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Vinyl Sulfonium as Novel Proteolytic Enzyme Inhibitor

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Abstract—Vinyl sulfoniums were synthesized from vinyl sulfides by methylation, and inhibited the proteolytic enzyme papain. Inhibition studies suggest a mechanism by which the vinyl sulfonium inhibitor covalently and irreversibly modifies the enzyme.

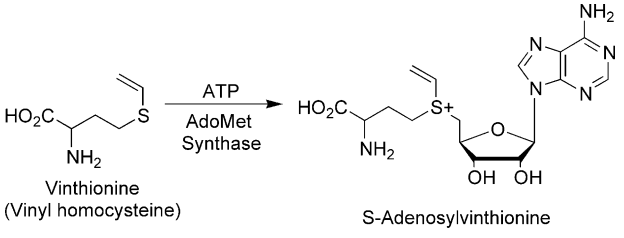
Enzymes play pivotal roles in biological processes, such as disease development and treatment. Selective modifications of enzymes by small molecules are important in understanding enzyme structure and mechanism, as well as in pharmaceutical discoveries. Screening a wide range of compounds—often containing different functionalities—is usually required to identify the desired inhibitors for a given enzyme. Therefore, developing new protein modification reagents based on novel functional groups will increase the chances to obtain enzyme inhibitors with tailored specificity and efficacy.

Vinyl sulfonium has been reported as an inhibitor for thioether methyltransferase (TEMTase) by Jerald Hoffman's group.[1] As shown in Scheme 1, ethylmethylvinyl sulfonium was generated from ethylvinyl sulfide and *S*-adenosylmethionine (AdoMet or SAM) by TEMTase. The vinyl sulfonium product covalently modified TEMTase, resulting in complete abolition of enzyme activity. Alternative substrates and their corresponding products, such as dimethyl sulfide and trimethyl sulfonium, protected the protein from such modification. These results suggested that the modification was likely to occur in the enzyme active site.[1]

S-Adenosylvinthionine is another vinyl sulfonium with intriguing biological activities.[2] It is produced from vinthionine (*S*-vinyl homocysteine) by *S*-adenosylmethionine synthetase, and is an analogue of *S*-adenosylmethionine, as shown in Scheme 2.[3] Radiolabeled [vinyl-^{14}C]vinthionine has been found to bind covalently to rat liver DNA, RNA and protein in vivo, but not in vitro.[2] *S*-Adenosylvinthionine has been postulated as the reactive alkylation reagent responsible for the covalent modification.[2]

Notwithstanding these interesting studies, vinyl sulfoniums have yet to be fully exploited for protein modification and enzyme inhibition. As outlined below, vinyl sulfoniums possess some unique chemical properties that make them excellent specific protein modification reagents. Firstly, vinyl sulfoniums are highly reactive towards nucleophiles.[4] Vinyl sulfoniums are more electrophilic than the corresponding vinyl sulfones, which have been used as cysteine protease inhibitors.[5,6] As illustrated in Scheme 3, Doering and Schreiber have suggested that the rapid reaction of vinyl sulfonium ion towards a nucleophile was attributed to the resonance stabilization of the carbanion by the sulfur atom of the sulfonium ylide intermediate.[4] They also have found that nucleophilic addition to the olefin was the predominant pathway, while nucleophilic

Scheme 1. TEMTase catalyzes the formation of vinyl sulfonium as its own inhibitor.



Scheme 2. Biosynthesis of *S*-adenosylvinthionine, a reactive analogue of *S*-adenosylmethionine.

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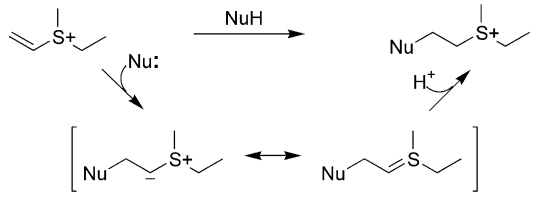


Scheme 3. Proposed reaction mechanism of nucleophilic addition to vinyl sulfonium.

substitution of the alkyl groups on the sulfur was insignificant.[4]

Secondly, reactivities of vinyl sulfoniums are sensitive to reaction environments. For example, both ethylmethylvinyl sulfonium and *S*-adenosylvinthionine are highly reactive towards nucleophiles in aprotic and nonpolar solvents, but are stable in aqueous solutions. They do not randomly react with proteins either.[1,2] The vinyl sulfonium reactant is highly charged and the transition-state of the addition reaction is less charged. As a result, relative to the transition state, the reactant is stabilized more in aqueous solutions than in nonpolar organic solvents.[7] Many protein binding sites are considered hydrophobic and nonpolar in nature.[8] In which case, vinyl sulfonium inhibitors that are inert in aqueous solutions can conceivably become extremely reactive once they bind to protein binding pockets. Lastly, various groups can be attached to the sulfur atoms in vinyl sulfoniums.[9] Several synthetic routes have been established. Structural variations of vinyl sulfoniums offer high probabilities to identify modification reagents, with tailored specificity and efficacy, to a given target protein. In summary, vinyl sulfoniums are highly reactive in nonpolar environments, but quite inert in aqueous solutions. These unique properties provide the chemical basis for vinyl sulfoniums as specific and potent inhibitors for a wide range of enzymes.

To test the generality of vinyl sulfonium as enzyme inhibitor, we synthesized several vinyl sulfoniums and tested their inhibitory activities against papain, a cysteine-protease. Many mechanism-based inhibitors for proteases contain electrophilic groups.[10,11] For this reason, we rationalized that vinyl sulfoniums would inhibit papain. Indeed, methylphenylvinyl sulfonium can completely abolish papain activity at low concentrations. Our studies suggest a mechanism by which the vinyl sulfonium inhibitor covalently and irreversibly modifies the enzyme. This work demonstrates the generality of vinyl sulfoniums as enzyme inhibitors.

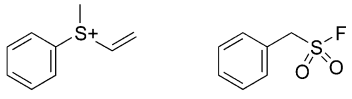
Inhibitor Design, Synthesis and Assay

Papain (EC 3.4.22.2) is an endopeptidase containing a nucleophilic cysteine in the active site.[11–13] The enzyme has a broad substrate specificity, accepting most of the amino acids at the P1 and P1′ positions.[11–13] As a result of its broad substrate specificity, papain is inhibited by a broad range of protease inhibitors with diverse structures, including phenylmethanesulfonyl fluoride (PMSF), tosyl phenylalanyl chloromethylketone (TPCK), tosyl lysyl chloromethylketone (TLCK), and L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64).[10–12] A majority of these mechanism-based inhibitors for proteases possess electrophilic groups (e.g., E-64 contains an epoxide group).[10–12] Naturally, we anticipated that papain would be a good model system to test the inhibitory activity of vinyl sulfoniums.

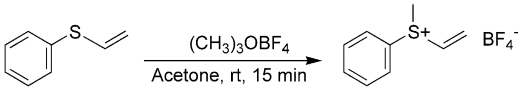
As mentioned earlier, Hoffman and co-workers[1] have reported that ethylmethylvinyl sulfonium inhibited the TEMTase enzyme, so we decided to test it for papain as well. Containing only simple alkyl groups and being highly charged, this compound might not bind to the papain active site with high affinity. On the other hand, methylphenylvinyl sulfonium and PMSF share similar spatial arrangement of the aromatic ring and the electrophilic center as shown in Scheme 4. For these reasons, we proposed that the aryl vinyl sulfonium was likely to bind to papain active site tighter and display higher inhibitory activity.

Following literature procedures, we have prepared vinyl sulfoniums from vinyl sulfides and various alkylation reagents, such as a combination of alkyl iodide and silver tetrafluoroborate.[9,14,15] However, there was a trace amount of residual silver ion in the final products when $AgBF_4$ was used. Because silver ion can inhibit papain by blocking the active site sulfydryl group,[16] we decided to use other methylation reagents in preparing vinyl sulfoniums. As depicted in Scheme 5, methylphenylvinyl sulfonium was synthesized from phenylvinyl sulfide and trimethyloxonium tetrafluoroborate in good yield.[17] Other sulfoniums used in this work were synthesized using the same method, and all gave satisfactory spectroscopic data.

Activity assay of papain was carried out according to a literature procedure.[18] In this assay, benzoyl-L-arginine *p*-nitroanilide (L-BAPA, 1 mM) was the substrate for papain, and reactions were monitored spectroscopically at 410 nm for the 4-nitroaniline product.[19] As shown in Figure 1, both ethylmethylvinyl sulfonium and methylphenylvinyl sulfonium reduced papain activity. The first vinyl sulfonium was, however, less active than the latter one. For example, at 400 μM, the ethylmethylvinyl sulfonium only inhibited 10% of papain activity, while methylphenylvinyl sulfonium inhibited more than 90%



Scheme 4. Structures of methylphenylvinyl sulfonium and papain inhibitor PMSF.



Scheme 5. Synthesis of vinyl sulfonium from vinyl sulfide using trimethyloxonium tetrafluoroborate as methylation reagent.

of the activity under the same condition. We hence focused our studies on methylphenylvinyl sulfonium.

We then investigated whether the inhibition was covalent and irreversible, as a nucleophilic addition mechanism depicted in Scheme 3 would suggest. Thus, 0.8 mL mixture of papain (1.1 mg/mL) and methylphenylvinyl sulfonium (1.2 mM) was extensively dialyzed against the assay buffer (3×100 mL) at 4 °C over a period of 12 h. In parallel, a solution of papain (1.2 mg/mL) was subjected to the same treatment. There was no precipitation in either of the samples after dialysis, and more than 80% of the proteins were recovered. The activities of the samples were determined using the standard assay, and the results are shown in Figure 2. There was no measurable activity loss for papain after dialysis, indicating the enzyme was stable under these conditions. There was no significant recovery of enzyme activity for the modified papain in 2 h (data not shown), suggesting that the inhibition was covalent and irreversible. On the other hand, prolonged dialysis regained about 20% of papain activity, suggesting the covalent modification was labile under these conditions. Another possible mechanism is that the methylphenylvinyl sulfonium acts as a tight-binding slow-release inhibitor to papain.

Given its simple structure, methylphenylvinyl sulfonium was not expected to be a tight-binding inhibitor of papain. Nevertheless, we decided to test this possibility by determining inhibitory activities of its structural analogues, dimethylphenyl sulfonium and ethylmethylphenyl sulfonium depicted in Scheme 6. As shown in Figure 3, these two sulfoniums only slightly reduced papain activities (less than 10%) at 800 μM, at which concentration the vinyl sulfonium counterpart abolished more than 90% of papain activity. With increased concentrations, these two sulfoniums did further reduce papain activities. Nonetheless, the effects were still small (e.g., in the presence of 4.0 mM of ethylmethylphenyl sulfonium, papain still maintained 80% of its activity). It is evident from these results that binding to papain alone cannot account for all the inhibitory activity of vinyl sulfonium, and covalent modification of papain by vinyl sulfonium must be involved in the inhibition.

There are two likely pathways for covalent modification of papain by vinyl sulfoniums. The first one involves nucleophilic attack on the vinyl group that is illustrated in Scheme 3; and the second possibility is a methyl transfer from the sulfonium to a nucleophile in papain. We have attempted to directly observe the inhibitor–papain adduct by mass spectroscopic analysis. However, the size heterogeneity of the commercial papain caused significant broadening of the signals for both modified and unmodified samples. We were, therefore, unable to unambiguously assess the nature of the modification by mass spectroscopic analysis. On the other hand, insight into the inhibition mechanism is provided from the studies on ethylmethylphenyl sulfonium and dimethylphenyl sulfonium. Prolonged incubation of these two sulfoniums with papain did not further enhance their inhibitory activities, implying that their inhibitory activities were not originated from covalent modification of papain. By analogy, it is indicative that modification of papain by methylphenylvinyl sulfonium involves nucleophilic addition to the vinyl group shown in Scheme 3.

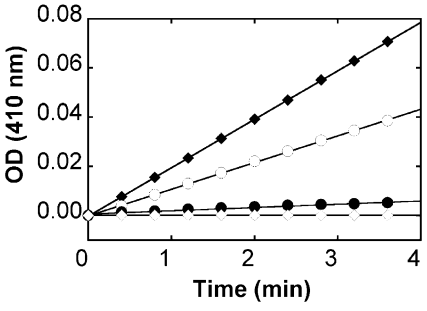
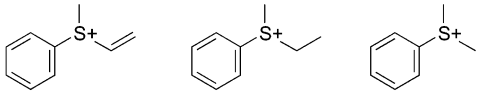


Figure 1. Time course of hydrolysis of L-BAPA (1 mM) at 25 °C by papain alone (1.3 μM, filled diamond); in the presence of ethylmethylvinyl sulfonium (10 mM, open circle) and methylphenylvinyl sulfonium (400 μM, filled circle); and without papain or inhibitors (open diamond).



Scheme 6. Structures of methylphenylvinyl sulfonium (left) and its analogues, ethylmethylphenyl sulfonium (center) and dimethylphenyl sulfonium (right).

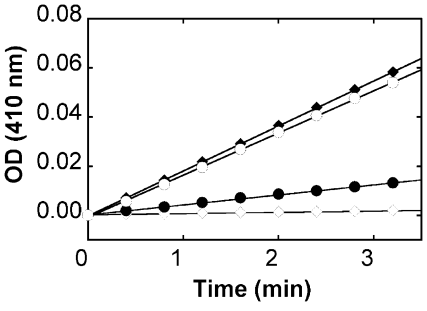


Figure 2. Total time course of hydrolysis of L-BAPA (1 mM) at 25 °C by unmodified papain before dialysis (filled diamond) and after dialysis (open circle), and methylphenylvinyl sulfonium-modified papain before dialysis (open diamond) and after dialysis (filled circle).

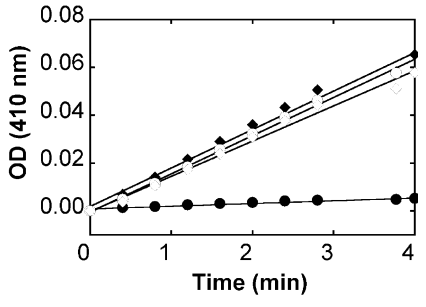


Figure 3. Papain-catalyzed hydrolysis of BAPA in the absence of inhibitor (filled diamond); in the presence of ethylmethylphenyl sulfonium (800 μM, open circle) and dimethylphenyl sulfonium (800 μM, open diamond); and in the presence of methylphenylvinyl sulfonium (400 μM, filled circle).

As expected from a nucleophilic addition to highly reactive vinyl sulfonium, the reaction between papain and methylphenylvinyl sulfonium appeared fast. Protein modifications were completed less than a minute, as shown in Figure 4. The compound was so reactive that it also reacted with dithiothreitol (DTT, 15 mM) present in the assay buffer. As shown in Figure 4, pre-incubation of the inhibitor in the assay buffer for 3 min before addition of the enzyme resulted in total loss of inhibitory activity. For these reasons, the concentration dependence of methylphenylvinyl sulfonium on papain inhibition that is shown in Figure 5 underestimates the efficiency of the inhibitor to a great extent. Considering the thiol concentration from DTT was 30 mM and the enzyme concentration was 1.3 μM (a ratio of 23,000 to 1), it is remarkable that the enzyme preferentially reacted with the inhibitor. This result agrees with our hypothesis that methylphenylvinyl sulfonium can bind to the papain active site, and desolvation enhances its reactivity toward the active site nucleophiles, most likely the catalytic cysteine residue.

Discussion and Conclusion

The work demonstrates that vinyl sulfonium is a novel enzyme inhibitor for proteolytic enzyme papain. Data gleaned from inhibition analyses support a mechanism by which the vinyl sulfonium binds to the papain, and then covalently and irreversibly modifies the enzyme via a nucleophilic addition reaction. The nucleophilic addition mechanism we proposed here is similar to that for vinyl sulfone, another type of mechanism-based cysteine protease inhibitor.[5,6] In contrast, a nucleophilic substitution reaction has been thought to account for enzyme modifications by peptidyl ketomethylsulfonium inhibitors, in which case the sulfoniums served as leaving groups.[20]

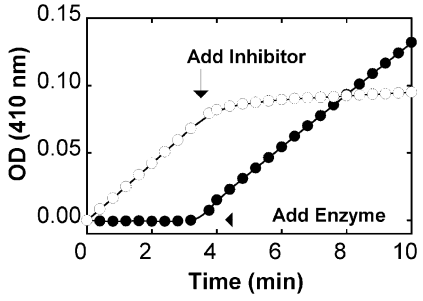


Figure 4. Time course of hydrolysis of L-BAPA. In one reaction, L-BAPA and papain were mixed first in the assay buffer, and then methylphenylvinyl sulfonium (400 μM) was added after 3.6 min (open circle). In the other reaction, L-BAPA and methylphenylvinyl sulfonium (400 μM) were mixed first in the assay buffer, and then papain was added after 3.6 min (filled circle).

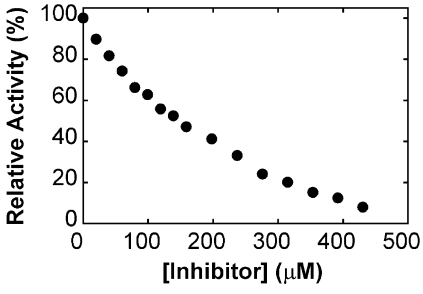
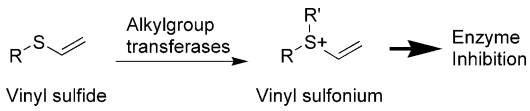


Figure 5. Concentration dependence of methylphenylvinyl sulfonium inhibitor on papain inhibition.



Scheme 7. Our proposal of using vinyl sulfide as mechanism-based inhibitor for alkyl transferases.

At this point, we have not yet been able to unambiguously assess the nature and sites of the modification. Further purification of the modified enzyme, followed by subsequent proteolytic digestion and protein mass spectroscopic analysis, is likely to pinpoint the precise mode of action. A better understanding of the inhibition mechanism will further improve the efficacy and specificity of vinyl sulfoniums as enzyme inhibitors. For instance, the reactivity of phenylvinyl sulfonium can be fine tuned by strategic placement of different substituents on the benzene ring.

The high intrinsic reactivity and structural diversity of vinyl sulfonium make it a promising candidate as a general mechanism-based enzyme inhibitor and pharmaceutical agent. Naturally, vinyl sulfoniums are likely to be excellent inhibitors for other cysteine proteases as well. Cysteine proteases play versatile and essential roles in disease formation and treatment.[10,11,13] For example, cathepsins are involved in progressive cartilage and bone degradation associated with arthritis;[21] and falcipain plays an indispensable role in hemoglobin degradation by *Plasmodium falciparum*, the most virulent human malaria parasite.[22] Design and synthesis of potent and selective inhibitors for these enzymes is an attractive approach to develop new chemotherapeutic treatments for these diseases.

In summary, we have shown that vinyl sulfoniums inhibit proteolytic enzyme papain. Our studies suggest a mechanism by which the vinyl sulfonium inhibitor covalently and irreversibly modifies the enzyme. This work also suggests the possibility of using vinyl sulfide as mechanism-based enzyme inhibitor (or suicide inhibitor) for enzymes that augment alkyl-group transfers, as illustrated in Scheme 7.

Acknowledgements

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17. Details for the synthesis of methylphenylvinyl sulfonium (CA Registry No. 63254-80-8): To a solution of phenylvinyl sulfide (675 μL, 5 mmol) in 2 mL anhydrous acetone, trimethyloxonium tetrafluoroborate (74 mg, 0.5 mmol) was added. The resulting mixture was stirred at rt for 15 min. The reaction was quenched by the addition of 0.5 mL methanol and stirred for 5 min. The solvent was evaporated in vacuo. Water (10 mL) was added to the residue and the aqueous solution was extracted with ether (10 mL×3). Solvent from the remaining aqueous solution was evaporated in vacuo. Dark brown sticky oil was obtained as the desired sulfonium (76 mg, 64% based on trimethyloxonium tetrafluoroborate). Dimethylphenyl sulfide was formed as the side product. Rearrangement of sulfonium has been reported in the literature (for an early report see: Ray, F. E.; Farmer, J. L. *J. Org. Chem.* **1943**, *8*, 391). Increasing the ratio of vinyl sulfide to methylation reagents minimized the side reaction. Under the above condition, the final product contained a trace amount (~1%) of dimethylphenyl sulfonium. ^{1}H NMR (acetone-d_6, 300 MHz): δ 8.09 (m, 2H), 7.80 (m, 3H), 7.19 (m, 1H), 6.18 (m, 2H), 3.61 (s, 3H). ^{13}C NMR (acetone-d_6, 75 MHz): δ 135.11, 134.54, 131.32, 130.35, 126.23, 125.32, 27.79. DEPT: CH, δ 134.54, 131.32, 130.35, 125.32; CH_2 δ 135.11; CH_3 δ 27.79. MALDI-MS (positive): calcd for $C_9H_{11}S^+$ 151.06, found 151.15. UV_{max} (H_2O): 223; and weak triplet at 266 nm.
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19. All kinetic measurements were performed at 25 °C in aqueous buffer containing 50 mM potassium phosphate, 2.5 mM ethylenediaminetetraacetate (EDTA) and 15 mM dithiothreitol (DTT). Stock solution of papain (1 mg/mL, P4762 from Sigma, 2×crystallized) was prepared in the assay buffer, incubated at rt for 60 min to activate the enzyme, and then stored on ice prior to all assays. Enzyme concentration was determined from the absorption at 278 nm ($\varepsilon = 2.5$ mg^{-1} cm^{-2}), and was 30 μg/mL (1.3 μM) in the reaction mixtures. Benzoyl-L-arginine *p*-nitroanilide (L-BAPA) was used as the substrate at a final concentration of 1 mM. Reaction courses were monitored spectroscopically at 410 nm for the 4-nitroaniline product. Stock solutions of sulfoniums were in water at concentrations of 20–80 mM, and were prepared freshly prior to all assays.
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