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Preparation of Peptide *p*-Nitroanilides Using an Aryl Hydrazine Resin

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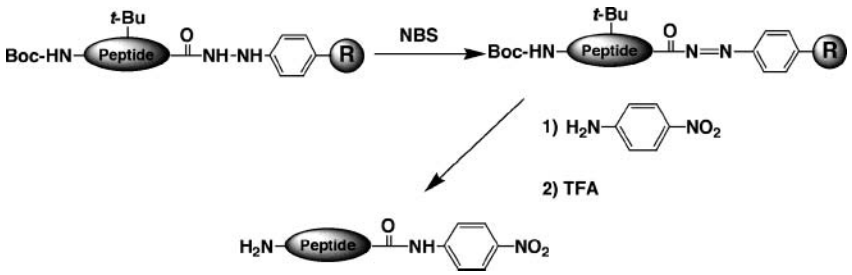
ABSTRACT



Peptide *p*-nitroanilides are useful compounds for studying protease activity; however, the poor nucleophilicity of *p*-nitroaniline makes their preparation difficult. We describe a new efficient approach for the Fmoc-based synthesis of peptide *p*-nitroanilides using an aryl hydrazine resin. Mild oxidation of the peptide hydrazide resin yields a highly reactive acyl diazene that efficiently reacts with weak nucleophiles. We have prepared several peptide *p*-nitroanilides, including substrates for the Lethal Factor protease from *B. anthracis*.

Peptide *p*-nitroanilides are useful chromogenic substrates for studying the activity and selectivity of proteolytic enzymes.[1] The preparation of a peptide *p*-nitroanilide on the solid-phase peptide synthesis (SPPS) platform is challenging due to the poor nucleophilicity of the aromatic amino group of *p*-nitroaniline (pNA), which is further deactivated by the electron-withdrawing nitro group.[2]

So far, several strategies have been reported for the preparation of peptide *p*-nitroanilides using SPPS. Burdick et al.[3] prepared a urethane-linked *p*-diaminobenzene resin for the synthesis of peptide 4-aminoanilides using Boc/benzyl chemistry. These peptide 4-aminoanilides were then oxidized with sodium perborate[4] to give the corresponding peptide *p*-nitroanilides. Mergler et al.[5] modified this protocol for Fmoc-based SPPS using Wang and Sasrin resins. However, this method required modifications of commercial resins prior to peptide synthesis. Abbenante et al.[6] improved Mergler's method by using commercially available trityl chloride resin (TCP resin) and the milder oxidizing reagent Oxone. However, this oxidation method is not compatible with amino acids sensitive to oxidative conditions.

Other investigators have used Fmoc-protected amino acid *p*-nitroanilides that were attached through side-chain functional groups (−SH, $-CO_2H$, $-NH_2$) to a chlorotrityl resin.[7,8] This approach, however, requires the preparation of Fmoc-N^{α}-protected amino acid *p*-nitroanilides, and it can be only used when the last residue of the peptide contains a suitable side-chain functional group (i.e., Cys, Asp, Glu, or Lys). Alsina et al.[9] modified this strategy by using a backbone amide linker (BAL) to attach an α-carboxylic group protected

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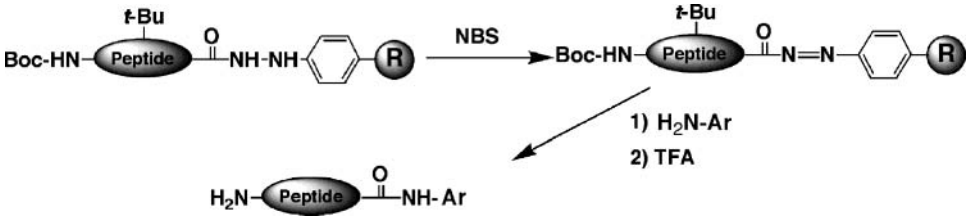
Scheme 1. Preparation of Peptide Anilides Using an Aryl Hydrazine Resin



amino acid to polystyrene resin through the α-amino group. After conventional Fmoc-SPPS, the carboxyl group was selectively deprotected, activated, and coupled with the appropriate amino acid *p*-nitroanilide. However, some epimerization on the penultimate residue can occur.

The methods presently available for the preparation of peptide *p*-nitroanilides either require synthetic manipulation steps or have limitations on the nature of amino acids that can be incorporated or have racemization problems. In the present work, we describe a novel and straightforward strategy for the synthesis of peptide *p*-nitroanilides using an aryl hydrazine linker that is compatible with Fmoc/t-Bu and Boc/Bz SPPS.[10−19]

Our new approach (see Scheme 1) involves the direct assembly of the peptide on an aryl hydrazine resin[12−19] using standard Fmoc-protocols. When the synthesis is completed, the fully protected peptide hydrazide resin is activated by mild oxidation with NBS in the presence of pyridine. The resulting acyl diazene resin is then cleaved with pNA. The fully deprotected peptide *p*-nitroanilide is finally obtained by treatment with trifluoroacetic acid (TFA).

Acyl diazenes are reactive intermediates that readily react with O- and N-nucleophiles.[13,17−19] However, Millington et al.[13] showed that highly inactivated or sterically hindered O-nucleophiles (such as *t*-BuOH or pentafluorophenol) are unable to react with acyl diazenes in an efficient way. Intrigued by this finding, we explored the reactivity of peptidyl diazenes to poorly reactive arylamines such a pNA (the pK_a of the conjugated acid of pNA's amino group is ∼1.0). Model tripeptide **1a** (Table 1) was synthesized on a hydrazinobenzoyl resin and then activated by oxidation with 2 equiv of NBS in the presence of anhydrous pyridine for 10 min at room temperature. When the oxidation was completed, the activated resin was washed with anhydrous dichloromethane (DCM) and treated with 40 equiv of pNA dissolved in DCM containing 5% dimethylformamide (DMF).[20] The cleavage reaction was monitored by analytical HPLC.[21] As anticipated, the cleavage reaction was slower than observed for aliphatic N-nucleophiles,[13,17−19] requiring 24 h for completion. The solvent was then removed and the residue was treated with trifluoroacetic acid (TFA) containing 5% H_2O. As shown in Figure 1A, the oxidation and cleavage reaction with pNA was very clean and the main product was identified by ES-MS as the corresponding peptide **1a** with a cleavage yield around 66% and purity around 95% (Table 1). We also reacted the same peptide diazene resin with 7-amino-4-methylcoumarin (AMC). Peptide AMC derivatives are also desirable chromogenic and fluorogenic substrates for exploring proteolytic activity.[1,22]

The cleavage reaction with AMC only took 6 h for completion. This result can be attributed to the higher nucleophilicity of the aromatic amino group in AMC (pK_a of the conjugated acid of the AMC's amino group is 3.8). Similar to the cleavage with pNA, the major product in the crude reaction mixture corresponded to the peptide-AMC **1b** (Figure 1B) with a cleavage yield of 77% and a purity of 96% (Table 1).

Table 1. Sequences, Molecular Weights, and Yields of Peptides Used in This Study.

peptide	sequence	M_w/Da		purity[c] (%)	yield[d] (%)
1a	H-LYA-pNA	485.5[a]	486.0[b]	95	66
1b	H-LYA-AMC	522.6[a]	522.0[b]	96	77
2	Ac-FA-pNA	398.4[a]	398.0[b]	98	46
3	Ac-F-D-A-pNA	398.4[a]	398.0[b]	97	46
4	H-LWA-pNA	508.6[a]	509.0[b]	95	81
5	H-LMYKA-pNA	744.9[a]	745.0[b]	85	60
6	Ac-RRRRVLR-pNA	1173.4[a]	1173.5[b]	90	43
7	Ac-LARRRPVLP-pNA	1239.5[a]	1239.3[b]	85	45

[a] Expected. [b] Found. [c] Based on HPLC purity. [d] Based on resin substitution.

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(20) The cleavage reaction was performed under dry N_2 atmosphere to minimize the hydrolysis of the acyl diazene resin.
(21) Small aliquots from the crude reaction were taken at various times. The solvent was removed under vacuum, the peptide *p*-nitroanilide deprotected with TFA (TFA/H_2O, 95:5 v/v) and then analyzed by analytical RP-HPLC.

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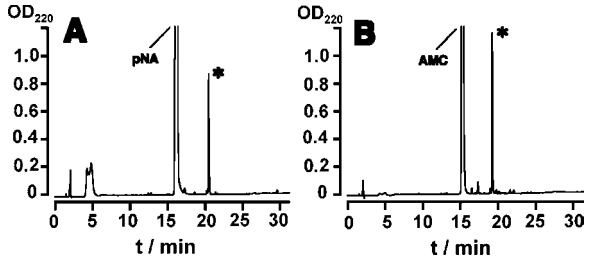

Figure 1. HPLC analysis of the crude product obtained by NBS oxidation and pNA/AMC cleavage. Peptide **1** was cleaved using pNA (A) and AMC (B). The asterisk denotes the corresponding peptide arylamide. Linear gradient of 0−70% buffer B (90% CH_3-CN, 10% H_2O, 0.1% TFA) in buffer A (H_2O, 0.1% TFA) over 30 min was used for HPLC.

Although peptidyl diazenes have been shown to be free of racemization during cleavage with aliphatic amines,[11,16,19,23] the slow cleavage reaction with pNA raised the possibility of racemization through oxazolone formation[24] during such a long reaction time (24 h). In order to exclude this possibility, two dipeptide diastereomers (peptides **2** and **3**) were assembled on the hydrazine resin, oxidized with NBS, and then cleaved with pNA for 24 h. As shown in Figure 2,

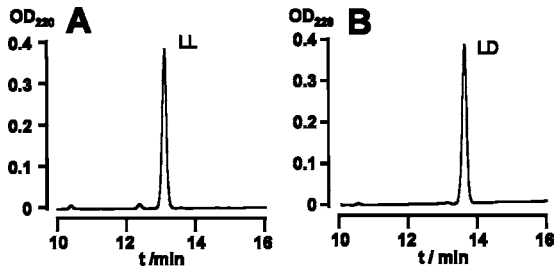

Figure 2. Epimerization studies of the C-terminal residue during activation and cleavage. HPLC traces of the crude products for the oxidation and cleavage with NBS and pNA of Ac-Phe(L)-Ala(L)-pNA (A) and Ac-Phe(L)-Ala(D)-pNA (B). Linear gradient of 30−70% buffer B (90% CH_3CN, 10% H_2O, 0.1% TFA) over 30 min was used for HPLC.

HPLC analysis of the crude cleavage reactions indicated no racemization of the C-terminal residue (<0.5% by HPLC analysis).

We have also recently shown that mild oxidation of peptide hydrazides with NBS is totally compatible with oxidative-sensitive residues (i.e., Tyr, Trp, Met, and Cys) when the appropriate protecting groups and oxidative conditions are employed.[19] Thus, peptide **4**, which contains an N^{im}-Boc protected Trp residue, was totally unaffected by the NBS oxidation and pNA cleavage, and it was obtained as the major component in the crude reaction mixture (Figure 3A, Table

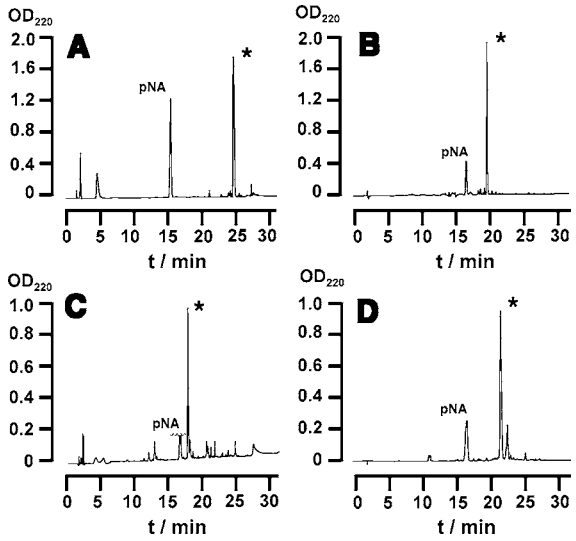

Figure 3. HPLC analysis of the crude product obtained by NBS oxidation and pNA cleavage: (A) peptide **4**, (B) peptide **5**, (C) peptide **6**, and (D) peptide **7**. The asterisk denotes the corresponding peptide arylamide. Linear gradient of 0−70% buffer B over 30 min was used for HPLC.

1). In the same way, Met-containing peptide **5** was also obtained in good yield (Figure 3B, Table 1). It is interesting to note that although the Met residue is initially oxidized by NBS to the corresponding sulfoxide, during the TFA deprotection step, the sulfoxide is reduced when the reaction is carried out for 3 h at room temperature in the presence of 2% EtSH. This result is notable because Met-containing peptide *p*-nitroanilides cannot be prepared by oxidation reactions performed with sodium perborate or Oxone.[3,4,6]

We also prepared peptides **6** and **7** (Figure 3C,D, Table 1). The sequence of these peptides contains the recognition sequence for the Lethal Factor (LF) protease,[25] and they have been fully characterized as substrates for this endometallo-protease.[1] The C-terminal modified peptides were obtained in good yield using the same protocol as for model peptide **1** (Table 1). In both cases, the crude material was purified by semipreparative RP-HPLC and used to test the activity of recombinantly generated LF protein (Figure 4).[26]

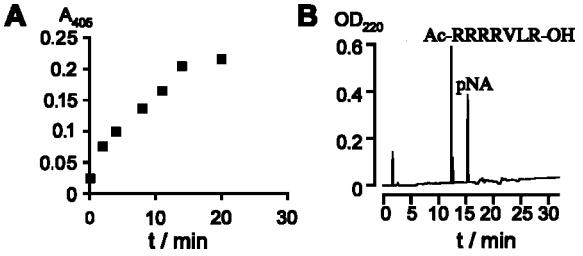

Figure 4. LF-catalyzed hydrolysis of peptide **6**. (A) Hydrolysis reaction was spectrophotometrically monitored at 405 nm. (B) HPLC analysis of the hydrolysis crude reaction after 20 min. HPLC conditions were similar to those employed in Figure 3.

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In conclusion, we have developed a straightforward method to prepare both peptide derivatives of pNA and AMC without limitation of size and amino acid composition. The mild oxidation procedure involved is totally compatible with sensitive amino acid residues under the conditions employed. Moreover, no detectable racemization is observed at the C-terminal residue during the activation and cleavage of the hydrazide linker by pNA. Finally, this new method requires neither premodification of commercial resins nor additional manipulation after the cleavage and deprotection steps.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(25) Lethal factor is a Zn-metalloprotease secreted by virulent strains of *B. anthacis* that targets the N-terminal region of mitogen-activated protein kinase kinases (MAPKKs).

(26) Recombinant LF was prepared by cloning the LF gene into the multiple cloning site of pTXB1 plasmid (New England Biolabs). LF was expressed as an intein fusion prtotein and purified using chitin beads.