Kinetic Characterization of Spiropyrans in Aqueous Media

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Electronic Supplementary Information

General Methods: UV spectra were recorded on a PerkinElmer Lambda 40 spectrometer at a scan speed of 480 nm s^{-1} and of bandwidth 1 nm. Fluorescence spectra were recorded on a PTI QM-7 fluorimeter, emission and excitation were internally corrected and both set to a bandwidth of 2 nm. Preparative HPLC was performed on a Waters 717plus autosampler. Linear gradients of eluent A (0.1% TFA / water) and eluent B (0.05 % TFA / acetronitrile) at a flow rate of 10 mL min^{-1} were performed on a Macherey-Nagel Nucleosil 100-7 C18 21×250 mm^2 column. LC-MS was performed on a Thermo Separation Products AS3000 autosampler equipped with a TSP P4000 quaternary pump and TSP UV6000LP photo diode array. Linear gradients of A (0.1 % formic acid / water) and B (acetonitrile) with a flow rate of 0.2 mL min^{-1} were performed on a Waters Atlantis dC18-3 3×100 mm^2 column. The flow from the HPLC was directly fed into the ESI source of a Finnigan LCQ Deca ion trap spectrometer. HR-ESI-MS were taken on an IonSpec Ultima 4.7 T FT Ion Cyclotron Resonance spectrometer.

Synthesis of peptide 1a: Peptide **1a** was synthesized according to literature[1] on 10 μM scale on NovaSyn TG Sieberamide resin (0.156 mmol/g load). Fmoc-Lys(Mtt)-OH, and Fmoc-Gly-OH were coupled via HOBt/HBTU activation while the spiropyran was introduced by coupling of a Fmoc-2-aminoethylglycine-spiropyran-OPfp pre-activated building block[1,2]. Final capping was realized with acetic acid anhydride / triethylamine / NMP 1:1:10, 3 min. Cleavage was achieved by dropping 20 ml 1 % trifluoroacetic acid / 4 % triisopropylsilane in hexafluoroisopropanol / dichloromethane 3:7 within 20 min over the resin. The obtained solution was evaporated to dryness, dissolved in 2 ml acetonitrile / water 1:1 and separated via preparative HPLC (5 % to 60 % in 60 min, t_R = 41.6 min) to obtain 5.4 mg [50 %] of pure product.

LC-MS (5 % to 70 % B in 30 min): $\mathbf{1a_{mc}}$ t_R = 12.54 min, $\mathbf{1a_{sp}}$ t_R = 15.8 min; both isomers: m/z = 971.5 $[M+Na]^+$ (90), 949.6 (100) $[M+H]^+$, 475.5 (50) $[M+2H]^{2+}$, 317.5 (30) $[M+3H]^{3+}$; **HR-ESI-MS**: calcd. 949.5254 for $[C_{46}H_{68}N_{12}O_{10}+H]^+$, found 949.5258; **UV-spectra**: 0.1 % TFA: $\mathbf{1a_{sp}}$ λ_{max} = 270 nm, 352 nm; $\mathbf{1a_{mc}}$ λ_{max} = 315 nm, 420 nm; pH 5.0 – 8.0: $\mathbf{1a_{sp}}$ λ_{max} = 270 (18 400 $M^{-1}cm^{-1}$) nm, 351 (11 400 $M^{-1}cm^{-1}$) nm; $\mathbf{1a_{mc}}$ 373 (20 500 $M^{-1}cm^{-1}$) nm, 520 (30 400 $M^{-1}cm^{-1}$) nm; isosbestic point $\mathbf{1a_{sp}}$ / $\mathbf{1a_{mc}}$ in 0.1% TFA: λ = 290 nm, pH 5.0 – 8.0 λ = 304 nm; isosbestic point 0.1 % TFA / pH 5.0 – 8.0: $\mathbf{1a_{mc}}$ λ = 343 nm, 390 nm, 460 nm; **fluorescence** emission of $\mathbf{1a_{mc}}$: excitation at 520 nm, 2 nm bandwith, λ_{em} = 603 nm.

[1] T. Stafforst, U. Diederichsen, *Eur. J. Org. Chem.*, 2007, 681.
[2] Fmoc-2-aminoethylglycine-spiropyran-OPfp was synthesized via the common route analog to literature. A. Samat, D. De Keukeleire, R. J. Guglielmetti, *Bull. Soc. Chim. Belg.*, 1991, **100(9)**, 679.

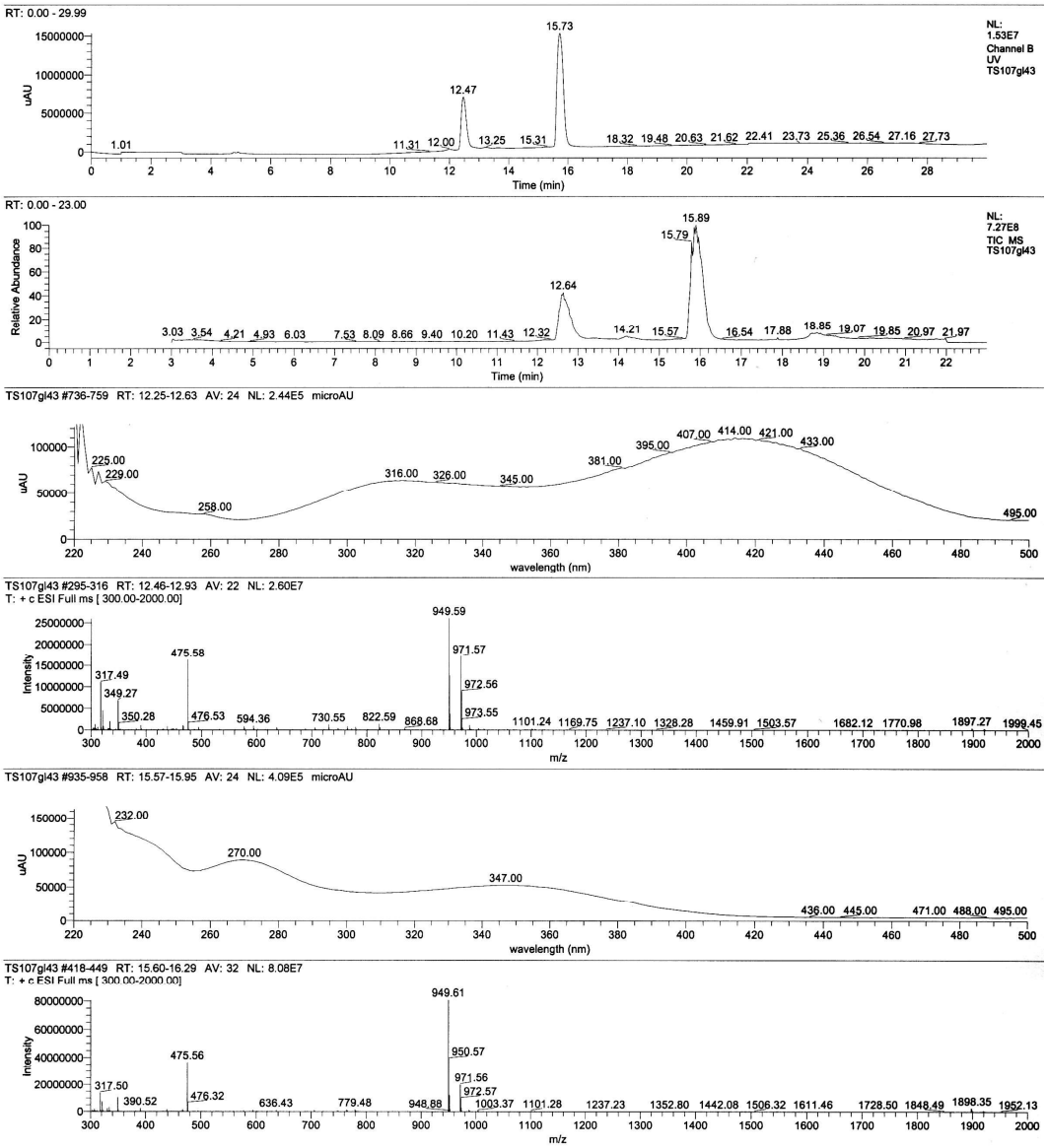


Fig 1. LC-MS of peptide **1a**: first row: UV-trace 254 nm, second row: mass-trace, third row: UV-spectra of $\mathbf{1a_{mc}}$, fourth row: mass spectra of $\mathbf{1a_{mc}}$, fifth row: UV-spectra of $\mathbf{1a_{sp}}$, sixth row: mass spectra of $\mathbf{1a_{sp}}$.

Synthesis of peptide 1b: Peptide **1b** was synthesized from **1a** by adding 10 equiv. 3-formylsalicylic acid to a ~ 2 millimolar solution of peptide **1a** in 10 mM sodium phosphate buffer, 100 mM NaCl pH 5.0, and keeping at 60 °C over night. The deep orange raw product was separated via preparative HPLC (5 % to 50 % in 60 min), t_R = 34.1 min.
LC-MS (5 % to 70 % B in 30 min): $\mathbf{1b_{mc}}$ t_R = 12.72 min, m/z = 948.6 (40) $[M+H]^+$, 475.3 (100) $[M+2H]^{2+}$, 317.7 (80) $[M+3H]^{3+}$; **HR-ESI-MS**: calcd. 948.5301 for $[C_{47}H_{69}N_{11}O_{10}+H]^+$, found 948.5315; **UV-spectra**: 0.1 % TFA, pH 5.0 – 8.0: $\mathbf{1b_{sp}}$ λ_{max} = 266- 320 nm (flat); $\mathbf{1b_{mc}}$ λ_{max} = 441 (12 900 $M^{-1}cm^{-1}$) nm; isosbestic point: $\mathbf{1b_{sp}}$ / $\mathbf{1b_{mc}}$ whole pH-range λ = 309 nm; **fluorescence** emission of $\mathbf{1b_{mc}}$: excitation at 440 nm, 2 nm bandwidth, λ_{em} = 601 nm.

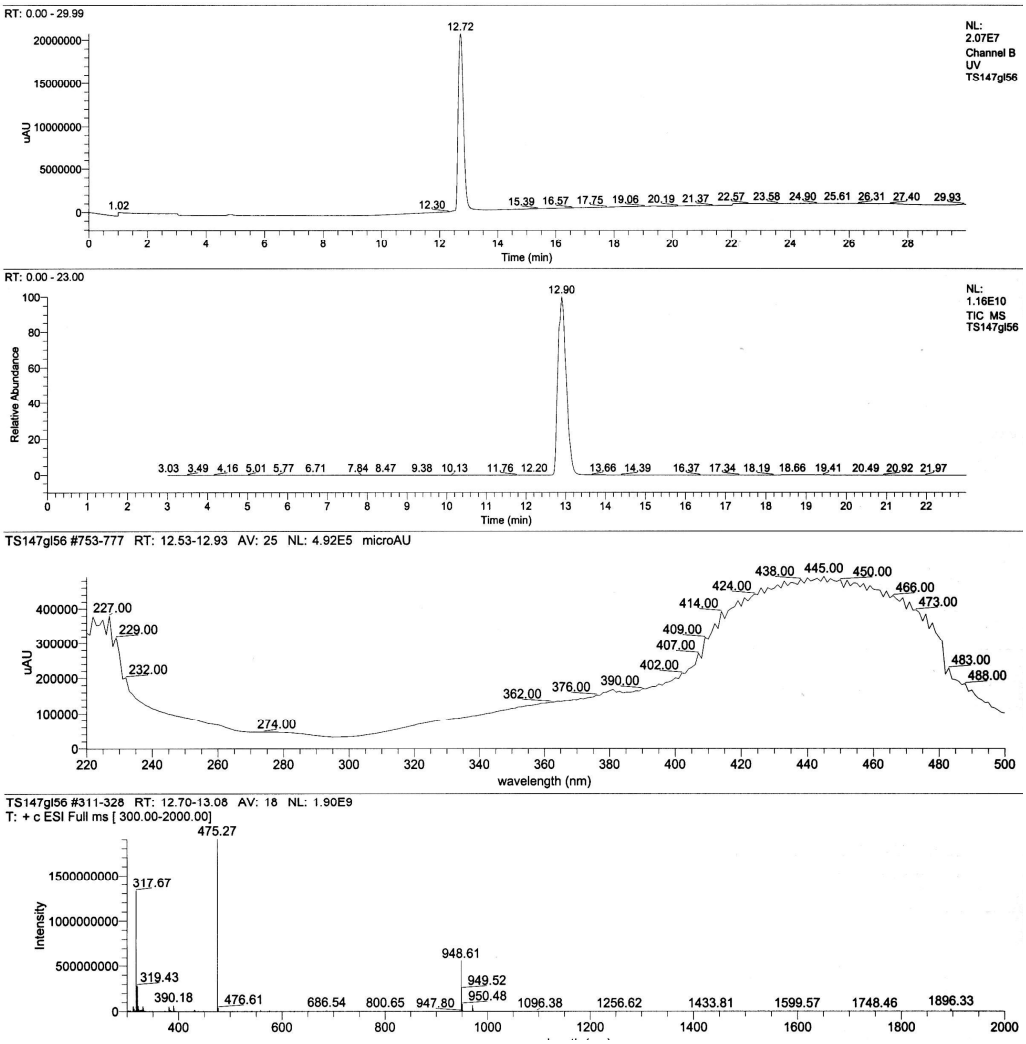


Fig 2. LC-MS of peptide **1b**: first row: UV-trace 254 nm, second row: mass-trace, third row: UV-spectra of $\mathbf{1b_{mc}}$, fourth row: mass spectra of $\mathbf{1b_{mc}}$.

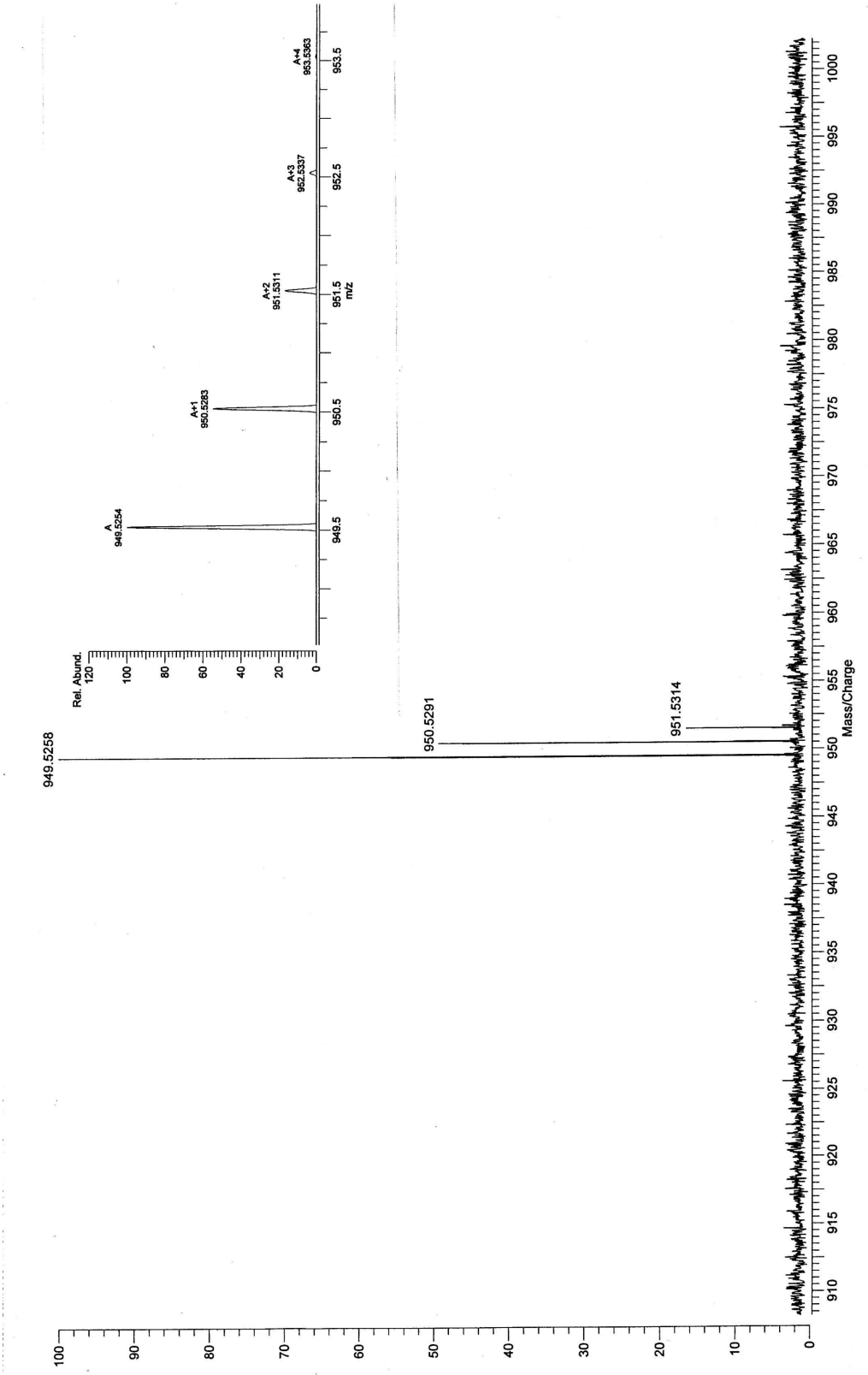


Fig 3. HR-ESI of peptide **1a**, and predicted signal pattern (inset) for $[C_{46}H_{68}N_{12}O_{10}+H]^+$.

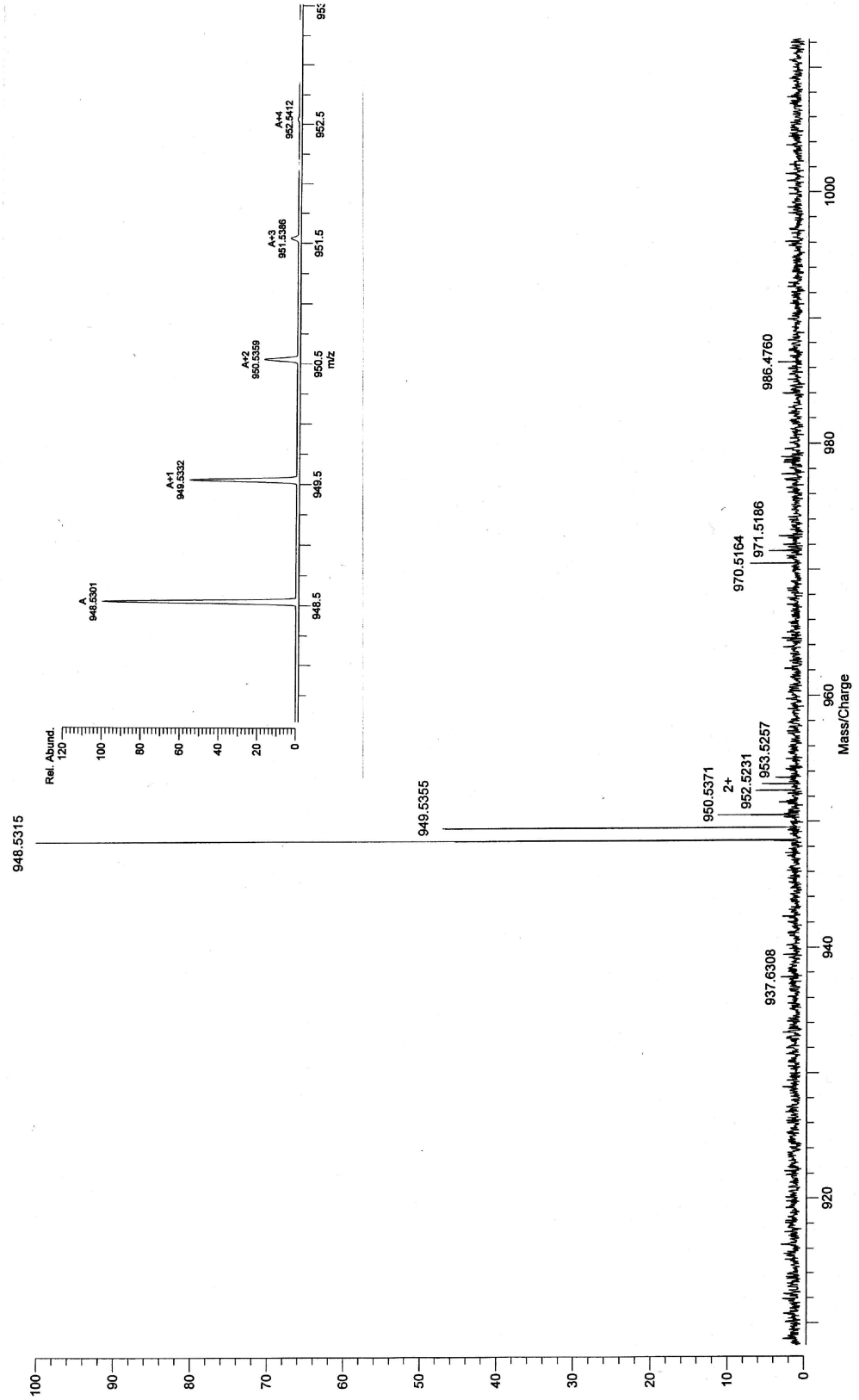
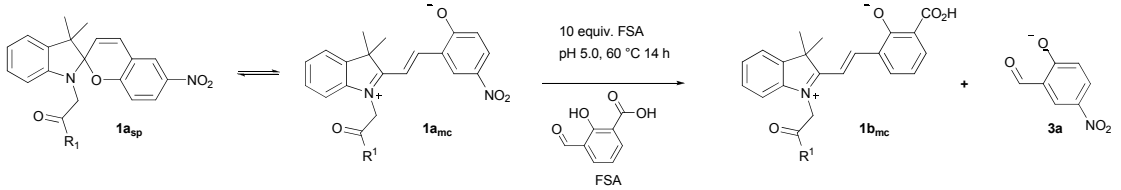


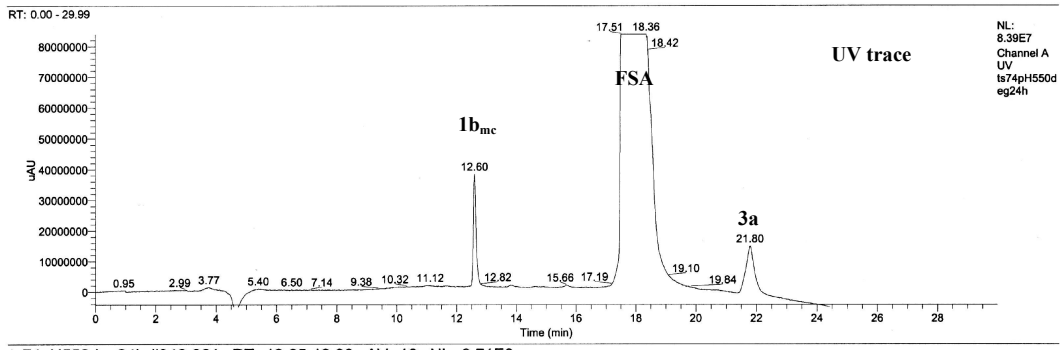
Fig 4. HR-ESI of peptide **1b**, and predicted signal pattern (inset) for $[C_{47}H_{69}N_{11}O_{10}+H]^+$.

Aldehyde Exchange Reaction 1a --> 1b



$C_{46}H_{68}N_{12}O_{10}$ | $C_{46}H_{68}N_{12}O_{10}$ | $C_8H_6O_4$ | $C_{47}H_{69}N_{11}O_{10}$ | $C_7H_5N_1O_4$
948.5 g/mol | 948.5 g/mol | 166.0 g/mol | 947.5 g/mol | 167.0 g/mol
t_R = 12.47 min | t_R = 15.80 min | t_R = 18.0 min | t_R = 12.60 min | t_R = 21.80 min
λ_{max} = 270; 357 nm | λ_{max} = 326; 420 nm | λ_{max} = 335 nm | λ_{max} = 445 nm | λ_{max} = 305 nm

R^1 = Ac-Lys-N(H)-N-CH$_2$-CO-Lys-Lys-Gly-NH_2



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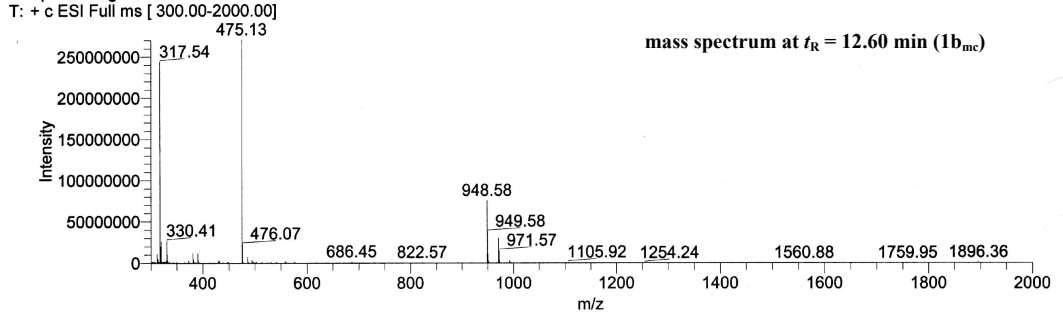
T: + c ESI Full ms [300.00-2000.00]



ts74pH550deg24h #1293-1326 RT: 21.53-22.08 AV: 34 NL: 6.78E4 microAU

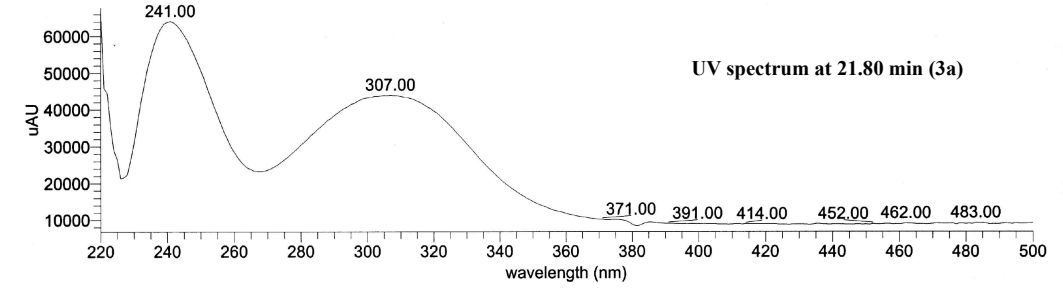


Fig. 5 Aldehyde exchange reaction: peptide **1a** was heated in presence of an excess of commercially purchased 3-formylsalicylic acid (FSA). The depletion of the starting material **1a**, the formation of the product **1b** and of the side product 5-nitrosalicylaldehyde **3a** was monitored by LC-MS. Given is the LC-MS after complete reaction (14 h): First row: UV-trace 254 nm; second row: mass spectrum at 12.60 min assigned to $\mathbf{1b}_{mc}$; third row: UV-spectra at 21.80 min assigned to **3a**. Conditions: 5% to 70% in 30 min

Degradation of Peptide 1a
Before Degradation

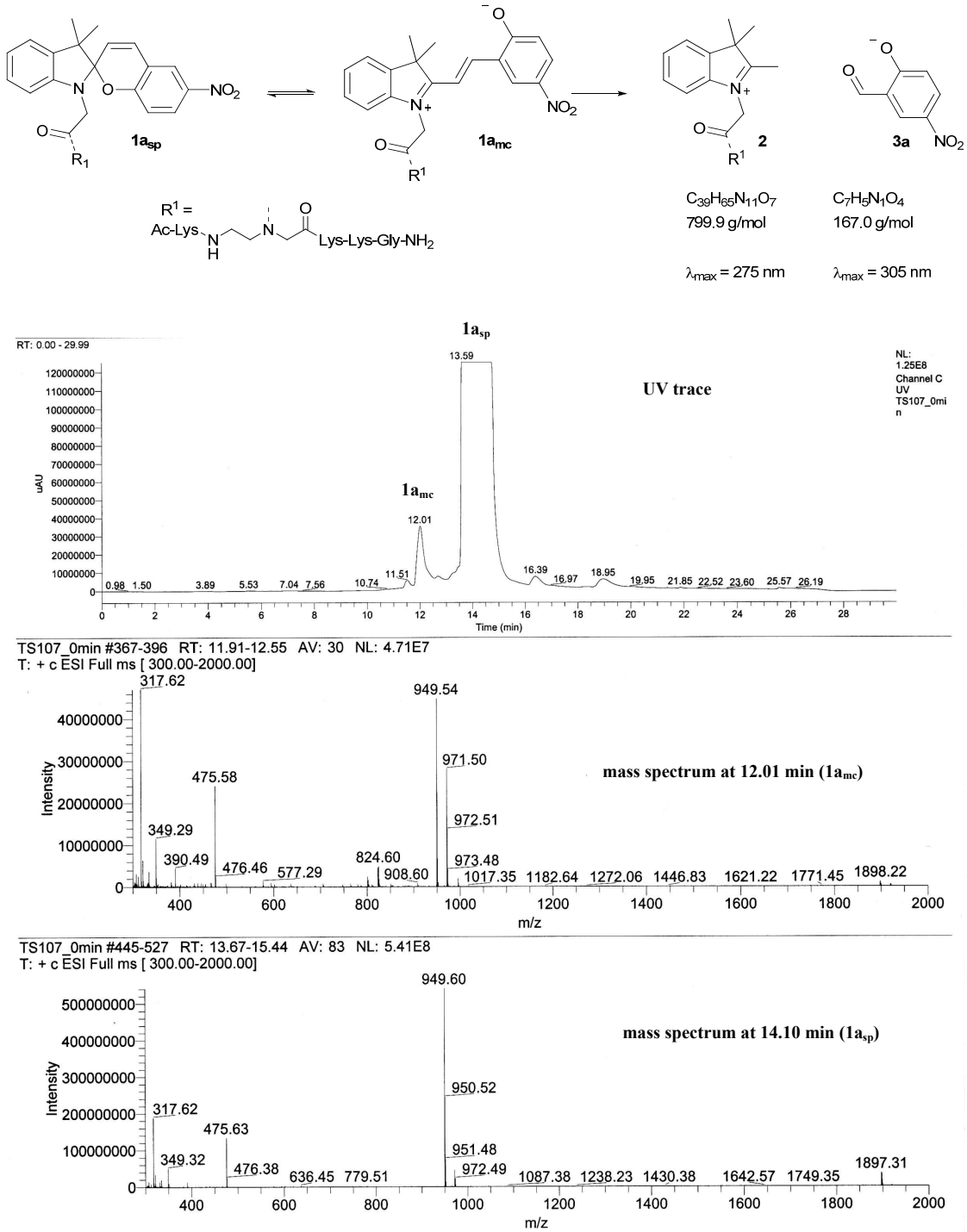
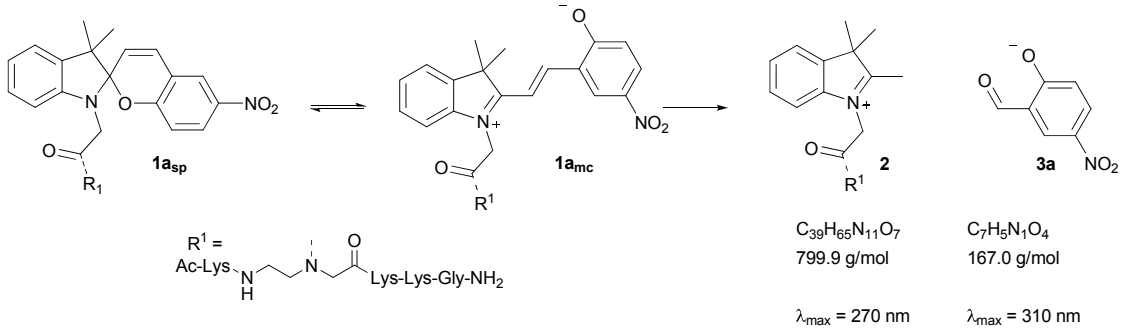


Fig 6. Degradation of peptide **1a**. LC-MS of peptide **1a** before start of degradation (0 min), buffer pH 7.0: first row: UV-trace 254 nm, second row: mass spectrum at 12.01 min assigned to $\mathbf{1a}_{mc}$, third row: mass spectra at 14.10 min assigned to $\mathbf{1a}_{sp}$. Conditions: 5 to 95% in 20 min

After Degradation of Peptide 1a



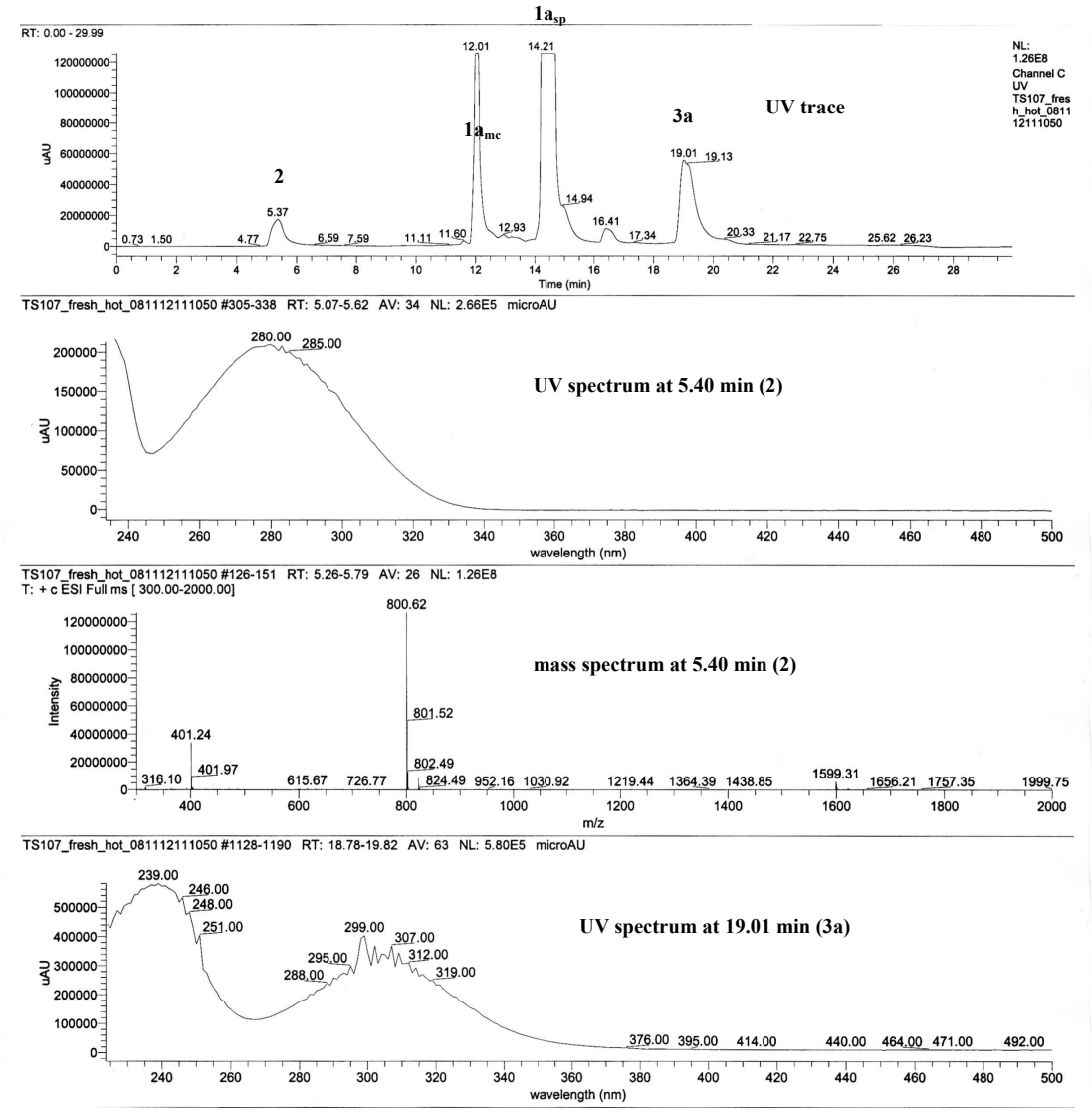


Fig 7. Degradation of peptide **1a**. LC-MS of peptide **1a** after 5 min at 90 °C in buffer pH 7.0: first row: UV-trace 254 nm, second row: UV spectra at 5.40 min assigned to **2**, third row: mass spectra at 5.40 min assigned to **2**; fourth row: UV-spectra at 19.01 min assigned to **3a**. Conditions: 5 to 95% in 20 min

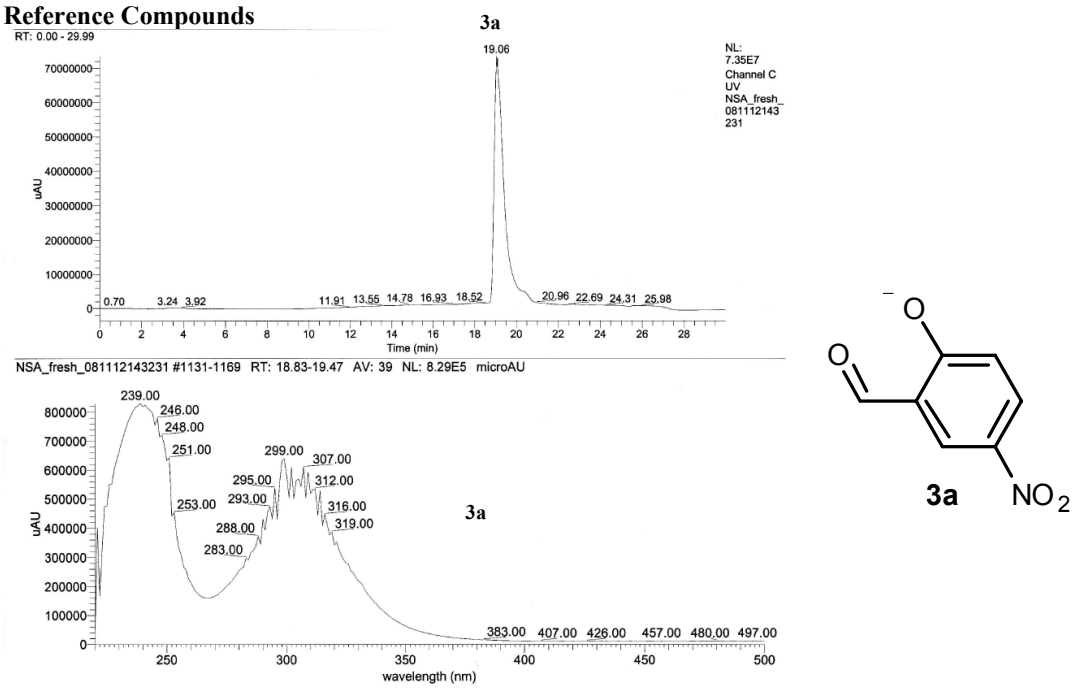
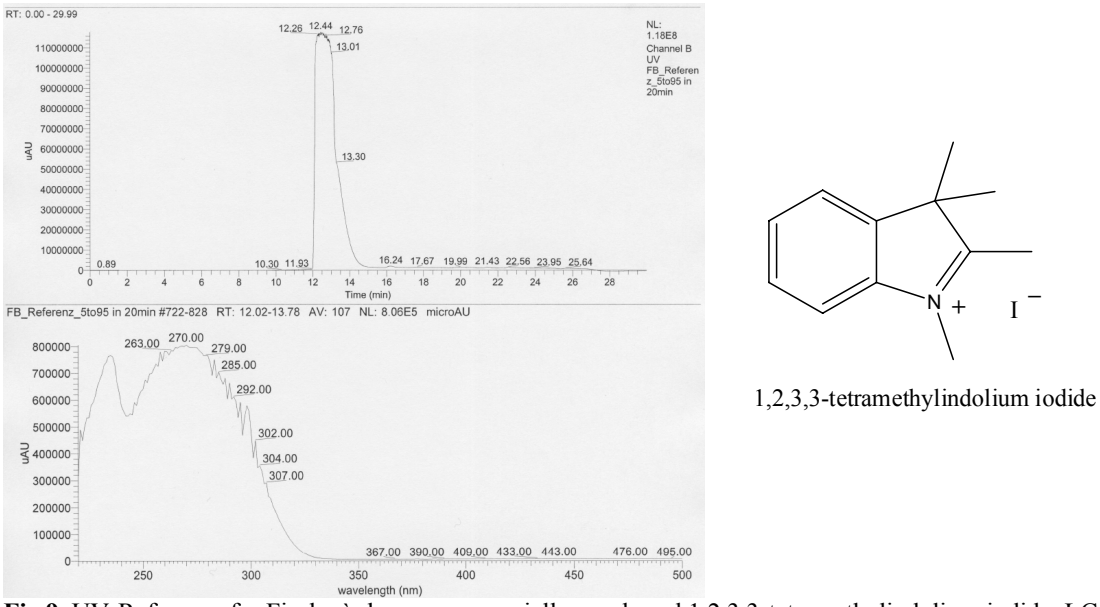


Fig 8. Reference: commercially purchased 5-nitrosalicylaldehyde = **3a**. LC-MS of peptide **3a** UV-trace 254 nm, second row: UV spectrum at 19.10 min (**3a**). Conditions: 5 to 95% in 20 min



1,2,3,3-tetramethylindolium iodide

Fig 9. UV-Reference for Fischer`s base: commercially purchased 1,2,3,3-tetramethylindolium iodide. LC-MS of 1,2,3,3-tetramethylindolium iodide: UV-trace 254 nm, second row: UV spectrum at 12.6 min. Conditions: 5 to 95% in 20 min

Coinjection of Degraded Peptide 1a + Aldehyde Reference

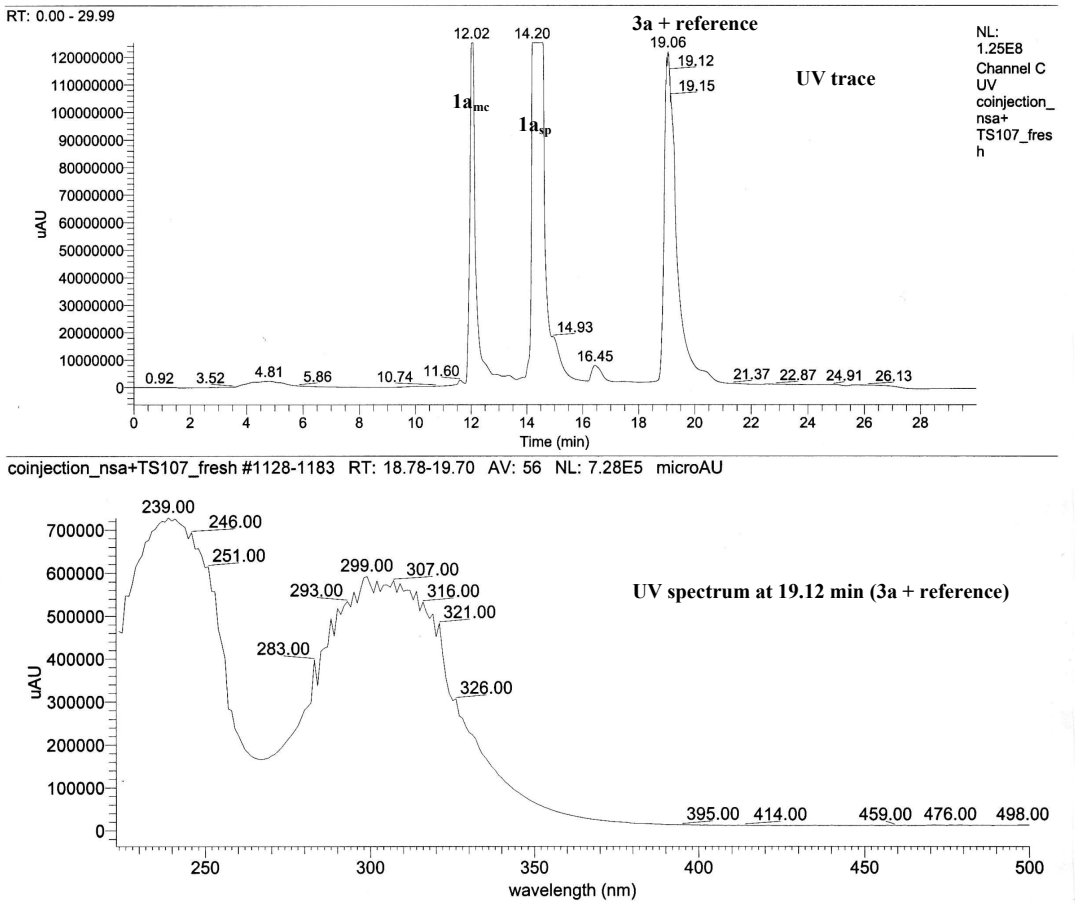


Fig 10. Coinjection of degraded peptide **1a** plus commercially purchased 5-nitrosalicylaldehyde as a reference for **3a**: LC-MS: first row: UV-trace 254 nm, second row: UV spectrum at 19.12 min assigned to **3a** + reference. Conditions: 5 to 95% in 20 min

Degradation of Peptide 1b
Before Degradation

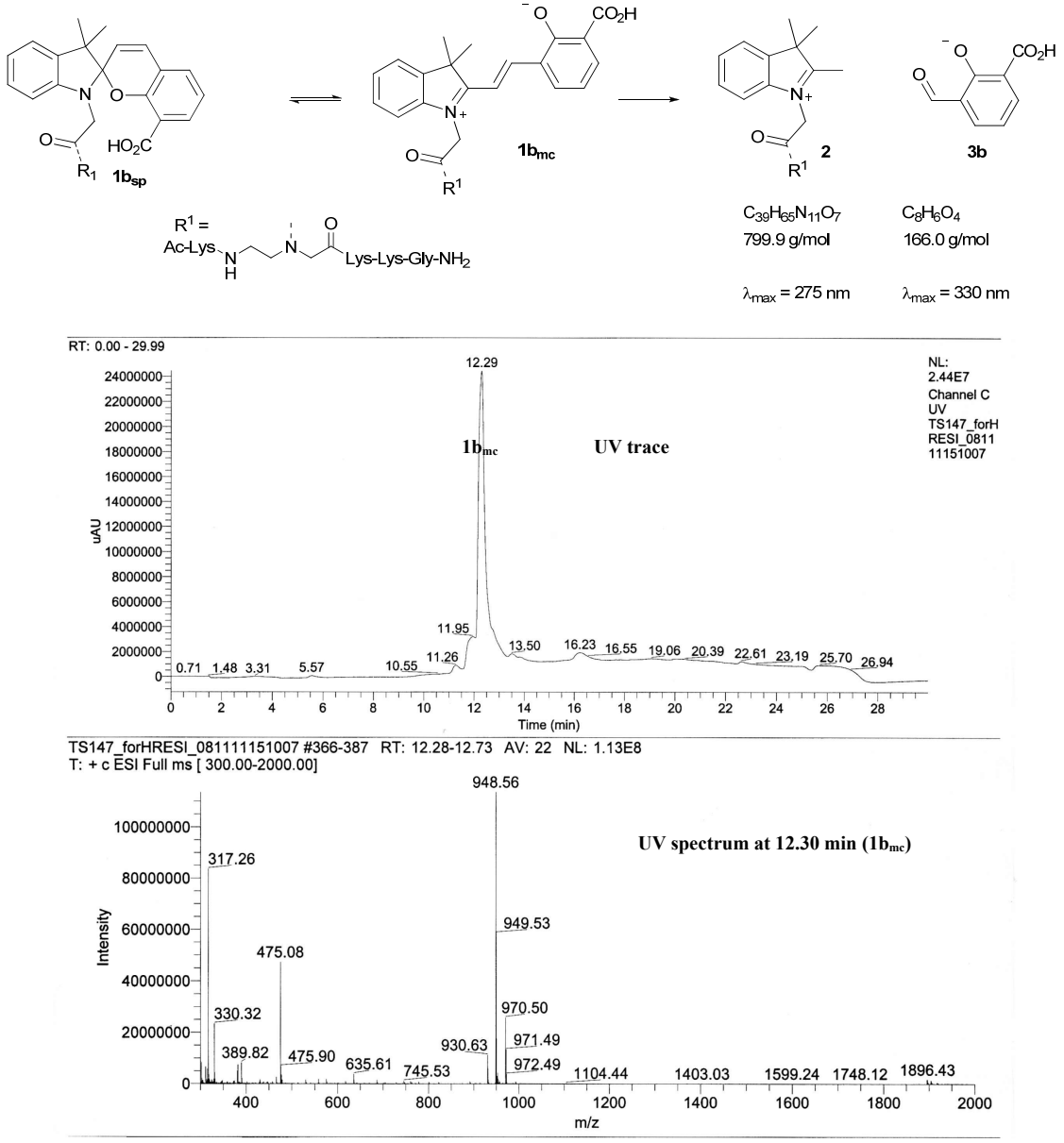


Fig 11. Degradation of peptide **1b**. LC-MS of peptide **1b** before start of degradation (0 min), buffer pH 7.0: first row: UV-trace 254 nm, second row: mass spectrum at 12.30 min assigned to $\mathbf{1b_{mc}}$. Conditions: 5 to 95% in 20 min

After Degradation of Peptide 1b

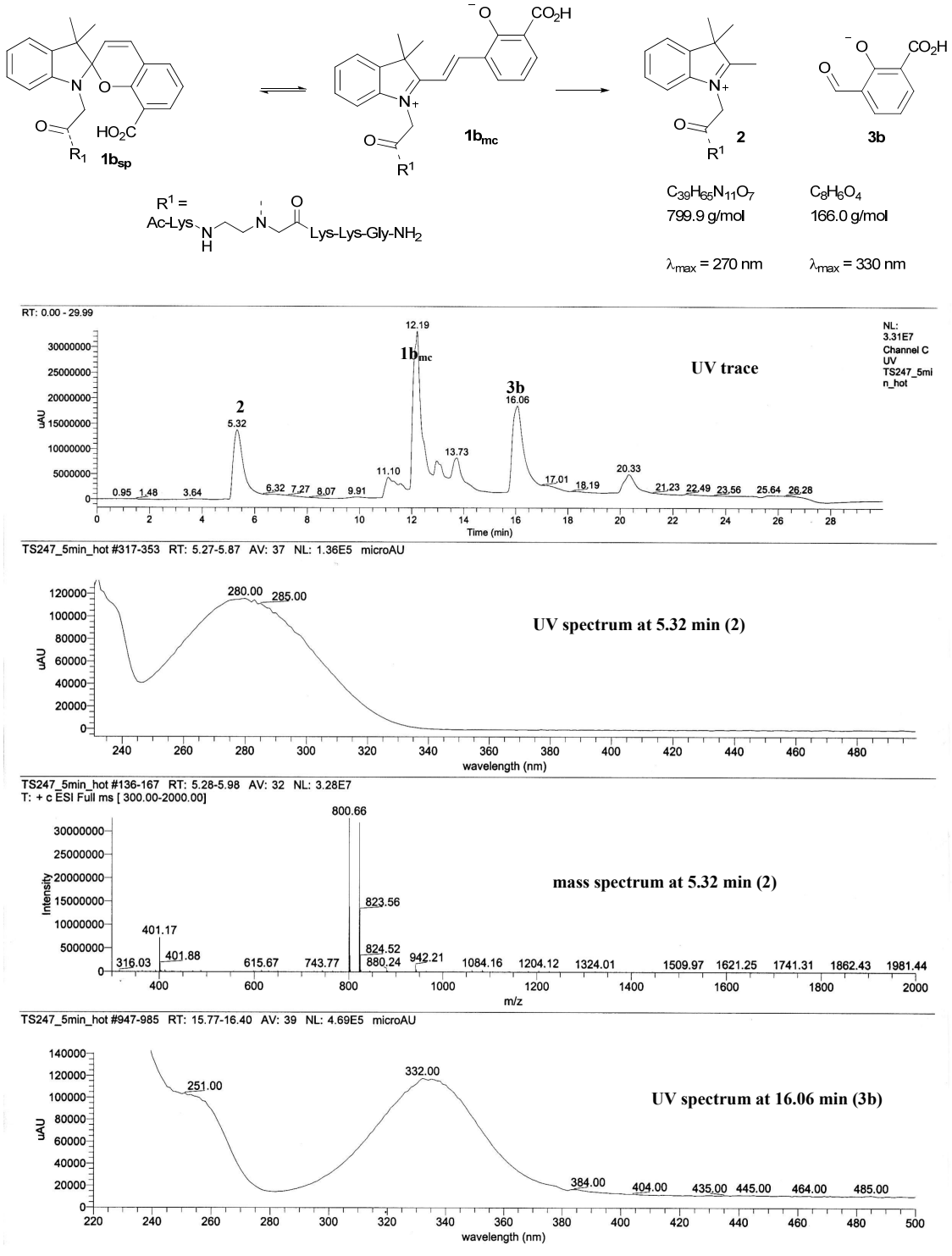


Fig 12. Degradation of peptide **1b**. LC-MS of peptide **1b** after 5 min at 90 °C in buffer pH 7.0: first row: UV-trace 254 nm, second row: UV spectrum at 5.32 min assigned to **2**, third row: mass spectrum at 5.32 min assigned to **2**, fourth row: UV spectrum at 16.06 min assigned to **3b**. Conditions: 5 to 95% in 20 min

Reference Compound

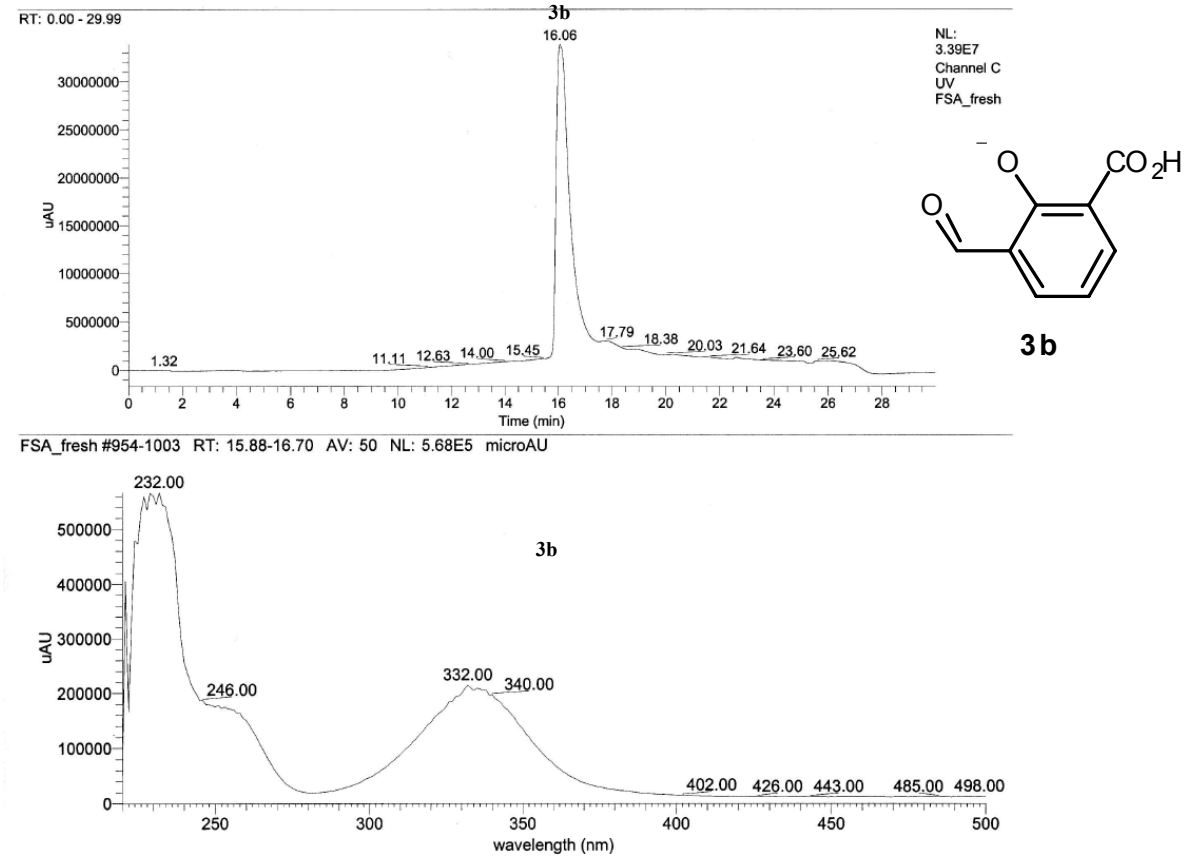


Fig 13. Reference: commercially purchased 3-formylsalicylic acid = **3b**. LC-MS of peptide **3b** UV-trace 254 nm, second row: UV spectrum at 16.10 min (**3b**). Conditions: 5 to 95% in 20 min

Coinjection of Degraded Peptide 1b + Aldehyde Reference

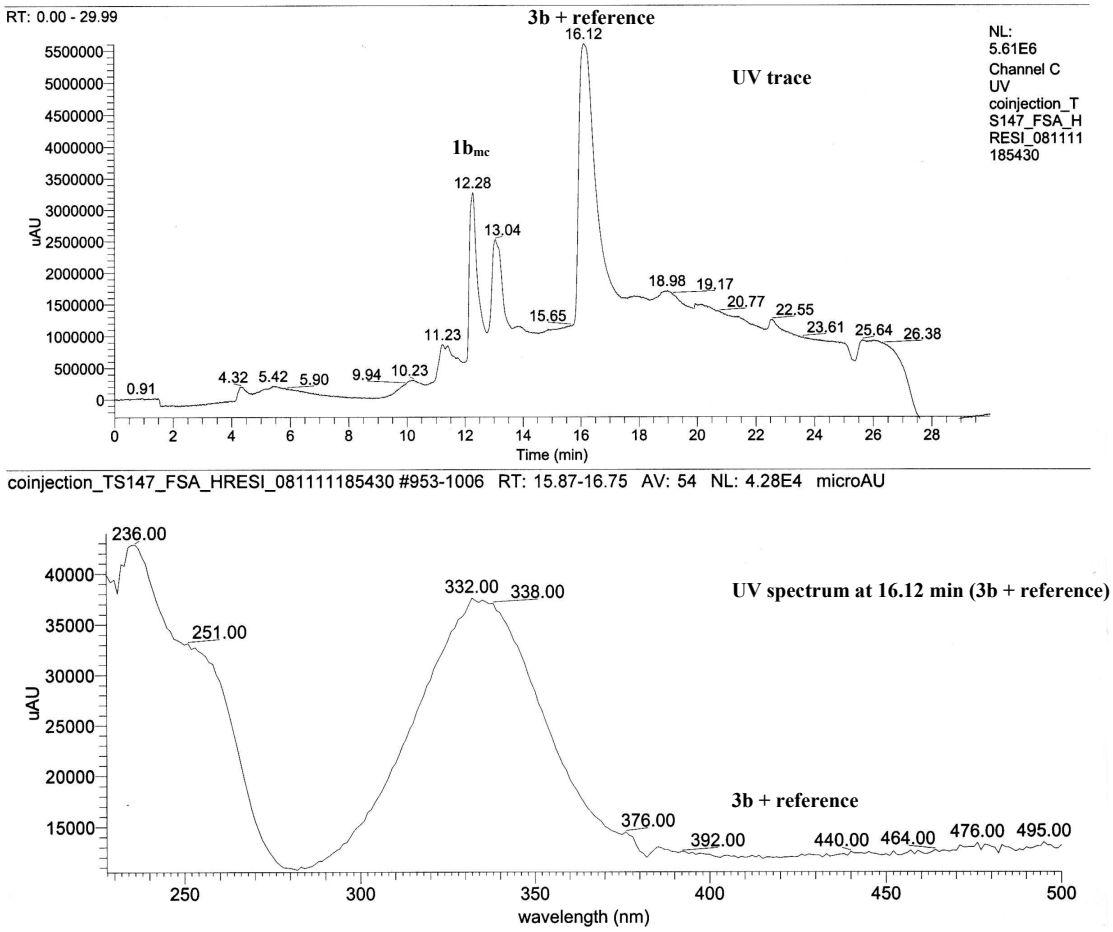


Fig 14. Coinjection of degraded peptide **1b** plus commercially purchased 3-formylsalicylic acid as a reference for **3b**: LC-MS: first row: UV-trace 254 nm, second row: UV spectrum at 16.12 min assigned to **3b** + reference. Conditions: 5 to 95% in 20 min

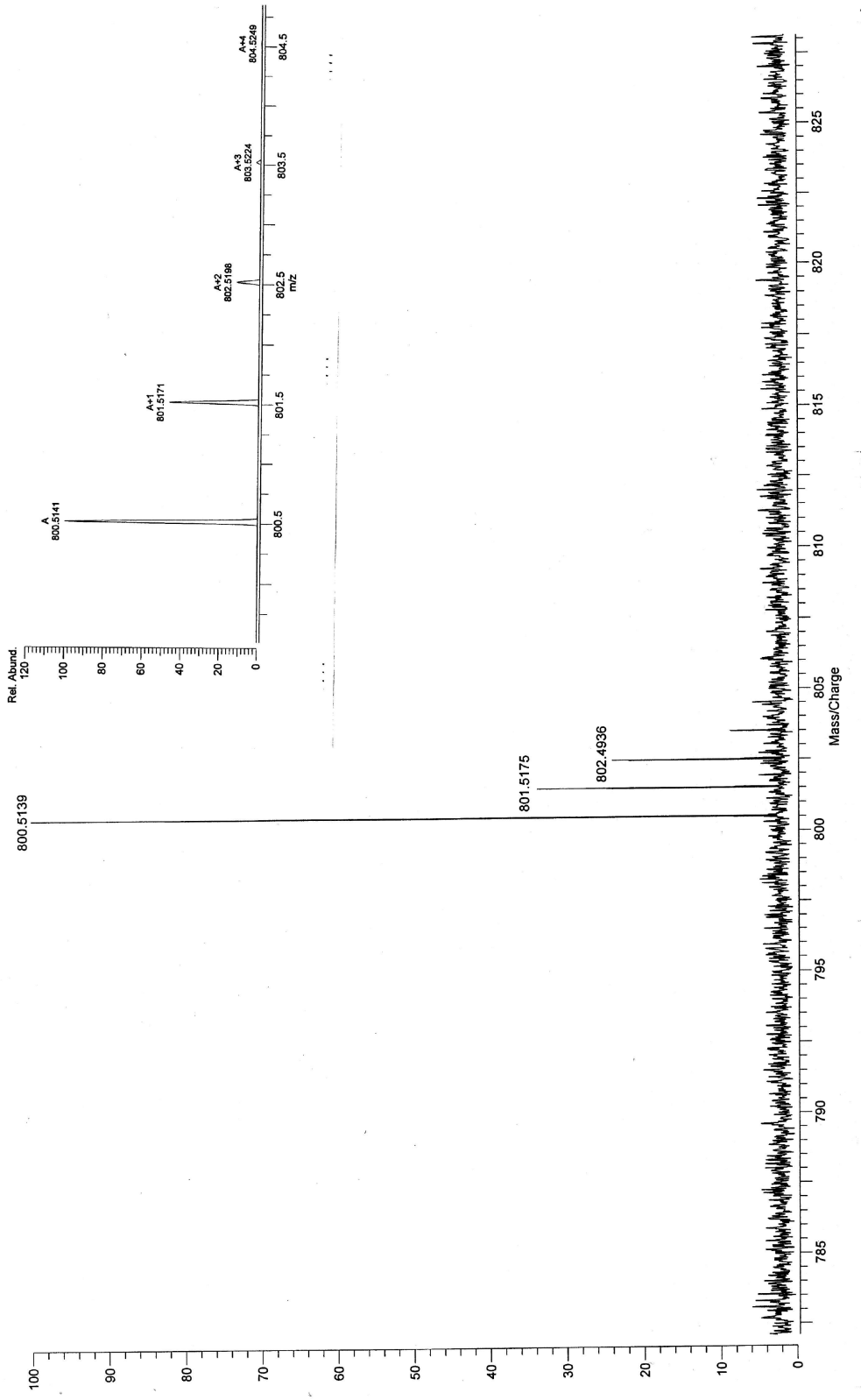


Fig. 15 High-resolution ESI-MS spectra of **2**. The inset shows the predicted signal pattern for the assumed molecular formula $[C_{39}H_{65}N_{11}O_7+H]^+$.

Kinetic measurements: A stock solution (~ 2 mM) of the respective peptide in 0.2 % TFA / water was heated up to 90 °C on a water bath in a light-protected amber Eppendorf-cup for 1 - 2 min to convert the peptides fully into the merocyanine form. These stock solutions were 100-fold diluted into 700 µL 0.1 % TFA or the respective 10 mM sodium phosphate buffer, 100 mM NaCl, pH 5.0, 6.0, 7.0 or 8.0 into 10 mm quartz glass UV cuvettes. A second set of samples was prepared as above but was subsequently irradiated with visible light (Conrad Electronic LED Spot Luxeon Green 3W 520 nm 17 nm halfwidth for **1a** and Conrad Electronic LED Spot Luxeon Blue 3W 465 nm 15 nm halfwidth for **1b**) in order to obtain the respective pure spiropyran form of the photoswitch. The UV absorbance traces (520 nm for **1a** in pH 5.0 – 8.0, 420 nm for **1a** in 0.1 % TFA, and 440 nm for **1b** in the whole pH range) were then taken at constant temperature under control of a Peltier element in a sample changer (**1a**$_{mc}$ and **1a**$_{sp}$ or **1b**$_{mc}$ and **1b**$_{sp}$ always in parallel). Prior to the start of the kinetic measurements, the content of the respective isomeric form was always controlled by UV-spectroscopy and for peptide **1a** also by LC-MS. The minor isomer was never detectable.
The three rates k_1, k_{-1} and k_2 were computed with the program DynaFit (version 3.28.054; P. Kuzmic, *Anal. Biochem.*, 1996, **237**, 260-273) by solving the following set of differential equations. For each pH the absorbance trace related to the merocyanine-form and the trace related to the spiropyran-form were fitted simultaneously to the set of differential equations, therefore concentration changes of the merocyanine form were related to the observed absorbance changes in the visible region (520 nm for **1a** and 440 nm for **1b**). The k_{-1} was set to 0 for **1b** and only k_1 and k_2 were computed.

$$\frac{d[sp]}{dt} = -k_1[sp] + k_2[mc]$$

$$\frac{d[mc]}{dt} = +k_1[sp] - k_{-1}[mc] - k_2[mc]$$

$$\frac{d[fb]}{dt} = +k_2[mc]$$

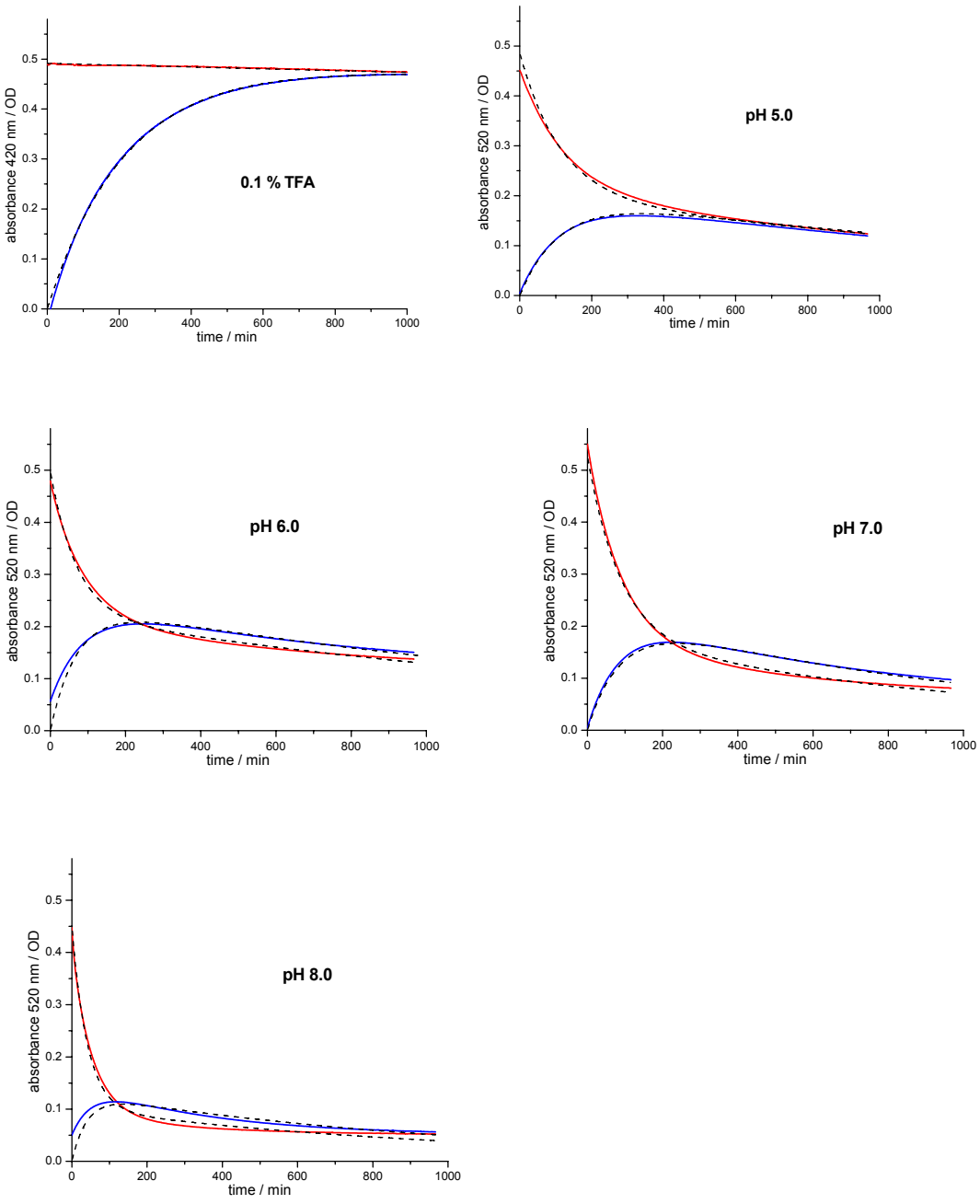


Fig. 16 Absorbance time traces for determination of k_1, k_{-1} and k_2. Traces were taken at 25.0 °C for $\mathbf{1a}_{sp}$ (blue) or $\mathbf{1a}_{mc}$ (red) each 20 µM in 10 mM sodium phosphate buffer, 100 mM NaCl, 25 °C. The kinetic parameters were obtained from fits (DynaFit) that are given as dashed black lines.

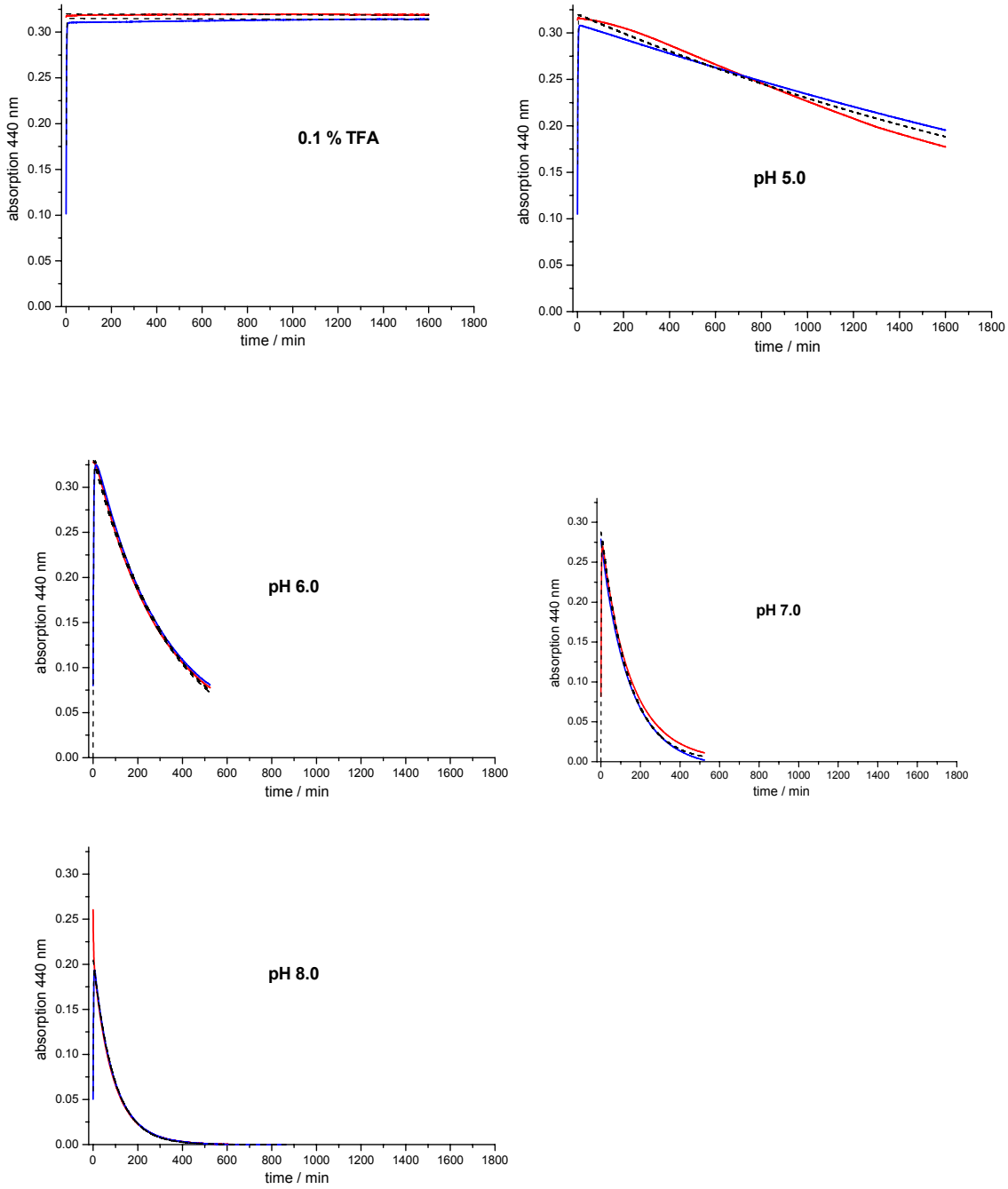


Fig. 17 Absorbance time traces for determination of k_1, k_{-1} and k_2. Traces were taken for **1b**$_{sp}$ (blue) or **1b**$_{mc}$ (red) each 25 µM in 10 mM sodium phosphate buffer, 100 mM NaCl, 29.0 °C. The kinetic parameters were obtained from fits (DynaFit) that are given as dashed black lines.