Anacardic acid derived salicylates are inhibitors or activators of lipoxygenases

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Abstract

Lipoxygenases catalyse the oxidation of unsaturated fatty acids, such as linoleic acid, which play a crucial role in inflammatory responses. Selective inhibitors may provide a new therapeutic approach for inflammatory diseases. In this study, we describe the identification of a novel soybean lipoxygenase-1 (SLO-1) inhibitor and a potato 5-lipoxygenase (5-LOX) activator from a screening of a focused compound collection around the natural product anacardic acid. The natural product anacardic acid inhibits SLO-1 with an IC$_{50}$ of 52 µM, whereas the inhibitory potency of the novel mixed type inhibitor 23 is five-fold enhanced. In addition, another derivative (21) caused non-essential activation of potato 5-LOX. This suggests the presence of an allosteric binding site that regulates the lipoxygenase activity.
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Introduction

Lipoxygenases (LOXs) are a family of non-heme iron-containing enzymes that catalyse the oxygenation of cis,cis-1,4-pentadiene moieties in lipids. In general, lipoxygenases (LOXs), which are widely distributed in both the plant and animal kingdom, are categorized into 5-LOX, 8-LOX, 12-LOX and 15-LOX based on the position of oxygenation of their substrates. Lipoxygenases convert their natural substrates, arachidonic acid, to hydroperoxy eicosatetraenoic acids (HPETEs) by a radical mechanism. Lipoxygenases found in plant oxygenate linoleic acid to generate 13-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13-HPOD). 5-HPETE is transformed into Leukotriene A4, which contains an unstable epoxide. Subsequently, Leukotriene A4 is converted either into Leukotriene B4 by an enzymatic hydrolysis or into Leukotriene C4 by glutathione S-transferase. The resulting Leukotriene C4 is further metabolized to Leukotriene D4 and Leukotriene E4. It has been reported that activities of lipoxygenases and their related products play an important role in numerous inflammatory and proliferative diseases such as asthma, atherosclerosis, and cancer. Leukotriene B4 activates inflammatory cells such as neutrophils and macrophages. This broad range of biological effects demonstrates the importance of lipoxygenases as a therapeutic target.

6-pentadecyl salicylic acid, commonly known as anacardic acid, is a natural product found in cashew nut shells. This compound is often associated with anti-inflammatory, anti-tumor, molluscicidal, and anti-microbial activities. Previous studies reported that anacardic acid inhibits peroxidation of linoleic acid by soybean lipoxygenase-1 (SLO-1) with an IC\textsubscript{50} of 85 µM. In addition, anacardic acid and its derivatives inhibit histone acetyl transferases (HATs) and cyclooxygenases, which are also involved in inflammation and cancer.

In this study, we investigate the affinity and selectivity of anacardic acid derived inhibitors for SLO-1 and potato 5-LOX as representatives of the lipoxygenase family. Although lipoxygenases are well-known drug targets, relatively little inhibitors with high selectivity and potency have been described (reviewed by Pergola and Werz). Currently, the selective 5-LOX inhibitor Zileuton (IC\textsubscript{50} = 0.5 µM) is marketed for treatment of asthma. Although there are no 15-LOX inhibitors available for the clinic, recent studies identified inhibitors with nanomolar affinity. Nevertheless, their efficacy in cell-based studies remains limited. Therefore, development of inhibitors of new-structural classes remains necessary in order to explore these enzymes further as drug targets.

Here, we describe the identification of lipoxygenase inhibitors and activators from a focused compound collection based on anacardic acid. Anacardic acid and its derivatives were synthesized following previous research and inhibition of SLO-1 and potato 5-LOX were investigated. We found that derivative 23 inhibits SLO-1 selectively to potato 5-LOX, whereas derivative 21 shows a three-fold activation on potato 5-LOX. The enzyme kinetic studies of these
modulators indicate the presence of an allosteric site that influences the activity of the lipoxygenase active site.

**Results and discussion**

**Design**

The fact that lipoxygenases are iron-containing enzymes combined with the fact that salicylates are known iron-binding compounds justifies the hypothesis that the iron-binding properties of salicylates are keys to their inhibitory potency. Based on this hypothesis a molecular modeling study was performed in order to propose a binding configuration of anacardic acid 18 to the soybean lipoxygenase-1 active site. A docking simulation shows that the salicylate from 18 can coordinate to the iron and that its hydrophobic tail fits well in the hydrophobic cavity in the active site of soybean lipoxygenase-1 (Figure 1). The hydrophobic interactions between the amino acid residues and the ligand are probably important for the interaction in the enzyme active site. Based on our proposed binding model we expect that the different 6-alkyl substituents in our focused compound collection fit, in principle, in the lipoxygenase active site. Therefore, we screened this focused compound collection for lipoxygenase inhibition.

![Figure 1. Binding pose of anacardic acid 18 (A) and inhibitor 23 (B) in a crystal structure of soybean lipoxygenase-1 (PDB files 1F8N). The iron atom is shown as a ball, the binding residues are shown as wireframes, and the ligand is shown as sticks. The hydrogen bonds are shown as cyan dashed lines. The iron coordination with the ligand and enzyme are illustrated with green lines.](image)

**Synthesis**

A compound collection was assembled around the natural product anacardic acid 18. This collection comprises the commercially available salicylates 12, 13 and 14, the previously published salicylates 15-23, 26-30, and 32 and the newly synthesized salicylates 24, 25, 31, and 33. The synthesis of anacardic acid and its derivatives 15-23, 26-30, and 32 were described previously by Ghizzoni et al. and compounds 24, 25, 31, and 33 were synthesized using similar procedures (Scheme 1). Triflate 1
Anacardic acid derived salicylates are inhibitors or activators of lipoygenase was synthesized as described by Uchiyama et al.\textsuperscript{20} Alkynes 2\textsuperscript{21} and 5 were prepared from triflate 1 or 3-iodobenzoic acid 3 by Sonogashira coupling with trimethylsilyl (TMS) acetylene and subsequent cleavage of the TMS protective group to yield the corresponding products (Scheme 1). Benzoazoles 7a and 7b were prepared from 2-amino-5-chlorophenol (6) and benzoic acid through amidation and cyclization.\textsuperscript{22} Subsequently, 7a was attached to 2 and 7b was attached to 5 to give the corresponding alkynyls, which were reduced using catalytic hydrogenation to give 24, and 31 (Scheme 1).

Sonogashira coupling of aryl chlorides has been described to proceed with PdCl\(_2\)(PPh\(_3\))\(_2\), P(tBu), and 1,8-Diazabicyclo [5.4.0]undec-7-ene (DBU) as catalysts, and Cs\(_2\)CO\(_3\) as a base to give >80% yields.\textsuperscript{23} However, our attempt to synthesize compounds 24, and 31 under these conditions yielded 20-32% of the desired products. These relatively low yields are possibly due to electronic properties of the benzoazole and/or the limited stability of the ester functionality in 2 or 5 under the basic coupling conditions.

Scheme 1. Reagent and conditions: a) Trimethylsilylacetylene, Cul, PdCl\(_2\)(PPh\(_3\))\(_2\), Et\(_2\)NH, PPh\(_3\), CH\(_3\)CN, (MW, 120 °C, 95 W), b) TBAF, THF, 0 °C, c) Cul, PdCl\(_2\)(PPh\(_3\))\(_2\), Et\(_2\)NH, HC≡CR, CH\(_3\)CN (MW, 100 °C, 70 W); d) H\(_2\), Pd/C, MeOH, 40 °C ; e) aqueous KOH 5 N, THF, 55 °C; f) benzyl bromide, K\(_2\)CO\(_3\), DMF, R.T. g) benzoic acid anhydride, pyridine, DMF, R.T.; h) SOCl\(_2\), 60 °C followed by 2-aminophenol, 0 °C; i) p-toluensulfonic acid, toluene, reflux; j) SOCl\(_2\), DMAP, DME, acetone, R.T; k) Br-Br, K\(_2\)CO\(_3\), DMF, R.T.; l) Br-O-Na+, THF, R.T.; m) Tf\(_2\)O, pyridine, CH\(_3\)Cl, 0 °C ; n) HC≡CR, aryl chloride, Cs\(_2\)CO\(_3\), PdCl\(_2\)(PPh\(_3\))\(_2\), P(tBu), DBU, DMF (MW, 150 °C, 95 W)

Benzoxazole 9 was prepared from commercially available 2-(4-bromophenyl)acetic acid (8) and 2-aminophenol following two reaction steps. Firstly, 2-(4-bromophenyl)acetic acid (8) was converted into the acyl chloride using thionyl chloride followed by amide bond formation with 2-aminophenol to give the amide with high yields (70%). Secondly, this amide was cyclized using p-toluensulfonic acid in toluene at reflux for 4 hours to give the product 9 with high yields (78-85%). Subsequently,
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The arylbromide (9) was coupled to trimethylsilyl acetylene by a Sonogashira coupling. The trimethylsilyl protective group was cleaved to give the corresponding alkyne 10. This alkyne was coupled to triflate 11 using a Sonogashira coupling to provide 55% yield of the corresponding alkyne (Scheme 1), which was hydrogenated to yield 25 in a single step.

Acetonide 13 was synthesized from 2,4-dihydroxybenzoic acid 12 as described by Tranchimand et al. Furthermore, acetonide 13 was converted to triflate 14 as described previously. Sonogashira reaction, a coupling of 1-ethynyl-4-heptyl benzene with triflate 14, was performed to give the alkyne with high yield (79%), which was hydrogenated to give compound 33.

Table 1. Compounds collection of anacardic acid and its derivatives

<table>
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<th>Compounds</th>
<th>R1</th>
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<th>R3</th>
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<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>14</td>
<td>C₂H₅</td>
<td>OH</td>
<td>CH₃</td>
</tr>
<tr>
<td>15</td>
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<td>30</td>
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<td>31</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>H</td>
<td>H</td>
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</tr>
<tr>
<td>33</td>
<td>H</td>
<td>OH</td>
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</tbody>
</table>
**SLO-1 and potato 5-LOX inhibition**

The compound collection (Table 1) was screened for inhibition of the lipoxygenase activity of soybean lipoxygenase-1 (SLO-1) and potato 5-lipoxygenase (potato 5-LOX). The lipoxygenase activity was monitored in real time using a spectrophotometric assay to monitor the formation of conjugated diene 13-hydroperoxy-cis-9-trans-11-octadecadienoic acid (13-HPOD) from linoleic acid.\(^\text{25, 26}\) The residual enzyme activity was monitored after 10 minutes of pre-incubation with 50 µM of the respective inhibitor. The enzymatic activity without inhibitor present was taken as reference and set to 100% and the activity without enzyme present was set to 0%. The percentages of the residual enzyme activity of SLO-1 and potato 5-LOX in the presence of the inhibitors are plotted in Figure 2.

![Figure 2](image.png)

**Figure 2.** Residual enzyme activity that was observed for the screening of the salicylate compound collection for inhibition of the lipoxygenase activity. The bars show the residual activity of potato LOX-5 (light grey) and soybean lipoxygenase-1 (SLO1) (dark grey) in the presence of 50 µM of the respective inhibitor. The percentage activity for each compound was calculated in comparison with the blank in which no inhibitor was present. The results were the average of three independent experiments with the standard deviations.

Anacardic acid 18 inhibits both potato 5-LOX and SLO-1 about 50% at 50 µM and the IC\(_{50}\) value for SLO-1 was 52 µM (Figure 3A, Table 2), which is in line with literature for SLO-1.\(^\text{10}\) In contrast, we observed an IC\(_{50}\) of 43 µM for potato lipoxygenase (Figure 3B), whereas literature reports a 30% activity inhibition at 6 µM.\(^\text{12}\) The difference is probably due to a difference in the type of substrate that was used. Salicylates 21, 23, 24 and 25 show 50% inhibition or more at 50 µM for SLO-1, whereas this is not observed for potato 5-LOX. In contrast, compounds 21 and 22 activate the potato 5-LOX enzyme at these concentrations. This demonstrates that these compounds activate or inhibit lipoxygenase activity and that selectivity between both lipoxygenases can be obtained by variation of the substitution in the 4- and/or 6- position of the salicylate.

This salicylate compound collection shows interesting structure activity relationships for SLO-1. The 6-pentadecyl substituent in 18 is important for inhibition, because inhibitors 15 and 16 with a 6-pentyl or 6-decyl substituent are less potent. The same is observed for the salicylates 12, 13, 14, 26...
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and 27 without an aliphatic substituent in the 6-position. These results demonstrate that the side chain length of the 6-alkyl chain is important for the SLO-1 inhibitory potency. This is possibly due to the hydrophobic interactions between the inhibitor and the binding pocket of the enzyme. In addition, a complete loss of inhibition was observed if the carboxylate of anacardic acid 18 was converted into a methyl ester 19, which demonstrates that the free carboxylate is also important for binding. Furthermore, the importance of the 2-hydroxyl functionality of the salicylate is demonstrated by the inactivity of the inhibitors 31-33, which show less than 50% inhibition 50 µM. These results demonstrate that the carboxylate and the hydroxyl of the salicylate core are both crucial for inhibition of SLO-1.

Salicylates 24 and 25 with a benzoxazole functionality in their side chain maintain their inhibition of SLO-1, whereas no inhibition of potato 5-LOX was observed. This demonstrates that selectivity between potato 5-LOX and SLO-1 can be obtained by variation of the 6-alkyl substituent in anacardic acid.

Figure 3. The IC$_{50}$ value for inhibition of (A) SLO-1 and (B) potato 5-LOX by anacardic acid (18). The results were the average of three independent experiments with error bars (±S.D.)

Inhibitor 23 shows an IC$_{50}$ of 11 µM (Table 2) for SLO-1, which is five-fold improved compared to anacardic acid 18, whereas no inhibition of potato 5-LOX was observed. Furthermore, inhibitor 23 also shows selectivity towards HATs, in which inhibitor 23 give no inhibitory effect on the activity of p300, and PCAF and modest effect on Tip60 at 200 µM. In comparison, the lower SLO-1 inhibition of 22 and 33 demonstrate that both hydroxyl groups of 23 are important for inhibition. In addition, the reduced potency of 20 shows that also the 1-ethyl-4-heptylbenzene substituent in 23 is important for inhibition.

Table 2. IC$_{50}$ values for inhibition of SLO-1 by anacardic acid derivatives

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>51.9 ± 5.0</td>
</tr>
<tr>
<td>21</td>
<td>55.4 ± 6.3</td>
</tr>
<tr>
<td>23</td>
<td>11.1 ± 0.8</td>
</tr>
<tr>
<td>25</td>
<td>58.5 ± 3.6</td>
</tr>
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In an attempt to improve the potency of inhibitor 23, we designed a novel compound 25 in which the aliphatic chain was replaced for a heterocycle. However, this derivative did not provide a better inhibition of SLO-1 (Table 2).

The influence of 23 on the Michaelis-Menten kinetics for conversion of the substrate linoleic acid was determined in order to establish the mechanism of SLO-1 inhibition. Inhibitor 23 causes both a reduction of $V_{\text{max}}^{\text{app}}$ and an increase of $K_{m}^{\text{app}}$ (Table 3), which indicates a mixed type of inhibition (Figure 4). The enzyme activity is expected to obey the model in Scheme 2, in which the inhibitor can bind to the free enzyme as well as to the substrate bound enzyme.

The values for $V_{\text{max}}$ and $K_{m}$ were determined from the Lineweaver-Burk plot using equation 1 (Figure 5) in the absence of inhibitor 23, respectively from the y-intercept and x-intercept. $V_{\text{max}}^{\text{app}}$ and $K_{m}^{\text{app}}$ for each inhibitor 23 concentration were calculated in the same manner as in the absence of inhibitor. $\alpha^\prime$ and $\alpha/\alpha^\prime$ values, which are the change in $V_{\text{max}}$ and $K_{m}$, are derived respectively from $V_{\text{max}}^{\text{app}}$ divided by $V_{\text{max}}$ and from $K_{m}^{\text{app}}$ divided by $K_{m}$. The inhibitor binding constant, $K_{i}$ and $K_{i}^\prime$ values, were calculated from the equation 2 and 3 (Figure 5) as described in the experimental sections. The binding constant for inhibition of the free enzyme ($K_{i}$) is 9.8 µM and the binding constant to the substrate bound enzyme ($K_{i}^\prime$) is 15.7 µM. The fact that inhibitor 23 binds to the free as well as the substrate bound enzyme as a mixed type inhibitor suggests the presence of an allosteric binding site.

![Figure 4](image-url)

**Figure 4.** Conversion of the substrate linoleic acid by the enzyme SLO-1 with no inhibitor present (♦), after preincubation with 6 µM inhibitor 23 (▲), and with 11 µM inhibitor 23 (■). The results were the average of three independent experiments with error bars (±S.D.)

**Table 3.** Enzyme kinetics parameter for SLO-1 inhibition

<table>
<thead>
<tr>
<th>[23] (µM)</th>
<th>$V_{\text{max}}^{\text{app}}$ (µM/s)</th>
<th>$K_{m}^{\text{app}}$ (µM)</th>
<th>$R^2$</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.437</td>
<td>12.4</td>
<td>0.963</td>
</tr>
<tr>
<td>6</td>
<td>0.321</td>
<td>14.3</td>
<td>0.967</td>
</tr>
<tr>
<td>12</td>
<td>0.251</td>
<td>15.5</td>
<td>0.979</td>
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Scheme 2. Kinetic model for mixed enzyme inhibition.

\[
\begin{align*}
E + S & \xrightleftharpoons[k_m']{K_i'} ESI \\
+ & \xrightarrow[k_{cat}]{[S]} ES + \\
& \xrightarrow[K_i]{[S]} E + \text{P}
\end{align*}
\]

**equation 1**

\[
\nu = \frac{V_{\text{max}}}{\alpha K_m + \alpha'[S]} \quad \text{equation 2}
\]

\[
\alpha = 1 + \frac{[I]}{K_i} \quad \text{equation 2}
\]

\[
\alpha' = 1 + \frac{[I]}{K_i'} \quad \text{equation 3}
\]

**Figure 5.** Equations for the enzyme kinetics according to the model in Scheme 2. \(v\) is the reaction velocity, \(V_{\text{max}}\) is the maximal reaction velocity, \([S]\) is the substrate concentration and \(K_m\) is the Michaelis-Menten constant. \(\alpha\) and \(\alpha'\), respectively, are the parameters to describe the change of substrate binding affinity to the enzyme and the change of the maximum velocities. \(K_i\) is the dissociation constant of inhibitor to the free enzyme and \(K_i'\) is the dissociation constant of inhibitor to the enzyme-substrate complex.

Interestingly, inhibitor 21, in which the 6-aliphatic side chain is replaced by an ether functionality, shows equal inhibition compared to anacardic acid 18 on SLO-1 (Table 2), whereas this inhibitor (and also inhibitor 22) shows about three-fold activation of potato 5-LOX. Compound 21 provided a concentration dependent activation with a maximal activation that is about 3 fold higher than the control activity at concentrations higher than 50 \(\mu\)M (Figure 6).

We decided to study the enzyme kinetics for the activation of potato 5-LOX by 21 in order to investigate the binding mechanism. The concentration dependent effect of 21 on the Lineweaver-Burk plot of potato 5-LOX activity was investigated (Figure 7A). Activator 21 causes an increase of the \(V_{\text{max}}\) and a decrease of the \(K_m\) values. This behavior, in which the reaction can occur in the presence or in the absence of the activator, indicates non-essential activation of potato 5-LOX 31 (Table 4). The enzyme kinetics for the activation of potato 5-LOX obeys the model shown in Scheme 3. The activator dissociation constants (\(K_A\)), the change in the affinity of substrate binding (\(\alpha\) value) and the change in the catalytic constant (\(\beta\) value) were determined from the re-plot of \(1/\Delta\text{slope}\) and the \(1/\Delta y\)-intercept (Figure 7B) according to equation 4 (Figure 8) as described in the experimental sections.

The kinetic analysis shows that \(\alpha = 0.013\), \(\beta = 1.4\) and \(K_A = 1.8\) mM. The \(\alpha\) value indicates that the substrate and the activator mutually enhance their binding by close to 100-fold. Consequently, the affinity of the activator for the substrate bound enzyme is 24 \(\mu\)M, which explains the observed activation at concentrations of 25 \(\mu\)M and higher. In addition, activator binding increases the catalysis rate by 1.4 fold. Taking this together, activator 21 shows non-essential activation and binds with relatively high affinity to substrate bound lipoxygenase and enhances the enzymatic conversion of the substrate.
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Figure 6. Compound 21 activates potato 5-LOX. The experiments were performed in the absence (control) or the presence of compound 21 with various concentrations. The results were the average of three independent experiments with error bars (±S.D.)

Figure 7. (A) Lineweaver-Burk plot of the potato 5-LOX activity with no activator present (●), after preincubation with 12.5 µM activator 21 (▲), with 25 µM activator 21 (■), and with 50 µM activator 21 (●). The results were the average of three independent experiments with error bars (±S.D.) (B) Re-plot of 1/Δslope (●) and the 1/Δy-intercept (♦)

Table 4. Enzyme kinetics parameter for potato 5-LOX activation

<table>
<thead>
<tr>
<th>[21] (µM)</th>
<th>$V_{max}^{App}$ (µM/s)</th>
<th>$K_{m}^{App}$ (µM)</th>
<th>$R^2$</th>
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<tr>
<td>0</td>
<td>1.597</td>
<td>1.160</td>
<td>0.998</td>
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<td>12.5</td>
<td>1.754</td>
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<td>25</td>
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<td>50</td>
<td>1.949</td>
<td>0.370</td>
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Scheme 3. Kinetic model for non-essential activation.

\[
\rho = \frac{V_{\text{max}} \times [S]}{K_m \left(1 + \frac{[A]}{K_A} \right) + [S] \left(1 + \frac{[A]}{\alpha K_A} \right)}
\]

Figure 8. Equation for the enzyme kinetics according to the model in Scheme 3. \(V_{\text{max}}\) is the maximal reaction velocity, \([S]\) is the substrate concentration and \(K_m\) is the Michaelis-Menten constant, \([A]\) is the activator concentration. \(\alpha\) and \(\beta\), respectively, are the parameters to describe the change in the affinity of substrate binding and the change in the catalytic constant.

Furthermore, the critical micelle concentrations (CMCs) for both anacardic acid (18) and 23 at the assay conditions (0.2 M borate buffer, pH 9.0, R.T) are, respectively, 390 µM and 436 µM (Figure 9B,D) whereas for compound 21 (in 0.1 M phosphate buffer pH 6.3, R.T), the CMC value is 208 µM (Figure 9C). These results indicate that micelle formation does not occur at concentrations employed for inhibition or activation. In addition, we found a CMC value for linoleic acid of 163 µM (Figure 9A), which demonstrates that the substrate concentration in the inhibition studies (100 µM) was below the CMC. Thus the enzyme kinetics was evaluated in a homogeneous system.

The kinetic studies on both potato 5-LOX and SLO-1 indicate the presence of an allosteric binding site that influences substrate binding and conversely is influenced by binding of the substrate. For potato 5-LOX we demonstrated activation of the enzyme activity and for SLO-1 we demonstrated inhibition. This behavior means that the described docking simulation as in the initial design (Figure 1) is not in line with the allosteric mechanism indicated from the kinetic studies of the modulator. These indications for an allosteric binding pocket are in line with previous studies. The observation of substrate inhibition of soybean lipoxygenase by linoleic acid indicates the presence of an allosteric binding site. It can be presumed that the salicylate based inhibitors resemble the lipid substrate linoleic acid and binds in a similar way to the enzyme, thereby inhibiting the enzyme activity via the allosteric binding pocket. In addition, mixed inhibition has been described previously for soybean lipoxygenase by the inhibitor oleyl sulfate, which also indicates the presence of an allosteric binding site. Non-essential activation has been described previously for 5-LOX by compound (R,S)-2-hydroxy-2-trifluoromethyl-trans-n-octadec-4-enoic acid (HTFOA) and phosphatidic acid. These findings also indicate the existence of allosteric site as enzyme activity regulator. Nevertheless,
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the location and structural properties of the allosteric binding pocket in these enzymes remain elusive. This demonstrates that structural characterization of the allosteric binding pocket remains a major challenge in lipoxygenase research.

![Figure 9](image)

**Figure 9.** Surface tensions of (A) Linoleic acid, (B) Anacardic acid 18, (C) compound 21, and (D) compound 23 against the logarithm of concentration. CMC values for A and C were measured in SLO-1 assay conditions, 0.2 M borate buffer, pH 9.0, R.T. (t = 19°C), whereas CMC value for B was measured in potato 5-LOX assay conditions, 0.1M phosphate buffer, pH 6.3, R.T. (t = 25°C).

This study identifies salicylate based molecules as allosteric regulators of lipoxygenase enzyme activity. Such inhibitors might find applications as starting points for development of therapeutic agents for asthma and inflammations. Nevertheless, the potency and selectivity of this compound class for human lipoxygenases remains to be investigated. It is encouraging that the anacardic acid derived salicylates show clear structure activity relationships, which indicates that further optimization of the affinity and selectivity of this compound class might be feasible.

**Conclusion**

This study demonstrates that anacardic acid 18 and its derivatives are lipoxygenase inhibitors and that their potency depends on the substitution pattern in the 4- and 6-position of the salicylate core. A novel inhibitor 23 was identified for soybean lipoxygenase-1 with a five-fold improved IC$_{50}$ value compared to anacardic acid 18. Enzyme kinetics reveal a mixed type inhibition pattern for 23 with a $K_i$ of 9.8 µM and a $K'_i$ of 15.7 µM (Scheme 3). In addition, an activator 21 of potato 5-lipoxygenase
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was identified for which $K_A$ is 1.8 mM, $\alpha$ is 0.013 and $\beta$ is 1.4 (Scheme 4). The affinity of 21 for the substrate bound enzyme ($\alpha K_A$) is 24 $\mu$M. To our knowledge, this is the first activator with micromolar potency for potato lipoxygenase. These inhibitors might provide valuable starting points for development of inhibitors that target lipoxygenases, which is relevant for inflammatory diseases.

**Experimental**

**General**

All reagent and solvent were purchased from commercial suppliers (Fluka, Sigma-Aldrich, Acros Organics) and were used without further purification unless stated otherwise. Dichloromethane was distilled over CaH$_2$ before use. Merck silica gel 60 F$_{254}$ plates were used for analytical thin layer chromatography (TLC) and spots were detected by UV light, or stained using KMNO$_4$ or ninhydrin solution. Column chromatography was performed with MP Ecocrom silica gel 32-63, 60Å using the flash chromatography technique. $^1$H (200 MHz) and $^{13}$C (50 MHz) NMR spectra were recorded on a Varian Gemini 200 spectrometer. $^{13}$C NMR spectra were recorded using the attached proton test (APT). Chemical shifts are reported in ppm (δ) relative to the solvent signals. Electrospray ionization mass spectra (ESI-MS) were recorded on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. High-resolution mass spectra (HR-MS) were recorded using a flow injection method on a LTQ-Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) with a resolution of 60,000 at $m/z$ 400. Protonated testosterone (lock mass $m/z$ = 289.2162) was used for internal recalibration in real time.

**Organic Synthesis of the focused compound collection of salicylates**

The synthesis of anacardic acid and its derivatives 15-23, 26-30, and 32 was described previously by Ghizzoni et al.¹⁸,¹⁹ and compounds 24, 25, 31, and 33 were synthesized using similar procedures according to Scheme 1.

**Synthetic procedure 1; Sonogashira coupling of aryl iodides or aryl triflates**

Freshly distilled diethylamine (0.15 mL, 1.5 mmol) and the alkyne (1.1 mmol) were subsequently added to a solution of the aryl iodide or aryl triflate (1.0 mmol), CuI (9.5 mg, 0.05 mmol), and PdCl$_2$(PPh$_3$)$_2$ (35 mg, 0.05 mmol) in degassed anhydrous acetonitrile (1.0 mL) under nitrogen atmosphere. The mixture was subjected to microwave irradiation for 20 min at 100 ºC (70 W). The reaction mixture was diluted with ethyl acetate (50 mL) and extracted with demi water (2 × 75 mL). The water phase was separated and washed with EtOAc (2 × 50 mL). The combined organic phases were washed with brine (2 × 75 mL), dried over Na$_2$SO$_4$ and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography.
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**Synthetic procedure 2; Sonogashira coupling of aryl chlorides**

The alkyne (1.2 mmol), the aryl chloride (1.3 mmol), and Cs$_2$CO$_3$ (0.47 g, 1.4 mmol) were dissolved in dry DMF (2 mL) under nitrogen atmosphere. Subsequently, 2 mol % PdCl$_2$(PPh$_3$)$_2$ (17 mg, 0.024 mmol), 4 mol % P(tBu)$_3$ (10 mg, 0.05 mmol), and 10% mol 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (18 mg, 0.12 mmol) were added to the reaction mixture. The mixture was subjected to microwave irradiation for 15 min at 150 °C (95 W). The mixture was diluted with 50 mL EtOAc and filtered over Celite. Then the filtrate was washed with brine (2 × 50 mL), and dried over MgSO$_4$ and the solvent was evaporated under reduced pressure. The product was obtained after purification using column chromatography.

**Synthetic procedure 3; reductive hydrogenation and or hydrogenolysis**

The starting material (0.5 mmol) was dissolved in MeOH and 10 mol% Pd/C (10%) (53 mg, 0.05 mmol) was added. The compound precursor of 25 named benzyl 2-((4-(benzo[d]oxazol-2-ylmethyl)phenyl)ethynyl)-4,6-bis(benzyloxy)benzoate was dissolved in EtOAc due to its low solubility in MeOH. The suspension was shaken with 3 atm H$_2$-pressure in a Parr apparatus at 40 °C overnight. Subsequently, the mixture was filtered through Celite. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography.

**Synthetic procedure 4; saponification of the acetonide**

The starting material (1.0 mmol) was dissolved in THF (10 mL) and an aqueous 5 N KOH solution (2 mL, 10 mmol) was added. The solution was stirred overnight at 55 °C. Subsequently, the reaction mixture was diluted with EtOAc (10 mL) and neutralized with 1 N HCl (20 mL). The mixture was extracted with EtOAc (3 × 30 mL). The organic phases were collected, washed with brine (1 × 50 mL), dried over Mg$_2$SO$_4$ and filtered. The solvent was evaporated and the product was purified by column chromatography.

**Synthetic procedure 5; benzoxazole synthesis**

2-amino-5-chlorophenol (1.4 g, 10 mmol) was dissolved in DMF (15 mL). Subsequently, pyridine (4.0 mL, 50 mmol) and (p-methoxy) benzoic acid anhydride (10 mmol) were added and the reaction mixture was stirred for 3 hours at room temperature. The reaction mixture was diluted with 100 mL water and extracted with EtOAc (2 × 100 mL). The organic layer was combined and extracted with 1 N HCl (2 × 150 mL) and saturated Na$_2$CO$_3$ (2 × 150 mL). The organic layer was washed with brine (1 × 150 mL), dried over Mg$_2$SO$_4$ and filtered. The solvent was evaporated to give a corresponding amide.

The amide (1 mmol) was dissolved in toluene (10 mL) and p-toluensulfonic acid (2 mmol) was added. The solution was heated to reflux for 4 hours. The reaction mixture was diluted with EtOAc
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(40 mL) and extracted with saturated NaHCO₃ (2 × 50 mL). The organic phases were collected, washed with brine (1 × 50 mL), dried over Mg₂SO₄ and filtered. The solvent was evaporated and the product was purified by column chromatography.

2-(4-bromobenzyl)benzo[d]oxazole (9)

4-bromophenyl acetic acid (2.15 g, 10 mmol) was dissolved in 10 mL SOCl₂ and heated to 60 °C for 30 minutes. The solvent was distilled off to obtain the acyl chloride as a yellow liquid. 2-aminophenol (1.09 g, 10 mmol) was suspended in dichloromethane (20 mL), Et₃N (2.77 mL, 20 mmol) was added and the mixture was cooled at 0 °C. The acyl chloride was added drop-wise to the mixture, which was stirred overnight while it warmed to room temperature. The reaction mixture was diluted with EtOAc (30 mL) and extracted with a saturated aqueous Na₂CO₃ solution (3 × 50 mL), 1 N aqueous HCl (3 × 50 mL) and brine (1 × 50 mL). The organic phase was dried with Mg₂SO₄ and evaporated to obtain the corresponding amide.

The amide (1.78 g, 5.8 mmol) was dissolved in toluene (40 mL) and p-toluensulfonic acid (2.21 g, 11.6 mmol) was added. The solution was heated to reflux for 4 hours. Subsequently, the reaction mixture was diluted with EtOAc (60 mL) and extracted with saturated aqueous NaHCO₃ (2 × 100 mL). The organic phase was collected, washed with brine (1 × 100 mL), dried over Mg₂SO₄ and filtered. The solvent was evaporated and the TLC confirmed product purity after the work up and therefore no further purification was performed. Yield 85%. Brown solid. Rf = 0.55 (heptane:EtOAc 1:1). ¹H NMR (200 MHz, DMSO) δ 4.32 (s, 2H); 7.31-7.36 (m, 4H); 7.55 (d, J = 8.3Hz, 2H); 7.62-7.70 (m, 2H). ¹³C NMR (50 MHz DMSO) δ 33.69; 110.86; 119.69; 120.57; 124.67; 125.23; 131.64; 131.78; 134.83; 141.04; 150.61; 165.19. MS ESI m/z 288.2; 290.2 [M+H]+.

2-(4-((trimethylsilyl)ethynyl)benzyl)benzo[d]oxazole

Into a dried 10-20 mL microwave vial, PdCl₂(PPh₃) (133 mg, 0.2 mmol), CuI (36 mg, 0.2 mmol), and PPh₃ (200 mg, 0.8 mmol) were suspended in CH₃CN (5 mL) under nitrogen atmosphere. Subsequently, the benzoazole 9 (1.10 g, 3.8 mmol), diethyl amine (Et₂NH) (5.9 mL, 57 mmol), and trimethylsilyl acetylene (TMSA) (402 mg, 4.2 mmol), were added to the mixture. The mixture was heated at 120 °C for 35 minutes via microwave irradiation (95 W). The reaction mixture was diluted with EtOAc (50 mL) and washed with water (3 × 50 mL). The water layer was washed with EtOAc (2 × 50 mL). The combined organic phases were extracted with brine (1 × 50 mL) and dried over
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Mg\(_2\)SO\(_4\). The solvent was evaporated under reduced pressure and the residue was purified by column chromatography with heptane/EtOAc 12:1 (v/v) as eluent. Yield 75%. Yellow to orange solid. R\(_f\) = 0.76 (heptane:EtOAc 1:1). \(^1\)H NMR (200 MHz, CDCl\(_3\)) \(\delta\) 0.09 (s, 9H); 4.08 (s, 2H); 7.05-7.12 (m, 4H); 7.21-7.27 (m, 2H); 7.47-7.52 (m, 2H). \(^{13}\)C NMR (50 MHz CDCl\(_3\)) \(\delta\) 0.16; 35.25; 104.84; 105.21; 110.79; 119.92; 122.60; 124.71; 125.27; 129.14; 132.63; 135.05; 151.15; 165.08. MS ESI m/z 306.2 [M+H]\(^+\).

2-(4-ethynylbenzyl)benzo[d]oxazole (10)

2-(4-((trimethylsilyl)ethynyl)benzyl)benzo[d]oxazole (611 mg, 2.0 mmol) was dissolved in THF (5 mL) and the solution was cooled to 0 \(^\circ\)C. Tetra butyl ammonium fluoride (TBAF) (2.6 mmol) in THF (1 M, 2.6 mL) was added and the reaction mixture was stirred for 10 minutes. The reaction mixture was diluted with EtOAc (50 mL), extracted with water (4 × 50 mL) and washed with brine (2 × 50 mL). The organic phase was dried over MgSO\(_4\) and filtered. The solvent was evaporated and the product was used without further purification. Yield 99%. Brown solid. R\(_f\) = 0.59 (heptane:EtOAc 1:1). \(^1\)H NMR (200 MHz, CDCl\(_3\)) \(\delta\) 3.07 (s, 1H); 7.29-7.36 (m, 4H); 7.44-7.50 (m, 2H); 7.68-7.72 (m, 2H). \(^{13}\)C NMR (50 MHz CDCl\(_3\)) \(\delta\) 35.07; 76.41; 104.09; 110.48; 119.83; 121.24; 124.31; 124.87; 129.02; 132.56; 135.45; 151.00; 164.57. MS ESI m/z 234.1 [M+H]\(^+\).

Benzyl 2-((4-(benzo[d]oxazol-2-ylmethyl)phenyl)ethynyl)-4,6-bis(benzyloxy)benzoate

The product was obtained using sonogashira coupling of benzyl 2,4-bis(benzyloxy)-6-(((trifluoromethyl)sulfonyl)oxy)benzoate 11 and 2-(4-ethynylbenzyl)benzo[d]oxazole 10 using synthetic procedure 1. The product was purified by column chromatography with heptane/EtOAc 4:1 (v/v) as eluent. Yield 51%. Yellow gum. R\(_f\) = 0.52 (heptane:EtOAc 1:1). \(^1\)H NMR (200 MHz, CDCl\(_3\)) \(\delta\) 4.29 (s, 2H); 5.02 (s, 2H); 5.05 (s, 2H); 5.34 (s, 2H); 6.56 (d, \(J=2.1\) Hz, 1H); 6.74 (d, \(J=2.1\) Hz, 1H); 7.17-7.22 (m, 4H); 7.32-7.36 (m, 15H); 7.46-7.50 (m, 2H); 7.68-7.73 (m, 2H). \(^{13}\)C NMR (50 MHz CDCl\(_3\)) \(\delta\) 35.25; 67.31; 70.54; 70.81; 87.23; 92.67; 102.19; 109.73; 110.81; 119.90; 122.18; 123.51; 124.76; 125.31; 127.30; 127.74; 128.14; 128.20; 128.45; 128.61; 128.76; 128.89; 129.25; 132.25; 132.42; 133.18; 135.15; 136.01; 136.32; 136.41; 157.28; 160.56; 166.80.
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2-(4-(benzo[d]oxazol-2-ylmethyl)phenethyl)-4,6-dihydroxybenzoic acid (25)

The product was obtained from benzyl 2-((4-(benzo[d]oxazol-2-ylmethyl)phenyl)ethynyl)-4,6-bis(benzyloxy)benzoate using synthetic procedure 3. The product was purified by column chromatography with heptane/EtOAc 1:2 (v/v) as eluent. Yield 86%. White solid. $R_f = 0.45$ (heptane:EtoAc 1:1). $^1$H NMR (200 MHz, DMSO) $\delta$ 2.67-2.75 (m, 2H); 2.94-3.02 (m, 2H); 4.26 (s, 2H); 6.14 (d, $J=2.4$ Hz, 1H); 6.20 (d, $J=2.4$ Hz, 1H); 7.16-7.35 (m, 6H); 7.60-7.68 (m, 2H). $^{13}$C NMR (50 MHz DMSO) $\delta$ 34.18; 37.53; 38.08; 101.26; 105.63; 110.62; 111.01; 119.77; 124.88; 125.40; 128.97; 129.39; 132.97; 141.12; 146.70; 150.73; 162.03; 164.16; 166.01; 173.06. HRMS: $m/z$ 390.1336 [M+H]+, calcd for C_{23}H_{20}O_{5}N_{1} 390.1331.

5-chloro-2-phenylbenzo[d]oxazole (7a)

The product was obtained from 2-amino-4-chlorophenol and benzoic acid using synthetic procedure 5. The product was purified by column chromatography with hexane/EtOAc 10:1 (v/v) as eluent. Yield 80%. Pink solid. $R_f = 0.44$ (hexane:EtOAc 3:1). $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 7.25 (dd, $J=8.5$; 1.9 Hz, 1H); 7.43-7.61 (m, 5H); 8.12-8.16 (m, 2H). $^{13}$C NMR (50 MHz CDCl$_3$) $\delta$ 104.98; 111.22; 120.45; 125.26; 126.69; 127.64; 128.95; 130.65; 131.78; 140.88; 150.90; 163.68. MS ESI $m/z$ 230.1; 232 [M+H]+.

2,2-dimethyl-5-((2-phenylbenzo[d]oxazol-5-yl)ethynyl)-4H-benzo[d][1,3]dioxin-4-one

The product was obtained from 5-ethynyl-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one 2 and 5-chloro-2-phenylbenzo[d]oxazole 7a using synthetic procedure 2. The product was purified by column chromatography with hexane/EtOAc 5:1 (v/v) as eluent. Yield 20%. Yellow solid. $R_f = 0.27$ (hexane:EtOAc 3:1). $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.7 (s, 6H); 6.96-7.01 (m, 3H); 7.53-7.61 (m, 5H); 7.82 (s, 1H); 8.24-8.29 (m, 2H). TOF MS ES+: $m/z$ 396.1233 [M+H]+, calcd for C_{25}H_{22}O_{3}N_{1} 396.1236.
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2,2-dimethyl-5-(2-(2-phenylbenzo[d]oxazol-5-yl)ethyl)-4H-benzo[d][1,3]dioxin-4-one

The product was obtained from 2,2-dimethyl-5-((2-phenylbenzo[d]oxazol-5-yl)ethynyl)-4H-benzo[d][1,3]dioxin-4-one using synthetic procedure 3. This intermediate was used for the next reaction without any further purification. Yield 81%. Yellow oil. \textit{R}\textsubscript{f} = 0.44 (hexane:EtOAc 3:1). \textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3}) \(\delta\) 1.63 (s, 6H); 2.95-3.03 (m, 2H); 3.34-3.42 (m, 2H); 6.75-6.83 (m, 2H); 7.28-7.36 (m, 2H); 7.44-7.48 (m, 5H); 8.17-8.22 (m, 2H). TOF MS ES+: \textit{m}/\textit{z} 340.1540 [M+H]\textsuperscript{+}, calcd for C\textsubscript{25}H\textsubscript{22}O\textsubscript{4}N\textsubscript{1} 340.1549.

2-hydroxy-6-(2-(2-phenylbenzo[d]oxazol-6-yl)ethyl)benzoic acid (24)

The product was obtained from 2,2-dimethyl-5-(2-(2-phenylbenzo[d]oxazol-5-yl)ethyl)-4H-benzo[d][1,3]dioxin-4-one using synthetic procedure 4. The product was purified by column chromatography with hexane/EtOAc 4:1 (v/v) as eluent. Yield 50%. White solid. \textit{R}\textsubscript{f} = 0.31 (EtOAc + 0.1% AcOH). \textsuperscript{1}H NMR (200 MHz, DMSO) \(\delta\) 2.93-3.04 (m, 4H); 6.72-6.78 (m, 2H); 7.15-7.28 (m, 2H); 7.61-7.64 (m, 5H); 8.17-8.22 (m, 2H). \textsuperscript{13}C NMR (50 MHz DMSO) \(\delta\) 35.93; 40.21; 110.70; 114.48; 119.82; 120.57; 120.76; 125.80; 126.99; 127.57; 129.75; 131.29; 132.24; 140.15; 140.31; 140.97; 150.89; 156.80; 162.38; 170.94. HRMS: \textit{m}/\textit{z} 344.1280 [M+H]\textsuperscript{+}, calcd for C\textsubscript{22}H\textsubscript{18}O\textsubscript{3}N\textsubscript{1} 344.1281

5-chloro-2-(4-methoxyphenyl)benzo[d]oxazole (7b)

The product was obtained from 2-amino-4-chlorophenol and 4-methoxybenzoic acid using synthetic procedure 5. The product was purified by column chromatography with hexane/EtOAc 8:1 (v/v) as eluent. Yield 48%. Pink solid. \textit{R}\textsubscript{f} = 0.62 (hexane:EtOAc 1:1). \textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3}) \(\delta\) 3.87 (s, 3H); 7.00 (d, \textit{J}= 8.9 Hz, 2H); 7.28 (dd, \textit{J}= 8.5; 1.9 Hz, 1H); 7.52-7.62 (m, 2H); 8.13 (d, \textit{J}= 8.9 Hz, 2H). \textsuperscript{13}C NMR (50 MHz CDCl\textsubscript{3}) \(\delta\) 55.68; 111.25; 114.64; 119.40; 120.24; 125.28; 129.67; 130.29; 141.30; 151.06; 162.75; 164.11. MS ESI \textit{m}/\textit{z} 260.1; 262.1 [M+H]\textsuperscript{+}. 
Anacardic acid derived salicylates are inhibitors or activators of lipoxygenase.

Benzyl 3-(2-(4-methoxyphenyl)benzo[d]oxazol-5-yl)ethynylbenzoate

The product was obtained from benzyl 3-ethynylbenzoate 5 and 5-chloro-2-(4-methoxyphenyl)benzo[d]oxazole 7b using synthetic procedure 2. The product was purified by column chromatography with hexane/EtOAc 6:1 (v/v) as eluent. Yield 32%. Yellow solid. R_f = 0.43 (hexane:EtOAc 1:1). ¹H NMR (200 MHz, CDCl₃) δ 3.81 (s, 3H); 5.31 (s, 2H); 6.95 (d, J=9.0 Hz, 2H); 7.30-7.46 (m, 7H); 7.58-7.67 (m, 3H); 7.95-8.17 (m, 4H). ¹³C NMR (50 MHz CDCl₃) δ 55.72; 67.22; 90.59; 113.78; 114.69; 119.25; 119.48; 119.70; 128.55; 128.60; 128.79; 128.88; 129.66; 129.84; 130.73; 133.00; 136.03; 143.05; 150.61; 154.37; 162.87; 164.64; 165.94. MS ESI m/z 460.3 [M+H]+.

3-(2-(2-(4-methoxyphenyl)benzo[d]oxazol-6-yl)ethyl)benzoic acid (31)

The product was obtained from benzyl 3-((2-(4-methoxyphenyl)benzo[d]oxazol-5-yl)ethynyl)benzoate using synthetic procedure 3. The product was obtained in high purity and no further purification was required. Yield 98%. White solid. R_f = 0.46 (EtOAc + 0.1% AcOH). ¹H NMR (200 MHz, CD₃OD) δ 3.00-3.06 (m, 4H); 3.85 (s, 3H); 7.05 (d, J=9.0 Hz 2H); 7.14-7.29 (m, 3H); 7.38 (s, 1H); 7.52 (d, J=8.1Hz, 1H); 7.72-7.81 (m, 1H); 7.84 (s, J/H); 8.08 (d, J=9.0 Hz 2H). ¹³C NMR (50 MHz CD₃OD) δ 39.24; 39.98; 56.22; 111.57; 115.79; 119.68; 126.46; 126.92; 128.17; 128.85; 130.45; 130.58; 131.71; 139.36; 141.02; 142.27; 152.18; 164.34; 164.37; 166.32. HRMS: m/z 374.1385 [M+H]+, calcd for C₂₃H₂₀O₄N₁ 374.1387.

7-(benzyloxy)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (13)

The acetonide 7-hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (558 mg, 2.9 mmol) was dissolved in DMF (10 mL) under nitrogen atmosphere. K₂CO₃ (955 mg, 6.9 mmol) and benzyl bromide (0.41 mL, 3. 5 mmol) were added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc (50 mL). The organic phase was washed with water (4 × 50 mL) and brine (3 × 20 mL). The organic phase was dried over MgSO₄ and the
solvent was evaporated. The residue was purified using column chromatography with heptane/EtOAc 4:1 (v/v) as eluent. Yield 84%. Colorless oil. R_f = 0.46 (heptane:EtOAc 1:1). ^1H NMR (200 MHz, DMSO) δ 1.68 (s, 6H); 5.20 (s, 2H); 6.75 (d, J=2.3 Hz, 1H); 6.84 (dd, J=8.7, 2.4 Hz, 1H); 7.31-7.48 (m,5H); 7.78 (d, J=8.7 Hz, 1H). ^13C NMR (50 MHz DMSO) δ 25.26; 70.02; 102.01; 105.75; 106.23; 111.20; 127.98; 128.18; 128.52; 130.76; 136.00; 157.39; 159.87; 165.16. MS ESI m/z 285.2 [M+H]^+.

**Benzyl 4-(benzyloxy)-2-(((trifluoromethyl)sulfonyl)oxy)benzoate (14)**

Sodium (0.2 g, 9.1 mmol) was added to a solution of benzyl alcohol (1.2 mL, 11.4 mmol) in THF (10 mL) under nitrogen atmosphere. The suspension was stirred until the sodium disappeared. Subsequently, the 7-(benzyloxy)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one 13 was added (0.65 g, 2.28 mmol) and the reaction mixture was stirred for 30 minutes at room temperature. The reaction mixture was diluted with EtOAc (50 mL) and washed with water (3 × 50 mL) and brine (3 × 20 mL). The organic phase was dried over MgSO_4_ and the solvent was evaporated. The product was purified using column chromatography to obtain benzyl 4-(benzyloxy)-2-hydroxybenzoate as a product.

Benzyl 4-(benzyloxy)-2-hydroxybenzoate (56 mg, 1.79 mmol) and pyridine (0.71 mL, 8.97 mmol) were dissolved in CH_2Cl_2 (10 mL) and cooled to 0°C under nitrogen atmosphere. Triflic anhydride (0.52 mL, 1.97 mmol) was added drop wise to the mixture and stirred for 1 hour. The mixture was poured onto solid NaHCO_3 and washed with 0.1 N HCl (3 × 30 mL), brine (2 × 15 mL), and dried over MgSO_4. The solvent was evaporated and the product was used without further purification. Yield 90%. Colorless solid. R_f = 0.57 (heptane:EtOAc 1:1). ^1H NMR (200 MHz, CDCl_3) δ 5.12 (s, 2H); 5.38 (s, 2H); 6.86 (d, J=2.4Hz, 1H); 7.0 (dd, J=8.9, 2.5 Hz, 1H); 7.31-7.49 (m, 10H); 8.05 (d, J=8.9 Hz, 1H). ^13C NMR (50 MHz, CDCl_3) δ 63.37; 71.09; 109.97; 114.40; 116.77; 117.31; 120.50; 127.75; 128.61; 128.76; 128.82; 128.84; 129.04; 134.29; 135.31; 135.68; 149.92; 163.27; 163.34. MS ESI m/z 467.1 [M+H]^+.

**Benzyl 4-(benzyloxy)-2-((4-heptylphenyl)ethynyl)benzoate**

The product was obtained from benzyl 4-(benzyloxy)-2-(((trifluoromethyl)sulfonyl)oxy)benzoate 14 and 1-ethynyl-4-heptylbenzene using synthetic procedure 1. The product was purified by column chromatography with heptane/EtOAc 21:4 (v/v) as eluent. Yield 79%. Orange oil. R_f = 0.64.
Anacardic acid derived salicylates are inhibitors or activators of lipoxygenase (heptane:EtOAc 1:1). $^1$H NMR (200 MHz, CDCl$_3$) δ 0.90 (t, $J=6.8$ Hz, 3H); 1.30-1.33 (m, 8H); 1.60-1.64 (m, 2H); 2.62 (t, $J=7.6$ Hz, 2H); 5.13 (s, 2H); 5.39 (s, 2H); 6.95 (dd, $J=8.8$, 2.6 Hz, 1H); 7.13 (d, $J=8.0$ Hz, 2H); 7.23 (d, $J=2.6$ Hz, 1H); 7.28-7.55 (m, 12H); 8.02 (d, $J=8.8$ Hz, 1H). $^{13}$C NMR (50 MHz CDCl$_3$) δ 14.31; 22.86; 29.36; 29.40; 31.46; 32.02; 36.14; 66.92; 70.40; 88.01; 95.06; 115.08; 119.66; 120.49; 124.24; 126.31; 127.72; 128.25; 128.46; 128.59; 128.72; 128.88; 131.88; 133.10; 136.23; 136.34; 143.92; 161.42; 165.86. MS ESI m/z 517.3 [M+H]$^+$. 

2-(4-heptylphenethyl)-4-hydroxybenzoic acid (33)

The product was obtained from benzyl 4-(benzyloxy)-2-((4-heptylphenyl)ethynyl)benzoate using synthetic procedure 3. No purification of the crude product was required. Yield 100%. White solid. R$_f$ = 0.41 (heptane:EtOAc 1:1). $^1$H NMR (200 MHz, CDCl$_3$) δ 0.86 (t, $J=6.3$ Hz, 3H); 1.26-1.30 (m, 8H); 1.54-1.61 (m, 2H); 2.55 (t, $J=7.7$ Hz, 2H); 2.83-2.91 (m, 2H); 3.23-3.31 (m, 2H); 6.66-6.73 (m, 2H); 7.05-7.16 (m, 4H); 8.05 (d, $J=8.3$ Hz, 1H). $^{13}$C NMR (50 MHz, CDCl$_3$) δ 14.34; 22.91; 29.44; 29.60; 31.84; 32.07; 35.84; 37.66; 113.32; 118.20; 120.63; 128.59; 134.93; 139.27; 140.71; 148.55; 159.50; 171.78. HRMS: m/z [M+H]$^+$, calcd for C$_{22}$H$_{29}$O$_3$ 341.2111, found 341.2112; m/z [M+Na]$^+$, calcd for C$_{22}$H$_{28}$O$_3$Na 363.1931, found 363.1931; m/z 379.1670 [M+K]$^+$, calcd for C$_{22}$H$_{28}$O$_3$K 379.1670.

Inhibition screening UV Assay

The enzyme potato 5-lipoxygenase (potato 5-LOX) and soybean lipoxygenase-1 (SLO-1) were obtained from Cayman Chemicals. Linoleic acid was obtained from Sigma.

Enzyme inhibition was measured by the residual enzyme activity after 10 minutes incubation with the inhibitor at room temperature. The enzyme activity was determined by conversion of the lipoxygenase substrate linoleic acid into hydroperoxy eicosatetraenoic acid (HPETE). The conversion rate was followed by UV absorbance of the conjugated diene at 234 nm ($\varepsilon = 25000$ M$^{-1}$ cm$^{-1}$) over a period of 20 minutes. The UV absorbance increase over time was used to determine the enzyme activity.

Phosphate buffer (NaH$_2$PO$_4$-Na$_2$HPO$_4$ 0.1 M) pH 6.3 was used as an assay buffer for all potato 5-LOX inhibitory experiments. The potato 5-LOX enzyme was diluted 1:2000 with the assay buffer. The inhibitor (100 mM in DMSO) was diluted with the assay buffer to 100.5 µM. The substrate, linoleic acid was diluted with EtOH to 20 mM. Subsequently, 1 mL of enzyme solution (1:2000) was mixed with 1 mL inhibitor solution (100.5 µM), which was incubated for 10 minutes. Subsequently, the linoleic acid solution (10 µL, 20 mM) was added to give a mixture with 1:4000 enzyme dilution,
Anacardic acid derived salicylates are inhibitors or activators of lipoxygenase

50 µM inhibitor and 100 µM linoleic acid. The conversion rate of the substrate was measured after 10 second reaction of the enzyme with the substrate. The reaction rate in absence of the inhibitor was used as positive control. In the positive control experiments the assay buffer was supplemented with a little amount of DMSO (3.0 µL of DMSO in 1.5 mL) in order to replace the DMSO inhibitor solution, which was also pre-incubated for 10 minutes.

Sodium borate buffer (H$_3$BO$_3$ 0.2 M) pH 9.0 was used as an assay buffer for all SLO-1 inhibition experiments. The enzyme SLO-1 was diluted 1:4000 using the SLO-1 assay buffer and the inhibitor (100 mM in DMSO) was diluted using the same buffer to 200 µM. The linoleic acid (20 mM in EtOH) was diluted with SLO-1 assay buffer to 200 µM. The enzyme (400 µL, 1:4000) was mixed with the inhibitor (400 µL, 200 µM) and the mixture was incubated for 10 minutes. Subsequently, the linoleic acid (800 µL, 200 µM) was added to give a mixture with 50 µM inhibitor and 100 µM linoleic acid. The UV absorbance at 234 nm was immediately measured after 10 seconds reaction of the enzyme with the substrate. The measurement in absence of inhibitor was used as a positive control. The assay buffer with a little amount of DMSO (3.0 µL of DMSO in 1.5 mL) was used to replace the inhibitor solution. This solution was also pre-incubated for 10 minutes.

**Lipoxygenase IC$_{50}$ determination**

Inhibitory concentration 50% (IC$_{50}$) for both potato 5-LOX and SLO-1 were determined using the same assay setup as the inhibitor screening. Various concentrations of inhibitor were added to the enzyme and the residual enzyme activity was determined (Table 2, Figure 2 and 10). These experiments were performed in triplicate. The calculation was performed with Excel 2010 and the non-linear curve fitting was performed with the Origin 8 software.

![Figure 10](image)

Figure 10. The IC$_{50}$ values for inhibition of SLO-1 by inhibitor 21 (A), inhibitor 23 (B) and inhibitor 25 (C). The results were the average of three independent experiments with error bars (±S.D.)

**Michaelis Menten Enzyme Kinetics**

The enzyme kinetics of potato 5-LOX and SLO-1 were also studied by the formation of the conjugated diene product at 234 nm (ε = 25000 M$^{-1}$ cm$^{-1}$) using the same experimental setup as for the IC$_{50}$ determination and the inhibitor screening. The substrate concentration was varied between 62.5 – 500 µM for SLO-1 kinetics analyses in absence or presence of fixed concentrations of inhibitor 23 (6
Anacardic acid derived salicylates are inhibitors or activators of lipoxygenase

µM, and 11 µM). The reaction velocities (v), which are the concentration change over time, were plotted against substrate concentration in Lineweaver-Burk plot (Figure 4) and the $K_m$ and $V_{max}$ and the apparent values ($K_m^{app}$ and $V_{max}^{app}$) in presence of the inhibitor were derived (Table 3).

The $V_{max}$ and $V_{max}^{app}$ were derived from the y-interception of Lineweaver-Burk plot for each inhibitor concentration, whereas $K_m$ and $K_m^{app}$ from the x-interception. $\alpha'$ which is the change in the $V_{max}$, was calculated from the $V_{max}^{app}$ divided by $V_{max}$. The change in the affinity of substrate binding represented by $\alpha/\alpha'$ was calculated from the $K_m^{app}$ divided by $K_m$. Furthermore, the $K_i$ and $K_i'$ were derived using equation 1.3 by substituting the $\alpha$ and $\alpha'$ values for each inhibitor concentration. The reported $K_i$ and $K_i'$ values are the averages of two inhibitor concentration (6 µM, and 11 µM).

Kinetic analyses of potato 5-LOX activation were performed in absence or presence of fixed concentrations of activator 21 (12.5 µM; 25 µM; 50 µM) and various concentration of substrate, ranging from 25-200 µM. Lineweaver-Burk plots (Figure 7A) were used to determine $K_m$ and $V_{max}$ and the apparent values ($K_m^{app}$ and $V_{max}^{app}$) in presence of the inhibitor were derived (Table 4). The slopes and y-intercepts were derived and re-plotted as $1/\Delta$slope or $1/\Delta$y-intercept versus $1/[activator]$ (Figure 7B). From these plots $\alpha$, $\beta$ and $K_A$ values were derived as described by Leskovac for non-essential activation. According to this method in the re-plot of $1/\Delta$y-intercept versus $1/[activator]$ the y intercept correspond to $\beta \times V_{max}/(\beta-1)$, whereas in the re-plot of $1/\Delta$slope versus $1/[activator]$ the y intercept correspond to $\beta \times V_{max}/K_m(\beta-\alpha)$. The x intersection point of two lines from the plot of $1/\Delta$intercept and $1/\Delta$slope is $-\beta/\alpha K_A$, which can be employed to derive the $K_A$ value if $\alpha$ and $\beta$ are known. All the experiments were performed in triplicate and the average triplicate values and their standard deviations are plotted. Calculations were performed with Excel 2010.

**Concentration-Dependent potato 5-LOX Activity**

Concentration-dependent potato 5-LOX activation profile was determined by comparing the enzyme activity in the presence of various concentration of activator. The experiment was performed using the same assay setup as for the inhibitor screening of potato 5-LOX. The potato 5-LOX enzyme was diluted 1:2000 with phosphate buffer (NaH$_2$PO$_4$-Na$_2$HPO$_4$ 0.1 M) pH 6.3. Various concentrations of activator, ranged from 12.5–200 µM, were used in the assay. The enzyme (1.0 mL) was pre-incubated with the activator (1.0 mL) for 10 minutes before the diluted substrate (10 µL, 20 mM) was added. The formation of HPETE was measured by the UV absorption at 234 nm. The experiments were performed in triplicate and the calculations were performed with Excel 2010.

**Critical Micelle Concentration (CMC) Determination**

The Critical Micelle Concentration (CMC) value is the upper limit of concentration range in which the substrate system is still homogenous. The CMC values were determined by measuring surface tension of the solutions as a function of the concentrations (1-1000 µM). The CMC values for anacardic acid, inhibitor 23, and linoleic acid were determined in a buffer containing 0.2 M H$_3$BO$_3$ at
Anacardic acid derived salicylates are inhibitors or activators of lipoxygenase.

pH 9.0, whereas for activator 21, the CMC value was determined in 0.1 M phosphate buffer (NaH$_2$PO$_4$-Na$_2$HPO$_4$) at pH 6.3. The surface tension of the solutions was measured using Du Noüy ring method with Pt-Ir ring of diameter 0.8 cm and Krüss tensiometer. The CMC was defined as the intersection of two linear lines extrapolating the surface tension as function of logarithm of the compounds concentration (Figure 9). The experiments were performed in triplicate and the calculations were performed with Excel 2010. We found that under these experimental conditions, the CMCs are 163 µM for linoleic acid, 390 µM for anacardic acid, 208 µM for 21 and 436 µM for 23.

**Molecular Docking**

The crystal structure of soybean lipoxygenase-1 was downloaded from Protein Data Bank (code 1F8N). All small molecules were drawn using Chemaxon MarvinSketch (www.chemaxon.com) and exported as mol2 files. The enzyme and small molecules were prepared for docking (structure recognition and protonation) using SPORES (www.tcd.uni-konstanz.de/research/spores.php). Molecular docking simulations were performed using PLANTS v1.6. The detailed of preparation using SPORES and the docking using PLANTS was done following the software instruction. The Fe$^{2+}$ in the SLO-1 active site was set as a docking site center. Fifteen poses were generated for each compound. The docking results were analyzed using Molegro Virtual Docker (www.molegro.com). The fitness of a pose is evaluated in terms of intermolecular interaction energy between the ligand and the enzyme, and the intramolecular interaction energy of the enzyme, which is represent by MolDock score (Table 5).

**Table 5.** The docking scores for the 15 poses of 18 and 23 in the SLO-1 active site.

<table>
<thead>
<tr>
<th>MolDock Score*</th>
<th>Compound 18</th>
<th>Compound 23</th>
</tr>
</thead>
<tbody>
<tr>
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*Pose 1 is the pose in which the salicylate group occupies the the position shown in Figure 1 (A); Pose 2 is the pose in which the salicylate group flips to the position shown Figure 1(B); The MolDock score is a calculated total energy for each pose (in arbitrary energy units)

References


