Supplementary Information

In Situ Maleimide Bridging of Disulfides and a New Approach to Protein PEGylation

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Supplementary Table 1 | Stopped-flow analysis of dihalomaleimide-mediated bridging of somatostatin.

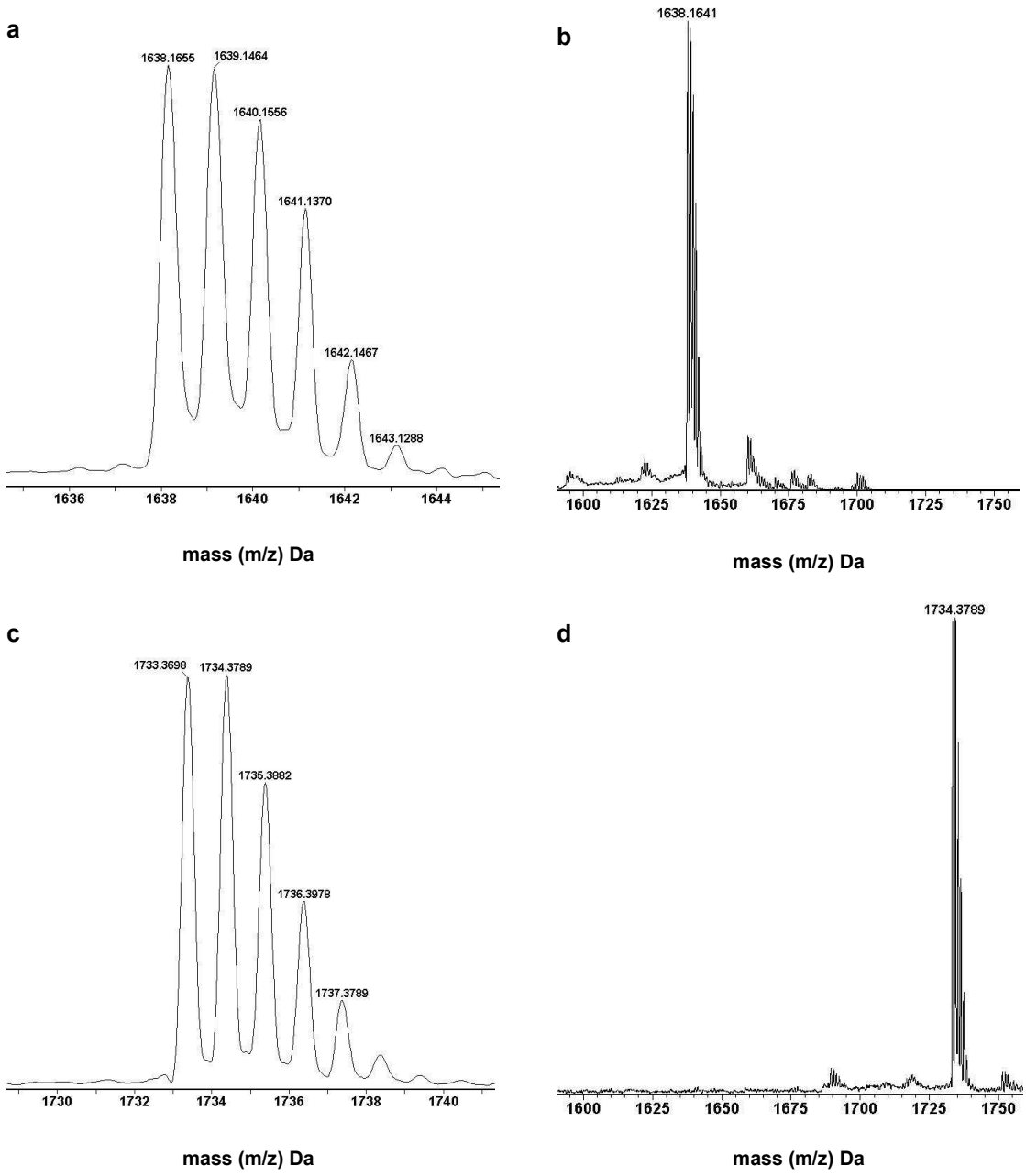
halogen	$k_{app}1$ [1 s^{-1}]	error $k_{app}1$	$k_{app}2$ [1 s^{-1}]	error $k_{app}2$
chlorine	0.080	± 0.001	0.013	± 0.001
bromine	0.148	± 0.003	0.018	± 0.001
iodine	0.204	± 0.002	0.019	± 0.002

Stopped-flow experiments were carried out as described in the supplementary methods, observing the formation of bridged somatostatin (**6)** by UV/Vis analysis. As the best results were obtained by a two-exponential fit two different rate constants are shown. The different reactivity of the two cysteines forming the disulfide bridge in somatostatin[1] could possibly give rise to two different reaction products resulting from the first addition of the dihalomaleimide to each of the free cysteines. This could account for the two observed rate constants in the formation of the final product, which is the only compound detected by the stopped-flow analysis.

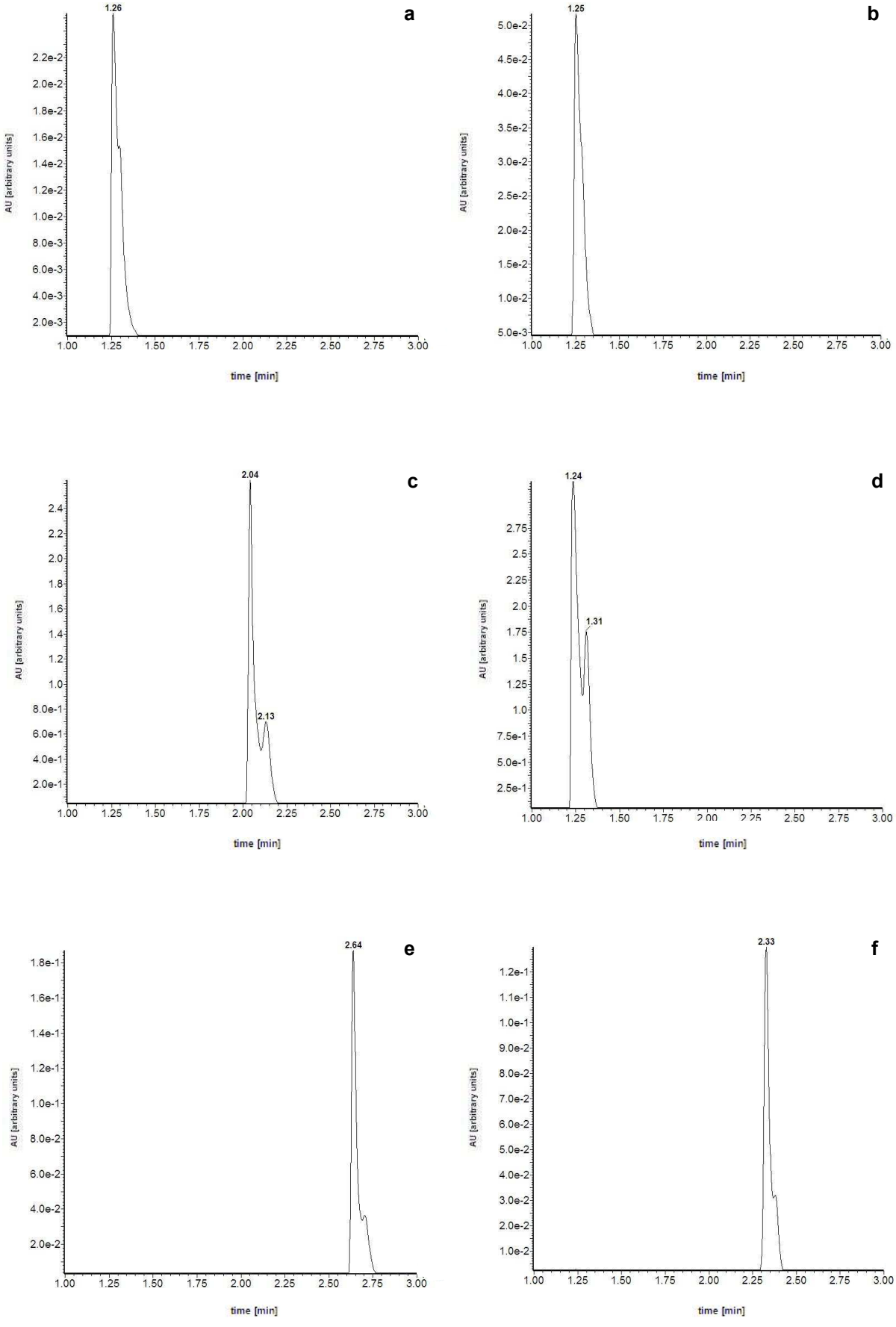
Supplementary Table 2 | Cross-reactivity of bridging reagents.

Compound	Normalised yield of recovered maleimide [%]
Dichloromaleimide (**2**)	74.7
Diiodomaleimide (**3**)	77.0
Dibromomaleimide (**1**)	83.7
Dithiophenolmaleimide (**5**)	88.3
Dimercapto-ethanolmaleimide (**4**)	100.0

TCEP cross-reactivity with bridging reagents was tested by treatment of each reagent with TCEP, followed by attempted recovery of the maleimide by purification (see supplementary methods). The yield of the sample reaction was normalised by the yield of the control reaction without TCEP to account for non TCEP mediated loss of reagent.



Supplementary Figure 1 | MALDI-TOF spectra of unmodified and bridged somatostatin. (**a**) Somatostatin and isotope peaks. (**b**) Somatostatin. (**c**) Somatostatin with inserted maleimide bridge (**6**) and isotope peaks. (**d**) Somatostatin with inserted maleimide bridge (**6**).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AU [arbitrary units]
time [min]
b
1.25
AU [arbitrary units]
time [min]
c
2.04
2.13
AU [arbitrary units]
time [min]
d
1.24
1.31
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time [min]
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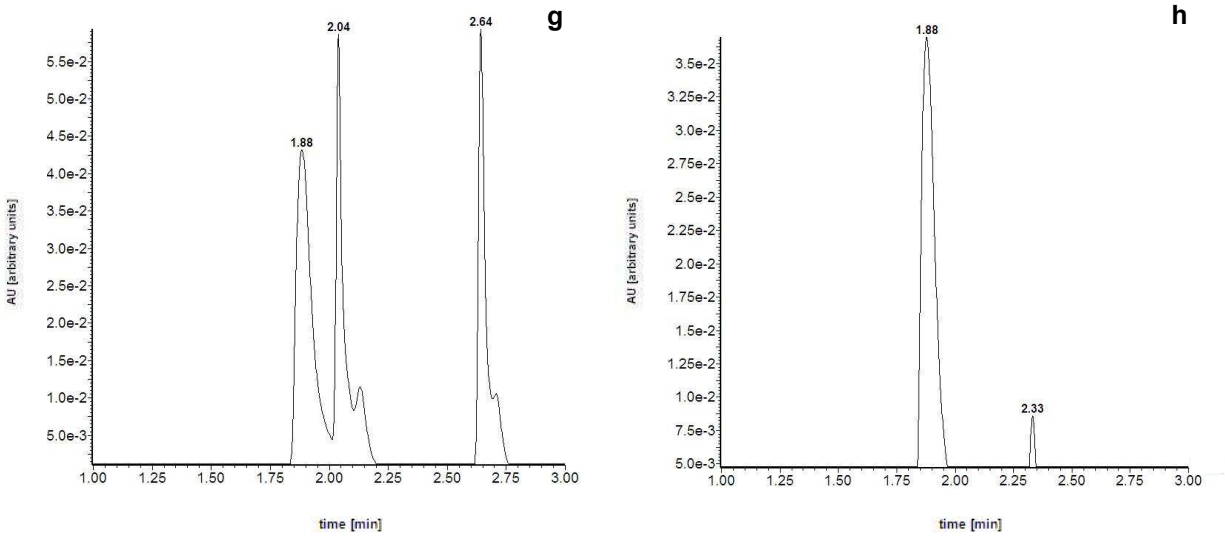
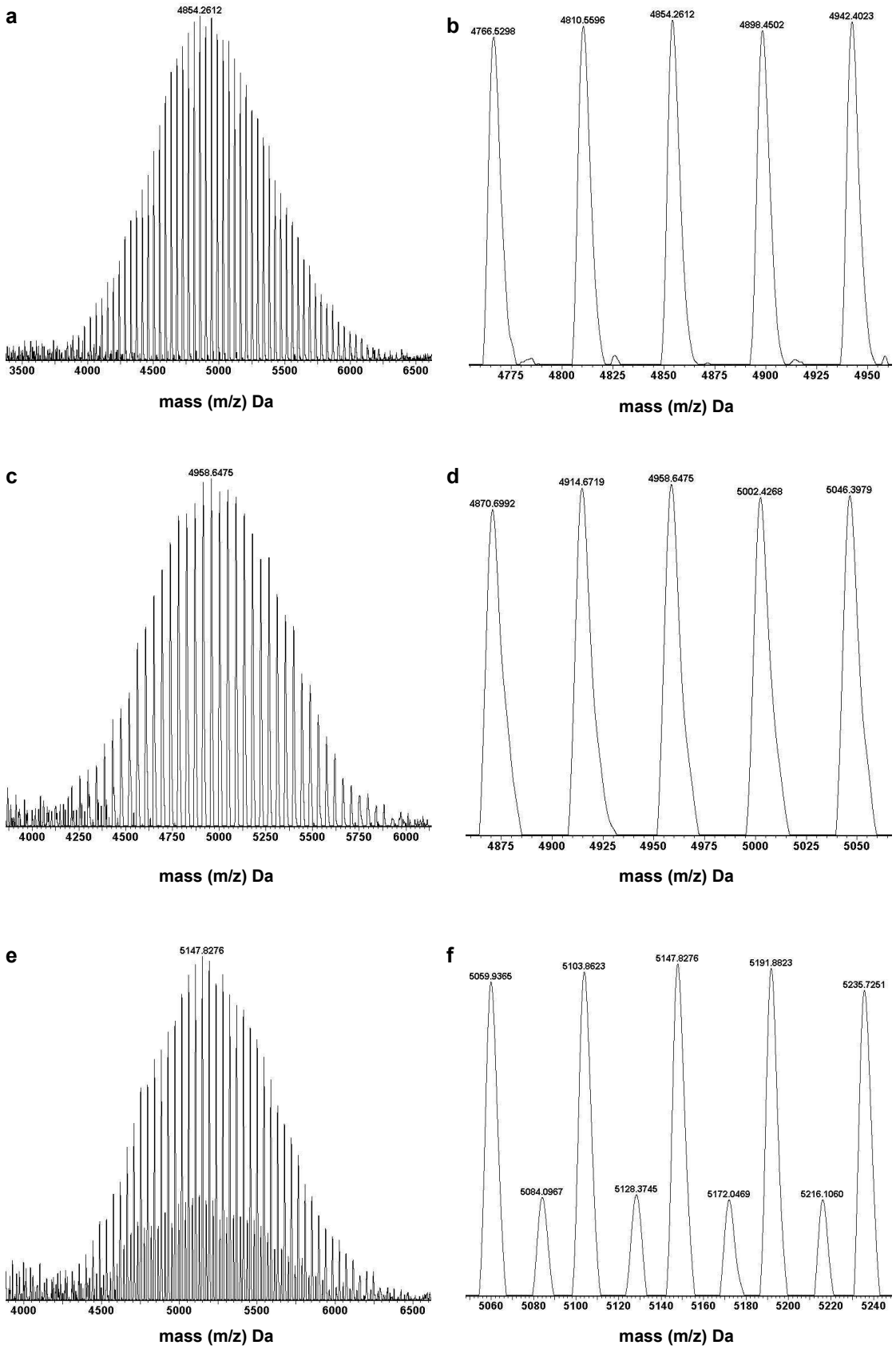
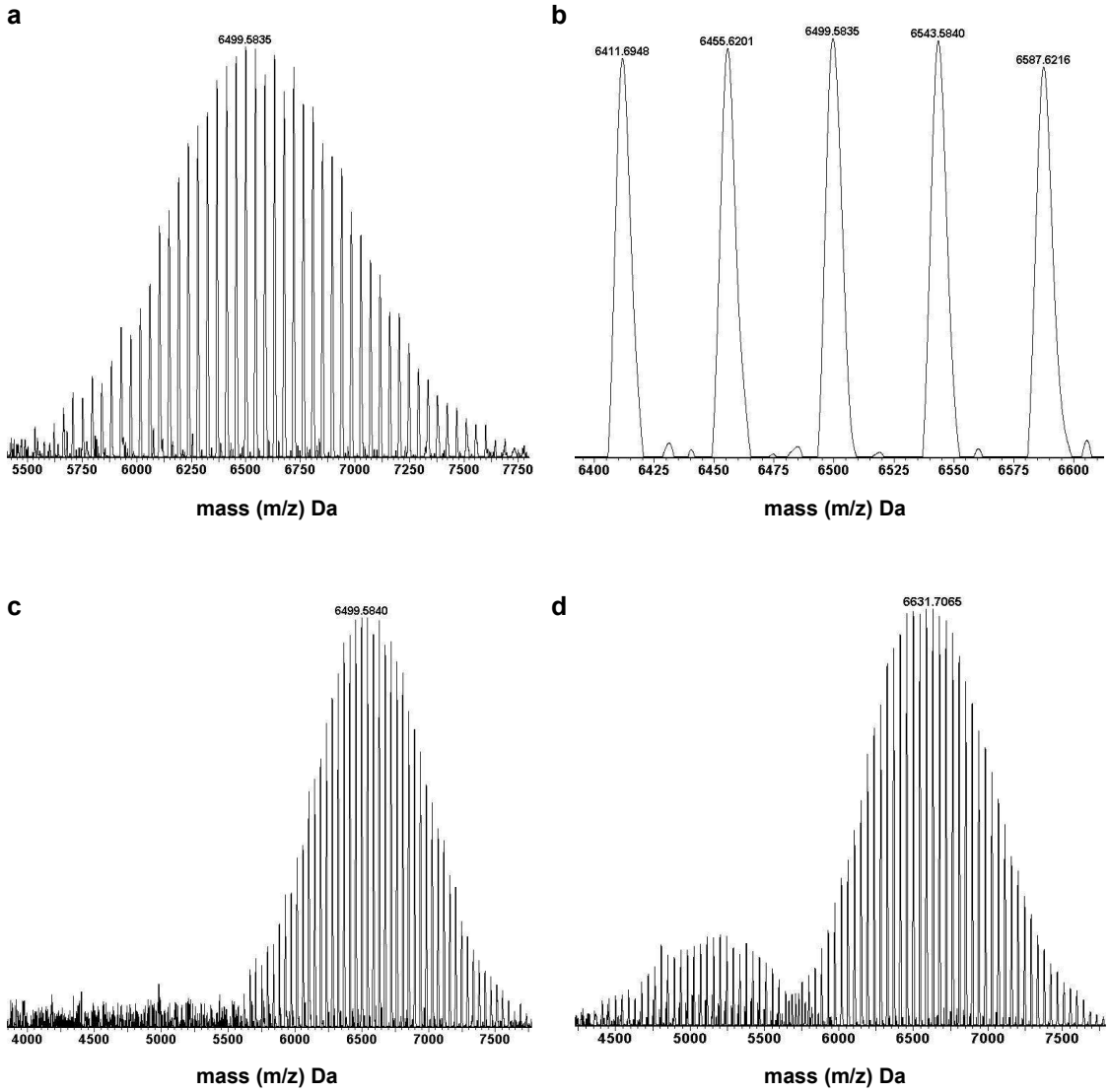
time [min]



Supplementary Figure 2 | LC elution profiles of somatostatin, its derivates, bridging and PEGylation reagents. Main peak shoulders and smaller double peaks are derived from salt interactions. Main compounds are shown at 152.6 µM except benzenethiol and dibromomaleimide which are shown at 15.3 mM. (**a**) Somatostatin. (**b**) Somatostatin with inserted maleimide bridge (**6**). (**c**) Benzenethiol. (**d**) Dibromomaleimide (**1**). (**e**) N-PEG-dithiophenolmaleimide (**8**). (**f**) N-PEG-dibromomaleimide (**7**). (**g**) PEGylated somatostatin (**9**) after reaction with N-PEG-dithiophenolmaleimide (**8**) (RT 1.88 min). Additional peaks are the free benzenethiol (RT 2.04 min) and residual PEGylation reagent (RT 2.64 min). (**h**) PEGylated somatostatin (**9**) after reaction with N-PEG-dibromomaleimide (**7**) (RT 1.88 min). The additional peak is the residual PEGylation reagent (RT 2.33 min).

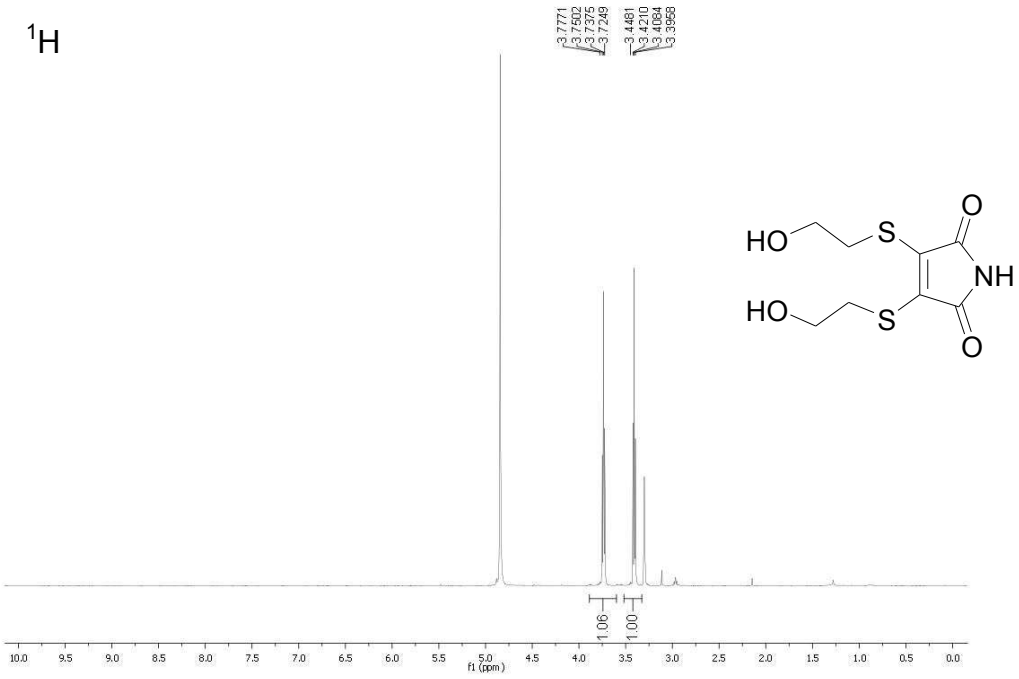


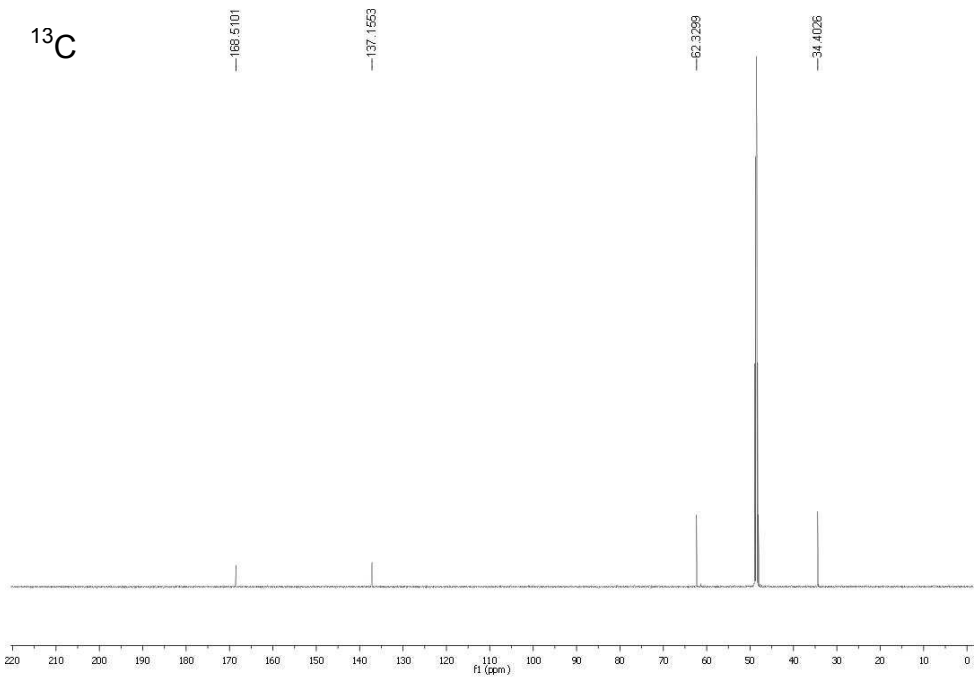
Supplementary Figure 3 | MALDI-TOF spectra of PEG5000 and synthesised PEGylation reagents. (**a**) PEG5000. (**b**) PEG5000 detail. (**c**) N-PEG-dibromomaleimide (**7**). (**d**) N-PEG-dibromomaleimide (**7**) detail. (**e**) N-PEG-dithiophenolmaleimide (**8**). (**f**) N-PEG-dithiophenolmaleimide (**8**) and salt peaks detail.



Supplementary Figure 4 | MALDI-TOF spectra of PEGylated somatostatin. No peptide species of higher MW were observed. (**a**) Somatostatin with inserted N-PEG-maleimide bridge (**9**). (**b**) Somatostatin with inserted N-PEG-maleimide bridge (**9**) detail. (**c**) Reaction product of reduced somatostatin with 1 equiv of the PEGylation reagent. (**d**) Reaction product of reduced somatostatin with 1.5 equiv of the PEGylation reagent.

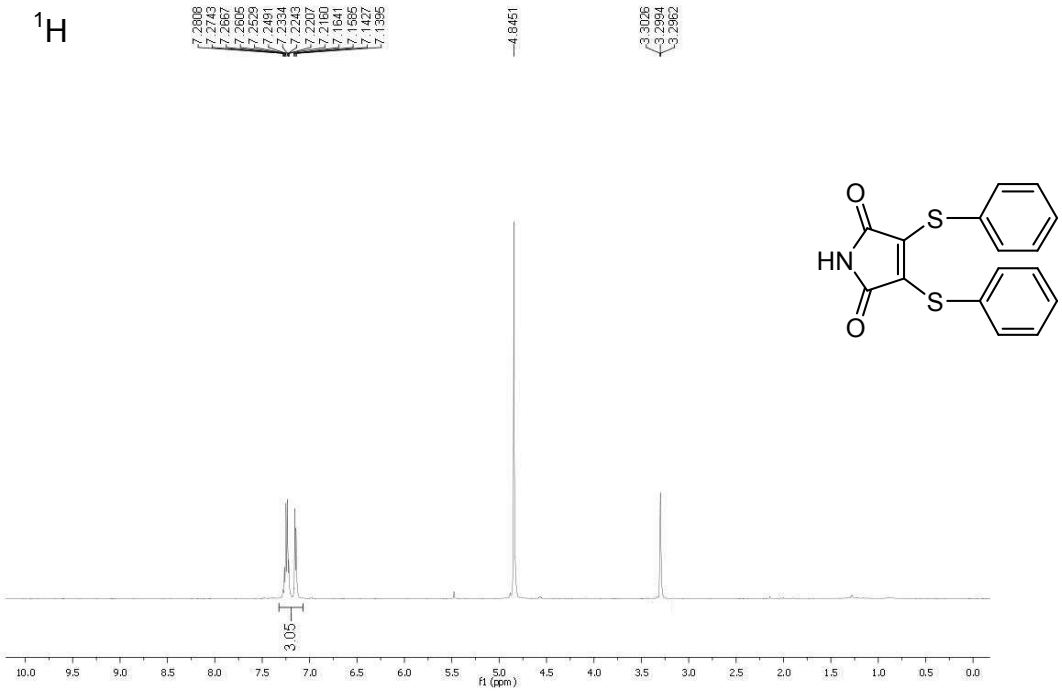
Supplementary Figure 5 | NMR spectra of synthesised compounds
(**a**) Dimercaptoethanolmaleimide (**4**)



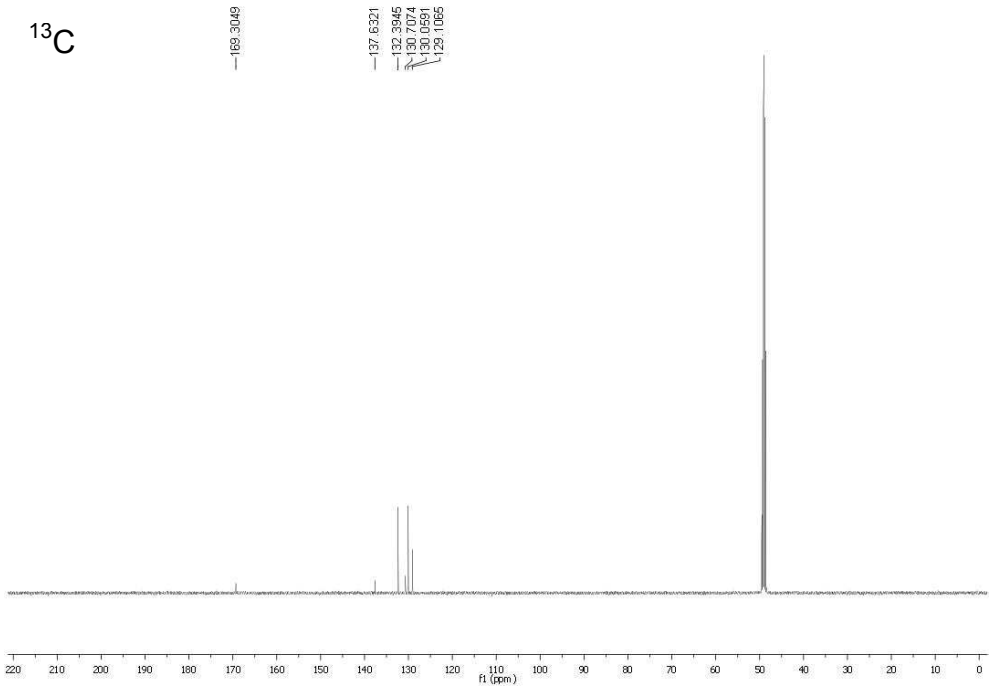


(**b**) Dithiophenolmaleimide (**5**)

^{1}H

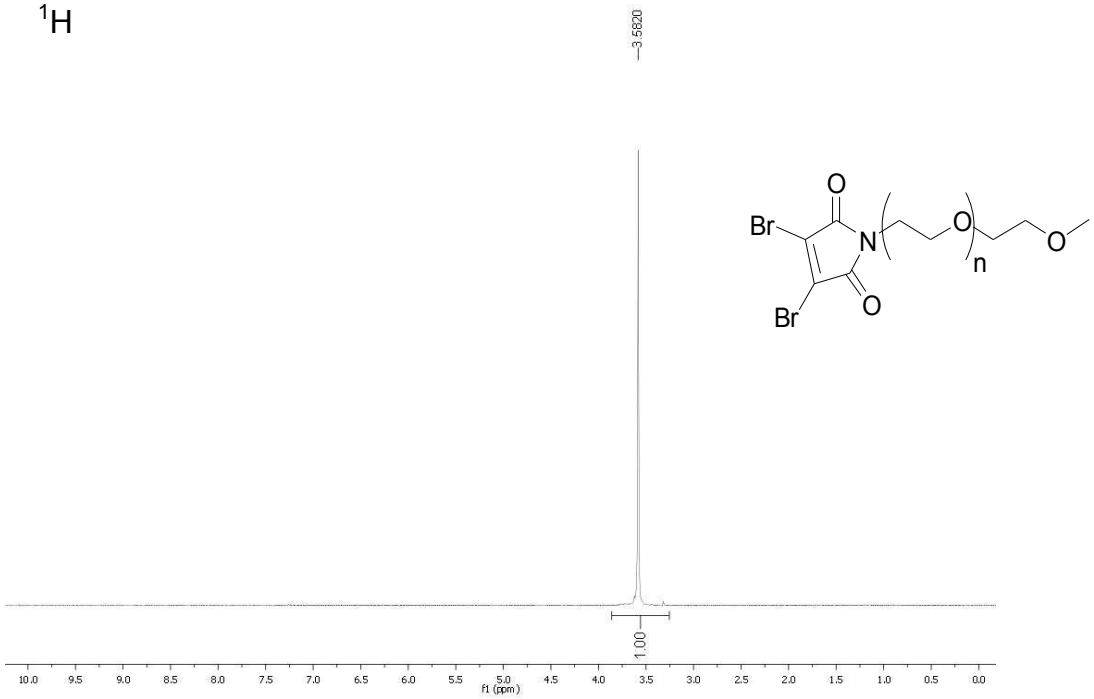


^{13}C

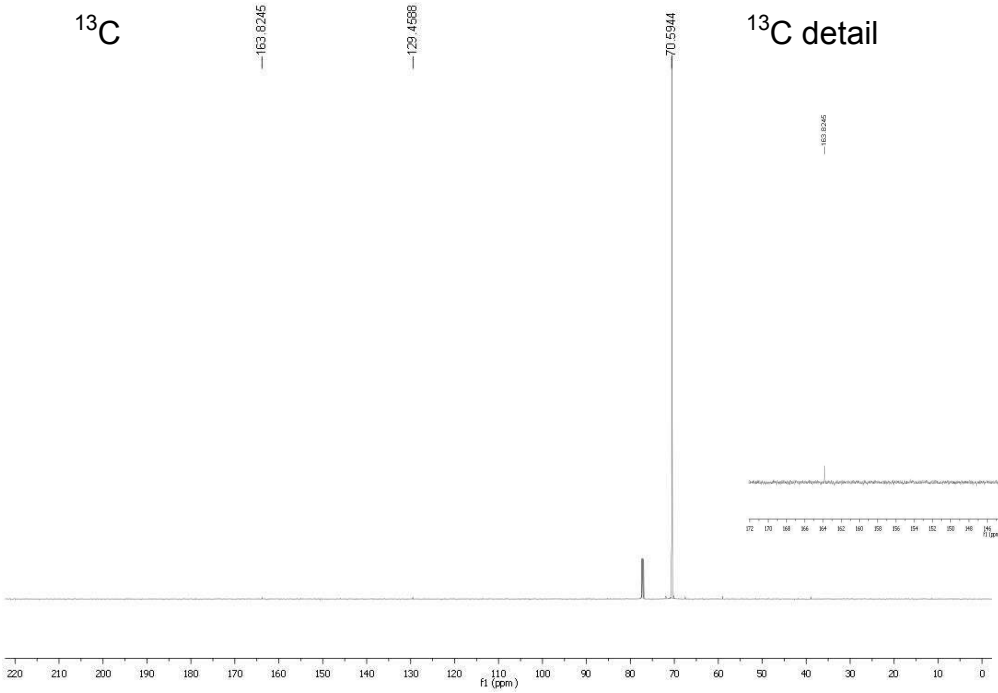


(**c**) *N*-PEG-dibromomaleimide (**7**)

^{1}H



^{13}C

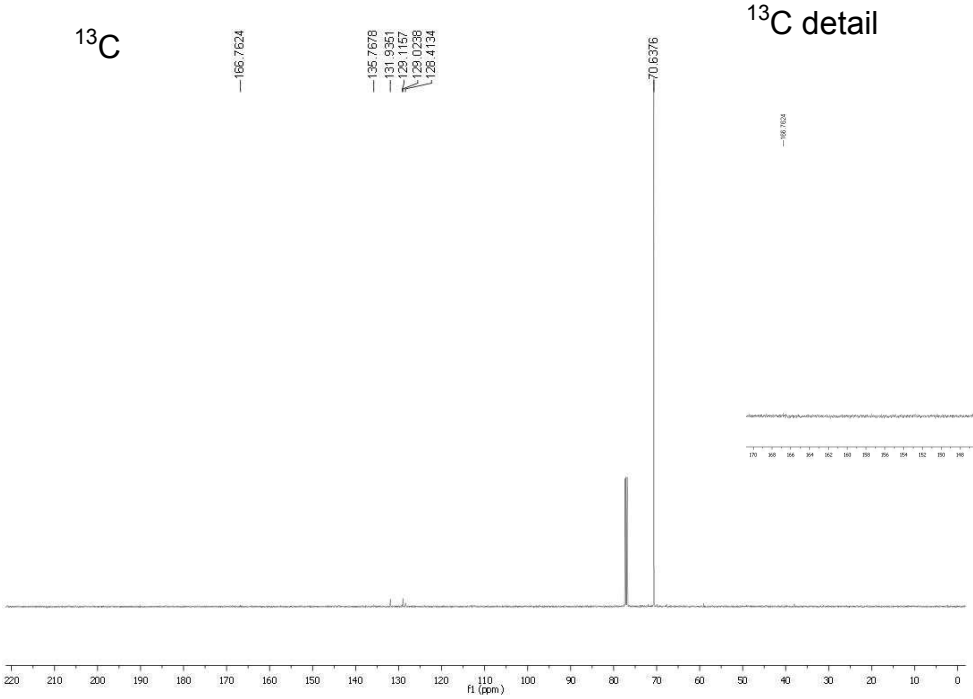


^{13}C detail

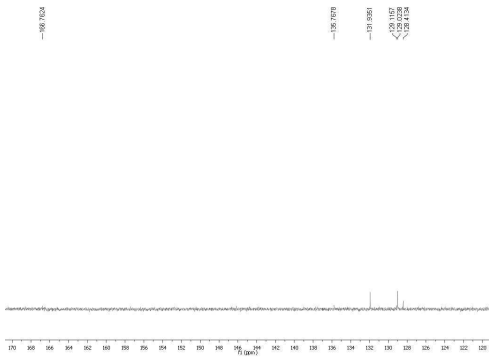
(**d**) *N*-PEG-dithiophenolmaleimide (**8**)

^{1}H

^{13}C



^{13}C detail



Supplementary Methods

General methods

Lyophilized somatostatin, PEG5000, TCEP, benzeneselenol and dibromomaleimide were purchased from Sigma-Aldrich and used without further purification. LCMS was performed on protein samples using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD) [column, Acquity uPLC BEH C18 1.7 μm 2.1 × 50 mm; wavelength, 254 nm; mobile phase, 95:5 water (0.1% formic acid):MeCN (0.1% formic acid), gradient over 4 min to 5:95 water (0.1% formic acid):MeCN (0.1% formic acid); flow rate, 0.6 mL/min; MS mode, ES+/-; scan range, m/z = 95-2000; scan time, 0.25 s]. Data were obtained in continuum mode. The electron spray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h.

MALDI-TOF analysis was performed on a MALDI micro MX (Micromass). Data was obtained in reflectron positive ion mode with a source voltage of 12 kV and a reflectron voltage of 5 kV at a laser wavelength of 337 nm. Samples were prepared as outlined below and those containing peptide were dialysed for 24 h in deionised H_2O. The peptide and its derivates (0.1-0.3 mg/ ml) were spotted onto a MALDI plate in 2 µl sinapinic acid (10 mg/ ml) after pre-spotting of trifluoroacetic acid (10 mg/ ml). ACTH (10 ng/ ml) was used for mass calibration.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 instrument operating at ambient temperature and a frequency of 500 MHz for ^{1}H (125 MHz for ^{13}C). ^{1}H NMR spectra were referenced to the $CDCl_3$ (7.26 ppm) signal and ^{13}C NMR spectra to the $CDCl_3$ (77.67 ppm) signal. Coupling constants are in Hz. Infra-red spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm^{-1}). Mass spectra and high resolution mass data were recorded on a VG70-SE mass spectrometer (EI mode and CI mode). Melting points were measured on a Gallenkamp heating block and are not corrected. All reactions were performed in an inert-gas environment if not stated otherwise. All buffer solutions were filter-sterilised.

General method for the bridging of somatostatin

Somatostatin was bridged following the procedure we have previously reported.[2] In short lyophilised somatostatin (mass = 1638) was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml) and reduced with 1.1 equiv of TCEP for 1 h at ambient temperature. Then either 1.1 equiv of the dihalomaleimides or various amounts of the dithiomaleimides (dissolved in the same buffer with 2.5-15.0 % DMF) were added and the reaction monitored at ambient temperature over 1 h. Quantitative insertion of the maleimide into the disulfide bond was confirmed by LC-MS and MALDI-TOF.

Stopped-flow kinetics of disulfide bridging

Ultra-fast kinetics for the reactions of dihalomaleimides with somatostatin were obtained with a stopped-flow device (Hi-Tech CW-61, Kinet Asyst) equipped with a 250 µl (loaded with the dihalomaleimide compound) and a 2.50 ml syringe (loaded with the peptide). Somatostatin (50 mM sodium phosphate, pH 6.2, 40 % ACN, 2.5 % DMF) was reduced with 1.1 eq of TCEP, completeness of reduction confirmed by LCMS and diluted as necessary in the same buffer. Dihalomaleimides were dissolved and diluted as necessary in the same buffer as somatostatin (7.5-15.0 % DMF).

Reactions were performed at 20 ºC and monitored via the increase of absorbance at a wavelength of 395 nm. Data was corrected by the absorbance of the solution containing peptide only. Final concentrations in the mixing chamber were 34.7 µM somatostatin and a 5x excess (173.5 µM) of the dihalomaleimide compound. Apparent rate constants were calculated using SigmaPlot (Systat Software). A two-exponential fit ($y = A - B^{(-x\,C)} - D^{(-x\,E)}$, with A = final absorbance, B = amplitude 1, C = k_{app1}, D = amplitude 2, E = k_{app2}) gave the best results. Errors were calculated from at least 3 experiments.

Cross-reactivity of bridging reagents with TCEP

TCEP (51.7 mg, 0.2 mmol) was dissolved in 50 ml buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) and 1 equiv of bridging reagent (in 1.25 ml DMF, final concentration 5 %) was added. The reaction mixture was stirred for 10 min at ambient temperature and 100 ml ethyl acetate was added. The organic phase was washed with saturated lithium chloride solution (4x 150 ml), water (100 ml), saturated sodium chloride solution (100 ml) and dried with magnesium sulphate. The solvent was removed *in vacuo*, the weight of the residual material quantified and its identity confirmed by NMR.

In the case of dimercaptoethanolmaleimide no DMF was present in the buffer and the aqueous phase was extracted with 5x 100 ml ethyl acetate. A control reaction without TCEP was performed for each sample and the yield normalised accordingly.

***In situ* bridging of somatostatin with TCEP**

Lyophilised somatostatin was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml). 5 equiv of either dibromomaleimide (**1**) or dithiophenolmaleimide (**5**) (dissolved in the same buffer with 7.5 % DMF) were added and the mixture incubated for 10 min at room temperature. Various amounts of TCEP were added and the formation of bridged somatostatin and side products monitored over 1 h at ambient temperature by LC-MS. Performed controls included samples without reducing agent and no reaction was observed when TCEP was not present.

Cleavage of the somatostatin disulfide bond with thiols

Lyophilised somatostatin was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml). 2 equiv of 2-mercaptoethanol or DTT (dissolved in the same buffer) were added at ambient temperature and cleavage of the disulfide bond monitored via the addition of an excess of dibromomaleimide (**1**) over 1 h. No bridged product (**6**) was observed in both cases indicating that no reduction of the disulfide bond had occurred.

Cleavage of the somatostatin disulfide bond with benzeneselenol

Lyophilised somatostatin was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml). 10 equiv of benzeneselenol (dissolved in the same buffer) were added at ambient temperature and cleavage of the disulfide bond monitored via the addition of an excess of dibromomaleimide (**1**). Bridged product (**6**) was observed only when 2 equiv of 2-mercaptoethanol had also been added and indicated that 30 % of the peptide disulfide bond had been reduced after 3 d.

In situ bridging of somatostatin with benzeneselenol

Lyophilised somatostatin was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml). 10 equiv of dithiophenolmaleimide (dissolved in the same buffer with 7.5 % DMF) were added and the mixture incubated for 10 min at room temperature. 5 equiv of freshly in the same buffer (7.5 % DMF) prepared benzeneselenol were added and the formation of bridged somatostatin and side products analysed over 2 h at ambient temperature by LC-MS.

PEGylation of somatostatin

Lyophilised somatostatin (mass = 1638) was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml) and reduced with 1.1 equiv of TCEP for 1 h at ambient temperature. Then 1.1 equiv of N-PEG- dibromomaleimide (**7**) (dissolved in the same buffer) was added and the reaction monitored by LC-MS at ambient temperature over 1 h. The identity of the product was confirmed by MALDI-TOF.

In situ PEGylation of somatostatin

Lyophilised somatostatin was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml). 5 equiv of N-PEG-dithiophenolmaleimide (**8**) (dissolved in the same buffer) were added and the mixture incubated for 10 min at room temperature. 3 equiv of TCEP were added and the reaction monitored over 1 h at ambient temperature by LC-MS. The identity of the product was confirmed by MALDI-TOF.

Cell culture

Cell-culture methods and the generation of stable cell lines were carried out as described.[3] HEK293 cells (human embryonic kidney cell line) stably expressing Kir3.1 and Kir3.2A channels were maintained in minimum essential medium supplemented with 10% foetal calf serum and 727 µg of G418 (Invitrogen), at 37 °C in humidified atmosphere (95% O_2, 5% CO_2). Cells were transiently transfected with SSTR2 DNA (Missouri S&T cDNA Resource Center) along with pEGFP-N1 (Clontech) for visualization of transfected cells using epifluorescence. Transfections were performed with 5 µl of Fugene HD (Roche) and 800 ng SSTR2-DNA and 40 ng EGFP-DNA per 97 µl of cell culture medium (containing no serum or antibiotics).

Preparation of somatostatin and analogues for patch-clamp experiments

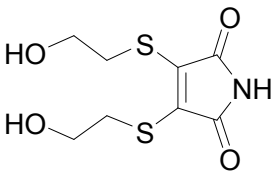
Bridged somatostatin (**6**), PEGylated somatostatin (**9**) and fluorescein-somatostatin were prepared as described above or elsewhere.[2] Somatostatin and its analogues were dialysed for 24 h at 4 ºC in buffer (50 mM sodium phosphate, pH 6.2) to remove the organic solvents. After dialysis the concentration was determined and the peptides stored at 4 ºC. A final concentration of 20 µM somatostatin and analogues were used (dilution was done in the extracellular patch-clamp buffer).

Electrophysiology

Whole cell patch-clamp current recordings were performed with an Axopatch 200B amplifier (Axon Instruments) using fire-polished pipettes with a resistance of 3-4 MΩ pulled from filamented borosilicated glass capillaries (Harvard Apparatus, 1.5 mm OD x 1.17 mm ID). Data was acquired and analysed via a Digidata 1322A interface (Axon Instruments) and pCLAMP software (version 8.1, Axon Instruments). A fast perfusion system was used to apply somatostatin and analogues (Rapid Solution

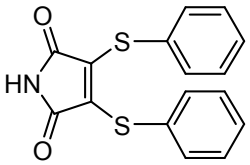
Changer, RSC-160, Bio-Logic France). Cells were clamped at -60 mV. The extracellular solution was (mM): NaCl 80, KCl 60, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, NaH_2PO_4 0.33, glucose 10, pH 7.4; while the intracellular solution was (mM): K gluconate 110, KCl 20, NaCl 10, $MgCl_2$ 1, MgATP 2, EGTA 2 GTP 0.3, pH 7.4. After agonist application, current activated with a delay "lag" followed by a rapid rise to peak amplitude "time to peak". After removal of the agonist, the current decays back to baseline. For each cell it was assessed if flow artefacts resulting from the pressure of drug application were present. This was done by applying bath solution from one of the sewer pipes at the beginning of the recordings. TertiapinQ, an inhibitor of GIRK current (Alomone), was used at a final concentration of 100 nM. Cells were incubated overnight with pertussis toxin (Sigma, 100 ng/ml), an inhibitor of Gi/o proteins. Drugs were prepared as concentrated stocks solutions and kept at -20°C.

Dimercaptoethanolmaleimide (4) (3,4-Bis-(2-hydroxy-ethylsulfanyl)-pyrrole-2,5-dione)



To 2-mercaptoethanol (683.8 µl, 9.8 mmol) in buffer (100 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0, 5.0 % DMF) was added dibromomaleimide (1 g, 3.9 mmol) in DMF (2.5 ml, final concentration DMF 7.5 %). The reaction was stirred for 30 min at ambient temperature and lithium chloride (20 g) was added. The aqueous reaction mixture was extracted with ethyl acetate (7 x 150 ml). The organic layers were combined, the solvent removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 1:1 to 1:9). Fractions containing the product were collected and the solvent were removed *in vacuo*. The still impure product was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5-10.0 % methanol) to afford dimercaptoethanolmaleimide as a yellow solid (518 mg, 53 %). λ_{max} (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF)/ 318 nm (ε/ dm^3 mol^{-1} cm^{-1} 1855); ^{1}H NMR (500 MHz, MeOD): δ = 3.74 (t, 4H, *J* = 6.4, 2x HO-C***H₂***), 3.41 (t, 4H, *J* = 6.3, 2x S-CH_2) ^{13}C NMR (125 MHz, MeOD): δ = 168.5 (C), 137.2 (C), 62.3 (CH_2), 34.4 (CH_2); IR (solid, cm^{-1}): 3344 (s), 2500 (m), 2078 (w); MS (EI) *m/z,* (%): 250 (M, 43), 232 (100), 161 (37); Mass calc. for $C_8H_{11}O_4NS_2$: 250.02077. Found: 250.02126; m.p. 46-50 °C.

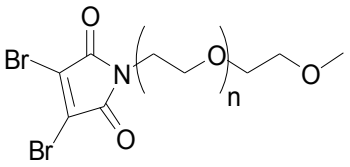
Dithiophenolmaleimide (5) (3,4-Bis-phenylsulfanyl-pyrrole-2,5-dione**)**



To dibromomaleimide (80.0 mg, 0.3 mmol) and sodium hydrogencarbonate (130.2 mg, 1.6 mmol) in methanol (6 ml) was slowly added benzenethiol (66.6 µl, 0.7 mmol) in methanol (1 ml). The reaction was stirred for 15 min at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether: ethyl acetate, gradient elution from 9 :

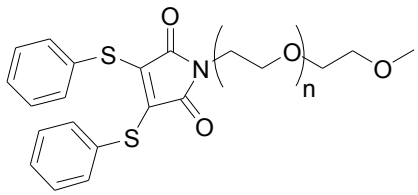
1 to 7 : 3) to afford the product as bright yellow crystals (73 mg, 75 %). λ_{max} (50 mM sodium phosphate, pH 6.2, 40 % MeCN, 2.5 % DMF)/ 412 nm (ε/ dm^3 mol^{-1} cm^{-1} 2245); ^{1}H NMR (500 MHz, MeOD): δ = 7.27-7.22 (m, 6H, Ar-H), 7.16-7.14 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, MeOD): δ = 169.3 (C), 137.6 (C), 132.4 (C), 130.7 (CH), 130.1 (CH), 129.1 (CH); IR (solid, cm^{-1}): 3285 (m), 3059 (w), 2924 (w), 1774 (m), 1715 (s); MS (CI) *m/z,* (%): 314 (M+H, 100), 206 (13), 111 (12); Mass calc. for $C_{16}H_{11}O_2NS_2$[+H]: 314.0231. Found: 314.0309; m.p. 102-104 °C (Literature: 123-126 °C).[4]

_N_-PEG-dibromomaleimide (7)



The reaction was carried out under strictly dry conditions. To triphenylphosphine (154.6 mg, 0.6 mmol) in a mixture of THF (8 mL) and DCM (3 mL) was added drop-wise diisopropyl azodicarboxylate (116.0 µl, 0.6 mmol) at -78 °C. The reaction was stirred for 5 min and mPEG5000 (2950.0 mg, 0.6 mmol) in dichloromethane (7 mL) was added drop-wise. The reaction was stirred for 5 min and neopentyl alcohol (26.5 mg, 0.3 mmol) in a mixture of THF (1 ml) and DCM (1 ml) was added. The reaction was stirred for 5 min and dibromomaleimide (150.0 mg, 0.6 mmol) in THF (2 ml) was added. The reaction was stirred for 5 min, the cold bath removed and stirred for 20 h at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5-5.0 % methanol). Fractions containing the product were collected and the solvent was removed *in vacuo*. The still impure product was purified by very slow flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5-6.0 % methanol) to afford *N*-PEG-dibromomaleimide as a slightly green crystalline powder (417 mg, 13 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.58 (s, 4x n H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 163.8 (C), 129.5 (C), 70.6 (2x n CH_2); IR (solid, cm^{-1}): 3517 (w), 2872 (s), 1977 (w), 1727 (m), 1641 (w); m.p. 51-55 °C.

_N_-PEG-dithiophenolmaleimide (8)



The reaction was carried out under strictly dry conditions. To triphenylphosphine (167.7 mg, 0.6 mmol) in a mixture of THF (8 ml) and DCM (3 ml) was added drop-wise diisopropyl azodicarboxylate (125.9 µl, 0.6 mmol) at -78 °C. The reaction was stirred for 5 min and mPEG5000 (1600.0 mg, 0.3 mmol) in DCM (7 ml) was added drop-wise. The reaction was stirred for 5 min and neopentyl alcohol (56.3 mg, 0.6 mmol) in a mixture of THF (1 ml) and DCM (1 ml) was added. The reaction was stirred for 5 min and dithiophenolmaleimide (200.0 mg, 0.6 mmol) in THF (3 ml) was added. The reaction was stirred for 5 min, the cold bath removed and stirred for 20 h

at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5-10.0 % methanol). Fractions containing the product were collected and the solvent was removed *in vacuo*. The still impure product was purified by flash chromatography on TLC grade silica gel (methanol : dichloromethane, gradient elution from 0.0-10.0 % methanol) to afford N-PEG-dithiophenolmaleimide as a bright yellow crystalline powder (1.24 g, 73 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.27-7.25 (m, 2H, Ar-H), 7.24-7.22 (m, 4H, Ar-H), 7.20-7.18 (m, 4H, Ar-H), 3.63 (s, 4x n H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.7 (C), 135.7 (C), 131.9 (CH), 129.1 (C), 129.0 (CH), 128.4 (CH), 70.6 (2x n CH_2); IR (solid, cm^{-1}): 3498 (w), 2881 (s), 1959 (w), 1711 (m); m.p. 57-59 °C.

The synthesis of N-fluoresceinmaleimide is described elsewhere.[2]

References

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