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Accelerated Fmoc solid-phase synthesis of peptides with aggregation-disrupting backbones†

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In this work, we describe an accelerated solid-phase synthetic protocol for ordinary or difficult peptides involving air-bath heating and amide protection. For the Hmsb-based backbone amide protection, an optimized acyl shift condition using 1,4-dioxane was discovered. The efficiency and robustness of the protocol was validated in the course of preparation of classical difficult peptides and ubiquitin protein segments.

Introduction

Total synthesis of peptides and proteins provides valuable molecular tools for chemical biology and medicinal chemistry.[1] Despite the introduction of routine solid phase peptide synthesis (SPPS) by Merrifield and the development of powerful native chemical ligation (NCL) by Kent,[2,3] the protein assembly process is often plagued by the presence of difficult peptide sequences. For Boc SPPS, repetitive TFA treatment of resins combined with *in situ* neutralization coupling can moderate the aggregation effect.[4] However, the toxicity of hydrogen fluoride as the cleavage reagent makes Boc SPPS less favorable to peptide chemists. While in the case of Fmoc SPPS, in which on-resin aggregation can easily occur through inter- or intramolecular chain interactions, difficult sequences pose a more severe bottleneck toward the preparation of high-quality peptides.[5] To address the issue of difficult sequences, many disaggregating strategies have been devised, which generally fall into two categories.

The first group of strategy (physical means) combat difficult sequences by adjusting the external environment during SPPS, which includes: (1) switching typical polystyrene resins to low-substitution high-swelling hydrophilic resins such as PEG-based resins,[6] (2) using alternative solvents or solvent mixtures (DMF, NMP, DMSO) for better swelling effects,[7] (3) adding chaotropic salts (LiCl, KSCN, $NaClO_4$) into the coupling solution,[8] (4) employing more aggressive coupling reagents, such as HATU/DIEA or COMU/DIEA single/multiple couplings, for a prolonged time,[9,10] and (5) applying a sophisticated microwave or conventional oil-bath heating apparatus.[11] The second group of strategy (chemical means) takes the advantage of backbone amide protection to suppress the amide N–H involved on-resin β-sheet formation (Fig. 1).[12] Kiso's group and Mutter's group designed an elegant aggregation-breaking method by the incorporation of "click peptides" or "switch peptides."[13] This method mostly relies on the synthesis of depsipeptides (*O*-acyl isopeptides) followed by in-solution O→N intramolecular acyl transfer to restore native Xaa-Ser/Thr peptide bonds. In addition to this depsipeptide approach,

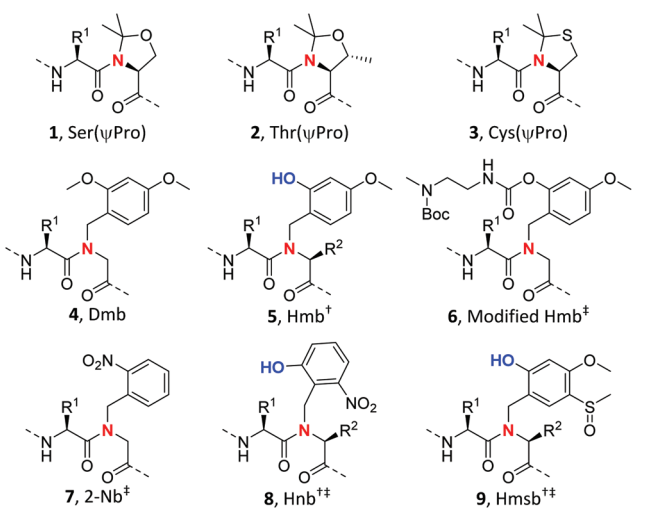

Fig. 1 Typical backbone protecting groups used during Fmoc SPPS. †These protecting groups are less sensitive to the steric hindrance of residue R^1 and R^2. ‡These protecting groups are stable under conventional TFA cleavage conditions.

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commercially available Fmoc-protected pseudoproline dipeptides have gained wide popularity as aggregation-breaking building blocks **1–3**.[14] An important feature is that pseudoproline structures can be converted to native amides during acidic cleavage. Unfortunately, their utility is limited to Xaa-Ser, Xaa-Thr or less frequently Xaa-Cys containing sequences. Another popular method exploits the acid lability of electron-rich benzyl protected amide. Both 2,4-dimethoxybenzyl (Dmb) **4** and 2-hydroxy-4-methoxybenzyl (Hmb) **5** groups were found to have considerable aggregation-disrupting effects.[15,16] These groups can also be cleaved with TFA to recover native amide bonds. Importantly, Fmoc-Asp(OtBu)-(Dmb)Gly-OH and Fmoc-Asp(OtBu)-(Hmb)Gly-OH have proven to be reliable building blocks to completely avoid the notorious aspartimide formation.[17] Recently, an Hmb derivative **6** was developed to allow the incorporation of an oligo-arginine tag, which is conducive for the synthesis of transmembrane peptides,[18] almost exclusively difficult sequences. The major drawback of these benzyl type protecting groups is the huge steric hindrance around the tertiary amide. The choice of R^2 is usually constricted to Gly and Ala. This is also the case for a photo-labile amide protecting group 2-nitrobenzyl (2-Nb) **7**.[19]

Interestingly, the phenol group in Hmb **5** permits fast phenol ester formation, and then tertiary amide generation through six-membered acyl migration.[16] This special mechanism slightly enhanced the residue (R^1 and R^2) tolerance of Hmb **5** compared with Dmb **4**. To speed up the rate-limiting acyl shift step, several electron-withdrawing modifications (**8** and **9**) on 2-hydroxybenzyl having better acyl shift kinetics were reported.[20,21] An additional advantage of these backbone protecting groups is that they are stable to standard acidic cleavage cocktails. This feature is instrumental because peptides containing tertiary amide backbones usually bear better HPLC elution behavior (shorter retention time and sharper peak) and greater solubility in the LC elution buffer (0.1% TFA, acetonitrile–water = 1/1, pH 1) or the NCL buffer (6 M Gn-Cl, pH 4–8).[18,22,23] After post-cleavage purifications and segment ligations of backbone-protected peptides, 2-hydroxy-6-nitrobenzyl (Hnb) **8** and 2-hydroxy-4-methoxy-5-methylsulfinylbenzyl (Hmsb) **9** were found to be cleavable by using UV illumination and sulfoxide-reducing TFA (Fig. S1, Scheme S1†), respectively, to generate free peptides. A similar safety-catch structure 2-methoxy-4-methylsulfinylbenzyl (Mmsb) was also very recently reported by the Albercio group.[24]

Previous work from Offer's group indicates that Hmsb **9** could improve the purity and solubility of on-resin aggregation-prone peptide sequences for both manual and automated SPPS.[21] However, the tolerance of **9** to β-branched amino acids was not investigated. In addition, to test the scope and feasibility of this method, we thought it was necessary to prepare longer peptides (>25 aa) with multiple Hmsb-protected amides. Herein, we discovered a new solvent incubation protocol, which exhibited better acyl shift kinetics to incorporate **9**. Our results showed that the new acyl transfer condition could tolerate couplings of sterically hindered β-branched amino acids (Ile, Val, Thr). Moreover, we successfully prepared several difficult sequences (70%–95% HPLC purities of crude products) using air-bath heating batch-based SPPS in conjunction with Hmsb-based backbone amide protection. The timescale of a single deprotection-washing-coupling SPPS cycle can be shortened by as much as 90 min using the new air-bath heating apparatus.

Results and discussion

Discovery of the optimized acyl shift condition in dioxane

On our route to extend the applicability of Fmoc SPPS with Hmsb-based backbone protection, we noticed Alewood group's report that O-acylation proceeds fast and quantitatively while the following acyl transfer step is sluggish. According to previous literature,[20a,21,25] the treatment of resins with nonpolar solvents could facilitate on-resin intramolecular acyl transfer. Therefore, we began our acyl shift optimization by screening various solvents or solvent mixtures.

Initially, we set two fundamental requirements for appropriate solvents: good resin-swelling capability and high boiling point. The first criterion is crucial for fast on-resin kinetics and the second is beneficial in case of high-temperature SPPS.[26] Based on this, we tested the effects of six solvents towards the on-resin acyl shift reaction on a model pentapeptide, H-Tyr-Leu-(Hmsb)Leu-Ser-Lys-NH_2 (Fig. 2). All deprotection and couplings were conducted under standard room temperature Fmoc SPPS conditions except the acyl shift step. Hmsb was synthesized using a slightly modified procedure compared with an earlier paper (Scheme S2†). For good comparison, all the reactions were conducted at 50 °C for 2 h in the presence of 5% (v/v%) DIEA (Table 2, entries 1–8). Modified cocktail K (TFA/H_2O/thioanisole/EDT = 87/5/5/3) was used as the cleavage reagent for sulfoxide-containing peptides.[27,28]

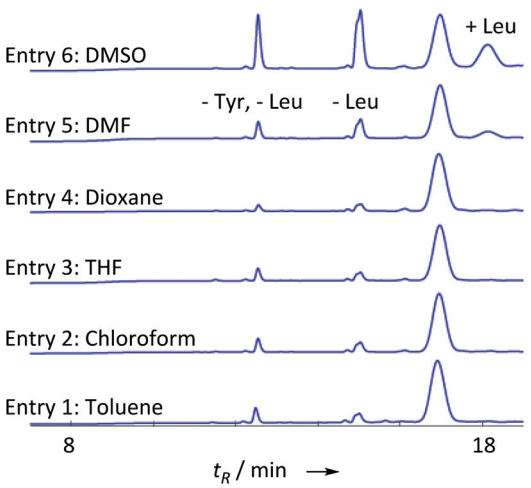
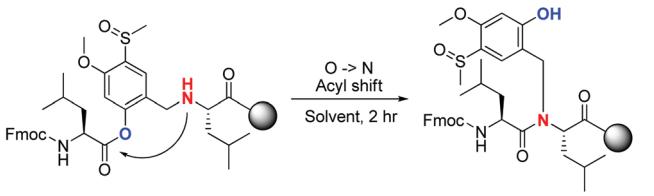


Fig. 2 HPLC traces of H-Tyr-Leu-(Hmsb)Leu-Ser-Lys-NH_2 synthesized under different acyl shift conditions. All acyl shift reactions were carried out in 5% (v/v) DIEA/solvent at 50 °C for 2 h.

Table 1 Optimization of the on-resin acyl shift reaction during Fmoc SPPS to prepare H-Tyr-Leu-(Hmsb)Leu-Ser-Lys-NH_2



Entry	Solvent (mixture)	DIEA [v/v %]	Temperature [°C]	HPLC purity [%]
1	Toluene	5	50	82
2	Chloroform	5	50	83
3	THF	5	50	84
4	1,4-Dioxane	5	50	90
5	DMF	5	50	74
6	DMSO	5	50	40
7	THF–MeOH = 4/1	5	50	67
8	THF–H_2O = 4/1	5	50	66
9	1,4-Dioxane	0	50	91
10	1,4-Dioxane	0	25	77
11	DMF	0	25	66
12	**1,4-Dioxane**	**0**	**75**	**97**
13	(Acyl shift step skipped)			52

As expected,[25] all of the nonpolar solvents afforded the crude Hmsb-protected peptide with good HPLC purity (Table 1, entries 1–4). Interestingly, 1,4-dioxane worked better than other less polar solvents (90% HPLC purity), possibly due to its excellent ability in resin-swelling and chain-solvation. Inferior results were obtained when aprotic polar solvents DMF and DMSO were used (Table 1, entries 5–6). In the case of DMSO, three byproducts were detected through HPLC and ESI-MS (Fig. 2), which corresponded to a Tyr^1 and Leu^2 deletion product, a Leu^2 deletion product, and a surprising Leu^2 addition product. The deletion product indicated an incomplete acyl shift reaction of Leu^3. The appearance of the additional byproduct might be caused by premature Fmoc deprotection by the N-terminal secondary amine of peptide chain in an aprotic polar solvent in the presence of base at higher temperature. Addition of water or methanol as a co-solvent during acyl shift failed to produce the product with a higher yield (Table 1, entries 7–8). Further experiments showed that additional base was unnecessary and the acyl shift happened more efficiently at higher temperature without DIEA (Table 1, entries 9–12). Therefore, neat dioxane (b.p. 101 °C) was chosen as the ideal solvent in the following acyl-shift experiments. Encouragingly, compared with Offer's previous protocol using DMF as the sole solvent during coupling and acyl shift, our new 1,4-dioxane involved acyl shift protocol showed a noticeable improvement (Table 1, entries 11–13).

Scope of Hmsb protection of backbone amides

To speed up Hmsb incorporation, we decided to perform both O-acylation and acyl shift reactions at elevated temperatures. Based on recent reports about microwave or oil-bath involved SPPS,[11c,d] we believed that manual SPPS at 50–90 °C could be attempted, which might afford comparable results to microwave-based automated SPPS.[29] Due to concerns of racemization and other side-reactions,[30] we carried out base-free DIC/Oxyma couplings at 75 °C (air-bath heating) for all the Fmoc amino acids except Arg, Cys and His.[31] Cysteine was coupled at 50 °C while Arg and His were coupled at room temperature. Next, we synthesized Hmsb protected sequences containing racemization-prone Phe, Ser, Cys residues (Fig. 3 and S2†). Note that both Ac-Ser[L]-(Hmsb)Ala-Lys-NH_2 and Ac-Ser[D]-(Hmsb)Ala-Lys-NH_2 showed double peaks on HPLC (peak volume ~ 1 : 1) with identical molecular weights (Fig. 3B). This was not caused by on-resin racemization of the building block but the fact that Hmsb was synthesized as a racemic sulfoxide compound. Interestingly, racemic Ac-Phe[L]-(Hmsb)Ala-Lys-NH_2 or Ac-Phe[D]-(Hmsb)Ala-Lys-NH_2 appeared inseparable using the same HPLC gradient. All of the LC co-injection traces confirmed that racemization ratio was quite low (<2%) during the manual high-temperature synthesis of Hmsb-protected peptides (Fig. 3).

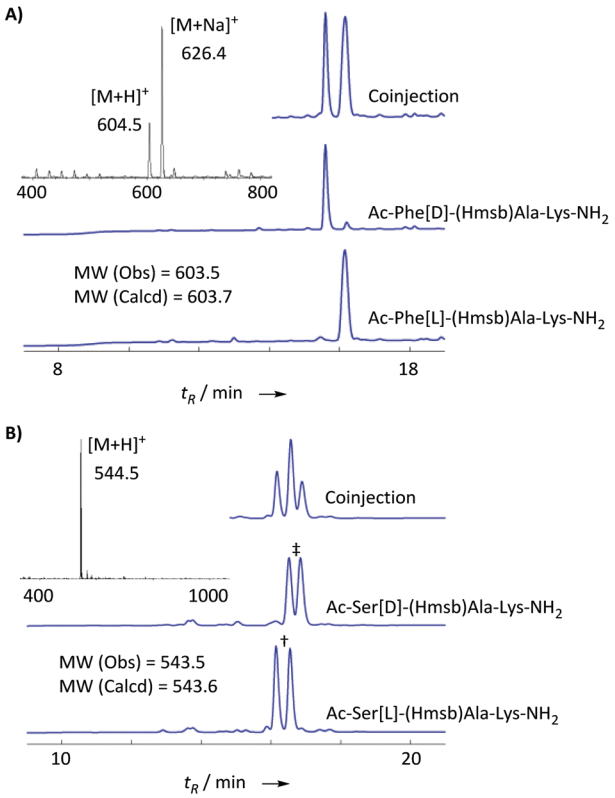


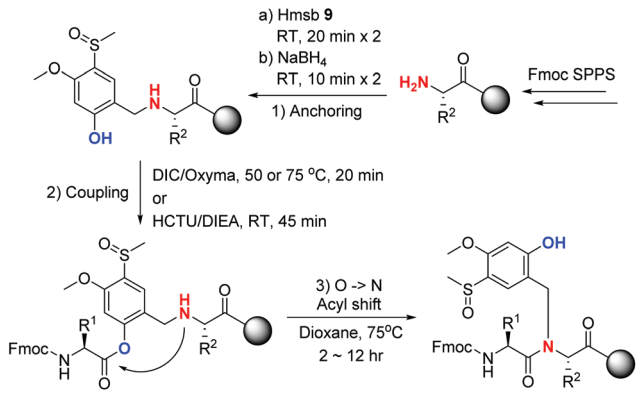
Fig. 3 Racemization tests of (A) Ac-Phe[L/D]-(Hmsb)Ala-Lys-NH_2 and (B) Ac-Ser[L/D]-(Hmsb)Ala-Lys-NH_2. †Ac-Ser[L]-(Hmsb)Ala-Lys-NH_2 containing racemic sulfoxides (~1 : 1) are separable by HPLC. ‡Ac-Ser[D]-(Hmsb)Ala-Lys-NH_2 containing racemic sulfoxides are separable by HPLC.

To study the reaction scope of the on-resin acyl shift reaction, we prepared another model pentapeptide H-Met(O)-Xaa-(Hmsb)Yaa-Ser-Lys-NH_2. The reaction efficiency was evaluated based on the HPLC purities of crude products using various Xaa/Yaa combinations (Fig. S3† and Table 2). We found that

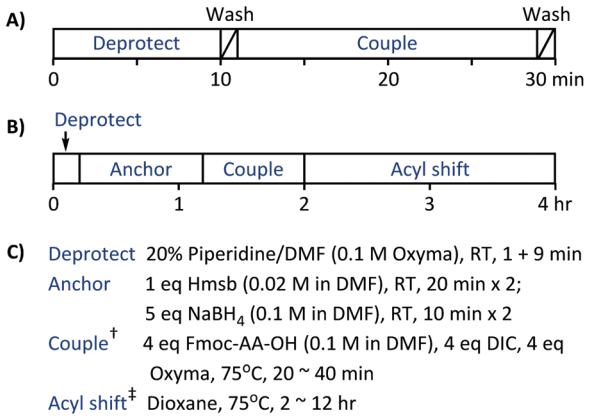
Table 2 Scope of the on-resin acyl shift reaction during Fmoc SPPS using a model sequence H-Met(O)-Xaa-(Hmsb)Yaa-Ser-Lys-NH_2

Entry	Xaa	Yaa	Acyl shift time [h]	HPLC purity [%]
1	Leu	Leu	2	95
2	Asn	Asn	2	96
3	Phe	Phe	2	78
4[a]	Arg	Arg	2	80
5	Pro	Gln	2	86
6	Trp	Tyr	2	70
7	Ile	Gly	12	90
8	Ile	Ala	12	86
9	Val	Ala	12	85
10	Thr	Ala	12	82
11	Val	Leu	24	75
12	Leu	Val	24	<5

[a] The acyl shift of Fmoc-Arg(Pbf)-OH was performed at room temperature rather than at 75 °C.



Scheme 1 Fully optimized incorporation of Hmsb **9** consists of three steps, (1) anchoring of salicylaldehyde onto free amino resin through reductive amination; (2) coupling of Fmoc-AA-OH to form a phenolate under room temperature or high temperature conditions; (3) on-resin acyl shift in 1,4-dioxane to make a protected tertiary amide bond.



Scheme 2 (A) Standard manual SPPS timeline. (B) Manual SPPS timeline with Hmsb incorporation. (C) The SPPS protocol includes four steps, deprotection, anchoring, coupling and acyl shift. †HCTU/DIEA (RT, 45 min) or DIC/Oxyma (RT, 90 min) coupling is chosen for Fmoc-Arg(Pbf)-OH to prevent δ-lactam formation of arginine. DIC/Oxyma (RT, 45 min) coupling is chosen for Fmoc-His(Trt)-OH to suppress racemization. DIC/Oxyma (50 °C, 20 min) coupling is chosen for Fmoc-Cys(Trt)-OH to suppress racemization. DIC/Oxyma (75 °C, 20 min) coupling is used for the rest of the 17 canonical amino acids. ‡The acyl shift step can be carried out for 2–12 h, depending on the steric bulkiness of both the acceptor and donor amino acids of the acyl transfer. For Fmoc-Arg(Pbf)-OH and Fmoc-His(Trt)-OH, the temperature should be set at RT to minimize any side reactions.

most β-linear amino acids are compatible with the on-resin acyl shift (Table 2, entries 1–6). The crude peptides displayed good to excellent HPLC purity. More importantly, Xaa seems to tolerate all the sterically hindered β-branched amino acids when Yaa = Gly or Ala (Table 2, entries 7–10). However, Val-(Hmsb)Leu and Leu-(Hmsb)Val obtained worse results indicating that the acyl shift kinetics are more sensitive to the steric bulkiness of Yaa than Xaa (Table 2, entries 11–12).[19,20a] We believed that these experimental data would be equally helpful when amino acid coupling and acyl shift were encountered after Hmb **5** and Hnb **8** incorporation.

With the scope of investigation of Hmsb protection of backbone amides, we updated the procedure of incorporation of Hmsb, (1) anchoring *via* on-resin two-step reductive amination; (2) coupling *via* O-acylation; (3) acyl shift *via* incubation in dioxane (Scheme 1). Furthermore, we depicted a timeline for Fmoc SPPS, which is based on air-bath heating (50 °C and 75 °C). Traditional Fmoc SPPS requires 45 to 120 min to introduce each amino acid building block. In comparison, our new coupling cycle (deprotection, washing and coupling) takes only 30 min to complete with an easily accessible air-bath shaker as the only additional piece of equipment for manual SPPS (Scheme 2A and Fig. S4†). The actual coupling time at high temperature is around 15 min (excluding 3–5 min for heat exchange). Piperidine deprotection was carried out at room temperature to suppress any base-induced side reactions.

Further tests of Fmoc SPPS assisted by air-bath heating

To evaluate the robustness of our new fast Fmoc SPPS protocol, we selected four difficult sequences from the literature, which were tested *via* air-bath heating Fmoc SPPS alone and another two sequences through high-temperature SPPS plus Hmsb incorporation. Four difficult peptides, ACP(65–74), ABC 20mer, PnlA(A10L) and HIV-1 PR(81–99) were synthesized on a 0.1 mmol scale using a 30 min coupling cycle (Scheme 2A). As shown in Fig. 4, crude peptide products were obtained at good to excellent HPLC purity (70%–95% based on peak area ratios, see Table 3). For all of the four peptides, the whole main peak on analytical HPLC was collected and characterized by direct injection ESI-MS.

Two additional sequences PolyL10mer and toxin protein Mambalgin-1(19–40) were further synthesized using both air-bath heating SPPS and Hmsb backbone protection. PolyL10 was found insoluble in common HPLC elution buffer H_2O/ACN = 1/1 when prepared through high-temperature SPPS alone. However, when Hmsb was incorporated between Leu^5 and Leu^6 as a backbone kink, we obtained the fully soluble

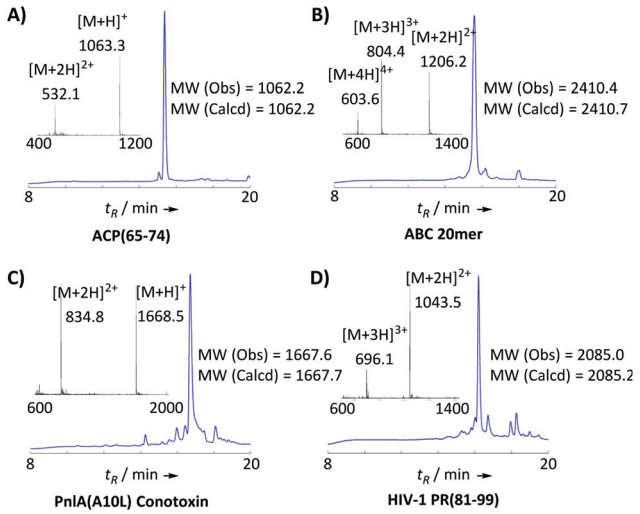


Fig. 4 Fmoc SPPS of difficult peptides using air-bath heating. (A) ACP (65–74): H-VQAAIDYING-NH_2; (B) ABC 20mer: H-VYWTSPFM(O)KLI-HEQCNRADG-NH_2; (C) PnlA(A10L) conotoxin: H-GCCSLPPCALNNP-DYC-NH_2; (D) HIV-1 PR(81–99): H-PVNIIGRNLLTQIGCTLNF-NH_2.

Table 3 Synthesis results

Entry	Peptide	Condition[a]	HPLC purity [%]	Crude yield[b] [%]
1	ACP(65–74)	A	95	86
2	ABC20mer	A	92	87
3	PnlA(A10L) conotoxin	A	76	82
4†	HIV-1PR(81–99)	A	70	91
5	PolyL10	A	89	85
6	PolyL10	B	N.D.	26
7	Mambalgin-1(19–40)	A	50	67
8	Mambalgin-1(19–40)	B	38	62
9	Mambalgin-1(19–40)	C	72	83
10	Mambalgin-1(19–40)	D	80	88
11	Ubiquitin(1–45)	A	42	73
12	Ubiquitin(1–45)	D	65	80
13	Ubiquitin(46–76)	A	31	55
14	Ubiquitin(46–76)	D	72	88

[a] A = air-bath heating SPPS; B = standard room temperature SPPS; C = air-bath heating SPPS combined with single Hmsb amide protection; D = air-bath heating SPPS combined with double Hmsb amide protection. [b] Crude yields were determined based on the weight of lyophilized crude cleavage product after ether precipitation.

and chemically homogeneous product (Fig. 5A and Table 3). In the same manner, we targeted another difficult sequence Mambalgin-1(19–40). In a control experiment, air-bath SPPS gives the crude peptide with average purity. In contrast, both one (between Leu^{34} and Gln^{35}) or two (between Leu^{30} and Lys;[31] Gln^{35} and Gly^{36}) amide-protected Mambalgin-1(19–40) crude peptides showed better HPLC purity than the former (Fig. 5B and Table 3).

Finally, we applied our new protocol to two protein segments, ubiquitin(1–45) and ubiquitin(46–76), to verify its robust performance. Both sequences were prepared with or without Hmsb incorporation using air-bath heating SPPS. As shown in Fig. 6 and Table 3, backbone amide protection significantly improved the qualities of the crude products. Two Hmsb deletion peaks could be identified from the mass spectrum of ubiquitin(1–45), which probably resulted from the partial decomposition of tertiary amide in the ionization room of the mass spectrometer. No significant deletion or truncation side-products could be found on the mass spectra of the main peaks. After semi-preparative HPLC purification, ubiquitin H-$M(O)^1$-F^{45}-$NHNH_2$ [G^{10}-(Hmsb)K^{11}, L^{15}-(Hmsb)E^{16}] and ubiquitin H-C^{46}-G^{76}-NH_2 [D^{52}-(Hmsb)G^{53}, L^{67}-(Hmsb)H^{68}] were deprotected in a modified TFA cocktail B (2.5% H_2O, 2.5% TIPS, 2.5% EDT, 1% Bu_4NI) to produce the target peptides H-M^1-F^{45}-NHNH2 and H-C^{46}-G^{76}-NH_2 (Fig. 6 and Scheme S1†) in >80% HPLC yield. Compared with previously reported additives, such as NH_4I/Me_2S,[32] NH_4I/TIPS and TMSBr or Bu_4NBr/thioanisole/EDT,[33,34] in our hands, Bu_4NI assisted sulfoxide reduction obtained essentially equal results. However, the Hmsb removal condition would yet need to be optimized to give cleaner deprotection results.

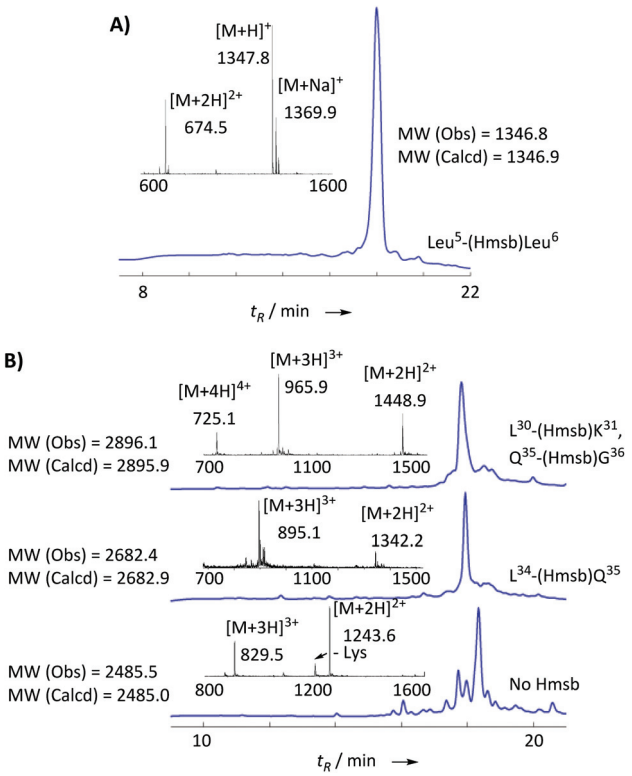


Fig. 5 Fmoc SPPS of difficult peptides using air-bath heating along with Hmsb backbone protection. (A) PolyL10: H-LLLLL(Hmsb)LLLLL-NH_2; (B) Mambalgin-1(19–40): H-CYHNTGMPFRNLKLILQGCSSS-NH_2.

Conclusions

In summary, we successfully improved the efficiency of Hmsb-based backbone amide protection using the key 1,4-dioxane involved acyl shift in an effort to tackle the synthesis of difficult peptides. To speed up the entire synthetic scheme

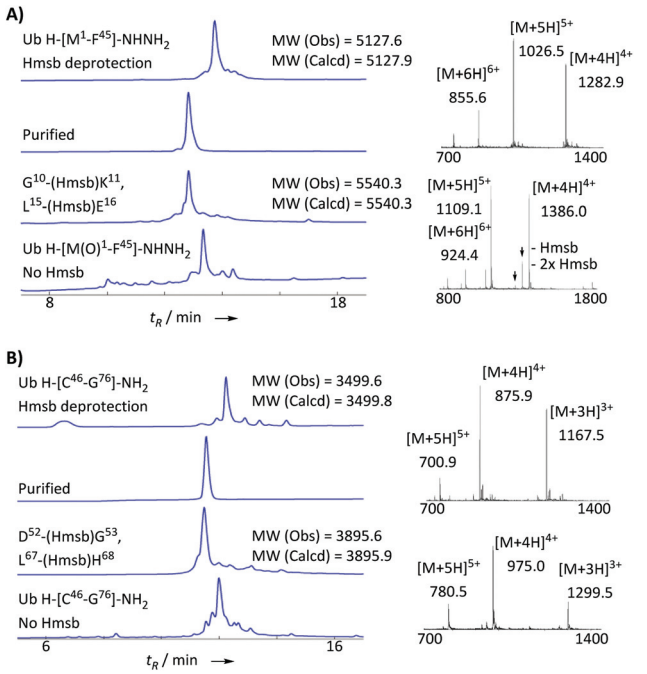


Fig. 6 Fmoc SPPS of ubiquitin fragments using air-bath heating along with Hmsb backbone protection. (A) Ubiquitin H-M(O)^{1}-F^{45}-NHNH$_2$; (B) Ubiquitin H-C^{46}-G^{76}-NH$_2$.

further, we devised a fast Fmoc SPPS protocol using a simple air-bath heating apparatus. Manual synthesis of an ordinary or difficult sequence containing 20–40 amino acid residues can be finished within 1–2 days (30 min for each coupling cycle). We considered that the heat and backbone amide protection worked synergistically to improve the peptide quality. The operability of this protocol was validated in the preparation of two protein segments ubiquitin(1–45) C-terminal α-hydrazide and ubiquitin(46–76) C-terminal α-amide, which are useful segment building blocks for assembling versatile ubiquitin derivatives through native chemical ligation.[35,36] Future projects would be focused on the combinatorial use of accelerated Fmoc SPPS and iterative peptide ligations.

Acknowledgements

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