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Design and Synthesis of Rhodamine 110 Derivative and Caspase-3 Substrate for Enzyme and Cell-Based Fluorescent Assay

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Abstract—*N*-Octyloxycarbonyl-R110 (**1**), with enhanced cell penetration and retention properties, was prepared from rhodamine 110. The tetrapeptide substrate *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) was prepared and shown to be efficiently cleaved by human recombinant caspase-3 and by apoptotic HL-60 cells. This substrate should prove useful in cell-based assays for apoptosis inducers and inhibitors.

Organisms eliminate unwanted cells by a process known as programmed cell death or apoptosis.[1,2] Apoptosis occurs as a normal aspect of animal development as well as in tissue homeostasis and aging. However, improperly regulated apoptosis can lead to many pathological conditions. Excessive apoptosis could lead to organ failure, including myocardial infarction, congestive heart failure, liver failure, ischemic stroke, and neurodegenerative diseases.[3] Inadequate apoptosis, on the other hand, can lead to other diseases such as cancer.[4] Apoptosis is mediated by a group of cysteine aspartyl proteases known as caspases.[5] Among these caspases, caspase-3 has been found to be the key member that cleaves many protein substrates in cells and leads to apoptosis.

We have been interested in the development of fluorogenic caspase substrates for cell-based high throughput screening of apoptosis inhibitors or inducers.[6] Ideally, a fluorogenic enzyme substrate used for this purpose should be cell permeable, should accumulate within the cell, and should be nonfluorescent and colorless before cleavage and highly fluorescent after cleavage. In addition, the fluorescent dye should be retained inside the cell after cleavage, and it should fluoresce at wavelengths >500 nm to avoid fluorescent signal from the screening compound. Due to their short emission wavelengths (< 500 nm), coumarin-based fluorogenic caspase-3 enzyme substrates, such as Ac-DEVD-AFC and Z-DEVD-AMC,[7] are not suitable for high throughput screening.

Traditional rhodamine 110 (R110) based protease substrates are bis-peptide-R110 derivatives,[8] such as (Z-Ala-Ala)$_2$-R110. Because these molecules contain two peptide groups, they are relatively large, which limits their ability to penetrate cells. The problem of cell penetration is even worse for caspase substrates containing one or more carboxylic acid groups since these charged groups further impede passage of the substrate across the cell membrane. In addition, the requirement that both peptide groups must be cleaved from the bis-substrates in order to generate a maximal signal limits the linear dynamic range of these substrates, as shown in the recently prepared (Z-DEVD)$_2$-R110 caspase-3 substrate.[9]

Since the mono-peptide R110 is fluorescent and it is not necessary to have two peptides to make a substrate, we took the approach of using one of the amino groups in R110 to introduce a blocking group as an *enhancer moiety*. The blocking group was designed to increase membrane penetration, resulting in the accumulation of the substrate inside the cell, and increased retention of the fluorescent moiety inside the cell after substrate cleavage. The other amino group was then used for the preparation of substrate. Herein we report the synthesis and characterization of *N*-octyloxycarbonyl-R110 (**1**) as a new R110 derived dye and *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) as a new caspase-3 substrate.

N-Octyloxycarbonyl-R110 (**1**) was prepared by reaction of R110 with octyl chloroformate (Scheme 1).[10] As expected, compound **1** was less fluorescent than R110 (relative fluorescence (Rf) = 0.16 versus R110 in 1:1

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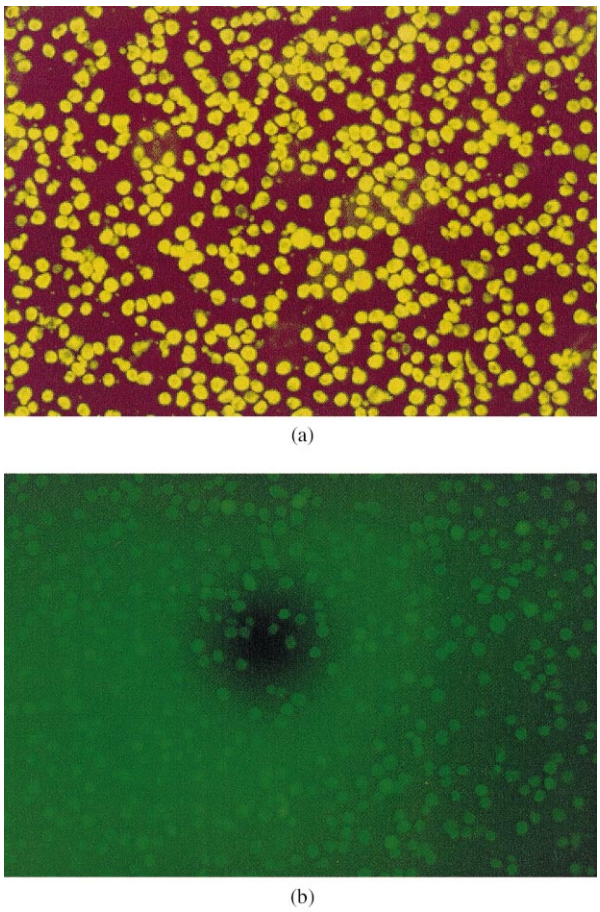


Figure 1. Staining of HL-60 cells by (a) *N*-octyloxycarbonyl-R110 (**1**); and (b) R110. HL-60 cells were placed in 5 mL of Iscove's medium (without serum or phenol-red) containing 10 μM *N*-octyloxycarbonyl-rhodamine 110 (**1**) or rhodamine 110. The cells were incubated for 2 h at 37 °C in a CO_2 incubator, recovered by centrifugation, and washed in 50 mL of ice-cold medium. The cells were re-centrifuged and the final pellet was resuspended in 50 μL of fresh medium. Aliquots of each cell suspension were placed in microslides and viewed on a Nikon inverted microscope with epifluorescent illumination using an FITC filter set.

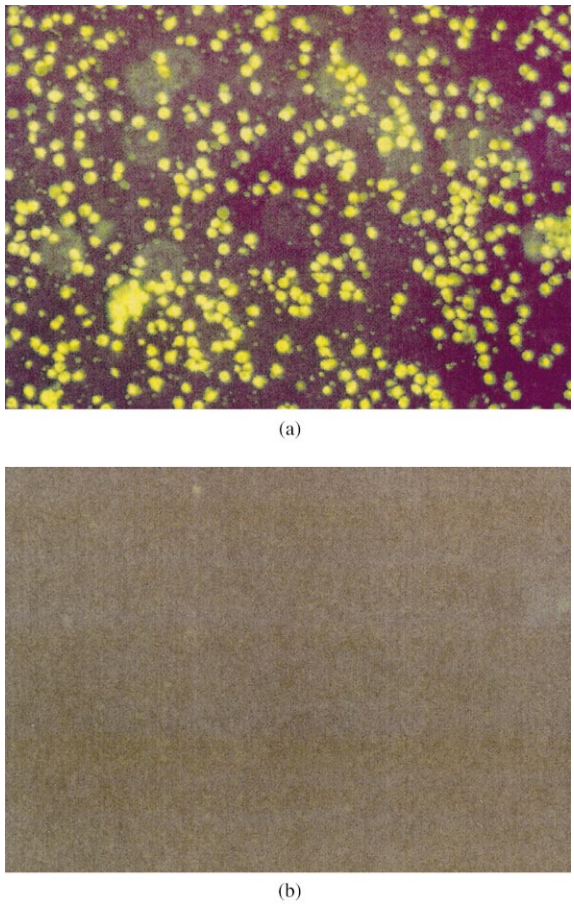


Figure 3. Staining of HL-60 cells by *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**): (a) Vinblastine-treated cells: and (b) Vinblastine-treated cells with 50 μM *N*-Ac-DEVD-CHO added in the assay stage. HL-60 cells were treated with 10 μg/mL vinblastine for 4 h to induce apoptosis. Following apoptosis induction, the cells were incubated with 50 μM *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) in caspase assay buffer (40 mM PIPES, pH 7.2; 100 mM NaCl; 10% sucrose; 1 mM EDTA; 10 mM DTT). The cells were then transferred to a glass microslide and viewed by epifluorescent illumination on a Nikon inverted microscope.

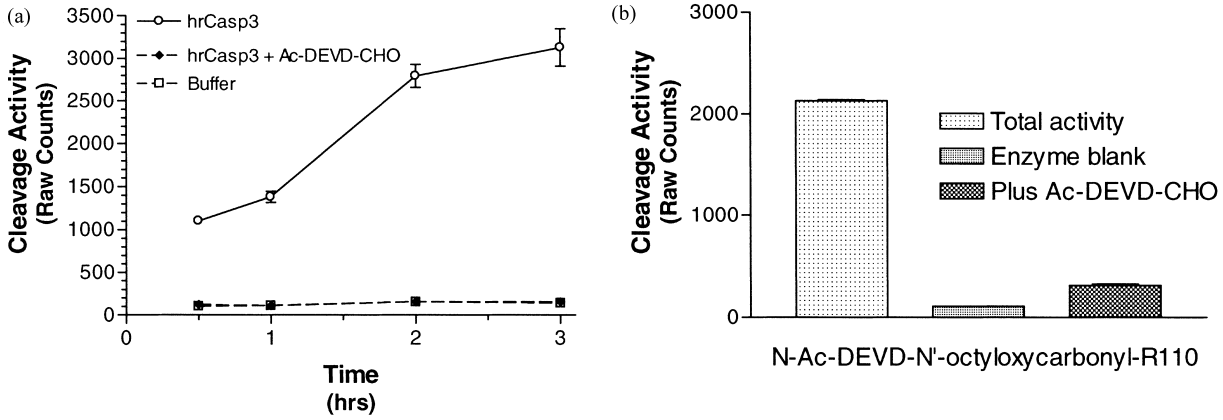
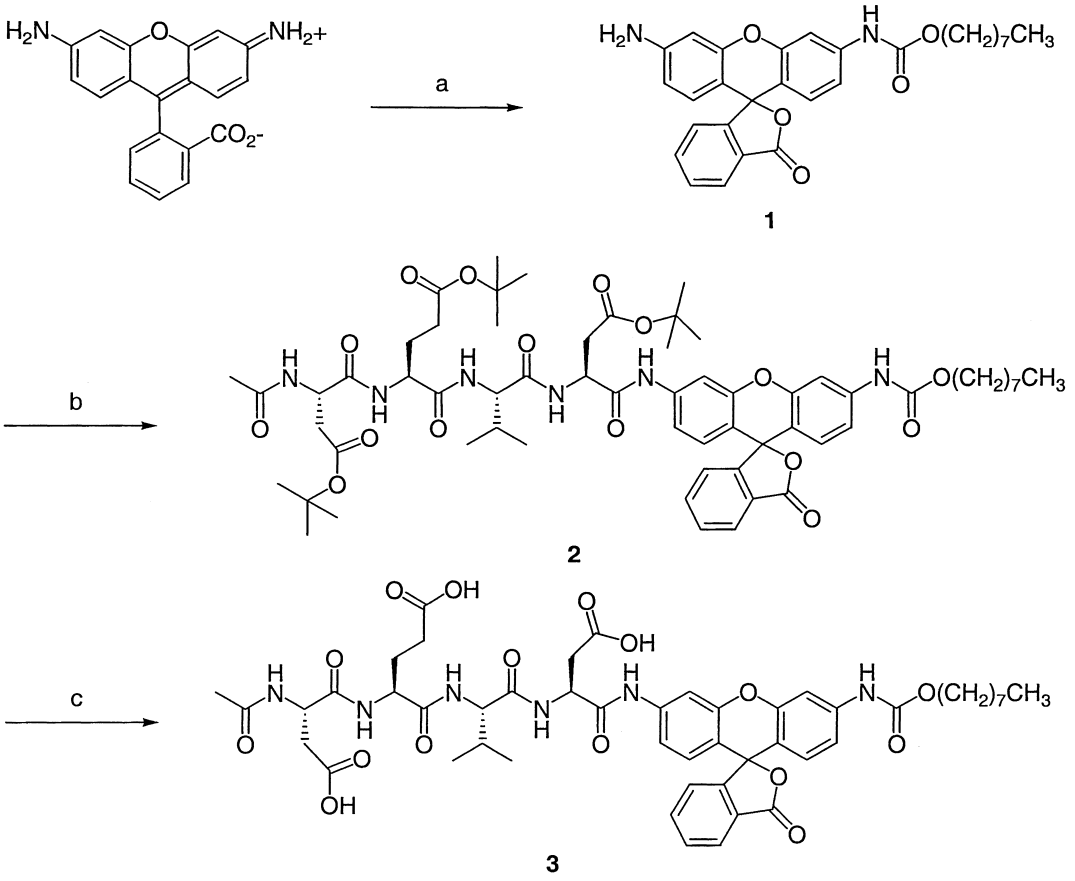


Figure 2. Cleavage of *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) by (a) hrCasp3; and (b) apoptotic lysate from vinblastine-treated HL-60 cells. The assays were carried out at 37 °C in 96-well plates in a 100 μL incubation containing 30 μL of hrCasp3 or cell lysate, 10 μM of substrate **3**, and caspase assay buffer (40 mM PIPES, pH 7.2; 100 mM NaCl; 10% sucrose; 0.1% CHAPS; 1 mM EDTA; 10 mM DTT). At the end of the incubation period (0.5, 1, 2, and 3 h for hrCasp3 and 3 h for apoptotic lysates), the fluorescence was measured on a Bio-Tek FL500 fluorescence microplate reader using excitation and emission wavelengths of 485 and 530 nm, respectively. Two different controls were run: (1) an enzyme blank consisting of samples containing substrate, without enzyme or cell lysate; and (2) an inhibitor control, consisting of samples which contained the caspase inhibitor, Ac-DEVD-CHO (10 μM). The controls gave essentially equivalent values in the hrCasp3 assay.



Scheme 1. Synthesis of *N*-octyloxycarbonyl-R110 (**1**) and *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**). (a) $CH_3(CH_2)_7OCOCl/(i\text{-}Pr)_2NEt/DMF$; (b) Ac-Asp(OBu-*t*)-Glu(OBu-*t*)-Val-Asp(OBu-*t*)/EDC/pyridine/DMF; (c) TFA/CH_2Cl_2.

methanol/1 mM Tris buffer). However, cells incubated with *N*-octyloxycarbonyl-R110 (**1**) were highly fluorescent (Fig. 1a). In contrast, cells incubated with R110 were not very fluorescent and there was a high degree of fluorescence in the medium (Fig. 1b). This suggests that compound **1** is more cell-permeable than R110 and is retained better by the cells.

The caspase-3 substrate *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**)[10] was prepared by coupling the *t*-Bu protected tetrapeptide Ac-DEVD-CO_2H with **1** to give **2**,[10] followed by deprotection of the *t*-Bu groups (Scheme 1). As expected, the reaction converted the fluorescent molecule of **1** into a nonfluorescent molecule **3**.

The cleavage of the caspase-3 substrate *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) was first tested using human recombinant caspase-3 (hrCasp3). Incubation of **3** with hrCasp3 gave a strong fluorescent signal, while incubation of **3** with buffer alone gave a very low background signal. The cleavage of **3** by hrCasp3 was inhibited by the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 2a). Substrate **3** also was cleaved by lysates prepared from HL-60 cells treated with the apoptosis-inducing mitotic inhibitor, vinblastine. Treatment of **3** with these lysates gave a strong fluorescent signal similar to hrCasp3 and this cleavage was inhibited by Ac-DEVD-CHO, suggesting that most of the cleavage was mediated by caspase-3-like proteases (Fig. 2b).

Incubation of vinblastine-treated, apoptotic cells with *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**) resulted in cells which were highly fluorescent (Fig. 3a). In the presence of Ac-DEVD-CHO, the cells showed almost no fluorescent signal (Fig. 3b), indicating that the cleavage of **3** in vinblastine treated cells was due to caspase-3-like proteases.

In conclusion, we have prepared a new R110-derived fluorescent dye, *N*-octyloxycarbonyl-R110 (**1**), which is more cell-permeable and has better cell retention properties than R110. We also have used **1** to prepare a novel, fluorogenic caspase-3 substrate, *N*-Ac-DEVD-*N*′-octyloxycarbonyl-R110 (**3**). Substrate **3** is an excellent substrate for hrCasp3 and it is efficiently cleaved by lysates prepared from apoptotic cells. In addition, substrate **3** strongly stains vinblastine-treated, apoptotic HL-60 cells. Substrate **3** should be useful for detecting apoptosis in whole cells and for cell-based high throughput screening of apoptosis inhibitors or inducers.

References and Notes

1. Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. *Annu. Rev. Biochem.* **1999**, *68*, 383.
2. Takahashi, A. *Int. J. Hematol.* **1999**, *70*, 226.
3. Wellington, C. L.; Hayden, M. R. *Clin. Genet.* **2000**, *57*, 1.

4. Lowe, S. W.; Lin, A. W. *Carcinogenesis* **2000**, *21*, 485.
5. Thornberry, N. A. *Chem. Biol.* **1998**, *5*, R97.
6. Weber, E.; Cai, S. X.; Keana, J. F. W.; Drewe, J. A.; Zhang, H.-Z. WO9918856, 1999; *Chem. Abstr.* **1999**, *130*, 308784.
7. Stennicke, H. R.; Salvesen, G. S. *J. Biol. Chem.* **1997**, *272*, 25719.
8. Leytus, S. P.; Patterson, W. L.; Mangel, W. F. *Biochem. J.* **1983**, *215*, 253.
9. Liu, J.; Bhalgat, M.; Zhang, C.; Diwu, Z.; Hoyland, B.; Klaubert, D. H. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3231.
10. The structure of compounds **1**–**3** was confirmed by NMR spectra, MS or elemental analysis. Compound **1**: ^{1}H NMR ($CDCl_3$) δ 7.99 (d, J = 7.2 Hz, 1H), 7.65–7.56 (m, 2H), 7.52 (bs, 1H), 7.15 (d, J = 7.5 Hz, 1H), 6.88–6.32 (m, 6H), 4.17 (t, J = 6.6 Hz, 2H), 3.9 (2H), 1.68 (m, 2H), 1.42–1.26 (m, 8H), 0.89 (t, J = 6.3 Hz, 3H). Anal. calcd for $C_{29}H_{30}N_2O_5$: C, 71.07; H, 6.53; N, 5.44. Found: C, 71.59; H, 6.21; N, 5.76. Compound **2**: ^{1}H NMR ($CDCl_3$) δ 9.01–8.53 (m, 3H), 8.07–7.99 (m, 1H), 7.77–6.78 (m, 9H), 6.72 (s, 1H), 6.69 (s, 1H), 4.93 (m, 1H), 4.76–4.64 (m, 1H), 4.39 (m, 1H), 4.16 (m, 2H), 4.06 (m, 1H), 3.08–2.02 (m, 9H), 2.10 (s, 3H), 1.47–1.29 (m, 39H), 1.05–0.96 (m, 6H), 0.88 (t, J = 5.7 Hz, 3H). Anal. calcd for $C_{61}H_{82}N_6O_{16}$: C, 62.92; H, 7.18; N, 7.22. Found: C, 62.96; H, 7.04; N, 7.63. Compound **3**: ^{1}H NMR (CD_3OD) δ 8.02–7.04 (m, 8H), 6.79–6.64 (m, 2H), 4.67 (m, 1H), 4.40 (m, 1H), 4.15 (t, J = 6.6 Hz, 2H), 4.15–3.94 (m, 2H), 3.10–2.10 (m, 6H), 2.01–1.94 (m, 3H), 1.69 (m, 2H), 1.29 (bs, 10H), 1.05–0.86 (m, 9H). MS, calcd for $C_{49}H_{58}N_6O_{16}$: 987. Found: 1009 ($M + Na^+$), 988 ($M + H^+$), 986 ($M - H^+$).