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Possible involvement of radical intermediates in the inhibition of cysteine proteases by allenyl esters and amides

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Dedicated to the memory of Professor Toshio Satoh of Tokushima Bunri University who made many lasting contributions to the field of medicinal chemistry.

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ABSTRACT

In order to investigate crystallographically the mechanism of inhibition of cysteine protease by α-methyl-γ,γ-diphenylallenecarboxylic acid ethyl ester **3**, a cysteine protease inhibitor having in vivo stability, we synthesized *N*-(α-methyl-γ,γ-diphenylallenecarbonyl)-L-phenylalanine ethyl ester **4**. Reaction of **4** with thiophenol, the SH group of which has similar pK_a value to that of cysteine protease, produced oxygen-mediated radical adducts **6** and **7** in ambient air but did not proceed under oxygen-free conditions. Catalytic activities of two thiol enzymes including cathepsin B were also lowered in the absence of oxygen. These results suggest that cysteine protease can act through an oxygen-dependent radical mechanism.

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Cysteine proteases are an important class of peptide-processing enzymes and, as the name implies, are characterized by having a cysteine residue within the active site.[1] These proteases are also ubiquitous enzymes that play important roles in many biochemical processes.[1,2] The presence of cysteine proteases in pathogenic microorganisms and roles in facilitating metastases of tumors are examples of many factors that make selective cysteine protease inhibitors important targets for drug design.[1] As a consequence of the wide therapeutic potential of these compounds, many cysteine protease inhibitors have been developed.[3] In much of the design of these inhibitors, it has been assumed that the catalytic mechanism of cysteine proteases is similar to that of serine proteases. Thus, the cysteinyl and histidinyl residues present in the active site form a thiolate/imidazolium ion pair that promotes the formation of an *S*-acyl-enzyme intermediate that is necessary for cleavage of the peptide bond.[4] However, despite the similarities of the proposed catalytic mechanism of cysteine and serine proteases, important differences have been revealed. Thus, the replacement of the active site serine with cysteine in two serine proteases (trypsin and subtilisin) abolished activity.[5] This result implies that cysteine proteases have another reaction mechanism, for example, a radical reaction mechanism that has been found in several enzymatic systems.[6]

Allenyl esters **2** derived from the hydrolysis and decarboxylation of diethyl α-alkynylmalonates **1** have been shown to inhibit the catalytic activity of cathepsin B in vitro, but do not inhibit

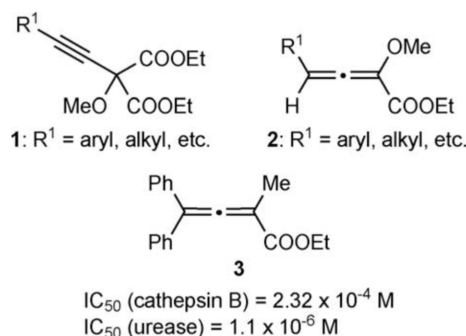

Figure 1. Structures of compounds **1**–**3** and inhibitory activities of **3**.[10]

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in vivo (Fig. 1). This lack of in vivo inhibition was ascribed to depletion of the inhibitor through competing reactions with endogenous low molecular weight thiol compounds such as cysteine and reduced glutathione.[7] As a result of our efforts to develop allene compounds which would be stable and active in vivo, we found that α-methyl-γ,γ-diphenylallenecarboxylic acid ethyl ester **3** could inhibit the catalytic activities of cathepsin B[8] ($IC_{50} = 2.32 \times 10^{-4}$ M) and urease[9] ($IC_{50} = 1.1 \times 10^{-6}$ M).[10] Compound **3** was stable in vivo and inhibited water immersion stress-induced ulcer formation in rats (Fig. 2).[10b] Thus we examined the reaction mechanism of **3** with such thiol enzymes.

In order to simplify the understanding of results, we employed low weight thiol compounds as model compounds of cysteine protease. The reactions of **3** with L-cysteine and reduced glutathione did not proceed in aqueous ethanol at 37 °C as we expected from the in vivo stability of **3**. These results were attributable to the relatively higher pK_a values of cysteine (pK_a 8.3)[11] and reduced glutathione (pK_a 8.7)[11] compared to cysteine proteases (pK_a 2.5–8.0)[12] which results from the formation of the ion pair with the histidine of the active site. We thus tried to examine the reaction of the conjugated allene system with the more acidic thiophenol (pK_a 6.6)[11] and to clarify the structure of the products unambiguously by X-ray crystallographic analysis. Since it was rather difficult to crystallize **3**, we designed amide **4** as a suitable model compound for this study because introduction of the L-phenylalanine ethyl ester unit through the amide bond was expected to make crystallization of the products easy due to the presence of the amide bond and the additional aromatic group.

Compound **3** was hydrolyzed with sodium hydroxide in aqueous EtOH to afford the corresponding carboxylic acid **5** in 60% yield (Scheme 1). Condensation of **5** with L-phenylalanine ethyl ester was carried out with 1,3-dicyclohexylcarbodiimide (DCC) in dichloromethane to give amide **4** in 60% yield. Preliminary biological evaluation of **4** showed that compound **4** has moderate inhibitory activity (56% at 10^{-3} M) toward cathepsin B. Reaction of **4** with thiophenol in benzene proceeded smoothly to give a mixture of diastereomers **6** and **7** isolated as crystals, as expected, in 43% and 23% yields, respectively.[13] The structure of **6** was confirmed by X-ray crystallographic analysis (Fig. 3).[14] If the addition of thiophenol to **4** occurs by simple nucleophilic addition, a proton will add to the α-carbon of the allene carboxylic acid moiety of the product. The presence of the hydroxyl group at this position in compounds **6** and **7** indicates that the reaction of **4** with thiophenol is not a simple nucleophilic addition reaction. This product is indicative of a radical reaction initiated by oxygen.

Based on the assumption that oxygen would be the initiator of any radical process in this system, we next examined the same reaction under an oxygen-free condition. Parallel reactions were carried out, one under an oxygen-free argon atmosphere, and the other in ambient air, and these were monitored by HPLC.[15] After 6 h, there was no apparent reaction in the absence of oxygen, whereas the reaction under ambient air had gone to completion. These results strongly indicate that the reaction of **4** with thiophenol is, in fact, a radical process that is dependent on the presence of oxygen. Indeed, Mueller and Griesbaum[16] reported that addition reaction of thiolate ions to allene derivatives may proceed via a radical mechanism, not a nucleophilic mechanism, from the studies on reaction of thiolate ions with some allene derivatives. They also indicated that reaction of thiolate ions to tetra-substituted allene derivatives is somewhat difficult because, unlike mono-, di-, and tri-substituted allene structures, a tetra-substituted allene structure cannot be isomerized to an acetylene form, which is assumed to be an active form to react with thiolate ions. Based on this evidence, in vivo stability of our allene derivatives in contrast to that of **2** may be due to their fully substituted structure.

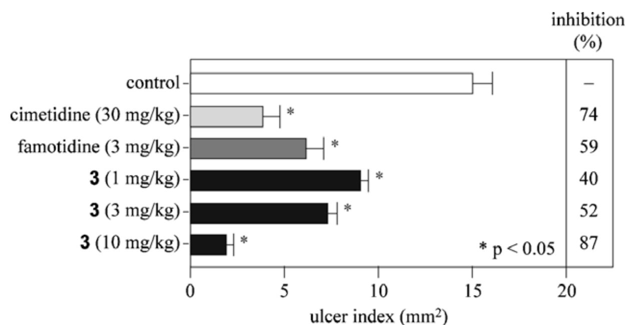
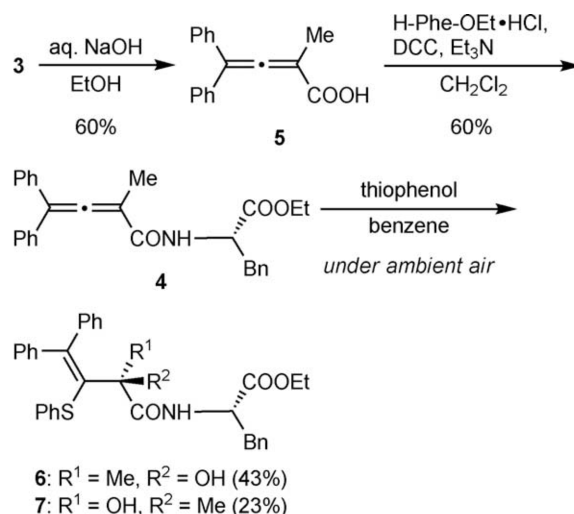


Figure 2. Effect of **3** on ulcer formation induced by water immersion stress in rats.[10b]



Scheme 1. Synthesis of amide **4** and reaction of **4** with thiophenol under ambient air.

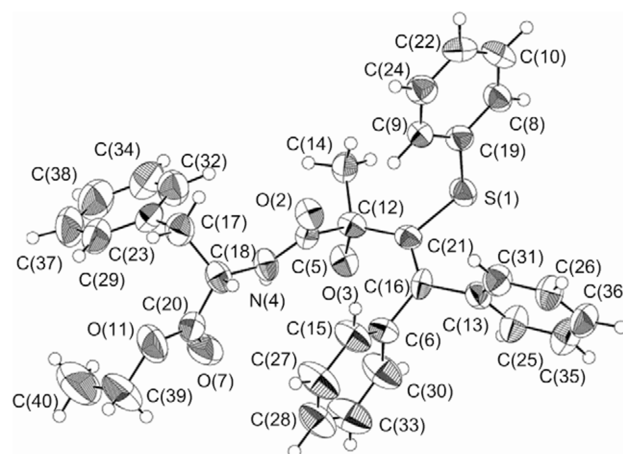


Figure 3. ORTEP drawing of compound **6**.

Table 1
Catalytic activities of cathepsin B and urease under oxygen-free condition

SH-enzyme	% Catalytic activity[a]
Cathepsin B[b]	72 (±6.50)
Urease[c]	60 (±10.5)

[a] Catalytic activity under ambient air taken as 100% (all values are means of at least three experiments, standard deviation is given in parentheses).
[b] The activity was measured by the method of Inubushi et al.[21]
[c] The activity was measured by the method of Smith et al.[22]

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