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N-DEVD-N′-morpholinecarbonyl-rhodamine 110: novel caspase-3 fluorogenic substrates for cell-based apoptosis assay

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Abstract—A novel caspase-3 substrate N-Ac-DEVD-N′-MC-R110, which is a fluorogenic substrate cleavable in a single step, has been prepared. It has a significantly higher enzyme turnover rate and sensitivity for detecting caspase-3 activity both in solution and living cells than existing fluorogenic substrates.

Apoptosis or programmed cell death is a normal physiological process that occurs during embryonic development, as well as in the maintenance of tissue homeostasis. Apoptosis plays a critical role in many diseases including cancer, AIDS, stroke, and many neurodegenerative disorders.[1,2] The activation of caspases is a hallmark of apoptosis. Caspase-3 has been identified to be a key member of the caspase family and is characterized as an effector or executioner caspase. During apoptosis, caspase-3 cleaves protein substrates inside the cell, and results in the formation of characteristic morphology of apoptotic cells.[3,4] In most of the caspase-3 substrates, the peptide recognition sequence is DEVD (the single-letter code for amino acids is used), and cleavage occurs after the second D.

Fluorometric methods based on the peptide substrates labeled with cleavable fluorophores have been widely used for assaying various proteases, including caspases, which are key enzymes for apoptosis. The early caspase fluorogenic substrates such as Ac-DEVD-AFC and Z-DEVD-AMC based on coumarin dye are commonly used for determining caspase-3 activity from cellular lysates.[5] However, their short wavelength, low extinction coefficient and high fluorescent background result in low assay sensitivity and limit their biological application. A sensitive caspase-3 substrate, $(Z\text{-DEVD})_2$-R110, based on a rhodamine dye was developed to overcome the limitations of the coumarin-based caspase substrates.[6] This rhodamine-labeled peptide substrate has a longer excitation and emission wavelength, low background signal, and being highly fluorescent. $(Z\text{-DEVD})_2$-R110, as a caspase-3 substrate, has been widely used in cell-based apoptosis assays for detection of caspase-3 activity. However, both peptide groups need to be cleaved from this bis-peptide substrate in order to generate maximal signal. This two-step cleavage limits its linear dynamic range. Further more, the bis-peptide substrate has poor cell permeability due to the two peptides that contain six carboxylic acid groups.

It is desirable to develop a rhodamine-based substrate with only one caspase-cleavable amide bond. The synthesis of N-Ac-DEVD-N′-octyloxycarbonyl-R110 as a cell-permeable caspase-3 substrate was reported by Cai's group.[7] More recently, N-Ac-DEVD-N′-(polyfluorobenzoyl)-R110 was also reported to be cell-permeable fluorogenic caspase substrates by the same group.[8] The disadvantage of these mono-peptide rhodamine substrates is much less assay sensitivity than $(Z\text{-DEVD})_2$-R110 after caspase cleavage. Here, we report a novel N-Ac-DEVD-N′-morpholinecarbonyl-R110 (N-Ac-DEVD-N′-MC-R110) as a cell-permeable caspase-3 substrate, which shows much higher sensitivity than $(Z\text{-DEVD})_2$-R110 for caspase-3 detection. More important, this novel fluorogenic caspase-3 substrate only requires a single step cleavage.

N-Ac-DEVD-N′-MC-R110 was designed to overcome both the poor cell penetration and the kinetic limitation of $(Z\text{-DEVD})_2$-R110. The new caspase-3 substrates should generate a highly fluorescent product upon a

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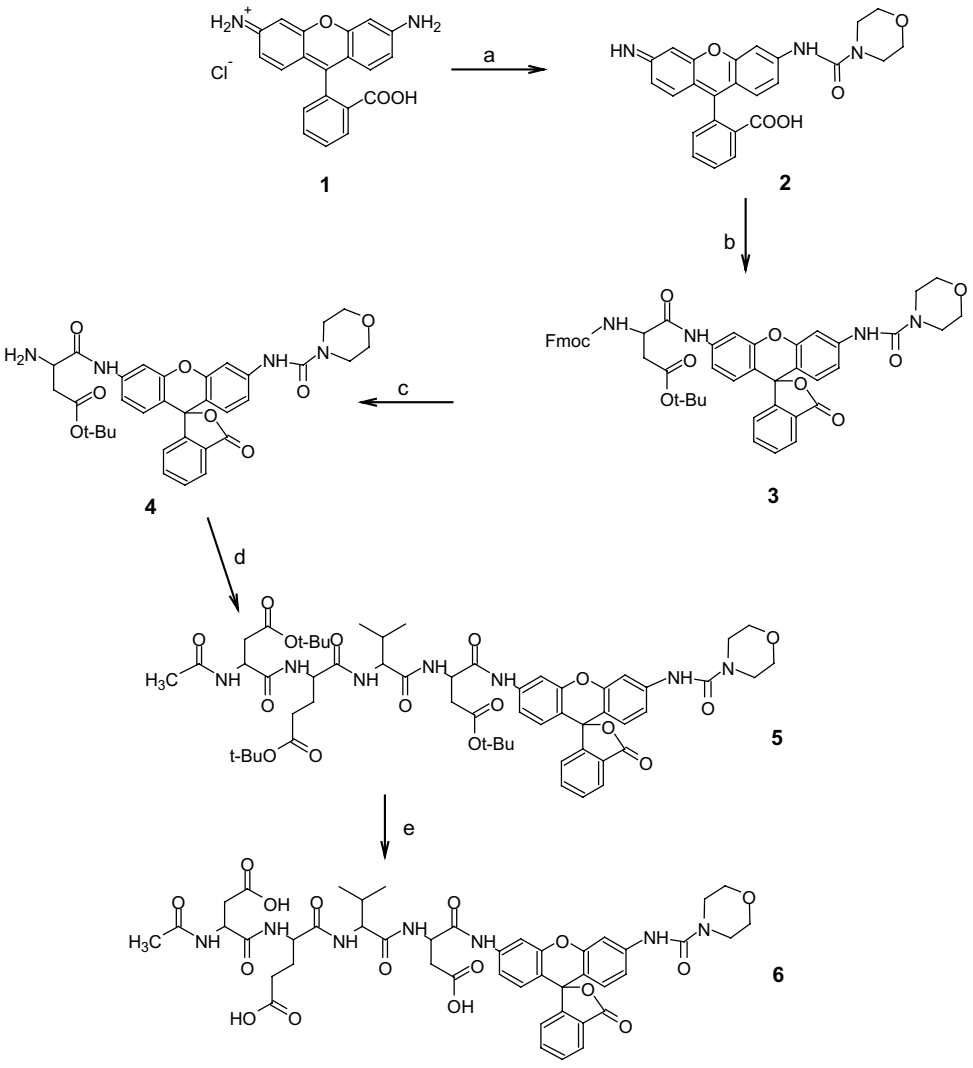
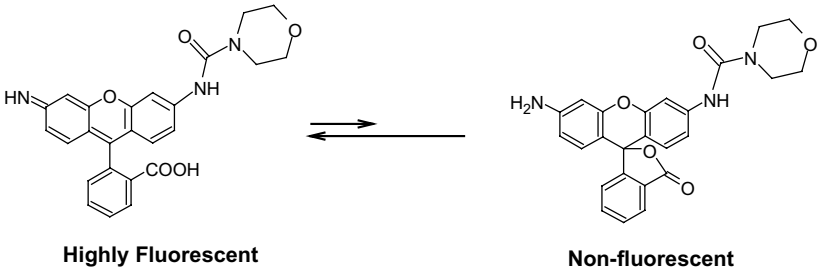
single cleavage by caspase-3. The relative fluorescence of N–R′–R110 (R′ = COOalkyl, COAr, etc.) reported by Cai's group is only approximately 5–16% of rhodamine 110.[7,8] In order to overcome the low fluorescence quantum yield of N–R′–R110, we synthesized and screened a number of blocking groups (N–R′) that are resistant to caspase hydrolysis. Morpholine moiety is identified to be a unique blocking group for developing R110 based caspase substrates. Morpholine moiety has been widely used as the building block in organic synthesis and drug-like compounds.[9,10]

Our synthetic approach is to attach a morpholine moiety to one of the primary amine groups in rhodamine 110. The desired compound, N-morpholinecarbonyl-R110 (N-MC-R110, **2**), was prepared by reaction of rhodamine 110 with morpholinecarbonyl chloride in anhydrous DMF with excellent yield (Scheme 1). Interestingly, N-MC-R110 (**2**) has very bright fluorescence in aqueous solution. The relative fluorescence measurement for N-MC-R110 (**2**) (quantum yield is 0.85) is 90% of that of rhodamine 110 (quantum yield is 0.95) in 1:1 mixture of methanol/1 mM Tris buffer. The relative fluorescence of N-MC-R110 (**2**) is almost 10 times higher than N–R′–Rh110 (R′ = COO alkyl, COAr).[7,8] The high fluorescent quantum yield of N-MC-Rh110 (**2**) suggests that N-MC-Rh110 (**2**) is a good fluorophore for developing fluorogenic caspase substrates.[11] This fluorophore has an equilibrium that favors the formation of a strong fluorescent emission for N-MC-R110 (**2**) as proposed in Scheme 2.

The caspase-3 substrate N-Ac-DEVD-N′-MC-R110 (**6**) was readily prepared by multiple steps as shown in Scheme 1. Initially, we attempted a one-step reaction to couple the tetrapeptide Asp-Val-Glu-Asp with **2** to make compound (**5**). But we only obtained **5** in a low yield. It appeared that sequential coupling that introduces aspartic acid first, then couple the tri-peptide Ac-Asp-Val-Glu gave the desired product (**5**) in good yield. The final TFA de-protection gave the desired substrate N-Ac-DEVD-N′-MC-R110 (**6**). N-Z-DEVD-N′-MC-R110 was also made through this stepwise coupling by using peptide Z-Asp-Val-Glu.



Scheme 1. Synthesis of N-Ac-DEVD-N′-MC-R110 (**6**). Reagents: (a) morpholinecarbonyl chloride/DMF/NEt(*i*-Pr)$_2$, yield 82%; (b) N-Fomc-Asp(O*t*Bu)/EDC/pyridine/DMF, yield 84%; (c) piperdine/chloroform, yield 88%; (d) Ac-Asp(O*t*Bu)-Glu(O*t*Bu)-Val/EDC/pyridine/DMF, yield 82%; (e) TFA/anisole/chloroform, yield 52%.



Scheme 2. Equilibration of N-MC-R110 (**2**).

The novel substrate N-Ac-DEVD-N′-MC-R110 (**6**) is non-fluorescent in buffer solutions. It becomes highly fluorescent due to the formation of N-MC-R110 (**2**) after the caspase-3 cleavage as shown in Figure 1. The dose response of N-Ac-DEVD-N′-MC-R110 (**6**) with various concentrations of purified active human recombinant caspase-3 is shown in Figure 2. This caspase-3 substrate (**6**) can detect as little as 0.6 ng of caspase-3/well/200 μL under our assay conditions.

The comparison of N-Ac-DEVD-N′-MC-R110 (**6**) with $(Z\text{-DEVD})_2$-R110, for the detection of caspase activity in vitro was studied. As shown in Figure 3, N-Ac-DEVD-N′-MC-R110 (**6**) is superior to $(Z\text{-DEVD})_2$-R110 for the detection of caspase-3. The enzymatic activity and sensitivity of this caspase-3 substrate (**6**) are much higher than $(Z\text{-DEVD})_2$-R110 in vitro. The higher detection sensitivity of substrate (**6**) than $(Z\text{-DEVD})_2$-R110 could be due to the fast formation of the highly fluorescent cleavage product N-MC-R110 (**2**) through a single-step enzymatic hydrolysis.

N-Ac-DEVD-N′-MC-R110 (**6**) was also compared with $(Z\text{-DEVD})_2$-R110 for the detection of caspase activity in JurKat cell. As shown in Figure 4, the fluorescence enhancement of N-Ac-DEVD-N′-MC-R110 (**6**) is three

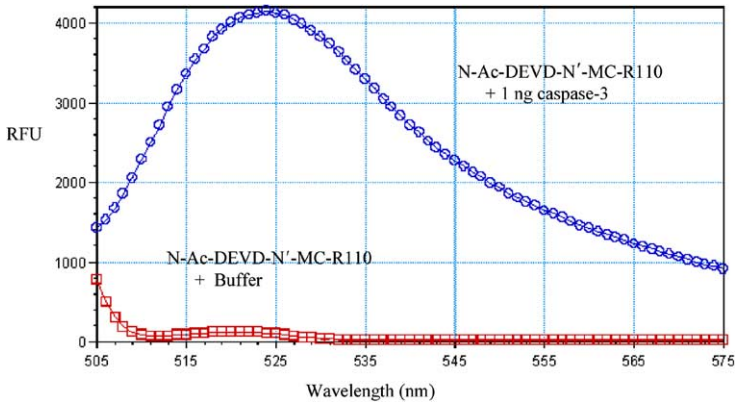


Figure 1. Fluorescence emission spectra of N-Ac-DEVD-N′-MC-R110 (**6**) with or without incubation of recombinant human caspase-3 (excitation at 485 nm).

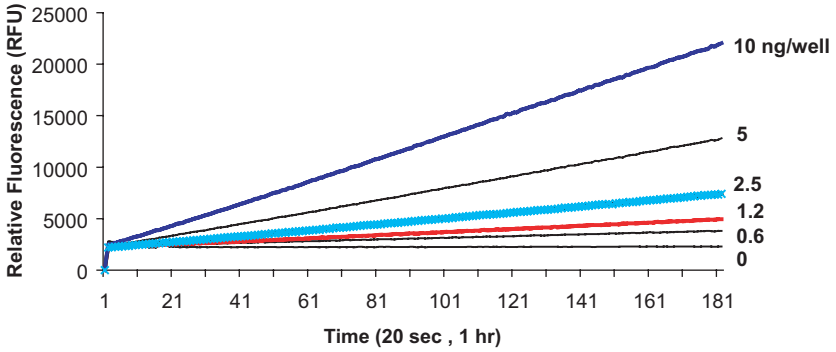


Figure 2. N-Ac-DEVD-N′-MC-R110 (**6**) is incubated with various concentrations of purified active human recombinant caspase-3 (Sigma) at room temperature. The caspase-3 is prepared in PBS buffer containing 0.1% BSA. One hundred microliters of diluted caspase-3 are transferred into a 96-well Costar black wall/clean bottom plate into each well of which an equal volume of 50 μM N-Ac-DEVD-N′-MC-R110 (**6**) in 2× reaction buffer is added. The enzyme kinetics are measured using a FLEXstation™ fluorescence microplate reader using excitation at 485 nm and emission detection at 525 nm for 1 h at 20 s interval.

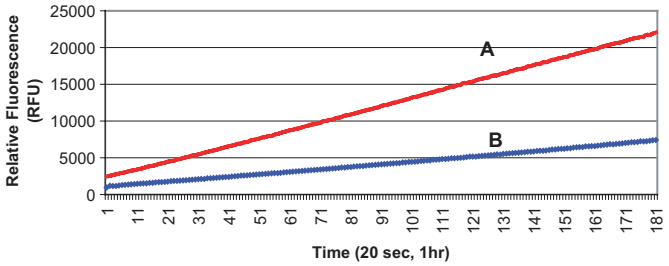


Figure 3. N-Ac-DEVD-N′-MC-R110 (**6**) (A) or $(Z\text{-}DEVD)_2$-R110 (B) is incubated with purified active human recombinant caspase-3 (Sigma) at room temperature. One hundred microliters of purified active human recombinant caspase-3 (10 ng/well, Sigma) in PBS buffer containing 0.1% BSA is treated with an equal volume of 50 μM N-Ac-DEVD-N′-MC-R110 (**6**) or $(Z\text{-}DEVD)_2$-R110 in 2× reaction buffer. The enzymes kinetics are measured using a FLEXstation™ fluorescence microplate reader using excitation at 485 nm and emission detection at 525 nm for 1 h at 20 s interval.

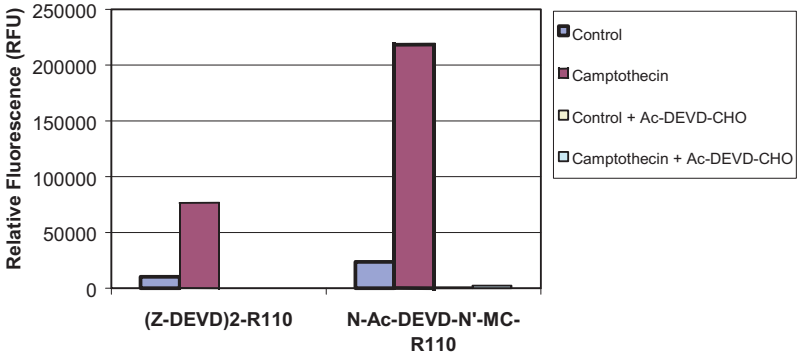


Figure 4. JurKat cells at a concentration of 100,000/well (100 μL) are treated with camptothecin (2 μM) for 6 h at 37 °C in a 5% CO_2 incubator to induce apoptosis, while untreated cells are used as a 'control'. Both the control and apoptotic cells are then incubated with an equal volume of 50 M N-Ac-DEVD-N′-MC-R110 (**6**) or $(Z\text{-}DEVD)_2$-R110 in 2× reaction buffer for 1 h. The incubations are performed either in the presence or absence of Ac-DEVD-CHO, a caspase-3 inhibitor (20 μM). The fluorescence is measured using a FLEXstation™ fluorescence microplate reader using excitation at 485 nm and emission detection at 525 nm.

times more than $(Z\text{-}DEVD)_2$-R110 in camptothecin-induced apoptotic JurKat cells. In the presence of Ac-DEVD-CHO, a caspase-3 inhibitor, the apoptotic JurKat cells show almost no fluorescent signal as in Figure 4. This result demonstrates that the cleavage of substrate (**6**) in camptothecin-induced apoptotic JurKat cells is due to caspase-3 activities.

In summary, we have developed a novel morpholine derived fluorescent dye N-MC-R110 (**2**), which shows fluorescent quantum yield close to rhodamine 110. This novel morpholine-modified dye N-MC-R110 (**2**) was successfully used for developing the ultra sensitive caspase-3 substrates: N-Ac-DEVD-N′-MC-R110 (**6**) and N-Z-DEVD-N′-MC-R110. The novel substrates only require a single step cleavage for detecting caspase-3 and eliminate the complications associated with $(Z\text{-}DEVD)_2$-R110. N-Ac-DEVD-N′-MC-R110 (**6**) has a significantly higher enzyme turnover rate and sensitivity for detecting caspase-3 activity both in solution and living cells compared to commercially available $(Z\text{-}DEVD)_2$-R110. The homogeneous fluorescent assays that are based on caspase-3 substrate (**6**) provide an excellent tool for high throughput screening of caspase-3 inhibitors and modulator(s).

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