Pergamon

Tetrahedron Letters 39 (1998) 7279–7282

TETRAHEDRON LETTERS

DIRECT INCORPORATION OF UNPROTECTED KETONE GROUPS INTO PEPTIDES DURING SOLID-PHASE SYNTHESIS: APPLICATION TO THE ONE-STEP MODIFICATION OF PEPTIDES WITH TWO DIFFERENT BIOPHYSICAL PROBES FOR FRET

Lisa A. Marcaurelle and Carolyn R. Bertozzi*

Department of Chemistry, University of California, Berkeley, CA 94720

Received 3 July 1998; revised 21 July 1998; accepted 22 July 1998

Abstract: An amino acid bearing an unprotected ketone group, (2*S*)-aminolevulinic acid, was incorporated into a synthetic peptide using standard Fmoc-based solid-phase methods. The ketone group remained unharmed during the synthesis and provided a uniquely reactive functional group for covalent modification of the peptide. The ketone and the sulfhydryl group of a cysteine residue elsewhere in the peptide were reacted simultaneously with two different biophysical probes, enabling the site-specific installation of a donor and acceptor pair for FRET in one step without the need for differential side chain protection.

The fluoresence resonance energy transfer (FRET) technique has found widespread use in the study of protein structure and dynamics.[1] The technique requires the installation of two biophysical probes, a donor and an acceptor, at defined locations within the peptide of interest. A number of methods are now available for the conjugation of small molecule probes to peptides and proteins. The majority of these methods are based on coupling electrophilic functional groups, such as α-iodoacetamides[2] and *N*-hydroxysuccinimido esters,[3] with exposed cysteine and lysine residues, respectively. However, in order achieve site-specific modification of a peptide in the presence of multiple copies of these nucleophilic residues, differential side chain protection must be employed during the synthesis. To avoid the need for extra protecting group manipulations during the conjugation of a synthetic peptide with two different probes, several groups have exploited selective N-terminal reactions,[1f] or incorporated non-native amino acids with suitable photophysical properties (e.g., *p*-nitrotyrosine) in place of their native counterparts.[4] These approaches are somewhat limited with regard to the site of residence and structure of the probe.

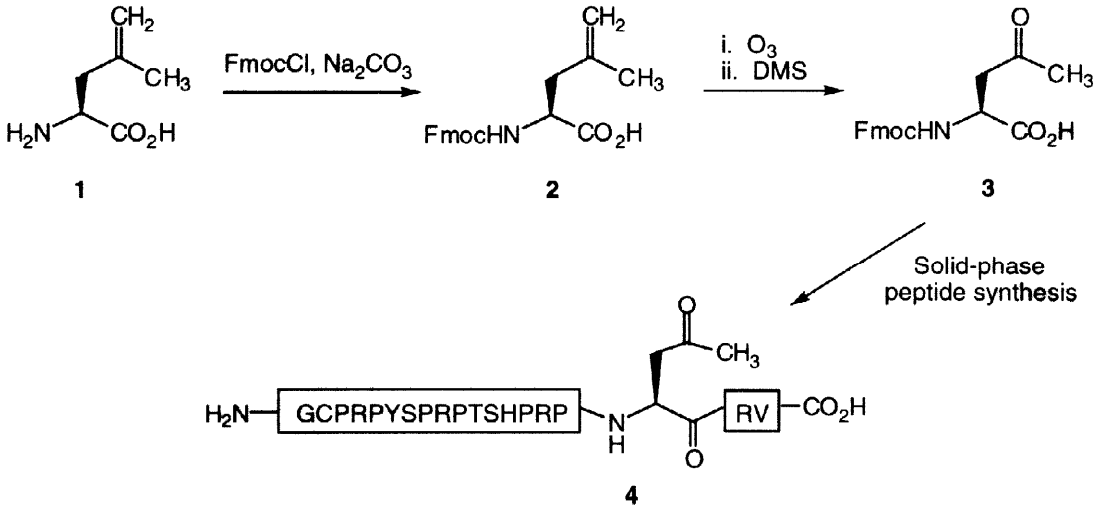
In recent years, the adornment of synthetic peptides with a uniquely reactive *electrophile* has become an increasingly popular tactic for site-specific modification. For example, ketone or aldehyde groups can be installed in synthetic peptides by conjugating levulinic acid (4-oxopentanoic acid) or masked aldehydes to the ε-amino groups of lysine side chains or to the N-terminus of the peptide.[5] Alternatively, aldehyde groups can be generated by the selective oxidation of N-terminal serine residues with periodate.[6] The ketones or aldehydes form stable covalent adducts with a complementary nucleophile, typically an aminooxy, hydrazide or thiosemicarbazide group, in aqueous milieu in the absence of side chain protecting groups. Still, these methods for decorating peptides with ketones and aldehydes require orthogonal side chain or N-terminal protection for site-specific introduction of the electrophile.

0040-4039/98/$ - see front matter
PII: S0040-4039(98)01588-3

Here we report that (2*S*)-aminolevulinic acid, an amino acid bearing an unprotected ketone group, can be directly incorporated into synthetic peptides using standard Fmoc-based solid-phase methods. The ketone group survives the synthesis without undergoing any apparent degradation or unwanted side reactions. Furthermore, the ketone is chemically orthogonal to all natural amino acid side chain functional groups. Thus, sulfhydryl groups of cysteine residues, for example, can be modified in the presence of the ketone and vice versa, allowing for the selective labeling of a peptide with two different probes in one synthetic step. The versatility of the ketone group is therefore two-fold: it can be installed at any site along the polypeptide backbone without need for extra protecting group steps, and it does not interfere with reactions involving the nucleophilic functional groups of native amino acid side chains.

Fmoc-protected (2*S*)-aminolevulinic acid (**3**) was synthesized in two steps from commercially available 4,5-dehydroleucine[7] (**1**) as depicted in Scheme 1. The α-amine was first protected with an Fmoc group to afford compound **2**,[8] which was then converted to keto-amino acid **3** by reductive ozonolysis.[9] We incorporated compound **3** into a 19-amino acid peptide (**4**) on an automated peptide synthesizer using standard Fmoc-based solid-phase methods.[10] The sequence of peptide **4** was derived from the anti-microbial glycopeptide drosocin, which is produced by insects in response to immune challenge.[11] The bacteriostatic potency of drosocin is enhanced five-fold by the presence of an O-linked glycan at Thr11, a feature that may reflect an altered conformational preference due to glycosylation. Thus, drosocin constructs labeled with biophysical probes may be useful tools for analyzing the conformation of the unglycosylated peptide compared to the glycosylated version. We chose to install the (2*S*)-aminolevulinic acid group in place of Ile17. In addition, a cysteine residue was incorporated in place of Lys2 to provide a second orthogonal site for covalent modification.

Scheme 1

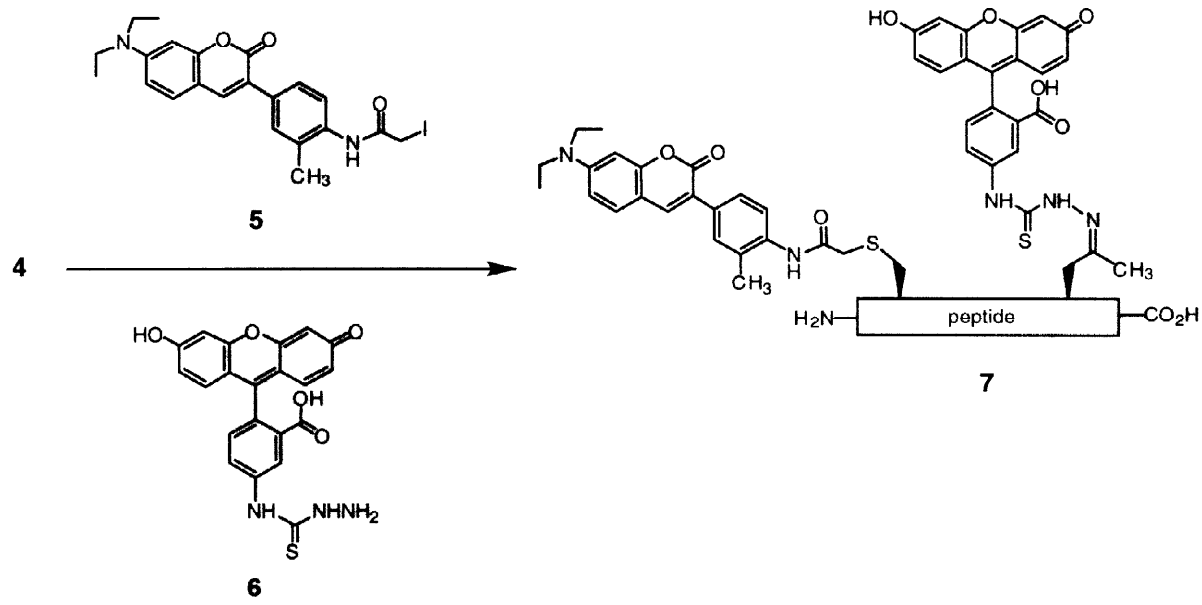


We were concerned that the ketone group might undergo unwanted side reactions, such as condensation with amines to form imines or enamines, or acid- or base-catalyzed aldol condensations, during the process of Fmoc-based solid-phase synthesis. However, the crude product obtained after synthesis and cleavage from the resin was a single peak by reversed-phase

HPLC (RP-HPLC) analysis, and its identity as the desired keto-peptide (**4**) was confirmed by mass spectrometry.[12] No other side products were obtained, indicating that the unprotected ketone group is fully compatible with the reagents of Fmoc-based synthesis, including DCC, piperidine and TFA. In essence, keto-amino acid **3** can be treated similarly to an alanine residue.

To demonstrate the versatility of the ketone group, we modified keto-peptide **4** with two biophysical probes, coumarin iodoacetamide (**5**) and fluorescein thiosemicarbazide (**6**), which are commonly used as a donor and acceptor pair for FRET.[13,14] The site-specific labeling of peptide **4** with these probes was achieved in one synthetic step (Scheme 2). Peptide **4** (5 mg) was incubated with 1.2 equivalents each of **5** and **6** in 900 μL DMF and 100 μL of 1.0 M sodium phosphate buffer, pH 7.0. The ligation reaction was complete after 24 hours and the fluorescently labeled peptide (**7**) was isolated by RP-HPLC and its identity confirmed by mass spectrometry.[15] This general strategy for attaching a pair of small molecules to synthetic peptides should be applicable to a wide range of targets.

Scheme 2



In summary, *N*-Fmoc-(2*S*)-aminolevulinic acid (**3**) can be incorporated into synthetic peptides in an unprotected form to provide a convenient electrophilic functional group, the ketone, for site-specific conjugation. The chemical orthogonality of the ketone to the sulfhydryl group of cysteine side chains allows for the selective modification of peptides with two different probes and facilitates the synthesis of constructs for FRET experiments. This method is therefore a useful complement to traditional protein modification techniques.

Acknowledgments: This research was supported by a CAREER Award from the National Science Foundation (CHE-9734430), and by generous funds from the W. M. Keck Foundation, the Exxon Education Fund, the Pew Scholars Program and the Burroughs Wellcome Fund.

References and notes

1. (a) Stryer, L. *Ann. Rev. Biochem.* **1978**, *47*, 819-846. (b) Lee, J. A.; Fortes, P. A. G. *Biochemistry* **1985**, *24*, 322-330. (c) Taniguchi, K.; Mardh, S. *J. Biol. Chem.* **1993**, *268a*, 15588-15594. (d) Wu, P.; Brand, L. *Anal. Biochem.* **1994**, *218*, 1-13. (e) Miki, M.; Kouyama, T. *Biochemistry* **1994**, *33*, 10171-10177. (f) Imperiali, B.; Rickert, K. W. *Proc. Natl. Acad. Sci.* **1995**, *92*, 97-101. (g) Xing, J.; Cheung, H. C. *Biochemistry* **1995**, *34*, 6475-6487. (h) Mehta, S.; Meldal, M.; Ferro, V.; Duus, J. O.; Bock, K. *J. Chem. Soc., Perkins Trans. 1* **1997**, 1365-1374. (i) Slevin, P. R. *Meth. Enzymol.* **1995**, *246*, 300-334.
2. (a) Sharma, J.; Luduena, R. F.; *J. Protein Chem.* **1994**, *13*, 165-176. (b) Wong, S. Y. C.; Guile, G.; Dwek, R.; Arsequell, G. *Biochem. J.* **1994**, *300*, 843-850. (c) Esmann, M.; Sar, P. C.; Hideg, K. Marsh, D. *Anal. Biochem.* **1993**, *213*, 336-348.
3. (a) Bradburne, J. A.; Godfrey, P.; Choi, J. H.; Mathis, J. N. *Appl. Environ. Microbiol*, **1993**, 59, 663-668. (b) Fan, J. G.; Pope, L. E.; Vitols, K. S.; Huennekens, F. M. *Biochemistry* **1991**, *30*, 4573-4580.
4. Meldal, M.; Breddam, K. *Anal. Biochem.* **1991**, *195*, 141-147.
5. (a) Canne, L. E.; Ferre-D'Amare, A. R.; Burley, S. K. ; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998-3007. (b) Shao, J.; Tam, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 3893-3899.
6. (a) Geoghegan, K. F.; Stroh, J. G. *Bioconjugate Chem.* **1992**, *3*, 138-146. (b) Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30-33. (c) Rose, K.; Zeng, W.; Regamey, P. -O.; Chernushevich, I. V.; Standing, K. G.; Gaertner, H. F. *Bioconjugate Chem.* **1996**, *7*, 552-556.
7. 4,5-Dehydroleucine (**1**) was purchased from Bachem.
8. Hardy, P. M.; Sheppard, P. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 723-729.
9. Preparation of *N*-Fmoc-(2*S*)-aminolevulinic acid (**3**): A solution of Fmoc-4,5-dehydroleucine (**2**)[8] (1.1 g, 3.1 mmol) in 9:1 CH_2Cl_2/methanol (15 mL) was cooled to -78 °C and purged with N_2 for 10 min. A stream of ozone was passed through the solution until a blue color persisted. The reaction mixture was then purged with N_2 (ca. 10 min) until the solution was no longer blue in color. Dimethylsulfide (0.58 mL, 7.9 mmol) was added and the solution was warmed to rt overnight. Excess dimethylsulfide and solvent were removed *in vacuo* and the resulting yellow syrup was purified by silica gel chromatography (10:1 $CHCl_3$/methanol, 0.1% AcOH) and crystallized from CH_2Cl_2/hexanes to afford 0.89 g (81%) of compound **3** as a white solid: mp 136-138 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, 2 H, *J* = 7.4), 7.58 (d, 2 H, *J* = 5.1), 7.39 (t, 2 H, *J* = 7.0), 7.30 (dt, 2H, *J* = 6.2, 1.2), 5.85 (d, 1 H, *J* = 7.8, N-H), 4.60 (m, 1H), 4.43-4.34 (m, 1H), 4.22 (t, 1 H, *J* = 6.9), 3.25 (dd, 1H, *J* = 18.3, 4.0), 2.99 (dd, 1H *J* = 15.5, 4.0), 2.19 (s, 3H, CH_3); C^{13} NMR (100 MHz, $CDCl_3$): δ 175.5, 156.2, 143.7, 143.6, 141.2, 127.7, 127.0, 125.0, 120.0, 119.8, 67.3, 49.5, 47.0, 45.0; FAB-HRMS calcd for $C_{20}H_{20}NO_5$ ($M+H^+$) 354.1341, found 354.1334.
10. Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161-214.
11. (a) Bulet, P.; Dimarcaq, J.-L.; Hetru, C.; Lagueux, M.; Charlet, M.; Hegy, G.; Dorsselar, A.V.; Hoffman, J. A. *J. Biol. Chem.* **1993**, *268*, 14893-14897. (b) Bulet, P.; Urge, L.; Ohresser, S.; Hetru, C.; Otvos, L. *Eur. J. Biochem.* **1996**, *238*, 64-69.
12. ES-MS: calcd for peptide **4** ($M+H^+$) 2173, found *m/z* 2173.
13. Coumarin iodoacetamide and flourescein thiosemicarbazide were purchased from Molecular Probes (cat. # C-404 and F-121, respectively).
14. (a) Haugland, R. *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*; 6th ed. 1996. (b) Theilen, T. P. G. M.; Maassen, J. A.; Kriek, J.; Moller, W. *Biochemistry* **1984**, *23*, 6668-6674. (c) Odom, O. W.; Deng, H. Y.; Dabbs, E.R.; Hardesty, B. *Biochemistry* **1984**, *23*, 5069-5076.
15. Reversed-phase (C_{18}) HPLC conditions: Elution gradient: CH_3CN (B) in H_2O (A), both with 0.1% TFA (10 → 60% B, over 50 min). ES-MS: calcd for peptide **7** ($M+H^+$) 2939, found *m/z* 2939.