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DESIGN AND SYNTHESIS OF NEW FLUOROGENIC HIV PROTEASE SUBSTRATES BASED ON RESONANCE ENERGY TRANSFER

Gary T. Wang, Edmund Matayoshi, H. Jan Huffaker, Grant A. Krafft*
Abbott Laboratories
Abbott Park, Illinois, 60064-3500

Summary: The design and synthesis of new fluorogenic substrate probes for HIV protease based on resonance energy transfer are described. These substrates permit sensitive, continuous measurement of HIV protease activity.

Specially tailored organic probe molecules are playing an increasingly significant role in efforts to elucidate important aspects of biological macromolecules. When coupled with spectroscopic techniques, probe molecules provide functional and mechanistic information on key enzymes, receptors, and structural proteins. Our recent interests have centered on the design of specific substrate probes for the human immunodeficiency virus (HIV) associated protease enzyme, now an important target for AIDS therapy.[1] Recently, we described a rapid and continuous assay for retroviral protease activity (including HIV) based on new fluorogenic substrate probes,[2,3] and in this report, we describe molecular design considerations, synthesis and purification aspects of these new substrates.

Early methods that were used to measure HIV protease (HIV PR) activity relied on tedious, time consuming methods such as western immunoblot analysis of the *gag* polyprotein and its cleavage products,[4] or HPLC[5] or thin-layer chromatographic[6] analysis of synthetic peptide cleavage fragments. These methods were impractical for screening or characterizing large numbers of inhibitors, and did not provide particularly accurate quantitation of enzyme activity for kinetic and mechanistic studies. We sought to develop substrate probe molecules that would allow rapid, efficient and quantitative measurement of HIV PR activity and evaluation of novel inhibitor molecules.

The major challenge associated with HIV PR substrate design was devising a strategy to obtain continuous signal generation resulting directly from the proteolytic process. Most protease assays rely on a cleavage-induced spectroscopic change in a departing chromogen or fluorogen located in the P1' position of a peptidic substrate, but HIV PR substrate recognition requires three or four amino acid residues on either side of the scissile bond,

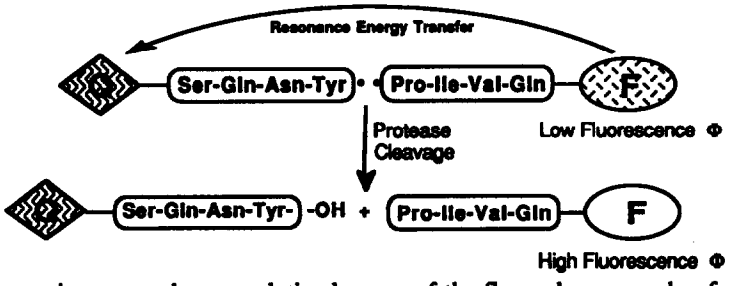
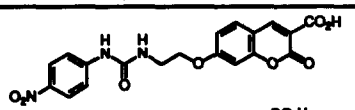
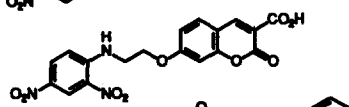
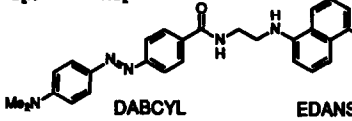


Fig. 1. Signal generation occurs by proteolytic cleavage of the fluorophore-quencher functionalized peptide. Resonant energy transfer (RET) from the fluorophore (F) excited state to the chromophore Q results in quenching.

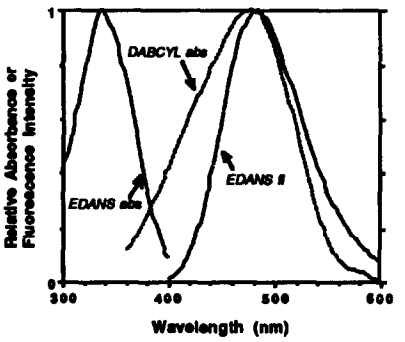
precluding this simple approach. Our substrate design was based on the concept of resonance energy transfer between a fluorophore and an acceptor chromophore linked by an HIV PR specific substrate peptide.[7] Energy transfer between the proximal chromophores in the intact peptide mediates a major reduction in the fluorophore quantum yield, while proteolytic cleavage of the connecting peptide liberates a tetrapeptide-fluorophore fragment with a fluorescence quantum yield restored to the level of the free fluorophore. The magnitude of the signal, and therefore the sensitivity of the assay, is dependent on the distance between the fluorophore and the acceptor chromophore, the spectral overlap of the two groups, and the transition dipole orientations. Since a peptide length of at least seven amino acids is required for substrate recognition by HIV protease,[5] limiting the proximity of the two chromophores, proper selection of a donor/acceptor pair with ideal spectral overlap was essential to maximize quenching efficiency. Good aqueous solubility of the chromophores also was desirable, due to the lipophilicity of the substrate peptides.

In order to determine a donor/acceptor combination with highly efficient quenching, we prepared conjugates of several chromophores and fluorophores and examined their fluorescence quenching efficiencies.[8] Some of these results are summarized in Table 1. The most effective fluorescence quenching was observed for the conjugate consisting of 4-(4'-dimethyl-aminobenzeneazo)benzoic acid (DABCYL) as the acceptor chromophore and 5-(2'aminoethyl)aminonaphthlene sulfonic acid (EDANS) as the donor fluorophore. The major characteristic responsible for the efficient quenching is the excellent overlap of the fluorophore excited state (λ_{max} at 490 nm) and the DABCYL absorption (pH dependent λ_{max} from 470-520 nm), as illustrated below. The relatively long EDANS lifetime (13 ns) and the large extinction coefficient of DABCYL contribute further to the quenching efficiency.

Table 1

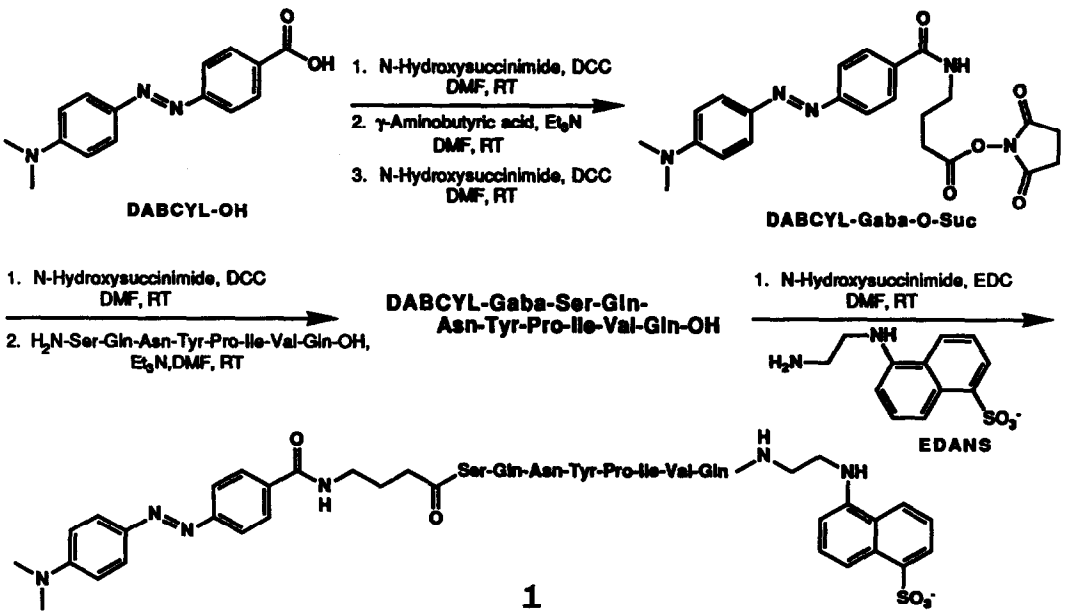
Quencher - Fluorophore Conjugate	Reduction of Fluorescence[a]
	14 fold
	25 fold
	>>200 fold

DABCYL EDANS

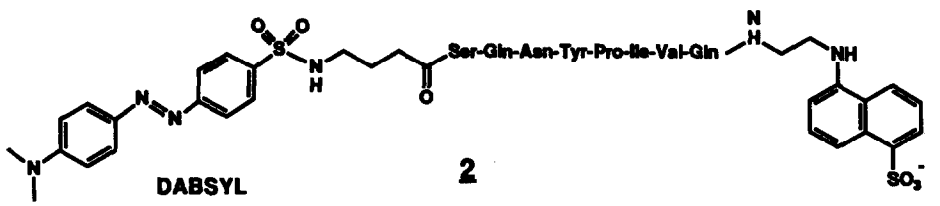


Scheme I outlines the synthetic route for one fluorogenic substrate with the sequence Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln. This octapeptide sequence represents one of several natural cleavage sites for HIV protease, and had been shown to be cleaved by recombinant HIV protease using an HPLC-based assay.[5] The DABCYL moiety was attached to the peptide N-terminus through a γ-aminobutyric acid spacer, incorporated to minimize potential steric problems involving the DABCYL group and the protease active site. This conjugation was accomplished via standard N-hydroxysuccinimide active ester chemistry.[9] Coupling of the EDANS amino group to the peptide C-terminus was achieved using 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (EDC) in the presence of N-hydroxysuccinimide. This second coupling step was not trivial, requiring careful attention to reaction time and temperature, since prolonged exposure to the reaction conditions resulted in significant product decomposition.[10] Careful HPLC purification of the ultimate product on a C_8 reverse phase column after this final coupling step was essential, since trace quantities of free EDANS fluorophore dramatically increased the background fluorescence.

SCHEME I



Initially, we also had synthesized the analogous sulfonamide substrate 2 with a DABSYL quencher group. However, final reverse phase HPLC purification (0.1% TFA/MeCN, pH=2) resulted in the formation of a contaminating fluorescent impurity. Hydrolysis of the DABSYL sulfonamide bond was suspected initially, but HPLC-MS analysis identified the fluorescent fragment as Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS, indicating that cleavage occurred at the amide bond between the Gaba and the serine.[11] Similar decomposition by hydrolysis also has been observed during HPLC of 1 at pH 2, but not at pH 4.9. The extent of decomposition was pH dependent in the range of pH=2-4, and was not detectable at pH>4. The HPLC-related decomposition occurred on C-8 silica gel columns and on polymeric resin-based reverse phase columns. No additional decomposition was observed when 1 or 2 were incubated in pH 2 buffered solution for 24 h. The extent of hydrolysis during HPLC was extremely low, but significant for this application due to the high fluorescence quantum yield of the impurity, relative to the intact difunctionalized peptide.



As expected, compound 1 is cleaved specifically at the Tyr-Pro amide bond by HIV protease (K_m=103 μM, k_{cat}=4.9 sec^{-1}),[2] Addition of purified recombinant HIV-1 protease to the substrate results in a steady increase in fluorescence intensity, permitting continuous monitoring of the enzyme activity. Exhaustive proteolysis of 1 produces a 40-fold fluorescence enhancement above the background fluorescence level, imparting to this assay the highest sensitivity of any method used to measure HIV protease activity.

The concept demonstrated here is extremely flexible since this donor/acceptor pair can be employed with essentially any peptide sequence. Similar probes have been prepared to measure protease activity associated with avian myeloblastosis virus,[2] and to measure human renin activity.[12] The HIV protease substrate has facilitated a systematic study of substrate specificity requirements for HIV protease, and systematic evaluation of potent inhibitors of this key viral enzyme. The results of these studies will be reported elsewhere.

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8. Fluorescence intensity measurements made in aqueous DMF at pH=4.7.
9. Coupling of the DABCYL chromophore to the peptide N-terminus proceeds as follows: To a solution of the octapeptide H_2N-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-OH (50 mg, 0.053 mmol) in 50 mL of anhydrous dimethylformamide (DMF) was added 30 mg (0.085 mmol) of the active ester, DABCYL-Gaba-O-Suc (Scheme I), and 200μL (excess) triethylamine. After stirring the mixture overnight at 25°C, the solvent and triethylamine were evaporated. The remaining red residue was washed several times with methylene chloride until the washings were colorless, in order to remove unreacted active ester and succinimde from the insoluble functionalized peptide. Filtration of the DABCYL-gaba-SQNYPIVQ from the last dichloromethane suspension provided 68 mg (100%) of dark red solid material that was sufficiently pure by HPLC analysis to be carried on to the next step. Mass spectrum (FAB+): $(M+1)^+$=1284, $(M+Na)^+$=1305; ^1H NMR (DMSO-d-6) resonances diagnostic for the DABCYL group: δ 3.10 (6H, s, $N(CH_3)_2$), 6.75 (2H, d, aryl), 7.80 (6H, d, aryl).
10. Coupling of DABCYL-Gaba-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-OH to EDANS proceeds as follows: DABCYL-Gaba-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-OH (50 mg, 0.039 mmol) was dissolved in 40 mL DMF. To this solution was added 5-(2'aminoethylamino)naphthalenesulfonic acid sodium salt (Sigma, 25 mg, 0.08 mmol), N-hydroxysuccinimide (30 mg, excess) and 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, 35 mg, 0.18 mmol). The reaction was stirred at 25°C and monitored by HPLC (C8 reverse phase). Complete conversion of starting material to a single new component was observed after 3 h. The mixture was filtered and purified directly by HPLC on a Rainin Dynamax C8 reverse phase column (25 x 2.1 cm, flow rate was 12 mL/min.) using a gradient consisting of 10 mM aqueous NaOAc buffer (pH 4.9) and acetonitrile (0% acetonitrile - 10min, 0-20% acetonitrile - 10 min, 20-50% acetonitrile - 35 min). The desired *product* eluted from the column with a retention time of 41 minutes. Evaporation of solvent gave a residue that was triturated with DMF to remove NaOAc. The DMF solution was concentrated to give 49 mg (81.0 % yield) of a red solid as the product. Mass spectrum (FAB+): $(M+1)^+$=1554, $(M+Na)^+$=1575. ^1H NMR (DMSO-d-6) resonances diagnostic for DABCYL: δ 3.10 (3H, s, $N(CH_3)_2$), 6.70 (2H,d, aryl), 7.75 (6H, d, aryl); and for EDANS: δ 3.0 (2H, t, CH_2), 3.35 (2H, t, CH2), 6.60 (1H, d, aryl), 7.4 (2H m, aryl), 8.10 (3H, m, aryl).
11. Aqueous acid has been shown to cleave amide bonds at serine amino groups more readily than at other amide bonds, perhaps via assistance by transacylation of the serine hydroxyl, and subsequent ester hydrolysis. See Nishimura, S., Jones, D. S., Khorana, H. G. *J. Mol. Biol.* **1965**, 13, 302-324.
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