Tetrahedron Letters, Vol.30, No.1, pp 39-42, 1989 0040-4039/89 $3.00 + .00
Printed in Great Britain Pergamon Press plc

METHODS FOR THE CARBOXYL-TERMINAL FLUORESCENT LABELING OF PEPTIDES USING SOLID PHASE PEPTIDE SYNTHESIS

Angelo P. Consalvo, Paul P. Tamburini, William Stern, and Stanley D. Young*

Unigene Laboratories, Inc.
110 Little Falls Road
Fairfield, NJ 07006

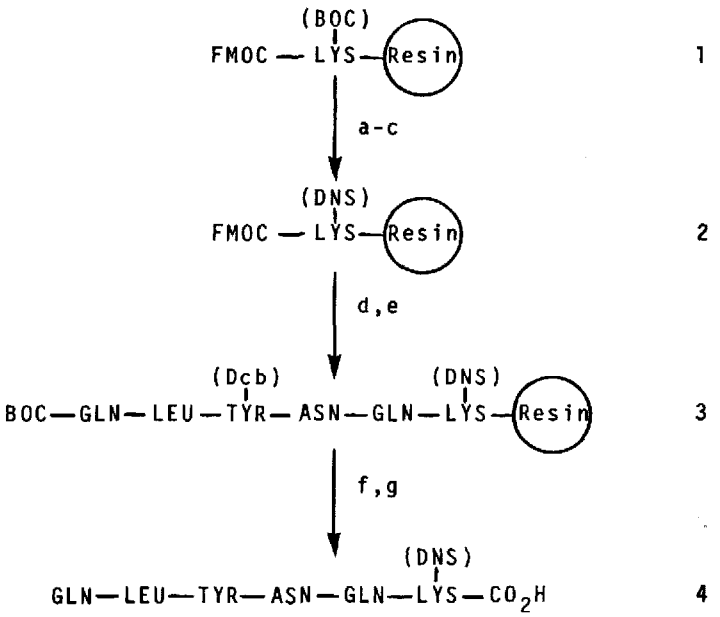
Summary: Two general methods for labeling synthetic peptides with a 5-dimethylamino-1-napthalenesulfonyl (dansyl) group at the C-terminal residue using solid phase peptide synthesis (SPPS) are described. Dansylated peptides are ideal substrates for fluorometric proteolytic enzyme assays.

We have shown the utility of peptides labeled at their amino termini with a fluorescent 5-dimethylamino-1-napthalenesulfonyl (dansyl) moiety as substrates for proteolytic enzyme assays. The method involves reverse-phase HPLC separation of substrate and enzymically generated product which are detected and quantified fluorometrically. An example is the assay used to follow the rates of conversion of model glycine-extended peptides to C-terminal peptide amides by the peptidyl α-amidation enzyme.[1] It is also possible to assay enzymes which perform amino terminal peptide modifications or endoproteolytic cleavages using peptide substrates dansylated at the C-terminus. This communication outlines two strategies for the specific labeling of peptides at the C-terminus using solid phase peptide synthesis (SPPS).[2] The syntheses of two peptide substrates are described: the first peptide (4) was used to assay the conversion of an amino terminal glutaminyl residue to pyroglutamic acid by glutaminyl cyclase; the second peptide (7) was used to assay the chymotryptic endoproteolysis of a C-terminal fragment of neurotensin (NT).

The strategy shown in Scheme I involves dansylation of the peptide on the ϵ-amino group of a C-terminal lysyl residue. Although the N-terminal NT fragment illustrated in Scheme I conveniently contained a lysyl residue, the procedure may be applied to any peptide of interest by simply extending its C-terminus by a single lysyl residue. It has been our experience that through a judicious selection of model peptide length, effects of the lysine extension or the dansyl moiety on the functional enzyme-peptide interaction may be avoided. N-α-FMOC-N-ϵ-BOC-lysine was covalently anchored to

Merrifield's chloromethylstyrene resin to obtain a loading of 0.2-0.3 mmol/g which was calculated from amino acid analysis.[3] After removal of the BOC group with TFA, the ε-amino group was dansylated to completion as determined by a ninhydrin test.[4] Following the removal of the FMOC group from the α-amine, the protected amino acids were added using conventional SPPS techniques to obtain 4. The dansyl amide is resistant to anhydrous HF which was used to cleave the peptide from the resin and remove the amino acid side chain protecting groups. The peptide 4 was purified by elution from a C-18 reverse-phase HPLC column with a linear gradient formed using 0.1% TFA and acetonitrile.

SCHEME I

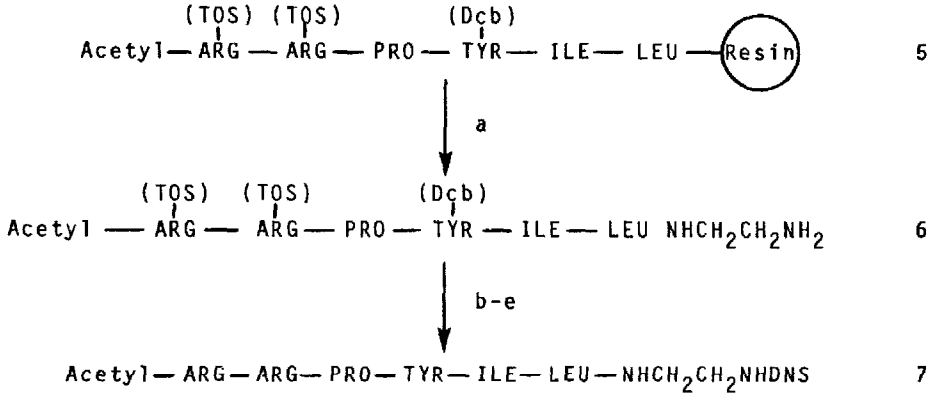


a) 35% TFA/CH_2CL_2 b) 10% DIPEA/CH_2CL_2 c) DNS-Cl, DIPEA, DMF
d) 20% Piperidine/DMF e) SPPS f) HF g) RP-HPLC.

The second strategy involves cleaving a fully-protected peptide from Merrifield's resin with ethylene diamine.[5,6] Other approaches to C-terminal aminoalkylamides either require classical solution synthesis[7] or do not leave the protecting groups intact,[8] an action which would lead to non-specific attachment of the dansyl tag. Scheme II outlines the synthesis of the protected C-terminal NT sequence, Arg-Arg-Pro-Tyr-Ile-Leu, linked to ethylene diamine. After dansylation of the crude cleavage mixture, the fully-protected peptide 6 was purified using C-18 reverse-phase HPLC and then deprotected in anhydrous HF to obtain 7. The purification step prior to HF removal of the side chain protecting groups was

found to be critical; otherwise the product mixture was complicated by several dansylated peptides which were indistinguishable by amino acid analysis.

SCHEME II

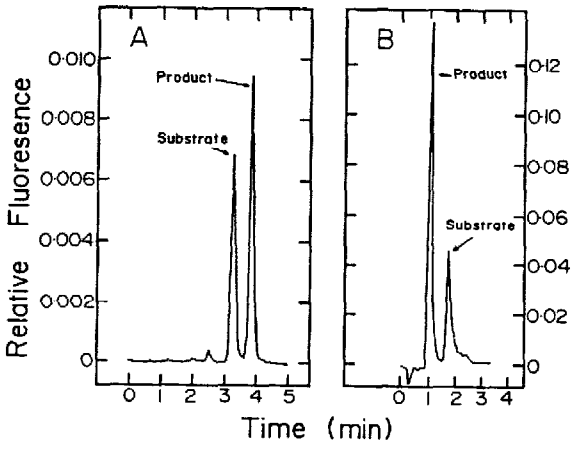


a) Ethylenediamine/MeOH b) DNS-Cl, DIPEA, DMF c) RP-HPLC d) HF e) RP-HPLC.

The peptide substrate 4 was used to develop an assay for glutaminyl cyclase.[9] The fluorescent substrate and product were separated in less than 4 min using isocratic elution on C-18 reverse-phase HPLC (Figure 1A). The detection limit for either peptide was 100 fmol, which is two orders of magnitude greater sensitivity than is possible using UV detection at 220 nm.[10] Fluorometric detection allows the direct assay of crude tissue homogenates without interference from the other components of the assay mixture. This assay offers several advantages over a more laborious radioimmunoassay (RIA) which used an antibody to detect the conversion of Gln-His-Pro-NH_2 to the pyroglutaminyl peptide, thyrotropin releasing hormone (TRH).[11] Another HPLC-based assay was developed using peptide 7 to analyze the endoproteolytic cleavage of NT at tyrosine (Figure 1B).

With the ability to fluorescently label either the N- or C-termini of peptide substrates in hand, it is now possible to develop rapid and sensitive assays for any sequence-specific proteolytic enzyme. The study of these enzymes is important for understanding post-translational protein modifications as well as protein metabolism.

FIGURE 1



A. Glutaminyl Cyclase Assay
Column: Hypersil 5 µm ODS, 4.6x100 mm
Eluant: 24% acetonitrile in 100 mM NaOAc, pH 6.5
Temperature: 50° C
Flow Rate: 1.2 mL/min
Fluorescent detection using a 352-360 nm excitation filter and a 482 nm cut-off emission filter.
Substrate: GlnLeuTyrAsnGln(ε-DNS)Lys, 80 pmol
Product: <GluLeuTyrAsnGln(ε-DNS)Lys, 130 pmol

B. Chymotrypsin Assay
Column: Vydac C_4 cartridge
Eluant A: 0.1% TFA
Eluant B: 80% acetonitrile, 0.08% TFA
Gradient: 15 to 40% B over 3 min
Temperature: 50° C
Flow Rate: 2 mL/min
Fluorescent detection using 220 nm excitation and 415 nm emission.
Substrate: AcArgArgProTyrIleLeuNHCH$_2$CH$_2$NH(DNS), 120 pmol
Product: IleLeuNHCH$_2$CH$_2$NH(DNS), 380 pmol

REFERENCES AND NOTES

1) Jones, B.N.; Tamburini, P.P.; Consalvo, A.P.; Young, S.D.; Lovato, S.J.; Gilligan, J.P.; Jeng, A.Y.; Wennogle, L.P., Analytical Biochemistry, 168, 272-279 (1988).

2) Merrifield, R.B., J. Am. Chem. Soc., 85, 2149-2154 (1963).

3) Amino acid composition analysis was performed by hydrolyzing the peptides with 6N HCl at 150°C for 1 hr using a Waters Pico-Tag system. The hydrolysates were derivatized with phenylisothiocyanate and the phenylthiocarbamyl amino acids were analyzed on C-18 RP-HPLC using gradient elution with 200 mM NaOAc, pH 6.5 and acetonitrile.

4) Kaiser, E.; Colescott, R.L.; Bossinger, C.D.; Cook, P.I., Analytical Biochemistry, 34, 595 (1970).

5) Merrifield, R.B., Biochemistry 3, 1385-1390 (1964).

6) Glass, J.D.; du Vigneaud, V., J. Med. Chem., 16, 160-161 (1973).

7) Geiger, R., Liebigs Ann. Chem., 750, 165-170 (1971). Geiger, R.; Gerhards, H.J.; Wiemer, G.; Hock, F.J., Peptides 1986, Proc. 19th Europ. Peptide Symp. (D. Theodoropoulos, ed.) Walter de Gruyter, 489-492 (1987).

8) Breipohl, G.; Knolle, J.; Geiger, R., Tetrahedron Lett., 5647-5650 (1987).

9) Consalvo, A.P.; Young, S.D.; Jones, B.N.; Tamburini, P.P., Analytical Biochemistry, In Press.

10) Fischer, W.H.; Spiess, J., Proc. Natl. Acad. Sci. USA, 84, 3628-3632 (1987).

11) Busby, W.H.; Quackenbush, J.H.; Youngblood, W.W.; Kizer, J.S., J. Biol. Chem., 262, 8532-8536 (1987).

(Received in USA 19 August 1988)