Structural Optimization and Biological Evaluation of 2-Substituted 5-Hydroxyindole-3-carboxylates as Potent Inhibitors of Human 5-Lipoxygenase

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Pharmacological suppression of leukotriene biosynthesis by inhibitors of 5-lipoxygenase (5-LO) is a strategy to intervene with inflammatory and allergic disorders. We recently presented 2-amino-5-hydroxy-1*H*-indoles as efficient 5-LO inhibitors in cell-based and cell-free assays. Structural optimization led to novel benzo[*g*]indole-3-carboxylates exemplified by ethyl 2-(3-chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (compound **11a**), which inhibits 5-LO activity in human neutrophils and recombinant human 5-LO with IC_{50} values of 0.23 and 0.086 μM, respectively. Notably, **11a** efficiently blocks 5-LO product formation in human whole blood assays ($IC_{50} = 0.83-1.6$ μM) and significantly prevented leukotriene B_4 production in pleural exudates of carrageenan-treated rats, associated with reduced severity of pleurisy. Together, on the basis of their high potency against 5-LO and the marked efficacy in biological systems, these novel and straightforward benzo[*g*]indole-3-carboxylates may have potential as anti-inflammatory therapeutics.

Introduction

Leukotrienes (LTs[a]) play established roles in the pathophysiology of inflammatory and allergic disorders (i.e., asthma and allergic rhinitis) but may also promote cancer and atherosclerosis.[1] LTs are formed from arachidonic acid (AA) by the initial introduction of molecular oxygen at C5, catalyzed by the 5-lipoxygenase (5-LO) enzyme. 5-LO is a nonheme iron-containing dioxygenase where the iron cycles between the inactive ferrous and the active ferric state.[2] Pharmacological interference with 5-LO is one strategy in the intervention with LT-mediated diseases.[3] There are at least four classes of LT synthesis inhibitors. Three of them are direct inhibitors of 5-LO that act by either reducing the active-site iron or uncoupling the redox cycle of the iron, those that chelate the iron, and finally compounds that act by nonredox mechanisms, presumably by competition with AA as substrate and/or with regulatory lipid hydroperoxides.[4] Besides direct 5-LO inhibitors, compounds that block the functional interaction between 5-LO and the 5-LO-activating protein (FLAP) are well characterized.[5] Such FLAP inhibitors block LT biosynthesis only in intact cells but do not directly interfere with 5-LO catalytic activity itself.[4]

The development of selective and potent inhibitors of LT biosynthesis for therapeutic application is a major challenge. Many synthetic compounds and natural products have been described as potent 5-LO inhibitors in cell-free or in cellular assays but often markedly lost efficacy in whole blood.[4,6] Moreover, most of them failed in animal studies or human clinical trials due to lack of efficacy or due to toxic side effects. Thus, despite intensive research, only compound **39** (zileuton, 1-(1-benzothiophen-2-ylethyl)-1-hydroxy-urea), an iron ligand-type 5-LO inhibitor with IC_{50} values of 0.5−1 μM in intact cells and whole blood,[7] could enter the market in 1996 as an antiasthmatic drug. It was presumed that, based on the poor efficacy of LT synthesis inhibitors, pharmacological intervention with LTs is of minor therapeutic value. However, LT antagonists are successfully used in asthma therapy and data from 5-LO deficient mice clearly show proof for a strong pathophysiological role of 5-LO products in several diseases.[3] Thus, the improvement of the pharmacological properties of 5-LO or FLAP inhibitors in terms of efficacy and safety is seemingly a major challenge and requires new leads with novel modes of molecular action.

We have recently discovered 2-amino-5-hydroxyindole esters as novel direct 5-LO inhibitors with unknown mode of 5-LO inhibition.[8] These novel structures inhibited 5-LO product synthesis in cell-based or cell-free assays with IC_{50} values of 2.4 and 0.3 μM, respectively,[8] and thus seem to be promising drug candidates. Here, we explored this class of compounds in order to optimize the structural features and to discover more potent derivatives with high efficacy in biological test systems. The annelation of a [*g*]benzene ring to the indole and replacement of the 2-amino moiety by a C1 to C3 alkyl chain essentially led to drug-like compounds that have a more than 10-fold higher potency than the parental compound ethyl 2-[(3-chlorophenyl)-amino]-5-hydroxy-1*H*-indole-3-carboxylate **37** and efficiently

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[a] Abbreviations: AA, arachidonic acid; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 5-HETE, 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; LPS, lipopolysaccharide; 5-LO, 5-lipoxygenase; LT, leukotriene.

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inhibited LT biosynthesis also in human whole blood and in pleural exudates of carrageenan-treated rats.

Results and Discussion

Chemistry. The Nenitzescu reaction[9] has proven to be the simplest entry into 5-hydroxyindoles. Essentially, stirring of the nucleophilic enaminoester **7** with 1.2 equiv of 1,4-quinone in ethanol at room temperature (RT) led to the expected 5-hydroxyindole derivatives (method a). We converted both 1,4-benzoquinone (**8**) and 1,4-naphthoquinone (**9**) with a variety of different enaminoesters **7** and the ketene aminal **13** successfully into the desired 5-hydroxyindoles **10** or the 5-hydroxybenzo[*g*-]indoles **11** and **14** (method a). Nevertheless, some of the title compounds synthesized in this manner were difficult to isolate due to contamination of numerous byproducts. Moreover, method a failed when using 6,7-dimethoxy-1,4-naphthoquinone (**15**), quinoline-5,8-dione (**16**), or the 2-aryl-1,4-benzoquinones **17** and **18** as reactants from enaminoester **7a**. Due to the fact that Lewis acid-promoted Nenitzescu reactions mostly provide better yields, we also used ZnI_2 as catalyst in a wide range of reactions (method b).[10] Moreover, the desired Nenitzescu products of the reported 1,4-quinones **15**−**18**[11−14] could be successfully synthesized according to method b.

Nenitzescu reactants **7** were prepared starting from carboxylic acids **1**, which were first activated to the appropriate imidazolides and then treated with the magnesium salt of 3-ethoxy-3-oxopropanoic acid **3a** or 3-benzyloxy-3-oxopropanoic acid **3b**, respectively. Subsequent acidic workup led both to the reported (**4a**−**i** and **k**−**p**) and the new β-ketoesters **4j**,**q**,**r**.[15] β-ketoesters **4** were converted into their appropriate enaminoesters **7** by refluxing them in toluene with an excess of ammonium acetate (**5**) or with amine **6p**, respectively.[16] Unfortunately, the hitherto unknown secondary enaminoester **7p** could not be separated from the remaining reactant and was thus employed as crude product for the subsequent Nenitzescu reaction. Eight of the enaminoesters **7** were treated with 1,4-benzoquinone (**8**) to form the 5-hydroxy-1*H*-indole-3-carboxylates **10a**−**c**, **k**−**n**, and **s**, and 18 of them were converted into 5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylates **11** by using 1,4-naphthoquinone (**9**) as reactant. A large part of indole-3-carboxylates **10** was still synthesized according to method a, and preparation of the **11** series was essentially carried out under Lewis acid promotion (method b) (Scheme 1).

Benzo[*g*]indole derivative **14** was also synthesized according method b. Ketene aminal **13**[17] was prepared from (*E*)-ethyl 3-amino-3-ethoxyacrylate (**12**) and 3-chloroaniline (**6a**) (Scheme 2).[18]

Using different 1,4-quinones for the Nenitzescu reaction, we were able to introduce various substituents in positions 6 and 7 of indole-3-carboxylate **10a**. Enaminoester **7a** was allowed to react with four additional 1,4-quinones **15**−**18** to give the 5-hydroxyindol-3-carboxylates **19**−**22** in moderate to good yields (method b) (scheme 3).

To eliminate redox activity,[8] we prepared the alkyl indole-3-carboxylates **27a**−**h** and **28**, which lack the 5-hydroxy group in contrast to the mentioned Nenitzescu products. All derivatives **27** and compound **28** could be synthesized in analogy to Bergman et al.[19] starting from alkyl indole-3-carboxylates **23a** or **23b** and the aryl-/arylalkyl or (di)allylamines **6a**−**h** (Scheme 4).

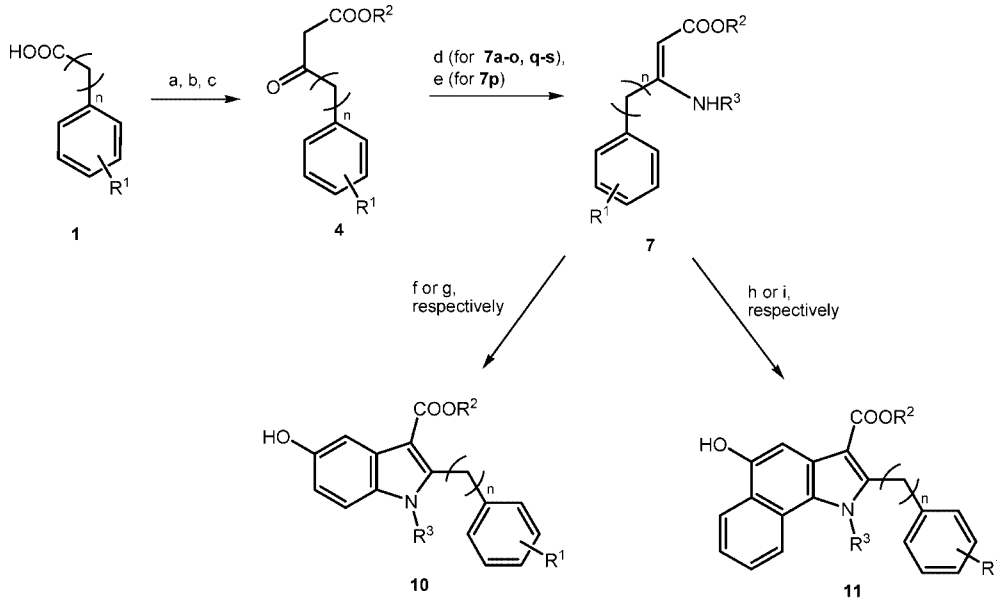
Further reactions were carried out starting from ethyl 2-(3-chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (**11a**) shown in Scheme 5: Acylation of **11a** with benzoylchloride (**29**) in an alkaline medium led to compound **30**.[20] Refluxing **11a** with methyliodide (**31**) in the presence of NaOH resulted in the doubly methylated compound **32**.[21] Finally, we synthesized the 5-phenyl substituted compound **36** via triflation of **11a** and subsequent Suzuki−Miyaura coupling of the generated triflate **34** with boronate **35** (Scheme 5).[22]

Structure−Activity Relationships Regarding Inhibition of 5-Lipoxygenase. Analysis of test compounds as 5-LO inhibitors was routinely carried out in two different test systems, that is, a cell-based assay using human neutrophils challenged by Ca^{2+} ionophore 5-methylamino-2-[[(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-[(2*S*)-1-*oxo*-1-(1*H*-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzooxazole-4-carboxylic acid (A23187) plus 20 μM exogenous AA as well as a cell-free assay using human recombinant 5-LO enzyme in high-speed (40000*g*) supernatants (S40) from lysates of transformed *Escherichia coli*. The 5-LO inhibitors **38** (*N*-(3-phenoxycinnamyl)-acetohydroxamic acid, BWA4C, routinely used as reference compound[23]) and **39**[7] suppressed 5-LO product formation in the cell-based assay with IC_{50} values of 0.16 ± 0.02 and 0.9 ± 0.1 μM, respectively, and in the cell-free assay with 0.038 ± 0.04 and 0.8 ± 0.2 μM, respectively.

5-Hydroxyindoles were shown to possess antioxidant activities.[24] Therefore, the 2-amino-5-hydroxy-1*H*-indole-3-carboxylates could act as redox-active 5-LO inhibitors by interfering with the redox cycle of the active-site iron of the 5-LO enzyme. To determine the requirement of the 5-hydroxy moiety as a part of the reducing structure, we analyzed several derivatives of the lead compound **37** devoid of the 5-OH group and, thus, lacking reducing properties (Table 1). The lead compound **37** potently inhibited 5-LO product synthesis with IC_{50} values of 2.4 ± 0.4 and 0.3 ± 0.09 μM in cell-based and cell-free assays, respectively.[8] In direct comparison to **37**, the corresponding analogue without the 5-hydroxy moiety (**27a**) is approximately 3-fold and about 27-fold less potent in intact cells and cell-free assays, respectively. Hence, the strong impact of the 5-hydroxy moiety on the potency, noticeable in cell-free assays, is only moderate in intact cells. Repositioning of the chlorine in ortho (**27b**) or para (**27c**) or variation of the halogen substituent (fluorine (**27d**), bromine (**27e**)) at the 2-(*N*)-aniline ring caused no substantial change in the potency versus **27a**, yielding compounds with IC_{50} values in the range of 3.5 ± 1.2 to 9.8 ± 1.9 μM for intact cells and 8.3 ± 1.4 to 13.4 ± 2.9 μM for cell-free assays. The replacement of the 5-hydroxy residue by chlorine (**28**) impaired the efficacy about 12-fold versus **37** in cell-free assays but only about 2-fold in intact cells. Finally, exchange of the aniline moiety by a benzylamino group (**27f**), did not significantly alter the efficacy versus **27a**. As found before[8] replacement of the *N*-aryl group in 2-position by one allyl moiety (**27g**) abrogated 5-LO inhibition, but introduction of two *N*-allyl residues (**27h**), which resembles a phenyl ring, led to similar potencies as obtained for **27a**.

Because the 5-hydroxy moiety increases the potency, we continued with the 5-hydroxy-indole backbone in order to obtain efficient 5-LO inhibitors. In our previous study, we found that the nature of the substituent in 2-position of the indole determines the efficacy. Thus, arylamine, arylethylamine, or a 4-arylpiperazin-1-yl group as well as a diallyl-amino residue in the 2-position of indole-3-carboxylates led to active compounds, whereas nonaromatic amine functions including primary amine, dimethylamine, pyrolidine, piperidine, or morpholine failed in this respect.[8] Moreover, chlorine substitution of the 2-(*N*)-aniline residue in 3′-position (e.g., in **37**) appeared advantageous.

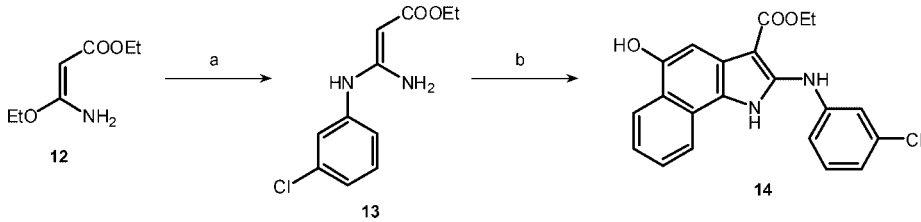
Scheme 1. Synthesis of Different 5-Hydroxy-1*H*-indole-3-carboxylates (**10**) and 5-Hydroxy-1*H*-benzo[*g*]indole-3-carboxylates (**11**)[a]



7	n	R^1	R^2	R^3
a	1	3-chloro	Et	H
b	1	2-chloro	Et	H
c	1	4-chloro	Et	H
d	1	3-fluoro	Et	H
e	1	4-fluoro	Et	H
f	1	3-bromo	Et	H
g	1	4-bromo	Et	H
h	1	3-methoxy	Et	H
i	1	4-methoxy	Et	H
j	1	4-trifluoromethyl	Et	H
k	0	3-chloro	Et	H
l	0	4-chloro	Et	H
m	2	3-chloro	Et	H
n	2	4-chloro	Et	H
o	3	H	Et	H
p	1	4-chloro	Et	Bn
q	1	3-chloro	Bn	H
r	1	4-chloro	Bn	H
s	2	H	Et	H

[a] Reagents and conditions: (a) *N*,*N*′-carbonyldiimidazole (**2**), DMF, RT; 1 h; (b) Mg-salt of 3-ethoxy-3-oxopropanoic acid (**3a**) or 3-benzyloxy-3-oxopropanoic acid (**3b**), RT; 4 h; (d) NH_4OAc (**5**), HOAc, toluene, reflux, 6 h; (e) benzylamine (**6f**), HCOOH, toluene, reflux, 6 h; (f) 1,4-benzoquinone (**8**), EtOH, reflux, 1 h; (g) 1,4-benzoquinone (**8**), CH_2Cl_2, ZnI_2, reflux, 40 min; (h) 1,4-naphthoquinone (**9**), EtOH, reflux, 1 h; (i) 1,4-naphthoquinone (**9**), CH_2Cl_2, ZnI_2, reflux, 40 min. All compounds **10** were synthesized via (f), **10a**,**c** and **k**−**n** additionally via (g); all compounds of **11** (except of **11o**) were synthesized via (i), **11a**,**c**,**k**−**m**,**o**, and **q** additionally or only via (h).

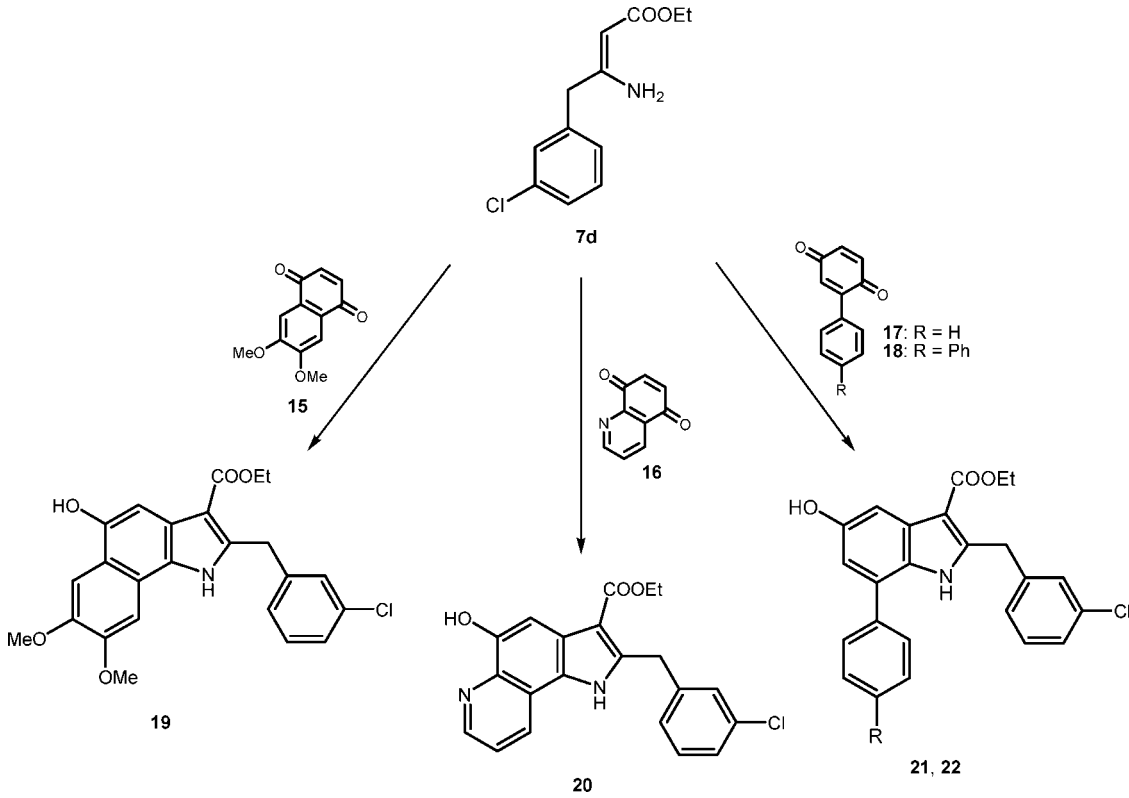
Scheme 2. Synthesis of Ethyl 2-(3-Chlorophenylamino)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (**14**)[a]



[a] Reagents and conditions: (a) EtOH, 3-chloroaniline (**6a**), reflux, 48 h; (b) 1,4-naphthoquinone (**9**), CH_2Cl_2, ZnI_2, reflux, 40 min.

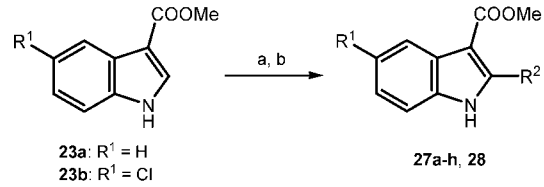
We studied whether nitrogen in the 2-position of the indole is actually a prerequisite for 5-LO inhibition. Compound **10a**, representing the methylene derivative of the parental compound **37**, was slightly more potent in neutrophils but more than 6-fold less active in cell-free assays (Table 2), implying that nitrogen in 2-position is actually not an absolute requirement but may govern the 5-LO inhibitory potency, at least in the cell-free assay. Repositioning of the chlorine in ortho (**10b**) did not alter the efficacy versus **10a**, whereas chlorine in para position (**10c**) increased the potency in intact cells about 2-fold. Moreover, deletion of the nitrogen bridge of **37** yielding the 2-(3-chlorophenyl)- or 2-(4-chlorophenyl)-indole derivatives **10k** and **10l**, respectively, led to active compounds with a moderate change of potency in neutrophils but markedly lower potency in the cell-free assay as compared to compound **37**. Finally, elongation to 1-(3-chlorophenyl)-ethyl (**10m**), 1-(4-chlorophenyl)ethyl (**10n**), or 1-phenylethyl (**10s**), essentially retained activity without substantial loss of potency versus corresponding phenyl- or benzyl-analogues lacking nitrogen in the 2-position.

Scheme 3. Synthesis of the 5-Hydroxy-1*H*-indole-3-carboxylates **19**−**22**[a]



[a] Reagents and conditions: 1,4-quinones **15**−**18**, CH_2Cl_2, ZnI_2, reflux, 40 min.

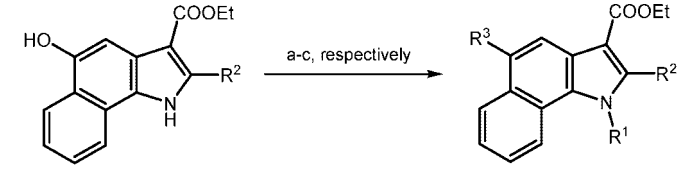
Scheme 4. Synthesis of the 2-Substituted Indole-3-carboxylates **27a**−**h** and **28**[a]



	R^1	R^2
27a	H	(3-chlorophenyl)amino
27b	H	(2-chlorophenyl)amino
27c	H	(4-chlorophenyl)amino
27d	H	(3-fluorophenyl)amino
27e	H	(3-bromophenyl)amino
27f	H	benzylamino
27g	H	allylamino
27h	H	diallylamino
28	Cl	(3-chlorophenyl)amino

[a] Reagents and conditions: (a) *N*,*N*′-dimethylpiperazine (**24**), *N*-chlorosuccinimide (**25**), CH_2Cl_2, 0 °C, 2 h; (b) trichloroacetic acid (**26**), amines **6a**−**h**, RT, 2 h.

Scheme 5. Modifications of Ethyl 2-(3-Chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (**11a**)[a]



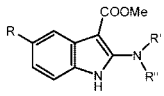
11	R^2
a	3-chlorobenzyl

	R^1	R^2	R^3
30	H	3-chlorobenzyl	OBz
32	Me	3-chlorobenzyl	OMe
36	H	3-chlorobenzyl	Ph

[a] Reagents and conditions: (a) **11a** → **30**: triethylamine, EtOAc, benzoylchloride (**29**), RT, 20 h. (b) **11a** → **32**: NaOH, methyliodide (**31**), THF, reflux, 10 h. (c) **11a** → **36** (i) **11d** → triflate **34**: Tf_2O (**33**), CH_2Cl_2, pyridine, 0 °C, 4 h; (ii) **34** → **36**: $Pd(OAc)_2$, PPh_3, DMF, phenylboronic acid pinacol ester (**35**), aq Na_2CO_3, reflux, 2 h.

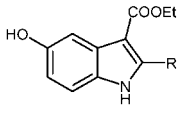
To obtain more potent 5-LO inhibitors, further structural variations based on the structure of the lead **37** were carried out. Another possibility for structural variation is given by annelation of benzene to the indole, yielding corresponding benzo[*g*]indole derivatives. Benzene annelation enlarges the hydrophobic core structure and increases the overall lipophilicity, a general determinant for potent 5-LO inhibitors.[25] Compound **14**, the benzo[*g*]indole analogue to **37**, is a potent inhibitor of 5-LO with IC_{50} values of 0.71 ± 0.25 μM (intact neutrophils) and 0.24 ± 0.06 μM (cell-free 5-LO) being superior over **37**, particularly in intact cells (Table 3). Interestingly, exchange of the nitrogen in 2-position of **14** by methylene,

Table 1. Inhibition of 5-LO Activity in Intact Neutrophils and in a Cell-Free Assay[a]

compound				inhibition of 5-LO activity (IC_{50} values [μM])	
	R	R'	R''	intact	cell-free
37	-OH	-H	3-chlorophenyl	2.4 ± 0.4	0.3 ± 0.09
27a	-H	-H	3-chlorophenyl	6.5 ± 1.9	8.1 ± 0.5
27b	-H	-H	2-chlorophenyl	9.8 ± 1.9	10.2 ± 0.5
27c	-H	-H	4-chlorophenyl	7.2 ± 0.9	9.8 ± 4
27d	-H	-H	3-fluorophenyl	7.9 ± 0.3	13.4 ± 2.9
27e	-H	-H	3-bromophenyl	3.5 ± 1.2	8.3 ± 1.4
27f	-H	-H	-Bn	4.0 ± 2.8	3.4 ± 1.8
27g	-H	-H	-allyl	> 30	> 30
27h	-H	-allyl	-allyl	12.5 ± 2.5	6.9 ± 1.4
28	-Cl	-H	3-chlorophenyl	5.5 ± 2.1	3.5 ± 0.9

[a] The IC_{50} values are given as mean ± SE of $n = 3-4$ determinations.

Table 2. Inhibition of 5-LO Activity in Intact Neutrophils and in a Cell-Free Assay[a]

compound		inhibition of 5-LO activity (IC_{50} values [μM])	
	R	intact	cell-free
37	3-chlorophenylamino	2.4 ± 0.4	0.3 ± 0.09
10a	3-chlorobenzyl	1.7 ± 0.8	2.0 ± 0.5
10b	2-chlorobenzyl	1.7 ± 0.4	1.6 ± 1.6
10c	4-chlorobenzyl	0.7 ± 0.4	1.2 ± 1.1
10k	3-chlorophenyl	3.3 ± 0.6	4.8 ± 0.9
10l	4-chlorophenyl	2.0 ± 0.05	2.1 ± 0.6
10m	1-(3-chlorophenyl)ethyl	2.8 ± 0.5	1.7 ± 0.6
10n	1-(4-chlorophenyl)ethyl	3.9 ± 1.8	0.7 ± 0.3
10s	1-phenylethyl	5.5 ± 1.4	7.3 ± 1.6

[a] The IC_{50} values are given as mean ± SE of $n = 3-4$ determinations.

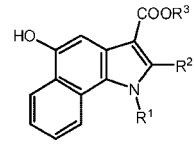
leading to compound **11a**, further increased the potency over **37** in intact cells about 10-fold ($IC_{50} = 0.23 \pm 0.07$) and in the cell-free assay more than 3-fold ($IC_{50} = 0.086 \pm 0.02$ μM), respectively. Therefore, we continued with compounds devoid of the nitrogen in 2-position of the indole. Variation of the positioning of the chlorine in the benzyl ring to the ortho (compound **11b**) or para (compound **11c**) positions as well as exchange of chlorine in **11a** by fluorine (**11d**), bromine (**11f**), or a methoxy moiety (**11h**) was essentially tolerated in cell-free assays, with a small loss of potency in intact cells for **11b** and **11c**. Also, substitution in the para position with fluorine (**11e**), bromine (**11g**), or a methoxy moiety (**11i**) caused no marked change of the potencies, whereas a trifluoromethyl group (**11j**) was rather detrimental.

Next, the effect of the distance between the phenyl ring in 2-position and the indole was evaluated. Both direct connection of the 3-chlorophenyl residue (**11k**) but also insertion of an ethylene bridge (**11m**) improved the potency for inhibition of cell-free 5-LO ($IC_{50} = 0.045 \pm 0.01$ and 0.031 ± 0.01 μM, respectively) versus the parental compound **11a** ($IC_{50} = 0.086 \pm 0.02$ μM). In contrast, the potency for inhibition of 5-LO activity in intact neutrophils was slightly reduced versus **11a**. Repositioning of the chlorine into para regardless of the distance of the aryl residue from the indole (compound **11l** and **11n**) did not significantly improve the potency versus **11a**. Substitution of the indole nitrogen of **11c** by a benzyl moiety (yielding **11p**) moderately reduced the potency. The corresponding benzyl esters **11q** and **11r** of compounds **11a** and **11c**, respectively, were similarly active as the ethyl esters, which indicates that also voluminous esters are tolerated.

Additional structural alterations of **11a** showed that annelation of a 2,3-dimethoxybenzene moiety (**19**) to the indole, instead of benzene, is clearly detrimental. On the other hand, when a pyridine ring was annelated to the indole (**20**) or when a phenyl moiety (**21**) or a biphenyl (**22**) was substituted in 7-position, such derivatives still blocked 5-LO activity with IC_{50} values 2.8 ± 0.7 to 5.7 ± 0.1 μM in intact cells and 0.33 ± 0.12 to 1.2 ± 0.2 μM in cell-free assays (Table 4). Finally, esterification of the 5-hydroxy group of **11a** with benzoic acid (leading to **30**) strongly reduced the potency. Also methylation of **11a** at the 5-hydroxy moiety and of the indole nitrogen (yielding **32**) or replacement of the 5-hydroxy group by phenyl (yielding **36**) led to inactive compounds. Taken together, several variations starting from **37** with IC_{50} values of 2.4 ± 0.4 (cell-based) and 0.3 ± 0.09 μM (cell-free) led to the novel benzo[*g*]indoles lacking the 2-amino functionality with IC_{50} values of 0.23 ± 0.07 (**11a**) in cell-based and 0.031 ± 0.01 μM (**11m**) in cell-free assays, respectively.

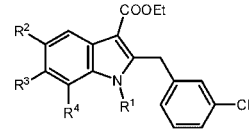
Studies on the Mode of Action of Benzo[*g*]indole-3-carboxylates as 5-LO Product Synthesis Inhibitors. Indole-based structures such as 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid (MK-886) suppress 5-LO product synthesis via inhibition of the AA-binding protein FLAP, which facilitates substrate supply toward 5-LO at the perinuclear region but does not inhibit 5-LO directly.[5] A direct interference of benzo[*g*]indole-3-carboxylates with 5-LO is evident based on the inhibition of the 5-LO enzyme in the cell-free assay. We addressed whether **11a** (the most potent derivative in intact neutrophils) shares also mechanistic properties with FLAP inhibitors. Typically, excessive supply of AA impairs the efficacy of FLAP inhibitors, and FLAP inhibitors block translocation of 5-LO from the cytosol to the perinuclear region in neutrophils. In contrast to MK-886, **11a** failed to block A23187-induced translocation of 5-LO in neutrophils (Figure 1A). Moreover, in contrast to MK-886,[26] no discrepancy in the efficacy of **11a** was obvious in neutrophils forming 5-LO products from relatively low amounts of endogenous AA as compared to conditions where ample exogenous AA (20 or 50 μM) was supplemented (Figure 1B). The lack of such a discrepancy in the efficacy of **11a** in intact neutrophils also might suggest that the compound does not act in a substrate competitive manner. Indeed, lowering (5 μM) or increasing (50 μM) the substrate concentration did also not significantly affect the inhibiton of 5-LO activity by **11a** in the cell-free assay (Figure 2A). Finally, wash-out experiments shown in Figure 2B support a reversible inhibition of cell-free 5-LO activity by **11a**, comparable to compound **39**. Together, we conclude that the benzo[*g*]indole-3-carboxylates, exemplified by **11a**, act as direct and reversible inhibitors of 5-LO, and despite some structural similarities (indole) with FLAP inhibitors, these compounds act independently of FLAP.

Table 3. Inhibition of 5-LO Activity in Intact Neutrophils and in a Cell-Free Assay[a]

compound				inhibition of 5-LO activity (IC_{50} values [μM])	
	R^1	R^2	R^3	intact	cell-free
14	-H	(3-chlorophenyl)amino	-Et	0.71 ± 0.25	0.24 ± 0.06
11a	-H	3-chlorobenzyl	-Et	0.23 ± 0.07	0.086 ± 0.02
11b	-H	2-chlorobenzyl	-Et	1.2 ± 0.07	0.097 ± 0.09
11c	-H	4-chlorobenzyl	-Et	1.2 ± 0.19	0.084 ± 0.05
11d	-H	3-fluorobenzyl	-Et	0.34 ± 0.03	0.14 ± 0.02
11e	-H	4-fluorobenzyl	-Et	0.50 ± 0.01	0.096 ± 0.02
11f	-H	3-bromobenzyl	-Et	0.45 ±0.14	0.15 ± 0.02
11g	-H	4-bromobenzyl	-Et	0.60 ± 0.24	0.095 ± 0.01
11h	-H	3-methoxybenzyl	-Et	0.52 ± 0.03	0.13 ± 0.15
11i	-H	4-methoxybenzyl	-Et	0.65 ± 0.3	0.15 ± 0.02
11j	-H	4-trifluoromethylbenzyl	-Et	1.7 ± 0.3	0.25 ± 0.18
11k	-H	3-chlorophenyl	-Et	0.52 ± 0.10	0.045 ± 0.01
11l	-H	4-chlorophenyl	-Et	0.32 ± 0.11	0.067 ± 0.02
11m	-H	3-chlorophenylethyl	-Et	0.49 ± 0.15	0.031 ± 0.01
11n	-H	4-chlorophenylethyl	-Et	2.8 ± 0.6	0.049 ± 0.04
11o	-H	phenylpropyl	-Et	0.44 ± 0.11	0.13 ± 0.02
11p	-Bn	4-chlorobenzyl	-Et	1.8 ± 0.05	0.65 ± 0.09
11q	-H	3-chlorobenzyl	-Bn	0.35 ± 0.09	0.13 ± 0.09
11r	-H	4-chlorobenzyl	-Bn	0.48 ± 0.13	0.17 ± 0.02

[a] The IC_{50} values are given as mean ± SE of $n = 3-4$ determinations.

Table 4. Inhibition of 5-LO Activity in Intact Neutrophils and in a Cell-Free Assay[a]

compound					inhibition of 5-LO activity (IC_{50} values [μM])	
	R^1	R^2	R^3	R^4	intact	cell-free
19	-H	-OH	2,3-dimethoxybenzo		7.0 ± 0.8	> 10
20	-H	-OH	[2,3]pyrido		4.3 ± 0.9	0.58 ± 0.15
21	-H	-OH	phenyl	-H	5.7 ± 0.1	1.2 ± 0.20
22	-H	-OH	4-biphenyl	-H	2.8 ± 0.7	0.33 ± 0.12
30	-H	-benzoyloxy	benzo		>10	3.4 ± 1.99
32	-Me	-OMe	benzo		>30	>10
36	-H	-phenyl	benzo		>30	>30

[a] The IC_{50} values are given as mean ± SE of $n = 3-4$ determinations.

Analysis of Benzo[*g*]indole-3-carboxylates in Biological and Pharmacological Relevant Test Systems. Cell-free assays and test systems based on isolated cells often neglect important parameters (i.e., albumin-binding, regulatory plasma components) that influence the bioactivity of test compounds in vivo, and inclusion of some of these variables might better

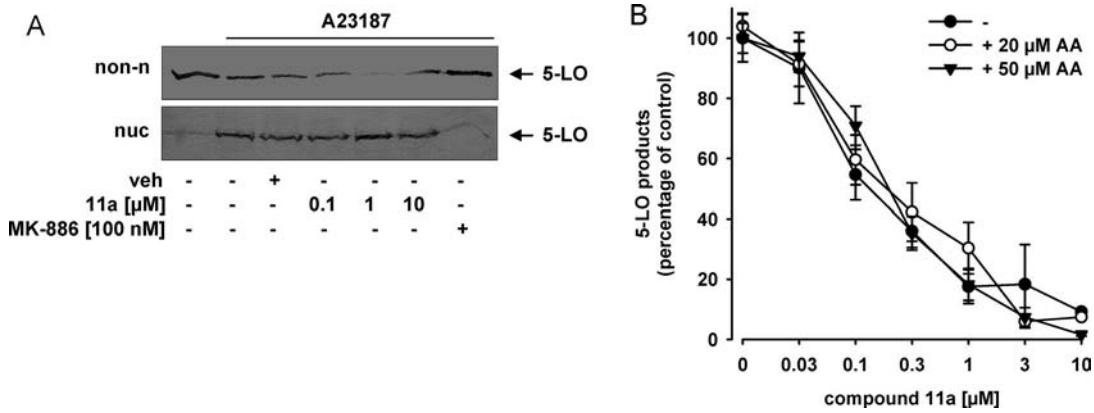


Figure 1. Effects of compound **11a** on 5-LO product synthesis and 5-LO translocation in human neutrophils. Isolated human neutrophils were preincubated with the test compounds (or DMSO as vehicle) for 15 min at 37 °C. (A) Assessment of 5-LO subcellular localization. Neutrophils were stimulated with 2.5 μM A23187 for 5 min at 37 °C, lysed by mild detergent (0.1% NP-40), and nuclear (nuc) and non-nuclear (non-n) fractions were separated. 5-LO protein was analyzed by Western Blot. Data are representatives for three independent experiments. (B) Assessment of 5-LO product synthesis. A23187 (2.5 μM) was added without (−) or with (+) 20 or 50 μM AA, as indicated, and after 10 min at 37 °C, 5-LO products were analyzed. Data are means ± SE; $n = 3-4$.

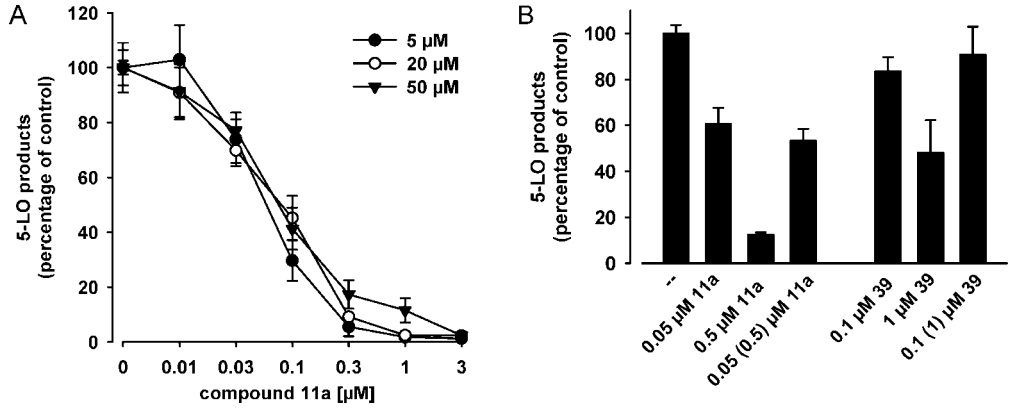


Figure 2. Effects of the substrate concentration on 5-LO inhibition by compound **11a** and wash-out experiments. (A) Aliquots of the supernatants of *E. coli* lysates were diluted in 1 mL of PBS, pH 7.4, and 1 mM EDTA, and preincubated with the test compounds for 10 min at 4 °C. Samples were prewarmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and AA (5, 20, or 50 μM) was added to start the 5-LO reaction. After 10 min, 5-LO products were analyzed. (B) Aliquots of the supernatants were incubated with 0.05 or 0.5 μM **11a**, 1 or 0.1 μM **39**, or vehicle (DMSO) for 10 min at RT. Then, one aliquot of the sample containing 0.5 μM **11a** and 1 μM **39** was diluted with assay buffer 10-fold (0.05 (0.5) μM **11a** and 0.1 (1) μM **39**, respectively), whereas the other aliquot was not altered. Samples were prewarmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and 20 μM AA was added to start the 5-LO reaction. After 10 min, 5-LO products were analyzed. Data are means ± SE; $n = 3-4$.

resemble the in vivo situation and anticipate in vivo outcomes.[4,6] To estimate the ability to inhibit 5-LO product formation in vivo in humans, compounds **11a** and **11l** were analyzed in a human whole blood assay using A23187 (30 μM) as stimulus. The formation of LTB_4 and of 5-HETE was suppressed by **11a** in a concentration-dependent manner with $IC_{50} = 1.6 \pm 0.3$ μM (Figure 3A) and also **11l** blocked 5-LO product formation efficiently ($IC_{50} = 1.3 \pm 0.15$ μM). Next, the efficacy of compound **11a** was assessed in human whole blood primed with lipopolysaccharide (LPS) and then challenged with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). In contrast to activation by A23187, stimulation of blood with fMLP following LPS-priming is considered to closely mimic pathophysiological conditions in the body.[27] Compound **11a** potently inhibited the formation of 5-LO products with an $IC_{50} = 0.83 \pm 0.07$ μM (Figure 3B).

The efficacy of compound **11a** was finally assayed for its ability to inhibit LTB_4 production in vivo by the use of an animal model of 5-LO-related inflammation, the carrageenan-induced pleurisy in rats.[28] The ip treatment of rats with 4 mg/kg of **11a**, 30 min before carrageenan administration, significantly reduced the inflammatory reaction measured as exudate volume (77%), inflammatory cell numbers (40%), and LTB_4 levels (49%) in the pleural exudates (Table 5). The well-recognized 5-LO inhibitor **39**[7] (10 mg/kg) also reduced exudate formation (77%), cell infiltration (41%), and LTB_4 exudate levels (66%), and its anti-inflammatory action was not significantly different from compound **11a** (Table 5).

Conclusions

Here we have synthesized and evaluated various series of indole-3-carboxylates as inhibitors of human 5-LO. Structural optimization led to 5-hydroxy-benzo[*g*]indole-3-carboxylates that suppress the formation of 5-LO products in cell-free and cellular assays, exemplified by **11a** with IC_{50} values of 0.086 and 0.23 μM, respectively. Under more physiological relevant conditions, e.g., in human whole blood assays, **11a** potently suppressed 5-LO product synthesis with IC_{50} values of 0.86 to 1.6 μM. Of interest, **11a** blocked LTB_4 generation in an in vivo model of inflammation, the carrageenan-induced rat pleurisy. This action might be responsible for the reduced inflammatory reaction measured as decrease of exudate volume and migrating cell numbers. Structure−activity relationships indicate that (I) the nitrogen in 2-position of the indole of the lead **37** is not absolutely essential but may increase the potency in cell-free assays, (II) direct linking of a halogen-substituted phenyl moiety

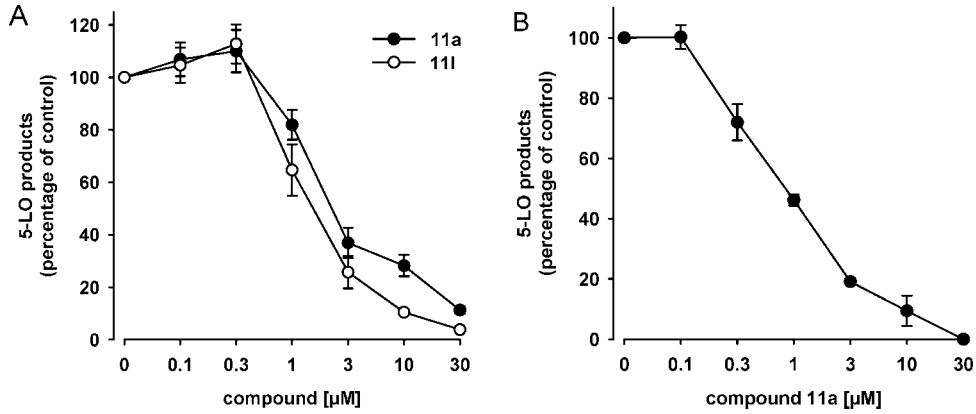


Figure 3. Concentration−response curves of compounds **11a** and **11l** for inhibition of 5-LO activity in human whole blood. Human whole blood was treated with the test compounds (or DMSO as vehicle) for 10 min at 37 °C and stimulated with (A) A23187 (30 μM) or (B) primed with LPS (1 μg/mL) for 30 min and then stimulated with fMLP (1 μM). After 15 min (LPS/fMLP) or 10 min (A23187) at 37 °C, the formation of 5-LO products was determined. Data are means ± SE; $n = 3-5$.

Table 5. Effect of Compound **11a** on Carrageenan-Induced Pleurisy in Rats[a]

treatment	exudate volume (mL)	inflammatory cells × 10^6	LTB_4 (ng/rat)
vehicle	0.48 ± 0.08	46.67 ± 3.53	1.17 ± 0.21
11a (4 mg/kg)	0.11 ± 0.0026***	28.00 ± 6.83*	0.60 ± 0.096*
zileuton (10 mg/kg)	0.11 ± 0.065**	27.54 ± 4.41**	0.40 ± 0.044**

[a] Thirty min before intrapleural injection of carrageenan, rats ($n = 10$ for each experimental group) were treated ip with 4 mg/kg **11a**, 10 mg/kg zileuton, or vehicle (DMSO 4%). Exudate volume, LTB_4 levels as well as inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean ± SE, $n = 10$. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$ vs vehicle.

in 2-position of the indole without bridging atoms is tolerated, (III) variations of halogen substituents regarding number, nature (F, Cl, Br), or positioning of the halogen at the aryl residue only slightly affect the potency, and (IV) the 5-hydroxy moiety may govern 5-LO inhibition, particularly in cell-free assays. A major step forward is the annelation of the benzene yielding benzo[*g*]indole structures that leads to increased potency, preferentially in intact cells. The benzo[*g*]indole ring system represents a unique, basic backbone structure of eicosanoid synthesis inhibitors. As pointed out in our previous report,[8] the free indole-3-carboxylic acids were not synthetically accessible and no conclusion can be drawn whether or not an ester moiety is necessary or if also the free acid may be active. The precise mode of action of how these compounds interfere with 5-LO activity remains unclear, and a definite assignment to a typical class of LT synthesis inhibitors is yet not possible. Nevertheless, our data suggest a noncompetitive and reversible mode of action on 5-LO and exclude FLAP as critical point of attack. In summary, on the basis of their ability to potently inhibit 5-LO product formation in various biological assays, associated with high efficacy in an animal model of (LT-mediated) inflammation comparable to zileuton (**39**), these novel 5-hydroxybenzo[*g*]indole carboxylates possess a high potential for therapeutic use and deserve further pharmacological analysis in other in vivo (animal) models.

Experimental Section

General Methods. Chromatography was carried out with 230−400 mesh silica gel (Merck, Darmstadt, Germany). The MPLC-apparatus consisted of the following segments: MPLC pump, BÜCHI (type B688); fraction collector, LKB (type 2111 Multirac and SuperFracTM); columns: BÜCHI, 15 mm × 460 mm, 26 mm × 460 mm, 36 mm × 460 mm, 49 mm × 460 mm. Melting points (mp): Büchi melting point apparatus, uncorrected. Mass spectra: Finnigan MAT TSQ 70 instrument. ^{1}H NMR spectra: Bruker AM 360 spectrometer at 360 MHz. Spectra were measured in DMSO-d_6 using TMS as internal standard. Elemental analyses were performed by the Organic Chemistry Department of the Friedrich Alexander University Erlangen; Apparatus: Carlo Erba (model 1108) and Heraeus (CHN-Rapid). Infrared spectra were recorded on the FTIR spectrometers Perkin-Elmer (type1740) and Jasco (Type 410).

Procedure for the Preparation of the β-Ketoesters 4j,q,r. To a solution of 12.0 mmol of 3-ethoxy-3-*oxo*-propanoic acid (for **4j**) or 3-benzyloxy-3-*oxo*-propanoic acid (for **4q**,**r**) in dry THF (15 mL), magnesium ethylate (687 mg, 6.0 mmol) was added and the reaction mixture was stirred at RT for 4 h. The solvent was evaporated, and the resulting residue was washed with ether, filtered off, and dried again under reduced pressure to give the magnesium salt of 3-ethoxy-3-*oxo*-propanoic acid (**3a**) or of 3-benzyloxy-3-*oxo*-propanoic acid (**3b**), respectively. *N*,*N*′-Carbonyldiimidazole (**2**, 1.78 g, 11.0 mmol) was slowly added to a solution of acid **1j**,**q**,**r** (10.0 mmol) in dry DMF (20 mL). After stirring the reaction mixture under nitrogen atmosphere for 1 h, the appropriate magnesium salt **3a** or **3b** was added and the mixture was allowed to stir for additional 4 h. The solution was acidified with 2 N HCl and extracted with ethylacetate. The combined organic layers were washed with 10% aqueous $NaHCO_3$ solution and water, dried over Na_2SO_4, filtered off, and evaporated to give a colorless oil crystallizing at <0 °C.

Procedure for the Preparation of the Unknown Enaminoesters (7). The appropriate β-ketoester **4** (3.0 mmol) and ammonium acetate (**5**, 1.16 g, 15.0 mmol) or amine **6p** (6.0 mmol), respectively, were dissolved in dry toluene (10 mL). After adding four drops of acetic acid (or formic acid in case of enaminoester **7p**), the reaction mixture was refluxed for 6 h under azeotropic removal of water. After cooling down to RT, the mixture was washed with saturated aqueous $NaHCO_3$ solution. The resulting organic layer was dried over Na_2SO_4, filtered, and evaporated to give a residue, which was purified by flash chromatography on silica gel.

(2*EZ*)-Ethyl 3-Amino-3-[(3-chlorophenyl)amino]acrylate (13). (2*E*)-Ethyl 3-amino-3-ethoxyacrylate (**12**, 401.2 mg, 2.52 mmol) was dissolved in ethanol (40 mL). After addition of 3-chloroaniline (**6a**, 321.5 mg, 2.52 mmol), the resulting mixture was heated under reflux for 48 h. Evaporation of the solvent resulted in a syrupy residue, which was purified via flash chromatography.

General Procedures for the Preparation of the 5-Hydroxy-1*H*-indole-3-carboxylates 10, 11, 14, and 19−22. To a solution of enamine **7** or ketene aminal **13** (1.0 mmol) in ethanol (4 mL), 1.2 mmol of 1,4-benzoquinone (**8**) or 1,4-naphthoquinone (**9**) were slowly added, respectively. After stirring for 1 h (under reflux using enamines **7**, at RT using ketene aminal **13**), the solvent was removed

under reduced pressure to give a black residue, which was purified by flash chromatography on silica gel (method a).

To a solution of the appropriate 1,4-quinone (1.0 mmol) in 3 mL CH_2Cl_2, ZnI_2 (32.0 mg, 0.1 mmol) was added and the resulting mixture was heated to boiling temperature. A solution of enamine **7** or ketene aminal **13**, respectively (1.0 mmol), in 2 mL of CH_2Cl_2 was added drop by drop under stirring for 5−10 min. After refluxing for additional 20 min, the mixture was cooled to 0−5 °C for 2−3 h. The precipitated crystals were filtered off and washed with CH_2Cl_2 and hexane. If necessary, crystallization of the compounds was performed in solvents (method b).

The method(s) applied is/are mentioned in Supporting Information in conjunction with the appropriate compounds **10**, **11**, **14**, and **19**−**22**.

Ethyl 2-(3-Chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (11a). Method a: 77.7 mg (0.205 mmol, 20%); method b: 197.2 mg (0.519 mmol, 52%); brown powder; mp 222 °C (cyclohexane/ethylacetate). ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.34 (t, 3H, J = 7.0 Hz, CH_2CH_3), 4.29 (q, 2H, J = 7.0 Hz, CH_2CH_3), 4.50 (s, 2H, CH_2), 7.21−7.38 (m, 4H, H-2′, H-4′, H-5′, H-6′), 7.41 (t, 1H, J = 7.5 Hz, H-7), 7.55 (s, 1H, H-4), 7.56 (t, 1H, J = 7.5 Hz, H-8), 8.17 (d, 1H, J = 7.9 Hz, H-6), 8.27 (d, 1H, J = 7.9 Hz, H-9), 9.66 (s, 1H, OH), 12.39 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.4 (CH_2CH_3), 32.4 (CH_2), 58.9 (CH_2CH_3), 101.5 (C-4), 104.2 (C-3), 120.4 (C-9), 121.8 (C-6), 122.4 (C-5a), 123.08 (C-3a), 123.15, 123.2 (C-7, C-9a), 124.0 (C-9b), 126.0 (C-4′), 126.1 (C-8), 127.0 (C-6′), 128.1 (C-2′), 130.2 (C-5′), 132.9 (C-3′), 141.9, 142.2 (C-1′, C-2), 148.3 (C-5), 165.0 (C═O). EI-MS: m/z (%) = 379 (88) [M$^+$], 350 (100), 270 (26), 241 (20), 121 (24). Anal. for $C_{22}H_{18}ClNO_3$: C, H, N.

Ethyl 2-(3-Chlorophenylamino)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (14). Method b: 133.3 mg (0.350 mmol, 35%); green powder; mp 204 °C (cyclohexane/ethylacetate). ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.36 (t, 3H, J = 7.2 Hz, CH_2CH_3), 4.30 (q, 2H, J = 7.2 Hz, CH_2CH_3), 7.01 (m, 1H, Ar), 7.20 (m, 1H, Ar), 7.29 (m, 1H, Ar), 7.34 (m, 2H, Ar), 7.49 (m, 2H, Ar), 8.15 (d, 1H, J = 8.3 Hz, Ar), 8.31 (d, 1H, J = 8.3 Hz, Ar), 8.82 (s, 1H, OH), 9.66 (s, 1H, NH′), 12.14 (s, 1H, NH). EI-MS: m/z (%) = 380 (58) [M$^+$], 334 (84) [M$^+$-OEt], 299 (100), 271 (21). Anal. for $C_{21}H_{17}ClN_2O_3$: C, H, N.

General Procedure for the Preparation of Indole-3-carboxylates 27a−h. First, 1.19 mmol of indole-3-carboxylate **23a** (or **23b** in case of indole **28**) was dissolved in CH_2Cl_2 (5 mL) and the solution was cooled down to 0 °C. Then, *N*,*N*′-dimethylpiperazine (**24**, 7.5 mg, 0.66 mmol) and *N*-chlorosuccinimide (**25**, 175 mg, 1.31 mmol) were added and the reaction mixture was allowed to stand at 0 °C for 2 h, whereupon a solution of trichloroacetic acid (**26**, 50 mg, 0.3 mmol) in CH_2Cl_2 (5 mL), including 2.34 mmol of the appropriate amines **6a**−**h**, was added. After 2 h at RT, the reaction mixture was washed with 10% aqueous $NaHCO_3$ solution, with 1 M aqueous hydrochloric acid, and finally with water. The resulting solution was dried over Na_2SO_4, filtered, and evaporated to give a residue, which was purified by flash chromatography on silica gel.

Ethyl 5-Benzoyloxy-2-(3-chlorobenzyl)-1*H*-benzo[*g*]indole-3-carboxylate (30). Benzoylchloride (**29**, 421.7 mg, 3.0 mmol) was added to a solution of ethyl 2-(3-chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (**11a**, 379.8 mg, 1.0 mmol) in ethylacetate (10 mL)/triethylamine (2 mL), and the mixture was stirred at RT for 20 h. After pouring into ice/water (100 mL) and extraction with ethylacetate, the organic layer was dried over Na_2SO_4. Removing the solvent in vacuo left the crude product, which was crystallized from ethylacetate/CH_2Cl_2, yielding the product as brown powder.

Ethyl 2-(3-Chlorobenzyl)-5-methoxy-1-methyl-1*H*-benzo[*g*]-indole-3-carboxylate (32). Ethyl 2-(3-Chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (**11a**, 189.9 mg, 0.50 mmol) was dissolved in a mixture of aqueous NaOH solution and THF. An excess of methyliodide (**31**) was added, and the solution was refluxed for 10 h. After neutralization with 2 N HCl, the residual methyliodide was emitted. The generated mixture of mono- and dimethyl product was extracted with ethylacetate, washed with water, and dried over Na_2SO_4. The desired dimethylated compound **32** was isolated via flash chromatography.

Ethyl 5-[[(Trifluoromethyl)sulfonyl]oxy]-2-(3-chlorobenzyl)-1*H*-benzo[*g*]indole-3-carboxylate (34). Ethyl 2-(3-Chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indol-3-carboxylate (**11a**, 949.6 mg, 2.5 mmol) was dissolved in 5 mL of CH_2Cl_2. After addition of triethylamine (506.0 mg; 5.0 mmol), trifluoroacetic anhydride (**33**, 1.76 g, 6.25 mmol) was added under cooling and the reaction mixture was stirred for 4 h at 0 °C. The solution was concentrated in vacuo to give the crude product, which was purified via flash chromatography.

Ethyl 2-(3-Chlorobenzyl)-5-phenyl-1*H*-benzo[*g*]indole-3-carboxylate (36). A mixture of 0.55 mmol of phenylboronic acid pinacol ester **35**, ethyl 5-[[(trifluoromethyl)sulfonyl]oxy]-2-(3-chlorobenzyl)-1*H*-benzo[*g*]indole-3-carboxylate (**34**), 256.0 mg, 0.50 mmol), $Pd(OAc)_2$ (3.4 mg, 0.015 mmol), PPh_3 (3.9 mg, 0.015 mmol), aqueous Na_2CO_3 solution, and DMF was heated under reflux for 2 h. The mixture was poured into water and the product was extracted with ethyl acetate. Removing the solvent in vacuo left the crude product, which was was purified via flash chromatography.

Preparation of Human Whole Blood and Cell Isolation. Fresh venous blood was collected in heparinized tubes (16 IE heparin/mL blood) by venipuncture from fasted (12 h) adult healthy volunteers, with consent (Blood Center, University Hospital, Tuebingen, Germany). The subjects had no apparent inflammatory conditions and had not taken anti-inflammatory drugs for at least ten days prior to blood collection.

For isolation of neutrophils, venous blood was subjected to centrifugation (4000*g*/20 min/20 °C) for preparation of leukocyte concentrates. Neutrophils were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes as described previously.[29] Neutrophils (purity >96−97%) were finally resuspended in ice-cold PBS plus 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer).

Determination of 5-LO Product Formation in Whole Blood and in Intact Neutrophils. For assays in whole blood, aliquots of 2 mL (A23187) or 3 mL (LPS and fMLP) were preincubated with the test compounds or with vehicle (DMSO) for 10 min at 37 °C, as indicated, and formation of 5-LO products was either started by addition of fMLP (1 μM) following priming with 1 μg/mL LPS for 30 min or by addition of A23187 (30 μM). The reaction was stopped on ice after 15 (LPS/fMLP) or 10 (A23187) min, and the samples were centrifuged (600*g*, 10 min, 4 °C). Aliquots of the resulting plasma (500 μL) were then mixed with 2 mL of methanol and 200 ng of prostaglandin B_1 were added as internal standard. The samples were placed at −20 °C for 2 h and centrifuged again (600*g*, 15 min, 4 °C). The supernatants were collected and diluted with 2.5 mL of PBS and 75 μL of HCl 1 N. Formed 5-LO metabolites were extracted and analyzed by HPLC as described.[29] 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans* isomers, and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl LTs C_4, D_4, and E_4 (<1% of total 5-LO products[30]) were not analyzed.

For assays of intact neutrophils, 10^7 freshly isolated neutrophils were resuspended in 1 mL of PGC buffer. After preincubation with the test compounds for 15 min at 37 °C, 5-LO product formation was started by addition of 2.5 μM ionophore A23187 with or without 20 μM AA (unless stated otherwise). After 10 min at 37 °C, the reaction was stopped with 1 mL of methanol. Then 30 μL of 1 N HCl, 200 ng prostaglandin B_1, and 500 μL of PBS were added and 5-LO products were analyzed as described.[8]

Determination of 5-LO Product Formation in Cell-Free Systems. *E. coli* MV1190 was transformed with pT3-5LO plasmid. Expression of recombinant 5-LO protein and preparation of 40000*g* supernatant (S40) for 5-LO activity assays was carried out as described.[31] For determination of the activity of 5-LO in S40, aliquots of the supernatants were diluted in 1 mL of PBS, pH 7.4, 1 mM EDTA. Samples were preincubated with the test compounds for 10 min at 4 °C, samples were prewarmed for 30 s at 37 °C,

and 2 mM $CaCl_2$ and 20 μM AA (unless stated otherwise) were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 mL of ice-cold methanol, and the formed metabolites were analyzed as described.[8]

Determination of Subcellular Localization of 5-LO in Neutrophils. Subcellular localization of 5-LO in neutrophils by cell fractionation was investigated as described previously.[29] In brief, freshly isolated neutrophils were preincubated with test compounds (or 0.3% DMSO as vehicle) for 15 min and 2.5 μM A23187 was added. The samples were incubated at 37 °C for 5 min, the reaction was stopped on ice, and nuclear and non-nuclear fractions were obtained after cell lysis by 0.1% NP-40. Aliquots of these fractions were immediately analyzed for 5-LO protein by SDS-PAGE and Western blotting using the 5-LO antiserum 1551, AK-7 (raised in rabbit, diluted 1:25, kindly provided by Dr. Olof Rådmark, Stockholm, Sweden) as described.[30]

Carrageenan-Induced Pleurisy in Rats. Test compounds were given ip to male Wistar Han rats (220−230 g) 30 min before carrageenan. Rats were anaesthetized and λ-carrageenan was injected into the pleural cavity. After four hours, the exudate was removed and the amount was measured. Infiltrated leukocytes in the exudate were counted by light microscopy after vital trypan blue staining. The amount of LTB_4 in the exudate was assayed by EIA according to manufacturer's protocol. The results are expressed as nanograms per rat and represent the mean ± SE of 10 rats. More methodological details can be found in Supporting Information.

Statistics. Data are expressed as mean ± SE. IC_{50} values, obtained from measurements at 4−5 different concentrations of the compounds, are approximations determined by graphical analysis (linear interpolation between the points at 50% activity). The program Graphpad Instat (Graphpad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post hoc tests. Where appropriate, Student's *t* test for paired and correlated samples was applied. A P value of <0.05 (*) was considered significant.

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Supporting Information Available: Elemental analyses or HRMS data, routine spectroscopic data (IR), general procedures for the preparation of the 2-substituted indole-3-carboxylates and ^{1}H NMR data, and carrageenan-induced pleurisy in rats. This material is available free of charge via the Internet at http://pubs.acs.org.

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