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Bicyclic carbamates as inhibitors of papain-like cathepsin proteases

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Abstract—A 6-oxa-1-aza-bicyclo[3.2.1]octan-7-one system inhibits the proteolytic activity of several cysteine proteases belonging to the papain family. In vitro mechanistic studies and in silico calculations suggest that the minimal π-overlap between the bridgehead nitrogen and the carbonyl leads to a considerable weakening of the urethane system, making it susceptible to nucleophilic attack from the active site thiol group. The resulting covalent adduct is slowly hydrolyzed, releasing the hydroxypiperidine product of the inhibitor. Synthesis and testing of a set of analogs led to variable protease subtype selectivities ranging from micromolar to nanomolar potencies.

Most cathepsins are endopeptidases belonging to the papain-like family of cysteine proteases.[1,2] Cathepsins are generally concentrated in lysosomal and endosomal compartments and play a role in a broad array of physiological processes. Among them are the rather non-specific 'house-keeping' functions such as degradation of both internalized and cellular proteins.[3] However, more specialized functions in the processing of enzymes and hormones have been recently attributed to cathepsins and they have been implicated in multiple disease processes.[4] Indeed, inhibition of specific cathepsins is believed to have therapeutic implications on pathological conditions associated with cellular homeostasis, apoptosis, tumor invasion and metastasis, bone resorption, and antigen presentation.[5–11]

Small molecule inhibitors of proteases have been the subject of several excellent and extensive reviews.[12–18] Many protease inhibitors, including those for cathepsins, consist of a peptide-substrate mimetic that mediates binding to specific pockets in the enzyme and an electrophilic 'warhead' that makes crucial contacts to the catalytic residues. The nature of the latter determines the classification of these small molecules into reversible and irreversible inhibitors. Several electrophilic groups have been routinely employed in the inhibition of proteases by covalent interaction of enzyme with inhibitor. Aldehydes, semicarbazones, nitriles, and ketones usually lead to reversible inhibition, whereas halomethylketones, epoxides, and Michael acceptors are among the functionalities used for irreversible inhibition.

To date relatively few protease inhibitors have successfully progressed through clinical trials. Major issues are the often non-drug-like properties of the peptidic portion of the inhibitor and/or the lack of selectivity, often attributed to non-specific interactions of the electrophilic functionality with endogenous nucleophiles in vivo.[16] In order to circumvent these drawbacks commonly seen with the classical approach of substrate-based drug design, novel molecular concepts need to be explored. We report the investigation of a strained bicyclic carbamate system discovered by high throughput screening (HTS).

We performed a HTS of an unbiased library of roughly 1 million compounds based on monitoring the cleavage rate of a coumarin-labeled synthetic tetrapeptide highly reactive to cathepsin S.[19] While many of the confirmed hits displayed an electrophilic moiety and/or a highly peptidic structure commonly seen in protease inhibitors, compound **1** stood out by its unusually low molecular weight and its non-peptidic nature. The structure of **1** was tentatively assigned as in Figure 1, by tracing the hit back to its origin. It is a cyclized

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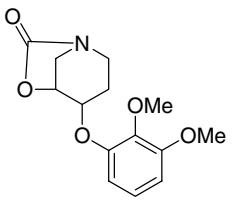

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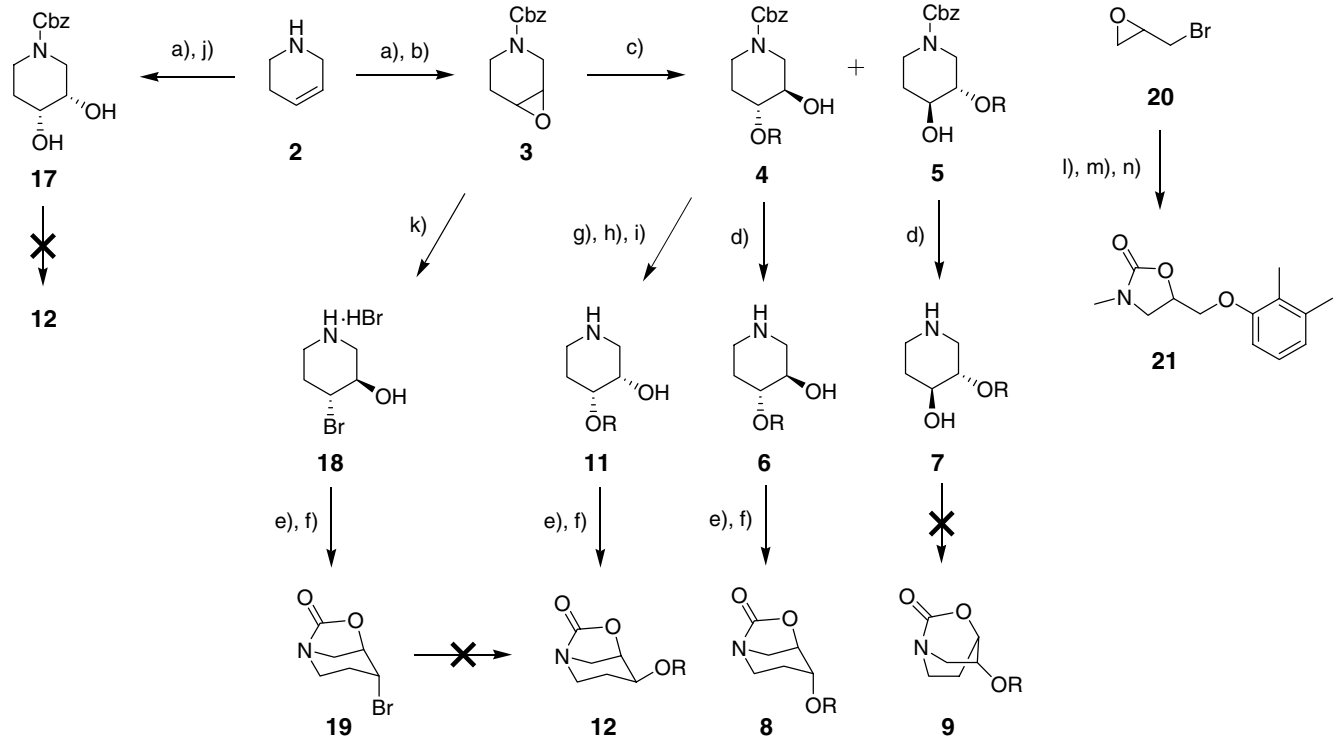
Figure 1. Compound **1**, the result of intramolecular cyclization of 4-aryloxy-3-hydroxypiperidines with phosgene.

derivative of 4-aryloxy-3-hydroxypiperidines such as ifoxetine, a selective serotonin reuptake inhibitor that has been studied more than 25 years ago at Ciba–Geigy as an antidepressant.[20–22]

First we resynthesized compound **1** to confirm its structural identity including its relative stereochemistry (Scheme 1).[23] According to synthetic procedures described by Paioni,[20] the tetrahydropyridine **2** was converted to the racemic Cbz-protected epoxide **3** in two steps. Opening of the epoxide **3** (ROH = 2,3-dimethylphenol) under basic aqueous conditions gave a mixture of regioisomers. As expected for a favored trans-diaxial ring-opening, the ratio of 4-aryloxy piperidines **4** versus 3-aryloxypiperidines **5** was about 5:1. The regioisomers were chromatographically separated and hydrogenolytically deprotected to give the 'open' forms **6** and **7**. The cyclization of **6** required a two step procedure: first the piperidine-nitrogen was selectively carbonylated to the corresponding chloroformate using triphosgene and triethylamine, then the 3-hydroxy group was deprotonated using sodium hydride, which led to spontaneous intramolecular cyclization to give racemic *trans*-4-(2,3-dimethyl-phenoxy)-6-oxa-1-aza-bicyclo[3.2.1]octan-7-one **8**. Attempts to cyclize the chloroformate intermediate of **7** to the bicycle **9** were unsuccessful, probably due to the unfavorable boat conformation of the piperidine ring required for intramolecular ring-closure.

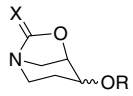
In order to invert the stereocenter at position three of compound **4**, we performed a Mitsunobu reaction using *p*-nitrobenzoic acid as the nucleophile. Alternatively, the inversion could be accomplished by an oxidation/reduction sequence using $Pyr{\cdot}SO_3$ and Red-Al, but with inferior yields. Saponification of intermediate **10** in methanolic base followed by deprotection gave piperidine **11** (cf. ifoxetine). Intramolecular cyclization analogous to the above described procedure gave *cis*-4-(2,3-dimethyl-phenoxy)-6-oxa-1-aza-bicyclo[3.2.1]octan-7-one **12**.

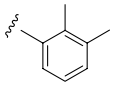
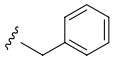
Analogs **13**–**16** (Table 1) were synthesized in a similar fashion to the synthesis of **12**. In the case of analog **13**, thiophosgene was used instead of phosgene in the cyclization step, for analogs **15** and **16** the corresponding alcohol (benzylalcohol for **15**, isopropanol for **16**) was used as solvent together with NaH in the opening of epoxide **3**.



Scheme 1. Reagents and conditions: (a) CbzCl, 0.5 M Na_2CO_3/dioxane 5:2, rt, 5 h; (b) *m*-CPBA, DCM, 0 °C–rt, 7 h; (c) ROH, 2 M NaOH/MeCN 1:4, reflux, 48 h; (d) 1 atm H_2, Pd/C (cat), EtOH, rt, 3 h; (e) triphosgene, NEt_3, DCM, 0 °C, 1 h; (f) NaH, rt, 18 h; (g) PPh_3, DEAD, *p*-nitrobenzoic acid, THF, 50 °C, 48 h; (h) NaOH, MeOH, rt, 1 h; (i) 1 atm H_2, Pd/C (cat), EtOH, rt, 3 h; (j) OsO_4, NMO, citric acid, *t*-BuOH/H_2O 1:1, 50 °C, 12 h; (k) HBr, HOAc, 0 °C–rt, 3 h; (l) 2,3-dimethylphenol, K_2CO_3, MeCN, 60 °C, 12 h; (m) $MeNH_2$, MeOH, 50 °C, 2 h; (n) CDI, DMAP, benzene, reflux, 4 h. Compounds **3**–**21** are racemic mixtures.

Table 1. Determined IC_{50} values in micromolar of compounds **1**–**21** for inhibition of various cathepsins[24]



Compound	X	St_{rel}	R	CatB	CatS	CatC	CatL	CatK	CatH	CatX	CatF	CatV
8	O	*trans*		0.046	0.473	1.727	1.270	2.438	>30	0.553	5.908	3.317
12	O	*cis*		0.001	0.016	0.018	0.022	0.038	2.653	0.197	0.331	0.131
13	S	*cis*		0.519	0.696	0.606	0.994	1.833	>100	7.149	>10	5.166
14	O	*cis*		0.005	0.017	0.029	0.026	0.104	6.591	0.326	0.487	0.158
15	O	*cis*		0.002	0.174	0.403	0.263	0.511	>10	0.547	3.760	1.289
16	O	*cis*		0.008	0.298	0.191	0.230	1.267	>10	1.102	1.826	0.894
19	O	*trans*	OR = Br	0.158	4.553	>10	5.816	>30	>100	>10	>10	>10
6				>100	>100	>100	>100	>100	>100	>100	>100	>100
11				>100	>100	>100	>100	>100	>100	>100	>100	>100
21				>100	>100	>100	>100	>100	>100	>100	>100	>100

St_{rel}, relative stereoconformation.

An alternative synthetic route via dihydroxylation led to the protected tetrahydropyridine **17**. Selective cyclization and alkylation of the deprotected diol failed to give the desired bicyclic carbamate analogs. Treatment of the epoxide **3** with HBr selectively gave bromide **18** which could be cyclized to the carbamate **19**, but any attempts to displace the bromide with phenols using inorganic bases in DMF (DMSO) were unsuccessful. The control compound **21** was synthesized in three steps starting from epibromohydrin **20**.

An in depth profiling[24] of analogs **8** and **12**–**16** revealed that these cyclic carbamates showed inhibitory activity across a panel of human cysteinyl cathepsin proteases (Table 1). Within this protease family, the determined IC_{50} values were lowest for cathepsin B, followed by cathepsins S, C, L, and K. Moderate activities were seen against cathepsins V, X, F, and H. The compounds were shown to be inactive against other families of cysteine proteases such as caspases and against members of the serine protease family such as thrombin and trypsin (data not shown).

Generally, there is a remarkable drop in IC_{50} values when comparing the *trans* to the *cis* configured stereoisomers (cf. **8** vs **12**). Also, analog **12**, which contains the more hydrophobic dimethylphenyl group, seemed to be consistently more active in the cathepsin panel when compared to the phenyl-substituted analog **14**. The thiocarbamate **13** had substantially less inhibitory effects when compared to the 'oxo'-carbamates. The alkyl-substituted analogs **15** (R = benzyl) and **16** (R = isopropyl) generally displayed about 10-fold higher IC_{50} values than **12** or **14**. An exception is the low single-digit nanomolar activity of the benzyl-substituted analog **15** against cathepsin B, representing the most selective inhibitor for cathepsin B in the series (100-fold).

Next we investigated the stability of these low molecular weight protease inhibitors. Both stereoisomers **8** and **12** were isolated as white, stable solids. The compounds were also stable in aqueous basic or neutral solutions, while spontaneous ring-opening was observed under fairly acidic conditions (pH < 2). To the best of our knowledge there are only two reports in the literature on similar 'anti-Bredt' bicyclic systems.[25,26] The bicyclic carbamate is predicted to be substantially less stable than usual carbamates due to substantial ring strain and minimal π-overlap between the bridgehead nitrogen and the carbonyl group. The relative thermodynamic stability of the bicyclic urethane **12** was calculated to be >20 kcal lower in comparison to the monocyclic analog **11**,[27] rendering it susceptible for nucleophilic attack. Besides other factors affecting inhibitory potency such as decreasing the degrees of rotational freedom in the bicycle **12**, this greatly enhanced susceptibility for nucleophilic attack may play a crucial role especially in the case of covalent inhibition.

To understand the mechanism of inhibition by these bicyclic carbamates, compound **12** was examined in a dialysis study (Fig. 2).[28] Briefly, cathepsin B was first incubated at 37 °C with 0.2 μM **12** ($200 \times IC_{50}$) for complete suppression of catalytic activity (2.9 rfu/sec for inhibitor treated vs 121 rfu/sec for vehicle control). Then the mixtures were dialyzed extensively at 4 °C to remove

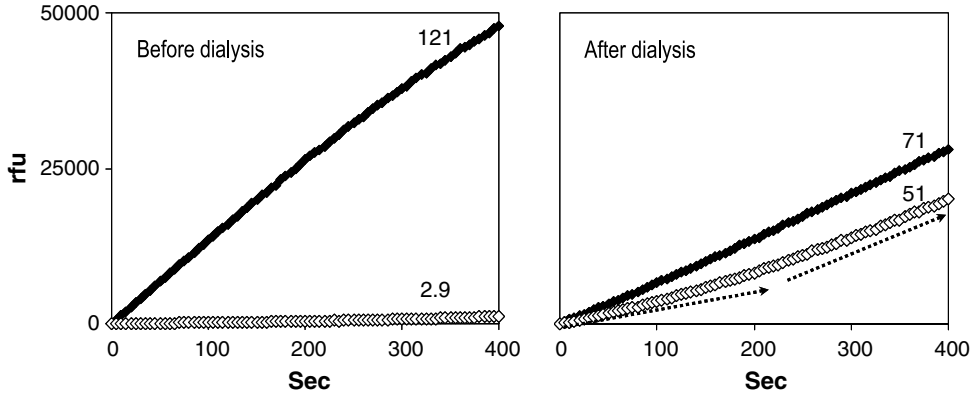


Figure 2. Reversibility of binding of compound **12** to cathepsin B. The rate of conversion of a fluorogenic substrate (Ac-His-Pro-Val-Lys-ACMC) is monitored using DMSO vehicle (solid diamond) and compound **12** treated (open diamond) cathepsin B before dialysis (left) and after dialysis (right). The number above each progress curve reflects the individual reaction rate (relative fluorescence units (rfu) per second). The dotted arrows on the right panel indicate the rate increase during the course of the protease activity assay for compound **12** treated cathepsin B.

unbound inhibitor before the cathepsin activities were re-measured at 37 °C (51 rfu/sec for inhibitor treated vs 71 rfu/sec for vehicle control). The apparent recovery of cathepsin B activity in compound **12** treated sample after dialysis suggests that compound **12** is a partially reversible inhibitor. However, a careful examination of the progress curves in Figure 2 revealed that the substrate conversion was accelerated over the course of the assay (indicated by the dotted arrowed lines). We speculated that this rate increase is likely caused by a temperature-sensitive release of covalently bound compound **12** from the active site, since the substrate conversion of the control was not affected by the incubation temperature.

To test this hypothesis, the potential adduct formation between cathepsin B and inhibitor (compound **12**) was analyzed by mass spectrometry following tryptic/chymotryptic digestion.[29] Two active site fragments of the test enzyme (cathepsin B) were identified as ^{19}EIRD QGSCGSC*W^{30} and ^{22}DQGSCGSC*W^{30} from the untreated cathepsin B sample.[30] For inhibitor treated cathepsin B, both fragments gained a mass of 247 amu, corresponding to a single covalent conjugation of **12** to the catalytic cysteine residues (C*). The stability of this adduct was indeed sensitive to temperature increase.[31] Compared to the control that remained on ice, a 2-h incubation at 37 °C promoted the dissociation of this enzyme-inhibitor adduct, resulting in a 4-fold increase in abundance of a metabolite. The multiple reaction monitoring (MRM) characteristics of the metabolite were identical to those of the suggested dissociation product **11**.

A potential binding mode of compound **12** with cathepsin B is shown in Figure 3. It was obtained using a MCMM/LLMOD conformational search strategy.[32] The hybridization of the carbonyl-carbon of **12** was changed from sp^2 to sp^3 and placed in covalent contact with the thiol group of the catalytic cysteine (Cys29). A formal negative charge was assigned to the carbonyl-oxygen. In this model the dimethylphenyl substituent occupies the hydrophobic P2-pocket, which is defined by aminoacid residues Gly27, Trp30, Gly73, Tyr75, Pro76, Ala173, Gly198, and Ala200. In addition the carbonyl-O of carbamate **12** forms hydrogen bonds with the side chains of His199 and Gln23. The calculation of the free energy of binding[33] revealed that the (*S*,*R*)-*cis* carbamate **12** is by ~6 kcal/mol more stable than the (*S*,*S*)-*trans* stereoisomer **8**. This agrees well with the observed lower IC_{50} value for the racemic *cis* stereoisomer.

In summary, a series of bicyclic carbamates was identified as novel, non-peptidic protease inhibitor scaffold starting from HTS. The scaffold is selective for the cathepsin family, with subtype selectivities following the order B > S > C,L,K > V,X,F,H. A preference for

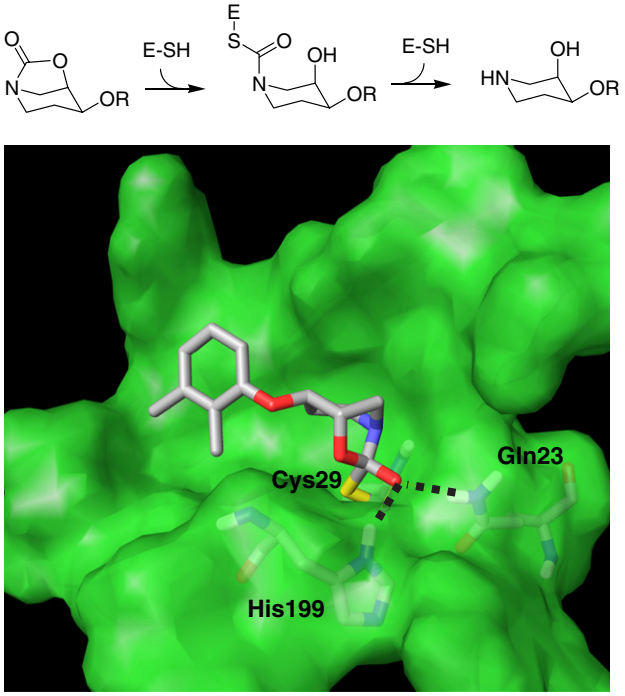


Figure 3. Proposed binding mode of compound **12** to cathepsin B (E-SH). The sulfur of Cys29 (yellow) is in covalent contact with the carbonyl-carbon of **12**. The dotted lines represent H-bonds between Gln23, His199 and the carbonyl-oxygen of **12**.

hydrophobic R-groups in *cis* configuration relative to the carbamate oxygen was observed, with determined IC_{50} values for cathepsin B in the single-digit nanomolar range. The carbamate functionality in the scaffold is substantially destabilized due to the 'anti-Bredt' nature of the bicycle, and offers a weak point for nucleophilic attack by the active site cysteine thiol. The bicycle subsequently undergoes ring-opening and covalently binds to the catalytic cysteine, leading to inhibition of the enzyme. This hypothesis is in accordance with the mass spectrometric analysis of digested enzyme. After incubation with compound **12**, the fragments which include the catalytic cysteine are covalently linked to the inhibitor. The non-linear rate recovery seen in dialysis studies and characterization of metabolites via MRM suggest that the inhibitor is slowly hydrolyzed off the enzyme as its ring-opened synthetic precursor **11** at ambient temperature. A molecular model of cathepsin B and covalently bound inhibitor **12** further strengthens the accordance of the proposed binding mode and the experimental observations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.014.

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23. Detailed synthetic procedures to compounds **3**–**21** can be found in Supplementary material.
24. Enzyme inhibition assays were performed as previously described (Tully D. C. et al. *Bioorg. Med. Chem. Lett.* **2006**, 16, 1975). Briefly, recombinant human cathepsin enzymes were used in all enzyme inhibition assays. The standard assay format contained 50 μM fluorogenic peptide substrate in 100 mM NaOAc, 1 mM EDTA, 0.01% Brij-35, and 5 mM DTT, pH 5.5, at 37 °C. The enzyme was preincubated with inhibitor for 20 min before substrate was added to initiate the reaction. The substrate hydrolysis was monitored by the increase in fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 450 nm on a Gemini EM fluorometer. The reaction progress curve was fitted to the Morrison equation using PlateKi (BioKin) and the apparent inhibition constants were reported as IC_{50} values.
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27. The strain energy was estimated from energy difference between open and closed forms of the bicyclic urethane (**11** and **12**) and its monocyclic analog **21**. The conformations were obtained via LMOD conformational search method (Macromodel, Schrodinger, Inc.), employing a MMFF94 force field and treating solvation implicitly via the Generalized Born method. The energy calculations were done using ab initio density functional method (Jaguar, Schrodinger, Inc.) in which the 6-31G** basis set and hybrid DFT functional B3LYP were employed. The aqueous solvation was treated with the Poisson–Boltzmann continuum-dielectric method.
28. Dialysis-based reversibility study: 2 nM recombinant cathepsin B was incubated with 0.2 μM compound **12** (IC_{50} = 1 nM) or DMSO (control) for 60 min at 37 °C with slow agitation on a shaker. Inhibition of the enzymatic activity was monitored according to the enzyme inhibition assay described in reference 24, by adding 50 μL of the preincubated enzyme-inhibitor mixture into 50 μL of 200 μM fluorogenic peptide substrate Ac-His-Pro-Val-Lys-ACMA. Alternatively, the enzyme-inhibitor mixture was injected into a 3-mL SlideDialyzer (Pierce, 7500-kDa cutoff) and dialyzed at 4 °C against 1 L of dialysis buffer (100 mM NaOAc, pH 5.5, 100 mM NaCl, 1 mM EDTA, 0.001% Brij-35, and 2 mM DTT). The dialysis buffer was changed three times in 8 h intervals. After dialysis, the cathepsin B activity was assayed as described above.
29. Enzymatic digestion of cathepsin B for mass spectrometric analysis: 1 nmol of cathepsin B was denatured in 3 M guanidine HCl. The cysteine residues were reduced with TCEP followed by treatment with iodoacetamide. Excess iodoacetamide was quenched with DTT. The solution was then diluted to 0.5 M guanidine with 50 mM Tris–HCl, pH 8, and 10 mM $CaCl_2$. Trypsin was added to the sample and proteolysis was carried out at 37 °C for 4 h. Then

chymotrypsin was added and the reaction was continued at rt for another 18 h.
30. Procedures and results for the mass spectrometric analysis of the cathepsin B digests can be found in Supplementary material.
31. Procedures and results for the temperature-dependent metabolite monitoring can be found in Supplementary material.
32. To model the interaction of the cyclic carbamates with cathepsin B, we used a mixed Monte Carlo Multiple Minimum (MCMM)/LLMOD (Large Scale LowMode) conformational search strategy available in MacroModel (version 8.0; Schrodinger, Inc.). The structural perturbation via the LLMOD method is alternated with the random changes in torsion angles from the MCMM method. During the LLMOD structural perturbation, protein residues within 4 Å from the bound ligand were allowed to move freely. The inhibitor was subjected to perturbations via TORS command available in MacroModel. The employed force field was OPLS-AA 2005 with distance-dependent dielectric constant $2r$ and no explicit solvation.
33. The free energy of binding of compounds was obtained by subtracting the ligand and receptor energies from that of the complex. For the free energy calculations an OPLS-AA force field (Macromodel, Schrodinger Inc.) was employed and the solvation was accounted for via Generalized Born method.