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Short Communication

On choosing the right ether for peptide precipitation after acid cleavage

BEATRIZ G. DE LA TORRE and DAVID ANDREU*
Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona Biomedical Research Park, 08003 Barcelona, Spain

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Abstract: Methyl *tert*-butyl ether (MTBE) and diethyl ether (DEE) tend to be regarded as interchangeable for the 'cold ether' workup concluding the final acidolytic cleavage and deprotection step of solid-phase peptide syntheses. However, the use of MTBE to precipitate peptides from strong acid solutions is shown to give rise to *t*-butyl alkylation byproducts, readily detectable by MALDI-TOF MS. The problem can attain undesirable dimensions in the cleavage of peptide resins containing high proportions of aromatic residues, particularly in peptide nucleic acid (PNA) syntheses. In those cases, DEE workup is advisable, as it consistently leads to cleaner products. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: acidolytic cleavage; diethyl ether; methyl *tert*-butyl ether; TFA; TFMSA

INTRODUCTION

The two main strategies of solid-phase peptide synthesis, the so-called Boc/benzyl and Fmoc/*t*-butyl chemistries, both rely on a final acidolytic step for removal of side-chain protecting groups and simultaneous cleavage of the peptide from the resin [1,2]. In Boc chemistry, such treatment involves strong acids such as anhydrous HF [3], TFMSA, [4,5] or trimethylsilyl trifluoromethane sulfonate (TMSOTf) [6]. Although HF has a long record of successful utilization, the latter two acids, and TFMSA in particular, are somewhat advantageous in that they do not require special, costly Teflon labware as the former, nor are they subject to the increasingly demanding safety and environmental regulations applicable to HF. In Fmoc chemistry, TFA (trifluoroacetic acid)-containing cocktails are used, practically with no exception, in the final acidolysis step.

In both Boc and Fmoc strategies, the final acidolysis step includes a 'cold ether' treatment, with slightly different purposes in each case. In HF cleavages, which typically conclude with an evaporation of HF under vacuum, the role of the cold ether is mainly to solubilize nonvolatile scavengers and organic byproducts (i.e. protecting group derivatives) remaining in the evaporation residue, while ensuring that the peptide remains precipitated, plus capturing (as a Lewis base) any residual traces of HF. In TFMSA and TFA cleavages, where evaporation of the acid is not feasible, the main goal of the cold ether step in the postcleavage workup is precipitating out the peptide from the acidolysis mixture.

Not much attention has been given over the years to which ether is best to use for these treatments. While diethyl ether (DEE) was usually the choice in earlier work, more recently a trend toward peroxide-free methyl *tert*-butyl ether (MTBE) can be observed. This switch from DEE to MTBE assumes the practical equivalence of both ethers as peptide precipitation agents, a notion implicit in several laboratory guidelines [7]. In this communication we show that such 'equivalence' must be regarded with caution, particularly in TFMSA and/or TFA cleavages, where MTBE workup tends to result in alkylation byproducts bearing one or more C_4H_9 units, detectable as $M+56$, $M+112$ peaks. Three relevant instances (Figure 1) of how MTBE workup complicates the final product are discussed below.

For the 14-residue peptide nucleic acid (PNA) sequence **1** in Figure 1, a Boc-based synthetic approach was chosen, with TFMSA cleavage (due to temporary unavailability of an HF line) and MTBE workup. Analysis of the cleavage product (Figure 2a) showed the target PNA to be accompanied by *ca* 40% of a byproduct of +56 mass units. In order to discard the interference of the only ^{t}Bu-based protecting group in the sequence, a cleavage with prior removal of the *N*-terminal Boc [8] was performed, but the problem persisted (Figure 2b). Use of different TFMSA/TFA ratios or scavengers did not eliminate the +56 peak (data not shown), whereas a cleavage cocktail containing ethanedithiol (EDT), allegedly a very efficient ^{t}Bu cation scavenger [9], considerably worsened the product distribution (Figure 2c).

A second example of the problem was found in a routine Fmoc synthesis of a 18-residue peptide

*Correspondence to: David Andreu, Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona Biomedical Research Park, Dr. Aiguader 88, 08003 Barcelona, Spain;
e-mail: david.andreu@upf.edu

1 cta cgt aga cca ct-Cys-Gly-NH_2

2 Ile-Trp-Lys-Ala-Ser-Phe-Thr-Thr-Phe-Thr-Val-Thr-Lys-Tyr-Trp-Phe-Tyr-Arg-NH_2

3a Lys-Trp-Lys(Me_3)-Leu-Phe-Lys(Me_3)-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-NH_2

3b Lys-Trp-Lys-Leu-Phe-Lys(Me_3)- Lys (Me_3)-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-NH_2

Figure 1 PNA and peptide sequences discussed in this study.

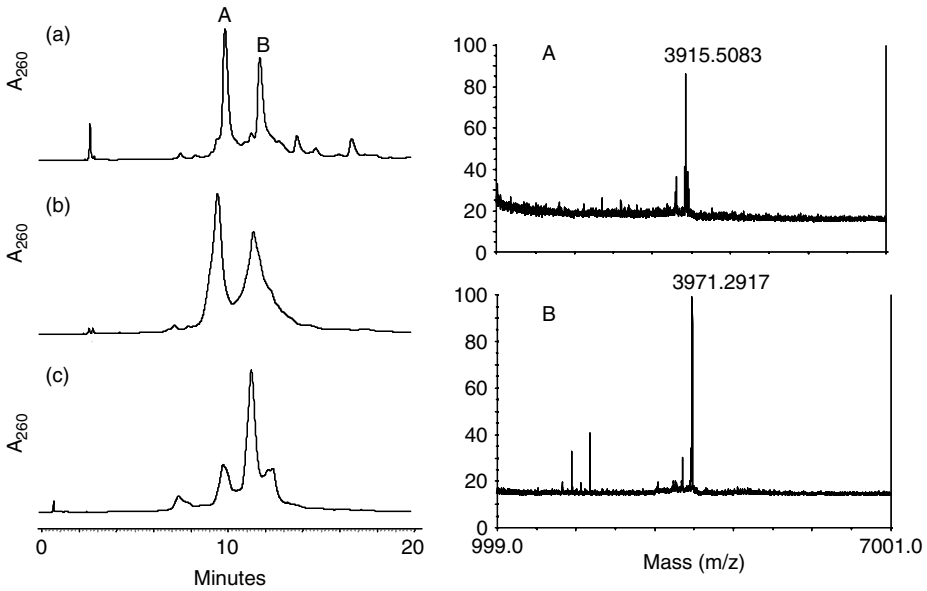

Figure 2 HPLC analysis of the cleavage mixture from a PNA-peptide oligomer synthesis (Figure 1, peptide **1** using MTBE workup. (2a) Standard TMFSA cleavage (see text), without prior removal of *N*-terminal Boc group. Insets: MALDI-TOF mass spectra of main peaks A (target compound) and B (+56 Da butyl byproduct). (2b) As above, with Boc group removed before cleavage. (2c) As in (2b), using an EDT-containing cleavage cocktail [TFA/TFMSA/$(iPr)_3SiH$/EDT, 8 : 3 : 1 : 1 v/v, 90 min]. Nucleosil column (4.6 × 250 mm, 5 μm), linear gradient from 5 to 35% B over 20 min.

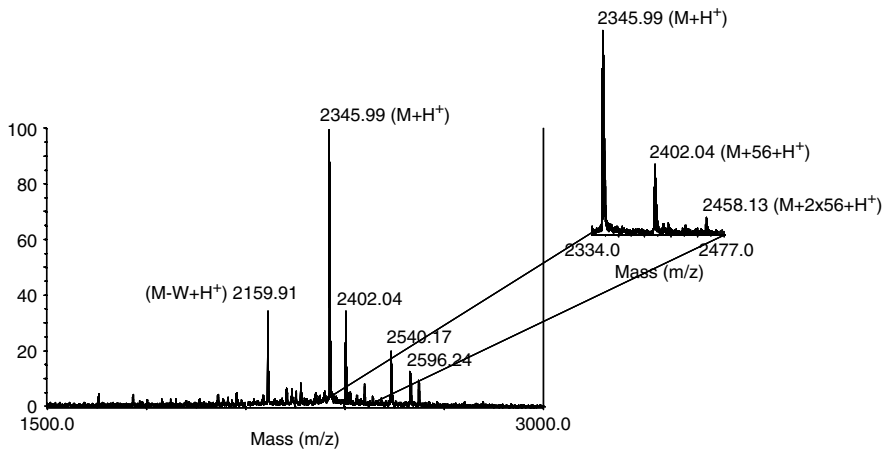

Figure 3 MALDI-TOF spectrum of the crude, cleaved product from peptide **2** synthesis (Figure 1) using MTBE workup. Expanded molecular ion area shows the presence of two ^{t}Bu adducts (+56 and +112 Da). The unassigned peak at *m/z* 2540 also has its +56 satellite.

2 containing six aromatic residues (Figure 1). Again, cleavage with a standard TFA/H_2O/$(iPr)_3SiH$ mixture followed by MTBE workup led to a crude material with quite significant M + 56 and M + 112 peaks in the MALDI-TOF mass spectrum (Figure 3). This MS observation has been repeated in several other Fmoc syntheses where MTBE postcleavage workup is used. The extent of *t*-butylation is not comparable to that

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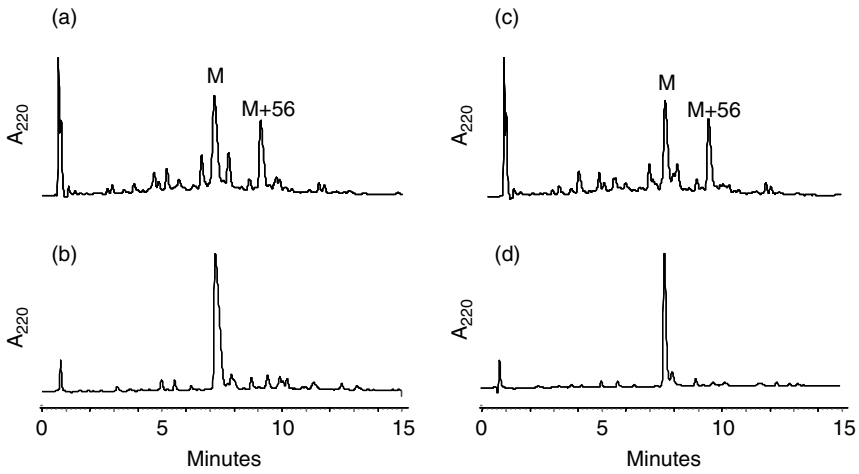

Figure 4 HPLC analysis of crude cleaved product from peptides **3a** (panels 4a, 4b) and **3b** (panels 4c, 4d) syntheses (Figure 1). Panels 4a, 4c: MTBE workup; panels 4b, 4d: DEE workup. Phenomenex Luna column (4.6 × 50 mm, 3 μm), linear gradient from 20 to 40% B over 15 min.

observed in TFMSA cleavages (i.e. usually a very minor HPLC impurity, though clearly detectable by MS) and appears to depend on the number of alkylation-prone residues (Trp, Tyr, Cys, Met).

Further illustration of the not-quite-equivalent role of the two ethers is provided by the two 15-residue peptides **3a** and **3b** in Figure 1, each containing a Trp residue and ε-trimethyllysine residues at various positions. Both peptides were assembled by Boc chemistry, N^{α}-deprotected (30% TFA), cleaved by TFA/TFMSA/$(iPr)_3SiH$ (8 : 3 : 1 v/v, 90 min), then worked up with either MTBE or DEE. In the first case, substantial peaks at +56 were again observed in the MALDI-TOF spectra of both crude materials (Figure 4). In contrast, the DEE-precipitated products gave each practically a single peak by both MS and HPLC.

The above results clearly indicate the nonequivalence of MTBE and DEE as promoters of peptide precipitation from TFMSA or TFA cleavage mixtures. In the first case, significant levels of ^{t}Bu cations are to be expected, not from butyl-based protecting groups in the peptide resin (discussed in some detail in a report on a multicenter study of peptide synthesis facilities, see Ref. 10, especially pp. 544–545) but from ^{t}Bu–O cleavage of MTBE itself under the prevailing strongly acidic conditions. For peptide sequences lacking the alkylation-prone residues, the problem may be minor and can go essentially undetected. However, for sequences rich in aromatic (including PNA nucleobases) or alkylation-prone residues, our data clearly support DEE as the peptide precipitation agent of choice.

EXPERIMENTAL PART

Boc–PNA monomers were purchased from Applied Biosystems (Foster City, CA); Boc- and Fmoc-protected amino acids were from Senn Chemicals (Dielsdorf, Switzerland). *p*-Methylbenzhydrylamine (MBHA) resin and Fmoc-Rink-amide (MBHA) resin were from Novabiochem (Laüfelfingen, Switzerland), HBTU and HOBt from Matrix Innovation (Montreal, Canada), and HATU from GenScript Corporation (Piscataway, NJ). HPLC-grade acetonitrile and peptide synthesis-grade solvents [*N*,*N*-dimethylformamide (DMF), dichloromethane (DCM)] and reagents (diisopropylethylamine (DIEA), TFA) were from SDS (Peypin, France). TFMSA and other reagents were from Sigma-Aldrich (Madrid, Spain).

Solid-phase Boc-based synthesis in the manual mode was carried out in polypropylene syringes fitted with a polyethylene frit. Boc-amino acids were coupled on MBHA resin using standard protocols with DIPCDI activation, and Boc–PNA monomers by means of HATU on an MBHA resin, following the synthesis cycle for the Boc/Z strategy described elsewhere [11]. Protected peptide- or PNA resins were cleaved by TFA/TFMSA/$(iPr)_3SiH$ (8 : 3 : 1 v/v, 90 min), precipitated by addition of chilled MTBE or DEE, solubilized in aqueous HOAc (10% v/v) and lyophilized.

Fmoc-based automated synthesis was performed in an Applied Biosystems (Foster City, CA, USA) model 433 peptide synthesizer employing standard 0.1-mmol-scale FastMoc protocols on Rink-amide MBHA resin. Eight-fold excesses of Fmoc-amino acids, HBTU, HOBt, and 16-folds of DIEA were used in the coupling steps. Protected peptide resins were N-deblocked with piperidine (20% v/v in DMF) prior to full deprotection and cleavage with TFA-water-$(iPr)_3SiH$ (95 : 2.5 : 2.5 v/v, 90 min, rt). Peptides were precipitated and worked up as above.

Analytical reversed-phase HPLC was performed on C_{18} columns. Solvents A and B were 0.045% TFA in H_2O and 0.036% TFA in acetonitrile (ACN), respectively. Linear gradients of solvent B into A were used for elution, at 1 ml/min flow rates, with UV detection at 220 nm (see figure captions for further details). Further characterization of the synthetic products was done by MALDI-TOF MS in a Voyager DE-STR system (Applied Biosystems, Foster City, CA) using sinapinic or α-hydroxycinnamic acid matrices.

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