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Biological Tuning of Synthetic Tactics in Solid-Phase Synthesis: Application to Aβ(1−42)

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Abstract: The β-amyloid(1−42) sequence has long been recognized as a challenging target for solid-phase peptide synthesis. We found that the known disaggregating role of Met-35 sulfoxide could be capitalized during stepwise solid-phase assembly of the Aβ(1−42) peptide chain to mitigate on-resin peptide chain aggregation, a presumed major source of synthetic difficulties. Furthemore, we demonstrate a hitherto-unreported on-resin reduction of the sulfoxide "aggregation protecting group" to allow for standard cleavage protocols, obviating a separate solution-phase sulfoxide reduction step.

The past several years have seen significant inroads toward an understanding of the molecular etiology of Alzheimer's Disease (AD). The β-amyloid (Aβ) (1−40) and (1−42) peptide isoforms are the primary constituents of the neurotoxic soluble[1−6] and insoluble[7−11] oligomers, respectively, that have been correlated with the progression of AD. A considerable amount of effort has therefore been focused on biophysical and pathological studies related to these and other Aβ isoforms. Recent evidence that the Aβ sequence plays a role in redox catalysis[12−15] and may coprecipitate in the form of metal-chelated clusters[14,16−18] has given new impetus to the investigation of the structure−activity relationships that underlie the true mechanism(s) of Aβ toxicity.

Reproducible synthetic access to full-length Aβ peptides and variants thereof incorporating various sequence modifications[9,19,20] and photoreactive probes[21,22] has been critical to the development of our understanding of the role of this peptide in AD research. Nevertheless, progress in this area has been hampered by the fact that few laboratories are equipped with the infrastructure and/or synthetic experience to prepare, on a routine basis, high-quality Aβ analogues for biophyiscal studies and neuropathological experiments. Our own recent efforts to develop conformationally defined Aβ peptide haptens were thwarted by the difficult synthesis and purification of the requisite Aβ peptide analogues. We therefore revisited the synthesis of the native Aβ(1−42) sequence to devise an improved synthetic route for the synthesis of the Aβ analogues required in our work.

In the Aβ sequence, the Met-35 residue has attracted considerable attention for a variety of reasons. While it has recently sparked interest for its newly identified role in redox catalysis,[13,15,20] it has been extensively studied for its potent effect on the solution-phase conformation and oligomerization kinetics of the full-length Aβ sequence.[23−26] Oxidation of the Met-35 side-chain thioether to the sulfoxide suppresses Aβ oligomerization by disruption of local hydrophobic and distant electrostatic interactions.[23−26] As the presence of higher-order structure in solution has long been correlated with "difficult sequences" during chain assembly on the solid phase,[27] we surmised that incorporation of Met-35 during chain assembly as the preformed sulfoxide derivative might improve the quality of the crude synthetic product, with the proviso that reduction of the sulfoxide moiety can subsequently be performed cleanly and quantitatively.

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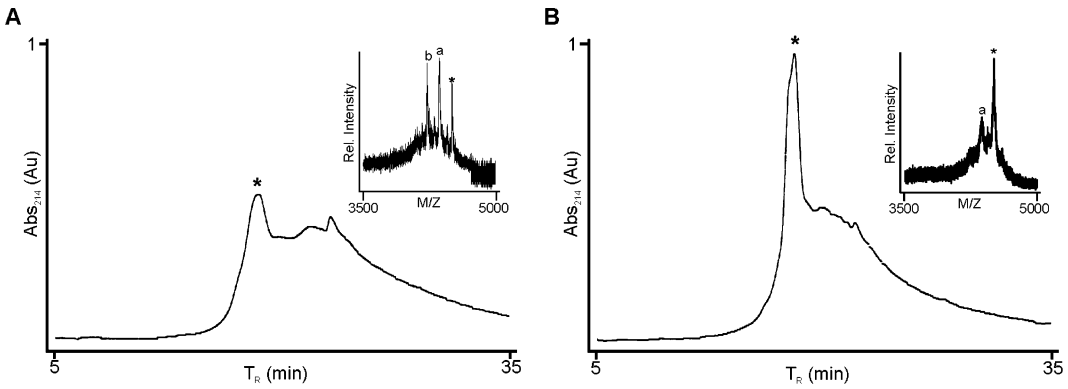

FIGURE 1. Analytical RP-HPLC and MALDI-TOF MS analyses of crude synthetic products following syntheses A (**A**) and B (**B**). Asterisk (*) denotes the desired full-length Aβ(1−42) product at 19.3 min; (a) and (b) in mass spectra denote deletion products corresponding to mono- and di-Phe deletions.

Furthermore, the use of Met-35 intentionally incorporated as the sulfoxide derivative allowed for the use of DMSO as a cosolvent during couplings (as the spectre of inadvertent Met oxidation during chain assembly is no longer an issue), which we presumed would have an additional disaggregating effect.

The advantages of DMSO as a valuable disaggregating solvent in SPPS have been known for some time, both as a neat coupling solvent and mixed with polar (e.g., DMF, NMP) and nonpolar (e.g., THF, toluene) cosolvents.[28−32] In addition, DMSO is a powerful cosolvent for the dissolution of poorly soluble amino acid derivatives and reagents. Although we employed a 0.5 M HBTU solution in both syntheses to allow for valid comparisons to be made, we found that a 3:1 DMF/DMSO (v/v) solution dissolved HBTU to a concentration of 0.6 M, as opposed to the 0.5 M saturation limit in DMF. Of additional practical importance, the 3:1 DMF/DMSO (v/v) solution rapidly dissolved even the normally stubbornly soluble amino acid derivatives such as Boc-Gln-OH and Boc-Arg(Tos)-OH, a key consideration for the preparation of dry-loaded amino acid cartridges on automated peptide synthesis equipment.

Despite the well-known benefits of DMSO in the disruption of resin-bound secondary stucture, its use in neat or cosolvent form for coupling reactions is contraindicated for most SPPS applications because of its known potential to oxidize thioether moieties in the resin-bound peptide chain at each repetitive cycle of chain elongation.[28,29] It must be emphasized that the problem of thioether oxidation is not confined to the Met side chain. As it is standard practice in both Boc and Fmoc SPPS to protect the sulfhydryl side chain of Cys as a substituted aryl thioether (*p*-methylbenzyl for Boc, trityl for Fmoc), oxidation of this functionality is an additional deleterious side reaction, as each (per cysteine residue) resulting racemic *S*-alkyl-*S*-aryl sulfoxide is stable to standard acidolytic cleavage/deprotection conditions and will therefore contaminate the cleaved crude product.[33,34] Thus, DMSO has found sparing application in SPPS as a result of due caution given that the majority of synthetic peptide targets contain Met and/or Cys residues.

We performed two syntheses in parallel using manual batchwise in situ neutralization protocols for Boc chemistry essentially as previously described.[35] Both syntheses employed the same batches of amino acids, resin, and other reagents, and all procedures from the initial linker loading step through cleavage workup were performed identically. The difference between these syntheses is as follows: in synthesis **A**, Met-35 was incorporated with the native thioether side chain and all couplings were performed with 0.5 M HBTU in DMF; in synthesis **B**, Met(O)-35 was incorporated as the preformed sulfoxide and the coupling milieu was exchanged for 3:1 DMF/DMSO (v/v). At the conclusion of both syntheses, the His-N^{im}-Dnp protections were removed, after which the sulfoxide moiety in the peptide-resin from synthesis **B** was reduced by a 15-min batchwise treatment with a cocktail of 5:5:90 (v/v) $SiCl_4$/anisole/TFA. Following standard HF cleavage and workup protocols, the crude lyophilizates obtained from both syntheses were inspected by analytical RP-HPLC (Figure 1).

The chromatogram obtained from synthesis **A** (Figure 1, panel A) is typical of the syntheses in our laboratory of Aβ(1−42) and analogues thereof; while RP-HPLC purification of the desired full-length product is possible, this is unusually difficult and mandates that preparative RP-HPLC runs be performed at a minimum of 50 °C. Specifically, deletion products corresponding to single (M^{1+} 4367.9 theoretical, 4368.6 observed) and double (M^{1+} 4220.7 theoretical, 4221.5 observed) phenylalanine deletions were found to coelute with the full-length Aβ(1−42) sequence (M^{1+} 4514.1 theretical, 4515.4 observed). In contrast, the combination of Met(O)-35 and a DMSO/DMF coupling cocktail in synthesis **B** afforded a marked improvement in product purity, which increased from 61.0% to 81.5% based on the area of the full-length product peak eluting at 19.3 min (Figure 1, panel B). This

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improvement in product purity was reflected in the yield of isolated, purified product, which increased from 11% (27 mg) to 18% (45 mg) based on theoretical peptide-resin loading per 400 mg HF cleavage. From a practical standpoint, RP-HPLC purification of the desired full-length product from the protocols developed in synthesis **B** is substantially less tedious and does not require the use of heated RP-HPLC equipment. The gains obtained by this procedure are now being employed in our laboratory for the routine preparation of Aβ analogues as well as other large peptides.

Clearly, the two differences in syntheses **A** and **B**—the use of Met-35-sulfoxide and the addition of 25% DMSO (v/v) to the coupling cocktail—beg the question of whether the enhanced quality of the synthetic product stems from DMSO disaggregation or the use of sulfoxide-protected Met. We believe that this is a somewhat moot point, as these two measures are symbiotically employed together; while Met(O) may be employed using a traditional DMF coupling cocktail, there is no reason to do so, given the myriad benefits of a mixed DMSO/DMF solvent system. At the same time, the use of DMSO can only be safely recommended when sulfoxides of both intentional and unintentional origin will be reduced in a single step after chain assembly.

Initially, we used the low-high HF cleavage method[36] to reduce Met(O)-35 in concert with the final side-chain deprotection/cleavage step; however, the time demands of the first phase of this two-step procedure motivated us to explore a more time-efficient alternative. Previous experience in our laboratory with the safety-catch amide linker (SCAL) pointed to the use of $SiCl_4$ in TFA for global reduction of sulfoxide moieties (of both intentional and unintentional origin) in the resin-bound peptide.[37,38] To minimize the potential for side reactions at the His-N^{im}-DNP protecting groups, we performed this reduction step after removal of the N^{im}-DNP and N-terminal Boc groups using a single batchwise treatment of the SCAL cleavage cocktail, which we reasoned would leave the highly acid-stable 4-(carboxyamidomethyl)benzyl ester peptide-resin anchorage intact. Standard HF cleavage of the reduced resin-bound peptide afforded material of a quality comparable to that obtained following the low-high HF cleavage method (data not shown).

In conclusion, the use of a biologically inspired sulfoxide protection strategy encompassing a side-chain protecting group and coupling solvent designed to suppress aggregation of the resin-bound peptide chain is recommended for the synthesis of Aβ isoforms and analogues and has potentially broad applicability in the synthesis of similar large, structure-ridden peptide targets. Of fundamental practical importance in this strategy is the use of a clean, quantitative, and practical method for reduction of sulfoxide moieties in the resin-bound peptide, both intentionally incorporated and unintentionally created upon repetitive exposure to DMSO during each coupling cycle.

Experimental Section

A Representative SPPS Cycle. The full-length human Aβ-(1−42) sequence (H_2N-DAEFRHDSGYEVHHQKLVFFAEDVG-SNKGAIIGLMVGGVVIA-COOH) was prepared using manual batchwise protocols for Boc SPPS which were modified from the procedure of Schnolzer et al.[35] Couplings were performed using 2.2 mmol of the appropriate N^{α}-Boc amino acid, 2.0 mmol of HBTU (0.5 M in the coupling solvent), and 5.8 mmol (1 mL) of DIEA. After preactivation for 90 s, the coupling cocktail was added to the drained resin bearing an N^{α}-trifluoroacetate salt. Couplings proceeded for 15 min with stirring, after which time a qualitative ninhydrin test was performed. The resin was then flow-washed with DMF (2 × 20 s) and treated with TFA (1 × 90 s), after which time it was flow washed with DMF (2 × 20 s) prior to the subsequent coupling.

On-Resin Sulfoxide Reduction. After removal of His N^{im}-Dnp protections by thiolysis in 1:2:7 (v/v) β-mercaptoethanol/Et_3N/DMF (3 × 10 min), the N^{α}-Boc-protected peptide resin was treated for 15 min in 5:5:90 (v/v) $SiCl_4$/anisole/TFA in a sealed vessel with shaking. The resin was then washed with CH_2Cl_2 and DMF (2 × 20 s each), neutralized with 1:9 Et_3N/DMF (5 min), washed with DMF and CH_2Cl_2 (2 × 20 s each), and dried in vacuo overnight prior to HF cleavage/deprotection.

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Supporting Information Available: Detailed resin loading and cleavage procedures and RP-HPLC conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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