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Synthesis of a macrocyclic rhodamine 110 enzyme substrate as an intracellular probe for caspase 3 activity

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Abstract

The synthesis of the macrocyclic rhodamine 110 caspase 3 substrate **8** is described. The key step is a high dilution intramolecular cyclization reaction of an in situ generated primary amine with a 4-nitrophenyl ester. Substrate **8** reacts with recombinant caspase 3 to yield a fluorescent signal but virtually no signal is detected in the absence of caspase 3 or in the presence of the caspase 3 inhibitor Ac-DEVD-CHO. Notably, **8** selectively stains live cells that have been induced to undergo apoptosis with etoposide.

Keywords: cyclization; enzymes and enzyme reactions; fluorescence; macrocycle.

Fluorogenic enzyme substrates offer a convenient and sensitive means to measure enzyme activity. Substrates incorporating the peptide sequence DEVD and a fluorophore such as 7-amino-4-trifluoromethylcoumarin[1] or rhodamine 110[2] (**1**, R110) have been prepared to measure caspase 3 activity in studies of apoptosis. However, these substrates cannot measure caspase 3 activity in intact cells since they are impermeable to the cell membrane. Masking the charge of the free carboxy groups of the glutamate and aspartate residues may improve substrate permeability of the cell membrane. We reasoned that the synthesis of a macrocyclic DEVD substrate may mask these charges since the carboxy groups should be more prone toward intramolecular hydrogen bonding compared to a non-cyclic analog. Therefore, we initiated studies into the synthesis of the macrocyclic DEVD-R110 substrate **8**.

Initially, the mono-protected R110 analog **2** was prepared by the reaction of **1** with 1 equiv. of FMOC chloride and *N*,*N*-diisopropylethylamine (DIEA) in DMF (35–40%, Scheme 1).[3a] Compound **2** was coupled to a suitably protected DEVD peptide. The α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz) group was chosen for protection of the terminal amine[4] while *t*-butyl esters were employed for the protection of the side chain carboxy groups.[5] The coupling of **2** and

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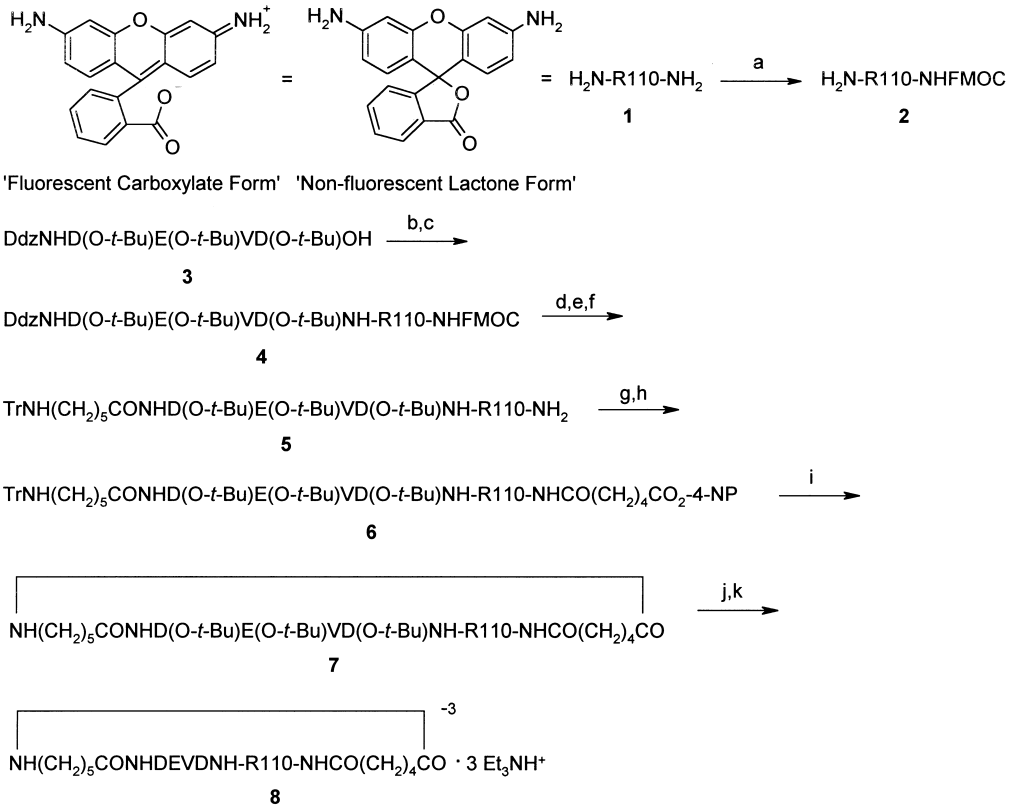
N-Ddz-DEVD tri-*t*-butyl ester (**3**) was accomplished via the mixed anhydride of **3** (isobutyl chloroformate (IBCF), *N*-methylmorpholine (NMM), THF) to give the disubstituted R110 **4** (55–61%).



Scheme 1. Reagents and conditions: (a) FMOC chloride, DIEA, DMF, rt; (b) IBCF, NMM, THF, –10°C; (c) **2**, THF, –10°C to rt; (d) 2.5% TFA in CH_2Cl_2 followed by NMM, rt; (e) mixed anhydride of $TrNH(CH_2)_5COOH$ in THF,[6] rt; (f) 2% piperidine in DMF, rt; (g) adipoyl chloride, collidine, THF, rt; (h) 4-nitrophenol, THF, rt; (i) 5% TFA in CH_2Cl_2 followed by CH_2Cl_2 and DIEA, rt; (j) 25% TFA in CH_2Cl_2, rt; (k) Et_3N, MeOH, rt

A spacer was inserted in two pieces to link the terminal amine of the peptide with the second amino group of the R110 molecule. The first piece (7 atoms) consisted of a 6-aminohexanoic acid residue that was *N*-protected with the triphenylmethyl (Tr) group.[3b] The Ddz protecting group of **4** was removed (2.5% TFA in CH_2Cl_2) and the amine salt was neutralized (NMM). The free amine was treated with the mixed anhydride of *N*-Tr-6-aminohexanoic acid.[6] Removal of the FMOC group (2% piperidine in DMF) provided the mono-substituted R110 **5**. A second spacer piece (6 atoms) was then added to complete the macrocyclic framework. Treatment of the aromatic amine of **5** with excess adipoyl chloride and collidine in THF afforded the non-fluorescent bis-amide (not shown). Reaction of the second acid chloride residue with 4-nitrophenol gave the active 4-nitrophenyl (4-NP) ester **6** (30%).[7]

The key cyclization step was now performed. Removal of the Tr group (5% TFA in CH_2Cl_2) gave the primary amine salt. The solution was diluted to a concentration of 1 mM and neutralized with DIEA. The reaction was stirred for four days at 25°C to give the cyclic triester **7** (37%).

MALDI mass spectral analysis of **7** gives the expected molecular ion [$(M+H)^+ = 1181$].[8] A second charged species [$(M+23)^+$] of near equal intensity is also present suggesting that polyamide **7** is able to coordinate with sodium ions thus lending further validity to the macrocyclic structural assignment. Removal of the *t*-butyl esters was accomplished with 25% TFA in CH_2Cl_2 to give **8**, which was isolated as the tris-Et_3NH^+ salt.[9]

Macrocycle **8** is virtually non-fluorescent in solution since diacyl R110 molecules are constrained to the lactone form. Treatment of **8** with recombinant caspase 3 induces formation of the fluorescent carboxylate form, presumably by cleavage of the DEVD link.[10] Essentially no fluorescence is observed under these conditions when the caspase 3 inhibitor Ac-DEVD-CHO is present.[11] Staining of live NIH3T3 and mouse melanoma D5 cell lines, induced to undergo apoptosis with etoposide, exhibited specific and localized intracellular substrate turnover.[12] Kinetics and specificity studies of such cyclic substrates are underway.

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3. (a) Greene, T. W. *Protective Groups in Organic Synthesis*; John Wiley & Sons: New York, 1981; pp. 225–226. (b) *ibid* pp. 273–274.
4. Schmitt, W.; Zanotti, G.; Wieland, T.; Kessler, H. *J. Am. Chem. Soc.* **1996**, *118*, 4380–4387.
5. Peptide **3** was purchased from BioMol Research Laboratories, Plymouth Meeting, PA, USA.
6. *N*-Tr-6-Aminohexanoic acid was prepared by the reaction of methyl 6-aminohexanoate hydrochloride with Tr-chloride (DMF, DIEA) followed by saponification. The corresponding mixed anhydride was prepared by treating the protected amino acid with IBCF in the presence of NMM in THF at –10°C.
7. The preparation of a cyclic peptide employing a 4-nitrophenyl ester has been reported. Studer, R. O.; Lergier, W. *Helv. Chim. Acta* **1965**, 460–470.
8. The mass spectral analysis was performed by Mass Consortium Corp., San Diego, CA, USA.
9. Compound **8**: ^{1}H NMR (D_2O) δ 0.97–1.80 (m, 6H), 1.21 (t, $J = 7$ Hz, 27H), 1.36–2.78 (m, 28H), 2.97 (q, $J = 7$ Hz, 18H), 4.06 (t, $J = 3$ Hz, 1H), 4.38 (bs, 1H), 4.68–4.80 (m, 1H), 6.64 (d, $J = 9$ Hz, 1H), 6.65 (d, $J = 9$ Hz, 1H), 7.11 (d, $J = 9$ Hz, 1H), 7.23 (d, $J = 8$ Hz, 1H), 7.50–7.83 (m, 4H), 7.88–7.93 (m, 1H), 8.02 (d, $J = 8$ Hz, 1H).
10. Assay conditions: [**8**] = 10 μM; Recombinant caspase 3 was prepared in bacterial cell cultures and crude preparations were diluted to give a measurable fluorescent signal. An 1:50 000 dilution gave about 1300 counts/h over 3 h. An 1:1000 dilution gave about 5000 counts/h over 12 h. Virtually no fluorescence was detected in the absence of recombinant caspase 3.
11. Garcia-Calvo, M.; Peterson, E. P.; Leiting, B.; Ruel, R.; Nicholson, D. W.; Thornberry, N. A. *J. Biol. Chem.* **1998**, *273*, 32608–32613. Experiments performed as described in Ref 10 but with the addition of Ac-DEVD-CHO (10 μM).
12. Assay conditions: NIH3T3 (mouse endothelial) and D5 (mouse melanoma) cell lines (ATCC, Rockville, MD), induced for apoptosis by treatment with etoposide (50 μg/mL in RPMI1640 media+10% CS for 18 h), with [**8**] = 50 μM (for 1 h) showed significant and cell-specific labeling. These same cell lines were also stained with the fluorescent caspase 3 cell permeable inhibitor FITC-VAD-FMK (Promega Corp., Madison, WI) to verify apoptosis induction. Non-induced cell lines showed little or no detectable labeling under identical conditions.