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Allicin and derivates are cysteine protease inhibitors with antiparasitic activity

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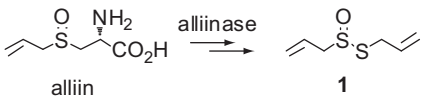
ABSTRACT

Allicin and derivatives thereof inhibit the CAC1 cysteine proteases falcipain 2, rhodesain, cathepsin B and L in the low micromolar range. The structure–activity relationship revealed that only derivatives with primary carbon atom in vicinity to the thiosulfinate sulfur atom attacked by the active-site Cys residue are active against the target enzymes. Some compounds also show potent antiparasitic activity against *Plasmodium falciparum* and *Trypanosoma brucei brucei*.

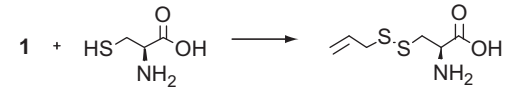
Allicin (*S*-allyl-2-propenyl thiosulfinate, **1**, Scheme 1) is the main biologically active component of garlic extracts. It is produced during the crushing of garlic by the interaction of alliin, (Scheme 1), a non-protein amino acid, with the pyridoxal phosphate containing enzyme, alliinase.[1] Allicin was found to inhibit the growth of a wide range of bacteria, it also shows antifungal, antiviral, and antiparasitic activity.[2–4]

Even though the antibacterial properties of allicin were already detected in 1940s, the mechanism of action was not elucidated in detail until 1998. NMR studies indicated that the particular structure of allicin makes it a good candidate for interaction with SH-groups of proteins and other biologically active molecules. In order to study the reaction of allicin with thiol containing proteins, the interaction of allicin with L-cysteine was used as a model. The isolated product was the *S*-allyl-mercapto cysteine, not its sulfoxide derivate (Scheme 2).[5]

It is reasonable to assume, that the broad-spectrum antimicrobial effects of allicin are due to inhibitory effects they may have on various thiol-dependent enzymes, for example, cysteine proteases.



Scheme 1. Biosynthesis of allicin (**1**).

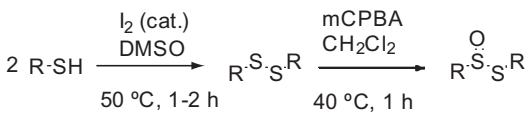


Scheme 2. Reaction of allicin with cysteine.[5]

This was already proven for the cytopathic effects of *Entamoeba histolytica*: the inhibition of parasite cathepsin-like proteases contributes to the antiparasitic activity of allicin.[3] Thus, allicin probably also inhibits related proteases from other parasites. In the present work we present synthesis of allicin and derivates thereof, the testing of the compounds against cathepsin-like cysteine proteases using fluorometric enzyme assays and results of microbial growth assays.

The synthesis of the potential inhibitors (as racemates) starts from symmetrical disulfides which were prepared from the corresponding thiols by oxidation with catalytic amounts of iodine in DMSO (Scheme 3).[6]

The thiosulfinates were synthesized by oxidation of the corresponding disulfides with an organic peracid. Peracetic, perbenzoic, perfuroic, perphtalic as well as percamphoric acid can be used.[7]



Scheme 3. Synthesis of allicin (**1**, R = allyl) and symmetrical derivatives **2**–**7** [R = *n*-propyl (**2**), benzyl (**3**), *tert*-butyl (**4**), cyclohexyl (**5**), 3-methylbuty-1-yl (isopentyl) (**6**), *n*-hexyl (**7**)].

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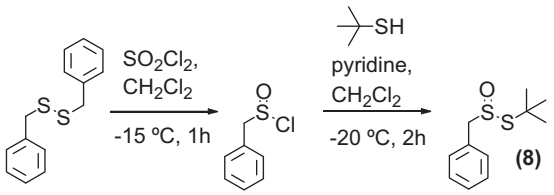
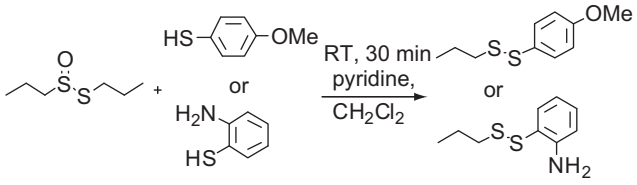


Scheme 4. Synthesis of mixed thiosulfinate (**8**).



Scheme 5. Model reaction of thiosulfinate (**2**) with thiols.

meta-Chloro perbenzoic acid (mCPBA) was the reagent of choice in the described work.

For the synthesis of the mixed thiosulfinate (**8**) dibenzyl disulfide was treated with sulfuryl chloride to yield benzyl sulfinyl chloride (Scheme 4).[8] Reaction with *tert*-butyl mercaptan yielded the mixed thiosulfinate (**8**) as single regioisomer.

The inhibitory effects of allicin and its derivates were tested against the parasite enzymes falcipain 2 from *Plasmodium falciparum*, the protozoa causing malaria, and rhodesain from *Trypanosoma brucei rhodesiense*, causing African sleeping sickness, and the mammalian cysteine proteases cathepsin B and L. These enzymes belong to the cysteine protease clan A, family C1 and have similar three-dimensional structures.[9] The parasite proteases are known to play essential roles in the pathogenicity of the protozoa.[10] Selected compounds were also tested against the respective parasites.

The postulated enzyme inhibition mechanism (Schemes 2 and 5[13]) involves a thiol-disulfide exchange reaction[5] yielding the cysteine residue of the active site of the enzyme blocked as disulfide. Due to subsequent disulfide exchange by DTT (dithiothreitol) which is necessarily present in the assay medium the disulfide-blocked enzyme can be reactivated yielding free enzyme and a mixed disulfide containing the inhibitor's thiol fragment and DTT. This new disulfide then can react as a reversible inhibitor which is well known for cysteine proteases.[15] In such a case, time-independent inhibition will be observed, that is, the degree of inhibition does not change over time (linear progress curves, Fig. 1, bottom). However, in some cases, namely for compounds **2**, **3**, and **7** time-dependent inhibition was observed, that is, inhibition potency increases with time. This is an indicator for irreversible inhibition, that is, the reactivation of the enzyme by DTT may not be possible in such cases. Examples for both, time-dependent and time-independent inhibition are shown in Figure 1.

Additionally, allyldisulfide (starting material for synthesis of allicin (**1**)) was tested against the enzymes. However, no decrease in enzyme activity was observed at 20 μM, showing that the thiosulfinate moiety is necessary for inhibition.

Table 1 summarizes the inhibition constants of the various cysteine proteases, and the IC_{50}-values for their antiparasitic activity.

With the exception of compounds **4** (*tert*-butyl), **5** (cyclohexyl), and the mixed thiosulfinate **8** (benzyl/*tert*-butyl) the synthesized thiosulfinates inhibited the proteases in the low micromolar range. The most active inhibitors were allicin (**1**) itself and derivatives **6** and **7** with larger lipophilic groups. These groups could address the hydrophobic S2 pockets of the enzymes. The inactivity or only weak activity of compounds **4**, **5** and **8** indicates that the inhibition reaction (Scheme 2 and 5[13]) may be sterically hindered if a second-

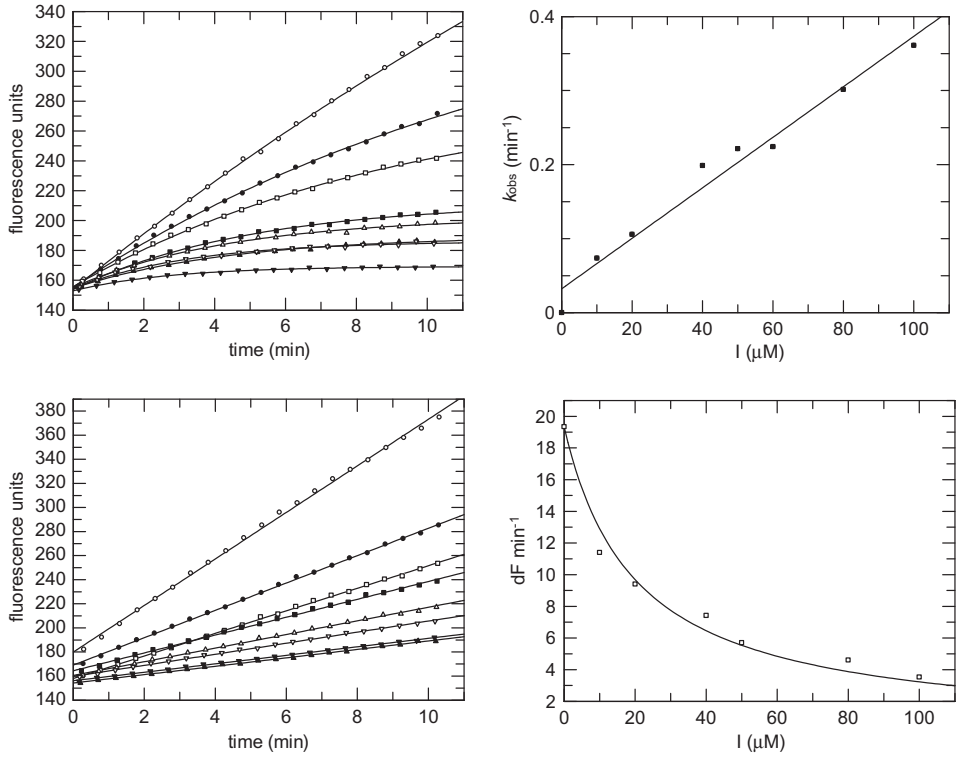


Figure 1. Inhibition of cathepsin L by compd **2** (top), left: progress curves at 0, 10, 20, 40, 50, 60, 80, 100 μM; right: k_{obs} versus [I] diagram for determination of second-order rate constant of inhibition k_{2nd}. Inhibition of cathepsin L by compd (**1**) (bottom), left: progress curves at 0, 10, 20, 40, 50, 60, 80, 100 μM; right: Dixon plot for determination of K_i.

Table 1
Biological activities of allicin and derivatives[14]

Compds	Cathepsin B Inhibition K_i, μM	Cathepsin L Inhibition K_i, μM	Falcipain 2 Inhibition K_i, μM	Rhodesain Inhibition K_i, μM	*P. falciparum* Inhibition IC_{50}, μM	*T. b. brucei* Inhibition IC_{50}, μM
Allicin (**1**)	8.6 ± 0.29	9.3 ± 0.39	1.04 ± 0.08	5.31 ± 0.83	5.21 ± 0.96	13.8 ± 0.06
2	15.6 ± 3.6	11.3 ± 2.4 (6664 ± 400 M^{-1} min^{-1})[b]	26.4 ± 2.3 (10,761 ± 680 M^{-1} min^{-1})[b]	4.44 ± 0.85 (7150 ± 1950 M^{-1} min^{-1})[b]	78.3 ± 23.3	>40
3	7.8 ± 2.2	31.7 ± 0.30	4.5 ± 1.2 (48,556 ± 1120 M^{-1} min^{-1})[b]	2.31 ± 0.07 (7800 ± 1300 M^{-1} min^{-1})[b]	30.3 ± 12.6	n.d.[a]
4	n.i.[a]	n.i.[a]	>100 (ca. 10)[a]	>100 (ca. 10)[a]	72.5 ± 14.7	>40
5	>100 (ca. 30)[a]	>100 (ca. 20)[a]	>100 (ca. 35)[a]	>100 (ca. 30)[a]	34.4 ± 13.3	n.d.[a]
6	18.9 ± 1.03	3.4 ± 1.1	3.04 ± 0.47	0.36 ± 0.05	54.7 ± 22.5	n.d.[a]
7	5.94 ± 1.30	10.4 ± 2.45	1.80 ± 0.05 (44,099 ± 3110 M^{-1} min^{-1})[b]	1.00 ± 0.25 (83,456 ± 19,089 M^{-1} min^{-1})[b]	10.9 ± 1.61	3.08 ± 0.11
8	n.i.[b]	>100 (ca. 15)[b]	n.i.[b]	>100 (ca. 20)[a]	52.9 ± 11.3	11.9 ± 4.33

[a] % Inhibition at 100 μM; n.i. no inhibition at 100 μM, n.d. not determined.
[b] Time-dependent inhibition, second-order rate constant k_{2nd}.

ary (compound **5**) or tertiary (compounds **4**, **8**) carbon atom is in vicinity to the sulfur atom of the thiosulfinate which is attacked by the active-site cysteine. Comparison of allicin (**1**) with its saturated derivative **2** shows that allicin is more active. This might be due to the reactivity of the allyl group of allicin which is known to undergo various fragmentation reactions yielding several reactive intermediates.[11]

The compounds do not inhibit one of the tested enzymes selectively, and also no selectivity between cathepsin L-like enzymes (falcipain 2, rhodesain, cathepsin L) and cathepsin B is observed. A slight preference for cathepsin L-like enzymes is found for inhibitor **6** only. The enzyme-inhibiting potencies of the compounds apparently contribute to their antiparasitic activity, as two of the most potent falcipain and rhodesain inhibitors (**1**, **7**) display potent antiplasmodial and antitrypanosomal activity.

However, with the exception of these two compounds, no clear correlation between antiprotease and antiparasitic activity is found. This indicates that the compounds may also address other targets (other proteases, e.g., falcipain 3, TbCatB,[12] or non-protease thiol-dependent enzymes, e.g., dehydrogenases) than the tested cysteine proteases, and that other properties such as cell permeability of the compounds may be important. Since the dihexyl derivative **7** is more stable in solution than allicin, which is decomposed in DMSO solution within several days due to its allyl groups, compound **7** may be a good starting point for designing allicin derivatives with enhanced affinities. The affinities could probably be optimized by moieties which better fit into the primed and non-primed substrate binding pockets of the target enzymes.

Acknowledgements

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Supplementary data

Supplementary data (synthesis, analytical data of the compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.062.

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13. In order to elucidate whether, besides allicin (Scheme 2),[5] other thiosulfinates react with low molecular weight thiols and thiol-dependent enzymes via a thiol-disulfide exchange we reacted thiosulfinate (**2**) with 4-methoxythiophenol and *o*-amino thiophenol, respectively. The products were isolated and analyzed by means of NMR spectroscopy. Indeed, the corresponding mixed disulfides, and not the mixed thiosulfinates emerged from these reactions (Scheme 5).
14. Enzyme assays and parasite growth assays were performed as described previously: (a) Breuning, A.; Degel, B.; Schulz, F.; Büchold, C.; Stempka, M.; Machon, U.; Heppner, S.; Gelhaus, C.; Leippe, M.; Ley, M.; Kisker, C.; Rath, J.; Stich, A.; Gut, J.; Rosenthal, P. J.; Schmuck, C.; Schirmeister, T. *J. Med. Chem.* **2010**, *53*, 1951; (b) Evers, A.; Heppner, S.; Leippe, M.; Gelhaus, C. *Biol. Chem.* **2008**, *389*, 1523.
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