Supplementary Information for:

Discovery of potent, proteolytically stable, and cell permeable human sirtuin peptidomimetic inhibitors containing N^{ε}-thioacetyl-lysine

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Contents:

Experimental

General. The following are the materials obtained for the compound synthesis. All the materials obtained from commercial sources were used as received without further purification. Sigma–Aldrich: silica gel (70–230 mesh, 60 Å), N-methylmorpholine (NMM), piperidine, phenol, thioanisole, ethanedithiol; EMD biosciences: dichloromethane (DCM), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), acetonitrile, acetic anhydride; Novabiochem: all the N^{α}-Fmoc-protected amino acids except (i) N^{α}-Fmoc-N^{δ}-acetyl-ornithine that was synthesized in the current study for the incorporation of acetyl-ornithine into compound **9** and (ii) N^{α}-Fmoc-N^{ε}-thioacetyl-lysine that was synthesized according to our previous procedure[1] and used in the current study for the incorporation of thioacetyl-lysine (ThAcK) into compounds **2**-**9**, Rink Amide AM resin for the solid phase peptide synthesis (SPPS), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU, the coupling reagent used for SPPS), N-hydroxybenzotriazole (HOBt, the additive used for SPPS); Bachem: N^{α}-Fmoc-ornithine • HCl; Fisher: anhydrous diethyl ether and anhydrous Na_2SO_4. Alfa Aesar: acetyl chloride.

^{1}H and ^{13}C NMR spectra were obtained on a Varian Mercury 300 spectrometer or a Varian GEMINI 300 spectrometer. Mass spectra were recorded on a Bruker Esquire-LC ion trap mass spectrometer (with electrospray ionization (ESI)). High resolution mass spectrometry (HRMS) was performed at the high resolution mass spectrometry facility of the University of California, Riverside. The samples were analyzed by the Agilent 6210 LCMS instrument that was operated in the flow injection mode and the "Multimode" ionization technique was used.

The following are the materials obtained for the biochemical assays used in the current study, including the sirtuin inhibition assay, digestion assays with pronase, HDAC8, and HeLa nuclear extract: GST-SIRT1 was expressed and purified from *Escherichia coli* as described

previously using pGEX2TK-P•SIRT1 (human full length) (a kind gift from Prof. Tony Kouzarides).[2] SIRT2 (human full length), SIRT3 (human amino acid 102-399), HDAC8 (human full length), and HeLa nuclear extract were purchased from Enzo Life Sciences (Product Nos. BML-SE251-0500, BML-SE270-0500, BML-SE145-0100, and BML-KI140-0100, respectively). The peptide substrates used in the current sirtuin inhibition assay were those we also used previously,[1-4] including the SIRT1 substrates H_2N-KKGQSTSRHK-AcK-LMFKTEG-COOH and H_2N-HK-AcK-LM-COOH corresponding to amino acids 372-389 and 380-384, respectively, of the K^{382}-acetylated human p53 protein; the SIRT2 substrate H_2N-MPSD-AcK-TIGG-COOH corresponding to amino acids 36-44 of the K^{40}-acetylated human α-tubulin; the SIRT3 substrate H_2N-KRLPKTRSG-AcK-VMRRLLRKII-COOH corresponding to amino acids 633-652 of the K^{642}-acetylated human acetyl-coenzyme A synthetase 2 (AceCS2). The sirtuin inhibition assay reference compound **10** was synthesized according to the literature procedure.[5] The sirtuin inhibition assay reference compounds EX-527 and AGK2 were obtained from Enzo Life Sciences (Product No. ALX-270-437-M001) and Sigma (Cat. No. A8231), respectively. The control peptide used in the digestion assays with HDAC8 and HeLa nuclear extract was the Fluor-de-Lys™ -HDAC8 deacetylase substrate (H_2N-RH-AcK-AcK-CONH-AMC, AMC stands for 7-amino-4-methylcoumarin) obtained from Enzo Life Sciences (Product No. BML-KI178-0005). The pronase from *Streptomyces griseus* was obtained from Roche Applied Science (Cat. No. 10165921001). We used the pentameric SIRT1 substrate (i.e. H_2N-HK-AcK-LM-COOH) as the positive control for the pronase digestion assay. NaCl, KCl, and NaH_2PO_4 were purchased from Fisher. Trizma, β-NAD^+, DMSO (molecular-biology grade), and a 1.0 M solution of $MgCl_2$ (molecular-biology grade) were purchased from Sigma. All the materials obtained from commercial sources were used as received without further purification.

Synthesis of N^{α}-Fmoc-N^{δ}-acetyl-ornithine. To a stirred suspension of N^{α}-Fmoc-ornithine • HCl (1.173 g, 3.0 mmole) in DCM (30 mL) was added dropwise at 0 °C a 0.4 M solution of NMM in DCM (30 mL), followed by acetyl chloride (320 μL, 4.5 mmole). After the addition was complete, the reaction mixture was stirred at room temperature for 2.5 h before the addition of 3 mL of 6 N HCl and 30 mL of ddH_2O at 0 °C to destroy excess acetyl chloride. The organic layer was isolated and washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure, affording an oily residue from which the desired product was isolated *via* silica gel column chromatography as a white solid (606 mg, 51%): ^{1}HNMR (300 MHz, DMSO-d_6): δ (ppm) 7.89-7.30 (m, 8H, H_{arom}), 4.30-4.20 (m, 3H, CH_2O and H_{alpha}), 3.98-3.90 (m, 1H, Fluorenyl H_9), 3.06-3.00 (m, 2H, CH_2N), 1.80 (s, 3H, CH_3), 1.80–1.40 (m, 4H, CH_2CH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 174.3 (COOH), 169.5 (C(=O)NH), 156.6 (NHC(=O)O), 144.2 (C_{arom}), 141.2 (C_{arom}), 128.1 (C_{arom}), 127.5 (C_{arom}), 125.7 (C_{arom}), 120.6 (C_{arom}), 66.1 (CH_2O), 54.2 (C_{alpha}), 47.1 (Fluorenyl C_9), 38.6 (CH_2N), 28.8 (CH_2), 26.4 (CH_2), 23.1 (CH_3).

Synthesis and purification of 2-9. Peptides **2-7** and peptidomimetics **8** and **9** were all synthesized using the Fmoc chemistry-based SPPS[6] on a PS3 peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) from Rink Amide AM resin. For each amino acid coupling reaction, 2 or 4 equivalents of a Fmoc-protected amino acid, 1.9 or 3.8 equivalents of the coupling reagent HBTU and the additive HOBt were used in the presence of 0.4 M NMM/DMF, and the coupling reaction was allowed to proceed at room temperature for 2 or 1 h respectively. A 20% (v/v) piperidine/DMF solution was used for Fmoc removal. The N-terminal α-amino group of each compound was acetylated on resin with acetic anhydride. All the compounds were cleaved from the resin by a TFA-containing cleavage cocktail (83.6% (v/v)

TFA, 5.9% (v/v) phenol, 4.2% (v/v) ddH_2O, 4.2% (v/v) thioanisole, 2.1% (v/v) ethanedithiol), precipitated in cold diethyl ether, and purified by the reversed-phase HPLC on a preparative C18 column (100 Å, 2.14 x 25 cm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 10mL/min and monitored at 214 nm. The pooled HPLC fractions were stripped of acetonitrile and lyophilized to give all the compounds (**2-9**) as puffy white solids. The purity of the purified **2-9** was >95% as verified by the reversed-phase HPLC on an analytical C18 column (100 Å, 0.46 x 25 cm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min and monitored at 214 nm. The exact masses of the purified peptides **2-7** were confirmed by electrospray ionization (ESI) mass spectrometric analysis. The exact masses of the purified peptidomimetics **8** and **9** were confirmed by the HRMS analysis.

Peptide **2**: MS (ESI) calcd. for $C_{36}H_{56}N_9O_7S_2$ ($[M + H]^+$) 790.4; found: 790.4.

Peptide **3**: MS (ESI) calcd. for $C_{36}H_{57}N_{10}O_7S_2$ ($[M + H]^+$) 805.4; found: 805.4.

Peptide **4**: MS (ESI) calcd. for $C_{39}H_{54}N_9O_8S_2$ ($[M + H]^+$) 840.4; found: 840.4.

Peptide **5**: MS (ESI) calcd. for $C_{35}H_{61}N_{10}O_7S_2$ ($[M + H]^+$) 797.4; found: 797.5.

Peptide **6**: MS (ESI) calcd. for $C_{35}H_{62}N_{11}O_7S_2$ ($[M + H]^+$) 812.4; found: 812.5.

Peptide **7**: MS (ESI) calcd. for $C_{37}H_{64}N_{11}O_8S_2$ ($[M + H]^+$) 854.4; found: 854.5.

Peptidomimetic **8**: MS (Multimode) calcd. for $C_{26}H_{47}N_7O_6NaS$ ($[M + Na]^+$) 608.3201; found: 608.3197. Calcd. for $C_{26}H_{48}N_7O_6S$ ($[M + H]^+$) 586.3387; found: 586.3367.

Peptidomimetic **9**: MS (Multimode) calcd. for $C_{24}H_{43}N_7O_6NaS$ ($[M + Na]^+$) 580.2888; found: 580.2895. Calcd. for $C_{24}H_{44}N_7O_6S$ ($[M + H]^+$) 558.3074; found: 558.3066.

Sirtuin inhibition assay. The HPLC-based sirtuin inhibition assay was performed as described previously.[1-3] Briefly, a sirtuin inhibition assay solution (100 μL) contained the following components: 25 mM (or 50 mM for the SIRT2 assay) Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA (Sigma Cat. No. A3803 with reduced fatty acid content, for the SIRT2 assay only), β-NAD^+ (0.5 mM for the SIRT1 and the SIRT2 assay, 3.5 mM for the SIRT3 assay), the peptide substrate (0.3 mM of the SIRT1 substrate: H_2N-KKGQSTSRHK-AcK-LMFKTEG-COOH for assaying compounds **2-7** as before for **1**[1,3] or H_2N-HK-AcK-LM-COOH for assaying compounds **8-10** and EX-527; 0.5 mM of the SIRT2 substrate: H_2N-MPSD-AcK-TIGG-COOH; 0.03 mM of the SIRT3 substrate: H_2N-KRLPKTRSG-AcK-VMRRLLRKII-COOH), an inhibitor with varied concentrations including 0, and an enzyme (GST-SIRT1, 350 nM; SIRT2, 150 nM; or SIRT3, 215 nM). Of note, the same [S]/Km ratios for both substrates (~3.2 for the peptide substrates and ~5.6 for NAD^+) were used for the inhibition assays with SIRT1, SIRT2, and SIRT3 when assessing the inhibitory selectivity of **8** and **9** among these three human sirtuins. An enzymatic reaction was initiated by the addition of an enzyme at 37 °C and was allowed to be incubated at 37 °C for 5 min (for the SIRT1 assay) or 60 min (for the SIRT2 assay) or 20 min (for the SIRT3 assay) until quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (100 Å, 5 μm, 0.46 x 25 cm) or a reversed-phase Pursuit XRs Ultra 2.8 C18 column (100 Å, 2.8 μm, 100 x 2.0 mm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min or 0.5 mL/min, respectively, and UV monitoring at 214 nm. Turnover of the limiting substrate was maintained at ≤13%. Stock solutions of **2-9** were prepared in ddH_2O, and those for **10**, EX-527, and AGK2 were prepared in DMSO. The final DMSO concentration in an assay

mixture was maintained at ≤ 5% (v/v). No deleterious effect on sirtuin's deacetylase activity was observed at this DMSO concentration. IC_{50} values were estimated from the Dixon plots ($1/v_0$ *vs.* [inhibitor])[7] as an indication of the inhibition potency.

Sirtuin time course assay. A sirtuin time course assay solution contained the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM NAD^+ (or 3.5 mM for the SIRT3 time course assay), 350 nM GST-SIRT1 (or SIRT2, SIRT3), and a test compound (**8** or **9**) (0.3 mM for the SIRT1 and the SIRT3 time course assays, 0.5 mM for the SIRT2 time course assay). An enzymatic reaction was initiated by the addition of the enzyme at 37 °C and was incubated at 37 °C until quenched at 0 and 4 h with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay mixtures were directly injected into a reversed-phase HPLC C18 column (100 Å, 0.46 x 25 cm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min, and UV monitoring at 214 nm.

Pronase digestion assay. The following procedure was modified from that used previously by Neffe et al.[8] To 200 µL of a 200 µM solution of a test compound (**8**, **9**, or the positive control) in ddH_2O was added 200 µL of a 8 ng/µL pronase solution in 100 mM Tris•HCl (pH 7.3), and the well-mixed solution was then incubated at 37 °C until quenched with a 1.0 M solution of acetic acid in ddH_2O at 0 and 30 min (for **8** and **9**) or at 0 and 5 min (for the positive control). One portion (40 µL) of the pronase digestion mixture was treated with 80 µL of the 1.0 M acetic acid solution, and the whole mixture was vigorously vortexed, centrifuged, and the supernatant was directly injected into a reversed-phase C18 analytical HPLC column (100 Å, 0.46 x 25 cm). The C18 column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min, and UV monitoring at 214 nm.

For the quenched samples not to be analyzed by HPLC immediately, they were snap frozen in liquid N_2 and stored at -80 °C until HPLC injection. The HPLC peak areas obtained for a given test compound at different time points were used to estimate the percentage remaining for this test compound as the function of the digestion time. The graph of the percentage remaining *versus* time was used to compare the proteolytic stability of different test compounds, as shown in **Fig. 4**.

HDAC8 time course assay. This assay was performed as described previously.[9] Briefly, a HDAC8 assay solution had the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA (Sigma Cat. No. A3803 with reduced fatty acid content), 0.3 mM of a test compound (**8**, **9**, or the control peptide), and 375 nM HDAC8. An enzymatic reaction was initiated by the addition of HDAC8 at room temperature and was allowed to be incubated at room temperature until quenched with the following stop solution at different time points (0, 1, 2, 3, 4h): 1.0 M HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase HPLC C18 column (100 Å, 0.46 x 25 cm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min, and UV monitoring at 214 nm. The HPLC peak areas obtained for a given test compound at different time points were used to estimate the percentage remaining for this test compound as the function of the HDAC8 incubation time. The graph of the percentage remaining *versus* time was used to compare the stability of different test compounds toward HDAC8, as shown in **Fig. S1**.

HeLa nuclear extract time course assay. The above-described HDAC8 assay procedure was followed except that HeLa nuclear extract (9 mg protein/mL) (12 µL) instead of HDAC8 was used and the enzymatic incubation was allowed to proceed at room temperature until

quenched at 0, 1, and 2h. The quenched assay mixtures were also analyzed with the reversed-phase HPLC. Again, the HPLC peak areas obtained for a given test compound (**8**, **9**, or the control peptide) at different time points were used to estimate the percentage remaining for this test compound as the function of the enzymatic incubation time. The graph of the percentage remaining *versus* time was also used to compare the stability of different test compounds toward the HDACs (primarily HDAC1 and HDAC2) present in the HeLa nuclear extract, as shown in **Fig. S1**.

Western blot analysis. HCT116 human colon cancer cells were cultured in McCoy5A culture medium containing 10% FBS with penicillin and streptomycin. HCT116 cells (5×10^5) were treated for 8 h with a test compound (**1**, **8**, or **9**) at different concentrations (**1**: 0, 5, 50, and 500 μM; **8**: 0, 3, 30, and 300 μM; **9**: 0, 5, 50, and 500 μM) and then collected and extracted with RIPA buffer (50mM Tris•HCl (pH 8.0), 0.5% triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM NaF). Equivalent amounts of proteins from each lysate were resolved in 10% SDS-polyacrylamide gel and then transferred onto PVDF membranes (Bio-Rad Laboratories). After having been blocked with Tris-buffered saline (TBS) containing 5% milk, the transblotted membrane was incubated overnight at 4 °C with acetylated p53 antibody (Cell Signaling) (1:1000 dilution). After washed twice with water, the membranes were incubated with the rabbit antimouse IgG-horseradish peroxidase conjugate (diluted 1:2500) for 2 h at room temperature, and again washed twice with water. The immunoblots were visualized by enhanced chemiluminescence. Stripping the membrane and then blot it with p53 antibody (Cell Signaling) (1:1000 dilution).

References:

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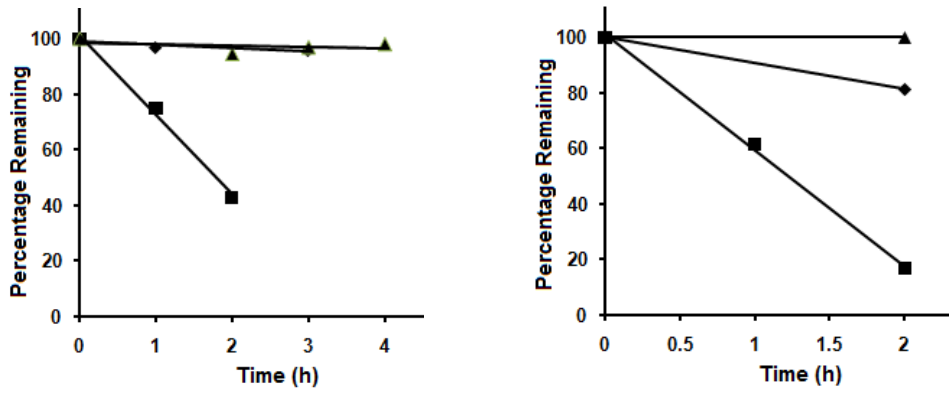
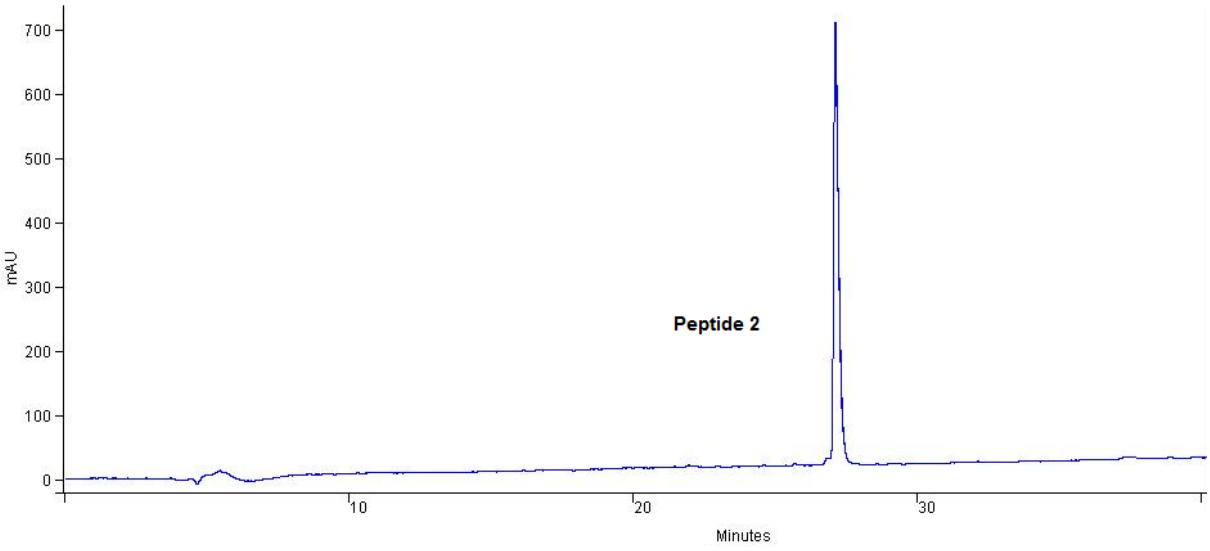


(A) (B)

Fig. S1. The stability of **8** and **9** toward the classical protein deacetylases. (A) HDAC8 time course assay: ♦, degradation profile of **8**; ▲, degradation profile of **9**; ■, degradation profile of the control peptide H_2N-RH-AcK-AcK-CONH-AMC. (B) HeLa nuclear extract time course assay: ♦, degradation profile of **8**; ▲, degradation profile of **9**; ■, degradation profile of the control peptide H_2N-RH-AcK-AcK-CONH-AMC.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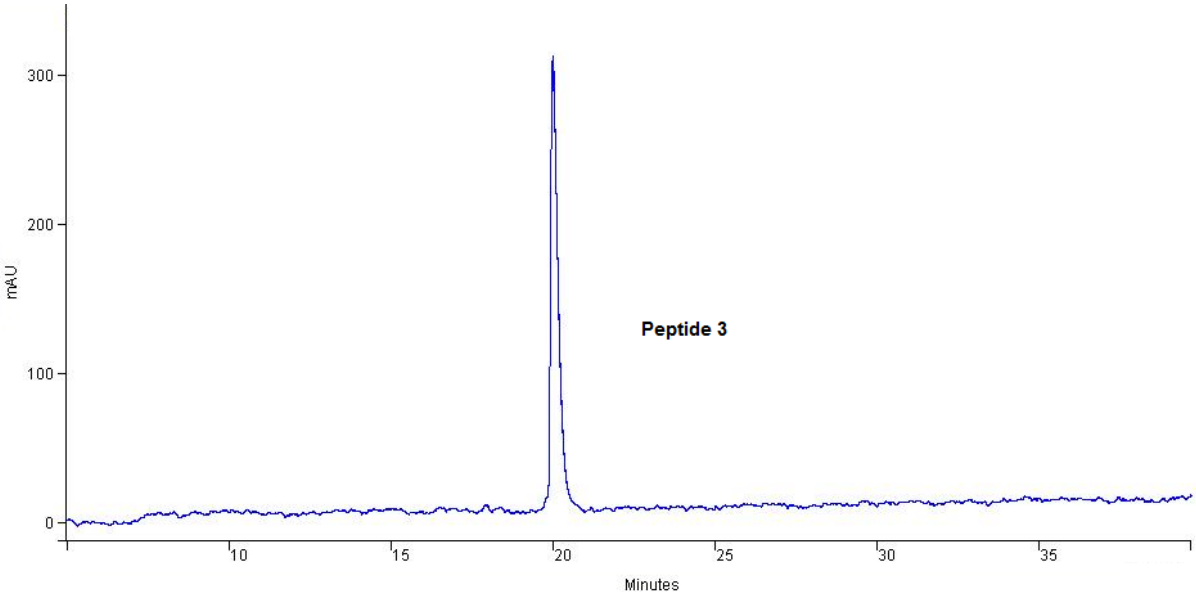
600
500
400
300
200
100
0
mAU
Peptide 2
10
20
30
Minutes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100
0
mAU
Peptide 3
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25
30
35
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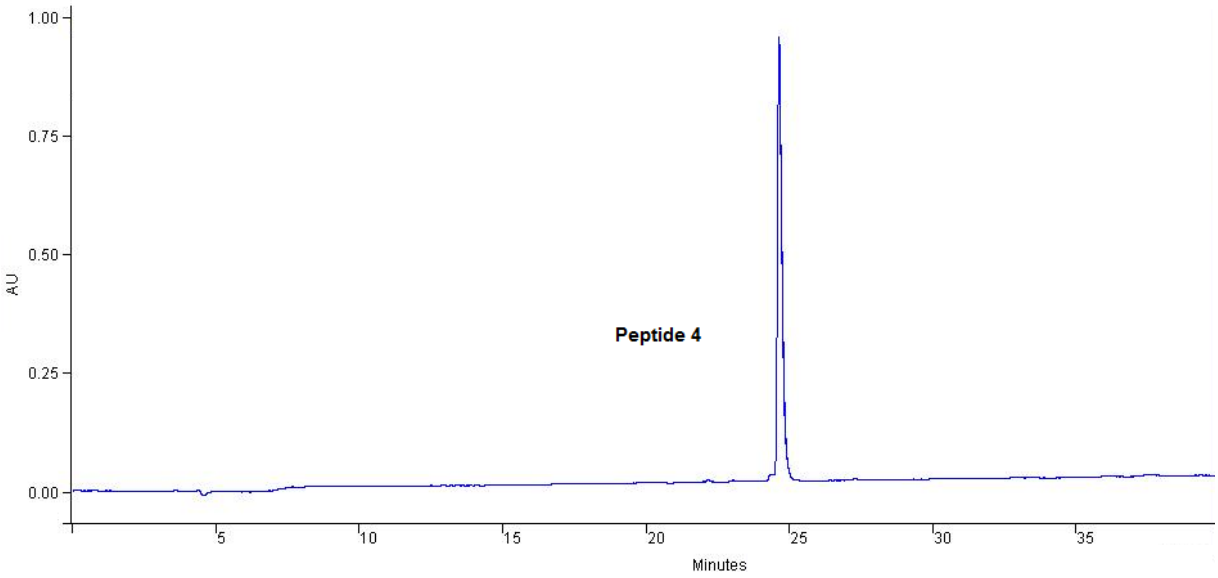
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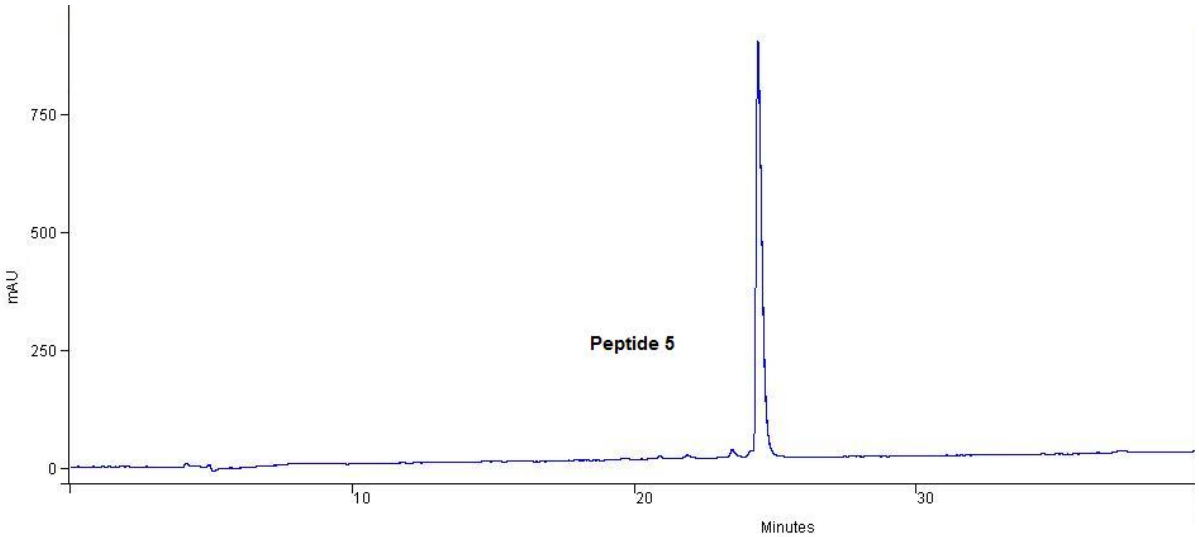
0.00
AU
Peptide 4
5
10
15
20
25
30
35
Minutes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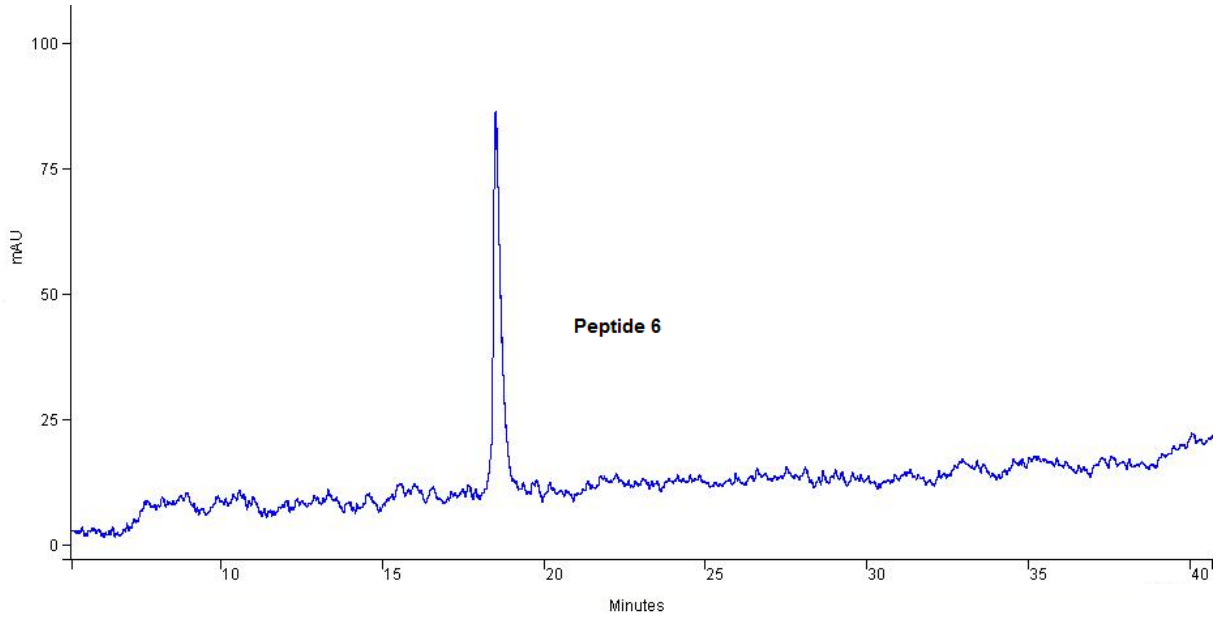
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35
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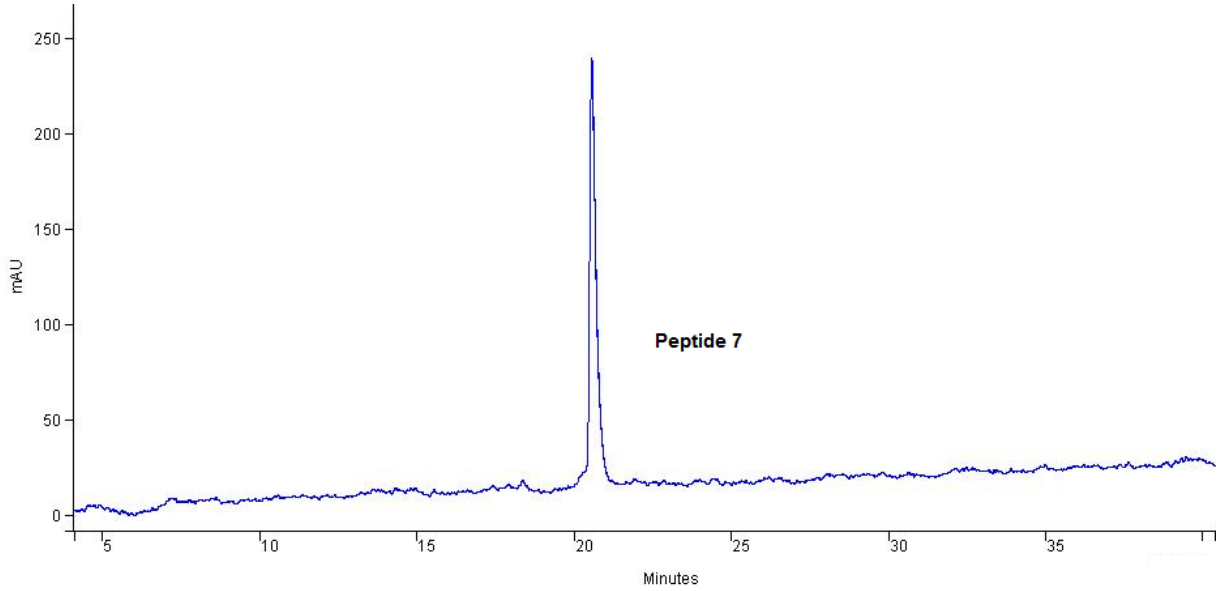
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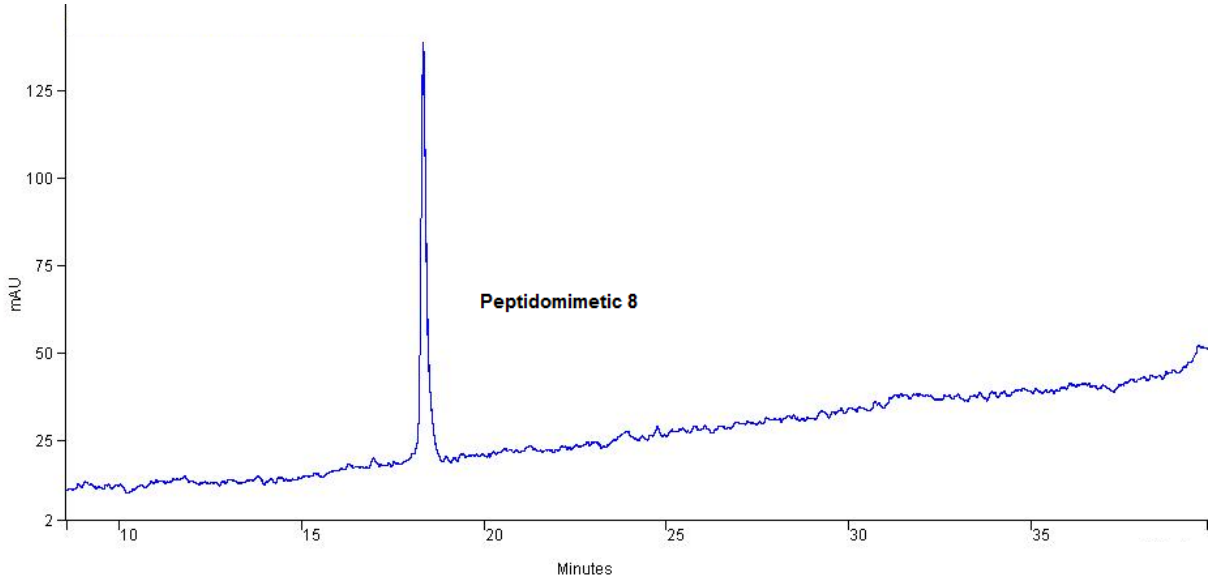
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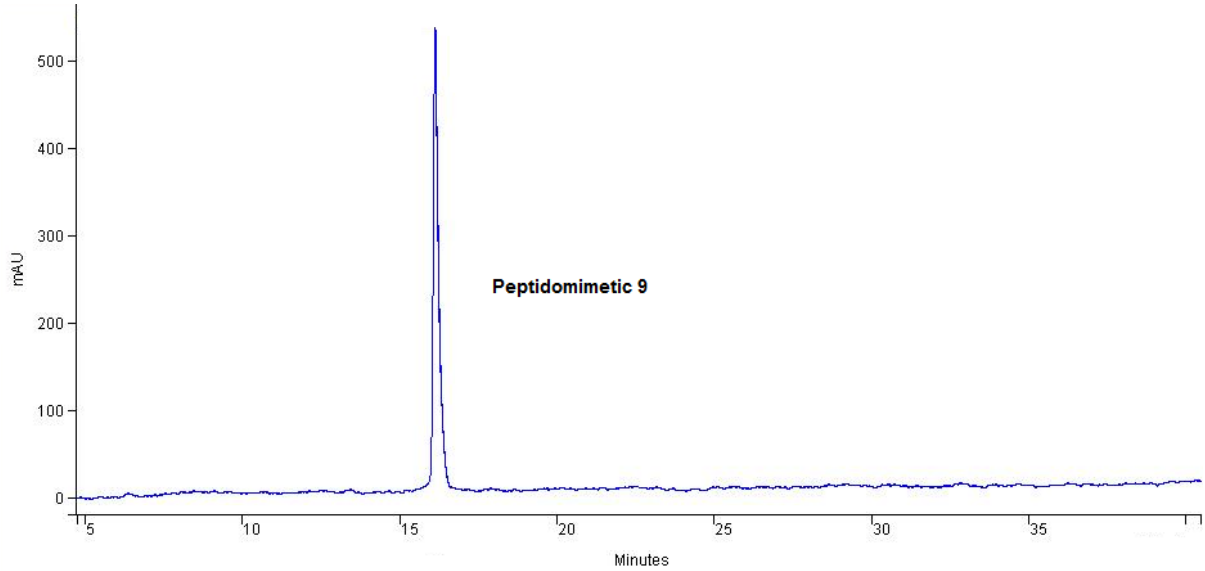
20
25
30
35
Minutes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10
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20
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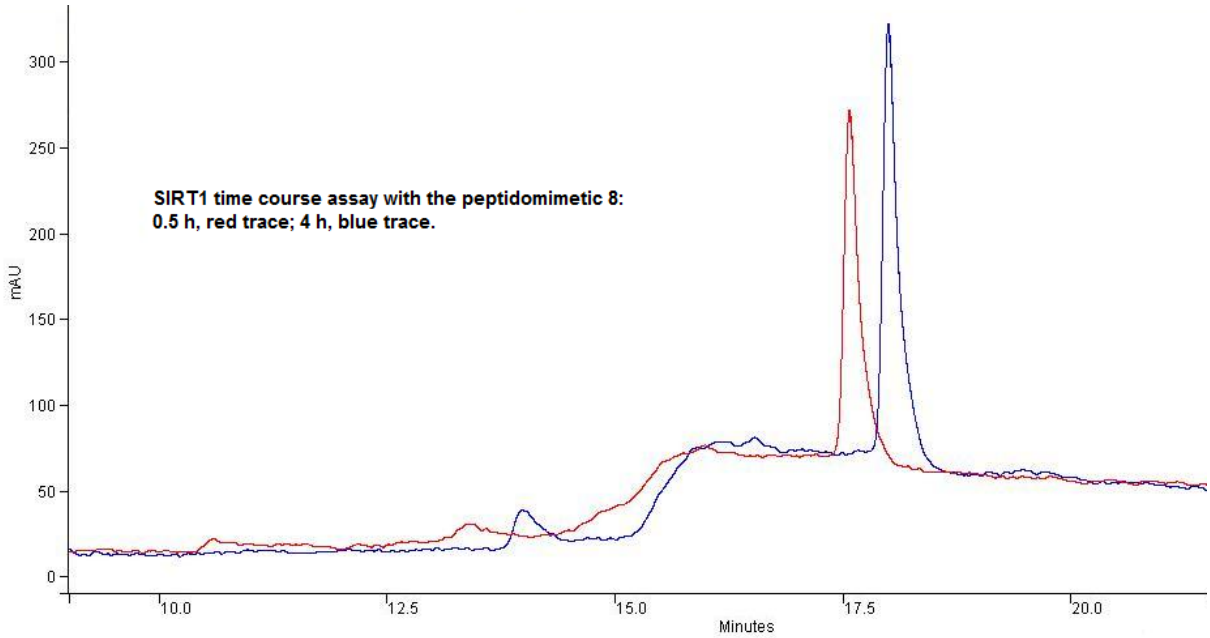
30
35
Minutes


SIRT1 time course assay with the peptidomimetic 8:
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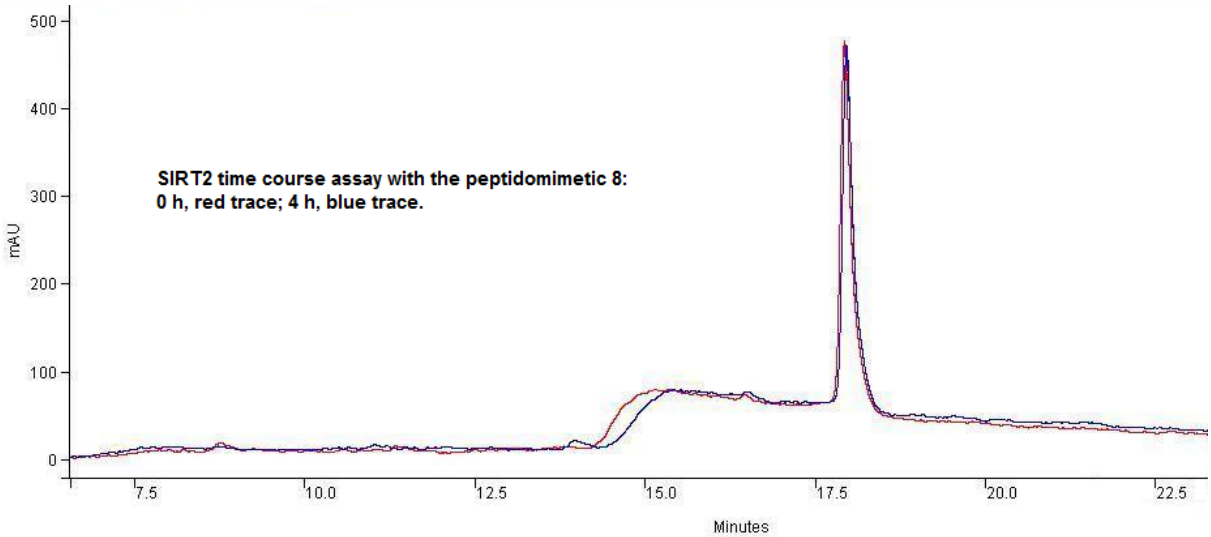
mAU
300
250
200
150
100
50
0
10.0
12.5
15.0
17.5
20.0
Minutes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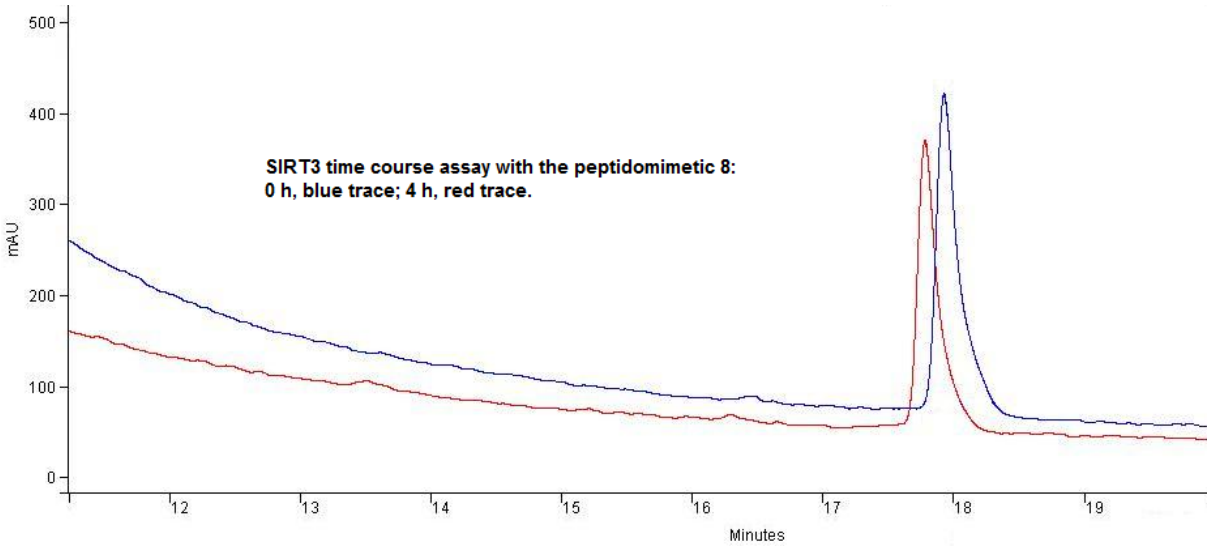
300
200
100
0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
Minutes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500
400
300
200
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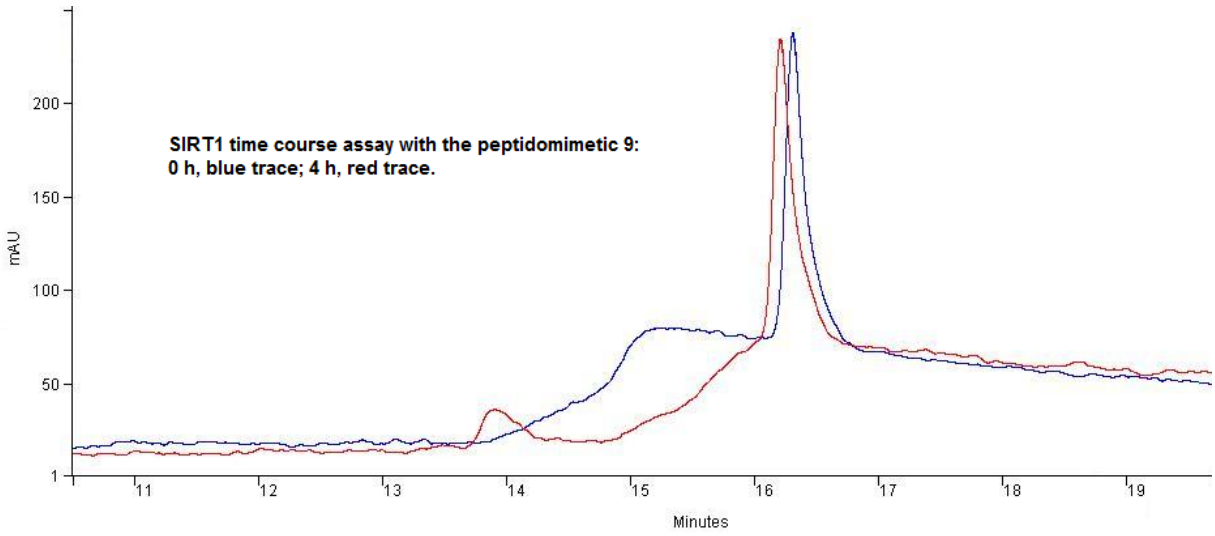
0
12
13
14
15
16
17
18
19
Minutes


SIRT1 time course assay with the peptidomimetic 9:
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1
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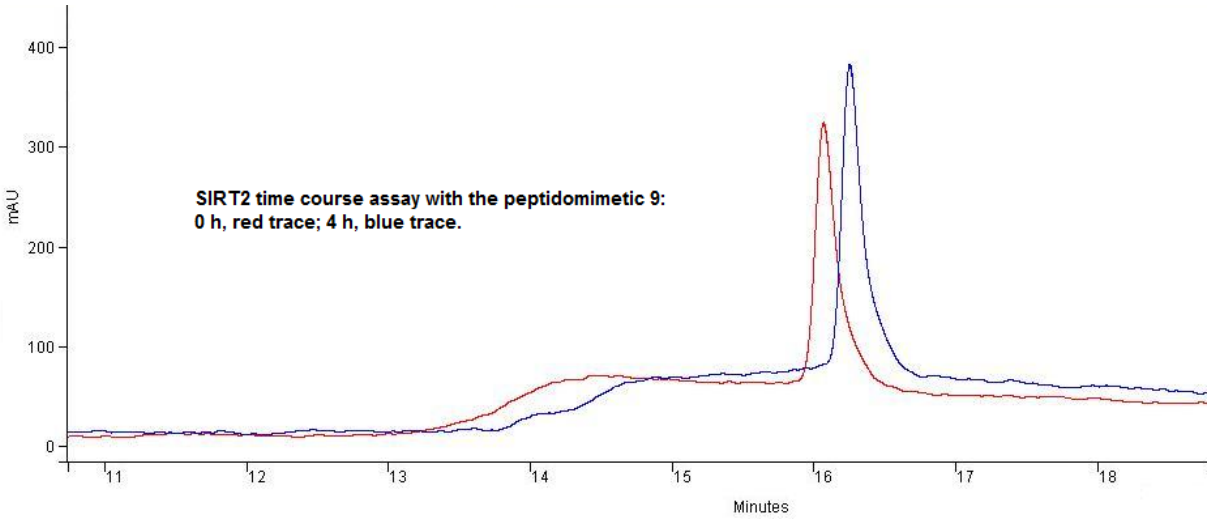
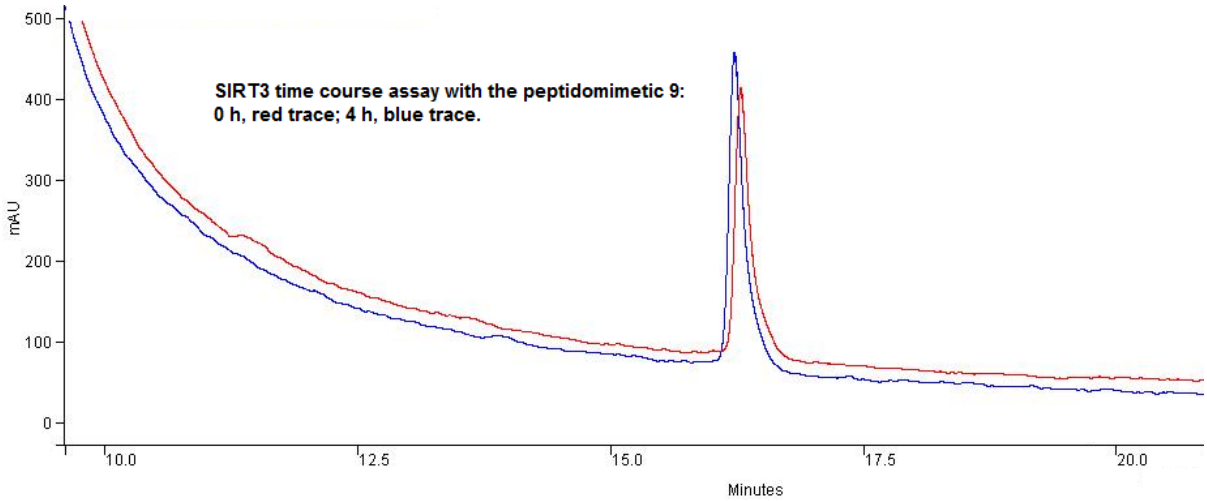
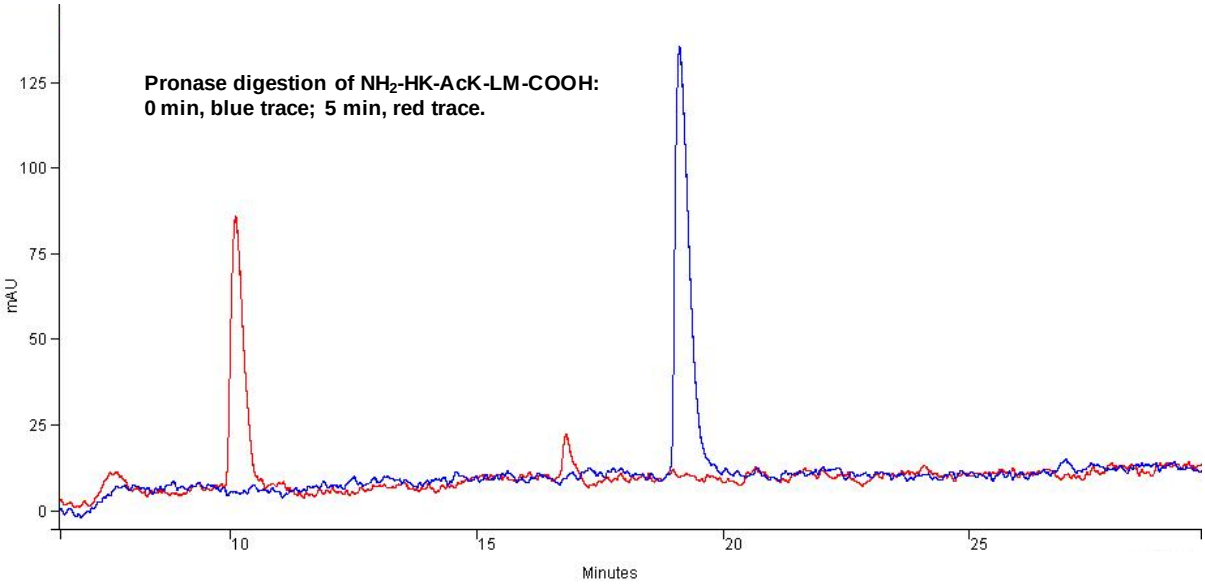
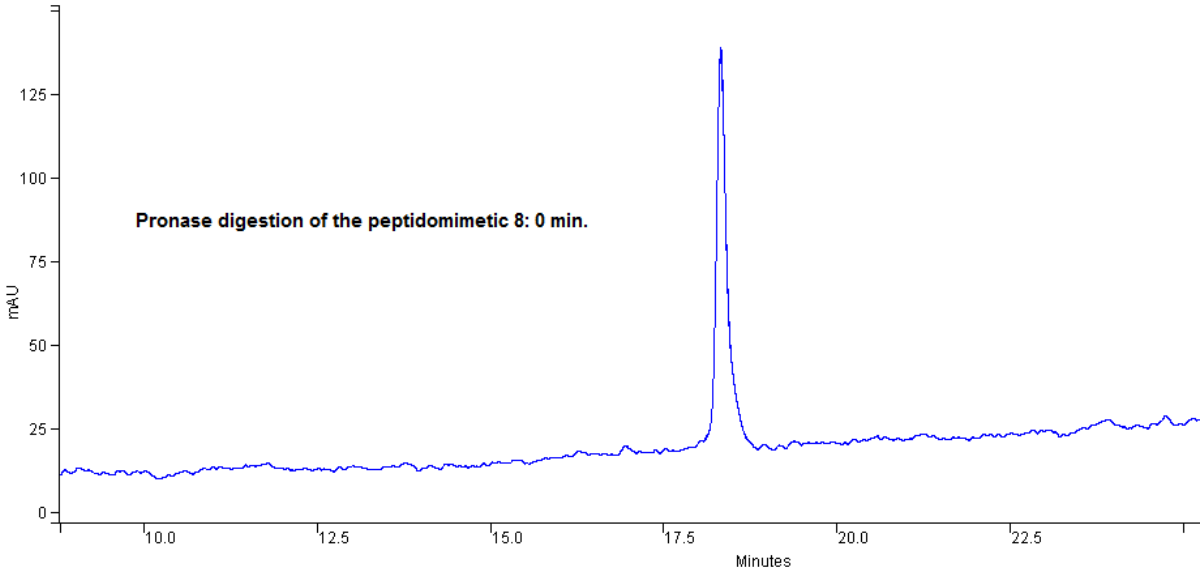
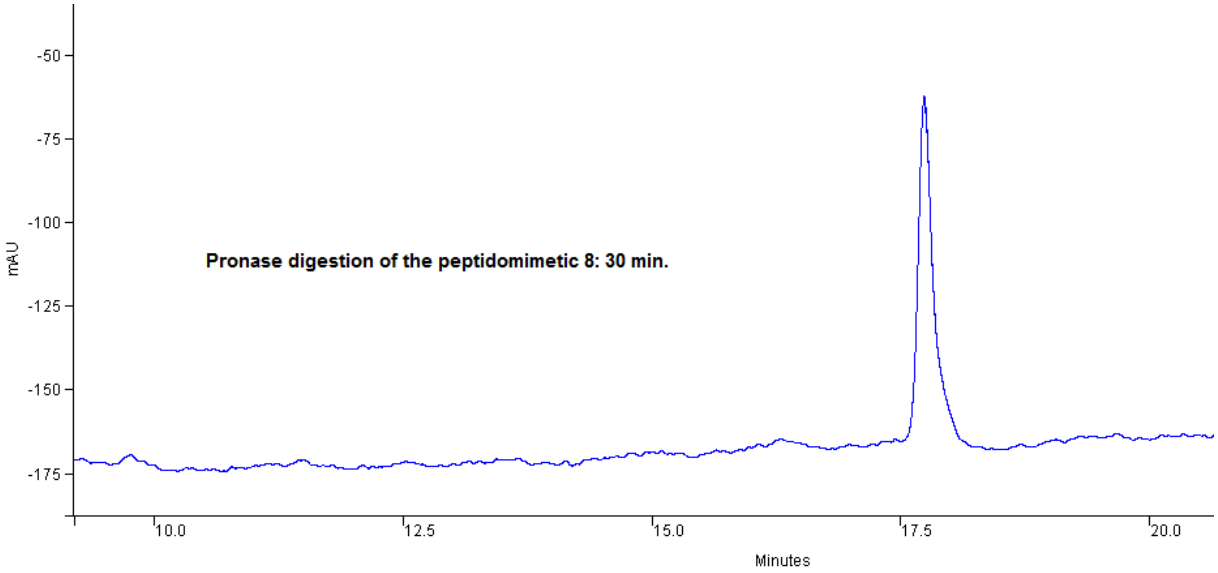
16
17
18
19
Minutes

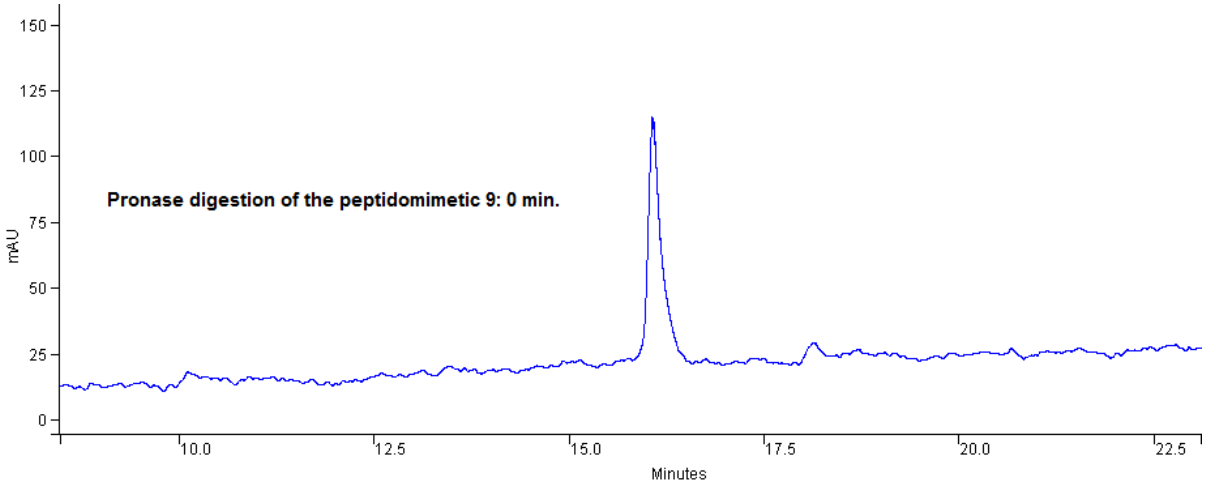

SIRT2 time course assay with the peptidomimetic 9:
0 h, red trace; 4 h, blue trace.
mAU
400
300
200
100
0
11
12
13
14
15
16
17
18
Minutes

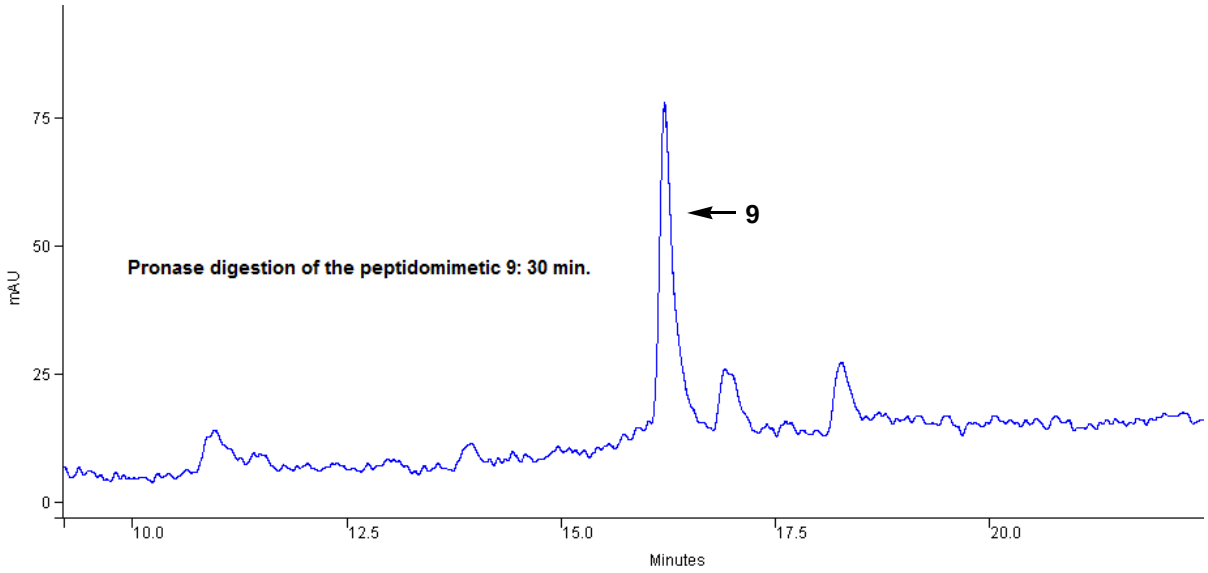

SIRT3 time course assay with the peptidomimetic 9:
0 h, red trace; 4 h, blue trace.
mAU
500
400
300
200
100
0
10.0
12.5
15.0
17.5
20.0
Minutes

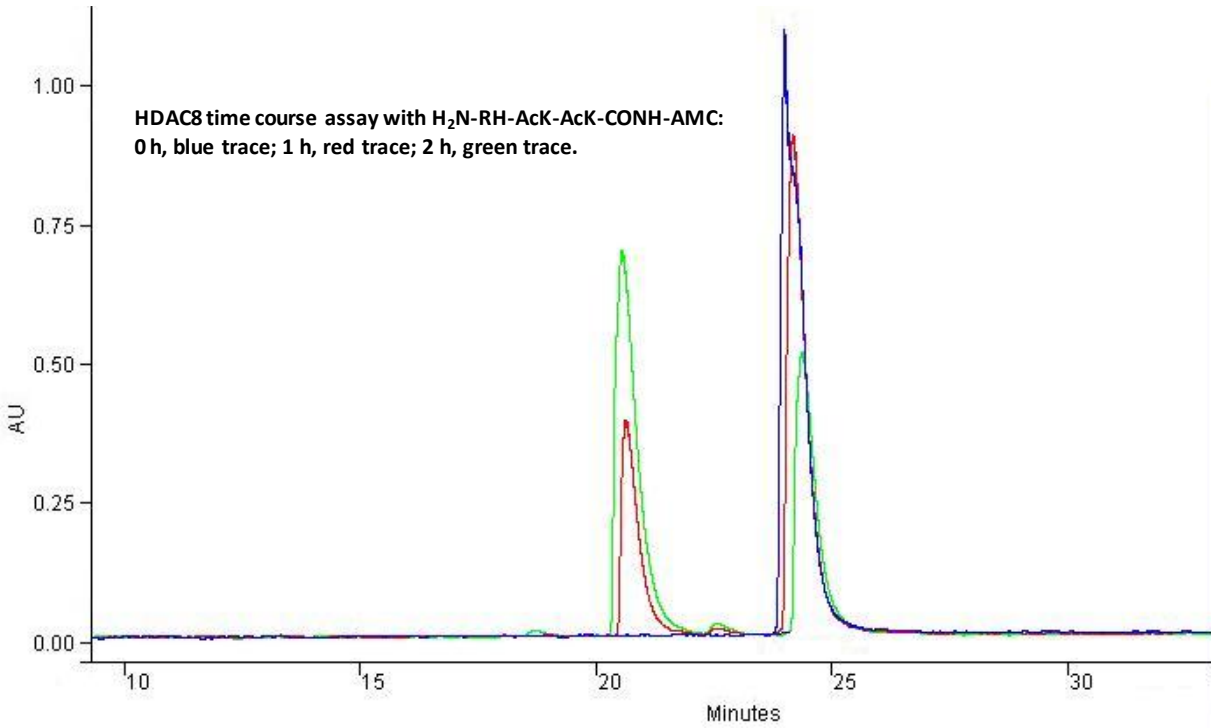

Pronase digestion of NH2-HK-AcK-LM-COOH:
0 min, blue trace; 5 min, red trace.
mAU
125
100
75
50
25
0
10
15
20
25
Minutes

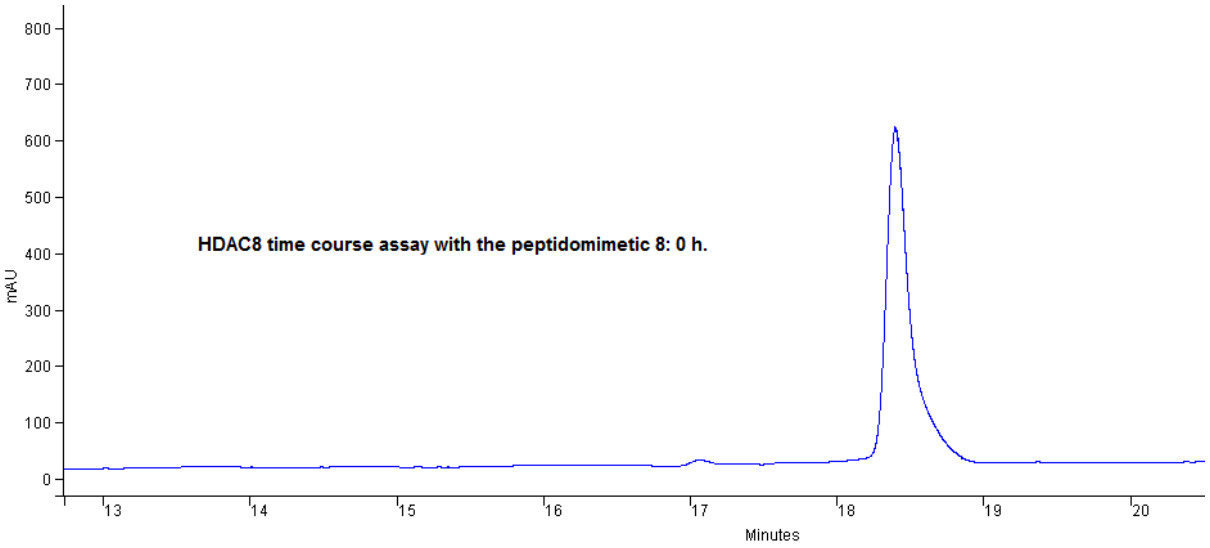

Pronase digestion of the peptidomimetic 8: 0 min.
mAU
125
100
75
50
25
0
10.0
12.5
15.0
17.5
20.0
22.5
Minutes

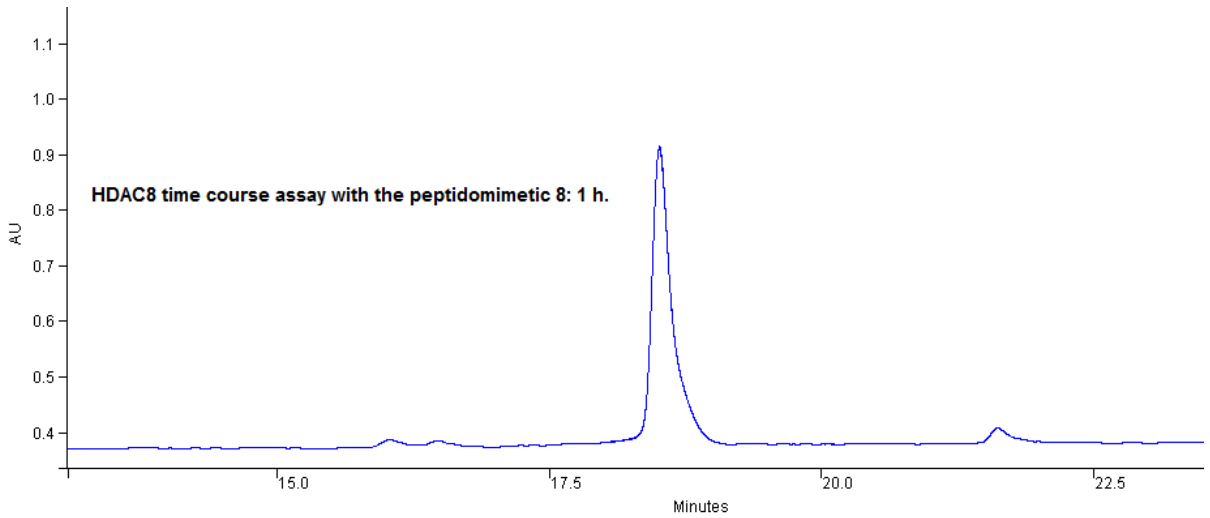

Pronase digestion of the peptidomimetic 8: 30 min.
mAU
-50
-75
-100
-125
-150
-175
10.0
12.5
15.0
17.5
20.0
Minutes

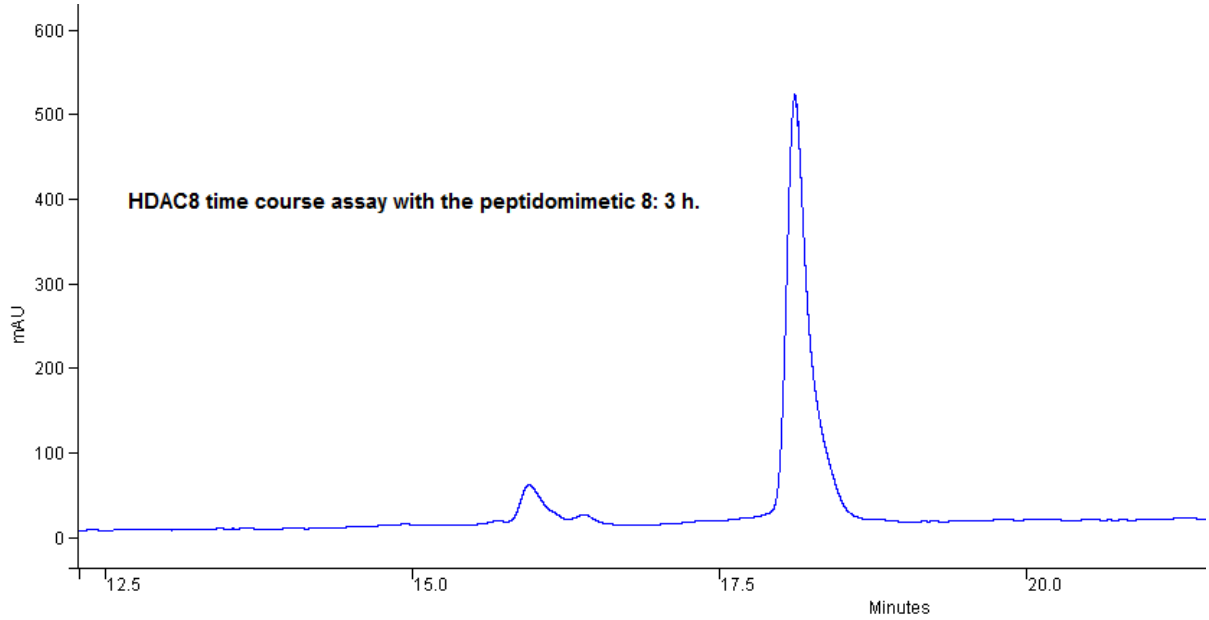

Pronase digestion of the peptidomimetic 9: 0 min.
mAU
150
125
100
75
50
25
0
10.0
12.5
15.0
17.5
20.0
22.5
Minutes

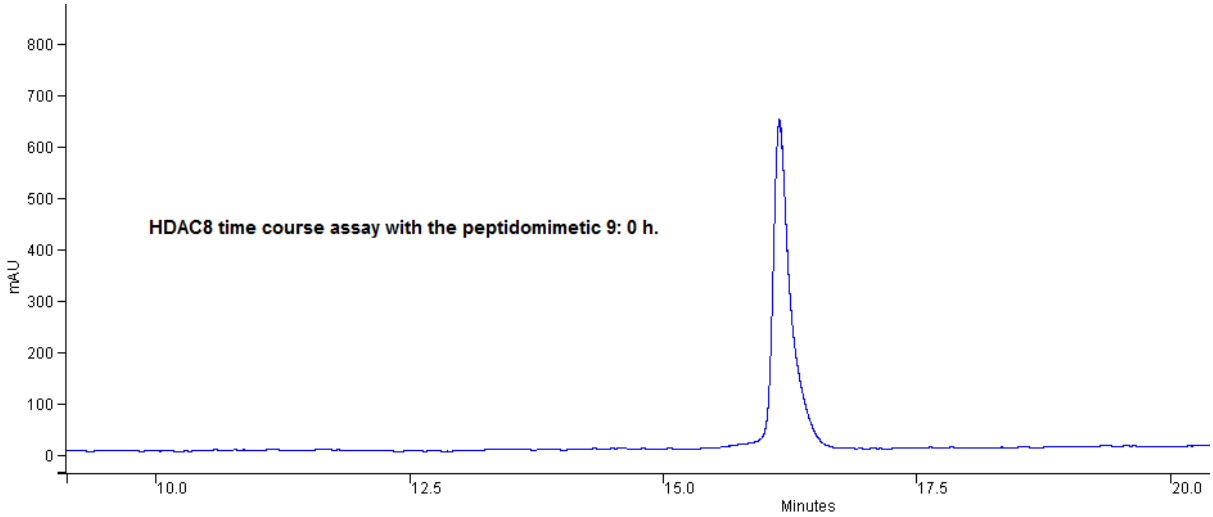

Pronase digestion of the peptidomimetic 9: 30 min.
9
mAU
75
50
25
0
10.0
12.5
15.0
17.5
20.0
Minutes

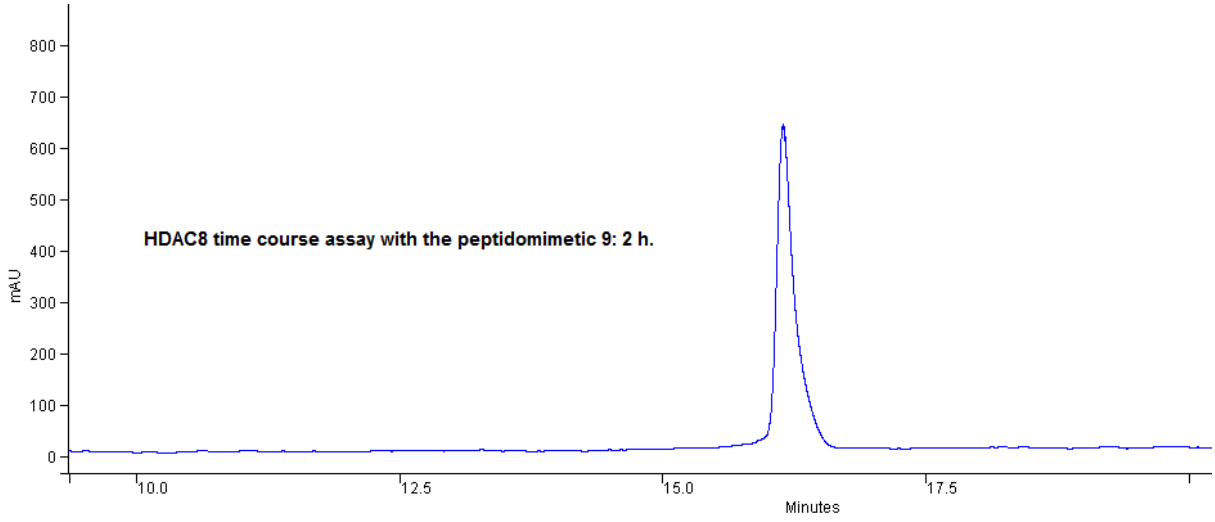

HDAC8 time course assay with H2N-RH-AcK-AcK-CONH-AMC:
0 h, blue trace; 1 h, red trace; 2 h, green trace.
AU
1.00
0.75
0.50
0.25
0.00
10
15
20
25
30
Minutes

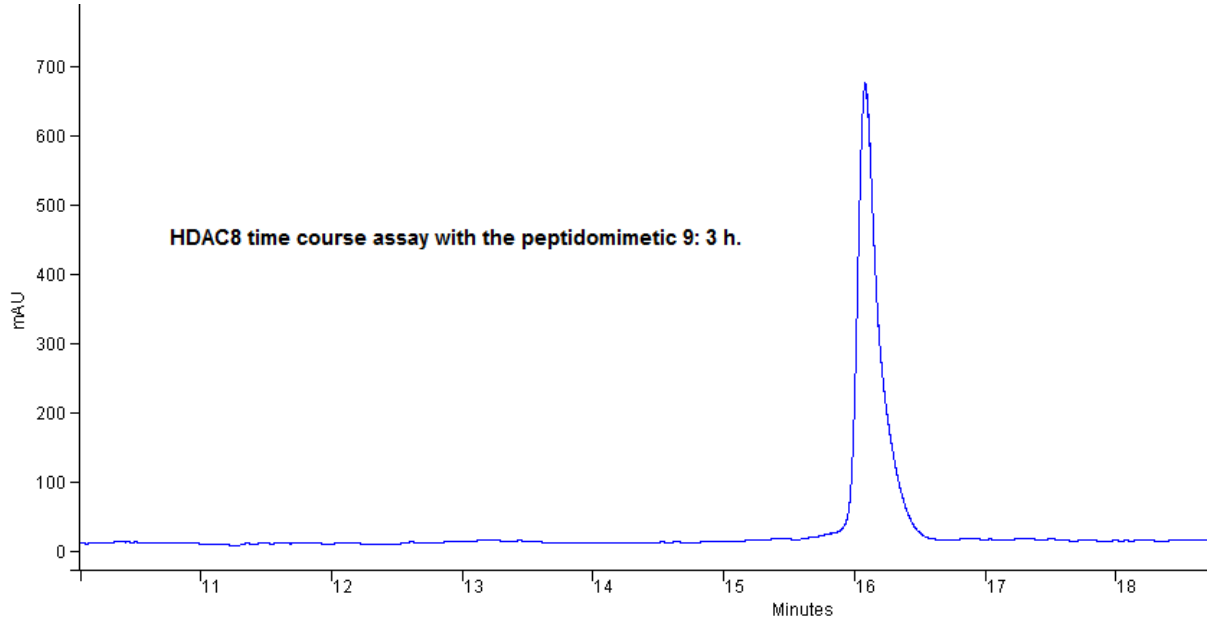

HDAC8 time course assay with the peptidomimetic 8: 0 h.
mAU
800
700
600
500
400
300
200
100
0
13
14
15
16
17
18
19
20
Minutes

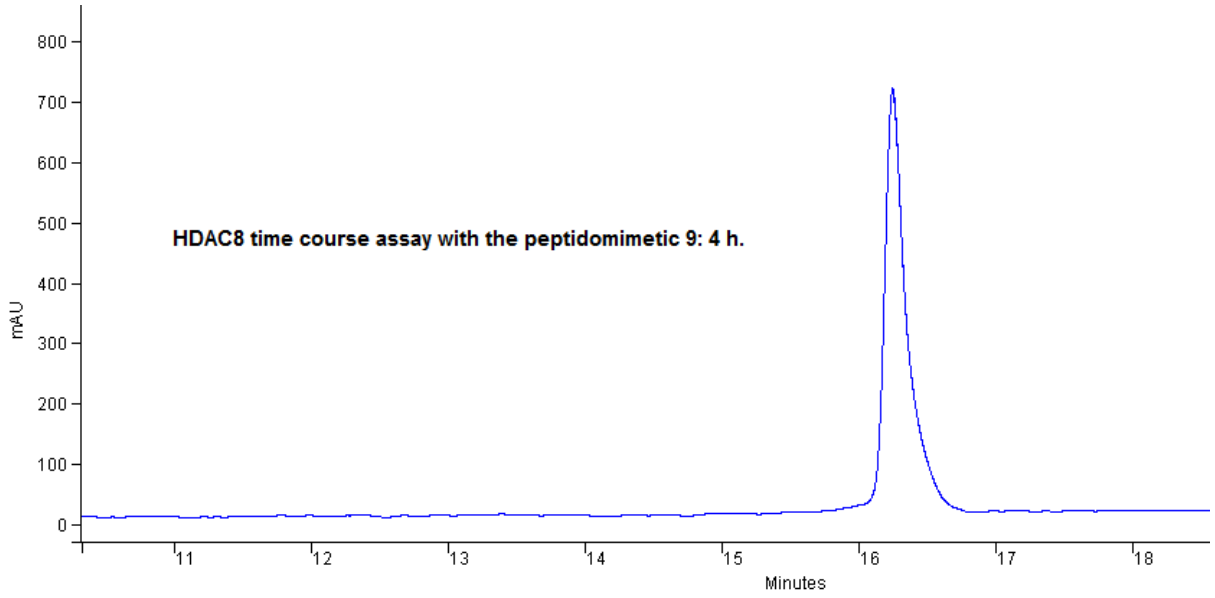

HDAC8 time course assay with the peptidomimetic 8: 1 h.
AU
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
15.0
17.5
20.0
22.5
Minutes

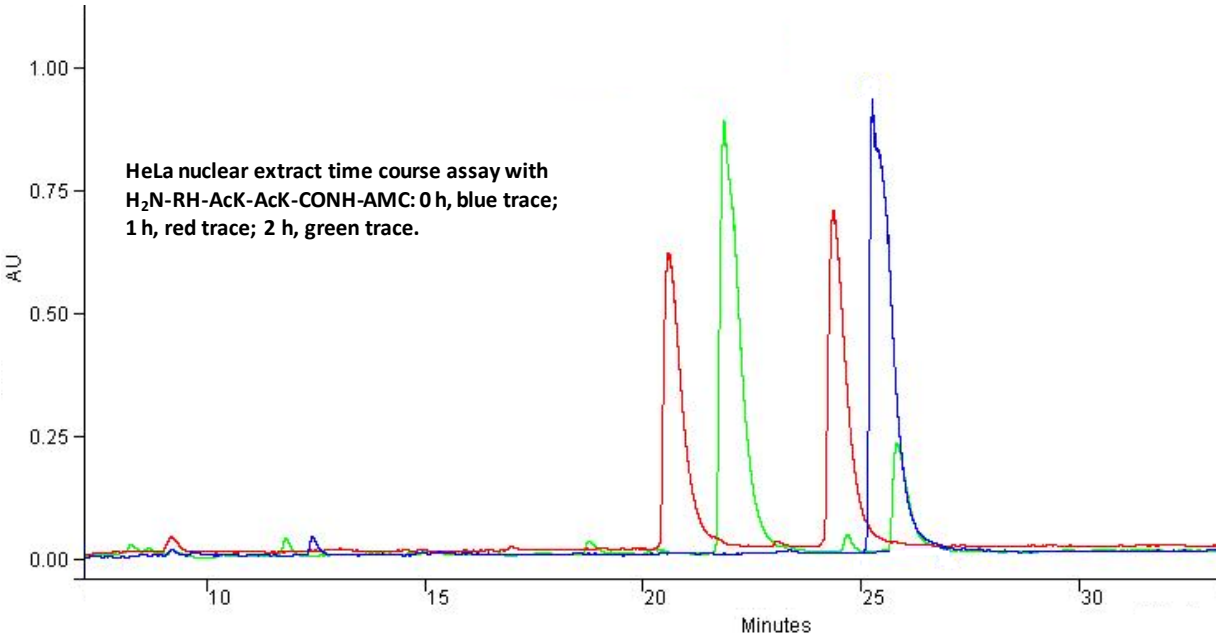

HDAC8 time course assay with the peptidomimetic 8: 3 h.
mAU
600
500
400
300
200
100
0
12.5
15.0
17.5
20.0
Minutes

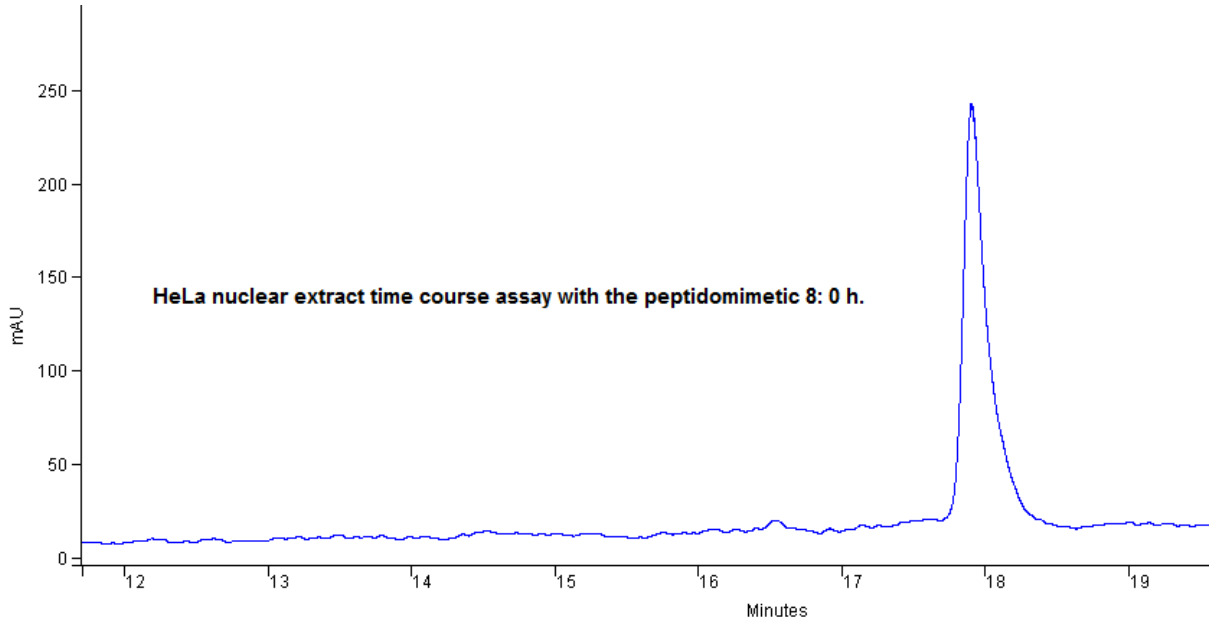

HDAC8 time course assay with the peptidomimetic 9: 0 h.
mAU
800
700
600
500
400
300
200
100
0
10.0
12.5
15.0
17.5
20.0
Minutes

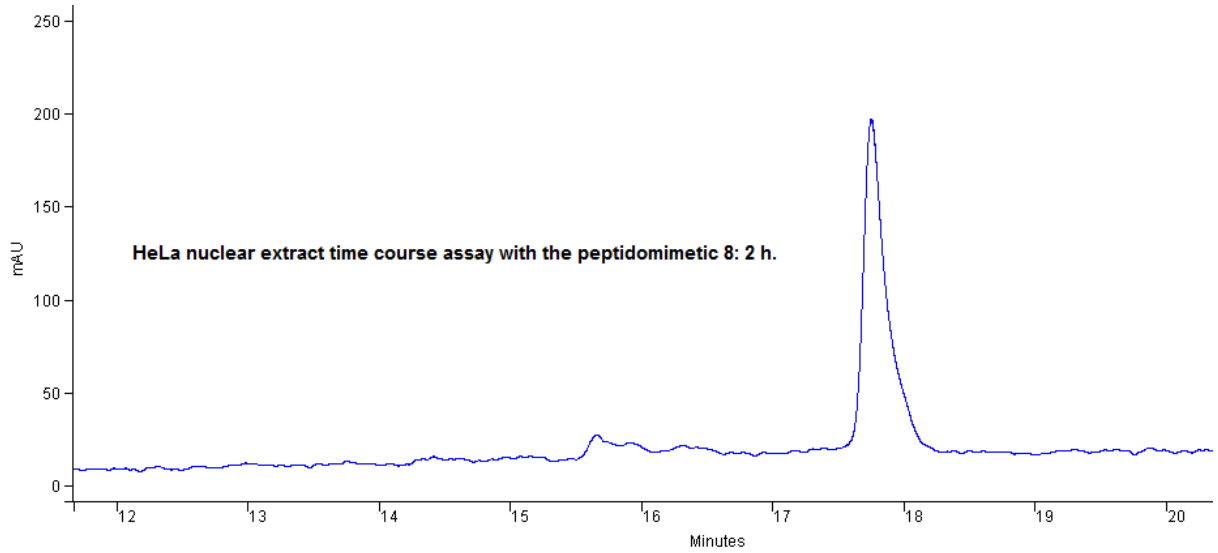

HDAC8 time course assay with the peptidomimetic 9: 2 h.
mAU
800
700
600
500
400
300
200
100
0
10.0
12.5
15.0
17.5
Minutes

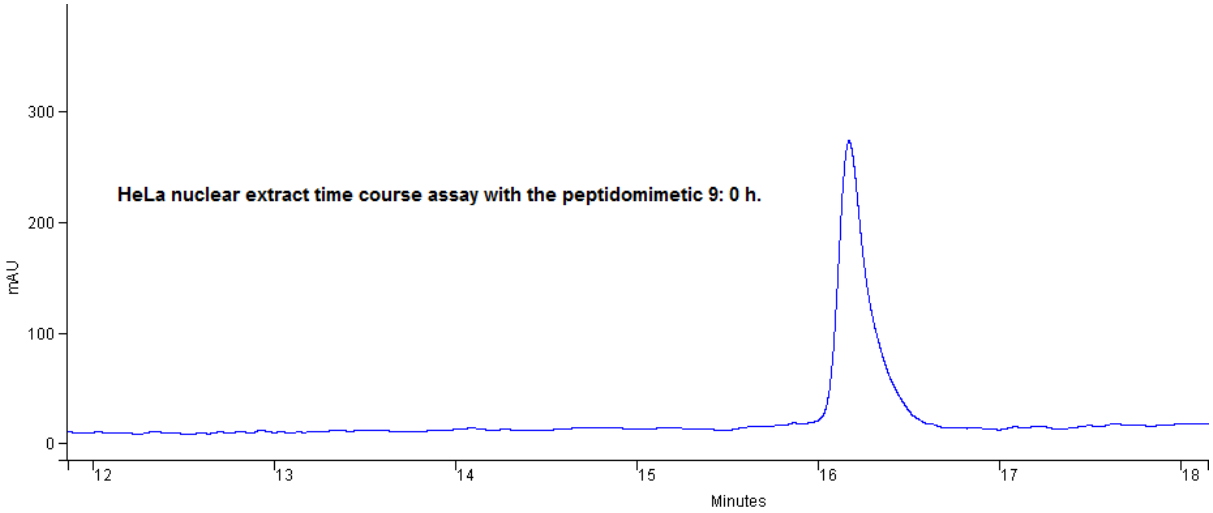

HDAC8 time course assay with the peptidomimetic 9: 3 h.
mAU
700
600
500
400
300
200
100
0
11
12
13
14
15
16
17
18
Minutes

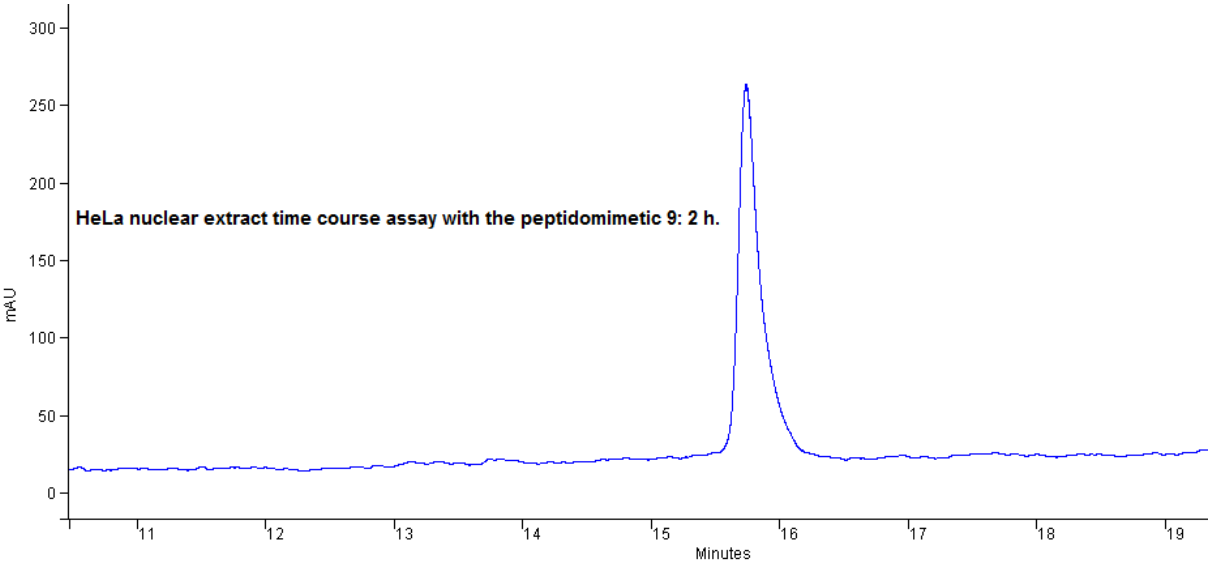

HDAC8 time course assay with the peptidomimetic 9: 4 h.
mAU
800
700
600
500
400
300
200
100
0
11
12
13
14
15
16
17
18
Minutes


HeLa nuclear extract time course assay with
H2N-RH-AcK-AcK-CONH-AMC: 0 h, blue trace;
1 h, red trace; 2 h, green trace.
AU
1.00
0.75
0.50
0.25
0.00
10
15
20
25
30
Minutes


HeLa nuclear extract time course assay with the peptidomimetic 8: 0 h.
mAU
250
200
150
100
50
0
12
13
14
15
16
17
18
19
Minutes


HeLa nuclear extract time course assay with the peptidomimetic 8: 2 h.
mAU
250
200
150
100
50
0
12
13
14
15
16
17
18
19
20
Minutes


HeLa nuclear extract time course assay with the peptidomimetic 9: 0 h.
mAU
300
200
100
0
12
13
14
15
16
17
18
Minutes


HeLa nuclear extract time course assay with the peptidomimetic 9: 2 h.
mAU
300
250
200
150
100
50
0
11
12
13
14
15
16
17
18
19
Minutes