Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

A Continuous, Fluorogenic Sirtuin 2 Deacylase Assay: Substrate Screening and Inhibitor Evaluation

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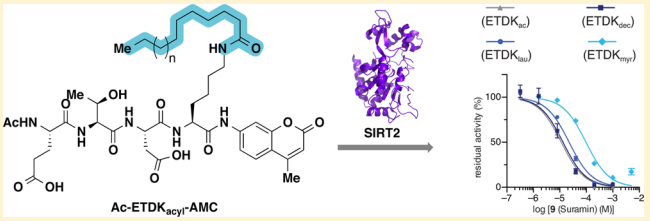
Supporting Information

ABSTRACT: Sirtuins are important regulators of lysine acylation, which is implicated in cellular metabolism and transcriptional control. This makes the sirtuin class of enzymes interesting targets for development of small molecule probes with pharmaceutical potential. To achieve detailed profiling and kinetic insight regarding sirtuin inhibitors, it is important to have access to efficient assays. In this work, we report readily synthesized fluorogenic substrates enabling enzyme-economical evaluation of SIRT2 inhibitors in a continuous assay format as well as evaluation of the properties of SIRT2 as a long chain deacylase enzyme. Novel enzymatic activities of SIRT2 were thus established in vitro, which warrant further investigation, and two known inhibitors, suramin and SirReal2, were profiled against substrates containing ε-*N*-acyllysine modifications of varying length.



INTRODUCTION

Humans have seven isoforms of the NAD$^+$-dependent sirtuin enzymes (SIRT1−7) that are homologous to yeast Sir2 (silent information regular 2).[1−3] Although these enzymes were originally classified as class-III histone deacetylases (HDACs), it has become evident that several members of this class should rather be denoted lysine deacylases (KDACs) as targeting of several ε-*N*-acyllysine posttranslational modifications (PTMs) have been discovered.[4−6] Thus, SIRT1−3 appear to be robust deacetylases but are not restricted to histone deacetylation as SIRT2 is primarily cytoplasmic and SIRT3 is mitochondrial.[2,7] Sirtuin 5 hydrolyzes ε-*N*-acyllysine modifications that are based on the dicarboxyl-derived groups malonyl (K_{mal}), succinyl (K_{suc}), and glutaryl (K_{glut}) modifications.[8−11] Sirtuin 6 is a histone deacetylase[12,13] but was also recently shown to demyristoylate TNF-α.[14] This led to a number of studies involving sirtuin-mediated hydrolysis of ε-*N*-acyllysine modifications of varying hydrocarbon length.[15−17] Interestingly, SIRT1−3 consistently exhibited potent activity against long chain acyl groups (e.g., C_8−C_{16}) in these studies, which may indicate that the physiologically relevant substrate space of these enzymes is broader than previously anticipated. Furthermore, a recent kinetic study revealed that NAD$^+$ dependence and inhibition of sirtuin deacylase activity by nicotinamide were dependent upon the identity of the acyl substrate.[18] Efficient assay platforms to assess inhibitors against both sirtuin deacetylase and long chain deacylase activity are therefore desirable.

In the present article, we describe the discovery of a highly efficient fluorogenic substrate for assessment of SIRT2 deacylase activity. We furthermore provide results of screening for deacylase activity against a series of acyl groups using this peptide sequence, and finally report evaluation of both inhibition of the deacetylase and longer chain deacylase activity of suramin as well as the recently reported SIRT2 inhibitor SirReal2.

RESULTS AND DISCUSSION

Preliminary Substrate Evaluation. We have previously reported efficient and enzyme-economical assay protocols for continuous assays employing SIRT5.[10] These were based on fluorogenic Ac-Leu-Gly-Lys-AMC peptide substrates [Ac-LGK-AMC (**1**); Figure 1a], which were applied in a trypsin-coupled assay format where release of the C-terminally linked 7-amino-4-methylcoumarin (AMC) fluorophore is monitored continuously. This design, however, was not directly transferable to substrates containing long chain acyl groups due to impaired aqueous solubility. Furthermore, known water-soluble sequences (QPKK (**2**) and TARK (**3**); Figure 1a), of which the former is commercially available in sirtuin deacetylase assay kits, proved incompatible with continuous assaying due to premature peptide cleavage by trypsin. Inspired by recent work demonstrating posttranslational acylation of K259 in dihydrolipoamide acetyltransferase [DLAT (**4**)],[19] we envisioned that the amino acids 256−259 of this protein could provide the desired solubility without encountering problems with trypsin cleavage. The deacylase activities of SIRT2 and SIRT6 were thus initially tested against substrates **1**, **2a**−**d**, **3a**−**d**, and **4a**−**d** (Figure 1b) since previous studies have shown

Received: October 1, 2015

DOI: 10.1021/acs.jmedchem.5b01532
J. Med. Chem. XXXX, XXX, XXX−XXX

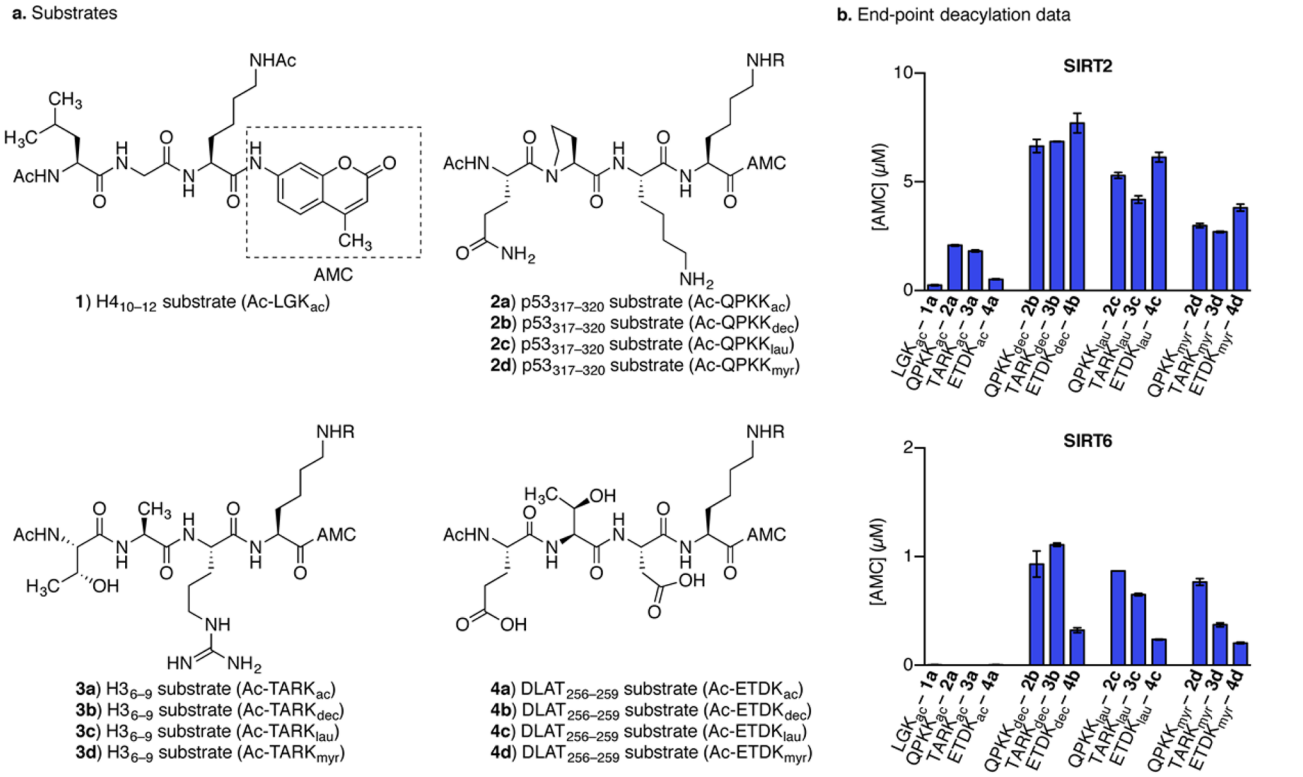


Figure 1. (a) Structures of substrates. (b) End-point deacylation data represented as conversion of substrate in micromolar based on a standard curve correlating relative fluorescence units (RFU) to concentration of AMC. The bar graphs represent conversion of substrate given in μM ± standard deviation based on at least two assays performed in duplicate.

high efficiency of SIRT2 against long chain ε-*N*-acyllysine substrates and that SIRT6 preferentially cleaves these modifications as well.[14,16]

Expectedly, SIRT6 consistently cleaved the long acyl chains (K_{dec}, K_{lau}, and K_{myr}) but not ε-*N*-acetyllysine (K_{ac}). The basic substrates (QPKK and TARK sequences) appeared to be more efficiently cleaved by SIRT6 than the acidic ETDK sequence. On the other hand, ETDK substrates containing long chains (**4b**–**d**) gave rise to the highest conversions exhibited by SIRT2 (Figure 1b). Interestingly, the opposite trend was observed for deacetylase activity of SIRT2, which was lower for the ETDK substrate (**4a**) compared to both QPKK (**2a**) and TARK (**3a**). This is in agreement with recent data indicating different kinetic behavior of the enzymes depending on substrate acyl group.[18] On the basis of these findings, the ETDK sequence was selected for further evaluation along two different avenues: (1) cleavage of potential ε-*N*-acyllysine modifications and (2) development of an efficient continuous SIRT2 deacylase assay.

Substrate Synthesis and Evaluation. It has been speculated that reactive acyl-CoA species may nonspecifically modify proteins without enzymatic catalysis.[20] With the high conversions recorded for deacylation applying the ETDK sequence, we therefore decided to apply this as a universal scaffold for screening of both long and short chain ε-*N*-acyllysine modifications inspired by acyl-CoA species from various biochemical pathways (Scheme 1). The substrates were prepared by acylation of a common precursor, compound **8**, prepared by coupling Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-OH to compound **6**, which was obtained employing a $POCl_3$-mediated AMC fluorophore coupling to the C-terminal of lysine (**5**).[10,21,22] In the substrate collection, it was decided to include acyl groups of varying length combined with differences in branching (**4e**,**f**) and oxidation state. Thus, α,β-unsaturated (**4h**–**j**), β-hydroxylated (**4k**–**m**), and β-keto (**4n**–**p**) functionalized substrates were included in the series (please consult the Supporting Information, Scheme S1 for syntheses of the carboxylic acids). Because of a combination of synthetic access as well as potential biological relevance, and because both stereoisomers exist as acyl-CoA species, it was decided to use the racemic β-hydroxy acids for the substrates in this initial screen (**4k**–**m**).

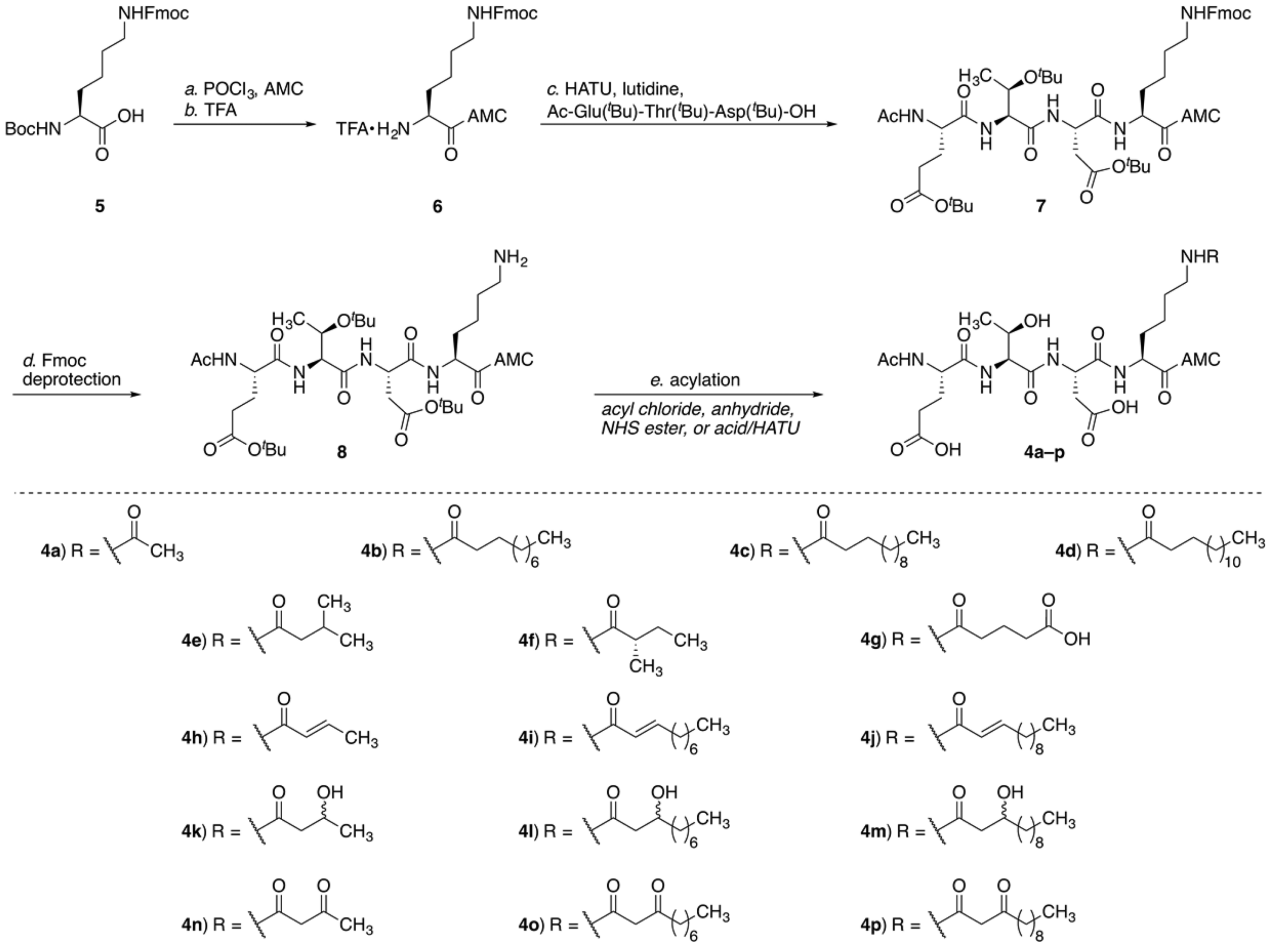
Compounds **4a**–**p** were then tested as substrates for sirtuins 1–3 and 6 (Figure 2). Sirtuins 1 and 3 exhibited insignificant cleavage of all substrates **4e**–**p**; however, this was also reflected in lower conversions of ETDK substrates **4a**–**d** compared to QPKK (**2a**–**d**) and TARK (**3a**–**d**) for both SIRT1 and SIRT3. Thus, the ETDK sequence appears to be particularly efficient for SIRT2-mediated long chain deacylation, whereas the QPKK and the TARK sequence were preferred for all deacylation reactions employing SIRT1, SIRT3, and SIRT6 (Figure 2).

Interestingly, SIRT2 was able to hydrolyze unsaturated (**4i**,**j**), β-hydroxylated (**4l**,**m**), and β-keto (**4o**,**p**) substrates with chain lengths of C10 and C12. For the unsaturated acyl groups (**4i**,**j**), the substrate conversion even rivaled that of SIRT2-mediated deacetylation of any tested peptide sequence (**1a**–**4a**). Whether this is just an in vitro finding associated with the efficient long chain deacylase activity of SIRT2 remains to be determined. Nevertheless, these observations are interesting and warrant further investigation of a possible physiological existence of proteins that are modified by unsaturated or oxidized acyl groups of varying lengths.

Optimization of Continuous SIRT2 Assay. To gain insight regarding the kinetics associated with SIRT2 cleavage of

DOI: 10.1021/acs.jmedchem.5b01532
J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 1. Substrate Synthesis and Structures of Acyl Modifications (4a–p)[a]



[a]Reagents and conditions: (a) $POCl_3$ (3.5 equiv), pyridine (10.3 equiv), 7-amino-4-methylcoumarin (AMC, 1.1 equiv), THF 0 °C → rt, 2 h (88%); (b) TFA–CH_2Cl_2 (15:85), 0 °C → rt, 3 h (94%); (c) Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-OH (0.95 equiv), HATU (1.05 equiv), lutidine (2.2 equiv), CH_2Cl_2, rt, 2 h (80%); (d) Fmoc deprotection with either (i) tris(2-aminoethyl)amine (20 equiv), CH_2Cl_2, rt, 210 min (**4a**) or (ii) piperidine (2 equiv), CH_2Cl_2–acetonitrile (20:15) 0 °C → rt, 14 h (**4b–p**); (e) (i) Acylation as outlined in the Experimental Section, (ii) CF_3COOH-CH_2Cl_2-H_2O (50:48:2).

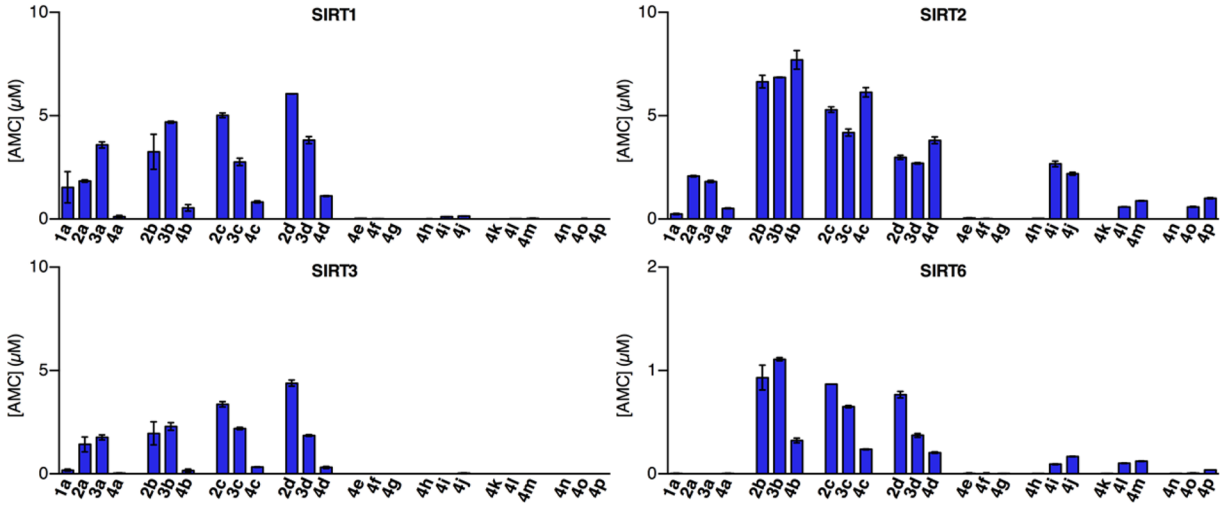


Figure 2. End-point deacylation data for incubation of substrates **4a–p** with sirtuins 1–3 and 6. The bar graphs represent conversion of substrate given in μM ± standard deviation based on at least two assays performed in duplicate.

substrates with varying acyl length, we detemined the rates of deacylation at varying substrate concentrations and fitted the data to the Michaelis–Menten equation. However, we found these experiments to be incompatible with the concentration of

DOI: 10.1021/acs.jmedchem.5b01532
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bovine serum albumin (BSA; 1 mg/mL) in the standard HDAC assay buffer used for the initial substrate screening, which might relate to sequestering of the lipidated substrates. Because SIRT2 is predominantly localized to the cytoplasm,[3] a lower pH would also be more physiologically relevant[23] and we therefore changed our buffer to one inspired by a previously applied HDAC assay buffer[24] (50 mM HEPES, pH 7.4, 0.05 mg/mL BSA, 100 mM KCl, 0.001% Tween-20, 200 μM TCEP). Gratifyingly, this buffer proved suitable for all substrates (**4a**–**d**), and we thus determined the rates of deacylation at varying substrate concentrations and fitted the data to the Michaelis–Menten equation. This was achieved employing an assay format where the secondary, fluorophore-releasing enzyme (trypsin) is included during the deacylation reaction. To avoid premature cleavage of the sirtuin during these assays, we therefore performed an optimization of SIRT2 to trypsin concentration enabling linear curves for the fluorophore release, which indicates steady-state kinetics. Employing these conditions, we then determined the Michaelis–Menten parameters (Figure 3 and Table 1).

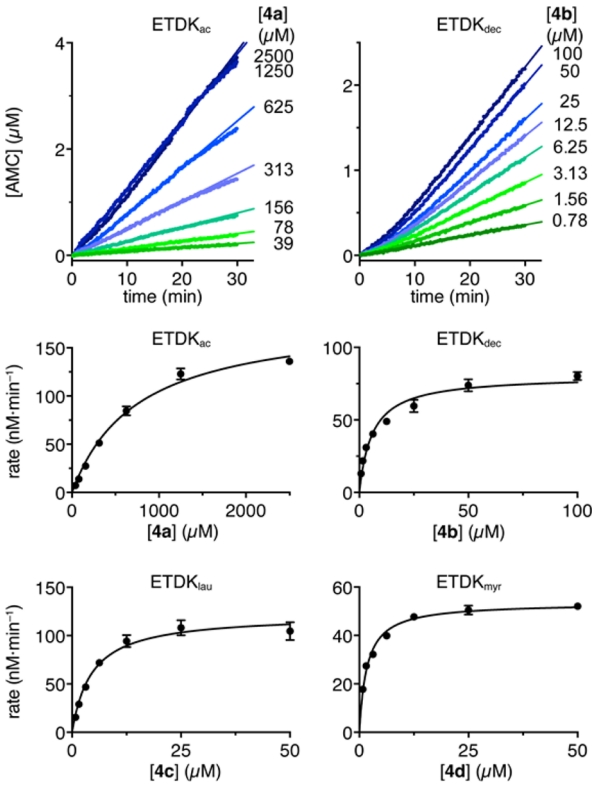

Figure 3. Progress curves for SIRT2-mediated deacylation of substrates **4a** and **4b** and Michaelis–Menten plots for SIRT2-dependent deacylation of substrates **4a**–**d** based on at least two assays performed in duplicate.

As previously reported using an LC-MS-based assay with longer peptide fragments,[17] the substrate affinity for SIRT2 was significantly higher for K_{myr} compared to K_{ac}, as indicated by 2 orders of magnitude difference in K_m values (Table 1).

Not surprisingly, the enzymatic efficiencies ($k_{cat} \times K_m^{-1}$) obtained in the current assay are lower than those reported with longer peptide fragments,[17] as also recently reported for SIRT5-based assays.[25] However, the protocol developed here is highly economical with respect to enzyme consumption and substrates are readily available in few synthetic steps. The differences in enzymatic efficiencies between substrates depended primarily on differences in K_m, but a 3–7-fold lower k_{cat} value for K_{ac} was also observed compared to the values for K_{dec}, K_{lau}, and K_{myr} (Table 1).

Investigation of the Inhibitory Effect of Suramin and SirReal2. With this new efficient and enzyme economical SIRT2 assay, we could then measure the potency of SIRT2 inhibitors against SIRT2-mediated hydrolysis of substrates with various lengths at the substrate K_m values, which would enable comparison of inhibitory activities between substrates. For these experiments, we chose suramin (**9**; Figure 4),[10,26,27] which is a well-known inhibitor of sirtuins 1, 2, and 5, together with the recently reported SIRT2 inhibitor, SirReal2 (**10**; Figure 4).[28]

For SirReal2 (**10**), limited solubility in the buffer prohibited full inhibition curves due to the need for large amounts of DMSO. The values recorded for inhibition at the highest concentration applied in this assay indicated somewhat lower potency than originally reported (0.4 μM),[28] which is not surprising because we used different substrates and applied them at their respective K_m values in the present study. Interestingly, however, these conditions enable comparison of inhibitor potencies against substrates with different chain lengths, which is relevant because nicotinamide inhibition of sirtuins was recently shown to vary in response to acyl chain length of the substrates.[18] For SirReal2, we observed similar inhibition of K_{ac}, K_{dec}, and K_{lau} deacylation but significantly lower effect against the demyristoylation activity (Figure 4 and Table 2). Suramin (**9**) was therefore included in the inhibitor evaluation, and the full dose–response experiments performed with this inhibitor recapitulated the substrate-specific inhibition indicated by the SirReal2 results (Figure 4 and Table 2). These initial observations regarding an acyl group preference of SIRT inhibitors are intriguing and the substrate design developed herein should enable further kinetic evaluation as well as effects on substrates containing additional types of acyl groups in future studies.

CONCLUSION

In the present study, we have identified a fluorophore-conjugated peptide sequence that enables continuous, trypsin-coupled assaying of SIRT2-mediated deacylase activity. Such

Table 1. Kinetic Parameters for SIRT2 Deacylation of Fluorogenic Substrates 4a–4d[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	$k_{cat}K_m^{-1}$ (s^{-1} M^{-1})
4a (ETDK$_{ac}$)	750 ± 90	(6.1 ± 0.1) × 10^{-3}	(8.1 ± 0.4) × 10^{1}
4b (ETDK$_{dec}$)	6.0 ± 0.8	(2.2 ± 0.1) × 10^{-2}	(3.8 ± 0.4) × 10^{3}
4c (ETDK$_{lau}$)	4.5 ± 0.6	(3.4 ± 0.3) × 10^{-2}	(7.5 ± 0.05) × 10^{3}
4d (ETDK$_{myr}$)	1.8 ± 0.1	(1.50 ± 0.05) × 10^{-2}	(8.5 ± 0.8) × 10^{3}

[a]The K_m value for NAD^+ measured with substrate **4d** at 20 μM concentration was determined to be 39 ± 2 μM. Michaelis–Menten plot is shown in Supporting Information, Figure S1.

DOI: 10.1021/acs.jmedchem.5b01532
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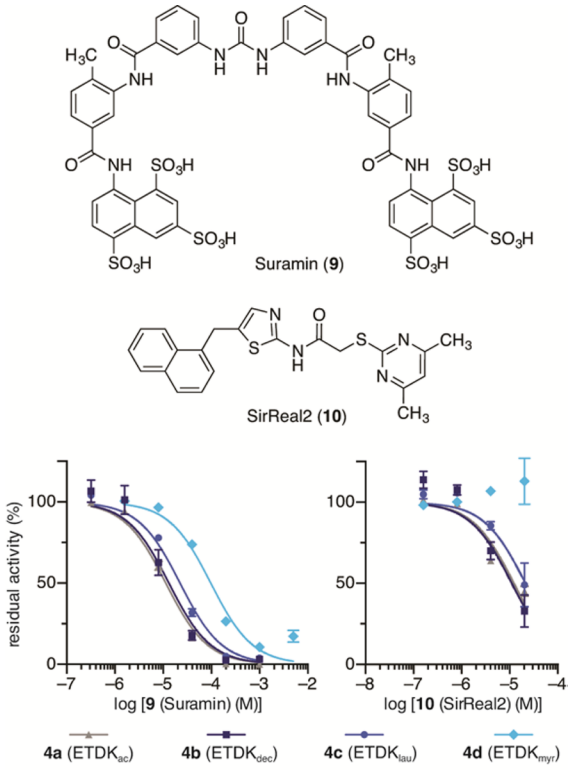

Figure 4. Structures of suramin (**9**) and SirReal2 (**10**) as well as dose–response curves for their inhibition of SIRT2-mediated deacylation of substrates **4a–c** (based on at least two individual assays performed in duplicate).

Table 2. Inhibition of SIRT2-Mediated Deacylation by Suramin and SirReal2[a]

substrate	Suramin (**9**) IC_{50} ± SD [K_i (μM)]	SirReal2 (**10**) inhibition at 20 μM (%)
4a (ETDK$_{ac}$)	11 ± 1 [5.7 ± 0.4]	55 ± 3
4b (ETDK$_{dec}$)	13 ± 1 [7 ± 2]	67 ± 10
4c (ETDK$_{lau}$)	22 ± 1 [11 ± 1]	51 ± 13
4d (ETDK$_{myr}$)	95 ± 1 [49 ± 3]	no inhibition

[a] K_i values were approximated from the IC_{50} values by using the Cheng–Prusoff equation [$K_i = IC_{50}/(1 + [\text{substrate}]/K_m)$].

substrates are useful for investigation of substrate acyl group specificity, which is an important research area in rapid development. A small collection of substrates containing unprecedented acyl groups that are potentially available through reaction of lysine side chains with the corresponding acyl-CoA species was synthesized, and new enzymatic activities of sirtuins were demonstrated in vitro using this collection. These findings now warrant further scrutiny to determine whether they are physiologically relevant. Furthermore, we established assay protocols for evaluation of sirtuin inhibitors in a continuous manner, which may reveal kinetic and mechanistic insight.

Whereas focus on activation of sirtuins has traditionally been dominating the drug discovery efforts in the field, the ability to inhibit these enzymes will be important to clarify the roles of sirtuins as either antitumor targets or tumor suppressors. Efficient assay protocols for screening regimes as well as kinetic investigations are therefore of pertinent interest and the results reported herein provide progress in this direction.

EXPERIMENTAL SECTION

General. All final substrates for biochemical investigation were purified by preparative reversed-phase HPLC on a C18 Phenomenex Luna column [250 mm × 20 mm, 5 μm, 100 Å] using an Agilent 1260 LC system equipped with a diode array UV detector and a gradient of eluent I (water–MeCN–TFA, 95:5:0.1) and eluent II (0.1% TFA in acetonitrile) rising linearly from 0% to 95% of eluent II during t = 5–45 min with a flow rate of 20 mL/min. All compounds were purified to >95% homogeneity as determined by analytical HPLC analysis, and lyophilization provided white fluffy solids, which were reconstituted in DMSO (>20 mM) before use. The concentrations were determined by UV spectroscopy using the extinction coefficient of Ac-Lys-AMC (ε_{326} = 17780 M^{-1} cm^{-1}).[29] Analytical reversed-phase HPLC was performed on a C18 Phenomenex Luna column [150 mm × 4.60 mm, 3 μm, 100 Å] using an Agilent 1100 LC system equipped with a diode array UV detector. UPLC-MS analyses were performed on a Waters Acquity ultra high-performance liquid chromatography system equipped with a C18 Phenomenex Kinetex column [50 mm × 2.1 mm, 1.7 μm, 100 Å]. A gradient with eluent III (0.1% HCOOH in water) and eluent IV (0.1% HCOOH in acetonitrile) rose linearly from 0% to 95% of eluent IV during t = 0.00–5.20 min.

General Acylation Procedure A. The desired acid (10 mg, 0.06 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL). Then HATU (23 mg, 0.06 mmol) and lutidine (12 mg, 0.12 mmol) were added and the resulting suspension was stirred for 10 min, before the mixture was cooled to 0 °C and Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-Lys-AMC (**8**, 40 mg, 0.05 mmol) was added. After the reaction reached completion as judged by LC-MS analysis, the solution was partitioned between CH_2Cl_2 (6 mL) and brine (5 mL). The aqueous phase was back extracted with CH_2Cl_2 (3 × 5 mL), and the combined organic layer was washed with aqueous HCl 0.5 M (2 × 10 mL). The acidic layer was back extracted with CH_2Cl_2 (2 × 8 mL), and the combined organic phase was washed with satd aqueous $NaHCO_3$ (2 × 10 mL). The basic aqueous phase was also extracted with CH_2Cl_2 (2 × 10 mL), and the resulting combined organic phase was washed with brine (30 mL). Again, the aqueous phase was back extracted with CH_2Cl_2 (2 × 10 mL), and the final combined organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give a residue, which was stirred in CF_3COOH–CH_2Cl_2–H_2O (2 mL, 50:48:2) for 90 min. The solution was then concentrated under reduced pressure and the crude residue was precipitated from ice-cold diethyl ether (15 mL). The crude residue was purified by reversed-phase preparative HPLC to afford the desired product.

General Acylation Procedure B. Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-Lys-AMC (**8**, 40 mg, 0.05 mmol) and *i*-Pr_2EtN (18 mg, 0.14 mmol) were suspended in anhydrous CH_2Cl_2 (2 mL) at 0 °C, and the desired anhydride, acid chloride, or NHS-ester (0.07 mmol) was added. The reaction reached completion as judged by LC-MS analysis, and the clear solution was diluted with CH_2Cl_2 (6 mL) and washed with aqueous HCl 0.5 M (2 × 5 mL). The aqueous phase was back extracted with CH_2Cl_2 (3 × 5 mL), and the combined organic layer was washed with brine (10 mL). Again, the aqueous phase was extracted with CH_2Cl_2 (3 × 8 mL) and the resulting combined organic layer was dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo. The resulting residue was dissolved in CF_3COOH–CH_2Cl_2–H_2O (2 mL, 50:48:2) and stirred for 90 min. The solution was then concentrated under reduced pressure and the crude residue was precipitated from ice-cold diethyl ether (15 mL). The crude residue was purified by reversed-phase preparative HPLC to afford the desired product.

Ac-Glu-Thr-Asp-Lys(Ac)-AMC (4a). Yield, 12 mg (36% from **7**). ^{1}H NMR (DMSO-d_6) δ 10.21 (s, 1H, NH_{AMC}), 8.23 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.5, 1H, NH_{Lys}), 7.78 (m, 2H, $NH\varepsilon_{Lys}$, $H8_{AMC}$), 7.71 (d, J = 8.7, 1H, $H5_{AMC}$), 7.68 (d, J = 8.0, 1H, NH_{Thr}), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.31 (m, 2H, $H\alpha_{Lys}$, $H\alpha_{Glu}$), 4.25 (dd, J = 7.9, 4.4, 1H, $H\alpha_{Thr}$), 4.01 (m, 1H, $H\beta_{Thr}$), 3.00 (q, J = 6.6, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.39 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.33–2.21 (m, 2H,

DOI: 10.1021/acs.jmedchem.5b01532
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$H\gamma_{Glu}$), 1.92 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.77 (s, 3H, $CH_3CONH\varepsilon_{Lys}$), 1.73 (m, 2H, $H\beta_{Lys\text{-}A}$, $H\beta_{Glu\text{-}A}$), 1.68−1.58 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.44−1.31 (m, 3H, $H\delta_{Lys}$, $H\gamma_{Lys\text{-}A}$), 1.27 (m, 1H, $H\gamma_{Lys\text{-}B}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($C\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.2 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.7 ($CONH_{Glu}$), 169.1 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.1 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.2 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.9 ($C\alpha_{Lys}$), 52.2 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.4 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.3 ($C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 28.8 ($C\delta_{Lys}$), 27.1 ($C\beta_{Glu}$), 22.9 ($C\gamma_{Lys}$), 22.6 ($CH_3CONH\varepsilon_{Lys}$), 22.5 ($CH_3CONH_{Glu}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$). HRMS calcd for $C_{33}H_{44}N_6NaO_{13}^+$ [M + Na]$^+$, 755.2864; found, 755.2851. UPLC-MS, t_R = 1.25 min.

Ac-Glu-Thr-Asp-Lys(Dec)-AMC (4b). Yield, 27 mg (67% from **8**). ^{1}H NMR (DMSO-d_6) δ 12.27 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (d, J = 7.5, 1H, NH_{Lys}), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.70 (m, 3H, NH_{Thr}, $NH\varepsilon_{Lys}$, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.31 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 7.9, 4.3, 1H, $H\alpha_{Thr}$), 4.02 (m, 1H, $H\beta_{Thr}$), 3.01 (q, J = 6.6, 3H, $H\varepsilon_{Lys}$), 2.74 (dd, J = 16.6, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.3, 1H, $H\beta_{Asp\text{-}B}$), 2.39 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.31−2.22 (m, 2H, $H\gamma_{Glu}$), 2.00 (t, J = 7.5, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.96−1.89 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.78−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.63 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.14 (m, 18H, $H\gamma_{Lys}$, $H\delta_{Lys}$, $CH_3(CH_2)_7$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.83 (t, J = 7.0, 3H, $CH_3(CH_2)_8$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 172.0 (2C, $CO\gamma_{Asp}$, $CONH\varepsilon_{Lys}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 (4_{AMC}-CH_3), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.1 ($C7_{AMC}$), 125.8 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.2 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.2 ($C\varepsilon_{Lys}$), 35.9 ($C\beta_{Asp}$), 35.4 ($CH_2CONH\varepsilon_{Lys}$), 31.3 ($CH_3(CH_2)_7/C\delta_{Lys}$), 31.2 ($C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 28.9−28.7 (5C, $CH_3(CH_2)_7/C\delta_{Lys}$), 27.1 ($C\beta_{Glu}$), 25.3−22.8 (2C, $CH_3(CH_2)_7/C\delta_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 22.1 ($C\gamma_{Lys}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$), 13.9 ($CH_3(CH_2)_8$). MS calcd for $C_{41}H_{61}N_6O_{13}^+$ [M + H]$^+$, 845.4; found, 845.3. HRMS calcd for $C_{41}H_{60}N_6NaO_{13}^+$ [M + Na]$^+$, 867.4116; found, 867.4124. Analytical HPLC gradient 10−35% eluent II in eluent I (25 min total run time), t_R = 15.0 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(Lau)-AMC (4c). Yield, 30 mg (73%, only part of the crude residue was purified). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.29 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.97 (d, J = 7.5, 1H, NH_{Lys}), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.75−7.66 (m, 3H, NH_{Thr}, $NH\varepsilon_{Lys}$, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.03 (br s, 1H, OH_{Thr}), 4.60 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.37−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.24 (dd, J = 7.9, 4.3, 1H, $H\alpha_{Thr}$), 4.02 (m, 1H, $H\beta_{Thr}$), 3.01 (q, J = 6.6, 2H, $H\varepsilon_{Lys}$), 2.74 (dd, J = 16.6, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.3, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.31−2.23 (m, 2H, $H\gamma_{Glu}$), 1.99 (t, J = 7.5, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.96−1.83 (m, 4H, CH_3CONH_{Glu}, $H\beta_{Glu\text{-}A}$), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.67−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.13 (m, 22H, $CH_3(CH_2)_9$, $H\delta_{Lys}$, $H\gamma_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.84 (t, J = 7.0, 3H, $CH_3(CH_2)_{10}$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 172.0 (2C, $CO\gamma_{Asp}$, $CONH\varepsilon_{Lys}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.1 ($C7_{AMC}$), 125.8 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.2 ($C\varepsilon_{Lys}$), 35.9 ($C\beta_{Asp}$), 35.4 ($CH_2CONH\varepsilon_{Lys}$), 31.3 ($CH_3(CH_2)_9/C\delta_{Lys}/C\gamma_{Lys}$), 31.2 ($C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 29.0−28.7 (7C, $CH_3(CH_2)_9/C\delta_{Lys}/C\gamma_{Lys}$), 27.1 ($C\beta_{Glu}$), 25.3−22.8 (2C, $CH_3(CH_2)_9/C\delta_{Lys}/C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 22.1 ($CH_3(CH_2)_9/C\delta_{Lys}/C\gamma_{Lys}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$), 13.9 ($CH_3(CH_2)_{10}$). MS calcd for $C_{43}H_{65}N_6O_{13}^+$ [M + H]$^+$, 873.5; found, 873.5. HRMS calcd for $C_{43}H_{64}N_6NaO_{13}^+$ [M + Na]$^+$, 895.4429; found, 895.4436. Analytical HPLC gradient 10−40% eluent II in eluent I (25 min total run time), t_R = 18.6 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(Myr)-AMC (4d). Yield, 31 mg (73%, only part of the crude residue was purified). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.43 (s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.96 (d, J = 7.5, 1H, NH_{Lys}), 7.79 (d, J = 2.0, 1H, $H8_{AMC}$), 7.76−7.68 (m, 3H, NH_{Thr}, $NH\varepsilon_{Lys}$, $H5_{AMC}$), 7.54 (dd, J = 8.7, 1.9, 1H, $H6_{AMC}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.04 (s, 1H, OH_{Thr}), 4.58 (q, J = 7.0, 1H, $H\alpha_{Asp}$), 4.32 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.23 (dd, J = 7.9, 4.3, 1H, $H\alpha_{Thr}$), 4.06−3.99 (m, 1H, $H\beta_{Thr}$), 3.01 (q, J = 6.5, 2H, $H\varepsilon_{Lys}$), 2.71 (dd, J = 16.6, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.58 (dd, J = 16.5, 7.1, 1H, $H\beta_{Asp\text{-}B}$), 2.39 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.31−2.23 (m, 2H, $H\gamma_{Glu}$), 2.00 (t, J = 7.5, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.95−1.84 (m, 4H, CH_3CONH_{Glu}, $H\beta_{Glu\text{-}A}$), 1.80−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.47−1.12 (m, 26H, $CH_3(CH_2)_{11}$, $H\delta_{Lys}$, $H\gamma_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.84 (t, J = 7.0, 3H, $CH_3(CH_2)_{12}$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 172.1 ($CONH\varepsilon_{Lys}/CO\gamma_{Asp}$), 172.0 ($CONH\varepsilon_{Lys}/CO\gamma_{Asp}$), 171.7 ($CO\alpha_{Glu}$), 171.1 ($CO_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO_{Thr}$), 169.5 ($CONH_{Glu}$), 159.98, 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.1 ($C7_{AMC}$), 125.8 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.6 ($C\beta_{Thr}$), 58.0 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.7 ($C\alpha_{Asp}$), 38.2 ($C\varepsilon_{Lys}$), 36.1 ($C\beta_{Asp}$), 35.4 ($CH_2CONH\varepsilon_{Lys}$), 31.3 ($CH_3(CH_2)_{11}/C\delta_{Lys}/C\gamma_{Lys}$), 31.2 ($C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 29.1−28.7 (9C, $CH_3(CH_2)_{11}/C\delta_{Lys}/C\gamma_{Lys}$), 27.1 ($C\beta_{Glu}$), 25.3−22.8 (2C, $CH_3(CH_2)_{11}/C\delta_{Lys}/C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 22.1 ($CH_3(CH_2)_{11}/C\delta_{Lys}/C\gamma_{Lys}$), 19.3 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$), 13.9 ($CH_3(CH_2)_{12}$). MS calcd for $C_{45}H_{69}N_6O_{13}^+$ [M + H]$^+$, 901.5; found, 901.5. HRMS calcd for $C_{45}H_{68}N_6NaO_{13}^+$ [M + Na]$^+$, 923.4742; found, 923.4748. Analytical HPLC gradient 15−50% eluent II in eluent I (25 min total run time), t_R = 19.2 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(isovaleryl)-AMC (4e). The title compound was synthesized according to general acylation procedure A. Reagents: isovaleric acid (6 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: 4 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(isovaleryl)-AMC (**4e**, 22 mg, 61% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.24 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (d, J = 7.5, 1H, NH_{Lys}), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.74−7.66 (m, 3H, NH_{Thr}, $NH\varepsilon_{Lys}$, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 5.03 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.02 (m, 1H, $H\beta_{Thr}$), 3.02 (q, J = 6.7, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.31−2.22 (m, 2H, $H\gamma_{Glu}$), 1.95−1.88 (m, 4H, $CHCH_2CONH\varepsilon_{Lys}$,$H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.78−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.67−1.60 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.43−1.31 (m, 4H, $H\delta_{Lys}$, $H\gamma_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.82 (dd, J = 6.3, 1.7, 6H, $(CH_3)_2CHCH_2CONH\varepsilon_{Lys}$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.3 ($CONH\varepsilon_{Lys}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 44.8 ($CH_2CONH\varepsilon_{Lys}$), 38.2 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.3 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 28.9 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 25.5 ($CHCH_2CONH\varepsilon_{Lys}$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 22.3 ($(CH_3)_2CHCH_2CONH\varepsilon_{Lys}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$). HRMS calcd for $C_{36}H_{50}N_6NaO_{13}^+$ [M + Na]$^+$, 797.3334; found, 797.3340. Analytical HPLC gradient 0−38% eluent II in eluent I (20 min total run time), t_R = 13.1 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*S*)-2-methylbutyryl)-AMC (4f). The title compound was synthesized according to general acylation procedure A. Reagents: (*S*)-2-methylbutyric acid (6 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: 7 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*S*)-2-methylbutyr-

DOI: 10.1021/acs.jmedchem.5b01532
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yl)-AMC (**4f**, 22 mg, 61% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.26 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.22 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (d, J = 7.5, 1H, NH_{Lys}), 7.78 (d, J = 1.9, 1H, $H8_{AMC}$), 7.74−7.66 (m, 3H, $NH\varepsilon_{Lys}$, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 1.9, 1H, $H6_{AMC}$), 6.26 (d, J = 0.9, 1H, $H3_{AMC}$), 5.05 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 7.9, 4.4, 1H, $H\alpha_{Thr}$), 4.05−3.98 (m, 1H, $H\beta_{Thr}$), 3.02 (m, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (s, 3H, 4_{AMC}-CH_3), 2.31−2.24 (m, 2H, $H\gamma_{Glu}$), 2.08 (dq, J = 13.5, 6.7, 1H, $CH(CH_3)CONH\varepsilon_{Lys}$), 1.96−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.49−1.19 (m, 6H, $CH_2CH(CH_3)CONH\varepsilon_{Lys}$, $H\gamma_{Lys}$, $H\delta_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.93 (d, J = 6.8, 3H, $CH_3CH_2CH(CH_3)CONH\varepsilon_{Lys}$), 0.75 (t, J = 7.4, 3H, $CH_3CH_2CH(CH_3)CONH\varepsilon_{Lys}$). ^{13}C NMR (151 MHz, DMSO) δ 175.3 ($CONH\varepsilon_{Lys}$), 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.9 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 41.3 ($CH(CH_3)CONH\varepsilon_{Lys}$), 38.2 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.3 ($C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 28.9 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 26.8 ($CH_2CH(CH_3)CONH\varepsilon_{Lys}$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$), 17.6 ($CH_3CH_2CH(CH_3)CONH\varepsilon_{Lys}$), 11.8 ($CH_3CH_2CH(CH_3)CONH\varepsilon_{Lys}$). HRMS calcd for $C_{36}H_{50}N_6NaO_{13}^+$ [M + Na]$^+$, 797.3334; found, 797.3339. Analytical HPLC gradient 0−25% eluent II in eluent I (25 min total run time), t_R = 17.0 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(glutaryl)-AMC (4g). The title compound was synthesized according to general acylation procedure B. The reaction was performed using 35 mg (0.04 mmol) of Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-Lys-AMC. Reagents: glutaric anhydride (7 mg, 0.06 mmol, 1.5 equiv), i-Pr_2EtN (16 mg, 0.12 mmol, 3.0 equiv). Reaction time: 1 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(glutaryl)-AMC (**4g**, 20 mg, 61% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.16 (br s, 3H, $COOH_{Asp}$, $COOH_{Glu}$, $HOOC(CH_2)_3CONH\varepsilon_{Lys}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.11 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.5, 1H, $NH\alpha_{Lys}$), 7.79−7.74 (m, 2H, $H8_{AMC}$, $NH\varepsilon_{Lys}$), 7.70 (d, J = 8.7, 1H, $H5_{AMC}$), 7.69 (d, J = 8.0, 1H, NH_{Thr}) 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (m, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Lys}$, $H\alpha_{Glu}$), 4.25 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.01 (m, 1H, $H\beta_{Thr}$), 3.01 (m, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.30−2.23 (m, 2H, $H\gamma_{Glu}$), 2.17 (t, J = 7.4, 2H, $CH_2(CH_2)_2CONH\varepsilon_{Lys}$), 2.06 (t, J = 7.5, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.95−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.78−1.59 (m, 5H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$, $CH_2CH_2CONH\varepsilon_{Lys}$, $H\beta_{Lys\text{-}B}$), 1.43−1.22 (m, 4H, $H\gamma_{Lys}$, $H\delta_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$). ^{13}C NMR (151 MHz, DMSO) δ 174.2 ($HOOC(CH_2)_3CONH\varepsilon_{Lys}$), 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CONH\varepsilon_{Lys}$/$CO\alpha_{Glu}$), 171.4 ($CONH\varepsilon_{Lys}$/$CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CH_3CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C4a_{AMC}$), 115.2 ($C6_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.3 ($C\delta_{Lys}$), 35.8 ($C\beta_{Asp}$), 34.5 ($CH_2CONH\varepsilon_{Lys}$), 33.0 ($CH_2(CH_2)_2CONH\varepsilon_{Lys}$), 31.3 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 28.8 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 20.7 ($CH_2CH_2CONH\varepsilon_{Lys}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$). HRMS calcd for $C_{36}H_{48}N_6NaO_{15}^+$ [M + Na]$^+$, 827.3075; found, 827.3062. Analytical HPLC gradient 0−30% eluent II in eluent I (20 min total run time), t_R = 11.9 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(crotonyl)-AMC (4h). The title compound was synthesized according to general acylation procedure B. Reagents: crotonyl chloride (7 mg, 0.07 mmol, 1.5 equiv), i-Pr_2EtN (18 mg, 0.14 mmol, 3.0 equiv). Reaction time: 1 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(crotonyl)-AMC (**4h**, 17 mg, 49% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.24 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.22 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.5, 1H, NH_{Lys}), 7.82 (t, J = 5.6, 1H, $NH\varepsilon_{Lys}$), 7.77 (d, J = 1.9, 1H, $H8_{AMC}$), 7.72 (d, J = 8.7, 1H, $H5_{AMC}$), 7.68 (d, J = 8.0, 1H, NH_{Thr}), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.60−6.52 (m, 1H, $CHCHCONH\varepsilon_{Lys}$), 6.27 (d, J = 1.1, 1H, $H3_{AMC}$), 5.86 (dd, J = 15.3, 1.7, 1H, $CHCONH\varepsilon_{Lys}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.01 (m, 1H, $H\beta_{Thr}$), 3.08 (q, J = 6.7, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.31−2.22 (m, 2H, $H\gamma_{Glu}$), 1.96−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 5H, $CH_3CHCHCONH\varepsilon_{Lys}$, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.33 (m, 3H, $H\delta_{Lys}$, $H\gamma_{Lys\text{-}A}$), 1.32−1.21 (m, 1H, $H\gamma_{Lys\text{-}B}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$). ^{13}C NMR (151 MHz, DMSO) δ 173.9 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.5 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 164.8 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 137.3 ($CHCHCONH\varepsilon_{Lys}$), 125.9 ($CHCONH\varepsilon_{Lys}$), 125.9 ($C5_{AMC}$), 115.3 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.2 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.2 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 28.8 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$), 13.9 ($CH_3CHCHCONH\varepsilon_{Lys}$). The *cis*- isomer could be detected (approx 10%, based on ^{1}H NMR). HRMS calcd for $C_{35}H_{46}N_6NaO_{13}^+$ [M + Na]$^+$, 781.3021; found, 781.3007. Analytical HPLC gradient 0−20% eluent II in eluent I (40 min total run time), t_R = 25.1 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*E*)-dec-2-enoyl)-AMC (4i). The title compound was synthesized according to general acylation procedure A. Reagents: (*E*)-dec-2-enoic acid (9 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: overnight. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*E*)-dec-2-enoyl)-AMC (**4i**, 19 mg, 48% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.25 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Thr}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.5, 1H, NH_{Lys}), 7.83 (t, J = 5.6, 1H), 7.77 (d, J = 2.0, 1H, $H8_{AMC}$), 7.73−7.67 (m, 2H, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.56 (dt, J = 15.3, 6.9, 1H, $CHCHCONH\varepsilon_{Lys}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.84 (d, J = 15.4, 1H, $CHCONH\varepsilon_{Lys}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.36−4.29 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 8.0, 4.3, 1H, $H\alpha_{Thr}$), 4.05−3.98 (m, 1H, $H\beta_{Thr}$), 3.08 (q, J = 6.7, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.30−2.24 (m, 2H, $H\gamma_{Glu}$), 2.08 (q, J = 6.9, 2H, $CH_3(CH_2)_5CH_2$), 1.96−1.89 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.60 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.19 (m, 14H, $H\gamma_{Lys}$, $H\delta_{Lys}$, $CH_3(CH_2)_5CH_2$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.85 (t, J = 7.0, 3H, $CH_3(CH_2)_6$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 164.9 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.2 ($CHCHCONH\varepsilon_{Lys}$/$C7_{AMC}$), 142.0 ($CHCHCONH\varepsilon_{Lys}$/$C7_{AMC}$), 125.9 ($C5_{AMC}$), 124.4 ($CHCONH\varepsilon_{Lys}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.3 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.2 (3C, $CH_3(CH_2)_5CH_2$, $C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 28.8−27.9 (4C, $CH_3(CH_2)_5$, $C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.8 ($CH_3(CH_2)_5$/$C\gamma_{Lys}$), 22.4 (CH_3CONH_{Glu}), 22.1 ($CH_3(CH_2)_5$/$C\gamma_{Lys}$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 13.9 ($CH_3(CH_2)_6$). HRMS calcd for $C_{41}H_{58}N_6NaO_{13}^+$ [M + Na]$^+$, 865.3960; found, 865.3964. Analytical HPLC gradient 15−40% eluent II in eluent I (25 min total run time), t_R = 14.7 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*E*)-dodec-2-enoyl)-AMC (4j). The title compound was synthesized according to general acylation procedure A. Reagents: (*E*)-dodec-2-enoic acid (11 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol,

DOI: 10.1021/acs.jmedchem.5b01532
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2.6 equiv). Reaction time: overnight. Purification of the crude residue in aliquots (~3 mg/run) by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*E*)-dodec-2-enoyl)-AMC (**4j**, 6 mg, 39% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.23 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.23 (s, 1H, NH_{AMC}), 8.23 (d, J = 7.7, 1H, NH_{Asp}), 8.13 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.5, 1H, NH_{Lys}), 7.84 (t, J = 5.6, 1H, $NH\varepsilon_{Lys}$), 7.78 (d, J = 1.9, 1H, $H8_{AMC}$), 7.75−7.66 (m, 2H, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 1.9, 1H, $H6_{AMC}$), 6.56 (dt, J = 15.2, 6.9, 1H, $CHCHCONH_{Glu}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.84 (d, J = 15.4, 1H, $CHCONH_{Glu}$), 5.05 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.37−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 7.9, 4.3, 1H, $H\alpha_{Thr}$), 4.06−3.98 (m, 1H, $H\beta_{Thr}$), 3.08 (q, J = 6.6, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (s, 3H, 4_{AMC}-CH_3), 2.31−2.23 (m, 2H, $H\gamma_{Glu}$), 2.07 (q, J = 6.9, 2H, $CH_3(CH_2)_7CH_2$), 1.96−1.89 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.69 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.69−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.17 (m, 18H, $H\gamma_{Lys}$, $H\delta_{Lys}$, $CH_3(CH_2)_7$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.85 (t, J = 7.0, 3H, $CH_3(CH_2)_8$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 164.9 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.2 ($CHCHCONH\varepsilon_{Lys}$), 142.1 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 124.5 ($CHCONH\varepsilon_{Lys}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.3 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 38.3 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.3−31.2 (3C, $CH_3(CH_2)_7CH_2$, $C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 28.9−27.9 (6C, $CH_3(CH_2)_7$, $C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.8 ($C\gamma_{Lys}$), 22.4 (CH_3CONH_{Glu}), 22.1 ($CH_3(CH_2)_7$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 13.9 ($CH_3(CH_2)_8$). HRMS calcd for $C_{43}H_{62}N_6NaO_{13}^+$ $[M + Na]^+$, 893.4273; found, 893.4275. Analytical HPLC gradient 15−40% eluent II in eluent I (25 min total run time), t_R = 14.7 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxybutyryl)-AMC (4k). The title compound was synthesized according to general acylation procedure A. Reagents: (*R*/*S*)-3-hydroxy-butyric acid (5 mg, 0.05 mmol, 1.0 equiv), HATU (19 mg, 0.05 mmol, 1.1 equiv), lutidine (10 mg, 0.09 mmol, 2.0 equiv). Reaction time: after 2 h, 3-hydroxy-butiryc acid (1 mg, 0.01 mmol, 0.2 equiv), HATU (4 mg, 0.01 mmol, 0.2 equiv), and lutidine (1 mg, 0.01 mmol, 0.2 equiv) were preincubated in CH_2Cl_2 (200 μL) then added to the reaction mixture. The reaction mixture was then stirred for 1 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxy-butyryl)-AMC (**4k**, 21 mg, 59% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.22 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.21 (d, J = 1.4, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (d, J = 7.4, 1H, NH_{Lys}), 7.77 (d, J = 1.9, 1H, $H8_{AMC}$), 7.75 (t, J = 5.6, 1H, $NH\varepsilon_{Lys}$), 7.71 (d, J = 8.7, 1H, $H5_{AMC}$), 7.68 (d, J = 8.0, 1H, NH_{Thr}), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.32 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.01 (m, 1H, $H\beta_{Thr}$), 3.93 (m, 1H, $CH_3CH(OH)CH_2$), 3.02 (m, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.31−2.22 (m, 2H, $H\gamma_{Glu}$), 2.17 (dd, J = 13.8, 7.2, 1H, $CH_{2,A}CONH\varepsilon_{Lys}$), 2.06 (ddd, J = 13.8, 5.9, 1.7, 1H, $CH_{2,B}CONH\varepsilon_{Lys}$), 1.95−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.69 (m, 2H, $H\beta_{Lys\text{-}A}$, $H\beta_{Glu\text{-}B}$), 1.63 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.43−1.22 (m, 5H, $H\gamma_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 1.02 (dd, J = 6.2, 3.8, 3H, $CH_3CH(OH)CH_2$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($C\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 170.6 ($CONH\varepsilon_{Lys}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.2 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 63.8 ($CH_3CH(OH)CH_2$), 57.9 ($C\alpha_{Thr}$), 53.9 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 45.3 ($CH_2CONH\varepsilon_{Lys}$), 38.2 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.2 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 28.8 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 23.3 ($CH_3CH(OH)CH_2$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$). HRMS calcd for $C_{35}H_{48}N_6NaO_{14}^+$ $[M + Na]^+$, 799.3126; found, 799.3139. Analytical HPLC gradient 0−35% eluent II in eluent I (20 min total run time), t_R = 12.4 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxydecanoyl)-AMC (4l). The title compound was synthesized according to general acylation procedure A. Reagents: 3-hydroxydecanoic acid (10 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: overnight. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxydecanoyl)-AMC (**4l**, 24 mg, 60% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.24 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.20 (d, J = 1.6, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.11 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (d, J = 6.4, 1H, NH_{Lys}), 7.77 (d, J = 1.4, 1H, $H8_{AMC}$), 7.74 (t, J = 5.5, 1H, $NH\varepsilon_{Lys}$), 7.72−7.66 (m, 2H, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.26 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.02 (m, 1H, $H\beta_{Thr}$), 3.79−3.72 (m, 1H, $(CH_2)_6CH(OH)CH_2$), 3.08−2.96 (m, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.39 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.30−2.23 (m, 2H, $H\gamma_{Glu}$), 2.11 (d, J = 6.6, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.96−1.89 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.69 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.58 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.44−1.14 (m, 16H, $H\gamma_{Lys}$, $H\delta_{Lys}$, $CH_3(CH_2)_6CH(OH)CH_2$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.84 (t, J = 7.0, 3H, $CH_3(CH_2)_6$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.8 ($CONH\varepsilon_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 67.4 ($CH(OH)CH_2CONH\varepsilon_{Lys}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 43.6 ($CH_2CONH\varepsilon_{Lys}$), 38.2 ($C\varepsilon_{Lys}$), 36.9 ($CH_3(CH_2)_6$), 35.8 ($C\beta_{Asp}$), 31.2 (2C, $CH_3(CH_2)_6$, $C\beta_{Lys}$), 30.3 ($C\gamma_{Glu}$), 29.1−28.7 (3C, $CH_3(CH_2)_6$, $C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 25.0 ($CH_3(CH_2)_6$), 22.8 ($C\gamma_{Lys}$), 22.4 (CH_3CONH_{Glu}), 22.1 ($CH_3(CH_2)_6$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 14.0 ($CH_3(CH_2)_6$). HRMS calcd for $C_{41}H_{60}N_6NaO_{14}^+$ $[M + Na]^+$, 883.4065; found, 883.4070. Analytical HPLC gradient 10−45% eluent II in eluent I (25 min total run time), t_R = 11.5 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxydodecanoyl)-AMC (4m). The title compound was synthesized according to general acylation procedure A. Reagents: (*R*/*S*)-3-hydroxydodecanoic acid (12 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: overnight. Purification of the crude residue in aliquots (~5 mg/run) by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys((*R*/*S*)-3-hydroxydodecanoyl)-AMC (**4m**, 22 mg, 53% from **7**) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.26 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.20 (d, J = 1.8, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.12 (d, J = 7.7, 1H, NH_{Glu}), 7.99 (d, J = 7.2, 1H, NH_{Lys}), 7.78 (d, J = 1.4, 1H, $H8_{AMC}$), 7.74 (t, J = 5.3, 1H, $NH\varepsilon_{Lys}$), 7.72−7.67 (m, 2H, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 1.8, 1H, $H6_{AMC}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.35−4.28 (m, 2H, $H\alpha_{Glu}$, $H\alpha_{Lys}$), 4.25 (dd, J = 8.0, 4.3, 1H, $H\alpha_{Thr}$), 4.05−3.99 (m, 1H, $H\beta_{Thr}$), 3.78−3.72 (m, 1H, $CH(OH)CH_2CONH\varepsilon_{Lys}$), 3.02 (m, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.3, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.30−2.23 (m, 2H, $H\gamma_{Glu}$), 2.11 (d, J = 6.6, 2H, $CH_2CONH\varepsilon_{Lys}$), 1.96−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.67−1.59 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.43−1.14 (m, 20H, $CH_3(CH_2)_8$, $H\gamma_{Lys}$, $H\delta_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.84 (t, J = 7.0, 3H, $CH_3(CH_2)_8$). ^{13}C NMR (151 MHz, DMSO) δ 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Asp}$), 171.1 ($CO\alpha_{Lys}$), 170.8 ($CONH\varepsilon_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 67.4 ($CH(OH)CH_2CONH\varepsilon_{Lys}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 49.6 ($C\alpha_{Asp}$), 43.6 ($CH_2CONH\varepsilon_{Lys}$), 38.2 ($C\varepsilon_{Lys}$), 36.9 ($CH_3(CH_2)_8$), 35.8 ($C\beta_{Asp}$), 31.3 ($C\beta_{Lys}$/$CH_3(CH_2)_8$), 31.2 ($C\beta_{Lys}$/$CH_3(CH_2)_8$), 30.3 ($C\gamma_{Glu}$), 29.1−28.7 (5C, $CH_3(CH_2)_8$, $C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 25.0 ($CH_3(CH_2)_8$), 22.8 ($C\gamma_{Lys}$), 22.4 (CH_3CONH_{Glu}), 22.1 ($CH_3(CH_2)_8$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 14.0 ($CH_3(CH_2)_8$).

DOI: 10.1021/acs.jmedchem.5b01532
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HRMS calcd for $C_{43}H_{64}N_6NaO_{14}^+$ [M + Na]$^+$, 911.4378; found, 911.4384. Analytical HPLC gradient 15−40% eluent II in eluent I (25 min total run time), t_R = 11.0 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(acetoacetyl)-AMC (4n). The title compound was synthesized according to general acylation procedure B. The reaction was performed on 35 mg of Ac-Glu(^{t}Bu)-Thr(^{t}Bu)-Asp(^{t}Bu)-Lys-AMC (0.04 mmol). Reagents: *N*-hydroxysuccinimidyl acetoacetate (10 mg, 0.05 mmol, 1.2 equiv), *i*-Pr$_2$EtN (10 mg, 0.08 mmol, 2.0 equiv). Reaction time: 1 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(acetoacetyl)-AMC (**4n**, 21 mg, 61% from **7**, keto−enol ratio 85:15 based on ^{1}H NMR) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.23 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.22 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.11 (d, J = 7.7, 1H, NH_{Glu}), 8.03−7.96 (m, 2H, $NH\varepsilon_{Lys}$, NH_{Lys}), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.71 (d, J = 8.7, 1H, $H5_{AMC}$), 7.68 (d, J = 8.0, 1H, NH_{Thr}), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.36−4.28 (m, 2H, $H\alpha_{Lys}$, $H\alpha_{Glu}$), 4.25 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.02 (td, J = 6.4, 1.8, 1H, $H\beta_{Thr}$), 3.26 (s, 2H, $CH_2CONH\varepsilon_{Lys}$), 3.05 (q, J = 6.7, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.40 (d, J = 1.1, 3H, 4_{AMC}-CH_3), 2.31−2.23 (m, 2H, $H\gamma_{Glu}$), 2.11 (s, 3H, $CH_3COCH_2CONH\varepsilon_{Lys}$), 1.96−1.88 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.80−1.70 (m, 2H, $H\beta_{Lys\text{-}A}$, $H\beta_{Glu\text{-}B}$), 1.68−1.60 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.45−1.33 (m, 3H, $H\delta_{Lys}$, $H\gamma_{Lys\text{-}A}$), 1.33−1.21 (m, 1H, $H\gamma_{Lys\text{-}B}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$). ^{13}C NMR (151 MHz, DMSO) δ 203.1 ($COCH_2CONH\varepsilon_{Lys}$), 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 165.9 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.1 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.2 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.8 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 51.3 ($CH_2CONH\varepsilon_{Lys}$), 49.6 ($C\alpha_{Asp}$), 38.5 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.2 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 29.9 ($CH_3COCH_2CONH\varepsilon_{Lys}$), 28.6 ($C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.8 ($C\gamma_{Lys}$), 22.4 ($CH_3CONH_{Glu}$), 19.2 ($C\gamma_{Thr}$), 18.0 ($4_{AMC}$-$CH_3$). HRMS calcd for $C_{35}H_{46}N_6NaO_{14}^+$ [M + Na]$^+$, 797.2970; found, 797.2949. Analytical HPLC gradient 0−30% eluent II in eluent I (20 min total run time), t_R = 12.8 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(3-oxodecanoyl)-AMC (4o). The title compound was synthesized according to general acylation procedure A. Reagents: 3-oxodecanoic acid (10 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: the reaction mixture was stirred overnight, then 3-oxodecanoic acid (5 mg, 0.03 mmol, 0.6 equiv), HATU (11 mg, 0.03 mmol, 0.6 equiv), lutidine (6 mg, 0.06 mmol, 1.2 equiv) were preincubated for 5 min in anhyd CH_2Cl_2 (250 μL), then added to the reaction. After 2 h, 3-oxodecanoic acid (2 mg, 0.01 mmol, 0.2 equiv), HATU (6 mg, 0.01 mmol, 0.2 equiv), and lutidine (3 mg, 0.03 mmol, 0.6 equiv) were preincubated in anhyd CH_2Cl_2 (100 μL) for 5 min then added to the reaction and stirred for 1 h. Purification of the crude residue by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(3-oxodecanoyl)-AMC (**4o**, 17 mg, 42% from **7**, keto−enol ratio 85:15, based on ^{1}H NMR) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.26 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.22 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.11 (d, J = 7.7, 1H, NH_{Glu}), 8.02−7.95 (m, 2H, $NH\varepsilon_{Lys}$, NH_{Lys}), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.74−7.67 (m, 2H, NH_{Thr}, $H5_{AMC}$), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.2, 1H, $H3_{AMC}$), 5.04 (br s, 1H, OH_{Thr}), 4.61 (q, J = 7.3, 1H, $H\alpha_{Asp}$), 4.36−4.28 (m, 2H, $H\alpha_{Lys}$, $H\alpha_{Glu}$), 4.25 (dd, J = 8.0, 4.3, 1H, $H\alpha_{Thr}$), 4.06−3.98 (m, 1H, $H\beta_{Thr}$), 3.25 (s, 2H, $CH_2CONH\varepsilon_{Lys}$), 3.04 (q, J = 6.7, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.3, 1H, $H\beta_{Asp\text{-}B}$), 2.45 (t, J = 7.3, 2H, $CH_2COCH_2CONH\varepsilon_{Lys}$), 2.40 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.30−2.23 (m, 2H, $H\gamma_{Glu}$), 1.95−1.90 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.60 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.46−1.14 (m, 14H, $CH_3(CH_2)_5CH_2$, $H\gamma_{Lys}$, $H\delta_{Lys}$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.83 (t, J = 7.1, 3H, $CH_3(CH_2)_6$). ^{13}C NMR (151 MHz, DMSO) δ 205.0 ($COCH_2CONH\varepsilon_{Lys}$), 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.7 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 165.9 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 50.5 ($CH_2CONH\varepsilon_{Lys}$), 49.6 ($C\alpha_{Asp}$), 42.0 ($CH_2COCH_2CONH\varepsilon_{Lys}$), 38.5 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.2−31.1 ($C\beta_{Lys}$, $CH_3(CH_2)_5$), 30.3 ($C\gamma_{Glu}$), 28.6−28.4 (3C, $CH_3(CH_2)_5$s, $C\delta_{Lys}$), 27.0 ($C\beta_{Glu}$), 22.9−22.8 ($CH_3(CH_2)_5$, $C\gamma_{Lys}$), 22.4 (CH_3CONH_{Glu}), 22.0 ($CH_3(CH_2)_5$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 13.9 ($CH_3(CH_2)_6$). HRMS calcd for $C_{41}H_{58}N_6NaO_{14}^+$ [M + Na]$^+$, 881.3909; found, 881.3915. Analytical HPLC gradient 15−40% eluent II in eluent I (25 min total run time), t_R = 9.9 min (>95% purity, UV_{230}).

Ac-Glu-Thr-Asp-Lys(3-oxododecanoyl)-AMC (4p). The title compound was synthesized according to general acylation procedure A. Reagents: 3-oxododecanoic acid (12 mg, 0.06 mmol, 1.2 equiv), HATU (23 mg, 0.06 mmol, 1.3 equiv), lutidine (12 mg, 0.12 mmol, 2.6 equiv). Reaction time: the reaction mixture was stirred overnight, then 3-oxododecanoic acid (2 mg, 0.01 mmol, 0.2 equiv), HATU (5 mg, 0.01 mmol, 0.2 equiv), and lutidine (1 mg, 0.01 mmol, 0.2 equiv) were preincubated for 5 min in anhyd CH_2Cl_2 (250 μL), then added to the reaction and stirred for 3 h more. Purification of the crude residue in aliquots (~3 mg/run) by preparative HPLC afforded Ac-Glu-Thr-Asp-Lys(3-oxododecanoyl)-AMC (the crude residue was partitioned in small batches and then purified: **4p**, 14 mg, 51% from **7**, keto−enol ratio 85:15, based on ^{1}H NMR) as a colorless fluffy solid after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.23 (br s, 2H, $COOH_{Asp}$, $COOH_{Glu}$), 10.22 (s, 1H, NH_{AMC}), 8.22 (d, J = 7.7, 1H, NH_{Asp}), 8.11 (d, J = 7.7, 1H, NH_{Glu}), 7.98 (t, J = 6.1, 2H, NH_{Lys}, $NH\varepsilon_{Lys}$), 7.78 (d, J = 2.0, 1H, $H8_{AMC}$), 7.71 (d, J = 8.7, 1H, $H5_{AMC}$), 7.68 (d, J = 8.0, 1H, NH_{Thr}), 7.52 (dd, J = 8.7, 2.0, 1H, $H6_{AMC}$), 6.26 (d, J = 1.1, 1H, $H3_{AMC}$), 5.04 (d, J = 4.6, 1H, OH_{Thr}), 4.61 (q, J = 7.4, 1H, $H\alpha_{Asp}$), 4.36−4.28 (m, 2H, $H\alpha_{Lys}$, $H\alpha_{Glu}$), 4.26 (dd, J = 8.0, 4.4, 1H, $H\alpha_{Thr}$), 4.05−3.98 (m, 1H, $H\beta_{Thr}$), 3.24 (s, 2H, $CH_2CONH\varepsilon_{Lys}$), 3.04 (q, J = 6.6, 2H, $H\varepsilon_{Lys}$), 2.75 (dd, J = 16.7, 5.7, 1H, $H\beta_{Asp\text{-}A}$), 2.59 (dd, J = 16.7, 7.4, 1H, $H\beta_{Asp\text{-}B}$), 2.45 (t, J = 7.3, 2H, $CH_2COCH_2CONH\varepsilon_{Lys}$), 2.40 (d, J = 1.0, 3H, 4_{AMC}-CH_3), 2.30−2.24 (m, 2H, $H\gamma_{Glu}$), 1.96−1.89 (m, 1H, $H\beta_{Glu\text{-}A}$), 1.86 (s, 3H, CH_3CONH_{Glu}), 1.79−1.70 (m, 2H, $H\beta_{Glu\text{-}B}$, $H\beta_{Lys\text{-}A}$), 1.68−1.60 (m, 1H, $H\beta_{Lys\text{-}B}$), 1.45−1.14 (m, 18H, $H\gamma_{Lys}$, $H\delta_{Lys}$, $CH_3(CH_2)_7CH_2$), 1.05 (d, J = 6.3, 3H, $H\gamma_{Thr}$), 0.84 (t, J = 7.0, 3H, $CH_3(CH_2)_8$). ^{13}C NMR (151 MHz, DMSO) δ 205.0 ($COCH_2CONH\varepsilon_{Lys}$), 174.0 ($CO\delta_{Glu}$), 171.9 ($CO\gamma_{Asp}$), 171.6 ($CO\alpha_{Glu}$), 171.1 ($CO\alpha_{Lys}$), 170.6 ($CO\alpha_{Asp}$), 169.9 ($CO\alpha_{Thr}$), 169.6 ($CONH_{Glu}$), 165.9 ($CONH\varepsilon_{Lys}$), 160.0 ($C2_{AMC}$), 153.6 ($C8a_{AMC}$), 153.0 ($C4_{AMC}$), 142.0 ($C7_{AMC}$), 125.9 ($C5_{AMC}$), 115.4 ($C6_{AMC}$), 115.1 ($C4a_{AMC}$), 112.4 ($C3_{AMC}$), 105.8 ($C8_{AMC}$), 66.7 ($C\beta_{Thr}$), 57.9 ($C\alpha_{Thr}$), 53.8 ($C\alpha_{Lys}$), 52.1 ($C\alpha_{Glu}$), 50.5 ($CH_2CONH\varepsilon_{Lys}$), 49.6 ($C\alpha_{Asp}$), 42.0 ($CH_2COCH_2CONH\varepsilon_{Lys}$), 38.5 ($C\varepsilon_{Lys}$), 35.8 ($C\beta_{Asp}$), 31.3 ($CH_3(CH_2)_7$), 31.2 ($C\beta_{Lys}$), 30.2 ($C\gamma_{Glu}$), 28.9−28.4 (5C, $C\delta_{Lys}$, $CH_3(CH_2)_7$), 27.0 ($C\beta_{Glu}$), 22.9−22.8 ($C\gamma_{Lys}$, $CH_3(CH_2)_7$), 22.4 (CH_3CONH_{Glu}), 22.1 ($CH_3(CH_2)_7$), 19.2 ($C\gamma_{Thr}$), 18.0 (4_{AMC}-CH_3), 13.9 ($CH_3(CH_2)_8$). HRMS calcd for $C_{43}H_{62}N_6NaO_{14}^+$ [M + Na]$^+$, 909.4222; found, 909.4225. Analytical HPLC gradient 15−50% eluent II in eluent I (25 min total run time), t_R = 12.2 min (>95% purity, UV_{230}).

Fluorescence Measurements. All microtiter plates were analyzed using a PerkinElmer Enspire plate reader with excitation at 360 nm and detecting emission at 460 nm. Fluorescence measurements (RFU) were converted to [AMC] concentrations based on an [AMC]−fluorescence standard curve and all data analysis was performed using GraphPad Prism.

Fluorogenic Sirtuin Substrate Screening. The initial screening for substrate deacylation activity was performed in Tris buffer (see Supporting Information) with end-point fluorophore release by trypsin. For a final volume of 25 μL per well, acyl substrates (**1a**, **2a−d**, **3a−d**, and **4a−p**, 50 μM) and NAD$^+$ (500 μM) were added to each well followed by a solution of sirtuin enzyme (250 nM). The reaction was incubated at 37 °C for 60 min, then 25 μL of a solution of trypsin and nicotinamide (25 μL, 5.0 mg/mL and 4 mM, respectively; final concentrations 2.5 mg/mL and 2 mM, respectively) was added, and the assay development was allowed to proceed for 90 min at room

DOI: 10.1021/acs.jmedchem.5b01532
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temperature before fluorescence analysis. The data were analyzed to afford [AMC] relative to control wells.

Determination of Kinetic Parameters for SIRT2-mediated Deacylation. Rate experiments for determination of kinetic parameters (substrates **4a**−**d** and NAD^+) were performed in HEPES buffer (see Supporting Information) in a final volume of 50 μL per well, where either acyl substrate in 2-fold dilution series [2.5−0.039 mM (for **2a**), 100−0.78 μM (for **2b**), and 50−0.78 μM (for **2c**−**d**) with NAD^+ (500 μM)] or NAD^+ in 2-fold dilution series [250−7.8 μM with substrate **4d** (20 μM)] were incubated with trypsin (40.0 ng/μL) and SIRT2 [500 nM (for **4a**) and 60 nM (for **4b**−**d**)]. In situ fluorophore release was monitored immediately by fluorescence readings recorded continuously every 10−15 s for 60 min at 25 °C to obtain initial rates ν_0 (nM s^{-1}) for each concentration. The data were fitted to the Michaelis−Menten equation to afford K_m (μM) and k_{cat} (s^{-1}) values.

Inhibition of SIRT2-Mediated Deacylase Activities. Hydrolase activities of sirtuin 2 against substrates **4a**−**d** were evaluated in HEPES buffer under varying concentrations of suramin (**9**) or SirReal2 (**10**) with end-point fluorophore release by trypsin. The sirtuin 2 enzyme (1000 nM for **4a**, 10 nM for **4b**−**d**) was incubated with the relevant substrate at the substrate K_m value (**4a** at 750 μM, **4b** at 6 μM, **4c** at 4.5 μM, **4d** at 1.8 μM), NAD^+ (200 μM), and inhibitor [suramin (**9**): 1000−1.6 μM for **4d**, 1000−0.32 μM for **4a**−**c** in 5-fold dilution series (final DMSO concentration ≤1%); SirReal2 (**10**): 20−0.16 μM in a 5-fold dilution series (final DMSO concentration ≤5%)] in a total volume of 25 μL. The reaction was incubated at 37 °C for 60 min, and then a solution of trypsin and nicotinamide (25 μL to give final concentrations of 0.2 mg/mL and 2 mM, respectively) was added. The assay development was allowed to proceed for 15 min at room temperature before fluorescence analysis. The data were fitted to the concentration−response equation with variable Hill slope to obtain IC_{50} values.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01532.

Michaelis−Menten plot for NAD^+, synthesis of (*Z*)-2-ene-, (*R*/*S*)-3-hydroxy-, and 3-oxo-carboxylic acids, additional experimental procedures, additional compound characterization data, definition of abbreviations used, as well as copies of ^1H NMR and ^{13}C NMR spectra (PDF)

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by The University of Copenhagen, The Lundbeck Foundation (Young Group Leader Fellowship, C.A.O.), The Danish Independent Research Council—Production and Technical Sciences (Sapere Aude grant no. 12-132328, A.S.M.), and The Carlsberg Foundation (equipment). We thank the COST action CM1406 (EPICHEMBIO) for support.

■ ABBREVIATIONS USED

ADPR, adenosine diphosphate ribose; AMC, 7-amino-4-methylcoumarin; BSA, bovine serum albumin; Dec, decanoyl; DIC, *N*,*N*′-diisopropylcarbodiimide; DLAT, dihydrolipoamide acetyltransferase; Glut, glutaryl; H3, histone 3 protein; H4, histone 4 protein; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HDAC, histone deacetylase; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; h, hour; KDAC, lysine deacylase; K_{ac}, ε-*N*-acetyllysine; K_{cr}, ε-*N*-crotonyllysine; K_{dec}, ε-*N*-decanoyllysine; K_{glut}, ε-*N*-glutaryllysine; K_{lau}, ε-*N*-lauryllysine; K_{mal}, ε-*N*-malonyllysine; K_{myr}, ε-*N*-myristoyllysine; K_{suc}, ε-*N*-succinyllysine; Lau, lauryl; MS, mass spectrometry; Myr, myristoyl; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; RFU, relative fluorescence units; rt, room temperature; SIRT, sirtuin; SPPS, solid-phase peptide synthesis; Suc, succinyl; TFA, trifluoroacetic acid; t_R, retention time; UPLC, ultrahigh performance liquid chromatography; VLC, vacuum liquid chromatography

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DOI: 10.1021/acs.jmedchem.5b01532
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