Triterpenoids as Novel Natural Inhibitors of Human Cathepsin L

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Cathepsins L (catL) and B play an important role in tumor progression and have been considered promising therapeutic targets in the development of novel anticancer agents. Using a bioactivity-guided fractionation, a series of triterpenoids was identified as a new class of competitive inhibitors towards cathepsin L with affinity values in micromolar range. Among the 14 compounds evaluated, the most promising were 3-epiursolic acid (**3**), 3-(hydroxyimino)oleanolic acid (**9**), and 3-(hydroxyimino)masticadienoic acid (**13**) with IC_{50} values of 6.5, 2.4, and 2.6 μM on catL, respectively. Most of the evaluated triterpenoids do not inhibit cathepsin B. Thus, the evaluated compounds exhibit a great potential to help in the design of new inhibitors with enhanced potency and affinity towards catL. Docking studies were performed in order to gain insight on the binding mode and SAR of these compounds.

Introduction. – Lysosomal cysteine proteases are characterized by having similar amino acid sequences, and they share a common papain-like folding structure [1][2]. Cathepsin L (catL) is an enzyme active only as endopeptidase, containing two domains, with Cys25, His163, and Asn187 forming the catalytic triad [3]. On the other hand, cathepsin B (catB) is a carboxypeptidase, which can act, depending on the pH, both as endopeptidase or exopeptidase. The main feature of catB is the occluding loop which comprises the His110 and His111 residues [1][3].

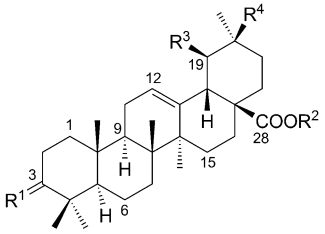
Increases in cysteine protease expression and activity have been associated with many types of human carcinomas such as colorectal [4], melanome [5], breast [6], and brain tumors [7]. Recently reported as important targets in tumor progression, catB and catL play an important role in angiogenesis, invasion, and metastasis [1][2][8][9] which have been considered the major cause of death from cancer [1]. It has been reported that cancer-associated proteases can be secreted by tumor cells acting on the degradation of extracellular matrix (ECM) and basement membrane [2][8]. Therefore, these cathepsins have been identified as promising therapeutic targets and are of special interest in the development of novel anticancer agents.

Plants are one of the largest sources of active compounds, and advances in cancer treatment require the continuing development of novel agents [10]. Over the past years, natural products have played an important role in medicine, as a source not only of potential chemotherapeutic agents, but also used as lead compounds in semisynthesis or total synthesis of new drugs [11][12]. Triterpenoids are a widespread group of

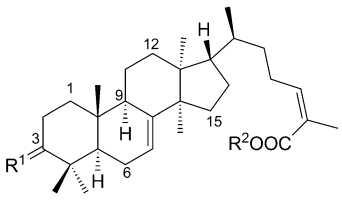
natural products occurring in several types of higher plants and organisms. These compounds present a broad range of pharmacological properties [13][14] such as anticancer [14], antibacterial [15], anti-inflammatory [16], anti-AIDS [17], and cytotoxic activities [18].

We have been interested in finding new cysteine protease inhibitors, and we previously described acridone alkaloids isolated from *Swinglea glutinosa* (BL.) MERR. (Rutaceae) as cathepsin V inhibitors [19]. Continuing the search for new enzyme inhibitors, in the present study we have screened several natural triterpenes and some of their derivatives against catB and catL. To the best of our knowledge, this is the first report describing these triterpenoids as inhibitors of human catL.

Results and Discussion. – *Chemistry.* Bioactivity-guided fractionation of the hexane active fraction from the stems of *Myrcia lingua* BERG. (Myrtaceae) resulted in the isolation of active acidic triterpenes such as 3-*O*-acetylursolic acid (**1**) [20], ursolic acid (**2**) [21], and 3-epiursolic acid (**3**; *Fig. 1*) [22]. To better investigate this class, some other compounds previously isolated in the laboratory of natural products were also evaluated against the proteases. 3-Oxoursolic acid (**4**) [23] was isolated from the stem bark of *Vochysia thyrsoidea* (Vochysiaceae). Compound **1** was treated with CH_2N_2 [24] to give the corresponding methyl ester **5** [24]. Oleanolic acid (*Aldrich O5504*, $\geq$97%; **6**) was purchased from *Aldrich Chemical Co.* and *Sigma*, and some other derivatives were obtained as indicated below. Compound **6** was acetylated with Ac_2O in pyridine [25], resulting in the 3-*O*-acetyloleanolic acid [25] (**7**). In addition, 3-oxooleanolic acid (**8**) [25] was obtained *via* oxidation of **6** with pyridinium chlorochromate (PCC) and CH_2Cl_2 [26]. Compound **8** was subjected to a reaction with $NH_2OH \cdot HCl$ in MeOH as described in [27] to afford the 3-(hydroxyimino) derivative **9** [27]. Masticadienoic acid (**10**) [28][29] and schinol (**11**) [29] were isolated from the fruit of *Schinus*



	R^1	R^2	R^3	R^4
1	β-AcO	H	Me	H
2	β-OH	H	Me	H
3	α-OH	H	Me	H
4	O	H	Me	H
5	β-AcO	Me	Me	H
6	β-OH	H	H	Me
7	β-AcO	H	H	Me
8	O	H	H	Me
9	HO–N	H	H	Me



	R^1	R^2
10	O	H
11	α-OH	H
12	O	Me
13	HO–N	H
14	HO–N	Me

Fig. 1. *Chemical structures of triterpenes and derivatives*

terebinthifolius (Anacardiaceae). Compound **10** was also treated with CH_2N_2 [24] to give the corresponding methyl ester **12** [28][29]. Compounds **10** and **12** were then subjected to a reaction with $NH_2OH \cdot HCl$ in MeOH [27] to afford the 3-(hydroxyimino) derivatives **13** [28][29], **14** [28][29], respectively. All compounds (*Fig. 1*) were isolated and identified by chromatographic and spectral methods, and all the obtained data were compared with those in the literature.

Bioactivity-Guided Fractionation. Using bioactivity-guided fractionation to find novel natural inhibitors of catB and catL, several native plants from the cerrado biome were screened against these enzymes, leading to a group of active triterpenes isolated from *Myrcia lingua* Berg. Among the obtained extracts from cerrado plants, the crude extracts from *M. lingua* showed significant inhibitions on the evaluated enzymes and were further selected to the isolation process. All extracts and fractions were evaluated at a concentration of 125 μg/ml. The inhibition of EtOH extract was higher than 91% on both enzymes, although the hexane fraction of the stems of *M. lingua* turned out to be the most active with inhibition higher than 90% on catL and 75% on catB.

The isolation procedure of hexane fraction led to the known triterpenes 3-*O*-acetylursolic acid (**1**), ursolic acid (**2**), and 3-epiursolic acid (**3**). The enzymatic assay on cysteine proteases revealed that acidic triterpenes inhibited catL.

Biochemical Evaluation of Natural Products and Derivatives. As a general procedure, the inhibition screen assay was first carried out at a concentration of 100 μM. To compare the potency and selectivity, we determined the IC_{50} values for both cathepsins. The evaluated compounds showed good selectivity towards the enzymes.

Eqn. 1 was used to calculate the percentage of inhibition:

$$\% \text{ Inhibition} = 100 \times (1 - V_i/V_0) \quad (1)$$

where V_i and V_0 are initial velocities (enzyme activities) determined in the presence and in the absence of the inhibitor, respectively.

Among the 14 compounds evaluated, the highest inhibitions were found for **3**, **9**, and **13** with IC_{50} values of 6.5, 2.4, and 2.6 μM on catL, respectively (*Table 1*). The majority of the evaluated triterpenoids did not inhibit catB.

It has been found that the more common structural modifications on triterpenes were at C(3) (OH group) and C(28) (COO group), and the obtained derivatives displayed enhanced pharmacological activities, especially anticancer properties [30]. Among the evaluated compounds, the presence of the C(28)OO group was important for the inhibitory potency of triterpenoids towards catL. It was also noticed that the presence of a hydroxyimino group at C(3) contributed significantly to the inhibition improvement. Evaluating the obtained data, we recognized that both triterpene skeletal types ursane and oleanane inhibited catL. Compounds **5**, **12**, and **14** completely lost their activity towards catL, when the COO group was transformed into the corresponding methyl ester, confirming the importance of a free C(28)OOH group for the activity towards catL.

The results by *Sporn et al.* showed that the presence of methyl ester, amides and CN groups at C(28) of compound **6** contribute to enhance the activity toward cancer cells and also to the ability to decrease inflammation [31]. In contrast, our results on catL demonstrated that the C(28)OOH is strongly required to inactivate the enzymes.

Table 1. *Inhibitory Activities of Triterpenoids towards CatL and CatB*

Compound	IC_{50} [μM][a])	
	Cathepsin L	Cathepsin B
1	12.3 ± 1.6	78.9 ± 6.4
2	39.5 ± 4.7	> 250
3	6.5 ± 0.9	> 250
4	8.3 ± 0.5	> 250
5	> 500	> 500
6	7.2 ± 1.1	> 250
7	9.7 ± 1.8	> 250
8	14.7 ± 0.9	> 250
9	2.4 ± 0.5	> 250
10	9.1 ± 2.3	> 250
11	8.2 ± 0.5	20.4 ± 2.0
12	> 250	> 250
13	2.6 ± 0.2	> 250
14	> 500	> 500
E-64[b])	0.03 ± 0.004	0.04 ± 0.004

[a]) The values represent means of three individual experiments ± SD. [b]) Positive control.

CatL and catB are the cysteine proteases strongly involved in metastasis, angiogenesis, and tumor invasion [1][2][8][9]. Recent studies described compound **2** as inhibitor of matrix metalloproteinases (MMPs) on rat C-6 glioma cells, and the IC_{50} value was 20 μM for MMP-9 inhibition. MMPs are proteases also overexpressed in tumor cells and related to cell invasion and degradation of ECM [30][32][33]. Interestingly, ursolic acid and its derivatives have been described as relatively nontoxic agents, which further focused our interest on this class of compounds [30][32]. To establish a correlation between anticancer and cathepsin activity, we found in the literature that several triterpenoids with saponin, ursane, and oleanane skeletons are being tested as anticancer agents, and some of them are already on clinical trials [30].

The most potent inhibitors were selected for further kinetic studies on catL to determine the type of inhibition and dissociation constants (K_i), although the kinetics of those compounds on catB were not investigated due to their low activity.

Mechanism of Inhibition. Triterpenes **3**, **6**, **7**, **9**, and **13** were selected to determine the type of inhibition towards catL. The inhibition kinetics analyzed by *Lineweaver–Burk* double reciprocal plots [34] revealed the series of triterpenoids as competitive inhibitors (*Fig. 2*); whereas the value of V_{max} remained constant at all inhibitor concentrations, the apparent value of K_m increased with increasing concentration of the inhibitor.

The K_i values for inhibitors **3**, **6**, **7**, **9**, and **13** are compiled in *Table 2*, and they confirm the evaluated triterpenoids as a new class of competitive inhibitors with affinity values in a μM range. Thus, these compounds display a potential to aid the design of new inhibitors with enhanced potencies and affinities towards catL.

Molecular-Docking Studies. To gain some insight on the experimental inhibition data, docking studies were undertaken, and the results were compared to the poses in

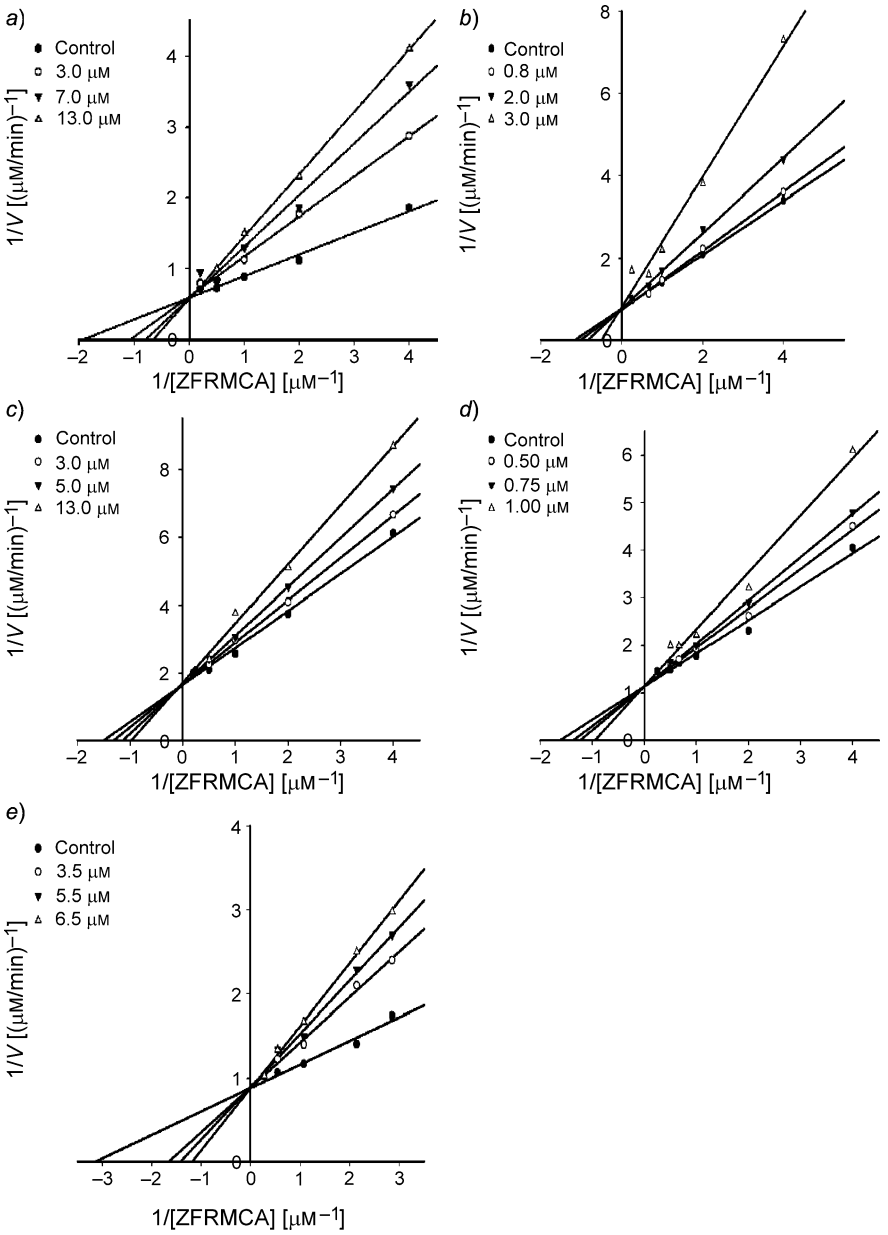


Fig. 2. K_i *Plots of compounds* **3** (*a*), **6** (*b*), **7** (*c*), **9** (*d*), *and* **13** (*e*) *on catL*. Kinetics measurements were conducted in the presence of increasing concentration of inhibitors. All data points are means of three experiments ±SD.

two crystal structures of the compounds with inhibitory activities in the nM order towards catL [35][36].

Table 2. *Inhibitory Effect of Triterpenoids on CatL*

Compound	K_i [μM][a])	Inhibition type
3	19.5	Competitive
6	6.15	Competitive
7	17.0	Competitive
9	2.0	Competitive
13	9.23	Competitive

[a]) The values represent means of three individual experiments ±SD.

In *Table 3*, the interactions of the different compounds with the amino acids of the subsites of catL are collated. It can be seen that the compounds in the crystallographic structures (pdb codes 2xu3 [35] and 2yj2 [36]) interact with amino acids of subsites S1, where the catalytic cys25 is located, S2, and S3. There are also a few interactions with amino acids of the S1′ and S2′ subsites (see *Fig. 3*).

The finding that compounds with four rings undergo only a few interactions with amino acids of subsites S2 and S3 may explain their rather low inhibitory activity; moreover, those with the lowest activity do not get close to the catalytic cys25 [37][38]. It should be noted that the five-membered ring compounds do not even interact with amino acids of the S2′ subsite.

Due to the size and rigidity of the triterpenoid ring systems, the molecules resemble in some way a cylinder that cannot pass through the narrow V-shaped channel that leads to the active site, preventing them from interacting with amino acids of subsites S2 and S3, which are important for the inhibitory activity [38].

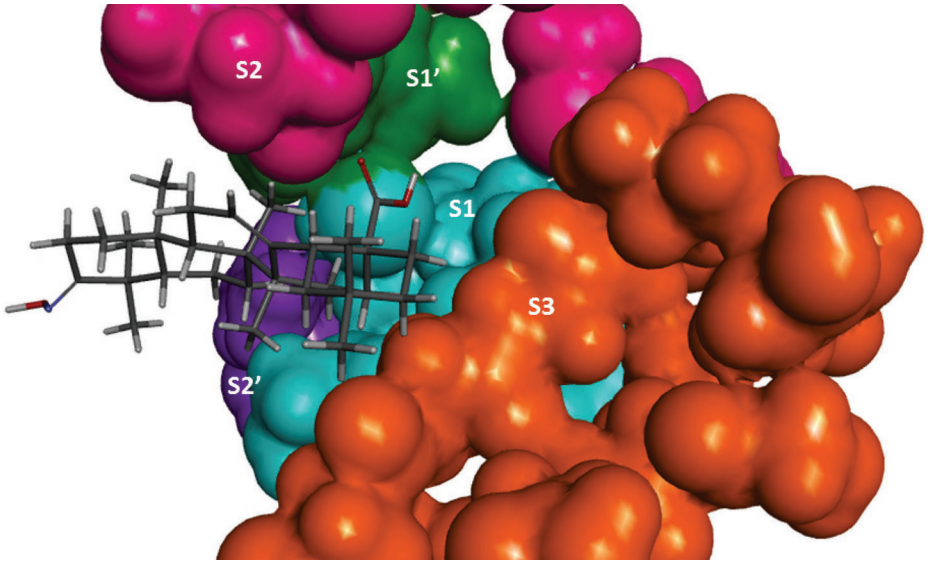


Fig. 3. *View of compound* **9** *docked into the active site of catL*. The residues are colored according to the subsite they are located: S3, orange; S2, magenta; S1, blue; S1′, green; and S2′, purple (see *Table 3*).

Table 3. *Interactions between the Ligands and CatL Residues and Their Inhibition Values.* A specific catL inhibitor should interact with the S2, S3, and S1′ subsites besides S1 which contains the catalytic cysteine [38]. It can be observed that compounds like **5** are not good inhibitors due to lack of interactions with S1′ subsite, neither would be compound **12** that do not interact with the catalytic cysteine (yellow). For comparison, the crystallographic results for two inhibitors in catL, pdb codes 2xu3 and 2xy2, are shown. The subsites are named according to the nomenclature of *Schechter* and *Berger* [39], and residues are colored according to their subsite location: S3, orange; S2, magenta; S1, blue; S1′, green; and S2′, purple.

Li-gands	Inhibition values (IC_{50}) [μM]	S3					S2							S1					S1′		S2′
		G61	N66	G67	G68	L69	M70	A214	A135	D160	M161	D162	G164	Q19	G23	S24	C25	W26	H163	G164	Q19
1	12.3		■	■	■						■	■			■		■		■		
2	39.5		■									■		■	■		■				
3	6.5		■		■						■	■			■		■		■		■
4	8.3											■		■	■		■				
5	> 500		■	■								■					■				
6	7.2			■	■						■	■			■	■	■		■		
7	9.7		■	■	■						■	■			■	■	■				
8	14.7											■					■				
9	2.4				■							■			■		■		■		
10	9.1		■		■							■		■			■		■	■	■
11	8.2		■		■			■				■					■		■	■	
12	> 250				■							■		■			■		■	■	
13	2.6		■		■							■					■		■	■	■
14	> 500				■							■	■	■			■		■		
2xu3	(0.160)	■			■	■	■	■	■		■	■					■		■	■	■
2yj2	(0.012)			■	■	■	■			■	■	■			■		■	■	■	■	■

CatB-docking studies showed that the compounds have no interactions with the catalytic residue Cys29. As previously reported by *Caracelli et al.*, a potent inhibitor for this protease should interact with Cys29 and residues of the S2′ subsite, in particular with His110 and His111 of the occluding loop [40][41].

Conclusions. – A series of natural triterpenoids and their derivatives were screened to evaluate their inhibitory activities towards cathepsins B and L. The promising results revealed that the compounds in which the COOH group remained unchanged were competitive inhibitors of catL. Docking studies on catL showed that the rigidity of the ring system prevents the compounds to reach the S2 and S3 subsites which are important for the inhibition. Finally, the achievement of the active compounds confirmed the proper functioning of the bioactivity-guided study, and these insights are important to aid the design of new inhibitors with enhanced potency and affinity towards the target enzyme.

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Experimental Part

General. All commercially available chemicals and reagents were purchased from *Aldrich Chemical Co.* and *Sigma*. Isolation procedures were carried out by anal. TLC on pre-coated aluminum silica gel *60* (SiO_2; *Merck*, 230–700 mesh). Solvents used in extracts preparation and chromatography fractionation were purchased from *Vetec*. ^{1}H- and ^{13}C-NMR spectra: *Bruker DRX-400* NMR spectrometer (400 and 100 MHz, resp.).

Enzyme Expression. The recombinant human catL was expressed in *Pichia pastoris* as described in [42]. CatB from human liver (*Aldrich* C8571) was purchased from *Aldrich Chemical Co.* and *Sigma*.

Extraction and Bioactivity-Guided Fractionation. The parts (leaves and stems) of seven different species from cerrado biome were dried (40°) for 10 d. The dried material was ground and extracted with EtOH at r.t. in three extractions (each 200 g) of vegetable mass. The EtOH extracts were filtered and concentrated under reduced pressure on a rotary evaporator, and then the dry extracts were evaluated on catL and catB. All 14 extracts were subjected to liquid–liquid partition resulting in hexane, AcOEt, and hydroalcoholic fractions.

The hexane fraction (MLCH; 117 g) from the stems of *M. lingua* Berg. showed good results on catL and was subjected to CC (SiO_2 *60* (4.0 × 40.0 cm); hexane/acetone 8:2) to afford six fractions, which were evaluated on catB and catL in order to assess the inhibitory potential of each one. Among them, the fractions MLCH-B (440.0 mg) and MLCH-C (249.0 mg) showed 95% and 96.7% inhibition on catL, resp. The fraction MLCH-B was separated by CC (SiO_2 *60* (2.5 × 35.0 cm); hexane/acetone 8:2) to give five subfractions. The obtained active fraction MLCH-BE (94.7 mg) was subjected to CC (SiO_2 *60* (1.5 × 30.0 cm); hexane/AcOEt 9:1) to afford the active triterpenes **1** (6.5 mg) and **3** (2.4 mg). MLCH-C was purified by CC (SiO_2 *60* (1.5 × 56.0 cm); hexane/acetone 8:2) to yield six subfractions. Finally, the active subfraction MLCH-CF (62.7 mg) was purified by CC (SiO_2 *60* (2.5 × 35.0 cm); hexane/acetone 9:1) to furnish the active triterpene **2** (1.2 mg). The bioactivity-guided study was monitored with TLC, and all pure compounds were fully characterized by NMR spectra (^{1}H, ^{13}C, and DEPT-135) and comparison with literature data.

Plant Material. The stems of *Myrcia lingua* Berg. (Myrtaceae) were collected in May 2011 in São Carlos, São Paulo state, and were identified by Dr. *Maria Inês Salgueiro Lima.* A voucher specimen (8366) was deposited with the Herbarium of Botany Department (HUFSCar) at Federal University of São Carlos, Brazil.

Kinectic Measurements. All commercially available chemicals and reagents were purchased from *Aldrich Chemical Co.* and *Sigma*, and kinetic measurements were carried out in a fluorimeter *Molecular Devices Spectra MAX M*3. Inhibitory activity was measured using the synthetic fluorometric substrate Z-Phe-Arg-AMC (benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide) at a concentration of 185 μM for catB (K_m 123 μM [43]: K_m 157.5 μM) and 10 μM for catL (K_m 1.2 μM [44]: K_m 2.4 μM)) [44]. The molar concentrations of all active cathepsins were determined by titration using the irreversible inhibitor E-64 [45]. CatB was assayed at 62 nM and catL was assayed at 55 nM. The enzyme was activated during 5 min with DTE (1,4-dithioerythritol) and acetate buffer (pH 5.5) at 37°, and then the mixture was incubated during 5 min with the sample. The experiments were carried out in triplicate (in 96-well black plates), and the final volume of the reaction mixture (200 μl) was kept under stirring (λ_{exc} 355 nm; λ_{em} 460 nm). All inhibitors were screened against catL and catB at an initial concentration of 100 μM. Control assays were performed without inhibitor (negative control) and in the presence of the irreversible inhibitor for cysteine peptidase, E-64 (positive control; 1-{*N*-[(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino}-4-guanidinobutane), which can irreversibly inhibit a wide range of cysteine peptidases and was first isolated and identified from *Aspergillus japonicas* in 1978 [46]. The IC_{50} values were determined by rate measurements with at least seven inhibitor concentrations. The inhibition type and K_i values were determined at the same experimental conditions. All kinetic parameters were determined by nonlinear regression employing the *SigmaPlot 12.0* enzyme kinetics module, and the type of inhibitor was established by *Lineweaver–Burk* plots of 1/*V vs.* 1/*S* at different inhibitor concentrations.

Molecular Docking. The three-dimensional structures of catL and catB were obtained from the *Protein Data Bank* [47] (PDB code 2XU3 [35] and 1GMY [48], resp.). Docking studies were performed using the GOLD program version 5.1 with the GoldScore function [49–51]. For the analysis of the results, the graphical program *Discovery Studio®* Visualizer 3.5 was used [52].

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