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Solid-Phase Synthesis

2-Methoxy-4-methylsulfinylbenzyl: A Backbone Amide Safety-Catch Protecting Group for the Synthesis and Purification of Difficult Peptide Sequences

Marta Paradís-Bas,[a, b] Judit Tulla-Puche,*[a, b] and Fernando Albericio*[a, b, c, d]

Abstract: The use of 2-methoxy-4-methylsulfinylbenzyl (Mmsb) as a new backbone amide-protecting group that acts as a safety-catch structure is proposed. Mmsb, which is stable during the elongation of the sequence and trifluoroacetic acid-mediated cleavage from the resin, improves the synthetic process as well as the properties of the quasi-unprotected peptide. Mmsb offers the possibility of purifying and characterizing complex peptide sequences, and renders the target peptide after NH_4I/TFA treatment and subsequent ether precipitation to remove the cleaved Mmsb moiety. First, the "difficult peptide" sequence H-$(Ala)_{10}$-NH_2 was selected as a model to optimize the new protecting group strategy. Second, the complex, bioactive Ac-$(RADA)_4$-NH_2 sequence was chosen to validate this methodology. The improvements in solid-phase peptide synthesis combined with the enhanced solubility of the quasi-unprotected peptides, as compared with standard sequences, made it possible to obtain purified Ac-$(RADA)_4$-NH_2. To extend the scope of the approach, the challenging Aβ(1-42) peptide was synthesized and purified in a similar manner. The proposed Mmsb strategy opens up the possibility of synthesizing other challenging small proteins.

Introduction

Solid-phase peptide synthesis (SPPS) is nowadays the most common strategy used to synthesize biopolymers in high yield and purity. This approach has been made possible thanks to great efforts of the scientific community to develop a wide range of functionalized resins,[1] coupling reagents,[2–5] and orthogonal protecting groups.[6,7] After extensive fine-tuning over the last 50 years, one would assume that chemists would have all the tools necessary to prepare a short sequence of any nature. However, this is not the case. The so-called "difficult sequences", which are short sequences that are apparently unreachable targets,[8–11] continue to pose a challenge.

These kinds of peptides are characterized by incomplete removal of the amino protecting group and poor incorporation of the protected amino acids at some stage of the synthesis, which prevents peptide elongation even after repeating reaction treatment. It is accepted that these difficulties are due to the formation of peptide secondary structure, such as β-sheets.[12,13] During the synthesis, these intra- or intermolecular interactions, which are usually sequence-dependent, spontaneously form aggregations. These assemblies are attributed to the formation of the backbone amide hydrogen bond with carbonyl groups of peptide chains also bound to the resin.

Self-interactions require the presence of the NH of the peptide bond, therefore, Pro-containing peptide sequences are relatively free of this problem. In this regard, Mutter described a revolutionary concept in protecting group strategy: the pseudoproline. This concept converts Ser, Thr, and Cys into their five-membered ring dimethyl derivatives, thi/oxazolidines, which, during the final global deprotection, render the native residues.[14,15] Following the same idea of masking the presence of the NH moiety, Kiso, Carpino, and Beyermann independently proposed the synthesis of Ser and Thr depsipeptides, which are more manageable (soluble building blocks) and can be converted into the corresponding peptides in basic medium.[16–19]

Parallel to the work of Mutter, Sheppard and co-workers put forward the use of an amide-protecting group, *N*-(2-hydroxy-4-methoxybenzyl) (Hmb).[20,21] However, during activation, the building block N^{α}-Fmoc-N^{α}-(Hmb)amino acid forms a 4,5-dihydro-8-methoxy-1,4-benzoxapin-2(3*H*)-one, which shows poor reactivity.[22] Thus, these residues should be incorporated in the

[a] *M. Paradís-Bas, Dr. J. Tulla-Puche, Prof. F. Albericio*
Institute for Research in Biomedicine (IRB Barcelona)
Baldiri Reixac 10, Barcelona, 08028 (Spain)
Fax: (+34) 93-4037126
E-mail: judit.tulla@irbbarcelona.org
albericio@irbbarcelona.org

[b] *M. Paradís-Bas, Dr. J. Tulla-Puche, Prof. F. Albericio*
CIBER-BBN, Networking Centre on Bioengineering
Biomaterials and Nanomedicine, Barcelona Science Park
Baldiri Reixac 10, Barcelona, 08028 (Spain)

[c] *Prof. F. Albericio*
Department of Organic Chemistry, University of Barcelona
Martí i Franquès 1-11, Barcelona, 08028 (Spain)

[d] *Prof. F. Albericio*
School of Chemistry & Physics, University of Kwazulu-Natal
University Road, Westville, X54001, Durban, 4001 (South Africa)

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form of *O*,*N*-bis(Fmoc) derivatives. Once these building blocks have been introduced, and after removal of both Fmoc groups, the coupling of the incoming amino acid is facilitated through a seven-membered ring O→N transacylation mechanism. In addition, the same authors proposed the use of a so-called "reversible backbone amide" protecting group, *N*-(3-methylsulfinyl-4-methoxy-6-hydroxybenzyl) (SiMB). In addition to the free hydroxyl group, SiMB contains an electron-withdrawing sulfoxide substituent *para* to the OH, with the unique purpose of enhancing the acylation step.[23] Global deprotection is carried out through a reductive acidolysis to convert the sulfoxide into the thioether and then allow the amide protecting group to be cleaved by trifluoroacetic acid (TFA). Similarly, the so-called "reversible" backbone protecting groups based on the nitro group, which is cleavable under photolysis, were proposed by Kent and co-workers.[24]

During recent years, our group has focused on the synthesis of these "difficult sequences". In this regard, we have used dimethoxybenzyl (Dmb) and developed 1-methyl-3-indolylmethyl (MIM) and 3,4-ethylenedioxy-2-thenyl (EDOTn)[25] to reduce intra- and inter-chain interaction, as well as prevent side reactions associated with the presence of the NH group, such as aspartimides[26–28] and internal diketopiperazine formation.[29,30]

The design of the last two protecting groups mentioned has shown some advantages over benzyl derivatives. However, we have focused our efforts on a more global solution for the synthesis and on the characterization and purification of complex sequences that is not limited to Ser/Thr/Cys but valid for a large number of residues. Here we describe a "safety-catch" backbone amide-protecting group[31] that is compatible not only with the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, but also with TFA treatment, with the latter being used to detach the peptide from the resin and remove the classical side-chain protecting groups. The synthesis of a quasi-protected peptide, which still bears the safety-catch protecting group, facilitates the characterization and purification of the peptide, and allows the target free-peptide to be rendered after a final and clean chemical treatment.

The Fmoc SPPS strategy is based on the use of a temporary protecting group that is removed by a base (piperidine) and cleavage upon global deprotection (by TFA), therefore, we chose not introduce any extra chemical reaction. We envisaged that the pair alkylsulfoxide/thioether would serve our requirements. Thus, the electron-withdrawing sulfoxide group introduces stability to a benzyl moiety, and it can be easily reduced to the corresponding electron-donating thioether, which confers acid lability to the same benzyl group. Specifically, 2-methoxy-4-methylsulfinylbenzyl (Mmsb), which is stable to TFA, was reduced to 2-methoxy-4-methylthiobenzyl (Mmtb), which is labile to TFA, thus perfectly matching our requirements (Figure 1).

The Mmsb moiety has been used for the preparation of the corresponding carbamate-based amine protection and integrated as a linker for the preparation of peptide acids.[32] The demethoxy (*p*-methylsulfinylbenzyl) version was also used as a carboxyl protecting group[33] and as a linker,[34] and its corresponding carbamate-based amine protection.[35] More

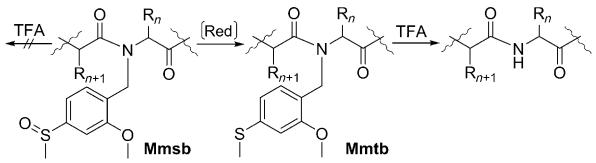


Figure 1. The concept of the new backbone amide-protecting group (Mmsb).

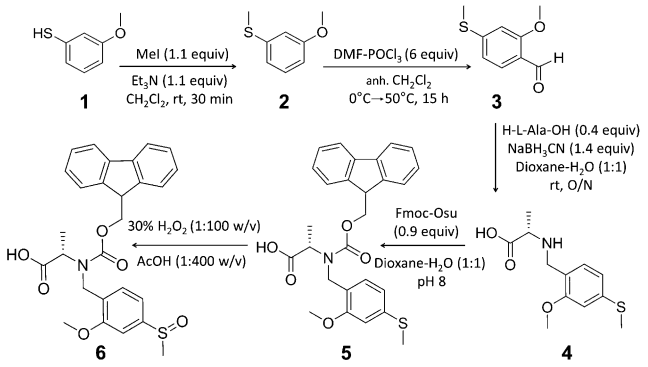
sophisticated linkers based on the same principle have also been used for SPPS.[36]

Here, we used the Mmsb group to prepare three "difficult sequences". After TFA global deprotection, the Mmsb-containing peptides were characterized and purified, when necessary, before performing a reductive TFA treatment, which reduces the sulfoxide to thioether and concomitantly removes it in a one-pot reaction.

First, the H-$(Ala)_{10}$-NH_2 sequence was selected as a model to study and optimize the new synthetic methodology proposed herein. Second, two interesting bioactive peptides, Ac-$(RADA)_4$-NH_2 (RADA-16) and Aβ(1-42), both with a high tendency to self-assemble, were synthesized for the validation of the Mmsb strategy, established in the first model. The first sequence is a challenging peptide for SPPS because of its hydrophobicity and its high tendency to form secondary structures that jeopardize the deprotection of the amino function and also acylation.[37] This peptide has been widely used to assay novel synthetic approaches.[38,39] The second peptide, RADA-16, is of interest because of its biomedical applications,[40,41] but it is again associated with a complex synthesis.[42] In this case, aggregation is related to its high content of alternating positively and negatively charged amino acids. Finally, we addressed the synthesis of the molecule considered to be responsible for amyloid formation in Alzheimer's disease, the extensively studied Aβ(1-42).[43,44] The synthesis of this peptide is challenging[18,45,46] because this molecule shows a strong tendency to form aggregates, which directly affects the elongation sequence and, at the same time, its solubility, thus complicating its purification.

Results and Discussion

To introduce the new Mmsb backbone amide-protecting group into the peptide sequence, the corresponding Fmoc derivative (Fmoc-*N*(Mmsb)-Ala-OH in our case) was prepared. The synthesis of this building block was accomplished in five steps (Scheme 1, see the Supporting Information for details).[47] Commercially available 3-methoxythiophenol (**1**) was methylated in practically quantitative yield with iodomethane (MeI) by careful dropwise addition of triethylamine to prevent dialkylation. Next, alkylated product **2** was formylated by reaction with freshly prepared Vilsmeier reagent,[48] which rendered the two possible isomers. 2-Methoxy-4-methylthiobenzaldehyde (**3**) was isolated (48% yield) after purification by reverse-phase chromatography on a C18 column. The reductive amination of aldehyde **3** with the amine of the unprotected alanine was reached in a one-step reaction with $NaBH_3CN$ in dioxane/H_2O (1:1).[25] *N*(Mmtb)-Ala-OH (**4**) was protected with the Fmoc



Scheme 1. Synthesis of Fmoc-*N*(Mmsb)-Ala-OH. O/N = overnight.

group by using a slight excess of Fmoc-OSu in basic media to afford Fmoc-*N*(Mmtb)-Ala-OH (**5**), which was easily purified by reverse-phase chromatography on a C18 column to remove the excess of unreacted alanine during the reductive amination step. Finally, oxidation of **5** with H_2O_2 rendered Fmoc-*N*(Mmsb)-Ala-OH (**6**) in excellent purity (98.0%) and without further purification; this building block was used directly for SPPS. The overall yield to convert **1** into **6** was 19%.

First, we tested our synthetic strategy on H-$(Ala)_{10}$-NH_2. As a control, the standard peptide was also synthesized with only the use of Fmoc-Ala-OH (**7**; Figure 2). Two modified H-$(Ala)_{10}$-NH_2 sequences were synthesized with one Fmoc-*N*(Mmsb)-Ala-OH at positions 6 (**8**) and 8 (**9**), respectively.

The three peptides were synthesized on Rink amide-polystyrene (PS) as a solid support and *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure)[49] as coupling reagent. In this regard, Fmoc-*N*(Mmsb)-Ala-OH was introduced with only 1.5 equiv (AA/coupling reagent), and the best conditions for the key incorporation of the incoming residue consisted of three couplings with Fmoc-Ala-OH (10 equiv), DIPCDI (10 equiv), and OxymaPure (10 equiv) at 45 °C, with MeCN/*N*,*N*-dimethylformamide (DMF) (3:1) as solvent. Once again, we observed that MeCN is a suitable solvent for incorporations onto hindered amino acids.[50]

Except for the coupling of the incoming Ala on the *N*-Mmsb residue, no double couplings were performed. However, the three peptide elongations were checked by the Kaiser test[51] after each coupling. The tests revealed that synthesis of the standard peptide took place with incomplete incorporations of the last four Ala residues.

The two modified Mmsb-containing peptides were cleaved from the resin by using TFA/CH_2Cl_2 (19:1), rendering both sequences with similar purity (Figures S3 and S4 in the Supporting Information), and the total stability of the Mmsb group was confirmed by MS analysis (*m*/*z* calcd for $C_{39}H_{63}N_{11}O_{12}S$: 909.44; found: 932.60 $[M+Na]^+$ (**8**) and 932.45 $[M+Na]^+$ (**9**)).

Finally, we used MS analysis to study the reductive acidolysis of the Mmsb-containing peptides caused by treatment with NH_4I/TFA (Figure 3 and Figure S12 in the Supporting Information). It was found that the Mmsb group was completely removed after 30 min in the case of **8** and after 2 h in the case of **9**. This deprotection was accomplished without the need for scavengers (some articles report the use of Me_2S)[23,32].

One proposed solution to prevent the main impurity detected in both modified Mmsb-containing peptide syntheses (9% in **8** and 6% in **9**) associated with the truncated peptide after coupling of the *N*-Mmsb residue involved preparing the dipeptide (in this case Fmoc-Ala-*N*(Mmsb)-Ala-OH) in solution (see the Supporting Information for details). This strategy was used

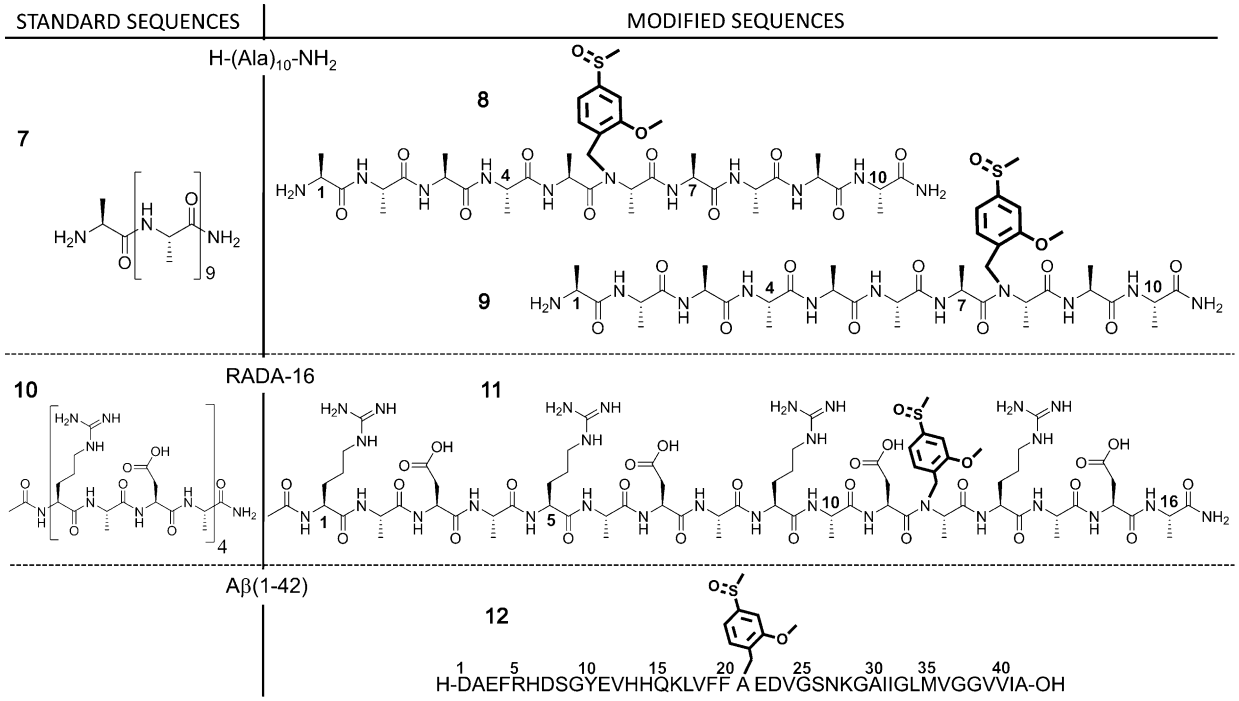


Figure 2. Structures of the synthesized peptides.

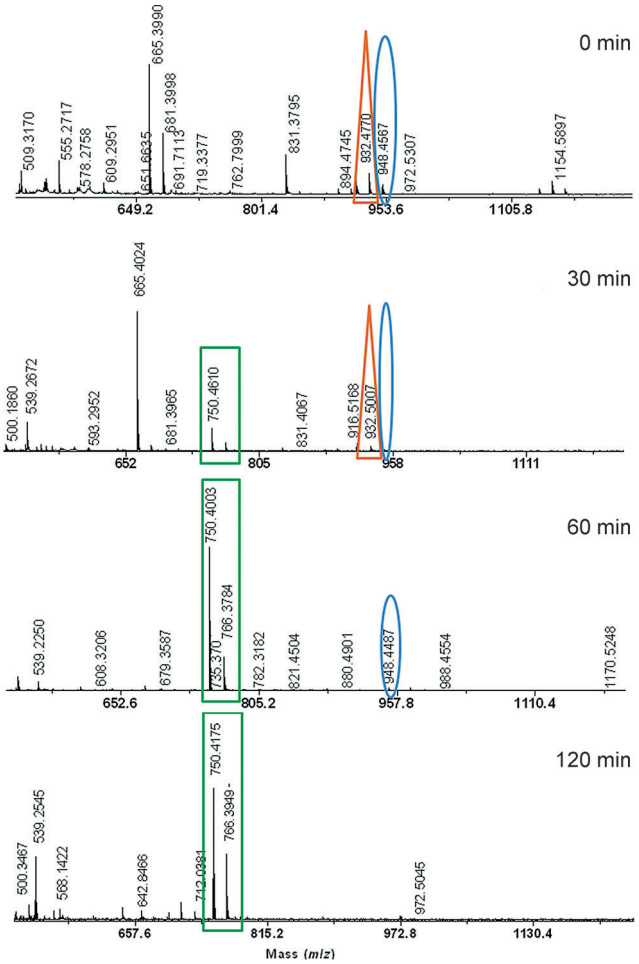


Figure 3. Monitoring of the Mmsb group removal by MALDI-TOF MS spectra. Starting material (Mmsb-protected H-(Ala)$_{10}$-NH$_2$) **8** is highlighted in blue, whereas the reaction intermediate (Mmtb-protected H-(Ala)$_{10}$-NH$_2$) and final product (unprotected H-(Ala)$_{10}$-NH$_2$) are shown in orange and green, respectively.

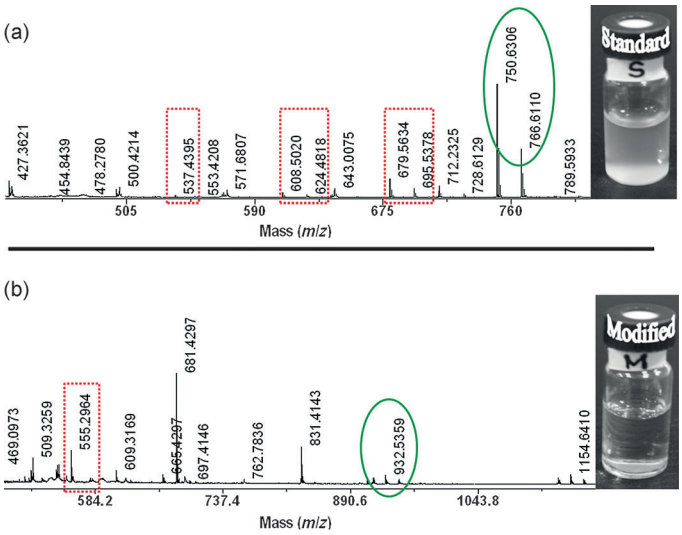


Figure 4. MALDI-TOF MS and solubility assays of H-(Ala)$_{10}$-NH$_2$ crude peptides synthesized: a) by using the common Ala, **7**, and b) by using the modified Fmoc-*N*(Mmsb)-Ala-OH, **8**. Concentration: 1 mg mL^{-1} in H$_2$O. Mass impurities assigned (red label), mass expected peptide (green label).

for the synthesis of modified H-(Ala)$_{10}$-NH$_2$ **8**, which showed an increased purity and the absence of the mentioned impurity (Figure S8 in the Supporting Information), thus confirming an approach to overcome the steric hindrance associated with the *N*(Mmsb) residue. The main conclusion drawn from these analyses is that the influence of the backbone protection has an effect that is apparent even six amino acids further down the backbone. Furthermore, in these two syntheses (**8** and **9**), no peptides lacking Ala residues were detected (Figure 4b and Figure S4b in the Supporting Information). The standard peptide synthesized with common Ala (**7**) showed four sequences lacking Ala residues, as shown by MS (Figure 4a); therefore, these results are consistent with those obtained from the qualitative Kaiser test. In addition, the increase in solubility of the modified sequences in common peptide solvents (H$_2$O or H$_2$O/MeCN, 1:1) was extraordinary (Figure 4b).

In addition to HPLC and MS analysis of standard and modified H-(Ala)$_{10}$-NH$_2$, and by means of circular dichroism (CD), we explored the effect on secondary structure when one Mmsb unit was introduced into the peptide sequence (Figure 5). The CD spectrum of the standard H-(Ala)$_{10}$-NH$_2$ peptide **7** showed a β-sheet structure profile (minimum at 215 nm), characteristic of self-assembling sequences, whereas the two modified peptides (**8** and **9**) showed a random coil structure (minimum at 200 nm). These data suggested the involvement of the Mmsb in the secondary structure of this peptide, possibly explaining the synthetic improvements observed in modified H-(Ala)$_{10}$-NH$_2$.

Encouraged by the results obtained in this peptide model using the new method, we explored the application of Mmsb to other "difficult peptides".

We recently reported that an acceptable quality of RADA-16 can be obtained only by coupling two protected octapeptides in solution.[42] In that study, we showed that neither the protected nor unprotected RADA-16 can be purified by chromatography. We learned that the difficulty arises after the incorporation of 4–5 amino acids. Thus, two syntheses, a standard

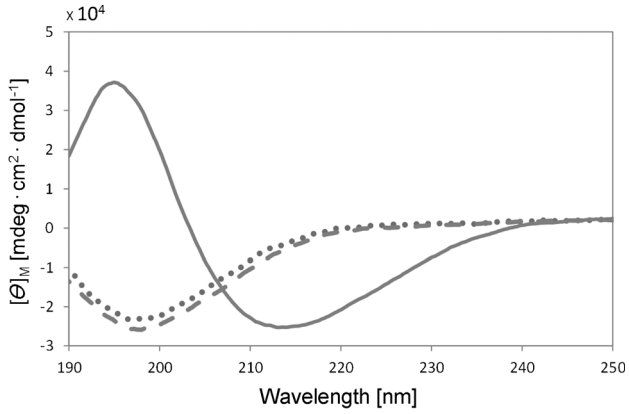


Figure 5. CD spectrum of a 25 μM solution of synthesized H-(Ala)$_{10}$-NH$_2$ in H$_2$O/2,2,2-trifluoroethanol (TFE) (9:1) at pH 5.2 of standard sequence **7** (solid curve) and modified sequences **8** and **9** (dotted and dashed curves, respectively).

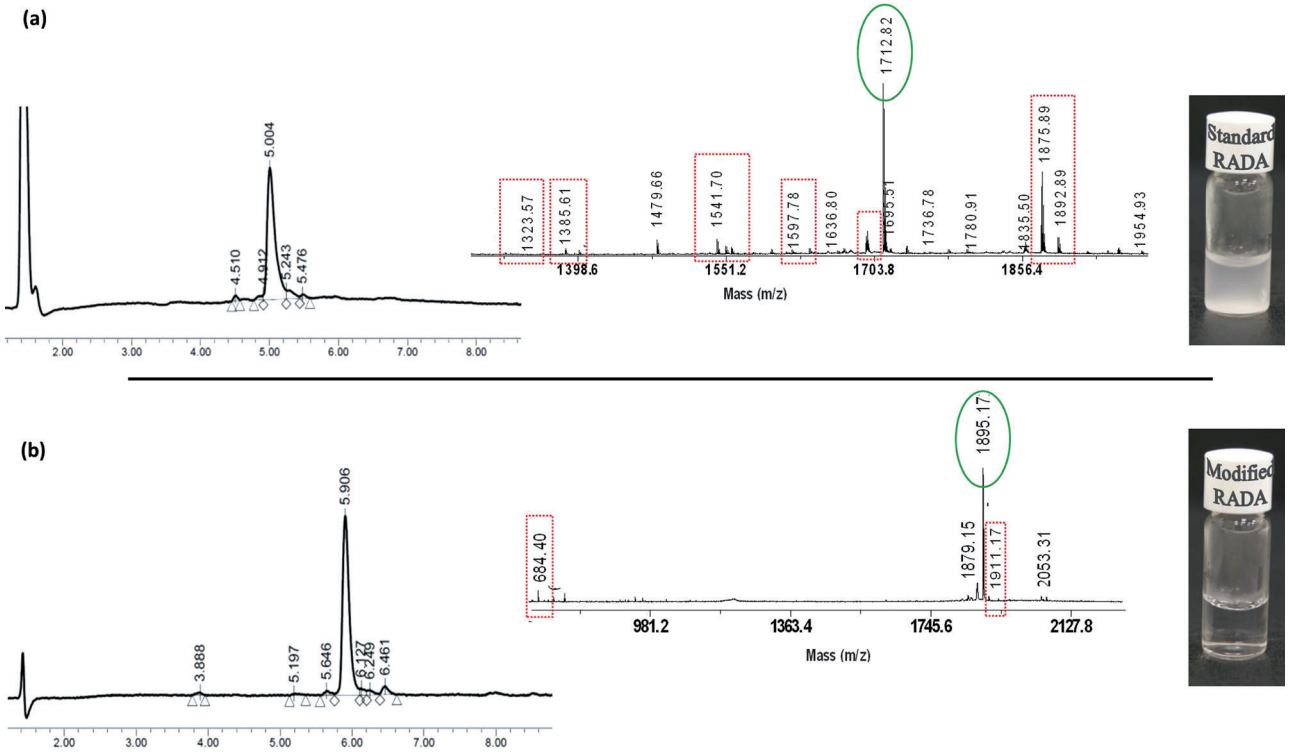


Figure 6. HPLC/MALDI-TOF MS/solubility assays of crude RADA-16 peptides synthesized: a) by using the common amino acids, and b) by using the modified Fmoc-*N*(Mmsb)-Ala-OH in position 12. Concentration: 1 mg mL^{-1} in H_2O. Mass impurities assigned (red label); mass expected peptide (green label).

RADA-16 (**10**) and a modified version (**11**), were carried out on Rink amide-ChemMatrix resin,[52] by using DIPCDI/OxymaPure as coupling reagent. On the modified peptide, Fmoc-*N*(Mmsb)-Ala-OH was introduced in position 12 (**11**). Peptide elongations were again checked qualitatively by the Kaiser test. Although no double couplings were performed, the synthesis of the standard RADA-16 showed, by Kaiser test, incomplete couplings in the last four residues. Fmoc-*N*(Mmsb)-Ala-OH was introduced as in the modified H-(Ala)$_{10}$-NH$_2$ examples. The coupling of the incoming Fmoc-Asp(*t*Bu)-OH was studied (Table 1 in the Supporting Information), concluding that the confluence of a MeCN/DMF (3:1) solvent mixture and three couplings at 45 °C using DIPCDI and OxymaPure, was once again crucial for satisfactory introduction of the incoming residue.

The final global deprotection/cleavage required prolonged acid treatment because of the high content of Pbf side-chain protecting groups of Arg [4 h with TFA/triisopropylsilane (TIS)/H_2O (38:1:1)]. Under these more demanding conditions, the acid-stability of the Mmsb group was confirmed. Although no significant differences between the two RADA-16 peptides were detected by HPLC (Figure 6), the MALDI-TOF MS analysis of these compounds revealed that standard RADA-16 contained several deletions (Figure 6a and Figure S2 in the Supporint Information; already anticipated by the Kaiser test results during synthesis), whereas the modified RADA-16 had none. Careful study of the HPLC and MS results concluded that the modified RADA-16 showed less than 1% of the impurity of

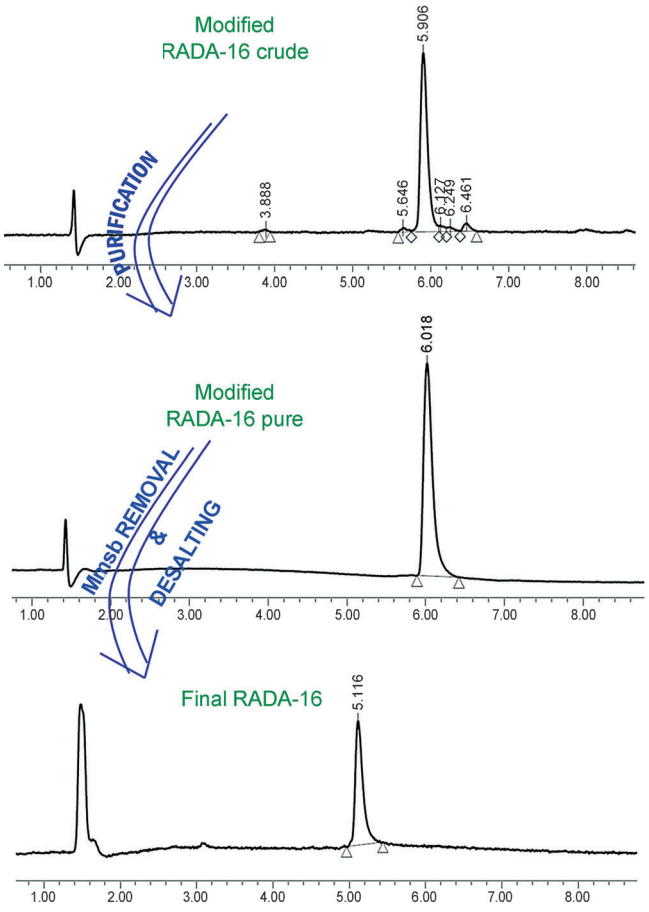


Figure 7. HPLC chromatograms of modified RADA-16 peptide: top) after peptide synthesis, middle) after purification, and bottom) final peptide after removing Mmsb group followed by desalting.

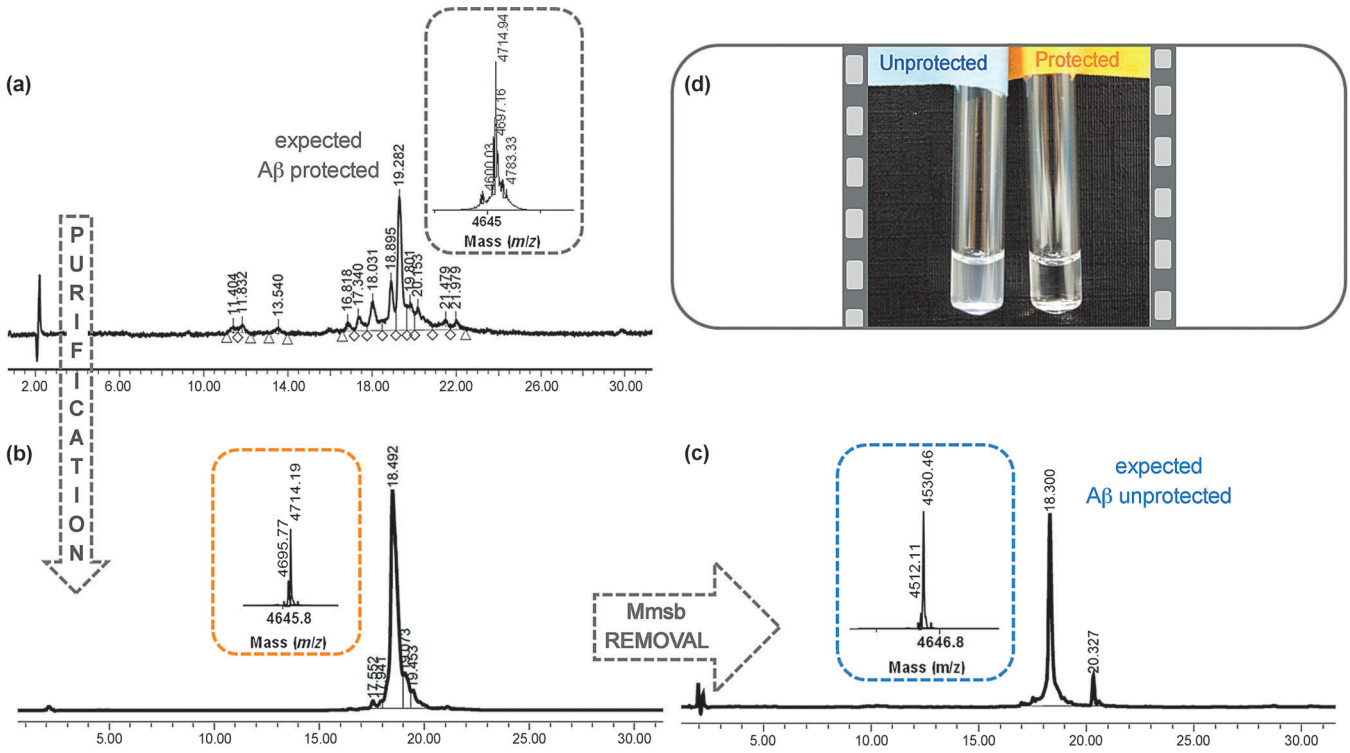


Figure 8. Analysis and characterization of synthesized modified Aβ(1-42): HPLC and MALDI-TOF MS of a) crude Mmsb-protected peptide after synthesis, b) purified Mmsb-protected peptide, c) unprotected peptide after Mmsb removal, and d) solubility of Aβ(1-42) at 1 mg mL^{-1} in H_2O of the Mmsb-protected analogue (orange label) and final unprotected Aβ(1-42) (blue label).

truncated peptide (5-mer, uncompleted incorporation of Asp after coupling of modified *N*(Mmsb) residue; Figure 6b and Figure S5 in the Supporting Information).[53] Most importantly, the solubility of the modified RADA-16 crude peptide in water was significantly higher than that of the standard peptide (Figure 6).

The lower solubility of the standard RADA-16, combined with the poor HPLC resolution of the expected peptide, for which the impurities eluted close to the desired product, prevented its purification. In contrast, the greater solubility of Mmsb-containing RADA-16 allowed its isolation. The final modified peptide was purified by HPLC analysis on a reverse-phase C18 column, and the Mmsb-protected peptide was isolated in excellent purity (99.9%) (*m/z* calcd for $C_{75}H_{123}N_{29}O_{27}S$: 1893.89, found: 1894.69 $[M+H]^+$; Figure 7 and Figure S9 in the Supporting Information). To remove the Mmsb group, the purified peptide was treated with a concentration of 1 mg mL^{-1} NH_4I/TFA and the backbone protecting group was completely removed after 45 min, with only a final desalting step (see the Supporting Information for details) required to reach the pure target RADA-16 peptide (Figure 7 and Figure S14 in the Supporting Information).

Inspired by the possibility of purifying RADA-16, we moved onto a second level, extending the application of the Mmsb backbone-protecting group to the Aβ(1-42) sequence. This amyloidogenic peptide was produced on an aminomethyl resin with a previous incorporation of 3-(4-hydroxymethylphenoxy)propionic acid as a linker in an automatic synthesizer with non-optimized conditions. In the middle of the sequence assembly (**12**, ^{21}AA), we manually inserted Fmoc-*N*(Mmsb)-Ala-OH, followed by the incoming Fmoc-Phe-OH. Both amino acids were coupled by following the same conditions described previously for other modified peptides. Once the elongation of the peptide was complete, the peptidyl resin was treated with TFA/TIS/H_2O (38:1:1) for 2 h, again confirming the acid-stability of Mmsb. Although the crude peptide obtained showed poor purity (35%; Figure 8a and Figures S6 and S7 in the Supporting Information) by HPLC analysis on a C4 column, our principal aim was to enhance the solubility of Aβ(1-42) and facilitate its purification.

The Mmsb-containing Aβ(1-42) was isolated by HPLC using the analogous semi-preparative C4 column. The enhanced solubility of the modified peptide (Figure 8d) allowed its purification (Figure 8b, and Figures S10 and S11 in the Supporting Information). Mmsb removal was completed after 2 h, checked by HPLC and MS (MALDI-TOF and HRMS-ESI), with both techniques confirming the synthesis of the natural Aβ(1-42) in 90% purity and with the expected mass (HRMS-ESI *m/z* calcd for $C_{212}H_{321}N_{55}O_{62}S_2$: 4693.3098, found: 1129.3273 $[M+4H]^+/4$, 903.6643 $[M+5H]^+/5$ and 753.3872 $[M+6H]^+/6$; Figure 8c and Figures S15 and S16 in the Supporting Information).

Conclusion

Mmsb is a new backbone amide-protecting group that is used in a safety-catch strategy. This new group is stable during global deprotection conditions. Mmsb represents a new concept for the synthesis and purification of "difficult peptides". Its

presence in a peptide confers several advantages over a standard synthesis. Thus, the elongation of the peptide sequence takes place smoothly and with better yields because of the disruption of the secondary structure. Furthermore, its use could prevent side-reactions, such as aspartimide and diketopiperazine formation, while facilitating others, such as cyclization. We have demonstrated that the presence of only one Mmsb molecule favors the incorporation of at least 10 amino acids in the case of H-$(Ala)_{10}$-NH_2 and RADA-16. In addition, the Mmsb-protected peptides are much more soluble than standard peptides, thus allowing better HPLC resolution and, most importantly, their purification. The improved solubility could be explained by the absence of secondary structure and also by the polarity of the sulfoxide. The strategy described here could be applied to any amino acid other than Ala. Preparation in solution of dipeptides (equivalent to pseudoprolines or depsipeptides) is advisable when using Mmsb-hindered amino acids, so as to facilitate incorporation in the peptide sequence. The removal of Mmsb takes place under conditions that do not damage the peptide and allows the desired peptide to be obtained after only one filtration. Notably, our results on the synthesis of Aβ(1-42), for which only one Mmsb group was introduced within 42 AA residues, support the proposed synthetic approach. The results show that this novel synthetic technique enhances the manipulation of the peptide, especially with regard to its solubility and purification. We envisage that this technology will have broad use not only for the synthesis of "difficult peptides", but also for the purification of a large range of other peptides.

Experimental Section

Synthesis of standard peptide sequences

H-$(Ala)_{10}$-NH_2 (**7**) and RADA-16 (**10**) peptides were synthesized on Rink-Amide AM Polystyrene resin (250 mg, 0.12 mmol, 0.48 mmol g^{-1}) and Rink-Amide AM ChemMatrix resin (100 mg, 0.053 mmol, 0.53 mmol g^{-1}), respectively. Polystyrene resin was conditioned by washing with DMF (3×1 min) and CH_2Cl_2 (3×1 min), and ChemMatrix resin was conditioned by initial washing with TFA/CH_2Cl_2 (1:99) (5×1 min), CH_2Cl_2 (5×1 min), DIEA/CH_2Cl_2 (1:19) (5×1 min) and CH_2Cl_2 (5×1 min). Commercial amino acids were coupled in both synthesis as follow: Fmoc-L-AA(PG)-OH (3 equiv), DIPCDI (3 equiv), and OxymaPure (3 equiv) in DMF, with a 5 min preactivation and with a total coupling time of 1 h. After every coupling, the resin was washed with DMF (3×1 min) and CH_2Cl_2 (3×1 min). Then, a Kaiser test was carried out to check which amino acid was not coupled completely [4 AA: Ala^1-Ala^4 in the case of H-$(Ala)_{10}$-NH_2 (**7**); and 4 AA: Ala^9, Ala^6, Ala^4, Arg^1 in the case of RADA-16 (**10**)]. No double couplings were performed independently of the test results. Next, the Fmoc removal was performed with piperidine/DMF (1:4) (25 mL g^{-1} resin, 1×1 min, 2×5 min), followed by resin washings with DMF (3×1 min), CH_2Cl_2 (3×1 min), and DMF (3×1 min). The cycle of AA coupling/Fmoc removal was performed until complete elongation of peptides, including the last Fmoc removal. In the case of RADA-16 (**10**), a final step of acetylation was accomplished by Ac_2O (10 equiv) with DIEA (10 equiv) in DMF for 30 min. Final resin washings were carried out with DMF (3×1 min) and CH_2Cl_2 (3×1 min). The peptides were subjected to cleavage treatment depending on the amino acid side chain-protecting groups: TFA/CH_2Cl_2 (19:1) for 1 h (H-$(Ala)_{10}$-NH_2, **7**) and TFA/TIS/H_2O (38:1:1) for 4 h (RADA-16, **10**). The mixture was partially evaporated under reduced pressure and the peptides were precipitated with cold diethyl ether. The liquid layer was removed by centrifugation and the solid was washed with cold diethyl ether to give white solids that were dissolved in H_2O/MeCN (1:1) and lyophilized. The crude peptides were analyzed by HPLC and MALDI-TOF MS (see Figures S1 and S2 in the Supporting Information).

Synthesis of modified peptide sequences

The modified peptide sequences [two analogues modified H-$(Ala)_{10}$-NH_2: one in position 6 (**8**), the second in position 8 (**9**); and the modified Ac-$(RADA)_4$-NH_2 in position 12 (**11**)] were synthesized on the same resins described in case of each standard peptide. The resin washings, commercial amino acid couplings, and Fmoc removal cycles were performed by following the same protocol described for the standard sequences synthetic methodologies. The Kaiser test was used to detect which amino acid was not completely coupled (neither in **8**, **9**, nor **11** were uncompleted couplings detected). No double couplings were performed except in the case of the incoming amino acid to H-*N*(Mmsb)-Ala-OH. The synthesis of modified Aβ(1–42) (**12**) was carried out by an automatic microwave peptide synthesizer (Discover, CEM corporation). The synthesis was performed with an Aminomethyl ChemMatrix resin (160 mg, 0.1 mmol, 0.62 mmol g^{-1}) with a previous incorporation of 3-(4-hydroxymethylphenoxy)-propionic acid as a linker, coupled with the same system described in standard sequences. Commercial and common Fmoc-L-AA(PG)-OH amino acids were used. The coupling system consisted of *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate (HBTU) with *N*,*N*-diisopropylethylamine (DIEA) and DMF as a solvent and the Fmoc removal was performed with piperidine/DMF (1:4).

Incorporation of the synthesized building block Fmoc-*N*(Mmsb)-Ala-OH

After Fmoc removal of the previous amino acid, the Fmoc-*N*(Mmsb)-Ala-OH (1.5 equiv) was coupled by using DIPCDI (1.5 equiv) and OxymaPure (1.5 equiv) in DMF for 1 h. Then, washings with DMF (3×1 min) and CH_2Cl_2 (3×1 min) were performed and Kaiser test confirmed the complete incorporation of the AA.

Incorporation of the incoming Fmoc-AA-OH to H-*N*(Mmsb)-Ala-OH

Modified RADA-16 (**11**) was selected to find the best coupling conditions to introduce the incoming amino acid (see Table S1 in the Supporting Information) and the extension of this reaction was evaluated by HPLC analysis of the crude peptide after an aliquot of peptidyl-resin was treated with a cleavage cocktail. The solvent was then evaporated and the peptide was precipitated with cold diethyl ether. Amino acid coupling consecutive to H-*N*(Mmsb)-Ala-OH was accomplished by following the same conditions used in all modified peptide sequences, at 45 °C for 2 h in DMF/MeCN (1:2), with the coupling reagent system DIPCDI (10 equiv) and OxymaPure (10 equiv) for Fmoc-Phe-OH (10 equiv). A total of three consecutive couplings (using the same coupling conditions) were performed.

Cleavage/global deprotection of side-chain protecting groups of modified peptides

After all the peptide elongation, cleavage, and post-cleavage treatments were performed by following specified conditions depending on the amino acid side chain-protecting groups nature, the peptides were subjected to cleavage treatment TFA/CH_2Cl_2 (19:1) for 1 h (**8** and **9**), TFA/TIS/H_2O (95:2.5:2.5) for 4 h (**11**) and TFA/TIS/H_2O (95:2.5:2.5) for 2 h (**12**). The mixture was partially evaporated under reduced pressure and the peptides were precipitated with cold diethyl ether. The liquid layer was removed by centrifugation and the solid was washed with cold diethyl ether to give white solids that were dissolved in H_2O/MeCN (1:1) and lyophilized to afford the modified sequences. The crude peptides were analyzed by HPLC and MALDI-TOF MS. Peptides were characterized by HPLC and MALDI-TOF MS (see Figures S3–S7 in the Supporting Information).

Removal of the Mmsb amide protecting group

Lyophilized modified crude peptides **8**, **9**, **11**, and **12** (1–5 mg) were dissolved in neat TFA (concentration 1 mg mL^{-1}) in a round-bottomed flask with constant stirring at RT. Ammonium iodine (30 equiv) was then added to the reaction mixture and the reaction was monitored by HPLC (an aliquot was taken from the reaction mixture, the TFA was removed by N_2 (g), and the peptide was precipitated in diethyl ether and redissolved in H_2O/MeCN (1:1)). When MALDI-TOF MS analysis revealed the completion of the reaction, removal of the iodine byproduct was carried out by filtration of the reaction mixture. The majority of volatiles were removed under reduced pressure and the precipitation of the peptide was performed by transferring the peptide solution dropwise to cold diethyl ether. After three times centrifugation and diethyl ether washings, the solid was lyophilized and the peptides were analyzed by MALDI-TOF MS (see Figures S12–16 in the Supporting Information).

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