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Fluorogenic ester substrates to assess proteolytic activity

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Abstract—The synthesis of a new type of fluorogenic ester substrates is described. Prepared from fluorescein in three steps with common commercially available precursors, they all generate bright green fluorescence upon proteolysis. Their particular structure allows the same substrate be used to report enzymatic activity of various proteases from serine and cysteine superfamilies. The substrate cleavage is sensitive to specific protease inhibitors providing a tool for inhibitor screening.

Proteases are involved in numerous crucial cellular processes varying from protein degradation and re-cycling to specific post-translational modifications.[1] Protease malfunctions often lead to pathologies, making them biomarkers and therapeutic targets of choice.[2] Assessing their catalytic activity is therefore essential.

Various methods for visualizing enzyme activity by using chromogenic or fluorogenic substrates have emerged recently.[3] For proteases, peptide substrates with internally quenched fluorescence have been intensively employed. There are more than forty papers referenced in the Pubmed database for the last 10 years.[4] These fluorogenic peptides, designed to perfectly match a substrate binding site,[5] are optimally suited to assess the substrate specificity of a given protease.[6] At the same time, short less-specific substrates are often useful for the simple detection of the catalytic activity of proteases. These substrates include one or two amino acid residues bound to amino-fluorophore such as amino-coumarins AMC[7] and ACC,[8] Rhodamine 110[9a] or cresyl violet.[9b] Z-F-R-AMC, the most widely used substrate for cysteine proteases of the papain family,[4a] shows how important such substrates can be.

It has been known for a long time that serine and cysteine proteases possess an esterase activity.[10] Due to ready transesterification of the scissile bond to the acyl–enzyme intermediate, the hydrolysis of peptide esters by these enzymes proceeds orders of magnitude faster than the corresponding enzymatic hydrolysis of peptide bonds.[10] This makes peptide esters a promising scaffold for the synthesis of fluorogenic protease substrates. To our knowledge, esters of fluorescein have often been employed for studying various esterase activities,[11] phosphodiesterases[12] and phosphatases,[13] but their use to sense protease activity has only been reported twice.[14]

We report here an easy, and low-cost, synthesis of new convenient short substrates based on single amino-acid fluorescein monoesters and demonstrate their use in universal and sensitive detection of protease activity.

Substrates were designed with the general formula PG-AA-MFE where PG is a protecting group (Boc or Fmoc), AA is an amino acid residue, and MFE stands for the 6′-methylfluorescein ether (Fig. 1). Due to the locked spiro-lactone structure, these compounds are non-fluorescent. Proteases were expected to hydrolyze

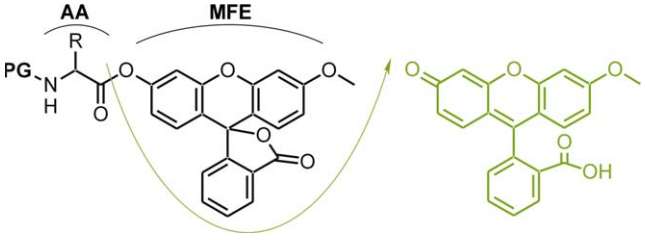

Figure 1. Schematic representation of fluorogenic ester substrates and protease cleaved product.

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the ester bond between AA and MFE thus 'unlocking' the fluorophore and releasing the fluorescent quinoid form (Figure 1, green arrow, determined quantum yield $\phi_{MFE} = 0.37$). In addition, three amino acid-free control compounds were synthesized, having PG-AA moiety replaced by an acetyl, a tosyl or a *p*-nitrobenzenesulfonyl group (Scheme 1).

Synthesis was started from fluorescein in open quinoid form **1**, which was methylated[15] by methyl iodide yielding dimethylfluorescein ether ester **2** (Scheme 1). The ester group was hydrolyzed by aqueous alkali and after acidic extraction, 6′-methylfluorescein ether **3** (MFE) was obtained in a lactone pale yellow form. Fluorogenic substrates **4a–e** were synthesized by acylation of MFE with protected amino acids and DCC as a coupling agent. Control molecules **4g–i** were obtained by reacting MFE with acetyl, tosyl or *p*-nitrobenzenesulfonyl chloride in the presence of DIEA in DCM. This three-step, fast, and cost-effective synthesis gave six amino acid substrates and three control molecules with good overall yield (30–50%).

There are four major classes of proteases: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. The cysteine protease cathepsin B (EC 3.4.22.1) is a papain-related enzyme involved in lysosomal protein processing.[16] The overexpression of this enzyme was found to be implicated in the tumor angiogenesis process[17] and linked with many etiologically different cancers.[18] This protease of medical interest[19] was chosen to test the synthesized MFE substrates.

The substrates were incubated at 37 °C with cathepsin B in a phosphate buffer (pH 6.7) containing EDTA and DTT, and the proteolysis was monitored by reading fluorescence emission at 515 nm ($\lambda_{exc} = 488$ nm). Since, like all known ester substrates, MFE esters undergo slow spontaneous hydrolysis, the rate of proteolysis was estimated as a difference of the substrate hydrolysis with and without protease (Fig. 2). Although all amino acid substrates were cleaved by cathepsin B, the comparison of initial reaction rates showed that Boc-protected-AA-MFE substrates are cleaved more readily than Fmoc-protected ones. The latter may be explained by higher steric hindrance of the Fmoc residue. The nature of AA residue influenced also the cleavage. Reaction rate order Gly, Ala > Abu > Leu seems to indicate that the smaller the amino acid hydrocarbon chain, the faster

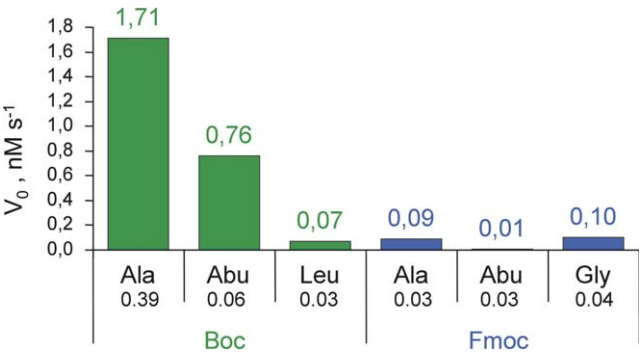


Figure 2. Comparison of initial rates of cathepsin B cleavage for six ester substrates. The values were calculated as a difference of the substrate hydrolysis rate with and without protease (the corresponding rates of spontaneous substrate hydrolysis are indicated below the histogram). The fluorescence units were converted into concentration equivalents by using a calibration curve made with known dilutions of MFE. All values were means of two or three experiments (average CVs < 0,2).

the hydrolysis. No enzymatic cleavage was observed with the control compounds Ac-MFE, Ts-MFE, and *p*-NO_2PhSO_2-MFE demonstrating that the presence of amino acid residue is essential for cathepsin B cleavage.

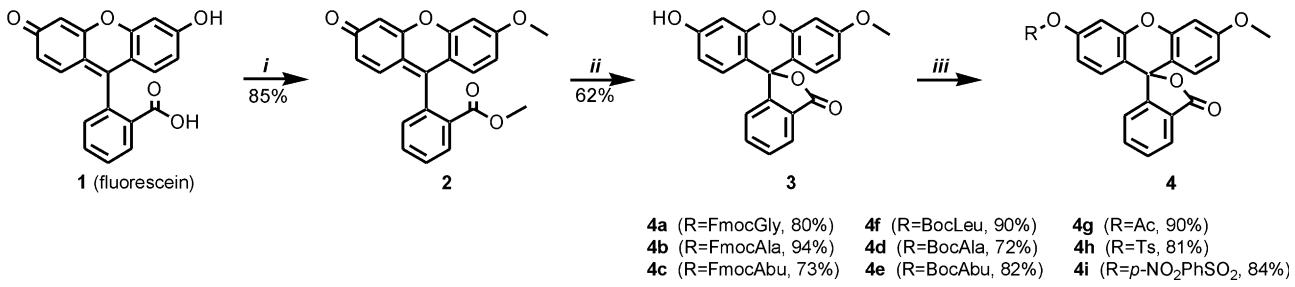
To address substrate cleavage in more detail, the initial reaction rates were measured with a substrate concentration ranging from 0 to 40 μM. The kinetic parameters were then obtained by fitting to Michaelis–Menten equation with OriginPro 7.5 software. The results listed in Table 1 show that hydrolysis of the Boc-protected substrates is approximately 10 times more efficient than

Table 1. Kinetic parameters for substrate cleavage by cathepsin B

Substrate	V_{max}[a] (nM s^{-1})	K_m[a] (μM)	k_{cat}[b] (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Boc-Ala-MFE	3.02 ± 0.48	4.5 ± 2.8	0.73 ± 0.12	2700
Fmoc-Ala-MFE	0.28 ± 0.04	7.6 ± 3.3	0.07 ± 0.01	150
Boc-Abu-MFE	1.05 ± 0.08	2.2 ± 0.6	0.25 ± 0.02	1910
Fmoc-Abu-MFE	0.11 ± 0.04	7.2 ± 6.9	0.03 ± 0.01	60
Boc-Leu-MFE	0.16 ± 0.48	2.7 ± 1.3	0.04 ± 0.01	240
Fmoc-Gly-MFE	0.37 ± 0.04	7.9 ± 3.3	0.09 ± 0.01	190

[a] Values obtained by fitting experimental data to Michaelis–Menten equation using OriginPro 7.5 software.

[b] Values calculated from V_{max} knowing concentration of cathepsin B (250 nM).



Scheme 1. Three-step synthesis of fluorogenic ester substrates. Reagents and conditions: i—MeI, K_2CO_3, DMF; ii—(1) 10% NaOH, MeOH, (2) 0.1 M HCl; iii—(**4a–e**) PG-AA, DCC, DCM, (**4g**) AcCl, (**4h**) TsCl, (**4i**) *p*-NO_2PhSO_2Cl, DIEA, DCM.

that of Fmoc analogues, mainly because of an order of magnitude higher turnover number. Close K_m values indicate that interaction of Boc-AA-MFE and Fmoc-AA-MFE with cathepsin B is similar, and Fmoc steric hindrance would then slow down the catalytic transformation once in the active site. The increase in size of the AA side chain also results in a decrease in k_{cat} without significant changes in K_m value.

Earlier k_{cat}/K_m values reported for cleavage of Z-FR-AMC by cathepsin B were of the order of 10^5–$10^6\ M^{-1}\ s^{-1}$, depending on reaction conditions.[20,4a] These values are two to three orders of magnitude higher than that obtained for Boc-Ala-MFE; probably because Boc-Ala does not fit S_1 and S_2 substrate-binding sites like Arg and Phe, known to be preferred P_1 and P_2 residues, respectively, for cathepsin B.[21]

We next examined whether Boc-Ala-MFE can be used to detect proteases distinct from cathepsin B. One other cysteine protease, papain (3.4.22.2), and two serine proteases, chymotrypsine (3.4.21.1) and hepatitis C virus (HCV) NS3 protease,[22] were tested. The proteolysis was analyzed with 5 μM Boc-Ala-MFE in appropriate buffers. As shown in Figure 3a, cathepsin B and serine proteases exhibited close cleavage rates, but papain proved to cleave this substrate considerably faster than other enzymes. This is consistent with exceptionally high activity and wide specificity of papain.[23] The observed difference in Boc-Ala-MFE cleavage may also be related to the organization of S_2–S_1 substrate-binding site of the proteases. The preferred residues in P_2–P_1 positions are F-G for papain,[24] F-R for cathepsin B,[21] and V-Y or A-F for chymotrypsin.[25] It is possible that P_1-Ala mimics better the nonpolar residue in the P_1-position of papain substrates than the charged P_1-residue of cathepsin B or bulky aromatic P_1-amino acid of chymotrypsin substrates. Indeed, comparison of the efficiency of the cleavage of three Boc substrates by these proteases showed that chymotrypsin has a reverse rate order compared to papain and cathepsin B preferring the substrates with bulkier side chains (Fig. 3b). Thus, formally, Boc-Leu-MFE could be considered as a chymotrypsin-specific substrate, while Boc-Ala-MFE is an universal substrate cleavable by all proteases including highly specific HCV NS3 enzyme.[26] The sensitivity obtained for papain (4 nM), for cathepsin B (54 nM), and for chymotrypsin (15 nM) was comparable with other analogous fluorimetric protease assays.[27] It should be noted, however, that for 500 nM NS3 the specific fluorescence increase showed by MFE-ester substrates was just above the defined limit of detection (S/N = 3).

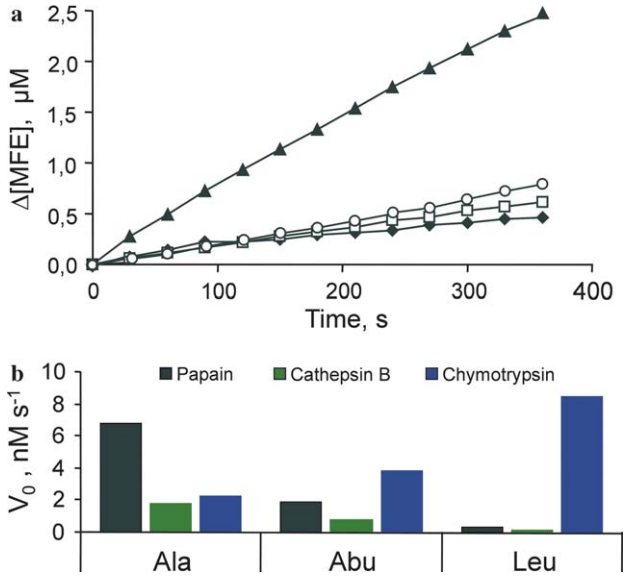


Figure 3. Cleavage of fluorogenic substrates by cysteine and serine proteases. (a) Cleavage of 5 μM Boc-Ala-MFE by 12.5 nM papain, $V_0 = 6.78$ nM/s (▲); 250 nM chymotrypsine, $V_0 = 2.23$ nM/s (○); 250 nM cathepsin B, $V_0 = 1.71$ nM/s (□); and 500 nM NS3, $V_0 = 1.19$ nM/s (♦). The values were calculated as a difference of the substrate hydrolysis rate with and without protease. All values are means of two or three experiments (average CVs < 0.2). (b) Comparison of the initial rates of protease cleavage for three Boc substrates.

One of the important applications of fluorogenic substrates is in screening protease inhibitors. We thus examined whether the proteolysis of Boc-Ala-MFE is sensitive to general cysteine protease inhibitor E-64 and to PMSF, the inhibitor of serine proteases. The proteolysis assay conditions were the same as described in Figure 3 except that 1 μM E-64 or 0.5 mM PMSF was present in the reaction buffer (Fig. 4).

Cysteine proteases were strongly inhibited by E-64, while PMSF had very moderate effect. On the other hand, E-64 did not affect at all the cleavage by chymotrypsin, while PMSF inhibited completely this serine protease. As expected, NS3 protease was not affected much by these inhibitors, probably due to its particularly shallow active site that renders inefficient an interaction with small molecule inhibitors.[26] The proteolysis of the substrate by NS3 could, however, be inhibited by 1 μM BILN 2061, a specific inhibitor of this enzyme[28] (data not shown). All together, the inhibition profile observed for Boc-Ala-MFE proteolysis suggests that the cleavage of this substrate represents correctly the enzyme catalysis and can be used for inhibitor assays. Moreover, such substrates are well suited for screening of chemical libraries because small molecules usually

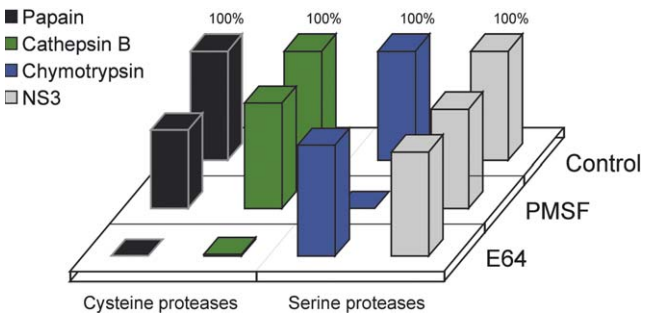


Figure 4. Effect of protease inhibitors on the initial rate of Boc-Ala-MFE proteolysis. The reaction conditions were the same as in Figure 3 except that 1 μM E64 or 0.5 mM PMSF were added where indicated.

display low absorption within the fluorescein excitation/emission range and will not interfere with fluorescence measurements.[29] Inhibitor assay could easily be performed in microtiter plates provided that purified enzymes with a high enough cleavage efficiency are used.

In conclusion, new fluorogenic MFE-ester substrates have been synthesized in a fast and convenient way. The substrates may be used as sensitive indicators of a wide range of proteolytic activities, and show promise for screening libraries of protease inhibitors.

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Supplementary data

Supporting information contains experimental information and characterization data for all new compounds. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.06.037.

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