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KELATORPHAN AND RELATED ANALOGS: POTENT AND SELECTIVE INHIBITORS OF LEUKOTRIENE A4 HYDROLASE

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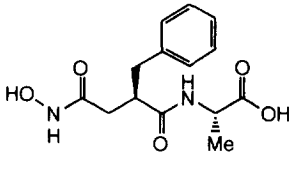
Abstract: The hydroxamic acid-containing peptide kelatorphan, a known inhibitor of enkephalin-degrading enzymes, is a potent, non-competitive inhibitor of leukotriene A4 (LTA4) hydrolase. Analogs of kelatorphan were prepared and several significantly and selectively inhibited both the hydrolase and aminopeptidase activity of the enzyme.

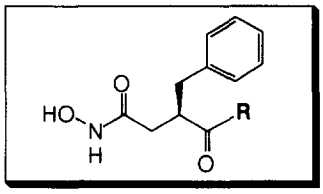
Leukotriene A4 (LTA4) hydrolase is the enzyme which catalyzes the hydrolysis of the epoxide-containing LTA4 to the potent pro-inflammatory diol, Leukotriene B4 (LTB4). Cloning of the 69 kD protein revealed substantial sequence homology between LTA_4 hydrolase and other zinc-containing aminopeptidases such as aminopeptidase N and thermolysin.[1] Subsequently, LTA_4 hydrolase was shown to contain one mole of zinc ion per mole of enzyme[2] and exhibit aminopeptidase activity in addition to its epoxide hydrolase activity.[3] Some known inhibitors of zinc-containing aminopeptidases are competitive, reversible inhibitors of both the aminopeptidase and hydrolase activity of LTA4 hydrolase, including bestatin[4] and the angiotensin-converting enzyme (ACE) inhibitor captopril.[5] A series of competitive, zinc-coordinating LTA4 hydrolase inhibitors has recently been described by Wong and Samuelsson[6] and several compounds were relatively potent inhibitors of both activities of the enzyme. Several amino acid hydroxamates, di- and tri-peptides[7] and opioid peptides such as enkephalins and dynorphins[8] also inhibit the peptidase activity of LTA4 hydrolase. As an adjunct to our LTB4 receptor antagonist program, we

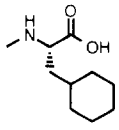


Kelatorphan (**1**)

were interested in examining inhibitors of LTA4 hydrolase for their potential utility as novel anti-inflammatory agents. The homology between the zinc-containing metalloproteases such as enkephalinase and LTA4 hydrolase prompted us to initiate a study of the LTA4 hydrolase inhibitory activity of the known, potent enkephalinase (NEP, neutral endopeptidase EC 3.4.24.11) inhibitor, kelatorphan[9] and several related dipeptides.

Table 1.

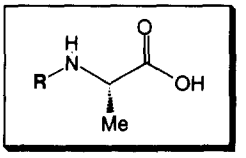


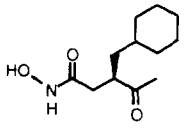
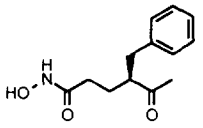
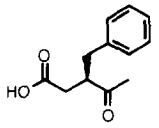
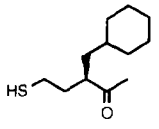
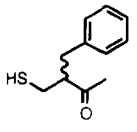
Cmpd	R	LTA4 Hydrolase IC_{50} (μM)	Aminopeptidase IC_{50} (μM)	NEP IC_{50} (μM)	ACE IC_{50} (μM)
Kelatorphan (**1**)	L-Ala	0.005	0.007	0.046	>10[a]
3	L-Pro	0.03	0.018	>36	3
4	D-Pro	20	15		>100
5	L-Met	0.012	0.002	61	20
6	L-Trp	0.01	0.005		
7	N-Me-L-Ala	0.003	0.001	3.4	>100
8		0.01	0.042	104	>100
9		0.008	0.01	17	40
10		1.8	2.3		>100

a) Literature[9] value. **Biochemical Methods** (Tables 1 and 2): **LTA_4 hydrolase:** Assays were performed as previously described[11] using purified recombinant human LTA_4 hydrolase. For IC_{50} determinations, LTB_4 was quantified using a commercially available ELISA assay (Caymen Chemicals). **Aminopeptidase:** Peptidase activity was monitored as previously described[11] using 1 mM leucine-p-nitroanilide as substrate and the formation of p-nitroaniline monitored spectrophotometrically at 405 nm over 15 min. **NEP:** Rabbit kidney NEP was assayed as previously described[12] using an HPLC assay which utilized ANP (99-126) as substrate. **ACE:** Rabbit lung ACE (Sigma) was assayed as previously described,[13] utilizing 2.5 mM N-(3-[2-furyl]acryloyl)-Phe-Phe-Gly-Gly as substrate and monitoring the decrease in absorbance at 340 nm over 10 min.

We have found that kelatorphan is a very potent inhibitor of both the epoxide hydrolase and peptidase activity of LTA4 hydrolase. Several hydroxamic acid-containing analogs of kelatorphan were prepared and activity against the enzyme assessed. As shown in Table 1, compounds in which **R** is an L-amino acid were potent inhibitors of both activities of the enzyme, while the two D-amino acid analogs, **4** and **10**, were only weak inhibitors. However, this site was accommodating to L-amino acids of varying degrees of steric bulk - from alanine to tryptophan and pipecolic acid. The compounds in Table 1 also demonstrated considerable selectivity against other metalloproteases such as NEP and ACE, whereas kelatorphan itself was a very potent inhibitor of NEP.

Table 2.



Cmpd	R	LTA4 Hydrolase IC_{50} (μM)	Aminopeptidase IC_{50} (μM)	NEP IC_{50} (μM)	ACE IC_{50} (μM)
11		0.005	0.01	2	>100
16		>10			20
17		0.3	0.14		>100
20		>3		>100	
23		>3	>10	0.0079	0.2
Captopril		10	2		

We also prepared a series of compounds in which the L-alanine portion of kelatorphan was maintained and modifications were carried out on the hydroxamate-containing portion of the molecule (Table 2). The cyclohexyl analog of kelatorphan (**11**) exhibited comparable activity against LTA4 hydrolase, while a chain extended analog **16**, was inactive at 10 μM. Replacement of the hydroxamic acid moiety with other potential zinc-binding groups, resulted in compounds with significantly diminished activity. Acid **17** was 60 times less potent than kelatorphan, while thiols **20** and **23** were inactive at 3 μM. Thiol **23**, however, was a potent inhibitor of NEP and ACE. The related thiol, captopril, a known potent inhibitor of ACE, was a weak inhibitor of both activities of LTA4 hydrolase. Although many of the compounds outlined in Tables 1 and 2 were potent inhibitors of LTA4 hydrolase, they all were relatively poor inhibitors of LTB4 biosynthesis in whole cells, specifically, HL-60s,[10]

with IC_{50}s ranging from 8 μM to >100 μM. Future synthetic efforts will focus on improving the ability of these analogs to penetrate whole cells via modifications such as peptide isosteres and conformational restriction.

The mechanism of inhibition of LTA_4 hydrolase by kelatorphan was also examined. Kelatorphan was found to be a reversible, non-competitive inhibitor using both the epoxide hydrolase assay (K_i = 18 nM) (Figure 1) and the aminopeptidase assay (K_i = 10 nM) (data not shown). These results are in contrast to the reported mechanism of inhibition by captopril, which demonstrated either a competitive or a mixed mechanism of inhibition under varying conditions.[5] Competitive inhibition was reported for the zinc-coordinating inhibitors synthesized by Wong.[6] These results suggest that kelatorphan may be binding in a different manner than the other zinc-coordinating inhibitors.

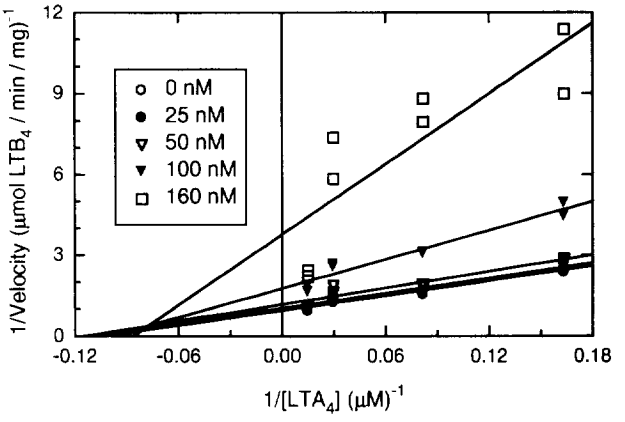
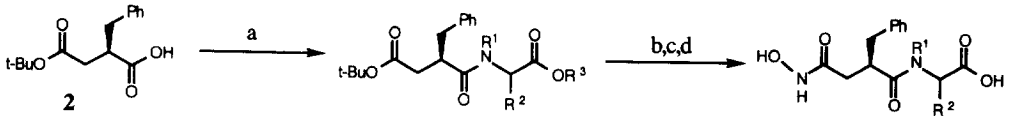


Figure 1. Inhibition of LTA_4 hydrolase activity by kelatorphan in the epoxide hydrolase assay. 1 μg enzyme was incubated with the indicated concentrations of kelatorphan in a final volume of 100 μL 50 mM pH 8 HEPES buffer, containing 1 mg/mL of fatty acid free BSA. The reaction was started by addition of varying concentrations of LTA_4 (7 - 70 μM) in ethanol, and quenched after 1 min as previously described.[11] LTB_4 was quantified in reference to PGB_1 using an HPLC assay.[11] Data was analyzed by the method of Dixon[14] to yield a K_i of 18 nM.

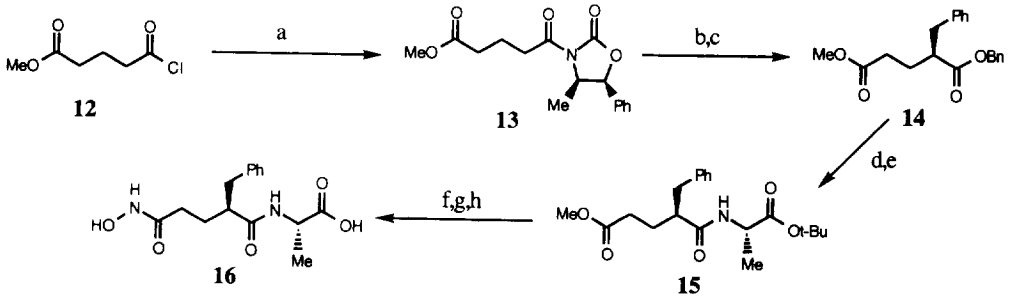
The compounds in Table 1 were all synthesized using the procedure recently described for the synthesis of kelatorphan[15] and outlined generically in Scheme 1. Acid **2** was synthesized in 3 steps from 3-phenylpropionic acid and was coupled to a variety of amino esters under standard conditions. Selective de-esterification, coupling of hydroxylamine and saponification provided hydroxamic acids **3-10**. Scheme 2 outlines the synthesis of **16**. Using Evans' oxazolidinone chemistry, acid chloride **12** was converted to diester **14** and further elaborated to provide hydroxamic acid **16**. Diacid **17** was prepared by simply eliminating the hydroxylamine coupling step of Scheme 1. Thiol **20** was synthesized as shown in Scheme 3. Acid **18** was converted to **19**, followed by reduction of the acid with diborane, Mitsunobu coupling with thioacetic acid and saponification to provide **20.** Hydroxamic acid **11** was also synthesized from acid **19**. Thiol **23** was synthesized as outlined in Scheme 4. Addition of thioacetic acid to acrylic acid **21**, followed by coupling of alanine t-butyl ester and de-esterification provided thiol **23**, which was tested as a 1:1 diastereomeric mixture.

Scheme 1.



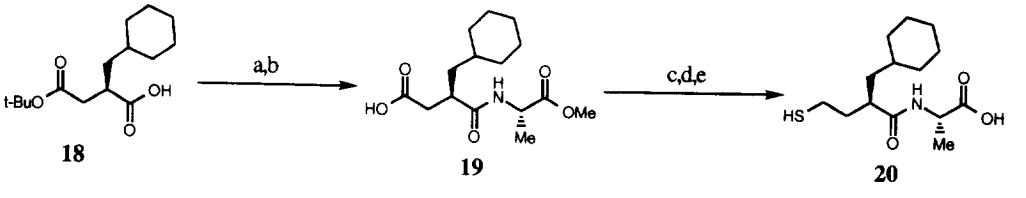
(a) amino ester, EDC, HOBt, NMM, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) $ClCO_2i$-Bu, NH_2OH•HCl, NMM, THF; (d) LiOH

Scheme 2.



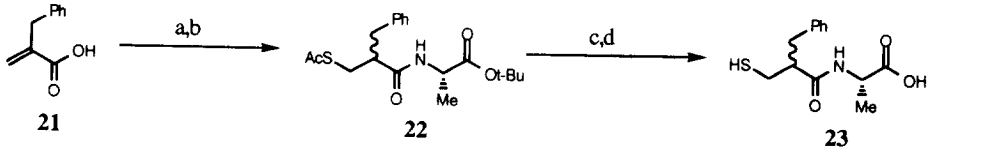
(a) (4R,5S)-(+)-4-methyl-5-phenyl-2-oxazolidinone/BuLi; (b) NaHMDS, BnBr; (c) BnOLi, THF; (d) H_2, Pd/C; (e) L-Ala-Ot-Bu•HCl, EDC, HOBt, NMM; (f) LiOH; (g) $ClCO_2i$-Bu, NH_2OH•HCl, NMM, THF; (h) TFA, CH_2Cl_2

Scheme 3.



(a) L-Ala-OMe, EDC, HOBt, NMM, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) BH_3•THF; (d) DIAD, Ph_3P, AcSH; (e) LiOH

Scheme 4.



(a) AcSH, 80°C; (b) L-Ala-Ot-Bu, EDC, HOBt, NMM, CH_2Cl_2; (c) TFA, CH_2Cl_2; (d) NaOMe, MeOH

In summary, we have found that several hydroxamic acid-containing dipeptides are potent and selective inhibitors of both the hydrolase and peptidase activity of LTA4 hydrolase. Furthermore, we have demonstrated that, in contrast to other zinc-chelating inhibitors, kelatorphan is a non-competitive inhibitor of both activities of the enzyme. Efforts are currently underway to develop analogs which are able to penetrate cells and which will ultimately demonstrate oral activity as potential anti-inflammatory agents.

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