Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Electronic Supporting Information

The CSY-protecting group in the microwave-assisted synthesis of aggregation-prone peptides

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1 Supplementary figures

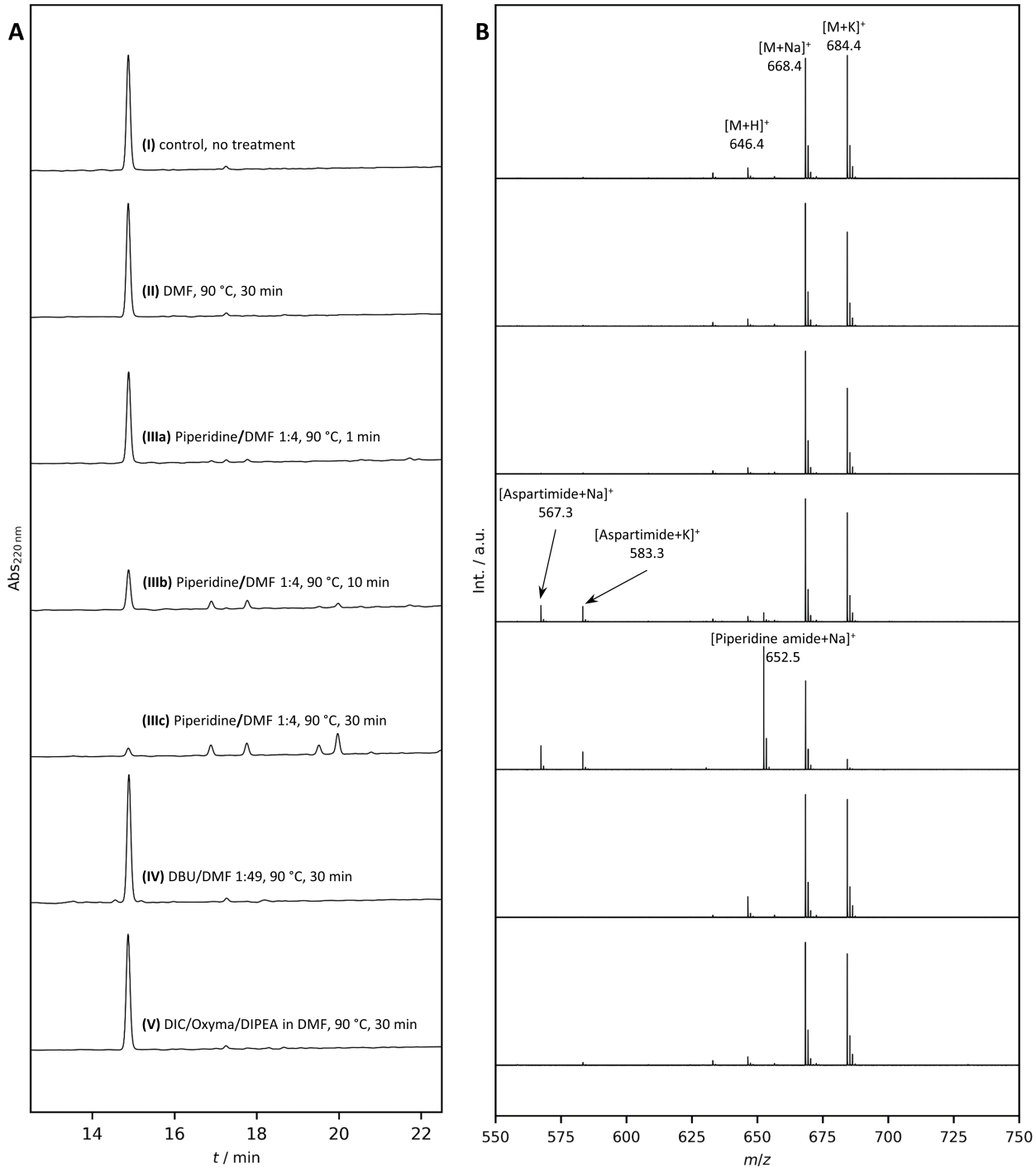


Figure S1. Treatment of resin-bound test peptide **4** under the different conditions of microwave-assisted solid-phase peptide synthesis. A: HPLC traces of crude peptide mixtures after acidic cleavage from the resin. B: MALDI-TOF-MS of crude peptide mixtures after acidic cleavage from the resin.

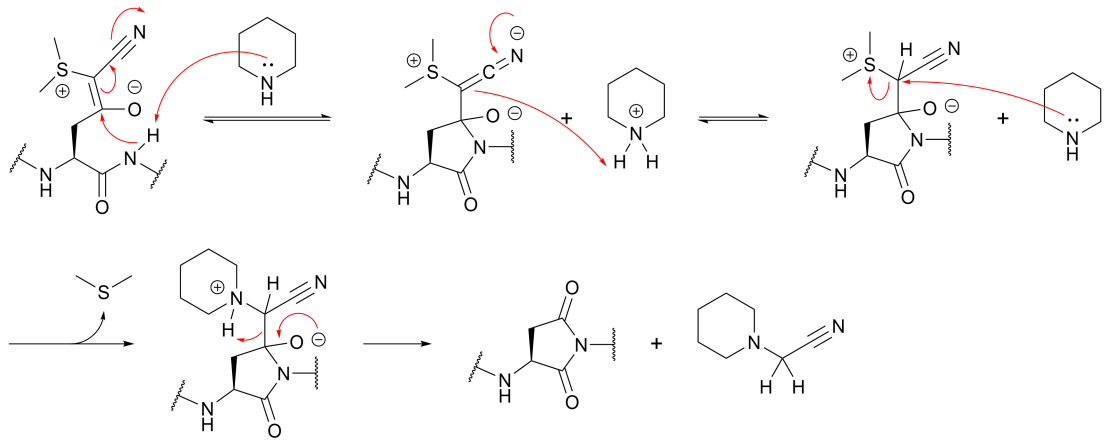


Figure S2. Postulated mechanism of the nucleophilic CSY cleavage with piperidine under microwave heating based on the observations from Figure S1.

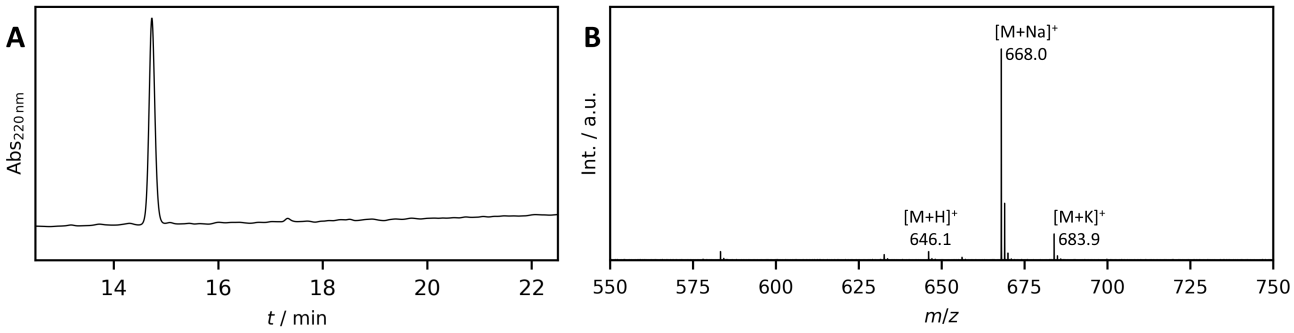


Figure S3. New microwave-assisted SPPS of test peptide **4** using the optimized synthesis protocol. A: HPLC trace of crude **4**. B: MALDI-TOF-MS of crude **4**.

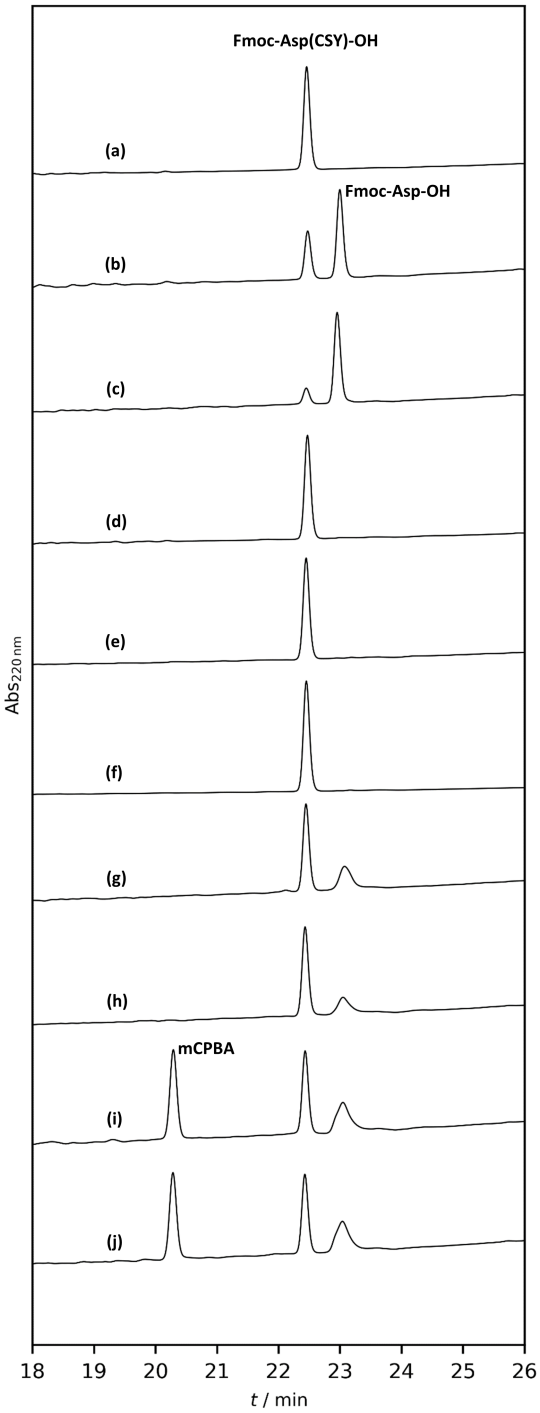


Figure S4. Screening of different cleavage conditions for the CSY protecting group from Fmoc-Asp(CSY)-OH with different oxidants (2.0 eq.). Conditions: a) Reference. b) NCS, MeCN + 0.1% TFA, water + 0.1% TFA. c) NCS, MeCN, acetate buffer pH 4.5. d) Iodine, MeCN + 0.1% TFA, water + 0.1% TFA. e) Iodine, MeCN, phosphate buffer pH 10.0. f) $NaIO_4$, MeCN, acetate buffer pH 4.5. g) Oxone, MeCN, water. h) Oxone, MeCN + 0.1% TFA, water + 0.1% TFA. i) mCPBA, MeCN, water. j) mCPBA, MeCN + 0.1% TFA, water + 0.1% TFA.

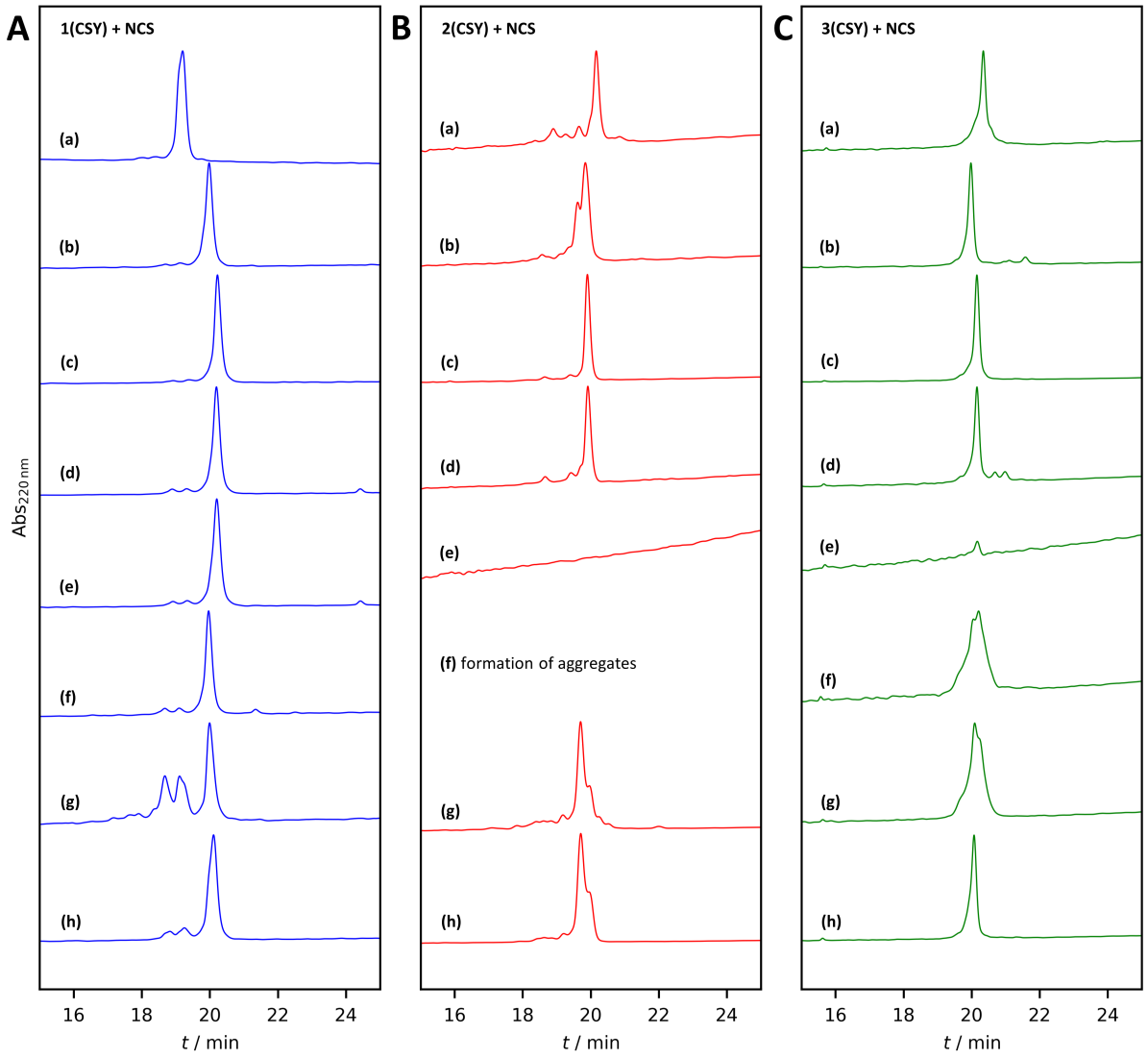


Figure S5. Screening of different cleavage conditions for the CSY protecting group from peptides **1**, **2**, and **3** with NCS (2.2 eq. regarding amount of CSY). Conditions: a) MeCN, acetate buffer pH 4.5 (conditions from Neumann *et al.*).[1] b) HFIP, MeCN + 0.1% TFA, water + 0.1% TFA. c) DMF, HFIP, water + 0.1% TFA. d) DMF, HFIP, TFE, water + 0.1% TFA. e) HFIP, TFE, MeCN + 0.1% TFA, water + 0.1% TFA. f) MeOH + 0.1% TFA, water + 0.1% TFA. g) GdnHCl, MeCN + 0.1% TFA, water + 0.1% TFA. h) GdnHCl, TFE, MeCN + 0.1% TFA, water + 0.1% TFA.

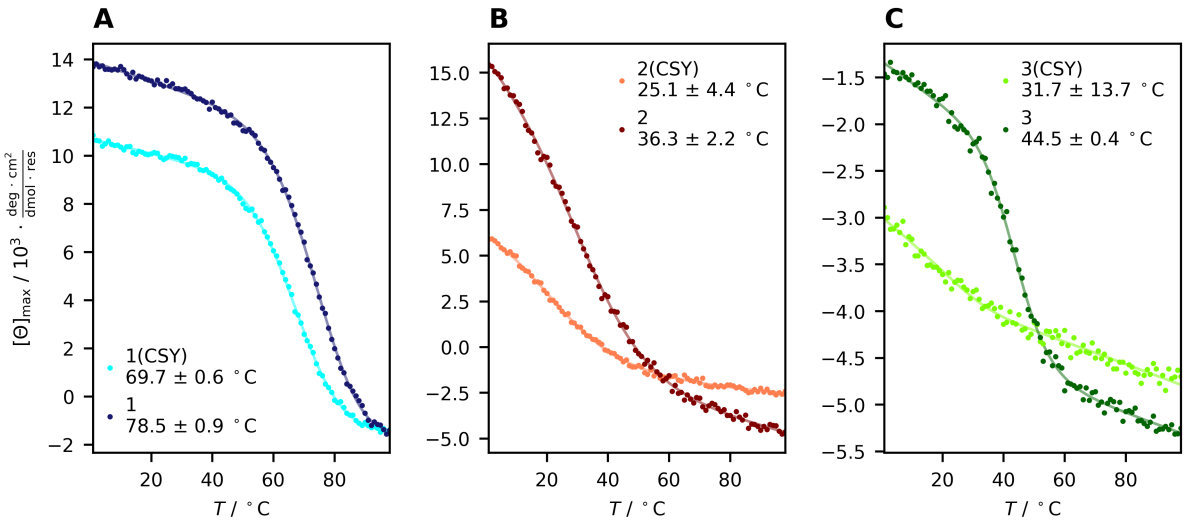


Figure S6. CD thermal denaturation profiles of CSY-protected and fully unprotected peptides **1**, **2** and **3**.

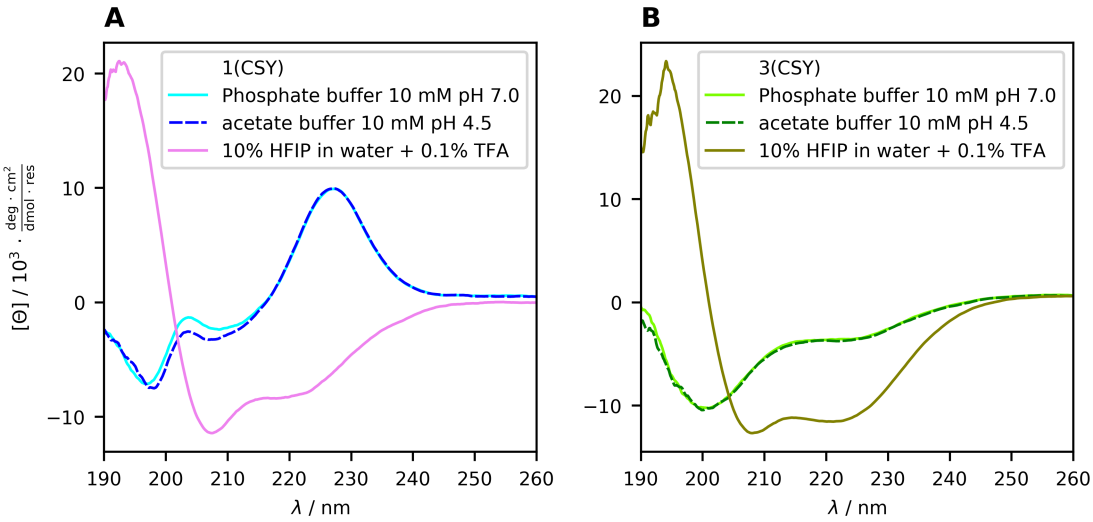


Figure S7. CD spectra of peptides **1**(CSY) and **3**(CSY) in different buffers.

2 Experimental procedures

2.1 Reagents and solvents

Standard Fmoc-protected amino acids and *N,N*-diisopropylcarbodiimide (DIC) were acquired from *Iris Biotech GmbH* (Marktredwitz, Germany). Fmoc-Nle-OH was purchased from *Carbolution Chemicals GmbH* (St. Ingebert, Germany). Oxyma®, *N,N*-diisopropylamine (DIPEA), piperidine, H-Gly-HMPB-ChemMatrix® resin, H-Rink amide ChemMatrix® resin, MeCN (HPLC grade), trifluoroacetic acid (TFA, HPLC grade) and triisopropylsilane (TIPS) were acquired from *Merck KGaA* (Darmstadt, Germany). DMF (peptide synthesis grade) was purchased from *Fisher Scientific* (Loughborough, United Kingdom). All other solvents and reagents were at least *pro analysis* grade quality and were acquired from *Carbolution Chemicals GmbH* (St. Ingebert, Germany), *Carl Roth GmbH + Co. KG* (Karlsruhe, Germany), *Fisher Scientific* (Loughborough, United Kingdom), *Grüssing GmbH* (Filsum, Germany), *Honeywell* (Seelze, Germany), *Merck KGaA* (Darmstadt, Germany), *neoFroxx GmbH* (Einhausen, Germany), *SERVA Electrophoresis GmbH* (Heidelberg, Germany), *Th. Geyer GmbH & Co. KG* (Renningen, Germany) and *VWR International* (Fontenay-sous-Bois, France). Reagents and solvents were used as received. Water was purified with a *Sartorius arium® mini* lab water system and degassed by flushing with N_2-gas for 10 min.

2.2 Software for data analysis and visualization

Data were analysed with *Anaconda 5.0.1* (*Python 2.7*), *OriginPro® 9.8.0.200* and *Microsoft Excel 2016* if not stated otherwise.

2.3 Peptide synthesis and purification

2.3.1 Solid-phase peptide synthesis and peptide isolation

Peptides were synthesized on a microwave-assisted peptide synthesizer (*CEM Liberty Blue*, see below for detailed settings). Preloaded H-Gly-HMPB ChemMatrix® resin or H-Rink-Amide ChemMatrix® resin was used as solid support. A loading density of 0.5 mmol/g was assumed for the synthesis. Prior to synthesis, the resin was swollen in DMF for at least 10 min. Fmoc protected amino acids were used as 0.2 M solution in DMF. Diisopropyl carbodiimide (DIC) dissolved in DMF (0.5 M) was used as activator, and Oxyma® solution in DMF (1.0 M) plus 0.1 M DIPEA was used as the activator base. A solution of piperidine/DMF 1:4 *v*/*v* was used to remove the Fmoc protecting group. For *N*-terminal acetylation the peptide resin was treated with acetic anhydride (0.5 mL) in DMF (4.5 mL) for 10 min, then washed with DMF (3 x 5 mL), MeOH (3 x 5 mL) and DCM (6 x 5 mL) and finally dried under reduced pressure. Cleavage of the peptide from the resin and final deprotection were carried out in trifluoroacetic acid (TFA) containing triisopropylsilane (TIPS) and water (detailed composition is described in chapter 2.3.5). After equilibration at rt for 4 h, the resin was filtered off and washed with TFA (2 x 5 mL). The TFA was removed in a stream of nitrogen and the peptide was precipitated from ice-cold Et_2O (25 mL). The peptide was isolated by centrifugation. The pellet was washed with Et_2O (2 x 25 mL) and dissolved in acetonitrile/water 1:4 *v*/*v* + 0.1% TFA (15 mL), filtered with a syringe filter (*MACHEREY-NAGEL CHROMAFIL® RC-45/15 MS*), and lyophilized (*Christ Alpha 2-4-LDplus* connected to a *VACUUBRAND RZ 6* pump).

2.3.2 High-performance liquid chromatography (HPLC)

The peptides were purified by reversed-phase HPLC on a *MACHEREY-NAGEL NUCLEODUR® C18ec* column (250 mm x 10 mm, 100 Å, 5 µm) at a flow rate of 3 mL/min at 50 °C. A *Jasco* chromatography system with a *PU-4180* pump, a *CO-4060* column thermostat and a *UV-4070* detector was used. Analytical HPLC was performed using a *Hitachi Primaide* chromatography system containing a *1110 Pump*, a *1210 auto sampler*, *1310 column oven* and a *1430 diode array detector*. A *VDS optilab VDSpher® PUR 100 C18-SE* (250 mm x 4.6 mm, 100 Å, 5 µm) column and a flow rate of 1 mL/min at 50 °C were used. Preparative chromatograms were monitored at 220 nm and 280 nm. Analytical chromatograms were monitored from 190 to 400 nm. Fractions containing pure peptide were identified by analytical HPLC and MALDI-TOF MS, pooled and lyophilized. As solvent system, water + 0.1% TFA (buffer A) and MeCN + 0.1% TFA (buffer B) was used. The gradients are described in Table S1.

Table S1: Gradients used for the purification and characterization of peptides **1** to **4** and Fmoc-Asp(CSY)-OH. The percentages of buffer B in buffer A (*v*/*v*) are given.

preparative	**Isocratic 1**	**linear gradient**	**Isocratic 2**	**Isocratic 3**
time / min	0 → 8	8 → 38	39 → 44	45 → 50
1	20%	20 – 40%	100%	20%
2	30%	30 – 70%	100%	30%
3 and 4	20%	20 – 70%	100%	20%
analytical				
time / min	0 → 5	5 → 35	36 → 41	42 → 50
1	20%	20 – 40%	95%	20%
2	30%	30 – 70%	95%	30%
3 and 4	20%	20 – 70%	95%	20%
Fmoc-Asp(CSY)-OH	20%	20 – 80%	95%	20%

2.3.3 Concentration determination

The peptides were dissolved in water and the concentration was determined by measuring the absorbance at 280 nm (ε_{280} [M^{-1} cm^{-1}]: **1**: 13980, **2**: 20970, **3**: 15470), calculated from the weighted sum of the extinction coefficients of Trp (5500 M^{-1} cm^{-1}) and Tyr (1490 M^{-1} cm^{-1}).[2] For the measurements an *Implen NP80* spectrophotometer was used. For peptides containing CSY the amount was determined by weighing.

2.3.4 Settings of the peptide synthesizer

The following Tables (S2-S20) contain the detailed settings for the microwave-assisted peptide syntheses performed in this study using the *CEM LibertyBlue* peptide synthesizer.

Table S2: Settings for Microwave Methods. Mixing of the reaction mixture was performed by bubbling nitrogen through the reaction vessel frit (bubble for 2 s, off for 3 s).

Microwave Method	**Temperature / °C**	**Power / W**	**Hold Time / s**	**DeltaT / °C**
Standard deprotection	75	155	15	2
	90	30	50	1
Conventional deprotection	25	0	300	2
Standard coupling	75	170	15	2
	90	30	110	1
Conventional coupling	25	0	3600	2
50 °C 10 min Coupling	25	0	120	2
	50	35	480	1

Table S3: Settings for **Resin Swelling**.

	Cycle Steps	**Parameter values**
1	Swell Resin	Main solvent volume: 15 mL, Time: 300 s

Table S4: Settings for **First Single Coupling**.

	Cycle Steps	**Parameter values**
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Wash	Volume: 4 mL, Drain Time: 5 s

Table S5: Settings for **Single Coupling**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
7	Wash	Volume: 4 mL, Drain Time: 10 s
8	Wash	Volume: 4 mL, Drain Time: 5 s

Table S6. Settings for **Single 50 °C 10 min Coupling**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Coupling	Reaction Method: 50 °C 10 min coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
7	Wash	Volume: 4 mL, Drain Time: 10 s
8	Wash	Volume: 4 mL, Drain Time: 5 s

Table S7: Settings for **Single RT Coupling**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Coupling	Reaction Method: Conventional coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Wash	Volume: 4 mL, Drain Time: 5 s

Table S8: Settings for **Single Coupling Cool Down**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
7	Wash	Volume: 4 mL, Drain Time: 10 s
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 10 s

Table S9. Settings for **Single Coupling CSY**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 10 s
11	Wash	Volume: 4 mL, Drain Time: 10 s

Table S10: Settings for **Single 50 °C 10 min Coupling CSY**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Coupling	Reaction Method: 50 °C 10 min coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: PositionACT, Activator Volume: 2 mL, Activator Base Position: PositionACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 10 s
11	Wash	Volume: 4 mL, Drain Time: 10 s

Table S11: Settings for **Double Coupling**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: Position ACT, Activator Volume: 2 mL, Activator Base Position: Position ACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
7	Wash	Volume: 4 mL, Drain Time: 5 s
8	Coupling	see step 6
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 5 s

Table S12: Settings for **Double Coupling Gly**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Wash	Volume: 4 mL, Drain Time: 10 s
8	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: Position ACT, Activator Volume: 2 mL, Activator Base Position: Position ACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 10 s
11	Coupling	see step 8
12	Wash	Volume: 4 mL, Drain Time: 10 s
13	Wash	Volume: 4 mL, Drain Time: 5 s
14	Wash	Volume: 4 mL, Drain Time: 5 s
15	Wash	Volume: 4 mL, Drain Time: 5 s

Table S13: Settings for **Double Coupling Cool Down**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 10 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Wash	Volume: 4 mL, Drain Time: 10 s
8	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: Position ACT, Activator Volume: 2 mL, Activator Base Position: Position ACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
9	Wash	Volume: 4 mL, Drain Time: 10 s
10	Wash	Volume: 4 mL, Drain Time: 10 s
11	Coupling	see step 8
12	Wash	Volume: 4 mL, Drain Time: 10 s
13	Wash	Volume: 4 mL, Drain Time: 10 s
14	Wash	Volume: 4 mL, Drain Time: 10 s
15	Wash	Volume: 4 mL, Drain Time: 10 s

Table S14: Settings for **Double Coupling CSY**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s
7	Wash	Volume: 4 mL, Drain Time: 5 s
8	Wash	Volume: 4 mL, Drain Time: 10 s
9	Coupling	Reaction Method: Standard coupling, Amino Acid: from method, Amino Acid Volume: 2.5 mL, Activator Bottle Position: Position ACT, Activator Volume: 2 mL, Activator Base Position: Position ACTB, Activator Base Volume: 1 mL, Delayed Reagent Time: 0 s, Delayed Reagent Bottle Position: PositionACTB, Delayed Reagent Volume: 0 mL, Manifold Wash Volume: 2 mL
10	Wash	Volume: 4 mL, Drain Time: 10 s
11	Wash	Volume: 4 mL, Drain Time: 10 s
12	Coupling	see step 9
13	Wash	Volume: 4 mL, Drain Time: 10 s
14	Wash	Volume: 4 mL, Drain Time: 10 s
15	Wash	Volume: 4 mL, Drain Time: 10 s
16	Wash	Volume: 4 mL, Drain Time: 10 s

Table S15: Settings for **Final Deprotection**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Standard Deprotection, Deprotection Volume: 4 mL
2	Wash	Volume: 4 mL, Drain Time: 5 s
3	Wash	Volume: 4 mL, Drain Time: 5 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s

Table S16: Settings for **RT Final Deprotection**.

	Cycle Steps	Parameter values
1	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
2	Deprotection	Reaction Method: Conventional Deprotection, Deprotection Volume: 4 mL
3	Wash	Volume: 4 mL, Drain Time: 10 s
4	Wash	Volume: 4 mL, Drain Time: 5 s
5	Wash	Volume: 4 mL, Drain Time: 5 s
6	Wash	Volume: 4 mL, Drain Time: 5 s

2.3.5 Specific synthetic methods for peptide 1 to 4

2.3.5.1 Peptide **1**

Resin: H-Gly-HMPB-ChemMatrix® 200 mg, 0.5 mmol/g, 0.1 mmol scale.
The peptide was not acetylated.
Cleavage cocktail: TFA (8.5 mL), TIPS (1 mL), water (0.5 mL).

Table S17: Settings for the synthesis of peptide **1**.

	Step	Used Cycles (Standard conditions)	Used Cycles (optimized conditions)
1	Resin swelling	Resin swelling	Resin swelling
2	G	already loaded	already loaded
3	S	First Single Coupling	First Single Coupling
4	P	Single Coupling	Single Coupling
5	R	Double Coupling	Double Coupling
6	E	Single Coupling	Single Coupling
7	W	Single Coupling	Single Coupling
8	Q	Single Coupling	Single Coupling
9	S	Single Coupling	Single Coupling
10	A	Single Coupling	Single Coupling
11	N	Single Coupling	Single Coupling
12	T	Single Coupling	Single Coupling
13	I	Single Coupling	Single Coupling
14	H	Single Coupling 50 °C	Single Coupling 50 °C
15	N	Single Coupling	Single Coupling
16	F	Single Coupling	Single Coupling
17	Y	Single Coupling	Single Coupling
18	Y	Single Coupling	Single Coupling
19	V	Single Coupling	Single Coupling
20	R	Double Coupling	Double Coupling
21	G	Double Coupling	Double Coupling Cool Down
22	D(CSY)	Single Coupling	Single Coupling CSY
23	A	Single Coupling	Single Coupling CSY
24	S	Single Coupling	Single Coupling CSY
25	Nle	Single Coupling	Single Coupling CSY
26	R	Double Coupling	Double Coupling CSY
27	K	Single Coupling	Single Coupling CSY
28	E	Single Coupling	Single Coupling CSY
29	W	Single Coupling	Single Coupling CSY
30	G	Double Coupling	Double Coupling CSY
31	P	Single Coupling	Single Coupling CSY
32	P	Single Coupling	Single Coupling CSY
33	L	Single Coupling	Single Coupling CSY
34	K	Single Coupling	Single Coupling CSY
35	Final deprotection	Final Deprotection	RT Final Deprotection

2.3.5.2 Peptide **2**

Resin: H-Rink-Amide-ChemMatrix® 200 mg, 0.5 mmol/g, 0.1 mmol scale.
The peptide was acetylated.
Cleavage cocktail: TFA (9.0 mL), TIPS (0.5 mL), water (0.5 mL).

Table S18: Settings for the synthesis of peptide **2**.

	Step	Used Cycles (optimized conditions)
1	Resin swelling	Resin swelling
2	D(CSY)	Double Coupling CSY
3	H	Single 50 °C 10 min Coupling CSY
4	P	Single Coupling CSY
5	S	Single Coupling CSY
6	L	Single Coupling CSY
7	W	Single Coupling CSY
8	S	Single Coupling CSY
9	V	Single Coupling CSY
10	L	Single Coupling CSY
11	D(CSY)	Single Coupling CSY
12	T	Single Coupling CSY
13	D(CSY)	Single Coupling CSY
14	A	Single Coupling CSY
15	N	Single Coupling CSY
16	W	Single Coupling CSY
17	Y	Single Coupling CSY
18	Y	Single Coupling CSY
19	P	Single Coupling CSY
20	L	Single Coupling CSY
21	G	Double Coupling CSY
22	S	Single Coupling CSY
23	S	Single Coupling CSY
24	P	Single Coupling CSY
25	D(CSY)	Single Coupling CSY
26	F	Single Coupling CSY
27	V	Single Coupling CSY
28	K	Single Coupling CSY
29	Y	Single Coupling CSY
30	W	Single Coupling CSY
31	S	Single Coupling CSY
32	P	Single Coupling CSY
33	P	Single Coupling CSY
34	L	Single Coupling CSY
35	G	Double Coupling CSY
36	Final deprotection	RT Final Deprotection

2.3.5.3 Peptide **3**

Resin: H-Rink-Amide-ChemMatrix® 100 mg, 0.5 mmol/g, 0.05 mmol scale (still amount of reagents were set for 0.1 mmol scale to drive reactions to completion).
The peptide was acetylated.
Cleavage cocktail: TFA (8.5 mL), TIPS (1 mL), water (0.5 mL).

Table S19: Settings for the synthesis of peptide **3**.

	Step	Used Cycles (optimized conditions)
1	Resin swelling	Resin swelling
2	Y	First Single Coupling
3	K	Single Coupling
4	E	Single Coupling
5	V	Single Coupling
6	Y	Single Coupling
7	P	Single Coupling
8	V	Single Coupling
9	P	Single Coupling
10	I	Single Coupling
11	Nle	Single Coupling
12	G	Double Coupling Gly
13	R	Double Coupling
14	K	Single Coupling
15	G	Double Coupling Gly
16	E	Single Coupling
17	S	Single Coupling Cool Down
18	D(CSY)	Single Coupling CSY
19	E	Single Coupling CSY
20	A	Single Coupling CSY
21	N	Single Coupling CSY
22	W	Single Coupling CSY
23	W	Single Coupling CSY
24	Q	Single Coupling CSY
25	E	Single Coupling CSY
26	E	Single Coupling CSY
27	P	Single Coupling CSY
28	K	Single Coupling CSY
29	D(CSY)	Single Coupling CSY
30	R	Double Coupling CSY
31	I	Single Coupling CSY
32	R	Double Coupling CSY
33	L	Single Coupling CSY
34	I	Single Coupling CSY
35	D(CSY)	Single Coupling CSY
36	G	Double Coupling CSY
37	K	Single Coupling CSY
38	K	Single Coupling CSY
39	F	Single Coupling CSY
40	P	Single Coupling CSY
41	L	Single Coupling CSY
42	D(CSY)	Single Coupling CSY
43	E	Single Coupling CSY
44	E	Single Coupling CSY
45	D(CSY)	Single Coupling CSY
46	N	Single Coupling CSY
47	G	Double Coupling CSY
48	N	Single Coupling CSY
49	F	Single Coupling CSY
50	D(CSY)	Single Coupling CSY
51	F	Single Coupling CSY
52	L	Single Coupling CSY
53	A	Single Coupling CSY

54	R	Double Coupling CSY
55	V	Single Coupling CSY
56	Y	Single Coupling CSY
57	E	Single Coupling CSY
58	A	Single Coupling CSY
59	Final deprotection	RT Final Deprotection

2.3.5.4 Peptide **4**

Resin: H-Rink-Amide-ChemMatrix® 200 mg, 0.5 mmol/g, 0.1 mmol scale.
The peptide was acetylated.
Cleavage cocktail: TFA (9.5 mL), TIPS (0.25 mL), water (0.25 mL).

Table S20: Settings for the synthesis of peptide **3**.

	Step	**Used Cycles (synthesis at room temperature)**	**Used Cycles (optimized method)**
1	Resin swelling	Resin swelling	Resin swelling
2	A	First Single Coupling	First Single Coupling
3	L	Single Coupling	Single Coupling
4	G	Double Coupling	Double Coupling Cool Down
5	D(CSY)	Single RT Coupling	Single Coupling CSY
6	F	Single RT Coupling	Single Coupling CSY
7	Final Deprotection	RT Final Deprotection	RT Final Deprotection

2.4 Screening for optimal Fmoc-deprotection and coupling conditions

To a *CEM* microwave pressure vessel (10 mL volume) containing a stirring bar resin-bound test peptide **4** (20 mg) and solutions (I) to (V) (2 mL) (see Table S21) were added and the vessel was then sealed. The filled vessel was placed into a *CEM Discovery* microwave reactor and reactions were performed under the following condition:

Dynamic mode, T = 90 °C, microwave power = 100 W, p up to 250 psi allowed, stirring speed: high, premixing: no, reaction time see Table S21.

Afterwards, the resin was filtered off, washed with DCM (10 x) and dried under reduced pressure. The dry resin was treated with TFA (950 µL), TIPS (25 µL) and water (25 µL) for 5 h. The resin was filtered off and washed with TFA (2 x 1 mL). The TFA was removed in a stream of nitrogen and the peptide was precipitated from cold Et_2O (10 mL) and isolated by centrifugation. The residue was washed with cold Et_2O (2 x 10 mL), briefly air-dried, dissolved in 20% MeCN in H_2O + 0.1% TFA (2 mL), and analyzed by MALDI-TOF-MS and analytical HPLC. For HPLC, an aliquot (5 µL) was mixed with buffer A (45 µL).

Table S21: Conditions for screening.

Reaction	Reagents	Reaction time (min)
I	-	-
II	DMF	30
IIIa	Piperidine/DMF 1:4 *v/v*	1
IIIb	Piperidine/DMF 1:4 *v/v*	10
IIIc	Piperidine/DMF 1:4 *v/v*	30
IV	DBU/DMF 1:49 *v/v*	30
V	DMF (2 mL) + 0.5 M DIC solution in DMF (2 mL) + 1 M Oxyma solution in DMF containing 0.1 M DIPEA (1 mL)	30

2.5 CD Spectroscopy

GENERAL: CD-measurements were performed using quartz cuvettes from *Starna* on a *Jasco J-810* CD spectrometer, equipped with *Jasco PTC-423S* Peltier thermostatted rectangular cell holder. The measuring chamber was flushed constantly with nitrogen. Settings: Data pitch: 0.1 nm; scanning speed: 100 nm/min, sensitivity: low, response: 2 s; bandwidth: 1 nm. The peptide concentration was 50 µM (20 µM for peptide **2**) and 10 mM sodium phosphate buffer at pH 7.0 was used.

SPECTRA MEASUREMENT: CD spectra were recorded from 190 to 260 nm in 0.1 cm cuvettes at 20 °C. After placing the cuvette into the holder, the sample was left to equilibrate for 5 min prior to measurement. 10 spectra were recorded and the data were accumulated. The measured ellipticity Θ (in mdeg) was converted to mean residue ellipticity [Θ] (in deg·cm^2·dmol^{-1}·res^{-1}) using Eq. 1 where Θ_{Blank} is the blank signal, c is the peptide concentration in mol/L, l is the pathlength in mm and n is the number of backbone peptide bonds.[3]

$$[\Theta] = \frac{\Theta - \Theta_{\mathrm{Blank}}}{c \cdot l \cdot n} \qquad \textbf{Eq. 1}$$

THERMAL DENATURATION: Melting curves were recorded from 2 to 98 °C in 0.1 cm cuvettes using [Θ] at the maximum, respectively (**1**(CSY) and **1**: 227 nm, **2**(CSY) and **2**: 228 nm, **3**(CSY) and **3**: 221 nm), with a temperature gradient of 1 °C/min. Data points were collected every 1 °C. The thermal denaturation data were fitted to Eq. 2 ($A = a \cdot T + b$, $B = c \cdot T + d$, linear fit of the upper and lower baseline, ΔH: molar enthalpy, R: universal gas constant) to determine the melting temperature T_m (in °C):[4]

$$[\Theta]_{227\,\mathrm{nm}}(T) = A + \frac{B - A}{1 + \exp\left(\left. -\Delta H \cdot \left(1 - \frac{T + 273.15\,\mathrm{K}}{T_\mathrm{m} + 273.15\,\mathrm{K}}\right) \right/ R \cdot (T + 273.15\,\mathrm{K}) \right)} \qquad \textbf{Eq. 2}$$

2.6 NMR spectroscopy

Products of organic synthesis were prepared for NMR spectroscopy by dissolving them in the deuterated organic solvent DMSO-d_6 (^{1}H-NMR spectra referenced at 2.50 ppm, ^{13}C-NMR spectra at 39.52 ppm) inside an NMR tube with a diameter of 5 mm. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY and ^{1}H-^{13}C-HSQC spectra were recorded using a *Bruker Avance III 600* NMR system for Fmoc-Asp(CSY)-O*t*Bu and a *Bruker Avance III 400* NMR measuring system for Fmoc-Asp(CSY)-OH. ^{1}H-NMR spectra of other purified fractions were recorded on a *Bruker Avance III 300* device to confirm purity.
Spectra processing and analysis was performed using *TopSpin 3.6.1* and *MestReNova 14.1.2*. Signal multiplicities were abbreviated with s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet).

2.7 IR-Spectroscopy

ATR-IR spectroscopy was performed on the *FT/IR-4600* Fourier transformation infrared spectrometer from *Jasco*. Spectra were recorded in a range from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.8 Mass spectrometry

Peptides were analysed by matrix-assisted laser desorption/ionisation (MALDI) combined with time of flight (TOF) analysis that was performed on a *Bruker Autoflex Speed* MALDI-TOF mass spectrometer. Spectra were measured in reflector mode (peptide **1** and **2**: 1000 to 8000 Da, **3**: 3 to 15 kDa, **4**: 20 to 1500 Da). Samples were prepared by applying a saturated 2,5-dihydroxybenzoic acid solution as a matrix (1 µL) together with the solubilized sample (1 µL) on a suitable target plate for MALDI-MS measurement and mixing on the plate.
The products of organic synthesis were analysed by electrospray-ionisation mass spectrometry (ESI) using a *ApexQe FT-ICR* mass spectrometer by *Bruker*.

Table S22: Important mass differences.

Modification	**Δ*m*/*z* (monoisotopic mass)**	**Δ*m*/*z* (average mass)**
native peptide + CSY	+ 83.0	+ 83.2
Aspartimide formation (native peptide – water)	– 18.0	– 18.0
Piperidine amide formation (native peptide – water + piperidine)	+ 67.1	+ 67.1

2.9 Melting point determination

The melting point of Fmoc-Asp(CSY)-OH was determined using a melting point meter *M5000* by *Krüss* in a 1 mm capillary tube.

2.10 Deprotection of CSY

The reactions were performed in polypropylene reaction tubes. After each addition of reagent, the mixture was vortexed and spun down with a small tabletop centrifuge. After completion of the reaction, an aliquot (10 µL) was taken, diluted with buffer A (10 µL) and analyzed with analytical HPLC.

(a) Conditions according to Neuman *et al*.[1]
CSY protected peptide (25 nmol) was dissolved in acetate buffer 10 mM (200 µL). NCS in MeCN (concentration of NCS in case of **1**(CSY): 1.25 mM, **2**(CSY): 5 mM, **3**(CSY): 7.5 mM) was added (4 x 11 µL, in total 2.2 equiv. per CSY group). Between each addition, the mixture was equilibrated for 10 min. The reaction was quenched with sodium ascorbate in 10% MeCN in water (concentration of sodium ascorbate in case of **1**(CSY): 7.5 mM, **2**(CSY): 30 mM, **3**(CSY): 45 mM) was added (44 µL, 6.0 equiv. regarding to NCS).

(b)
CSY protected peptide (250 nmol) was dissolved in buffer A (1800 µL) and HFIP (200 µL). NCS dissolved in buffer B (concentration of NCS in case of **1**(CSY): 1.25 mM, **2**(CSY): 5 mM, **3**(CSY): 7.5 mM) was added (4 x 110 µL, in total 2.2 equiv. per CSY group). Between each addition, the mixture was equilibrated for 10 min. The reaction was quenched with sodium ascorbate in 10% B in A (concentration of sodium ascorbate in case of **1**(CSY): 7.5 mM, **2**(CSY): 30 mM, **3**(CSY): 45 mM) was added (440 µL, 6.0 equiv. regarding to NCS). The samples were frozen in liquid nitrogen and freeze-dried to obtain a colorless foam, which was further purified by HPLC.

(c) preparative scale
2(CSY) (250 nmol) was dissolved in buffer A (1 mL), DMF (800 µL) and HFIP (200 µL). NCS in DMF (5 mM, 4 x 110 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 5 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, 110 µL, 5.0 equiv. regarding to NCS) and buffer A:DMF 1:1 (1450 µL) was added. The mixture was directly injected into the HPLC. Due to high content of DMF several peaks can occur, which all contain peptide. These were collected, pooled and re-purified after freeze drying.

(c) analytical scale
CSY protected peptide (25 nmol) was dissolved in buffer A (100 µL), HFIP (20 µL) and DMF (80 µL). NCS in DMF (5 mM, **1**(CSY): 4 x 2.75 µL, **2**(CSY): 4 x 11 µL, **3**(CSY): 4 x 16.5 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 5 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 2.75 µL, **2**(CSY): 11 µL, **3**(CSY): 16.5 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 186.25 µL, **2**(CSY): 145 µL, **3**(CSY): 117.5 µL) was added.

(d)
CSY protected peptide (25 nmol) was dissolved in buffer A (100 µL), HFIP (20 µL) and TFE (80 µL). NCS in DMF (5 mM, **1**(CSY): 4 x 2.75 µL, **2**(CSY): 4 x 11 µL, **3**(CSY): 4 x 16.5 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 5 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 2.75 µL, **2**(CSY): 11 µL, **3**(CSY): 16.5 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 186.25 µL, **2**(CSY): 145 µL, **3**(CSY): 117.5 µL) was added.

(e)
CSY protected peptide (25 nmol) was dissolved in buffer A (100 µL), HFIP (20 µL) and TFE (80 µL). NCS in MeCN (5 mM, **1**(CSY): 4 x 2.75 µL, **2**(CSY): 4 x 11 µL, **3**(CSY): 4 x 16.5 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 5 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 2.75 µL, **2**(CSY): 11 µL, **3**(CSY): 16.5 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 186.25 µL, **2**(CSY): 145 µL, **3**(CSY): 117.5 µL) was added.

(f)
CSY protected peptide (250 nmol) was dissolved in buffer A (1800 µL) and HFIP (200 µL). NCS in MeOH + 0.1% TFA (5 mM, **1**(CSY): 4 x 27.5 µL, **2**(CSY): 4 x 110 µL, **3**(CSY): 4 x 165 µL, in total 2.2 equiv.

per CSY group) was added. Between each addition, the mixture was equilibrated for 10 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 27.5 µL, **2**(CSY): 110 µL, **3**(CSY): 165 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 1862.5 µL, **2**(CSY): 1450 µL, **3**(CSY): 1175 µL) was added.

(g)
CSY protected peptide (250 nmol) was dissolved in buffer A (500 µL) and guanidine hydrochloride in buffer A (6.0 M, 1500 µL). NCS in buffer B (5 mM, **1**(CSY): 4 x 27.5 µL, **2**(CSY): 4 x 110 µL, **3**(CSY): 4 x 165 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 10 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 27.5 µL, **2**(CSY): 110 µL, **3**(CSY): 165 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 1862.5 µL, **2**(CSY): 1450 µL, **3**(CSY): 1175 µL) was added.

(h)
CSY protected peptide (250 nmol) was dissolved in buffer A (1050 µL), guanidine hydrochloride in buffer A (6.0 M, 750 µL) and TFE (200 µL). NCS in buffer B (5 mM, **1**(CSY): 4 x 27.5 µL, **2**(CSY): 4 x 110 µL, **3**(CSY): 4 x 165 µL, in total 2.2 equiv. per CSY group) was added. Between each addition, the mixture was equilibrated for 10 min. The reaction was quenched with sodium ascorbate in buffer A (100 mM, **1**(CSY): 27.5 µL, **2**(CSY): 110 µL, **3**(CSY): 165 µL, 5.0 equiv. regarding to NCS) and buffer A (**1**(CSY): 1862.5 µL, **2**(CSY): 1450 µL, **3**(CSY): 1175 µL) was added.

2.11 CSY Deprotection of Fmoc-Asp(CSY)-OH

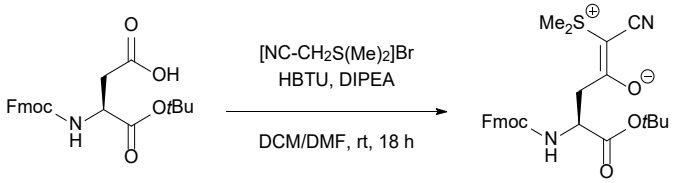
The reactions were performed in polypropylene reaction tubes. After each addition of reagent, the mixture was vortexed and spun down with a small tabletop centrifuge. A solution of Fmoc-Asp(CSY)-OH in MeCN or buffer B (2 mM, 10 µL, 1 equiv.) was mixed with the solvent system summarized in Table S23. The oxidant solution (4 mM, 2 x 5 µL, 2 equiv.) (Table S22) was then added. Between the additions, the mixture was equilibrated for 5 min. The reaction was quenched by addition of sodium ascorbate in 10% MeCN in water (20 mM, 10 µL, 10 equiv.). An aliquot (10 µL) was taken, diluted with buffer A (10 µL) and analysed with analytical HPLC.

Table S23: Conditions for the deprotection of Fmoc-Asp(CSY)-OH.

	Fmoc-Asp(CSY)-OH (2 mM) dissolved in	**solvent system**	**oxidant solution (4 mM)**
a	buffer B	buffer B (10 µL), buffer A (180 µL)	buffer B, no oxidant
b	buffer B	buffer B (10 µL), buffer A (180 µL)	NCS in buffer B
c	MeCN	MeCN (10 µL), acetate buffer pH 4.5 (50 mM, 40 µL), H_2O (140 µL)	NCS in MeCN
d	buffer B	buffer B (10 µL), buffer A (180 µL)	Iodine in buffer B
e	MeCN	MeCN (10 µL), phosphate buffer pH 10.0 (50 mM, 40 µL), H_2O (140 µL)	Iodine in MeCN
f	MeCN	MeCN (10 µL), acetate buffer pH 4.5 (50 mM, 40 µL), H_2O (140 µL)	$NaIO_4$ in water
g	MeCN	MeCN (10 µL), water (180 µL)	Oxone in water
h	buffer B	buffer B (10 µL), buffer A (180 µL)	Oxone in buffer A
i	MeCN	MeCN (10 µL), water (180 µL)	mCPBA in water
j	buffer B	buffer B (10 µL), buffer A (180 µL)	mCPBA in buffer A

3 Organic Synthesis

3.1 Synthesis of Fmoc-Asp(CSY)-O*t*Bu



Fmoc-Asp-O*t*Bu **Fmoc-Asp(CSY)-O*t*Bu**

All reactions were performed in a nitrogen atmosphere. A mixture of dimethyl sulfide (2.27 g, 2.66 mL, 36.46 mmol,1.50 equiv.) and 1-bromoacetonitrile (4.37 g, 2.54 mL, 36.46 mmol, 1.50 equiv.) was stirred for 18 h at room temperature.

The resulting (cyanomethyl)-di-methyl sulfonium bromide was suspended in dry DCM (40 mL) using ultrasonication for 15 min and stirred for another 10 min. For better solvation DMF (20 mL) was added to the suspension.

Separately, Fmoc-Asp-O*t*Bu (10.00 g, 24.30 mmol, 1.00 equiv.) was dissolved in DMF (60 mL). HBTU (13.83 g, 36.46 mmol, 1.50 equiv.) was added and the suspension was stirred for 15 min. Then DIPEA (9.42 g, 12.40 mL, 72.90 mmol, 3.00 equiv.) was added and stirring continued for 10 min. The solution was transferred to the suspension of (cyanomethyl)-di-methyl sulfonium bromide and the flask was washed with DMF (30 mL), which also was transferred to the suspension. The reaction mixture was stirred at room temperature for 18 h.

The reaction mixture was washed with 10% citric acid (200 mL) and the aqueous phase was then extracted with DCM (3 x 20 mL). The combined organic phases were concentrated *in vacuo*, then dissolved in ethyl acetate (200 mL) and washed with a saturated $NaHCO_3$ solution (100 mL), deionized H_2O (3 x 100 mL) and brine (100 mL), respectively. The organic phase was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure.

The product-containing fractions were combined and the solvent was removed under reduced pressure to give an oily residue which was lyophilised to give Fmoc-Asp(CSY)-O*t*Bu as a yellowish foam (11.46 g, 23.17 mmol, 95%).

TLC: R_f = 0.12 (SiO_2, DCM:MeOH, 40:1).

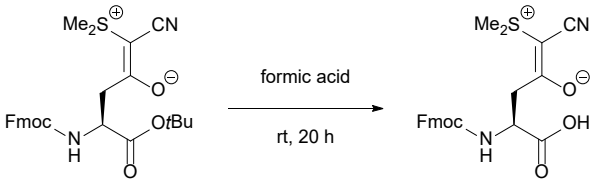
MS HR-ESI: *m*/*z* 495.1950 (calculated: 495.1948 for $C_{27}H_{31}N_2O_5S^+$ $[M+H]^+$), 517.1769 (calculated: 517.1768 for $C_{27}H_{30}N_2NaO_5S^+$ $[M+Na]^+$).

IR (ATR): $\tilde{\nu}$ [cm^{-1}] 3324.7, 2975.6, 2930.3, 2171.5 (*v* CN), 1714.4 (*v* CO), 1580.9 (*v* CO), 1499.8 (*v* CO), 1447.8, 1367.3, 1308.5, 1247.7, 1222.6, 1148.5, 1040.4, 988.3, 841.8, 759.8, 738.6, 619.2, 532.3.

^{1}H-NMR (600 MHz, DMSO-d$_6$): δ [ppm] 7.89 (d, *J* = 7.5 Hz, 2H, H_{ar}), 7.71 (d, *J* = 7.4 Hz, 2H, H_{ar}), 7.56 (d, *J* = 8.4 Hz, 1H, NH), 7.42 (t, *J* = 7.5 Hz, 2H, H_{ar}), 7.34-7.31 (m, 2H, H_{ar}), 4.40-4.37 (m, 1H, $C_\alpha H$), 4.33-4.25 (m, 2H, CH_2), 4.21 (t, *J* = 7.0 Hz, 1H, CH), 2.78 (s, 6H, $S(CH_3)_2$), 2.72 (t, *J* = 6.4 Hz, 2H, $C_\beta H_2$), 1.37 (s, 9H, $C(CH_3)_3$).

^{13}C-NMR (151 MHz, DMSO-d$_6$): δ [ppm] 185.65 (COON), 170.91 (COOC), 155.94 (CO), 143.93 (2 x C_{ar}), 140.73 (2 x C_{ar}), 127.66 (2 x CH_{ar}), 127.09 (2 x CH_{ar}), 125.27 (2 x CH_{ar}), 120.14 (2 x CH_{ar}), 119.37 (CN), 80.58 (C), 65.65 (CH_2), 55.19 (C), 50.84 ($C_\alpha H$), 46.62 (CH), 39.57 ($C_\beta H_2$), 27.60 (5 x CH_3).

3.2 Synthesis of Fmoc-Asp(CSY)-OH



Fmoc-Asp(CSY)-O*t*Bu **Fmoc-Asp(CSY)-OH**

Fmoc-Asp(CSY)-O*t*Bu (5.00 g, 10.11 mmol, 1.00 equiv.) was dissolved in formic acid (150 mL) and stirred at room temperature for 20 h. The organic acid was removed under reduced pressure. The oily residue was coevaporated with methanol (150 mL) and DCM (10 mL) yielding a yellow foam, which was again coevaporated with petrol ether (3 x 50 mL). The substance was dried *in vacuo* over $NaHCO_3$ overnight.
The yellow foam was dissolved in ethyl acetate (80 mL). Immediately a colourless precipitate was formed. The suspension stirred at room temperature for 3 h giving a thick slurry mixture. It was diluted with ethyl acetate (30 mL) and heated under reflux for 30 min. After cooling to room temperature, the colourless precipitate was filtered off, washed with ethyl acetate and dried under reduced pressure. The mother liquor was concentrated *in vacuo* to give a yellow foam again and was recrystallised from ethyl acetate (20 mL). Fmoc-Asp(CSY)-OH was yielded as a colourless powder (3.90 g, 8.89 mmol, 88%).

Melting point: 145 to 146 °C.
TLC: R_f = 0.55 (SiO_2, DCM:MeOH:HCOOH, 10:1:0.1).
MS HR-ESI: *m*/*z* 439.1325 (calculated: 439.1322 for $C_{23}H_{23}N_2O_5S^+$ $[M+H]^+$).
IR (ATR): $\tilde{\nu}$ [cm^{-1}] 3520.5, 3475.7, 3377.3, 3025.4, 2930.0, 2181.4 (*v* CN), 1701.3 (ν_{as} CO), 1522.4 (*v* CO), 1444.8 (*v* CO), 1325.5, 1259.9, 1221.2 1149.6, 1045.2, 997.5, 917.0, 779.8, 735.1, 633.7, 618.8, 583.0, 535.3.
^{1}H-NMR (400 MHz, DMSO-d$_6$): δ [ppm] 12.61 (s, 1H, COOH), 7.89 (d, *J* = 7.6 Hz, 2H, H_{ar}), 7.71 (d, *J* = 7.3 Hz, 2H, H_{ar}), 7.51 (d, *J* = 8.4 Hz, NH), 7.42 (t, *J* = 7.5 Hz, 2H, H_{ar}), 7.35-7.30 (m, 2H, H_{ar}), 4.48-4.41 (m, 1H, $C_\alpha H$), 4.28-4.20 (m, 3H, CH, CH_2), 2.89-2.73 (m, 8H, $S(CH_3)_2$, $C_\beta H_2$).
^{13}C-NMR (101 MHz, DMSO-d$_6$): δ [ppm] 185.81 (COON), 173.27 (COOH), 155.78 (CO), 143.81 (2 x C_{ar}), 140.68 (2 x C_{ar}), 127.61 (2 x CH_{ar}), 127.06 (2 x CH_{ar}), 125.27 (2 x CH_{ar}), 120.08 (2 x CH_{ar}), 119.27 (CN), 65.65 (CH_2), 55.05 (C), 50.06 ($C_\alpha H$), 46.60 (CH), 39.67 ($C_\beta H_2$), 27.54 ($S(CH_3)_2$).

4 Calculation of the cost of the synthesis of Fmoc-Asp(CSY)-OH

The cost of the synthesis of Fmoc-Asp(CSY)-OH is calculated for the here presented procedure and is compared to the synthesis reported by Neumann *et al.*.[1] Prices (Table S24) are given without taxes. Only reagents are included in the calculation because the usage of solvent and silica gel is comparable in both procedures.

Table S24: Prices of the used reagents for the synthesis of Fmoc-Asp(CSY)-OH.

Reagent	Supplier	Package	Price / €	Price/g or mL
dimethyl sulfide	Merck (Sigma-Aldrich)	250 mL	35.77	0.14308 €/mL
bromo acetonitrile	Merck (Sigma-Aldrich)	25 g	70.20	2.80800 €/g
Fmoc-Asp-O*t*Bu	Carbolution	25 g	152.50	6.10000 €/g
T3P in EtOAC	Merck (Sigma-Aldrich)	10 mL	33.60	3.36000 €/mL
HBTU	Carbolution	100 g	49.50	0.49500 €/g
DIPEA	Merck (Sigma-Aldrich)	500 mL	103.6	0.20720 €/mL
TFA	Merck (Sigma-Aldrich)	500 mL	86.02	0.17204 €/mL
formic acid	VWR	1000 mL	11.34	0.01134 €/mL

Cost of the synthesis reported in this work:

First step: Fmoc-Asp(CSY)-O*t*Bu
Used Reagents: dimethyl sulfide (2.66 mL), bromo acetonitrile (4.37 g), Fmoc-Asp-O*t*Bu (10 g), HBTU (13.83 g), DIPEA (12.4 mL).
Total cost: 83.07 €
Yield: Fmoc-Asp(CSY)-O*t*Bu (11.46 g)
price per g: 7.25 €/g

Second step: Fmoc-Asp(CSY)-OH
Used Reagents: Fmoc-Asp(CSY)-O*t*Bu (5 g), formic acid (150 mL).
Total cost: 37.94 €
Yield: Fmoc-Asp(CSY)-OH (3.90 g)
price per g: 9.73 €

Cost of the synthesis reported by Neumann *et al.*:[1]

First step: [NC-CH_2-SMe_2]Br
Used reagents: dimethyl sulfide (7.53 mL), bromo acetonitrile (12.35 g)
Total cost: 35.76 €
Yield: [NC-CH_2-SMe_2]Br 16.8 g
Price per g: 2.13 €/g

Second step: Fmoc-Asp(CSY)-O*t*Bu
Used Reagents: [NC-CH_2-SMe_2]Br (0.53 g), Fmoc-Asp-O*t*Bu (1 g), T3P(2.01 mL), DIPEA 1.29 mL).
Total cost: 14.25 €
Yield: Fmoc-Asp(CSY)-O*t*Bu (1.07 g)
price per g: 13.32 €/g

Third step: Fmoc-Asp(CSY)-OH
Used Reagents: Fmoc-Asp(CSY)-O*t*Bu (1 g), TFA (10 mL).
Total cost: 15.04 €
Yield: Fmoc-Asp(CSY)-OH (0.85 g)
price per g: 17.69 €

5 Characterization of peptides

5.1 Peptide 1(CSY)

H-KLPPGWEKRXS-AD(CSY)GRVYYFNHITNASQWERPSG-OH

Chemical formula: $C_{179}H_{264}N_{52}O_{48}S$, molar mass: 3944.5 g/mol, exact mass: 3942.0 Da

After final cleavage the crude product was obtained as a yellowish powder (143.5 mg). A part of it (42.6 mg) was purified by HPLC and after freeze-drying a colorless powder (12.1 mg, 3.07 µmol, 28%) was obtained.

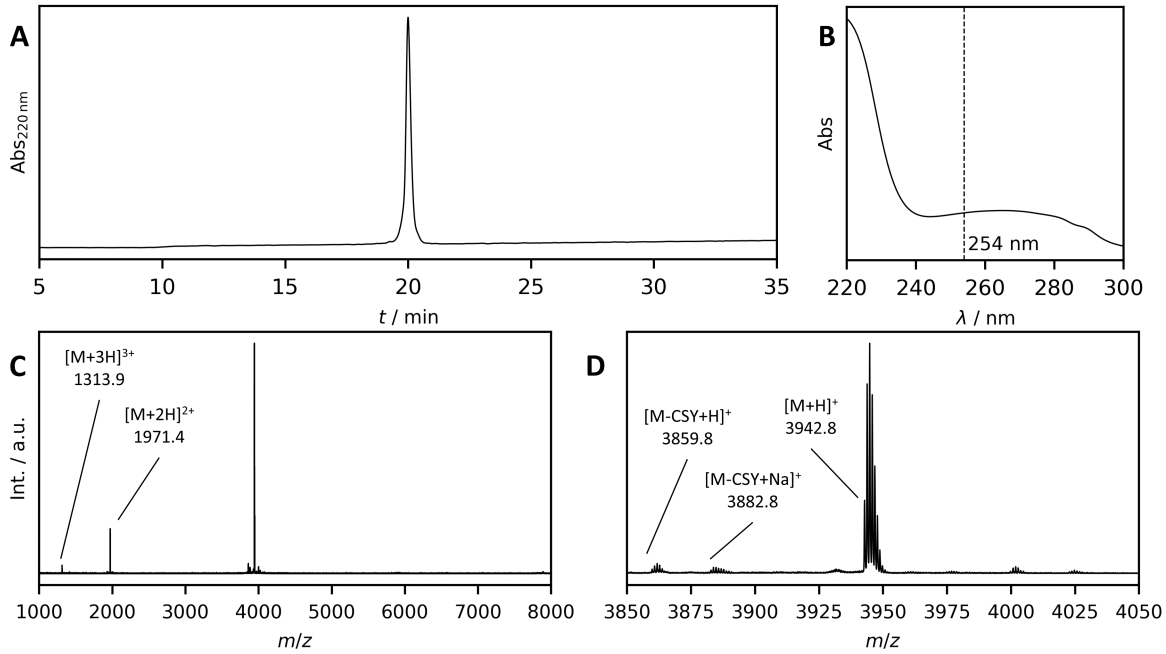


Figure S8: Analytical data for **1(CSY)**. A: analytical HPLC trace t_R = 20.0 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom).

5.2 Peptide 1

H-KLPPGWEKRXS-ADGRVYYFNHITNASQWERPSG-OH

Chemical Formula: $C_{175}H_{259}N_{51}O_{49}$, molar mass: 3861.3 g/mol, exact mass: 3858.9 Da

Peptide **1(CSY)** (250 nmol) was CSY-deprotected with method (b). Unprotected peptide **1** was obtained as a colorless powder (112.2 nmol, 45%).

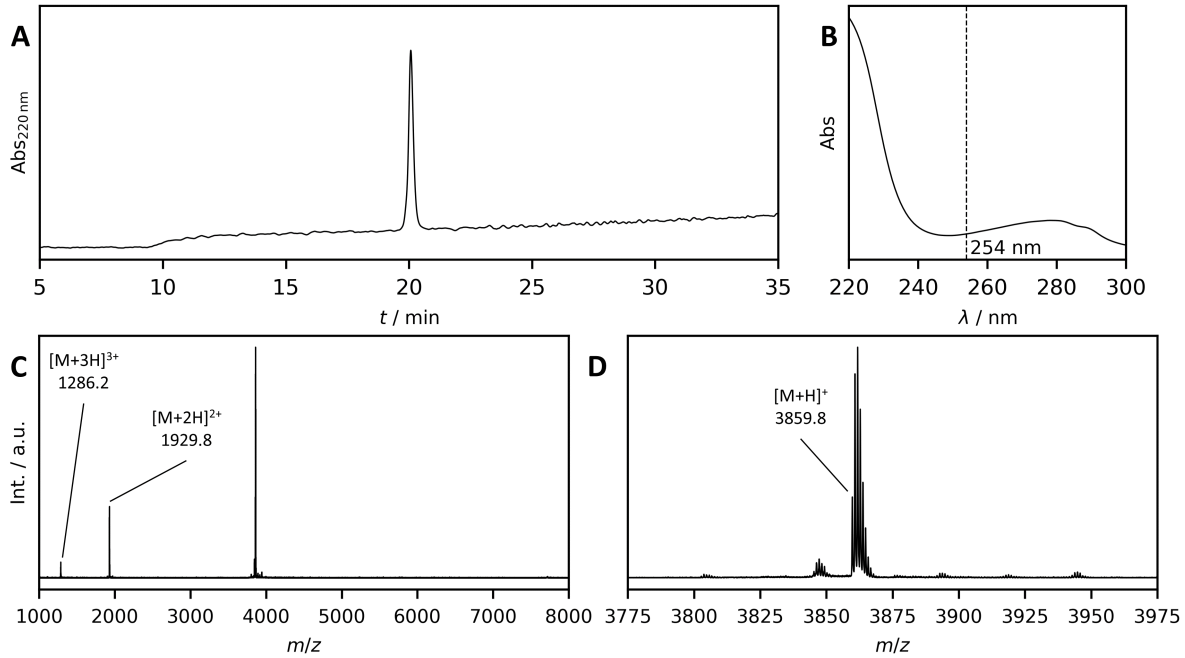


Figure S9: Analytical data for **1**. A: analytical HPLC trace t_R = 20.1 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom).

5.3 Peptide 2(CSY)

Ac-GLPPSWYKVFD(CSY)PSSGLPYYWNAD(CSY)TD(CSY)LVSWLSPHD(CSY)-NH_2
Chemical formula: $C_{204}H_{276}N_{46}O_{49}S_4$, molecular mass: 4285.0 g/mol, exact mass: 4281.9 Da
After final cleavage the crude product was obtained as a yellowish powder (157.1 mg). A part of it (41.0 mg) was purified by HPLC and after freeze-drying a colorless powder (11.2 mg, 2.61 µmol, 27%) was obtained.

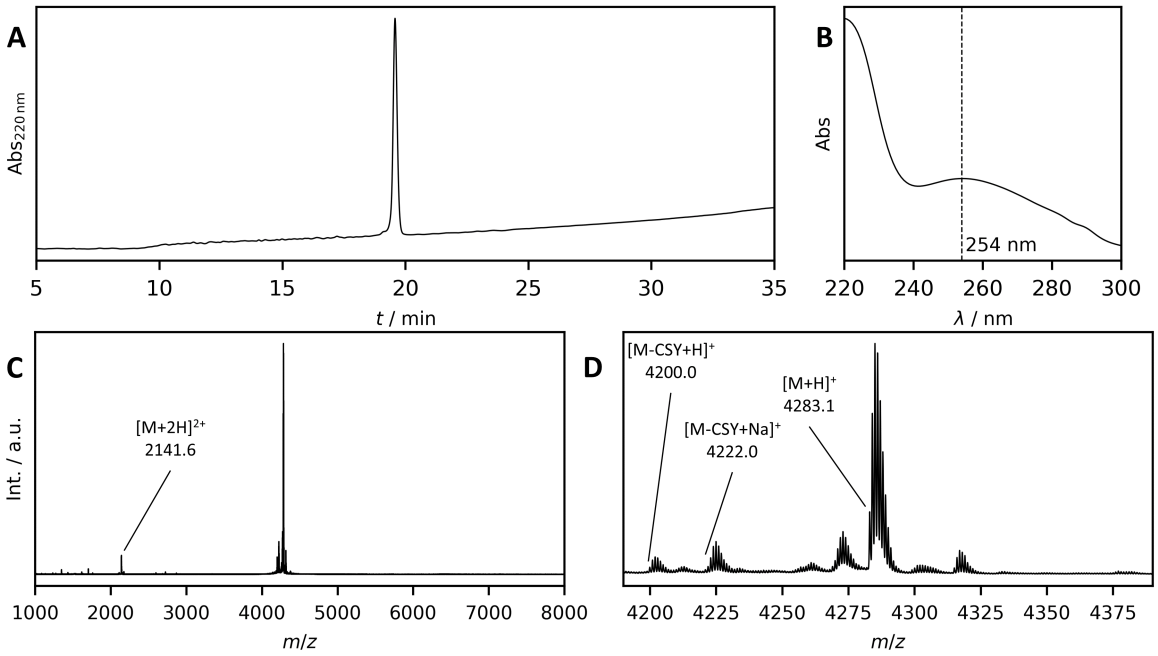


Figure S10: Analytical data for **2(CSY)**. A: analytical HPLC trace t_R = 19.6 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom).

5.4 Peptide 2

Ac-GLPPSWYKVFDPSSGLPYYWNADTDLVSWLSPHD-NH_2
Chemical formula: $C_{188}H_{256}N_{42}O_{53}$, molecular mass: 3952.4 g/mol, exact mass: 3949.9 Da
Peptide **2(CSY)** (250 nmol) was CSY-deprotected with method (c). Unprotected peptide **2** was obtained as a colorless powder (41.5 nmol, 17%).

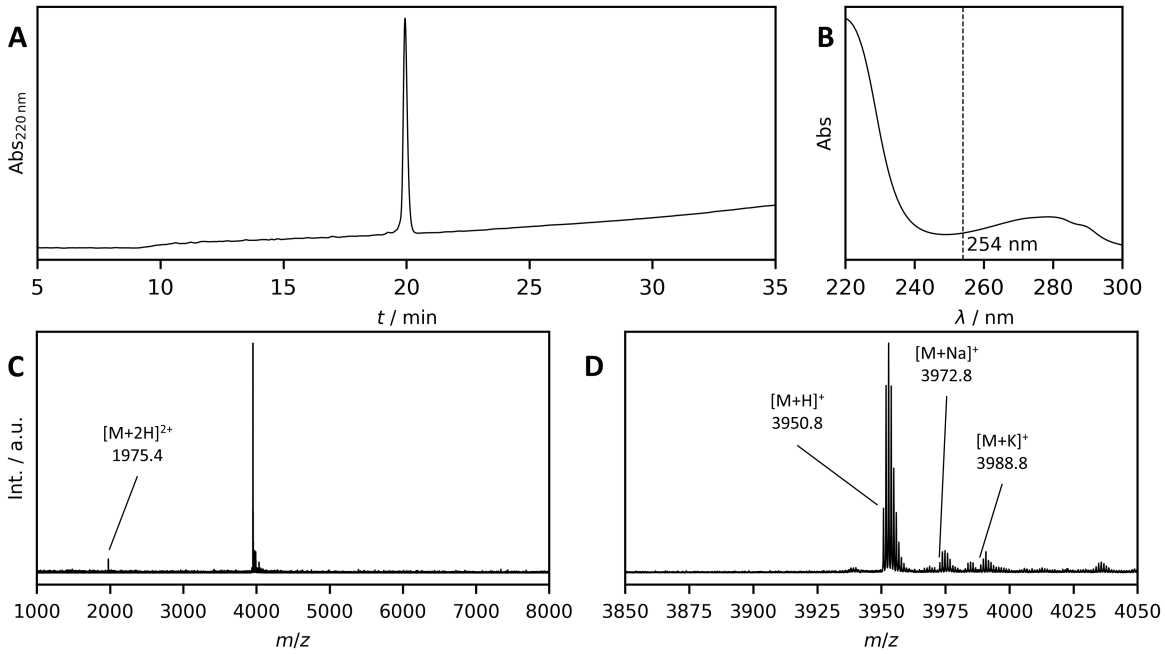


Figure S11: Analytical data for **2**. A: analytical HPLC trace t_R = 19.9 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom).

5.5 Peptide 3(CSY)

Ac-AEYVRALFD(CSY)FNGND(CSY)EED(CSY)LPFKKGD(CSY)ILRIRD(CSY)KPEEQWWNAED(CSY)SEG KRGXIPVPYVEKY-NH_2

Chemical formula: $C_{334}H_{493}N_{87}O_{88}S_6$, molecular mass: 7327.5 g/mol, exact mass: 7322.5 Da

After final cleavage and precipitation from diethyl ether the crude product was obtained as a yellowish powder (155.3 mg). A part of it (76.2 mg) was purified by HPLC and after freeze-drying a colorless powder (14.2 mg, 1.94 µmol, 19%) was obtained.

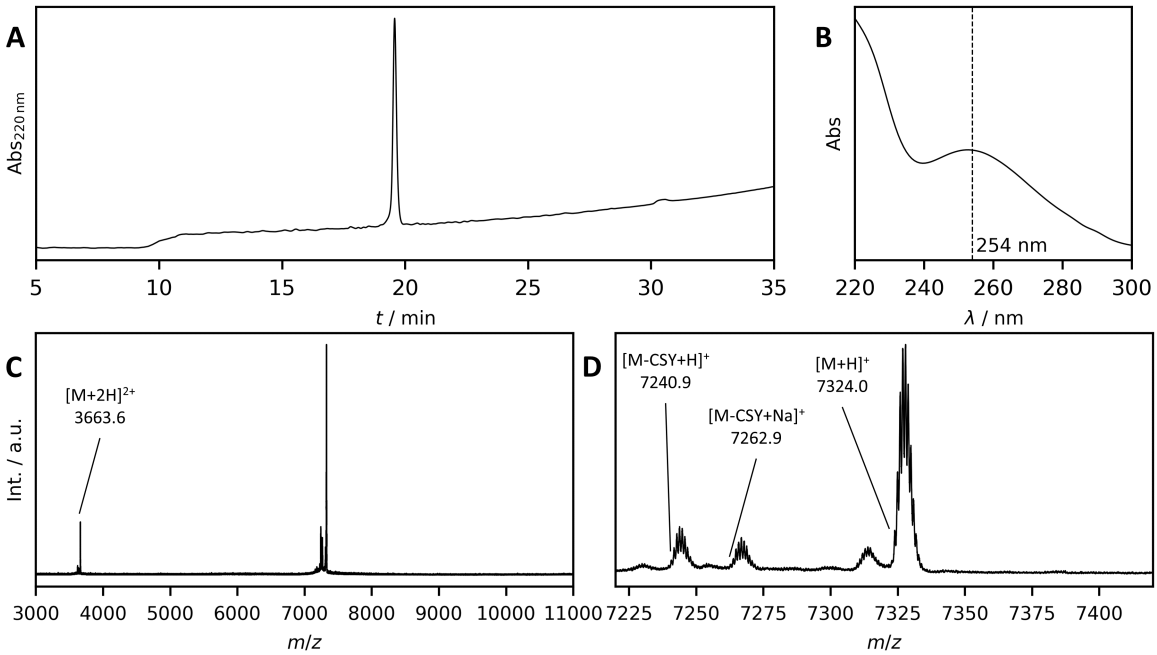


Figure S12: Analytical data for **3(CSY)**. A: analytical HPLC chromatogram t_R = 19.6 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom).

5.6 Peptide 3

Ac-AEYVRALFDFNGNDEEDLPFKKGDILRIRDKPEEQWWNAEDSEGKRGXIPVPYVEKY-NH_2

Chemical formula: $C_{310}H_{463}N_{81}O_{94}$, molecular mass: 6828.6 g/mol, exact mass: 6824.4 Da

Peptide **3(CSY)** (250 nmol) was CSY-deprotected with method (b). Unprotected peptide **3** was obtained as a colorless powder (101.4 nmol, 41%).

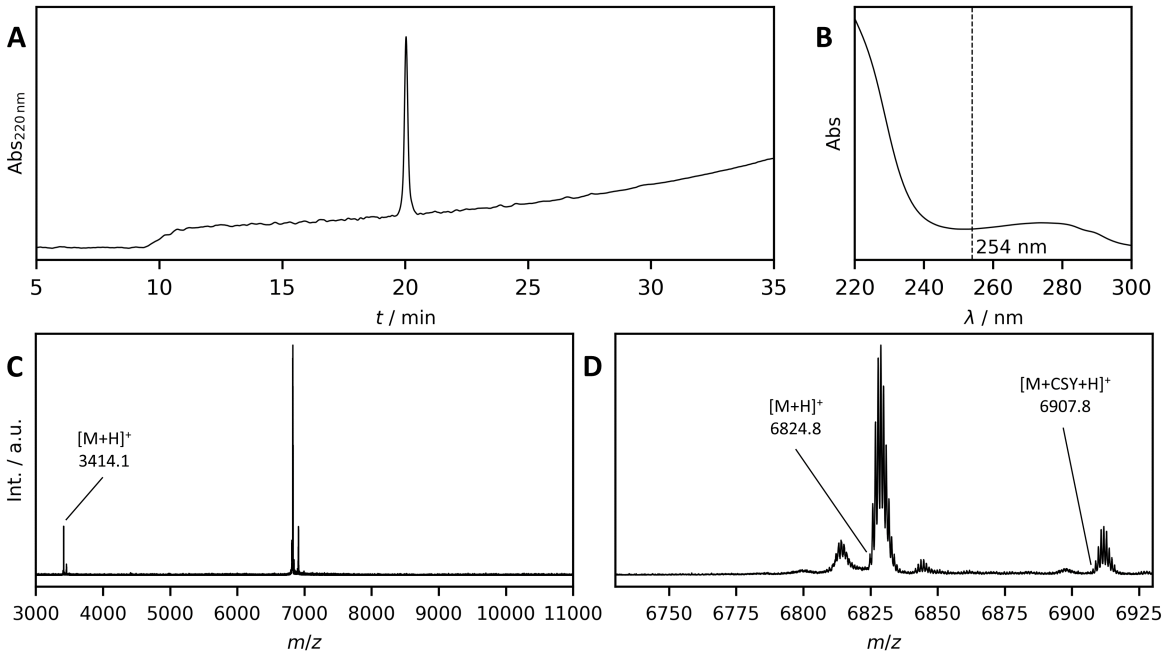


Figure S13: Analytical data for **3**. A: analytical HPLC trace t_R = 20.0 min. B: UV/Vis spectrum of the main peak at t_R. MALDI-TOF MS spectrum (C: full, D: zoom). Traces of partially CSY-protected **3** were detected in the

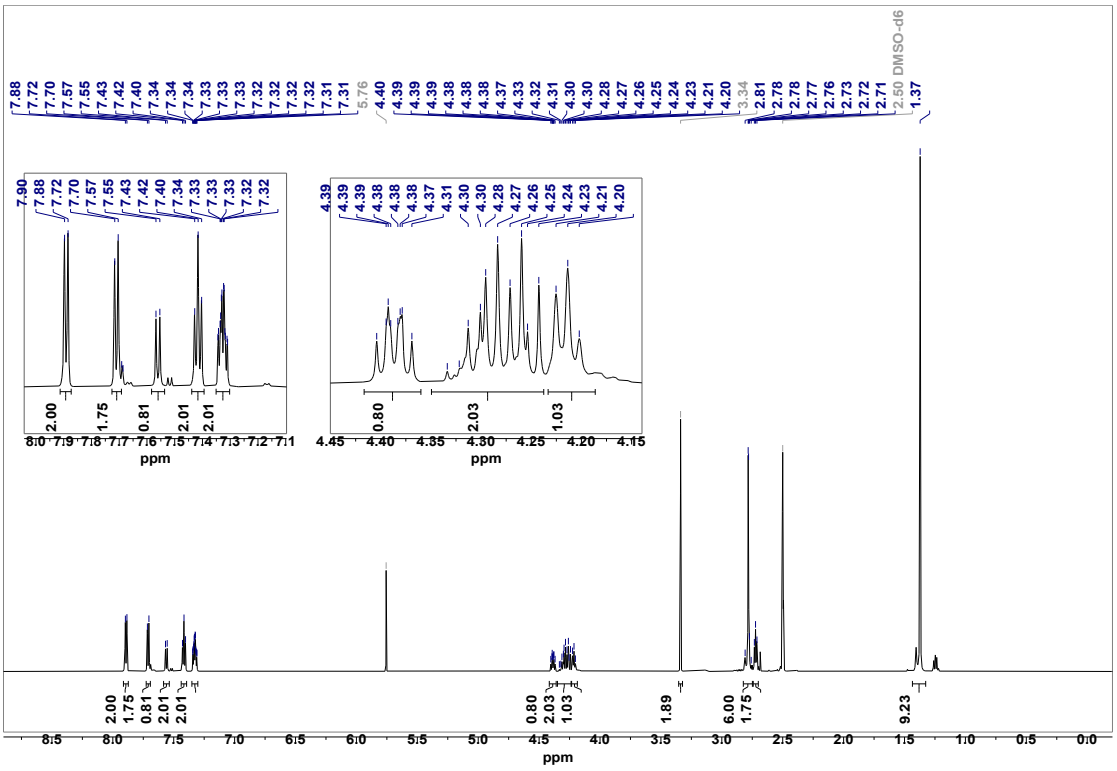
MS spectrum. Positively charged CSY flies extremely well in MALDI-TOF. No CSY-protected species were found in the UV/Vis spectrum and HPLC.

6 Additional Spectra

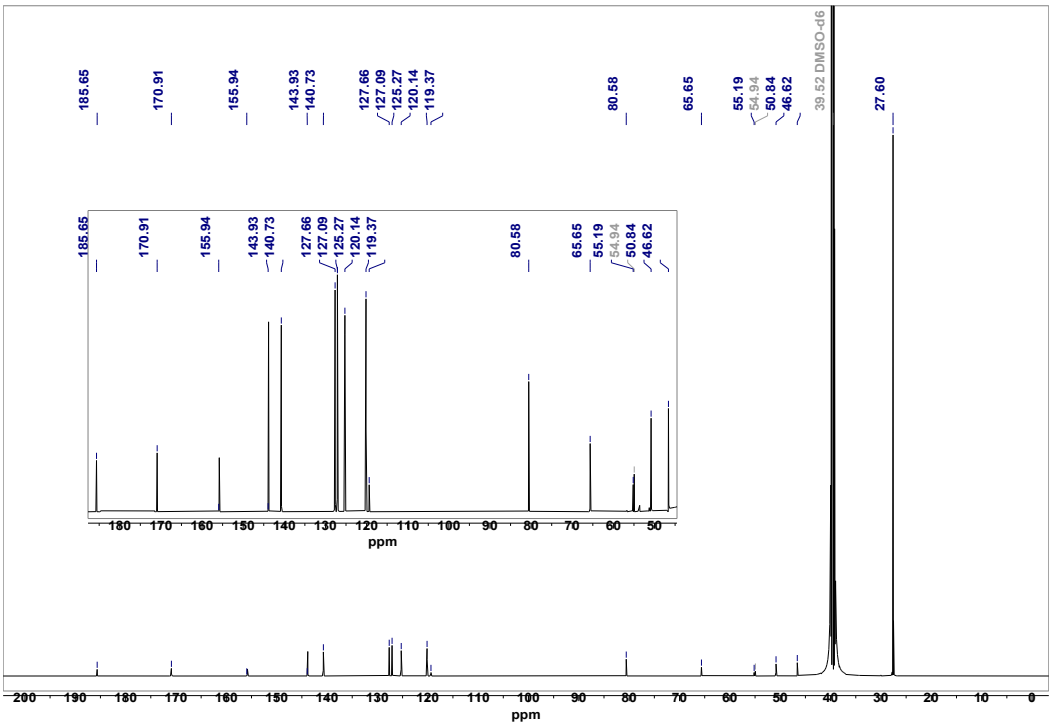
6.1 NMR

6.1.1 Fmoc-Asp(CSY)-O*t*Bu

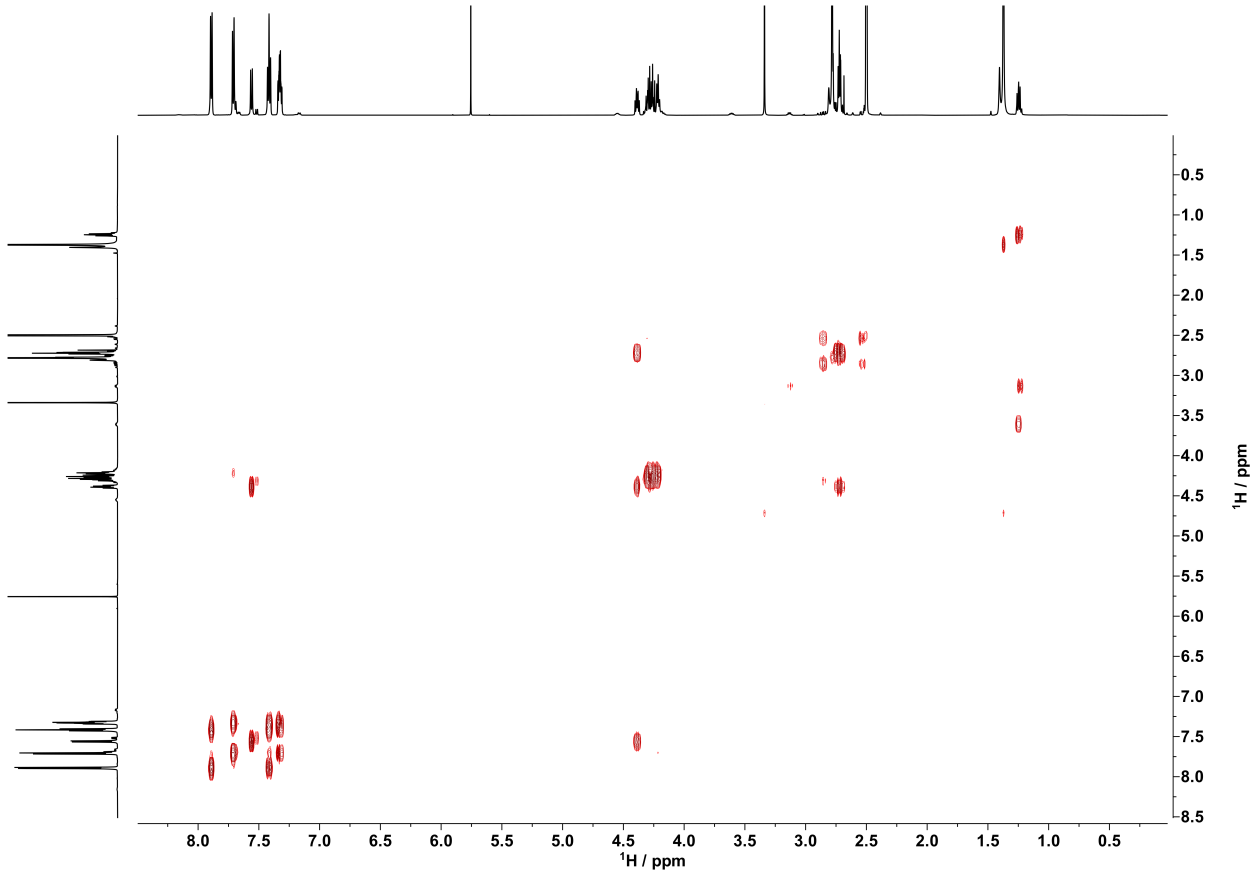
^{1}H-NMR (600 MHz, DMSO-d_6)



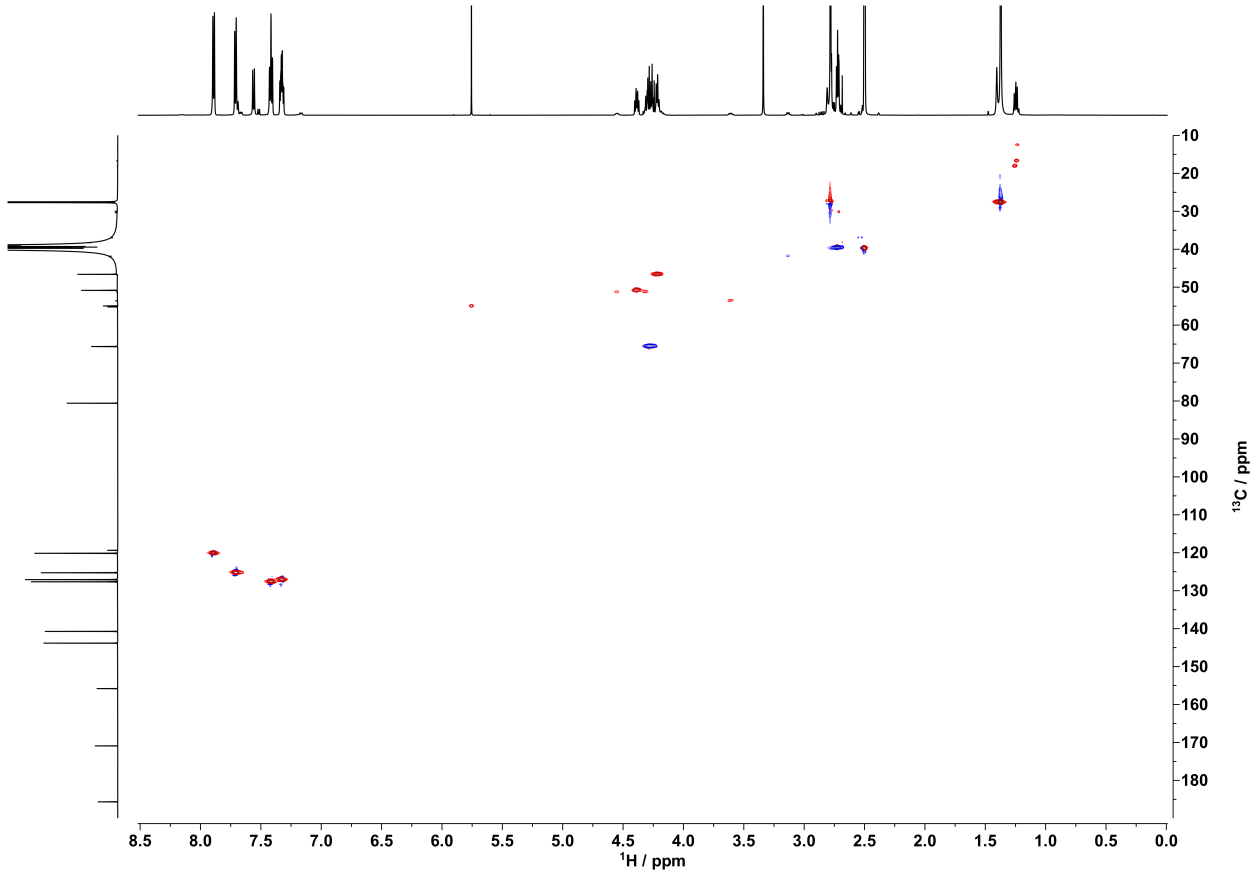
^{13}C-NMR (151 MHz, DMSO-d6)



^{1}H-^{1}H COSY (600 MHz, 600 MHz, DMSO-d$_6$)

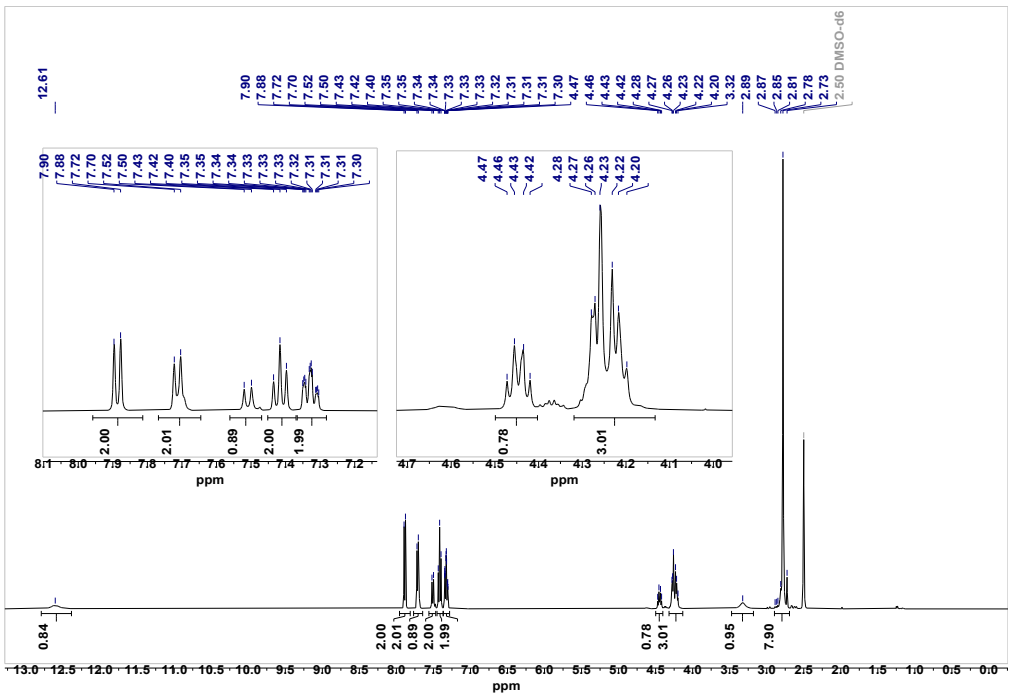


^{1}H-^{13}C HSQC (600 MHz, 151 MHz, DMSO-d$_6$)

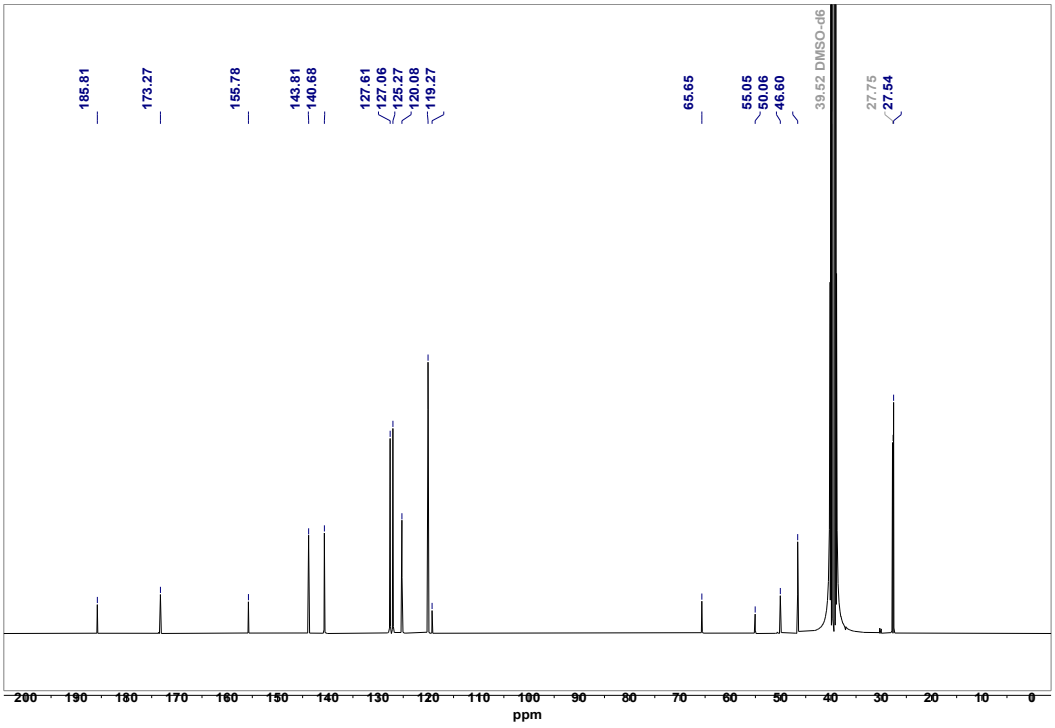


6.1.2 Fmoc-Asp(CSY)-OH

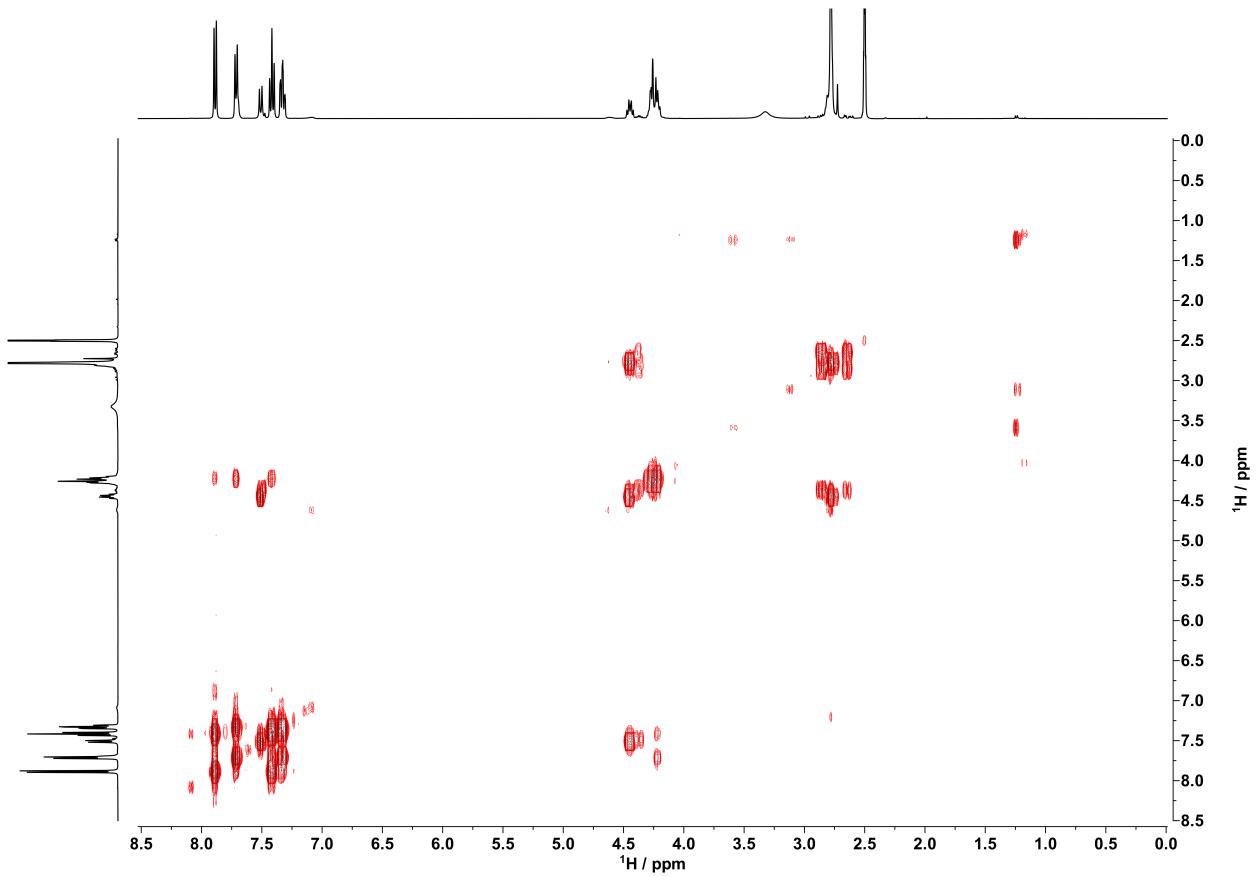
^{1}H-NMR (400 MHz, DMSO-d_6)



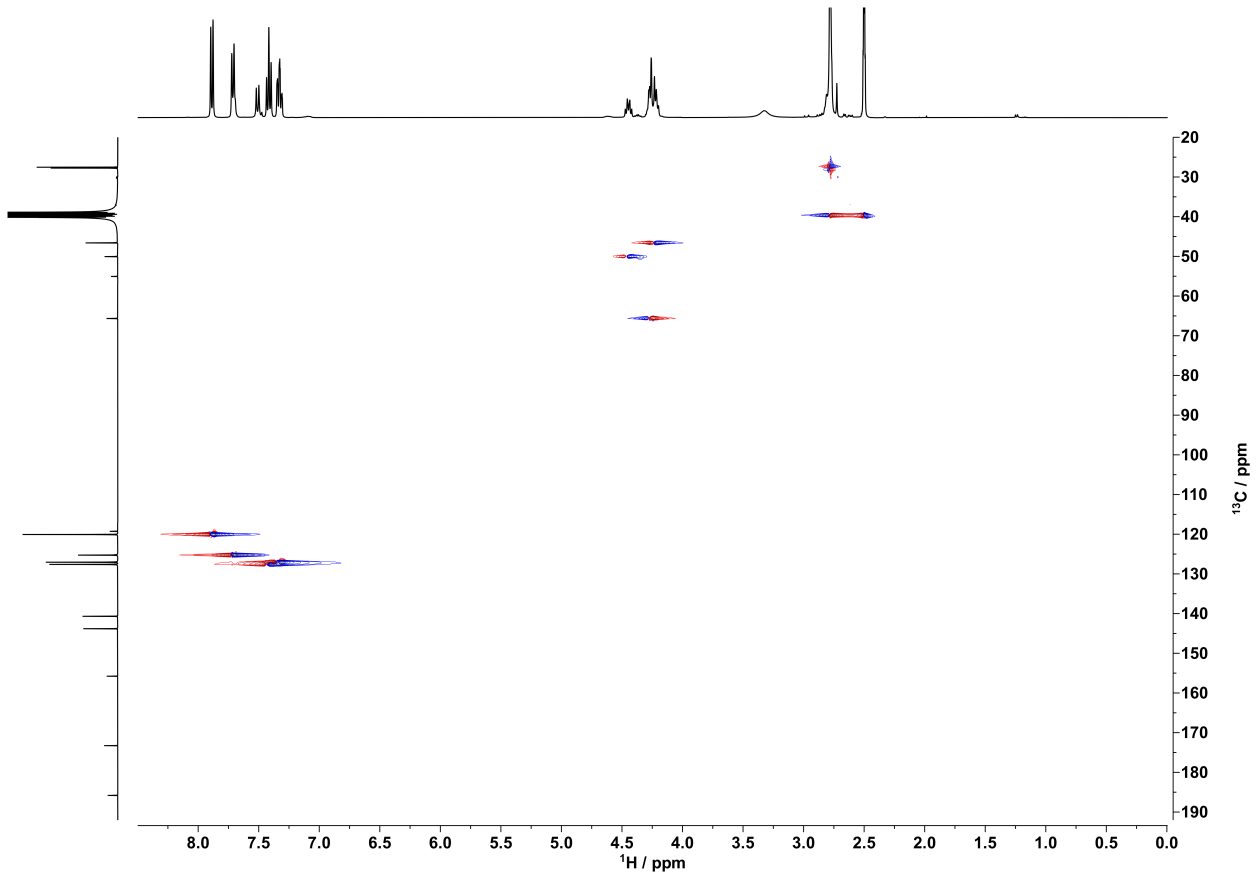
^{13}C-NMR (101 MHz, DMSO-d_6)



$^{1}H,^{1}H$ COSY (400 MHz, 400 MHz, DMSO-d_6)

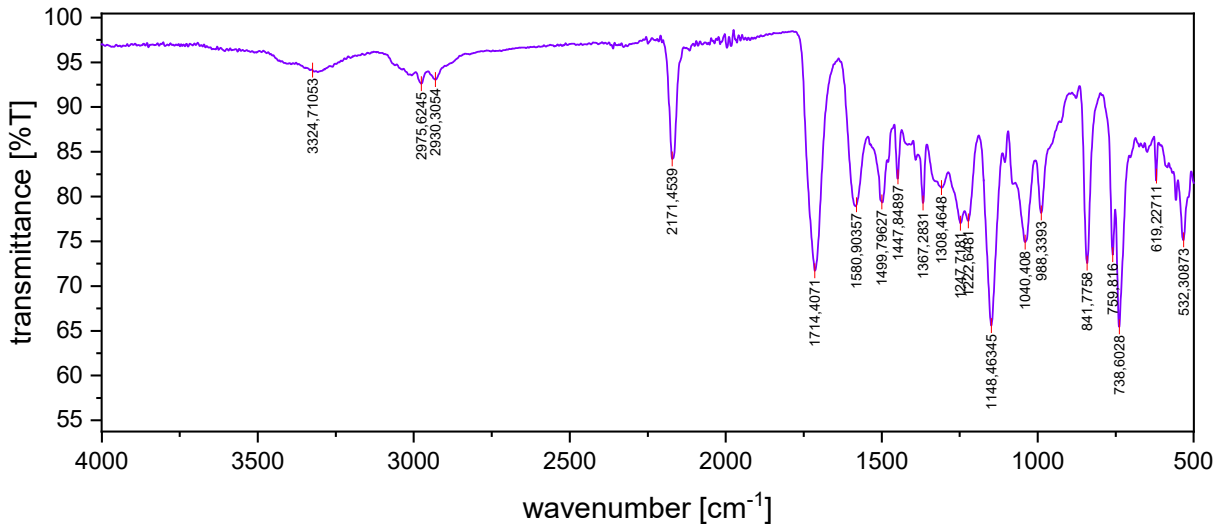


$^{1}H,^{13}C$ HSQC (400 MHz, 101 MHz, DMSO-d_6)

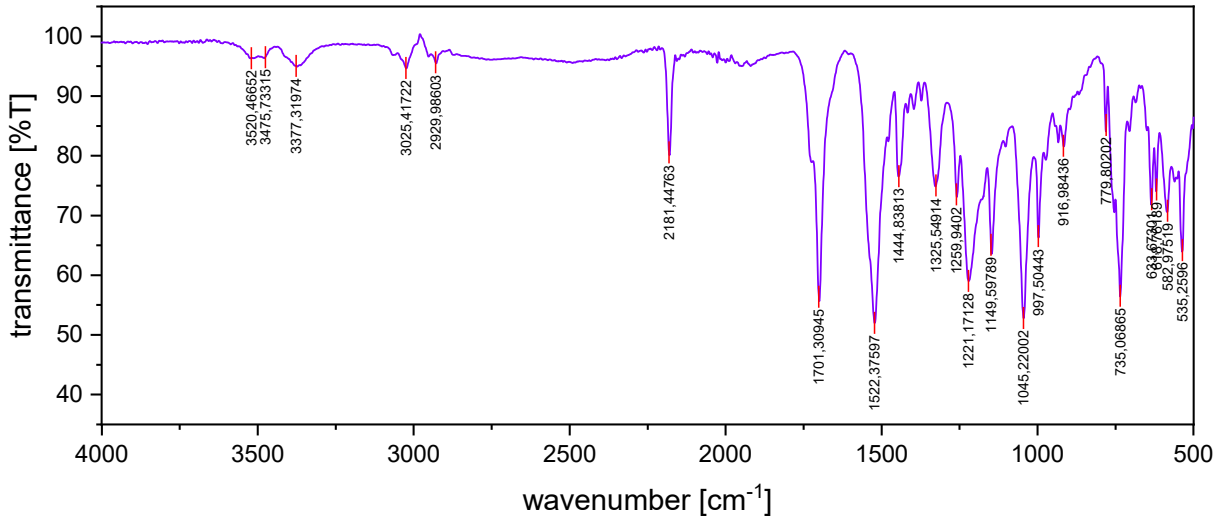


6.2 FT-IR

6.2.1 Fmoc-Asp(CSY)-O*t*Bu



6.2.2 Fmoc-Asp(CSY)-OH



6.3 ESI-MS

6.3.1 Fmoc-Asp(CSY)-O*t*Bu

Mass Spectrum Formula Report

Analysis Info

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Acquisition Date 4/23/2021 8:37:47 AM

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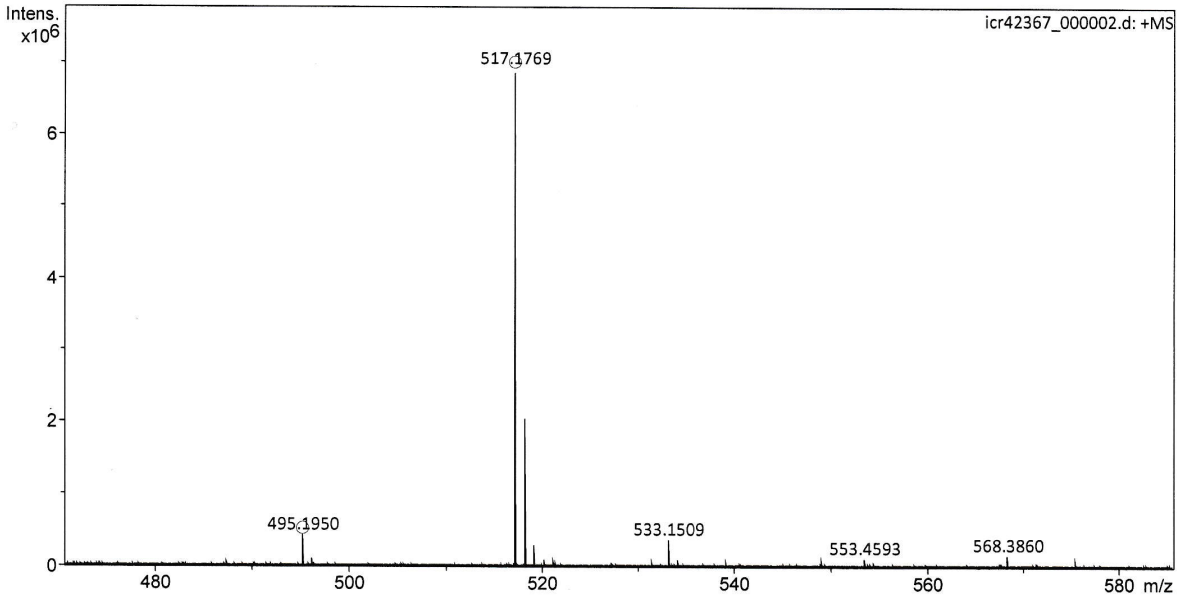

Meas. m/z	Ion Formula	m/z	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
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	C20H33NO13	495.1946	-0.7	23.7	5.0	odd	ok
	C19H27N8O8	495.1946	-0.7	26.6	10.5	even	ok
	C20H23N12O4	495.1960	2.0	27.0	15.5	even	ok
	C26H25N9S	495.1948	-0.3	29.1	19.0	odd	ok
	C27H31N2O5S	495.1948	-0.3	29.9	13.5	even	ok
	C18H21N15O3	495.1946	-0.7	38.9	16.0	odd	ok
	C33H25N3O2	495.1941	-1.7	52.1	23.0	odd	ok
	C35H27O3	495.1955	1.0	71.1	22.5	even	ok
517.1769	C27H30N2NaO5S	517.1768	-0.3	7.1	13.5	even	ok
	C26H31NO8S	517.1765	-0.9	8.9	12.0	odd	ok
	C27H27N5O4S	517.1778	1.7	10.5	17.0	odd	ok
	C26H24N9NaS	517.1768	-0.3	16.4	19.0	odd	ok
	C25H25N8O3S	517.1765	-0.9	16.6	17.5	even	ok
	C21H28N5NaO9	517.1779	1.9	28.3	10.0	odd	ok
	C33H24N3NaO2	517.1761	-1.7	30.4	23.0	odd	ok
	C33H21N6O	517.1771	0.4	31.4	26.5	even	ok
	C20H29N4O12	517.1776	1.4	32.4	8.5	even	ok
	C20H32NNaO13	517.1766	-0.7	35.0	5.0	odd	ok
	C20H22N12NaO4	517.1779	1.9	39.5	15.5	even	ok
	C35H26NaO3	517.1774	0.9	46.4	22.5	even	ok

Bruker Compass DataAnalysis 4.3 printed: 4/23/2021 8:41:34 AM Page 1 of 1

6.3.2 Fmoc-Asp(CSY)-OH

Mass Spectrum Formula Report

Analysis Info Acquisition Date 4/28/2021 8:15:38 AM

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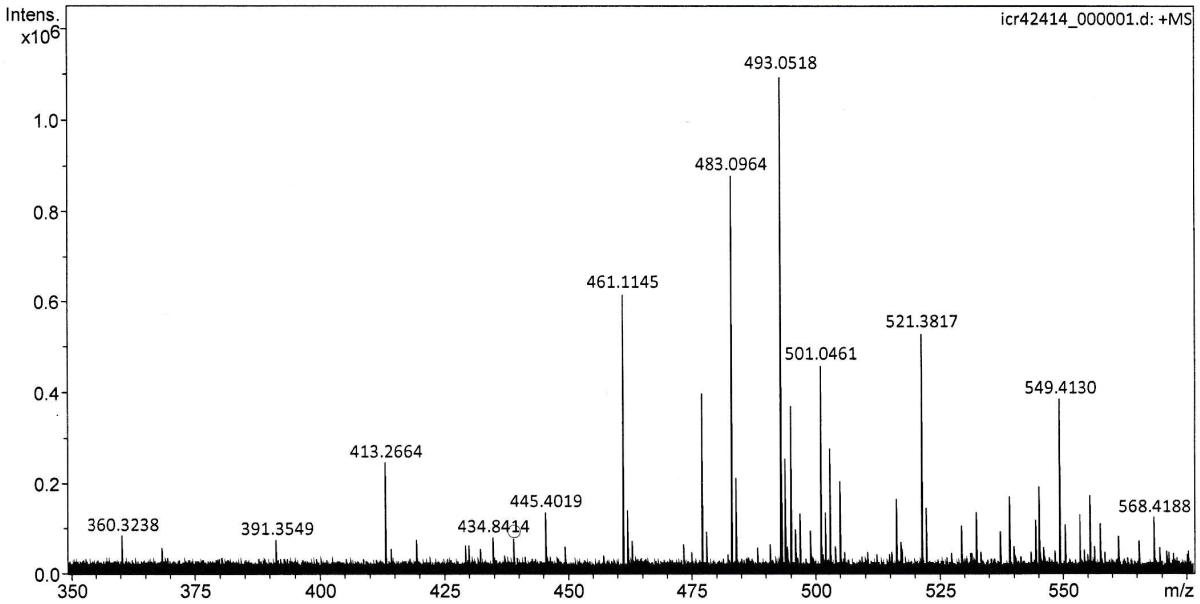

Meas. m/z	Ion Formula	m/z	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
439.1325	C23H23N2O5S	439.1322	-0.6	n.a.	13.5	even	ok
	C31H19O3	439.1329	0.9	n.a.	22.5	even	ok

7 Abbreviation

CSY	Cyanosulfurylide
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DIC	*N*,*N'*-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
Gdn HCl	Guanidine hydrochloride
HFIP	1,1,1,3,3,3-Hexafluoroisopropanol
mCPBA	*meta*-Chloroperoxybenzoic acid
NCS	*N*-Chlorosuccinimide
Oxone	Potassium peroxymonosulfate
TFE	2,2,2-Trifluoroethanol
TIPS	Triisopropyl silane

8 Literature

1. K. Neumann, J. Farnung, S. Baldauf and J. W. Bode, *Nat. Commun.*, 2020, **11**, 982.
2. C. N. Pace, F. Vajdos, L. Fee, G. Grimsley and T. Gray, *Protein Sci.*, 1995, **4**, 2411-2423.
3. N. J. Greenfield, *Nat. Protoc.*, 2006, **1**, 2876-2890.
4. N. J. Greenfield, *Nat. Protoc.*, 2006, **1**, 2527-2535.