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Selective, On-Resin *N*-Methylation of Peptide *N*-Trifluoroacetamides

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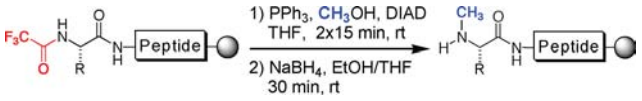
ABSTRACT



Mild and efficient methods for site-specific methylation of peptide backbone amides are important tools for chemists seeking to modulate the pharmacokinetic properties of peptide drugs. The Mitsunobu reaction was used to selectively methylate *N*-trifluoroacetamide (Tfa) protected peptides on-resin. The Tfa group was removed quickly and completely by reduction with excess $NaBH_4$, and it was shown to be orthogonal to many of the protecting groups used in solid-phase peptide synthesis.

Backbone amide methylation is a hallmark feature of cyclic peptide natural products and influences both the physical properties and the conformational states of these molecules. For example, the orally bioavailable cyclic peptide immunosuppresant drug cyclosporin A is *N*-methylated at seven of its 11 backbone amides, at least three of which are essential for its bioactivity and bioavailability.[1] Several researchers have shown that *N*-methylation of backbone amides can enhance the potency, selectivity, and bioavailability of cyclic peptides relative to their unmethylated precursors.[2,3] Consequently, the growing demand for selectively *N*-methylated peptides has spawned an interest in the development of mild and efficient methods for site-specific methylation of peptide backbone amides on the solid phase.

Even though the demand for *N*-methylated amino acids has increased significantly in recent years, only a few of these building blocks are commercially available. Methods exist for the solution-phase conversion of unprotected,[4] Boc-protected,[5] or Fmoc-protected[6] α amino acids into their *N*-methyl cognates; however, coupling *N*-protected *N*-methyl amino acids onto resin-bound peptides can be especially challenging when there are multiple, contiguous *N*-methylated residues in the sequence.[7] As a result, methods that enable on-resin backbone amide *N*-methylation are highly desirable, even though they add additional steps to a synthesis.

Virtually all of the methods reported for on-resin, site-specific backbone amide methylation have utilized the *N*-arylsulfonamide moiety to generate a nucleophilic anion

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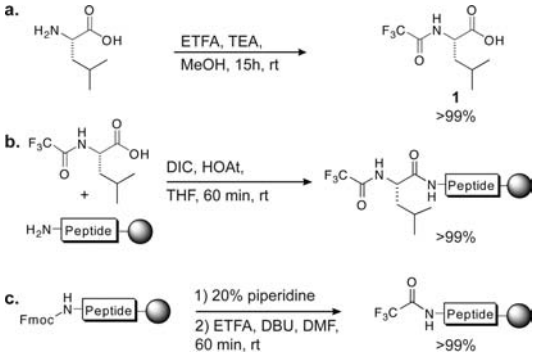
that can be alkylated using methyl *p*-nitrobenzenesulfonate,[8] dimethyl sulfate,[9] alkyl halides,[10] or Mitsunobu conditions.[9b,10a,10b,11] Following on the initial reports of Miller and Scanlan,[8] the Kessler group has published widely on the use of the *o*-nitrobenzenesulfonamide (*o*-NBS) protection group to facilitate selective methylation of peptide backbone amides using the Mitsunobu reaction (see ref 12 for a review). We have found that selective methylation of the *o*-NBS group using Mitsunobu conditions is indeed efficient; however, removal of the *o*-NBS from *N*-methylated residues is often unpredictable and sequence dependent. Herein, we report the uprecedented use of *N*-trifluoroacetamide (Tfa) as an *N*-terminal protecting group to facilitate rapid and efficient on-resin *N*-methylation via the Mitsunobu reaction. Importantly, the Tfa group is readily removed using conditions that are orthogonal to most standard SPPS protecting groups, making this a significant advance in the area of site-selective peptide *N*-methylation.

We investigated other amine protection groups that are reactive toward Mitsunobu methylation while still affording mild and reliable cleavage conditions. The *N*-trifluoroacetamide is known to form a stabilized anion,[13] and its use has been demonstrated in a solution-phase, intramolecular Mitsunobu reaction.[14] It is also reputed to be one of the most labile amides used as a protection group.[15] These reports led us to test the reactivity and selectivity of the Tfa group in the site-specific *N*-methylation of resin-bound peptides, which has not been reported in the literature.

The dipeptide Tfa-L-Leu-L-Leu was synthesized on the 2-chlorotrityl resin using two different methods. In the first method, Tfa-protected L-leucine was synthesized using the procedure reported by Curphey (Scheme 1a).[16] The product was isolated via extraction and subsequently coupled onto a resin-bound leucine using base-free conditions in order to prevent epimerization (Scheme 1b). The second method entailed on-resin installation of the Tfa group via protection group exchange. A Leu-Leu dipeptide was synthesized on the 2-chlorotrityl chloride resin using standard Fmoc chemistry. The Tfa group was installed by first removing the *N*-terminal Fmoc group, followed by treatment with triethylamine (TEA, 10 equiv) and ethyl trifluoroacetate (ETFA, 12 equiv; Scheme 1c). At room temperature, on-resin trifluoroacetylation using TEA was slower than desired and we found that the use of a microwave reactor hastened the process significantly (20W, 75 °C, 10 min). Substituting DBU for TEA (12 equiv DBU, 10 equiv ETFA, 60 min) also enhanced the rate of the reaction at room temperature.

Scheme 1. Synthesis of *N*-Tfa Amino Acids and Peptides: (a) Synthesis of Tfa-L-leucine;[16] (b) Coupling of Tfa-L-leucine to a Resin-Bound Peptide; (c) Installation of Tfa Group via Protection Group Exchange



The Tfa-protected dipeptide was subject to the same Mitsunobu reaction conditions reported for the selective *N*-methylation *o*-NBS protected peptides.[9b,11b,12] Triphenylphosphine (5 equiv) and methanol (10 equiv) were dissolved in minimal anhydrous THF and added to a reaction vial containing the resin-bound, Tfa-protected dipeptide. Diisopropyl azodicarboxylate (DIAD, 5 equiv) was added slowly with vigorous agitation, and the reaction vessel was capped and shaken for an additional 15 min. The *N*-methylated peptide was obtained in good to excellent yields of 80−99% (compound **2**; see the Supporting Information, Figure S3), with the best results obtained when precautions were taken to exclude water from the reactants, solvent, and the resin. When necessary, a second treatment pushed the reaction to >99% methylation without undesirable side reactions.

We examined the conditions previously reported to cleave the Tfa group on the solid phase. Contrary to literature reports, we found that the Tfa group was quite robust. Of the many cleavage conditions that have been reported (K_2CO_3 in methanol,[17] aqueous piperidine,[18] hydrazine,[19] reduction[13,14,20]), the only method that removed

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the Tfa group completely within 30 min was reduction with sodium borohydride in THF/ethanol (1:1 v/v; Table 1). These results indicate that the Tfa group is sufficiently stable for use in SPPS and that rapid and reliable removal of the Tfa group can be accomplished using mild conditions that are compatible with SPPS.

Table 1. Reaction Conditions Investigated To Remove the Tfa Group from a Resin-Bound Tfa-Leu-Leu Dipeptide

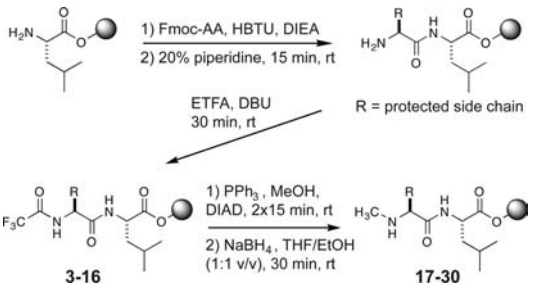
reaction conditions	time (h)	% Tfa group removed[a]
15% N_2H_4, 15% MeOH, 70% DMF (v/v)	1	0
15% N_2H_4, 15% MeOH, 70% THF (v/v)	1	0
5% H_2O, 25% MeOH, 70% THF (v/v), excess K_2CO_3	1	0
10% H_2O, 10% piperidine, 80% DMF (v/v)	1	0
10% piperidine, 90% DMF (v/v)	1	0
5% H_2O, 95% THF, 1 equiv of LiOH	1	0
5 equiv of DBU, 10 equiv of BME in NMP	1	0
10 equiv of NaOEt in THF	1	100[b]
10 equiv of $NaBH_4$ in EtOH/THF (1:1 v/v)	0.5	100

[a] Percent removal of Tfa group was determined by integrating the peaks in the HPLC trace (λ = 220 nm). [b] Deprotection with NaOEt resulted in ~10% epimerization of the *N*-terminal residue.

In order to assess the scope of the Tfa-Mitsunobu method for broader application in SPPS, we examined both the regioselectivity of the methylation reaction and the stability of various side-chain protection groups toward treatment with $NaBH_4$. Fourteen dipeptides were synthesized in parallel by adding different Fmoc-protected amino acids to the 2-chlorotrityl resin preloaded with leucine (Scheme 2). The amino acids Arg(Mtr), Arg(Pbf), Cys(Trt), Glu(O-*t*-Bu), Glu(OBnz), Gln(Trt), His(Trt), Lys(Boc), Lys(Dde), Lys(Mtt), Orn(Alloc), Ser(OBnz), Trp(Boc), and Tyr(O-*t*-Bu) were deprotected, and each dipeptide was trifluoroacetylated using ETFA and DBU. The Tfa-dipeptides were subjected to two sequential Mitsunobu reactions. An aliquot of each dipeptide was set aside for LCMS and NMR analysis, and the remainder of each resin was treated with excess $NaBH_4$ for 30 min. In all cases, the LCMS and NMR spectra showed complete methylation that was regioselective for the backbone amide. Moreover, there was no evidence of side chain modifications nor loss of side chain protection groups during the methylation step (see the Supporting Information, Figures S3–S27).

Virtually all of the side chain protection groups examined in this study were found to be stable to treatment with excess $NaBH_4$ (see the Supporting Information, Figures S28–S41). Even though $NaBH_4$ is known to reduce methyl and allyl esters to the corresponding alcohols, we found that *tert*-butyl and benzyl esters were stable to these conditions. Likewise, the trityl ester linking the peptide to the solid phase was stable to these conditions. Of the 14 amino acids investigated, the only side chain protection group affected by treatment with $NaBH_4$ was the Lys-Dde protection group, which was removed completely. This is

Scheme 2. Synthesis of Dipeptides Used To Evaluate the Regioselectivity of the Tfa-Mitsunobu *N*-Methylation



noteworthy because the use of $NaBH_4$ for the cleavage of Dde has not been reported in the literature.

The discovery of a rapid and mild Dde cleavage protocol is fortuitous because Dde is only quasi-orthogonal to Fmoc. Dde is stable to the bases used to remove Fmoc, but Fmoc is quickly cleaved by hydrazine. The discovery that Dde can be removed quickly with $NaBH_4$ led us to verify the stability of Fmoc to treatment with $NaBH_4$. We were pleased to find that Fmoc was completely unreactive to $NaBH_4$ (**31**, see the Supporting Information, Figure S42). These findings reveal that Fmoc and Dde are fully orthogonal when $NaBH_4$ is used to deprotect Dde. To date, there has been only one other report of Dde cleavage conditions that enable orthogonality with Fmoc ($NH_2OH \cdot HCl$, imidazole);[21] however, this method requires a 3 h reaction time, and it is unknown whether these conditions are combatible with the various protection groups used in SPPS.

We also evaluated the efficacy of the Tfa-Mitsunobu *N*-methylation method relative to that of the *o*-NBS method by challenging both with a tri-*N*-methylated tetrapeptide that contained sterically demanding residues such as the bulky, β-branched Thr(O-*t*-Bu). H-*N*-MeTyr(O-*t*-Bu)-*N*-MeTrp(Boc)-*N*-MeThr(O*t*Bu)-Leu-OH was synthesized using both methods (Scheme 3), and upon completion of both syntheses the crude products were analyzed by LCMS (Figure 1; see the Supporting Information, Figures S43 and S44).

The HPLC trace for the Tfa-Mitsunobu synthesis showed the desired tri-*N*-methylated tetrapeptide as the major product (Figure 1a, t_R 8.44). The two most significant byproducts represent truncations (-Trp and -Tyr, respectively), and the peak at 7.49 min represents the full tetrapeptide with the Tyr *tert*-butyl ether removed. The LCMS data for the intermediate peptides showed that each Tfa deprotection was complete, indicating that these truncations resulted from incomplete amino acid couplings.

The *o*-NBS-Mitsunobu synthesis, however, provided the desired tetrapeptide as only one among four major components (Figure 1b, t_R 8.42), with the major byproducts arising from incomplete removal of the *o*-NBS group at different steps (see the Supporting Information for a more

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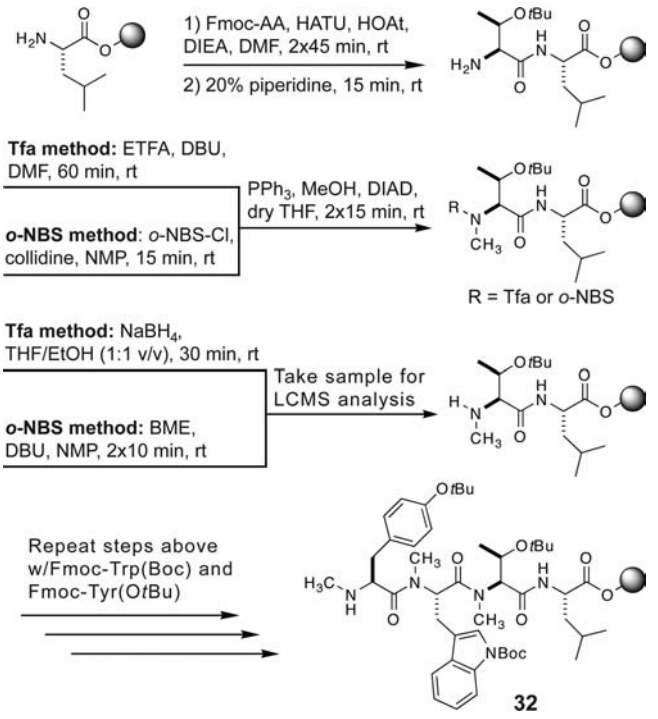
Scheme 3. Synthesis of Tri-*N*-methylated Tetrapeptides Using Tfa- and *o*-NBS-Mitsunobu Methods



complete analysis of these data). We used the standard conditions reported for efficient removal of *o*-NBS (5 equiv of DBU, 10 equiv of BME, 2 × 10 min in NMP); however, these results suggest that repeated rounds of deprotection and LCMS monitoring may be warranted for some sequences to ensure complete deprotection and avoid unwanted truncations. In contrast, the Tfa method was consistently efficient with respect to both the *N*-methylation chemistry and the deprotection steps.

The *o*-NBS-Mitsunobu method is regarded as the standard protocol for on-resin, site-specific backbone amide methylations. One advantage that has been attributed to *o*-NBS is the removal of incompletely *N*-methylated byproducts, presumably due to the inability to remove *o*-NBS from non-*N*-methylated residues.[2d,8,9b] In principle, this enables purification of the resulting byproducts as protected truncations. In this model system, we found that *N*-methylation was equally efficient using both methods, however, *the removal of the protection group from N-methylated residues* was the problematic step for the *o*-NBS

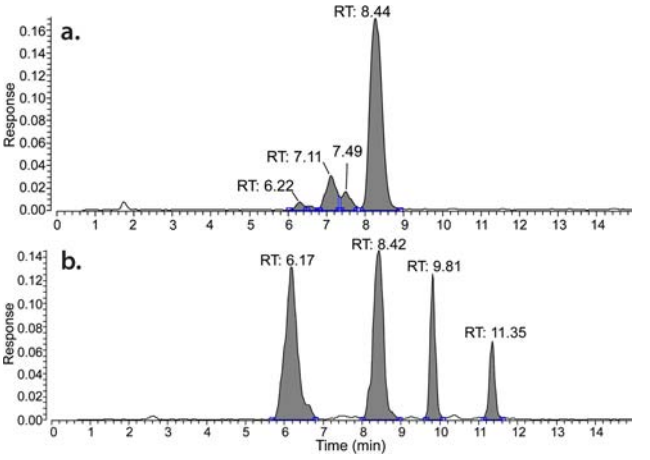


Figure 1. HPLC traces of tri-*N*-methylated tetrapeptids synthesized via the (a) Tfa-Mitsunobu method and (b) the *o*-NBS-Mitsunobu method.

strategy. The efficiency and consistency with which the Tfa group is both *N*-methylated and removed thus represents a potential improvement over the conventional methodology.

We have demonstrated that the Tfa protection group enables site-specific *N*-methylations under Mitsunobu conditions, with no side-chain reactivity or protection group losses. The Tfa group was shown to be stable to a variety of conditions, but was removed quickly and reliably using excess $NaBH_4$ in THF/EtOH. The Tfa cleavage conditions were determined to be orthogonal to virtually all of the protection groups commonly used in SPPS, indicating that the Tfa group may have more utility as a general protection group in SPPS than has previously been reported.

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Supporting Information Available. Experimental procedures and ^1H NMR and LCMS (ESI) spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.