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Efficient Acylation of the *N*-Terminus of Highly Hindered $C^{\alpha,\alpha}$-Disubstituted Amino Acids via Amino Acid Symmetrical Anhydrides

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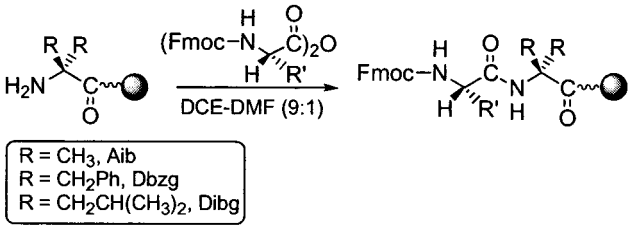
ABSTRACT



Fmoc amino acid symmetrical anhydrides are efficient and readily available reagents for acylation of the *N*-terminus of highly hindered $C^{\alpha,\alpha}$-dialkylated α-amino acids. Comparison of a variety of coupling protocols showed that the symmetrical anhydride method always provided the superior results. This method was successfully applied to the solid-phase synthesis of a peptide containing three ααAAs at alternating positions.

The incorporation of $C^{\alpha,\alpha}$-disubstituted α-amino acids (ααAAs) is a recognized method of inducing secondary structure into peptides.[1,2] The ααAAs with short side chains such as Aib,[3] Deg, and Dpg have been incorporated into peptides by a variety of methods,[4] including standard carbodiimide protocols,[5] urethane-protected *N*-carboxy anhydrides,[6] activated oxazolones,[7] amino acid fluorides,[8] and HOAt-based uronium (e.g., HATU)[9] and phosphonium salts

(1) For recent examples of ααAAs in highly structured peptides, see: (a) Formaggio, F.; Crisma, M.; Rossi, P.; Scrimin, P.; Kaptein, B.; Broxterman, Q. B.; Kamphuis, J.; Toniolo, C. *Chem.-Eur. J.* **2000**, *6*, 4498−4504. (b) Vijayalakshmi, S.; Rao, R. B.; Karle, I. L.; Balaram, P. *Biopolymers* **2000**, *53*, 84. (c) Yokum, T. S.; Gauthier, T. J.; Hammer, R. P.; McLaughlin, M. L. *J. Am. Chem. Soc.* **1997**, *119*, 1167−1168.

(2) For examples of antimicrobial active peptides containing ααAAs, see: (a) Epand, R. F.; Epand, R. M.; Formaggio, F.; Crisma, M.; Wu, H.; Lehrer, R. I.; Toniolo, C. *Eur. J. Biochem.* **2001**, *268*, 703−712. (b) Yokum, T. S.; Elzer, P. H.; McLaughlin, M. L. *J. Med. Chem.* **1996**, *39*, 3603−3604.

(3) Abbrevations: Aib, α-aminoisobutyric acid; Boc, *tert*-butoxycarbonyl; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; Dbzg, $C^{\alpha,\alpha}$-dibenzylglycine; DCE, 1,2-dichloroethane; Deg, $C^{\alpha,\alpha}$-diethylglycine; Dibg, $C^{\alpha,\alpha}$-diisobutylglycine; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dpg, $C^{\alpha,\alpha}$-dipropylglycine; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, hydroxy-7-azabenzotriazole; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; PAL, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid; PEG-PS, poly(ethylene glycol)polystyrene graft; PyAOP, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxylic acid; TPS, triisopropylsilane.

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Table 1. Coupling of Fmoc-Lys(Boc)-OH onto (A) H-Dbzg-Ala-Dpg-Glu(OtBu)-PAL-PEG-PS[a] and (B) H-Dibg-Ala-PAL-PEG-PS[b]

						coupling yield (%)[d]	
entry	coupling method[c]	base	solvent	time (h)	temp (°C)	A	B
1	PyAOP	DIEA	DMF	8	50	<10	–
2	PyAOP	DIEA	DCE–DMF (1:1)	8	50	20[a]	22
3	PyAOP–HOAt	DIEA	DCE–DMF (1:1)	8	25	25[a]	26
4	PyAOP–HOAt	DIEA	DCE–DMF (1:1)	8	50	–	35
5	HATU	DIEA	DMF	8	25	18[a]	32
6	HATU	DIEA	DCE–DMF (1:1)	8	50	–	46
7	Fmoc-Lys(Boc)-F	DIEA	DCE–DMF (1:1)	8	50	26	28
8	Fmoc-Lys(Boc)-F	DIEA	DCE–DMF (9:1)	8	50	46	50
9	symmetrical anhydride[e]	no base	CH_2Cl_2	8	25	–	62
10	symmetrical anhydride	no base	DCE–DMF (9:1)	3	50	95[a]	91
11	symmetrical anhydride	no base	DCE–DMF (1:1)	3	50	81[a]	73
12	symmetrical anhydride	no base	DMF	3	50	52[a]	34
13	BOP-Cl[f]	no base[g]	CH_2Cl_2	8	25	49	53
14	BOP-Cl[f]	no base[g]	DCE–DMF (9:1)	8	50	88	–

[a] Coupling results under certain conditions were previously described in ref 15. [b] Peptide H-Lys-Dbzg-Ala-Dpg-Glu-$CONH_2$ and H-Lys-Dibg-Ala-$CONH_2$ were manually synthesized on Fmoc-PAL-PEG-PS resin. All couplings before acylation of Dbzg or Dibg were efficiently carried out using PyAOP in DCE–DMF (1:1) at 50 °C. Conditions: PyAOP, 4 equiv; DIEA, 8 equiv; Fmoc amino acids, 4 equiv (0.3 M). Deblock, piperidine: DBU:DMF (5:2:93). [c] Coupling conditions: (i) for entries 1–2, PyAOP, 4 equiv; DIEA, 8 equiv; Fmoc amino acids, 4 equiv (0.3 M); (ii) for entries 3–4, PyAOP, 4 equiv; HOAt, 4 equiv; DIEA, 8 equiv; Fmoc-Lys residue, 4 equiv (0.3 M); (iii) for entries 5–6, HATU, 4 equiv; DIEA, 8 equiv; Fmoc-Lys residue, 4 equiv (0.3 M); (iv) for entries 7–8, the preformed amino acid fluoride[17] was used in the coupling. Coupling conditions, DIEA, 4 equiv; Fmoc-Lys(Boc)-F, 4 equiv (0.3 M); (v) for entries 9–12, Fmoc-Lys(Boc)-symmetrical anhydride, 3 equiv (0.2 M); (vi) for entries 13–14, Fmoc-Lys(Boc)-BOP mixed anhydride, 4 equiv (0.3 M). [d] The coupling yield was determined by UV analysis of the Fmoc-deprotection.[13] [e] For entries 9–12, the preformed Fmoc-Lys(Boc)-symmetrical anhydride was used in the coupling. Symmetrical anhydride was prepared by treatment of 2 equiv of Fmoc-Lys(Boc)-OH with 1 equiv of DCC in CH_2Cl_2 at room temperature for 2 h and the precipitated DCU was removed by filtration. For entries 10–12, the filtrate was concentrated and the symmetrical anhydride was taken up in proper solvent for coupling. [f] For entries 13–14, preformed mixed anhydride was used in the coupling. The mixed anhydride was prepared by treatment of 1 equiv of Fmoc-Lys(Boc)-OH with 1 equiv of BOP-Cl with the presence of 1 equiv of DIEA in CH_2Cl_2 at 0 °C for 1 h.[19] The concentrated mixed anhydride was taken up in proper solvent for coupling. [g] For entries 13–14, DIEA was used in the formation of mixed anhydrides, no base was used in the coupling.

(e.g., PyAOP).[10] Incorporation of more sterically demanding ααAAs such as Dbzg, Dibg, or TOAC into peptides is more problematic. For example, incorporation of Dbzg or TOAC onto a growing peptide chain was readily accomplished by activated ααAA oxazolones or traditional HBTU coupling reagent.[11] In contrast, acylation of the *N*-terminus of Dbzg or TOAC with DCC/HOBt, mixed anhydride, or HATU only gave low to moderate yields.[11,12] Thus the difficulties in incorporating sterically hindered ααAAs into internal positions of peptide chains mainly stem from the inefficient acylation of the ααAA *N*-termini. Herein we report a convenient method for the high yield acylation of the *N*-terminus of sterically hindered ααAAs using amino acid symmetrical anhydrides in the absence of base.

As part of our ongoing program aimed at conformationally restricted peptides containing ααAAs at alternating sequence positions, Dbzg, Dibg, or Dpg was incorporated into peptides using Fmoc solid-phase synthesis.[13] We have found that PyAOP is a powerful coupling reagent for incorporation of less hindered ααAAs such as Dpg into peptide internal positions at elevated temperature. In the synthesis of a series of pentapeptides which have the sequence H-xxx-Dpg-Tyr-Dpg-xxx-$CONH_2$ (xxx = Lys and/or Glu),[14] the coupling of *C*-terminal Fmoc-Dpg-OH to the resin using PyAOP in DMF at room temperature was straightforward. In contrast, attempted acylation of Dpg with Fmoc-Tyr-OH under the same conditions gave poor coupling (30%). When the synthesis was performed at 50 °C, a much improved coupling yield (>90%) was obtained (data not shown). Our recent synthetic study of peptides containing highly hindered ααAAs such as Dbzg and Dibg shows that coupling of Fmoc-Dbzg-OH or Fmoc-Dibg-OH onto the *N*-terminus of unhindered amino acids such as Ala using PyAOP at elevated temperature was relatively efficient.[15] However, once Dbzg or Dibg is at the *N*-terminus of the peptide, the following acylation of the ααAAs became very difficult (Table 1, entries 1–8). In our work, coupling of Fmoc-Lys(Boc)-OH onto the *N*-terminus of Dbzg or Dibg using PyAOP in DMF was ineffective (Table 1, entry 1). When the coupling was performed in a low polarity solvent mixture (DCE–DMF, 1:1),[16] improved

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(14) Synthesis of peptides H-xxx-Dpg-Tyr-Dpg-xxx-$CONH_2$ (xxx = Lys and/or Glu) were performed on Perseptive Biosystem Peptide Synthesizer 9050 with Fmoc-PAL-PEG-PS resin as solid support. Solvent, DMF; PyAOP, 4 equiv; DIEA, 8 equiv; Fmoc amino acids, 4 equiv. Acylation of the *N*-terminal Dpg was performed at 50 °C, while all other couplings were carried out without heating. Deblock, piperdine:DBU:DMF (5:2:93).

(15) For synthesis of peptide H-Lys-Dbzg-Ala-Dpg-Glu-$CONH_2$, see: Fu, Y.; Hammarström, L. G. J.; Miller, T. J.; Fronczek, F. R.; McLaughlin, M. L.; Hammer, R. P. *J. Org. Chem.* **2001**, *66*, 7118–7124.

(16) PyAOP and HATU are not soluble in DCE or DCE-DMF (9:1); couplings in these solvents were not attempted.

but still low yields were obtained (Table 1, entries 2−4). The attempted acylation of *N*-terminus of Dbzg or Dibg using HATU also gave a low yield at room temperature, while the acylation of Dibg at 50 °C resulted in a moderate yield (Table 1, entry 6). When an amino acid fluoride[17] protocol was utilized, the acylation in DCE−DMF (1:1) gave low yields for both Dbzg and Dibg. Interestingly, when the acylations were carrried out in a less polar solvent mixture of DCE−DMF (9:1), improved yields were obtained (Table 1, entries 7−8).

Previous studies showed symmetrical anhydrides could readily acylate *N*-substituted secondary amino acids in nonpolar solvent such as CH_2Cl_2.[18] In our hands we found that the symmetrical anhydride of Fmoc-Lys(Boc)-OH in CH_2Cl_2 in the absence of base gave an improved yield (62%) in the acylation of the Dibg-resin at room temperature (Table 1, entry 9). On the basis of our previous work, we found that the coupling could be improved at elevated temperatures. Thus, the symmetrical anhydride we prepared in CH_2Cl_2 was concentrated and redissolved in the higher boiling DCE−DMF mixture. Now a 3 h coupling in DCE−DMF (9:1) at 50 °C gave an acylating yield of 95% for the Dbzg-resin and 91% for the Dibg-resin (Table 1, entry 10). As before, increases in solvent polarity gave dramatically reduced coupling yields (Table 1, entries 11−12). It was also observed that activated mixed anhydride formed by BOP-Cl[19] and Lys gave very good coupling yield at elevated temperatures (Table 1, entry 14).

To verify the utility of the symmetrical anhydride method in the assembly of difficult sequence peptides, a highly conformationally constrained analogue of *β*-amyloid peptide core, AMY-1, was synthesized. In this peptide sequence,

AMY-1: H-Lys-**Dibg**-Val-**Dbzg**-Phe-**Dpg**-$(Lys)_6$-$CONH_2$

Phe, Val, or Lys is at either the *C*- or *N*-terminus of Dbzg and Dibg, which could make efficient amide bond formation at both termini of the ααAAs difficult. To optimize the yield of the peptide, three activation methods were utilized during the peptide assembly.[21] The incorporation of the first six lysine residues, Dpg, and Phe into the peptide using the PyAOP protocol at room temperature was straightforward. Coupling of Fmoc-Dbzg-OH onto Phe was also performed using PyAOP at 50 °C in which an 89% yield was obtained after 8 h. In contrast, the coupling of Fmoc-Dibg-OH onto Val using PyAOP/HOAt at 50 °C only gave a 58% yield after 12 h. When the coupling of Fmoc-Dibg-OH onto Val was performed under HATU/HOAt conditions, an improved yield (81%) was obtained.

As we experienced before, once the Dbzg or Dibg was at the *N*-terminus of the peptide, the following acylation of the ααAAs was very difficult (Figure 1). Monitoring of the

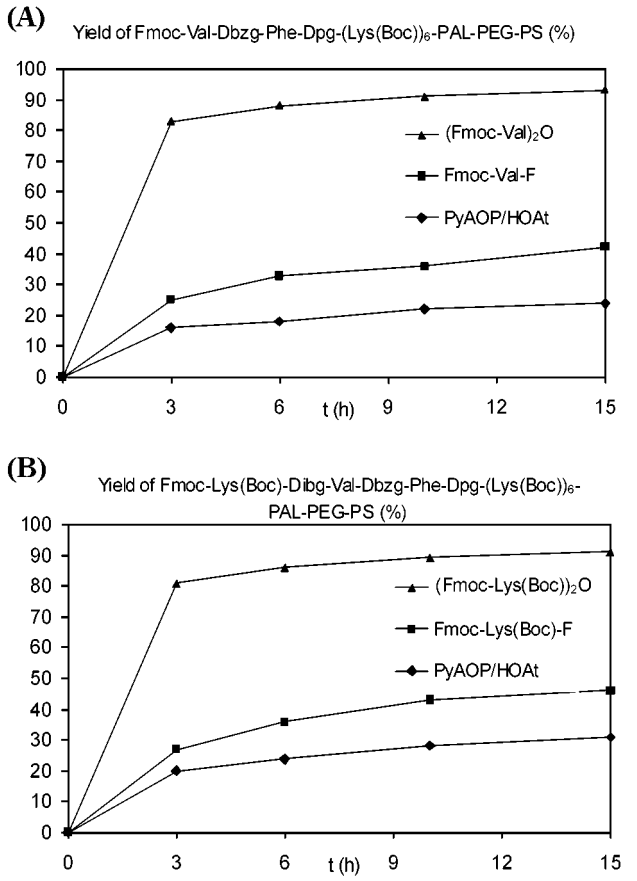

Figure 1. (A) Coupling of Fmoc-Val-residue onto H-Dbzg-Phe-Dpg-$(Lys(Boc))_6$-PAL-PEG-PS;[20] (B) coupling of Fmoc-Lys(Boc)-residue onto H-Dibg-Val-Dbzg-Phe-Dpg-$(Lys(Boc))_6$-PAL-PEG-PS.

coupling of Fmoc-Val or Fmoc-Lys to the highly hindered *N*-terminus of Dbzg or Dibg, respectively, using PyAOP/HOAt or amino acid fluorides showed that only low to moderate yields were obtained after prolonged reaction time. Attempted coupling of Fmoc-Val-OH or Fmoc-Lys-OH to the *N*-terminus of Dbzg or Dibg under HATU/HOAt conditions also gave low yields (32%, 35%) after 15 h. In contrast, the acylation using readily available Fmoc amino acid symmetrical anhydrides in low polarity solvent without base resulted in excellent coupling yields. The deblocked peptide was obtained in high quality and good yield as verified by HPLC (Figure 2) and by MALDI-MS.

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(20) Acylation using PyAOP/HOAt, via amino acid fluorides or symmetrical anhydrides, were performed under the same conditions as described in Table 1, note c.

(21) The first six Lys residues were coupled to PAL-PEG-PS resin under PyAOP/DIEA/DMF coupling conditions on Perseptive Biosystem Peptide Synthesizer 9050. The rest of amino acid residues were incorporated manually into the sequence. Dpg, Phe, and Dbzg were incorporated into sequence using PyAOP/DIEA in DCE−DMF (1:1); Val residue was coupled to *N*-terminus of Dbzg via amino acid symmetrical anhydride method; Dibg was coupled to Val using HATU (4 equiv), HOAt (4 equiv), DIEA (8 equiv), and Fmoc-Dibg-OH (4 equiv, 0.3 M) in DCE/DMF (1:1) at 50 °C; Lys residue was coupled to Dibg via symmetrical anhydride at 50 °C.

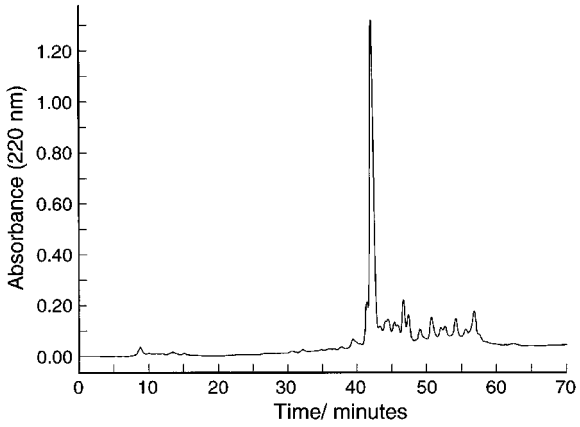

Figure 2. HPLC profile of crude peptide AMY-1 (H-Lys-Dibg-Val-Dbzg-Phe-Dpg-$(Lys)_6$-$CONH_2$). Column: Delta-Pak C_{18}, 15 μm 300 Å, 8 × 100 mm. Eluent A: 0.05% TFA in H_2O; eluent B: 0.05% TFA in CH_3CN. Gradient: 10−70% B over 60 min; flow rate 1 mL/min.

It is of great interest to note the remarkable coupling efficiency with symmetrical anhydrides, especially when the attempted conventional activation methods for difficult couplings failed to yield satisfactory results. Our study shows that symmetrical anhydrides couple more efficiently at the *N*-terminus of ααAAs in nonpolar solvents than in polar solvents. As we previously proposed,[15] the reactivity of amino acid symmetrical anhydrides may be enhanced by the anchimeric assistance from intermolecular H-bonding between the NH of ααAA and the anhydride carbonyl oxygen, which would be more pronounced in nonpolar solvents.

In summary, we present an efficient method for incorporating amino acid residues onto the *N*-terminus of highly hindered ααAAs. Fmoc amino acid symmetrical anhydrides are readily available acylating agents and the lack of base could effectively prevent a variety of side reactions. This convenient coupling method may have general application for synthesis of conformationally constrained peptides containing a variety of sterically hindered ααAAs.

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Supporting Information Available: HPLC profile of crude peptide DIBG-1 (H-Lys-Dibg-Ala-$CONH_2$), MALDI mass spectra of peptide DIBG-1 and peptide AMY-1 (H-Lys-Dibg-Val-Dbzg-Phe-Dpg-$(Lys)_6$-$CONH_2$). This material is available free of charge via the Internet at http://pubs.acs.org.

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