A continuous sirtuin activity assay without any coupling to enzymatic or chemical reactions

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Sirtuins are NAD+ dependent lysine deacylases involved in many regulatory processes such as control of metabolic pathways, DNA repair and stress response. Modulators of sirtuin activity are required as tools for uncovering the biological function of these enzymes and as potential therapeutic agents. Systematic discovery of such modulators is hampered by the lack of direct and continuous activity assays. The present study describes a novel continuous assay based on the increase of a fluorescence signal subsequent to sirtuin mediated removal of a fluorescent acyl chain from a modified TNFα-derived peptide. This substrate is well recognized by human sirtuins 1–6 and represents the best sirtuin 2 substrate described so far with a k_{cat}/K_M-value of 176 000 M\(^{-1}\)s\(^{-1}\). These extraordinary substrate properties allow the first determination of K_i-values for the specific Sirt2 inhibitory peptide S2iL5 (600 nM) and for the quasi-universal sirtuin inhibitor peptide thioxo myristoylTNFα (80 nM).

Reversible acylation of protein lysine residues is one of the most abundant posttranslational modifications (PTMs) involved in several cellular processes like metabolic regulation, cell cycle control and epigenetics1,2. Lysine acetylation is determined by the enzymatic activity of lysine acetyltransferases and lysine deacylases. Recent studies detect alternative acylations as in vivo PTMs, including propionylations3,4, succinylations5,6, malonylations6,7, glutarylations8, crotonylations9, butyrylations2, 2-hydroxyisobutyrylations10, phosphoglycerations11 and myristoylations12. The generation of these PTMs is not fully understood, but it is evident that some of these acyl-transfers represent spontaneous reactions with acyl-CoAs or acylphosphates as acyl-donors forming stable amide bonds13–17. Removal of such acyl moieties from lysine side chains is catalyzed by either zinc ion dependent lysine deacylases or by a conserved family of NAD+ dependent lysine deacylases, known as sirtuins. The mitochondrial sirtuin 5 (Sirt5) has over hundred-fold higher catalytic efficiency for succinylated and glutarylated lysine residues as compared to acetylated lysines7,8,18,19, whereas Sirt6 prefers long acyl chains, such as myristoylated lysine side chains20,21. Recently, it has been demonstrated that Sirt4 is able to remove lipoyl and biotinyl residues from lysine side chains both in vitro and in vivo22 while Sirt3 seems to be an in vivo decrotonylase, in addition to its established deacetylase function23. Furthermore, it is known that Sirt2 exhibits demyristoylase24,25 and depalmitoylase activity26. Sirtuin mediated deacytlation regulates several metabolic processes, such as fatty acid synthesis, glucose homeostasis and stress response27. Moreover, sirtuins are involved in diseases like diabetes, cancer and neurodegeneration28, making these enzyme attractive targets for pharmacological modulation. However, few compounds for sirtuin inhibition and activation are available, and the unavailability of sensitive and reliable assays also suitable for high-throughput screens has contributed to this lack of modulators29. In fact, assay artifacts contributed to a controversy about the general possibility to activate sirtuins, but more recent work

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involveing robust yet time-demanding low-throughput mass spectrometry-based sirtuin assays confirmed the controversial Sirt1 activation and revealed the possibility to activate Sirt5 and Sirt6.

For the efficient development of sirtuin effectors, reliable and ideally continuous high-throughput assays are indispensable. Several existing, and in most cases discontinuous activity assays (reviewed in 28,30) are based on the separation of products and substrates by HPLC/CE, by mass spectrometry or spectrophotometric detection of one reaction component. Nevertheless, continuous activity assays are known which couple the sirtuin reaction to either an additional enzymatic reaction, a chemical reaction such as intramolecular trans-esterification, or to fluorescence enhancement by aggregation-induced emission.

For microtiter plate (MTP)-based assay formats the sirtuin reaction is currently coupled to enzymatic reactions either sensing the released nicotinamide, the remaining NAD+, or the deacetylated peptide product. One advantage of monitoring sirtuin-mediated release of nicotinamide is the compatibility with any substrate including proteins and also with any type of lysine acylation. However, the enzymatic cascade needed for signal generation, limits the linear range of the assay and makes it more sensitive to interference in compound tests as observed for GW5074, a Sirt5 inhibitor that also affects GDH activity. Hubbard et al. substituted the last enzymatic step by a chemical reaction sensing ammonia allowing more accurate but discontinuous activity determinations.

Assays sensing the deacetylated product of the sirtuin reaction utilize the substrate specificity of proteases like Trypsin, which have a strong preference for positively charged side chains in the P1-position and thus do not cleave the acetylated substrates of the sirtuin-mediated reaction. This principle has been introduced using peptidyl -7-amino-4-methyl-coumarin derivatives. Subsequent to deacylation of the peptidyl moiety the bond between the C-terminus of the peptidyl moiety and the amino-coumarin derivative is hydrolyzed by the helper protease hereby releasing the highly fluorescent 7-amino-4-methyl-coumarin.

This assay is very sensitive but makes use of sirtuin substrates with suboptimal K_M values and it often has to be performed discontinuously because of the susceptibility of sirtuins to digestion by the helper protease. Appropriate substrates have been synthesized for assaying sirtuin isoforms against acetylated, succinylated, glutarylated, adipoylated or myristoylated lysine residues. The fluorophore replacing the C-terminal peptide part renders these substrates highly artificial and has been reported to cause artifacts in compound tests. Improved substrates for Sirt1 and Sirt2 have been reported using FRET by introducing tetramethylrhodamine as a fluorophor and QSY-7 as a quencher at the N- and C-terminus, respectively, of a p53-derived peptide. For Sirt5 and Sirt6 activity measurements the fluorophore/quencher pair Dabcyl and EDANS was used in a glutamate dehydrogenase derived peptide sequence. Recently, we were able to show that use of 2-aminobenzoylamides as fluorophores and 3-nitrotyrosines as quenchers in a carbamoyl phosphate synthetase 2-aminobenzoylamides as fluorophores and 3-nitrotyrosines as quenchers in a carbamoyl phosphate synthetase derived peptide allows sensitive detection of Sirt5 activity in a continuous format.

Relatively high amounts (up to 4 μM) of sirtuin have been used in activity assays to correct for suboptimal substrate properties. This limits the applicability of the Michaelis-Menten-equation, which is valid only if enzyme concentration is much lower than substrate concentration. Additionally, due to the high enzyme concentrations, reliable estimation of I_C50- or K_i-values is difficult for inhibitors with affinities far below the enzyme concentration.

As previously established, sirtuins 1–6 are able to remove mid-chain acyl residues like octanoyl, decanoyl- and myristoyl-moieties from lysine side chains in histone H3 derived model peptides. Recently, using a similar histone H3 peptide substrate, it could be demonstrated that Sirt1-3 are able to remove myristoyl residues from lysine side chains and Sirt2 represents a very efficient demyristoylase. This fact and inspection of several crystal structures of a myristoylated/thioxy myristoylated peptides in complex with Sirt6/Sirt2 prompted us to test if the hydrophobic channel on the surfaces of Sirt6/Sirt2 can accommodate small fluorophores, like 2-aminobenzoylamidates, to create a continuous sirtuin activity assay.

If accepted by sirtuins, replacement of one amino acid residue within the TNFα derived substrate by a 3-nitrotyrosine residue as a quencher moiety should yield a peptide derivative increasing its fluorescence subsequent to sirtuin treatment in the presence of NAD+ (Fig. 1).

Results

We synthesized peptides derived from the TNFα sequence which are used as model substrates or inhibitors for different sirtuin isoforms (Figs 2 and 3). Nosyl-protection at one lysine residue for selective on-resin
modification of this side chain and Fmoc-based solid phase peptide chemistry was employed. The peptide 1a is the best Sirt6 substrate described so far.  

1a and 2a were subjected to HPLC-based activity assay to assess their substrate properties and to determine if the quencher moiety is accepted by sirtuin 6. Negative controls without NAD⁺ under identical conditions yielded
pared to but interestingly yielded an improved substrate for Sirt2 with a specificity constant of \(1.8 \times 10^6 \text{M}^{-1} \text{s}^{-1}\). Further elongation of the spacer resulted in slightly decreased substrate properties for sirtuins 1, 3, 4, 5, and 6 as compared to 1a. Peptide 3 was not a substrate for sirtuins 1 and 3–7 but showed some activity for Sirt2 in an HPLC based end-point activity assay. However, increasing the number of methylene groups to place the fluorophore to a different position was not a substrate for sirtuins 1 and 3–6. Substrate properties for Sirt2 resulted in >1000-fold decrease of substrate properties for Sirt2 (Table 1) and complete loss of activity for sirtuin isoforms 1–6. The development of Sirtuin mediated transformation of 3 into 2b could be followed directly and continuously \(\lambda_{\text{Em}} = 310 \text{ nm}, \lambda_{\text{Ex}} = 405 \text{ nm}\) using a fluorescence spectrometer (Supplementary Fig. S5). Without NAD\(^+\) in the presence of sirtuin enzyme or without sirtuin in the presence of NAD\(^+\) no significant change in fluorescence signal over time could be observed (Supplementary Fig. S5). This indicated that the observed fluorescence change results directly from sirtuin-mediated decylation and not from unspecific interactions between NAD\(^+\) and/or sirtuin and 3.

The slope of change in fluorescence intensity is linearly dependent on the enzyme concentration (Supplementary Fig. S5). Progress curves at different concentrations were linear below 25% conversion of the substrate. We used a completely converted assay solution (controlled by LC-MS) for the generation of appropriate standard curves (Supplementary Fig. S9). Additionally, we were able to demonstrate that the activity assay is compatible with 96- and 384-well microtiter plate-based equipment yielding \(Z’\)-factors of 0.85 for 3 at 25 \(\mu\text{M}\) concentration. Kinetic constants determined with either HPLC based assay or with the assay performed in both MTP fluorescence readers and spectrophotometers yielded comparable results (Supplementary Table S2).

Due to the relatively low \(k_{\text{cat}}\)-values of the known substrates, “classical” sirtuin activity assays are done in time-frames between 30 and 120 min and at enzyme concentrations between 0.5 and 4 \(\mu\text{M}\) to generate sufficient signal changes. At these conditions the basic assumption of the Michaelis-Menten-equation \(\frac{v}{[E]} = \frac{k_{\text{cat}}[S]}{K_M + [S]}\) is not valid. Moreover, the high amount of enzyme prevents the correct determination of \(K_M\)-values for sirtuin inhibitors with affinities below half of the enzyme concentrations used. With substrate 3 we were able to follow enzymatic activities down to 10 nM sirtuin concentration (Supplementary Fig. S8). We used a 96-well MTP fluorescence reader for the determination of the \(K_M\)-values for different compounds (Fig. 3) including inhibitors with high affinities to sirtuin isoforms (Table 3).

The first product of the sirtuin reaction, nicotinamide (NAM), is known to be a non-competitive inhibitor with respect to both acylated peptide substrate and NAD\(^+\) co-substrate by re-binding to the active site and attacking the sirtuin bound O-alkylimidate reforming NAD\(^+\). For Sirt6 an IC\(_{50}\) value of 2.2 \(\mu\text{M}\) was reported for NAM indicating that this isoform is not influenced by physiological NAM concentrations\(^{38}\).

### Table 1. Kinetic constants for 3, 4, 4α and 5 and Sirt2.

| Substrate | \(k_{\text{cat}} [\mu\text{M}]\) | \(10^{-3}k_{\text{cat}} [\text{s}^{-1}]\) | \(k_{\text{cat}}/K_M [\text{M}^{-1} \text{s}^{-1}]\) | Distance (No. of bonds) |
|-----------|------------------|-----------------|-----------------|------------------|
| 1         | 17.7 ± 1.5       | 4.0 ± 0.1       | 224\(^a\)        | 6                |
| 1a        | 1.2 ± 0.1        | 45.4 ± 1.8      | 58 600\(^b\)     | 8                |
| 3         | 0.1 ± 0.02       | 23.8 ± 0.8      | 176 000\(^a\)    | 11               |
| 5         | 15.3 ± 2.7       | 2.4 ± 0.1       | 150\(^b\)        | 13               |

\(^a\)mean ± s.d. (n = 2).

### Table 2. Kinetic constants for 3 and Sirt1-6.

| Enzyme   | \(k_{\text{cat}} [\mu\text{M}]\) | \(10^{-3}k_{\text{cat}} [\text{s}^{-1}]\) | \(k_{\text{cat}}/K_M [\text{M}^{-1} \text{s}^{-1}]\) | \(c (\text{Sirt}) [\text{nM}]\) |
|---------|------------------|-----------------|-----------------|------------------|
| Sirt1   | 0.7 ± 0.08       | 2.1 ± 0.1       | 287\(^a\)        | 500              |
| Sirt2   | 0.12 ± 0.02      | 23.8 ± 0.8      | 176 000\(^a\)    | 10               |
| Sirt3   | 3.3 ± 0.4        | 9.1 ± 0.4       | 2 800\(^b\)       | 100              |
| Sirt4   | 49.5 ± 7.5       | 0.4 ± 0.02      | 7\(^a\)           | 1000             |
| Sirt5   | 46.1 ± 7.2       | 3.2 ± 0.2       | 69\(^a\)          | 500              |
| Sirt6   | 23.5 ± 4.9       | 0.9 ± 0.1       | 39\(^a\)          | 500              |

\(^a\)mean ± s.d. (n = 2).
We determined the $K_i$-values of NAM for Sirt3 and Sirt6 to be 93 μM and 451 μM, respectively, using NAD$^+$ at saturating conditions (Supplementary Fig. S13). Under peptide substrate saturating conditions $K_i$-values were found to be 45 μM and 415 μM, respectively (Supplementary Fig. S13). The $K_i$-value for NAM was lower than expected for Sirt6, but still higher than for other isoforms. Recently, it was shown that the IC_{50}-values for NAM are dependent on the chemical acyl moiety and that different sirtuin isoforms have different acyl-dependent susceptibilities to NAM inhibition. Our substrate closely resembles the physiological myristoyl substrate hence our value should reflect the sensitivity of this substrate modification. Recently, compounds Quercetin and Ex-527 were reported as Sirt6 inhibitors with inhibition of enzymatic activity of 52% and 56%, respectively, if used at 200 μM concentration. We determined $K_i$-values for these two small molecules and found considerable non-competitive inhibition with respect to the peptide substrate (Table 3). The cyclic peptide derivative S2IL5, containing a trifluoroacetylated lysine side chain as a warhead for inhibition of sirtuin catalysis was claimed to be a Sirt2 specific inhibitor with affinities to the active site in the low nanomolar range as determined by iso thermal calorimetric measurements. Using 3 as substrate the determined $K_i$-value is 560 nM and the cyclic inhibitor behaved non-competitive for the peptide substrate (Supplementary Fig. S17). Replacement of the amide bond formed by the acyl chain and the ε-amino function of the lysine side chain by a thioxy amide bond transforms substrates into extremely slow substrates/inhibitors by generation of a stalled intermediate resembling sirtuin bi-substrate inhibitors. Thioc derivative S2IL5, containing a trifluoroacetylated lysine side chain as a warhead for inhibition of sirtuin catalysis was claimed to be a Sirt2 specific inhibitor with affinities to the active site in the low nanomolar range as determined by iso thermal calorimetric measurements. Using 3 as substrate the determined $K_i$-value is 560 nM and the cyclic inhibitor behaved non-competitive for the peptide substrate (Supplementary Fig. S17). Replacement of the amide bond formed by the acyl chain and the ε-amino function of the lysine side chain by a thioxy amide bond transforms substrates into extremely slow substrates/inhibitors by generation of a stalled intermediate resembling sirtuin bi-substrate inhibitors. Thioc derivative S2IL5, containing a trifluoroacetylated lysine side chain as a warhead for inhibition of sirtuin catalysis was claimed to be a Sirt2 specific inhibitor with affinities to the active site in the low nanomolar range as determined by iso thermal calorimetric measurements. Using 3 as substrate the determined $K_i$-value is 560 nM and the cyclic inhibitor behaved non-competitive for the peptide substrate (Supplementary Fig. S17). Replacement of the amide bond formed by the acyl chain and the ε-amino function of the lysine side chain by a thioxy amide bond transforms substrates into extremely slow substrates/inhibitors by generation of a stalled intermediate resembling sirtuin bi-substrate inhibitors. Thioc derivative S2IL5, containing a trifluoroacetylated lysine side chain as a warhead for inhibition of sirtuin catalysis was claimed to be a Sirt2 specific inhibitor with affinities to the active site in the low nanomolar range as determined by iso thermal calorimetric measurements. Using 3 as substrate the determined $K_i$-value is 560 nM and the cyclic inhibitor behaved non-competitive for the peptide substrate (Supplementary Fig. S17). Replacement of the amide bond formed by the acyl chain and the ε-amino function of the lysine side chain by a thioxy amide bond transforms substrates into extremely slow substrates/inhibitors by generation of a stalled intermediate resembling sirtuin bi-substrate inhibitors.}

### Table 3. $K_i$-values for different inhibitors.

| Inhibitor | Enzyme | $K_i$ (3) [μM] | $K_i$ (NAD$^+$) [μM] |
|-----------|--------|---------------|-----------------|
| NAM       | Sirt3  | 93.0 ± 8.5    | 45.0 ± 14.2     |
|           | Sirt6  | 451.0 ± 60.7  | 415.0 ± 45.1    |
| Ex-527    | Sirt6  | 100.0 ± 11.0  | n.d.            |
| Quercetin | Sirt6  | 21.0 ± 3.4    | n.d.            |
| S2IL5     | Sirt2  | 0.6 ± 0.2     | n.d.            |
| 8         | Sirt2  | 0.08 ± 0.02   | n.d.            |
|           | Sirt3  | 0.1 ± 0.02    | n.d.            |
|           | Sirt6  | 0.4 ± 0.1     | n.d.            |
|           | Sirt6* | 1.1 ± 0.1*    | n.d.            |
|           | Sirt60 | 1.9 ± 0.2*    | n.d.            |
| 9         | Sirt6/ | 0.6 ± 0.1*    | n.d.            |
|           | Sirt6# | 1.7 ± 0.5#    | n.d.            |
| 10        | Sirt2  | 0.3 ± 0.1     | n.d.            |
| 11        | Sirt2  | 50.0 ± 9.9    | n.d.            |

Identification of small molecule modulators of sirtuin activity using 3 could be hampered by absorbance/fluorescence of the effectors in the range between 320 nm and 400 nm. Consequently, the known sirtuin activity modulator resveratrol could not be analyzed because of the high extinction coefficient in that range. Using HPLC-based activity assay we found no significant influence of resveratrol on Sirt1 mediated decylation of 3 (Supplementary Fig. S4). To be able to analyze small molecules with absorption/fluorescence in the range of 2-aminobenzoylamide fluorescence, we exchanged the 2-aminobenzoylamide fluorophore by (4-N,N-dimethylamino-1,8-naphthalimido)-acetamide resulting in derivative 6 with fluorescence excitation at 471 nm. Surprisingly, this fluorophore could be used in combination with the 3-nitro-L-tyrosine quencher despite non-optimal overlap of the spectra. Compound 6 was not a substrate for sirtuins 1, 3, 5 and 6 as determined by HPLC-based assays and was a weak substrate for Sirt2 with an about 500-fold lower $k_{cat}/K_m$-value as compared to 3. Interestingly, Sirt4 recognized 6 better than 3 resulting in an about 10-fold improved $K_m$-value (Supplementary Table S2) indicative of differences in the flexibility of the hydrophobic channel accommodating the acyl chain between Sirt4 and the other sirtuin isoforms. However, these results showed that the development of substrates with different spectral properties is possible enabling the simultaneous detection of enzymatic activity using substrate mixtures. We analyzed kinetics for Sirt2 and Sirt4 using a mixture of substrates 3 and 6 using...
the suboptimal substrate properties of N-terminal amino function could be fitted, but the published coordinates are given in a conformation resembling the cis conformation of peptide bonds. In the recently reported structures of Sirt2 complexed with a thioxo myristoylated inhibitor closely related to 8 (PDB ID 4Y6Q) or a thioxo myristoylated peptide derived from Histone H3 (PDB IDs 4Y6L and 4R8M) the conformation of the thioxo amide bond was in trans conformation which is the preferred conformation of secondary amide/thioxo amide bonds in aqueous solutions. We were able to enrich the faster migrating isomer to 72.4% and the cis isomer of 8, respectively, pointing to a small preference for the cis conformation of the amide bond (Supplementary Fig. S36). Nevertheless, because of the suboptimal substrate properties of 3 for Sirt6 the assay duration was 30 min which allows significant re-equilibration of the photo-induced change of the cis/trans equilibrium. Therefore, we introduced an additional methyl group at the lysine nitrogen resulting in a tertiary thioxo amide 9 which is an inhibitor with similar affinities to the active site of Sirt6 as compared to 8 (Supplementary Table S4). The rate constant for the cis/trans isomerization of the tertiary thioxo amide bond of 9 (5.4 × 10⁻⁴ s⁻¹ at 20°C) was much slower than that of the secondary thioxo amide bond of 8 (Supplementary Fig. S35). HPLC analyses revealed a cis content of about 50% and there was no change detectable subsequent to photo-excitation at the π−π* transition of the tertiary thioxo amide bond. We tested several different organic solvents to change the cis content but found no sufficient differences (Supplementary Table S5). Therefore, the two isomers were separated by HPLC at low temperatures (4°C).

We were able to enrich the faster migrating cis isomer to 72.4% and the trans isomer to 70.3% (Fig. 4). The frozen isomers (−70°C) were stable for several days (Supplementary Fig. S39).

Determination of inhibition of Sirt6 by 9 using samples with different cis content showed minimal preference for the cis isomer (IC₅₀ values of 0.6 μM and 1.7 μM for 72.4% and 29.7% of cis of 9, respectively Supplementary Fig. S38). These results again demonstrated the plasticity within the active site of sirtuins, at least for Sirt6, enabling both isomers to bind with similar affinities. Inspection of the electron density maps of PDB ID 3ZG6 suggest that there is sufficient space around the lysine side chain amide/thioxo amide bond to fit both isomers. Recently, Sirt6 coordinates of 3ZG6 were re-refined by Denus lab and it was established that the myristoylated peptide should be in a trans conformation regarding the amide bond between the lysine side chain and the acyl moiety.

Here we present a continuous sirtuin activity assay allowing convenient measurement of highly accurate data. The sensitivity of the activity assay enables the reliable determination of IC₅₀ values for inhibitors with affinities below 100 nM. Because of the demonstrated compatibility with 384-well MTP readout we expect that this assay principle will find widespread application in drug discovery projects. Additionally, the superior substrate properties of 3 allow the investigation of isomer specificity in the binding of inhibitors to the active site of sirtuins enlarging the portfolio of tools in sirtuin research.
Methods

Chemicals and general methods. All chemicals were purchased from Sigma (Saint Louis, USA) if not denoted otherwise. Rink amide MBHA resin was obtained from Iris Biotech (Marktredwitz, Germany). 9-fluorenylmethoxy-carbonyl- (Fmoc) protected amino acid derivatives and O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethylyuronium hexafluorophosphate (HBTU) were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Roth (Karlsruhe, Germany).

For HPLC separations solvents consisting of water (solvent A) and ACN (solvent B), both containing 0.1% TFA, were used. Analytical runs were performed on an Agilent 1100 HPLC (Boeblingen, Germany) with a quaternary pump, a well-plate autosampler and a variable wavelength detector. Separations were performed on a 3.0 × 50 mm reversed phase column (Phenomenex Kinetex XB C-18, 2.6 μm) with a flow-rate of 0.6 mL/min. A Merck-Hitachi High Speed LC system (Darmstadt, Germany) with a Merck Hibar Li Chrospher® RP-8 column (250–25 mm, 5 μm) was used for preparative separations (flow-rate: 8 mL/min). Eluted compounds were analyzed by MALDI mass spectrometry. NMR spectroscopy was carried out using Varian Gemini 2000 spectrometer in deuterated chloroform.

Synthesis of Fmoc-Lys(Nosyl)-OH (N-α-(9-Fluorenylmethoxy carbonyl)-N-ε-(2-nitrobenzenesulfonyl)-L-lysine). The solution of L-lysine hydrochloride (20 mmol) and NaHCO₃ (20 mmol) in 20 mL of H₂O was combined with CuSO₄·5H₂O (10 mmol) solution in 40 mL of H₂O. The vigorously stirred purple solution was cooled in an ice bath and a solution of 2-nitrobenzenesulfonyl chloride (30 mmol) in acetone (60 mL) was added. Next solid NaHCO₃ (75 mmol) was added in portions over 1 hour. The stirred reaction mixture was left in a melting ice bath overnight. The blue precipitate was filtered, subsequently washed with H₂O, ethanol and diethyl ether (Et₂O). After air drying yield of the complex was 87%. To the copper complex of ε-nosyl lysine (5 mmol) a solution of ethylenediaminetetraacetic acid (EDTA) disodium salt (6.5 mmol) in 40 ml of H₂O was added. This suspension was stirred and heated at 70–80 °C until no blue complex was left and then cooled to room temperature. Afterwards, solid NaHCO₃ (75 mmol) was added in portions over 1 hour. The stirred reaction mixture was left in a melting ice bath overnight. The blue precipitate was filtered, subsequently washed with H₂O, ethanol and diethyl ether (Et₂O). After air drying yield of the complex was 87%. To the copper complex of ε-nosyl lysine (5 mmol) a solution of ethylenediaminetetraacetic acid (EDTA) disodium salt (6.5 mmol) in 40 ml of H₂O was added. This suspension was stirred and heated at 70–80 °C until no blue complex was left and then cooled to room temperature. Afterwards, solid NaHCO₃ (10.5 mmol) was added to the formed suspension of ε-nosyl lysine and followed by solution of Fmoc-N-hydroxsuccinimide ester (Fmoc-OSu) (10.5 mmol) in 30 mL of acetone. The mixture was stirred vigorously overnight, diluted with 250 mL of 1% solution of NaHCO₃, and extracted with Et₂O (3 × 100 mL). Ether washings were back extracted with diluted NaHCO₃ solution and discarded. Combined aqueous phases were acidified with 10% HCl and extracted with dichloromethane (DCM) (3 × 50 mL). Combined organic phases were washed with water and dried over Na₂SO₄. Solvent was evaporated to afford target compound as white foam. Yield: 91%.
Synthesis of carboxymethyl dithiomyristoate. Carboxymethyl dithioester was prepared in accordance to Leon et al.88. To the solution of myristic acid (4 mmol), HBTU (4 mmol) and N,N-diisopropylethylamine (DIPEA) (8 mmol) in DCM (30 mL) was added piperidine (4.2 mmol). After 4 hours reaction mixture was diluted with water and extracted with DCM. Extracts were washed with a solution of HCl, diluted NaHCO₃, and with water, dried over Na₂SO₄ and DCM was evaporated. To the residue toluene (10 mL) was added followed by Lawesson's reagent (2 mmol). Reaction was heated at 106 °C for 3.5 hours. Solvent was evaporated and the residue flash-chromatographed (silica gel, ethyl acetate (EtOAc)/petr.ether 1:9) (Yield: 74%).

The solution of N-thiomyristoyl piperidine (3 mmol) and bromoacetic acid (3.2 mmol) in 7 mL of dry DCM was left at room temperature overnight. The solution was saturated with dry H₂S for 30 min and DCM was evaporated in vacuo. Flash-chromatography of the residue (silica gel, DCM/AcOH 10:0.05) afforded the pure product as a yellow solid (Yield: 25%).

Synthesis of methyl 3-[(methylthio)thiocarbonyl]propanoate. A solution of succinic anhydride (30 mmol) and piperidine (50 mmol) in EtOAc (10 mL) was refluxed for 10 min. On the next day, precipitated product was filtered, washed with EtOAc and air dried (Yield: 90%).

N,N-pentamethylenesuccinamic acid (10 mmol) was refluxed in anhydrous MeOH (15 mL) containing 2 drops of H₂SO₄ for 4 hours. Solvent was evaporated and residual oil dissolved in EtOAc, washed with NaHCO₃ solution, water and dried over Na₂SO₄. Evaporation of EtOAc afforded product as a colorless oil (Yield: 84%).

Methyl N,N-pentamethylenesuccinamate (8.4 mmol) and Lawesson's reagent (5.1 mmol) were refluxed in tetrahydrofuran (THF) (10 mL) for 1 hour. THF was evaporated in vacuum and the residue was flash-chromatographed (silica gel, EtOAc/petr.ether 1:5). Yield of slightly yellowish oil 86%.

To a solution of Methyl 3-(N,N-pentamethylenethiocarbamoyl)propanoate (2 mmol) in anhydrous THF (8 mL) Mel (10 mmol) was added. The reaction was conducted for 48 h in darkness Yellow-colored THF was decanted, the crystals were briefly washed with dry THF and dissolved in dried DCM (3 mL). Dried H₂S was bubbled into solution for 2 h and mixture was left at 0 °C for 24 h. After addition of H₂O (100 mL), product was extracted with EtOAc, washed several times with water, brine and dried over Na₂SO₄. Evaporation of EtOAc in vacuo gave the crude product as a yellow oil (Yield: 81.5%).

Synthesis of TNF-α peptide derivatives. The peptide Ac-EALPKK(NS)XGG-NH₂ (X = T, Y(NO₂)₃ or Mcm) was synthesized by standard manual solid-phase-peptide synthesis using Fmoc-protected amino acid derivatives. Rink amide MBHA resin was treated with N,N-diisopropylethylamine (DIPEA) in DMF at RT (60 min). The solution of triphenylphosphine (5 eq) instead of triethylamine (5 eq) was bubbled into solution for 2 h and mixture was left at 0 °C for 24 h. After addition of H₂O (100 mL), product was treated with EtOAc, washed several times with water, brine and dried over Na₂SO₄. Evaporation of EtOAc in vacuo gave the crude product as a yellow oil (Yield: 81.5%).

Peptides 10 and 11 are based on a CPS1-peptide (Bz-GVLKEYGV-NH₂). To a DMF solution of the CPS1 peptide, ethyl dithioacetate (1.2 eq) and triethylamine (5 eq) (10) or methyl 3-[(methylthio)thiocarbonyl]propanoate (1.1 eq) and triethylamine (5 eq) (11) were added. Reaction mixture was stirred for 3–5 h. For 11 1 M NaOH (6 eq) was added and stirring continued for another 2 h.

The cyclic peptide inhibitor S2IL5 was synthesized by standard Fmoc-based solid phase peptide synthesis as described by Yamagata et al.60. All 3-nitrotrosine containing peptides (2a, 2b, 3, 4, 4a, 5 and 6) require an additional piperidine treatment step (20% piperidine in DMF, 2 × 10 min) to remove acyl-group from 3-nitrotrosine before cleavage.

The resin was washed with DCM (5 × 4 min), methanol (3 × 4 min) and DCM again and treated with TFA/H₂O (98:2) (2 × 60 min). Combined TFA solutions were evaporated in vacuum and re-dissolved in ACN/H₂O solution (50:50). HPLC purification and subsequent lyophilization yielded pure peptides.

1H NMR (400 MHz, CDCl₃) δ ppm: 8.15 – 8.06 (m, 1H), 7.85 – 7.78 (m, 1H), 7.75 (d, J = 7.5 Hz, 2H), 7.71 – 7.63 (m, 2H), 7.59 (d, J = 7.3 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 5.48 (t, J = 5.8 Hz, 1H), 3.41 (d, J = 7.8 Hz, 1H), 4.48 – 4.29 (m, 3H), 4.21 (t, J = 6.6 Hz, 1H), 3.16 – 3.0 (m, 2H), 1.94 – 1.34 (m, 6H). 13C NMR (100 MHz, CDCl₃) δ ppm: 176.0, 156.1, 148.0, 143.8, 143.7, 141.3, 133.5, 132.7, 131.0, 127.7, 127.1, 125.3, 125.1, 120.0, 67.2, 53.3, 47.1, 43.3, 31.6, 28.9, 21.9.
Expression and purification of human sirtuins. Sirt1, Sirt2, Sirt3, Sirt5 and Sirt6 were expressed and purified as described before.\textsuperscript{26,30,70,71}

To obtain the expression plasmid of human (His\textsubscript{6})-SUMO-Sirt4(29–314), the respective DNA fragment was PCR-amplified using gene-specific primers from the plasmid pET101-Sirt4, which carries the Sirt4 gene, and cloned into the Bsal, Xhal sites of pET-SUMO yielding the plasmid pET-SUMO-Sirt4(29–314).

The protein was overexpressed in \textit{E. coli} BL21 (DE3) cells at 18 °C. The purification of the protein was performed using affinity chromatography on Ni-NTA resin in 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl. The matrix-bound (His\textsubscript{6})-SUMO-Sirt4(29–314) was eluted by imidazole in the buffer and further purified by gel filtration in 10 mM HEPES, pH 7.8, 150 mM KCl, 1.5 mM MgCl\textsubscript{2}, and stored at −20 °C for use.

pQE-80L (Qiagen, Valencia, CA) His-tagged Sirt6 (1–355) was transformed into the competent \textit{E. coli} strain, BL21 DE3 and overexpressed at room temperature. For purification a nickel resin affinity chromatography was used in 50 mM NaPO\textsubscript{4}, pH 7.2, 250 mM NaCl, 5 mM imidazol, 1 mM BME and eluted by 250 mM imidazol in the buffer. Furthermore, Sirt6 was purified secondarily via a HiTrap SP-Sepharose Fast Flow column (GE Healthcare) using a linear gradient from 50–750 mM NaCl in 50 mM NaPO\textsubscript{4}, pH 7.2, 1 mM BME. Afterwards fractions containing purified Sirt6 were pooled, concentrated and dialyzed into 50 mM Tris, 100 μM TCEP and 5% (w/v) glycerol and stored at −70 °C.

HPLC based activity assay. For the determination of kinetic constants for all sirtuin mediated reactions solutions containing 20 mM TRIS/HCl pH 7.8, 150 mM NaCl, 5 mM MgCl\textsubscript{2} (assay-buffer), 500 μM NAD\textsuperscript{+} and varying substrate concentrations (0.5–100 μM) were used. Deacylation was started by adding human sirtuin to reach a final concentration of 0.01–0.5 μM. Enzyme-catalyzed reaction was stopped using TFA (1% final concentration) after 1 min to 180 min of incubation at 37 °C depending on substrate reactivity. The cleavage rate of the peptide concentration was fixed (5, 25 or 200 μM) and the NAD\textsuperscript{+} was determined by linear regression of 1/apparent V\textsubscript{max} against the corresponding inhibitor concentration. The resulting plots were analyzed by a competitive inhibition and non-competitive inhibition model using the program SigmaPlot 8 (Systat Software, San Jose, USA). All measurements were done in duplicates.

Continuous fluorescence assay. The fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) at λ\textsubscript{ex} = 310 nm and λ\textsubscript{em} = 405 nm (slit\textsubscript{ex} = 5 nm, slit\textsubscript{em} = 2.5 nm, PMT = 700 V for 3, 4 and 5 as well as slit\textsubscript{ex} = 10 nm, slit\textsubscript{em} = 10 nm, PMT = 950 V for 4a). Each reaction mixture contained assay-buffer, 0.5 mM NAD\textsuperscript{+} and various peptide concentrations (0.1–100 μM) and was preincubated for 5 minutes at 37 °C. The reaction was started by adding human sirtuin (0.1–0.5 μM) and observed for 5–10 minutes. Product formation could be monitored by increase of relative fluorescence. This signal was converted into product concentration via calibration lines. The slope of the linear regression of product formation against time yielded the reaction velocity rates in μM/s. K\textsubscript{cat} and k\textsubscript{cat} were obtained by non-linear regression according to Michaelis-Menten. All measurements were done in duplicates. For determination of reaction velocity rates in μM/s calibration lines were necessary. Therefore a reaction mixture was prepared, containing assay-buffer, 2 μM Sirt1, 500 μM NAD\textsuperscript{+} and 100 μM of 3, 4, 4a or 5 was incubated overnight at 37 °C. The reaction mixture was analyzed with HPLC, to control if the entire peptide substrate was turned to product. Additionally the mixture was diluted (0.1–25 μM) and measured with Hitachi F-4500 fluorescence spectrophotometer at the same conditions as described above.

The microtiter plate fluorescence measurements were performed on a Tecan Infinite M200 microplate reader (Männedorf, Switzerland) at λ\textsubscript{ex} = 320 nm and λ\textsubscript{em} = 408 nm (lag time 9 μs, integration time 20 μs, gain 160, 170 or 182). The reactions (total volume 100 μl) were measured in black low-binding 96- well microtiter plates (NUNC). Assay-buffer, 500 μM NAD\textsuperscript{+} and 0.07–200 μM peptide substrate were pre-incubated at 37 °C for 5 min. The reaction was started by adding human sirtuin (0.01–0.5 μM). The signals were converted into product concentration via calibration lines and the resulting data were evaluated as described above (single fluorescence measurement). The determination for the kinetic constants of NAD\textsuperscript{+} was performed in the same way, except that the peptide concentration was fixed (5, 25 or 200 μM) and the NAD\textsuperscript{+} concentration was varied (10–1500 μM). All measurements were done in duplicates. The reaction mixture for the calibration lines was prepared as described for the single fluorescence measurements. After complete turnover of peptide substrate 3, the solution was diluted (0.2–20 μM) and measured on Tecan infinite M200 microplate reader at λ\textsubscript{ex} = 320 nm and λ\textsubscript{em} = 408 nm (lag time 9 μs, integration time 20 μs, gain 182 (G182), 170 (G170) and 160 (G160)) K\textsubscript{v} values of the inhibitors were determined by recording k\textsubscript{cat} and K\textsubscript{cat} values for 3 in the presence of varying inhibitor concentrations (0.01–600 μM). The resulting plots were analyzed by a competitive inhibition and non-competitive inhibition model using the program Sigma Plot 8. The linear regression of the apparent K\textsubscript{cat}-values against the corresponding inhibitor concentration yielded the inhibitor constant K\textsubscript{i} for competitive inhibition. The K\textsubscript{i} for non-competitive inhibition was determined by linear regression of 1/apparent V\textsubscript{max} against the corresponding inhibitor concentration. The negative K\textsubscript{i} value can be determined as intersection with the X-axis from these plots.

Photo-induced change of cis content of thioxo peptides. Excitation experiments of thiopeptide were done in a cuvette at 254 nm under stirring with a UV-lamp (UV handheld lamp, Carl Roth). For irradiation a distance of 5 cm between cuvette and UV-lamp was chosen. UV-spectra were recorded between 230 and 325 nm using a spectrophotometer (Specord M500).
For determination of temperature dependent cis/trans isomerization a 50 μM solution of thiopeptide was incubated for 10 min at different temperatures (10–70 °C). UV-spectra were recorded at ground state (GS) and after irradiation at 254 nm (irradiation time 45 s to 5 min) at photostationary state (PSS). Several UV-spectra over time were recorded to determine rates of cis/trans isomerization. Using a differential spectrum (UV spectrum GS – UV spectrum PSS) activation parameter and isomerization velocity could be examined.

The cis/trans content of a 50 μM thiopeptide solution was changed by 5 min irradiation at 254 nm and the resulting solution was analysed by HPLC. Additionally, several solvents were tested to enhance cis content. As solvents HzO, acetic acid, TFA, trifluoro ethanol (TFE), 0.5 M LiCl in HzO/ethanol (EtOH)/TFE, methanol (MeOH), formic acid, N-methyl pyrolidon (NMP), DMF, Dimethylsulfoxid (DMSO) and tetrahydrofuran (THF) were chosen. Cis content was determined via HPLC of a 500 μM solution of 9.

For the separation of isomers, 5–6 mg of 9 were dissolved in 50% ACN and equilibrated overnight. For better separation HPLC-solvents were cooled down to 4°C and a linear gradient of 45% solvent B to 55% solvent B was used. Fractions were immediately frozen in liquid nitrogen. HPLC-based determination of cis content was done directly after preparative separation.

The examination of the isomer specific inhibition of 8, 9, 10 and 11 was examined via HPLC using reaction solutions composed of 500 μM NAD+, 30 μM peptide, 0.5 μM sirtuin and 0.5–40 μM inhibitor in GS, PSS or GS* in assay buffer. After 30 min incubation at 20 °C reaction was stopped using 10% TFA solution. Inhibitor solutions were irradiated at 254 nm for 5 min. Separated isomers were applied in concentrations from 1 to 10 μM. The influence of cis content on sirtuin inhibition using fluorescence spectrometer was determined with 500 μM NAD+, 5 μM 3, 0.1 μM Sir2 and 0.1 μM 9. Reactions were done at 20 °C with 9 in GS and PSS (after 5 min irradiation at 254 nm). 9 in PSS was applied immediately after irradiation (transfer time ~5 s) and started directly by adding sirtuin. Reactions were measured within 1 min to avoid re-isomerization.

Z’ factor analysis. The Z’ factor is a dimensionless, simple statistic parameter for high-throughput screening assays[2]. It is defined as the ratio of separation band to signal dynamic range of the assay and used the signal variation at the two extremes of the activity range (0 and 100% activity).

\[
Z' = 1 - \left( \frac{(3 \cdot SD_{100\%} + 3 \cdot SD_{0\%})}{(mean_{100\%} - mean_{0\%})} \right)
\]

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Author Contributions
M.S., S.S. and C.R. designed the assay and wrote the manuscript. S.S., C.R. and M.M. wrote supplementary information. S.S., M.M. and P.Z. synthesized peptides. S.S. and C.R. performed kinetic analysis with HPLC-based assay. S.S. and Z.S. performed kinetic measurements with direct fluorescence assay. P.Z. and C.R. performed photo-induced cis/trans isomerization of thioxo peptides. P.Z. and C.R. expressed and purified Sirt5, C.K. and C.S. Sirt1, Sirt2, Sirt3 and Sirt6. C.S.-F. performed expression and purification of Sirt4 and M.O.H. of Sirt7.

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