Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2291–2296

BIOORGANIC &
MEDICINAL CHEMISTRY
LETTERS

A RAPID METHOD TO IDENTIFY *EXO*-PROTEASE INHIBITORS

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Received 27 April 1999; accepted 26 May 1999

Abstract: A new approach to the evaluation of *exo*-protease inhibitor candidates is presented. The application of new water-soluble substrates that release organic-soluble fluorescent groups upon proteolytic cleavage allows amplification of the assay signal via concentration of the cleavage product. A combinatorial library of disubstituted xanthenes designed to resemble a known inhibitor was screened and a new HLE inhibitor (K_i = 79 μM) was identified.

Introduction

Small molecule protease inhibitors have become an integral facet of pharmaceutical development.[1] Current advances arising from arrayed and combinatorial methods pose an increasing demand for tools that can efficiently differentiate large numbers of candidates.[2] Here, we describe an approach that rapidly addresses *exo*-protease inhibition through the implementation of efficient combinatorial and assay technologies.

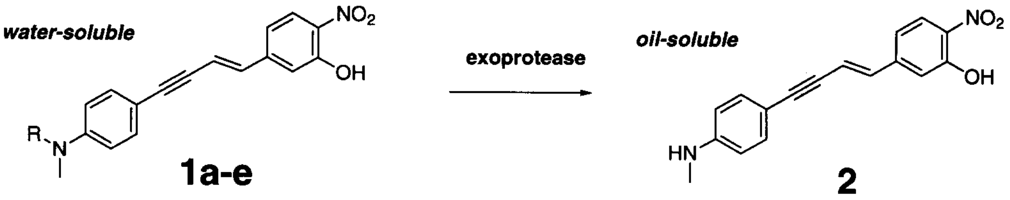
Proteolytic screening is currently monitored using a combination of substrates that change color, electrochemistry, or fluorescence upon reaction.[3] Several of these materials, such as bis-amides of rhodamine 110,[4] can be screened at high-throughput by combining visual analysis with photographic digitization. Unfortunately, this approach currently requires at least μM product formation.[5]

Assay development

We reduced this limit to the nanomolar level by developing a new class of substrates whose solubility and spectroscopic properties invert with proteolysis. A system was designed such that substrates (water-soluble and nonfluorescent) release an oil soluble fluorophore upon hydrolysis.[6] Using this approach, we could assay trypsin, kallikrein, cathepsin B, and elastase activity at enzyme levels reduced by more than 30-fold and in a fraction of the time required using the conventional substrates.[7,8] Alkenyne (**2**) is sparingly soluble and nonfluorescent, $\Phi_f \sim 10^{-7}$, in aqueous media, and highly soluble and fluorescent in mineral oil, $\lambda_F = 511$ nm with $\Phi_f \sim 0.11$ (Figure 1).[9] The origin of this enhancement arises from the solvent-induced stabilization of twisted-

PII: S0960-894X(99)00387-X

intramolecular charge transfer (TICT) states. When excited to the singlet state, materials presenting charge transfer undergo facile relaxation from planar-conjugated to orthogonally-twisted or TICT states. Due to their perpendicular orbital alignment, TICT states present greater dipole character than their planar hosts and no longer relax to the ground state through fluorescent mechanisms. Therefore, factors that increase their expression, such as solvent polarity, also present a net loss in fluorescence.



enzyme	peptide (R)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($10^5\ M^{-1}s^{-1}$)	visual limit (μU)[10]
trypsin	Z-L-Ile-L-Pro-L-Arg -	97.0	72.5	7.48	22±9
kallikrein	Z-L-Pro-L-Phe-L-Arg -	92.2	65.5	7.13	49±9
cathepsin B	Z-L-Arg-L-Arg -	129.0	121.0	9.37	37±9
elastase	$CH_3O(CH_2CH_2O)_4CO$-L-Ala-L-Ala -	325.0	145.0	4.46	29±6
HLE	L-Tyr-L-Leu-L-Val-	16.1	3.6	2.25	15+3

Figure 1. Application of two-phase colorimetry to monitor *exo*-proteases.[7,8] Hydrolysis of the water-soluble peptides **1a–e** releases **2**, which becomes fluorescent as it is extracted into a trace amount of mineral oil. The visual limit indicates the lowest level of enzyme required for detection within 1h. Under the conditions used for each assay, derivatives of rhodamine 110 required ≥ 1 mU.[10]

When functionalized with water-soluble peptides, conjugates **1a–e** of **2** become insoluble in mineral oil and rapidly partition into aqueous media, thereby destroying their fluorescence.[11] The extent of this regulation is apparent by the fact that fluorescence was not detected when aqueous solutions of these conjugates were placed under a conventional handheld UV lamp (Mineralight UVGL-58). When mixed with the appropriate enzyme, hydrolysis of the C-terminal amide releases **2**, which is subsequently extracted into mineral oil. Like rhodamine 110, the concentration of indicator **2** must be greater than ~1 μM. However, **2** is now concentrated by the use of two phases. Using 1 μL of mineral oil per 200 μL of aqueous buffer, the analyte is concentrated ~200-fold, therein reducing the detection limit to require 5 nM of product formation.

Additionally, the new substrates beneficially require the cleavage of only one amide per signal, therein simplifying their reaction kinetics as compared to commonly used bis-substituted derivatives such as rhodamine 110.[4] In all cases, catalysis was comparable to that observed using known substrates (Figure 1),[7,8] indicating that the phase transfer was not rate limiting. The strength of the assay is demonstrated in Figure 2. Here, a selection of unknowns was assayed for trypsin inhibition. The first three wells (top left) were loaded with known inhibitors for comparison purposes.[12] As indicated, only one additional well lacked fluorescence, thereby indicating that its contents, *N*-phenylmaleimide, was a weak inhibitor (K_i ~ 0.8 mM).[13]

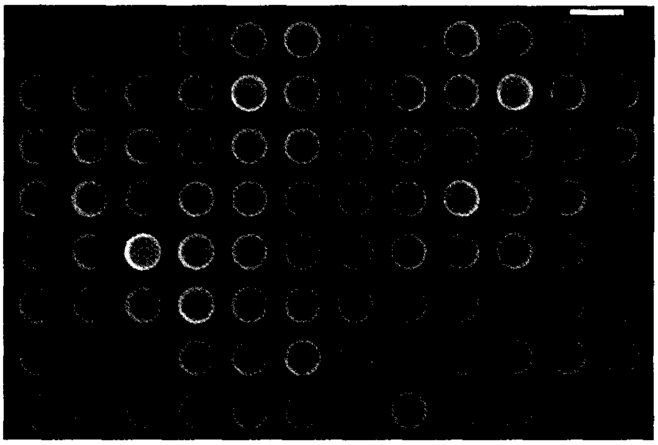


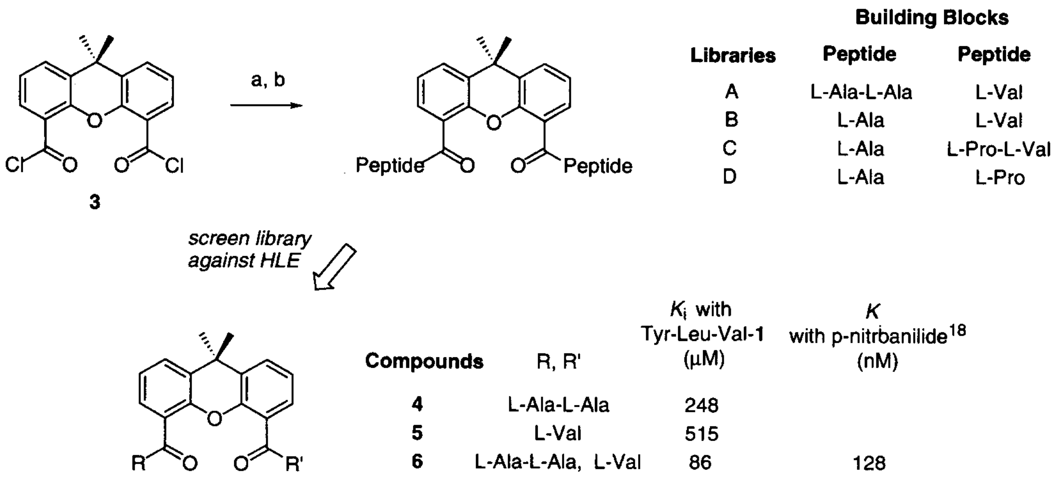
Figure 2. Application of two-phase colorimetry to screen for trypsin inhibition. Ninety-two shelf chemicals were screened by comparison with background (well at bottom right corner) and three known inhibitors (first three trials on top row).[12,14] Inhibition is indicated by the lack of emitted visible light. Fluorescence was obtained by excitation at 366 nm with a hand-held UV lamp. White bar in upper left indicates 1 cm.

Results and Discussion

With an efficient screen in hand, we then devised a series of small libraries against human leukocyte elastase (HLE). Each library was produced by a solution-phase core molecule approach[15] and was designed to resemble a peptide-sequence recognized by HLE, Ala-Ala-Pro-Val.[16] Accordingly, 9,9-dimethylxanthene-4,5-dicarboxylic acid chloride (**3**) was reacted with left- and right- termini of Ala-Ala-Pro-Val, with the intention replacing internal amino acid(s) with the core.[17] A hit was found in a series of four libraries produced by incorporating two building blocks per core (Scheme 1). The only library of this group exhibiting micromolar inhibition was that which contained Ala-Ala and Val, library A. Notably, library B was inactive, indicating the importance of the two terminal alanines. Subsequent resynthesis of the individual components **4**–**6** showed that, indeed, the mixed Ala-Ala/Val adduct was responsible for the greatest activity (K_i = 79 μM), exceeding the activity of the bis(Ala-Ala) and bis(Val) adducts by factors of 2 and 6.5, respectively.

Insertion of the core altered both the position of the two amino acid side chains as well as inverting the head-to-tail orientation of the Ala-Ala residue. Interestingly, this modification induced only a modest loss in inhibition. As apparent upon overlaying **6** into a crystal structure of HLE complexed with MeO-Suc-Ala-Ala-Pro-ValCH_2Cl,[19] the enzymatic pocket not only allows the critical encapsulation of the terminal Val residue but also readily accommodates the bulky dimethylxanthene.

Application of two-color colorimetry to solution-based core molecule display has rapidly identified an inhibitor of HLE. While applied here for a specific core and protease, the methods described herein should be directly applicable to a variety of medicinally-significant *exo*-proteases.



Libraries	Building Blocks Peptide	Building Blocks Peptide
A	L-Ala-L-Ala	L-Val
B	L-Ala	L-Val
C	L-Ala	L-Pro-L-Val
D	L-Ala	L-Pro

Compounds	R, R'	K_i with Tyr-Leu-Val-1 (μM)	K with p-nitrbanilide[18] (nM)
4	L-Ala-L-Ala	248	
5	L-Val	515	
6	L-Ala-L-Ala, L-Val	86	128

Scheme 1. Dimethylxanthene core-based libraries targeting HLE (EC 3.4.21.37). Each library was prepared by coupling a mixture of two peptides onto **3**. Each library (A–D) consists of a mixture of bis(peptide$_1$), bis(peptide$_2$), and peptide$_1$-peptide$_2$. Screening using the method described in Figure 1 indicated that only library A presented μM inhibition. Using this information, the activity of each component was determined independently. Conditions: (a) *t*-butyl ester of peptide$_1$ (1.1 equiv), *t*-butyl ester of peptide$_2$ (1.1 equiv), Et_3N (3 equiv), CH_2Cl_2, 1.5 h. (b) neat TFA, 3 h.

Acknowledgment: We thank Julius Rebek, Jr. for his insight and guidance, Mike Pique for molecular modeling experimentation, Ellen Choi for her invaluable technical assistance, Andrea Schliep for proofreading, Alan McPhee for photography, and the Skaggs Foundation for generous financial support.

References and Notes

1. (a) Whittaker, M. *Curr. Opin. Chem. Biol.* **1998**, *2*, 386. (b) Dolle, R. E. *Mol. Diversity* **1997**, *2*, 223. (c) Kennedy, A. R. *Pharmacol. Theraputics* **1998**, *78*, 167. (d) Amzel, L. M. *Curr. Opin. Biotech.* **1998**, *9*, 366.

2. (a) Cargill, J. F.; Lebl, M. *Curr. Opin. Chem. Biol.* **1997**, *1*, 67. (b) Czarnik, A. W. *Curr. Opin. Chem. Biol.* **1997**, *1*, 60. (c) Weber, L. *Curr. Opin. Chem. Biol.* **1998**, *2*, 381. (c) Amzel, L. M. *Curr. Opin. Biotech.* **1998**, *9*, 336. (d) Veber, D. F.; Drake, F. H.; Gowen, M. *Curr. Opin. Chem. Biol.* **1997**, *1*, 151. (e) Houston, J. G.; Banks, M. *Curr. Opin. Biotech.* **1997**, *8*, 734. (f) Brown, D. *Mol. Diversity* **1997**, *2*, 217.

3. (a) In *Topics in Fluorescence Spectroscopy: Probe Design and Chemical Sensing*; Lakowicz, J. R., Ed. Plenum: New York, 1994, vol 4. (b) Mason, W. T. *Fluorescent and Luminescent Probes for Biological Activity*; Academic: San Diego, 1993.

4. (a) Leytus, S. P.; Toledo, D. L.; Mangel, W. F. *Biochim. Biophys. Acta.* **1984**, *788*, 74. (b) Leytus, S. P.; Patterson, W. L.; Mangel, W. F. *Biochem. J.* **1983**, *215*, 253. (c) Leytus, S. P.; Melhado, L. L.; Mangel, W. F. *Biochem. J.* **1983**, *203*, 299.

5. Even the most robust chromaphores (i.e., $\varepsilon \geq 90{,}000\ \mathrm{cm^{-1}M^{-1}}$) require a concentration greater than 1 μM to appear colored.

6. La Clair, J. J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 325.

7. All enzymes were purchased from Sigma [product number] and standardized as follows (enzyme): (trypsin) One unit or 16.6 μg of Type IX from porcine pancreas [T 0134] converted 1 μmol/min of BAEE to *N*-α-benzoylarginine at pH 7.6 at 23 °C. (kallikrein) One unit or 25.6 μg of kallikrein from porcine pancreas [K 3627] converted 1 μmol/min of BAEE at pH 8.7 at 25 °C. (cathepsin B) One unit or 30.3 μg of cathepsin B from bovine spleen [C 6286] hydrolyzed 1 μmol/min of *N*-α-CBZ-lysine-p-nitrophenylester at pH 5.0 at 25 °C. (elastase) One unit or 1.2 μg of elastase from porcine pancreas [E 0127] solublized 1 mg of elastin in 20 min at pH 8.8 at 37 °C. (HLE) One unit or 1.2 μg of elastase from human leukocytes [E 8140] released 1 μmol/min of *p*-nitrophenol from *N*-*t*-BOC-L-alanine-*p*-nitrophenyl ester at pH 6.5 at 37 °C. Enzymes were examined in the following media: (1) trypsin in 10 mM Tris-HCl pH 7.8; (2) kallikrein in 50 mM TRIS pH 9.0; (3) cathepsin B in 100 mM potassium phosphate pH 6.0; (4) elastase in 10 mM Tris-HCl pH 7.5 and (5) HLE in 50 mM NaOAc, 100 mM NaCl, 8 mM $\mathrm{NaN_3}$ pH 6.0. Each assayed was developed in a 300 μL 96-well Teflon plate by adding 20 μL of enzyme stock to a μL of mineral oil and 180 μL of 12 μM substrate in buffer. The plate was covered with a thin sheet of Teflon and vigorously shaken at 37 °C.

8. Kinetics were determined by diluting with 100 μL of spectral grade n-heptane, transferring the organic layer to a quartz 96-well plate quantifying **2** (λ_{max} = 424 nm, $\varepsilon = 67{,}400\ \mathrm{M^{-1}cm^{-1}}$) relative to the amount of mineral oil (λ_{max} = 272 nm) using a conventional plate reader (Molecular Dyamics SpectraMax 250).

9. La Clair, J. J. *J. Am. Chem. Soc.* **1997**, *119*, 7676.

10. A unit (U) is defined throughout as the quantity of enzyme required to produce 1 μmol of product per minute.

11. Peptide substrates **1 a–e** were prepared by coupling the appropriate protected peptide onto **2**. This was readily accomplished exposing a mixture of **2** (1.3 equiv) and peptide (1 equiv) to EDC (2 equiv) and DMAP (0.01 equiv) in anhydrous DMF. Pure substrate was readily obtained after flash chromatography. Each compound was verified by LRMS (CI) and their purity was shown to be >95% by analytical HPLC.

12. Known trypsin inhibitors were displayed along the top row (left to right): 4-aminobenzamidine, phenylmethylsulfonylfluoride and *N*-tosyl-L-phenylalanine chloromethyl-ketone.

13. Hall, P. L.; Anderson, C. D. *Biochem.* **1997**, *13*, 2082.

14. The assay was determined by presenting 100 µL of a 10 µM stock of each unknown in 10 mM Tris-HCl containing 1% DMSO to 0.1 mU trypsin in 100 µL of 10 mM Tris-HCl at pH 7.8, and then developing after 30 min of incubation at 37 °C by shaking with 10 µL mineral oil and 50 µL of 80 µM substrate (Z-Ile-Pro-Arg-1) in 10 mM Tris-HCl at pH 7.8 containing 1% DMSO. The entire assay including a 30 min pre-incubation was complete within 40 min.

15. (a) Pryor, K. E.; Shipps, G. W., Jr.; Skyler, D. A.; Rebek, J., Jr. *Tetrahedron* **1998**, *54*, 4107. (b) Shipps, G. W., Jr.; Pryor, K. E.; Xian, J.; Skyler, D. A.; Davidson, E. H.; Rebek, J., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11833. (c) Carell, T.; Wintner, E. A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2061. (d) Carell, T.; Wintner, E. A.; Bashir-Hashemi, A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2059.

16. (a) Gutschow, M.; Neumann, U. *J. Med. Chem.* **1998**, *41*, 1729. (e) Buynak, J. D.; Rao, A. S.; Ford, G. P.; Carver, C.; Adam, G.; Geng, B.; Bachmann, B.; Shobassy, S.; Lackey, S. *J. Med. Chem.* **1997**, *40*, 3423. (f) Regan, J.; McGarry, D.; Bruno, J.; Green, D.; Newman, J.; Hsu, C. Y.; Kline, J.; Barton, J.; Travis, J.; Choi, Y. M.; Volz, F.; Pauls, H.; Harrison, R.; Zilberstein, A.; Ben-Sasson, S. A.; Chang. M. *J. Med. Chem.* **1997**, *40*, 3408.

17. All libraries were prepared similarly by adding triethylamine (0.3 mL) and a solution of xanthene diacidchloride **3** (30 mg, 0.090 mmol) in 1 mL CH_2Cl_2 to a solution of two building blocks (0.099 mmol each) in 3 mL CH_2Cl_2. This solution was stirred for 1.5 h, then diluted with CH_2Cl_2 and was washed with water (3 x). The organic phase was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation and the residue was deprotected with neat TFA (10 mL) for 3 h. The TFA was removed by rotary evaporation and the residue freeze-dried, yielding the library as a pale tan foam. Single compounds **4–6** were isolated from library **A** by preparative HPLC. Each compound was identified by LRMS (ESI +) and their purity was shown to be >95% by analytical HPLC.

18. ICI 200880, an established HLE inhibitor, had a K_i = 182 nM using Tyr-Leu-Val-**2**. Inhibition was also compared using MeO-Suc-Ala-Ala-Pro-Ala-*p*-nitroanalide indicated that **6** and ICI 200,355 had a respective K_i of 128 nM and 4 nM.[16b]

19. Our approach was modeled after this inhibitor as a crystal structure of its complex to HLE (pdb file = 1ppg) has been determined. Wei, Z.; Mayr, I.; Bode, W. *FEBS Lett.* **1988**, *234*, 376.