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Rational Design of Aziridine-Containing Cysteine Protease Inhibitors with Improved Potency: Studies on Inhibition Mechanism

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To enable a rational design of improved cysteine protease inhibitors, the present work investigates trends in the inhibition potency of aziridine derivatives with a substituted nitrogen center. To predict the influence of electron-withdrawing substituents, quantum chemical computations of the ring opening of N-formylated, N-methylated, and N-unsubstituted aziridines with thiolate were performed. They revealed that the N*-formyl group leads to a strong decrease of the reaction barrier and a considerable increase in exothermicity due to stabilization of the transition state. In contrast, a nucleophilic attack at the carbonyl carbon atom is characterized by very low reaction barriers, suggesting a reversible reaction, thus providing the theoretical background for the reversible inhibition of cysteine proteases by peptidyl aldehydes. Reactions of aziridine building blocks (diethyl aziridine-2,3-dicarboxylate* ***1****, diethyl 1-formyl aziridine-2,3-dicarboxylate* ***2****) with a model thiolate in aqueous solution which were followed by NMR spectroscopy and mass spectrometry, showed the N-formylated compound* ***2*** *to readily undergo a ring-opening reaction. In contrast, the reaction of* ***1*** *with the thiolate is much slower. Enzyme assays with the cysteine protease cathepsin L showed* ***2*** *to be a 5000-fold better enzyme inhibitor than* ***1****. Dialysis assays clearly proved irreversible inhibition. These experiments, together with the results obtained with the model thiolate, indicate that the main inhibition mechanism of the N-formylated aziridine* ***2*** *is the ring-opening reaction rather than the reversible attack of the active site cysteine residue at the carbonyl carbon atom.*

Introduction

Cysteine proteases are attractive targets for the development of new drugs, as they play pivotal roles in many diseases.[1–6] Epoxide- and aziridine-containing cysteine protease inhibitors[3,6,7] have been developed that are analogous to the naturally occurring inhibitor E-64, an epoxysuccinyl peptide discovered in the late 1970s.[8] The inhibition mechanism is the irreversible alkylation of the Cys residue of the protease active site, initiated by nucleophilic attack of the negatively charged Cys thiolate at one of the electrophilic ring carbon atoms followed by irreversible opening of the three-membered ring.[3,7] While reversible protease inhibition is considered to be more appropriate for chronic diseases in which long-term treatment with an inhibitor is necessary, irreversible inhibition could be especially useful in the treatment of infectious diseases in which a protease of an infectious agent is targeted.[3]

Besides the common principal inhibition mechanism, several differences can be found between the epoxide and aziridine heterocycles. These include alkylation rates, the influence of medium pH, substituent effects, and stereoselectivity of inhibition.

In previous publications,[9,10] we reported quantum chemical calculations concerning the ring-opening reaction of the three-membered heterocycles aziridine, oxirane, and thiirane with the nucleophile methyl thiolate. The influence of environment and substituents at the ring carbon atoms on the kinetics and thermodynamics of the thiolate alkylation were discussed. These calculations indicated that the aziridine ring opening is influenced by acidic media to a greater extent than the epoxide or thiirane ring opening, confirming the experimental data relating to aziridines as inhibitors of cysteine proteases.[9] In addition, our computations suggested that a free carboxylic acid attached to the three-membered ring enhances inhibition potency for different reasons.[10] For epoxides and thiiranes, the ionic interaction between negatively charged carboxylate and the active site histidinium ion may be the main reason for improved inhibition potency of acids relative to esters. However, the aziridines are probably much more potent as uncharged free carboxylic acids because of intramolecular water-mediated acid catalysis. Our computations[10] also nicely explained the observed regioselectivity of the ring opening of epoxides with methyl thiolate.[11]

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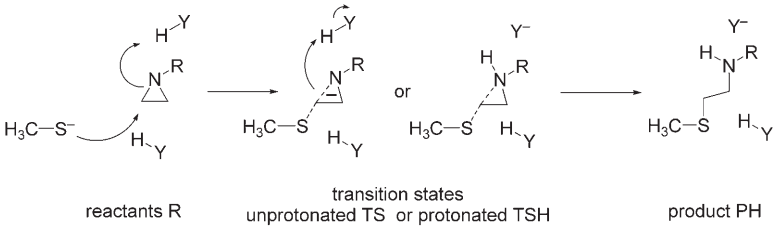
Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author: Synthesis and analytical data of inhibitor **2**, description of the enzyme and NMR assays, ^{1}H NMR spectra of the reaction of **2** with 4-methoxythiophenol, ratios of reaction products of the reaction of compound **2** with 4-methoxythiophenol.

In contrast to epoxides and thiiranes, the aziridine ring allows additional substitution at the aziridine nitrogen atom. This may be done to introduce either affinity-enhancing substituents targeting additional enzyme subsites or to introduce moieties that modulate the properties of the aziridine ring relating to the nucleophilic ring-opening reaction. Comparison of aziridine-based inhibitors showed that the N-acylated derivatives without a free acid group at the aziridine ring carbon atoms[12] are, in general, more potent than corresponding N-unsubstituted or N-alkylated derivatives.[13,14] This should be due to the electron-withdrawing property of the acyl group attached to the aziridine nitrogen atom activating the three-membered ring towards nucleophilic attack. To investigate in detail the influence of such substituents on the kinetics and thermodynamics of the ring-opening reaction, and to provide a rational background for the design of aziridine-based inhibitors with improved inhibition potency, we extended our studies on aziridines containing different substituents at the aziridine nitrogen atom.

Results and Discussion

To understand the influence of electron-withdrawing substituents at the nitrogen center on the inhibition potency of substituted aziridines, we employed model systems similar to those which were successfully used to understand differences in the inhibition mechanisms of aziridines and epoxides, and to elucidate the various effects connected with variations in the pH value of the environment.[9,10] They are depicted in Scheme 1.



Scheme 1. Model reaction of the ring opening of differently substituted aziridines with methyl thiolate. NR = NH, NCH_3, or NCHO.

The trends, reaction barriers, and energies of the ring-opening reactions of three-membered rings ($(CH_2)_2NR$ for which R = H, CH_3, and CHO) with methyl thiolate were computed and compared. For these computations the density functional theory was used.[15] In our model system, two water molecules were employed to mimic the molecular effects of environments with weak-proton donating ability. For environments possessing a higher proton-donating ability two ammonium ions were used. Bulk effects of the environment were taken into account by a continuous approach.[16] More information about the model and a detailed analysis of its accuracy can be found in the Experimental Section and in the literature.[9,10] Table 1 characterizes the computed reaction paths with the computed electronic energies. The corresponding enthalpies are uniformly higher by about 4 kJ mol^{-1}. Free energies are not given, as entropic effects arise mainly within the formation of the reversible enzyme–inhibitor complex produced beforehand.[9,10] Figure 1 depicts the geometries of transition states and products of entries 1, 2, 4, and 5 of Table 1.[17]

Table 1. Influence of N-substituents on the reaction profiles of the ring-opening reactions as shown in Scheme 1.

Entry	NR	Solvent HY	TS[a]	TSH[a]	P[a]	PH[a]
1	NH	2 H_2O	+118	–[b]	–[b]	−26
2	NH	2 NH_4^+	–[b]	+ 60	–[b]	−100
3	NCH_3	2 H_2O	+129	–[b]	–[b]	−11
4	NCHO	2 H_2O	+60	–[b]	−106	−97
5	NCHO	2 NH_4^+	+55	–[b]	–[b]	−171

[a] TS denotes the transition states. Proton transfer from the explicitly considered solvent molecules during the ring opening is indicated by the abbreviation PH for protonated products and TSH for protonated transition states. All electronic energy values are relative to the reactants and are in kJ mol^{-1}. The corresponding enthalpies are about 4 kJ mol^{-1} higher (see text). [b] Could not be localized.

For an environment with low proton-donating ability (two water molecules to capture molecular effects of the solvent) our computations predict a very high reaction barrier for the ring-opening reaction of the unsubstituted aziridine (entry 1 of Table 1: 118 kJ mol^{-1}, R = H). A proton transfer from one of the water molecules occurs after the transition state (TS) leading to a protonated product (PH, Figure 1 a). The reaction is computed to be slightly exothermic (entry 1 of Table 1: −26 kJ mol^{-1}).[9] For an environment with high proton-donating ability, modeled using two ammonium ions as solvent molecules (Table 1, entry 2, R = H), the strongly basic character of the evolving anionic nitrogen center leads to proton transfer from an ammonium ion before the transition state is reached (TSH, Figure 1 b). As a consequence, the reaction barrier drops from 118 kJ mol^{-1} (Table 1, entry 1) to 60 kJ mol^{-1} (Table 1, entry 2) and the reaction gets considerably more exothermic (−100 kJ mol^{-1} instead of −26 kJ mol^{-1}).[9]

The influence of alkyl substituents ($R = CH_3$) was computed for solvents with weak proton-donating ability ($HY = H_2O$). In comparison to the nonsubstituted aziridine, a methyl group slightly increases the reaction barrier (129 kJ mol^{-1} for entry 3 versus 118 kJ mol^{-1} for entry 1), and the reaction gets slightly less exothermic. (−11 kJ mol^{-1} for entry 3 versus −26 kJ mol^{-1} for entry 1). This change is in line with the +I effect of alkyl groups which increases basicity and energy content of the evolving amine anion. Due to the similarities between R = H and $R = CH_3$, computations within a more protic environment were not performed.

A substitution of the aziridine NH by an *N*-formyl unit (Table 1, entry 4) has a much larger impact. It halves the reaction barrier (60 kJ mol^{-1} as opposed to 118 kJ mol^{-1}) and increases the exothermicity of the reaction to −97 kJ mol^{-1}. The magnitude of the influence of CHO is similar to the influence

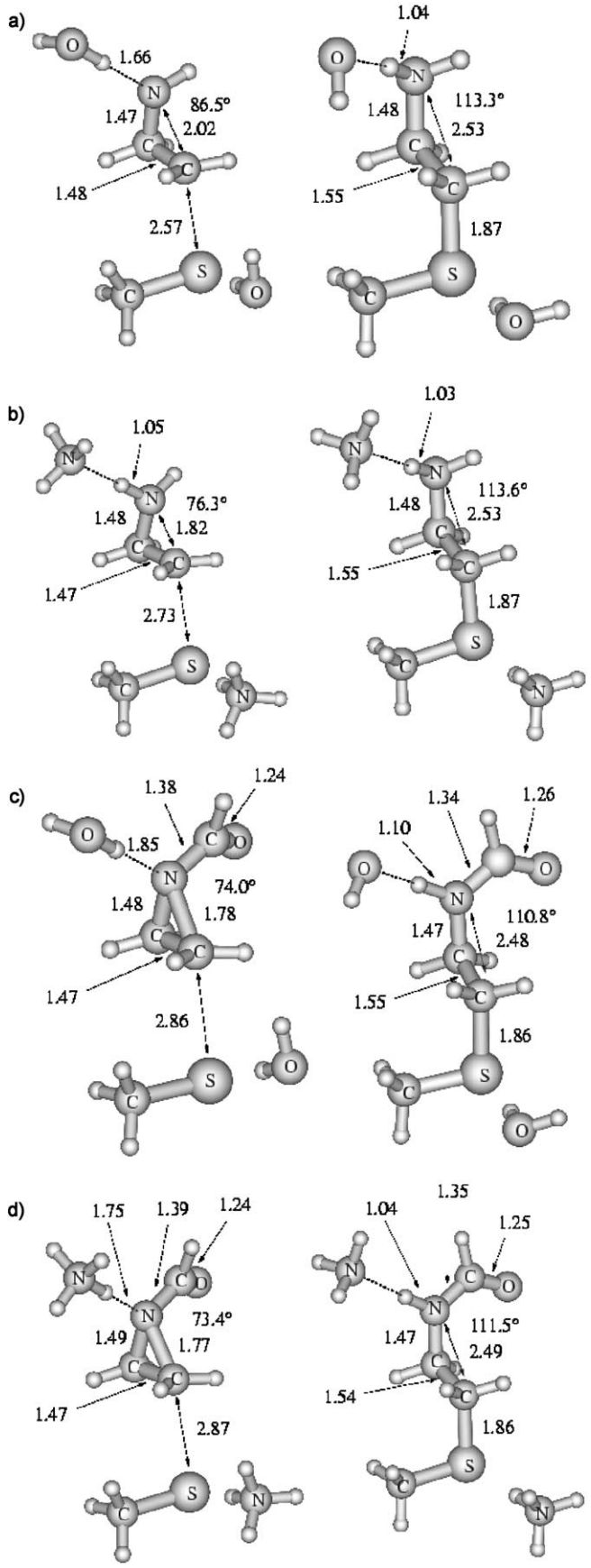


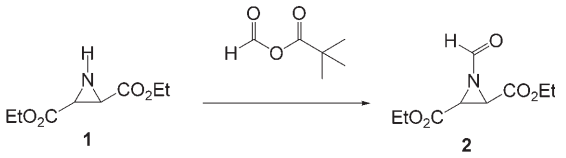
Figure 1. Geometrical arrangements of transition states (left-hand side) and products (right-hand side) for a) entry 1, b) entry 2, c) entry 4, and d) entry 5 of Table 1. Bond angles and distances (Å) are indicated.

of environments with strong donating strength.[9] This huge influence results from the stabilization of the negative charge of the evolving anion by the formyl substituent. The stabilization effect is also seen in the calculations for an environment with high proton donating ability (Table 1, entries 2 and 5; two ammonium ions as solvent). For R = H, a proton transfer from an ammonium ion to the nitrogen center takes place before the transition state (Figure 1 b),[9] whereas for R = CHO, the transition state remains nonprotonated (Figure 1 d). This explains why the reaction barrier for R = CHO decreases by only 5 kJ mol^{-1} going from two water molecules to two ammonium ions (entry 4: 60 kJ mol^{-1} versus entry 5: 55 kJ mol^{-1}). On the product side, the protonated (PH) and the nonprotonated (P) forms represent local minima (Table 1, entry 4) if molecular solvent effects are taken into account by two water molecules. Both are very similar in energy, again reflecting the strongly stabilizing effect of the CHO group. In comparison to the reaction with two water molecules, product formation is predicted to be thermodynamically favored for the reaction with two ammonium ions (Table 1, entry 5, geometry shown in Figure 1 d). This is in line with the basicity of the nonprotonated product and the higher acidity of ammonium, which in comparison with water enables an easier protonation of the product.

Figure 1 shows that the Hammond postulate[15] holds for the studied ring-opening reaction. The geometrical arrangement of the TS of the ring-opening reaction of the unsubstituted aziridine in an environment with low proton-donating ability (entry 1 of Table 1, $\Delta E_{reac} = -26$ kJ mol^{-1}; Figure 1 a, left-hand side) resembles the product geometry (Figure 1 a, right-hand side), for example, the ring is already considerably opened ($d_{N-C} = 2.02$ Å; $\sphericalangle NCC = 86^\circ$). For the reactants values of $d_{N-C} = 1.48$ Å and $\sphericalangle NCC = 60^\circ$ are computed. If the reaction becomes more exothermic, the TS increasingly resembles the reactants. For an environment with a high proton-donating ability (entry 2 of Table 1, $\Delta E_{reac} = -100$ kJ mol^{-1}; Figure 1 b) geometrical parameters of $d_{N-C} = 1.82$ Å and $\sphericalangle NCC = 76^\circ$, the TS are computed, whereas $d_{N-C} = 1.77$ Å and $\sphericalangle NCC = 73^\circ$ are predicted for the substituted aziridine in an environment with a high proton-donating ability (entry 5 of Table 1, $\Delta E_{reac} = -171$ kJ mol^{-1}; Figure 1 d).

The computations also clearly show that all substituents that are able to delocalize the emerging negative charge at the nitrogen center, will considerably improve the ring-opening reaction. That is, in the case of the reaction with an enzyme, the alkylation rate constant k_i should be enlarged. However, for quantitative predictions it is necessary to include influences arising from possible protonation from either the protein environment or the solvent, that is, the effect should be smaller than expected from a comparison of entry 1 and 5 of Table 1.

To prove these predictions we synthesized the N-formylated aziridine-2,3-dicarboxylate **2** by reaction of diethyl aziridine-2,3-dicarboxylate **1**[18] with pivaloyl formyl anhydride (Scheme 2).[19] The compound was tested in fluorimetric assays on the CAC1[20] cysteine protease cathepsin L and related proteases.

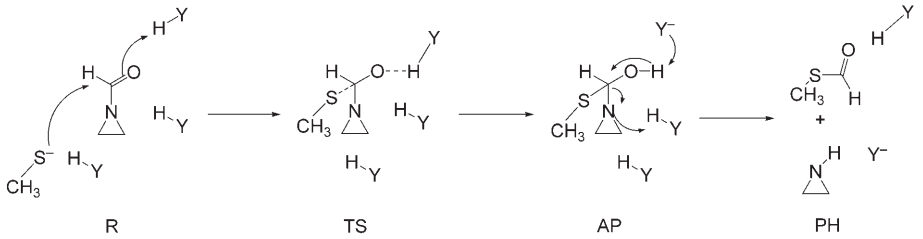


Scheme 2. Synthesis of *N*-formyl aziridine-2,3-dicarboxylate **2**.

In agreement with the proposed improvement of inhibition, we found an inhibition constant which is about 5000-fold higher than the one obtained with the non-formylated inhibitor **1**. Compound **1** is a very weak inhibitor ($k_2 \approx k_{obs}/[I] = 11\ M^{-1}\,min^{-1}$) whereas the *N*-formyl derivative **2** exhibits a k_2 value of $57{,}143\ M^{-1}\,min^{-1}$ against cathepsin L ($K_i = 0.5 \pm 0.1\ \mu M$, $k_i = 0.028 \pm 0.002\ min^{-1}$). Similar inhibition constants are found for the inhibition of the cathepsin L-like cysteine proteases, falcipain 2 from *Plasmodium falciparum* ($K_i = 1.9 \pm 0.6\ \mu M$; $k_i = 0.0064 \pm 0.0001\ min^{-1}$) and rhodesain from *Trypanosoma brucei rhodesiense* ($K_i = 0.06 \pm 0.008\ \mu M$).

Compound **2** exhibits extreme hydrolytic instability in DMSO, but not in less nucleophilic solvents like water, acetonitrile, or methanol. Within six hours, **2** is quantitatively decomposed in DMSO to **1** and formic acid,[21] whereas in water alone no hydrolysis takes place within 4 days, and in slightly alkaline aqueous medium (pH 7.6) a four-day reaction time is needed for complete hydrolysis of **2** to **1** and formic acid, as examined by ^1H NMR spectroscopy. This observation could indicate that **2** probably inhibits cysteine proteases not only by a ring-opening reaction but also by transfer of the formyl group to a nucleophilic center of the enzyme or by a reversible reaction of the active site cysteine with the formyl group. The latter reaction would correspond to the known inhibition mechanism of peptidyl aldehydes.[22]

To get information about the kinetics and thermodynamics of the nucleophilic attack at the formyl carbon atom (Scheme 3), further quantum chemical computations were performed. The results are shown in Table 2. The methods of the computations are identical to those used for the ring-opening reaction. However, to include all possible molecular effects of the solvent, a third ammonium ion had to be included in the vicinity of the carbonyl oxygen center.



Scheme 3. Nucleophilic attack of methyl thiolate at the formyl carbon atom of *N*-formyl aziridine.

A comparison of the reaction profiles of Table 2, entry 2 with Table 1, entry 5 is depicted in Figure 2. Going from the reactants (R) to the right-hand side, the reaction profile of the ring-opening reaction is depicted (entry 5, Table 1). R denotes the reactants methyl thiolate + *N*-formyl aziridine, TS1 the transition state of the ring-opening reaction, and PH1 depicts the product of the ring-opening reaction. Going from the reactants to the left-hand side, the reaction profile of the nucleophilic attack at the formyl carbon atom is given (entry 2, Table 2). TS2 indicates the transition state of the nucleophilic attack at the formyl carbon atom, AP, the intermediate of the nucleophilic attack at the formyl carbon (hemithioacetal/thioaminal), and PH2, the products of the nucleophilic attack at the formyl carbon atom, *S*-formyl thiol + aziridine.

Table 2. Computed reaction profiles of the reactions of *N*-formyl aziridine with methyl thiolate as shown in Scheme 3.

Entry	NR	Solvent HY	TS[a]	TSH[a]	AP[a]	PH[a]
1	NCHO	2 H_2O	_[b]	_[b]	_[b]	+91
2	NCHO	3 NH_4^+	+34	+32	+21	+27

[a] TS and PH denote the transition states and products, respectively. AP denotes the hemithioacetal/thioaminal intermediate. All values are relative to the reactants and are in $kJ\,mol^{-1}$. [b] Transition states (TS or TSH) and intermediate AP could not be localized.

This figure clearly shows that the barrier for the attack at the formyl carbon (TS2) is much lower than that for the attack at the ring carbon (TS1) (34 $kJ\,mol^{-1}$ versus 55 $kJ\,mol^{-1}$). In contrast, the reaction passing over the intermediate hemithioacetal (AP) to the reaction products aziridine + *S*-formyl thiol (PH2) is predicted to be endothermic. The low barrier of attack at the formyl carbon leading to the intermediate AP (TS2), and the low-energy differences between TS2, the intermediate AP, and the products PH2, indicate that this reaction pathway is reversible. These predictions are in line with the experimental finding that the cysteine protease inhibition mechanism of peptidyl aldehydes is based on the reversible hemithioacetale formation between active site thiolate and aldehyde. Thus, our calculations also provide the kinetic and thermodynamic background for this inhibition mechanism.[22]

Notably PH2 represents a formylated thiol. This can only be formed if a proton transfer to the aziridine unit takes place. Otherwise a deprotonated aziridine anion which is too high in energy would form. The high energy content of such a species may be the reason why structures corresponding to TS2 or AP could not be localized when two water molecules were included instead of three ammonium molecules.

To experimentally examine these findings we performed a model reaction with 4-methoxythiophenolate in D_2O at pH 7.6 (Scheme 4, Table 3).[23] The thiophenol was chosen because it represents a relatively acidic thiol ($pK_a = 7.4$–7.8)[24,25] with half the concentration deprotonated at pH 7.6. This model system

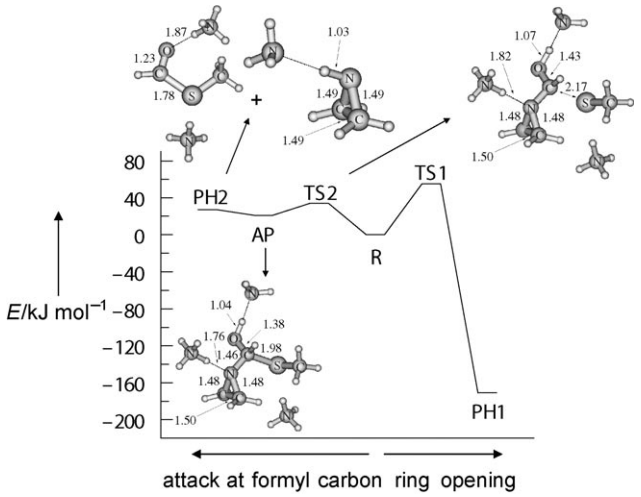
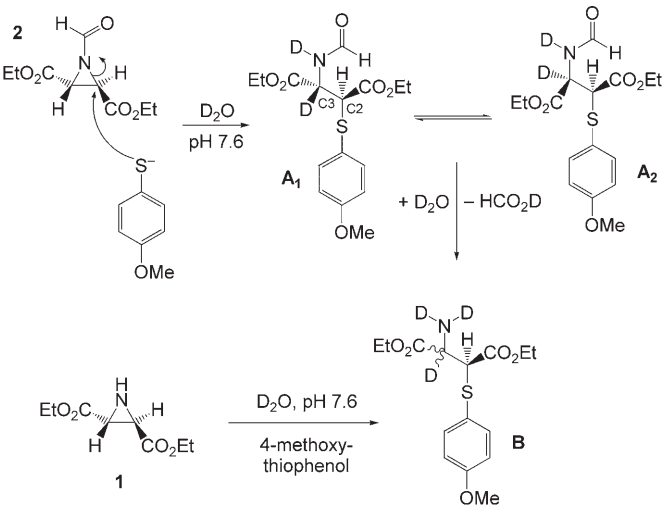


Figure 2. Reaction profiles and geometric arrangements of the model reactions of N-formylated aziridine with methyl thiolate. Going from the reactants (R) to the right-hand side the reaction profile of the ring-opening reaction is depicted (entry 5, Table 1). To the left-hand side, the reaction profile of the nucleophilic attack at the formyl carbon atom is given (entry 2, Table 2). R: reactants methyl thiolate + *N*-formyl aziridine; TS1: transition state of the ring-opening reaction; PH1: product of the ring-opening reaction; TS2: transition state of the nucleophilic attack at the formyl carbon atom; AP: intermediate of the nucleophilic attack at the formyl carbon atom (thiohemiacetal/thioaminal); PH2: products of the nucleophilic attack at the formyl carbon atom, *S*-formyl thiol + aziridine.



Scheme 4. Reactions of compounds **2** and **1** with 4-methoxythiophenol at pH 7.6.

Table 3. ^{1}H NMR data of the reactions of **2** and **1** with 4-methoxythiophenol in D_2O, (pH 7.6) as shown in Scheme 4.[a]

Protons	**2**	4-methoxythiophenol	$\mathbf{A_1}$	$\mathbf{A_2}$	**B**[b]	HCO_2D
at C2	s, 3.73	–	first d, then s, 5.20	s, 5.03	s, 4.80	–
at formyl-C	s, 8.86	–	s, 8.30	s, 8.36	–	s, 8.56
OCH_3	–	s, 3.88	s, 3.98	[c]	s, 3.96	–
aromatic	–	d, 6.85, 7.36	d, 7.15, 7.67	[c]	d, 7.12, 7.62	–

[a] Chemical shifts are given in δ ppm. [b] Peaks were assigned by comparison with the spectra obtained from the reaction of **1** with 4-methoxythiophenol; [c] Peaks could not be detected, they are probably covered by the corresponding signals of $\mathbf{A_1}$ or **B**; s, singlet, d, doublet.

resembles the active site cysteine in CAC1 proteases which is known to exist precatalytically as thiolate.[3–6] The reaction was followed for four days by NMR spectroscopy and mass spectrometry. In addition, the behavior of **2** alone in pH 7.6 buffer (see above), and 4-methoxythiophenol alone at pH 7.6, as well as the reaction of **1** with 4-methoxythiophenol were examined.[26] While **2** readily[27] reacts with the model thiol to give the ring-opened product **A** (identified by the signals of the formyl proton at $\delta = 8.30$ ppm and the proton at C2 at $\delta =$ 5.20 ppm), the reaction of **1** with the thiol is much slower, leading to only low concentrations of the corresponding ring-opened product **B** within 4 days. No peaks for the deformylated aziridine **1** (ring protons: s at $\delta = 2.84$ ppm) or a possibly emerging intermediate hemithioacetal could be found during the reaction of **2** with the thiol, additional confirmation that the nucleophilic attack occurs at the ring carbon and not at the formyl carbon atom. The ring-opened product **A** slowly hydrolyzes to formic acid and **B** as indicated by the peaks at 4.80 ppm (C2 proton), 3.96 ppm (methoxy group), and 7.12/7.62 ppm (aromatic protons) that were assigned by comparison to the spectra obtained from the reaction of **1** with the thiol. Besides the clarification of the reaction mechanism, our NMR studies also showed that the formylated ring-opened product **A** epimerizes in the slightly alkaline solution. In addition to the successive doubling of the signals for the formyl proton and the proton at C2, the NMR spectra showed that the doublet for the proton at C2 (5.2 ppm) changes into a singlet because of a hydrogen–deuterium exchange at position C3. This epimerization is due to the acidity of the C-3 proton, and is in agreement with our previous studies concerning the mechanism of epimerization of corresponding compounds which also contain two vicinal carboxylic ester groups.[18] The twofold deuteration of **A** was also confirmed by MS studies that revealed mass peaks at 358 $[M_{D2}+H]^+$ and 380 $[M_{D2}+Na]^+$ for the ring-opened compound **A**.

These results confirm our predictions insofar as the nucleophilic attack at the aziridine ring carbon atom takes place with a model thiol in solution. The results, however, cannot prove whether this reaction takes place with cysteine proteases. In such a system, the situation would change if the enzyme formylated at the cysteine sulfur atom, slowly hydrolyzed to release free enzyme and formic acid. As a consequence this reaction, which is endothermic if stopped at the formylated cysteine, would become exothermic and, because of its low reaction barriers, would probably become favorable. A hint for

such an inhibition mechanism is given by the inhibition constants found for cathepsin L and related proteases: The greatly improved k_2 value (approximately 5000 times higher) results mainly from a very low dissociation constant K_i (0.06–1.9 μM). This is remarkable, as the inhibitor does not contain any affinity-enhancing peptide sequences which could explain the low K_i values. In view of the instability of compound **2** against nucleophiles like DMSO (leading to the formation of formic acid) and this low dissociation constant, the double-step inhibition mechanism shown in Scheme 5 might be possible.

After formation of the noncovalent enzyme inhibitor complex EI_{CHO}, both an irreversible ring-opening reaction (pathway A) leading to an alkylated enzyme E-I_R and a simultaneously occurring reversible hemithioacetale formation (pathway B) could occur. The latter would lead to the intermediate E-I_{AP}, the enzyme inhibitor adduct produced by an attack at the formyl carbon. This intermediate would yield the formylated enzyme E_{CHO} and deformylated inhibitor I, and finally free enzyme E and formic acid resulting from slow hydrolysis (pathway C). These latter pathways could explain the very low K_i value, then being the product of K_{i1}, K_{i2}, and K_{i3}.

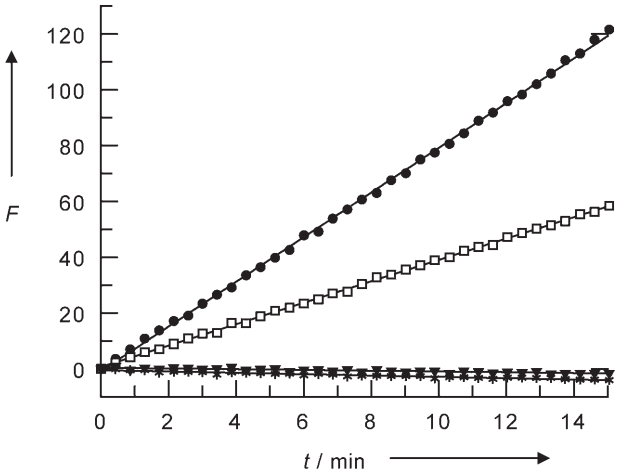
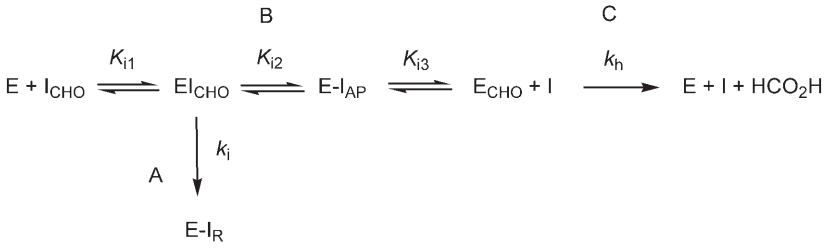


Figure 3. Results of the dialysis assays of **2** with cathepsin L. The assays show that the inhibition of cathepsin L by **2** is fully irreversible. From top to bottom: ● cathepsin L+substrate; □ cathepsin L→3 h dialysis→addition of substrate; ▼ cathepsin L+**2**→30 min incubation→addition of substrate; * cathepsin L+**2**→30 min incubation→3 h dialysis→addition of substrate; *F*, fluorescence units.



Scheme 5. Possible mechanisms of the inhibition of the cysteine protease cathepsin L by **2**. I_{CHO} = *N*-formylated aziridine **2**; EI_{CHO} = reversible, noncovalent enzyme inhibitor complex; I = aziridine **1**; E = enzyme; E-I_R = enzyme, alkylated by ring opening of **2**; E-I_{AP} = enzyme–inhibitor adduct (intermediate of the attack at the formyl carbon atom); E_{CHO} = *S*-formylated enzyme.

To evaluate if inhibition of cathepsin L by **2** is fully irreversible or if a partial reactivation of the enzyme may occur after removal of the inhibitor, dialysis assays were performed. These clearly proved the fully irreversible inhibition (Figure 3) underlining that the ring-opening pathway A also occurs in the reaction with the enzyme.

However, we cannot exclude that the reversible attack at the carbonyl carbon (pathway B) might also occur simultaneously with the irreversible pathway. If the reversible reaction occurs, the final hydrolysis step (pathway C) must be extremely slow as we did not find any reactivated enzyme after dialysis.

For the final clarification of the inhibition mechanism, NMR studies using the enzyme and a ^{13}C-labeled inhibitor could be a useful tool. Thus, our future efforts are directed toward the synthesis and NMR studies of ^{13}C-labeled inhibitors.

Summary

Aziridine-based peptides and peptidomimetics have proven to be irreversible inhibitors of cysteine proteases. To investigate in detail the influence of different substituents at the aziridine nitrogen atom on the kinetics and thermodynamics of the ring-opening reaction, we performed quantum chemical calculations with small model systems. These computations explain the enhanced inhibition potency of N-acylated aziridines. Substitution of the aziridine nitrogen atom with a formyl moiety, the smallest possible acyl group, halves the computed reaction barrier and increases the exothermicity of the reaction in comparison with the unsubstituted derivative. This influence results from the formyl substituent strongly stabilizing the negative charge of the evolving anion.

Reactions with diethyl 1-formyl aziridine-2,3-dicarboxylate (**2**) which were performed to verify the predictions, showed a 5000-fold enhanced inhibition potency relative to the N-unsubstituted analogue **1,** against the cysteine protease cathepsin L and related enzymes. This inhibition increase is mainly a result of very low dissociation constants K_i. Together with the hydrolytic instability of **2** in DMSO, this suggested a possible inhibition mechanism involving nucleophilic attack at the carbonyl carbon atom, as is also found for aldehyde-based inhibitors. Computations showed this pathway to exhibit very low reaction barriers, permitting a reversible attack at the carbonyl carbon of **2**. Thus, the computations nicely explain the reversibility of inhibition of cysteine proteases by aldehyde-based inhibitors. The model reaction with a thiolate, observed by NMR spectroscopy and mass spectrometry, and dialysis assays with cathepsin L, proved that the nucleophilic attack takes place at the aziridine ring carbon atoms and is fully irreversible.

Experimental Section

Computational details

The geometric parameters of the relevant stationary points of the potential energy surface were computed with the BLYP[28–30] functional in combination with a TZV + P basis set.[31] All stationary points were checked by frequency calculations. All energies were obtained from B3LYP[28] single-point calculations in combination with a TZV + P basis.[31] For gas phase this combined approach showed an excellent agreement with CCSD(T) results, while the BLYP underestimated the barrier heights considerably.[9] All calculations were performed with the TURBOMOLE[32] or the GAUSSIAN98 program package.[33]

In our model system, the attacking cysteine is mimicked by a methyl thiolate ($H_3C–S^-$) while the inhibitors are modeled by the three-membered ring systems $(H_2C)_2NR$ with R = H, CH_3, and CHO. Bulk effects of the environment are accounted for by employing the COSMO approach[34,35] with the standard parameter settings used in TURBOMOLE.[35–38] Molecular effects of the solvent are captured by a series of model systems in which solvent molecules with increasing proton-donating ability are placed in the vicinity of the heteroatom of the three-membered rings, and in the vicinity of the methyl thiolate. Water molecules were employed to mimic environments with weak proton-donating ability ($pK_a = 15.74$), while NH_4^+ ($pK_a = 9.25$) molecules were used to simulate environments with higher proton-donating abilities.

Computations showed[9] that in our model systems, entropy effects mainly influence the energy difference between reactant and transition state. This indicates that these effects are more connected with the association process (K_i) than with the reaction itself (k_i). Therefore in the present study, electronic energies instead of free energies are used.

Supporting Information Available: Synthesis and analytical data of inhibitor **2**, description of the enzyme and NMR assays, ^{1}H NMR spectra of the reaction of **2** with 4-methoxy thiophenol, ratios of reaction products of the reaction of compound **2** with 4-methoxy thiophenol.

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Keywords: cysteine proteases · inhibitors · quantum chemical computations

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