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Chemoselective Alkylation of *N*-Alkylaminooxy-Containing Peptides

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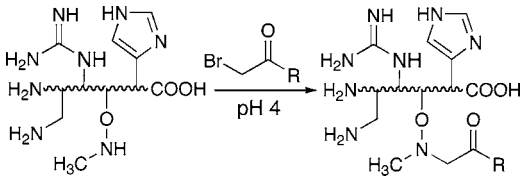
ABSTRACT



Peptides containing *N*-alkylaminooxy amino acids were chemoselectively alkylated with allylic, benzylic, and α-carbonyl bromides, *N*-ethylmaleimide, and hexyl acrylate in mildly acidic aqueous/organic solutions. Alkylation at the aminooxy nitrogen proceeds in good yields with excellent to complete chemoselectivity in the presence of all common amino acids except cysteine. This reaction complements the selective glycosylation and acylation of *N*-alkylaminooxy groups and provides an avenue for the synthesis of peptide arrays comprising a wide variety of neoglycopeptides and neolipopeptides.

Chemoselective reactions to attach small molecules to peptides and proteins serve as the synthetic equivalent of post-translational modifications in natural systems. Such reactions generally rely on the insertion of an amino acid with noncoded side chain functionality into a peptide sequence. Ideally, the chosen unnatural functional group would be capable of multiple types of reactions that mirror nature's post-translational modifications, which include glycosylation, palmitoylation (acylation), prenylation (alkylation), phosphorylation, and sulfonation.

N-Alkylaminooxy groups are proving to meet this multireaction ideal for "post-synthetic" modifications of peptides because they remain nucleophilic in acidic, aqueous solutions. They are extremely useful for glycosylation,[1,2] largely because the attached sugars maintain cyclic conformations,[1a,2b] and they have been shown to undergo chemoselective acylation with succinimidyl esters.[3] We now report the selective alkylation of *N*-alkylaminooxy groups in peptides. This new reactivity significantly broadens the range of selective modifications possible at *N*-alkylaminooxy moieties and enables the synthesis of combinatorial peptide arrays with diverse postsynthetic modifications.

To investigate whether peptidic *N*-alkylaminooxy groups could undergo chemoselective alkylation, we synthesized peptides with one of three previously reported *N*-alkylaminooxy amino acids, **1**−**3** (Figure 1). As their protected derivatives **1b**−**3b**, they were incorporated into peptides using standard in situ neutralization protocols for Boc-chemistry-based solid-phase peptide synthesis (SPPS).[4] *N*-Alkylaminooxy-containing peptides may also be synthesized by Fmoc-chemistry-based SPPS using derivatives **1c**−**3c**.[1e,5]

(1) (a) Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269−12278. (b) Carrasco, M. R.; Nguyen, M. J.; Burnell, D. R.; MacLaren, M. D.; Hengel, S. M. *Tetrahedron Lett.* **2002**, *43*, 5727−5729. (c) Peri, F.; Venturini, S.; Nicotra, F. In *Peptides 2002: Proceedings of the 27th European Peptide Symposium*, Aug 31−Sep 6, 2002, Sorrento, Italy; Benedetti, E., Pedone, C., Eds.; Edizioni Ziino: Napoli, Italy, 2002; pp 252−253. (d) Carrasco, M. R.; Brown, R. T. *J. Org. Chem.* **2003**, *68*, 8853−8858. (e) Filira, F.; Biondi, B.; Biondi, L.; Giannini, E.; Gobbo, M.; Negri, L.; Rocchi, R. *Org. Biomol. Chem.* **2003**, *1*, 3059−3063. (f) Matsubara, N.; Oiwa, K.; Hohsaka, T.; Sadamoto, R.; Niikura, K.; Fukuhara, N.; Takimoto, A.; Kondo, H.; Nishimura, S.-I. *Chem. Eur. J.* **2005**, *11*, 6974−6981.

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(4) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.

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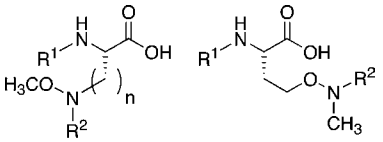

1 n=1
2 n=2

3

1a, **2a**, and **3a** R^1=R^2=H
1b, **2b**, and **3b** R^1=Boc, R^2=Z(2Cl)
1c, **2c**, and **3c** R^1=Fmoc, R^2=Boc

In peptide sequences, **1a**=Z^1, **2a**=Z^2, **3a**=Z^3

Figure 1. *N*-Alkylaminooxy amino acids used for model peptides.

For our initial studies, we synthesized the peptide H-LAZ^3-SK-NH_2, **4**, and reacted it with a variety of common alkylating agents in 0.1 M NaOAc, pH 4.0 buffer at 37 °C with up to 50% CH_3CN (Table 1). Our criterion for a successful reaction was high conversion to a single, major product. We quickly found that activated halides were required. Ordinary alkyl bromides showed no reaction (entries 1 and 2), and even methyl iodide led to very slow addition (entry 8). However, allyl, benzyl, and α-carbonyl bromides reacted efficiently (entries 3−7). The α-carbonyl bromides (entries 5−7) proved ideal and led to both high conversions and high yields. By contrast, benzyl and allyl bromide led to lower conversions and modest yields with the appearance of multiple double-addition products. We also found that the *N*-alkylaminooxy group underwent rapid, complete conjugate addition with *N*-ethylmaleimide, which bodes well for its use with a wide variety of commercially available maleimide-based labeling reagents (entry 10). Conjugate addition to hexyl acrylate also proceeded cleanly but more slowly (entry 11).

Table 1. Alkylation Reactions with H-LAZ^3SK-NH_2, **4**

entry	electrophile	time (h)	conv[a] (%)	yield[b] (%)
1	bromobutane	47	0	0
2	bromocyclopentane	47	0	0
3	benzyl bromide	1	76	70
4	allyl bromide	7	64	57
5	bromoacetic acid	19	>95	>95
6	methyl bromoacetate	23	92	88
7	bromoacetyl Val-OMe	23	>95	>95
8	methyl iodide	23	16	16
9	cyclohexene oxide	16	<3	<3
10	*N*-ethylmaleimide	7	>98	>98[c]
11	hexyl acrylate	48	82	77

[a] Conversion determined by HPLC integration (UV detection at 214 nm) of all peptide products relative to starting peptide. [b] Yield determined by HPLC integration of major monoalkylated peptide product compared to starting peptide and other peptide products. [c] Product was an approximately equal mixture of the two possible diastereomers.

(5) (a) Carrasco, M. R.; Brown, R. T.; Serafimova, I. M.; Silva, O. *J. Org. Chem.* **2003**, *68*, 195−197. (b) Carrasco, M. R.; Brown, R. T.; Doan, V. H.; Kandel, S. M.; Lee, F. C. *Biopolymers (Peptide Science)* **2006**, *84*, 414−420.

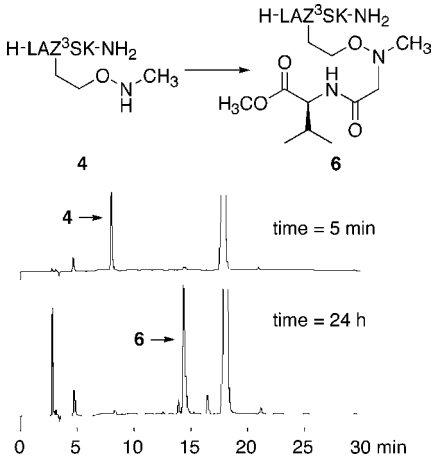

Figure 2. HPLC chromatographs for the reaction of **4** with bromoacetylated Val-OMe. HPLC conditions: Kromasil C18, 0−65% B in A over 30 min, A = H_2O + 0.1% TFA, B = CH_3CN + 0.08% TFA.

To assess the chemoselectivity of the reactions, corresponding control reactions were run under identical conditions with the peptide H-LASK-NH_2, **5**. With the α-bromocarbonyl substrates, we observed no products whatsoever. The acrylate and maleimide substrates led to <3% addition products, and reactions of **5** with allyl and benzyl bromide yielded 6−9% of addition products. From the control reactions with **5**, we infer that all the observed alkylations with **4** were selective for the alkylaminooxy nitrogen. Further, even though there is some nonselective reaction of **4** with benzyl and allyl bromide, the major double-addition products in these reactions are attributed to a second alkylation at the alkylaminooxy nitrogen and not alkylation of a competing functional group.

HPLC data for entry 7 are shown in Figure 2. Here, a 8 mM solution of **4** in 0.1 M NaOAc, pH 4.0 buffer at 39 °C was treated with 1000 mol % of bromoacetylated Val-OMe. At 23 h, both the conversion of **4** (calcd for $(M + H)^+$ 547.4, found 547.4) and the yield of a single, monoalkylated product (calcd for $(M + H)^+$ 718.5, found 718.4) are >95%.[6]

We next synthesized the three peptides H-ELFHSIZ^3-EKAR-NH_2, **7**, H-ELFHSIZ^2EKAR-NH_2, **8**, and H-ELFHSIZ^1-EKAR-NH_2, **9**, to assess alkylation chemoselectivity in the presence of carboxylates and imidazoles and whether the varying alkylaminooxy side chains could all be alkylated similarly. Alkylation reactions of **7** led to conversions as high as those with **4** but lower overall yields (Table 2, entries 1−4). Here, in addition to a major peak corresponding to the desired single-addition product, we always observed multiple byproducts in small amounts that can be attributed to additional, nonselective additions at other locations.

(6) The two peaks flanking **6** in the bottom trace were identified by ESI-MS as hydroxyacetyl Val-OMe and acetoxyacetyl Val-OMe, which result from background reactions of the bromide with the buffer and were seen in a control reaction where **4** was omitted. For the chromatograph of the control reaction of **5** with bromoacetylated Val-OMe, see the Supporting Information.

Table 2. Alkylation Reactions of H-ELFHSIZEKAR-NH_2 Peptides **7**-**9**

entry	peptide	electrophile	time (h)	conv[a] (%)	yield[b] (%)
1	**7**	methyl bromoacetate	24	>95	75
2	**7**	bromoacetyl Val-OMe	24	>98	68
3	**7**	hexyl acrylate	48	77	49[c]
4	**7**	*N*-ethylmaleimide	13	93	84
5	**8**	methyl bromoacetate	24	85	51
6	**8**	bromoacetyl Val-OMe	22	76	48
7	**8**	hexyl acrylate	48	48	12
8	**8**	*N*-ethylmaleimide	24	74	61
9	**9**	bromoacetyl Val-OMe	48	78	40
10	**9**	*N*-ethylmaleimide	43	44	28

[a] Conversion determined by HPLC integration (UV detection at 214 nm) of all peptide products relative to starting peptide. [b] Yield determined by HPLC integration of major monoalkylated peptide product compared to starting peptide and other peptide products. [c] The low yield in this reaction was predominantly a result of decomposition of the aminooxy side chain rather than nonspecific alkylation. See the Supporting Information.

Importantly, because the conversions remained high, the nonchemoselective reactions led predominantly to multiply alkylated products from which the desired monoalkylated product could be easily separated. Overall, chemoselectivity was higher with the conjugate additions than with the α-bromocarbonyl derivatives.

We also found that as the distance between the aminooxy nitrogen and the peptide backbone decreased, so did the rate of alkylation. Reactions of the shorter *N*-alkylaminooxy side chains of peptides **8** and **9** proceeded more slowly (entries 5−10). In fact, reaction with the aminooxy side chain of **9** was slow enough to be impractical: the nonselective alkylations now proved to have very competitive rates.

HPLC data for entry 1 are shown in Figure 3. Here, a 10 mM solution of **7** in 0.1 M NaOAc, pH 4.0 buffer at 40 °C was treated with 1000 mol % of methyl bromoacetate. At 24 h, the conversion of **7** (calcd for $(M + 2H)^{2+}$ 679.9, found 679.8) to products is essentially complete, and the yield of a single, monoalkylated product (calcd for $(M + 2H)^{2+}$ 715.9, found 716.0) is 75%. At longer retention times, however, peaks corresponding to more than one addition of electrophile can be seen. The three largest of these were identified by ESI-MS as two distinct doubly alkylated products (peaks **A** and **B**) and a singly alkylated peptide with pyroglutamic acid at the N-terminus (peak **C**). We infer that the His imidazole and both Glu carboxylates undergo small amounts of alkylation, and when an ester is formed at the N-terminal Glu, rapid cyclization to pyro-Glu occurs.[7] Importantly, though, at 24 h only one peak had a mass corresponding to the desired, singly alkylated product. As in the corresponding experiment with **4**, reaction at the aminooxy nitrogen was essentially complete. Thus the nonchemoselective reactions led to multiply alkylated products, which eluted at retention times significantly distinct from the desired product.

(7) Control experiments conducted under identical conditions with the peptide H-ELFHSIEKAR-NH_2, **11**, confirmed these background alkylations. See the Supporting Information.

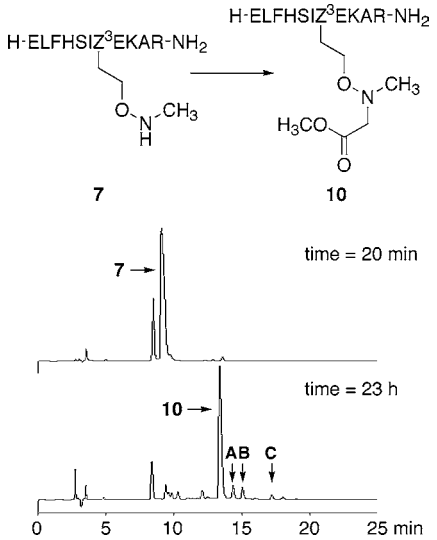

Figure 3. HPLC chromatographs for the reaction of **7** with methyl bromoacetate. HPLC conditions: Kromasil C18, 15−45% B in A over 25 min, A = H_2O + 0.1% TFA, B = CH_3CN + 0.08% TFA

Our last test of the chemoselectivity of the alkylation involved the cysteine-containing peptide H-FAZ[3]RLEGFCSK-NH_2, **12**, and its des-Z^3 control peptide H-FARLEGFCSK-NH_2, **13**. Control reactions with **13** quickly showed that our standard reaction conditions led to cysteine alkylation. However, we also observed homodimerization of **13** via the disulfide. We thus devised a simple procedure to effect selective alkylation of **12**. A 5 mM solution of **12** in 0.1 M sodium acetate, pH 4 buffer was basified to pH >8 and allowed to stir at room temperature. After disulfide formation was complete (24 h), the mixture was acidified to pH 4, charged with 1000 mol % of bromoacetyl-Val-OMe, and allowed to stand at 40 °C. When neither non- nor mono-alkylated disulfide remained (24 h), reduction of the disulfide and quenching of the excess alkylating agent was accomplished by the addition of large excesses of both dithiothreitol and tris(carboxyethyl)phosphine hydrochloride to yield the desired monoalkylated product. In addition to demonstrating an avenue for selective alkylation in cysteine-containing peptides, this result highlights that *N*-alkylaminooxy alkylations can be effected in disulfide-containing peptides and proteins.

In general, maintaining high peptide concentrations is critical for high alkylation rates. We favor using 10 mM solutions of peptide in 0.1 M NaOAc, pH 4.0 buffers. In cases where the electrophile has very limited aqueous solubilty, like with hexyl acrylate, solutions of 50:50 buffer/CH_3CN are effective. We find that heating the reactions at 38−40 °C improves conversion rates without reducing yields. We have also found that the concentration of electrophile is less important, but we generally use a large amount (1000 mol %) relative to the peptide. The one exception is with the extremely reactive *N*-ethylmaleimide, where we only use 150 mol % to minimize nonspecific alkylations.

We have demonstrated that *N*-alkylaminooxy groups in peptides can be chemoselectively modified by alkylation reactions. In particular, alkylations with α-bromocarbonyl compounds, maleimides, and acrylates all proceed with excellent conversions and high chemoselectivities. Considering how many organic molecules can be derivatized with bromoacetic acid and/or acrylic acid and via *N*-alkylation by maleimide, these *N*-alkylaminooxy reactions open the possibility for the attachment of a wide variety of substrates to peptides and proteins. Alkylation of *N*-alkylaminooxy peptides provides an important complement to the established glycosylation and acylation reactivity and greatly enhances the range of "post-translational" modifications that can be facilitated by *N*-alkylaminooxy moieties.

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Supporting Information Available: Representative synthetic procedures, analytical data for peptides **4**, **5**, **7**−**9**, and **11**−**13**, and data for additional reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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