Electronic Supplementary Material (ESI) for Chemical Communications

The ββα fold of zinc finger proteins as a "natural" protecting group. Chemoselective synthesis of a DNA-binding zinc finger derivative †

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Abbreviations

TFA: trifluoroacetic acid

EDT: ethanedithiol

TIS: triisopropylsilane

HATU: 2-(1H-7-aza- benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HBTU: 2-[(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

DEDTC: diethyldithiocarbamate

General peptide synthesis procedures

All peptide synthesis reagents and amino acid derivatives were purchased from GL Biochem (Shanghai) and Novabiochem; amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Glu(O*t*-Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH and Fmoc-Asp(O*t*-Bu)-OH except for the orthogonally protected Fmoc-Lys(Alloc)-OH, which was purchased from *Bachem*. All other chemicals were purchased from *Aldrich* or *Fluka*. All solvents were dry and synthesis grade, unless specifically noted.

Peptides were synthesized using an automatic peptide synthesizer from *Protein Tecnologies PS3 PeptideSynthesizer*. Peptide synthesis was performed using standard Fmoc solid-phase method on a PAL–PEG–PS resin (0.19 mmol/g) using HBTU as coupling agent, DIEA as base and DMF as solvent. In peptide **1**, the Pro of the position 162 was changed for D-proline to avoid that alkylation of the cysteine would disrupt the correct folding of the peptide. The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 20 min. The cleavage/deprotection step was performed by treatment of the resin-bound peptide for 1.5–2h with the following cleavage cocktail: 940 µL TFA, 25 µL EDT, 25 µL H_2O and 10 µL TIS (1 mL of cocktail / 40 mg resin).

High-Performance Liquid Chromatography (HPLC) was performed using an *Agilent 1100* series Liquid Chromatograph Mass Spectrometer system. Analytical HPLC was run using a *Eclipse XDB-C18 analytical column (4.6 x 150 mm, 5 µm)*, 1 ml/min, gradient 5 to 75% B over 30 min. Purification of the peptides was performed on a semipreparative *Phenomenex Luna–C18* (250 × 10 mm) reverse-phase column. Purification of the monoalkylated products was performed on an analytical *Phenomenex Luna-C18* reverse-phase column. Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an *Agilent 1100* Series LC/MSD VL G1956A model in positive scan mode.

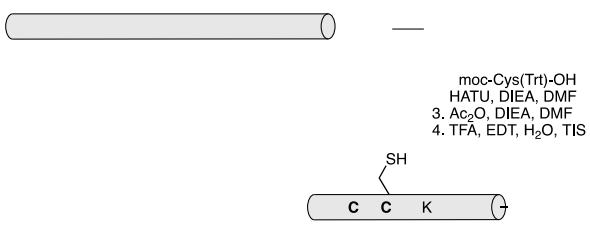


Fig. S1 Synthesis of model peptides **1** and **2**. Arrows point to the thiols that are selectively modified in the presence of Zn(II) ions.

Side chain elaboration to make peptide 2

Side chain deprotection: The resin containing the alloc peptide (75 mg, aprox. 0.015 mmol) was suspended in DMF and shaken for 1 h to ensure a good swelling. Then $Pd(OAc)_2$ (1 mg, 0.3 equiv), 4-methylmorpholine (16 µL, 10 equiv), PPh_3 (6 mg, 1.5 equiv), $PhSiH_3$ (18 µL, 10 equiv) and 1 mL CH_2Cl_2 were added, and the mixture shaken overnight. The resin was then filtered and washed with DMF, DEDTC and DMF again.

Coupling of the cysteine: A solution of Fmoc-Cys(Trt)-OH (35 mg, 6×10^{-2} mmol, 4 equiv), HATU (23 mg, 6×10^{-2} mmol, 4 equiv) and DIEA (460 µL, 0.195 M in DMF, 9×10^{-2} mmol, 6 equiv) in 205 µL DMF were added. The reaction mixture was shaken for 1 h. The resin was washed with DMF (3 ×, 5 min) and Et_2O (2 ×, 5 min).

Cleavage/deprotection of the resin-bound peptides under standard conditions afforded major products that were purified by RP−HPLC, 4 ml/min, gradient 10 to 50% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA) and identified as the desired peptides.

Peptide **1** was isolated with an approx. yield of 20%, while **2** was obtained with an approx. yield of 17%.

Analytical Data of the purified peptides:

Peptide 1: **EM–ESI$^+$ (m/z):** Calcd. for $C_{147}H_{241}N_{53}O_{39}S_3$: 3471.01. Found: 1157.9 $[M+3H]^{3+}$; 868,5 $[M+4H]^{4+}$; 695.2 $[M+5H]^{5+}$; 579.3 $[M+6H]^{6+}$.

Peptide 2: **EM–ESI$^+$ (m/z):** Calcd. for $C_{166}H_{268}N_{54}O_{47}S_3$: 3868.43. Found: 1290.4 $[M+3H]^{3+}$; 967.7 $[M+4H]^{4+}$; 774.3 $[M+5H]^{5+}$.

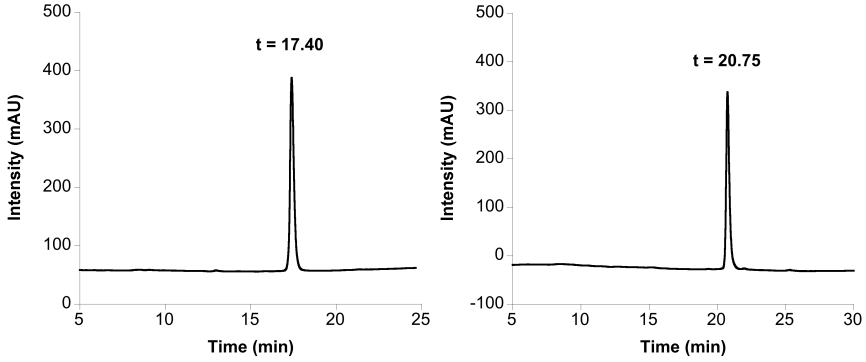
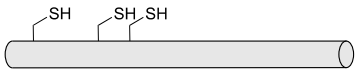


Fig. S2 HPLC chromatograms of the purified peptides. Left: peptide **1**. Right: peptide **2**.

Procedure for monoalkylation of the peptide 1:



The peptide (0.1 mg, $3x10^{-5}$ mmol) was dissolved in desoxygenated phosphate buffer (130 µL, 10 mM, pH = 7.5). A $ZnSO_4$ solution ($4.5x10^{-5}$ mmol, 9 µL of a 5 mM solution in Milli-Q water, 1.5 equiv) was added, and the mixture was stirred at room temperature for 10 minutes under argon atmosphere. Benzyl bromoacetate ($12x10^{-5}$ mmol, 16 µL of a 7.6 mM solution in desoxygenated CH_3CN, 4 equiv) was added, and the solution was stirred again at room temperature for 10 minutes under argon atmosphere.

The monoalkylated product was purified by RP−HPLC, 1 mL/min, gradient 10 to 50% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA, retention time = 18.42 min), and identified as product **3** by mass spectrometry.

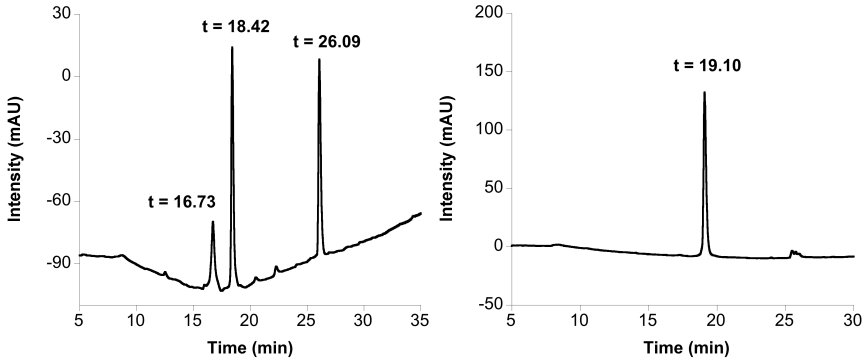


Fig. S3 HPLC chromatograms of the reaction with Zn(II) (left) and the purified monoalkylated product (right).

Left:

t = 16.73 starting peptide **1**.

t = 18.42 monoalkylated product. **EM–ESI$^+$ (m/z):** Calcd. for $C_{156}H_{249}N_{53}O_{41}S_3$: 3619.17. Found: 1207.3 $[M+3H]^{3+}$; 905.8 $[M+4H]^{4+}$.

t = 26.09 alkylating agent.

Right:

t = 19.10 monoalkylated product. **EM–ESI$^+$ (m/z):** Calcd. for $C_{156}H_{249}N_{53}O_{41}S_3$: 3619.17. Found: 1207.3 $[M+3H]^{3+}$; 905.8 $[M+4H]^{4+}$.

Alkylation of the peptide 1 without zinc: the procedure was the same as described above but without adding $ZnSO_4$.

t = 19.45 monoalkylated product (different than that obtained from the reaction with Zn(II)).

t = 22.36 trialkylated product: **EM–ESI$^+$ (m/z):** Calcd. for $C_{174}H_{265}N_{53}O_{45}S_3$: 3912.9. Found: 1305.6 $[M+3H]^{3+}$; 979.1 $[M+4H]^{4+}$; 783.5 $[M+5H]^{5+}$.

t = 26.03 alkylating agent.

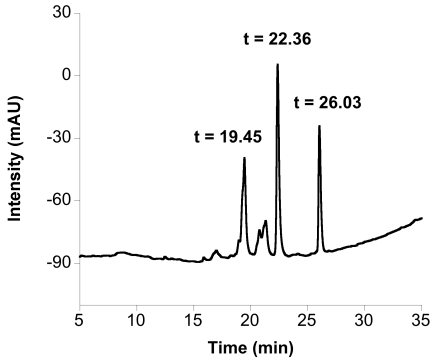


Fig. S4 HPLC chromatogram of the alkylation of the peptide **1** without Zn(II).

Co-injection of monoalkylated products

In order to confirm that monoalkylated products obtained in the presence and absence of zinc were monoalkylated derivatives at different positions, we carried out a co-injection of the crudes obtained in both reactions, in the presence and absence of zinc, and assigned the peaks according to its mass (LC-ESI/MS).

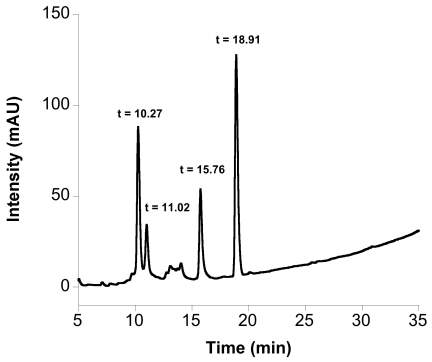


Fig. S5 HPLC chromatogram of the co-injection of the reactions with and without Zn(II), gradient 25 to 75% B over 30 min.

t = 10.27 monoalkylated product.

t = 11.02 monoalkylated product.

t = 15.76 trialkylated product.

t = 18.91 alkylating agent.

Co-injection of the reaction crude coming from the alkylation of peptide 1, with excess of alkylating agent

To further confirm that the peak at 26 min corresponds to the alkylating agent, we carried out a coinjection of alkylating agent with the crude of the reaction of the alkylation of peptide **1**. The results show that the peak at 26 min is the alkylating agent because this peak increases its size.

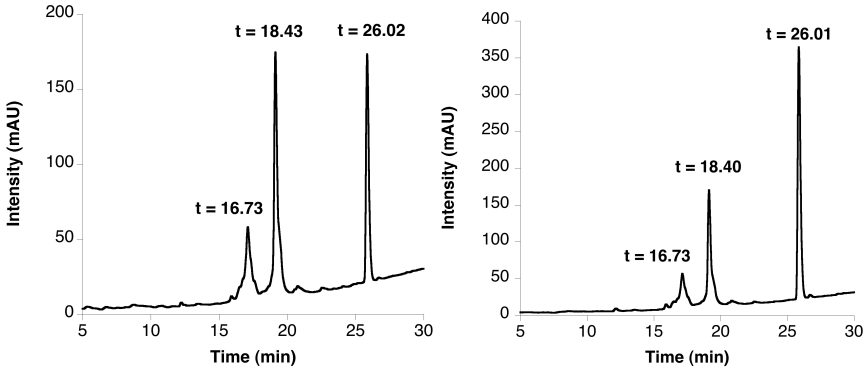
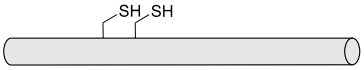


Fig. S6 HPLC chromatograms of alkylation of the peptide **1** (left) and the co-injection of the reaction crude with 2 equiv of alkylating agent added.

Procedure for monoalkylation of the peptide 2



The peptide (0.06 mg, $1.6x10^{-5}$ mmol) was dissolved in desoxygenated phosphate buffer (65 µL, 10 mM, pH = 7.5). A $ZnSO_4$ solution ($2.5x10^{-5}$ mmol, 5 µL of a 5 mM solution in Milli-Q water, 1.5 equiv) was added, and the mixture was stirred at 5 ºC (or room temperature) for 10 minutes under argon atmosphere. Benzyl bromoacetate ($6x10^{-5}$ mmol, 8 µL of a 7.6 mM solution in desoxygenated CH_3CN, 4 equiv) was added, and the solution was stirred again at 5 ºC (or room temperature) for 5 minutes under argon atmosphere.

The monoalkylated product was purified by RP−HPLC, 1 mL/min, gradient 10 to 50% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA), and identified as product **5** by mass spectrometry. Analytical Data of the purified product: **EM–ESI$^+$ (m/z):** Calcd. for $C_{175}H_{276}N_{54}O_{49}S_3$: 4016.59. Found: 1338.9 $[M+3H]^{3+}$; 1004.8 $[M+4H]^{4+}$.

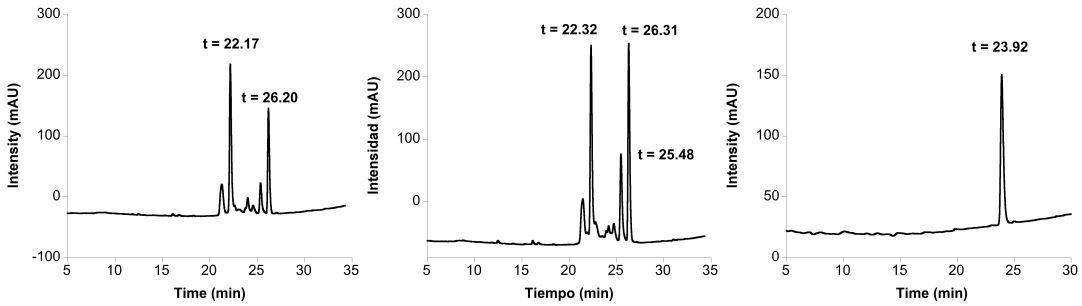


Fig. S7 HPLC chromatogram of the reaction with Zn(II) at 5 ºC (left), at room temperature (center) and the purified monoalkylated product (right). Left: t = 22.17 desired monoalkylated product, t=26.20 benzyl bromoacetate. Center: t=22.32 desired monoalkylated product, t=25.48 trialkylated product, t=26.31 benzyl bromoacetate.

Alkylation of the peptide 2 without zinc: the procedure was the same as described above but without adding $ZnSO_4$.

t = 25.09 trialkylated product: **EM–ESI$^+$ (m/z):** Calcd. for $C_{193}H_{292}N_{54}O_{53}S_3$: 4312.91. Found: 1438.4 $[M+3H]^{3+}$; 1079.4 $[M+4H]^{4+}$.

t = 26.14 alkylating agent

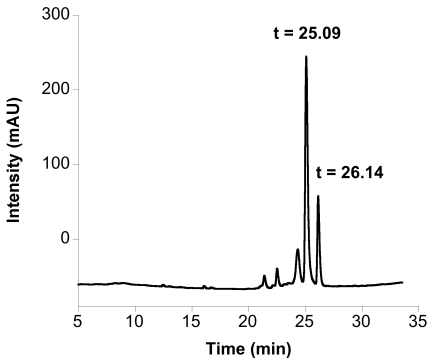


Fig. S8 HPLC chromatogram of alkylation of the peptide **2** without Zn(II).

Analysis of the kinetics of the monoalkylation of peptide 2

A more detailed HPLC analysis of the reaction of peptide **2** with benzyl bromoacetate in the presence of Zn (II) is shown in the next figure. The reaction was quenched with thiophenol before LC-MS analysis.

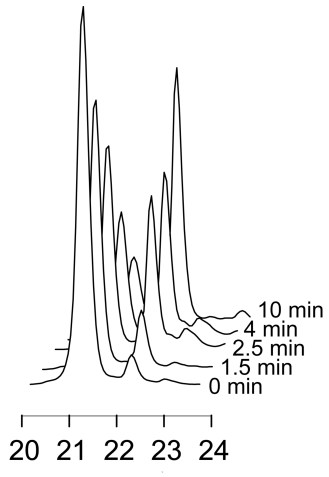
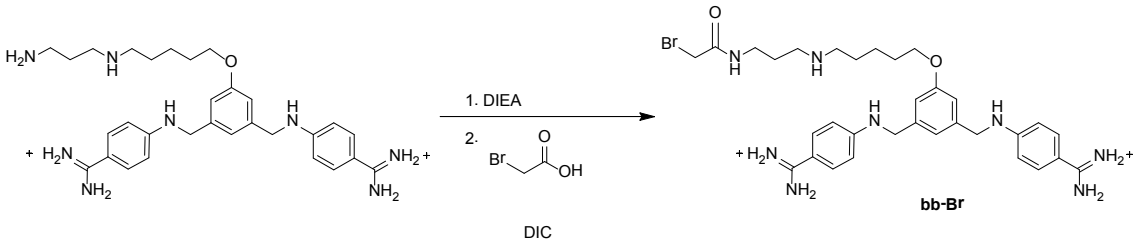


Fig. S9 Monoalkylation of peptide **2** at different reaction times. Reaction carried out at 15ºC. *Conversions*: initial: 4%, 1.5 min: 13%, 2,5 min: 37%, 4 min: 53%, 10 min: 76%. *Conversions* refer to the proportion of product with respect to the total amount of product and starting peptide.

Synthesis of bb-Br

Synthesis of the **bb1** fragment was carried out as previously described.[S1]



bb1 (1 mg, 1x10^{-3} mmol) was mixed withDIEA (5 µL, 0.195 M in DMF, 1×10^{-3} mmol, 1 equiv) and coupled with bromoacetic acid activated with diisopropylcarbodiimide (DIC) in DMF (3 µL of a 0.3 M solution in DCM, 1 equiv of the preactivated compound). The mixture was stirred at room temperature for 12 hours, after which HPLC analysis showed a conversion of over 40%. The product was purified by RP-HPLC, 4 mL/min, gradient 5 to 60% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA), and identified as the desired product by mass spectrometry and NMR. **^{1}H NMR** (500 MHz, D_2O): δ = 1.76 (m, 4H), 1.92 (m, 2H), 3.05 (m, 4H), 3.33 (t, *J*=6.6Hz, 2H), 3.89 (s, 2H), 4.06 (t, *J*=6.3Hz, 2H), 4.38 (s, 4H), 6.56 (m, 4H), 6.88 (d, *J*=10.4 Hz, 3H), 7.39 (m, 4H). **^{13}C NMR** (500 MHz, D_2O): δ = 22.28 (CH_2), 25.12 (CH_2), 25.31 (CH_2), 27.72 (CH_2), 27.86 (CH_2), 36.48 (CH_2), 44.68 (CH_2), 45.43 (CH_2), 47.42(CH_2), 111.93 (CH), 112.47 (CH), 113.29 (C), 117.13 (CH), 129.21 (CH), 141.20 (C), 153.10 (C), 158.73 (C), 165.00 (C), 170.46 (C). **EM–ESI$^+$:** Calcd. for $C_{32}H_{43}BrN_8O_2$: 651.27. Found: 651.27.

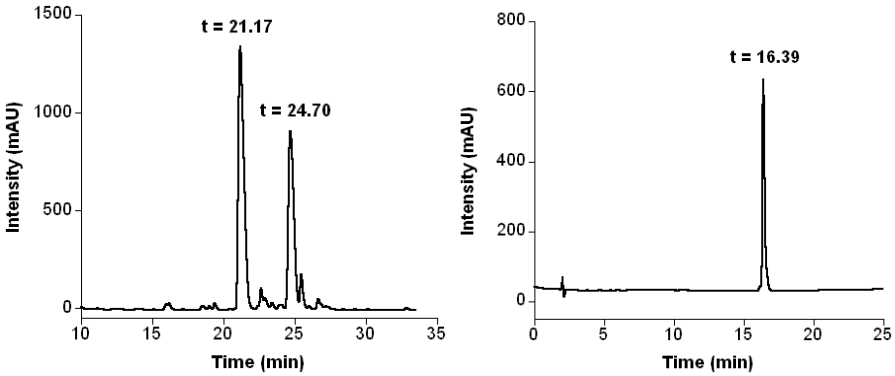


Fig. S10 Left: HPLC chromatogram of the reaction, gradient 0 to 40% B over 40 min (t = 21.17 **bb1**, t=24.70 **bb-Br**). Right: HPLC chromatogram of the purified product, gradient 5 to 75% B over 30 min.

[S1] M. I. Sánchez, O. Vázquez, M. E. Vázquez and J. L. Mascareñas, *Chem. Eur. J.*, 2013, **19**, 9923.

Synthesis of conjugate 4

Peptide **2** (0.3 mg, $7.2x10^{-5}$ mmol) was dissolved in desoxygenated phosphate buffer (300 µL, 10 mM, pH = 7.5). A $ZnSO_4$ solution ($1.1x10^{-4}$ mmol, 2.2 µL of a 50 mM solution in Milli-Q water, 1.5 equiv) was added, and the mixture was stirred at room temperature for 10 minutes under argon atmosphere. **bb-Br** ($7.2x10^{-5}$ mmol, 36 µL of a 2 mM solution in desoxygenated Milli-Q H_2O, 1 equiv) was added, and the solution was stirred again at room temperature for 60 minutes under argon atmosphere.

The conjugate was purified by RP−HPLC, 1 mL/min, gradient 5 to 75% B over 30 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA), and identified as product **4** by mass spectrometry. Analytical Data of the purified product: **EM–ESI$^+$ (m/z):** Calcd. for $C_{198}H_{309}N_{61}O_{50}S_3$: 4437.15. Found: 740.2 $[M+6H]^{6+}$; 888.8 $[M+5H]^{5+}$, 1110.8 $[M+4H]^{4+}$; 1479.6 $[M+3H]^{3+}$.

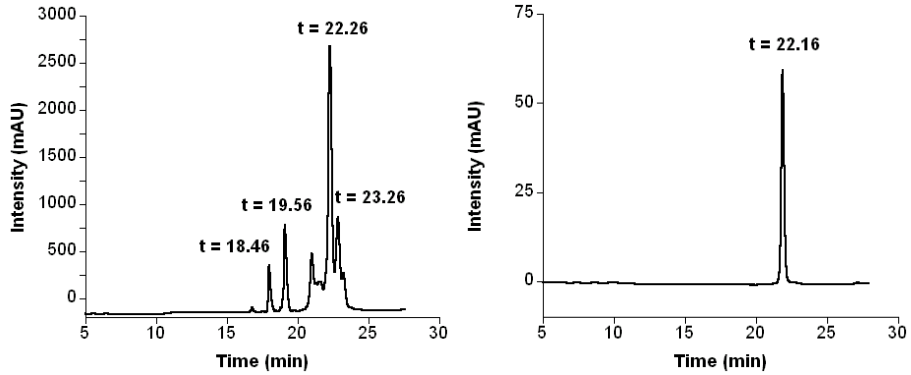


Fig. S11 Left: HPLC chromatogram of the reaction: t = 18.46 hydrolyzed **bb-Br**, t=19.56 **bb-Br**, t=22.26 conjugate **4**, t=23.26 peptide **2**. Right: HPLC chromatogram of the purified product.

Oligonucleotide sequences: Double stranded oligonucleotides used for EMSA experiments with conjugate **4** were supplied by *Thermo Fischer* and their sequences were (only one strand is shown):

AT•GAG	5'−GACGGAATTTGAGAGCGTCG−3'
AT•GAG^{m}	5'−GACAAATTTCATAGTACGCT−3'

EMSA experiments: Binding reactions were performed over 45 min. in 18 mM Tris-HCl (pH 7..5), 90 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM TCEP, 9% glycerol, 0.11 mg/mL BSA, 2.2% NP-40 and 0.02 mM of $ZnCl_2$. In the experiments we used 50 nM of the ds−DNAs. After incubation for 45 min products were resolved by PAGE using a 10% non-denaturing polyacrylamide gel and 0.5x TBE buffer for 60 min at 4 ºC, and analyzed by staining with SyBrGold (Molecular Probes: 5 µL in 50 mL of 1x TBE) for 10 min. and visualized by fluorescence.

5x TBE buffer (0.445M Tris, 0.445 M Boric acid)

EMSA with the DNA mutated at the peptide binding site **AT•GAG^{m}**

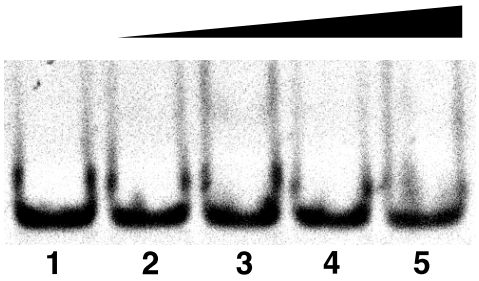


Fig. S12 EMSA results for conjugate **4**. Lanes 1-5: [**4**] = 0, 600, 800, 900, 1000 with 50 nM of **AT•GAGA^{m}** dsDNA.

MS/MS study of monoalkylated product 3

Trypsin Digestion: 20 □g of peptide were suspended in 20 □L of a 8 M urea solution in water. 20 □L of a 150 mM DTT (Dithiothreitol) solution in 50 mM NH_4HCO_3 were added and the mixture was incubated for 30 min at r.t. To the mixture, 20 □L of a 500 mM IAA (Iodoacetamide) solution in 50 mM NH_4HCO_3 were added and the mixture was incubated again for 60 min at r.t. in the dark. To this solution, 2 □L of a 0.2 □g□□□L trypsin solution were added, and the mixture incubated at 37ºC. Aliquots of the digestion were quenched at different times by addition of 1 □L of 20% (v/v) formic acid.

LC-MS/MS analysis: 3 □L of the above partially digested monoalkylated peptide were injected onto Ultimate 3000 nanoHPLC equipped with a C-18 reverse-phase micro-column (300 □m ID x 5 mm PepMap™) (Thermo) to remove salts. Digested solutions were analyzed in a continuous gradient consisting of 30-40 % B in 15 min, 40-80% B in 0.1 min (A= H_2O 0.1% formic acid, B= ACN 0.1% formic acid) on reversed phase nanocolumn (75 □m x 15 cm C18, 3□m 100 Å, Acclaim® PepMap100) (Thermo). A flow rate of ca. 300 nL/min was used to elute peptides from the column to a PicoTip™ emiter nano spray needle (New Objetive, Woburn, MA) for real time ionization and peptide fragmentation on a 7T APEX-Qe hybrid Qh FTICR mass spectrometer (Bruker Daltonics, Bremen, Germany). Data-dependent tandem mass spectra (MS/MS) were acquired by precursor ion selection in the quadrupole followed by collisional activation and storage in the adjacent hexapole trap. The most abundant multiple charged ion was automatically selected for MS/MS, excluding background signals and previously selected ions. Data acquisition was performed using ApexControl3.0 software and data analysis was performed using DataAnalysis 4.0 and Biotools 3.2 software from Bruker.

We only collected the initial eluting peptides, as they contain the N-terminal fragments required for the identification.

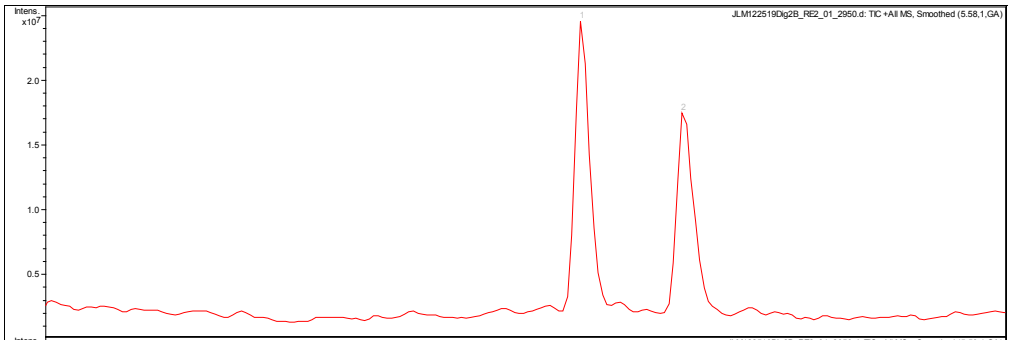


Fig. S13 Initial peaks collected after digestion for 2 h.

Peak 1 **m/z:** Calcd. for $C_{72}H_{108}N_{20}O_{22}S_3$: 1700.7. Found: 851.4 $[M+2H]^{2+}$.

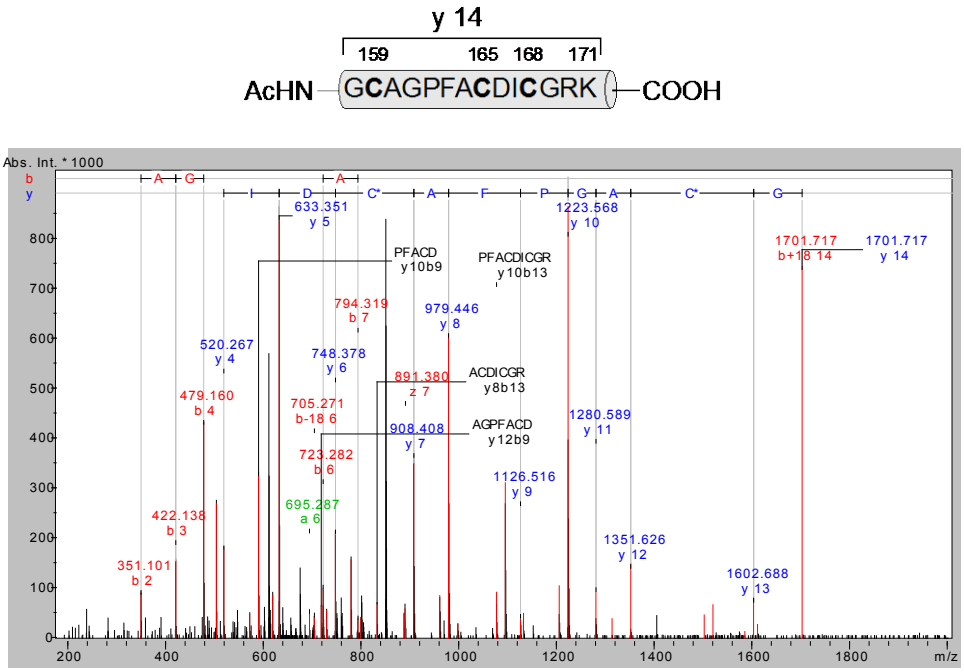
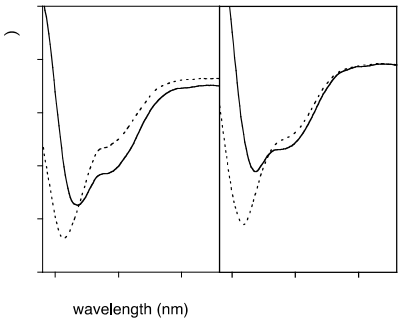


Fig. S14 Deconvoluted MS/MS spectra of the first chromatographic peak showing the masses of the different fragments of the y ion (blue serie). C# indicates Carboxybenzylmethyl cysteine and C* indicates Carbamidomethyl cysteine.

Peak 2 **m/z:** Calcd. for $C_{66}H_{96}N_{18}O_{21}S_3$: 1572,6. Found: 787.3 $[M+2H]^{2+}$.

CD measurements were made in a 2 mm cell at 5 ºC. Samples contained 10 mM phosphate buffer pH 7.5 and 10 µM peptide. The mixtures were incubated for 5 min before registering. The spectra are the average of 5 scans and were processed using the “smooth” macro implemented in the program *Kaleidagraph* (v 3.5 by Synergy Software).



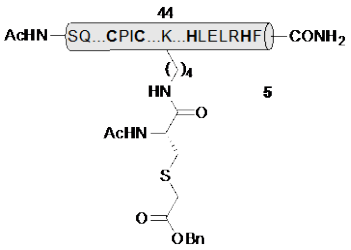
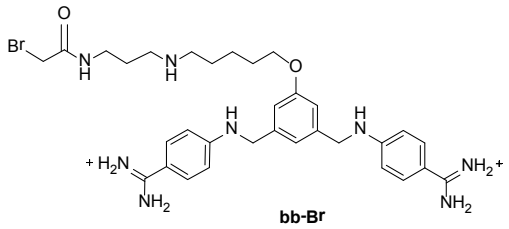
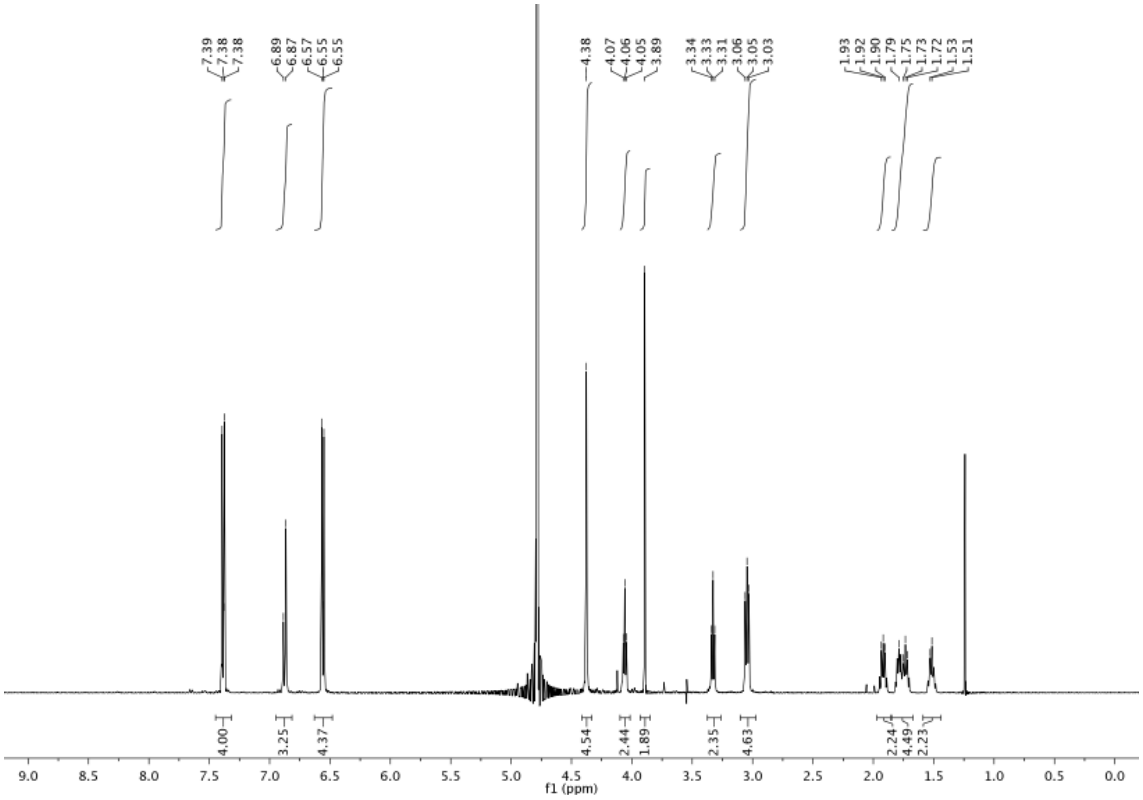


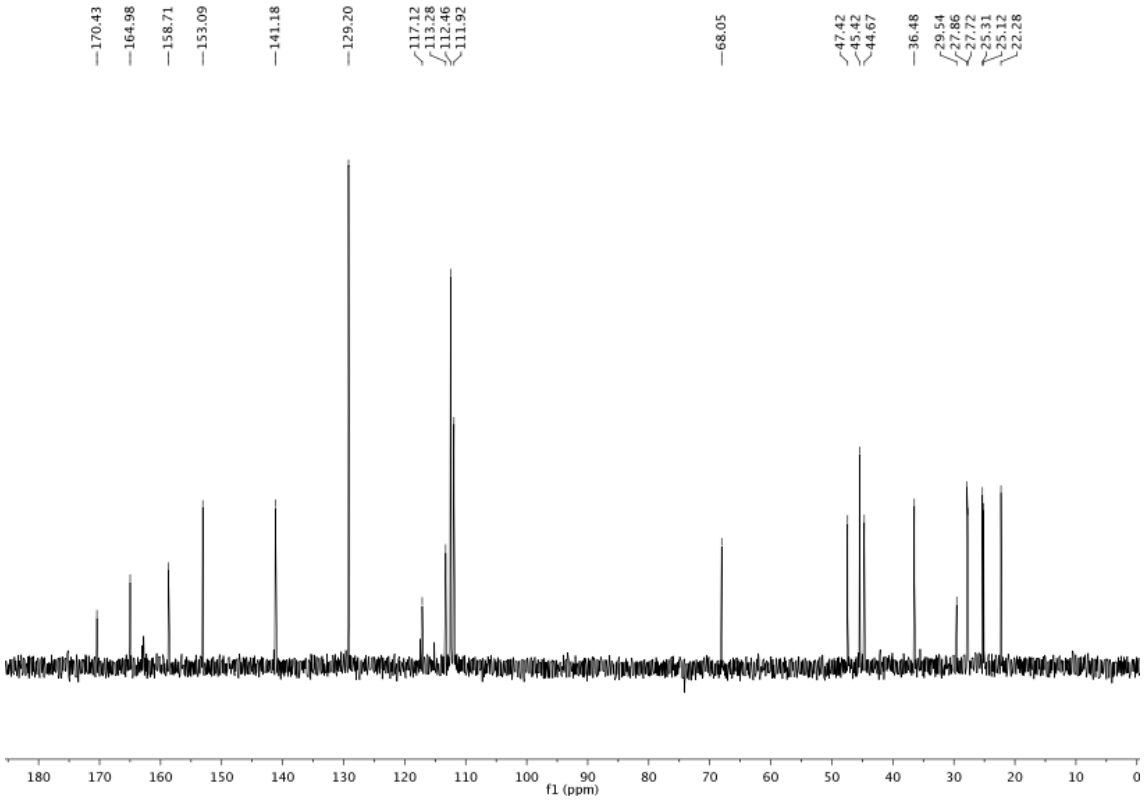
Fig. S15 Left: CD spectra of peptide **2** in absence of $ZnSO_4$ (dashed line) and in the presence of 5 equiv of $ZnSO_4$ (solid line); Center: CD spectra of the monoalkylated product obtained by reaction of peptide **2** with benzylbromoacetate in presence of Zn(II). Right: structure of the monoalkylated peptide **5**.

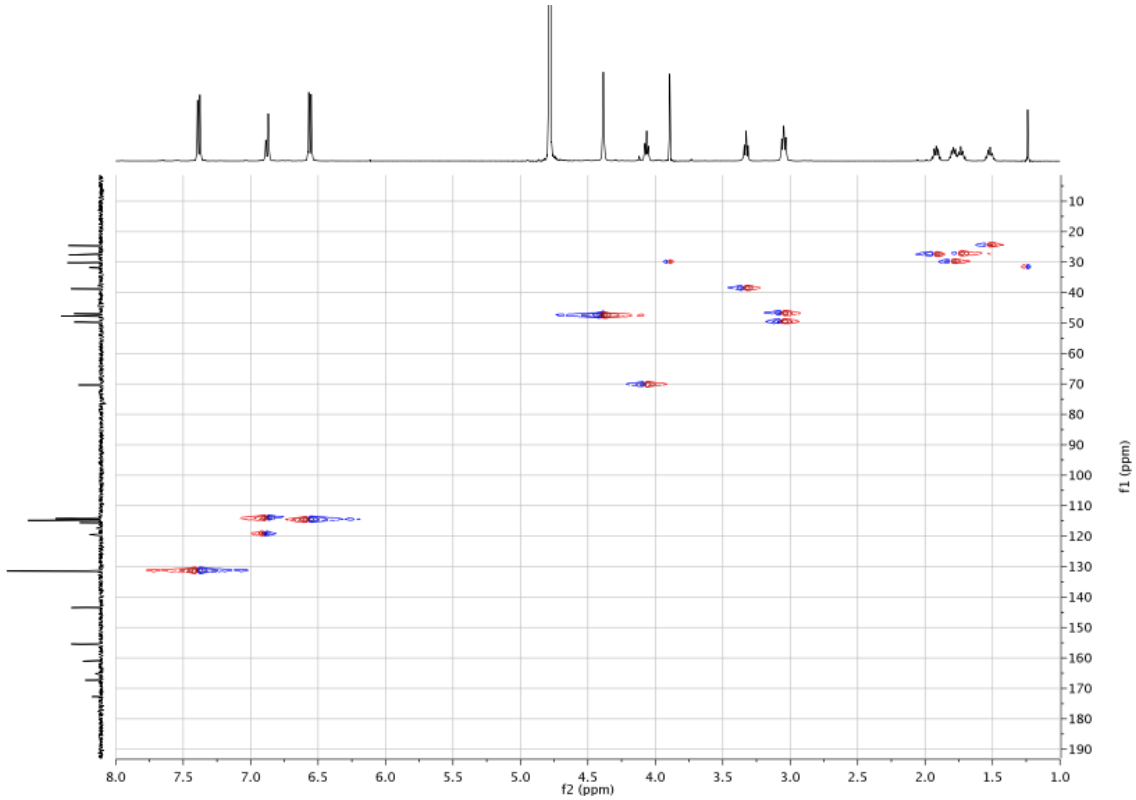
NMR and ESI spectra

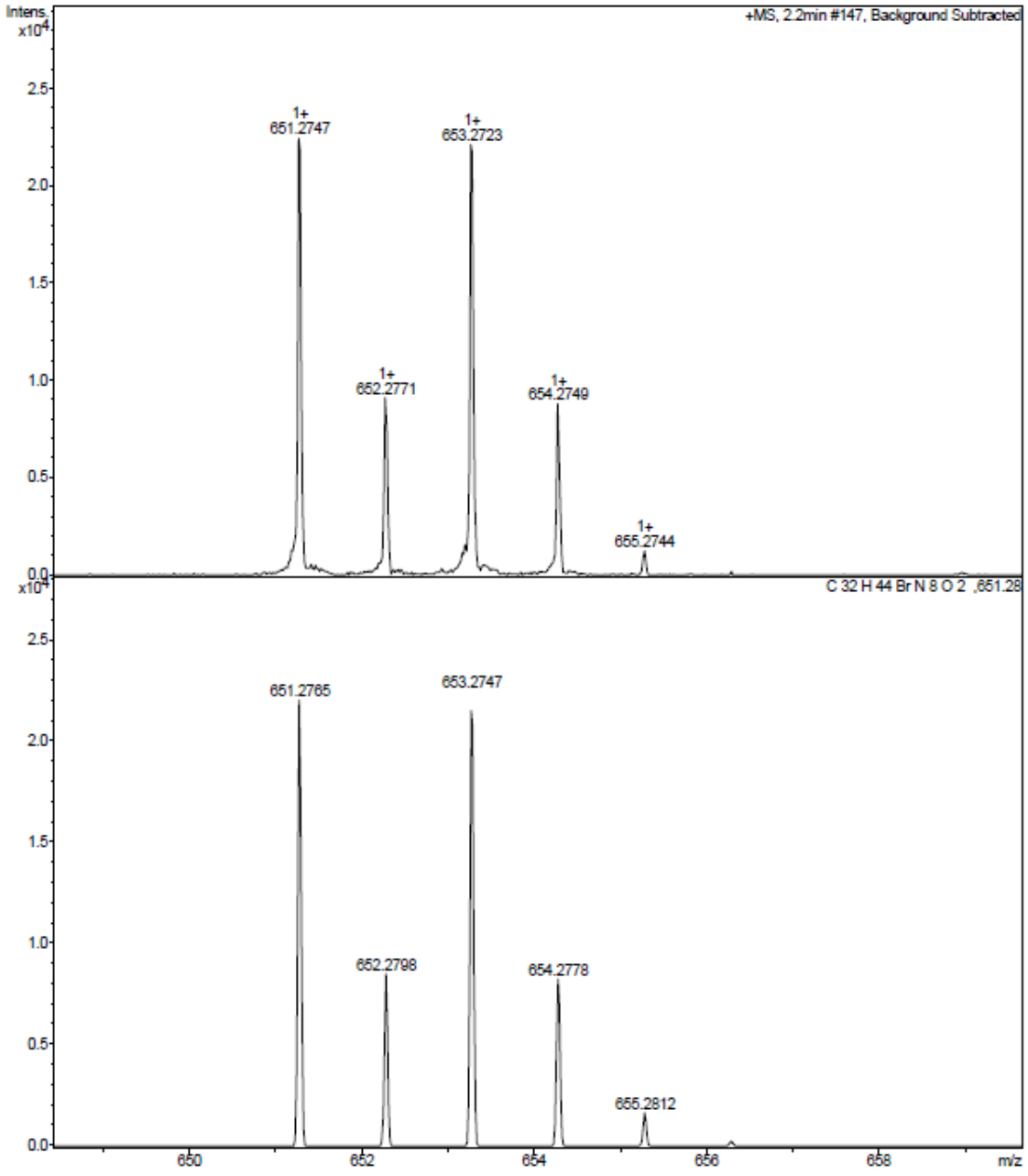


bb-Br









Electronic Supplementary Material (ESI) for Chemical Communications