# Articles

## A General Method for the Preparation of Internally Quenched Fluorogenic Protease Substrates Using Solid-Phase Peptide Synthesis†,[1]

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A general scheme for obtaining a fluorescent donor/acceptor peptide substrate via solid-phase synthesis methodology is presented. The key feature of this method is the design of a glutamic acid derivative that has been modified on the carboxyl side chain with a 5-[(2′-aminoethyl)-amino]naphthelenesulfonic acid (EDANS) to create a fluorescent donor moiety that can be incorporated near the C-terminus of the peptide substrate. The corresponding fluorescent acceptor group containing a 4-[[4-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) can then be attached to the resin-bound peptide at the N-terminus while all side-chain groups are still fully protected. Substrates for renin and HIV proteinase are synthesized as examples.

Research on proteolytic enzyme purification and kinetics, as well as enzyme inhibitor identification, is greatly facilitated by the availability of a sensitive, continuous enzyme assay. Such a real-time assay can be achieved through the use of a substrate that exhibits a spectrophotometric change proportional to its rate of cleavage by the enzyme. One of the most common designs for such a substrate relies on the placement of a chromogenic moiety at the site of proteolytic cleavage. However, this strategy is limited if the enzyme does not readily accommodate the chromophoric group in the catalytic center of its active site. An alternative approach, which allows for the design of a substrate with optimal residues at the enzyme active site, is one in which a fluorescent donor is placed near one end of the substrate and an acceptor group is placed near the other end.[2] The fluorescence of this type of substrate is initially quenched by intramolecular resonance energy transfer between the donor and acceptor, but as the enzyme cleaves the substrate the fluorescence increases. The sensitivity of this type of assay, as well as the maximum spacing between the donor/acceptor pair allowed for efficient quenching, is critically dependent on the spectral overlap of the fluorescent emission of the donor with the absorption of the acceptor.[3] One very effective donor/acceptor pair, recently reported for use in a substrate designed for an HIV protease assay, is the 5-[(2′-aminoethyl)amino]naphthelenesulfonic acid (EDANS) and 4-[[4′-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) pair shown in Figure 1.[3] In the study by Matayohsi and coworkers[3] the DABCYL group was attached to the peptide, and then the DABCYL–peptide was coupled to the EDANS group.[4] Such a synthetic strategy would usually preclude the use of peptide substrates with amine or carboxylic acid side-chain groups and could lead to racemization of the C-terminal amino acid.

We report here a general method for incorporating the EDANS/DABCYL pair into any peptide substrate using standard Boc-derived solid-phase peptide synthesis methodology.[5] The key to the procedure is the attachment of the EDANS fluorescent donor group to the γ-carboxylate of a suitably protected glutamic acid residue, as shown in Scheme I, to give compound 1. Ultimately, this leaves the α-amino and α-carboxyl groups available for subsequent solid-phase coupling procedures. It also is possible to customize other desirable features, such as solubility, into the substrate by having additional amino acids C-terminal to the Glu(EDANS) residue. In the examples given here, an arginine or lysine residue was used as the amino acid to anchor the peptide to the solid-phase resin and to provide enhanced solubility to the substrate. When the Boc protecting group was removed by trifluoroacetic acid treatment from the Glu(EDANS) residue attached to the resin, chain termination with loss of EDANS, probably through pyroglutaminyl formation, was observed. To circumvent this difficulty γ-aminobutyric acid (γAbu) as its Boc-amino derivative was attached to Glu(EDANS)-OBzl in solution after removal of the Boc group from the glutamate residue by HCl/dioxane. The Abu group was added as a spacer to reduce the possibility of the EDANS moiety having a deleterious effect on the interaction of the substrate with subsites of the enzyme. The dipeptide derivative 3 could thus be purified and thoroughly characterized before attachment to the solid-phase resin.

The three fluorogenic enzyme substrates described in Table I were synthesized on an ABI 430A peptide

† This paper is dedicated to Prof. Hirschmann on the event of his 70th birthday.

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commision on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *158*, 9). All optically active amino acids are of the L variety unless otherwise specified. Additional abbreviations used: BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DABCYL, 4-[[4′-(dimethylamino)phenyl]azo]benzoic acid; DIPEA, *N,N*-diisopropylethylamine; EDANS, 5-[(2′-aminoethyl)amino]-naphthelenesulfonic acid; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; DMF, dimethylformamide; γAbu, γ-aminobutyric acid; PAM, (phenylacetamido)methyl; TFA, trifluoroacetic acid.

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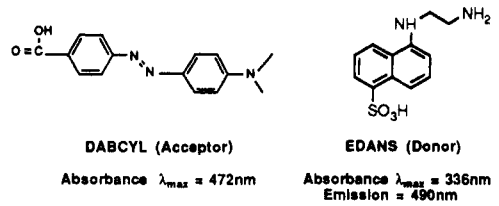


**Figure 1.** Donor/acceptor pair used for internal resonance energy transfer.

**Scheme I**

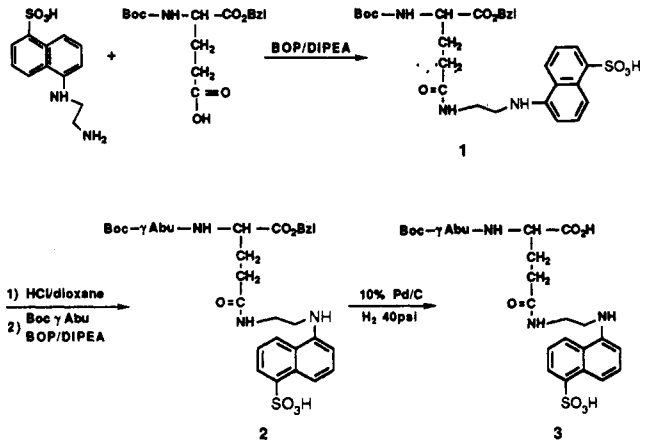


**Table I.** Kinetic Parameters for Enzyme-Catalyzed Hydrolysis of Fluorogenic Substrates

| enzyme | substrate[a] | $k_{cat}$, $s^{-1}$ | $K_M$, μM |
|---|---|---|---|
| HIV-1 protease | γAbu-S-Q-N-Y-P-I-V-γAbu-Glu-R (ACCEPTOR / DONOR) | 6 | 120 |
| HIV-1 protease | γAbu-K-A-R-V-Nle-F-E-A-γAbu-Glu-R (ACCEPTOR / DONOR) | 20 | 1 |
| renin | γAbu-H-P-F-H-L-L-I-H-γAbu-Glu-K (ACCEPTOR / DONOR) | 4 | 50 |

[a] An arrow indicates the cleavage point of the substrates by the enzyme.

synthesizer. Similar to the secondary amine of the reduced-bond pseudopeptide Xaaψ[$CH_2NH$]Yaa, the secondary amine of the EDANS group did not require protection during solid-phase synthesis.[6] However, as a precaution, no capping steps with acetic anhydride were used during the synthesis to preclude the possibility of acetylating the 2′-aminoethyl group. The final synthetic step for each substrate was coupling with the DABCYL group using [(benzotriazolyl)oxy]tris(dimethylamino)-phosphonium hexafluorophosphate (BOP).[7] The substrates were cleaved from the resin and the side-chain-protecting groups were removed by reaction with HF. It should be noted that the DABCYL and EDANS groups were stable to the HF treatment. Final products were

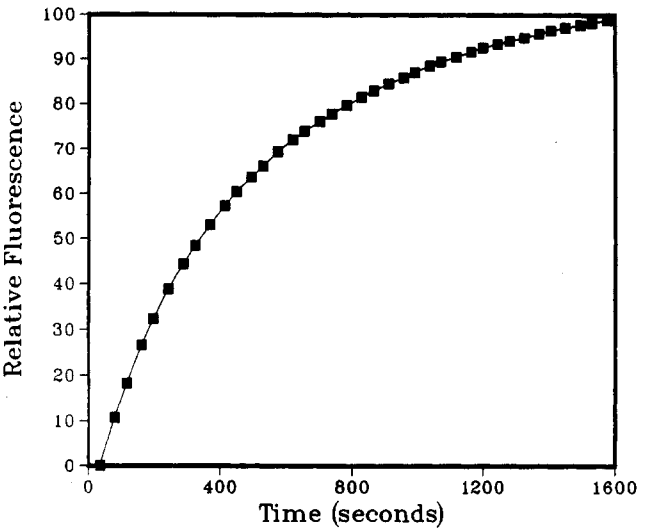


**Figure 2.** Time course of the change in fluorescence during hydrolysis of substrate 4 by recombinant human renin. The reaction conditions were as follows: pH 7, 37 °C, 50 mM 3,3-dimethylglutarate, 50 mM *n*-octyl β-glycopyranoside, and 50 mM NaCl with $[S_0]$ = 1.97 μM and [renin] = 12.7 nM.

obtained by purification by preparative high performance reverse-phase chromatography on C-18 derivatized silica gel.

The kinetic data for the three substrates are listed in Table I. Each substrate was cleaved at only one position by its respective enzyme. That the cleavage site was the expected one was verified by sequencing the C-terminal fragment. A typical time course of the change in fluorescence during enzyme-catalyzed hydrolysis of one of the substrates is given in Figure 2 for the action of recombinant human renin[8] on the renin substrate. Interestingly, the $K_m$ values for the two fluorogenic substrates of the HIV-1 protease are about 1 order of magnitude better than those reported in the literature for analogous unlabeled substrates, i.e., 60 μM for KARVLFEA-Nle-G-$NH_2$[9] and 1500 μM for SQNYPIVQ.[10] The renin fluorogenic substrate has a 2-fold better $K_m$ value compared to its unlabeled substrate, i.e., 95 μM for PHPFHLLIHK. Thus, the addition of the donor/acceptor pair to the substrates has not been deleterious to substrate/enzyme binding.

Until the very recent work of Meldal and Breddam,[11] who introduced the anthranilic acid/3-nitrotyrosine donor acceptor pair into solid-phase synthesis, only the dansyl/tryptophan pair, with poorly efficient long range energy transfer properties,[2] could be utilized in this convenient synthetic methodology. We have demonstrated a method of introducing the extremely efficient EDANS/DABCYL pair into substrates using conventional solid-phase synthesis. With this pair there is no problem with interfering

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fluorescence from any natural amino acid residues in the substrate or enzyme, because the EDANS fluorescence is near 500 nm. The large spectral overlap between DABCYL and EDANS[3] allows for substrates with many residues between the donor/acceptor pair. In the case of the renin substrate, nine amino acid residues and two spacer groups separate the donor and acceptor. The favorable spectroscopic properties of the EDANS/DABCYL groups, when combined with the convenient method of synthesis described here, should provide many useful substrates to study a wide variety of enzymes.

## Experimental Section

An ABI 430A peptide synthesizer was used for solid-phase peptide synthesis. NMR spectra were recorded on a Brucker 300-MHz instrument. Chemical shifts are expressed with tetramethylsilane as internal standard. Fluorescent measurements were made on a P.E. LS-50. Mass spectra, combustion analyses, and optical rotations were obtained by the Physical and Analytical Chemistry Unit of Upjohn Laboratories. Amino acid analyses were carried out on a Beckman 6300; peptide hydrolysis conditions: constant-boiling HCl/5% phenol, 110 °C/24 or 48 h. Analytic HPLC was performed with a Vydac C18 reverse-phase column using a Varian Vista 5500 instrument equipped with a Waters 490 variable-wavelength detector. The analytical HPLC of all final products (4–6) showed a single symmetrical peak when monitored at three wavelengths (220, 340, 470 nm). Preparative HPLC was performed with Vydac C18 purchased from the Separations Group packed in a 3 × 30 cm column. The flow rate was typically 2.5 mL/min with a gradient of eluant formed by an Autochron gradient maker. Flash column chromatography was carried out on Merck silica gel (0.040–0.063 mm particle size).[12] DMF was dried over preactivated 4-Å molecular sieves. Trifluoroacetic acid (TFA) and 4 N HCl/dioxane in 10-mL ampules were purchased from Pierce Chemical Co. $N^{\alpha}$-Boc-L-glutamic acid α-benzyl ester and $N^{\alpha}$-Boc-γ-aminobutyric acid (γAbu) were purchased from Bachem. BOP and Boc-$N^{im}$-[(benzyloxy)methyl]histidine and Boc-norleucine were purchased from Advanced Chem Tech. DABCYL and EDANS were purchased from Sigma. $N^{\alpha}$-Boc-amino acids, $N^{\alpha}$-Boc-ε-(2-chlorobenzyloxycarbonyl)lysine–[[4-(oxymethyl)-phenyl]acetamido]methyl (PAM) resin (0.65 mmol/g), and $N^{\alpha}$-Boc-$N^{\gamma}$-(*p*-tolylsulfonyl)arginine–PAM resin (0.5 mmol/g) were purchased from Applied Biosystems Inc. The following amino acid side-chain-protecting groups were used: *p*-tolylsulfonyl (Tos) for Arg, (benzyloxy)methyl (Bom) for His, 2-chlorobenzyloxycarbonyl (Cl-Z) for Lys, and 2-bromobenzyloxycarbonyl (Br-Z) for Tyr.

**Boc-Glu(EDANS)-OBzl (1).** EDANS (300 mg, 1.13 mmol), Boc-Glu-OBzl (381 mg, 1.13 mmol), and BOP (489 mg, 1.13 mmol) were mixed with 10 mL of dry DMF. *N,N*-Diisopropylethylamine (DIPEA) (392 μL, 2.25 mmol) was added to the suspension, and the mixture was stirred at room temperature under a nitrogen atmosphere for 4 h, yielding a clear solution. HPLC analysis of an aliquot of the reaction showed essentially no Boc-Glu-OBzl starting material remaining. DMF was removed by rotary evaporation under reduced pressure, and the crude residue was purified twice by flash chromatography, once in MeOH (saturated with $NH_3$)/$CH_2Cl_2$ (1:4), followed by MeOH/HOAc/EtOAc (6:1:43) to yield 486 mg (71%) of **1** as a pale golden solid after lyophilization from water: $^1$H NMR ($CD_3OD$) δ 1.4 (s, 9 H), 1.9 (m, 1 H), 2.2 (m, 1 H), 2.3 (t, 2 H), 3.4 (t, 2 H), 3.5 (t, 2 H), 4.2 (m, 1 H), 5.1 (d, 2 H), 6.7 (d, 1 H), 7.3 (m, 8 H), 8.1 (m, 2 H), 8.2 (d, 1 H); FAB/MS *m/z* 586 (M + H$^+$). $[\alpha]^{20}_D$ −14° (c 3.48, MeOH). For elemental analysis, an aliquot of sample was further purified by chromatography on a C18 reverse-phase column using 20 mM ammonium acetate as counterion and a gradient from 100% $H_2O$ to 60/40 $H_2O$/$CH_3CN$. The ammonium counterion on the sulfonate was converted to sodium by passage of the sample down a Dowex 50W-X2 ion exchange column. Anal. ($C_{29}H_{34}N_3O_8S_1Na_1 \cdot 2H_2O$) C, H, N.

**Boc-GABA-Glu(EDANS)-OBzl (2).** Compound **1** (200 mg, 0.3 mmol) was treated with 4 N HCl/dioxane (20 mL) for 1 h. The solvent was removed by rotary evaporation under reduced pressure, and to the residue were added Boc-γAbu (132 mg, 0.64 mmol), BOP (181 mg, 0.64 mmol), DIPEA (282 μL, 1.62 mmol), and 10 mL of DMF. The reaction was stirred for 2 h under a nitrogen atmosphere at room temperature. The DMF was removed by rotary evaporation under reduced pressure, and the residue was purified twice by flash chromatography in the solvent systems used for **1** to yield 156 mg (69%) of **2** after lyophilization from $H_2O$: $^1$H NMR ($CD_3OD$) δ 1.4 (s, 9 H), 1.7 (m, 2 H), 1.9 (m, 1 H), 2.2–2.3 (m, 5 H), 3.0 (t, 2 H), 3.4 (t, 2 H), 3.5 (t, 2 H), 4.5 (m, 1 H), 5.1 (d, 2 H), 6.7 (d, 1 H), 7.3 (m, 8 H), 8.1 (m, 2 H), 8.2 (d, 1 H); FAB/MS *m/z* 671 (M + H$^+$). $[\alpha]^{20}_D$ −7° (c 0.83, MeOH). For elemental analysis, an aliquot of sample was purified by chromatography on a preparative C18 reversed-phase column using 2 mM ammonium acetate buffer and a gradient from 100% $H_2O$ to 60/40 $H_2O$/$CH_3CN$. Anal. ($C_{33}H_{45}N_5O_9S_1 \cdot H_2O$) C, H, N.

**Boc-GABA-Glu(EDANS)-OH (3).** Compound **2** (113 mg, 0.164 mM) was dissolved in 30 mL of MeOH in a Parr bottle. Pd/C (10% 33 mg) was added and the reaction was shaken in a Parr apparatus under an $H_2$ atmosphere at 40 psi for 8 h. The catalyst was removed by filtration, and the solvent was removed by evaporation under reduced pressure to yield 69 mg (71%) of **3** which was used immediately for the next reaction: $^1$H NMR ($CD_3OD$) δ 1.4 (s, 9 H), 1.8 (m, 2 H), 1.9 (m, 1 H), 2.2–2.4 (m, 6 H), 3.1 (m, 2 H), 3.4 (t, 2 H), 3.5 (m, 2 H), 4.4 (m, 1 H), 6.7 (d, 1 H), 7.4 (m, 2 H), 8.1 (m, 3 H).

**General Procedure for the Synthesis of Peptides.** Compound **3** (312 mg, 0.44 mmol) was added to 0.40 mmol of the appropriate $N^{\alpha}$-deprotected and neutralized amino acid–PAM solid-phase resin in a manual synthesis apparatus. BOP (195 mg, 0.44 mmol), 15 mL of DMF, and DIPEA (153 μL, 0.88 mmol) were added, and the mixture was stirred overnight. At this time, if the ninhydrin test[13] was positive, a recoupling was done with one-half the amount of **3**, BOP, and DIPEA for 4 h. The derivatized resin was then washed with $CH_2Cl_2$ and EtOH, dried under vacuum, and transferred to an ABI reaction vessel. The standard amino acids were incorporated using $N^{\alpha}$-Boc-amino acids with the ABI double-coupling protocol. After removal of the N-terminal Boc group and neutralization, the peptide–resin was returned to a manual synthesizer and Boc-γAbu (202 mg, 1 mmol), BOP (442 mg, 1 mmol), DIPEA (522 μL, 2 mmol), and 20 mL of DMF were added and the mixture was stirred overnight. After thorough washing of the peptide–resin the Boc group was removed by reaction with 40% TFA/$CH_2Cl_2$ for 30 min. After rinsing, the peptide–resin was neutralized by three washings with 10% DIPEA/$CH_2Cl_2$ for 5 min each. The DABCYL group was added by reacting with DABCYL (291 mg, 1 mmol), BOP (442 mg, 1 mmol), and DIPEA (523 μL, 2 mmol) in DMF overnight. If the ninhydrin test was positive, the coupling was repeated on one-half the scale as above for 4 h. The dried peptide–resin was then treated at −5 °C for 1 h with 10 mL of HF with 10% anisole added as a cation scavenger. After removal of the solvent the peptide–resin mixture was washed with $CH_2Cl_2$, and the peptide was dissolved away from the resin with 50% acetic acid/water. The crude peptide was lyophilized from glacial acetic acid. Each peptide was purified by preparative reversed-phase chromatography on a Vydac C18 column with a gradient of water (0.1% TFA) to 50% water/acetonitrile (0.1% TFA).

**DABCYL-γAbu-His-Pro-Phe-His-Leu-Leu-Ile-His-γAbu-Glu(EDANS)-Lys (4).** The peptide was synthesized and purified by the standard protocol, except resorcinol (100 mg) was added to the HF reaction mixture as an additional scavenger for the Bom protecting group on histidine.[14] The purified peptide was lyophilized from glacial acetic acid to give a red powder:

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amino acid analysis, Glu (1.04), Ile (1.12), Leu (2.09), Phe (0.94), His (2.94), Lys (0.86), Pro (1.40); FAB/MS $m/z$ 1940 ($M + H^+$).

**DABCYL-γAbu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-γAbu-Glu-(EDANS)-Arg (5).** The peptide was synthesized by the standard protocol. The purified peptide was lyophilized from glacial acetic acid to give a red powder: amino acid analysis, Asp (0.93), Ser (0.93), Glu (2.17), Val (0.92), Ile (0.92), Tyr (0.99), Arg (1.12), Pro (0.88); FAB/MS $m/z$ 1775 ($M + H^+$).

**DABCYL-γAbu-Lys-Ala-Arg-Val-Nle-Phe-Glu-Ala-γAbu-Glu(EDANS)-Arg (6).** The peptide was synthesized and purified by the standard procedure. The purified peptide was lyophilized from glacial acetic acid to give a red powder: amino acid analysis, Glu (2.14), Ala (1.81), Val (0.96), Nle (1.00), Phe (1.01), Lys (0.99), Arg (2.09); FAB/MS $m/z$ 1887 ($M + H^+$).

**Kinetic Measurements of Substrates.** The renin substrate, 4, was investigated at pH 7, 37 °C, in 50 mM 3,3-dimethylglutarate, 50 mM *n*-octyl β-glycopyranoside, and 50 mM NaCl with initial substrate concentration ($[S_0]$) = 1.97 μM and renin concentration = 12.7 nM. The HIV protease substrates were investigated at pH 5.0, 37 °C, in 0.1 M NaCl and 1 mM EDTA, with $[S_0]$ = 49.6 or 5 μM and recombinant HIV-1 protease[15] concentration equal to 0.15 μM for substrate **5** and $[S_0]$ = 10 μM and [HIV-1] = 23 nM for substrate **6**. The substrate concentrations were determined by amino acid analysis of stock solutions. The entire time course of the reactions was recorded by monitoring the fluorescence at 490 nm with an excitation wavelength at 340 nm.[3] The kinetic parameters were calculated by fitting the fluorescence vs time data points using a modified nonlinear least-square-fit program[16] to an integrated form of the Michaelis–Menton equation. The $K_m$ value for the unlabeled renin substrate, PHPFHLLIHK, was determined according to the procedure described by Heinrikson and Poorman.[17] Briefly, the hydrolysis of the substrate was monitored at 231 nm, where the absorbance decreases as the Leu–Leu bond is cleaved by the enzyme. The concentration of substrate was 218 μM, and the concentration of enzyme was 0.19 nM with the same buffer as for the assay of the fluorogenic substrate. Kinetic parameters were determined as above.

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