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FLUORESCENT MOLECULAR PROBES V: A SENSITIVE CASPASE-3 SUBSTRATE FOR FLUOROMETRIC ASSAYS

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Abstract: (Z-Asp-Glu-Val-Asp)$_2$-Rhodamine 110 [(Z-DEVD)$_2$-Rh 110] was prepared and characterized as a sensitive fluorogenic substrate for the determination of caspase-3 activity.

Programmed cell death, the so-called apoptosis, has recently received intensive attention. It is a normal physiological process that occurs during embryonic development as well as in maintenance of tissue homeostasis. However, improperly regulated apoptosis can contribute to several pathological conditions including cancer, Alzheimer's disease, spinal muscular atrophy, ischemic cardiac damage, and autoimmue syndromes.[1,2] One of the earliest and most consistently observed features of apoptosis is the induction of a series of cytosolic proteases, the caspases, which cleave protein substrates and lead to apoptotic morphology.[1-4] Caspase-3, also called appopain or CPP32, has been identified to be a key member of this caspase family of proteases.[2] It has been suggested that activation of the ICE-family proteases and caspase-3 activity are required for several phenotypes associated with apoptosis in mammalian cells.[3,4]

Currently two coumarin-based fluorogenic substrates, Ac-DEVD-AFC and Z-DEVD-AMC, are predominantly used to determine caspase-3 activity.[1-4] However, the low extinction coefficients and short excitation and emission wavelengths of the enzymatic products released from the two coumarin substrates seriously limit the assay sensitivity. Additionally, the residual fluorescence of the two existing substrates and poor wavelength separation of their enzymatic products from the cellular autofluorescence also tend to give high background in microscopic assays.

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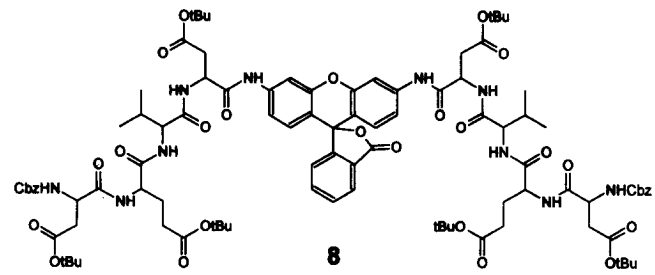

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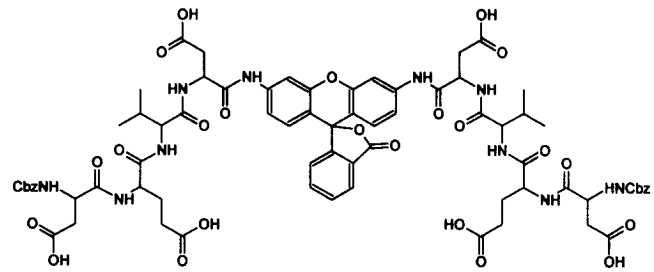
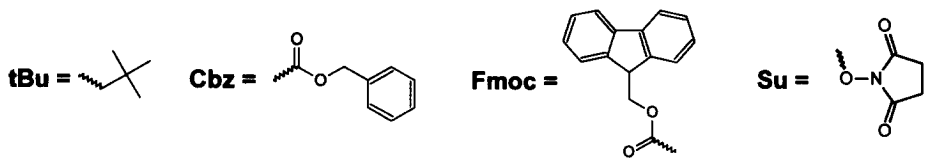
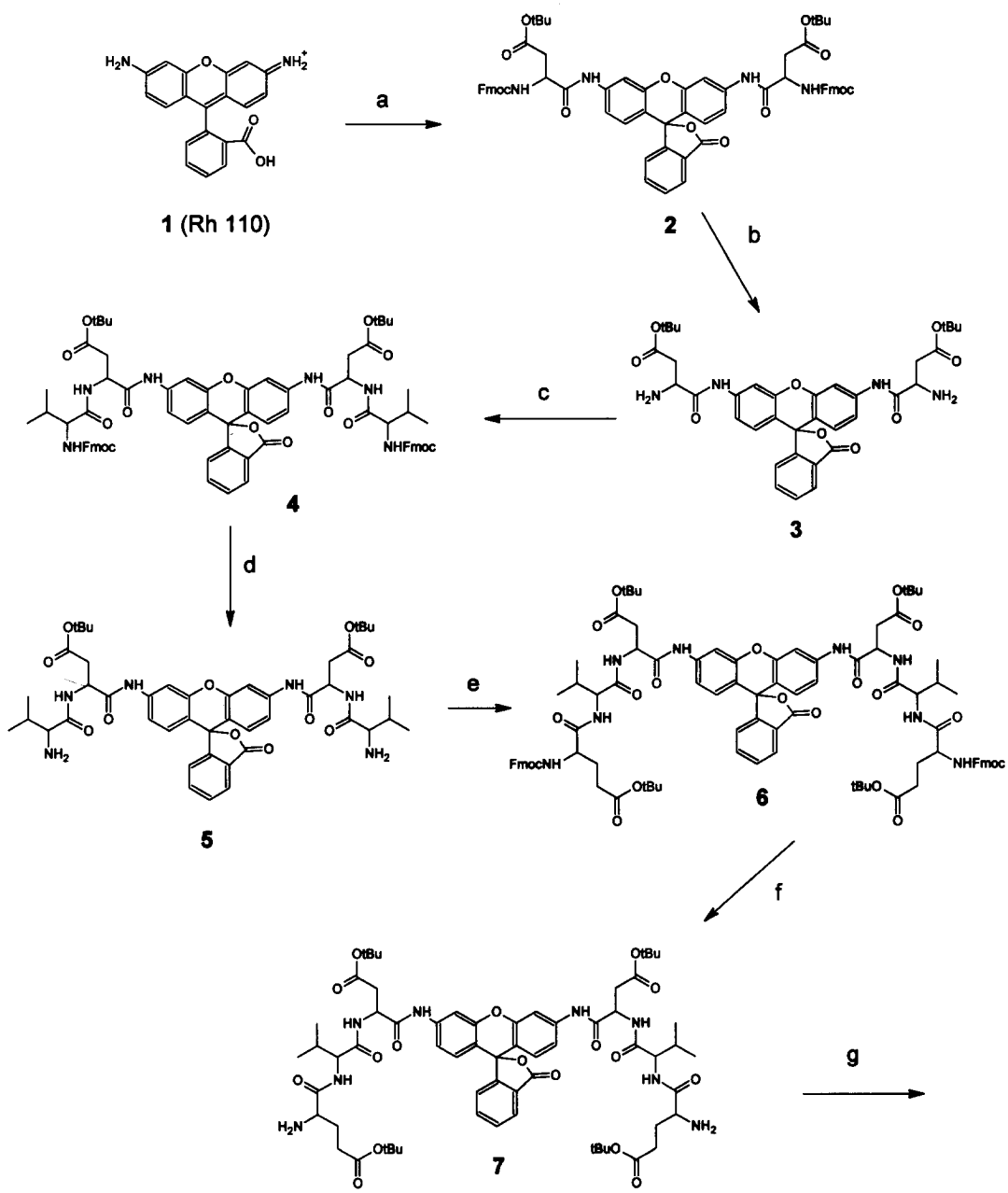


9 (Z-DEVD)$_2$-Rh 110



Scheme 1. Synthesis of (Z-DEVD)$_2$-Rh 110.

(a). N-Fmoc-Asp(OtBu)/1-(3-dimethylamino)propyl-3-ethylcarbodiimide hydrochloride (EDC)/pyridine/DMF, yield = 86%; (b). piperidine/chloroform, yield = 93%; (c). N-Fmoc-Val/EDC/pyridine/DMF, yield = 81%; (d). piperidine/chloroform, yield = 92%; (e). N-Fmoc-Glu(OtBu)/EDC/pyridine/DMF, yield = 83%; (f). piperidine/chloroform, yield = 92%; (g). N-Cbz-Asp(OSu)/chloroform, yield = 80%; (h). trifluoroacetic acid/anisole/chloroform, yield = 45%.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$(Z\text{-DEVD})_2$-Rh 110 (rhodamine 110) was designed to overcome the limitations of the existing coumarin-based caspase substrates, as mentioned above. The substrate was readily prepared as shown in Scheme 1.[5] Initially we prepared tetrapeptide Asp-Val-Glu-Asp, and attempted to couple the peptide with Rh 110 in a single step to give the desired substrates. However, we had difficulties in the coupling of the tetrapeptide with Rh 110 although a variety of conditions were tried. Even coupling of the dipeptide Asp-Val with Rh 110 was a low-yielding reaction. In our hands, it appeared that sequential coupling is the most effective method to prepare the substrate that has been commercialized recently. In general, we found that Rh 110 is much less reactive with bulky acids (such as peptides) compared with smaller acids (such as single amino acids).

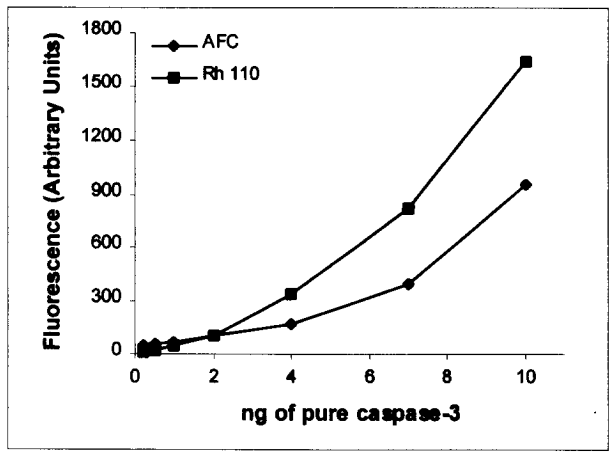

Figure 1. Detection limit of caspase-3 using different DEVD substrates. For the AFC substrate, 44 μL of 25X reaction buffer (250 mM PIPES, pH 7.4, 50 mM EDTA, 2.5% CHAPS, 125 mM DTT) was diluted with 934 μL water. To the reaction solution was added 22.4 μL of a 4.9 mM Z-DEVD-AFC stock solution; for the Rh 110 substrate, 1.79 μL of 12.3 mM $(Z\text{-DEVD})_2$-Rh 110 stock solution was added to the reaction solution. To the AFC and Rh 110 substrate solutions, various dilutions of the enzyme were added, and incubated at room temperature. The fluorescence data were recorded on a Perkin-Elmer HTS-7000 plate reader with settings: for the AFC assay, Ex = 360 nm, Em = 535 nm, Gain = 40; For the Rh 110 assay, Ex = 485 nm, Em = 535 nm, Gain = 35.

$(Z\text{-DEVD})_2$-Rh 110 was compared with Z-DEVD-AFC, a fluorogenic caspase-3 substrate that is currently used in a variety of caspase-3 assays. As shown in Figs. 1 and 2, $(Z\text{-DEVD})_2$-Rh 110 exhibits much higher turnover rate and sensitivity than the AFC substrate. Under our assay conditions, $(Z\text{-DEVD})_2$-Rh 110 is at least 10-fold more sensitive than Z-DEVD-AFC. It can detect less than 1 ng caspase-3/mL.[6] However, the enzymatic hydrolysis of $(Z\text{-DEVD})_2$-Rh 110 is a two-step process while the hydrolysis of Z-DEVD-AFC is a single step reaction. The intermediate product, Z-DEVD-Rh 110, is less fluorescent than the final product (Rh

110). The two-step hydrolysis limits the linear dynamic range of $(Z\text{-DEVD})_2$-Rh 110 substrate as seen in Figure 1. We are in the process of developing a new Rh 110-based substrate that contains only one protease-hydrolyzable amide group.

Table 1. Spectral properties of Z-DEVD-AFC, $(Z\text{-DEVD})_2$-Rh 110 and their enzymatic products*

	λ_{abs} (nm)	$\varepsilon \times 10^{-2}$ ($cm^{-1}M^{-1}$)	λ_F (nm)	Φ_F
Z-DEVD-AFC	336	139	440	0.44
AFC	376	182	499	0.54
$(Z\text{-DEVD})_2$-Rh 110	234	551	N/A	Not detected
Rh 110	499	912	519	0.92

*The fluorescence quantum yields were determined in PBS buffer (pH 7.2) as described in reference [7]. For the AFC compounds, quinine sulfate was used a reference standard while fluorescein was used as a reference standard for Rh 110.

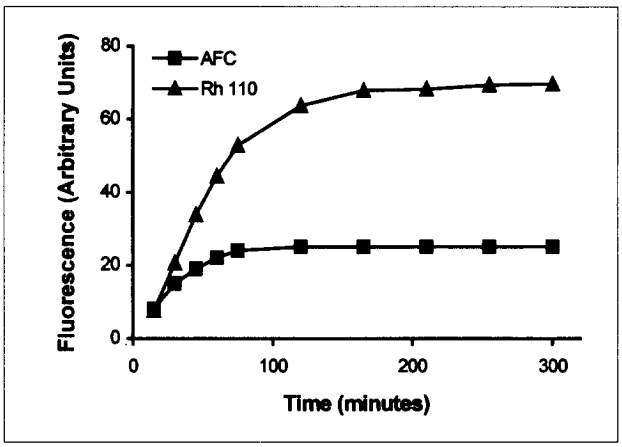

Figure 2. Turnover of $(Z\text{-DEVD})_2$-Rh 110 (6.25 μM) and Z-DEVD-AFC (25 μM). The enzymatic reactions were run as described in Figure 1, and followed by measuring the fluorescence changes in a CytoFluor II microplate reader using the settings in Figure 1.

As shown in Table 1, Rh 110, the caspase-cleaved product of $(Z\text{-DEVD})_2$-Rh 110, has maximum absorption at 499 nm that matches very well with 488 nm line of the argon-ion laser. The argon-ion laser is the most popular excitation light source for flow cytometers, fluorescence microscopes and other fluorescence

equipment, such as various microplate readers. The enzymatic product of $(Z\text{-DEVD})_2$-Rh 110 has a much higher extinction coefficient, fluorescence quantum yield and photostability compared with the product of Z–DEVD-AFC. Additionally, $(Z\text{-DEVD})_2$-Rh 110 is nonfluorescent, and generates highly fluorescent Rh 110 product upon cleavage of the DEVD blocking groups of $(Z\text{-DEVD})_2$-Rh 110 by the caspase, while Z-DEVD-AFC itself still has strong residual fluorescence. In summary, these characteristics make $(Z\text{-DEVD})_2$-Rh 110 a convenient and sensitive substrate for caspase-3. We have successfully used $(Z\text{-DEVD})_2$-Rh 110 in flow cytometric analysis of apoptosis (data not shown). The flow cytometry analysis indicated that $(Z\text{-DEVD})_2$-Rh 110 is at least 10 times more sensitive than Z-DEVD-AFC, which is consistent with our microplate assays, as described above.[8]

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References and Notes

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4. Stennicke, H. R.; Salvesen, G. S. *J. Biol. Chem.* **1997,** *272*, 25719.
5. $(Z\text{-DEVD})_2$-Rh 110 was purified by HPLC using the following conditions: Prep LC2000 (Waters Corporation, Milford, MA); Prodigy Prep C18 reverse phase column (Phenomenex Corporation, Torrance, CA); linear gradient from 14% to 28% acetonitrile in 25 mM NH_4OAc (pH = 7); 65 mL/min flow rate. The structure was confirmed to elemental analysis, mass and NMR spectra.
6 Purified active recombinant human caspase-3 (Pharmingen, San Diego, CA) was used in the microplate assays.
7. Diwu, Z.; Lu, Y.; Zhang, C.; Klaubert, D. H. *Photochem. Photobiol.* **1997,** *66*, 424.
8. Jurkat cells (American Type Culture Collection Co., Rockville, MD) were used in our flow cytometric assays. 10 μM camptothecin was used to induce apoptosis.