

Supporting Information

Synthetic Strategies to Prepare Bioactive Lysine and Peptide Conjugates with Triazolium Derivatives

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Materials and Methods

Reagents. Fmoc protected amino acids Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH and Fmoc-Val-OH were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Peptide-synthesis grade *N,N'*-Dimethylformamide (DMF) and acetonitrile (ACN) were purchased from Carlo Erba (Milan, Italy). Dichloromethane (DCM), trifluoroacetic acid (TFA), triisopropylsilane (TIS), methanol (MeOH), piperidine, *N,N'*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM), 1,2-ethanedithiol (EDT), acetic anhydride, potassium hydroxide, hydrochloric acid, hydroxylamine hydrochloride, ethyl cyanoacetate, 2,4-pentanedione (acetylacetone), formaldehyde, secondary amines: 4-methylpiperidine, pyrrolidine, morpholine were purchased from Sigma-Aldrich (Milan, Italy). *N*-ethylisopropylamine was purchased from TCI Chemicals (Zwijndrecht, Belgium). Rink amide AM resin (100-200 mesh, loading: 0.74 mmol/g) was purchased from CBL (Patras, Greece). Fmoc-Lys(Mtt) Wang resin (100 200 mesh, loading: 0.54 mmol/g) was purchased from Novabiochem (Merck, Darmstadt, Germany). All the reagents for elastase activity assays were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany; enzyme – porcine pancreas elastase, PPE, EC 3.4.21.36; substrate – *N*-succinyl-Ala-Ala-Ala-p-nitroanilide, SucAla3-pNA; control inhibitor – MeOSuc-Ala-Ala-Pro-Val-chloromethylketone, MeOSuc-AAPV-CMK, SPCK). Reagents and solvents were purchased at the highest commercial quality and used without additional purification, unless otherwise stated.

Purification methods. Crude peptides were purified by Reverse-Phase Flash Liquid Chromatography on Isolera One Flash Chromatography (Biotage, Uppsala, Sweden) using a SNAP Ultra C18 column (40 g) at 20 mL/min flow. Crude *Safirinium*-lysine conjugates and building blocks were purified by semipreparative RP-HPLC on a HPLC system Waters Alliance 2695 Separations Module (column Phenomenex Aqua C18 (25 cm × 3 mm × 5 μm)), coupled to a UV 2996 PDA detector. Eluent systems: 0.1% TFA in H₂O (A), 0.1% TFA in ACN (B) was used in all cases.

Characterization methods

HPLC-MS. Characterization was performed by analytical RP-HPLC using a Thermo Scientific Ultimate 3000 UHPLC coupled with Thermo Scientific MSQ plus, supplied with a CSH C18 Acquity UPLC® column (2.1×100 mm × 1.7 μm, 45°C), or using an HPLC system Waters Alliance 2695 Separations Module (column Bioshell: A160 Peptide C18 (10 cm × 3 mm × 2.7 μm)), coupled to a UV 2996 PDA detector and to an ESI-MS single quadrupole Micromass ZQ detector. For both instruments solvent systems A (0.1% TFA in H₂O) and B (0.1% TFA in ACN) were used.

Nuclear Magnetic Resonance (NMR). The ¹H and ¹³C NMR spectra were recorded on a 600 MHz Bruker Avance spectrometer. ¹H NMR of **1** was recorded with a 200 MHz Varian Gemini spectrometer. Spectra were recorded at a temperature of 300K and calibrated relative to TSP (0.00 ppm) as internal standard. 1D NMR spectra were recorded in the Fourier mode with quadrature detection. The water signal was suppressed with the watergate 3-9-19 pulse sequence.^[55] 1D NMR spectra were processed and analyzed using SpinWorks. 2D NMR (including 2D COSY^[56-57] and TOCSY^[58]) spectra were analyzed with NMRFAM Sparky software.^[61] Splitting patterns are indicated as follows: bs, broad singlet; s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet.

Synthesis of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**)

The synthesis was carried out according to literature procedures.^[46-47, 26]

Yield: 41% (3.0 g, 0.018 mol). ¹H NMR (200MHz, DMSO-*d*₆) δ 12.70 (bs, 1H, NH); 6.39 (s, 1H, CH); 2.45 (s, 3H, CH₃); 2.38 (s, 3H, CH₃). Spectral data are in accordance with literature data.^[26]

Synthesis of 8'-carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (2)

4-Methylpiperidine (**A**) (0.504 mL, 4.27 mmol, 1 equiv), was added to a 37% v/v aqueous solution of formaldehyde (37% v/v, 0.32 mL, 1equiv) and the solution was incubated at room temperature for 15min in an Eppendorf tube. The obtained solution was added to a solution of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (700 mg, 4.27 mmol) in MeOH (13 mL). The reaction mixture was stirred overnight, resulting in a yellowish solution strongly absorbing UV light ($\lambda = 366$ nm). MeOH was evaporated, then crude *Safirinium* derivative **2** was lyophilized from H₂O, recrystallized from MeOH/Acetone 1:4, and lyophilized from water again. 8'-Carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**2**) was obtained as a light yellow powder in 73% yield (868 mg, 3.14 mmol) as mixture of two products (peak A, Rt = 3.65 min and peak B, Rt = 3.92 min) displaying the same mass (Figure S1), thus corresponding to two diastereoisomers. After HPLC purification, it was possible to recover only the pure (1*r*, 4*r*) diastereoisomer (peak A) characterized by HPLC and ESI-MS (Figure S2), ¹H NMR (Figure S3, lower panel) and ¹³C NMR (Figure S4).

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3398, 2924, 1678, 1639, 1568, 1433, 1363, 1226, 1200, 1134, 835, 802, 723, 621, 532

¹H NMR (600MHz, 10% D₂O) δ 6.15 (s, 1H), 5.66 (s, 2H), 3.71 – 3.68 (m, 2H), 3.51 (dt, *J* = 13.38 Hz, *J* = 2.88 Hz, 2H), 2.26 (s, 3H), 2.24 (s, 3H), 1.94 – 1.91 (m, 2H), 1.80 – 1.72 (m, 1H), 1.58 – 1.51 (m, 2H), 0.93 (d, *J* = 6.61 Hz, 3H)

¹³C{¹H} NMR (151MHz, 10% D₂O) δ 171.8, 155.2, 148.6, 140.8, 115.8, 111.2, 75.4, 68.7, 21.3, 19.0, 17.6.

HR-ESI-MS: [*M*]⁺ 277.1790 (found), [*M*]⁺ 277.1790 (calc)

RP-HPLC-MS (gradient: 3-60% B in A in 5 min): Rt = 3.65 min and 3.92 min, MS: [*M*]⁺ 276.27 and 276.26 (found), [*M*]⁺ 276.17 (calcd).

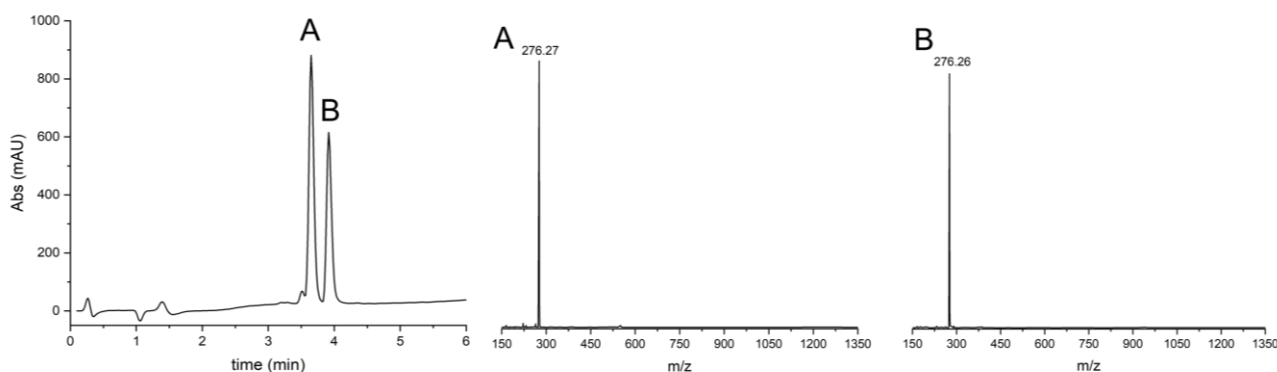


Figure S1. RP-HPLC-MS of the mixture of the two diastereoisomers of Safirinium derivative 8'-carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**2**), gradient: 3-60% B in A in 5 min: Rt = 3.65 min (peak A) and 3.92 min (peak B), MS: [*M*]⁺ 276.27 and 276.26 (found), [*M*]⁺ 276.17 (calcd.).

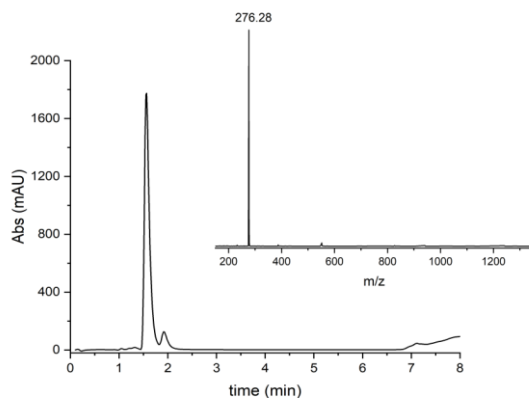


Figure S2. RP-HPLC-ESI-MS of *Safirinium* derivative (1*r*,4*r*)-8'-carboxy-4,5,7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (peak A in Figure S1), isocratic 15% B in A in 5 min: $R_t = 1.57$ min. MS: $[M]^+$ 276.28 (found), $[M]^+$ 276.17 (calcd.).

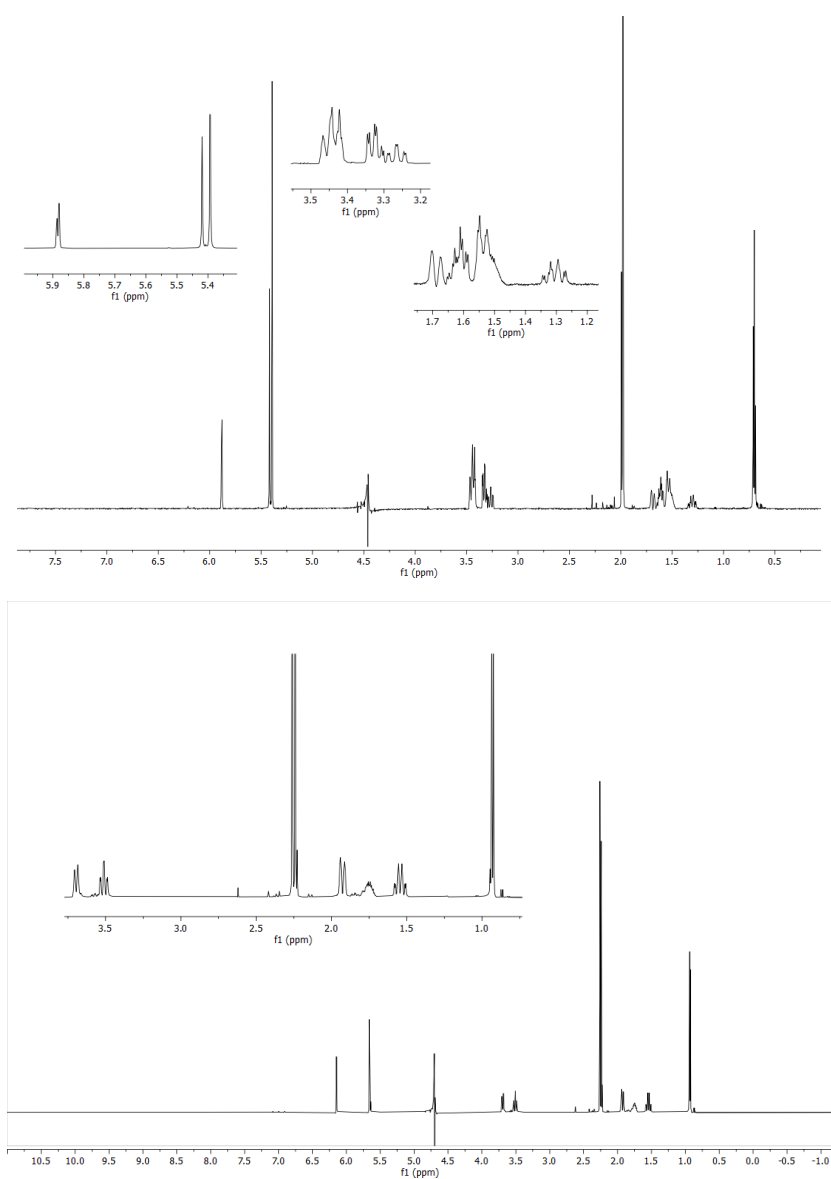


Figure S3. ^1H NMR of *Safirinium* derivative 8'-carboxy-4,5,7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**2**). Upper panel: ^1H NMR of the mixture containing both diastereoisomers. Lower panel: ^1H NMR of (1*r*,4*r*)-8'-carboxy-4,5,7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt.

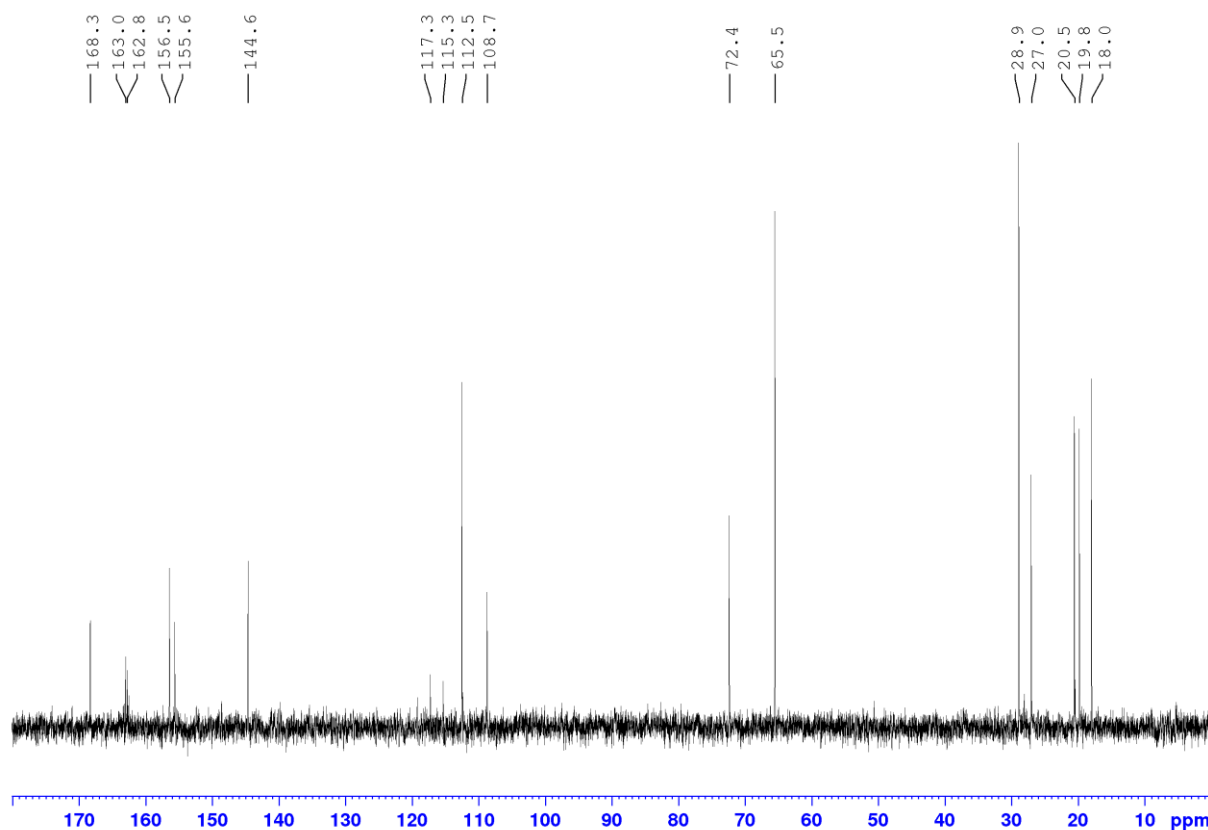


Figure S4. ^{13}C NMR of Safirinium derivative (1*r*,4*r*)-8'-carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt.

Synthesis of 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[pyrrolidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (3)

The synthesis was carried out according to the procedure reported in the literature.^[26]

Yield: 85% (905 mg, 3.65 mmol).

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3381, 3013, 2962, 2955, 2926, 1634, 1591, 1560, 1518, 1477, 1458, 1433, 1375, 1343, 1227, 1188, 1098, 1030, 935, 908, 797, 779, 717, 664, 594, 581, 554, 536

^1H NMR (600MHz, 10% D_2O) δ 6.05 (s, 1H), 5.72 (s, 2H), 3.78 – 3.72 (m, 4H), 2.29 – 2.22 (m, 2H), 2.18 (s, 3H), 2.17 – 2.10 (m, 2H), 2.08 (s, 3H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 171.8, 155.2, 148.6, 140.8, 115.9, 111.2, 75.4, 68.7, 21.3, 19.0, 17.6

HR-ESI-MS: $[\text{M}]^+$ 248.1395 (found), $[\text{M}]^+$ 248.1399 (calcd).

RP-HPLC-MS (gradient: 3-60% B in A in 5 min): R_t = 3.05 min, MS: $[\text{M}]^+$ 248.19 (found), $[\text{M}]^+$ 248.14 (calcd).

Spectral data are in accordance with literature data.^[26]

Synthesis of 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[morpholine-4,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-4-ium inner salt (4)

The synthesis was carried out according to the procedure reported in the literature.^[26]

Yield: 81% (914 mg, 3.46 mmol).

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3397, 3011, 2965, 1632, 1562, 1585, 1522, 1518, 1447, 1377, 1346, 1233, 1194, 1119, 1059, 961, 853, 802, 779, 702, 644, 594.

^1H NMR (600MHz, 10% D_2O) δ 6.08 (s, 1H), 5.74 (s, 2H), 4.22 (ddd, $J = 13.51$ Hz, $J = 7.90$ Hz, $J = 2.82$ Hz, 2H), 3.93 (ddd, $J = 13.57$ Hz, $J = 5.47$ Hz, $J = 3.09$ Hz, 2H), 3.78 – 3.74 (m, 2H), 3.67 – 3.63 (m, 2H), 2.21 (s, 3H), 2.09 (s, 3H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 171.7, 155.4, 148.9, 140.8, 116.1, 111.5, 76.4, 63.9, 61.7, 19.0, 17.6

HR-ESI-MS: $[\text{M}]^+$ 264.1341 (found), $[\text{M}]^+$ 264.1348 (calcd)

RP-HPLC-MS (gradient: 3-60% B in A in 5 min): $R_t = 1.72$ min, MS: MS: $[\text{M}]^+$ 264.20 (found), $[\text{M}]^+$ 264.13 (calcd).

Spectral data are in accordance with literature data.^[26]

Synthesis of 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**)

N-ethyl 2-propanamine (**D**) (0.517 mL, 4.27 mmol, 1 equiv), was added to a 37% v/v aqueous solution of formaldehyde (37% v/v, 0.32 mL, 1equiv) and the solution was incubated at room temperature for 15min in an Eppendorf tube. The obtained solution was added to a solution of 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (700 mg, 4.27 mmol) in MeOH (13 mL). The reaction mixture was stirred overnight, resulting in a yellowish solution strongly absorbing UV light ($\lambda = 366$ nm). MeOH was evaporated, then crude *Safirinium* derivative **5** was lyophilized from H_2O , recrystallized from MeOH/Acetone 1:4, and lyophilized from water again. 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**) was obtained as a light yellow powder in 87% yield (980 mg, 3.71 mmol).

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3410, 3275, 2986, 1636, 1589, 1555, 1528, 1458, 1429, 1369, 1341, 1225, 1196, 1184, 1153, 1121, 1070, 1032, 797, 777, 687, 619, 602

^1H NMR (600MHz, 10% D_2O) δ 6.03 (s, 1H), 5.63 (d, $J^2 = 9.85$ Hz, 1H), 5.56 (d, $J^2 = 9.85$ Hz, 1H), 3.85 (hept, $J^3 = 6.61$ Hz, 1H), 3.65 (s, $J = 6.85$ Hz, 1H), 3.47 (s, $J = 6.95$ Hz, 1H), 2.20 (s, 3H) 2.07 (s, 3H), 1.32 (d, $J = 6.06$ Hz, 3H), 1.30 (d, $J = 6.60$ Hz, 3H), 1.24 (t, $J = 7.15$ Hz)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 171.4, 155.2, 148.2, 140.2, 115.8, 111.1, 69.8, 68.9, 60.3, 18.8, 17.5, 15.7, 15.2, 7.2

HR-ESI-MS: $[\text{M}]^+$ 264.1701 (found), $[\text{M}]^+$ 264.1712 (calcd).

RP-HPLC-MS (gradient: 3-60% B in A in 5 min): $R_t = 3.35$ min, MS: $[\text{M}]^+$ 264.25 (found), $[\text{M}]^+$ 264.17(calcd). The peak at $m/z = 222.21$ corresponds to fragmentation of the ^iPr group in the ion source.

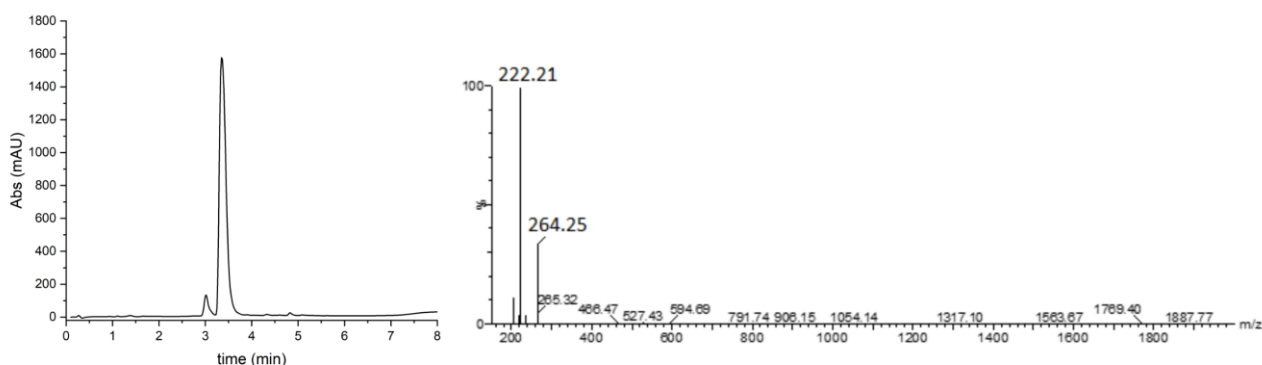


Figure S5. RP-HPLC-MS of Safirinium derivative 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**), gradient: 3-60% B in A in 5 min: Rt = 3.35 min, MS: [M]⁺ 264.25 (found), [M]⁺ 264.17(calcd). The peak at m/z = 222.21 corresponds to fragmentation of the ¹Pr group in the ion source.

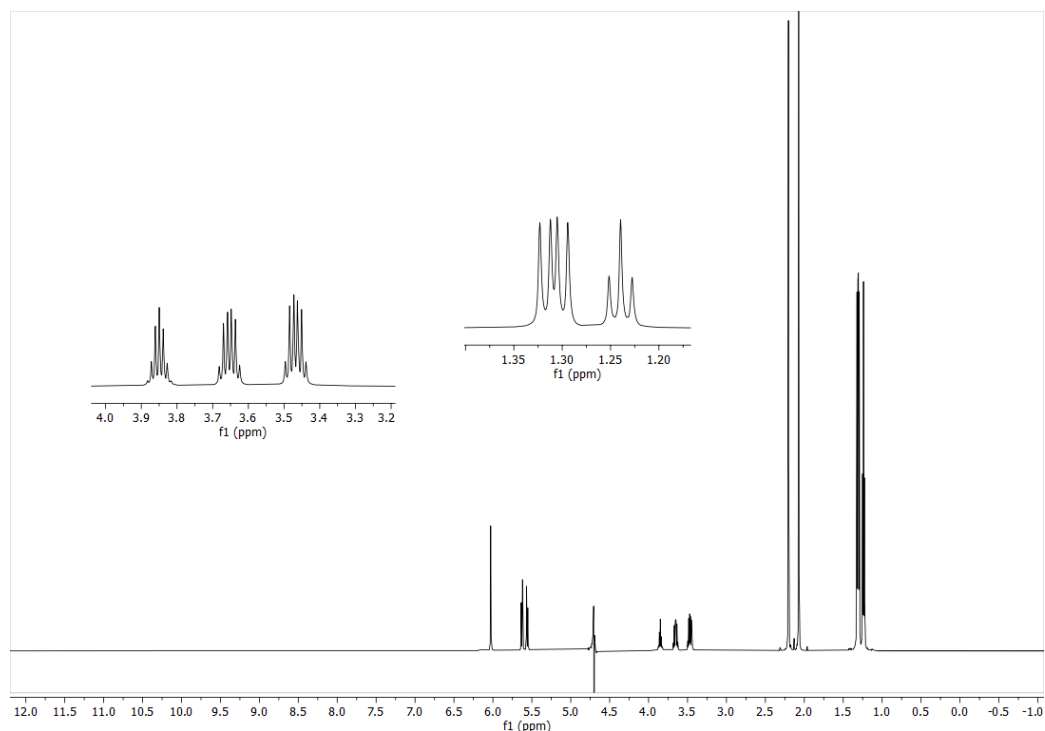


Figure S6. ¹H NMR of Safirinium derivative 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**).

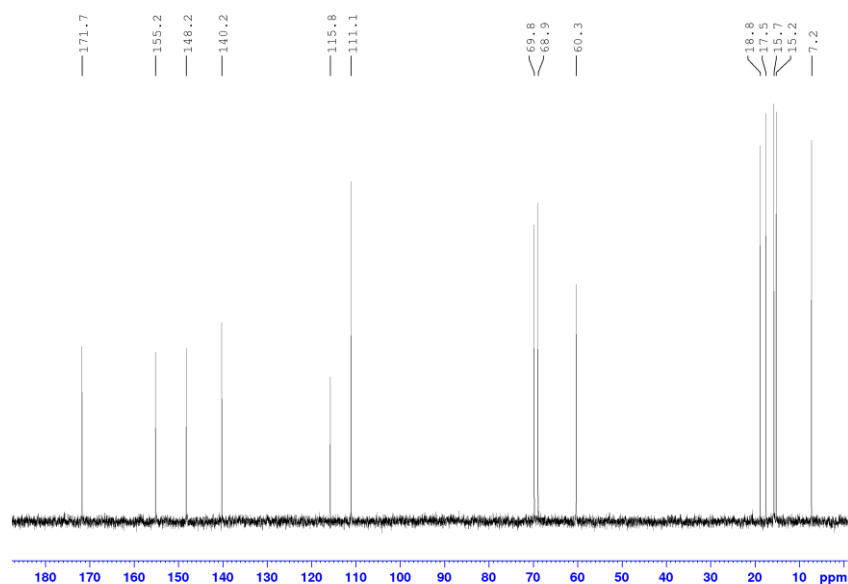
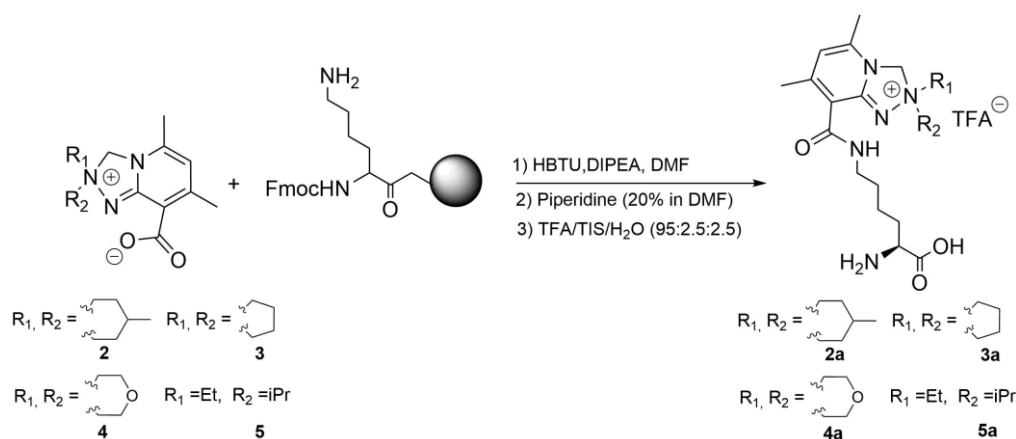


Figure S7. ¹³C NMR of Safirinium derivative 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**).

Deprotection of the Mtt protecting group on Fmoc-Lys(Mtt)-Wang resin

Deprotection of the Mtt protecting group on Fmoc-Lys(Mtt)-Wang resin (1 g, loading: 0.54 mmol/g) was performed suspending the resin in a solution of TFA/TIS/DCM, 1/5/94, v/v/v (1 mL/100 mg of resin) for 25 min. After filtration, the resin was shaken repeatedly with a solution of TFA/TIS/DCM, 1/5/94, v/v/v (1 mL/100 mg of resin) (3×10 min, then a series of 5 min cycles until complete discoloration), washing with DCM after each deprotection step. The deprotected aminoacyl resin was washed repeatedly with pure DCM, until the solvent was transparent and pH value was neutral. The deprotected resin was dried under vacuum and then used for the preparation of *Safirinium*-lysine conjugates **2a-5a** and of building blocks **2a'-5a'** following general procedures A and B respectively.

General procedure A



Fmoc-Lys-Wang resin (65 mg) was swelled in DMF for 30 min. A solution of *Safirinium* derivative (**2**, **3**, **4**, **5**), HBTU and DIPEA (2.5 equiv, 2.5 equiv and 4.5 equiv respectively) in DMF (1 mL) was added to the resin and shaken for 1 h. After filtration, a second coupling was performed using a solution of *Safirinium* derivative (**2**, **3**, **4**, **5**), HBTU, and DIPEA (1.5 equiv, 1.5 equiv, 2.8 equiv respectively) in DMF (1 mL). The resin was washed with DMF (3 x 3 mL) and DCM (2 x 3 mL) and dried under vacuum.

The obtained Fmoc-Lys[*N*ε(**2-5**)]-Wang resin was swelled in DMF for 20 min, then treated with piperidine (20% v/v in DMF, 1 x 3 mL for 3 min, then 1 x 3 mL for 7 min) to remove the Fmoc protecting group, washed with DMF (3 x 3 mL) and dried. Addition of the cleavage cocktail TFA/TIS/H₂O (95/2.5/2.5, v/v/v) to the resin and shaking for 3h at room temperature removed the H-Lys[*N*ε(**2-5**)]-OH from the resin. The solution was filtered off, the resin was washed additionally with 1 mL of cleavage cocktail. Then, cold diethyl ether was added to precipitate the crude product, which was centrifuged, additionally washed with cold diethyl ether, lyophilized, and purified by semipreparative RP-HPLC (gradient: 0%-30% B in A in 30 min) giving the products (**2a**, **3a**, **4a**, **5a**) as follows.

Synthesis of *N*⁶-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a**)

Following the **general procedure A**, **2a** was obtained as a mixture in 37% yield (3 mg, 0.007 mmol) from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**2**) (56.3 mg, 0.20 mmol). The mixture of the two products (R_t = 3.23 min and R_t = 3.67 min) was analysed by ¹H NMR (Figure S9). Moreover, considering that HR-ESI-MS (Figure S10) of the mixture displayed a single peak, we could hypothesize that we obtained the

two diastereoisomers. HPLC purification allowed to recover both the pure (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers that were analysed independently.

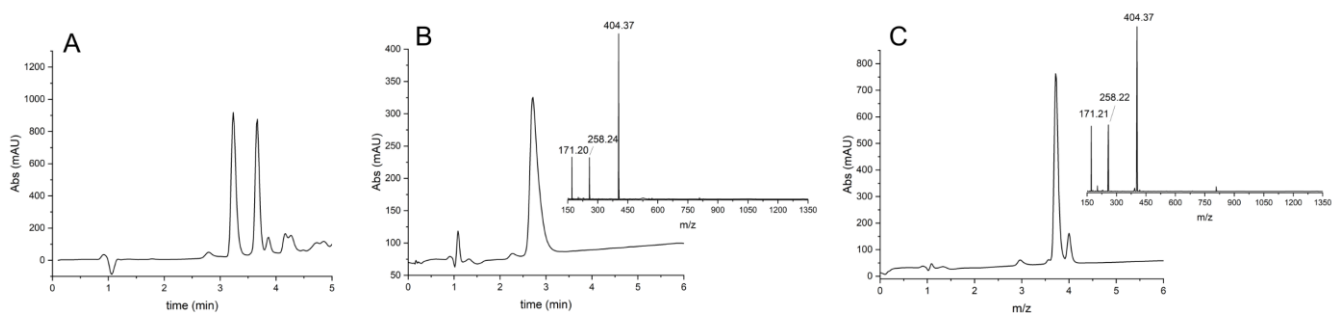


Figure S8. RP-HPLC-MS of *Safirinium*-lysine conjugate *N*⁶-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a**). (A) The chromatogram (gradient 5-40% B in A in 5 min) of the crude product shows two peaks corresponding to the two diastereoisomers. MS: [M]⁺ 404.37 (found), [M]⁺ 404.53.17(calcd). (B): HPLC-MS (gradient 5-30% B in A in 5 min) of the isolated peak at Rt = 3.23 min in chromatogram A. (C): HPLC-MS (gradient 5-30% B in A in 5 min) of the isolated peak at Rt = 3.67 min in chromatogram A.

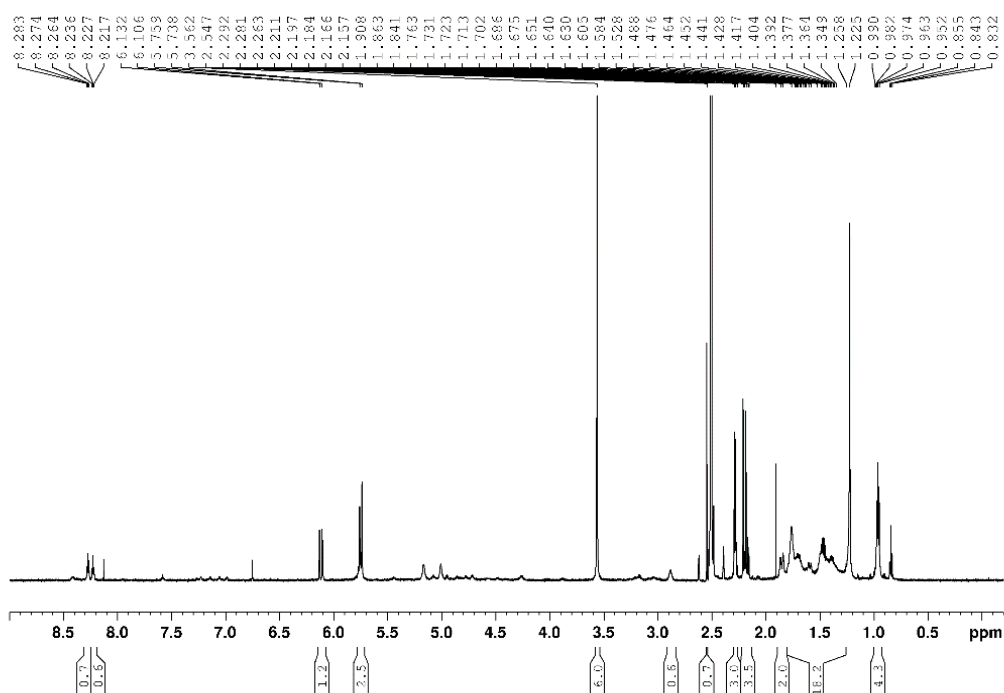


Figure S9. ¹H NMR (DMSO-*d*₆) of *Safirinium*-lysine conjugate *N*⁶-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a**). The measurement was carried out on the mixture containing both the (1*r*,4*r*) and the (1*s*,4*s*) diastereoisomers.

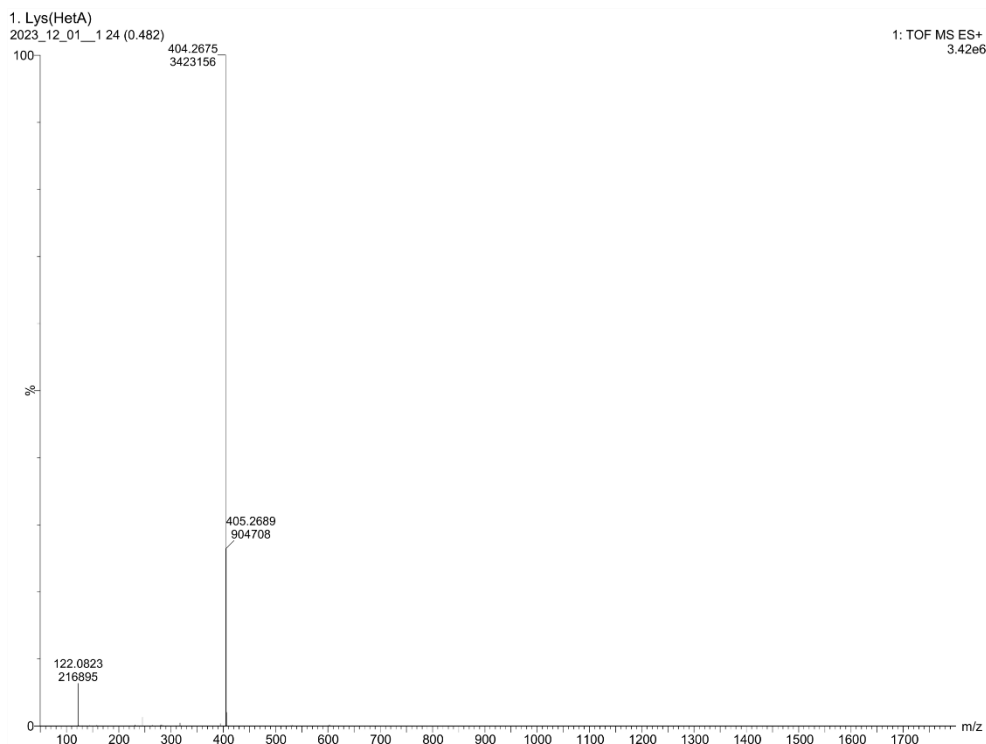


Figure S10. HR-ESI-MS spectrum of *Safirinium*-lysine conjugate *N*⁶-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a**), MS: [M]⁺ 404.2675 (found) [M]⁺ 404.2650 (calcd). The measurement was carried out on the mixture containing both the (1*r*,4*r*) and the (1*s*,4*s*) diastereoisomers.

Safirinium-lysine conjugate *N*⁶-[(1*s*,4*s*)-4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate

$[\alpha]_D^{20} = 400.8$ (*c* = 0.29, water)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3310, 3234, 2916, 2851, 1680, 1647, 1470, 1393, 1196, 1179, 1134, 1047, 721, 631.

¹H NMR (600MHz, 10% D₂O) δ 8.51 (t, *J* = 5.83 Hz, 1H), 7.74 (bs, 2H), 6.10 (s, 1H), 5.63 (s, 2H), 3.70 – 3.64 (m, 4H), 3.55 (dt, *J* = 11.68 Hz, *J* = 3.51 Hz, 2H), 3.29 (k, *J* = 6.89 Hz, 2H), 2.21 (s, 3H), 2.12 (s, 3H), 1.87 – 1.72 (m, 4H), 1.60 – 1.55 (m, 2H), 1.46 – 1.36 (m, 2H), 0.93 (d, *J* = 6.42 Hz, 3H) (Figure S11).

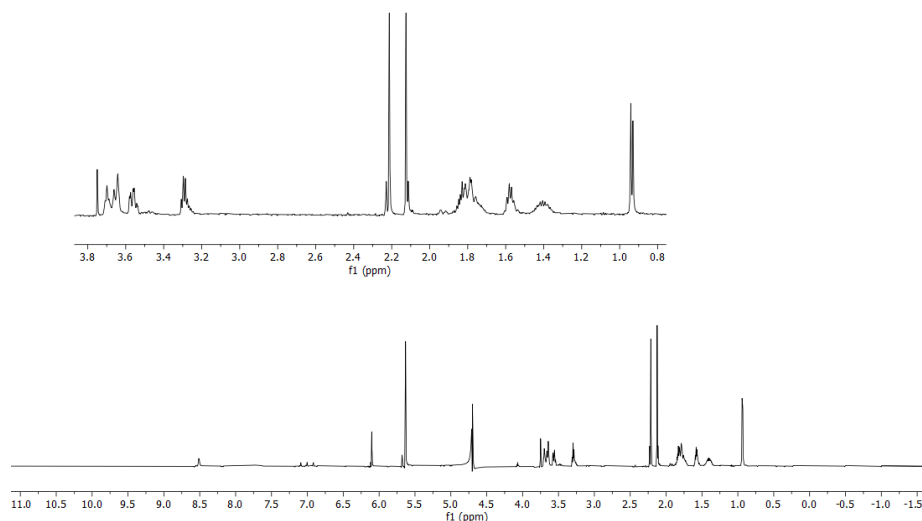


Figure S11. ^1H NMR (10% D_2O) of Safirinium-lysine conjugate N^6 -[(1*s*,4*s*)-4,5',7'-trimethylspiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate.

Synthesis of N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (3a**)**

Following the **general procedure A**, **3a** was obtained from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[pyrrolidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**3**) (51.5 mg, 0.21 mmol) in 72% yield (6 mg, 0.016 mmol, based on crude product).

$[\alpha]_D^{20} = 157.5$ ($c = 0.44$, water)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3394, 2913, 2849, 1672, 1641, 1566, 1425, 1368, 1200, 1182, 1134, 631, 536

^1H NMR (600MHz, $\text{DMSO}-d_6$) δ 8.22 (t, $J = 5.6\text{ Hz}$, 1H), 6.12 (s, 1H), 5.86 (s, 2H), 2.28 (s, 3H), 2.18 (m, 7H), 1.80-1.69 (m, 3H), 1.55-1.26 (m, 9H)

*- some signals overlapped with residual solvent signals

^1H NMR (600MHz, 10% D_2O) δ 8.53 (s, 1H), 7.75 (bs, 2H), 6.11 (s, 1H), 5.76 (s, 2H), 3.77 – 3.75 (m, 4H), 3.26 (k, $J = 6.54\text{ Hz}$, 2H), 2.24 (m, 2H), 2.21 (s, 3H), 2.15 (m, 2H), 2.11 (s, 3H), 1.83 – 1.81 (m, 2H), 1.56 (q, $J = 7.14\text{ Hz}$, 2H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 174.2, 166.3, 163.1, 162.8, 155.4, 152.6, 143.3, 117.3, 115.3, 111.3, 111.1, 75.3, 68.9, 54.5, 44.7, 39.4, 30.0, 27.8, 22.3, 21.8, 21.3, 18.8, 17.8, 16.3

HR-ESI-MS: $[\text{M}]^+$ 376.2329 (found), $[\text{M}]^+$ 376.2337 (calcd)

RP-HPLC-MS (gradient: 5-30% B in A in 5 min): $R_t = 1.72\text{ min}$ (purity: 98.3%), MS: MS: $[\text{M}]^+$ 376.33 (found), $[\text{M}]^+$ 376.24 (calcd)

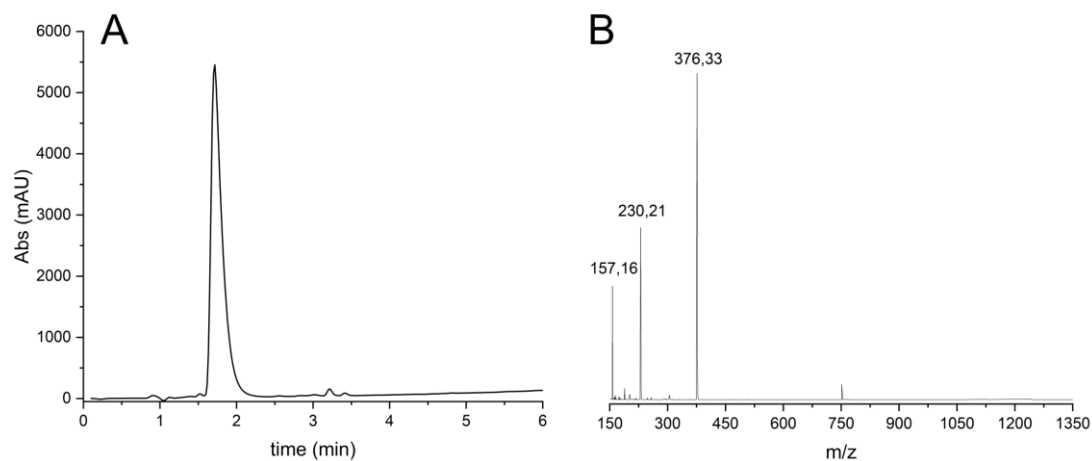


Figure S12. RP-HPLC-ESI-MS of Safirinium-lysine conjugate N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a**). A) RP-HPLC chromatogram (gradient 5-30% B in A, in 5 min), r.t. = 1.72 min. B) MS: $[M]^+$ 376.33 (found), $[M]^+$ 376.24 (calcd).

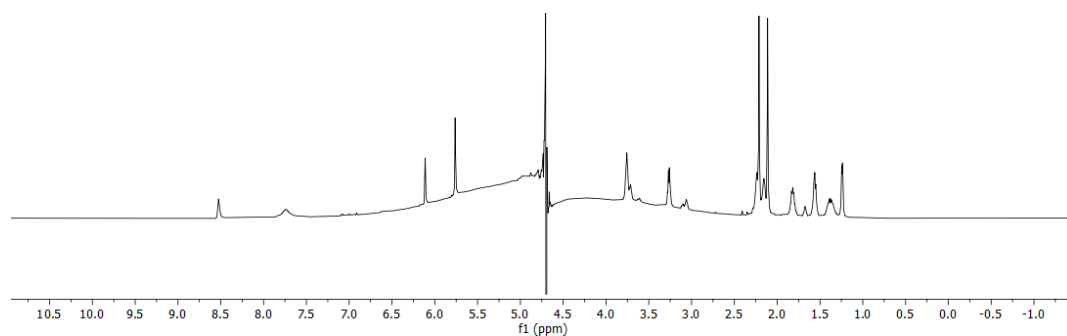


Figure S13. ^1H NMR spectrum (10% D_2O) of Safirinium-lysine conjugate N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a**).

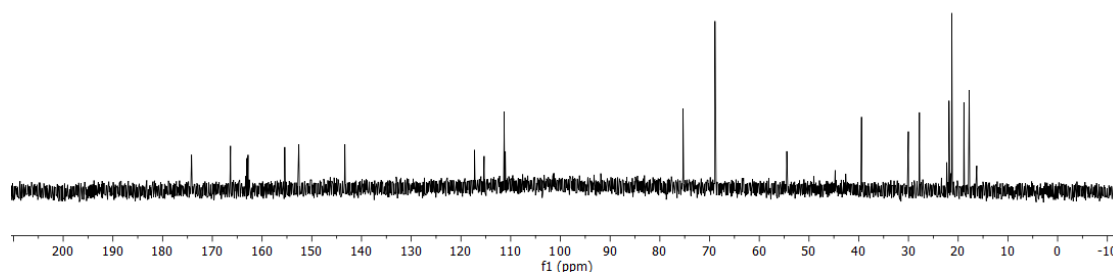


Figure S14. ^{13}C NMR spectrum (10% D_2O) of *Safirinium*-lysine conjugate *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a**).

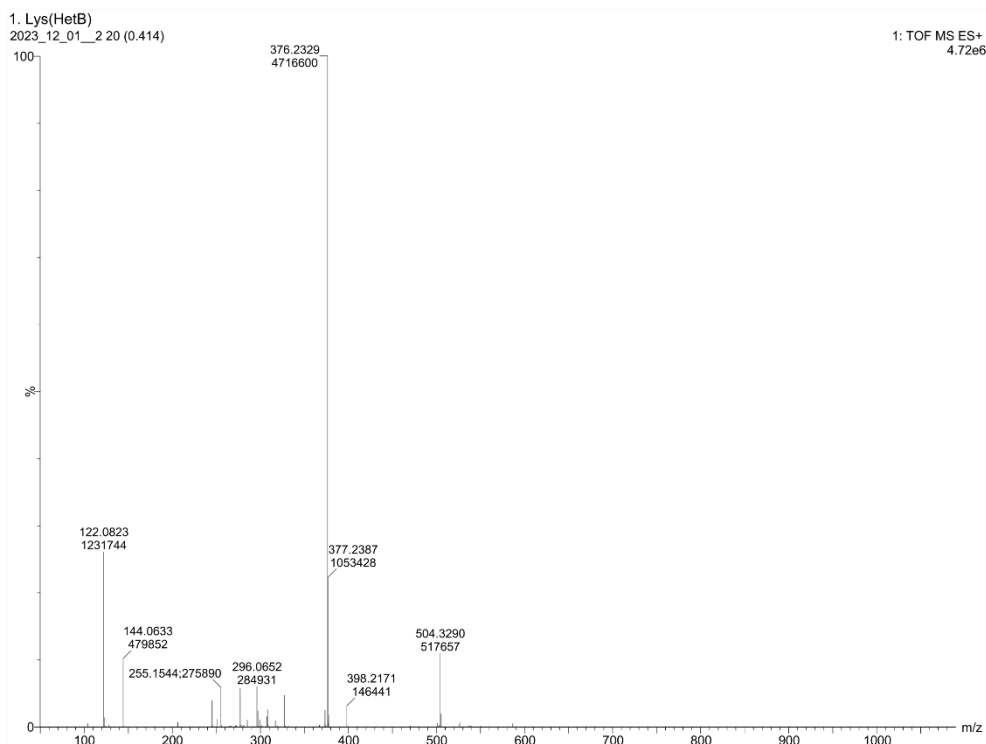


Figure S15. HR-ESI-MS spectra of *Safirinium*-lysine conjugate *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a**), MS: $[\text{M}]^+ 376.2329$ (found), $[\text{M}]^+ 376.2337$ (calcd).

Synthesis of *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a**)

Following the **general procedure A**, **4a** was obtained from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[morpholine-4,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-4-ium inner salt (**4**) (54.7 mg, 0.21 mmol) in 68% yield (5 mg, 0.013 mmol, based on crude product).

$[\alpha]_D^{20} = 78.1$ (c = 0.71, water)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3362, 2916, 2851, 1703, 1680 1369, 1202, 1182, 632, 534.

^1H NMR (600MHz, DMSO- d_6) δ 8.24 (t, J=5.7Hz, 1H), 6.17 (s, 1H), 5.85 (s, 2H), 2.30(s, 3H), 2.20 (s, 3H), 1.83 – 1.26 (m, 11H)

*- some signals overlapped with residual solvent signals

^1H NMR (600MHz, 10% D_2O) δ 8.55 (t, J = 5.44 Hz, 1H), 6.15 (s, 1H), 5.79 (s, 2H), 4.20 – 4.16 (m, 2H), 3.97 – 3.93 (m, 2H), 3.81 – 3.75 (m, 2H), 3.67 – 3.64 (m, 2H), 3.31 – 3.28 (k, J = 6.54 Hz, 2H), 2.24 (s, 3H), 2.13 (s, 3H), 1.87 – 1.82 (m, 2H), 1.61 – 1.56 (m, 2H), 1.44 – 1.38 (m, 2H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 173.9, 166.3, 155.6, 153.1, 143.5, 115.3, 111.7, 111.3, 64.1, 61.7, 54.2, 39.4, 29.9, 27.9, 21.8, 18.9, 17.8

HR-ESI-MS: found: 392.2303Da $[\text{M}]^+$, calcd: 392.2287Da $[\text{M}]^+$

RP-HPLC-MS (gradient: 5-30% B in A in 5 min): $R_t = 1.23$ min (purity: 98.4%), MS: $[\text{M}]^+ 392.34$ (found), $[\text{M}]^+ 392.23$ (calcd)

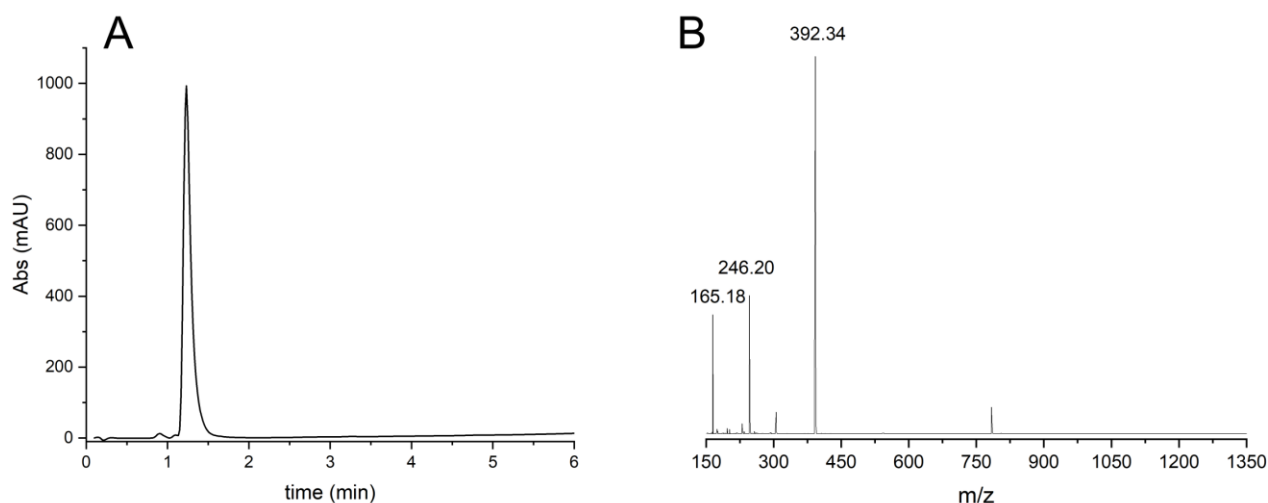


Figure S16. RP-HPLC-ESI-MS of Safirinium-lysine conjugate N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a**). A) RP-HPLC chromatogram (gradient 5-30% B in A), in 5 min, $r.t. = 1.23$ min. B) MS: $[\text{M}]^+ 392.34$ (found), $[\text{M}]^+ 392.23$ (calcd).

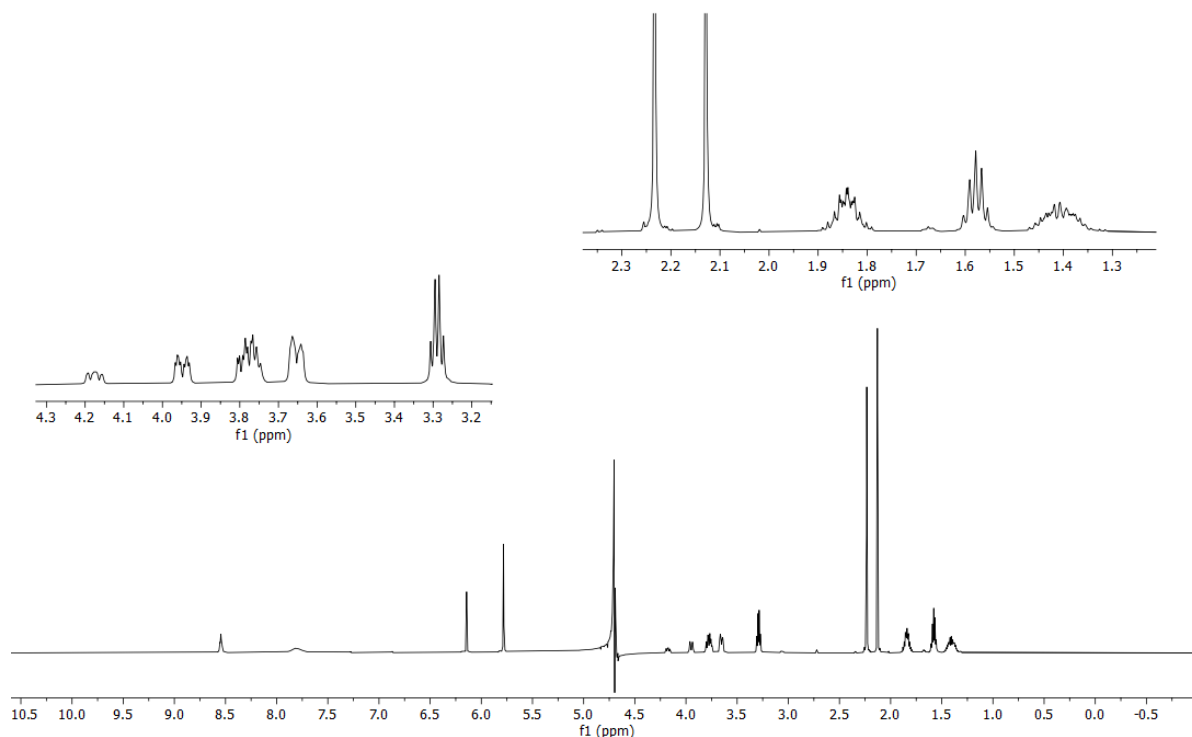


Figure S17. ^1H NMR (10% D_2O) spectrum of *Safirinium*-lysine conjugate *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a**).

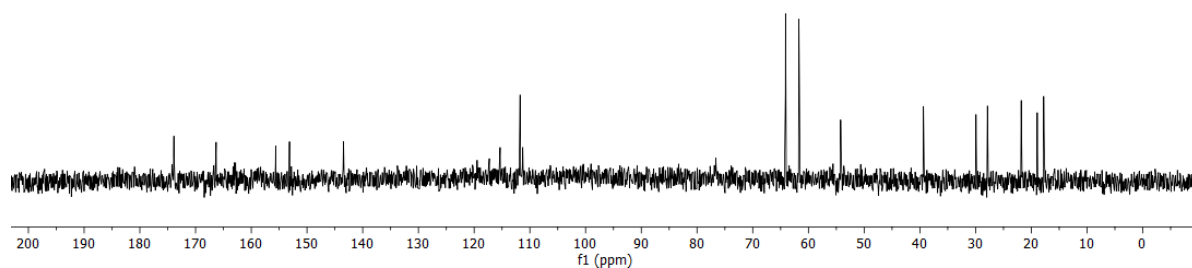


Figure S18. ^{13}C NMR (10% D_2O) spectrum of *Safirinium*-lysine conjugate *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a**).

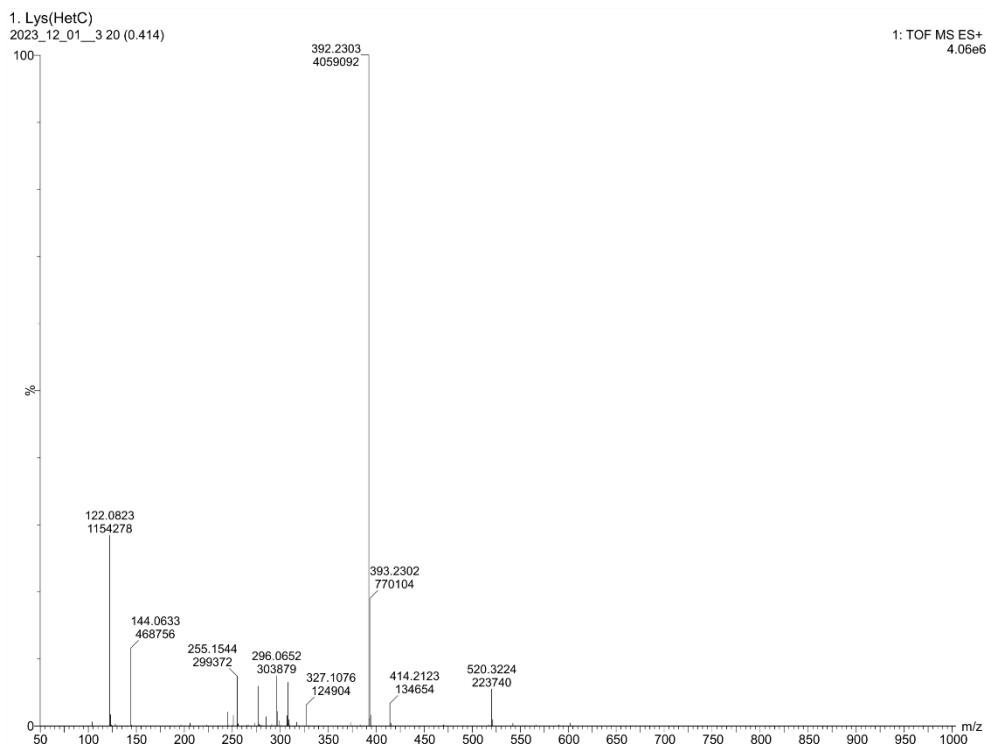


Figure S19. HR-ESI-MS spectrum of *Safirinium*-lysine conjugate *N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a**), MS: [M]⁺ 392.2303 (found) [M]⁺ 392.2287 (calcd).

Synthesis of *N*⁶-[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**)

Following the **general procedure A**, **5a** was obtained from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**) (54.8 mg, 0.21 mmol) in 75% yield (6.3 mg, 0.016 mmol, based on crude product).

$[\alpha]_D^{20} = 133.2$ (*c* = 0.58, water)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3277, 2916, 2851, 1670, 1640, 1557, 1433, 1262, 1179, 1126, 833798, 719, 546.

¹H NMR (600MHz, DMSO-*d*₆) δ 8.34 (t, *J*=5.6Hz, 1H), 6.09 (s, 1H), 5.74 (dd, *J*=10.0Hz *J*=55.3Hz, 2H), 2.32 (s, 3H), 2.16 (s, 3H), 1.74 – 1.65 (m, 4H), 1.54 – 1.30 (m, 17H)

*- some signals overlapped with residual solvent signals

¹H NMR (600MHz, 10% D₂O) δ 8.63 (t, *J* = 5.22 Hz, 1H), 6.09, (s, 1H), 5.67 (d, *J* = 9.88 Hz, 1H), 5.60 (d, *J* = 9.52 Hz, 1H), 3.88 (q, *J* = 6.54 Hz, 1H), 3.75 (t, *J* = 6.03 Hz, 1H), 3.66 (sext, *J* = 6.71 Hz, 1H), 3.52 – 3.47 (m, 1H), 3.30 – 3.26 (m, 2H), 2.23 (s, 3H), 2.09 (s, 3H), 1.89 – 1.78 (m, 2H), 1.60 – 1.55 (m, 2H), 1.44 – 1.39 (m, 2H), 1.32 (d, *J* = 6.56 Hz, 3H), 1.29 (d, *J* = 6.55 Hz, 3H), 1.23 (t, *J* = 7.21 Hz, 3H)

¹³C{¹H} NMR (151MHz, 10% D₂O) δ 173.9, 166.5, 163.0, 162.8, 155.4, 152.2, 142.9, 130.2, 126.8, 117.3, 111.1, 69.9, 69.1, 60.4, 54.3, 39.5, 30.0, 27.8, 21.9, 18.6, 17.7, 15.7, 15.2, 7.2

HR-ESI-MS: found: 392.2663Da [M]⁺, calcd: 392.2651Da [M]⁺

RP-HPLC-MS (gradient: 5-30% B in A in 5 min): *R*_t = 2.37 min (purity: 95.5%), MS: [M]⁺ 392.36 (found), [M]⁺ 392.52 (calcd)

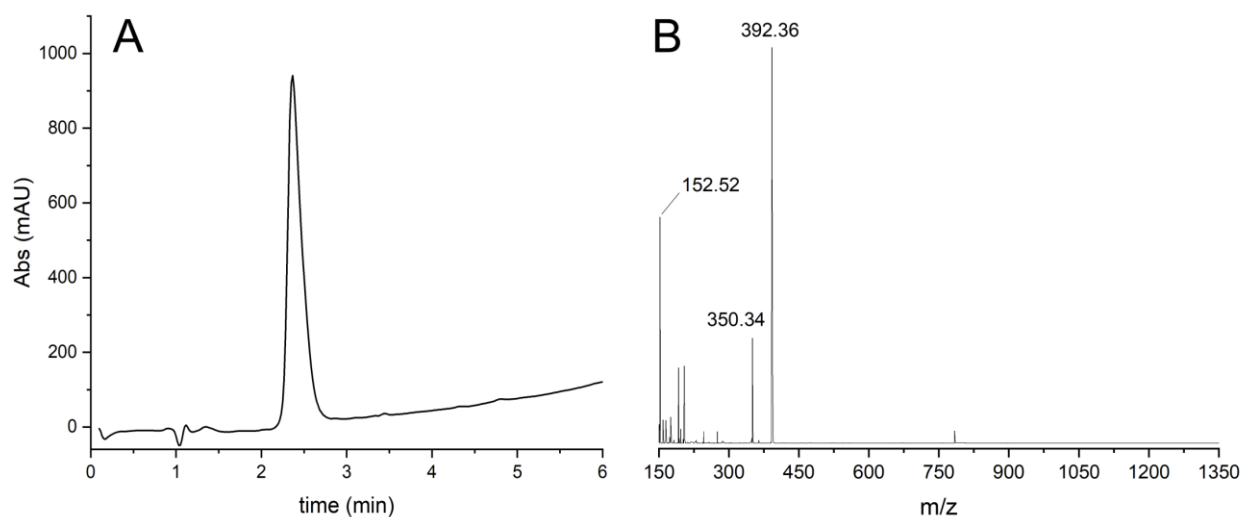


Figure S20: RP-HPLC-ESI-MS of *Safirinium*-lysine conjugate *N*⁶-[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-a]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**). A) RP-HPLC chromatogram (gradient 5-30% B in A, in 5 min), *R*_t = 2.37 min. B) MS: [*M*]⁺ 392.36 (found), [*M*]⁺ 392.52 (calcd).

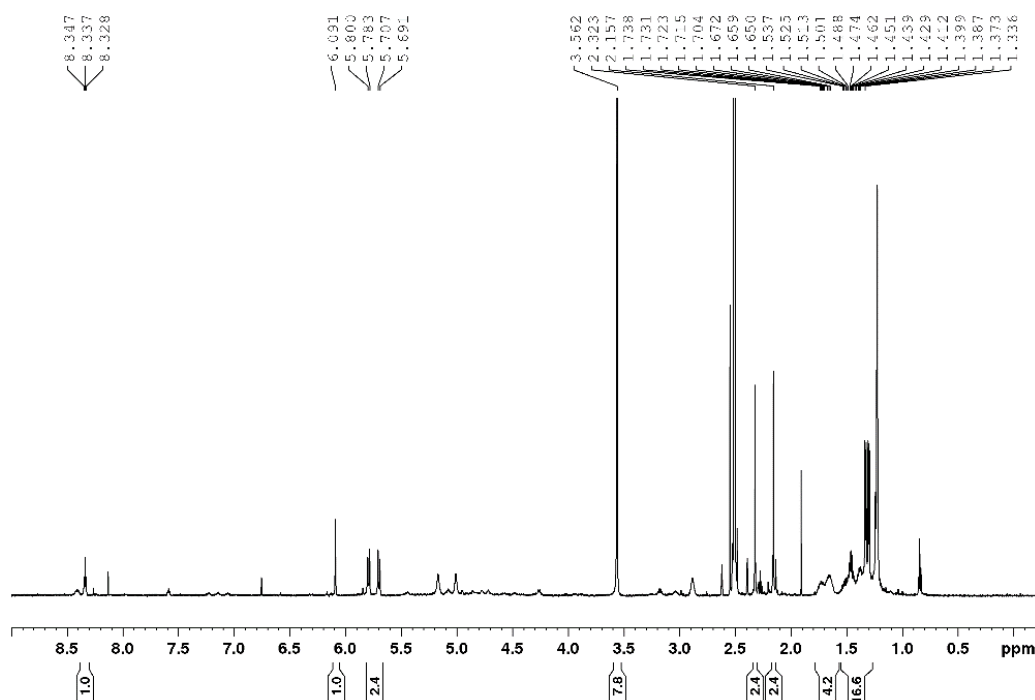


Figure S21. ¹H NMR (DMSO-*d*₆) spectrum of *Safirinium*-lysine conjugate *N*⁶-[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-a]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**).

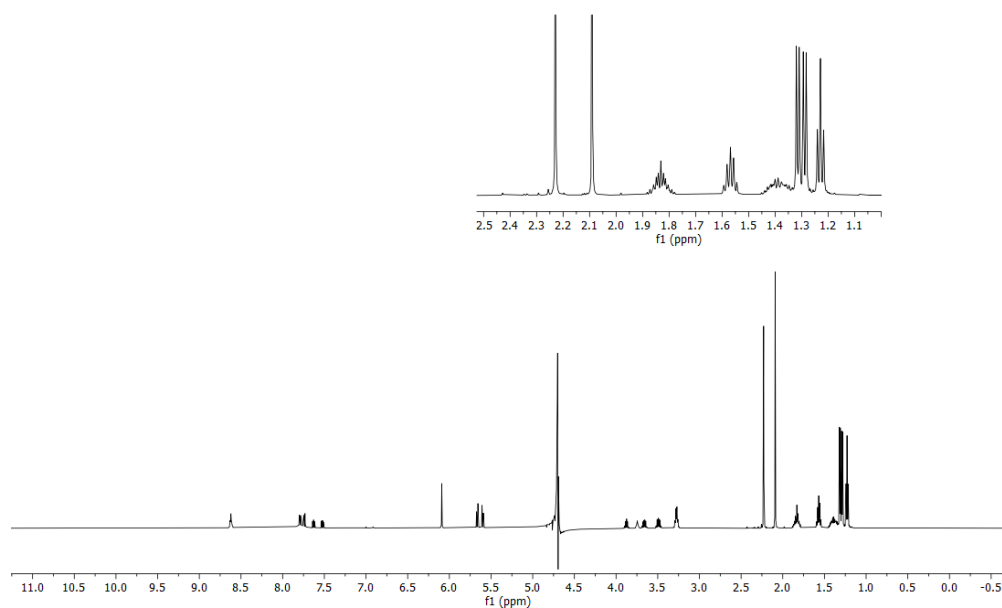


Figure S22. ^1H NMR (10% D_2O) spectrum of *Safirinium*-lysine conjugate N^6 -[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-a]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**).

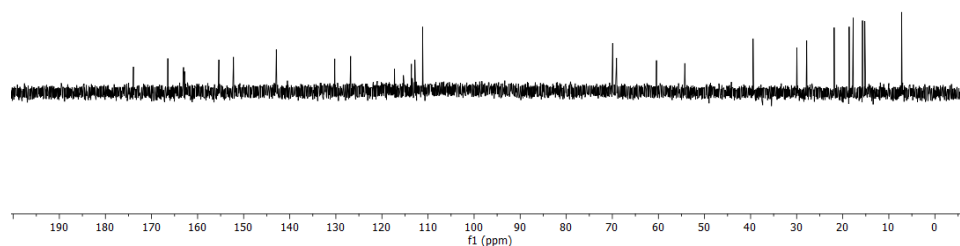


Figure S23. ^{13}C NMR (10% D_2O) spectrum of *Safirinium*-lysine conjugate N^6 -[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-a]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**).

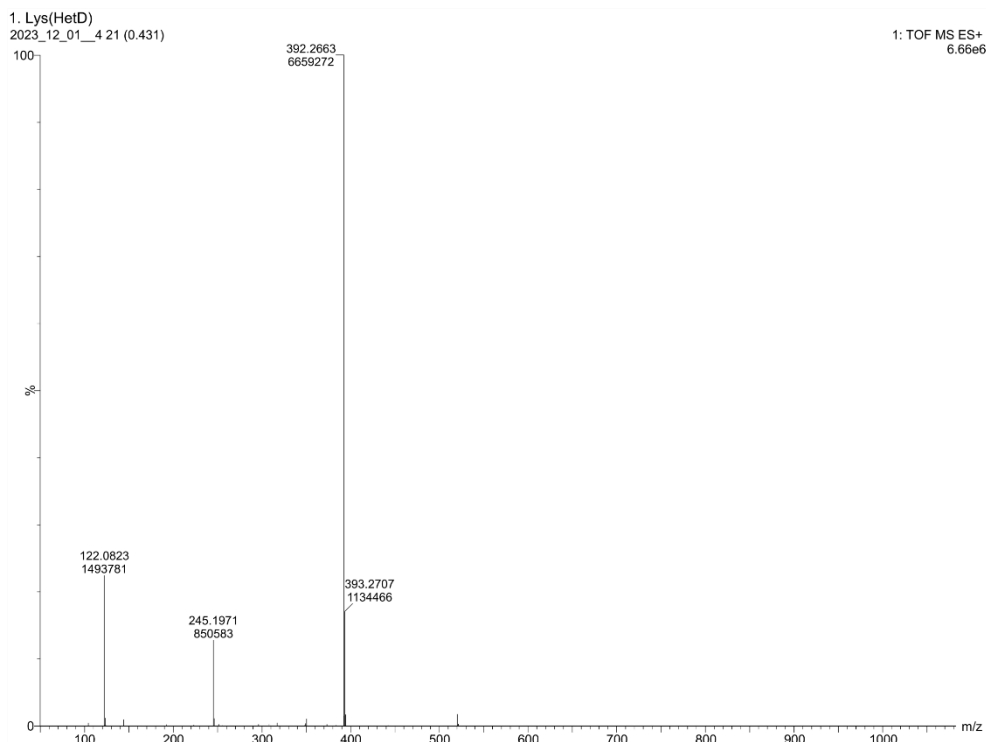
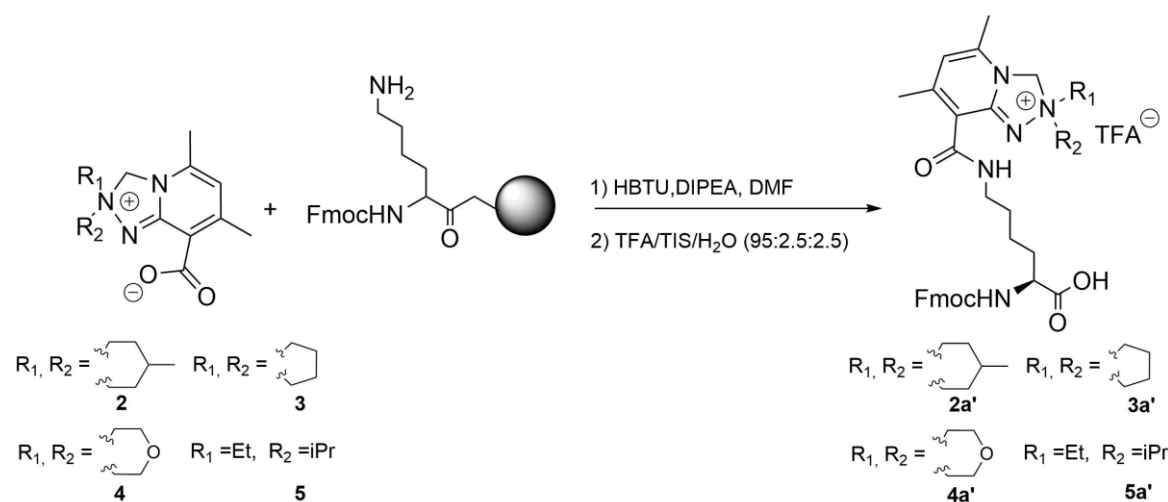


Figure S24. HR-ESI-MS spectrum of *Safirinium*-lysine conjugate *N*⁶-[2-ethyl-2-(1-methylethyl)-5,7-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-a]pyridin-2-ium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a**), MS: [M]⁺ 392.2663 (found), [M]⁺ 392.2651 (calcd).

General procedure B



Fmoc-Lys-Wang resin (150 mg) was swelled in DMF for 30 min. A solution of *Safirinium* derivative (**2**, **3**, **4**, **5**), HBTU and DIPEA (2.5 equiv, 2.5 equiv and 4.5 equiv respectively) in DMF (1 mL) was added to the resin and shaken for 1 h. After filtration, a second coupling was performed using a solution of *Safirinium* derivative (**2**, **3**, **4**, **5**), HBTU, and DIPEA (1.5 equiv, 1.5 equiv, 2.8 equiv respectively) in DMF (1 mL). The resin was washed with DMF (3 x 3 mL) and DCM (2 x 3 mL) and dried under vacuum. The obtained Fmoc-Lys[Nε(**2-5**)]-Wang resin was shaken with the cleavage cocktail TFA/TIS/H₂O (95/2.5/2.5, v/v/v) for 3h at room temperature to remove the building block Fmoc-Lys[Nε(**2-5**)]-OH from

the resin. The solution was filtered off, the resin was washed additionally with 1 mL of cleavage cocktail. Then, cold diethyl ether was added to precipitate the crude product, which was centrifuged, additionally washed with cold diethyl ether, lyophilized and purified by semipreparative RP-HPLC (gradient 30%-90% B in A in 30 min) giving the desired product (**2a'**, **3a'**, **4a'**, **5a'**) as follows.

Synthesis of N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (2a'**)**

Following the **general procedure B**, **2a'** was obtained in 85% yield (32 mg, 0.05 mmol, based on crude product) from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-4,5',7'-trimethyl-3'*H*-spiro[piperidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**2**) (55 mg + 33.5 mg, 0.32 mmol).

$[\alpha]_D^{20} = 105.1$ (c = 0.53, DMSO)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3298, 2916, 2851, 1643, 1557, 1450, 1180, 1130, 1043, 831, 798, 719, 544.

^1H NMR (600MHz, DMSO- d_6) δ 8.27 (t, *J* = 5.6 Hz, 1H)#, 8.22 (t, *J* = 5.7 Hz, 1H)*, 7.91 (d, *J* = 7.6 Hz, 4H)#, 7.75 – 7.73 (m, 4H)#, 7.68 (d, *J* = 8.1 Hz)#, 7.66 (d, *J* = 8.0 Hz, 1H)*, 7.46 (t, *J* = 4.7 Hz, 4H)#, 7.34 (t, *J* = 7.3 Hz, 4H)#, 6.16 (s, 1H)#, 6.13 (s, 1H)*, 5.81 (s, 2H), 5.79 (s, 2H), 4.35 – 4.25 (m, 6H)#, 3.99 – 3.93 (m, 4H)#, 3.71 – 3.68 (m, 2H)3*, 3.30 – 3.17 (m, 4H)#, 2.32 (d, *J* = 1.4 Hz, 6H), 2.25 (s, 3H), 2.21 (s, 3H), 1.82 – 1.39 (H, 13H) 0.95 (t, *J* = 6.7 Hz, 3H)

* - second proton overlapped by residual water signal

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, DMSO- d_6) δ 175.4, 163.4#, 163.3*, 156.6(8)#, 156.6(6)*, 155.5#, 155.4*, 152.4#, 151.5*, 144.3, 144.2(5)#, 144.2(2)*, 143.4#, 143.2*, 141.2(1)#, 141.1(9)*, 128.3#, 128.2*, 127.5(7)#, 125.8#, 125.7*, 120.6, 112.5,# 112.3*, 111.0#, 110.5*, 66.1#, 66.0*, 65.1#, 64.9*, 54.3#, 54.1*, 47.1, 38.9#, 38.7*, 30.9#, 30.8*, 29.5, 29.1#, 29.0*, 28.9#, 28.8*, 27.5, 23.5#, 23.4#, 23.2*, 21.2#, 20.7*, 20.3#, 20.1*, 18.5(4)#, 18.4(8)*

#, * - doublet signals (2 diastereoisomers).

(In all cases, for ^1H spectra the intensity of the signals is disturbed due to water suppression with the 3-9-19 watergate sequence, so the signal intensity is based on the interpretation of the 2D spectra)

RP-HPLC-MS: (gradient: 30-90% B in A in 5 min): R_t = 3.98 min, MS: MS: $[\text{M}]^+$ 626.48 (found), $[\text{M}]^+$ 626.78 (calcd).

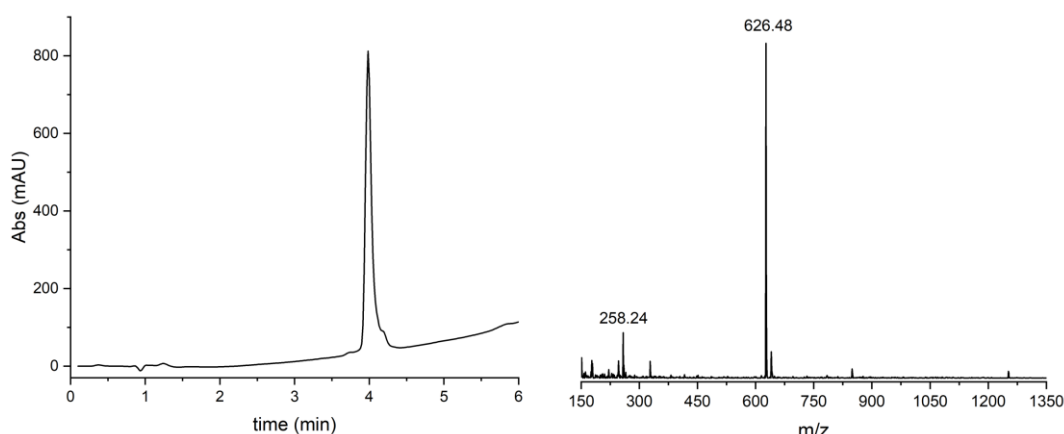


Figure S25. RP-HPLC-ESI-MS of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a'**), mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers. Left panel: RP-HPLC chromatogram

(gradient 30-90% B in A, in 5 min), Rt = 3.98 min. Right panel: MS: $[M]^+$ 626.48 (found), $[M]^+$ 626.78 (calcd).

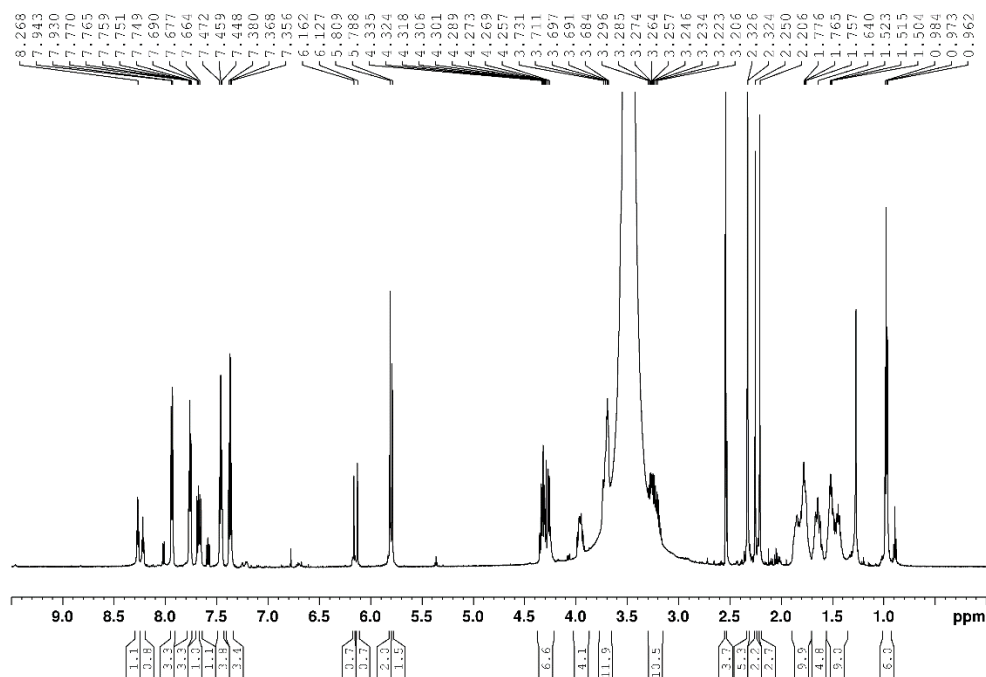


Figure S26. ^1H NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[4,5,7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a'**), mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers.

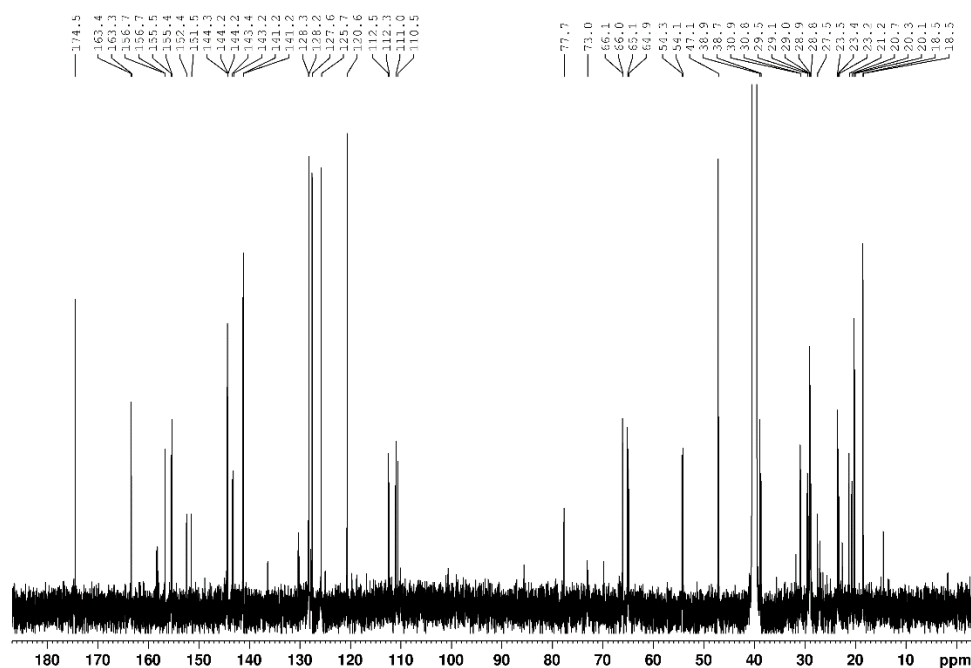


Figure S27. ^{13}C NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[4,5,7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**2a'**), mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers.

Synthesis of N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (3a'**)**

Following the **general procedure B**, **3a'** was obtained in 96% yield (33 mg, 0.06 mmol, based on crude product) from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[pyrrolidine-1,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-1-ium inner salt (**3**) (50.2 mg + 25.1 mg, 0.32 mmol).

$[\alpha]_D^{20} = 96.0$ ($c = 0.51$, DMSO)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3279, 2916, 1643, 1567, 1450, 1179, 1130, 1041, 833, 800, 762, 741, 719, 644, 600, 544.

^1H NMR (600MHz, DMSO- d_6) δ 8.19 (t, $J = 5.6$ Hz, 1H), 7.91 (d, $J = 7.5$ Hz, 2H), 7.75 – 7.73 (m, 2H), 7.64 (d, $J = 8.1$ Hz, 1H), 7.43 (t, $J = 7.3$ Hz, 2H), 7.34 (t, $J = 7.4$ Hz, 2H), 6.12 (s, 1H), 5.89 (s, 1H), 4.32 – 4.22 (m, 4H), 3.94 – 3.90 (m, 1H), 3.81 – 3.78 (m, 4H), 3.25 – 3.15 (m, 2H), 2.29 (s, 3H), 2.19 (s, 3H), 2.18 – 2.10 (m, 4H), 1.77 – 1.71 (m, 1H), 1.66 – 1.59 (m, 1H), 1.52 – 1.38 (m, 4H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, DMSO- d_6) δ 174.5, 163.4, 158.3, 158.1, 156.7, 155.6, 151.8, 144.3(2), 144.2(6), 143.1, 142.2(1), 142.1(9), 130.3, 128.1, 127.6, 127.5, 125.7, 120.6(1), 120.6(0), 112.3, 110.7, 74.7, 68.5, 66.1, 54.3, 47.1, 40.5, 30.9, 28.9, 23.5, 21.8, 20.1, 18.5

RP-HPLC-MS: (gradient: 30-90% B in A in 5 min): $R_t = 3.47$ min, MS: $[\text{M}]^+ 598.47$ (found), $[\text{M}]^+ 598.72$ (calcd)

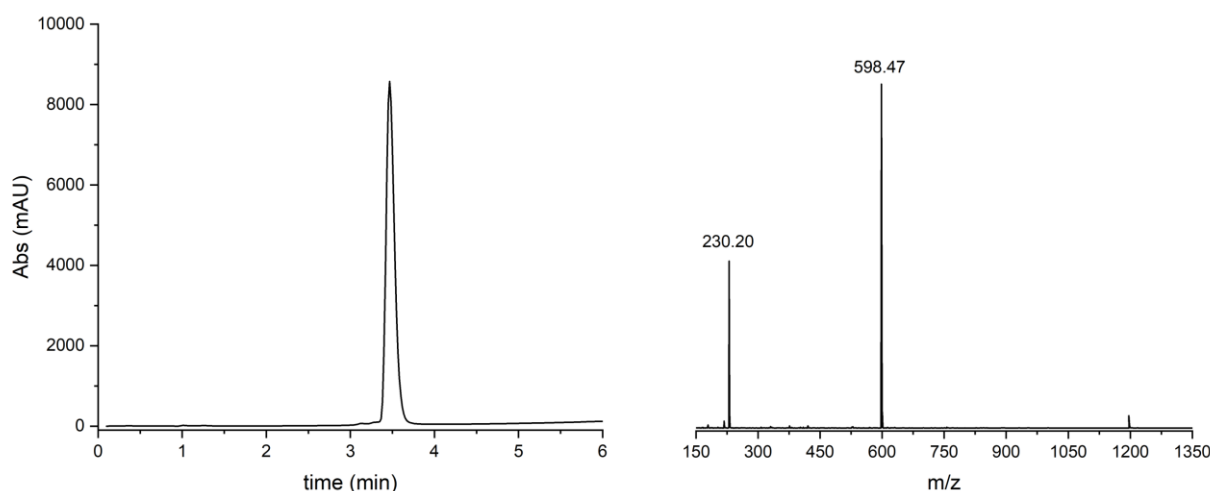


Figure S28. RP-HPLC-ESI-MS of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a'**). Left panel: RP-HPLC chromatogram (gradient 30-90% B in A, in 5 min), $R_t = 3.47$ min. Right panel: MS: $[\text{M}]^+ 598.47$ (found), $[\text{M}]^+ 598.72$ (calcd).

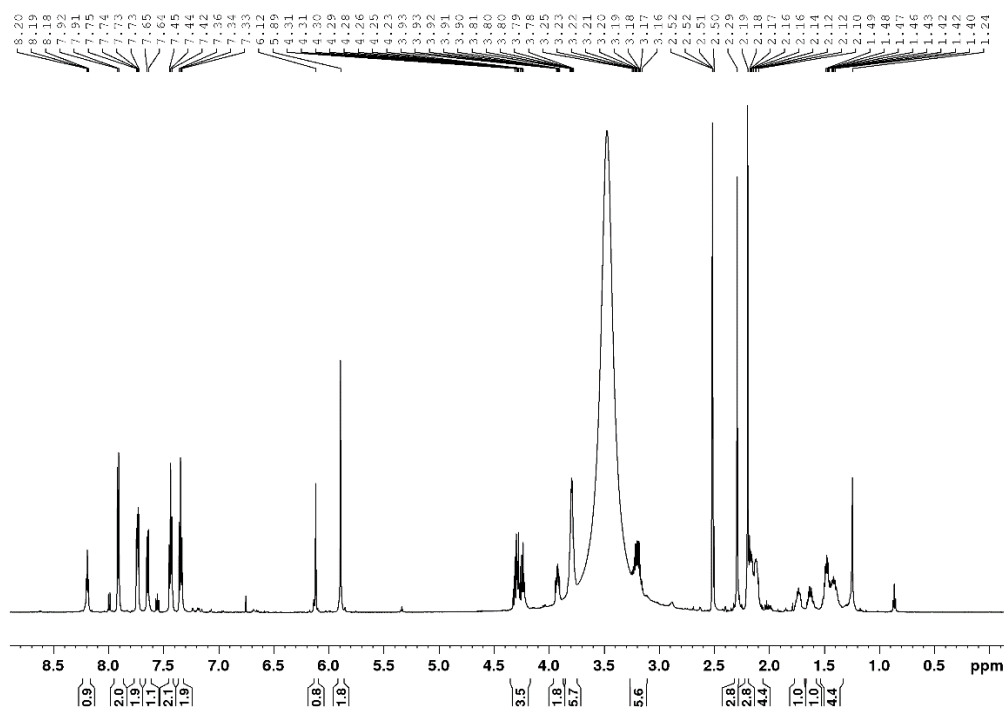


Figure S29. ^1H NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a'**).

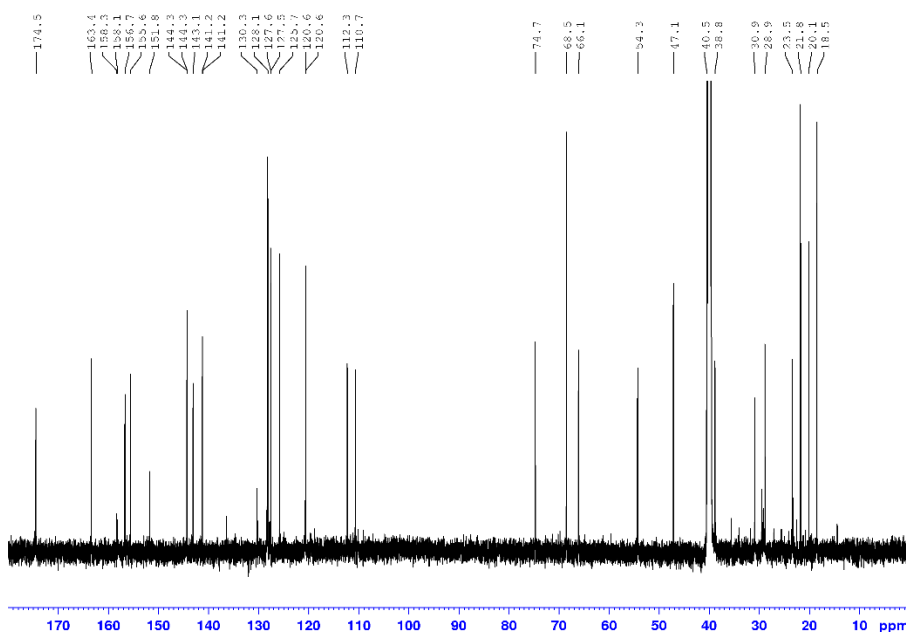


Figure S30. ^{13}C NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**3a'**).

Synthesis of N^2 -((9*H*-fluoren-9-yl)methoxycarbonyl)- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (4a'**)**

Following the **general procedure B**, **4a'** was obtained in 90% yield (30 mg, 0.05 mmol, based on crude product) from the reaction of Lys Wang resin with 8'-carboxy-5',7'-dimethyl-3'*H*-spiro[morpholine-4,2'-[1,2,4]triazolo[4,3-*a*]pyridin]-4-ium inner salt (**4**) (53.5 mg + 32 mg, 0.32 mmol).

$[\alpha]_D^{20} = 82.3$ ($c = 0.51$, DMSO)

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3296, 2916, 2851, 1651, 1557, 1180, 1128, 1069, 833, 800, 741, 719, 644, 602, 584.

^1H NMR (600MHz, DMSO- d_6) δ 8.21 (t, $J = 5.6$ Hz, 1H), 7.91 (d, $J = 7.5$ Hz, 2H), 7.75 – 7.73 (m, 2H), 7.64 (d, $J = 8.1$ Hz, 1H), 7.43 (t, $J = 7.4$ Hz, 2H), 6.17 (s, 1H), 5.87 (s, 2H), 4.32 – 4.22 (m, 4H), 4.09 – 5.05 (m, 2H), 3.96 – 3.92 (m, 4H), 3.84 – 3.81 (m, 2H), 3.65 – 3.64 (m, 2H), 3.26 – 3.10 (m, 1H), 2.88 (bs, 2H), 2.31 (s, 3H), 2.21 (s, 3H), 1.79 – 1.73 (m, 1H), 1.67 – 1.61 (m, 1H), 1.56 – 1.41 (m, 5H),

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, DMSO- d_6) δ 174.5, 163.3, 156.7, 155.6, 152.2, 144.3(3), 144.2(7), 143.2, 141.2(2), 141.1(9), 130.3, 128.3, 128.1, 127.6, 127.5, 125.8, 120.6(2), 120.6(1), 112.6, 111.0, 66.1, 64.3, 61.8, 54.2, 54.0, 47.1, 44.6, 40.5, 38.9, 30.9, 29.5, 23.5, 20.1, 18.5

RP-HPLC-MS: (gradient: 30-90% B in A in 5 min): $R_t = 3.40$ min, MS: $[\text{M}]^+ 614.46$ (found), $[\text{M}]^+ 614.72$ (calcd).

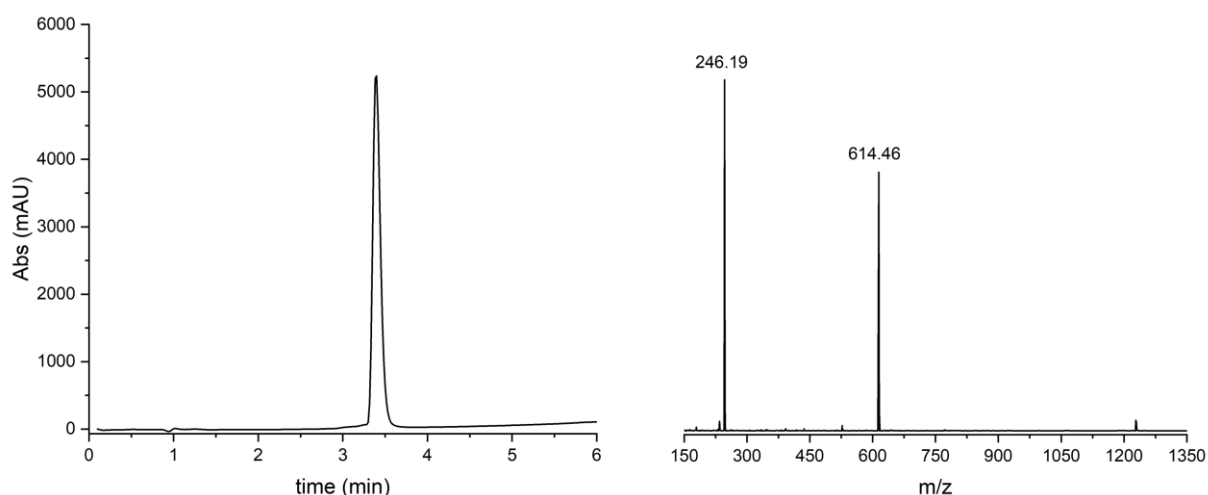


Figure S31. RP-HPLC-ESI-MS of building block N^2 -((9*H*-fluoren-9-yl)methoxycarbonyl)- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a'**). Left panel: RP-HPLC chromatogram (gradient 30-90% B in A, in 5 min.), $R_t = 3.70$ min. Right panel: MS: $[\text{M}]^+ 614.46$ (found), $[\text{M}]^+ 614.72$ (calcd).

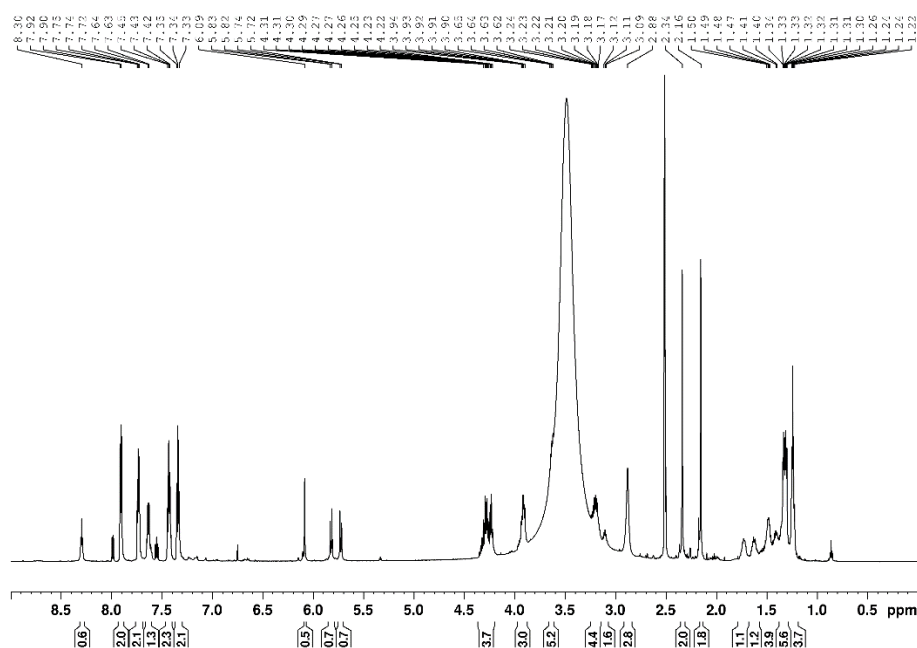


Figure S32. ^1H NMR spectrum (DMSO- d_6) of building block N^2 -((9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a'**).

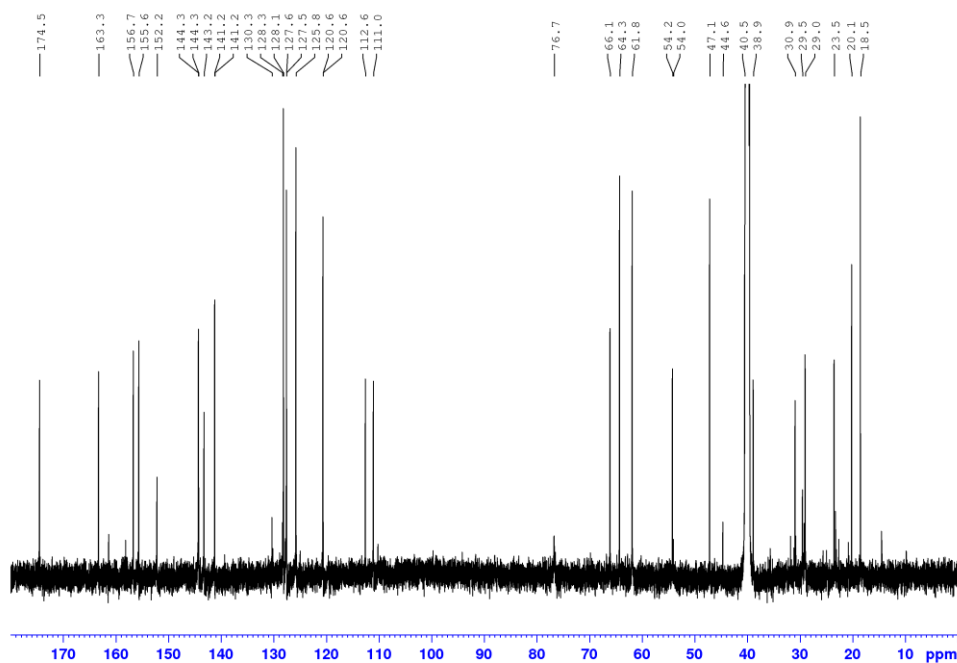


Figure S33. ^{13}C NMR spectrum (DMSO- d_6) of building block N^2 -((9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysine 2,2,2-trifluoroacetate (**4a'**).

Synthesis of N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (5a'**)**

Following the **general procedure B**, **5a'** was obtained in 81% yield (27 mg, 0.04 mmol, based on crude product) from the reaction of Fmoc-Lys-Wang resin with 8'-carboxy-2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium inner salt (**5**) (55 mg + 31 mg, 0.33 mmol).

$[\alpha]_D^{20} = 107.8$ ($c = 0.51$, DMSO).

IR (neat) $\tilde{\nu}/\text{cm}^{-1}$: 3289, 2916, 2849, 1643, 1557, 1450, 1179, 1128, 1042, 833, 800, 762, 741, 719, 644, 621, 609.

^1H NMR (600MHz, DMSO- d_6) δ 8.30 (t, $J = 5.6$ Hz, 1H), 7.91 (d, $J = 7.5$ Hz, 2H), 7.74 (t, $J = 6.5$ Hz, 2H), 7.64 (d, $J = 8.0$ Hz, 1H), 7.43 (t, $J = 7.1$ Hz, 2H), 7.34 (t, $J = 7.4$ Hz, 2H), 6.1 (s, 1H), 5.82 (d, $J = 10.0$ Hz, 1H), 5.73 (d, $J = 10.0$ Hz, 1H), 4.33 – 4.22 (m, 3H), 3.94 – 3.90 (m, 2H), 3.65 – 3.62 (m, 2H), 3.24 – 3.18 (m, 2H), 3.13 – 3.08 (m, 1H), 2.88 (bs, 2H), 2.34 (s, 3H), 2.16 (s, 3H), 1.75 – 1.71 (m, 1H), 1.66 – 1.59 (m, 1H), 1.58 – 1.39 (m, 4H), 1.34 – 1.30 (m, 6H), 1.26 – 1.22 (m, 3H)

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, DMSO- d_6) δ 174.5, 163.5, 161.3, 156.7, 155.7, 150.9, 144.3, 144.2, 142.6, 141.2(0), 141.1(9), 130.3, 128.1, 127.5(7), 127.5(5), 125.8, 120.6, 112.7, 110.4, 70.0, 68.3, 66.0, 59.8, 54.3, 47.1, 44.6, 38.9, 30.9, 28.9, 23.5, 23.2(1), 19.9, 18.4, 16.4, 16.0

RP-HPLC-MS: (gradient: 30-90% B in A in 5 min): $R_t = 3.76$ min, MS: $[\text{M}]^+ 614.49$ (found), $[\text{M}]^+ 614.77$ (calcd).

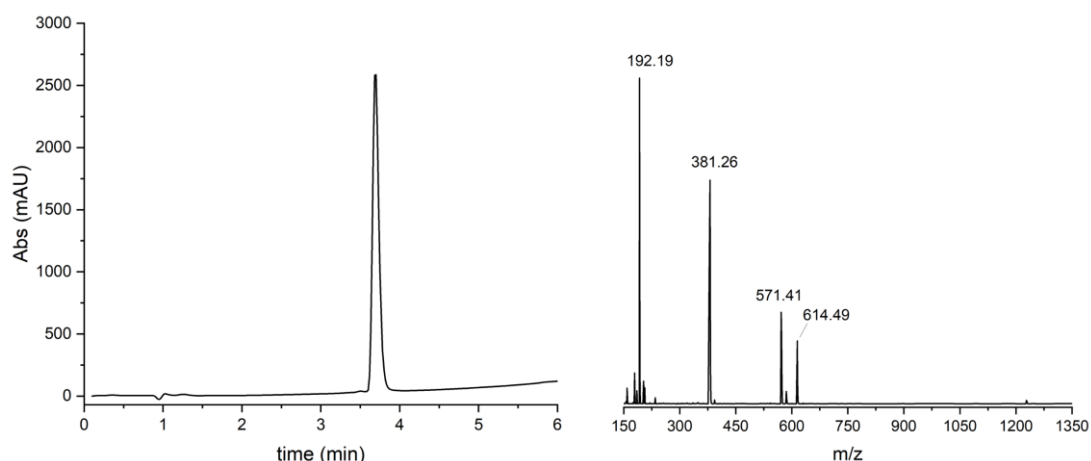


Figure S34. RP-HPLC-ESI-MS of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a'**). Left panel: RP-HPLC chromatogram (gradient 30-90% B in A, in 5 min), $R_t = 3.76$ min. Right panel: MS: $[\text{M}]^+ 614.49$ (found), $[\text{M}]^+ 614.77$ (calcd).

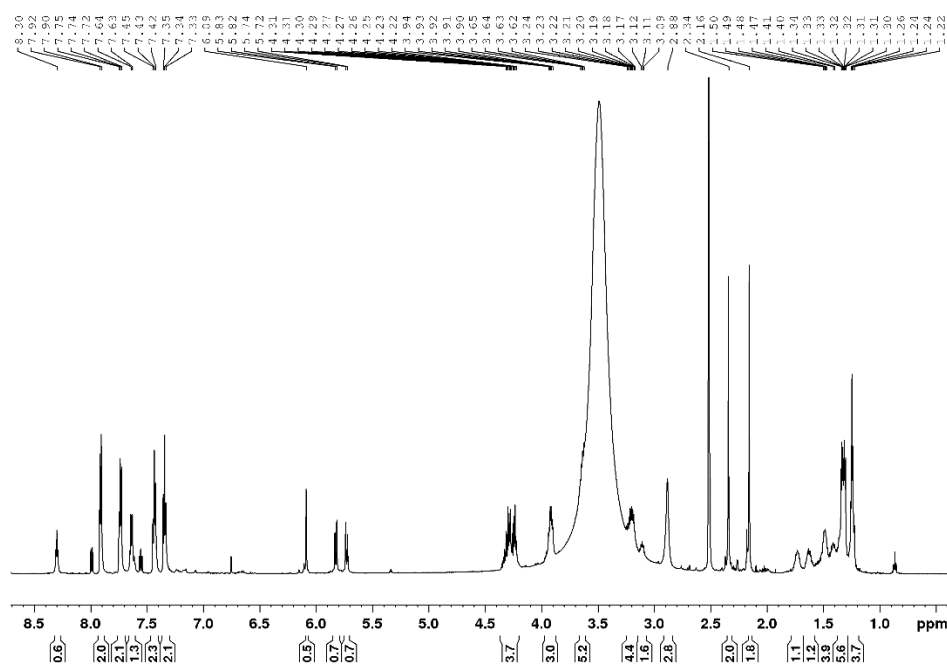


Figure S35. ^1H NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a'**).

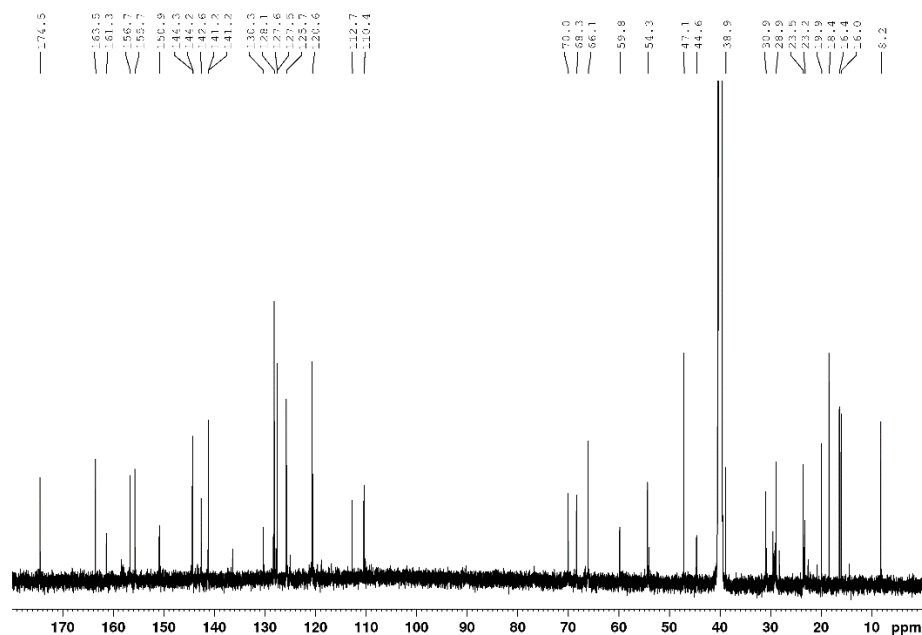
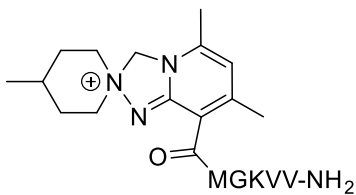
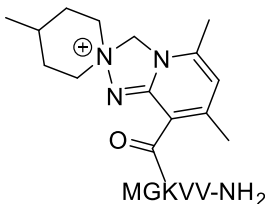
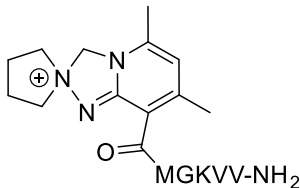
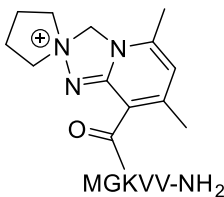
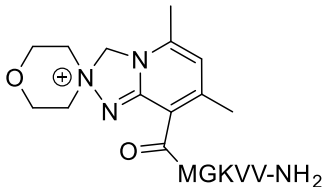
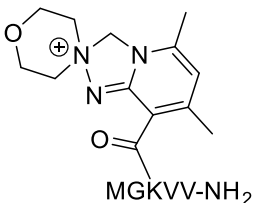
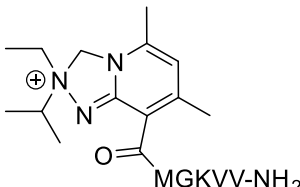
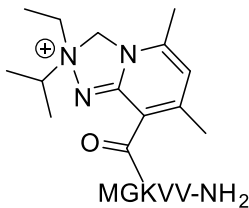


Figure S36. ^{13}C NMR spectrum (DMSO- d_6) of building block N^2 -[(9*H*-fluoren-9-yl)methoxycarbonyl]- N^6 -[2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**5a'**).

Table S1. Sequence of pentapeptide **P** and structures of its *Safirinium* conjugates. Amino acids are represented with a single-letter code.

Ac-MGKV V -NH $_2$ (P)	
 <p>P2</p>	 <p>P2a</p>
 <p>P3</p>	 <p>P3a</p>
 <p>P4</p>	 <p>P4a</p>
 <p>P5</p>	 <p>P5a</p>

General procedure C

All peptides were synthesized manually on solid phase following the Fmoc/*t*Bu orthogonal strategy reported in the main text (**Solid Phase Peptide Synthesis**). Dry Rink amide AM resin (100-200 mesh, loading: 0.74 mmol/g, 1 g) was swelled for 40 min in DMF and elongated following the procedure mentioned above using the appropriate amino acids orthogonally protected as follow: Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH and Fmoc-Val-OH, or building block N²-((9H-fluoren-9-yl)methylcarbonyl)-N⁶-[4,5,7'-trimethyl-3'*H*-spiro[[[1,2,4]triazolo[4,3-*a*]pyridine-1,2'-piperidin]-1-ium-8'-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**2a'**), N⁶-[5',7'-dimethyl-3'*H*-spiro[[1,2,4]triazolo[4,3-*a*]pyridine-1,2'-pyrrolidin]-1-ium-8'-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**3a'**), N²-((9H-fluoren-9-yl)methylcarbonyl)-N⁶-[5',7'-dimethyl-3'*H*-spiro[[1,2,4]triazolo[4,3-*a*]pyridine-4,2'-morpholin]-4-ium-8'-carbonyl]-L-lysine 2,2,2-trifluoroacetate (**4a'**), N²-((9H-fluoren-9-yl)methylcarbonyl)-N⁶-[2-ethyl-2-(1-methylethyl)-5',7'-dimethyl-2,3-dihydro-[1,2,4]triazolo[4,3-*a*]pyridin-2-ium-8'-carbonyl]-L-lysine 2,2,2-trifluoroacetate **5a'**.

After acetylation on the N-terminus, performed using a solution of Ac₂O (20 equiv) and NMM (20 equiv) in DCM (1mL/100 mg resin) for 2×45 min, the peptidyl resin (Ac-Met-Gly-Lys(Boc)-Val-Val-NH-Rink amide resin, Ac-Met-Gly-Lys(**2-5**)-Val-Val-NH-Rink amide resin) was incubated with a solution of TFA/H₂O/EDT/TIS (94/2.5/2.5/1, v/v/v/v, 1mL/100 mg resin) to remove the peptide from the resin, and the obtained crude peptide was precipitated by addition of cold diethyl ether and recovered by centrifugation.

Synthesis of *N*-Acetyl-L-methionylglycyl-L-lysyl-L-valyl-L-valinamide (**P**)

Peptide **P** was synthesized, N-acetylated, cleaved from the resin and recovered solid as described in the General procedure C. After a first purification via RP-flash chromatography, using a gradient of 30-90% B in A in 18 min, 65 mg of the obtained products were again purified (RP-flash chromatography, gradient of 10-25% B in A in 15 min) to get pure Ac-Met-Gly-Lys-Val-Val-NH₂ (**P**) (37 mg, 57% yield (purification)) as a white solid.

¹H NMR (600MHz, DMSO-*d*₆) δ 8.26 (t, *J* = 5.8 Hz, 1H), 8.18 (d, *J* = 7.3 Hz, 1H), 7.96 (d, *J* = 8.7 Hz, 1H), 7.89 (d, *J* = 8.0 Hz, 1H), 7.71 (bs, 2H), 7.63 (d, *J* = 8.9 Hz, 1H), 7.39 (s, 1H), 7.08 (s, 1H), 4.38 – 4.34 (m, 1H), 4.31 – 4.28 (m, 1H), 4.18 (dd, *J* = 8.26, *J* = 7.2, 1H), 4.14 (dd, *J* = 8.9, *J* = 6.6, 1H), 3.78 (dd, *J* = 16.6, *J* = 5.9, 1H), 3.70 (dd, *J* = 16.6, *J* = 5.7, 1H), 2.78 (t, *J* = 6.5, 1H), 2.52 – 2.46 (m, 2H), 2.7 (s, 3H), 2.04 – 1.91 (m, 3H), 1.90 (s, 3H), 1.84 – 1.79 (m, 1H), 1.71 – 1.66 (m, 1H), 1.58 – 1.51 (m, 3H), 1.35 – 1.25 (m, 2H), 0.89 – 0.86 (m, 12H)

¹³C{¹H} NMR (151MHz, DMSO-*d*₆) δ 173.2, 172.3, 171.9, 171.2, 170.3, 169.1, 58.6, 57.8, 52.6(9), 52.6(5), 42.5, 39.2, 31.9, 31.8, 31.0, 30.6, 30.0, 27.2, 23.0, 22.6, 19.7(3), 19.7(0), 18.7, 18.4, 15.0

RP-HPLC-MS (gradient: 5-95% B in A in 10 min): Rt = 3.69 min, MS: [M + H]⁺ 574.6 (found), [M + H]⁺ 574.8 (calcd). Purity: 97%

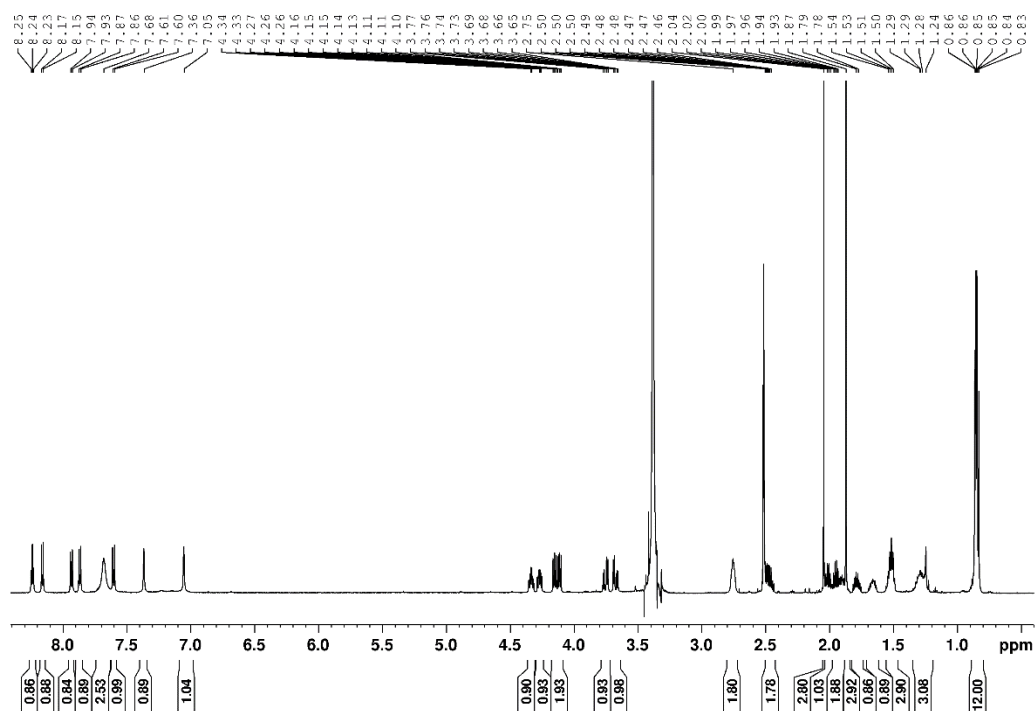


Figure S37. ¹H NMR spectrum of Peptide **P** (Ac-MGKV-V-NH₂).

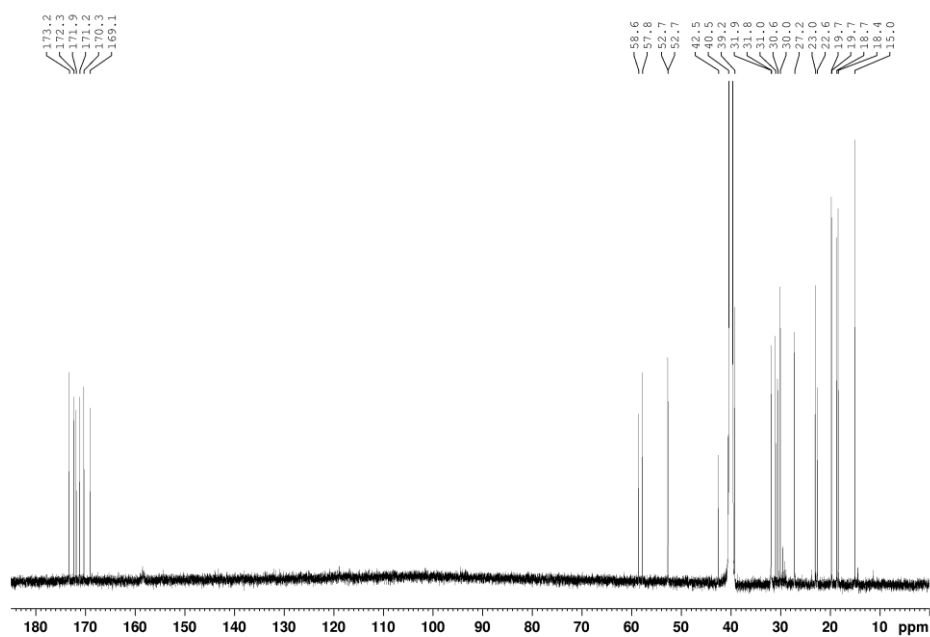


Figure S38. ¹³C NMR spectrum of Peptide **P** (Ac-MGKV-V-NH₂).

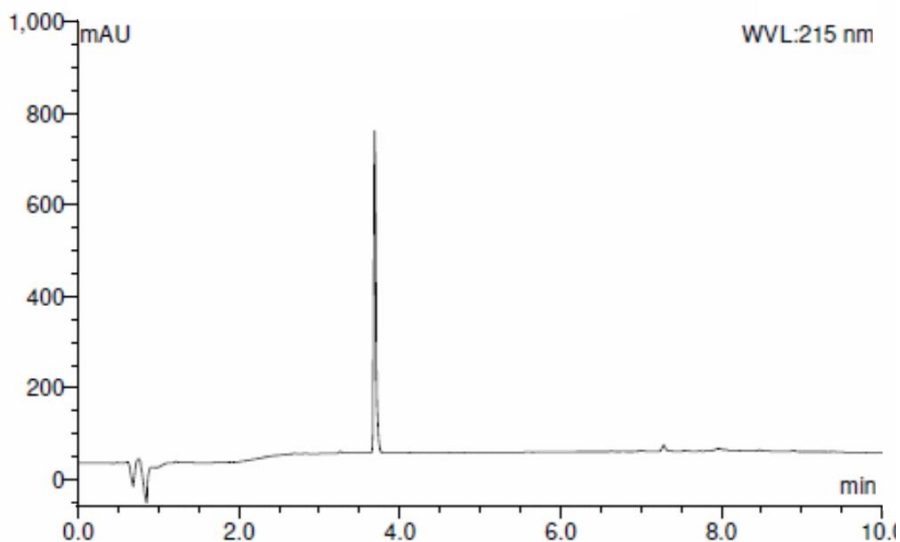


Figure S39. RP-HPLC chromatogram (gradient: 5-95% B in A in 10 min) of peptide **P** (Ac-MGKV-V-NH₂).

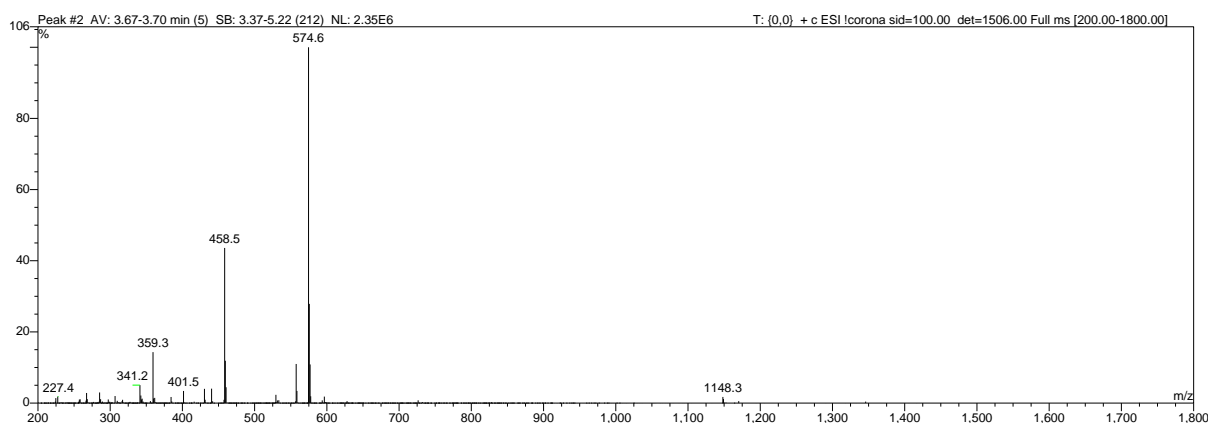


Figure S40. MS spectrum of peptide **P** (peak at $R_t = 3.70$ min in the chromatogram), MS: $[M + H]^+$ 574.6 (found), $[M + H]^+$ 574.8 (calcd).

Synthesis of *N*-Acetyl-L-methionylglycyl-*N*⁶-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysyl-L-valyl-L-valinamide (**P2a**)

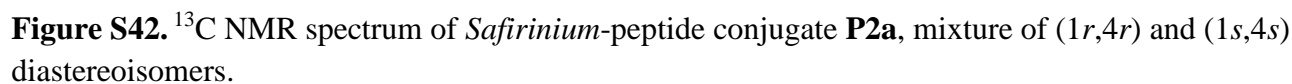
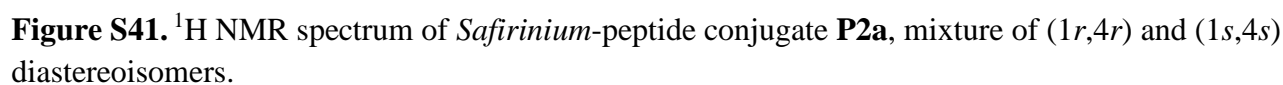
Peptide **P2a** was synthesized, N-acetylated, cleaved from the resin and recovered as solid as described in the **general procedure C**. The crude product was purified via RP-flash chromatography (gradient: 10%-35% B in A in 25 min) giving **P2a** in 3% yield (3.3 mg) as a white solid.

¹H NMR (600MHz, 10% D₂O) δ 8.50 (t, $J = 5.6$ Hz) + 8.46 – 8.42 (m, 2H), 3.32 – 3.31 (m, 1H), 8.16 9t, $J = 7.8$ Hz, 1H), 8.12 (t, $J = 7.4$ Hz, 1H), 7.89 – 7.96 (m, 1H), 7.56 (s, 1H), 7.03 (s, 1H), 6.12 + 6.11 (2 x s, 1H)*, 5.68 + 5.64 (2x s, 2H), 4.33 (m, 1H), 4.23 (m, 1H), 4.05 (t, $J = 8.1$, 1H), 4.00 (t, $J = 7.9$ Hz, 1H), 3.85 – 3.84 (m, 2H), 3.70 – 3.64 (m, 2H), 3.58 – 3.54 (m, 2H), 3.51 – 3.46 (m, 2H), 3.29 – 3.22 (m, 2H), 2.58 – 2.53 (m, 4H), 2.24 + 2.22 (2 x s 3H)*, 2.14+2.12 (2 x s, 3H)*, 2.02 – 1.88 (m, 8H), 1.83 – 1.64 (m, 5H), 1.59 – 1.50 (m, 3H), 1.39 – 1.21 (m, 2H), 0.95 – 0.92 (m, 3H), 0.87 – 0.82 (m, 12H)

* - doublet signals (2 diastereoisomers).

- some signals overlapped by the residua water signal

RP-HPLC-MS: (gradient: 5-95% B in A in 10 min): Rt = 4.43 min and 4.51 min, MS: [M]⁺ 831.8 (found), [M]⁺ 831.5 (calcd). Purity: 95%



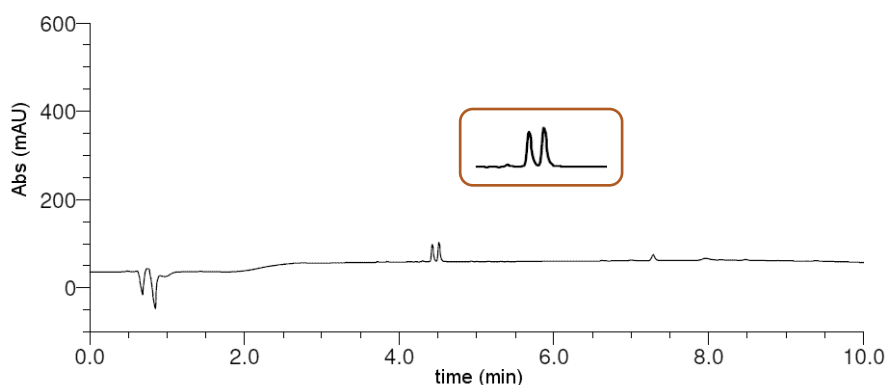


Figure S43. RP-HPLC chromatogram (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P2a**, mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers.

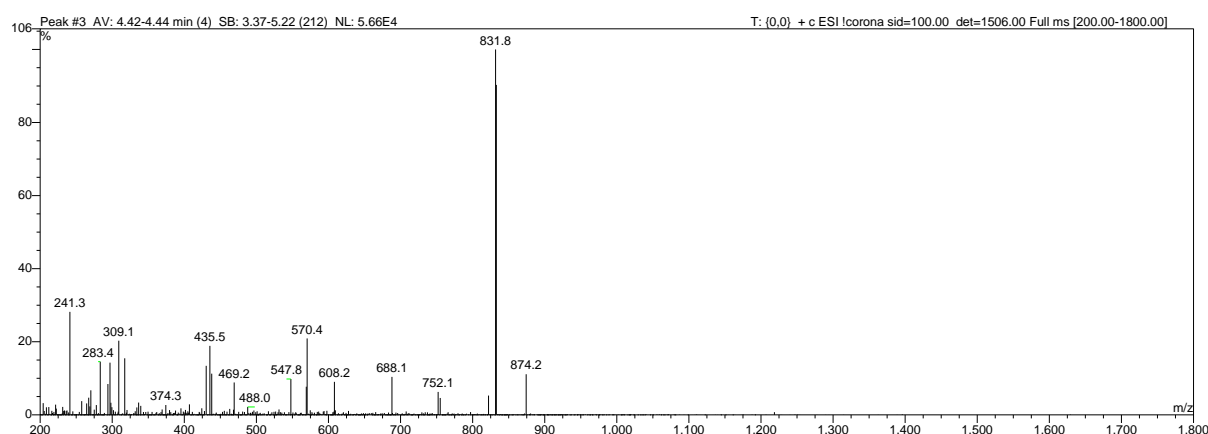


Figure S44. MS spectrum of *Safirinium*-peptide conjugate **P2a**, mixture of (1*r*, 4*r*) and (1*s*, 4*s*) diastereoisomers, $[M]^+$ 831.8 (found), $[M]^+$ 831.5 (calcd).

Synthesis of *N*-Acetyl-L-methionylglycyl-*N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-lysyl-L-valyl-L-valinamide (**P3a**)

Peptide **P3a** was synthesized, N-acetylated, cleaved from the resin and recovered as solid as described in the **general procedure C**. The crude product was purified via RP-flash chromatography (gradient: 10%-35% B in A in 25 min) giving **P3a** in 7% yield (4 mg) as a white solid.

¹H NMR (600MHz, 10% D₂O) δ 8.48 (q, *J* = 5.2 Hz, 1H), 8.45 (t, *J* = 5.7 Hz, 1H), 8.32 – 8.31 (m, 1H), 8.17 (d, *J* = 7.9 Hz, 1H), 8.13 (d, 7.9 Hz, 1H), 7.97 (d, *J* = 6.8 Hz, 1H), 7.57 (s, 1H), 7.03 (s, 1H), 6.12 (s, 1H), 5.78 (d, *J* = 2.4 Hz, 2H), 4.37 – 4.32 (m, 1H), 4.25 – 4.22 (m, 1H), 4.11 – 3.99 (m, 2H), 3.85 – 3.84 (m, 2H), 3.79 – 3.75 (m, 4H), 3.29 – 3.19 (m, 2H), 2.58 – 2.53 (m, 1H), 2.51 – 2.46 (m, 1H), 2.25 – 2.10 (m, 7H), 2.02 (s, 3H), 2.00 – 1.90 (m, 9H), 1.77 – 1.72 (m, 1H), 1.69 – 1.59 (m, 1H), (m, 1.55 – 1.50 (m, 2H), 1.35 – 1.26 (m, 3H), 0.93 (dd, *J* = 12.8 Hz, *J* = 7.0, 2H), 0.90 (dd, *J* = 6.8 Hz, *J* = 3.3 Hz, 2H), 0.87 – 0.83 (m, 12H)

¹³C{¹H} NMR (151MHz, 10% D₂O) δ 175.9, 174.5(9), 174.5(5), 173.9, 173.4, 171.2, 170.5, 166.2, 155.4, 152.6, 143.4, 111.3, 75.3, 68.9, 59.7, 59.4, 55.1, 53.8, 53.2, 42.6, 39.6, 30.6, 30.1(3), 30.0(8), 29.9, 29.3, 27.6, 22.5, 21.8, 21.3, 18.9, 18.4(0), 18.3(7), 18.3, 17.9, 17.8, 17.7, 14.2

RP-HPLC-MS (gradient: 5-95% B in A in 10 min): Rt = 4.20 min, MS: $[M]^+$ 803.5 (found), $[M]^+$ 803.5 (calcd). Purity: 95%

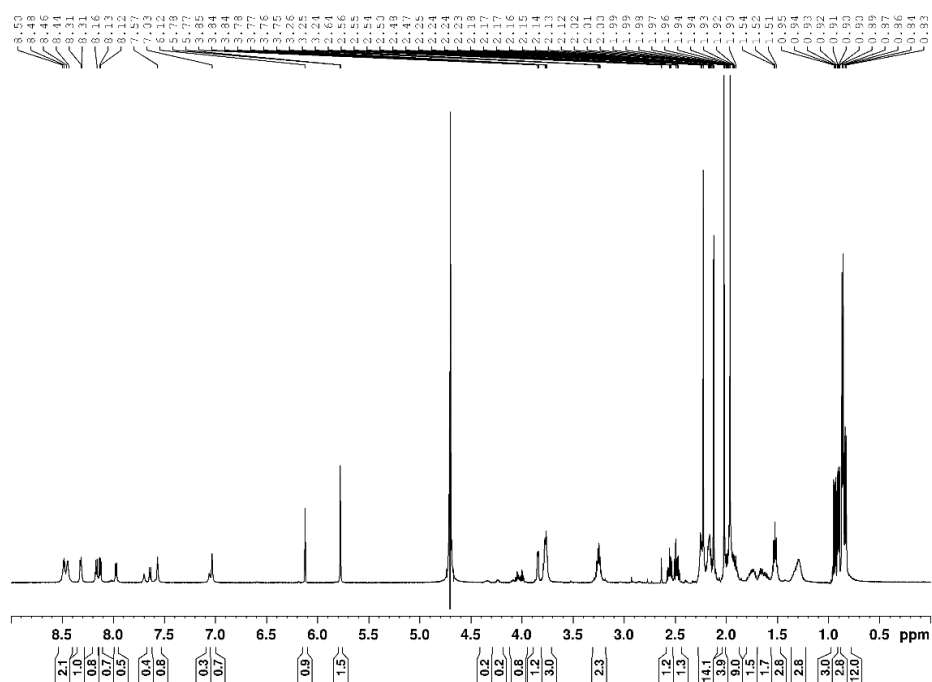


Figure S45. ^1H NMR spectrum of *Safirinium*-peptide conjugate **P3a**

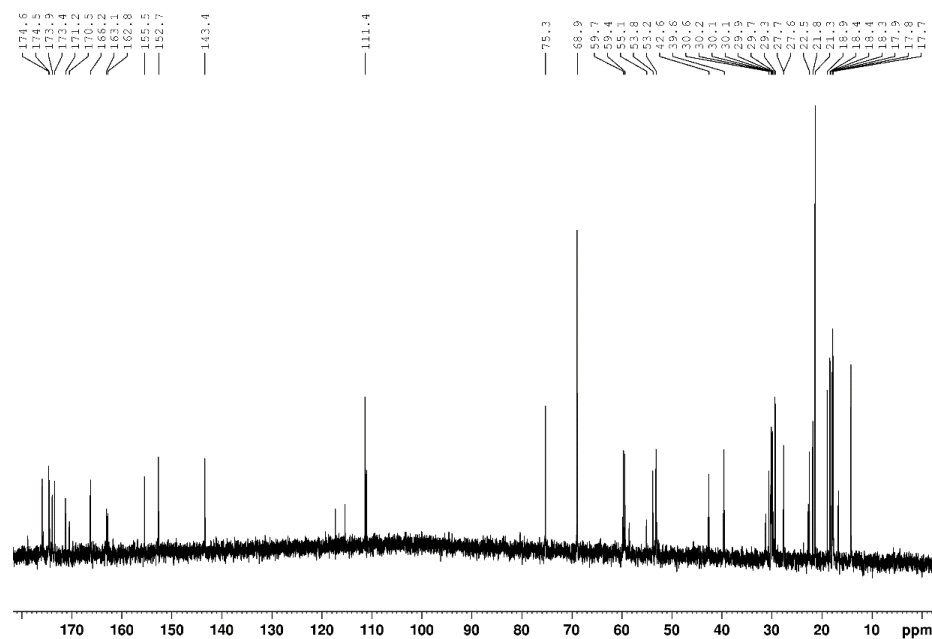


Figure S46. ^{13}C NMR spectrum of *Safirinium*-peptide conjugate **P3a**

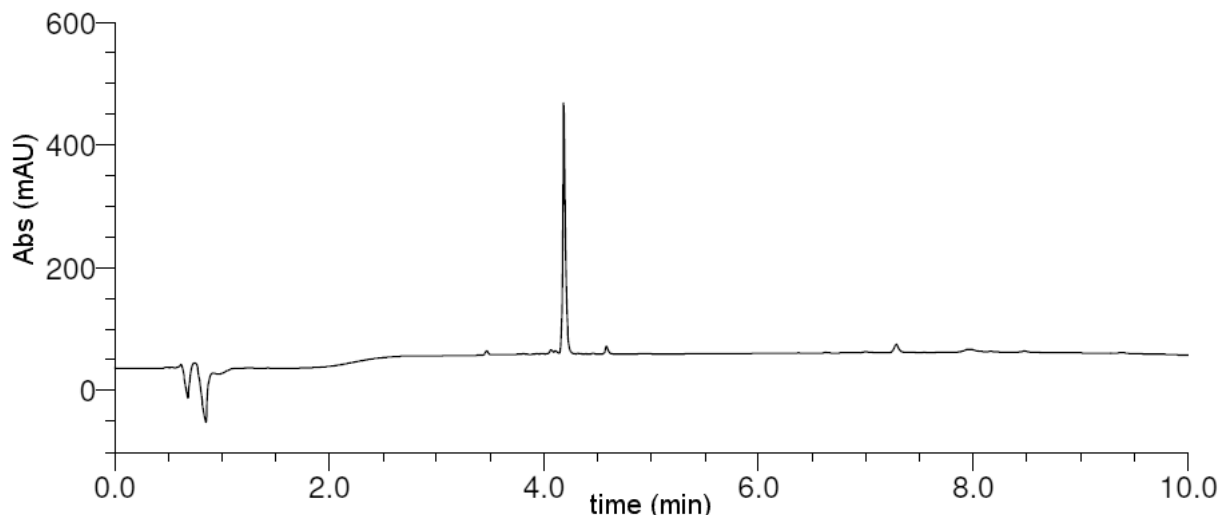


Figure S47. RP-HPLC chromatogram (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P3a**, $R_t = 4.20$ min.

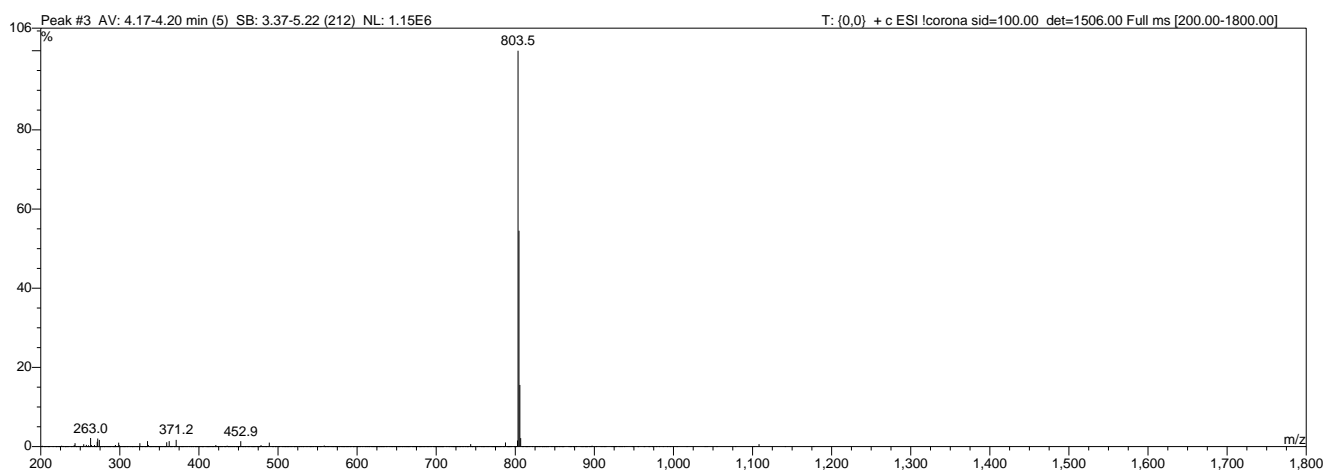


Figure S48. MS spectrum of *Safirinium*-peptide conjugate **P3a** ($R_t = 4.20$ min), MS: $[M]^+$ 803.5 (found), $[M]^+$ 803.5 (calcd).

Synthesis of *N*-Acetyl-L-methionylglycyl-*N*⁶-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]morpholinium-8'-carbonyl]]-L-lysyl-L-valyl-L-valinamide (P4a**)**

Peptide **P4a** was synthesized, N-acetylated, cleaved from the resin and recovered as solid as described in the **general procedure C**. The crude product was purified via RP-flash chromatography (gradient: 10%-35% B in A in 25 min) giving **P4a** in 9% yield (5.5 mg) as a white solid.

¹H NMR (600MHz, 10% D₂O) δ 8.50 (t, $J = 5.7$ Hz, 1H), 8.44 (t, $J = 5.9$ Hz, 1H), 8.17 (d, $J = 7.9$ Hz, 1H), 8.16 (d, $J = 7.9$ Hz, 1H), 7.57 (s, 1H), 7.03 (s, 1H), 6.16 (s, 1H), 5.79 (s, 2H), 4.35 – 4.32 (m, 1H), 4.26 – 4.22 (m, 1H), 4.20 – 4.17 (m, 2H), 4.05 (t, $J = 8.3$ Hz, 1H), 4.00 (t, $J = 7.9$ Hz, 1H), 3.98 – 3.94 (m, 2H), 3.85 (d, $J = 5.8$ Hz, 2H), 3.82 – 3.78 (m, 2H), 3.67 – 3.64 (m, 2H), 3.30 – 3.24 (m, 2H), 2.58 – 2.53 (m, 1H), 2.51 – 2.46 (m, 1H), 2.25 (s, 3H), 2.14 (s, 3H), 2.02 (s, 3H), 2.01 – 1.89 (m, 8H), 1.78 – 1.72 (m, 1H), 1.70 – 1.64 (m, 1H), 1.54 (p, $J = 7.3$ Hz, 2H), 1.40 – 1.27 (m, 2H), 0.87 – 0.83 (m, 12H)

¹³C{H} NMR (151MHz, 10% D₂O) δ 175.9, 174.5(9), 174.5(4), 173.9, 173.4, 171.2, 166.2, 163.0, 162.8, 155.6, 153.2, 143.5, 111.8, 111.3, 76.9, 64.1, 61.7, 59.7, 59.4, 53.8, 53.2, 42.6, 39.5, 30.6, 30.1(3), 30.0(8), 29.9, 29.3, 27.7, 22.5, 21.8, 19.0, 18.4(1), 18.3(8), 17.9, 17.8, 17.7, 14.2

RP-HPLC-MS (gradient: 5-95% B in A in 10 min): Rt = 4.13 min, MS: [M]⁺ 819.8 (found), [M]⁺ 819.5 (calcd). Purity: 96%

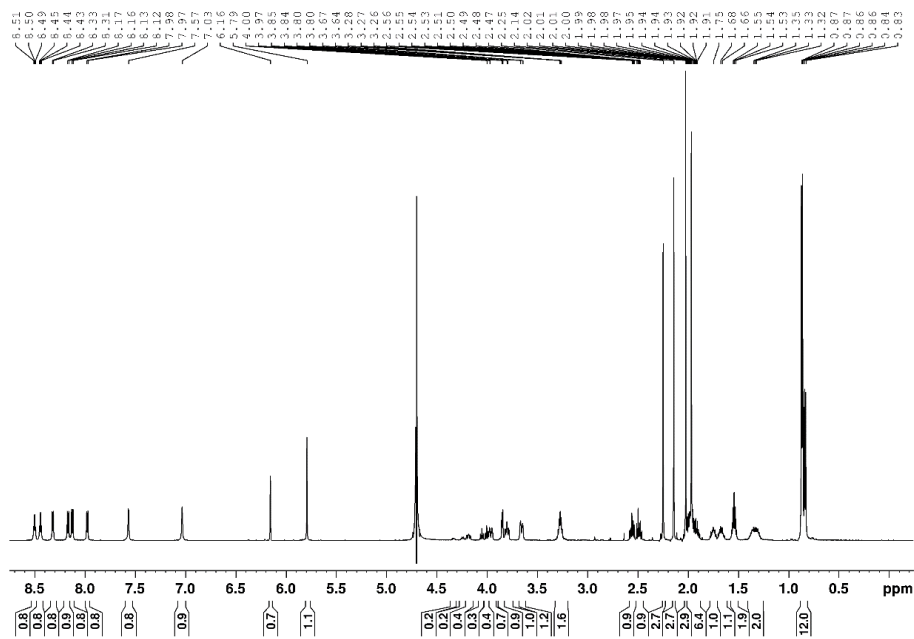


Figure S49. ^1H NMR spectrum of *Safirinium*-peptide conjugate **P4a**

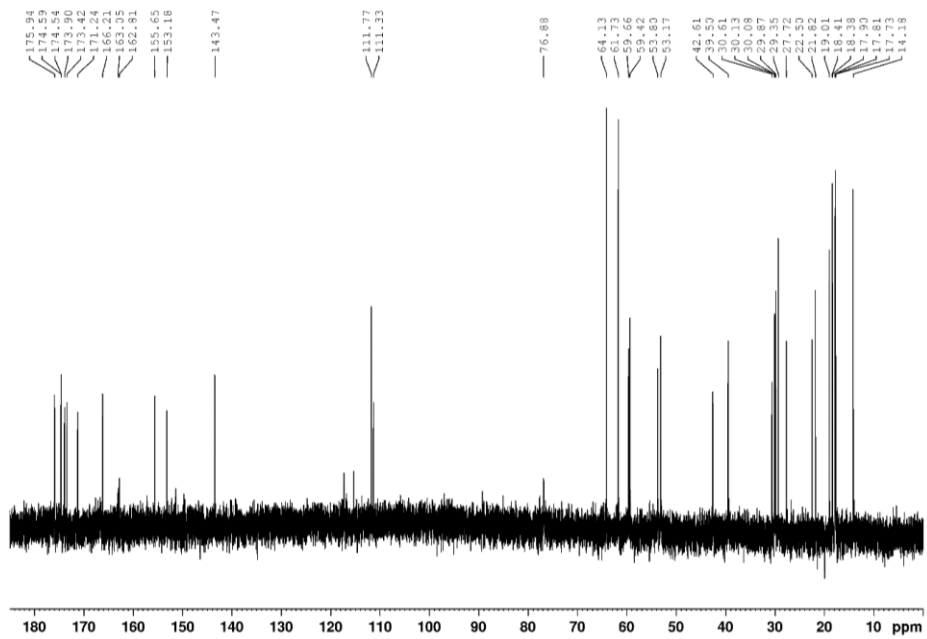


Figure S50. ^{13}C NMR spectrum of *Safirinium*-peptide conjugate **P4a**

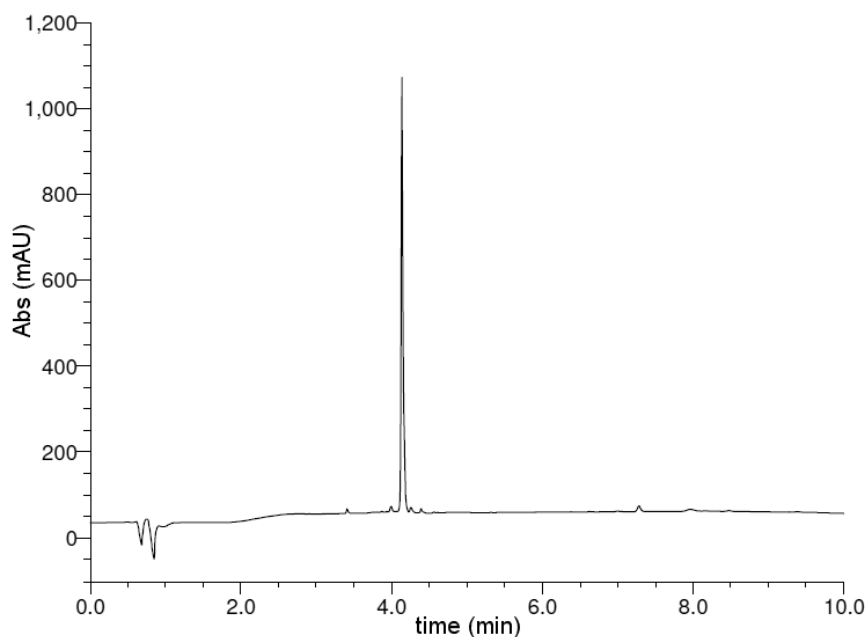


Figure S51. RP-HPLC (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P4a**, $R_t = 4.13$ min

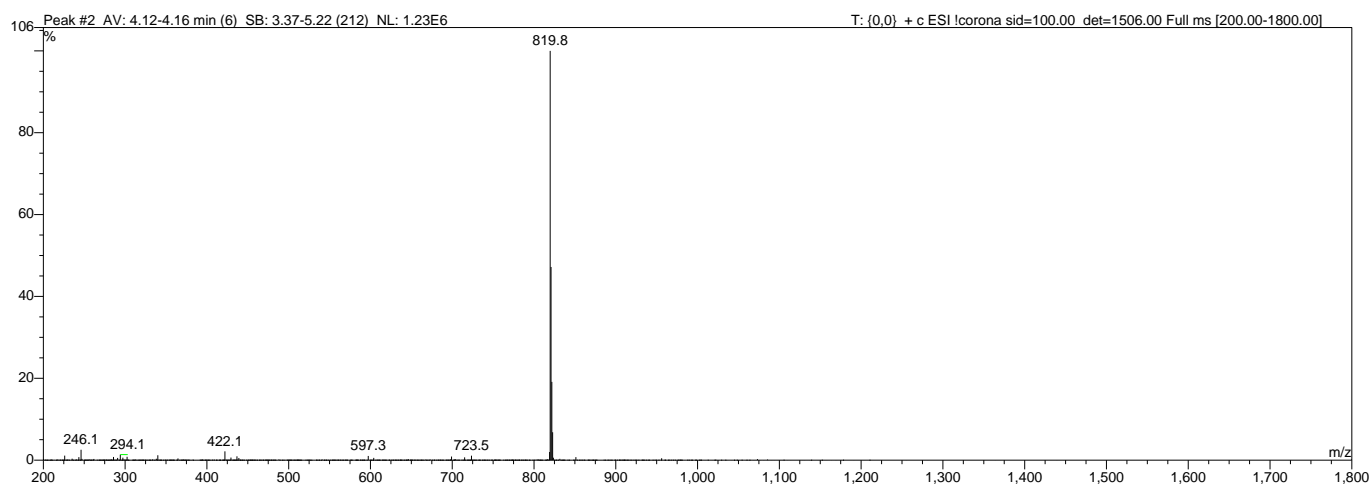


Figure S52. MS spectrum of *Safirinium*-peptide conjugate **P4a** ($R_t = 4.13$ min in the chromatogram), MS: $[M]^+$ 819.8 (found), $[M]^+$ 819.5 (calcd)

Synthesis of *N*-Acetyl-L-methionylglycyl-*N*⁶-[2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-lysyl-L-valyl-L-valinamide (P5a**)**

Peptide conjugate **P5a** was synthesized, *N*-acetylated, cleaved from the resin and recovered as solid as described in the **general procedure C**. The crude product was purified via RP-flash chromatography (gradient: 10%-35% B in A in 25 min) giving **P5a** in 5% yield (3 mg) as a white solid.

¹H NMR (600MHz, 10% D₂O) δ 8.57 (t, *J* = 5.2 Hz, 1H), 8.44 (t, *J* = 5.8 Hz, 1H), 8.32 (d, *J* = 6.5 Hz, 1H), 8.16 (d, *J* = 7.9 Hz, 1H), 8.12 (d, *J* = 8.0 Hz, 1H), 7.97 (d, *J* = 6.7 Hz, 1H), 7.56 (s, 1H), 7.03 (s, 1H), 6.09

(s, 1H), 5.67 (d, J = 10.0 Hz, 1H), 5.60 (d, J = 10 Hz, 1H), 4.34 – 4.31 (m, 1H), 4.24 – 4.22 (m, 1H), 4.04 (t, J = 8.3 Hz, 1H), 3.99 (t, J = 7.9 Hz, 1H), 3.89 – 3.80 (m, 3H), 3.66 (se, J = 6.8 Hz, 1H), 3.49 (se, J = 6.8 Hz, 1H), 3.29 – 3.21 (m, 2H), 2.57 – 2.52 (m, 1H), 2.50 – 2.43 (m, 1H), 2.23 (s, 3H), 2.09 (s, 3H), 2.01 (s, 3H), 2.00 – 1.87 (m, 6H), 1.76 – 1.70 (m, 1H), 1.68 – 1.62 (m, 1H), 1.52 (p, J = 7.2 Hz, 2H), 1.35 – 1.26 (m, 8H), 0.86 – 0.82 (m, 12H)

¹³C{¹H} NMR (151MHz, 10% D₂O) δ 175.9, 174.6, 174.5, 173.9, 173.4, 171.2, 166.4, 155.4, 152.2, 142.9, 111.2, 70.0, 69.0, 60.4, 59.6, 59.4, 53.8, 53.1, 42.6, 39.6, 30.6, 30.1(0), 30.0(7), 29.9, 29.3, 27.6, 22.5, 21.8, 18.7, 18.3(9), 18.3(6), 17.9, 17.7(5), 17.7(2), 15.7, 15.2, 14.1, 7.2

RP-HPLC-MS (gradient: 5-95% B in A in 10 min): Rt = 4.30 min, MS: [M]⁺ 819.9 (found), [M]⁺ 820.1 (calcd). Purity: 95%

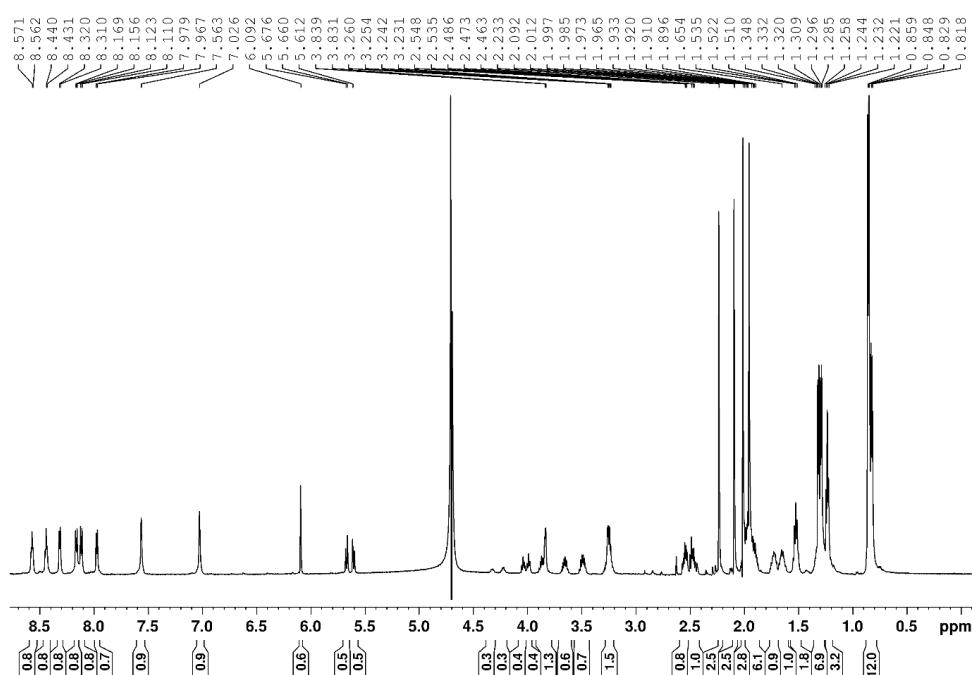


Figure 53. ¹H NMR spectrum of *Safirinium*-peptide conjugate **P5a**

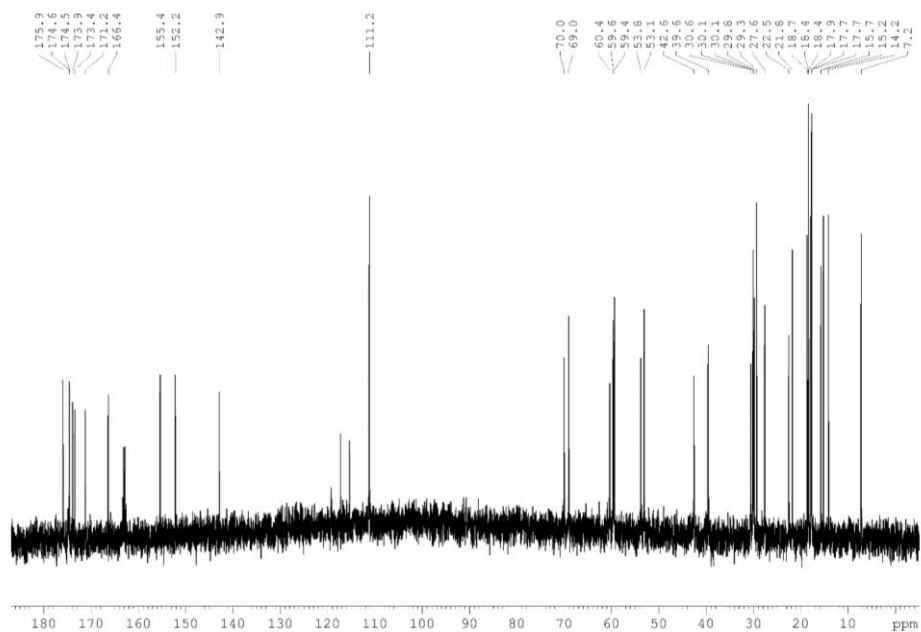


Figure S54. ^{13}C NMR spectrum of *Safirinium*-peptide conjugate **P5a**

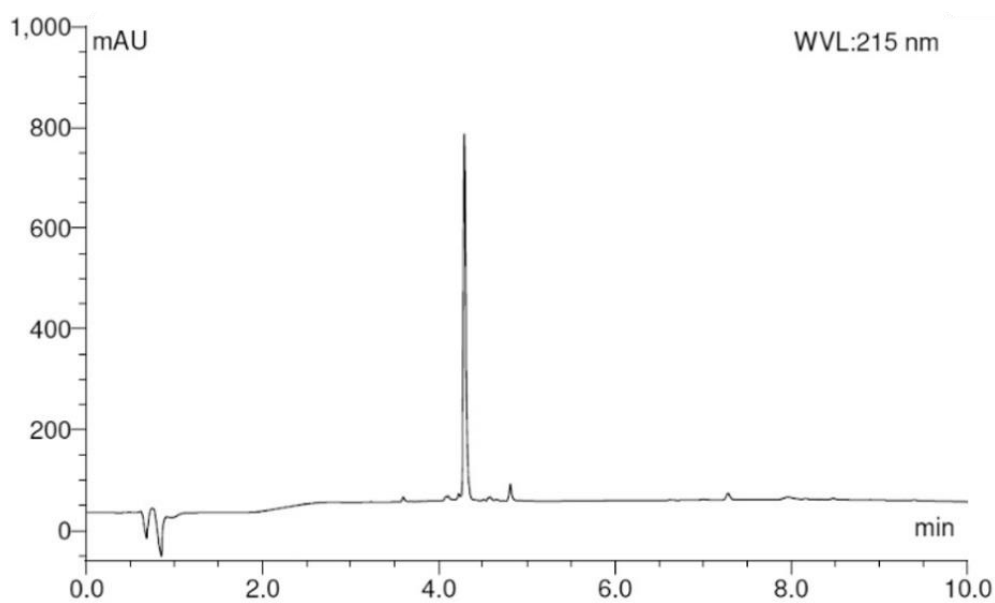


Figure S55. RP-HPLC chromatogram (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P5a** ($R_t = 4.30$).

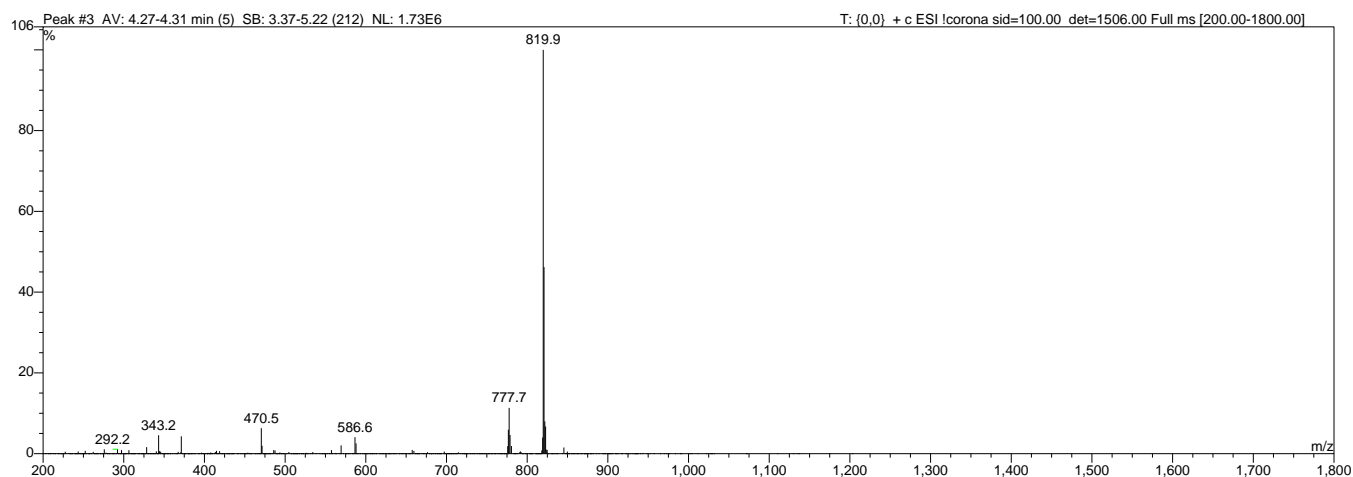


Figure S56. MS spectrum of *Safirinium*-peptide conjugate **P5a** ($R_t = 4.30$ min), $[M]^+$ 819.9 (found), $[M]^+$ 820.1 (calcd).

General procedure D

The peptide was synthesized manually on solid phase following the Fmoc/*t*Bu orthogonal strategy reported in the main text (**Solid Phase Peptide Synthesis**). Dry Rink amide AM resin (100-200 mesh, loading: 0.74 mmol/g, 1 g) was swelled for 40 min in DMF and elongated following the procedure mentioned above using the appropriate amino acids orthogonally protected as follow: Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH and Fmoc-Val-OH. A solution of the *Safirinium* derivative (**2**, **3**, **4**, **5**), HBTU and DIEA (2.5 equiv, 2.5 equiv and 4.5 equiv, respectively) in DMF (1mL) was added to H-Met-Gly-Lys(Boc)-Val-Val-NH-Rink amide AM resin and shaken for 1 h, to enable coupling of the derivative to the N-terminus of the peptide. Cleavage from the resin and final side chain deprotections were performed shaking the resin with the cleavage cocktail TFA/H₂O/EDT/TIS (94/2.5/2.5/1, v/v/v/v, 1mL) for 3 h. After filtration, cold diethyl ether was added and the crude was recovered by centrifugation, washed twice with cold diethyl ether, redissolved in Milli-Q water and freeze-dried. Purification via RP-flash chromatography (gradient: 5%-40% B in A in 30 min) gave the pure peptide conjugate (**P2**, **P3**, **P4**, **P5**) as follows.

Synthesis of *N*-[4,5',7'-trimethyl-spiro[piperidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]- L-methionylglycyl-L-lysyl-L-valyl-L-valinamide (**P2**)

Peptide conjugate **P2** was synthesized, cleaved from the resin and purified as described in the **general procedure D**. The product was obtained as a white powder in 37% yield (27 mg, 0.034 mmol).

¹H NMR (600MHz, 10% D₂O) δ 8.92 – 8.89 (m, 1H), 8.49 (dd, $J = 48.6$ Hz, $J = 4.3$ Hz, 1H), 8.18 (bs, 2H), 8.11 (bs, 1H), 7.57 (s, 1H), 7.45 (bs, 2H), 7.04 (s, 1H), 6.143 (s, 1H), 5.68 (d, $J = 8.8$ Hz, 1H), 4.29 – 4.25 (m, 1H), 4.05 – 4.03 (m, 1H), 4.00 – 3.98 (m, 1H), 3.94 – 3.87 (m, 2H), 3.72 – 3.45 (m, ??H), 2.90 (s, 2H), 2.66 – 2.55 (m, 2H), 2.24 – 2.10 (m, 6H), 2.05 (s, 3H), 2.04 – 1.93 (m, 4H), 1.8 – 1.72 (m, 4H), 1.65 – 1.55 (m, 4H), 1.32 – 1.29 (m, 2H), 0.95 – 0.94 (m, 3H), 0.87 – 0.82 (m, 12H)

¹³C{¹H} NMR (151MHz, 10% D₆O) δ 175.9, 173.9*, 173.8#, 173.7(0)*, 173.6(8)#, 173.5, 170.9(2)*, 170.8(9)#, 166.5*, 166.4#, 155.7, 155.5(2)*, 155.4(8)#, 1542, 144.4*, 144.1#, 112.1*, 111.8#, 109.9#, 109.5*, 77.8, 65.6, 65.5, 65.4, 59.7*, 59.6(7)#, 59.4, 53.5(5)*, 53.5(2)#, 53.1*, 52.9#, 42.5(3)*, 42.4(6)#,

39.4*, 39.3[#], 30.6(2)*, 30.5(9)[#], 30.5, 30.1(2)*, 30.0(9)[#], 29.9, 29.2(9)*, 29.2(7)[#], 28.7(9)*, 28.7(6)[#], 28.5, 26.4(1), 26.3(7), 22.0(1)*, 22.0(0)[#], 19.9, 19.3, 17.9(2), 17.9(0), 17.8(8), 17.8(7), 17.7, 14.1(5), 14.1(2)
 *,[#] - doublet signals (2 diastereoisomers).

RP-HPLC-MS: (gradient: 5-95% B in A in 10 min): Rt = 4.02 min and 4.14 min, MS: [M]⁺ 789.7 (found), [M]⁺ 789.5 (calcd). Purity: 95%

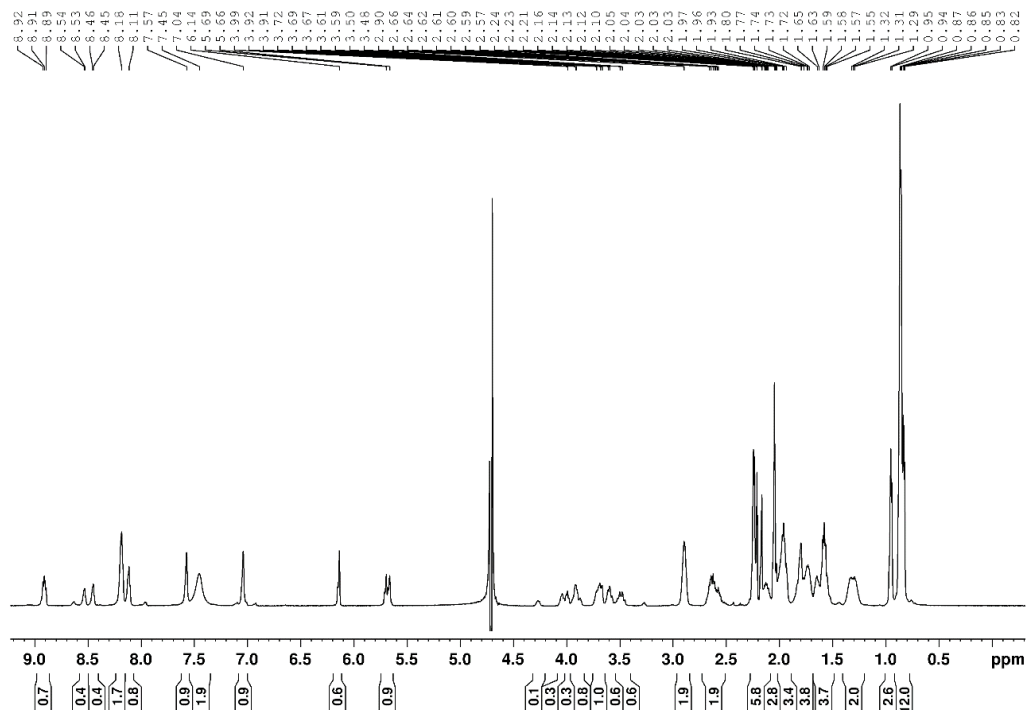


Figure S57. ¹H NMR spectrum of *Safirinium*-peptide conjugate **P2**, mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers.

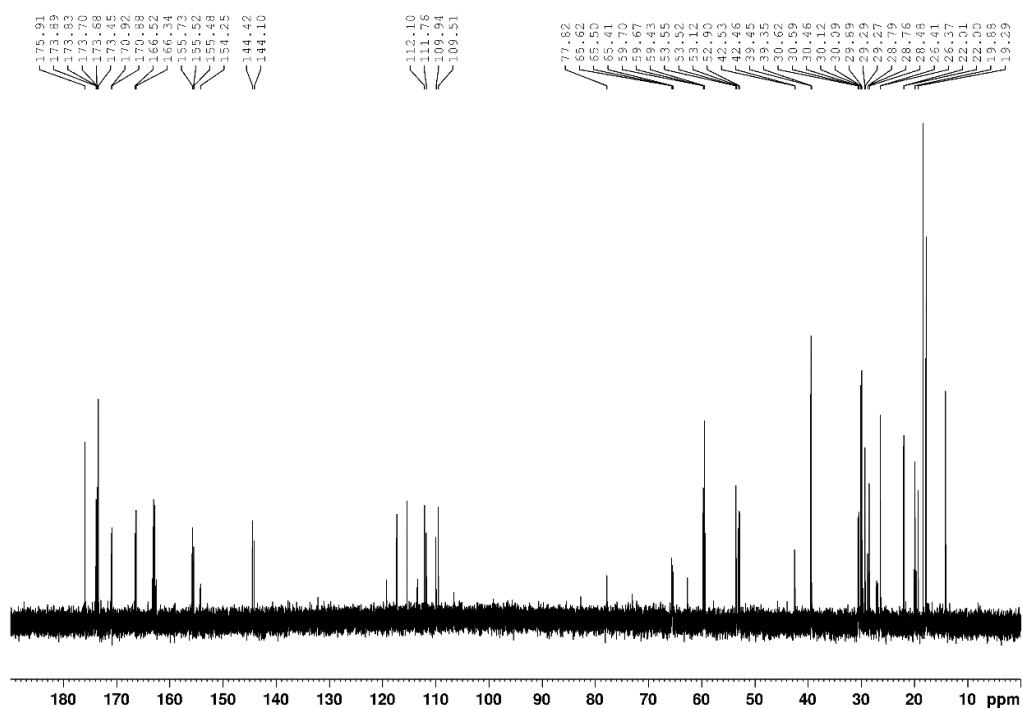


Figure S58. ^{13}C NMR spectrum of *Safirinium*-peptide conjugate **P2**, mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers.

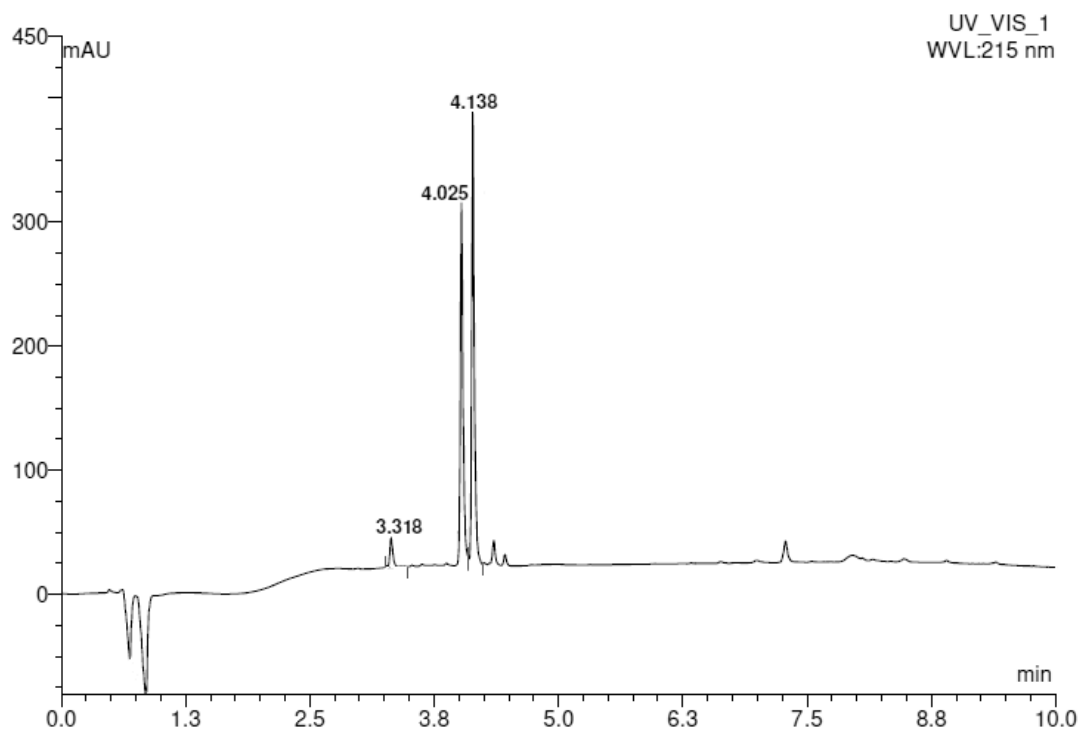


Figure S59. RP-HPLC (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P2**, mixture of (1*r*,4*r*) and (1*s*,4*s*) diastereoisomers ($R_t = 4.03$ min and 4.14 min).

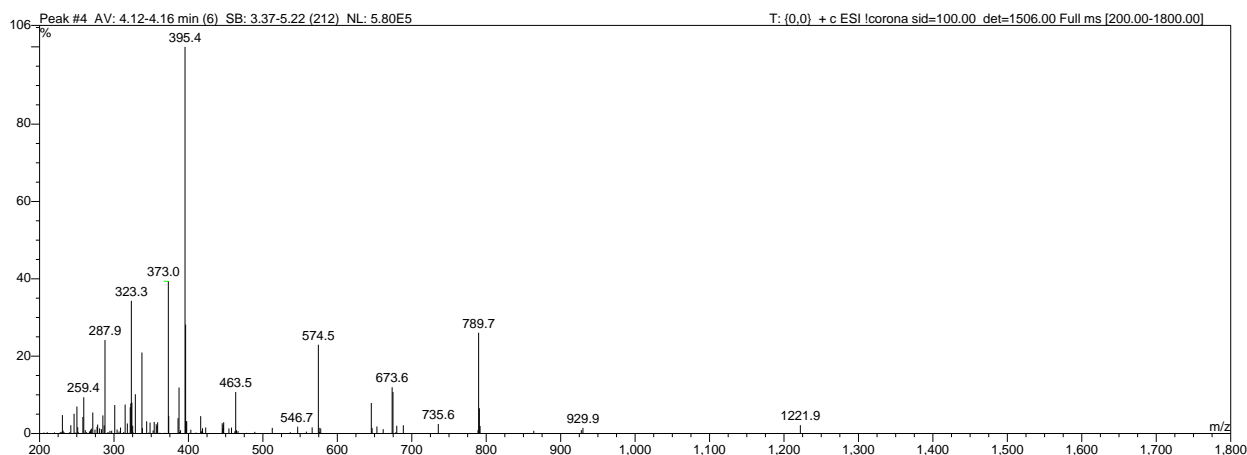


Figure S60. MS spectrum of *Safirinium*-peptide conjugate **P2**, mixture of (1*r*, 4*r*) and (1*s*, 4*s*) diastereoisomers, $[M]^+$ 789.7 (found), $[M]^+$ 789.5 (calcd).

Synthesis of *N*-[5',7'-dimethyl-spiro[pyrrolidine-1,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]- L-methionylglycyl-L-lysyl-L-valyl-L-valinamide (P3**)**

Peptide conjugate **P3** was synthesized, cleaved from the resin and purified as described in the **general procedure D**. The product was obtained as a white powder in 27% yield (19 mg, 0.025 mmol).

^1H NMR (600MHz, 10% D_2O) δ 8.88 (d, J = 6.9 Hz, 1H), 8.48 (t, J = 5.8 Hz, 1H), 8.20 – 8.18 (m, 2H), 8.12 (d, J = 6.8 Hz, 1H), 7.58 (s, 1H), 7.45 (bs, 2H), 7.04 (s, 1H), 6.14 (s, 1H), 5.81 (d, J = 2.0, 2H), 4.29 (4.27 (q, J = 7.1 Hz, 1H), 4.05 (t, J = 8.1 Hz, 1H), 4.00 (t, J = 7.9 Hz, 1H), 3.94 (dd, J = 6.9 Hz, J = 5.8 Hz, 1H), 3.88 (dd, J = 6.9 Hz, J = 5.8 Hz, 1H), 3.81 – 3.75 (m, 4H), 2.91 – 2.88 (m, 2H), 2.66 – 2.55 (m, 2H), 2.24 – 2.18 (m, 10H) 2.15 – 2.09 (m, 1H), 2.05 (s, 3H), 2.01 – 1.93 (m, 3H), 1.79 – 1.69 (m, 1H), 1.68 – 1.62 (m, 1H), 1.61 – 1.56 (m, 2H), 1.38 – 1.25 (m, 2H), 0.87 – 0.83 (m, 12H)

* - some signals overlapped with the residua water signal

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 175.9, 173.9, 173.7, 173.5, 170.9, 166.5, 155.7, 154.5, 144.1, 111.8, 109.6, 75.1, 69.1, 69.0, 59.7, 59.4, 53.5, 53.1, 42.5, 39.5, 30.6, 30.2, 30.1, 29.9, 29.2, 26.4, 22.0, 21.4(4), 21.4(1), 19.4, 18.4, 17.9(1), 17.8(9), 17.7, 14.1

RP-HPLC-MS: (gradient: 5-95% B in A in 10 min): R_t = 3.74 min, MS: $[M]^+$ 761.7 (found), $[M]^+$ 761.5 (calcd). Purity: 95%

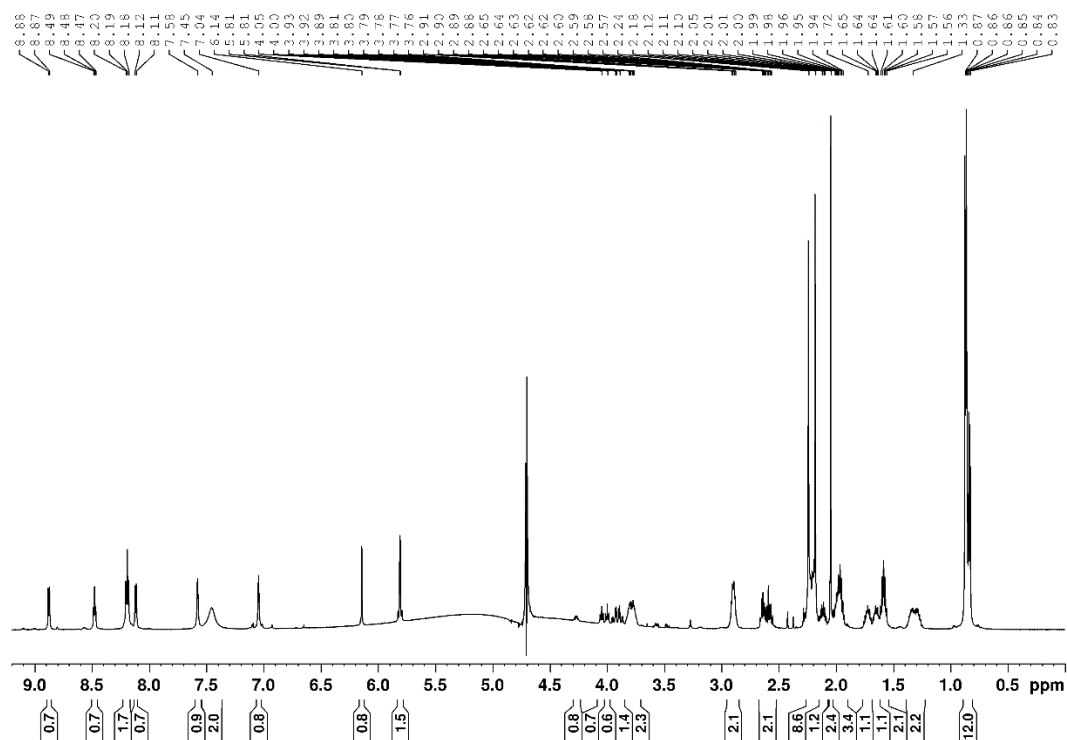


Figure S61. ^1H NMR of *Safirinium*-peptide conjugate **P3**

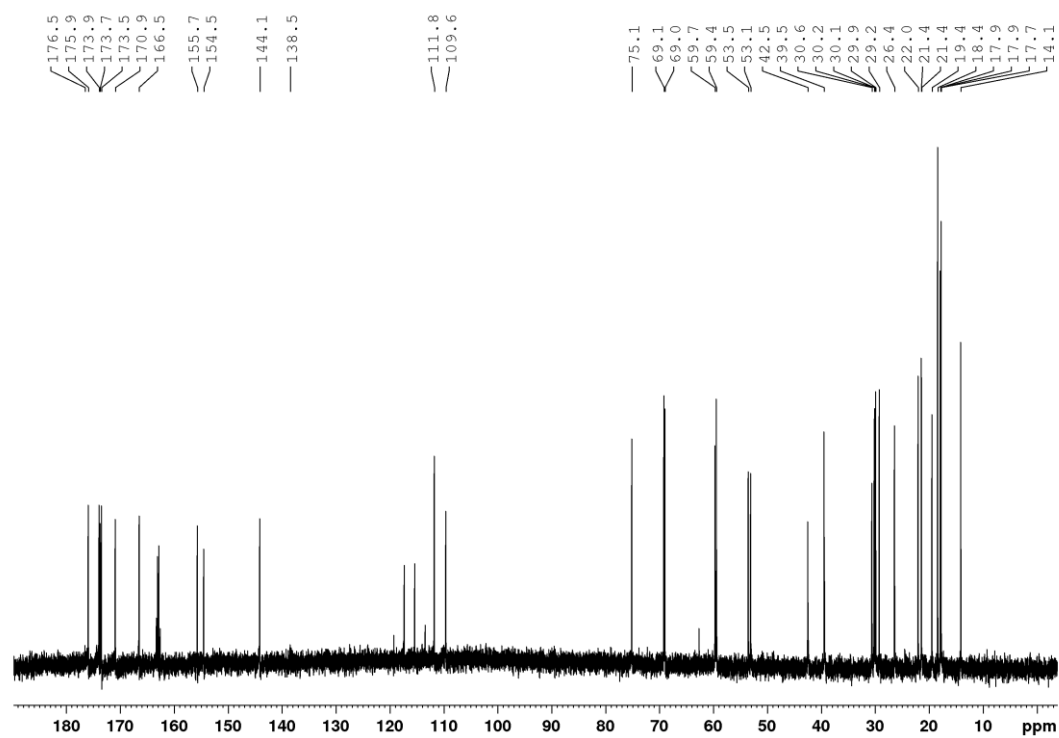


Figure S62. ^{13}C NMR of *Safirinium*-peptide conjugate **P3**

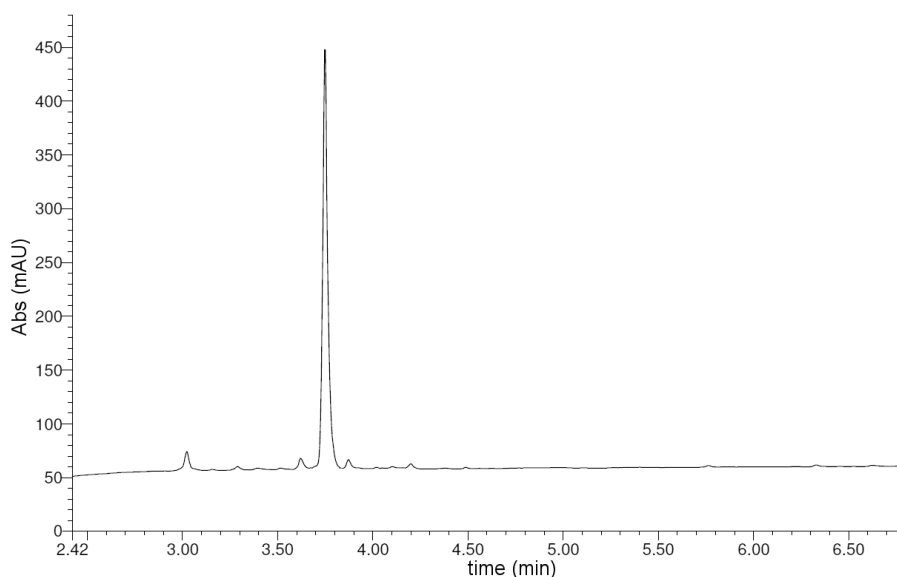


Figure S63. RP-HPLC (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P3** ($R_t = 3.74$ min).

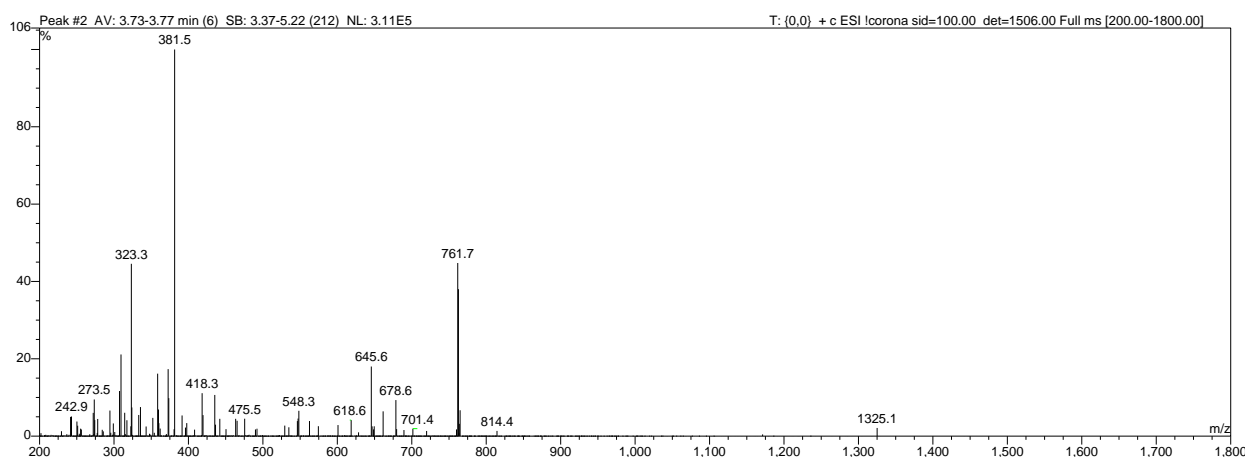


Figure S64. MS spectrum of *Safirinium*-peptide conjugate **P3** ($R_t = 3.74$ min) $[M]^+$ 761.7 (found), $[M]^+$ 761.5 (calcd).

Synthesis of *N*-[5',7'-dimethyl-spiro[morpholine-4,2'(3'*H*)-[1,2,4]triazolo[4,3-*a*]pyridinium-8'-carbonyl]]-L-methionylglycyl-L-lysyl-L-valyl-L-valinamide (**P4**)

Peptide conjugate **P4** was synthesized, cleaved from the resin and purified as described in the **general procedure D**. The product was obtained as a white powder in 38% yield (27 mg, 0.035 mmol).

^1H NMR (600MHz, 10% D_2O) δ 8.91 (d, $J = 7.2$ Hz, 1H), 8.47 (t, $J = 5.9$ Hz, 1H), 8.20 (d, $J = 5.1$ Hz, 1H), 8.18 (d, $J = 4.9$ Hz, 1H), 8.14 (d, $J = 6.8$ Hz, 1H), 7.58 (s, 1H), 7.45 (bs, 2H), 7.04 (s, 1H), 6.18 (s, 1H), 5.80 (d, $J = 2.0$ Hz, 2H), 4.28 – 4.25 (m, 1H), 4.21 – 4.17 (m, 2H), 4.04 (t, $J = 8.1$ Hz, 1H), 4.00 – 3.87 (m, 5H), 3.84 – 3.81 (m, 2H), 3.69 – 3.64 (m, 2H), 2.92 – 2.87 (m, 2H), 2.69 – 2.58 (m, 2H), 2.26 (s, 3H), 2.20 (s, 3H), 2.16 – 2.12 (m, 1H), 2.05 (s, 3H), 2.04 – 1.92 (m, 4H), 1.74 – 1.69 (m, 1H), 1.67 – 1.62 (m, 1H), 1.58 (q, $J = 7.8$ Hz, 2H), 1.38 – 1.25 (m, 2H), 0.87 – 0.82 (m, 12H)

* - Halfa Met1 overlapped with residual water signal

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 175.9, 173.7(4), 173.7(2), 173.5, 170.9, 166.3, 155.8, 155.1, 144.2, 112.4, 112.2, 109.9, 77.1, 64.2, 61.8, 59.7, 59.4, 53.5, 52.9, 42.5, 39.4, **39.3**, 30.6, 30.3, 30.1, 29.9, 29.3, 26.4(1), **26.3(6)**, 22.0, 19.6, 18.3(8), 18.3(7), 17.9(0), 17.8(9), 17.8(0), 14.1

RP-HPLC-MS: (gradient: 5-95% B in A in 10 min): R_t = 3.65 min, MS: $[\text{M}]^+$ 777.8 (found), $[\text{M}]^+$ 777.4 (calcd). Purity: 96%

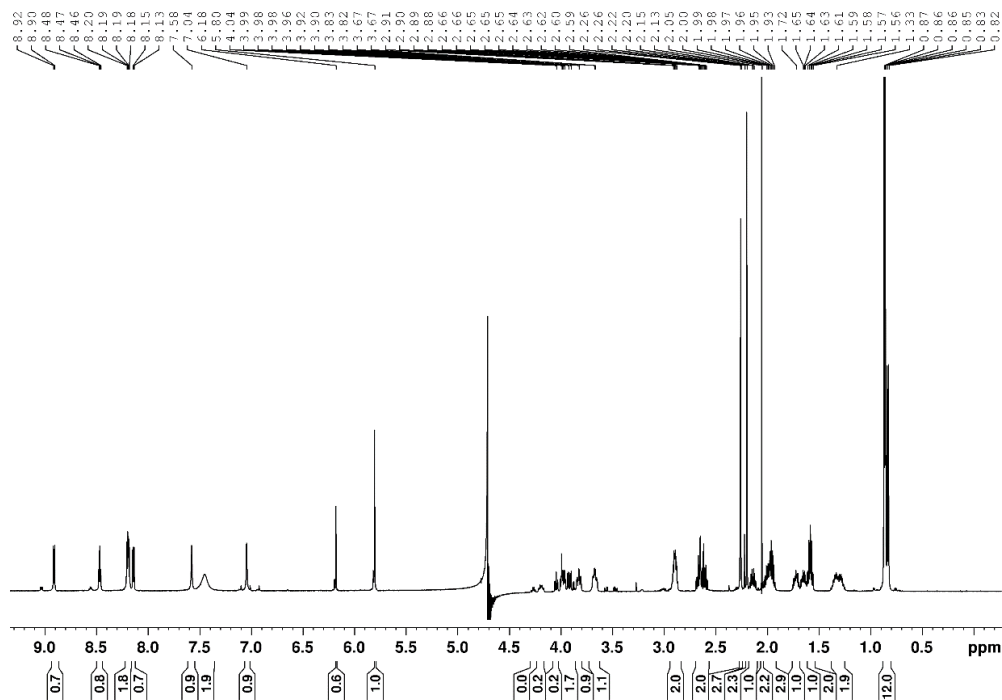


Figure S65. ^1H NMR of *Safirinium*-peptide conjugate **P4**.

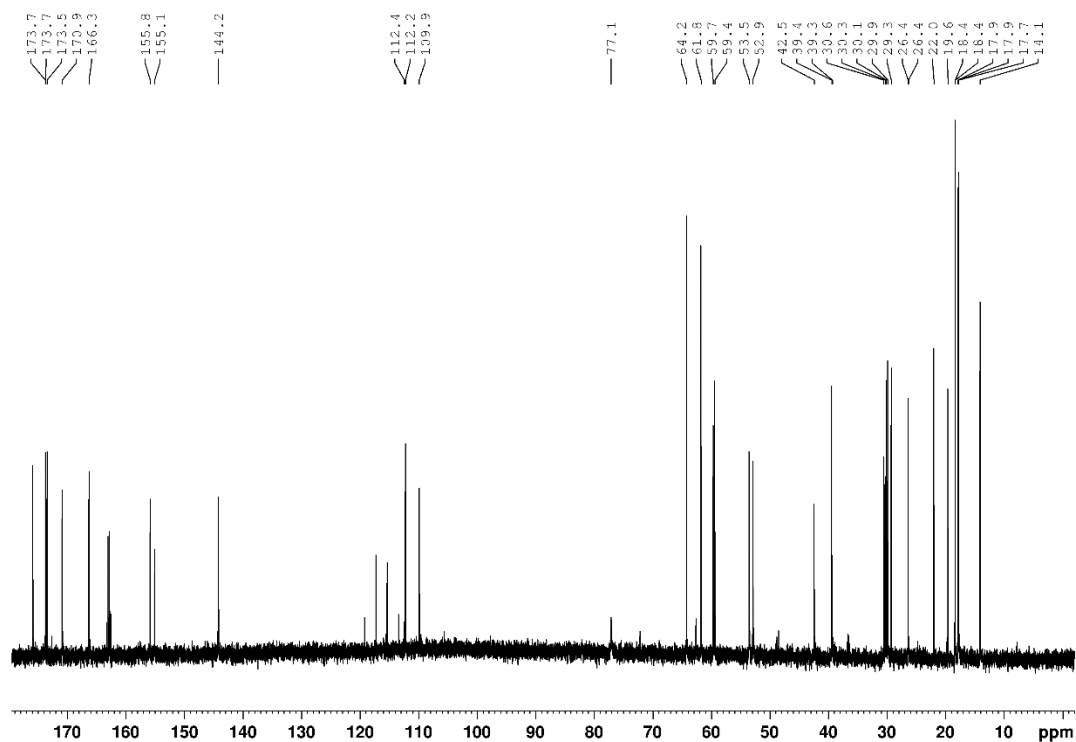


Figure S66. ^{13}C NMR of *Safirinium*-peptide conjugate **P4**.

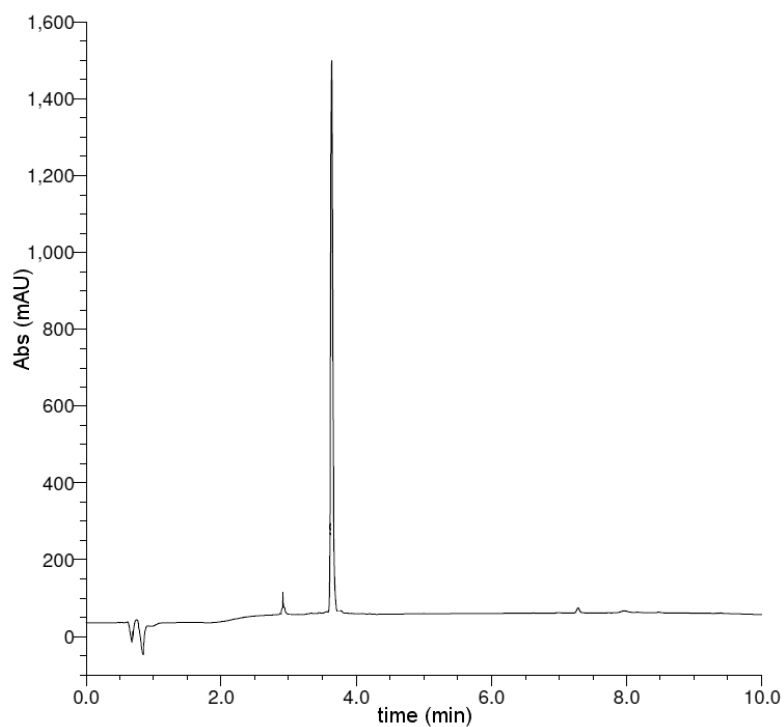


Figure S67. RP-HPLC (gradient: 5-95% B in A in 10 min) of *Safirinium*-peptide conjugate **P4** ($R_t = 3.65$ min).

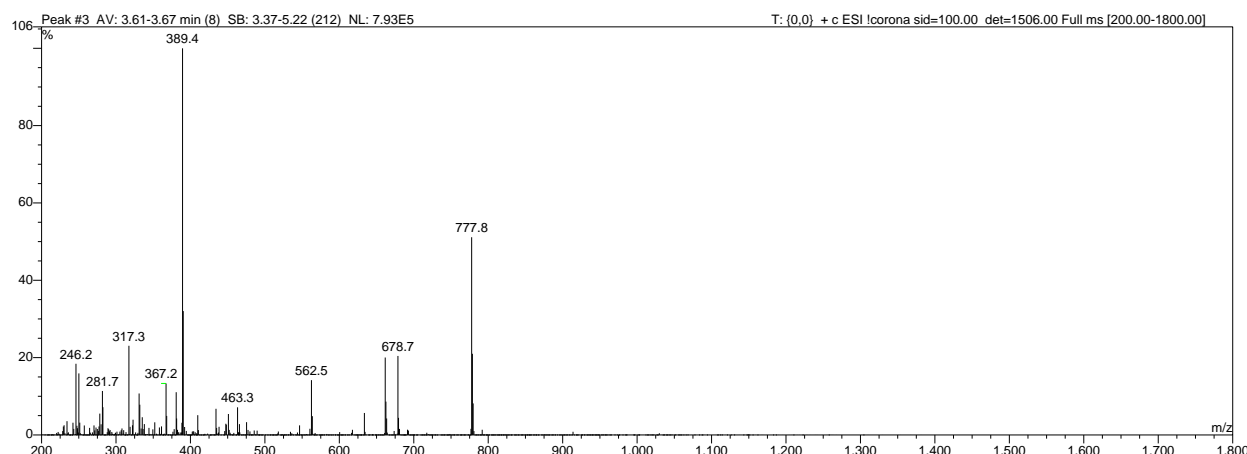


Figure S68. MS spectrum of *Safirinium*-peptide conjugate **P4** (Rt = 3.65 min), $[M]^+$ 777.8 (found), $[M]^+$ 777.4 (calcd).

Synthesis of *N*-[2-ethyl-2,3-dihydro-5,7-dimethyl-2-ethyl-2-(1-methylethyl)-1,2,4-triazolo[4,3-*a*]pyridinium-8-carbonyl]-L-methionylglycyl-L-lysyl-L-valyl-L-valinamide (P5**)**

Peptide conjugate **P5** was synthesized, cleaved from the resin and purified as described in the **general procedure D**. The product was obtained as a white powder in 15% yield (11 mg, 0.014 mmol).

^1H NMR (600MHz, 10% D_2O) δ 8.97 (d, J = 7.0 Hz, 1H), 8.50 (t, J = 5.9 Hz, 1H), 8.20 (d, J = 3.5 Hz, 1H), 8.19 (d, J = 3.3 Hz, 1H), 8.11 (d, J = 6.8 Hz, 1H), 7.58 (s, 1H), 7.45 (bs, 2H), 7.04 (s, 1H), 6.12 (s, 1H), 5.69 (dd, J = 9.9 Hz, J = 3.7 Hz, 1H), 5.64 (dd, J = 9.9 Hz, J = 6.2 Hz, 1H), 4.27 (q, J = 7.1 Hz, 1H), 4.04 (t, J = 8.1 Hz, 1H), 3.99 (t, J = 7.9 Hz, 1H), 3.96 – 3.75 (m, 2H), 3.67 (se, J = 6.8 Hz, 1H), 3.56 – 3.49 (m, 1H), 2.92 – 2.87 (m, 2H), 2.65 – 2.54 (m, 2H), 2.25 (s, 3H), 2.17 (s, 3H), 2.13 – 2.09 (m, 1H), 2.04 (s, 3H), 2.01 – 1.93 (m, 2H), 1.75 – 1.69 (m, 1H), 1.67 – 1.62 (m, 1H), 1.58 (p, J = 7.7 Hz, 2H), 1.36 – 1.28 (m, 8H), 1.25 (t, J = 7.1 Hz, 3H), 0.87 – 0.85 (m, 12H), 0.83 (d, J = 6.8 Hz, 3H)

* - Halfa Met1 overlapped with residual water signal

$^{13}\text{C}\{\text{H}\}$ NMR (151MHz, 10% D_2O) δ 175.9, 173.8, 173.7, 173.4, 170.9, 166.6, 155.7, 154.0, 143.7, 111.6, 109.8, 70.2, 68.9, 60.4, 59.7, 59.4, , 53.5, 53.1, 52.5, 42.3, 39.4, 30.6, 30.2, 30.1, 29.9, 29.25, 26.4, 22.0, 19.2, 18.3(8), 18.3(7), 17.9(2), 17.8(5), 17.7, 15.8, 15.3, 14.1, 7.3

RP-HPLC-MS: (gradient: 5-95% B in A in 10 min): Rt = 3.89 min, MS: $[M]^+$ 777.8 (found), $[M]^+$ 778.0 (calcd). Purity: 95%

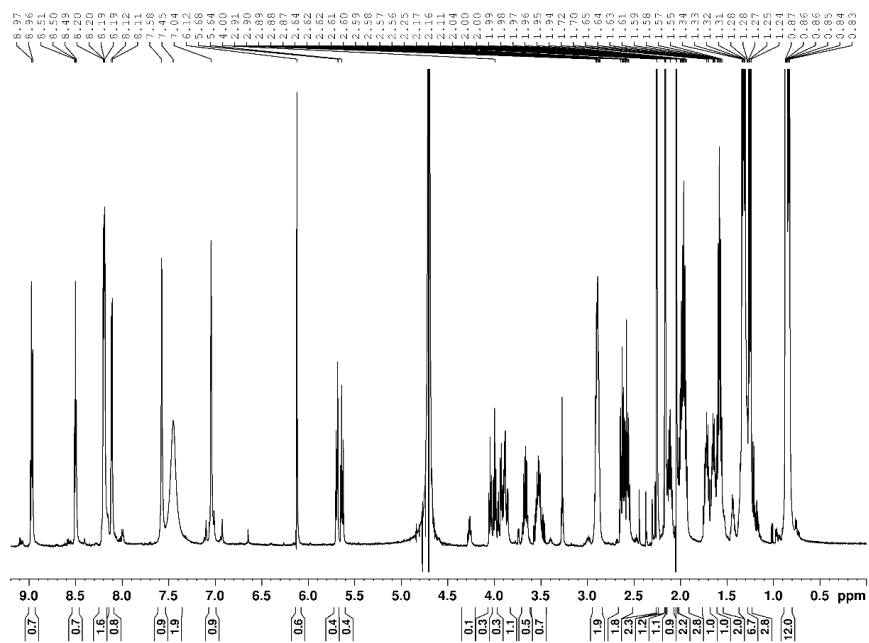


Figure S69. ^1H NMR of *Safirinium*-peptide conjugate **P5**.

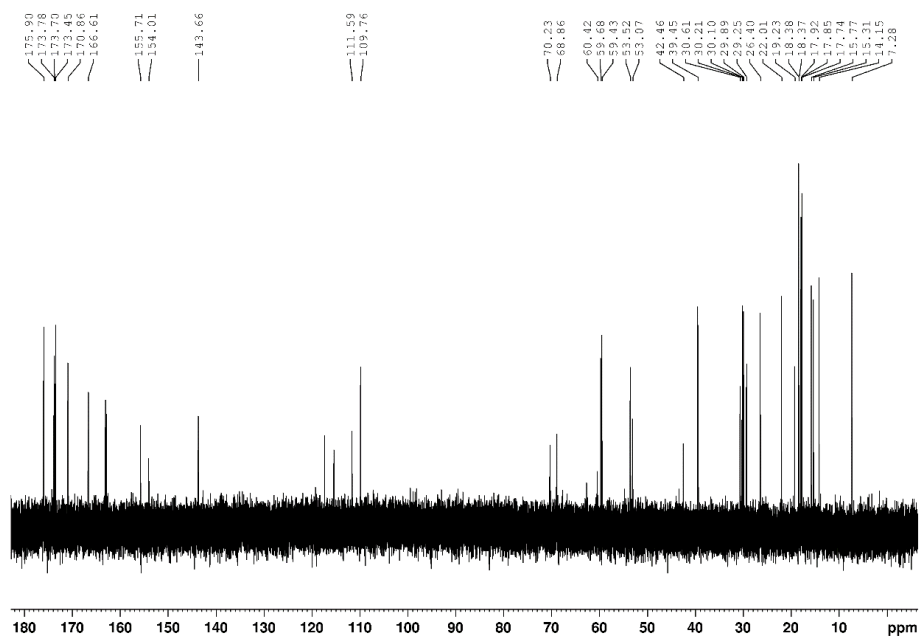


Figure S70. ^{13}C NMR of *Safirinium*-peptide conjugate **P5**.

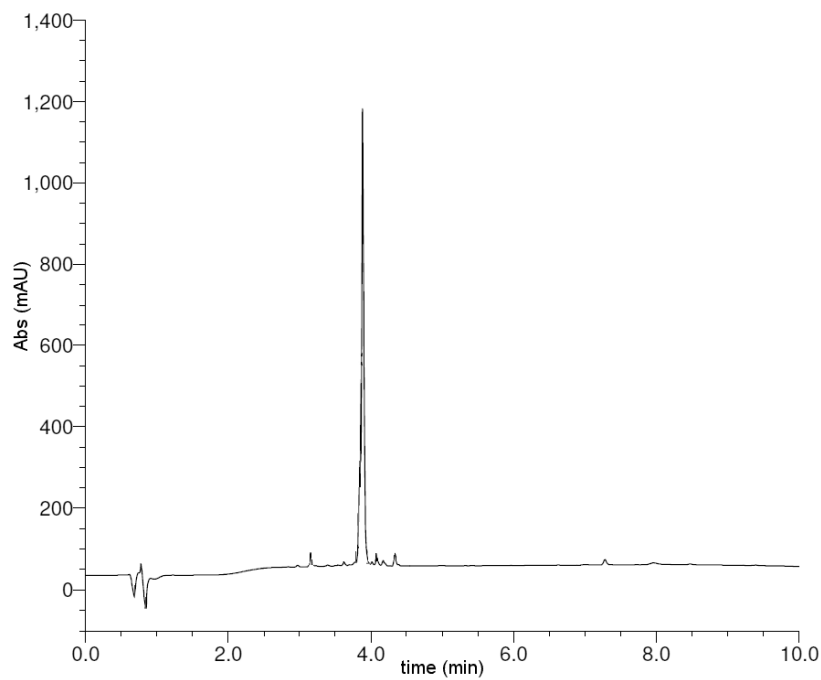


Figure S71. RP-HPLC of *Safirinium*-peptide conjugate **P5** ($R_t = 3.89$ min).

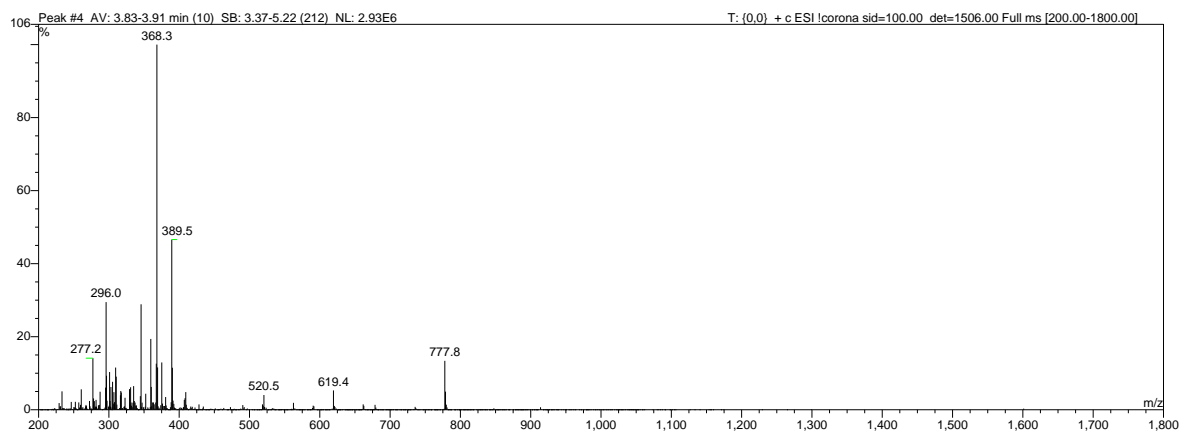


Figure S72. MS spectrum of *Safirinium*-peptide conjugate **P5** ($R_t = 3.89$ min), $[M]^+$ 777.8 (found), $[M]^+$ 778.0 (calcd).

Enzymatic assays (inhibitory activity against porcine pancreas elastase)

Porcine pancreas elastase activity assay: reagents preparation. Peptides **P**, **P2-P5** and **P2a-P5a** were prepared as stock solutions ($C_M=500\ \mu\text{M}$) in the buffer without DMSO, due to their good solubility in water at this concentration. Tris-HCl (pH=8.0, 0.1 M) buffer used for the assay was prepared in H_2O with an addition of 1% v/v DMSO for enhanced solubility of all compounds in the well. The percentage of DMSO, optimal for elastase highest activity and viability, was adjusted experimentally. There was no significant difference in the enzyme activity between 0, 0.5, and 1% DMSO percentage in the assay buffer; 5% of DMSO slightly decreased the PPE activity, and should be only used in case of poorly soluble compounds. To balance the best solubility and optimal enzyme activity, an addition of 1% of DMSO in the Tris-HCl buffer was then applied for each assay.

PPE powder (1 mg, 4.8 units/mg) was dissolved in Tris-HCl buffer (1 mL) and the solution was divided into aliquots of 50 μL for storage at -80°C . Shortly before the assay, 50 μL aliquot was reconstituted with 1150 μL of assay buffer to achieve 0.2 unit/mL concentration.

The substrate, *N*-Suc-AAA-*p*-nitroanilide, was prepared as stock solution by dissolving it (10 mg) in 764 μL of Tris-HCl buffer ($C_M=29\ \text{mM}$) and the obtained solution was divided into aliquots of 80 μL for storage at -80°C . Shortly before an assay, 80 μL aliquot was reconstituted with 720 μL of assay buffer to achieve 2.9 mM concentration.

Porcine pancreas elastase activity assays: Protocol description. The total volume in the well was adjusted to 100 μL to decrease the consumption of enzyme. The enzyme stock solution (50 μL , 4.8 units/mL) was dissolved in assay buffer (624 μL), achieving the concentration of 0.35 units/mL. 100 μL of the substrate stock solution (29 mM) was dissolved in assay buffer (887 μL), achieving 1.45 mM concentration.

10 μL of enzyme solution was added into each well (0.035 units/mL in the well). Inhibitor control, SPCK, was added into IC (inhibitor control) well at the final concentration of 5 μM . All tested compounds – peptides, modified peptides, Fmoc-deprotected building blocks, and triazolopyridinium moieties – were added in different volumes of 100 μM solution to achieve 5 different tested concentrations in the range of 10-50 μM . The plate was agitated for 1 min and incubated for 10 min. 20 μL of substrate solution was added into each well (0.29 mM in the well) to start the reaction. The absorbance in each well was measured with 12 s interval in a kinetic mode for 10 min ($\lambda = 410\ \text{nm}$). Details are summarized in **Table S2**. The well with enzyme only was used as a blank.

Table S2. Reagents of the PPE activity assay. Total volume in each well = 100 μ L. IC: Inhibitor control: test of the enzymatic activity in the presence of the inhibitor SPCK. EC= enzyme control: test of the enzymatic activity run without inhibitor

	Blank [μ L]	IC [μ L]	EC [μ L]	C _{final} =10 μ M [μ L]	C _{final} =20 μ M [μ L]	C _{final} =30 μ M [μ L]	C _{final} =40 μ M [μ L]	C _{final} =50 μ M [μ L]
Tested compound (100 μ M)	-	-	-	10	20	30	40	50
SPCK, inhibitor control (50 μ M)	-	10	-	-	-	-	-	-
PPE, enzyme (0.35 unit/mL)	10	10	10	10	10	10	10	10
<i>N</i> -Suc-AAA- <i>p</i> -NA, substrate (1.45 mM)	-	20	20	20	20	20	20	20
Assay buffer	90	60	90	60	50	40	30	20

Porcine pancreas elastase activity assays: Data elaboration. PPE activity in the presence of the tested compounds was calculated by the Equation SE1, where Sample_{slope}: the slope of the reaction progress curve, in its initial linear segment, for the reaction mixture with the tested sample; EC_{slope}: the slope of the reaction progress curve, in its initial linear segment, for the reaction mixture without inhibitor (EC).

$$\% \text{ of } PPE_{\text{activity}} = \frac{\text{Sample}_{\text{slope}}}{\text{EC}_{\text{slope}}} \times 100 \quad (\text{SE1})$$

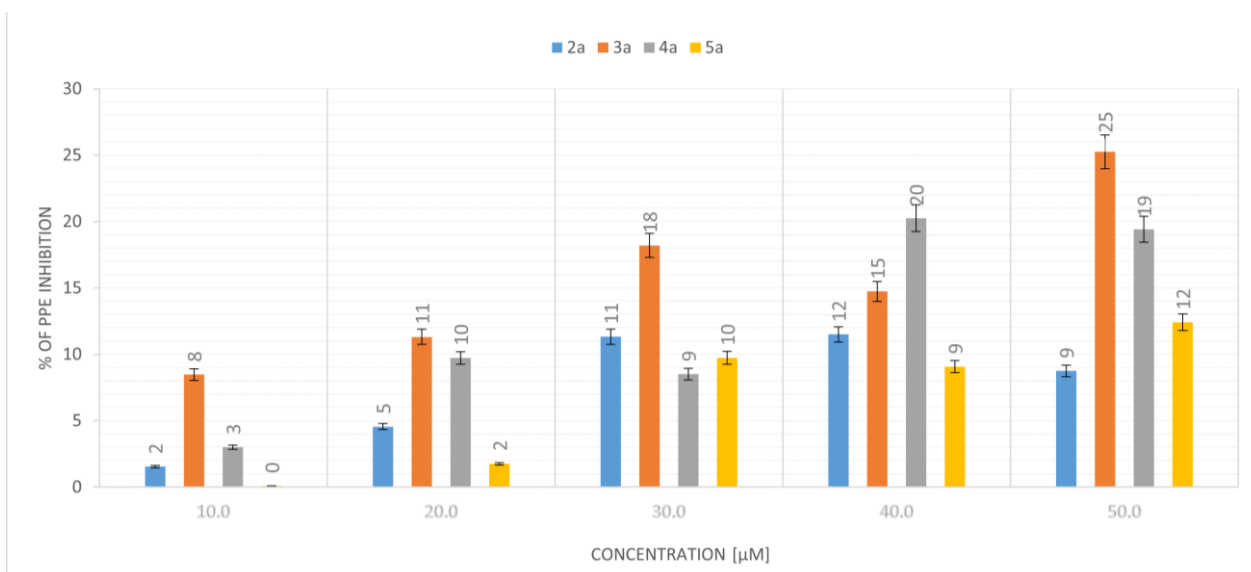


Figure S73. Averaged values ($n = 2$) of PPE inhibition in the presence of *Safirinium*-lysine conjugates **2a-5a**

Molecular modelling

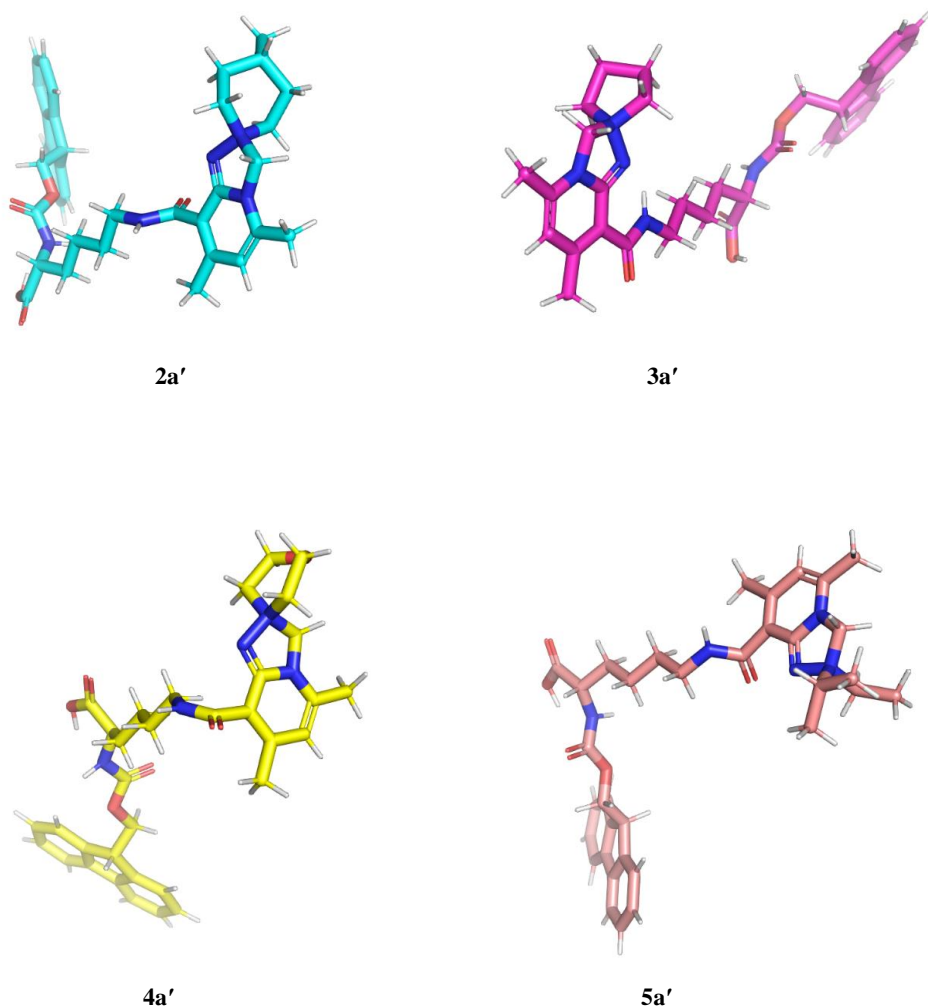


Figure S74. Optimized three-dimensional structures of **2a'-5a'**

Table S3. List of dihedral angles of optimal structures of **P2-P5** and **P2a-P5a** obtained during molecular modeling calculation.

compound	Met1		Gly2		Lys3		Val4		Val5	
	ϕ [°]	ψ [°]	ϕ [°]	ψ [°]	ϕ [°]	ψ [°]	ϕ [°]	ψ [°]	ϕ [°]	ψ [°]
P2	57,0	-139,3	-177,1	-177,6	-136,6	155,1	-128,8	132,0	-130,7	134,9
P3	57,4	-138,8	-172,6	-179,7	-136,8	154,2	-128,8	131,0	-131,6	134,8
P4	57,3	-137,3	-175,2	180,0	-137,2	155,5	-139,6	132,2	-130,7	134,6
P5	57,3	-137,3	-172,0	178,4	-137,6	153,4	-129,8	131,7	-131,4	134,7
P2a	57,7	-141,7	-173,4	-179,5	-136,0	153,4	-129,6	133,2	-129,3	134,1
P3a	57,5	-142,1	-173,8	-178,5	-135,9	152,2	-129,8	131,9	-130,7	134,8
P4a	57,9	-141,7	-174,8	-178,7	-136,0	154,4	-129,4	131,1	-131,3	135,3
P5a	57,6	-141,9	-175,1	-178,2	-136,6	153,4	-129,3	131,2	-132,1	135,5

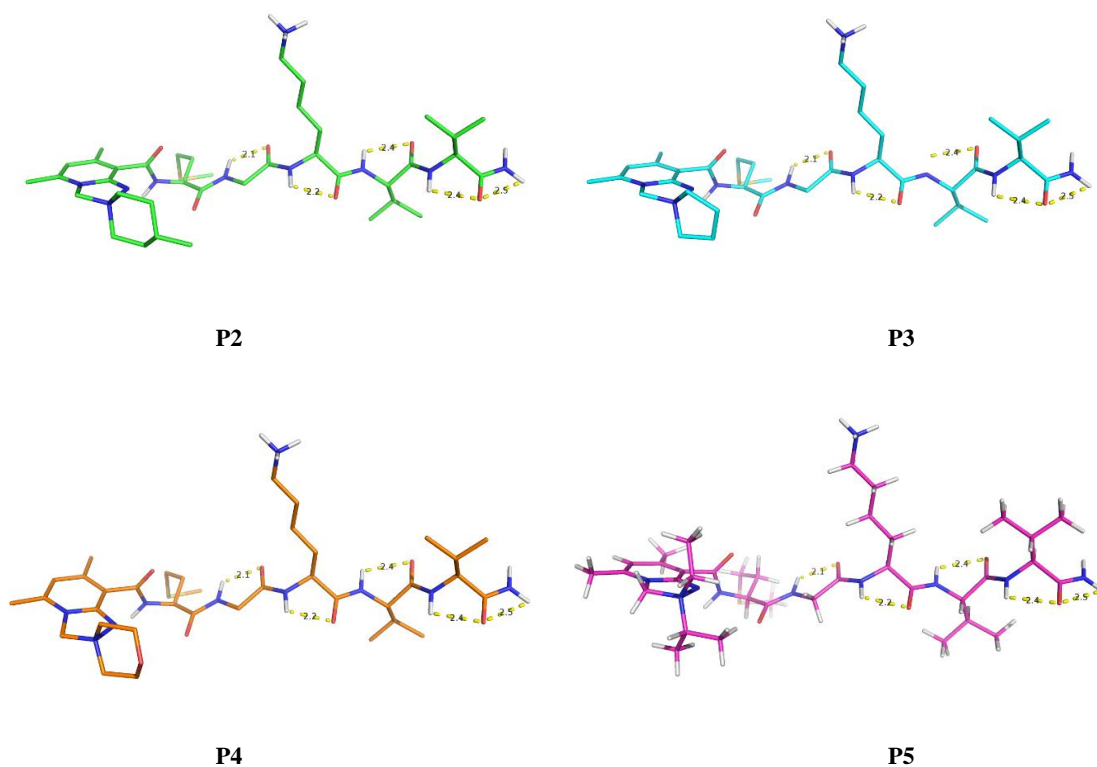


Figure S75. Optimized three-dimensional structures of **P2-P5**.

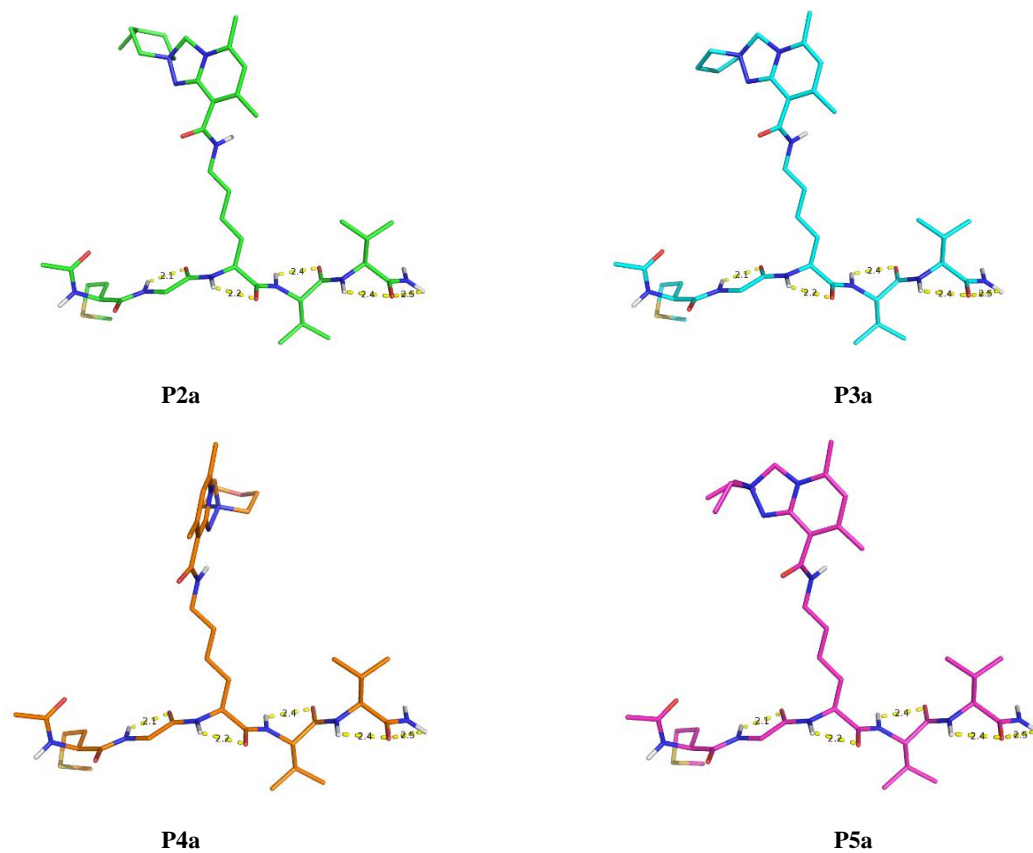


Figure S76. Optimized three-dimensional structures of **P2a-P5a**.

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