Electronic Supplementary Material (ESI) for Chemical Communications
This journal is © The Royal Society of Chemistry 2013

A Broad Spectrum Dark Quencher: Construction of Multiple Colour Protease and Photolytic Sensors

*Finith E. Jernigan and David S. Lawrence**

The Department of Chemistry, the Division of Chemical Biology and Medicinal Chemistry, and the Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599

Supporting Information

General Procedures

General reagents and solvents were purchased from Fisher or Sigma-Aldrich. Novasyn TGR Resin, Fmoc-Lys(Mtt)-OH (N-α-Fmoc-N-ε-4-methyltrityl-L-lysine), and all Fmoc protected natural amino acids were purchased from EMD Biosciences Inc. HCTU [1H-benzotriazolium-1 [bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate (1),3-oxide] was purchased from Peptides International (Louisville, KY, U.S.A.). TAM (5-carboxytetramethylrhodamine), FAM (5-carboxyfluorescein), and ROX (5-carboxy-X-rhodamine, triethylammonium salt) were purchased from Chempep, Inc. (Wellington, Fl, U.S.A). DECou (7-(diethylamino)coumarin-3-carboxylic acid), Cou343 ([11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinoline-10-carboxylic acid), atto610 NHS-ester, and atto700 NHS-ester were purchased from Sigma-Aldrich. Fmoc-Photolabile Linker (Fmoc-Ø-OH, **S3**) (4-{4-[1-(9-fluorenylmethyloxycarbonyl)ethyl]-2-methoxy-5-nitrophenoxy}butanoic acid) was purchased from Advanced ChemTech (Louisville, KY, U.S.A.). Flash Chromatography was performed on a Biotage (Charlotte, N.C., U.S.A.) Isolera One System using a 120 g C18 reverse phase column. Trypsin from bovine pancreas was purchased from Sigma-Aldrich.

Synthesis of *c*AB40 (4)

The sodium salt of bromaminic acid **2** (1-amino-4-bromo-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid sodium salt, 0.500 g, 1.23 mmol), 4-aminophenylacetic acid **3** (0.166 g, 1.1 mmol), sodium carbonate (0.197 g, 1.58 mmol), copper (II) sulfate (28 mg, 0.179 mmol) and water (50 mL) were added to a 100 mL flask. The color of the solution turned from a bright red to a dark blue after stirring and heating the mixture to reflux for 24 h, indicating product formation. The aqueous mixture was washed with

This journal is © The Royal Society of Chemistry 2013

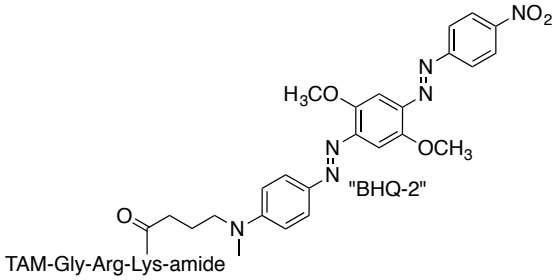
dichloromethane (DCM) (3 x 50 mL) and the solvent removed via rotary evaporation. The resulting solid was dissolved in methanol, filtered, and the solvent removed via rotary evaporation. The furnished solid was purified via reverse-phase flash chromatography using an acetonitrile to water gradient (3% to 95%) over 30 min to furnish the desired material as a dark blue solid in 37.2% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.62 (broad, 5H), 8.212 (t, J = 9.2 Hz, 1H), 8.208 (t, J = 8.8 Hz, 1H), 8.044 (s, 1H), 7.787 (t, J = 3.2 Hz, 1H), 7.787 (t, J = 4.4Hz, 1H), 7.236 (d, J = 8.4 Hz, 2H), 7.240 (d, J = 8.4 Hz, 2H), 3.611 (s, 2H), 2.537 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.506, 181.971, 172.859, 144.138, 142.695, 141.011, 137.861, 134.155, 133.613, 133.202, 132.858, 131.349, 130.815, 126.090, 126.017, 123.034, 122.735, 111.513, 109.543; HRMS (ESI-) m/z calc'd for $C_{22}H_{15}N_2O_7S^-$ [M-]: 451.0605, m/z found: 451.0607.

Synthesis of Fluorophore/Dye-Labeled Peptides

Peptides were synthesized using standard Fmoc solid-phase synthesis on a Prelude peptide synthesizer (Protein Technologies Inc., Tucson, AZ, U.S.A.). Novasyn TGR resin was swelled for 30 min in DCM before synthesis. Fmoc-Lys(Mtt)-OH was then coupled using 5.0 equiv AA, 4.9 equiv of HCTU, 20 equiv of diisopropylethylamine (DIPEA) in N,N-dimethylformamide (DMF) (2 × 5 min) followed by a DMF wash (6 × 30 s). The Lys 4-methyltrityl protecting group was then deprotected using 7% TFA/7% TIS/86% DCM. The deprotected side chain amine was then acylated using 1.1 equiv *c*AB40, 1.0 equiv HCTU, and 20.0 equiv DIPEA in DMF (2 x 2 h). The Fmoc-protecting group was removed using 20% piperidine in DMF (2 × 2.5 min) followed by a DMF wash (6 × 30 s). Fmoc-Arg-OH and Fmoc-Gly-OH were then to the free N-terminus of the peptide using the conditions described for the coupling and deprotection of Fmoc-Lys(Mtt)-OH. In the case of **11**, the photolabile amino acid **S3** was coupled instead of Fmoc-Arg-OH. The N-terminal Fmoc was then removed using 20% piperidine in DMF (2 × 2.5 min) and fluorophores were coupled using one of two methods. (1) The fluorophores DECou, Cou343, 5'-FAM, TAM, and ROX were coupled to the N-terminal amine using 5.0 equiv of fluorophore, 4.9 equiv of HCTU, and 20 equiv of DIPEA in DMF (1× 60 min) followed by a wash (3× DMF, IPA, DCM). (2) The fluorophores atto610-NHS ester and atto700-NHS ester were coupled using 1.0 mg of dye and 20 equiv of DIPEA in DMF (1 × 60 min) followed by a wash (3X DMF, IPA, DCM). The synthesis of peptides **13**, **15**, and **16** followed the exact same procedure as above, but the Mtt protecting group deprotection and *c*AB40 coupling was skipped. The synthesis of peptide **14** followed the exact same procedure as above, but fluorophore coupling was skipped to leave a free N-terminal amine. After synthesis was complete, the peptide was cleaved from the resin and deprotected using a 95:2.5:2.5 TFA:H_2O:triisopropylsilane (TIPS) cleavage cocktail. The peptides were isolated via filtration, precipitated with ice-cold ether, and centrifuged. The precipitates were air-dried, dissolved in DMSO, and purified using HPLC (3% to 95% acetonitrile to water gradient with 0.1% TFA over 30 min using a 25 cm x 21.2 mm C18 column). Peptides **5** - **11** were collected, freeze-dried, and characterized by matrix-assisted laser desorption/ionization mass spectrometry (Matrix: alpha cyano-4-hydroxycinnaminic acid, mono-isotopic): DECou-GRK(*c*AB40)-amide **5** [Exact Mass

This journal is © The Royal Society of Chemistry 2013

calculated: 1035.391, found: 1035.355 $(M+H)^+$], Cou343-GRK(*c*AB40)-amide **6** [Exact Mass calculated: 1059.391, found: 1059.351 $(M+H)^+$], FAM-GRK(*c*AB40)-amide **7** [Exact Mass calculated: 1150.349, found: 1150.333 $(M+H)^+$], TAM-GRK(*c*AB40)-amide **8** [Exact Mass calculated: 1205.451, found: 1205.476 $(M+H)^+$], atto610-GRK(*c*AB40)-amide **9** [Exact Mass calculated: 1165.529, found: 1165.556 $(M+H)^+$], atto700-GRK(*c*AB40)-amide **10** [The structure of atto700 has not been published, exact mass could not be calculated, found: 1340.512], TAM-G-Ø-K(*c*AB40)-amide **11** [Exact Mass calculated: 1329.456, found: 1329.427 $(M+H)^+$]. Peptides **12** – **16** were collected, freeze-dried, and were characterized by positive electrospray ionization quadrupole mass spectrometry (ESI^+-QMS): TAM-GRK(BHQ-2)-amide **12** [Exact Mass calculated: 1259.575, found: 1259.606 $(M+H)^+$], DEAC-GRK(NH_2)-amide **13** [Exact Mass calculated: 602.3, found: 602.3 $(M+H)^+$], NH_2-GRK(*c*AB40)-amide **14** [Exact Mass calculated: 793.3, found: 793.3 $(M+H)^+$], Cou343-GRK(NH_2)-amide **15** [Exact Mass calculated: 626.3, found: 626.3 $(M+H)^+$], TAM-GRK(NH_2)-amide **16** [Exact Mass calculated: 771.4, found: 771.4 $(M+H)^+$].



12

Photolysis Protocol

The photolabile peptide **11** (1 μM) was incubated in 1 mM dithiothreitol and 25 mM Tris pH 7.4 buffer. An aliquot was removed to collect a base line fluorescence measurement using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). The aliquot was returned to stock solution and mixed thoroughly. The sample was then photolyzed using 290 nm to 390 nm (365 nm maximum light intensity) light by an Oriel Hg arc lamp (power supply model 69907 with a 200 Watt Hg lamp, NewPort, North Billerica, MA) using a UG.1 UV bandpass filter (Newport, North Billerica, MA) at various time points at room temperature. After the photolysis period, an aliquot was removed and the fluorescence of the photolyzed material determined. This cycle was repeated until three consecutive fluorescence measurements remained constant to establish a maximum fluorescence enhancement.

Trypsinolysis Protocol

Peptides **5** - **10** (1 μM) were incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once

This journal is © The Royal Society of Chemistry 2013

a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added and the fluorescence enhancement was monitored. Experiments were performed in triplicate. Initial rates (<10% of substrate converted to product) were calculated by converting change in fluorescence units/sec to μM product formed/sec.

Absorbance Spectra

Peptides **5**, **6**, and **8** (15 μM) were incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette. After 5 min, the absorbance was collected.

Viscosity and Hydroxypropyl-ß-cyclodextrin Experiments

Peptides **5**, **6**, and **8** (1 μM) were incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol, 0, 10, 20, 30, 40, or 50 (w/w) PEG400 or 0, 5, 10, 15, 20, 25, or 30 mM hydroxypropyl-ß-cyclodextrin in a 200 μL quartz cuvette at 30 ˚C. After 20 min, fluorescence was collected for each well using a Molecular Devices SpectraMAX Gemini EM fluorescent plate reader (Sunnyvale, CA).

This journal is © The Royal Society of Chemistry 2013

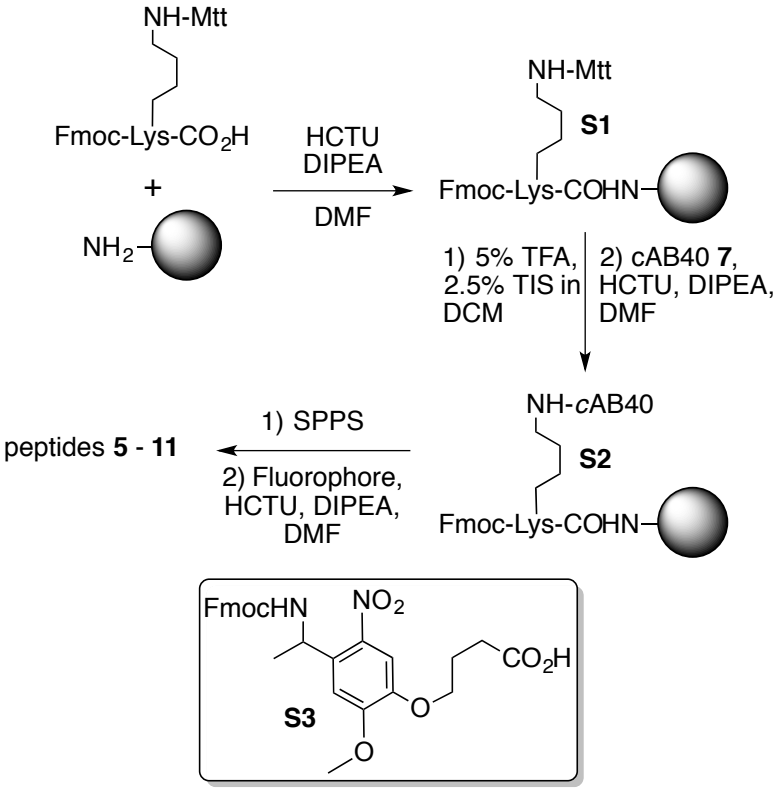


Figure S1. Synthesis of trypsin substrates 5 – 10 and the photolabile cassette 1. Fmoc-Lys(Mtt)-OH was coupled to the Novasyn TGR-resin to afford **S1**. The 4-methyltrityl (Mtt) protected amine was subsequently deprotected and acylated with HCTU [1H-benzotriazolium-1 [bis(di-methylamino)-methylene]-5-chloro-hexafluoro-phosphate (1),3-oxide]-activated *c*AB40 to furnish **S2**. Trypsin substrates **5** - **10** were then prepared by sequentially coupling Fmoc-Arg-OH and Fmoc-Gly-OH to **S2** via solid phase peptide synthesis. The photosensitive reporter **11** was constructed in an analogous fashion by sequentially coupling the Fmoc-photolabile linker **S3** and Fmoc-Gly-OH. Finally, in each case the fluorophore was coupled using HCTU in DIPEA/DMF.

This journal is © The Royal Society of Chemistry 2013

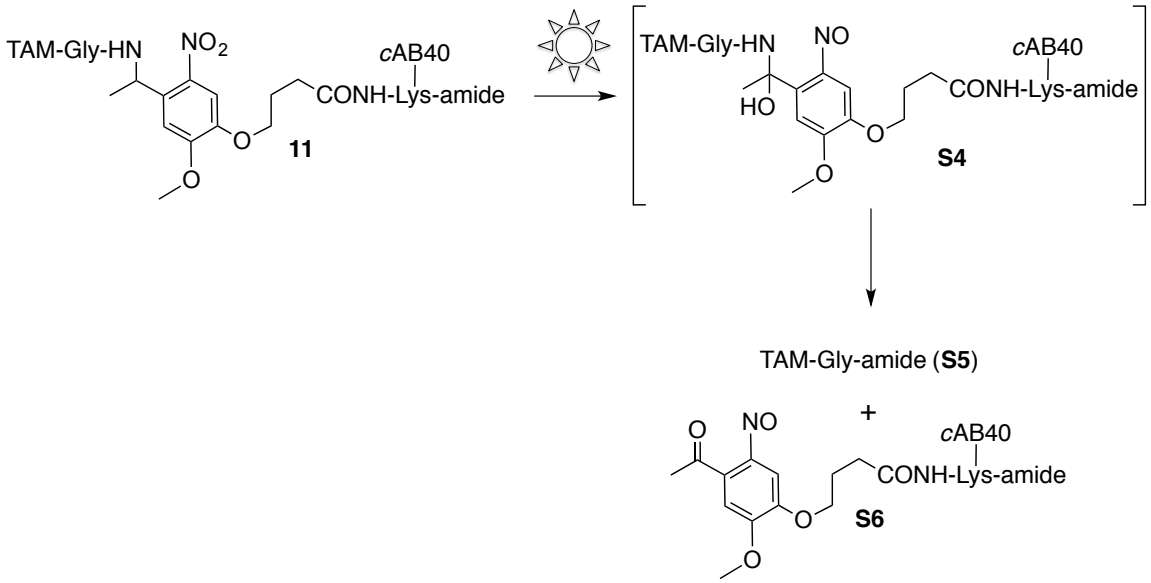


Figure S2. Photolysis of TAM-G-Ø-K(*c*AB40) 11. Illumination at 360 nm induces a well-established transfer of one of the oxygens of the nitro functionality to the nearby benzylic position. The intermediate hemiaminal **S4** decomposes, generating two fragments, one with the now highly fluorescent TAM fluorophore **S5** and the other with the AB40 quenching partner **S6**.

This journal is © The Royal Society of Chemistry 2013

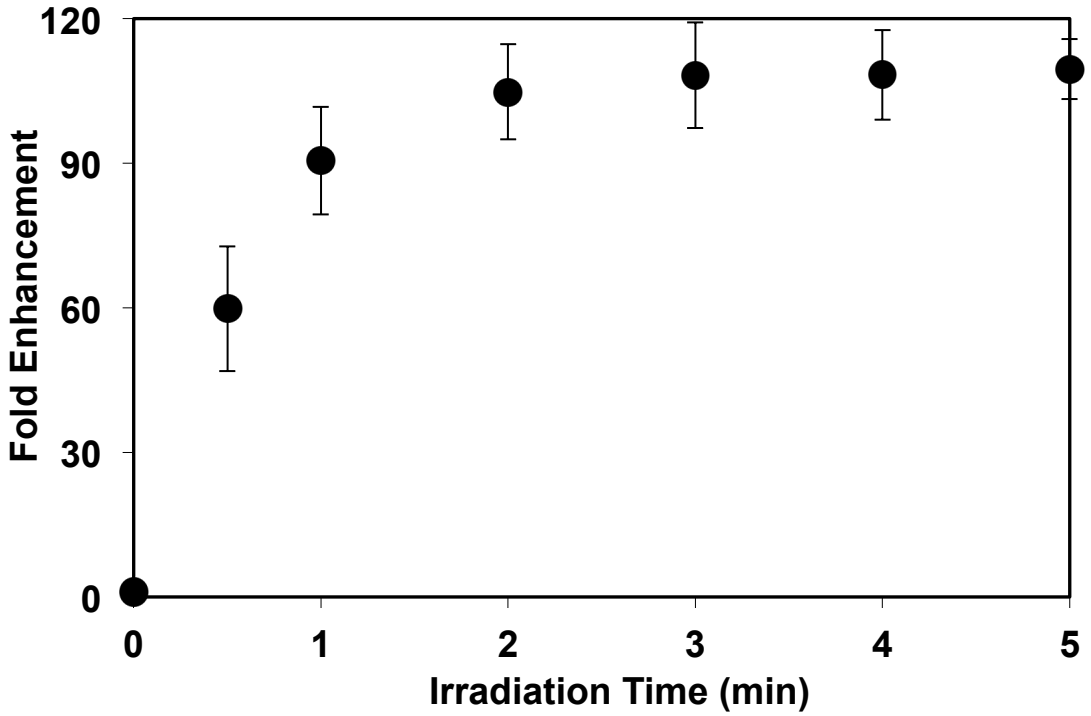


Figure S3. Change in fluorescence, during photolysis of 11, as a function of illumination time.

This journal is © The Royal Society of Chemistry 2013

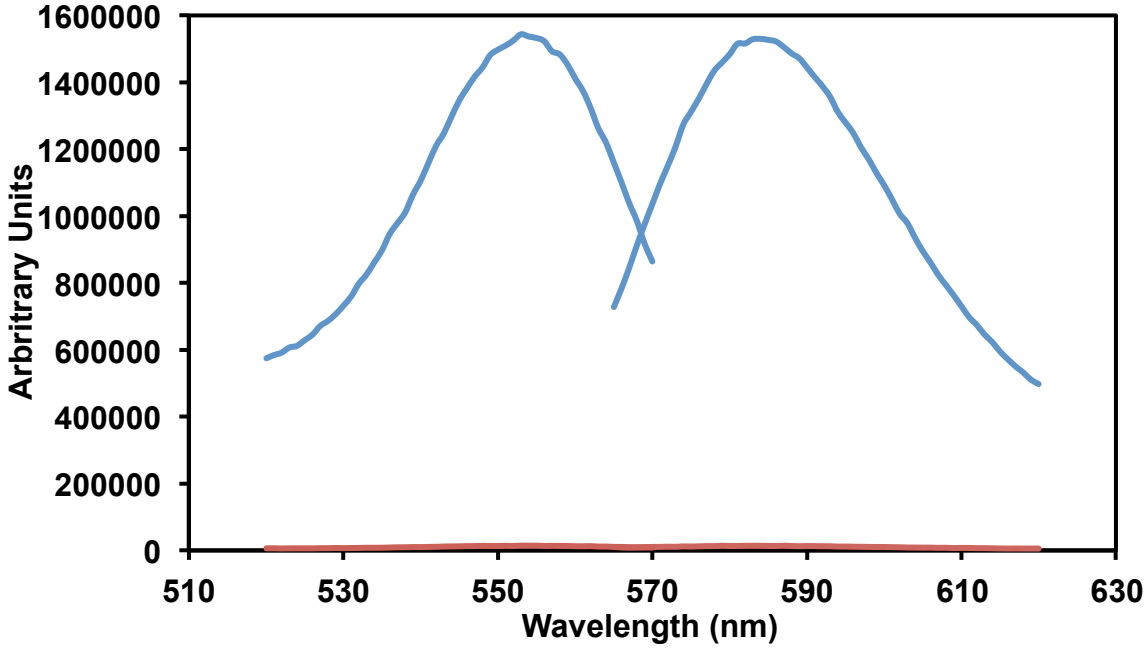


Figure S4. Fluorescence Spectra of TAM-G-Ø-K(*c*AB40) 11 before (Red) and after (Blue) photolysis.

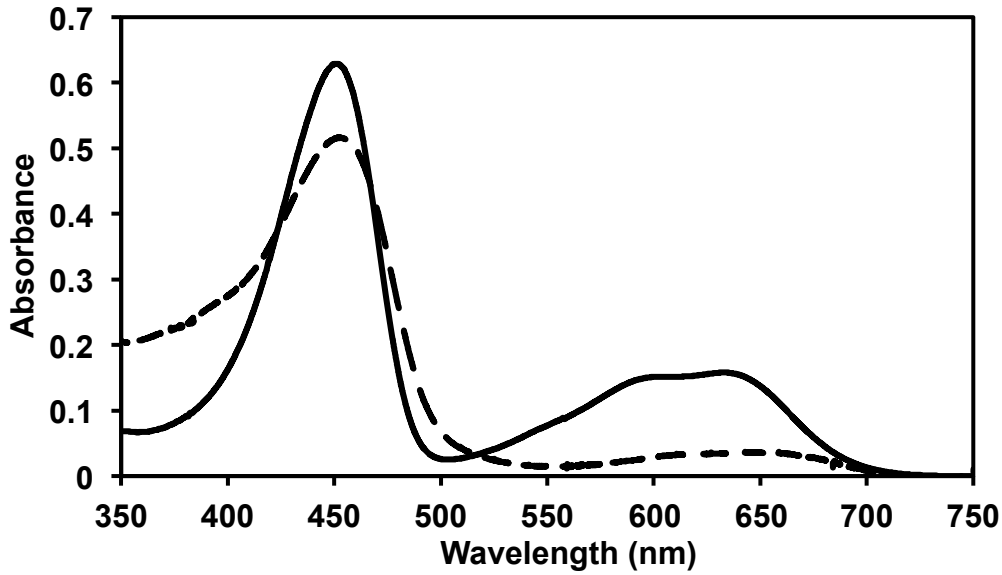
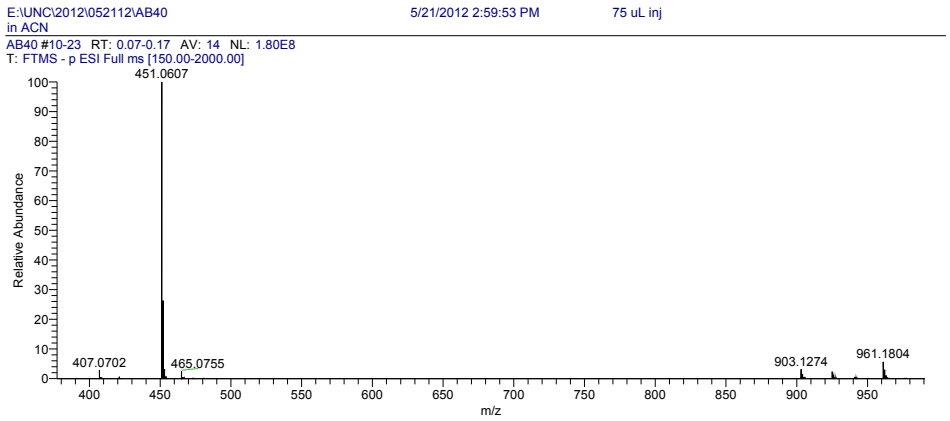


Figure S5. Absorbance spectrum of Cou343-GRK(*c*AB40)-amide 6 (solid) and the combined spectrum (dotted) of Cou343-GRK(NH_2)-amide 15 and NH_2-GRK(*c*AB40)-amide 14.

AB40 (negative ion mode, accurate mass; detail of preceding spectrum):



Assignments of additional confirmatory peaks:

m/z	*formula*	*r+db*	*measurement error (ppm)*	*assignment*
407.0702	$C_{21}H_{15}O_6N_2S$	15.5	-1.168	$[M\text{-}H\text{-}CO_2]^-$
903.1274	$C_{44}H_{31}O_{14}N_4S_2$	31.5	-1.103	$[2M\text{-}H]^-$
925.1095	$C_{44}H_{30}O_{14}N_4S_2Na$	31.5	-0.855	$[2M\text{-}2H\text{+}Na]^-$
941.0831	$C_{44}H_{30}O_{14}N_4S_2K$	31.5	-1.177	$[2M\text{-}2H\text{+}K]^-$
961.1804	$C_{46}H_{37}O_{14}N_6S_2$	31.5	-1.055	$[2M\text{-}2H\text{+}NH_4\text{+}CH_3CN]^-$

Novatia, LLC · 11 Deerpark Drive, Suite 202 · Monmouth Junction, NJ 08852
732.274.9933 (p) · 732.274.9935 (f)
http://www.enovatia.com

Figure S6. HRMS of *c*AB40 4. m/z calc'd for $C_{22}H_{15}N_2O_7S^-$ [M-]: 451.0605, m/z found: 451.0607.

This journal is © The Royal Society of Chemistry 2013

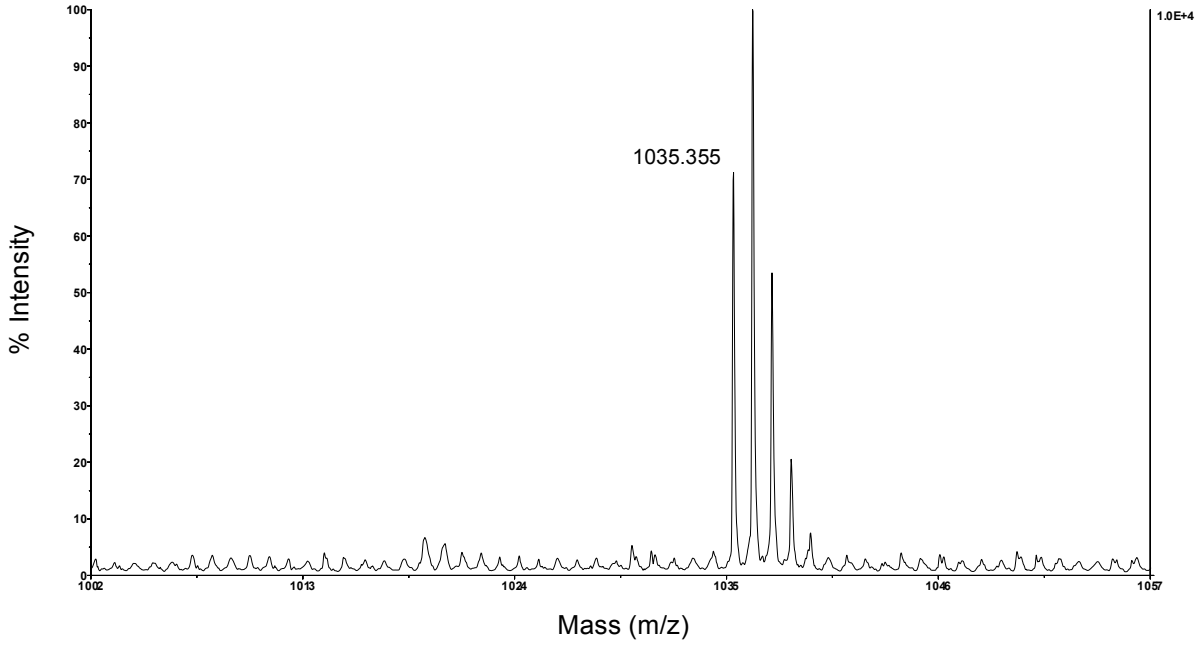


Figure S7. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of DECou-GRK(*c*AB40)-amide 5. m/z calc'd for $C_{50}H_{57}N_{11}O_{12}S$ $[M+H]^+$: 1035.391, m/z found: 1035.355.

This journal is © The Royal Society of Chemistry 2013

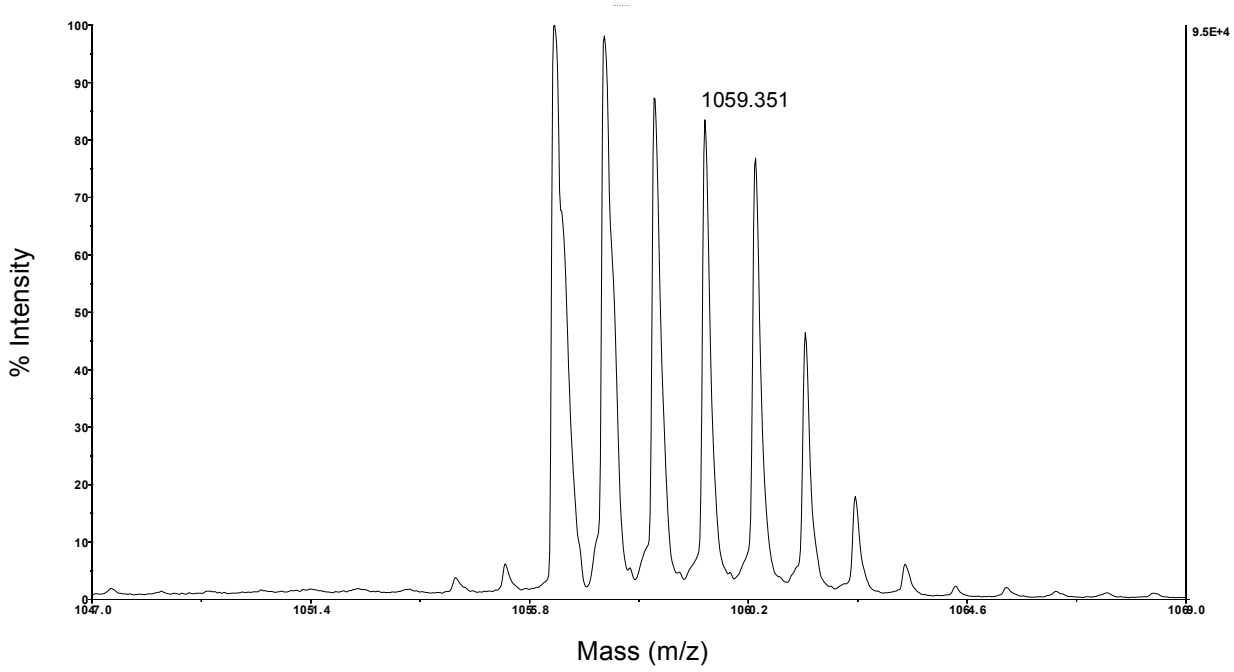


Figure S8. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of Cou343-GRK(*c*AB40)-amide 6. m/z calc'd for $C_{50}H_{57}N_{11}O_{12}S$ $[M+H]^+$: 1059.391, m/z found: 1059.351

This journal is © The Royal Society of Chemistry 2013

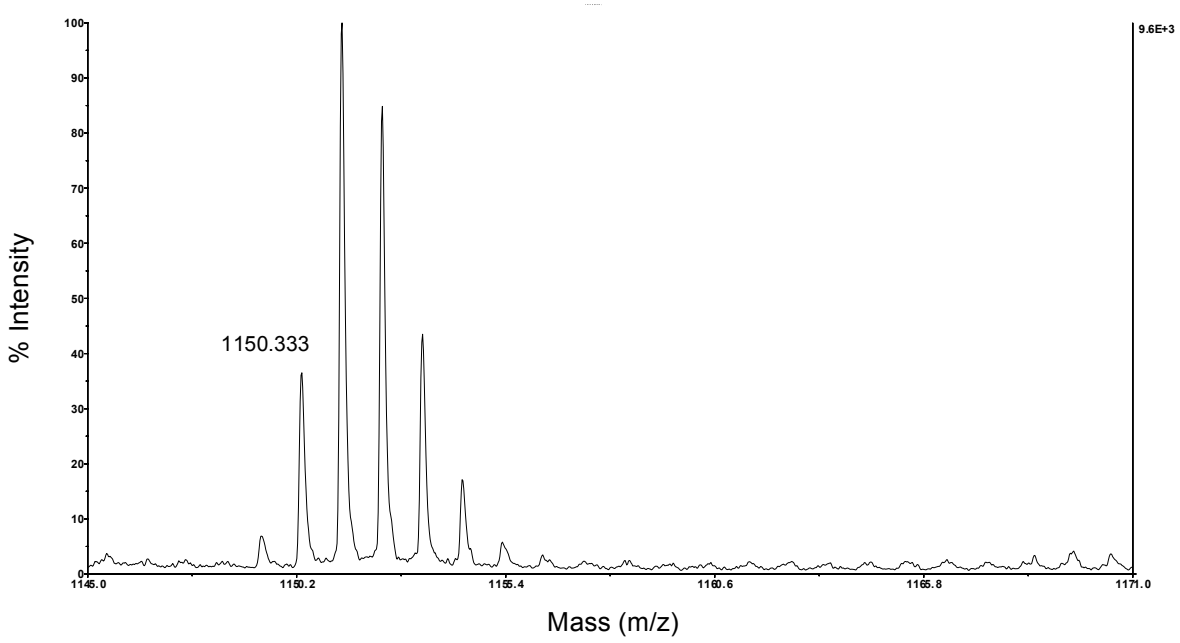


Figure S9. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of FAM-GRK(*c*AB40)-amide 7. m/z calc'd for $C_{57}H_{54}N_{10}O_{15}S$ $[M+H]^+$: 1150.349, m/z found: 1150.333.

This journal is © The Royal Society of Chemistry 2013

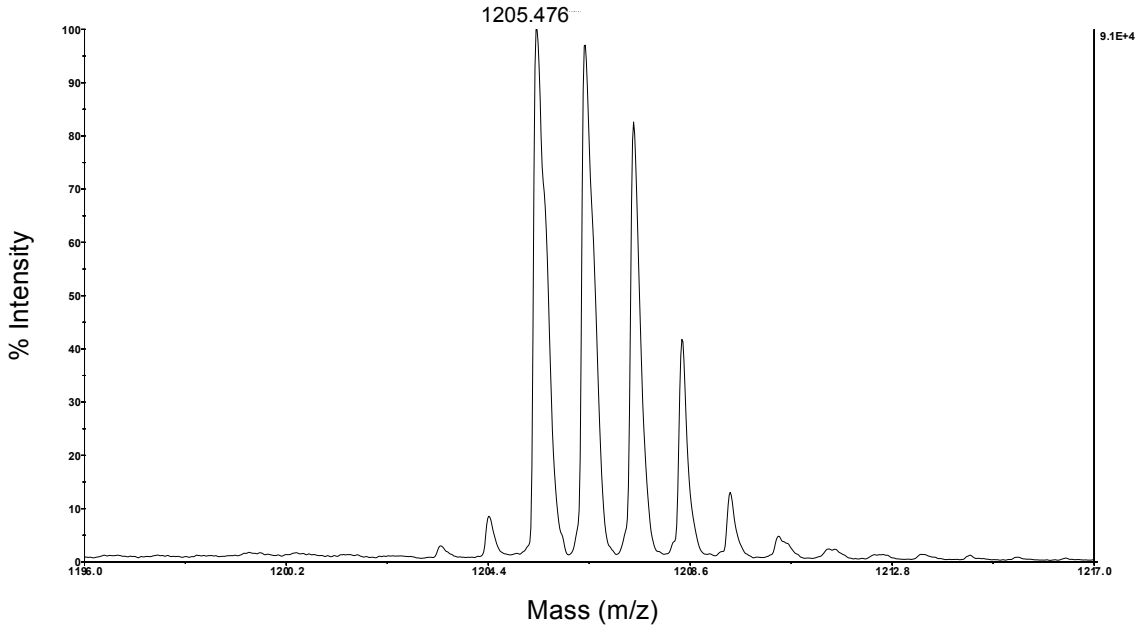


Figure S10. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of TAM-GRK(*c*AB40)-amide 8. m/z calc'd for $C_{61}H_{65}N_{12}O_{13}S$ $[M+H]^+$: 1205.451, m/z found: 1205.476.

This journal is © The Royal Society of Chemistry 2013

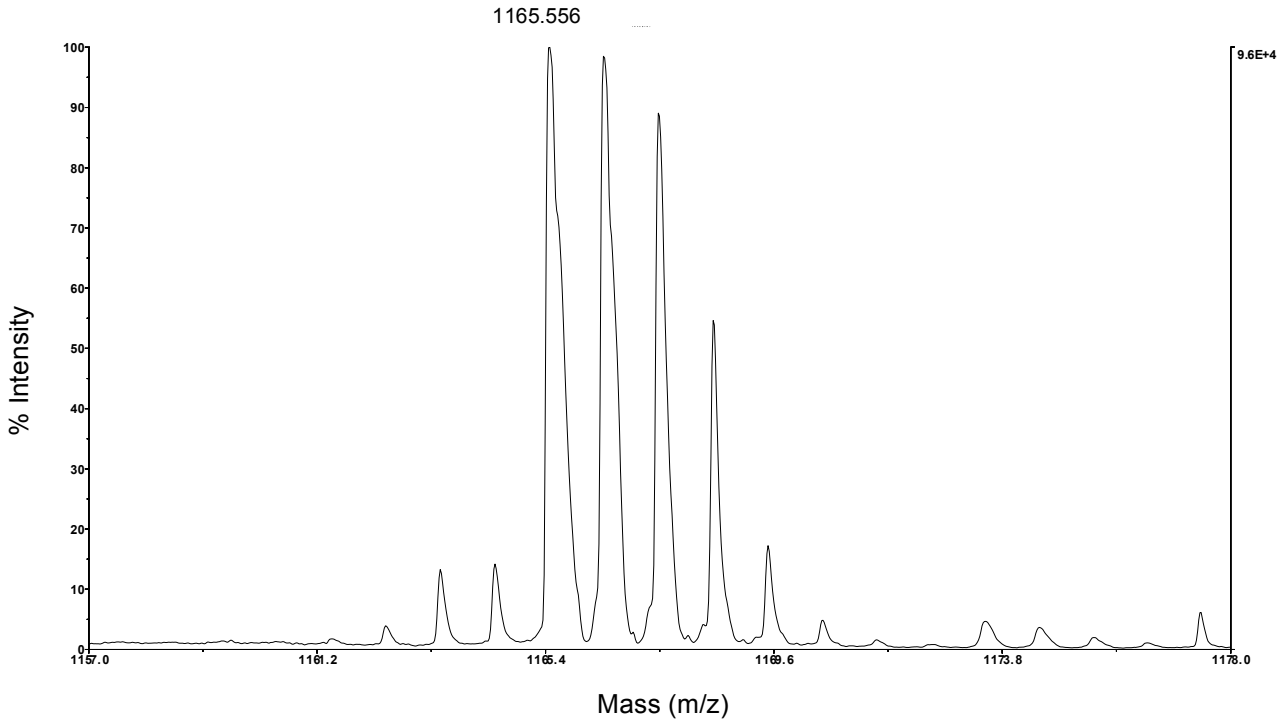


Figure S11. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of atto610-GRK(*c*AB40)-amide 9. m/z calc'd for $C_{61}H_{73}N_{12}O_{10}S$ $[M+H]^+$: 1165.529, m/z found: 1165.556.

This journal is © The Royal Society of Chemistry 2013

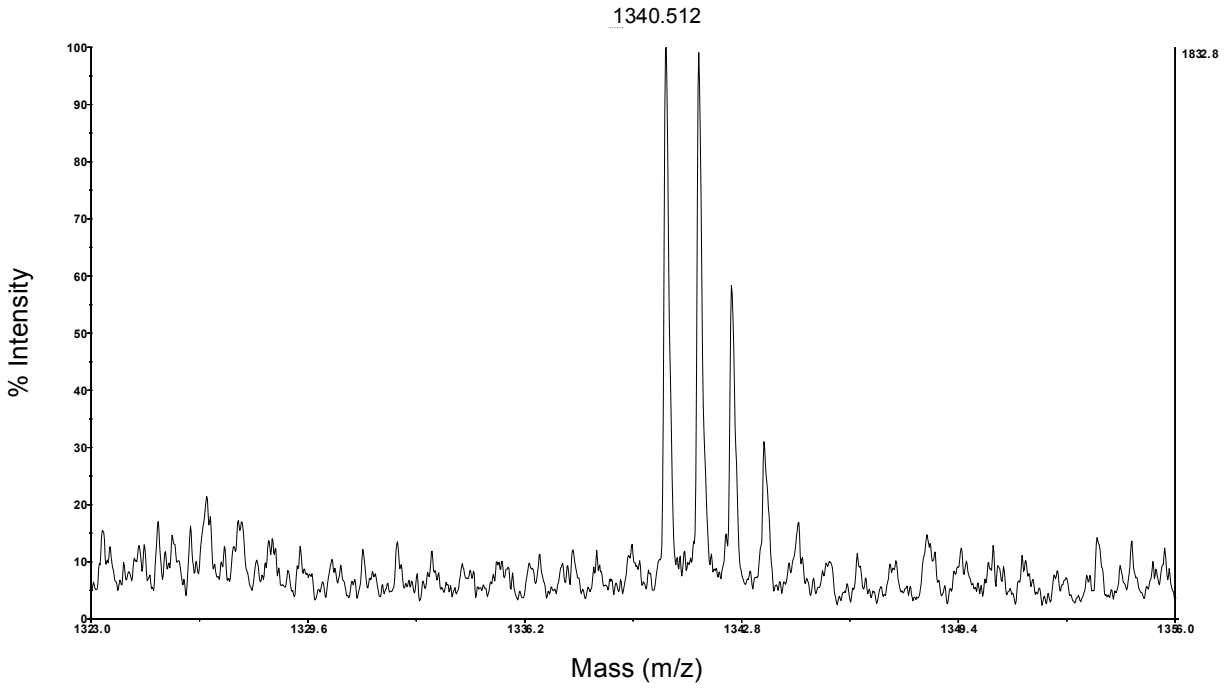


Figure S12. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of Atto700-GRK(*c*AB40)-amide 10. Unable to calculate exact mass due to unpublished atto 700 structure, m/z found: 1340.512.

This journal is © The Royal Society of Chemistry 2013

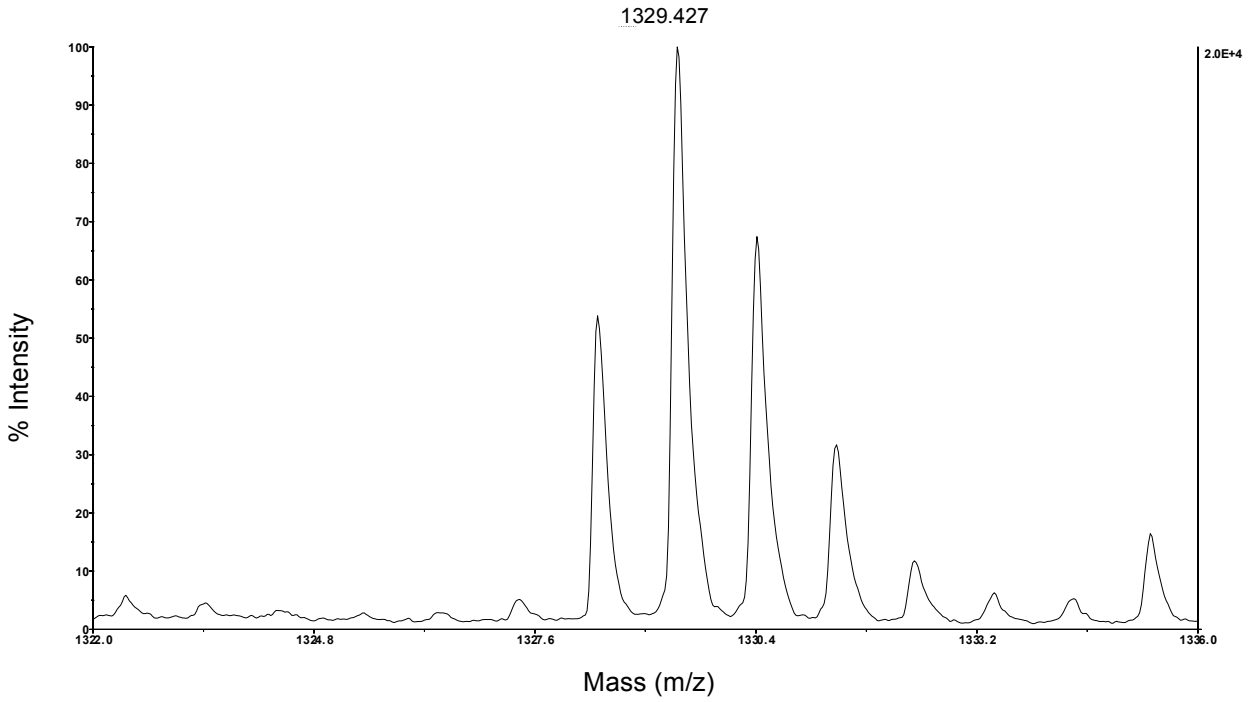


Figure S13. MALDI-TOF (alpha cyano-4-hydroxycinnaminic acid, mono-isotopic) of TAM-G-Ø-K(*c*AB40)-amide 11. m/z calc'd for $C_{78}H_{84}N_{13}O_{11}S$ $[M+H]^+$: 1329.456, m/z found: 1329.427.

This journal is © The Royal Society of Chemistry 2013

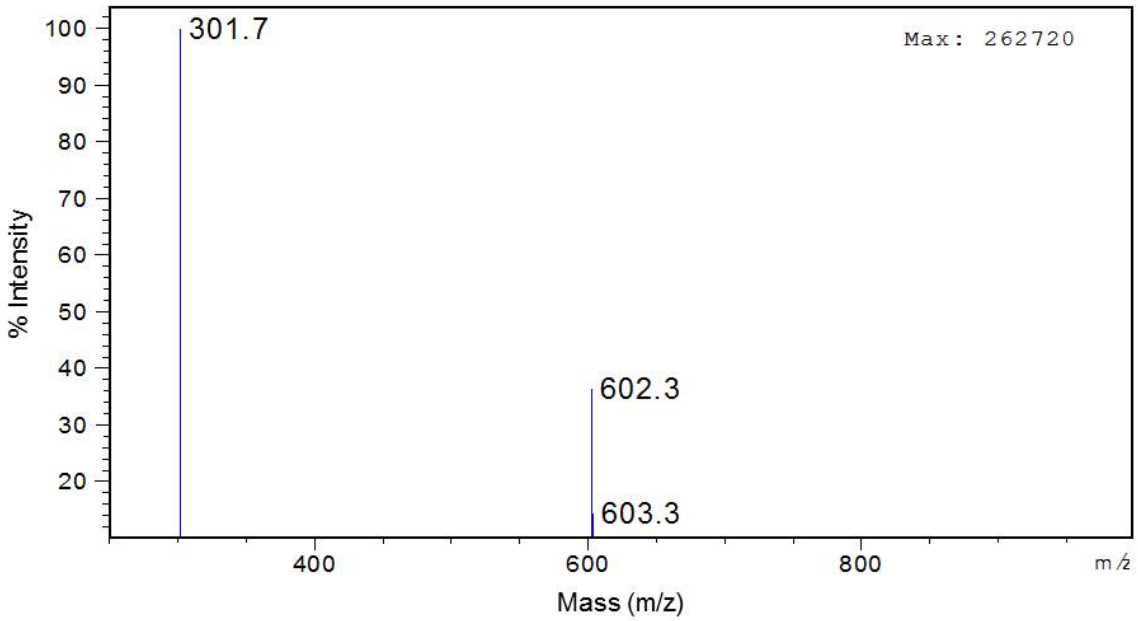


Figure S14. ESI(+)-QMS of DEAC-GRK(NH_2)-amide 13. m/z calc'd for $C_{28}H_{44}N_9O_6^+$ $[M]^+$: 602.3, m/z found: 602.3.

This journal is © The Royal Society of Chemistry 2013

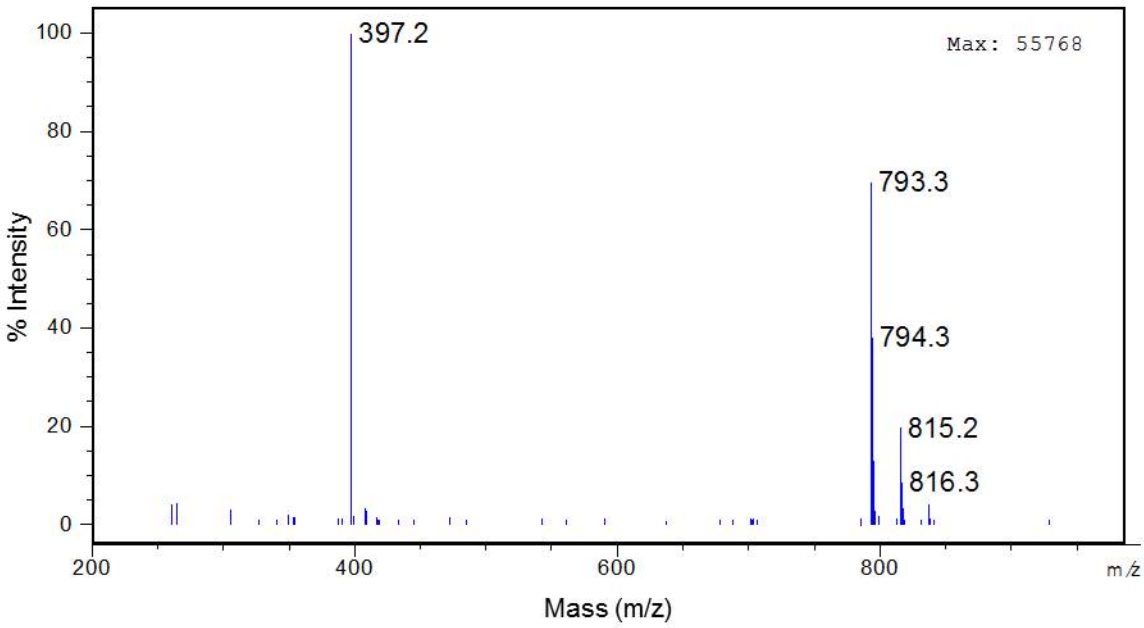


Figure S15. ESI(+)-QMS of H_2N-GRK(*c*AB40)-amide 14. m/z calc'd for $C_{36}H_{45}N_{10}O_9S^+$ $[M]^+$: 793.3, m/z found: 793.3

This journal is © The Royal Society of Chemistry 2013

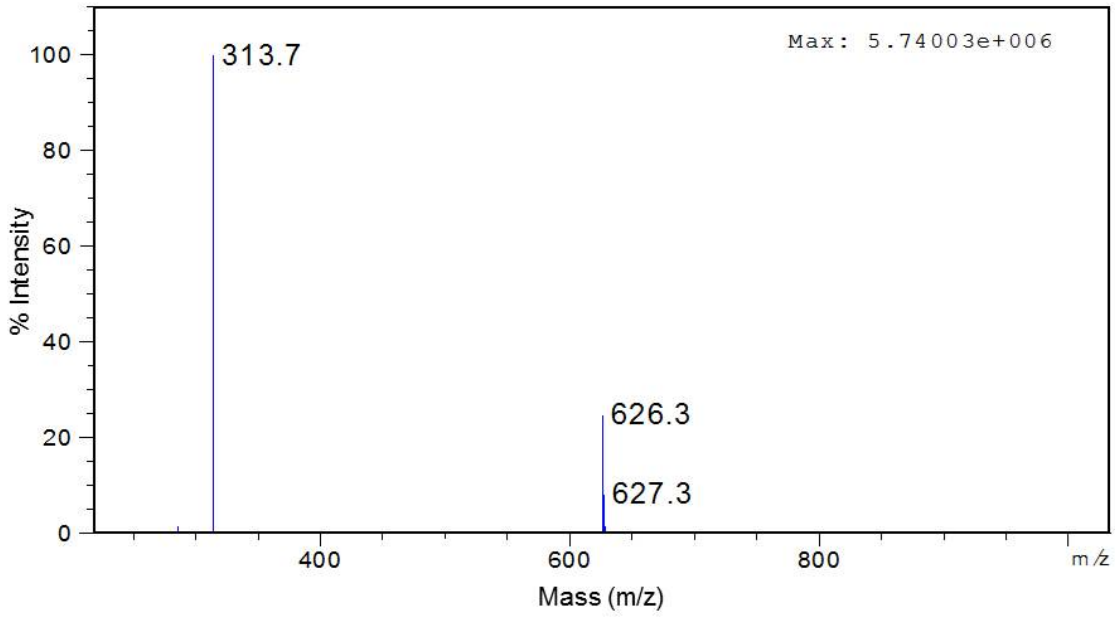


Figure S16. ESI(+)-QMS of Cou343-GRK(NH_2)-amide 15. m/z calc'd for $C_{30}H_{44}N_9O_6^+$ $[M]^+$: 626.3, m/z found: 626.3.

This journal is © The Royal Society of Chemistry 2013

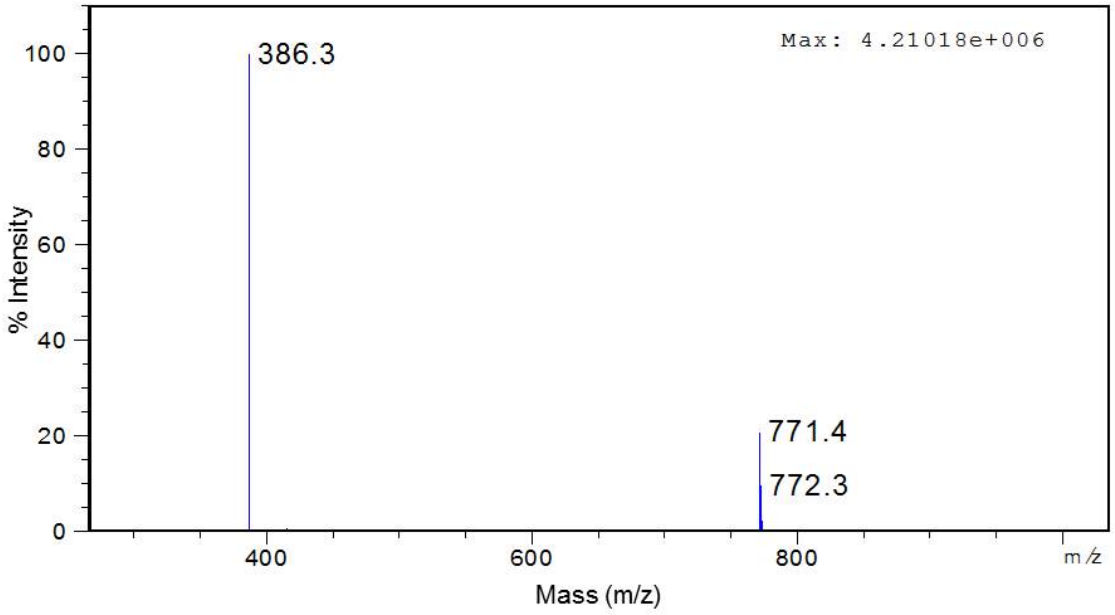


Figure S17. ESI(+)-QMS of TAM-GRK(NH_2)-amide 16. m/z calc'd for $C_{39}H_{51}N_{10}O_7^+$ $[M]^+$: 771.4, m/z found: 771.4.

This journal is © The Royal Society of Chemistry 2013

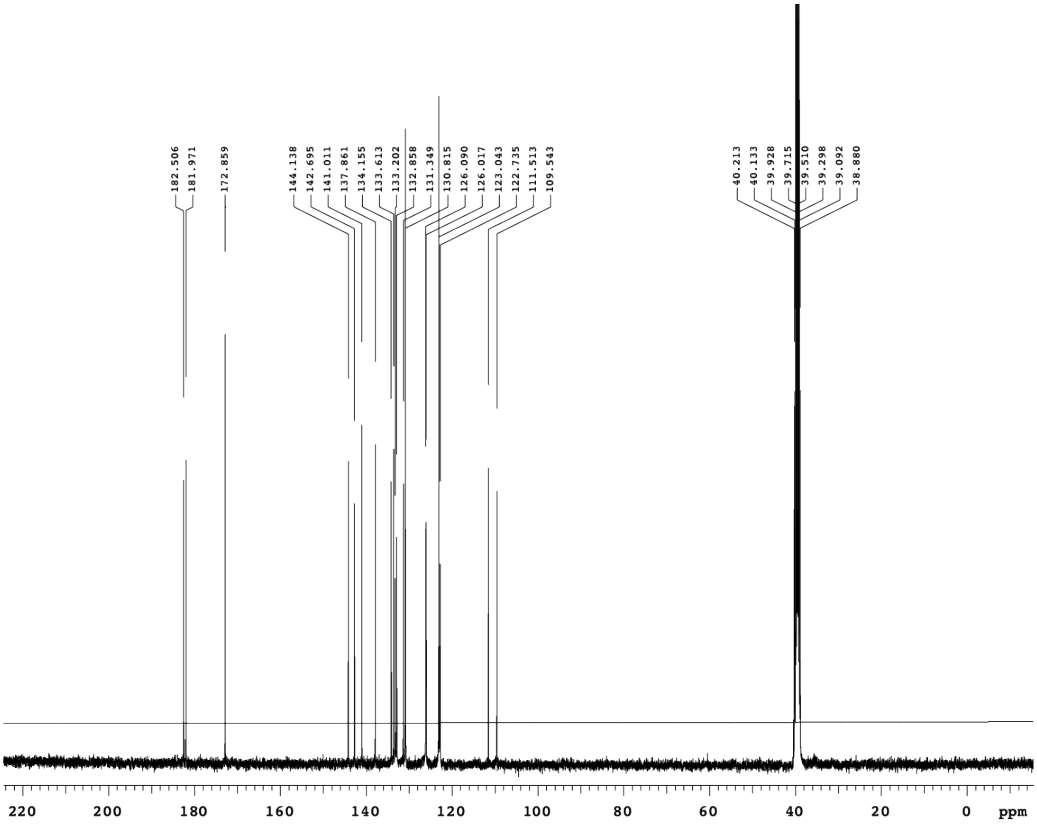


Figure S18. ^{13}C NMR (400 MHz, $CDCl_3$) of *c*AB40 (4).

This journal is © The Royal Society of Chemistry 2013

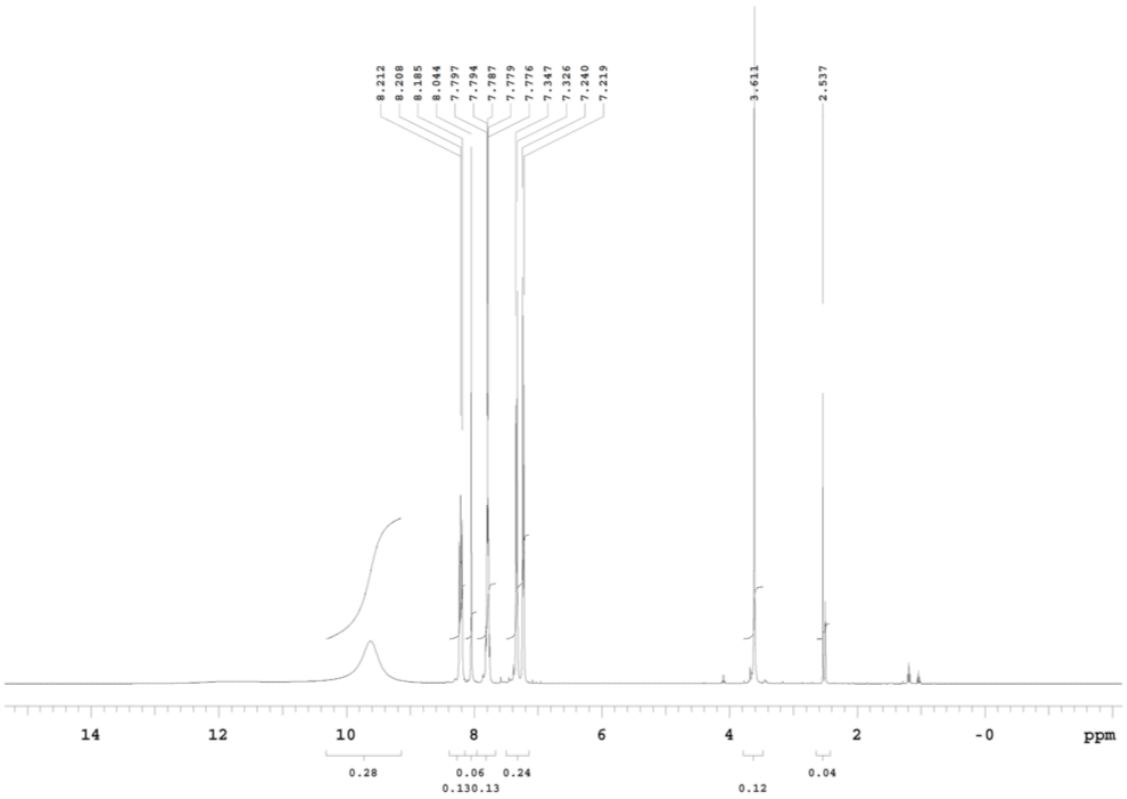


Figure S19. ^{1}H NMR (400 MHz, DMSO-d$_6$) of *c*AB40 (4).

This journal is © The Royal Society of Chemistry 2013

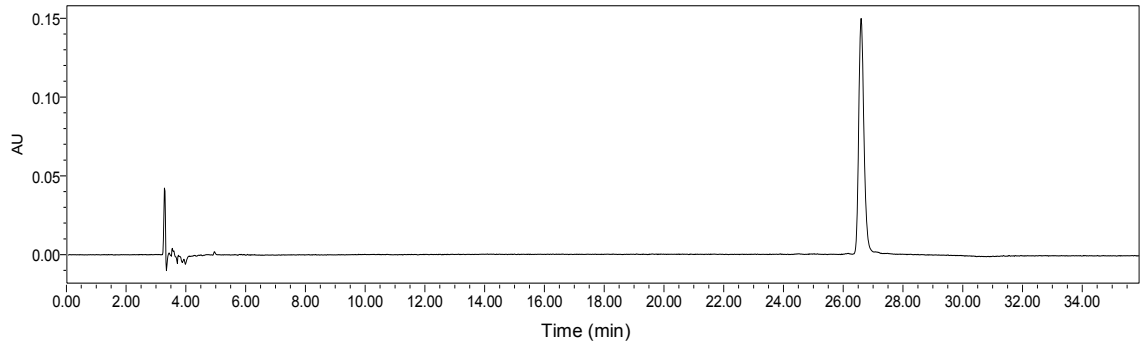


Figure S20. Assessments of purity of DECou-GRK(*c*AB40)-amide 5. RP-HPLC injection of DECou-GRK(*c*AB40)-amide **5** on a 250 mm x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min. A small amount of DMSO was present in the sample, which eluted at 3 min.

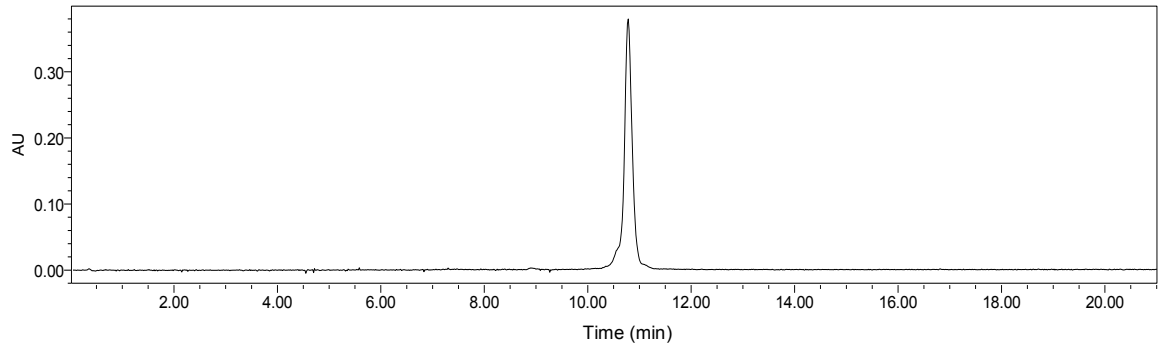


Figure S21. Assessment of purity of DECou-GRK(*c*AB40)-amide 5. RP-HPLC injection of DECou-GRK(*c*AB40)-amide **5** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 20 min.

This journal is © The Royal Society of Chemistry 2013

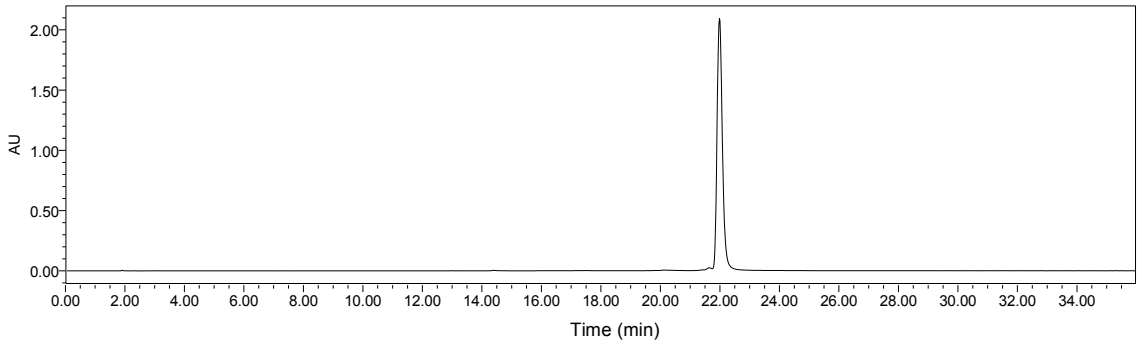


Figure S22. Assessment of purity of Cou343-GRK(*c*AB40)-amide 6. RP-HPLC injection of Cou343-GRK(*c*AB40)-amide **6** on an Alltech Apollo 250 mm x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min.

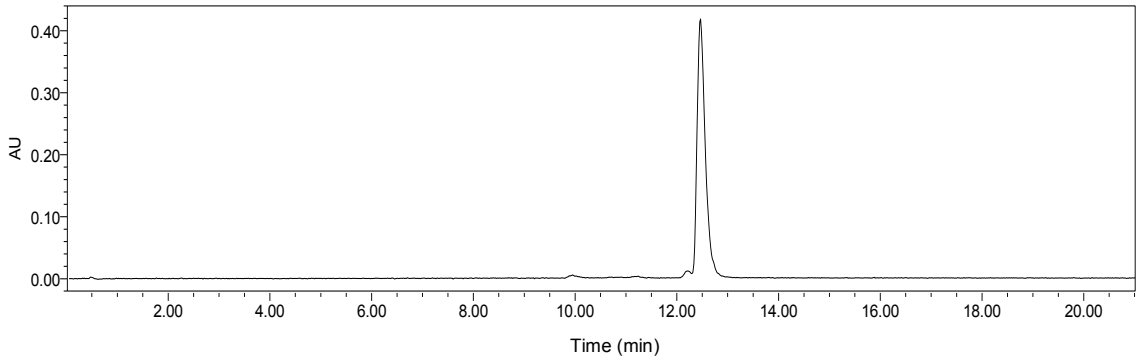


Figure S23. Assessment of purity of Cou343-GRK(*c*AB40)-amide 6. RP-HPLC injection of Cou343-GRK(*c*AB40)-amide **6** on a Restek Viva 50mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 20 min.

This journal is © The Royal Society of Chemistry 2013

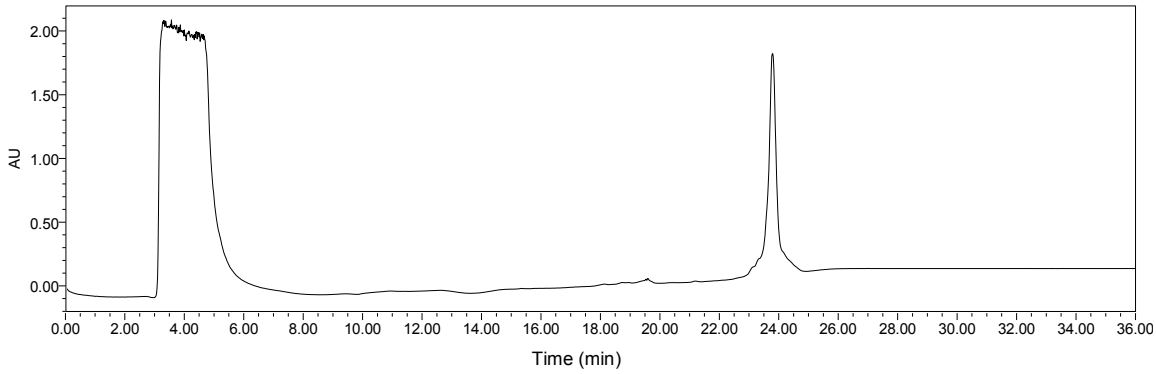


Figure S24. Assessment of purity of FAM-GRK(*c*AB40)-amide 7. RP-HPLC injection of FAM-GRK(*c*AB40)-amide **7** on an Alltech Apollo 250 mm x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min. DMSO was present in the sample, which eluted at 3 min.

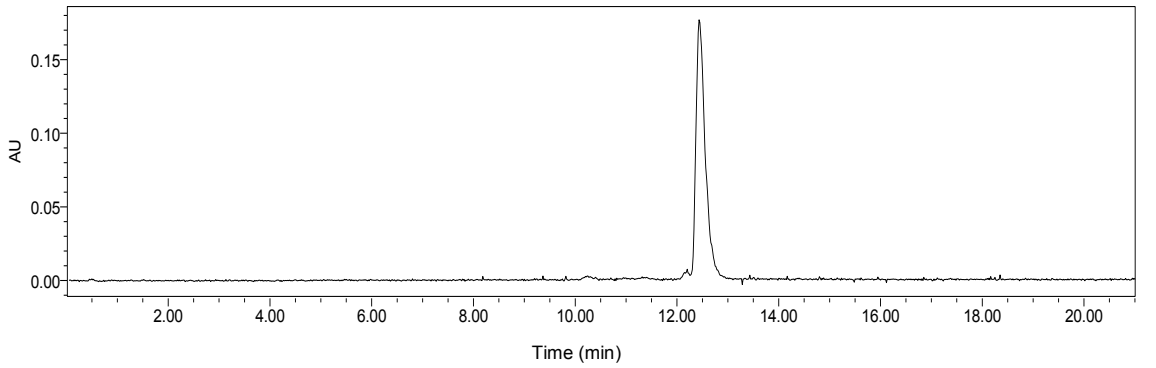


Figure S25. Assessment of purity of FAM-GRK(AB40)-amide 7. RP-HPLC injection of FAM-GRK(*c*AB40)-amide **7** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 20 min.

This journal is © The Royal Society of Chemistry 2013

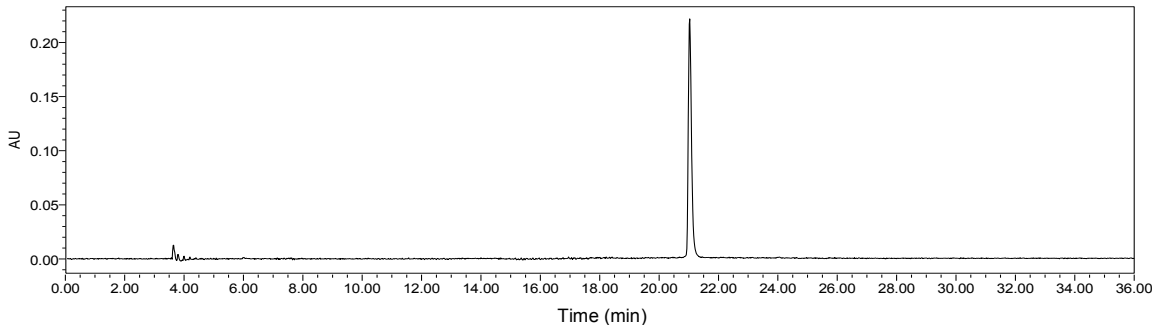


Figure S26. Assessment of purity of TAM-GRK(*c*AB40)-amide 8. RP-HPLC run of TAM-GRK(*c*AB40)-amide **8** on an Alltech Apollo 250 x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min.

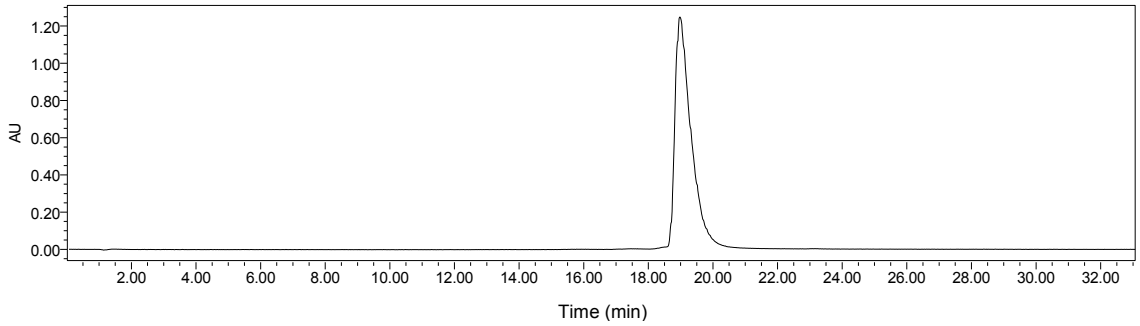


Figure S27. Assessment of purity of TAM-GRK(*c*AB40)-amide 8. RP-HPLC injection of TAM-GRK(*c*AB40)-amide **8** on a Restek Viva 50 x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 30 min.

This journal is © The Royal Society of Chemistry 2013

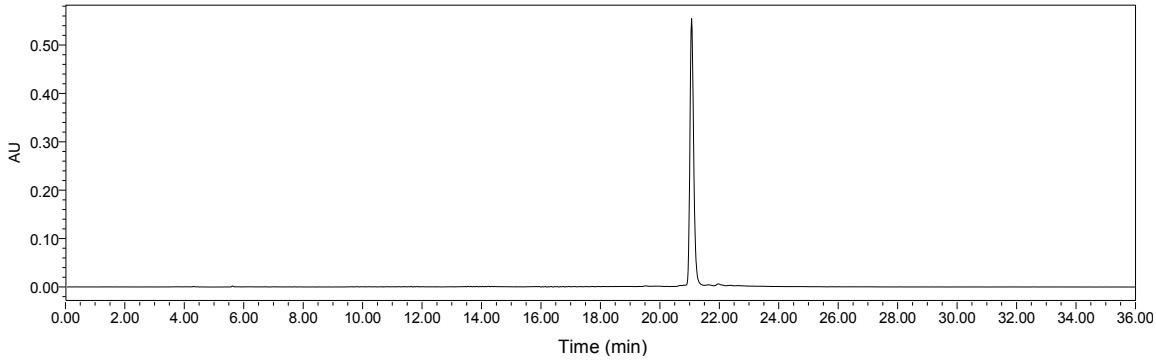


Figure S28. Assessment of purity of atto610-GRK(*c*AB40)-amide 9. RP-HPLC injection of atto610-GRK(*c*AB40)-amide **9** on an Alltech Apollo 250 x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min.

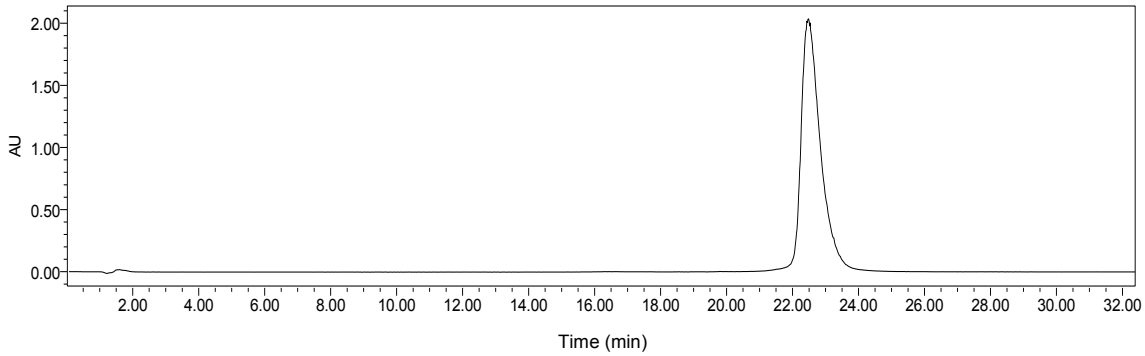


Figure S29. Assessment of purity of atto610-GRK(*c*AB40)-amide 9. RP-HPLC injection of atto610-GRK(*c*AB40)-amide **9** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 30 min.

This journal is © The Royal Society of Chemistry 2013

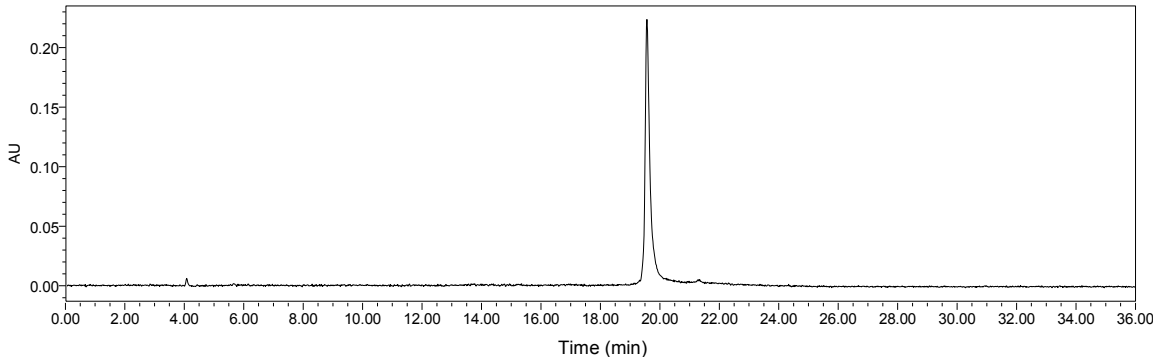


Figure S30. Assessment of purity of atto700-GRK(*c*AB40)-amide 10. RP-HPLC injection of atto700-GRK(*c*AB40)-amide **10** on an Alltech Apollo 250 mm x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min.

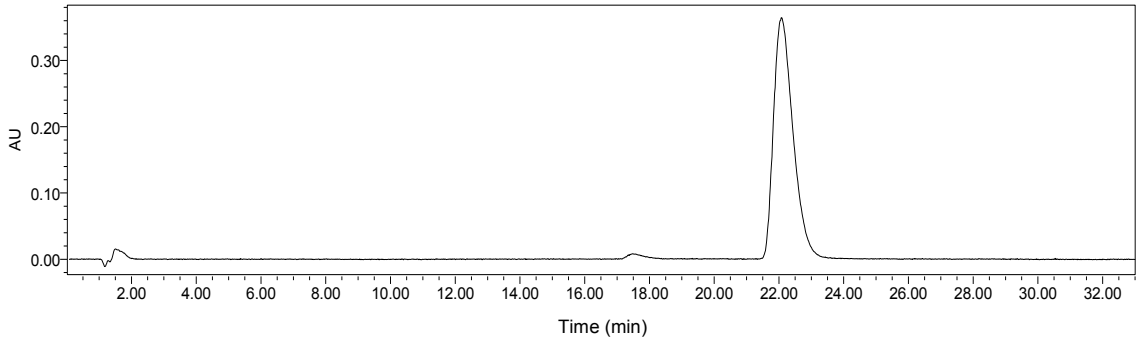


Figure S31. Assessment of purity of atto700-GRK(*c*AB40)-amide 10. RP-HPLC injection of atto700-GRK(*c*AB40)-amide **10** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 30 min.

This journal is © The Royal Society of Chemistry 2013

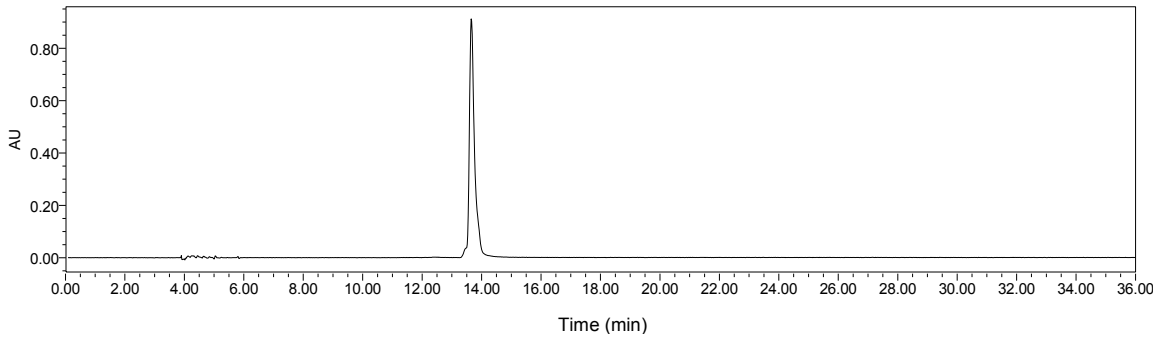


Figure S32. Assessment of purity of TAM-G-Ø-K(*c*AB40)-amide 11. RP-HPLC injection of TAM-G-PL-K(*c*AB40)-amide **11** on an Alltech Apollo 250 mm x 4.6 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 35 min.

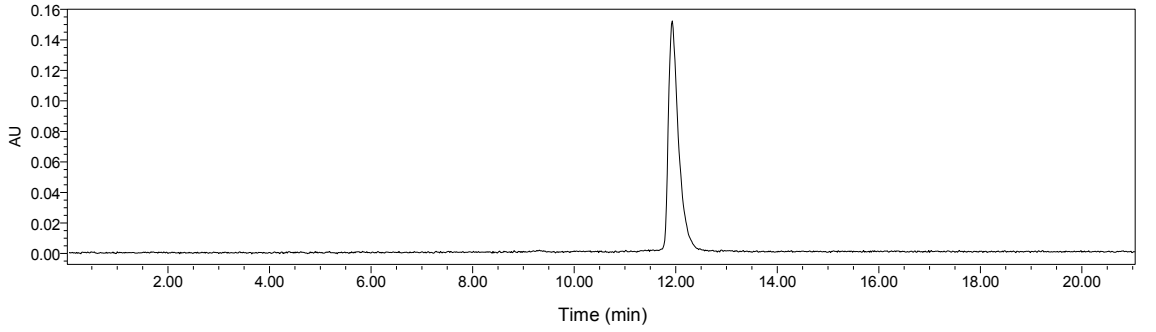


Figure S33. Assessments of purity of TAM-G-Ø-K(*c*AB40)-amide 11. RP-HPLC injection of TAM-G-PL-K(*c*AB40)-amide **11** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 20 min.

This journal is © The Royal Society of Chemistry 2013

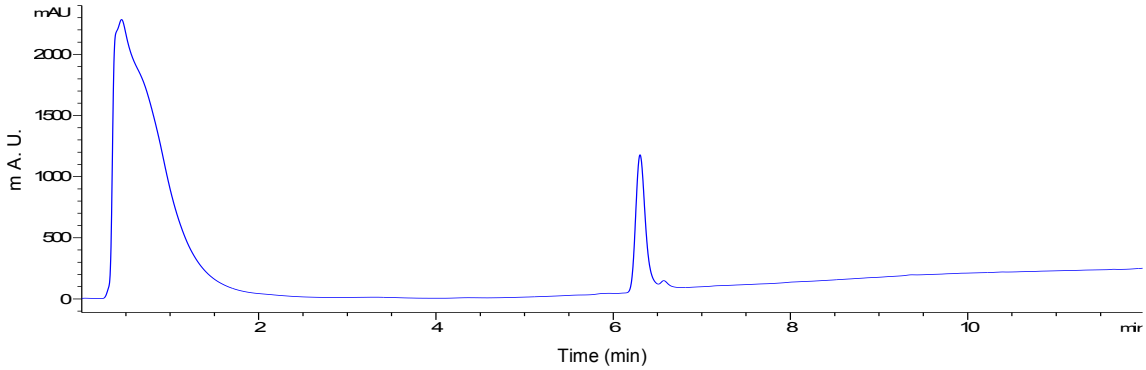


Figure S34. Assessment of purity of DEAC-GRK(NH_2)-amide 13. RP-HPLC injection of DEAC-GRK(NH_2)-amide **13** on an Alltech Apollo 50 mm x 2.1 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 12 min.

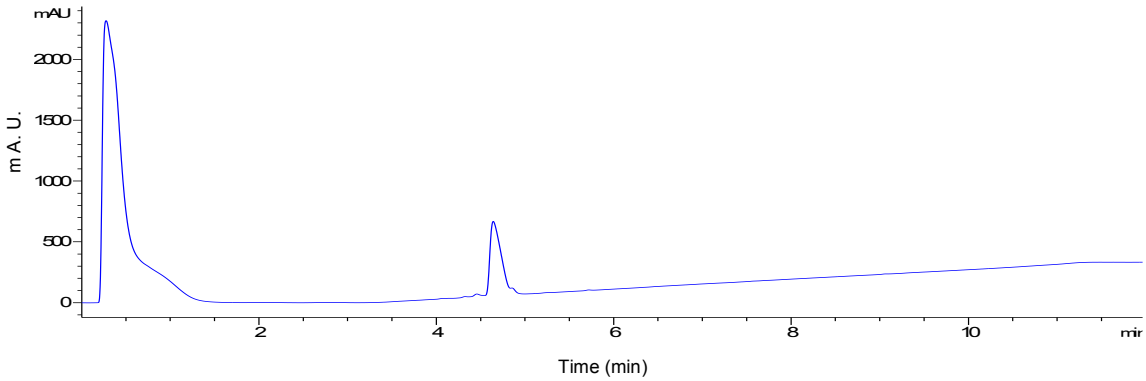


Figure S35. Assessment of purity of DEAC-GRK(NH_2)-amide 13. RP-HPLC injection of DEAC-GRK(NH_2)-amide **13** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 12 min.

This journal is © The Royal Society of Chemistry 2013

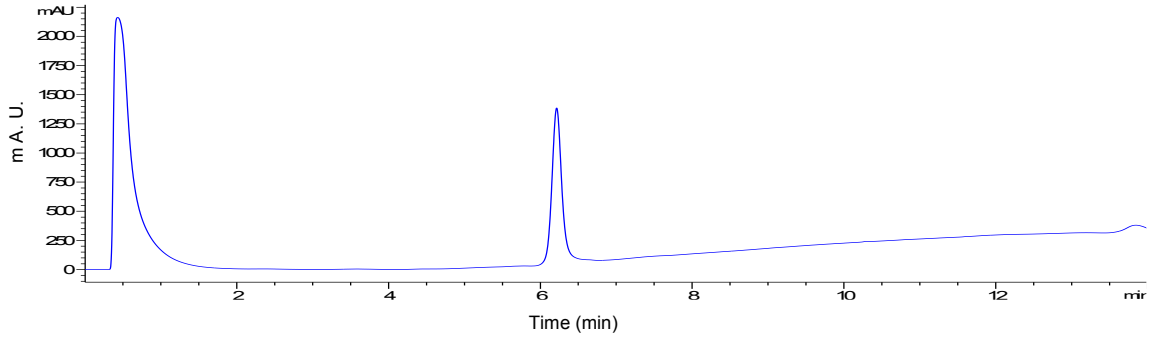


Figure S36. Assessment of purity of NH_2-GRK(*c*AB40)-amide 14. RP-HPLC injection of NH_2-GRK(*c*AB40)-amide **14** on an Alltech Apollo 50 mm x 2.1 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 12 min.

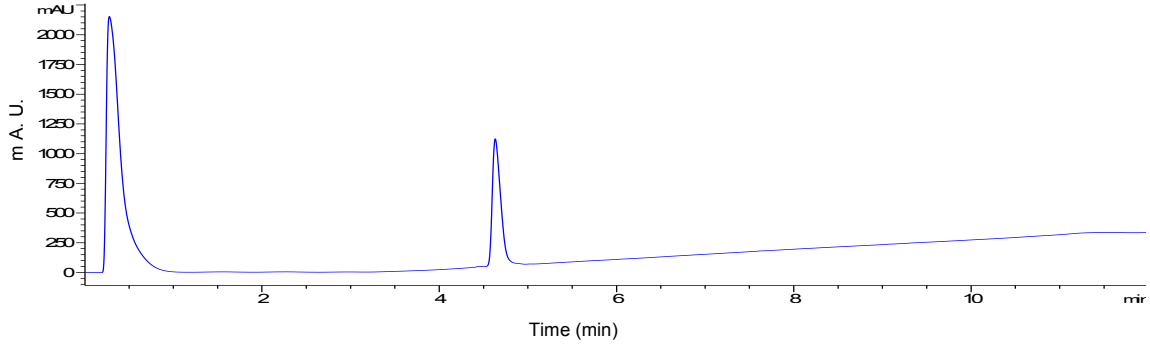


Figure S37. Assessment of purity of NH_2-GRK(*c*AB40)-amide 14. RP-HPLC injection of NH_2-GRK(*c*AB40)-amide **14** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 12 min.

This journal is © The Royal Society of Chemistry 2013

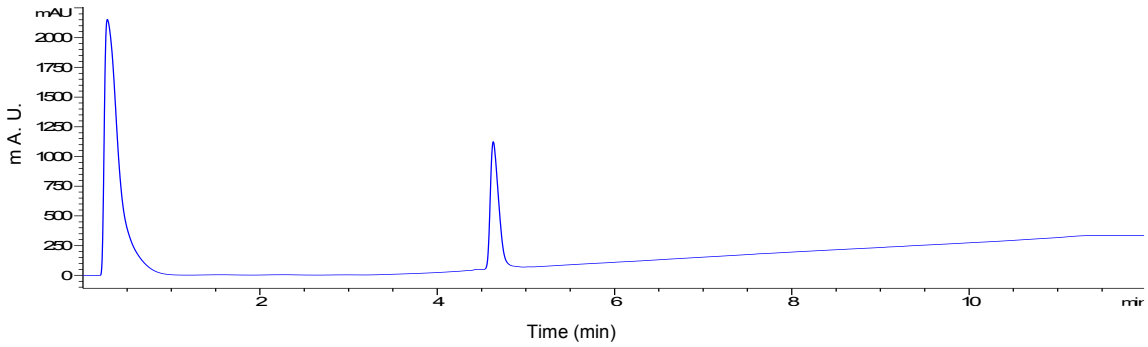


Figure S38. Assessment of purity of Cou343-GRK(NH_2)-amide 15. RP-HPLC injection of Cou343-GRK(NH_2)-amide **15** on an Alltech Apollo 50 mm x 2.1 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 12 min.

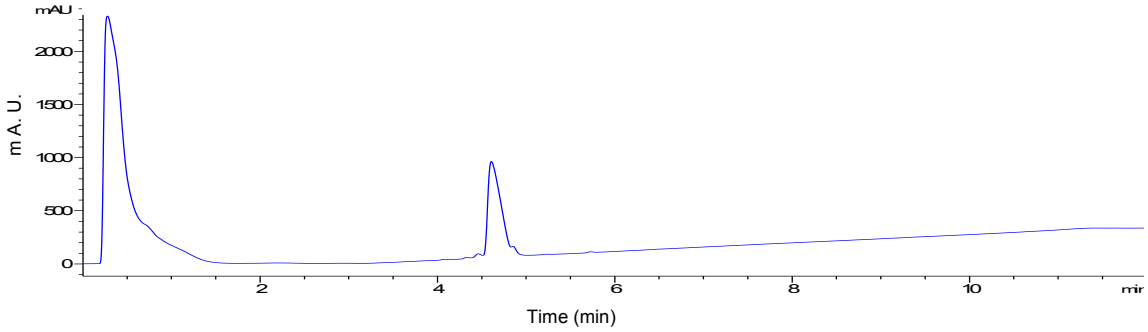


Figure S39. Assessments of purity of Cou343-GRK(NH_2)-amide 15. RP-HPLC injection of Cou343-GRK(NH_2)-amide **15** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 12 min.

This journal is © The Royal Society of Chemistry 2013

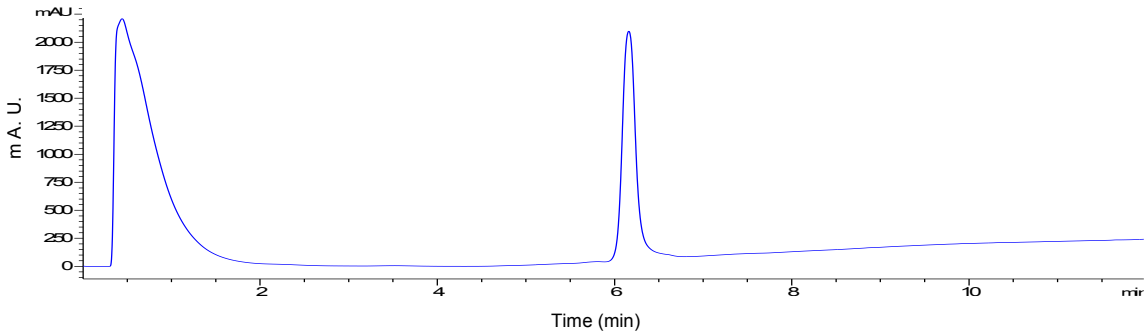


Figure S40. Assessment of purity of TAM-GRK(NH_2)-amide 16. RP-HPLC injection of TAM-GRK(NH_2)-amide **16** on an Alltech Apollo 50 mm x 2.1 mm, 5 µm C18 column. The injection was monitored at 220 nm using a 0 - 97% linear acetonitrile to water gradient over 12 min.

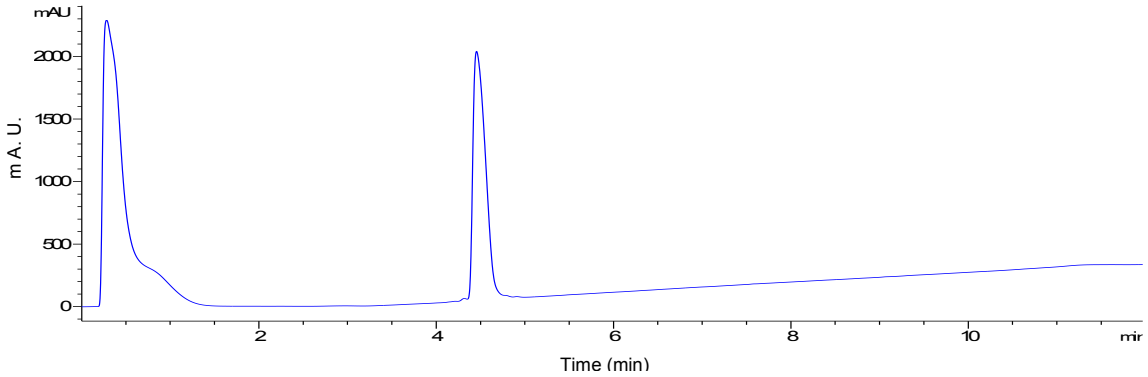


Figure S41. Assessments of purity of TAM-GRK(NH_2)-amide 16. RP-HPLC injection of TAM-GRK(NH_2)-amide **16** on a Restek Viva 50 mm x 2.1 mm, 5 µm C4 column. The injection was monitored at 220 nm using a 0 – 97% linear acetonitrile to water gradient over 12 min.

This journal is © The Royal Society of Chemistry 2013

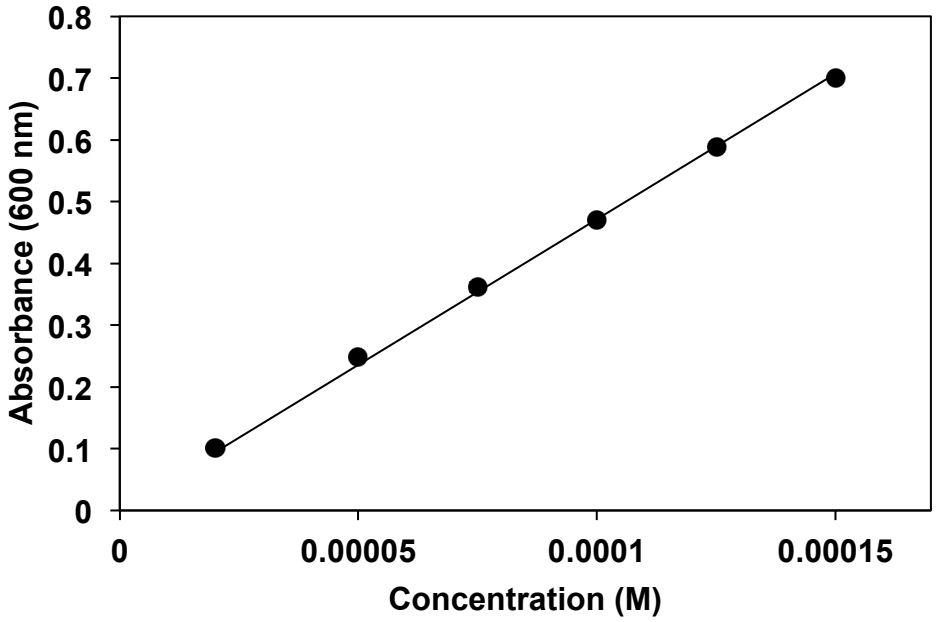


Figure S42. Beer's law plot of absorbance as a function of [*c*AB40]. The experiment was performed in triplicate. Errors bars are present in the figure, however they are too tight to observe. The molar absorptivity (ε) of *c*AB40 (**4**) is 4715 ± 35 $M^{-1}cm^{-1}$ at 600 nm.

This journal is © The Royal Society of Chemistry 2013

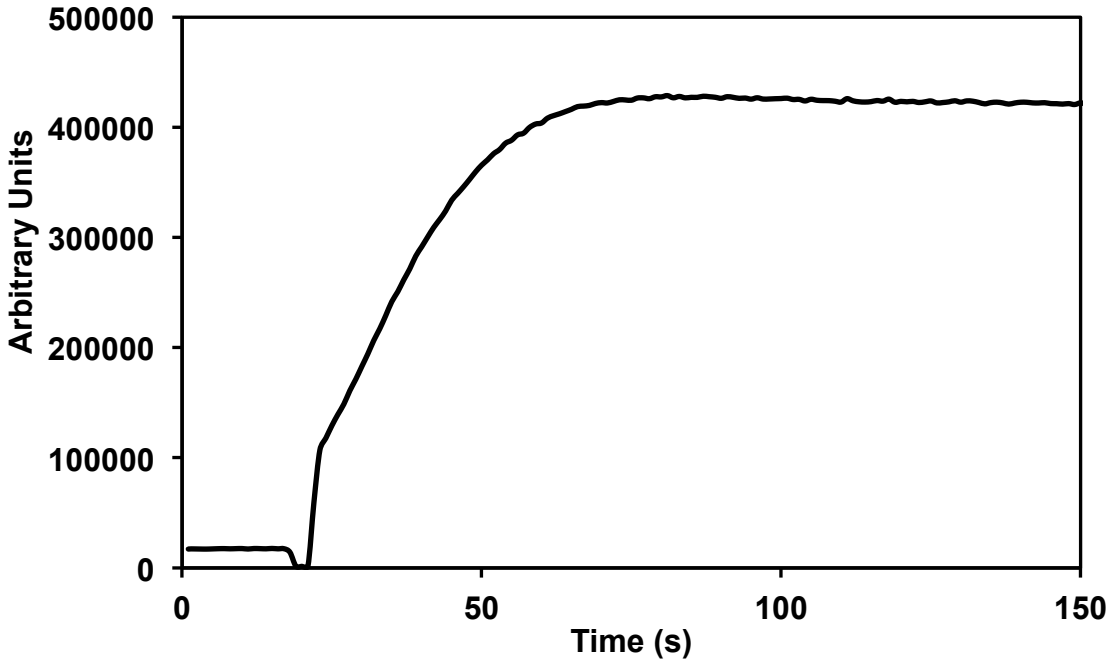


Figure S43. Trypsinolysis of DEAC-GRK(*c*AB40)-amide 5. Peptide **5** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (50 μM; final concentration 500 nM) was added (at 15 s) and the fluorescence monitored at 479 nm (excitation = 430 nm). Initial rate = 0.0284 ± 0.0022 μM/s.

This journal is © The Royal Society of Chemistry 2013

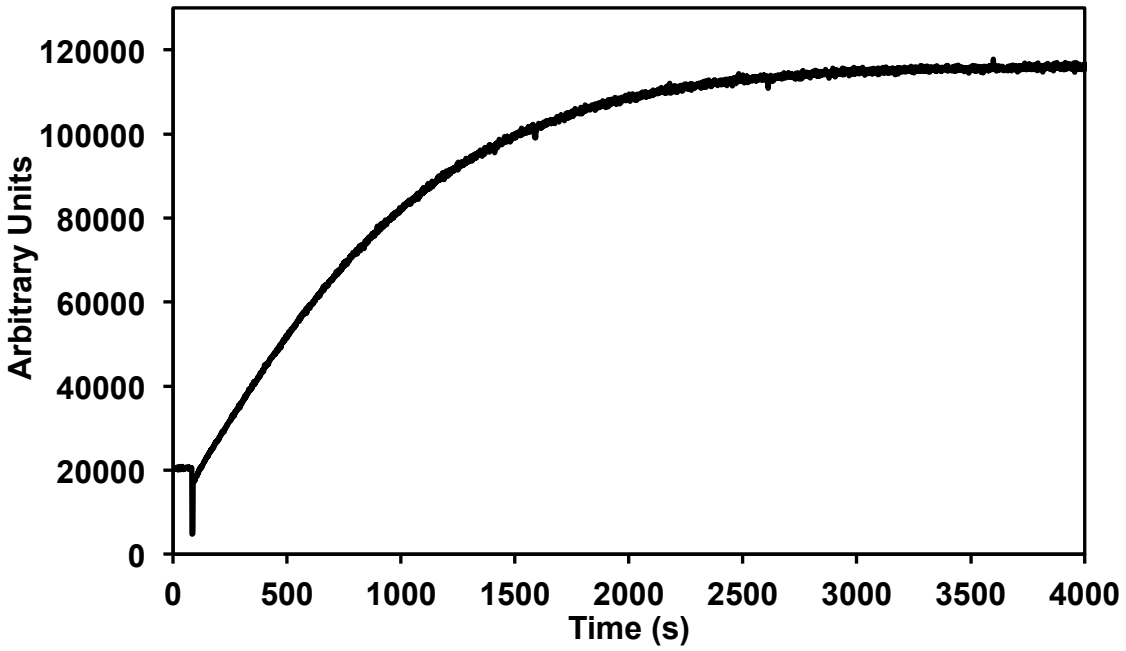


Figure S44. Trypsinolysis of Cou343-GRK(*c*AB40)-amide 6. Peptide **6** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added (at 30 s) and the fluorescence monitored at 490 nm (excitation = 445 nm). Initial rate = 0.000832 ± 0 0.000034 μM/s.

This journal is © The Royal Society of Chemistry 2013

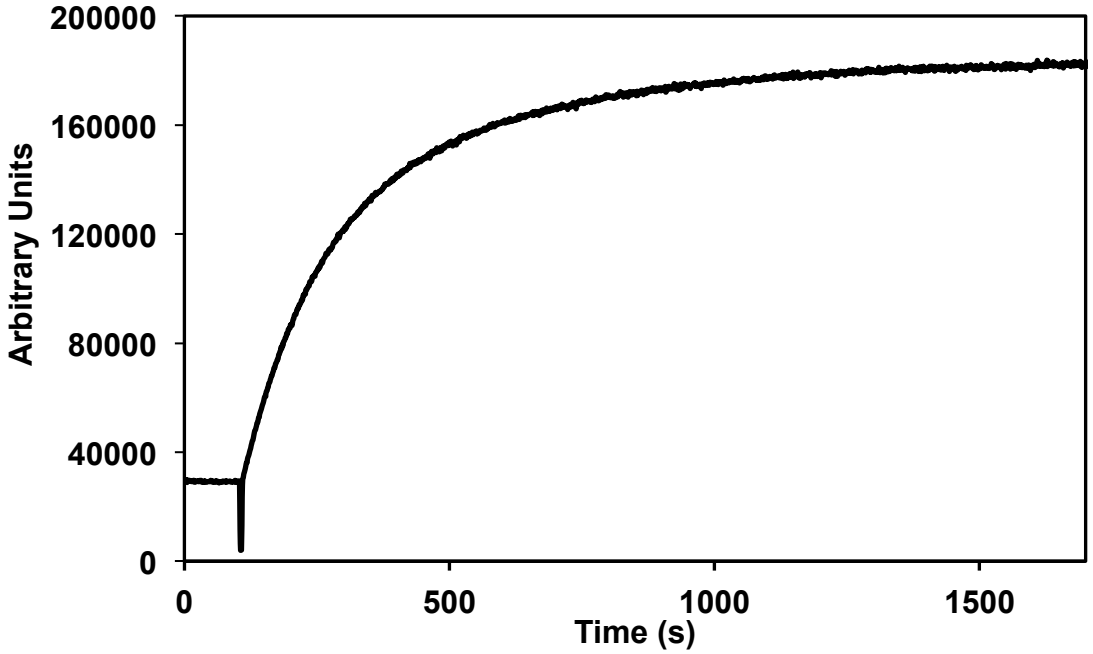


Figure S45. Trypsinolysis of FAM-GRK(*c*AB40)-amide 7. Peptide **7** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added (at 80 s) and the fluorescence monitored at 494 nm (excitation = 520 nm). Initial rate = 0.00270 ± 0.000212 μM/s.

This journal is © The Royal Society of Chemistry 2013

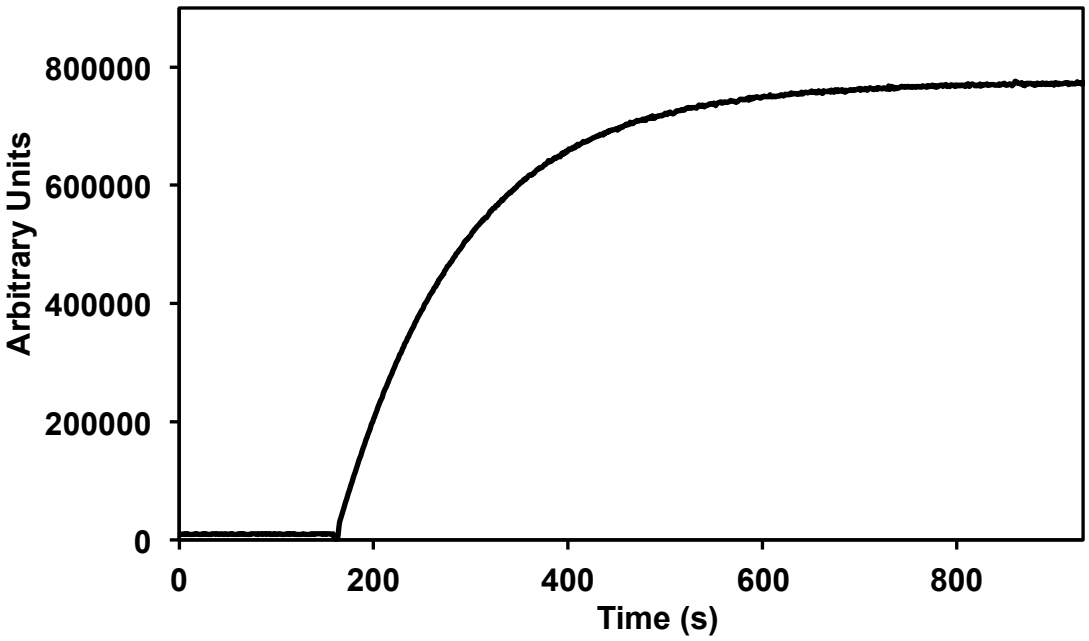


Figure S46. Trypsinolysis of cassette TAM-GRK(*c*AB40)-amide 8. Peptide **8** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added (at 180 s) and the fluorescence monitored at 550 nm (excitation = 580 nm). Initial rate = 0.004904 ± 0.000715 μM/s.

This journal is © The Royal Society of Chemistry 2013

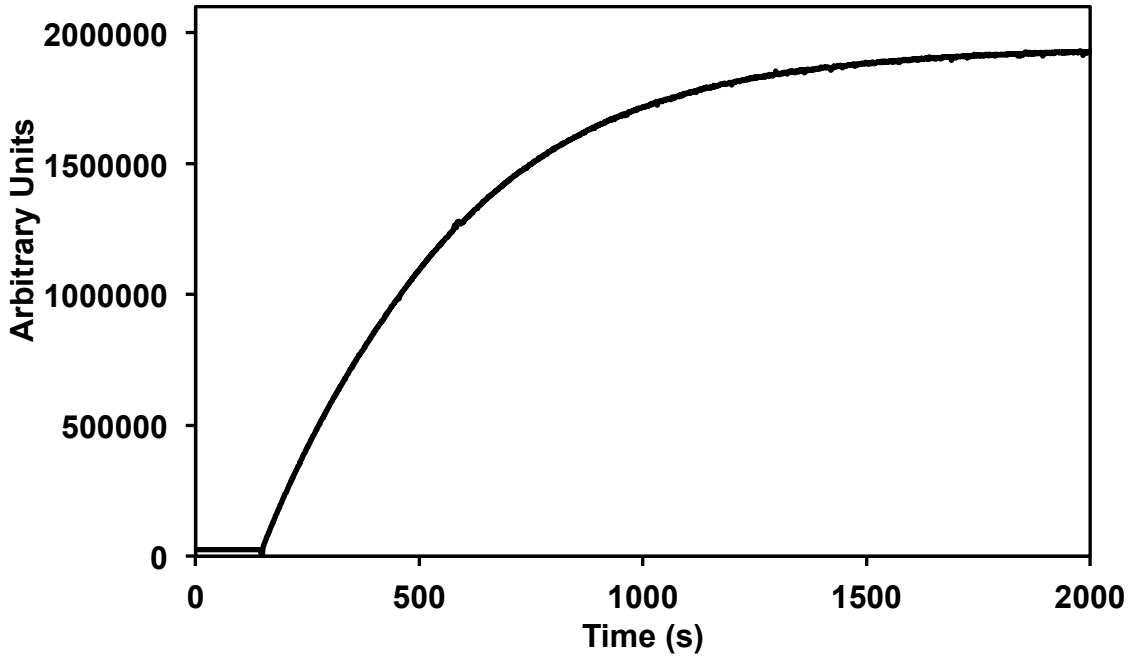


Figure S47. Trypsinolysis of Atto610-GRK(*c*AB40)-amide 9. Peptide **9** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added (at 125 s) and the fluorescence monitored at 635 nm (excitation = 610 nm). Initial rate = 0.001583 ± 0.000122 μM/s.

This journal is © The Royal Society of Chemistry 2013

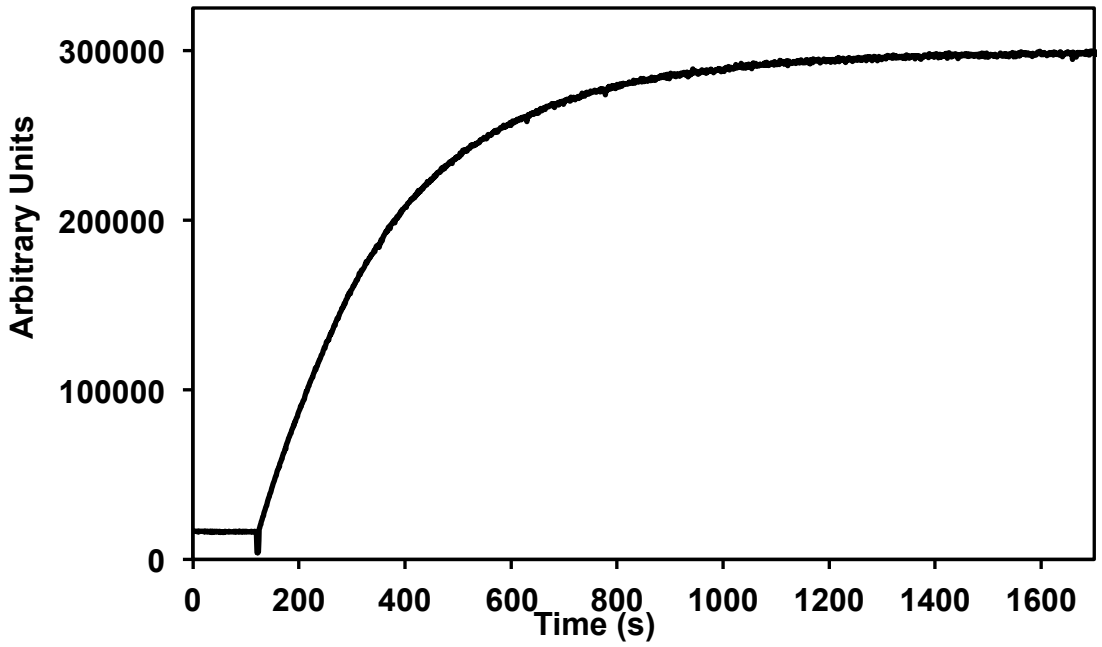


Figure S48. Trypsinolysis of Atto700-GRK(*c*AB40)-amide 10. Peptide **10** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, 2 μL of trypsin (5 μM; final concentration 50 nM) was added (at 120 s) and the fluorescence monitored at 714 nm (excitation = 681 nm). Initial rate = 0.002835 ± 0.000225 μM/s.

This journal is © The Royal Society of Chemistry 2013

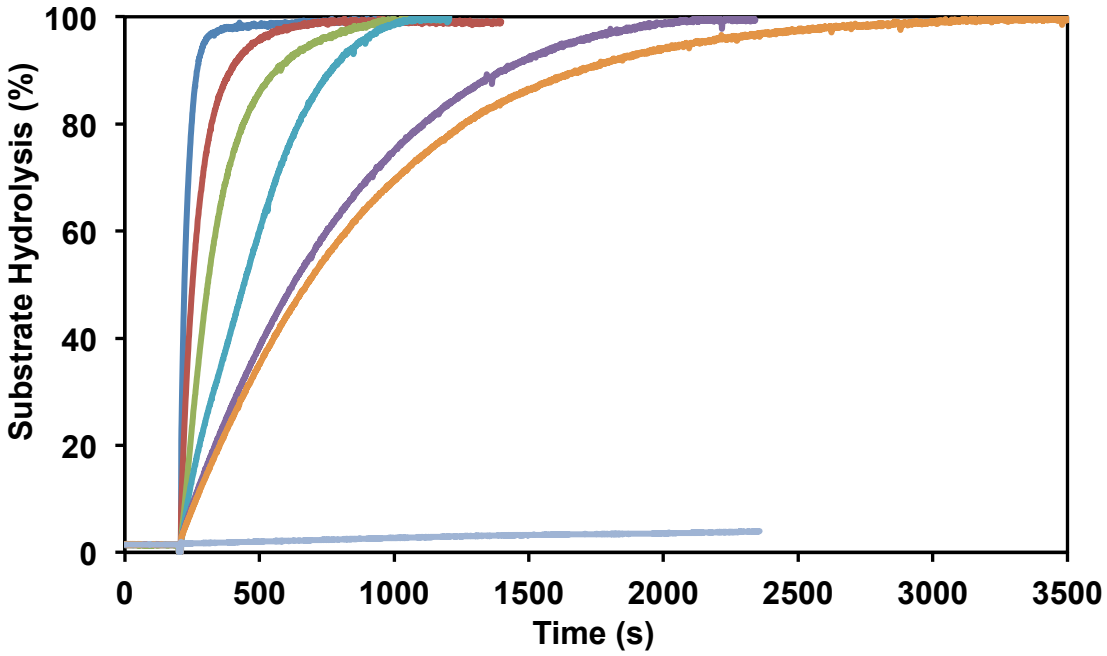


Figure S48. Hydrolysis of TAM-GRK(*c*AB40)-amide 8 at various serine protease concentrations. Peptide **8** (1 μM) was incubated in 25 mM Tris pH 7.4 buffer containing 1 mM dithiothreitol in a 200 μL quartz cuvette at 30 ˚C. After 5 min, a fluorescent baseline was collected using a Photon Technology QM-4 spectrofluorimeter (Birmingham, NJ). Once a stable baseline had been established, a serine protease was added. Final concentration: 200 nM trypsin (dark blue), 100 nM trypsin (red), 50 nM trypsin (green), 25 nM trypsin (cyan), 10 nM trypsin (purple), 5 nM trypsin (orange), and 50 nM chymotrypsin (azure) and the fluorescence emission monitored at 550 nm (excitation = 580 nm).