Computational methods

The molecular structures of all compounds analyzed in the present study were generated using the MOE 2011.10 modeling software (Chemical Computing Group, Montreal, Canada). The OpenEye (OpenEye Scientific Software Inc., Santa Fe, USA) utilities flipper and pkatyper were used to enumerate all E/Z isomers and relevant protonation states. Initial ligand conformations resulted from an energy minimization using the MMFF94x force field as implemented in MOE.

The crystal structures of human Sirt5 in complex with succinylated and malonylated peptides were retrieved from the Protein Databank (PDB code 3RIY and 3RIG). For the subsequent docking studies, all water and ligand molecules were removed and both structures were protonated and minimized using the Amber99 force field.

At the beginning we evaluated the docking performance of the GOLD5.1 docking program (Cambridge Crystallographic Data Centre, Cambridge, UK) by re-docking the co-crystallized ligands NAD^+ and succinylated lysine substrate to one selected Sirt5 structure (PDB 3RIY). The obtained docking accuracy (RMSD to crystal structure) was sufficient for all four scoring functions implemented in GOLD (e.g. RMSD for NAD^+: 0.88ang (GoldScore), 1.14ang (ChemScore), 0.96ang (ASP) and 1.60ang (PLP)).

Ensemble docking of all compounds to both protein structures was carried out with all four scoring functions and default search settings. The binding cavity was defined by the co-crystallized succinylated peptide.

Each inhibitor was docked 15 times with each scoring function and the resulting 60 poses per ligand were analyzed by calculating protein-ligand interaction fingerprints (PLIF) as implemented in MOE.

Docking solutions generated by GoldScore and ChemScore showed a significant bias (overrepresented fingerprint) towards one particular binding mode that is presented in the current work. This binding mode got also the highest GoldScore and/or ChemScore rank for 12 out of 15 active thiobarbiturates. For 3 thiobarbiturates the selected poses were among the five top-ranked ones. PLP and ASP scoring functions could not produce a consistent binding mode and were not further considered.

Recombinant protein expression and purification of Sirtuin isoforms

Vectors containing the human sirtuin genes were kindly provided by Prof. Dr. A. Salminen (University Kuopio, Finland, modified pGEX2T comprising Sirt1 gene coding for $Sirt1_{133-747}$) and Prof. Dr. E. Verdin (Gladstone Institute of Virology and Immunology, San Francisco, USA. Sirt2: modified pGEX2T vector comprising Sirt2 gene coding for $Sirt2_{25-389}$, Sirt3: pTrcHis2C comprising Sirt3 gene coding for $Sirt3_{101-399}$, Sirt5: pTrcHis2C comprising Sirt5 gene coding for $Sirt5_{39-310}$). All sirtuin isoforms were expressed in *E. coli* BL21 DE3 strains (Sirt1: BL21 DE3 Codonplus RIPL overnight at 18 °C, Sirt2/3: BL21 DE3 pLysS for 4 h at 37 °C, Sirt5: BL21 DE3 overnight at 18°C). Overexpression was induced at an OD_{600} of 0.5-0.7 by adding IPTG (Sirt1: 0.1 mM, Sirt2/3: 1 mM, Sirt5: 0.15 mM). After centrifugation, the bacterial pellet was resuspended in lysis buffer (Sirt1: Tris*HCl 50 mM, NaCl 400 mM, glycerol 10% (v/v), β-ME 10 mM, EGTA 1 mM, Protease inhibitor tablets (Roche), pH 8.0; Sirt2/3: Tris*HCl 50 mM, NaCl 300 mM, imidazole 20 mM, β-ME 2 mM, Protease inhibitor tablets (Roche), pH 8.0; Sirt5: PBS, Tween 20 1% (v/v), glycerol 10% (v/v), Protease inhibitor tablets (Roche), pH 7.4) and lysed using a microfluidizer (Microfluidics, Newton, USA). Cell debris was removed via centrifugation and cell lysate was applied to an affinity chromatography column (Sirt1: GSTrapFF 5 mL, Sirt2/3/5: HisTrapHP 5 mL, GE Healthcare, Freiburg, Germany). After immobilisation, the tagged protein was washed intensively and eluted using GSH (Sirt1, 25 mM) or imidazole (Sirt2/3/5, 300 mM). Fractions containing the appropriate sirtuins were pooled, concentrated and applied to a gel filtration column (Sirt1: Superdex 26/60 S200, Sirt2/3/5: Superdex 26/60 S75, GE Healthcare, Freiburg, Germany) equilibrated in gel filtration buffer (Tris*HCl 50 mM, NaCl 50 mM, glycerol 10% (v/v), DTT 0.2 mM, pH 8.0). Fractions after the gel filtration were analyzed via SDS-PAGE and the fractions containing the appropriate sirtuin isoform were pooled, concentrated and flash-frozen. The purity of the concentrated sirtuin solution was above 75% (Sirt1) or above 90% (Sirt2/3/5).

HPLC method to determine purity of compounds

HPLC purity determinations were performed on a JASCO HPLC system under isocratic conditions, using a Phenomenex Synergi Hydro RP-C18 column (250 mm x 4.6 mm, 4 µm particle size) (A) or Phenomenex Synergi 4µ MAX-RP 80 Å (150 mm x 4.6 mm) (B). Elution was performed using 0.035% of TFA in H_2O/ACN 55/45 (v/v) (A) or 0.05% of TFA in H_2O/ACN 70/30 (v/v) (B) at room temperature. Injection volumes were 2 µL, flow rate was 1 $mLmin^{-1}$ (A) and 0.5 $mLmin^{-1}$ (B), detection was performed with UV (λ=210 nm (A) and 254 nm (B)). ZK(s)A purity was measured via method (A), thiobarbiturates via method (B). The purity of all tested compounds were ≥ 95%, as measured by HPLC.

Synthesis of benzyl N-(5-{[(tert-butoxy)carbonyl]amino}-1-[(4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl]pentyl)carbamate: ZK(b)A

Compound **Z-*L*-Lys(Boc)-OH** (400 mg, 1.05 mmol) was solved in 10 mL of dry DMF at 0 °C, together with 7-amino-4-methylcoumarin (221 mg, 1.26 mmol) and DIPEA (460 µL, 2.63 mmol). Propylphosphonic anhydride (T3P®) (50% w/w solution in DMF, 2 mL) was added dropwise and the reaction stirred for 30 min at the same temperature, then left overnight at room temperature. When TLC indicated complete consumption of starting material, the reaction mixture was poured into a separating funnel containing an aqueous solution of NaOH 1 M. The mixture was extracted with EtOAc. The combined organic phases were collected and washed with a citric acid 5% solution, and brine. The organic layer was dried over $MgSO_4$, filtered, and evaporated to give a

crude reaction mixture, which was treated with diethyl ether. The precipitate was filtrated and purified by flash column chromatography on silica gel with 2% methanol in DCM as eluent to furnish the titled compound **ZK(b)A** (Figure SI3) as a white solid (400 mg, 71%): mp 111 °C. ^{1}H NMR (DMSO-d_6) **δ** 10.47 (s, 1H), 7.77 (d, *J* = 1.7 Hz, 1H), 7.73 (d, *J* = 8.7 Hz, 1H), 7.63 (d, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 1.7, 8.7 Hz, 1H), 7.39-7.14 (m, 5H), 6.76 (t, *J* = 5.4 Hz, 1H), 6.27 (s, 1H), 5.04 (s, 2H), 4.18-4.09 (m, 1H), 2.93-2.85 (m, 2H), 2.41 (s, 3H), 1.73-1.56 (m, 2H), 1.43-1.27 (m, 13H); ^{13}C NMR (DMSO-d_6) **δ** 172.4, 160.4, 156.6, 156.4, 154.1, 153.5, 142.6, 137.3, 128.7, 128.3, 128.2, 126.3, 115.7, 115.6, 112.7, 106.1, 77.7, 65.9, 56.0, 31.7, 29.6, 28.7, 23.3, 18.4; LRMS (ESI) *m/z* 538 $[M + H]^+$.

Synthesis of benzyl N-{5-amino-1-[(4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl]pentyl}carbamate: ZKA

Compound **ZK(b)A** (250 mg, 0.46 mmol) was solved at 0 °C in 3 mL of dry DCM, and trifluoroacetic acid (175 µL, 2.3 mmol) was added dropwise. When addition was complete, the cooling bath was removed, and the mixture stirred for 3 h. The solvent was evaporated to dryness and the residue was dissolved with EtOAc and washed with a saturated $NaHCO_3$ aqueous solution. The organic phase was dried over anhydrous Na_2SO_4, evaporated to dryness and the residue was washed with Et_2O. The precipitate was filtered and purified by flash column chromatography on silica gel with 10% methanol in DCM as eluent to furnish the titled compound **ZKA** (Figure SI4) as a white solid (170 mg, 83%): mp 170 °C. ^{1}H NMR (DMSO-d_6) **δ** 10.56 (s, 1H), 7.84-7.56 (m, 5H), 7.51 (dd, *J* = 1.8, 8.5 Hz, 1H), 7.40-7.13 (m, 5H), 6.28 (s, 1H), 5.05 (s, 2H), 4.21-4.04 (m, 1H), 2.77 (t, *J* = 7.5 Hz, 2H), 2.40 (s, 3H), 1.79-1.61 (m, 2H), 1.60-1.50 (m, 2H), 1.47-1.31 (m, 2H); ^{13}C NMR (DMSO-d_6) **δ** 172.2, 160.4, 156.5, 154.1, 153.5, 142.7, 137.4, 128.8, 128.3, 128.2, 126.4, 115.7, 115.5, 112.7, 106.2, 66.0, 55.9, 39.0, 31.4, 27.1, 22.9, 18.4; LRMS (ESI) *m/z* 438 $[M + H]^+$.

Syntheses of #13, #14, #15 were published in [1]. Compounds were used with the purity as stated in the original manuscript.

Synthesis of 5-[4-(4-Methoxyphenyl)benzylidene]-2-thiobarbituric acid, #10

Synthesis of the starting material 4-(4-Methoxyphenyl)benzaldehyde: 4-Methoxyphenylboronic acid (0.61 mmol), 4-bromobenzaldehyde (0.45 mmol), Pd/C 10% (3.5 mol% [aldehyde]) and sodium carbonate (0.171 mmol) were placed in a Schlenk tube and suspended in a mixture of water/ethanol (1:1). After evacuation and nitrogen gassing (3x), the suspension was stirred for 5 hours at room temperature. After filtering with a microfilter, the mixture was extracted three times with brine and diethyl ether, dried over sodium sulfate and evaporated in vacuo.[2] Yield: 93% of a white solid; ^{1}H NMR (DMSO-d_6) **δ** 10.03 (s, 1H), 7.98 - 7.94 (m, 2H), 7.90 - 7.86 (m, 2H), 7.77 - 7.72 (m, 2H), 7.10 - 7.05 (m, 2H), 3.82 (s, 3H); no mp, ^{13}C NMR spectrum or mass spectrometry were performed

#10 was synthesized according to standard methods.[1] Yield: 53% of an orange powder; mp: 320 °C. ^{1}H NMR (DMSO-d_6) **δ** 12.46 (s, 1H), 12.36 (s, 1H), 8.31 - 8.34 (m, 2H), 8.30 (s, 1H), 7.76 - 7.82 (m, 4H), 7.05 - 7.10 (m, 2H), 3.83 (s, 3H); ^{13}C NMR (DMSO-d_6) **δ** 178.87, 162.23, 160.37, 160.01, 155.81, 144.57, 135.39 (2C), 131.39, 131.36, 128.66 (2C), 126.00 (2C), 118.58, 115.06 (2C), 55.73 (CH_3); MS (EI) *m/z* 312.1 $[M]^+$

Synthesis of 5-[5-(2,3-Dichlorophenyl)-furyl-2-idene]-2-thiobarbituric acid, #1

Yield: 54% of a red substance; mp: dec. ^{1}H NMR (DMSO-d_6) **δ** 12.34, 12.28 (2 s, 2H), 8.62-8.55 (m, 1H), 8.13 (s, 1H), 8.09-8.03 (m, 1H), 7.80-7.74 (m, 1H), 7.62-7.58 (m, 1H), 7.57-7.51 (m, 1H); ^{13}C NMR: no data due to deficient solubility; MS (APCI, NH_4) *m/z* 366.0(Cl^{35} + Cl^{35}) $[M - H]^-$, 368.0(Cl^{35} + Cl^{37}) $[M - H]^-$, 370.0 (Cl^{37} + Cl^{37}) $[M - H]^-$.

Synthesis of 5-[4-(4-Bromobenzyloxy)benzylidene]-2-thiobarbituric acid, #11

#11 was synthesized according to published methods.[1] Yield: 79% of a yellow solid; mp: 296 °C. ^{1}H NMR (DMSO-d_6) **δ** 12.40 (s, 1H), 12.30 (s, 1H), 8.44 - 8.39 (m, 2H), 8.27 (s, 1H), 7.64 - 7.59 (m, 2H), 7.47 - 7.42 (m, 2H), 7.18 - 7.13 (m, 2H), 5.25 (s, 2H); ^{13}C NMR (DMSO-d_6) **δ** 178.71, 163.18, 162.60, 160.36, 156.22, 138.26 (2C), 136.18, 131.89 (2C), 130.45 (2C), 125.99, 121.69, 116.28, 115.24 (2C), 69.30; MS (CI, NH_3) *m/z* 416.9 $[M + H]^+$ (Br^{79}, 100), 418.9 $[M + H]^+$ (Br^{81}, 100).

Synthesis of N-Methyl-2-thiobarbituric acid (Scheme SI2)

The compound was synthesized according to standard procedures.[3] Yield: 82% of a slightly rose substance.

Synthesis of N-Ethyl-2-thiobarbituric acid (Scheme SI2)

The compound was synthesized according to standard procedures.[3] Yield: 52% of a pastel pink substance.

Synthesis of N-Allyl-2-thiobarbituric acid (Scheme SI2)

The compound was synthesized according to the same methods described above. Yield: 67%.

General Procedure for the synthesis of arylidene-N-alkyl-2-thiobarbituric acids using the example of the synthesis of N-Methyl-5-(3-indolydene)-2-thiobarbituric acid, #4 (Scheme SI2)

N-methyl thiobarbituric acid (2 mmol) was solved in 26 mL HCl in ethanol and heated to 50 °C. 2 mmol of Indole-3-carboxaldehyde in 9 mL HCl in ethanol was added and stirred until an orange precipitate was formed. The precipitate that formed was filtered, washed two times each with ethanol, water and diethyl ether and dried in vacuo to give 387 mg (67%) of a shiny orange powder. NMR spectroscopy showed cis/trans isomers in a ratio of approx. 1/1; mp: dec. ^{1}H NMR (DMSO-d_6) **δ** 13.02, 13.00 (2 br s, 1H), 12.38, 12.35 (2 s, 1H), 9.62, 9.60 (2 m, 1H), 8.78 (s, 1H), 7.93-7.87 (m, 1H), 7.65-7.59 (m, 1H), 7.40-7.31 (m, 2H), 3.63, 3.60 (2 s, 3H); ^{13}C NMR (DMSO-d_6) **δ** 179.10, 179.01, 163.30, 161.60, 161.20, 159.95, 146.34, 145.62, 141.80, 141.63, 137.07, 129.57, 129.51, 124.56, 123.62, 118.31, 118.27, 113.85, 113.18, 112.88, 109.13, 109.07, 34.10, 33.64; MS (APCI, NH_4) *m/z* 284.3 $[M - H]^-$.

Synthesis of N-Ethyl-5-(3-indolydene)-2-thiobarbituric acid, #5

Yield: 387 mg (67%) of a shiny orange powder; mp: dec. ^{1}H NMR (DMSO-d_6) **δ** 13.00, 12.98 (2 br s, 1H), 12.33, 12.29 (2 s, 1H), 9.62, 9.60 (2 m, 1H), 8.79, 8.78 (2 s, 1H), 7.94-7.87 (m, 1H), 7.65-7.58 (m, 1H), 7.38-7.32 (m, 2H), 4.38 (dq, 2H, J= 11.2, 7.0 Hz), 1.22 (dt, 3H, J= 7.0, 7.0 Hz); ^{13}C NMR (DMSO-d_6) **δ** 178.45, 178.38, 162.69, 161.53, 160.67, 159.86, 146.33, 145.66, 141.83, 141.72, 137.10, 137.09, 129.58, 129.51, 124.55, 123.62, 118.32, 118.29, 113.87, 113.85, 113.25, 112.92, 109.21, 109.19, 41.73, 41.29, 12.83, 12.77; MS (APCI, NH_4) *m/z* 298.2 $[M - H]^-$.

Synthesis of N-Methyl-5-(4-benzyloxybenzylidene)-2-thiobarbituric acid, #8

Yield: 387 mg (51%) of a shiny yellow powder; mp: 235 °C. ^{1}H NMR (DMSO-d_6) **δ** 12.53, 12.46 (2 br s, 1H), 8.46-8.41 (m, 1H), 8.40-8.34 (m, 1H), 8.32, 8.30 (2 s, 1H), 7.51-7.33 (m, 5H), 7.19-7.13 (m, 2H), 5.26 (s, 2H), 3.57, 3.56 (2 s, 3H); ^{13}C NMR (DMSO-d_6) **δ** 179.73, 179.63, 163.55, 163.45, 162.86, 161.02, 160.43, 159.01, 157.67, 156.88, 138.39, 138.16, 136.65, 128.96, 128.56, 128.35, 125.98, 125.79, 116.34, 116.28, 115.27, 115.23, 70.17, 34.26, 33.87; MS (APCI, NH_4) *m/z* 351.1 $[M - H]^-$.

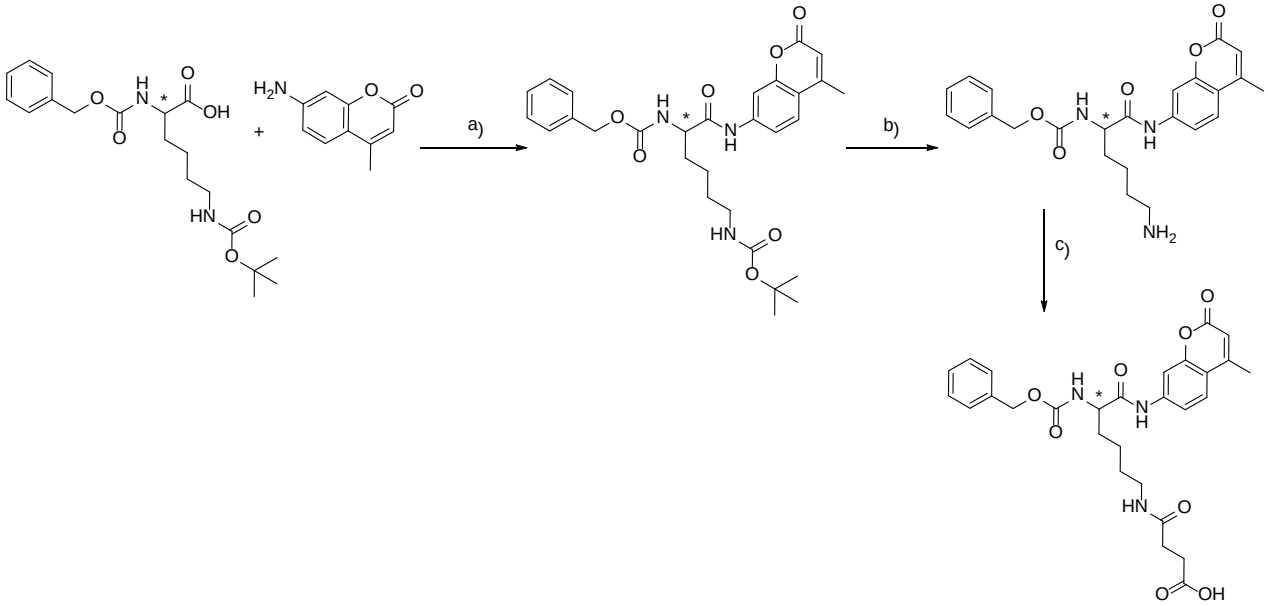
Synthesis of N-Ethyl-5-(4-benzyloxybenzylidene)-2-thiobarbituric acid, #9

Yield: 230 mg (63%) of a shiny yellow powder; mp: 223 °C. ^{1}H NMR (DMSO-d_6) **δ** 12.49, 12.42 (2 br s, 1H), 8.45-8.36 (m, 2H), 8.33, 8.30 (2 s, 1H), 7.51-7.33 (m, 5H), 7.20-7.14 (m, 2H), 5.27 (s, 2H), 4.32 (q, 3H, J= 6.9 Hz), 1.19 (t, 2H, J= 6.9 Hz); ^{13}C NMR (DMSO-d_6) **δ** 179.04, 178.97, 163.54, 163.48, 162.24, 160.96, 159.86, 158.93, 157.64, 156.96, 138.37, 138.22, 136.66, 128.96, 128.56, 128.36, 126.01, 125.80, 116.46, 116.38, 115.25, 70.17, 41.98, 41.64; MS (APCI, NH_4) *m/z* 365.2 $[M - H]^-$.

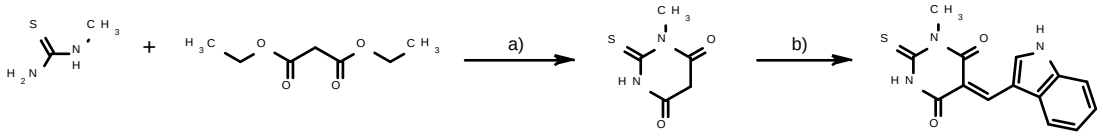
Synthesis of N-Allyl-5-(4-Benzyloxybenzylidene)-2-thiobarbituric acid, #10

Yield: 8% of a red substance; mp: 205 °C. ^{1}H NMR (DMSO-d_6) **δ** 12.46, 12.37 (2 br s, 1H), 8.48-8.30 (m, 2H), 8.30 (s, 1H), 7.54-7.31 (m, 5H), 7.25-7.12 (m, 2H), 5.80-5.92 (ddd, 1H, J= 17.4 Hz, J= 10.6 Hz, J= 4.9 Hz), 5.27 (s, 2H), 5.18 (dd, 2H, J= 17.4 Hz, J= 10.6), 4.93 (d, 2H, J= 4.9 Hz); ^{13}C NMR (DMSO-d_6) **δ** 191.72, 174.17, 174.00, 163.71, 162.84, 161.52, 156.39, 137.76, 136.73, 133.28, 132.22, 130.20, 128.93, 128.81, 128.78, 128.50, 128.35, 128.28, 128.16, 128.11, 128.05, 127.82, 115.72, 115.29, 114.71, 114.68, 114.42, 70.09, 69.57, 47.99; MS (APCI, NH_4) *m/z* 379.1 $[M + H]^+$.

Scheme SI1. Reagents and conditions: a) T3P®, DIPEA, dry DMF, r.t., 8h, 71%; b) TFA, dry CH_2Cl_2,



0°C, 3h, 83%; c) Succinic anhydride, DIPEA, dry THF, r.t., 8h , 76%.



Scheme SI2. Reagents and conditions: a) Na, EtOH, 4h reflux; b) HCl in ethanol, Indole-3-carbox-aldehyde, 50 °C

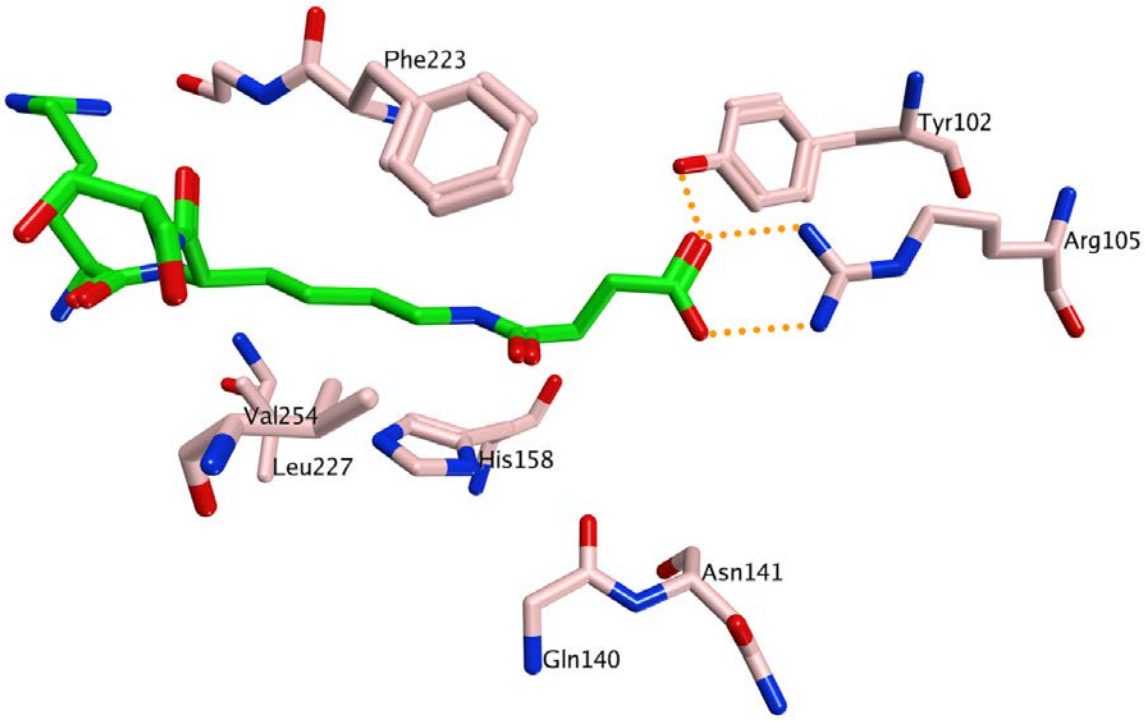


Figure SI1: Succinylated lysine peptide bound to Sirt5 (PDB code: 3RIY). The succinyl group shows strong electrostatic interactions with the side chains of Tyr102 and Arg105 (dashed orange lines).

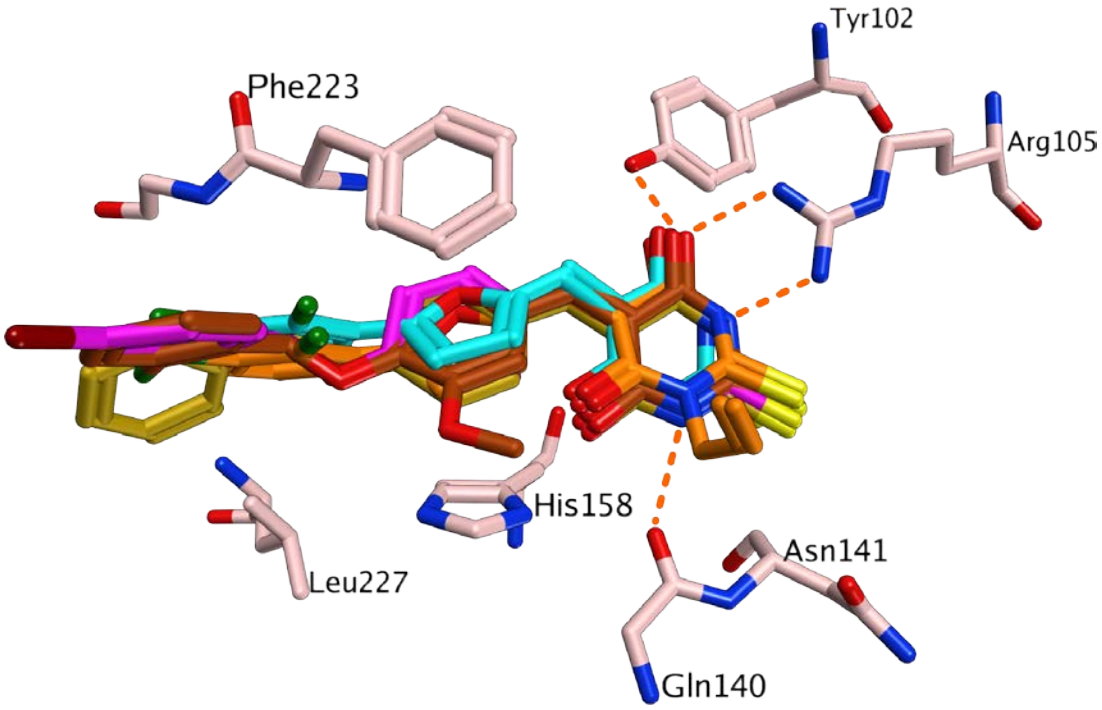


Figure SI2: Docking poses of the five most potent thiobarbiturates discovered in the current work. The acidic thiobarbiturate is mimicking the succinyl group of the substrate (dashed orange lines). Only interacting amino acid residues of Sirt5 are displayed.

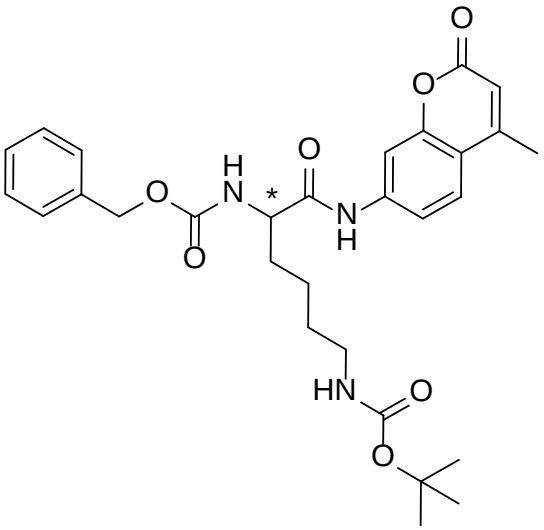


Figure SI3: Synthesis intermediate ZK(b)A

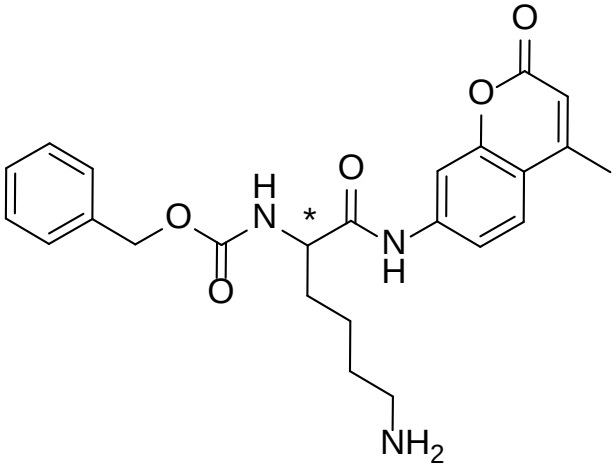


Figure SI4: Desuccinylated resp. deacetylated sirtuin metabolite ZKA

Compound	Chembridge # (CB)
#2	CB 5966223
#6	CB 5665891
#7	CB 5553831
#12	CB 5539622

Table SI1: Chembridge order numbers for commercially available inhibitors.

References for supplemental information

[1] Uciechowska, U., et al., Thiobarbiturates as sirtuin inhibitors: virtual screening, free-energy calculations, and biological testing. *ChemMedChem.*, **2008**, 3 (12), 1965-76.

[2] Zhang, G.L., Efficient protocol for the phosphine-free Suzuki-Miyaura reaction catalyzed by palladium on carbon at room temperature. *Synthesis-Stuttgart*, **2005** (4), 537-542.

[3] Dickinson, R.G. and N.W. Jacobsen, Hydrazinolysis of Heterocyclic-Compounds .2. 6-Amino-4-Hydroxy-1-Methylpyrimidine-2(1h)-Thione and Related Compounds. *Aust. J. Chem.*, **1975**, 28 (11), 2435-2446.