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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.

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

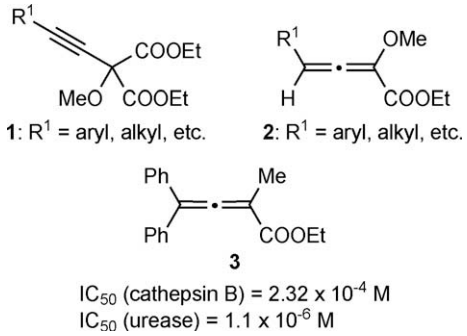

Figure 1. Structures of compounds **1**–**3** and inhibitory activities of **3**.[10]

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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,

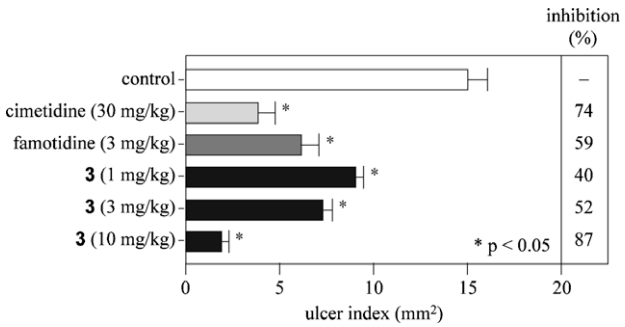
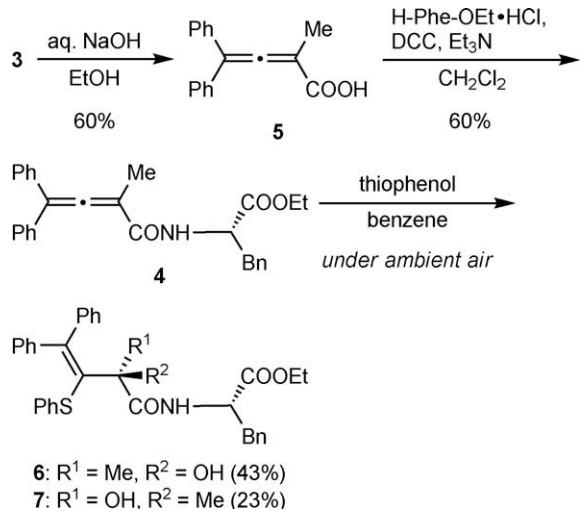


Figure 2. Effect of **3** on ulcer formation induced by water immersion stress in rats.[10b]



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

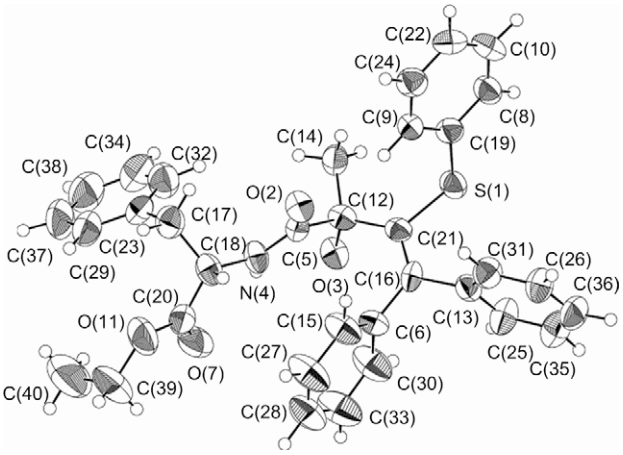


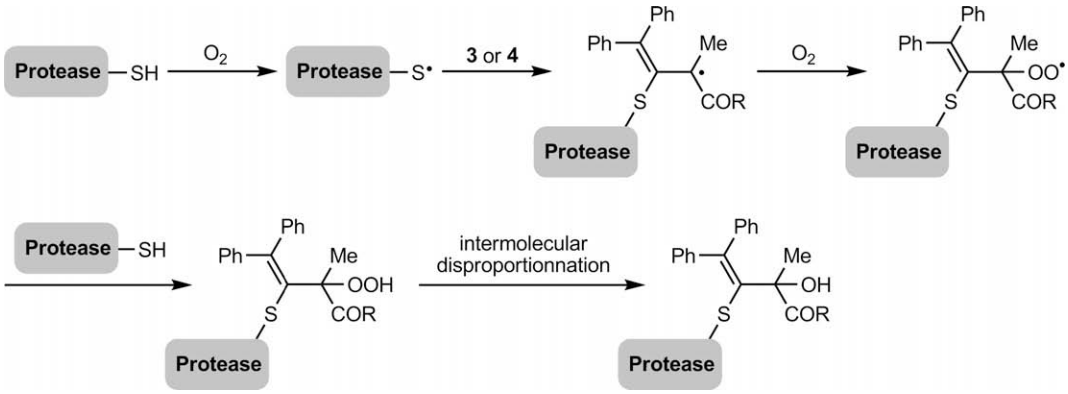
Figure 3. ORTEP drawing of compound **6**.

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.

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]



Scheme 2. Proposed mechanism for inhibition of cysteine protease by allenyl ester **3** (R = OEt) and amide **4** (R = NHCH(Bn)COOEt).

There are several reports describing the oxidative inhibition of caspases,[17] a family of cysteine proteases, with oxidants such as nitric oxide,[18] hydrogen peroxide,[19] or peptidyl peroxide.[20] However, we have found no report that describes the oxidative activation of cysteine proteases with an exogenous oxidant. Activation resulting from the presence of oxygen should necessarily result from oxygen functioning as an initiator of a radical-mediated reaction. However, to our knowledge, such a radical-mediated reaction of cysteine proteases previously has never been considered. If the reaction of cysteine proteases with **3** were to proceed via a mechanism similar that of thiophenol, then a decreased catalytic activity of the protease should be observed under oxygen-free conditions. Thus, we examined the catalytic reaction of cathepsin B under an oxygen-free argon atmosphere. Under the oxygen-free condition, the catalytic activity of cathepsin B was, in fact, lowered, but not totally lost (Table 1). This result strongly indicates that a radical intermediate, which is initiated with oxygen, is involved in the catalytic reaction of cysteine proteases. The fact that oxygen depletion results in only partial inactivation also suggests that cysteine proteases may include both radical and nucleophilic components to the mechanism of the catalytic reaction, although it is also possible that residual traces of oxygen in the solution may be a factor in this result. It is also interesting that the catalytic activity of urease was lowered under an oxygen-free argon atmosphere (Table 1).

In summary, based on similarity of the pK_a value of the SH group to cysteine protease, we employed thiophenol as a model compound to investigate the mechanism of the inhibition of cysteine protease by allenyl ester **3**. Reaction of amide **4**, a more highly crystalline derivative of allene compound **3**, with thiophenol proceeded by a mechanism involving radical species, formation of which was initiated by oxygen. It should be noted that the catalytic activities of cathepsin B and urease also depended on the presence or absence of oxygen. These results suggest that cysteine proteases can act as radical catalysts using oxygen as an initiator. On the basis of these results and on information from the literature,[23] we propose the mechanism for inhibition of cysteine proteases by **3** and **4** in Scheme 2. Further studies on the involvement of radical species in reaction of cysteine protease, including direct observation of radical intermediates by ESR, are currently underway.

Acknowledgments

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13. To a solution of **4** (2.00 g, 4.7 mmol) in dry benzene (20 mL) was added thiophenol (5.0 mL, 47 mmol). After stirring at room temperature for 48 h, the resulting mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (benzene/EtOAc 10/1) to give **6** (1.05 g, 43%) and **7** (0.56 g, 23%). (*S*)-2-Hydroxy-2-methyl-4,4-diphenyl-3-thiophenyl-3-butenecarbonyl-L-phenylalanine ethyl ester (**6**): White prisms; mp 127–129 °C; $[\alpha]_D^{20}$ −4° (*c* 1.35, $CHCl_3$); IR (KBr) 3057, 1740, 1657 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.22 (3H, t, *J* = 7.1 Hz), 1.58 (3H, s), 3.06 (2H, m), 3.32 (1H, s), 4.14 (2H, q, *J* = 7.3 Hz), 4.41 (1H, q, *J* = 6.2 Hz), 6.94–7.31 (20H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 27.9, 29.7, 37.8, 53.6, 61.5, 78.5, 126.7, 127.1, 127.6, 127.9, 128.5, 129.1, 129.3, 135.6, 136.1, 137.3, 142.1, 143.9, 167.7, 171.2, 174.1; MS (EI) *m*/*z* 551 (M^+). (*R*)-2-Hydroxy-2-methyl-4,4-diphenyl-3-thiophenyl-3-butenecarbonyl-L-phenylalanine ethyl ester (**7**): White prisms; mp 146–149 °C; $[\alpha]_D^{20}$ +37° (*c* 0.91, $CHCl_3$); IR (KBr) 3406, 1738, 1658 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.23 (3H, t, *J* = 7.3 Hz), 1.57 (3H, s), 3.01 (2H, d, *J* = 5.9 Hz), 3.60 (1H, s), 4.17 (2H, q, *J* = 7.2 Hz), 4.53 (1H, q, *J* = 5.9 Hz), 6.99–7.26 (20H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 27.9, 29.7, 38.0, 53.4, 61.4, 78.9, 125.9, 126.8, 127.1, 127.6, 127.9, 128.5, 129.1, 129.4, 135.7, 136.0, 137.5, 141.8, 144.0, 170.9, 173.8, 195.9; MS (EI) *m*/*z* 551 (M^+).
14. Crystal data of **6**: $C_{34}H_{33}NO_4S$, orthorhombic, $P2_12_12_1$, *M* = 551.705, *a* = 5.9840 (3) Å, *b* = 20.514 (2) Å, *c* = 24.393 (3) Å, *V* = 2994.4 (5) $Å^3$, *Z* = 4, *Dc* = 1.224 Mg/m^3, μ(MoKα) = 0.146 mm^{-1}, *T* = 298 K, colorless prism (0.35 × 0.2 × 0.15 mm), 4253 measured, 4242 independent, *R* = 0.073, *wR* = 0.157 for 2477 observed reflections [$I > 2\sigma$ (*I*)]. CCDC 699505 contains the supplementary crystallographic data for this molecule. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.
15. Compound **4** (7.7 mg, 0.018 mmol) was dissolved in dry benzene (0.77 mL) under an oxygen-free argon atmosphere or ambient air. The oxygen-free condition was obtained by bubbling argon through the solution. Thiophenol (12.8 μL, 0.125 mmol) was added to the solution and aliquots were taken after stirring at room temperature for 1, 3, and 6 h and analyzed by HPLC using a Chiralcel OD-R column (4.6 × 250 mm) (Daicel Chemical Industries, Ltd., Tokyo, Japan), a mixture of acetonitrile/water (6/4) was used for the mobile phase. The UV absorptions of the products were measured at 254 nm.
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21. Cathepsin B enzyme assay: To a solution of 4 mM sodium ethylenediaminetetraacetate–0.4 M sodium acetate buffer (pH 5.5) in water (250 μL) was added water (350 μL), 0.2 M L-cysteine (40 μL), 2 U/mL cathepsin B (10 μL, BioPur, Bubendorf, Switzerland) under an oxygen-free argon atmosphere or ambient air. The oxygen-free condition was obtained by bubbling argon through all solutions except the water solution of cathepsin B. After incubating at room temperature for 3 min, a solution of 80 μM Z-Phe-Arg-MCA (Peptide Institute, Osaka, Japan) in water (250 μL) was added to the mixture. Aliquots were taken after incubating at 37 °C for 5 min, and then monochloroacetic acid (1 mL, pH 4.3) was added to terminate the reaction. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin in the mixture was measured at excitation 370/emission 460 nm on a HITACHI F-3000 fluorescence spectrophotometer (Tokyo, Japan). See also: Inubushi, T.; Kakegawa, H.; Kishino, Y.; Katunuma, N. *J. Biochem.* **1994**, *116*, 282.
22. Urease enzyme assay: To a solution of 2.4 U/mL urease (Toyobo Co. Ltd., Osaka, Japan) in water (100 μL) was added 0.1 M phosphate buffer (650 μL, pH 7.0) under an oxygen-free argon atmosphere or ambient air. The oxygen-free condition was obtained by bubbling argon through all solutions except the water solution of urease. After incubating at 37 °C for 10 min, a solution of 2 mg/mL urea in water (100 μL) was added to the mixture. The mixture was incubated at 37 °C for 15 min. To the mixture was added solutions of 10% potassium carbonate in water (500 μL) and 0.5% dansyl chloride in acetone (600 μL). The mixture was incubated at 55 °C for 90 min, and then a solution of 5% glycine in water (3 mL) was added. After incubating at 55 °C for 30 min, water (5 mL) was added to the mixture. The mixture was allowed to cool to room temperature and extracted with chloroform (2 mL). The organic layer was concentrated to give dansyl amide. The obtained dansyl amide was dissolved in a solution of 1 μM dansyl methyl amide in acetonitrile (1 mL). Aliquots were taken and analyzed by HPLC using a YMC-Pack ODS-A (6.0 × 150 mm) (Yamazen Co., Osaka, Japan), a solution of 50% acetonitrile in 17.2 mM phosphate buffer (pH 7.2) was used for the elution phase. The fluorescence intensity of the obtained dansyl amide was measured at excitation 340/emission 530 nm on a JASCO FP-920 fluorescence detector (Tokyo, Japan). See also: Smith, J. R. L.; Smart, A. U.; Hancock, F. E.; Twigg, M. V. *Chem. Ind.* **1989**, 353.
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