SUBJECT: Utility of Trifluoromethane Sulfonic Acid As a Cleavage Reagent In Solid-Phase Peptide Synthesis

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Forward

TFMSA: An alternative method to hydrogen fluoride cleavage/deprotection of synthetic peptides.

The following paper discusses research and development performed at Applied Biosystems concerning the use of trifluoromethanesulfonic acid (TFMSA) as a cleavage/deprotection reagent in solid phase peptide synthesis. TFMSA is clearly a viable option for those laboratories desiring a routine and economical alternative to hydrogen fluoride. Some features of this method are:

♦ TFMSA requires no specialized apparatus for containment during the cleavage procedure;
♦ This method may avoid many of the undesirable aspects associated with HF cleavage;
♦ Large number of TFMSA cleavages can be performed simultaneously; and
♦ Both large-scale and small-scale cleavages can be achieved with a minimum of development time.

In meeting our commitment to support the research needs of our customers, TFMSA-compatible amino acids and resins are now available from Applied Biosystems. We will also be providing high quality TFMSA in both 10-gram and 1-gram quantities very soon. A list of currently available TFMSA-compatible reagents can be found at the end of this publication.
WARNING  TFMSA is extremely corrosive and must be handled with great caution. All procedures must be conducted in an efficient* fume hood. Use of safety glasses, proper rubber gloves, and safety clothing is imperative.

* An efficient fume hood is defined as one that has a minimum face velocity of 0.51 meters/second (100 ft/min) as the average velocity of the open area. This can be measured using an anemometer. Determine that velocity meets or exceeds local government regulations. Fumes from the hood must be safely directed out of the building.
Introduction

An earlier User Bulletin described our initial experiences with trifluoromethanesulfonic acid (TFMSA) as an alternative to liquid hydrogen fluoride (HF) in cleavage/deprotection of peptides synthesized according to automated ("t-Boc") solid phase protocols. Since that time we have developed the technique further, more fully defining the role of thiol scavengers, and clarifying requirements for amino acid side-chain protection. The results are presented in this paper, which details our progress to date and offers specific examples of peptides obtained from TFMSA cleavage, including comparative HF data where applicable.

Materials and Methods

Chemicals and reagents were as stated previously. Boc-Trp (N\textsuperscript{0}-MTS) and Boc-Arg (N\textsuperscript{\textalpha}-MTS) [MTS = Mesitylene-2-Sulfonyl-] were supplied by Applied Biosystems as the dicyclohexylamine and cyclohexylamine salts respectively. Before use, each salt was converted into its free acid by partitioning 10 mM lots between 100 mL 0.5M aqueous HCl (chilled) and 200 mL CH\textsubscript{2}Cl\textsubscript{2}, washing the organic phase with 3x20 mL portions of the dilute HCl solution, then 2x50 mL saturated NaCl. The separated CH\textsubscript{2}Cl\textsubscript{2} solution was dried over anhydrous MgSO\textsubscript{4}, filtered, and dried down on a rotary evaporator under vacuum. The resulting glass was pulverized and stored in a desiccator at -15°C until needed.

Peptides were assembled on an Applied Biosystems Model 430A Peptide Synthesizer. Chemicals and reagents were supplied by the manufacturer including t-Boc amino acids with side-chain protection as summarized in Table 1, or as otherwise noted in the text. Attachment of the C-terminus amino acid to the polystyrene resin was achieved through either phenylacetoamidomethyl (PAM) or 4-methylbenzhydridylamino (MBHA) linkages.

Cleavage Procedure

A. Small Scale (100 mg.)

1. Place the resin-bound peptide into a 10-mL round-bottom flask supplied with a glass stopper and micro stir-bar.

2. Add 150 µL of thioanisole: EDT (2:1) and stir for 10 minutes at room temperature.

3. Add 1 mL of neat TFA and stir 5-10 minutes.

4. Slowly add 100 µL of TFMSA dropwise with vigorous stirring.

Note: Addition of TFMSA produces heat. This can be minimized by careful addition of the TFMSA.
5. Allow the mixture to react for 15-30 minutes. Peptides anchored by amide bonds to MBHA resin require 1 1/2 - 2 1/2 hours reaction time for maximum cleavage yields.

6. Pipette the contents of the vial to a sintered glass funnel (coarse porosity) that has been fitted with a collection flask containing approximately 25 mL of methyl t-butyl ether (MBE). The resin will be trapped on the filter allowing the TFA solution to pass through. The peptide will precipitate, and acidic scavenger materials will remain in solution.

7. Rinse the reaction vial three times with 0.5 mL TFA. Transfer each rinse to the glass funnel and re-suspend the solid residue to ensure complete washing. Combine filtrates with the peptide and refrigerate briefly to complete peptide deposition.

8. Collect the precipitate, washing the solid thoroughly with small portions of MBE.

9. Dissolve the dried crude powder in an appropriate aqueous solvent, lyophylize resultant filtered solution. Prior to lyophilization, the crude peptide solution can also be desalted by gel filtration in the usual manner.

B. Large Scale (1 gram)

1. Place the resin in a 100-mL stoppered round-bottom flask equipped with a stir-bar.

2. Add 1.5 mL scavenger mix, and stir 5-10 minutes.

3. Slowly add 15 mL of a freshy prepared solution of TFMSA-TFA (1:10 v/v).

   **Note** If convenient, the reaction flask may be immersed in an ice-bath to facilitate more rapid addition of acidic reagent without undue evolution of heat.

4. Stir 1/2 hour (or about 2 hours in the case of amide-linked peptide-resin) at room temperature (20°C).

5. Contents of the flask are then transferred to a sintered glass funnel (coarse porosity). Place over a collection flask containing approximately 250 mL MBE, and then treat as in steps 7 through 9 in the small scale protocol. Adjust volumes for larger scale.

All manipulations must be carried out in a ventilated fume hood to minimize exposure to volatile acid and foul-smelling thiol fumes.
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To scale up or down from either of the two methods, alter the amount of reagents to the weight of resin in direct proportion to the recommended ratios described above.

To assess the degree of cleavage achieved, the recovered resin is neutralized with a dilute solution of diisopropylethylamine in CH₂Cl₂, rinsed thoroughly, dried, then subjected to the quantitative ninhydrin procedure to determine residual amine content².

Chromatography
All analyses were performed on an Applied Biosystems Series 4 Liquid Chromatograph, fitted with an Applied Biosystems ISS-100 autosampler and LCI-100 integrator. The analytical column was a 25 x 0.46 cm Vydac C₁₈ (300 Angstrom, 5µm), operated at 45°C and 1.0 mL/min flow rate. Peaks were detected at either 215 or 280 nm, using a Kratos Model 773 variable wavelength UV detector. A ternary gradient program was utilized (Method 11), where solvent A was 25:75 MeOH-H₂O, solvent B was 1% aqueous TFA, and solvent C was CH₃CN. The gradient was developed from 0 to 40°C over 14 minutes followed by a ramp to 60°C over an additional 2 minutes while holding B constant at 10% throughout the run (i.e., mobile phase @ 0.1% TFA). A modification of Method 11 is detailed on pertinent chromatograms.

Results and Discussion

Representative Cleavages
Table 1 lists the peptides to which TFMSA cleavage methodology has been applied. Some of these peptides have also been prepared with various combinations of protecting groups, resulting in a total of 35 peptides exposed to the TFMSA technique. All peptides were successfully cleaved with the exception of peptide 24, (porcine insulin 1-14). This peptide required approximately a 5-hour reaction time to liberate >90% of the theoretically available peptide from the MBHA resin support. The crude product itself was difficult to characterize due to considerable solubility problems.

Table 1. Peptides Subjected to TFMSA Cleavage

1. H-Leu-Ala-Gly-Val-OH
2. H-Leu-Ala-Gly-Val-HN₂
3. H-Glu-Asp-Gly-Thr-OH
5. [ASP¹⁰]-ACP
6. [Ser¹⁰]-ACP
Table 1. Peptides Subjected to TFMSA Cleavage (continued)

7. [Thr\textsuperscript{10}]-ACP
8. H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH (ACTH 1-10)
9. H-Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH\textsubscript{2}
   (Substance P)
12. H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH\textsubscript{2} (Oxytocin)
13. H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-NH\textsubscript{2}
   (Somatostatin amide)
14. [Pro\textsuperscript{7}]-ACP
15. [Gly\textsuperscript{7}]-ACP
17. Somatostatin-OH
18. H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-OH
19. H-Ser-Tyr-Ser-Met-Glu-His-Phe-Trp-Gly-OH
20. H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-OH
21. H-Ser-Tyr-Ser-Met-Glu-His-Phe-NH\textsubscript{2}
   Cys-Asn-Ser-Phe-Arg-OH (ANF-23)
23. p Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH\textsubscript{2}
   (Gastrin I)
24. H-Gly-Ile-Val-Glu-Cys-Ser-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-NH\textsubscript{2}
   (porcine insulin 1-14)

Chromatograms of 100 mg, small-scale, and 1.3 g, large-scale, cleavages of peptide 4
(ACP 65-74) are shown (Figs. 1a, 1b). In each case >99% of available peptide was
removed, as determined by quantitative ninhydrin assay of the recovered resin. This
assay is a reliable indication of cleavage efficiency, because crude (lyophilized)
product weight usually exceeds theoretical weight due to the presence of water
soluble salts not removed prior to freeze drying. (Note the virtual identity of the crude
product profile between small and large scale cleavages).
Figure 1. HPLC analysis of TFMSA cleavage of peptide 4, ACP 65-74. Conditions: gradient Method 11, 215 nm, Peak A = peptide 4; (a) small scale (100 mg), (b) large-scale (1.3 g).

Figures 2 through 7 show HPLC assays of crude products from selected cleavages. Products were obtained by the TFMSA methodology described in the Materials and Methods section, or modified as noted in the respective legends. Chromatograms of the corresponding HF cleavage products, where available, are also included; the HF crudes were generated under standard conditions (HF-anisole 10:1 v/v, 0°C, 1 hr), except where noted. No optimization of HF cleavage conditions was carried out.

In some instances, the protected amino acids used in chain assembly which were compatible with TFMSA chemistry may be less favored for HF applications.

Deprotection

Current results reaffirms our previous conclusion regarding side-chain protection of Arg and Cys as their obligatory MTS and 4-methoxybenzyl (MOB) derivatives respectively. It must be emphasized that use of side-chain protecting groups for Arg and Cys, other than those described, will yield substantially inferior products under TFMSA conditions.

Trp (N\textsuperscript{in}-MTS) is the preferred substrate for TFMSA chemistry (Fig. 2a), though the (N\textsuperscript{in}-CHO) derivative can also be efficiently deprotected using the thioanisole-EDT cocktail (Fig. 3a). However, we have found that formylated Trp-peptides sometimes generate insoluble nonpeptidic polymeric material on TFMSA cleavage which interferes with subsequent sample work-up by clogging the fritted disk during filtration. Also, the isolated crude peptides are usually somewhat colored (yellow to pale
yellow-orange) compared to the white to off-white crudes obtained from cleavages of the analogous MTS-protected Trp peptides.

Figure 2. HPLC analysis of TFMSA and HF cleavage products of peptide 8, ACTH 1-10, where Arg and Trp had MTS side chain protection, and Met was incorporated as either its reduced form (a, b) or its sulfoxide (c).

**Conditions:** gradient Method 11, 215 nm, Peak A = Met (0)–8, Peak B = Met–8; (a) std. TFMSA, (b) std. HF (c) TFMSA on Met(0)–peptide resin using a 1:1:1 mixture of anisole–thioanisole–m–cresol as scavengers.

Histidine can be readily deprotected in either its (Nim–Tosyl) or (Nim–Z, carbobenzyloxy) derivative. The (Nim–DNP, dinitrophenyl) was not evaluated because of its well-known resistance to acidic cleavage. Methionine should be incorporated as its sulfoxide [Met(0)], both to avoid alkylation during chain assembly\(^3\) and to minimize side products from cleavage. Normally, one can expect 10-40% of the (reduced) Met peptide to be formed under standard TFMSA cleavage of Met(0)–peptides (e.g., Fig. 3a). However, we find that exposure of certain Met(0) peptides to a TFMSA–TFA solution containing dimethyl sulfide quantitatively reduces Met(0)– to Met–peptide (Fig. 4b). Data on this will be presented in a future publication. Methods which have been developed previously for reduction of Met(0) peptides include incubation with thioglycolic acid\(^4\) or N–methylmercaptoacetamide\(^5\). More recently, the use of dimethyl sulfide in the presence of low concentrations of HF\(^6\) has led to successful reduction during cleavage.
Figure 3. HPLC analysis of TFMSA and HF cleavages of peptide 19.
Conditions: gradient Method 11, 215 nm, Trp(CHO) and Met(0) protection,
Peak A = Met(0)–, Trp(H)– 19, Peak B = Met(0)–, Trp(CHO)– 19, Peak C = Met, Trp(H)– 19,
Peak D = Met, Trp(CHO)– 19; (a) std. TFMSA (b) Std. HF, (c) TFMSA using 1:1:1 mixture of
anisole–thioanisole–m–cresol as scavengers. Note absence of Trp deprotection.
Figure 4. HPLC analyses of TFMSA and HF cleavages of Peptide 23, Gastrin-I. Conditions: Modified gradient (0 to 40% C in 16 min, then 50% C in 4 min), 280 nm, Met(0) and Trp(CHO) protection, Peak A = Gastrin I; (a) “low-high” HF cleavage (b) TFMSA/dimethyl sulfide followed by std. TFMSA cleavage.

We also found that protection of Asp as its β-cyclohexyl ester renders the residue refractory to efficient side-chain deprotection (Fig. 5). Such protection is not recommended in the context of TFMSA cleavages. Cyclohexyl (or cyclopentyl) esters were conceived as a stratagem in classical solid phase synthesis/HF cleavage methodologies to avoid sequence-dependent “aspartimide” (or glutarimide) formation. In the sole case where appreciable rearrangement of an Asp-peptide on TFMSA cleavage was noted, (peptide 15, Fig. 6a), reduction of the cleavage reaction temperature significantly suppressed the incidence of this unwanted by-product (Fig. 6b).
Figure 5. HPLC analysis of TFMSA cleavage of peptide 10, where Asp, Cys, and Arg have O–cyclohexyl (OcHx), S–(4–Methyl)benzyl (MBzl), and N[^alpha]-MTS protection respectively. Conditions: gradient method 11, 215 nm, Peak A = authentic peptide, Peak B = Asp (O–cyclohexyl)–10, Peak C = Cys [S–(4–Methyl)benzyl]–10, Peak D = Asp (O–cyclohexyl, Cys [S–(4–Methyl)benzyl])–10.

Identity of the rearrangement product was confirmed by quantitative N–terminus sequencing of a preparatively purified sample of Peak B, which showed blocking of Edman degradation at the sixth cycle. The major product, Peak A, (Figs. 6a, 6b), sequenced normally to the carboxy terminus. Low-temperature reaction, as a method for minimizing β–carboxyl cyclization, was noted also by Yajima and Fujii in a study on TFMSA and methane sulfonic acid (MSA) deprotection of model Asp-containing dipeptides."
Figure 6. HPLC analysis of TFMSA and HF cleavages of Peptide 15, where Asp had O–Bz protection. Conditions: gradient Method 11, 215 nm, Asp (Bz) protection, Peak A = [Gly\(^7\)]–ACP, Peak B = “aspartimide” (or B–peptide) rearrangement product; (a) std. TFMSA, (b) std. TFMSA, conducted at -10°C, 1 hr.

Table 2 summarizes the appropriate side-chain protection found experimentally to be compatible with TFMSA cleavage/deprotection chemistry.

**Table 2. Amino Acid Side-Chain Protection Compatible with TFMSA Cleavage**

<table>
<thead>
<tr>
<th>Protection</th>
<th>Protection</th>
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<tr>
<td>Glu(OBzl)</td>
<td>Lys(Cl–Z)</td>
</tr>
<tr>
<td>Asp(OBzl)</td>
<td>Arg(MTS)</td>
</tr>
<tr>
<td>His(Z), His(Tos)</td>
<td>Cys(Mob)</td>
</tr>
<tr>
<td>Ser(Bzl)</td>
<td>Met(0)</td>
</tr>
<tr>
<td>Thr(Bzl)</td>
<td>Trp(MTS)</td>
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<tr>
<td>Tyr(Br–Z)</td>
<td>Trp(CHO)</td>
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The Role of Thiolated Adjuvants

After performing hundreds of cleavages on more than 35 differently substituted and/or protected peptides on solid phase support, it is concluded that the type and composition of so-called scavengers used in the reaction mixture profoundly affect product yield both quantitatively and qualitatively. Aryl thiol ethers and thiols serve as reaction promoters as well as mere carbocation acceptors. Indeed, we find that
thioanisole is a requirement for efficient cleavage from both PAM and, especially, MBHA-type supports. Thioanisole functions as a “push-pull” catalyst for scission of benzylic substrates. Typically we can cleave 90-99% of the theoretically available peptide from PAM resin and 70-80% from MBHA resin using thioanisole. Substituting phenols or phenolic ethers for thioanisole reduces overall cleavage yields by 25-90%, based upon quantitative ninydrin assay of recovered resin.

As a corollary to the above, we note that the presence of dialkylthioethers severely retards the scission of peptide from the support, despite inclusion of thioanisole under the conditions as described. Ethylmethyl sulfide, dimethyl sulfide, tetrahydrothiophene, etc., when present in amounts equal to or as low as one-half (v/v) the thioanisole level in the reaction mixture, all suppress peptide cleavage to varying degrees (40-80%).

Ethanedithiol (EDT) is an absolute requirement for removal of the formyl group in Trp (N\textsuperscript{\text{\textalpha}}-CHO) residues (Figs. 3a, 5b). Its usefulness in catalyzing deformylation of Trp (N\textsuperscript{\text{\textalpha}}-CHO) peptides has been observed previously in HF cleavages. Also, EDT apparently serving as a reducing agent under the strongly acidic cleavage condition, significantly minimizes formation of dimers or other types of -S–S– aggregates in cysteinyl peptides.

Finally, when cleaving cysteinyl peptides, our data show that such adjuvants as m– or p– thiocresol or p–methoxythiophenol are contraindicated because their use produces either S–adducts or other –S–S– dimerization. Although thiophenols also catalyze deformylation of Trp(N\textsuperscript{\text{\textalpha}}-CHO), their deleterious effects on cysteinyl peptides further recommended EDT as the thiol of choice for deprotection of Trp(N\textsuperscript{\text{\textalpha}}-CHO). Anisole, or the cresols, used in conjunction with thioanisole, appear to be neutral in their effects, neither enhancing nor degrading product quality.

Table 3 summarizes the properties of the thiol adjuvants used in TFMSA cleavages.

**Table 3. Role of Thio Additives**

| Thioanisole | 1. Carboxation acceptor (cation scavenger)  
| 2. Accelerates acidolysis |
| Ethanedithiol | 1. N\textsuperscript{\text{\textalpha}}-formyl deprotection of Trp(CHO)  
| 2. Reducing properties minimizes formation of Cys–Cys dimers  
| 3. Partial reduction of Met(0) |
| Dimethylsulfide | 1. Reduces Met(0) to Met |
Conclusion

The data in this study support the conclusion that highly efficient peptide chain assembly on a solid support will be reflected in the quality of crude product obtainable from TFMSA cleavage in most instances (e.g., Fig. 7b). Crude product profiles compare favorably to those obtained from classical HF approaches, including peptides generally regarded as difficult to prepare, such as peptide 13, or to isolate, such as peptides 22 and 23. However, a less than optimal chain assembly (Fig. 7c), incorrect choice of protected amino acid precursor for synthesis, or injudicious use of scavenger types on cleavage will result in a complex mixture with low product recovery.

Figure 7. HPLC analysis of TFMSA and HF cleavages of peptide 22, ANF–23. Conditions: gradient Method 11, 215 nm, Peak A = (linear) authentic peptide, other peaks not identified; (a) Std. HF, where Arg and Cys have N14–Tos and S–(40–Methyl)benzyl protection respectively, and coupling efficiencies >99% for all residues, (b) std. TFMSA, where Arg and Cys have N33–MTS and S–Mob protection respectively, and coupling efficiencies >99% for all residues, (c) std. TFMSA, where Arg and Cys protected as in (b), and coupling efficiencies at several residues were only 80-90%.

While our studies strongly support general application of the standard TFMSA method described in this Bulletin, it is recommended that cleavages first be conducted on a small scale for those resin-bound peptides known or suspected to be particularly labile to strong acid exposure. Changes in reaction conditions could well induce more
tractable crude product profiles. As previously discussed, the cleavage chemistry is dramatically influenced by thiol adjuvant composition. This allows manipulation of reaction conditions in a variety of ways that may lead to higher product recoveries in certain cases. For example, one could retain formyl protection of acid-sensitive Trp-peptides by eliminating EDT from the cleavage reaction, then subsequently complete deprotection of the resultant crude peptide through gentle base-catalyzed hydrolysis of the formyl moiety.

Once the cleavage conditions are defined for a particular peptide application, there is a high degree of reproducibility of crude product profile on subsequent cleavages. Any variability noted was usually ascribed to differences in chain assembly coupling yields between duplicated syntheses.

Addendum

Tam et al, recently described in great detail the use of TFMSA in deprotection of resin-bound peptides14. Refer to this article for further elaboration on other aspects of thiol-mediated strong-acid cleavage techniques.

References