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(*S*)-Thiirancarboxylic Acid as a Reactive Building Block for a New Class of Cysteine Protease Inhibitors

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Abstract—For (*S*)-thiirancarboxylic acid a second-order rate constant of $k_{2nd} = 222\ M^{-1}\ min^{-1}$ for the irreversible inhibition of papain was determined. The ethyl and methyl ester do not inhibit the enzyme time-dependently. An improved synthesis of enantiomerically pure thiirancarboxylic acid is described. It is shown that thiirancarboxylates can be substrates for serine proteases (α-chymotrypsin) and esterases (pig liver esterase) and even for metallo proteases (thermolysin).

Since the isolation of E-64 [(*S,S*)-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane] from *Aspergillus japonicus*[1] and its discovery as a selective and irreversible cysteine protease inhibitor, epoxysuccinyl peptides ((**I**); Fig. 1) have become one of the most investigated substance classes in the cysteine protease inhibitors research field.[2] The inhibition of cysteine proteases by this type of inhibitor takes place via nucleophilic epoxide ring opening by the cysteinate residue of the enzyme's active site leading to alkylation and therefore inactivation of the enzyme. Replacement of the electrophilic epoxide ring by an aziridine ring which is also susceptible to nucleophilic ring opening has led to a new class of selective and irreversible cysteine protease inhibitors (aziridinyl peptides (**II**); Fig. 1).[3] Peptides derived from (*S*)-aziridine-2-carboxylic acid have also been synthesized ((**III**); Fig. 1).[4] The building block itself ((*S*)-aziridine carboxylic acid; Table 2)[5] as well as C- and N-protected peptides[4b] containing the aziridine carboxylic acid are weak irreversible papain inhibitors. Besides epoxides and aziridines, thiiranes are also known to react with nucleophiles.[6] Thus, the thiirane ring could be a useful building block for peptidic cysteine protease inhibitors as well. However, the synthesis of epithiosuccinyl peptides by conversion of the reversed configured epoxysuccinyl peptides failed.[7] Although (*S,S*)-*trans*-diethyl epithiosuccinate itself ((**IV**); Fig. 1) could be synthesized, further reactions and enzyme assays were not possible, since this building block rapidly decomposes to fumaryl derivatives.[8] Among the known thiiranes, enantiomerically pure (*S*)-thiirancarboxylic acid esters are readily available from the reversed configured cysteine esters.[9] Within the above listed series of inhibitors, peptides containing this head group as the reactive building block with thiol-alkylating properties are missing ((**V**); Fig. 1).

In this paper we report on both the synthesis of (*S*)-thiirancarboxylic acid as a potential building block for peptidic cysteine protease inhibitors as well as on the results of inhibition assays with papain, as the best known example of cysteine proteases. Furthermore, enzymatic hydrolyses of thiirancarboxylic acid esters with several hydrolases as a new synthetic approach to thiirancarboxylic acid are described.

(*S*)-Ethyl thiirancarboxylate (**1**) and (*S*)-methyl thiirancarboxylate (**2**) were prepared by reaction of ethyl

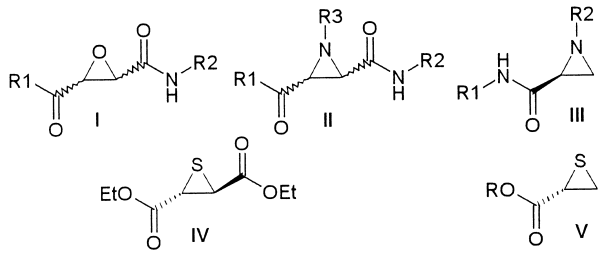

Figure 1. Three-membered heterocycles as reactive building blocks for peptidic cysteine protease.

Abbreviations: α-CTR, α-chymotrypsin; PLE, pig liver esterase; BAPA, benzoyl arginyl-*p*-nitroanilide; *p*NA, *p*-nitroanilide; FAGLA, 3-(2-furyl)-acryloyl-glycyl-leucine amide; TL, thermolysin.

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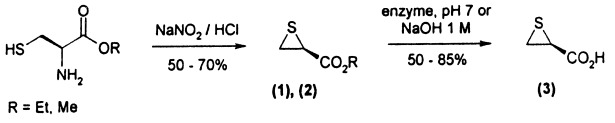
and methyl (*R*)-cysteinate, respectively, with $NaNO_2$/HCl (Scheme 1).[9]

For the synthesis of thiirancarboxylic acid (**3**) two different pathways exist: (1) alkaline hydrolysis of esters;[9a] and (2) direct cyclization of the amino acid cysteine with $NaNO_2$/HCl.[9a] The alkaline hydrolysis of (*S*)-esters with equimolar amounts of NaOH leads to the (*S*)-acid with a chemical yield of about 50% without any unhydrolyzed esters being recovered. This low yield results from polymerization.[10] The direct cyclization of (*R*)-cysteine with $NaNO_2$/HCl does not proceed stereospecifically and leads to (*R*)-thiirancarboxylic acid (57–60%ee) with a chemical yield of 25–30%.[9a] To improve the access to (*S*)-thiirancarboxylic acid, hydrolysis assays of the ethyl and methyl ester (**1**, **2**) with several hydrolases were carried out under pH-stat conditions. In contrast to the enzymatic hydrolyses of aziridine-,[11] oxirane-,[12] cyclopropane-,[13] and oxaziridine-carboxylates,[14] such reactions have yet not been carried out with thiiranes. The results of these assays are summarized in Table 1.

PLE, a serine esterase, and the serine protease α-chymotrypsin as well as the Zn-containing metallo protease thermolysin accept (*S*)-ethyl and methyl thiirancarboxylates (**1**, **2**) as substrates. The reaction progress can be followed by consumption of NaOH (0.1 mol/L) and TLC and the (*S*)-acid (**3**) can be isolated in good yields. Unhydrolyzed esters can be recovered almost quantitatively since polymerization byproducts are only produced to a minimal extent.[10]



Scheme 1. Synthesis and hydrolysis of (*S*)-thiirancarboxylates.

The hydrolysis of the ethyl ester (**1**) by the serine protease trypsin does not exceed the hydrolysis without enzyme indicating that thiirancarboxylates are not substrates for this protease.

Thiirancarboxylic acid esters (**1**), (**2**) and the acid (**3**) were tested against the model cysteine protease papain with L-BAPA as substrate.[15] Hydrolysis of this substrate by papain leading to release of *p*-nitroaniline was monitored spectrophotometrically at 405 nm. Dilution[16] and continuous[17] assays were carried out to determine the inhibition constants. In both assay types, a non-time-dependent inhibition of papain was found for the thiirancarboxylic acid esters (**1**) and (**2**) indicating reversible inhibition[25] (Fig. 2, dilution assays with papain and thiiranes).

This was confirmed by dialysis assays carried out with methyl thiirancarboxylate (**2**) and papain. Dixon plots revealed dissociation constants $K_I = 0.57 \pm 0.03$ mM for the ethyl ester (**1**) and $K_I = 0.20 \pm 0.015$ mM for the methyl ester (**2**). In contrast, the free acid (**3**) is an irreversible inhibitor of papain as shown by time-dependent loss of enzyme activity in dilution (Fig. 2) and continuous assays (Fig. 3). Since no saturation conditions could be achieved (Fig. 4), due to solubility problems and a high K_I value, the individual inactivation constants k_i (min^{-1}) (first-order rate constant) and K_I (mM) (dissociation constant) could not be determined. Therefore, the second-order rate constant $k_{2nd} = k_i/K_I$ (M^{-1} min^{-1}) was calculated from $k_{2nd} = k_{obs}/[I]$ (M^{-1} min^{-1}) (Fig. 4) with the pseudo-first-order inactivation rates k_{obs} [min^{-1}] obtained either from the dilution or continuous assays (Fig. 3). A second-order rate constant of inactivation $k_{2nd} = 222 \pm 8$ (M^{-1} min^{-1}) was calculated for the thiirancarboxylic acid (**3**).

To examine the reaction of the thiiranes with the nucleophile cysteine included in the assay buffer, methyl thiirancarboxylate (**2**) and the acid (**3**) were incubated

Table 1. Enzymatic and alkaline hydrolysis of (*S*)-thiirancarboxylates (**1**) and (**2**)[a]

Ester	Enzyme[b]	Hydrolysis conditions	Turnover (%)[c]	Acid/ester (%)[d]
(**1**) 2 mmol	—	pp 50 mL, rt	5/7/10[e]	
(**1**) 2 mmol	—	tris 50 mL, rt	6/8.5/12[e]	
(**1**) 2 mmol	PLE, 100 μL (150 U mg^{-1})	pp 50 mL, rt	42/nd/72	61/20
(**2**) 2 mmol	PLE, 250 μL (150 U mg^{-1})	pp 50 mL, rt	100[f]	85/0
(**1**) 2 mmol	α-CTR, 250 mg (10 mU mg^{-1})	pp 50 mL, rt	89[g]	65/5
(**1**) 2 mmol	Trypsin, 25 mg (500 mU mg^{-1})	pp 50 mL, rt	9/nd/15[e]	
(**1**) 4 mmol	TL, 150 mg (100 mU mg^{-1})	tris 100 mL, rt	29/40/50[e]	
(**1**) 7.5 mmol	—	NaOH (1 M), 0 °C	100[h]	50/0

[a]pp, phosphate buffer pH 7.0; tris, TRIS buffer pH 7.2, all buffer solutions contained 10% acetone; rt, room temperature; nd, not determined.
[b]The specific activities of the enzymes were determined against the following substrates: PLE/ethylbutyrate, α-CTR/AcPhe-*p*NA, trypsin/L-BAPA, thermolysin (TL)/FAGLA.
[c]Calculated by consumption of NaOH (0.1 M) after 48 h/72 h/96 h.
[d]Yield (%) of isolated (*S*)-thiirancarboxylic acid and isolated recovered (*S*)-ester after extraction at pH 7 (ester) and pH 1 (acid).
[e]Turnover, calculated by NaOH consumption, fits with acid/ester ratio calculated from ^{1}H NMR spectra after extraction at pH 1.
[f]24 h reaction time.
[g]48 h reaction time.
[h]2 h reaction time, addition of 1 eq NaOH.

with buffer solution 1 h prior to enzyme and substrate addition. No differences in inhibition constants could be found compared to assays without preincubation of inhibitor and cysteine-containing buffer. Therefore, reaction of cysteine as a low molecular weight thiol with the thiiranes can be excluded.

To investigate the selectivity of inhibition of cysteine proteases, continuous inhibition assays were carried out with the serine proteases α-chymotrypsin and trypsin as well as with the metallo protease thermolysin. The ethyl ester (**1**) and the acid (**3**) were used at [I] = 1.2 mM (**1**) and [I] = 1.0 mM (**3**) concentrations. In all cases no inhibition could be observed indicating that thiirancarboxylates are selective inhibitors of cysteine proteases. In contrast, as shown by the hydrolysis assays (Table 1), thiirancarboxylates can function as substrates for these types of proteases.

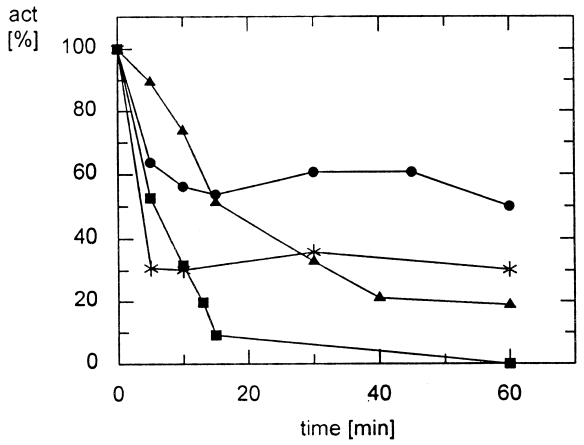


Figure 2. Dilution assays with papain and thiiranes. Plot of remaining enzyme activity (act (%)) versus time for thiiranes (**1**–**3**) at following inhibitor concentrations: ● 0.6 mM (**1**); ∗ 1.0 mM (**2**); ▲ 0.3 mM (**3**); ■ 1.0 mM (**3**).

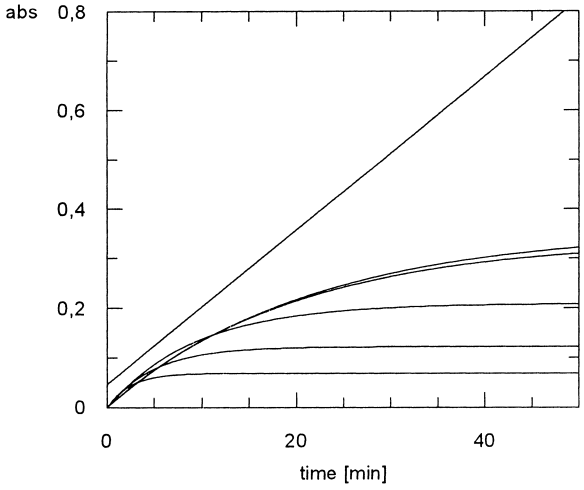


Figure 3. Continuous assays with papain and thiirancarboxylic acid (**3**). Progress curves (absorption (abs) versus time) at increasing inhibitor concentrations [I]: 0.0 mM; 0.2 mM; 0.3 mM; 0.7 mM; 0.9 mM; 1.0 mM.

Statements concerning the reactivity of three-membered heterocycles (C_2X; X = O, NH, S) towards nucleophilic ring opening can only be general and shallow,[6] since only few systematic studies have been carried out so far. Systematic studies with thiiranes are often prevented by polymerization, since the thiol or thiolate produced during reaction is itself strongly nucleophilic. The mechanisms of nucleophilic ring opening under either acidic or basic conditions (A2, S_N2)[6,18] are the same for these three ring systems. A study with the 2-methyl-substituted compounds (propylene oxide, propylene imine, propylene sulfide) showed a decreasing reaction rate of ring opening with HCl[19] and H_3CCO_2H,[20] respectively, in the order: oxirane ≥ aziridine >> thiirane.[19,20] A similar result (O > S) has been observed for the ring opening with aniline.[21] This order of decreasing reactivity (O > N > S) is in accordance with the decreasing ring strain as well as with the decreasing electronegativity of the heteroatom X.[22]

Within the series of electrophilic cysteine protease inhibitory three-membered heterocycles (Table 2) a direct comparison is only possible for *the aziridine* versus *the thiirane* (2-carboxylic acid-substituted compounds) and for *the oxirane* versus *the aziridine* (2,3-dicarboxylic acid-substituted compounds), respectively. The inhibition constants (Table 2) clearly show that the enzymatic nucleophilic ring opening follows the same order of reactivity established for chemical ring opening with the nucleophiles Cl^- or aniline: O > N > S.

Comparing the thiirane derivatives with the oxirane and aziridine inhibitors, the free carboxylic acid function seems to be an essential feature for good inhibition. Accordingly, the same inhibition mechanism, with the carboxylate anion of the inhibitor interacting with the histidinium ion of the enzyme's active site,[2] can be postulated.

Another similarity of the three electrophilic building blocks is the selectivity towards cysteine proteases. Thus, serine and metallo proteases are not inhibited and a reaction with unactivated nucleophiles (cysteine in buffer solution) does not occur under the assay conditions.

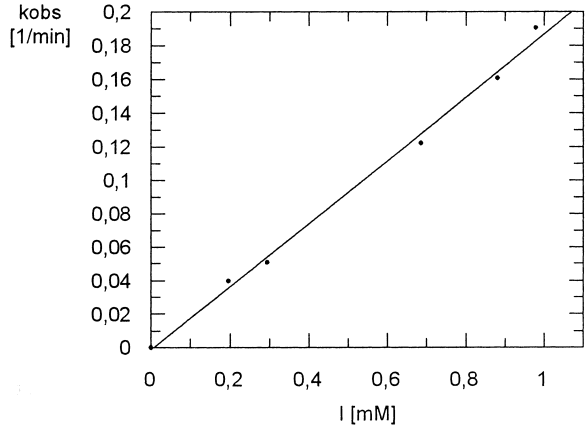
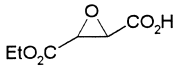
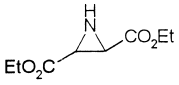
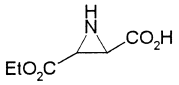
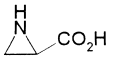
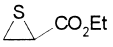
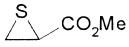
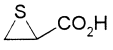


Figure 4. Inactivation of papain with thiirancarboxylic acid (**3**). Plot of pseudo-first-order inactivation rates k_{obs} versus inhibitor concentrations [I].

Table 2. Inhibition of papain by oxirane-, aziridine- and thiirancarboxylic acids and esters

Inhibitor	Structure	Configuration	k_{2nd} [M^{-1} min^{-1}]	IC_{50} [μM]	K_I [mM]	ref
Diethyl epoxysuccinate		*R*, *R* *R*, *R*+*S*, *S* *S*, *S*	101±20 104±31	 0.207 		24 1 3b
3-Ethoxycarbonyl-oxirane-2-carboxylic acid		*R*, *R*+*S*, *S*		0.0105		1
Diethyl aziridine-2,3-dicarboxylate		*R*, *R* *S*, *S*	12±0.5 11±0.5[a]			3b 3b
3-Ethoxycarbonyl-aziridine-2-carboxylic acid		*R*, *R* *S*, *S*	3525±408 4692±1000[b]			24 3c
Aziridinecarboxylic acid		*S*	1020[c]			5
Ethyl thiirancarboxylate		*S*		0.57±0.03		
Methyl thiirancarboxylate		*S*		0.2±0.015		
Thiirancarboxylic acid		*S*	222±8			

[a]pH 4: 61±5.
[b]pH 4: 24772±7000.
[c]The carboxamide is reported to be less potent by orders of magnitude than the free acid.[5]

The data obtained in this first comparison of oxirane-, aziridine-, and thiirancarboxylic acid derivatives show that the thiirancarboxylic acid represents a new electrophilic unit for irreversible and selective cysteine protease inhibitors. Since a peptide structure containing this building block is reported to be stable[23] further studies with this thiirane are promising. It is shown herein for the first time that thiirancarboxylates can be substrates for serine proteases and esterases and even for metallo proteases.

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