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Semipermanent *p*-nitrobenzyloxycarbonyl (*p*NZ) protection of Orn and Lys side chains: prevention of undesired α-Fmoc removal and application to the synthesis of cyclic peptides

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Abstract—Semipermanent side-chain protection of Orn and Lys with *p*-nitrobenzyloxycarbonyl (*p*NZ) for Fmoc/^{t}Bu chemistry does not result in the unwanted removal of α-Fmoc that occurs when groups such as Alloc are used for the same application. Furthermore, *p*NZ can be used in conjuction with *p*-nitrobenzyl ester (*p*NB) to prepare cyclic peptides.
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1. Introduction

Solid phase has become the preferred mode for peptide synthesis.[1] The success of these syntheses depends to a great extent on the coupling reagents used[1,2] as well as the temporary and permanent protecting strategies employed.[1,3] Among the most useful temporary protection groups for amino acid side chains is fluorenylmethoxycarbonyl (Fmoc), which is removed after the incorporation of each protected amino acid. In contrast, the *tert*-butyl (^{t}Bu), which is removed at the end of the synthesis, is the permanent group of choice for most peptides.[4]

In the case of cyclic[5] or branched[6] peptides, semipermanent protecting groups must also be used. These groups must be stable to the conditions used to remove the temporary protecting group (i.e., piperidine for Fmoc) and should be removed without affecting any permanent protecting groups. Currently, the semipermanent protecting groups most widely used in tandem with the Fmoc/^{t}Bu strategy are allyl derivatives.[7] However, the palladium complex used in their removal is expensive. Furthermore, deprotection of allyloxycarbonyl (Alloc) from the side chains of Orn and Lys generates a highly basic free amine that causes premature removal of Fmoc groups.[8] This side reaction can have disastrous effects for a synthesis, cleaving up to 20% of Fmoc groups present.

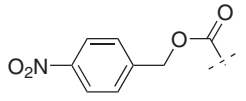

Figure 1. Structure of the *p*NZ protecting group.

Herein reported is the use of *p*-nitrobenzyloxycarbonyl (*p*NZ) as an alternative to Alloc for semipermanent side-chain protection of Orn and Lys. The application of *p*NZ to avoid undesired α-Fmoc removal and in the synthesis of cyclic peptides is also discussed (Fig. 1).

2. Results and discussion

The *p*NZ group was first described by Carpenter and Gish as an alternative to benzyloxycarbonyl (Z).[9] It has also been used for the protection of the ε-amino group of Lys.[10] Very recently, the *p*NZ has been described as a temporary protecting group for the α-functionalities in solid-phase peptide synthesis.[11] *p*NZ is orthogonal to the most common solid-phase peptide synthesis protecting groups such as ^{t}Bu/*tert*-butyloxycarbonyl (Boc), Fmoc, and Alloc, and is removed under

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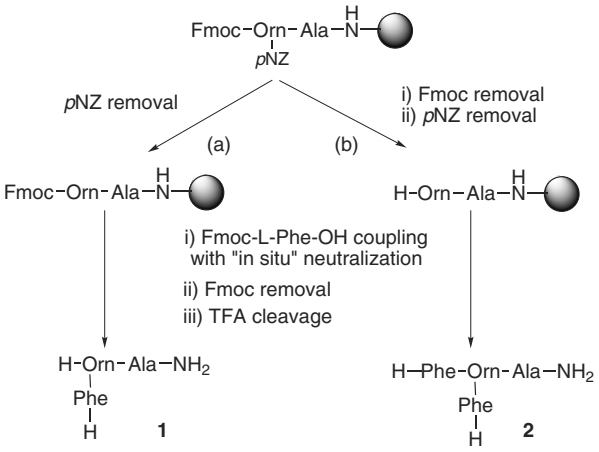
reductive and nearly neutral conditions with $SnCl_2$ in the presence of low concentrations of acid (1.6 mM HCl/dioxane).[11] Furthermore, its use circumvents typical side reactions associated with piperidine, such as the formation of aspartiimides or diketopiperazines (DKP).[11]

2.1. Avoiding undesired α-Fmoc removal

The basis for using *p*NZ to avoid DKP formation is that its removal implies catalytic amounts of acid. Thus, the deprotected amino acid is obtained as an ammonium salt, leaving the basicity and nucleophilicity of the amine function masked.[11] The same principle was thus applied to circumvent the unwanted removal of Fmoc caused by the primary amines of Lys or Orn side chains upon deprotection during solid-phase synthesis.

To demonstrate the utility of *p*NZ as a temporary protecting group for the side chains of Orn and Lys, the peptide {[H-Orn(&)-Ala-NH_2][H-Phe&]}[12] (**1**) was prepared from Fmoc-Orn(*p*NZ)-OH (Scheme 1, pathway **a**).[13] After *p*NZ removal in mildly acidic conditions, Fmoc-Phe-OH was directly coupled without a prior neutralization step. Instead, an in situ neutralization method similar to that used to avoid DKP formation was used.[11,14] If the α-Fmoc of Orn had been prematurely removed, then the byproduct {[H-Phe-Orn(&)-Ala-NH_2][H-Phe&]} (**2**) would have been generated, as was observed upon following pathway **b** of Scheme 1. As expected, peptide **2** was not detected by HPLC (Fig. 2) in crude **1**, indicating that premature Fmoc removal had been avoided by using *p*NZ.



Scheme 1. Strategy used to demonstrate that premature α-Fmoc elimination does not occur when *p*NZ is used to protect amino acid side chains.

2.2. Use of *p*NZ/*p*NB protection for the solid-phase synthesis of cyclic peptides

Similarly to the pair Alloc/Allyl used for the side-chain protection of Lys or Orn and Glu or Asp, *p*NZ can be used in conjunction with the related *p*-nitrobenzyl (*p*NB) for the synthesis of side chain to side-chain cyclic peptides.[15] This was demonstrated for the synthesis of the cyclic peptide H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2, a conformationally restricted analog of deltorphin.[16]

The peptide was synthesized on a Rink amide resin using Fmoc-Lys(*p*NZ)-OH and Fmoc-Glu(O*p*NB)-OH[17] together with other Fmoc/*t*Bu amino acids as shown in Scheme 2. The elongation of the peptide chain proceeded smoothly using *N*,*N*-diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt) as coupling agents. The semipermanent *p*NZ and *p*NB groups were removed with $SnCl_2$ and catalytic HCl. The cyclization was then carried out using benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP)/(*N*,*N*-*d*iisopropylethyl amine (DIEA)). Finally, cleavage of the peptidyl resin with trifluoroacetic acid (TFA) rendered the target peptide with a yield of 14.1% after HPLC purification (Fig. 3).

3. Conclusions

The use of *p*NZ as a temporary protecting group of Orn and Lys avoids premature α-Fmoc removal after side-chain deprotection of the ω-amino function. Furthermore, *p*NZ has been used in combination with its derivative *p*NB to synthesize a cyclic peptide via side-chain to side-chain cyclization. The *p*NZ/*p*NB combination has thus been validated as a new member

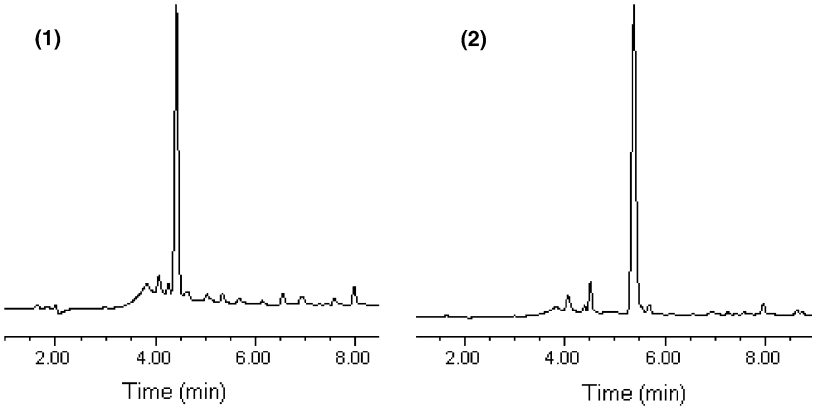
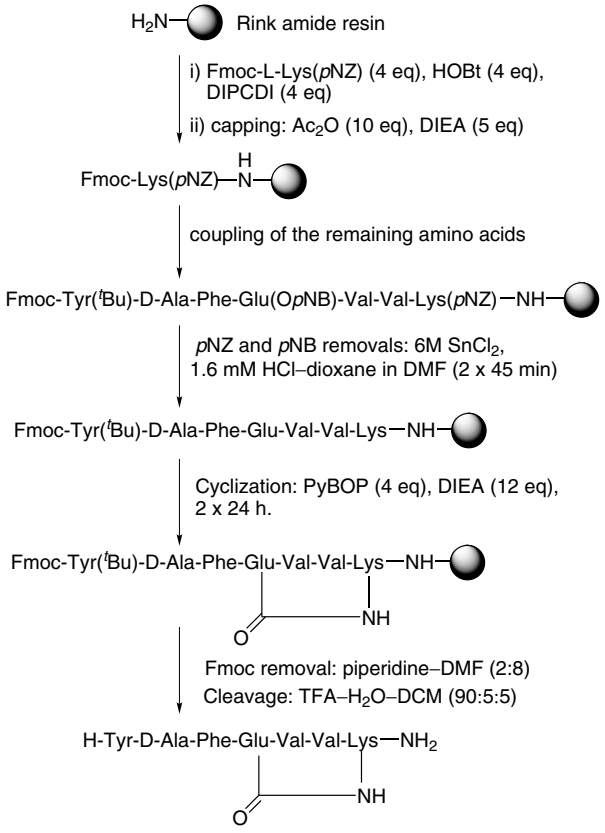


Figure 2. HPLC chromatograms of H-Orn(Phe)-Ala-NH_2 (**1**) and H-Phe-Orn(Phe)-Ala-NH_2 (**2**).



Scheme 2. Solid-phase synthesis of the deltorphin cyclic analog, H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2.

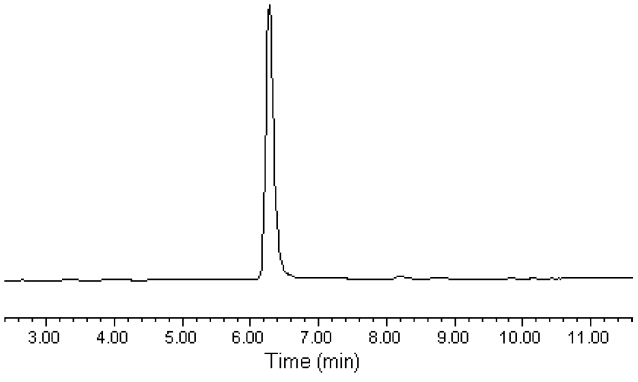


Figure 3. HPLC chromatogram of the cyclic deltorphin analog H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2.

of the family of orthogonal protecting group pairs[18] used in the synthesis of cyclic peptides as well as other complex peptides and organic molecules. Using the *p*NZ/*p*NB pair in conjunction with the Alloc/Allyl pair could enable regioselective synthesis of bicyclic peptides.

4. Experimental section

Analytical HPLC was carried out in a Waters instrument comprising two solvent-delivery pumps and automatic injector (Waters Separations Module 2695) and a variable-wavelength detector (model Waters 996 Photodiode Array). UV detection was performed at 220 nm, and linear gradients of CH_3CN (0.036% TFA) into H_2O (0.045% TFA) were run at a flow rate of 1.0 mL/min (Gradient A: from H_2O to CH_3CN in 15 min; Gradient B: from CH_3CN–H_2O (80:20) CH_3CN–H_2O (65:35) in 15 min).

4.1. Avoiding undesired α-Fmoc removal

To a Rink amide resin ($f = 0.66$ mmol/g, 100 mg), placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disk, was added Fmoc-Ala-OH (4 equiv) in the presence of equimolar amounts of DIPCDI and HOBt in *N*,*N*-dimethylformamide (DMF). The mixture was stirred for 1 h, at which point the Fmoc group was removed with piperidine–DMF (1:4). Fmoc-Orn(*p*NZ)-OH (4 equiv) was then coupled as described above. The resin was dissolved in DMF and divided into two equal portions (**a**,**b**).

The *p*NZ group of resin **a** was removed using 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF (2×30 min), and Fmoc-Phe-OH was subsequently coupled with PyBOP (5 equiv) and DIEA (8 equiv) in DMF. The Fmoc groups were removed, the peptidyl resin was cleaved with TFA/DCM/H_2O (90:5:5), and crude **1** was concentrated by evaporation, then characterized by HPLC ($t_R = 5.39$ min, gradient A) and ESMS (calcd for $C_{26}H_{36}N_6O_4$, 496.3, found *m*/*z*, 497.5 $[M+H]^+$).

The Fmoc and *p*NZ groups of **b** were removed as previously described and Fmoc-Phe-OH was coupled as above. Then, Fmoc group was removed and the peptide submitted to the same protocol as above to give **2**, which was dissolved in CH_3CN–H_2O and characterized by HPLC ($t_R = 4.42$ min, gradient A) and ESMS (calcd for $C_{17}H_{27}N_5O_3$, 349.2, found *m*/*z*, 350.4 $[M+H]^+$).

4.2. Preparation of the cyclic deltorphin analog H-Tyr-D-Ala-Phe-Glu(&)-Val-Val-Lys(&)-NH_2

Fmoc-Lys(*p*NZ)-OH (0.9 equiv), HOBt (0.9 equiv), and DIPCDI (0.9 equiv) in DMF were added to Rink amide resin (0.2 g, $f = 0.68$ mmol/g) and stirred for 1 h. The resin was then treated with Ac_2O (10 equiv) and DIEA (5 equiv) in DMF for 30 min in order to acetylate the free amino groups. The new loading of the resin was calculated by UV titration of the Fmoc group ($f = 0.49$ mmol/g). The remaining amino acids were coupled using 4 equiv each of Fmoc-Aaa-OH/DIPCDI/HOBt in DMF. Once the linear peptide was assembled, the Fmoc group of the last residue (Tyr) was left on, and the *p*NZ and *p*NB groups were removed by treating the resin with 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF (2×45 min). An aliquot of the resin was then treated with piperidine/DMF (1:4) to remove the Fmoc group, treated with TFA/H_2O/DCM (90:5:5) to cleave the peptide from the resin, and analyzed by HPLC ($t_R = 6.08$ min, gradient A) and ESMS (calcd for $C_{42}H_{63}N_9O_{10}$, 853.5, found *m*/*z*, 854.2 $[M+H]^+$).

The remaining resin was treated with PyBOP (4 equiv) and DIEA (12 equiv) in DMF for 24 h to cyclize the

peptide. An aliquot of the resin was then treated with piperidine/DMF (1:4), TFA/H_2O/DCM (90:5:5), and analyzed by HPLC and HPLC–MS, which indicated that the crude product comprised 65% linear peptide and 35% cyclic peptide. The remaining resin was re-treated with the same reagents and after 24 h an aliquot of the resin was analyzed as described above, indicating 50% linear peptide and 50% cyclic peptide. The cyclization was completed by stirring the resin with PyBOP (4 equiv), DIEA (12 equiv), and HOAt (8 equiv) in DMF for 72 h. The Fmoc group was then removed and the peptide was cleaved from the resin with TFA/H_2O/DCM (90:5:5). The TFA was removed by evaporation and the crude was dissolved in CH_3CN/H_2O (7:3) and washed three times with $CHCl_3$. H_2O was added to the aqueous layer, which was lyophilized and found to contain a 1:1 mixture of the linear and cyclic peptides among other impurities. The crude product was purified by semipreparative HPLC to afford 1.8 mg of the cyclic peptide, which was characterized by HPLC (t_R = 6.26 min, 99% of purity, gradient B) and ESMS (calcd for $C_{42}H_{63}N_9O_{10}$, 835.5, found m/z, 836.7 $[M+H]^+$).

Acknowledgments

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Fmoc-L-Glu(ONB)-O′Bu (1.68 g, 3 mmol) was dissolved in TFA/DCM (1:1) (20 mL) and stirred for 1 h. The solvent was then eliminated in vacuo and diethyl ether was added and evaporated four times in order to ensure that the TFA was removed. After drying the product in vacuo, a white solid was obtained (1.51 g, 3 mmol, nearly quantitative yield).
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