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 / acetonitrile) 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 ×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 \(\mu\text{M}\) 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, \(t_R = 41.6\text{ min}\)) to obtain 5.4 mg [50 %] of pure product.

**LC-MS** (5 % to 70 % B in 30 min): \(1_{\text{amc}}\) \(t_R = 12.54 \text{ min}\), \(1_{\text{asp}}\) \(t_R = 15.8 \text{ min}\); 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**: calcld. 949.5254 for [C\(_{46}\)H\(_{68}\)N\(_{12}\)O\(_{10}\) + H]\(^+\), found 949.5258; **UV-spectra**: 0.1 % TFA: \(1_{\text{asp}} \lambda_{\text{max}} = 270\text{ nm}, 352\text{ nm}; 1_{\text{amc}} \lambda_{\text{max}} = 315\text{ nm, 420 nm}; \text{pH } 5.0 – 8.0: 1_{\text{asp}} \lambda_{\text{max}} = 270 (18 400 M\(^{-1}\)cm\(^{-1}\)) nm, 351 (11 400 M\(^{-1}\)cm\(^{-1}\)) nm; \(1_{\text{amc}}\) 373 (20 500 M\(^{-1}\)cm\(^{-1}\)) nm, 520 (30 400 M\(^{-1}\)cm\(^{-1}\)) nm; isosbestic point \(1_{\text{asp}} / 1_{\text{amc}}\) in 0.1% TFA: \(\lambda = 290\text{ nm}, \text{pH } 5.0 – 8.0 \lambda = 304\text{ nm}; \text{isosbestic point 0.1 % TFA / pH } 5.0 – 8.0: 1_{\text{amc}} \lambda = 343\text{ nm, 390 nm, 460 nm}; \text{fluorescence} \text{ emission of } 1_{\text{amc}}\text{ excitation at } 520\text{ nm, 2 nm bandwidth, } \lambda_{\text{em}} = 603\text{ nm.}**

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Fig 1. LC-MS of peptide 1a: first row: UV-trace 254 nm, second row: mass-trace, third row: UV-spectra of 1a_mic, fourth row: mass spectra of 1a_mic, fifth row: UV-spectra of 1a_ap, sixth row: mass spectra of 1a_ap.
**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 overnight. 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): $1b_{mc}$ $t_R = 12.72$ 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 [C47H69N11O10+H]+, found 948.5315; **UV-spectra**: 0.1 % TFA, pH 5.0 – 8.0: $1b_{dp}$ $\lambda_{\text{max}} = 266$- 320 nm (flat); $1b_{mc}$ $\lambda_{\text{max}} = 441$ (12 900 M⁻¹cm⁻¹) nm; isosbestic point: $1b_{dp}$ / $1b_{mc}$ whole pH-range $\lambda = 309$ nm; **fluorescence** emission of $1b_{mc}$: excitation at 440 nm, 2 nm bandwidth, $\lambda_{\text{em}} = 601$ nm.

![Fig 2. LC-MS of peptide 1b](image-url)
Fig 3. HR-ESI of peptide 1a, and predicted signal pattern (inset) for [C₄₆H₆₈N₁₂O₁₀+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 $\rightarrow$ 1b

C$_4$H$_6$N$_2$O$_2$

$\text{t}_R = 12.47 \text{ min}$

$\lambda_{\text{max}} = 270; 357 \text{ nm}$

C$_4$H$_6$N$_2$O$_2$

$\text{t}_R = 15.80 \text{ min}$

$\lambda_{\text{max}} = 326; 420 \text{ nm}$

C$_7$H$_5$N$_1$O$_4$

$\text{t}_R = 18.0 \text{ min}$

$\lambda_{\text{max}} = 335 \text{ nm}$

C$_{47}$H$_69$N$_{11}$O$_{10}$

$\text{t}_R = 12.60 \text{ min}$

$\lambda_{\text{max}} = 445 \text{ nm}$

C$_8$H$_6$O$_4$

$\text{t}_R = 18.0 \text{ min}$

$\lambda_{\text{max}} = 335 \text{ nm}$

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 $\text{t}_R = 12.60 \text{ min}$ (1b$_{\text{mc}}$); third row: UV-spectra at 21.80 min assigned to 3a. Conditions: 5% to 70% in 30 min
Degradation of Peptide 1a
Before Degradation

![Chemical structure 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 1a

- mass spectrum at 12.01 min (1a

- mass spectrum at 14.10 min (1asp)

Conditions: 5 to 95% in 20 min
After Degradation of Peptide 1a

![Chemical structures and UV spectra](image)

**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
**Electronic Supplementary Information**

**Reference Compounds**

**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

**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

![Chemical structures and UV traces](image)

**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 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**

![UV Trace](image1)

**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 $[\text{C}_{39}\text{H}_{65}\text{N}_{11}\text{O}_{7}+\text{H}]^+$. 
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 (1ama and 1asa or 1bma and 1bsp 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 1a$_{p}$ (blue) or 1a$_{m}$ (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 $1b_{hp}$ (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.