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Sensitive luciferin derived probes for selective carboxypeptidase activity

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ABSTRACT

Highly selective luminescent probes, QLUC-TYR and LUC-GLU, for detection of carboxypeptidase activity were synthesized. Caged substrates were first cleaved by corresponding carboxypeptidases, and then they were activated by luciferase to emit light. Enzymatic activities of biologically important carboxypeptidases can be determined using this technology.

Luciferase has been widely used as an image reporter to study numerous biological events in vivo.[1–3] This light-producing system gives high signal with low background in the presence of O_2, Mg^{2+}, ATP, and D-luciferin. Luciferase specifically oxidizes luciferin; however, this substrate selectivity limits its application to a small number of suitable light-emitting compounds and its utility as a readout of this biochemical reaction. Modifications at the carboxyl or hydroxyl groups of luciferin often prohibit its conversion to the key intermediate, oxyluciferin, and abolish the luminescence.[4] Recently, few modified luciferins, whose carboxyl or hydroxyl groups were blocked by various chemical moieties, have been reported to yield additional biological information.[5–9] Removing blocking moieties through reactions either enzymatically catalyzed or photoactivated released free luciferin for subsequent bioluminescence. Such strategies have extended the luciferase platform to study other enzymes. In this study, we reported a new type of 'caged' luciferins to investigate carboxypeptidase activities.

Carboxypeptidases are a family of proteases that cleave at specific C-terminal amino acid residues in polypeptides and proteins with critical roles in normal biological processes and in diseases.[10] For example, carboxypeptidase A (CPA) and B (CPB), mainly produced by the pancreas, are metalloenzymes which are similar in primary amino acid structure and substrate specificity.[11,12] It has been shown that CPA in mast cells can enhance the resistance to snake and honeybee venoms.[13] In plasma, the precursor proteins, procarboxypeptidase A, exhibit only low catalytic activity before activation by trypsin cleavage.[14,15] CPA activity was found to be upregulated in pancreatitis and pancreatic cancer; therefore, it has been proposed as a biomarker for early detection.[16,17] In addition to CPA and CPB, carboxypeptidase G, a bacterial enzyme that hydrolyzes folic acid, has been proposed as a potential drug activator to convert an anticancer prodrug for enhanced potency.[18,19] These observations provide the motivation for developing new protease-specific agents that can provide important information of biological processes.

The current assay for CPA activity is based on the hydrolysis of *N*-(4-methoxyphenylazoformyl)-Phe-OH by monitoring the absorption decrease at 350 nm.[20] This method is light-sensitive[21] and not suitable for cell assay. Since the light-emitting luciferase assay is more sensitive and potentially useful for cell or in vivo imaging, new luciferin derivatives with tyrosine (QLUC-TYR) and glutamate (LUC-GLU) residues were designed to detect carboxypeptidase activities for CPA and CPG, respectively (Fig. 1).[22]

Aiming to distinguish different carboxypeptidases, the substrates were designed to report enzyme selectivity by luminescence of the specific wavelength. Quinolylluciferin (QLUC), a luciferase substrate, was selected because of its known property of producing light at long wavelength.[23] QLUC was synthesized from 6-methoxy-2-quinoline-carbonitrile, which was demethylated by pyridine hydrochloride and followed by cycloaddition with D-cysteine to give the desired compound, using a previously reported procedure.[23] QLUC-TYR was prepared by solid-phase synthesis[24] using Wang resin. Fmoc-Tyr(2-ClTrt)-OH was activated by 1-methylimidazole and 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole and linked to the resin (Scheme 1). The Fmoc group was removed by piperidine/DMF (1:3) and then reacted with quinolylluciferin catalyzed by HOBt/HBTU/DIPEA. The resin was treated

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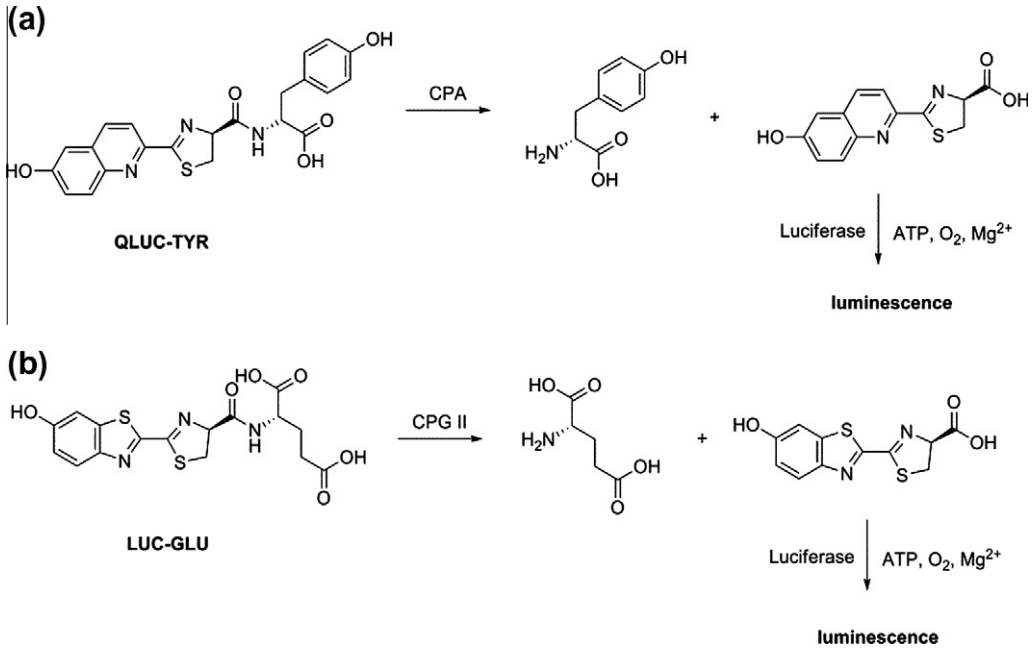
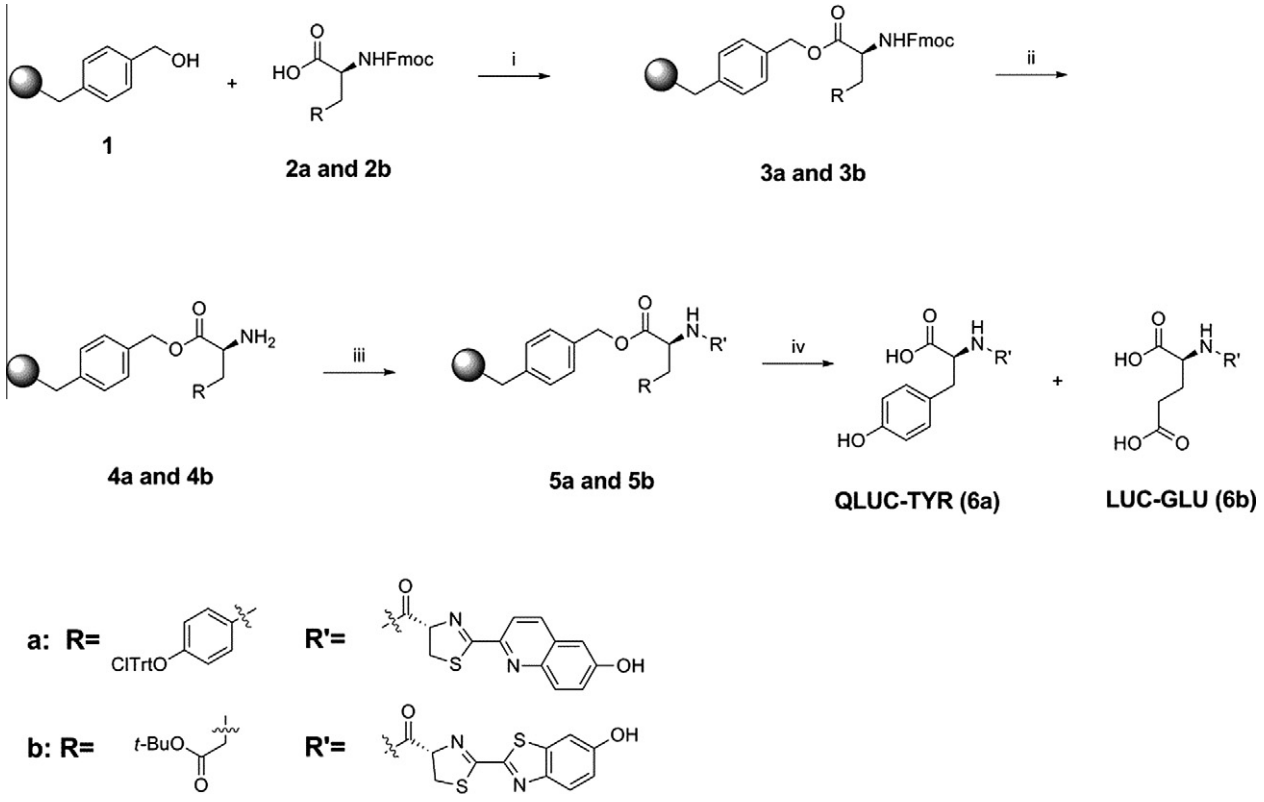


Figure 1. The structures of QLUC-TYR (a) and LUC-GLU (b) and their enzymatic degradation are shown.



Scheme 1. Reagents and synthetic conditions: (i) **2a** or **2b**, Wang resin, 1-methylimidazole, 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole, CH_2Cl_2, rt, 2.5 h; (ii) 25% piperidine in DMF, rt, 25 min, 2 times; (iii) quinolylluciferin or D-luciferin, HOBt, HBTU, DIPEA, CH_2Cl_2, rt, 2 h; (iv) TFA/H_2O/TIS (38:1:1), rt, 2 h.

with H_2O/TRIS/TFA (1:1:38) solution to give QLUC-TYR (**6a**). Same procedure was utilized to prepare LUC-GLU (**6b**) using Fmoc-Glu(O*t*Bu)-OH (Scheme 1). Although both derivatives can be prepared by solution phase synthesis, the solid phase synthesis provides high efficiency without multiple purification steps.

The normalized fluorescence emission spectra of QLUC-TYR and LUC-GLU were recorded in water with excitation at 340 nm, and the observed λ_{max} value was 524 and 540 nm for QLUC-TYR and LUC-GLU, respectively (Fig. 2). It shifted slightly from the parent molecules, the λ_{max} of QLUC and LUC was 518 and 531 nm,

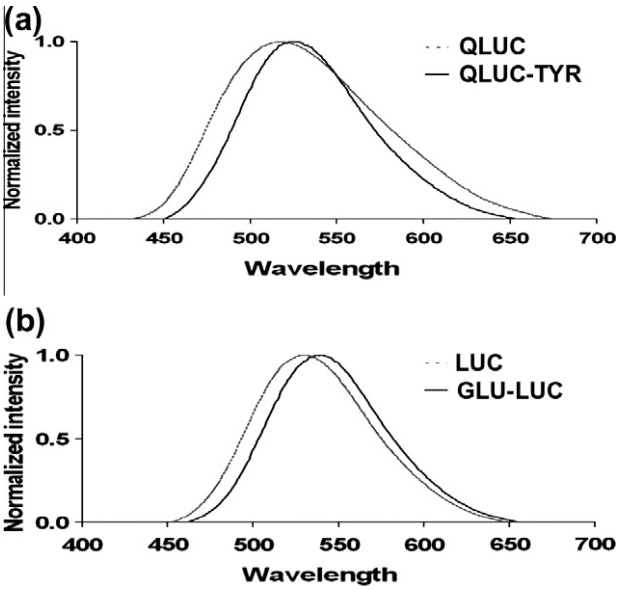


Figure 2. The fluorescent emission spectra of QLUC-TYR & QLUC (a) and LUC-GLU & LUC (b) with excitation at 340 nm in water are compared.

respectively, suggesting that the attachment of amino acids has a small effect on the fluorescent wavelength.

To investigate the enzyme activation systems, QLUC-TYR and LUC-GLU (22.8 mM, 2 μL) were first treated with CPA and CPG at 30 °C for 4 h, respectively. The reaction mixture was then treated with luciferase (39,800 units, 10 μL), and luminescence was measured with a luminometer. This observation indicated that the enzymatic hydrolysis of QLUC-TYR and LUC-GLU released quinolylluciferin and luciferin, which were subsequently hydrolyzed

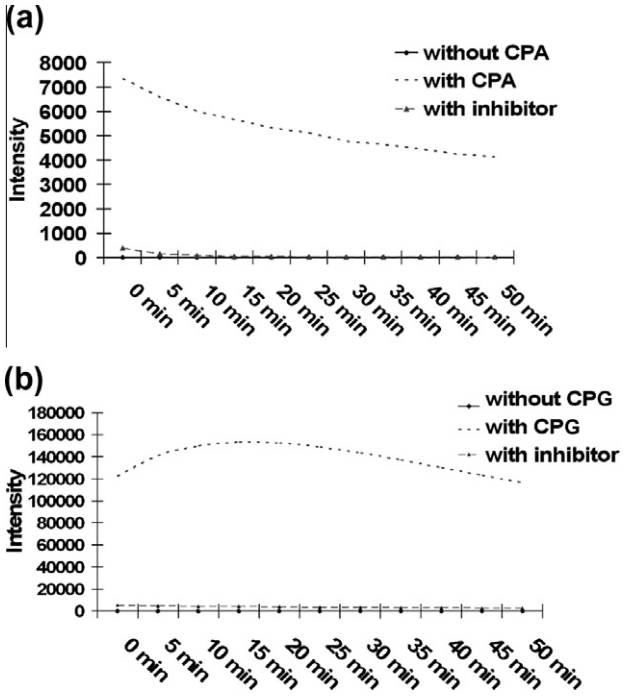


Figure 3. The luminescent emission spectra of QLUC-TYR (a) and LUC-GLU (b) are compared. These two derivatives were treated with CPA in (a) and CPG in (b) that gave the luminescent emission (gray dashed line). It showed no luminescence without enzyme activation (black hard line) and low emission with addition of luciferase essay inhibitor (gray dashed line with triangles).

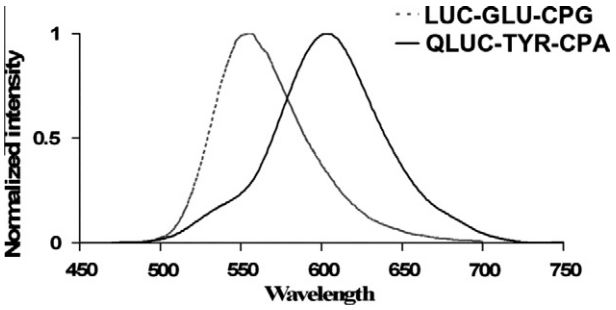


Figure 4. The luminescent wavelength spectra of (a) QLUC-TYR system with CPA and (b) LUC-GLU with CPG are compared.

by luciferase to yield luminescence (Fig. 3). Without carboxypeptidases, luciferase alone was not able to generate luminescence, because the caged luciferins containing amino acid groups were not substrates for luciferase. To further evaluate the enzyme specificity in this process, a reported luciferase inhibitor, methyl ether of luciferin,[25] was added (22.8 mM, 2 μL) to the assay under the same reaction conditions. It resulted in a significant suppression of the luminescence signal of QLUC-TYR and LUC-GLU (Fig. 3), supporting the specificity of the luminescence to luciferase and luciferin. Titration experiments showed that these probes are detectable even at sub-nM concentration (Fig. S1). CPA and CPG could detect their corresponding substrates as low as 0.9 and 0.09 nM, respectively. Further quantitative measurement of CPA and CPG both showed a dose dependent activation (Figs. S2 and S3).

While examining the luminescent intensity, the luminescence of the QLUC-TYR system was found significantly lower than that of the LUC-GLU system. Several experimental conditions, such as addition of Zn^{2+}, reaction temperature (to 37 °C), enzyme concentration, and incubation time were all varied; however, the relative intensity of luminescence was not significantly improved (data not shown). It has been reported that addition of FBS or BSA could facilitate the hydrolysis[19]; however, their inclusion in our reaction

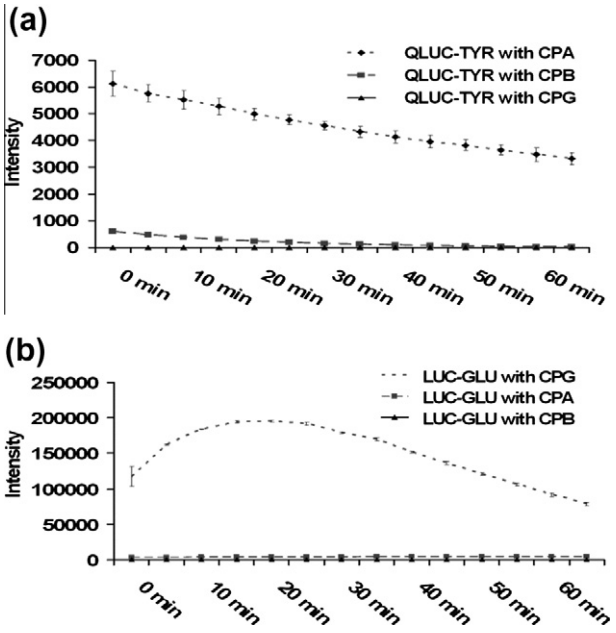


Figure 5. The luminescent emission spectra of (a) QLUC-TYR system with CPA (dashed line), CPB (dashed line with square), and CPG (solid line with triangle); (b) LUC-GLU system with CPG (dashed line), CPA (dashed line with square), and CPB (solid line with triangle) are compared.

mixture did not alter the luminescent signal (data not shown). These observations may suggest that the low intensity of luminescence is due to the nature of the quinolylluciferin but not the enzyme activity. For efficient luminescence, the structure of luciferin appears to be critical.[26]

In contrast to fluorescent characteristics, the luminescent wavelength emitted by QLUC is different compared to that produced by luciferin. This distinction provides a practical way to monitor enzyme activities using a single compound or a mixture of these two derivatives. When QLUC-TYR was incubated with CPA and treated with luciferase, the emission λ_{max} was 603 nm (Fig. 4). Conversely, when LUC-GLU was underwent the same condition with CPG, the emission λ_{max} was 556 nm (Fig. 4). These readings were similar to the direct measurement using QLUC and LUC with luciferase, their luminescence λ_{max} were 600 and 554 nm, respectively, which are consistent with reported findings.[23] This data suggests that the emission wavelength was not affected by the neighboring amino acid residues, and a specific enzyme could be detected using a mixture of substrates. However, we have not been able to achieve this yet since only luciferin-specific wavelength at 556 nm was clearly observed, probably due to the dramatic difference in the luminescent intensity and substrate specificity of the luciferase.

Since the enzyme-substrate specificity is crucial for the system, the correlation between the analogs and three carboxypeptidases was further investigated. QLUC-TYR was treated with CPA, CPB, and CPG separately under the same condition (Figs. 5a and S4). The luminescence of QLUC-TYR/CPA combination was significantly increased as described previously. In contrast, the results in QLUC-TYR/CPB (<10% compared to QLUC-TYR/CPA) and QLUC-TYR/CPG both showed low signal. When the LUC-GLU system was evaluated, LUC-GLU/CPG gave the most intensive signal relative to LUC-GLU/CPA (2% compared to LUC-GLU/CPG) and LUC-GLU/CPB (Fig. 5b). The result further confirmed the high selectivity between the enzymes and substrates in our design.

Luciferin has been extensively used to measure luciferase activity both in vitro and in vivo. Our design has broadened the application of the luciferase assay to carboxypeptidases. Since the reaction required two co-existing enzymes, in vivo imaging might not be practical. However, the in vitro detection of carboxypeptidases would be effective. High selectivity between the enzyme and substrate makes this system potentially useful for monitoring different enzyme activities. Although the attempt of using bioluminescence at specific wavelengths to measure the different enzyme activity was not successful in our experimental system, this concept is technically feasible.

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Supplementary data

Supplementary data (experimental details and characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.023.

References and notes

1. Contag, C. H.; Bachmann, M. H. *Annu. Rev. Biomed. Eng.* **2002**, *4*, 235.
2. Contag, P. R.; Olomu, I. N.; Stevenson, D. K.; Contag, C. H. *Nat. Med.* **1998**, *4*, 245.
3. Edinger, M.; Cao, Y. A.; Hornig, Y. S.; Jenkis, D. E.; Verneris, M. R.; Bachmann, M. H.; Negrin, R. S.; Contag, C. H. *Eur. J. Cancer* **2002**, *38*, 2128.
4. White, E. H.; Steinmetz, M. G.; Miano, J. D.; Wildes, P. D.; Morland, R. *J. Am. Chem. Soc.* **1980**, *102*, 3199.
5. Jones, L. R.; Goun, E. A.; Shinde, R.; Rothbard, J. B.; Contag, C. H.; Wender, P. A. *J. Am. Chem. Soc.* **2006**, *128*, 6526.
6. Shao, Q.; Jiang, T.; Ren, G.; Cheng, Z.; Xing, B. *Chem. Commun.* **2009**, 4028.
7. Zhou, W.; Andrews, C.; Liu, J.; Shultz, J. W.; Valley, M. P.; Cali, J. J.; Hawkins, E. M.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ChemBioChem* **2008**, *9*, 714.
8. Zhou, W.; Shultz, J. W.; Murphy, N.; Hawkins, E. M.; Bernad, L.; Good, T.; Moothart, L.; Frackman, S.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *Chem. Commun.* **2006**, 4620.
9. Wehrman, T. S.; von Degenfeld, G.; Krutzik, P. O.; Nolan, G. P.; Blau, H. M. *Nat. Med.* **2006**, *3*, 295.
10. Anson, M. L. *J. Gen. Physiol.* **1937**, *20*, 663.
11. Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3875.
12. Coll, M.; Guash, A.; Aviles, F. X.; Huber, R. *EMBO J.* **1991**, *10*, 1.
13. Metz, M.; Piliponsky, A. M.; Lammel, V.; Abrink, M.; Pejler, G.; Tsai, M.; Galli, S. J. *Science* **2006**, *313*, 526.
14. Stewart, J. D.; Gilvarg, C. *Clin. Chim. Acta* **1999**, *281*, 19.
15. Stewart, J. D.; Gilvarg, C. *Clin. Chim. Acta* **2000**, *292*, 107.
16. Shamamian, P.; Marcus, S.; Deutsch, E.; Maldonado, T.; Liu, A.; Stewart, J.; Eng, K.; Gilvarg, C. *Gastroenterology* **1998**, 114.
17. Brand, R. *Cancer J.* **2001**, *7*, 287.
18. Goldman, P.; Levy, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 1299.
19. Mancini, L.; Davies, L.; Friedlos, F.; Falck-Miniotis, M.; Dzik-Jurasz, A. S.; Springer, C. J.; Leach, M. O.; Payne, G. S. *NMR Biomed.* **2009**, *22*, 561.
20. Mock, W. L.; Liu, Y.; Stanford, D. J. *Anal. Biochem.* **1996**, *239*, 218.
21. Kanstrup, A.; Buchardt, O. *Anal. Biochem.* **1991**, *194*, 41.
22. McGullough, J. L.; Chabner, B. A.; Bertino, J. R. *J. Biol. Chem.* **1971**, *246*, 7207.
23. Branchini, B. R.; Hayward, M. M.; Bamford, S.; Brennan, P. M.; Lajiness, E. J. *Photochem. Photobiol.* **1989**, *49*, 689.
24. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.
25. Denburg, J. L.; Lee, R. T.; McElroy, W. D. *Arch. Biochem. Biophys.* **1969**, *134*, 381.
26. Matthews, J. C.; Hori, K.; Cormier, M. J. *Biochemistry* **1977**, *16*, 5217.