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Synthesis, stability and optimized photolytic cleavage of 4-methoxy-2-nitrobenzyl backbone-protected peptides†

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We demonstrate the potential of 4-methoxy-2-nitrobenzyl as a Boc chemistry-compatible fully reversible backbone modification for synthetic peptides.

Backbone protection has been shown to increase the efficiency of stepwise chain assembly for the solid phase synthesis (SPPS) of certain aggregating peptides.[1] Backbone modification has also been shown to enhance the solubility of sparingly soluble peptides by disrupting H-bond driven aggregation, and thus disfavoring the aggregated state.[2–4] Because beta-sheet formation is mediated by inter-strand hydrogen bonding between the backbone carbonyl oxygen and amide proton, a number of strategies focused on deleting the amide proton have been developed in order to prevent formation of backbone hydrogen bond networks. For Fmoc chemistry, the Hmb[1–3,5] and other similar protecting groups,[6,7] as well as pseudoproline[8–10] amino acids, have been used successfully to disrupt beta sheet formation and improve the quality of the crude synthetic product and the solubility of the free peptide.

Unfortunately, these strategies are not compatible with the strong acids used for chain assembly and/or side-chain deprotection in Boc chemistry-based synthetic strategies. Some backbone protecting groups, such as *N*-(2-hydroxybenzyl)[11] and *N*-(2-methoxybenzyl),[12] are stable to the conditions of chain assembly by Boc chemistry and have been shown to improve the synthesis of difficult sequences, but are not stable to the treatment with liquid HF used to remove side chain protecting groups. What is lacking in the armamentarium of Boc chemistry SPPS is a backbone protecting group that is stable both to the conditions of chain assembly and to deprotection/cleavage—preventing aggregation during chain assembly and workup—but that can be removed cleanly post-synthesis, at will, to recover the native peptide. Here, we report the development of a photolabile backbone protecting group that satisfies these requirements.

The 2-nitrobenzyl (2-Nb) group, introduced by Schofield,[13] can be cleaved by UV irradiation at ~360 nm, and methoxy substituents on the aromatic ring have been shown to increase the kinetics of photocleavage.[14] The 2-Nb group has previously been introduced into the peptide backbone using Fmoc chemistry.[15] Because of the electron-withdrawing *o*-nitro ring substitution, we also expected it to be stable to the the anhydrous hydrofluoric acid (HF) used for side-chain deprotection and cleavage from the resin in Boc SPPS. To test this, we synthesized a model peptide Met-Gly-(2-Nb)Gly-Phe-Leu using standard Boc/benzyl chemistry protocols.[16] We also synthesized the same test peptide with 4-methoxy-2-Nb and 4,5-dimethoxy-2-Nb backbone protecting groups to gauge the effect of electron donating substituents on the stability of the 2-Nb group to HF cleavage (Fig. 1). All 2-Nb-based protecting groups were completely stable to HF, with negligible amounts of deprotected peptide observed by LC-MS. This indicated that these 2-Nb groups could be stably incorporated into peptides using Boc chemistry.

To understand the reactivity of *N*-(2-nitrobenzyl)-aminoacyl-peptides during SPPS, we undertook a systematic study of the acylation of N(2-Nb)-glycine-, N(2-Nb)-alanine-, N(2-Nb)-leucine-, and N(2-Nb)-valine-peptides. Acylation of the N(2-Nb)-amino acyl-peptide was expected to become increasingly difficult as the bulk of the side chain on the alpha carbon increased, with the beta-branched N(2-Nb)-valine-peptide expected to be the most difficult. For activation of the incoming Boc-amino acid, we used an HOAT active ester (HOAT/DIC and HATU)‡, symmetric anhydride (DIC), or acid chloride (BTC).[17] Each coupling was performed for 3 hours. The results are shown in Table 1. N(2-Nb)-glycine- and N(2-Nb)-alanine-peptides were easily acylated by Boc-glycine

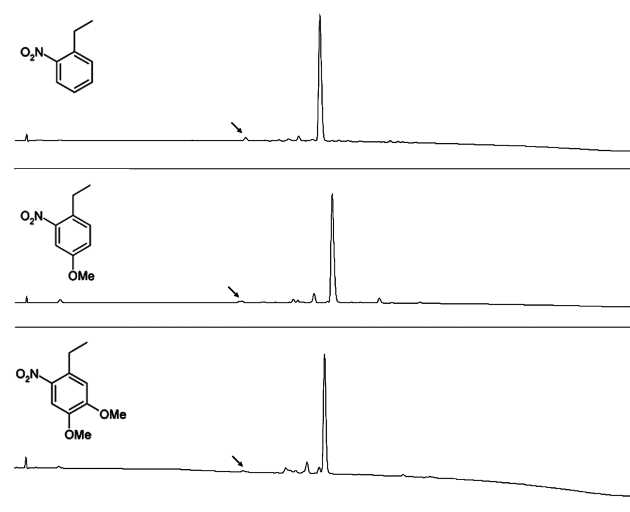

Fig. 1 Stability of N(2-Nb)-based backbone protection to Boc chemistry. Test peptides MG(X-G)FL, where X is the backbone protecting group shown in the upper left-hand corner of each panel, were synthesized by Boc SPPS and the crude products from HF treatment were analyzed by analytical RP-HPLC with online mass detection, using a 5–65% gradient of solvent B *vs* solvent A where A = H_2O + 0.1% TFA and B = CH_3CN + 0.08% TFA. UV detection was at 214 nm. The major peaks in each panel correspond to the expected protected peptides. The arrow in each panel indicates the deprotected peptide, MGGFL.

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Table 1 Coupling to N(2-Nb)-aminoacyl peptides[a]

	G(nG)F	G(nA)V	V(nA)V	G(nL)V	V(nL)V	G(nV)V	V(nV)V
HOAT/DIC	>99%	>99%	69%	4%	<1%	<1%	<1%
HATU	>99%	>99%	63%	22%	<1%	<1%	<1%
DIC	>99%	>99%	82%	30%	<1%	<1%	<1%
BTC	>99%	>99%	97%	66%	<1%	<1%	<1%

[a] Glycine or valine activated as either the HOAt active ester (with HOAT/DIC and HATU), the symmetric anhydride (with DIC), or the acid chloride (with BTC) were coupled to 2-Nb amino acids during the synthesis of the test peptide MD(G/V)(X)VK, where X is either N(2-Nb)Gly ('nG'), N(2-Nb)Ala ('nA'), N(2-Nb)Leu ('nL'), or N(2-Nb)Val ('nV'). The test peptide for the N(2-Nb)Gly coupling was MG(X)FL. For clarity, only the amino acids preceding and following the N(2-Nb)-substituted amino acid are shown. Coupling yields were determined by analytical HPLC of the crude product.

using all methods of activation. Coupling Boc-valine to N(2-Nb)-alanine was more difficult, although near quantitative coupling could be achieved with the amino acid chloride. Acylation of N(2-Nb)-Leu was only partially successful with Boc-glycine, and was not successful with Boc-valine regardless of activation method used. Essentially no acylation of N(2-Nb)-valine by Boc-glycine or Boc-valine was observed with any of the activated species. While not as serious as N(2-Nb)-valine, coupling to N(2-Nb)-leucine showed lower yields. Compared to Hmb-protected amino acids used in Fmoc chemistry, the 2-Nb-protected amino acids show a similar trend but slightly lower overall reactivity to incoming acylating reagents. The increased reactivity of the Hmb group is likely due to the ability of the 2-hydroxy group to catalyze intramolecular acyl transfer to the amine,[1,18] a role the 2-Nb group cannot perform. Despite limitations on where 2-Nb groups could be incorporated, it is still potentially useful for our objective of enhancing the solubility of synthetic peptides made by Boc chemistry.

With an understanding of where 2-Nb groups can be placed into peptides, we turned to the study of removing the N-substitution. Given the proposed mechanism of photocleavage of the N(2-Nb) group,[19,20] we reasoned that electron-donating substitution on the phenyl ring would accelerate photolysis through resonance-stabilization of the positive charge formed on the benzylic carbon during photolysis, and increase the yield of deprotected peptide. To test this, we synthesized a test peptide Met-Gly-(Y)Gly-Phe-Leu where Y was either 2-nitrobenzyl, 4-methoxy-2-nitrobenzyl, or 4,5-dimethoxy-2-nitrobenzyl. Each peptide was then subjected to low-intensity UV irradiation§ at 366 nm and the cleavage reaction followed by RP-HPLC (Fig. 2). The 2-Nb and 4-methoxy-2-Nb protecting groups showed similar cleavage kinetics and reaction profiles, with cleavage complete within 1 hour. Both were substantially better than the 4,5-dimethoxy-2-Nb group, consistent with results from others using substituted 2-Nb groups for caging studies.[21]

A possible side reaction during UV irradiation is oxidation of methionine residues, and for this reason we included a methionine in our model peptide to test for this reaction. We observed no significant methionine oxidation during our photolysis experiments. However, a number of other side-products accumulated during the photolysis reactions that reduced the yield of deprotected peptide. Mass analysis of these side products showed that they were very similar in mass to the starting protected peptide. We reasoned that these side-reactions were caused by reaction of the nitrosobenzaldehyde, formed during photolysis of the protecting groups, with the peptide. This reactive species is often avoided through the use of compounds which are substituted on the benzylic carbon to give an unreactive ketone upon photocleavage. Substitution on the benzylic carbon also stabilizes the positive charge on this atom formed during the cleavage process, increasing the kinetics of cleavage.[14,22] However, we decided to avoid substitution on the benzylic carbon because such a modification would likely hinder acylation of the secondary amine by an incoming amino acid. Therefore, to suppress side reactions during photocleavage we scavenged any aldehyde formed using a 200-fold molar excess of cysteine (Fig. 3), which we reasoned could trap the aldehyde as a thiazolidine. Inclusion of

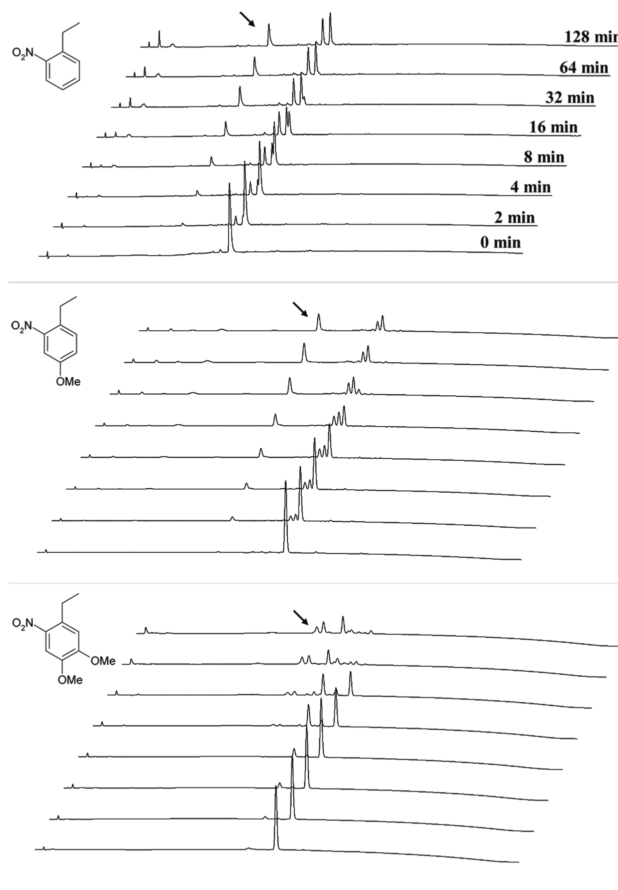

Fig. 2 Photolysis of backbone protecting groups. The group being cleaved from the test peptide MG(X-G)FL is shown in the upper left-hand corner of each panel. The arrows indicate the deprotected peptide, MGGFL. Cleavage was monitored by RP-HPLC on a C4 column using a 5–65% gradient of solvent B *vs* solvent A where A = H_2O + 0.1% TFA and B = CH_3CN + 0.08% TFA. Detection was at 214 nm.

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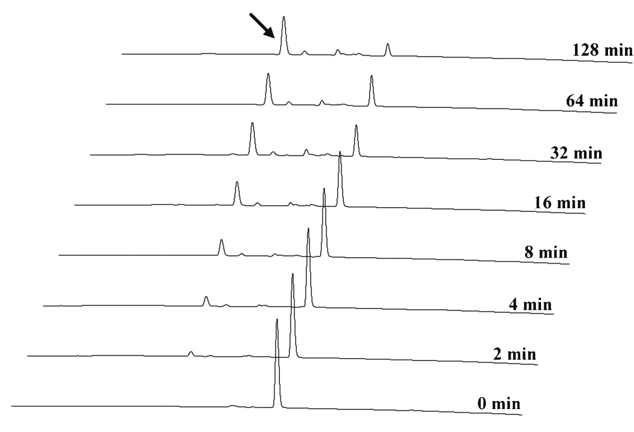

Fig. 3 Photocleavage of 4-methoxy-2-nitrobenzyl-peptide (1 mM) with cysteine (200 mM) added as a scavenger. The arrow indicates the deprotected peptide, MGGFL. Cleavage was monitored by RP-HPLC on a C4 column using a 5–65% gradient of solvent B or solvent A where A = H_2O + 0.1% TFA and B = ACN + 0.08% TFA. Detection was at 214 nm.

cysteine as a scavenger greatly reduced the formation of side-products during the reaction, and the deprotected peptide was recovered near quantitatively. Because cysteine is both a natural and mild scavenger, we expect it to be compatible with photolytic deprotection of N(2-Nb)-protected peptides in biological contexts.

In this report we have demonstrated the stability of N(2-Nb) protecting groups to the conditions of Boc chemistry SPPS, described conditions for the incorporation of N(2-Nb) groups during peptide synthesis, and optimized photolytic cleavage of 4-methoxy-2-nitrobenzyl from the peptide backbone. We are currently using 2-Nb as a backbone protecting group to improve the synthesis and handling of sparingly soluble peptides.

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Notes and references

‡ The method of formation of the HOAT active ester has previously been shown to affect acylation yields,[23] and we therefore tried both DIC and *in situ* activation with HATU.
§ UV irradiation was performed using a hand-held UV lamp for thin-layer chromatography. For details, see experimental procedures†.

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