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Substrates for Efficient Fluorometric Screening Employing the NAD-Dependent Sirtuin 5 Lysine Deacylase (KDAC) Enzyme

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ABSTRACT: The class III lysine deacylases (KDACs), also known as the sirtuins, have emerged as interesting drug targets for therapeutic intervention in a variety of diseases. To gain a deeper understanding of the processes affected by sirtuins, the development of selective small molecule modulators of individual isoforms has been a longstanding goal. Essential for the discovery of novel modulators, however, are good screening protocols and mechanistic insights with regard to the targets in question. We therefore evaluated the activities of the seven human sirtuin hydrolases against a panel of fluorogenic substrates. Both commonly used, commercially available substrates and novel chemotypes designed to address recent developments in the field of lysine post-translational modification were evaluated. Our investigations led to the discovery of two new fluorogenic ε-N-succinyllysine-containing substrates that enable highly efficient and enzyme-economical screening employing sirtuin 5 (SIRT5). Furthermore, optimized protocols for facile kinetic investigations were developed, which should be valuable for enzyme kinetic investigations. Finally, these protocols were applied to a kinetic analysis of the inhibition of SIRT5 by suramin, a potent sirtuin inhibitor previously shown by X-ray crystallography to bind the substrate pocket of the human SIRT5 KDAC enzyme.

INTRODUCTION

The sirtuins, silent information regulator 2 (Sir2) enzymes, are a family of NAD-dependent KDACs comprising seven human isoforms (SIRT1−7).1−4 These enzymes have been linked to a variety of phenotypes and are therefore considered targets for therapeutic intervention in a broad spectrum of diseases, including various cancers, diabetes, cardiovascular disease, and neurodegeneration.5−8 The human genome encodes for a total of 18 different KDACs, 11 of which are Zn²⁺-dependent isoforms (classes I, II, and IV), while the sirtuins constitute the class III KDACs (Figure 1a).1,2 Contrary to the Zn²⁺-dependent isoforms, the sirtuins utilize NAD as a cofactor in a deacylation event that has been studied mechanistically9 as well as supported by several X-ray cocrystal structures with substrates and/or cofactor bound to the enzyme10−12 (see Figure 1b for overall reaction). Only sirtuins 1−3 and 5 have been shown to harbor robust deacetylase activity, while sirtuins 6 and 7 were significantly less active and SIRT4 showed no activity.18,19 Sirtuin 4 has been shown to facilitate ADP-ribosylation20 and has therefore been suggested to be an ADP-ribosyl transferase enzyme rather than a deacetylase. Moreover, SIRT5 was recently shown to be a potent demalonylase and desuccinylase of lysine residues in mitochondrial proteins.21 The precise roles of the individual KDAC isoforms in cellular function are not yet fully understood, however, and new tools for accurate and efficient profiling of their enzymatic activity are therefore highly desirable. Furthermore, development of protocols for high-throughput screening efforts aimed at the discovery of new inhibitor candidates is an important objective.

In light of the recent discovery of novel post-translational acylations of lysine ε-amino groups,22 we envisioned that performance of a systematic screening of the specificities of the complete panel of recombinant human sirtuins 1−7 against a series of fluorogenic substrates accommodating variations in both peptide sequence and identity of the ε-N-acyl group might yield interesting new tools for some of these enzymes.

RESULTS AND DISCUSSION

Substrate Collection. For the profiling, we selected the collection of substrates shown in Scheme 1a. Fluorogenic peptide fragments 1−3 (based on p53 fragments), 5a (based on a histone 4 fragment), and 6a are commercially available substrates used for assays employing the various KDAC enzyme isoforms. Substrate 4 was designed based on part of the amino acid sequence of histone 3 (amino acids 6−9), and it was included in our series because of work demonstrating activity of SIRT6 against a H3K9Ac peptide in vitro23 (see Supporting Information Scheme S1 for synthetic route). Substrates 5b, 5c, 6b, and 6c were designed and included in our panel to address the recently discovered activities of SIRT5 against ε-N-succinyllysine (Ksuc) and ε-N-malonyllysine (Kma) containing substrates. Finally, we included a recently developed substrate for efficient profiling of class IIa KDACs (5d),24 as well as a crotonylated peptide (5e) to address the recent discovery of ε-N-crotonyllysine (Kcr) residues in histone tails.25

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The synthesis of substrate 5d has been reported previously,\(^2\) however, we were interested in devising an efficient strategy that would deliver 5a-e in a convergent manner from inexpensive starting materials. As several attempts to introduce the AMC fluorophore at a late-stage failed, we applied the protocol of Jung and co-workers\(^2\) followed by mild Fmoc removal to prepare intermediate 8 in high yield. Subsequent standard peptide coupling using DIC and HOBt as coupling reagents and Boc group removal furnished peptide 10, which could be readily functionalized to give substrates 5a-e (Scheme 1b). The intermediate 8 was also used for preparation of substrates 6b and 6c (Supporting Information Scheme S2).

Substrate Profiling with SIRT1–7. For comparison we first tested for deacetylation using 1 μg of enzyme per reaction. The deacetylations were allowed to proceed for 1 h before adding trypsin “developer” to release the fluorophore of deacetylated substrate molecules and thereby obtain end point assay readings (see Supporting Information Figure S1 for schematic representation of the assay). Not surprisingly, on the basis of the literature,\(^19\) SIRT1–3 generally exhibited good activities toward the acetylated (Kac) substrates, whereas no measurable activities were observed for sirtuins 4, 6, and 7 (Figure 2). Sirtuin 5 exhibited poor activity against the Kac substrates in our collection, with only a poor signal-to-background ratio observed against substrate 2, which is somewhat surprising because SIRT5 has been classified as an active deacetylase enzyme.\(^19\) This, however, agrees with more recent investigations reporting low affinity for Kac substrates with SIRT5.\(^2\)

On the other hand, SIRT5 exhibited very efficient desuccinylase activity with substrate 5b, which is also in agreement with recent contributions in the literature using nonfluorogenic substrates.\(^2\) Unfortunately, substrate 4, which was designed in the hopes of achieving activity of SIRT6, did not furnish a measurable signal under the applied conditions. This indicates that more elaborate H3K9Ac-containing fragments are necessary in order to obtain SIRT6 deacetylase activity, as the reported activity was observed with a ∼2700 Da H3 peptide.\(^2\) In addition, the reported activity was obtained with >4 times the amount of enzyme used in the present study, which would be considered a prohibitively large amount of enzyme for screening purposes.

The activities of sirtuins 1–3 and 5 were then measured against substrates 5a–c and 6a–c, which confirmed a selective demalonylase and desuccinylase activity of SIRT5, while SIRT1–3 showed deacetylase activity exclusively (Figure 3).

The deacetylation reactions catalyzed by SIRT1 were performed with two different commercially available preparations of the enzyme to test whether this would influence the substrate specificity. Thus, the six acetylated substrates were treated with full-length SIRT1 enzyme\(^2\) or a truncated SIRT1-GST fusion protein,\(^2\) respectively (Supporting Information Figure S2). Under the applied conditions, the efficacies of these two different enzymes followed the same general trend but with higher activities observed for the full-length enzyme except against substrate 4 where equally good activities were observed (Supporting Information Figure S2).

Finally, the activities of SIRT1–3, SIRT5, and SIRT6 against substrates 5d and 5e were recorded under the same conditions as described vide supra. The very low activities observed against substrate 5d are in agreement with previous findings.\(^30\)

Decrotonylase activity was investigated briefly using substrate 5e because of the very recent work showing that crotonylation of ε-amino groups of lysine residues should now be considered a possible post-translational modification in histone proteins.\(^25\)

A weak signal was observed for SIRT1, which may warrant further investigations with more elaborate substrates in the future, while a lack of activity was observed for sirtuins 2, 3, 5, and 6 (Supporting Information Figure S3). As such, our results do not indicate that sirtuins harbor potent decrotonylase activity, albeit based on a limited data set.

Optimization of SIRT5 Desuccinylase Assays. Because of the high efficacy of SIRT5 observed against succinylated substrates 5b and 6b (Figure 3), we envisioned that these substrates might potentially prove useful for efficient screening of putative inhibitor compounds and decided to address these substrates in further detail.\(^3\) First, we performed titrations to determine appropriate amounts of enzyme necessary for SIRT5 screening with 5b or 6b, which showed that an enzyme loading as low as 50 ng of SIRT5 per reaction (30 nM) was sufficient for end point readings with both substrates (Supporting Information Figure S4). Then kinetic constants were determined by measuring initial rate velocities as a function of substrate concentration and fitting the data to the Michaelis–Menten equation to give the \(K_m\) and \(V_{max}\) values as shown in Table 1. The \(k_{cat}\) values were calculated from the relationship \(k_{cat} = \frac{V_{max}}{[enzyme]}\), based on enzyme stock concentration and purity given by the enzyme suppliers.

The turnover numbers \(k_{cat}\) and catalytic efficiencies \(k_{cat}/K_m^{-1}\) obtained for both of these substrates were somewhat lower than those reported for longer non-fluorophore-containing peptide sequences (about 5- to 50-fold).\(^2\) The detection of a released fluorophore, however, enables significantly higher signal sensitivity compared to HPLC-based screening methods, thus enabling the low enzyme loading of 30 nM, as compared to the 1 μM recently reported for an HPLC-based assay.\(^7\) The fluorometric method furthermore allows for easy performance of continuous rate experiments as long as an appropriate secondary enzyme is chosen. The secondary enzyme in the assay, which serves to
“Reagents and conditions: (a) POCl₃ (3 equiv), pyridine (10.6 equiv), 7-amino-4-methylcoumarin (AMC, 1.15 equiv), THF, 0 °C → rt, 2.5 h (quant). (b) Piperidine (1.1 equiv), MeCN, CH₂Cl₂, 0 °C → rt, 24 h (88%). (c) Ac-Leu-Gly-OH (1.02 equiv), diisopropylcarbodiimide (DIC, 1.1 equiv), hydroxybenzotriazole (1.1 equiv), CH₂Cl₂, rt, 50 min (80%). (d) Trifluoroacetic acid-CH₂Cl₂ (1:9, v/v), rt, 30 min. (e) Ac₂O (1.5 equiv), i-Pr₂NEt (6 equiv), CH₂Cl₂, rt, 1 h (34%, two steps). (f) Succinic anhydride (1.7 equiv), i-Pr₂NET (6 equiv), CH₂Cl₂, rt, 55 min (70%, 2 steps). (g) β-Potassium methyl malonate (2 equiv), i-Pr₂NET (2.5 equiv), HATU (2 equiv), MeCN, 0 °C, 20 min; (2) LiOH (7.3 equiv), THF–H₂O, rt, 15 min (49%, 3 steps from 9). (h) Trifluoroacetic anhydride (9 equiv), i-Pr₂NET (14 equiv), CH₂Cl₂, 0 °C → rt, 22 h (60% from 9). (i) trans-Crotonic anhydride (1.8 equiv), i-Pr₂NET (6 equiv), CH₂Cl₂, rt, 10 min (59% from 9).

**Figure 2.** Results of sirtuin deacetylation and desuccinylation of substrate 5b. The data were obtained as end point readings after incubation for 1 h with each substrate at 37 °C and represent at least two individual experiments performed in duplicate. The amount of recombinant enzyme added to each reaction was 1 μg/50 μL as determined from stock solution concentrations and purities given by the suppliers (SIRT1-GST fusion protein was used as SIRT1 source). The RFU values are normalized to control wells without sirtuin enzyme present. See Supporting Information Figures S2 and S3 for additional profiling data.

**Figure 3.** Bar graph for deacylation of substrates 5a–c and 6a–c. The data were obtained as end point readings after incubation for 1 h with each substrate at 37 °C and represent at least two individual experiments performed in duplicate.

**Table 1.** Kinetic Parameters for SIRT5 Desuccinylation of Fluorogenic Substrates 5b and 6b

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μM s⁻¹)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5b</td>
<td>33 ± 1.8</td>
<td>1.9 × 10⁻⁴</td>
<td>3 × 10⁻⁴</td>
<td>9.2 × 10²</td>
</tr>
<tr>
<td>6b</td>
<td>84 ± 22</td>
<td>5.6 × 10⁻⁴</td>
<td>9 × 10⁻⁴</td>
<td>1.1 × 10²</td>
</tr>
</tbody>
</table>

$^a$The $K_m$ value for NAD measured with substrate 6b at 200 μM was 150 ± 45 μM. Michaelis–Menten plot is shown in Figure S5a.
instantaneously process the intermediate product to give a readout, and second, it is essential that the sirtuin enzyme will not be degraded under the assay conditions because of the inherent peptidase activity of the developer.

For the present protocol, we considered the Lys-C protease, which has been used as a mild nondegrading option in HDAC experiments, and trypsin, which is known from end point assays to be very potent. Initial rate experiments for Lys-C at 4 milliunits of enzyme per reaction revealed a $V_{\text{max}}$ that was considerably higher than those of SIRT5 against 5b and 6b, indicating that Lys-C could be a useful option (Supporting Information Figure S6). With trypsin our concern was the possibility of premature inactivation of SIRT5 due to degradation. At a final concentration of 10 μg/mL of trypsin in the assay, however, the rate increase in fluorescence signal was stable for recordings up to one hour (Supporting Information Figure S7). Protocols were thus established for end point assays, where interference of the developer enzyme is absent, as well as for continuous assays employing two different in situ developer enzymes.

**Evaluation of Inhibitors.** Though substrate 6b furnished slightly lower catalytic efficacy than 5b, its simple chemical structure and hence easy accessibility was appealing, and it was therefore decided to perform inhibition experiments using substrate 6b. Initially, IC$_{50}$ values were determined by nonlinear regression of dose--response data obtained by end point assays. The selected sirtuin inhibitors were the SIRT1 inhibitor sirtinol, the SIRT2 inhibitor AGK2, and suramin, a drug used to treat sleeping sickness. The IC$_{50}$ values were all in agreement with previously published data obtained using an HPLC-based assay (Figure 4a, top).

In an attempt to achieve binding affinity by interaction of the active site arginine (Arg-105, Figure 4b) with a carboxylic or hydroxamic acid functionality, three known HDAC inhibitors that are all in clinical use were tested as well (Figure 4a, bottom panel). This proved fruitless with the simple structures tested herein, although evidence that a carboxylic acid moiety may contribute to the binding affinity of SIRT5 inhibitors was recently described for ε-$N$-thiosuccinyllysine-containing inhibitors, which were designed by analogy to ε-$N$-thioacetyllysine-containing SIRT1 inhibitors.

Because of previously reported assay artifacts related to fluorophore-containing substrates, which has come under scrutiny with respect to activation of SIRT1, we performed kinetic inhibition experiments using the continuous assay methods discussed vide supra to further validate the developed substrates and protocols. We chose the commercially available inhibitor suramin, which conveniently has been cocrystallized with SIRT5, for our kinetic studies.

First, we measured steady-state velocities from a titration of substrate 6b at saturating NAD concentration and varying concentrations of suramin to give a secondary double reciprocal plot (Lineweaver–Burk plot) as shown in Supporting Information Figure S8. Intersection of the lines with the y-
Using the progression curve method, K.22 Convergent synthetic routes for preparation of these furnished similar Kcr.22

Development allowed for the system to reach steady state after a lag time of almost 30 min before the system reached steady state. On the other hand, the resulting progress curves remained linear up to 2 h (Figure 5a). Trypsin reached a steady state. When using Lys-C as the coupled secondary enzyme, we observed a lag time of almost 30 min before the system reached a steady state. However, the resulting progress curves remained linear up to at least 50 min (Figure 5b). Linear graphs in these plots represent a constant deamalonylase and desuccinylase activities as recently described for nonfluorogenic peptide substrates.

In summary, we have evaluated the activities of the seven human sirtuin hydrolases against a series of fluorogenic substrates in vitro. The tested substrate collection was designed to address enzyme specificity toward the most commonly used commercial substrates as well as the recently discovered lysine post-translational modifications (i.e., Kma, Ksuc, and Kcr).22 Convergent synthetic routes for preparation of these new chemotypes were devised to gain easy access to the substrate collection.

Our screening confirmed that SIRT1–3 all exhibit relatively broad-spectrum deacetylase activities. Sirtuins 4, 6, and 7 were practically inactive under our conditions, which is in agreement with previous literature reporting lack of SIRT4 activity and significantly lower deacetylase activities of SIRT6 and SIRT7 compared to SIRT1–3.18 These findings, however, do not rule out that these enzymes may deacetylate substrates not yet discovered. Sirtuin 5, on the other hand, exhibited somewhat surprisingly low deacetylase activities but very potent demalonylase and desuccinylase activities as recently described for nonfluorogenic peptide substrates.

Thus, two new substrates were discovered, which enable highly efficient SIRT5 desuccinylase activity at significantly lower enzyme loading than previously described. As such, these readily prepared substrates should be suitable for screening efforts toward the discovery of novel SIRT5 inhibitor compounds. Furthermore, optimized conditions for facile kinetic investigations of SIRT5 interaction with substrates and/or inhibitors were developed, which will be valuable for mechanistic investigations. The well-known inhibitor suramin, which has been cocryrstallized with SIRT5, was used to validate our two different continuous assay protocols by performing progression curve experiments.

Additionally, the results of the first deconylation experiments performed with sirtuin enzymes indicated that sirtuins are not likely to be potent deconylases.

**EXPERIMENTAL METHODS**

**Materials.** Recombinant SIRT1-GST fusion protein, SIRT2, and Lys-C (Lysobacter enzymogenes) were purchased from Merck-Millipore (Darmstadt, Germany), and SIRT1 (full length His-tagged), SIRT3, SIRT5, and SIRT6 were from Enzo Life Sciences (Postfach, Switzerland). Recombinant SIRT4 and SIRT7 were from Abnova (Taipei, Taiwan). Sirtuin assay buffer was prepared as described in the Biomed International product sheets BML-AK-555 (Tris-Cl (50 mM), NaCl (137 μM), KCl (2.7 μM), MgCl2 (1 μM), pH 8.0, and bovine serum albumin (1 mg/mL)). The SIRT2 substrate (3), suramin, sirtinol, and AGK2 inhibitors were from Enzo Life Sciences (Postfach, Switzerland). 4-Phenylbutyric acid, valproic acid, NAD, and trypsin (10 000 units/mg, TPCK treated from bovine pancreas) were from Sigma-Aldrich (Steinheim, Germany).

**Sirtuin Deacylation Assays.** In brief, all reactions were performed in black low binding NUNC 96-well microtiter plates. End point assays were performed by incubation of the appropriate substrate, NAD, sirtuin, and inhibitor (if applicable) in assay buffer (50 μL, final volume). Control wells without enzyme were included in each plate. After 1 h at 37 °C, a developer mixture (50 μL) containing nicotinamide (2 mM) and trypsin (0.4 mg mL⁻¹) was added, and the assay was incubated for 15–30 min at room temperature before the
plates were analyzed using a Perkin-Elmer Enspire plate reader with excitation at 360 nm and emission at 460 nm. Rate experiments for determination of kinetic parameters were performed by incubation of substrate, NAD, and sirtuin in assay buffer (100 μL final volume) with in situ fluorophore cleavage by Lys-C or trypsin. Fluorescence readings were recorded every 30 s for at least 30 min at 25 °C to obtain initial linear rates (RFU min⁻¹) for each concentration. The data were analyzed using GraphPad Prism to afford $V_\text{max}$, $K_m$ (RFU min⁻¹) values. The $V_\text{max}$ values were calculated based on the [7-amino-4-methylcoumarin]–RFU standard curve (Supporting Information Figure S5) and the enzyme purities and concentrations given by the suppliers.

Chemistry. General Methods and Materials. 2-Chlorotrityl chloride resin was from Sigma-Aldrich (Milwaukee, WI). All α-amino acids, chemicals, and solvents were analytical grade and used without further purification as obtained from commercial suppliers. Anhydrous THF was stored over sodium and freshly distilled prior to use. Anhydrous MeCN was dried by storage over 3 Å molecular sieves. Anhydrous CH2Cl2, i-Pr2NET, and pyridine were dried by storage over 4 Å molecular sieves. Dry column vacuum chromatography was performed on silica gel 60 (particle size 0.045–0.35 mm) and commercial plates were analyzed using a Perkin-Elmer Enspire plate reader with excitation at 360 nm and emission at 460 nm. Rate experiments for determination of kinetic parameters were performed by incubation of substrate, NAD, and sirtuin in assay buffer (100 μL final volume) with in situ fluorophore cleavage by Lys-C or trypsin. Fluorescence readings were recorded every 30 s for at least 30 min at 25 °C to obtain initial linear rates (RFU min⁻¹) for each concentration. The data were analyzed using GraphPad Prism to afford $V_\text{max}$, $K_m$ (RFU min⁻¹) values. The $V_\text{max}$ values were calculated based on the [7-amino-4-methylcoumarin]–RFU standard curve (Supporting Information Figure S5) and the enzyme purities and concentrations given by the suppliers.

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Chemistry. General Methods and Materials. 2-Chlorotrityl chloride resin was from Sigma-Aldrich (Milwaukee, WI). All α-amino acids, chemicals, and solvents were analytical grade and used without further purification as obtained from commercial suppliers. Anhydrous THF was stored over sodium and freshly distilled prior to use. Anhydrous MeCN was dried by storage over 3 Å molecular sieves. Anhydrous CH2Cl2, i-Pr2NET, and pyridine were dried by storage over 4 Å molecular sieves. Dry column vacuum chromatography was performed on silica gel 60 (particle size 0.045–0.35 mm) and commercial plates were analyzed using a Perkin-Elmer Enspire plate reader with excitation at 360 nm and emission at 460 nm. Rate experiments for determination of kinetic parameters were performed by incubation of substrate, NAD, and sirtuin in assay buffer (100 μL final volume) with in situ fluorophore cleavage by Lys-C or trypsin. Fluorescence readings were recorded every 30 s for at least 30 min at 25 °C to obtain initial linear rates (RFU min⁻¹) for each concentration. The data were analyzed using GraphPad Prism to afford $V_\text{max}$, $K_m$ (RFU min⁻¹) values. The $V_\text{max}$ values were calculated based on the [7-amino-4-methylcoumarin]–RFU standard curve (Supporting Information Figure S5) and the enzyme purities and concentrations given by the suppliers.
NH₂), 8.11 (d, J = 7.2, 1H, NH₅), 7.99—8.08 (m, 2H, NH₅ and NH₆), 7.80 (d, J = 1.9, 1H, H₈M), 7.72 (d, J = 8.7, 1H, H₉MAC), 7.52 (dd, J = 8.7 and 2.0, 1H, H₆MAC), 6.27 (d, J = 1.2, 1H, H₃MAC), 4.32—4.43 (m, 1H, H₃MAC), 4.22 (q, J = 7.4, 1H, H₄LAM), 3.74 (marm3)], J = 16.6 and 5.7, 1H, H₈Gly), 3.71 (marm3)], J = 16.6 and 5.6, 1H, H₆Gly), 3.10 (s, 2H, CH₂(CO)), 3.04 (q, J = 6.5, 2H, CH₂Gly), 2.39 (dd, J = 10.6 and 1.2, 3H, CH₃Gly), 1.45—1.79 (m, 6H, CH₃LAM and CH₂LAM), 1.19—1.51 (m, 6H, CH₂LAM and CH₂LAM), 0.88 (d, J = 6.5, 3H, CH₃LAM), 0.83 (d, J = 6.4, 3H, CH₃LAM). ¹³C NMR (DMSO-d₆) δ = 173.0 (COO⁻), 171.5 (COO⁻), 169.8 (CH.CO), 169.1 (COO⁻), 165.6 (CONH), 161.0 (C₃AMC), 153.7 (C₈AMC), 153.1 (C₇AMC), 144.2 (C₇AMC), 126.0 (C₅AMC), 115.3 (C₉MAC), 115.2 (C₅AMC), 112.4 (C₇AMC), 105.8 (C₆AMC), 53.6 (C₄bAMC), 51.5 (C₈bAMC), 42.6 (CH₂(CO)), 42.1 (C₄bAMC), 31.4 (C₄bAMC), 28.7 (C₈bAMC), 24.2 (C₉MAC), 23.0 (C₉MAC), 22.9 (C₆bAMC), 22.5 (CH₂CO), 21.7 (CH₂LAM), 18.0 (CH₃LAM). UPLC—MS tᵣ = 1.49 min, m/z 602.283 [(M + H)⁺, C₇H₅N₂O₆⁺] calc 602.281.

**Ac-Leu-Gly-Lys(Tfa)-(7-amino-4-methylcoumarin) (5d)**. Crude Ac-Leu-Gly-Lys(Tfa)-(7-amino-4-methylcoumarin) trifluoroacetate salt (10, 52 mg) was suspended in anhydrous CH₂Cl₂ (4 mL) and MeCN (3 mL) under N₂ at 0 °C. i-Pr₂NET (62 mg, 477 mmol) and (CF₃CO)₂O (127 mg, 0.61 mmol) were added over a time course of 20 h to drive the reaction to completion. Then the reaction mixture was taken up in aqueous HCl (1 M, 10 mL) and CH₂Cl₂ (20 mL). The aqueous phase was extracted with CH₂Cl₂ (3 × 20 mL), and the combined organic phase was washed with aqueous HCl (1 M, 10 mL), after which the aqueous phase was back-extracted with CH₂Cl₂ (3 × 20 mL). The organic phase was then washed with saturated aqueous NaHCO₃ (10 mL), dried over MgSO₄, and evaporated to dryness. The residue was purified by column chromatography (0—5% MeOH in CH₂Cl₂), affording the desired Ac-Leu-Gly-Lys(Tfa)-(7-amino-4-methylcoumarin) (5d) as a white solid (26 mg, 60% from 9). The ¹³C NMR data did not correspond fully with previously reported data, since we did not observe additional peaks attributed to a mixture of conformers. ¹¹H NMR (DMSO-d₆) δ = 10.39 (s, 1H, NH₅), 9.42 (t, J = 5.7, 1H, NHCOCF₃), 8.34 (t, J = 5.8, 1H, NH₆), 8.10 (d, J = 7.3, 1H, NH₉), 8.05 (d, J = 7.5, 1H, NH₅), 7.79 (d, J = 2.0, 1H, H₈M), 7.72 (d, J = 8.7, 1H, H₉M), 7.51 (dd, J = 8.7 and 2.0, 1H, H₆M), 6.27 (d, J = 1.4, 1H, H₃M), 4.32—4.44 (m, 4H, H₃M), 4.22 (q, J = 7.5, 1H, H₄LAM), 3.74 (marm3)], J = 16.8 and 5.4, 1H, H₈Gly), 3.71 (marm3)], J = 16.8 and 5.7, 1H, H₆Gly), 3.16 (q, J = 6.7, 2H, CH₂Gly), 2.40 (d, J = 1.4, 1H, CH₃Gly), 1.45—1.79 (m, 6H, CH₃LAM and CH₂LAM), 1.19—1.51 (m, 6H, CH₂LAM and CH₂LAM), 0.88 (d, J = 6.5, 3H, CH₃LAM), 0.83 (d, J = 6.4, 3H, CH₃LAM). ¹³C NMR (DMSO-d₆) δ = 172.9 (COO⁻), 171.4 (COO⁻), 169.7 (CH₂CO), 169.0 (COO⁻), 160.0 (C₃AMC), 156.1 (q, J = 35.9, COCF₃), 153.6 (C₈AMC), 153.1 (C₇AMC), 142.1 (C₆AMC), 126.0 (C₄AMC), 116.0 (q, J = 288.0, CF₃), 115.3 (C₉MAC), 115.2 (C₅AMC), 112.4 (C₇AMC), 105.8 (C₆AMC), 53.5 (C₄₅bAMC), 51.5 (C₈₅bAMC), 42.0 (C₇bAMC), 40.5 (C₆bAMC), 39.0 (C₅bAMC), 31.4 (C₄bAMC), 28.0 (C₉bAMC), 24.2 (C₉MAC), 22.9 (C₉MAC), 22.5 (CH₂CO), 21.7 (CH₂LAM), 18.0 (CH₃LAM). UPLC—MS tᵣ = 1.62 min, m/z 584.4 [(M + H)⁺, C₇H₅N₂O₄⁺] calc 584.3. HRMS m/z 584.3079 [(M + H)⁺, C₇H₅N₂O₄⁺] calc 584.3079.

**ASSOCIATED CONTENT**

### Supporting Information

Additional figures, additional experimental details, and compound characterization data, as well as ¹H, ¹³C, and ¹⁹F NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

ADPR, adenosine diphosphate ribose; AMC, 7-amino-4-
methylcoumarin; DIC, N,N'-diisopropylcarbodiimide; H₃, histone 3 protein; H₄, histone 4 protein; HATU, O-(7-azabenzoazol-1-yl)-N,N',N',N'-tetramethyluronium hexafluorophosphate; HDAC, histone deacetylase; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; h, hour; KDAC, lysine deacetylase; Kac, ε-N-acetyllysine; Ksc, ε-

succinyllysine; Kna, ε-N-malonyllysine; Ktfα, ε-N-trifluoroacetyl-
lysylne; Kcr, ε-N-crotonyllysine; Ma, malonyl; MS, mass spectrometry; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; RFU, relative fluorescence unit; rt, room temperature; SIRT, sirtuin; Suc, succinyl; TFA, trifluoro-
acetic acid; tᵣ, retention time; UPLC, ultrahigh performance liquid chromatography.
REFERENCES

(44) Tornheim, K. Kinetic applications using high substrate and competitive inhibitor concentrations to determine $K_{\text{m}}$ or $K_{\text{i}}$. Anal. Biochem. 1994, 221, 53−56.