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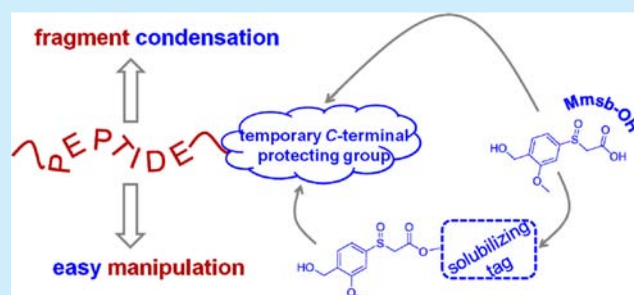
Semipermanent *C*-Terminal Carboxylic Acid Protecting Group: Application to Solubilizing Peptides and Fragment Condensation

Marta Paradís-Bas,[†,‡] Judit Tulla-Puche,*[,†,‡] and Fernando Albericio*[,†,‡,§,∥,⊥]

[†]Institute for Research in Biomedicine (IRB Barcelona) Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain
[‡]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain
[§]Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain
[∥]School of Chemistry & Physics, University of Kwazulu-Natal, Durban 4001, South Africa
[⊥]School of Chemistry, Yachay Tech, Yachay City of Knowledge, Urcuquí 100119, Ecuador

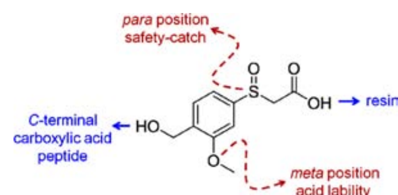
Supporting Information

ABSTRACT: The 2-methoxy-4-methylsulfinylbenzyl alcohol (Mmsb-OH) safety-catch linker has been described as a useful tool to overcome two obstacles in peptide synthesis: the solubility and fragment condensation of peptides. The incorporation of the linker into an insoluble peptide target, thereby allowing the conjugation of a poly-Lys as a "solubilizing tag", notably enhanced the solubility of the peptide. The selective conditions that remove that linker favored its incorporation as a semipermanent *C*-terminal protecting group, thereby allowing fragment condensation of peptides.



Peptides with a large number of nonpolar amino acids, self-assembling tendency, and highly structured or merely difficult sequences remain a challenge in terms of synthesis. One of the proposed strategies used to attain the synthesis of such molecules tackles the common limitation of insufficient solubility. In recent years, several synthetic modes aimed to enhance solubility have been described. Most are focused on the disruption of β-sheet interactions, which is achieved by using mainly backbone amide protecting groups, such as pseudoprolines[1] or others based on substituted benzyl group[2−4] or the depsipeptides grounded on the *O*−*N* intramolecular acyl migration strategies,[5] among others. Expensive presynthesized building blocks or the limitation of purification conditions associated with these strategies highlight the need to explore new tools to facilitate such syntheses. Although less studied, another peptide approach to increase solubility consists of the incorporation of a temporary *C*-terminal cationic peptide fragment attached to the native sequence by a cleavable linker, known as a "solubilizing tag". Although this kind of method has been used in the field of recombinant proteins for years,[6−8] only a few examples have been described for peptides.[9,10] There is currently no robust synthetic tool to address the increasing demand for peptide targets with inherent insolubility. In order to open the spectrum of applications in this area, and taking advantage of our previous experience in enhancing peptide solubility,[11] herein we sought to exploit the 2-methoxy-4-methylsulfinylbenzyl alcohol (Mmsb-OH) linker[12] (Scheme 1) as a *C*-terminal semipermanent protecting group.

Scheme 1. Mmsb-OH Linker Structure



Previous studies initiated by Englebretsen and Alewood[9] proposed the (Gly-Arg)$_4$ motif as a "solubilizing tag" linked to a sequence by the glycolic acid, and later Englebretsen[13] again, followed by Wade,[14] described a five Lys sequence to solubilize peptides linked through the base-labile 4-hydroxymethyl-benzoic acid (HMBA) linker. Brimble[15] also used that last linker connected to a six Arg "solubilizing tag". However, the use of those linkers can lead to the formation of undesired aspartimide side products, which are favored by the basic cleavage conditions. To circumvent this issue and to increase the solubility of a given peptide, here we attached the sequence to a six Lys mer, as a "solubilizing tag", through the Mmsb-OH linker (Scheme 2). The peculiarity of this linker is that, in addition to being compatible with Boc/Bzl and Fmoc/*t*Bu solid phase peptide synthesis (SPPS) strategies, as a safety-catch[16,17] linker its cleavage is mediated after a selective chemical modification (in that case reductive acidolysis), thereby

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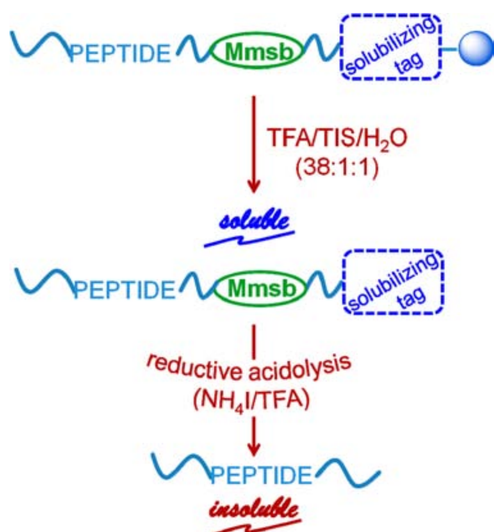
Scheme 2. Proposed Solubility Peptide Answer by Using a "Solubilizing Tag" Attached to the Mmsb-OH Linker



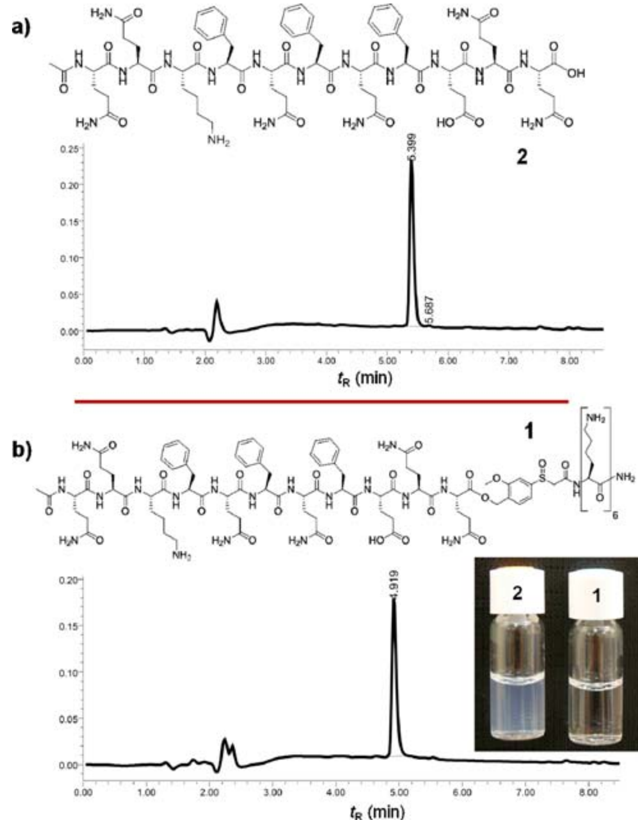
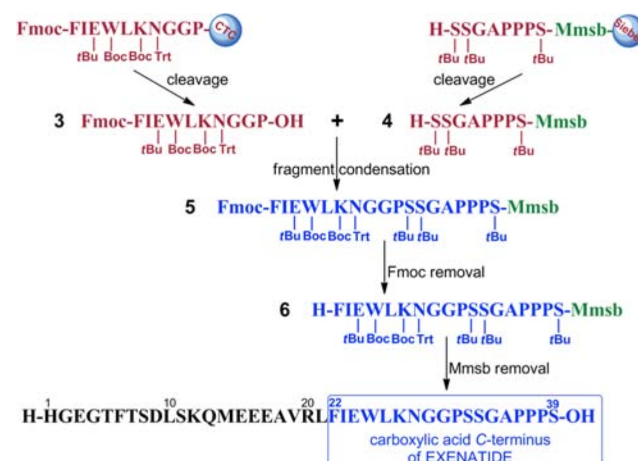


Figure 1. Structures and HPLC chromatograms, gradient from 10 to 60% B, of standard Q11 **2** (a) and solubilizing tag-linked Q11 peptide **1** (b). Solubility assays were done at 1 mg/mL in water.

Scheme 3. Synthetic Design of Fragment Condensation to Obtain the Exenatide Fragment H-(22Phe-39Ser)-OH



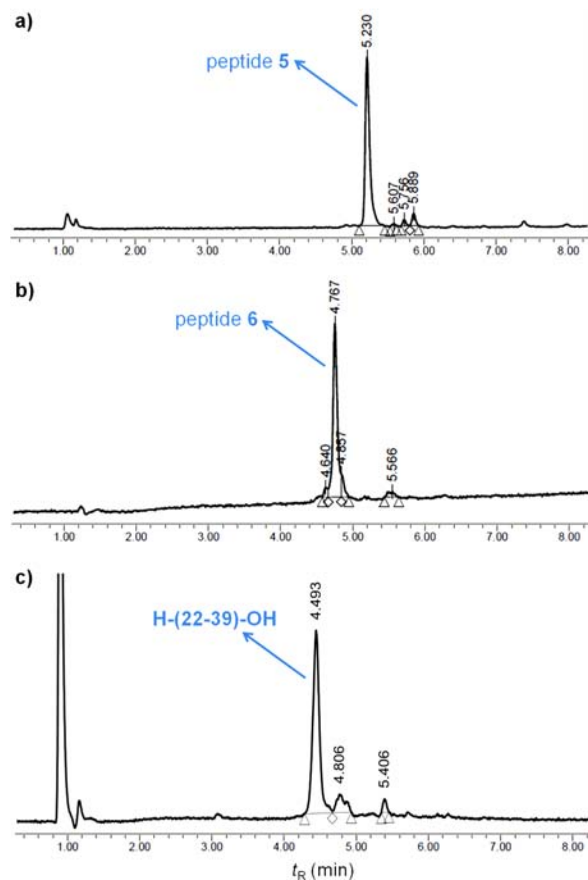


Figure 2. HPLC chromatograms of peptide crude products with unprotected side chains after fragment condensation, gradient from 30 to 80% B (a); Fmoc removal, gradient from 10 to 60% B (b); and Mmsb removal, gradient from 10 to 60% B (c).

avoiding basic conditions and consequently side reactions related to the same. Herein, we described for the first time its incorporation by the Fmoc/*t*Bu SPPS strategy. The insoluble target sequence Q11 (Ac-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2)[18] was selected to explore our proposal. This peptide is a self-assembling molecule with the capacity to form hydrogels. Extensive biomedical Q11 applications have been reported; therefore, synthetic methods to achieve the sequence are required.[19,20]

The Mmsb-OH linker was synthesized by optimizing the strategy proposed by Thennarasu and collaborators[12] (see details in the Supporting Information). Only one purification step was required, and an overall yield of 28% was obtained.

Initially, the solubilizing tag-linked Q11 peptide (**1**) was synthesized by SPPS (see details in the Supporting Information) on a Rink-amide resin, after the coupling of six Fmoc-L-Lys(Boc)-OH amino acids using OxymaPure (3 equiv)

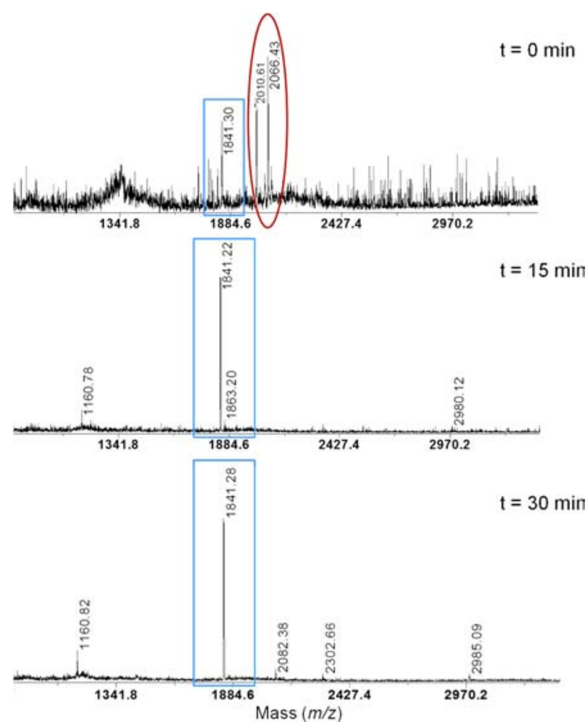


Figure 3. MS (MALDI-TOF) spectra of Mmsb removal monitored for peptide **6**. Starting material (**6**) (red) and final product H-(22−39)-OH (blue).

and DIPCDI (3 equiv) over 1 h. The Mmsb linker was introduced under mild conditions with HOSu (1.5 equiv) and DIPCDI (1.5 equiv) to avoid multiple incorporations. To complete the Q11 amino acid sequence, the same conditions used to couple the Lys were applied. After the final acetylation [by Ac_2O (5 equiv) and DIEA (5 equiv) over 30 min], the peptide was cleaved with TFA/TIS/H_2O (38:1:1) for 1 h, rendering the expected sequence with high purity (99.9%) (Figure 1b and Figure S2 in the Supporting Information), thereby confirming the stability of the Mmsb linker under the acidic cleavage conditions. Parallel to this synthesis, the carboxylic acid version of Q11, assigned as a standard (**2**), was synthesized on the same type of resin using the same coupling reagent conditions described before. After cleavage with TFA/TIS/H_2O (38:1:1) for 1 h, the peptide was precipitated with ether and lyophilized to afford the sequence, also with high purity (99.4%) (Figure 1a and Figure S1 in the Supporting Information). Although the HPLC chromatograms do not show big differences in polarity, the modified Q11 sequence (Figure 1b) showed enhanced solubility, which was attributed to the strategy used to attach the sequence to the Mmsb-$(Lys)_6$ "solubilizing tag".

In order to obtain the native Q11 from the solubilizing tag-linked Q11 peptide, the crude product was subjected to a reductive acidolysis treatment by NH_4I (30 equiv) in neat TFA (1 mg/mL) for 30 min. The ensuing MS analysis confirmed the total release of the Q11 sequence (Figure S8 in the Supporting Information) with high peptide purity (99.9%).

In addition to demonstrating the capacity of Mmsb to improve peptide properties, thereby facilitating its manipulation, and motivated by the possibility to have in our hands a potential *C*-terminal protecting group, we wanted to contribute with those already known carboxylic protecting groups for peptide synthesis.[21] Thereby, we addressed one of the SPPS's most important applications: fragment condensation, where the Mmsb would act as a *C*-terminal protecting group. Large peptide sequences sometimes require the use of sophisticated methods to assemble the peptide fragments in solution in order to reach the final target. One of the most common strategies for this purpose is to prepare peptide fragments for ensuing coupling in solution.[22] The need for an appropriate combination of protecting groups is crucial, and in this regard, the Mmsb-OH, as a carboxylic protecting group, is highly suitable for this strategy. We selected the well-known peptide Exenatide to test the conjugation. This compound is of relevance because of its use in diabetes therapy.[23] Specifically, the synthesized sequence was the acid carboxylic version of *C*-terminal Exenatide (NH_2-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-OH), which was assembled from two presynthesized peptide fragments (Scheme 3).

The separation of the fragments, in terms of retrosynthetic analysis, was chosen between ^{31}Pro and ^{32}Ser. First, Pro is a cyclic amino acid that does not allow racemization during fragment condensation; second, we took advantage of preceding literature regarding Exenatide synthesis.[24] Initially, peptide fragments Fmoc-(22−31)-OH (**3**) and H-(32−39)-Mmsb (**4**) were synthesized separately by the *t*Bu/Fmoc SPPS strategy on CTC[25] and Sieber[26] resin (see Supporting Information for details), respectively. Both resins allow synthesis of peptides with fully protected side chains. After cleavage with a reduced amount of TFA (1% in the case of CTC and 3% in the case of Sieber), the two peptides were lyophilized and analyzed by HPLC and MS, both techniques confirming the expected sequences were properly protected (see Figures S3 and S4 in the Supporting Information). Fragment condensation of the two peptides was performed using H-(32−39)-Mmsb (1.5 equiv) and Fmoc-(22−31)-OH (1 equiv) coupled to PyBOP (1 equiv) and HOBt (1 equiv) at pH 8−9 in DMF. The reaction was stopped at 7 h. After lyophilizing the crude product (**5**), the expected peptide was obtained with 92% HPLC purity (Figure 2a and Figure S5 in the Supporting Information).

Fmoc was removed with diethylamine to facilitate the analysis, rendering the expected protected sequence (**6**) with 91% HPLC purity (Figure 2b and Figure S6 in the Supporting Information). Finally, in a one-pot reaction by reductive acidolysis, the side chains and the Mmsb *C*-terminal protecting groups were removed (Figure 3) to afford the expected H-(22−39)-OH peptide fragment with 85% HPLC purity (Figure 2c and Figure S9 in the Supporting Information). In this case, because of the presence of Trp (an amino acid that is known to be susceptible to alkylation), diisopropyl sulfide was added as a scavenger.[27] Again, the total stability of Mmsb during fragment condensation and also its application in convergent strategies to attain large peptide sequences were demonstrated.

In summary, we synthesized the Mmsb-OH linker in a straightforward manner in five steps and only one purification step. The linker was later incorporated into a peptide sequence by SPPS, the first time applied in the Fmoc/*t*Bu strategy. Mmsb-OH has been exploited in two important aspects focused on the development of new tools for peptide synthesis. First, Mmsb-OH serves as a tool to enhance the solubility of peptides, such as Q11, through the incorporation of a "solubilizing tag". Second, based on the carboxylic acid protection, the linker has allowed the synthesis of a large fragment of Exenatide by fragment condensation. This

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achievement opens the possibility of using the Mmsb linker strategy to synthesize other peptides with difficult sequences.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR characterization of Mmsb synthesis, HPLC and MS peptides characterization, and solubility data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: judit.tulla@irbbarcelona.org.
*E-mail: albericio@irbbarcelona.org.

Notes

The authors declare no competing financial interest.

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