SUPPLEMENTAL INFORMATION

Sensitive versatile fluorogenic transmembrane peptide substrates for rhomboid intramembrane proteases

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Running title: *Fluorogenic substrates for rhomboid proteases*

ABBREVIATIONS

ACN	Acetonitrile
AcOH	Acetic acid
DCM	Dichloromethane
DIC	Diisopropylcarbdodiimide
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
DTT	Dithiothreitol
FA	Formic acid
HATU	2-(1H-(7-azabenzotriazol-1-yl))-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazol
iPrOH	Isopropylalcohol
TCEP	*Tris*(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chemical Synthesis

EDANS/Dabcyl labelled peptides

The major part of peptide synthesis was performed on Tenta Gel S Rinkamide resin (substitution 0.24 mmol/g) on PS3 peptide synthesizer (Protein technologies, USA), using Fmoc-chemistry protocol at a scale of 0.1 mmol. The coupling of Fmoc-Glu(EDANS)-OH and Fmoc-Lys(Dabcyl)-OH was performed manually at the same scale in fourfold excess, using HBTU (4 eqv) / HOBt (4 eqv) / DIEA (6 eqv) in 3-4 ml DMF. The peptide was cleaved from the resin by the mixture of trifluoroacetic acid / triisopropylsilane / water = 95:2.5:2.5 for 3 hours. The mixture was concentrated under nitrogen and the crude peptide was precipitated with cold ether. The precipitate was washed by ether, dried and purified by RP HPLC.

TAMRA/QXL labelled peptides

Synthesis of TAMRA

Trimellitic anhydride (**1)** (4 g, 21 mmol) and 3-dimethylaminophenol **(2)** (5.8 g, 42 mmol) were refluxed in toluene (220 mL) for 12 hours as described (1). Upon cooling to room temperature, the resulting precipitate was collected by filtration, and further purified by flash chromatography (silica gel, dichloromethane/methanol/acetic acid = 8:1.9:0.1) to afford pure mixture of 5(6)-carboxytetramethylrhodamine (TAMRA) as a dark purple solid.

Coupling of TAMRA to ε-amino group of the lysine at 7th position.
Fmoc-Lys(Mmt)-OH was coupled overnight manually to the rest of the peptide on the resin in 4-fold excess, using DIC (7 eqv) / HOBt (5 eqv) method in 3 mL DMF. The peptide-containing resin was washed with DMF (6 × 2 mL), isopropanol (6 × 2 mL), DMF (6 mL) and DCM (10 mL). Mmt as a very acid labile orthogonal protecting group was removed by treatment of the resin with a mixture of DCM / TFE / AcOH (7:2:1) in 4-5 cycles by 15 min each and washing with pure DCM between cycles until no red-orange methoxytrityl cations in the eluate were detected (2). The resin was washed with DCM (10 mL) and dried at vacuum. The ε-deprotected lysine was proved by the ninhydrin test. After soaking with DMF (3-4 mL) for 10 min, the resin was ready for coupling of TAMRA (4 eqv), using HATU (4 eqv) / HOBt (4 eqv) / DIEA (6 eqv) method in 3 – 4 mL DMF for 4-6 h. Then the resin was washed with DMF (6 × 2 mL), isopropanol (6 × 2 mL), DCM (10 mL) and was dried in the vacuum. Peptide synthesis was continued in the automated peptide synthesizer, and the peptides were cleaved off the resin by 5-6 mL of the TFA/TIS/ethanedithiol/thioanisole/water mixture (90:2:2.5:3:2.5) for 3.5 h. The peptides were processed and purified as described for the EDANS-Dabcyl labelled ones above.

Labelling with QXL610 vinylsulfone
The purified deprotected peptide was used for labelling of the cysteine at 15 position with a dark quencher QXL610 with vinylsulfone (VS) reactive group as described (3). The 37-mer TAMRA-peptide (13 mg, 2.9 μmol) was dissolved in 14 mL Tris-buffer (pH 7.8), containing 0.24 M TCEP (48 μL). The QXL610VS (3 mg, 5.9 μmol), dissolved in 360 μL DMSO was added and the mixture was stirred for 2 – 3 h at room temperature. When the starting peptide was depleted (80 – 90 % conversion as judged by HPLC analysis), the reaction was quenched by addition of DTT (22 mg, 0.14 mmol) to a final concentration 10 mM and stirred for 20 min. The solution was frozen and lyophilized and the double labelled TAMRA/QXL peptide was isolated by RP HPLC on a C_{18} Watrex Reprosil semipreprative column 250 × 10 (gradient 30 – 80 % B) at flow 3 mL/min.

HPLC analysis and purification
Chromatographic conditions: Module Jasco PU 1580 Series (Jasco, Japan) with a preparative C_{18} column (250 × 20 mm, 10 μm particle size , Watrex International, Inc, San Francisco, California, USA), using water + 0.1 % trifluoroacetic acid (A) / acetonitrile (B) as mobile phases, at 10 mL/min flow rate. Elution gradient: from 30 % B to 80 %B over 60 minutes at room temperature. The elution was monitored by absorption at 210 nm, using UV – VIS 1575 detector. The fractions corresponding to the major peak (desired peptide) were combined, frozen and lyophilized, giving the pure peptide. Analytical runs were done on the same module using Watrex C_{18}, 250 × 4.6 mm, 5 μm particle size column (Watrex International, Inc, San Francisco, California, USA) at 1 mL/min flow rate using various solvent gradients ranging from solvent A to solvent B as defined above over 30 minutes at room temperature.

LC-MS
Agilent Technologies Liquid Chromatograph coupled with TOF 6230 ESI-MS detector. Gradient: 2 %B to 100 %B over 10 min on a 1.7 μm particle size C_{18}, 100 × 2.1 mm RP HPLC column (Waters) at a flow rate of 0.3 mL/min, where A = water + 0.1%FA and B = ACN + 0.1%FA.

NMR spectroscopy
NMR spectra were acquired on a Bruker AV-400 MHz at room temperature.

SUPPLEMENTAL RESULTS

Compound characterisation data

5(6)-Carboxytetramethylrhodamine; 5(6)-TAMRA

Yield 1.3 g (14 %)

^{1}H NMR (401 MHz, DMSO-d6) δ (ppm) = 8.69 (s, 1H, aromatic), 8.40 – 8.28 (m, 1H, aromatic), 7.59 (d, J = 7.9 Hz, 1H, aromatic), 7.19 – 6.85 (m, 6H, aromatic), 3.24 (s, 12H, CH3).

ESI-MS: Monoisotopic mass 430.15; found $[M+H]^+$ 431.2, $[M+Na]^+$ 453.2.

KSp31 peptide

Sequence: KRHDINHISKSDTGIIFAAISLFSLLFQPLFGLLSKK

Purified by RP HPLC using gradient of 50 – 100 % B; (A = 1 % CH_3CN, 1 % iPrOH, 98 % water + 0.1 % TFA; B = 35 % CH_3CN, 60 % iPrOH, 5 % water + 0.1 % TFA). The product elutes at 27 min.

Yield: 20.3 mg

LC-MS: One peak at 9.58 min, m/z 4153.36 calculated for $C_{195}H_{313}N_{51}O_{49}$; found 4153.3: $[M+3H]^{3+}$1385.4, $[M+4H]^{4+}$ 1039.34, $[M+5H]^{5+}$ 831.66.

KSp35 peptide

Sequence: KRHDINE(Edans)ISKSDTGK(Dabcyl)IFAAISLFSLLFQPLFGLSKK

Yield: 10.4 mg

LC-MS: One peak at 9.39 min, m/z 4546.44 calculated for $C_{215}H_{328}N_{54}O_{53}S_1$; found 4546.45: $[M+4H]^{4+}$ 1137.62, $[M+5H]^{5+}$ 910.30, $[M+6H]^{6+}$ 758.75, $[M+7H]^{7+}$.

KSp64 peptide

Sequence: KRHRIK(Tamra)RVRHADTGC(QXL610)IFAAISLFSLLFQPLFGLSKK

Yield: 2.5 mg

LC-MS: One peak at 5.34 min, m/z calculated for $C_{223}H_{346}N_{64}O_{48}S$ plus QXL610 fragment (formula not released by the manufacturer) as 5204.6; found 5204.7: $[M+4H]^{4+}$ 1302.18, $[M+5H]^{5+}$ 1041.94, $[M+6H]^{6+}$ 868.45.

KSp76 peptide

Sequence: KRHDINK(Tamra)ISKSDTGC(QXL610)IFAAISLFSLLFQPLFGLSKK

Yield: 4.2 mg;

LC-MS: One peak at 9.45 min, m/z calculated for $C_{207}H_{321}N_{51}O_{52}S$ plus QXL610 fragment as 4916.8; found 4917.28: $[M+4H]^{4+}$ 1230.33, $[M+5H]^{5+}$ 984.47, $[M+6H]^{6+}$ 820.55.

SUPPLEMENTAL REFERENCES

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