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Activation and inhibition of leukotriene A_4 hydrolase aminopeptidase activity by diphenyl ether and derivatives

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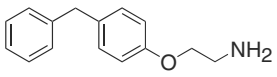
ABSTRACT

The synthesis and biological evaluation of a series of diphenyl ether derivatives were described. The compounds can either activate or inhibit the aminopeptidase activity of leukotriene A_4 hydrolase, while at the same time do not influence the hydrolase activity. Further enzyme kinetics and molecular modeling investigation on these novel chemical activators revealed their possible activation mechanism. These compounds can be used as probes to regulate the aminopeptidase activity of leukotriene A_4 hydrolase.

Leukotriene A_4 hydrolase (LTA4H) is a zinc-containing enzyme that converts leukotriene A_4 (LTA_4) into the proinflammatory substance leukotriene B_4 (LTB_4), which mainly functions as a chemoattractant and a stimulator of inflammatory cells.[1,2] LTA4H is believed to play an important role in inflammatory progress because the action of this enzyme is the rate limiting step in the biosynthesis of LTB_4 in the 5-lipoxygenase pathway.[3] Therefore, LTA4H has long been regarded as an anti-inflammatory target, and numerous inhibitors of LTA_4 hydrolase have been reported over the past decades.[4]

Besides epoxide hydrolase activity, LTA4H was also discovered possessing an aminopeptidase activity,[5,6] according to its high sequence homology to other zinc aminopeptidase. As an aminopeptidase, LTA4H can cleave amino acid from the N-terminus of various di- and tripeptide substrates.[7] It also hydrolyzes a number of chromogenic *p*-nitroanilide or β-naphthylamide derivatives of various amino acids, especially alanine and arginine. Interestingly, the aminopeptidase activity of LTA4H is activated by monovalent anions[8] and albumin.[9] However, organic compounds that can activate the aminopeptidase activity of LTA4H have not yet been found.



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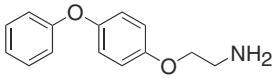

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The crystal structure of human LTA4H was solved in 2001,[10] which reveals that LTA_4 locates in a L-shaped pocket, and the two catalytic activities of LTA4H use specific but overlapping active sites. As a result, most of the reported inhibitors not only reduce the formation of LTB_4, but also act as antagonists for the aminopeptidase activity. Although these inhibitors have been characterized in great detail, compounds that specifically regulate the aminopeptidase activity of the enzyme have so far received much less attention. We are interested in developing chemical compounds that enable the regulation of the peptidase activity of LTA4H. The special structural features of LTA4H made the discovery of such modulator possible. The void region in the pocket of aminopeptidase activity can be used to bind other small molecules in addition to the peptide substrate. Such modulator will provide us novel insights into the mechanism of aminopeptidase activity of LTA4H.

Previous study of Penning et al. discovered a series of phenoxyethylamino analogs as potent LTA_4 hydrolase inhibitors.[11]

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Compound **1** was one of them with excellent potency in inhibiting LTB_4 production (IC_{50} = 9 nM). In the present study, the linker atom between two aryl rings of **1** was changed from carbon to oxygen for the convenience of synthesis, resulting compound **2**. We tested the biological activities of the starting and intermediate compounds in synthesis, and surprisingly found that 4-phenoxyphenol (**3**) can activate the aminopeptidase activity of LTA4H. Furthermore, several derivatives of **3** with longer hydrophobic carbon chains were synthesized, and the activities of these compounds, as well as diphenyl ether (**4**), which can be seen as a parent compound of **3**, were tested.

Our general synthetic approach to the title compounds is shown in Scheme 1. 4-phenoxyphenol (**3**) was used as starting material to synthesize compound **2**. With nucleophilic substitute reaction of excessive dibromoethane, **5** was obtained in high yields. Through sealed tube reaction method, compound **2** was obtained by mixing **5** and ammonia together. Preparations of compound **6a–6e** and **8a–8e** were started from **3** and **7**, respectively, with bromoalkane using nucleophilic substitute reaction.

Both the aminopeptidase activity and the epoxide hydrolase activity of the compounds were evaluated using recombinant human LTA4H. The human LTA4H gene (Invitrogen) was inserted into pET 21a plasmid, and then transformed into *Escherichia coli* for expression. The protein was purified by ammonium sulfate precipitation, anion-exchange chromatography and gel filtration. The aminopeptidase activity was determined spectrophotometrically by the release of the colorimetric product *p*-nitroanilide (*p*NA) from Ala-*p*NA. The epoxide hydrolase activity was determined using an RP-HPLC assay to quantify the amount of LTB_4.[12] Detailed procedure of expression, purification, and activity test of LTA4H were described in Supplementary material.

Compound **2** demonstrated good inhibitory potency against recombinant human LTA_4 hydrolase (IC_{50} = 35 ± 2 nM), as well as good activity for aminopeptidase (IC_{50} = 228 ± 14 nM). For other compounds, at concentration of 100 μM, **3**, **4**, **6a–6e** and **8a–8e** had no apparent influence on LTB_4 formation. Alternatively, we surprisingly found that these compounds took different effects on the aminopeptidase activity of LTA4H. As shown in Figure 1, the effects of **3**, **4**, **6a**, **6b**, **8a** and **8b** were all activation. **6a** exhibited the highest stimulation of the activity in all compounds by about 12-fold higher than LTA4H alone. However, when the length of the carbon chain was extended to three, the effect changed from activation to inhibition. The relative activity was no compound < **4** < **6a** > **6b** > **6c** > **6d** > **6e**, and no compound < **3** < **8a** > **8b** > **8c** > **8d** > **8e**.

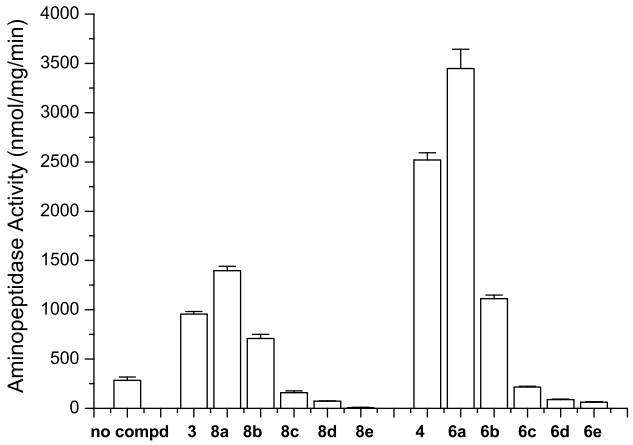


Figure 1. Effects of **3**, **4**, **6a–6e**, and **8a–8e** at 100 μM on the aminopeptidase activity of hydrolyzing Ala-*p*NA.

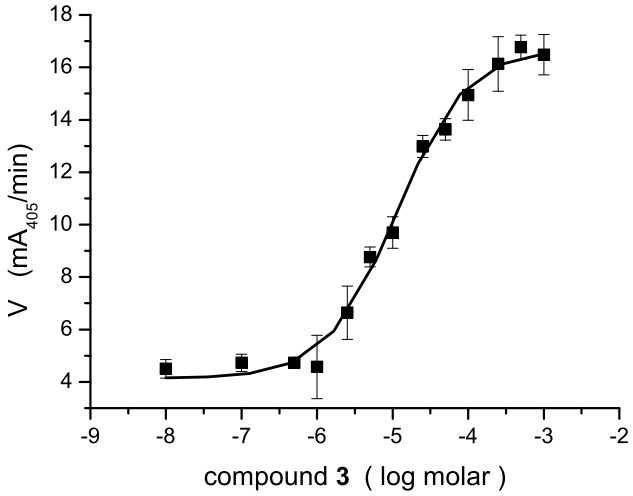
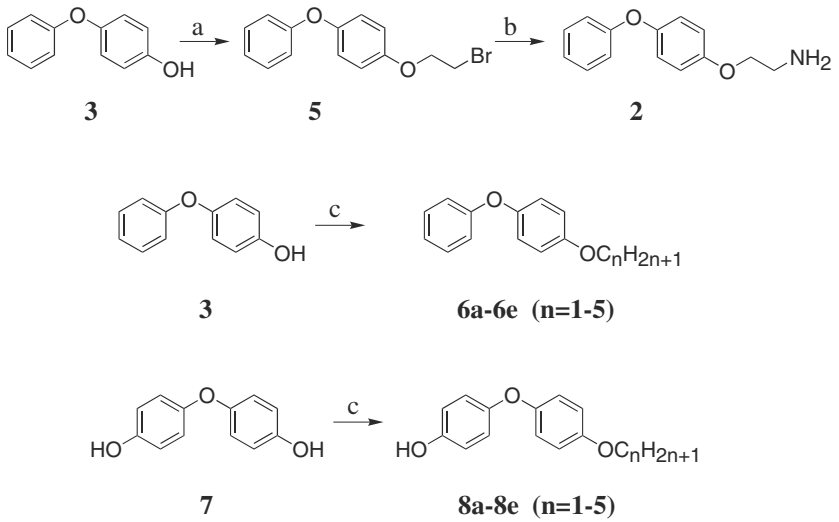
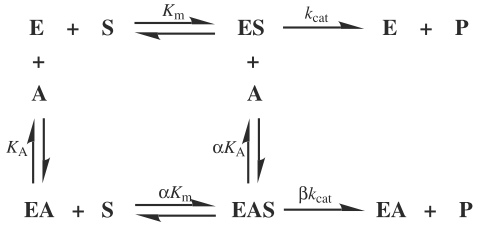


Figure 2. Activation of aminopeptidase activity as a function of the compound **3** concentration.

Since the activation of the aminopeptidase activity of LTA4H by chemical compounds has not been reported before, we investigated this process in detail by studying the activation of LTA4H



Scheme 1. Synthetic approach. Reagents: (a) $BrCH_2CH_2Br$, K_2CO_3; (b) $NH_3{\cdot}H_2O$, Δ; (c) $C_nH_{2n+1}Br$ (*n* = 1–5), KOH, DMF.



Scheme 2. Activation model of LTA4H by **3**.

by **3**. Dose–response measurements were performed, and the result showed that the activation of LTA4H by **3** was concentration-dependent, with an AC_{50} = 11.1 μM (Fig. 2).

Furthermore, kinetic parameters for activation effect were evaluated. As the aminopeptidase activity is always detectable, even in the absence of **3**, suggesting a nonessential, mixed-type mechanism. The kinetic mode of activation is described in Scheme 2, where A is the activator molecule and K_A is the activator dissociation constant. As shown in Figure 3, Lineweaver–Burk plots crossed at *X*-axis, so constant α equals to 1. Other kinetic parameters were estimated by non-linear fitting using the equation:

$$\left(\frac{K_m}{V_{max}}\right)_{app} = \left(\frac{K_m}{V_{max}}\right)_0 \times \left(\frac{K_A + [A]}{K_A + \beta[A]}\right),$$

where K_m is the Michaelis constant, V_{max} is the maximum reaction velocity, and the subscript 'app' represents apparent values with compound, while the subscript '0' represents constants without compound. The values were calculated, that is K_A = 1.8 ± 0.3 μM, β = 4.5 ± 0.4, as well as α = 1, indicating that the reaction predominantly proceeded via the enzyme–compound–substrate complex which had a considerably higher turnover number, whereas the binding ability of LTA4H and Ala-*p*NA remained constant.

Table 1 gives the constants of AC_{50} or IC_{50} of these compounds. There is a clear tendency that both IC_{50} and AC_{50} values decrease along with the elongation of the carbon chain length. In addition, compounds with hydroxyl substituent showed higher AC_{50} values than that without it, for example, **3** > **4**, **8a** > **6a** and **8b** > **6b**. This is probably due to the hydrophobic environment of the enzyme pocket did not match to the additional hydroxyl group.

It should be mentioned that **6c** is an exception. Its effect on LTA4H depends on the substrate concentration. When the Ala-*p*NA concentration is higher than 0.65 mM, **6c** activates the aminopeptidase activity, however, when the concentration is less than 0.65 mM, **6c** turns into inhibitor. Lineweaver–Burk plots of **6c** un-

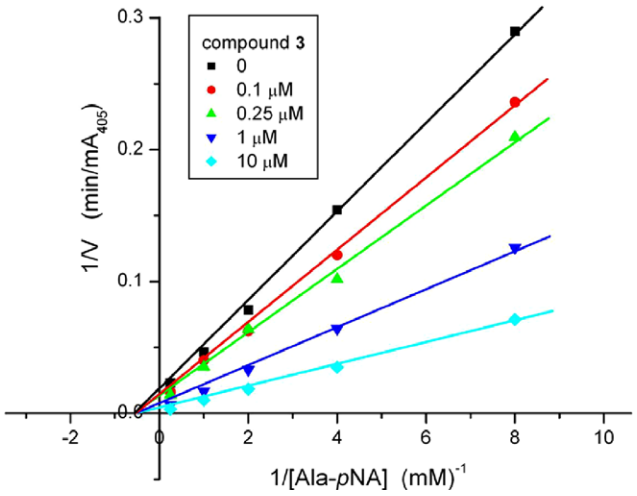


Figure 3. Lineweaver–Burk plots for LTA4H catalyzed Ala-*p*NA as a function of concentration of compound **3**.

Table 1
AC_{50} or IC_{50} values of aminopeptidase activity by **3**, **4**, **6a**–**6e**, and **8a**–**8e**

Compound	AC_{50} (μM)[a]	IC_{50} (μM)[a]
3	11.1 (±1.6)	–[b]
4	6.01 (±0.47)	–
6a	3.39 (±0.30)	–
6b	2.82 (±0.17)	–
6c	–	–
6d	–	5.72 (±0.23)
6e	–	4.13 (±0.36)
8a	17.0 (±2.5)	–
8b	14.8 (±2.1)	–
8c	–	5.69 (±0.62)
8d	–	5.56 (±0.29)
8e	–	2.48 (±0.08)

[a] Average of three experiments, standard deviation is given in parentheses.
[b] Not available.

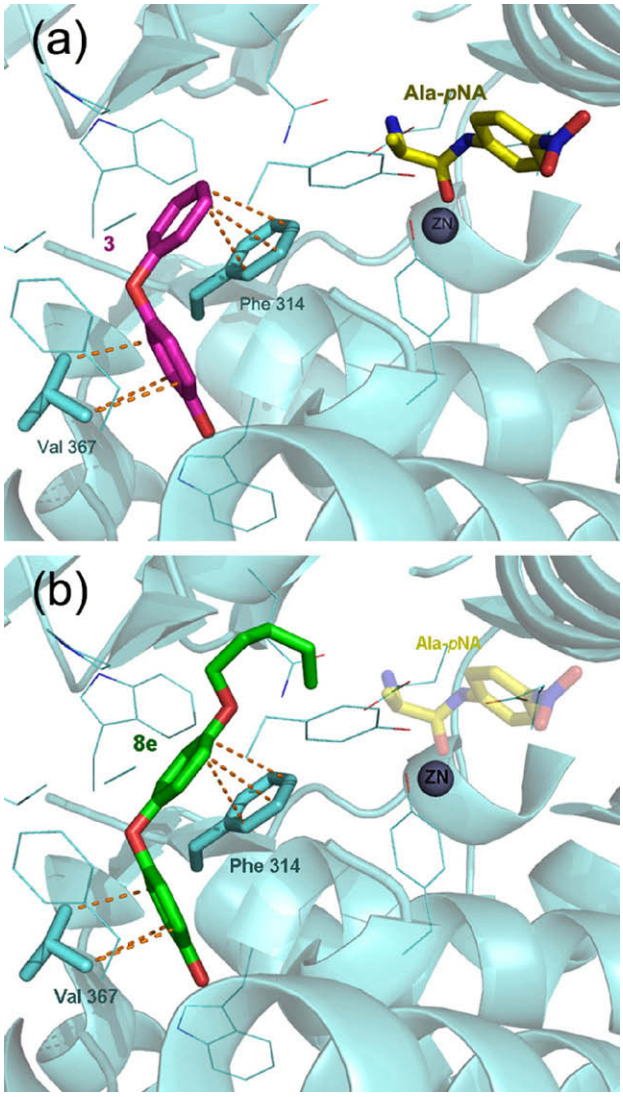


Figure 4. (a) Model of **3** and Ala-*p*NA binding at the active site of LTA4H. (b) Model of **8e** binding with LTA4H; Ala-*p*NA is shown in light yellow only for indicating the substrate binding position, and it cannot bind with LTA4H with **8e** at the same time. Hydrophobic clusters are shown in orange dashes.

der various concentrations crossed at the first quadrant, and the apparent K_m of LTA4H with **6c** was larger than that without **6c** (Fig. S1 in Supplementary material). According to Scheme 2, we deduced that the kinetic parameters of **6c** is $\alpha > \beta > 1$. So **6c** is a compound which has dual tendency of both activation and inhibition.

In addition to experimental study, docking simulations were performed to obtain the most possible model of the enzyme–compound–substrate complex. Ala-*p*NA and **3** were separately docked into 1HS6[10] using Autodock 3.05[13], and the docking results are shown together in Figure 4a. The docking simulations showed that both Ala-*p*NA and **3** locate in the pocket of LTA4H. Ala-*p*NA locates in the substrate binding site of aminopeptidase activity, while compound **3** holds the hydrophobic part of the pocket without extending to the aminopeptidase substrate binding part, and thus has no direct contact with Ala-*p*NA. The hydrophobic interaction between phenyl groups of **3** and Phe-314, Val-367 of LTA4H contribute most to the binding. The orientations of **3** and Ala-*p*NA in LTA4H is reasonable to form the enzyme–compound–substrate complex (**'EAS'** in Scheme 2), which shows high catalytic turn over number. Moreover, as most of the known monofunctional aminopeptidase do not have large void in their substrate binding pocket as LTA4H does, the complex of LTA4H binding with **3** might mimic the structure features of these normal aminopeptidases to some extent. So we concluded that **3** locates at a proper position for activating the aminopeptidase activity when Ala-*p*NA is substrate.

We also analyzed the binding modes of **4**, **6a**–**6e**, **8a**–**8e** with LTA4H. They bind at the same position as **3** does. The carbon chains of **6a**–**6e** and **8a**–**8e** extend into the pocket towards aminopeptidase substrate binding part depending on the number of carbons. For example, Figure 4b shows the model of **8e** binding with LTA4H. This compound partly occupies the peptide binding site, and the peptide cleaving activity is inhibited. It needs to be clarified that the substrate Ala-*p*NA and **8e** cannot bind with LTA4H at the same time, and Ala-*p*NA shown in Figure 4b is just to give us an impression where the substrate binding pocket is. Whether the effect on aminopeptidase activity is activation or inhibition depends on the relative position of compounds against Ala-*p*NA. The experimental AC_{50} or IC_{50} values correlate well with the number of carbons chains, verifying that the modeled complex structures are reasonable.

In summary, a series of diphenyl ether derivatives were designed and synthesized based on a known LTA4H inhibitor analogue. The compounds showed regulation capability of the aminopeptidase activity of LTA4H, either activation or inhibition. The activation mechanism was further investigated by kinetic studies and molecular modeling. Compound **3** was found to bind in the narrow hydrophobic part of the LTA_4 binding pocket, and enhance the peptidase cleaving activity by increasing the turnover number. The discovery of specific LTA4H aminopeptidase activators and inhibitors can modulate the peptidase activity in two directions, either by activating or by blocking the target activity. Such regulations give us more information than inhibitors only. As the physiological function of the peptidase activity of LTA4H is unclear, these modulators may provide us novel tools to probe the possible roles of LTA4H as aminopeptidase in vivo.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.044.

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