Site-Selective N-Methylation of Peptides on Solid Support

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Conformational restriction of peptides can yield useful information about their bioactive conformation.[1] *N*-Methyl amino acid substitutions have often been used to increase the potency or selectivity of a peptide ligand.[2] This modification largely restricts the affected residue and the amino acid preceding it to an extended conformation.[3] However, few *N*-methyl amino acids are commercially available[4] and their synthesis is tedious.[2a] Thus, it is often impractical to explore the effects of N-methylation at every residue in a peptide (a procedure we term "*N*-methyl scanning"). Herein, we describe the first general solid phase method for site-selective N-methylation at any position in a peptide.[5] The practical utility of this method is demonstrated by the *N*-methyl scanning of the thrombin receptor agonist peptide H_2N-SFLLRN-$CONH_2$.[6]

The simplest approach to N-methylation of a peptide would involve direct methylation of the N-terminal residue of the resin-bound peptide. However, monomethylation of primary amines is a difficult synthetic problem.[7a] Several strategies have been reported for their synthesis,[7] but these schemes are not readily amenable to solid phase synthesis, and the conditions are not compatible with sensitive functionality and racemizable stereocenters.

Our initial attempts to achieve chemoselective N-alkylation of the amino terminus of a resin-bound peptide employed a three-step procedure involving trifluoroacetylation, selective alkylation via the Mitsunobu reaction,[8] and deprotection to afford the *N*-alkylamine. Unfortunately, we were unable to achieve any reaction using Mitsunobu conditions on solid support. Attempted alkylation of an *N*-trifluoroacetyl peptide using the "enhanced" Mitsunobu conditions of Tsunoda and coworkers[9] also failed, as did the attempted alkylation of the more acidic *N*-toluenesulfonyl and *N*-methanesulfonyl peptide derivatives.[10]

The treatment of an *N*-toluenesulfonyl peptide on solid support with alkylating agents and base was subsequently investigated.[11] Methylation of the sulfonamide in DMF with dimethyl sulfate in the presence of the hindered, nonionic guanidinium base MTBD,[12] was selective and complete in less than 2 h. The use of MTBD as base was critical to achieve high yields, since weaker bases gave poor or no yield of methylated product and stronger bases resulted in methylation of the amide backbone.[13]

On the other hand, attempts to alkylate the aforementioned *N*-trifluoroacetyl peptide using excess dimethyl sulfate and MTBD gave only 50% product after 24 h. This result prompted us to explore the use of a sulfonamide activating group that could be selectively removed after alkylation. The cleavable alkyl sulfonyl chlorides we synthesized[14] formed sulfonamides with amines in solution, but failed to yield the sulfonamide when added to the resin-bound peptide free amine in methylene chloride. This failure is presumably related to the ready formation of reactive sulfenes.

The failure of our alkyl sulfonyl chlorides to couple to the resin led us to explore the possibility of using aryl sulfonyl chlorides, which cannot form sulfenes. Fukuyama and coworkers have recently described the cleavage of nitrobenzenesulfonamides via nucleophilic aromatic substitution with thiophenol.[15] We were pleased to find that *o*- and *p*-nitrobenzenesulfonyl chlorides (*o*-NBS and *p*-NBS) readily coupled to the free amine of a support-bound peptide. Both peptides were readily methylated using the conditions described above. Quantitative cleavage of *o*-NBS and *p*-NBS was effected with thiophenol and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in DMF. The *o*-NBS group cleaved more readily than *p*-NBS and was used for all subsequent reactions. β-Mercaptoethanol was substituted for thiophenol, since it cleaved the sulfonamides faster and is less toxic, less noxious, and more commonly found in the typical laboratory. Finally, methyl *p*-nitrobenzenesulfonate, a commercially available crystalline solid, was substituted for dimethyl sulfate on the basis of handling and toxicity considerations.

Our procedure (Scheme 1) thus involves (1) protection of the resin-bound peptide free amine as the *o*-nitrobenzenesulfonamide by treatment with the corresponding sulfonyl chloride in CH_2Cl_2 containing collidine for 2 h, (2) selective deprotonation of the sulfonamide with the strong, nonionic base MTBD and alkylation with methyl *p*-nitrobenzenesulfonate in DMF for 30 min, and (3) selective removal of the *o*-NBS group with β-mercaptoethanol and DBU in DMF for 30 min.[16] The final deprotection step is easily followed via the formation of a bright yellow color, presumably due to the release of 2-(2-nitrophenylthio)ethanol. This three-step procedure did not cause any detectable racemization of the peptide stereocenters.[17]

In order to demonstrate that our method could be used to "*N*-methyl scan" each amino acid in a peptide, standard Fmoc

(1) For reviews, see: (a) Rizo, J.; Gierasch, L. M. *Ann. Rev. Biochem.* **1992**, *61*, 387−418. (b) DeGrado, W. F. *Adv. Protein Chem.* **1988**, *39*, 51−124.

(2) For examples, see: (a) Ali, F. E.; Bennett, D. B.; Calvo, R. R.; Elliott, J. D.; Hwang, S.-M.; Ku, T. W.; Lago, M. A.; Nichols, A. J.; Romoff, T. T.; Shah, D. H.; Vasko, J. A.; Wong, A. S.; Yellin, T. O.; Yuan, C.-K.; Samanen, J. M. *J. Med. Chem.* **1994**, *37*, 769−780. (b) Schmidt, R.; Kalman, A.; Chung, N. N.; Lemieux, C.; Horvath, C.; Schiller, P. W. *Int. J. Pept. Protein Res.* **1995**, *46*, 47−55.

(3) Manavalan, P.; Momany, F. A. *Biopolymers* **1980**, *19*, 1943−1973. N-Methylation also allows left-handed α-helical structures, promotes *cis*−*trans* isomerization about the peptide bond, disrupts hydrogen bonding to the amide nitrogen, and increases the hydrophobicity of the peptide.

(4) Usually limited to those amino acids without side chain functionality (i.e., Gly, Ala, Leu, Ile, Val, Phe).

(5) Our method is compatible with Fmoc solid-phase peptide synthesis (SPPS). For another approach to N-methylation of peptides on a solid support, see: Kaljuste, K.; Unden, A. *Int. J. Pept. Protein Res.* **1993**, *42*, 118−124. However, this approach is incompatible with Fmoc SPPS. Nonselective permethylation of peptides on solid support has also been reported [Ostresh, J.M.; Husat, G. M.; Blondelle, S. E.; Dorner, B.; Weber, P. A.; Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11138−11142].

(6) Scarborough, R. M.; Naughton, M. A.; Teng, W.; Hung, D. T.; Rose, J.; Vu, T.-K. H.; Wheaton, V. I.; Turck, C. W.; Coughlin, S. R. *J. Biol. Chem.* **1992**, *267*, 13146−13149. All peptides are referred to using the standard one-letter amino acid code.

(7) (a) Krishnamurthy, S. *Tetrahedron Lett.* **1982**, *23*, 3315−3318 and references therein. (b) Henry, J. R.; Marcin, L. R.; McIntosh, M. C.; Scola, P. M.; Harris, G. D., Jr.; Weinreb, S. M. *Tetrahedron Lett.* **1989**, *30*, 5709−5712. (c) Grieco, P. A.; Bahsas, A. *J. Org. Chem.* **1987**, *52*, 5747−5749.

(8) (a) Mitsunobu, O. *Synthesis* **1981**, 1. (b) Hughes, D. *Org. React.* **1992**, *42*, 335−656.

(9) (a) Tsunoda, T.; Yamamiya, Y.; Ito, S. *Tetrahedron Lett.* **1993**, *34*, 1639−1642. (b) Tsunoda, T.; Yamamiya, Y.; Kawamura, Y.; Ito, S. *Tetrahedron Lett.* **1995**, *36*, 2529−2530.

(10) Formed from H_2N-LLONSF-$CONH_2$ on Tentagel resin (Bayer, E. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 113−129) treated with the corresponding sulfonyl chloride in CH_2Cl_2 for 2 h with collidine as base.

(11) The reactions were all followed by cleaving a small portion of the resin and analyzing it with RP-HPLC and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

(12) See: Schwesinger, R. *Chimia* **1985**, *39*, 269−271. MTBD is 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene and is sold by Aldrich under the name 1,3,4,6,7,8-hexahydro-1-methyl-2*H*-pyrimido[1,2-a]pyrimidine.

(13) For example, Proton Sponge [Aldrich] failed to give any product, DBU gave only modest yields, and the iminophosphorane base BEMP [Schwesinger, R. *Chimia* **1985**, *39*, 269−271] was not selective, causing methylation of the peptide backbone.

(14) This work will be reported elsewhere.

(15) Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373−6374.

(16) The *o*-NBS group cleaves much more slowly if it has not been alkylated, thus capping unalkylated sequences.

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Scheme 1. Selective N-Methylation of Peptides

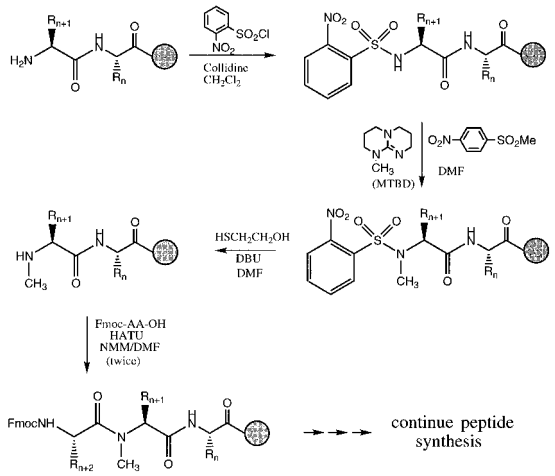

Table 1. Crude Purity and Purified Peptide Yield

peptide amide	crude purity (%)[a]	isolated yield (%)[b]
(*N*-Me)SFLLRN	86	67
S(*N*-Me)FLLRN	64	33[c]
SF(*N*-Me)LLRN	84	65
SFL(*N*-Me)LRN	84	59
SFLL(*N*-Me)RN	89	64
SFLLR(*N*-Me)N	89	48

[a] Crude purity assessed by RP-HPLC at 215nm (10−50% CH_3CN in H_2O containing 0.1% trifluoroacetic acid). [b] Purified by preparative RP-HPLC (same solvent system) to >99% by HPLC (215 nm). Yield based on manufacturer's stated resin substitution. [c] Only 91% pure. The lower yield of the (*N*-Me)Phe peptide was primarily due to poor coupling of the final serine residue. Purification was difficult due to a closely-eluting impurity arising from this coupling step.

((fluorenylmethoxy)carbonyl) solid-phase peptide synthesis (SPPS) of the thrombin receptor agonist peptide H_2N-SFLLRN-$CONH_2$ was performed on polystyrene resin[18] (Table 1). Peptide synthesis was carried out to the position where N-methylation was desired, the Fmoc group was then removed, and the resin-bound peptide was treated as shown in Scheme 1. The next amino acid residue was activated with the recently described azabenzotriazole-based coupling reagent, HATU,[19] and standard Fmoc SPPS was continued.

The introduction of the *o*-NBS group was quantitative, and methylation was 95% complete in all cases;[20] however, a small amount (5−11% by HPLC) of a side product was formed in the arginine-containing peptide fragments from modification of the Arg(Pmc) residue.[21] This posed no problem for the *N*-methyl scanning of this peptide, since the small amount of side product is easily separated by reverse-phase (RP) HPLC. Its formation could also be eliminated entirely by the substitution of arginine with an orthogonally-protected ornithine,[22] which can be converted to arginine by guanylation at the completion of the synthesis.[23] Removal of the *o*-NBS group by treatment with β-mercaptoethanol and DBU in DMF proceeded rapidly and quantitatively.

The six different N-methylated peptides synthesized have identical masses, differing only in the location of the *N*-methyl group. However, high-energy collision-induced dissociation (CID) analysis with a tandem mass spectrometer[24] and matrix-assisted laser desorption ionization postsource decay (MALDI-PSD) mass spectrometry[25] both allowed unequivocal assignment of the site of methylation in each peptide. The N-methylated residue is readily identified by its often prominent *N*-methylimmonium ion peak. Analysis of the peptide fragments was used to corroborate this assignment and to confirm its location in the peptide sequence.

We have thus demonstrated a powerful new method for site-specific N-methylation of peptides on solid support using commercially available chemicals. This method provides the first general procedure for *N*-methyl scanning of bioactive peptides and should aid in the discovery of more active and selective ligands for pharmaceutically-interesting receptors. It also opens the door to other modifications of the amide backbone by employing different alkylating agents and electrophiles[26] and for combinatorial strategies for producing large libraries of N-modified peptides. In addition, we anticipate that this method may also be of general use for the chemoselective N-alkylation of support-bound polyfunctional nonpeptidyl molecules.[27] Further studies of the applications and compatibility of this method will be forthcoming.

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Supporting Information Available: A detailed experimental procedure and HPLC and mass spectra for the N-methylated peptides (28 pages). See any current masthead page for ordering and Internet access instructions.

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(17) To determine whether these conditions cause any racemization, the dipeptide Phe-Leu was synthesized and subjected to the sequence outlined in Scheme 1. The diastereomer D-Phe-Leu was similarly prepared. These diastereomers are readily resolved by reverse-phase HPLC. No racemization was detected (<0.5%).

(18) Rink amide MBHA resin [Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787−3788].

(19) *O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate [Carpino, L.A. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398]. This step was performed twice to ensure complete coupling.

(20) The phenylalanine residue in *o*-NBS-FLLRN-$CONH_2$ had to be subject to two cycles of methylation in order to achieve >95% methylation (after the first cycle, 17% of the sulfonamide remained unmethylated). This appears to be a function of the peptide sequence, not an intrinsic problem with the phenylalanine residue, since the dipeptide Phe-Leu was easily methylated using this protocol.

(21) Yields: 5−11% by HPLC, <5% isolated. The 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) protecting group [Ramage, R.; Green, J. *Tetrahedron Lett.* **1987**, *28*, 2287−2289], a sulfonamide, presumably renders the arginine side chain nitrogens slightly susceptible to electrophilic attack under these basic conditions. This side product appeared to be somewhat labile to the conditions of amino acid coupling and/or Fmoc deprotection. On the basis of the mass spectral data, we postulate that the side product is due to a reaction with methylated DMF [Bredereck, H.; Effenberger, F.; Simchen, G. *Angew. Chem.* **1961**, *73*, 493]. We are currently exploring methods to eliminate its formation.

(22) The peptide H_2N-LLONSF-$CONH_2$ was methylated as in Scheme 1 with no formation of side product.

(23) Bodanszky, M.; Ondetti, M. A.; Bikhimer, C. A.; Thomas, P. L. *J. Am. Chem. Soc.* **1964**, *86*, 4452−4459.

(24) For a review, see: Papayannopoulos, I. A. *Mass Spectrom. Rev.* **1995**, *14*, 49−73.

(25) Spengler, B.; Kirsch, D.; Kaufmann, R.; Jaeger, E. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 105−108. Different fragmentation is seen in MALDI-PSD than in high-energy CID. Both methods were independently sufficient, however, to unambiguously assign the site of N-methylation in our peptides.

(26) For example, we have effected N-allylation of peptides on solid support using Pd(0) chemistry.

(27) See: Thompson, L. A.; Ellman, J. A. *Chem. Rev.* **1996**, *96*, 555−600.