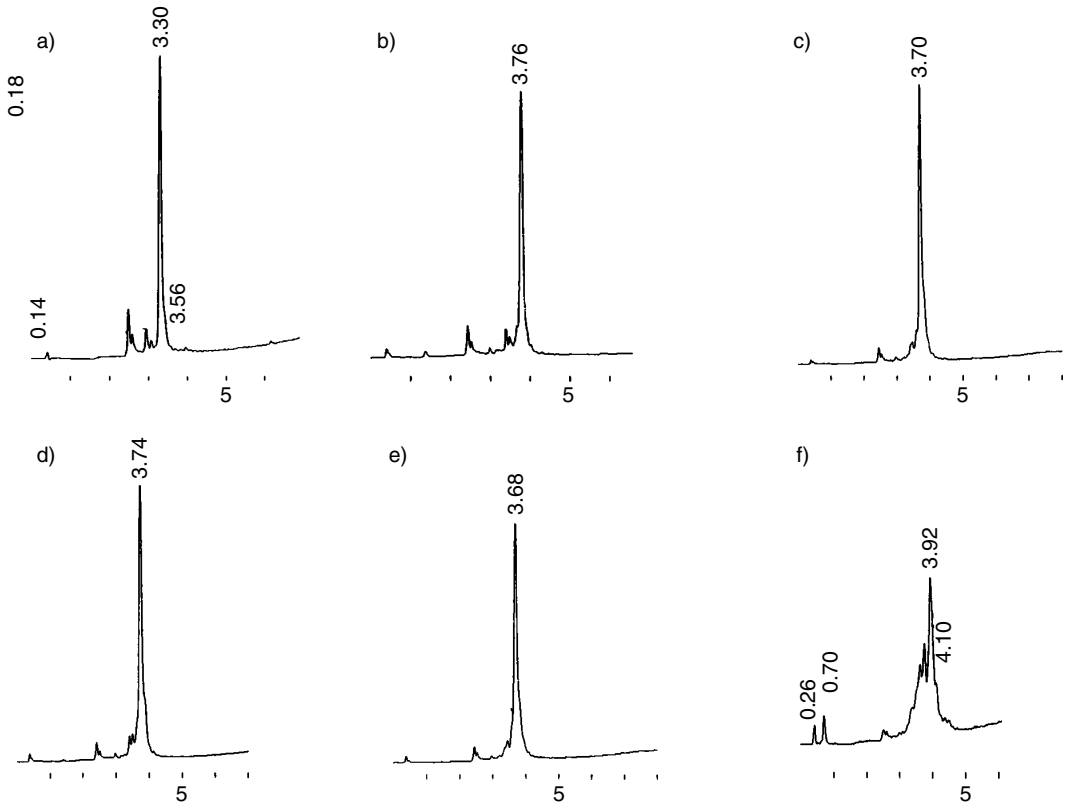

Figure 5 HPLC elution profiles of crude peptides obtained with the Chromolith column. Conditions were as described in materials and methods. (a) Peptide **1**, 30mer; (b) peptide **2**, 46mer; (c) peptide **3**, 64mer; (d) peptide **4**, 80mer; (e) peptide **5**, 95mer; (f) peptide **6**, 50mer made with standard amino acid building blocks.

the consistency of the peak heights throughout the latter stages of assembly. It is our experience that during the assembly of long peptides the heights of the Fmoc deblock peaks gradually reduced as the synthesis progressed.

The HPLC elution profiles of the crude peptides are given in Figure 5. As a general rule, compounds that elute earlier than the target arise from deletions and truncations, whereas those eluting later result from modifications and incomplete protecting group removal. The crude product from the synthesis of the 50-residue peptide **6** prepared using standard Fmoc building blocks was highly heterogenous. On HPLC, this material eluted as a very broad peak with a pronounced leading edge, indicative of the presence of many deletion and truncation by-products. As the presence of any of the target peptide in the sample could not be detected by ESI-MS, the synthesis was terminated at this point. By comparison the 46-residue product **2**, made with oxazolidine dipeptides, appeared quite homogenous. The major component was identified by ESI-MS as the desired peptide.

The HPLC profiles of the 64, 80 and 95 residue peptides indicated the products to be remarkably homogenous considering their length. Similar results were also obtained using different column packing materials and elution buffers. In every case the identity of the major component was confirmed by ESI-MS as the desired peptide (Table 2).

Table 2 ES-MS Results

Peptide	Found	Monoisotopic	Average
1	3399.1	3398.8	3400.8
2	5216.6	5215.8	5218.9
3	7412.3	7410.0	7414.5
4	9198.4	9193.9	9199.4
5	10854.0	10849.7	10856.2

Values quoted are for the protonated molecular ion in the deconvoluted spectrum. Expected masses calculated using applet located at www.medlib.med.utah.edu/masspec/mole.htm.

The ESI-MS spectra of the 80 and 95 residue peptides both contained a significant peak at minus 17–18 daltons (Figure 6) consistent with the presence of deamidated or dehydrated by-products in the crude products; the resolution of the deconvoluted spectra was insufficient to distinguish unequivocally between the loss of water or ammonia. These contaminants could either arise from aspartimide formation during piperidine-mediated Fmoc removal [21] or during post-synthetic handling as a result of Asn degradation [22]. The absence of evidence for piperidide by-products [21], which invariably

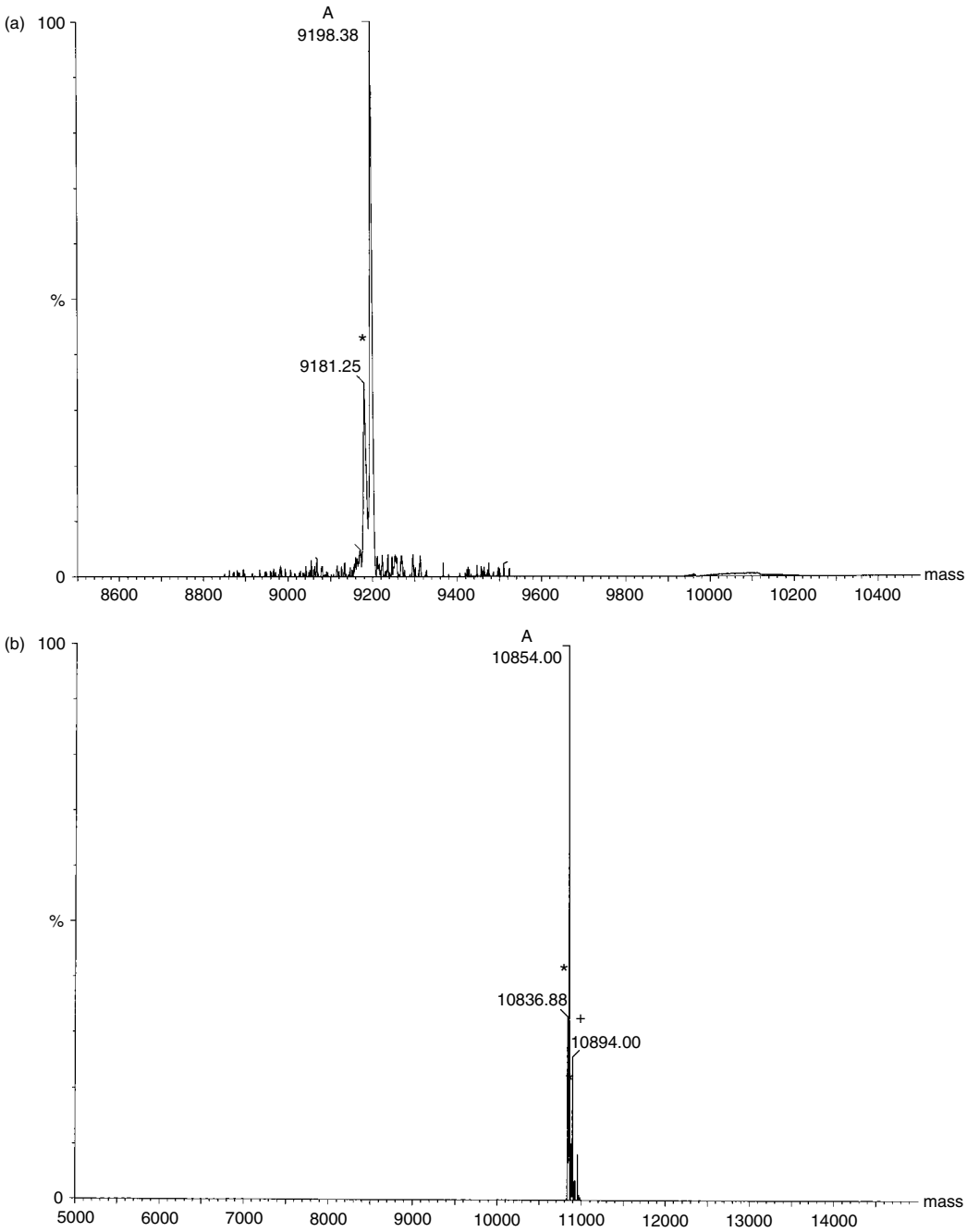

Figure 6 Deconvoluted ESI-MS spectra obtained from samples collected during HPLC elution of (a) peptides **4** and (b) **5**. *Peaks due to dehydrated or deamidated by-products. +Peak due to product/s containing one unopened pseudoproline residue ($M + H^+ + 40$ (C_3H_4)).

accompany aspartimide formation in Fmoc SPPS, would tend to mitigate against the former explanation. Although in the mass spectrum of synthetic HIV protease prepared by Boc chemistry, a similar peak was indeed ascribed by Kent [1] to the sum of all dehydration side-reactions that had occurred during synthesis.

In addition, the ESI-MS spectrum of the 95-residue peptide contained a peak at $(M + H^+)$ plus 40 daltons, which most likely corresponded to a peptide or peptides containing a single unopened oxazolidine ring, or side-reaction arising from liberated acetone. HPLC-ESI-MS analysis indicated this material was associated with the trailing edge of the HPLC peak. The crude 95 peptide was further characterized by digestion with trypsin, followed by MS/MS analysis on the total digest mixture. Fragments accounting for the majority of the sequence could be identified with only the expected low molecular weight fragments being absent (Table 3).

A sample of the crude peptide was purified by HPLC on a Chromolith column using a linear gradient of 30%–45% buffer B over 12 min. The analytical HPLC profile and ESI-MS spectrum of this material are shown in Figure 7.

Table 3 Mass of Protonated Molecular Ions of Predicted and Expected Fragments from Tryptic Digest

Peptide	Expected	Found
1–5	574.3	nd
6–9	478.3	nd
11–17	731.4	nd
18–33	1859.9	1860.4
23–33	1261.6	1261.7
34–38	628.4	628.5
39–46	1019.5	1019.6
47–55	1080.6	1080.7
48–55	952.5	952.6
59–68	1074.6	1074.7
60–68	946.5	946.6
69–75	828.6	nd
76–88	1475.6	1475.7
76–93	2033.9	2034.1

CONCLUSIONS

The routine use of pseudoproline dipeptides appeared to be a very promising strategy for the synthesis of long serine- and threonine-rich peptides by

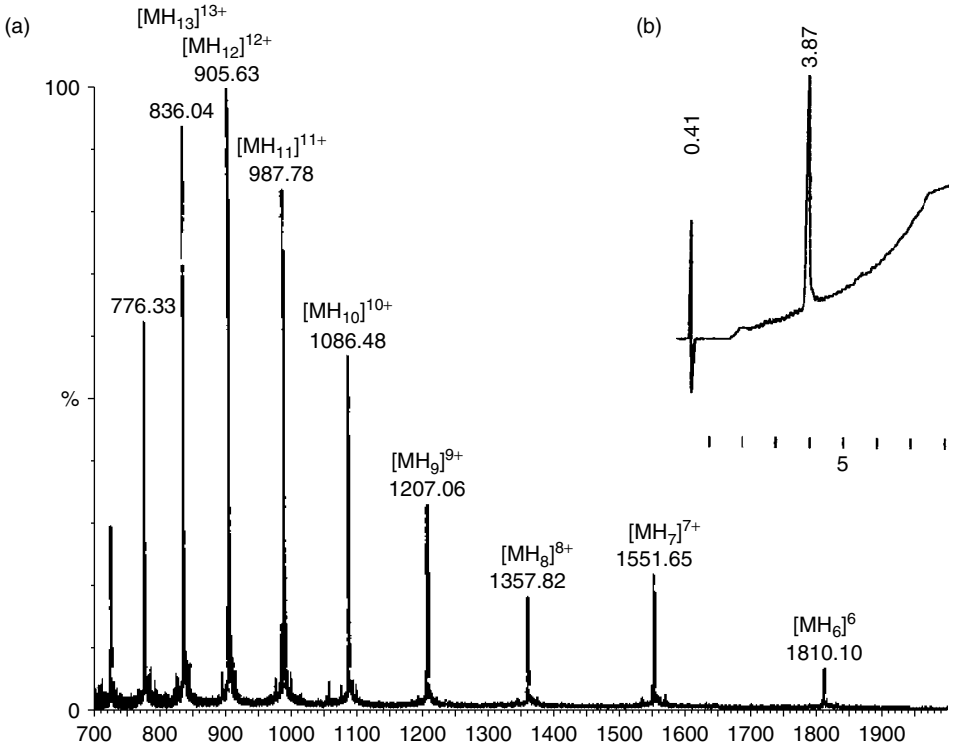

Figure 7 (a) ESI-MS spectrum and HPLC elution profile (b) of purified peptide **5**.

Fmoc solid phase methods. Further investigations are presently underway to see if such remarkable results can also be obtained for peptides of similar length or longer. If the approach is found to be generally applicable, the milder and cleaner conditions employed for side-chain deprotection in Fmoc synthesis should make it the method of choice for such applications. One obvious limitation is the requirement for the target peptide to contain serine and threonine residues; however, this could be ameliorated through the use of other turn-inducing dipeptides, such as Fmoc-Aaa-(Y)Gly-OH where Y is an acid-labile protecting group.

The problem of incomplete pseudoproline ring opening or acetone modification is currently under investigation. Aspartimide formation is likely to be an important issue in the synthesis of long peptides by Fmoc methods, resulting from the long exposures of vulnerable sequences to piperidine. Fortunately, for the most problematic sequence, Asp-Gly, a solution is at hand in the form of Fmoc-Asp(OtBu)-(Hmb)Gly-OH [23], as protection of the Asp-Gly amide bond by Hmb is known to completely prevent this side reaction [24].

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