Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information:

Facile synthesis of colorimetric histone deacetylase substrates

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Experimental Section

General methods and reagents:

Amino acid (AA) derivatives were purchased from GLS (Shanghai, China). Coupling reagents were bought from Merck Novabiochem (Darmstadt, Germany), Tentagel RAM resin from Rapp Polymere (Tübingen, Germany). *N*ε-Acetyl-Lysine and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). Analytical RP-HPLC was performed on a Varian ProStar 210 HPLC system with a Nucleosil C18 column (5 µm, 4,6 x 250 mm, Machery-Nagel), with 0.1% TFA in water (A) and 80% ACN, 0.1% TFA in water (B), as eluents. The analytical gradient was 5-95% B over 40 min. with a flow rate of 1 mL/min. Preparative purifications were conducted on a Varian ProStar 210 HPLC system equipped with a preparative Dynamax C18 column (10 µm, 21,4 x 250 mm, Varian) and a flow rate of 13 mL/min. The DNBS protected lysine building block was characterized by NMR spectroscopy, ESI-MS and analytical RP-HPLC. NMR spectra were recorded on a Bruker AV 300 MHz instrument. ESI-MS analyses were conducted on a Mariner ESI-TOF (Applied Biosystems). All peptides were analyzed by MALDI-MS on a MALDI-TOF-TOF, 4700 Proteomics Analyzer (Applied Biosystems) and by analytical RP-HPLC. Recombinant human SIRT1 was purchased from Biomol (Hamburg, Germany).

The optical purity of amino acid derivatives was determined with GC-MS by C.A.T. GmbH & Co. Chromatographie und Analysentechnik KG (Tübingen, Germany).

Synthesis of DNBS-Lys(Ac)-OH (5):

H-Lys(Ac)-OH **4** (2 mmol, 376 mg) was suspended in acetonitrile (6ml), BSA (*N,O*-bis(trimethylsilyl)acetamide, 4.32 mmol, 1.056 ml) was added and the reaction was heated to 58°C for 15 min until **4** had dissolved completely. The reaction was cooled to room temperature, DNBS-Cl (2,4-dinitrobenzensulfonyl chloride, 1.96 mmol, 523 mg) was added and the solution was stirred over night. The yellow solution was concentrated and the resulting oil was solved in ethyl acetate (30 ml). The organic phase was washed 4 times with each 10 ml of hydrochloric acid (16%*)* and dried over sodium sulfate*.* The solvent was removed and the solid was dried under vacuum to yield DNBS-Lys(Ac)-OH **5** (1.94 mmol, 810 mg, 98%) as a light yellow solid.

ESI-MS: m/z = $[M+H]^+$ calculated: 419.39, found: 419.02

^{1}H-NMR (DMSO-d6, 300 MHz): δ = 12.78 (br, 1H, CO_2H), 8.90 (d, 1H, *J* = 8.8 Hz DNBS-NH), 8.86 (s, 1H, *J* = 2.0 Hz H_{Ar}), 8.61 (d, 1H, *J* = 8.7 Hz, *J* = 2.0, Hz H_{Ar}), 8.25 (d, 1H, *J* = 8.7 Hz, H_{Ar}), 7.73 (t, 1H, NH-Ac), 3.84 (m, 1H, H-2), 2.92 (m, 2H, H-6), 1.74 (s, 3H, $COCH_3$), 1.64 (m, 2H, H-3), 1.37-1.17 (m, 4H, H-4, H-5); see Fig. S4

^{13}C-NMR (DMSO-d6, 75.5 MHz): δ =172.6 (**C**O_2H), 169.2 (**C**OCH_3), 149.9 (C_{ar}-DNBS), 147.5 (C_{ar}-DNBS), 138.6 (CH_{ar}-DNBS), 132.1 (CH_{ar}-DNBS), 127.2 (CH_{ar}-DNBS), 120.0 (CH_{ar}-DNBS), 56.2 (C-2), 38.5 (C-6), 31.5, 28.7, 22.9, 22.9; see Fig. S5

RP-HPLC: t_R = 23.5 min.

Solid-phase peptide synthesis, *in situ* formation and coupling of DNBS-Lys(Ac)-Cl (6):

Peptids were synthesized up to the Lys(Ac) moiety with standard Fmoc-based solid-phase chemistry on an Intavis Respep XL synthesizer. TentaGel R RAM resin (cap. : 0.19 mmol/g) was used as solid support and amino acid side-chains were protected as follows: Arg(Pbf), His(Trt), Lys(Boc), Ser(tBu) and Trp(Boc). 5-amino-2-nitro-benzoic acid (5,2-ANB) was coupled without any protection. Coupling reactions were performed with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro-phosphate (HBTU) as the activation agent and *N*-Methylmorpholine (NMM) in DMF/NMP as base. Each successive amino acid was introduced by double coupling with a 5-fold molar excess. Removal of the Fmoc group was carried out with 20 % piperidine in DMF.

Formation of DNBS-Lys(Ac)-Cl (**6**): DNBS-Lys(Ac)-OH (**5**) was suspended in dry DCM (0.3 M), thionyl chloride (2 eq.) and pyridine (1 eq.) was added subsequently and the mixture was stirred at 45°C under reflux for 30 min until all components had dissolved completely. After cooling down to room temperature tert-butanol (3 eq.) was added and the solution was stirred for an additional 15 minutes. The resin (1/3 eq.) was added to the solution and dry DCM was added dropwise until a homogeneously suspended slurry was obtained. Afterwards *N,N*-Diisopropylethylamine (DIPEA, 3 eq.) was added and the suspension was stirred for 1 hour. The resin was washed with DMF and DCM. The DNBS group was cleaved off with a freshly prepared 5% thiophenolate solution. Therefore, thiophenole (10 eq.) was added to a suspension of K_2CO_3 (21 eq.) in DMF and shaken. The resin was agitated with the filtered solution for 15 min. which lead to complete removal of the protection group. All further amino acid couplings were performed as described

above on the peptide synthesizer. Peptides were cleaved off the resin with complete removal of the side-chain protection groups with a cleavage cocktail that contained TFA / Phenol / Triisopropylsilane / H_2O (85 : 5 : 5 : 5) for four hours. Cleaved peptides were precipitated in cold diethyl ether, centrifuged and washed with diethyl ether, dissolved in H_2O and lyophilized. Crude peptides were HPLC-purified on a preparative C18 column to approximately 95% purity (see Fig. S6).

To evaluate the coupling efficiency of DNBS-Lys(Ac)-Cl **6**, resin-bound peptide **S1** was subjected to a series of test coupling reactions (Table S1): *In situ* formed **6** was coupled to the resin-bound **S1** as mentioned above. Resin samples were taken after 5, 10, and 30 minutes. Peptides **S1** and **S2** were cleaved off the resin, precipitated in cold diethyl ether, dissolved in H_2O, lyophilized and analyzed by analytical HPLC to determine the conversion of **S1** to **S2**. Additionally, **S1** was treated with **6** without previous scavenging of thionyl chloride with tert-butanol. The coupling efficiency was determined after 60 min. To test conventional coupling methods DNBS-Lys(Ac)-OH **5** (3 eq.) was coupled to **S1** with 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HATU) (2,5 eq.) and DIPEA (9eq.) in DCM (0.5 ml) for 60 min.

Expression and purification of Sir2.1:

His_6-tagged Sir2.1 was expressed in BL21 pLysS cells (Stratagen). The pET21 expression vector for Sir2.1 was kindly provided by Wolfgang Fischle. Expression was induced at an OD_{600} of 0.6 with 0.5 mM IPTG. After 4h at 25°C the cells were harvested by centrifugation, resuspended in washing buffer (50 mM Phosphate, pH 8.0, 100 mM NaCl) and stored at -20°C until usage. After thawing and addition of protease inhibitor (PMSF 0.2 mM final concentration), the cells were lysed (EmulsiFlex-C3; Avestin, Ottawa, Canada). 2 ml of washed Ni-NTA superflow slurry (Qiagen) was added to the cleared lysate and incubated for 1h at 8°C. After washing with 50 ml of washing buffer, Sir2.1 was eluted off the beads with 2 x 3 ml of elution buffer (50 mM Phosphate, pH 8.0, 100 mM NaCl, 300 mM Imidazol). Combined fractions were buffer exchanged to storage buffer (50 mM Phosphate, pH 8.0, 100 mM NaCl, 25 µM $ZnCl_2$, 10% glycerol, 1mM DTT) on HiTrap Desalting columns (Amersham Pharmacia Biotech), concentrated (Amicon ultra, MWCO 10 kD, Millipore) to 100 µM and stored at -80°C until usage.

Deacetylation Assays with Sir2.1:

All used stock solutions were prepared with assay buffer (50mM Phosphate, pH 8.0, 100 mM NaCl, 25 µM $ZnCl_2$). 60 µl of assay buffer were mixed with 62.5 µl of a 10 mM solution of NAD^+ and 1.25 µl of the 100 µM Sir2.1 stock solution. The reaction was started by adding 1.25 µl of a 10 mM solution of substrate **7**. The reaction was stopped after 5 or 20 min by adding 10 µl of a 3 M nicotinamide solution. Control reactions contained no enzyme or 10 µL of a 3 M nicotinamide solution right from the start. Tryptic digests were performed by adding 40 µL of a trypsin solution (5 mg/ml

trypsin, 50 mM Phosphate, pH 8.0, 100 mM NaCl, 25 µM $ZnCl_2$, 20 % glycerol) for 1 h at 37°C.

The assays were analyzed by HPLC and spectrophotometric readout at 405 nm on a V-560 spectrophotometer (Jasco) in 100 µL cuvettes, referenced on a reaction mixture without **7**. Each reaction was performed at least four times.

Deacetylation Assays with human SIRT1:

All used stock solutions were prepared with assay buffer (see above). 62.5 µl of 10 mM solution of NAD^+ were mixed with 12.5 µl of commercially available SIRT1 (6.5 µM), 12,5 µl of vehicle (10% DMSO (v/v) in assay buffer) and 25 µl assay buffer. When p53-AMC (**1a**) or Boc-Lys(Ac)-AMC (**1b**) were used as substrates, the vehicle was substituted by assay buffer without DMSO. The reactions were started by adding 12.5 µl of either substrate **7** (1 mM in assay buffer), p53-AMC (**1a**) (1 mM in assay buffer 10% DMSO) or Boc-Lys(Ac)-AMC (**1b**) (1 mM in assay buffer 10% DMSO). For kinetic analysis the reactions were stopped at various time points by adding 10 µl Nicotinamide (3 M) and analyzed by HPLC as described below. The SIRT1 activation assays were performed as above. When resveratrol was added the vehicle was replaced by 12.5 µl of Resveratrol (2 mM in 10% DMSO/assay buffer (v/v)). The reactions were stopped after 20 min (40 min in case of Boc-Lys(Ac)-AMC as substrate) by addition of 10 µl a 3M nicotinamide solution. Tryptic digests were performed by adding 10 µL of a 20 µg/mL solution (in 50 mM acetic acid/water) of sequencing grade modified Trypsin (Promega) for 1 h at 37°C.

Assays with substrate **7** were analyzed as described above. Assays with substrates **1a** and **1b** were analyzed on a Jasco FP-6500 fluorescence spectrometer.

LC and LC-MS analysis of the deacetylation assays:

Samples were analyzed on an Agilent 1100/1200 System equipped with an EC 250/4 Nucleodur 100-5 C18ec column. Peptides were eluted over 20 minutes with a gradient of 5 – 95 % of solvent A (Water/ 0,1 % TFA) to solvent B (80% ACN/ 20% Water/ 0,1 % TFA) with a flow rate of 1 ml/min. The chromatograms were recorded at 340 nm. Deacetylation levels were calculated from integrated peak intensities of the deacetylation reactions before tryptic digest.

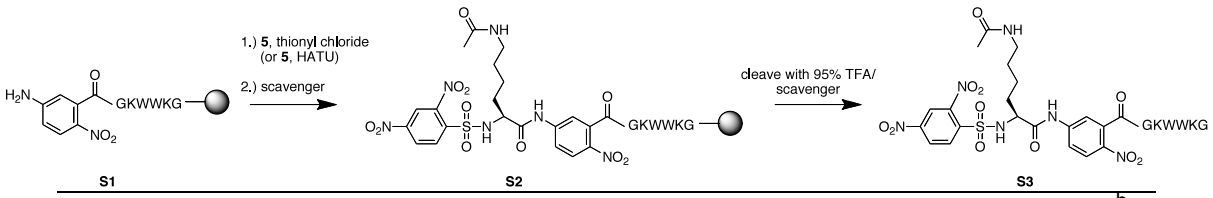
The identity of each eluted peptide was confirmed by LC-MS (Acuity UPLC, ESI, LCT Premier-TOF, Waters):

Compound:	Mass (calculated)	Mass (detected)
7	1251.46	1252.6
8	1209.42	1210.6
9	742.89	743.42
10	572.68	573.29

Supplementary Experiments:

In situ formation and optimizing the coupling reaction of DNBS-Lys(Ac)-Cl (6):

DNBS-Lys(Ac)-Cl (**6**) was freshly prepared by treating DNBS-Lys(Ac)-OH (**5**) with 2 eq thionyl chloride for 30 min at 45°C under reflux. We observed 40 % coupling when freshly formed **6** was added directly to resin-bound model peptide **S1** (Table S2). The coupling yields were determined by cleaving the formed product **S2** and non-converted **S1** off the resin followed by HPLC analysis (Table S1). We observed that the coupling yields increased when excess thionyl chloride was scavenged by adding 3 eq of tert-butanol. Under these optimized conditions a conversion of 72% was achieved within five minutes of reaction time. Extended reaction times of **6** with **S1** did not result in a significant increase of coupling yields (Table S1). In contrast the formation of **S3** was not detected when **5** was activated with the coupling reagent 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU) instead of thionyl chloride (Table S1).



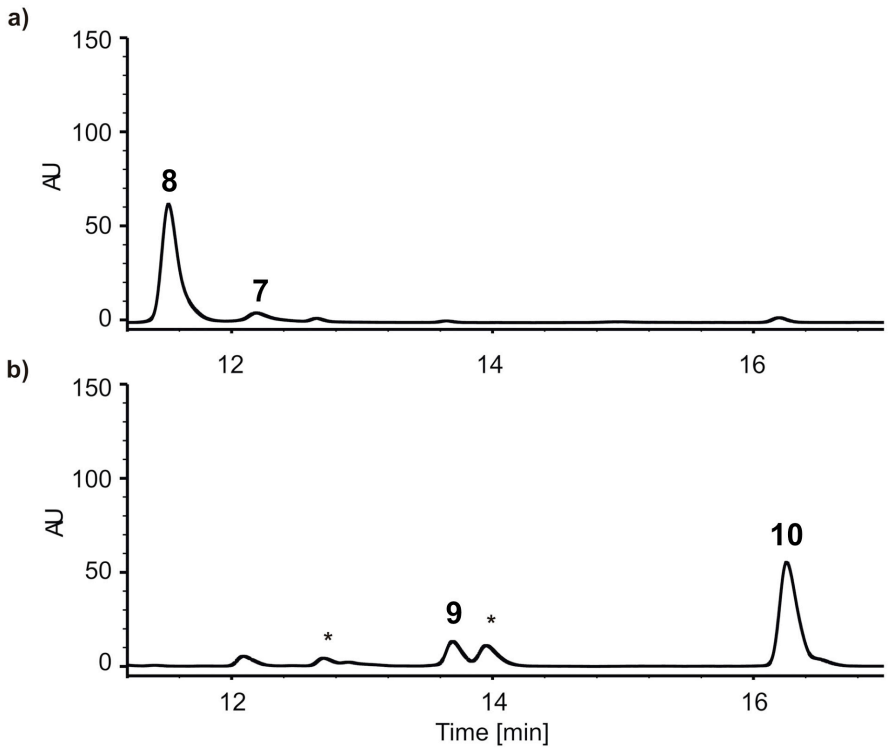
Coupling Time (min)	Activation	Scavenger	Conversion [%][b]
5	TC [a]	tBuOH	72
10	TC [a]	tBuOH	72
30	TC [a]	tBuOH	74
60	TC [a]	-	40
60	HATU	-	-

[a] thionyl chloride. [b] after treating model peptide **S2** with activated **5** the peptide was cleaved off the resin and analyzed by HPLC

Supplementary Table S1: Coupling yields under different reaction conditions.

Extended deacetylation of 7 by Sir2.1:

We further analyzed the deacetylation reaction of **7** by Sir2.1 after 20 min of reaction time. Under these conditions 90 % of **7** is deacetylated to **8** (Fig. S1). Trypsinization converts **7** and **9** to **8** and **10** as observed for the reaction at 5 min (Fig. 1). We adjusted the trypsin amount and incubation time for complete cleavage of **8** based on 90 % of deacetylation to ensure that the trypsinization reaction is not rate-limiting for the optical readout. These optimized trypsination protocols were used for all assays (see page 6 and 7)



Supplementary Fig. S1: Deacetylation reaction after 20 minutes: (a) Before tryptic digestion. Under these conditions 90 % of Sir2.1-catalyzed deacetylation is observed. (b) After tryptic digestion. Peaks marked by * are cleavage products formed by chymotrypsin impurities of the commercially available trypsin.

Optical readout of the deacetylation reaction with 7 and Sir2.1:

After analyzing the Sir2.1-catalyzed deacetylation of **7** by HPLC we followed the same reaction by optical readouts at 405 nm. A significant increase in absorbance was observed after 5 minutes of Sir2.1-catalyzed deacetylation followed by tryptic digest (Fig. S2). In contrast omission of Sir2.1 or addition of inhibitory nicotinamide lead to no significant increase in absorbance. An extended reaction time of 20 minutes resulted in a further increase of absorbance, which correlated with the degree of deacetylation monitored by HPLC (Fig. 1 and Fig. S1).

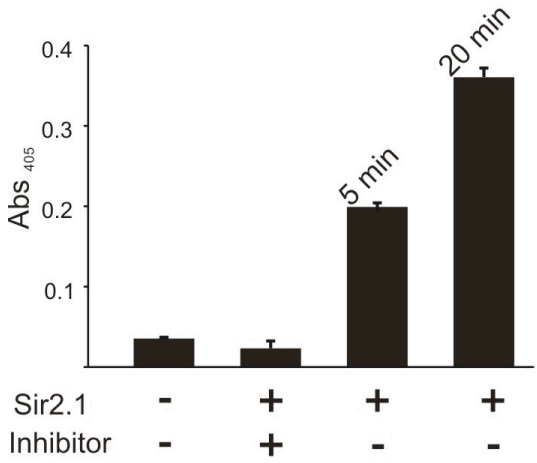
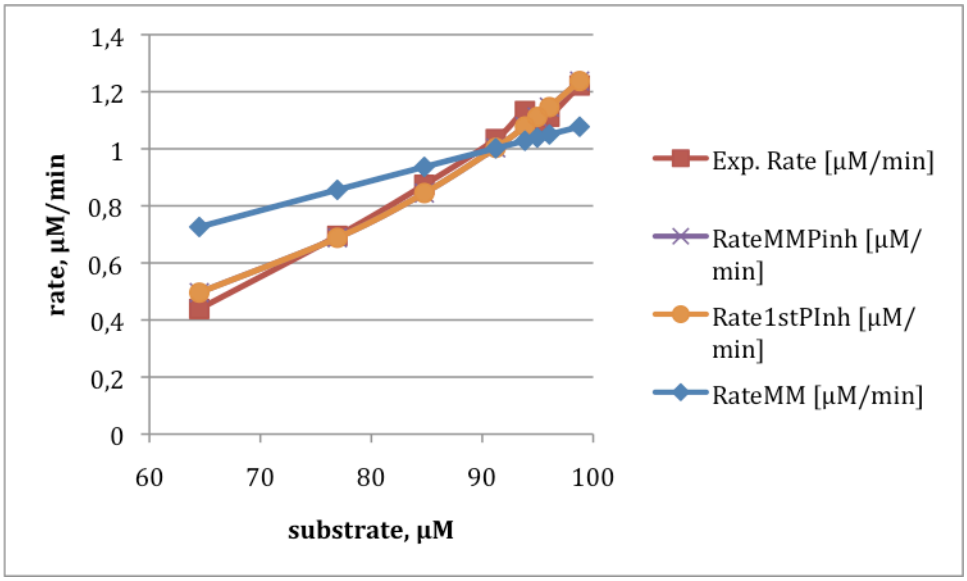


Figure S2. Spectrophotometric readout of the Sir2.1-catalyzed deacetylation assays. No significant increase in absorbance at 405 nm is detected when Sir2.1 is omitted or if inhibitory nicotinamide is present during the reaction. The uninhibited reaction results in increase of absorbance after 5 and 20 minutes of reaction time.

Determining the kinetic parameters for the deacetylation of 7 and 1a with SIRT1:

The kinetic parameters were derived from the progress curves of the SIRT1-catalyzed deacetylation of p53-5,2-ANB (**7**) and p53-AMC (**1a**) at 100 µM substrate concentration as described previously for the formate oxidation by formate dehydrogenase.[26] The used incremental method retrieves rate data from progress curves by differentiation of the concentrations using difference quotient established on the time intervals' mid. Once the substrate and product concentrations pass the relevant concentration range over the reaction progress, this method provides the same information content as initial rate measurements where the initial concentrations are adjusted manually by varying the start concentrations of the substrates.

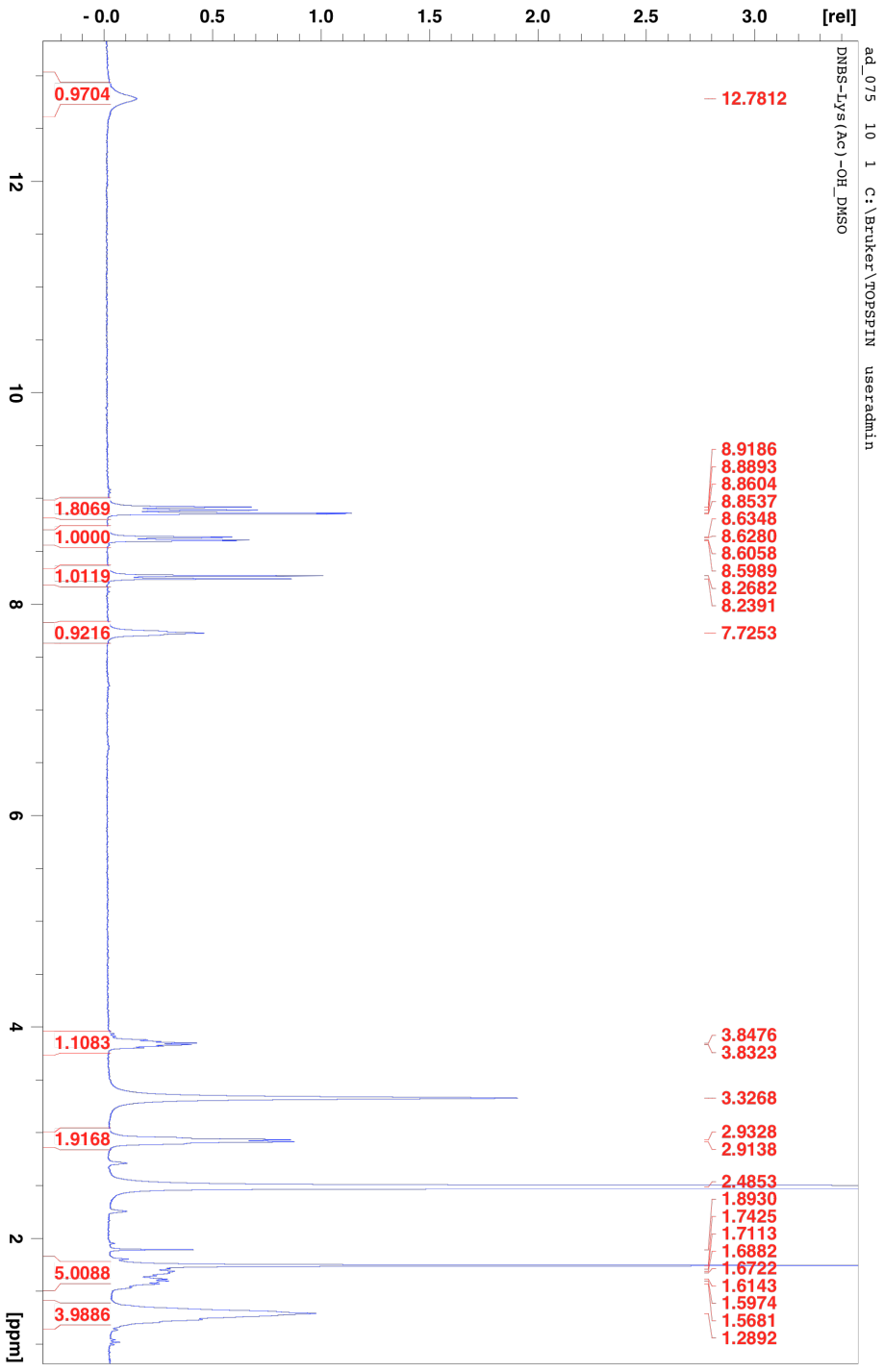
The resulting sets of rate vs. concentration data were analyzed by minimizing the sum of square errors as objective function for the estimation of the kinetic parameters. The minimization was achieved by using the multi-start GRG-nonlinear algorithm in the Solver add-in of Microsoft Excel Professional 2010 (version 14.0.6112.2000). As illustrated in Fig. S3 for the SIRT1-catalyzed deacetylation of p53-5,2-ANB (**7**) the progress curves were fitted to Michalis-Menten kinetics (MM; $v = V_{max} * S / (K_m + S)$), Michaelis-Menten kinetics with product inhibition (MMPInh; $v = V_{max} * S / (K_m + S) / (1 + P / K_{iP})$), and first order kinetics with product inhibition (1^{st}PInh; $v = V_{max} / K_m * S / (1 + P / K_{iP})$). In agreement with the observation that SIRT1 is inhibited by two of its products, the deacetylated peptide and nicotinamide, the best fit was obtained for first order kinetics with product inhibition, which were used to retrieve the kinetic parameters.



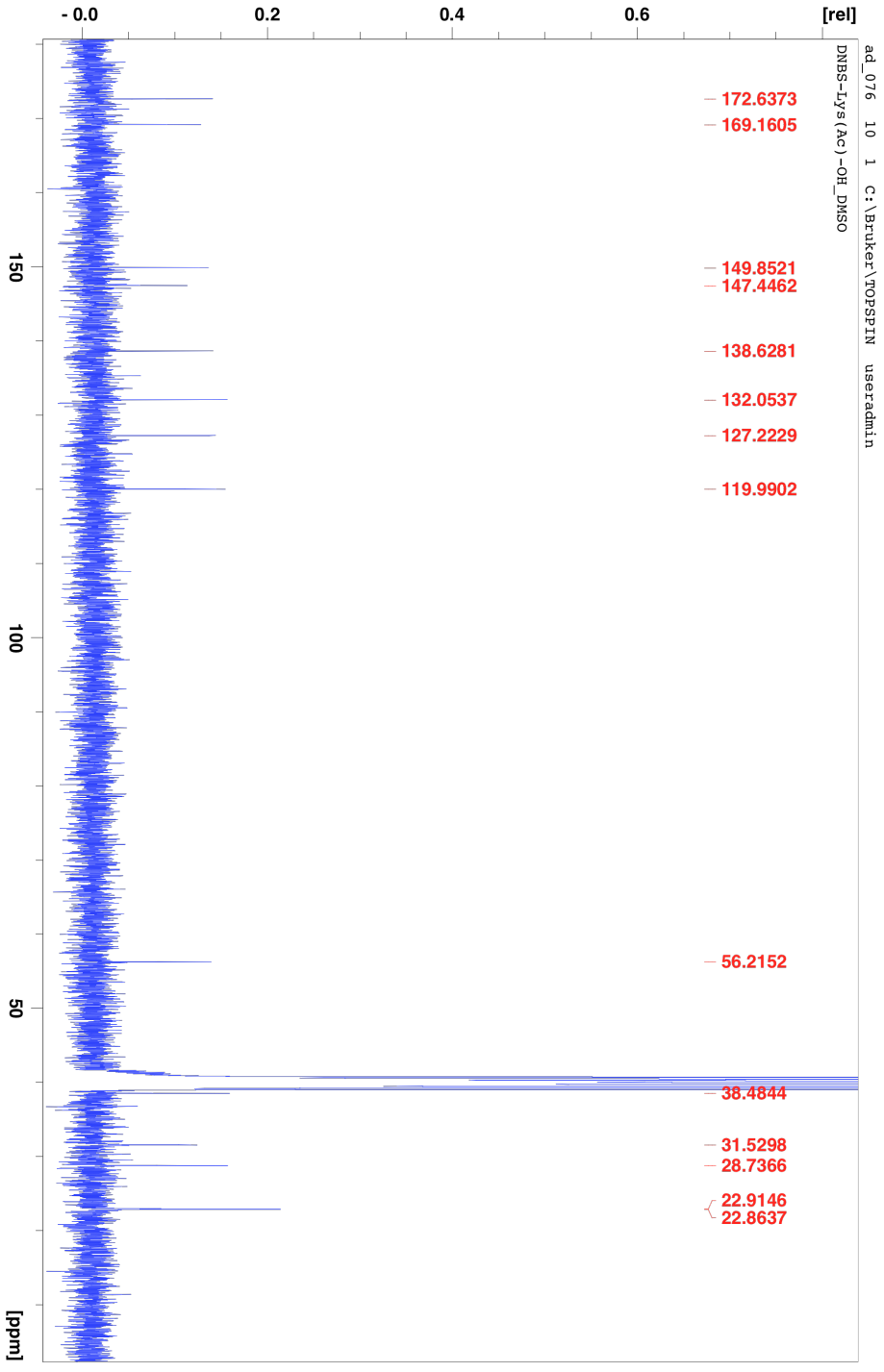
Supplementary Fig. S3: Michaelis-Menten plots (rate vs. substrate concentration) for the p53-5,2-ANB (**7**) conversion by SIRT1 including best fits for three different rate law assumptions.

	V_{max}/K_m			K_{iP}		
	1/min		rel. 95% Conf.	µM		rel. 95% Conf.
p53-AMC	0.0121 ±	0.0013	10.8%	20.6 ±	3.4	16.3%
p53-5,2-ANB	0.0128 ±	0.0013	10.3%	53 ±	26	49.3%

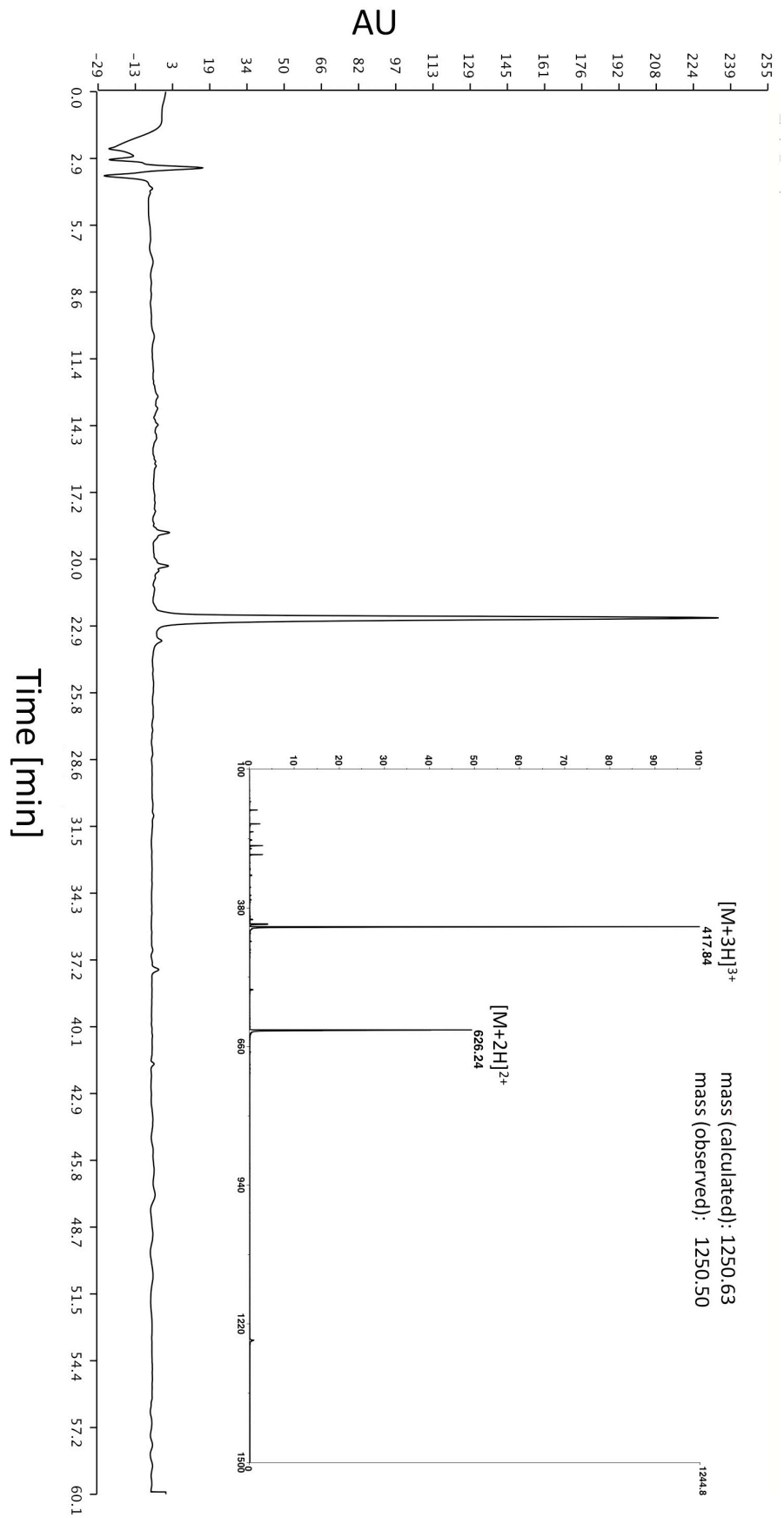
Supplementary Table S2: Estimated kinetic parameters and 95% confidence intervals for first order reaction with product inhibition of the conversion of p53-AMC (**1a**) and p53-5,2-ANB (**7**). The 95% confidence intervals were estimated using the linearized parameter sensitivity and the average measurement error of all raw data of ± 10.6%.



Supplementary Fig. S4: ^{1}H NMR spectrum of DNBS-Lys(Ac)-OH (**5**) in DMSO-d6



Supplementary Fig. S5: ^{13}C NMR spectrum of DNBS-Lys(Ac)-OH (**5**) in DMSO-d6



Supplementary Fig. S6: Characterization of p53-5,2-ANB (**7**)