Molecular Design of Potent Inhibitor Specific for Cathepsin B Based on the Tertiary Structure Prediction

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To design a potent inhibitor specific for cathepsin B (rat liver), the tertiary structure was predicted based on the crystal structure of the papain complexed with (+)-(2*S*,3*S*)-3-{1-[*N*-(3-methylbutyl)amino]leucylcarbonyl}oxirane-2-carbolylic acid (E-64-c), a thiol protease inhibitor. Taking advantage of the structural characteristics of the predicted active site, seventeen inhibitors were chemically synthesized by molecular modeling, and one of them, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) was shown to be the first potent inhibitor specific for cathepsin B. The relationship between the structure and inhibitory activity is discussed based on the model structure of the cathepsin B–inhibitor complex.

Keywords CA-074; molecular design; cathepsin B; tertiary structure prediction; papain–E-64-c complex; restrained molecular dynamics simulation

Introduction

Cathepsin B (EC 3.4.22.1) is one of the lysosomal thiol proteases found in mammalian cells,[1,2] and has major roles not only in the breakdown of intracellular protein but also in the degradation of extracellular-matrix proteins including collagen and elastin. Since the overexpression of cathepsin B has been implicated in a number of diseased states such as muscular dystrophy,[3] osteoporosis,[4] pulmonary emphysema[5,6] and tumor invasion,[7] it is of special importance to develop the potent inhibitors specific for this protease; they are of critical importance in establishing the biological role of this enzyme involved in these diseases and in developing therapeutic drugs. Although several classes of inhibitors such as leupeptin and antipain[8,9] have already been developed as inhibitors for thiol proteases, none of them can specifically distinguish cathepsin B from other thiol proteases.

We recently analyzed the X-ray crystal structure of papain–E-64-c complex at 2.1 Å resolution,[10] where the E-64-c (**1**) is a potent irreversible inhibitor for thiol proteases developed from the parent compound E-64 (**2**),[11,12] and the inhibitory mechanism of E-64-c was elucidated at the atomic level.[13,14] A conventional method to estimate the tertiary structure of a target protein from the X-ray crystal structure of a homologous protein was also developed using restrained molecular dynamics (RMD) simulation.[15] Since both the cathepsin B and papain belong to the thiol proteases, the tertiary structures around their catalytic sites

TABLE I. Comparison of the Amino Acid Sequence from Rat Liver Cathepsin B and Papain

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                1         10                  20         30         40         50
Papain      :IPEYVDWRQKGAVTPVKNQ----GSCGSCWAFSAVVTIEGIIKIRTGNLNQYSE
              ||   | |        |    |     |||||||||  ||       | | | | |
Cathepsin B :LPESFDAREQWSNCPTIAQIRDQGSCGSCWAFGAVEAMSDRICIHT-NVNVEVS
              1         10         20         30         40         50

                          60         70         80         90
Papain      :QE-LLDC------DRRSYGCNGGYPWSALQLVAQYGIHY-RNTYPYEGVQRY--
              | || |       |     |||||||  |         |     |        ||
Cathepsin B :AEDLLTCCGIQCGD----GCNGGYPSGA-------GNFWTRKGLVSGGVYNSHI
                    60         70                  80         90

                   100        110        120
Papain      :-CRSREKGPYAAKTDGVRQVQPYNQGALL-----------YS------------
              |       |       | |    |    |                 ||
Cathepsin B :GCLPYTIPPCEHHVNGSR---PPCTGEGDTPKCNKMCEAGYSTSYKEDKHYGYT
               100        110        120        130        140

                                  130                 140        150
Papain      :---------------IA-NQPV-------SVVLQAAGKDFQLYRGGIFVGPCGNK
                           |   | ||       |         ||   |   |       |
Cathepsin B :SYSVSDSEKEIMAEIYKNGPVEGAFTVFS--------DFLTYKSGVYKHEAGDV
              150        160        170                 180        190

                 160                 170        180        190              200
Papain      :VD-HAVAAVGYG-----PNYILIKNSWGTGWGENGYIRIKRGT---GN-SYGVC
               ||      | |       | | |   |||    ||  ||    | ||      |   |   |
Cathepsin B :MGGHAIRILGWGIENGVP-YWLVANSWNVDWGDNGFFKILRGENHCGIESEIVA
                    200        210        220        230        240

                   210
Papain      :GLYTSSFYPVKN
              |
Cathepsin B :GIPRTQ
               250
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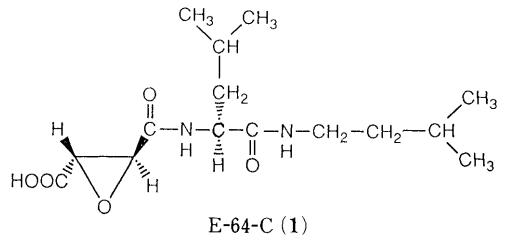
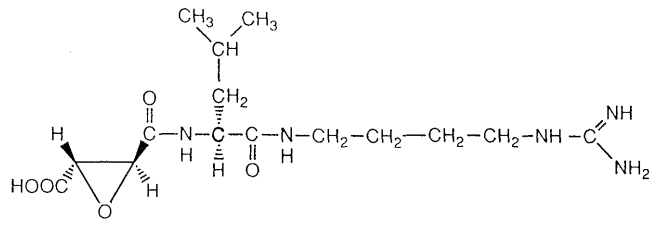
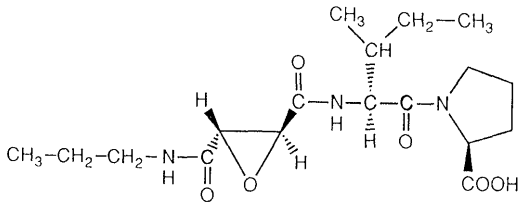


E-64-C (1)



E-64 (2)



CA-074 (3)

Fig. 1. Chemical Formulae of Three Important Epoxysuccinylpeptides Considered for Molecular Design

1, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucylamino-3-methylbutane (E-64-c); **2**, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucylagmatine (E-64); **3**, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074).

may be similar, although the homology between them is relatively low (about 29%); Table I shows the sequence alignment of papain and cathepsin B (rat liver).[16] Since no three-dimensional structure on a cathepsin B active site is available at present, the prediction of a possible tertiary structure by the RMD simulation method was attempted to design the specific inhibitors.

L-*trans*-Epoxysuccinic acid group was used as a fundamental chemical structure to develop the inhibitors, because it has the advantage of forming an irreversible covalent bond with the S^{γ} atom of catalytic Cys residue, and of being able to utilize much information on the structure–activity relationships concerning E-64 and its analogues.[11,12,17,18] Based on the molecular fitting to the cathepsin B active site, seventeen compounds were designed, and *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline, CA-074 (**3**), was shown to be a potent inhibitor specific for cathepsin B *in vitro* and *in vivo* in rats.[19,20] This paper deals with the details leading to the molecular design of CA-074. The chemical structures of **1**—**3** are shown in Fig. 1.

Experimental

Materials All compounds designed were chemically synthesized according to the method of Tamai *et al.*[18] with some modification, and their purities were checked by nuclear magnetic resonance (NMR) measurements and thin layer chromatography (TLC). The details of the syntheses will be published elsewhere. Rat liver cathepsin B was purified as described previously[21-23] with the additional purification steps of high pressure liquid chromatography (HPLC) on TSK gel G 3000 SW and concanavalin A (Con A)-Sepharose. Papain was purchased from Sigma Co. (U.S.A.), and purified as described by Kimmel and Smith.[24]

TABLE II. Structural Modification of P Site and Inhibitory Activity Values (IC_{50}, nM) of Epoxysuccinyl Peptides for Cathepsin B and Papain

Compound	Structure	Cathepsin B	Papain
Ioxistatin	EtO–tES–Leu–NH–CH_2–CH_2–$CH(CH_3)_2$	3.4	13
4	EtO–tES–Ile–OH	24000	132000
5	EtO–tES–Ile–Pro–OH	3	16000
6	EtO–tES–Pro–Pro–OH	50	>100000
7	EtO–tES–Ile–Ala–OH	23	5800
8	EtO–tES–Gly–Pro–OH	15300	95400
9	EtO–tES–Ile–Pro–OBzl	930	2000

tES: L-*trans*-epoxysuccinyl.

TABLE III. Structural Modification of Epoxysuccinyl Peptide P′ Site and Inhibitory Activity Values (IC_{50}, nM) for Cathepsin B and Papain

Compound	R	Cathepsin B	Papain
RO–tES–Ile–Pro–OH			
10	H	30.4	250
11	Me	20.0	589
5	Et	3	16000
12	iso-Pr	1.5	62200
13	iso-Bu	1.4	3300
14	cyclo-Hex	1.1	5400
R–tES–Ile–Pro–OH			
15	Et_2N	2080	111000
16	EtNH	6.9	53000
17	iso-PrNH	4.6	55000
3(CA-074)	*n*-PrNH	2.2	57400
18	iso-BuNH	1.8	60000
19	cyclo-HexNH	2.2	14200

tES, L-*trans*-epoxysuccinyl; iso-Pr, isopropyl; iso-Bu, isobutyl; cyclo-Hex, cyclohexyl; *n*-Pr, *n*-propyl.

Measurements of Inhibitory Activities Inhibitory activities of compounds synthesized were expressed as the 50% inhibitory concentration values (IC_{50}, nM), according to the experimental procedure in previous papers.[19,20] The specificities for cathepsin B and papain were judged from respective IC_{50} values.

Prediction of Cathepsin B Tertiary Structure The RMD simulation method[15] we recently developed was applied to predict the tertiary structure of cathepsin B based on the X-ray crystal structure of papain–E-64-c complex, where the united-atom parameters[25] were used for the MD calculations.

The complete amino acid sequence of rat liver cathepsin B[16] was used to construct the tertiary structure. Matching between the papain and cathepsin B sequences was done so as to achieve maximal similarity employing the program BION (IntelliGenetics, Inc., CA), and the resultant alignment is presented in Table I.[26] Treatment of the insertion/deletion regions between the predicting and reference enzymes and the actual computational procedure for constructing possible tertiary structure by the RMD simulation method were discussed in detail previously,[15] and the same methodology was used. For the common regions of the two enzymes, energy restraints were imposed on the interatomic C_{α} distances and backbone torsion angles of cathepsin B in order to construct the same spatial orientations as those of papain, while no restraints were applied on the insertion or deletion regions. Solvent water molecules were not included in the structure generation of cathepsin B, but the effect of solvent was approximated by the distance-dependent dielectric constant. The RMD simulation was carried out during 120 ps, where the system was treated as having a constant temperature of 310 K, time step of 0.5 fs, nonbonded cutoff of 8 Å, and temperature relaxation time of 0.02 ps. After generating the tertiary structure of cathepsin B including four disulfide bonds according to the computational protocol previously proposed,[15] the structure was simulated during 10 ps without imposing any restraint. Since the energy profile indicated that the structure was satisfactorily equilibrated, the final structure at 10 ps (total of 130 ps from beginning)

was selected as representative of the predicted structure of cathepsin B. The adaptability of this approach to estimate the tertiary structure of a protein from the X-ray crystal structure of a family reference protein has already been ascertained by comparison between the predicted and X-ray analyzed structures of *Crotalus atrox* venom phospholipase A_2 and of bovine pancrestic β-trypsin.[15]

Modeling of Inhibitor–Cathepsin B Complex L-*trans*-Epoxysuccinic acid was assumed to be a key group for the molecular modeling of the inhibitor, because of its advantage of forming an irreversible covalent bond with Cys S^{γ} atom and its extensive experimental insights on E-64 (**2**) and its derivatives.[11,12,17,18] The molecular design was made through (1) the construction of the energy-minimized tertiary structure of epoxysuccinyl peptide, (2) the visual molecular fitting of it to the cathepsin active site, and (3) the total energy-minimization and short MD simulation during 20 ps for the complex structure, where the covalent bond formation of oxirane C2 atom to Cys-29 S^{γ} atom with *R* configuration was postulated, as was formed in papain.[28] The inhibitory activities of seventeen modeled peptides are summarized in Tables II and III.

Results and Discussion

Tertiary Structure Prediction of Cathepsin B and Comparison with Papain Tertiary structure prediction of rat liver cathepsin B based on the crystal structure of papain–E-64-c complex[10] is shown in Fig. 2. Cathepsin B, like papain, has two domains: one consists of 12—134, 153—158 and 250—252 residues (L-domain) and the other of the remaining residues (R-domain). The catalytic center of Cys-29 is located at the cleft between these two domains. Although the overall structure of cathepsin B might be strongly similar to papain, some characteristic differences are also observable between them, especially concerning the tertiary structures around active centers and these appear to be important for distinguishing them from each other. The following differences are noticeable in the overall structure. (A) Out of a long sequence (residue 123—165) of cathepsin B, which corresponds to the deletion site of papain, residues 157—165, shown in red color, are located near the active site, and are able to participate in the interaction with the inhibitor or substrate, while residues 123—156, marked in blue, are far from the active center. (B) Residues 26—29 and 69—71, traced in green, which are located at the S site,[29] consist of hydrophilic amino acids rather than those of papain (residues 21—25 and 63—66, respectively).

Observations noted as remarkable in the tertiary structures of cathepsin B and papain active sites shown in Fig. 3 are the following differences. (I) The S site of cathepsin B, which has a neutral and hydrophobic environment, is spatially smaller and shallower than that of papain. (II) The S′ site in cathepsin B is spatially wider than that in papain. (III) The side chain of acidic Glu-161 is located near the S1 subsite of cathepsin B, but this is not true in papain. (IV) The side chain of the catalytic His-197 residue of cathepsin B is much more flexible than that of His-159 of papain, due to the existence of two flexible Gly residues (Gly-196 and Gly-195). These four differences are important in designing the potent inhibitors specific for cathepsin B. It is also worthwhile noting that, in contrast to cathepsin B, tertiary structures of cathepsin H and L, predicted from the papain complexed with E-64-c, show S and S′ sites of similar sizes to papain (the corresponding figures are not shown).

Design of Inhibitors and Specificity for Cathepsin B Our strategy to develop the inhibitors specific for cathepsin B from a starting inhibitor of E-64-c ethylester (loxistatin) is based on four premises: (i) the modification of P site of inhibitor molecule because of the smaller and shallower S site in cathepsin B than that in papain, (ii) the elongation of P′ site because of the wider S′ site groove of cathepsin B than that of papain, (iii) the hydrogen bond formation with the side chain of acidic Glu-161 located near the S1 subsite of cathepsin B, and (iv) the fixation of the movable side chain of His-197 by the hydrogen bond formation.

(1) Structural Modification of P Site (Table II) The investigation was begun with a survey of the residues which

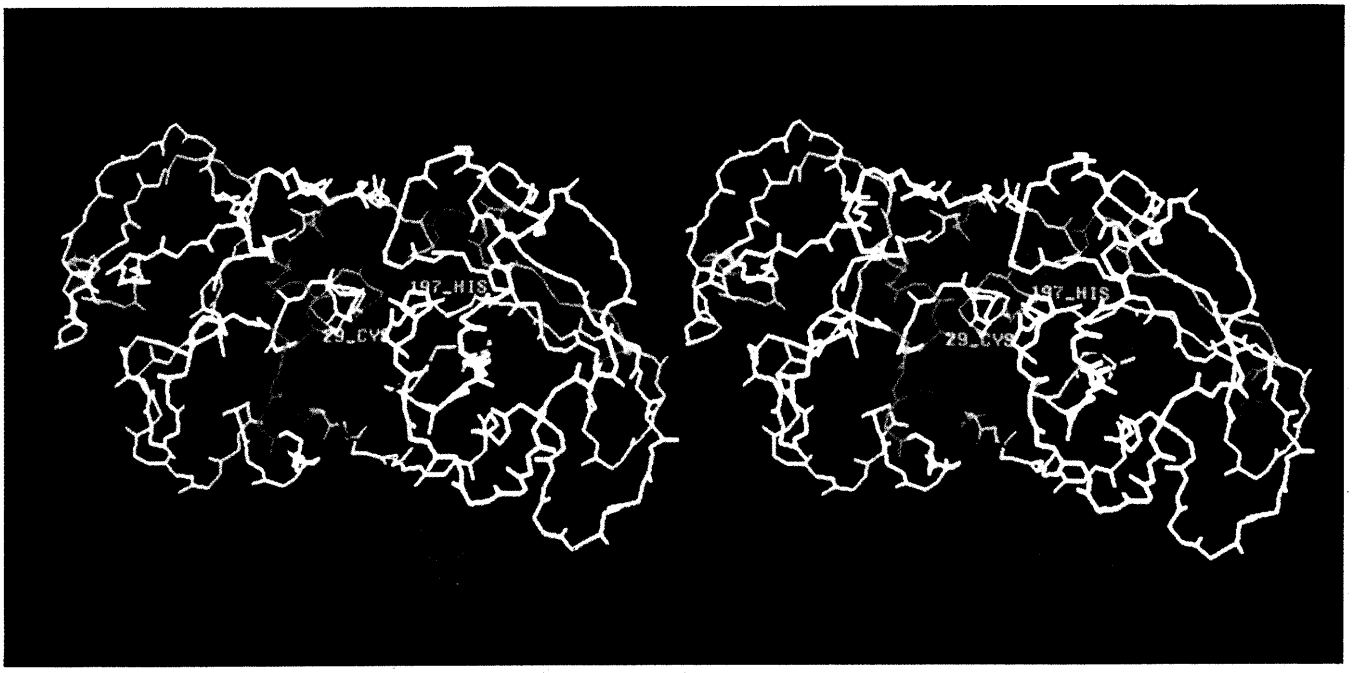


Fig. 2. Stereoscopic View of a Predicted Tertiary Structure of Cathepsin B by RMD Simulation

Residues 123—165 of cathepsin B correspond to the deletion site of papain, and are shown in blue (123—156) and red (157—165), respectively. The deletion/insertion sites in the domains are shown in green.

could be best matched at the pocket in the cathepsin B S site (indicated by asterisk in Fig. 3). Because this pocket has a neutral and hydrophobic environment, five kinds of amino acids (Gly, Ser, Thr, Ile and Trp) were applied to the visual and energy-minimized modeling of the complex structure. Ile was selected as the best fitting amino acid; the molecular volumes of Gly, Ser and Thr were too small for the tight binding with its binding pocket, while that of Trp, in contrast, was large. However, as shown in Table II, compound **4** as well as several compounds possessing P1 residue alone, shows no significant inhibitory activity for either papain or cathepsin B. Using MD simulations, investigation was made of the P2 residue exhibiting the tight binding with the S2 subsite of cathepsin B, keeping in mind that the residue is also able to form a hydrogen bond with the His-197 imidazole ring. Pro was suggested as a suitable residue. In fact, among compounds consisting of P1 and P2 residues so far investigated (data are given for compounds **5**—**8**), compound **5** showed the most potent inhibitory activity. To reveal the specificity for cathepsin B, it was essential that the C-terminal of the compound have a free form of carboxyl group; the specificity for cathepsin B was

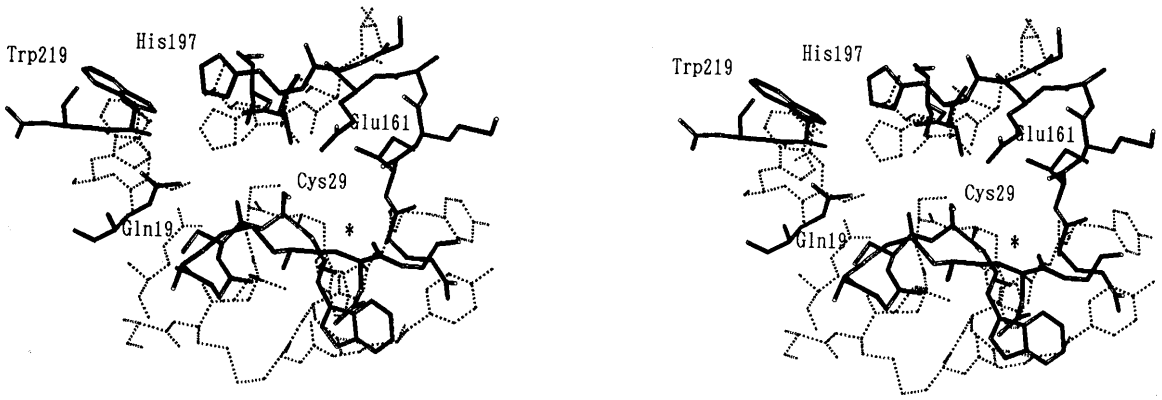


Fig. 3. Superimposition of Active Sites of Cathepsin B (Solid Lines) and Papain (Dotted Lines)

The Cys-29 of cathepsin B or the Cys-25 of papain (shown at center) forms the catalytic site, and to the right and left of it are the active S and S′ sites, respectively. The charge-relay network for the catalysis is formed in the S′ site (Gln-19, Cys-29, His-197 and Asn-217). The catalytic Cys is irreversibly inactivated by the covalent bond formation with the epoxysuccinyl group of inhibitors. The labelled amino acids correspond to those of cathepsin B.

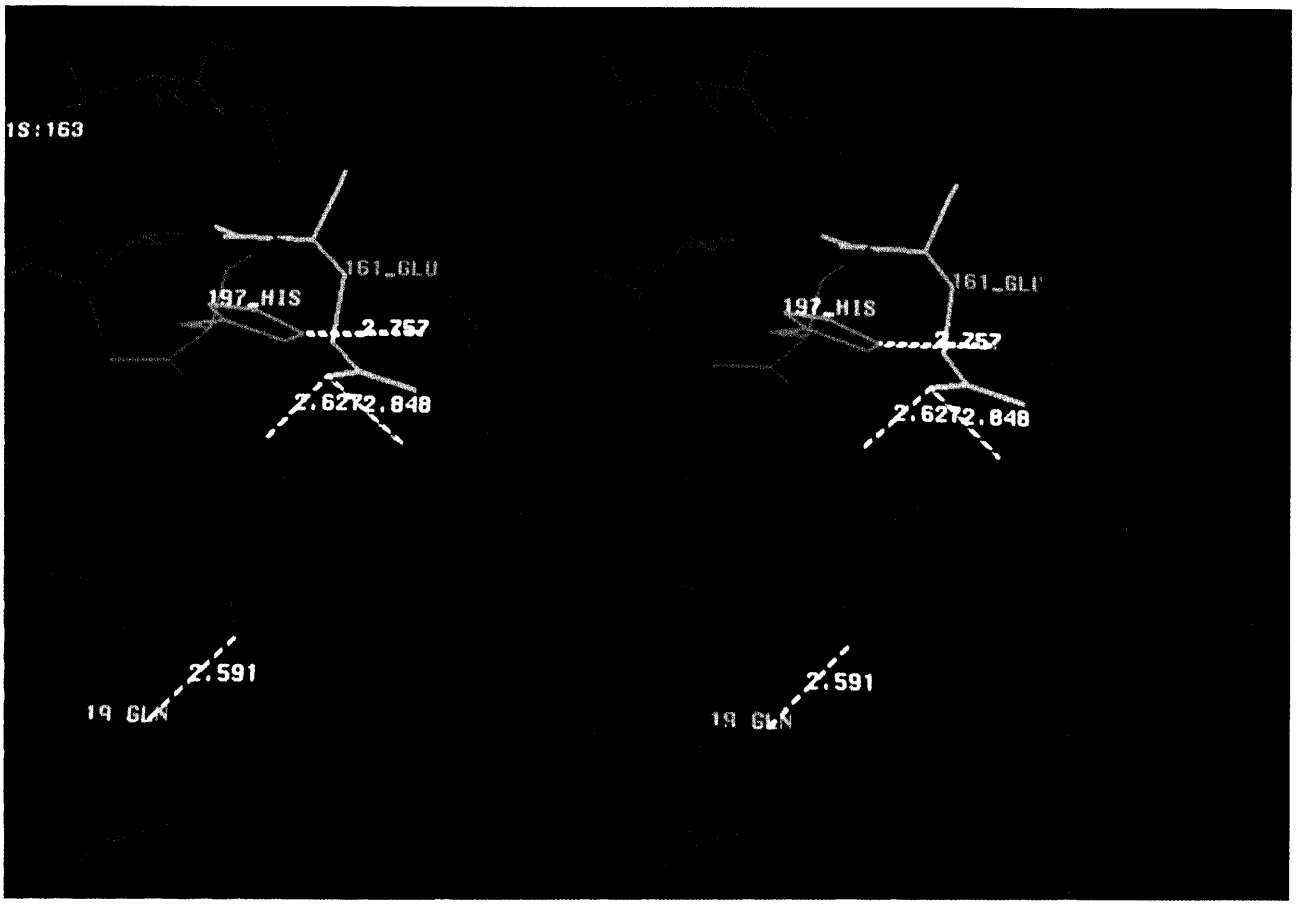


Fig. 4. Possible Binding Mode of CA-074 to Active Site of Cathepsin B

CA-074 is shown in red. The dotted lines represent possible hydrogen bonds along with their distances in angstroms.

significantly decreased by benzylation (compound **9**). These findings are in accordance with premises (i) and (iv).

(2) Structural Modification of P′ Site (Table III) Since the S′ site of cathepsin B was suggested to have a wider groove than that of papain, a modification was attempted by which to elongate the P′ site of inhibitor molecule, so as to further increase the specificity for cathepsin B. Compound **10** or **11** with, respectively, the carboxyl or methylester group at the P′-end, showed weaker activity and lower specificity against cathepsin B than did compound **5**. The existence of bulky alkyl group such as isopropyl (**12**), isobutyl (**13**) or cyclohexyl (**14**) ester strongly inactivated cathepsin B, together with demonstrating sufficient specificity. This result suggests the premise of (ii).

On the other hand, these ester inhibitors are easily hydrolyzed when they are given to animals, as exemplified by the easy transformation of loxistatin to E-64-c in blood.[30] To design more stable inhibitors for hydrolysis in a living cell, amido derivatives were considered (Table III). Although the substitution of the ester group with the corresponding amide group resulted in a slightly weakened inhibitory activity, the specificity for cathepsin B was significantly increased. It is important to note that CA-074 (**3**), *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline, is an excellent inhibitor which is specific only for cathepsin B; its inhibitory activities (IC_{50} nM) for cathepsin L and H and calpain II are 1.72×10^5, 4.20×10^5 and $> 2.0 \times 10^5$, respectively, and details have been published.[19,20]

Possible Binding Mode of CA-074 with Cathepsin B The probable binding mode of CA-074 with cathepsin B was simulated by the MD method using AMBER 3.0.[31] A stereoscopic view of the complex is shown in Fig. 4. Four kinds of hydrogen bond formations are possible in the binding. As intended in the molecular design, the Glu-161 carboxyl group can participate in bifurcated hydrogen bond formation to OH and NH groups of the backbone of CA-074, supporting premise (iii). C-terminal carboxyl oxygen plays a role in the fixation of His-197 by a hydrogen bond formation. The amino group of Gln-19 side chain forms a hydrogen bond with the CA-074 succinyl oxygen atom, as was observed in the papain–E-64-c complex. Furthermore, the *n*-propyl (P′ site) and L-isoleucyl (P site) moieties of CA-074 are tightly held by the van der Waals contacts with Trp-219 and Ile-158, respectively.

We succeeded in designing CA-074 as the first compound selectively inhibiting cathepsin B *in vivo* as well as *in vitro*.[19,20] This could be useful for studies on the selective role of cathepsin B in biological phenomena and in intracellular protein degradation processes; little is yet known of the biological function of cathepsin B. Our success offers encouragement that the methodology described here can provide a powerful approach for designing compounds specific for a target enzyme.

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