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A Novel Continuous Assay for the Deacylase Sirtuin 5 and Other Deacetylases

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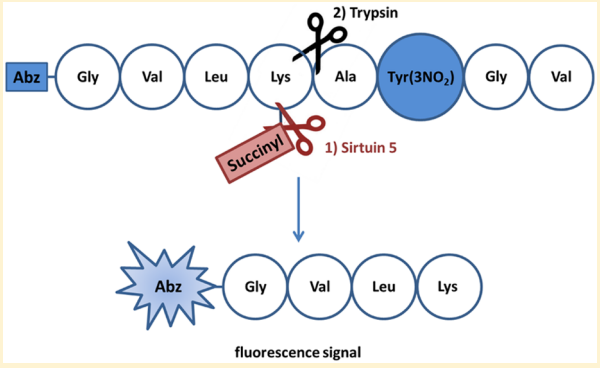
Supporting Information

ABSTRACT: Sirtuins are NAD^+ dependent lysine deacylases involved in many regulatory processes like control of metabolic pathways, DNA repair, and stress response. Modulators of sirtuin activity are needed as tools for uncovering the biological function of these enzymes and as potential therapeutics. Systematic discovery of such modulators is hampered by the lack of efficient and simple continuous activity assays running at low sirtuin concentrations in microtiter plates. Here we describe an improved continuous sirtuin 5 assay based on the coupling of the sirtuin reaction to a proteolytic cleavage using internally fluorescence-quenched substrates. Systematic optimization of a carbamoyl phosphate synthetase 1 derived, glutarylated peptide yielded a Sirt5 substrate with k_{cat}/K_M value of 337 000 M^{-1} s^{-1}, which represents the best sirtuin substrate described so far. These extraordinary substrate properties allowed reliable determination of K_i values for different inhibitors in the presence of only 10 nM sirtuin in microtiter plate format. Assay conditions could be transferred effectively to other lysine deacetylases, like sirtuin 2 and sirtuin 3, which now enables more efficient development of sirtuin targeting drugs.



INTRODUCTION

Protein lysine acetylation is one of the most abundant posttranslational modifications (PTM), involved in many cellular processes.[1,2] The level of lysine acetylation is regulated by acetyltransferases and deacetylases. Besides acetylation, recent studies found other acylations as *in vivo* PTMs. This includes propionylation,[3,4] succinylation,[5,6] malonylation,[6,7] glutarylation,[8] crotonylation,[9] butyrylation,[3] and myristoylation.[10] The generation of these PTMs is enigmatic at least in mitochondria because there were no classical acetyltransferases found in this compartment. Recent work demonstrated that these acyl transfers might happen spontaneously with acyl-CoAs or acylphosphates as acyl donors.[11−13] More is known about the removal of such acyl moieties. Sirtuins, a conserved family of NAD^+-dependent lysine deacetylases, have different deacylation activities besides removing acetyl residues.[14,15] For example, the mitochondrial sirtuin 5 (Sirt5) has a ∼1000-fold higher catalytic efficiency for succinylated and glutarylated lysine residues compared with acetylated lysines,[7,8,15,16] whereas Sirt6 prefers long acyl chains, like myristoylated lysine side chains.[14] Additionally, it was shown that Sirt4 is able to remove lipoic acid and biotin residues from active site lysines both *in vitro* and *in vivo*[17] and Sirt3 seems to be the *in vivo* decrotonylase.[18] Sirtuin-dependent deacetylation regulates many metabolic processes, such as fatty acid synthesis, glucose homeostasis, and stress response,[19] and sirtuins are involved in diseases like diabetes, cancer, and neurodegeneration,[19] making these enzyme attractive targets for drug discovery.

For the systematic development of sirtuin effectors, reliable and efficient high-throughput assays are necessary. Several assays were developed based on the separation of product and substrate (HPLC, CE)[20−22] or spectrometric detection of one reaction compound,[23−26] which are in most cases discontinuous.[27] Nevertheless, there are continuous sirtuin activity assays described, coupling the sirtuin reaction to additional enzymatic reactions,[25,28] chemical reactions like intramolecular transesterifications,[29] DNA interaction,[30] or fluorescence enhancement by oligomerization and precipitation.[31] One of the most common assays determines released nicotinamide, which is transformed to nicotinic acid and ammonia by nicotinamidase. Ammonia can be converted with NAD(P)H and α-ketoglutarate by glutamate dehydrogenase (GDH) leading to a UV signal.[25] This complex enzymatic cascade and the many substrates involved, however, limit the measurement range of the assay and make it more sensitive to interference in compound tests. Generally, the deacetylated peptide product could be quantified by coupling to a lysine-recognizing protease reaction.[28] Subsequent to sirtuin-mediated deacylation of the respective lysine residue the used helper protease is able to hydrolyze the lysinyl-chromophore/fluorophore amide bond

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leading to a signal increase. This principle is used in the so-called Fluor-de-Lys assay, but the fluorophore replacing the C-terminal peptide part renders this substrate highly artificial and has been reported to cause artifacts in compound tests.[32] Because of the protease needed for the assay, it is mostly used discontinuously, and because of the suboptimal substrate properties for the sirtuins, it is extremely time-consuming (up to 2 h for both the sirtuin and the protease reaction). Madsen and Olsen used this assay principle in a continuous manner,[33] but the substrates showed very low catalytic efficiencies for Sirt5 (Table 1, entries H4 K12-Succ and Ac-K(Succ)-AMC).

Table 1. Kinetic Constants for Sirt5 Mediated Desuccinylations

substrate	K_M [μM]	k_{cat} [10^{-3} s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]
H3 K9-Succ[a15]	5.8 ± 2.7	25 ± 2	4300
GDH K503-Succ[b15]	14 ± 4	28 ± 2	2000
ACS2 K628-Succ[c15]	450 ± 150	268 ± 51	600
H4 K12-Succ[d33]	33 ± 1.8	30	920
Ac-K(Succ)-AMC[33]	84 ± 22	9	110
Z-K(Succ)-AMC[34]	14.7 ± 1.8	*f*	*f*
CPS1 K527-Succ (peptide **1**)[e22]	3.8 ± 0.6	53 ± 2	14000

[a]Histone H3; H-KQTARK(Succ)STGGWW-OH. [b]Glutamate dehydrogenase, H-SGASEK(Succ)DIVHSGWW-OH. [c]Acetyl-CoA synthetase 2, H-KTRSGK(Succ)VMRRWW-OH. [d]Histone H4, Ac-LGK-(Succ)-AMC. [e]Carbamoyl phosphate synthetase 1, Bz-GVLK(Succ)-EYGV-NH_2. [f]Not determined.

To shorten reaction times in such assays based on weak substrates, high amounts (up to 4 μM) of sirtuin are often used in activity assays.[14] This limits the validity of the Michaelis–Menten equation, which has the prerequisite that enzyme concentration has to be much lower than substrate concentration. Therefore, measurements with sirtuin concentrations in the range of the used substrate concentration will yield correct kinetic constants in special cases only. Moreover, reliable determination of IC_{50} or K_i values is impossible for strong binding inhibitors under such conditions. Therefore, much better sirtuin substrates are needed for reliable determination of K_i values for strong binding inhibitors. Such assays would ideally allow real-time monitoring for increased data accuracy and be applicable in a parallel measurement mode for convenient collection of larger sets of activity data for enzymatic studies and drug development campaigns.

RESULTS AND DISCUSSION

For substrate optimization, we started with a succinylated peptide (**1**) derived from carbamoyl phosphate synthetase 1 (CPS1), which was discovered to be a supersubstrate for Sirt5 using a peptide microarray approach[35] and which represents in the N-terminally benzyolated form the best substrate described so far for Sirt5 (Scheme 1).[22]

We wanted to introduce fluorophore and quencher moieties into the CPS1 peptide by minimal modification of the structure avoiding unfavorable interaction of the substrate with the active site of Sirt5. Inspection of Sirt5 cocrystallized with **1** (pdb 4UTN) revealed that the benzoyl moiety of **1** does not contact Sirt5 and that there is some space around the tyrosine side chain in +2 position for additional functionalities. Thus, we substituted the *N*-terminal benzoyl moiety by 2-aminobenzoyl-residue (Abz) generating a fluorophore (**2**). Alternatively, we introduced a nitro group to the tyrosine side chain in the meta position (**3**), transforming this residue into an efficient quencher (Y(NO_2)) without changing its α-amino acid nature within the peptide. Finally, we combined both modifications in one sequence resulting in an internally fluorescence-quenched peptide derivative (**4a**). This combination of fluorophore and quencher within one peptide sequence results in a very efficient assay for protease activity.[36] Obviously, these modifications of **1** did not negatively influence the substrate properties for Sirt5 as shown in Figure 1. The helper protease trypsin disfavors

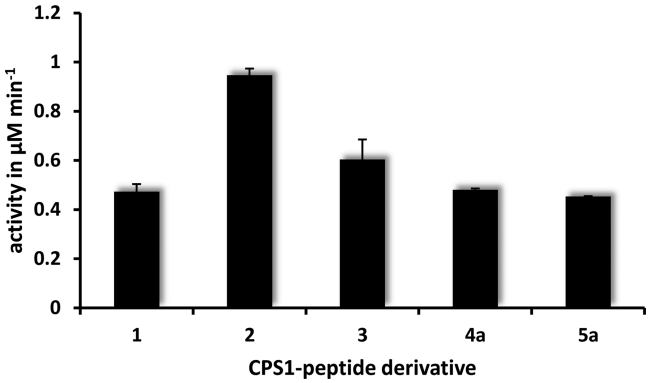
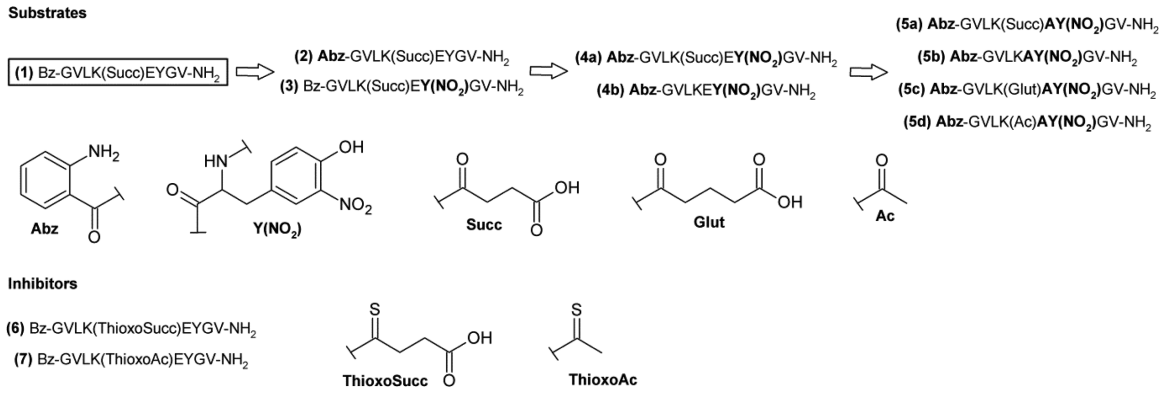


Figure 1. Sirt5-activity against different CPS1 peptide derivatives (**1**–**5a**). Data were obtained by end point measurements after 1 h reaction time. Peptide (100 μM), 500 μM NAD^+, and 0.1 μM Sirt5 were incubated at 37 °C. All measurements were done in duplicates.

Scheme 1. CPS1 Peptide Derivatives



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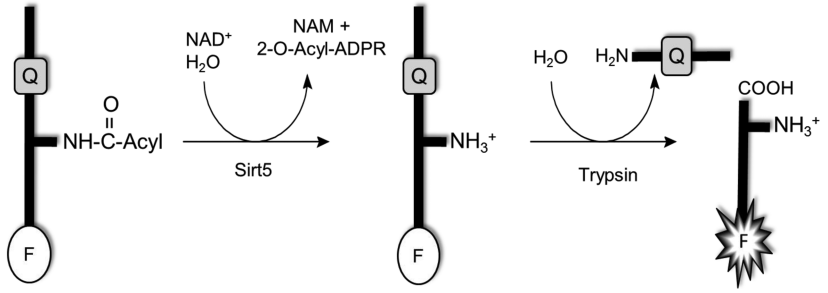
Scheme 2. Assay Principle[a]



[a]Sirt5 removes acyl-residue from lysine side chain. Trypsin hydrolyzes peptide C-terminal of lysine thereby separating fluorophore and quencher.

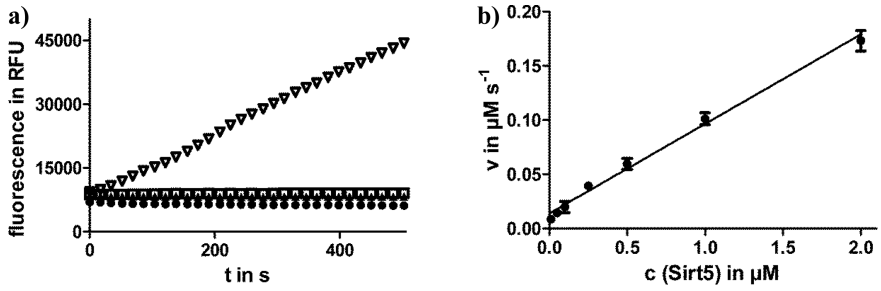


Figure 2. (a) Change of fluorescence intensity under different conditions. Data were obtained for **5a** (●) without trypsin, (□) without Sirt5, (▲) without NAD^+, and (▽) with all components included. (b) Reaction velocity is dependent on Sirt5 concentrations (10−200 nM). All reactions were done in triplicate.

negatively charged side chains in P1′-position,[37] leading to high K_M value for **4b**, the product of the Sirt5 mediated desuccinylation of **4a** (Figure S1). Therefore, we exchanged the glutamate by an alanine leading to the derivatives **5a** and **5b**. This modification has no influence on Sirt5 catalysis (Figure 1) but clearly improves both K_M and k_{cat} value for trypsin (Table S1). This optimized substrate should allow the efficient coupling of the deacetylation reaction and the detection reaction (Scheme 2) possibly even in a continuous assay (see below).

Optimal emission and excitation wavelengths of the reaction product mixture for fluorescence detection were determined via a 3D-spectrum, which was obtained from fully hydrolyzed **5b** (Figure S2). The reaction could be followed at an excitation wavelength of 320 nm and an emission wavelength of 420 nm. A fluorescence signal change could be observed only when all components of the reaction are added. Reactions without trypsin, NAD^+, or sirtuin showed no change in fluorescence intensity (Figure 2a).

A Sirt5 concentration series showed linear correlation between enzyme amount and activity against **5a**, confirming that the assay signal is an accurate measure of sirtuin catalysis. The results showed that already 10 nM Sirt5, more than 20-times less than recommended for the FdL assay, is sufficient to get a reliable fluorescence change in less than 5 min, which represents a paramount improvement of existing sirtuin activity assays. For trypsin, a concentration of 0.01 mg mL^{-1} was chosen to ensure that trypsin reaction is not limiting in the coupled assay (Figure S3). Additionally, we tested whether trypsin is able to hydrolyze Sirt5 during the reaction. Treatment of Sirt5 with trypsin for different times followed by sirtuin activity measurements with HPLC indicates that Sirt5 activity is not influenced significantly for up to 5 h exposure to protease (Figure S4). Additionally, we analyzed the stability of Sirt5 against trypsin the presence and absence of substrate by SDS-PAGE and silver staining (Figure S12a). Proteolytically stable fragments are formed. Without the substrate, Sirt5 is degraded within 1 h. The protective effect of the substrate is caused by the interaction with Sirt5 and not by inhibition of trypsin activity because **1** did not show any inhibitory effect on the trypsin-mediated hydrolysis of either benzoyl-arginine-4-nitroanilide or **5b** measured by UV−vis or fluorescence spectrometry, respectively. This stability against trypsin and the improved substrate properties of **5b** for the helper protease enabled us to monitor the kinetics of Sirt5 mediated desuccinylation of **5a** in a continuous format. Subsequent to preincubation of NAD^+ and **5a** in buffer at 37 °C for 5 min, trypsin was added followed by Sirt5, and the reaction was then monitored continuously through fluorescence detection. This setup enabled sensitive real-time monitoring of Sirt5-dependent substrate deacylation to generate **5b**, which is instantly hydrolyzed by trypsin separating fluorophore and quencher, thereby leading to increased fluorescence (Scheme 2). Progress curves were analyzed through linear regression resulting in initial velocities for different substrate concentrations. Relative fluorescence units (RFU) could be transformed into molecular reaction velocity with a calibration curve (Figure S5). The initial velocity rates were plotted against substrate concentrations, and kinetic values were determined using the Michaelis−Menten equation (Figure 3, Table 2). The catalytic efficiency for **5a** is 17.5-fold higher compared with the best known succinylated Sirt5 substrate **1**.[22] We confirmed this result by determining the kinetic constants for **5a** using an HPLC assay (Table 2). Additionally, we determined kinetic constants for cosubstrate NAD^+ using the novel fluorescence-based assay and **5a** at saturating conditions (Table 2, Figure S6). The relatively low K_M value of 26 μM is comparable to the described K_M value for NAD^+ determined at saturating conditions with **1** using an HPLC-based assay.[22] Recently, Feldman et al. revealed a correlation between acyl chain length

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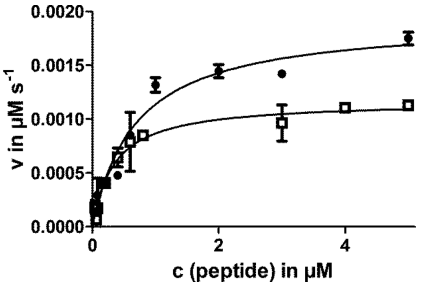

Figure 3. Michaelis–Menten plot for Sirt5 mediated deacylation of **5a** (●) and **5c** (□). Fluorescence was measured in 96-well microtiter plates. Solid lines represent fit to Michaelis–Menten equation. All reactions were done in duplicate.

Table 2. Kinetic Constants for 5a, 5c, and NAD$^+$ Measured with Sirt5

	K_M [μM]	k_{cat} [10^{-3} s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]
5a	0.69 ± 0.1[a]	200 ± 10[a]	290000[a]
	0.9 ± 0.2[b]	103 ± 34[b]	114000[b]
NAD$^+$[c]	26 ± 6[a]	91 ± 7[a]	3400[a]
5c	0.35 ± 0.07[a]	117 ± 7[a]	337000[a]

[a]Determined using 96-well microtiter plate reader. [b]Determined by HPLC. [c]Kinetic values for NAD$^+$ were measured with saturated substrate **5a**.

and NAD$^+$ K_M values for Sirt1–3 and Sirt6 and suggested that sirtuin-mediated removal of a long-chain acyl modification is not influenced by NAD$^+$ levels within a cell.[38] A similar relationship could be applicable to Sirt5 while using succinylated substrates.

Sirt5 was shown to be an efficient deglutarylase *in vitro* and *in vivo*.[8,22] Therefore, we combined this acyl moiety with our assay principle leading to **5c**. The kinetic efficiency of **5c** is 1.2-times higher than that for **5a** (Table 2). Compared with recently published FRET-based Sirt6 and Sirt5 substrates, which were used in a discontinuous assay approach,[39] **5a** and **5c** show improved K_M (up to 25-fold) as well as k_{cat} values (>60-fold) resulting in dramatically improved specificity constants. This enables for the first time activity measurements at low enzyme concentrations in very short time. Additionally, in both cases of the published FRET-based sirtuin assays,[39] the aspartic acid residue in the +1 position leads to suboptimal substrate properties for the helper protease resulting in high amounts of protease. This high protease concentration prevents continuous measurements.

We tested the performance of this novel Sirt5 assay in parallel, higher-throughput setups by determining K_i values for inhibitors. It was shown that Sirt5 is insensitive to nicotinamide (NAM) inhibition if analyzed with an acetylated substrate but is inhibited by NAM with a succinylated peptide as substrate.[40] Nevertheless, there are no K_i values published for NAM. We determined the inhibition constant for NAM against peptide substrate **5a** and NAD$^+$ analyzing kinetic constants in the presence of different inhibitor concentrations ranging between 0 to 200 μM. For peptide **5a**, V_{max} values decrease with inhibitor concentration, whereas K_M values are similar (Figure 4). These results indicate a noncompetitive type of inhibition. Linear fitting of the V_{max}^{-1} against inhibitor concentration plot leads to a K_i value of 15 ± 1.5 μM as an x-axis intercept (Figure S7), which is comparable to published IC_{50} values.[40] In contrast, NAM inhibition against NAD$^+$ showed a competitive

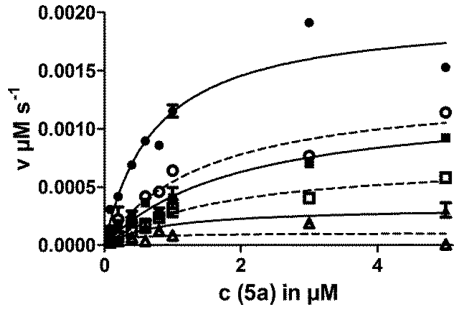

Figure 4. Michaelis–Menten plot for the inhibition by nicotinamide. Reactions were performed with varying concentrations of **5a**, 0–200 μM NAM, and saturating NAD$^+$. Fluorescence was measured using microtiter plate reader. All reactions were done in duplicate. NAM concentrations: (●) 0 μM; (○) 25 μM, (■) 50 μM; (□) 100 μM; (▲) 150 μM, (△) 200 μM.

behavior with a K_i value of 3.7 ± 0.3 μM. This value was received from a plot of K_M value versus inhibitor concentration, where also the x-axis intercept represents the negative K_i value (Figure S7). Generally, the determined K_i values for NAM and Sirt5 are relatively low compared with other sirtuin isoforms. There was no effect of NAM on the helper enzyme trypsin ruling out that inhibition of the protease generates false good K_i values.

Replacement of the acyl oxygen atom by a sulfur transforms sirtuin substrates into inhibitors by the formation of a "stalled" intermediate with ADP-ribose, which resembles a bisubstrate inhibitor.[41,42] Previously it was shown that peptides containing thiosuccinylated lysines are specific Sirt5 inhibitors.[43,44] This prompted us to synthesize CPS1 derivatives with either thioacetyl (**7**) or thiosuccinyl (**6**) moieties at the lysine side chain. The K_i values for peptide derivatives **6** and **7** were measured using **5a** as a substrate. As shown in Figure S8, **7** is not able to inhibit Sirt5 up to 150 μM (K_i value 666 μM), whereas **6** represent a strong and potent Sirt5 inhibitor with a K_i value of 1.2 μM, demonstrating again the strong Sirt5 preference for succinylated over acetylated acyl residues.

Recently Li et al. showed that Sirt1 can be activated by treatment with 500 μM leucine,[45] which is responsible for the prevention of metabolic diseases in high-fat diet-induced obese mice. To analyze whether Sirt5 might also be amenable to activation by leucine, we treated Sirt5 with 0–500 μM leucine and followed the desuccinylation of **5a**. There is no significant influence of leucine on the Sirt5 reaction visible (Figure S9).

Encouraged by the extraordinary catalytic efficiencies of **5a** and **5c** for Sirt5, we wondered whether this peptide sequence would yield similar results for other sirtuin isoforms and thus allow comparable assays, with sensitive and continuous detection. Therefore, we synthesized an acetylated version of our substrate peptide (**5d**). Peptide **5d** is a substrate for Sirt1, Sirt2, and Sirt3 but not a substrate for Sirt4, Sirt6, and Sirt7. The stability of Sirt1, Sirt2, and Sirt3 against trypsin was analyzed in a similar manner as for Sirt5. Again Sirt2 and Sirt3 form stable fragments for at least 30 min (Figure S12b,c), which are catalytically active (Figure S10), but Sirt1 was immediately cleaved by trpysin. Determination of the kinetic constants showed similar values for Sirt2 compared with Sirt5 (Table 3, Figure S11), and an about 100-fold lower catalytic efficiency for Sirt3. Nevertheless, the substrate is more efficiently recognized than the widely used Fluor-de-Lys substrate by Sirt2 and also Sirt3, indicated by K_M values for

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Table 3. Kinetic Constants for Sirt2 and Sirt3 with 5d

	K_M [μM]	k_{cat} [10^{-3} s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]
Sirt2	3 ± 0.5	130 ± 7	43000
Sirt3	4.1 ± 0.7	12 ± 0.7	3000

5d that are 60-fold (Sirt2) and 8-fold (Sirt3) lower compared with the FdL substrate.[46] These results demonstrate that our improved, sensitive, and continuous Sirt5 assay is also applicable to Sirt2 and Sirt3 and enables simple, continuous, and sensitive measurement of their activity. These assays now enable the efficient and accurate characterization of larger sets of sirtuins and their modulators as required, for example, in drug development campaigns.

CONCLUSION

In summary, we created a continuous assay using internally fluorescence-quenched peptides **5a** and **5c**, which are highly specific and efficient substrates for Sirt5. The catalytic efficiencies of the developed Sirt5 substrates are several hundred-fold higher compared with the commercially available Sirt5 substrate (Table 1), and the specificity constant for **5c** represents the best substrate described for a sirtuin so far. This high specificity constant makes it possible to use sirtuin concentrations down to 10 nM saving both enzyme and assay time and allowing reliable kinetics and inhibition studies even for tight binding ligands. The assay principle could be transferred to following Sirt2 and Sirt3 activity by changing the acyl moiety at the lysine side chain to acetyl (**5d**). The sensitivity of the substrates **5a**, **5c**, and **5d** enables effective screenings for novel effectors of sirtuin activity in microtiter plate format in a continuous manner. No complex liquid handling steps are needed for stopping reactions. They thus enable a simple and robust setup for screening and analyzing of modulators for sirtuins, a highly interesting target family for which currently only few specific modulators are available.

EXPERIMENTAL SECTION

Chemicals and General Methods. All chemicals were purchased from Sigma-Aldrich if not stated otherwise. For HPLC measurements, 0.1% TFA in H_2O (solvent A) and 0.1% TFA in ACN (solvent B) were used. Compounds were detected at 260 nm. An Agilent 1100 HPLC was used for analytical analysis with a quaternary pump, a well-plate autosampler, and a variable wavelength detector. Separations were performed on a Phenomenex Kinetex XB C-18 (2.6 μm, 3.0 mm × 50 mm) column with a linear gradient from 25% to 55% solvent B in 4 min (flow rate: 0.6 mL min^{-1}). For purification of peptides, a Merck Hitachi Speed LC system with a Phenomenex Kinetex 5 μM XB-C18 column (100 Å, 21.2 mm × 250 mm) was used. Separations were performed with a linear gradient from 20% to 50% solvent B in 60 min (8 mL min^{-1}). Identity of eluted compounds was analyzed by MALDI mass spectrometry on Bruker Ultraflex-II-TOF/TOF with MALDI source. NMR spectroscopy was carried out using Varian Gemini 2000 spectrometer in deuterated chloroform.

Solid Phase Peptide Synthesis. All peptides were synthesized using standard Fmoc chemistry on a MBHA Rink amide resin (IRIS Biotech). Fmoc-Lys(Nosyl)-OH was used as an orthogonally protected building block allowing selective on-resin modification of the lysine side chain. Resin was treated with DMF for 10 min. Fmoc deprotection was performed for 15 min with 20% piperidine in DMF followed by washings with DMF (5 min, 5 times). Amino acid derivatives (4 equiv, Merck) were activated with 4 equiv of HBTU and 8 equiv of DIPEA in DMF. Finally, the amino group of glycine was modified with 4 equiv of benzoic acid (**1**, **3**, **6**, and **7**) and 8 equiv of DIPEA in DMF or 4 equiv of 2-aminobenzoic acid, 4 equiv of HBTU, and 8 equiv of DIPEA in DMF (**2**, **4a**,**b**, and **5a**−**d**). Lysine protection group Nosyl (2-nitrobenzenesulfonyl) was removed using thiophenol and DBU in DMF. The free lysine side chain was modified with 4 equiv of succinic anhydride (**1**−**3**, **4a**, and **5a**), glutaric acid (**5c**), or acetic anhydride (**5d**) and 8 equiv of DIPEA in DMF. For compounds **6** and **7**, methyl-dithiomethyl-succinate and ethyl-dithioacetate, respectively, were used for thioacylation of deprotected lysine side chains. After washing with DCM, methanol, and again DCM (3 min, 5 times each), peptides were cleaved from resin with 98% TFA (Roth) and precipitated with cold diethyl ether. After purification by reversed phase HPLC, purity and identity were checked with analytical HPLC and MALDI-MS (purity >95%). ^{13}C NMR of **6** (400 MHz, d_6-DMSO) δ ppm: 202.32, 174.39, 173.90, 173.24, 172.45, 171.81, 171.33, 169.71, 168.96, 167.06, 156.22, 134.33, 131.84, 130.50, 128.78, 128.02, 127.68, 115.31, 58.14, 57.85, 54.80, 52.83, 52.27, 51.69, 45.63, 43.20, 42.56, 33.32, 31.67, 31.07, 30.78, 30.51, 27.86, 27.18, 24.63, 23.33, 22.01, 19.66, 18.33. ^{13}C NMR of **7** (400 MHz, d_6-DMSO) δ ppm: 199.20, 174.39, 173.22, 172.43, 171.80, 171.33, 169.70, 168.96, 167.05, 156.22, 134.34, 131.84, 130.50, 128.77, 128.02, 127.68, 115.31, 58.13, 57.84, 54.79, 52.28, 51.67, 45.81, 43.20, 42.55, 37.21, 33.27, 31.69, 31.07, 30.78, 30.51, 27.86, 27.23, 24.63, 23.34, 22.01, 19.67, 18.33.

HPLC Activity Assay. Deacylation of CPS1 peptides was performed at 37 °C in 20 mM TRIS buffer, pH 7.8, containing 150 mM NaCl, 5 mM $MgCl_2$, and 5% DMSO (assay buffer). Reaction mixtures consist of 500 μM NAD^+, 0.5−10 μM peptide, and 0.01 μM Sirt5. After several time points, reaction was stopped with 10% TFA (v/v), and samples were measured by HPLC. Stability of Sirt2, Sirt3, and Sirt5 was determined by incubating 0.01 μM sirtuin with 0.01 mg mL^{-1} trypsin at 37 °C followed by activity measurements after different pretreatment times with 5 μM **5a** or **5d** in the presence of 500 μM NAD^+. All measurements were done in duplicate.

Fluorescence Activity Assay. Reactions were measured in black low-binding 96-well microtiter plates (NUNC) and contained 500 μM NAD^+, 0.04−5 μM peptide, 0.01−0.1 μM sirtuin, and 0.01 mg mL^{-1} trypsin in assay buffer for the determination of kinetic values. Fluorescence was measured at an excitation wavelength of 320 nm and emission wavelength of 420 nm. The analysis of kinetic values for NAD^+ was performed equally. The reactions contained 10 μM **5a** and varying concentrations of NAD^+ (5−500 μM). Nicotinamide concentrations from 25−200 μM were used for determination of K_i value. The influence of leucine on sirtuin reaction was checked by end point measurements with 0−500 μM leucine, 500 μM NAD^+, 5 μM **5**, 0.01 μM Sirt5, and 0.01 mg mL^{-1} trypsin. Calibration curve was determined after overnight digestion of **5b** at concentration from 0.04 to 5 μM with trypsin. Optimal enzyme concentrations for the assay were obtained by changing trypsin concentration from 0.05 to 2 μM and Sirt5 concentrations from 0.01 to 2 μM and measuring activity with 500 μM NAD^+ and 50 μM **5a**.

Enzymes. Human Sirt2, Sirt3, and Sirt5 were expressed and purified as described by Gertz and co-workers.[32] Porcine trypsin was purchased from Sigma-Aldrich.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00293.

Additional figures and tables showing determination of kinetic constants, stability of sirtuins against trypsin, and NMR and MS spectra of synthesized peptides (PDF)

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Notes

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■ ABBREVIATIONS

Abz, 2-amino benzoic acid; ACS2, acetyl-coenzyme A synthetase 2; AMC, 7-amino-4-methylcoumarin; CE, capillary electrophoresis; CPS1, carbamoyl phosphate synthetase 1; DIPEA, *N*,*N*-diisopropylethylamine; FdL, Fluor-de-Lys; Fmoc, 9-fluorenylmethoxy carbonyl; GDH, glutamate dehydrogenase; Glut, glutaryl; H3, histone 3; H4, histone 4; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MBHA, 4-methylbenzylhydrylamine; NAM, nicotinamide; PTM, posttranslational modification; RFU, relative fluorescence unit; Sirt2, sirtuin 2; Sirt3, sirtuin 3; Sirt5, sirtuin 5; Succ, succinyl; TFA, trifluoroacetic acid; Thioac, thioacetyl; Thiosucc, thiosuccinyl; Y(NO_2), 3-nitro-tyrosine; Z, benzyl oxycarbonyl

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