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Stereochemical Structure Activity Relationship Studies (S-SAR) of Tetrahydrolipstatin

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Supporting Information Placeholder

ABSTRACT: Tetrahydrolipstatin (THL), its enantiomer, and an additional six diastereomers were evaluated as inhibitors of the hydrolysis of *p*-nitrophenyl butyrate by porcine pancreatic lipase. IC_{50}s were found for all eight stereoisomers ranging from a low of 4.0 nM for THL to a high of 930 nM for the diastereomer with the inverted stereocenters at the 2,3,2'-positions. While the enantiomer of THL was also significantly less active (77 nM) the remaining five stereoisomers retained significant inhibitory activity (IC_{50}s = 8.0 to 20 nM). All eight compounds were also evaluated against three human cancer cell lines (human breast cancers MCF-7 and MDA-MB-231, human large-cell lung carcinoma H460). No appreciable cytotoxicity was observed for THL and its seven diastereomers, as their IC_{50}s in a MTT cytotoxicity assay were all greater than three orders of magnitude of campothecin.

KEYWORDS: *tetrahydrolipstatin, pancreatic lipase, diastereomers, inhibitory activity, cytotoxicity, structure activity relationship*

Tetrahydrolipstatin (THL, **1**)[1,2] is an FDA approved oral anti-obesity drug that inhibits pancreatic lipase digestive enzyme.[3,4,5] Inhibition of pancreatic lipase in the intestinal lumen results in the inability to digest dietary triacylglycerides (fat) and diacylglycerides to free fatty acids and monoacylglycerides that can be absorbed into the bloodstream and lymphatic system. Dietary fat and diacylglycerides are then eliminated as bodily waste without the caloric consequences of their taste and consumption. As part of an effort aimed at developing a novel treatment for pancreatitis, we became interested in structure activity space around (THL) **1**. Pancreatitis is believed to result from the excessive release of active pancreatic digestive enzymes.[6] As THL (**1**) is known to inhibit pancreatic lipase by covalent modification, it was identified as lead structure in our program aimed at capturing and removing excess pancreatic lipase.

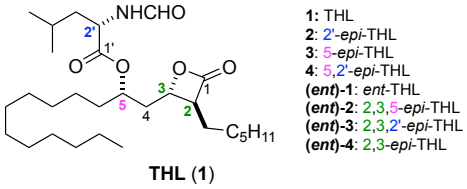
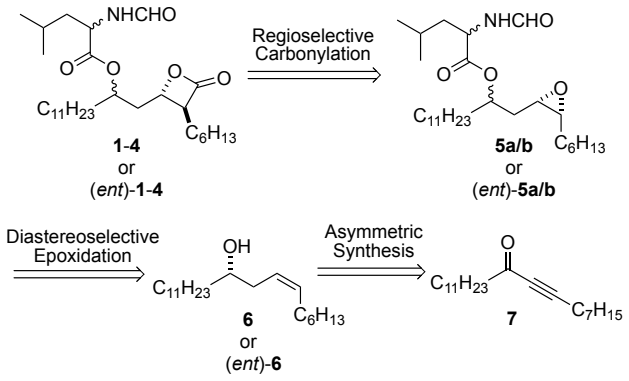


Figure 1. Structures of THL (**1**) and seven diastereomers

THL (**1**) is known to be a potent inhibitor of porcine pancreatic lipase hydrolysis of triolein (fat) emulsion (IC_{50} of 360 nM).[1] The mechanism of activity for THL (1) has been shown to occur via ring opening of the β-lactone by a lipase active site serine to form a stable ester.[7,8,9] When the hydrolysis of the esterified serine is slow, this covalent modification of the lipase enzyme leads to essentially irreversible inactivation.

Previously, THL has been investigated *in vivo* for the treatment of pancreatitis by intravenous,[10] intraperitoneal,[11,12,13] and direct injection into pancreatic tissue.[9,11,12,13,14] In addition, THL has been found to inhibit the thioesterase domain of fatty acid synthase (FAS),[9,15,16] and has been investigated as anticancer chemotherapy.[15,16,17,18,19,20,21,22,23,24] THL has been shown to inhibit the *in vitro* growth of Giardia duodenalis, a gastrointestinal parasite.[25] THL may also have value as an antifungal agent against fungi that lack lipase enzymes and rely on free fatty acids for their nutrition and growth.[26,27]

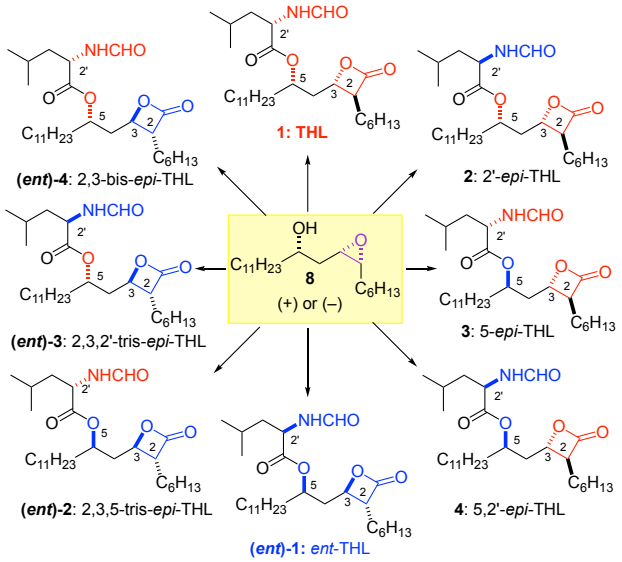


Scheme 1. Retrosynthetic Analysis for THL

In an effort to find novel structures that covalently modified pancreatic lipase, we decided to explore the structural space around THL (**1**). While there had been some structure activity relationship studies (SAR) of the lipstatins, there have been fewer studies of its stereoisomers. The syntheses of some THL diastereomers have been reported (*e.g.*, **2**, **3**, *ent*-**2,** *ent*-**4,** and the 3-*epi*-THL), along with a smattering of lipase activities (e.g., **2**, **3,** and the 3-*epi*-THL).[16,23,28,29,30] For instance, the C2' epimer **2**, was reported to be less potent than THL (**1**) in a diacylglycerol lipase assay.[29] Similarly, the *cis*-β-lactone isomer (3-*epi*-THL)[23] has been evaluated for lipase inhibitory activity (IC_{50} = 15 nM). However, no systematic study of the *trans*-β-lactone stereoisomers has been reported. Thus, we identified seven *trans*-β-lactone stereoisomers of THL (**2**-**4** and (*ent*)-**1**-**4**) as compounds as a convenient method to identify the structural space around the enzyme active site (Figure 1). Importantly, these type of comprehensive stereochemical-

SAR studies (S-SAR) of natural products and their derivatives, require an asymmetric synthetic approach to the structure of interest.[31,32,33,34,35,36,37]

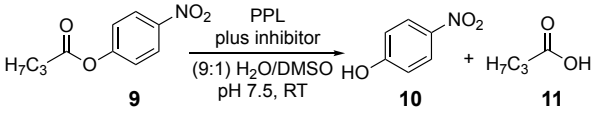
Over the years there have been numerous syntheses of THL and related molecules.[38,39,40,41] Our own successful synthesis of THL was of the *de novo* asymmetric variety and as a result was stereodivergent in its approach. Specifically, it used a catalytic asymmetric reduction of achiral ynone **7** to install the initial stereocenter (Scheme 1), followed by a diastereoselective epoxidation of **6** and regioselective Coates carbonylation[42,43,44,45,46,47,48] of **5** to afford either enantiomer of the four diastereomers (**1**-**4**).[49] Key to the success of this stereodivergent approach was the recognition that all eight THL stereoisomers (**1**-**4** and (*ent*)-**1**-**4**) could be diastereo- and regio-selectively prepared from the homo-allylic epoxide **8** and its enantiomer in only three steps (Scheme 2).



Scheme 2. Asymmetric approach to THL and 7 stereoisomers

Herein we report the evaluation of the eight THL diastereoisomers (**1**-**4** and (*ent*)-**1**-**4**). All the stereoisomers were evaluated for their lipase inhibitory properties using porcine pancreatic lipase (PPL). To ensure that the various stereochemical inversions on THL did not introduce any unwanted toxicities, the cytotoxicity of all eight stereoisomers were determined against three cancer cell lines (H460, MCF-7 and MDA-MB-231).

Porcine pancreatic lipase is frequently used,[7,50,51] since the active site of the porcine lipase is highly homologous to the human lipase (Scheme 3).[52] Porcine pancreatic lipase enzyme concentrations were varied between the range of 0.50 to 10.0 µg/200 µL in buffer solution to determine the optimal enzyme concentration of 5.0 µg/200µL. This concentration was consistent with values used for a related assay.[51] The concentration of the lipase substrate, *p*-nitrophenyl butyrate (*p*-NPB),[53] in DMSO/buffer solutions were varied from 0.25 mM to 1.0 mM without any deleterious effects on IC_{50} values. Solubility issues became apparent at higher concentration, as such, all assays were run with a concentration of 0.25 mM *p*-NPB. A 10% DMSO concentration was used, which was found to be sufficient for maintaining homogeneous solutions at all assay concentrations. Using this assay, the K_m (Michaelis-Menten constant) for our PPL was determined to be 2.41 mM (see supporting information) which corresponds well with a related assay.[54]



Scheme 3. Hydrolysis of *p*-nitrophenyl butyrate (*p*NPB) to *p*-nitrophenol (*p*NP) by porcine pancreatic lipase (PPL) in the presence of tetrahydrolipstatin and its diastereomers (**1**-**4** and (*ent*)-**1**-**4**).

THL was found to be a very potent inhibitor of PPL with an IC_{50} of 4.0 nM, which correlates well with known IC_{50} from related assays with 1,2-diacyl-*sn*-glycerol as the substrate.[23,29,51] None of the other seven diastereomers were more potent PPL inhibitors, with IC_{50} values ranging from 8.0 to 77 nM (cf, Figure 2, Table 1). One diastereomer, ***ent*-3**, did not show any significant inhibition of lipase activity below 100 nM, with an $IC_{50} > 900$. The error bars reflect 95% confidence limits for run in triplicates on three separate occasions ($N = 9$) and are consistent with slight variations in pH, enzyme concentration, and timing the start of the assays.

Table 1. IC_{50} of THL diastereomers against pancreactic lipase [a]

Cpd	Epimerization	IC_{50} (nM)
1	THL	4.0 (±1.3)
2	2'-*epi*-THL	15.4 (±2.6)
3	5-*epi*-THL	17.4 (±3.1)
4	5,2'-*epi*-THL	8.0 (±1.5)
***ent*-1**	*ent*-THL	76.9 (±17.6)
***ent*-2**	2,3,5-*epi*-THL	20 (±7.2)
***ent*-3**	2,3,2'-*epi*-THL	930 (±340)
***ent*-4**	2,3-*epi*-THL	14.7 (±2.9)

[a] The results are an average of three independent triplicate experiments ($N = 9$). The 95% confidence limits are indicated in parentheses.

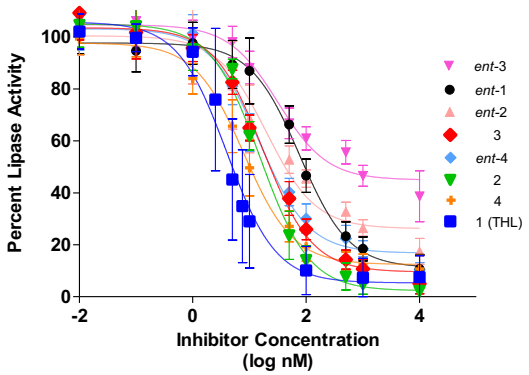


Figure 2. IC_{50} curves for THL and seven stereoisomers against pancreatic lipase using inhibitor concentrations between 0.01 nM and 10 µM.

The eight stereoisomers were assayed for cytotoxicity against three human cancer cell lines: two human breast adenocarcinoma cancer cell lines MCF-7 and MDA-MB-231 and one human large-cell lung carcinoma cell line H460 (Table 2 and Figures 3-5). A standard 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay of cell viability was used.[55,56] The MDA-MB-231 was chosen as it has been previously used in several studies of THL cytotoxicities.[16, 23]

Table 2. IC_{50} of THL diastereomers against cancer cells (nM)[a]

Compound	H460	MDA-MB-231	MCF-7
1 to *(ent)*-4	$>10^5$	$>10^5$	$>10^5$
camptothecin	110	540	160

[a] The cell line was grown in 96-well plates at a density of 5 × 103 cell/well for 12 to 16 h. IC_{50} values were determined via MTT assay from a 72 h drug treatment (0.1 nM to 100 μM), with untreated wells as controls. The viability was measured by UV absorbance at 570 nm. The dose-dependent experiment was performed in 2 replicate wells of each compound for a single concentration with 3 independent experiments ($N = 6$).

Of the three cell lines we tested, MDA-MB-231 was most sensitive to THL compounds. The IC_{50} value we observed for THL against the MDA-MB-231 was ~100 μM, which was significantly higher than previously reported (IC_{50} 13 μM).[23] This difference may be due to variations in the culture method and assay.[57,58,59] For the MCF-7 cell line, the IC_{50}'s of all of the THL diastereomers were at or above 100 μM, with the lowest being for THL **2** (see Figure 4). In general, varying the stereochemistry of THL had little effect on the cytotoxicity. Interestingly, *ent*-**3**, the least potent PPL inhibitor, demonstrated no significant difference in cytotoxicity across the three cell lines.

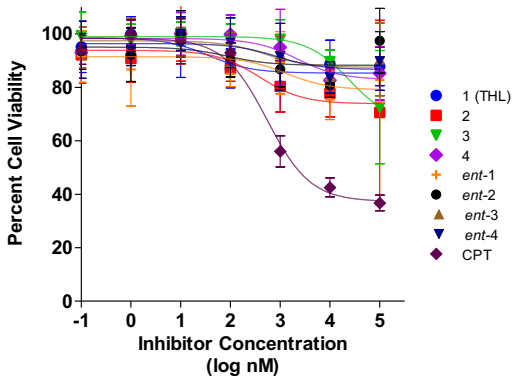


Figure 3. IC_{50} curves for THL and seven stereoisomers against MDA-MB-231 cell line using inhibitor concentration from 0.1 nM to 100 μM. CPT = camptothecin.

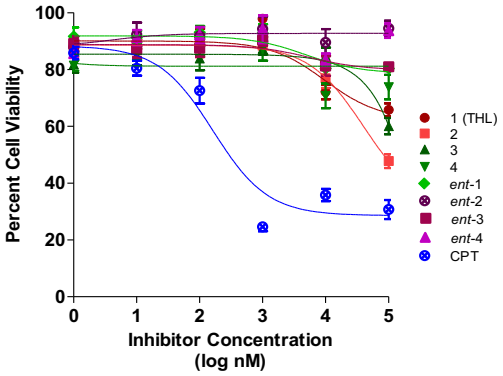


Figure 4. IC_{50} curves for THL and seven stereoisomers against MCF-7 cell line using inhibitor concentration from 0.1 nM to 100 μM. CPT = camptothecin.

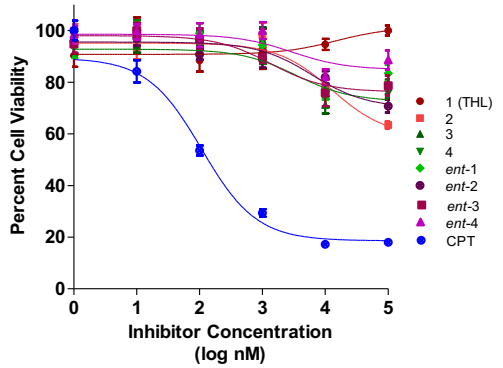


Figure 5. IC_{50} curves for THL and seven stereoisomers against H460 cell line using inhibitor concentration from 0.1 nM to 100 μM. CPT = camptothecin.

In conclusion, synthetic THL and seven of its stereoisomers were evaluated *in vitro* as porcine pancreatic lipase inhibitors. This study found that the stereochemistry of THL plays a modulating role on inhibitory activity, with the IC_{50}'s of THL and its diastereomers ranging from 4.0 to 930 nM. While THL proved to be the most potent inhibitor (4.0 nM), in this assay, several diastereomers retained significant inhibitory activity. Of these, diastereomers, (*ent*)-**2** had the most surprising PPL-inhibitory activity (20 nM), as it is enantiomeric at the key β-lactone portion of the molecule. For comparison, the enantiomer of THL, (*ent*)-**1**, was much less active (77 nM). Interestingly, this systematic inversion of stereochemistry around the THL structure had no deleterious effects on the cytotoxicity of these molecules. These S-SAR findings suggest that there is potential for future medicinal chemistry around the β-lactone structural motif of THL. Our efforts along these lines will be reported in due course.

ASSOCIATED CONTENT

Supporting Information. Experimental details for synthetic procedure and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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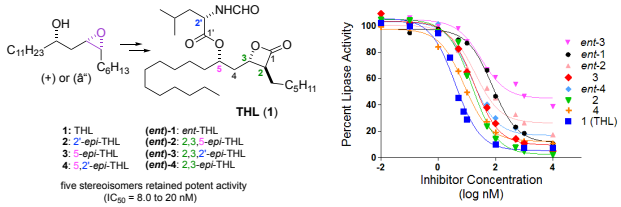
Lay Summary:

Tetrahydrolipstatin (THL) is a natural product derivative that has been approved by the FDA as an oral anti-obesity drug, which acts by the inhibition of the pancreatic lipase enzyme in the digestive track. Synthetic access to THL and seven diastereomers enabled a novel stereochemistry based structure activity relationship study of this class of biologically active natural products. Several stereoisomers of THL were discovered with significant pancreatic lipase inhibitory activity.

TOC:



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