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2-Methoxy-4-methylsulfinylbenzyl Alcohol as a Safety-Catch Linker for the Fmoc/tBu Solid-Phase Peptide Synthesis Strategy

K. P. Nandhini, Fernando Albericio,* and Beatriz G. de la Torre*

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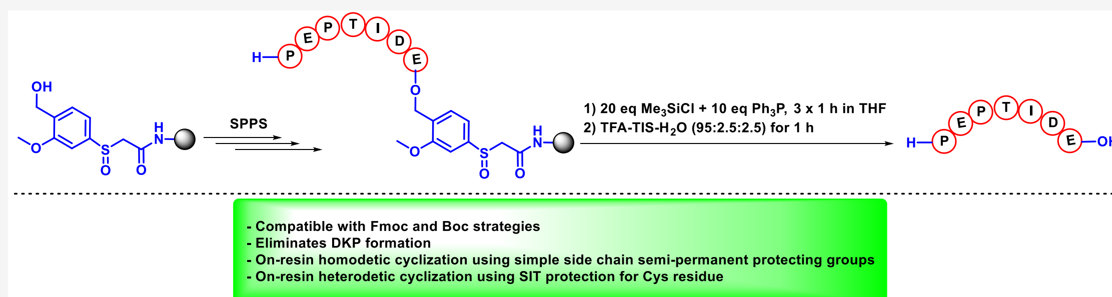

ABSTRACT: Fmoc and Boc group are the two main groups used to protect the α-amino function in Solid-Phase Peptide Synthesis (SPPS). In this regard, the use of the Mmsb linker allows the combination of these two groups. Peptide-O-Mmsb-Resin is stable to the piperidine and trifluoroacetic acid (TFA) treatment used to remove Fmoc and Boc, respectively. The peptide is detached in a two-step protocol, namely reduction of the sulfoxide to the sulfide with Me_3SiCl and Ph_3P, and then treatment with TFA. The advantage of this strategy has been demonstrated by the following: preparation of peptide with no diketopiperazine formation in sequences prone to this side reaction; on-resin cyclization without the concourse of common organic reagents such as Pd(0) but of difficult use in a biological laboratory; and on-resin disulfide formation in a total side-chain unprotected peptide. The use of Mmsb linker together with Msib (4-(methylsulfinyl)benzyl) and Msbh (4,4′-bis(methylsulfinyl)benzhydryl) described in the accompanying manuscript add a fourth dimension to the SPPS protecting group scheme.

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■ INTRODUCTION

Peptides are key biomolecules for the pharmaceutical industry.[1] In this context, more than 100 peptides have been approved as drugs by the US Food and Drug Administration (FDA).[2] Furthermore, peptides are the base for diagnostic tools[3] and drug delivery systems.[4] In addition, several personalized treatments with peptide-based neoantigen vaccines have been administered with great medical success.[5,6] This impressive panorama was unthinkable 50 years ago when the synthesis in solution of a short peptide for research purposes could involve several months of work and production of the same peptide could even take years. This paradigm shift has been possible thanks to the implementation of the solid-phase peptide synthesis (SPPS) strategy on the part of research laboratories and, importantly, by industry. First described by the Nobel Prize laureate R. Bruce Merrifield in the 1960s,[7] SPPS has greatly expedited the first (discovery) and the last (production) step of the drug discovery process.

Briefly, SPPS relies on the use of a solid polymeric protecting group for the C-terminal carboxyl group, and the elongation of the peptide chain is then done in the C → N direction by successive removal of the protecting group of the α-amino group and coupling of the next amino acid with both the α-amino and the side chain, which can be protected if required. At the end of the synthetic process, the peptide is detached from the resin and the side-chain protecting groups are removed, very often in a single step, referred to as global deprotection.[7] For the simplest linear peptides, two kinds of protecting groups are required, one protecting the α-amino and the other the side chains of the trifunctional residues and linking the C-terminal amino acid to the solid support. Merrifield used *tert*-butyloxycarbonyl (Boc) for the α-amino and benzyl (Bzl)-type protecting groups for the rest in the so-called Boc/Bzl strategy. Boc is an excellent protecting group because it can be efficiently and cleanly removed by solutions of trifluoroacetic acid (TFA) in a trace-less mode. In this regard, in addition to removing the Boc group, TFA strongly solvates the solid support, thereby minimizing interchain interactions, which often lead to the formation of deletion peptides.[7,8] On the other hand, Bzl-type protecting groups are not always totally stable to the TFA, and their removal and the

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cleavage of the peptide from the solid support requires the concourse of the hazardous anhyd HF, which is rarely available in research laboratories and, in addition, jeopardizes peptide production.[9] As an alternative, the so-called fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy has become the method of choice for peptide research and production purposes. Fmoc and *t*Bu [alkxoxybenzyl or trityl (Trt) for the linker) are orthogonal protecting groups that are removed by different chemical mechanisms (base for Fmoc and TFA for the rest) without the concourse of strong acids.[10]

The synthesis of more complex molecules such as cyclic and/or branched peptides requires a third level of protection, which is accomplished by Trt (removed by a low concentration of TFA), allyl (removed by Pd(O)),[11] or *p*-nitrobenzyl (removed by $SnCl_2$)[12] groups. Pd(0) and $SnCl_2$ are commonly used in organic chemistry laboratories but are more difficult to find in laboratories devoted to biology. In this regard, it would be useful to have another kind of protecting group that is stable to TFA treatment but removable by acids of moderate strength.

This idea is materialized in the "safety catch" concept, in which a stable group is chemically (or photochemically) converted to its labile form in response to its exposure to a given reagent before the cleavage reaction.[13] As described in the preceding manuscript[14] and references cited therein,[15,16] arylalkyl sulfoxides are useful entries for the development of novel "safety-catch" protecting groups. These compounds are stable to both Fmoc and Boc chemistry, but as sulfoxide groups are converted into sulfide-based ones, acidolytic cleavage occurs smoothly and with increased reaction rates as part of the electronic effects conferred by the substituent on the ring (Figure 1).

Figure 1. Structures of (methylsulfinyl)benzene-(methylthio)benzene duo for safety-catch protection.

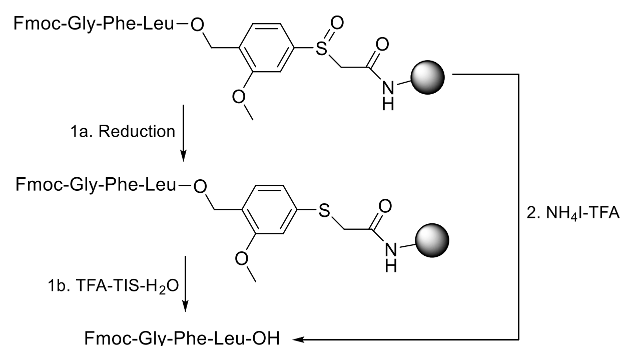
The 2-methoxy-4-methylsulfinylbenzyl alcohol (Mmsb) linker was first proposed by Thennarasu and Liu for the synthesis of peptides using Boc-amino acids.[17] At the end of the elongation, the peptide was cleaved from the resin through reductive cleavage. Patek and Lebl[18−21] described the SCAL linker (4,4′-bis(methylsulfinyl)-2-(4-carboxybutoxy)-*N*-Fmoc-benzhydrylamine), which is based on the bis(methylsulfinyl)-benzydrylamine moiety mimicking the Rink-amide linker, for the preparation of C-terminal amide peptides. A few years later, our group proposed the Mmsb linker as a semipermanent protecting group of the C-terminal carboxylic acid for connecting the desired peptide to a solubilizing tag of $(Lys)_6$.[22] This linker was specially designed for the synthesis and purification of hydrophobic peptides. The peptidic constructs are built on a regular acid-labile support, such as Rink-amide-Resin, starting with $(Lys)_6$, then the Mmsb linker, and finally the target peptide. TFA treatment of the peptide resin renders the target peptide bounde to $(Lys)_6$ through the Mmsb linker. This construct is easier to purify because it is more soluble than the target peptide due to the presence of $(Lys)_6$. Once the peptide has been purified, the Lys tag is removed from the construct by reductive acidolysis. Herein, we report on the use of the Mmsb linker bound to a solid support for the synthesis of peptides using mostly Fmoc-amino acids, but also Boc-amino acids if required, and Boc/*t*Bu side-chain protecting groups, which can be removed by TFA when the peptide is still anchored to the resin. Furthermore, we propose a two-step process to cleave the peptide from the solid support. The first step involves the reduction of the sulfinyl to the thio moiety, and the second comprises peptide cleavage with TFA in the presence of scavengers.

RESULTS AND DISCUSSION

HO-Mmsb Sulfoxide Moiety As a Linker in Fmoc/*t*Bu SPPS. The precedents in the literature, including our own work, for peptide cleavage from the HO-Mmsb linker involved only one step, namely reductive acidolysis using 30 equiv of NH_4I in 5% Me_2S-TFA.[19,23] The use of Me_2S is not pleasant, and the presence of the NH_4I in the final solution cleavage requires an extra purification step. Furthermore, we envisaged the possibility of carrying out this process in two steps, namely reduction to the thio derivative, followed by peptide cleavage by treatment with TFA, thus introducing a considerable degree of versatility to the synthetic strategy.

Of the two steps, the reduction is key for accomplishing efficient cleavage. In this regard, the reduction step was broadly studied using the tripeptide Fmoc-Gly-Phe-Leu-O-Mmsb-Resin as a model (Scheme 1).

Scheme 1. Strategy Followed for the Optimization of the Cleavage in Two Steps



The Fmoc-Gly-Phe-Leu-O-Mmsb-Resin was treated with the reducing cocktail (Table 1) and then with TFA-TIS-H_2O (95:2.5:2.5) for 1 h, and the peptide obtained was then analyzed by HPLC. Following this approach, only peptide bound to the reduced linker was cleaved. Finally, the previously treated resin was treated again with 30 equiv of NH_4I in TFA[22] (3 treatments) to ensure the reduction of the entire Mmsb linker and to cleave the remaining peptide from the resin. Comparison of the amount of Fmoc-Gly-Phe-Leu-OH obtained from the two cleavages indicates the efficiency of the reducing cocktail.

Following some of the methods described independently by Lebl and co-workers[19] and Samanen and Brandeis[24] for the reduction of various sulfoxide moieties, we first studied NH_4I (30 equiv) in AcOH, with the idea of simply reducing the sulfoxide but without concomitant cleavage of the peptide from the resin (Table 1, no. 1). Although the method seemed to work, the reduction was not complete, and the large amount of salts present hinders the practical application of this approach. Next, we used Me_3SiCl in combination with *N*-

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Table 1. Sulfoxide Reduction Conditions on Solid Phase

no.	reduction conditions	reduction of Mmsb linker[a]
1	30 equiv of NH_4I in AcOH (1 h) (×3)	+ + +
2	10 equiv of Me_3SiCl + 10 equiv of Ac-Met, 1 h, THF	+
3	10 equiv of Me_3SiCl + 10 equiv of Ac-Met, 1 h, DCM	−
4	5 equiv of Me_3SiCl + 10 equiv of Me_2S, 1 h, THF	+
5	30 equiv of Me_3SiCl + 30 equiv of Ac-Met, 1 h, THF	+
6	10 equiv of Me_3SiCl + 10 equiv of Ac-Met (40 °C), 1 h, THF	−
7	10 equiv of Me_3SiCl (30 min) + 10 equiv of Ac-Met (30 min) (x 3), THF	−
8	10 equiv of Me_3SiCl + 10 equiv of TEA (30 min) + 10 equiv of Ac-Met (30 min) (×3), THF	−
9	10 equiv of Me_3SiCl + 10 equiv of Ac-Met in AcOH (1 h)	−
10	7 equiv of AcCl + 4.5 equiv of KI in AcOH (1 h)	+[b]
11	20 equiv of Me_3SiCl + 10 equiv of Ph_3P (3 × 1 h) in THF	+ + + +
12	20 equiv of Me_3SiCl + 2 equiv Ph_3P (3 × 1 h) in THF	+ + + +
13	30 equiv of NH_4I + TFA[c]	+ + + +

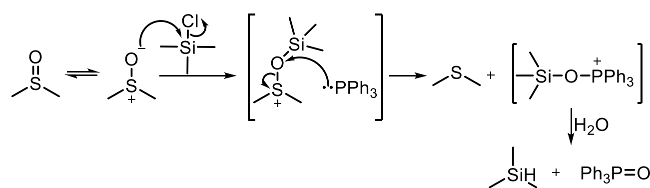
[a]+: reducing capacity. −: negligible, [b]Reduce and cleave the peptide at the same time. [c]Reduction and acidolysis.

acetyl-L-methionine (Ac-Met) (nonvolatile dialkylsulfide exempt of smell) or Me_2S. Thus, 10 equiv of Me_3SiCl and Ac-Met, each in THF (Table 1, no. 2) for 1 h rendered some of the target peptides. When DCM (Table 1, no. 3) was used instead of THF, a negligible amount of peptide was obtained. Given this finding, THF, which is a friendlier solvent than DCM, was adopted for future work. The use of Me_2S gave a low amount of a peptide accompanied by an unknown complex mixture after cleavage (Table 1, no. 4). A large excess (30 equiv) of Me_3SiCl (Table 1, no. 5) did not improve the reduction, and even a slightly higher temperature (40 °C) gave poorer results (Table 1, no. 6). Next, to favor the reaction of the reagent with the sulfoxide, Me_3SiCl was added first followed by Ac-Met after 30 min. However, the results were very poor, both in the absence and in the presence of base (triethylamine, TEA) (Table 1, nos. 7 and 8). The reaction was then tested under the same conditions but in an acid medium, yielding negative results once again (Table 1, no. 9). The use of AcCl in the presence of KI led to some reduction but without room for improvement because these conditions also caused premature cleavage of the peptide from the resin (Table 1, no. 10). Finally, satisfactory results were obtained when Ph_3P was used in THF (Table 1, no. 11). Further work has demonstrated that the amount of Ph_3P can be reduced from 10 to 2 equiv (Table 1, no. 12). As a control, the one-step protocol (reduction and acidolysis) was carried out (Table 1, no. 13).

The plausible reaction mechanism is depicted in Scheme 2. The deoxygenation of sulfoxide to the corresponding sulfide possibly acts first through the attack of Me_3SiCl forming sulfonium ion (similar to the Pummerer reaction).[25] Then the presence of Ph_3P might attack the oxygen atom in the sulfonium ion to form the corresponding sulfide plus an intermediate phosphonium ion. The phosponium ion in turn forms phosphonium oxide as a byproduct. Minimizing the amount of Ph_3P in the reduction conditions greatly minimized the byproduct.

After screening the reduction, we tested the best conditions [20 equiv Me_3SiCl + 10 equiv Ph_3P, (3 × 1 h) in THF, Table 1, no. 11] again on H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-O-Mmsb-Gly-Phe-Leu-NH-Rink-amide-Resin. The idea behind this multicleavable resin was to have an internal reference to double check that the reduction and cleavage of the target peptide H-Tyr-Gly-Gly-Phe-Leu-OH take place quantitatively and that the Mmsb linker is stable under conventional cleavage conditions (Scheme 3).

Scheme 2. Plausible Mechanism for the Deoxygenation of Sulfur Using Me_3SiCl and Ph_3P



Thus, the direct cleavage (I) of the full peptide resin with TFA−TIS−H_2O (95:2.5:2.5, 1 h) took place only through the Rink-amide linker (>99% of stability of the Mmsb linker to the TFA conditions), as indicated by LC−MS (Figure 2, I). In contrast, when exposed to the two-step protocol, namely, reduction followed by TFA cleavage (II), the same full peptide resin rendered only H-Tyr-Gly-Gly-Phe-Leu-OH (<1% of the full peptide, the HO-Mmsb-Gly-Phe-Leu-NH_2 was not observed by HPLC because presumably it did not precipitate during the workup), as shown by LC−MS (Figure 2, II).

Study of DKP Formation. The high stability of five- or six-membered ring cycles drives several side reactions in peptide synthesis.[26,27] The highly stable DKP is the smallest cyclic peptide likely to be formed. DKP is especially favored when the first or two first amino acids are Pro and/or Gly, as these are prone to induce a cis-conformation, thereby facilitating the cyclization.[28] Chirality of the two first amino acids, meaning one L and one D, also facilitates the side reaction because the corresponding DKP is more stable.[29−31] In all these cases, DKP formation is very severe and it can ruin a synthetic process. Although DKP formation can happen in a range of scenarios (see Figure 3 for some examples), it most commonly occurs during the elongation of the peptide chain on an ester-based resin [hydroxymethylbenzyl- (Merrifield, Wang) or chlorotrityl-(CT) resins].

In all cases, the cyclization step (DKP formation) takes place during the removal of the N^{α}-protecting group of the second amino acid. Although it can be catalyzed by acids, the reaction in base is much more important. In this regard, the Fmoc/*t*Bu strategy is more detrimental than the Boc/Bzl approach. In the latter, DKP formation occurs mostly during the neutralization step carried out with 5% *N*,*N*-diisopropylethylamine (DIEA) in the corresponding solvent. In this case, the side reaction can be minimized or even avoided if, after removal of the Boc group, neutralization is carried out in situ during the coupling.[36] In this case, there is competition between coupling and DKP formation, and once acylation has taken place, the side reaction cannot occur. The most efficient way to minimize DKP formation in a Fmoc/*t*Bu strategy is by using the hindered CTC resin. However, even these cases are not exempt from the side reaction. If Wang resin is used, then the second amino acid can be protected with a Trt group with an in situ neutralization coupling step,[37] or allyloxycarbonyl (Alloc) with a tandem deprotection-coupling reaction using Fmoc-aa-F as active species. These two strategies are still tedious because they require the preparation of N^{α}-protected

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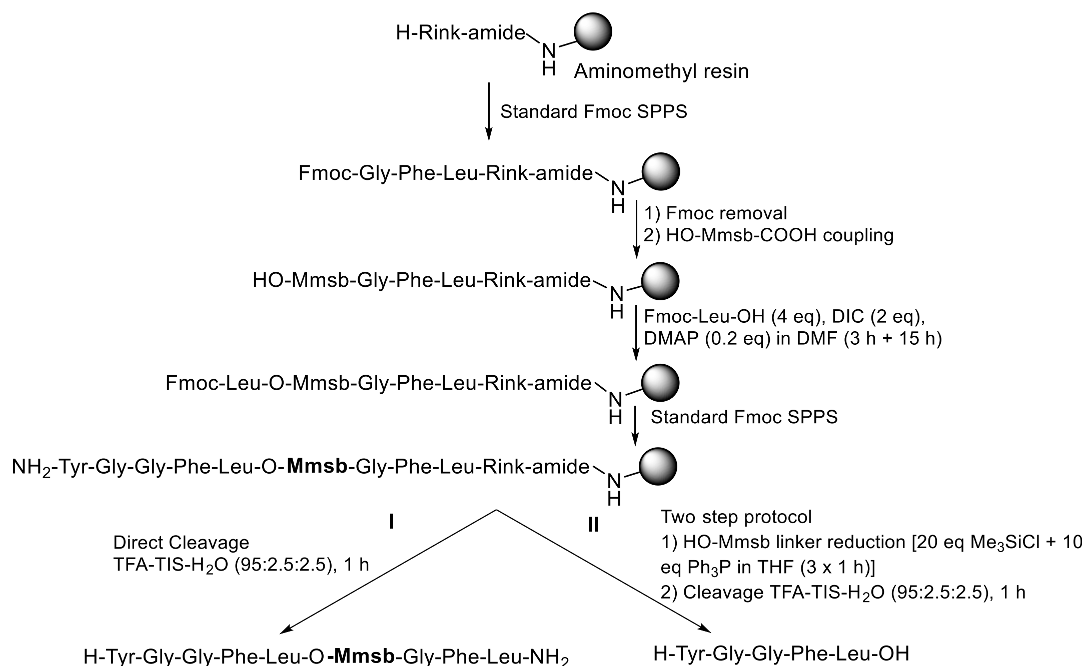
Scheme 3. Study of Stability (I) and Reduction (II) of the Mmsb Linker



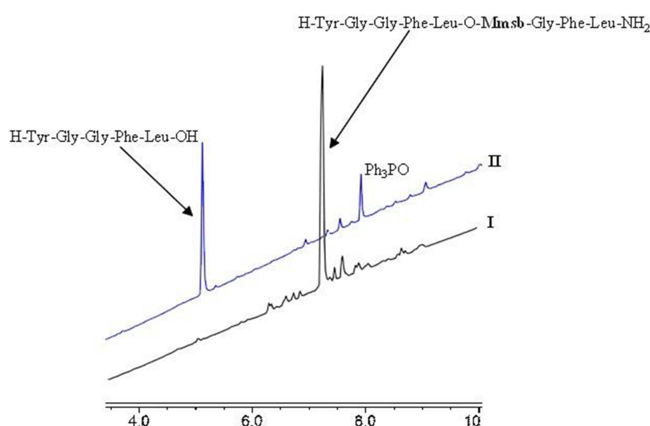


Figure 2. HPLC comparison of H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-O-Mmsb-Gly-Phe-Leu-AM Resin before and after reduction of the Mmsb linker, followed by TFA treatment. I: direct cleavage using TFA mixture. II: two-step protocol (reduction and cleavage).

amino acids with Trt and Alloc groups and also the preparation of the corresponding fluorides. The beauty of the HO-Mmsb-Resin is that it is compatible with both the Fmoc and Boc strategies. To minimize/prevent DKP formation, Boc is used to protect the second amino acid, followed by Fmoc for the first and remaining amino acids.

We studied DKP formation on the model peptide Fmoc-DVal-Pro-OH, which was prepared on HO-Mmsb-Resin. This Fmoc-dipeptide was synthesized via two strategies I and II. Strategy I involved the use of Fmoc-amino acids for the whole synthesis (Scheme 4, right), and strategy II involves the use of Boc-DVal-OH in place of Fmoc-DVal-OH as the second amino acid, followed by in situ neutralization and coupling of the next Fmoc-Phe-OH (Scheme 4, left). Analysis of the Fmoc-Phe-DVal-Pro-OH obtained from strategies I and II after reduction and cleavage of the peptide from the resin revealed that strategy I showed no indication of the desired peptide. In contrast, strategy II yielded the desired Fmoc-tripeptide. To confirm the analysis, we further proceeded to add Fmoc-Leu-OH to the Fmoc-Phe-DVal-Pro-O-Mmsb-Resins by esterification in the presence of DMAP (from strategies I and II) without the removal of the Fmoc group from the third amino acid (Phe) in the peptide chain (Scheme 4).

After the reactions shown in Scheme 4, in the case of strategy I, the analysis after the reduction and cleavage revealed the presence of the desired peptide Fmoc-Phe-DVal-Pro-OH (Figure 4, strategy I), while the strategy II yielded only Fmoc-Leu-OH (Figure 4, strategy II). The presence of only Fmoc-Leu-OH and no desired peptide on the resin (strategy II) after the addition of Fmoc-Leu-OH indicated 100% DKP formation because Fmoc-Leu-OH was incorporated to the hydroxy group of the Mmsb linker liberated during DKP formation (Figure 4, strategy II). For strategy I, Fmoc-Leu-OH was not observed after its addition, thereby confirming the presence of 100% Fmoc-Phe-DVal-Pro-OH with 0% DKP formation (Figure 4, strategy II). To conclude, the stability of the HO-Mmsb linker toward acid permitted the use of Boc-amino acids during peptide chain elongation (via HO-Mmsb-Resin), which in turn helped to prevent DKP formation in SPPS.

On-Resin Homodetic Cyclization on a Model Peptide Using *t*Bu-Based Side-Chain Protecting Groups. In nature, peptide cyclization renders more rigid structures, which is often translated into greater stability and activity.[38,39] There are two kinds of cyclic peptides, namely homodetic, where all connections are through amide/peptide bonds, and heterodetic, where other chemical functions are present [disulfide, thioether, (thio)ester]. Homodetic cyclization can be head-to-tail (N- and C-terminal amino acids), side chain-to-side chain (usually Lys and Glu/Asp residues), or a combination of both head/tail-to-side chain. In the laboratory, homodetic cyclization can be carried out in solution once the peptide has been elongated in the solid phase and after its cleavage from the resin or in the solid phase while the peptide is still anchored on resin and therefore before the cleavage.[40,41] Both strategies require protection of all chemical functions

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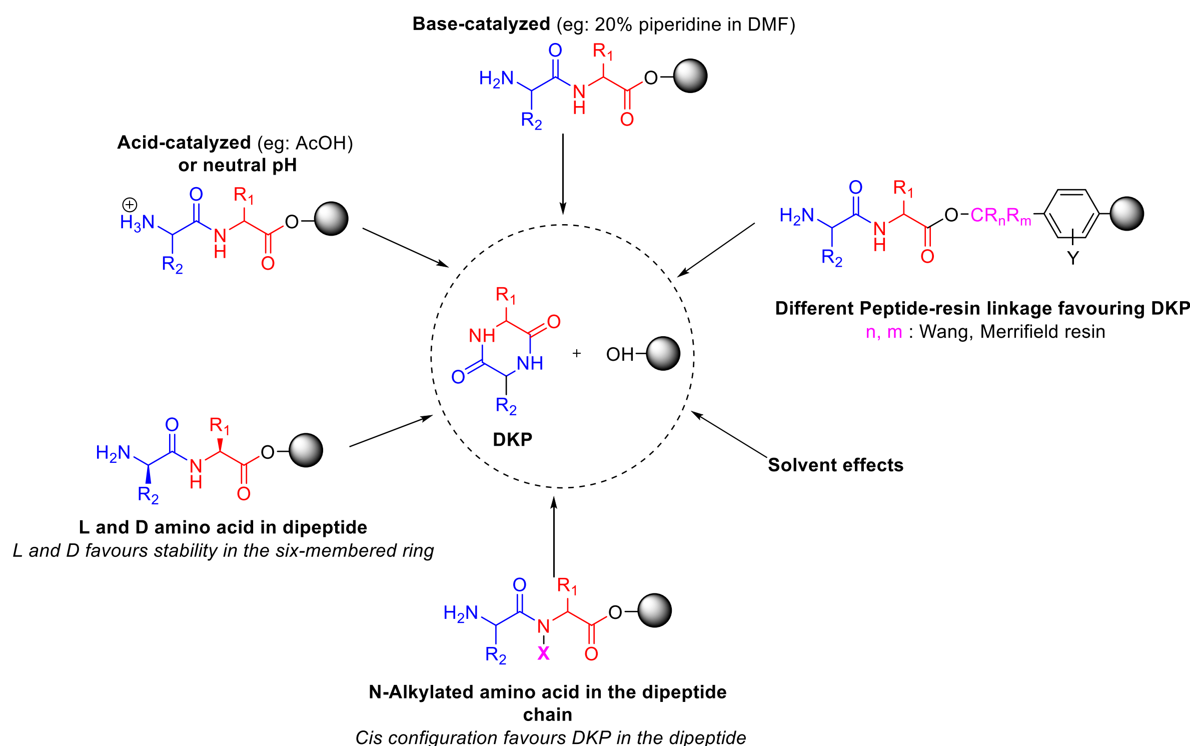
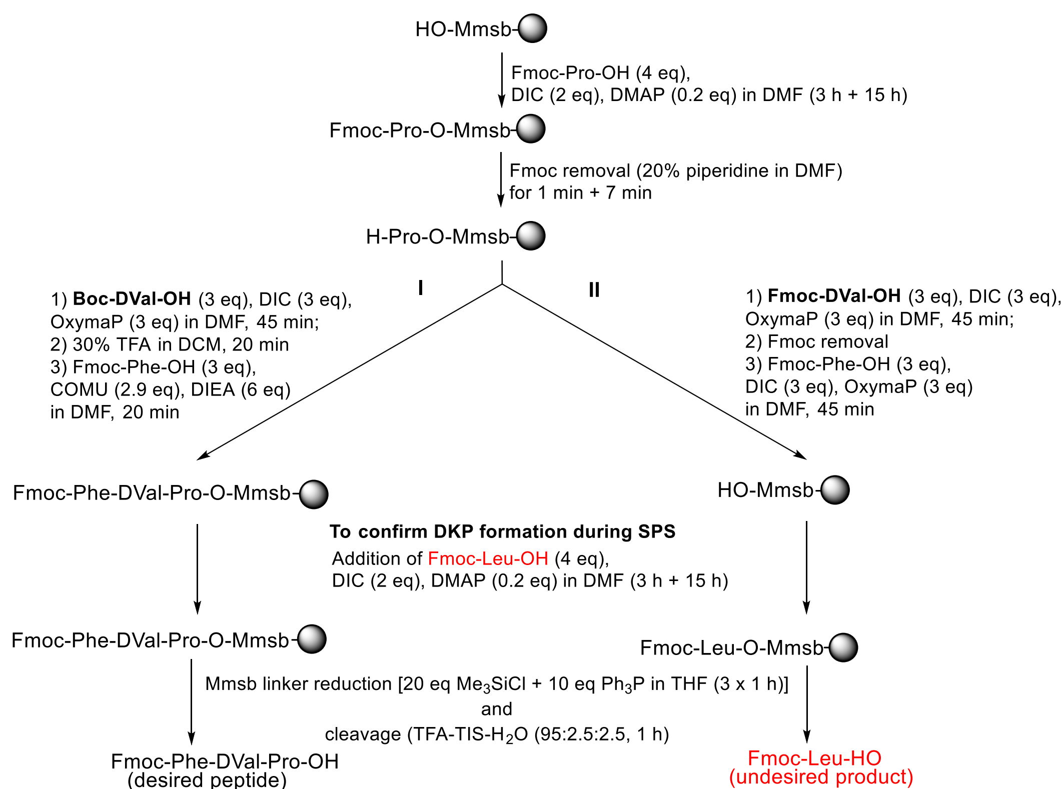


Figure 3. Factors leading to DKP formation include acid-catalyzed reaction,[27,29] base-catalyzed reaction,[30] neutral pH,[32] cis−trans configuration,[31] *N*-alkylated amino acid,[33] L- and D-amino acid,[29,30] different peptide-Resin linkages,[30] and solvent effects.[34,35]

Scheme 4. Study of DKP Formation on Fmoc-DVal-Pro-O-Mmsb-Resin



except the carboxyl and the amine, which render the final amide bond. On-resin cyclization has several advantages. First, it benefits from the pseudodilution effect associated with solid-phase chemistry,[42,43] thus overcoming the high dilution required in solution, which can be an issue. In addition, in the case of side chain-to-side chain, on-resin cyclization does not require reprotection of the carboxylic acid group when acid peptides are synthesized in solution. Usually, for such cyclizations, the concourse of an orthogonal protection scheme is required, mainly the allyl-based protecting groups (semi-

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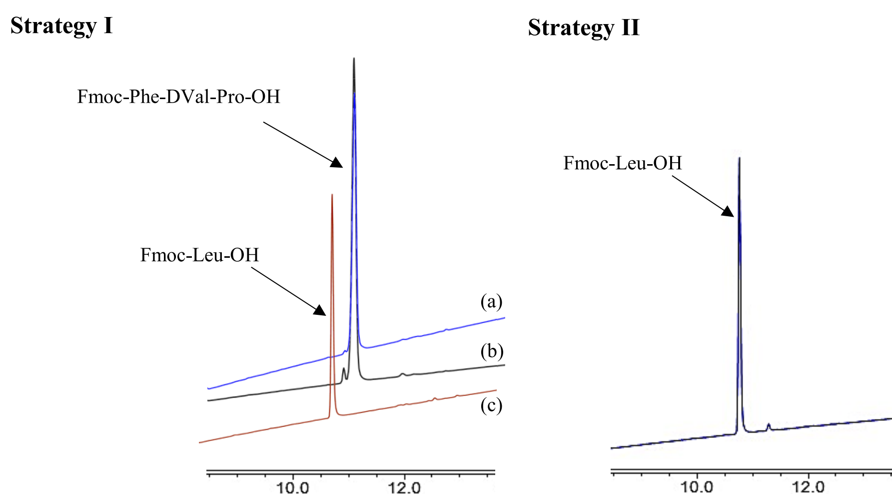

Figure 4. Studies toward avoiding the DKP formation using Mmsb linker. Analysis of cleavage solution after the experiment of Scheme 4, Strategy I: (a) before Fmoc-Leu-OH addition and (b) after Fmoc-Leu-OH addition (0% DKP formation); (c) Fmoc-Leu-OH reference. Strategy II: after Fmoc-Leu-OH addition (100% DKP formation).

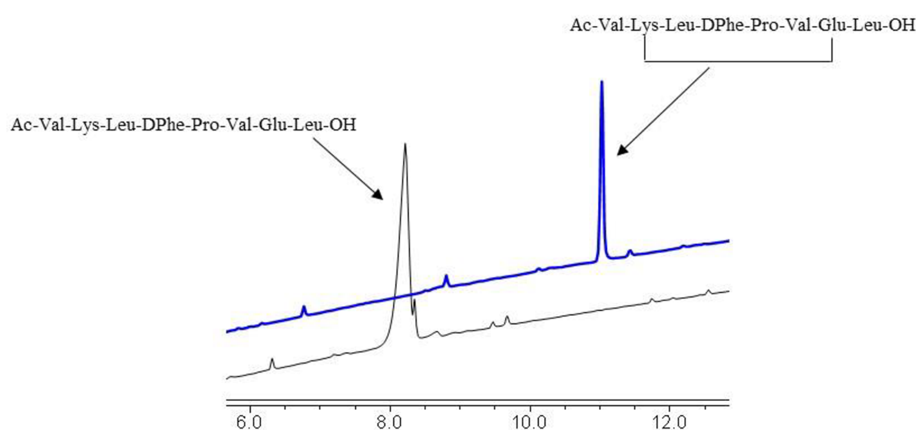

Figure 5. Solid-phase lactam cyclization using Boc and O*t*Bu as semipermanent protecting groups.

permanent protecting group) in combination with the Fmoc (temporary protecting group)/*t*Bu (permanent protecting group) scheme.[11,44] Allyl groups are removed by Pd(0) chemistry, which is not friendly in most nonorganic chemistry laboratories. In this context, the *t*Bu-based protecting groups could be considered the friendliest as semipermanent protecting groups, but they are not compatible with regular acid-labile resins. In this regard, the use of *t*Bu-based groups as semipermanent protecting groups is proposed in combination with HO-Mmsb-Resins (permanent protecting group).

We studied on-resin cyclization using the peptide Ac-Val-Lys(Boc)-Leu-DPhe-Pro-Val-Glu(O*t*Bu)-Leu-O-Mmsb-Resin as a model and following the standard Fmoc/*t*Bu strategy. The on-resin removal of the side-chain protecting groups Boc and O*t*Bu from Lys and Glu amino acids from the peptidyl resin was performed using 50% TFA in DCM for 30 min at rt. After reduction and cleavage of the deprotected linear peptide from the resin, its integrity was confirmed using HPLC and LCMS (Figure 5). After side-chain removal, the peptidyl resin was neutralized and the on-resin cyclization was performed using PyOxime (3 equiv) and DIEA (3 equiv) in DMF for 1 h at rt. After this time, the peptidyl resin was reduced and cleaved to analyze the product by HPLC and LCMS, which confirmed the presence of the desired cyclized peptide (Figure 5). These results confirmed that, by synthesizing the peptide using HO-Mmsb-Resin, the acid-labile side-chain protecting groups can be successfully removed to facilitate on-resin cyclization of the peptide.

On-Resin Heterodetic (Disulfide) Cyclization of Somatostatin. As mentioned earlier, on-resin cyclization has considerable advantages over classical solution cyclization. In the case of heterodetic disulfide peptides, an additional benefit is that oxidation (cyclization) in solution usually requires a very high dilution because it is a thermodynamic process, while on-resin cyclization is carried out with the concourse of only a little amount of solvent. Our group recently developed *sec*-isoamyl mercaptan (SIT) for the protection of the thiol of Cys.[45,46] This protecting group, with less steric hindrance than S*t*Bu, can be better removed by mild reducing agents such as 1,4-dithiothreitol (DTT), which in many cases does not remove the S*t*Bu.[45] It was later demonstrated that the SIT protecting group for the side chain of Cys allows for chemoselective disulfide formation in peptides through a thiol−disulfide interchange method. Basically, this consists of the use of a SIT and a Trt protecting group for each Cys. During the global deprotection, the Trt group of a Cys residue is removed, while the SIT group remains on the second Cys residue. Finally, under mild basic

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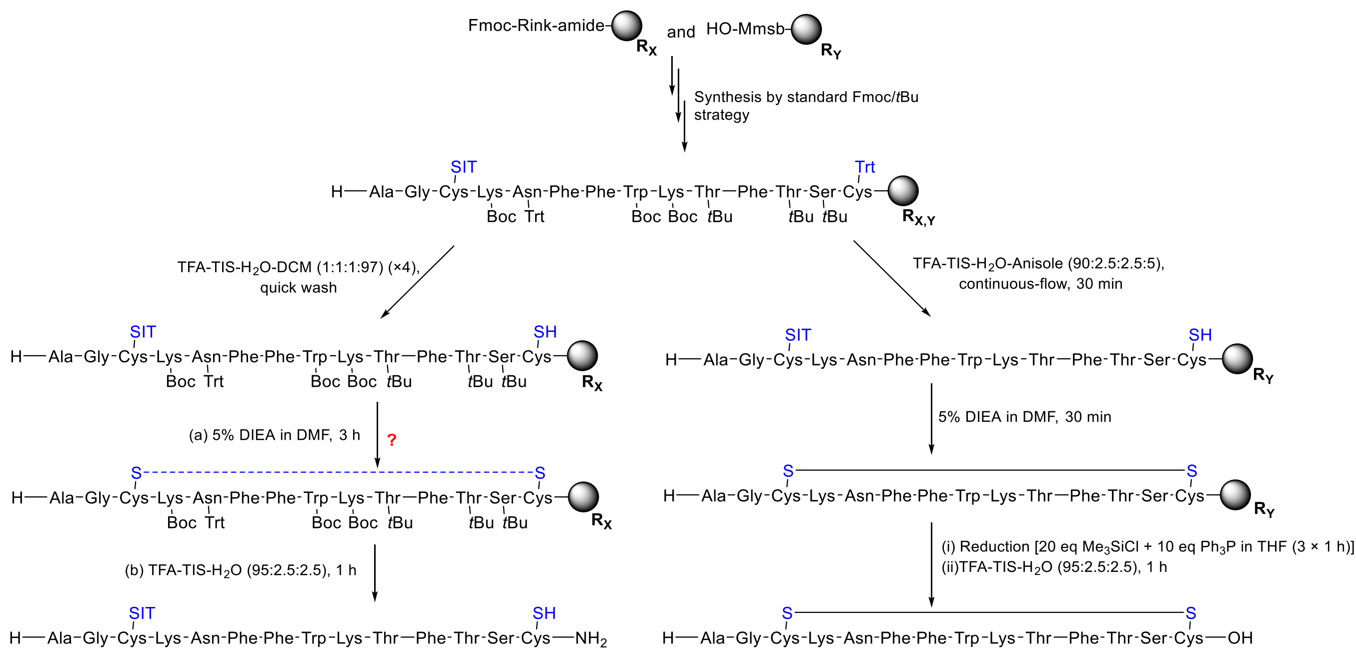
Scheme 5. Cyclization of Somatostatin Peptide (a) on R_x = Fmoc-Rink-amide-AM-Resin and (b) on R_y = HO-Mmsb-Resin



pH, the thiol formed on the first Cys attacks the "S" holding the SIT of the second Cys, rendering the disulfide bridge. In an attempt to expand this chemistry, we assayed this chemoselective disulfide formation through a thiol−disulfide interchange in solid phase. Given its industrial value, somatostatin was chosen as a model peptide. First, the sequence was elongated on a Fmoc-Rink-amide-AM-Resin using classical Fmoc/*t*Bu chemistry (Scheme 4 left). The thiol of the Cys C-terminal was protected with Trt, while SIT was used to protect the Cys residue close to the N-terminal. Global deprotection of an aliquot of the peptide resin and posterior analysis of the cleaved peptide by HPLC and LC−MS showed that the elongation took place properly (Figures S8 and S20). The Trt protecting group of the Cys residue was then removed by four treatments with dichloromethane (DCM)−TFA−TIS−H_2O (97:1:1:1), and the protected peptide resin was treated with 5% DIEA in *N*,*N*-dimethylformamide (DMF) for 3 h. After global deprotection of the peptide using a high concentration of a TFA-containing cleavage cocktail, analysis by HPLC revealed the presence of only linear somatostatin-NH_2, with no presence of the target cyclic peptide.

This poor result could be attributable to steric hindrance between the two Cys residues. Thus, there was one Trt, three Boc, and three *t*Bu protecting groups, in addition to the aromatic rings of two Phe and one Trp groups. The presence of these bulky groups could jeopardize the formation of the disulfide bridge.

Next, the same sequence was elongated on HO-Mmsb-Resin (Scheme 5 right). The reduction of the linker followed by the global deprotection of an aliquot of the peptide resin showed good quality of the peptide, as revealed by HPLC and LC−MS (Figure 6a). Furthermore, the protected peptide-O-Mmsb-Resin was treated with a solution containing a high concentration of TFA (continuous-flow conditions) to remove all the side-chain protecting groups on the peptidyl resin. Reduction of the Mmsb linker was followed by the cleavage of the peptide resin, yielding the linear peptide (Figure 6b). Of note, although the reactions were carried out one after the other, the cyclization step had already started, as shown by the corresponding HPLC analysis (Figure 6b).

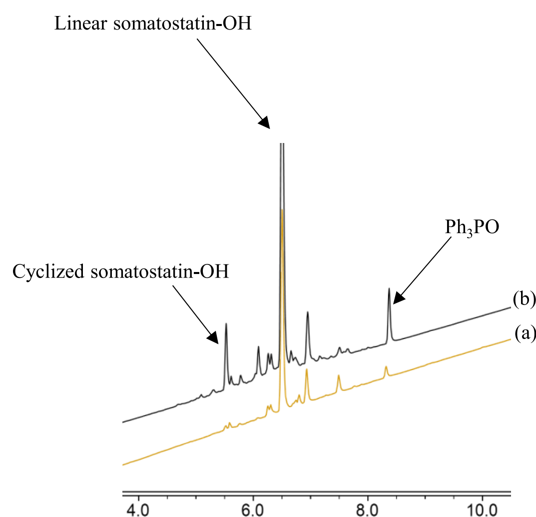


Figure 6. Linear somatostatin (SIT)-OH after (a) linker reduction and cleavage and (b) side-chain removal, linker reduction, and cleavage.

Finally, another aliquot of protected peptide-O-Mmsb-Resin was treated with TFA to remove the side-chain protecting groups, and the SIT-protected peptide was treated with 5% DIEA in DMF for 30 min. The peptide-O-Mmsb-Resin was then reduced using 20 equiv of Me_3SiCl + 10 equiv of Ph_3P in THF (3 × 1 h), and the peptide was cleaved from the resin using TFA-TIS-H_2O (95:2.5:2.5) for 1 h. HPLC and LC−MS analyses indicated the presence of the cyclic target peptide accompanied by some dimers (Figure 7).

■ CONCLUSIONS AND PERSPECTIVES

The use of the Mmsb linker allows the combination of the best features of Fmoc and Boc chemistry, the two major protecting schemes in SPPS. The attachment of the first amino acid with

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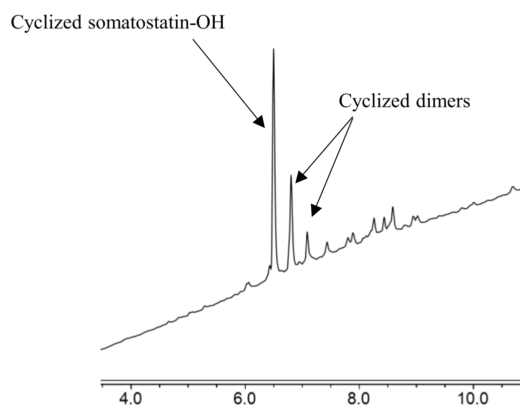

Figure 7. On-Resin cyclization on Somatostatin-O-Mmsb-Resin.

the Mmsb linker is stable to the conditions used to remove Fmoc (piperidine) and Boc (TFA). These acidic conditions are also used to remove the side-chain protecting groups in the Fmoc strategy. At the end of the stepwise elongation and further manipulation, the peptide is cleaved from the Mmsb-Resin in two steps, namely reduction of the sulfoxide to sulfide, followed by detachment of the free peptide from the acid-labile resin using TFA. This two-step protocol, in addition, of allowing a better control of the reactions, renders the peptide aafter cleavage without salts. On the other hand, the large amount of ammonium iodide present in the one-step protocol is difficult to remove. This two-step cleavage developed herein is key for an extension of this "safety-catch" technology to other applications, such as the side-chain protecting amino acids described in the companion paper.[14]

The combination of the two strategies has several advantages. It allows the fine-tuning of some of the side reactions associated with the use of base (piperidine), such as the DKP formation. Furthermore, as the cleavage of the peptide from the Mmsb-Resin is done with standard reagents (Me_3SiCl, Ph_3P, and then TFA), this strategy could be used for the synthesis of cyclic or branched peptides without the concourse of Pd chemistry, which is common in the organic chemistry laboratories but of the more difficult use devoted to biology. The use of Mmsb opens up the possibility of performing the global deprotection in two steps, namely removal of the TFA-labile side-chain protecting groups and then reduction and final TFA cleavage. Both steps require TFA treatment. However, importantly, the first one can be performed in a continuous-flow mode. This could have the further advantage of minimizing the presence of the carbocations formed from the side-chain protecting groups with unprotected sensitive amino acids, such as Trp, Tyr, Cys, and Met, thus reducing the risk of back alkylation reactions.

Although not addressed in this study, Mmsb is also compatible with other sets of protecting groups such as allyl *p*-nitrobenzyl. In this regard, Mmsb together with the Msib (4-(methylsulfinyl)benzyl) and Msbh (4,4′-bis(methylsulfinyl)-benzhydryl) described in the accompanying manuscript[14] add a fourth dimension to the SPPS protecting group scheme.

■ EXPERIMENTAL SECTION

Materials and Methods. Aminomethyl polystyrene resin (AM resin) (0.92 mmol/g) was procured from Purolite, while Fmoc-L-amino acids were purchased from Iris Biotech Gmbh, Germany. DIC and OxymaPure were a gift from Luxembourg Industries, Ltd., Tel Aviv, Israel. Other common chemicals and solvents were purchased from common commercial suppliers like Merck. For analytical HPLC, an Agilent 1100 system was used with a Phenomenex AerisC18 column (3 μm, 4.6 × 150 mm) over a 5−95% gradient of MeCN (0.1% TFA)/H_2O (0.1% TFA) for 15 min, unless stated otherwise (flow rate 1.0 mL/min and UV detection at 220 nm). Data obtained from analytical HPLC were processed by Chemstation software. All mass spectrometry data were obtained from a Thermo Fisher Scientific UltiMate 3000 UHPLC-ISQ EC single quadrupole mass spectrometer in positive ion mode over a 5−95% gradient of MeCN (0.1% HCOOH)/H_2O (0.1% HCOOH) for 15 min, if not stated otherwise.

Solid-Phase Peptide Synthesis. The peptides were synthesized on AM resin (0.92 mmol/gram) using the standard Fmoc/*t*Bu strategy. The resin was first conditioned by washing with DMF (2 × 1 min), DCM (2 × 1 min), and DMF (2 × 1 min). Fmoc-Ala-OH (3 equiv) was as first coupled to the AM resin using DIC (3 equiv) and OxymaPure (3 equiv) in DMF for 45 min. Fmoc was removed by treatment with 20% piperidine/DMF (1 × 1 min and 1 × 7 min) followed by washing the resin with DMF, DCM, and DMF. The resin was then functionalized by coupling the HO-Mmsb linker. HO-Mmsb (1.5 equiv) was incorporated on the AM resin using *N*-hydroxysuccinimide (HOSu) (1.5 equiv) and DIC (1.5 equiv) in DMF for 1 h. The coupling of the first amino acid onto the HO-Mmsb-Ala-AM-Resin (the HO-Mmsb-Ala-AM Resin is generally referred to as HO-Mmsb-Resin throughout this article) was achieved through esterification using Fmoc-AA-OH (4 equiv), DIC (2 equiv) and a catalytic amount of DMAP (0.2 equiv) in DMF for 3 h, followed by double coupling using the same coupling cocktail for 15 h. Fmoc removal and coupling of next amino acids were carried out using DIC (3 equiv) and OxymaPure (3 equiv) in DMF, as a coupling system, for 45 min at rt. This was repeated until the final peptides were achieved. Washes between couplings and deprotections were performed with DMF (3 × 1 min), DCM (3 × 1 min), and DMF (3 × 1 min). For Boc amino acids, the standard coupling condition as for the Fmoc strategy was used, after which Boc was removed using 50% TFA in DCM for 30 min. Boc removal was followed by in situ neutralization and coupling of the next Fmoc-amino acid using Fmoc-aa-OH (3 equiv) and using COMU (2.9 equiv) and DIEA (6 equiv) as coupling conditions. In some cases, acetylation on the free *N*-terminal end of the peptidyl resin was achieved by using acetic anhydride (10 equiv) and DIEA (20 equiv) in DMF for 20 min at rt. The final peptidyl resins were then washed and dried well to continue with reduction and final cleavage of the desired peptides.

General Synthesis for the Mmsb Linker. The first step involved the alkylation of 3-methoxythiophenol (71 mmol) with ethyl-2-bromoacetate (99.4 mmol) in 25 mL of DCM treated with TEA (71 mmol) at 0 °C, and then the reaction mixture was allowed to warm to room temperature for 1 h. The crude mixture was further diluted with DCM and washed with (×2) H_2O. The organic layer was collected, dried over $MgSO_4$, filtered, and evaporated to dryness to afford **1** in 96% yield, which was reasonably pure for the next step. In the following step, the compound **1** (86.47 mmol) in DCM (15 mL) was treated with Vilsmeier reagent $POCl_3$ (51.88 mmol)/DMF (43.24 mmol) in 90 mL of DCM at 0 °C under argon atmosphere, followed by reflux until the reaction was considered complete by HPLC. The reaction mixture was quenched with ice, extracted with (3×) DCM, collected, and dried over $MgSO_4$. Upon column purification using EtOAc/Hex. (0−5% EtOAc), the product **2** was obtained as a white solid in 40% yield. Of note, a mixture of isomers or a single isomer was observed. Next, $NaBH_4$ (9.053 mmol) was added to a solution of **2** in THF/H_2O (60:30) and stirred at rt for 30 min, and then LiOH (18.07 mmol) was added portionwise and left to stir overnight. Upon completion of the reaction, the mixture was diluted with H_2O (90 mL) and extracted with (3×) EtOAc. The aqueous layer was acidified with 2 N HCl to pH 1, furnishing product **3** as a white solid in 90% yield. Finally, the last step consisted of the oxidation of **3** from the sulfide to sulfoxide group using 30% H_2O_2 (17.65 mmol) in acetic acid (6 mL) for 1 h. The mixture was diluted with H_2O (54 mL) and then freeze-dried to a white solid, Mmsb (**4**), in 99% yield. White solid, yield = 2 g (75%). HPLC [5−95% of

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MeCN (0.1% HCOOH)/H_2O (0.1% HCOOH) for 15 min] t_R = 3.26 min. ^{1}H NMR (400 MHz, CD_3CN, few drops of MeOD for solubility): δ 7.58−7.56 (d, J = 8.2 Hz, ArH), 7.28 (br, ArH), 4.63 (s, CH_2), 3.89 (s, CH_3) 3.83−3.82 (s, CH_2). $^{13}C\{^1H\}$ NMR (100 MHz, CD_3CN, few drops of MeOD for solubility): δ 167.3, 158.3, 143.5, 134.9, 128.8, 117.1, 106.3, 61.9, 59.5, 56.2. MS m/z calcd for $C_{10}H_{12}O_5S$: $[M + H]^+$ = 244.04; found $[M + H]^+$ = 245.14.

The "Two-Step" Protocol for the Deprotection of HO-Mmsb-Resin. *Step 1: Reduction of the HO-Mmsb Linker on Peptidyl Resin.* After the synthesis of the desired peptide on the linker-resin, the linker was reduced using 20 equiv of Me_3SiCl and 10 equiv of Ph_3P in THF (3 × 1 h). In some cases, different ratios of Ph_3P were also used to improve the reduction conditions. After the reduction, the resin was washed using THF (×2), DCM (×2), DMF (×2), and DCM (×2). After washing, the resin beads were dried.

Step 2: Cleavage of Reduced Peptidyl Resin. The peptide was cleaved from dried resin using a TFA-TIS-H_2O (95:2.5:2.5) cleavage cocktail for 1 h. Next, the mixture was partially evaporated under reduced pressure and then precipitated using cold diethyl ether. The precipitate was washed with cold diethyl ether and collected after centrifugation, dissolved in ACN/H_2O, and analyzed by HPLC and LCMS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.2c01057.

Characterization of the peptides on HPLC and LCMS; NMR characterization for Mmsb linker (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Fernando Albericio − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4000, South Africa; Institute for Advanced Chemistry of Catalonia (IQAC−CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain;* orcid.org/0000-0002-8946-0462; Email: albericio@ukzn.ac.za

Beatriz G. de la Torre − *KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa;* orcid.org/0000-0001-8521-9172; Email: garciadelatorreb@ukzn.ac.za

Author

K. P. Nandhini − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4000, South Africa; KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.joc.2c01057

Author Contributions

The strategy was designed by all of the authors. Experimental works were executed by K. P. Nandhini. All of the authors discussed the results and prepared the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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