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Photochromic Indolyl Fulgimides as Chromo-Pharmacophores Targeting Sirtuins

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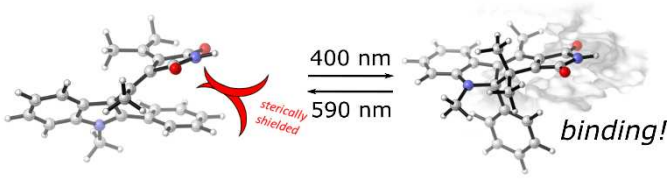


ABSTRACT: Sirtuins are involved in epigenetic regulation, the pathogenesis of cancer and, several metabolic and neurodegenerative diseases. Though being a promising drug target, only one small molecule passed class II clinical trials so far. Deriving a better mechanistic understanding is hence crucial to find new modulators. We previously reported on a series of dithienyl maleimides as photochromic tool compounds. However, their photochromic behavior was limited. To improve the interconversion and stability of both photoisomers, we replaced the dithienyl maleimide with a fulgimide as photochromic core to result in biologically active compounds reversibly addressable with purple and orange light. We characterize the obtained compounds regarding their spectroscopic properties, their photostability and binding characteristics towards sirtuins resulting in a fully remote-controllable Sirtuin modulator using visible light as the external stimulant.

INTRODUCTION

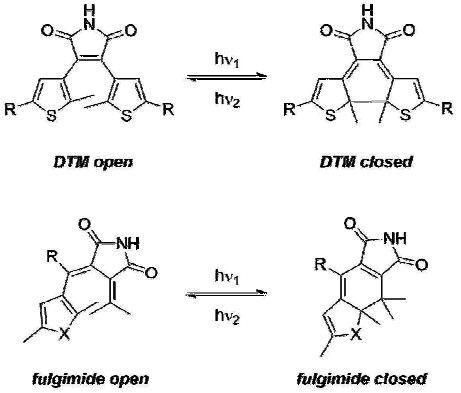
Histone deacetylases (HDACs) are an enzyme family involved in epigenetic regulation but have also been associated with the pathogeneses of cancer and several further human illnesses, like metabolic or neurodegenerative diseases. Hence, HDACs became an interesting target in both anticancer research and epigenetics. The enzyme family is dived in four subclasses. Class I, II and IV are zinc-dependent amidohydrolases. The members of class III (sirtuins) require NAD^+ as a co-substrate.[1-5] In the human genome, seven different sirtuin isotypes are encoded (Sirt1-7).[6] HDACs became an interesting target in both anticancer research and epigenetics. Indeed, several anticancer drugs targeting HDAC class I and II are already in clinical trial or approved therapeutics.[7] However, for sirtuins, only one small molecule inhibitor passed class II clinical trials so far, the indole-based inhibitor Selisistat (EX-527).[8] The most promising classes of small molecule inhibitors towards sirtuins up to this date include their endogenous inhibitor, namely nicotinamide and its analogues, splitomicins,[3] suramins,[9] chromon-4-ones, chromones,[10, 11] the human Sirt2 (hSirt2) selective SirReals, that induce a rearrangement in the active site of hSirt2,[12] thienopyrimidinones[13] and bisindolylmaleimides (BIMs).[2] To find new modulators of the sirtuins, a deeper mechanistic insight regarding their bioactivity is needed.

In the last decade, the development of small photochromic molecules in biological research has increased rapidly due to their highly diverse applicability. Moreover, the usage of light as an external control element in a biological context is very appealing as it is orthogonal to most cellular processes and can be easily regulated regarding dose, space and time.[14, 15] Especially for the investigation of enzymatic processes, photoswitches have been incorporated into larger biomolecules as non-natural amino-acids or have been used as photo-controllable enzyme ligands.[16] In this fashion, photoswitchable modulators for *e.g.* ATP synthase,[17] the respiratory chain,[18] kinases,[19, 20] proteasomes,[21] acetylcholinesterase,[22] the dopamine receptor,[23] and, HDACs[24, 25]are reported.

Diarylethenes (DAEs), fulgides and fulgimides are frequently employed as photochromic moiety since they usually form thermally very stable photoisomers. The pivotal photo-reaction of both photoswitches is a reversible ring closure and ring opening electrocyclization, interconverting a flexible open (less colored) and a rigid closed (more colored) photo-isomer (*cf.* Scheme 1).[16]

In order to control a biochemical process by means of a photoswitchable ligand, the photochromic moiety of the modulator should be in close proximity to a site that interacts strongly with the biomolecule.[26] Optimally, the interaction is fully intact in one photoisomeric state, but not in the other. We intended to fully exploit this concept by merging the biochemically interacting and the photochromic site in the same moiety, to form a so-called *chromo-pharmacophore*,[25] where the photochemical changes influence the bio interaction the most.

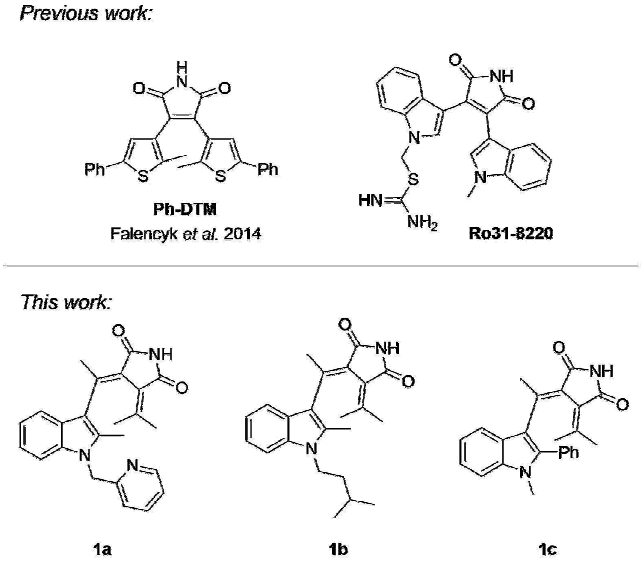
Scheme 1 Light-induced photo-isomerization of dithienylmaleimides (DTMs) and fulgimides: Irradiation with $h\nu_1$ triggers the ring-closure reaction to result in the more colored, rigid closed form. Through the application of suitable lower-energetic light ($h\nu_2$) the reaction can be reversed.



Previously, we reported the synthesis and biological evaluation of dithienylmaleimides (DTMs) as photocontrollable inhibitors for sirtuins (**Ph-DTM**, Chart 1).[25] The structure was inspired by Ro31-8220, which has been identified as a hSirt2 inhibitor.[2] Indeed, both molecules have in common a maleimide core. Whereas the parent inhibitor exhibits two indole substituents, the photoswitch has two thiophene groups attached to the core. It can be photoisomerized with the light of 312 nm and 530 nm exhibiting selectivity towards hSirt2 with one photoisomer showing a 22-fold better affinity than the other.

Although being a promising starting point in the development of photochromic sirtuin ligands, **Ph-DTM** faced two main limitations: 1) photo-isomerization has to be triggered applying highly energetic UV-light, which can be tolerated in enzyme assays, but could be a serious drawback for future application in more complex cellular- or tissue-based- assays; 2) photoinduced ring closure could only be facilitated in non-polar solvents due to a twisted intramolecular electron charge transfer within the photoswitch in polar environment (*e.g.* buffered aqueous systems). Thus, under assay conditions, photoswitching was only feasible in one direction.[25, 27-29]

Chart 1 Top: Previously developed dithienylmaleimide switch by Falencyk *et al.* inspired by the sirtuin bisindolylmaleimide (BIM) inhibitor Ro31-8220 (Published by the Royal Society of Chemistry).[25] Bottom: Indolyl fulgimides investigated in this work regarding improved photochromic behavior.



Here, we envisioned to replace the DTM core through indolyl fulgimides (**1a**-**c**, Chart 1) that are known to photoisomerize in aqueous systems.[23, 24] The indole moiety causes a bathochromic shift in the absorption spectrum of the molecule, allowing to trigger the ring-closing reaction of the switch with purple light (400 nm). In addition, indole allows further substitutions through functionalized alkyl chains as present in Ro31-8220 and many other BIMs.[2] Hence, we report the development of a small series of indolyl fulgimides as chromo-pharmacophores. We will investigate the compounds regarding their spectroscopic characteristics as well as their activity towards sirtuins.

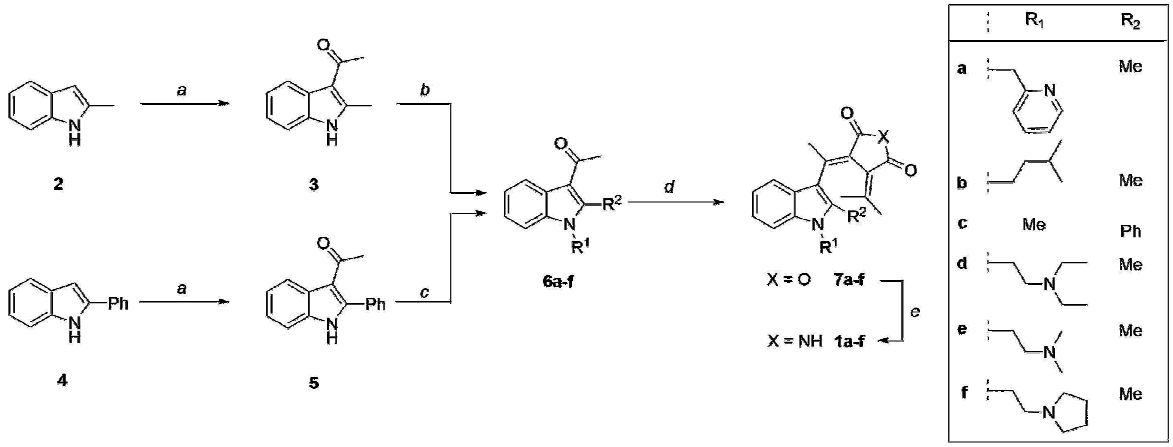
RESULTS AND DISCUSSION

Design and Synthesis. We sought to synthesize a small library of indolyl based fulgimides as the indole core is known to induce a bathochromic shift in the compound's absorption profile and can be *N*-alkylated with various side-chains (**1a**-**f**, Scheme 1).[30] Position 2 of the indole core is substituted with either a methyl group (**1a**, **b**, **d**-**f**) or a phenyl group (**1c**) to prevent oxidation upon ring-closure. As this substituent is involved in the electrocyclic ring-closure, usually a methyl group is used representing the smallest possible choice.[1] However, we envisioned a larger geometrical difference upon isomerization if a phenyl ring is part of the rearrangement. Hence, we designed compound **1c** bearing this novel feature.

The synthesis towards fulgimides **1a**-**f** involved a Stobbe-condensation as key step.[24] We started from commercially available 2-Me or 2-Ph indole (**2** and **4**, Scheme 2). An acetyl group was installed in position 3 (83% or 64% yield, respectively) *via* a Vilsmeier-Haack reaction (path *a*, Scheme 2).[31] Compound **3** was treated with KOH and alkylated using various functionalized alkyl chlorides to generate substituted indoles **6a**, **b**, **d**-**f** (path *b*, 15-79%

yield). Similarly, compound **5** was treated with NaH and MeI to obtain derivative **6c** (path *c*, 79%). Subsequently, compounds **6a-f** were employed in a Stobbe condensation with diethyl 2-(propan-2-ylidene)succinate, resulting in fulgides **7a-c** (*d*, 16-19%). Additionally, **7a** and **7c** were successfully crystallized and their X-Ray structure was measured (Figure 1). Compounds **7d-f** turned out to be highly unstable during the isolation attempts (see Experimental Section), probably due to the tertiary aliphatic amine in the side chain. Thus, we decided to use the crude mixtures of **7d-f** in the last reaction step, involving the aminolysis of the fulgides with NH_3 and a subsequent ring-closure to the form the central pyrrolin-2,5 dinone ring using (trimethylsilyl)diazomethane and NaH (*e*, Scheme 2).[32] Fulgimides **1a-c** could be isolated after column chromatography and subsequent preparative HPLC (2-10% yield). A crystal structure of compound **1c** could be obtained (see Supporting Information). However, the formation of compounds **1d-f** was not observed.

Scheme 2 Synthesis of fulgimides 1a-f: *a*: $POCl_3$, DMA, 80 °C, 2h (3: 83%, 5: 64%). *b*: alkyl chloride, KOH, DMF, 80 °C, 20h (15-79%). *c*: MeI, NaH, DMF, 0→r.t., 16h, 79%. *d*: diethyl 2-(propan-2-ylidene)succinate, LDA, THF, N_2, -105 °C→r.t., 18h; then, KOH, EtOH, 70 °C, 24h; then, AcCl, 40 °C, 18h (16-19%, 7d-f directly converted). *e*: NH_3 (2M in MeOH), N_2, 18h; then, (trimethylsilyl)diazomethane, MeOH; then, NaH, THF (2-10%, 1d-f decomposed during the preparation).



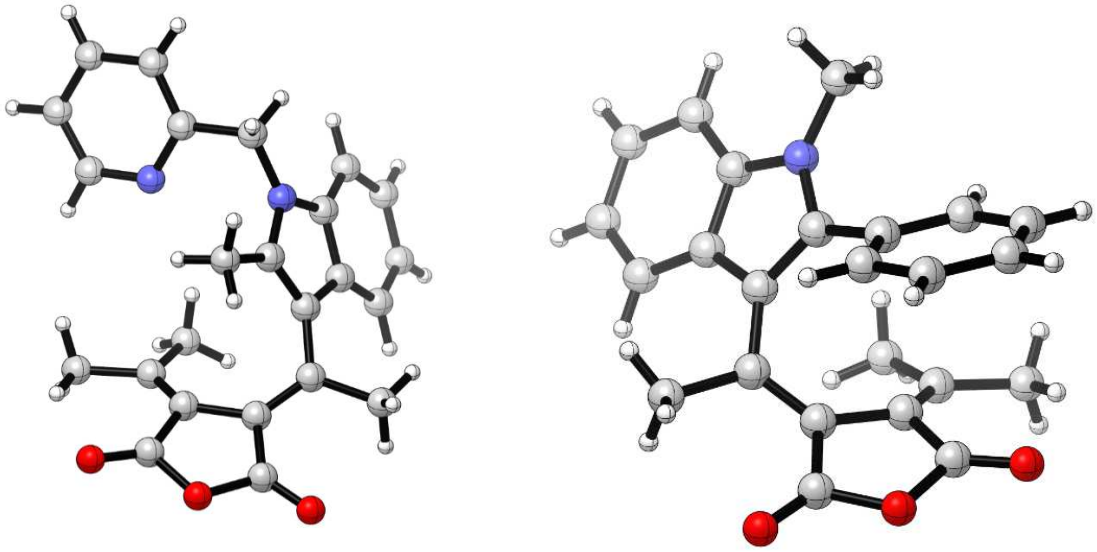
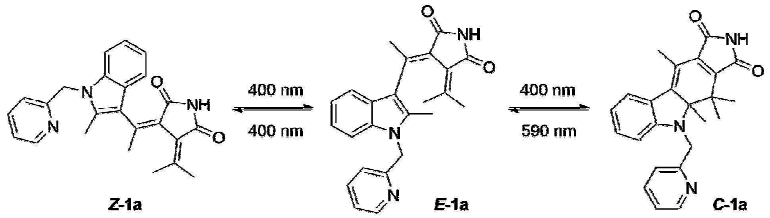


Figure 1. X-Ray structures of compounds **7a** (left) and **7c** (right).

Photochromic Properties. Fulgimides exhibit three distinct photoisomers: the two ring-opened isomers (in their *E* and *Z configuration*) and the ring-closed isomer (*C*). Scheme 3 exemplary shows the light-triggered isomerizations of compound **1a**. The open photoisomers interconvert between each another through irradiation with light of 400 nm.[30] However, only the *E*-isomer can cyclize under these conditions to form the closed isomer ***C*-1a**. The ring-closed photoisomer ***C*-1a** is thermally stable (stability test at 37 °C showed hardly any ring-opening of compounds ***C*-1a** and ***C*-1c** over four hours, which are comparable to assay conditions, but **1b** resulted in decomposition; *vide infra* and the Supporting Information) and can be re-opened photochemically through irradiation with light of 590 nm.

Scheme 3 Photoisomers of compound 1a: *Z*-1a can isomerize to *E*-1a under irradiation with 400 nm light reversibly. The same wavelength triggers the ring-closure reaction to form *C*-1a, which can be back-isomerized to its open form *E*-1a through irradiation with 590 nm light.



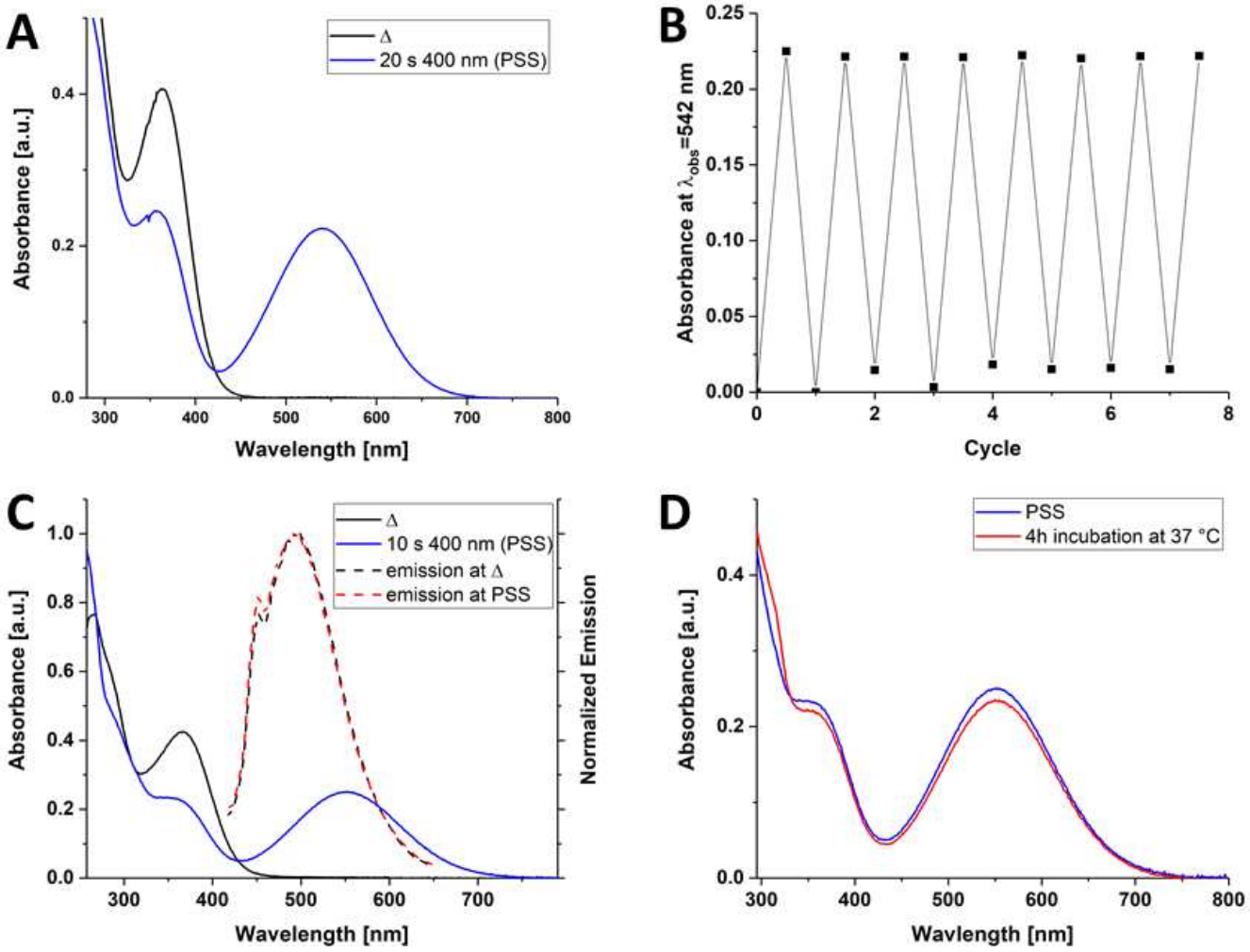


Figure 2. Spectroscopic properties of compound **1a** in various solvents. **A:** UV/Vis spectrum of **1a** 100 µM in DMSO (black: open form, blue: PSS). **B:** Fatigue resistance of **1a** 100 µM in DMSO over eight cycles (λ_{obs}= 542 nm). **C:** Spectra of **1a** 100 µM in assay buffer/DMSO 1:2 (solid black: UV/Vis spectrum of open form, solid blue: UV/Vis spectrum of PSS, dashed black: fluorescence emission spectrum of open form, dashed red: fluorescence emission spectrum at PSS). **D:** Temperature stability of ***C*-1a** at 37 °C.

The photochemical behavior of fulgimides **1a-c** was investigated in DMSO and buffered aqueous solutions (ZMAL assay buffer,[33] *vide infra* and the Supporting Information). The spectra of **1a** are shown exemplarily in Figure 2. The open isomers of **1a-c** exhibit the main absorption band around 380 nm, which is tailing into the visible region giving the compounds a bright yellow color at the thermodynamic equilibrium (see Figure 2 and the Supporting Information for further spectra as well as the composition of the thermodynamic equilibrium). Upon irradiation with light of 400 nm, the band around 380 nm decreases and a new, broad absorption band around 580 nm arises. The new band can be attributed to the ring-closed isomer, which UV/Vis spectrum is bathochromically shifted due to

its extended π-conjugation throughout the scaffold.[34] Hence, the solution at the photostationary state (PSS) appears violet in color.

Table 1 summarizes the photochromic properties of fulgimides **1a-c**. The PSS of the compounds was reached in 10-35 seconds and contained 19-73% of the closed isomer (determined through analytical HPLC, see Supporting Information for traces and Table S1 for a wider range of measurements). The quality of the PSS appears to strongly depend on the substitution pattern of the indole moiety. Indeed, the phenyl substituent in position 2 of **1c** seems to sterically hinder the ring-closure reaction. All fulgimides show excellent fatigue resistance in repetitive cycle performance (alternated irradiation with light of 400 nm and 590 nm, see Supporting Information). Noteworthy, compounds **1a-c** can be photoisomerized similar in DMSO and aqueous buffer DMSO mixtures, which is an important characteristic for application in a biological environment.

Another interesting spectroscopic characteristic was observed. While irradiating the samples at 400 nm we found that the compounds exhibit fluorescence. Hence, we recorded the emission spectra of compounds **1a-c** in a mixture of the assay buffer and DMSO (2:1) exciting the sample at 390 nm (*cf.* assay read-out, *vide infra*). All compounds showed fluorescence maxima around 480 nm in both photoisomers. Additionally, compounds **1a** and **1b** exhibit a second maximum at 450 nm.

Table 1. **Photochromic properties of fulgimides 1a-c (DMSO and buffer-DMSO-mixture at a concentration of 100 µM).**

Compound	Solvent	λ_{max}^{Δ} [nm]	λ_{max}^{closed} [nm]	$\varepsilon_{\lambda max}^{closed}$	Isosbestic Point [nm]	PSS[a] E/Z/C [%]
1a	DMSO	363	542	n.d.	420	n.d.[b]
	Buffer/DMSO (2:1)	366	552	n.d.[b]	431	n.d.[b]
	Buffer (5% DMSO)[c]	261	564	5599	421	62:19:19[d]
1b	DMSO	366	563	5803	421	23:10:66
	Buffer/DMSO (2:1)	274, 375	588	4451	335, 416	23:4:73
1c	DMSO	296, 364	583	3115	408	45:24:31
	Buffer/DMSO (2:1)	296, 372	606	5408	272, 436	53:18:29
	Buffer (5% DMSO)	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	65:3:32[d]

[a]after irradiation with λ=400 nm determined by HPLC. [b]n.d. = not determined. [c]the concentration was 25µM. [d]The solution was irradiated with 400 nm for one minute.

Biological Evaluation. The inhibition of three human sirtuin isoforms (hSirt1-3) by **1a** and **c** was tested with the fluorescence-based ZMAL assay (Compound **1b** did not appear to be thermally stable and was hence excluded from biological evaluation).[33] The photoswitches were incubated in a 96-well plate and tested at the thermodynamic equilibrium and at the PSS. Therefore, photoisomerization to the PSS was initiated directly in the assay mixture using a 96-LED irradiation setup (400 nm for 15 min, setup see Supporting Information). Comparison of the maximum activity of the sirtuins without inhibitor showed that irradiation alone did not have any influence on the enzymatic activity. Since the compounds are fluorescent, the intrinsic fluorescence under assay conditions was measured and subtracted from the total fluorescence intensity detected. Table 2 shows the inhibition of the three sirtuin isotypes at 100 µM in percent and the IC_{50} values for the most active compounds **1a** and **1c**.

For **1a** a 1.5-fold difference in inhibition towards hSirt 2 between the thermodynamic equilibrium and the PSS was detected. The 2-phenyl group in **1c** is untypical in classical fulgimides and was expected to result in a greater steric demand difference of the two photoisomers. Indeed, exhibiting an IC_{50} value of 29.6 µM a higher inhibition of hSirt2 was detected at the PSS as compared to the thermodynamic equilibrium. Considering that at the PSS only around 30 % of the closed isomer can be achieved (*vide supra*), the closed form of **1c** appears to be a significantly better inhibitor of hSirt2 than the open one. Indeed, it was not possible to calculate an IC_{50} value for the open isomer, since the maximum inhibition for concentrations up to 500 µM was too low (<50 %).

To visualize these findings, we optimized the structures of both photoisomers of **1a** and **1c** at the M06-2x/def2TZVP level of theory (Figure 3, see Supporting Information for details). Like in the X-ray structure of its precursor (**7c** in Figure 1) the phenyl substituent in ***O*-1c** is orientated parallel to the succinimide moiety. As latter is essential for binding towards the enzyme, we suppose that the phenyl-ring could shield this interaction. In contrast, in ***C*-1c** the phenyl ring is orientated in an approximately 90-degree angle with respect to the succinimide moiety, hence, facilitating an interaction towards the active site. In both ***O*-1a** and ***C*-1a**, an interaction is possible. Changes in geometry take place mainly in the photochromic moiety of

the compound leading to slightly altered binding properties.

Table 2: Percentage inhibition of sirtuins at 100 µM or IC_{50} in µM.

	hSirt1		hSirt2		hSirt3	
Comp.	**Δ[a]**	**PSS**	**Δ[a]**	**PSS**	**Δ[a]**	**PSS**
1a	20.7 %	20.0 %	19.9 ± 1.6 µM	30.4 ± 3.5 µM	22.6 %	n.i.[b]
1c	n.i.[b]	n.i.[b]	28.8 %	29.6 ± 4.8 µM	4.7 %	n.i.[b]

[a] Δ = thermodynamic equilibrium. [b]n.i. = no inhibition at 100 µM.

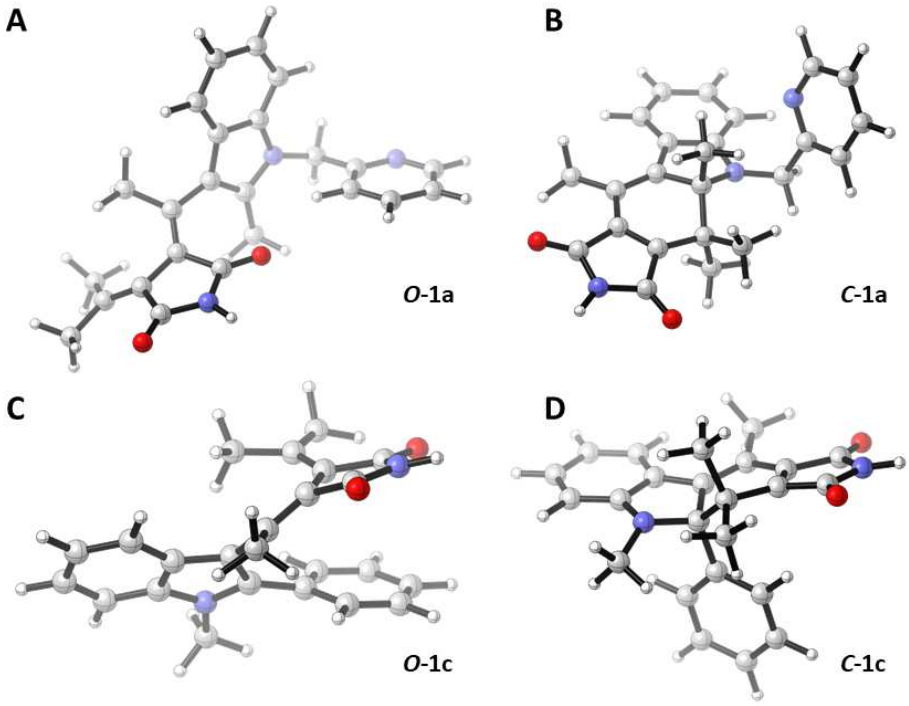


Figure 3: Simulated structures of **1a** (**A:** open isomer, **B:** closed isomer) and **1c** (**C:** open isomer, **D:** closed isomer) at the M06-2x/def2TZVP level of theory.

CONCLUSIONS

In summary, we attempted to improve the photochromic properties, *i.e.* the reversible photo-isomerization in aqueous environment and bathochromic shift of the irradiation wavelength, of previously developed chromo-pharmacophores. Thus, we replaced the previously used DTM-based scaffold with a fulgimide. Spectroscopic characterization of three novel fulgimides showed good photoisomerization properties in both DMSO and DMSO buffer mixtures. Photoisomerization cycles could be induced with purple (400 nm) and orange (590 nm) light several times, without significant loss of performance. Moreover, the fulgimides exhibit fluorescence emission when irradiated with 390 nm or 400 nm light. Testing of **1a** and **c** towards hSirt1-3 in the well-established ZMAL-assay showed inhibition of hSirt2. For compound **1a** an IC_{50} value of 19.9 µM was obtained at the thermodynamic equilibrium. Inhibition at the PSS was approximately 1.5-fold lower. Also, **1c** showed a difference in affinity. At the thermodynamic equilibrium, the maximum inhibition by **1c** at the highest concentration tested (500 µM) was still below 50 % and only the IC_{50} value at the PSS could be determined to be 29.6 µM. This behavior could be visualized simulating the structures of both photoisomers. We found that the parallelly oriented phenyl moiety in the ***O*-1c** is shielding the succinimide moiety and consequently preventing binding to the active site. Hence, we could improve the photochromic properties of chromo-pharmacophores towards sirtuins as desired. Albeit succeeding in our goal, we partially lost affinity and difference in inhibition of both photoisomers compared to our previously reported series. Further structural optimizations of the presented compounds could finally lead to a reversibly *in situ* controllable sirtuin ligand using visible light.

EXPERIMENTAL SECTION

General Remarks. All reactions were performed using magnetic stirring under ambient atmosphere unless otherwise specified. Reactions requiring anhydrous conditions were carried out under dry nitrogen or argon atmosphere using standard Schlenk techniques. All reagents and solvents were obtained from commercial suppliers and used without further purification if not otherwise stated. For TLC, silica-coated aluminum plates (Macherey-Nagel ALUGRAM Xtra SIL G, 0.2 mm) were utilized. Visualization was done with UV light at 254 or a 400 nm LED (to induce photo-isomerization on the TLC plate). For MPLC a Biotage Isolera One Flash Purification System with manually packed columns using Macherey- Nagel GmbH & Co. KG 60M (0.04-0.063 mm, 230-400 grain diameter) was utilized. For preparative HPLC, a 1260 Infinity LC System from Agilent with a reverse phase Phenomenex Luna® 10µ C18(2) column (250 × 21.2 mm, 100 Å) was utilized. HPLC conditions were the following: solvent A = water (Millipore), solvent B = MeCN (Fisher scientific, gradient grade); flow rate = 22 mL/min. NMR measurements were carried out using a Bruker Avance 400 MHz spectrometer (^{1}H: 400 MHz, ^{13}C: 101 MHz, T = 300K) or a Bruker Avance 600 MHz spectrometer (^{1}H: 600 MHz, ^{13}C: 151 MHz, T = 300 K). Chemical shifts are reported in δ [ppm] relative to an internal standard (solvent residual peak). The used solvents are indicated for each spectrum. Coupling constants are reported in Hertz [Hz]. Characterization of the signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet, dd = doublet of doublet, dt = doublet of triplet. Integration is directly proportional to the number of the protons. Characterization of the ^{13}C-NMR signals: (+) for CH_3 or CH, (-) for CH_2 and (q) for quaternary C-atoms. The assignment resulted from DEPT135 and HSQC experiments. High resolution mass spectra (HR-MS) were recorded with an Agilent Tech 6540 UHD Accurate Mass Q-TOF LC/MS spectrometer. IR spectroscopy was done using an Agilent Cary 630 FTIR.

For UV/Vis measurements, an Agilent 8453 spectrometer was employed. For temperature control, a Varian Cary Single cell Peltier apparatus was used. The used solvent is stated for each experiment. UV-induced isomerizations were performed using an Edison Edixeaon LED (395-405 nm, 0.35 W) or an OSRAM Oslon SSL 80 (590 nm, 0.9 W). Sample volume was 3 mL. The purity of the compounds was analyzed through separation on a 1220 Infinity LC System from Agilent with a reverse phase Phenomenex Luna® 3µ C18(2) column (150 × 2.0 mm, 100 Å) thermostatted at 25 °C. HPLC conditions were the following: solvent A = water (Millipore)/TFA (0.05% v/v), solvent B = MeCN (Fisher scientific, gradient grade); flow rate = 0.3 mL/min; injection volume 5 µL, elution with a gradient of 5% to 98% MeCN in 20 min (for purity: 220 nm and 254 nm; for PSS: respective isosbestic point of the compound; DAD detector).

Synthesis. *General procedure for fulgimide formation.* Fulgide **7** (1.0 eq.) was dissolved in NH_3 (2M in MeOH, 1 mL per 0.2 mmol) under nitrogen atmosphere and stirred at ambient temperature for 18 h. The solvent was removed under reduced pressure. The residue was dissolved in toluene (1 mL per 0.4 mmol). MeOH (1 mL per 0.2 mmol) and (trimethylsilyl)diazomethane (2M in hexane, 1.5 eq.) were added and the reaction mixture was stirred for 1.5 h at ambient temperature. Then, the solvent was removed *in vacuo*. The crude mixture was dissolved in dry THF (1 mL per 0.4 mmol) and added to a stirred suspension of NaH (60% in paraffin, 5.4 eq.) in dry THF (1 mL per 0.4 mmol) at 0 °C. The mixture was stirred for 24 h at room temperature and quenched with water. The mixture was extracted with EA (three times, *ca.* 2 mL per 0.1 mmol). The combined organic layers were washed with water (*ca.* 2 mL per 0.1 mmol) and brine (*ca.* 2 mL per 0.1 mmol) and dried over Na_2SO_4. The solvent was removed under reduced pressure. Compound **1a**-**1c** were purified through MPLC (NH-capped column, 20-100% EA in PE) and preparative HPLC (MeCN in 0.05% TFA/water 5-95% over 20 min). Compounds **1d**-**f** decomposed during the purification process and could not be isolated.

*3-(1-(2-Methyl-1-(pyridin-2-ylmethyl)-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)pyrrolidine-2,5-dione (**1a**).* The product was obtained as yellow powder (6.9 mg, 0.018 mmol, 3% yield). ^{1}H NMR (300 MHz, Methanol-d_4) δ =8.51 (ddd, *J* = 4.9, 1.8, 0.9 Hz, 1H), 7.71 (td, *J* = 7.7, 1.8 Hz, 1H), 7.51 – 7.42 (m, 1H), 7.40 – 7.33 (m, 1H), 7.29 (ddd, *J* = 7.7, 4.9, 1.1 Hz, 1H), 7.18 – 7.08 (m, 2H), 6.88 (dd, *J* = 7.8, 1.1 Hz, 1H), 3.63 (s, 2H), 2.75 (s, 3H), 2.15 (s, 3H), 2.08 (s, 3H), 0.96 (s, 3H). ^{13}C{^{1}H}-NMR (151 MHz, Methanol-d_4) δ =170.2 (q), 169.9 (q), 157.2 (q), 148.9 (q), 147.7 (q), 143.5 (q), 137.5 (+), 137.1 (q), 134.1 (q), 125.7 (q), 124.7 (+), 124.2 (+), 122.7 (+), 121.6 (+), 120.9 (+), 120.2 (+), 119.1 (q), 117.4 (q), 109.3 (+), 70.2 (-), 25.1 (+), 21.3 (+), 20.3 (+), 10.8 (+). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{24}H_{23}N_3O_2^+$ 386.1863; found 386.1861. IR was not performed due to the small amount of compound obtained. HPLC: purity: >99%. R_t= 5.67 min and 6.14 min (73% *E* and 27% *Z* isomer).

*3-(1-(1-Isopentyl-2-methyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)pyrrolidine-2,5-dione (**1b**).* The compound was obtained as yellow solid (19 mg, 0.052 mmol, 10% yield). ^{1}H NMR (300 MHz, Methanol-d_4) δ =7.36 (ddt, *J* = 10.8, 8.0, 1.0 Hz, 2H), 7.16 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 7.10 – 7.01 (m, 1H), 4.15 (s, 2H), 2.72 (s, 3H), 2.19 (s, 3H), 2.11 (s, 3H), 1.70 – 1.60 (m, 1H), 1.60 – 1.51 (m, 2H), 1.01 (d, *J* = 6.4 Hz, 6H), 0.88 (s, 3H). ^{13}C{^{1}H}-NMR (75 MHz, Methanol-d_4) δ =171.7 (q), 171.4 (q), 148.6 (q), 145.5 (q), 137.9 (q), 135.0 (q), 126.9 (q), 126.4 (q), 125.2 (q), 122.6 (+), 121.2 (+), 120.4 (+), 118.3 (q), 110.5 (+), 42.6 (-), 40.1 (-), 27.2 (+), 26.3 (+), 23.0 (+), 22.9 (+), 21.8 (+), 12.1 HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{23}H_{29}N_2O_2^+$ 365.2224; found: 365.2224. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2956 (w), 2318 (w), 1737 (m), 1684 (m), 1580 (m), 1316 (m), 1245 (m), 734 (m). HPLC: purity: >99%. R_t= 5.75 min and 6.49 min (94% *E* and 6% *Z* isomer).

*3-(1-(1-Methyl-2-phenyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)pyrrolidine-2,5-dione (**1c**).* The compound was obtained as yellow powder (10.5 mg, 0.028 mmol, 2 % yield). ^{1}H NMR (400 MHz, Acetonitrile-d_3) δ =7.58 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.45 (dd, *J* = 8.2, 0.9 Hz, 2H), 7.42 – 7.34 (m, 1H), 7.27 (ddt, *J* = 8.1, 7.0, 1.0 Hz, 1H), 7.22 (dt, *J* = 7.0, 1.4 Hz, 2H), 7.16 (ddt, *J* = 8.0, 7.0, 1.0 Hz,

1H), 3.61 (s, 3H), 2.69 (s, 3H), 1.89 (s, 3H), 0.91 (s, 3H). $^{13}C\{^1H\}$-NMR (151 MHz, Acetonitrile-d_3) δ =170.4 (q), 168.8 (q), 147.5 (q), 143.4 (q), 139.4 (q), 139.1 (q), 132.7 (+), 130.8 (+), 129.4 (+), 129.4 (+), 126.7 (q), 125.8 (q), 125.4 (q), 123.3 (+), 121.5 (+), 121.0 (+), 117.9 (q), 111.3 (+), 31.9 (+), 26.1 (+), 22.7 (+), 21.7 (+). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{24}H_{23}N_2O_2^+$ 371.1754; found 371.1754. IR (neat, cm^{-1}) $\tilde{\nu}$ = 3168 (w), 3053 (w), 2952 (w), 1737 (s), 1689 (s), 1364 (s), 1326 (m), 1200 (m), 734 (s), 701 (s). HPLC: purity: >99%. R_t=3.41 min and 3.77 min (98% *E* and 2% *Z* isomer).

*1-(2-Phenyl-1H-indol-3-yl)ethan-1-one (**5**).* *N,N*-Dimethyl acetamide (DMA, 4.9 mL) was cooled to 0 °C. Then, $POCl_3$ (2.4 mL, 13.45 mmol) and 2-phenyl indole (2.0 g, 10.35 mmol) in DMA (2.6 mL) were added via syringe. The reaction mixture was heated to 80 °C for two hours. The cooled solution was then poured onto water (500 mL) and washed with diethyl ether (3x200 mL). The aqueous phase was treated with NaOH (2M, aq) to precipitate the product. The solids were isolated through vacuum filtration over a Büchner funnel and then dried at high vacuum. The product was obtained as beige powder (2.02 mg, 8.60 mmol, 64% yield). ^{1}H NMR (400 MHz, Acetone-d_6) δ =10.92 (*b*s, 1H), 8.37 – 8.25 (m, 1H), 7.67 – 7.59 (m, 2H), 7.57 – 7.46 (m, 3H), 7.45 – 7.40 (m, 1H), 7.26 – 7.07 (m, 1H), 2.04 (s, 3H). $^{13}C\{^1H\}$-NMR (101 MHz, Acetone-d_6) δ =193.4 (q), 144.6 (q), 135.8 (q), 133.3 (q), 130.0 (+), 129.3 (+), 128.5 (+), 127.7 (q), 123.0 (+), 122.2 (+), 121.8 (+), 115.1 (q), 111.2 (+), 29.5 (+). HR-MS (EI) m/z: [M$^{\bullet+}$] calculated for $C_{16}H_{13}NO$ 235.0997; found 235.0991. IR (neat, cm^{-1}) $\tilde{\nu}$ = 3176 (w), 3041 (w), 1610 (m), 1580 (m), 1408 (m), 1197 (m), 957 (m), 745 (s), 701 (s). R_f: 0.45 (40% EA in PE). Melting Point: 224 °C.

*General procedure for alkylation of indole derivatives (**6a**, **b**, **d-f**).* The acetylated indole **3** (1.0 eq.), KOH (5.0 eq.) and the appropriate alkylation reagent (1.5 eq.) were dissolved in DMF (1.5 ml per mmol). The reaction mixture was stirred for 20 h at 80 °C. The reaction mixture was diluted with water and extracted with EA (three times, each *ca.* 2 mL per mmol). The solution was washed with water (*ca.* 2 mL per mmol). The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified through automated flash column chromatography (conditions specified for each compound).

*1-(2-Methyl-1-(pyridin-2-ylmethyl)-1H-indol-3-yl)ethan-1-one (**6a**).* Purification through automated flash column chromatography (35-100% EA in PE). The product was obtained as yellowish powder (1014 mg, 3.84 mmol, 31% yield). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =8.60 (ddd, *J* = 4.9, 1.8, 0.9 Hz, 1H), 8.05-7.99 (m, 1H), 7,52 (td, *J* = 7.7, 1.8 Hz, 1H), 7.31-7.28 (m, 1H), 7,24 (dd, *J* = 13.5, 1.1 Hz, 1H), 7.22-7.19 (m, 1H), 7.19-7.15 (m, 1H), 6.59 (d, 7.9 Hz, 1H), 5.48 (s, 2H), 2.76 (s, 3H), 2.71 (s, 3H). $^{13}C\{^1H\}$-NMR (101 MHz, Chloroform-*d*) δ =194.8 (q), 156.0 (q), 149.7 (+), 144.8 (q), 137.2 (+), 136.3 (q), 126.5 (q), 122.7 (+), 122.4 (+), 122.2 (+), 120.9 (+), 120.1 (+), 114.9 (q), 109.8 (+), 48.39 (-), 31.8 (+), 12.7 (+). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{17}H_{17}N_2O^+$ 265.1335; found 265.1339. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2922 (m), 1625 (s), 1416 (s), 752 (s). R_f: 0.29 (50% EA in PE). Melting Point: 187 °C.

*1-(1-Isopentyl-2-methyl-1H-indol-3-yl)ethan-1-one (**6b**).* Purification through automated flash column chromatography (35-100% EA in PE, then up to 10% MeOH in EA). The product was obtained as beige solid (2141 mg, 8.80 mmol, 63% yield). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =8.09 – 7.76 (m, 1H), 7.33 (ddt, *J* = 6.3, 4.0, 2.0 Hz, 1H), 7.29 – 7.05 (m, 2H), 4.28 – 3.82 (m, 2H), 2.78 (s, 3H), 2.69 (s, 3H), 1.78 – 1.70 (m, 1H), 1.67 – 1.54 (m, 2H), 1.03 (d, *J* = 6.5 Hz, 6H). $^{13}C\{^1H\}$-NMR (101 MHz, Chloroform-*d*) δ =194.6 (q), 144.5 (q), 135.8 (q), 126.5 (q), 121.9 (+), 121.8 (+), 120.7 (+), 114.2 (q), 109.6 (+), 41.5 (-), 38.4 (-), 31.7 (+), 26.3 (+), 22.5 (q), 12.6 (+). HR-MS (EI) m/z: [M$^{\bullet+}$] calculated for $C_{16}H_{21}NO$ 243.1623; found 243.1615. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2956 (m), 2870 (m), 1638 (s), 1513 (m), 1461 (s), 1409 (s), 1372 (s), 1107 (m), 138 (s). R_f: 0.83 (EA). Melting Point: 152 °C.

*1-(1-Methyl-2-phenyl-1H-indol-3-yl)ethan-1-one (**6c**).* Compound **5** (886 mg, 5.0 mmol) was dissolved in DMF (12.5 mL) and cooled to 0 °C. NaH (210 mg, 5.25 mmol, 60% on paraffin oil) and MeI (342 µL, 5.50 mmol) were added in portions. The reaction was stirred at ambient temperature for 16 h. Then, the reaction mixture was poured onto water (100 mL) and extracted with ethyl acetate (3x50 mL). The combined organic phases were washed with NaOH (2M, aq, 50 mL) and dried over Na_2SO_4. The volatiles were removed *in vacuo*. Purification through automated flash column chromatography (25-85% EA in PE) efforted the product as pink powder (985 mg, 3.94 mmol, 79% yield). ^{1}H-NMR (400 MHz, Acetone-d_6) δ =8.64 (ddd, *J* = 7.7, 1.5, 0.8 Hz, 1H), 7.85 – 7.78 (m, 3H), 7.79 – 7.70 (m, 2H), 7.67 (dt, *J* = 8.1, 0.9 Hz, 1H), 7.55 – 7.38 (m, 2H), 3.72 (s, 3H), 2.04 (s, 3H). $^{13}C\{^1H\}$-NMR (101 MHz, Acetone-d_6) δ =192.7 (q), 146.4 (q), 136.8 (q), 132.4 (q), 130.5 (+), 129.6 (+), 128.8 (+), 126.9 (q), 123.0 (+), 122.4 (+), 122.1 (+), 115.4 (q), 109.9 (+), 30.3 (+), 29.3(+). HR-MS (EI) m/z: [M$^{\bullet+}$] calculated for $C_{17}H_{15}NO$ 249.1154; found 249.1155. IR (neat, cm^{-1}) $\tilde{\nu}$ = 3063 (w), 2922 (m), 2855 (m), 1617 (m), 1577 (w), 1461 (m), 1386 (m), 1100 (m), 932 (m), 701 (s), 742 (s). R_f: 0.58 (40% EA in PE). Melting Point: 103 °C.

*1-(1-(2-(Diethylamino)ethyl)-2-methyl-1H-indol-3-yl)ethan-1-one (**6d**).* Purification through automated flash column chromatography (35-100% EA in PE, 0-20% MeOH in EA). The product was obtained as brown oil (463 mg, 1.70 mmol, 15%). ^{1}H-NMR (300 MHz, Chloroform-*d*) δ =8.04-7.88 (m, 1H), 7.40-7.28 (m, 1H), 7.27-7.18 (m, 2H), 4.21 (t, *J* = 7.4 Hz, 2H), 2.79 (s, 3H), 2.75-2.65 (m, 5H), 2.58 (q, *J* = 7.1 Hz, 4H), 1.00 (t, *J* = 7.1 Hz, 6H). $^{13}C\{^1H\}$-NMR (75 MHz, Chloroform-*d*) δ =194.6 (q), 144.9 (q), 135.9 (q), 126.5 (q), 121.9 (+), 121.8 (+), 120.7 (+), 114.2 (q), 109.5 (+), 51.9 (+), 47.7 (+), 42.1 (+), 31.7 (-), 12.7 (-), 12.0 (-). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{17}H_{25}N_2O^+$ 273.1961; found 273.1967. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2967 (m), 2806 (m), 1636 (s), 1408 (s), 738 (s). R_f: 0.29 (80% EA in PE).

*1-(1-(2-(Dimethylamino)ethyl)-2-methyl-1H-indol-3-yl)ethan-1-one (**6e**).* Purification by automated flash col-

umn chromatography (35-100% EA in PE, 0-20% MeOH in EA) the product as brown oil (1180 mg, 4.83 mmol, 42%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =8.00-7.90 (m, 1H), 7.36-7.27 (m), 7.28-7.18 (m), 4.20 (t, *J* = 7.5 Hz, 2H), 2.75 (s, 3H), 2.64 (s, 3H), 2.59 (t, *J* = 7.5 Hz, 2H), 2.31 (s, 6H). ^{13}C{^{1}H}-NMR (101 MHz, Chloroform-*d*) δ =194.6 (q), 144.6 (q), 135.9 (q), 126.5 (q), 122.0 (+), 121.9 (+), 120.8 (+), 114.4 (q), 109.5 (+), 58.0 (-), 45.8 (+), 41.4 (-), 31.6 (+), 12.5 (+). ESI-MS: calculated: 244.1576, found: 245.1 (MH^+, 100%). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{15}H_{21}N_2O^+$ 245.1648; found 245.1652. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2784 (m), 1613 (s), 1408 (s), 752 (s). R_f:0.16 (50% EA in PE).

*1-(2-Methyl-1-(2-(pyrrolidin-1-yl)ethyl)-1H-indol-3-yl)ethan-1-one (**6f**).* Purification through automated flash column chromatography (35-100% EA in PE, 0-20% MeOH in EA). The product was obtained as brown oil (697 mg, 2.58 mmol, 22%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =8.04-7.93 (m), 7.40-7.35 (m), 7.28-7.23 (m), 4.31 (t, *J* = 7.8 Hz, 2H), 2.84-2.76 (m), 2.67 (s), 2.63 (t, *J* = 6.7 Hz), 1.83 (quintet, *J* = 3.3 Hz). ^{13}C{^{1}H}-NMR (101 MHz, Chloroform-*d*) δ =194.6 (q), 144.6 (q), 135.9 (q), 126.5 (q), 122.0 (+), 121.9 (+), 120.8 (+), 114.4 (q), 109.6 (+), 54.8 (-), 54.5 (-), 42.3 (-), 31.7 (+), 23.5 (-), 12.6 (+). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{17}H_{23}N_2O^+$ 271.1805; found: 271.1809. IR (neat, cm^{-1}) $\tilde{\nu}$ = 2959 (m), 1628 (s), 1405 (s), 741 (s). R_f: 0.14 (80% EA in PE).

General procedure for fulgide formation. A solution of diethyl 2-(propan-2-ylidene)succinate (1.0 eq.) in dry THF (1.25 mL per mmol) was cooled to -105 °C under nitrogen atmosphere. A solution of LDA (1.0 eq.) was freshly prepared by adding a solution of ^{n}BuLi (1.0 eq.) to a solution of diisopropylamine (1.0 eq.) in anhydrous THF (0.5 mL per mmol) at -78 °C. The LDA-solution was added dropwise to the succinate -105 °C and was stirred for 30 min at the same temperature. A precooled (-78 °C) solution of compound **4** (1.0 eq.) in dry THF (0.4 mL per mmol) was added dropwise. The reaction mixture was warmed to room temperature for 18 hours. The reaction was acidified with aqueous HCl (2M) and the aqueous layer was extracted with EA (three times, *ca.* 4 mL per mmol). The combined organic layers were washed with brine (*ca.* 4 mL per mmol), dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in EtOH (5 mL per mmol) and a saturated aqueous solution of KOH (0.4 mL per mmol) was added. The mixture was stirred at 70 °C for 24 h, poured onto ice and acidified with aqueous HCl (2M). The aqueous layer was extracted with EA (three times, *ca.* 4 mL per mmol) and the solvent was removed under reduced pressure. The residue was dissolved in acetyl chloride (1.7 mL per mmol) and the solution was stirred at 40 °C for 18 hours. The solvent was evaporated *in vacuo* and the crude product was purified by automated flash column chromatography (35-100% EA in PE, 0-25% MeOH in EA) and preparative HPLC (in the case of **7a-b**; **7c** crystallized after normal phase chromatography which used for structure determination and the compound was converted after that; **7d-f** turned out to be highly instable under HPLC conditions, NEt_3 treated silica gel or NH-caped silica gel. Thus, they were converted directly after identification through ESI-MS).

*(E)-3-(1-(2-Methyl-1-(pyridin-2-ylmethyl)-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)dihydro-furan-2,5-dione (**7a**).* The pure product was obtained as yellow solid (245 mg, 0.63 mmol, 19%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =8.58 (d, *J* = 5.0 Hz, 1H), 7.63 (td, *J* = 7.7, 1.8 Hz), 7.44 (dd, *J* = 7.1, 1.6 Hz), 7.30-7.26 (m, 1H), 7.25-7.13 (m), 6.78 (d, *J* = 7.9 Hz, 1H), 5.45 (s, 2H), 2.83 (s, 3H), 2.18 (d, *J* = 1.3 Hz, 6H), 1.02 (s, 3H). A set of small signals from closed photoisomer could not be avoided. ^{13}C{^{1}H}-NMR (101 MHz, Chloroform-*d*) δ =164.0 (q), 163.7 (q), 156.1(q), 153.8 (q), 149.2 (+), 149.1 (q), 137.6 (+), 136.8 (q), 135.1 (q), 125.2 (q), 123.0 (+), 122.5 (+), 121.3 (q), 121.1 (+), 120.7 (+), 119.7 (+), 119.5 (q), 117.3 (q), 109.6 (+), 48.3 (-), 26.4 (+), 23.6 (+), 22.7 (+), 12.3 (+). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{24}H_{23}N_2O_3^+$ 387.1703; found 387.1712 (MH^+, 100%, closed form), 387.1706 (MH^+, 100%, opened form). IR (neat, cm^{-1}) $\tilde{\nu}$ = 2922 (w), 1800 (s), 1748 (s), 1408 (s), 1222 (s), 920 (s), 745 (s). R_f: 0.41 (50% EA in PE).

*(E)-3-(1-(1-Isopentyl-2-methyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)dihydrofuran-2,5-dione (**7b**).* The pure product was obtained as yellow solid (182 mg, 0.498 mmol, 16%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ =7.42 – 7.37 (m, 1H), 7.31 – 7.27 (m, 1H), 7.22 (ddd, *J* = 8.3, 7.0, 1.2 Hz, 1H), 7.14 (ddd, *J* = 8.0, 6.9, 1.1 Hz, 2H), 4.18 – 3.92 (m, 2H), 2.81 (s, 3H), 2.20 (d, *J* = 0.9 Hz, 6H), 1.63 – 1.50 (m, 3H), 1.02 (dd, *J* = 6.6, 1.4 Hz, 8H), 0.93 (s, 3H). A set of signals from closed photoisomer could not be avoided. Assignment was done through ppm calculation and comparison with the precursor. Due to the complexity a carbon NMR was not recoded of this intermediate. ESI-MS: calculated: 365.1991, found: 753.4 ($2MNa^+$, 45%), 388.2 (MNa^+, 20%), 366.2 (MH^+, 100%). HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{23}H_{28}NO_3^+$ 366.2064; found 366.2065 (MH^+, 100%,). IR (neat, cm^{-1}) $\tilde{\nu}$ = 2982 (w), 1804 (w), 1737 (s), 1712 (s), 1367 (m), 1282 (m), 1223 (m), 1175 (s), 1077 (s), 1029 (s). R_f: 0.85 (40% EA in PE).

*(E)-3-(1-(1-Methyl-2-phenyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)dihydrofuran-2,5-dione (**7c**).* The product was obtained as crystalline solid (498 mg, 1.348 mmol, 16% yield). The structure was confirmed through X-Ray analysis and converted directly. HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{24}H_{22}NO_3^+$ 372.1594; found 372.1594.

*(E)-3-(1-(1-(2-(Diethylamino)ethyl)-2-methyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)dihydro-furan-2,5-dione (**7d**).* The product was obtained as yellow oil and was directly converted to fulgimide **1d**. HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{24}H_{31}N_2O_3^+$ 395.2329; found 395.2335.

*(E)-3-(1-(1-(2-(Dimethylamino)ethyl)-2-methyl-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)di-hydrofuran-2,5-dione (**7e**).* The product was obtained as yellow oil and directly converted to fulgimide **1e**. HR-MS (ESI$^+$) m/z: [M+H$^+$] calculated for $C_{22}H_{27}N_2O_3^+$ 367.2016; found 367.2021.

(E)-3-(1-(2-Methyl-1-(2-(pyrrolidin-1-yl)ethyl)-1H-indol-3-yl)ethylidene)-4-(propan-2-ylidene)dihydro-furan-2,5-dione

(7f). The product was obtained as yellow solid and directly converted to fulgimide **1f**. ESI-MS: calculated: 392.2100, found: 393.2 (MH+, 100%). HR-MS (ESI+) m/z: [M+H+] calculated for $C_{24}H_{29}N_2O_3^+$ 393.2173; found 393.2177.

Biological evaluation. The biological activity of compounds **1a-c** was tested employing an *in vitro* activity assay based on the deacetylation of the substrate Z-Lys(Ac-AMC) (ZMAL).[34] A detailed description of the procedure can be found in the Supporting Information. To determine the influence of the intrinsic fluorescence of the compounds a no-conversion control was performed. For preliminary testing, each compound was diluted to 100 μM final assay concentration. For compounds scoring an inhibition higher that 50 % at 100 μM for either of the isomers an IC_{50} value was determined.

ASSOCIATED CONTENT

Supporting Information. Detailed information regarding spectroscopic and biological experiments, NMR experiments, HPLC traces, X-Ray data, computational analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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