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A Combinatorial Approach for the Discovery of Drug-Like Inhibitors of 15-Lipoxygenase-1

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Abstract

Human 15-lipoxygenase-1 (15-LOX-1) is a mammalian lipoxygenase which plays an important regulatory role in several CNS and inflammatory lung diseases. To further explore the role of this enzyme in drug discovery, novel potent inhibitors with favorable physicochemical properties are required. In order to identify such new inhibitors, we established a combinatorial screening method based on acylhydrazone chemistry. This represents a novel application of combinatorial chemistry focusing on the improvement of physicochemical properties, rather than on potency. This strategy allowed us to efficiently screen 44 reaction mixtures of different hydrazides and our previously reported indole aldehyde core structure, without the need for individual synthesis of all possible combinations of building blocks. Our approach afforded three new inhibitors with IC_{50} values in the nanomolar range and improved lipophilic ligand efficiency.

Introduction

Over the past two decades, dynamic combinatorial chemistry (DCC) has been applied for the identification of novel ligands for various protein targets.[1–4] In DCC, building blocks or fragments equipped with functional groups to form reversible covalent bonds are combined to generate libraries of potentially bioactive molecules. Library generation in the presence of pharmacologically relevant targets enables the selective amplification of high-affinity binders. Although the method has the potential to accelerate the drug-discovery process significantly, it critically depends on the availability of relatively large amounts of a stable protein target. In practice, many pharmacologically relevant targets cannot be expressed in high quantities and lack the stability required for DCC. In these cases, DCC can be reformatted to generate single reaction mixtures in which building blocks or fragments are linked to provide diverse libraries of functionalized molecules in one step without further purification.[5] We aim to apply this strategy to address the lipoxygenases, which is a class of pharmacologically highly relevant enzymes that lack stability to enable DCC in its classical form.[6]

The lipoxygenases are a group of enzymes that have attracted a lot of attention in drug discovery over the past decades. They are non-heme iron-containing enzymes, which catalyze the regio- and stereospecific insertion of molecular oxygen (O_2) into polyunsaturated fatty acids, such as arachidonic acid (AA) and linoleic acid (LA). This results in the formation of hydroperoxy fatty acids, which are an important source of oxidative stress and can eventually cause cell death via a process denoted ferroptosis.[7] The hydroperoxy fatty acids are further metabolized into lipid signaling molecules, such as leukotrienes and lipoxins, that play key regulatory roles in many diseases.[8]

A lipoxygenase that is gaining interest in drug discovery is human 15-lipoxygenase-1 (15-LOX-1). Its metabolites have been implicated in several diseases with a large impact on modern society, such as asthma, diabetes, stroke, Alzheimer's disease, Parkinson's disease, and cancer.[9,10] Recent evidence shows that 15-LOX-1 plays key roles in the molecular mechanisms contributing to the pathophysiology of these diseases. Although several lipoxygenase inhibitors have been reported, it proved to be challenging to generate potent small-molecule inhibitors with diverse structures and favorable physicochemical properties.[11] Many lipoxygenase inhibitors reach log *P* values of 5 and higher and calculated lipophilic ligand efficiencies (LLEs) are usually poor. In this perspective, it would be highly relevant to discover completely novel substitution patterns for previously identified lipoxygenase binding core structures. This would enable optimization of these inhibitors in completely novel directions to enable, ultimately, inhibitors with favorable physicochemical properties for *in vivo* applications.

In 2015, we applied substitution-oriented fragment screening (SOS), which resulted in the identification of hit compounds with good potency and clear structure–activity relationships (SARs) for 15-LOX-1 inhibition.[12] Optimization of one of these hits, aldehyde **1**, resulted in inhibitor **2** with a K_i of 36 nM (Figure 1). Compound **2** has a high potency but an unfavorable calculated log *P* (clog*P*) value of 7.0 (ACD/ChemSketch),[13] which can be attributed to the aliphatic lipid tail at the indole 3-position. This inhibitor sets the stage to search for completely new substituents at the indole 3-position using acylhydrazone chemistry, as applied in DCC, to generate a compound collection with high diversity at this position. Linkage of the indole aldehyde **1** with diverse hydrazides enables convenient generation of a diverse compound collection that is screened for 15-LOX-1 inhibition without prior purification. Here, we describe a novel application of combinatorial chemistry focusing on the improvement of physicochemical properties rather than on inhibitory potency.

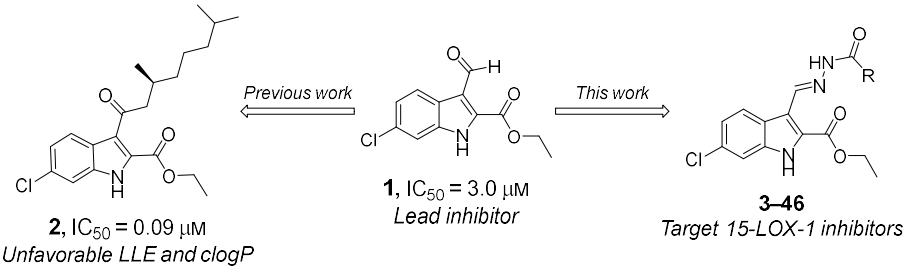


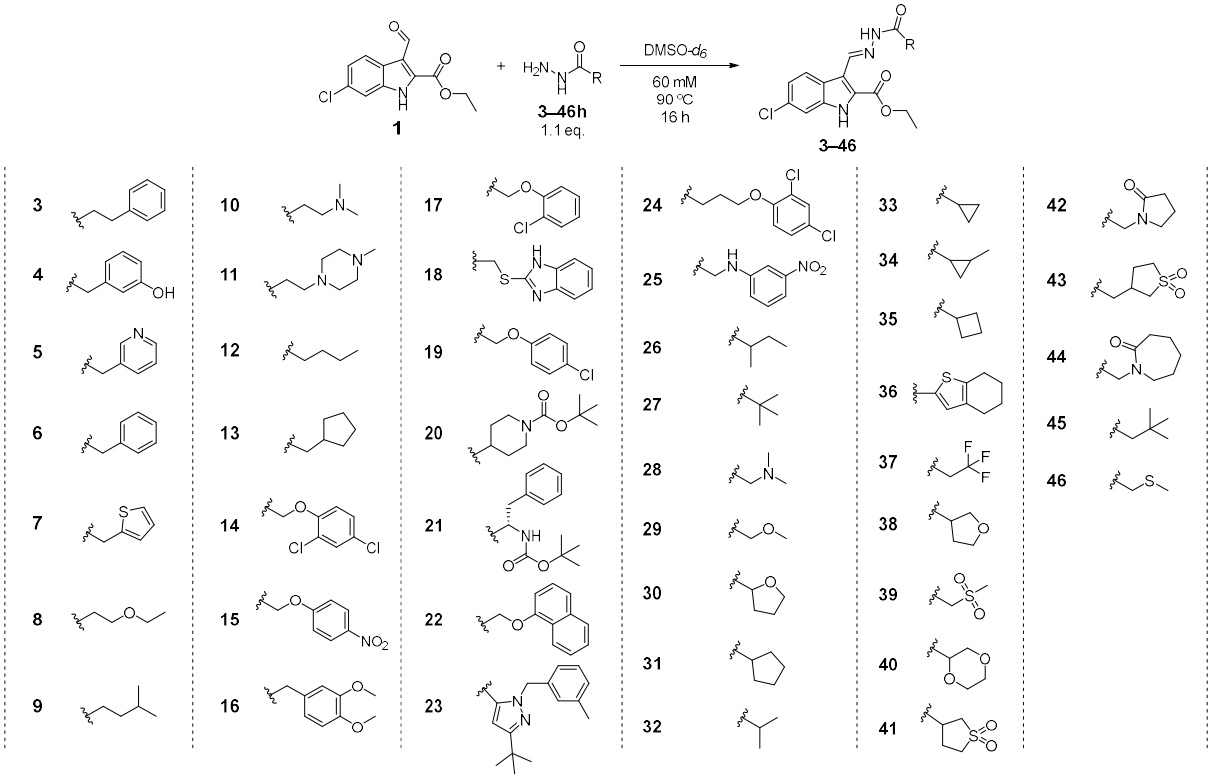
Figure 1 Rational design of the new acylhydrazone-based inhibitors against human 15-LOX-1.

Results and Discussion

Generation and screening of a focused compound collection

We generated a diverse compound collection of acylhydrazones by mixing 3-formyl indole **1** with a collection of hydrazides (Scheme 1). Assembly of the compound collection proceeded with 0.1 eq. excess of hydrazide and heating overnight. Subsequently, small aliquots of the mixtures were analyzed by NMR spectroscopy, which showed for all reactions disappearance of the aldehyde peak and

appearance of the corresponding acylhydrazone signals around 11.5 and 9.0 ppm, thus indicating full conversion. Furthermore, NMR analysis showed exclusively the expected product and the excess of the hydrazide, confirming the absence of side-products.



Scheme 1 Compound collection of *in situ* formed acylhydrazones that are screened for 15-LOX-1 inhibitory activity.

Screening the reaction mixtures for 15-LOX-1 inhibition was done using the UV absorption assay as reported before.[6,12] In order to see a potential increase in potency relative to compound **1** (IC_{50} = 2.97 μM), **1** was dissolved and diluted under the same conditions and added to the screening. The residual enzyme activity was measured after incubation with 0.50 μM of each reaction mixture (Figure 2). During initial studies, we screened at 5 and 1 μM, whereupon many compounds were giving full or close to full inhibition. Upon lowering the concentration to 0.50 μM, we could find a better distinction between highly and less active compounds.

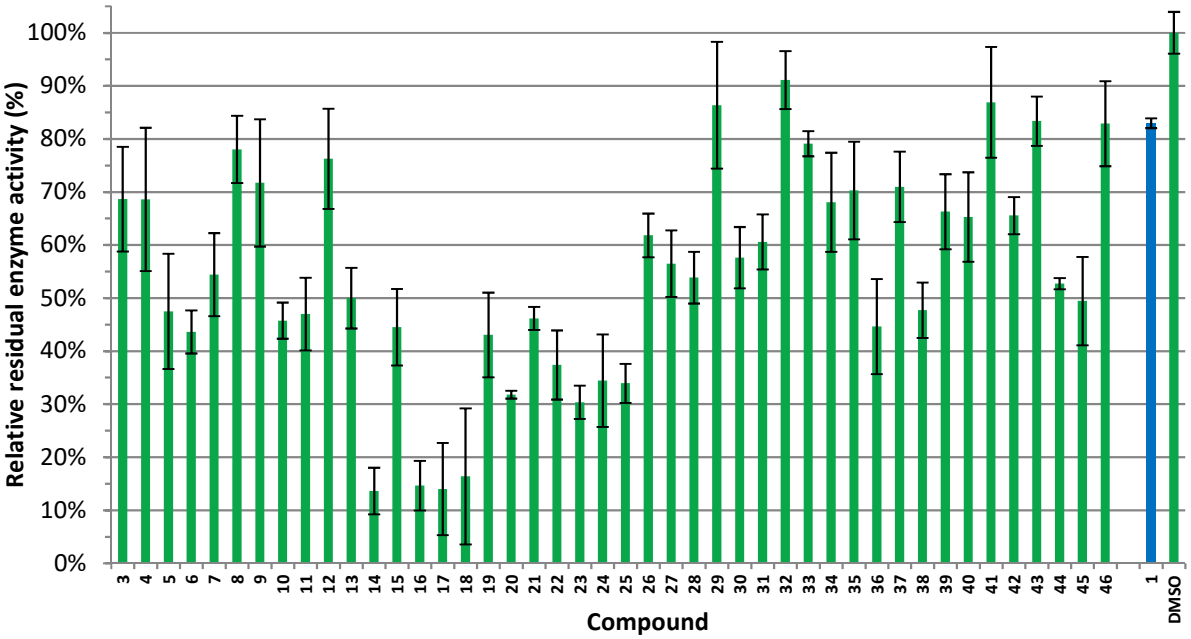
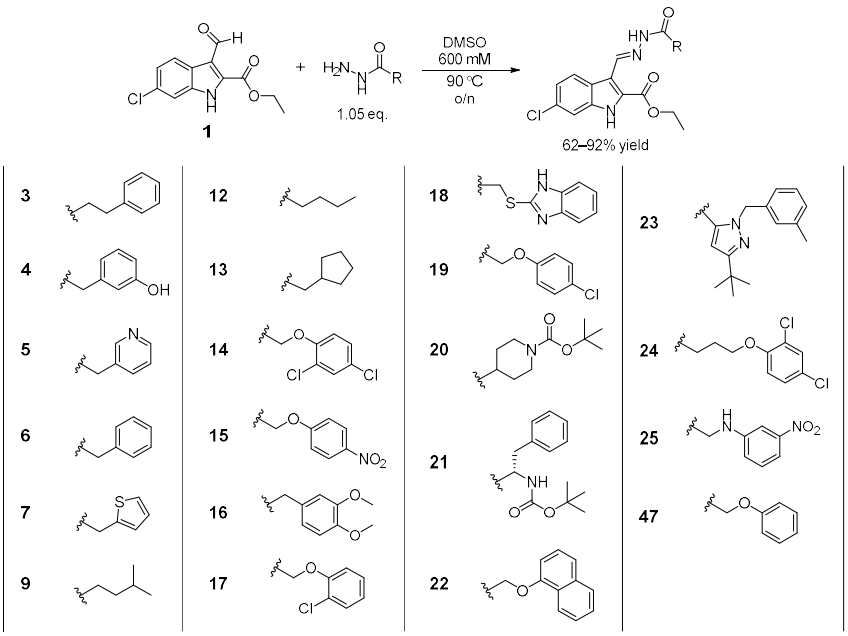


Figure 2 Bar graph representing the relative residual enzyme activity after eight minutes incubation with 0.50 µM of reaction mixtures **3**–**46**. The aldehyde starting material (**1**) is shown as reference in blue. The experiment was performed in triplicate and the standard error is shown. DMSO was used to determine the activity in the absence of inhibitor. The residual activity was determined by dividing the rate observed in the presence of compound by the rate observed for the DMSO control.

The applied reaction conditions of the acylhydrazone formation assured full conversion for all reaction mixtures. However, the use of 0.1 eq. excess of hydrazide and the lack of further purification may influence the residual enzyme activity. A control assay with 50 nM of the corresponding hydrazides showed that this low concentration of hydrazide does not significantly decrease the activity of 15-LOX-1 (Figure S1). The measured decrease in activity caused by most of the reaction mixtures is likely to originate solely from the acylhydrazone products, since incubation with aldehyde starting material **1** resulted in 83% of residual enzyme activity (Figure 2). Incubation with the different reaction mixtures gave a residual enzyme activity between 14% and 91%. Among these mixtures, four samples decreased the residual enzyme activity by more than 80% (**14** and **16**–**18**), indicating the presence of potent inhibitors of 15-LOX-1.

Synthesis and IC_{50} determination of selected acylhydrazones

As a next step, we therefore synthesized and purified these four compounds as well as 17 other inhibitors (**3**–**7**, **9**, **12**–**25** and in addition compound **47** to complete a small SAR, Scheme 2). After synthesis and purification, the final products were isolated in high yields of 62–92% and ≥95% purity as confirmed by quantitative ^{1}H-NMR spectroscopy (qHNMR).[14] The acylhydrazone products exist mainly in the *E*-isomer, giving *syn*- and *antiperiplanar* conformers (E_{syn} and E_{anti}).[15] In all cases, the imine is found primarily in E_{syn} configuration, except for **23** in which the bulky substituent favors the E_{anti} isomer. Subsequently, the acylhydrazone inhibitors were subjected to IC_{50} determination (Table 1, Figures S2 and S3).



Scheme 2 Synthesis of selected acylhydrazones, which were tested for 15-LOX-1 inhibitory activity.

Table 1 IC_{50} values of the acylhydrazones for inhibition of 15-LOX-1.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
3	>> 10	**17**	0.23 ± 0.02
4	1.80 ± 0.23	**18**	0.14 ± 0.02 [α]
5	0.78 ± 0.12	**19**	0.74 ± 0.05
6	> 10	**20**	0.47 ± 0.09 [α]
7	> 10	**21**	> 10
9	>> 10	**22**	> 10
12	>> 10	**23**	4.22 ± 0.54 [β]
13	> 10	**24**	> 10
14	0.99 ± 0.14 [α,β]	**25**	> 10
15	> 10	**47**	> 10
16	0.92 ± 0.13 [α,β]	**1** [12]	2.97 ± 0.50

Experiment performed in triplicate. IC_{50} values are reported including standard error of the fit. [α] Observed Hill-slope of around 0.5, [β] inhibition reaches a maximum of 80%.

Table 1 summarizes the inhibitory activity of the acylhydrazones against 15-LOX-1. Since the goal of this project was to identify new substituents at the indole 3-position of **1** (IC_{50} = 2.97 μM) an IC_{50} cut-off value of 10 μM was employed.

The acylhydrazones bearing a phenyl ring in the side chain exhibit an IC_{50} value of more than 10 μM (**3** and **6**). However, with an alcohol substituent at the *meta*-position (**4**) or methoxy groups at the *meta*- and *para*-positions (**16**), an increase in potency is observed. When the phenyl moiety is substituted by a 3-pyridinyl ring (**5**), a large increase in activity is observed. Replacing the 3-pyridinyl with a thiophenyl ring (**7**) or short aliphatic substituents, such as isopentyl, butyl and cyclopentyl (**9**, **12** and **13**, respectively), leads to a loss of activity. In contrast to previous work, in which the aliphatic tail was favored,[12] it seems that the combination of the acylhydrazone moiety and aliphatic R groups is not tolerated. However, carbamate **20** (IC_{50} = 0.47 ± 0.09 μM) is a potent inhibitor of 15-LOX-1. We observed an interesting SAR after comparing the IC_{50} values of compounds **14**, **17** and **19**. Introduction of a phenoxy moiety bearing one or two electron-withdrawing chlorine substituents increases the

potency against 15-LOX-1. Interestingly, the potency is higher when the chlorine substituent is at the *ortho*-position (**17**) than at the *para*-position (**19**). When a chlorine substituent is located at both the *ortho*- and *para*-position (**14**), the potency is similar to that when a chlorine substituent is at the *para*-position alone. But when the carbon linker is extended by two more carbon atoms (**24**), the compound loses its activity. Substitution of the *para*-Cl by a nitro group (**15**) leads to a loss in potency. Replacing the phenoxy by a phenylamine and use of a nitro group at the *meta*- instead of *para*-position (**25**), did not give an improvement in activity. Additionally, expanding the ring system from phenoxy to naphthyloxy (**22**) also led to a loss of potency, possibly caused by the larger ring size. To investigate the role of the oxygen atom of the phenoxy group and the substituents on the ring, we also synthesized compound **47** and subjected it to biochemical testing. Acylhydrazone **47** turned out to be inactive against 15-LOX-1, indicating the importance of a substituent on the ring.

The size of the R-groups, which are accommodated by the enzyme could give valuable information about the size of the active site, assuming that the inhibitors adopt a similar binding mode as proposed before.[12] Of the compounds with very bulky substituents (**21**–**23**), only the compound with a substituted pyrazole (**23**) is active against 15-LOX-1. The IC_{50} value of 4.22 ± 0.54 μM is in the same range as indole fragment **1**. Compared to **19**, compound **18** bears a slightly larger benzimidazole, but turns out to be the most potent inhibitor in this study. The substituted pyrazole and the benzimidazole compound could indicate that the active site can host larger moieties than assumed to date.

Whereas inhibitors **4**, **5**, **17**, **19** and **23** display Hill-slopes of around 1.0 in the curve fitting for IC_{50} determination, inhibitors **14**, **16**, **18** and **20** show Hill-slopes below 1.0. Lower Hill-slopes suggest binding models more complicated than 1:1 binding to the active site of the enzyme.[16] The best compound with a Hill-slope of around 1.0 is acylhydrazone **17** (IC_{50} = 0.23 ± 0.02 μM), that has a potency in the same range as the previously identified inhibitor **2** (IC_{50} = 0.09 ± 0.03 μM).[12] Even though there is in some cases a discrepancy in the ranking of the inhibitors using the screening protocol and the determined IC_{50} values, the screening turned out to be effective at identifying the most potent inhibitors.

After having identified the acylhydrazones as inhibitors of 15-LOX-1, two control experiments containing a high concentration (25 μM) of the eight most potent inhibitors (**4**, **5**, **14**, **16** and **17**–**20**) were performed (Figures S4 and S5). The control experiment without LA, but with enzyme and the control experiment without enzyme, but with LA both showed that the UV absorption at 234 nm is stable over time. This indicates that the compounds are stable during the time of the assay (in agreement with a reported NMR study)[17] and are not prone to oxidation events that modify the inhibitor chromophore or other assay components such as the LA substrate. Additionally, using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) and a ferric reducing antioxidant power (FRAP) assay, we showed that the compounds did not exhibit any detectable antioxidant properties (Figures S6 and S7).[18–21]

Docking of acylhydrazones

The acylhydrazones from Table 1 were docked into PDB ID: 1LOX.[22] Initially, we performed the docking study using the updated PDB ID 2P0M.[23] But due to the high root-mean-square deviation (RMSD) value for the re-docking of the X-ray ligand in 2P0M, we chose 1LOX for our docking study (Figure S8). Compounds **21** and **23** could not be docked due to steric clashes with the protein. The acylhydrazones were predicted to bind primarily with the tail in the pocket and the indolyl moiety situated at the entrance of the active site. This is also in line with the experimental evidence, showing a large effect on the potency upon changing (substituents on) the tail. In most cases (except for

acylhydrazones **5**, **7**, **12** and **13**) poses in which Arg403 is addressed by the carbonyl group of the indole ethyl ester were obtained. The indolyl moiety is engaged in hydrophobic interactions with Ile414 and Leu597. According to the results of our docking study, all acylhydrazones are predicted to inhibit more or less in the same order of magnitude, while this is clearly not observed *in vitro*. Representative docking poses of compounds **14**, **17**–**19**, and **47** are shown in Figures S9A–E.

Ligand-Efficiency Metrics

Ligand-efficiency metrics quantify how effectively a molecule uses its structural features in binding to a drug target. In hit-to-lead optimization, the use of efficiency metrics to guide multiparameter optimization is therefore recommended over focusing only on potency.[24] In order to compare the acylhydrazone inhibitors developed in this study to the previously reported inhibitor **2**, we used the two most important ligand-efficiency metrics: ligand efficiency (LE) and lipophilic ligand efficiency (LLE) (Table 2). The binding-efficiency index (BEI) and surface-efficiency index (SEI) of the acylhydrazones are rather comparable to the previously reported inhibitor.

The LE is defined as the ratio of the binding affinity of a ligand divided by the number of non- hydrogen (*i.e.*, heavy atoms (HAs)) and follows the equation: LE = (1.37/HA) x pIC_{50}.[25] Inhibitor **17** contains 29 HAs and its IC_{50} value is 0.23 μM, so the LE is 0.31 kcal per mol per HA. A proposed acceptable value of LE is: LE >≈ 0.3 kcal per mol per HA.[26] All potent inhibitors in this study, except compound **23**, exhibit LE values of around 0.3 kcal per mol per HA.

LLE combines potency and lipophilicity using the equation: LLE = pIC_{50} – clog*P*.[27] The clog*P* value of compound **17** is 5.1 (Table S2), resulting in an LLE value of 1.6. Since the clog*P* value of the compounds is much lower, the acylhydrazones show an improvement of a factor 2–29 in LLE in comparison to **2** (clog*P* = 7.0, LLE = 0.10). Compound **5** (LLE = 2.9) has by far the highest LLE of all the acylhydrazones and compared to **2**, a 29-fold improvement in LLE was observed. A proposed acceptable value of LLE for drug candidates is >≈ 5.[27] Which is a criterion that most previously published inhibitors do not meet.[12] The LLE value of compound **5** is near the acceptable range, indicating there is still room for optimization.

In summary, the LEs of the acylhydrazones with IC_{50} < 10 μM are around the acceptable value of 0.3, slightly lower than the previously identified inhibitor **2**. A major improvement was accomplished with respect to LLE. The clog*P* value of inhibitor **2** is 7.0, while the acylhydrazone inhibitors exhibit clog*P* values between 3.2 and 5.8 (Tables 2 and S2). Although the acylhydrazones are slightly less potent than **2**, the LLE was increased by a factor 2–29. Taking into account the IC_{50} value and LLE, three new inhibitors (**17**, **18** and **20**) were found with improved LLE and an IC_{50} value below 0.5 μM. Of these three compounds, only acylhydrazone **17** shows a Hill-slope of around 1.0 and falls into an interesting SAR. Therefore, we consider compound **17** as most promising inhibitor.

Table 2 15-LOX-1 inhibition values and ligand-efficiency metrics of the most potent acylhydrazone inhibitors.

Inhibitor	IC_{50} (μM)	LE[α]	LLE	clog*P*[β]
4	1.8	0.28	1.8	3.95 ± 0.96
5	0.78	0.31	2.9	3.19 ± 0.96
14	0.99[γ]	0.27	0.23	5.77 ± 1.01
16	0.92[γ]	0.27	1.6	4.42 ± 0.97
17	0.23	0.31	1.6	5.07 ± 1.01
18	0.14[γ]	0.30	1.5	5.31 ± 1.01
19	0.74	0.29	0.92	5.21 ± 0.98
20	0.47[γ]	0.26	2.1	4.24 ± 1.12
23	4.2	0.20	0.37	5.00 ± 0.95

2 [12]	0.09	0.37	0.10	6.95 ± 0.41

[α] The unit of LE is kcal/(mol x HA), [β] ACD/ChemSketch,[13] [γ] Observed Hill-slope of around 0.5.

Conclusions

In this study, we used a combinatorial approach as an alternative to DCC to identify new inhibitors for human 15-LOX-1, which is an important drug target in many diseases with large impact on modern society. By combining various hydrazides bearing different substituents with an indole aldehyde core, reaction mixtures containing different acylhydrazones were screened against 15-LOX-1. After synthesis and purification of four distinct hits from the screening and various other library members, three new inhibitors were found with IC_{50} values below 0.5 μM and improved LLE. Of these three, inhibitor **17** emerged as the most promising candidate. Compound **17** inhibits 15-LOX-1 with an IC_{50} value of 0.23 ± 0.02 μM, falls into an interesting SAR and has an improved LLE in comparison to the reference inhibitor. In the future, we hope that our combinatorial chemistry approach can be applied to develop new inhibitors for challenging drug targets, which cannot be used under typical DCC conditions.

Experimental Section

Synthesis and characterization

General methods

All reagents were purchased from Sigma-Aldrich, Acros Organics or TCI Europe, and were used without further purification, unless noted otherwise. Hydrazides **4h**–**7h** and **13h** and aldehyde **1** were synthesized as previously reported.[28,29] Hydrazides **12h**, **14h**–**25h** and **26h**–**46h** were obtained from Apollo Scientific Ltd. or Enamine Ltd. All solvents were reagent-grade, and if necessary, dried and distilled prior to use. Reactions were monitored by thin-layer chromatography (TLC) on silica-gel-coated aluminum foils (silica gel 60/Kieselguhr F254, Merck). Flash-column chromatography was performed on silica gel (SiliCycle 40–63 μm). Melting points were determined with a Büchi B-545 apparatus using a heating rate of 1 °C/min. NMR spectra were recorded on an Agilent 400 MR, Varian Inova 500 MHz or Bruker Ascend™ 600 MHz spectrometer at 25 °C, unless indicated otherwise. Chemical shifts (δ) are reported in ppm relative to the residual solvent peak. Splitting patterns are indicated as (s) singlet, (d) doublet, (t) triplet, (q) quartet, (qt) quintet, (m) multiplet and (br.) broad. Coupling constants (J) are reported in Hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap-XL mass spectrometer (mass accuracy <4 ppm).

*General procedure 1 – Synthesis of hydrazides **3h** and **8h**–**11h***

To a solution of ester precursor in ethanol (0.3–0.4 M), was added an aqueous hydrazine solution (55%, 3–4 eq.). The reaction mixture was heated to reflux overnight, subsequently the solvent was removed under reduced pressure, and the crude was purified using flash column chromatography (DCM/MeOH 9:1).

Reaction-mixture screening

Acylhydrazones **3**–**46** were formed using aldehyde **1** and 1.1 eq. of structurally different hydrazides. Stock solutions were prepared in DMSO-d_6 at concentrations of 120 and 132 mM, respectively. Then, 50 μL of aldehyde and 50 μL of hydrazide solution were added to a 500 μL Eppendorf tube, mixed and heated overnight in a heating block at 90 °C. By taking small aliquots of the reaction mixture, full conversion of the reaction mixtures was confirmed by ^{1}H-NMR (no more aldehyde was observed).

General procedure 2 – Synthesis of selected acylhydrazones

After analysis of the reaction mixture screening, several acylhydrazones were selected for larger-scale synthesis and purification. The required hydrazides (14.7–35.0 mg, 0.0932–0.138 mmol) were weighed into 0.5 mL Eppendorf tubes. To indole aldehyde **1** (285 mg, 1.13 mmol), was added DMSO-d_6 (1.885 mL, 600 mM). Then, the aldehyde stock solution was added to each hydrazide, resulting in 1.05 equivalents of hydrazide with respect to aldehyde. The Eppendorf tubes were mixed, shortly centrifuged and then placed in a pre-heated 90 °C aluminum heating block overnight. The yellow solutions were allowed to cool down to room temperature, mixed, shortly centrifuged and then added dropwise to ice-cold demi water (1500 µL). After mixing, the suspensions were centrifuged at 12.5 x 10^3 g at 5 °C for 10 min. The pale yellow aqueous DMSO layer was removed and in order to remove the remaining DMSO, the solids were washed with ice-cold demi water (2 x 1200 µL; including mixing, sonication and centrifugation). Then, the remaining hydrazide was removed by washing the solids with ice-cold absolute EtOH (3 x 1.2 mL, same procedure as with water). The solids were dried under high-vacuum until no more weight loss was observed, resulting in product (yield 62–92%) with purity ≥ 95% as confirmed by qHNMR (see supporting information).[14]

The acylhydrazone products exist mainly in the *E*-isomer, giving *syn*- and *antiperiplanar* conformers (E_{syn} and E_{anti}). The NMR shift of nuclei around the acylhydrazone moiety is different for both conformational isomers.[15] This results in a shift of characteristic signals, such as the imine -N=C<u>H</u>-, amide -N<u>H</u>- and, if applicable, methylene group alpha to the acylhydrazone carbonyl -NH-(C=O)-C<u>H</u>$_2$-, but for example also the ethyl ester of the indole is often affected. In case of the ethyl ester, the signals of both conformers are partially overlapping. Although the *J*-coupling is the same in each case, the shift between the peaks of both conformers and the ratio between E_{syn} and E_{anti} is different for each compound. Instead of reporting a multiplet, the coupling constants were measured after peak dissection and reported as one signal. In all cases, except for **23**, the imine -N=C<u>H</u>- is found primarily in E_{syn} configuration. Acylhydrazone **23** contains a bulky side-group, which causes the conformation to be fixed in approximately 86% of the E_{anti} isomer. 2D NOE analysis and the corresponding proposed 3D structures of compounds **14**, **18** and **23** are shown in Figures S10–S15.

3-Phenylpropanehydrazide (3h) The product was synthesized according to general procedure 1, starting from ethyl 3-phenylpropanoate (522 mg, 2.93 mmol). Purification by column chromatography afforded hydrazide **3h** as white solid (370 mg, 2.25 mmol, 77% yield). The spectroscopic data are consistent with those previously reported.[30] M.p. 102–103 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.14 (m, 5H), 3.70 (br. s, 2H), 2.96 (t, J = 7.7 Hz, 2H), 2.49 – 2.41 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 140.5, 128.6, 128.3, 126.4, 36.3, 31.4. HRMS (ESI+) calculated for $C_9H_{13}N_2O$ [M + H]$^+$ 165.1022, found 165.1020.

3-Ethoxypropanehydrazide (8h) The product was synthesized according to general procedure 1, starting from ethyl 3-ethoxypropanoate (557 mg, 3.81 mmol). Purification by column chromatography afforded hydrazide **8h** as pale yellow oil (426 mg, 3.22 mmol, 85%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.89 (br. s, 1H), 3.79 (br. s, 2H), 3.59 (t, J = 5.9 Hz, 2H), 3.43 (q, J = 7.0 Hz, 2H), 2.38 (t, J = 5.9 Hz, 2H), 1.12 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3, 66.5, 66.1, 35.3, 15.0. HRMS (ESI+) calculated for $C_5H_{13}N_2O_2$ [M + H]$^+$ 133.0972, found 133.0970.

4-Methylpentanehydrazide (9h) The product was synthesized according to general procedure 1, starting from methyl 4-methylpentanoate (513 mg, 3.94 mmol). Purification by column chromatography afforded hydrazide **9h** as pale yellow solid (458 mg, 3.52 mmol, 89%). M.p. 53–54 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.91 (br. s, 1H), 4.12 (br. s, 2H), 2.04 – 1.96 (m, 2H), 1.56 – 1.42 (m, 1H), 1.41 – 1.32 (m, 2H), 0.84 (d, J = 6.5 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.8, 34.3, 31.5, 27.2, 22.2. HRMS (ESI+) calculated for $C_6H_{15}N_2O$ [M + H]$^+$ 131.1179, found 131.1178.

3-(Dimethylamino)propanehydrazide (10h) The product was synthesized according to general procedure 1, starting from methyl 3-(dimethylamino)-propanoate (330 mg, 2.52 mmol). Purification by column chromatography (DCM/MeOH 9:1, with 0.5% v/v 7 M NH_3 in methanol) afforded hydrazide **10h** as yellow oil (179 mg, 1.36 mmol, 54%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.40 (br. s, 1H), 3.83 (br. s, 2H), 2.52 (t, J = 5.9 Hz, 2H), 2.39 (t, J = 5.9 Hz, 2H), 2.25 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 54.8, 44.7, 31.8. HRMS (ESI+) calculated for $C_5H_{14}N_3O$ [M + H]$^+$ 132.1131, found 132.1130.

3-(4-Methylpiperazin-1-yl)propanehydrazide (11h) The product was synthesized according to general procedure 1, starting from methyl 3-(4-methylpiperazin-1-yl)propanoate (300 mg, 1.61 mmol). Purification by column chromatography (DCM/MeOH 9:1, with 0.5% v/v 7 M NH_3 in methanol) afforded hydrazide **11h** as yellow waxy, hygroscopic solid (179 mg, 0.961 mmol, 60%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.20 (br. s, 1H), 3.86 (br. s, 2H), 2.64 (t, J = 6.0 Hz, 2H), 2.57 (br. s, 8H), 2.43 (t, J = 6.1 Hz, 2H), 2.35 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 55.1, 53.5, 52.6, 46.0, 31.0. HRMS (ESI+) calculated for $C_8H_{19}N_4O$ [M + H]$^+$ 187.1553, found 187.1551.

Ethyl (*E*)-6-chloro-3-((2-(3-phenylpropanoyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (3) This compound was synthesized according to synthetic procedure 2, starting with 3-phenylpropanehydrazide (20.6 mg, 0.125 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (56:44) as white solid (40.5 mg, 0.102 mmol, 85%). M.p.: >244 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.21 (br. s, 2H), 11.45 (br. s, 1H), 11.41 (br. s, 1H), 8.92 (s, 1H), 8.81 (s, 1H), 8.44 (d, J = 8.7 Hz, 1H), 8.22 (d, J = 8.7 H, 1H), 7.50 – 7.49 (m, 2H), 7.35 – 7.25 (m, 8H), 7.23 (dd, J = 8.7, 2.0 Hz, 1H), 7.21 – 7.17 (m, 2H), 7.13 (dd, J = 8.7, 2.0 Hz, 1H), 4.40 (q, J = 7.1 Hz, 4H), 3.04 – 2.94 (m, 4H), 2.93 (t, J = 7.7 Hz, 2H), 2.55 (dd, J = 8.3, 7.1 Hz, 2H), 1.40 (t, J = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.2 (C), 167.6 (C), 160.67 (C), 160.66 (C), 141.5 (CH), 141.3 (C), 141.1 (C), 139.2 (CH), 136.93 (C), 136.85 (C), 130.3 (C), 130.2 (C), 128.3 (4xCH), 128.23 (2xCH), 128.19 (2xCH), 127.3 (C), 127.2 (C), 125.95 (CH), 125.91 (CH), 125.7 (CH), 124.8 (CH), 123.1 (C), 122.8 (C), 122.01 (CH), 121.95 (CH), 116.3 (C), 115.9 (C), 112.2 (CH), 112.1 (CH), 61.24 (CH_2), 61.17 (CH_2), 35.8 (CH_2), 33.7 (CH_2), 30.7 (CH_2), 30.1 (CH_2), 14.2 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{21}H_{21}ClN_3O_3$ [M + H]$^+$ 398.127, found 398.127.

Ethyl (*E*)-6-chloro-3-((2-(2-(3-hydroxyphenyl)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (4) This compound was synthesized according to synthetic procedure 2, starting with methyl 2-(3-hydroxyphenyl)acetate (21.5 mg, 0.129 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (57:43) as white solid (36.5 mg, 0.0913 mmol, 74%). M.p.: >247 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.22 (br. s, 2H), 11.66 (s, 1H), 11.50 (s, 1H), 9.31 (2 br. s overlap, 2H), 9.00 (s, 1H), 8.83 (s, 1H), 8.42 (d, J = 8.7 Hz, 1H), 8.23 (d, J = 8.7 Hz, 1H), 7.50 – 7.48 (m, 2H), 7.22 (2dd overlap, J = 8.7, 1.3 Hz, 2H), 7.13 – 7.06 (m, 2H), 6.81 – 6.71 (m, 4H), 6.65 – 6.89 (m, 2H), 4.41 (q, J = 7.1 Hz, 4H), 3.94 (s, 2H), 3.46 (s, 2H), 1.40 (t, J = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-

d$_6$) *δ* 172.0 (C), 166.3 (C), 160.69 (C), 160.65 (C), 157.29 (C), 157.27 (C), 142.1 (CH), 139.5 (CH), 137.02 (C), 136.98 (C), 136.93 (C), 136.87 (C), 130.3 (C), 130.2 (C), 129.21 (CH), 129.16 (CH), 127.4 (C), 127.3 (C), 125.6 (CH), 125.1 (CH), 123.1 (C), 122.9 (C), 122.1 (CH), 122.0 (CH), 119.8 (CH), 119.7 (CH), 116.2 (C), 116.0 (CH), 115.83 (CH), 115.80 (C), 113.5 (CH), 113.4 (CH), 112.13 (CH), 112.09 (CH), 61.3 (CH_2), 61.2 (CH_2), 41.3 (CH_2), 39.1 (CH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{19}ClN_3O_4$ [*M* + H]$^+$ 400.106, found 400.106.

Ethyl (*E*)-6-chloro-3-((2-(2-(pyridin-3-yl)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (5) This compound was synthesized according to synthetic procedure 2, starting with methyl 2-(pyridine-3-yl)acetate (20.9 mg, 0.138 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (62:38) as white solid (38.4 mg, 0.100 mmol, 76%). M.p.: >222 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-*d*$_6$) *δ* 12.25 (br. s, 2H), 11.76 (br. s, 1H), 11.61 (br. s, 1H), 9.00 (s, 1H), 8.87 (s, 1H), 8.54 (d, *J* = 1.9 Hz, 1H), 8.53 (d, *J* = 1.9 Hz, 1H), 8.47 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.44 (dd, *J* = 4.8, 1.7Hz, 1H), 8.41 (d, *J* = 8.7 Hz, 1H), 8.31 (d, *J* = 8.7 Hz, 1H), 7.76 (dt, *J* = 7.9, 2.0 Hz, 1H), 7.72 (dt, *J* = 7.9, 2.0 Hz, 1H), 7.51 (d, *J* = 2.0 Hz, 1H), 7.49 (d, *J* = 2.0 Hz, 1H), 7.39 – 7.32 (m, 2H), 7.23 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.21 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 4.11 (s, 2H), 3.63 (s, 2H), 1.40 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) *δ* 171.4 (C), 165.8 (C), 160.7 (C), 160.6 (C), 150.5 (CH), 150.1 (CH), 147.8 (CH), 147.6 (CH), 142.3 (CH), 139.8 (CH), 137.1 (CH), 137.0 (C), 136.9 (C), 136.7 (CH), 131.5 (C), 131.5 (C), 130.3 (C), 130.2 (C), 127.6 (C), 127.4 (C), 125.6 (CH), 125.1 (CH), 123.4 (CH), 123.3 (CH), 123.1 (C), 122.9 (C), 122.2 (CH), 122.1 (CH), 116.1 (C), 115.7 (C), 112.2 (CH), 112.1 (CH), 61.3 (CH_2), 61.2 (CH_2), 38.1 (CH_2), 36.3 (CH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{19}H_{18}ClN_4O_3$ [*M* + H]$^+$ 385.106, found 385.107.

Ethyl (*E*)-6-chloro-3-((2-(2-phenylacetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (6) This compound was synthesized according to synthetic procedure 2, starting with 2-phenylacetohydrazide (18.9 mg, 0.126 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (58:42) as white solid (37.8 mg, 0.0985 mmol, 82%). M.p.: >255 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-*d*$_6$) *δ* 12.23 (br. s, 2H), 11.70 (s, 1H), 11.53 (s, 1H), 9.00 (s, 1H), 8.85 (s, 1H), 8.41 (d, *J* = 8.7 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 1H), 7.49 (d, *J* = 1.9 Hz, 1H), 7.39 – 7.28 (m, 8H), 7.27 – 7.19 (m, 4H), 4.40 (q, *J* = 7.1 Hz, 4H), 4.05 (s, 2H), 3.57 (s, 2H), 1.40 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) *δ* 172.0 (C), 166.3 (C), 160.7 (C), 160.6 (C), 142.1 (CH), 139.5 (CH), 136.9 (C), 136.8 (C), 135.8 (C), 135.7 (C), 130.3 (C), 130.2 (C), 129.3 (2xCH), 129.0 (2xCH), 128.3 (2xCH), 128.2 (2xCH), 127.4 (C), 127.3 (C), 126.5 (CH), 126.3 (CH), 125.6 (CH), 125.0 (CH), 123.1 (C), 122.9 (C), 122.2 (CH), 122.0 (CH), 116.2 (C), 115.8 (C), 112.2 (CH), 112.1 (CH), 61.3 (CH_2), 61.2 (CH_2), 41.2 (CH_2), 39.0 (CH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{19}ClN_3O_3$ [*M* + H]$^+$ 384.111, found 384.111.

Ethyl (*E*)-6-chloro-3-((2-(2-(thiophen-2-yl)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (7) This compound was synthesized according to synthetic procedure 2, starting with 2-(thiophen-2-yl)acetohydrazide (14.7 mg, 0.0941 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (61:39) as white solid (29.4 mg, 0.0754 mmol, 84%). M.p.: >258 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-*d*$_6$) *δ* 12.25 (br. s, 2H), 11.73 (s, 1H), 11.57 (s, 1H), 8.99 (s, 1H), 8.86 (s, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.33 (d, *J* = 8.7 Hz, 1H), 7.51 (d, *J* = 1.8 Hz, 1H), 7.49 (d, *J* = 1.8 Hz, 1H), 7.40 (dd, *J* = 4.9, 1.5 Hz, 1H), 7.36 (dd, *J* = 5.2, 1.2 Hz, 1H), 7.24 (dd, *J* = 3.0, 2.0 Hz, 1H), 7.22 (dd, *J* = 3.0, 2.0 Hz, 1H), 7.02 – 6.92 (m, 4H), 4.41 (q, *J* = 7.1 Hz, 4H), 4.25 (s, 2H), 3.80 (s, 2H), 1.40 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) *δ* 170.80 (C), 165.3 (C), 160.7 (C), 160.6 (C), 142.4 (CH), 139.8 (CH), 137.0 (C),

136.88 (C), 136.86 (C), 136.84 (C), 136.80 (C), 136.76 (C), 130.3 (C), 130.2 (C), 127.6 (C), 127.4 (C), 126.7 (CH), 126.6 (CH), 126.5 (CH), 126.3 (CH), 125.6 (CH), 125.12 (CH), 125.06 (CH), 125.04 (CH), 123.1 (C), 122.9 (C), 122.2 (CH), 122.1 (CH), 116.1 (CH), 115.7 (CH), 112.2 (C), 112.1 (C), 61.3 (CH_2), 61.2 (CH_2), 35.5 (CH_2), 33.7 (CH_2), 14.3 (CH_3), 14.2 (CH_3). HRMS (ESI+) calculated for $C_{18}H_{17}ClN_3O_3S$ [*M* + H]$^+$ 390.067, found 390.067.

Ethyl (*E*)-6-chloro-3-((2-(4-methylpentanoyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (9) This compound was synthesized according to synthetic procedure 2, starting with 4-methylpentanehydrazide (16.4 mg, 0.126 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (58:42) as brown solid (32.1 mg, 0.0882 mmol, 74%). M.p.: >244 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.20 (br. s, 2H), 11.42 (s, 1H), 11.33 (s, 1H), 8.94 (s, 1H), 8.81 (s, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 1H), 7.49 (d, *J* = 1.9 Hz, 1H), 7.22 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.19 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 2.69 – 2.65 (m, 2H), 2.25 – 2.20 (m, 2H), 1.71 – 1.47 (m, 6H), 1.40 (t, *J* = 7.1 Hz, 6H), 0.93 (d, *J* = 6.3 Hz, 6H), 0.90 (d, *J* = 6.3 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.3 (C), 168.6 (C), 160.71 (C), 160.69 (C), 141.4 (CH), 139.0 (CH), 137.0 (C), 136.9 (C), 130.3 (C), 130.2 (C), 127.3 (C), 127.2 (C), 125.7 (CH), 124.8 (CH), 123.1 (C), 122.9 (C), 122.0 (CH), 121.9 (CH), 116.4 (C), 115.9 (C), 112.3 (CH), 112.1 (CH), 61.24 (CH_2), 61.16 (CH_2), 34.1 (CH_2), 33.4 (CH_2), 32.3 (CH_2), 30.4 (CH_2), 27.5 (CH), 27.8 (CH), 22.3 (4xCH_3), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{18}H_{23}ClN_3O_3$ [*M* + H]$^+$ 364.142, found 364.142.

Ethyl (*E*)-6-chloro-3-((2-pentanoylhydrazineylidene)methyl)-1*H*-indole-2-carboxylate (12) This compound was synthesized according to synthetic procedure 2, starting with pentanehydrazide (14.9 mg, 0.128 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (57:43) as white solid (35.8 mg, 0.102 mmol, 84%). M.p.: >236 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.18 (br. s, 2H), 11.41 (s, 1H), 11.33 (s, 1H), 8.94 (s, 1H), 8.80 (s, 1H), 8.45 (d, *J* = 8.7 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.8 Hz, 1H), 7.49 (d, *J* = 1.8 Hz, 1H), 7.23 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.22 (dd, *J* = 8.7, 1.9 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 2.67 (t, *J* = 7.5 Hz, 2H), 2.23 (t, *J* = 7.5 Hz, 2H), 1.66 – 1 .54 (m, 4H), 1.44 – 1.29 (m, 10H), 0.92 (t, *J* = 7.4 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.1 (C), 168.4 (C), 160.69 (C), 160.67 (C), 141.3 (CH), 138.9 (CH), 136.94 (C), 136.85 (C), 130.3 (C), 130.2 (C), 127.2 (C), 127.1 (C), 125.7 (CH), 124.8 (CH), 123.1 (C), 122.9 (C), 122.03 (CH), 121.97 (CH), 116.4 (C), 116.0 (C), 112.2 (CH), 112.0 (CH), 61.24 (CH_2), 61.17 (CH_2), 33.9 (CH_2), 32.0 (CH_2), 27.2 (CH_2), 26.5 (CH_2), 22.1 (CH_2), 21.9 (CH_2), 14.3 (CH_3), 14.1 (CH_3), 13.8 (CH_3), 13.7 (CH_3). HRMS (ESI+) calculated for $C_{17}H_{21}ClN_3O_3$ [*M* + H]$^+$ 350.127, found 350.126.

Ethyl (*E*)-6-chloro-3-((2-(2-cyclopentylacetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (13) This compound was synthesized according to synthetic procedure 2, starting with 2-cyclopentylacetohydrazide (18.9 mg, 0.133 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (55:45) as white solid (35.7 mg, 0.0950 mmol, 75%). M.p.: >244 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.19 (br. s, 2H), 11.39 (s, 1H), 11.33 (s, 1H), 8.94 (s, 1H), 8.80 (s, 1H), 8.45 (d, *J* = 8.7 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 1H), 7.49 (d, *J* = 1.9 Hz, 1H), 7.23 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.24 (t, *J* = 2.2 Hz, 1H), 7.22 (t, *J* = 2.2 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 2.68 (d, *J* = 7.4 Hz, 2H), 2.36 – 2.19 (m, 4H), 1.83 - 1.70 (m, 4H), 1.65 – 1.56 (m, 4H), 1.56 – 1.46 (m, 4H), 1.40 (t, *J* = 7.1 Hz, 6H), 1.32 – 1.12 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.7 (C), 168.0 (C), 160.71 (C), 160.69 (C), 141.4 (CH), 138.8 (CH), 137.0 (C), 136.9 (C), 130.3 (C), 130.2 (C), 127.3 (C), 127.2 (C), 125.7 (CH), 124.8 (CH), 123.1 (C), 122.9 (C), 122.01 (CH), 121.96 (CH), 116.4 (C), 116.0 (C), 112.2 (CH), 112.1

(CH), 61.2 (CH_2), 61.2 (CH_2), 40.4 (CH_2), 38.3 (CH_2), 36.5 (CH), 36.0 (CH), 32.1 (2xCH_2), 31.9 (2xCH_2), 24.5 (4xCH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{19}H_{23}ClN_3O_3$ [*M* + H]$^+$ 376.142, found 376.142.

Ethyl (*E*)-6-chloro-3-((2-(2-(2,4-dichlorophenoxy)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (14) This compound was synthesized according to synthetic procedure 2, starting with 2-(2,4-dichlorophenoxy)acetohydrazide (29.2 mg, 0.124 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers and a third isomer (72:25:3) as white solid (45.3 mg, 0.0966 mmol, 82%). M.p.: >252 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.28 (br. s, 2H), 11.77 (br. s, 2H), 9.03 (s, 1H), 8.85 (s, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.28 (d, *J* = 8.7 Hz, 1H), 7.61 (d, *J* = 2.6 Hz, 1H), 7.58 (d, *J* = 2.6 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 2H), 7.39 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.34 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.24 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.21 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.15 (d, *J* = 8.9 Hz, 1H), 7.09 (d, *J* = 8.9 Hz, 1H), 5.37 (s, 2H), 4.83 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.9 (C), 163.2 (C), 160.64 (C), 160.60 (C), 152.9 (C), 152.7 (C), 143.4 (CH), 140.5 (CH), 136.91 (C), 136.85 (C), 130.4 (C), 130.2 (C), 129.4 (CH), 129.2 (CH), 128.1 (CH), 127.9 (CH), 127.7 (C), 127.6 (C), 125.5 (CH), 125.4 (CH), 125.1 (C), 124.4 (C), 123.1 (C), 122.9 (C), 122.5 (C), 122.3 (CH), 122.2 (CH), 122.1 (C), 115.9 (C), 115.5 (C), 115.4 (CH), 115.1 (CH), 112.2 (CH), 112.1 (CH), 67.1 (CH_2), 65.8 (CH_2), 61.33 (CH_2), 61.27 (CH_2), 14.2 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{17}Cl_3N_3O_4$ [*M* + H]$^+$ 468.028, found 468.028.

Ethyl (*E*)-6-chloro-3-((2-(2-(4-nitrophenoxy)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (15) This compound was synthesized according to synthetic procedure 2, starting with 2-(4-nitrophenoxy)acetohydrazide (26.6 mg, 0.126 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (72:28) as white solid (48.9 mg, 0.110 mmol, 92%). M.p.: >230 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.28 (br. s, 2H), 11.81 (br. s, 2H), 9.07 (s, 1H), 8.87 (s, 1H), 8.43 (d, *J* = 8.7 Hz, 1H), 8.30 (d, *J* = 8.7 Hz, 1H), 8.26 – 8.20 (m, 4H), 7.51 (d, *J* = 1.6 Hz, 2H), 7.26 – 7.19 (m, 3H), 7.20 – 7.14 (m, 3H), 5.42 (s, 3H), 4.89 (s, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.8 (C), 163.7 (C), 163.2 (2xC), 160.64 (C), 160.59 (C), 143.6 (CH), 141.3 (C), 140.9 (C), 140.7 (CH), 136.92 (C), 136.86 (C), 130.34 (C), 130.26 (C), 127.70 (C), 127.66 (C), 125.8 (2xCH), 125.7 (2xCH), 125.6 (CH), 125.3 (CH), 123.1 (C), 122.9 (C), 122.4 (CH), 122.2 (CH), 115.9 (C), 115.44 (C), 115.35 (2xCH), 115.2 (2xCH), 112.2 (CH), 112.1 (CH), 66.7 (CH_2), 66.6 (CH_2), 61.34 (CH_2), 61.27 (CH_2), 14.3 (CH_3), 14.2 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{18}ClN_4O_6$ [M + H]$^+$ 445.091, found 445.090.

Ethyl (*E*)-6-chloro-3-((2-(2-(3,4-dimethoxyphenyl)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (16) This compound was synthesized according to synthetic procedure 2, starting with 2-(3,4-dimethoxyphenyl)acetohydrazide (26.6 mg, 0.127 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (58:42) as white solid (47.8 mg, 0.108 mmol, 89%). M.p.: >234 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.23 (br. s, 2H), 11.63 (br. s, 1H), 11.47 (br. s, 1H), 8.99 (s, 1H), 8.84 (s, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.32 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.49 (d, *J* = 2.0 Hz, 1H), 7.23 (2dd overlap, *J* = 8.7, 2.0 Hz, 2H), 6.95 (2d overlap, *J* = 2.0 Hz, 2H), 6.90 (d, *J* = 8.2 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 6.85 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.82 (dd, *J* = 8.2, 2.0 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 3.96 (s, 2H), 3.76 (s, 3H), 3.73 (s, 3H), 3.70 (s, 3H), 3.62 (s, 3H), 3.48 (s, 2H), 1.40 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 172.2 (C), 166.6 (C), 160.7 (C), 160.6 (C), 148.54 (C), 148.46 (C), 147.7 (C), 147.5 (C), 142.0 (CH), 139.4 (CH), 136.9 (C), 136.8 (C), 130.3 (C), 130.2 (C), 128.1 (C), 128.0 (C), 127.4 (C), 127.2 (C), 125.6 (CH), 125.0 (CH), 123.1 (C), 122.8 (C), 122.2 (CH), 122.0 (CH), 121.2 (CH), 121.0 (CH), 116.3 (C), 115.8 (C), 113.2 (CH), 113.0 (CH), 112.2 (CH), 112.1 (CH), 111.9 (CH), 111.8

(CH), 61.3 (CH_2), 61.2 (CH_2), 55.6 (CH_3), 55.51 (CH_3), 55.45 (CH_3), 55.2 (CH_3), 40.8 (CH_2), 38.7 (CH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{22}H_{23}ClN_3O_5$ [*M* + H]$^+$ 444.132, found 444.132.

Ethyl (*E*)-6-chloro-3-((2-(2-(2-chlorophenoxy)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (17) This compound was synthesized according to synthetic procedure 2, starting with 2-(2-chlorophenoxy)acetohydrazide (25.5 mg, 0.127 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (72:28) as white solid (46.5 mg, 0.107 mmol, 88%). M.p.: >266 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.27 (br. s, 2H), 11.77 (br. s, 1H), 11.75 (br. s., 1H), 9.04 (s, 1H), 8.86 (s, 1H), 8.43 (d, *J* = 8.7 Hz, 1H), 8.28 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 2H), 7.46 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.44 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.31 (ddd, *J* = 8.3, 7.5, 1.6 Hz, 1H), 7.27 (ddd, *J* = 8.4, 7.4, 1.6 Hz, 1H), 7.24 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.20 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.12 (dd, *J* = 8.4, 1.4 Hz, 1H), 7.04 (dd, 8.4, 1.3 Hz, 1H), 7.00 (td, *J* = 7.7, 1.4 Hz, 1H), 6.96 (td, *J* = 7.6, 1.4 Hz, 1H), 5.34 (s, 2H), 4.80 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.2 (C), 163.5 (C), 160.64 (C), 160.60 (C), 153.6 (C), 153.5 (C), 143.3 (CH), 140.5 (CH), 136.90 (C), 136.85 (C), 130.4 (C), 130.2 (C), 130.1 (CH), 129.9 (CH), 128.3 (CH), 128.1 (CH), 127.63 (C), 127.61 (C), 125.6 (CH), 125.3 (CH), 123.1 (C), 122.9 (C), 122.3 (CH), 122.2 (CH), 122.1 (CH), 121.5 (CH), 121.5 (C), 121.0 (C), 115.9 (C), 115.5 (C), 114.2 (CH), 113.7 (CH), 112.14 (CH), 112.10 (CH), 67.0 (CH_2), 65.5 (CH_2), 61.33 (CH_2), 61.27 (CH_2), 14.2 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{18}Cl_2N_3O_4$ [*M* + H]$^+$ 434.067, found 434.067.

Ethyl (*E*)-3-((2-(2-((1H-benzo[d]imidazol-2-yl)thio)acetyl)hydrazineylidene)methyl)-6-chloro-1*H*-indole-2-carboxylate (18) This compound was synthesized according to synthetic procedure 2, starting with 2-((1H-benzo[d]imidazol-2-yl)thio)acetohydrazide (28.6 mg, 0.129 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (68:32) as pale yellow solid (42.4 mg, 0.0930 mmol, 76%). M.p.: >217 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.59 (br. s, 2H), 12.29 (br. s, 2H), 11.95 (br. s, 1H), 11.75 (br. s, 1H), 9.00 (s, 1H), 8.88 (s, 1H), 8.41 (d, *J* = 8.7 Hz, 1H), 8.36 (d, *J* = 8.7 Hz, 1H), 7.55 – 7.29 (m, 6H), 7.22 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.16 – 7.08 (m, 5H), 4.67 (s, 2H), 4.42 (q, *J* = 7.1 Hz, 4H), 4.20 (s, 2H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.7 (C), 163.7 (C), 160.7 (2xC), 149.9 (C), 149.6 (C), 143.6 (C), 143.5 (C), 142.7 (CH), 140.2 (CH), 136.94 (C), 136.85 (C), 135.6 (C), 135.5 (C), 130.4 (C), 130.3 (C), 127.7 (C), 127.6 (C), 125.6 (CH), 125.2 (CH), 123.1 (C), 122.9 (C), 122.19 (CH), 122.17 (CH), 121.8 (CH), 121.6 (CH), 121.2 (CH), 121.1 (CH), 117.3 (2xCH), 115.9 (C), 115.6 (C), 112.2 (CH), 112.1 (CH), 110.5 (CH), 110.3 (CH), 61.4 (CH_2), 61.3 (CH_2), 34.3 (CH_2), 33.8 (CH_2), 14.3 (CH_3), 14.2 (CH_3). HRMS (ESI+) calculated for $C_{21}H_{19}ClN_5O_3S$ [*M* + H]$^+$ 456.089, found 456.089.

Ethyl (*E*)-6-chloro-3-((2-(2-(4-chlorophenoxy)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (19) This compound was synthesized according to synthetic procedure 2, starting with 2-(4-chlorophenoxy)acetohydrazide (25.8 mg, 0.129 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (65:35) as white solid (47.7 mg, 0.110 mmol, 90%). M.p.: >239 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.28 (br. s, 2H), 11.73 (br. s, 2H), 9.08 (s, 1H), 8.85 (s, 1H), 8.44 (d, *J* = 8.7 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 2H), 7.38 – 7.36 (m, 2H), 7.35 – 7.31 (m, 2H), 7.24 (dd, *J* = 8.7, 1.9Hz, 1H), 7.21 (dd, 8.7, 1.9Hz, 1H), 7.06 – 7.02 (m, 2H), 6.99 – 6.95 (m, 2H), 5.22 (s, 2H), 4.70 (s, 2H), 4.42 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.4 (C), 163.8 (C), 160.7 (C), 160.6 (C), 157.2 (C), 156.8 (C), 143.5 (CH), 140.4 (CH), 136.94 (C), 136.88 (C), 130.3 (C), 130.2 (C), 129.3 (2xCH), 129.1 (2xCH), 127.7 (2xC), 125.6 (CH), 125.2 (CH), 124.9 (C), 124.4 (C), 123.1 (C), 122.9 (C), 122.3 (CH), 122.2 (CH), 116.5 (2xCH), 116.3 (2xCH), 116.0 (C), 115.5 (C), 112.1

(2xCH), 66.7 (CH_2), 65.2 (CH_2), 61.3 (CH_2), 61.2 (CH_2), 14.2 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{18}Cl_2N_3O_4$ [*M* + H]$^+$ 434.067, found 434.067.

Ethyl (*E*)-3-((2-(1-(tert-butoxycarbonyl)piperidine-4-carbonyl)hydrazineylidene)methyl)-6-chloro-1*H*-indole-2-carboxylate (20) This compound was synthesized according to synthetic procedure 2, starting with tert-butyl 4-(hydrazinecarbonyl)piperidine-1-carboxylate (31.3 mg, 0.129 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (52:48) as white solid (36.0 mg, 0.0755 mmol, 62%). M.p.: >231 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.22 (br. s, 2H), 11.49 (br. s, 1H), 11.37 (br. s, 1H), 8.96 (s, 1H), 8.82 (s, 1H), 8.44 (d, *J* = 8.7 Hz, 1H), 8.22 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.49 (d, *J* = 1.8 Hz, 1H), 7.31 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.22 (dd, *J* = 8.7, 1.8 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 4.06 – 3.95 (m, 4H), 3.34 – 3.27 (m, 1H, partial overlap with water), 3.01 – 2.69 (m, 4H), 2.43 (tt, *J* = 11.5, 3.8 Hz, 1H), 1.82 (d, *J* = 11.4 Hz, 2H), 1.75 (d, *J* = 10.7 Hz, 2H), 1.56 – 1.44 (m, 4H), 1.43 – 1.37 (m, 24H). ^{13}C NMR (126 MHz, DMSO-d_6, 60 °C) δ 175.1 (C), 170.1 (C), 160.4 (2xC), 153.8 (2xC), 141.9 (CH), 139.1 (CH), 136.8 (C), 136.7 (C), 130.1 (2xC), 127.3 (C), 127.1 (C), 125.5 (CH), 124.5 (CH), 123.0 (C), 122.8 (C), 122.0 (CH), 121.8 (CH), 116.3 (C), 115.8 (C), 112.0 (CH), 111.9 (CH), 78.4 (2xC), 61.0 (2xCH_2), 42.9 (2xCH_2), 42.7 (2xCH_2), 40.4 (CH), 37.7 (CH), 27.92 (6xCH_3), 27.87 (2xCH_2), 27.2 (2xCH_2), 14.0 (CH_3), 13.9 (CH_3). In order to observe all CH_2 signals of the piperidinyl moiety, the ^{13}C-NMR spectrum was recorded at 60 °C instead of 25 °C. HRMS (ESI+) calculated for $C_{23}H_{30}ClN_4O_5$ [*M* + H]$^+$ 477.190, found 477.190.

Ethyl (*E*)-3-((2-((tert-butoxycarbonyl)-L-phenylalanyl)hydrazineylidene)methyl)-6-chloro-1*H*-indole-2-carboxylate (21) This compound was synthesized according to synthetic procedure 2, starting with tert-butyl (*S*)-(1-hydrazineyl-1-oxo-3-phenylpropan-2-yl)carbamate (35.0 mg, 0.125 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (52:48) as white solid (49.9 mg, 0.0973 mmol, 82%). M.p.: >222 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.26 (br. s, 2H), 11.63 (br. s, 1H), 11.50 (br. s, 1H), 9.00 (s, 1H), 8.85 (s, 1H), 8.44 (d, *J* = 8.7 Hz, 1H), 8.21 (d, *J* = 8.7 Hz, 1H), 7.51 (d, *J* = 1.9 Hz, 2H), 7.39 – 7.15 (m, 12H), 7.10 (d, *J* = 8.7 Hz, 1H), 6.99 (dd, *J* = 8.6, 2.0 Hz, 1H), 5.30 – 5.17 (m, 1H), 4.42 (q, *J* = 7.1 Hz, 4H), 4.28 – 4.22 (m, 1H), 3.09 – 2.70 (m, 4H), 1.41 (t overlap, *J* = 7.1 Hz, 6H), 1.36 – 1.14 (m, 18H). ^{13}C NMR (151 MHz, DMSO-d_6) 173.0 (C), 168.2 (C), 160.6 (2xC), 155.6 (C), 155.4 (C), 142.6 (CH), 140.2 (CH), 138.04 (C), 138.00 (C), 136.91 (C), 136.85 (C), 130.3 (C), 130.2 (C), 129.2 (2xCH), 128.9 (2xCH), 128.11 (2xCH), 128.06 (2xCH), 127.7 (C), 127.4 (C), 126.3 (2xCH), 125.6 (CH), 124.9 (CH), 123.0 (C), 122.7 (C), 122.1 (CH), 121.7 (CH), 116.2 (C), 115.6 (C), 112.3 (CH), 112.1 (CH), 78.1 (C), 78.0 (C), 61.3 (CH_2), 61.2 (CH_2), 55.1 (CH), 51.9 (CH), 37.3 (CH_2), 36.0 (CH_2), 28.18 (6xCH_3), 14.3 (CH_3), 14.2 (CH_3). HRMS (ESI+) calculated for $C_{26}H_{30}ClN_4O_5$ [*M* + H]$^+$ 513.190, found 513.189.

Ethyl (*E*)-6-chloro-3-((2-(2-(naphthalen-1-yloxy)acetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (22) This compound was synthesized according to synthetic procedure 2, starting with 2-(naphthalen-1-yloxy)acetohydrazide (27.4 mg, 0.127 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (66:34) as white solid (45.9 mg, 0.102 mmol, 85%). M.p.: >247 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.28 (br. s, 2H), 11.83 (br. s, 1H), 11.80 (br. s, 1H), 9.10 (s, 1H), 8.89 (s, 1H), 8.46 (d, *J* = 8.7 Hz, 1H), 8.36 – 8.26 (m, 3H), 7.93 – 7.87 (m, 2H), 7.59 – 7.47 (m, 8H), 7.43 (td, *J* = 8.0, 6.8 Hz, 2H), 7.25 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.19 (dd, *J* = 8.7, 2.0 Hz, 1H), 6.98 (dd, *J* = 7.8, 0.9 Hz, 1H), 6.91 (dd, *J* = 7.8, 0.9 Hz, 1H), 5.42 (s, 2H), 4.90 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.5 (C), 163.9 (C), 160.7 (C), 160.6 (C), 153.7 (C), 153.5 (C), 143.5 (CH), 140.4 (CH), 136.93 (C), 136.87 (C), 134.1 (2xC), 130.3 (C), 130.2 (C), 127.6 (C),

127.6 (C), 127.4 (2xCH), 126.6 (CH), 126. 4 (CH), 126.1 (CH), 126.1 (CH), 125.6 (CH), 125.34 (CH), 125.25 (CH), 125.2 (CH), 124.92 (C), 124.89 (C), 123.1 (C), 122.9 (C), 122.3 (CH), 122.2 (CH), 122.0 (CH), 121.7 (CH), 120.7 (CH), 120.2 (CH), 116.0 (C), 115.5 (C), 112.1 (2xCH), 105.6 (CH), 105.3 (CH), 66.9 (CH_2), 65.4 (CH_2), 61.33 (CH_2), 61.25 (CH_2), 14.22 (CH_3), 14.15 (CH_3). HRMS (ESI+) calculated for $C_{24}H_{21}ClN_3O_4$ [*M* + H]$^+$ 450.122, found 450.121.

Ethyl (*E*)-3-((2-(3-(tert-butyl)-1-(3-methylbenzyl)-1*H*-pyrazole-5-carbonyl)hydrazineylidene)methyl)-6-chloro-1*H*-indole-2-carboxylate (23) This compound was synthesized according to synthetic procedure 2, starting with 3-(tert-butyl)-1-(3-methylbenzyl)-1*H*-pyrazole-5-carbohydrazide (26.7 mg, 0.0932 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (14:86) as white solid (36.2 mg, 0.0696 mmol, 78%). M.p.: >237 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.26 (br. s, 2H), 11.91 (br. s, 2H), 9.18 (s, 1H), 8.92 (s, 1H), 8.50 (d, *J* = 8.7 Hz, 1H), 8.07 (d, *J* = 8.7 Hz, 1H), 7.51 (d, *J* = 1.9 Hz, 2H), 7.26 (dd, *J* = 8.7, 2.0 Hz, 2H), 7.18 (t, *J* = 7.6 Hz, 1H), 7.11 (t, *J* = 7.9 Hz, 1H), 7.05 (d, *J* = 7.4 Hz, 2H), 6.98 (s, 2H), 6.96 (s, 2H), 6.88 (d, *J* = 7.7 Hz, 2H), 5.70 (s, 2H), 5.55 (s, 2H), 4.43 (q, *J* = 7.1 Hz, 4H), 2.25 (s, 3H), 2.12 (s, 3H), 1.40 (t, *J* = 7.1 Hz, 6H), 1.31 (2s overlap, 18H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.6 (C), 159.6 (C), 155.7 (C), 143.6 (CH), 138.3 (C), 137.4 (C), 136.9 (C), 134.1 (C), 130.3 (C), 128.3 (CH), 127.8 (CH), 127.6 (C), 127.4 (CH), 125.7 (CH), 123.9 (CH), 123.1 (C), 122.2 (CH), 116.3 (C), 112.1 (CH), 104.4 (CH), 61.2 (CH_2), 53.5 (CH_2), 31.8 (C), 30.4 (3xCH_3), 21.0 (CH_3), 14.3 (CH_3). HRMS (ESI+) calculated for $C_{28}H_{31}ClN_5O_3$ [*M* + H]$^+$ 520.211, found 520.211.

Ethyl (*E*)-6-chloro-3-((2-(4-(2,4-dichlorophenoxy)butanoyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (24) This compound was synthesized according to synthetic procedure 2, starting with 4-(2,4-dichlorophenoxy)butanehydrazide (32.3 mg, 0.123 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (63:37) as white solid (49.2 mg, 0.0990 mmol, 85%). M.p.: >221 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.22 (br. s, 2H), 11.50 (br. s, 1H), 11.42 (br. s, 1H), 8.94 (s, 1H), 8.82 (s, 1H), 8.45 (d, *J* = 8.7 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 1H), 7.56 (d, *J* = 2.6 Hz, 1H), 7.55 (d, *J* = 2.6 Hz, 1H), 7.49 (2d overlap, *J* = 1.9 Hz, 2H), 7.36 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.34 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.23 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.20 (2d overlap, *J* = 8.9 Hz, 2H), 7.12 (dd, *J* = 8.7, 1.9 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 4H), 4.18 (t, *J* = 6.3 Hz, 2H), 4.13 (t, *J* = 6.3 Hz, 2H), 2.90 (t, *J* = 7.2 Hz, 2H), 2.44 (t, *J* = 7.2 Hz, 2H), 2.15 – 2.03 (m, 4H), 1.40 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.4 (C), 167.8 (C), 160.7 (2xC), 153.03 (C), 153.00 (C), 141.5 (CH), 139.3 (CH), 136.95 (C), 136.88 (C), 130.3 (C), 130.1 (C), 129.23 (CH), 129.20 (CH), 128.11 (CH), 128.09 (CH), 127.4 (C), 127.2 (C), 125.7 (CH), 124.9 (CH), 124.4 (C), 124.3 (C), 123.1 (C), 122.9 (C), 122.44 (C), 122.41 (C), 122.0 (2xCH), 116.3 (C), 115.9 (C), 115.2 (CH), 115.1 (CH), 112.2 (CH), 112.1 (CH), 68.5 (CH_2), 68.4(CH_2), 61.24(CH_2), 61.17(CH_2), 30.3 (CH_2), 28.5 (CH_2), 24.3 (CH_2), 23.6 (CH_2), 14.2 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{22}H_{21}Cl_3N_3O_4$ [*M* + H]$^+$ 496.059, found 496.059.

Ethyl (*E*)-6-chloro-3-((2-((3-nitrophenyl)glycyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (25) This compound was synthesized according to synthetic procedure 2, starting with 2-((3-nitrophenyl)amino)acetohydrazide (26.2 mg, 0.125 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (68:32) as yellow solid (43.7 mg, 0.0985 mmol, 83%). M.p.: >251 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.26 (br. s, 2H), 11.70 (br. s, 1H), 11.68 (br. s, 1H), 9.02 (s, 1H), 8.87 (s, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.36 (d, *J* = 8.7 Hz, 1H), 7.52 (d, *J* = 1.9 Hz, 1H), 7.50 (d, *J* = 1.9 Hz, 1H), 7.44 – 7.32 (m, 6H), 7.28 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.22 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.11 (ddd, *J* = 8.0, 2.4, 1.1 Hz, 1H), 7.05 (ddd, *J* = 8.0, 2.3, 1.2 Hz, 1H), 6.78 (t, *J* = 6.1 Hz, 1H, -NH), 6.66 (t, *J* = 5.8

Hz, 1H, -NH), 4.47 – 4.37 (m, 7H), 3.96 (d, *J* = 6.1 Hz, 1H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.3 (C), 165.9 (C), 160.7 (C), 160.6 (C), 149.7 (C), 149.6 (C), 148.78 (C), 148.76 (C), 142.6 (CH), 140.1 (CH), 137.0 (C), 136.9 (C), 130.31 (C), 130.25 (C), 130.0 (CH), 129.8 (CH), 127.6 (C), 127.4 (C), 125.6 (CH), 125.1 (CH), 123.1 (C), 122.9 (C), 122.3 (CH), 122.1 (CH), 118.9 (CH), 118.7 (CH), 116.1 (C), 115.7 (C), 112.2 (CH), 112.1 (CH), 110.5 (CH), 110.2 (CH), 105.6 (CH), 105.4 (CH), 61.3 (CH_2), 61.2 (CH_2), 45.2 (CH_2), 43.9 (CH_2), 14.24 (CH_3), 14.15 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{19}ClN_5O_5$ [*M* + H]$^+$ 444.107, found 444.106.

Ethyl (*E*)-6-chloro-3-((2-(2-phenoxyacetyl)hydrazineylidene)methyl)-1*H*-indole-2-carboxylate (47) This compound was synthesized according to synthetic procedure 2, starting with 2-phenoxyacetohydrazide (21.3 mg, 0.128 mmol). The product was isolated as a mixture of E_{syn} and E_{anti} conformers (62:38) as white solid (42.8 mg, 0.107 mmol, 88%). M.p.: >267 °C (decomposition). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.27 (br. s, 2H), 11.72 (br. s, 2H), 9.09 (s, 1H), 8.86 (s, 1H), 8.45 (d, *J* = 8.7 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 7.50 (d, *J* = 1.8 Hz, 2H), 7.35 – 7.28 (m, 4H), 7.24 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.20 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.04 – 6.92 (m, 6H), 5.19 (s, 2H), 4.68 (s, 2H), 4.42 (q, *J* = 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.7 (C), 164.1 (C), 160.7 (C), 160.6 (C), 158.2 (C), 157.9 (C), 143.5 (CH), 140.3 (CH), 136.93 (C), 136.86 (C), 130.3 (C), 130.2 (C), 129.5 (2xCH), 129.4 (2xCH), 127.61 (C), 127.59 (C), 125.6 (CH), 125.2 (CH), 123.1 (C), 122.9 (C), 122.3 (CH), 122.2 (CH), 121.2 (CH), 120.7 (CH), 116.0 (C), 115.5 (C), 114.7 (2xCH), 114.4 (2xCH), 112.11 (CH), 112.12 (CH), 66.5 (CH_2), 64.8 (CH_2), 61.3 (CH_2), 61.2 (CH_2), 14.3 (CH_3), 14.1 (CH_3). HRMS (ESI+) calculated for $C_{20}H_{19}ClN_3O_4$ [*M* + H]$^+$ 400.106, found 400.106.

Enzyme-activity studies

Human 15-LOX-1 Screening UV Assay and compound potency

The 15-LOX-1 enzyme was expressed and purified as described before.[6] Using a BioTek Synergy H1 hybrid plate reader, the activity of 15-LOX-1 was determined by the conversion of linoleic acid (LA) to 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(*S*)-HpODE) (λ_{max} of 234 nm) in the assay on which we reported before.[6,12]

The substrate, LA (Sigma-Aldrich, L1376), was diluted in ethanol to 500 μM. The reaction mixture (60 mM in DMSO-d_6) was diluted to 10 μM over two steps. The inhibitor solution of 10 μL was mixed with 130 μL of assay buffer (25 mM HEPES, pH 7.5) and 50 μL of 1:160 enzyme solution and incubated for 8 min at rt. After incubation, 10 μL of 500 μM LA was added to the mixture, which resulted in a mixture with a final enzyme dilution of 1:640, 25 μM LA and 0.50 μM inhibitor (1.0 vol.% DMSO). The linear increase in absorption at 234 nm was used to determine the enzyme activity. The linear part covered eight data points during the first five minutes, while excluding the first two points. In order to calculate the percentage inhibition the slope of the linear part of each curve was calculated and divided by the DMSO control without presence of inhibitor. In both cases the blank reaction (without enzyme and inhibitor) was subtracted. The optimum concentration of 15-LOX-1 was determined by an enzyme activity assay (640 times dilution). All experiments were performed in triplicate, and the average triplicate values and their standard errors are plotted. Data analysis of all experiments was performed using the Microsoft Excel Professional 2010 and Graphpad Prism 5.00 software. In addition to the 44 reaction mixtures, the aldehyde hit compound was dissolved and diluted under the same conditions. The residual enzyme activity after incubation with 0.50 μM of each reaction mixture is shown in Figure 2.

The half-maximal inhibition concentration (IC_{50}) of the inhibitors for 15-LOX-1 was determined using the same assay, but with a final enzyme dilution of 1:120. Using a serial dilution with buffer, the desired concentrations of the inhibitor were obtained, ranging from 0.098 to 50 μM or 0.049 to 25 μM, depending on the inhibitory potency. The IC_{50} curves can be found in the supporting information.

Control assays using the eight most potent acylhydrazones

Without LA: 3.57 μL 10 mM inhibitor solution was added to 1 mL of buffer. 140 μL of this mixture was added to a 96-well plate in triplicate. As control, the inhibitor solution was replaced by a DMSO/buffer solution. Then 10 μL of ethanol was added to each well. Subsequently, 50 μL of enzyme solution was added to all wells, except for the blank in which the enzyme solution was replaced by buffer. Then the absorption at 234 nm was measured over time.

Without 15-LOX-1: 3.57 μL 10 mM inhibitor solution was added to 1 mL of buffer. 140 μL of this mixture was added to a 96-well plate in triplicate. Then 50 μL of buffer was added to each well. As control, the inhibitor solution was replaced by a DMSO/buffer solution. Then 10 μL of 500 μM LA solution was added to each well, except for the blank in which the LA solution was replaced by ethanol. Then the absorption at 234 nm was measured over time.

DPPH antioxidant assay

To investigate whether the acylhydrazone inhibitors exhibit antioxidant potential, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was employed in triplicate.[18,19] The inhibitors* and DPPH were dissolved in MeOH containing 10% DMSO to obtain a 2* mM stock solution of each inhibitor and a 0.2 mM stock solution of DPPH. Then, 111.1 μL of inhibitor was added to the first well of the 96-well plate, after which 11.1 μL was transferred to the next well in a 10X serial dilution. Subsequently, 100 μL 0.2 mM DPPH was added, resulting in 1 mM to 1 nM inhibitor concentration** and a constant DPPH concentration of 0.1 mM. The mixtures were incubated for 15 min. at ambient temperature protected from light. As a control, a compound dilution series in MeOH (containing 10% DMSO) was used. The absorbance at 517 nm (λ_{max} as determined beforehand) was measured using a BioTek Synergy H1 hybrid plate reader. The control was subtracted and the data were normalized to the absorbance without inhibitor. The concentration that gave 50% reduction in absorbance (EC_{50}) was determined by a dose response inhibition non-linear fit in Graphpad 5.00. In addition to the acylhydrazone inhibitors, the antioxidant potencies of **1** and ascorbic acid were evaluated.

** **16** and **17** were dissolved to obtain 0.5 mM; **19** and **47** were dissolved to obtain 1 mM, ** the starting concentration in the assay was 250 μM for **16** and **17**, and 500 μM for **19** and **47**.*

FRAP antioxidant assay

In order to determine if the acylhydrazone inhibitors exhibit electron-donating properties, a ferric reducing antioxidant power (FRAP) assay was employed as described previously with minor modifications.[20,21] The following stock solutions were prepared; Acetate buffer: anhydrous sodium acetate (1870 mg, 22.8 mmol) was dissolved in MilliQ (800 mL) and glacial acetic acid (15–16 mL) was added until a pH of 3.6 was obtained. Then, MilliQ was added to obtain a total volume of 1000 mL and a final concentration of 0.30 M acetate. $FeCl_3$: Iron(III) chloride hexahydrate (270.8 mg, 1.002 mmol) was dissolved in MilliQ (50 mL). TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine (156.1 mg, 0.500 mmol) was dissolved in HCl (50 mL, 40 mM). $FeSO_4$: Iron(II) sulfate heptahydrate (27.8 mg, 0.100 mmol) was added to a 100 mL volumetric flask, which was subsequently filled with MilliQ until a total volume of 100 mL. The FRAP reagent was formed by adding $FeCl_3$ (1000 μL, 20 mM) and TPTZ (1000 μL, 10 mM) to 10.0 mL

of 300 mM acetate buffer. The reagent was incubated in a 37 °C water bath for 20 minutes and freshly prepared before each experiment.

The acylhydrazone inhibitors (1.87–2.72 mg, 4.21–8.30 μmol) were dissolved in DMSO and subsequently diluted to obtain 1.00 mM stock solutions. Ascorbic acid (3.50 mg, 0.0199 mmol) was dissolved in MilliQ, subsequently diluted to obtain a 1.00 mM stock solution and used immediately. Then, to a 96-well plate was added to each well (in duplicate) 10 μL of MilliQ, 1.0 mM $FeSO_4$ or 1.0 mM ascorbic acid. Subsequently, 10 μL of DMSO or 1.0 mM acylhydrazone was added. Then, 300 μL of pre-warmed FRAP reagent was added using a multipipette. The absorbance at 595 nm (λ_{max} as determined beforehand) was measured every 15 seconds using a BioTek Synergy H1 hybrid plate reader at 37 °C for 5 minutes. The measurements were repeated using a blank FRAP reagent, lacking the TPTZ ligand. The average absorbance around 4 minutes after addition of the FRAP reagent (or FRAP reagent without TPTZ) was used for the calculations. First, the blank measurements were subtracted from the FRAP assay, after which the FRAP value was calculated based on the $FeSO_4$ controls.

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Appendix A. Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2019.04.021.

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