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Obviation of hydrogen fluoride in Boc chemistry solid phase peptide synthesis of peptide-$^{\alpha}$thioesters

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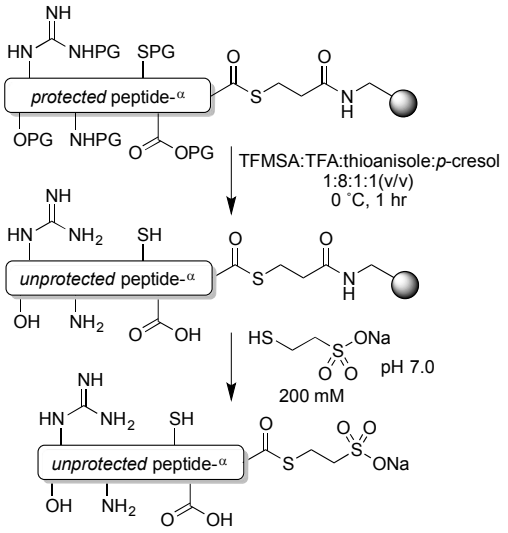
Under suitable conditions, trifluoromethanesulfonic acid performs comparably to hydrogen fluoride for the on-resin global deprotection of peptides prepared by Boc chemistry solid phase peptide synthesis (SPPS). Obviation of hydrogen fluoride in Boc chemistry SPPS enables the straightforward synthesis of peptide-$^{\alpha}$thioesters for use in native chemical ligation.

Peptide-$^{\alpha}$thioesters are the key building blocks for the total synthesis of proteins by native chemical ligation[1,2]. But, for many researchers, preparation of peptide-$^{\alpha}$thioesters *via* Boc chemistry SPPS is the principal obstacle to applying the chemical ligation approach. Preparation of peptide-$^{\alpha}$thioesters is straightforward using '*in situ* neutralization' Boc chemistry SPPS[3], with the use of a preformed thioester attached to the resin by a cleavable linker[4]. However, the use of anhydrous hydrogen fluoride (HF)[5,6], which is perceived as mandatory for the global deprotection and cleavage of peptides prepared by Boc chemistry SPPS, is hazardous and requires specialized equipment, and extreme stringent safety precautions[7]. Because of the technical challenges and hazards associated with the use of HF, a variety of Fmoc chemistry SPPS methods has been devised and used to access peptide-$^{\alpha}$thioesters[8].

Recently, there has been a resurgence of interest in Boc chemistry SPPS in conjunction with non-HF deprotection for the synthesis of peptide-thioesters.[9] In particular, the use of trifluoromethanesulfonic acid[10] (TFMSA) has been applied to the deprotection and cleavage of peptide-$^{\alpha}$thioesters. To support and extend this strategy, we have directly compared HF to TFMSA deprotection, and show that under suitable conditions, TFMSA can be employed for final 'global' deprotection of peptide-$^{\alpha}$thioesters prepared by Boc chemistry SPPS, and that results qualitatively similar to those obtained with HF are possible. Keys to success with TFMSA deprotection were the use of strict temperature control, appropriately acid-labile side chain protecting groups, and the additive thioanisole.

One of the documented challenges with TFMSA deprotection/cleavage of peptides prepared by SPPS is efficient cleavage of the peptide-resin linkage[11,12]. The peptide-resin linkage is engineered to be one of the most stable 'protecting groups' that must be cleaved during the final global deprotection step, because premature cleavage causes both chain loss and chronic trifluoroacetylation during chain assembly by Boc chemistry SPPS[13]. For this reason, as well as to simplify the removal of TFMSA from peptide products†, we chose to employ acid-stable thioester peptide-resin linkers, and to cleave peptide products from the resin *after* side chain deprotection, by treatment with the thiol nucleophile sodium 2-mercaptoethanesulfonate (MESNA) in 6 M guanidine hydrochloride, 200 mM phosphate buffer, pH 7 (**Scheme 1**)[9a,b].



Scheme 1. On-resin global deprotection, followed by nucleophilic cleavage for recovery of peptide products in Boc chemistry SPPS. A full list of TFMSA-compatible protecting groups (PGs) and their abbreviations is given in **Table 1**.

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This two-step strategy afforded reasonable isolated yields of peptide-αthioesters after solid phase extraction and lyophilisation, and enabled us to test the utility of flow conditions for minimizing side reactions during deprotection. TFMSA gave products of comparable quality to those obtained with HF when used under the following conditions[14]: TFMSA:TFA:thioanisole:*p*-cresol 1:8:1:1 (v/v), 0 °C, 1 h. Comparative deprotections of the human growth hormone releasing hormone (GHRH) segment 1-29 (**Figure 1**) are illustrative:

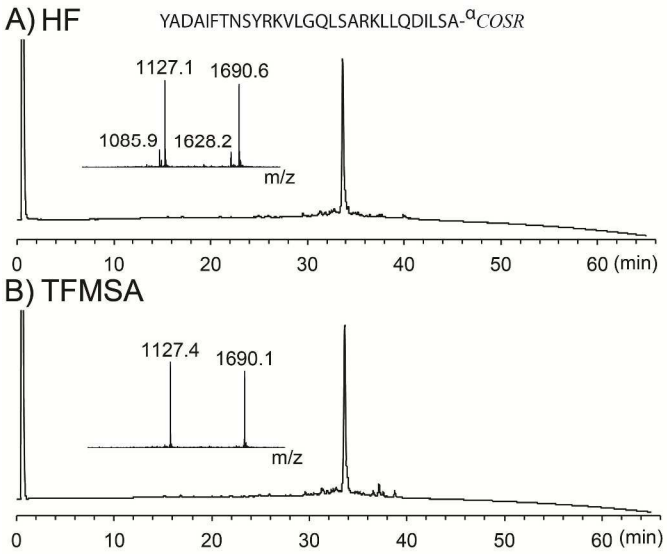


Figure 1. LC-MS data for crude products obtained by nucleophilic cleavage of GHRH-α*COSCH*$_2$*CH*$_2$*CO*-resins with 2-mercaptoethanesulfonate (MESNA), after side chain deprotection with: (A) HF:*p*-cresol 10:1 (v/v), 0 °C, 1 h; or (B) TFMSA:TFA:thioanisole:*p*-cresol 1:8:1:1 (v/v), 0 °C, 3 x 5 min, 1 x 15 min, 1 x 30 min. Reaction products were: GHRH-α*COSCH*$_2$*CH*$_2$SO$_3$H (exact mass: 3377.73 Da; average mass: 3379.92 Da; found: (A) 3378.7 Da; (B) 3378.8 Da). A side product evident in the mass spectra of (A) was GHRH-α*COOH* (exact mass: 3253.77; average mass: 3255.74; found: 3254.53 Da).

Temperature control was crucial for minimizing acid-catalyzed aspartimide formation during TFMSA deprotections. In our hands, this side reaction was severe at ambient temperature, giving as much as ~50% conversion to aspartimide after 1 h (see SI, **Table S6**). Fortunately, model studies corroborated literature precedent[14] that showed aspartimide formation could be effectively suppressed by maintaining the deprotection temperature at 0 °C. Therefore, an important consideration in the development of generally applicable TFMSA deprotection conditions was the identification of side chain protecting groups that are efficiently removed without resorting to the use of ambient temperature, as has been advocated in some protocols[12]. Protecting groups that for this reason were found to be necessary included: Arg(Mbs), Asp(OBzl), Glu(OBzl), and Cys(Mob). A list of TFMSA-compatible protecting groups (and abbreviations) using the conditions described above is given in **Table 1**.

N$^{\omega}$-*p*-methoxybenzenesulfonyl-Arginine	Arg(Mbs)
Aspartic acid β-benzyl ester	Asp(OBzl)
S-acetamidomethyl-Cysteine	Cys(Acm)*
S-(4-methoxybenzyl)-Cysteine	Cys(Mob)
1,3-thiazolidine-4-carboxylic acid	Thz*
Glutamic acid ω-benzyl ester	Glu(OBzl)
N$^{\pi}$-(2,4-dinitrophenyl)-Histidine	His(Dnp)
N$^{\varepsilon}$-(2-chloro-benzyloxycarbonyl)-Lysine	Lys(Cl-Z)
Serine O-benzyl ether	Ser(Bzl)
Threonine O-benzyl ether	Thr(Bzl)
N^{in}-cyclohexyloxycarbonyl-Tryptophan	Trp(Hoc)
O-(2-bromobenzyloxycarbonyl)-Tyrosine	Tyr(Br-Z)

Table 1. Side chain-protected amino acids employed in this work, and found to be compatible with TFMSA deprotection conditions. Note that His(Dnp) is presumably stable to TFMSA conditions, but is cleaved by thiol during the subsequent cleavage reaction. All other protecting groups are removed efficiently as described, except where denoted by an asterisk.

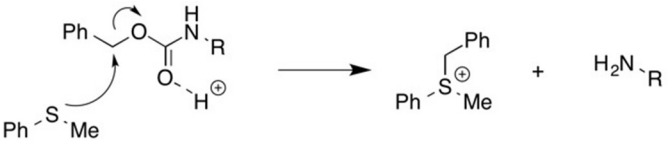
In general, no obvious issues (e.g., exacerbated aspartimide formation from Asp(OBzl)) due to the use of alternative protecting groups were encountered[††]. However, one comment on the use of Arg(Mbs) is warranted. In the absence of thioanisole, migration of the sulfonyl group to tyrosine reproducibly occurred at a level of ~30%, at least in one model system (see SI, **Table S7**). Use of thioanisole effectively suppressed this known side reaction, consistent with literature precedent[15].

A second finding further highlights the importance of thioanisole as an additive in TFMSA deprotections: at 0 °C, thioanisole was required for the efficient deprotection of Lys(Cl-Z) (**Table 2**):

Deprotection Conditions	Product Distribution (%)	
	Lys	Lys(Cl-Z)
TFMSA:TFA:*p*-cresol 1:8:1 0 °C, 1 hr	5	95
TFMSA:TFA:thioanisole:*p*-cresol 1:8:1:1 0 °C, 1 hr	>95	<5

Table 2. Effect of thioether additives on the efficiency of Lys(Cl-Z) deprotection, as determined by treatment of GFK(Cl-Z)AD(OcPent)AL-α*COSCH*$_2$*CH*$_2$*CO*-resins with select deprotection conditions. Deprotected peptide products were cleaved by treatment with MESNA buffer, and were characterized by LC-MS. The ratios of N$^{\varepsilon}$-Lys products were approximated by integration of the resulting UV chromatograms.

These observations are consistent with a "push-pull" mechanism, in which thioanisole accelerates the dealkylation of benzyl ethers, benzyl esters, and carbamates[16]:



The stability of Lys(Cl-Z) to TFMSA in the absence of thioanisole was surprising, given that Tyr(Br-Z) was

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deprotected efficiently under identical conditions (see SI), and that Lys(Cl-Z) and Tyr(Br-Z) exhibit comparable deprotection rates in TFA/dichloromethane[17].

Methionine alkylation by protecting group byproducts has been documented as a side reaction in TFMSA deprotections[12,18], and we were curious whether flow deprotections could alleviate this problem. To address this question, comparative flow and batch deprotections of the peptide MLK(Cl-Z)MK(Cl-Z)T(Bzl)T(Bzl)FY(Br-Z), which is reported to undergo extensive methionine alkylation in both HF (~30%) and TFMSA (~90%)[18], were carried out. Surprisingly, in no case was significant methionine alkylation observed (trace amounts of benzyl adducts are evident in the mass spectra of TFMSA-derived products), and comparable yields of crude peptides of similar quality were obtained in each case (**Figure 2**, **Table 3**). These results suggest that methionine alkylation is no more severe in TFMSA than in HF when conditions are chosen correctly, and that little if any benefit is conferred by the use of flow deprotections.

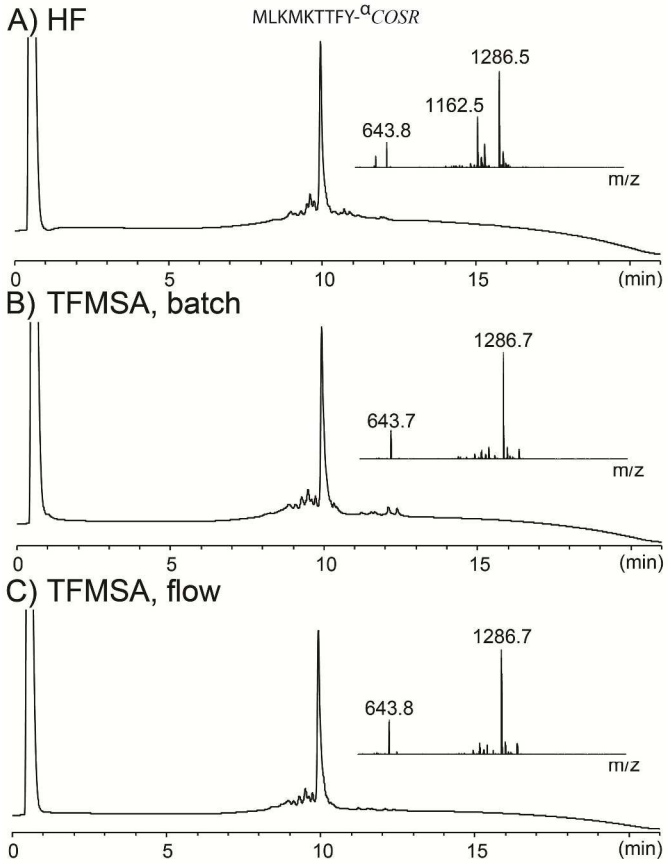
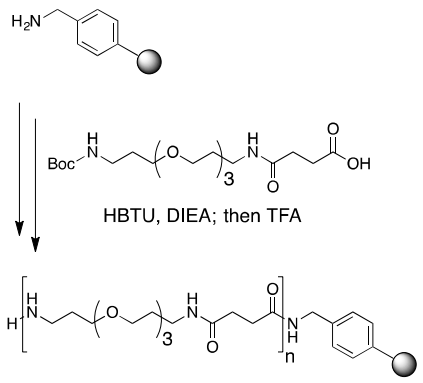


Figure 2. LC-MS data for nucleophilic cleavage of MLKMKTTFY-α*COSCH*$_2$*CH*$_2$*CO*-resins with 2-mercaptoethanesulfonate (MESNA), after side chain deprotection with: (A) HF:*p*-cresol 10:1 (v/v), 0 °C, 1 h; (B) TFMSA:TFA:thioanisole:*p*-cresol 1:8:1:1, 0 °C, 1 h; and (C) TFMSA:TFA:thioanisole:*p*-cresol 1:8:1:1, 0 °C, 3 x 5 min, 1 x 15 min, 1 x 30 min. Reaction products were: MLKMKTTFY-α*COSCH*$_2$*CH*$_2$*SO*$_3$*H* (exact mass: 1285.6 Da; found: (A) 1285.6 Da; (B) 1285.6 Da; (C) 1285.7 Da). A side product evident in the mass spectra of (A) was MLKMKTTFY-α*COOH* (exact mass: 1161.58 Da; average mass: 1162.46 Da; found: 1161.5 Da).

Further comparative deprotection studies were carried out on peptides spanning the sequence of the model protein crambin. In no case was there a significant benefit to the use of HF over TFMSA, nor of flow over batch TFMSA deprotection (**Table 3**; SI **Figures S4, S5, S6**).

The nucleophilic cleavage strategy described herein necessitated the use of resins compatible with *both* organic and aqueous solvents. For this purpose, we have employed aminomethyl copoly(styrene-1% divinylbenzene) resin functionalized with N-succinyl-4,7,10-trioxa-1,13-tridecanediamine ("TTD-Succ") monomers **(Scheme 2)**. Reliable resin preparations free of side reaction-promoting functional groups are key to effective SPPS[19], and the use of resins derived from aminomethyl-polystyrene and well-characterized small molecule linker moieties[20] was desirable in this regard. TTD-Succ-polystyrene resins[21] were conveniently prepared, gave excellent results in SPPS, and were sufficiently aqueous-compatible to enable the efficient thiolytic cleavage of resin-bound, deprotected peptides under aqueous conditions on the timescale of hours (**Table 3**). Two TTD-Succ units were generally sufficient to confer aqueous compatibility; in one model system, a single TTD-Succ monomer sufficed (see SI).



Scheme 2. Preparation of "TTD-Succ"-polystyrene resins for use in SPPS, followed by on-resin global side chain deprotection with TFMSA, and nucleophilic cleavage with the thiol reagent MESNA.

The guidelines reported here provide a framework toward achieving the long-sought goal of non-HF deprotections in Boc chemistry SPPS. Specifically, the two-step on-resin TFMSA deprotection/thiolytic cleavage strategy appears to be suitable for routine use for the preparation of peptide-α thioesters, which are essential starting materials for the synthesis of proteins by native chemical ligation. For specialized labs routinely employing the HF strategy, obviation of HF can increase the throughput of deprotections following Boc SPPS, as many reactions can be conducted in parallel (unlike for HF deprotections, which have limited throughput due to the specialized equipment required for safe handling). More generally, obviation of HF lowers the barrier to the use of Boc SPPS by non-specialists, and may stimulate renewed interest in Boc chemistry SPPS for applications where Fmoc SPPS proves unsatisfactory[22], or where the incorporation of base-sensitive functionalities during SPPS is required.

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Peptide-$^{\alpha}$thioester	Deprotection	Isolated yield
GHRH-$^{\alpha}$*COSR*	HF	47%
GHRH-$^{\alpha}$*COSR*	TFMSA "flow"	44%
MLKMKTTFY-$^{\alpha}$*COSR*	HF	43%
MLKMKTTFY-$^{\alpha}$*COSR*	TFMSA "flow"	49%
MLKMKTTFY-$^{\alpha}$*COSR*	TFMSA "batch"	46%
ACNRVYIHPFW-$^{\alpha}$*COSR*	HF	23%
ACNRVYIHPFW-$^{\alpha}$*COSR*	TFMSA "flow"	29%
Crambin 1-15-$^{\alpha}$*COSR*	HF	79%
Crambin 1-15-$^{\alpha}$*COSR*	TFMSA "flow"	79%
Crambin 1-15-$^{\alpha}$*COSR*	TFMSA "batch"	74%
Crambin 16-25-$^{\alpha}$*COSR*	HF	85%
Crambin 16-25-$^{\alpha}$*COSR*	TFMSA "flow"	78%
Crambin 16-25-$^{\alpha}$*COSR*	TFMSA "batch"	78%
Crambin 32-46-$^{\alpha}$*COSR*	HF	79%
Crambin 32-46-$^{\alpha}$*COSR*	TFMSA "flow"	74%
Crambin 32-46-$^{\alpha}$*COSR*	TFMSA "batch"	75%

Table 3. Isolated yields of peptide-$^{\alpha}$*thioesters* obtained from comparative deprotection/nucleophilic cleavage experiments. Crude reaction mixtures were de-salted by solid phase extraction (Grace-Vydac C18 cartridges), and lyophilized to yield the peptide-$^{\alpha}$*thioesters* as stable, non-hygroscopic powders.

Notes and references

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† Unlike HF, TFMSA remains strongly acidic in aqueous solution, and upon storage can cause degradation of lyophilized peptides derived from ether precipitation from TFMSA/TFA mixtures. On-resin deprotection minimizes this issue, and streamlines the separation of peptide products from deprotection reactants (which is a hazardous procedure, even for TFA deprotection/cleavage following Fmoc SPPS).
†† One observed side reaction *not* due to the use of alternative protecting groups was trifluoroacetylation, which reproducibly occurred to varying extent for N-terminal Ser and Thr peptides. This known side reaction[23] could be eliminated by acetylation of the N-terminus prior to side chain deprotection (see SI, **Figures S9, S10**). Where a free N-terminus Ser or Thr is required, use of a protecting group, stable to TFMSA deprotection, and subsequently removable under different conditions is recommended.

Electronic Supplementary Information (ESI) available: Experimental details, LC-MS chromatograms/spectra, and supporting data tables. See DOI: 10.1039/c000000x/

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