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Introduction of a Highly Photodurable and Common-laser Excitable Fluorescent Amino Acid into a Peptide as a FRET Acceptor for Protease Cleavage Detection

Masumi Taki,* Yoshito Yamazaki, Yuto Suzuki, and Masahiko Sisido
Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530

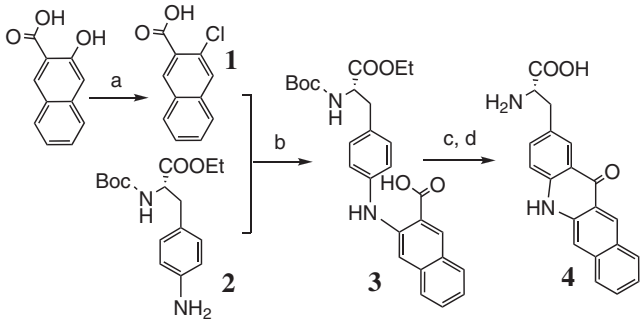
(Received May 6, 2010; CL-100431; E-mail: taki@biotech.okayama-u.ac.jp)

Synthesis and photochemical properties of a benzoacridone-containing fluorescent amino acid (badAla) which is photodurable and excitable by widely applicable 488 nm lasers was described. The amino acid carries a relatively small fluorophore that shows absorption around 450–500 nm, emission above 500 nm with a high fluorescence quantum yield (0.65), and relatively long fluorescence lifetime (17 ns). BadAla can be used in solid-phase peptide synthesis without any precaution. A peptide-containing badAla was used to measure caspase activity via fluorescence resonance energy transfer (FRET).

Fluorescent probes are widely used in peptide chemistry. In particular, introducing donor and acceptor fluorescence resonance energy transfer (FRET) pair in a single peptide is a versatile technique to evaluate specific protease activity. Several intramolecular FRET peptide substrates have been designed and reported.[1] The design generally includes D–X–A structure, in which D is the fluorescent donor group and A is the acceptor group, separated by a peptide sequence (X) containing the protease cleavage site. If the proteolysis occurs, separation of the donor and the acceptor results in a loss of energy transfer and subsequent enhancement of donor fluorescence.

Usually, peptides are derivatized with fluorescent probes at specific positions after solid-phase peptide synthesis (SPPS) and purification. The reactive groups are typically free amine at the N-terminus and thiol group at the side chain. However, the presence of other reacting groups in the same peptide makes the labeling process difficult in many cases. Direct introduction of the chromophores into the peptide sequence by using fluorescent nonnatural amino acids can avoid the problem. However, there are few fluorescent amino acids as monomers which are applicable for SPPS. Recently, Deleris et al. synthesized two different fluorescence nonnatural amino acids as tools for protease cleavage detection by using FRET.[2] However, severe fluorescence quenching between the two fluorophores took place, probably because they are prone to aggregate in aqueous conditions. Once they aggregated, the emission of the fluorophore is highly quenched. For this reason, we tried to find an appropriate FRET pair of two fluorescent nonnatural amino acids.

An acridone-containing fluorescent nonnatural amino acid (acrydonylalanine: acdAla), which was initially reported by Szymanska et al.,[3] is a promising candidate; its small size may minimize steric disturbance in the substrate recognition by proteases. Moreover, it shows many excellent photophysical properties such as high quantum yield, long fluorescence lifetime, high photostability, excitability by a violet laser (405 nm), and moderate solubility in aqueous solutions.[3,4] Here we synthesized a novel benzoacridone-containing amino acid (badAla (**4**)) which is excitable by widely applicable 488 nm lasers and act as an efficient acceptor from acdAla (Scheme 1).



Scheme 1. Synthesis of badAla (**4**): (a) PCl_5, $POCl_3$; (b) Cu/Cu_2O, K_2CO_3, 2-methoxyethanol; (c) phosphoric acid; and (d) LiOHaq., THF.

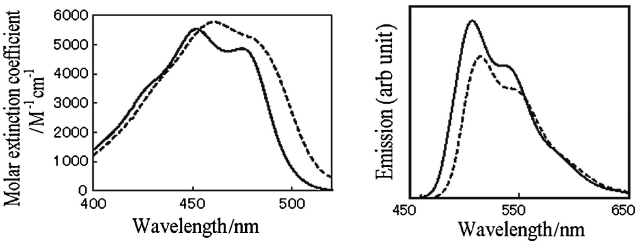


Figure 1. Absorption (left) and emission (right) spectra of badAla (**4**) in EtOH (solid line) and in 50% EtOH/PBS buffer (dotted line).

First, we synthesized 3-chloro-2-naphthoic acid (**1**), and then it was coupled with Boc-protected aminophenylalanine–ethyl ester **2**. Initially, the coupling reaction was attempted under reported conditions for the Ullmann–Jourdan reaction with 2-chlorobenzoic acid and **2**.[3] However, only a trace of the coupled product **3** was obtained. We found the reaction yield was greatly improved when 2-methoxyethanol was used as the solvent and Cu_2O cocatalyst was present in the reaction mixture. Finally, badAla (**4**) was successfully synthesized in 38% yield from **2**.

Absorption and fluorescence spectra of badAla (**4**) are typical of benzoacridone derivative (Figure 1). As we expected, **4** can be excited at longer wavelengths than that of acdAla; the absorption band spans the region of 450–500 nm. When **4** was excited at 488 nm, fluorescence emission above 500 nm was observed. The absorption and emission bands allow us to excite and detect the benzoacridone chromophore selectively, without any interference from other natural chromophores of peptides and proteins, or those inherently existing in living cells. As shown in the Supporting Information,[5] the overlap of the emission spectrum of acdAla and the absorption spectrum of

Table 1. Fluorescence quantum yields of various amino acids and Boc-derivatives containing acridone moieties in MeOH[a]

	Xaa			Boc-Xaa		
	acdAla	sacdAla	badAla	acdAla	sacdAla	badAla
Non-degassed	0.61	0.31	0.51	0.61	0.54	0.49
Degassed	0.74[3]	0.40	0.65			

[a]For the structures of amino acids, see Supporting Information.[5]

badAla (**4**) shows that FRET can occur between these two probes if they are attached onto the same peptide.

Fluorescence decay time of *tert*-butoxycarbonyl–badAla (Boc–badAla) in PBS buffer was 17 ns (see Supporting Information[5]), which is close to that of Boc–acdAla (16 ns)[4] or Boc–sacdAla (14 ns).[6] The fluorescence decay time is long enough for eliminating inherent fluorescence from living cells.

The photodurability of badAla (**4**) was compared with those of other fluorescent groups frequently used in biochemical experiments. BadAla (**4**) retained more than 85% of its original fluorescence intensity after 1 h irradiation, whereas fluorescence intensities of anthranylalanine, BODIPY, and fluorescein decreased more rapidly under the same irradiation conditions.[5] These data indicate that the benzoacridonyl group is tough enough for prolonged measurement on fluorescence imagers or confocal microscopes equipped with common-lasers.

Absolute values of fluorescence quantum yield for the acridone-containing amino acids and their Boc-derivatives were measured by a C9920-02 Absolute PL Quantum Yield Measurement System (Hamamatsu photonics K. K.), and are shown in Table 1. In every case, the quantum yield of the fluorescence was sufficiently high compared to conventional fluorophores, even when the solvent (MeOH) was not degassed.

The fluorescent nonnatural amino acids, acdAla and badAla (**4**) as a FRET pair, were introduced into a peptide by conventional SPPS. Without doing any HPLC purification, we could observe a single peak in the HPLC measurement, and the corresponding m/z value was found in MALDI-TOF-MS. This suggests that side-reaction seldom occurred and these amino acids are applicable to conventional SPPS. The sequence of synthesized peptide was Glu–badAla–**Asp**–**Glu**–**Val**–**Asp**–acdAla–Glu, which contains the caspase-3 cleavage site (**bold**) to detect the enzymatic activity. Caspase-3 is a key enzyme secreted during apoptosis, and measurement of caspase-3 activity would provide valuable information about the apoptotic mechanism. The two Glu units increased the solubility of the peptide and their electric repulsion prevented aggregation of the fluorophores. Without adding Glu units at both ends, the fluorophores easily aggregated. The synthesized peptide showed yellow fluorescence of the acceptor (badAla) when the donor (acdAla) was excited, because intramolecular FRET occurred from acdAla to badAla. The FRET quenching efficiency was 80%. In contrast, a mixture that contained an equimolar amount of the monomeric donor (acdAla) and the acceptor (badAla) showed acdAla fluorescence when the former was excited.[5]

When the peptide was incubated with caspase-3, the cleavage site could be cleaved, resulting in the separation of the donor–acceptor distances, leading to the recovery of the donor fluorescence. The proteolysis-induced fluorescence recovery is shown in Figure 2. With an increased incubation time, the emission peak of the donor around 450 nm greatly recovered.

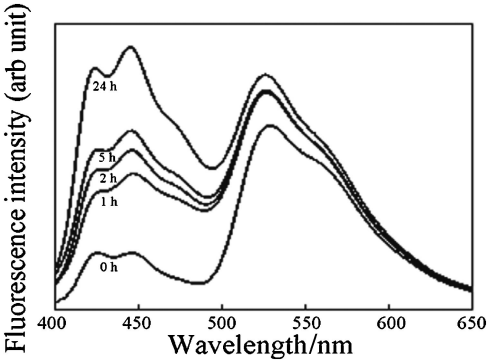


Figure 2. Continuous fluorometric assay of caspase-3 (0.3 μg, Sigma) with Glu–badAla–Asp–Glu–Val–Asp–acdAla–Glu (0.2 μM) in a buffer (50 mM HEPES (pH 7.4), 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, and 1 mM EDTA) at 25 °C. Around 450 nm, from bottom to top, fluorescence spectra were recorded at $t = 0$, 1, 3, 5, and 24 h after addition of the protease ($\lambda_{ex} = 360$ nm).

Along with this moment, we also observed slight enhancement of the acceptor fluorescence around 530 nm. We assume that quenching occurred to some extent in the double-labeled peptide concomitantly with FRET prior to the caspase-3 cleavage.[2,7]

In conclusion, the new nonnatural amino acid (badAla (**4**)) was useful as a FRET acceptor for protease cleavage detection. To the best of our knowledge, it is the smallest fluorescent amino acid which can be excitable by widely applicable lasers at 488 nm and applicable to SPPS without any protection/deprotection. We also found that **4** shows moderately high fluorescence quantum yield, long lifetime, and is durable to prolonged photoirradiation. The acridone-containing amino acids,[8] when introduced into peptides and proteins as the FRET pair, will be a useful probe for monitoring protease cleavage under currently available laser equipments such as fluorescence imagers or confocal microscopes.

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References and Notes

1 H. Y. Sun, R. C. Panicker, S. Q. Yao, *Biopolymers* **2007**, *88*, 141.
2 T. Berthelot, J.-C. Talbot, G. Laïn, G. Déleris, L. Latxague, *J. Pept. Sci.* **2005**, *11*, 153.
3 A. Szymańska, K. Wegner, L. Łankiewicz, *Helv. Chim. Acta* **2003**, *86*, 3326.
4 H. Hamada, N. Kameshima, A. Szymańska, K. Wegner, L. Łankiewicz, H. Shinohara, M. Taki, M. Sisido, *Bioorg. Med. Chem.* **2005**, *13*, 3379.
5 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.
6 M. Taki, Y. Suzuki, Y. Yamazaki, M. Sisido, in *Peptide Science 2006*, ed. by H. Ishida, H. Mihara, The Japanese Peptide Society, **2006**, p. 12.
7 S. Mizukami, K. Kikuchi, T. Higuchi, Y. Urano, T. Mashima, T. Tsuruo, T. Nagano, *FEBS Lett.* **1999**, *453*, 356.
8 Boc- or Fmoc-protected fluorescent amino acids (acdAla and badAla) are currently available from Watanabe Chemical Industries.

