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PROBING THE INHIBITION OF LEUKOTRIENE A_4 HYDROLASE BASED ON ITS AMINOPEPTIDASE ACTIVITY

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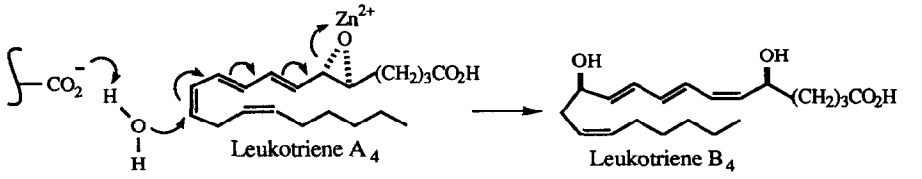
Abstract: Several inhibitors of LTA_4 hydrolase from human leukocytes were prepared to probe the active site of the enzyme.

Leukotriene (LT) A_4 hydrolase catalyzes the hydrolysis of the arachidonic acid-derived allylic epoxide LTA_4 (5(S)-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) to produce the dihydroxy fatty acid leukotriene(LT) B_4 (5S,12R-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid) (eq 1),



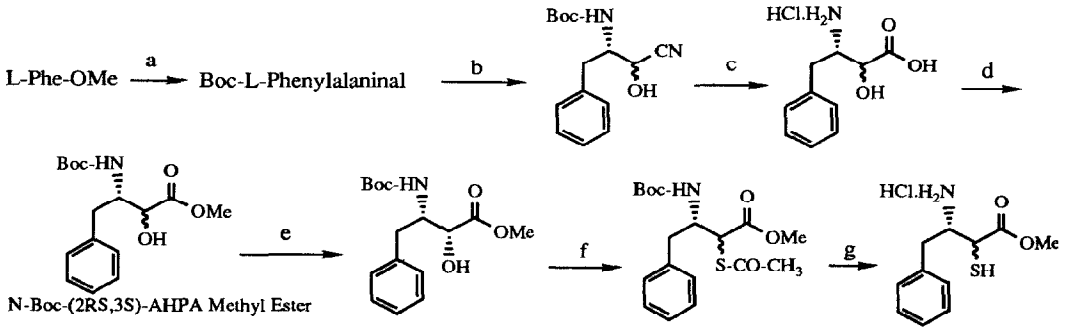
(1)

one of the physiologically important terminal products in the arachidonic acid biosynthetic pathway.[1,2] The enzyme has been purified to homogeneity from various sources as a water-soluble, monomeric protein with a molecular weight of about 70000.[3] The genes coding for the human enzyme from placenta and spleen have been cloned and sequenced.[4] The mechanism of LTA_4 hydrolase has not been well documented, but the recent studies have shown the similarity between this enzyme and some zinc metalloenzymes as the LTA_4 hydrolase contains one zinc ion[5,6] essential for the activity, and also exhibits aminopeptidase activity.[6] Further studies suggest that the peptidase and epoxide hydrolase activities of this enzyme occur at the same active site.[6]

It is of great interest to develop inhibitors of LTA_4 hydrolase as potential antiinflammatory agents since LTB_4 is a proinflammatory mediator which stimulates adhesion of circulating neutrophils to vascular endothelium and directs their migration toward sites of inflammation. LTA_4 hydrolase is irreversibly inhibited by its substrate LTA_4[7,8] and substrate analogues, e.g. LTA_3 and LTA_5.[8-10] Bestatin, a naturally occurring, D-phenylalanine-derived norstatine-type aminopeptidase dipeptide inhibitor,[11] was reported[12] as a reversible inhibitor of LTA_4 hydrolase. It showed inhibition for both aminopeptidase and epoxide hydrolase activities of this enzyme. Among other inhibitors tested, only captopril had the same inhibitory potency as bestatin.[12]

The inhibition activity of bestatin against LTA_4 hydrolase prompted us to study the other L- and D-phenylalanine-derived norstatine-type of compounds as inhibitors. We speculate that the peptidase activity of LTA_4 hydrolase is like that of Zn^{++}-containing thermolysin mechanically. Norstatine type of peptide isosteres, therefore, should be good inhibitors. The inhibition of captopril may be due to a strong interaction between the mercapto group and the zinc ion at the enzyme's active site. We envisage that better inhibitors may be designed if this binding/coordination interaction can be optimized.

A representative synthesis of N-t-Butyloxycarbonyl-(2RS,3S)-3-amino-2-hydroxy-phenylbutanoic acid (N-BOC-(2RS,3S)-AHPA) methyl ester is shown in Scheme I. (2RS,3S)-



Scheme I . a, 1) $(Boc)_2O$, 2) DIBAL-H (2.5 eq.), - 77 °C. b, KCN, $NaHSO_3$. c, 25% HCl, 80 °C. (ref. 13a). d, 1) $(Boc)_2O$, 2) MeI, $KHCO_3$, 55% overall yield. e, Silica G column. f, 1) MsCl, 2) Potassium thioacetate, DMF, RT, 60%. g, 1) LiOH (1eq.), MeOH: THF (5:1), 2) HCl (gas), Ether, 50%.

AHPA was converted[14] to N-BOC-(2RS,3S)-AHPA methyl ester and separated on silica gel to obtain the two diastereomers N-BOC-(2R,3S)-AHPA and N-BOC-(2S,3S)-AHPA methyl esters. The

stereochemistry at the 2-position was assigned by comparison of (2S,3R)-AHPA isopropyl ester to the published data.[13a,b] Syntheses of thiol compounds **12** and **13** were also outlined in the scheme. Since racemization of 2-position took place easily, they were made as a mixture of two diastereomers.

The DL-phenylalanine phosphonate analogue **14** was prepared to be tested as transition-state analog inhibitor.[15] The difluoroketone compound **15**, another type of transition-state analog inhibitor of proteases,[16] was prepared by first coupling cinnemaldehyde with ethyl difluorobromoacetate[17] followed by oxidation[18] to yield the final product.

Several amino acid amides were tested as substrates for LTA_4 hydrolase. It was found that only L-enantiomers were substrates, and L-alanine *p*-nitroanilide was the best (Table 1). D-Alanine *p*-nitroanilide was not acceptable. The synthetic compounds were then tested as inhibitors of LTA_4

Table 1. Comparison of kinetic parameters for LTA_4 hydrolase-catalyzed hydrolysis of LTA_4 and amide substrates.

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	k_{cat}/K_m ($M^{-1}s^{-1}$)
LTA_4	7.6	572	0.9×10^5
L-Lysine *p*-nitroanilide	100	30	3.5×10^2
L-Ala *p*-nitroanilide	500	530	1.2×10^3
D-Ala *p*-nitroanilide	---	0	---
L-Arg *p*-nitroanilide	200	140	7.5×10^2
L-Pro *p*-nitroanilide	100	130	1.5×10^3
L-Leu *p*-nitroanilide	300	130	5×10^2

Determined in 50 mM Tris-Cl, pH 7.6, in the presence of 0.1 M NaCl. $\varepsilon_{410\ nm}$ for *p*-nitroaniline = 8850 $M^{-1}cm^{-1}$. For LTA_4 substrate analysis see ref 12.

hydrolase from human leukocytes[5,6] with L-alanine *p*-nitroanilide as substrate (Table 2). Study of the four AHPA methyl ester stereoisomers **1**, **2**, **3** and **4** revealed that the configurations at both 2- and 3- positions are important for inhibition activity, and the isomer (2S,3S)-AHPA methyl ester **2** was the most potent with an inhibition constant $K_i = 50$ μM. The other three isomers, (2R,3S), (2S,3R) and (2R,3R)-AHPA methyl esters, showed poor or no inhibition. A free amino group is necessary as N-Boc-(2S,3S)-AHPA shows no activity. Hydrolysis of the methyl esters to free acids

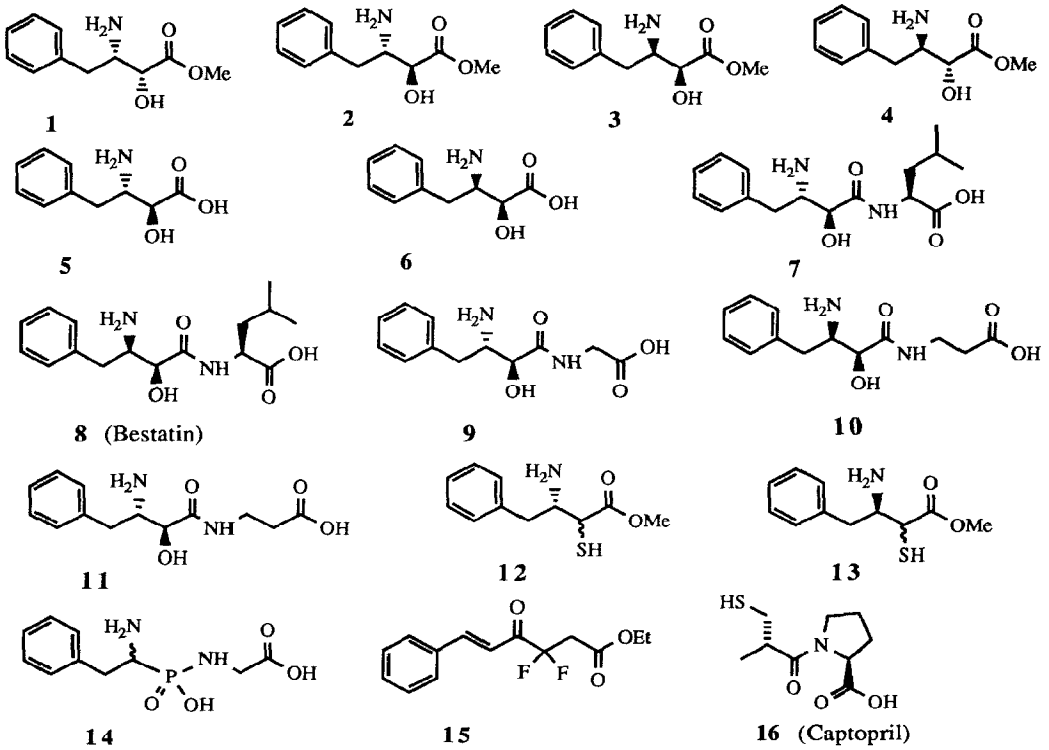


Table 2. Inhibition Constants of the Listed Compounds for LTA_4 hydrolase[a]

Compd.	K_i	Compd.	K_i
1	$IC_{50} > 0.5$mM[b]	**9**	15 μM
2	50 μM	**10**	$IC_{50} \approx 100$ μM
3	NI[c]	**11**	$IC_{50} \approx 80$ μM
4	NI	**12**	$IC_{50} \approx 250$ μM
5	$IC_{50} > 1$ mM	**13**	$IC_{50} > 250$ μM
6	NI	**14**	$IC_{50} \approx 200$ μM
7	$IC_{50} \approx 20$ μM	**15**	$IC_{50} > 5$ mM
8	$IC_{50} = 4$ μM (0.2 μM[d])	**16**	$IC_{50} = 0.07$ μM[d]

[a] The enzymatic assays were performed in Tris-HCl buffer (0.05 M, PH 8.0) with L-alaninyl *p*-nitroanilide (1.5 mM) as substrate. 1.5 μg of the enzyme purified from human leukocytes was added to each assay (final volume = 1.0 mL). Dixon plots were used to determine the K_i values. IC_{50} = The concentration for half maximal inhibition.

[b] Less than 50% inhibition was observed at the specific concentration.

[c] NI, no inhibition observed with 1 mM inhibitor in the assay.

[d] Vs L-lysine *p*-nitroanilide substrate, reference 12.

(see compounds **5** and **6**) lost their inhibition potency. The inhibition activity was improved in the case where the C-terminal of (2S,3S)-AHPA was coupled to L-leucine or glycine (**7** or **9**), but became worse when coupled to β-alanine (see compound **11**). It is interesting to note that though (2S,3R)-AHPA methyl ester **3** shows no inhibition to this enzyme, its amide derivative of L-leucine (bestatin) is a more potent inhibitor than the amide derivative of **2**. Coupling of **2** with D-leucine did not improve the potency. Surprisingly, the two thiol compounds **12** and **13** are poor inhibitors, so are the transition-state analogues phosphonate **14** and difluoroketone **15**. Work is in progress to develop better inhibitors based on the stereochemistry of **2**. We believe the hydroxy group occupies the position of the water molecule involved in the hydrolysis of the peptide bond assisted by a general base (perhaps $-CO_2^-$), and the Zn^{++} ion is coordinated to the amino and the carbonyl groups. Perhaps better inhibitors can be developed with the introduction of appropriate groups (R_1,R_2) to the two stereogenic centers of **2** so that a better binding and coordination would result in a strong inhibition (Figure 1).

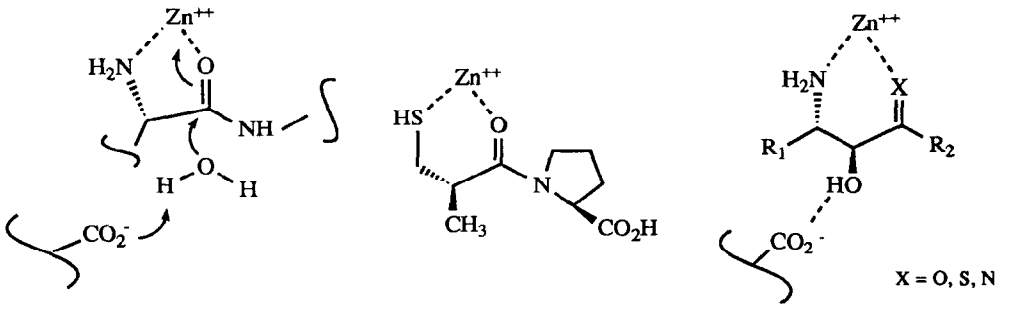


Figure 1 Mechanism (left), captopril complex (center), and possible new inhibitors of LTA $_4$ hydrolase (right)

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