

Proceedings

# A New Fluorogenic Substrate for Granzyme B Based on Fluorescence Resonance Energy Transfer †

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**Abstract:** The synthesis and characterization of a new fluorogenic substrate for Granzyme B (GzmB) is reported. The substrate design was based on the fluorescence resonance energy transfer (FRET) principle using 5-(2'-aminoethyl)aminonaphthalene sulfonic acid (Edans) and 4-[[4'-(*N,N*-dimethylamino)phenyl]diazenyl]benzoic acid (Dabcyl) as donor-acceptor pair, linked to a specific sequence for GzmB (AAD), with an additional amino acid as anchoring point (K). The tetrapeptide was synthesized by microwave-assisted solid phase peptide synthesis (MW-SPPS) and coupled to Dabcyl and Edans at its *N*- and *C-termini*, respectively. The obtained probe was purified by semi-preparative HPLC and characterized by NMR, UV/Vis absorption and fluorescence spectroscopy and mass spectrometry.

**Keywords:** donor-acceptor pair; FRET; granzyme B; fluorescent probes; peptides

## 1. Introduction

The serine protease Granzyme B (GzmB) is a potent inducer of apoptosis in target cells when released by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells, representing one of the two dominant mechanisms by which T cells mediate cancer cell death [1,2]. Considering the GzmB's preference for cleaving after aspartic acid, several substrates containing IEPD and IETD sequences coupled to chromogenic or fluorescent leaving groups have been successfully used for GzmB detection [3–5]. However, these two sequences lack specificity as they can also be cleaved by some caspases, a family of cysteine proteases that are also activated during apoptosis [6,7]. Alternatively, recent studies have revealed a specific substrate for GzmB, Boc-AAD-SBzl, which cannot be cleaved by any caspase [4].

The use of a fluorescence resonance energy transfer (FRET) strategy is widely used to design probes for the detection of enzyme activity. In these probes, a fluorescent donor and an acceptor group are attached to either end of a cleavable enzyme substrate. Upon enzymatic cleavage of the substrate, the donor and acceptor are separated and emission from the fluorophore is restored, resulting in an increase of fluorescence intensity proportional to enzyme activity [8–10]. Among the many possible combinations of donor-acceptor, the pair 5-(2'-aminoethyl)aminonaphthalene sulfonic acid (Edans) and 4-[[4'-(*N,N*-dimethylamino)phenyl]diazenyl]benzoic acid (Dabcyl) is largely used to monitor serine proteases activity, with Edans acting as the acceptor and Dabcyl as the donor. In fact, this donor-acceptor pair has been successfully applied to fluorescence-based assays of SARS coronavirus 3C-like protease [11], human immunodeficiency virus protease [12], human neutrophil elastase [13], human cytomegalovirus protease [14], hepatitis C virus protease [15], among others.

Having these facts in mind and considering our interest in using FRET-based techniques to monitor GzmB activity in a controlled release system of immunostimulating drugs applied in the treatment of colorectal cancer [16], we report herein the synthesis and characterization of a new fluorogenic GzmB substrate. For this, our substrate design was based on the FRET principle using Edans/Dabcyl as donor-acceptor pair, linked to a specific sequence for GzmB (AAD), with an additional amino acid as anchoring point (K).

## 2. Experimental Section

### 2.1. Reagents and Solvents

All Fmoc-amino acids and 2-chlorotriyl chloride resin (with a degree of functionalization of 1.3 mmol/g) were purchased from AAPPTec (Louisville, KY, USA). The coupling reagents and additives ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma) and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from AAPPTec; *N*-hydroxysuccinimide (NHS) and *N,N'*-diisopropylcarbodiimide (DIC) were purchased from Acros Organics (Geel, Belgium). Additional reagents and solvents were purchased from Merck KGaA (Darmstadt, Germany) and Acros Organics and used as received. The fluorophore Edans was acquired from Sigma-Aldrich (St. Louis, MO, USA) and the chromophore/quencher Dabcyl was synthesized according to standard azo-coupling reactions [17].

### 2.2. Instrumentation

TLC analyses were carried out on 0.25 mm thick silica plates coated with fluorescent indicator F<sub>254</sub> (Merck KGaA, Darmstadt, Germany) and spots were visualised in a CN15 viewing cabinet under UV lamp at 365 nm (Vilber Lourmat, Marne-la-Vallée, France). NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for <sup>1</sup>H and 100.6 MHz for <sup>13</sup>C using the solvent peak as an internal reference. The solvents are indicated in parentheses before the chemical shift values ( $\delta$  relative to TMS and given in ppm). Assignments were supported by two-dimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. Unidad de Espectrometria de Masas" at the University of Vigo, Spain. Microwave-assisted solid phase peptide synthesis was carried out on a CEM Discover SPS equipment (CEM Corp., Matthews, GA, USA). Probe purification was performed by semi-preparative HPLC on a Shimadzu LC-8A, UV/Vis JASCO 875-UV detector and a Shimadzu C-RGA Chromatopac register (Shimadzu Europa GmbH, Duisburg, Germany) on a Europa Peptide 120 C18 (5 mm) column (Teknokroma, Barcelona, Spain) using ACN/water (3:1, *v/v*) ( $\lambda_{\text{det}} = 432$  nm). UV/Vis absorption spectra were obtained using a Shimadzu UV/2501PC spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) and fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer (HORIBA Europe GmbH, Darmstadt, Germany) in standard quartz cuvettes.

### 2.3. Methods

#### 2.3.1. Synthesis of H-Ala-Ala-Asp(OtBu)-Lys(Boc)-OH on Resin (1)

The peptide was synthesized by a microwave-assisted solid phase peptide synthesis protocol on a manual microwave-assisted peptide synthesizer using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy.

*Attachment of the first amino acid to the resin.* The 2-chlorotriylchloride resin (1 g) was swollen in DCM for 15 min and filtered. The protected C-terminal amino acid residue, Fmoc-Lys(Boc)-OH (2 equiv on the degree of functionalization of the resin), was attached to the resin in the presence of 4 equiv of *N,N*-diisopropylethylamine (DIPEA) (based on the amino acid) under anhydrous conditions in DCM solution (10 mL). The mixture was stirred for 5 h at room temperature. The resin was filtered and washed sequentially with a mixture of DCM/MeOH/DIPEA (25.5:3.0:1.5, *v/v*; 3 × 10 mL), DCM (3 × 10 mL), DMF (3 × 10 mL), MeOH (3 × 10 mL) and diethyl ether (2 × 10 mL). After, the

resin was dried in vacuo. A standard protocol was applied to determine the loading of the resin, resulting in a loading of 0.4872 mmol/g. The peptide chain was elongated in consecutive cycles of deprotection and coupling.

*Deprotection of Fmoc-amino acids.* Deprotection was performed with 20% piperidine in DMF (5 mL/g resin) for 30 s, under microwave irradiation (power = 50 W, temperature = 75 °C). The resin was filtered, then a fresh solution of 20% piperidine in DMF (7 mL/g resin) was added and allowed to react for 3 min under microwave irradiation (power = 50 W, temperature = 75 °C). The resin was washed with DMF (3 × 10 mL) and MeOH (3 × 10 mL) and this washing cycle was repeated four times.

*Coupling of Fmoc-amino acids.* The Fmoc-amino acid (5 equiv on the loading) was dissolved in dry DMF (6 mL/g resin) and DIC (5 equiv) and Oxyma (5 equiv) dissolved in dry DMF (2 mL/g) were added. The reaction mixture was allowed to stir at room temperature for 5 min before added to the resin. The suspension was then subjected to two microwave irradiation cycles for 5 min (power = 25 W, temperature = 75 °C). The resin was washed with DMF (3 × 10 mL) followed by MeOH (3 × 10 mL) and this washing cycle was repeated three times. Part of the resin was subjected to cleavage to afford the free peptide (see details below) for characterization by NMR.

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ = 1.16–1.38 (28H, m, 2 × CH<sub>3</sub> Ala, γ-CH<sub>2</sub> Lys, δ-CH<sub>2</sub> Lys, 3 × CH<sub>3</sub> Boc and 3 × CH<sub>3</sub> OtBu), 1.45–1.58 (2H, m, β-CH<sub>2</sub> Lys), 2.39–2.48 (1H, m, β-CH<sub>2</sub> Asp), 2.69 (1H, dd, *J* = 5.6 Hz and 17.0 Hz, β-CH<sub>2</sub> Asp), 2.81–2.88 (2H, m, ε-CH<sub>2</sub> Lys), 3.82–3.90 (2H, m, α-CH Lys and α-CH Ala), 4.28 (1H, s, α-CH Ala), 4.51–4.56 (1H, m, α-CH Asp), 6.65 (1H, s, NH Boc), 7.50 (1H, d, *J* = 6.8 Hz, NH Lys), 8.35 (1H, d, *J* = 7.2 Hz NH Asp or NH Ala 2), 8.43 (1H, s, NH Asp or NH Ala 2) ppm.

<sup>13</sup>C NMR (100.6 MHz, DMSO-*d*<sub>6</sub>): δ = 18.36 (CH<sub>3</sub> Ala), 18.50 (CH<sub>3</sub> Ala), 22.26 (γ-CH<sub>2</sub> Lys), 27.68 (CH<sub>3</sub> Boc or CH<sub>3</sub> OtBu), 28.29 (CH<sub>3</sub> Boc or CH<sub>3</sub> OtBu), 29.40 (δ-CH<sub>2</sub> Lys), 31.54 (β-CH<sub>2</sub> Lys), 37.38 (β-CH<sub>2</sub> Asp), 39.50 (ε-CH<sub>2</sub> Lys), 48.55 (α-CH Ala 1 or α-CH Ala 2), 48.84 (α-CH Ala 1 or α-CH Ala 2), 49.65 (α-CH Asp), 53.33 (α-CH Lys), 77.34 (C(CH<sub>3</sub>) Boc or C(CH<sub>3</sub>) OtBu), 80.15 (C(CH<sub>3</sub>) Boc or C(CH<sub>3</sub>) OtBu), 155.55 (C=O Boc), 169.44 (C=O Asp), 171.45 (C=O Ala 1), 171.92 (C=O Ala 2), 172.14 (C=O OtBu), 173.30 (C=O Lys) ppm.

### 2.3.2. Synthesis of DabcyL-Ala-Ala-Asp(OtBu)-Lys(Boc)-OH (2)

After the last Fmoc group had been removed from the peptide-resin **1** with 20% piperidine in DMF, DabcyL (5 equiv on the loading) was coupled to the N-terminal alanine of the peptide-resin **1** with the aid of DIC (5 equiv) and Oxyma (5 equiv) in dry DMF. The mixture was shaken at room temperature for 15 min before being added to the resin. The suspension was subjected to four microwave irradiation cycles for 5 min (power = 25 W, temperature = 75 °C). The resin was washed with DMF (3 × 10 mL) followed by MeOH (3 × 10 mL) and this washing cycle was repeated six times. The resin with DabcyL-peptide was stirred for 2 h in a mixture of acetic acid (AcOH)/2,2,2-trifluoroethanol (TFE)/DCM (1:2:7, *v/v*; 10 mL) at room temperature. This mixture was left overnight at room temperature. The solution was filtered, and the resin was washed with TFE/DCM (2:8, *v/v*; 2 × 10 mL). The solvent was removed under reduced pressure in a rotary evaporator and the product was precipitated with diethyl ether and stored at 4 °C overnight. The product was collected by centrifugation, three times suspended in diethyl ether, collected by centrifugation and dried in vacuo. DabcyL-Ala-Ala-Asp(OtBu)-Lys(Boc)-OH **2** (0.082 g, yield 30%) was obtained as orange solid.

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ = 1.05–1.29 (28H, m, 2 × CH<sub>3</sub> Ala, γ-CH<sub>2</sub> Lys, δ-CH<sub>2</sub> Lys, 3 × CH<sub>3</sub> Boc and 3 × CH<sub>3</sub> OtBu), 1.49–1.59 (1H, m, β-CH<sub>2</sub> Lys), 1.62–1.70 (1H, m, β-CH<sub>2</sub> Lys), 2.39–2.50 (2H, m, β-CH<sub>2</sub> Asp), 2.78–2.89 (2H, m, ε-CH<sub>2</sub> Lys), 3.06 (6H, s, 2 × CH<sub>3</sub> DabcyL), 3.92–3.97 (1H, m, α-CH Lys), 4.24–4.31 (1H, m, α-CH Ala 2), 4.45–4.59 (2H, m, α-CH Ala 1 and α-CH Asp), 6.69 (1H, t, *J* = 5.2 Hz, NH Boc), 6.84 (2H, d, *J* = 9.2 Hz, H-3' and H-5'), 7.55 (1H, d, *J* = 7.2 Hz, NH Lys), 7.81 (4H, dd, *J* = 2.8 Hz and 9.2 Hz, H-3, H-5, H-2' and H-6'), 8.03 (2H, d, *J* = 8.8 Hz, H-2 and H-6), 8.12 (1H, d, *J* = 7.2 Hz, NH Ala 2 or NH Asp), 8.23 (1H, d, *J* = 8.4 Hz, NH Ala 2 or NH Asp), 8.64 (1H, d, *J* = 6.8 Hz, NH Ala 1) ppm.

$^{13}\text{C}$  NMR (100.6 MHz, DMSO- $d_6$ ):  $\delta$  = 17.79 (CH<sub>3</sub> Ala), 18.31 (CH<sub>3</sub> Ala), 22.34 ( $\gamma$ -CH<sub>2</sub> Lys), 27.64 (CH<sub>3</sub> Boc or CH<sub>3</sub> OtBu), 28.29 (CH<sub>3</sub> Boc or CH<sub>3</sub> OtBu), 29.33 ( $\delta$ -CH<sub>2</sub> Lys), 31.40 ( $\beta$ -CH<sub>2</sub> Lys), 37.37 ( $\beta$ -CH<sub>2</sub> Asp), 39.55 ( $\varepsilon$ -CH<sub>2</sub> Lys), 48.29 ( $\alpha$ -CH Ala 2), 49.26 ( $\alpha$ -CH Ala 1 or  $\alpha$ -CH Asp), 49.46 ( $\alpha$ -CH Ala 1 or  $\alpha$ -CH Asp), 52.93 ( $\alpha$ -CH Lys), 64.95 (2  $\times$  CH<sub>3</sub> DabcyI), 77.36 ( $\underline{\text{C}}$ (CH<sub>3</sub>) Boc or  $\underline{\text{C}}$ (CH<sub>3</sub>) OtBu), 80.18 (C(CH<sub>3</sub>) Boc or  $\underline{\text{C}}$ (CH<sub>3</sub>) OtBu), 111.61 (CH-3' and CH-5'), 121.44 (CH-3 and CH-5), 125.15 (CH-2' and CH-6'), 128.73 (CH-2 and CH-6), 134.27 (C4), 142.68 (C4'), 152.90 (C1'), 154.12 (C1), 155.57 (C=O Boc), 165.73 (C=O Asp), 169.36 (C=O Ala 1), 169.42 (C=O Ala 2), 172.23 (C=O OtBu), 173.56 (C=O Lys) ppm.

MS  $m/z$  (ESI, %): 811 ([M + H]<sup>+</sup>, 19), 356 (19), 145 (20), 134 (34), 329 (39), 132 (74), 328 (100). HRMS:  $m/z$  (ESI) calc. for C<sub>40</sub>H<sub>59</sub>N<sub>8</sub>O<sub>10</sub> 811.4349; found 811.4338.

### 2.3.3. Synthesis of DabcyI-Ala-Ala-Asp(OtBu)-Lys(Boc)-Edans (3)

The attachment of Edans to the peptide C-terminal was performed using a solution method with EDC/NHS [18]. For this, DabcyI-peptide **2** (0.078 g, 0.096 mmol) was dissolved in dry DMF (5 mL) and placed in an ice bath. After stirring for 5 min, NHS (0.074 g, 0.643 mmol) was added, followed by EDC (0.085 g, 0.443 mmol) and Et<sub>3</sub>N (62  $\mu$ L). The reaction mixture was stirred for 1 h, then Edans (0.052 g, 0.197 mmol) and Et<sub>3</sub>N (27  $\mu$ L) were added. The mixture was stirred at room temperature for 5 h. The solvent was removed under reduced pressure in a rotary evaporator. The crude product was purified by semi-preparative HPLC using ACN/water mixture (3:1) as eluent and a flow of 1 mL/min. The desired product eluted from the column with a retention time of 16 min. The probe **3** (0.005 g, yield 5%) was obtained as orange solid.

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  = 1.22–1.35 (28H, m, 2  $\times$  CH<sub>3</sub> Ala,  $\gamma$ -CH<sub>2</sub> Lys,  $\delta$ -CH<sub>2</sub> Lys, 3 $\times$ CH<sub>3</sub> Boc and 3 $\times$ CH<sub>3</sub> OtBu), 1.47–1.65 (1H, m,  $\beta$ -CH<sub>2</sub> Lys), 1.66–1.68 (1H, m,  $\beta$ -CH<sub>2</sub> Lys), 2.44–2.54 (2H, m,  $\beta$ -CH<sub>2</sub> Asp), 2.58–2.71 (2H, m,  $\varepsilon$ -CH<sub>2</sub> Lys), 3.07 (6H, s, 2  $\times$  CH<sub>3</sub> DabcyI), 3.33–3.43 (4H, m, CH<sub>2</sub>-1' and CH<sub>2</sub>-2'), 4.15–4.19 (1H, m,  $\alpha$ -CH Lys), 4.26 (1H, t,  $J$  = 7.2 Hz,  $\alpha$ -CH Ala 2), 4.46–4.56 (2H, m,  $\alpha$ -CH Ala 1 and  $\alpha$ -CH Asp), 6.63–6.68 (2H, t,  $J$  = 5.2 Hz, NH Boc and 2'-NH Edans), 6.84 (2H, d,  $J$  = 9.2 Hz, H-3' and H-5' DabcyI), 7.17 (1H, d,  $J$  = 8.0 Hz, H-4 Edans), 7.27–7.34 (2H, m, H-3 and H-7 Edans), 7.57 (1H, d,  $J$  = 8.0 Hz, NH Lys), 7.61–7.83 (6H, m, H-3, H-5, H-2', H-6' DabcyI and H-6, H-8 Edans), 8.02 (2H, dd,  $J$  = 2.0 Hz and 6.8 Hz, H-2 and H-6 DabcyI), 8.10–8.14 (2H, m, NH Ala 2 or NH Asp and H-2 Edans), 8.17 (1H, d,  $J$  = 8.0 Hz, NH Ala 2 or NH Asp), 8.24 (1H, s, 5-NH Edans), 8.59 (1H, d,  $J$  = 7.2 Hz, NH Ala 1) ppm.

MS  $m/z$  (ESI, %): 1059 ([M + H]<sup>+</sup>, 7), 132 (8), 445 (8), 513 (10), 142 (14), 542 (17), 481 (19), 453 (20), 541 (28), 481 (48), 453 (20), 541 (28), 481 (48), 453 (52), 480 (83), 452 (100). HRMS:  $m/z$  (ESI) calc. for C<sub>52</sub>H<sub>71</sub>N<sub>10</sub>O<sub>12</sub>S 1059.4968; found 1059.4965.

### 2.3.4. UV/Vis Absorption and Fluorescence Spectroscopy of Peptides 2–3

The spectroscopic characterization of peptides **2–3** was carried out by UV/Vis absorption spectroscopy (300–700 nm). Solutions of the compounds (1  $\times$  10<sup>-5</sup> M) were made in phosphate buffer at pH 7.5. Fluorescence spectra of probe **3** and Edans were obtained by excitation at the wavelength of maximum absorption, with a 5 nm slit.

## 3. Results and Discussion

### 3.1. Synthesis of the Fluorogenic Substrate for Granzyme B

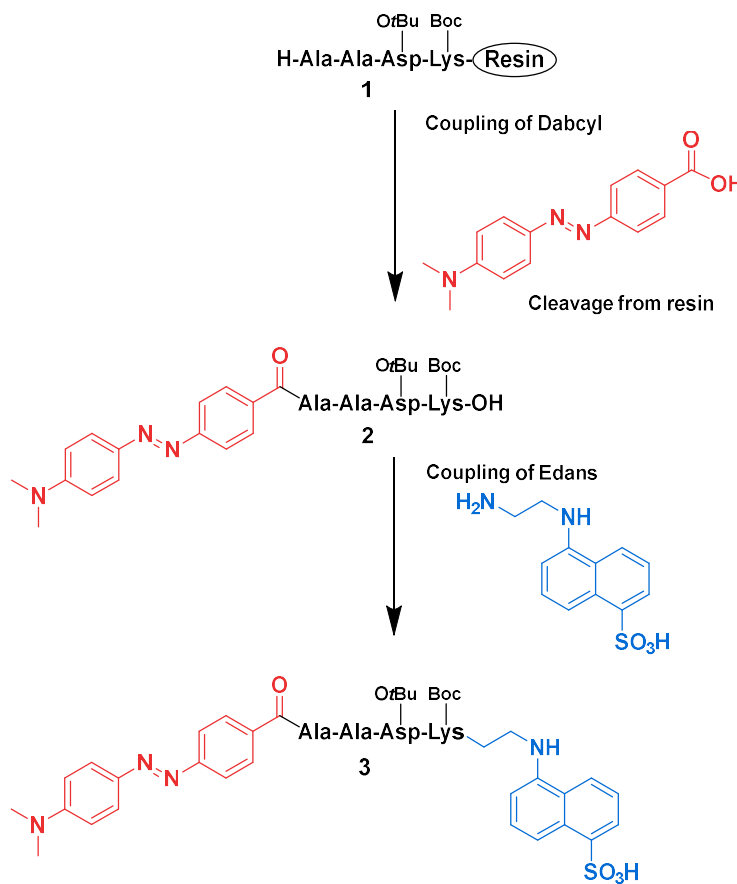
Considering our interest in using FRET-based techniques to monitor GzmB activity in a controlled release system of immunostimulating drugs applied in the treatment of colorectal cancer, we have synthesized and fully characterized a new fluorogenic GzmB substrate. For this, our substrate design was based on two criteria: (1) the specific sequence for GzmB (Ala-Ala-Asp, AAD) should be attached to an additional amino acid as anchoring point (Lys, K); and (2) upon enzymatic cleavage of this substrate, the fluorophore had to be linked to Lys residue. Taking into account the above-mentioned aspects and considering the GzmB's preference for cleaving after aspartic acid at the P1 position (nomenclature according to Schechter and Berger) [19], in our substrate the fluorophore was coupled to the C-terminus of the tetrapeptide (AADK) and the quencher was

attached to the N-terminus of this peptide. GzmB would cleave the substrate between residues Asp (P1) and Lys (P1'), -AADK- (the scissile Asp-Lys bond is underlined).

Therefore, the synthesis of the tetrapeptide and the introduction of Dabcyl were obtained by solid phase, while the incorporation of Edans was performed in solution synthesis (Scheme 1). The side chain amino and carboxyl groups of lysine and aspartic acid protected with Boc and OtBu groups, respectively, were maintained during the inclusion of the FRET pair on the peptide to avoid cross reactions.

The required tetrapeptide AADK in its protected form (H-Ala-Ala-Asp(OtBu)-Lys(Boc)-OH 1) was synthesized by microwave-assisted solid phase peptide synthesis (MW-SPPS) applying the Fmoc strategy. The protected derivative of C-terminal amino acid residue, Fmoc-Lys(Boc)-OH, was attached to the 2-chlorotriylchloride resin through a nucleophilic substitution reaction using an excess of Fmoc-amino acid and a base, DIPEA, resulting in a loading of 0.487 mmol/g. The peptide chain was elongated in consecutive cycles of deprotection and coupling of the required amino acids.

The free N-terminal of peptide 1, still attached to the resin, was then coupled to the quencher Dabcyl by microwave-assisted using DIC/Oxyma in dry DMF. The labelled peptide, Dabcyl-Ala-Ala-Asp(OtBu)-Lys(Boc)-OH 2, was cleaved from the solid support by treatment with AcOH/TFE/DCM (1:2:7, *v/v*). The desired product was obtained with a global yield of 30% and characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy and high resolution mass spectroscopy. The obtained data was in agreement with the expected structure.



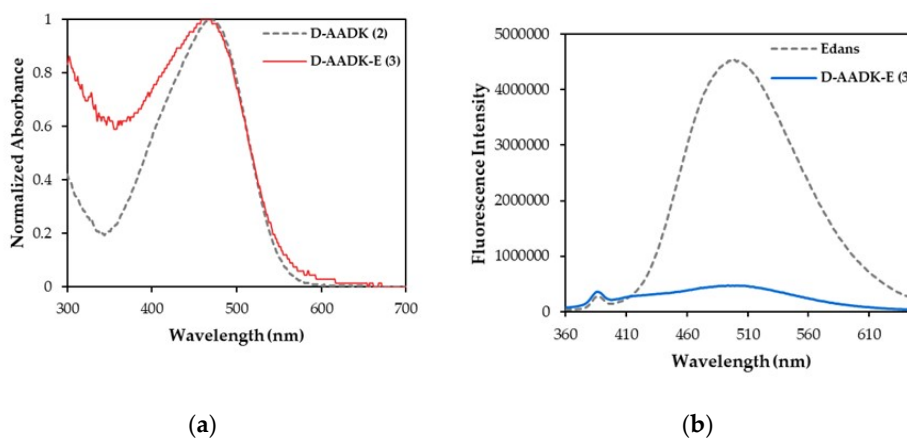
**Scheme 1.** Synthesis of Dabcyl-Ala-Ala-Asp(OtBu)-Lys(Boc)-Edans (3).

Finally, the fluorophore Edans was coupled in a solution reaction to the C-terminal lysine using EDC/NHS in dry DMF according to the method reported by Krafft et al. [18]. The obtained product was carefully purified by semi-preparative HPLC to ensure proper separation from free Edans which could disturb the subsequent fluorescence measurements. Thus, the desired probe Dabcyl-Ala-Ala-Asp(OtBu)-Lys(Boc)-Edans 3 was obtained in 5% yield and characterized by  $^1\text{H}$

NMR spectroscopy and high resolution mass spectroscopy. The obtained data was in agreement with the expected structure.

### 3.2. UV/Vis Absorption and Fluorescence Spectroscopy

To evaluate the photophysical properties, the absorption and fluorescence spectra of labelled peptide **2** (with the quencher attached) and peptide probe **3** (with both quencher and fluorophore attached) were measured in phosphate buffer at pH 7.5. As depicted in Figure 1a, Dabcyl-AADK **2** and Dabcyl-AADK-Edans **3**, showed absorption bands with similar wavelengths of maximum absorption (470 nm and 462 nm, respectively), related to the Dabcyl chromophore. As expected, the fluorescence spectrum of probe **3** exhibited a very low intensity emission band at 494 nm, upon excitation at 342 nm, thus confirming the existence of intramolecular quenching between Edans (acceptor) and Dabcyl (donor) in the probe **3**. To further confirm this observation, a fluorescence spectrum of free Edans was obtained the same conditions. Comparison of the fluorescence spectrum of probe **3** with that of free Edans (Figure 1b) clearly showed that the acceptor Dabcyl efficiently quenches the donor Edans' fluorescence in probe **3**, as predicted.



**Figure 1.** UV/Vis absorption and fluorescence spectroscopy: (a) normalized UV/Vis spectra of peptides **2–3** ( $1 \times 10^{-5}$  M) at pH 7.5 in phosphate buffer; (b) fluorescence spectra of free Edans and peptide probe **3** ( $1 \times 10^{-5}$  M) at pH 7.5 in phosphate buffer, upon excitation at 432 nm.

## 4. Conclusions

In summary, based on FRET principle, a new fluorogenic substrate for Granzyme B was designed, synthesized, and fully characterized. We have demonstrated that microwave-assisted solid phase synthesis accompanied by solution synthesis can be effectively used to obtain the desired fluorogenic substrate bearing a donor/acceptor pair as Edans/Dabcyl. Moreover, future work will be developed to optimize the synthesis method to increase the overall synthesis yield.

By comparison of the fluorescence spectra of the synthesized probe and free Edans, the existence of intramolecular fluorescence quenching between Edans and Dabcyl in the probe was confirmed, thus enabling it to be a very promising candidate as probe for the subsequent activity assays with GzmB.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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