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Improved Preparation of Amyloid-β Peptides Using DBU as N^{α}-Fmoc Deprotection Reagent

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Abstract: Previous studies have shown the amyloid peptides, Aβ 1-40/42, to be exceptionally difficult to assemble by Fmoc-solid phase peptide synthesis due to the high hydrophobicity of the *C*-terminal segment and resulting on-resin aggregation. We found that the use of the stronger and more efficient base, DBU, at a concentration of 2% in DMF for N^{α}-Fmoc deprotection allowed substantially improved continuous flow solid phase assembly of the model peptide Aβ 29-40/42 fragments. This suggested that, at least for these sequences, incomplete deprotection was a greater problem than incomplete amino acid acylation. This base was then used during the synthesis of both Aβ 1-40 and Aβ 1-42, up to and including Ser[8], from which point 20% piperidine in DMF was utilized so as to avoid potential aspartimide formation at Asp[7]. By this means, the deprotection efficiency through the difficult *C*-terminal portion of the sequence was much improved and resulted in increased availability of terminal amino groups for acylation. This simple strategy that obviates the need for special conditions significantly improved crude peptide quality and allowed considerable facilitation of subsequent purification. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; amyloid-β peptides; aspartimide; continuous flow Fmoc solid phase peptide synthesis; DBU; purification

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia affecting today's society [1,2]. It is a neuro-degenerative disease that is clinically characterized by the gradual onset and progressive decline in memory and other cognitive functions. While the aetiology of AD is not yet known, the histopathological hallmarks of the disease include the extracellular deposition of amyloid-β peptide (Aβ) and loaded senile plaques in the brain parenchyma and blood vessels [3]. Aβ is excised from the amyloid precursor protein (APP) (Figure 1) by the sequential action of β- and γ-secretase enzymes, resulting in a peptide fragment that spans the extracellular and transmembrane regions of APP [4]. Aβ, a 39 or *C*-terminally sequentially extended to 43 residue peptide, has been shown to be neurotoxic [5] as a consequence of its propensity to aggregate and form fibrils. Such aggregation involves a number of characteristics of the peptide; these include the β-pleated secondary structure in the peptide and low solubility in physiological conditions [6,7]. Aβ 1-42 forms a β-sheet structure and amyloid fibrils much more readily than does Aβ 1-40 [8].

Recently there has been much interest in reports outlining the use of Aβ peptides for apparently successful immunization against AD in mouse models of the disease. That the burden of brain amyloid can be reduced in this manner provides convincing evidence that abnormal Aβ processing plays a large role in AD [9–11]. Consequently, there has been renewed interest in the rapid and efficient production of large quantities of Aβ. However, the Aβ sequence is noted for the significant challenges that it

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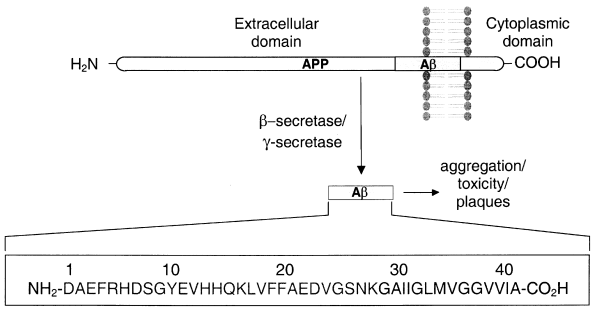

Figure 1 Schematic representation of the APP and Aβ cleavage by the secretase enzymes, including the Aβ sequence in single letter code.

presents to the synthetic chemist [12]. As a so-called 'difficult sequence' it has received much attention from various laboratories interested in improving the yields and crude purity of peptides prepared by solid phase synthesis particularly using Fmoc chemistry. Recent attempts to improve synthetic quality have focused on incorporating backbone protection into the peptide to ease 'on-resin' aggregation during the synthesis, and subsequently improve the permeability of reagents through the resin [13–15]. Alternatively, attention has also been directed at employing potent acylation reagents, such as HATU, to more efficiently couple amino acids through the troublesome areas of the sequence [16]. Such approaches, while apparently effective, are somewhat cumbersome and expensive. Additionally, these have principally focused on improved acylation as the primary remedial action. Little work has examined the effect of improved deprotection procedures on synthesis efficiency. Consequently, we undertook to investigate this aspect and to subsequently develop an improved, simple experimental protocol to synthesize Aβ 1-40/42 by Fmoc-SPPS with full automation and at reduced cost. We describe herein our successful efforts to modify the synthetic methodology to accommodate the use of the more effective N^{α}-Fmoc deprotection reagent, DBU.

MATERIALS AND METHODS

Chemicals

All Fmoc-protected amino acids were of the L-configuration and, together with DMF and HBTU, were obtained from Auspep (Melbourne, Australia). PAC-PS-PEG resin was obtained from PerSeptive Biosystems (Foster City, CA, USA). DBU was obtained from Aldrich (St Louis, MO, USA) and used without further purification. All organic solvents were of HPLC grade and all other reagents were of the highest quality available.

Peptide Synthesis

Continuous flow Fmoc-SPPS was used throughout [17]. The β-amyloid peptides and fragments were assembled using an Applied Biosystems Pioneer Synthesiser (Foster City, CA, USA) and PAC-PEG-PS supports that had been derivatized with *C*-terminal residues as previously described [17]. Synthesis scale was 0.1 mmol and Fmoc-amino acids were activated with HBTU/DIEA in DMF [18]. Amino acid side chain protecting groups were: Trt for Asn, Gln, His; *t*Bu for Ser, Asp, Glu, Tyr; *t*Boc for Lys and pentamethyldihydrobenzofuransulfonyl (Pbf) for Arg. After each synthesis, resin-bound peptides were washed and dried, and then subjected to global cleavage and deprotection in a mixture of TFA/H_2O/ethanedithiol (EDT)/triethylsilane (TIS) (94:2.5:2.5:1 v/v, 10 mL). After 2–3 h, the resin was removed by filtration and washed twice with TFA (5 mL). The total volume of the combined TFA filtrate was reduced to less than 5 mL under a stream of N_2, and the peptide precipitated with cold diethyl ether, centrifuged and triturated once more. The product was then dissolved in a solution of H_2O/CH_3CN/TFA (90:10:0.1 or 80:20:0.1 v/v/v) and lyophilized.

Fmoc Deprotection during SPPS

Aβ 29-40 was synthesized once using 20% piperidine/DMF throughout and deprotection cycles of 5 min duration. For all other amyloid peptides 2% DBU/DMF solution was used in a deprotection cycle of 5 min duration until residue Ser^8 at which point the solution was changed to 20% piperidine/DMF and the deprotection cycle time extended to 10 min.

Aspartimide Formation Study

Twenty-milligram aliquots of resin-bound Aβ 1-42 were treated separately with 2% DBU/DMF solution for 40 min and 24 h. After washing and drying these were then cleaved and deprotected by treatment with TFA/H_2O/EDT/TIS (94:2.5:2.5:1, 2 mL) for 2.5 h. Each peptide was then lyophilized and analysed by reverse phase (RP)-HPLC and MALDI-TOF MS.

Characterization

RP-HPLC was performed on a Waters (Milford, USA) instrument controlled by Millennium software equipped with photo-diode array (PDA) detection. The solvent system used throughout this study (except where mentioned) was buffer A: 10 mM aqueous ammonium bicarbonate, buffer B: CH_3CN. Analytical RP-HPLC was performed using a Vydac C4 analytical column (4.6 × 250 mm, 5 μm) (Hesperia, USA), at a flow rate of 1 mL/min using a linear gradient of CH_3CN. For MALDI-TOF MS analysis, samples were mixed with α-cyano-4-hydroxycinnamic acid as matrix and examined using a Bruker BIFLEX instrument (Bremen, Germany) in linear mode at 19.5 kV, or reflector mode at 19.5 kV, 20 kV gradient reflector.

RESULTS AND DISCUSSION

During Fmoc-SPPS, aggregation, through interchain association, of the growing resin-bound peptides is known to contribute to the difficulty of the synthesis [12,19]. Aggregation typically results in a decrease in the rates of acylation and deprotection, and consequently leads to the production of deletion products of varying length and composition. Such a decrease in the purity of the crude material often results in subsequent difficult purification. Strategies to combat Fmoc-SPPS resin-bound aggregation and improve synthetic product yield include the use of more potent acylation reagents [16], backbone amide protection [14,15], incorporation of pseudoproline [20,21], microwave irradiation to enhance coupling efficiency [22] and SPPS at elevated temperatures [23]. However these strategies, while apparently effective, can become prohibitively expensive, involve extra steps and particular care to ensure that the conditions are suitable and complete acylation occurs.

Comparatively little work has been carried out on another consequence of on-resin aggregation, that of slow or inefficient removal of the Fmoc group that can also contribute to synthesis failure [24,25]. A solution of 20% piperidine in DMF is traditionally and commonly employed to effect N^{α}-Fmoc deprotection, a base and concentration that has proven to be effective in most situations. However, this base solution is often inadequate during on-resin aggregation as is often evidenced by the onset of slow broad Fmoc-piperidine adducts by UV monitoring of the reaction effluent during continuous flow SPPS. When this occurs, the usual course of remedial action is extension or repetition of the deprotection cycle of synthesis. However, it has been shown that under such circumstances, the tertiary amidine, DBU, is markedly more effective than piperidine [26,27]. In order to determine if slow Fmoc deprotection was a significant problem in the assembly of Aβ peptides, we undertook to examine the use of this base. To assist this study, the fragment Aβ 29-40 was used as a model sequence and test of solid phase synthetic chemistry. Its high hydrophobicity has previously made this a challenging sequence to assemble [28]. To confirm the difficult synthesis of Aβ 29-40, we first synthesized this peptide using standard Fmoc-continuous flow methodology throughout together with 20% piperidine/DMF solution to effect N^{α}-deprotection. RP-HPLC and MALDI-TOF MS analysis of the resulting crude product showed the presence of several deletion peptides (Figure 2(a)). One of these was a single glycine deletion that co-elutes with the target

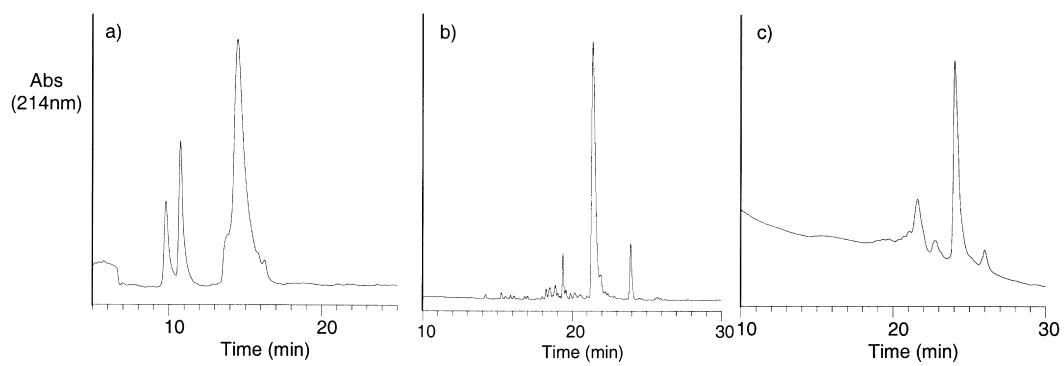

Figure 2 RP-HPLC profiles of the crude Aβ 29-40/42 fragments. (a) Aβ 29-40 synthesized using 20% piperidine/DMF, gradient: 10–60% B (30 min) on Vydac C4 analytical column at 60°C. (b) Aβ 29-40 synthesized using 2% DBU/DMF, RP-HPLC conditions same as for (a). (c) Aβ 29-42 synthesized using 2% DBU/DMF, gradient: 10–90%B (30 min) on Vydac C4 analytical column at 60°C.

peptide under the RP-HPLC conditions employed. Other side products included combinations of valine and glycine deletions. This led us to conclude that slow coupling of Val[39] onto Val[40] was one source of potential problems and that an additional source would be the incomplete deprotection of valine, a residue that has a known propensity for slow N^{α}-Fmoc deprotection [29]. The abundance of glycine and valine deletion products in this crude sample clearly makes the target peptide a useful model for improved synthetic method development.

A repeat assembly of the Aβ 29-40 peptide was undertaken using a solution of 2% DBU/DMF for N^{α}-Fmoc deprotection. This concentration of base has been previously recommended as appropriate for use in 'difficult' peptide synthesis [26,27]. Fmoc removal at this concentration is rapid and side chain modification is unknown except at sensitive Asp-X sites leading to imide formation [30]. The substantially improved quality of Aβ 29-40 in this second synthesis (Figure 2(b)) suggested that, at least for this peptide, incomplete Fmoc group removal may be a more significant problem in Fmoc-SPPS than incomplete acylation, contrary to the focus of others [16]. In order to confirm this result, we synthesized Aβ 29-42, the *C*-terminal region of the historically more 'difficult' peptide, Aβ 1-42, again utilizing the more effective Fmoc-deprotection ability of DBU. The synthesis of this fragment was also successful (Figure 2(c)), indicating that the assembly of the full-length peptide would likely be improved by use of this base.

While the *C*-terminal region of the Aβ peptide contains no residues that are susceptible to side chain modification during SPPS, the amino terminal region has an Asp-Ser pair. This sequence is known to be prone to base-mediated imide formation [30,31]. This side reaction involves cyclization of the aspartic acid side chain and the amide backbone nitrogen of the carboxyl residue, resulting in the loss of one water molecule, and in the presence water, subsequent hydrolysis of the imide to form either the α- (native) or β-peptide (non-native). Attempts to minimize imide formation by use of more bulky side chain protection of the aspartic acid β-carboxyl, such as 1-adamantyl ester and β-3-methylpent-3-yl esters [32], are limited by their incomplete compatibility with Fmoc-SPPS or are commercially unavailable. Furthermore Karlstrom *et al.* [33] have shown that these measures are not completely effective in preventing imide formation. In contrast, Hmb-backbone amide protection has been shown to be completely effective, but is expensive [30,31,34]. Use of alternative bases such as piperazine in the presence of 1-hydroxybenzotriazole has been successful but are less efficient than DBU [31]. In the case of the Aβ peptide, the Asp[7]-Ser[8] pair can be expected to be susceptible to DBU-mediated imide formation so this was avoided by prudent replacement of the deprotection reagent before the synthesis reached this point. Piperidine was used for the remainder of the synthesis although this base is itself not entirely innocuous [30]. For cycles where the deprotection reagent was piperidine, the deprotection cycle time was increased from 5 to 10 min, in order to ensure complete reaction. Using this simple deprotection protocol, synthesis of the full-length peptide, Aβ 1-40, was carried out using single 30 min coupling times throughout for the addition of each amino acid. The assembly proceeded smoothly and the deprotection peaks collated at the completion of the synthesis, showed good consistency. The crude cleaved product was obtained in a purity of 45% (estimated by RP-HPLC peak integration), an excellent result for a peptide of this length and complexity (Figure 3(a)). Use of the same synthetic process was repeated to obtain Aβ 1-42, which was produced in 40% yield (Figure 3(b)). Both peptides could be readily purified by conventional RP-HPLC in overall yields of 24 and 17%, respectively, relative to starting crude peptide. This synthetic protocol is now in use for the ready preparation of analogues of Aβ for the systematic examination of aggregation and amyloid formation behaviour.

Confirmation of Aspartimide Formation in the Presence of DBU

With resin-bound Aβ 1-42 in hand, we undertook to establish conclusively if the precautions taken in the synthetic preparation of the peptides to avoid DBU-mediated imide formation were necessary. RP-HPLC time course analyses of cleaved peptides were thus performed with aliquots of 2% DBU base-treated resin samples removed at 40 min and 24 h. After 40 min, corresponding to eight cycles of N^{α}-deprotection, it is evident that the crude peptide was undergoing modification (Figure 4(b), cf. (a)). After 24 h of base treatment, substantial alteration of the integrity of native Aβ 1-42 took place (Figure 4(c), Table 1). While the presence of the cyclic imide was confirmed by MALDI-TOF MS (Figure 5), the β-peptide could not be positively identified in the

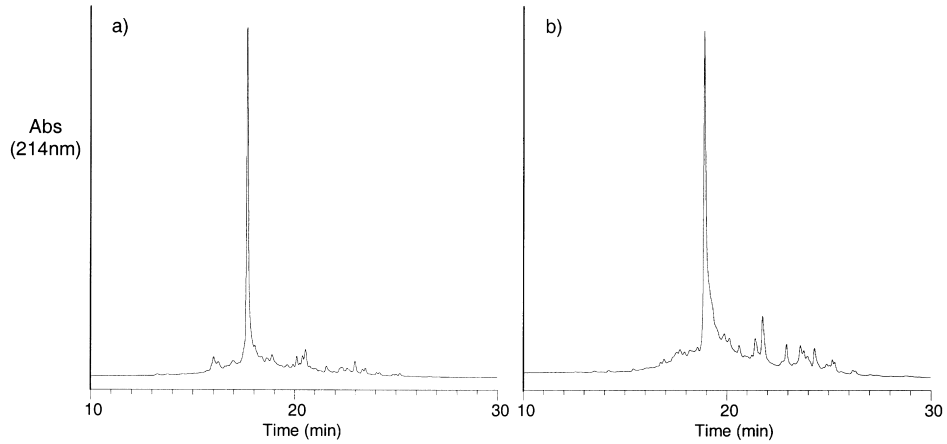

Figure 3 Crude cleaved (a) Aβ 1-40 and (b) Aβ 1-42. Both peptides were synthesized using 2% DBU/DMF until Ser^8 and the remainder was completed using 20% piperidine/DMF. Gradient: (a) 10–60% B (30 min), and (b) 15–50% B (30 min).

Table 1 Cyclic Imide Formation from 2% DBU/DMF Treated Resin-bound Aβ 1-42 Determined by Integration of the Respective RP-HPLC Chromatograms after MALDI-TOF Analysis Confirmed Identity of Each[a]

Test exposure time	Aβ 1-42-unmodified peptide (%)	Cyclic Aβ 1-42 (%)
40 min	82	18
4 h	63	37
24 h	38	62

[a] Values presented are expressed as percentages of the total area of the relevant peaks.

RP-HPLC profile of the peptide. This is probably because it co-elutes with the α-peptide under the chromatography conditions. Nevertheless, the presence of the imide, which is also vulnerable to epimerization [34], confirmed earlier studies [31,35] and the need to replace the DBU with the less detrimental piperidine for the final stages of the synthesis of Aβ.

Optimizing the Preparative Purification of Aβ Peptides

A less appreciated area of difficulty in the preparation of synthetic Aβ peptides is the post-synthesis characterization and purification by RP-HPLC. Typical chromatography conditions involve the use of TFA buffers in concentrations of 0.05–1% in aqueous and organic phases. Buffers of this type have a pH value which can be too acidic for some peptides and proteins, leading to lowered solubility, poor interaction with and decreased recoveries from the RP-HPLC column. Phosphate buffer systems can also be employed, but present difficulties if a

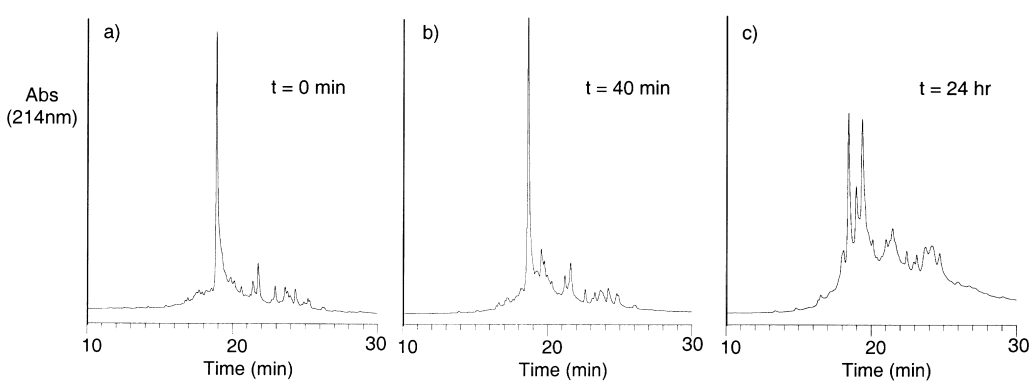

Figure 4 Time course analysis of aspartic acid side chain modification in the presence of DBU. Resin-bound Aβ 1-42 peptide was suspended in a solution of 2% DBU in DMF for (a) 0 min, (b) 40 min, (c) 24 h. RP-HPLC analysis was conducted using Vydac C4 column with Buffers A: 10 mM aq NH_4HCO_3, and B: CH_3CN, gradient 15–50% B (30 min).

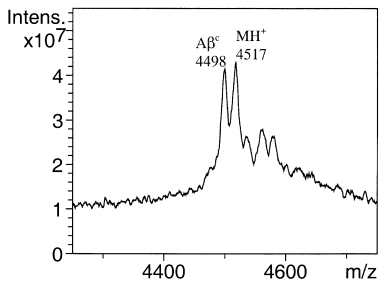

Figure 5 MALDI-TOF mass spectrum of crude Aβ 1-42 peptide after 24 h exposure to 2% DBU/DMF solution. MH^+ observed 4517 (expected 4515); $A\beta^c$ (cyclic imide) observed 4498 (expected 4497).

salt-free product is required. Previous work in our laboratory using the fragment Aβ 1-28 showed that basic buffers systems allow better characterization and isolation of Aβ peptides [A. Tickler, unpublished]. We therefore used our synthetic Aβ 29-40/42 peptides, which presented serious challenges for post-synthesis characterization, to assess a variety of RP-HPLC buffer conditions. Ammonium bicarbonate buffers (10 mM concentration) were shown to be particularly beneficial, and conveniently, are also volatile which allows removal by lyophilization. Best results were obtained with a C4 support at elevated temperature (60°C). The behaviour, however, of peptides purified with in these buffer conditions remains to be investigated. Kaneko *et al.* [36] have shown significant differences in the CD spectrum of the TFA and HCl salts of Aβ regarding the propensity of the Aβ peptide to form β-sheet/β-turn structures, illustrating the importance of residual salts in the formation of secondary structure.

CONCLUSION

An inexpensive and readily implemented variation of existing Fmoc-SPPS was developed that enabled rapid and efficient synthesis of the notoriously difficult Aβ peptides. Improved Fmoc removal rather than improved acylation is most critical to the high purity synthesis of these peptides. The improved synthetic method provides a simple cost-effective preparation of these peptides and analogues for biophysical and clinical studies.

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REFERENCES

1. Carr DB, Goate A, Morris JC. Current concepts in the pathogenesis of Alzheimer's disease. *Am. J. Med.* 1997; **103**: 3s–10s.
2. Clippingdale AB, Wade JD, Barrow CJ. The Aβ peptide and its role in Alzheimer's disease. *J. Peptide Sci.* 2001; **7**: 227–249.
3. Behl C. Alzheimer's disease and oxidative stress: Implications of novel therapeutic approaches. *Prog. Neurobiol.* 1999; **57**: 301–323.
4. Wilson CA, Doms RW, Lee VM-Y. Intracellular APP processing and Aβ production in Alzheimer's disease. *J. Neuropath. Exp. Neurol.* 1999; **58**: 787–794.
5. Forloni GE, Angeretti LN, Della Torre P, Salmona M. Amidation of β-amyloid peptide strongly reduced the amyloidogenic activity without alteration of neurotoxicity. *J. Neurochem.* 1997; **69**: 2048–2054.
6. Li L, Darden TA, Bartolotti L, Kominos D, Pederson LG. An atomic model for the pleated β-sheet structure of Aβ amyloid protofilaments. *Biophys. J.* 1999; **76**: 2871–2878.
7. Gursky O, Aleshkov S. Temperature-dependent β-sheet formation in β-amyloid Aβ(1-40) peptide in water: Uncoupling β-structure folding from aggregation. *Biochim. Biophys. Acta* 2000; **1476**: 93–102.
8. Barrow CJ, Zagorski MG. Solution structures of β peptide and its constituent fragments: Relation to amyloid deposition. *Science* 1991; **253**: 179–182.
9. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. Immunisation with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse [see comments]. *Nature* 1999; **400**: 173–177.
10. Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HTJ, Nixon RAMM, Bergson C, Fraser PE, St Geroge-Hyslop P, Westaway D. Aβ peptide immunisation reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* 2000; **408**: 979–982.
11. Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW. Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 2000; **408**: 982–985.

12. Hyde C, Johnson T, Owen D, Quibell M, Sheppard RC. Some 'difficult sequences' made easy. *Int. J. Peptide Protein Res*. 1994; **43**: 431–440.
13. El-Agnaf OMA, Harriott P, Guthrie DJS, Irvine BG, Walker B. The synthesis of some peptides related to the amyloid β peptide 25-35: Use of *N*-(2-hydroxy-4-methoxybenzyl) protection. *Lett. Peptide Sci*. 1994; **1**: 135–141.
14. Quibell M, Turnell WG, Johnson T. Preparation and purification of β-amyloid (1-43) via soluble, amide backbone protected intermediates. *J. Org. Chem*. 1994; **59**: 1745–1750.
15. Quibell M, Turnell WG, Johnson T. Improved preparation of β-amyloid (1-43): Structural insight leading to optimised positioning of *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection. *J. Chem. Soc. Perkins Trans*. 1995; **16**: 2019–2024.
16. El-Agnaf OMA, Goodwin H, Sheridan JM, Frears ER, Austen BM. Improved solid phase synthesis of amyloid proteins associated with neurodegenerative diseases. *Protein Peptide Lett*. 2000; **7**: 1–8.
17. Atherton E, Sheppard RC. *Solid Phase Peptide Synthesis — A Practical Approach*. IRC Press at Oxford University Press: Oxford, 1989.
18. Albericio F, Kates SA. Coupling methods: Solid-phase formation of amide and ester bonds. In *Solid Phase Synthesis: A Practical Guide*, Kates SA, Albericio F (eds). Marcel Dekker: New York, 2000; 275–330.
19. Bedford J, Hyde C, Johnson T, Jun W, Owen D, Quibell M, Sheppard RC. Amino acid structure and 'difficult sequences' in solid phase peptide synthesis. *Int. J. Peptide Protein Res*. 1992; **40**: 300–307.
20. Mutter M, Nefzi A, Sato T, Sun X, Wahl F, Wahl T. Pseudo-prolines for accessing 'inaccessible' peptides. *Peptide Res*. 1995; **8**: 145–153.
21. Sampson WR, Patsiouras H, Ede N. The synthesis of 'difficult' peptides using 2-hydroxy-4-methoxybenzyl or pseudoproline amino acid building blocks: A comparative study. *J. Peptide Sci*. 1999; **5**: 403–409.
22. Yu H-M, Chen S-T, Wang K-T. Enhanced coupling efficiency in solid-phase peptide synthesis by microwave irradiation. *J. Org. Chem*. 1992; **57**: 4781–4784.
23. Varanda LM, Miranda M, Theresa M. Solid-phase peptide synthesis at elevated temperatures: A search for an optimised synthesis of unsulfated cholecystokinin-12. *J. Peptide Res*. 1997; **50**: 102–108.
24. Larsen BD, Holm A. Incomplete Fmoc deprotection in solid-phase synthesis of peptides. *Int. J. Peptide Protein Res*. 1994; **43**: 1–9.
25. Fontenot JD, Ball JM, Millar MA, David CM, Montelaro RC. A survey of potential problems and quality control in peptide synthesis by fluorenylmethoxycarbonyl procedure. *Peptide Res*. 1991; **4**: 19–25.
26. Wade JD, Bedford J, Sheppard RC, Tregear GW. DBU as N^{α}-deprotecting reagent for the fluorenylmethoxycarbonyl group in continuous flow solid phase peptide synthesis. *Peptide Res*. 1991; **4**: 194–199.
27. Dettin M, Pegoraro S, Rovero P, Biccato S, Bagno A, di Bello C. SPPS of difficult sequences. A comparison of chemical conditions, synthetic strategies and on-line monitoring. *J. Peptide Res*. 1997; **49**: 103–111.
28. El-Agnaf OMA, Mahil DS, Patel BP, Austen BM. Oligomerization and toxicity of β-amyloid-42 implicated in Alzheimer's disease. *Biochem. Biophys. Res. Commun*. 2000; **273**: 1000–1007.
29. Kent SBH. Chemical synthesis of peptides and proteins. *Ann. Rev. Biochem*. 1988; **57**: 957–989.
30. Lauer JL, Fields CG, Fields GB. Sequence dependence of aspartimide formation during 9-fluorenylmethyoxycarbonyl solid-phase peptide synthesis. *Lett. Peptide Sci*. 1995; **1**: 197–206.
31. Wade JD, Mathieu MN, Macris M, Tregear GW. Base induced side reactions in Fmoc-solid phase peptide synthesis: Minimisation by use of piperizine as N^{α}-deprotection reagent. *Lett. Peptide Sci*. 2000; **7**: 107–112.
32. Karlstrom A, Unden A. A new protecting group for aspartic acid that minimises piperidine-catalysed aspartimide formation in Fmoc solid-phase peptide synthesis. *Tetrahedron Letts*. 1996; **37**: 4243–4246.
33. Karlstrom A, Unders A. Design of protecting groups for the [beta]-carboxylic group of aspartic acid that minimize base-catalysed aspartimide formation. *Int. J. Peptide Protein Res*. 1996; **48**: 305–311.
34. Quibell M, Owen D, Packman LK, Johnson T. Suppression of piperidine-mediated side product formation for Asp(OBu^t)-containing peptides by the use of *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection. *J. Chem. Soc., Chem. Commun*. 1994: 2343–2344.
35. Dolling R, Beyermann M, Haenel J, Kernchen F, Krause E, Franke P, Brudel M, Bienert M. Piperidine-mediated side product formation for Asp(OBu^t)-containing peptides. *J. Chem. Soc., Chem. Commun*. 1994: 853–854.
36. Kaneko I, Tutumi S. Conformations of β-amyloid in solution (Reply). *J. Neurochem*. 1997; **68**: 438–439.

