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BRIEF ARTICLE

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A Class of 5-Benzylidene-2-phenylthiazolinones with High Potency as Direct 5-Lipoxygenase Inhibitors

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Supporting Information

ABSTRACT: A novel class of potent direct 5-lipoxygenase (5-LO) inhibitors bearing a thiazolinone-scaffold identified by virtual screening is presented. A range of substitutions and the importance of the 2-phenyl moiety were evaluated. This series is characterized by high potency in intact polymorphonuclear leukocytes and a cell-free system, exemplified by (*Z*)-2-(4-chlorophenyl)-5-(4-methoxybenzylidene)-5*H*-thiazol-4-one (**18**, IC_{50} = 0.28 and 0.09 μM). These disubstituted thiazolinones may possess potential for intervention with inflammatory and allergic diseases and certain cancer types.

INTRODUCTION

5-Lipoxygenase (5-LO), a non-heme-iron-containing dioxygenase, catalyzes the biosynthesis of leukotrienes (LTs), which are lipid mediators of inflammatory and allergic responses and play a key role in host defense reactions. LTs exert their biological effects via specific G-protein-coupled receptors and play a pivotal role in inflammatory and allergic disorders and cardiovascular diseases and cancer.[1,2] 5-LO catalyzes the first steps in the conversion of arachidonic acid (AA) into LTA_4 under the aid of the nuclear membrane-bound 5-LO-activating protein (FLAP).[3] LTA_4 is further converted by LTA_4 hydrolase into LTB_4 which is a potent chemotactic agent and activator for phagocytes. Alternatively, conjugation of LTA_4 with reduced glutathione by LTC_4 synthases yields the cysteinyl-containing LTs C_4, D_4, and E_4 that cause bronchoconstriction and vascular permeability.[1] Because of the key role of 5-LO in LT biosynthesis, 5-LO inhibitors are supposed to be of therapeutic value for the treatment of asthma, allergic rhinitis, atherosclerosis, and certain types of cancer.[2,4] Also, some novel indications came up, e.g., chronic myeloid leukemia (CML),[5] malaria,[6a,6b] and cancer chemoprevention.[7]

Recently, we presented the use of ligand-based virtual screening to identify new inhibitors of 5-LO product formation.[8] Briefly, two similarity search methods, "Charge3D"[9a,9b] and "TripleCharge3D",[9a,9c] were applied for virtual screening. With these methods, two molecules are compared based on their three-dimensional distribution of partial atom charges. This led among others to the discovery of a thiazolinone structure series, represented by **1** (5-(4-ethoxybenzylidene)-2-phenyl-5*H*-thiazol-4-one, Figure 1A and Table 1), having low micromolar 5-LO inhibitory activity in a cellular assay. Here, we describe a structure−activity relationship (SAR) of this class of direct 5-LO inhibitors based on the scaffold of **1**. Characteristic structural features of **1** are two phenyl residues connected to the central thiazolinone. Our strategy was aimed at exploring the influence of different substituents at these phenyl residues (Figure 1B) and assessing the role of the 2-phenyl residue on 5-LO activity.

RESULTS AND DISCUSSION

In our previous report, we presented the 5-benzylidene-2-phenyl-5*H*-thiazol-4-one **1** (Figure 1A) as an inhibitor of 5-LO product formation with low micromolar activity.[8] The thiazolinone core by itself is known in 5-LO inhibitors; in 1994 Unangst et al. reported dual 5-LO/COX inhibition by 5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-methylene]-2-imino-4-thiazolidinone (Figure 2A).[10a] Recently, Geronikaki et al. identified 2-thiazolylimino-5-arylidene-4-thiazolidinones (Figure 2B) with moderate activity against dual soybean 13-LO/COX-1.[10b] Nevertheless, theses structures differ from our class in the 2-phenyl moiety and the substitution pattern at both phenyl rings that is unique to our compound class. Notably, for **1** no inhibition of COX-2 was observed.[8] Thus, it is possible that the 2-phenyl substituent in this scaffold class is responsible for the selective 5-LO activity. To our knowledge, this moiety has not been described as a 5-LO/COX dual inhibitor.

To further evaluate the potential of **1**, we assessed its effectiveness in a cell-free assay. For LT biosynthesis, AA is released by $cPLA_2$ and converted by activated 5-LO after the transfer by FLAP.[3] Activation of 5-LO in the cell requires Ca^{2+} mobilization and/or 5-LO phosphorylation by protein kinases that causes translocation of 5-LO from soluble cellular compartments to the nuclear membrane close to FLAP.[3] For conclusive analysis of the effectiveness of the test compounds, a cell-based test system using isolated human polymorphonuclear leukocytes (PMNL) and a cell-free assay utilizing the 100000*g* supernatant (S100) of PMNL homogenates were applied. In the cell-based assays, many

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possibilities aside from direct interference with 5-LO exist (e.g., FLAP or $cPLA_2$ inhibition, suppression of 5-LO kinases or Ca^{2+} mobilization, inhibition of 5-LO translocation) eventually suppressing LT synthesis.[11] To evaluate the compounds in intact PMNL, the 5-LO products 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(P)ETE) and the *trans*- and epi-*trans*-isomers of LTB_4, as well as LTB_4 were analyzed. Cells were preincubated with the compounds, and subsequently 5-LO product formation was elicited by 2.5 μM ionophore A23187. Exogenous AA (20 μM) was supplemented to circumvent the necessity of $cPLA_2$-mediated endogenous substrate supply. For evaluation of the effectiveness of the test compounds in the cell-free assay, 5-LO products were analyzed in PMNL S100 after preincubation with the compounds and subsequent stimulation of 5-LO product formation by addition of 1 mM $CaCl_2$ and 20 μM AA. The 5-LO inhibitor **35** (BWA4C)[12] was taken as reference compound showing IC_{50} of 0.05 μM in PMNL S100 and IC_{50} of 0.08 μM in intact PMNL (Table 1), which is similar to the literature.[12] Because we were mainly interested in SARs regarding the direct interaction with 5-LO, the following discussion is focused on the activity of the compounds in cell-free S100. Nevertheless, all inhibitors were active in the whole cell assay even though slightly less potent. Their potency in intact PMNL is given for completeness.

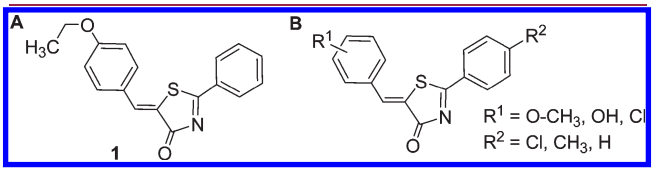
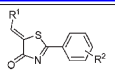
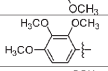
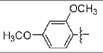
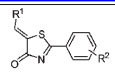
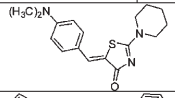
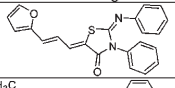
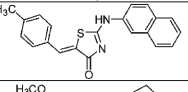
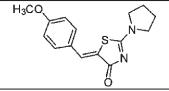
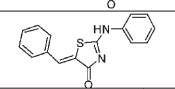
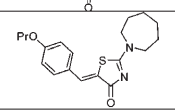
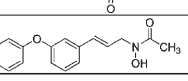


Figure 1. Structures of parent compound **1** (A) and herein reported active compounds (B, best hits).

Compound **1** caused potent inhibition of 5-LO product formation in intact PMNL and in cell-free PMNL S100 with IC_{50} of 2 and 0.5 μM, respectively (Table 1), which identifies the compound as a direct 5-LO inhibitor. As the first hit, **1** served as the structural template for a follow-up virtual screening round to identify analogues bearing the thiazolinone scaffold. Our study aimed at exploring the influence of different substituents at both phenyl residues (Figure 1 B) and assessing the role of the 2-phenyl residue on 5-LO inhibitory activity. Thus, we screened the ASINEX (Moscow, Russia) and SPECS (Delft, The Netherlands) compound collections for 5-benzylidene-2-phenylthiazolinones

Table 1. IC_{50} for Inhibition of 5-LO and 5-LO Product Formation by Test Compounds in Cell-Free S100 and Intact PMNL[c]

Compound			5-LO product formation IC_{50} [μM]	
	R^1	R^2	S100	PMNL
1		H	0.5	2
2		*p*-CH_3	0.3	0.58
3		H	0.3	0.65
4		H	0.54	0.48
5		H	3	9.5
6		H	3	4.4
7*		*p*-CH_3	0.3	0.66
8		*p*-CH_3	0.13	0.35
9		*p*-CH_3	0.4	2.45
10		*p*-CH_3	0.98	2.02
11		*p*-CH_3	1.3	0.8
12		*p*-CH_3	2.7	>10[a]
13		*p*-CH_3	1.25	2.9
14		*p*-CH_3	0.35	0.73
15		*p*-CH_3	0.23	0.4
16		H	0.3	0.91
17		H	0.3	4.3
18*		*p*-Cl	0.09	0.28

Compound			5-LO product formation IC_{50} [μM]	
	R^1	R^2	S100	PMNL
19*		H	0.15	0.53
20*		*p*-CH_3	0.19	2.11
21*		*p*-$OCH_2C(O)OCH_3$	0.58	0.86
22*		*p*-OH	0.65	1.69
23*		*p*-OCH_3	4	0.32
24*		*p*-NH_2	0.63	1.93
25*		*p*-$C(O)CH_3$	0.11	0.55
26*		*m*-$C(O)CH_3$	0.13	0.38
27*		*m*-F	0.12	0.24
28*		*p*-Cl	0.11	0.4
29			>30[b]	20
30			>30[b]	30
31			>30[b]	>30[b]
32			>30[b]	>30[b]
33			>30[b]	>30[b]
34			14	12
35 (BWA4C)			0.05	0.08

[a] Residual activity (percentage of control) is >60% at 10 μM . [b] Residual activity (percentage of control) is >60% at 30 μM. [c] The asterisk (*) indicates synthesized compounds.

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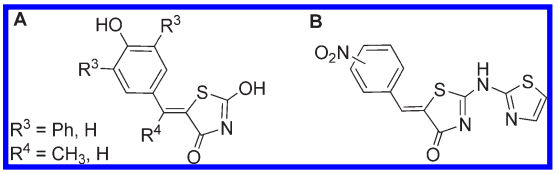

Figure 2. Sample structures of known thiazolinone-based 5-LO inhibitors (A,[10a] B[10b]): best (A, R^3 = Ph, R^4 = H; B, *m*-NO_2) and worst derivative (A, R^3, R^4 = H; B, *p*-NO_2) of each series is shown.

using MACCS substructure keys.[13a,13b] This involved the generation of MACCS keys from a given set of 166 predefined substructures. A bit is set whenever one of these substructures is present in a molecule. Bit strings of the query and the database compounds were compared using the Tanimoto coefficient *T*.[14a,14b] *T* ranges from 0 to 1, where 1 indicates bit string identity. Molecules were retained with a Tanimoto coefficient of >0.69 (indicating structural similarity to **1**). From this list, 19 commercially available substances (**2**−**17**, **30**, **31**, and **33**, Table 1) were selected and tested with three compounds derived from our previous screening[8] (**29**, **32**, and **34**) to obtain a preliminary SAR. We selected compounds bearing different substitution patterns at the 5-benzylidene and compounds having the 2-phenyl substituted to examine the role of these moieties. Substances with substitutions at the 5-benzylidene residue all bear *p*-methyl or no substitution at the 2-phenyl residue (**2**−**13**). The direct 5-LO inhibitory activity determined in S100 varied about an order of magnitude (0.13 μM (**8**) and 3 μM (**5** and **6**)) despite the variation in substitution pattern performed (**2**−**13**). Within this series, *o*-OH seems to be more potent than *p*-OH, which is most prominent if there is no substitution at R^2 present (**5** and **3**), enhancing the inhibitory activity from 3 to 0.3 μM. Replacement of *m*-chloro of **5** by another electron-withdrawing but less lipophilic group, nitro (**6**), did not alter efficiency (IC_{50} = 3 μM for both). The chloride substitution alone (**2**) produced one of the most potent compounds of this series but did not significantly alter the potency compared to the unsubstituted derivative **14** (IC_{50} = 0.35 μM). Elongation with a vinyl group (**15**) was also well tolerated (IC_{50} = 0.23 μM). Different groups are accepted at the 5-position of the thiazolinone, notably also lipophilic, voluminous ones like *tert*-butylphenyl (**16**, IC_{50} = 0.3 μM) or anthracene (**17**, IC_{50} = 0.3 μM), even though **17** may get trapped in highly lipophilic areas in the cell, as it is less potent in intact PMNL.

To explore the influence of substitutions at the 2-phenyl moiety, we synthesized several thiazolinones with substitutions at R^2 (**18**−**28**), as only derivatives with substitutions at the 5-benzylidene were available commercially. The starting point was **7** bearing a *p*-methoxy group at the 5-benzylidene which was kept for all compounds except **20** and **28**. The following general synthetic procedure was applied (Figure 3): a solution of the corresponding benzonitrile (1 equiv, 0.07−0.5 mmoL), thioglycolic acid (1−1.1 equiv), the corresponding benzaldehyde (1 equiv), and TEA in methanol was refluxed overnight. The reaction mixture was evaporated under reduced pressure, and the pure product was recrystallized from ethanol and washed with acetone.[15]

The introduction of electron-withdrawing halogens at the 2-phenyl, *p*-chloride (**18**), or *m*-fluoride (**27**) did not alter the inhibitory potency (IC_{50} = 0.09 and 0.12 μM, respectively). Another electron withdrawing group, *p*-amino (**24**), led to a

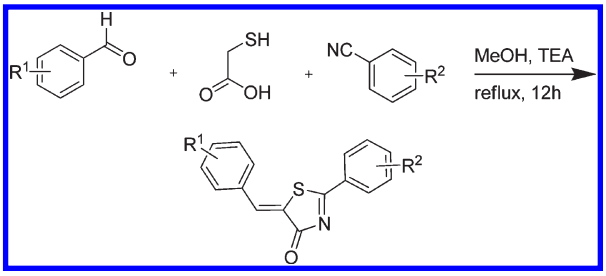

Figure 3. General synthetic procedure for synthesized compounds.[15]

slight decrease of inhibitory activity (IC_{50} = 0.63 μM). *p*- and *m*-acetaldehyde (**25** and **26**) were equipotent (IC_{50} = 0.11 and 0.13 μM), whereas a *p*-hydroxy group (**22**) led to a somewhat decreased potency (IC_{50} = 0.65 μM). Exchange of the *p*-methoxy residue of **18** by a hydroxyl group (**28**) was without effect (IC_{50} = 0.09 and 0.11 μM). Addition of a hydrophilic methyl ester at R^1 (**20**) and R^2 (**21**) was also well tolerated with IC_{50} of 0.19 and 0.58 μM. The only exception was a methoxy substitution at R^2 (**23**), which was surprisingly less potent in S100 (IC_{50} = 4 μM) but highly potent in intact PMNL (IC_{50} = 0.32 μM).

To examine the role of the 2-phenyl substituent for 5-LO inhibitory activity, we evaluated the potency of compounds having this moiety replaced by different aliphatic heterocyles (pyrrolidine, piperidine, or azepane) or by an N-linker (**29**−**34**). These structural changes were detrimental with an almost total loss of activity, e.g., **33**. Only the replacement of the 2-phenyl by an azepane (with a propoxy substituent at R^1, **34**) preserved moderate activity (IC_{50} = 14 μM). This highlights the directly coupled 2-phenyl residue as being superior to the examined heterocycles and N-linkers for the tested compounds regarding 5-LO inhibitory activity.

Taken together, we found that by keeping the core scaffold, different substituents cause only small changes in the compounds potencies. Different groups were tolerated at the 5-position, notably also lipophilic voluminous ones (e.g., **17**). High overall lipophilicity is known as a general determinant for many potent 5-LO inhibitors.[16] In contrast, changes in the scaffold to aliphatic cyclic amines (pyrrolidine, piperidine or azepane) or N-containing linkers instead of the 2-phenyl residue (**29**−**34**) showed impaired (**34** and **29**) or no 5-LO inhibitory activity (**30**−**33**). Thus, it can be deduced that the 2-phenyl residue is crucial for the 5-LO inhibitory activity among this compound class. Regarding different substitutions at the 5-benzylidene moiety, inhibitory activity of these thiazolinones seems to be robust against alterations at that residue. Obviously, these compounds presented here show a flat, so-called "continuous structure−activity relationship".[17] LO inhibitors are already known to show a continuous SAR:[18] In a systematic analysis of SARs over different compound classes, Wawer et al. identified that LO inhibitors, taken from the molecular drug data report (MDDR, version 2005.2, Symyx Software, San Ramon, CA, U.S.), display a "continuous" SAR.[18] Continuous SARs are characterized by tolerance against structural variations.[17] The spectrum of active compounds for a given target thereby includes similar structures that show similar activity and structures with different scaffolds having nearly the same biological activity. The latter could (in the case of 5-LO) be ascribed to the well-known three types of 5-LO inhibitors (iron ligand, redox type, and nonredox type).[16] On the basis of their structures, for the compounds reported herein iron-chelating or reducing properties are unlikely. Thus, they

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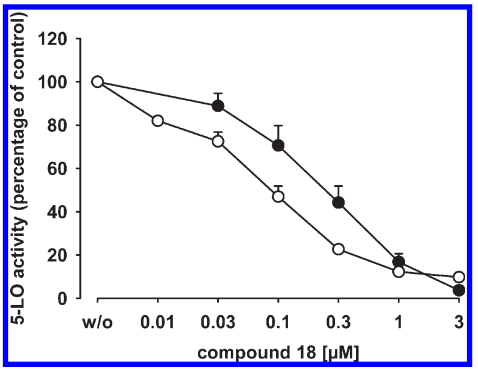

Figure 4. Inhibition of 5-LO product formation by **18** in intact PMNL (●) and in PMNL 100000*g* supernatants (○). Error bars give the standard error of the mean (n = 3−5).

might be designated nonredox type inhibitors or even belong to a novel class.

Comparison with the known thiazolinone 5-LO inhibitors[10a,10b] (Figures 1 and 2) revealed the 2-phenyl moiety as a major structural difference of the compounds presented herein. Both already published inhibitor classes have the 5-benzylidene-2-phenyl-5*H*-thiazol-4-one core in common but apart from this have a quite distinct scaffold. Furthermore, only limited structures are available. Thus, comparison to other SARs of thiazolinone based 5-LO inhibitors is quite difficult. The structures published by Geronikaki et al.[10b] exhibit weaker 5-LO inhibitory potency ($IC_{50} \approx 90\ \mu M$) than those by Unangt et al.[10a] and by us (both $IC_{50} < 1\ \mu M$). Moreover, the least active compound by Unangst et al. (Figure 2A, $IC_{50} > 10\ \mu M$[10a]) resembles **28** except the 2-chlorophenyl. Thus, it can be hypothesized that the 2-phenyl of our scaffold class is important for 5-LO inhibitory activity in this structural context.

To compare our compounds with already known 5-LO inhibitors, we performed a MACCS keys similarity search against publicly available 5-LO inhibitors. We screened the ChEMBLdb (https://www.ebi.ac.uk/chembldb), a database of bioactive druglike small molecules with annotated bioactivities abstracted from the literature, using **1** as query. Among the 20 most similar unique structures bearing no thiazolinone moiety (Supporting Information Table S1), scaffolds comprising diverse heterocycles and phenyl rings are found. The potencies (determined in comparable cell based assays) range from IC_{50} = 2 nM to IC_{50} = 9200 nM. Therewith our structures are well situated in the activity range of known 5-LO inhibitors. Our structures presented herein are characterized by direct, selective, stimulus independent, and noncytotoxic 5-LO inhibitory mechanisms (personal communication), which might be advantageous over other inhibitors.

With substitutions at R^1 position we were able to slightly improve the activity of the lead structure, exemplified by **18** which concentration-dependently inhibited 5-LO in the cell-free assay and in intact PMNL with IC_{50} of 0.09 and 0.28 μM, respectively (Figure 4). Our SAR studies revealed a clear SAR in the 2-position of the thiazolinone, where replacement of benzene by aliphatic heterocycles or introduction of an N-linker led to a complete loss of activity. In contrast, the 5-benzylidene showed high tolerance toward structural modifications in terms of introduced substituents. Taken together, this series represents the first class of 5-benzylidene-2-phenylthiazolinones with potent direct 5-LO inhibitory activity.

CONCLUSIONS

We show that 5-benzylidene-2-phenylthiazolinones act as novel and potent direct 5-LO inhibitors, exemplified by **18** with IC_{50} in intact PMNL and cell-free S100 of 0.28 and 0.09 μM, respectively. The high effectiveness in whole cells and cell-free systems encourages further investigations of this compound class. Future experiments to resolve the binding mode will allow for structure-based lead optimization to further increase their inhibitory activity at 5-LO. Furthermore, studies addressing the effectiveness in vivo using animal models of inflammation are necessary and may reveal the therapeutic potential of these thiazolinone derivatives.

MATERIALS AND METHODS

Compounds and Chemistry. Compounds **1**−**6**, **8**−**17**, and **29**−**34** were purchased from Asinex (Moscow, Russia) or SPECS (Delft, The Netherlands) and exhibit ≥95% purity determined by supplier using ^{1}H NMR and LC−MS. Compounds **7** and **18**−**28** were not found to be commercially available and thus synthesized according to Zayed et al.[15] (By now, **19** is commercially available.) Melting points for synthesized compounds were determined on a Buchi 510 melting point apparatus (Buchi, Switzerland) and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker DPX 250 (250 MHz) spectrometer (Bruker, Germany). ^{1}H NMR data are reported in the following order: chemical shift (d) in ppm downfield from tetramethylsilane as internal reference; multiplicity (br, broad; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet); approximate coupling constant (J) in hertz (Hz); number and assignment of protons. ^{13}C NMR spectra were recorded on a Bruker DPX 250 (63 or 75 MHz) spectrometer (Bruker, Germany). ESI-MS was performed on a Fisons Instruments VG Platform II (Manchester, Great Britain) in positive polarity. Data are listed as mass number ($[M + H]^+$) and relative intensity (%). Elemental analysis results (C, H, N) were measured on a CHN-Rapid (Heraeus, Germany) and were within ±0.4% of the theoretical values for all final compounds, which corresponds to ≥95% purity. Educts and all other reactants were commercially obtained from Sigma-Aldrich, ABCR, Alfa Aesar, and Acros Organics and were used without further purification unless otherwise stated. Analytical TLC (thin layer chromatography) was performed with TLC plates (F254, Merck) with detection using a UV lamp. For the Knoevenagel condensation, only the *Z*-isomer was obtained.

General Procedure. A solution of the corresponding benzonitrile (1 equiv, 0.001−0.05 mol), thioglycolic acid (1−1.1 equiv), the corresponding benzaldehyde (1 equiv), and TEA in methanol was refluxed for at least 12 h (Figure 3). The mixture was evaporated under reduced pressure. The pure product was recrystallized from ethanol and washed with acetone.[15] Detailed synthesis and analytical data of all synthesized compounds are provided in the Supporting Information.

Assay Systems. Materials. Arachidonic acid and calcium ionophore A23187 were from Sigma (Deisenhofen, Germany). HPLC solvents were from Merck (Darmstadt, Germany).

Determination of 5-LO Product Formation. Determination of 5-LO product formation was performed as previously described.[19] A detailed description is provided in the Supporting Information. For calculation of IC_{50}, samples were treated with three increasing concentrations of each compound in three to five independent experiments and the mean and standard error of the mean were calculated. Measurements from each drug concentration were normalized to DMSO control condition. IC_{50} values are approximations determined by graphical analysis (linear interpolation between points between 50% activity) using SigmaPlot2004 (SSI Inc.).

Computational Methods. Similarity Searching Using MACCS Keys. The similarity search for commercially available

structural similar derivatives of compound **1** was performed with MOE (version 2006.08, Chemical Computing Group Ltd., Montreal, Canada) using MACCS substructure keys[13a,13b] and the Tanimoto coefficient.[14a,14b] The ASINEX Gold (version Nov 05; 231812 molecules, ASINEX, Moscow, Russia) and SPECS compound collections (version Feb 08; ~200000 molecules, SPECS, Delft, The Netherlands) were screened.

■ ASSOCIATED CONTENT

Supporting Information. Chemical synthesis; ^{1}H and ^{13}C NMR, MS, and elemental analysis data of synthesized compounds; X-ray structure of 7; 5-LO activity assay; and data of MACCS keys similarity search against known 5-LO inhibitors. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

5-LO, 5-lipoxygenase; AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; 5-H(P)ETE, 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; $LT(B_4)$, leukotriene (B_4); PMNL, polymorphonuclear leukocyte; S100, 100000*g* supernatant; SAR, structure−activity relationship

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