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A simple, continuous fluorometric assay for HIV protease

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Novel fluorogenic substrates for human immunodeficiency viral protease have been developed based on the principle of fluorescence energy transfer. Starting from a p24/p15 cleavage site-derived hexapeptide substrate, Ac-Thr-Ile-Nle-Nle-Gln-Arg-NH_2, incorporation of 2-aminobenzoic acid in place of the acetyl group as the donor and *p*-NO_2-Phe at the P1′ position as acceptor gave the intramolecularly quenched fluorogenic substrate. Cleavage of the substrate by HIV protease released the fluorescent *N*-terminal tripeptide from its close apposition to the quenching nitrobenzyl group, resulting in enhanced fluorescence. An automated assay based on 96-well microtiter plates and a fluorometric plate reader have been developed, which allow high throughput of compounds in the search for HIV protease inhibitors.

Key words: fluorogenic substrate; fluorometric assay; HIV protease inhibitors

Human immunodeficiency viral (HIV) protease is a logical therapeutic target in the search for AIDS drugs. In order to allow the exploration of potential inhibitors of HIV protease and to optimize lead compounds, a convenient assay to kinetically characterize the inhibitor which allowed for automation and high throughput was necessary. Initial assays (1–3) used in the characterization of HIV protease were based on HPLC separation of products and substrate at fixed time intervals. This HPLC assay quickly became the rate-limiting step in the development of HIV protease inhibitors with desirable therapeutic properties. A continuous assay which allowed for quantitative kinetic characterization of the interaction of the inhibitors with HIV protease was needed. The strategy of developing either a chromogenic or a fluorogenic substrate was chosen based on work characterizing short peptide substrates of known HIV protease cleavage sites (4).

Our initial attempts focused on developing a chromogenic substrate. Because of the known accommodation of Phe and Tyr at position P1 in HIV protease substrates, we prepared a series of potential substrate analogs containing *p*-NO_2-Phe at either P1 or P1′. Of the compounds we examined which were octapeptides or smaller, only the P1′ analogs were substrates and the changes in absorbance upon hydrolysis were insufficient for a sensitive, continuous assay. Recently, Nashed *et al.* (5) and Tomaszek *et al.* (6) have reported a spectrophotometric assay based on cleavage of chromogenic substrates containing *p*-NO_2-Phe at position P1. In these cases, the substrates were an octapeptide, a nonapeptide, and a decapeptide. Richards *et al.* (7) have recently reported a nonapeptide substrate with *p*-NO_2-Phe at the P1′ site. A high-throughput, radiometric assay for screening for HIV protease inhibitors, which is not continuous, has been reported by Hyland *et al.* (8). Tamburini *et al.* (9) have developed a sensitive HPLC assay based on a dansylated heptapeptide substrate and fluorescence detection.

Fluorogenic substrates for hydrolytic enzymes have been widely used in biochemistry because of their high sensitivity. In many cases, the fluorophore, e.g. *C*-terminal peptide anilides, is linked directly to the bond undergoing cleavage, resulting in a highly fluorescent group upon hydrolysis. As the minimum length for a substrate of HIV protease from our studies was a hexapeptide, which is cleaved into two tripeptides, such a strategy was not applicable. The idea of using intramolecularly quenched fluorogenic substrates containing a donor and an acceptor chromophore in

Abbreviations: HIV, human immunodeficiency virus; DMF, dimethylformamide; CH_2Cl_2, dichloromethane; HPLC, high performance liquid chromatography; Abz, 2-aminobenzoic acid, or anthranilic acid; DMSO, dimethylsulfoxide; TFA, trifluoroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

the same molecule which would become separated upon hydrolysis has been widely used (10, 11). Recent examples of applications include a bacterial collagenase (12), vertebrate collagenase and gelatinase (13), enkephalinase (14), and atrial dipeptidyl carboxyhydrolase (15). As we had demonstrated that a substrate for HIV protease could contain *p*-NO_2-Phe, a known acceptor, at position P1′, incorporation of a fluorescent donor moiety in the *N*-terminal segment was attempted. The peptide, Ac-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, was modified by replacement of the *N*-terminal acetyl group with various fluorescent groups based on anthranilic acid, 2-aminobenzoic acid (Abz). A suitable fluorogenic substrate resulted, and an automated assay utilizing microtiter plates and a fluorescence plate reader has been developed. During the course of preparing this paper, we became aware of similar efforts to develop such an assay by Weidner *et al.* (16), Matayoshi *et al.* (17), and Geoghegan *et al.* (18) utilizing different fluorogenic substrates.

MATERIALS AND METHODS

Solid phase synthesis of HIV protease substrates

Acetyl hexapeptide amides were prepared by solid phase peptide synthesis using the *p*-methylbenzhydrylamine resin (Sigma, St. Louis, MO). For each synthesis, 0.5 g of polymer (1 mmol free amine/g of resin) was used. The following BOC-amino acids were used (Bachem, Torrance, CA): Boc-Arg(Tos), Boc-Gln, Boc-Thr(Bzl), Boc-Asp(OBzl), Boc-Lys(Cl-Z), Ser(Bzl), Boc-Leu, Boc-Ile, Boc-Val, Boc-Nle, and Boc-Phe(*p*-NO_2). The Phe(*m*-NO_2) used was kindly provided by Drs. John Talley and Dan Getman of Monsanto (St. Louis) and protected by reaction with di-*t*-butylpyrocarbonate. The following synthetic protocol was used for incorporation of the Boc-amino acids:

Deprotection. 50% trifluoroacetic acid/CH_2Cl_2 5 min and 25 min

CH_2Cl_2	2 × 1 min
Isopropanol	2 × 1 min
CH_2Cl_2	2 × 1 min

Neutralization. 10% diisopropylethylamine/CH_2Cl_2 3 min and 5 min

CH_2Cl_2	2 × 1 min
DMF	2 × 1 min

Coupling. 4 equiv. of Boc-amino acid and 4 equiv. of diisopropylcarbodiimide in the presence of 4 equiv. of hydroxybenzotriazole in DMF for 2 h. Coupling in DMF was repeated if the Kaiser test (19) was positive. Acetylation was performed with acetic anhydride in DMF in the presence of an equivalent amount of DIPEA for 30 min. Completed peptides were cleaved with HF/anisole, 9:1. Crude peptides were dissolved in 20–50% acetic acid and lyophilized. They were purified by reversed-phase HPLC on a C_{18} semipreparative column using a 0.05% TFA/H_2O and 0.05% TFA/acetonitrile gradient. Their identity was confirmed by high-resolution mass spectrometry, NMR, and amino acid analyses.

Synthesis of 2-aminobenzoyl-Thr-Ile-Nle-Phe (p-NO_2)-Gln-Arg-NH_2, Abz-NF-6*

The protocol was essentially that used for the other substrates with the following modifications. Abz was coupled using an equivalent amount of BOP reagent (20) and 3 equiv. of DIPEA in DMF for 2 h. The cleaved peptide was dissolved in glacial acetic acid, diluted with water, and lyophilized. Small portions of the crude peptide were dissolved in glacial acetic acid, and purified by a Vydac C_{18} semipreparative HPLC column (1 × 25 cm) using a 25–45% acetonitrile gradient (0.05% TFA) for 40 min. The main peak at 35% acetonitrile was collected and lyophilized. The structure of the peptide was confirmed by FABMS (MH_+ = 940), by amino acid analysis (Beckman System Gold), and by UV and fluorescence spectroscopy.

HPLC assay

The HPLC HIV protease assay was conducted using either synthetic HIV protease (1) in which the two Cys residues (Cys^{67}, Cys^{95}) had been replaced by the isosteric α-aminobutyric acid to eliminate the complications of free sulfhydryl groups (21), or cloned material expressed in *E. coli* supplied by Dr. George Glover of Monsanto (St. Louis). In all cases examined, the cleavage patterns and inhibition results were identical. Synthetic HPLC-purified protease was dissolved in a buffer of 6M guanidine·HCl, 100 mM Tris, pH 7.5, 40% glycerol at a concentration of 50 μg/mL. It was dialyzed versus a buffer of 2M guanidine·HCl, 100 mM Tris, pH 7.5, 40% glycerol (100 × volume, 2 × 1 h, room temp.), followed by dialysis against 20 mM phosphate buffer, pH 7.5, 40% glycerol, 0.1% CHAPS (100 × volume, 2 × 1 h, room temp.), and finally a buffer (1 mM phosphate buffer, pH 7.5, 40% glycerol, 0.1% CHAPS) according to J. Schneider and S.B.H. Kent (personal communication). The precipitated solid material was removed by centrifugation, and the refolded HIV protease was stored at −70°. Twenty microliters of 0.1 mM substrate (dissolved in DMSO and diluted with assay buffer) was mixed with 20 μL assay buffer (20 mM phosphate buffer, pH 6.4, 20% glycerol, 0.1% CHAPS). Ten microliters of HIV protease stock was added, and the mixture was incubated at 25° for the desired time. The reaction was stopped by the addition of 60 μL of 10% TFA and the sample was applied to an HPLC column (Vydac C18,

0.46 × 25 cm), developed with 0.05% TFA for 5 min, followed by a gradient of 0–40% acetonitrile in 40 min. For inhibitor studies, 10 μL of the protease solution was preincubated at 25° for 10 min with 20 μL of 0.1 mM inhibitor (dissolved in DMSO and diluted to 0.1 mM with assay buffer). Then 20 μL of test substrate, Ac-Thr-Ile-Met-Met-Gln-Arg-NH_2, or Abz-Thr-Ile-Nle-Phe-(*p*-NO_2)-Gln-Arg-NH_2 (Abz-NF*-6), was added in order to determine inhibition of cleavage. Reactions were stopped and cleavage rates were monitored by HPLC as above. In order to confirm the cleavage pattern of Abz-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, cleavage was allowed to finish as judged by HPLC and the two product peaks were isolated by HPLC. Incubation with either synthetic HIV protease or enzyme expressed and purified from *E. coli* (kindly supplied by Dr. George Glover of Monsanto) gave the same HPLC pattern (Fig. 1). Retention time of the substrate was 42.1 min, while Abz-Thr-Ile-Nle-OH was 37.4 min and H-Phe(*p*-NO_2)-Gln-Arg-NH_2 was 17.7 min. Identity of the product peptides was confirmed by FABMS and amino acid analysis.

Fluorescence spectra

The excitation and emission spectra were measured on an SLM 8000 C spectrofluorometer. Both the substrate, Abz-NH*-6, and the *N*-terminal product, Abz-Thr-Ile-Nle-OH, show absorption maxima at 337 nm and broad emission maxima between 390 and 440 nm. The comparison between substrate and product at the same concentration shows dramatically the tenfold increase in excitation and sixfold increase in emission upon enzymatic hydrolysis. Initial kinetics were monitored at 25° with magnetic stirring at optimal conditions (excitation = 337 nm and emission = 410 nm).

UV spectra

The absorption spectra of the substrate, Abz-NH*-6, and the *N*-terminal product, Abz-Thr-Ile-Nle-OH, were recorded on a Beckman DU-8 spectrophotometer. The substrate shows maxima at 284 nm and 258 nm, while the cleavage product has maxima at 318 nm and 252 nm.

Fluorescence assay

Fluorescence measurements on 96-well ELISA plates were made with the Titertek Fluoroskan II, version 3.1. An excitation filter of 355 nm (bandwidth 35 ± 4 nm) and an emission filter of 430 nm (bandwidth 25 ± 3 nm) were used. Ten microliters of a stock solution (0.05 mg/mL) of HIV protease were incubated with five different concentrations of Abz-NF*-6 in a final volume of 100 μL of assay buffer at 37° with the increase in fluorescence monitored in each well every 2 min for 20 min. A stock solution of 1 mM Abz-NF*-6 in DMSO was diluted to 0.1 mM with assay buffer and used for the assay. The highest concentration of DMSO tested was 5%, which shows no effects of cleavage. A standard curve (Fig. 2) relating

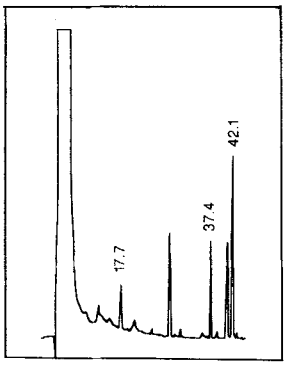

FIGURE 1

HPLC profile of elution pattern of Abz-Thr-Ile-Nle-Phe-(*p*-NO_2)-Gln-Arg-NH_2 (Abz-NF*-6, elution time = 42.1 min) and products, Abz-Thr-Ile-Nle-OH (elution time = 37.4) and H-Phe(*p*-NO_2)-Gln-Arg-NH_2 (elution time = 17.7 min). Forty microliters of 50 μM Abz-NF*-6 in assay buffer were incubated with 10 μL of HIV protease solution for 60 min at room temperature. Cleavage was stopped by addition of 60 μL of 10% TFA and the mixture was analyzed by reversed phase HPLC (Vydac C_{18}, 0.46 × 25 cm column). Mobile phase 0.05% TFA/H_2O (5 min), 0–40% acetonitrile (0.05% TFA) at 1 mL/min, detection at 220 nm. Other peaks are due to buffer components. UV absorption (aufs: 0.2) plotted versus time.

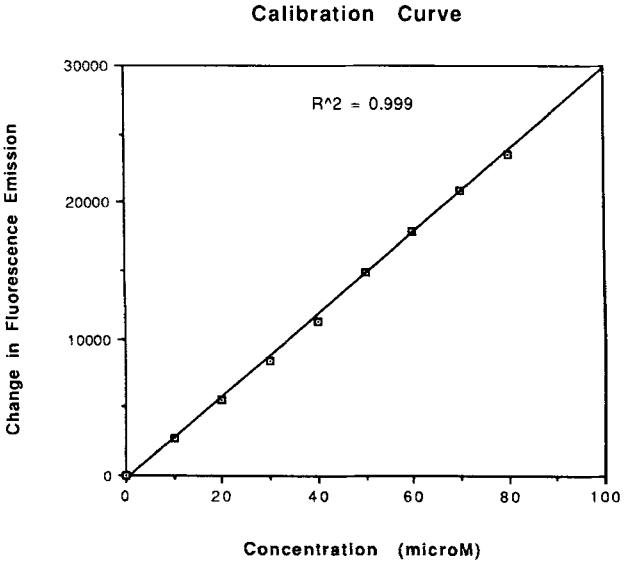

FIGURE 2

Standard calibration curve of fluorescence increase versus concentration. The fluorogenic substrate (Abz-NleF*-6) at eight different concentrations was incubated overnight with 0.5 μg of HIV protease in assay buffer (final volume = 100 μL) on a microtiter plate. The changes in fluorescence emission were measured and are plotted versus the concentration of substrate.

changes in fluorescent intensity to changes in concentration of product was used to convert fluorescence changes into molar velocities. In order to predetermine the concentration range for K_i determination of inhibitors, we do a preliminary screening assay by HPLC. Twenty microliters of 0.1 mM inhibitor in assay buffer (pH 6.4) and 10 μL of HIV protease (stock solution of 0.05 mg/mL) were preincubated for 5–10 min at 25°. Twenty microliters of 0.1 mM Abz-NF*-6 were added and the reaction continued for 1 h. Sixty microliters of 10% TFA were added to stop the reaction and the amount of cleavage determined by HPLC. In the absence of inhibition, almost complete cleavage occurs. By the amount of substrate remaining, an estimated K_i allows appropriate choice of concentration ranges for the inhibitor during the fluorescence assay.

RESULTS AND DISCUSSION

Six peptides were prepared as potential chromogenic substrates with the following sequences:

H-Ser-Phe-Asn-Phe(*p*-NO_2)-Pro-Gln-Val-Thr-OH
H-Arg-Lys-Ile-Leu-Phe(*p*-NO_2)-Leu-Asp-Gly-OH
H-Thr-Leu-Asn-Phe(*p*-NO_2)-Pro-Ile-Ser-Pro-OH
Ac-Leu-Asn-Phe(*m*-NO_2)-Pro-Ile-Ser-NH_2
Ac-Thr-Ile-Phe(*p*-NO_2)-Nle-Gln-Arg-NH_2
Ac-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2

Of these six peptides, only the last with the Phe(*p*-NO_2) residue in the P1′ position showed cleavage under our conditions of HPLC assay and with incubation times of 1 h. Tomaszek *et al.* (6) have reported a chromogenic octapeptide substrate, Ac-Arg-Lys-Ile-Phe(*p*-NO_2)-Leu-Asp-Gly-NH_2, which is similar to those we tried, but with blocked amino and carboxyl terminals. Nashed *et al.* (5) have reported two chromogenic peptide substrates, Ac-Lys-Ala-Ser-Gln-Phe(*p*-NO_2)-Pro-Val-Val-NH_2 and H-Thr-Phe-Gln-Ala-Phe(*p*-NO_2)-Pro-Leu-Arg-Ala-OH, which can form the basis of a spectrophotometric assay. In these three cases, the hydrolysis leaves the chromogenic residue at the *C*-terminus, resulting in greater spectral changes. Because of the small spectral changes seen with the chromogenic residue at the *N*-terminus, we decided to try the fluorogenic substrate strategy. By preparing a longer substrate, Lys-Ala-Arg-Val-Leu-Phe(*p*-NO_2)-Glu-Ala-Met, with the chromogenic residue at the P1′ position, Richards *et al.* (7) have developed a spectrophotometric assay.

Modification of the hexapeptide substrate, Ac-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, with the acceptor residue in the *C*-terminal product only required the addition of a donor fluorescent group to the *N*-terminal product. Replacing the acetyl group with 2-aminobenzoic acid (Abz) resulting in the fluorogenic peptide, Abz-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, gave the same combination of donor and acceptor used by Carmel & Yaron (10) in their substrate for angiotensin converting enzyme. Since those authors had shown that quenching efficiency depends on the proximity of the donor and acceptor chromophores, we synthesized an analog with the Abz group at position P3, Abz-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, but the cleavage efficiency was dramatically reduced. The hexapeptide substrate above referred to as Abz-NF*-6

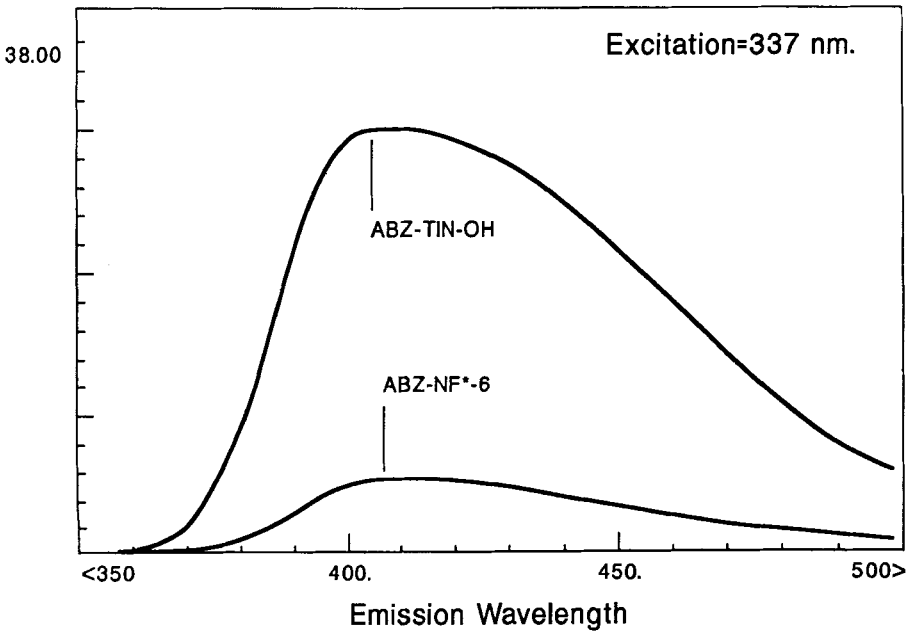

FIGURE 3
Fluorescence emission spectra of 0.1 mM substrate (Abz-NF*-6) and 0.1 mM *N*-terminal cleavage product (Abz-Thr-Ile-Nle-OH) in assay buffer, showing quenched fluorescence in substrate due to proximity of donor (Abz) and acceptor (Phe-(*p*-NO_2)) chromophores. Fluorescence magnitude plotted versus emission wavelength at 337 nm excitation wavelength.

shows a small amount of fluorescent background due to incomplete quenching of the fluorophore, but an approximate sixfold increase in fluorescence upon hydrolysis with a broad maximum between 390 and 440 nm as seen in Fig. 3. A variety of other fluorescent groups can be incorporated in tha P4 position consistent with its surface location as seen in the X-ray crystal structure (22) of the complex of synthetic HIV protease with MVT-101, an inhibitor (4) of similar structure, Ac-Thr-Ile-Nleψ[CH_2NH]-Nle-Gln-Arg-NH_2.

Kinetic measurements of the cleavage of Abz-NF*-6 by synthetic HIV protease showed typical Michaelis-Menten behavior with linear kinetics over the 20 min of reaction when less than 10% of the substrate is cleaved by the enzyme (Fig. 4). A Lineweaver-Burk plot (1/v versus 1/[S]) of the initial velocities, calculated from the linear phase of the reactions, gave a $K_m = 37 \pm 8\,\mu M$ (Fig. 5). V_{max} was calculated to be $690 \pm 70\,nmol \cdot min^{-1} \cdot mg\,protease^{-1}$. These results are similar to those reported for longer peptide substrates using synthetic HIV protease (2). The $K_{cat} = 0.29 \pm 0.03\,s^{-1}$ was calculated from V_{max}, assuming 100% activity of the starting HIV protease. This number is underestimated, however, since we observe precipitation during refolding of HIV protease, and we do not know the specific activity of an authentic standard. The chromogenic substrates of Nashed *et al.* (5) had K_m's estimated to be greater than 450 mM. The ratio K_{cat}/K_m for Abz-NF*-6 with synthetic HIV protease is $7.8 \pm 0.3\,mM^{-1}s^{-1}$. This value is somewhat lower than for longer substrates with recombinant HIV protease expressed in *E. coli* (23), while the ratio for the nonapeptide chromogenic substrate of Nashed *et al.* (5) was estimated to be $23\,mM^{-1}s^{-1}$.

Since this work was completed, Matayoshi *et al.* (17), and Geoghegan *et al.* (18) have published similar assays for HIV protease. Matayoshi *et al.* (16) prepared 4-(4-dimethylaminophenylazo)benzoic acid-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid with a $K_m = 103 \pm 8\,\mu M$, and reported a 40-fold increase in fluorescence yield on cleavage. This is somewhat surprising as quenching is related to distance, which

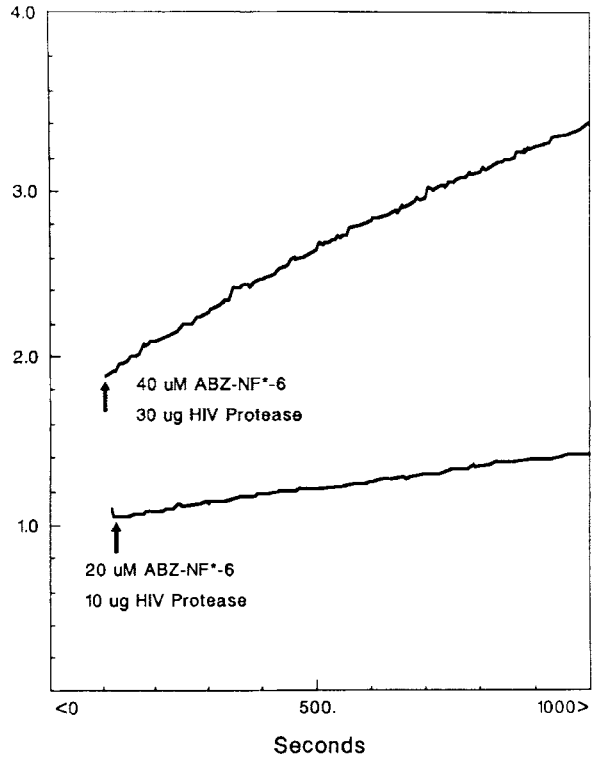

FIGURE 4

Fluorescence emission increase as function of time showing linear portion of assay under conditions used. Initial kinetics were monitored on the SLM 8000 C fluorometer at 25° with magnetic stirring, at optimal excitation (337 nm) and emission (410 nm) wavelength. Arrows indicate the addition of HIV protease (10 μg and 30 μg from HIV protease stock solution, respectively) to two different concentrations of Abz-NF*-6 fluorogenic substrate (20 μM lower and 40 μM upper line) in final volume of 2 mL assay buffer. After the addition of the HIV protease, the substrate is immediately cleaved, and the fluorescence emission linearly increases for up to 20 min. Fluorescence emission plotted versus time.

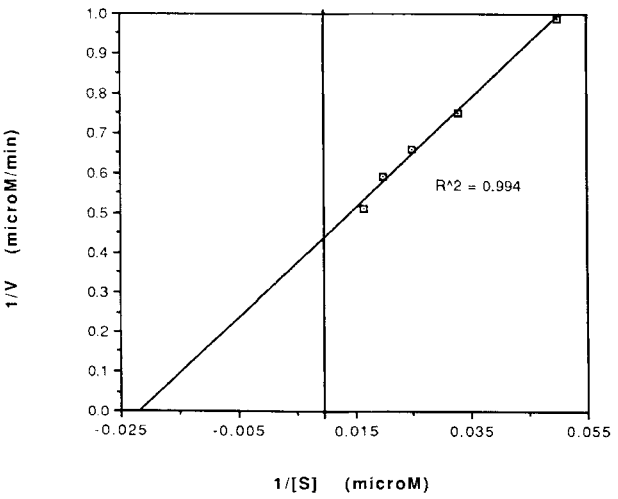

FIGURE 5

Lineweaver-Burk plot of substrate kinetics. The fluorogenic substrate (Abz-NleF*-6) at five different concentrations was incubated with 0.5 μg of HIV protease in assay buffer (final volume = 100 μL) on a microtiter plate. Fluorescence increases were monitored every 2 min for 20 min (there is no increase in fluorescence in the absence of enzyme, data not shown) and converted to velocity using the standard curve (Fig. 2). A plot of 1/v versus 1/[S] is shown. The K_m of Abz-NleF*-6 was calculated to be $37 \pm 8\,\mu M$ based on the average of four measurements.

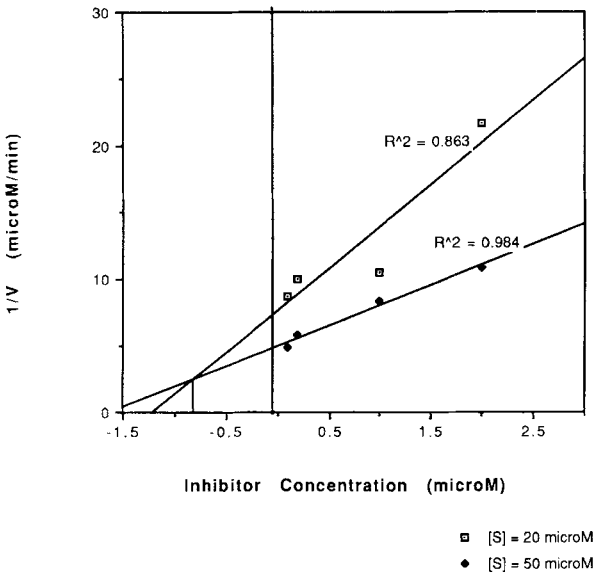

FIGURE 6

K_i determination of MVT-101 (Ac-Thr-Ile-Nleψ[CH_2NH]-Nle-Gln-Arg-NH_2). Initial velocities were calculated at two different substrate concentrations (20 and 50 μM) in the presence of the inhibitor at four different concentrations (2, 1, 0.2, and 0.1 μM) with 0.5 μg of HIV protease in a final volume of 100 μL of assay buffer. 1/v versus [I] was plotted (Dixon plot) and the K_i was calculated to be 810 $\pm$ 40 nM (HPLC assay gave a K_i = 780 $\pm$ 80 nM).

suggests that a folded conformation must predominate for the substrate. Geoghegan *et al.* (18) prepared 5-dimethylaminonaphthalene-1-sulfonyl-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Trp, but were unable to determine the K_m, which was estimated at greater than 100 mM. The increase in the Trp emission peak upon hydrolysis was estimated to be 2.5-fold. The fluorescent substrate reported here has a K_m = 37 $\pm$ 8 μM and an increase in fluorescence of sixfold. While this may make it somewhat less sensitive than that reported by Matayoshi *et al.* (17), its smaller size and ease of synthesis recommends it. Clearly, further work to optimize the donor-acceptor pair could be done if enhanced sensitivity were a major goal.

Utilizing the fluorogenic substrate, Abz-NF*-6, a screening procedure for potential HIV protease inhibitors was established which allowed the determination of inhibitor affinity. Eight wells allowed the determination of the effect of four different concentrations of inhibitor on two substrate concentrations—which was minimally sufficient to calculate a K_i value (Fig. 6) based on a Dixon plot (1/v vs. inhibitor concentration). Fluorescence was measured every 2 min for 20 min. Thus, it is feasible to determine the affinity of 12 inhibitors in 20 min. This is to be contrasted with the HPLC analysis, which requires about 30 separate HPLC runs, each of which takes approximately 1 h, for the determination of one K_i. Under routine conditions, however, one would probably run determinations in duplicate and include an inhibitor standard, which would reduce the number of K_i determinations to 15 per hour. Utilizing this assay procedure, a number of inhibitors of HIV protease have been quantitated (24, 25).

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