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Caulerpenyne and Related Bis-enol Esters Are Novel-Type Inhibitors of Human 5-Lipoxygenase

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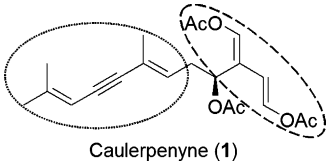
Caulerpenyne (CYN) is a sesquiterpene from green algae with known inhibitory properties against soybean lipoxygenase. Here we introduce a detailed structure–activity study elucidating the inhibitory effects of CYN and a library of six synthetic CYN analogues on isolated human 5-lipoxygenase (5-LO) and cellular 5-LO in polymorphonuclear leukocytes. Essential structural elements are identified and a structurally simplified inhibitor is introduced. The modes of 5-LO inhibition by CYN and the synthetic inhibitors cannot be assigned to any of the known categories of lipoxygenase inhibitors. These compounds clearly interfere directly with 5-LO and represent rather small and flexible molecules, with unique structures among 5-LO inhibitors identified thus far.

Caulerpenyne (CYN (**1**)) is a sesquiterpene found in several green algae belonging to the genus *Caulerpa*. It often represents the major secondary metabolite in Caulerpales with concentrations reaching up to 1.3% of the fresh weight of the alga.[1] The acetylated sesquiterpene plays a central role in the rapid wound closure of the siphonous green alga *Caulerpa taxifolia* and other members of this genus.[2] During this process, CYN (**1**) is enzymatically deacetylated and thereby transformed to a highly reactive 1,4-bis-aldehyde. Following up reports on the inhibitory activity of CYN (**1**) on microtubule protein polymerization, first synthetic CYN analogues in form of colchicine hybrids were generated, however with limited activity.[3] CYN (**1**) itself is also active as inhibitor of telomerase,[4] amylase,[5] xanthine oxidase[6] and plant lipoxygenases (LOs).[7] We reported that CYN (**1**) inhibits soybean LO with an IC_{50} value of 5.1 μM in an uncompetitive manner.[7b] This plant LO has long been used as a model enzyme for mammalian LOs due to some functional similarities.[8] However, recent mechanistic and structural investigations as well as comparative inhibitor studies with human LOs revealed significant differences between these LOs.[8a,9] In the present study, we assess the effects of CYN and a library of synthetic CYN analogues on isolated human 5-LO and cellular 5-LO in polymorphonuclear leukocytes (PMNL).

For the library design, structural motives of CYN were considered separately. Initial experiments using CYN derivative **4** revealed that the acetate group at C4 is not essential for activity. Therefore, the synthetic strategy focused on modification of the dienyne terminus and of the bis-enolacetate functionality (dotted and dashed in Figure 1, respectively). Modifications at the terminus allowed systematically addressing the influence of the sesquiterpene backbone of the natural product in comparison to smaller and less functionalized substituents. Complete truncation of the terminus results in the commercially available 1,4-diacetoxy-(1*E*,3*E*)-butadiene (**7**). The other test compounds were synthesized according to a strategy based on a route to **4** introduced by Richter et al.[10] Starting from 1,4-but-2-ynediol carbomagnesation allows introduction of different alkyl residues or the protected akynyl at position 2. Lipase-mediated acetylation and oxidation using Dess–Martin periodane provides the central intermediate that can finally be acetylated under basic conditions using acetic acid anhydride (Scheme 1, compounds **2**, **3** and **4**). To evaluate the role of the bis-enolacetate structural element, the acetates were replaced with linear carboxylic acids of longer chain length. In these cases vinyl capronate and *Candida rugosa* lipase or vinyl stearate and *Rhizopus arrhizzus* lipase were selected for the enzymatic acylation reactions. Acetic acid anhydride was replaced with anhydrides of the longer chain length fatty acids in the second acylation (Scheme 1, compounds **5** and **6**).



Caulerpenyne (**1**)

Figure 1. The sesquiterpene caulerpenyne (**1**), dotted and dashed parts indicate groups modified for SAR studies, the secondary OAc group could be omitted without significant loss of activity.

To assess the effects of CYN on 5-LO activity, well-established cell-free and cell-based assays were applied.[11] The cell-free assays allow analysis of direct inhibition of 5-LO. They are

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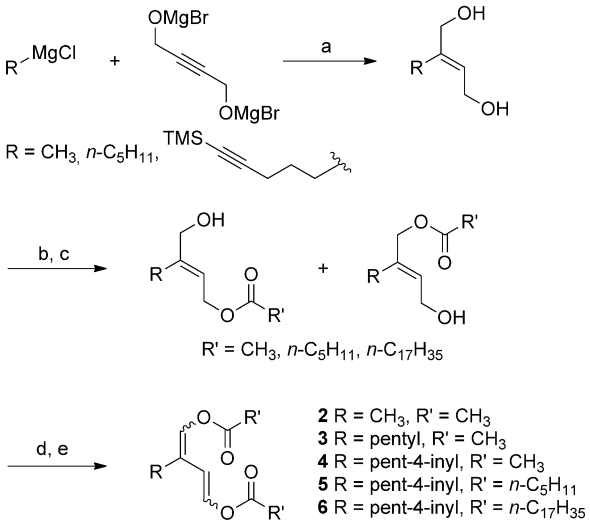


Scheme 1. Synthesis of bis-enol esters with different side chains and acyl moieties. *Reagents and conditions*: a) Et_2O, reflux; b) vinyl carboxylic ester, lipase, diisopropyl ether; c) for synthesis of **4**, **5** and **6**, tetrabutylammonium fluoride, THF; d) Dess–Martin periodinane, CH_2Cl_2; e) carboxylic acid anhydride, 4-(dimethylamino)pyridine, Et_3N. See the Supporting Information for detailed procedures.

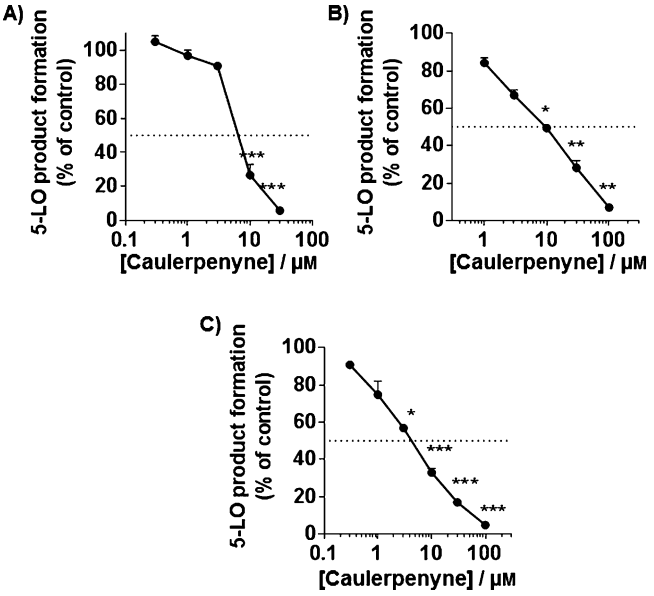


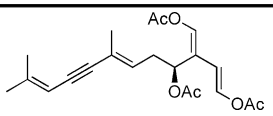
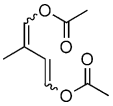
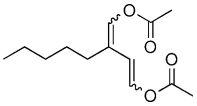
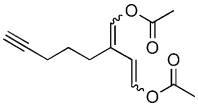
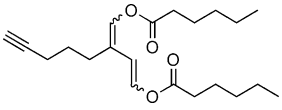
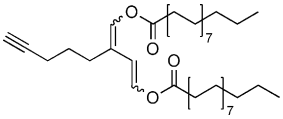
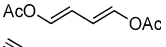
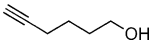
Figure 2. Inhibition of 5-LO activity by caulerpenyne (CYN (**1**)). Inhibition of the formation of 5-LO products (LTB_4 and its *trans* isomers and 5-H(P)ETE) in A) intact human PMNL and in B) PMNL homogenates. C) Inhibition of the activity of isolated human recombinant 5-LO. Data are given as mean + SE, $n = 3$. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle (100%).

based on incubation of isolated human recombinant 5-LO or PMNL homogenates as enzyme source in the presence or absence of inhibitors with arachidonic acid (AA, 20 μM) as substrate. The cell-based assay was performed in the presence or absence of exogenous AA as substrate.[11] This assay uses Ca^{2+}-ionophore A23187-stimulated human PMNL and can reveal indirect inhibitory effects on 5-LO product formation (e.g., block of substrate supply, influence on the 5-lipoxygenase-activating protein (FLAP), and on 5-LO activation by phosphorylation). For both assays, formation of 5-LO products (i.e., LTB_4 and its trans-isomers and 5-H(P)ETE) derived from AA were determined by RP-HPLC.[12]

In the cell-based assay, CYN (**1**) potently and concentration-dependently suppressed 5-LO product formation with an IC_{50} of 5.9 μM (Figure 2A, Table 1). For the reference 5-LO inhibitor zileuton (approved as drug for asthma therapy), the IC_{50} value was determined at 0.6 μM (not shown). In the cell-free assay, using PMNL homogenates as source for 5-LO, CYN (**1**) inhibited 5-LO activity with an IC_{50} value of 9.5 μM (Figure 2B), and for isolated human recombinant 5-LO an IC_{50} value of 4.2 μM was obtained (Figure 2C). These data demonstrate that CYN (**1**) is a 5-LO inhibitor with effectiveness in intact cells.

The chemical structure of the active CYN (**1**) cannot be categorized into known classes of 5-LO inhibitors.[13] We thus decided to undertake structure–activity relationship (SAR) studies in order to dissect the pharmacophore of CYN (**1**). Truncation of the entire terminus results in the bis-enolacetate 1,4-diacetoxy-(1*E*,3*E*)-butadiene (**7**), which led to loss of po-

Table 1. IC_{50} values [μM] of the bisenolacylates and a control compound for 5-LO inhibition.

Compd		Purified 5-LO[a]	PNML−AA[b]	PNML + AA[c]
1		4.2	5.9	21.0
2		39.1	18.4	n.d.
3		> 30[d]	2.1	n.d.
4		11.8	5.1	18.8
5		48.6	1.0	n.d.
6		21.0	> 100	n.d.
7		> 100	> 100	n.d.
8		> 100	> 100	n.d.

[a] Purified 5-LO incubated with 20 μM AA as substrate. [b] A23187-activated intact polymorphonuclear leukocytes (PNML) without AA. [c] A23187-activated intact PNML with 20 μM AA; PNML were preincubated with test compounds for 15 min and then activated to induce 5-LO product formation for 10 min. Data are means, $n = 3$–4. n.d. = not determined. [d] Not soluble at > 30 μM.

tency in PMNL and completely failed to inhibit isolated 5-LO up to 100 μM. Hence, the alkylation in 2-position of the butadiene moiety is essential for bioactivity. Introduction of a methyl group at this position (**2**) recovered 5-LO inhibition, although the potency was almost 10-fold lower as compared to CYN (**1**) (Table 1). Similarly, substitution with *n*-pentyl (**3**) that at least partially recovers the hydrophobicity of the dienyne terminus of CYN (**1**) resulted in a moderate inhibitor of 5-LO in the cell-free assay with an $IC_{50} > 30$ μM. It is surprising that this compound caused strong suppression of 5-LO activity in intact cells ($IC_{50} = 2.1$ μM), which might be related to interference with 5-LO regulatory processes (e.g., block of substrate supply, 5-LO phosphorylation or FLAP) or unspecific (acute cytotoxic) effects. Interestingly, introduction of a pent-4-inyl residue at 2-position of the butadiene (**4**) led to comparable potency as found for CYN (**1**) (Table 1). Unsaturation of the hydrophobic terminus thus seems essential for activity. The pent-4-inyl moiety itself (in hept-6-in-1-ol **8**) was completely inactive in the cell-based and the cell-free assay (Table 1).

The fact that a substantially simplified molecule compared to CYN (**1**) exhibited comparable activity opened up new possibilities for further modifications based on the structure of **4**. The synthetic strategy to **2**–**4** could be adapted to exchange the acetyl moieties by longer chain length fatty acids. Capronyloxy (**5**) and stearyloxy derivatives (**6**) were thus accessible for testing. In the cell-based assay a pronounced influence of the length of the fatty acid residues on 5-LO inhibition was observed. The bis-capronyloxy derivative **5** was even more potent compared to CYN (**1**) itself and inhibited 5-LO in the cell-based assay with an IC_{50} value of 1 μM, while the bis-enol stearate (**6**) completely failed to inhibit in the cell-based assay at 100 μM. Interestingly, **5** was a poor inhibitor of isolated 5-LO ($IC_{50} = 48.6$ μM) and also **6** was less active ($IC_{50} = 21$ μM) as compared to CYN (**1**) and **4**. This observation would be in accordance with a mechanism where **5** may, in analogy to **3**, target 5-LO regulatory events in the cell. The long C-18 alkyl chains of **6** decrease the solubility and might hamper cell penetration into the cytosol where 5-LO resides. In all above assays it has to be considered that due to the synthetic procedures, all four configurational isomers of **2**–**6** are present and might differentially contribute to inhibition of 5-LO, as compared to CYN (**1**) as stereochemically defined structure.

The mode of 5-LO inhibition by the most potent compounds CYN (**1**) and the alkynyl derivative **4** was studied in more detail. Because many direct 5-LO inhibitors act as antioxidants due to interference with the active site iron by uncoupling its redox cycle or by radical scavenging during enzymatic catalysis,[13a] we first assessed the radical scavenging activity of CYN (**1**) and **4** using the well-established di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay.[14] Neither compound caused significant radical scavenging activity at concentrations up to 200 μM, whereas L-cysteine and ascorbic acid (used as reference compounds) clearly blocked DPPH radical formation (Figure 3). Therefore, and supported by the lack of redox-active

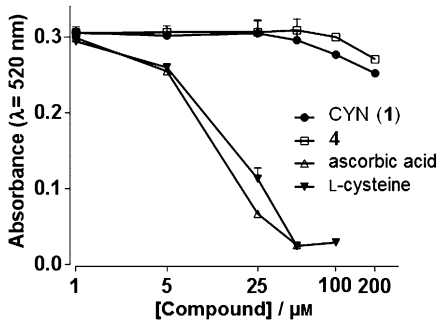


Figure 3. Radical scavenging activity. Test compounds were incubated with 5 nmol DPPH for 30 min at RT, and the absorbance was measured at 520 nm. Ascorbic acid and L-cysteine were used as controls. Values are given as percentage of control (100%) mean + SE, $n = 3$.

structural elements, we suggest that CYN (**1**) and **4** may indeed inhibit 5-LO in a non-redox fashion.

To investigate if the compounds inhibit 5-LO in a reversible manner, wash-out experiments using isolated 5-LO enzyme were performed. 5-LO activity was inhibited by 1 μM and 10 μM CYN (**1**) to 25% and 75%, respectively (Figure 4A). A

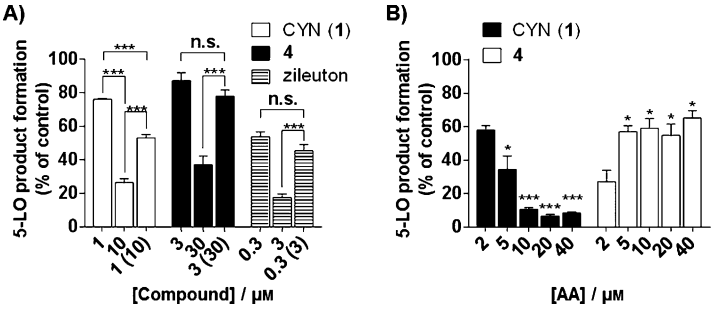


Figure 4. Reversibility of 5-LO inhibition and influence of substrate concentration. A) Wash-out experiments by 10-fold dilution in incubations of isolated 5-LO and test compounds. B) Inhibition of isolated 5-LO by test compounds (10 μM, each) at various AA concentrations. Values, given as percentage of control (vehicle, 100%), are means + SE, $n = 4$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus vehicle (100%).

10-fold dilution of the sample containing 10 μM CYN (**1**) (final concentration 1 μM) partially reduced 5-LO inhibition to about 50%. These data suggest an incomplete reversion of 5-LO suppression by CYN (**1**) after wash-out. In contrast to CYN (**1**), the inhibitory effect of compound **4** after 10-fold dilution from 30 μM to 3 μM corresponds to that of an initially administered 3 μM solution (Figure 4A), indicating that the inhibitory effects are immediately reversible. As expected, Inhibition of 5-LO by the reference compound zileuton was also reversible in comparable wash-out experiments (Figure 4A).[11]

Next, we analyzed how 5-LO inhibition by CYN (**1**) and **4** is affected by the substrate (AA) concentration. Previous studies showed that 5-LO activity is maximal at about 10–20 μM AA but then declines at higher concentrations due to substrate inhibition.[13b] As shown in Figure 4B, the magnitude of 5-LO inhibition by CYN was enhanced by increasing the AA concentra-

tion (from 2 to 40 μM), implying an uncompetitive mode of inhibition. Thus, at 2 μM AA almost 60% 5-LO activity remained in the presence of 10 μM CYN (**1**), while at 40 μM AA about less than 10% activity was detectable. In contrast, for **4**, increasing the substrate concentration impaired the potency against 5-LO (Figure 4B). Here, 5-LO activity at 2 μM AA was suppressed down to 25%, but at 40 μM AA 5-LO inhibition was moderate and 65% enzyme activity remained. These data suggest that CYN (**1**) acts in an uncompetitive manner, while in sharp contrast **4** inhibits 5-LO in an AA competitive way.

In addition, we analyzed the ability of CYN (**1**) and **4** to inhibit the 5-LO-related human 12-LO and 15-LO-1 that are present in PMNL-adherent platelets and eosinophils, respectively.[15] As shown in Figure 5, CYN (**1**) concentration-dependently suppressed the activity of both 12-LO and 15-LO-1 in PMNL homogenates albeit with reduced potency ($IC_{50}=36$ and 42 μM, respectively) as compared to 5-LO ($IC_{50}=9.5$ μM) under the same assay conditions. In contrast, compound **4** (up to 100 μM) failed to significantly repress the activities of these LOs.

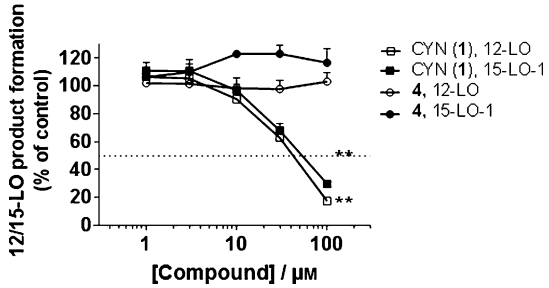


Figure 5. Effects of CYN (**1**) and compound **4** on the activity of 12-LO and 15-LO-1 in homogenates of PMNL. Data are given as means + SE, $n=3$. $^{**}p<0.01$ versus vehicle (100%).

Taken together, despite similar potencies against 5-LO, the mode of 5-LO inhibition by CYN (**1**) and **4** is substantially different: while 5-LO inhibition by **4** is reversible, impaired by increasing AA concentrations and selective for 5-LO, inhibition of 5-LO by CYN (**1**) is only partially reversible, markedly improved at increasing substrate concentrations and unselective as also 12/15-LOs are inhibited. The molecular mechanisms underlying 5-LO inhibition by these compounds are still elusive. 5-LO inhibitors have been intensively developed since 1983 in order to be used as anti-inflammatory or anti-allergic drugs, and a vast number of compounds had been introduced.[16] These substances can be categorized as (1) redox-active agents, (2) iron-ligands, (3) fatty acid competitors that act at the active 5-LO site, or (3) represent agents that interfere with phospholipid binding of 5-LO via the C2-like domain.[13a] Neither CYN (**1**) nor compound **4** can be assigned to any of these categories. Both compounds clearly interfere directly with 5-LO and represent rather small and flexible molecules, with unique structures among 5-LO inhibitors identified thus far.

Experimental Section

Details on materials, synthetic procedures and spectroscopic data can be found in the Supporting Information.

Biological assays

Human PMNL: Human polymorphonuclear leukocytes (PMNL) were freshly isolated from peripheral blood (Institute for Transfusion Medicine, University Hospital Jena, Germany) as described.[11] In brief, venous blood from healthy adult donors was centrifuged (4000 g/20 min/20 °C) to prepare leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation and centrifugation on Nycoprep cushions. The pellet was resuspended, erythrocytes were lysed under hypotonic conditions, and PMNL were recovered by centrifugation (purity > 96–97%).

Determination of 5-LO activity in intact cells: PMNL (1×10^7 mL^{-1}) were pre-incubated with test compounds for 15 min at 37 °C. Then, 5-LO product formation was started by addition of 2.5 μM Ca^{2+}-ionophore A23187 with or without 20 μM AA. After 10 min at 37 °C, 5-LO metabolites (LTB_4 and its all-*trans* isomers and 5-H(P)ETE) were extracted and analyzed by RP-HPLC as described.[11]

Analysis of LO activities in cell-free assays: Human recombinant 5-LO was expressed in *E. coli* BL21 transformed with pT3–5LO plasmid, and isolated by affinity chromatography on an ATP-agarose column as described.[11] Alternatively, PMNL were homogenized by sonication and used as source for 5-LO, 12-LO and 15-LO. Isolated 5-LO or PMNL homogenates were pre-incubated with test compounds for 10 min at 4 °C and pre-warmed for 30 s at 37 °C. 5-LO product formation was initiated by addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min at 37 °C, formed 5-LO metabolites (all-*trans* isomers of LTB_4 and 5-H(P)ETE), 12-H(P)ETE and 15-H(P)ETE were analyzed by RP-HPLC as described.[11]

Determination of radical scavenging activity: Compounds were dissolved in EtOH and combined with an equal volume of 100 mM di-(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) in EtOH (final volume: 200 μL). After incubation for 30 min, absorbance was measured at 520 nm as described.[11]

Statistics: Data are expressed as mean + standard error (SE). Statistical evaluation was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post hoc tests or by Student's *t* test for paired and correlated samples. *P* values <0.05 were considered statistically significant. All statistical calculations were performed using GraphPad InStat 3.10 (GraphPad Software Inc., La Jolla, CA, USA). IC_{50} values were determined by graphical analysis using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA).

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Keywords: caulerpenyne · inhibition · lipoxygenase · structure activity · synthetic analogues

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