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AUTOMATED SYNTHESIS OF FLUOROGENIC PROTEASE SUBSTRATES: DESIGN OF PROBES FOR ALZHEIMER'S DISEASE-ASSOCIATED PROTEASES

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Summary: A facile automated solid phase method for the synthesis of internally quenched, fluorogenic protease substrates is described. Substrates specifically tailored to represent putative cleavage sites for the normal secretory processing pathway and the amyloidogenic processing pathway(s) of the Alzheimer's disease amyloid precursor protein (APP) have been prepared by this method, and utilized to identify neural protease activities in normal and Alzheimer's disease brain tissue.

Fluorogenic protease substrates have emerged as important tools in enzymology of known, purified proteases, and in screening of inhibitors of these proteases as potential therapeutics. Efforts in our laboratories over the past several years have successfully exploited internally quenched fluorogenic protease substrates in studies of HIV protease[1-3] and renin,[4-6] and in studies of inhibitors for these therapeutic drug targets. Another promising area of application for these internally quenched fluorogenic substrates is the discovery of new protease activities from tissue specimens or cultured cells based on the selective recognition and cleavage of extended peptide-based substrates. In this paper we describe an automated solid phase method that permits synthesis of internally quenched fluorogenic substrates based on virtually any peptide sequence, and we describe the synthesis of substrates, the peptide sequences of which encompass the secretory and N-terminal amyloidogenic proteolytic processing sites of the Alzheimer's disease (AD) amyloid precursor protein (APP).

In a previous report,[2] we described the incorporation of the Edans fluorophore and Dabcyl quenching chromophore at the carboxyl and amino termini, respectively, to generate useful fluorogenic substrates for HIV protease. The major drawback associated with this synthetic chemistry was the limitation on peptide side chain functionality to those groups that would not interfere with the condensation reactions. This limitation precluded the synthesis of substrates for many proteases of interest. To circumvent this difficulty, we have synthesized Fmoc-substituted amino acid reagents which incorporate the Edans or Dabcyl groups as part of the side chain, as shown in Figure 1. The synthesis of N_{α}-Fmoc-N_{ε}-Dabcyl-L-lysine **1** proceeded in a single step (95% yield), while the synthesis of the N_{α}-Fmoc-L-Glutamic acid-γ-(Edans) amide **2** required two steps (71% yield). With these reagents in hand, a completely automated, solid phase synthetic protocol based on Fmoc chemistry was developed to synthesize substrates shown in Figure 2 which represent putative cleavage sites for the amyloidogenic processing pathway (**3**) and the normal secretory processing pathway (**4**) of APP.

The automated process is illustrated in Figure 3.[7] Several aspects of this automated process are noteworthy. It is advantageous to incorporate the N_{α}-Fmoc-L-Glutamic acid-γ-(Edans) amide near the amino terminus and the N_{α}-Fmoc-N_{ε}-Dabcyl-L-lysine at the carboxy terminus, since we have detected some chemical instability associated with the Edans group. This positioning of the fluorophore limits its exposure to the repetitive conditions of the automated synthetic protocol. Direct attachment of N_{α}-Fmoc-N_{ε}-Dabcyl-L-

lysine to the SAMBHA resin [8] is not highly efficient, and it is advantageous to insert a glycine or other natural amino acid to the resin first, or to use a resin pre-functionalized with a first residue. Finally, the use of a radical scavenger mixture during the cleavage step[7] is essential to avoid decomposition of the Edans group.

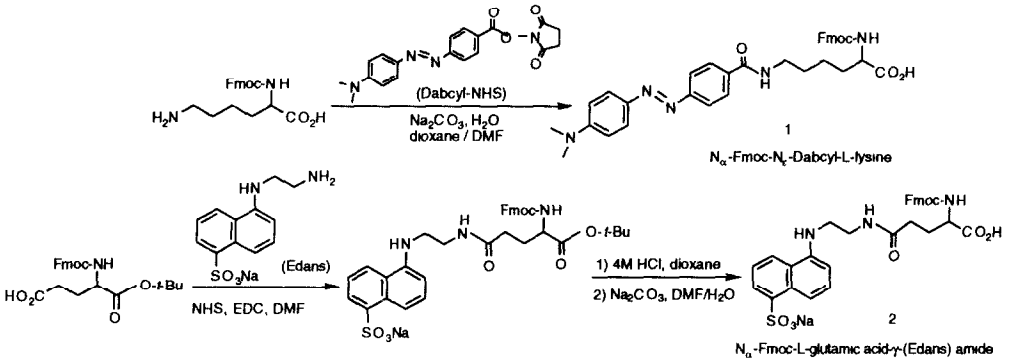

Figure 1. Synthesis of derivatized amino acids, N_α-Fmoc-N_ε-Dabcyl-L-lysine (1), containing the quenching Dabcyl moiety, and N_α-Fmoc-L-glutamic acid-γ-(Edans) amide (2), containing the fluorophore Edans moiety.

Using this automated synthetic route, the substrate format can be configured such that the desired peptide substrate sequence is flanked directly at its termini with **1** and **2**, enabling incorporation of additional charged residues at either end to enhance solubility. This positions the fluorophore and quencher groups as close as possible for optimal energy transfer, but permits incorporation of multiple charged amino acids. The two termini may be left unprotected as free carboxyl and amino groups, or they may be prepared as carboxamide and N-acetamide to protect against exopeptidases potentially present in cell or tissue-derived samples.

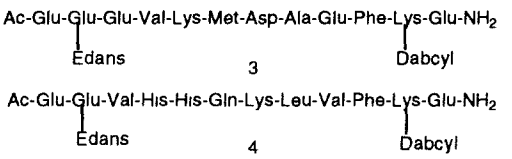

Figure 2. Fluorogenic substrates represent putative cleavage sites for the amyloidogenic processing pathway (**3**) and the normal secretory processing pathway (**4**) of the Alzheimer's disease amyloid precursor protein (APP).

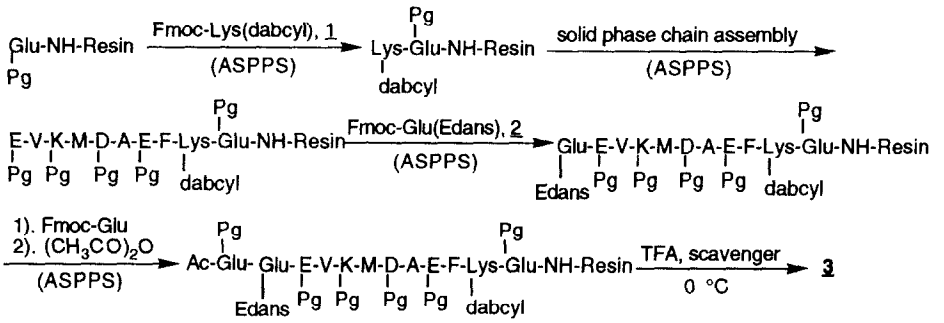

Figure 3. Automated solid phase synthesis of fluorogenic substrates.[7] Abbreviations: ASPPS, automated solid phase peptide synthesis; Pg, protecting group. Single letter notations for natural amino acids are as follows: A, Ala; D, Asp; E, Glu; F, Phe; K, Lys, M, Met; V, Val.

This automated synthetic procedure gives excellent results. Figure 4A presents the high performance liquid chromatography (HPLC) analysis of the crude amyloidogenic substrate **3**, which indicates that the unpurified material contains at least 85% the desired material, identified by electrospray ionization mass spectrometry after HPLC purification. We have synthesized more than thirty substrates for a variety of proteases with comparable results.

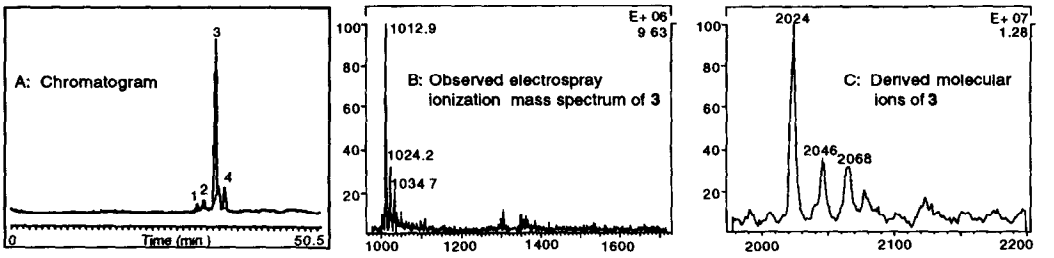

Figure 4. A: Reverse phase HPLC chromatogram of crude substrate for amyloidogenic protease(s) (**3**) obtained using HPLC conditions described in reference 7. Peak #3 was isolated and identified as the desired substrate. **B**: Observed electrospray mass spectrum of **3** showing the double-charged ions. A solution of purified **3** in 50% aqueous acetonitrile with 0.1% TFA was used for this experiment.[14] **C**: Computer-derived molecular ions of **3** based on the observed data shown in Fig. 4B. The calculated molecular weight of **3** is 2046, assuming that the sulfonate group of Edans moiety exists as sodium salt.

This automated protocol has proved extremely valuable in the synthesis of a series of fluorogenic protease substrates that resemble putative proteolytic processing sites from the Alzheimer's amyloid precursor protein (APP). APP has been identified as the progenitor of the amyloid ß protein (a 39-43 residue peptide also known as ß/A4 amyloid), which is the principle component of neuritic plaques, one of the hallmarks of Alzheimer's disease (AD).[9] These amyloid plaques have been speculated to play a causative role in AD.[10] The ß/A4 amyloid peptide arises by proteolytic processing of amyloid precursor protein (APP) which exists in brain tissue as three distinct forms containing either 695, 751 or 770 amino acid residues. These forms are the result of alternate mRNA splicing from a single APP gene.[11] The processing pathway connecting ß/A4 amyloid and APP has not been elucidated completely, however it has been demonstrated in several cell culture systems transfected with an APP gene that a distinct constitutive secretory processing pathway exists. Membrane-bound APP can be proteolytically cleaved within the β/A4 sequence at the Lys16-Leu17 bond of β/A4 by a yet unidentified membrane-bound protease known as the secretase.[12] This cleavage results in secretion of the N-terminal portion of APP, and precludes β/A4 peptide generation. In cell culture systems, some fraction of APP has been shown to avoid this secretory cleavage, instead trafficking to lysosomal compartments where β/A4 generating proteolytic cleavages may occur.[13] Whatever the venue for these amyloidogenic cleavage events, the data obtained to this point clearly indicate that ß/A4 amyloid results by a pathway distinct from the secretory pathway, probably involving distinct proteases. Inhibition of these β/A4 amyloid generating proteases may provide the basis for therapeutic intervention in early stages of Alzheimer's disease. These results have prompted intense efforts to identify the proteases associated with the amyloidogenic and secretory processing of APP. Future reports will describe results in which these fluorogenic substrates have been used as valuable tools for identification, characterization and purification of these enzymes.

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7. The automated solid phase synthesis was carried out on an Applied Biosystems (Foster City, CA.) Model 431A peptide synthesizer using the Fmoc chemistry, which involves activation of protected amino acids through dicyclohexylcarbodiimide (DCC) catalyzed formation of hydroxybenzotriazole (HOBT) active ester in N-methylpyrolidone (NMP) . The following side chain protecting groups are used: Asp and Glu, *t*-butyl ester; Lys, *t*-butyloxycarbonyl; His and Gln, triphenylmethyl (trityl). The natural amino acids (using 4- or 10-fold excess) were coupled using standard methods as specified by the instrument manufacturer. The derivatized amino acids **1** and **2** (using 3-fold excess) was pre-dissolved in 2-3 mL of 20% DMSO in NMP and coupled using the same chemistry but extensively modified instrumental modules, including elimination of NMP addition to the amino acid cartridge, deletion of all mixing cartridge action and extension of coupling time. Following the synthesis, the dried substrate-resin was treated with 20 mL of 93% TFA and 7% of a scavenger cocktail (anisole, ethylene dithiol and ethyl methyl sulfide, 1:3:3) at 0 °C for 4 hours. After removing the resin by filtration, the TFA solution was concentrated and the crude product was precipitated with diethyl ether. The crude substrate was dissolved in DMSO and purified by reverse phase HPLC on a Rainin 41.4 mm ID x 25 cm C_{18} column (300 Angstrom pore and 12 μm particle size) using a flow rate of 40 mL/min. Solvent gradient: 15% solvent B (acetonitrile with 0.1% TFA) for 10 minutes then linear gradient of 15 to 50% solvent B in 35 minutes (solvent A was 0.1% aqueous TFA). The pooled HPLC fractions were then lyophilized to give the final product.
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14. We have observed peaks corresponding to apparent mass of (M+16) in addition to peaks corresponding to the expected molecular weight if a solution of **3** in DMSO was used in electrospray ionization mass spectroscopic analysis, indicating oxidation of the methionine. This is apparently due to oxygen transfer from DMSO sensitized during ionization. Experience from our laboratory has indicated that this is a phenomena common to many peptides containing methionine. Therefore DMSO should be used as solvent for electrospray ionization mass spectroscopic analysis of these compounds with caution.

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