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Direct Solid-Phase Synthesis of the β-Amyloid (1−42) Peptide Using Controlled Microwave Heating

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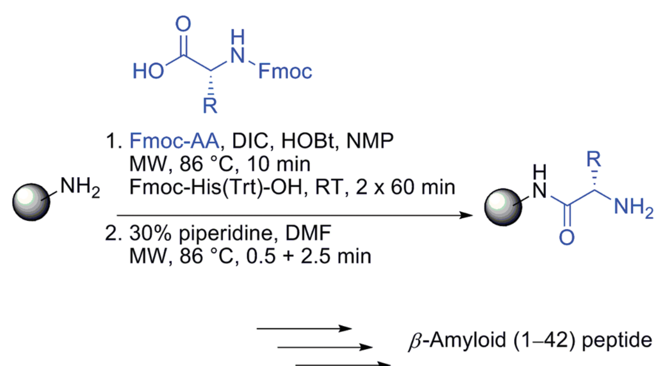

Standard linear Fmoc/*t*-Bu solid-phase synthesis of the 42-mer β-amyloid (1−42) peptide was achieved under controlled microwave conditions at 86 °C using inexpensive DIC/HOBt as coupling reagent on ChemMatrix resin. In order to avoid racemization of the sensitive amino acids, the coupling of the three His residues in the difficult peptide sequence was performed at room temperature. The desired peptide was obtained within 15 h overall processing time in high yield and purity (78% crude yield).

Alzheimer's disease is a neurodegenerative disorder characterized by extracellular accumulation of amyloid senile plaques within the brain tissue.[1] The major proteinaceous component of amyloid deposits consists of aggregates of sparingly soluble β-amyloid peptides (Aβ 1−39, 1−40, 1−42, 1−43), which are derived from proteolytic cleavage of the amyloid protein precursor.[2] Aβ (1−42) (Figure 1) plays a crucial role in the pathogenesis of Alzheimer's disease in that it forms β-sheet structures and amyloid fibrils which induce neurotoxicity in both in vitro[3] and in vivo experiments.[4]

The Aβ (1−42) peptide has received a considerable amount of attention in the peptide community during the last decades, and as one of the most prominent examples of a so-called "difficult peptide" is well-known for its complexity of preparation.[5] Two major factors make this sequence a problematic target for stepwise solid-phase peptide synthesis (SPPS). First, the hydrophobic composition of the C-terminal region results in an association between resin-bound peptide chains. This aggregation leads to gross steric hindrance and a significant decrease in the rates of coupling and deprotection reactions. Second, failure to achieve near-quantitative reactions results in amino acid deletion sequences. This may lead to major difficulties in the purification and analysis of the target peptide due to solubility/aggregation problems and coinciding chromatographic mobilities.

FIGURE 1. Sequence of β-amyloid (1−42) peptide.

A number of research groups have been involved in overcoming the difficulties in the assembly of the biologically important Aβ (1−42) peptide, generally applying SPPS using either Boc/Bzl or Fmoc/*t*-Bu orthogonal protection strategies.[5−14]

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Improvements both in terms of yield and purity have been achieved by, for example, employing highly reactive acylation agents (HATU,[6] Fmoc-aminoacyl fluorides[7]), solvent additives[8,9] in a coupling step, or stronger base (DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene) for deprotection of the Fmoc group.[10] Other methods to improve the synthetic quality of A*β* peptides have focused on introducing specific reversible modifications at the peptide backbone, for example, using Hmb (2-hydroxy-4-methoxybenzyl)-protected amino acids,[11] pseudoproline dipeptide derivatives,[12] and the use of an O–N intramolecular acyl migration reaction of the corresponding *O*-acyl isopeptide.[13] These strategies were designed to ease aggregation during the assembly of the peptide chain on the solid support and to improve the permeability of reagents through the resin. However, such indirect approaches, while apparently effective, are somewhat cumbersome and expensive.[11,12]

Therefore, the development of efficient direct and linear methods for the solid-phase synthesis of the A*β* (1–42) peptide are still of great importance. A recent advance in this context has been the development of a fully poly(ethylene glycol)-based solid support (ChemMatrix resin) that allows the stepwise preparation of complex peptides, including A*β* (1–42), with relative ease on the solid phase.[14] Herein, we report a rapid and simple protocol for the preparation of the *β*-amyloid (1–42) peptide employing a microwave-assisted Fmoc/*t*Bu solid-phase method that utilizes standard *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) coupling reagents.

During the past few years, a number of reports have highlighted the benefits of applying microwave heating toward solid-phase peptide synthesis,[15] with most of the recent attempts focusing on difficult to prepare α-peptides,[16] *β*-peptides,[17] and peptoids[18] using conventional Fmoc/*t*-Bu orthogonal protection strategies. In 2008, our group reported a microwave protocol for the preparation of several difficult peptide sequences (a 9-mer, 15-mer, and 24-mer) where both the coupling and deprotection steps were carried out under controlled microwave conditions in a dedicated single-mode microwave peptide synthesizer.[19,20] This optimized Fmoc/*t*-Bu SPPS method utilized ChemMatrix resin[14] and standard DIC/HOBt coupling conditions,[21] employing a 3 molar excess of activated Fmoc-amino acid at a temperature of 86 °C with 10 min reaction time. For the deprotection step, 30% piperidine in DMF was used in a two-step cleavage protocol (0.5 and 2.5 min) at the same reaction temperature. These reaction conditions were found to be far superior to conventional coupling/deprotection at room temperature and provided the difficult α-peptides in high purity and yield.[19] However, applying these elevated temperature in solid-phase peptide synthesis a significant amount of racemization was found for the sensitive amino acids His (ca. 7% D-His) and Cys (ca. 2% D-Cys).[19,22]

Herein we report an improved protocol for the preparation of the A*β* (1–42) peptide. Compared to our previous method,[19] we have implemented two changes in the synthetic protocol. First, the molar excess of Fmoc-amino acid and coupling reagents was increased from 3 to 5 equiv to improve the efficiency of the coupling reactions. Importantly, in order to avoid the racemization of sensitive amino acids at high temperatures,[19,22] the coupling of the three His residues in the sequence (H in Figure 1) was performed at room temperature without microwave irradiation.

Microwave-assisted SPPS was performed using a dedicated 300 W single-mode manual microwave peptide synthesizer (Discover SPS).[20] The DIC/HOBt couplings and Fmoc-deprotection steps were carried out in a solid-phase reaction vessel under atmospheric conditions, while the reaction temperature was measured continuously with a fiber-optic probe inserted into the reaction vessel.[23] The reaction vessel is a polypropylene tube with a frit attached and designed for solid-phase synthesis allowing for bottom filtration. Both the coupling and the deprotection steps under microwave conditions were performed applying a pulsed temperature control program using comparatively small maximum microwave power levels (10 W for coupling and 20 W for deprotection) in order to rapidly achieve the desired reaction temperatures (typically 86 °C for both coupling and deprotection).[19]

Following the general experimental conditions described above, the A*β* (1–42) peptide was assembled on 0.075 mmol Rink Amide ChemMatrix resin employing conventional Fmoc/*t*-Bu orthogonal protection strategy. In the coupling steps, a 5 molar excess of the corresponding Fmoc-amino acid in combination with the standard coupling cocktail DIC/HOBt in 1-methyl-2-pyrrolidone (NMP) was used (single couplings). Applying our optimized microwave heating conditions,[19] the coupling steps were performed at 86 °C (10 min). Microwave-assisted Fmoc-deprotection with piperidine/DMF was achieved in two steps: after an initial 30 s of microwave irradiation (86 °C), the resin was washed and subsequently exposed to a fresh portion of the cleavage cocktail and irradiated for an additional 2.5 min (86 °C). In all three cases, the sensitive Fmoc-His(Trt)-OH residues (H in Figure 1) were built into the sequence at room temperature in order to minimize racemization using an extended reaction time of 60 min and applying a double coupling strategy.[24]

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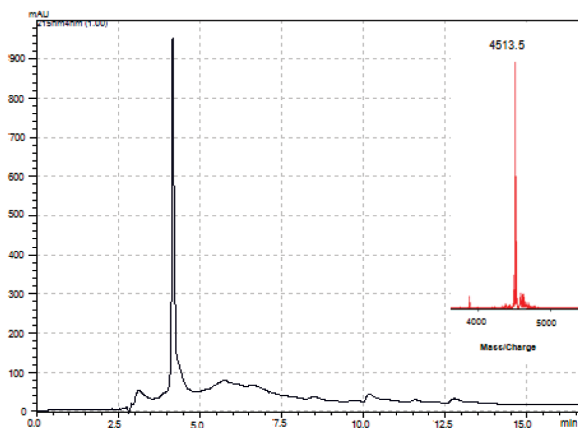

FIGURE 2. HPLC chromatogram and MALDI-TOF spectrum (insert) of crude Aβ (1−42) peptide synthesized under microwave conditions. Calculated mass: 4513.1 Da. The full mass spectrum is shown in the Supporting Information (Figure S1).

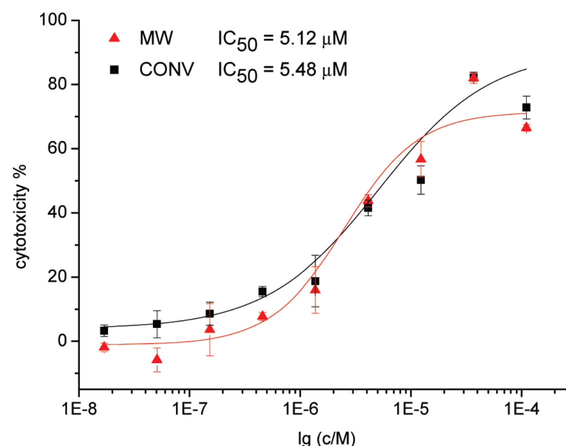

FIGURE 3. Cytotoxic effect of β-amyloid (1−42) peptide synthesized under microwave (red, MW) and conventionally heated conditions (black, CONV) on SH-SY5Y neuroblastoma cell lines. Cytotoxicity is expressed as percentage of the untreated control.[24]

Following final Fmoc removal, the Aβ (1−42) peptide was cleaved from the resin using a mixture of TFA/dithiothreitol/H_2O (95:2.5:2.5) at room temperature and precipitated with cold diethyl ether to obtain the crude peptide in 78% yield. The cleaved product was stored at −20 °C to prevent ^{35}Met(O) formation[5] and further aggregation.

In agreement with previous investigations,[5,9,14] the characterization of the Aβ (1−42) peptide by HPLC and MALDI-TOF proved to be a nontrivial affair due to strong aggregation phenomena. Ultimately, the crude peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP),[5,9,14] and analytical HPLC analysis was performed at 60 °C using a Phenomenex Jupiter C4 column.[24] The chromatogram of the cleaved peptide (Figure 2) showed essentially a single peak at 4.14 min, and the identity of the crude Aβ (1−42) was confirmed by MALDI-TOF MS (M_{calc}: 4513.1 Da, M_{obs}: 4512.5 Da). Further evidence for the constitution of the synthesized Aβ (1−42) peptide was derived by standard acidic amino acid analysis (Table S2, Supporting Information) and by comparison of the ^{1}H NMR spectrum of the crude peptide with published data (Figure S2, Supporting Information).[24]

Problems in handling the Aβ (1−42) peptide can often result from minor impurities, small quantities of peptides with partial racemization, and/or the presence of ^{35}Met(O), the latter having an effect on aggregation rates and toxicity.[5] If aggregated, Aβ (1−42) peptide is extremely insoluble in *aqueous* media which leads to difficulties in the use of this material in biological and biophysical tests.[5] Gratifyingly, no Met(O) was observed from the data obtained from amino acid analysis and the MALDI-TOF spectrum.

An obvious concern when dealing with peptide couplings and deprotection reactions at elevated temperatures is the racemization of amino acids at the α-carbon atom and/or in the side chain. The biological properties of proteins and peptides are often critically dependent on the configuration of the backbone chiral centers, maintaining the integrity of these centers is therefore of significant importance in peptide synthesis. In our previous study on microwave-assited SPPS we have observed that while most amino acids are not sensitive to racemization under DIC/HOBt coupling conditions at 86 °C, considerable levels of racemization for both His (ca. 7% D-His) and Cys (ca. 2% D-Cys) were observed.[19,22] Gratifyingly, in the synthesis of Aβ (1−42) switching from microwave (86 °C) to a room temperature coupling step for the three Fmoc-His(Trt)-OH residues the racemization levels for His were very low (0.3% D-His) as evidenced by GC−MS analysis after derivatization of the His residue (Table S3, Supporting Information).[24]

The synthesis of the Aβ (1−42) peptide was additionally evaluated using conventional heating instead of microwave heating at the same 86 °C temperature. Analogous to the results we have obtained for shorter difficult peptide sequences,[19] also for the 42-mer Aβ (1−42) similar peptide purities, yields, and His racemization levels were obtained when the microwave coupling and deprotection steps were replaced by conventional heating protocols under otherwise identical conditions (Table S1, Figure S3, Supporting Information).[24,25] Furthermore, no significant differences in the peptide content of the two differently synthesized β-amyloid (1−42) were detected (Table S2, Supporting Information).

Finally, the in vitro neurotoxicitiy of Aβ (1−42) peptide synthesized under different SPPS methods was evaluated. Therefore, SH-SY5Y human neuroblastoma cells were treated with the two differently prepared Aβ (1−42) peptides (synthesized under microwave and conventionally heated conditions) at a 1.69×10^{-2}−1.10×10^{2} μM concentration range, and the viability of the cells was determined by using the MTT assay.[26] The data summarized in Figure 3 demonstrate that the two differently synthesized Aβ (1−42) peptides show almost identical cytotoxicity effects on SH-SY5Y human neuroblastoma cells. Since irradiation of peptides with microwave energy has been claimed to result in a deaggregation of the peptide backbone via direct interaction of the peptide chain with the electric field,[15a,b] these results are important in demonstrating the bioequivalency of conventionally and microwave-heated synthetic peptides, in particular as the toxicity of the Aβ (1−42) peptide is known to depend strongly on aggregation phenomena.[5a]

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In conclusion, we have developed an improved synthetic protocol for the direct microwave-assisted synthesis of the 42-mer β-amyloid peptide. While standard solid-phase protocols typically result in peptides of poor quality, the application of controlled microwave heating provides the Aβ (1−42) peptide in high purity in only 15 h of total processing time. Our best conditions utilized 5 equiv of activated Fmoc-amino acid at a temperature of 86 °C for 10 min coupling and 3 min deprotection time. The coupling of His residues was performed at room temperature to eliminate the effect of racemization for this sensitive amino acid. The results of in vitro neurotoxicity assay indicate that the microwave-synthesized peptide is equal in potency to the peptide synthesized under conventional heating conditions at the same temperature.

Experimental Section

Synthesis of Aβ (1−42) Peptide Using Microwave Irradiation at 86 °C. To a 10 mL bottom-filtration reaction vessel 0.075 mmol (150 mg, loading 0.50 mmol/g) of RAM ChemMatrix resin was transferred and swollen in 4 mL of DCM for 30 min. The resin was washed with NMP (2 × 4 mL). In a separate vial the corresponding Fmoc-amino acid (0.375 mmol), DIC (0.375 mmol, 58 μL), and HOBt (0.375 mmol, 61 mg) were combined in 1 mL of NMP. The preactivated coupling cocktail was added to the resin after 2 min, and the reaction mixture was irradiated at 86 °C for 10 min using the SPS program (maximum power 10 W, ΔT = 3 °C, 75 °C nominal temperature setting). Incorporation of Fmoc-His(Trt)-OH residue was performed at room temperature; see below for detailed experimental conditions. The suspension was then washed with DMF, DCM (5×4 mL each), and DMF (2×4 mL). To the resin was added 2 mL of 30% piperidine in DMF in a deprotection cycle. After the ^{8}Ser residue 30% piperidine in DMF containing 0.1 M HOBt was utilized to avoid potential aspartimide formation at ^{7}Asp. The reaction vessel was placed into the microwave cavity of a CEM SPS manual peptide synthesizer and irradiated for 30 s at 86 °C (SPS mode, maximum power 20 W, ΔT = 3 °C, 75 °C nominal temperature setting). The resin was subsequently washed with 4 mL of DMF, and 2 mL of 30% piperidine in DMF was added to the sample and irradiated for an additional 2.5 min at 86 °C (SPS mode, maximum power 20 W, ΔT = 3 °C, 75 °C nominal temperature setting). The suspension was then washed with DMF and DCM (5×4 mL each) and NMP (2×4 mL). After the last deprotection step, the peptidyl resin was washed with DMF and DCM (5 × 4 mL each) and dried under reduced pressure.

Incorporation of Fmoc-His(Trt)-OH residue. In a separate vial, Fmoc-His(Trt)-OH (0.375 mmol, 232 mg), DIC (0.375 mmol, 58 μL), and HOBt (0.375 mmol, 61 mg) were combined in 1 mL of NMP. The preactivated coupling cocktail was added to the resin after 2 min, and then the reaction mixture was placed in the PLS 4 × 6 synthesizer and shaken for 1 h at room temperature. After the reaction time, the resin was washed with NMP (2×4 mL) and the coupling step was repeated with freshly prepared coupling cocktail.

Final Cleavage. The peptide was cleaved from the solid support with a cleavage cocktail (10 mL) of TFA/DTT/water (95: 2.5: 2.5) at ambient temperature for 5 h. The resin was filtered and washed with a small amount of cleavage cocktail. The residual product was precipitated with ice-cold diethyl ether, and the peptide was collected by filtration, dissolved in deionized water and lyophilized (78% yield of crude peptide).

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Supporting Information Available: Description of experimental procedures, chromatograms, NMR and MS spectra, and amino acid analysis of peptides. This material is available free of charge via the Internet at http://pubs.acs.org.