Supporting Information

L,D-transpeptidase Specific Probe Reveals Spatial Activity of Peptidoglycan Crosslinking

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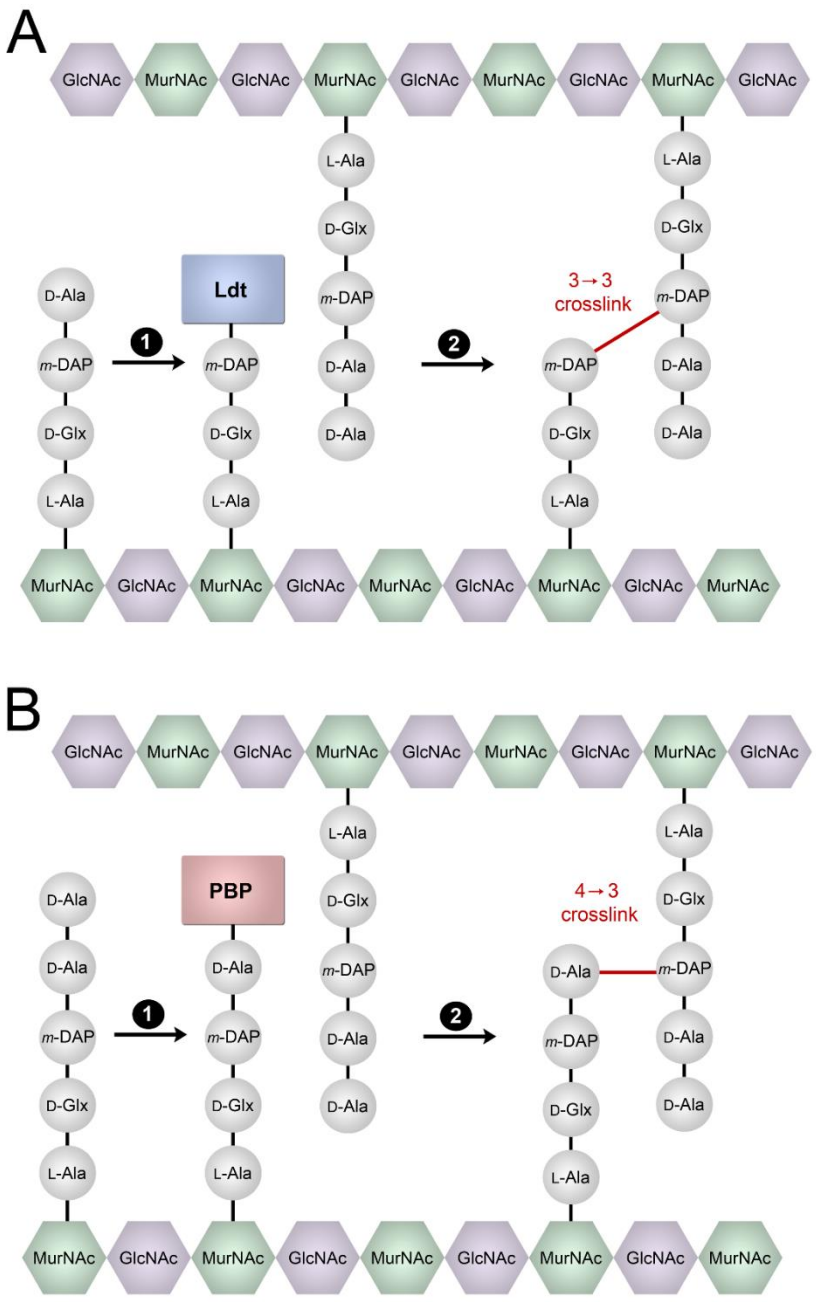


Figure S1. L,D-transpeptidase (Ldt) vs D,D-transpeptidase (DD-TPs) crosslinking reactions (A). A neighboring amino group from the 3rd position (ex. *m*-Dap) captures the acyl intermediate created by the tetrapeptide substrate of Ldts to generate a 3-3 crosslink. (B) A neighboring amino group from the 3rd postion (ex. *m*-Dap) captures the acyl intermediate created by the pentapeptide substrate of DD-TPs to generate a 4-3 crosslink.

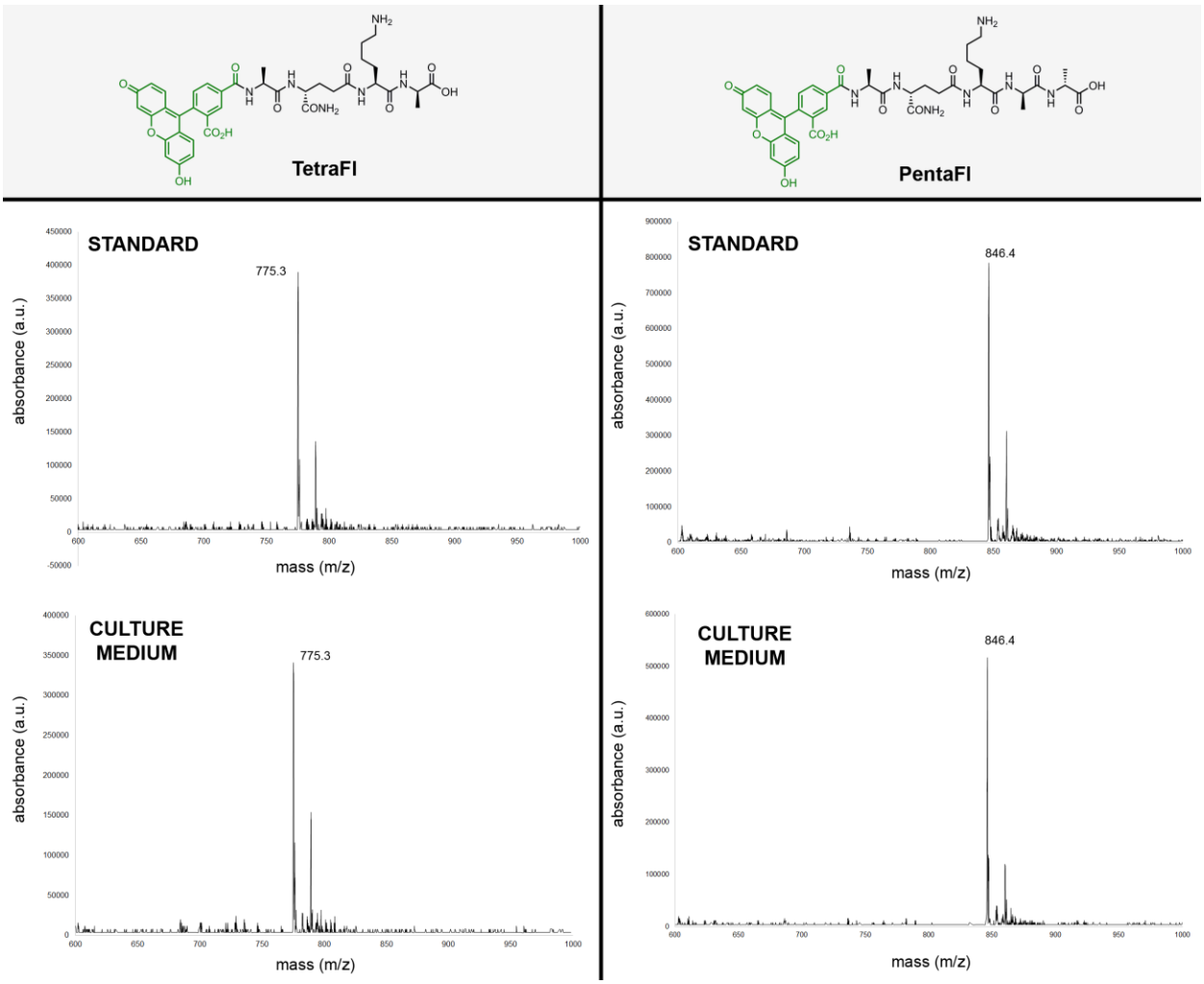
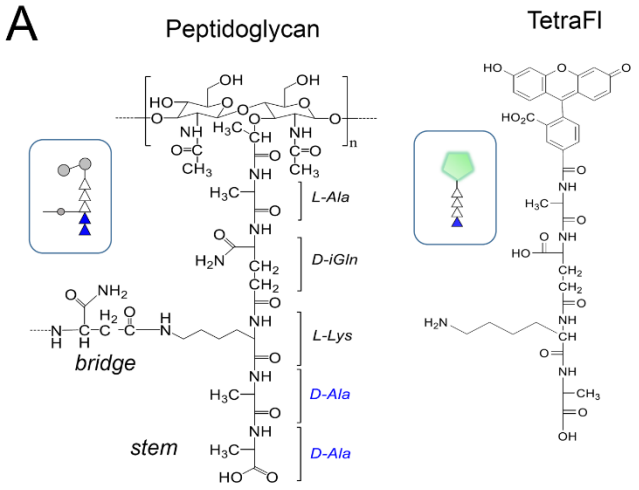
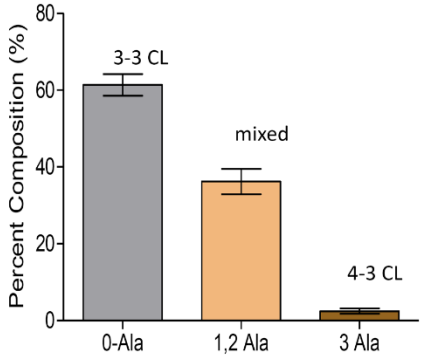
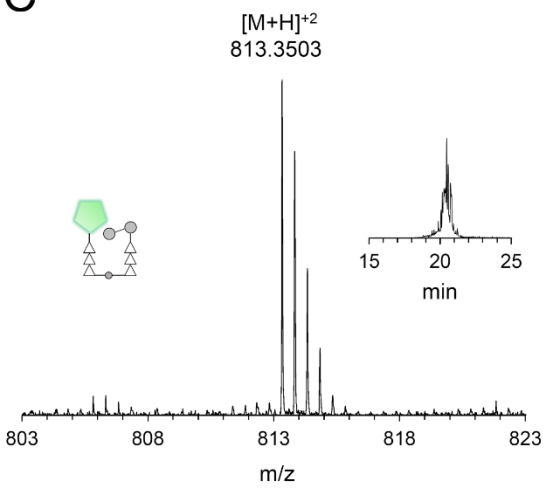
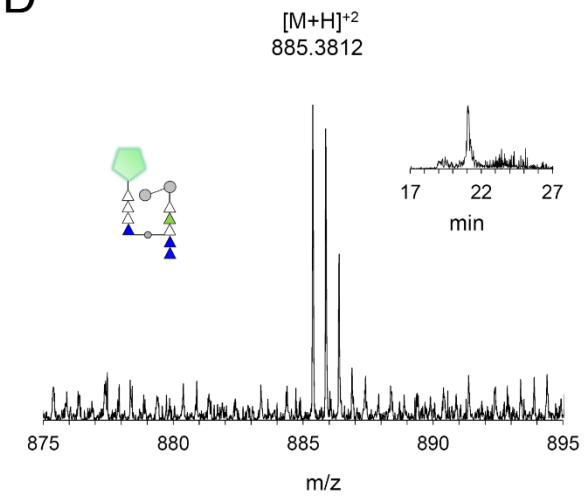


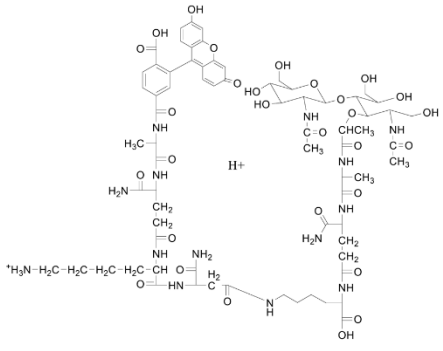
Figure S2. Mass spectrometry analysis of stem peptides incubated with *E. faecium*. **TetraFl** or **PentaFl** (100 μM) were incubated overnight with *E. faecium* D344S. After incubation, the cells were harvested and supernatant analyzed by LC-MS. Mass spectrometry analysis showed no alteration of the stem peptides before (standard) and after bacteria treatment (culture medium).



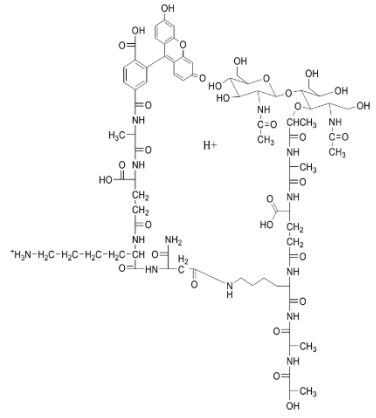








Chemical Formula: $C_{72}H_{102}N_{14}O_{29}^{2+}$
Exact Mass: 1626.69262
Molecular Weight: 1627.67590
m/z: 813.34631 (100.0%), 813.84799 (77.9%), 814.34966 (29.9%), 814.85134 (6.7%), 814.34843 (6.0%), 813.84483 (5.2%), 814.85011 (4.6%), 814.34650 (3.5%), 815.35179 (1.8%), 814.84818 (1.5%), 815.35302 (1.4%), 813.84945 (1.2%), 813.84842 (1.1%)
Elemental Analysis: C, 53.13; H, 6.32; N, 12.05; O, 28.51



Chemical Formula: $C_{78}H_{110}N_{14}O_{33}^{2+}$
Exact Mass: 1770.73
Molecular Weight: 1771.80
m/z: 885.37 (100.0%), 885.87 (84.4%), 886.37 (35.1%), 886.87 (8.8%), 886.37 (6.8%), 886.87 (5.7%), 885.87 (5.2%), 886.37 (4.4%), 887.37 (2.4%), 886.87 (1.8%), 887.37 (1.6%), 885.87 (1.3%), 886.37 (1.1%)
Elemental Analysis: C, 52.88; H, 6.26; N, 11.07; O, 29.80

Figure S3. TetraFl PG analysis. (A) Chemical structure of *E. faecium* peptidoglycan (PG) repeat unit and **TetraFl**. (B) **TetraFl** incorporated PG dimers were quantified by integrating the extracted ion chromatograms (XICs) for the selected ions as shown in the figure inset. All error bars represent standard errors of means (n = 3). (C) Mass spectrum and XIC of **TetraFl**-PG with 3-3 CL with observed $[M+H]^{+2}$ m/z of 818.3503. (D) Mass spectrum and XIC of **TetraFl**-PG with 4-3 CL with observed $[M+H]^{+2}$ m/z of 818.8754. (E) The chemical structure for **TetraFl**-PG with 3-3 CL shown in C. (F) The chemical structure of **TetraFl**-PG with 4-3 CL shown in D).

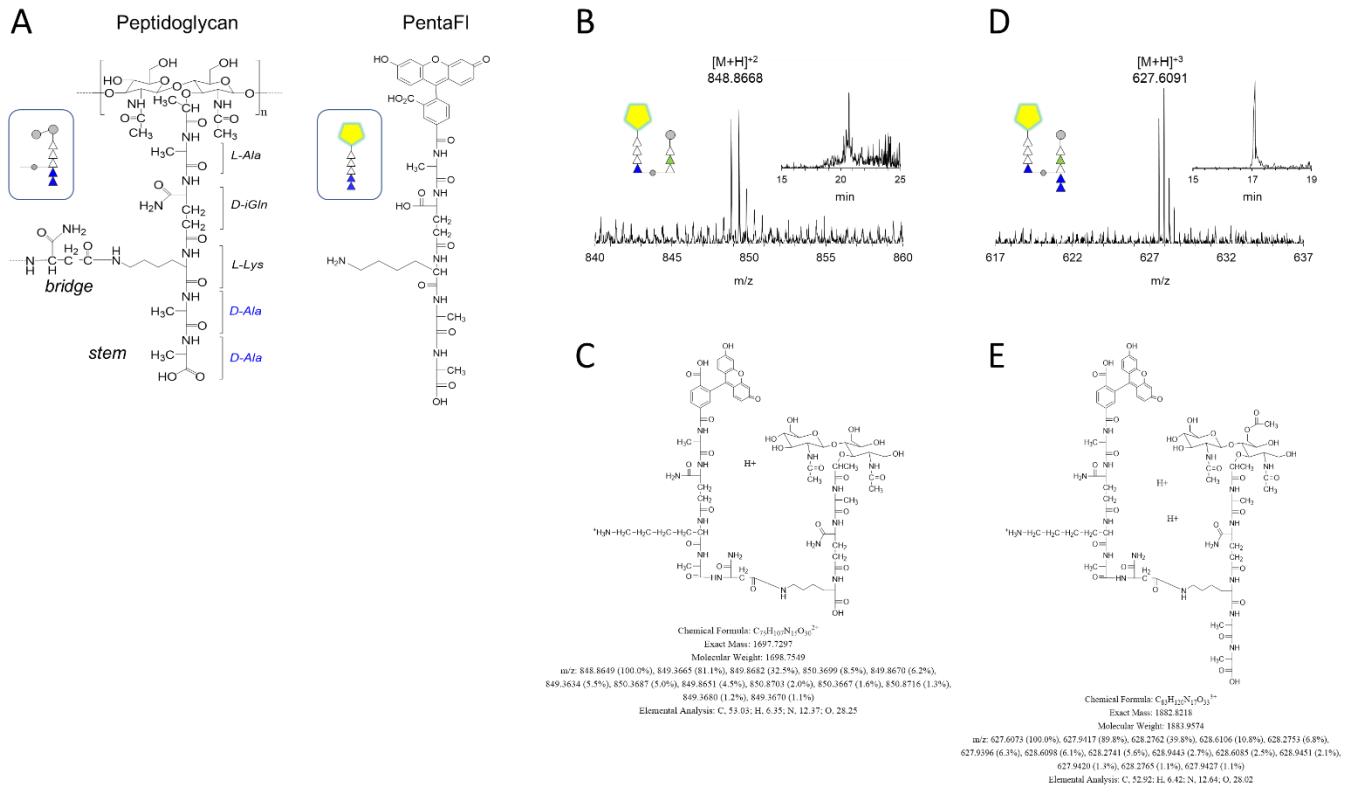


Figure S4. PentaFl PG analysis. (A) Chemical structure of *E. faecium* peptidoglycan (PG) repeat unit and **PentaFl**. (B) Mass spectrum and XIC of **PentaFl**-PG dimer with 4-3 CL with observed $[M+H]^{+2}$ m/z of 848.8668. (C) The chemical structure for **PentaFl**-PG DIMER shown in B. (D) Mass spectrum and XIC of **PentaFl**-PG dimer with 4-3 CL with observed $[M+H]^{+3}$ m/z of 627.6091. (E) The chemical structure of **PentaFl**-PG shown in D).

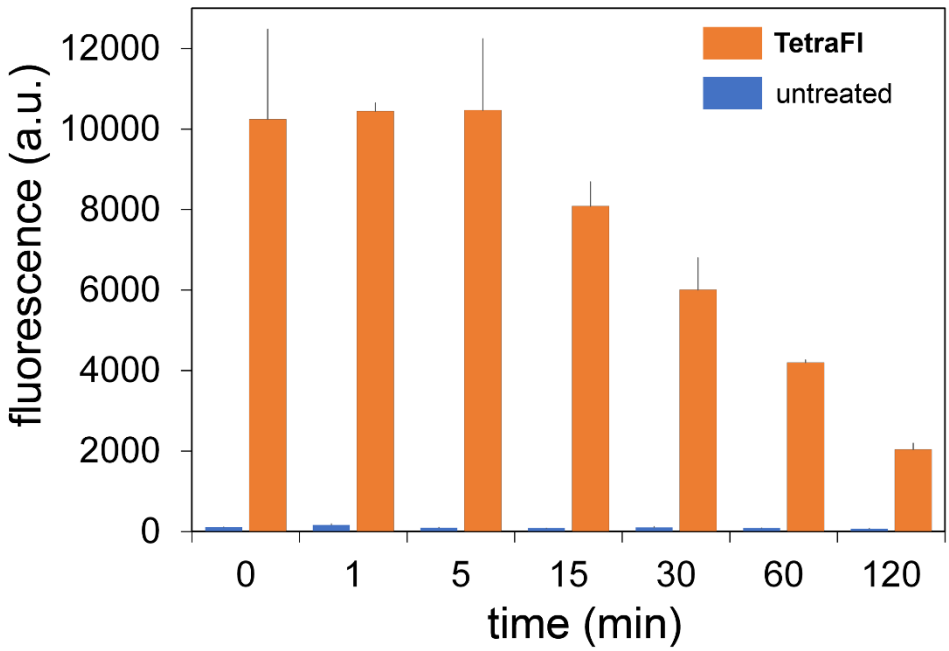


Figure S5. PG digestion of *E. faecium* D344S. Cells were incubated overnight with 100 μM **TetraFl** or untreated (media only). Cells were fixed with formaldehyde, washed 3X (PBS), then incubated at 37 °C with 500 μg/mL lysozyme. At various time points, cell samples were collected and cells were analyzed by flow cytometry.

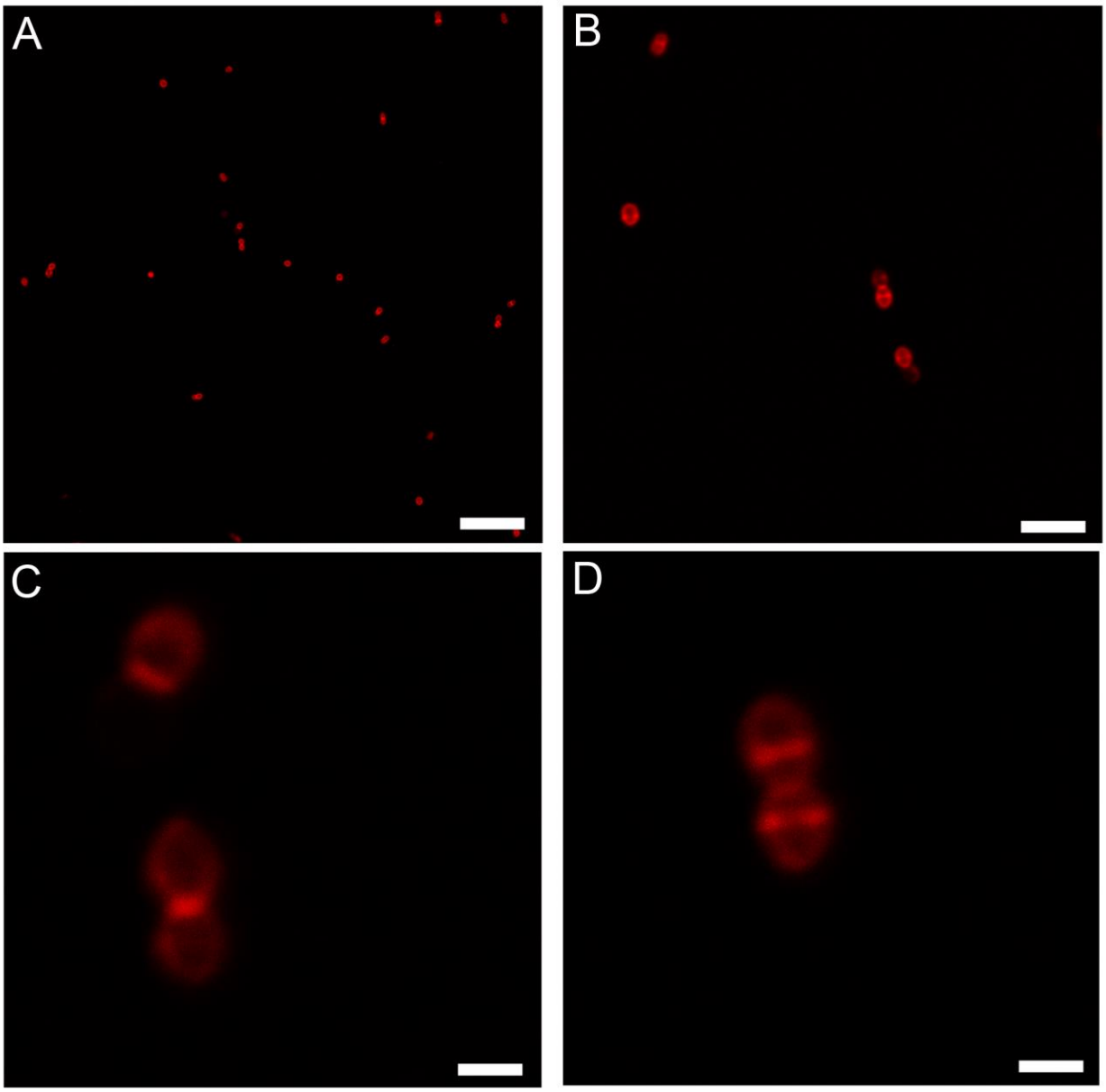


Figure S6. Confocal imaging of sacculi isolated from *E. faecium*. *E. faecium* cells were treated overnight with 500 µM **TetraRh-3**, sacculi was isolated, and imaged by confocal microscopy. Scale bar: 50 µm (A), 10 µm (B), 1 µm (C and D).

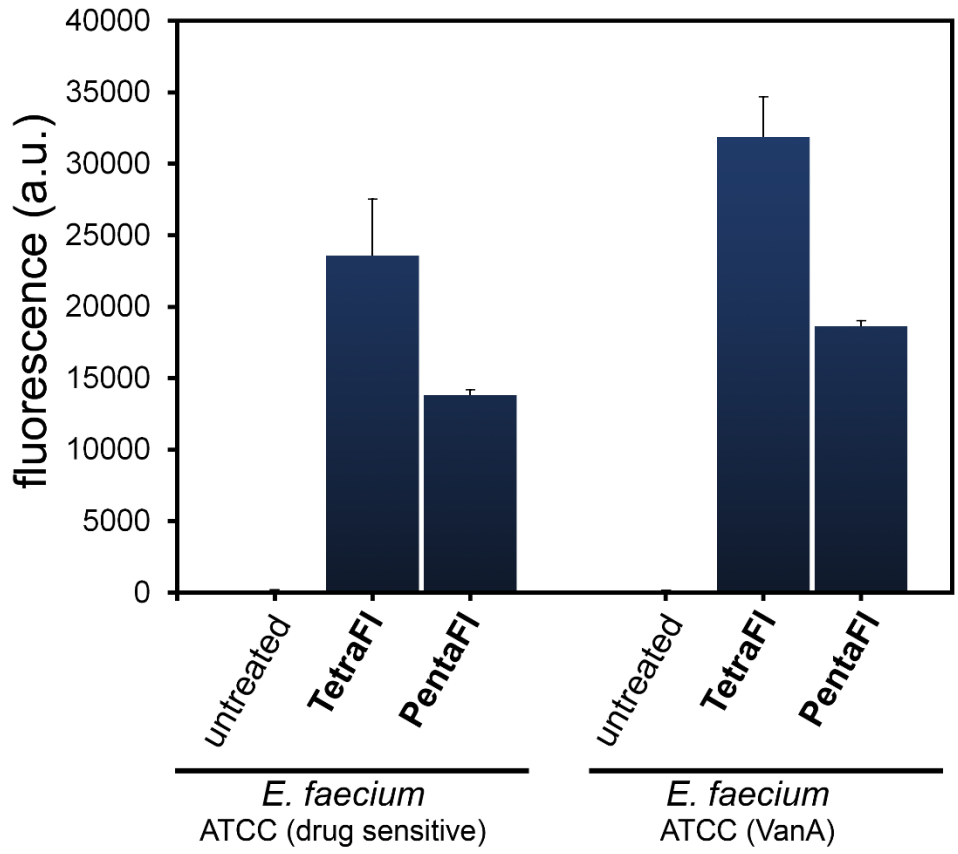


Figure S7. Flow cytometry analysis of additional *E. faecium* strains ATCC BAA-2317 (VanA) and ATCC BAA-2127 (drug sensitive) incubated overnight with 100 µM **TetraFl** or **PentaFl**. Data are represented as mean + SD (n = 3).

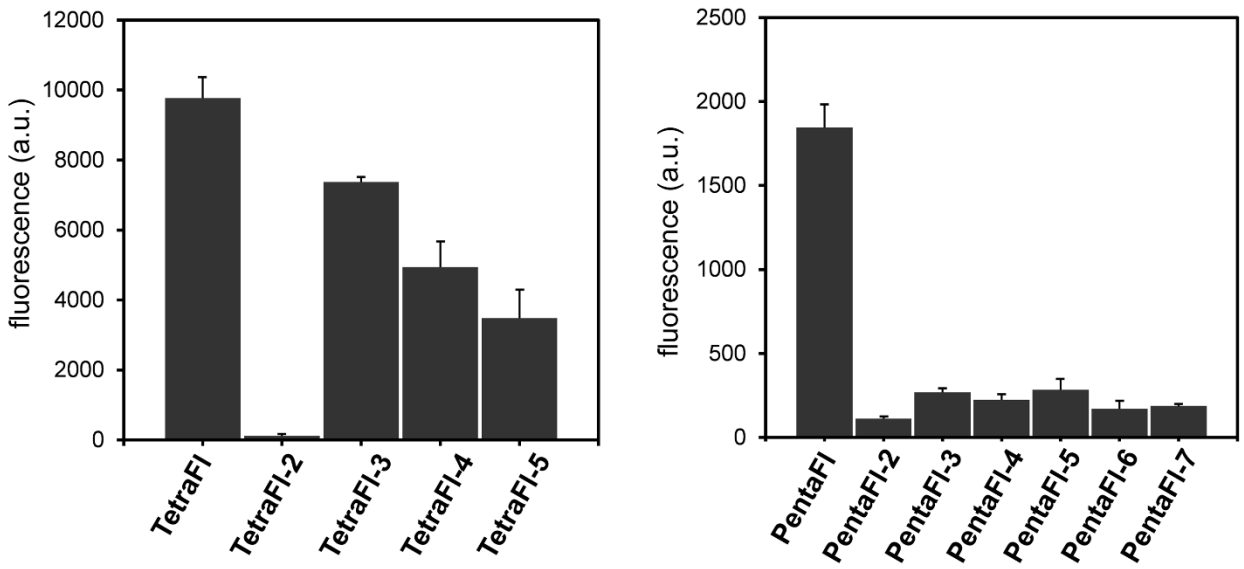


Figure S8. Flow cytometry analysis of *E. faecium* (D344S) incubated overnight with 100 µM tetrapeptide or pentapeptide with variations (see Figure 3). Data are represented as mean + SD (n = 3).

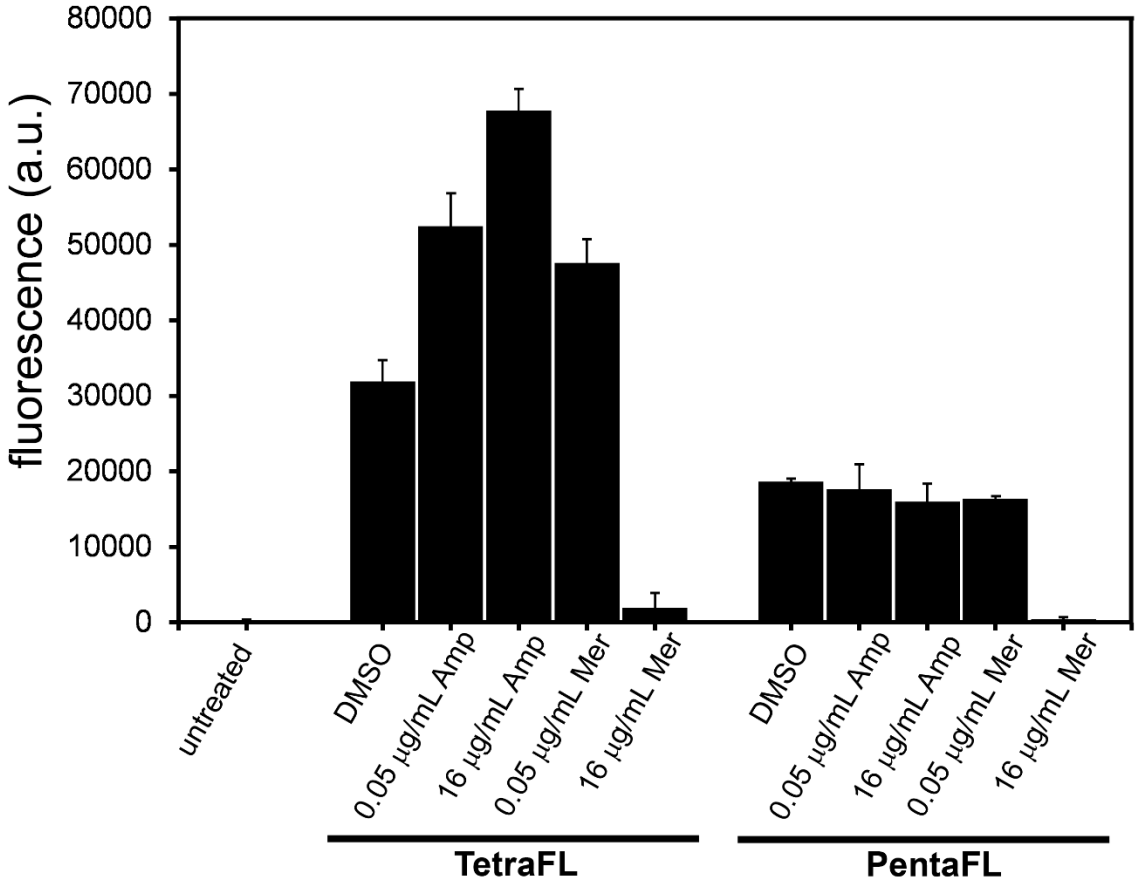


Figure S9. Flow cytometry analysis of *E. faecium* ATCC BAA-2317 (VanA) incubated overnight with 100 µM **TetraFl** or **PentaFl** with varying concentrations of ampicillin or meropenem. Data are represented as mean + SD (n = 3).

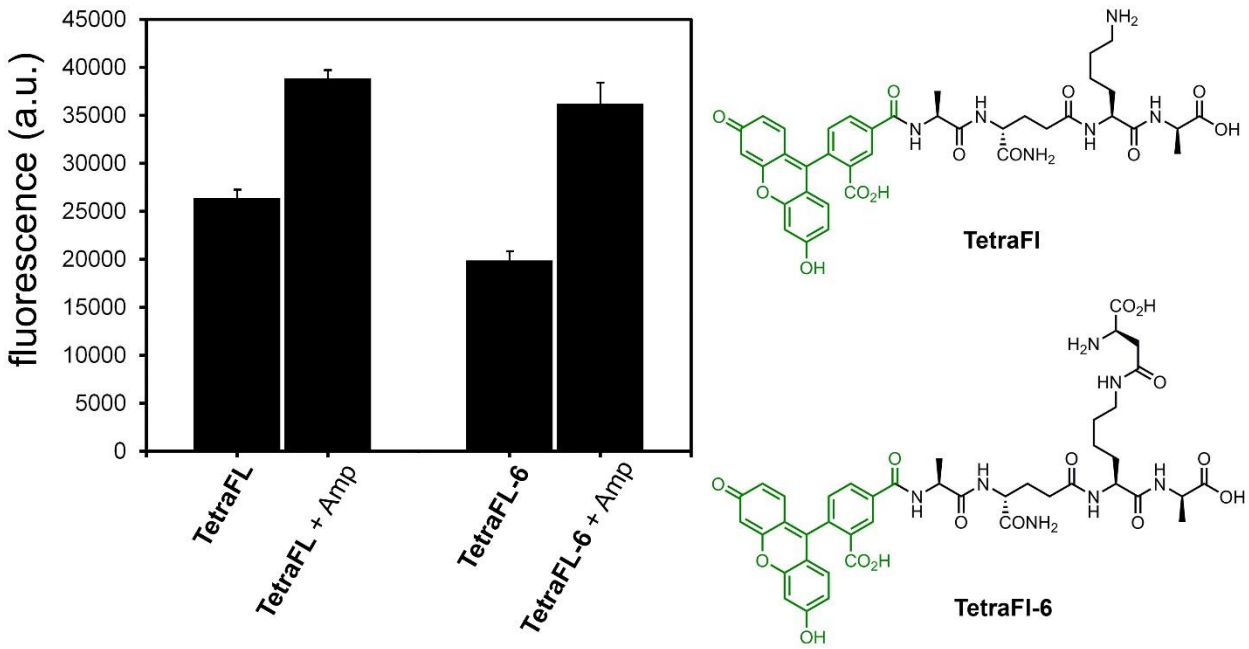


Figure S10. Flow cytometry analysis of *E. faecium* BAA-2317 (VanA) incubated overnight with 100 µM **TetraFl** or **TetraFl-6** with or without 16 µg/mL ampicillin (see Figure 3). Data are represented as mean + SD (n = 3).

MIC values		
Ampicillin: >64 ug/mL	**Imipenem**: >64 ug/mL	**Vancomycin**: >64 ug/mL
Amoxicillin: >64 ug/mL	**Ceftriaxone**: 64 ug/mL	**Erythromycin**: 64 ug/mL
Meropenem: 32 ug/mL	**Aztreonam**: >64 ug/mL	

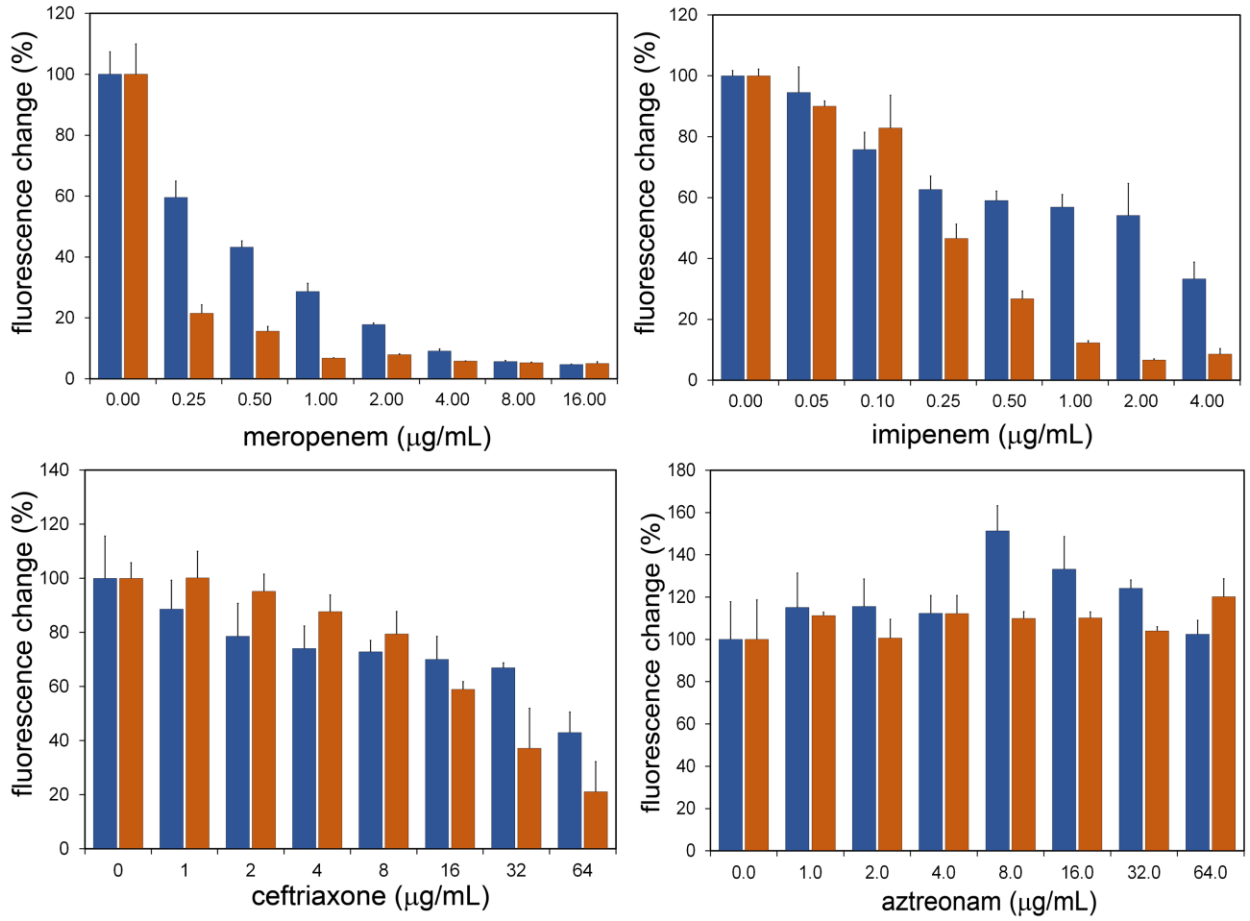


Figure S11. *Top:* MIC values for the antibiotics tested. *Bottom:* Flow cytometry analysis of *E. faecium* (M9) treated overnight with 100 µM **TetraFl** (blue bars) or **PentaFl** (orange bars) and increasing concentrations of meropenem, imipenem, ceftriaxone, or aztreonam. Data are represented as mean + SD (n = 3).

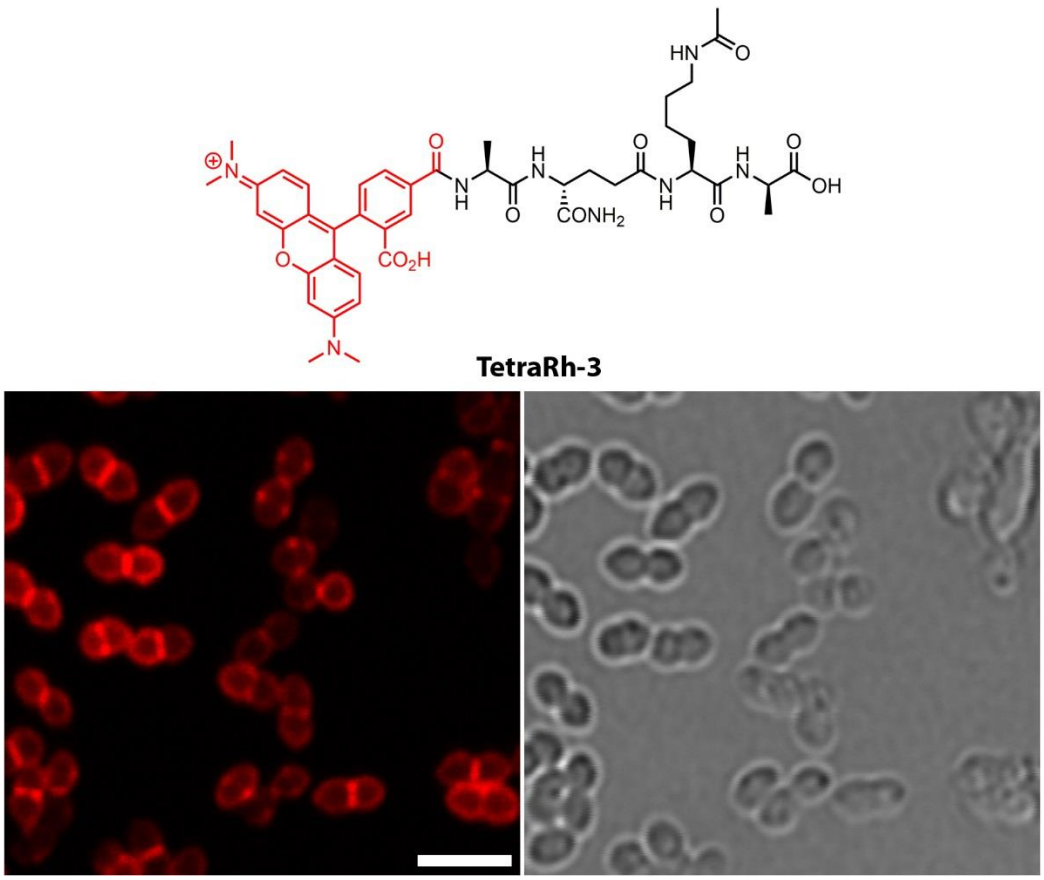


Figure S12. *Above*: chemical structure of **TetraRh-3**. *Below*: confocal microscopy image of *E. faecium* (WT) treated with 5-minute pulse of 500 µM **TetraRh-3** (scale bar: 3 µm).

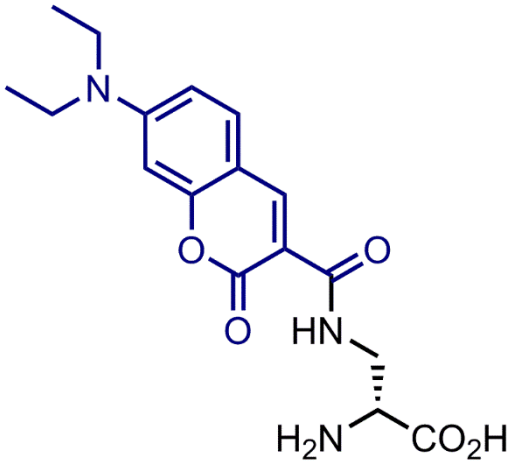
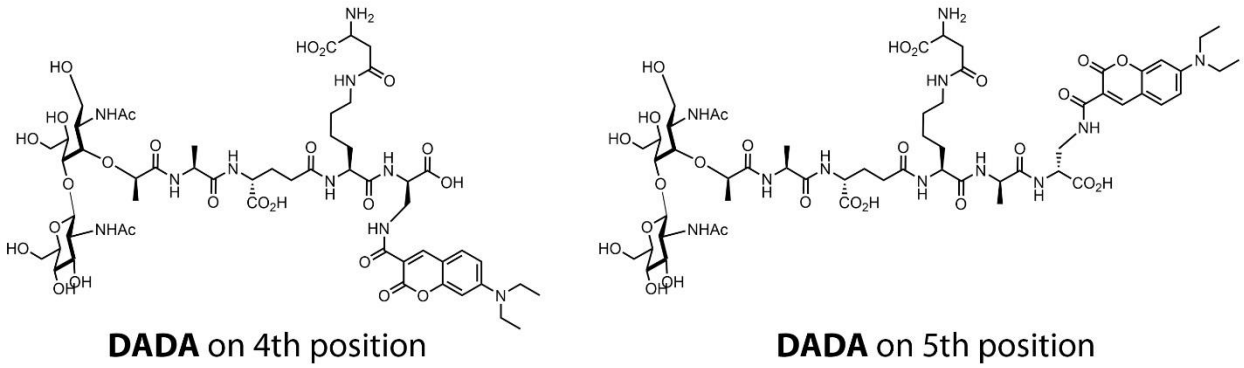


Figure S13. Structure of single D-amino acid derivative **D**iethyl-**A**mino-coumarin-**D**-**A**lanine (**DADA**).



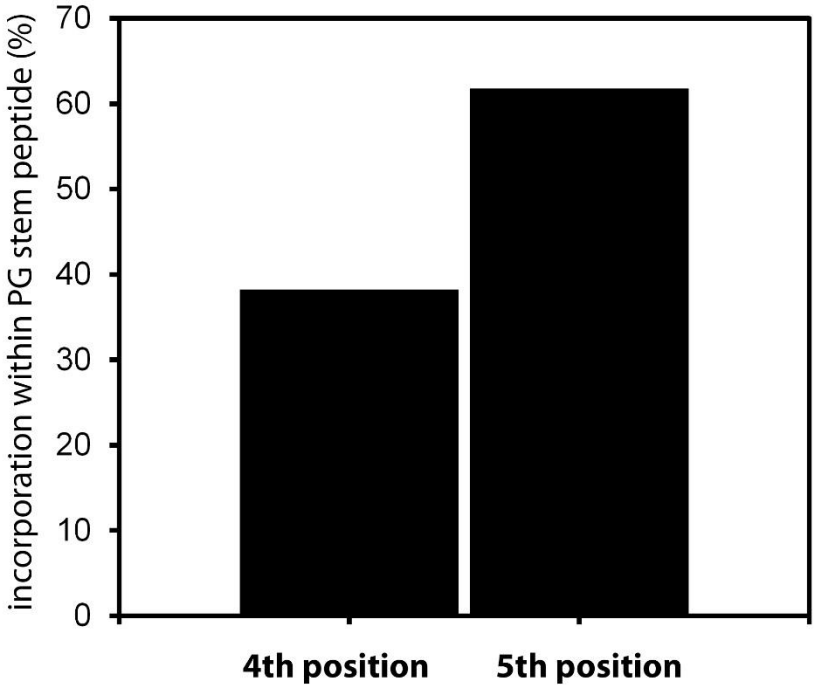


Figure S14. *Above*: chemical structure of PG monomers with **DADA** either on the 4th or 5th position within the stem peptide. *Bottom*: Compositional percentage of incorporation of **DADA** in *E. faecium* cells that were treated with 2 mM of **DADA** for 16 h. The sacculi was isolated and the composition of the monomeric strands was analyzed via LC-MS. The area of each individual peak was used to calculate the percentage of 4th vs. 5th position (100 % is total area of the peaks corresponding to the structures shown above).

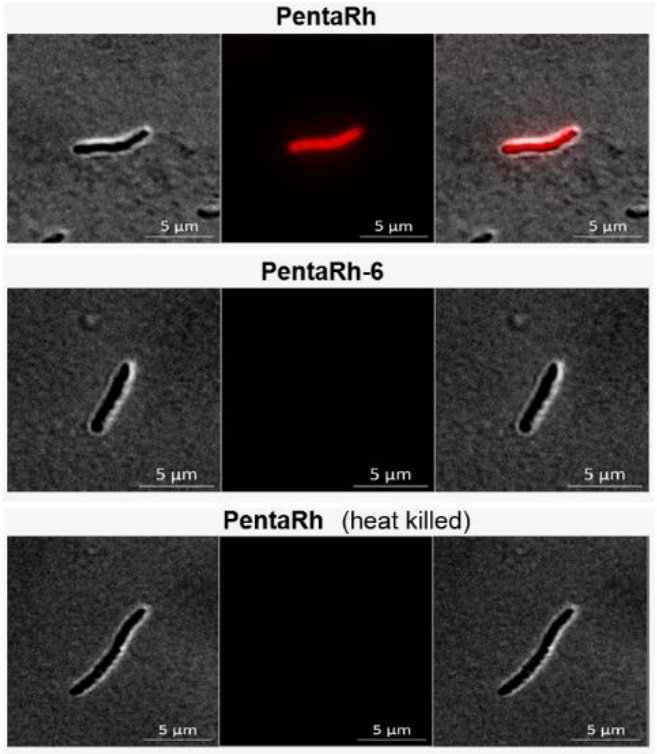


Figure S15. Staining of *M. smegmatis* mc^2155 for 3 h with 50 µM **PentaRh** or **PentaRh-6**. (Scale bar: 5 µm).

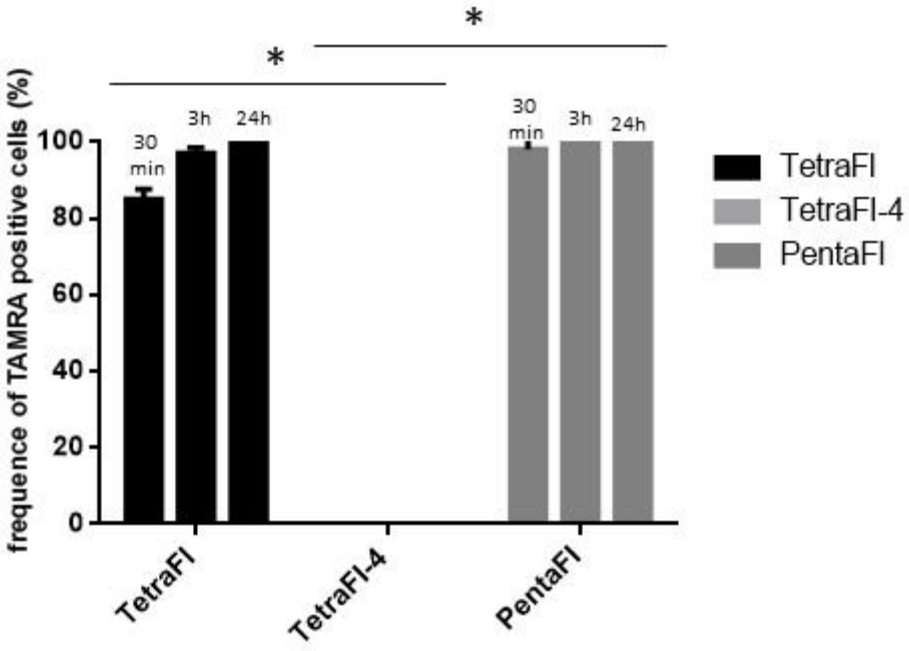


Figure S16. Amidation of the stem peptide probes at D-glutamate is required for probe incorporation in M. *tuberculosis*. *M. tuberculosis* cells were labelled with 50 μM of amidated probes (**TetraFl** and **PentaFl**) and non-amidated probe (**TetraFl-4**) for 30 min, 3 h and 24 h. Data are represented as a mean of the count of cells (n=300) of 3 independent replicas of the experiments. The student t test was used to detect statistical significance. * represents p values of < 0.0001.

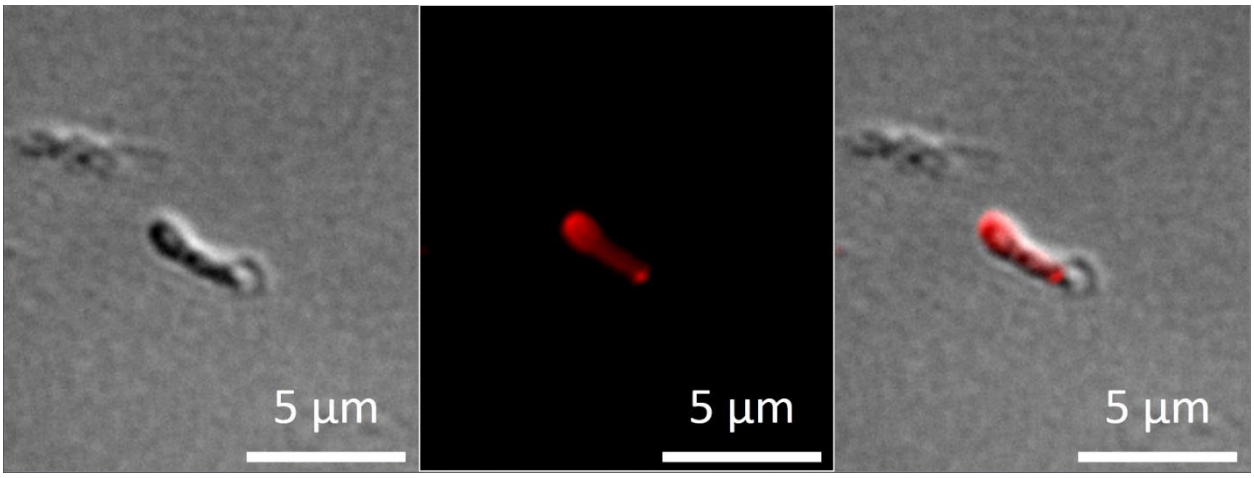


Figure S17. Confocal microscopy image of *M. tuberculosis* H37Rv treated for 24 h with 50 µM **TetraRh** with **100 ug/mL** meropenem. (Scale bar: 5 µm).

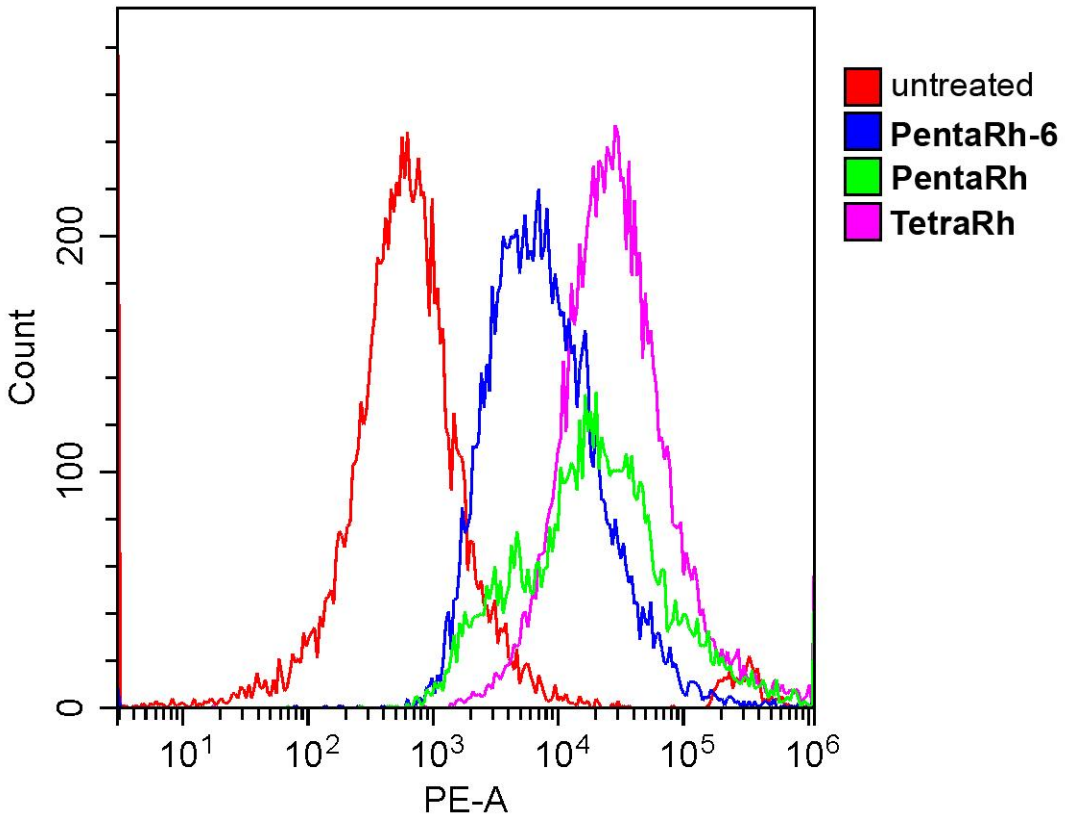


Figure S18. Flow cytometry analysis of *M. tuberculosis* H37Rv treated overnight with 50 μM of tetrapeptide or pentapeptide with variations. The Rhodamine signal is indicated on the X-axis as detected by the PE-A channel (excitation/ emission maxima ~546/579).

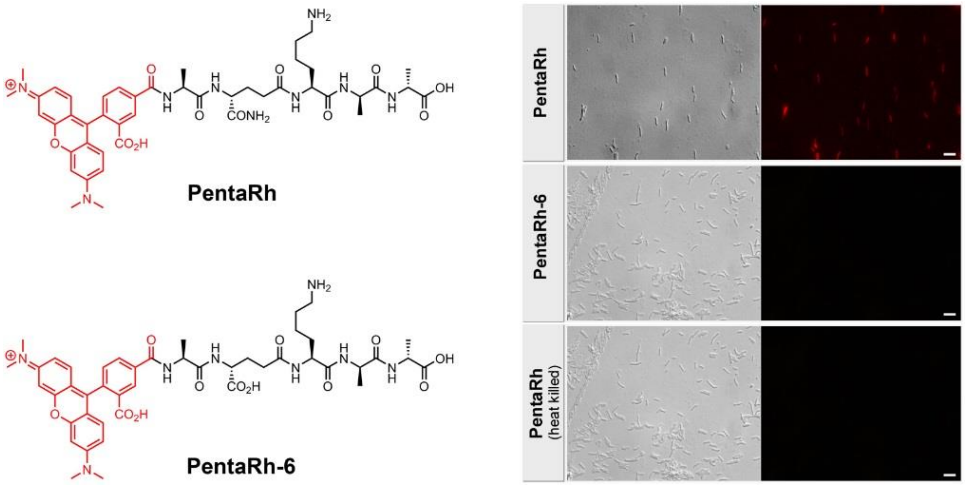


Figure S19. Staining of *M. tuberculosis* for 24 h with 50 µM **PentaRh** or **PentaRh-6**. (Scale bar: 5 µm).

Materials. All peptide related reagents (resin, coupling reagent, deprotection reagent, amino acids, and cleavage reagents) were purchased from ChemImpex. All other reagents were purchased from Sigma and were used without purification. Bacterial strains *E. faecium* D344S and M9 were grown in BHI for all experiments. D344S and M9 strains were kind gift from Dr. Arthur (Centre National de la Recherche Scientifique, Institut Pasteur). While D344S was characterized as a drug-sensitive strain, M9 was found to be highly drug-resistant. The main mode of drug-resistance related to M9 involves a D,D-carboxypeptidase step in the intracellular space that leads to both cross-resistance to glycopeptide and β-lactam antibiotics. *E. faecium* ATCC BAA-2317 (VanA) and *E. faecium* ATCC BAA-2127 (drug sensitive) were grown in Trypticase soy broth (TSB). All *Mycobacterium smegmatis* strains were grown in lysogeny broth (LB) containing 0.05% Tween-80 unless noted otherwise.

Confocal microscopy analysis of *E. faecium* labeled with TetraRh, PentaFl, and DADA. Brain heart infusion (BHI) broth containing 500 µM **TetraRh,** 500 µM **PentaFl,** and 5 mM **DADA** was prepared. *E. faecium* (D344S) from an overnight growth was added to the medium (1:10 dilution) and incubated at 37 ºC with shaking at 250 rpm for 5 minutes. The bacteria were immediately harvested at 6,000g and washed three times with original culture volume of 1x PBS followed by fixation with 2% formaldehyde in 1x PBS for 30 min at ambient temperature. The cells were washed once more to remove formaldehyde and then analyzed using a Nikon C2 confocal microscope. For *Mycobacterium smegmatis* ATCC 14468 (WT), the previous procedure was repeated except using LB (0.05% tween) as the growth media and a 30 minute incubation.

***In vivo* labeling of *E. faecium* with TetraRh in live *C. elegans*.** N2 *Caenorhabditis elegans* were maintained by standard protocol using nematode growth agar with bacterial lawns of *E. coli* OP50 (source) on a 60mm x 15mm cell culture dish. *C. elegans* were grown to contain primarily L4 larval stage nematodes by incubation at ambient temperature for ~48-52 h, washed off the plates with M9 buffer, and washed three times with M9 buffer. For washing steps, the *C. elegans* were pelleted at 1000g. The *C. elegans* were resuspended in 400 µL of M9 buffer containing 10% BHI broth and transferred to a sterile 24 multiwell plate. *E. faecium* (100 µL from overnight growth) was washed and added to the 400 µL suspension of *C. elegans*. The *C. elegans* were incubated at ambient temperature for 4 h, harvested at 1000g and washed three times with M9 buffer to remove bacteria on the outside of the *C. elegans*. The *C. elegans* were then resuspended in 500 µL of M9 buffer containing 10% BHI broth and 50 µM **TetraRh**, and incubated at ambient temperature for 2 h. The *C. elegans* were harvested at 1000g and washed three times with M9 buffer and resuspended in 10mM sodium azide in M9 buffer and analyzed by confocal microscopy.

Bacterial growth conditions for *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv. *Mycobacterium smegmatis* mc²155 was grown at 37 ºC in Middlebrook 7H9 broth supplemented with 0.2% glucose, 0.2% glycerol, 0.085% NaCl and 0.05% Tween80. *M. tuberculosis* H37Rv was grown at 37 °C in Middlebrook 7H9 broth supplemented with 10% OADC. for *M. smegmatis*, broth cultures were incubated 37 ºC with shaking at a 100 rpm. The antibiotics used for media supplementation were at the following concentrations: Meropenem (100 µg/ml) and clavulanate (100 µg/ml) which have sub-lethal effects on mycobacteria.

Microscopy and Flow cytometry analysis of *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv labeled with TetraRh, PentaRh and PentaRh6 probes. Five militres cultures of *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv were grown at 37 °C to an OD_{600nm} of 0.8 and 1, respectively, in Middlebrook 7H9 broth. The 5 ml cultures of *M. smegmatis* and *M. tuberculosis* H37Rv were pelleted by centrifugation at 4000 xg for 5 min. The supernatant was discarded and the cells were resuspended in 2.5 ml of Middlebrook 7H9 broth. Four microliters of either the

TetraRh, **PentaRh** and **PentaRh6** (5 mM) PG probe was added to 396 µl of *M. smegmatis* mc^{2}155 and *M. tuberculosis* H37Rv cells (making a final concentration of 50 µM **TetraRh**, **PentaRh** or **PentaRh6** probe). As controls, Meropenem-Clavulanate (100 µg/ml) treated - and heat killed - *M. smegmatis* mc^{2}155 and *M. tuberculosis* H37Rv were also used for the labeling experiment. Heat killing was performed on a heating block set at 65 ºC and inserted into a BioSafety Cabinet for 24 h prior to addition of the PG probes. The *M. smegmatis* and *M. tuberculosis* labelling experiment was performed for 24 h, however, sampling for analysis of probe incorporation was done after 30 min, 3 h, 6 h, 9 h and 24 h of incubation at 37°C. Thereafter, the cells were washed in 1x PBS (500 µl) three times to remove unincorporated probe and the cells were then resuspended in 100 µl of PBS followed by fixation with 2.5% glutaraldehyde for 24 h. The cells were washed three times with 1x PBS (500 µl) to remove the glutaraldehyde and then resuspended in 500 µl 1x PBS. For microscopy, 5 µl of the cells was spotted on glass slides and viewed with the Zeiss Observer Z1 inverted fluorescence microscope under the DS-red channel (excitation/ emission maxima ~546/579) and the images were analyzed with the ZEN lite software (Zeiss). For flow cytometry analysis of incorporation of the different probes, 100 µl of the cells was transferred to a 96 well plate and the Cytoflex flow cytometer (Beckman Coulter) was used for analysis of probe incorporation. The PE-A channel (excitation/ emission maxima ~470/585) was used for detection of the Rhodamine signal (excitation/ emission maxima ~561/578). The gain for the PE-A channel was reduced from 370 to 120.

Peptidoglycan Isolation. BHI medium (200 mL) containing 500 µM **TetraFl** was prepared. *E. faecium* D344S bacteria were added to the BHI medium (1:100) and allowed to grow overnight at 37 ^{o}C with shaking at 250 rpm. The cells were harvested and washed with 1x phosphate buffer saline (PBS) (3 × 50 mL each). The cells were then resuspended in 1x PBS and boiled for 7 min and then centrifuged at 14,000g for 8 min at 4 °C. Cells were then placed in 25 mL of 5% (w/v) sodium dodecyl sulfate (SDS) and boiled for 25 min followed by centrifugation at 14,000g for 8 min at 4 °C. Following centrifugation, cells were boiled again in 25 mL of 4% (w/v) SDS for 15 min followed by centrifugation using same parameters as before. Cells were then washed 5 times with 60 °C DI water to remove all SDS. After washing, cells were resuspended in 6 mL of 20 mM Tris buffer (pH 8.0). The cells were treated with 800 µg DNase for 24 hrs followed by Trypsin (800 ug) for another 24 hrs (37 ^{O}C shaking at 80 rpm). Cells were then boiled for 30 min. Lysozyme (250 ug/mL) was added and cells incubated for 24 hrs at 37 ^{o}C shaking at 250 rpm. Following lysozyme digestion, cells were frowzen and lyophilized, then dissolved in 0.375 M sodium borate buffer (pH 9.0) prepared in HPLC grade water. Sodium borohydride (10 mg) in 1 mL borate buffer was added for 30 min, then quenched with 125 uL phosphoric acid. The cell wall digest was filtered using a 0.45 um and 30K centrifuge filter respectively, freezed and lyophilized. The digest was then analyzed by LC-MS.

Mass Spectrometry Analysis of Stem Peptides incubated with *E. faecium*. BHI medium was prepared with 100 µM **TetraFl** or **PentaFl**. *E. faecium* D344S bacteria were added to the BHI medium (1:100) and allowed to grow overnight at 37 ^{o}C with shaking at 250 rpm. Samples of 100 µM TetraFl or PentaFl were also prepared in BHI medium and incubated overnight 37 ^{o}C with shaking at 250 rpm without the addition of bacteria (standards). The bacteria were harvested, supernatant collected and analyzed by Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

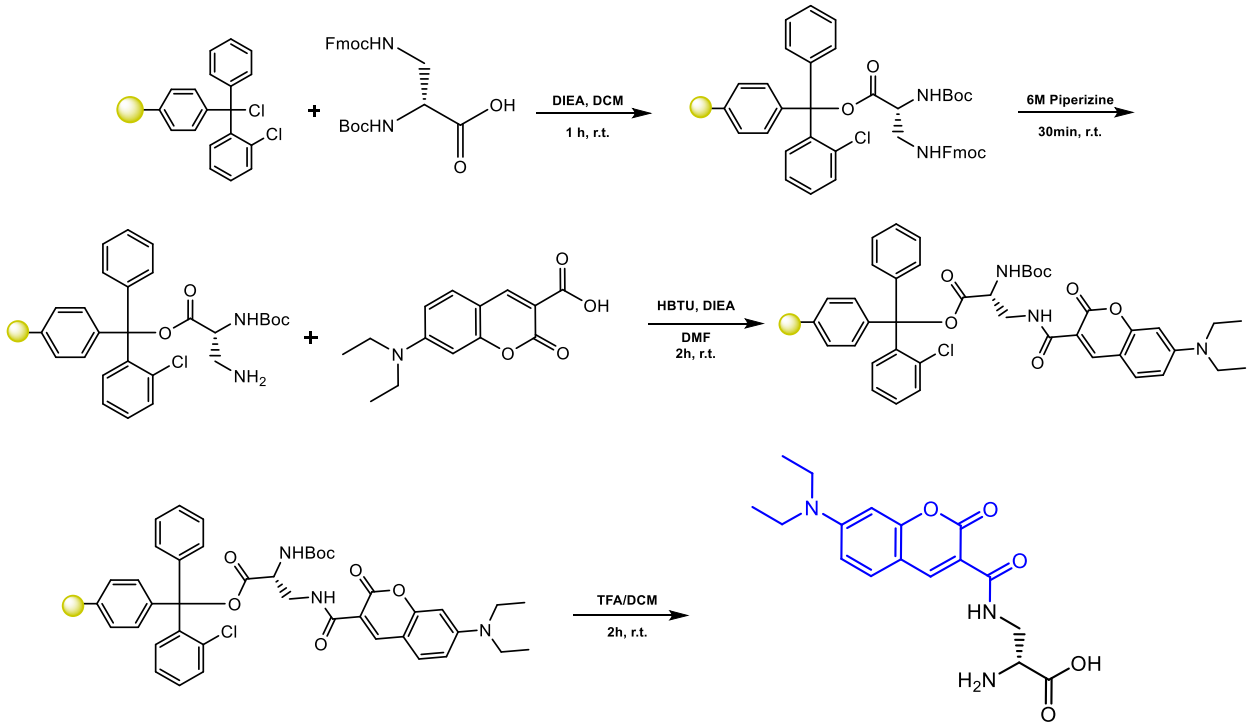
PG Digestion/Lysozyme Treatment of Labeled Peptidoglycan. *E. faecium* D344S (1:100) were added to the BHI medium or BHI medium containing 100 µM **TetraFl** and allowed to grow overnight at 37 ^{o}C with shaking. The bacteria were harvested at 6,000g and washed three times with original culture volume of 1x PBS followed by fixation with 2% formaldehyde in 1x PBS for 30 min at ambient temperature. Cells were washed once more to remove the formaldehyde. Cells

were washed three times with 1x PBS. The bacteria were resuspended in 1x PBS containing 500 μg/mL lysozyme (MP biomedicals) and incubated at 37°C. A portion of the cells were taken at 1, 5, 30, 60, and 120 min. At each time point, the collected bacteria were washed three times with 1x PBS and resuspended in a final solution of 1x PBS containing 4% formaldehyde to quench the lysozyme reaction. Cells were analyzed using a BDFacs Canto II flow cytometer using the previously stated parameters.

Confocal Imaging of Sacculi. Peptidoglycan Isolation. BHI medium (**1**00 mL) containing 500 μM **TetraFl-3** was prepared. *E. faecium* D344S bacteria were added to the BHI medium (1:100) and allowed to grow overnight at 37 °C with shaking at 250 rpm. The cells were harvested and washed with 1x phosphate buffer saline (PBS) (3 × 50 mL each). The cells were then resuspended in 1x PBS and boiled for 7 min and then centrifuged at 14,000*g* for 8 min at 4 °C. Cells were then placed in 25 mL of 5% (w/v) sodium dodecyl sulfate (SDS) and boiled for 25 min followed by centrifugation at 14,000g for 8 min at 4 °C. Following centrifugation, cells were boiled again in 25 mL of 4% (w/v) SDS for 15 min followed by centrifugation using same parameters as before. Cells were then washed 6 times with 60 °C DI water to remove all SDS. After washing, cells were resuspended in 6 mL of 20 mM Tris buffer (pH 8.0). Pellets were treated with 800 μg DNase for 24 hrs followed by trypsin (800 μg) for another 24 hrs (37 °C shaking at 80 rpm). Pellets were boiled for 25 min followed by centrifugation at 14,000g for 8 min at 4 °C, resuspended in 5 mL of 1X PBP, and imaged on a glass slide.

MIC Determination. MIC reported for the eight antibiotics were carried out using the microdilution method according to the Clinical and Laboratory Standards Institute recommendations with the change in medium (Brain-Heart Infusion).

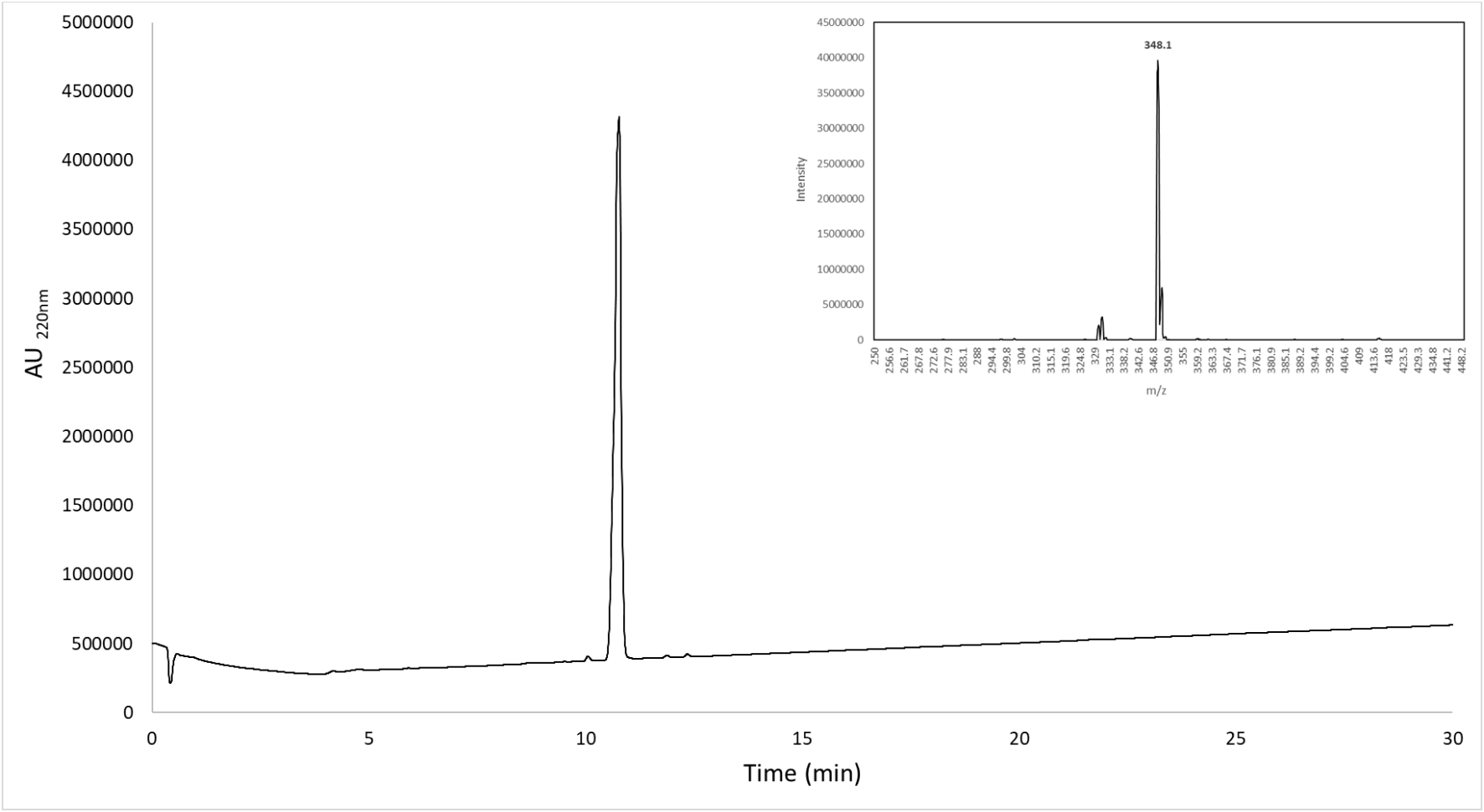
Scheme S1. Synthesis of (Diethylamino)coumarin-carbonyl-amino-D-alanine (DADA)



DADA

A 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added N^{α}-Boc-N^{β}-Fmoc-D-2,3-diaminopropionic acid (1.1 eq, 258 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was shaken for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min then washed as before. 7-(Diethylamino)coumarin-3-carboxylic acid (3 eq, 430 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **DADA**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

^{1}H NMR (400 MHz, DMSO) : δ 1.15 (t, 6H, CH_2-C*H*$_3$), 3.80 (m, 2H, -CH-C*H*$_2$-NH), 4.15 (t, 1H, -C*H*-CH_2-NH-), 6.65 (s, 1H, -C-C*H*-C-), 6.85 (d, 1H, -C-C*H*-CH-), 7.70 (d, 1H, -CH-C*H*-C-), 8.70 (s, 1H, -C-C*H*-C-) ^{13}C NMR (DMSO) : δ 12.76, 44.82, 52.43, 96.28, 108.00, 109.28, 110.70, 132.22, 148.41, 153.09, 157.79, 162.01, 163.84, 169.92 ESI-MS: $[M+H]^+$ calculated: 348.1 found: 348.1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25
30
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Intensity
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35000000
30000000
25000000
20000000
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5000000
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267.8
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277.9
283.1
288
294.4
299.8
304
310.2
315.1
319.6
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329
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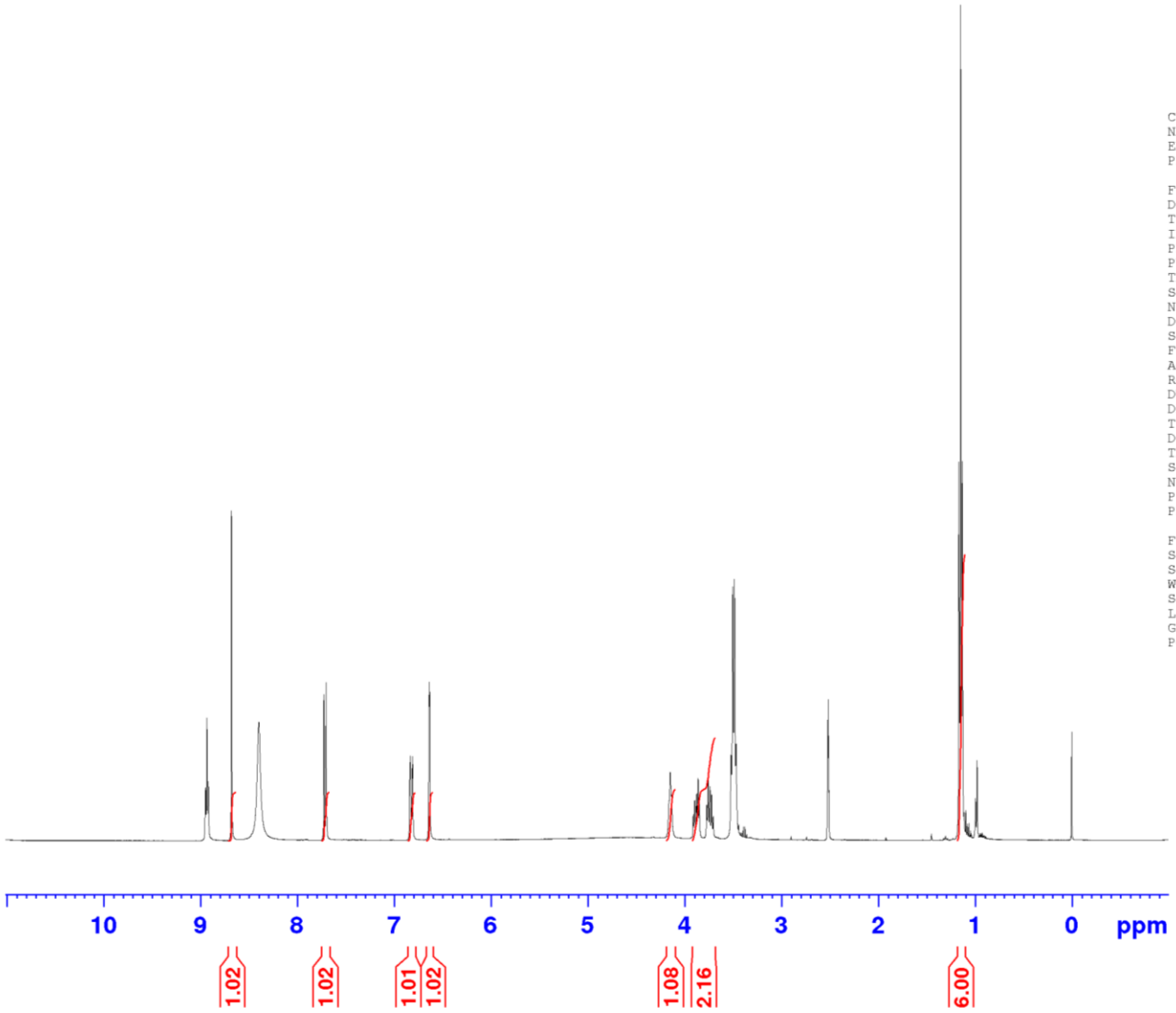
m/z

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DE                 6.50 usec
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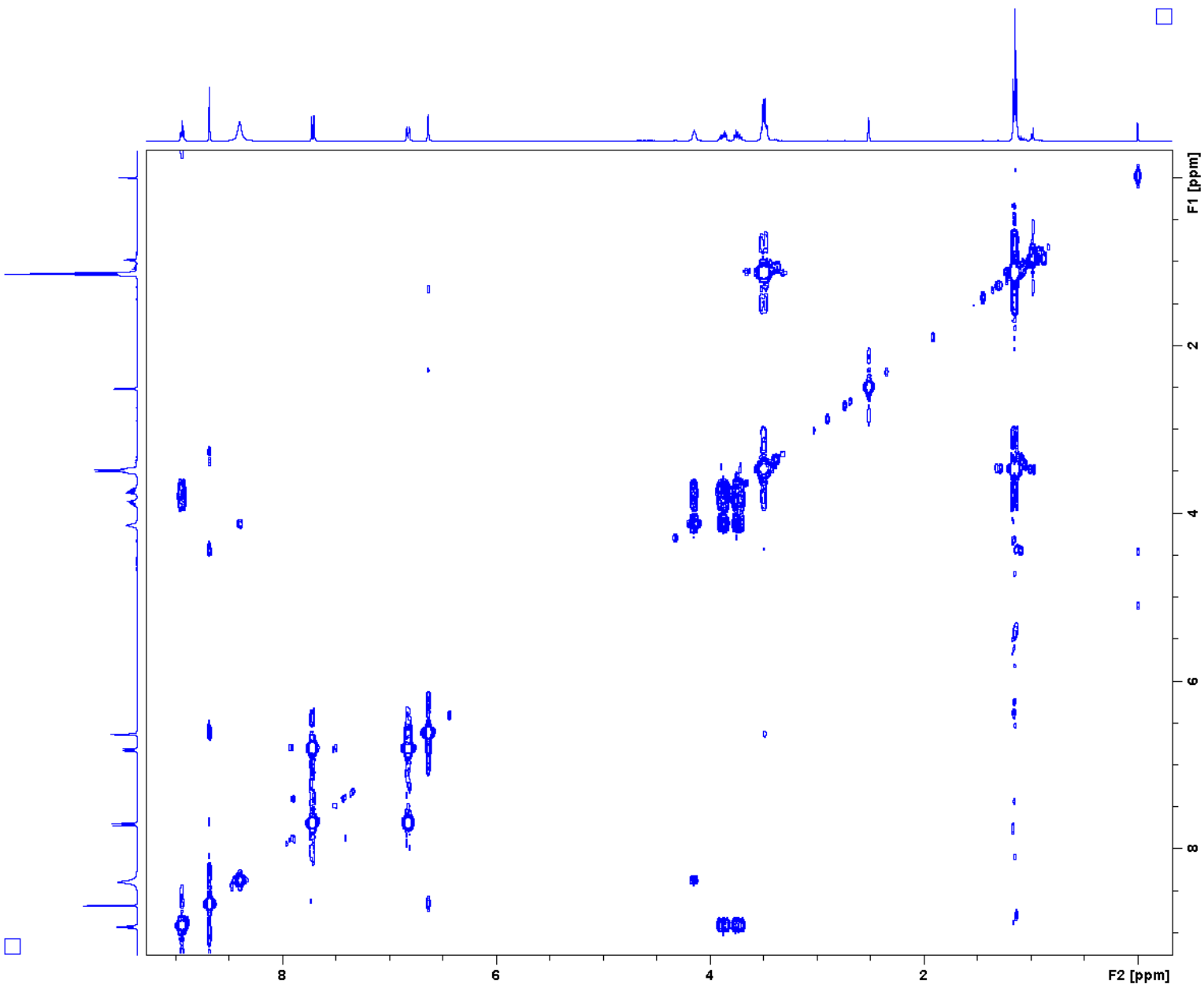
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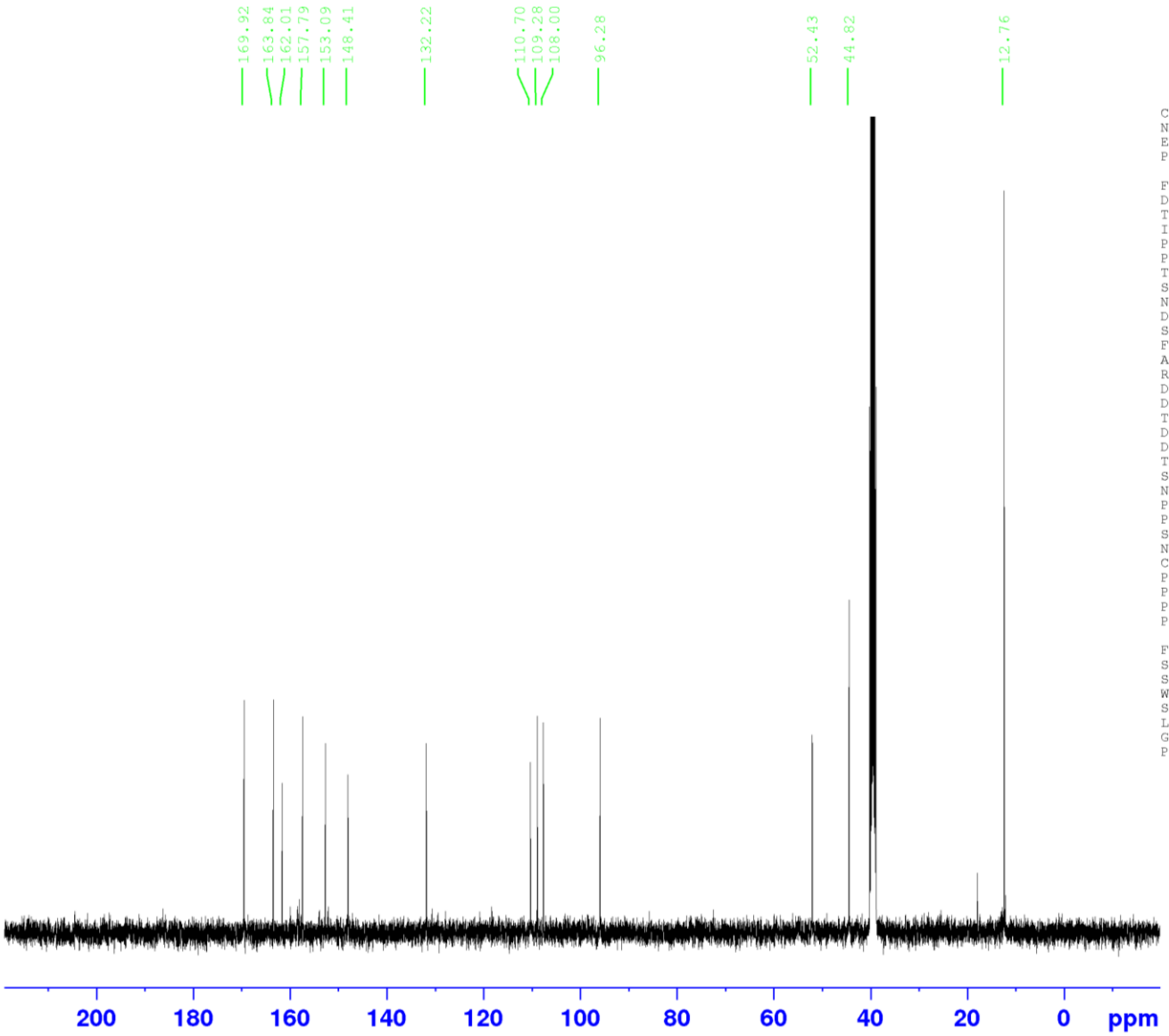


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NUC2                 1H
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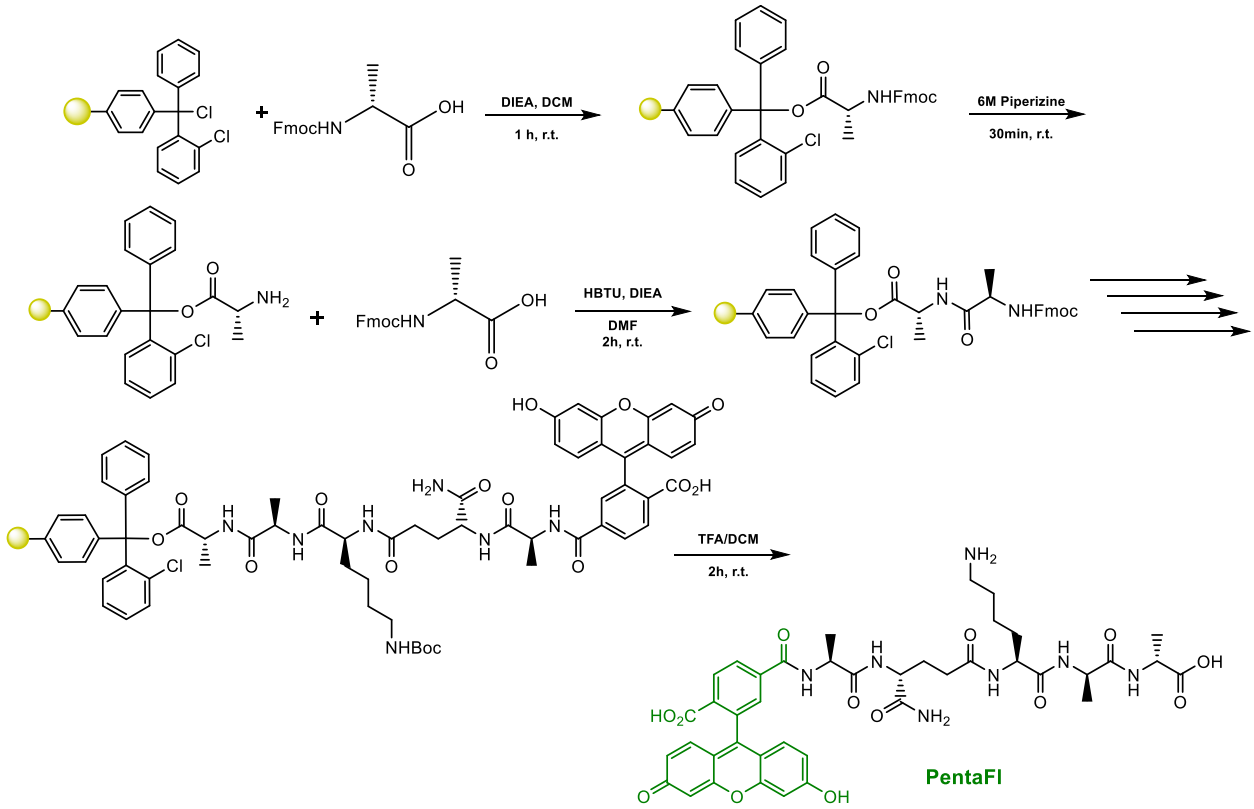
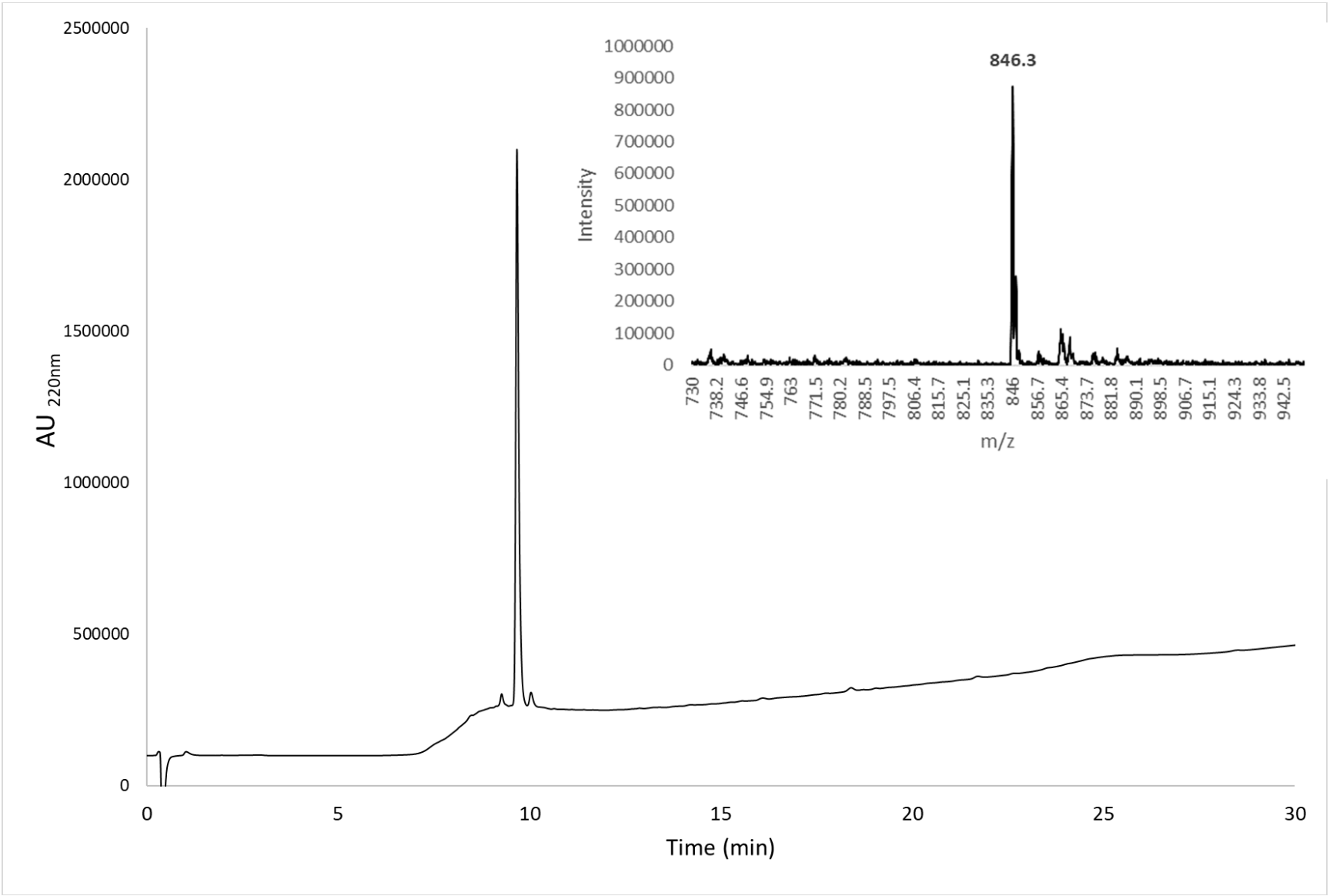
Scheme S2. Synthesis of PentaFl



A 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 846.3 found: 846.3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800000
700000
600000
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300000
200000
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746.6
754.9
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771.5
780.2
788.5
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806.4
815.7
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865.4
873.7
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915.1
924.3
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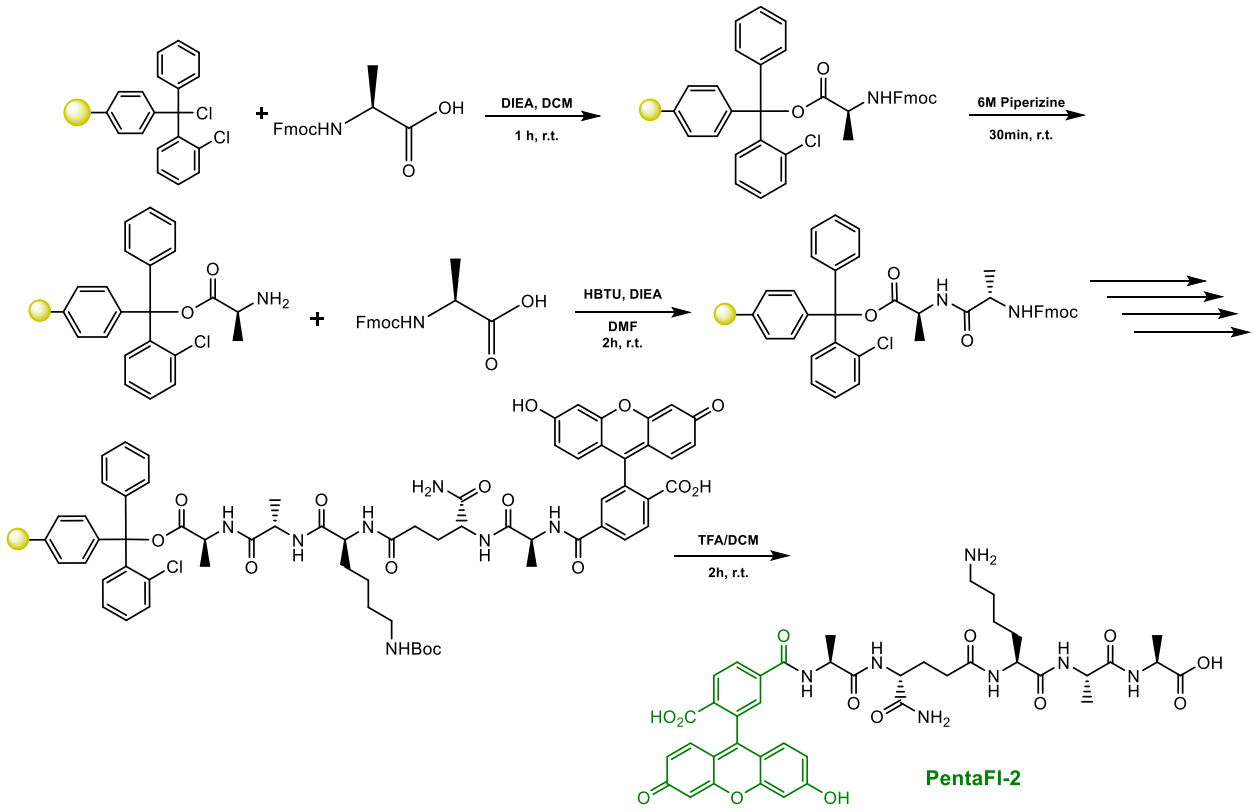
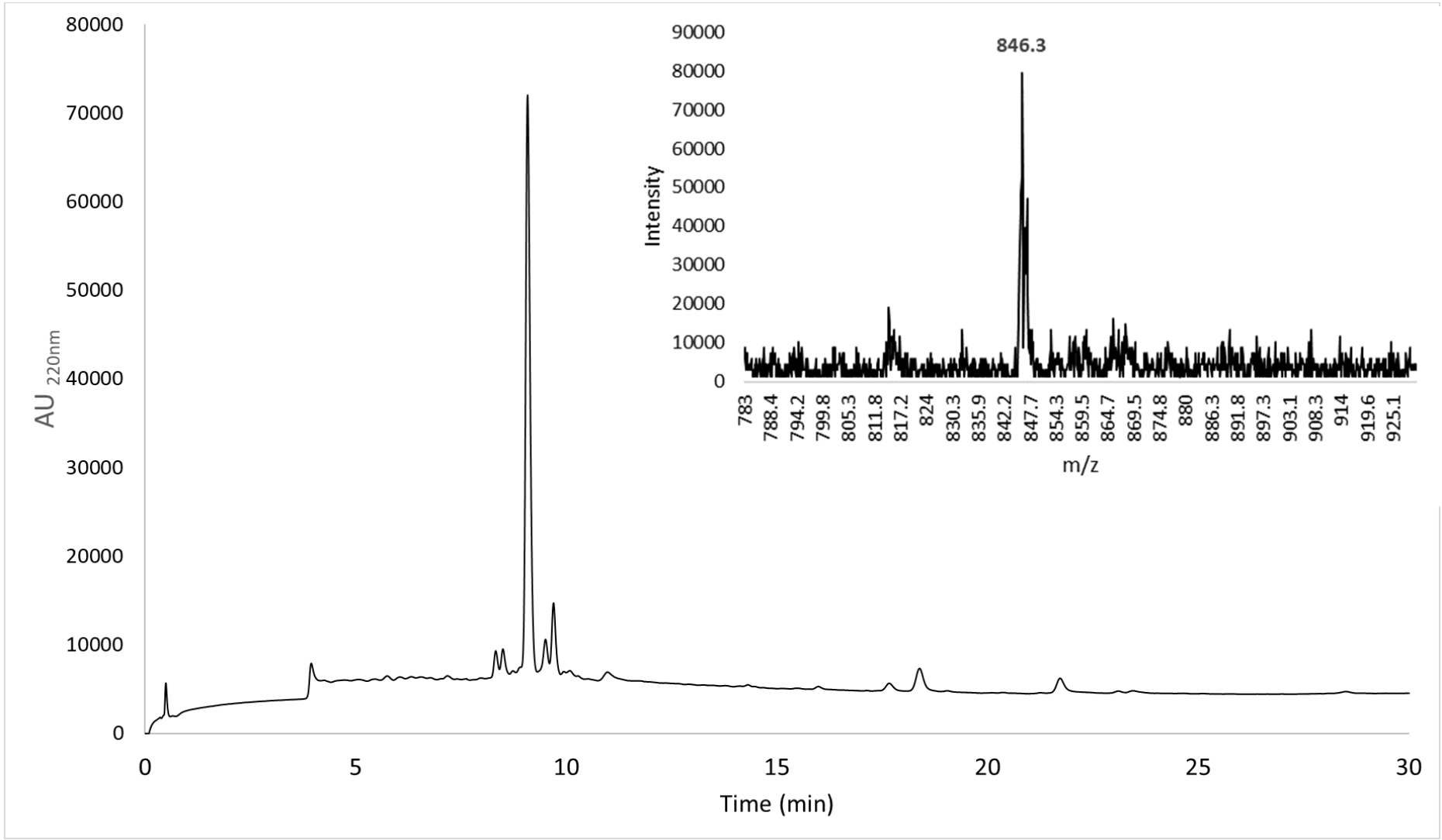
942.5
m/z

Scheme S3. Synthesis of PentaFl-2



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-L-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl-2**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 846.3 found: 846.3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Intensity
90000
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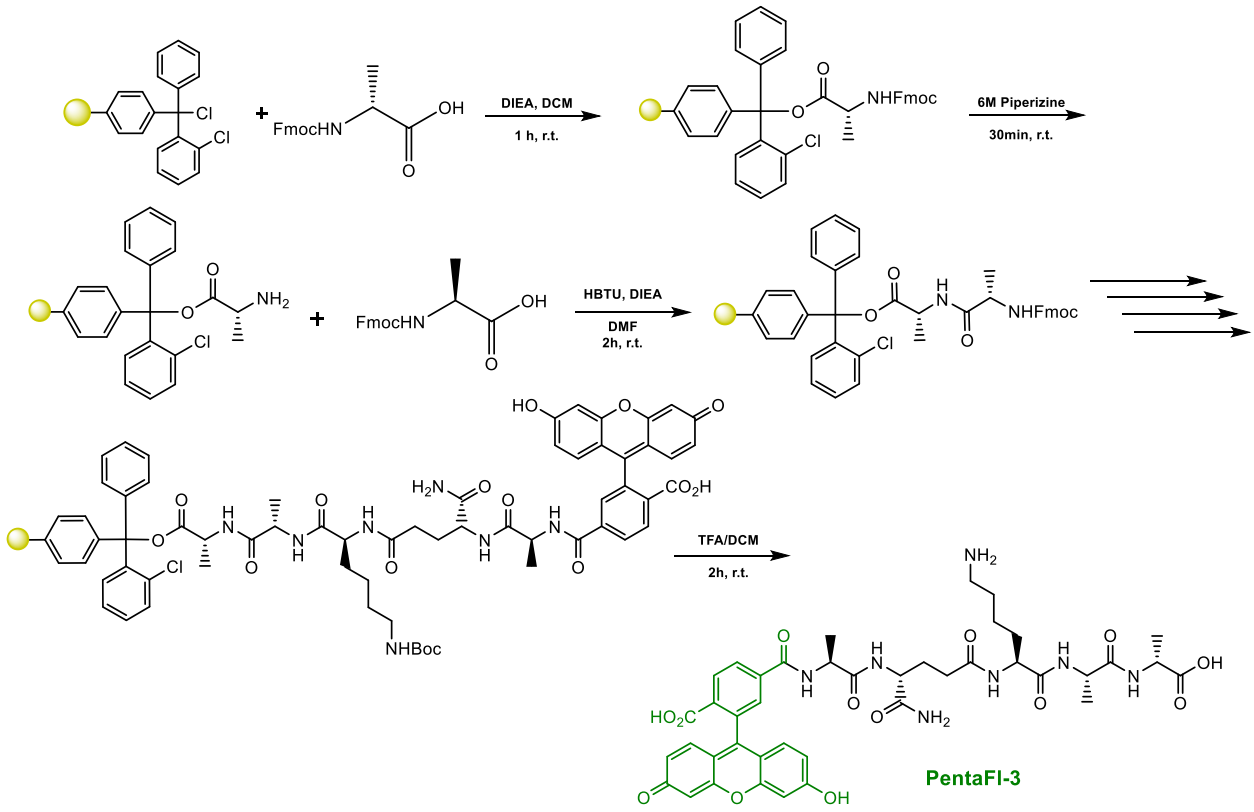
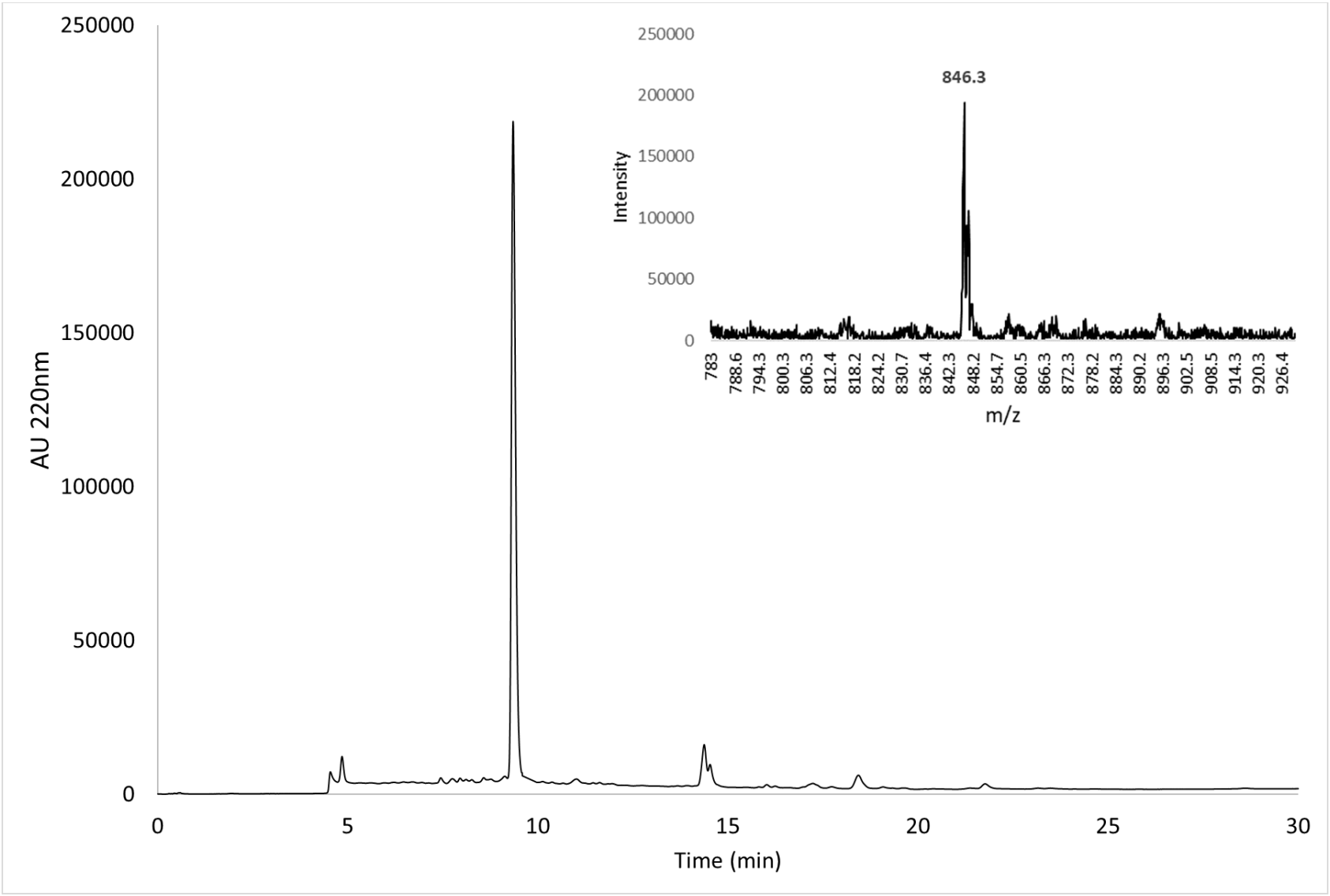
919.6
925.1
m/z

Scheme S4. Synthesis of PentaFl-3



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl-3**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 846.3 found: 846.3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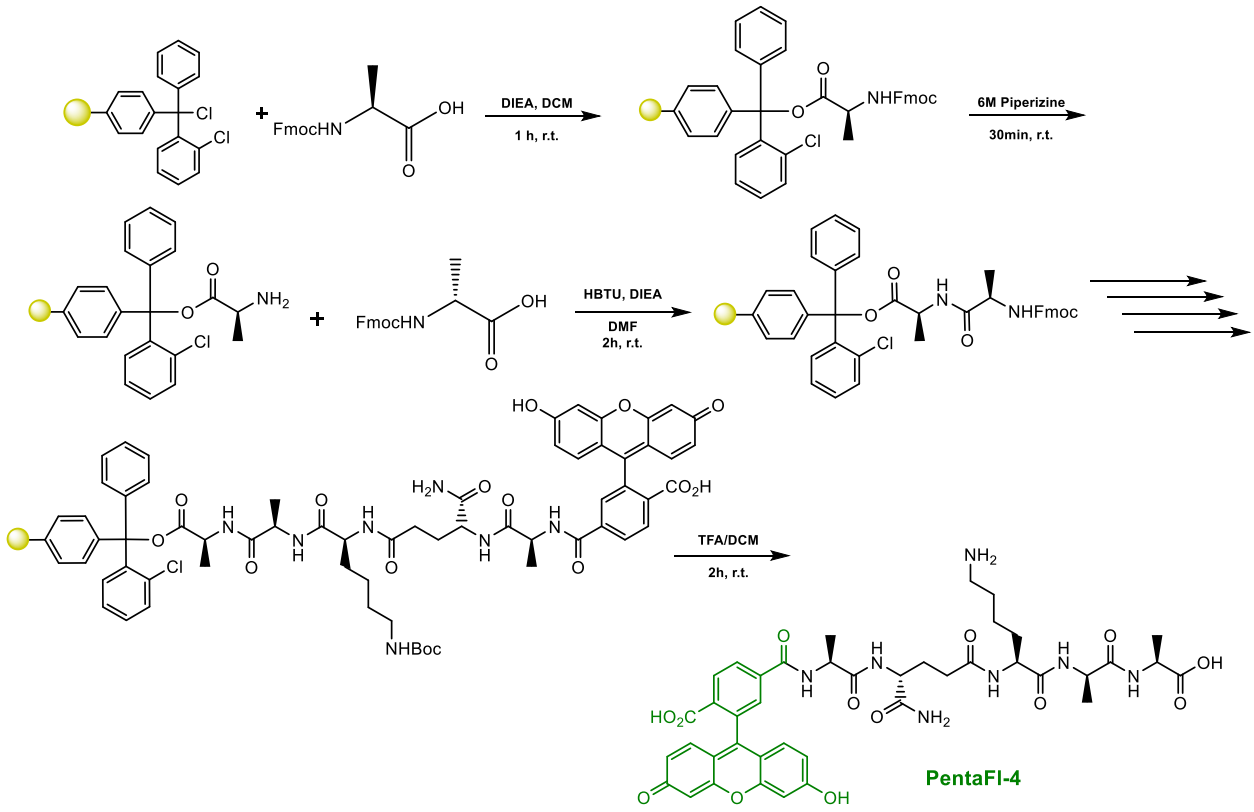
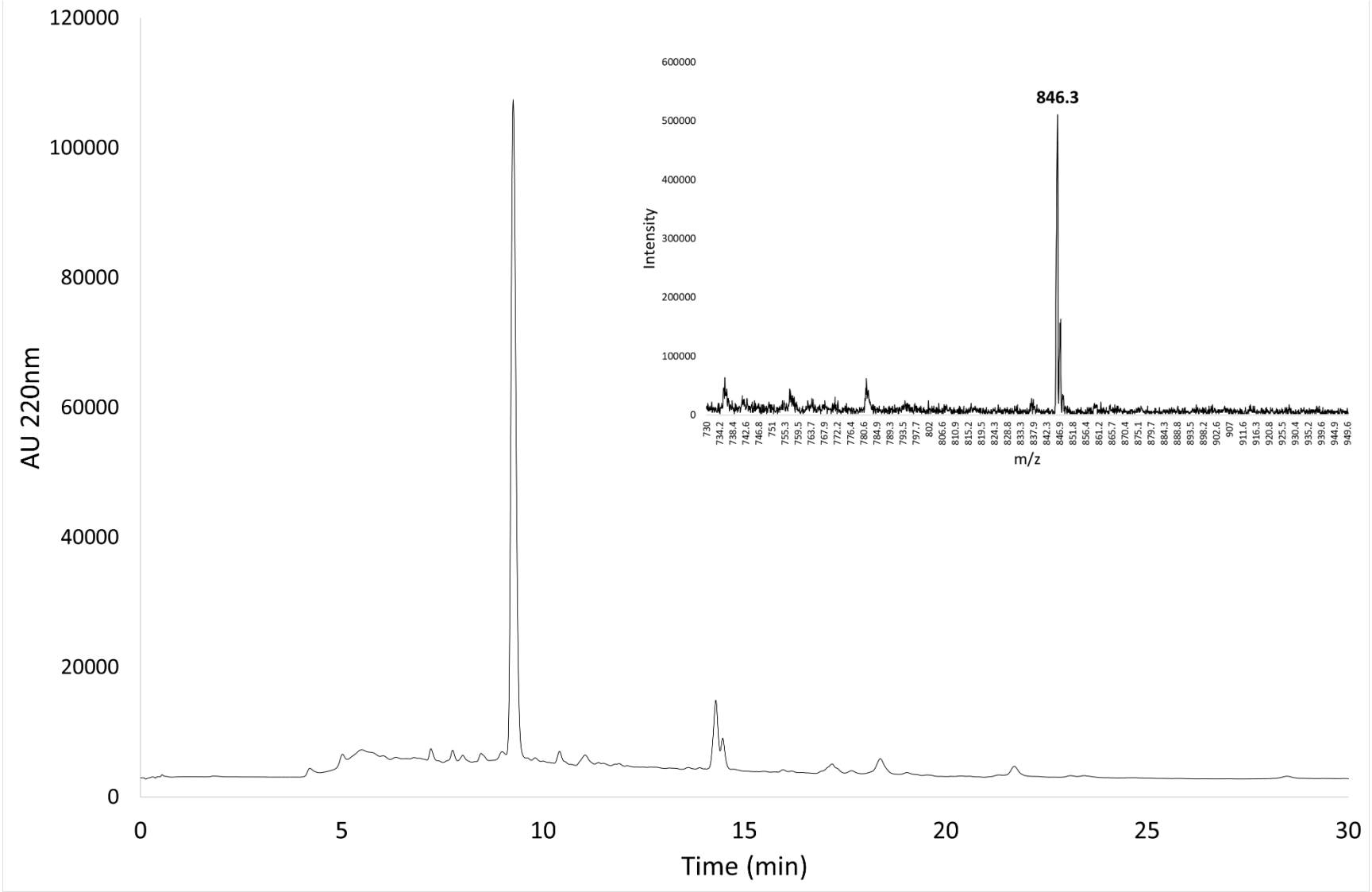
Scheme S5. Synthesis of PentaFl-4



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-L-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl-4.** The sample was analyzed using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 846.3 found: 846.3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Intensity
m/z
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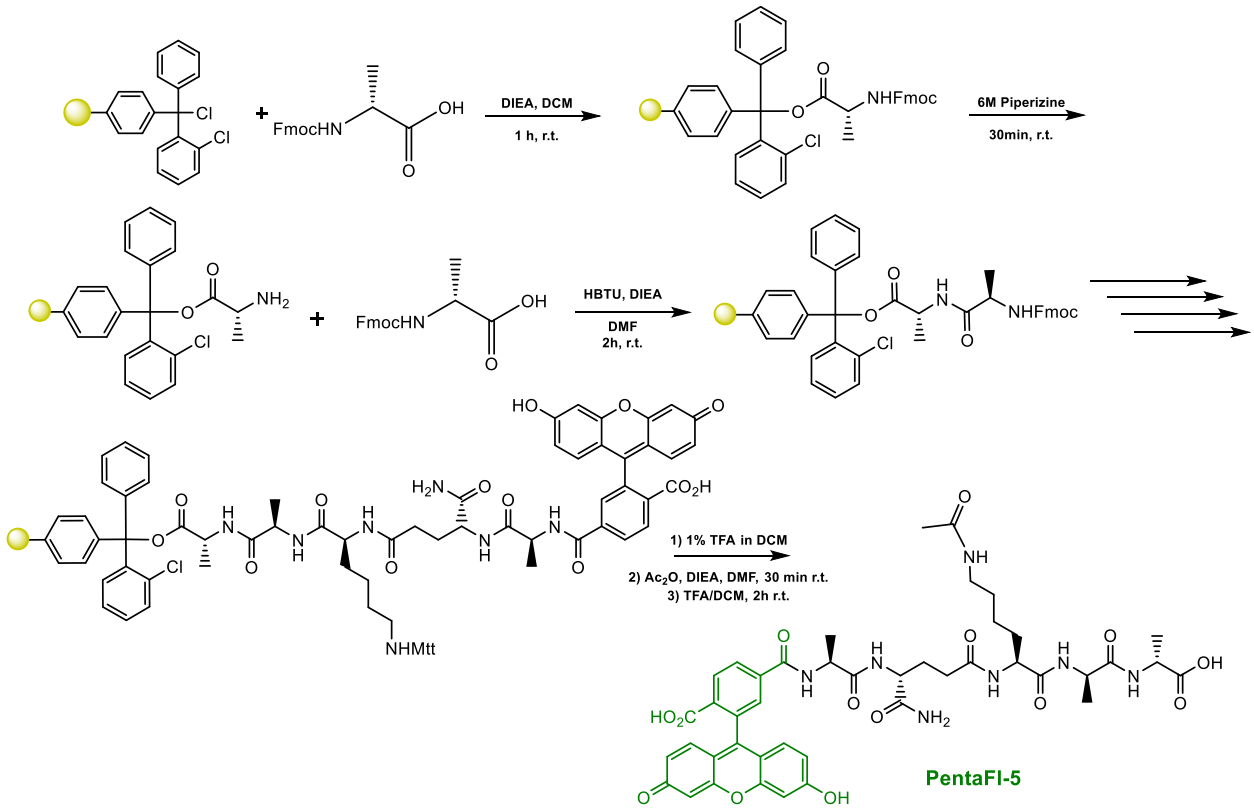
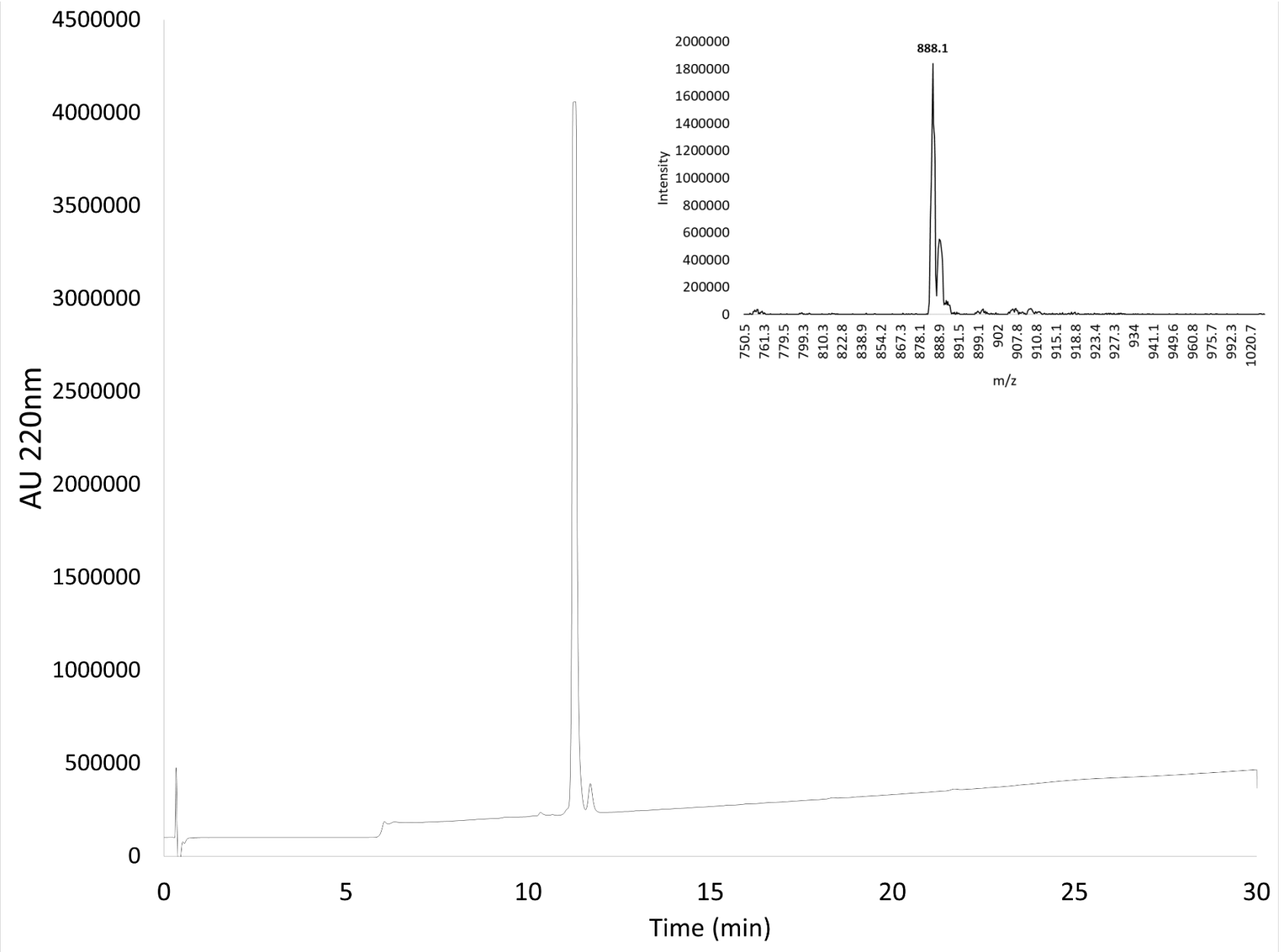
Scheme S6. Synthesis of PentaFl-5



To a 25 mL peptide synthesis vessel charged with Fmoc-D-Alanine Wang resin (950 mg, 0.55 mmol). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed with MeOH and DCM (3 x 15 mL each). Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) was added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Mtt)-OH, Fmoc-D-glutamic acid α-amide and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of 1% TFA / 5% TIPS in DCM and shaken for 10 min and washed. The step was repeated five times for removal of the Mtt group. Acetic anhydride (5 eq, 0.260 mL) and DIEA (10 eq, 0.956 mL) in DMF was added and resin shaken for 30 min at ambient temperature. The resin was washed and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl-3.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 888.3 found: 888.1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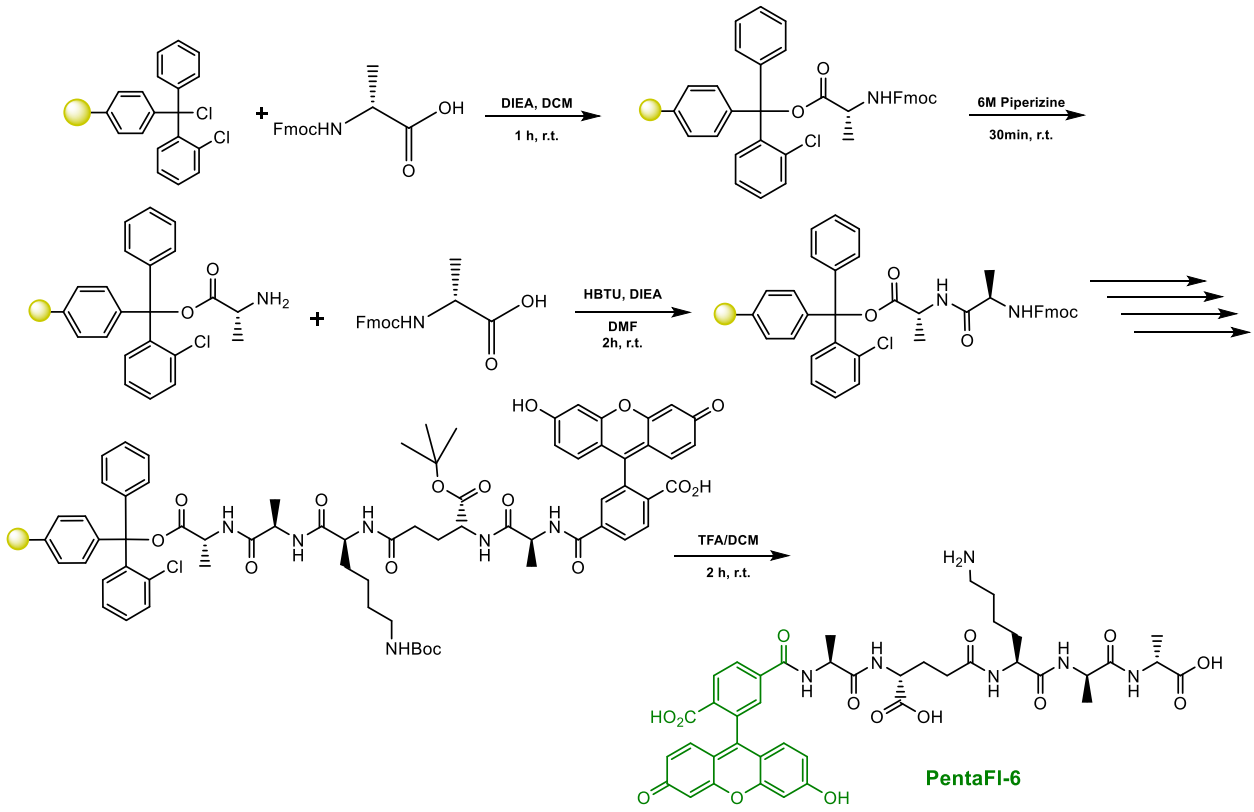
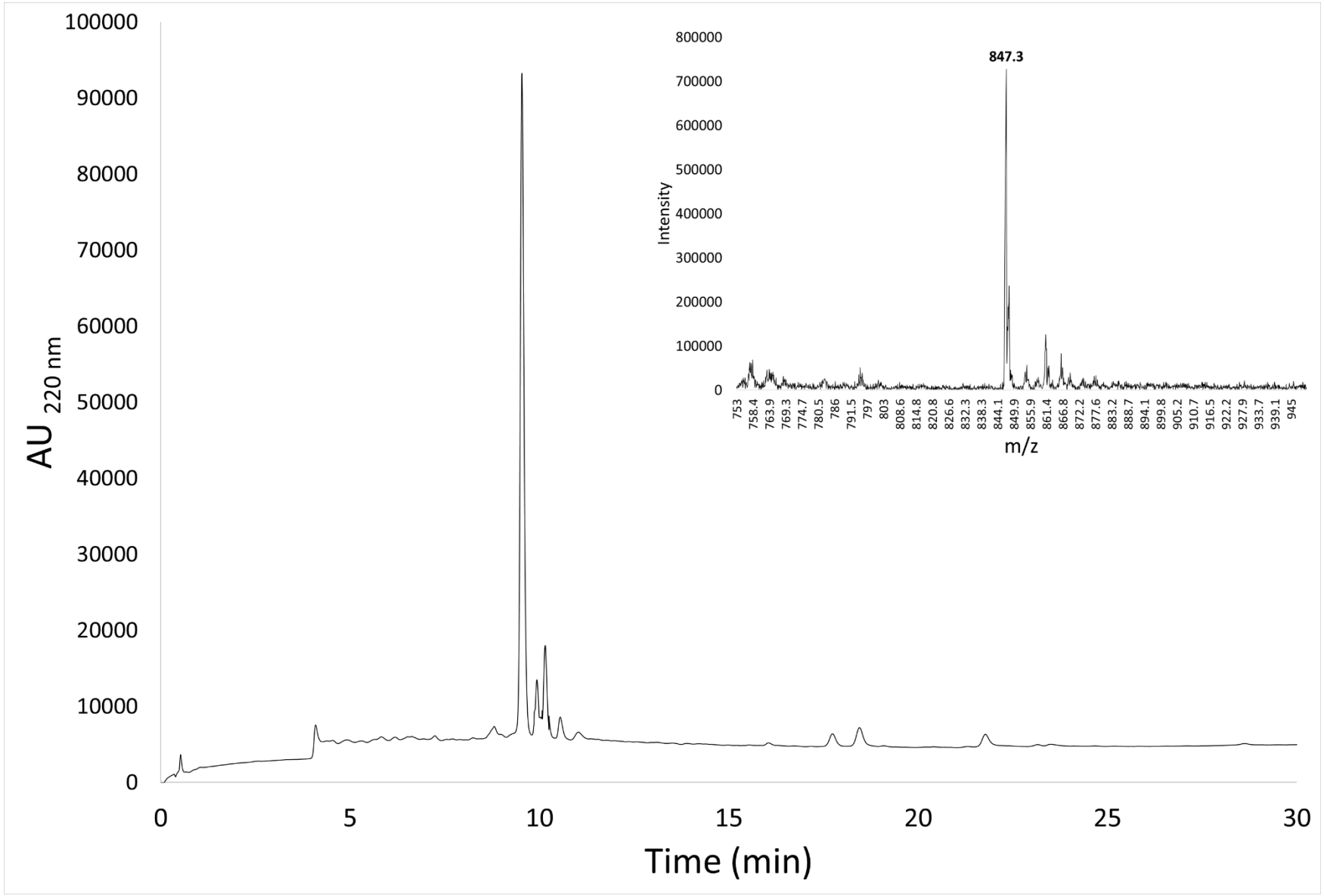
m/z

Scheme S7. Synthesis of PentaFl-6

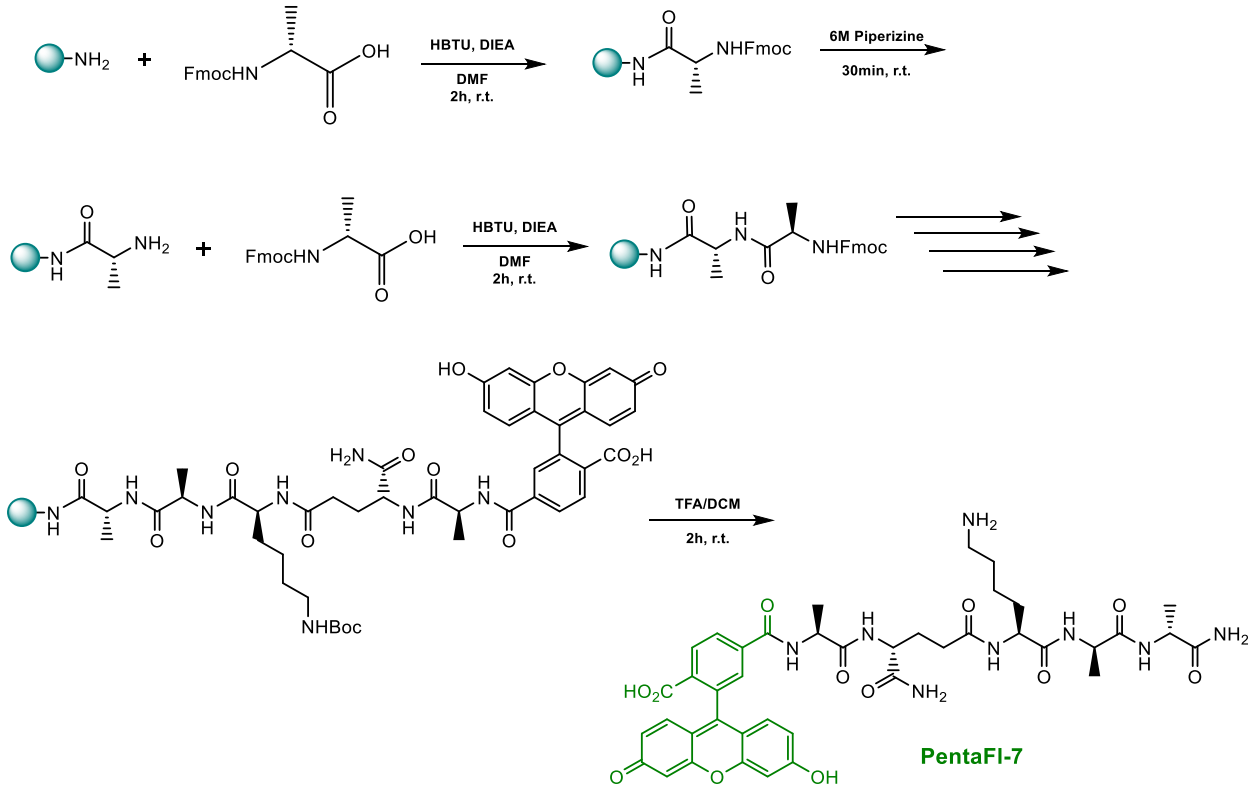


To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-Glu(OtBu)-OH, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl-6.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 847.3 found: 847.3

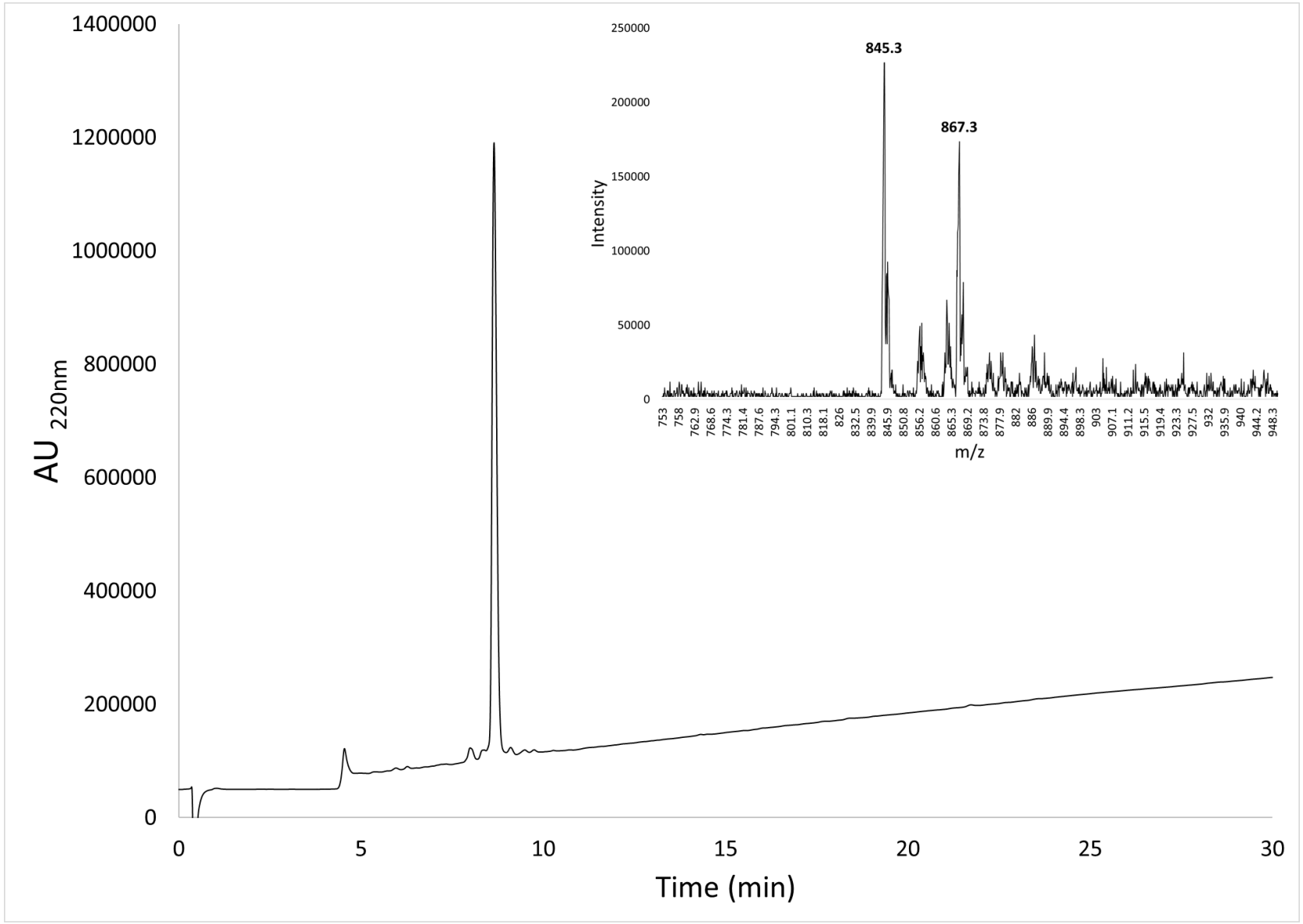

AU 220 nm
Time (min)
Intensity
m/z
847.3

Scheme S8. Synthesis of PentaFl-7

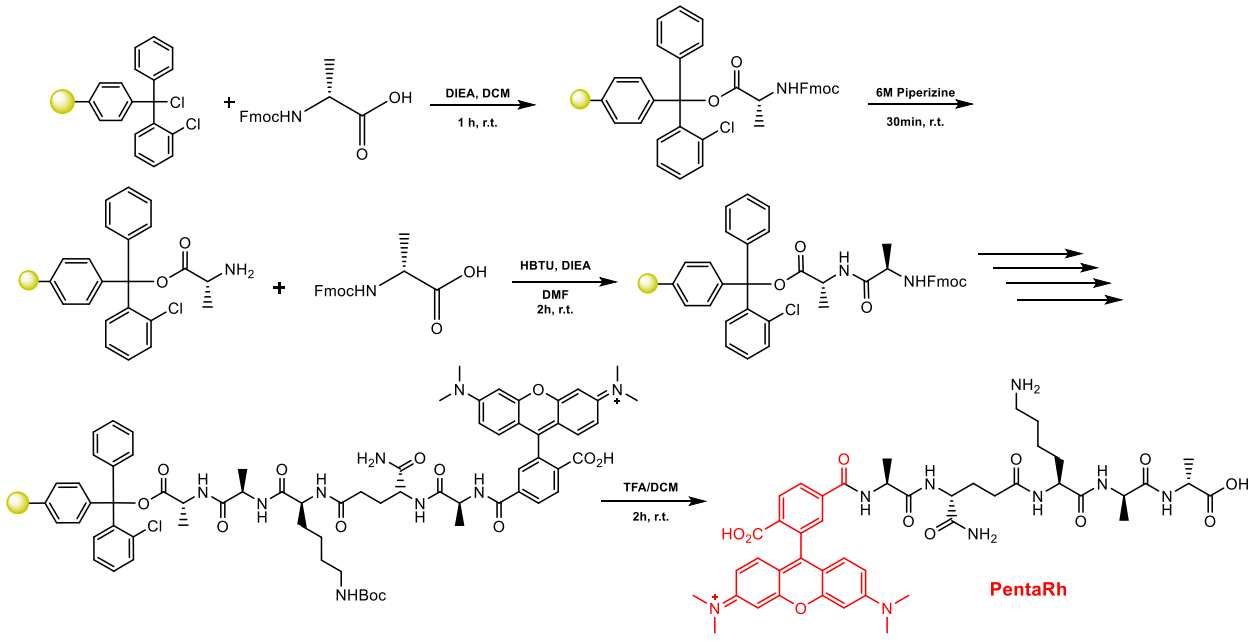


To a 25 mL peptide synthesis vessel charged with Rink Amide Resin (500mg, 0.30mmol). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed with MeOH and DCM (3 x 15 mL each). Fmoc-D-alanine (3 eq, 280 mg, 0.90 mmol), HBTU (3 eq, 341 mg, 0.90 mmol), and DIEA (6 eq, 0.314 mL, 1.80 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-alanine, Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 226 mg, 0.60 mmol), HBTU (2 eq, 228 mg, 0.60 mmol) and DIEA (6 eq, 0.314 mL, 1.80 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaFl-7.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 845.3 found: 845.3

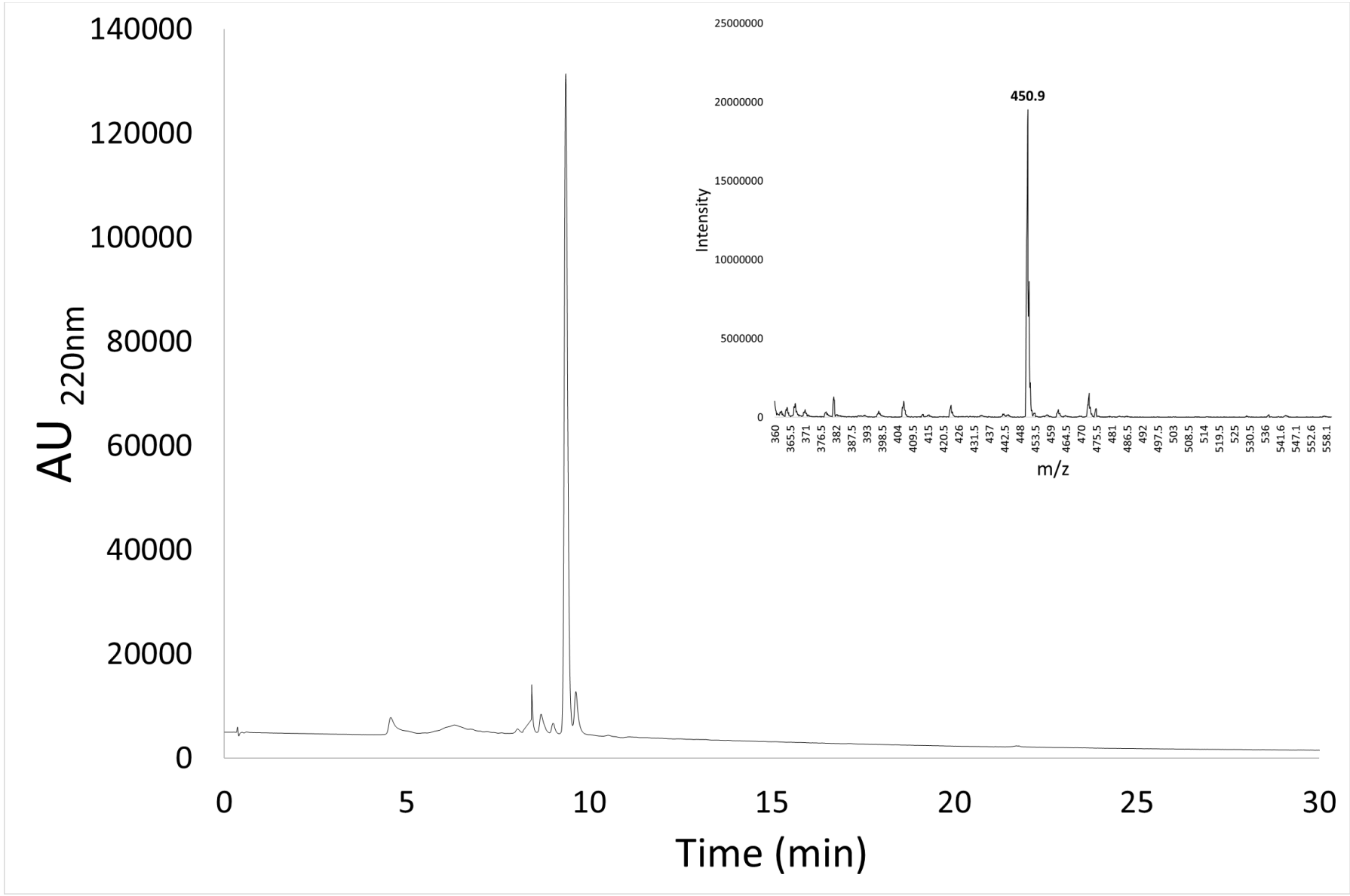

AU 220nm
Time (min)
Intensity
m/z
845.3
867.3

Scheme S9. Synthesis of PentaRh



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxy-tetramethylrhodamine (2 eq, 474 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaRh-6**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+2H]^{2+}$ calculated: 451.2 found: 450.9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80000
60000
40000
20000
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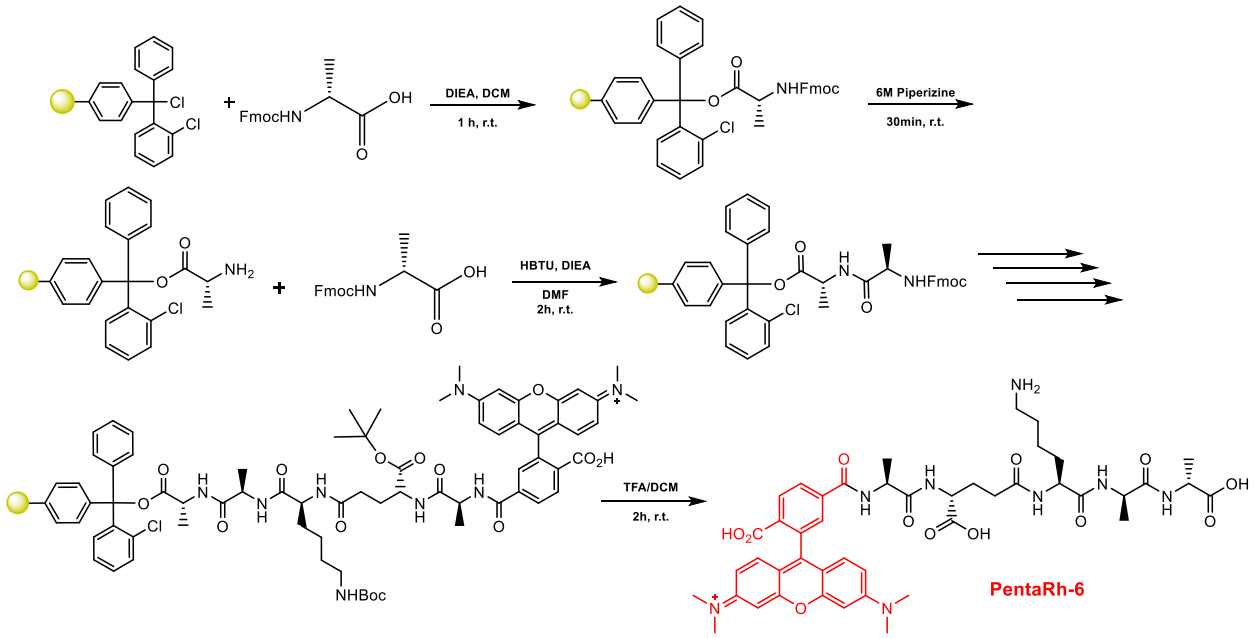
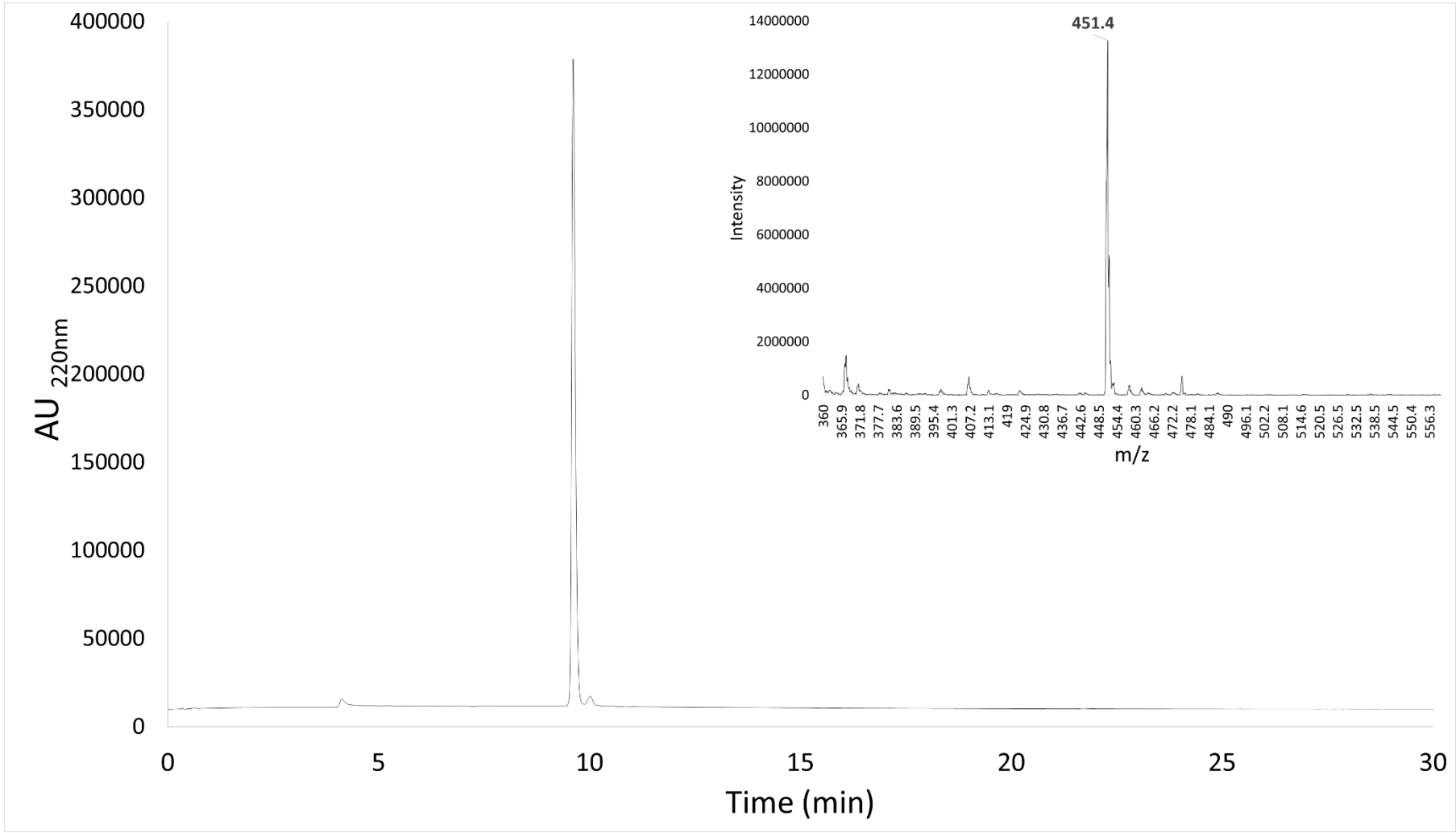
0
5
10
15
20
25
30
Time (min)
Intensity
25000000
20000000
15000000
10000000
5000000
0
450.9
360
365.5
371
376.5
382
387.5
393
398.5
404
409.5
415
420.5
426
431.5
437
442.5
448
453.5
459
464.5
470
475.5
481
486.5
492
497.5
503
508.5
514
519.5
525
530.5
536
541.6
547.1
552.6
558.1
m/z

Scheme S10. Synthesis of PentaRh-6



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-D-alanine (3 eq, 513 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-Glu(OtBu)-OH, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxy-tetramethylrhodamine (2 eq, 474 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **PentaRh-6**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+2H]^{2+}$ calculated: 451.7 found: 451.4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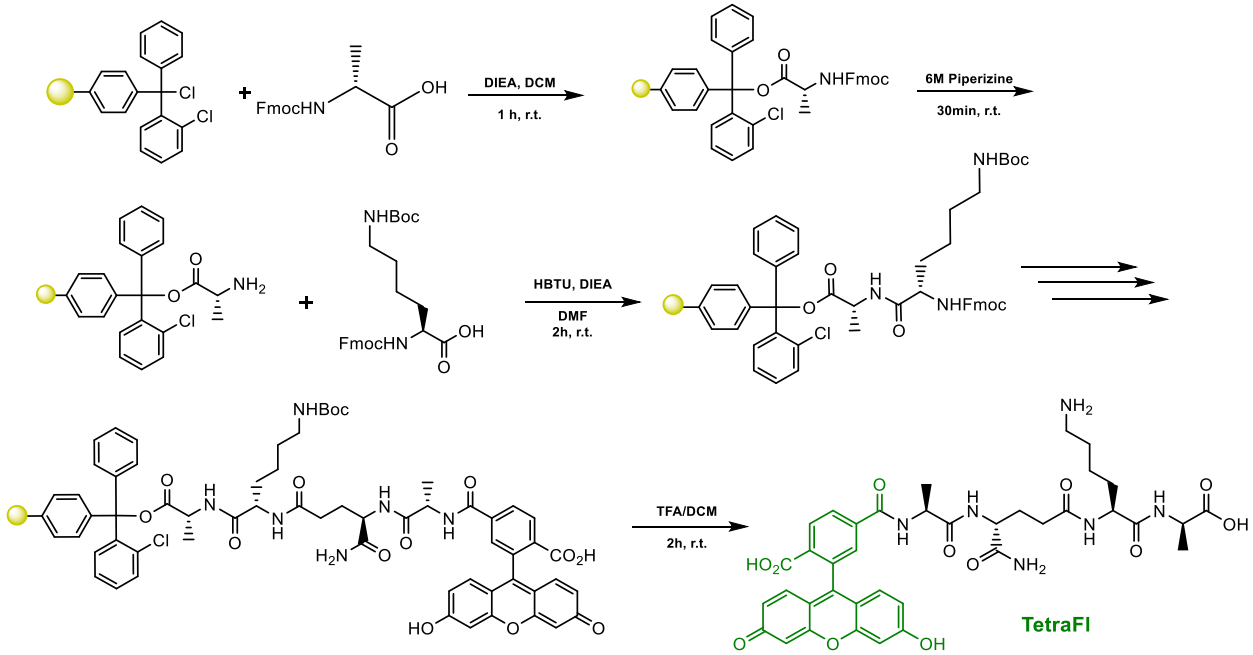
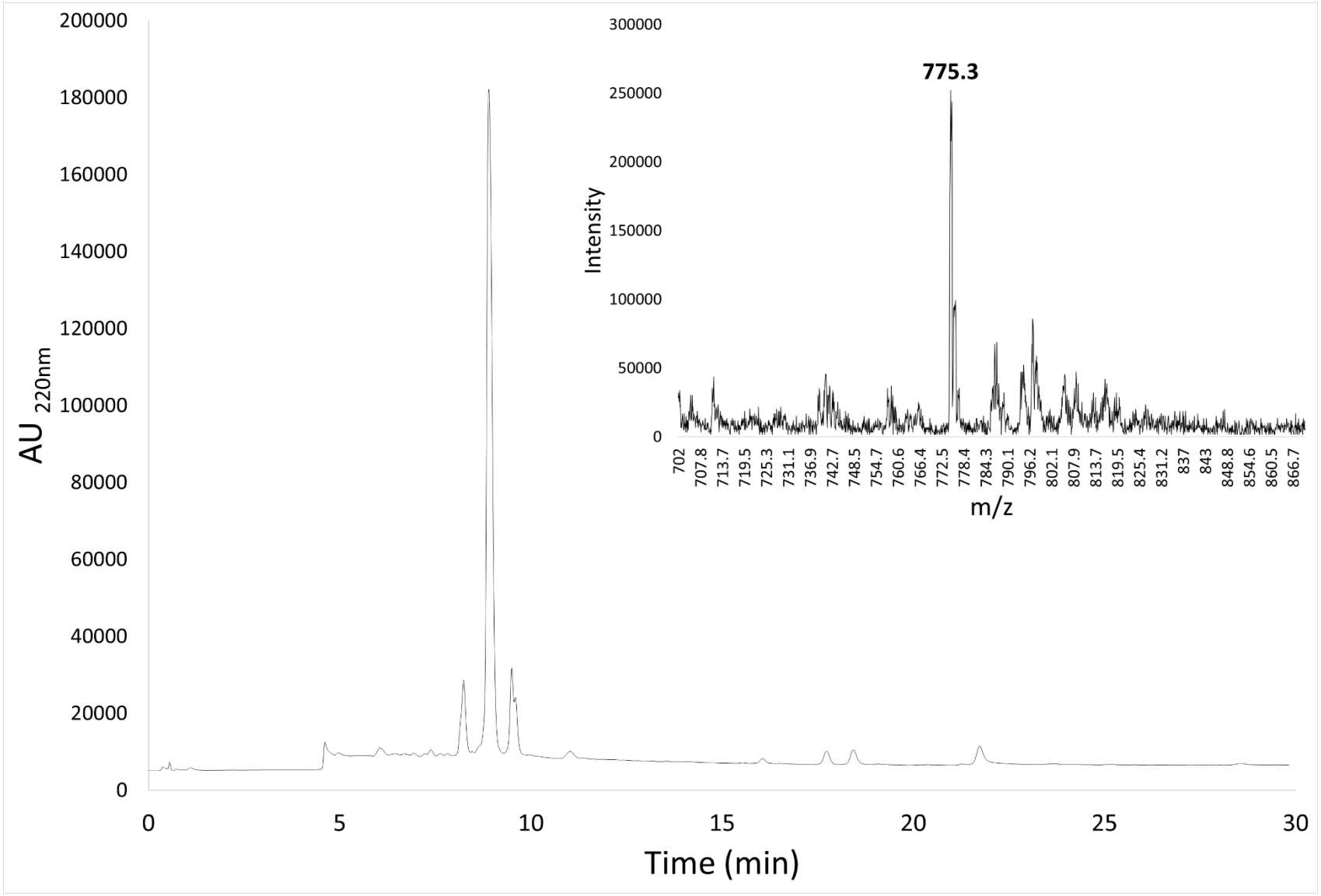
50000
0
0
5
10
15
20
25
30
Time (min)
Intensity
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
451.4
360
365.9
371.8
377.7
383.6
389.5
395.4
401.3
407.2
413.1
419
424.9
430.8
436.7
442.6
448.5
454.4
460.3
466.2
472.2
478.1
484.1
490
496.1
502.2
508.1
514.6
520.5
526.5
532.5
538.5
544.5
550.4
556.3
m/z

Scheme S11. Synthesis of TetraFl

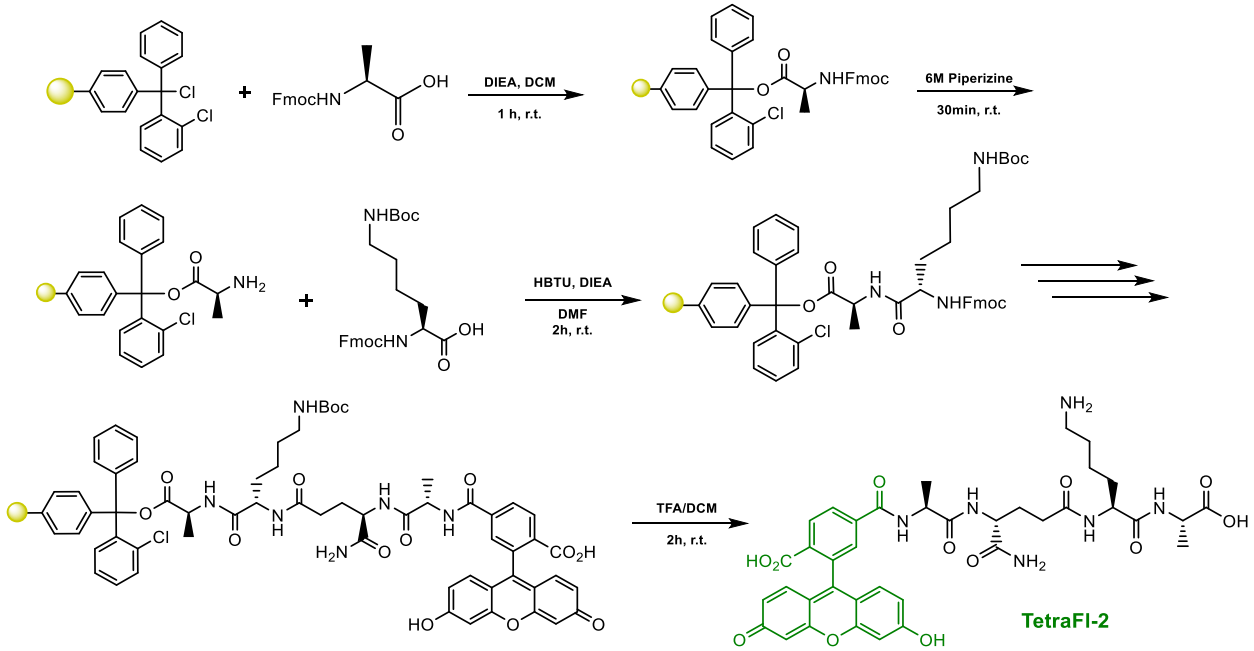


To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-Lys(Boc)-OH (3 eq, 773 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-glutamic acid α-amide and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 775.3 found: 775.3

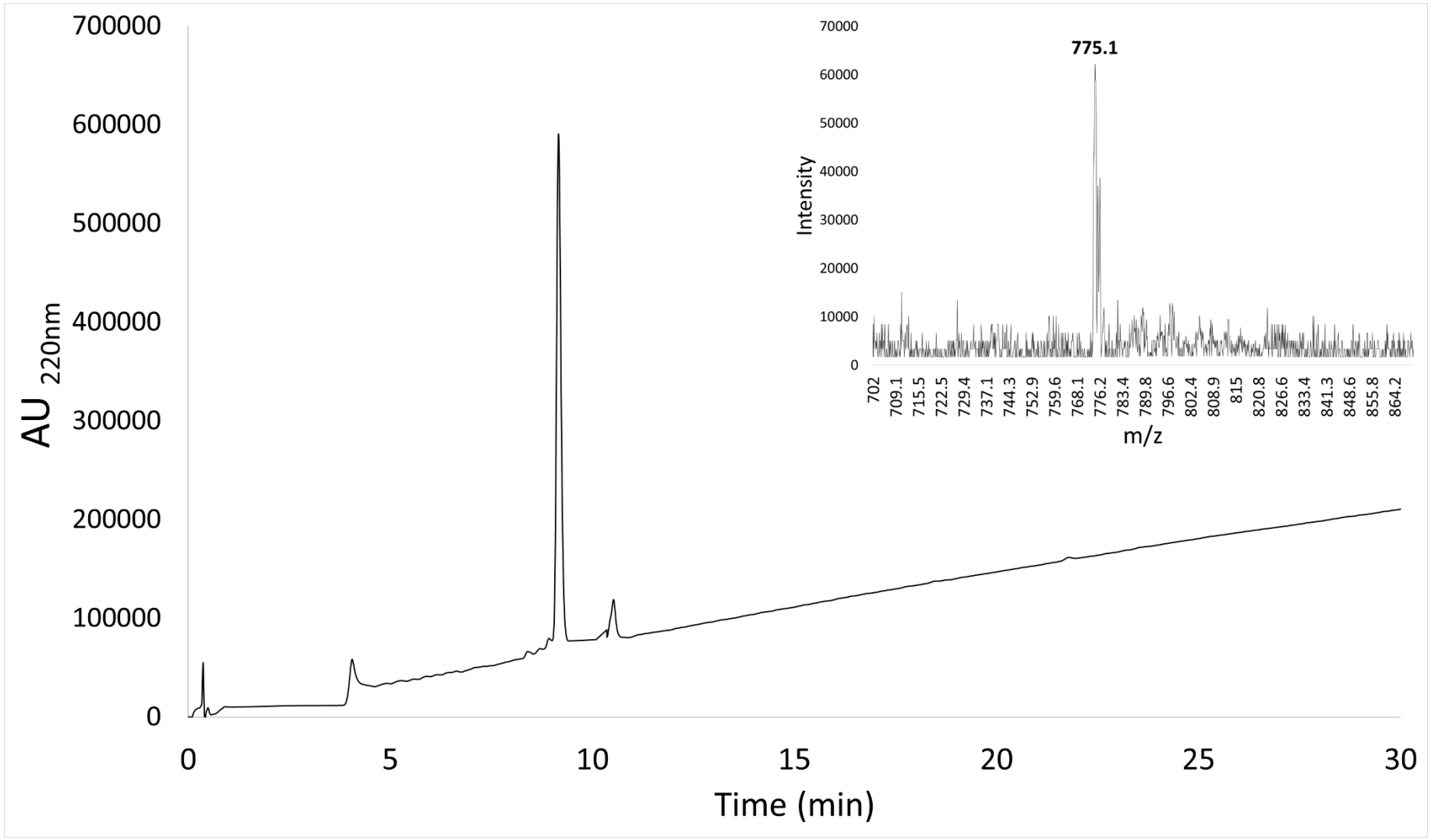

AU 220nm
Time (min)
Intensity
m/z
775.3

Scheme S12. Synthesis of TetraFl-2



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-L-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-Lys(Boc)-OH (3 eq, 773 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-glutamic acid α-amide and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl-2.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 775.3 found: 775.1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AU 220nm
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5
10
15
20
25
30
Time (min)
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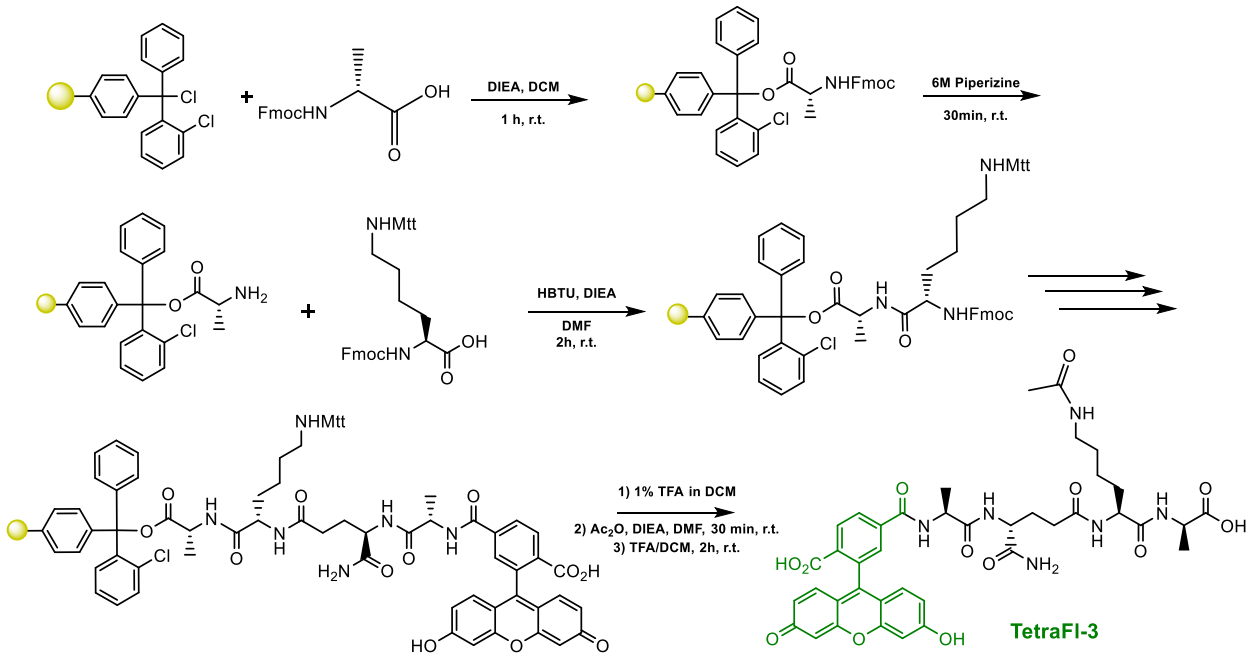
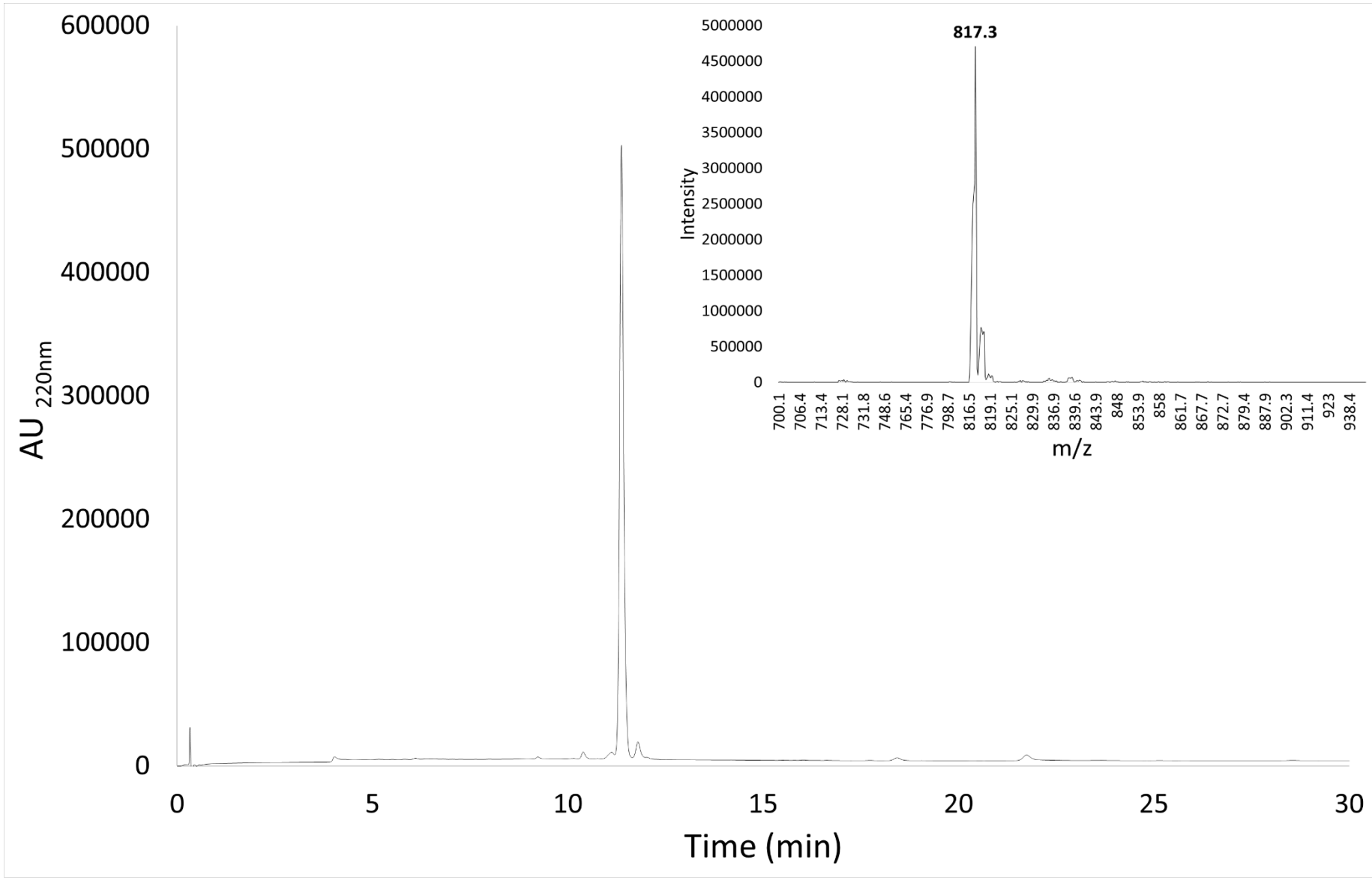
70000
60000
50000
40000
30000
20000
10000
0
Intensity
702
709.1
715.5
722.5
729.4
737.1
744.3
752.9
759.6
768.1
776.2
783.4
789.8
796.6
802.4
808.9
815
820.8
826.6
833.4
841.3
848.6
855.8
864.2
m/z

Scheme S13. Synthesis of TetraFl-3



To a 25 mL peptide synthesis vessel charged with Fmoc-D-Alanine Wang resin (950 mg, 0.55 mmol). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed with MeOH and DCM (3 x 15 mL each). Fmoc-L-Lys(Mtt)-OH (3 eq, 1.02 g, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) was added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-glutamic acid α-amide and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of 1% TFA / 5% TIPS in DCM and shaken for 10 min and washed. The step was repeated five times for removal of the Mtt group. Acetic anhydride (5 eq, 0.260 mL) and DIEA (10 eq, 0.956 mL) in DMF was added and resin shaken for 30 min at ambient temperature. The resin was washed and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl-3.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 817.3 found: 817.3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5
10
15
20
25
30
Time (min)
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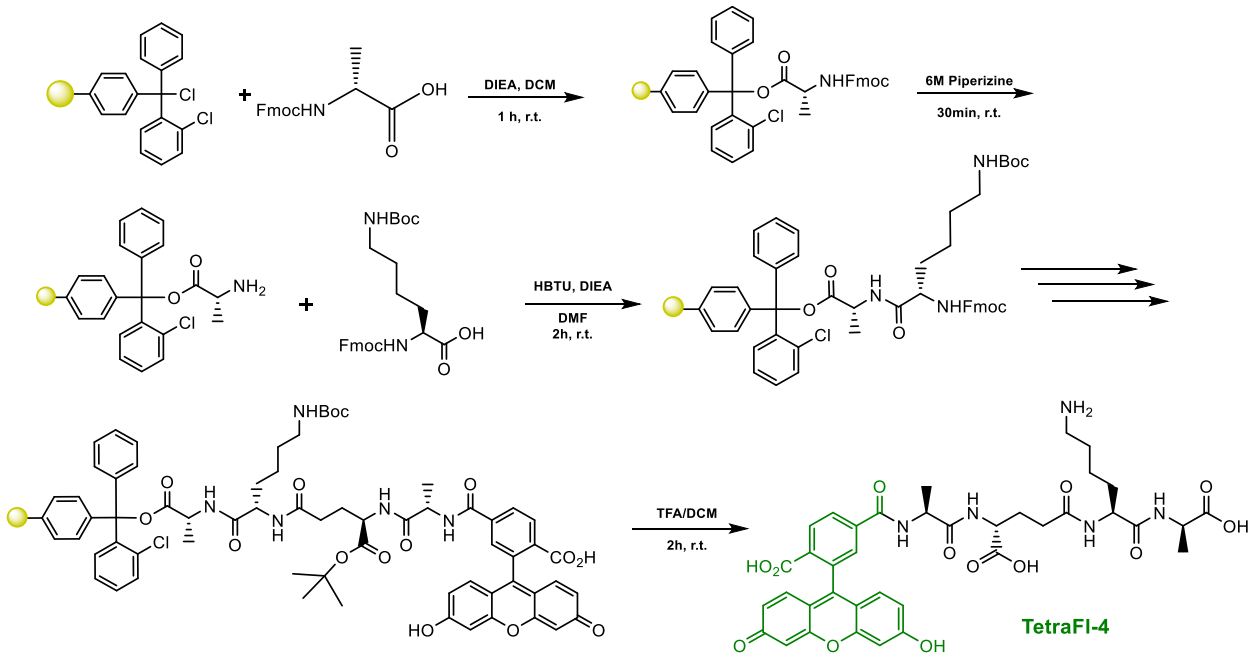
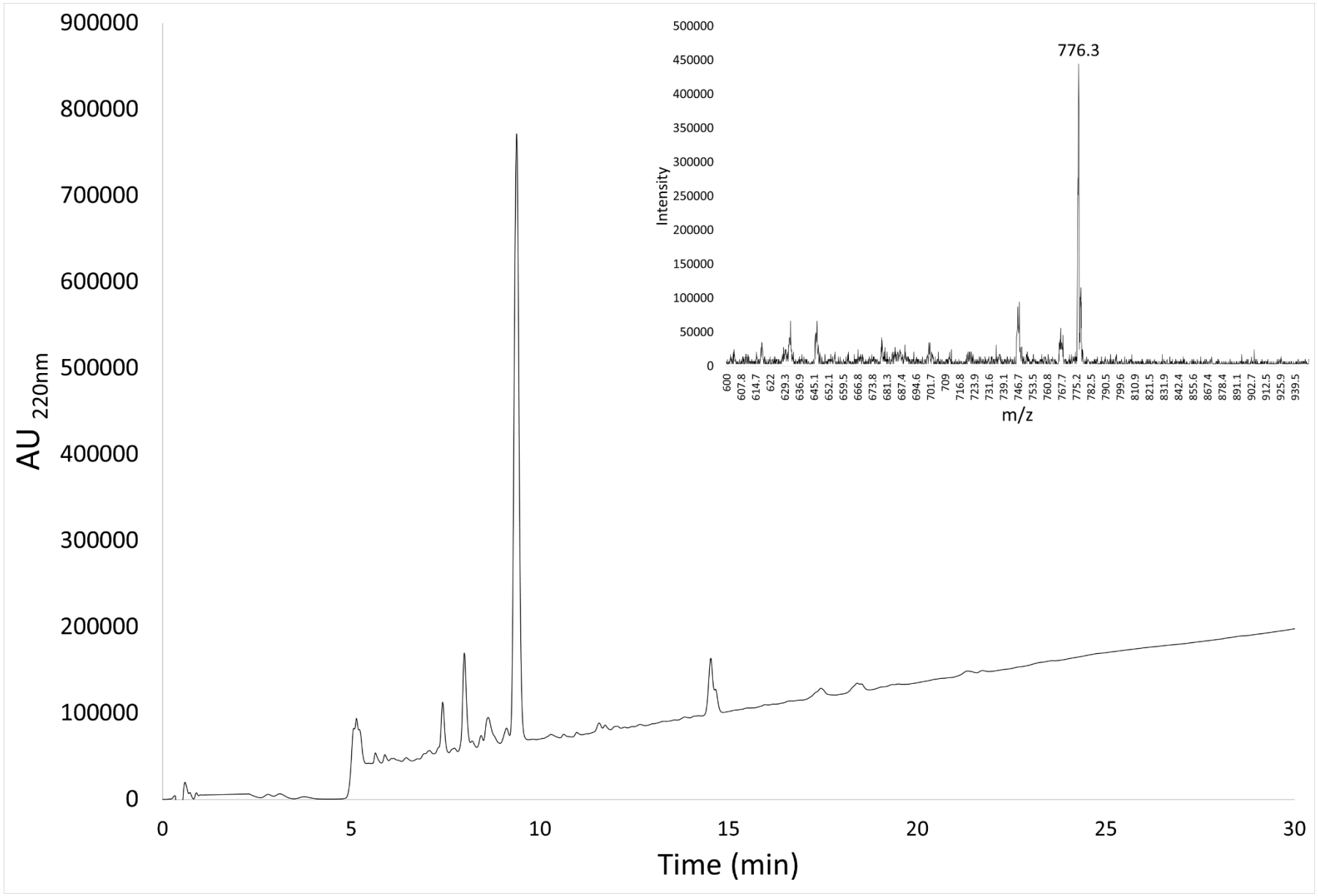
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Intensity
817.3
700.1
706.4
713.4
728.1
731.8
748.6
765.4
776.9
798.7
816.5
819.1
825.1
829.9
836.9
839.6
843.9
848
853.9
858
861.7
867.7
872.7
879.4
887.9
902.3
911.4
923
938.4
m/z

Scheme S14. Synthesis of TetraFl-4



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-Lys(Boc)-OH (3 eq, 773 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-Glu(OtBu)-OH and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 413 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl-4.** The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 776.2 found: 776.3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0
5
10
15
20
25
30
Time (min)
Intensity
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
776.3
600
607.8
614.7
622
629.3
636.9
645.1
652.1
659.5
666.8
673.8
681.3
687.4
694.6
701.7
709
716.8
723.9
731.6
739.1
746.7
753.5
760.8
767.7
775.2
782.5
790.5
799.6
810.9
821.5
831.9
842.4
855.6
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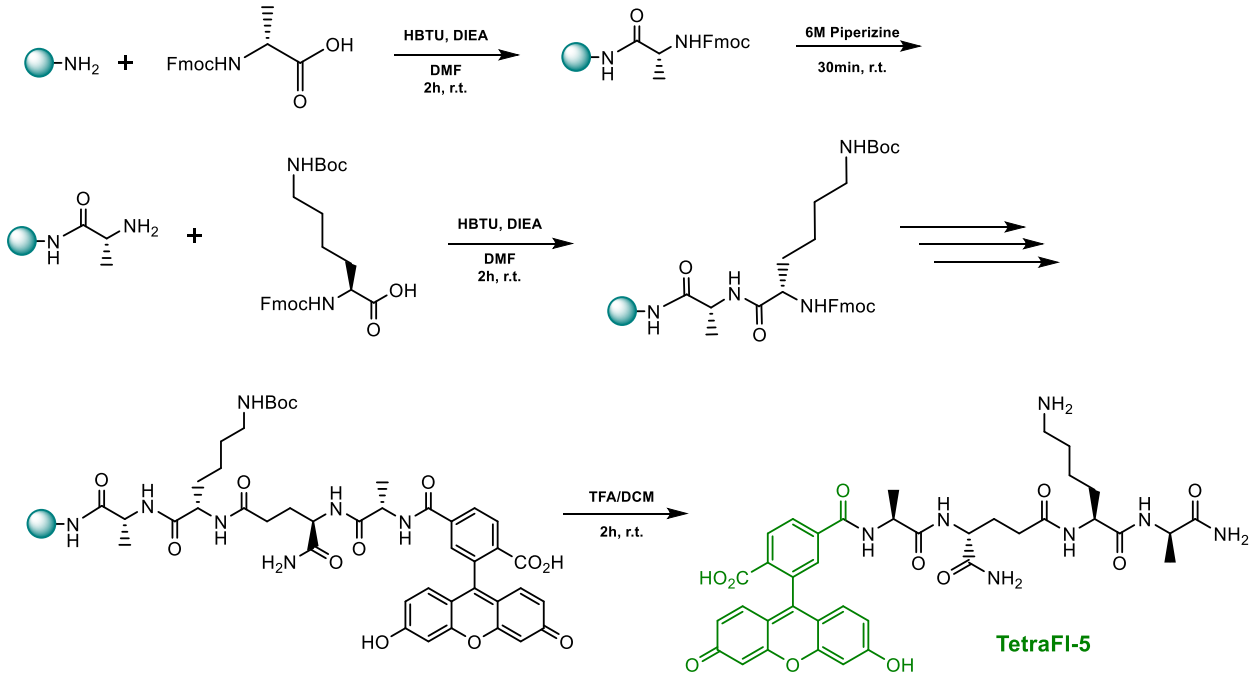
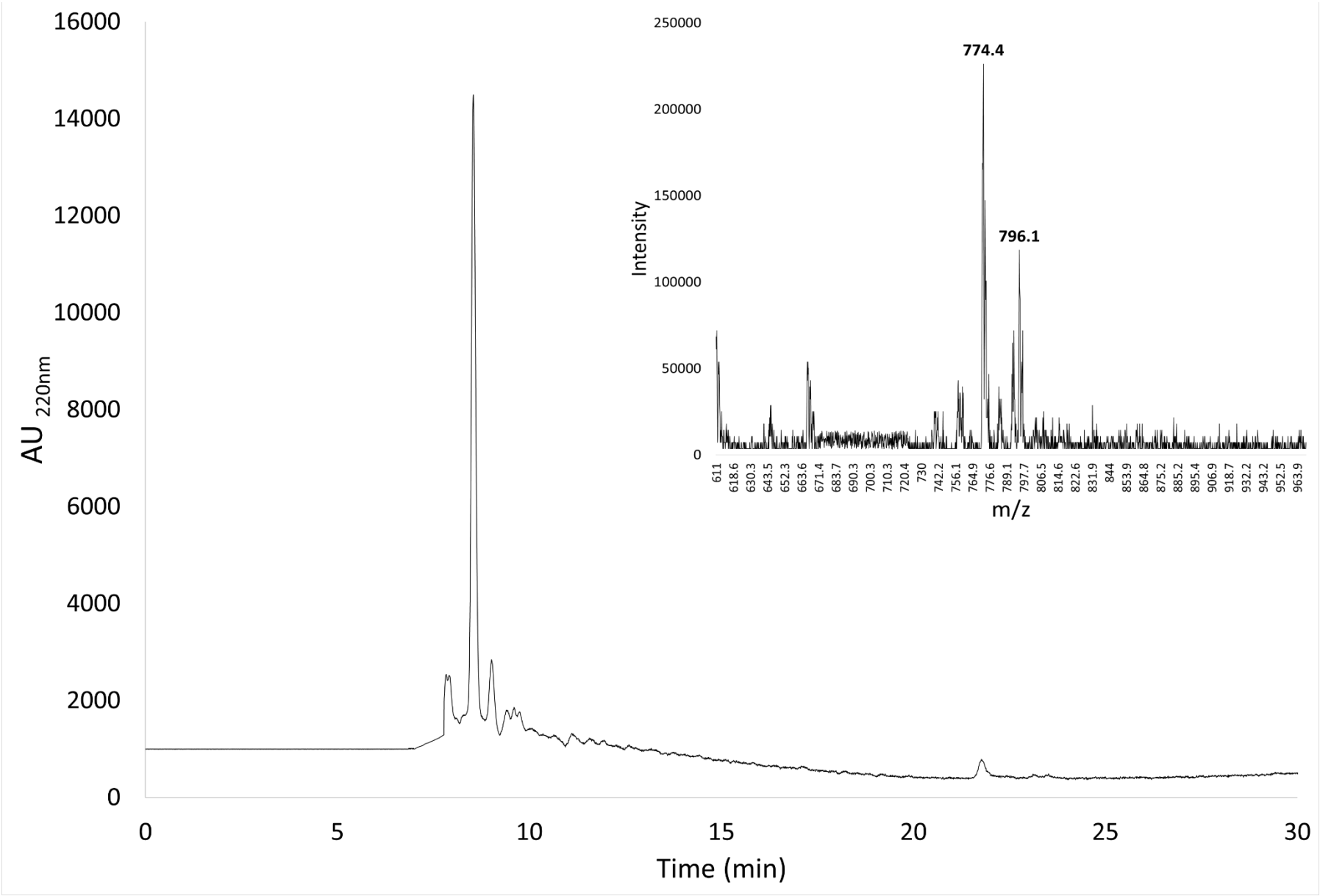
891.1
902.7
912.5
925.9
939.5
m/z

Scheme S15. Synthesis of TetraFl-5

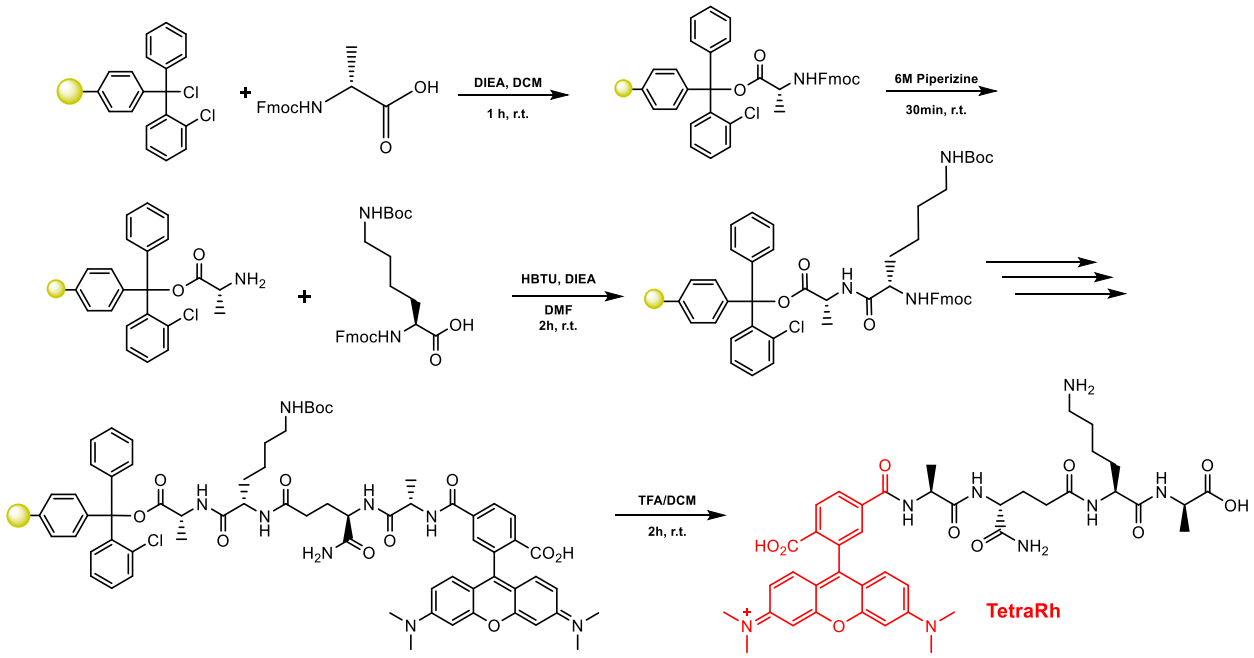


To a 25 mL peptide synthesis vessel charged with Rink Amide Resin (500mg, 0.30mmol). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed with MeOH and DCM (3 x 15 mL each). Fmoc-D-alanine (3 eq, 280 mg, 0.90 mmol), HBTU (3 eq, 341 mg, 0.90 mmol), and DIEA (6 eq, 0.314 mL, 1.80 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-L-Lys(Boc)-OH, Fmoc-D-glutamic acid α-amide, and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxyfluorescein (2 eq, 226 mg, 0.60 mmol), HBTU (2 eq, 228 mg, 0.60 mmol) and DIEA (6 eq, 0.314 mL, 1.80 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraFl-5**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+H]^+$ calculated: 774.3 found: 774.4

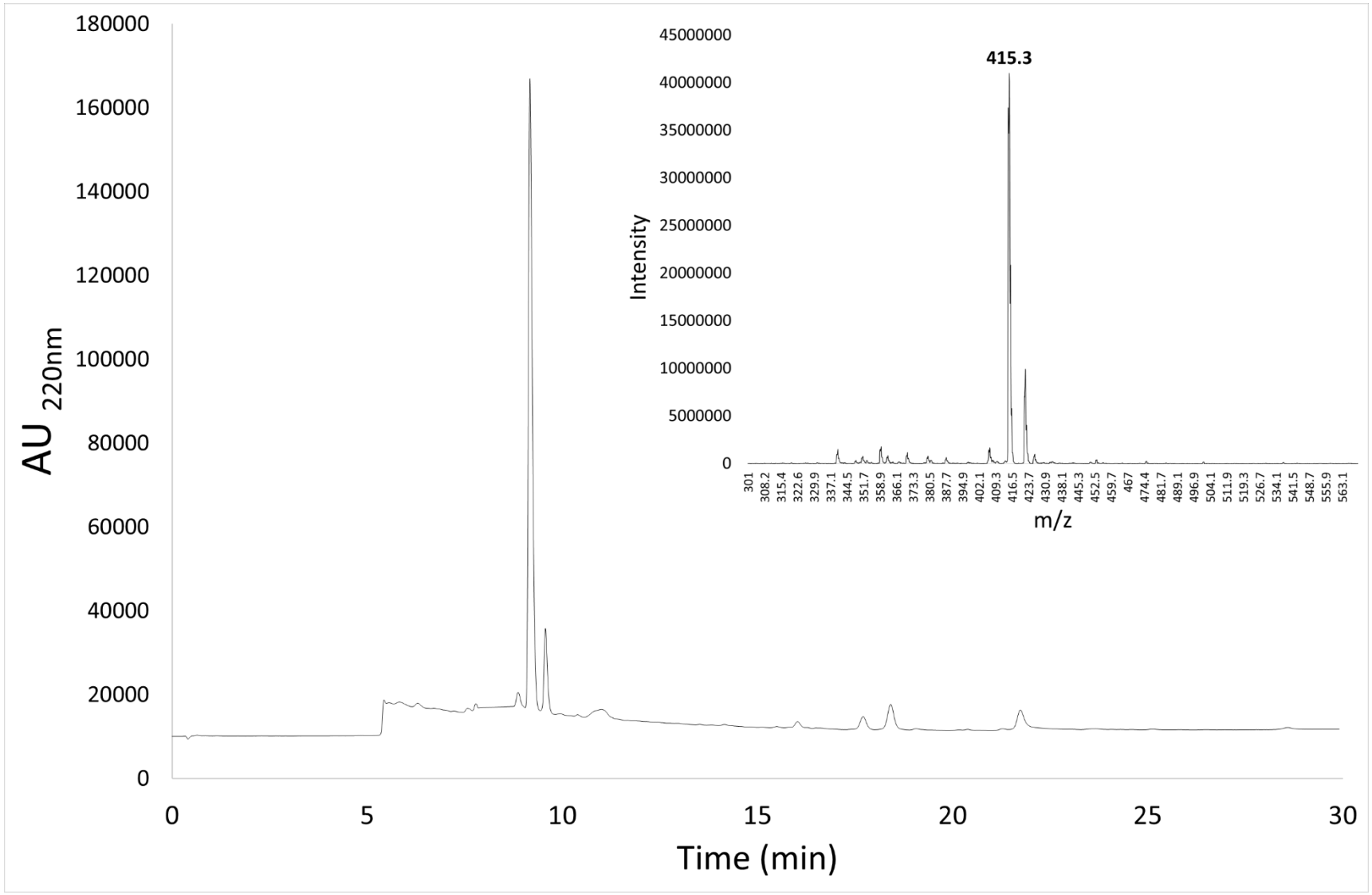

AU 220nm
Time (min)
Intensity
m/z
774.4
796.1

Scheme S16. Synthesis of TetraRh



To a 25 mL peptide synthesis vessel charged with 2-Chlorotrityl chloride resin (500mg, 0.55mmol) was added Fmoc-D-alanine (1.1 eq, 188 mg, 0.605 mmol) and DIEA (3 eq, 0.286 mL, 1.65 mmol) in dry DCM (15 mL). The resin was agitated for 1 h at ambient temperature and washed with MeOH and DCM (3 x 15 mL each). The Fmoc protecting group was removed with 6 M piperazine/100 mM HOBt in DMF (15 ml) for 30 min at ambient temperature, then washed as before. Fmoc-L-Lys(Boc)-OH (3 eq, 773 mg, 1.65 mmol), HBTU (3 eq, 625 mg, 1.65 mmol), and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) were added to the reaction flask and agitated for 2 h at ambient temperature. The Fmoc deprotection and coupling procedure was repeated as before using the same equivalencies with Fmoc-D-glutamic acid α-amide and Fmoc-L-alanine. The Fmoc group of L-alanine was deprotected and resin coupled with 5(6)-carboxy-tetramethylrhodamine (2 eq, 474 mg, 1.1 mmol), HBTU (2 eq, 416 mg, 1.1 mmol) and DIEA (6 eq, 0.574 mL, 3.30 mmol) in DMF (15 mL) shaking overnight. The resin was washed as before and added to a solution of TFA/DCM (2:1, 20 mL) with agitation for 2 h at ambient temperature. The resin was filtered and resulting solution concentrated *in vacuo*. The residue was trituated with cold diethyl ether and purified using reverse phase HPLC using H_2O/MeOH to yield **TetraRh**. The sample was analyzed for purity using a Shimadzu LC 2020 with a Phenomenex Luna 5µ C18(2) 100Å (30 x 2.00 mm) column; gradient elution with H_2O/CH_3CN.

ESI-MS: $[M+2H]^{2+}$ calculated: 415.7 found: 415.3


AU 220nm
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
30
Time (min)
Intensity
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
415.3
301
308.2
315.4
322.6
329.9
337.1
344.5
351.7
358.9
366.1
373.3
380.5
387.7
394.9
402.1
409.3
416.5
423.7
430.9
438.1
445.3
452.5
459.7
467
474.4
481.7
489.1
496.9
504.1
511.9
519.3
526.7
534.1
541.5
548.7
555.9
563.1
m/z